INVESTIGATION OF TNFR2 EXPRESSING T REGULATORY CELLS AND INFLAMMATORY MEDIATORS IN CHRONIC RHINOSINUSITIS WITH NASAL POLYPS' PATIENTS ATTENDING HOSPITAL UNIVERSITI SAINS MALAYSIA

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UNIVERSITI SAINS MALAYSIA

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by

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LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell.
CD-	Cluster of Differentiation, e.g.: CD25, CD127.
CD4	Co-receptor molecule involved in MHC-II adhesion, found in T helper.
CD8	Co-receptor molecule involved in MHC-I adhesion, found in cytotoxic T cell.
CD25	The alpha chain of the IL-2 receptor expressed on activated T cell.
CD127	IL-7 receptor alpha chain, found in human Tregs.
CTLA-4	Cytotoxic T-lymphocyte Associated protein 4.
DC	Dendritic Cell.
CCL	Chemokine (C-C motif) Ligand.
CRS	Chronic Rhinosinusitis.
CRSwNP	Chronic Rhinosinusitis with Nasal Polyps.
CT	Computed Tomography.
FBC/DC	Full Blood Count with White Blood Cells Differential.
FCS	Flow Cytometry Standard.
FMO	Fluorescence Minus One.
FDA	Food Drug Administration.
Foxp3	Forkhead box P3.
GITR	Glucocorticoid-Induced TNF receptor-Related protein
ECRSwNP	Eosinophilic type of CRSwNP.
EDTA	Ethylenediaminetetraacetic acid, anti-coagulant for blood tube.
ELISA	Enzyme Linked Immunosorbent Assay.
EPOS	European Position Paper on Rhinosinusitis and Nasal Polyps.

HPE	Histopathological Examination.
HPF	High Power of Field.
IL	Interleukin.
ILC2s	Group 2 Innate Lymphoid Cells.
IFN	Interferon.
IgE	Immunoglobulin E.
INCS	Intranasal Corticosteroid.
Ki67	Nuclear protein associated with cellular proliferation.
mAb	Monoclonal Antibody.
LK	Lund Kennedy.
LM	Lund Mackay.
MHC-II	Major Histocompatibility Complex Class II.
MFI	Median Fluorescence Intensity.
NECRSwNP	Non-eosinophilic type of CRSwNP.
NF-κB	Nuclear factor-κβ.
NP	Nasal Polyp.
OX40	A member of the tumor necrosis factor receptor family.
PBMC	Peripheral Blood Mononuclear Cell.
PBS	Phosphate-buffered Saline.
PMT	Photomultiplier Tube, detectors that commonly in flow cytometry used to detect fluorescence in sample.
QoL	Quality of Life.
SNOT-22	Sino-nasal Outcome Test-22
T cell	T lymphocytes, a nucleated white blood cell made in the thymus.
T convs	T conventional cells.

T effs	T effector cells.
TGF-β	Tumour Growth Factor-β
Th	T helper cell (e.g.: Th1 and Th2).
Th1	T lymphocytes making cytokines to help inflammation and anti-viral responses.
Th2	T lymphocytes making cytokines to help antibody.
Th17	T lymphocytes making predominantly IL-17.
Tregs	T regulatory cells.
TNFR1	Tumour Necrosis Factor Receptor Type 1.
TNFR2	Tumour Necrosis Factor Receptor Type 2.
TNF-α	Tumour Necrosis Factor.
TSDR	Tregs-Specific Demethylated Region

LIST OF APPENDICES

SIASATAN SEL T REGULATORI YANG MENGEKPRESI TNFR2 DAN MEDIATOR RADANG DALAM PESAKIT KRONIK RHINOSINUSITIS DENGAN POLIPS HIDUNG YANG MENGHADIRI HOSPITAL UNIVERSITI SAINS MALAYSIA

ABSTRAK

Penyakit Kronik Rhinosinusitis dengan Polip Hidung (CRSwNP) ialah penyakit keradangan kronik mukosa sinus paranasal yang kebanyakannya dikaitkan dengan keradangan jenis-2. Patofisiologi CRSwNP dipercayai berpunca daripada ketidakseimbangan imun keradangan kronik dengan sel pengawalseliaan berfungsi yang tidak mencukupi untuk mengekalkan keseimbangan imunologi. Sel T Kawal Selia (Tregs) ialah pengawal selia kritikal toleransi imun. Treg yang dijumpai reseptor faktor nekrosis tumor 2 (TNFR2) baru-baru ini dicirikan sebagai populasi Treg yang paling kuat sekatan. Kajian ini bertujuan untuk menentukan bahagian subset $TNFR2^+$ Tregs dalam Sel Mononuklear Darah Periferal (PBMC) pesakit CRSwNP berbanding kawalan sihat. Di samping itu, tahap pengeluaram sitokin; IL-4, IL-10 dan TNF telah diakses untuk menyokong penemuan pada sel Treg $TNFR2^+$. Dua puluh lima pesakit CRSwNP (n = 25) dan dua puluh lima kawalan sihat (n = 25) telah direkrut dalam kajian ini. Sitometer aliran lima warna digunakan untuk menentukan perkadaran ungkapan subset Tregs. PBMC telah diasingkan daripada darah periferi menggunakan sentrifugasi kecerunan Lymphoprep dan diwarnai dengan antibodi berlabel florophore. Subset Tregs dikenal pasti menggunakan penanda CD4, CD25, CD127, TNFR2 dan Foxp3, dalam satu koktel antibodi. Sitokin daripada serum diukur menggunakan immunoassay berasaskan sitometrik LegendPlex ®. Keputusan menunjukkan pesakit CRSwNP

mempamerkan peningkatan ketara bagi bahagian TNFR2 dalam kedua-dua fenotip sel T $CD4^{+}Foxp3^{+}$ ($p < 0.05$) dan $CD4^{+}CD25^{+}CD127^{-}Foxp3^{+}$ T sel ($p < 0.05$) berbanding individu yang sihat. Begitu juga, TNFR2 juga menunjukkan ekspresi yang jauh lebih tinggi dalam Tregs berbanding Tconvs dalam kedua-dua pesakit dan individu yang sihat, menunjukkan TNFR2 lebih banyak dihasilkan pada Tregs berbanding Tconvs, $p < 0.001$. Di samping itu, peningkatan ketara tahap TNF juga diperhatikan dalam pesakit CRSwNP berbanding individu yang sihat, ($p < 0.001$) yang mencadangkan untuk mempunyai kesan imunosupresif dalam keradangan yang berpanjangan, melalui laluan timbal balik TNF-TNFR2 Tregs. Walaubagaimanapun, tiada perkaitan ditemui di antara kriteria klinikal pesakit CRSwNP dengan sel kumpulan-kumpulan $TNFR2^{+}$ Tregs. Ringkasnya, kami mencadangkan pengawalseliaan $TNFR2^{+}$ Tregs sebagai subset pengawal yang paling kuat dan tahap tinggi TNF, terdedah untuk menunjukkan sekatan imun yang berfungsi untuk mewujudkan toleransi imun dalam pesakit CRSwNP yang dirawat.

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ABSTRACT

Chronic Rhinosinusitis with Nasal Polyps (CRSwNP) is an inflammatory disease of the paranasal sinus mucosa predominantly driven by chronic type 2 inflammation. CRSwNP pathophysiology is believed to result from immune dysregulation with insufficient regulatory cells to sustain immunological homeostasis. Regulatory T cells (Tregs) are critical regulators of immune tolerance. Tregs expressing tumor necrosis factor receptor 2 (TNFR2) have been recently characterized as the most potently suppressive Tregs population. This study aimed to determine the proportion of $TNFR2^+$ Tregs subsets in Peripheral Blood Mononuclear Cells (PBMC) of the CRSwNP patients compared to healthy controls. The secretion levels of cytokines; IL-4, IL-10 and TNF were accessed to support the findings on $TNFR2^+$ Treg cells. Twenty-five CRSwNP patients (n = 25) and twenty-five healthy controls (n = 25) were recruited in this study. A five-color flow cytometer was used to determine the proportion of the expression of Tregs subsets. PBMC was isolated from peripheral blood using Lymphoprep gradient centrifugation and stained with fluorophore-labelled antibodies. Tregs subsets were identified using markers of CD4, CD25, CD127, TNFR2 and Foxp3, in one antibody cocktail. The cytokines from the serum were measured using Legend Plex ® cytometric-based immunoassays. The result showed CRSwNP patients exhibited a significant upregulation of TNFR2 proportion in both phenotype of

$CD4^+Foxp3^+$ T cells ($p < 0.05$) and $CD4^+CD25^+CD127^-Foxp3^+$ T cells compared to healthy individuals, ($p<0.05$). Similarly, TNFR2 also showed a significantly higher expression in Tregs than Tconvs in both patients and healthy individuals, indicating TNFR2 was preferentially expressed on Tregs over Tconvs, $p < 0.001$. In addition, significant increase of TNF level also observed in CRSwNP patients compared to healthy individuals, ($p < 0.001$). However, no correlation was found between the clinicocharacteristics of CRSwNP patients and $TNFR2^+$ Tregs subsets. In conclusion, this study suggested the up regulation of $TNFR2^+$ Tregs as the most potent suppressive subset and high level of TNF, prone to indicate a functioning immune suppression to establish immune tolerance in treated CRSwNP patients, partly via TNF-TNFR2 axis.

CHAPTER 1

INTRODUCTION

1.1 Background of Research

The sinonasal tract is continuously exposed to the various environmental stimuli such as foreign particulates, inhaled allergens and other airborne irritants which leading to robust immune response e.g., immune activation or suppression. Normal consequence of an acute inflammatory process is complete resolution which encountered and typically cleared with minimal tissue reaction. However, a chronic inflammatory infiltrate in the nasal mucosa is feasible, creating an exaggerated inflammatory response with failed and deficient resolution resulting in the symptoms, physical features, and radiographic changes associated with pathologic airway diseases. Hence, the sinonasal tract must sustain a good immune tolerance to keep airway homeostasis and avoid immunopathology such as Chronic Rhinosinusitis with Nasal Polyps (CRSwNP).

CRSwNP is an inflammatory disorder of the nasal mucosa and paranasal sinuses that is accompanied by the formation of nasal polyps (NP). The disease is characterized by clinical symptoms of nasal blockage, anterior or posterior rhinorrhea, facial pain and hyposmia that persists more than 12 weeks. It affects 1- 4% of the world population (Chen et al., 2020), and has a profound impact on patient's quality of life (QoL) (Kazi et al., 2021, Mullol et al., 2022) . CRSwNP has a prominent type 2 mediated inflammation, in Western population and growing clinical cases in Asia (Zhang et al., 2017, Laidlaw et al., 2021). This endotype tends to be severe and recurrent

in half of CRSwNP patients, particularly in those having type 2 inflammation and hypereosinophilia (Fokkens et al., 2019).

The adaptive immunity is controlled by the diametrical activities of conventional T-cells (Tconvs) and regulatory T-cells (Tregs). $CD4^+$ Tconvs modulate adaptive immune response, meanwhile Tregs repress the response to protect body from inflammatory diseases and autoimmunity (Mensink et al., 2022). Their diametrical activities of Tconvs and Tregs depend on the extent of the immune response and their environment, with coordinated role for cytokine and costimulatory receptors (Mensink et al., 2022). In context of inflammation, it is imperative to maintain immune homeostasis. Otherwise, alteration in this delicate balance of immune homeostasis can lead to pathological condition, especially when it cannot be resolved and cause chronicity.

Tregs, a discrete population of immunosuppressive $CD4^+$ T cells can suppress other immune cells, hence become crucial mediator of peripheral self-tolerance (Sakaguchi et al., 2008). Functional Tregs eliminate autoreactive T-cells, induce self-tolerance and hindering the inflammatory processes to maintain normal state of self-tolerance (Grover et al., 2021). In CRSwNP, the protective adaptive pathways reported to be titled, usually with an excessive and persistent inflammatory response (Tan et al., 2017). In support of this, the numbers of regulatory T cells (Tregs) are generally reduced in CRSwNP, and this has been interpreted as declining in Treg activity causing chronicity (Palmer et al., 2017). For Tconvs population, however, flow cytometric analysis revealed elevated numbers have been associated with the disease (Bruaene et al., 2009, Derycke et al., 2014).

Recently, tumour necrosis factor receptor 2 (TNFR2) was found to play a decisive role in the activation, expansion, survival, and phenotypic stability of Tregs

(Chen & Plebanski, 2019, Lubrano di Ricco et al., 2020, He et al., 2022). Furthermore, TNFR2 expression identified the most suppressive subset of Tregs in mouse and humans (Chen et al., 2008, Chen et al., 2010). Tregs generally identified as the immunosuppressive population expressing high level of CD25, $CD127^{low}$ compared to human Teffs. Tregs lineage is regulated by unique transcription marker of Forkhead box p3 (Foxp3) which dictate the development and function of Tregs. Several studies have been reported on Foxp3 expression are highly confined to TNFR2 marker on Tregs, indicating that TNFR2 as the superior marker than CD25, CD127 in Tregs phenotype (Zhang et al., 2018, Ye et al., 2020).

Promoting TNFR2 activity on Tregs has been promising approach which affirmed by numerous studies in immune-related disease such as autoimmune disease and allergy. In contrast, TNFR2 inhibition has been established as one of the strategies in cancer therapy coinciding with high expression of TNFR2 observed on certain cancers and on tumour associated Tregs (Yang et al., 2018, Medler et al., 2022, Li et al., 2022). While TNFR2 has been actively explored in their role on agonist and antagonist in the existing disease, which potentially opening new treatment path, the role of TNFR2 in CRSwNP, particularly Tregs activity, however, has been understudied.

Multicolour flow cytometry enables an extensive and detailed characterization of lymphocytes subsets. Conducting immunophenotyping by flow cytometry is believed to provide clearer understanding the state of immune tolerance in CRSwNP. Therefore, by investigating the proportion of TNFR2 expressing Tregs subset in CRSwNP patients and its association with clinical characteristics, the finding of immunophenotyping study potentially provide future direction in CRSwNP treatment by targeting immunomodulatory mechanism for the induction of immune tolerance.

1.2 Rational of the study

Subtle balance of immune homeostasis is modulated by the interaction between cytokines and their cell surface signalling receptors. Tregs as abovementioned generally identified with the phenotype $CD4^{+}CD25^{+}CD127^{low}$ for activated human Tregs. The combination of CD4, CD25 and CD127 was increasingly used to isolate Tregs in adoptive immunotherapy and functional studies. The definitive marker for human Tregs has become an issue a Tregs population revealed heterogeneity with multiple cell subset and has plastic phenotype depending on the nature of microenvironment (Qiu et al., 2020). Unremitting expression of Foxp3 in Tregs is vital for their stability, metabolism, and suppressive function. In inflammatory microenvironment, some Tregs can produce pro-inflammatory cytokines such as IL-17 and IFN-γ and earn an aberrant effector-like phenotype (plasticity) or even diminish Foxp3 expression (Piconese et al., 2021). The maintenance of Tregs stability is paramount for proper Tregs function to govern the immune balance.

Recently, it has been explicated that TNFR2 signalling on Tregs is critical to maintain Tregs activation, expansion, and phenotypic stability. Compelling evidence demonstrated TNFR2 co stimulation receptor on Tregs identified as the most potently suppressive Tregs population (Chen et al., 2010, Chen et al., 2012, Chen et al., 2021,). CRSwNP has been reported as disease with decreased and impaired Tregs functions that resulted to the dysregulation of immune tolerance (Van Bruaene et al., 2008, Palmer et al., 2017). Few studies also reported on the elevated number of Tregs in NP and peripheral blood of CRSwNP, hence indicated on inconsistency of Tregs status in CRSwNP. Besides, as the endotype and immune profile of CRSwNP varied across ethnicity, the study on Tregs population in CRSwNP across Malaysian population remained sparse.

Hence, this study was conducted to investigate on TNFR2^{+} Tregs as the maximally suppressive subset in exploring tolerance status in CRSwNP. Tregs phenotyping on TNFR2 expression together with cytokines assessment are crucial for the basic understanding of CRSwNP disease complexity. Figure 1 showed a hypothetical model for Tregs-mediated immune suppression in CRSwNP investigated in the study.

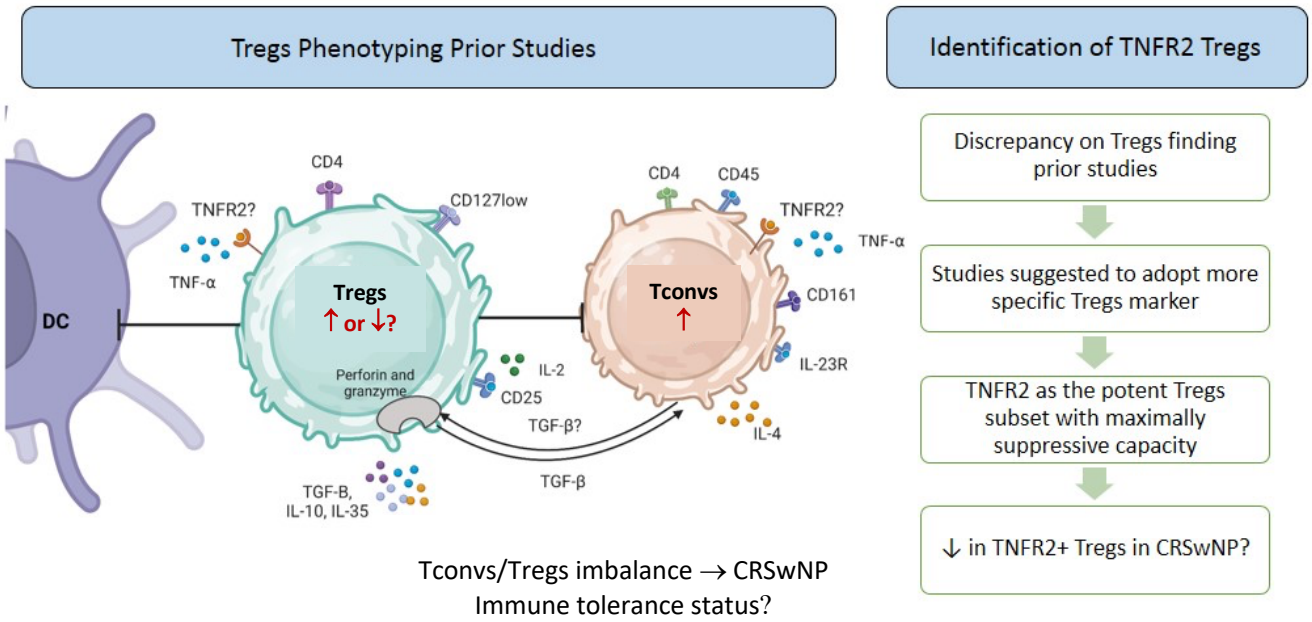


Figure 1.1 Hypothetical model for Tregs-mediated immune suppression is shown. The phenotype of prior studies is defined by the surface marker identification of Tconvs and Tregs respectively (Li et al., 2008, Sharma et al., 2010, Sharma et al., 2012, Rai et al., 2020). Prior exposure to antigens, sinonasal homing-dendritic cells will capture the allergen, migrate to the lymph node, and undergo maturation. Tregs modulate immune system using their various surface receptors (CD4, CD25, $CD127^{low}$) and Foxp3 transcription factor through the inhibition of dendritic cell (DC) function and maturation, via the secretion of anti-inflammatory cytokines such as IL-10, IL-35, and/or through direct inhibition of Teffs (Sakaguchi et al., 2020). Moreover, Tregs and Tconvs share reciprocal pathway due to their plasticity in inflammatory environment depending on cytokine milieu. The exact mechanism of TNFR2 axis in Tregs as the maximally suppressive and stable phenotype in CRSwNP context, however yet to be investigate.

1.3 Research Questions

1) Do the demographic and clinical characteristics of CRSwNP patients and healthy controls are different?
2) Do the proportion of peripheral TNFR2^{+} Tregs and TNFR2^{+} Teffs in CRSwNP patients are different than those in healthy individuals?
3) Do the level of cytokines IL-4, IL-10, TNF-α in the serum of CRSwNP patients are different than those in the healthy individuals?
4) What are the associations of TNFR2 cell subsets with the clinicopathological characteristics in CRSwNP patients?

1.4 Research Objectives

1.4.1 General Objectives

To determine the proportion of Tregs subsets in PBMC of CRSwNP patients and healthy controls.

1.4.2 Specific objectives

1) To determine the demographic and clinical characteristics of CRSwNP patients and healthy controls
2) To evaluate the expression of TNFR2 on Tregs and Teffs in CRSwNP patients and healthy individuals
3) To evaluate cytokine secretion of IL-4, IL-10 and TNF-α in serum samples taken from CRSwNP patients and healthy individuals.

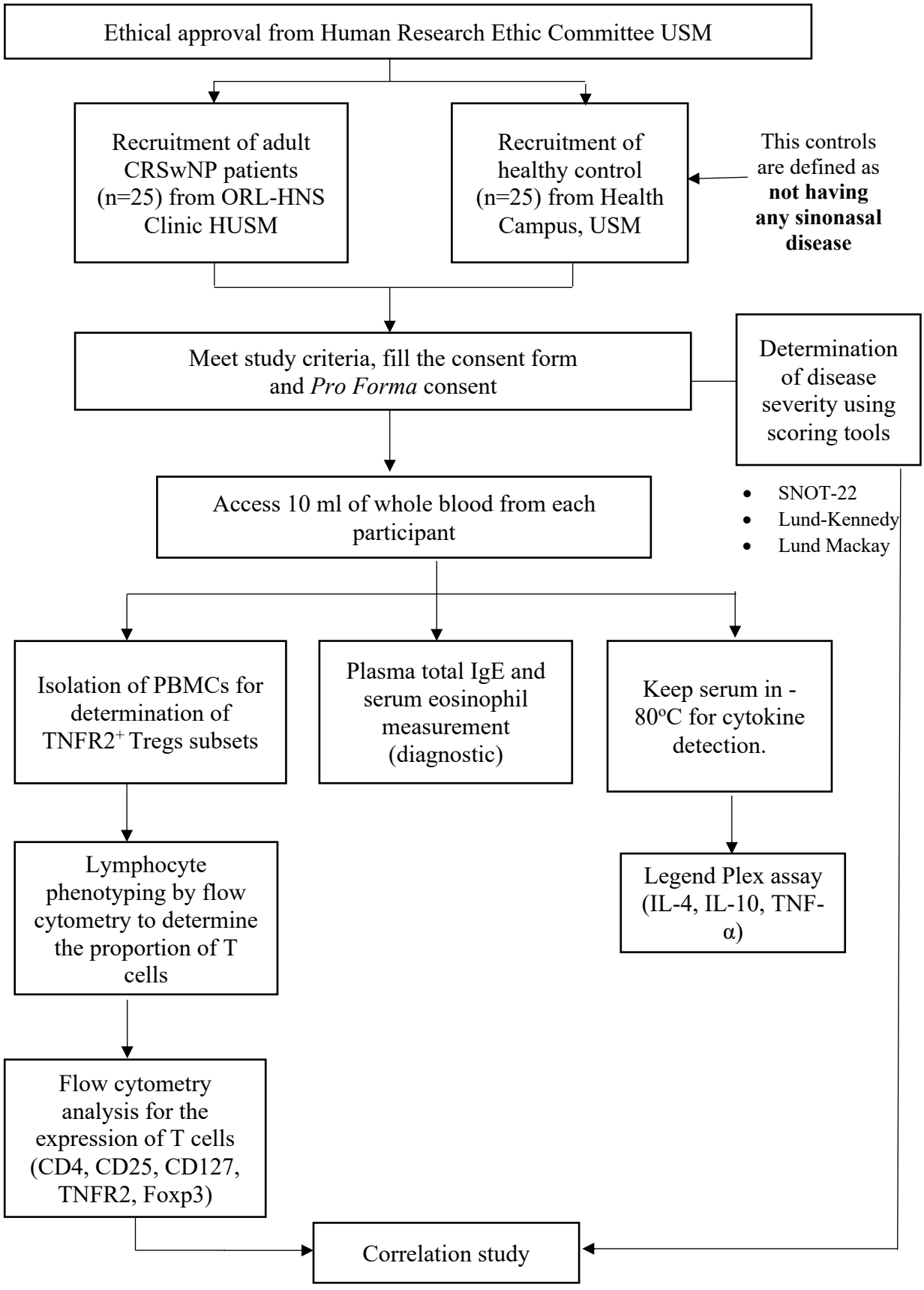
4) To determine the relationship between the expression of TNFR2^{+} cell subsets and the clinicopathological characteristics of CRSwNP patients

1.5 Research Hypothesis

1. There is difference in the demographic and clinical characteristics between CRSwNP patients and healthy controls.
2. There is difference in the proportion of TNFR2 expression Tregs subsets in CRSwNP patients and healthy controls. We expected lower proportion of Tregs-expressing TNFR2 in CRSwNP patients compared to healthy control due to the lower Tregs activity which deficient to induce immune tolerance and balancing homeostasis condition.
3. There is difference in the cytokine levels of CRSwNP patients and healthy individuals. We expected lower expression of inhibitory IL-10 expression and elevated level of IL-4 and TNF-α in CRSwNP.
4. There is a positive relationship between the proportion of TNFR2 cell subsets with the clinicopathological characteristics of CRSwNP patients.

1.6 Flow chart of the Study

The flowchart of the overall study is depicted as below:



CHAPTER 2

LITERATURE REVIEW

2.1 Chronic Rhinosinusitis with Nasal Polyps

Nasal polyps (NP) are the most prevalent mass lesion of nose and was first reported 4000 years ago at the time of ancient Egypt (El Banhawy et al., 2016). Hundred years later Hippocrates described the term “polyps”, as it derived from the Greek (pôlupos), which means by many feet (like an octopus) (Mudry, 2020). He also introduced the first surgical techniques for the removal such as cauterization, removal by pulling a sponge ball and snare in his book of Disease II (Mudry, 2020). NP are known as an outgrowth of inflammatory masses that have grown into the middle meatus, causing nasal obstruction (Toro & Portela, 2023). The aetiology is complex, nonetheless generally it is associated with the consequence of chronic nasal inflammation (Chalermwatanachai et al., 2018, Mudry, 2020).

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a chronic inflammatory disease of the sinonasal mucosa accompanied by polyp formation (Fokkens et al., 2020). The disease affects 1 – 4% of the world population (Chen et al., 2020) and is characterized by nasal blockage, rhinorrhoea, facial pain, and hyposmia that persist longer than 12 weeks (Epperson et al., 2020). Apart from physical symptoms, patients also exhibit psychological symptoms, particularly depression and anxiety, impairing the patient’s ability to work (Chung et al., 2015, Fokkens et al., 2020). A recent meta-analysis reveals that apart from rhinology symptoms, olfactory dysfunction and psychological symptoms also have the most impact on quality of life (QoL) whereas sleep disorder is rarely exacerbated by the disease (Mullol et al., 2022).

CRSwNP appears on condition of middle age with the average age of onset being 42 years, often diagnosed ranging from 40–60 years (Stevens et al., 2016). The disease tends to be refractory compared to another phenotype of chronic rhinosinusitis (CRS) despite of medical and surgical therapy given. Patients may have recurrences of their sinus disease (Fokkens et al., 2020) with recurrence rate of 55% – 66% reported after surgery (Lou et al., 2015, Riva et al., 2022). Besides, patients with nasal polyposis often have other comorbidities such as allergy, asthma, and aspirin hypersensitivity, sinobronchial syndrome or cystic fibrosis (Pawankar & Nonaka, 2007, Stevens et al., 2016, Chen et al., 2020).

2.1.1 Epidemiology of CRSwNP

The global prevalence is estimated to affect relatively 1 – 4 % of the general population, and 25 – 30% among CRS patients (De Conde et al., 2016, Zhang et al., 2017, Chen et al., 2020). Epidemiologic studies have revealed on wide variations of CRS across geographical regions, with region in Europe and USA up to 10.9% and 12% respectively, whereas region in Korea reached 6.9% of the population, consisted with 2.5% of CRSwNP cases from National Health Survey. The prevalence of CRS in different regions worldwide is shown in Figure 2.1 and the prevalence of CRSwNP in different studies in Asia and Europe population is described in Figure 2.2.

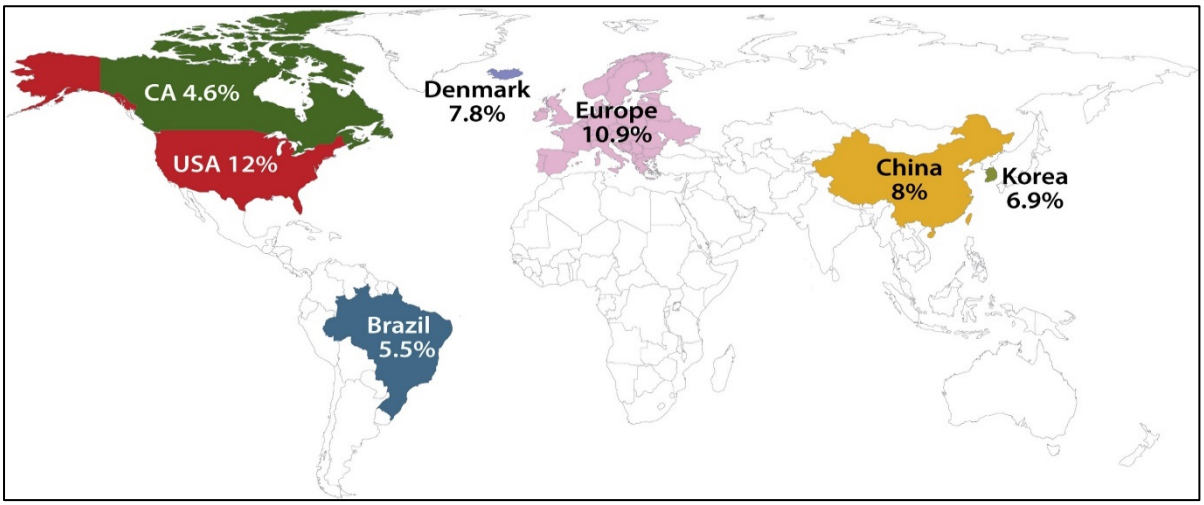


Figure 2.1 Prevalence of CRS in different regions of the world (adapted from (Bachert et al., 2015)

TABLE I. Studies documenting variation in prevalence of CRSsNP and CRSwNP between Asian and European countries

Asia/Europe	Publication year	Region/country	Population characteristics	Study design/method for assessing prevalence	Prevalence of CRS or CRSwNP (%)	Reference
Asia	2016	Eighteen major cities in mainland China	36,577 respondents	Telephone interview	2.1% CRS	12
	2015	Seven Chinese cities	10,636 respondents	Face-to-face interview	8% CRS; 1.1% CRSwNP	13
	2011	KNHANES	4,098 participants	Interview regarding nasal symptoms and endoscopic examination	6.95% CRS	14
	2016	KNHANES (2008-2012)	28,912 adults (aged ≥20 y)	Interview regarding nasal symptoms and endoscopic examination	8.4% CRS; 2.6% CRSwNP	15
	2015	KNHANES (2009-2011)	19,152 participants (aged ≥ 20 y)	Interview regarding physical examinations and endoscopic examination	2.5% CRSwNP	16
Europe	2011	Nineteen centers in 12 countries in Europe	57,128 adults aged 15-75 y	Postal questionnaire	10.9% CRS	1
	2005	France	10,033 subjects (aged ≥18 y)	A kind of validated questionnaire/ algorithm (90% specificity and sensitivity)	2.1% CRSwNP	9
	1999	Southern Finland	4,300 adults aged 18-65 y	Postal questionnaire	4.4% CRSwNP	10
	2003	Skövde, Sweden	1,387 subjects (aged >20 y)	Interview regarding questions and endoscopic examination	2.7% CRSwNP	17

KNHANES, Korean National Health and Nutrition Examination Survey

Figure 2.2 Studies documenting variation in prevalence of CRSsNP and CRSwNP between Asian and European countries. Adapted from (De Conde et al., 2016, Yuan Zhang et al., 2017, Chen et al., 2020)

2.1.2 Aetiology of CRSwNP

CRSwNP has a complex aetiology, which can be summarized with dysregulated interaction between body (comprising genetic and immunological factors) and environment (including microbiome) (Figure 2.3), leading to aberrant and persistent upper airway inflammation (Laidlaw et al., 2020, Xu et al., 2020). The establishment of CRSwNP according to specific endotypes is essential to resolve these delicate myriads of mechanisms underlying the disease process, hence improving treatment management and strategy. Figure 2.3 illustrates on the summary of the complex interaction of the contributing factors to the development of CRSwNP.

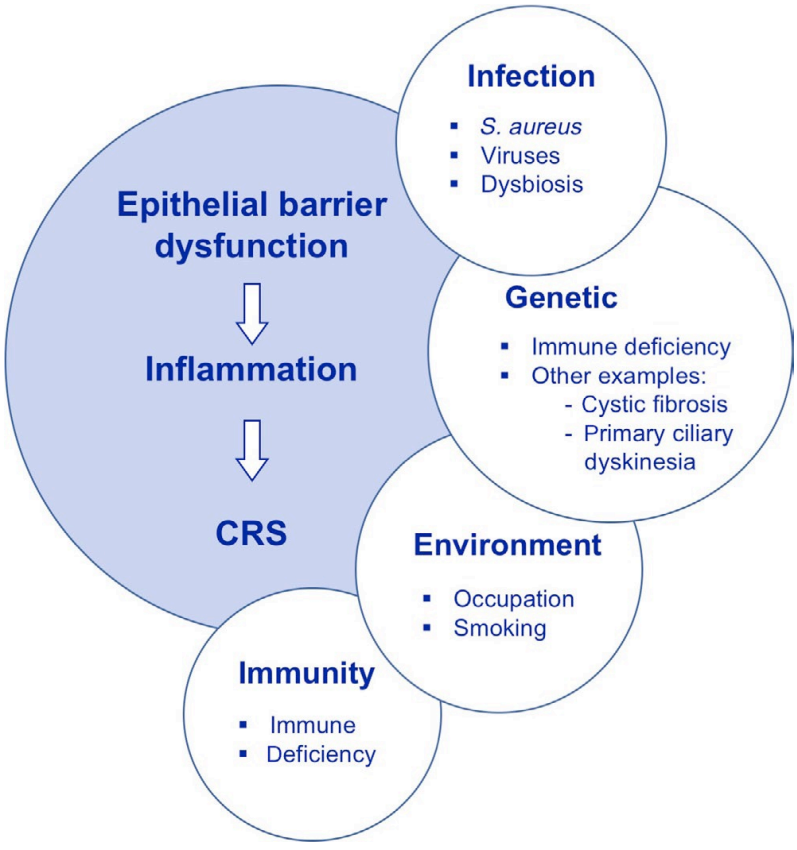


Figure 2.3 The outcome of complex interaction between immune, genetic, and environmental factors contributing to CRS process, adapted from (Xu et al., 2020).

2.1.3 Endotype and Disease Severity

Coinciding with the latest edition of European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS 2020), a new classification of CRSwNP has been emerged, which is useful for the improvement of treatment strategy (Fokkens et al., 2020). The disease is classified coarsely into the two groups of nasal polyposis: localized and diffused. Localized NP is linked with serious concern on sinonasal malignancy notably in elderly patients, thus further investigations should be comprehended to strategize appropriate treatment plan. Meanwhile diffused NP is often associated with CRSwNP with two distinct endotypes of type 2 inflammation and non-type 2 inflammation (Fokkens et al., 2020).

Type 2 inflammation demonstrates with high level of Th2 cytokines, such as IL-4, IL-5, and IL-13, and infiltrating eosinophils, meanwhile non-type 2 inflammation is related to Th1/Th17 immune responses characterized by cytokine IL-17 and IL-8 as well as excess neutrophilic inflammation (Figure 2.4) (Wang et al., 2016, Schleimer, 2017). Tissue eosinophilia in CRSwNP patients have been strongly correlates with type 2 inflammation which demonstrated severe symptoms, high recurrent rate, more recalcitrant towards therapy compared to non-type 2 inflammation (Schleimer, 2017, Ahern & Cervin, 2019). In addition, compelling evidence also portray olfactory dysfunction, a prominent CRS symptom is significantly affected by type 2 inflammatory endotype (Rombaux et al., 2016, Kohli et al., 2017). A recent multi-centric study revealed that 86.1% of type 2 CRSwNP patients were greatly affected by the dysfunction of olfactory function meanwhile only small percentage of non-type 2 patients involve with this impairment (Macchi et al., 2023).

2.1.4 Diagnosis of CRSwNP

The disease can be diagnosed by the evidence of both subjective and objective assessment of sinonasal inflammation. By clinical definition, the patient must report the presence of anterior or posterior rhinorrhoea, nasal congestion, nasal obstruction, hyposmia and/or facial pain which lasting for more than 12 weeks duration (Fokkens et al., 2020). In addition to subjective (symptom) assessment, the nasal endoscopy and paranasal computed tomography (CT) scan must be conducted to confirm the disease.

According to EPOS 2020, CRSwNP patient must show the endoscopic sign of nasal polyps and/or mucopurulent discharge primarily from middle meatus and/or oedema. Meanwhile for CT finding, there must be the indication of mucosal changes within the ostiomeatal complex and/or sinuses (Fokkens et al., 2020). Patients with CRSwNP generally have greater extent of sinus disease than CRSsNP patients as signified by severe sinus CT and endoscopic score (Deal & Kountakis, 2004). Even after the sinus surgery, patients with CRSwNP often have disease recurrence and more likely to have revision sinus surgery (Lou et al., 2015, Riva et al., 2022). Figure 2.5 shows the overview of the anatomy and objective findings of endoscopy and computed tomography of a healthy individuals and CRSwNP patients as comparison.

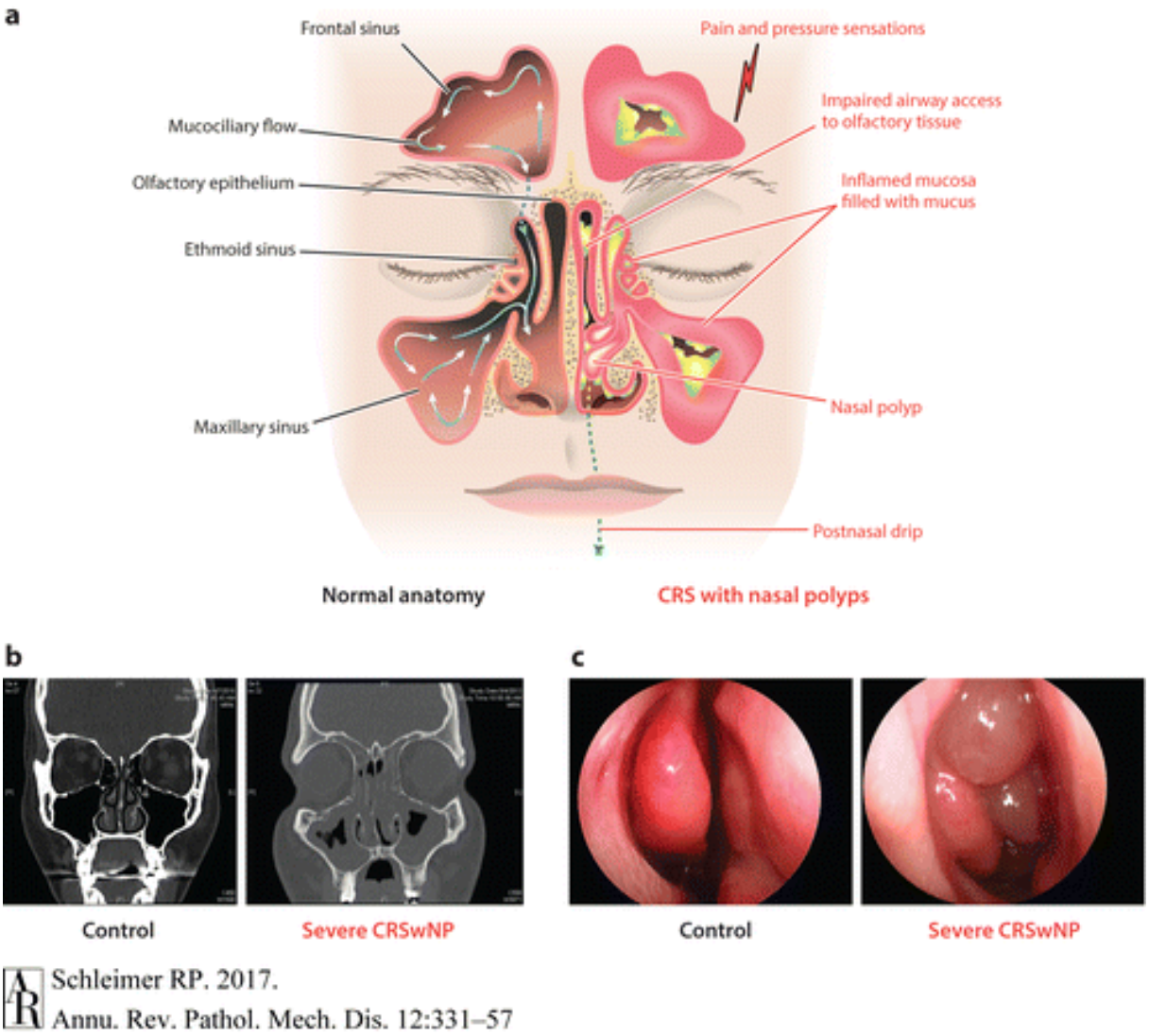


Figure 2.4 Overview of anatomy and objective finding for CRSwNP evaluation (A) Gross anatomical and inflammatory changes of CRSwNP and healthy one (B) Computed tomography (CT) finding of a healthy person and a severe CRSwNP patient (C) Endoscopic finding from a healthy person and a severe CRSwNP patients. Adapted from (Schleimer, 2017)

2.1.5 Treatment of CRSwNP

The first-line treatment for CRSwNP is medical treatment; however, if medical treatment fails, surgical intervention is necessary. Topical and systemic corticosteroids are both the cornerstones of CRSwNP treatment (Fokkens et al., 2020). Corticosteroids mitigate inflammation by modulating various immune responses, including hindering the production of proinflammatory cytokines, lymphocyte proliferation and delayed hypersensitivity (Benninger et al., 2003). Topical intranasal corticosteroids (INCSs) are reported to be effective in improving nasal symptoms and reducing polyp scores and polyp recurrence postoperatively (Swords et al., 2021). A meta-analysis indicated that moderate to high evidence of long-term use of INCS resulted in improvement in nasal obstruction via different delivery methods with minimal adverse effects (Bognanni et al., 2022).

In addition, a short course of systemic steroids is prescribed for patients with re-exacerbation symptoms (Fokkens et al., 2020). However, the administration of systemic steroids is not advised for a prolonged period, as it may implicate adverse effects even with low doses of steroids (Fokkens et al., 2020, De Corso et al., 2022). Another CRSwNP biologic is macrolide antibiotics. Multiple studies have examined the efficacy of macrolide therapy for CRSwNP as adjunctive therapy, which has resulted in variable success (Vidler et al., 2011, Amali et al., 2015, Fokkens et al., 2020). Macrolide is believed to exert anti-inflammatory properties by inhibiting proinflammatory cytokines, including IL-8, which is known as a potent neutrophil chemoattractant (Suzuki et al., 1997). Two meta-analysis studies conducted on the efficacy of macrolides in CRSwNP patients indicated that there is limited scientific evidence to support the long-term use of macrolides in ameliorating quality of life and disease severity; however, it may play

a role in improving endoscopic scores postoperatively in CRS patients (Pynnonen et al., 2013, Shu et al., 2023).

Monoclonal antibodies (mAbs) target type 2 inflammatory components, altering the pathologic function of IgE and specific cytokines. mAbs show encouraging results in controlling severe CRSwNP, which is unattainable with medication and surgery (Brown & Senior, 2020). Dupilumab is the first monoclonal antibody approved for CRSwNP therapy and the only biologic advised by the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) 2020 panel to be administered among CRSwNP patients (Fokkens et al., 2020). Dupilumab binds specifically to interleukin (IL)-4 receptor alpha to inhibit IL-4 and IL-3 signal transduction, thus suppressing the inflammation mediated by Th2. Other immunotherapeutic approved include omalizumab and mepolizumab, which block IgE and IL-5 signalling, respectively (Tai et al., 2022). Despite the clinical advantage, mAbs remain costly compared to the current standard care of medical therapy and surgery (Parasher et al., 2022, Van der Lans et al., 2022). To date, there are minimal data on the cost-effectiveness of mAbs used in chronic medication (Brown & Senior, 2020). However, the use of mAbs in asthma has resulted in a constant and considerable improvement in quality of life, and it remains an option for difficult-to-treat CRSwNP (Harada et al., 2021).

Table 2.1 presents a list of the existing biologics and emerging immunotherapies for CRSwNP, their binding target, FDA approval or stage of study and the effect on Tregs. Figure 2.6 represents the approaches in existing biologics and emerging immunotherapy in CRSwNP.

Table 2.1 List of existing biologics and emerging immunotherapies for CRSwNP therapy.

	Approaches	**Name**	**Approval or stage of the study**	**Underlying mechanism or Effect**	**Reference**
Existing Biologics	Macrolides	Azithromycin	Randomized Controlled Trial	• Immunomodulatory effect by suppressing inflammatory cytokine IL-8.	(Suzuki et al., 1997, Bergström et al., 2019)
		Clarithromycin	Randomized Controlled Trial	• Immunomodulatory effect via their antioxidative properties.	(Zimmermann *et al.*, 2018)
	Steroid therapy	Corticosteroid	Randomized Controlled Trial	• Reduce eosinophil and Th2 cells in local tissue post therapy. • Inhibit proinflammatory cytokines and lymphocyte proliferation.	(Zhang et al., 2019, Bognanni et al., 2022)
	Probiotic therapy	Recombinant *L.plantarum*	Preclinical	• Suppress Th2 responses of IL-4 & IL-5 and increase levels of IFN-y. • Counterregulatory Th1 responses by upregulation of FoxP3 in spleen cells.	(Mukerji et al., 2009)
		Oral Probiotic (*L.rhamnosus*)	Randomized Controlled Trial	• In CRS patients, the adjuvant of oral probiotic was found no effect on long term SNOT-22 score compared to placebo	(Schwarzer et al., 2011)

Table 2.1 Continued

Emerging Immunotherapeutic	Adoptive Cell Transfer	iTregs injection	Preclinical	• Enhancement of Tregs via iTregs injection. • Inhibit eosinophilic inflammation by hindering inflammatory cytokines of IL-4, IL-5, and IL-13.	(Chang et al., 2020)
	Immuno modulators	Dupilumab	Approved by FDA in 2019	• Regulates Th2 inflammation by inhibiting IL-4 and IL-13 signal transduction.	(Matsuyama et al., 2023)
		Omalizumab	Approved by FDA in 2020	• Inhibit IgE interaction with receptors on the cell surface. • Bind to the Fc receptor to prevent the basophils and mast cell activation.	(Amat et al., 2016, López-Abente et al., 2021)
		Mepolizumab	Approved by FDA in 2021	• Hinders IL-5 from binding to receptors expressed on eosinophils and basophils. • Inhibits eosinophilic inflammation.	(Bergantini et al., 2020, Bergantini et al., 2021)
		Benralizumab	Clinical Trial Phase 3	• Binds to IL-5 receptor on eosinophils surface to activate anti-IL-5 receptor α-subunit. • Diminish signal transduction and apoptosis of eosinophils.	(Sandhu et al., 2023)
		Reslizumab	Clinical Trial Phase 2	• Inhibits IL-5 from binding to IL-5 receptors. • Limits eosinophil differentiation.	(Harada et al., 2021)

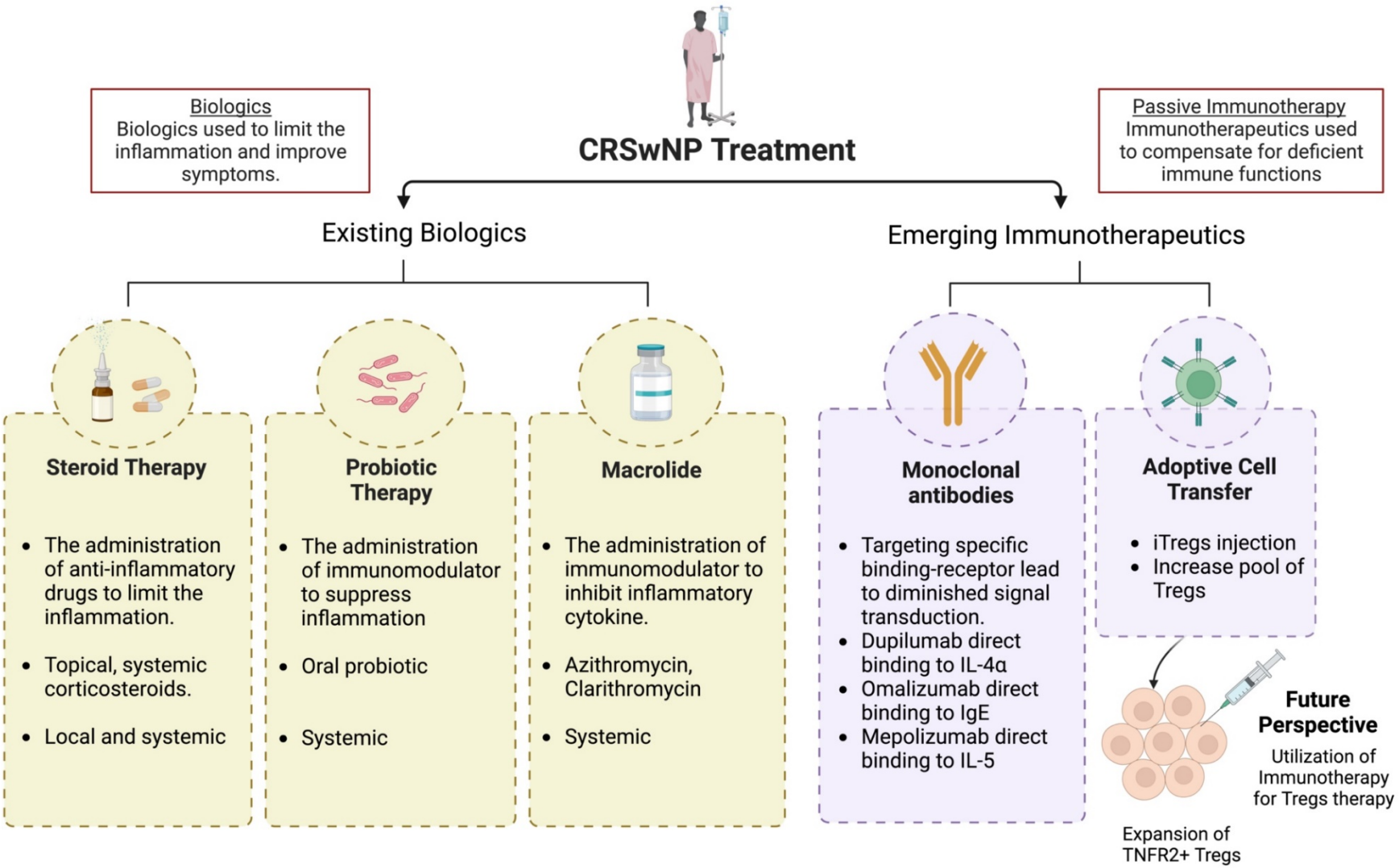


Figure 2.5 Approaches in existing biologics and emerging immunotherapy in CRSwNP. Adapted from (M Yusoff et al., 2024)

2.2 Immunopathogenesis of CRSwNP

2.2.1 T cell Physiology

To elucidate immunopathogenesis aspect of CRSwNP, some general immunological topics need to be known. The immune system comprises two intertwining arms of protection, against pathogenic particulates or allergens. The first arm constitutes innate defence which involves with non-specific killing of pathogens, utilized by the host immediately or within hours to combat with antigens (Marshall et al., 2018). Besides, second arm of adaptive defence involves with more specific recognition, with selective expansion of cells governed to target specific pathogens or allergens and consequently evolve becoming immunological memory (Cano & Lopera, 2013, Gray & Gibbs, 2022). Two prime cells of adaptive immunity comprise: T cells and B cells. T cells are matured from thymus, activated to proliferate via the recognition of antigen on the surface of APCs meanwhile B cells are developed from the bone marrow, differentiated into plasma cells to generate antibodies (Marshall et al., 2018). The focus regarding CRSwNP immunopathogenesis is on T cells.

Generally, T cells are recognized by the expression of surface receptors into $CD4^+$ and $CD8^+$. $CD8^+$ T cells usually cytotoxic, directly disrupt cancer cells via perforin and granzyme release hence compromise cells via T-cell Receptor (TCR) interaction with MHC class I molecules. On the contrary, $CD4^+$ T cells indirectly subscribe to cellular disruption (Gray & Gibbs, 2022). They “mediate” the immune response by commanding other cells to conduct and regulate the immune response. T helper cells (Th) are activated through TCR identification of antigen bound to MHC class II molecules (Marshall et al., 2018). Following these events, subsequent differentiation and polarization occur between these cells, depending on cytokines

milieu and antigens in the microenvironment, contributed to a highly specific adaptive immune response (Gray & Gibbs, 2022). The activation and differentiation of T cells occur within three conditions, first there are interaction of the TCR with the peptide presented by the HLA molecule, second there are signalling through co-stimulatory molecule such as CD28 and third there are participation of cytokines that inaugurate clonal expansion (Cano & Lopera, 2013, Adams et al., 2020). Moreover, the cytokines also abetted this activation which will define the fate of response later. Subsets identified upon the T cell differentiation currently include T helper 1 (Th1), Th2, Th17, Th22, follicular helper T (Tfh) and regulatory T cells (Tregs), which each of them has unique role in mediating protection in the adaptive immunity (Cano & Lopera, 2013).

2.2.2 Type 2 Response in CRSwNP

When an allergen breaches the upper airway, it encounters dendritic cells (DC) if not expulsed by defensive mucociliary. DC then internalize the allergen, migrate to the lymph nodes, and present the foreign antigen to naïve T cells (Figure 2.6). Upon this event, it activates the differentiation of $CD4^+$ T cells into several types of Th cells, depending on the cytokine production (Madore & Laprise, 2010). Evidence shows that inflammation in CRSwNP contributed by several T cells subsets such as Th1 (type 1 immune response), Th2 (type 2 immune response) and Th17 (type 3 immune response), indicating on heterogeneity of CRSwNP (Vlaminck et al., 2021). Although CRSwNP involved with different direction of immune response, type 2 skewed immunity has been implicated eminently in CRSwNP in most Causian and increasing Asian CRSwNP patients (Zhang et al., 2017, Laidlaw et al., 2020).

Type 2 skewed immune response characterized by an increased numbers of Th2 cells, which linked with mucosal eosinophilia, promoted by the upregulation of IL-4,