THE INNATE IMMUNE RESPONSE TO STAPHYLOCOCCUS AUREUS BIOFILMS ON HUMAN SINONASAL EXPLANTS

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"We shall not cease from exploration,
and the end of all our exploring will be
to arrive where we started
and to know the place for the first time."

T.S. Elliot (1888-1965)

Dedicated to my beloved wife Renata who first crossed the Andes Mountain Range from Brazil to be with me in Chile and now has crossed The Pacific Ocean to accompany me in Australia

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Abstract

Chronic Rhinosinusitis (CRS) is the persistent and symptomatic inflammation of the mucosa of the nose and paranasal cavities. It is a prevalent condition severely affecting the quality of life of around 10% of the population in Western countries. Its pathogenesis involves environmental factors such as viruses or bacteria on predisposed hosts triggering local mucosal inflammation. *Staphylococcus aureus* (*S. aureus*) is the most common isolated bacterium in CRS and, when forming biofilms, increases its resistance to antibiotics, being correlated with recalcitrant cases and higher rates of mucosal inflammation. The local inflammation can be explained by virulence factors from *S. aureus,* and also by innate and adaptive immune mechanisms of the host immune response. Although some researchers have explored the late or adaptive immune response associated with *S. aureus* biofilms, less is known about the initial or innate immune response that *S. aureus* biofilms trigger in the mucosa.

This thesis aimed to study part of the mucosal innate immune response to *S. aureus* biofilms. We have challenged human sinonasal tissues—from normal donors undergoing transnasal pituitary surgery—with *S. aureus* biofilms *ex vivo* using an explant model. This model mimics *in vivo* conditions because it allows biofilms to grow at the air-liquid interface. Also, the biofilm-mucosa interaction is more physiological than primary cell cultures, because the communication between different host cells is preserved in the explant model. After the interaction with *S. aureus* biofilms, explant tissues produced IL-6 and other cytokines polarised to a Th1/Th17 type of immune response. The observed Th1/Th17 immune response differs from previous reports in eosinophilic CRS patients with nasal polyps (CRSwNP) showing a predominant Th2 response. Apparently there is an evolution from early Th1/Th17 immune responses to late Th2 in *S. aureus* biofilm associated infections. The turning point between these two types of immune responses seems critical in CRS because it could explain the origin of the Th2 inflammation. In the

future, the use of long-term animal models could help to illustrate the progression from an initial Th1/Th17 to a late Th2 type of immune response in the sinonasal mucosa.

S. aureus biofilms also induced apoptosis in the mucosa as demonstrated by the up-regulation of cleaved caspase-3 in our settings. We also demonstrated the induction and activation of the Nod2 receptor and downstream pathway secondary to *S. aureus* biofilms. The Nod2 receptor recognises a small portion of peptidoglycan that is available during early phases of *S. aureus* biofilm formation. The role of Nod2 in CRS and biofilm infections should be evaluated in future studies.

In conclusion, we demonstrated that early *S. aureus* biofilms induce a proinflammatory response in the sinonasal mucosa. This proinflammatory response seems to be crucial for biofilm attachment and persistence, and its modulation could represent an alternative to prevent *S. aureus* biofilm infections. Secreted staphylococcal products such as alpha (α) toxin and staphylococcal protein A (SpA) are two virulence factors critical during early biofilm growth. These proteins are also able to generate immune responses and represent targets for potential therapeutic intervention during biofilm infections in the sinonasal mucosa.

Statement

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Daniel Anibal Cantero Cajas
November 2014

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I would like to acknowledge everyone who encouraged and helped me to engage in the PhD program and finish this thesis. As an ENT surgeon dedicated mainly to dealing with rhinological problems in Chile, I commonly faced Chronic Rhinosinusitis (CRS) patients that, despite medical and/or surgical treatment, continued to suffer debilitating symptoms and had a poor quality of life. While working in Chile, the biofilm theory in CRS was in full swing and the Department of ENT Surgery of The Queen Elizabeth Hospital in Adelaide, directed by Professor PJ Wormald, was one of the most prolific in the world in terms of research and publications related to CRS. New opportunities to understand and treat CRS were emerging. I have to thank first Professor Wormald for giving me the opportunity to work in his Department and develop my research project under his supervision. Professor Wormald has offered me the ideal balance between freedom and responsibility to develop original research on a fascinating and relevant subject. Professor Wormald is an inspiring leader in terms of hard work and discipline, commitment to excellence, mentoring, and teamwork. Besides research skills, I have learnt from the clinical, surgical, and academic activities ongoing in the Department. Working in this Department has been an amazing and growing experience, professionally and personally.

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Publications generated during the candidature

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A human nasal explant model to study *Staphylococcus aureus* biofilm in vitro

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Cantero D, Wormald PJ.

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***Staphylococcus aureus* biofilms induce apoptosis and expression of interferon-gamma, interleukin-10 and interleukin-17A on human sinonasal explants**

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Presentations during the candidature

Department Presentation, TQEH, Adelaide, August 2011

"The host immune response to *S. aureus* biofilm"

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Research Proposal: "The immune response to *S. aureus* biofilms"

Department Presentation, TQEH, Adelaide, February 2012

"Antibacterial Response to *S. aureus* biofilms on sinus mucosa"

Research Day BHI, Adelaide, October 2012:

"Characterization of the acute inflammatory response to *Staphylococcus aureus* biofilm on a human sinonasal explant model."

BHI Postgraduate Seminar, Adelaide, November 2012

"Initial Immune response of Explant tissues to *S. aureus* biofilms."

Department Presentation, TQEH, Adelaide, November 2012

"Staphylococcus aureus: Host bacterium interactions in the sinonasal mucosa. Update and future perspectives"

Presentation at the University of Adelaide, Discipline of Surgery Research Dinner, May 2013.

"Early inflammatory Response to *Staphylococcus aureus* biofilm on a human nasal explant model."

Presentation at the American Rhinological Society Meeting, September 2013, Vancouver, Canada.

"*S. aureus* biofilm activates the Nod2 pathway on a human sinonasal explant model.

Department Presentation, TQEH, Adelaide, October 2013

"Innate Immnune Response to *S. aureus* biofilms."

Research Day BHI October 2013:

"*S. aureus* Biofilms induce a Th1/Th17 response on human sinonasal explants."

End of PhD Department Presentation, TQEH, Adelaide, June 2014

"The immune response to *S. aureus* biofilms using a human nasal explant model"

Final BHI Postgraduate Seminar, Adelaide, June 2014

"Innate immnune response to *S. aureus* biofilms on sinonasal tissue explants."

List of Abbreviations used in this thesis

ACP: Antrochoanal Polyp

ADAM10: A disintegrin and metalloproteinase domain-containing protein 10

ADEPs: Acyldepsipeptides

AERD: Aspirin-Exacerbated Respiratory Disease

AFRS: Allergic Fungal Rhinosinusitis

AIM2: Absent in melanoma protein 2

AIP: Auto inducing peptide

AKT1: V-akt murine thymoma viral oncogene homolog 1

AMPs: Antimicrobial peptides

APCs: Antigen Presenting Cells

α-toxin: Alpha toxin or alpha haemolysin

BAI: Biofilm Associated Infections

β-haemolysin: beta haemolysin or beta toxin

BPE: Bovine Pituitary Extract

CA-MRSA: Community-acquired MRSA

Can: Collagen binding surface protein

CASP1: Caspase 1

CASP3: Caspase 3

CBD: Calgary Biofilm Device

CCL2: Chemokine (C-C) ligand 2

CCL5: Chemokine (C-C motif) ligand 5 (RANTES)

cDNA: Complementary DNA

CHIPS: Chemotaxis inhibitory protein of staphylococci

CF: Cystic Fibrosis

CFTR: Cystic Fibrosis Trans-membrane Conductance Regulator

CFU: Colony Forming Unit

ClfA: Clumping factor A

ClfB: Clumping factor B

CLRs: C-type Lectin Receptors

CpG: Cytosine-phosphate-Guanine. Areas of DNA where these sequences occur.

CRS: Chronic Rhinosinusitis

CRSsNP: Chronic Rhinosinusitis Without Nasal Polyps

CRSwNP: Chronic Rhinosinusitis With Nasal Polyps

COPD: Chronic Obstructive Pulmonary Disease

CSF: Cerebrospinal Fluid

CSLM: Confocal Scanning Laser Microscope

CT: Computer Tomography

CVID: Common Variable Immune Deficiency

CXCL1: Chemokine (C-X-C) motif ligand 1

CXCL2: Chemokine (C-X-C) motif ligand 2

Cy3: Cyanine dye number 3

C5aR: C5a receptor

DAB: Diaminobenzidine

DAP: Diaminopimelic acid

DAPI: 4', 6-diamidino-2-phenylindole

DCs: Dendritic Cells

DMEM: Dulbecco's Modified Eagle's Medium

dsRNA: Double-Stranded RNA

dsDNA: Double-Stranded DNA

δ toxin: Delta toxin or delta haemolysin

ECP: Eosinophilic Cationic Protein

eDNA: extracellular DNA

EGFR: Epidermal growth factor receptor

ELISA: Enzyme-Linked Immunosorbent Assay

EM: Eosinophilic Mucus (Mucin)

EPOS: European Position paper on Rhinosinusitis and Nasal Polyps

EP(3)OS: European Position paper on Rhinosinusitis and Nasal Polyps, 1997

EPS: Extracellular Polymeric Substances

ESS: Endoscopic Sinus Surgery

Fab: Antigen-binding fragment

FACS: Fluorescence-activated cell sorting

FADD: Fas (TNFRSF6)-associated via death domain

Fc: Fragment cristallizable or gamma fraction of immunoglubulins

FOXP3: Forkhead box P3

FPR: Formyl peptide receptor

FnbpA: Fibronectin binding protein A

FnbpB: Fibronectin binding protein B

GATA-3: Transcription factor differentiating to Th2 responses. GATA is a family of

transcription factors. “GATA” represents a DNA sequence that these proteins bind.

G-CSF: Granulocyte Colony Stimulating Factor (CSF3)

GM-CSF: Granulocyte Macrophage Colony Stimulating Factor (CSF2)

γ hemolysin: Gamma haemolysin or gamma toxin

HAMsF12: Ham’s nutrient mixture number 12

HE: Hematoxylin and Eosin

Hla: Alpha haemolysin

Hlg: Gamma hemolysin

ICD-9: Ninth Revision codes

IFN-γ: Interferon gamma

Ig: Immunoglobulin

IgE: Immunoglobulin E

IgG: Immunoglobulin G

IHC: Immunohistochemistry

IL-1: Interleukin 1

IL-1β: Interleukin 1 beta

IL-2: Interleukin 2

IL-4: Interleukin 4

IL-5: Interleukin 5

IL-6: Interleukin 6

IL-7: Interleukin 7

IL-8: Interleukin 8 (or CXCL8)

IL-10: Interleukin 10

IL-12: Interleukin 12

IL-13: Interleukin 13

IL-17: Interleukin 17

IL-17A: Interleukin 17A

IL-17C: Interleukin 17C

ILC: Innate Lymphoid Cell

INCSs: Intranasal corticosteroids

IRF5: Interferon regulatory factor 5

IRF7: Interferon regulatory factor 7

IPAF: Ice protease-activating factor

INCSs: Intranasal corticosteroids

ITF: Insulin Transferrin Selenium

LPS: Lipopolysaccharide

LAS AF: Leica Application Suite Advance Fluorescence Software

LTA: Lipoteichoic acid

MAPK3: Mitogen-Activated Protein Kinase 3

MASP-1: Mannose Associated Serine Protease 1

MASP-2: Mannose Associated Serine Protease 2

MBL: Mannose Binding Lectin

MCP-1 (MCAF): Monocyte Chemoattractant Protein 1

MDP: Muramyl Dipeptide

MDSCs: Myeloid-Derived Suppressor Cells

MFI: Mean Fluorescence Intensity

MFU: Mc Farland Unit

MIC: Minimum Inhibitory Concentration

MIP-1β: Macrophage Inflammatory protein 1β

MLST: Multilocus Sequence Typing

mRNA: Messenger RNA

MRSA: Methicillin-resistant *S. aureus*

MSCRAMM: Microbial Surface Components Recognising Adhesive Matrix Molecules

MyD88: Myeloid Differentiation primary response gene (88)

NADPH: Nicotinamide Adenine Dinucleotide Phosphate (reduced form)

NALT: Nasal Associated Lymphoid Tissue

NCBI: National Centre for Biotechnology Information

NFκB: Nuclear factor kappa light chain enhancer of activated B cells

NFκB1: Subunit 1 of NFκB. The gene encodes a 105 kD protein

NLRs: Nucleotide binding and oligomerization domain (NOD)-like receptors

NLRP1: NOD-, LRR- and pyrin domain-containing 1

NLRP3: NOD-, LRR- and pyrin domain-containing 3

NO: Nitric Oxide

Nod1: Nucleotide-binding Oligomerization Domain containing 1 protein

Nod2: Nucleotide-binding Oligomerization Domain containing 2 protein

NSAID: Non-steroidal anti-inflammatory

ODN: Oligodeoxinucleotides

OME: Otitis Media with Effusion

P. aeruginosa*: Pseudomonas aeruginosa*

PAMPs: Pathogen Associated Molecular Patterns

PBS: Phosphate-Buffer Saline Solution

PBP2a: Penicillin Binding Protein 2a

PCR: Polymerase Chain Reaction

PGN: Peptidoglycan

PIA: Polysaccharide Intercellular Adhesin

PMN: Polymorphonuclear

PNAG: Poly-N-Acetylglucosamine

PRRs: Pattern Recognition Receptors

PROMS: Patient Reported Outcome Measures

PSM: Phenol soluble modulins

PVL: Panton Valentine leukocidin

qRT-PCR: Quantitative Reverse-Transcriptase Polymerase Chain Reaction

RIPK2: Receptor-interacting serine/threonine-protein kinase 2

RLRs: Retinoic Acid-Inducible Gene Receptors

ROR-γt: RAR-related orphan receptor gamma

S. aureus*: Staphylococcus aureus*

SAgs: Superantigens

SCCmec: Staphylococcal chromosome cassette *mec*

SCV: Small Colony Variants

SDHA: Succinate Dehydrogenase Complex, subunit A

SEA: Staphylococcus enterotoxin A

SEB: Staphylococcus enterotoxin B

SEC: Staphylococcus enterotoxin C

SED: Staphylococcus enterotoxin D

SEE: Staphylococcus enterotoxin E

SEG: Staphylococcus enterotoxin G

SEM: Scanning Electron Microscopy

SET: Staphylococcus enterotoxin T

SE*l*s: Staphylococcal enterotoxin-like proteins

SE*l*J: Staphylococcal enterotoxin-like protein J

SE*l*V: Staphylococcal enterotoxin-like protein V

SLE: Systemic Lupus Erythematosus

SLPI: Secretory leukocyte proteinase inhibitor

SNOT: Sino-Nasal Outcome Test

SpA: *Staphylococcus aureus* protein A

SP-D: Surfactant protein D

ssRNA: Single-Stranded RNA

STAT3: Activator of Transcription 3

Tc: T cytotoxic cell

T-bet: T-box transcription factor differentiating to Th1 responses

TCR: T cell receptor

TEM: Transmission Electron Microscopy

TGF-β1: Transforming growth factor beta 1

Th: T helper cell

Th1: T helper cell 1

Th2: T helper cell 2

Th17: T helper cell 17

TIR: Toll/Interleukin (IL)-1 receptor

TLRs: Toll Like Receptors

TLR2: Toll Like Receptors number 2

TLR3: Toll Like Receptors number 3

TNF: Tumor Necrosis Factor (formerly known as TNF-alpha)

TNF-β: Tumor Necrosis Factor beta

TNFR1: Tumor necrosis factor receptor 1

TSST: Toxic Shock Syndrome Toxin

Treg: T regulatory cells

VAS: Visual analogue scores

VRSA: Vancomycin Resistant Staphylococcus Aureus

vWF: Von Willebrand factor

USA: United States of America

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