The role of immune cells in Chronic Rhinosinusitis

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B.Med.Pharm.Biotech

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for the degree of Doctor of Philosophy
in
The Discipline of Surgery, School of Medicine
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Declaration

I, Dijana Miljkovic, certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Dijana Miljkovic
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Publications arising from thesis


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Presentations arising from thesis

T regulatory and Th17 cells in Chronic Rhinosinusitis with polyps
Basil Hetzel Institute, Adelaide Australia, October 2015

T regulatory and Th17 cells in Chronic Rhinosinusitis with polyps
American Rhinologic Society Meeting, Dallas, Texas USA, September 2015

Discordant frequencies of tissue-resident and circulating CD180-negative B cells in chronic rhinosinusitis
American Rhinologic Society Meeting, Dallas, San Diego USA, September 2016

Naïve and effector b-cell subtypes are increased in chronic rhinosinusitis with polyps
University of Adelaide, Dept of Surgery Research Meeting, Adelaide Australia, August 2016

Discordant frequencies of tissue-resident and circulating CD180-negative B cells in chronic rhinosinusitis
Basil Hetzel Institute, Research Day Meeting, Adelaide Australia, October 2016

CRSwNP characterised by Th17 effector cytokines in mucosa and peripheral blood
Basil Hetzel Institute, Research Seminar, Adelaide Australia, October 2016

Awards arising from thesis

Discordant frequencies of tissue-resident and circulating CD180-negative B cells in chronic rhinosinusitis
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<th>Description</th>
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<tbody>
<tr>
<td>AHC</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ATPs</td>
<td>Adenosine triphosphates</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptors</td>
</tr>
<tr>
<td>Bregs</td>
<td>Regulatory B cells</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane regulator</td>
</tr>
<tr>
<td>CRS</td>
<td>Chronic rhinosinusitis</td>
</tr>
<tr>
<td>CRSsNP</td>
<td>CRS without nasal polyps</td>
</tr>
<tr>
<td>CRSwNP</td>
<td>CRS with nasal polyps</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
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<td>DAMPS</td>
<td>Danger associated molecular patterns</td>
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<td>DC</td>
<td>Dendritic cell</td>
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<td>dsRNA</td>
<td>Double stranded RNA</td>
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<tr>
<td>ECP</td>
<td>Eosinophil cationic protein</td>
</tr>
<tr>
<td>ECRS</td>
<td>Eosinophilic chronic rhinosinusitis</td>
</tr>
<tr>
<td>EPO</td>
<td>Eosinophil peroxidase</td>
</tr>
<tr>
<td>EPOS</td>
<td>European position paper</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony stimulating factor</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>Interleukin 12</td>
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<td>ILCs</td>
<td>Innate lymphoid cells</td>
</tr>
<tr>
<td>INF-α</td>
<td>Interferon α</td>
</tr>
<tr>
<td>iTreg</td>
<td>Inducible Tregs</td>
</tr>
<tr>
<td>Lin-</td>
<td>Lineage marker negative origin</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LTI</td>
<td>Lymphoid tissue inducer cells</td>
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<td>Major basic proteins</td>
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<td>Myeloid differentiation factor 2</td>
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<td>mDC</td>
<td>Myeloid DC</td>
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<td>Myeloid differentiation primary response gene 88</td>
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<td>MZ</td>
<td>Marginal zone</td>
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<td>NETS</td>
<td>Neutrophil extracellular traps</td>
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<td>Nuclear factor Kappa B</td>
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<td>nTregs</td>
<td>Natural Tregs</td>
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<td>PAMPS</td>
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<td>RAR-related orphan receptor gamma t</td>
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<tr>
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<td>Radioprotective 105</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>T-bet</td>
<td>T-box transcription factor</td>
</tr>
<tr>
<td>Th1</td>
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</tr>
<tr>
<td>Th2</td>
<td>T helper 2</td>
</tr>
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<td>TIR</td>
<td>Toll/interleukin-1 receptor</td>
</tr>
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<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cells</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR domain-containing adaptor inducing interferon-B</td>
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Abstract

Chronic rhinosinusitis (CRS) is a heterogenous disease characterised by the symptomatic inflammation of the nose and paranasal sinuses for more than 12 weeks. These symptoms include nasal obstruction, nasal discharge, facial pain and pressure, resulting in a considerable impairment of a patients’ quality of life. CRS is sub-categorised into two types based on the absence (CRSsNP) and presence of nasal polyps (CRSwNP) visualised within the middle meatus. Interestingly, although CRSsNP patients may lack easily identifiable polyps, the mucosa of these patients may show variable degrees of polypoid change. This raises the question as to whether the proposed classification system is an over simplification and that CRSsNP and CRSwNP in fact only represent two extremes of phenotype along a broader spectrum of immunologically different disease processes. Recently, research into CRS has identified a dysregulated immune response as a major contributor to the aetiopathology of disease, however few studies have utilised flow cytometry to phenotype the cells present. This thesis examines both the local and systemic populations of different adaptive and innate immune cells in the tissue and blood of CRSsNP and CRSwNP patients along different degrees of polypoid change within the same patient.
1.1 Chronic Rhinosinusitis

CRS is characterised by the persistent inflammation of the nose and paranasal sinuses for more than 12 weeks.\(^1\) Two or more of the symptoms present should include nasal blockage/obstruction/congestion, nasal discharge, facial pain or pressure and the reduction or total loss of sense of smell. This is confirmed by either endoscopy; showing signs of nasal polyps, mucopurulence, oedema and nasal obstruction and/or by inflammation on radiological examination where the Computed Tomography (CT) shows mucosal changes within the osteomeatal complex or paranasal sinuses.\(^1\)

The prevalence of CRS has been shown to be among the ten most disabling chronic conditions; with sufferers having a significantly impaired quality of life.\(^2\)\(^3\)\(^4\) Up to 16\% of the population suffer from the condition in the United States.\(^3\) In the Asia-Pacific and Europe prevalence is lower with only 1\% suffering from the disease in a nationwide survey in Korea, and 10\% in Australia and in 19 centres around Europe.\(^5\)\(^6\)
1.2 Pathology of CRS

CRS is considered a multifactorial inflammatory condition where intrinsic and extrinsic factors play a role in its initiation and manifestation.

**Figure 1.1 Pathology of CRS.** Chronic rhinosinusitis is a multifactorial inflammatory condition which involves intrinsic factors such as anatomy, genetics, allergy, asthma and the immune system interplaying with extrinsic allergens and pathogens.

1.2.1 Extrinsic factors

**Tobacco**

Tobacco smoking, both active and passive has been clinically associated with CRS. Zhou et al reported the negative effect that smoking can have on mucociliary clearance.
Although in a transient event cilia beat frequency is thought to be protective, the increased exposure of an irritant leading to increases in cilia beat frequency is thought to lead to a pro-inflammatory state. They observed that cilia beat frequency is increased both in smokers and non-smokers exposed to second-hand smoke when compared to non-smokers not exposed to smoke at all. Goblet cell hyperplasia is decreased in CRS patients’ sinonasal mucosa whereas squamous cell metaplasia is increased in patients exposed to tobacco smoke in CRSwNP patients. Olfactory function is also found to be adversely impaired with increased exposure to tobacco smoke further adding to the growing evidence of tobacco smoking’s negative effects on sinonasal mucosal functions.

**Pathogens**

**Viruses**

Patients presenting to the clinic with CRS often report that their symptoms developed after infection with a common cold. Although rhinovirus is linked to damage of the epithelial barrier, studies investigating the precise role in CRS haven’t yet been able to reach consensus in the part it plays either in the pathophysiology of CRS.

**Fungi**

Fungi are thought to play a role in a small proportion of CRS patients (Cleland et al, Zhou et al, in press). However, Ponikau’s group has advocated a greater role for fungi suggesting that they may play a role in the majority of CRS patients. In his study most patients with CRS had detectable fungal hyphae in the mucus that was obtained from their sinuses. However in their study fungus was found in nasal secretions regardless of whether the patient had CRS or was a healthy control. Current thinking is that *Aspergillus* and *Alternaria* play the most important role in CRS especially in allergic
fungal sinusitis, which is associated with nasal polyps and double densities on CT scan. In immune compromised patients fungus can become invasive resulting in vasculitis, ischaemic necrosis and life-threatening disease.\textsuperscript{14}

Bacteria

Bacterial colonisation in the sinonasal cavity is common. Commensal bacteria found in control samples include \textit{coagulase negative Staphylococcus, corynebacterium sp. and S.aureus}.\textsuperscript{15} Cultures in CRS report a predominance of \textit{Heamophilus influenzae, Streptococcus pneumoniae} and \textit{Moraxella catarrhalis} in the absence of antibiotics, whereas other studies report a predominance of \textit{S. aureus and P. aeruginosa}.\textsuperscript{16} Since the yield of anaerobic bacterial cultures in CRS has been quite variable, non-culture dependent techniques such as pyrosequencing are now being used to study the bacterial abundance in health and disease.\textsuperscript{17}

Bacterial biofilms

Biofilms are made up of a polymeric extracellular matrix encasing communities of bacteria and are one of the main survival techniques utilized by bacteria to protect them from environmental stress.\textsuperscript{18} Bacteria living in biofilms are also known to have enhanced resistance to antibiotics compared to their planktonic counterparts.\textsuperscript{19} In CRS, the presence of biofilms has been linked to more severe disease pathology.\textsuperscript{20} Interestingly, patients with biofilms not only have a more severe disease phenotype preoperatively but also have worse outcomes postoperatively, reflecting the importance of biofilms in sinus disease.\textsuperscript{21}

Superantigens

Viruses, fungi and bacteria all have a role in the superantigen hypothesis; the peptides they contain on their surface as well as excrete are able to activate and induce a large
proportion of T lymphocytes. *S.aureus* enterotoxins have been shown to be a pathogenic factor in CRS.\textsuperscript{22} These enterotoxins are able to induce an inflammatory response characterised by potent Th2 type inflammation and expansion of polyclonal IgE formation.\textsuperscript{23}

### 1.2.2 Intrinsic factors

**Anatomy**

Half of all patients diagnosed with CRS have two or more anatomic variations, whereas a third have a single anatomic variation.\textsuperscript{24} A deviated nasal septum is most common amongst disease sufferers, followed by unilateral concha bullosa and a bent middle turbinate.\textsuperscript{25} These anatomic variations can contribute to the blockage of the osteomeatal units, impaired drainage and ventilation pathways of the sinuses and can inevitably increase the risk of sinus mucosal disease.\textsuperscript{26}

**Mucociliary clearance**

The mucociliary system responsible for mucus movement across the beating cilia is an integral part of the host’s mechanical defence mechanism. It is believed that when ciliary function is impaired, respiratory secretions stagnate resulting in the ineffective clearance of pathogens. Consequentially in the paranasal sinuses, this stagnation may lead to inflammation that ultimately develops into a chronic inflammatory state, which can exist both with and without the presence of an active infection.\textsuperscript{27}

**Genetics**

Genetic susceptibility is thought to play a role in CRS. There is evidence for family groups that have an abnormally high prevalence of CRS.\textsuperscript{28} In addition patients with CRS are more likely to report a family history of CRS compared to those without CRS.\textsuperscript{6,29} The cystic fibrosis transmembrane regulator (CFTR) gene, which encodes a
chloride channel and regulatory protein in cystic fibrosis sufferers, has also been found mutated in patients with CRS and thought to be associated with the development of CRS.\textsuperscript{30} Human Leukocyte Antigen (HLA) genes that function in antigen presentation by B cells, dendritic cells and macrophages have had alleles associated with disease in CRS.\textsuperscript{31} Although other studies have looked at genetic variations in genes thought to play a role in the innate and adaptive immunity in CRS such as a single-nucleotide polymorphisms in pattern recognition taste receptors; there are very few and the results haven’t yet been replicated.\textsuperscript{32,33}

There is, however, an abundance of studies looking at gene expression differences between CRS with and without nasal polyps.\textsuperscript{34} The majority of these have focused on the adaptive immune response, especially the genes involved in the T cell response. In CRS with polyps, increases in messenger RNA expression for genes linked to the Th2 type inflammation and the eosinophil recruitment pathway have been described as key players in the disease.\textsuperscript{35,36} Innate immune responses have also been a focus with Jardeleza et al reporting an increase in genes regulating the nitric oxide pathway in polyp patients.\textsuperscript{37} NOD-like receptor mRNA, responsible for the response to microbial and host-derived danger signals, has also been found to be higher in nasal polyps compared to the nasal mucosa.\textsuperscript{38} Toll-like receptor molecule mRNA levels have been reported down-regulated in patients with polyps, resulting in the reduced ability of the host to sense danger signals from foreign antigens. This dysregulation of receptor signalling indicates a genetic link to an impaired innate immune pathway in CRS.\textsuperscript{39}

**Immune function**

Abnormalities in immune function may play a profound role in CRS, with initial studies finding that the disease is prevalent in individuals with human immunodeficiency virus.\textsuperscript{40} It is also found in people with common variable immunodeficiency disorder
and has been thought to play an important role in its aetiology.\textsuperscript{41} This coupled with abnormal T cell function testing and low IgA in some patients accounts for the immunocompromised state in CRS.\textsuperscript{42}

**Allergy**

Although there are numerous studies reporting the involvement of allergy in CRS the results are varied with some studies claiming allergy as an important factor and potentially even a subtype of CRS whereas others claim it to be unrelated to the disease process.\textsuperscript{43,44} Currently, the term allergy is being used based on a clinical history of reactions to any environmental agents as well as rhinoconjunctivitis, drug hypersensitivity, and food allergy.\textsuperscript{45} The term is used alongside with atopy, defined as skin prick test and/or specific IgE positivity to at least one common aeroallergen. Allergic rhinitis is defined as a symptomatic presentation of allergy defined by sneezing, runny/itchy/blocked nose and itchy eyes when exposed to an allergen.\textsuperscript{45,46} The lack of a common definition of allergy at least in part results in the varied reports of its importance in CRS.

**Asthma**

Asthma has been associated with upper airway diseases since the 1990’s.\textsuperscript{47} It is thought to be present in at least half the diagnosed CRS population and histopathologically appears to be quite a similar disease process.\textsuperscript{48,49} Eosinophilic inflammation, basement thickening and the erosion of the epithelium that cause prominent damage to the epithelial layer are similar characteristics of the two diseases that are absent in healthy controls.\textsuperscript{49} Most recently, in a cross-sectional survey, The Korean National Health and Nutrition Examination Survey listed asthma as the only significant associated risk factor in nasal polyposis.\textsuperscript{43} Rhinosinusitis is more common in patients with severe steroid dependent asthma compared to those with mild to moderate asthma, 84% of
patients with severe asthma have abnormal CT scans.\(^{50,51}\) Interestingly, endoscopic sinus surgery for the alleviation of symptoms of CRS has been shown to improve asthma based on both a quality of life questionnaire and an asthma control test.\(^{52}\)

![Computed tomography showing an asymptotic patient with no mucosal thickening of the sinuses (A) and a patient with mucosal thickening of ethmoid and maxillary sinuses (B).](image)

**Figure 1.2. Computed tomography of healthy and diseased sinus.** Computed tomography showing an asymptotic patient with no mucosal thickening of the sinuses (A) and a patient with mucosal thickening of ethmoid and maxillary sinuses (B).

### 1.2.3 Sinonasal Polyps

CRS is divided into two disease phenotypes based on the presence or absence of nasal polyps. According to the current European position paper (EPOS)\(^{54}\) on CRS, nasal polyps should be visualised endoscopically, arising bilaterally from the middle meatus into the nasal cavity, allowing the classification of CRS patients into CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP).\(^{53}\) Nasal polyps are grape-like structures which are composed of edema, inflammatory cells, connective tissue, mucous glands and capillaries encased in pseudostratified epithelium.\(^{54}\) In western
countries, CRSwNP is thought to differ from CRSsNP by having a more eosinophilic Th2 type disease as opposed to Asian CRSwNP counterparts that have more of a neutrophilic inflammatory infiltrate.\textsuperscript{55,56}

**Figure 1.3. Sinonasal polyps.** CRS is divided into two disease phenotypes based on the absence (A) of nasal polyps or presence of nasal polyps visualised bilaterally in the middle meatus (B).

The current medical practice involves antibiotics, systemic and topical steroids as the appropriate treatment in the management of CRS.\textsuperscript{1} Topically administered glucocorticoids have been studied immensely and a pooled meta-analysis of eight studies comparing intranasal corticosteroids to placebo demonstrated significant benefit in terms of symptom scores for the topical steroid group.\textsuperscript{36} Maximum medical therapy often includes a 10-day course of antibiotics, bidaily topical corticosteroids as well as a three-week tapering dose of systemic corticosteroids. Patients that fail maximal medical therapy have the option of undergoing endoscopic sinus surgery if symptoms persist.
Surgery allows for the removal of polyps, polypoid mucosa and diseased tissue with opening of the sinus drainage pathways and an improvement in nasal and sinus aeration.\textsuperscript{1}

1.2 Innate Immune System

1.2.1 Overview

The innate immune system is the body’s first line of defence against inhaled irritants, allergens and commensal organisms. The sinonasal epithelium plays a major role in this defence by not only providing a physical barrier to these foreign particles but also by being able to sense danger signals and coordinate an appropriate immune response. The cells that lie within the epithelium and subepithelial layers, as well as the vast array of protein receptors they express and cytokines they are able to release, are crucial in the immune response. In a healthy state the epithelium surveys danger and battles foreign antigens and danger signals by releasing cytokines and chemokines which are able to activate and recruit immune cells to the site of tissue damage. In CRS we see considerable differences in the different components that make up this innate response leading to the hypothesis that the innate immune system is deregulated in CRS patients and accounts at least in part to the disease process by supporting the ongoing inflammation essential to the disease process.\textsuperscript{57}

1.2.2 Sinonasal Epithelium

The sinonasal epithelium consists of ciliated pseudostratified columnar and cuboidal cells scattered with goblet cells.\textsuperscript{58} The cells are held together by tight junctions and rest on a basement membrane consisting of collagen. The intercellular junctional complexes composed of tight junctions, adherence junctions and desmosomes maintain
the structural cohesive integrity of the epithelial layer. In contrast, gap junctions allow for communication between cells and propagate signals across the epithelial surface.

Figure 1.4. Sinonasal epithelium. Ciliated pseudostratified columnar and cuboidal epithelial cells scattered with goblet cells resting on a collagen basement membrane.

1.2.3 Mucociliary system

Mucus is produced predominantly by the mucous glands present in the submucosa and to a lesser extent by goblet cells. It covers the sinonasal epithelium assisting the mucociliary function of the innate immune system by trapping foreign particles. Ciliated columnar cells contribute directly to the mucociliary function. They trap inhaled agents in the mucus and then propel the trapped particles to the pharynx where it is swallowed and enters the stomach in order to be further degraded by gastric acidity and gastric enzymes. The mucociliary transport system is impaired in patients with
CRS. This may be due in part of the increase seen in mucus viscosity following the release of mediators of inflammation together with the altered function of the periciliary stratum, which in turn slows down the mucociliary transport wave as detected by experimental charcoal powder.\textsuperscript{62} Recently studies of the epithelium have found that pendrin, an epithelial anion transporter protein expressed by surface epithelial cells is increased in CRSwNP and correlated with the mucus component protein Muc5AC. This indicates that pendrin might modulate the mucus production in these patients.\textsuperscript{63}

**1.2.4 Pattern Recognition Receptors**

Germline-encoded pattern recognition receptors (PRRs) are responsible for the recognition of danger and damage signals.\textsuperscript{64} They are able to recognise highly conserved microbial molecular structures termed pathogen-associated molecular patterns (PAMPS) and danger associated molecular patterns (DAMPS) released during host tissue injury and death. Their activation leads to unique signalling pathways that lead to the transcription of genes that regulate inflammation as well as more specific immune responses, activating arms of the adaptive immune system.\textsuperscript{65}

**Toll-Like Receptors**

Toll-like receptors (TLR) are a group of highly conserved PRRs found on epithelial, fibroblast, endothelial and innate and adaptive immune cells such as dendritic cells and macrophages, B-cells and T-cells.\textsuperscript{66,67} They are a type I transmembrane domain glycoprotein comprised of an amino (N) terminal ectodomain that contains leucine-rich repeats that mediate ligand recognition, a single transmembrane spanning domain that determines cellular localisation and a carboxyl-terminal globular cytoplasmic Toll/interleukin-1 (IL-1) receptor (TIR) domain that mediates downstream signalling.\textsuperscript{67} 10 TLRs (TLR1-10) have been identified in humans.\textsuperscript{68} Of those TLR2, TLR3, TLR4
and TLR9 have been shown to be expressed on airway mucosa and may play a contributing role to the development of CRS.\(^1\)

TLR2 expression is found to be higher in CRS patients compared to controls. It is also relatively higher in CRS patients with biofilms compared to those without, which is not surprising considering this TLR is essential in detecting bacterial and fungal wall components.\(^6^9\) In addition, TLR2 is found on dendritic cells, T cells and B cells and it combines with TLR1 or TLR6 in order to form TLR1/2 and TLR6/2 heterodimers. Activation of these receptors results in signalling, through the myeloid differentiation primary response gene 88 (MyD88) dependent pathway, activating the transcription of Nuclear Factor Kappa B (NF-kB).\(^7^0\) The outcome of this activation is a multitude of downstream effects. In a mouse model of asthma, intranasal TLR2 activation has been shown to promote the expansion of allergen-specific regulatory T cells.\(^7^1\) In TLR2-/- animals, the intraepithelial cell tight junctions are compromised leading to an increase in mucosal inflammation.\(^7^0\) TLR2 expression and activation is also correlated with TGF-\(\beta\)1 and collagen deposition in CRS and gives further insight into the importance of TLR2 in the regulation of mucosal immunity.\(^7^2\)

TLR3 is located on the intracellular or cell surface of fibroblasts and epithelial cells as well as in the endosomes of myeloid dendritic cells and macrophages.\(^7^3\) It recognises double stranded ribonucleic acid (RNA) (dsRNA) from viruses as well as RNA released from damaged cells.\(^7^4\) Activation of TLR3 can lead to a response in both the MyD88-dependent and MyD88-independent pathway. In this pathway, TLR3 is able to directly lead to the production of type I interferons and inflammatory cytokines.\(^7^5\)

In mice, TLR3 has been shown to be upregulated following influenza exposure. When challenged with influenza TLR3-/- mice have significantly reduced inflammatory
mediators including RANTES, interleukins as well as a lower number of CD8+ lymphocytes in the bronchioalveolar airspace.\textsuperscript{76} Although most of the focus of TLR3 has been on viral dsRNA, airborne fungi have also been shown to induce TLR3 expression on nasal epithelial cells.\textsuperscript{77} Recently data has suggested an opposing effect of ds DNA to TLR3; \textit{Alternaria sp.} were found to inhibit the production of Interferon-B by suppressing TLR3 expression.\textsuperscript{78} Allergen exposure in symptomatic allergic rhinitis also down-regulates TLR3 expression. Together this data indicates that TLR3 is involved in both protective immunity and pathogenic inflammatory tissue damage.\textsuperscript{79}

TLR4, a cell surface TLR, was the first mammalian TLR identified. It is present on monocytes, macrophages, neutrophils, immature dendritic cells and B cells. It was initially found to be responsible for the recognition of lipopolysaccharide (LPS), a component of the gram-negative bacterial outer membrane.\textsuperscript{80} TLR4 associates with CD14, an anchored membrane protein which is necessary for LPS recognition to form a functional LPS receptor complex. The TLR4/LPS complex forms a heterodimer with myeloid differentiation factor 2 (MD-2) in order to recognise a common pattern in structurally diverse LPS molecules.\textsuperscript{81} The sensing of LPS by the heterodimer leads to the activation of the MyD88-dependant pathway activating (NfK-B) resulting in the immense production of inflammatory cytokines and chemokines.\textsuperscript{82} TLR4 is also activated by viral and fungal proteins as well as endogenous ligands such as necrotic cells, heat shock proteins and fibrinogen.\textsuperscript{83,84} Responding to these ligands can result in activation of both the MyD88-dependent pathway and MyD88-independent pathway. The MyD88-independent pathway needs TRIF-related adaptor molecule (TRAM) for the activation of TIR domain-containing adaptor inducing interferon-B (TRIF) proteins in order to result in the production of interferons.\textsuperscript{82}
TLRs were first studied in CRS in 2005 with the finding by Dong et al that TLR4 mRNA was expressed in nasal epithelium as well as overexpressed in the epithelium of CRS compared to controls. Since then other studies have looked at TLR4 proteins confirming the increase found in CRS compared to control patients. TLR4 increases in the nasal epithelium could result in an increase in inflammation seen in CRS patients and a recent study has also addressed that the increase in TLR4 plays an important role in the remodelling of CRSsNP. The increase in TLR4 was correlated with upregulated TGF-B1 production and collagen deposition as well as neutrophil infiltration and a T helper 1 (Th1) skewed inflammatory response.

TLR9 is expressed by numerous immune cells including B cells, NK cells and plasmacytoid dendritic cells and responds to both bacterial and viral DNA. This results in the activation of the MyD88 downstream signalling cascade and potently induces inflammatory cytokines such as interleukin 12 (IL-12), tumour necrosis factor α (TNF-α) and antiviral cytokine interferon α (INF-α). In the respiratory system TLR9 is thought to reduce T helper 2 (Th2) dependent inflammation by the induction of an increased Th1 response. Apart from its role in host defence against invading pathogens, TLR9 is also involved in the pathogenesis of a range of autoimmune disorders. In systemic lupus erythematosus (SLE) has been well documented that unlike other most other TLRs playing a mostly detrimental role in disease, TLR9 has a protective effect with the absence of TLR9 leading to more a more extreme disease phenotype. This is also apparent in CRS where functional TLR9 protein has been found decreased in CRSwNP compared to non-diseased controls.

**NOD-like Receptors**

NOD-like receptors (NLR) contain a central nucleotide binding and oligomerization domain and are a group of highly conserved cytosolic receptors. As an integral part of
the innate immune system, they function by sensing DAMPS. In microbes, they recognise viral RNA, fungal hyphae and peptidoglycan. In the environment, they recognise asbestos, silica and alloy particles as well as ultraviolet radiation. They also recognise antigens from self, including adenosine triphosphates (ATPs), cholesterol crystals and uric acid.\(^9_6\) NLRs are present on macrophages, monocytes, dendritic cells, neutrophils and epithelial cells.\(^9_7,9_8\) In humans, 22 NLR proteins have been identified. Their activation leads to the induction of an immune cascade resulting in distinct functions that can be divided into four groups; inflammasome formation, signal transduction, transcription activation and autophagy.\(^9_9\)

Inflammasomes are multimolecular protein complexes that assemble in the cytosol after immune activation. NLRs form inflammasomes once activated and result in the activation of caspase-1 activity. The caspase activation implements the activation of an array of downstream effectors, each of which lead to inflammation or cell death.\(^1_0_0\) The role of inflammasomes in chronic inflammation has been a research interest in recent years. Genome-wide studies have found associations with single nucleotide polymorphisms within inflammasomes to be associated with Crohn’s disease.\(^1_0_1\) Deficiencies in inflammasomes have also been linked with increased susceptibility to colitis suggesting an important role for inflammasomes in mucosal diseases.\(^1_0_2\) The involvement of inflammasomes has been documented in CRS, with both the inflammasome and signalling pathways being upregulated in CRS patients with \textit{S. aureus} biofilms, implying that \textit{S. aureus} biofilms and subsequent inflammasome activation play a role in triggering an inflammatory response in these patients.\(^1_0_3\)

\textbf{CD180}

CD180, also called radioprotective 105 (RP105) is a TLR-like receptor that has a similar structure to other TLRs, consisting of conserved leucine-rich repeat domains.\(^1_0_4\)
It is the most recent receptor to be identified and as yet has no natural known ligand. It is found on B cells, dendritic cells and macrophages. It acts similarly to TLR4 in its dependency on another molecule for its expression and signalling, MD1. Just like the TLR4/MD2 complex, MD1 is associated with the extracellular domain of CD180. Interestingly, it has recently been discovered that CD180 is a physiological regulator of TLR4 signalling. The direct interaction of the CD180/MD1 to the TLR4/MD2 receptor complex inhibits LPS binding. It is thus thought to be important in preventing over amplification of the TLR4 response which can lead to endotoxin shock.

CD180 has been studied mostly in autoimmune diseases. CD180 negative B cells have been shown to be increased in the peripheral blood of SLE patients and this correlates with SLE disease activity and Immunoglulin (Ig)G production. Interestingly, CD180 antigen binding results in receptor internalisation and these CD180 negative B cells produce IgM and IgG antibodies suggesting that CD180 negative B cells represent pathogenic and possibly autoreactive B cell subsets. These CD180 negative B cells are also significantly increased and suggested hallmarks of other autoimmune diseases including Sjögren syndrome and dermatomyositis.
The complement system is a group of 30 membrane-bound and circulating proteins that play a major role in the innate immune systems host defence.\(^{112}\) The proteins are activated in a cascade-like fashion through three different pathways; the classical pathway, the lectin pathway and the alternative pathway. The classical pathway is initiated by IgM or IgG and referred to as the antibody dependent pathway.\(^{113}\) The lectin pathway is initiated by specific C-type lectins which recognise patterns found in the outer layers of yeast and fungal microorganisms. The alternative pathway resembles the innate immune system and is activated by large complex polysaccharides such as those in microbial walls.\(^{114}\)

**Figure 1.5. TLR function in CRS.** TLR2 responds to lipoteichoic acid, TLR3 to viral DNA, TLR4 with co-accessory molecule MD2 to LPS, TLR9 to bacterial and viral DNA and TLR-like receptor CD180 forms with co-accessory molecule MD1.

**1.2.5 Complement**

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Together these pathways are responsible for the body’s ability to fight infection by providing an immediate response against invading pathogens. This causes the lysis of pathogens both by antibody-independent and dependent mechanisms, chemotaxis, phagocytosis and cell adhesion. Complement is also involved in the bridging of innate and adaptive immunity by promoting B cell activation and the clearance of apoptotic and necrotic cells.

Complement proteins have also been shown to be involved in disease processes. In asthma, they are responsible for inflammatory cell infiltration, mucus secretion and increases in vascular permeability. Complement has been made known to account for autoimmune disease causing inflammation and tissue damage particularly in the case of SLE. Deficiencies in complement have also been shown to account for microbial reinfections with S. aureus, a prominent microbe in CRS with the bacterial proteins being able to block complement receptors and in turn result in the evasion of phagocytosis.

1.3 Cells of the Innate Immune System

1.3.1 Dendritic cells

Dendritic cells (DCs) are highly specialised antigen-presenting cells (APC) that have a key role in the immune system due to their ability to bridge both the innate and adaptive immune arms. They do this by capturing antigens in response to both pathogen or self danger signals via PAMPS and DAMPS that are recognised by the various PRR they contain on their surface. Upon sensing these pathogens the dendritic cell engulfs particles, slowly degrades the matter and in turn matures. This results in the upregulation of co-stimulatory and MHC I and II molecules allowing the antigen to be presented to naïve T cells causing their activation and proliferation. DCs are present
in parts of the body that are exposed to the environment as well as organ entry points. They are found in large quantities in lymphoid tissues (lymph nodes, tonsils, spleen, thymus, bone marrow and Peyer’s patches) but may also be found in small amounts in non-lymphoid organs and make up 0.0 to 0.59% of all leukocytes in peripheral blood. Phenotypically they can be described by the expression of integrins CD11c, CD103 and CD24. These classical DCs can further be subcategorized into three types based on both their function and phenotype; plasmacytoid DC (pDC) and two types of myeloid DCs, mDC1, and mDC2.

pDCs express the key marker CD303, a type 2 transmembrane lectin receptor that is involved in cell adhesion, capture, and processing of antigens. The antigens that stimulate pDCs include viruses such as herpes simplex virus, human immunodeficiency virus type 1 and influenza virus through TLR7 and TLR9. The cells are also stimulated by bacteria such as *S. aureus* and microbial DNA. In response to these stimuli activated pDCs produce a range of cytokines and chemokines including INF-α/β, TNF-α, IL-1 and IL-6. This attracts and activates Th1 polarized cells and NK cells. It also induces B cell activation and antibody production, further instigating a role for pDCs in the activation of both host innate and adaptive immune systems.

Considerable amounts of pDCs have been detected in the nasal epithelium. Hartmann et al. have been able to show both pDCs and SDF-1, the pDC major chemoattractant, to be expressed in the nasal epithelium. pDC numbers were shown to be further increased in patients that had recently had an upper respiratory tract infection. The pDC numbers were lower in patients with allergies compared to healthy individuals and absent in patients undergoing treatment with glucocorticoids. In CRS, numbers of pDC cells are significantly increased in nasal polyp tissue compared to healthy non-inflamed mucosa.
Interestingly, in the sinonasal epithelium, patients with polyps and allergy had lower pDC numbers than those with polyps alone.\textsuperscript{130} mDC1 and mDC2 can be differentiated by the expression of c-type lectins BDCA-1 and BDCA-3 respectively.\textsuperscript{131} mDCs express TLR1, TLR2, TLR4, TLR5 and TLR6 suggesting that they are more responsive to bacterial antigens.\textsuperscript{132} mDC1 are found more frequently than mDC2 in tissues. They produce high levels of pro- and anti-inflammatory cytokines such as interleukins IL-10, IL-12 and IL-23 causing the activation of innate and adaptive immune cells, pDCs, cytotoxic T cells as well as Th1 cells.\textsuperscript{133,134} mDC2 in contrast mostly produce interferons and high levels of antiviral cytokines resulting in the activation of Th2 type immunity.\textsuperscript{135,136} Kirsche et al have findings to support this theory in CRS. They found a Th2 skew in CRSwNP patients based on a decreased Th1/Th2 ratio in CRSwNP compared to CRSsNP and controls and correlated this to a lower mDC1/pDC ratio in these patients.\textsuperscript{137} Other studies, however, found an increase in mDCs in CRSwNP and have attributed this as one of the many causes of inflammation of the disease.\textsuperscript{130,138}

### 1.3.2 Macrophages

Macrophages play an important role in maintaining tissue homeostasis. They are one of the major effectors of the innate immune system and are located in all tissues, have great plasticity as well as anatomical and functional diversity.\textsuperscript{139} They were initially classified as making up part of the mononuclear phagocyte system, which consisted of a family of cells comprising bone marrow progenitors, blood monocytes, and tissue macrophages.\textsuperscript{140} Monocytes and macrophages express CD14 on their surface, a specific pattern recognition receptor.\textsuperscript{141} Macrophages can be differentiated from monocytes based on their expression of 25f9, a marker of macrophage maturation.\textsuperscript{142} It was for a long time thought that macrophages residing in tissue were relying on bone
marrow-derived blood monocytes for their replenishment. However, in recent years, evidence suggests that tissue macrophages are from two origins, one is established prenatally from the primitive yolk sac and the other monocyte-derived.\textsuperscript{143} Embryonic macrophages are derived from the mesoderm and initially responsible for the homeostatic functions associated with developmental tissue remodeling and vascularisation.\textsuperscript{144,145} These tissue macrophages are found in cellular compartments within the tissue they reside in and are thought to then be self-sufficient and independent from further hematopoietic input.\textsuperscript{146,147}

Alveolar macrophages are of specific interest because much like in the sinonasal mucosa these macrophages reside on the epithelial surface and are more in contact with the direct environment than macrophages in other tissues. Embryonic macrophages appear to colonise the lung after birth and differentiate into alveolar macrophages and live independently of blood monocyte input. If these resident tissue macrophages are depleted, a second type of macrophage can develop in adulthood from tissue infiltrating monocytes.\textsuperscript{148} This pathway is generally considered more pathological but also has homeostatic and inflammatory functions.\textsuperscript{149}

Macrophages, like the name suggests, function in phagocytosing apoptotic cells and cellular debris both in developmental stages and in adult life. They also have an immunological role and the ability to adapt to their environment. This has led to their further classification of M1 classically activated and M2 alternatively activated macrophages.\textsuperscript{150,151} Mimicking T cell nomenclature, the pro-inflammatory M1 macrophages were labeled because of their differentiation in response to the Th1-derived IFN-\(\gamma\) in cell-mediated immunity to intracellular infection. M2 macrophages, considered anti-inflammatory, differentiate mainly in response to Th2 cytokines such as IL-4 in response to extracellular parasitic infection.\textsuperscript{151}
In CRS, macrophages came to light by a study done by Shun et al looking at local chemokines that may contribute to disease pathogenesis. They suggest that nasal polyp fibroblasts may contribute to nasal polyp development by the production of CCL2 to promote macrophage recruitment. In the gut mucosa, resident macrophages stimulated by their PRR secrete cytokines and show strong bactericidal activity. In inflammatory bowel disease (IBD), CD14+ macrophages have recently been implicated in the contribution to disrupting the epithelial barrier through the deregulation of tight junction proteins as well as the induction of epithelial cell apoptosis.

Interestingly, the same plasticity that defines a macrophages function, the ability to change phenotype according to their tissue environment, is what has proven troublesome in their characterisation. Most studies have been carried out in mice and there is a great divergence in cellular markers between not only animal models but also diseases and tissue states. In the intestinal mucosa for instance, macrophages in the large and small intestine differ in phenotype, as they do from healthy bowels and those with diseases such as ulcerative colitis and Crohn disease. Due to these difficulties, research on macrophages is still scarce, especially in diseases such as chronic rhinosinusitis.

### 1.3.3 Eosinophils

Eosinophils are circulating leukocytes distinguished from their bone marrow derived counterparts by the cytoplasmic specific granules they contain. From the bone marrow, eosinophils are released and continually replenished into the blood stream making up 1-3% of the cells in the periphery. Although they express a multitude of surface receptors common to other innate cells, eosinophils are also host to the unique surface receptors IL-5Ra, CCR3 and Siglec proteins.
The IL-5Ra receptor binds to T cell cytokine IL-5 which has the most prevalent impact in the production and regulation of eosinophils. IL-5 production is attributed to Th2 cells as well as the epithelium which is capable of secreting the cytokines TSLP and IL25. These cytokines activate Th2 cells and lead to the production of IL-5, resulting in the promotion of eosinophilia. The resulting IL-5 signalling is responsible for the generation of eosinophils from progenitor cells, eosinophil activation and survival. Interestingly, IL5 also promotes the further release of IL-5 from eosinophils, resulting in a positive feedback loop. IL-5 is mandatory in the chemotaxis of eosinophils from the blood to tissue and is also responsible for the recruitment of eosinophils into tissues in inflammatory diseases.\textsuperscript{160-162}

Once stimulated, recruited eosinophils elicit their bactericidal response by the extracellular release of their cytoplasmic granules. This secretory pathway occurs by picmeal degranulation; the vesicular transport of small particles that are then released to the cells surface. This resulting response to cytokines such as INF\textsubscript{γ} leads to the development of these granule protein vesicles while leaving eosinophils viable and able to respond to further stimuli.\textsuperscript{163} Four of the major eosinophil granule proteins released include eosinophil cationic protein (ECP), major basic proteins (MBP1 and MBP2), eosinophil-derived neurotoxin, and eosinophil peroxidase (EPO). These proteins activate other immune cells and are major contributors to the toxicity of microorganisms through the generation of reactive oxidants as well as direct killing of bacteria.\textsuperscript{164}

Eosinophils also play an essential role in the pathology of disease, especially chronic inflammation in diseases such as asthma, eosinophilic oesophagitis as well as CRS. Allergic asthma has been extensively studied both in humans and mice models and it has been shown that allergic asthma is associated with eosinophilic inflammation in the
The proinflammatory mediators derived by eosinophils are major contributors to airway epithelial damage, hyperresponsiveness, mucus secretion and airway remodelling, hallmarks of chronic respiratory and sinonasal inflammation. In allergy the antigens are responsible for the cascade of signalling which marks the Th2 cell recruitment and cytokine production resulting in the attraction of eosinophils to the airways.  

It has long been thought that eosinophils are central in the inflammation seen in the sinonasal mucosa. Polyp development has been of particular interest with many studies finding increased numbers of eosinophils in CRSwNP patients compared to those without any polypoid change. Polyps from Caucasian cohorts have shown to be largely eosinophilic in nature compared to Asian counterparts that are mostly neutrophilic. CRSsNP on the other hand has remained for the most part absent of eosinophil infiltration seen in CRSwNP. Interestingly although not common, patients with CRSsNP and an abundance of eosinophils have been shown to be highly unresponsive to medical and surgical interventions.

Evidence that eosinophils play a role in the ongoing inflammation in CRS is further described by studies showing that RANTES, a key attractant cytokine for T cells and monocytes is found in all nasal polyps predominantly in eosinophils and epithelial cells and at a forty times higher abundance than in control tissues. The increase of IL-5 in nasal polyps as well as chemokines and eotaxins that are able to recruit eosinophils directly even in the absence of IL-5 to the site of inflammation are also increased in CRSwNP patients further adding to the pathophysiology of the disease.

Eosinophilia is so prevalent in CRS that CRSwNP patients that present with thick mucus production, loss of smell, recurrent bacterial infections and long term
inflammation that are found to have an abundance of eosinophils in their polyps are further subclassified into Eosinophilic Chronic Rhinosinusitis (ECRS) and non-ECRS.\textsuperscript{175} The majority of these patients are Caucasian patients that have recurrence after surgery and are most difficult to treat both medically and surgically.\textsuperscript{176}

### 1.3.4 Mast cells

Mast cells originate from haematopoietic cells in the bone marrow as mast cell precursors. They travel through the circulatory system by transendothelial migration into tissues and finally mature in the tissue microenvironment in which they reside.\textsuperscript{177} They are found in most vascularised tissue and are most abundant at body surfaces that interact with the environment such as mucosal tissue in the respiratory system, the gut as well as the skin.\textsuperscript{178} Mast cells express a wide variety of cell surface receptors including immunoglobulin Fc receptors required for defence through antigens of acquired immunity; complement receptors, NLRs and TLRs for defence through antigens of innate immunity. They are differentiated from other immune cells such as the phenotypically similar basophil based on the expression of c-kit stem cell factor CD117.\textsuperscript{179}

Upon activation, cross-linking of B-cell derived IgE to high-affinity IgE receptors leads to the release of an array of potent mediators including the degranulation of preformed mediators stored in the cell’s cytoplasmic granules.\textsuperscript{180} These include vasoactive amines, histamine, proteases, proteoglycans as well as cytokines and growth factors. This reactivity to antigens identifies mast cells as key effector cells or in IgE-mediated hypersensitivity and important in allergic diseases.\textsuperscript{181} Asthma is characterised by ongoing mast cell activation. Gene clustering studies show increases of expression of mast cell proteases in airway epithelial brushings in asthmatic subjects.\textsuperscript{182} Elevated levels of mast cell secreted proteins tryptase and prostaglandin D2 are elevated in
bronchoalveolar lavage fluid of asthmatics compared to those without asthma. Ultrastructural analysis of mast cells in lung tissue also shows that asthmatics have more degranulation than people without atopy and asthma.

Interestingly, mast cells have a longer lifespan than the average immune cell in tissues and hold onto their function by being able to replenish the contents of their secreted granules allowing them to be activated repeatedly. These tissue-resident mast cells are also able to expand at sites of inflammation and are responsible for the maintenance of epithelial barriers, regulating cell turnover, permeability, and progenitor recruitment. In the digestive system, mast cells are thought to have both a pathogenic and a protective role. They produce immunosuppressive cytokines such as TNF-a and IL-10 which promote tolerance mediated by Treg cells. They are also able to produce cytokines that promote inflammation and inhibit tumor cell growth by releasing TGF-B and interleukins. In CRS, although mast cell numbers are unchanged between CRSwNP patients and healthy nasal mucosa, mast cell degranulation has been found elevated in CRSwNP compared to both matched inferior turbinate samples and ARS inferior turbinates. This is confirmed by Patou et al. that reported enhanced mast cell mediators in CRSwNP compared to controls when tissue fragments are stimulated by anti-IgE on a human nasal challenge model. Whether the mast cell effectors play a protective role against pathogens or are contributing to the ongoing inflammatory state in CRSwNP still needs to be elucidated.

1.3.5 Neutrophils

Neutrophils are leukocytes with a polymorphic nucleus that have critical roles in innate immune defence. They are one of the most produced cells in the bone marrow and depend on the cytokine granulocyte colony stimulating factor (G-CSF) for their maturation. Circulating neutrophils are the first line of defence and not surprisingly
they are the most prominent circulating lymphocyte in humans. Neutrophils enter tissues in a process termed neutrophil recruitment which is made up of a cascade consisting of transmigration of the neutrophil into tissue. However, they have a short lifespan of up to five days after recruitment. Mature neutrophils exert their function by degranulation, respiratory burst and the generation of neutrophil extracellular traps (NETS). They protect the host from microbial pathogens and minimize side effects from apoptotic and injured cells. Interestingly, neutrophils are thought to be fairly unresponsive to single stimuli. The exposure to initial stimuli primes the cell allowing a rapid and maximum neutrophil activation such as radical oxygen generation and phagocytosis upon activation by a secondary stimulus.

Once activated neutrophils phagocytose particles, which are frequently opsonised by IgG at a rapid pace. This uptake marks in the fusion of the phagocytic vacuole with preformed granules within the cell to form the phagosome. This result in the concurrent increase in oxygen consumption associated with the ROS generated by the activation of NAPDH oxide which results in the killing of engulfed pathogens. Degranulation also occurs during pathogen engulfment or neutrophil activation via neutrophil-associated PAMPs, resulting in the release of extracellular vesicles containing proteinases and antimicrobial peptides. The discovery of NETS has paved the way for neutrophils to once again be in the research spotlight as the most recent area of current lymphoid research. This method of host defence involves neutrophils extruding a meshwork of chromatin fibres containing granule-derived antimicrobial peptides and enzymes over pathogens resulting in neutrophil cell death and pathogen entrapment. Most recently neutrophils have also been implicated in the regulation of adaptive immunity. They have long been known to be capable of producing cytokines and play a role in T cell responses. Neutrophils have also been shown to produce BAFF and are able to
accelerate plasma cell generation and antigen-specific B-cell IgG and IgM production.\textsuperscript{204-206}

Neutrophils were first considered to have an important role in CRS after a study using IHC showing neutrophils to be activated in CRSwNP patients with cystic fibrosis compared to CRSwNP without cystic fibrosis.\textsuperscript{207} Van Zele et al. later showed that CRSwNP had an increased numbers of neutrophils as well as MPO, the most abundant neutrophil enzyme, compared to controls.\textsuperscript{55} On the contrary levels of MPO are lower in CRS with biofilm compared to CRS without biofilm suggesting the absence of neutrophils a detriment in CRS disease.\textsuperscript{208} This recruitment of neutrophils to mucosal sites is driven by IL-8, derived from nasal epithelial cells in response to PRR stimulation by antigens.\textsuperscript{209} Neutrophil accumulation in CRS depends on both ethnicity and the absence or presence of nasal polyps. Neutrophil infiltration is observed to be higher in CRSwNP than in CRSsNP, however, due to a higher eosinophilic/neutrophilic ratio in CRSwNP disease than CRSsNP, CRSsNP is termed the CRS subtype of neutrophilic nature.\textsuperscript{1,210} In studies of polyps from Chinese patients, neutrophilic and eosinophilic infiltration appears to be less than in Caucasian polyps, however, the ratio of eosinophilic/neutrophilic infiltration was markedly reduced in Chinese patients terming these ‘neutrophilic’ polyps.\textsuperscript{56,170} With neutrophils being able to play a role in the resolution of chronic inflammation as well as pathology in the inflammatory state their role in CRS is not yet clear.

\textbf{1.3.6 Innate Lymphoid Cells}

Innate lymphoid cells (ILCs) are the most recent group of innate cells to be identified. They comprise a host of subsets including cytotoxic NK cells, lymphoid tissue inducer cells (LTi) and more recent ILC1, ILC2 and ILC3. They are characterised by classical lymphoid morphology but lack the cell surface expression of surface molecules
stemming from the same lineage giving them lineage marker negative origin (Lin-)
nomenclature. The non-cytotoxic ILC subsets release cytokines responsible for the
initial immune regulation before an adaptive immune response can be mounted. The
different ILCs have been defined by Th cell nomenclature based on the differential
requirements they have for transcription as well as the effector cytokines they release
that lead to their distinct functions.\textsuperscript{211,212}

ILC1 cells were only definitively discovered and named in 2013\textsuperscript{213}. They are called
ILC1 due to having a cytokine expression profile similar to Th1 cells. Their
transcription is controlled by transcription factor T-bet, and in response to IL-12, they
produce INF\textsuperscript{gamma}. They are able to be distinguished from NK cells as they lack the
cytotoxic granules perforin and granzyme B and NK markers CD16 and CD94.\textsuperscript{213} The
frequency of ILC1 is thought to be in low amounts in the steady state, however, in the
same study that initially described them they were found at high frequencies in the
inflamed mucosa of people with Crohn’s disease. ILC1 have also been implicated in
pathogenic infections: an elevated population was found in patients with chronic
hepatitis B along with the elevated ILC1 transcription factors and effector cytokine
INF\textsuperscript{gamma} and cytokine IL-12.\textsuperscript{214}

ILC2 cells are important mediators of allergic inflammation. Upon discovery, they were
considered members of the Th17 family, IL-17 cytokine family that respond to IL-25 in
a similar fashion to Th2 cells.\textsuperscript{215} We are now aware that ILC2 rely on GATA3 for their
transcriptional regulation and that they are activated by IL-25, IL-33, and TSLP, the
cytokines that are otherwise prominently produced by epithelial cells.\textsuperscript{216,217} ILC2 are
defined as lineage negative cells that express various cell surface markers listed in
Table 2.\textsuperscript{211} ILC2 are found at minute frequencies in the blood and are mostly
abundant at mucosal barriers whereupon stimulation they rapidly expand producing vast
amounts of IL-5 and IL-13. Mjosberg et al. first studied them in humans in nasal polyps, the gut, and the lung. Since this initial discovery ILC2 have further been implicated in CRS with studies suggesting their enrichment in CRSwNP and CRS patients with allergy. This enrichment of ILC2 in CRSwNP has also been linked with Th2 cell amounts and high tissue and blood eosinophilia. In allergic rhinitis, ILC2 are increased in the periphery of patients with seasonal allergic rhinitis during pollen season as well as in patients with allergic rhinitis after cat allergen nasal challenge.

The ILC3 population consists of ILC3 and previously mentioned LTi cells that are present from embryogenesis and an essential inducer of the development of prenatal lymph nodes and peyer’s patches. The ILC3 are differentiated from other ILC based on their dependence on transcriptions factors RAR-related orphan receptor gamma t (Rorγt) and aryl hydrocarbon receptor (AHR). In the adult, the ILC3 are formed in the bone marrow and reside mostly in the mucosa. After activation mostly through epithelial-derived cytokines IL-23, ILC3 are a major source of IL-22 and IL-17. In the mucosa of IBD patients, ILC3s have been shown to accumulate and promote tissue inflammation by the uncontrolled overproduction of IL17A and IL-22. Interestingly in other recent studies ILC3 have been implicated in being involved in shaping the commensal microbiota in the gut and preventing microbial dissemination that could drive an inflammatory response. Although they are yet to be established in CRS, ILC3 are emerging as an important subset of inflammatory disease such as in the pathogenesis of psoriasis. Immunotyping of psoriasis patients has revealed that IL-17A and IL-22 producing ILC3 are found in greater numbers in both the skin and peripheral blood of these patients compared to healthy individuals and thought to contribute to the pathogenesis of this disease.
Figure 1.6. ILC function in mucosa homeostasis. ILC effector functions promote immunity at mucosal surfaces. ILC1 protect against viruses and intracellular bacteria by producing INFγ. ILC2 protect against helminthes by producing IL-4, IL-5, IL-9 and IL-13. ILC3 protect against extracellular bacteria by producing IL-17A and IL-22. Adapted from Artis et al. Nature, 2015.
1.4 Adaptive Immune System

1.4.1 Overview
The adaptive immune system’s main function is to provide defence against invading pathogens. The majority of responses are destructive and hence it is vital that the system is able to distinguish what is foreign to self. The adaptive immune response is mediated by lymphocytes with two main responses; antibody mediated by B cells and cell mediated by T cells, which will be discussed in detail in this section.

1.4.2 B Cells
B cells and the antibodies they produce are crucial in the adaptive immune response. B-lymphocytes arise haematopoetic precursor cells in the bone marrow. During their development, they are structured along the functional rearrangement process of immunoglobulin gene segments which ultimately enables them to give rise to a B cell repertoire that is responsive to more than $5 \times 10^{13}$ antigens.$^{230,231}$ Once this rearrangement is complete B cells termed immature B cells migrate from the bone marrow to the spleen to differentiate into naïve, follicular or marginal zone B cells.$^{232}$ Naïve cells are characterised by the expression of the IgD protein on their surface and lack CD27, a member of the tumour necrosis factor superfamily and implicated in B cell activation.$^{233}$ These B cells are termed naïve because they are yet to encounter their antigen. Once antigen activation occurs, naïve B cells can develop into antibody-secreting plasmablasts, plasma cells or memory B cells.$^{234,235}$

The initial and rapid B cell antibody response are dominated by plasmablasts located in peripheral immune organs. They undergo rapid clonal expansion which leads to the generation of large amounts of terminally differentiated short lived antibody producing plasmablast cells in the periphery.$^{236}$ Combined with CD19+, plasmablasts express
memory antigen CD27 and CD38, a molecule involved in cell adhesion and signal transduction.\textsuperscript{237} They are identified as CD19+IgD-CD27+CD38high. Plasmablasts after antibody production are then able to survive longer and differentiate into long living plasma cells that lose the expression of CD19 but are identified based on their expression of CD38 and CD27.\textsuperscript{238,239}

Memory B cells classified as CD19+CD27+IgD-CD38- cells, are able to remember encounters with antigens. This allows them to respond to antigens at a faster rate and with a more robust antibody response than naïve B cells encountering an antigen for the first time.\textsuperscript{240 241}

\textbf{Figure 1 7. B cell maturation.} Naïve B cells have left the bone marrow yet to encounter an antigen. Upon antigen encounter they can differentiate into short-lived antibody producing plasmablasts, long lived antibody producing plasma cells or memory B cells that are able to produce a more robust response upon encounter with the same antigen the naïve B cell was exposed to.
in the lymphoid follicles of the spleen and lymph nodes and are responsible for presenting antigens to activated T cells.\textsuperscript{242} Follicular B cells express monoreactive B cell receptors (BCR) or the IgD or IgM isotype composed of membrane immunoglobulin and heavy and light chain aminoacids.\textsuperscript{243} They participate in T cell dependent immune responses; activation of their receptors by antigens leads to the internalisation, processing and presentation of the antigen to T cells.\textsuperscript{243} They are also able to participate in T cell independent responses in times that they recirculate the bone marrow and respond to blood borne pathogens.\textsuperscript{244} MZ B cells are found in the marginal zones of the spleen, ready to screen pathogens and antigens found in blood. They express monoreactive BCRs as well as high levels of TLRs, providing a B cell link to the innate and adaptive immune systems.\textsuperscript{245} B1 cells are located in peritoneal and pleural cavities and at mucosal sites facilitating the screening of pathogens in tissues most susceptible to damage from the environment. They are the main producers of naturally occurring antibodies.\textsuperscript{246}

Aside from antibody production and antigen presentation, B cells are also capable of suppressor functions. Regulatory B cells (Bregs) carry out this suppressive function. Mizoguchi et al. initially discovered them in mouse models of disease as a small subset of B cells that are able to produce IL-10.\textsuperscript{247,248} Later, studies suggesting their presence in humans emerged where they were considered to limit inflammation. These CD19+ B cells have been found to have a high expression of glycoproteins CD24 and CD38 functioning in cell adhesion and signal transduction.\textsuperscript{249,250} They use IL-10 or direct cell to cell contact to suppress T cell proliferation and the production of pro inflammatory cytokines.\textsuperscript{250}

B cells have been implicated in various inflammatory and autoimmune diseases. In CRS, total B cells as well as further subtyped naïve, plasma and memory cells, have
shown to be enriched in nasal polyps compared to tissue from control patients. This is thought to account for the elevation of antibodies seen in these patients further adding to the deregulated local inflammatory state in CRS. Systemic Lupus Erythematosus SLE is a B-cell mediated autoimmune disease thought to be due to autoreactive B cells surviving in the periphery. The B cell persistence, and production of autoantibodies accounts for the inflammatory response, which is characteristic of in the immunopathology of the disease. Inflammatory bowel disease (IBD) is another disease associated with deregulated B cell responses. The activation of B cells is increased, causing an abnormal accumulation of antibody secreting plasma cells in the intestinal mucosa. This is thought to not only contribute to the inflammation of intestinal tissue but also account for evident mucosal tissue damage.

1.4.3 T cells

Each T lymphocyte expresses a unique TCR on the surface as the result of developmental selection upon maturation in the thymus. These T cells start out residing in secondary lymphoid organs and are termed naïve T cells as they have yet to encounter a foreign antigen. Once they encounter this antigen by a presentation with an APC such as a dendritic cell they display them on the surface by MHC protein. Once activated T cells undergo clonal expansion and migrate through the tissues to sites of antigen presence. These activated T cells capable of effector function include cytotoxic T cells, CD8+ T cells and helper T cells, CD4+ cells. Once the antigen is eliminated most T cells die, leaving behind memory T cells that may survive for years in lymphoid organs and resident tissues, able to be activated once again in response to the same antigen that initiated the naïve T cell they initially mounted a response from.
Th1 and Th2 cells

Naïve CD4+ T cells differentiate into effector cells. Initially two subsets were identified, Th1 and Th2. Th1 cells differentiate as a result of the interaction of TCR and the CD4 receptor along with MHC II complex presented by APC. Once activated these APCs continue to be involved in Th1 cell differentiation by the production of IL12, a dominant cytokine in the development of IFN-gamma producing T cells. Several transcription factors are important in coordinating the differentiation of Th1 cells, the main one being T-box transcription factor (T-bet). Not only does T-bet promote the differentiation of Th1 cells, it also suppresses the development of Th17 cells which have opposing functions. Th1 cells are involved in the elimination of intracellular pathogens, mostly due to their production of IFN-gamma. IFN-gamma causes the activation and recruitment of phagocytes such as macrophages to sites of infection resulting in the clearing of pathogenic microbes. Th1 cells can also produce TNF-alpha T cell growth factor IL2, responsible for the development of Treg and primary and memory CD8+ T cells leading to the enhanced overall adaptive immune response.

Th2 differentiation is dependant on cytokines IL-4 and IL-2. The major transcription factor involved in the Th2 lineage differentiation includes IL-4 induced STAT6, which up-regulates the expression of the master regulator GATA3. GATA3 is also capable of enhancing Th2 cytokine production whilst suppressing Th1 differentiation by down-regulating STAT4. Th2 cells are known to be involved in the response to extracellular parasites such as helminths and play a major role in allergic disease. Key effector cytokines include a range of interleukins (IL-4, IL-5, IL-9, IL-13, IL-10, IL-15) and amphiregulin leading to an eosinophilic response characteristic of inflammatory and allergic disease.
The first description of T cells in CRS showed elevated levels of both Th1 and Th2 cytokines in CRS, with a further abundance of Th2 cytokines associated with atopy. A follow-up study reported that IL-5, a prominent Th2 cytokine in CRS was independent of atopic status. Interestingly this discrepancy has now been thought to be due to ethnic differences in the sample population. In Caucasians, CRSsNP is skewed towards a Th1 phenotype with high levels of INF-gamma whereas CRSwNP is considered Th2 skewed with an abundance of IL-5. A comparative study on both Chinese and Belgian polyps concluded that there is a Th2 bias in Caucasian polyps compared to a Th1/Th17 bias in Chinese polyps. CRSsNP, however, remains to be Th1 biased regardless of ethnicity, with the Th1 cytokine as well as the TGF-beta receptor being increased in CRSsNP compared to CRSwNP.

**Th17 cells**

Th17 cells emerged ten years ago as a newly discovered CD4+ T cells that produced IL-17 by a lineage distinct to that of Th1 and Th2. They are considered as a cell with both protective and pathogenic functions. They play an important part in the adaptive immune system by the generation of inflammation. However, this inflammation, if sustained, may result in inflammation-associated pathologies such as tissue damage and the disruption of mucosal homeostasis. Th17 cells are characterised by CCR6+, a CC motif 6 chemokine receptor protein. The main cytokines they produce are IL-17A, IL-17-F, IL-21, and IL-22. Both IL-17A and IL17F cause the up-regulation of pro-inflammatory cytokines and chemokines. IL-17A is thought to have a more protective and regulatory role than its’ IL-17F counterpart. In the airways, IL-17A has been shown to cause a release of chemokines that recruit neutrophils and fungicidal peptides. In a mouse model of ulcerative colitis, IL-17A has been shown to mediate an effect on T cell driven mucosal inflammation by reducing the maturation of
Th1 cells. The cytokine also strengthens tight junctions by inducing claudin and mucin expression further implying its ability to function in a protective role. IL-21 functions in the production of proinflammatory cytokines TNF, IL-6 and INFgamma in the mucosa as well as aiding in the recruitment of neutrophils. In the gut it is considered to be pathogenic with neutralising antibodies to the cytokine being protective and reducing inflammation in mice. It is also implicated in a range of autoimmune diseases. IL-21 has been shown to be increased in active SLE and could be responsible for the generation of plasma B cells in the disease state. Stimulation of mucosal T cells with IL-21 results in an increase in Th1 and Th17 responses further adding to the inflammatory state in patients with IBD. IL-22, by contrast, has been found to induce intraepithelial cell activation and survival by moderating Treg cell subsets. IL-22 is capable of inducing proliferative and anti-apoptotic pathways as well as producing antimicrobial peptides, which help prevent tissue destruction and promote repair. Apart from IBD Th17 responses have been implicated in the pathophysiology of a number of inflammatory disorders, including rheumatoid arthritis, systemic lupus erythematosus, asthma and CRS. In CRS, studies have assessed Th17 abundance by IL17 expression. Although the early research did not find an increase in IL17 production in CRS, two more recent studies in the adult Chinese population have reported increased IL-17 in CRS, suggesting a possible role of Th17 cells in this condition. Further research is needed in order to characterise the presence as well as the nature of the immune function of Th17 cells in CRS.

Treg
T regulatory cells (Treg) are commonly differentiated based on their developmental function. Inducible Tregs (iTreg), also called adaptive Tregs, are developed in the peripheral lymphoid organs after antigen priming, whereas natural Treg (nTreg) are
released from the thymus as a distinct lineage resulting in the expression of Foxp3 that iTregs sometimes lack. Both Treg cell subsets are CD4+ cells that co-express high levels of CD25, a high affinity IL-2 receptor alpha-chain and contain transcription factor Foxp3, although some iTreg may or may not express Foxp3. There is still a lot of discrepancy surrounding Treg, with nTregs being the most understood and the main focus of further discussion here.

Treg cells characterized by CD4+CD25+CD127low surface marker expression have a distinct suppressor function, downregulating downstream T cell effector immune responses. They mediate their activity by direct cell-to-cell contact and through the production of TGF-B and IL10 cytokines. These cytokines downgrade immune responses and assist in self-tolerance. Tregs have been implicated in a range of mucosal and autoimmune diseases such as ulcerative colitis and rheumatoid arthritis. More recently differences in Treg cell populations have been observed in CRS, although some discrepancy does exist in the published literature. Two studies have reported a decrease in these cells in patients with CRS. Both studies defined Tregs using only Foxp3 expression whereas a more recent more definitive study defining Treg as CD4+CD25+FoxP3+ cells, has observed an increase in mucosal Tregs in CRS. This increase in Tregs in the mucosa could account for a defect in the immune defence against pathogens by the suppression of Th1 and cytotoxic T cells. However, as this is the first definitive study of Tregs in CRS further studies would be beneficial to elucidate whether they are contributing to the ongoing inflammation in the sinuses, as well as to determine which sinonasal tissue types they are found in.
Figure 1.8. T helper subsets. Naïve T cells in the presence of IL-2 and TGF-β differentiate into Tregs that promote immune tolerance. In the presence of IL-12 they differentiate into Th1 subsets that protect against intracellular pathogens. In the presence of IL-2 they differentiate into Th2 cells which protect the host against extracellular pathogens. In the presence of TGF-β, IL-6 and IL-21 they differentiate into Th17 cells that protect against extracellular bacteria and cause inflammation. Adapted from Bailey et al. Front. Immunol. 2014.
Chapter 2: T regulatory and Th17 cells in Chronic Rhinosinusitis with polyps
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2.1 Abstract

**Introduction:** Chronic Rhinosinusitis (CRS) is categorised into two types based on the absence (CRSsNP) and presence of nasal polyps (CRSwNP). Although CRSsNP patients lack nasal polyps, the mucosa may show variable degrees of polypoid change. This raises the question of whether or not the classification system is an oversimplification and that CRSsNP and CRSwNP only represent two phenotypic extremes along a broader spectrum of immunologically different disease processes. To investigate this, adaptive and innate immune cells were compared in the different tissue types within CRSsNP and CRSwNP patients.

**Methods:** Tissue from 15 CRSwNP, 6 CRSsNP and 8 healthy control patients was obtained prospectively. Non-polypoid mucosa, polypoid tissues and polyps were obtained at the time of endoscopic sinus surgery and analysed using Flow Cytometry for various adaptive and innate immune cell subsets.

**Results:** In the polyps from CRSwNP patients there were significantly more T regulatory (Treg) cells (12.86 +/- 12.60 vs 2.83 +/- 4.68) and Th17 cells (16.12 +/- 11.75 vs 2.31 +/- 2.13) compared to the polypoid tissue from CRSsNP patients. Cellular infiltrates in the non-polypoid or polypoid mucosa of the different patient categories showed no difference in CRSwNP, CRSsNP and control groups.

**Conclusion:** This observational study identified an increase in Treg and Th17 cells in CRSwNP patients implying that these cells may be implicated in polyp development. Importantly it also identified a similar inflammatory infiltrate in non-polyp or polypoid mucosa across control, CRSsNP and CRSwNP groups inferring that polyps should be sampled when studying CRSwNP.
2.2 Introduction

T helper (Th) cells, key players in the adaptive immune system, have recently been implicated in a variety of chronic diseases. In a non-disease state, cells of the adaptive immune system work together with cells of the innate immune system to mount an effective immune response. The dysregulation of this response causes immune dysfunction and prohibits the clearance of invading pathogens resulting in an ongoing inflammatory state. Th cells, once stimulated, have the capacity to differentiate into Th1, Th2, Th17, follicular helper T cells and Treg each of which has a specific immune-modulatory function.

Treg cells characterized by CD4+CD25+CD127low surface marker expression have a suppressor function, down regulating downstream T cell effector immune responses. They mediate their activity by direct cell-to-cell contact and through the production of TGF-B and IL10 cytokines. These cytokines downgrade immune responses and assist in self-tolerance. In humans, these cells have been implicated in a range of mucosal and autoimmune diseases such as ulcerative colitis and rheumatoid arthritis. More recently differences in Treg cell populations have been observed in chronic rhinosinusitis (CRS), although some discrepancy does exist in the published literature. Two studies have reported a decrease in these cells in patients with CRS, whereas a more recent study has observed an increase in mucosal Tregs in CRS.

Th17 cells have a crucial role in the induction of immune-related tissue injury and are characterized by the production of IL-17A, IL-17F, IL-6, TNF-α, and IL-22. Pathogenic Th17 responses have been implicated in the pathophysiology of a number of inflammatory disorders, including rheumatoid arthritis, systemic lupus erythematosus,
asthma and CRS. In CRS, studies have assessed Th17 abundance by IL17 expression. Although early research did not find an increase in IL17 production in CRS, two more recent studies in the adult Chinese population have reported increased IL17 in CRS, suggesting a possible role of Th17 cells in this condition.

CRS is considered an inflammatory disease that is regulated by T cell subsets. Currently, the disease is sub-categorised into two types based on the absence (CRSsNP) and presence of nasal polyps (CRSwNP) visualised within the middle meatus. Interestingly, although CRSsNP patients may lack easily identifiable polyps, the mucosa of these patients may show variable degrees of polypoid change. This raises the question as to whether or not the proposed classification system is an oversimplification and that CRSsNP and CRSwNP in fact only represent two extremes of phenotype along a broader spectrum of immunologically different disease processes.

To investigate this, we examined the local and systemic populations of different adaptive and innate immune cells in the tissue and blood of CRSsNP and CRSwNP patients. Furthermore, we examined tissue with different degrees of polypoid changes in the same patient.

### 2.3 Materials and Methods

#### Patient Groups

This study was approved by the Human Research Ethics Committee of the Queen Elizabeth Hospital, Adelaide, Australia. Tissue samples and blood specimens were prospectively collected at the time of endoscopic sinus surgery from control patients, CRSsNP and CRSwNP patients. Control patients were patients undergoing endoscopic sinonasal procedures without clinical or radiological evidence of sinus disease. CRS
patients were patients who fulfilled the diagnostic criteria set out in the recent position papers by the American Academy of Otolaryngology and Head and Neck Surgery and the European Position Statement on Chronic Rhinosinusitis\textsuperscript{1,315}. Patients with CRS were further sub-classified according the absence or presence of visible polyps present within the middle meatus on nasal endoscopy. Exclusion criteria included minors < 18 years, pregnancy, malignancy, immune disorders and the use of antibiotics or oral corticosteroids in the month preceding surgery. All patients provided informed written consent prior to enrollment. Patients were classified as atopic if they had positive RAST and/or skin prick testing. Demographic and clinical data was collected on all patients prior to the commencement of the study.

**Specimen collection**

Tissue samples were collected at the time of endoscopic sinus surgery from the ethmoid sinuses. Structures were defined as polyps when they extended into the middle meatus as defined by the EPOS guidelines\textsuperscript{53}. In contrast, polypoid structures were more confined and did not extend into the middle meatus. They had a typical cobblestone macroscopic appearance. Differentiation between polyp, polypoid or mucosa was done by the operating surgeon (PJW, AP). From CRSwNP patients, polyps, polypoid tissue and non-polypoid mucosa was collected. Polypoid and non-polypoid mucosa was collected from CRSsNP patients, and in control patients, non-diseased mucosal tissue was obtained as part of the endoscopic approach. Peripheral blood samples were taken from each patient just prior to the operation.

**Immunophenotyping of tissue and peripheral blood using flow cytometry**

Heparinized peripheral blood was lysed for 15 minutes using Pharmlyse (Becton Dickinson Biosciences, San Jose, CA, USA). Tissue samples were washed and dissected into pieces ≤ 2mm before being prepared into a single cell suspension by
enzymatic digestion with 2 mg/ml collagenase type II (Sigma-Aldrich, MO, USA) and 0.04 mg/ml DNase I (Roche Applied Sciences, Vilvoorde, Belgium) for 45 min at 37°C. Cell suspensions were filtered through a 100 µm nylon mesh and washed in PBS. All cells were stained with Fixable Viability Dye eFluor® 780 (eBioscience, San Diego, CA, USA) at 4°C for 30 minutes to exclude dead cells before staining with the following antibodies from Becton Dickinson Biosciences (San Jose, CA, USA) listed in Table 2.1. The antigens used to describe individual cell types are listed in Table 2.2. Eight colour flow cytometry was performed using a gating strategy based on fluorescence minus one controls as specified in Figure 2.1, 2.2, 2.3 and 2.4.
Table 2.1. Flow cytometry antibodies

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<td>CD4</td>
<td>SK3</td>
<td>FITC</td>
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<tr>
<td>CD25</td>
<td>M-AZ51</td>
<td>BV421</td>
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<td>CCR4</td>
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<td>PECY7</td>
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<td>CCR6</td>
<td>11A9</td>
<td>BV510</td>
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<td>HIL-7R-M21</td>
<td>PE</td>
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</tr>
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<td>SIC3</td>
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<tr>
<td>CD8</td>
<td>RPA-T8</td>
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<td>CRTH2</td>
<td>BM16</td>
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<td>HLA-DR</td>
<td>G46-6</td>
<td>BV510</td>
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<tr>
<td>Lineage Cocktail</td>
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<td>25F9</td>
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Table 2.2. Markers used for cell classification

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<tr>
<td>Th2</td>
<td>CD45+CD4+CCR4+</td>
</tr>
<tr>
<td>Th17</td>
<td>CD45+CD4+CCR6+</td>
</tr>
<tr>
<td>Treg</td>
<td>CD45+Cd4+Cd25+CD127low</td>
</tr>
<tr>
<td>GammaDelta T cell</td>
<td>CD45+CD3+TCRgd+</td>
</tr>
<tr>
<td>T cell</td>
<td>CD45+CD3+</td>
</tr>
<tr>
<td>B cell</td>
<td>CD45+CD19+</td>
</tr>
<tr>
<td>Natural Killer Cell</td>
<td>CD45+CD3-CD16+Cd56+</td>
</tr>
<tr>
<td>Plasmacytoid Dendritic cell</td>
<td>CD45+CD123+HLADR+Lin-CD11c-</td>
</tr>
<tr>
<td>Myeloid Dendritic cell</td>
<td>CD45+CD123-HLADR+Lin-CD11c+</td>
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<tr>
<td>Basophil</td>
<td>CD45+Cd123+HLADR-</td>
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<td>Mast cell</td>
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<td>Macrophage</td>
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</tr>
<tr>
<td>Neutrophil</td>
<td>CD45+CD16+CD66B+</td>
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Figure 2.1. Flow cytometry gating strategy of Treg, Th1, Th2 and Th17 cells. Live cells were identified (A) and CD45+CD4+ Th cells gated (B) to identify CD25+CD127low Treg (C), CXCR3+ Th1 (D), CCR4+ Th2 (E), and CCR6+ Th17 cells (F).
Figure 2.2. Flow cytometry gating strategy of total T cells, CD4 T cells, CD8 T cells, TCR gamma delta T cells, NK cells and B cells. CD3+ T cells were selected from Live CD45+ cells (A), then separated into CD4+ and CD8+ T cells (B) and TCR gamma delta cells (D). CD3-CD19+ gating identified B cells (E). CD16+CD56+ gating identified NK cells (C) from live CD45+ cells.
Figure 2.3. Flow cytometry gating strategy of mast cells, basophils, plasmacytoid and myeloid dendritic cells. CD45+ live cells gated on CD117+ identify mast cells (A) and CD123+HLADR- identify basophils (B). Lineage negative HLADR+ (C) cells are further gated to identify CD11c+CD123- myeloid and CD123+CD11c- plasmacytoid dendritic cells (D).
Figure 2.4. Flow cytometry gating strategy of eosinophils, macrophages and neutrophils.

High side scatter CD45+ live cells are further divided into CD16- to identify eosinophils (D), and CD16+CD66b+ neutrophils (C). CD45+ live cells gated on CD14+CD163+ identify macrophages (B).

Statistical analysis

The Kruskal-Wallis test was used for the comparison of data from the three independent disease groups of patients. Mann-Whitney test was used for comparison of two independent disease groups. For within group comparisons, data was analysed using a linear mixed effects model with specimens treated as random factors. Log transformations were applied to the data due to violations of the distributional assumptions of a linear model. The results were back transformed to the numeric scale.
prior to reporting. As a result, subgroup comparisons represent the ratio of two geometric means. All data were analysed using SAS v9.3 (SAS Institute Inc., Cary, NC, USA). Tests were two-tailed and significance was assessed at the 5% alpha p< 0.05)

2.4 Results

Patients

Twenty-nine patients used for this study included 15 CRSwNP, 6 CRSsNP and 8 Controls. Their demographic information is summarized in Table 2.3. Tissue and blood specimens were examined from each of the three patient groups by means of flow cytometry using cell surface markers that define Th1, Th2, Th17, Treg, Gamma Delta T cells, total T cells, B cells, natural killer cells, plasmacytoid dendritic cells, myeloid dendritic cells, basophils, mast cells, macrophages, eosinophils and neutrophils.

Table 2.3. Patient demographics for T cell study

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>CRSsNP</th>
<th>CRSwNP</th>
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<tbody>
<tr>
<td>Number</td>
<td>8</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>Median Age (IQR)</td>
<td>66 (50-73)</td>
<td>53 (42-70)</td>
<td>50 (44-68)</td>
</tr>
<tr>
<td>Male/Female</td>
<td>1/7</td>
<td>4/2</td>
<td>8/7</td>
</tr>
<tr>
<td>Asthmatic/non-asthmatic</td>
<td>1/7</td>
<td>1/5</td>
<td>7/8</td>
</tr>
<tr>
<td>Allergic/non-allergic/NA</td>
<td>2/6</td>
<td>3/3</td>
<td>4/4/7</td>
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<tr>
<td>Previous ESS: 0/1≥ 2</td>
<td>n/a</td>
<td>2/2/2</td>
<td>7/7/1</td>
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</table>
Cellular comparison between the non-polypoid mucosa of CRSwNP, CRSsNP and control patients

Cellular infiltrates in the mucosa of the different patient categories showed no difference between the three groups.

Cellular comparison between the CRSwNP polyp, CRSwNP polypoid and CRSsNP polypoid tissue

CRSwNP polypoid tissue had significantly more Th17 cells compared to CRSsNP polypoid tissue (14.64 +/- 16.87 vs 2.313 +/- 2.134, Mann-Whitney test p≤0.05, Figure 2.5). In polyps from CRSwNP patients there were significantly more Treg cells (12.86 +/- 12.60 vs 2.83 +/- 4.68) and Th17 cells (16.12 +/- 11.75 vs 2.31 +/- 2.13) compared to the polypoid tissue from CRSsNP patients. There were no differences observed in the other cell types.
Figure 2.5. Th17 cells are increased in polypoid CRSwNP tissue compared to polypoid CRSsNP tissue. Graph shows CRSwNP and CRSsNP polypoid Th17 numbers with mean. p≤0.05 Mann-Whitney test.

**Cellular comparison within CRSsNP group**

Cellular infiltrate between the non-polypoid mucosa and polypoid mucosa of the CRSsNP group showed no statistically detectable difference.

**Cellular comparison within CRSwNP group**

CRSwNP patients however had significantly more eosinophils (6.10 +/- 5.83 vs 2.53 +/- 2.75), macrophages (7.22 +/- 5.49 vs 4.35 +/- 3.72), mast cells (6.81 +/- 7.54 vs 2.53 +/-
natural killer cells (11.86 +/- 12.53 vs 8.31 +/- 19.23) and TCR gamma delta T cells (19.61 +/- 18.07 vs 11.85 +/- 18.31) in the polyps compared to non-polypoid mucosa from the same patient. CRSwNP polypoid tissue had significantly more mast cells (7.45 +/- 6.89 vs 2.53 +/- 2.48) and natural killer cells (12.84 +/- 18.21 vs 8.31 +/- 19.23) compared to non-polypoid mucosa of the same patient (Kruskal-Wallis p≤0.05, Table 2.4). There were no differences seen between the cellular infiltrate of polypoid vs polyp tissue in CRSwNP patients.

Table 2.4. Cellular comparison using Kruskal Wallis Test between conventional Control, CRSsNP and CRSwNP groups as a % of CD45+ cells.

<table>
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<tr>
<td>T regulatory</td>
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<tr>
<td>T helper 1</td>
<td>0.727</td>
</tr>
<tr>
<td>T helper 2</td>
<td>0.104</td>
</tr>
<tr>
<td>T helper 17</td>
<td>0.011</td>
</tr>
<tr>
<td>CD4 T cell</td>
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<tr>
<td>CD8 T cell</td>
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<tr>
<td>Bcell</td>
<td>0.125</td>
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<tr>
<td>Gamma Delta T cell</td>
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<tr>
<td>Natural Killer</td>
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<td>Mast Cell</td>
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<td>Plasmacytoid Dendritic cell</td>
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<td>Basophil</td>
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<td>Eosinophil</td>
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</tr>
<tr>
<td>Neutrophil</td>
<td>0.342</td>
</tr>
</tbody>
</table>

**Cellular comparison between CRSwNP, CRSsNP and control groups**

Due to finding no statistical difference within the different tissue types in CRSsNP and within the CRSwNP polyp and polypoid tissue we compared the three patient groups.
using conventional methods; non-polypoid mucosa from Control and CRSsNP patients and polyps from CRSwNP patients.

CRSwNP patients demonstrated a significantly higher number of Treg cells (12.86 +/− 12.60 vs 2.41 +/− 3.24), Th17 (16.12 +/− 11.75 vs 2.870 +/− 2.512), natural killer cells (11.68 +/− 12.53 vs 0.9454 +/− 1.012) and eosinophils (12.92 +/− 12.29 vs 0.9892 +/− 1.111) compared to CRSsNP patients (Kruskal-Wallis p ≤ 0.05, Figure 2.6). All other cell types assessed were similar between patient groups. No differences were observed in the peripheral blood between the different patient groups in any of the cell types.

Figure 2.6. Treg, Th17, NK cells and eosinophils are enriched in CRSwNP polyps compared to CRSsNP mucosa. Graph shows CRSwNP polyp, CRSsNP mucosa and control mucosa Treg (A), Th17 (B), NK cell (C) and Eosinophil (D) medians with interquartile range. p ≤ 0.05 Kruskal-Wallis.
2.5 Discussion

In this study, we found that there is a greater number of resident Tregs, Th17 cells, natural killer cells and eosinophils in CRSwNP patients compared to CRSsNP patients. Importantly, we found that these cellular differences were not present in either the peripheral blood or the non-polypoid mucosal tissue across the different patient groups or between polypoid and non-polypoid mucosa of CRSsNP patients or between polyp and polypoid tissue of CRSwNP patients. Our findings also show that there is no significant difference between the inflammatory infiltrate between controls and CRSwNP tissue leading us to speculate that perhaps in a normal situation there is a variation of inflammatory cells composed of an intermediate number of eosinophils, NK cells, Th17 cells and Tregs.

CRSwNP has been characterised by a dysregulation of T cells, including Tregs. Tregs are involved in suppressive functions that lead to a down regulation in Th1 responses and cellular cytotoxicity. Our study found an increased number of Tregs in CRSwNP compared to CRSsNP suggesting that they play a role in the impaired immunity in patients with polyps. Excessive suppression of Th1 and cytotoxic cells results in a defect in the immune defence against pathogens and could contribute to the ongoing inflammation, accounting for the excessive inflammatory state in these polyp patients. Our findings support those previously published studies by Sharma et al. who reported the mucosa of CRSwNP patients have a higher proportion of Tregs compared to CRSsNP patients. Pant et al have also reported an increase in Tregs in CRS mucosa compared to controls although they did not observe a difference between CRSsNP and CRSwNP subgroups. Other studies investigating the role of Tregs...
have however, reported a decrease in CRSwNP compared to CRSsNP and control subjects which is contradictory to our results\textsuperscript{298,299,316}. In these studies they determined Tregs using either Foxp3 mRNA expression \textsuperscript{298,299,316,318} or protein levels as measured by Immunohistochemistry (IHC) \textsuperscript{317,322}. We defined Tregs using flow cytometry as CD45+CD4+CD25+CD123\textsuperscript{low}, which is reportedly a more specific natural Treg population with suppressor capabilities\textsuperscript{323}. Furthermore this is one of the largest studies using a Caucasian cohort, and differences in inflammatory cell accumulation in nasal polyps are reported to be affected by ethnicity\textsuperscript{56,298}.

To our knowledge this is the first study that has used flow cytometry to examine for resident Th17 cells in the mucosa of CRS patients based on cell surface expression of CD45+CD4+CCR6+. We found that Th17 cells were increased in CRSwNP compared to CRSsNP. Our study supports previous studies that found an increase in Th17 in CRSwNP defined by IL17 mRNA expression \textsuperscript{324}, mRNA expression of the aryl hydrocarbon receptor, a receptor supressing Th17 differentiation in CRSwNP \textsuperscript{300} and IL17 protein \textsuperscript{318,325-327}. Contrary to our findings two independent studies by Derycke et al.\textsuperscript{328} and Hu et al (32) reported a similar Th17 amount in CRSwNP and CRSsNP with the former study also showing no significant difference between CRS patients and controls. It should be mentioned however, that unlike our study, both these studies used the expression of IL17 as a surrogate marker of Th17 cell presence. Furthermore in the Derycke et al study, IL17 levels were recorded following stimulation of their cells rather than in a natural unstimulated basal state. Although IL-17 is a prominent cytokine of Th17 cells, it has recently been shown to be non-specific to Th17 cells, also being produced by gamma delta T cells, lymphoid tissue inducer cells, natural killer cells and macrophages\textsuperscript{225,329-331}. Thus it is not a specific marker for the Th17 cell population and could be the reason for differing reports in Th17 cell abundance in CRS. In our study
we did not employ surrogate indicators of Th17 presence but rather defined Th17 cells using the expression of their specific cell surface marker CCR6+. This surface marker is uniquely expressed on Th17 cells. Th17 cells have been shown to increase the amount of neutrophils as well as eosinophils to areas of inflammation and even account for the inflammatory lesions in ulcerative colitis. This increase in Th17 cells we found may be one of the factors driving polyp formation and further studies need to be undertaken to characterize the intracellular cytokine profiles of these Th17 cells to get a better understanding of their function in CRS.

This study is also the first to comprehensively characterize and compare the cellular infiltrates between phenotypically different tissues in the same patient groups. We compared the cellular infiltrates of sinus mucosa from control, CRSsNP and polyp patients to that of polypoid mucosa in CRSsNP and CRSwNP patients and polyps of CRSwNP patients. Interestingly the cellular infiltrate between the non-polypoid mucosa of patient categories were shown to be similar in CRSwNP, CRSsNP and control groups. Differences were most apparent within the CRSwNP group. Polypoid lesions had an abundance of mast cells and natural killer cells compared to non-polypoid mucosa. Polyps also had an abundance of mast cells and natural killer cells and in addition they also had significantly more eosinophils, macrophages and TCR gamma delta T cells compared to non-polypoid mucosa. This study supports other studies that have found an increase in eosinophils and macrophages when comparing nasal polyp tissue to mucosa in CRSwNP patients. This increase of inflammatory cells in polyps is thought to be a part of the disease process and accounts for the persistent inflammation seen in these patients. Although these studies have reported mostly similar inflammatory cell dispersal between nasal polyps and mucosa, unlike our study they have also reported an increase in neutrophils, CD8 T cells and plasma cells.
These differences could be due the differing experimental techniques used as well as differences in inflammatory cell accumulation in nasal polyps due to ethnicity\textsuperscript{56,298}. Interestingly our study showed no difference between the cellular infiltrate of polypoid vs polyp tissue in CRSsNP patients. This finding together with the reports that polyps differ in terms of inflammatory infiltrate to non-polypoid mucosa in CRSwNP patients suggests that care must be taken when sampling tissue from CRSwNP patients. The CRS patient classification based on the standardised European position paper guidelines to group CRSwNP patients and CRSsNP patients classifies the polyp not only from an anatomical perspective but one that is distinct in immune cell abundance as well\textsuperscript{1}.

### 2.6 Conclusion

In summary, our study identified an increase in Treg and Th17 cells in CRSwNP patients implying that these cells may be implicated in polyp development. Importantly it also identified a similar inflammatory infiltrate in non-polypoid mucosa across control, CRSsNP and CRSwNP groups, which infers that polyps should be sampled when sampling CSRwNP patients.
Chapter 3: CRSwNP is characterised by Th17 effector cytokines in mucosa and peripheral blood

Statement of Authorship

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<tr>
<th>Title of Paper</th>
<th>CRSwNP is characterised by Th17 effector cytokines in mucosa and peripheral blood</th>
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| Publication Status | Published  
☑ Accepted for Publication  
☑ Submitted for Publication  
☒ Unpublished and Unsubmitted work written in manuscript style |
| Publication Details | Submitted in the Journal of Allergy and Clinical Immunology |

Principal Author

| Name of Principal Author (Candidate) | Dijana Miljkovic |
| Contribution to the Paper | Collection of samples  
Experiments conducted  
Data analysis and Paper writing |
| Overall percentage (%) | 100% |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. |
| Signature | Date | 02/02/2017 |
Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

iv. the candidate’s stated contribution to the publication is accurate (as detailed above);

v. permission is granted for the candidate in include the publication in the thesis; and

vi. the sum of all co-author contributions is equal to 100% less the candidate’s stated contribution.

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3.1 Abstract

Introduction: Recent studies have implied a role for Th17 cells in CRS with nasal polyposis (CRSwNP) patients. However, Th17 cytokine production in CRS is still unknown. Here we sought to quantify IL-17A, IL17-F, IL-21 and IL-22 cytokines produced by Th17 cells in mucosal tissue and peripheral blood of CRSwNP, CRS without nasal polyops (CRSsNP) and control patients.

Methods: Samples were prospectively collected from CRS patients and non-CRS controls. We used flow cytometry to characterise the Th17 cells and their cytokines in sinonasal tissue and peripheral blood.

Results: A total of 36 patients were recruited to the study. CRSwNP patients had significantly more IL-17A (9.53 +/- 2.71 vs 1.11 +/- 0.43 vs 0.77 +/- 0.07), IL-17F (4.96 +/- 1.48 vs 0.88 +/-0.31 vs 0.56 +/- 0.04), IL-21 (5.55 +/- 2.01 vs 1.60 +/- 0.71 vs 1.53 +/- 0.55) and IL-22 (4.73 +/- 1.58 vs 0.70 +/-0.28 vs 0.88 +/- 0.26) producing Th17 cells in the polyps compared to CRSsNP and control mucosa per mg of tissue respectively. Allergic CRSwNP patients had decreased numbers of IL-21 producing Th17 cells compared to non-Allergic CRSwNP. (1.69 +/- 0.57 vs 9.41 +/-3.23) per mg of tissue respectively, (Kruskal-Wallis p<0.05)

Conclusion: In summary, our study identified increased amounts of Th17 derived cytokines IL-17A, IL-17F, IL21 and IL22 in CRSwNP patient polyps and peripheral blood suggesting a local and systemic role for Th17 cells in CRS. Atopic CRSwNP had decreased amounts of Th17 derived IL-21 in their polyps implying a potential protective role for IL-22 in CRSwNP allergic inflammation.
3.2 Introduction

T helper 17 (Th17) cells are characterised by CC motif 6 chemokine receptor (CCR6) expression and produce the signature cytokines interleukin-17A (IL-17A), IL-17F, IL-21, and IL-22. Th17 cells play a significant role in the adaptive immune system by generating inflammation in response to infection, in particular to Candida albicans and Staphylococcus aureus. Although vital in protecting the host against pathogens, dysregulated inflammation, if sustained, may result in inflammation-associated pathologies such as tissue damage and the disruption of mucosal homeostasis. Both IL-17A and IL17F cause the up-regulation of pro-inflammatory cytokines and chemokines such as IL-6, granulocyte colony-stimulating factor (GCSF) and CXCL1 and CXCL2.

In the airways, IL-17A has been shown to cause a release of chemokines that recruit neutrophils and fungicidal peptides. On the contrary, excessive IL-17A production in the synovial fluid of rheumatoid arthritis patients has been demonstrated to have a role in the progression of the disease. Antibodies targeting IL-17A result in a reduction of clinical symptom severity, further highlighting the importance of aberrant cytokine responses in differing immune microenvironments.

IL-21 intensifies the production of pro-inflammatory cytokines in the mucosa as well as aiding in the recruitment of neutrophils. In the gut mucosa it is considered to be pathogenic and antibodies targeted to neutralising the cytokine have a protective and anti-inflammatory effect. IL-21 is also implicated in a range of autoimmune diseases. It has been shown to be increased in active SLE and could be responsible for the generation of plasma cells in the disease state and epithelial cell activation and survival by moderating T regulatory cell subsets. IL-22 is also capable of inducing proliferative and anti-apoptotic pathways as well as producing antimicrobial peptides, which help prevent tissue destruction and promote repair.
Th17 responses have been implicated in the pathophysiology of a number of inflammatory disorders, including rheumatoid arthritis, SLE, and asthma. In chronic rhinosinusitis (CRS), studies have assessed Th17 abundance by measuring IL-17 expression. Although early studies did not find an increase in IL-17 production in CRS, two more recent studies in the adult Chinese population have reported increased IL-17 in eosinophilic CRSwNP, suggesting a possible role of Th17 cells in the inflammatory condition. However, it has been shown that IL-17 can also be produced by different immune cell types, including neutrophils and gammadelta T cells and thus, differential IL-17 abundance does not necessarily imply changes in Th17 cell frequencies. Further research is needed in order to characterise the presence as well as the nature of the immune function of Th17 cells in CRS. Recently, we have reported an increase in Th17 cell numbers in CRS with polyp (CRSwNP) patients. In this present study we further characterise Th17 cells by immunophenotyping the IL-17A, IL-17F, IL-21 and IL-22 cytokines they produce in the mucosa and periphery of CRSwNP, CRS without nasal polyposis (CRSsNP) and non-CRS controls.

3.3 Methods

Patient Sample Collection

This study was approved by the Human Research Ethics Committee of the Queen Elizabeth Hospital, Adelaide, Australia. Specimens were prospectively collected at the time of endoscopic sinus surgery from non-diseased controls, CRSsNP and CRSwNP. Ethmoid mucosa was used for controls and CRSsNP patients, polyps from CRSwNP patients and peripheral blood was collected from each patient prior to their operation. Control patients were undergoing endoscopic sinonasal procedures for pituitary tumour resections and were without clinical or radiological evidence of past or present sinonasal disease. CRS patients fulfilled the diagnostic criteria set out in the recent
position papers by the American Academy of Otolaryngology and Head and Neck Surgery and the European Position Statement on Chronic Rhinosinusitis.\textsuperscript{1,315} Patients with CRS were further sub-classified according to the absence or presence of visible polyps present within the middle meatus on nasal endoscopy. Exclusion criteria included minors < 18 years of age, pregnancy, malignancy, immune disorders and the use of antibiotics or oral corticosteroids in the month preceding surgery. All patients provided informed written consent prior to enrollment. Patients were classified as atopic if they had positive RAST and/or skin prick testing. Demographic and clinical data was collected from all patients prior to the commencement of the study.

**Cell Preparation**

Tissue samples were washed and dissected into pieces ≤ 2mm before being prepared into a single cell suspension by enzymatic digestion with 2 mg/ml collagenase type II (Sigma-Aldrich, MO, USA) and 0.04 mg/ml DNAse I (Roche Applied Sciences, Vilvoorde, Belgium) for 45 min at 37°C. Cell suspensions were filtered through a 70 μm nylon mesh and washed in PBS. Lymphocytes were isolated from heparinized peripheral blood by Ficoll-Paque PLUS (GE Healthcare, Chicago, USA).

**Flow Cytometric Immunophenotyping**

Cells stimulated with cell stimulation cocktail (eBioscience, San Diego, CA, USA) for six hours were washed and stained with Fixable Viability Dye eFluor® 506 (eBioscience, San Diego, CA, USA) at 4°C for 30 minutes to exclude dead cells before cell surface staining with CD4+CCR6+CD45+ to define Th17 cells. Cells were fixed and permeabilised before staining with intracellular cytokines IL-17A, IL-17F, IL-21 and IL-22 (eBioscience, San Diego, CA, USA). Antibody details are listed in supplementary Table 3.1. Gates were set on unstimulated and fluorescent minus one controls.
Table 3.1. Th17 cell flow cytometry antibodies

<table>
<thead>
<tr>
<th>Target Antigen</th>
<th>Clone</th>
<th>Conjugation</th>
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<tr>
<td>CD45</td>
<td>H130</td>
<td>APC-eFluor-780</td>
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<tr>
<td>CD4</td>
<td>RPA-T4</td>
<td>Ef-450</td>
</tr>
<tr>
<td>CCR6</td>
<td>R6H1</td>
<td>Pe-Cy7</td>
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<tr>
<td>IL-17A</td>
<td>EBio64DEC17</td>
<td>FITC</td>
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<td>IL-17F</td>
<td>SHCR17</td>
<td>PE</td>
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<tr>
<td>IL-21</td>
<td>EBio3A3-N2</td>
<td>eFluor-660</td>
</tr>
<tr>
<td>IL-22</td>
<td>22URTI</td>
<td>Percp-eFluor-710</td>
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</table>

**Statistical analysis**

The data were summarised using means with standard deviations and medians with range. The Kruskal-Wallis test was used for the comparison of data from the three independent disease groups of patients. Mann-Whitney test was used for comparison of two independent disease groups. All tests were two-tailed and significance was assessed at the 5% alpha.

**3.4 Results**

**Patients**

Samples from a total 36 patients (12 CRSwNP, 19 CRSsNP and 5 controls) were used for this study. Patient demographic information is summarized in Table 3.2.
Table 3.2. Patient demographics for Th17 study

<table>
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<tr>
<th></th>
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<th>CRSwNP</th>
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<td>Number</td>
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<td>19</td>
<td>12</td>
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<tr>
<td>Median Age (IQR)</td>
<td>54 (52-55)</td>
<td>59 (50-65)</td>
<td>56 (51-61)</td>
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<tr>
<td>Male/Female</td>
<td>2/3</td>
<td>13/6</td>
<td>10/2</td>
</tr>
<tr>
<td>Asthmatic/non-asthmatic</td>
<td>0/5</td>
<td>6/13</td>
<td>6/6</td>
</tr>
<tr>
<td>Allergic/non-allergic</td>
<td>4/9</td>
<td>4/12</td>
<td>8/8</td>
</tr>
<tr>
<td>Previous ESS: 0/1/≥ 2</td>
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<td>10/6/3</td>
<td>6/2/4</td>
</tr>
</tbody>
</table>

**Th17 cells are increased in CRSwNP mucosa**

CRSwNP patients had significantly more total CD4+ T helper cells in the polyps compared to CRSsNP and control mucosa (90.73 +/- 20.47 vs 39.72 +/- 11.14 vs 22.24 +/- 10.07 per mg of tissue respectively, Kruskal-Wallis p<0.01) (Figure 3.1A). CRSwNP patients had more Th17 cells in the polyps compared to CRSsNP and control mucosa (40.39 +/- 7.75 vs 16.19 +/- 4.49 vs 9.26 +/- 4.32 per mg of tissue respectively, Kruskal-Wallis p<0.01) (Figure 3.1B). No differences were seen in CD4+ or Th17 + numbers in the peripheral blood of control, CRSsNP or CRSwNP patients.
Th17 cytokines are increased in mucosal CRSwNP Th17 cells.

CRSwNP patients had significantly more IL-17A, IL-17F and IL-21 cytokines produced by Th17 cells in the polyps compared to CRSsNP and control mucosa (Figure 3.2A-C). CRSwNP patients also had more IL-17F producing Th17 cells in the polyps compared to CRSsNP and control mucosa. (Figure 3.2B). CRSwNP patients have more IL-21 producing Th17 cells in the polyps compared to CRSsNP and control mucosa. (Figure 3.2C). CRSwNP patients have more IL-22 producing Th17 cells in the polyps compared to CRSsNP and control mucosa per mg of tissue respectively, Kruskal-Wallis p<0.01 significant only compared to CRSsNP) (Figure 3.2D). Table 3.3 details cytokine frequencies for controls, CRSsNP and CRSwNP patients.
Figure 3.2. Percentage of IL-17A+Th17+ (A), IL-17F+Th17 (B), IL-21+Th17 (C) and IL-122+Th17 (D) cells per mg of tissue in control, CRSsNP and CRSwNP mucosa. Medians with interquartile range. p≤0.05 Kruskal-Wallis.
Table 3.3. Flow cytometry analysis of IL-17A, IL17-F, IL-21 and IL-22 expressing Th17 cells in mucosa (Kruskal-Wallis mean +/- SEM)

<table>
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<tr>
<th></th>
<th>Controls</th>
<th>CRSsNP</th>
<th>CRSwNP</th>
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<tbody>
<tr>
<td>Th17+IL-17A (per mg of tissue)</td>
<td>0.77 +/- 0.07</td>
<td>.11 +/- 0.43</td>
<td>9.53 +/- 2.71</td>
</tr>
<tr>
<td>Th17+IL-17F (per mg of tissue)</td>
<td>0.56 +/- 0.04</td>
<td>0.88 +/- 0.31</td>
<td>4.96 +/- 1.48</td>
</tr>
<tr>
<td>Th17+IL-21 (per mg of tissue)</td>
<td>1.53 +/- 0.55</td>
<td>1.60 +/- 0.71</td>
<td>5.55 +/- 2.01</td>
</tr>
<tr>
<td>Th17+IL-22 (per mg of tissue)</td>
<td>0.88 +/- 0.26</td>
<td>0.70 +/- 0.28</td>
<td>4.73 +/- 1.58</td>
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**Th17 cytokines are increased in peripheral CRSwNP Th17 cells.**

In the peripheral blood CRSwNP patients have significantly more IL-17A producing Th17 cells compared to CRSsNP and control patients. (Figure 3.3A). CRSwNP patients have more IL-17F producing Th17 cells in the polyps compared to CRSsNP and patients (Figure 3.3B). CRSwNP patients have significantly more IL-21 producing Th17 cells in the polyps compared to CRSsNP and control patients. (Figure 3.3C). CRSwNP patients have significantly more IL-22 producing Th17 cells in the polyps compared to CRSsNP and control patients. (Figure 3.3D). Table 3.4 details cytokine frequencies for controls, CRSsNP and CRSwNP patients.
Figure 3.3. Percentage of IL-17A+Th17+ (A), IL-17F+Th17 (B), IL-21+Th17 (C) and IL-22+Th17 (D) cells as a % of CD45+ cells in control, CRSsNP and CRSwNP peripheral blood. Medians with interquartile range. p ≤ 0.05 Kruskal-Wallis.
Table 3.4. Flow cytometry analysis of IL-17A, IL17-F, IL-21 and IL-22 expressing Th17 cells in peripheral blood (Kruskal-Wallis mean +/- SEM)

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>CRSsNP</th>
<th>CRSwNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th17+IL-17A (% CD45)</td>
<td>0.73 +/- 0.33</td>
<td>1.55 +/- 0.62</td>
<td>3.31 +/- 0.93</td>
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<tr>
<td>Th17+IL-17F (% CD45)</td>
<td>0.56 +/- 0.04</td>
<td>0.88 +/- 0.31</td>
<td>4.97 +/- 1.48</td>
</tr>
<tr>
<td>Th17+IL-21 (% CD45)</td>
<td>1.43 +/- 0.57</td>
<td>2.46 +/- 0.88</td>
<td>5.48 +/- 1.62</td>
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<tr>
<td>Th17+IL-22 (% CD45)</td>
<td>0.39 +/- 0.16</td>
<td>1.32 +/- 0.74</td>
<td>3.01 +/- 1.12</td>
</tr>
</tbody>
</table>

Th17 cytokines were equally produced within control, CRSsNP and CRSwNP mucosa and peripheral blood.

There were no differences observed between the amounts of any cytokine produced, IL-17A, IL-17F, IL-21 and IL-22 within control, CRSsNP or CRSwNP mucosa (Figure 3.4A-C) or peripheral blood. Kruskal-Wallis (Figure 3.4D-F).
Th17 cells produce less IL-21 in Allergic CRSwNP

Allergic CRSwNP patients had decreased numbers of IL-21 producing Th17 cells compared to non-allergic CRSwNP. (1.69 +/- 0.57 vs 9.41 +/- 3.23) per mg of tissue respectively, Kruskal-Wallis p<0.05 (Figure 3.5). No differences were seen in CRSsNP patients or in the peripheral blood of any of the patient groups.
Figure 3.5. Percentage of IL-21n allergic vs non-allergic CRSwNP mucosa.
Medians with interquartile range. $p \leq 0.05$ Kruskal-Wallis.

3.5 Discussion

This study for the first time demonstrates the cytokine production of Th17 cells in CRS.
It supports our previous study where we showed an increase in Th17 cells in CRSwNP patients compared to CRSsNP and controls. By further defining the cytokines that these Th17 cells produce we were able to get a clearer picture of the complex immune microenvironment we see in CRSwNP patients. Th17 cells producing IL-17A were increased in CRSwNP patient polyps and peripheral blood compared to CRSsNP and controls. This IL-17A elevation in Th17 cells could account for the IL-17A abundance seen in CRSwNP patients in various studies.\textsuperscript{343,344} In Japanese populations CRSwNP increases in IL-17A cytokine production are correlated with eosinophil numbers.\textsuperscript{343,345} A different cohort of Chinese patients has linked IL-17A abundance with elevated amounts of neutrophils in CRSwNP.\textsuperscript{344} IL-17A is known to cause the induction and activation of neutrophils and neutrophil activating cytokines and in the airways by
releasing chemokines that can recruit neutrophils and fungicidal peptides.\textsuperscript{285,286} Most recently, dysregulated IL-17A production was also found to promote neutrophil infiltration resulting in the delay of wound healing and tissue repair in mouse models, and could account for the inflammation and airway remodelling seen in certain CRSwNP patient subtypes.\textsuperscript{346} IL-17F, although newly discovered and less studied, is very similar in structure to IL-17A and has a similar role.\textsuperscript{347} This is evident in the accumulation of neutrophils in allergic diseases with high IL-17A and IL-17F production.\textsuperscript{348} In our study, IL-17F, much like its IL-17A counterpart was increased in the polyps and peripheral blood of CRSwNP patients. Recently, however, in vivo studies of an induced model of colitis have shown that IL-17A and IL-17F can have differing roles. Mice deficient in IL-17F but not IL-17A have defective airway neutrophils in response to allergen challenge demonstrating an important functional difference in the IL-17F immune response which warrants further research.\textsuperscript{349}

Studies in CRS have reported increased levels of IL-21 mRNA and protein using ELISA in polyp tissues and peripheral blood. Our study adds to this research suggesting the increase may, in fact, be due to increases of IL-21 producing Th17 cells in CRSwNP patients.\textsuperscript{350} IL-21 regulates T and B lymphocyte survival, activation and proliferation and in-vitro IL-21 has been shown to have an effect on polyp B cell differentiation and IgG and IgA production.\textsuperscript{350-352} In our study, CRSwNP patients that were atopic had remarkably less IL-21 cytokine producing Th-17 cells as evidenced by flow cytometry compared to non-atopic CRSwNP. These results are in agreement with studies in allergic rhinitis: IL-21 administered at the time of antigen challenge in ovalbumin-induced mice reduces allergic symptoms and antigen-specific IgE levels.\textsuperscript{353} Further to this, signaling through the IL-21 receptor mediates house dust mite airway hyper-responsiveness by enhancing Th2 cytokine production.\textsuperscript{354} There are
discrepancies, however, with other in vivo animal studies suggesting that IL-21 doesn’t affect airway remodeling. Further studies, focusing on airway remodeling in CRS will be needed to elucidate the role of the Th17 cytokine IL-21 in allergic patients.

Few studies have investigated the role of IL-22 in CRS. Ramanthan et al confirmed the presence of the IL-22 receptor IL-21R1 on the surface of nasal epithelial cells and discovered lower receptor quantities in recalcitrant CRSwNP compared to CRSsNP and controls. This was confirmed by another study by Wang et al however, there are conflicting reports about cytokine IL-22 levels, with one study showing no statistical difference in IL-22 levels between patient groups and the other finding IL-22 was significantly higher in CRSsNP mucosa compared to controls. IL-22 is produced by activated T cells as well as innate lymphoid cells and innate immune cells. In our study, we show that Th17 derived IL-22 is increased in CRSwNP patients polyps and peripheral blood. The cytokine is a key mediator of mucosal host defence and protects against infections of extracellular antigens. IL-22 promotes keratinocyte migration and innate immune function as well as tissue repair. Interestingly although IL-22 plays a protective role in mucosal diseases such as inflammatory bowel disease by enhancing barrier integrity of the intestinal tract, in other diseases such as psoriasis it has been eluded to synergise with proinflammatory cytokines and induce disease progression. It is evident that cytokines may have opposing effects in diverse tissue microenvironments and further functional studies are needed in order to see whether the Th-17 derived cytokines we see elevated in CRSwNP patients are in fact playing a protective or pathogenic role in CRS.
3.6 Conclusion

In summary, although Th17 cells were increased in CRSwNP polyps and not in the periphery, Th17 derived cytokines IL-17A, IL-17F, IL21, and IL22 are higher in abundance in CRSwNP patient polyps and peripheral blood suggesting both a localised and systemic role in the disease process. Interestingly, atopic CRSwNP has decreased amounts of Th17 derived IL-21 in their polyps implying a potential protective role for IL-22 in CRSwNP allergic inflammation.
## Chapter 4: Naïve and effector b-cell subtypes are increased in chronic rhinosinusitis with polyps

### Statement of Authorship

<table>
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<tr>
<th>Title of Paper</th>
<th>Naïve and effector b-cell subtypes are increased in chronic rhinosinusitis with polyps</th>
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| Publication Status | Published  
☑ Accepted for Publication  
☑ Submitted for Publication  
☐ Unpublished and Unsubmitted work written in manuscript style |
| Publication Details | Submitted in the American Journal of Rhinology and Allergy |

### Principal Author

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<tr>
<th>Name of Principal Author (Candidate)</th>
<th>Dijana Miljkovic</th>
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| Contribution to the Paper | Collection of samples  
Experiments conducted  
Data analysis and Paper writing |
| Overall percentage (%) | 100% |

**Certification:**
This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.

**Signature**

| Date | 02/02/2017 |
Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

vii. the candidate’s stated contribution to the publication is accurate (as detailed above);
viii. permission is granted for the candidate in include the publication in the thesis; and
ix. the sum of all co-author contributions is equal to 100% less the candidate’s stated contribution.

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<td>Peter-John Wormald</td>
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<td>Sarah Vreugde</td>
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Signature: Date 02/02/17
4.1 Abstract

**Background:** Recent studies have demonstrated that B cells as well as their chemo-attractants are elevated in the nasal mucosa of CRS with nasal polyposis (CRSwNP) patients. However, the presence of different effector B cell subsets in the mucosa and periphery of CRS patients is yet to be characterised.

**Objective:** Here we sought to quantify naïve, plasmablasts, plasma and memory B-cells in mucosal tissue and peripheral blood of CRSwNP, CRS without nasal polyps (CRSsNP) and controls patients.

**Methods:** Polyps, mucosa and peripheral blood samples were prospectively collected from CRS patients and non-CRS controls. We used flow cytometry to distinguish between naïve, plasmablast, plasma and memory B-cells in sinus tissue and peripheral blood.

**Results:** A total of 45 patients were recruited to the study. CRSwNP patients had significantly increased mucosal B-cell numbers compared to controls (3.39 +/- 4.05 vs 0.39 +/- 1.05 % of live cells respectively, Kruskal-Wallis p<0.01). This included naïve B-cells (0.61 +/- 0.94 vs 0.11 +/- 0.24 of live cells, Kruskal-Wallis p<0.03) plasmablasts (0.06 +/- 0.26 vs 0.00 +/- 0.00, Kruskal-Wallis p<0.055), and memory B cells (0.62 +/- 1.26 vs 0.05 +/- 0.15, Kruskal-Wallis p<0.02). Within the patient groups, plasma cells were the most frequent observed cell type.

**Conclusion:** In summary, our study identified increased frequencies of different B-cell subtypes in CRSwNP patient mucosa but not in peripheral blood. We also found that CRSwNP patients had significantly increased B-cell subtypes than CRSsNP and controls. These results imply a potential role for mucosal B-cells in the ongoing inflammation in CRSwNP patients.
4.2 Introduction

B cells play a fundamental role in the adaptive immune response at mucosal surfaces. Once activated, naïve B cells develop into antibody-secreting plasmablasts, plasma cells or memory B cells. The initial and rapid B cell antibody responses are dominated by plasmablasts located in the peripheral immune organs. They undergo clonal expansion which leads to the generation of large amounts of terminally differentiated short lived antibody producing plasmablast cells. These plasmablast cells are able to differentiate further into long lived plasma cells that continue to produce antibodies long-term and are capable of increased survival and circulation throughout the body. Memory B cells are long surviving cells which, upon secondary encounter with the same antigen the naïve B cell was exposed to, respond at a faster rate and with a more robust antibody response.

Chronic rhinosinusitis (CRS) is an inflammatory disease characterised by a unique inflammatory microenvironment. Recent studies have demonstrated that B cells as well as their chemo-attractants are elevated in the nasal mucosa of CRS with nasal polyposis (CRSwNP) patients. It is documented that CRSwNP patients contain elevated local IgE antibodies as well as autoantibodies in polyps, however, the study of B cell subsets in CRS is yet to be elucidated. Using flow cytometry, in this study, we identified naïve and effector B cell subsets in the mucosa and periphery of CRSwNP, CRS without nasal polyposis (CRSsNP) and non-CRS controls.

4.3 Methods

Patient Sample Collection

This study was approved by the Human Research Ethics Committee of the Queen Elizabeth Hospital, Adelaide, Australia. Representative tissue samples (polyps for
CRSwNP and ethmoid mucosa for CRSsNP and controls) and blood specimens were prospectively collected at the time of endoscopic sinus surgery from control, CRSsNP and CRSwNP. Control patients were undergoing endoscopic sinonasal procedures for pituitary tumour resections and were without clinical or radiological evidence of past or present sinus disease. CRS patients fulfilled the diagnostic criteria set out in the recent position papers by the American Academy of Otolaryngology and Head and Neck Surgery and the European Position Statement on Chronic Rhinosinusitis. Patients with CRS were further sub-classified according to the absence or presence of visible polyps present within the middle meatus on nasal endoscopy. Exclusion criteria included minors < 18 years of age, pregnancy, malignancy, immune disorders and the use of antibiotics or oral corticosteroids in the month preceding surgery. All patients provided informed written consent prior to enrollment. Patients were classified as atopic if they had positive RAST and/or skin prick testing. Demographic and clinical data was collected on all patients prior to the commencement of the study.

**Cell Preparation**

Tissue samples were washed and dissected into pieces ≤ 2mm before being prepared into a single cell suspension by enzymatic digestion with 2 mg/ml collagenase type II (Sigma-Aldrich, MO, USA) and 0.04 mg/ml DNase I (Roche Applied Sciences, Vilvoorde, Belgium) for 45 min at 37°C. Cell suspensions were filtered through a 100 µm nylon mesh and washed in PBS. Heparinized peripheral blood was lysed for 15 minutes using Pharmlyse (Becton Dickinson Biosciences, San Jose, CA, USA).

**Flow Cytometric Immunophenotyping**

Cells were stained with Fixable Viability Dye eFluor® 780 (eBioscience, San Diego, CA, USA) at 4°C for 30 minutes to exclude dead cells before staining with the following antibodies listed in Table 4.1. Eight-colour flow cytometry was performed
using a gating strategy based on fluorescence minus one controls as specified in supplementary Figure 4.1. Naïve B cells were defined as CD19+CD27-IgD+; Plasmablasts were defined as CD19+CD27+IgD-CD38high; Plasma cells were defined as CD19-CD27+CD38+; Memory B cells were defined as CD19+CD27+ IgD-CD38-.

Table 4.1. B cell flow cytometry antibodies

<table>
<thead>
<tr>
<th>Target Antigen</th>
<th>Clone</th>
<th>Conjugation</th>
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<td>IgD</td>
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<tr>
<td>CD19</td>
<td>HIB19</td>
<td>APC</td>
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</table>
Figure 4.1. B cell gating strategy. Live cells were identified (A) and CD19+IgD+ (B) CD27- (C) cells gated to identify CD19+CD27-IgD+ Naïve B cells. CD19+CD27+ (D) cells identified and gated on IgD- CD38high and CD38- (E) to identify CD19+ CD27+IgD-CD38high Plasmablasts and CD19+CD27+ IgD-CD38-. Memory B cells. Plasma cells were gated on CD19- (F) and CD27+CD38+ (G).

Statistical analysis

The data were summarised using means with standard deviations and medians with range. The Kruskal-Wallis test was used for the comparison of data from the three independent disease groups of patients. Mann-Whitney test was used for comparison of two independent disease groups. All tests were two-tailed and significance was assessed at the 5% alpha.
4.4 Results

Patients

Samples from a total 45 patients (16 CRSwNP, 16 CRSsNP and 13 controls) were used for this study. Patient demographic information is summarized in Table 4.2.

Table 4.2. Patient demographics for B cell study

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>CRSsNP</th>
<th>CRSwNP</th>
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<tbody>
<tr>
<td>Number</td>
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<td>16</td>
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<tr>
<td>Median Age (IQR)</td>
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<td>Male/Female</td>
<td>5/8</td>
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B cells are increased in CRSwNP mucosa

CRSwNP patients had significantly more CD19+ B-cells in their sinus mucosal polyps compared to controls (11.9 fold increase, 3.39 +/- 4.05 vs 0.39 +/- 1.05 % of live cells respectively Kruskal-Wallis p<0.01) (Figure 4.2). Further sub-classification of total CD19+ B-cell numbers showed that all B-cell subtypes were significantly increased in CRSwNP patient tissue compared to controls. This included CD19+CD27-IgD+ naïve B cells (13.3 fold increase, 0.61 +/- 0.94 vs 0.11 +/- 0.24 of live cells respectively, Kruskal-Wallis p<0.03) (Figure 4.3A), CD19+CD27+CD38highIgD+ plasmablasts
(1000 fold increase, 0.06 +/- 0.26 vs 0.00 +/- 0.00 respectively, Kruskal-Wallis p<0.055) (Figure 4.3B), CD19+CD27+CD38-IgD- memory B cells (1000 fold increase, 0.62 +/- 1.26 vs 0.05 +/- 0.15 respectively, Kruskal-Wallis p<0.02) (Figure 4.3C) in CRSwNP patients compared to controls. No differences were seen in plasma cell numbers across the patient groups (Figure 4.3D). No differences were seen in CD19+ cell numbers or B cell subtypes in the peripheral blood of the different patient groups (Figure 4.4).

![CD19+ B cells](image)

**Figure 4.2.** Percentage of CD19+ cells out of total live cell numbers in controls, CRSsNP and CRSwNP mucosa. Medians with interquartile range. p≤0.05 Kruskal-Wallis.
Figure 4.3. Percentage of Naïve B cells (A), Plasmablasts (B), Memory B cells (C) and Plasma cells (D) out of total live cell numbers in controls, CRSsNP and CRSwNP mucosa.

Medians with interquartile range. $p \leq 0.05$ Kruskal-Wallis.
Plasma cells are the most abundant B cell type in mucosa and CRSwNP peripheral blood.

In all tissue samples from control, CRSsNP, and CRSwNP patients, plasma cells were the most abundant cell type followed by naïve B cells (Figure 4.5).

In the peripheral blood of control and CRSsNP patients there were more naïve cells than plasmablasts and memory B cells (25.4 and 3.4 fold more naïve cells in control and 21.8 and 8.01 fold more in CRSsNP respectively) (Figure 4.6A-B). In the peripheral blood of CRSwNP patients there were more plasma cells than plasmablasts and memory B-cells. (25.4 and 27.1 fold increase respectively) (Figure 4.6C)
in CRS patients and showed increased numbers of mucosal B cell numbers in CRSwNP patients. Earlier studies using flow cytometry and immunohistochemistry have found increased mucosal B cell numbers in CRSwNP patients. Utilizing flow cytometry and a combination of lineage markers, we further defined B-cell subsets in CRS patients and showed increased numbers of naïve B-cells, plasmablasts and

Figure 4.5. Percentage of Naïve B cells, Plasmablasts, Memory B cells, and Plasma cells out of total live cell numbers in controls (A), CRSsNP (B) and CRSwNP (C) mucosa. Medians with interquartile range. p≤0.05 Kruskal-Wallis.

Figure 4.6. Percentage of Naïve B cells, Plasmablasts, Memory B cells, and Plasma cells out of total live cell numbers in controls (A), CRSsNP (B) and CRSwNP (C) peripheral blood. Medians with interquartile range. p≤0.05 Kruskal-Wallis.

4.5 Discussion

CRS is an inflammatory condition characterised in part by a dysregulated adaptive immune response. Earlier studies using flow cytometry and immunohistochemistry have found increased mucosal B cell numbers in CRSwNP patients. Utilizing flow cytometry and a combination of lineage markers, we further defined B-cell subsets in CRS patients and showed increased numbers of naïve B-cells, plasmablasts and
memory B-cells in CRSwNP tissue but not in peripheral blood. This is in support of studies by Psaltis et al that showed an increase in total B cell numbers as well as different B cell subpopulations in CRSwNP tissue compared to controls. In addition, the B cell cytokine B cell Activating Factor (BAFF), important in B cell IgG class switching, and its receptor Transmembrane Activator And CAML Interactor (TACI, alias TNFRSF13B), required for the survival of activated B cells and plasmablasts in vitro, are found elevated in CRSwNP compared to healthy subjects.

We observed that plasma cells were the most abundant cell type compared to other B-cells in the CRSwNP mucosa, however, although elevated, we didn’t find any statistically significant differences in plasma cell numbers between patient groups. Other studies show that CRSwNP patients harbor an increased number of plasma cells as well as increased concentrations of immunoglobulins IgA, IgG and IgE within the polyps but not in the blood. These discrepancies on the number of plasma cells between the different studies could be due to the different methods used. For example, Hulse et al cultured their biopsies for four days and used CD138+ to characterize plasma cells unlike our use of fresh biopsies and the use of CD19-CD27+CD38+ markers.

Most recently, a study investigating B cells in non-atopic CRSsNP patients found an influx of IgE-expressing plasmablasts present in the mucosa that were virtually absent in control tissue or peripheral blood. Our study also demonstrates that plasmablasts are increased in numbers within CRSsNP and CRSwNP tissue further indicating the presence of an ongoing active immune response in these patients. The accumulation of BAFF as well as the elevation of plasma and plasmablast cells in CRSwNP patients supports the theory of a secondary lymphoid microenvironment, which favours the activation of naïve B cells in polyp patients.
Although effector B cell presence and antibody production has historically thought to be protective, studies have shown that the accumulation of antibodies such as IgA and IgG results in the accumulation and degranulation of eosinophils, one of the main factors associated with polyp formation. Together, our finding of increased B cell subtypes in the tissue but not in the blood of CRSwNP patients supports the hypothesis that there is a local immune microenvironment within the chronically inflamed sinonasal mucosa that contributes to the ongoing inflammation in CRS, and potentially to polyp formation.

4.6 Conclusion

In summary, our study identified increased frequencies of different B-cell subtypes in CRSwNP patient mucosa but not in peripheral blood. In CRSwNP we also found plasma cells were the most abundant effector B cell. These results imply a potential role for B cells in the chronic inflammation in CRSwNP patients.
Chapter 5: Discordant frequencies of tissue-resident and circulating CD180-negative B cells in chronic rhinosinusitis
# Statement of Authorship

**Title of Paper:** Discordant frequencies of tissue-resident and circulating CD180-negative B cells in chronic rhinosinusitis

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**Publication Details**

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**Principal Author**

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<th>Name of Principal Author (Candidate)</th>
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**Contribution to the Paper**

- Collection of samples
- Experiments conducted
- Data analysis and paper writing

**Overall percentage (%)**

- 100%

**Certification:**

This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would compromise its inclusion in this thesis. I am the primary author of this paper.

**Signature**

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**Co-Author Contributions**

By signing the Statement of Authorship, each author certifies that:

1. The candidate’s stated contribution to the publication is accurate (as detailed above);
2. Permission is granted for the candidate to include the publication in the thesis; and
3. The sum of all co-author contributions is equal to 100% less the candidate’s stated contribution.

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<tr>
<th>Name of Co-Author</th>
<th>C. A. Kies, PhD</th>
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**Contribution to the Paper**

- Confocal immunofluorescence experiments detecting B cells and visualising using confocal microscope

**Signature**

*Date: 1 February 2017*

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<th>E. Hauben, PhD</th>
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**Contribution to the Paper**

- Contributed to the writing of the manuscript and to data analysis.

**Signature**

*Date: 5/3/47*

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5.1 Abstract

**Background:** The unconventional Toll Like Receptor (TLR) CD180 is implicated in chronic inflammatory diseases, however, its role in chronic rhinosinusitis (CRS) has yet to be investigated. Here we study the expression of CD180, its homologue TLR4 and Myeloid Differentiation factor MD1 on mucosal and systemic immune cell populations in relation to serum IgG levels.

**Methods:** A total of 70 patients were recruited to the study. Mucosal and peripheral blood samples were prospectively collected from CRS patients and non-CRS controls without evidence of sinus disease. The expression of TLR4, MD1 and CD180 was investigated using qRT-PCR, immunohistochemistry and flow cytometry. Serum IgG levels were determined using ELISA.

**Results:** CRS with nasal polyp (CRSsNP) patients had significantly increased mRNA expression of CD180 and MD1 compared to controls (5.54 and 2.1fold respectively, P<0.01). B-cells lacking CD180 were lower in CRSsNP tissue compared to CRSsNP and controls (21.07 +/- 6.41 vs 41.61 +/- 7.82 vs 40.06 +/- 8.06, P<0.01) but higher in blood (39.18 +/- 8.3 vs 17.95 +/- 7.82 and 12.49 +/- 4.92, P≤0.05).

**Conclusion:** Changes in mucosal and peripheral CD180 expressing B-cells were identified in CRSsNP patients compared to CRSsNP and controls. This suggests a role for these cells in the dysregulated immune response in these patients.
5.2 Introduction

Toll like receptors (TLRs) play a critical role in the initial activation of the innate immune system and in the promotion of adaptive immunity. They represent a group of pattern recognition receptors responsible for sensing highly conserved microbial motifs. Of the 10 human TLRs, TLR4 was the first to be identified. By forming a heterodimer with the secreted protein Myeloid Differentiation factor 2 (MD2), TLR4 is responsible for the recognition of lipopolysaccharide (LPS), a component of the gram-negative bacterial outer membrane. CD180 (alias RP105/LY64) is an orphan member of the TLR family with a role in B-cell activation and is most closely related to TLR4. Containing conserved extracellular leucine-rich repeat (LRR) domains, CD180 acts similarly to TLR4 in its dependency on another molecule for its membrane localisation and signalling, Myeloid Differentiation factor 1 (MD1). Unlike TLR4 and other TLRs, CD180 lacks a cytoplasmic Toll/ interleukin-1 (IL-1) receptor (TIR) domain, which is a key signaling domain unique to the TLR system. Regardless, CD180-ligation initiates a B-cell Receptor (BCR)-like signaling cascade that is independent from TLR signaling adaptors and induces affinity maturation and antibody responses that are partially T cell independent.

CD180 is found most abundantly on immune cells, including B-cells, dendritic cells and macrophages, and, as such, has been implicated mainly in diseases involving dysregulation of the immune system. CD180 ligation leads to internalization of CD180 and potent antigen-specific IgG responses. B cells lacking CD180 surface expression are elevated in the peripheral blood of patients with autoimmune diseases including Sjögren syndrome and dermatomyositis. In patients with SLE, CD180 negative B cells have been associated with disease activity and IgG production.
suggesting that these cells represent pathogenic, and possibly autoreactive, B cell subsets.\textsuperscript{108}

Chronic rhinosinusitis (CRS) is considered an inflammatory disease with a dysregulated immune response. Of the 10 human TLRs, TLR2, TLR3, TLR4 and TLR9 have been shown to be expressed in airway epithelium and may play a contributing role to the development of CRS.\textsuperscript{1} Previous studies have demonstrated that B cells are elevated in the nasal mucosa of CRSwNP patients\textsuperscript{252} however, the expression and localisation of CD180 within the sinonasal mucosa is yet to be identified. Here we sought out to identify whether CD180 was expressed in nasal tissue and investigate the local and systemic populations of immune cells expressing TLR4MD1 and CD180 in relation to IgG levels in serum.

5.3 Methods

Patient Sample Collection

This study was approved by the Human Research Ethics Committee of the Queen Elizabeth Hospital, Adelaide, Australia. Tissue samples and blood specimens were prospectively collected at the time of endoscopic sinus surgery from control, CRS without nasal polyps (CRSsNP) and CRS with nasal polyp patients (CRSwNP). Control patients were undergoing endoscopic sinonasal procedures without clinical or radiological evidence of sinonasal disease. CRS patients were patients who fulfilled the diagnostic criteria set out in the recent position papers by the American Academy of Otolaryngology and Head and Neck Surgery and the European Position Statement on Chronic Rhinosinusitis.\textsuperscript{1,315} Patients with CRS were further sub-classified according to the absence or presence of visible polyps present within the middle meatus on nasal endoscopy. Exclusion criteria included minors < 18 years, pregnancy, malignancy,
immune disorders and the use of antibiotics or oral corticosteroids in the month preceding surgery. All patients provided informed written consent prior to enrollment. Patients were classified as atopic if they had positive RAST and/or skin prick testing. Demographic and clinical data was collected on all patients prior to the commencement of the study.

**Cell Preparation**

Tissue samples were washed and dissected into pieces ≤ 2 mm before being prepared into a single cell suspension by enzymatic digestion with 2 mg/ml collagenase type II (Sigma-Aldrich, MO, USA) and 0.04 mg/ml DNAse I (Roche Applied Sciences, Vilvoorde, Belgium) for 45 min at 37°C. Cell suspensions were filtered through a 100 μm nylon mesh and washed in PBS. Heparinized peripheral blood was lysed for 15 minutes using Pharmlyse (Becton Dickinson Biosciences, San Jose, CA, USA).

**Flow Cytometric Immunophenotyping**

Cells were stained with Fixable Viability Dye eFluor® 780 (eBioscience, San Diego, CA, USA) at 4°C for 30 minutes to exclude dead cells before staining with the following antibodies listed in Table 5.1 as per manufacturer recommendations. Eight-colour flow cytometry was performed using a gating strategy based on fluorescence minus one controls as specified in Figures 5.1 and 5.2 (for B-cells). B-cells were defined CD19+, dendritic cells CD11c+CD14+ and epithelial cells cytokeratin+. Naïve B cells were defined as CD19+CD27-IgD+. Plasmablasts were defined as CD19+CD27+IgD-CD38high. Memory B cells were defined as CD19+CD27+ IgD-CD38-. 
## Table 5.1. B cell and CD180 flow cytometry antibodies

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<td>CD19</td>
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Figure 5.1. **B cell CD180 Gating strategy.** Live cells were identified (A) and CD19+IgD+ (B) CD27- (C) cells gated to identify CD19+CD27-IgD+ Naïve B cells. CD19+CD27+ (D) cells identified and gated on IgD- CD38high and CD38- (E) to identify CD19+ CD27+IgD-CD38high Plasmablasts and CD19+CD27+ IgD-CD38-. Memory B cells. CD180 expression then gated on per cell type (F).

Figure 5.2. Gating strategy for CD180 PE expressing B-cells shown for control (A), CRSsNP (B), and CRSwNP (C) mucosa.
mRNA expression analysis

Nasal mucosa (for control and CRSsNP patients) and polyps (for CRSwNP patients) were collected intra-operatively. The specimens were stored in RNALater at -80°C prior RNA extraction. Total RNA extraction was performed using the RNeasy Lipid Tissue Mini Kit (74106, Qiagen, Hilden, Germany) as per manufacturer’s instruction.

Complementary DNA (cDNA) synthesis was performed using the QuantiTect Reverse Transcription kit (205313, Qiagen, Hilden, Germany) according to the manufacturer’s instructions. TaqMan real-time PCR assays (Thermo Fisher Scientific, Massachusetts, USA) MD-1 (Hs00169454_m1), CD180 (Hs00194403_m1) and TLR4 (Hs00152939_m1) were used. The cDNA was pre-amplified with pooled Taqman gene expression assays for 16 cycles of 95°C for 15 sec and 60°C for 4 min. The pre-amplified cDNA and Taqman gene assays were prepared as per Fluidigm BioMark HD System Protocol (Fluidigm Corporation, CA, USA). Both samples and assays were loaded on to a 48.48 Dynamic Array chip (BMK-M-48.48, Fluidigm Corporation, CA, USA) and primed into the matrix using IFC Controller MX (Fluidigm Corporation, CA, USA). Real-time PCR was conducted using Biomark HD Platform (Fluidigm Corporation, CA, USA) and programmed as follows: 95°C for 1 min and 35 cycles of 96°C for 5 sec and 60°C for 20 sec. Data acquisition and analysis was performed using Fluidigm Real-Time PCR Analysis Software v4.1.2 (Fluidigm Corporation, CA, USA). Delta-cycle threshold (ΔCt) values were calculated in reference to an endogenous control gene, hypoxanthine phosphoribosyltransferase (HPRT) 1. Delta-ΔCt (ΔΔCt) values for CRSsNP and CRSwNP patients were normalised to non-CRS controls. All gene expression data was presented as fold ratio ($2^{-\Delta\Delta Ct}$).
ELISA

Serum IgG levels were determined using a Ready SET Go ELISA kit according to the manufacturer’s instructions (ABCAM, Cambridge, UK). Results were expressed in pg/ml.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue blocks were sectioned in 4µm thickness. Slides were de-paraffinized and serially rehydrated. Antigen retrieval was performed by submerging the slides in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) in a pressure cooker for 10 minutes. After cooling at room temperature (RT), tissue sections were incubated in 3% H$_2$O$_2$ for 10 minutes to block endogenous peroxidases. Sections were then sequentially blocked in 2.5% normal horse serum for 10 minutes, then incubated in a humidified chamber with antibodies targeting CD20 (1:20, ab9475, ABCAM, Cambridge, UK) and CD180 (1:100, ab12627, ABCAM, Cambridge, UK) at 4°C overnight. Slides were incubated with 1:200 diluted secondary anti-rabbit AF488 and anti-mouse CY3 antibodies (Jackson ImmunoResearch Labs Inc., West Grove, PA, USA) for 1 hour at RT in the dark. 200 ng/mL of DAPI (Sigma-Aldrich, MO, USA) was added to resolve nuclei. Slides were mounted and visualized by using a LSM700 confocal laser scanning microscope (Zeiss Microscopy, Germany).

Statistical analysis

The data were summarised using means with standard deviations and medians with range. Pearson's correlation coefficient was used to describe associations between CD180 negative B cells and IgG levels. The Kruskal-Wallis test was used to assess the effect of patient groups on CD180 negative B cells; post hoc comparisons were made using the Mann-Whitney test. One-way ANOVA and Games-Howell post hoc analysis used to analyse gene expression data using IBM SPSS.
Statistics, Version 23.0 (IBM Corp., Armonk, NY, USA). All tests were two-tailed and significance was assessed at the 5% alpha.
5.4 Results

Patients

Samples from a total of 70 patients were used for the flow cytometry study. These included samples from 25 patients (9 CRSwNP, 12 CRSsNP and 4 controls) to determine CD180 expression on B cells, dendritic cells and epithelial cells, and samples from a further 45 patients (16 CRSwNP, 16 CRSsNP and 13 controls) to phenotype CD180-expressing B cells. Patient demographic information is summarized in Tables 5.2 and 5.3. For the gene expression analysis, a total of 45 patients were included (21 CRSwNP patients, 17 CRSsNP patients and 7 controls).

Table 5.2. Patient demographics CD180 expression on B cells, dendritic cells and epithelial cells

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Table 5.3. Patient demographics CD180 on B cells

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**Increased mRNA expression levels of CD180 and MD1 in CRSwNP patients**

We first determined whether there were differences in CD180 expression in the nasal mucosa of our different patient groups. In CRSwNP patients, there was a significant increase in the mRNA expression of CD180 and MD1 in CRSwNP compared to control mucosa (5.54 and 2.1-fold respectively, Kruskal-Wallis p≤0.005). No difference was observed in TLR4 expression. A comparison of the fold-changes between CRSwNP and CRSsNP relative to controls is shown in Figure 5.3.
Figure 5.3. Graph showing the relative mRNA expression of MD1 and CD180 in control, CRSsNP and CRSwNP patient sinonasal mucosa, normalised to an endogenous control gene (HPRT1). Error bars indicate SD.

**Immunofluorescence shows CD180+ and CD180- B cells within CRSwNP tissue**

We next used immunofluorescence analysis to determine the localisation of CD180 expressing cells within the nasal tissue. CD180 expression was detected in immune cells within the submucosa. Co-staining with the B-cell marker CD20 showed that CD180 was expressed in some, but not all B-cells (Figure 5.4).
CRSwNP patients show increased numbers of CD180+CD19+ B-cells and CD180+CD14+CD11c+ dendritic cells.

We next quantified the levels of CD180-expressing cells in nasal mucosa. Flow cytometry showed that CRSwNP patients had significantly more CD180 expressing cells compared to CRSsNP and controls (Figure 5.5A). There were significantly more CD180+CD19+ B-cells in both CRSwNP (2.1 fold increase) and CRSsNP (1.6 fold increase) vs control (Figure 5.5B) and more CD180+CD14+CD11c+ dendritic cells in CRSwNP (2.2 fold increase) compared to CRSsNP patients (Figure 5.5C). In contrast, CD180+cytokeratin+ epithelial cells were less frequent in CRSwNP compared to CRSsNP and controls (Figure 5.5D). Table 5.4 details cell frequencies for controls, CRSsNP and CRSwNP patients for each of these cell types. There were no differences observed in the total, or relative, expression of MD1, and TLR4 in any of the cell types.
Figure 5.5. Percentage of CD180+ cells out of total live cell numbers in controls, CRSSNP and CRSwNP sinonasal mucosa (A). Percentage of CD180+ CD19+ B-cells (B), CD14+CD11c+ dendritic cells (C) cytokeratin+ epithelial cells (D) in controls, CRSSNP and CRSwNP. Medians with interquartile range. $p \leq 0.05$ Kruskal-Wallis.
Table 5.4. Flow cytometry analysis of CD180 expression on CD19+, CD14+CD11c+ and cytokeratin+ cells in in mucosa (Kruskal-Wallis mean +/- SEM)

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<tr>
<td>CD180+ cells (%live cells)</td>
<td>0.98 +/- 0.33</td>
<td>0.76 +/- 0.21</td>
<td>2.42 +/- 0.58</td>
<td>p ≤ 0.01</td>
</tr>
<tr>
<td>CD180+CD19+ (%CD180)</td>
<td>25.43 +/- 3.36</td>
<td>41.94 +/- 4.964</td>
<td>53.36 +/- 5.92</td>
<td>p ≤ 0.05</td>
</tr>
<tr>
<td>CD180+CD14+CD11c+ (%CD180)</td>
<td>5.37 +/- 1.46</td>
<td>5.30 +/- 1.33</td>
<td>12.00 +/- 2.46</td>
<td>p ≤ 0.05</td>
</tr>
<tr>
<td>CD180+cytokeratin+ (%CD180)</td>
<td>12.45 +/- 3.30</td>
<td>7.94 +/- 1.59</td>
<td>3.93 +/- 1.18</td>
<td>p ≤ 0.05</td>
</tr>
</tbody>
</table>

**B cells lacking CD180**

B cells lacking CD180 surface expression were decreased in CRSwNP mucosa compared to CRSsNP and controls (1.9 fold). In contrast, in the peripheral blood, CD180 negative B cells were elevated in CRSwNP patients compared to CRSsNP patients and controls (3.1 fold) (Figure 5.6A-B). Cell numbers for each of these patient groups for CD180 negative cells are shown in Table 5.5. B cells lacking the CD180 receptor were most likely to be naïve B cells in control mucosa and plasmablasts in CRSsNP and in CRSwNP mucosa, although this was only statistically significant for the control and CRSwNP group. (Figure 5.7A-C) Table 5.6 details cell numbers for each of the patient subgroups.

In the peripheral blood there was an even spread of CD80 negative CD19+ cells on the naïve, plasmablast and memory B cells. (Figure 5.7D-F) There was a positive
correlation between peripheral blood B cells lacking the CD180 receptor and serum IgG levels in all groups (control $r = 0.36$, CRSsNP $r = 0.33$ and CRSwNP $r = 0.17$)

Figure 5.6. Percentage of CD180- B cells in mucosa (A) and blood (B) in controls, CRSsNP and CRSwNP. Medians with interquartile range. $p \leq 0.05$ Kruskal-Wallis.
Table 5.5. Flow cytometry analysis of CD180 expression on B cells in mucosa and blood* (Kruskal-Wallis mean +/- SEM)

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>CRSsNP</th>
<th>CRSwNP</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19+ B-cell (%live cells)</td>
<td>0.58 +/- 0.29</td>
<td>0.89 +/- 0.29</td>
<td>3.82 +/- 1.35</td>
<td>≤0.01</td>
</tr>
<tr>
<td>Memory B cells (%live cells)</td>
<td>0.05 +/- 0.04</td>
<td>0.12 +/- 0.09</td>
<td>0.68 +/- 0.39</td>
<td>≤0.05</td>
</tr>
<tr>
<td>Plasmablast cells (%live cells)</td>
<td>0.01 +/- 0.00</td>
<td>0.01 +/- 0.00</td>
<td>0.11 +/- 0.07</td>
<td>≤0.05</td>
</tr>
<tr>
<td>Naïve B cells (%live cells)</td>
<td>0.11 +/- 0.07</td>
<td>0.19 +/- 0.10</td>
<td>0.61 +/- 0.23</td>
<td>≤0.05</td>
</tr>
<tr>
<td>CD180 negative B-cell+ (% B-cell)</td>
<td>40.06 +/- 8.06</td>
<td>41.61 +/- 7.82</td>
<td>21.07 +/- 6.41</td>
<td>≤0.01</td>
</tr>
<tr>
<td>CD180 negative B-cell+ (%B-cell)*</td>
<td>12.49 +/- 4.92</td>
<td>17.95 +/- 7.82</td>
<td>39.18 +/- 8.3</td>
<td>≤0.05</td>
</tr>
</tbody>
</table>
Table 5.6. Flow cytometry analysis of CD180 negative B cell subtypes in mucosa

(Kruskal-Wallis mean +/- SEM)

<table>
<thead>
<tr>
<th></th>
<th>Naïve B cells (%live cells)</th>
<th>Plasmablasts (%live cells)</th>
<th>Memory B cells (%live cells)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>21.35 +/- 7.04</td>
<td>11.10 +/- 11.10</td>
<td>3.64 +/- 2.77</td>
<td>p≤0.05</td>
</tr>
<tr>
<td>CRSsNP</td>
<td>26.26 +/- 6.85</td>
<td>57.93 +/- 15.55</td>
<td>12.32 +/- 3.91</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>CRSwNP</td>
<td>14.09 +/- 3.86</td>
<td>43.94 +/- 11.43</td>
<td>8.62 +/- 2.91</td>
<td>p≤0.05</td>
</tr>
</tbody>
</table>

**Figure 5.7.** Percentage of Naïve B-cells, Plasmablasts and Memory B cells lacking the CD180 receptor in control (A), CRSsNP (B) and CRSwNP (C) mucosa. Percentage of Naïve B-cells, Plasmablasts and Memory B cells in control (D), CRSsNP (E) and CRSwNP (F) blood. Medians with interquartile range. p≤0.05 Kruskal-Wallis.
5.5 Discussion

The innate immune system, through conventional and unconventional TLRs, plays a critical role in the initiation and maintenance of inflammatory diseases. The unconventional TLR CD180 has largely been studied as a B-cell receptor, but it is expressed by a wide variety of cells including monocytes, macrophages, dendritic and epithelial cells. Our study aligns with these findings and confirms that CD180 is expressed mainly on B-cells, but also on dendritic cells and epithelial cells within the sinonasal mucosa. CD180 is critically involved in the host immune response to infection by modulating the activity of TLR2 and TLR4. Consequently, aberrant expression of CD180 has detrimental effects on establishing immune responses particularly to infections with pathogens that are sensed by TLR2 and TLR4. This is exemplified in CD180-/- mice that are shown to be impaired in their ability to control low-grade *Mycobacterium tuberculosis* infections, which are dependent on TLR2 sensing for immune response induction. *Staphylococcus aureus* infections, which are potentially involved in CRS disease recalcitrance, also activate immune cells mainly through TLR2 sensing, and it has been shown that the concerted action of TLR2, TLR4 and CD180 is required to shape the inflammatory response against *S. aureus* infections.

In B-cells, CD180 activation leads to receptor internalisation and signalling resulting in B cell proliferation and activation. Our data indeed shows that CD180 is expressed on all B-cell subtypes and that those subtypes, in particular plasmablasts, are increased in number in CRSwNP tissue. This suggests that there is an ongoing recruitment of naïve B-cells into the tissue in CRS accompanied by an ongoing activation of those cells into plasmablasts in association with elevated CD180 expression in those cells.
Our study also shows that CD19+ B cells lacking CD180 surface expression are decreased in CRSwNP tissue but increased in the systemic circulation compared to CRSsNP and controls. When investigating B cell sub-populations we found that these mucosal CD180 negative B cells were mainly plasmablasts in CRSwNP and CRSsNP tissue, whereas CD180 was uniformly expressed in the different B-cell subtypes in the periphery. Since CD180 activation leads to receptor internalisation and signalling, it could be postulated that the reduced surface expression of CD180 in blood B-cells in CRSwNP patients reflects B-cell activation. A lack of CD180 receptor expression on a significant proportion of circulating B cells has been found in autoimmune syndromes including SLE, dermatomyositis and Sjögren’s syndrome and is positively correlated with severity of disease and levels of autoantibodies in SLE patients. In addition, injection of activating anti-CD180 mAb into mice has been shown to result in intrinsic B-cell proliferation and differentiation, accompanied by rapid increases in IgG levels. Whilst we did not identify differences in total systemic IgG levels between CRS patients and controls as reported by others, our study did find a moderate positive correlation of peripheral CD180 negative B-cell numbers with total IgG amounts in serum. Further research is needed to determine whether CD180 negative blood B cells in CRSwNP reflect disease activity and may account for increased IgG amounts in those patients.

5.6 Conclusion

In summary, our study identified decreased tissue resident CD180 negative B-cells in CRSwNP patients compared to CRSsNP and controls. In contrast, CD180 negative B cells were elevated in CRSwNP blood compared to CRSsNP and controls. These results imply a potential role for CD180 B cells in the antibody-mediated immunity in CRSwNP patients.
Chapter 6: Discussion

CRS is considered a multifactorial inflammatory condition of heterogeneous nature. It is broadly subcategorised into two types based on the absence and presence of nasal polyps visualised in the middle meatus.\textsuperscript{53} Although broad, this definition has been useful in a clinical setting.\textsuperscript{390} In Western countries CRSwNP have been thought to be an eosinophilic driven Th2 disease which is steroid responsive and has hence been able to guide therapy.\textsuperscript{56,391} In the past, clinical subtypes of CRS have been considered including allergic fungal rhinosinusitis and eosinophilic mucus chronic rhinosinusitis.\textsuperscript{392,393} The emergence of studies in non-Western populations reporting a wide spectrum of immunologic disease profiles such as the involvement of both Th1 and Th2 subtypes in CRSwNP, historically believed to be a Th2 type driven disease, further warranted the need to look at CRS subtypes perhaps not only from a clinical perspective but from an immune perspective as well.\textsuperscript{56} We raised the question as to whether or not the proposed classification system was an over simplification and that CRSsNP and CRSwNP in fact only represent two extremes of phenotype along a broader spectrum of immunologically different disease processes. To investigate this, we examined the local and systemic populations of different adaptive and innate immune cells in the tissue and blood of CRSsNP and CRSwNP patients. Furthermore, we examined tissue with different degrees of polypoid changes in the same patient.

Our results showed that the cellular infiltrate between the non-polypoid mucosa of patient categories were shown to be similar in CRSwNP, CRSsNP and control groups. Differences were most apparent within the CRSwNP group. CRSwNP polypoid lesions had an abundance of mast cells and natural killer cells compared to non-polypoid mucosa. Polyps also had an abundance of mast cells and natural killer cells compared to
non-polypoid mucosa and in addition they also had significantly more eosinophils, macrophages and TCR gamma delta T cells compared to non-polypoid mucosa. Our study supports other studies that have found an increase in eosinophils and macrophages when comparing nasal polyp tissue to mucosa in CRSwNP patients. The accumulation of inflammatory cells in polyps is thought to be a part of the disease process and accounts for the persistent inflammation seen in these patients. Interestingly our study showed no difference between the cellular infiltrate of polypoid vs polyp tissue in CRSsNP patients. This finding together with the reports that polyps differ in terms of inflammatory infiltrate to non-polypoid mucosa in CRSwNP patients suggests that care must be taken when sampling tissue from CRSwNP patients. The CRS patient classification based on the standardised European position paper guidelines to group CRSwNP patients and CRSsNP patients classifies the polyp not only from an anatomical perspective but one that is distinct in immune cell abundance as well.

With this knowledge, we next evaluated the presence of adaptive immune cells in CRS by sampling mucosa from controls and CRSsNP patients and polyps from CRSwNP patients. CRSwNP has been characterised by a dysregulation of T cells, including Tregs. Tregs are involved in suppressive functions that lead to a down regulation in Th1 responses and cellular cytotoxicity. Our study found an increased number of Tregs in CRSwNP compared to CRSsNP suggesting that they play a role in the impaired immunity in patients with polyps. Excessive suppression of Th1 and cytotoxic cells results in a defect in the immune defence against pathogens and could contribute to the ongoing inflammation, accounting for the excessive inflammatory state in these polyp patients. Our findings support those previously published studies by Sharma et al. who reported the mucosa of CRSwNP patients have a higher proportion of Tregs compared to CRSsNP patients. Pant et al have also reported an increase in Tregs in
CRS mucosa compared to controls although they did not observe a difference between CRSsNP and CRSwNP subgroups\textsuperscript{319}. Other studies investigating the role of Tregs have however, reported a decrease in CRSwNP compared to CRSsNP and control subjects which is contradictory to our results,\textsuperscript{298,299,316} In these studies they determined Tregs using either Foxp3 mRNA expression or protein levels as measured by Immunohistochemistry (IHC). \textsuperscript{317,322} We defined Tregs using flow cytometry as CD45\textsuperscript{+}CD4\textsuperscript{+}CD25\textsuperscript{+}CD123\textsubscript{low}, which is reportedly a more specific natural Treg population with suppressor capabilities\textsuperscript{323}. Furthermore this is one of the largest studies using a Caucasian cohort, and differences in inflammatory cell accumulation in nasal polyps have been reported to be affected by ethnicity.\textsuperscript{56,298}

We for the first time using flow cytometry examined resident Th17 cells in the mucosa based on cell surface expression of CD45\textsuperscript{+}CD4\textsuperscript{+}CCR6\textsuperscript{+}. We reported that Th17 cells were increased in CRSwNP compared to CRSsNP. This was able to settle some discrepancies found in other studies that reported increases in Th17 in CRSwNP defined by IL-17 mRNA expression \textsuperscript{324}, in CRSwNP \textsuperscript{300} and IL-17 protein,\textsuperscript{318,325-327} and those that found similar Th17 amounts in CRSwNP and CRSsNP.\textsuperscript{328} Even though IL-17 is a key cytokine of Th17 cells, it has recently been shown to be non-specific to Th17 cells, also being produced by gamma delta T cells, lymphoid tissue inducer cells, natural killer cells and macrophages.\textsuperscript{225,329-331} Thus it is not a specific marker for the Th17 cell population and could be the reason for differing reports in Th17 cell abundance in CRS. In our study we did not employ surrogate indicators of Th17 presence but rather defined Th17 cells using the expression of their specific cell surface marker CCR6\textsuperscript{+}. This surface marker is uniquely expressed on Th17 cells.\textsuperscript{328} Th17 cells have been shown to increase the amount of neutrophils as well as eosinophils to areas of inflammation and even account for the inflammatory lesions in ulcerative
This increase in Th17 cells we found may be one of the factors driving polyp formation and hence further studies needed to be undertaken to characterize the intracellular cytokine profiles of these Th17 cells to get a better understanding of their function in CRS.

We demonstrated the IL-17A, IL-17F, IL-21 and IL-22 cytokine production of Th17 cells in CRS. Th17 cells producing IL-17A and IL-17F were increased in CRSwNP patient polyps and peripheral blood compared to CRSsNP and controls. IL-17F, although newly discovered and less studied, is very similar in structure to IL-17A and has a similar role. The accumulation of both cytokines is found in allergic diseases with high neutrophil production. IL-17A is known to cause the induction and activation of neutrophils and neutrophil activating cytokines and in the airways by releasing chemokines that can recruit neutrophils and fungicidal peptides. Most recently, dysregulated IL-17A production was also found to promote neutrophil infiltration resulting in the delay of wound healing and tissue repair in mouse models, and could account for the inflammation and airway remodelling seen in certain CRSwNP patient subtypes.

Few studies have investigated the role of IL-22 in CRS. Ramanthan et al confirmed the presence of the IL-22 receptor IL-21R1 on the surface of nasal epithelial cells and discovered lower receptor quantities in recalcitrant CRSwNP compared to CRSsNP and controls. This was confirmed by another study by Wang et al however, there are conflicting reports about cytokine IL-22 levels, with one study showing no statistical difference in IL-22 levels between patient groups and the other finding IL-22 was significantly higher in CRSsNP mucosa compared to controls. IL-22 is produced by activated T cells as well as innate lymphoid cells and innate immune cells. In our study we show that Th17 derived IL-22 is increased in CRSwNP patients polyps and
Interestingly although IL-22 plays a protective role in mucosal diseases such as inflammatory bowel disease by enhancing barrier integrity of the intestinal tract, in other diseases such as psoriasis it has been eluded to synergise with pro-inflammatory cytokines and induce disease progression.\textsuperscript{361,362}

Our study further added to cytokine research in CRS that reported increased levels of IL-21 mRNA and protein using ELISA in polyp tissues and peripheral blood. We suggest that the increase may in fact be due to increases of IL-21 producing Th17 cells in CRSwNP patients.\textsuperscript{350} IL-21 regulates T and B lymphocyte survival, activation and proliferation and in-vitro IL-21 has been shown to have an effect on polyp B cell differentiation and IgG and IgA production.\textsuperscript{350-352} In our study, CRSwNP patients that were atopic, had remarkably less IL-21 cytokine producing Th17 cells as evidenced by flow cytometry compared to non-atopic CRSwNP. These results are in agreement with studies in allergic rhinitis: IL-21 administered at the time of antigen challenge in ovalbumin-induced mice reduces allergic symptoms and antigen specific IgE levels.\textsuperscript{353}

Further to this, signalling through the IL-21 receptor mediates house dust mite airway hyper-responsiveness by enhancing Th2 cytokine production.\textsuperscript{354} There are discrepancies however, with other in vivo animal studies suggesting that IL-21 doesn’t affect airway remodelling.\textsuperscript{355} Further studies, focusing on airway remodelling in CRS will be needed to elucidate the role of the Th17 cytokine IL-21 in allergic patients. It is evident that cytokines may have opposing effects in diverse tissue microenvironments and further functional studies, such as those using IL-21 to stimulate B cell activation and proliferation are needed in order to see whether the Th-17 derived cytokines we see elevated in CRSwNP patients are in fact playing a protective or pathogenic role in CRS.

Utilizing flow cytometry and a combination of lineage markers, we defined B-cell subsets in CRS patients and showed increased numbers of naïve B-cells, plasmablasts
and memory B-cells in CRSwNP tissue but not in peripheral blood. We defined B cells further based on their expression of toll like receptor CD180. Once ligated with antigen CD180 internalizes and produces potent antigen-specific IgG responses. We showed that CD180 is expressed on all B-cell subtypes and that those subtypes, in particular plasmablasts, are increased in number in CRSwNP tissue. This suggests that there is an ongoing recruitment of naïve B-cells into the tissue in CRS accompanied by an ongoing activation of those cells into plasmablasts in association with elevated CD180 expression in those cells.

Our study identified decreased tissue resident CD180 negative B cells in CRSwNP patients compared to CRSsNP and controls. In contrast, CD180 negative B cells were elevated in CRSwNP blood compared to CRSsNP and controls. A lack of CD180 receptor expression on a significant proportion of circulating B cells has been found in autoimmune syndromes including SLE, dermatomyositis and Sjögren’s syndrome and is positively correlated with severity of disease and levels of autoantibodies in SLE patients. In addition, injection of activating anti-CD180 mAb into mice has been shown to result in intrinsic B-cell proliferation and differentiation, accompanied by rapid increases in IgG levels. Whilst we did not identify differences in total systemic IgG levels between CRS patients and controls as reported by others, our study did find a moderate positive correlation of peripheral CD180 negative B-cell numbers with total IgG amounts in serum. Further research is needed to determine whether CD180 negative blood B cells in CRSwNP reflect disease activity and may account for increased IgG amounts in those patients.
Chapter 7: Conclusion

In conclusion, this PhD thesis has provided novel insights into the role of the immune system in CRS. Firstly, we have identified differences within the mucosal and polypoid immune microenvironment of a polyp patient and have determined that the polyp is the hub of cellular activity that should be sampled when studying CRSwNP patients. We have also identified increases in B cells, Tregs as well as Th17 cells and their cytokines in CRSwNP patients, implying that these cells may be implicated in polyp development. We hope that continued research in this area will ultimately result in a better understanding of the complex endotypes of the disease and direct investigations into a targeted therapeutic approach.
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