Defects In Cellular Immunity In Patients With Chronic Rhinosinusitis

Saud R Alromaih, MD, FRCSC

Department of Otolaryngology-Head and Neck Surgery, McGill University, Montreal

December 2015

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science in Otolaryngology-Head and Neck Surgery

It is my genuine gratefulness and warmest regard that I dedicate this work to my beloved parents. Without you, none of my success would be possible. To Maha Alsugair, my beloved wife, your patience and support during my career are unforgettable. To my daughters, Jory and Yara, your presence in my life keeps me happy, hopeful and ambitious.

TABLE OF CONTENTS

ABSTRACT

Chronic Rhinosinusitis (CRS) represents persistent inflammation of sinonasal mucosa for more than 12 weeks. It is one of the most common chronic diseases worldwide affecting all age groups with an estimated prevalence of 12.5% in the United States. It has huge impact on health expenditure and quality of life, with an estimated annual health care expenditure in USA of $12.8 billion. Heterogeneous in pathophysiology, clinical presentation, and therapeutic response, CRS represents a spectrum of disease entities with variable pathophysiology. Although the presence of intracellular pathogens is a hallmark of impaired cellular immunity, the observation of intraepithelial S. aureus in sinonasal biopsies taken from CRS patients has led us to suspect cellular immune dysfunction, represented by Major Histocompatibility Complex-I (MHC-I) deficiency and/or CD8+ (Cluster of Differentiation 8+) cytotoxic lymphocytes, as possible pathophysiology.

In this series of studies, we have explored a potential role of CD8+ lymphocyte deficiency in the development of CRS, exploring the subject from a genetic, demographic population based and translational aspects to propose possible mechanisms.

In my first manuscript, we aimed to verify whether genetic factors associated with MHC-I deficiency are present in CRS. Previous results from a genome-wide association study of chronic rhinosinusitis (1) were screened for polymorphisms in the CD8A (Cluster of Differentiation 8 Antigen), TAP1 (Tapasin 1), TAP2 (Tapasin 2) and TAPBP (Tapasin binding-protein) genes associated with MHC-I immunodeficiency syndrome(s). We identified that polymorphisms in the CD8A (rs3810831) and TAPBP (rs2282851) genes were significantly

associated with CRS, suggesting that modified CD8A or TAPBP gene function may contribute to the development of CRS.

In my second manuscript, we aimed to verify in-vivo whether CD8+ lymphocytes decrease was present in CRS and whether this was related to the presence of intraepithelial pathogens, notably S. aureus. Using biopsies of sinonasal mucosa taken from patients and controls, we assessed levels of CD8+ lymphocytes and intraepithelial S. aureus using immunohistochemistry. We extended this by staining for other subsets of lymphocytes and also neutrophils as markers of innate immunity. We observed lower levels of CD8+ cells in CRS patients, which were strongly and inversely related to the increased presence of S. aureus in sinonasal epithelium. Given the ubiquitous nature of this observation, we suggested that this might not necessarily be of genetic origin but instead suggested that S. aureus might be modulating the level of MHC-I immune activity locally at the level of the sinus mucosa as a form of immune evasion. In addition, as innate immunity and Major Histocompatibility Complex –II (MHC-II) processes remained conserved, as evidenced by persistence of neutrophils and CD16/56+ (Cluster of Differentiation 16/56+) natural killer, and CD4+ (Cluster of Differentiation 4+) T-helper lymphocyte, respectively, we postulated that this immune modulation was specific to MHC-I mediated immunity.

In my third manuscript, we aimed to determine the phenotype of CRS in patients with low serum CD8+ lymphocytes in comparison to that of conventional CRS. Sixty-seven CRS patients identified with low CD8+ lymphocytes were compared with an existing population

of 480 patients having CRS with nasal polyposis previously recruited for genetic association studies. Evolution of disease was somewhat different in CRS with low CD8+. Fewer patients required surgery and their first surgery was performed at a more advanced age, but antibiotic use was higher, which should help predict the evolution of future patients with low CD8+ lymphocytes.

This thesis thus reports for the first time the existence of CD8+ lymphocyte dysfunction in CRS patients and further suggests both genetic and bacterial factors as potential causative mechanisms. Practical implications of this work include:

1) The recommendation of routine assessment for identification of patients with CD8+ lymphopenia via lymphocyte immunophenotyping in severe CRS as assistance to determining an appropriate and personalized management plan.

2) The emphasis of a need to reconsider the pathogenic mechanisms of S Aureus to consider the possibility of bacterially-induced local immune modulation as a bacterial evasion strategy, suggesting a need to incorporate strategies for managing this into future treatment algorithms.

RÉSUMÉ

La rhinosinusite chronique (RSC) représente une inflammation persistante de la muqueuse sino-nasale de plus de 12 semaines. Elle est l'une des maladies chroniques les plus répandues dans le monde entier affectant tous les groupes d'âge avec une prévalence estimée à 12.5% aux États-Unis. Elle a un énorme impact sur les dépenses de la santé et la qualité de vie, avec une dépense annuelle estimée en soins de santé aux États-Unis à 12.8 milliards de dollars. Hétérogène dans la physiopathologie, la présentation clinique, et la réponse thérapeutique, la RSC représente un spectre d'entités morbides avec physiopathologie variable. Bien que la présence de pathogènes intracellulaires soit une caractéristique de l'immunité cellulaire affaiblie, l'observation intra-épithéliale de S. aureus dans les biopsies sino-nasales prélevés chez des patients atteints de RSC nous a conduit à soupçonner un dysfonctionnement cellulaire de l'immunité, représentée par la déficience de CMH-I (Complexe majeur d'histocompatibilité-I) et/ou des lymphocytes cytotoxiques CD8+ (Cluster de Différenciation 8+), comme physiopathologie possible.

Dans cette série d'études, nous avons exploré un rôle potentiel de la déficience des lymphocytes CD8+ dans le développement de RSC, en explorant le sujet selon les aspects génétique, basé sur la démographie de la population et des aspects translationnels pour proposer des mécanismes possibles.

Dans mon premier manuscrit, nous avons cherché à vérifier si les facteurs génétiques associés à la déficience en CMH-I sont présents dans la RSC. Des résultats précédents d'une étude d'association génétique de la rhinosinusite chronique (1) ont été triés pour les

polymorphismes dans les gènes CD8A (Cluster de Différenciation 8A) , TAP1 (Tapasin1), TAP2 Tapasin 2) et TAPBP (Tapasin binding-protien) associés au(x) syndrome(s) d'immunodéficience du CMH-I. Nous avons identifié que les polymorphismes dans les gènes CD8A (rs3810831) et TAPBP (rs2282851) sont associés de façon significative avec la RSC, suggérant que la fonction modifiée du gène CD8A ou du gène TAPBP peut contribuer au développement de la RSC.

Dans mon deuxième manuscrit, nous avons cherché à vérifier in-vivo si la baisse des lymphocytes CD8+ était présente dans la RSC et si cela était lié à la présence d'agents pathogènes intra-épithéliaux, notamment S. aureus. En utilisant des biopsies de la muqueuse sino-nasale prélevées sur des patients et des contrôles, nous avons évalué les niveaux de lymphocytes CD8+ et de S. aureus intra-épithélial par immunohistochimie. Ceci a été étendu à la coloration d'autres sous-unités de lymphocytes et aussi de neutrophiles comme marqueurs de l'immunité innée. Nous avons remarqué des niveaux inférieurs de cellules CD8+ chez les patients atteints de la RSC, qui étaient fortement et inversement liées à la présence accrue de S. aureus dans l'épithélium sino-nasal. Compte tenu du caractère propre de cette observation, nous avons suggéré que ceci n'était pas nécessairement d'origine génétique, mais plutôt suggérait que S. aureus pourrait être en train de moduler le niveau de l'activité immunitaire du CMH-I localement au niveau de la muqueuse des sinus comme une forme d'évasion immunitaire. De plus, comme les procédés de l'immunité innée et du CMH-II (Complexe majeur d'histocompatibilité-II) sont restés conservés, comme en témoigne la persistance de neutrophiles et de cellules ''tueuses naturelles'' CD16/56+ (Cluster de Différenciation 16/56+), et de lymphocytes T-helper CD4+ (Cluster de Différenciation 4+), respectivement, nous avons postulé que cette modulation immunitaire était spécifique au CMH-I médiant l'immunité.

Dans mon troisième manuscrit, nous avons cherché à déterminer le phénotype de la RSC chez les patients à faible taux sérique de lymphocytes CD8+ en comparaison à celui de la RSC conventionnelle. Soixante sept patients atteints de la RSC identifiés avec un faible taux de lymphocytes CD8+ ont été comparés à une population existante de 480 patients atteints de la RSC avec polypose nasale précédemment recrutés pour des études d'association génétique. L'évolution de la maladie était quelque peu différente dans la RSC avec un faible taux de CD8+. Peu de patients ont eu besoin de chirurgie et leur première chirurgie a été effectuée à un âge plus avancé, mais l'utilisation d'antibiotiques était plus élevée, ce qui devrait aider à prédire l'évolution de futurs patients à faible taux de lymphocytes CD8+.

Cette thèse rapporte ainsi pour la première fois l'existence d'un dysfonctionnement des lymphocytes CD8+ chez les patients atteints de la RSC et suggère en outre des facteurs génétiques et bactériens comme des mécanismes causals potentiels. Les implications pratiques de ce travail comprennent:

1) La recommandation de l'évaluation de routine pour l'identification des patients atteints de lymphopénie CD8+ via l'immuno-phénotypage des lymphocytes chez les patients atteints de RSC grave comme une aide à la détermination d'un plan de gestion approprié et personnalisé.

2) L'emphasis de reconsidérer les mécanismes pathogéniques de S. aureus à envisager la possibilité d'une modulation immunitaire locale bactérienne induite comme une stratégie d'évasion bactérienne, ce qui suggère la nécessité d'intégrer des stratégies pour gérer ceci dans les futurs algorithmes de traitement.

LIST OF ABBREVIATIONS:

Abbreviation	Meaning
AAO-HNS	American Academy of Otolaryngology-Head and Neck Surgery
ABRS	Acute bacterial rhinorinusitis
AKT	Ak strain transforming
ANA	Anti-nuclear antibody
ANCA	Anti-neutrophil cytoplasmic antibody
APC	Allophycocyanin
ASA	Acetylsalicylic acid
BD	Becton-Dickinson Company
CD	Cluster of Differentiation
CD 16/56+	Cluster of Differentiation 16/56+
CD 3+	Cluster of Differentiation 3+
CD 4+	Cluster of Differentiation 4+
CD 8+	Cluster of Differentiation 8+
CD8A	Cluster of Differentiation 8 Antigen
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CI	Confidence Interval
CNS	Coagulase-negative staphylococcus
CRCHUM	Centre de Recherche du Centre Hospitalier de l'Université de Montréal
CRP	C-reactive protein
CRS	Chronic rhinosinusitis
CRSsNP	Chronic rhinosinusitis without nasal polyposis
CRSwNP	Chronic rhinosinusitis with nasal polyposis
CRSwoNP	Chronic rhinosinusitis without nasal polyposis
CT	Computerized Tomography
CTL	Control
DAB	Diaminobenzidine
DNA	Deoxyribonucleic Acid
DOCK8	Dedicator Of Cytokinesis 8

DPC	Diagnostic Products Corporation
ESS	Endoscopic Sinus Surgery
FESS	Functional Endoscopic Sinus Surgery
FITC	Fluorescein isothiocyanate
HLA	Human leukocyte antigen
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL-10	Interleukin-10
IQR	Interquartile range
IRAK4	Interleukin-1 receptor-associated kinase 4
MAb	Monoclonal Antibody
MHC-I	Major histocompatibility complex-1
MR	Magnetic resonance
MRI	Magnetic resonance imaging
OCT	Optimal cutting temperature
OR	Odd ratio
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
pGWAS	pooling-based Genome-Wide Association Study
PI3K	Phosphatidylinositol 3-kinase
PLINK	A free open-source whole genome association analysis toolset
PNA-FISH	Peptide Nucleic Acid-Fluorescence In Situ Hybridization
Sag	Superantigens
SNP	Single Nucleotide Polymorphism
STAT3	Signal Transducer and Activator of Transcription 3
TABP	Tapasin binding-protein
TAP1	Tapasin 1
TAP2	Tapasin 2
TCR	T cell receptor
Th1	T-helper 1

Th2	T-helper 2
TLR	Toll-like receptor
TLR2	Toll-like receptor 2
TYK2	Tyrosine Kinase 2

ACKNOWELDGEMENTS

First of all, I am deeply indebted to Prof. Martin Desrosiers who introduced me to basic science research and connected me to the research world. He provided me with unlimited and continuous professional support during my enrichment year at McGill University during my residency program in Otolaryngology. He has an invaluable input in every aspect of this thesis. Moreover, he supported me in pursuing my career in Rhinology and Skull-Base Surgery while I was his clinical fellow at L'Université de Montréal.

On the same time, I would like to take this opportunity to thank the government of King Abdullah Ibn Abdulaziz and then King Salman Ibn Abdulaziz represented by King Saud University in Riyadh and the Saudi Arabian Cultural Bureau in Ottawa for sponsoring my scholarship.

I would like to thank Antoine Turmel Foundation for funding my research projects and to extend my thanks to Le Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM) as well as the Genome Quebec Innovation Centre for their invaluable support and assistance.

I would like to thank Ms. Leandra Mfuna-Endam for her help with ethical approvals, patients' recruitment and data collection, my colleagues Dr. Joseph Schwartz and Dr. Nathalie Gabra for their help in writing the second and third manuscripts, respectively, Mrs. Sawsan Al-Mot for her patience while teaching me immunohistochemistry, Dr. Ali Alfilali-Mouhim for his expertise in statistical analysis, Mrs. Joanna Aroutian and Mrs. Ilhem Douagui for their help during my master application and subsequent registrations.

I would like to thank Dr. Yohan Bossé, Dr. François Larivière, Dr. Françoise LeDeist and Ms. Jose Marie Brito for their input in various manuscripts.

I would like to thank Prof. Saul Frenkiel and Dr. John Manoukian for their support in getting me into this invaluable master program and Dr Simon Rousseau for his general guidance and support throughout my projects.

I am greatly thankful to Prof. Bernard Segal who encouraged me to explore basic science research as well and his consistent support and guidance in writing this thesis.

PREFACE & CONTRIBUTION OF AUTHORS

This thesis represents a collaborative work. I reviewed the literature and then I stated the rationale behind my projects. I established my objectives after reviewing what the team of my clinical supervisor Prof. Desrosiers had done already. I wrote the overall discussion, overall conclusion as well as edited this thesis in its entirety. For the first manuscript "Chapter 3", I reviewed the existing data and retrieved the relevant SNPs for further analysis, interpreted the data and wrote the manuscript in its entirety. For the second manuscript "Chapter 4", I reviewed patients' data, performed immunohistochemistry on tissue slides and counted targeted cells, interpreted results and wrote the manuscript. For the third manuscript "Chapter 5", I retrieved and reviewed patients' data, interpreted results and wrote the manuscript conjointly with Dr. Nathalie Gabra. Dr. Joseph Schwartz contributed in writing the third manuscript. Ms. Leandra Mfuna-Endam helped with ethical approvals, patients' recruitment and data collection. Mrs. Sawsan Al-Mot taught me and supervised me with immunohistochemistry and helped in data interpretation in the second and third manuscripts. Dr. Ali Alfilali-Mouhim was consulted for statistical analysis in all manuscripts. Dr. Yohan Bossé was involved immensely in patient recruitment and data interpretation of the original article (1), which our first manuscript was based on. Dr. Joaquin Madrenas was involved in data interpretation of the second manuscript. Dr. François Larivière, Dr. Françoise LeDeist, and Ms. Jose Marie Brito were involved in data interpretation of the third manuscript. Prof. Martin Desrosiers provided enormous supervisory guidance and support in stating the rationale, designing studies' protocols, data interpretation, writing all manuscripts, as well as in writing this thesis.

1. INTRODUCTION

Chronic rhinosinusitis (CRS) remains a complex disease with heterogeneous pathophysiology. CRS is characterized by inflammatory cell infiltrates and bacterial colonization of the mucosa. Research in this area has focused on the eosinophil, potential stimulation of the immune system by staphylococcal super antigens, and bacterial persistence via the formation of bacterial biofilms or intra-epithelial penetration. Immune dysfunction, whether humoral or cellular, is implicated in CRS as well (2-4).

In an attempt to extend these findings, my supervisors have previously explored CRS using genetic association studies (1, 5). They have identified associations with numerous genetic polymorphisms in signaling and regulatory mechanisms of innate immunity, suggesting that alterations in mucosal immunity may be implicated in the development of disease. The possibility of an immune deficiency contributing to the development of CRS was further supported by the clinical presentation of numerous genetically determined immunodeficiency syndromes, where development of CRS was part of the clinical picture.

They have explored mucosal immunity in CRS by performing histologic examination and expression studies of surgical samples. Expression studies have demonstrated a down regulation of expression of various antimicrobial proteins, further supporting the notion that reduced local immunity defenses may facilitate bacterial colonization. However, the inflammation associated with this has been highly variable, with some specimens demonstrating intense neutrophil infiltrates, and others demonstrating a near total absence, suggesting that different pathogenic mechanisms may underlie the common clinical phenotype of chronic rhinosinusitis.

In an attempt to identify constitutive factors implicated in the susceptibility to the development of CRS, they have used an in-vitro model of differentiated epithelial cell cultures raised from normal subjects and patients with CRS with, and without, nasal polyposis. Cluster analyses of expression studies have suggested the existence of two different CRS groups, one with a high degree of inflammatory activity, and the second, with a low degree of inflammation. While these two groups present two quite distinct expression patterns, there is a marked overlap between the group with nasal polyps and the group without nasal polyps, suggesting the presence of different pathogenic mechanisms underlie these clinical phenotypes.

They have further shown that, in the hypo-inflammatory group, there is a reduced sensitivity of cultured epithelial to innate immune stimulation with TLR ligands. This phenomenon appeared to be limited to cells from within the sinus, as circulating T cells obtained from the same patients respond normally to stimulation, suggesting the presence of local factors within the sinus environment that were modulating the local immunity to decrease bacterial clearance by reduction of inflammation, apoptosis and pyroptosis. They have also identified a possible mechanism for this by the demonstration of an immunomodulatory effect by Staphylococcus aureus, where an addition of a filtrate prepared from isolates reduced T-lymphocyte responsiveness to stimulation (6-8).

In a translational component, they have attempted to verify whether these molecular signatures can be used to differentiate clinical patients independently of their usual clinical phenotypes. To this end, they performed a translational study comparing the expression signature from epithelial brushings obtained from CRS patients three months post surgery. These were more pertinent to clinical disease as they sampled not only the epithelium, but also inflammatory cell infiltrates, epithelial and subepithelial areas, thus furnishing information on differences in inflammatory cell markers and/or activation. Moreover, on cluster analysis of the expression results, they again demonstrated two distinct expression signatures, characterized by high and low expression of pro-inflammatory cytokines. However, for the first time, they have noted a pronounced difference in the expression of various markers of CD8+ lymphocytes (cytotoxic T-cells, T- suppressor cells or simply CD8+ cells), suggesting either reduced levels of CD8+ cells, or else a dysfunction within CD8+ lymphocyte (data not published yet).

1.1. Thesis Rationale:

Taken together, these findings suggest that in a subpopulation of CRS, local mucosal immunity is impaired and may impair clearance of bacteria from the mucosal surface or from within those cells composing the epithelium. This may also represent a survival strategy for bacteria, where the bacteria themselves suppress local immunity in order to reduce cellular destruction by apoptosis and T-cell dependent mechanisms as the presence of intracellular pathogens is very suggestive of CD8+ cytotoxic lymphocyte dysfunction (4). These observations led to the thesis objectives listed in Section 1.2.

1.2. Objectives:

1. To identify whether CD8A gene polymorphism is associated with CRS
2. To determine the level of CD8+ T-lymphocytes at the sinonasal mucosa and to correlate it with the presence of intracellular S. aureus bacteria.
3. To determine whether CRS patients have low CD8+ lymphocytes.

1.3. Thesis Organization:

- Chapter 2: Literature review of chronic rhinosinusitis
- Chapters 3-5: Three separate manuscripts related to each of the above objectives. Manuscripts have been edited and modified to meet the requirements of Graduate and Postdoctoral Studies at McGill University.
- Chapter 6: Overall discussion
- Chapter 7: Overall conclusion
- Chapter 8: References

2. LITERATURE REVIEW

Chronic Rhinosinusitis is normally defined as persistent inflammation of sinonasal mucosa for more than 12 weeks (9). It is one of the most common chronic diseases worldwide affecting all age groups, with an estimated prevalence of 12.5% in the United States (10). Its economic burden is significant: The annual productivity-loss cost of refractory CRS is about $10.000 per patient, exceeding costs of severe asthma, chronic migraine or diabetes; an average of 18 full days is missed per year; the associated annual health care expenditure in USA is estimated to be $12.8 billion (11).

These patients are three times more likely to report their health as poor (4.6% vs. 1.7%). They have more depression (8.4% vs. 4.1%); they use more antidepressants (9.1% vs. 4.6%), and they have more visits to mental-health professionals (11.8% vs. 7.0%) (12). High levels of anxiety and depression are common in patients who undergo evaluation for CRS. Psychiatric comorbidity is associated with increased symptoms in CRS and increased health-care utilization (13). Quality of life was reduced by chronic rhinosinusitis in 94% of patients scheduled for endoscopic sinus surgery and 74% of them ranked their disease as severe or intolerable (14).

Chronic rhinosinusitis is a multifactorial problem involving numerous host and non-host factors. CRS can present with nasal polyposis “CRSwNP”, or without nasal polyposis “CRSwoNP or CRSsNP”. Heterogeneous in pathophysiology, clinical presentation, and therapeutic response, CRS represents a spectrum of disease entities with variable pathophysiology. Infections, allergies, pollutants, anatomic variations, autoimmune-related, immune deficiency, odontogentic, cystic fibrosis and ciliary dyskinesia are well known etiologies. Common bacterial pathogens are different in CRS than the usual bacteria involved in acute bacterial rhinosinusitis “ABRS”. Staphylococcus aureus, Coagulase-Negative Staphylococcus “CNS” and Pseudomonas Aeruginosa are the most commonly recovered bacteria in CRS. Different pathogen survival strategies have been described, including biofilms, superantigens and the presence of intracellular pathogens; showing interplay between host and pathogens (2, 3, 9, 15). Apart from Cystic Fibrosis and Primary Ciliary Dyskinesia, genetic bases

are postulated but remain elusive and deserve attention (1, 5). Increased knowledge of cellular and molecular derangements in CRS suggests potential etiologies and targets for therapy.

Patients commonly present with a combination of facial pressure, nasal congestion, nasal obstruction, rhinorrhea, hyposmia or even anosmia. Other complaints include fatigue, tiredness, halitosis, earache, toothache and headaches. Nasal endoscopy may show polyps, secretions, mucosal edema and/or inflammation (2, 3, 9, 16, 17). One of the widely accepted diagnostic criteria for CRS is the one proposed by the American Academy of Otolaryngology-Head and Neck Surgery multidisplinary Rhinosinusitis Task Force in 2004 (9).

CRS complications are uncommon but nevertheless could still be life threatening. Orbital infections are the most common complication but intracranial complications like meningitis, encephalitis and intracranial abscesses are the most serious (18).

The work up is usually directed to explore possible etiologies, extent of disease and presence of unusual anatomic variations and/or complications. This includes conventional sinonasal endoscopies, cultures, and imaging studies. CT scans are very useful but MR imaging might be necessary. Biopsies, brushings, allergy testing can be utilized as well. In some patients, immune deficiency and autoimmune work-up can be requested including lymphocyte phenotyping, immunoglobulins, rheumatoid factors, CRP, ANA and ANCA studies (2, 3, 16, 17, 19).

The treatment is directed toward identifying possible etiological factors to control the disease and alleviate patient's symptoms. Removing environmental factors, like allergens, irritants, pollutants should be considered. Medical treatments commonly include local treatments such as saline rinses and intranasal corticosteroids. Systemic therapy involves using short-term oral steroids and antimicrobial therapy. Decongestants, antihistamines and leukotriene receptor antagonists could be used in some patients as an adjunct therapy. Surgical treatment aims at establishing sinus drainage and ventilation, addressing possible pathological anatomical variations as well as possible complications. Functional Endoscopic Sinus Surgery (FESS) is considered the standard surgical approach, although external approaches may be needed in certain occasion addressing complicated and/or refractory CRS. Disease may not be controlled

after surgery and care of this challenging group of patients has driven much of the recent rhinological research (2, 3, 16, 17, 20).

2.1. Linking Statement To The First Manuscript:

This thesis is concerned with cellular immunity represented by CD8+ cytotoxic T-lymphocytes and its relation to CRS as suggested by the presence of intracellular staphylococcus aureus in sinonasal epithelium. The next chapter will consider whether CD8A gene polymorphisms might predict the severity of CRS.

3. MANUSCRIPT 1: CD8A GENE POLYMORPHISMS PREDICT SEVERITY FACTORS IN CHRONIC RHINOSINUSITIS*

3.1. Title Page:

Saud Alromaih, MD, FRCSC[1-2]; Leandra Mfuna Endam, MSc[3]; Ali Alfilali-Mouhim, PhD[3]; Yohan Bosse, PhD[4]; Martin Desrosiers, MD, FRCSC[1-3].

[1]Division of Otolaryngology-Head and Neck Surgery, Faculty of Medicine, University of Montreal, Montreal, Quebec, Canada

[2]Department of Otolaryngology-Head and Neck Surgery, McGill University, Montreal, Quebec Canada

[3]Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Montreal, Quebec, Canada;

[4]Institut Universitaire de Cardiologie et de Pneumologie de l'Université Laval, Québec, QC, Canada and Department of Molecular Medicine, Laval University, Québec, QC, Canada

Key words:

MHC-I immunodeficiency, chronic rhinosinusitis, genetic association study, CD8A, TABP phenotyping, MHC-I deficiency

Published In:

International Forum of Allergy and Rhinology. 2013 Aug;3(8):605-11.

doi: 10.1002/alr.21174. Epub 2013 May 2.

*This project was presented at the Annual Meeting of American Rhinologic Society, Washington, DC; 2012.

3.2. Abstract

Introduction:

A genetic basis to chronic rhinosinusitis (CRS) is postulated, but remains elusive. We have recently identified low levels of circulating CD8+ (Cluster of differentiation 8+) lymphocytes as a frequent finding in difficult-to-treat or refractory CRS. In MHC-I (major Histocompatability Complex-I) deficiency, low circulating levels of CD8+ lymphocytes secondary to mutations in the CD8A) Cluster of differentiation 8A), TAP1 (Tapasin 1), TAP2 (Tapasin 2) or TAPBP (Tapasin binding-protein) genes lead to a clinical syndrome, which is associated with severe CRS.

Objective:

To identify whether genetic factors associated with MHC-I deficiency are present in CRS.

Methods:

Previous results from a genome-wide association study of chronic rhinosinusitis (1) were screened for polymorphisms in the CD8A, TAP1, TAP2 and TAPBP genes associated with MHC-I immunodeficiency syndrome. Significant polymorphisms were tested for associations with clinical picture characterizing severe CRS.

Results:

Polymorphisms in the CD8A (rs3810831) and TAPBP (rs2282851) genes were significantly associated with CRS. Major allele homozygosity for CD8A (rs3810831) was associated with a higher frequency of affected relatives ($p=0.052$), increased severity as characterized by age at diagnosis ($p=0.009$), age at 1st surgery ($p=0.004$) and number of surgeries ($p=0.008$), while TAPBP (rs2282851) was associated increased risk for CRS (OR= 2.48, $p= 0.0076$)

Conclusion:

Modified CD8A or TAPBP gene function may contribute to the development of refractory CRS via altered MHC-1 function and reduction of circulating CD8+ lymphocytes.

Significance:

Identification of markers in the CD8A or TAPBP genes via sequencing may offer a basis for genetic testing in CRS.

3.3. Introduction:

Chronic rhinosinusitis (CRS) is an inflammatory disease of the upper airway characterized by persistent inflammation and bacterial colonization of the paranasal sinuses (3). While significant progress is being made in documenting immunologic and bacteriologic alterations present in active disease, predisposing factors contributing to susceptibility to chronic rhinosinusitis remain mostly unknown (21).

It is believed that genetic factors contribute to the development of chronic sinusitis. A frequently cited example is cystic fibrosis; where mutations in the CFTR gene create disordered chloride transport in epithelium. Among the other manifestations, this leads to development of CRS in the large majority of affected patients (22). Genetically modified mouse models suggest potential importance of other genes in the development of CRS, where several single-gene knockout models include development of chronic sinusitis as part of their phenotype (23).

Using both candidate gene approaches and modified genome-wide association techniques, our group and others have identified polymorphisms in genes associated with innate immunity and regulation of inflammatory responses, suggesting that dysregulated immunity may play an important role in susceptibility to this disorder (5).

Reduction in immune surveillance and pathogen clearance may conceivably play a role in the development of CRS and explain the persistent bacterial presence in these individuals. While the most frequently considered immunodeficiency in chronic sinusitis is reduced gamma globulin levels of different causes, other types of impaired immunity may also be considered for potential role in CRS as well (24). In individuals with deficient cellular immunity, decreased pathogen clearance from intercellular locations is a frequent feature of the disorder. This parallels with the persistence of bacterial pathogens within the epithelium of CRS patients (25). As intracellular presence of pathogens is a hallmark of impaired cellular immunity, this has led us to incorporate assessment of cellular immunity in the assessment of difficult-to-treat CRS patients, leading to the observation that decreased CD8+ circulating lymphocyte subpopulations are relatively frequent in this diseased population (26).

While multiple mechanisms for adaptive immune defects are known, one of potential interest to CRS is the broad category of MHC-I deficiencies caused by genetic mutations within the CD8A, TAP1, TAP2 or TAPBP genes. The reduced clearance of infected cells by cytotoxic CD8+ lymphocytes culminates in a common clinical phenotype characterized by otitis media, purulent rhinitis, pansinusitis evolving to inflammatory lung disease (27).

Given the potential similarities between MHC-I deficiencies and CRS, we wished to evaluate whether genetic factors associated with MHC-I deficiency were present in a population of patients with severe CRS. In order to assess whether a potential role for MHC-I deficiency is present in CRS, data from our previously performed pooling-based genome-wide association study of patients with severe CRS were screened for polymorphisms within genes identified with MHC-I deficiency.

3.4. Methods:

The pooling-based genome-wide association study on CRS has been reported in detail elsewhere (1) and is only summarized here briefly.

3.4.1. Patient Population And Sample Collection:

206 subjects with severe CRS (with or without polyposis, or recurrent acute sinusitis) and 196 controls were recruited prospectively. Severe CRS was defined as either: 1) persistent signs/symptoms of CRS despite previous endoscopic sinus surgery (ESS), or 2) a history of more than one ESS for CRS regardless of outcome. Definitions are according to the 2004 American Academy of Otolaryngology-Head and Neck Surgery guidelines (9). All subjects completed a standardized questionnaire assessing age, gender, presence of affected first-degree blood relatives, smoking, history of perennial or seasonal allergies, physician diagnosed asthma and acetylsalicylic acid (ASA) intolerance. Information on disease related factors including age at diagnosis, age at first surgery and number of previous surgeries were recorded. Initial diagnoses of CRS with or without nasal polyposis, or recurrent acute sinusitis, were taken from patient records, and classified according to the 2004 American Academy of Otolaryngology-Head and Neck Surgery guidelines (9). All patients with early onset nasal polyposis (first surgery at less than 25 years of age) had previously undergone sweat chloride testing performed to rule out a diagnosis of cystic fibrosis.

Blood samples were collected in BD Vacutainer Serum Separator Tubes (BD Diagnostics, Franklin Lakes, NJ), and stored at 4°C until analysis for DNA extraction. Total IgE measurements were performed using a DPC Immulite System (Diagnostic Products Corporation – Siemens, Los Angeles, CA).

3.4.2. Controls And Sample Collection:

Controls were recruited following two strategies: either spouses or non-blood relatives living in the same household and individuals recruited by random telephone screening matched to the patient's postal code. The only attempt at matching subjects and control is their geographical location to minimize differences in potential environmental exposures. Nevertheless, a standardized questionnaire assessing age, sex and ethnic origin (but not smoking, history of atopy or physician diagnosed asthma) was obtained for controls. The Oragene kit (DNA Genotek, Ottawa, Ontario) was used for saliva collection and sent to controls with prepaid

return postage. Saliva samples were stored at room temperature until genotyping as recommended by the manufacturer.

3.4.3. DNA Extraction

DNA was isolated from peripheral blood leucocytes. Blood was collected in citrate treated tubes, and DNA was isolated using the Puregene DNA isolation kit (Gentra System, QIAGEN), following the high throughput protocol for 10 ml whole blood provided with this kit. DNA extracted from saliva was performed using the Oragene DNA Purification Protocol (DNA Genotek, Ottawa, Ontario). Isolated DNA from both blood and saliva was stored at -80°C prior to use.

3.4.4. Ethical Considerations:

The Institutional Review Boards for the Ethical Conduct of Research of the McGill University Health Center and of the Centre Hospitalier de l'Université de Montréal both approved the study and all subjects signed an informed consent.

3.4.5. Pooling-Based Genome-Wide Association Study:

Results from a previously performed pooling-based genome-wide association study (pGWAS) of severe CRS published by our group (1) were used for this study. In the pGWAS approach, instead of genotyping each subject individually and then calculating allelic frequencies, genotyping is performed on a pool of an equal amount of DNA from each subject in either affected or control populations and then tested using high throughput chips. In our pGWAS, this was performed on the Illumina Sentrix® HumanHap550 Genotyping BeadChip (Illumina Corporation, San Diego, CA) which interrogates 555 175 SNPs distributed across the whole human genome. High priority SNPs were identified based on differences in allelic frequencies estimated from the DNA pools between affected and control populations in a hypothesis-free fashion, and individual genotyping was performed as part of a panel of 1536 SNPs genotyped using the Illumina GoldenGate assay (Illumina Corporation, San Diego, CA)(28).

3.4.6. Statistical Analysis:

Genetic association with CRS was tested with allelic and genotypic models. Odds ratios (OR) and 95% confidence intervals (95% CI) were given. In order to determine nominal p-

values, case and control labels were randomly permuted to generate 10,000 replicates under the global null hypothesis of no genetic association. Logistic regression models were performed to control for sex as covariates and to calculate odds ratios for homozygous and heterozygous. SNP association tests were performed using PLINK program version v1.02 (29).

3.4.7. Identification And Assessment Of SNPs In Genes Associated With MHC1 Deficiency Syndromes:

Our 1536 SNPs panel was screened for associations in SNPs from the following genes associated with MHC-I immune deficiency syndromes: i) CD8A (Cluster of Differentiation 8A), ii) TAP1 (Tapasin 1), iii) TAP2 (Tapasin 2) and iv) TAPBP (Tapasin binding-protein). Furthermore, in an attempt to identify the impact of selected polymorphisms on development of disease, the phenotype of different allele subpopulations within CRS population were analyzed using one-way ANOVA and Fisher Exact test, considering a p-value of ≤ 0.05 as statistically significant.

3.5. Results:

Demographic and basic clinical data of the pGWAS population are summarized for patients and controls in Table 3.1.

Among the 1536 SNPs panel derived from our pGWAS, 492 SNPs were associated with CRS at uncorrected p-values of $p \leq 0.05$ after genomic correction. Of the screened genes, only the CD8A (SNP rs3810831) and TAPBP (SNP rs2282851) were significantly associated with CRS. No polymorphism in the TAP1 or TAP2 genes were associated with CRS in this pGWAS and thus had not been individually genotyped.

The CD8A (SNP rs3810831) suggested a protective effect of the minor allele on development of CRS (patients: 21%; controls 27%; OR: 0.706; p=0.047, Table 3.2). Assessment according to genotype groups suggested recessive transmission, with a pronounced protective effect (OR = 0.370; p=0.012) associated with the heterozygous TC genotype (Table 3.3).

Table 3.1. Demographic and clinical characteristics of CRS and control populations:

	CRS cases	Controls
Total	206	196
Age (mean in years ± SD)	52.3 ± 13.0	48.8 ± 15.0
Male/Female ratio	1.10 (108/98)	0.79 (86/110)
Ethnic group:		
White	85.2	89.5
Middle East	4.9	4.4
Jewish	5.4	1.1
Asian	1.5	1.1
First Nation	1.5	0.6
Black	1.0	0.6
Hispanic	0.5	1.1
Pacific Islander	0.0	1.7
Initial diagnosis CRS, No. (%)		
CRSwNP	154 (74.8)	-
CRSsNP	52 (25.2)	-
Age at first CRS diagnosis (years)	33.4 ± 14.3	-
Age at first sinus surgery (years)	38.1 ± 15.1	-
History of asthma (%)	63.7	-
History of allergy (%)	65.5	-
History of ASA sensitivity (%)	28.6	-
Smoking (non/ex/current) (%)	48.1/40.8/11.2	-
IgE (IU/L) (median ± IQR)	87.0 ± 174.5	-
≥120 UI/mL (%)	41.7	-
White-blood-cell count (median ± IQR)	3.6 ± 4.4	-

Table 3.2: Allelic associations of CD8A and TAPBP in CRS:

Gene Symbol	Chromosome	SNP	Location	Case; Control Frequencies	Major allele	Minor allele	OR (95% CI)	P value
CD8A	2	rs3810831	8687258	0.21; 0.27	T	C	0.706 (0.5001 - 0.996)	0.047
TAPBP	6	rs2282851	33388287	0.34; 0.25	C	T	1.533 (1.111 - 2.114)	0.009

Table 3.3: Genotype associations of CD8A and TAPBP in CRS:

Symbol	SNP	Homo-zygote	Hetero-zygote	Case; Control Homozygote %	Case; Control Heterozygote %	OR Homo	P-value Homo	OR Hetero	P value Hetero
CD8A	rs31810832	CC	CT	0.041; 0.056	0.332; 0.422	0.370	0.212	0.370	0.004
TAPBP	rs2282851	TT	TC	0.136; 0.077	0.412; 0.365	2.670	0.042	1.119	0.721

Certain demographic and disease-specific factors differed between genotypes. Homozygosity for the major TT allele in CD8A (rs3810831) was associated with a higher frequency of affected relatives (p=0.051) and increased markers of disease severity as characterized by younger age at diagnosis (p=0.0009), younger age at first surgery (p=0.004) and greater number of surgeries (p=0.008) as would be expected as the opposite of the protective CC allele. There was no statistically significant difference in the incidence of nasal polyposis among different genotypes (Tables 3.4a and 3.4b).

The TABP (rs2282851) was associated with an increased risk for CRS (Patients: 34.2%; controls 25.3%; OR: 1.53; p=0.009, Table 3.2). Assessment according to genotype group suggested a recessive mode of transmission, with a pronounced increased risk (OR = 2.67; p=0.042) associated with the homozygous TT genotype; see Table 3.3. In contrast to the findings for CD8A, minor allele homozygosity for the TT risk genotype was associated an increased history of eczema and a higher total IgE level (Tables 3.5a and 3.5b).

Table 3.4a: Phenotypes of different genotypes of CD8A:

Genotype	CC (n=8)		CT (n=65)		TT (n=123)		p-value
	Average	SD	Average	SD	Average	SD	
Age (years)	55	14	52	13	52	13	0.083
Age at 1st diagnosis (years)	37	14	35	14	28	17	0.009
Age at first operation (years)	41	14	40	16	30	20	0.004
Age at most recent surgery (years)	50	15	49	13	50	15	0.821
Number of surgeries	2.25	1	2.6	1.4	3.4	2	0.008

Table 3.4b: Phenotypes of different genotypes of CD8A, using Exact Fisher test:

Genotype	CC (n=8)	CT (n=65)	TT (n=123)	P-value
Gender	Males: 62% (5/8) Females 38% (3/8)	Males 55% (36/65) Females 45% (29/65)	Males 49.6% (61/123) Females 50.4% (62/123)	0.531
CRSwNP	7/8 (87.5%)	47/65 (72.3%)	93/123 (75.61%)	0.729
Relatives with CRS	2/7 (29%)	14/58 (24%)	47/111 (42%)	0.052
History of Asthma	5/8 (62.5%)	42/65 (64.62%)	78/123 (63.41)	0.929
ASA intolerance	2/8 (25%)	24/65 (36.92%)	31/121(25.62%)	0.256
COPD	0/8 (0%)	8/65 (12.31%)	9/123 (0.74%)	0.386
Active smoker	1/8 (12.5%)	10/65 (15.38%)	9/123 (7.32%)	0.166
Ex-smoker	5/8 (62.5%)	33/65 (50.77%)	57/123 (46.34%)	0.618
Allergies	7/8 (87.5%)	40/65 (61.54%)	82/123 (66.67%)	0.356
Eczema	2/8 (25%)	11/65 (16.92%)	25/123 (20.33%)	0.717

Table 3.5a: Phenotypes of different genotypes of TAPBP, using one-way ANOVA:

Genotype	TT (n=27)		CT (n=82)		CC (n=90)		p-value
	Average	SD	Average	SD	Average	SD	
Age in years	54	10.85	52	13,87	52	12.94	0.791
Age at 1st diagnosis (years)	32	11.65	32	15.01	35	14	0.194
Age at first operation (years)	39	13.04	37	16.21	39	14.63	0.730
Age at most recent surgery (years)	50	9.87	48	13.70	47	13.51	0.656
Number of surgeries	3.66	2.73	2.96	2.71	3.14	3.15	0.557
Eosinophil absolute count x10E9/L (0-0.8)	0.29	0.25	0.34	0.37	0.32	0.29	0.779
IgE serum level kUI/L (<100)	170.56	284.07	206.76	273.45	107.3	98.81	0.010

Table 3.5b: Phenotypes of different genotypes of TAPBP, using Exact Fisher test:

Genotype	TT (n=27)	CT (n=82)	CC (n=90)	p-value
Gender	Males 48% (13/27) Females 52% (14/27)	Males 59% (48/82) Females 41% (34/82)	Males 48% (43/90) Females 52% (47/90)	0.326
CRSwNP	71/90 (78.88%)	62/82 (75.61%)	17/27 (62.69%)	0.253
Relatives with CRS	10/25 (40%)	24/74 (32.4%)	32/80 (40%)	0.596
Asthma	16/27 (59.25%)	53/82 (64.63%)	57/90 (63.33%)	0.879
ASA intolerance	9/26 (34.61%)	23/82 (28.05%)	26/89 (29.21%)	0.843
COPD	3/27 (11.11%)	8/82 (9.76%)	6/90 (6.66%)	0.646
Active smoker	4/27 (14.81%)	9/82 (10.8%)	7/90 (7.77%)	0.468
Ex-smoker	14/27 (51.85)	41/82 (50%)	41/90 (45.56%)	0.818
Allergies	22/27 (81.48%)	54/82 (65.85%)	55/90 (61.11%)	0.144
Eczema	8/27 (29.63%)	21/82 (25.61%)	10/90 (11.11%)	0.016

3.6. Discussion:

Recent studies in molecular mechanisms underpinning immune deficiencies have led to an explosion in our molecular understanding of diseases. Immune deficiencies previously identified mainly on the basis of clinical phenotype can now be characterized as to their underlying genetic basis (30).

In this study, we identified that polymorphisms within the CD8A and TAPBP genes implicated in MHC-I immunodeficiency syndromes are associated with the population of severe CRS. This association is most acutely pronounced for the CD8A gene. Homozygotes for the risk-associated genotype of this SNP are more likely to present with a more severe disease characterized by development of disease at an earlier age, an earlier need for surgical therapy and a higher tendency to recurrent disease is indicated by an increased number of surgeries. Further support for a potential genetic basis beyond the early age of development of disease is afforded by a greater frequency of affected first-degree relatives with CRS. Of most pronounced interest for this is the frequency of homozygotes with the TT genotype associated with the severe form of CRS. In association studies of complex diseases, it is unusual to identify a frequent genetic variation that has such a significant effect, which strengthens the potential importance of this association. However, despite this strong link, it remains unknown how this genetic polymorphism contributes to development of disease and it would be interesting to know the extent of association in less severe forms of disease.

While we have not documented a direct functional impact of this polymorphism on the function of the CD8A gene, association of the homozygotes status with severe disease suggest that there could be an effect on function. Mechanisms by which altered CD8A gene function may contribute to CRS disease and relates to reduced clearance of diseased epithelial and inflammatory cells infected with bacteria or viruses. These may facilitate persistence, allowing these agents to persist and behave as biological modifiers (31).

The SNP rs2282851 of the TAPBP gene is also associated with CRS, but genotype-specific effects on demographic parameters and disease-specific markers are less pronounced. Nevertheless, associations of the at-risk allele for eczema and total serum IgE may be pertinent to

our hypotheses for CRS pathogenesis. Eczema is characterized by frequent Staphylococcus aureus colonization, which is a frequent feature of CRS. In both disorders it is believed to exert its effect by exerting an immunomodulatory role via secretion of exotoxins, which may increase IgE production. This would be consistent with the observation of increased IgE also associated with this genotype, and suggest that this SNP may potentially exert its influence via reduced clearance of S Aureus from infected cells.

While we suggest that these polymorphisms may be influencing CD8+ lymphocyte levels or activity, we are not surprised that this population does not follow a more severe course with greater morbidity and mortality. It can be expected that these adult forms of disease be of reduced intensity than that of the extreme pediatric phenotypes where they were initially described. Additionally, immune deficiency does not necessarily portend a dismal clinical prognosis for CRS. In prospective studies of patients with recognized and well-managed immune deficiencies, outcomes were similar as for immunocompetent CRS patients (32).

The current work, while exciting in terms of its implications, nevertheless suffers from a number of limitations, which must be validated by further work and replication in other populations. Identification of causative mutations will require sequencing of the CD8A and TAPBP genes, which has not been performed on the samples. Despite intriguing associations, these results nevertheless demonstrate an association between observed polymorphisms and disease, and do not identify mutations within the gene as such responsible for the disorder. As well, it would be of interest to compare genotypes circulating CD8A levels to identify whether these could potentially be responsible for other published clinical observations documenting low circulating CD8A levels in a subpopulation of patients with CRS. Additionally, the results obtained in this distinct subpopulations of patients with severe CRS cannot be generalized to a more general population commonly seen in the office and do not thus for the moment have any direct clinical implications.

Additionally, there are clearly recall biases in the reporting of the number of operation and dates by patients and future prospective studies would more appropriate. While lack of old hospital records and absence of a centralized health care record database made it impossible to

verify these parameters against an outside reference, every attempt was nevertheless made to ensure maximal possible accuracy within these limitations. All data was collected by using a standardized questionnaire, by the same experienced clinical trial monitor at the time of recruitment, and thus any recall variation should be similar for all three genotypes.

Despite these limitations, the differential clustering of patients according to genotype, with one group clearly presenting demographic factors suggestive of more severe disease is nevertheless highly interesting and suggests a potential contribution of immune defects to the pathogenesis of certain forms of CRS. Studies of genes implicated in immune-deficiency may thus serve as a basis for further studies in our understanding of the disorder. While in this study we have concentrated on MHC-I deficiency, it can be speculated that other immune deficiencies may play a role in the development of CRS as well. Immunodeficiency with the IRAK4 is responsible for recurrent pneumococcal or Haemophilus infections during childhood. In previous work from our group, we have identified polymorphisms within the IRAK4 gene associated with CRS, and more interestingly, as having a genotype specific impact on IgE level, suggesting a link between innate immunity and the biochemical observations in certain forms of CRS (33). Additional forms of other immune deficiencies may also be implicated in CRS given similarities between observations and those disorders in CRS. Of particular potential interest is Job syndrome or hyper-IgE syndrome (34). This syndrome is of particular potential interest to CRS given the clinical phenotype, which incorporates high IgE levels, and persistent colonization with hypodeveloped responses against this pathogen. Intriguingly, diseases with a distinctive phenotype as this can be traced to mutations within either the TYK2, STAT3 or DOCK8 genes, suggesting that defect at function of multiple genes may lead to common clinical phenotype, and suggesting one of the potential difficulties in interpreting genome-wide association studies.

3.7. Conclusion:

The results of this study suggest that modified CD8A or TAPBP gene function may contribute to the development of CRS via altered MHC-I function and/or reduction of circulating CD8 lymphocytes. These early exploratory findings suggest the potential role for adaptive immune defects in the pathogenesis of CRS. While these findings are not directly applicable for clinical management of patients at present, they suggest that in the future, assessment of immune function and /or gene re-sequencing may eventually play a role in the management the patients with CRS.

3.8. Acknowledgments

Y. Bossé is a research scholar from the Heart and Stroke Foundation of Canada

3.9. Linking Statement To The Second Manuscript:

The above manuscript explored CD8A gene polymorphisms and its relationship to the phenotypes of CRS. The next chapter will examine whether CD8+ lymphocytes at the level of the sinonasal mucosa might influence intracellular pathogens or not.

4. Manuscript 2: LOW INTRAEPITHELIAL CD8+ LYMPHOCYTE SUBPOPULATION AND INCREASED STAPHYLOCOCCUS AUREUS PRESENCE IN SINUS MUCOSA SUPPORT A LOCAL IMMUNE DYSFUNCTION IN THE PATHOPHYSIOLOGY OF CHRONIC RHINOSINUSITIS*

4.1. Title Page:

Saud Alromaih, MD, FRCSC[1, 2,3], Joseph S Schwartz, MD, FRCSC[1, 4], Sawsan Al-Mot, MSc[2], Leandra Mfuna Endam, MSc[2], Abdelali Filali-Mouhim, PhD[2], Joaquin Madrenas, MD, PhD[5] and Martin Desrosiers, MD, FRCSC[2, 6]

[1]Department of Otolaryngology - Head & Neck Surgery, McGill University, Montreal, Canada
[2] Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Montreal
[3]Dept of Otolaryngology – Head & Neck Surgery, King Saud University, Riyadh, Saudi Arabia
[4]Dept of Otolaryngology - Head & Neck Surgery, University of Pennsylvania, Philadelphia, USA
[5]Department of Microbiology & Immunology, McGill University, Montreal, Canada
[6]Dept of Otolaryngology - Head & Neck Surgery, Université de Montréal, Montreal, Canada

Corresponding Author:
Dr. Martin Desrosiers
Department of Otolaryngology - Head & Neck Surgery, Centre Hospitalier de l'Université de Montréal, Pavilion Marie-Morin, #4-42, 3840 Rue Saint-Urbain, Montréal, Canada, H2W1T8
Tel: (514) 890-8000 ext. 14170 Fax: (514) 412-7246 desrosiers_martin@hotmail.com

Key words: Pathophysiology of chronic rhinosinusitis, bacterial immune evasion, inflammatory cells in CRS, CD8+ lymphocyte, and staphylococcus aureus

Financial disclosure: Financially supported by the Fondation Antoine Turmel

Manuscript will be submitted to the International Forum of Allergy and Rhinolog
*Presented at the Annual Meeting of European Rhinologic Society, Toulouse, France; 2012.

4.2. Abstract:

Introduction:

CD8+ (Cluster of Differentiation 8+) cytotoxic lymphocytes are important element of the cellular adaptive immunity responsible for killing damaged or dysfunctional cells, including cells infected with pathogens. As a consequence, it is unusual to observe pathogens present intracellularly within the sinus mucosa, and their presence is suggestive of an immune deficiency.

Staphylococcus aureus is a frequently implicated pathogen in chronic rhinosinusitis (CRS), and in CRS, can frequently be detected not only on the surface but also within the epithelial cells of the sinus mucosa. Identification of intracellular pathogens in CRS patients with otherwise normal immunity suggests that *S. aureus* may be modulating host immunity locally at the mucosal level to elude destruction by CD8+ lymphocytes. To support this hypothesis, we wished to evaluate the relationship between intracellular S. aureus and CD8+ lymphocyte subpopulation in CRS patients.

Methods:

Endoscopic sinonasal mucosal biopsies were taken intraoperatively from the ethmoid bulla of 13 CRS patients undergoing endoscopic sinus surgery (ESS) and 8 control subjects undergoing trans-nasal approaches for non-CRS diseases such as lacrimal, orbital or skull base non-hormonally active lesions. Immunohistochemistry for CD8+ lymphocytes and *S. aureus* was performed and extended to include neutrophils as markers of innate immune activation and other lymphocyte subsets including CD4+ (Cluster of Differentiation 4+), CD16/56+ (Cluster of Differentiation 16/56+) natural killer and δγ TCR lymphocytes. Unpaired student test and Pearson's correlation coefficient were used for statistical analysis.

Results:

A lower level of CD8+ lymphocytes in the epithelium of sinus biopsies from CRS patients was noted (CRS: 37 cells/field vs. controls "CTL": 178 cells/field; p=0.001). Presence of intraepithelial S. aureus was significantly higher in CRS biopsy samples (CRS: 259 cells/field vs. CTL: 110 cells/field; p=0.001), with a strong inverse correlation noted between intraepithelial

S. aureus and CD8+ lymphocyte levels (r = -0.63). Neutrophils and CD4+ lymphocytes were significantly higher in CRS samples than controls; however, levels of other cells were similar.

Conclusion:

Presence of increased levels of intracellular S. aureus in sinus mucosa is a feature of CRS and is associated with significant depressed infiltration of CD8+ lymphocytes. As innate and MHC-II (Major Histocompatability Complex-II) mediated responses appear conserved, this suggests that S. aureus may be selectively modulating MHC-I immune responses locally to escape destruction by CD8+ cytotoxic lymphocytes.

4.3. Introduction:

Chronic rhinosinusitis (CRS) is an epidemiologically important chronic inflammatory disease process, with significant well-documented economic implications (35, 36). Moreover, CRS confers a significant negative impact on patient quality of life analogous to that of congestive heart failure and chronic obstructive pulmonary disease (12). Yet, despite the commonality and severity of CRS, little is known about the exact pathophysiologic mechanism underlying this disease process.

Presence of innate and adaptive defense mechanisms within the sinus mucosa are essential to protecting against invasive pathogens and clearing infected cells. We suspect that defects in immunity contribute to CRS pathogenesis, interfering with pathogen detection and effector functions thereby allowing persistence of pathogens within infected cells.

CD8+ lymphocytes are an important element of the cellular adaptive immunity, acting via MHC-I signaling to kill damaged or dysfunctional cells, including cells infected with pathogens. How their reduction in number and/or function could contribute to the development of disease remains unknown. However, evidence is emerging that this could be via facilitating persistent bacterial infection, which then behaves as a biologic modifier. Recent CRS literature is increasingly highlighting immune evasion by bacteria as another factor contributing to development of disease.

The earliest suggestion of bacteria employing immune evasion strategies to facilitate their persistence dates back more than 10 years with the first reported publication demonstrating the presence of bacterial biofilms in CRS (37). Although the discovery of bacterial biofilms is centuries old, the notion that bacteria are evading immune detection by forming organized communities shrouded in a protective polysaccharide matrix has strongly resonated within the scientific community directing considerable research into diagnostic strategies and therapeutic eradication. Additional research suggests that bacterial immune evasion extends below the nasal mucosal surface with evidence of intramucosal bacterial microcolonies (*S. aureus* in particular) in CRS patients in the absence of a local immune response (31, 38, 39).

S. aureus is a frequently implicated pathogen in CRS. As a pathobiont (bacteria with the potential to behave as a pathogen or as a symbiotic organism), *S. aureus* is capable of colonizing hosts asymptomatically but can also cause severe infections under selected circumstances. The mechanisms underlying pathobiosis are unknown, although certain theories have been suggested. The Madrenas group has shown that *S. aureus* may promote commensalism by triggering a TLR-2 dependent; PI3K/AKT mediated IL-10 response that down regulates pro-inflammatory T cell host responses (8, 40-42). Taken together, the above findings suggest that *S.* Aureus may be locally modulating host immunity to elude destruction by host response.

4.4. Objectives:

We believe that impaired local immunity at the level of the sinus mucosa is implicated in S Aureus persistence in CRS. In order to verify this hypothesis, we aimed to evaluate the relationship between intracellular S. aureus and lymphocyte subpopulations in CRS patients.

4.5. Methods:

Endoscopic sinonasal mucosal biopsies were taken intraoperatively from the ethmoid bulla of 13 CRS patients and 8 control subjects. Immunohistochemistry for CD8+ lymphocytes and *S. aureus* was performed and extended to include neutrophils as markers of innate immune activation and other lymphocyte subsets including CD4+, CD16/56+ natural killer and δγ TCR lymphocytes.

4.5.1. Ethical approval

The study was approved by the ethical review board at Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM). All subjects were aged 18 years or more and voluntarily participated following informed consent.

4.5.2. Study Subjects

13 patients who met criteria for CRS as per published AAO-HNS guidelines (43) and who had previously failed at least one course of maximal medical therapy were recruited. 8 Non-CRS controls who were undergoing a trans-nasal endoscopic approach for access to structures of the orbit, lacrimal system or skull base were also recruited. Exclusion criteria included a history of immune deficiency or cystic fibrosis. No patient received corticosteroids or antibiotics (oral or topical) in the 30 days prior to surgery.

4.5.3. Biopsy preparation for Immunohistochemistry

Surgical biopsy samples were taken at the level of the anterior ethmoid bulla. Biopsy samples were immediately transported on dry ice in a moist sterile compress in a sterile container to the pathology lab situated within the same hospital. Samples were then cut into $5mm^2$ sections, submerged in optimal cutting temperature compound (OCT) and stored at -80°C. Frozen biopsies were cut into 5 micron sections then mounted and fixed on glass slides with ethanol/methanol 60/40%. Slides were stored at -80°C until use (44).

4.5.4. Immunohistochemistry (IHC) Staining

Immunohistochemistry (IHC) staining was performed on frozen sections obtained from the CRS surgical biopsies and control groups. A modified immunoperoxidase method of immunohistochemistry was performed. Five-micron frozen sections were thawed and rinsed in Phosphate Buffered Saline (PBS) followed with 0.2% Triton X100 in PBS for 15 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 minutes at room temperature. The slides were washed in PBS and pretreated with universal blocking solution (Dako, Toronto, ON, Canada) for 30 minutes. Section slides were incubated overnight at 4°C with primary diluted CD8+ antibody (goat anti-human, Dako, Toronto, Canada) and S. *aureus* (rabbit anti-human, ABCAM, Toronto, Canada). The slides were rinsed and incubated with the appropriate biotinylated secondary antibody for 30 min at room temperature. After washing in PBS, Streptavidin/Horse Radish Peroxidase complex (Vector, Canada) was applied for 30 min at room temperature. The reaction result was visualized with DAB/hydrogen peroxide (DAB "3,3'-diaminobenzidine" Kit, Dako, Toronto, Canada). Sections were rinsed in distilled water, lightly stained with hematoxylin, dehydrated, cleared, and cover slipped (44). In a similar fashion, we elected to stain for neutrophils (with mouse anti-human neutrophil elastase, Dako, Toronto, Canada), CD4+ lymphocytes (with mouse anti-human, BioLegend, San Diego, California) CD16/56 natural killer lymphocytes (with mouse anti-human, Dako, Toronto, Canada) as well as δγ TCR lymphocytes (with mouse anti-human, Dako, Toronto, Canada).

4.5.5. Immunohistochemistry Scoring and Statistical analysis

Five randomly selected fields with intact nasal mucosa were examined under light microscope (Olympus CX31, Waltham, MA, USA) at 20X magnification by two separate observers. Positively staining epithelial and subepithelial cells were counted and the average was taken. Results were compared and re-analyzed when greater than 5% difference was seen between observers. Unpaired student test and Pearson's correlation coefficient were used for statistical analysis.

4.6. Results:

All 21 patients (13 CRS patients and 8 control subjects) underwent surgical biopsies at the time of surgery. Only biopsies with sufficient areas of undamaged attached epithelium to allow analysis of 5 non-adjacent fields were retained for analysis. Luckily, all subjects had sufficient mucosa to be analyzed. Basic demographic and laboratory data is presented in Table 4.1.

Table 4.1: Basic demographic and laboratory data:

	Patients (n=13)		Controls (n=8)		
	Average	SD	Average	SD	p-value
Age	47.92	10.05	42.75	12.95	0.318
Males: Females (Using Fisher's test)	5:8	-	4:4	-	0.673
WBC (4.0-11.0 x10E9/L)	7.2	2.65	7.6	2.76	0.744
Neutrophils (1.4-7.7 x10E9/L)	4.47	2.33	5.25	2.20	0.456
Eosinophils (0.0-0.8 x10E9/L)	0.28	0.17	0.05	0.08	0.002
Lymphocytes 1.0-4.0 x10E9/L)	1.9	0.53	1.9	0.77	1.000
Total serum IgE (Less than 100kIU/l)	179.70	196.70	107.71	214.36	0.441

Cell counts for CRS (13 Patients and Control subjects are summarized in Table 4.2 and Table 4.3. More epithelial cells contained S. aureus in patients than controls (258.54 cells/field vs. 110.38 cells/field; p-value: 0.001). Despite this high level of bacterial presence, CRS patients had lower levels of intra-epithelial CD8+ lymphocytes when compared to controls (43.46 cells/field vs. 177.63 cells/field; p-value: 0.001) with a strong inverse correlation between the two (r= -0.63). Brief subgroup analysis (6 patients with nasal polyposis and 7 patients without polyps) showed similar results except that mucosa of patients without polyposis have even less amounts of CD8+ lymphocytes and more total IgE.

Table 4.2: Cell counts/field; taken as the average of five **epithelial** fields at 20X magnification:

	Patients (n=13)		Controls (n=8)		p-value
	Average	SD	Average	SD	
Cells containing S. aureus	258.54	69.08	110.38	50.72	0.0001
CD8+ lymphocytes	43.46	43.57	177.63	42.41	0.0001
Neutrophils	103.77	50.84	18.38	13.29	0.0020
CD4+ lymphocytes	17.33	6.1	11.5	3.25	0.0240
CD16/56+ lymphocytes	16.83	15.14	13.63	3.29	0.5667
δγ TCR lymphocytes	7.42	5.25	3.63	2.2	0.0710

Table 4.3: Cell counts/field; taken as the average of five **sub-epithelial** fields at 20X magnification:

	Patients (n=13)		Controls (n=8)		p-value
	Average	SD	Average	SD	
Neutrophils	363.92	245.04	63.64	34.48	0.0029
Cells containing S. aureus	12.77	5.15	15.86	8.71	0.3166
CD8+ lymphocytes	543.38	221.63	570	332.07	0.8272
CD4+ lymphocytes	292.83	66.79	171	85.36	0.0017
CD16/56+ lymphocytes	41.08	24.17	44.75	11.67	0.6944
δγ TCR lymphocytes	4.23	1.17	2	2.51	0.0119

For demonstration purposes, Figures 4.1 and 4.2 show selected fields with extreme counts. At the subepithelial level, we noted no significant difference, neither in CD8+ lymphocytes, nor in S. aureus.

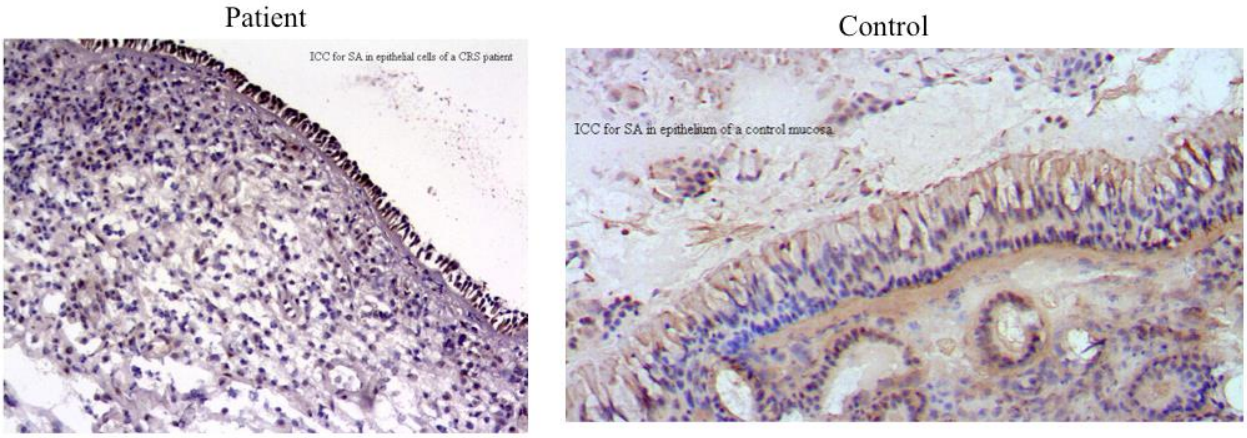

Figure 4.1: Immunohistochemistry for *S. aureus* in a specimen taken from a CRS patient (left) and in a control specimen (right); shown at 100X. Cells in brown represent cells infected by S. aureus. Note the difference between the specimen taken from a patient when compared to that of a control subject.

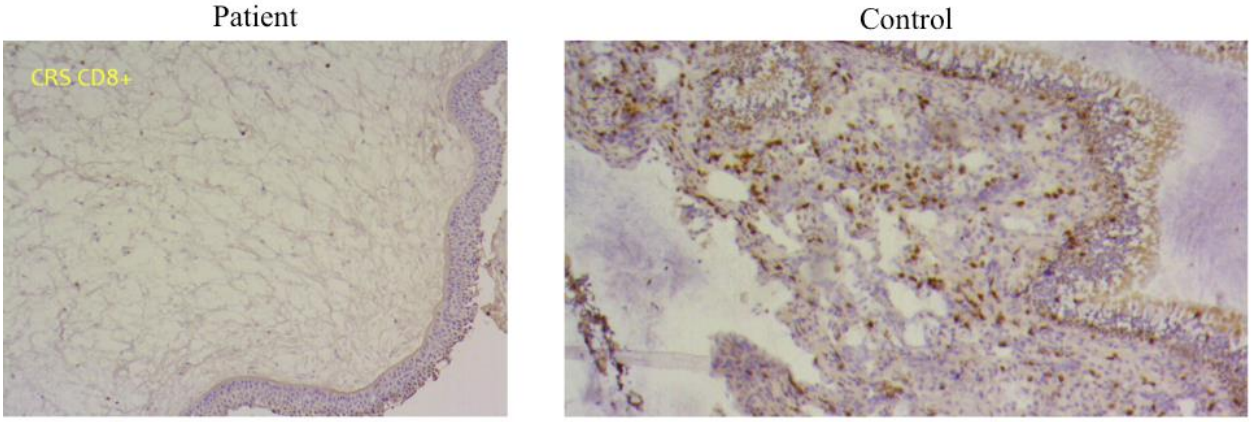

Figure 4.2: Immunohistochemistry for CD8+ lymphocytes in a specimen taken from a CRS patient (left) and in a control specimen (right); shown at 100X. Cells in brown represent CD8+ lymphocytes. Note the difference between the specimen taken from a patient when compared to that of a control subject.

As what we would expect with a chronic inflammatory process, neutrophils were higher in the CRS group. At the subepithelial level, a greater number of CD4+ T-helper lymphocytes were seen, as were δγ TCR lymphocytes. In epithelium, levels of CD4+ T-helper lymphocytes were higher, but there was no difference in δγ TCR lymphocytes. There was no significant difference in CD16/56+ ('natural killer') lymphocytes at either epithelial or subepithelial levels.

4.7. Discussion:

The above results showed that decreased levels in CD8+ lymphocytes were present at the epithelial, but not at the subepithelial level in CRS sinus mucosal biopsies, suggesting the presence of localized MHC-I mediated immune dysfunction. This suggests that, as these decreases were inversely correlated with the levels of S. aureus present in the epithelium, that there was an MHC-I immunity defect limited to the epithelium, which was responsible for the increased bacterial burden.

This appears limited to the MHC-I signaling as innate and adaptive immunity, as reflected by levels of neutrophils and NK cells as markers of innate inflammation, and of CD4+ lymphocytes as surrogates of MHC-II activation, appearred conserved. Whether this is primary and subsequent to a pre-existing immune defect, or locally induced by bacterial modulation of host immune responses is currently unknown. Nevertheless, this is a novel observation and suggests a new facet to the elaboration of pathophysiologic mechanisms in CRS, by suggesting that bacteria may manipulate their local microenvironment to avoid detection.

The concept of S. Aureus modulating immune activity in CRS using secreted virulence factors is not new (45) but has to date implicated staphylococcal enterotoxins or "superantigens" stimulating non-specific T-cell activation. The concept suggested here, that it might act as a biological modifier to elude inflammatory responses, is a novel one but articulates well with our evolving concepts of CRS.

CRS has been described as a feature of humoral immune deficiencies, but more recently, deficiencies in innate and adaptive immune signaling have also been described. Variations in genes coding for the elements of innate immunity have been described in CRS and have a functional impact. Variations in genes for IRAK-4, an important intermediary in TLR signaling, are seen in CRS and have an impact on total serum IgE(33). More recently, we described that polymorphisms in the CD8A gene, which codes for CD8+ cytotoxic lymphocytes, were associated with a refractory CRS population (46). In a follow up study confirming the potential role of CD8+ cells in the pathogenesis of CRS, we demonstrated that 16% of CRS patients had low circulating levels CD8+ lymphocytes when assed by lymphocyte subtyping (47).

While the possibility of a genetically-determined deficiency in immune function is supported by the our previous work, the frequency of these deficiencies is nevertheless low, and fails to account for the somewhat ubiquitous reduction in CD8+ lymphocytes at the mucosal level seen in this study. In addition, this defect appears specific to CD8+ cells, as these patients otherwise demonstrated intact innate immune responses with conserved neutrophilic and CD16+56+ Natural Killer cells responses and intact MHC II immunity as reflected by levels of CD4+ T-helper cells which were comparable in both CRS and CTL groups.

S. aureus is frequently implicated intraepithelial pathogen in chronic rhinosinusitis (31, 48). We suggest that in CRS, S. aureus may exert an effect by modulating local host immunity to elude destruction by CD8+ lymphocytes (42). Its persistence locally may allow it to behave as a biologic modifier ensuring chronicity of the disease process. While further research confirming this effect and exploring implicated mechanisms is required, results from this study nevertheless suggest that we may need to expand the strategies of S. aureus in CRS beyond superantigens production so that it includes immune evasion as survival mechanism.

At this moment, with limited number of patient in this study and given the fact that there is no clear definition of normal limits of CD8+ lymphocytes in sinonasal mucosa, it is difficult to correlate its percentage to the percentage of patients with low systemic CD8+ lymphocytes that is going to be addressed in the next manuscript.

Emerging evidence from other organ systems supports the notion that bacterial flora is a crucial element for conditioning and influencing immune responses in both health and disease. This enhanced appreciation of the importance of pathogenic behaviors, such as bacterial evasion, suggests that modifications of current concepts and strategies are required to treat this disease beyond the surface biofilm and to target intraepithelial bacteria deep in the mucosal surface. Novel anti-bacterial strategies, either using novel antibiotic treatments, or attempts to re-engineer the sinus microbiome using healthy bacteria may offer novel therapeutic strategies in the future.

4.8. Conclusion:

Presence of increased levels of intracellular S. aureus in sinus mucosa was a feature of CRS and was associated with significantly depressed infiltration of CD8+ lymphocytes. As innate and MHC-II mediated responses appear conserved, this suggested that S. aureus may be selectively modulating MHC-I immune responses locally to escape destruction by CD8+ cytotoxic lymphocytes. This may require reconsideration of our current understanding of the role of S. auerus in CRS, which may have important potential therapeutic applications.

4.9. Linking Statement To The Third Manuscript:

The laboratory methodologies used in the studies presented in chapters 3 and 4 could not be applied in typical clinical practices. The next chapter will attempt to move from bench to bedside. It will try to determine whether some patients with CRS might exhibit low CD8+ lymphocytes. It will do this by using a more readily available blood test that could be used in day-to-day practice and then comparing their clinical presentation and outcomes to the rest of CRS patients.

5. MANUSCRIPT 3: CLINICAL FEATURES OF CYTOTOXIC CD8+ T-LYMPHOCYTE DEFICIENCY IN CHRONIC RHINOSINUSITIS PATIENTS: A DEMOGRAPHIC AND FUNCTIONAL STUDY

5.1. Title Page:

Nathalie Gabra, MD[1*]; Saud Alromaih, MD, FRCSC[2*]; Leandra Mfuna Endam, MSc[3]; Rose Marie Brito, MSc[4]; Sawsan Al-Mot, MSc[3]; Francois Larivière, MD, Françoise LeDeist, MD, PhD[4-6]; Martin Desrosiers, MD, FRCSC[3, 7].

[1]Faculty of Medicine, University of Montreal, Montreal, Quebec, Canada;

[2]Department of Otolaryngology-Head and Neck Surgery, McGill University, Montreal, Quebec Canada;

[3]Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Montreal, Quebec, Canada;

[4]Centre Hospitalier Universitaire (CHU) Sainte-Justine, Research Center, Montreal, Quebec, Canada;

[5]Department of Microbiology and Immunology, University of Montreal, Montreal, Quebec, Canada;

[6]Department of Pediatrics, University of Montreal, Montreal, Quebec, Canada;

[7]Division of Otolaryngology-Head and Neck Surgery, Centre Hospitalier de l'Université de Montréal (CHUM), Montreal, Quebec, Canada.

Published in:

International Forum of Allergy and Rhinolog. 2014 Jun;4(6):495-501.

doi: 10.1002/alr.21313. Epub 2014 Mar 17.

*These two authors contributed equally to this work.

This project was presented at the Annual Meeting of American Rhinologic Society, San Francisco, CA in September 2011.

5.2. Abstract:

Introduction And Objectives:

Identification of Staphylococcus Aureus (S. auereus) intracellularly in chronic rhinosinusitis (CRS) suggests an underlying cellular immunodeficiency. Supporting this, we have previously reported low CD8+ (Cluster of Differentiation 8+) cytotoxic T-lymphocyte levels in a sub-population of CRS patients (26) and identified polymorphisms in the CD8A (Cluster of Differentiation 8A) gene associated with CRS (46). In order to better understand the role of low CD8+ in CRS, we wished to determine the phenotype for CRS/low CD8+ in comparison to that of conventional CRS.

Methods:

Sixty-seven CRS patients identified with low CD8+ lymphocytes were compared with an existing population of 480 patients with CRS with nasal polyposis previously recruited for genetic association studies. They were compared for demographics; disease evolution and bacteriology on endoscopic culture.

Results:

Mean level of CD8+ lymphocytes in the CRS/low CD8+ population was 0.15x109/L (N=0.20-1.5x109)/L. There was no difference between both groups in terms of history of allergy, asthma, eczema, Acetylsalicylic acid (ASA) intolerance or smoking. The bacteriology was similar between both groups (S Aureus: CRS/low CD8+: 35 %; CRS 32 %, p=0.643). Evolution of disease was somewhat milder in CRS/low CD8+, with fewer patients requiring surgery, and first surgery performed at a more advanced age. However, antibiotic use was higher in CRS/low CD8+. Subgroup analysis restricted to CRSwNP/low CD8 or CRSsNP/low CD8+ phenotypes did not substantially alter these results.

Conclusion:

Low CD8+ levels are often identified in CRS patients; however, these patients have disease remarkably similar to those with conventional CRS. This suggests that immune deficiency, whether systemic or locally mediated, can be well tolerated and may be present in CRS. CRS patients with low CD8+ levels may possibly require antibacterial therapies as part of ongoing management.

5.3. Introduction:

The lining of the paranasal sinuses represents a mucosal interface between the internal and external environments where antigens and pathogens are encountered and cleared with a minimal inflammatory reaction. However, in a considerable proportion of individuals, persistent inflammation of the sinus mucosa, with intense cellular infiltrate and bacterial colonization occurs, resulting in chronic rhinosinusitis (43).

While current efforts attempt to understand the pathology of the disease by describing the immunologic and bacteriologic factors encountered in surgical biopsy specimens, early events in pathogenesis and etiologic factors predisposing to development of CRS remain mostly unknown (21). However, current thinking is evolving to suggest that CRS is a multifactorial disease, possibly secondary to presence of microbes within the sinus mucosa in a host with an altered immune function or mechanical epithelial barrier of genetic or toxic origin (49).

In 2010, using a PNA-FISH method, Sachse et al. demonstrated the presence of intracellular Staphylococcus Aureus (S Aureus) in the sinus epithelium of chronic rhinosinusitis with nasal polyposis (CRSwNP) (50), a finding which has since been confirmed by other authors (51). Among other mechanisms, S Aureus is believed to contribute to development of CRS via bacterial secretion of enterotoxins or superantigens (SAg) which result in a massive activation of T cells, with Th2-dominated cytokine release and subsequent eosinophil recruitment and a local IgE response (52-54).

However, this ignores the question of how S Aureus establishes residence within the sinus mucosa. As the presence of intracellular pathogens is a hallmark of some innate and adaptive immune deficiencies (55), the presence of intracellular S Aureus in CRS suggests these may contribute to the development of disease.

Evidence for a role in immune deficiency in CRS has already been suggested by studies of innate immunity in CRS by the documentation of reduced number of TLR2 receptors in patients with CRS (56-58) and a reduced function of TLR2 receptors in epithelial cells cultured from CRS subjects (6, 7). Altered function of major histocompatibility complex-I (MHC-I)

molecules and/or of CD8+ lymphocyte functions may also play a role. Children with MHC-I deficiency present with respiratory infections and purulent sinusitis as part of their phenotype (59). In support of a possible role for MHC-I/CD8+ lymphocyte dysfunction as an etiologic factor in CRS, we have previously documented that polymorphisms in genes coding for CD8A and Tapasin binding-protein (TAPBP) are associated with CRS (46). A functional role for the identified polymorphisms was suggested by the demonstration of a genotype specific difference in evolution of sinus disease in this population.

In the light of the emerging importance of immunodeficiencies extending beyond what was previously suspected and their potential implications in CRS pathogenesis, we have over the past years incorporated blood lymphocyte sub-population as part of our investigation for patients with CRS coming to our tertiary care center.

In an earlier study, we initially described a group of patients with low CD8+ T-Lymphocytes levels in a selected population of severe CRS patients screened with lymphocyte immunophenotyping as part of assessment of CRS refractory to medical and surgical therapy with possible symptoms of immune deficiency as suggested by of pneumonia or IV antibiotic use for infection (26, 60). In these selected refractory CRS population, below normal levels of CD8+ lymphocytes were identified in 13% of assessed patients. Given the unexpectedly high frequency of low CD8+ T-lymphocytes levels seen in this group, lymphocyte immunophenotyping has since become a routine part of assessment of patients consulting for CRS.

In order to better understand the consequences of CD8+ T-lymphocytes lymphopenia in CRS, we aimed to determine the CRS phenotype associated with low blood CD8+ T-lymphocyte in comparison to that of conventional CRS. Additionally, we aimed to explore whether low circulating CD8+ T-lymphocytes levels involved a peculiar CD8+ lymphocyte population.

5.4. Methods:

5.4.1. Ethical Consideration:

Approval for this project was obtained from the institutional review board of the Centre Hospitalier de l'Université de Montréal. All patients undergoing functional studies gave informed consent.

5.4.2. Study Population:

Lymphocyte immunophenotyping is routinely performed in patients consulting for CRS in our tertiary sinus center. Patients with CRS and low CD8+ T lymphocytes were identified between June 2009 and January 2012 and a retrospective chart review of their demographic factors was performed. In an attempt to understand the importance of this finding, a subpopulation of 8 patients with CRS/low circulating CD8+ lymphocytes was assessed for a more complete lymphocyte subpopulation study.

5.4.3. Demographics And Clinical Features:

Demographic parameters, medical history, disease characteristics, laboratory results and bacteriology on endoscopic sinus culture were assessed. This population was then compared to an existing database of 480 patients with CRS with nasal polyposis (CRSwNP) recruited over a two-year period from 2007-2009 for genetic association studies, for which similar demographic information had been recorded.

5.4.4. Lymphocyte Subpopulation Study:

The following monoclonal antibodies (MAb) were used in the immunofluorescence studies: anti-HLA ABC: G46-2.6 (IgG1), anti-CD3: Leu4 (IgG2a;), anti-CD4: Leu 3a (IgG1,), anti-CD8: Leu 2a (IgG1), anti-CD19: HIB (IgG1), anti-CD56: MY31 (IgG1), anti –CD197: 3D12 (IgG2a) from Becton Dickinson, San Diego, CA and anti-TCR αβ: BMA031 (IgG1,); anti-TCR γδ: IMMU 510 (IgG1), anti-CD45RA ALB11 (IgG1) anti-CD31: WM59 (IgG1), anti-TCRBV1 (IgG1), anti-TCRBV2 (IgM) from Beckman Coulter, Mississauga, Canada. Fluorescence staining was done with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)- or allophycocyanin (APC)- or PE-Cy7 -conjugated mAbs on whole blood. Cells were analysed on FACSCanto flow cytometer (Becton Dickinson) using FACSDiva software.

5.4.5. Statistical analysis

Statistical study was performed using SPSS (version 20) software (SPSS, Inc, an IBM Company, Chicago, Illinois). Normal distribution of the population was verified using Shapiro-Wilk tests. The comparisons of the two groups were studied using Student T-tests, ANOVA test and Pearson Chi-Square tests. A p-value ≤ 0.05 was considered statistically significant.

5.5. Results:

5.5.1. Demographics And Phenotyping:

A total of 67 CRS patients with low CD8+ T lymphocytes level (CRS/low CD8+) were identified during this period. The average peripheral blood count of CD8+ T lymphocytes was 0.15x10E9/L. CD4+ T cells, the overall lymphocytic count and eosinophilia, were within normal limits (Table 5.1). Determination of levels of immunoglobulins IgA, IgG and IgM was performed simultaneously with lymphocyte immunotyping in all subjects. In all low CD8 subjects save one this was within normal ranges.

Table 5.1: Laboratory values in the CRS/ low CD8+ T-lymphocytes group:

Parameter	Average	Normal range
CD4+ T cells	0.63± 0.23	0.4-2.0 x10E9/L
CD8+ T cells	0.15± 0.04	0.2-1.5 x10E9/L
CD4/CD8 ratio	4.2	1.0-3.5
Total serum IgE	286± 1223	0-100 kUI/L
Lymphocytes	1.22± 0.34	1.0-4.0 x10E9/L
Eosinophils	0.28± 0.33	0.0-0.8 x10E9/L

In the CRS/low CD8+ population, 68 % had CRSwNP. When compared to the 480 patients with CRSwNP, the CRS/Low CD8+ population was significantly older ($p<0.001$). When the age factor was controlled for, there is no difference between the two populations groups in terms of gender, history of asthma, ASA intolerance, eczema or smoking (Table 5.2). The

bacteriology on sinus endoscopic culture was also similar between both groups with no difference in the prevalence of S. Aureus (p=0.643) (Table 5.3).

Table 5.2: Baseline demographic data:

Parameter	CRS/Low CD8+	CRSwNP	p-value
Age in years** N	56.7±11.42 67	49.5±12.5 480	<0.001*
Male N	47.8 % 67	41.5 % 480	0.328
CRSwNP N	68 % 45	100 % 480	-
History of Asthma N	55.4 % 65	57.7 % 478	0.719
ASA intolerance N	16.1 % 56	27.4 % 471	0.069
Smoking N	4.3 % 46	9.7 % 476	0.233

Statistical analysis using Chi-Square test and Student t-test.

*Age factor controlled with ANOVA test.

Table 5.3: Bacteriology on endoscopically-obtained sinus culture:

Parameter	CRS/Low CD8+ N= 67	CRSwNP N= 383	P-value
E. Coli	4 %	4.4 %	0.810
H. Influenzae	2 %	1.8 %	0.988
P. Aeruginosa	7 %	7.5 %	0.970
S. Aureus	35 %	32 %	0.643
S. Pneumoniae	12.9 %	6 %	0.108

Statistical analysis using Chi-Square test.

Evolution of the disease however appears to be different in both groups. Severity of the disease as assessed by need for endoscopic sinus surgery to gain disease control was milder in the CRS/low CD8+ group (Table 5.4). Only 51% of the CRS/low CD8+ group underwent endoscopic sinus surgery, as compared to 75% of the CRSwNP group (p=0.001). In addition, age at first surgery was higher in the CRS/low CD8 group, again suggesting lesser degree of severity of the disease. In patients with low CD8+ having previously undergone ESS, there was trend to a lower lifetime number of sinus surgeries, which did not attain significance. However, management strategies appear to differ, with a significantly higher rate in the use of oral antibacterial therapies received over the past year in the CRS/Low CD8+ group (Table 5.4).

Table 5.4: Evolution of disease of the CRS/low CD8 vs. reference CRSwNP groups:

Parameter	CRS/Low CD8+	CRSwNP	p-value
History of Sinus Surgery N	51 % 60	75 % 358	0.001
Age at 1st surgery N	48.4± 12.9 48	38.3 357	<0.001
Number of surgeries N	1.28± 1.24 67	2.28 373	0.116
Number of oral antibiotics prescribed in past years N	1.24± 1.69 46	1.06± 1.92 476	0.019
Intermittent Antibiotics N	34 % 56	5 % 472	<0.001
Continuous Antibiotics N	22 % 58	1 % 472	<0.001

Statistical analysis using Chi-Square test and Student t-test.

Subgroup analysis restricted to the CRSwNP/low CD8+ lymphocytes or CRSsNP/low CD8+ lymphocyte phenotypes did not modify these results. Apart from the previously noted age difference, there are no differences between the CRS/low CD8+ and reference CRSwNP group, nor between the CRSsNP/low CD8+ and CRSwNP/low CD8+ groups. While use of intermittent antibiotics was slightly greater in the CRSsNP/low CD8+ group as compared to CRSwNP/low CD8 group, in both instances these remained higher than in the reference population (Tables 5.5, 5.6 and 5.7).

Table 5.5: Subgroup analysis according to CRS phenotype: Demographic data and circulating CD8+ level.

Parameter	CRwNP/Low CD8+	CRSwNP	p-value	CRSsNP/Low CD8+	CRSwNP	p-value	CRSsNP/Low CD8+	CRwNP/Low CD8+	p-value
CD8+ T cells	0.15±0.04	-	-	0.16±0.02	-	-	0.16±0.02	0.15±0.04	0.874
	45			22			22	45	
Age in years	56.7±13.1	49.5±12.5	<0.001*	55.1±10.4	49.5±12.5	<0.001*	55.1±10.4	56.7±13.1	0.832
N	45	480		22	480		22	45	
Male	44 %	41.5 %	0.678	55.6 %	41.5 %	0.258	55.6 %	44 %	0.534
N	45	480		22	480		22	45	
History of Asthma	60 %	57.7 %	0.769	40.9%	57.7 %	0.493	40.9%	60 %	0.248
N	45	478		22	478		22	45	
ASA intolerance	16.6%	27.4%	0.242	14%	27.4%	0.174	14%	16.6%	0.691
N	42	471		14	471		14	42	
Smoking	3%	9.7 %	0.164	7.6%	9.7 %	0.759	7.6%	3%	0.113
N	33	476		13	476		13	33	

Statistical analysis using Chi-Square test and Student T-test.

*Age factor controlled with ANOVA test.

Table 5.6: Frequency of recovery for Staphylococcus aureus according to CRS phenotype.

Parameter	CRwNP/Low CD8+	CRSwNP	p-value	CRSsNP/Low CD8+	CRSwNP	p-value	CRSsNP/Low CD8+	CRwNP/Low CD8+	p-value
S. Aureus	36 % 45	32 % 480	0.632	37% 22	32% 480	0.617	37% 22	36% 45	0.852

Statistical analysis using Chi-Square test and Student T-test.

Table 5.7: Evolution of the disease: Sub group analysis.

Parameter	CRwNP/Low CD8+	CRSwNP	p-value	CRSsNP/Low CD8+	CRSwNP	p-value	CRwNP/Low CD8+	CRSsNP/Low CD8+	p-value
History of Sinus Surgery	54 %	75 %	0.017*	47%	75 %	0.012	58 %	47%	0.628
N	43	358		17	358		43	17	
Age at 1st surgery	49.26	38.3	<0.001*	47.3	38.3	<0.001*	49.26	47.3	0.824
N	26	357		22	357		26	22	
Number of surgeries	1.18	2.28	0.044*	1.24	2.28	0.035*	1.18	1.24	0.075
N	38	373		19	373		38	19	
Number of oral antibiotics prescribed in past year N	1.34± 1.6 31	1.06± 1.92 476	0.019*	1.22 15	1.06± 1.92 476	0.039	1.34± 1.6 31	1.22 15	0.091
Intermittent Antibiotics	25 %	5 %	<0.001*	56%	5 %	<0.001*	25 %	56%	0.038
N	40	472		16	472		40	16	*
Continuous Antibiotics	21 %	1 %	<0.001*	28%	1 %	<0.001*	21 %	28%	0.256
N	42	472		16	472		42	16	

Statistical analysis using Chi-Square test and Student T-test.

5.5.2. Lymphocyte Subpopulation Study:

In 9 patients with previously documented CD8+ T-lymphopenia, we repeated the CD8+ lymphocytes measurements and analysed the other blood lymphocyte populations including the CD4+, the B (CD19+) and NK (CD56+CD3-) lymphocytes (Table 5.8). In order to exclude a more global immunodeficiency, naïve thymic (CD45RA+CD31+) CD4+ lymphocytes, memory (CD27+) B-lymphocytes, VB1and VB2 expression on CD4+ and CD8+ T lymphocytes, and HLA Class I expression on lymphocytes and monocytes were studied (Table 5.9).

Table 5.8: Lymphocyte subpopulations in the CRS/low CD8 group. Values indicate the blood population count/mm^3.

Patient	Age	Lymphocytes	CD3+	CD4+	CD8+	CD19+	CD56+CD3-
1	66	1190	869	726	107	179	145
2	70	1530	918	765	107	306	171
3	46	920	690	580	101	74	86
4*	48	1320	1162	977	172	98	3
5	52	1980	1208	1089	59	416	455
6	74	740	459	311	96	107	159
7	78	740	481	392	74	30	142
8**	46	1550	1225	868	310	6	202
9	64	800	480	296	96	104	160

*Patient 4 had a profound natural killer cell (CD56+CD3-) lymphopenia.

**Patient 8 had normal CD8 + T cells level on testing and was excluded from further analysis.

Table 5.9: B-lymphocyte assessment in the CRS/low CD8 group. Values indicate the percentage of Naïve thymic among CD4 T cells (CD45RA+CD31+) and of the memory (CD27+) among B (CD19+) cells.

Patient	Memory B lymphocytes	Thymic naive CD4 lymphocytes
Normal ranges (40-70 years)	12-43	11-40
1	16	14
2	11	14
3	10	24
4	36	32
5	29	11
6	11	5**
7	5	3**
9	16	17

*Patient number 8 had normal CD8 + T cells level and was excluded from analysis.

**Patients were older than 70 years (74 and 78 yrs).

According the decrease of this population in elderly, these values can be considered as normal.

In one patient, CD8+ lymphocyte levels were within normal ranges and no further testing was performed. However, in the other 8 subjects persistently low CD8+ levels were seen. These 8 subjects underwent further functional testing. The blood count of all lymphocyte populations was found to be normal in all patients except two patients; one presented a profound NK lymphopenia (P4) and another one presented low memory B cell counts (P7) (Table 5.6). The MHC-I expression was not different from that observed in healthy controls in lymphocytes (median 28668; SEM 2538 vs. 26108, 2380) and monocytes (31816; 3352 vs. 29813; 2760). The presence of the different functional subsets of CD8+ T cells based on expression of CD45RA and CCR7 detected no major anomaly in the distribution of CD8+ T subsets (Naïve CD45RA+ CCR7+, central memory CD45RA- CCR7+, effector memory CD45RA- CCR7- and CD45RA+ effector memory CD45RA+CCR7-) (Table 5.10).

Table 5.10: Characterization of CD8+ T- cell population in the CRS/low CD8 group. Values indicate the percentage of Naive (CD45RA+CCR7+), TCM (Central memory ;CD45RA+CCR7-). TEM (effector memory CD45RA-CCR7-) and TEMRA (CD45RA+ effector memory CD45RA+CCR7-) among CD8+ T lymphocytes.

Patient (age in years)	Naïve	T_{CM}	T_{EM}	T_{EMRA}
Normal value	2-46	3-23	22-76	12-64
1 (66)	18	6	61	16
2 (70)	9	4	58	29
3 (46)	43	4	37	16
4 (48)	59	5	19	17
5 (52)	36	10	45	10
6 (74)	13	6	63	18
7 (78)	14	4	31	51
9 (64)	35	11	42	12

*Patient number 8 had normal CD8+ T cells levels and was excluded from analysis.

5.6. Discussion:

In this study we report for the first time the presence of CD8+ lymphopenia in a subset of CRS patients, and we assessed the impact of this finding by comparing (a) the phenotype and clinical presentation of CRS in patients with CD8+ T lymphopenia with (b) a reference group of patients with CRSwNP. Our results suggest that while there are no differences between both groups in terms of demographic parameters or bacteriology, however evolution of disease and management strategies differ somewhat. In patients with CRS/low CD8+, evolution of disease is somewhat milder with less patients requiring surgery, and with first surgeries being performed at a more advanced age. In an observation with potential therapeutic implications, antibiotic use was higher in the CRS/low CD8+ T lymphocytes population. These observations remained similar for both CRSwNP/low CD8 and CRSsNP/lowCD8 subtypes when subgroups for each phenotype were assessed separately.

It is initially somewhat surprising that while patients with CD8+ T lymphopenia can be identified in a subset of CRS patients, this does not appear to predict more severe disease. However, this is supported by our previous work on CD8A gene polymorphisms in CRS, where homozygotes for the minor allele presented a milder form of disease as evidenced by a lower frequency of surgery and a later presentation age (46). Taken together, these two findings suggest that immunosuppression is well tolerated at the level of the sinus mucosa. Further support for this concept is furnished by more favorable outcomes following endoscopic sinus surgery for CRS in patients with immune deficiencies (32).

While this study does not establish a direct causal link between CD8+ T lymphopenia and development of CRS, the results are nevertheless intriguing, with potential implications for understanding of CRS. In our current understanding of CRS pathophysiology, questions remain as to how bacteria persist in the sinus mucosa and how they influence inflammation. These topics are widely debated. The possibility that CRS patients may suffer from deficient adaptive immunity may offer a link between these two seemingly unrelated conditions.

CD8+ T lymphopenia may contribute to the development of CRS via impaired adaptive immunity, leading to defective clearance of infected cells due to alterations in the expression and/or function of the MHC-I system. Usually, antigens processed within the cell are presented at the cell surface by MHC-I receptors, allowing recognition and destruction of infected cells by CD8+ T lymphocytes. However, individuals with CD8+ T cell lymphopenia may either have a defect in expression of MHC-I, or an intrinsic defect of CD8+ T lymphocyte differentiation, proliferation and/or survival, possibly allowing the persistence of infected cells at the sinus mucosa. Interestingly, in our patients, despite the identified CD8+ T cell lymphopenia, the expression of MHC-I and functional differentiation of CD8+ T cells were nevertheless normal, suggesting that an intrinsic inability of migration by CD8+ T lymphocytes is *a priori* unlikely (46). The reduction in circulating CD8+ T lymphocytes may impact disease via other functional mechanisms.

Defective clearance of bacteria in infected cells may lead to bacterial persistence, with implications for the inflammation seen in CRS. The bacteria found in sinuses are increasingly

believed to play an important role to the development of CRS by behaving as disease modifiers rather than as primary etiologic agents in the development of CRS (49). These immunomodulatory characteristics have been particularly well described for S. aureus, which is recognized as being capable of infiltrating the nasal mucosa and modulating cellular immune responses via multiple mechanisms (8, 40, 61, 62). Thus, persistence of bacteria via defective clearance of bacteria in infected cells may contribute to the development or persistence of CRS.

In the light of the results of our study, we hypothesize that patients with low CD8+ T lymphocytes in peripheral blood may have low levels of CD8+ T lymphocytes in the nasal mucosa. This may lead to perturbations in the continual interplay between immune effector cells and the intracellular pathogens present in the nasal mucosa of CRS patients. Additionally, the presence of intracellular immunomodulatory pathogens might downregulate the infiltration of CD8+ T lymphocytes locally and a CD8+ lymphopenia would prevent the activation of inflammatory cascades leading to severe CRS. Hence, patients with CD8+ lymphopenia might present with a milder form of CRS initially. However, following endoscopic sinus surgery, the sinus mucosa is more in contact with external bacteria and more severe disease could possibly occur. This intriguing possibility needs to be verified experimentally in a patient group with low circulating CD8+ lymphocytes. Previous reports assessing CD8+ lymphocyte levels in the sinus mucosa have reported variable results.

Our study, despite its potential interest, nevertheless has several limitations. First, because of the retrospective nature of this study, it is difficult to analyze the qualitative clinical data and the symptomatology. Further prospective studies would be more appropriate. Second, we chose to compare our heterogeneous population of CRS/CD8+ lymphopenia with and without nasal polyps (NP) with a CRSwNP population in which immunophenotyping had not been performed as it includes a ready database of huge number of patients. This may lead to possible criticisms that either (a) the reference population of CRSwNP may harbor a large number of unsuspected CD8+ lymphopenic patients, or (b) the CRSsNP subgroups might exhibit different behavior.

Questions as to the incidence of CD8+ lymphopenia in our CRSwNP reference population cannot be answered because this information is unavailable. The use of lymphotyping

as an assessment of immune function in CRS patients has not previously been reported, and thus we have not assessed this parameter during the development of our CRS populations. Also, there is no literature available from which to draw meaningful comparisons. However, even in the absence of lymphocyte subtyping, while our CRS population will necessarily include a certain number of individuals with low CD8 counts, this percentage will likely remain low overall. Based on our previous experience, 13% of selected CRS patients have a lower than normal CD8 level (data not published). Thus the 'reference' CRS population for comparison can be predicted as having 13% of patients with a low CD8 level – also predicting that 87% would have normal values. Thus, the overwhelming majority of cases can be expected to have normal lymphocyte differentials.

As regards potential differences between the CRSwNP and CRSsNP subgroups, in this study, there were no meaningful differences observed on subgroup analysis according to this clinical phenotype. In future studies, affected subgroups may be better explored using an analysis of markers of underlying mechanisms instead (63, 64).

Additionally, lymphocyte levels may fluctuate over time due to illness, cyclical variations, or medication use such as oral corticosteroids. Also, it is possible that we have identified a number of subjects with transitory decreases in lymphocyte levels which may normalize over time (65). Nevertheless, in our population, low CD8+ lymphocyte levels appear to be stable. As 8 of the 9 subjects initially identified with low CD8+ on screening had persistently low CD8+ levels on the retesting performed as part of additional lymphocyte function testing.

Despite these limitations, this study is of interest as it is the first report in the literature to address the phenotypic implication of CD8+ lymphopenia in CRS patients. It contributes to our rapidly expanding understanding of the many facets of immunodeficiency, and such better understanding could be clinically relevant in developing new patient management algorithms according to each case.

5.7. Conclusion:

In summary, these results offer a novel and intriguing potential pathophysiologic mechanism to add to the rapidly improving understanding of CRS. Patients with CD8+ T lymphocytes lymphopenia express a different phenotype when compared to patients with conventional CRS. However, it seems that this immune deficiency, whether systemically or locally mediated, is well tolerated and the clinical presentation is milder than usual. In terms of clinical implications, our results suggest that these patients may occasionally benefit from antibacterial therapies; thus identification of patients with CD8+ lymphopenia using lymphocyte immunophenotyping should be helpful in determining an appropriate and personalized management of the patients.

5.8. Acknowledgements

This study is funded by Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM).

6. OVERALL DISCUSSION:

Polymorphisms within the CD8A genes are implicated in MHC-I immunodeficiency syndromes and are associated with the population of severe CRS. Modified CD8A or TAPBP gene function may contribute to the development of CRS via altered MHC-I function and/or reduction of circulating CD8+ lymphocytes. This was evident by the more severe CRS phenotype associated with the major allele homozygosity.

However, despite this strong link, it remains unknown how this genetic polymorphism contributes to development of disease. Altered CD8A gene function may contribute to the CRS by reducing the clearance of infected intracellular cells. Defective clearance of these infected cells will allow their persistence, which is increasingly believed to play an important role to the development of CRS by behaving as disease modifiers rather than primary etiologic agents in the development of CRS.

While it suggests that these polymorphisms may be influencing CD8+ lymphocyte levels or activity, it is not surprising that this polymorphism does not always follow a more severe course, as immune deficiency does not necessarily predict a worse clinical prognosis for CRS. Interplay between immune effector cells and the intracellular pathogens presents in the nasal mucosa of CRS patients. The presence of intracellular immunomodulatory pathogens might downregulate the infiltration of CD8+ T lymphocytes locally and a CD8+ lymphopenia would prevent the activation of inflammatory cascades leading to severe CRS.

Patients with low CD8+ T lymphocytes in peripheral blood may have low levels of CD8+ T lymphocytes in the nasal mucosa. In patients with CRS/low CD8+, evolution of disease is somewhat milder with fewer patients requiring surgery and with first surgeries being performed at a more advanced age. In an observation with potential therapeutic implications, antibiotic use was higher in the CRS/low CD8+ T lymphocytes population. This immune deficiency, whether systemically or locally mediated, is thus well tolerated and the presentation is milder than usual.

The previous studies have limitations. There are clearly recall biases in the reporting of the number of operations and dates by patients. Every attempt was nevertheless made to ensure maximal possible accuracy within these limitations. All data was collected by using a standardized questionnaire, by the same experienced clinical trial monitor at the time of recruitment. Our patient population did not represent a heterogeneous population while the disease itself differs widely due to regional differences in terms of genetics as well as bacteriology.

7. OVERALL CONCLUSION:

This thesis reports for the first time (a) the impact of CD8A gene polymorphisms on CRS, (b) the inverse relationship between the CD8+ lymphocytes and the intracellular staphylococcus aureus in sinonasal mucosa and, (c) the presence of a subpopulation of CRS patients with low systemic CD8+ cells who behave differently.

It is the first comprehensive report discussing the potential role of CD8+lymphocyte dysfunction in CRS supported by genetic variations and low serum levels of CD8+ lymphocytes in a subpopulation of CRS patients. The demonstration of a link between CD8+ lymphocyte and intracellular staphylococcus aureus in the sinonasal mucosa suggests that local modulation of CD8+ lymphocytes, possibly via secreted mediators, may offer an insight to this observation.

Identification of patients with CD8+ lymphopenia via lymphocyte immunophenotyping is feasible during day-to-day practice and should be helpful in determining an appropriate and personalized management plan for patients identified in this manner. Such patients should receive lower doses of corticosteroids and may benefit more from a long-term antimicrobial therapy.

8. REFERENCES:

1. Bosse Y, Bacot F, Montpetit A, Rung J, Qu HQ, Engert JC, et al. Identification of susceptibility genes for complex diseases using pooling-based genome-wide association scans. Human genetics. 2009;125(3):305-18.
2. Bachert C, Pawankar R, Zhang L, Bunnag C, Fokkens WJ, Hamilos DL, et al. ICON: chronic rhinosinusitis. The World Allergy Organization journal. 2014;7(1):25.
3. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. Rhinology. 2012;50(1):1-12.
4. Henkart PA, Catalfamo M. CD8+ effector cells. Advances in immunology. 2004;83:233-52.
5. Mfuna-Endam L, Zhang Y, Desrosiers MY. Genetics of rhinosinusitis. Current allergy and asthma reports. 2011;11(3):236-46.
6. Desrosiers M MEL, Divoy C, et al. . Bioinformatic Analysis Of Epithelial Cell Gene Expression In Chronic Rhinosinusitis Identifies Constitutive Differences Between CRSwNP And CRSsNP. The Journal of Allergy and Clinical Immunology 2011;127(2-supplement).
7. Divoy C RS, Berube J et al. . Epithelial Cells From Chronic Rhinosinusitis Patients Are Hyporesponsive To Bacteria: Evidence Of Innate Immune Dysfunction In CRS. . The Journal of allergy and clinical immunology. 2011;127(2-supplement):AB2–AB3.
8. Chau TA, McCully ML, Brintnell W, An G, Kasper KJ, Vines ED, et al. Toll-like receptor 2 ligands on the staphylococcal cell wall downregulate superantigen-induced T cell activation and prevent toxic shock syndrome. Nature medicine. 2009;15(6):641-8.
9. Meltzer EO, Hamilos DL, Hadley JA, Lanza DC, Marple BF, Nicklas RA, et al. Rhinosinusitis: establishing definitions for clinical research and patient care. The Journal of allergy and clinical immunology. 2004;114(6 Suppl):155-212.
10. Hamilos DL. Chronic rhinosinusitis: epidemiology and medical management. The Journal of allergy and clinical immunology. 2011;128(4):693-707; quiz 8-9.
11. Rudmik L, Smith TL, Schlosser RJ, Hwang PH, Mace JC, Soler ZM. Productivity costs in patients with refractory chronic rhinosinusitis. The Laryngoscope. 2014;124(9):2007-12.

12. Macdonald KI, McNally JD, Massoud E. The health and resource utilization of Canadians with chronic rhinosinusitis. The Laryngoscope. 2009;119(1):184-9.
13. Wasan A, Fernandez E, Jamison RN, Bhattacharyya N. Association of anxiety and depression with reported disease severity in patients undergoing evaluation for chronic rhinosinusitis. The Annals of otology, rhinology, and laryngology. 2007;116(7):491-7.
14. Damm M, Quante G, Jungehuelsing M, Stennert E. Impact of functional endoscopic sinus surgery on symptoms and quality of life in chronic rhinosinusitis. The Laryngoscope. 2002;112(2):310-5.
15. Patel NA, Ferguson BJ. Odontogenic sinusitis: an ancient but under-appreciated cause of maxillary sinusitis. Current opinion in otolaryngology & head and neck surgery. 2012;20(1):24-8.
16. Desrosiers M, Evans GA, Keith PK, Wright ED, Kaplan A, Bouchard J, et al. Canadian clinical practice guidelines for acute and chronic rhinosinusitis. Executive summary. Journal of otolaryngology - head & neck surgery = Le Journal d'oto-rhino-laryngologie et de chirurgie cervico-faciale. 2011;40 Suppl 2:S91-8.
17. Desrosiers M, Evans GA, Keith PK, Wright ED, Kaplan A, Bouchard J, et al. Canadian clinical practice guidelines for acute and chronic rhinosinusitis. Allergy, asthma, and clinical immunology : official journal of the Canadian Society of Allergy and Clinical Immunology. 2011;7(1):2.
18. Hicks CW, Weber JG, Reid JR, Moodley M. Identifying and managing intracranial complications of sinusitis in children: a retrospective series. The Pediatric infectious disease journal. 2011;30(3):222-6.
19. Mafee MF, Tran BH, Chapa AR. Imaging of rhinosinusitis and its complications: plain film, CT, and MRI. Clinical reviews in allergy & immunology. 2006;30(3):165-86.
20. Georgalas C, Cornet M, Adriaensen G, Reinartz S, Holland C, Prokopakis E, et al. Evidence-based surgery for chronic rhinosinusitis with and without nasal polyps. Current allergy and asthma reports. 2014;14(4):427.
21. Tewfik MA, Bosse Y, Al-Shemari H, Desrosiers M. Genetics of chronic rhinosinusitis: a primer. Journal of otolaryngology - head & neck surgery = Le Journal d'oto-rhino-laryngologie et de chirurgie cervico-faciale. 2010;39(1):62-8.

22. Wang X, Cutting GR. Chronic rhinosinusitis. Advances in oto-rhino-laryngology. 2011;70:114-21.

23. Fung-Leung WP, Schilham MW, Rahemtulla A, Kundig TM, Vollenweider M, Potter J, et al. CD8 is needed for development of cytotoxic T cells but not helper T cells. Cell. 1991;65(3):443-9.

24. Srinivasa BT, Alizadehfar R, Desrosiers M, Shuster J, Pai NP, Tsoukas CM. Adult primary immune deficiency: what are we missing? The American journal of medicine. 2012;125(8):779-86.

25. Wood AJ, Douglas RG. Pathogenesis and treatment of chronic rhinosinusitis. Postgraduate medical journal. 2010;86(1016):359-64.

26. Alromaih S, Mfuna-Endam L, Lariviere F, Begin P, Desrosiers M. Cd8+ Lymphocyte Immunodeficiency: An Important Unrecognized Cause Of Refractory Chronic Rhinosinusitis. American Rhinologic Society meeting; San Francisco, USA2011.

27. Rezaei N. Primary Immunodeficiency Diseases: Definition, Diagnosis, and Management. 1st ed: Springer; 2008.

28. Shen R, Fan JB, Campbell D, Chang W, Chen J, Doucet D, et al. High-throughput SNP genotyping on universal bead arrays. Mutation research. 2005;573(1-2):70-82.

29. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. American journal of human genetics. 2007;81(3):559-75.

30. Casanova JL, Abel L. The human model: a genetic dissection of immunity to infection in natural conditions. Nature reviews Immunology. 2004;4(1):55-66.

31. Corriveau MN, Zhang N, Holtappels G, Van Roy N, Bachert C. Detection of Staphylococcus aureus in nasal tissue with peptide nucleic acid-fluorescence in situ hybridization. American journal of rhinology & allergy. 2009;23(5):461-5.

32. Khalid AN, Mace JC, Smith TL. Outcomes of sinus surgery in ambulatory patients with immune dysfunction. American journal of rhinology & allergy. 2010;24(3):230-3.

33. Tewfik MA, Bosse Y, Lemire M, Hudson TJ, Vallee-Smejda S, Al-Shemari H, et al. Polymorphisms in interleukin-1 receptor-associated kinase 4 are associated with total serum IgE. Allergy. 2009;64(5):746-53.

34. Holland SM, DeLeo FR, Elloumi HZ, Hsu AP, Uzel G, Brodsky N, et al. STAT3 mutations in the hyper-IgE syndrome. The New England journal of medicine. 2007;357(16):1608-19.
35. Anand VK. Epidemiology and economic impact of rhinosinusitis. The Annals of otology, rhinology & laryngology Supplement. 2004;193:3-5.
36. Bhattacharyya N. Incremental health care utilization and expenditures for chronic rhinosinusitis in the United States. The Annals of otology, rhinology, and laryngology. 2011;120(7):423-7.
37. Cryer J, Schipor I, Perloff JR, Palmer JN. Evidence of bacterial biofilms in human chronic sinusitis. ORL; journal for oto-rhino-laryngology and its related specialties. 2004;66(3):155-8.
38. Clement S, Vaudaux P, Francois P, Schrenzel J, Huggler E, Kampf S, et al. Evidence of an intracellular reservoir in the nasal mucosa of patients with recurrent Staphylococcus aureus rhinosinusitis. The Journal of infectious diseases. 2005;192(6):1023-8.
39. Wood AJ, Fraser JD, Swift S, Patterson-Emanuelson EA, Amirapu S, Douglas RG. Intramucosal bacterial microcolonies exist in chronic rhinosinusitis without inducing a local immune response. American journal of rhinology & allergy. 2012;26(4):265-70.
40. Frodermann V, Chau TA, Sayedyahossein S, Toth JM, Heinrichs DE, Madrenas J. A modulatory interleukin-10 response to staphylococcal peptidoglycan prevents Th1/Th17 adaptive immunity to Staphylococcus aureus. The Journal of infectious diseases. 2011;204(2):253-62.
41. Mele T, Madrenas J. TLR2 signalling: At the crossroads of commensalism, invasive infections and toxic shock syndrome by Staphylococcus aureus. The international journal of biochemistry & cell biology. 2010;42(7):1066-71.
42. Peres AG, Madrenas J. The broad landscape of immune interactions with Staphylococcus aureus: from commensalism to lethal infections. Burns : journal of the International Society for Burn Injuries. 2013;39(3):380-8.
43. Benninger MS, Ferguson BJ, Hadley JA, Hamilos DL, Jacobs M, Kennedy DW, et al. Adult chronic rhinosinusitis: definitions, diagnosis, epidemiology, and pathophysiology. Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery. 2003;129(3 Suppl):S1-32.

44. Al-Mot S. Molecular Signatures as a New Classification Scheme for Chronic Rhinosinusitis: McGill University; 2012.

45. Bachert C, Zhang N, Patou J, van Zele T, Gevaert P. Role of staphylococcal superantigens in upper airway disease. Current opinion in allergy and clinical immunology. 2008;8(1):34-8.

46. Alromaih S, Mfuna-Endam L, Bosse Y, Filali-Mouhim A, Desrosiers M. CD8A gene polymorphisms predict severity factors in chronic rhinosinusitis. International forum of allergy & rhinology. 2013;3(8):605-11.

47. Gabra N, Alromaih S, Endam LM, Brito RM, Lariviere F, Al-Mot S, et al. Clinical features of cytotoxic CD8+ T-lymphocyte deficiency in chronic rhinosinusitis patients: a demographic and functional study. International forum of allergy & rhinology. 2014;4(6):495-501.

48. Quinn GA, Tarwater PM, Cole AM. Subversion of interleukin-1-mediated host defence by a nasal carrier strain of Staphylococcus aureus. Immunology. 2009;128(1 Suppl):e222-9.

49. Kern RC, Conley DB, Walsh W, Chandra R, Kato A, Tripathi-Peters A, et al. Perspectives on the etiology of chronic rhinosinusitis: an immune barrier hypothesis. American journal of rhinology. 2008;22(6):549-59.

50. Sachse F, Becker K, von Eiff C, Metze D, Rudack C. Staphylococcus aureus invades the epithelium in nasal polyposis and induces IL-6 in nasal epithelial cells in vitro. Allergy. 2010;65(11):1430-7.

51. Tan NC, Foreman A, Jardeleza C, Douglas R, Vreugde S, Wormald PJ. Intracellular Staphylococcus aureus: the Trojan horse of recalcitrant chronic rhinosinusitis? International forum of allergy & rhinology. 2013;3(4):261-6.

52. Bachert C, Gevaert P, Holtappels G, Johansson SG, van Cauwenberge P. Total and specific IgE in nasal polyps is related to local eosinophilic inflammation. The Journal of allergy and clinical immunology. 2001;107(4):607-14.

53. Seiberling KA, Grammer L, Kern RC. Chronic rhinosinusitis and superantigens. Otolaryngologic clinics of North America. 2005;38(6):1215-36, ix.

54. Zhang N, Gevaert P, van Zele T, Perez-Novo C, Patou J, Holtappels G, et al. An update on the impact of Staphylococcus aureus enterotoxins in chronic sinusitis with nasal polyposis. Rhinology. 2005;43(3):162-8.

55. Foreman A, Holtappels G, Psaltis AJ, Jervis-Bardy J, Field J, Wormald PJ, et al. Adaptive immune responses in Staphylococcus aureus biofilm-associated chronic rhinosinusitis. Allergy. 2011;66(11):1449-56.

56. Ramanathan M, Jr., Lee WK, Dubin MG, Lin S, Spannhake EW, Lane AP. Sinonasal epithelial cell expression of toll-like receptor 9 is decreased in chronic rhinosinusitis with polyps. American journal of rhinology. 2007;21(1):110-6.

57. Ramanathan M, Jr., Lee WK, Spannhake EW, Lane AP. Th2 cytokines associated with chronic rhinosinusitis with polyps down-regulate the antimicrobial immune function of human sinonasal epithelial cells. American journal of rhinology. 2008;22(2):115-21.

58. Ramanathan M, Jr., Spannhake EW, Lane AP. Chronic rhinosinusitis with nasal polyps is associated with decreased expression of mucosal interleukin 22 receptor. The Laryngoscope. 2007;117(10):1839-43.

59. Carneiro-Sampaio M, Coutinho A. Immunity to microbes: lessons from primary immunodeficiencies. Infection and immunity. 2007;75(4):1545-55.

60. Alromaih S, Mfuna-Endam L, Desrosiers M. CD8+ cytotoxic lymphocyte infiltration of the sinus mucosa is reduced in CD8+ deficient patients. . Combined Otolaryngology Spring Meeting-ARS section; San Diego, USA2012.

61. Patou J, Gevaert P, Van Zele T, Holtappels G, van Cauwenberge P, Bachert C. Staphylococcus aureus enterotoxin B, protein A, and lipoteichoic acid stimulations in nasal polyps. The Journal of allergy and clinical immunology. 2008;121(1):110-5.

62. Peters AT, Kato A, Zhang N, Conley DB, Suh L, Tancowny B, et al. Evidence for altered activity of the IL-6 pathway in chronic rhinosinusitis with nasal polyps. The Journal of allergy and clinical immunology. 2010;125(2):397-403 e10.

63. Akdis CA, Bachert C, Cingi C, Dykewicz MS, Hellings PW, Naclerio RM, et al. Endotypes and phenotypes of chronic rhinosinusitis: a PRACTALL document of the European Academy of Allergy and Clinical Immunology and the American Academy of Allergy, Asthma & Immunology. The Journal of allergy and clinical immunology. 2013;131(6):1479-90.

64. Lam M, Hull L, McLachlan R, Snidvongs K, Chin D, Pratt E, et al. Clinical severity and epithelial endotypes in chronic rhinosinusitis. International forum of allergy & rhinology. 2013;3(2):121-8.
65. Hong MS, Dan JM, Choi JY, Kang I. Age-associated changes in the frequency of naive, memory and effector CD8+ T cells. Mechanisms of ageing and development. 2004;125(9):615-8.