UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Academic Unit of Clinical and Experimental Sciences

Characterising the role of *Staphylococcus aureus* and its toxins in chronic rhinosinusitis

by

Timothy Charles Biggs

Thesis for the degree of Doctor of Philosophy (PhD)

April 2019

iv

UNIVERSITY OF SOUTHAMPTON
ABSTRACT
FACULTY OF MEDICINE
Infection and Immunity
Doctor of Philosophy
CHARACTERISING THE ROLE OF STAPHYLOCOCCUS AUREUS AND ITS TOXINS IN CHRONIC RHINOSINUSITIS
by Timothy Charles Biggs

Chronic rhinosinusitis (CRS) is a chronic inflammatory condition affecting the lining of the nose and paranasal sinuses. It is the second most common chronic disease worldwide, and impacts significantly on patients' quality of life and healthcare resources. CRS is subdivided into two main disease categories: CRS with and without nasal polyps (CRSwNP and CRSsNP respectively). It is well established that bacteria, most notably Staphylococcus aureus (*S. aureus*), play an important role in the pathogenesis of CRS. Whilst much emphasis has been placed on surface bacteria, intracellular bacteria are gaining more prominence in relation to resistant disease. The intracellular environment may provide a protective niche for pathogenic bacteria to evade host immunity, and provide a reservoir for re-infection. Recently published findings from the Southampton Upper Airway Research Group reported the novel finding of intracellular *S. aureus* within nasal polyp mast cells, in addition to epithelial cells. Furthermore, the presence/addition of *Staphylococcus aureus* Enterotoxin B (SEB) further promoted the internalisation of *S. aureus* into mast cells. The aim of this work was to further characterise the host response towards *S. aureus* and its toxins, with particular emphasis on *S. aureus*-mast cell interactions.

A prospective study was performed using ex-vivo sinonasal mucosa and nasal polyp tissue from CRS patients, and sinonasal mucosa from non-CRS patients undergoing trans-sphenoidal pituitary surgery as a control. Pro-inflammatory cytokine profiles of these tissue sub-sites were measured using real time quantitative polymerase chain reaction (RT-qPCR). An explant tissue model was developed to study the immune response of nasal polyps, and control samples to SEB, with the resultant host immune response measured using RT-qPCR and Luminex. An in vitro cell culture model was developed to characterise interactions between a CRS-specific *S. aureus* isolate and the RPMI-2650 epithelial cell line, HMC-1 mast cell line and LAD2 mast cell line. *S. aureus* was cultured to the mid-log phase and combined with all three cell lines, at set time-points and multiplicity of infection ratios. Intracellular uptake of *S. aureus* was examined using confocal laser scanning microscopy, intracellular viability and its release was examined using colony forming unit (CFU) enumerations, and the associated host immune response was measured using RT-qPCR and Luminex. Study of the potential phenotypic change to *S. aureus*, from its continual uptake and release from mast cells, was undertaken in both HMC-1 and LAD2 cell lines, utilising CFUs, RT-qPCR and Luminex. For the study of IgE sensitisation and its effect on the activation of *S. aureus* infected LAD2 cells, the following techniques were used; LDH assays, degranulation (β-hexosaminidase) assays, protein kinase phosphorylation, RT-qPCR and Luminex.

In comparison to control patients, CRSwNP patients display upregulated pro-inflammatory cytokines (IL-5, IL-8) toll-like receptors (TLR-4) and matrix metalloproteinases (MMP-28). In many cases the non-polypoidal sinonasal mucosa of CRSwNP and nasal polyps display strikingly similar immune profiles, with no statistical difference found between the two sites. SEB was found to significantly upregulate nasal polyp gene expression ratios of IL-5, IL-17A, TNFα, TGF-β, and supernatant protein concentrations of IFNγ, IL-5, IL-17A, and TNFα. In cell line culture experiments, RPMI-2650 epithelial cells, as well as HMC-1 and LAD2 mast cells, readily internalised *S. aureus*. This intracellular uptake was significantly enhanced in the presence of SEB for both epithelial and mast cells at 24 hours. Intracellular *S. aureus* was found to be viable and capable of release, rapidly replenishing previously eradicated extracellular bacterial populations. Upon *S. aureus* exposure, both mast cells (HMC-1) and epithelial cells (RPMI-2650) contributed to inflammation through pro-inflammatory cytokine release

into the culture supernatant (IFNγ, TNFα, IL-17A, IL-1β and IL-6). *S. aureus* was able to significantly downregulate both the gene expression (IL-8, IL-1β, TNFα, TGF-β1 and IL-5) and protein release (TNFα) of LAD2 mast cells through the sequential repeated intracellular uptake and release of *S. aureus*. In the presence of prior IgE sensitisation, *S. aureus* infected LAD2 mast cells were able to limit degranulation, the host immune response (TNFα gene expression and protein release), and the phosphorylation of Atk2 and GSK-3α/β.

These findings demonstrate the importance of *S. aureus* and its toxins in the development of a chronic inflammatory reaction in CRS patients. Epithelial and mast cells appear to provide a protective niche, shielding *S. aureus* from immune mediated clearance, as well as acting as a reservoir of infection that can replenish eradicated bacterial populations. Through prior IgE sensitisation of mast cells, *S. aureus* was able to manipulate the subsequent host immune response, favouring its own survival. A similar response was also seen following repeated uptake and release of *S. aureus* from mast cells, suggesting bacterial phenotypic change. This is of particular relevance in patients with elevated IgE levels, as is seen in patients with *S. aureus* colonisation where IgE forms against both *S. aureus* and its enterotoxins. This may well facilitate the ongoing survival and submucosal persistence of *S. aureus*, and could contribute towards the development of a chronic disease process. These patients are likely to benefit from aggressive medical therapy, particularly in the post-operative period, to eradicate *S. aureus*, in order to improve long-term treatment outcomes.

PRESENTATIONS

International Oral Presentations

1. Biggs T.C., Hayes S.M., Harries P.G., Walls A., Pender S., Salib R.J. Characterisation of interactions between *S. aureus* and mast cells in chronic rhinosinusitis. Proceedings of the European Rhinology Society Meeting, London, April 2018. Invited presentation, 'Best of British' symposium.

2. Biggs T.C., Abadalkareem R.S., Hayes S.M., Harries P.G., Pender S., Walls A., Salib R.J. Allergic sensitisation of mast cells promotes the survival of *S. aureus* - Implications for chronic rhinosinusitis. Proceedings of the European Rhinology Society Meeting, London, April 2018.

3. Biggs T.C., Hayes S.M., Harries P.G., Pender S., Salib R.J. Investigation of the Th17 inflammatory response in chronic rhinosinusitis with nasal polyps. Proceedings of the Symposium on Experimental Rhinology and Immunology of the Nose, Dusseldorf, April 2017.

4. Biggs T.C., Hayes S.M., Harries P.G., Pender S., Salib R.J. The possible role of bacterial linked pro-inflammatory cytokines in the formation of nasal polyps? Proceedings of the European Rhinology Society Meeting, Stockholm, July 2016.

5. Biggs T.C., Hayes S.M., Lamont H., Harries P.G., Pender S., Salib R.J. Do bacteria contribute to the formation of nasal polyps in patients with chronic rhinosinusitis? Proceedings of the European Rhinology Society Meeting, Amsterdam, June 2014.

National Oral Presentations

6. Biggs T.C., Hayes S.M., Holding R., Harries P.G., Allan R.N., Pender S., Walls A.F., Salib R.J. Intracellular localisation of S. aureus in mast cells - A potential bacterial survival mechanism in

chronic rhinosinusitis. Proceedings of the Royal Society of Medicine, Laryngology and Rhinology Section, London, March 2019. *Winner of the best oral presentation prize

7. Biggs T.C., Hayes S.M., Harries P.G., Pender S., Salib R.J. British Rhinological Society research grant update: Intracellular *S. aureus* within mast cells. Proceedings British Rhinological Society Meeting, Jersey, May 2017.

8. Biggs T.C., Hayes S.M., Harries P.G., Pender S., Salib R.J. The role of bacteria in the formation of nasal polyps. Proceedings of the Rosetrees Trust PhD Conference, London, Sept 2016

9. Biggs T.C., Hayes S.M., Harries P.G., Pender S., Salib R.J. Investigation of the Th17 inflammatory response in chronic rhinosinusitis with nasal polyps. Proceedings of the British Rhinological Society Meeting, Leeds, May 2016.

10. Biggs T.C., Hayes S.M., Harries P.G., Pender S., Salib R.J. British Rhinological Society research grant proposal, Dragons Den: Intracellular *S. aureus* within mast cells. Proceedings of the British Rhinological Society Meeting, Leeds, May 2016.

11. Biggs T.C., Hayes S.M., Lamont H., Harries P.G., Pender S., Salib R.J. Analysis of bacterial linked pro-inflammatory cytokines in patients with chronic rhinosinusitis, and their possible role in the formation of nasal polyps. Proceedings of the British Rhinological Society Meeting, Manchester, May 2015

12. Biggs T.C., Hayes S.M., Lamont H., Harries P.G., Pender S., Salib R.J. Do bacteria contribute to the formation of nasal polyps in patients with chronic rhinosinusitis? Proceedings of the Society of Academic Research in Surgery Meeting, Cambridge, Jan 2014.

RESEARCH GRANTS

1. Royal College of Surgeons Research Training Fellowship, (Oct 2016)

2. British Rhinological Society Research Grant, (May 2016)

3. Rosetrees Trust Research Grant, (Nov 2015)

4. Royal Society of Medicine, Laryngology and Rhinology Equipment Grant, (Nov 2015)

5. Primerdesign Ltd 'Silver' Research Student Sponsorship, (March 2014)

6. Otorhinolaryngological Research Society (ORS) Research Grant, (March 2014)

7. Rosetrees Trust Research Grant, (Jan 2014)

8. University of Southampton, Faculty of Medicine, Research Management Council Research Grant, (Jan 2014)

9. Royal Society of Medicine, Laryngology and Rhinology Equipment Grant, (Nov 2013)

10. Spire Hospitals Research Grant, (Aug 2012)

RESEARCH PRIZES

- Best presentation prize, Rhinology section meeting of the Royal Society of Medicine, March 2019

- Basic science research paper prize, European Rhinologic Society, April 2018

- Travelling fellowship prize, Foundation Rhinology of Utrecht, April 2018

- Junior travel prize, European Rhinologic Society, June 2014

- Junior travel prize, NIHR Southampton Respiratory BRC, June 2014

- Runner-up prize for best oral presentation, British Rhinological Society Meeting, May 2013

  Biggs TC, Vavatsikou E, Hayes SM, Harries PG, Pender S, Salib RJ. Do bacteria contribute to the formation of nasal polyps; analysis of the Th17 pathway, Defensin 5 and MMP 7 in Chronic Rhinosinusitis.

x

Table of Contents

List of Figures

List of Tables

Academic Thesis: Declaration Of Authorship

I, Timothy Charles Biggs, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research:

Characterising the role of Staphylococcus aureus and its toxins in chronic rhinosinusitis

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission.

Signed:

Date:

Timothy C Biggs

Timothy C Biggs

Acknowledgements

Timothy C Biggs

Timothy C Biggs

Definitions and Abbreviations

AMPs	Antimicrobial peptides
APCs	Antigen presenting cells
BMMC	Bone marrow-derived murine mast cells
CBA	Colombia blood agar
CF	Cystic fibrosis
CFU	Colony forming unit
CLSM	Confocal laser scanning microscopy
CRS	Chronic rhinosinusitis
COPD	Chronic obstructive pulmonary disease
COX-1	Cyclooxygenase
CRSsNP	Chronic rhinosinusitis without nasal polyps
CRSwNP	Chronic rhinosinusitis with nasal polyps
DCs	Dendritic cells
ELISA	Enzyme-linked immunosorbent assay
EMTU	Epithelial-mesenchymal unit
FBS	Foetal bovine serum
FESS	Functional endoscopic sinus surgery
FISH	Fluorescence *in situ* hybridization
GA²LEN	The Global Allergy and Asthma Network of Excellence
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBSS	Hanks' Balanced Salt Solution
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
LDH	Lactate dehydrogenase
LMS	Lund-Mackay score
MCP	Monocyte chemoattractant protein
MMP	Matrix metalloproteinase
MMR	Macrophage mannose receptor
MOI	Multiplicity of infection
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	National Health Service

OR	Odds ratio
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
PCD	Primary ciliary dyskinesia
PRRs	Pattern recognition receptors
RANTES	Regulated on activation normal T cell expressed and secreted
RORγT	Retinoid-related orphan receptor gamma t
RT-qPCR	Real-time quantitative polymerase chain reaction
S. aureus	*Staphylococcus aureus*
SAE	*Staphylococcal aureus* enterotoxin
SAgs	Superantigens (bacterial)
SE	Staphylococcal enterotoxins
SEM	Standard error of the mean
SCF	Stem cell factor
SEB	*Staphylococcal aureus* enterotoxin B
SpA	Staphylococcal surface protein A
SSLs	Staphylococcal superantigen-like proteins
TGF-β	Transforming growth factor-beta
Th	T helper cells
TIMPs	Tissue inhibitors of metalloproteinases
TNFα	Tumour necrosis factor-alpha
TLRs	Toll-like receptors
Tregs	T regulatory cells
ZO1	Zonula occludens-1
β-actin	Beta-actin

1 Introduction

1.1 Anatomy of the nose and sinuses

1.1.1 Nasal cavity

The nasal cavity extends from the nostrils anteriorly (external nares) to the choanae posteriorly (posterior nares) opening into the nasopharynx. It comprises paired cavities separated by a nasal septum. The medial wall, the nasal septum, is comprised of the membranous septum, septal cartilage (quadrangular cartilage), medial crus of the greater alar cartilages, vomer, crest of the maxillary bone and the crest of the palatine bone. The lateral walls are formed by three or four sets of turbinates (conchae) that project into the nasal cavity, and are named from bottom to top, comprising of the inferior, middle and superior conchae/turbinates (**Figure 1.1**). A supreme turbinate, located above the superior turbinate, is found in approximately 60% of subjects (1). Surrounding these structures are the openings of the sinus cavities into the nose, known as ostia or meati.

The nasal cavity is subdivided into two main regions, the olfactory and respiratory region. The olfactory region comprises the superior turbinate and corresponding septum (olfactory slit). It is covered in less vascular, non-ciliated epithelium (schneiderian membrane), and contains nerve cell bodies that give rise to the olfactory nerve fibres that mediate the sense of smell. The respiratory region represents the remainder of the nasal cavity. Except for the vestibule, the nasal cavity is covered by pseudostratified columnar ciliated epithelium (respiratory epithelium).
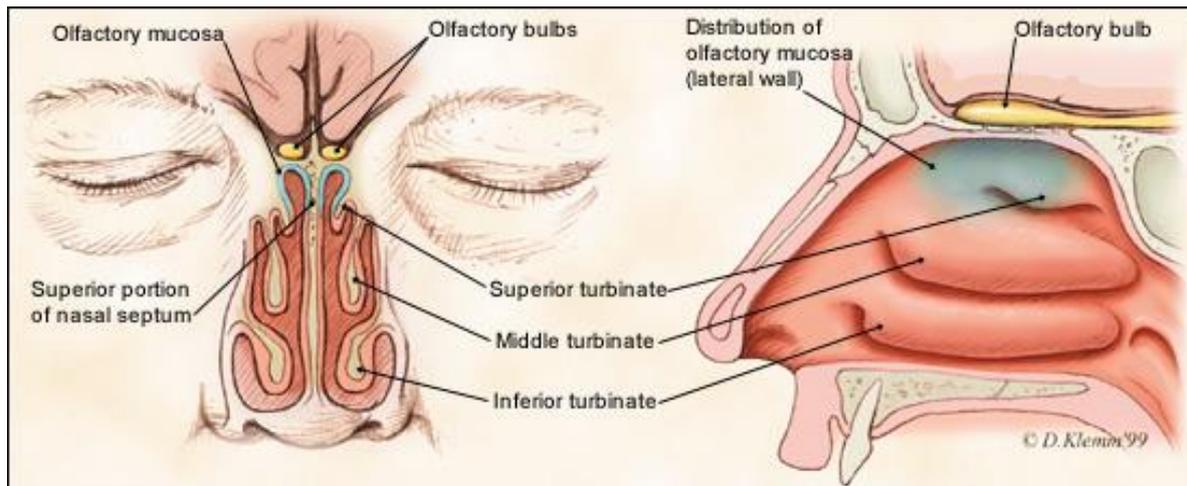
Figure 1.1 Coronal and sagittal images of the superior, middle and inferior turbinates of the lateral wall of the nasal cavity. Image taken from, ENT for medical students (2).

1.1.2 Paranasal sinuses

There are four sets of paired sinuses surrounding the nasal cavity, the frontal, ethmoid, maxillary and sphenoid sinuses (**Figure 1.2**). The sinuses are air-containing cavities within the skull. Various theories about their role exist, including weight reduction, humidification, resonance of speech, and protection of intracranial contents (crumble zones). The sinuses drain into the lateral walls of the nasal cavity. The frontal sinuses, which are absent at birth but develop during childhood, drain into the frontal recess. This is a space located posterior to the agger nasi, the most anterior ethmoidal air cell, and between the caudal edge of the middle turbinate and lamina papyracea. The nasofrontal duct, the extension of the inferior funnel below the ostium of the frontal sinus, enters the anterior end of the middle meatus, the hiatus semilunaris (or semilunar hiatus), which is located superiorly to the bulla and inferiorly to the uncinate process. The anterior ethmoidal cells and maxillary sinus ostium open into the ethmoidal infundibulum, which is a space extending from the semilunar hiatus, downwards and posteriorly, between the lateral wall and the uncinate process. The posterior ethmoidal sinuses drain into the superior meatus, located inferior to the superior turbinate. The sphenoid sinus ostium drains into the sphenoethmoidal recess, located between the superior or supreme turbinate and the anterior surface of the body of the sphenoid bone.

3
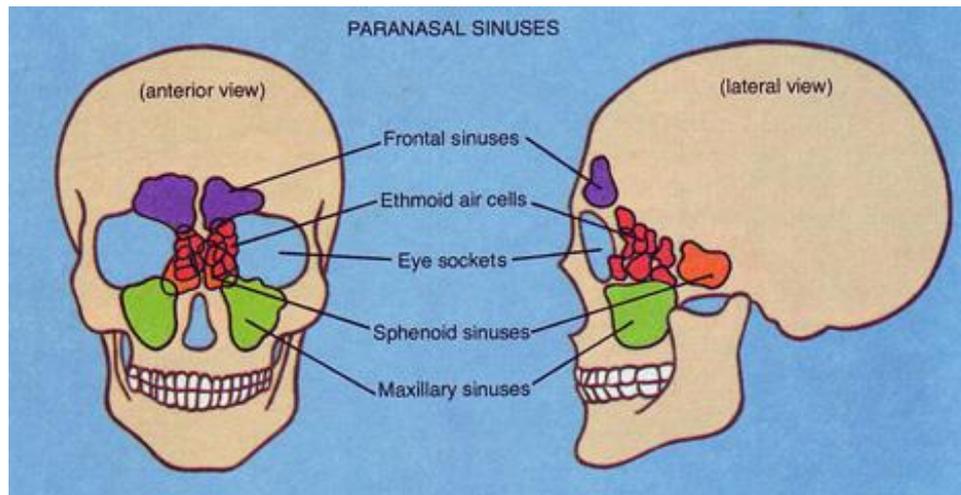
Figure 1.2 Anatomical location of the paranasal sinuses. Image taken from, ENT for medical students (2).

1.1.3 Blood supply

The nasal cavity has a rich arterial blood supply, receiving branches from both the internal and external carotid arteries (**Figure 1.3**). The main branches anastomose in an anterior area of the nasal septum, called Little's area or Kiesselbach's plexus. The most anterior vessels supplying the nasal cavity are the anterior and posterior ethmoidal arteries, branches of the ophthalmic artery, itself a branch of the internal carotid artery. The anterior ethmoidal artery, the larger of the two, travels via its correspondingly named canal before turning inferiorly over the cribiform plate. It supplies the anterior one third of the lateral wall and septum. The posterior ethmoidal artery supplies the superior turbinate and superior part of the septum. The anterior ethmoidal artery is at risk of injury in endoscopic sinus surgery and can cause significant bleeding, often retracting within the orbit. This can result in significant complications including blindness, due to optic nerve ischaemia, if not dealt with promptly.

The sphenopalatine artery, the terminal branch of the maxillary artery, from the external carotid, enters the nasal cavity through the sphenopalatine foramen and divides into the lateral posterior nasal artery and septal posterior nasal artery. The lateral posterior nasal artery, running along the middle and inferior turbinates, supplies the middle and inferior turbinates. The septal posterior nasal artery,

running across the face of the sphenoid, divides into branches supplying the posterior septum. The superior labial artery from the septal branch of the facial artery, supplies the vestibule of the nose and anterior septum, anatomising within Little's area.

The venous system within the nose controls the turbinate erectile tissues, and the nomenclature closely follows that of the arterial supply.
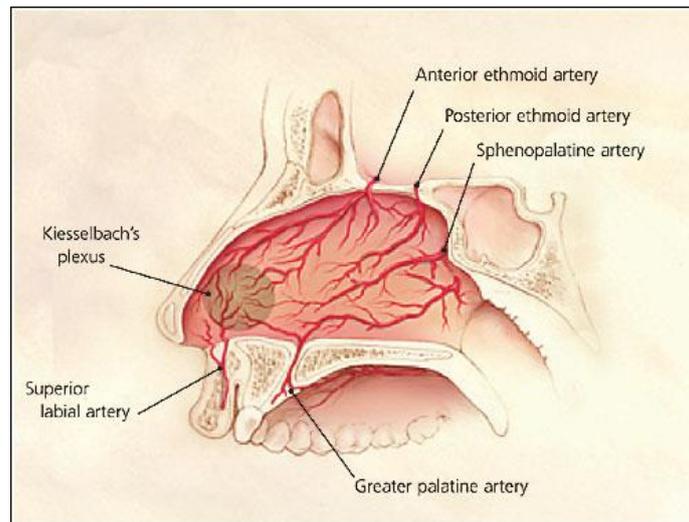
Figure 1.3 The arterial blood supply of the nasal cavity. Image taken from, ENT for medical students (2).

1.1.4   Physiology

The physiological functions of the nose include conditioning of inspired air and nasal resistance, vocal resonance, olfaction, and drainage of the sinuses (3). It also serves to prime the immune system through antigen binding at mucosal surfaces.

1.1.4.1   Air conditioning

The majority of the air we breathe passes through our nose. The nasal mucosa, which has a rich blood supply and large surface area, helps to adjust the humidity and temperature of the inspired air. Blood and autonomic supply to the nose controls mucus production and the degree of nasal congestion. The nose goes through a nasal cycle, of alternating congestion and decongestion which causes reciprocal

fluctuations in nasal airflow. This cycle is repeated throughout the day, approximately every 4 hours, and is thought to help with smell detection, humidity and maintaining effective cilia function.

The nose and paranasal sinuses are lined by respiratory epithelium (pseudostratified columnar ciliated epithelium). Cilia on the surface of the mucosa beat in an organised fashion which produces a mucociliary transport system. This allows mucous, produced by numerous goblet cells, and inhaled debris, to be transported to the back of the nasal cavity and into the nasopharynx to be swallowed. This system relies on the effective mucus production and cilia function. Cilia are programmed to beat in a specific direction, posteriorly towards the oropharynx. Microbes attacking the epithelium and their secreted toxins can alter cilia structure, movement and function. This, combined with inflammatory responses and the development of chronic rhinosinusitis (CRS), can lead to ciliary dysfunction and even loss (**Figure 1.4**).
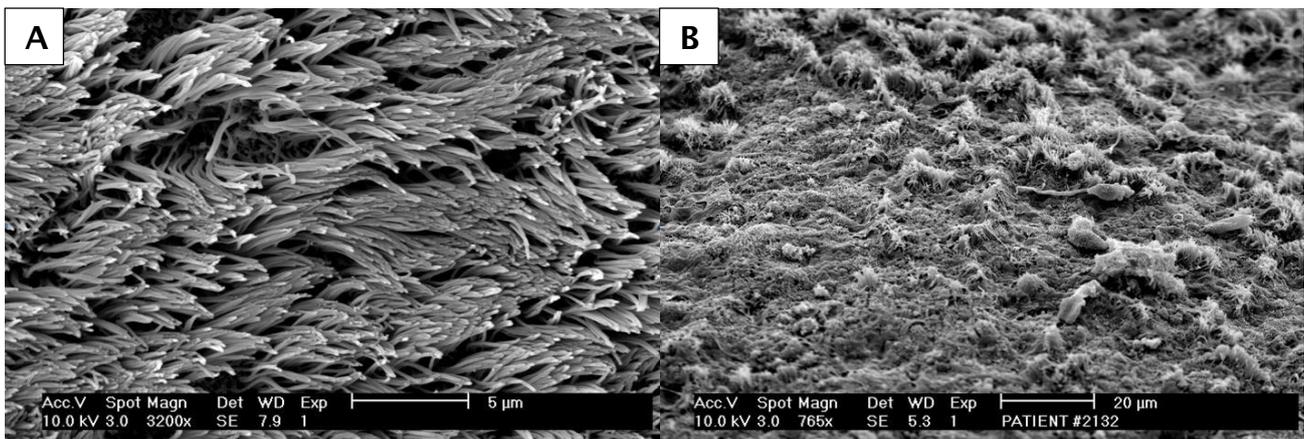
Figure 1.4 Scanning electron microscopy images of cilia overlying the nasal mucosa. A) Normal cilia structure in a healthy individual, B) Cilia loss in a patient with CRS. Images taken from, Jeremiah et al (3).

1.1.4.2 Vocal resonance

The nose is thought to be a resonating chamber for certain consonants during speech. The sounds, M, N and NG, pass up through the nasopharynx and are emitted through the nose. The significance of

this is highlighted in nasal airway obstruction, where the quality and character of the voice is often altered.

1.1.4.3 Olfaction

Odour detection is a result of input from the olfactory and trigeminal nerves (4). Smells reach the olfactory epithelium in one of two ways, firstly through nasal breathing and secondly through retronasal airflow during eating. The olfactory epithelium is made up of 3 main cell types; basal, supporting and olfactory receptor cells (**Figure 1.5**). Basal cells are stem cells, and regenerate olfactory receptor cells. Supporting cells have numerous microvilli and secretory granules, and empty their contents onto the mucosal surface. Receptor cells are bipolar neurons, possessing a dendritic rod that contains specialized cilia extending from the olfactory vesicle, and a long central process that forms the fila olfactoria (unmyelinated olfactory axons). The cilia provide the transduction surface for odorous stimuli, aided by odor molecules dissolving within the overlying mucus.
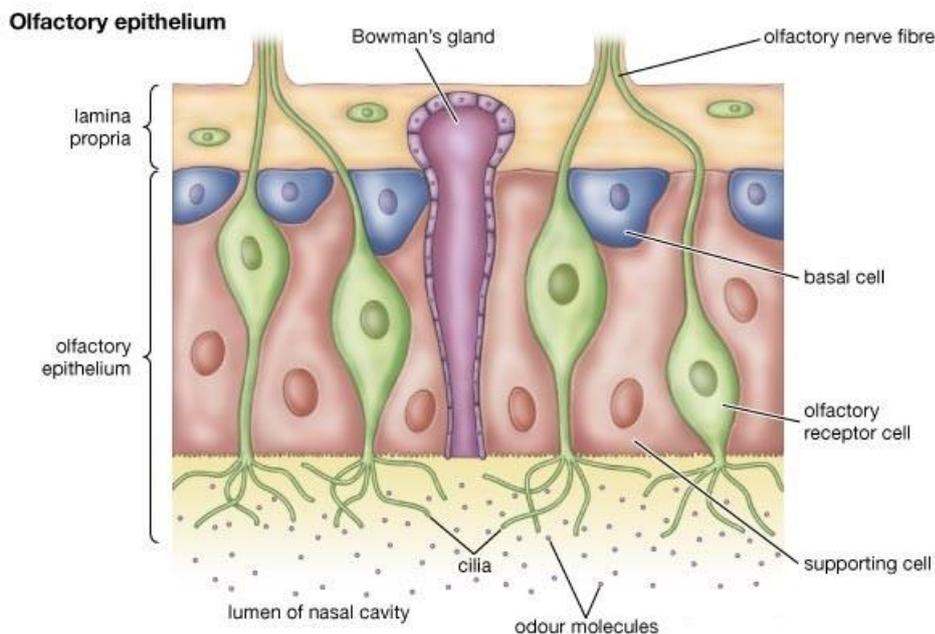
Figure 1.5 Schematic representation of the olfactory epithelium. Image taken from, the Encyclopaedia Britannica (5).

Odorous molecules stimulate the olfactory axons directly, sending signals back through the olfactory bulb, and then to the primary olfactory cortex. The trigeminal nerve sends fibres to the olfactory epithelium to detect caustic chemicals, such as ammonia. The most common causes of olfactory loss include CRS and nasal polyps, upper respiratory tract infections, head trauma, and aging.

1.2 Chronic rhinosinusitis

1.2.1 Background

Chronic rhinosinusitis (CRS) in adults is defined as an inflammatory condition affecting the mucosal lining of the nasal cavities and paranasal sinuses (6). It is a chronic condition with symptoms lasting more than 12 weeks, without complete resolution. It is characterised by clinical symptoms and either endoscopic signs and/or computed tomography (CT) changes (**Figure 1.6**). The diagnostic criteria based on the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) are presented in **Table 1.1**.
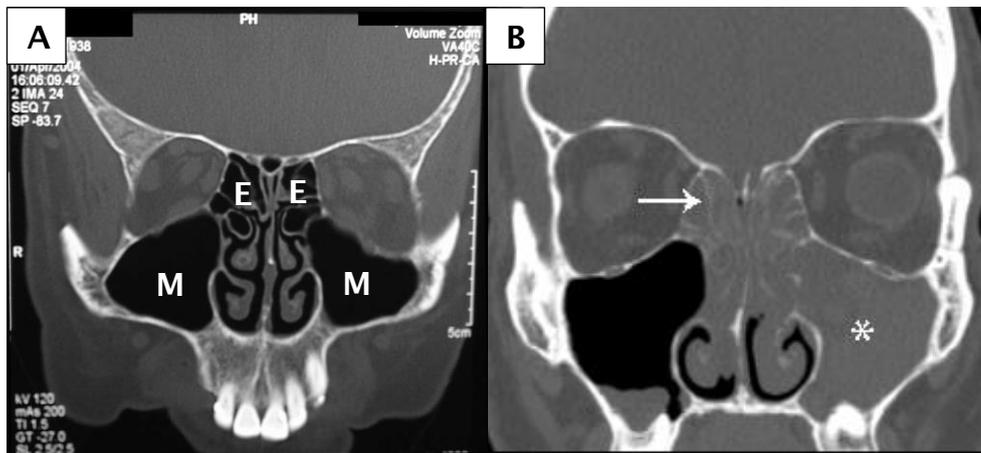
Figure 1.6 CT images of the nasal cavity and paranasal sinuses. A) Normal coronal CT image of the paranasal sinuses demonstrating paired maxillary sinuses (M) and ethmoidal sinuses (E). B) A coronal CT image of a patient with CRS demonstrating an opacified maxillary sinus (white star) and ethmoidal sinus (white arrow). Images taken from, Adelglass et al (7) and Ferguson et al (8).

CRS with or without nasal polyps, is a common chronic disease affecting approximately 15% of the UK population (6). It is an inflammatory condition of the nose and paranasal sinuses causing localised and systemic symptoms, impacting significantly on the quality of life of afflicted individuals, placing a huge financial burden on an already over-stretched National Health Service (NHS). It is estimated that medical and surgical treatments associated with CRS cost the NHS in the region of £100 million annually (6).

Table 1.1 EPOS diagnostic criteria for CRS. Taken from, Fokkens et al (6).

Primary symptoms	➢ Inflammation of the nose and the paranasal sinuses characterised by 2 or more symptoms, one of which should be either nasal blockage/obstruction/congestion or nasal discharge (anterior/posterior nasal drip) ➢ +/- Facial pain or facial pressure ➢ +/- Reduction or loss of smell **For ≥12 weeks**
and either	**endoscopic signs of** ➢ Polyps and/or ➢ Mucopurulent discharge from the middle meatus and/or ➢ Oedema/mucosal obstruction primarily in the middle meatus
and or	**CT changes** ➢ Mucosal changes within the ostiomeatal complex and/or sinuses

There are many theories regarding the aetiopathogenesis of CRS, including the role of superantigens (9-11), abnormal cell-mediated immune responses (12, 13), remodelling (14-16), epithelial defects (17, 18), osteitis of the sinus walls (19, 20), and viral (21), bacterial (22-25), and fungal factors (26, 27). However, the exact cause remains unclear and further research into the pathogenesis of CRS is needed. Currently the mainstay of CRS treatment is the use of topical and systemic steroids, antibiotics and surgical clearance when maximal medical therapy fails. Many CRS patients require multiple operations in their lifetime costing the NHS millions of pounds each year. Some of the unanswered questions include what role do bacteria have in driving the disease process, and in particular, the development and growth of nasal polyps. A better understanding of disease mechanisms would enable better targeted therapeutic strategies to be developed. It would also aid understanding of the mechanisms of progression from non-polyp to polyp disease.

The severity of the disease can be divided into mild, moderate and severe based on a total severity analogue scale (0-10cm, Likert scale). Furthermore, the disease can be classified into two distinct groups based on the presence or absence of nasal polyps (CRSwNP and CRSsNP respectively).

1.2.4 Chronic rhinosinusitis with and without nasal polyps

It is important to differentiate CRS into CRSwNP and CRSsNP as they have very relevant clinical, pathological and immunological differences. Nasal polyps are essentially bags of fluid filled with inflammatory cells, predominantly eosinophils, lined by nasal epithelial cells and congruous with adjacent nasal mucosa. These grape-like structures (**Figure 1.7**) cause physical blockage within the nose and contain numerous cytokines important in disease modifying processes. It is unclear what causes these structures to grow in some patients. Undoubtedly, an allergic tendency is one factor, but it is likely that bacteria and their toxins also play an important role.
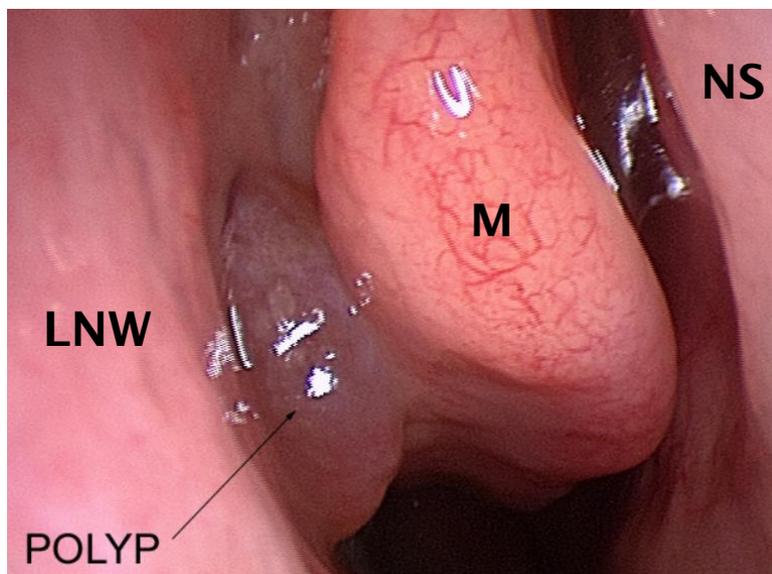
Figure 1.7 Nasal polyp within the right nasal cavity. This picture shows a nasal polyp between the middle turbinate (M) and lateral nasal wall (LNW). Nasal septum (NS) also shown. Image amended from, Roth et al (28).

Current evidence suggests that CRSwNP displays a primarily T-helper (Th) 2 directed response, characterised by eosinophilic inflammation, driven by interleukin (IL) 5 and eotaxin, resulting in the chemotaxis, activation and survival of eosinophils (10). CRSwNP is characterised by low levels of tumour necrosis factor-alpha (TNF-α) and transforming growth factor-beta (TGF-β), high rates of tissue remodelling with pseudocyst formation, excessive infiltration of inflammatory cells (mainly

eosinophils) and reduced levels of collagen deposition within the extracellular matrix (29). In contrast, CRSsNP is primarily Th1 driven, involving high levels of Interferon-gamma (IFN-γ), TNFα and TGF-β with collagen deposition and fibrosis. In CRSwNP, enterotoxins from *Staphylococcus aureus* (*S. aureus*) act as superantigens, inducing activation of T-cells and exacerbation of on-going inflammation (10, 29).

1.3 Epidemiology of CRS

The quality of epidemiological data in CRS is poor, due to the heterogeneity of CRS and the difficulties in disease phenotyping. However, the available evidence clearly shows that CRS is a very common condition affecting up to 15% of the worldwide population (**Table 1.2**) (30).

A national health survey conducted in Canada (77,364 participants) assessing the prevalence of CRS in the general population found an incidence of 3.4% in men and 5.7% in females (30). A similar survey conducted in the US (220,267 participants) revealed an estimated incidence of 14% (31). In contrast a study in Korea, using clinical and endoscopic diagnostic criteria, found a reported incidence of 1% (32). Within Europe the most definitive data regarding disease incidence comes from The Global Allergy and Asthma Network of Excellence (GA[2]LEN) study, a large questionnaire based project spanning 19 European centres (57,128 participants) using the EPOS defined clinical diagnosis of CRS (33). This produced an overall prevalence of CRS at 10.9%, with marked changes across differing countries. The lowest reported prevalence of 6.9% was found to be Germany with the highest of 27.1% found within Portugal. Smokers had a higher incidence, with an odds ratio of 1.7. Those of increasing age were found to have a lower incidence of CRS (66-74 years old), compared to younger patients (15-24 years old), odds ratio (OR) 0.64. Females were slightly more likely to suffer from CRS than males, OR of 1.1.

In a further paper evaluating the GA[2]LEN study data, the association of asthma in those with CRS was assessed (34). There was a very high correlation of asthma in those with CRS (adjusted OR 3.47) at all ages, and this association was higher in those with CRS and allergic rhinitis (adjusted OR 11.85). There were also geographical variations in similarity with the CRS incidence data.

As well as being a very common disease, CRS patients often respond poorly to medical and surgical intervention, with an associated elevated rate of disease recurrence. A recent CRS Epidemiological Study (CRES) involving 1,249 CRS patients from across the UK, found a revision surgical rate of 55% in CRSwNP patients (35). Indeed, 20% of these required more than two operations, with a mean of 2.98 procedures per patient. Revision surgery rates were lower in CRSsNP patients (13%), but those with

Timothy C Biggs

associated fungal disease had much higher rates of surgical revision (82%), and 58% had undergone

more than one surgical procedure in their lifetime.

Table 1.2 Summary of CRS prevalence data.

STUDY	YEAR	COUNTRY	PREVALENCE
Chronic Rhinosinusitis without nasal polyps (CRSsNP)			
Min *et al* (32)	1991	Korea	1%
Chen *et al* (30)	1997	Canada	5%
Collins (36)	1997	USA	15.5%
Blackwell *et al* (37)	2002	USA	16%
Ahsan *et al* (38)	2004	Scotland	9.6%
Shashy *et al* (39)	2004	USA	2%
Pleis *et al* (31)	2008	USA	14%
Hastan *et al* (33), GA2LEN study	2011	Europe	10.9%
Pilan *et al* (40)	2012	Brazil	5.5%
Gao *et al* (41)	2016	China	8%
Hirsch *et al* (42)	2016	USA	11.9%
Chronic Rhinosinusitis with nasal polyps (CRSwNP)			
Settipane (43)	1996	USA	4.2%
Hedman *et al* (44)	1999	Finland	4.3%
Johansson *et al* (45)	2003	Sweden	2.7%
Klossek *et al* (46)	2005	France	2.1%
Kim *et al* (47)	2011	Korea	0.5%
Ahn *et al* (48)	2016	Korea	2.6%

1.4 Aetiology

1.4.1 Background

The aetiopathogenesis of CRS is not currently well understood. Suggested theories include the role of super-antigens, abnormal cell-mediated immune responses, changes in the inflammatory cytokine cascade, osteitis of the sinus walls, and viral, bacterial and fungal factors.

1.4.2 Ciliary impairment

The nose and paranasal sinuses are lined by ciliated pseudostratified columnar epithelium, as are nasal polyps (**Figure 1.8**). These columnar cells have around 200-300 cilia per cell and beat in an organised fashion. With the addition of mucus, produced by adjacent globlet cells, cilia form a mucociliary transport mechanism to trap airborne particles, bacteria and fungi; transporting them to the oropharynx to be swallowed. Ciliary function is a highly important factor in maintaining clearance of the sinuses and the prevention of chronic inflammation. There is a higher likelihood of CRS in conditions affecting the mucociliary clearance mechanisms, such as cystic fibrosis (CF) and primary ciliary dyskinesia (PCD) (6). Approximately 40% of patients with CF go on to develop nasal polyps (6), and ciliary function has been shown to be impaired in nasal polyp tissue compared to turbinate tissue within control patients (18). However, not all patients with ciliary dysfunction go onto develop CRS, and therefore ciliary dysfunction may be a primary cause or a secondary phenomenon of CRS.
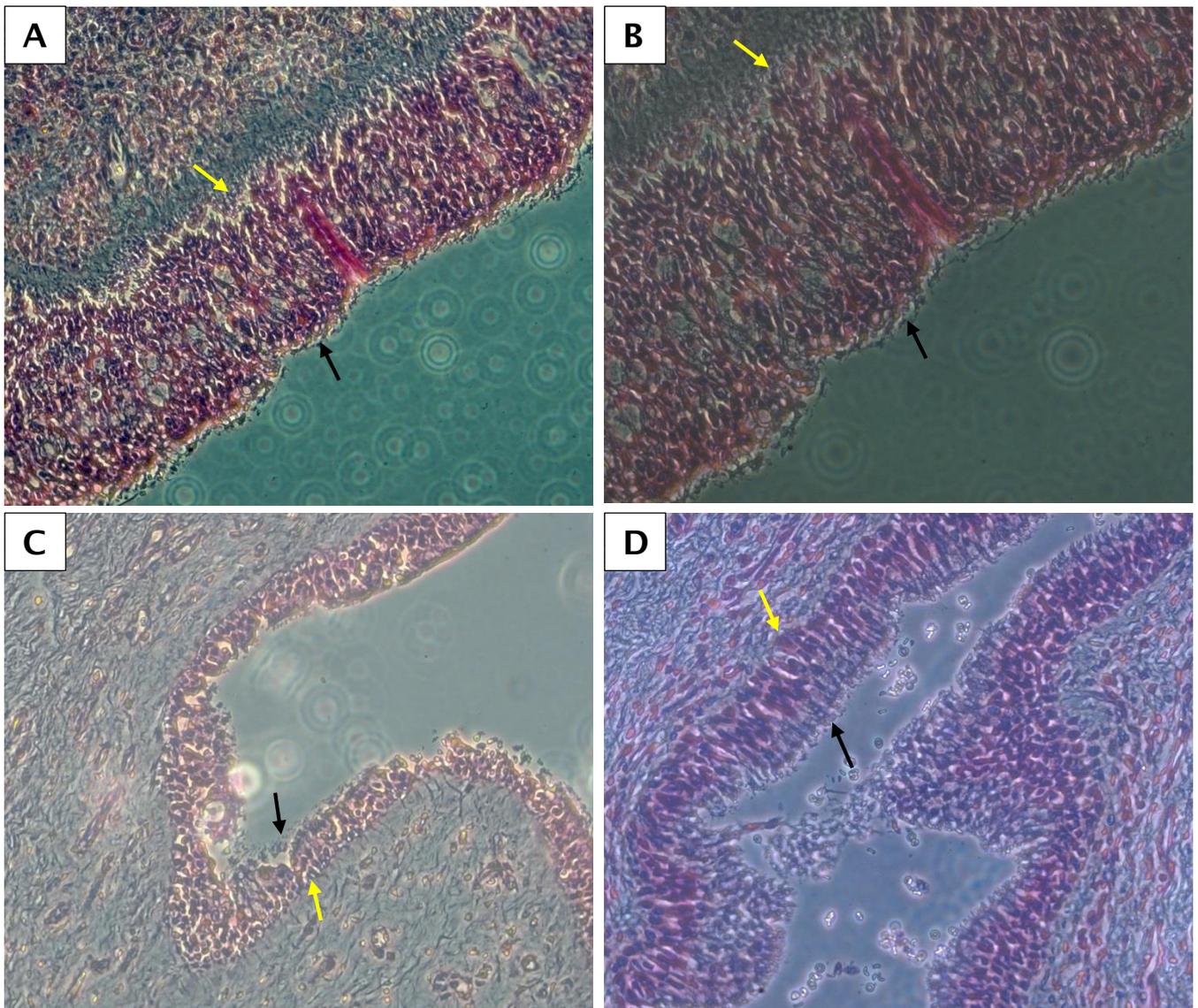
Figure 1.8 Photomicrograph of haematoxylin and eosin (H&E) stained sections of sinonasal mucosa and nasal polyp. A) Nasal polyp histology, x20 magnification. B) Nasal polyp histology, x32 magnification. C) Non-polypoidal mucosa from CRSwNP patient, x20 magnification. D) Control mucosa, x20 magnification. Images taken by author. Yellow arrow represents the basement membrane, with the black arrow representing the cilia.

1.4.3 Allergy

A number of studies suggest an increased prevalence of allergy in patients with CRS (6). However, allergy is not considered the primary determining factor in the development of CRS, but may contribute to mucosal inflammation. Amongst CRS patients undergoing sinus surgery the prevalence of allergy based on positive skin prick testing ranges from 50% to 84% (6, 49). In patients with nasal polyp disease allergy prevalence has been reported between 10% and 64% (6). However, in contrary,

other reports have failed to show any increased incidence of allergy in nasal polyp disease (43). Allergy is likely to be a contributing factor in CRS development in some patients, and treating it can help control symptoms or prevent exacerbations (50).

1.4.4 Asthma

Asthma and CRS frequently co-exist, but this relationship is poorly understood (6). The GA2LEN study, a large questionnaire based project spanning 19 European centres (57,128 participants), found a very high correlation of asthma in those with CRS (adjusted OR 3.47) at all ages, and that this association was higher in those with allergic rhinitis (adjusted OR 11.85) (34). Nasal polyps have been found to be closely associated with asthma, and nasal polyps have been found to develop more quickly, mean of 2 years, in patients with aspirin exacerbated respiratory disease (51).

1.4.5 Aspirin Exacerbated Respiratory Disease

Aspirin exacerbated respiratory disease (AERD), often referred to as Samter's Triad, is the association of salicylate sensitivity, nasal polyps and asthma. The mechanisms of disease are linked to the infiltration of mast cells and eosinophils in to the respiratory epithelium. These cells cause synthesis of leukotrienes and prostaglandins as well as histamine/tryptase release, which causes upper and lower airway inflammation. Inflammation within this condition is partially inhibited by synthesis of prostaglandin E2 through cyclooxygenase 1 (COX-1) (52). However, when aspirin or NSAIDs are taken, prostaglandin E2 synthesis stops, as COX-1 is inhibited, and there is a subsequent release of histamine and leukotrienes. This further exacerbates inflammation and leads to significant worsening of upper and lower airway disease.

This condition relates to a small subset of CRSwNP patients, but when present patients have aggressive polyp disease and difficult to treat asthma. Up to 36-96% of aspirin sensitive patients have CRSwNP (6). They tend to be non-atopic with late onset asthma, with increased prevalence over the age of 40.

Aspirin desensitisation is a potential treatment option, and has been shown to improve symptom scores, and reduce the need for repeated surgery and asthma medications (53, 54).

1.4.6 Anatomical variations

Many CRS patients will have subtle anatomical variations, as do many asymptomatic patients within the general population (55). It has been proposed that certain anatomical variations, such as concha bullosa, nasal septal deviation, displaced uncinate, etc. can lead to mucosal thickening, and the development of CRS (56). However, there have never been any proven correlations (6, 57). It is likely there is no significant causal relationship between subtle anatomical defects and the development of CRS. However, structural defects impeding the drainage of secretions within the ostiomeatal complex may increase the risk of disease development, persistence, and chronicity.

1.4.7 Biofilms

Bacteria exist in two distinct forms. The first is in the planktonic form, well known to be associated with numerous infections including pneumonia and pyelonephritis (58). The second form, genotypically and phenotypically unique from the first, is the biofilm structure. A biofilm is defined as a community of bacteria that are encased in an excreted exopolysaccharide matrix and are irreversibly attached to a surface (59). It is now recognised that the majority of pathogenic bacteria exist as a biofilm (59).

Bacterial biofilms have an enhanced ability to evade host defences and have reduced sensitivity to traditional antimicrobial agents. Bacteria in this biofilm state transform through a well-established life cycle, intermittently shedding to the planktonic form (**Figure 1.9**), which could explain the low bacterial culture rates using traditional culture techniques (60). Reduced susceptibility to antimicrobial agents is a common feature of all bacterial biofilms and is one aspect that creates the biggest clinical challenge in their long-term eradication.
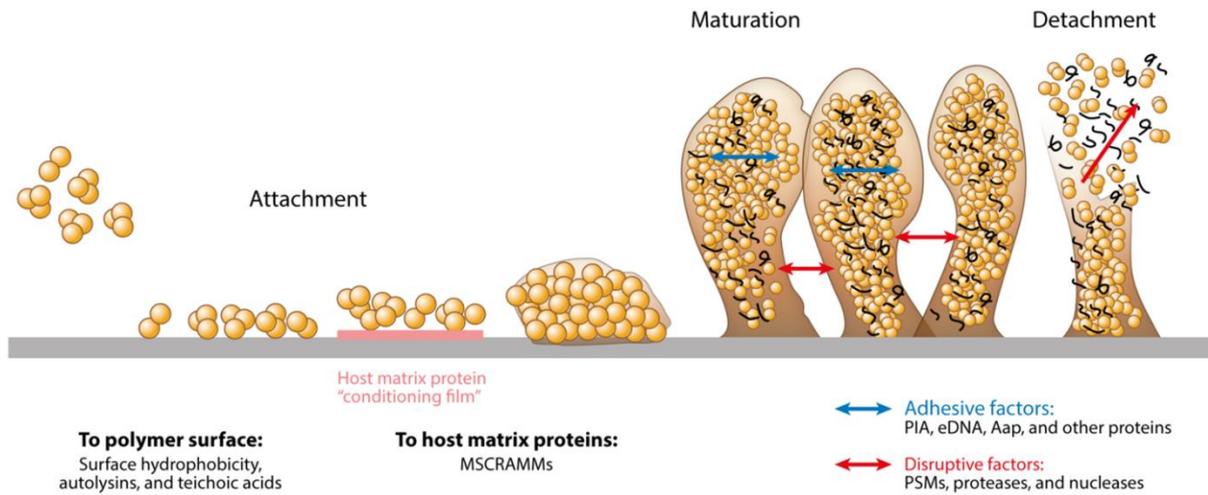
Figure 1.9 Representation of the biofilm cycle. Stage 1 reversible attachment, Stage 2 irreversible attachment, Stage 3 & 4 maturation, Stage 5 detachment/dispersal. Image taken from, Otto et al (60).

The bacterial biofilm hypothesis in CRS is now well established, with *S. aureus* being the most abundant and clinically relevant of all the pathogens identified (61-66). Work conducted by my predecessor, Mr Stephen Hayes (PhD Clinical Research Fellow), revealed biofilms on the surface of non-polypoidal mucosa of CRSwNP patients and the sinonasal mucosa of CRSsNP patients. In contrast, control mucosa was not found to contain these bacterial aggregates (**Figure 1.10**). These surface biofilms were found to contain *S. aureus* in 70% of CRS patients, P. aeruginosa and H. influenza in 30%. Furthermore, he discovered that nasal polyps contained intracellular bacteria in 90% of cases studied, in addition to other authors (22, 23, 61, 63, 64, 66-75), but was the first to discover that epithelial and mast cells were harbouring these bacteria - (**Figures 1.11 & 1.12**).
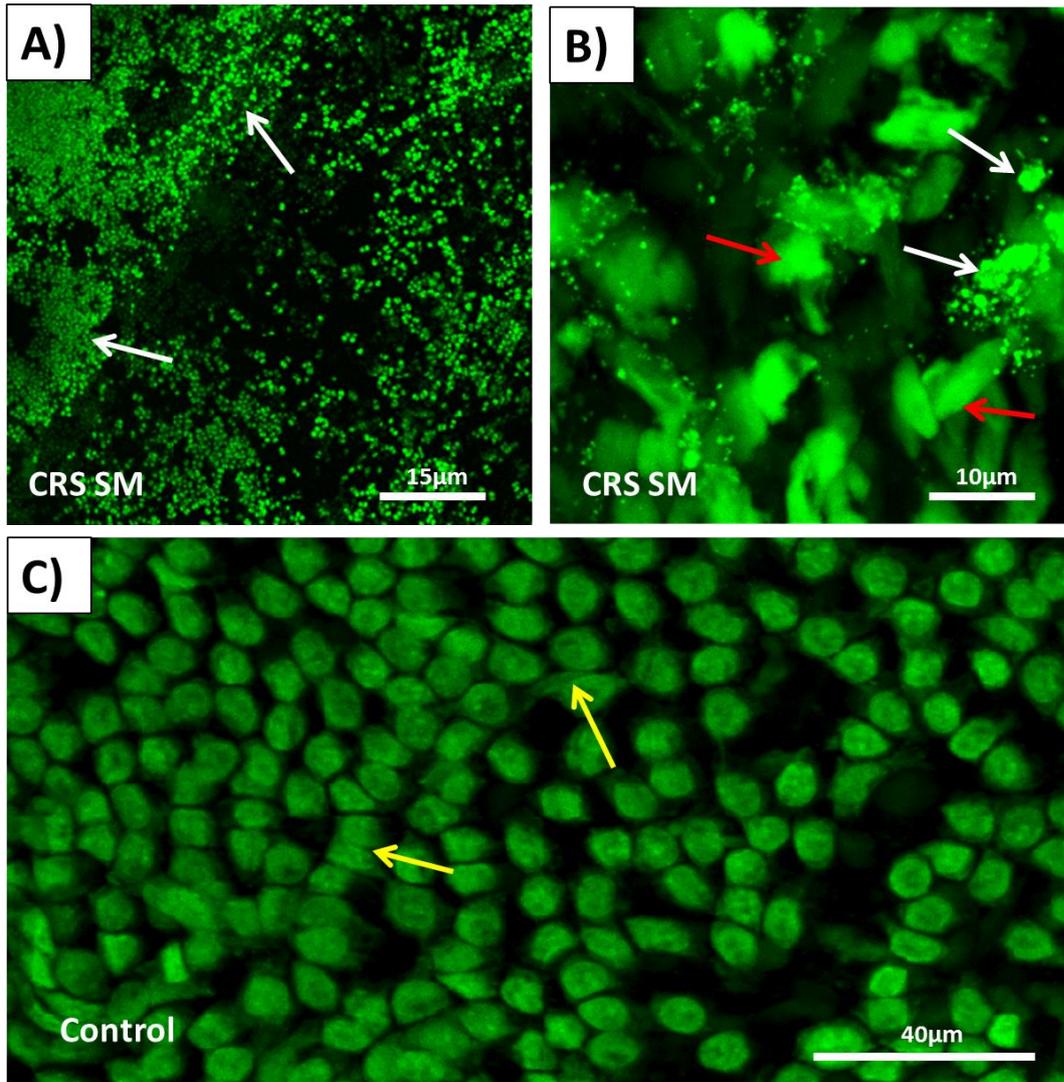
Figure 1.10 Bacterial biofilms on the surface of CRS sinonasal mucosa. Representative CLSM images of, A-B) CRS sinonasal mucosa showing viable aggregates of bacterial biofilms (white arrows) attached to the underlying surface epithelium (red arrows), C) control sinonasal mucosa showing epithelial cells (yellow arrows) with no evidence of any associated bacterial biofilms. Image taken from, Hayes SM, unpublished PhD thesis.
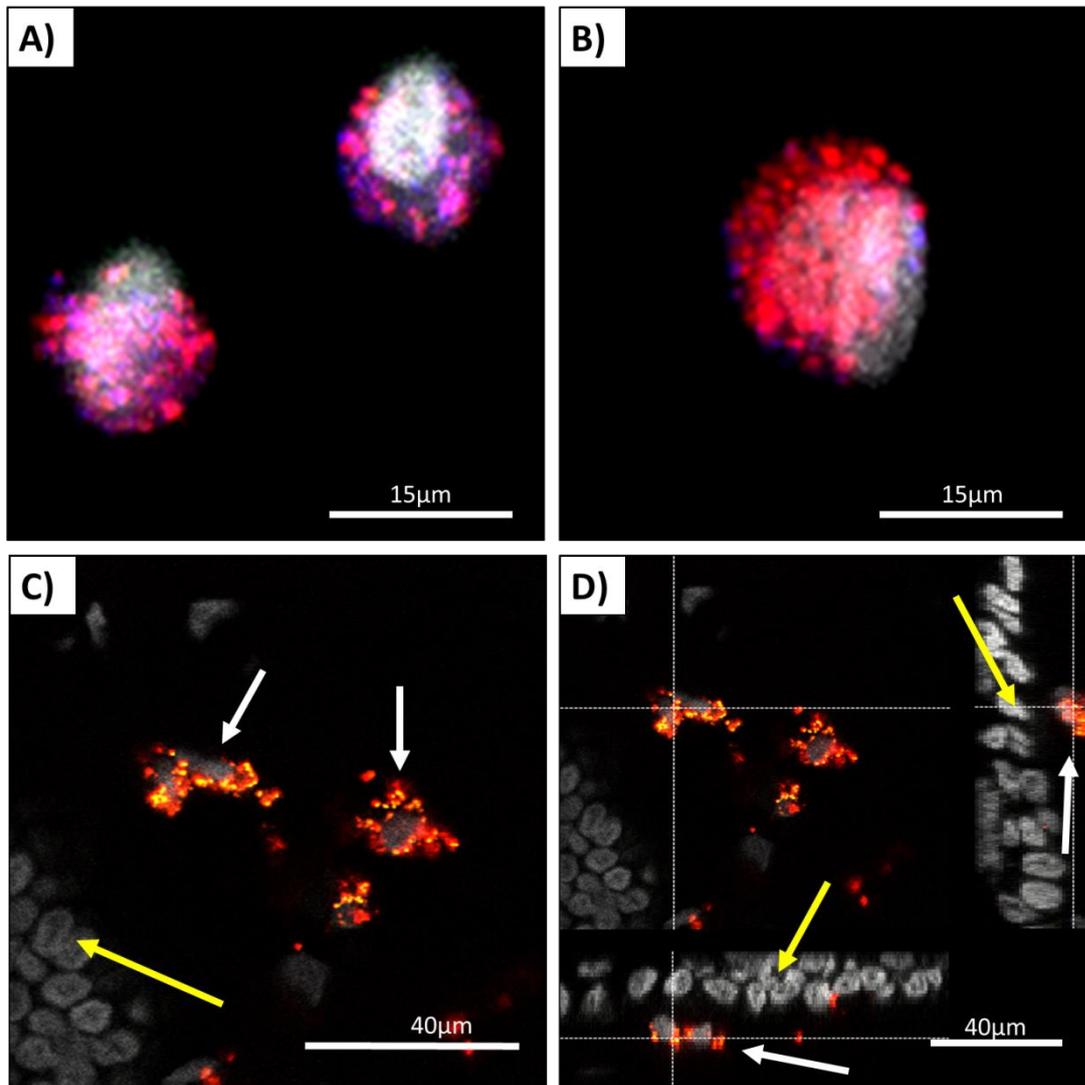
Figure 1.11 Intracellular *S. aureus* in ex vivo nasal polyp (NP) tissue. A-B) Representative high resolution CLSM images of NP tissue hybridised with 16S rRNA FISH probes (Sau/Sta) showing intracellular *S. aureus* reservoirs (pink) colocalised with DAPI-stained host nuclei (grey). C) Intracellular *S. aureus* aggregates (white arrows) beneath the epithelial surface (yellow arrows). D) XYZ view: lower magnification image clearly demonstrating intracellular *S. aureus* aggregates (white arrows) beneath the epithelial surface (yellow arrows). Image taken from, Hayes et al (25).
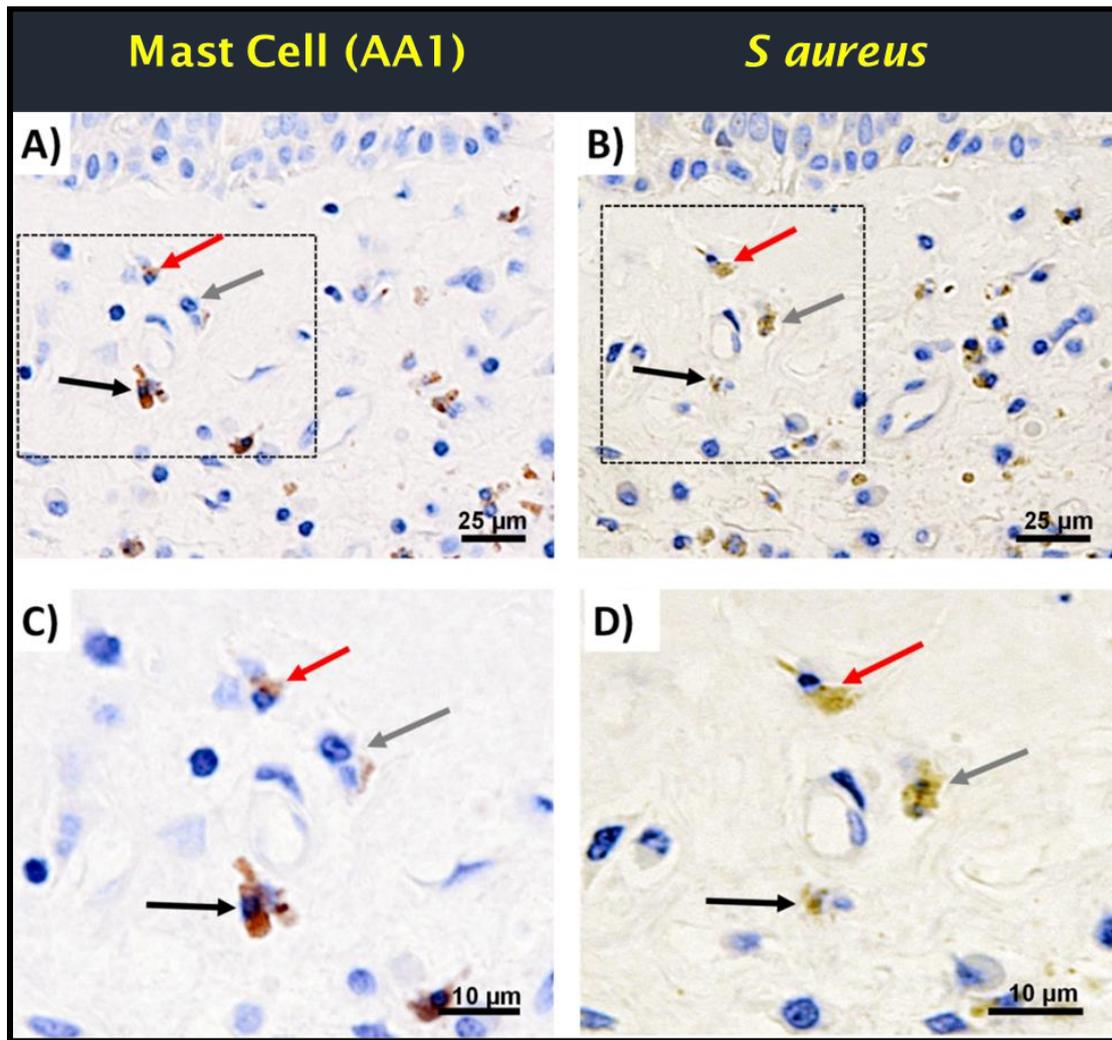
Figure 1.12 Immunohistochemical co-localisation of *S. aureus* in nasal polyp mast cells. Photomicrographs of sequential 2µm sections of NP tissue stained with monoclonal anti-MC tryptase, and mouse monoclonal anti-*S. aureus*, demonstrating A&B) sub-epithelial intracellular *S. aureus* within MC (arrows) (x20 magnification). Both images are shown at higher magnification (C & D x40 magnification). Image taken from, Hayes et al (25).

By existing within cells it is hypothesised that bacterial pathogens may be protected from the immune system (76). Traditional antibacterial therapies are tested for their *in vitro* activity against extracellular bacteria, thus intracellular bacteria my continue to survive and even act as a reservoir for re-infection following treatment (23). In this way bacteria can continue to mediate on-going inflammatory effects, and re-seed eradicated surface populations, thus acting as the Trojan horse of recalcitrant CRS (23). Further work evaluating the direct effect of surface and intracellular bacterial residency are required, to better define the relevance of bacteria biofilms in the development of CRS.

1.4.8 *Staphylococcus aureus* superantigens

The most frequently identified pathogen in patients with CRS is *S. aureus* (61-66). Bacterial superantigens (SAgs), which includes staphylococcal enterotoxins (SE), belong to a family of staphylococcal superantigen-like proteins (SSLs). These are secreted by *S. aureus* and are potent initiators of the innate and adaptive immune response, most notably the activation and stimulation of T-cells.

These molecular proteins are able to activate T cells via the T cell receptor on the MHC class II complex, independent from the antigen-specific groove, by binding to the variable β-chain (10). Once activated, T cells can orchestrate a severe inflammatory reaction, including the polyclonal activation of B cells and recruitment of eosinophils (**Figure 1.13**) (77).
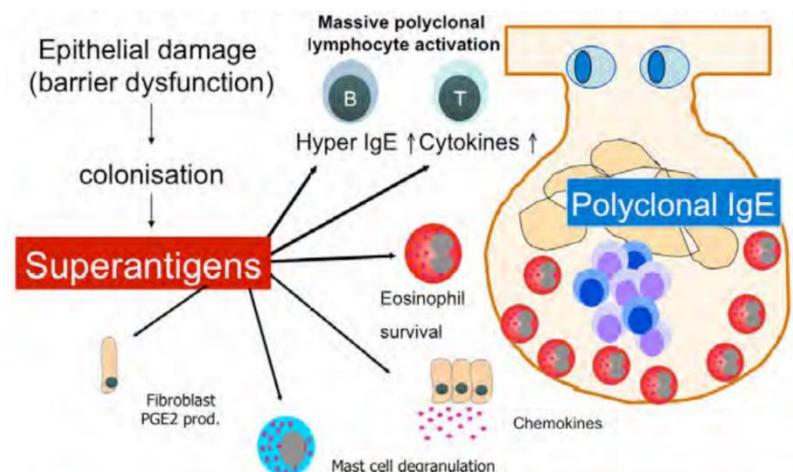
Figure 1.13 Superantigens (SAgs) in CRS. Image taken from, Bachert C et al (78).

Current evidence suggests that CRSwNP is characterised by eosinophilic inflammation, driven by IL-5 and eotaxin, which results in the chemotaxis, activation and survival of eosinophils (10). This is in contrast to CRSsNP in which IFNγ and TGF-β1 are key players, with a Th1 skewed inflammatory response (10). CRSwNP is thought to be a Th2 driven disease, with enterotoxins from *S. aureus* acting as superantigens, inducing activation of T-cells and the exacerbation of on-going inflammation (29).

Indeed, based on these differing immunogenic profiles CRSwNP and CRSsNP have been regarded as two separate disease entities, although this concept is not universally accepted (79).

S. aureus, both surface and intracellular, have been found to be associated with nasal polyps (25). Furthermore, IgE against *S. aureus*, and increased numbers of T-cells expressing the T cell receptor β-chain variable region, known to stimulate T cells through superantigens, have been found in CRSwNP patients compared to CRSsNP and controls (10, 80, 81). In culture, SEB stimulates a variety of pro-inflammatory cytokines. In a study conducted by Patou et al (2008) *Staphylococcal aureus* enterotoxin B (SEB) was used to stimulate nasal polyp tissue and controls (11). Tissue was fragmented and placed in media, with subsequent SEB stimulation for 24 hours. Results revealed significant increases of IL-1β, TNFα, IFN-γ, IL-2, -4, -5, -10, and -13 in both groups, with this increase significantly higher in nasal polyps compared with controls. They concluded that enterotoxins induce the release of cytokines, with a Th2-skewed pattern in nasal polyps (two-fold increase in IL-2, -4 and -5). These results provide evidence for the stimulatory role of SAgs in those with nasal polyps (11). Of interest, such polarisation was observed in Th2-skewed mucosal tissue but not in control tissue, highlighting the importance of the cytokine environment in a given tissue in determining the interaction between SAgs on T effector cells (11).

The direct effects of SEB on the potential formation of nasal polyps has been examined within an ovalbumin (OVA) induced allergic rhinitis mouse model. In a study performed by Kim et al (2011), OVA with SEB was introduced into the nasal cavities of mice over an 8 week period (82). Results revealed that the group treated with OVA with SEB had significantly increased epithelial disruption and nasal polypoid lesions than mice treated with OVA only. Furthermore, there was a significant increase in inflammatory cells, eosinophils and lymphocytes. Levels of IL-5, eotaxin, and OVA-specific IgE in nasal lavage fluid were increased in the OVA with SEB group. Therefore low-dose SEB induced nasal polypoid lesions with an increased eosinophilic infiltration in an allergic rhinosinusitis murine model (82).

There is mounting evidence that SE, as well as activating T-cells, also increase IgE production and B-cell repertoire in nasal polyps (10), together with lower airway effects too (78). SE skew the cytokine

milieu in a Th2 direction, increasing IL-5 and eosinophil survival. SE also increase local formation of IgE antibodies, which can stimulate mast cell degranulation. Furthermore, SE suppress natural and induced T-regulatory cells, which could be relevant in severe eosinophilic inflammation (10).

1.4.9 Osteitis

Osteitis, inflammation within the bone, is a common feature of CRS patients, evident on the pre-operative CT scans (**Figure 1.14**). There is growing evidence to support the presence of bacterial biofilms in patients with CRS, and most recently these have been correlated with in increased likelihood of osteitis (22, 68-71, 83). Enhanced ability to evade host defences, reduced susceptibility to antimicrobial agents and low bacterial culture rates using traditional methods are common features of bacterial biofilms, partially explaining why surgical management is the mainstay of treatment in severe CRS disease. However, even after surgery, a significant subset of patients with resistant disease will have recurrence, and this often occurs in patients with co-existing *S. aureus* colonisation (65, 67, 84). Intracellular bacteria have now additionally been identified within the sinus mucosa of CRS patients (22, 23, 75). Thus, bacteria appear to be intrinsically linked to severe inflammation within the paranasal sinuses. However, although the presence of bacterial biofilms seems to be correlated with increasing evidence of osteitis, evidence of bacterial penetration into the bone surrounding the paranasal sinuses is limited (85, 86). Rather than the bone acting as a harbourer of bacteria initiating an inflammatory response, it seems that the bone may act as a reservoir for inflammatory cytokines, facilitating the development of chronicity, even after the overlying mucosa has been removed (87).

Figure 1.14 Osteitis of the left sphenoid sinus walls. Coronal CT scan of thickened and inflamed bone (red arrow) surrounding an opacified left sphenoid sinus (red star). Image taken from, Schubert et al (88).

There are a number of studies within the literature suggesting that osteitis is associated with more significant and recalcitrant disease within CRS patients (19, 87, 89, 90). Indeed, it occurs in up to 88% of patients undergoing multiple sinus surgery operations compared to approximately 30% undergoing primary surgery (19, 20, 87, 89). However, though osteitis has been correlated with CRS, and predominantly in those with severe and/or recurrent disease, there is limited evidence suggesting a direct role if any in the pathogenesis of the disease. A number of recent studies have shown elevated tissue inflammation and cytokines within the bone of osteitis patients, giving credence to the theory that osteitis could be acting as a cytokine reservoir for on-going inflammation within the sinonasal mucosa. A study published by Detwiller et al (2013), revealed up-regulation of the protease, Matrix Metalloprotease (MMP) 9 in the bone taken from the ethmoidal sinuses of patients with osteitis (91). In addition, Snidvongs et al (2012), highlighted increased mucosal eosinophilia in CRS patients with osteitis (92), with Tuszynska et al (2010) revealing increased pro-inflammatory cytokines in those with osteitis (93). Although this research suggests some interesting correlations, it falls short of explaining a distinct disease pathway and crucially, for translational benefits in clinical care to occur, a lack of

research suggesting future treatment paradigms for managing the presence of osteitis in severe and recalcitrant CRS disease.

Overall, current evidence suggests that osteitis is a disease modifier rather than a disease initiator, increasing the requirements for future medical care and surgery. A significant number of patients remain resistant to conventional therapies, and further funded research into the causes and effects of osteitis could be critical for future clinical and therapeutic advances in this area.

1.5 The immune response in chronic rhinosinusitis

1.5.1 Background

CRS is a disease characterised by chronic inflammation of the sinonasal mucosa. According to the EPOS guidelines, it can be subdivided into two main disease categories, CRSwNP and CRSsNP, which display differing immune profiles (6).

1.5.2 The epithelial barrier

In addition to secreting antimicrobial products in response to host invasion, the nasal epithelium provides a physical barrier to invading pathogens, most commonly *S. aureus* in the case of CRS. This barrier between cells is maintained by intercellular junctional complexes composed of occluding junctions (or tight junctions), adhering junctions, desmosomes (and hemidesmosomes) and gap junctions (**Figure 1.15**).

Occluding junctions (also known as tight junctions or zonula occludens) surround cells forming a belt like structure that acts as a physical barrier. Claudins and occludin associate with intracellular peripheral membrane proteins called zonula occludens (ZO) proteins, which anchor the strands to the cytoplasmic actin cytoskeleton. The permeability of these junctions varies in different sites, thus creating a semi-permeable membrane.

Adhering junctions (e.g. cadherin) are located below occluding junctions, and connect to the tension bearing filaments of the cytoskeleton (i.e. actin filaments). The actin filaments are located below the apical surface circumferentially around the cell, as a 'marginal' band.

Desmosomes are points of intercellular contact that 'rivet' cells together. They connect to intermediate filaments, which form a network of great tensile strength. In addition to these junctional proteins that form cell to cell connections, there are proteins linking cells to the extracellular matrix. These consist of integrin proteins, of which hemidesmosomes represents one such structure.

Hemidesmosomes connect the basal surface of an epithelial cell to the underlying basal lamina, binding intracellularly to the intermediate filaments (keratin in epithelial cells).

Finally, gap junctions formed of proteins, called connexions, form a transport connection between cells allowing inorganic ions and other small water-soluble molecules to pass directly from the cytoplasm of one cell to the cytoplasm of the other. These connections are controllable and form a signalling network between adjacent cells.
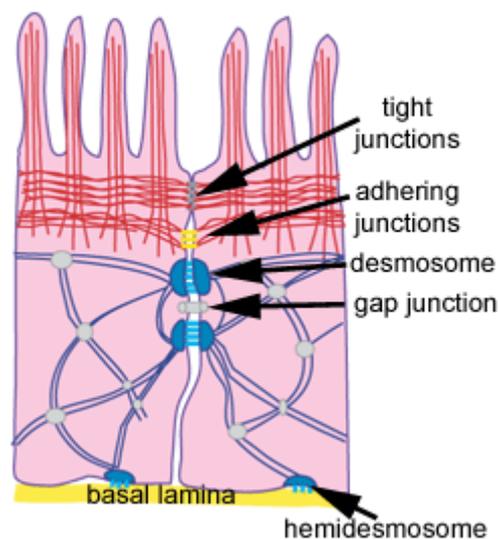
Figure 1.15 Epithelial cell intercellular junctional complexes. Image taken from, The histology guide, University of Leeds (94).

There are limited studies to date which have examined the role of epithelial tight junctional defects in the upper airway, and the development of CRS. A study published by Malik et al (2015) examining human primary nasal epithelial cell lines grown in an air liquid interface (ALI), and exposed to *S. aureus* conditioned media (95), revealed a reduction in transepithelial electrical resistance (TEER) and disruption of zona occluden-1 (ZO-1) measured with immunofluorescence. Results indicated the disruption of tight junctions maintaining epithelial integrity, hypothesising that this could be used as a method for bacteria to enter the subepithelium. Further studies have identified CRS patients to have reduced TEER values compared to controls, when extracted epithelial cells were grown in ALI cultures (96). There is also evidence that pro-inflammatory cytokines, from the Th1, Th2 and Th17 pathway,

can cause a degree of tight junction disruption and the potential for increased epithelial permeability (96-98). There is also some early evidence that steroids can help to improve barrier function through their action on junctional proteins (99, 100). However, the true clinical and pathophysiological importance of this is not yet fully understood. A definitive mechanism for bacterial invasion into the subepithelium is currently lacking within the literature.

1.5.3 Toll-like receptors

Toll-like receptors (TLRs) are present on epithelial cells and recognise pathogen-associated molecular patterns (PAMPs) on the surface of invading pathogens. In response, TLRs stimulate direct transcription and/or translation of mucins and antimicrobial peptides (AMPs). They also activate epithelial cells to secrete defence molecules and cytokines, through the action of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). This activates the immune pathway, and may contribute to persistent inflammation within CRS mucosa. Sinonasal epithelial cells express TLRs 1 to 10, with TLR2 having the greatest diversity of ligands to recognise a wide array of gram-positive and gram-negative bacteria as well as fungi (101, 102). TLR4 is important in host responses to gram-negative bacterial infections within the airway (103). Conflicting profiles of TLR expression have been reported in CRS patients (104). Studies have shown significantly increased expression of TLRs 2, 5, 6, 7, 8 and 9 in patients with CRSwNP compared to CRSsNP and controls (104). This provides evidence of the possible effects of bacteria within the pathogenesis of CRS and formation of nasal polyps, and the concept of disease subtypes. However, further work is required to define the significance of these findings in the future (25, 105).

1.5.4 Tissue remodeling

Tissue remodelling describes a process of modification to the normal composition and structure of the epithelium and underlying structures. Transforming growth factor beta (TGF-β) and matrix

metalloproteinases (MMPs) are thought to be major players in the remodelling processes of upper and lower airway disease.

1.5.4.1 Transforming growth factor beta

CRSsNP is driven by fibrosis, with basement membrane thickening, goblet cell hyperplasia and mononuclear cell infiltration. In contrast, CRSwNP is characterised by oedematous stroma, albumin deposits, pseudocyst formation, and subepithelial and perivascular inflammatory cell infiltration (106). Within the upper airway, elevated TGF-β1 levels have been correlated with CRSsNP, with low levels found in CRSwNP (**Figure 1.16**) (14). TGF-β is implicated in extracellular matrix deposition in the lower airways, and within the upper airways low levels of TGF-β in CRSwNP decrease tissue repair, resulting in tissue oedema and collagen defects. Whilst high levels in CRSsNP contribute to basement membrane thickening, excessive collagen deposition and fibrosis (106). TGF-β1 represents a master switch for inflammation and remodelling in both upper and lower airways diseases, but further research is necessary to define its exact role in the pathogenesis of CRS (106).
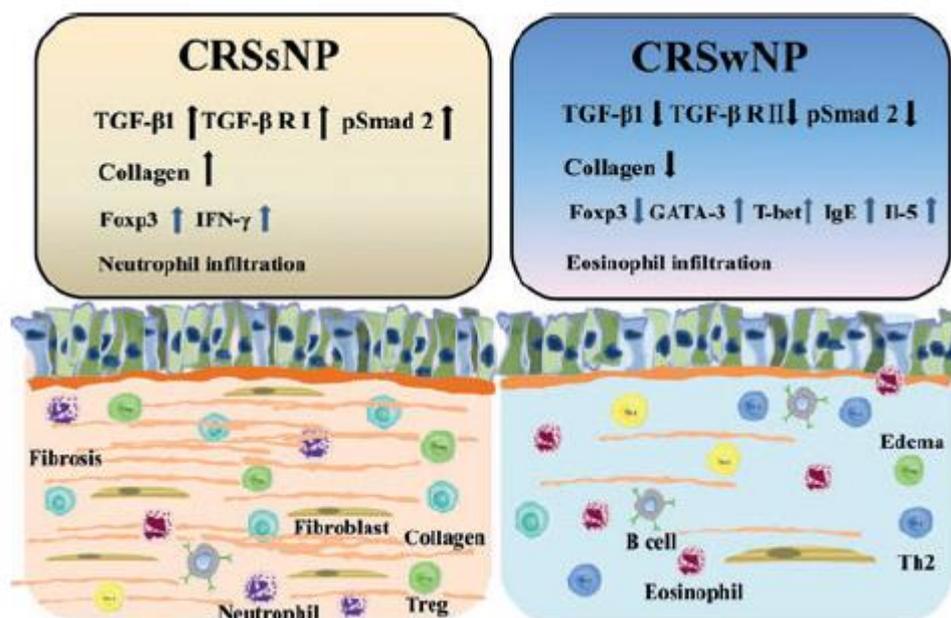
Figure 1.16 Remodelling processes in chronic rhinosinusitis. Image taken from, Yang Y et al (106).

1.5.4.2 Matrix metalloproteinases

A family of 24 zinc dependent endopeptidases comprise the human MMPs, capable of degrading extracellular matrix and basement membranes (107). The natural tissue inhibitors of metalloproteinases (TIMPs) maintain the balance between extracellular-matrix deposition and degradation. There are four known types of TIMPs, all which inhibit MMPs. TIMPs also exhibit additional functions including pro- and anti-apoptotic activity, cell-growth and tumour growth reducing functions (107). MMPs are excreted in an inactivated pro-enzyme (Zymogen) form. They become activated in the extracellular environment, through interaction between the thiol group of a pro-domain cysteine residue and the zinc ion of the catalytic site, thus allowing their catalytic properties to become active (cysteine-switch mechanism) (108). MMP activity is regulated at four points - gene expression, pericellular accumulation of enzymes, pro-enzyme (or zymogen) activation and enzyme inactivation. Therefore, measurement of gene expression only can be misleading, as further steps are required for MMPs to display their proteolytic properties (108).

There are some conflicting data regarding MMP levels in CRS, but the majority of studies report increased expression and activation of MMPs, with an associated decrease in TIMPs, in CRSwNP (16, 107, 109-113). The strongest correlations have revealed increased expression of MMP-9 in CRSwNP patients with a reduction in TIMP-1, measured using various techniques including immunohistochemistry, enzyme-linked immunosorbent assay (ELISA) and real time quantitative polymerase chain reaction (RT-qPCR) (16, 107, 110-113). Increased levels of MMPs are thought to be involved in remodelling of the extracellular matrix, aiding the formation of loose stroma, tissue oedema, pseudocyst formation and epithelial defects.

1.5.5 Dendritic cells

Dendritic cells (DCs) are recognised as professional antigen-presenting cells (APCs), important in initiating and coordinating cell-mediated adaptive immune responses (114). They produce pro-inflammatory cytokines in response to pathogens, which induce naïve T cell differentiation into Th1,

Th2, Th17 and Treg cell linages. DCs have been described in the nasal mucosa, with multiple subsets present (114). A recent study examining myeloid DCs in control tissue (turbinate) compared to nasal polyps found a significant increase in the number of DCs within nasal polyp tissue, potentially contributing to tissue inflammation (115). These observations were mirrored in a number of other studies, comparing nasal polyp tissue to turbinate tissue from non-CRS control patients (116-118).

1.5.6 Macrophages

Macrophages are innate immune cells which have a variety of roles including pathogen response, inflammation, tissue repair, and removal of particulates. Macrophage responses can be subdivided into two main types, M1 the classical activation pathway, which is driven by Th1 cytokines and involves a pro-inflammatory response to kill intracellular pathogens. M2, the alternative pathway, is Th2 driven and is important in defense against helminthes, humoral immunity and tissue repair (6). M2 macrophages, expressing elevated macrophage mannose receptor (MMR) are present in high levels in CRSwNP patients compared to CRSsNP and controls (6, 119, 120). Furthermore, phagocytosis of *S. aureus* by human derived macrophages was reduced in CRSwNP as compared to macrophages from the inferior turbinate. This could be of relevance to the intracellular residency of *S. aureus* and its possible role in mediating ongoing pro-inflammatory effects (25).

1.5.7 T cells

Within the nasal mucosa, antigen presenting cells (APCs) cause naïve T lymphocytes to differentiate into one of several T cell lineages: Th1, Th2, Th17, and T regulatory cells (Tregs) (6). For Th1 responses the key transcriptional factor is T-bet, which produces a macrophage rich cellular infiltrate with raised IFN-γ. For Th2, GATA-3 is the transcriptional factor with associated cytokines IL-4, IL-5, IL-13 and an eosinophilic rich cellular response. Th1 responses are designed to be effective against viruses and intracellular bacteria, with Th2 responses aimed at parasites, particularly those too large for phagocytosis. The transcriptional factor for Th17 cells is Retinoid-related orphan receptor gamma t

(RORγT). IL-23 has been shown to drive the differentiation and expansion of Th17 cells as they acquire

expression of the IL-23 receptor, with activated cells secreting IL-17 (121). Tregs are characterised by

the transcriptional factor forkhead box p3+ (FoxP3) with the purpose of limiting the differentiation of

other T cell linages and providing peripheral tolerance (**Figure 1.17**) (6).

CRSwNP is regarded as a Th2 driven process, in contrast to CRSsNP which is predominantly Th1 (6, 29,

122-125). Th1/Th17 involvement has been found in Asian nasal polyps (124-129), which typically have

a neutrophilic infiltrate. There is limited data regarding a similar Th17 response in Caucasian patients

(124, 130-132). However, there are some recently published studies which do suggest a Th17 response

in some Caucasian patients (133, 134).
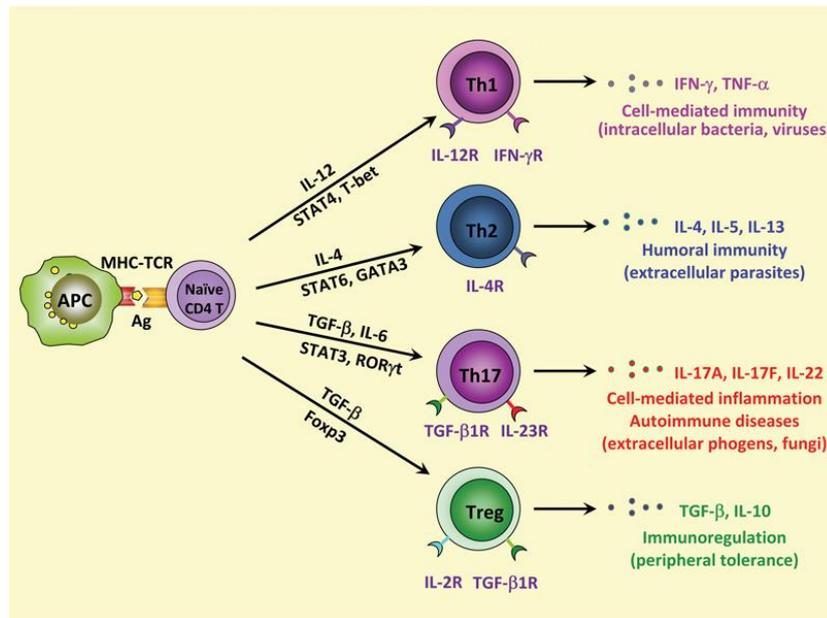
Figure 1.17 T-cell differentiation factors. APC, antigen-presenting cell; Foxp3+, forkhead box p3+; IFN, interferon; MHC–TCR, major histocompatibility complex–T-cell receptor; ROR, retinoid-related orphan receptor; TGF, transforming growth factor; Th, T helper; Treg, regulatory T. Image taken from, Leung S (135).

1.5.8 Eosinophils

Eosinophils are circulating granulocytes whose primary function is mucosal immune defence. They

also play a role in tissue remodelling and repair (6). Eosinophils have long been associated with CRS as

they were the original histological hallmark of the disorder (136). Nasal polyps were deemed to be

produced as a direct result of allergy. This was partly due to an overlap with allergic rhinitis, and a perception that nasal polyp development could be a continuation of the same disease process. Later research however, established CRS as a separate disease entity, with two distinct disease subtypes, CRSwNP and CRSsNP (6).

Eosinophilia predominates in Th2 driven CRSwNP, although geographical differences do exist, with Asian polyps being more neutrophilic in nature. These Asian neutrophilic nasal polyps display a Th1/Th17 inflammatory profile, as opposed to the Th2 driven Caucasian eosinophilic polyps. Furthermore, CF-related nasal polyps tend also to be more neutrophilic. Therefore, whilst eosinophils are not essential to the formation of nasal polyps, their presence indicates a significant pro-inflammatory drive. Indeed, both disease severity and the requirements for revision surgery have been correlated with increasing nasal polyp eosinophilia (137-139).

In order to exert pro-inflammatory effects eosinophils require attraction, priming and activation. Eosinophils are attracted through the expression of eosinophil-attracting chemokines, regulated on activation normal T cell expressed and secreted (RANTES), Eotaxin 1-3 and monocyte chemoattractant protein (MCP) 1-4. Once attracted to the relevant area, the cells are primed and activated through Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5. Th2 cytokines, via these mechanisms, cause the recruitment, activation and survival of eosinophils in CRS (140). It is thought that SAgs promote mucosal eosinophilia primarily by accentuating local Th2 responses, but it has also been suggested that biofilms may drive eosinophilic inflammation through alternative unrelated mechanisms (24, 141).

1.5.9 Neutrophils

Neutrophils are important in the early phagocytosis and killing of extracellular microbes. Neutrophils are driven to mucosal sites by microbial stimulation of pattern recognition receptors (PRRs), as well as the chemokine IL-8 (CXCL8) (6). A relative tissue neutrophilia is seen in CF and Asian nasal polyps. Although neutrophilic in nature, Asian polyps contain varying degrees of other inflammatory cells

(125). However, when eosinophils are present in Asian polyps, there appears to be a higher rate of recurrence following surgery (13). The exact role of neutrophils in CRS, and indeed nasal polyp development, is not well understood especially as traditionally they have been considered as an acute response cell with short lived survival times. However, recent studies have investigated their possible role in disrupting the resolution of disease and driving chronic inflammatory states, as seen in neutrophilic lung diseases such as CF and chronic obstructive pulmonary disease (COPD) (142). Thus, neutrophils are likely to play a role in the development of some nasal polyp subsets, including CF and Asian nasal polyps.

1.5.10 Mast cells

Mast cells are sentinel cells that form part of the innate immune system, patrolling sites constantly exposed to the environment and frequently colonised by pathogens (143). Mast cells are best known for their role in allergic sensitisation and reactions, via the high affinity IgE receptor (FcεRI), resulting in degranulation and release of histamine and other immunogenic precursors. However, in addition, studies have demonstrated their role in host defence, through their phagocytic actions as well as secretion of pro-inflammatory cytokines that signal other key effector cells (143).

Increased levels of mast cells have been reported in CRSwNP, compared to CRSsNP and controls (6, 144, 145). It was suggested that mast cells could contribute to nasal polyp formation through induction, augmentation and maintenance of eosinophilic inflammation, via IgE-dependent and independent processes (146). It had been shown that mast cell degranulation can be triggered by Staphylococcal surface protein A (SpA), and that mast cell prostaglandins could be involved in the recruitment and activation of Th2 lymphocytes in nasal polyps (11, 147). However, despite this work, the relative importance of mast cells in the pathogenesis of CRSwNP remained unclear. Further work, related to the role of mast cells in the pathogenesis of CRS and nasal polyps has come to light since the publication of the EPOS guidelines (6).

An important paper outlining the potential for *S. aureus* to internalise within mast cells and subvert its defensive mechanisms was published by Abel et al (2011) (143). Using bone marrow-derived murine mast cells (BMMC) and a human mast cell line (HMC-1), it was shown that mast cells released extracellular traps and antimicrobial compounds to internalise and kill *S. aureus*. Furthermore, in a subset of mast cells rather than being killed *S. aureus* was able to survive within the intracellular milieu, by increasing cell wall thickness and by gaining access into the mast cell cytosol. The significance of this study was made relevant by a later study published by Hayes et al (2015) (25). This work revealed the relative abundance of mast cells in CRS patients (**Figure 1.18**, Hayes SM, unpublished PhD thesis) and reported the novel finding of intracellular *S. aureus* within mast cells in nasal polyps (**Figure 1.12**).

It was suggested that mast cells may be acting as a double-edged sword, by promoting innate immunity against microbial pathogens, but also providing a safe haven for *S. aureus* by protecting it from extracellular antimicrobial compounds. This not only avoids clearance of the bacteria, but also facilitates the establishment of an intracellular microbial reservoir that could lead to seeding of bacteria, persistence and chronic carriage. This could lead to disease chronicity, higher rates of recurrence, and the development of resistant disease. A further study, published recently using a mouse model, has shed light on the significance of mast cells in the development of CRS and nasal polyps (148). This study utilised a wild type and mast cell deficient mouse model of CRS, using allergen sensitisation and chronic challenge. It found that the model deficient of mast cells did not go on to develop cystic nor polypoidal changes within the nose, suggesting a critical role of mast cells in the disease pathogenesis. This study does provide some insight into the potential significance of mast cells in the formation of nasal polyps, and taken with other evidence highlights the importance of further research into this area.
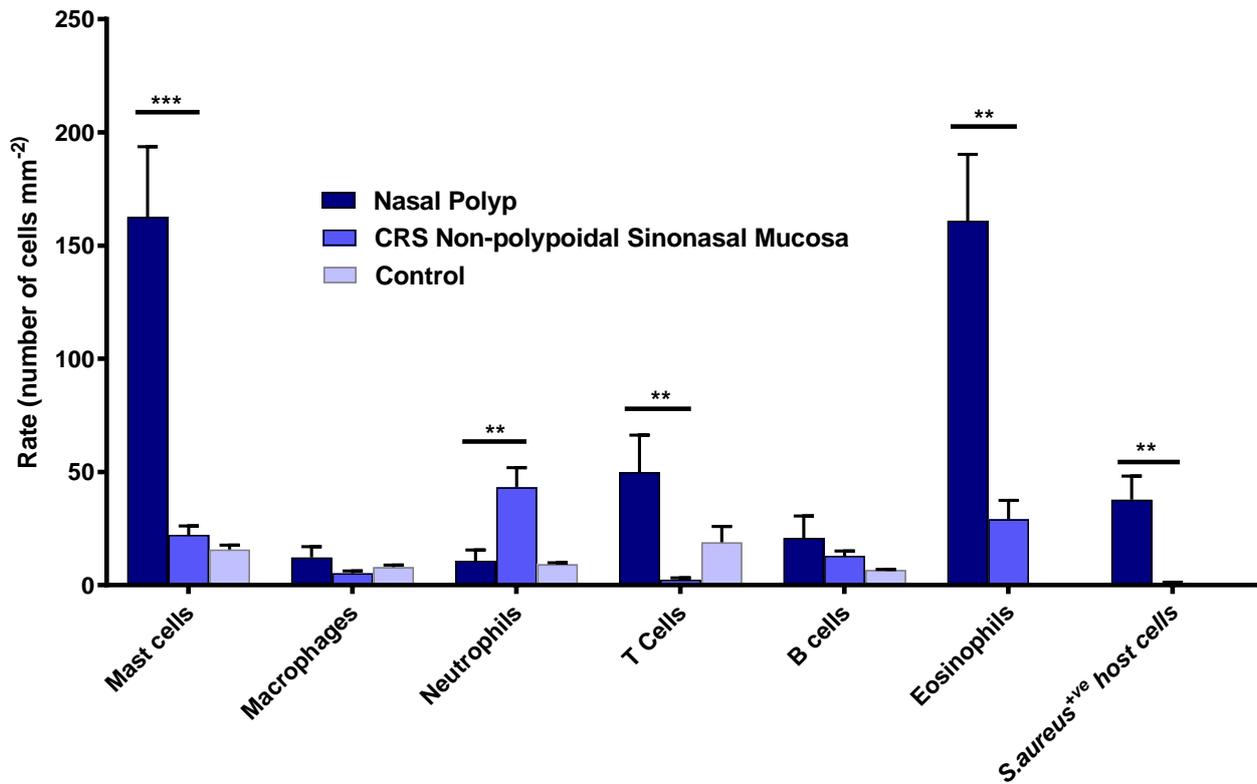
Figure 1.18 Host cell profiling in nasal polyp, CRS non-polypoidal sinonasal mucosa and control tissue. Nasal polyp & adjacent non-polypoidal CRS sinonasal mucosa (n=10), controls (n=7). Only significant p-values shown; error bars represent mean + 1SD. ** p < 0.001, *** p < 0.0001. Figure taken from, Hayes SM, unpublished PhD thesis.

1.6 Hypothesis

Bacteria, and the toxins they produce, promote chronic inflammation. Epithelial and mast cells aid this process, through the provision of a protected intracellular niche, and through the upregulation and release of pro-inflammatory cytokines upon bacterial exposure and uptake.

1.7 Aims

The overall aims of this research are:

1. To compare the differences in the immune profiles of nasal polyps compared to adjacent non-polypoidal sinonasal mucosa, within the same patients. As a means of understanding the factors related to the growth and recurrence of nasal polyps.

2. To study the immune response of nasal polyps to SEB stimulation, to further clarify its possible role in the development of chronic inflammation and nasal polyp formation.

3. To determine whether epithelial cells uptake *S. aureus*, and whether this remains viable and capable of propagating ongoing infection and inflammation.

4. To further understand the significance of *S. aureus* uptake into mast cells, and what effect this has on mast cell activation, survival, and the associated immune response.

Timothy C Biggs

2   Materials and Methods

2.1   Ethical approval

Full ethical approval was obtained for this study from the Southampton and South West Hampshire Research Ethics Committee (Ethics Reference Code: REC 09/H0501/74). Patients were fully counselled on all aspects of the study, and given reasonable time to make a non-pressurised, informed decision. Patients undertook formal written consent (**Appendix I**) prior to enrolment with provision of a written information leaflet (**Appendix II**).

2.2   Subjects

Patients meeting the diagnostic criteria set out in the EPOS guidelines for surgery, were included in the study (6), together with suitable control patients undergoing trans-sphenoidal pituitary surgery. CRSwNP and CRSsNP patients, undergoing functional endoscopic sinus surgery (FESS) were operated on by Mr Rami Salib (Director of Upper Airway Research, University of Southampton; Associate Professor of Rhinology, University of Southampton; Principle Academic Supervisor and Consultant Rhinologist) or Mr Philip Harries (Consultant Rhinologist). Patients were listed for surgery after having failed a course of maximal medical therapy, consisting of two months of nasal and/or oral steroids, saline washes (douche), and antibiotics. Exclusion criteria included age under 18 years, cystic fibrosis (CF) or primary ciliary dyskinesia (PCD) patients, those with other immune deficiency syndromes, and those on steroid or antibiotic therapy within 8 weeks of surgery.

Control tissue was obtained from patients undergoing endoscopic trans-sphenoidal pituitary surgery, with absence of clinical symptoms of CRS. Mr Ashok Rokade (Consultant Rhinologist & Anterior Skull Base Surgeon) and Mr Nijaguna Mathad (Consultant Neurosurgeon) operated on these patients. Control tissue was classified as material which would have been discarded as clinical waste after the surgery. This material represented healthy sinonasal mucosa surgically removed from the nasal cavity in order to gain access to the pituitary gland.

2.3 Phenotyping

All enrolled patients underwent skin-prick allergy testing to a standard set of aero-allergens (ALK-Abello, Reading, Berkshire, UK) prior to surgery, as per normal clinical practice. All patients underwent computerised tomography (CT) scanning prior to surgery and radiological disease severity was graded using the Lund-Mackay scoring system (LMS) (149). Demographic data was collected pre-operatively including sex, age, allergy, asthma, aspirin insensitivity, previous nasal surgery, past medical history, and smoking status. The perioperative endoscopic appearance of the sinonasal cavity was also noted, as well as the presence or absence of nasal polyps.

2.4 Tissue culture

2.4.1 Tissue collection, culture and storage

Tissue was collected at source and transported to the laboratory in Hanks' Balanced Salt Solution (HBSS). Tissue was either processed for live culture or frozen for later use. Tissue was cut into small samples (2x2mm) and snap frozen in liquid nitrogen and stored at -80°C for future processing. Fresh tissue was placed within a nasal explant model, as detailed below, for the analysis of *Staphylococcal aureus* enterotoxins outlined in Chapter four.

2.4.2 *Ex-vivo* nasal explant model

Following tissue collection and transport in HBSS, polyp and mucosal controls were cut into small explants (3mm^2), with four pieces placed on a 100μm cell strainer (VWR International, Radnor, PA, USA) within a six well tissue culture plate (Greiner Bio-One International, Kremsmunster, Austria) containing 1ml of synthetic interstitial fluid AQIX RS-1 serum free medium (AQIX Ltd, London, UK) (**Figure 2.1**). Tissue explants were semi-submerged within this culture, thereby maintaining the physiological air/fluid interface. The explants were cultured for 24 hours at 37°C (5% CO_2) with and without the addition of SEB (10μg/ml, Sigma-Aldrich, Gillingham, UK). This dose of SEB was selected as it was a standard dose used within the literature, allowing the study of the immunological effects of *S. aureus* exposure combined with limitation of tissue toxicity (150-153). Tissue was collected following culture, snap frozen in liquid nitrogen and stored at -80°C for future real-time quantitative polymerase chain reaction (RT-qPCR) processing. Culture supernatants were collected, centrifuged (250g for 5 minutes) to remove cell debris, aliquoted and stored at -80°C for Luminex processing.

A

| Tissue Explants 3mm^2 | Aqix media +/- SEB | Cell strainer |

B
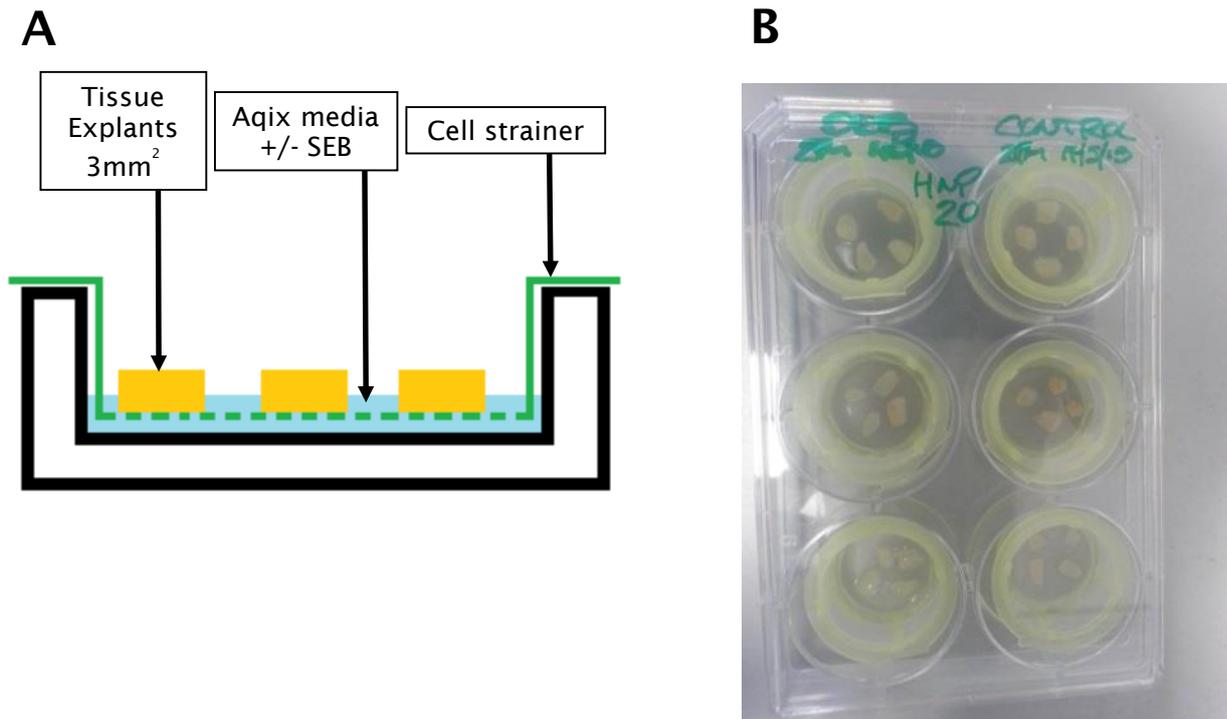
Figure 2.1 Nasal explant culture model.

2.4.2.1 Viability testing of the Ex-vivo nasal explant model

Six explant tissue models were set up, over 6 days to measure tissue viability using an LDH assay. Six separate samples were cultured with and without SEB (10μg/ml), as well as in 1% triton X-100 over 24 hours to derive the maximal production of LDH. The supernatant of separate explant cultures were collected at days 1, 2, 3 and 6, centrifuged (250g for 5 minutes) to remove cell debris, aliquoted and stored at -80°C for measurement of LDH release.

2.5 Immortalised cell line culture

For *S. aureus* co-infection experiments, outlined in chapter five and six, three immortalised cells lines were used as detailed below.

2.5.1 HMC-1 mast cell line

The HMC-1 mast cell line was originally established from a patient with mast cell leukaemia (a kind gift from Dr JH Butterfield, Mayo Clinic, Rochester, MN, USA) (154). This cell line represents an immature mast cell line, with associated quick doubling rate (approx. 18 hours) that do not express the high affinity IgE receptor (FcεRI) and are also poorly granulated (155). Mast cells were taken from this original stock and split, with cell lines grown for a maximum of six months before reviving fresh aliquots. Mast cells were grown in Iscove's Modified Dulbecco's Medium (IMDM, Sigma-Aldrich Ltd, Gillingham, UK) supplemented with 10% foetal bovine serum (FBS, Sigma-Aldrich Ltd, Gillingham, UK) and 2% Penicillin/Streptomycin (Sigma-Aldrich Ltd, Gillingham, UK). HMC-1 cells were fed by media exchange undertaken twice weekly, and centrifuged to pellet cells (250g, 5 mins) with full media exchange every other week. HMC-1 cells were grown in 250ml culture flasks (Greiner Bio-One International, Kremsmunster, Austria) in 20mls of media, at between 250 thousand and 30 million cells per flask.

2.5.2 LAD2 mast cell line

The LAD2 cell line, developed in the Laboratory of Allergic Diseases at NIAID/NIH from a patient with mastocytosis, represents a more mature mast cell line (156) with associated slower doubling rates (approx. 2 weeks). LAD2 cells express FcεRI and contain granules that degranulate in response to antigen (155), making them more representative of tissue resident mast cells. LAD2 cells were cultured in StemPro – 34 SFM media (Life Technologies Ltd, Carlsbad, CA, USA) supplemented with stem cell factor (SCF) and 0.2% Gentamycin, Penicillin and Streptomycin (Sigma-Aldrich Ltd, Gillingham, UK).

LAD2 cells were fed by media exchange once weekly, and centrifuged to pellet cells (250g, 10 mins) with full media exchange undertaken every other week. LAD2 cells were grown in 250ml culture flasks (Greiner Bio-One International, Kremsmunster, Austria) in 20mls of media, at between 250 thousand and 10 million cells per flask.

2.5.3 RPMI 2650 nasal epithelial cell line

The RPMI 2650 nasal (septal) epithelial cell line was harvested from the pleural effusion of a patient with nasal septal squamous cell carcinoma (157). It is a widely used immortalised cell line, which is representative of the nasal epithelium and can be differentiated into an air liquid interface culture (158, 159). RPMI 2650 epithelial cells were grown in minimum essential medium eagle (MEM, Sigma-Aldrich, Gillingham, UK) supplemented with 10% FBS, 2% L-glutamine and 1% non-essential amino acids. Epithelial cells were fed by media exchange every week, and passaged when they had formed a confluent monolayer within a 250ml culture flask (Greiner Bio-One International, Kremsmunster, Austria). For passage, cells were incubated with 10mls of Trypsin-EDTA (0.25%) and incubated for 5 mins (37°C with 5% CO_2). Following incubation, cells were agitated to aid in detachment and 10mls of media added to neutralise the Trypsin. Cells were then centrifuged (250g, 5 mins) and reconstituted if fresh media with 500 thousand cells placed in a culture flask for attachment and growth. Experiments were conducted on epithelial cells between passages 25 to 35.

2.4   Lactate dehydrogenase cytotoxicity assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme, present in numerous types of cells. Damage to the cell membrane causes release of LDH into the extracellular space. As such, LDH assays enable the detection of this enzyme as a surrogate marker of cell stress, damage and death.

An LDH cytotoxicity assay (Sciencell Research Laboratories, Corte Del Cedro, CA, USA) was used as per the manufacturer's instructions. A typical LDH standard curve was constructed by adding 2µL of LDH stock to 498µL of PBS (500mU/ml of LDH), and sequentially diluted to a final solution concentration of 7.8mU/ml (range of detection 500mU/ml to 7.8mU/ml). Culture supernatants, LDH standards and controls were placed within a 96 well plate (Greiner Bio-One International, Kremsmunster, Austria) in triplicate. A final working volume of 60µL was added to each well and incubated at room temperature in the dark for 20 minutes. The reaction was stopped with 20µL of sodium oxymate solution per well, and absorbance measured at 490nm. A standard curve was constructed (**Figure 2.2**) and LDH levels measured by extrapolating the absorbance using the equation y=mx+c.
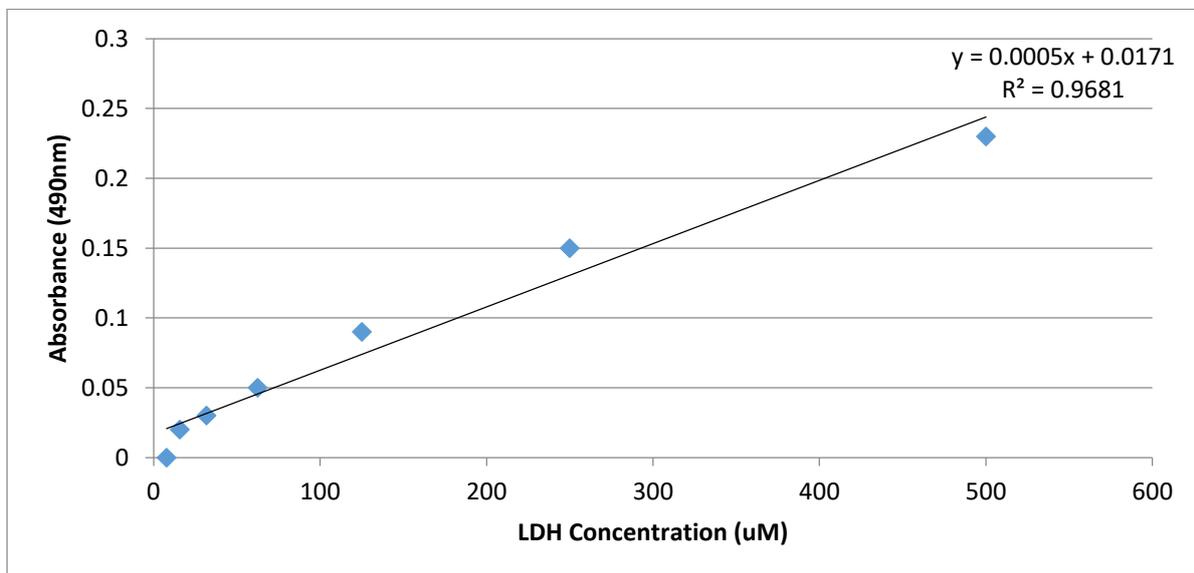
Figure 2.2 LDH assay standard curve.

2.5 Real-time quantitative polymerase chain reaction

This was conducted in an RNA/RNase and DNA/DNase free environment.

2.5.1 Tissue disruption

Frozen tissue was disrupted for 45 seconds using 0.5g of 1.0mm zirconia beads (Thistle Scientific, Glasgow, Scotland, UK) using a MagNA-lyser (Roche, Basel, Switzerland) set at 4,500rpm, and chilled on ice for 5 minutes.

2.5.2 RNA extraction and quantification

Column extraction with Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany), was chosen over phenol extraction using TRIzol® (Life Technologies Ltd, Carlsbad, CA, USA), due to superior RNA quantity and quality. **Table 2.1** reveals the NanoDrop® ND-1000 spectrophotometer (Life Technologies Ltd, Carlsbad, CA, USA) results of the phenol (TRIzol®) extraction in comparison to column extraction (Qiagen RNeasy Mini Kit). **Figure 2.3** shows the spectrophotometer absorbance trace for a column extraction sample, revealing high purity and low levels of contamination.

Table 2.1 Comparison of phenol vs column RNA extraction.

	Mean (range) RNA yield	Mean 260/280nm absorbance ratio	Mean 260/230nm absorbance ratio
Phenol extraction (TRIzol®) n=12	41 (17-86) ng/μL	1.47	0.31
Column extraction (Qiagen RNeasy Mini Kit) n=24	62 (21-126) ng/μL	1.97	1.42
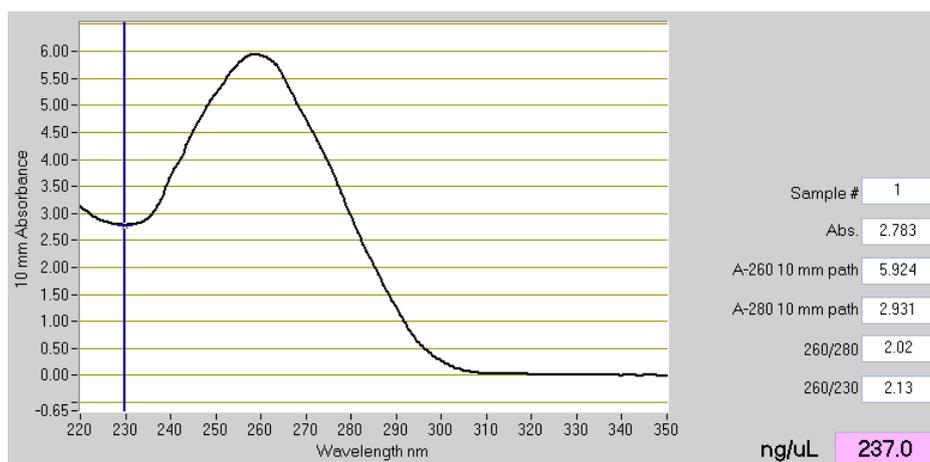
Figure 2.3 Column extraction spectrophotometer trace.

Following tissue disruption, in 350µL of buffer RLT, ribonucleic acid (RNA) extraction was undertaken using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Following a spin step (3 minutes at >8000 x g), and transfer of the supernatant into a fresh tube, 350µL of 70% ethanol was added to the lysate and mixed by pipetting. The resultant mixture, 700µL, was then transferred to a RNeasy Mini spin column, placed within a 2mL collection tube and centrifuged for 15 seconds at >8000 x g. The flow-through was discarded and the process repeated with RW1 and RPE buffers. These steps culminated in a drying step, where the column was air-spun for 1 minute, followed by an RNase-free water eluting spin. The eluting spin was repeated with the flow-through of the final step to increase RNA yield.

RNA was quantified, and assessed for purity, using the NanoDrop® ND-1000 spectrophotometer (Life Technologies Ltd, Carlsbad, CA, USA). Based on these results, some samples with low yield or purity were excluded. This also served to standardise the RNA volumes for the reverse transcription stage.

2.5.3 Reverse transcription

Reverse transcription was performed using the Thermofisher Scientific high capacity RNA-to-cDNA kit following the manufacturer's instructions (Life Technologies Ltd, Carlsbad, CA, USA). In brief, 500ng of

RNA template was added to RNase-free 0.5mL microfuge tubes (Life Technologies Ltd, Carlsbad, CA, USA) and made up to a volume of 9μL and kept on ice. Following this, 10μL of RT Buffer Mix was added together with 1μL RT Enzyme Mix to each tube, making 20μL per reaction in total. Tubes were centrifuged for 30 seconds (250g) and then placed within a thermal cycler for 60 minutes at 37°C, 5 minutes at 95°C, then held at 4°C. cDNA was stored at -20°C for later use.

2.5.4 Quantitative polymerase chain reaction

Due to standardisation of the volume of RNA template, all samples contained the same amount of cDNA, but differing ratios of target genes depending on the experimental conditions. cDNA samples were diluted by 1/20 in RNase-free water prior to plating.

Using a MicroAmp Optical 384-well reaction plate (Life Technologies Ltd, Carlsbad, CA, USA) 4.5μL of diluted cDNA was placed into each well in duplicate. Following which, 5μL of SYBR Green PCR Master Mix (Primerdesign Ltd, Southampton, Hampshire, UK) was added to 0.5μL of primer (Primerdesign Ltd, Southampton, Hampshire, UK), before added to each corresponding well, making a final reaction volume of 10μL. Once complete, the PCR plate was firmly sealed with a plastic cover (Life Technologies Ltd, Carlsbad, CA, USA), centrifuged (2 minutes at 250g) and the reaction undertaken (ABI 7900HT, Applied Biosystems, Foster City, CA, USA). The PCR protocol consisted of 2 mins at 95°C, 40 cycles of 95°C for 15 seconds and 60°C for 1 min, followed finally by a melting curve of 95°C for 15 seconds, 60°C for 15 seconds and 95°C for 15 seconds. The expression of a housekeeping gene, Beta-actin (β-actin), was used to normalise for transcription and amplification variations among samples (160).

2.5.5 Real time quantitative polymerase chain reaction analysis

Results were analysed using the SDS v2.4 software (Applied Biosystems, Foster City, CA, USA). Prior to calculating the absolute quantification values, and C_T values, the baseline and threshold were set to account for any variations in the data. The dissociation curve of each primer was analysed prior to

exporting the data, examining for the presence of primer dimers. **Figure 2.4** highlights the formation of a primer dimer, indicating the potential for non-specific binding. Such data was excluded, and the experiments repeated with new primers. Following export of the C_T values from the SDS 2.4 software into excel, the comparative C_T method ($2^{-\Delta\Delta C_T}$) was used to calculate normalised relative fold change compared to controls (161).
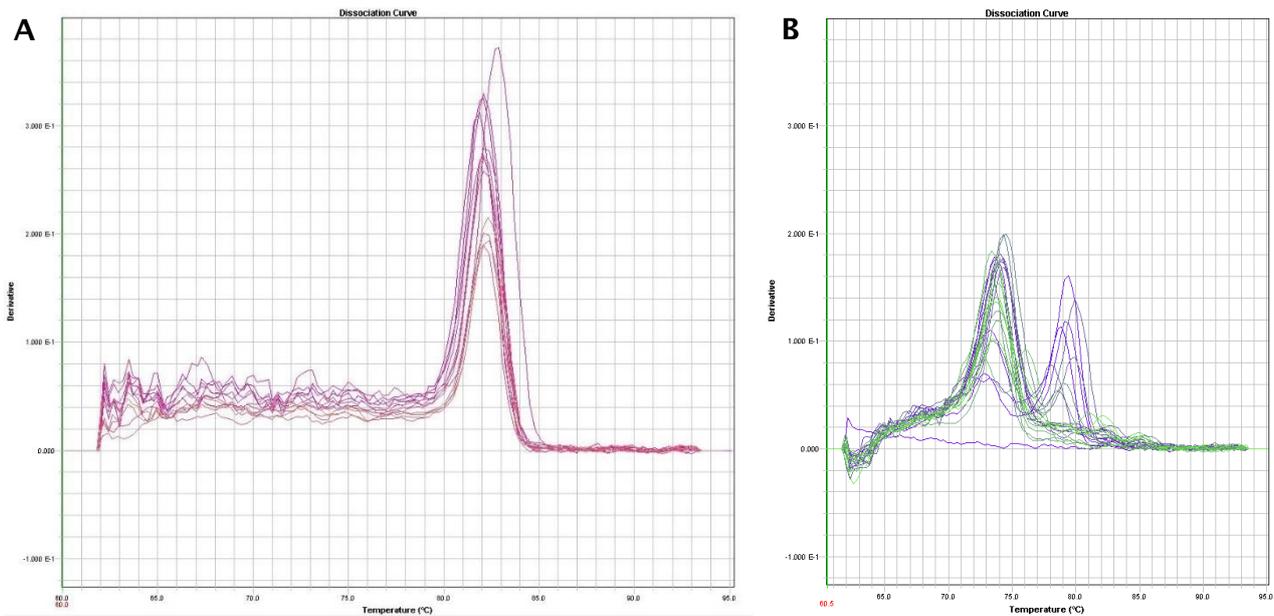
Figure 2.4 Primer dissociation curves. A) Normal melting curve, B) Abnormal melting curve indicating the formation of primer dimers.

2.5.6 Primer selection

Primer targets were selected in order to cover all of the transcriptional factors and effector cytokines of the main T-cell pathways. They were also selected to study the immune response to bacteria, together with remodelling and general inflammatory responses. Primer targets, and their main functions, are outlined in **Table 2.2**. Primer sequences are outlined in **Appendix III**.

Table 2.2 Primer targets and their main functions.

Gene name	Function
T-bet	Transcriptional factor for Th1 pathway
IFNγ	Th1 cytokine
GATA-3	Transcriptional factor for Th2 pathway
IL-5	Th2 cytokine
RORγT	Transcriptional factor for Th17 pathway
IL-17A	Th17 cytokine
FoxP3	Transcriptional factor for Treg pathway
MMP-9	Remodelling
MMP-28	Remodelling
IL-6	Immune response to infection
IL-1β	Anti-bacterial pro-inflammatory response
TGF-β1	General immune response/Remodelling
TNFα	General immune response
MMP-7	Remodelling/Anti-bacterial actions through the action of defensins
Defensin 5	Alpha-defensin/Antibacterial peptide
IL-8	Chemotaxis of immune cells in response to infection (Mainly Neutrophils)
TLR-2	Bacterial pattern recognition and activation of innate immunity
TLR-4	Bacterial pattern recognition and activation of innate immunity

2.6 Protein analysis techniques

Luminex was used to study protein content in culture supernatants, following nasal explant culture (outlined in 2.4.1). Analytes were selected in order to provide protein data for Th1 (IFNγ), Th2 (IL-5) and Th17 (IL-17A) differentiation. They were also selected to examine bacterial linked immune response (IL-1β, MMP-7 and IL-6), together with general inflammatory response (TNFα).

2.6.1 Luminex

Culture supernatant protein analytes (IFNγ, IL-1β, IL-5, IL-6, MMP-7, TNFα and IL-17A) were quantified using Luminex (R&D systems Inc, Minneapolis, MN, USA), as per the manufacturer's instructions. Within a 96 well microplate, 50µL of the microparticle cocktail was added to each well. Following this, 50µL of each standard and experimental sample (in duplicate) was added. The plate was securely covered with a foil plate sealer and incubated at room temperature for 2 hours on a horizontal orbital microplate shaker (800rpm). Wells were washed three times with wash buffer (100µL), whilst utilising a microplate magnetic device attached to the bottom. Following which, 50µL of diluted Biotin Antibody Cocktail was adding to each well, sealed, incubated and shaken (800rpm) for a further 1 hour. Each well was washed, as previously detailed, prior to the addition of 50µL diluted Steptavidin-PE. The plate was sealed, incubated and shaken (800rpm) for 30 minutes at room temperature. Wash steps were repeated, prior to plate reading using a Luminex analyser (Bio-Plex® 200 System, Bio-Rad Laboratories Ltd, Hercules, CA, USA). Total protein was measured for tissue samples, to standardise results, as detailed in section 2.6.2. **Table 2.3** outlines the cytokine concentration range within the standard curve, with **Figure 2.5** representing an example of a standard curve for IL-17A.

Timothy C Biggs

Table 2.3 Range of Luminex cytokine concentrations within the standard curve.

Cytokine	Lowest detectable concentration within the standard curve (pg/ml)	Highest detectable concentration within the standard curve (pg/ml)
IFNγ	4.66	11,322
IL-1β	1.54	3,922
IL-5	0.66	476
IL-6	1.64	1,176
MMP-7	44.11	33,136
TNFα	0.86	2,149
IL-17A	2.47	2,093
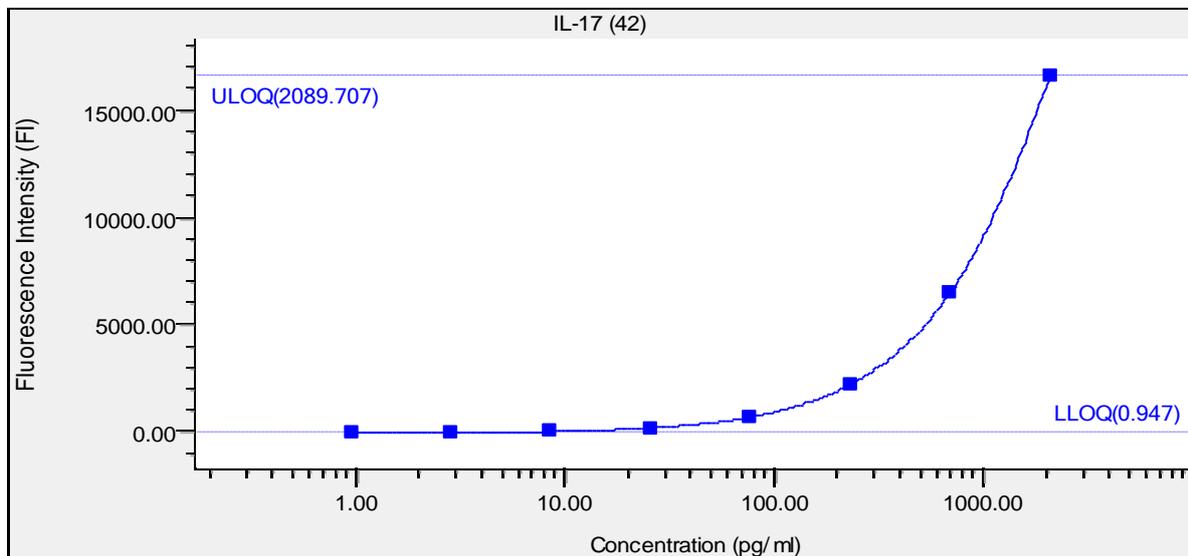
Figure 2.5 IL-17A Luminex standard curve.

2.6.2 Quantification of total protein

When examining stimulated tissue samples, Luminex results were standardised by measuring the total protein content, thereby representing pg/ml per µg of total protein for each analyte measured. This was due to the potential for slight variations in the size of samples when cutting prior to culture. When undertaking Luminex on epithelial and mast cell lines, cells were counted to ensure consistency and standardisation, thus total protein quantification was unnecessary.

Total protein was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd, Hercules, CA, USA) as per the manufacturer's instructions. A BSA standard curve was constructed by serial diluting the standard from a concentration of 1mg/ml to 0.05mg/ml. Experimental samples, BSA standards and controls were combined with dye reagent and added in triplicate into a 96 well plate (Greiner Bio-One International, Kremsmunster, Austria). Samples were incubated at room temperature for 15 minutes. The absorbance was measured at 595nm, and a standard curve constructed (**Figure 2.6**). Total protein levels were measured by extrapolating measured absorbance using the equation y=mx+c.
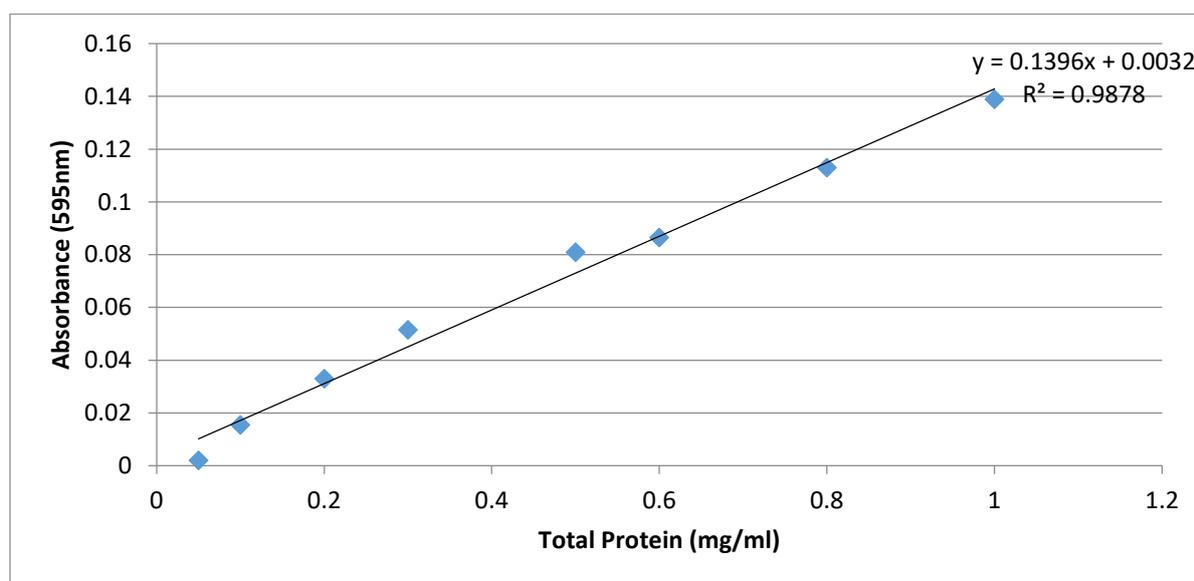
Figure 2.6 Total protein assay standard curve.

2.7 *S. aureus* bacterial culture experiments

S. aureus was isolated from CRS tissues by Mr Stephen Hayes (**detailed methods in appendix IV**). *S. aureus* stored in the mid-log growth phase as a glycerol stock, was streaked on to a Colombia Blood Agar (CBA) plate (Life Technologies Ltd, Carlsbad, CA, USA) prior to overnight culture (37°C, 5% CO_2). Following incubation, eight colony forming units were placed in 20mls of RPMI media and *S. aureus* grown to the mid-log phase. Serial optical density (OD) measurements were taken and plotted against CFU counts (**Figures 2.7**). These experiments were repeated separately four times, with linear correlation calculated (**Figure 2.8**).
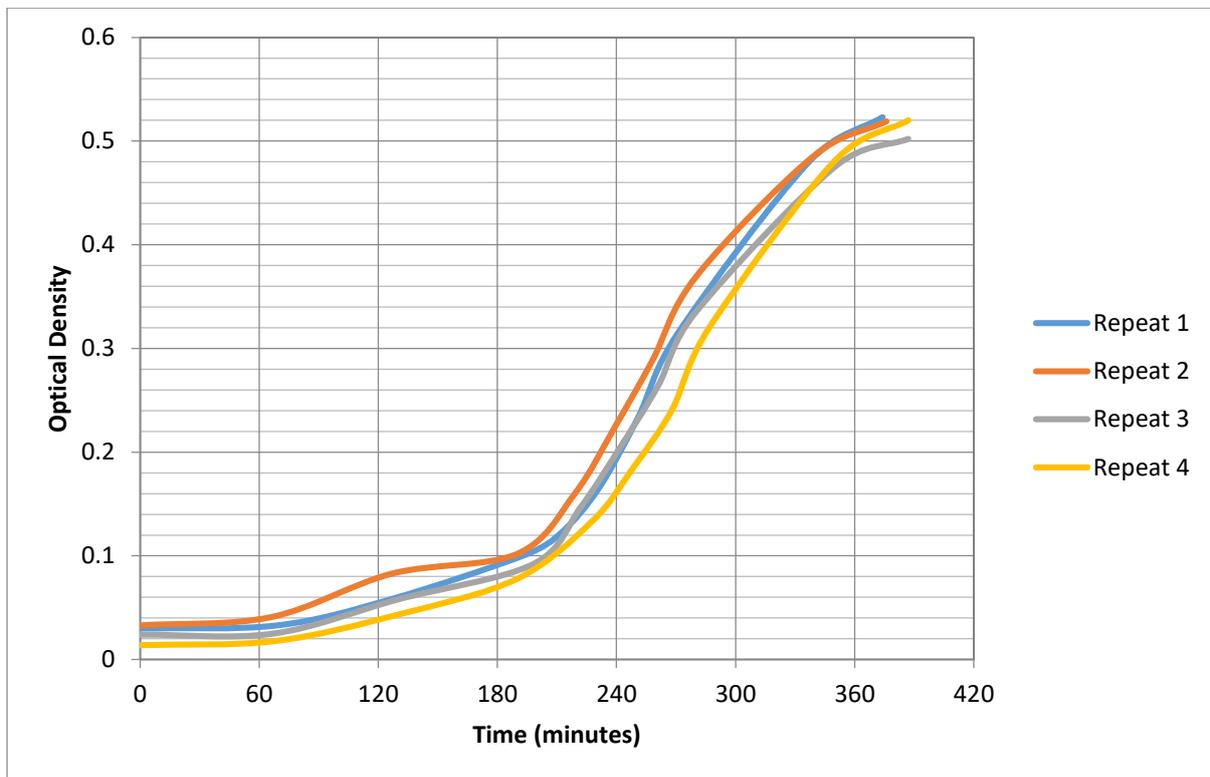
Figure 2.7 *S. aureus* optical density time course. *S. aureus* (P3 strain) was grown in RPMI media (37°C, 5% CO_2) and optical density measurements taken every 30 minutes over 6 hours.
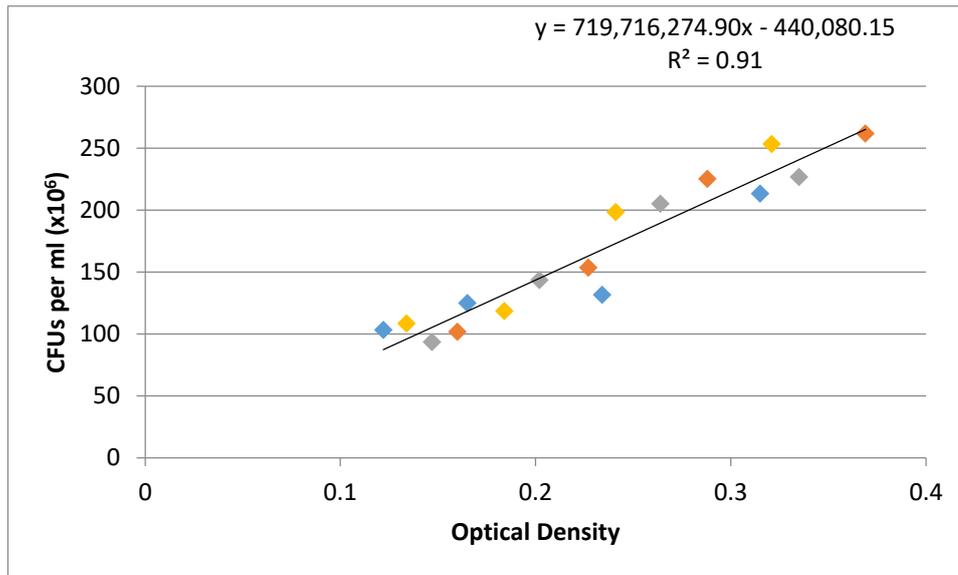
Figure 2.8 *S. aureus* optical density vs CFUs per ml (x10^6). *S. aureus* (P3 strain) was grown in RPMI media (37°C, 5% CO_2) to the mid-log phase, with optical density measurements and CFU enumeration undertaken every 30 minutes over 6 hours.

2.7.1 Dead *S. aureus*

Dead, but structurally intact, *S. aureus* was required to investigate whether the viability of *S. aureus* altered the immune response. The same strain of *S. aureus* (P3) was used and grown to the mid-log phase (37°C, 5% CO_2) in RPMI medium. The number of viable *S. aureus* were calculated, using the equation y = mx + c, using the linear correlation performed as in **Figure 2.8**. *S. aureus* was centrifuged (12,000g for 10 minutes) and immersed in 16% paraformaldehyde solution for 1 hour. The non-viability of the culture was determined after streaking on CBA plates, confirming no *S. aureus* growth following 48 hours incubation (37°C, 5% CO_2).

2.7.2 *S. aureus* co-infection model

S. aureus was cultured with a number of cell lines to further our understanding of the uptake of intracellular bacteria and the associated immune response. *S. aureus* was grown to the mid-log phase with the optical density measured, and the number of viable *S. aureus* calculated using the equation y = mx + c (**Figure 2.8**). *S. aureus* was washed twice and resuspended in HBSS. A known quantity of *S.*

aureus was then added to mast (HMC-1 and LAD2) and epithelial (RPMI 2650) cell lines, in a number of different multiplicity of infection ratios (MOI) and time-points. When examining infection at 24 hours, extracellular *S. aureus* was eradicated with 20µg/ml of Lysostaphin (incubation at 37°C for 30 minutes) at six hours, followed by continuation of the culture with fresh media over a further 18 hours. This was undertaken due to the virulence of *S. aureus* and the significant amount of cell death observed over a 24-hour continuous culture. In addition, this technique also enabled us to examine the re-seeding potential of *S. aureus* released from mast and epithelial cells following eradication of extracellular bacterial colonies.

2.7.3 *S. aureus* intracellular viability

As detailed above, *S. aureus* was grown to the mid-log phase and cultured with mast cell (HMC-1 and LAD2) and epithelial cell (RPMI 2650) lines. Prior to bacterial co-culture, cells were washed three times in media containing no antibiotics.

For measurement of CFU viability of extracellular and intracellular *S. aureus* growth, cell lines were co-cultured with *S. aureus* for up to 24 hours. Following co-culture, epithelial cells were treated with Trypsin (Sigma-Aldrich Ltd, Gillingham, UK) for 5 minutes, agitated and diluted with an equal volume of media prior to centrifugation. Mast cells were centrifuged directly from culture. Cells and *S. aureus* were centrifuged at 250g for 5 minutes, thus pelleting the cells but keeping the majority of *S. aureus* within the supernatant. Following centrifugation, 200µL of supernatant was removed and serially diluted, with CFU enumeration undertaken on CBA plates with subsequent overnight incubation (37°C, 5% CO_2). Pelleted cells were resuspended in media containing 20µg/ml of Lysostaphin, and incubated at 37°C for 30 minutes. Once extracellular *S. aureus* has been eradicated (separately tested by steaking on CBA plates with overnight incubation) the cells were washed three times and then vortexed for 10 minutes in 1% Triton X-100, releasing intracellular bacteria. Samples were then serially diluted with CFU enumeration undertaken on CBA plates with subsequent overnight incubation (37°C, 5% CO_2). This method is outlined in **Figure 2.9**.
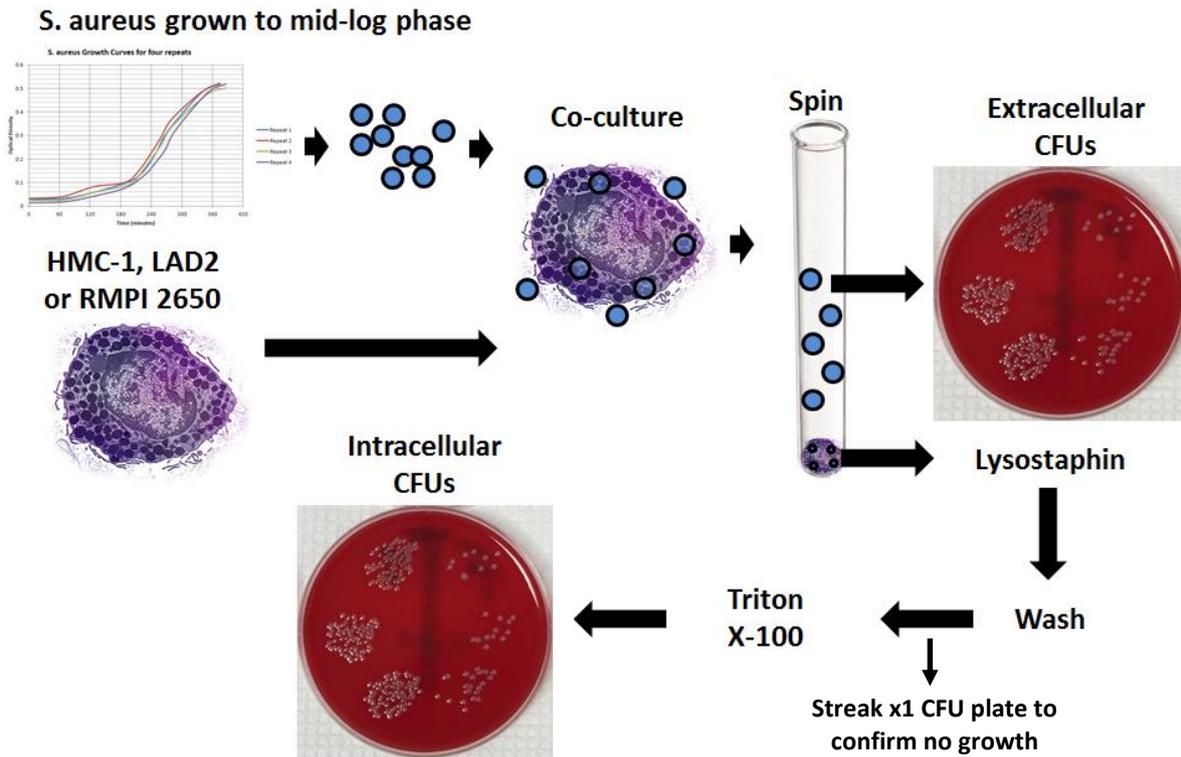
Figure 2.9 *S. aureus* co-culture method.

2.7.4 The immune response of *S. aureus*

As previously detailed, *S. aureus* was grown to the mid-log phase and co-cultured with mast and epithelial cell lines at an MOI of 1:1, using 5M cells and 5M *S. aureus* per well. Cells were grown in six different groups, control, live *S. aureus*, live *S. aureus* with 10μg/ml of SEB, dead *S. aureus*, dead *S. aureus* with 10μg/ml of SEB, and finally 10μg/ml of SEB alone. Co-culture was undertaken over 4 hours, prior to trypsinisation (for epithelial cultures), centrifugation (250g, 5 mins) and storage. RNAlater (Life Technologies Ltd, Carlsbad, CA, USA), stored at -20°C, was used to preserve RNA from cell samples. Supernatants were snap frozen and stored at -80°C. RT-qPCR and Luminex was carried out on cells and supernatant samples as previously outlined.

2.7.5 Study of *S. aureus* internalised strains

In order to study the possible effects of phenotypic changes occurring in *S. aureus* following its internalisation and subsequent release and re-infection of mast cells, stocks were created for a number of new *S. aureus* strains. The original stock strain (P3) of CRS specific *S. aureus* was co-cultured with HMC-1 and LAD2 cells separately (1 million cells to 1 million *S. aureus* over 4 hours). Cells and *S. aureus* were centrifuged at 250g for 5 minutes, and the supernatant discarded. Cells were washed and suspended in media containing 20μg/ml of Lysostaphin, and incubated at 37°C for 30 minutes. Cells were washed three times and then vortexed for 10 minutes in 1% Triton X-100, releasing intracellular *S. aureus* into the supernatant. Following this, 10μL of *S. aureus* supernatant was streaked on to a CBA plate and incubated overnight (37°C, 5% CO_2). This once internalised *S. aureus* (designated P3.1) was then grown to the mid-log phase and combined with 25% glycerol solution (Sigma-Aldrich Ltd, Gillingham, UK) and stored at -80°C. This process was then repeated with the P3.1 strain, followed by subsequent mast cell internalised *S. aureus* up to the 5th internalisation (P3.5). Separate strains for each cell line were created (HMC-1 and LAD2) to ensure consistency. Glycerol stocks of P3 to P3.5 were stored in -80°C. These stock strains were then used for later mast cell co-infection, for the study of intracellular viability and internalisation potential (CFUs) and immune response (RT-qPCR and Luminex), for which detailed methods are presented above.

2.7.6 Study of IgE sensitisation on *S. aureus* infected LAD2 cells

2.7.6.1 Joint collaboration with Rana Abadalkareem

Experiments in sections 6.6.5 were undertaken as a joint collaboration between myself and Rana Abadalkareem (PhD Student, University of Southampton) under the supervision of Dr Andrew Walls. I undertook the bacterial culture associated with these experiments, whilst Rana undertook the cell culture. We both co-infected the LAD2 cells, and collected the cells and supernatants. Rana independently carried out LDH assays, β-hexosaminidase experiments and protein kinase

phosphorylation experiments, whilst I independently undertook RT-qPCR and Luminex experiments. We analysed the results and constructed the presented figures. We have both used the same figures in each of our theses, but have critically apprised the results independently, forming our own thoughts and conclusions. This collaboration allowed the generation of additional results, maximising available time and resources, and involved an equal 50% contribution.

2.7.6.2 IgE co-infection model

For this experiment LAD2 cells were divided into four different experimental groups (three experimental repeats), each containing 5 million cells; IgE sensitised non-infected, non-IgE sensitised non-infected, non-IgE sensitised infected and IgE sensitised infected. Sensitised LAD2 cells were exposed to 1.5µg Human Myeloma IgE (Merck Millipore, Hertfordshire, UK) overnight, washed three times and resuspend in fresh media.

As previously detailed, *S. aureus* was grown to the mid-log phase and co-cultured with infected LAD2 cell groups at an MOI of 1:1, using 5M cells and 5M *S. aureus* per well. Following culture cells were centrifuged (250g for 5 minutes), and split into three groups; no stimulation, anti-IgE stimulation and calcium ionophore stimulation. Goat anti-human IgE (Merck Millipore, Hertfordshire, UK) at 3µg/ml was added to cells over 2 hours, with calcium ionophore groups exposed to 0.03µM of Calcium Ionophore (Sigma-Aldrich Ltd, Gillingham, UK) over 2 hours. All groups were then centrifuged and stored in RNAlater (Life Technologies Ltd, Carlsbad, CA, USA) at -20°C, with RT-qPCR carried out at a later date. Supernatants were snap frozen and stored at -80°C. RT-qPCR and Luminex was carried out on cells and supernatant samples as previously outlined.

2.8 Confocal laser scanning microscopy

2.8.1 Co-culture experiments

An epithelial cell line (RPMI 2650) was grown as a monolayer in an eight well glass chamber slide (Thistle Scientific, Glasgow, UK). Cells were seeded at 200,000 cells per chamber and left for 48 hours to form a confluent monolayer. Following culture with antibiotic supplemented media (Penicillin and Streptomycin), cells were washed three times with antibiotic free media, to remove non-adherent cells and provide the correct environment for bacterial co-culture. Cells in a representative single test well were trypsinised, counted and the corresponding number of *S. aureus* added to remaining wells, at an MOI of 1:1. Cells were cultured for up to 6 hours. The extracellular and intracellular bacterial groups were co-cultured and fixed. The intracellular CLSM images were obtained by substituting the media for media containing 20µg/ml Lysostaphin (Sigma-Aldrich Ltd, Gillingham, UK) to eradicate all extracellular *S. aureus*, leaving only the intracellular *S. aureus* visible. Following fixation cells were permeabilised (0.5% Triton X-100), blocked (3% BSA) and then stained.

Epithelial and mast cell lines were both stained with the same protocol. A 1/200 dilution of a mouse monoclonal anti-staphylococcus primary antibody (AB37644, Abcam, Cambridge, UK) in PBS with 3% bovine serum albumin (BSA) and 0.5% Triton X-100 was incubated overnight at 4°C. Cells were washed three times, then incubated at room temperature for 2 hours with a 1/500 dilution of a polyclonal donkey anti-mouse Alexa Fluor® 568 (AB175472, Abcam, Cambridge, UK) in PBS with 3% BSA and 0.5% Triton X-100. Cells were then washed three times with PBS and incubated with 0.5µg/ml of 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) with 3% BSA and 0.5% Triton X-100. Cells were then finally washed and stored in PBS for imaging. Cells were imaged with a Leica TCS SP5/8 inverted confocal microscope (Leica Microsystems, Milton Keynes, UK) using a 63x glycerol immersion lens. Sequential scanning was used to eliminate interference. Images were collected and analysed using Leica LAS-AF software.

For the study of *S. aureus* infected mast cells with the addition of a cell tracker, to visualise the cell cytosol, cells were washed and treated with Lysostaphin as detailed above. Following further washing steps, cells were incubated in a 25µM solution of CellTracker™ Deep Red fluorescent dye (Life Technologies Ltd, Carlsbad, CA, USA) for 30 minutes. Cells were then stained for the visualisation of intracellular *S. aureus* and cell nuclei as outlined above, prior to imaging using the confocal microscope.

2.9   Statistical analysis

Statistical analysis was performed using Graph Pad Prism 7.0 software (Graph Pad Software Inc., San Diego, CA, USA). Significance was accepted at $p<0.05$, as per normal scientific practice. Data were assessed for normality using a histogram plot and normality testing was also performed to select the use of a parametric or non-parametric statistical test. The D'Agostino Pearson test for normality was used to test normality for five or more samples/subjects, and the Shapiro-Wilk test for less than five samples/subjects. Parametric tests were selected when the data was assessed to be of a normal distribution, with non-parametric tests used for skewed data. In some chapters, where multiple cytokines were used, normality testing was undertaken and the best test selected based on a majority assessment across all cytokines studied. Statistical tests used are detailed in the individual figure legends.

In general, paired tests were used to study differences between the same tissue sample or cell line, across differing treatment conditions. Cell line cultures consist of millions of clone cells, and for the purpose of these experiments can be classified as paired. For parametric data, t-tests were used. For parametric paired data, paired t-tests were used. For non-parametric data, Mann-Whitney U tests were used. For non-parametric paired data, Wilcoxon tests were used. Two-way ANOVA was used in the study of IgE sensitisation and *S. aureus* co-infection experiments, Chapter 6.6.5, to account for multiple groups.

Timothy C Biggs

3 Immunological profiling of key inflammatory drivers associated with chronic rhinosinusitis

3.1 Introduction

Chronic rhinosinusitis (CRS) can be broadly classified into two main categories based on the presence (CRSwNP) or absence (CRSsNP) of nasal polyps. CRSwNP is characterised by increased IL-5, eotaxin, tissue oedema, low levels of TGF-β1 and a Th2 skewed inflammatory prolife, with effects from *S. aureus* and its enterotoxins (6). In contrast, CRSsNP has IFN-γ and TGF-β1 as major players, involving a much more fibrosis driven Th1 biased inflammatory process (6).

Whilst the cytokine profiles of these broad disease subsets have been well characterised over the last decade, relatively little is known about what drives nasal polyp formation specifically. What is also unclear is how nasal polyps recur following their surgical removal, and whether this is related to the immune response present in the retained non-polypoidal mucosa. Limited studies reveal that these non-polypoidal tissues, although seeming macroscopically normal, do have elevated numbers of inflammatory cells (118, 123, 134, 162). However, there have been no studies to date which investigate the inflammatory gene expression of these sites. Further research investigating the potential of the non-polypoidal sinonasal mucosa to drive nasal polyp formation is of high clinical importance, as this would further emphasise the importance of aggressive post-operative treatments to prevent nasal polyp recurrence.

S. aureus has been identified in CRS patients, and found intracellularly within nasal polyp epithelial and mast cells (17, 23, 25, 75, 163). It is currently unclear how *S. aureus* evades immune detection, and whether the cytokine profile of these patients contribute towards its presence and continued survival. This chapter therefore aims to provide further insight into the factors related to nasal polyp growth and recurrence, and whether there is an environment in CRSwNP patients that facilitates the potential survival of *S. aureus* within sinonasal tissues.

3.2 Hypothesis

The non-polypoidal mucosa of CRSwNP patients contributes towards the formation and recurrence of nasal polyps, by acting as a cytokine reservoir with a persistent pro-inflammatory drive. Furthermore, *S. aureus* is likely to contribute to nasal polyp formation, facilitated through a disordered bacterial clearance mechanism present in CRSwNP tissues.

3.3 Aims

To examine the cytokine profiles of CRSwNP, CRSsNP and control patients, and to compare the non-polypoidal sinonasal mucosa of CRSwNP patients with adjacent nasal polyps.

3.4 Objectives

1. To study the differing immune profiles of CRSwNP, CRSsNP and control patients, through the analysis of cytokine gene expression ratios.

2. To compare the differences in the immune profiles of nasal polyps to the adjacent non-polypoidal sinonasal mucosa, within the same patients.

3. To identify evidence of a disordered bacterial clearance mechanism in CRSwNP patients, that may facilitate the survival of bacteria and the development of a chronic inflammatory state.

3.5   Methods

For detailed methods please refer to chapter 2, pages 41-65. To answer the proposed questions of this chapter, cytokines and transcriptional factors associated with the 4 major T-cell pathways were studied; Th1 (T-bet and IFNγ), Th2 (GATA-3 and IL-5), Th17 (RORγT and IL-17A), Treg (FoxP3). In addition, bacterial related toll like receptors (TLR-2 and TLR-4), bacterial associated cytokines and peptides (IL-8, IL-6, IL-1β and defensin 5), general pro-inflammatory cytokines (TNFα and TGF-β1) and remodelling factors (MMP7, -9 and -28) were studied, looking for evidence of a disordered immune response against bacteria and the development of a chronic inflammatory state.

3.5.1   Subjects and sampling

In total 30 patients were included in this part of the study. These were 20 consecutive patients with CRSwNP and CRSsNP undergoing functional endoscopic sinus surgery, who met the inclusion and exclusion criteria. In addition, 10 control patients, undergoing trans-sphenoidal pituitary surgery were also enrolled. Non-polypoidal sinonasal mucosa and nasal polyp samples were obtained from CRSwNP patients, with sinonasal mucosa obtained from CRSsNP and controls. Sinonasal mucosa was obtained from the uncinate process in CRSwNP and CRSsNP patients, and from the inferior turbinate of controls. Nasal polyp tissue was harvested from the middle meatus of CRSwNP patients. **Figure 3.1** highlights the location of paired nasal biopsies taken from CRSwNP patients.
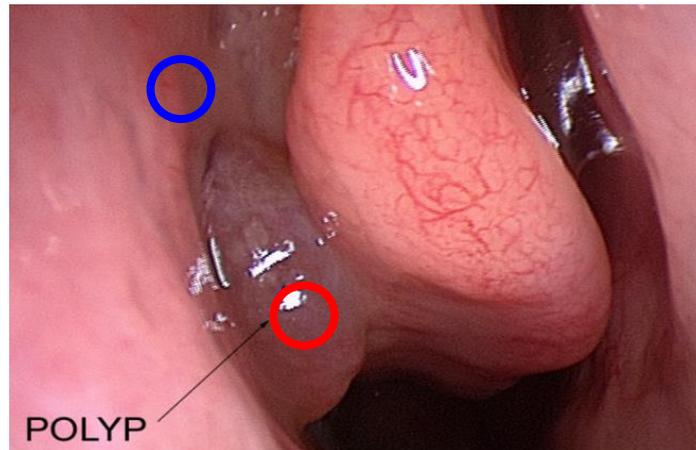
Figure 3.1 Location of nasal biopsies in CRSwNP. Endoscopic image of the right sinonasal cavity from a patient with CRSwNP. Nasal polyp biopsy is taken from the middle meatus (red circle), with non-polypoidal mucosa taken from the uncinate process (blue circle). Image adapted from, Roth et al (28).

3.6 Results

3.6.1 Patient Demographics

Demographic data for study participants is shown in **Table 3.1**.

Table 3.1 Study patient demographics.

Characteristics	CRSwNP	CRSsNP	Control
Subject numbers	12	8	10
Mean age (range)	43 (31-67)	41 (38-50)	56 (46-75)
Sex	6 Male/6 Female	3 Male/5 Female	5 Male/5 Female
Current smokers	4	2	0
Asthma	7	2	0
Positive SPT	4	2	0
Previous Surgery	3	1	0
SNOT-22 score (range)	62 (20-101)	46 (15-67)	22 (5-61)
Mean LMS (range)	14.5 (3-24)	7.8 (2-13)	0

3.6.2   The immune response of sinonasal mucosa

3.6.2.1   The cytokine and chemokine response of sinonasal tissues

Nasal polyps and the non-polypoidal sinonasal mucosa of CRSwNP patients displayed very similar cytokine/chemokine gene expression ratios (**Figure 3.2**). Indeed, there was no statistical difference in gene expression measured between them.

The gene expression of IFNγ was significantly (p≤0.01) upregulated within nasal polyps compared to control samples (**Figure 3.2**). IL-5 gene expression was significantly (p≤0.01) upregulated within paired CRSwNP samples (nasal polyps and non-polypoidal sinonasal mucosa) compared to CRSsNP. Furthermore, IL-5 gene expression was significantly (p≤0.05) upregulated in nasal polyps compared to control mucosa. IL-17A was significantly upregulated in CRSsNP samples, in comparison to nasal polyps (p≤0.05) and controls (p≤0.001).

There was no statistically significant difference in TGF-β1 or TNFα gene expression across any of the disease subtypes or tissue sites (nasal polyp vs non-polypoidal sinonasal mucosa) (**Figure 3.2**). IL-1β gene expression was significantly downregulated in paired CRSwNP samples compared to both CRSsNP (p≤0.01) and controls (p≤0.001). IL-6 gene expression was significantly (p≤0.05) downregulated in paired CRSwNP, and CRSsNP, samples compared to controls. Gene expression of the chemokine IL-8 (CXCL8) was significantly (p≤0.05) upregulated in paired CRSwNP samples compared to CRSsNP, and in nasal polyps compared to controls.
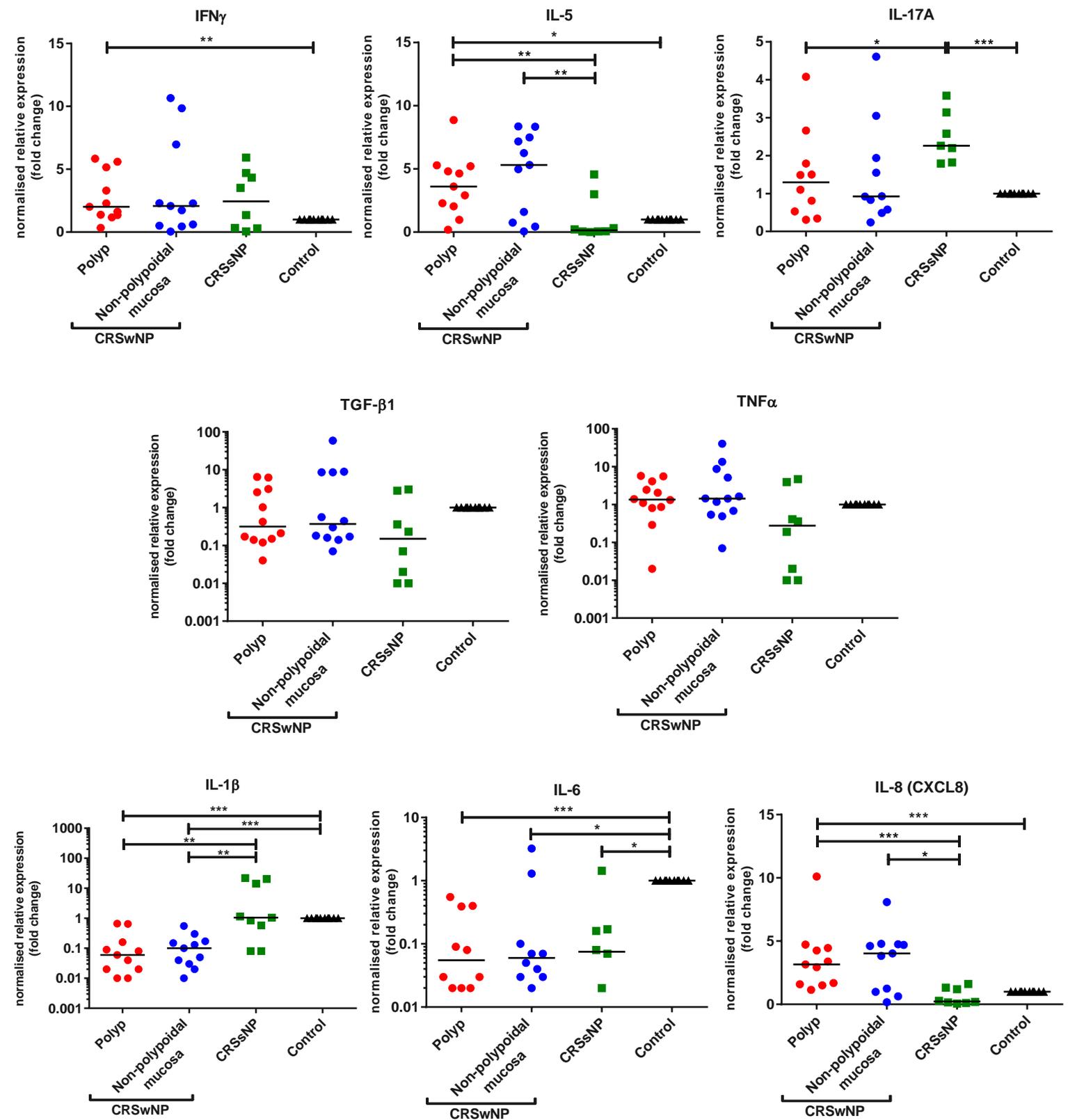
Figure 3.2 IFNγ, IL-5, IL-17A, TGF-β1, TNFα, IL-1β, IL-6 and IL-8 normalised gene expression of sinonasal tissue samples. RT-qPCR undertaken using nasal polyp (n=12), non-polypoidal sinonasal mucosa (n=12), CRSsNP sinonasal mucosa (n=8) and control (n=10) samples. Individual sample points and median lines. Wilcoxon tests performed for paired samples (polyp and non-polypoidal sinonasal mucosa) and Mann-Whitney U-tests for all other analyses, $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

3.6.2.2 T cell transcriptional factor expression in sinonasal tissues

T-bet (**Figure 3.3**) gene expression was significantly (p≤0.01) downregulated in paired CRSwNP samples (nasal polyp and non-polypoidal sinonasal mucosa) compared to both CRSsNP and controls. The gene expression of T-bet was significantly (p≤0.05) downregulated in nasal polyps when compared to the adjacent non-polypoidal sinonasal mucosa. CRSsNP T-bet gene expression was found to be significantly (p≤0.05) upregulated in comparison to paired CRSwNP samples and controls.

GATA-3 gene expression was significantly (p≤0.05) downregulated in paired CRSwNP samples compared to CRSsNP, and in nasal polyp samples compared to controls (**Figure 3.3**). There were no statistically significant differences in RORγT gene expression across different disease subtypes or tissue sites. FoxP3 gene expression was significantly (p≤0.01) downregulated in the non-polypoidal mucosa of CRSwNP patients in comparison to controls.
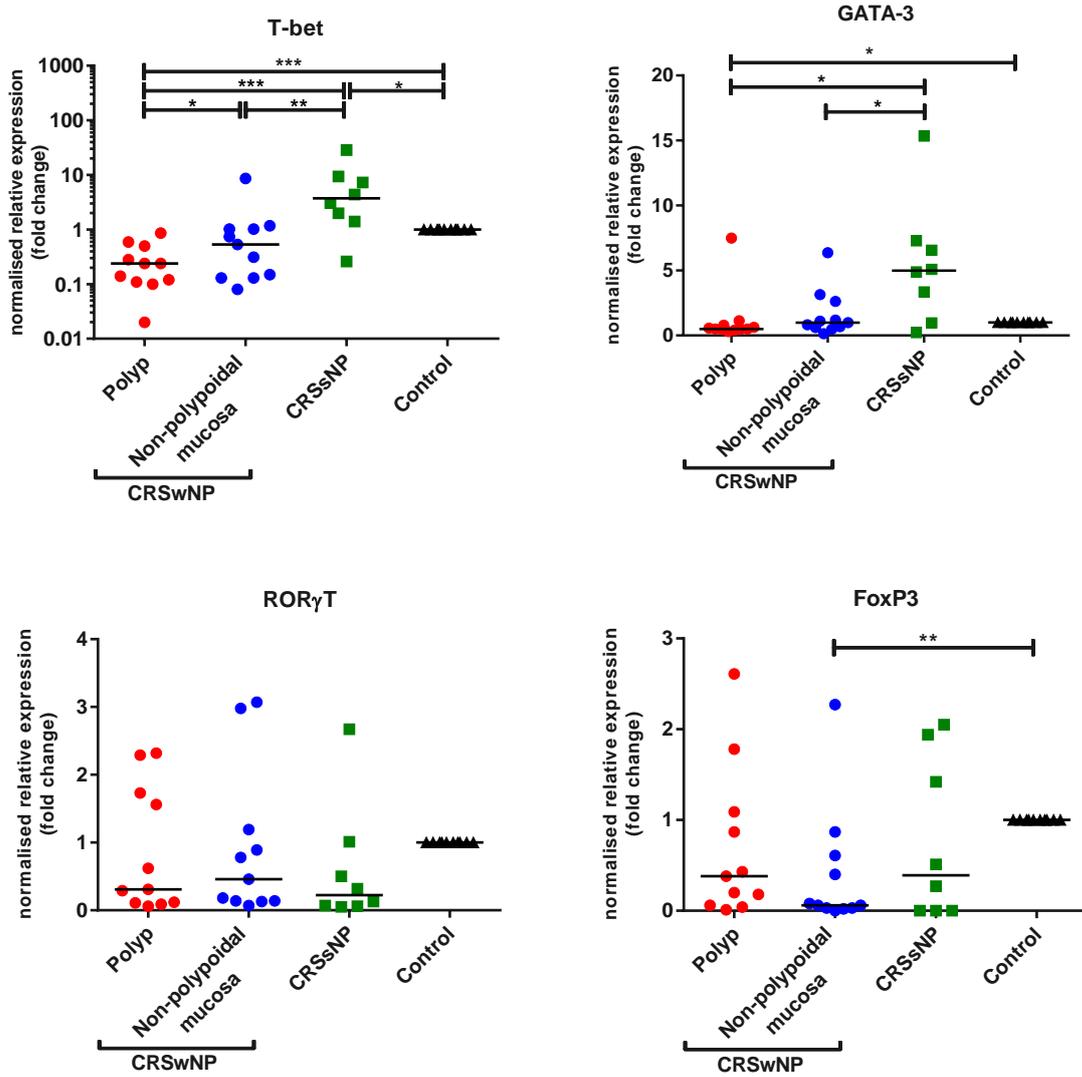
Figure 3.3 T-bet, GATA-3, RORγT and FoxP3 normalised gene expression of sinonasal tissue samples. RT-qPCR undertaken using nasal polyp (n=12), non-polypoidal sinonasal mucosa (n=12), CRSsNP sinonasal mucosa (n=8) and control (n=10) samples. Individual sample points and median lines. Wilcoxon tests performed for paired samples (polyp and non-polypoidal sinonasal mucosa) and Mann-Whitney U-tests for all other analyses, $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

3.6.2.3 The gene expression of toll like receptors in sinonasal tissues

TLR-4 gene expression was significantly (p≤0.05) upregulated in paired CRSwNP samples (nasal polyp and non-polypoidal sinonasal mucosa) compared to controls (**Figure 3.4**). There was no significant change in TLR-2 gene expression across disease subtypes or tissue sites.
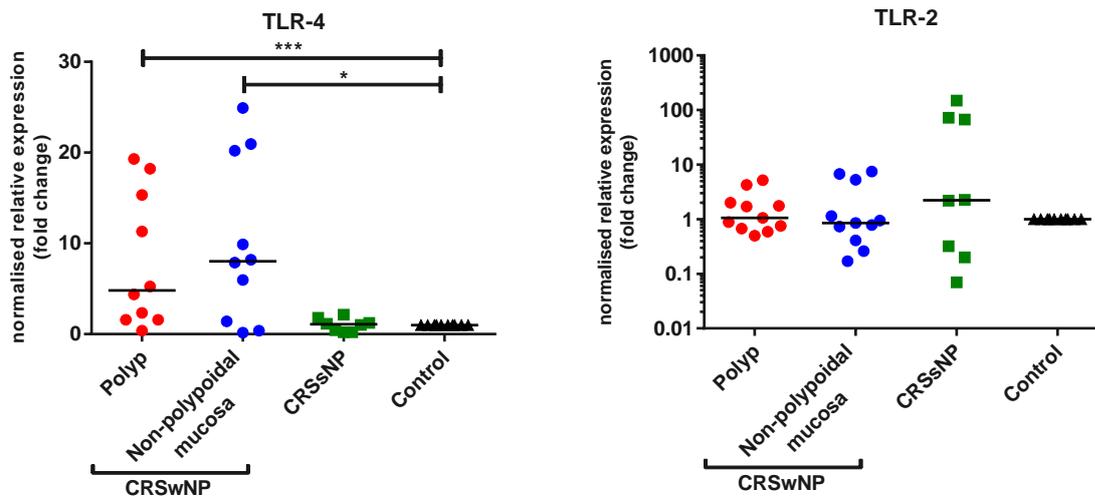
Figure 3.4 TLR-4 and -2 normalised gene expression of sinonasal tissue samples. RT-qPCR undertaken using nasal polyp (n=12), non-polypoidal sinonasal mucosa (n=12), CRSsNP sinonasal mucosa (n=8) and control (n=10) samples. Individual sample points and median lines. Wilcoxon tests performed for paired samples (polyp and non-polypoidal sinonasal mucosa) and Mann-Whitney U-tests for all other analyses, p≤0.05 (*) and p≤0.001 (***).

3.6.2.4 Matrix metalloproteinase and defensin expression in sinonasal tissues

Results (**Figure 3.5**) show statistically significant (p≤0.01) upregulation of MMP-28 in paired CRSwNP samples (nasal polyp and non-polypoidal sinonasal mucosa) compared to CRSsNP, and in nasal polyps in comparison to controls. There was no significant differences in MMP-9, MMP-7 or defensin-5 gene expression ratios across disease subtypes or tissue sites.
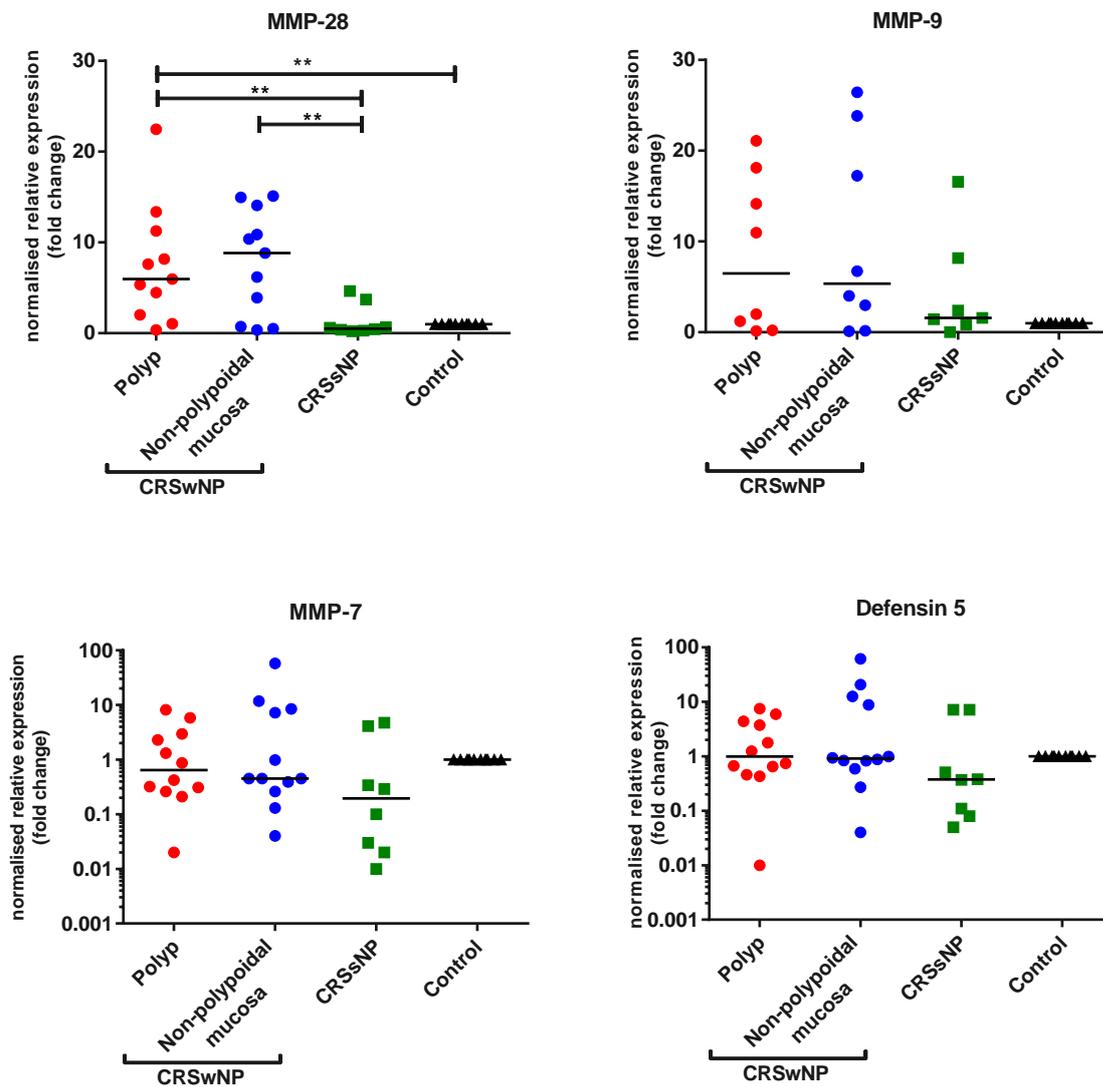
Figure 3.5 MMP-28, -9, -7 and defensin 5 normalised gene expression of sinonasal tissue samples. RT-qPCR undertaken using nasal polyp (n=12), non-polypoidal sinonasal mucosa (n=12), CRSsNP sinonasal mucosa (n=8) and control (n=10) samples. Individual sample points and median lines. Wilcoxon tests performed for paired samples (polyp and non-polypoidal sinonasal mucosa) and Mann-Whitney U-tests for all other analyses, p≤0.01 (**).

3.7 Discussion

Patient demographics

Mean ages of the study populations were comparable, and sex ratios evenly distributed. There was a higher ratio of males in CRSwNP and females in CRSsNP, which is in keeping with the literature (6). Smoking rates for CRS patients were higher than those quoted (25% vs 10-15%), which may represent the prevalence of smoking in the local area of recruitment (164). Asthma and allergy rates were similar to those found within the literature, with a rate of 58% for asthma compared with 26% (ranging from 10% to 64%), and 33% for positive allergy skin prick testing compared with approximately 54% (ranging from 50 to 84%) (6). Previous surgery in the CRSwNP group (25%) was higher than that in the CRSsNP group (12.5%) and matches that seen within the literature, suggesting a revision surgery rate of between 19% to 57% in CRSwNP, and 13% for CRSsNP (35). Aspirin sensitivity was not reported in any patients included in this study, as per the inclusion/exclusion criteria. Sino-nasal outcome test (SNOT-22) scores, a disease specific quality of life assessment (165), were higher in CRSwNP compared to those with CRSsNP and controls. The control group had elevated SNOT-22 scores due to symptoms secondary to the pituitary pathology, including tiredness, lack of sleep etc., impacting upon quality of life. The mean SNOT-22 of non-CRS patients within the literature is 16.4 (166). A radiological grading system assessing the severity of disease on CT scanning was used, the Lund-Mackay score (LMS) (149). As expected, CRSwNP patients had a higher score than those with CRSsNP, due to the presence of nasal polyposis.

T-cell profiling of sinonasal tissues

Historically, CRSwNP has been regarded primarily as a T-helper (Th) 2 mediated disease, characterised by IL-5 driven eosinophilic inflammation, with contributory effects from *S. aureus* and its enterotoxins (10, 29, 167). Results from this study further confirm a Th2 profile in CRSwNP samples, with upregulation of the Th2 effector cytokine IL-5. However, paradoxically results reveal downregulated GATA-3, the transcription factor for the Th2 linage. This would ordinarily go against a Th2 response.

However, there is evidence of downregulated GATA-3 gene expression in CRSwNP tissues in similar studies (168, 169). GATA-3 may be low due to an already strongly polarised Th2 environment, thus limiting the further differentiation of these cells on the genome level. This study has also shown upregulated IFN-γ in CRSwNP tissues, typically regarded as a Th1 cytokine. Elevated IFNγ has been found to enhance the anti-bacterial and pro-inflammatory response of human mast cells to *S. aureus* infection (170). Thus, elevated IFN-γ may indicate a response to bacterial biofilms within CRSwNP tissues.

In contrast to the Th2 mediated pro-inflammatory response of CRSwNP, CRSsNP results revealed upregulation of the Th1 cytokine (IFNγ) and transcription factor (T-bet). This supports previous studies suggesting a Th1 response in CRSsNP patients (6, 29, 124, 140). However, in addition, presented results revealed evidence of a Th17 response in CRSsNP samples. Th17 responses are present in normal mucosal homeostasis, and as a defensive mechanism for pathogenic invasion. It is well established that CRSwNP samples from Asian patients as well as cystic fibrosis patients are skewed towards the Th17 pathway (6, 124-126, 128, 129, 131, 171). The identification of a Th17 response in this study is interesting, as limited evidence of this exists within Caucasian patients. A recent multicentre study by Wang et al (2016), which included 573 patients from Caucasian and Asian populations, found limited evidence of a Th17 response in Caucasian nasal polyps, but did find elevated levels in one centre for both CRSwNP and CRSsNP (132). Despite smaller numbers, two recently published studies examining T-cell responses in Caucasian nasal polyps identified elevated IL-17A, consistent with Th17 cell activation (133, 134). Results presented, support the association of a Th17 response in Caucasian CRSsNP mucosa. CRSsNP mucosa contains surface biofilms, with nasal polyps containing intracellular colonies of bacteria in addition to surface biofilm (22, 23, 25, 75). The Th17 response of CRSsNP could constitute an early response against these surface biofilms, or as a result of T-cell dysregulation causing an ongoing fibrosis driven inflammatory state.

The immune response of nasal polyps vs the non-polypoidal sinonasal mucosa

There is a paucity of data comparing the immune profiles of nasal polyps with adjacent non-polypoidal sinonasal mucosa in CRSwNP patients (118, 123, 134, 162). Studies that have been undertaken have examined the cellular composition of immune cells between these sites using flow-cytometry, identifying a diffuse mucosal involvement (118, 123, 134, 162). This study complements these findings using an alternative technique (RT-qPCR), but provides additional insight into the immune response and how this could be contributing to nasal polyp development.

Despite their very different macroscopic appearances, the non-polypoidal sinonasal mucosa of CRSwNP and nasal polyps display strikingly similar immune profiles. Indeed, in many cases the non-polypoidal mucosa displayed higher pro-inflammatory gene expression ratios than that of the nasal polyps. This is significant in the propensity of these areas to develop into de-novo nasal polyps, and clinically relevant when considering the management of these patients following endoscopic sinus surgery, and their requirement for ongoing medical treatment to prevent recurrence. When examining the gene expression of FoxP3, a Treg transcriptional factor, there was significant downregulation within the non-polypoidal sinonasal mucosa in comparison to controls. This may represent evidence of a disordered T cell response of this tissue which could further exacerbate the development of a chronic inflammatory state, and the propensity to nasal polyp development at this site. These findings raise the possibility of a spectrum of disease, with progression from non-polypoidal mucosa to nasal polyps, rather than two distinct disease processes (CRSwNP and CRSsNP). Recent studies are starting to move away from the traditional rigid clinical and immunological classification of CRS as two distinct disease categories. Rather, it is suggested that patients can be phenotyped based on their pro-inflammatory cytokine profiles (13, 132, 172, 173). This is in keeping with our results which highlight significant heterogeneity in cytokine levels.

Disordered bacterial immune responses, related to the downregulation of IL-1β

The downregulation of IL-1β and IL-6 gene expression in CRSwNP samples compared to controls is significant, and has not been previously reported. Although, decreased IL-1β has been observed in CRSwNP subcategorised into those with high IL-5 expression, which could be significant as these patients tend to have severe nasal polyp disease with worse post-operative treatment outcomes (172, 174). IL-1β, a cytokine implicated in a number of inflammatory conditions and produced by monocytes and macrophages, is important in the defence against bacterial infections at mucosal surfaces (175). Moreover, when its activity is inhibited in animal models there is increased susceptibility to bacterial infections, including *S. aureus* (176, 177). This study has revealed IL-1β to be significantly downregulated in CRSwNP samples (nasal polyp and non-polypoidal sinonasal mucosa), which are known to harbour submucosal and intracellular bacteria (25). These results may indicate evidence of disordered bacterial immune response which could contribute to subepithelial and intracellular bacterial survival. This is further supported by the findings of IL-6 gene expression, involved in phagocytic cell activation and acute phase response to infection, which was also found to be downregulated in CRSwNP samples. IL-6 deficiency can lead to impaired immunity to viral, parasitic and bacterial infections (178). In combination with other presented results, highlighting upregulation of IL-8 and TLR-4, these results indicate upregulation of cytokines involved in the detection of bacteria (TLR-4) and recruitment of defensive cell types (IL-8), but downregulation of cytokines involved in the immune mediated clearance of bacteria (IL-6 and IL-1β). This may represent a mechanism by which bacteria use to subvert the local immune response ensuring their ongoing survival. Although these theories are speculative, they do raise significant questions regarding the immune response against bacteria in CRSwNP patients. Is the localised immune response of CRSwNP patients facilitating the survival of bacteria, or are bacteria modulating the local immune response to aid their own survival?

3.8 Conclusions

Although appearing macroscopically normal, the non-polypoidal mucosa represents a reservoir of pro-inflammatory cytokines that may contribute to de-novo nasal polyp growth, but also the recurrence of nasal polyps following surgical clearance. These findings highlight the relevance and importance of continued medical therapy following nasal polyp clearance, in order to prevent the growth and recurrence of nasal polyps. They also suggest the possibility of a disordered immune response towards bacteria, which may facilitate the survival of bacteria and development of chronic inflammation. Further work examining the role of bacterial toxins in chapter 4 would be of benefit, in elucidating their possible contributory role in the development of nasal polyps.

85

4 Investigating the role of *Staphylococcus aureus* enterotoxin B in the development of nasal polyps

4.1 Introduction

S. aureus has been shown to be present on the sinonasal mucosa of CRSwNP and CRSsNP patients, and found intracellularly within nasal polyps (17, 23, 25, 75, 163). The presence of intracellular bacteria is clinically relevant, as it is associated with significantly worse post-operative outcomes following endoscopic sinus surgery (23). *S. aureus* secretes a number of toxins, which module immune responses and could contribute toward the development of nasal polyps. However, there remain large gaps in our knowledge regarding the exact mechanisms involved.

A family of 23 pyrogenic toxin superantigens (SAgs) are produced by *S. aureus*. Amongst these, *Staphylococcal aureus* enterotoxin B (SEB) remains the most potent, and has been associated with CRSwNP due to its superantigenic properties (10, 179). *Staphylococcal aureus* enterotoxins (SAEs) are able to activate T cells, via the T cell receptor (TCR) MHC class II complex, independent from the antigen specific grove, by the variable β-chain. In this way SAEs, of which SEB is one, can activate as much as 20% to 25% of the T cell population, rather than 0.1% through conventional binding (180). Specific IgEs to SAEs have been detected in nasal polyps, with the correlation of increased levels of IL-5, eotaxin and eosinophilic cationic protein (144). Increased numbers of T cells expressing the TCR β-chain variable region have also been found in nasal polyps, and associated with specific IgE to SAEs (81). Furthermore, a study examining nasal polyp and control tissues found increased Th1/Th2-derived pro-inflammatory cytokines in response to SEB stimulation (11). Levels were highest within nasal polyps, favouring a Th2 response, with increases in IL-4, IL-5 and IL-13. SAEs are thought to be associated with the possible development of CRSwNP, which partly explains the rationale for the use of the anti-staphylococcal antibiotic Doxycycline in these patients (6).

As highlighted in chapter 3, nasal polyps are characterised by a Th2 dominated response, involving upregulated Th2 cytokines (IL-5) and downregulated Th1 responses. A Th17 response was not found within nasal polyp samples, but has been shown in Asian nasal polyps, which display a largely neutrophilic infiltrate (124-129). The absence of a Th17 response in Caucasian populations is surprising, given that Th17 cells represent a distinct T-cell linage involved in the host defence against

86

extracellular pathogens, and CRS patients have surface and intracellular biofilms (17, 23, 25, 75, 163). Recent studies have started to show upregulated Th17 cytokines in Caucasian CRSwNP and CRSsNP tissues (98, 132, 134). Further work examining T-cell responses are required to clarify the host immune responses, mediated by bacteria and their toxins, and more importantly their possible role in the formation of nasal polyps.

4.2 Hypothesis

SEB induces a potent pro-inflammatory response within nasal polyp tissues, likely contributing towards their growth.

4.3 Aims

To examine the cellular response of nasal polyp and control mucosa to SEB stimulation using an *ex vivo* nasal explant model.

4.4 Objectives

1. To study the immune response of SEB, in order to clarify its role in the development of nasal polyps.

2. To investigate the ability of SEB to amplify pre-existing immune responses, further skewing the T-cell repertoire.

3. To further investigate the role of SEB in promoting a disordered anti-bacterial response, through the downregulation of IL-1β and IL-6.

4.5 Methods

For detailed methods please refer to chapter 2, pages 41-65.

4.5.1 Subjects and sampling

In total, 20 consecutive patients were enrolled into this part of the study. 15 CRSwNP patients undergoing functional endoscopic sinus surgery and 5 control patients undergoing trans-sphenoidal pituitary surgery, who met the inclusion and exclusion criteria, were enrolled. Polyp tissue was harvested from the middle meatus of CRSwNP patients and mucosa from the inferior turbinate of control patients at the time of surgery.

It was not possible to study the effect of SEB on the non-polypoidal sinonasal mucosa of CRSwNP patients. Very little non-polypoidal tissue was harvested at the time of surgery, in order to maintain mucosal coverage. This was further compounded by the paucity of non-polypoidal sinonasal mucosa in patients with advanced disease presenting to this tertiary referral centre. The explant model requires greater amounts of tissue to set up. Therefore, this part of the study was limited to the investigation of the immune response in nasal polyp and control tissues only.

4.6   Results

4.6.1   Patient Demographics

Demographic data for study participants is shown in **Table 4.1**.

Table 4.1 Patient demographics.

Characteristics	CRSwNP Polyp	Control Mucosa
Subject numbers	15	5
Mean age (range)	49 (31-82)	60 (49-75)
Sex	6 Male/9 Female	3 Male/2 Female
Current smokers	4	0
Asthma	7	0
Positive SPT	4	0
Previous Surgery	7	0
SNOT-22 score (range)	55 (20-101)	25.2 (6-61)
Mean LMS (range)	14.5 (3-24)	0

4.6.2   Viability testing of the nasal explant culture model

4.6.2.1   LDH assay results of nasal polyp explant cultures

Viability testing, through the measurement of LDH levels, was undertaken on explant cultures over six days (**Figure 4.1**). There was a statistically significant difference between the positive control, and the unstimulated and SEB stimulated explant cultures. There was no significant difference in LDH levels between unstimulated and SEB stimulated explants.
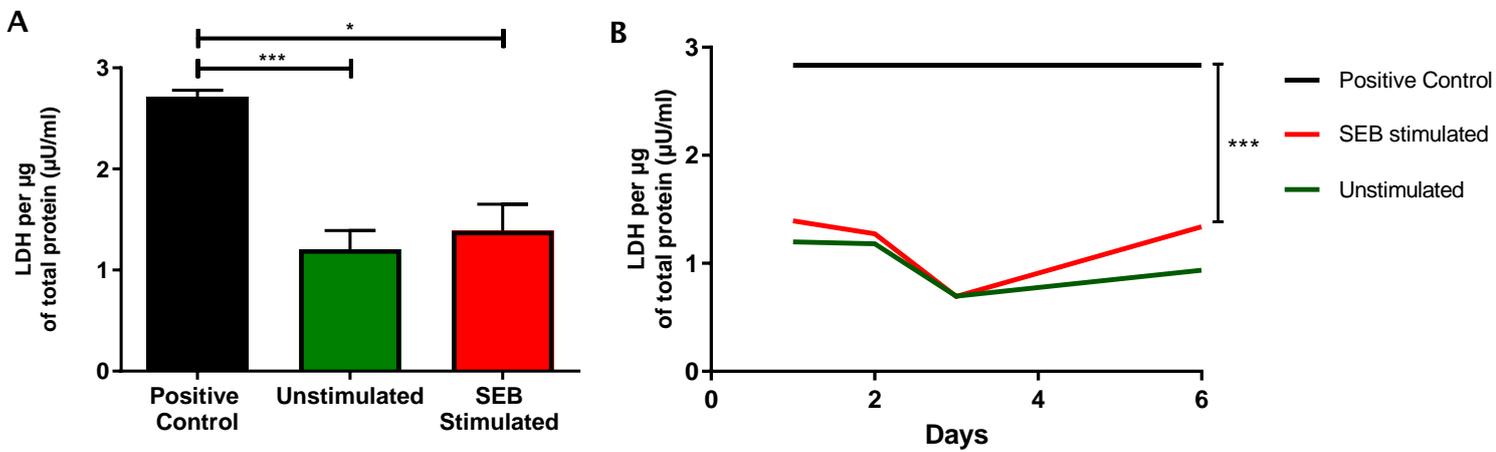
Figure 4.1 Viability testing of nasal explant cultures. Unstimulated and SEB stimulated (10µg/ml) nasal polyp explants (n=6), A) at 24 hours of culture, B) over 6 days. The positive control was obtained by culturing a nasal polyp sample in 1% Triton X-100, in order to derive the maximal LDH production. Statistical analysis was performed using paired t-tests, p≤0.05 (*) and p≤0.001 (***).

4.6.3   Nasal explant inflammatory profiles

4.6.3.1   Regulation of cytokine and chemokines in response to SEB stimulation

When nasal polyps were stimulated with SEB (**Figure 4.2**) there was significantly ($p \leq 0.05$) upregulated gene expression of IL-5, IL-17A, TNFα and TGF-β1. In contrast, there was significant downregulation of IL-1β gene expression upon SEB stimulation of nasal polyp explants. The gene expression of IL-6 and IL-8 (CXCL8) did not change significantly following nasal polyp SEB stimulation. There was a significant ($p \leq 0.05$) difference in the gene expression of IFNγ in SEB stimulated controls compared to SEB stimulated nasal polyps (**Figure 4.2**).

There was a significant increase in IFNγ ($p \leq 0.001$), IL-5 ($p \leq 0.05$), IL-17A ($p \leq 0.05$) and TNFα ($p \leq 0.01$) protein concentrations within the explant culture supernatant following SEB stimulation of nasal polyps (**Figure 4.3**). For cytokines IL-5, IL-17A and TNFα, there was both significant upregulation of gene expression and protein concentration, when nasal polyps were stimulated with SEB. Concentrations of IL-1β and IL-6 remained unchanged following nasal polyp SEB stimulation. Control mucosa stimulated with SEB resulted in a significant ($p \leq 0.05$) rise in only one cytokine measured, IFNγ. When control mucosa was stimulated with SEB (**Figure 4.2**) there was a small upregulation of gene expression for all cytokines and chemokines studied (IFNγ, IL-5, IL-17A, TNFα, TGF-β1, IL-1β, IL-6 and IL-8), but this change was not deemed to be of statistical significance. There was a slight increase in protein concentrations of IL-5, IL-17A, TNFα, IL-1β and IL-6 when control mucosa was stimulated with SEB (**Figure 4.3**). However, these changes were not statistically significant.
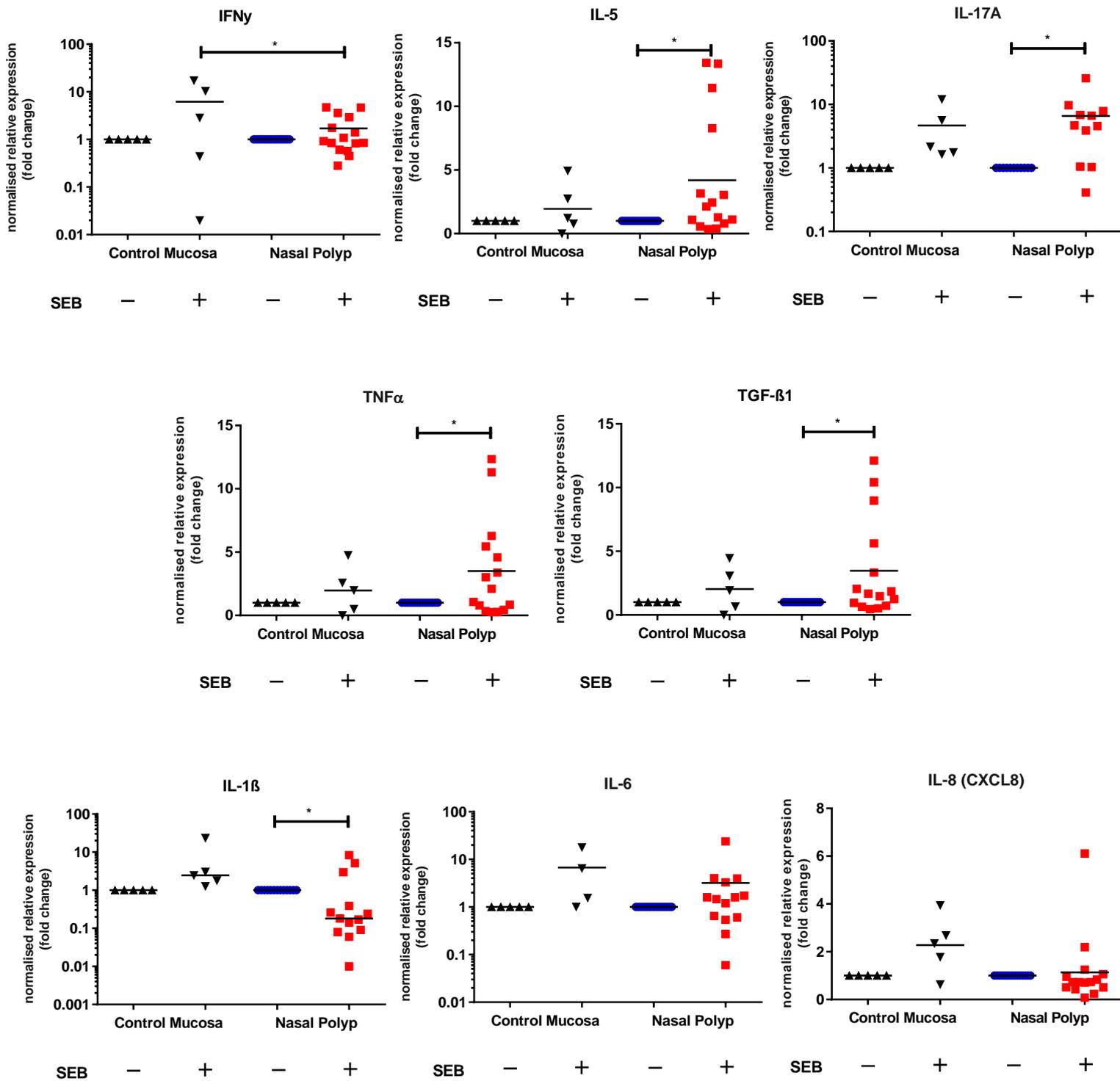
Figure 4.2 IFNγ, IL-5, IL-17A, TNFα, TGF-β1, IL-1β, IL-6 and IL-8 normalised gene expression of SEB stimulated sinonasal tissue explants. RT-qPCR undertaken using unstimulated and SEB stimulated (10μg/ml) control sinonasal mucosa (n=5) and nasal polyp (n=15) explants over 24 hours. Individual sample points and mean lines. Paired t-tests were performed between SEB stimulated and unstimulated samples, and t-tests between control and nasal polyps, p≤0.05 (*).
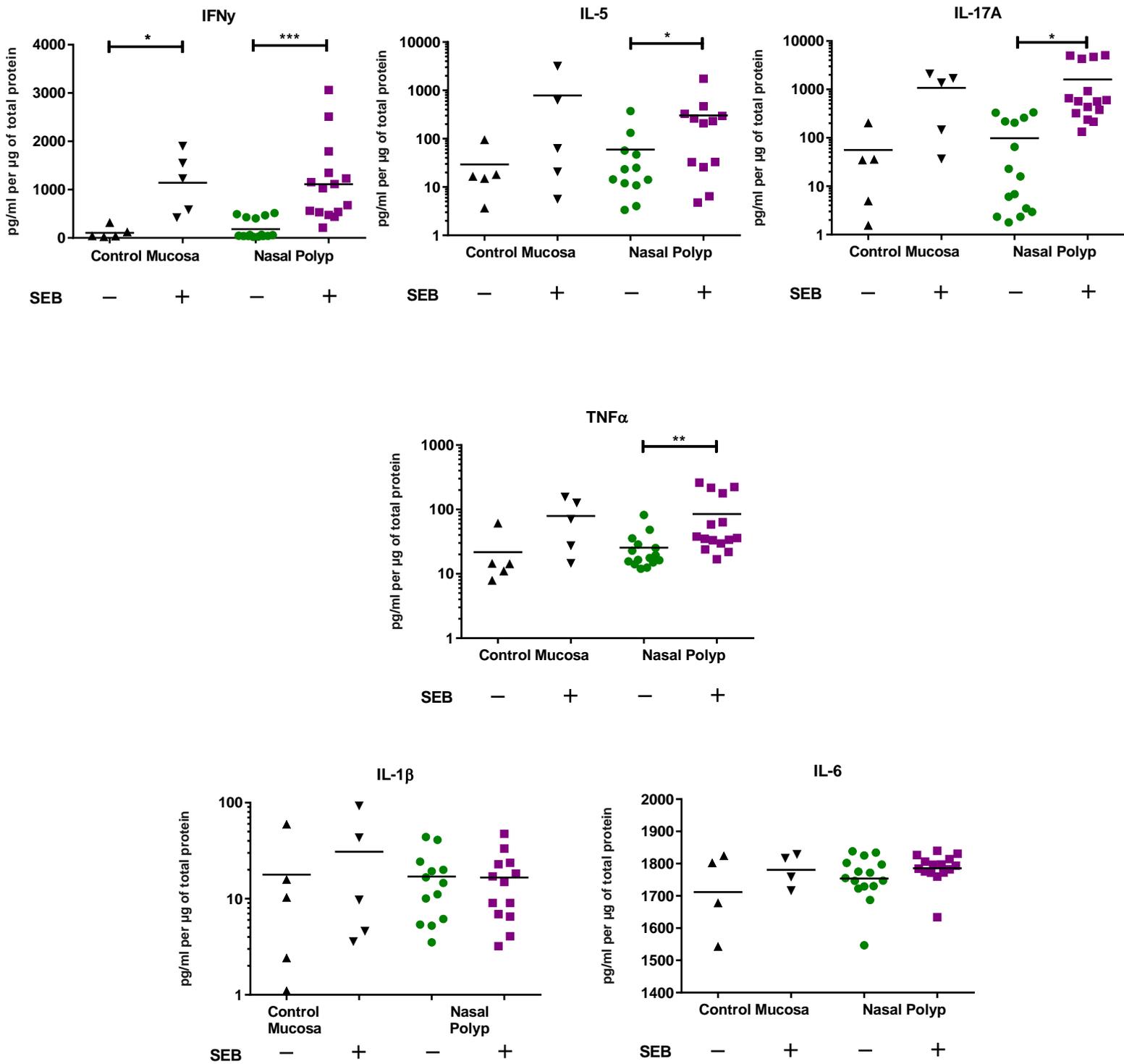
Figure 4.3 IFNγ, IL-5, IL-17A, TNFα, IL-1β and IL-6 culture supernatant cytokine concentrations of SEB stimulated sinonasal tissue explants. Luminex undertaken using unstimulated and SEB stimulated (10μg/ml) control sinonasal mucosa (n=5) and nasal polyp (n=15) explant culture supernatants over 24 hours. Individual sample points and mean lines. Paired t-tests were performed between SEB stimulated and unstimulated samples, and t-tests between control and nasal polyps, p≤0.05 (*), p≤0.01 (**) and p≤0.001 (***).

4.6.3.2   Regulation of T cell transcriptional factors in response to SEB stimulation

There was no significant change in the gene expression of the four T cell transcriptional factors (**Figure 4.4**), when nasal polyp or control samples were stimulated with SEB. There was a significant ($p \leq 0.05$) difference in the gene expression of T-bet when comparing the response of SEB stimulated control samples to SEB stimulated nasal polyps.
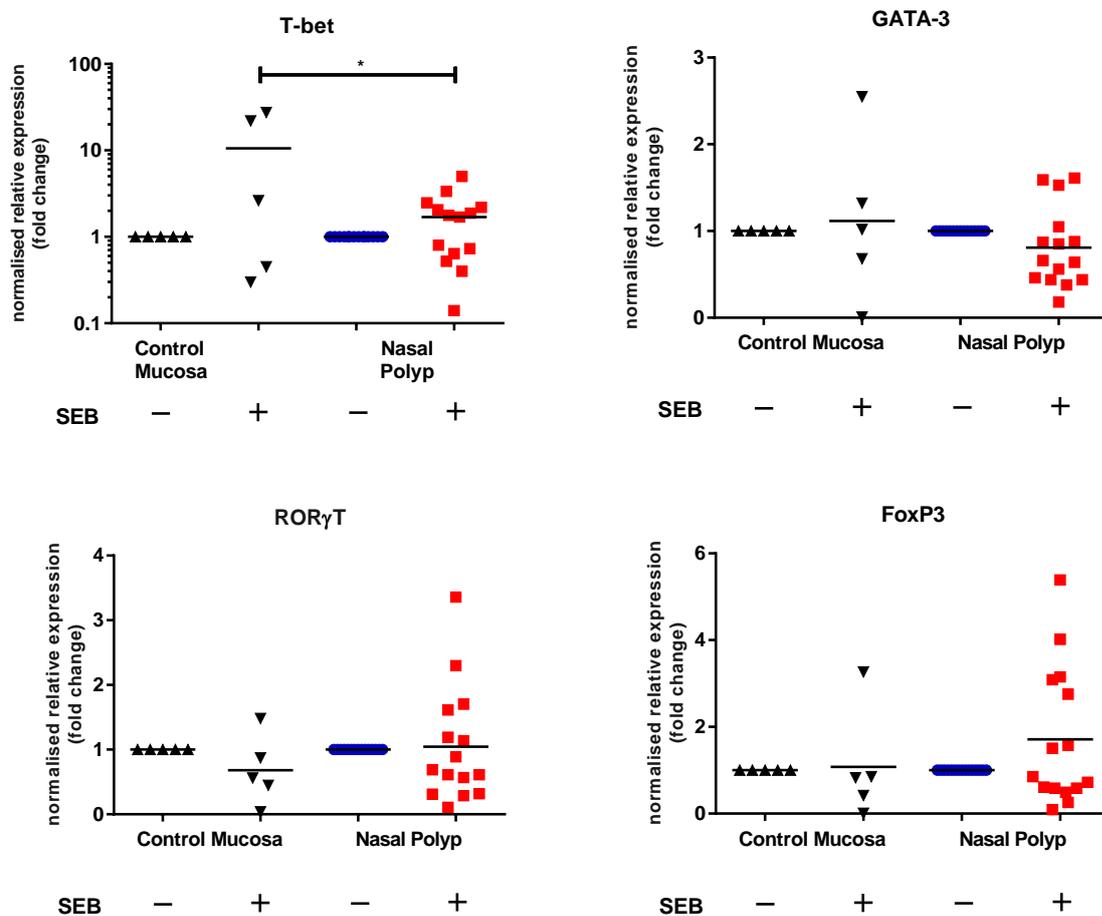
Figure 4.4 T-bet, GATA-3, RORγT and FoxP3 normalised gene expression of SEB stimulated sinonasal tissue explants. RT-qPCR undertaken using unstimulated and SEB stimulated (10µg/ml) control sinonasal mucosa (n=5) and nasal polyp (n=15) explants over 24 hours. Individual sample points and mean lines. Paired t-tests were performed between SEB stimulated and unstimulated samples, and t-tests between control and nasal polyps, $p \leq 0.05$ (*).

4.6.3.3   Regulation of Toll like receptors in response to SEB stimulation

There was no significant change in the gene expression of TLR-2 or TLR-4 in control mucosa or nasal polyps when stimulated with SEB (**Figure 4.5**).
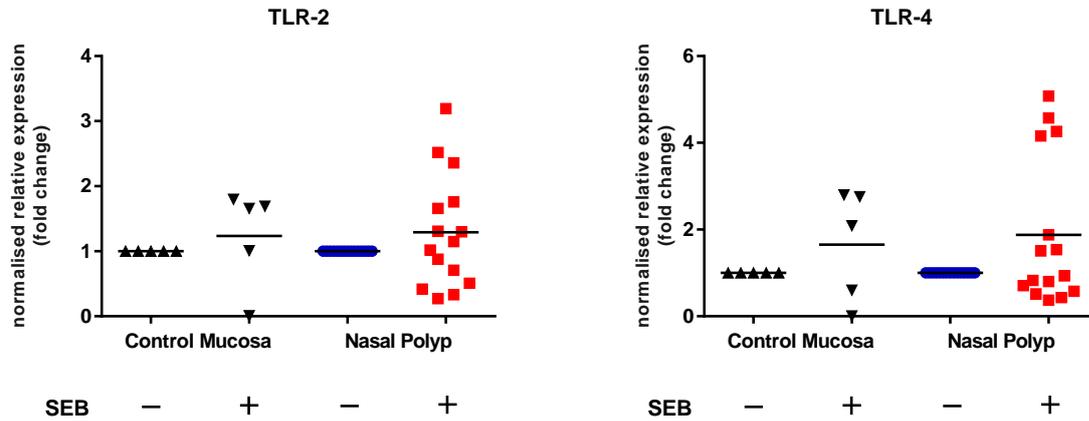
Figure 4.5 TLR-2 and TLR-4 normalised gene expression of SEB stimulated sinonasal tissue explants. RT-qPCR undertaken using unstimulated and SEB stimulated (10µg/ml) control sinonasal mucosa (n=5) and nasal polyp (n=15) explants over 24 hours. Individual sample points and mean lines. Paired t-tests were performed between SEB stimulated and unstimulated samples, and t-tests between control and nasal polyps.

4.6.3.4 Regulation of Matrix metalloproteinase and defensin in response to SEB stimulation

Results (**Figure 4.6**) show statistically significant (p≤0.05) upregulation of defensin 5 and matrix metalloproteinase (MMP) -7 and -28 gene expression in SEB stimulated nasal polyps. There was no significant change in the gene expression of MMP-9 in SEB stimulated nasal polyps. There was no significant difference in gene expression of MMPs and defensins when stimulating control mucosa with SEB. Culture supernatant protein levels of MMP-7 were found to be significantly (p≤0.05) elevated following nasal polyp SEB stimulation (**Figure 4.7**), which matched a similar upregulation of MMP-7 gene expression (**Figure 4.6**).
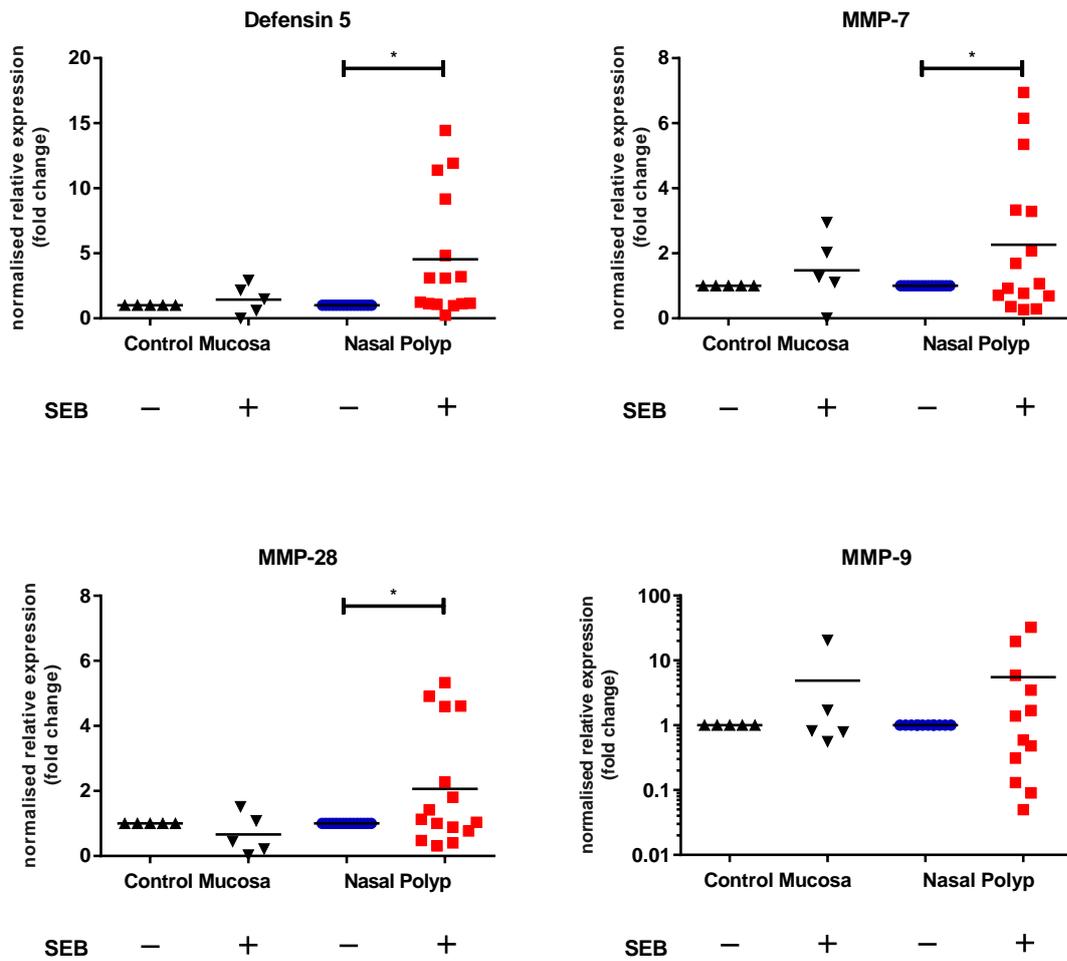
Figure 4.6 Defensin 5, MMP-7, -28 and -9 normalised gene expression of SEB stimulated sinonasal tissue explants. RT-qPCR undertaken using unstimulated and SEB stimulated (10μg/ml) control sinonasal mucosa (n=5) and nasal polyp (n=15) explants over 24 hours. Individual sample points and mean lines. Paired t-tests were performed between SEB stimulated and unstimulated samples, and t-tests between control and nasal polyps, p≤0.05 (*).
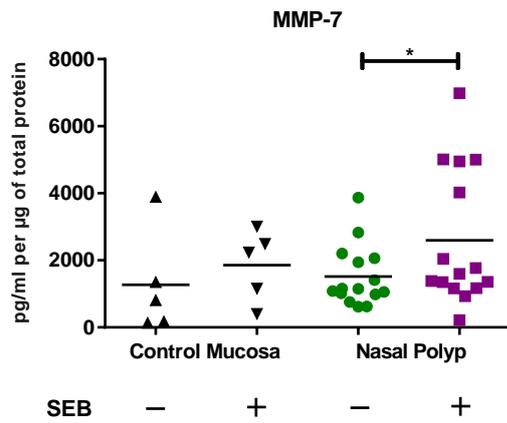
Figure 4.7 MMP-7 culture supernatant cytokine concentrations of SEB stimulated sinonasal tissue explants. Luminex undertaken using unstimulated and SEB stimulated (10µg/ml) control sinonasal mucosa (n=5) and nasal polyp (n=15) explant culture supernatants over 24 hours. Individual sample points and mean lines. Paired t-tests were performed between SEB stimulated and unstimulated samples, and t-tests between control and nasal polyps, p≤0.05 (*).

4.7 Discussion

Patient demographics

Patient demographics were evenly distributed throughout the datasets, with no significant phenotypic skewing seen within any one category. Sex ratios, age, asthma, allergy, SNOT-22 and Lund Mackay scores (LMS) broadly reflect those seen within the literature, outlined in detail in section 3.7 (6).

SEB skews the T-cell repertoire of nasal polyps towards the Th2 and Th17 pathway

Findings show that when compared to controls, SEB stimulation of nasal polyps results in upregulation of the Th2 derived pro-inflammatory cytokine IL-5, both on the genome and protein level. In addition, and in contrast to a similar study undertaken by Patou et al (11), results suggest a Th17 response in nasal polyps following SEB simulation. It is currently unclear whether nasal polyps from Caucasian patients display a Th17 skewed inflammatory profile. Most historical studies found no evidence (124-126, 128, 129, 131, 132, 171), but there are a few recently published studies that suggest otherwise (132-134). The absence of a Th17 response would be surprising given the Th17 cells role in the host defence against extracellular pathogens, and the abundance of surface and intracellular bacteria (75). Interestingly, a study undertaken by Ramezanpour et al (98) found evidence of significant epithelial barrier disruption when CRS epithelial cells were exposed to Th17 cytokines (IL-17, IL-22 and IL-26). This was evidenced by the breakdown of zonula occludens-1 (ZO-1) and reduced transepithelial electrical resistance (TEER) values. These results highlight the significance of bacteria and their toxins in driving an inflammatory response in CRSwNP patients, and that this inflammatory response could be promoting further bacterial entry.

MMPs, as their name suggests, cleave a variety of structural extracellular matrix proteins (181). MMP-7 is important in the activation of alpha defensins, of which defensin 5 is one. These peptides, secreted by neutrophils, act as antibacterial peptides against gram-positive and gram-negative bacteria, but also possess important immunomodulatory functions, including the chemotaxis of innate immune cells such as macrophages and mast cells (182). Defensin 5, originally thought to act only within the

gut, has now been identified in other areas too, namely the reproductive and respiratory tract (183).

Results in this chapter have provided evidence of upregulated gene expression of MMP-7 and defensins 5 in nasal polyps, and increased protein production of MMP-7 in nasal polyps when stimulated with SEB. TLR-2 and -4 were not upregulated following SEB stimulation, which was expected given their role in the recognition of bacterial lipopolysaccharides. The remodelling peptide MMP-28 was also found to be upregulated within SEB stimulated nasal polyps, supporting the role of bacteria in the remodelling process. These findings support the notion that bacterial toxins contribute to persistent mucosal inflammation and remodelling, potentially mediating further polyp growth and chronicity of the disease process.

SEB amplifies disordered bacterial immune responses, through the downregulation of IL-1β

IL-1β is a potent pro-inflammatory cytokine that is crucial for host-defence responses to infection and injury (184). It is a cytokine released by activated phagocytic cells in response to the detection of pathogen associated molecular patterns (PAMPs), which helps form an immune response and clear bacterial infection. In chapter 3 the significant downregulation of IL-1β gene expression in CRSwNP tissues was discovered, suggesting possible evidence of dysfunctional bacterial responses. Findings here have showed further downregulation of IL-1β gene expression in SEB stimulated nasal polyps. This is a significant finding, as it suggests that SEB, secreted by *S. aureus*, would seem to contribute towards the development of a disordered bacterial immune response in nasal polyp tissues. This could compromise the ability of these tissues to effectively clear bacteria, with the subsequent promotion of Th2/Th17 mediated inflammation. In this way SEB is likely to contribute to a chronic inflammatory state, promoting tissue inflammation and oedema, and the formation of nasal polyps.

4.8   Conclusion

Evidence has been presented of a Th2/Th17 skewed response of nasal polyps following exogenous SEB stimulation. These results are significant in highlighting the importance of bacteria and their toxins in driving inflammation in CRSwNP patients. It is likely that bacteria, and in particular *S. aureus*, play an important role in the development of chronicity and recalcitrance in CRS.

5   The immunological response of the nasal epithelium to

S. aureus infection

5.1 Introduction

The epithelium is the first line of defence against invading pathogens, and represents an important element of the immune response. Defects in epithelial function and integrity can result in increased incidence of infection. In cystic fibrosis, where there is impaired mucociliary clearance, this can manifest as CRS or chest infections (185). Furthermore, research has revealed evidence of epithelial barrier dysfunction in CRS, including diminished tight junctional proteins in response to the secretion of pro-inflammatory cytokines and bacterial proteases (96, 97, 186, 187). These defects are associated with increased permeability across the epithelial barrier, allowing bacteria to enter the submucosal space (97, 188). The epithelium is also important in secreting defensive enzymes (e.g. lysozyme), peptides (e.g. defensins) and molecules (e.g. ROS), as well as representing an important step in the activation of the innate and adaptive immune response.

The intracellular residency of *S. aureus* into epithelial cells has been observed in CRS patients previously, and this has been correlated with poorer treatment outcomes (163). Interestingly, this is not exclusive to CRS, but is seen in many other chronic diseases including periodontitis, *Campylobacter jejuni* induced infectious-diarrhoea, listeriosis and tuberculosis to name but a few (189, 190). Bacteria are able to enter both professional phagocytes such as monocytes or macrophages, and non-professional phagocytes such as epithelial and mast cells (25, 143, 189). For entry into non-professional phagocytes, bacteria bind to surface receptors resulting in the activation of a signalling cascade which leads to reorganisation of the actin cytoskeleton and bacterial uptake within a vacuole via the zipper or trigger mechanism (189). Following internalisation, bacteria are then able to replicate in three main compartments: lysosome-like vacuoles, non-acidic intracellular vacuoles, and/or the cell cytosol (189, 190). Bacteria are also able to directly infect neighbouring cells, including release into the extracellular compartment. Whilst many of these bacteria are likely killed, some will survive and promote the development of a chronic inflammatory reaction. This may further aid bacterial propagation, by further compromising epithelial integrity and allowing entry of more bacteria. Despite

recent advances in this area, there remains limited knowledge of the immunological response of the

epithelium to bacterial infection, and in particular *S. aureus*.

5.2 Hypothesis

The epithelium not only facilitates the survival of *S. aureus*, through intracellular uptake and subsequent release, but also contributes towards the development of a chronic inflammatory state.

5.3 Aims

To characterise the immunological response of the epithelium to *S. aureus* infection using a representative cell line.

5.4 Objectives

1. To investigate uptake of *S. aureus* into epithelial cells including viability of the bacteria.

2. To characterise epithelial inflammatory responses to *S. aureus*.

5.5 Methods

For detailed methods please refer to chapter 2, pages 41-65.

5.6 Results

5.6.1 Confocal laser scanning microscopy

An RPMI 2650 cell line was used to demonstrate intracellular uptake of *S. aureus* (**Figure 5.1**). The number of intracellular *S. aureus* and proportion of infected cells increased over the studied time course (**Figure 5.1 and 5.2**). Epithelial cell sizes increased at 2 hours, but then decreased significantly ($p \leq 0.05$) at 6 hours (**Figure 5.3**).
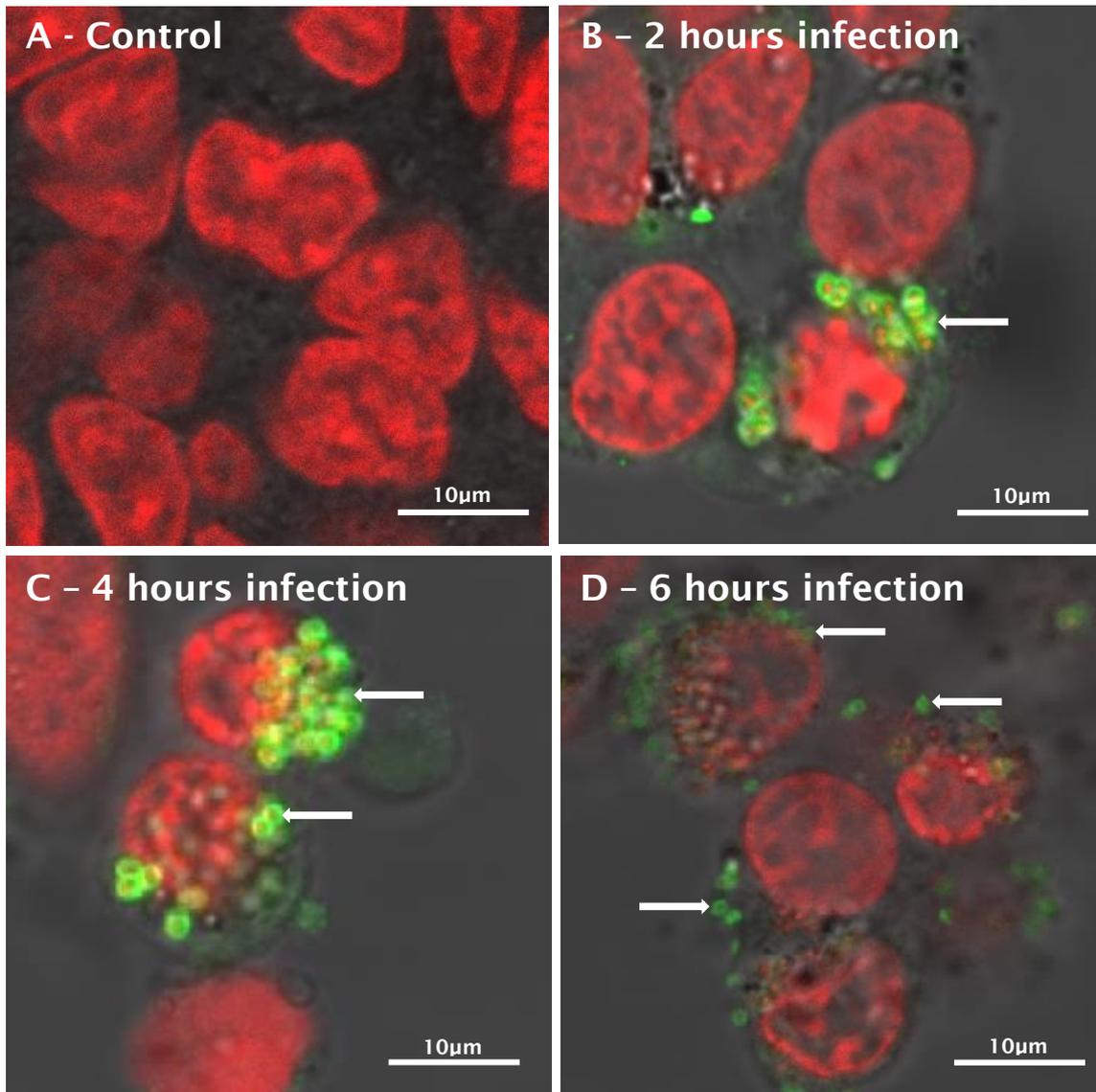
Figure 5.1 Intracellular uptake of *S. aureus* into nasal epithelial cells. Representative CLSM images of epithelial cells (RPMI 2650) co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 6 hours. Staining undertaken with an anti-*S. aureus* antibody and secondary Alexa 568 (green), and DAPI (red). Images demonstrate, A) cell nuclei (red) with no *S. aureus*, B-D) intracellular *S. aureus* (green, highlighted with white arrows) at increasing intervals of infection. Extracellular *S. aureus* was eradicated with 20μg/ml Lysostaphin.
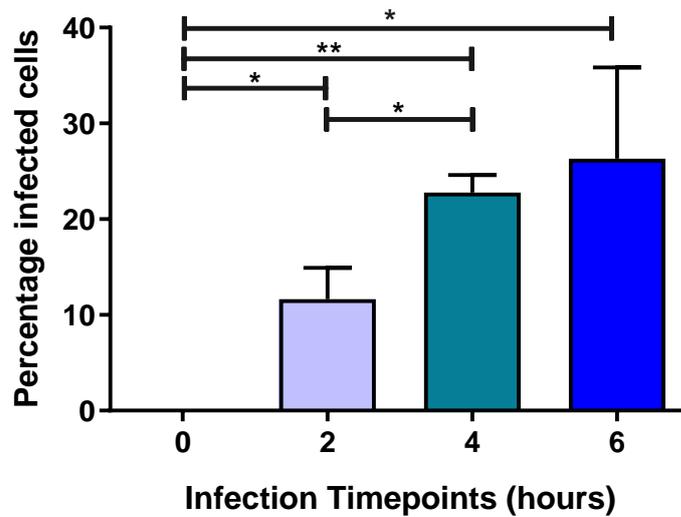
Figure 5.2 Proportion of epithelial cells containing intracellular *S. aureus*. Epithelial cells (RPMI 2650) were co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 6 hours. Data obtained using CLSM, 50 cells counted in each of three experimental repeats, with bars representing the mean and error bars representing SEM. Statistical analysis was performed using paired t-tests, p≤0.05 (*) and p≤0.01 (**).
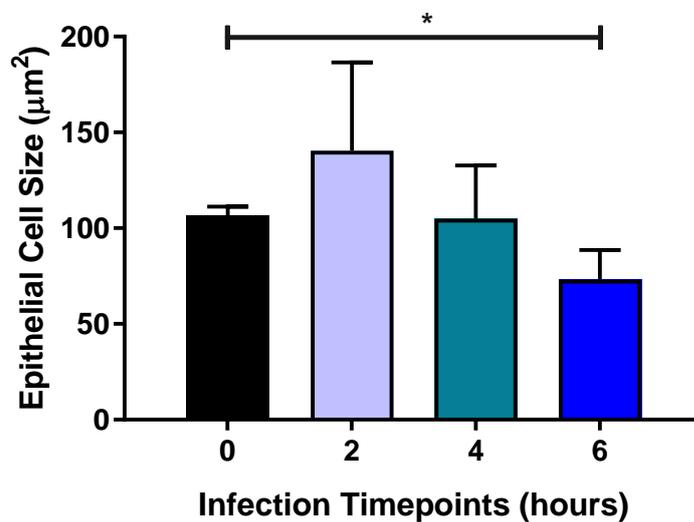
Figure 5.3 Epithelial cell size following *S. aureus* co-infection. Epithelial cell (RPMI 2650) size was measured following co-infection with a CRS *S. aureus* isolate (MOI 1:1) over 6 hours. Size calculated using the largest cross section of multiple sequential CLSM images, 20 cells counted in each of three experimental repeats, with bars representing the mean and error bars representing SEM. Statistical analysis was performed using paired t-tests, p≤0.05 (*).

5.6.2 Viability of *Staphylococcus aureus*

Intracellular *S. aureus* was found to be viable within epithelial cells (RPMI 2650), and capable of forming new colonies (**Figure 5.4**). Viable intracellular *S. aureus* was enumerated from 2 hours onwards. There was an initial drop in intracellular *S. aureus* (between 2 and 4 hours), followed by an increase (between 4 and 6 hours). Following eradication of extracellular *S. aureus* at 6 hours, and further culture for 24 hours, there were still a large number of viable intracellular *S. aureus* (1.32×10^8 *S. aureus* per ml), but significantly less ($p \leq 0.001$) than at 6 hours.

Between 2 and 6 hours the number of extracellular *S. aureus* grew steadily (**Figure 5.5**). There was no significant difference in the number of *S. aureus* grown with and without the addition of nasal epithelial cells. Following eradication of all extracellular *S. aureus* at 6 hours, and re-culture for a further 24 hours, there was evidence of an accumulation of viable extracellular *S. aureus*. When *S. aureus* was grown in the presence of SEB, there was no statistically significant difference in the rate of extracellular growth.

Between 2 and 6 hours of culture SEB had no significant effect on the number of viable intracellular *S. aureus* (**Figure 5.6**). However, at 24 hours there was a statistically significant ($p \leq 0.01$) difference in the number of viable intracellular *S. aureus*, with higher CFUs found in the SEB stimulated group (**Figure 5.6**).
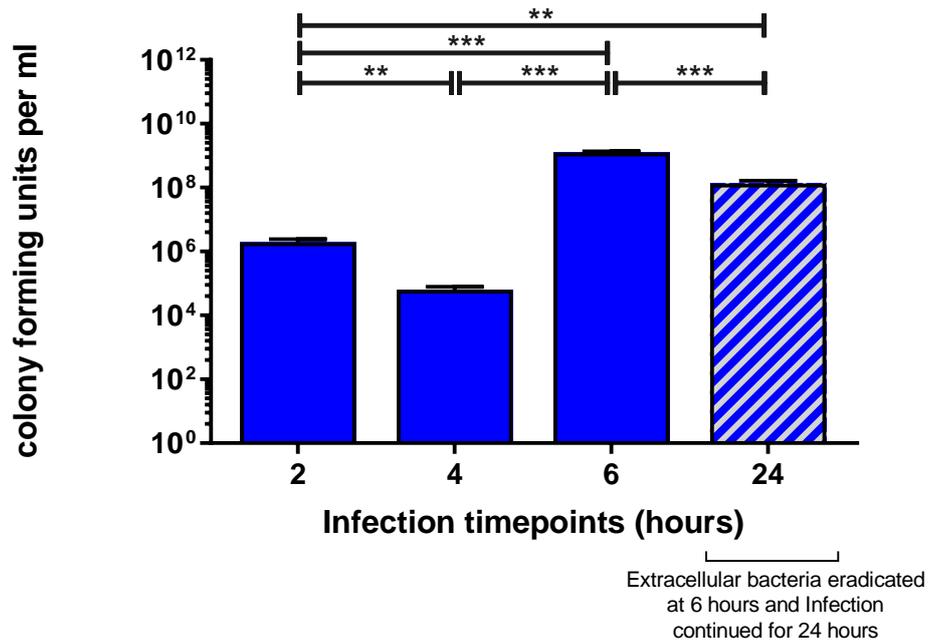
Figure 5.4 Intracellular *S. aureus* CFU time-course from nasal epithelial cell co-cultures. RPMI 2650 nasal epithelial cells (2 million) were co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 24 hours. Intracellular CFUs were enumerated on CBA plates following eradication of extracellular *S. aureus* (20µg/ml Lysostaphin). Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Statistical analysis was performed using paired t-tests, p≤0.01 (**) and p≤0.001 (***).
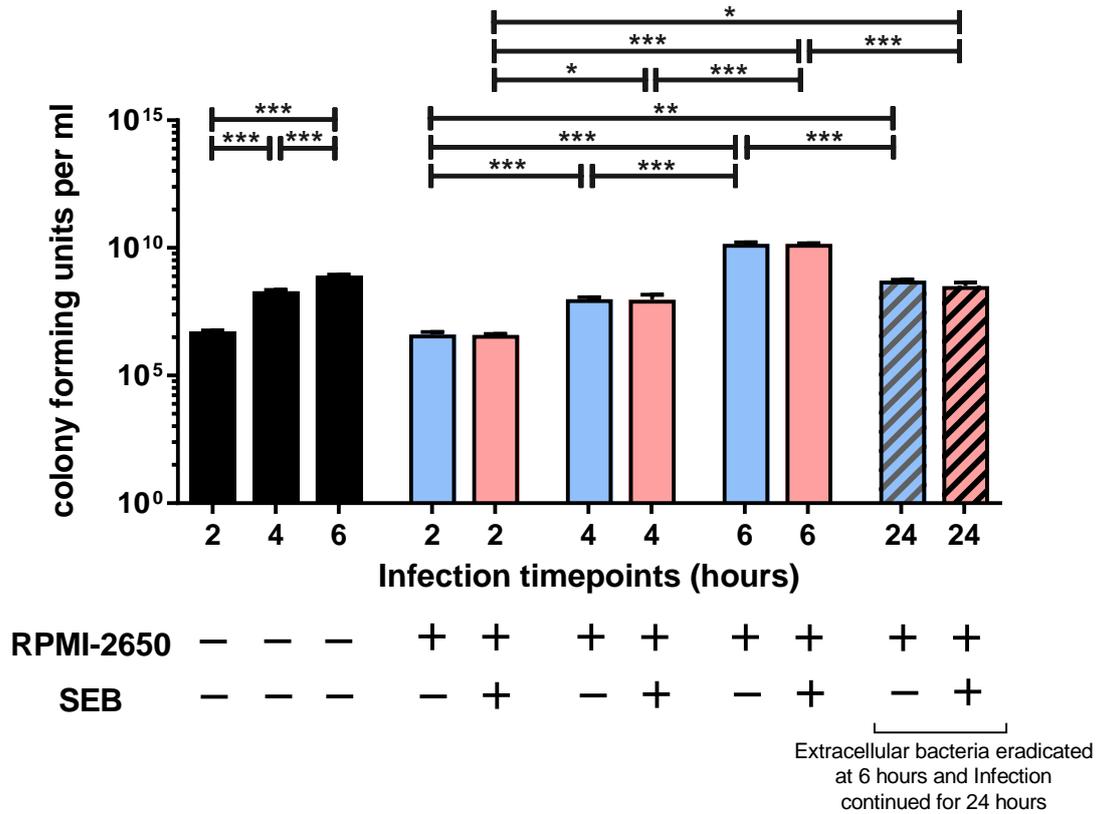
Figure 5.5 Extracellular *S. aureus* CFU time-course, with and without nasal epithelial cells and SEB. RPMI 2650 nasal epithelial cells (2 million) were co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 24 hours. Extracellular CFUs were enumerated on CBA plates. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Statistical analysis was performed using paired t-tests (time course within RPMI 2650 groups) and unpaired t-tests (between groups with and without RPMI 2650 cells), p≤0.05(*), p≤0.01 (**) and p≤0.001 (***).
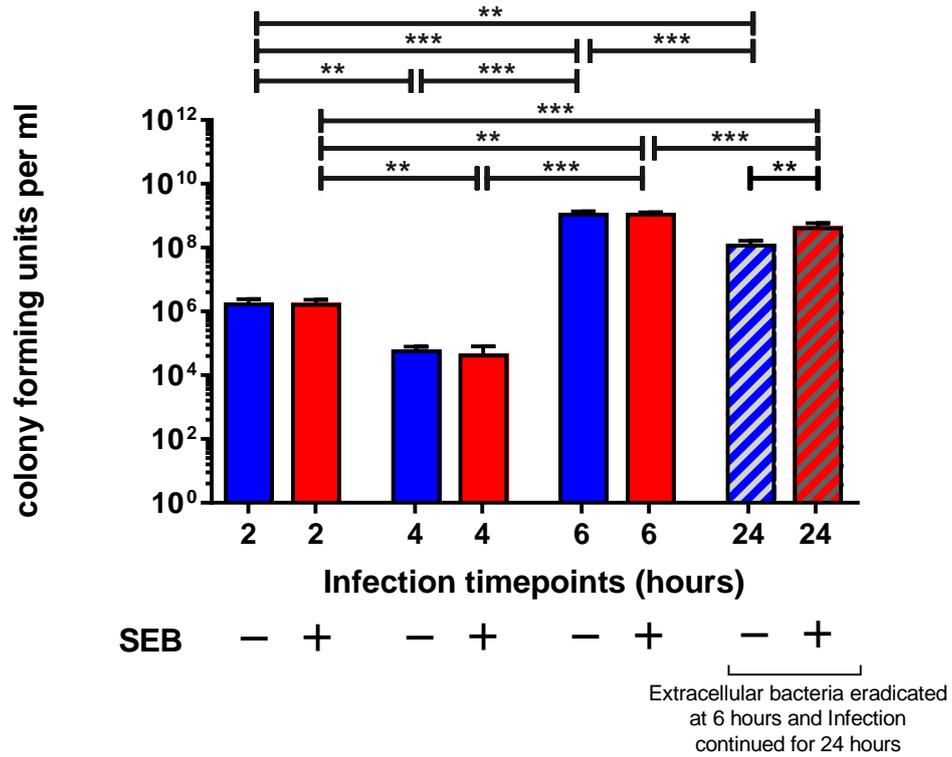
Figure 5.6 Intracellular *S. aureus* CFUs from nasal epithelial cell co-cultures, with and without the addition of SEB. RPMI 2650 nasal epithelial cells (2 million) were co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 24 hours, with and without the addition of SEB (10μg/ml). Intracellular CFUs were enumerated on CBA plates following eradication of extracellular *S. aureus* (20μg/ml Lysostaphin). Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Statistical analysis was performed using paired t-tests, p≤0.01 (**) and p≤0.001 (***).

5.6.3 The epithelial barrier in response to SEB and *S. aureus*

When control tissue and nasal polyp explants were exposed to SEB, there was a statistically significant ($p \leq 0.05$) downregulation of the tight junctional protein zonula occludens 1 (ZO1) gene expression, but no statistically significant downregulation of occludin (**Figure 5.7**). When a nasal epithelial cell line (RPMI 2650) was co-cultured with live *S. aureus* over 6 hours, there was a statistically significant ($p \leq 0.05$) downregulation of ZO1 observed at 4 hours (**Figures 5.8 and 5.9**). In addition, (**Figure 5.9**) the stimulation of epithelial cells with SEB also resulted in a significant ($p \leq 0.05$) downregulation of ZO1 gene expression.
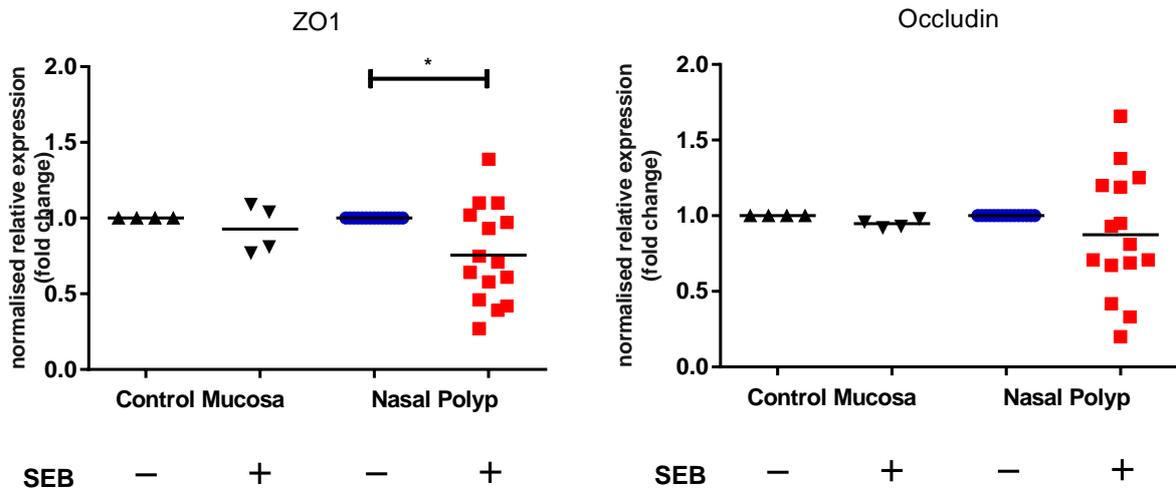
Figure 5.7 ZO1 and occludin normalised gene expression of SEB stimulated sinonasal tissue explants. RT-qPCR undertaken using unstimulated and SEB stimulated (10µg/ml) control sinonasal mucosa (n=5) and nasal polyp (n=15) explants over 24 hours. Individual sample points and mean lines. Paired t-tests were performed between SEB stimulated and unstimulated samples, and t-tests between control and nasal polyps, p≤0.05 (*).
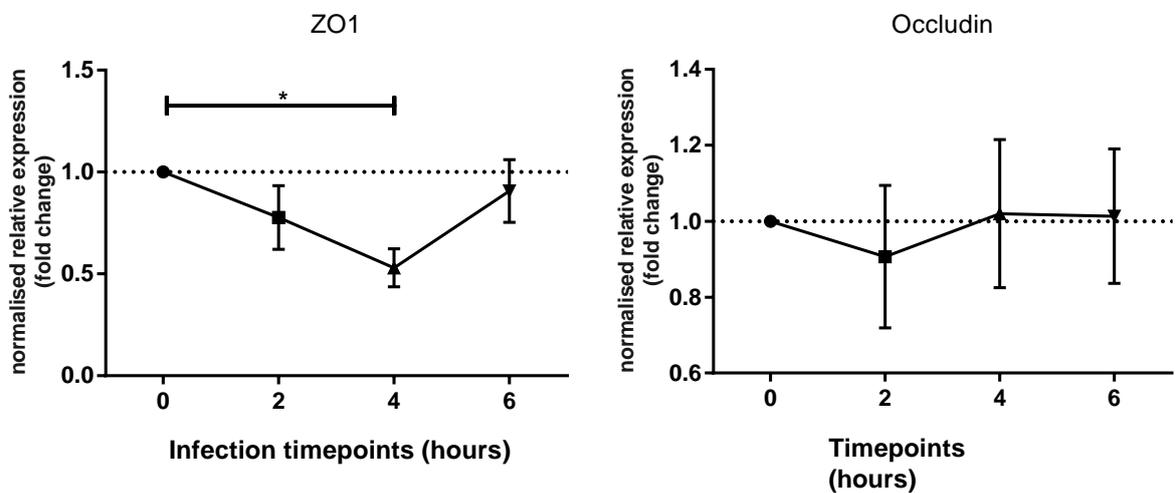
Figure 5.8 Nasal epithelial cell normalised gene expression time-course of ZO1 and occludin following *S. aureus* infection. Epithelial cells (RPMI 2650) were co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 6 hours, with RT-qPCR undertaken. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*).
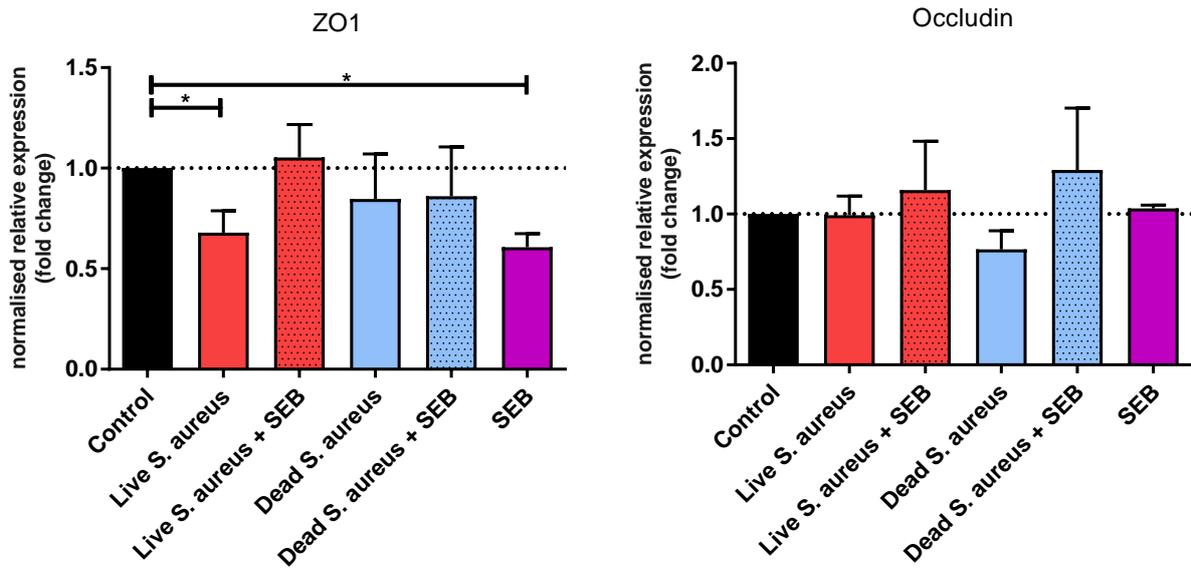
Figure 5.9 Nasal epithelial cell normalised gene expression of ZO1 and occludin in SEB stimulated/*S. aureus* infected groups. RT-qPCR undertaken using an epithelial cell line (RPMI 2650) exposed to SEB (10µg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*).

5.6.4   The nasal epithelial immune response towards *S. aureus*

5.6.4.1   Cytokine response of nasal epithelial cells to *S. aureus* and SEB

When a nasal epithelial cell line (RPMI 2650) was exposed to live *S. aureus*, there was significant (p≤0.05) upregulation of TNFα, IL-5, and TGF-β1 gene expression, in comparison to controls (**Figure 5.10**). The addition of dead *S. aureus* did not significantly alter the gene expression of measured cytokines, with the exception of IL-17A and IL-5, which saw significant (p≤0.05) downregulation in comparison to controls. The addition of SEB did not significantly alter the gene expression of pro-inflammatory cytokines in live and dead *S. aureus* groups, except with regard to TGF-β1. SEB alone resulted in a significant downregulation of IL-5 (p≤0.001) and IL-1β (p≤0.05) gene expression in comparison to controls. There was no significant change in the gene expression of IFNγ to *S. aureus* or SEB. Gene expression of IL-5 and TNFα peaked at 4 hours following live *S. aureus* infection (**Figure 5.11**).

There was significant (p≤0.05) elevation of IFNγ, IL-17A, TNFα, IL-1β and IL-6 cytokine protein content in culture supernatants when epithelial cells were exposed to live *S. aureus* (**Figure 5.12**). The addition of SEB to live *S. aureus* did not significantly alter the level of secreted cytokine. Dead *S. aureus*, dead *S. aureus* with SEB, and SEB alone did not result in significant alteration in cytokine release into the culture supernatant.
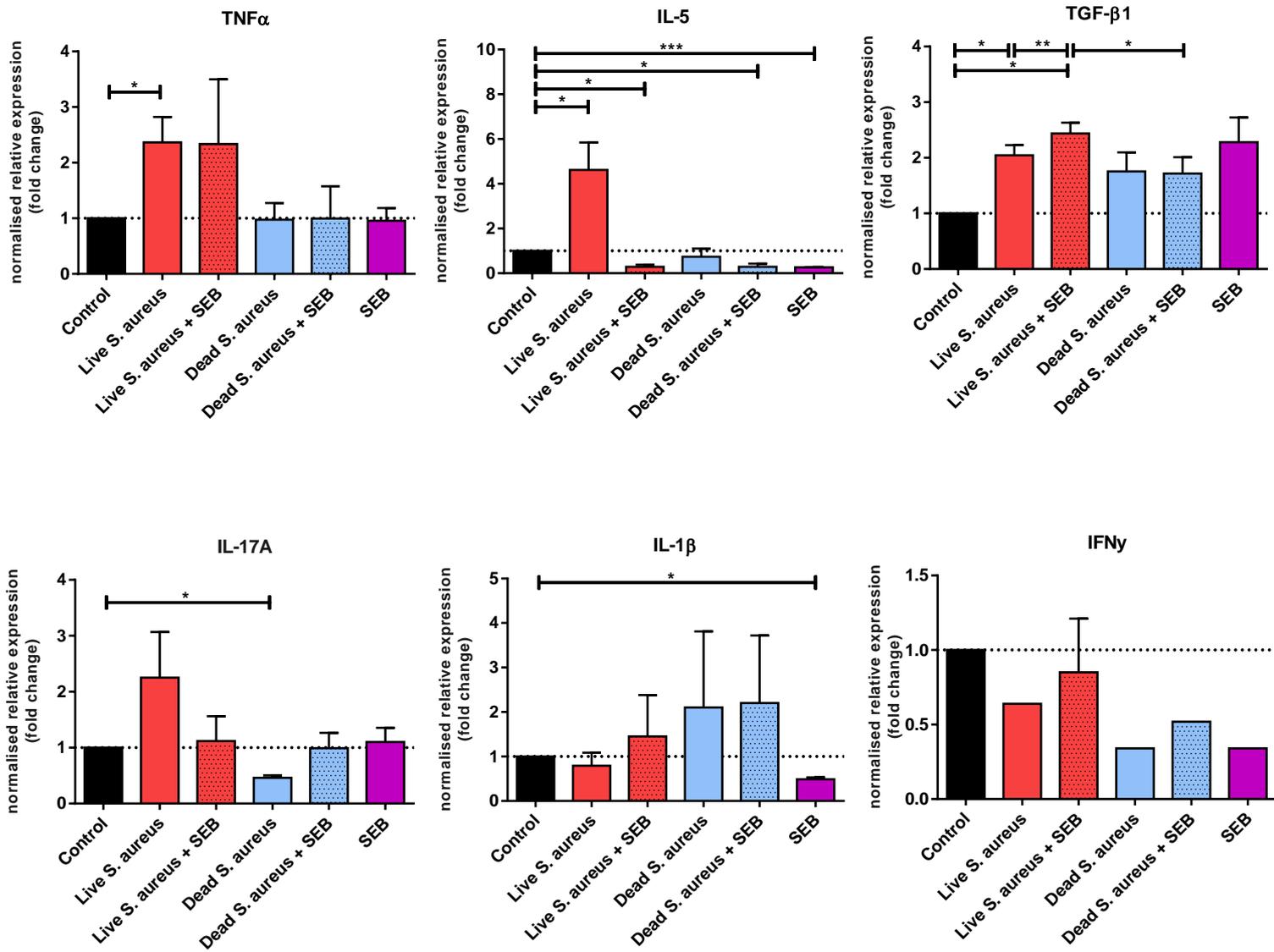
Figure 5.10 Nasal epithelial cell normalised gene expression of TNFα, IL-5, TGF-β1, IL-17A, IL-1β, and IFNγ in SEB stimulated and/or *S. aureus* infected groups. RT-qPCR undertaken using an epithelial cell line (RPMI 2650) exposed to SEB (10μg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*), p≤0.01 (**) and p≤0.001 (***).
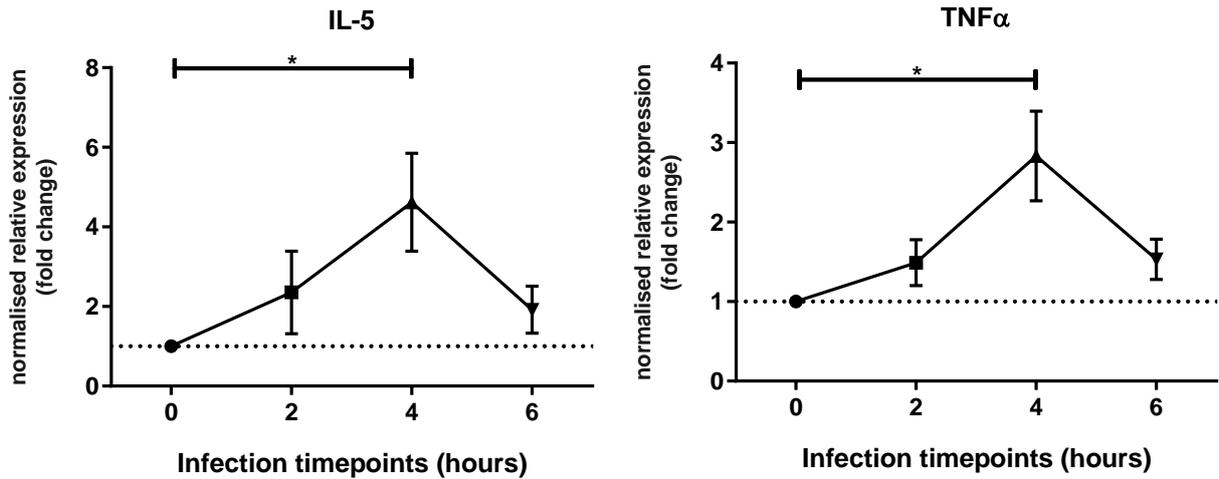
Figure 5.11 Nasal epithelial cell normalised gene expression time-course of IL-5 and TNFα following *S. aureus* infection. Nasal epithelial cells (RPMI 2650) were co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 6 hours, with RT-qPCR undertaken. Three experimental repeats are displayed, with bars representing the mean and error bars representing +/-SEM. Paired t-tests were performed, p≤0.05 (*).
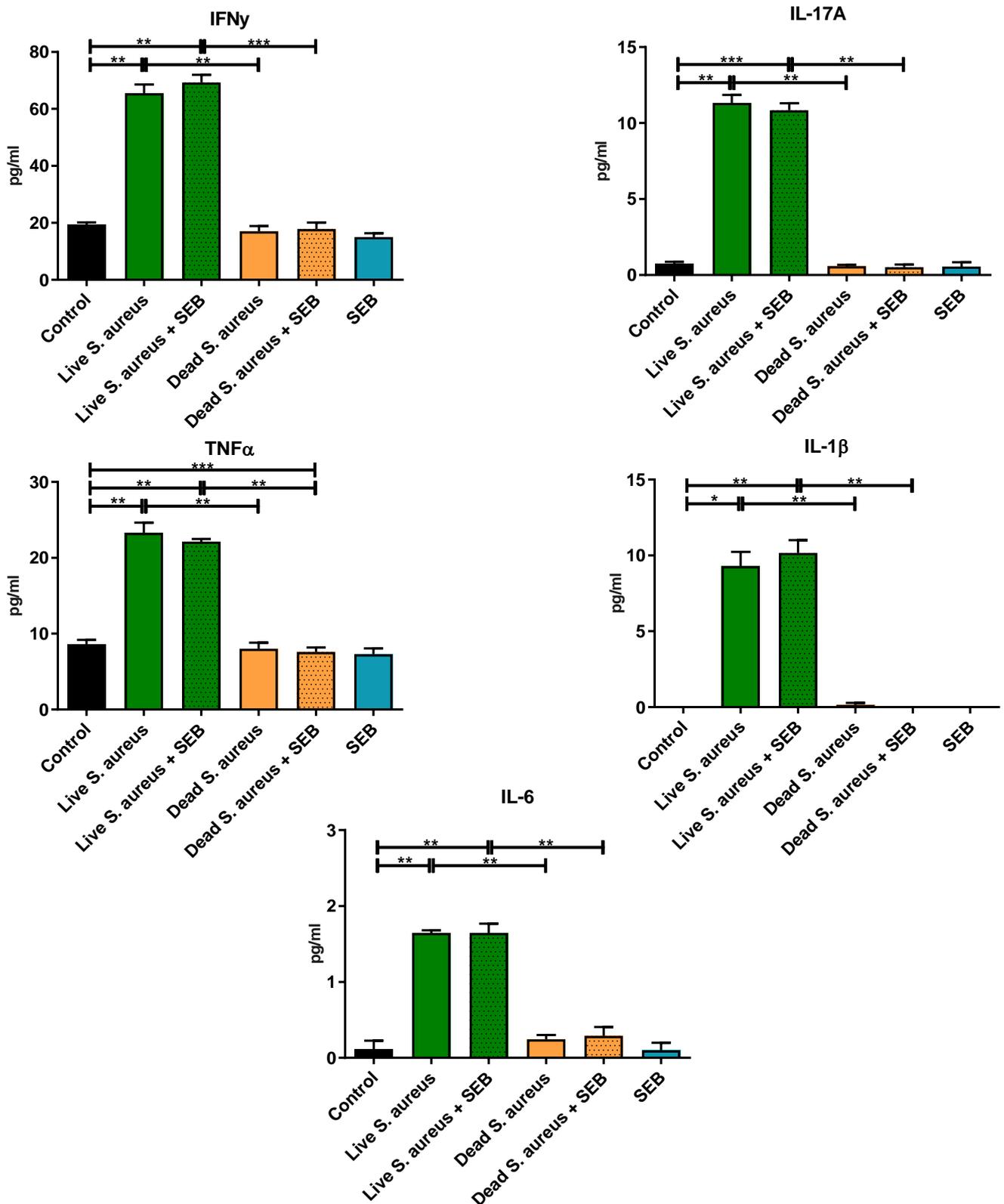
Figure 5.12 Nasal epithelial cell culture supernatant cytokine concentrations of IFNγ, IL-17A TNFα, IL-1β and IL-6, in SEB stimulated and/or *S. aureus* infected groups. Luminex undertaken using an RPMI 2650 nasal epithelial cell line (2 million cells per ml) exposed to SEB (10µg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*), p≤0.01 (**) and p≤0.001 (***).

Timothy C Biggs

5.6.4.2 T cell transcriptional factor regulation within nasal epithelial cells exposed to *S. aureus* and SEB

The gene expression of the transcriptional factor FoxP3 was significantly (p≤0.05) downregulated in response to live and dead *S. aureus* infection (**Figure 5.13**). SEB did not further enhance the effect of *S. aureus*.
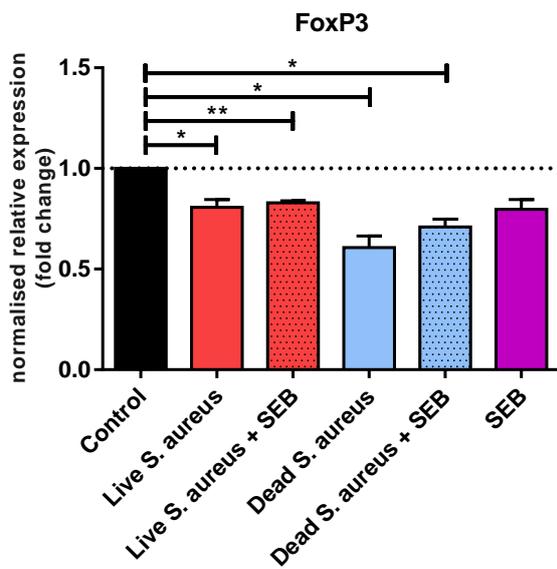
Figure 5.13 Nasal epithelial cell normalised gene expression of FoxP3 in SEB stimulated and/or *S. aureus* infected groups. RT-qPCR undertaken using a nasal epithelial cell line (RPMI 2650) exposed to SEB (10µg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*) and p≤0.01 (**).

5.6.4.3 Gene expression of toll like receptors in nasal epithelial cells exposed to *S. aureus* and SEB

There was significant ($p \leq 0.05$) downregulation of TLR-2 and TLR-4 gene expression when epithelial cells were exposed to live *S. aureus*, independent of the presence of SEB (**Figure 5.15**). Dead *S. aureus*, with and without the addition of SEB, together with SEB alone resulted in no statistically significant changes in TLR-2 and -4 gene expression ratios.
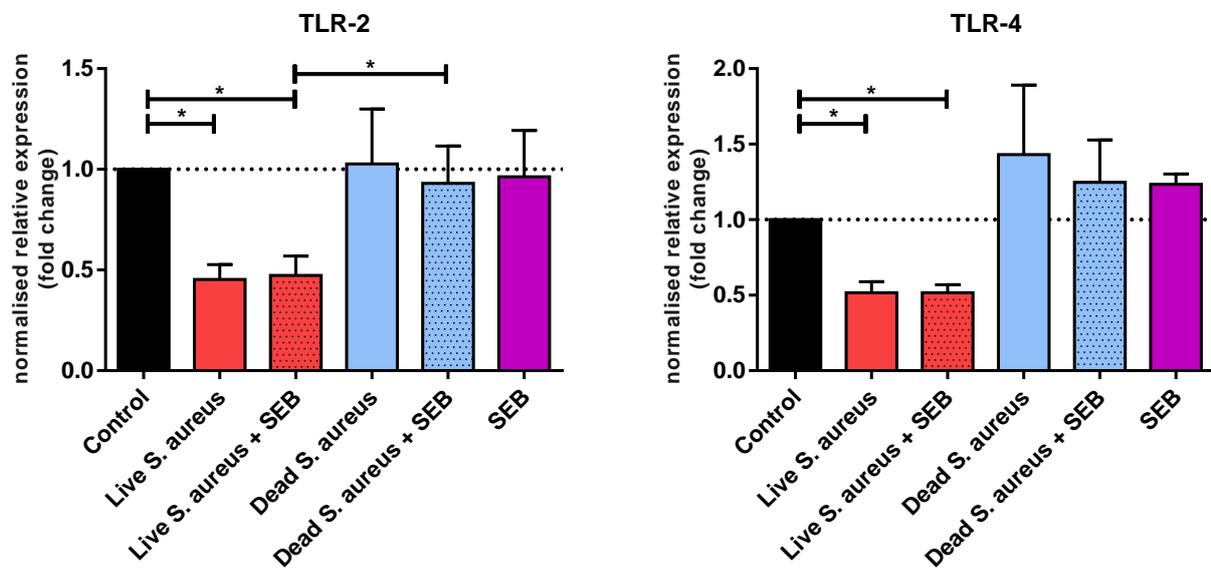
Figure 5.14 Nasal epithelial cell normalised gene expression of TLR-2 and TLR-4 in SEB stimulated and/or *S. aureus* infected groups. RT-qPCR undertaken using a nasal epithelial cell line (RPMI 2650) exposed to SEB (10μg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, $p \leq 0.05$ (*).

5.6.4.4 Regulation of matrix metalloproteases and defensins, in nasal epithelial cells exposed to

S. aureus and SEB

There was no significant change in the gene expression of MMP-7 or defensin 5 when exposed to live *S. aureus*, dead *S. aureus*, SEB or a combination of them all (**Figure 5.15**). However, there was a significant ($p \leq 0.01$) increase in MMP-7 protein concentration in the culture supernatant, when nasal epithelial cells were exposed to live *S. aureus* and live *S. aureus* plus SEB (**Figure 5.16**).
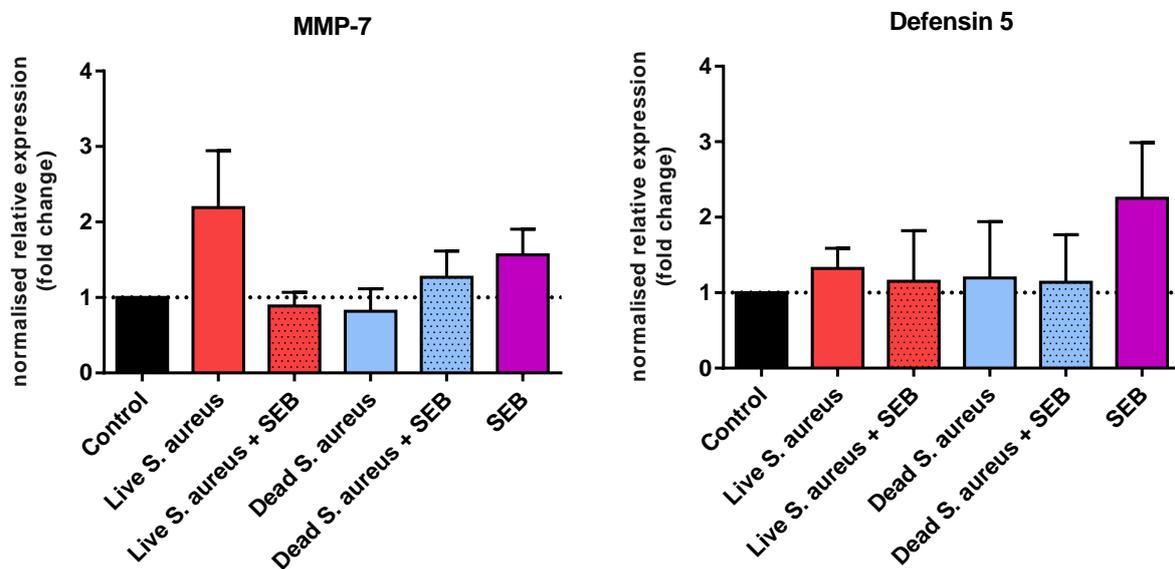
Figure 5.15 Nasal epithelial cell normalised gene expression of MMP-7 and defensin 5 in SEB stimulated and/or *S. aureus* infected groups. RT-qPCR undertaken using a nasal epithelial cell line (RPMI 2650) exposed to SEB (10µg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed.
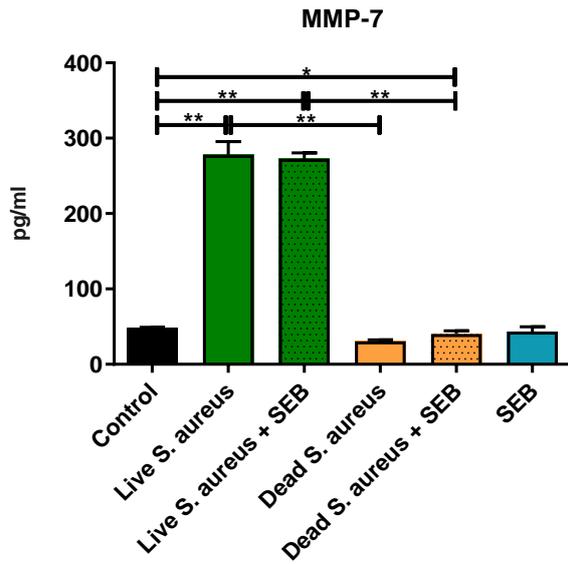
Figure 5.16 Nasal epithelial cell culture supernatant cytokine concentrations of MMP-7 in SEB stimulated and/or *S. aureus* infected groups. Luminex undertaken using an RPMI 2650 nasal epithelial cell line (2 million cells per ml) exposed to SEB (10μg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*) and p≤0.01 (**).

5.6.4.5 Overview of the epithelial immune response to *S. aureus* infection and SEB exposure in the RPMI-2650 cell culture model

Table 5.1 details a summary of the gene expression cytokine changes associated with live and dead *S. aureus* infection, and/or SEB exposure, of RPMI-2650 epithelial cells. **Table 5.2** details a summary of the supernatant cytokine concentrations of associated experiments.

Table 5.1 Summary table of nasal epithelial cytokine gene expression data and signifiance levels.

	Live *S. aureus*	Live *S. aureus* + SEB	Dead *S. aureus*	Dead *S. aureus* + SEB	SEB
TNFα	2.4	2.3	1.0	1.0	1.0
IL-5	4.6	0.3	0.7	0.3	0.3
TGF-β1	2.0	2.4	1.8	1.7	2.3
IL-17A	2.3	1.1	0.5	1.0	1.1
IL-1β	0.8	1.5	2.1	2.2	0.5
IFNγ	0.6	0.9	0.3	0.5	0.3
FoxP3	0.8	0.8	0.6	0.7	0.8
TLR2	0.5	0.5	1.0	0.9	1.0
TLR4	0.5	0.5	1.4	1.2	1.2
MMP-7	2.2	0.9	0.8	1.3	1.6
Defensin 5	1.3	1.2	1.2	1.1	2.3

Gene expression fold change ratios of figures 5.10, 5.13, 5.14 and 5.15. Green boxes represent significantly upregulated gene expression compared to controls (p≤0.05), with red boxes denoting significantly downregulated gene expression compared to controls (p≤0.05). Paired t-tests were performed.

Table 5.2 Summary table of nasal epithelial cytokine data and signifiance levels.

	Control	Live *S. aureus*	Live *S. aureus* + SEB	Dead *S. aureus*	Dead *S. aureus* + SEB	SEB
IFNγ	19.4	65.5	69.3	17.0	17.9	15.0
IL-17A	0.7	11.3	10.9	0.6	0.5	0.6
TNFα	8.6	23.3	22.1	8.0	7.6	7.3
IL-1β	0	9.3	10.2	0	0	0
IL-6	0.1	1.6	1.6	0.2	0.3	0.1
MMP-7	47.8	277.8	272.3	30.4	40.2	43.4

Table of collated Luminex culture supernatant cytokine concentrations (pg/ml) of figures 5.12 and 5.16. Green boxes represent significantly increased cytokine concentrations compared to controls ($p \leq 0.05$). Paired t-tests were performed.

5.7 Discussion

***S. aureus* uptake, survival and release from nasal epithelial cells**

The nasal epithelial line (RPMI-2650) internalises CRS specific isolates of *S. aureus*, with the proportion of infected epithelial cells increasing over time. Furthermore, the internalised *S. aureus* remain viable and capable of forming new colonies. This translates to survival of *S. aureus* and its ability to propagate infection. When examining CFUs of internalised *S. aureus* within epithelial cells, there was a significant drop in viable intracellular *S. aureus* between 2 and 4 hours, as well as 6 and 24 hours. The internalisation of *S. aureus* into the epithelial cells was evident from 2 hours, and this drop seen at 4 hours may represent an attempt to clear intracellular *S. aureus*. However, the number of *S. aureus* then appeared to rise, possibly due to replication of *S. aureus* within the epithelial cells as well as continued uptake of extracellular *S. aureus*. Once extracellular *S. aureus* had been eradicated at 6 hours, not only was further *S. aureus* found within the culture supernatant at 24 hours, indicating extracellular *S. aureus* seeding, but there was also a drop in intracellular *S. aureus* colonies. This could be secondary to cell lysis, resulting in the seeding of extracellular *S. aureus* and propagation of extracellular infection. There was a reduction in the volume of epithelial cells following infection, which was significant at 6 hours. This may represent a degree of cell shrinkage prior to apoptosis, and may further enhance the ability of *S. aureus* to invade the subepithelial space.

The addition of SEB resulted in a significant increase in viable intracellular *S. aureus* at 24 hours. This could be related to the function of *S. aureus* enterotoxins as bacterial virulence factors, with elevated levels allowing more effective entry into the epithelial cells (191). When examining the effect of experimental therapies to target SEB, such as mAb 20B1, *S. aureus* infected skin in mice treated with this therapy had reduced invasion depth, improved *S. aureus* clearance and reduced levels of inflammation compared to controls (191). This highlights the importance of SEB as a virulence factor for *S. aureus* infection, and its significance in promoting internalisation within epithelial cells. The addition of epithelial cells to *S. aureus* culture had no effect on its growth in supernatant, suggesting the inability of these cells to combat bacterial infection independently.

Live S. aureus promotes the development of a chronic inflammatory state

Results outline the ability of live *S. aureus* to drive a significant pro-inflammatory response, both on the gene and protein level. This inflammatory response was skewed towards the Th2 pathway, involving upregulation of IL-5 gene expression and IL-6 release into the culture supernatant. These responses to *S. aureus* are likely to contribute towards the development of chronic inflammation, remodelling and localised tissue oedema, which could drive the formation and growth of nasal polyps. The addition of SEB to Live *S. aureus* did not seem to significantly enhance the inflammatory response of epithelial cells, indicating that SEB may be acting on other cell types to produce the inflammatory effects outlined previously in nasal polyp explants. However, SEB did downregulate the gene expression of one studied cytokine, IL-1β. This may be relevant given earlier results in chapters 3 and 4, outlining the significant downregulation of IL-1β in CRSwNP tissues and the further significant downregulation in SEB stimulated nasal polyps. These results support an important role of IL-1β in the development of CRS, through disordered bacterial clearance mechanisms. Results presented highlight the importance of live bacterial infection in driving chronic inflammation, and that this is skewed towards the Th2 pathway, as previously seen with SEB in chapter 3. Thus, the interaction and internalisation of *S. aureus* is likely to be important in the development of chronic inflammation, re-emphasising the importance of strategies aimed at eradicating bacteria in CRS.

Unusually, *S. aureus* infection was seen to significantly downregulate the gene expression of TLR-2 and -4. TLRs are a family of pattern recognition receptors that detect pathogenic ligands on the surface of bacteria, fungi and viruses and play an important role in innate immunity, signalling the activation and recruitment of other immune cells to the site of infection. Lack of TLR-2 responses increase the susceptibility of the host to *S. aureus* infection (192). Therefore, the downregulation of TLRs seems counterintuitive in combating infection, unless this is a *S. aureus* mediated response to stop the recruitment of immune cells that would otherwise clear ongoing infection. This may be yet another example of a disordered bacterial response, increasing the susceptibility of mucosal tissues to bacterial invasion and the development of chronic inflammation.

Overall dead *S. aureus* stimulation of epithelial cells did not result in a significant pro-inflammatory response, with or without the addition of SEB. However, in the case of FoxP3, the transcriptional factor for Treg cell development, live and dead *S. aureus* exposure significantly downregulated FoxP3 gene expression. This is interesting, given the earlier findings in chapter 3 which reported downregulated FoxP3 gene expression in the non-polypoidal mucosa of CRSwNP patients. *S. aureus* could be facilitating the development of a disordered T-cell environment, favouring Th2-mediated chronic inflammation, through the dysregulation of Treg cells. This could potentially promote the transition between non-polypoidal to polypoidal mucosa, with frank polyp development down-stream.

5.8 Conclusions

Evidence has been presented of the ability of *S. aureus* to internalise within nasal epithelial cells whilst maintaining its viability and ability to repopulate eradicated extracellular colonies. Furthermore, there is evidence of disordered bacterial clearance and a polarised T cell response. These changes contribute towards the development of a chronic inflammatory reaction, which further promotes bacterial survival and propagation of infection.

6 Characterisation of interactions between *S. aureus* and mast cells using a cell culture model

6.1 Introduction

Mast cells are found in connective (e.g. skin) and mucosal tissues throughout the body, most commonly those of the respiratory, gastrointestinal and urinary tracts. Their role in the development of allergic reactions, mediated through the high-affinity IgE receptor (FCεR1), are well documented. Mast cells are classically activated through the binding of an allergen to a pre-IgE sensitised cell (193). This results in the degranulation, releasing pre-formed mediators and cytokines, including histamine, TNFα, IL-8 and proteases (β-hexosaminidase). *De-novo* production of lipid mediators (leukotrienes and prostaglandins), cytokines, chemokines, and growth factors also occur in response to FCεR1-dependent activation (194). This results in localised tissue oedema and the development of an acute inflammatory response. Non-IgE mediated activation occurs in response to TLR, pattern recognition receptor, cytokine, neuropeptide, histamine and complement binding, amongst others (195). Mast cells were initially thought to be only associated with allergic diseases, however the discovery of bacteria harbouring mast cells in various disease states has highlighted their possible role in disease pathogenesis.

Mast cells have now been found to be of importance in a number of bacterial infections, recently highlighted through the development of a mast cell deficient mice model. Experiments reveal that mice deficient of mast cells are unable to clear *Klebsiella pneumonia* infection, which is subsequently reversed through reconstitution of mast cells into the peritoneal cavity (196). Furthermore, mast cell deficient mice infected with *Mycoplasma pulmonis* and *Listeria monocytogenes* have poorer survival outcomes compared to infected wild-type mice (197, 198). A shared mechanistic pathway is thought to be due to a lack of TNFα release from mast cells, and thus reduced neutrophil recruitment to areas of active infection (198). Mast cell TNFα release has been shown to be effective in recruiting not only neutrophils but also dendritic cells, shown specifically in the response to *S. aureus*-derived peptidoglycan and *E. coli* infection (199, 200). *In vitro* experiments using primary mast cells and mast cell lines have shown mast cells to phagocytoses a variety of bacterial strains including *E. coli*, *Klebsiella pneumonia*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Mycobacteria tuberculosis* and *S. aureus*

(194). In general, incubation of mast cells with bacteria causes activation and release of pre-formed mediators, however not all bacteria cause this effect, therefore the response to bacterial infection seems specific (194). For example, pathogenic strains of *E. coli*, *Streptococcus pneumonia*, *Staphylococcus pneumonia*, *Pseudomonas aeruginosa*, *Mycoplasma pneumonia*, and *Mycobacterium tuberculosis* result in the release of preformed and de novo synthesized mediators, whereas incubation with commensal non-pathogenic strains such as *Lactobacillus*, and *Bifidobacteria* does not (194). Recently *S. aureus* harbouring mast cells have been found in nasal polyps, raising the possibility that they may be important in the pathogenesis of CRS or development and growth of nasal polyps (25).

Research conducted to date has shown that mast cells lines (HMC-1) and primary human mast cells internalise laboratory strains of *S. aureus*, and that this intracellular *S. aureus* remains viable (143). Mast cells have also been found to undergo degranulation in response to *S. aureus* infection, secreting pre-formed mediators and cytokines including IL-6 and TNFα, which are thought to aid in the recruitment of further defensive cell types (194). Mast cells have also been found to have some anti-bacterial activity against *S. aureus*, through the secretion of extracellular traps, although it seems that they are eventually overwhelmed, compromising their ability to deal with further ongoing infection (143). Despite this, little work has been carried out examining pathogenic strains of *S. aureus* obtained from CRS patients. It is unclear exactly how CRS specific strains of *S. aureus* interact with mast cells and what pro-inflammatory effects they produce. This chapter aims to characterise these interactions and clarify possible mechanisms, responsible for *S. aureus* internalisation and survival in mast cells and the resultant development of a chronic inflammatory state.

6.2   Hypothesis

The process of *S. aureus* internalisation within mast cells, and then survival by evasion of immune detection, provides a mechanism for seeding bacteria into the extracellular compartment. The end result is a chronic inflammatory state which further promotes the survival of bacteria.

6.3   Aims

To characterise interactions between mast cells and *S. aureus* using a cell culture model.

6.4   Objectives

1. To determine whether mast cells are capable of internalising CRS-related *S. aureus* strains.

2. To investigate the viability of intracellular *S. aureus* and its ability to propagate ongoing infection.

3. To characterise the pro-inflammatory response of *S. aureus* infected mast cells.

4. To investigate the effects of *S. aureus* infection on mast cell activation through IgE dependent and

    independent mechanisms.

6.5 Methods

For detailed methods please refer to chapter 2, pages 41-65. For experimental methods undertaken in sections 6.6.5.1, 6.6.5.2 and 6.6.5.4, please see **appendix IV**. These experiments were undertaken as a joint collaboration with Rana Abadalkareem (PhD student), under the supervision of Dr Andrew Walls.

6.6 Results

6.6.1 Confocal laser scanning microscopy

A *S. aureus* co-infection model using the HMC-1 mast cell line revealed intracellular uptake of *S. aureus* into mast cells (**Figure 6.1**). At 2 hours of co-infection *S. aureus* could be seen binding to the surface of mast cells (**Figure 6.2B**). At 4 hours, surface bound and intracellular *S. aureus* could be identified (**Figure 6.2C**). From 6 to 24 hours the number of *S. aureus* present within the mast cells continued to increase (**Figure 6.2D-F**). Intracellular *S. aureus* was further confirmed with the use of an intracellular cell tracker (**Figure 6.3**), highlighting the location within the cell cytosol. LAD2 cells, similarly internalised *S. aureus* (**Figure 6.4**). HMC-1 mast cell size increased steadily throughout the measured time points (**Figure 6.5**).
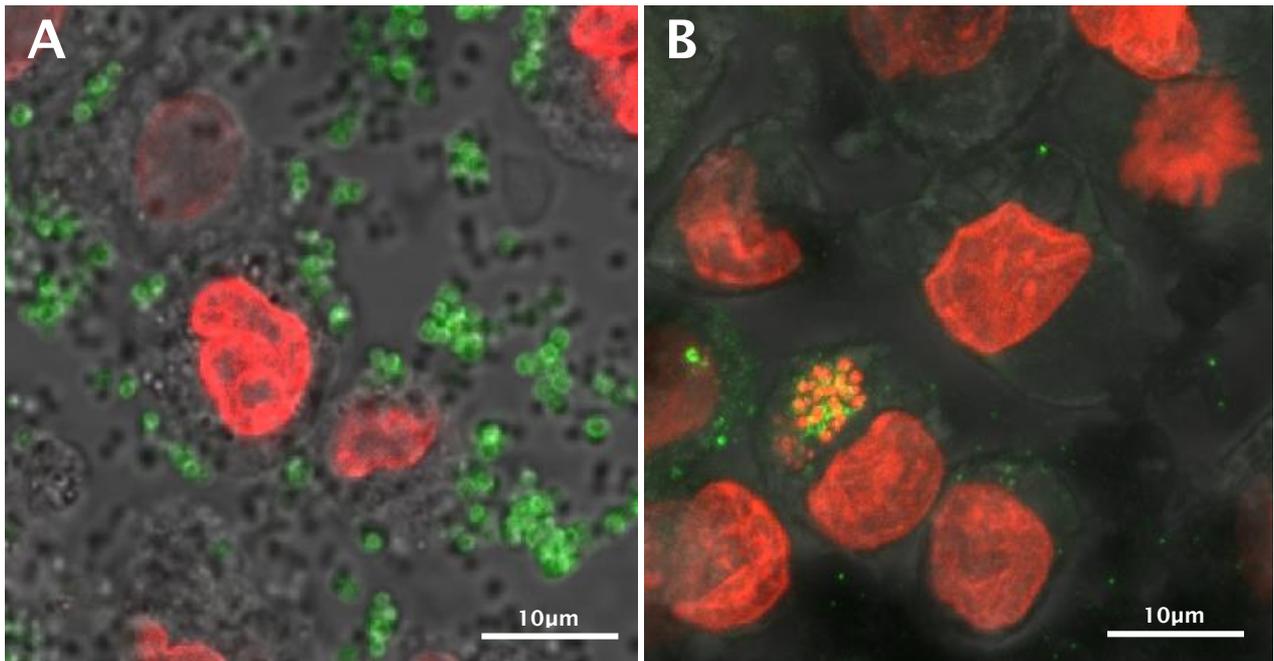
Figure 6.1 Binding and subsequent uptake of intracellular *S. aureus* into HMC-1 mast cells. Representative CLSM images of mast cells (HMC-1) co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Staining undertaken with an anti-*S. aureus* antibody and secondary Alexa 568 (green), and DAPI (red). A) Mast cells with surface bound and intracellular *S. aureus* (green). B) Mast cells with intracellular *S. aureus* only (smaller green/red circles). Extracellular *S. aureus* was eradicated with 20µg/ml Lysostaphin.
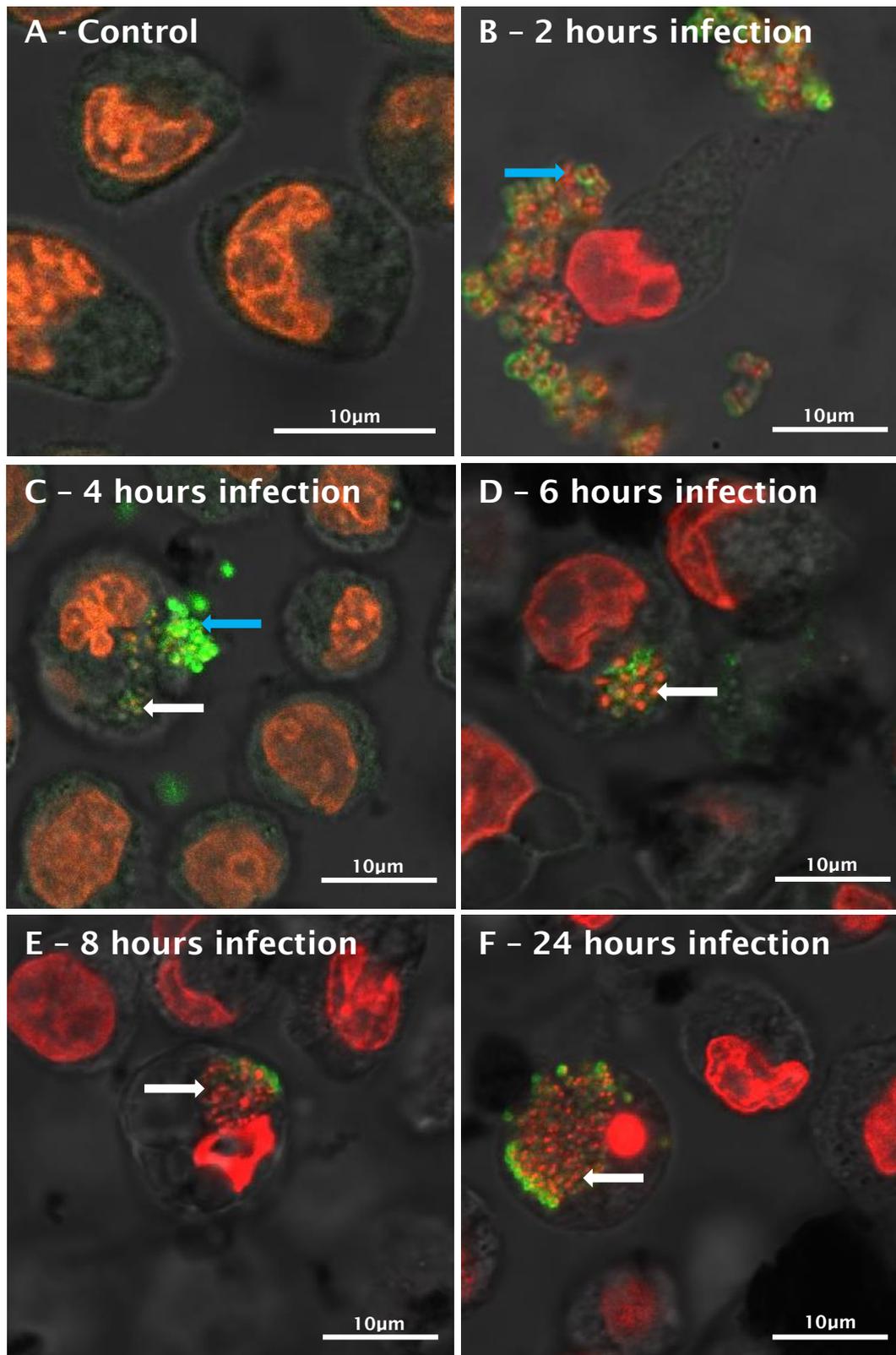
Figure 6.2 Internalisation of *S. aureus* into HMC-1 mast cells over 24 hours. Representative CLSM images of mast cells (HMC-1) co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 24 hours. Staining undertaken with an anti-*S. aureus* antibody and secondary Alexa 568 (green), and DAPI (red). Images demonstrate, A) no *S. aureus*, B) surface binding *S. aureus* (smaller green/red circles), C) mast cells internalising *S. aureus* (surface bound - blue arrow, intracellular - white arrow), C-E) Increasing number of intracellular *S. aureus* (white arrows) within mast cells. Extracellular *S. aureus* was eradicated with 20µg/ml Lysostaphin.
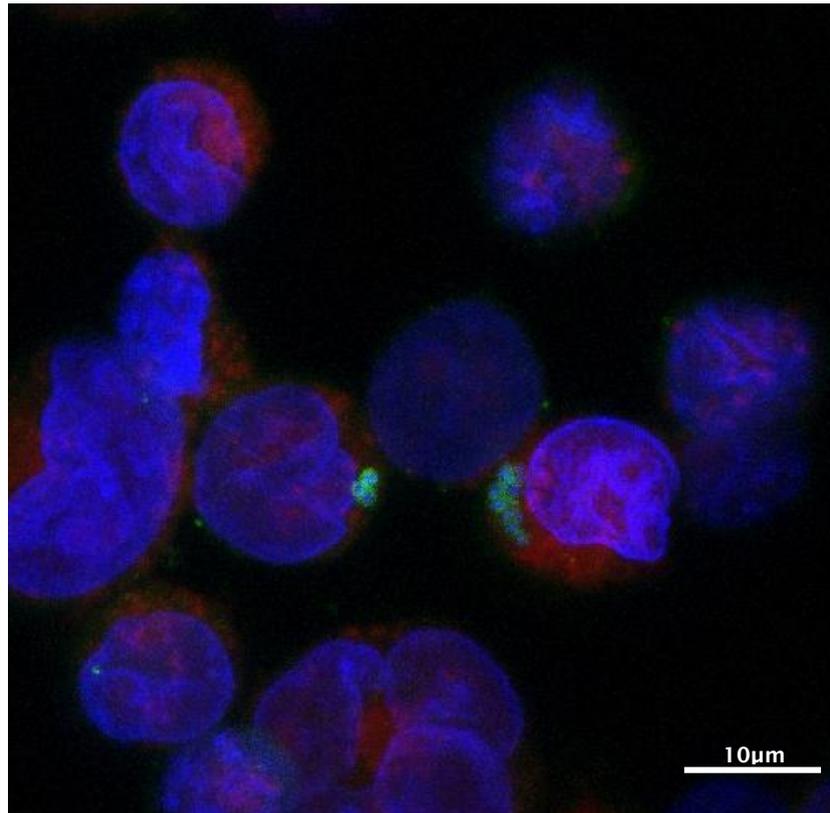
Figure 6.3 I**ntracellular *S. aureus* in HMC-1 mast cells, visualised using a cell tracker**. Representative CLSM images of mast cells (HMC-1) co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Staining undertaken with an anti-*S. aureus* antibody and secondary Alexa 568 (green), DAPI (blue) and deep red fluorescent CellTracker™ (red). Extracellular and bound *S. aureus* eradicated with 20µg/ml Lysostaphin.
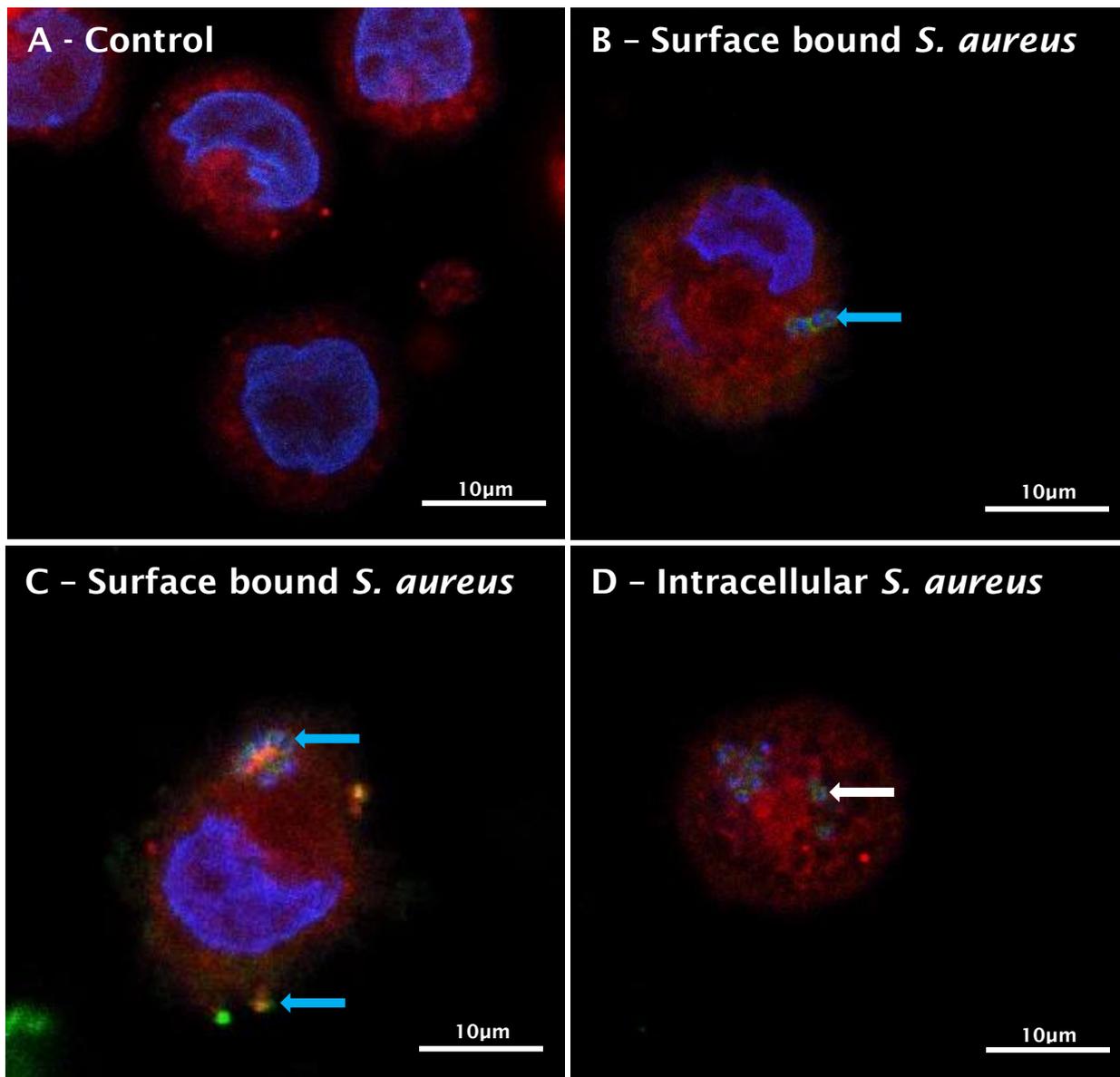
Figure 6.4 Internalisation of *S. aureus* into LAD2 mast cells. Representative CLSM images of mast cells (LAD2) co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Staining undertaken with an anti-*S. aureus* antibody and secondary Alexa 568, combined with DAPI (blue) and Deep Red fluorescent CellTracker™ (red). Images demonstrate, A) no *S. aureus*, B,C) surface binding *S. aureus* (blue arrow), D) Intracellular *S. aureus* (white arrow). Extracellular and bound *S. aureus* eradicated following 20µg/ml Lysostaphin.
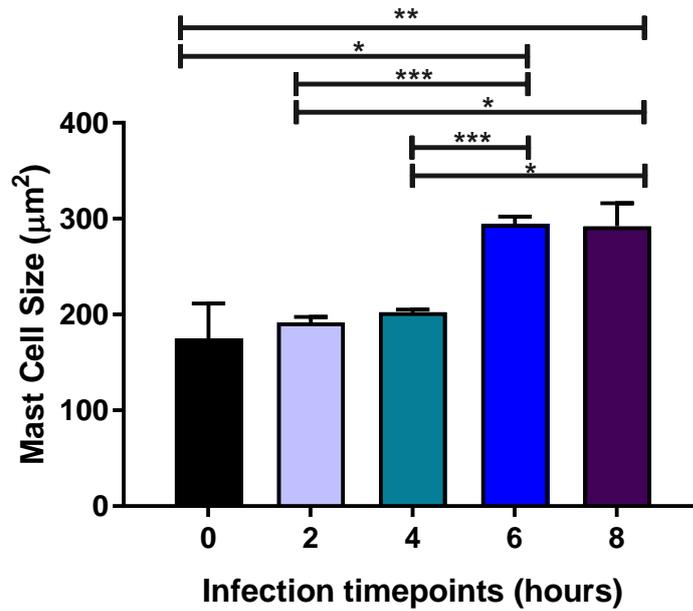
Figure 6.5 HMC-1 mast cell size following _S. aureus_ co-infection. HMC-1 mast cells were co-cultured with a CRS _S. aureus_ isolate (MOI 1:1) over 8 hours. Size calculated using the largest cross section of multiple sequential CLSM images, and calculated using Image J (n=20). Three experimental repeats displayed, bars represent the mean and error bars represent SEM. Statistical analysis was performed using paired t-tests, p≤0.05 (*), p≤0.01 (**) and p≤0.001 (***).

6.6.2 Viability of intracellular *Staphylococcus aureus*

Following *S. aureus* co-culture with HMC-1 and LAD2 cell lines, there was uptake and survival of viable intracellular *S. aureus*, capable of forming new colonies (**Figure 6.6**). The HMC-1 cell line was associated with significantly (p≤0.01) higher numbers of intracellular *S. aureus* compared to the LAD2 cell line. The extracellular growth of *S. aureus* was significantly (p≤0.01) higher in the LAD2 co-culture compared to the HMC-1 co-culture.
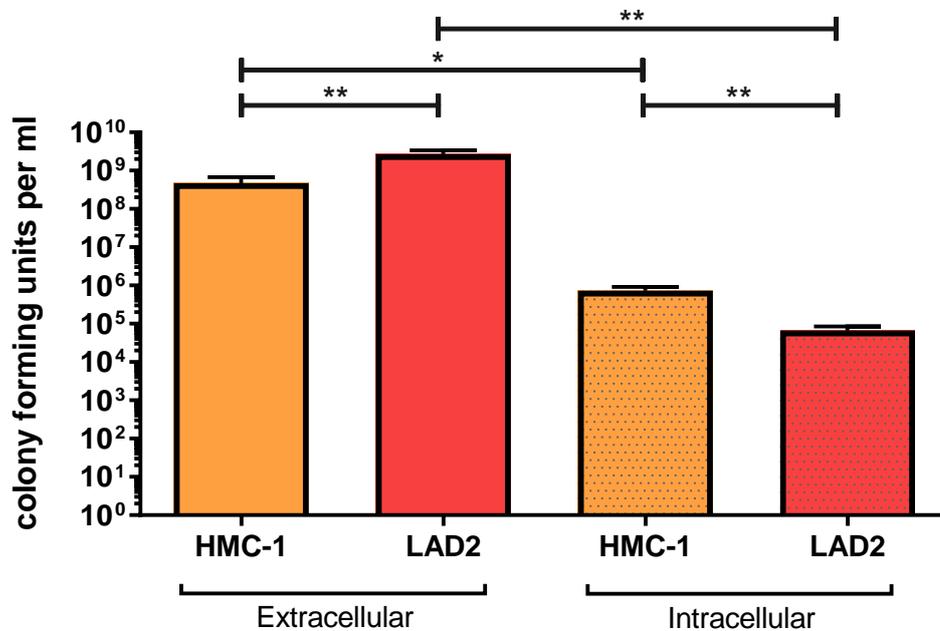
Figure 6.6 Intracellular and extracellular *S. aureus* enumerated from HMC-1 and LAD2 cells. HMC-1 and LAD2 mast cells (2 million) were co-cultured with a CRS *S. aureus* isolate (MOI 20:1) over 4 hours. Extracellular CFUs were enumerated on CBA plates, with intracellular CFUs enumerated on CBA plates following extracellular *S. aureus* eradication (20μg/ml Lysostaphin). Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Statistical analysis was performed using t-tests, p≤0.05 (*) and p≤0.01 (**).

There was a steady increase in the number of viable intracellular *S. aureus* enumerated from HMC-1 mast cells over 24 hours (**Figure 6.7**). There was also a gradual increase in the number of viable extracellular *S. aureus* in the HMC-1 co-cultures (**Figure 6.8**). The presence of mast cells or SEB did not alter the rate of extracellular *S. aureus* growth. Following extracellular *S. aureus* eradication at 6 hours, there was re-infection and growth of *S. aureus* in the culture supernatant, and associated significant (p≤0.001) increase in the number of viable intracellular *S. aureus* within HMC-1 mast cells (**Figure 6.7 and 6.8**).
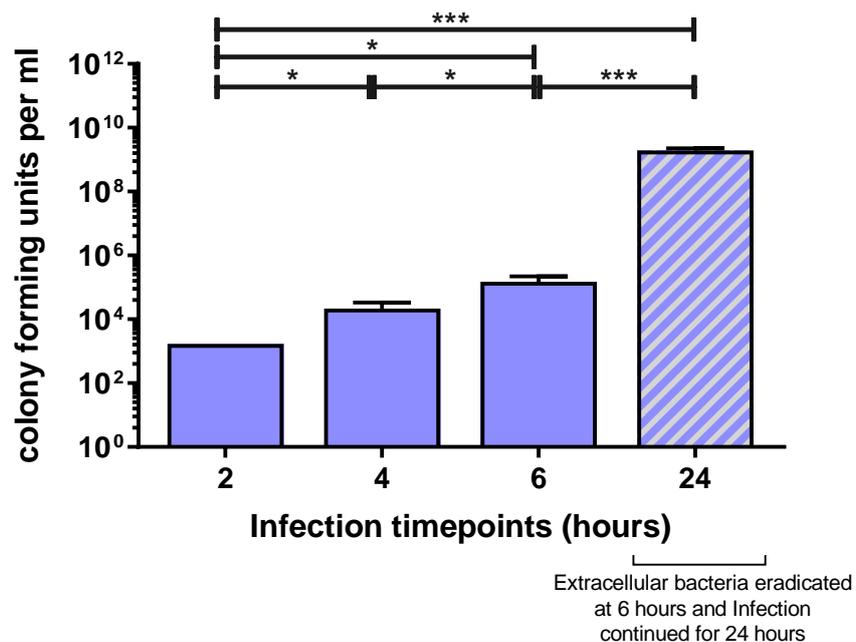
Figure 6.7 Intracellular *S. aureus* CFU time-course, enumerated from mast cell co-cultures. HMC-1 mast cells (2 million) were co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 24 hours. Intracellular CFUs were enumerated on CBA plates following eradication of extracellular *S. aureus* (20µg/ml Lysostaphin). Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Statistical analysis was performed using paired t-tests, p≤0.05 (*) and p≤0.001 (***).
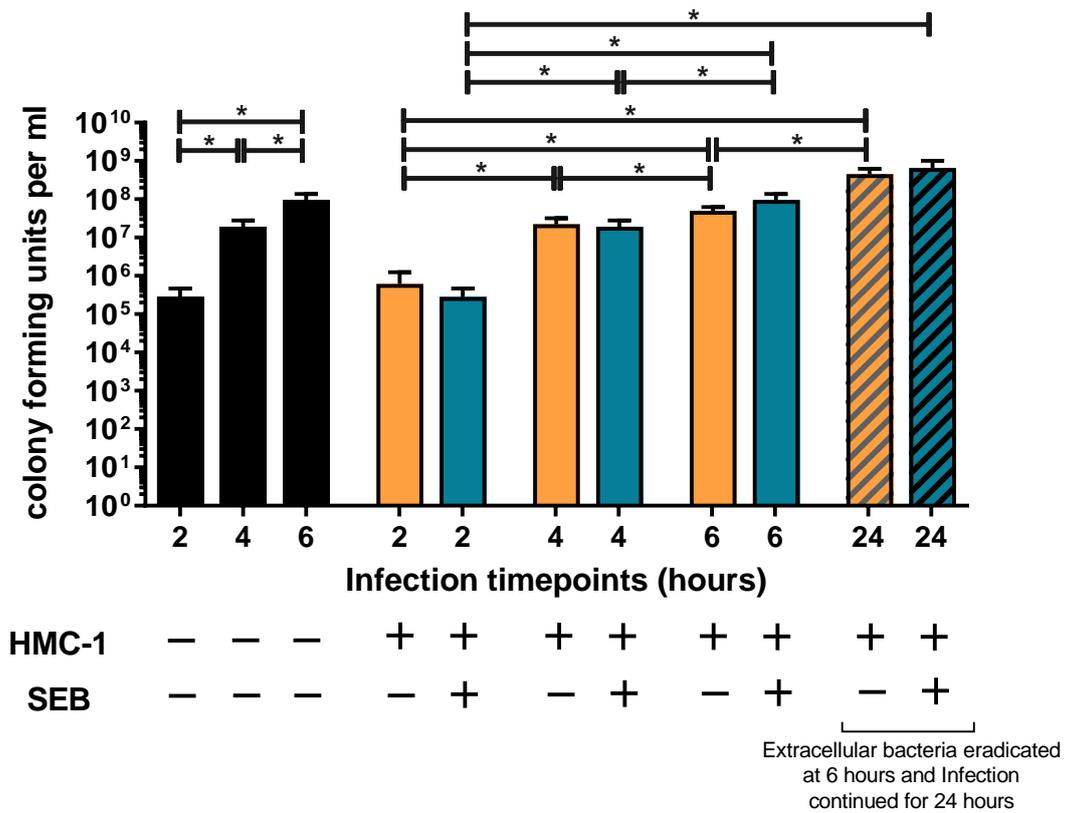
Figure 6.8 Extracellular *S. aureus* CFU time-course, with and without mast cells and SEB. HMC-1 mast cells (2 million cells) were co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 24 hours. Extracellular CFUs were enumerated on CBA plates. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Statistical analysis was performed using paired (time course within HMC-1 groups) and unpaired (between groups with and without the HMC-1 cells) t-tests, p≤0.05(*).

There was a significant (p≤0.01) increase in the number of viable intracellular *S. aureus* within HMC-1

mast cells when stimulated with SEB over 24 hours (**Figure 6.9**).
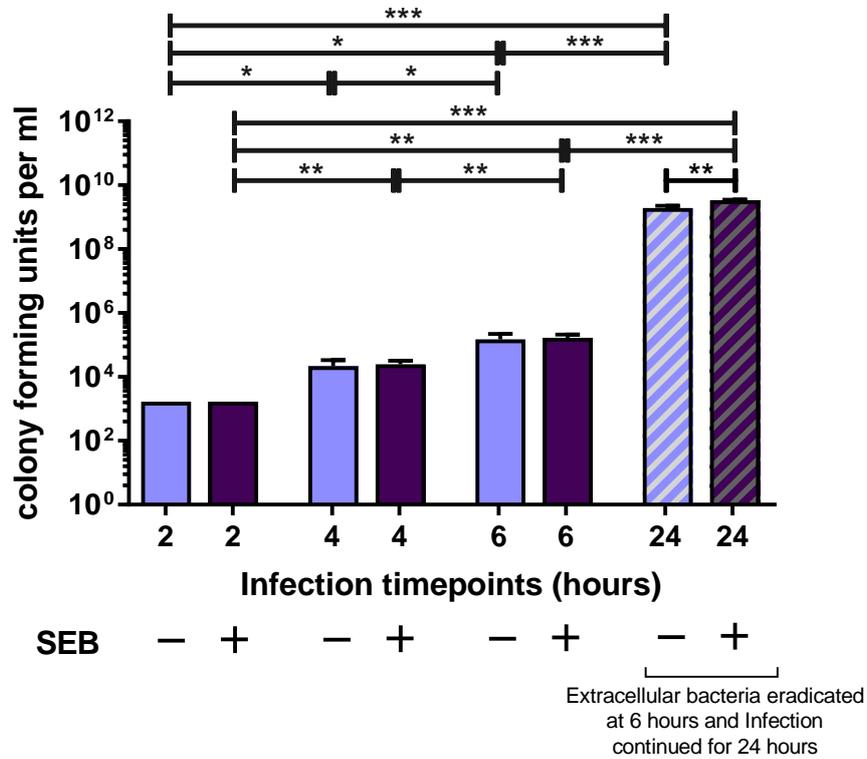
Figure 6.9 Intracellular *S. aureus* CFUs enumerated from mast cell co-cultures, with and without the addition of SEB. HMC-1 mast cells (2 million) were co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 24 hours, with and without the addition of SEB (10µg/ml). Intracellular CFUs were enumerated on CBA plates following eradication of extracellular *S. aureus* (20µg/ml Lysostaphin). Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Statistical analysis was performed using paired t-tests, p≤0.05 (*), p≤0.01 (**) and p≤0.001 (***).

When mast cells were infected with *S. aureus*, followed by eradication of extracellular colonies, and the infection continued in the presence of antibiotic supplemented media (20µg/ml Lysostaphin), there was no associated increase in viable intracellular *S. aureus* (**Figure 6.10**).
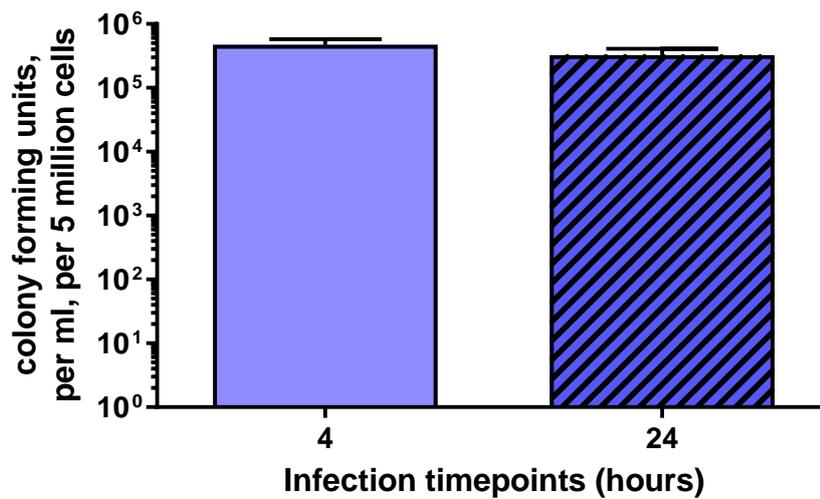
Figure 6.10 Intracellular *S. aureus* proliferation experiment. HMC-1 mast cells (5 million) were co-cultured with a CRS *S. aureus* isolate (MOI 1:1), over 4 hours. Following which, extracellular *S. aureus* were eradicated and the cells cultured in Lysostaphin (20µg/ml) enriched media for a further 18 hours, eradicating any subsequent *S. aureus* release. Intracellular *S. aureus* CFUs were enumerated on CBA plates following eradication of extracellular *S. aureus* (20µg/ml Lysostaphin). Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Statistical analysis was performed using paired t-tests.

6.6.3 The immune response of *S. aureus* infected mast cells

6.6.3.1 Cytokine response of mast cells to *S. aureus* and SEB

HMC-1 mast cells

Exposure of HMC-1 mast cells to live *S. aureus* resulted in a significant (p≤0.05) downregulation of IFNγ, IL-5, IL-1β and IL-6 (**Figure 6.11**). The addition of dead *S. aureus* significantly downregulated the gene expression of IFNγ and TGF-β1 (combined with SEB). SEB did not seem to significantly alter the gene expression of cytokines in live and dead *S. aureus* groups. SEB alone did result in the significant downregulation of one cytokine measured, IL-1β. There was no significant change in IL-17A or TNFα gene expression following *S. aureus* or SEB exposure of HMC-1 mast cells (**Figure 6.11**).

Over 2 to 6 hours of HMC-1 and live *S. aureus* co-culture, there was a progressive downregulation of TNFα, IL-1β and IFNγ gene expression (**Figure 6.12**). These results were reproducible, with 3 experimental repeats undertaken, and were in line with earlier observations (**Figure 6.11**).

Measurement of cytokine protein content in HMC-1 culture supernatants showed statistically significant elevation of secreted IFNγ, IL-17A, TNFα, IL-1β and IL-6 in response to live *S. aureus* (**Figure 6.13**). The addition of SEB to live *S. aureus* did not significantly alter the level of secreted cytokine. The addition of dead *S. aureus* and/or SEB did not result in a significant change in the levels of cytokines measured in the culture supernatant. Over 2 to 6 hours of HMC-1 and *S. aureus* co-culture, there was a steady and progressive increase in the measured concentration of IL-17A, which peaked at 24 hours (**Figure 6.14**).
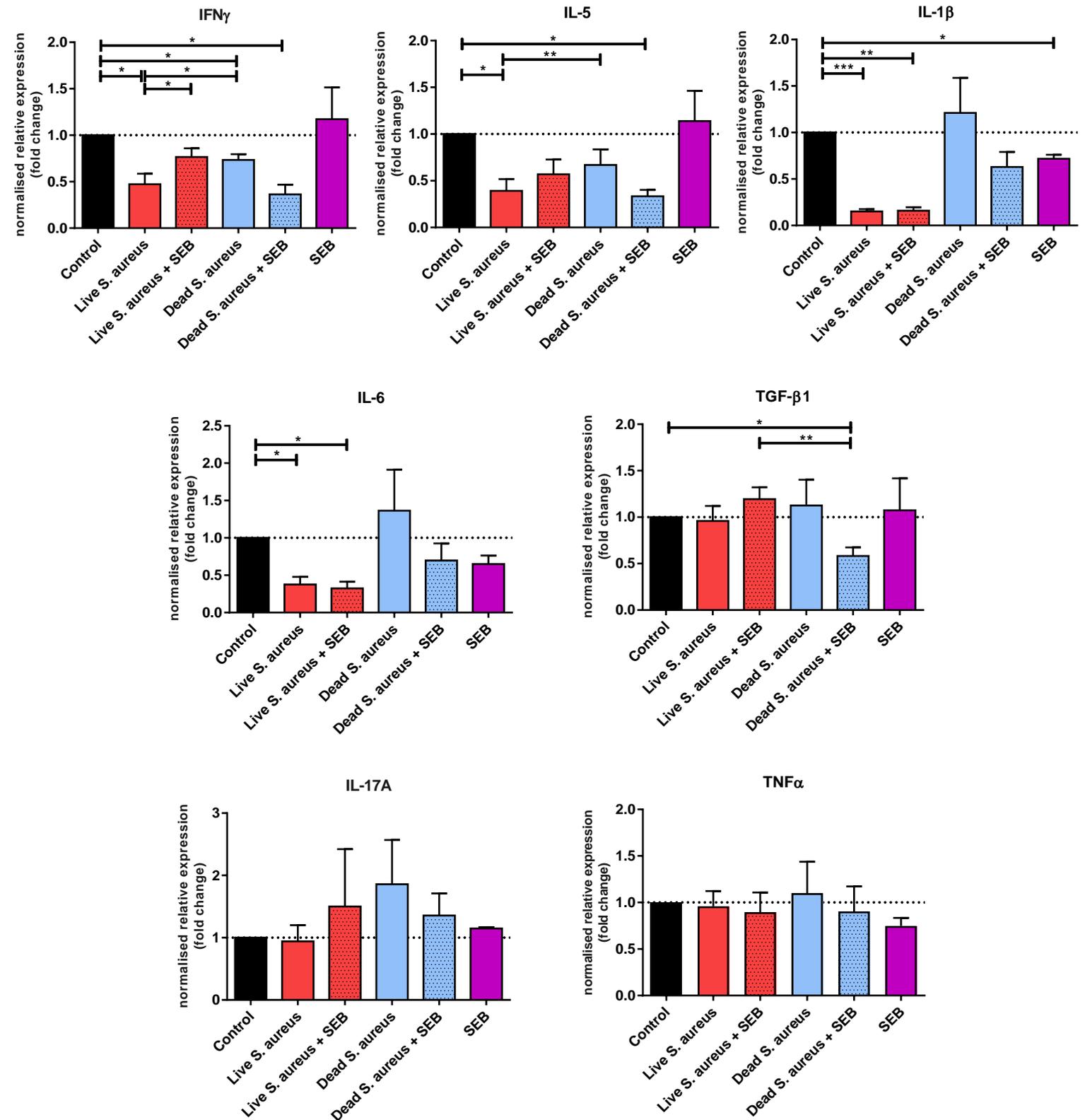
Figure 6.11 HMC-1 mast cell normalised gene expression of IFNγ, IL-5, IL-1β, IL-6, TGF-β1, IL-17A and TNFα, in SEB stimulated and/or *S. aureus* infected groups. RT-qPCR undertaken using a mast cell line (HMC-1) exposed to SEB (10µg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*), p≤0.01 (**) and p≤0.001 (***).
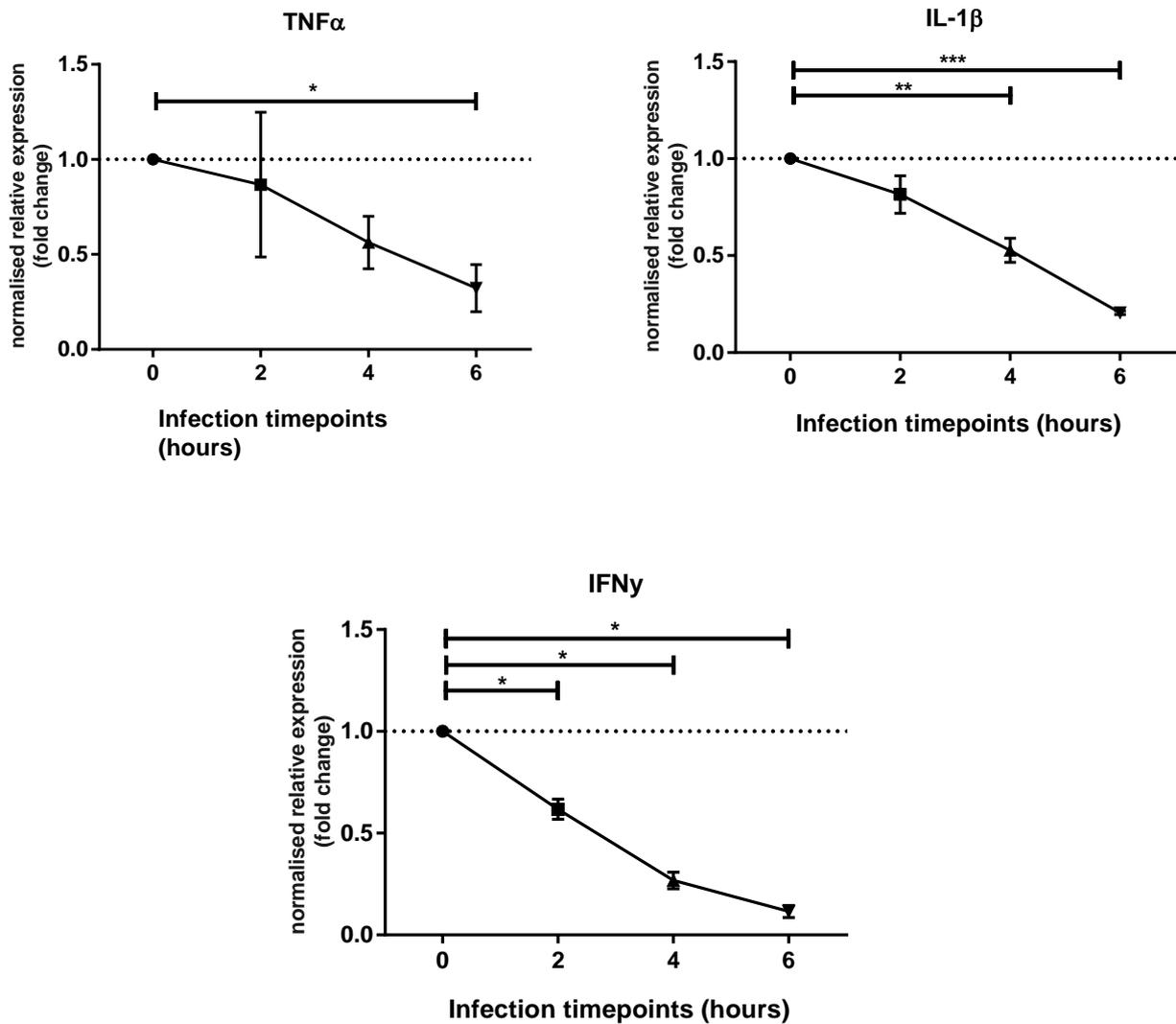
Figure 6.12 HMC-1 mast cell normalised gene expression time-course of TNFα, IL-1β and IFNγ following *S. aureus* infection. Mast cells (HMC-1) were co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 6 hours, with RT-qPCR undertaken. Three experimental repeats are displayed, with bars representing the mean and error bars representing +/-SEM. Paired t-tests were performed, p≤0.05 (*), p≤0.01 (**) and p≤0.001 (***).
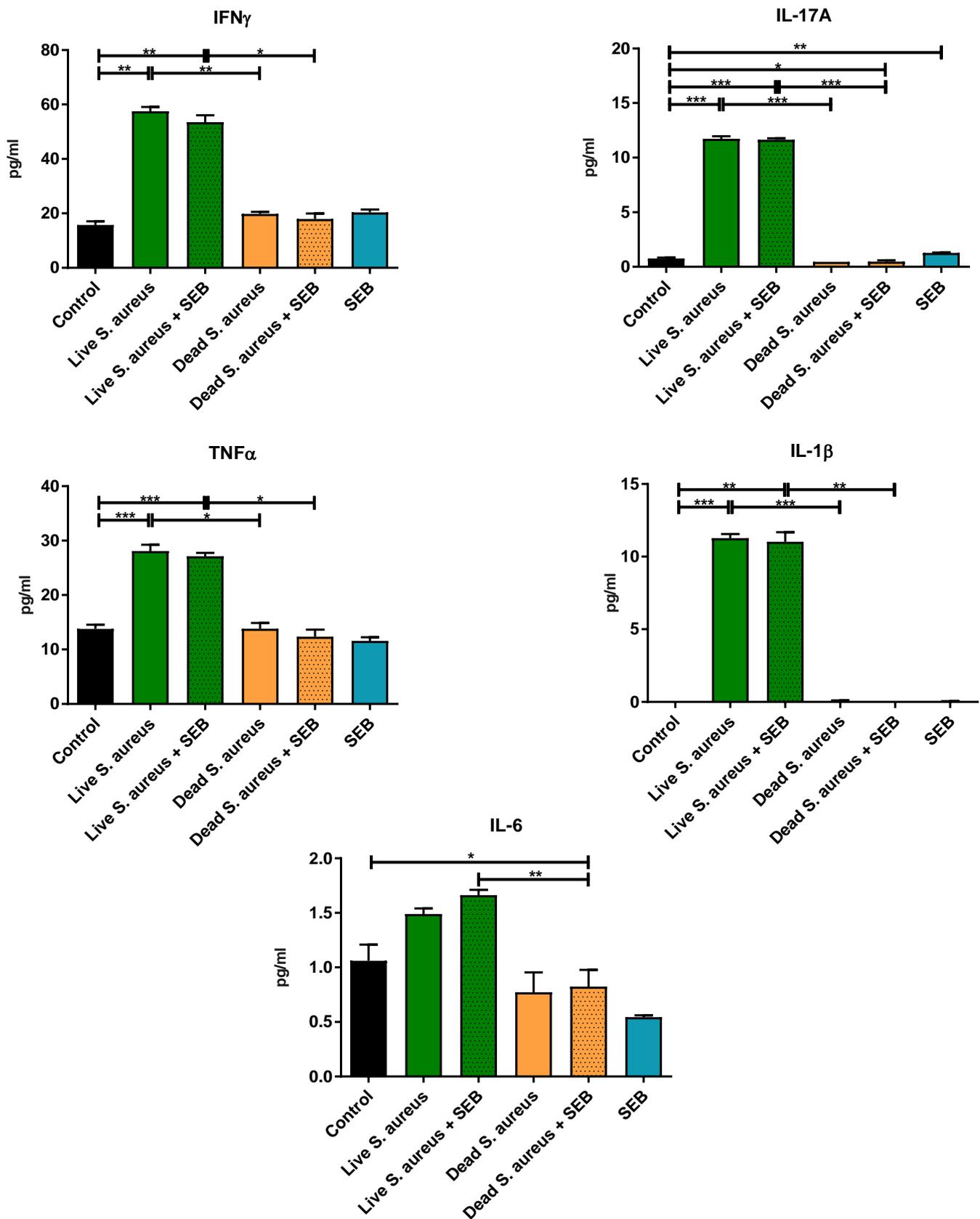
Figure 6.13 HMC-1 mast cell culture supernatant cytokine concentrations of IFNγ, IL-17A TNFα, IL-1β and IL-6, in SEB stimulated and/or *S. aureus* infected groups. Luminex undertaken using a HMC-1 mast cell line (5 million cells per ml) exposed to SEB (10μg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*), p≤0.01 (**) and p≤0.001 (***).
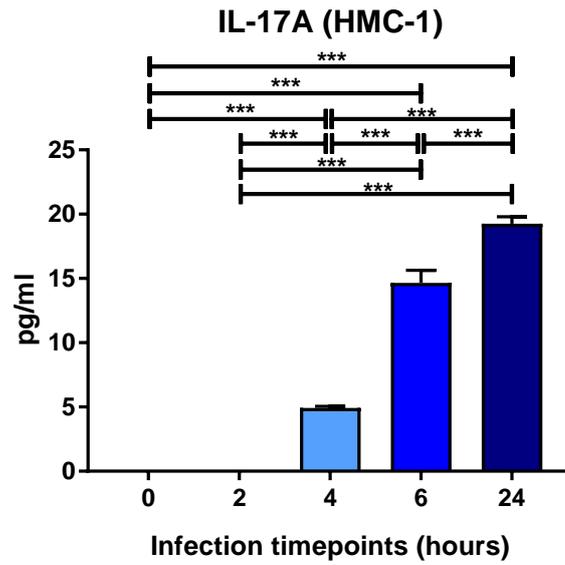
Figure 6.14 HMC-1 mast cell supernatant cytokine concentration time-course of IL-17A following *S. aureus* infection. HMC-1 mast cells (5 million cells per ml) were co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 24 hours, with Luminex undertaken on culture supernatants. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*), p≤0.01 (**) and p≤0.001 (***).

LAD2 mast cells

Exposure of LAD2 mast cells to live *S. aureus* resulted in a significant (p≤0.05) upregulation of IL-5 and TNFα gene expression (**Figure 6.15**). The addition of dead *S. aureus* and/or SEB was not associated with significant changes in gene expression. There was no significant change in gene expression for IL17A, IFNγ, TFG-β1 or IL-1β when LAD2 cells were exposed to *S. aureus* and/or SEB.

Measurement of cytokine protein content in LAD2 culture supernatants showed significant (p≤0.05) elevation of secreted TNFα in response to live *S. aureus* infection over 4 hours (**Figure 6.16**). Peak concentrations of supernatant TNFα and IFNγ were reached following 6 hours of *S. aureus* co-infection, with IL-17A peaking maximally at 24 hours (**Figure 6.17**). IL-6 and IL-1β were not detected within the culture supernatants of LAD2 co-cultures.
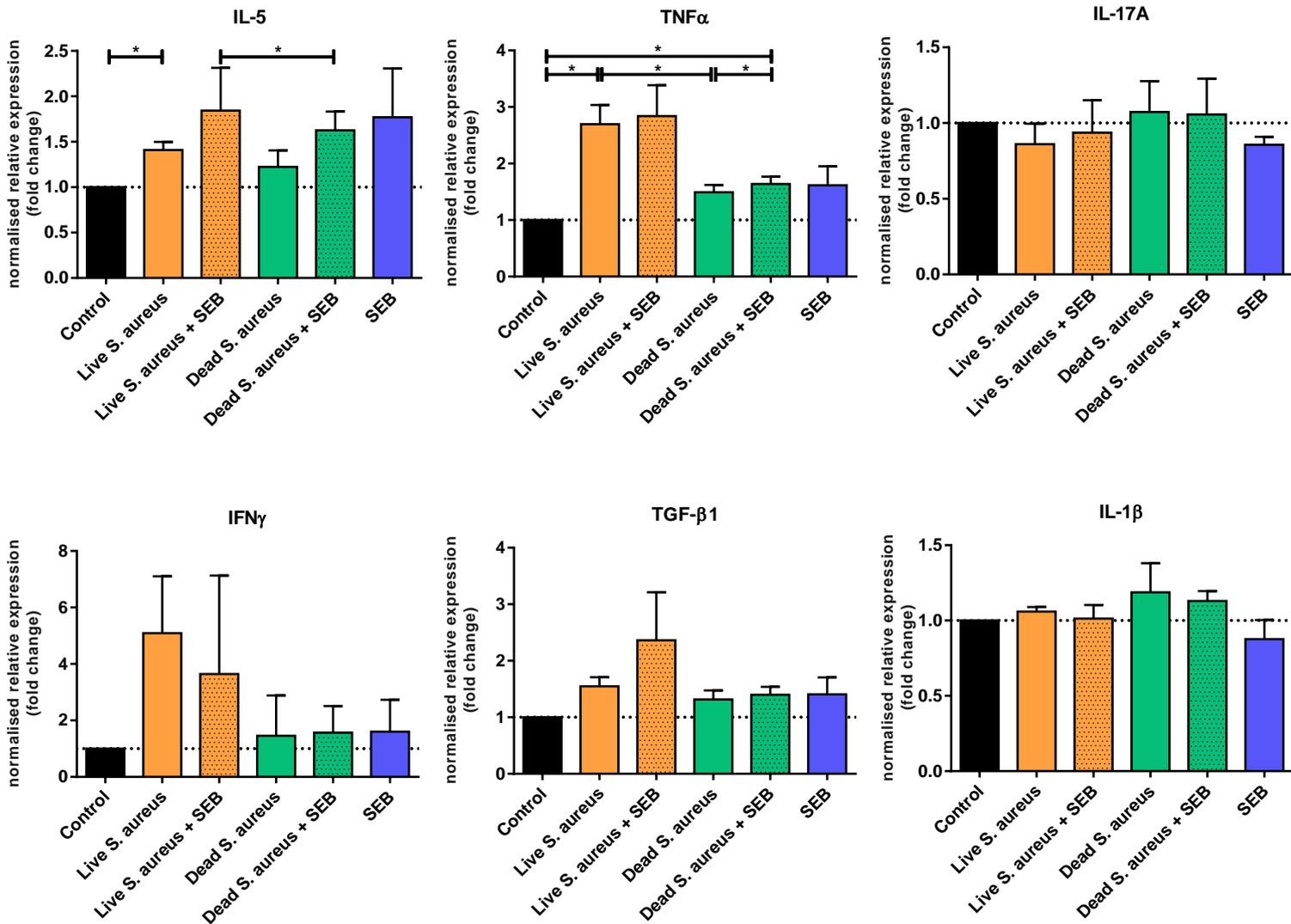
Figure 6.15 LAD2 mast cell normalised gene expression of IL-5, TNFα, IL-17A, IFNγ, TGF-β1, and IL-1β, in SEB stimulated and/or *S. aureus* infected groups. RT-qPCR undertaken using a mast cell line (LAD2) exposed to SEB (10µg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*).
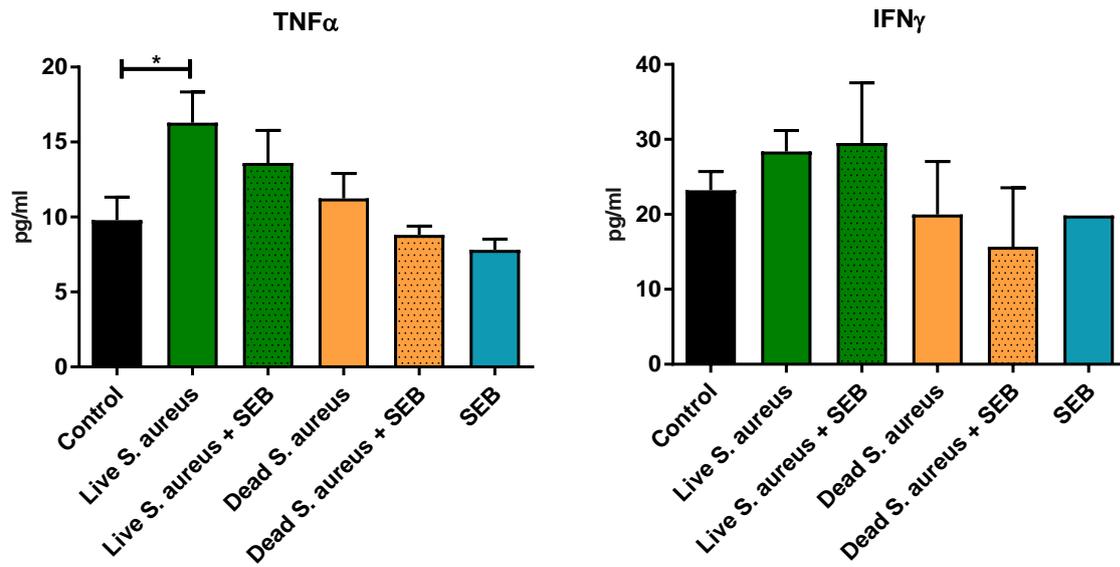
Figure 6.16 LAD2 mast cell culture supernatant cytokine concentrations of TNFα, IFNγ and IL-1β in SEB stimulated and/or *S. aureus* infected groups. Luminex undertaken using a LAD2 mast cell line (5 million cells per ml) exposed to SEB (10µg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*).
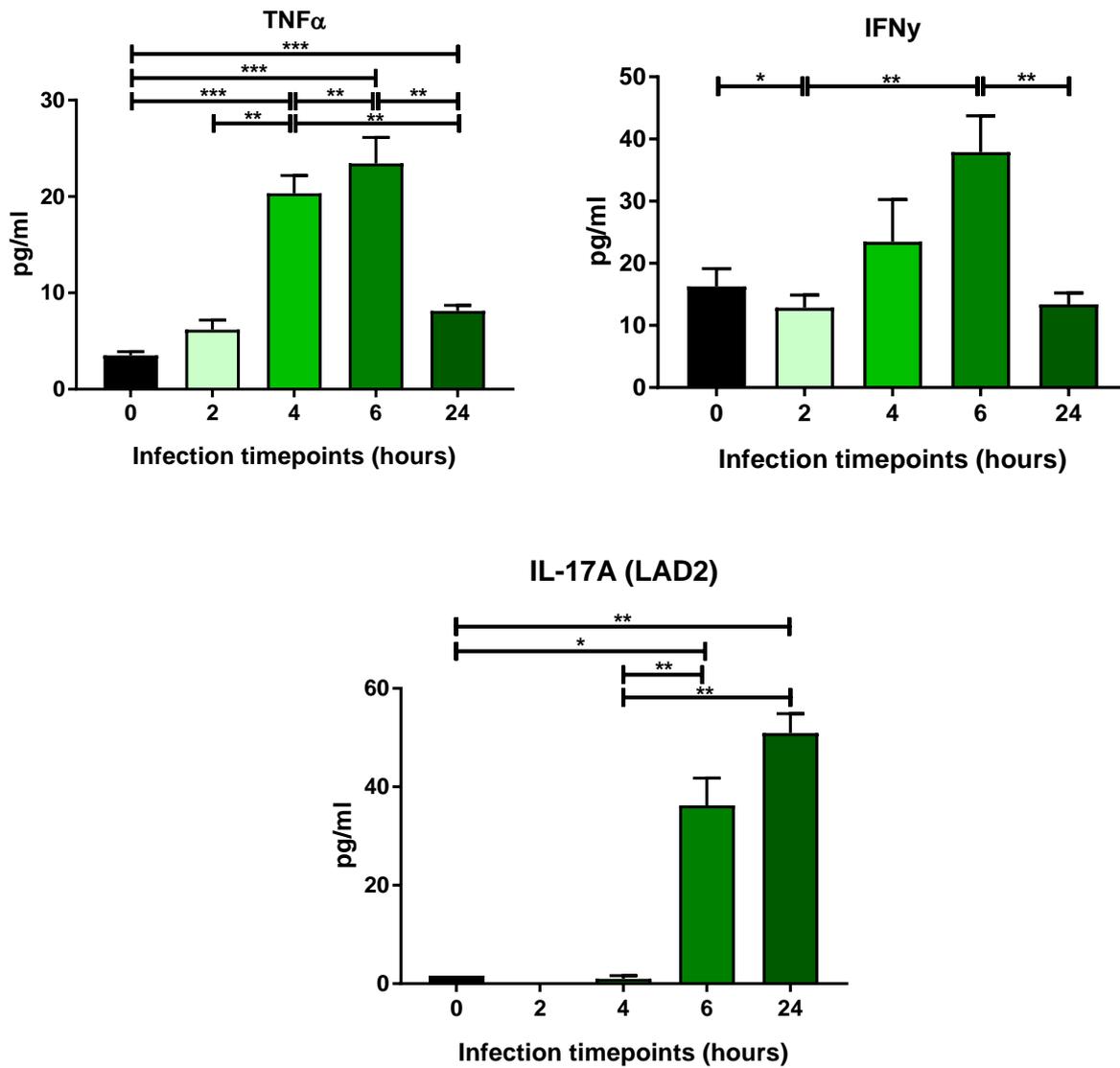
Figure 6.17 LAD2 mast cell supernatant cytokine concentration time-course of TNFα, IFNγ and IL-17A following *S. aureus* infection. LAD2 mast cells (5 million cells per ml) were co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 24 hours, with Luminex undertaken on culture supernatants. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*), p≤0.01 (**) and p≤0.001 (***).

6.6.3.2   T cell transcriptional factor regulation within mast cells exposed to *S. aureus* and SEB

There was significant (p≤0.05) downregulation of the Treg transcriptional factor, FoxP3, when comparing live *S. aureus* to dead *S. aureus* infection, within HMC-1 mast cells (**Figure 6.18**). There was no significant change in FoxP3 gene expression when examining LAD2 cells infected with live/dead *S. aureus* or stimulated with SEB (**Figure 6.19**).
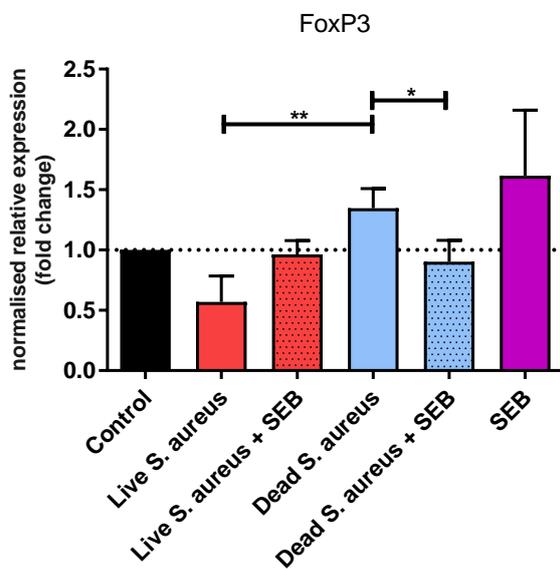
Figure 6.18 HMC-1 mast cell normalised gene expression of FoxP3 in SEB stimulated and/or *S. aureus* infected groups. RT-qPCR undertaken using a mast cell line (HMC-1) exposed to SEB (10μg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*) and p≤0.01 (**).
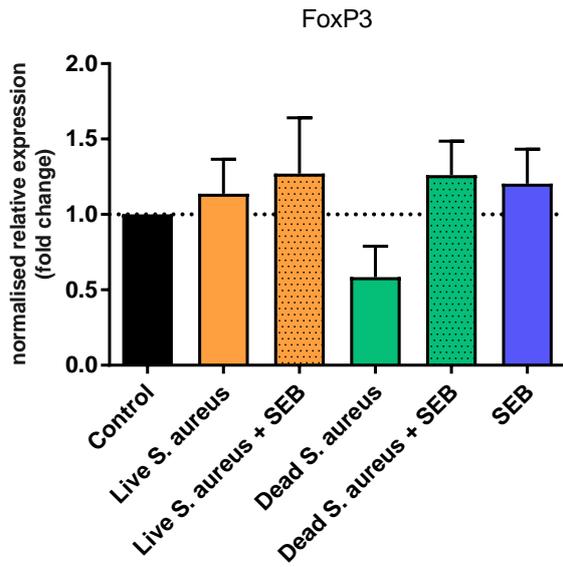
Figure 6.19 LAD2 mast cell normalised gene expression of FoxP3 in SEB stimulated and/or *S. aureus* infected groups. RT-qPCR undertaken using a mast cell line (LAD2) exposed to SEB (10µg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed.

6.6.3.3   Gene expression of toll like receptors in mast cells exposed to *S. aureus* and SEB

Exposure of HMC-1 mast cells to live *S. aureus* resulted in a significant (p≤0.05) downregulation of TLR-2 gene expression (**Figure 6.20**). Dead *S. aureus* combined with SEB resulted in significant downregulation of TLR-2 gene expression compared to controls. There was a progressive downregulation of TLR-2 gene expression over 2 to 6 hours following *S. aureus* infection of HMC-1 mast cells (**Figure 6.21**).

There was no significant change in TLR-2 gene expression when examining LAD2 live/dead *S. aureus* infected or SEB stimulated groups (**Figure 6.22**). TLR-4 gene expression was examined in both HMC-1 and LAD-2 cell lines, but was undetectable.
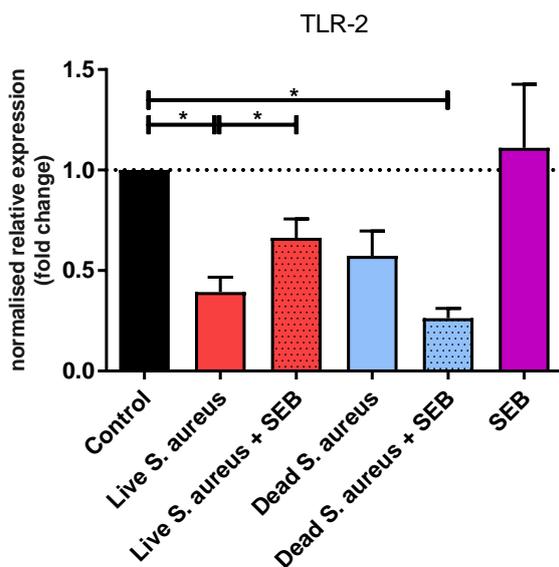
Figure 6.20 HMC-1 mast cell normalised gene expression of TLR-2 in SEB stimulated and/or *S. aureus* infected groups. RT-qPCR undertaken using a mast cell line (HMC-1) exposed to SEB (10µg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*).

Figure 6.21 HMC-1 mast cell normalised gene expression time-course of TLR-2 following *S. aureus* infection. Mast cells (HMC-1) were co-cultured with a CRS *S. aureus* isolate (MOI 1:1) over 6 hours, with RT-qPCR undertaken. Three experimental repeats are displayed, with bars representing the mean and error bars representing +/-SEM. Paired t-tests were performed, p≤0.05 (*).
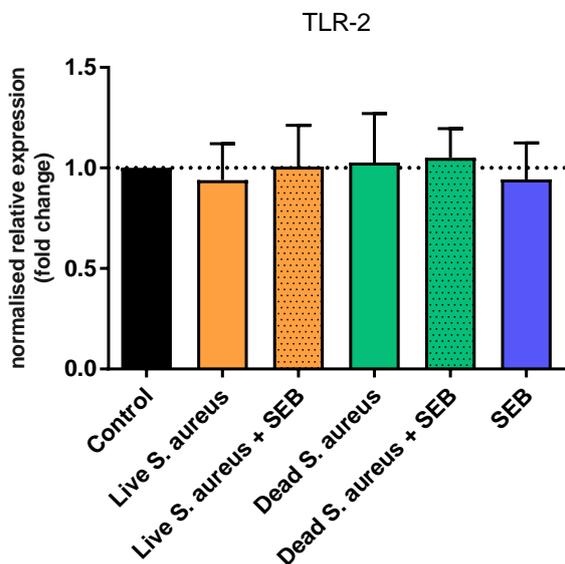
Figure 6.22 LAD2 mast cell normalised gene expression of TLR-2 in SEB stimulated and/or *S. aureus* infected groups. RT-qPCR undertaken using a mast cell line (LAD2) exposed to SEB (10μg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed.

6.6.3.4 Regulation of matrix metalloproteases and defensins, in HMC-1 mast cells exposed to *S. aureus* and SEB

Exposure of HMC-1 mast cells to live *S. aureus* resulted in a significant (p≤0.05) downregulation of MMP-7 and defensin 5 gene expression (**Figure 6.23**). There was a significant downregulation of MMP-7 gene expression in live compared to dead *S. aureus* exposure. Defensin 5 revealed significantly downregulated gene expression to dead *S. aureus* combined with SEB, in addition to that of live *S. aureus*.

Measurement of cytokine protein concentration in HMC-1 culture supernatants showed significant elevation of MMP-7 in response to live *S. aureus* infection (**Figure 6.24**). The addition of SEB to live *S. aureus* did not significantly alter the level of secreted cytokine. Dead *S. aureus* or SEB alone, did not result in significant alteration in cytokine secretion.
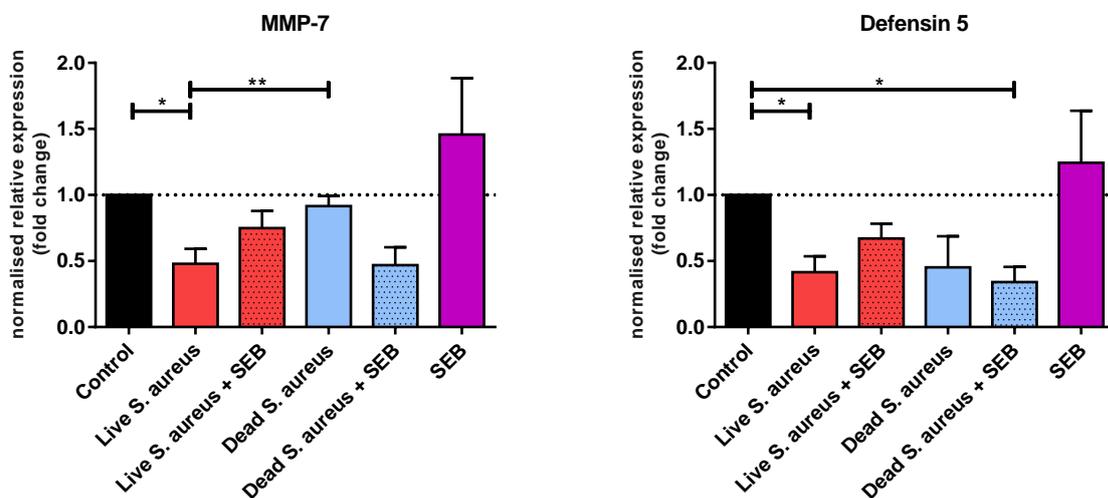
Figure 6.23 HMC-1 mast cell normalised gene expression of MMP-7 and defensin 5 in SEB stimulated and/or *S. aureus* infected groups. RT-qPCR undertaken using a mast cell line (HMC-1) exposed to SEB (10µg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.01 (**) and p≤0.001 (***).
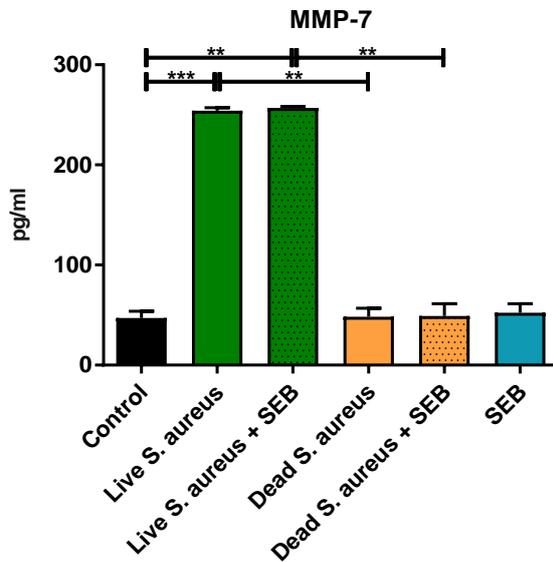
Figure 6.24 HMC-1 mast cell culture supernatant cytokine concentrations of MMP-7 in SEB stimulated and/or *S. aureus* infected groups. Luminex undertaken using a HMC-1 mast cell line (5 million cells per ml) exposed to SEB (10µg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 1:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.01 (**) and p≤0.001 (***).

6.6.3.5 Overview of the mast cell immune response to *S. aureus* infection and SEB exposure in HMC-1 and LAD2 cell culture models

Table 6.1 details a summary of the gene expression cytokine changes associated with live and dead *S. aureus* infection, and/or SEB exposure, of HMC-1 and LAD2 mast cells. **Table 6.2** details a summary of the supernatant cytokine concentrations of associated experiments.

In summary, presented results confirm the significant ($p \leq 0.05$) release of pro-inflammatory cytokines (IFNγ, IL-17A, TNFα, IL-1β and IL-6) into the culture supernatant of *S. aureus* exposed HMC-1 mast cells. On the genome level there was widespread significant ($p \leq 0.05$) downregulation of pro-inflammatory gene expression (IFNγ, IL-5, IL-1β and IL-6) of *S. aureus* exposed HMC-1 mast cells.

In contrast, presented results show significant ($p \leq 0.05$) release of TNFα and IL-1β into the culture supernatant of *S. aureus* exposed LAD2 mast cells. On the genome level there was significant ($p \leq 0.05$) upregulation of IL-5 and TNFα gene expression of *S. aureus* exposed LAD2 mast cells.

Table 6.1 Summary table of HMC-1 and LAD2 mast cell gene expression ratios and significance levels.

	HMC-1 Mast Cells					LAD2 Mast Cells				
	Live *S. aureus*	Live *S. aureus* + SEB	Dead *S. aureus*	Dead *S. aureus* + SEB	SEB	Live *S. aureus*	Live *S. aureus* + SEB	Dead *S. aureus*	Dead *S. aureus* + SEB	SEB
IFNγ	0.5	0.8	0.7	0.4	1.2	5.1	3.6	1.5	1.6	1.6
IL-5	0.4	0.6	0.7	0.3	1.1	1.4	1.8	1.2	1.6	1.7
IL-1β	0.2	0.2	1.2	0.6	0.7	1.1	1.0	1.2	1.1	0.9
IL-6	0.4	0.3	1.4	0.7	0.7	6.9	6.3	3.1	5.5	3.3
TGF-β 1	1.0	1.2	1.1	0.6	1.1	1.6	2.4	1.3	1.4	1.4
IL-17A	1.0	1.5	1.9	1.4	1.1	0.9	0.9	1.1	1.1	0.9
TNFα	1.0	0.9	1.1	0.9	0.7	2.7	2.8	1.5	1.6	1.6
FoxP3	0.6	1.0	1.3	0.9	1.6	1.1	1.3	0.6	1.3	1.2
TLR2	0.4	0.7	0.6	0.3	1.1	0.9	1.0	1.0	1.0	0.9
MMP-7	0.5	0.8	0.9	0.5	1.5	1.4	3.8	2.4	1.1	1.3
Defensin 5	0.4	0.7	0.5	0.3	1.3	0.9	1.0	1.0	1.0	0.9

Gene expression fold change ratios of figures 6.11, 6.15, 6.18, 6.19, 6.20, 6.22 and 6.23. Green boxes represent significantly upregulated gene expression compared to controls (p≤0.05), with red boxes denoting significantly downregulated gene expression compared to controls (p≤0.05). Paired t-tests were performed.

Table 6.2 Summary table of HMC-1 and LAD2 mast cell cytokine data and significance levels.

	HMC-1 Mast Cells						LAD2 Mast Cells					
	Control	Live *S. aureus*	Live *S. aureus* + SEB	Dead *S. aureus*	Dead *S. aureus* + SEB	SEB	Control	Live *S. aureus*	Live *S. aureus* + SEB	Dead *S. aureus*	Dead *S. aureus* + SEB	SEB
IFNγ	15.8	57.5	53.5	19.9	17.9	20.4	23.2	28.4	29.5	20.0	15.7	19.9
IL-17A	0.8	11.7	11.6	0.5	0.5	1.3	N/A	N/A	N/A	N/A	N/A	N/A
TNFα	13.8	28.1	27.1	13.8	12.4	11.6	9.8	16.3	13.6	11.2	8.8	7.8
IL-1β	0	11.3	11.0	0	0	0	2.3	11.4	7.3	0	0	6
IL-6	1.1	1.5	1.7	0.8	0.8	0.5	N/A	N/A	N/A	N/A	N/A	N/A
MMP-7	47.1	254.1	257.0	48.3	49.2	52.3	N/A	N/A	N/A	N/A	N/A	N/A

Table of collated Luminex culture supernatant cytokine concentrations (pg/ml) of figures 5.12 and 5.16. Green boxes represent significantly upregulated gene expression compared to controls (p≤0.05). Paired t-tests were performed.

6.6.4 Down-stream immunomodulatory effects of repeated *S. aureus* internalisation and release in mast cells

6.6.4.1 Repeated internalisation does not render *S. aureus* more susceptible to mast cell uptake

As previous outlined in chapter 2, five separate *S. aureus* strains were obtained that had been through one to five successive mast cell uptake and release cycles (P3.1 to P3.5). Viable intracellular *S. aureus* was found in HMC-1 and LAD2 cells from both the unaltered original stock strain (P3) as well as the once internalised strain (P3.1) (**Figure 6.25 and 6.26**). There was no significant difference in the number of internalised viable bacteria when examining the original CRS-specific isolate (P3) compared to the P3.1 and P3.5 strains in HMC-1 and LAD2 mast cells (**Figure 6.27**, p>0.05).
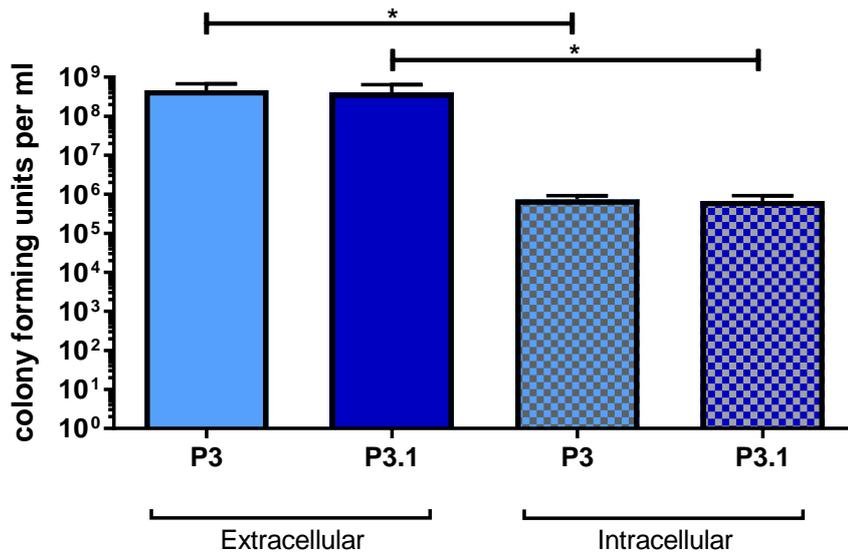
Figure 6.25 Intracellular and extracellular P3 and P3.1 *S. aureus* strains enumerated from HMC-1 cells. HMC-1 mast cells (2 million cells) were co-cultured with P3 and P3.1 CRS *S. aureus* isolates (MOI 20:1) over 4 hours. Extracellular CFUs were enumerated on CBA plates, with intracellular CFUs enumerated on CBA plates following extracellular *S. aureus* eradication (20µg/ml Lysostaphin). Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Statistical analysis was performed using t-tests, p≤0.05 (*).
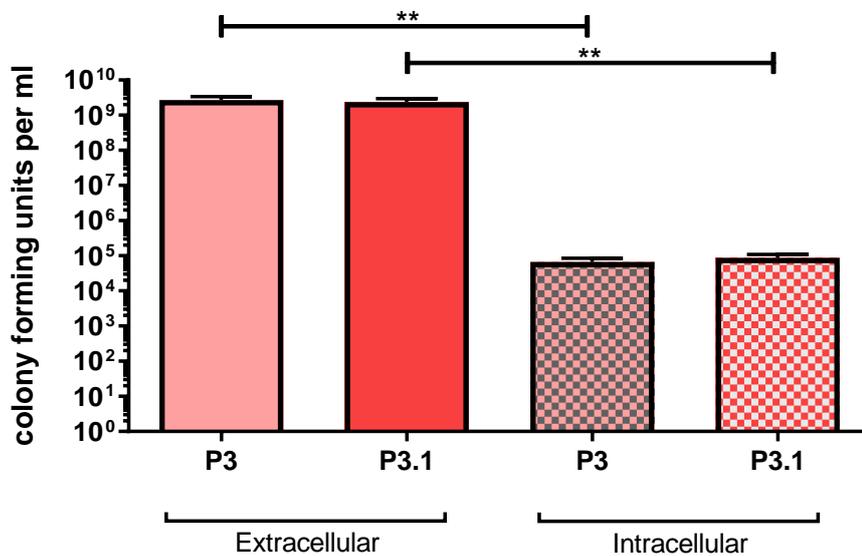
Figure 6.26 Intracellular and extracellular P3 and P3.1 *S. aureus* strains enumerated from LAD2 cells. LAD2 mast cells (2 million cells) were co-cultured with P3 and P3.1 CRS *S. aureus* isolates (MOI 20:1) over 4 hours. Extracellular CFUs were enumerated on CBA plates, with intracellular CFUs enumerated on CBA plates following extracellular *S. aureus* eradication (20µg/ml Lysostaphin). Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Statistical analysis was performed using t-tests, p≤0.01 (**).

A

B
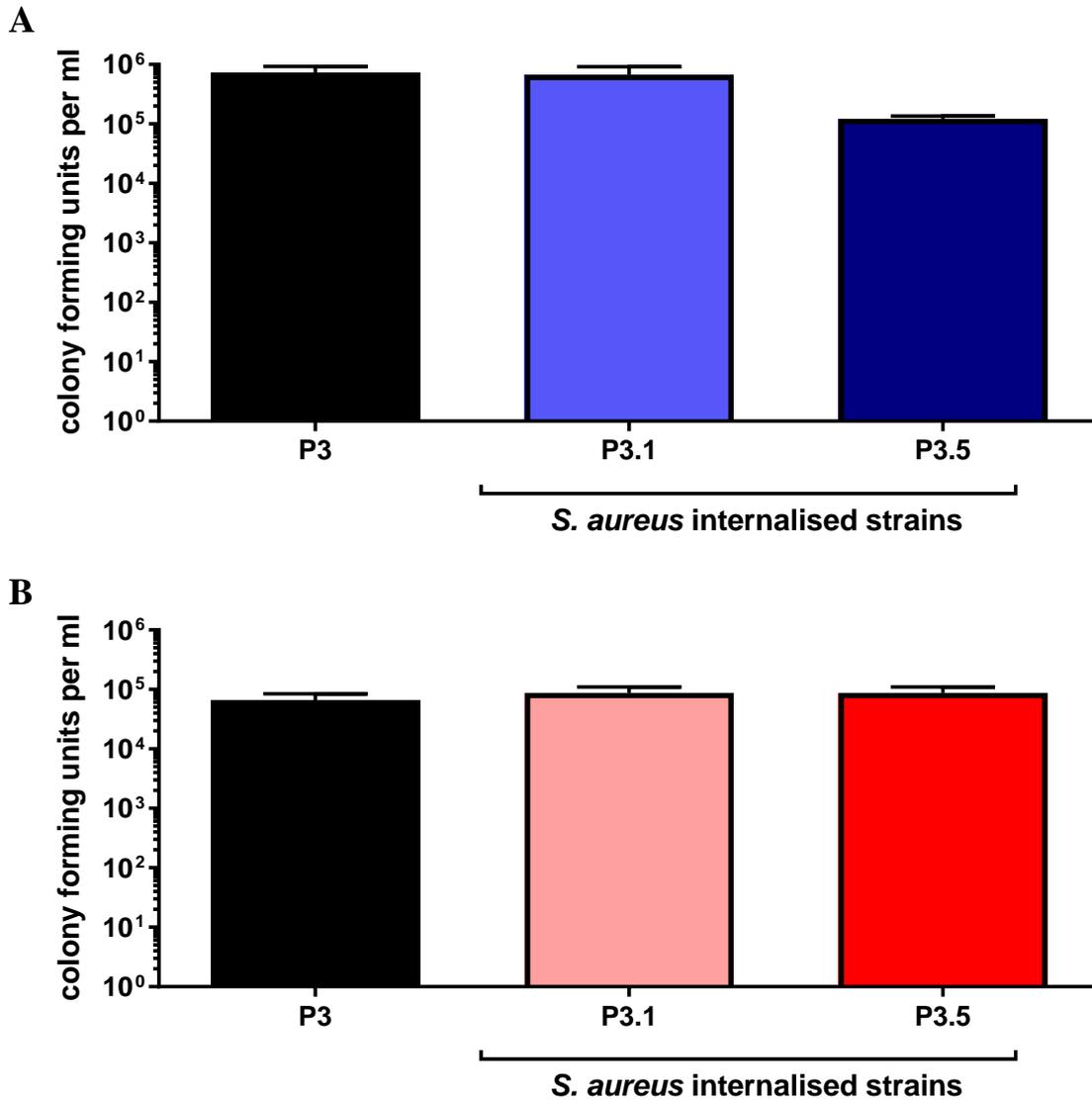
Figure 6.27 Enumeration of intracellular *S. aureus* from HMC-1 and LAD2 cells using a CRS-specific *S. aureus* isolate (P3), and P3.1, P3.5 internalised strains. HMC-1 (A) and LAD2 (B) (2 million cells) were co-cultured with *S. aureus* isolates (MOI 20:1), over 4 hours. Intracellular CFUs were enumerated on CBA plates following extracellular *S. aureus* eradication (20μg/ml Lysostaphin). Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Statistical analysis was performed using paired t-tests.

6.6.4.2   Immune response of *S. aureus* internalised strains

HMC-1 mast cells

Exposure of HMC-1 mast cells to P3 *S. aureus* resulted in upregulated IL-8 and TGF-β1, and downregulated IL-6, IL-1β and IL-5 gene expression (**Figure 6.28**). These results are consistent with those obtained in previous experiments (**Figure 6.11**). Infection of HMC-1 mast cells with the P3.1 and P3.5 *S. aureus* strains resulted in a progressive downregulation of IL-8, IL-1β and TGF-β1 gene expression. In contrast, there was an associated upregulation of IL-5. There was a statistically significant downregulation of IL-8 ($p \leq 0.05$) and IL-1β ($p \leq 0.01$), when comparing the immune response of the P3.1 vs P3.5 *S. aureus* in HMC-1 mast cells. The gene expression of TNFα did not change significantly across *S. aureus* strains.

Exposure of HMC-1 mast cells to P3 *S. aureus* resulted in increases in TNFα and IFNγ culture supernatant cytokine concentrations (**Figure 6.29**). Infection of HMC-1 mast cells with P3.1 and P3.5 strains was associated with a decrease in the concentrations of TNFα and IFNγ, with this reaching statistical significance for TNFα ($p \leq 0.01$). IL-17A, IL-6 and IL-1β were not detected within the culture supernatants of HMC-1 co-infections.
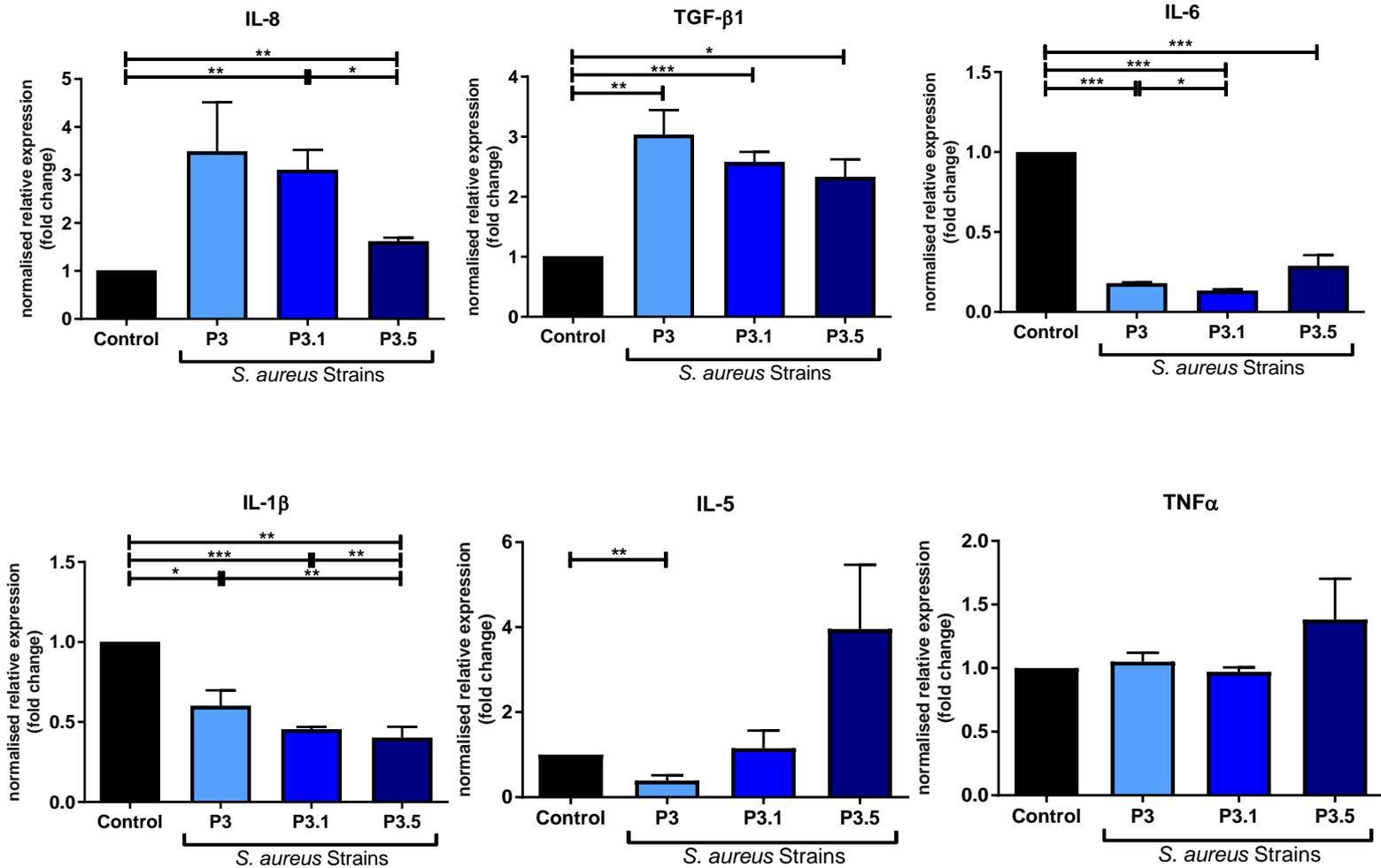
Figure 6.28 HMC-1 mast cell normalised gene expression of IL-8, TGF-β1, IL-6, IL-1β, IL-5 and TNFα following co-infection using different internalised *S. aureus* strains. HMC-1 mast cells were co-cultured with P3, P3.1 and P3.5 CRS *S. aureus* isolates (MOI 20:1), over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*), p≤0.01 (**) and p≤0.001 (***).
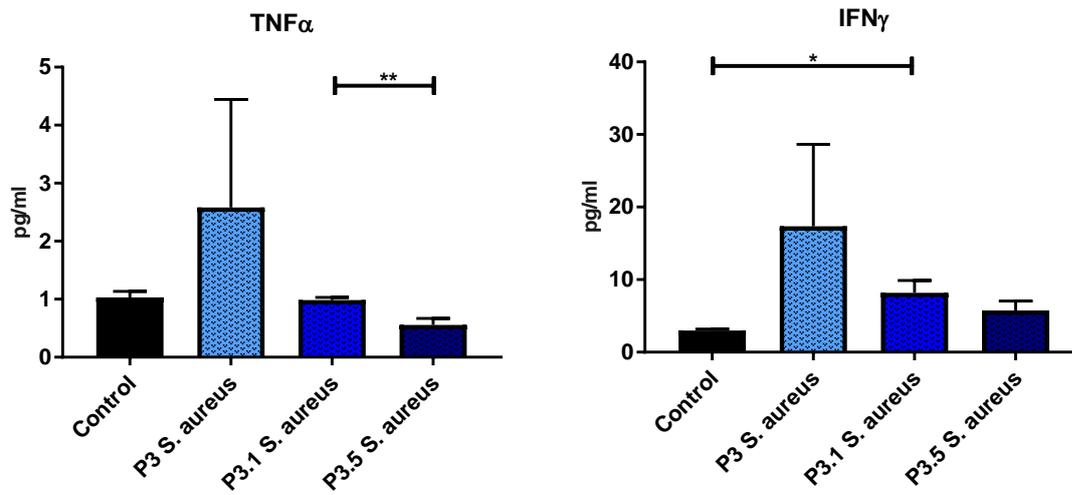
**Figure 6.29 HMC-1 mast cell culture supernatant cytokine concentrations of TNFα and IFNγ following co-infection using different internalised *S. aureus* strains**. Luminex undertaken using a HMC-1 mast cell line (5 million cells per ml) exposed to SEB (10µg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 20:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*) and p≤0.01 (**).
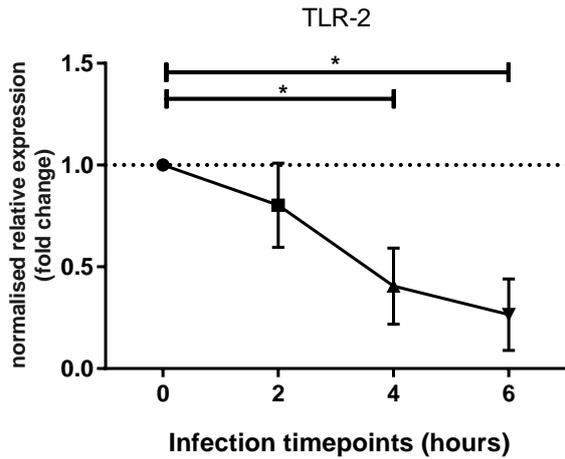
LAD2 mast cells

Exposure of LAD2 mast cells to P3 *S. aureus* resulted in upregulation of IL-8, TNFα, TGF-β1 and IL-5 gene expression (**Figure 6.30**), in consistency with previously presented results (**Figure 6.15**). The use of P3.1 *S. aureus* resulted in further upregulation of IL-8, IL-1β, TNFα, TGF-β1 and IL-5 gene expression. In contrast, results revealed an associated downregulation of IL-8, IL-1β, TNFα, TGF-β1 and IL-5 gene expression when LAD2 mast cells were exposed to P3.5 *S. aureus*. This downregulation reached statistical significance for TNFα (p≤0.01), TGF-β1 (p≤0.05) and IL-5 (p≤0.05).

Exposure of LAD2 mast cells to P3 *S. aureus* resulted in an increase in TNFα, IL-17A and IFNγ culture supernatant cytokine concentrations (**Figure 6.31**). Infection of LAD2 mast cells with P3.1 *S. aureus* showed a similar response to the P3 strain, indeed there was no statistically significant difference between these groups. However, the P3.5 *S. aureus* was associated with a reduction in TNFα, IL-17A and IFNγ culture supernatant cytokine concentrations and this drop was statistically (p≤0.01) significant with regards to TNFα and IL-17A. IL-6 and IL-1β were not detected within the culture supernatants of LAD2 co-cultures.
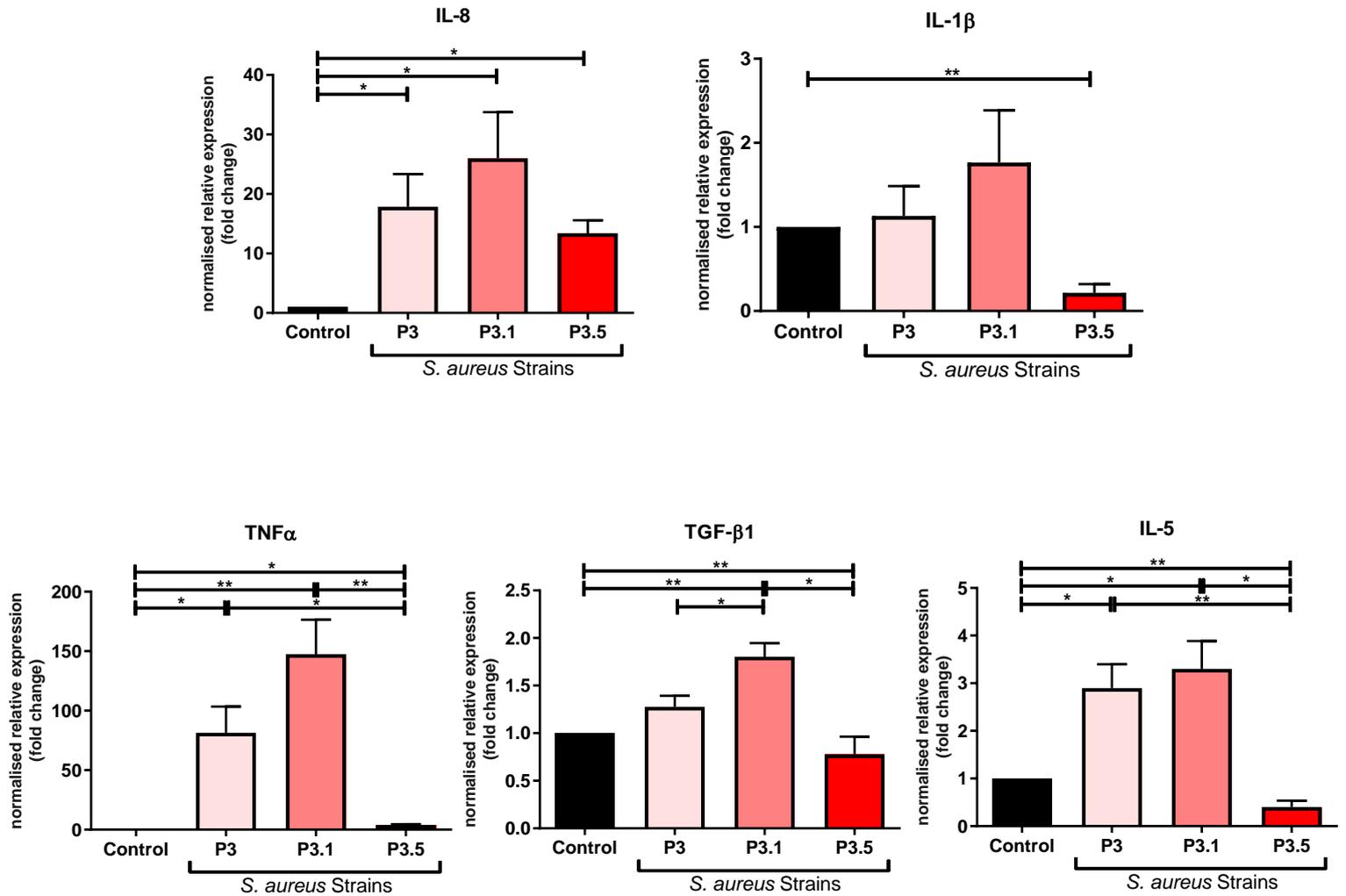
Figure 6.30 LAD2 mast cell normalised gene expression of IL-8, IL-1β, TNFα, TGF-β1 and IL-5 following co-infection using different internalised *S. aureus* strains. LAD2 mast cells were co-cultured with P3, P3.1 and P3.5 CRS *S. aureus* isolates (MOI 20:1), over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*) and p≤0.01 (**).
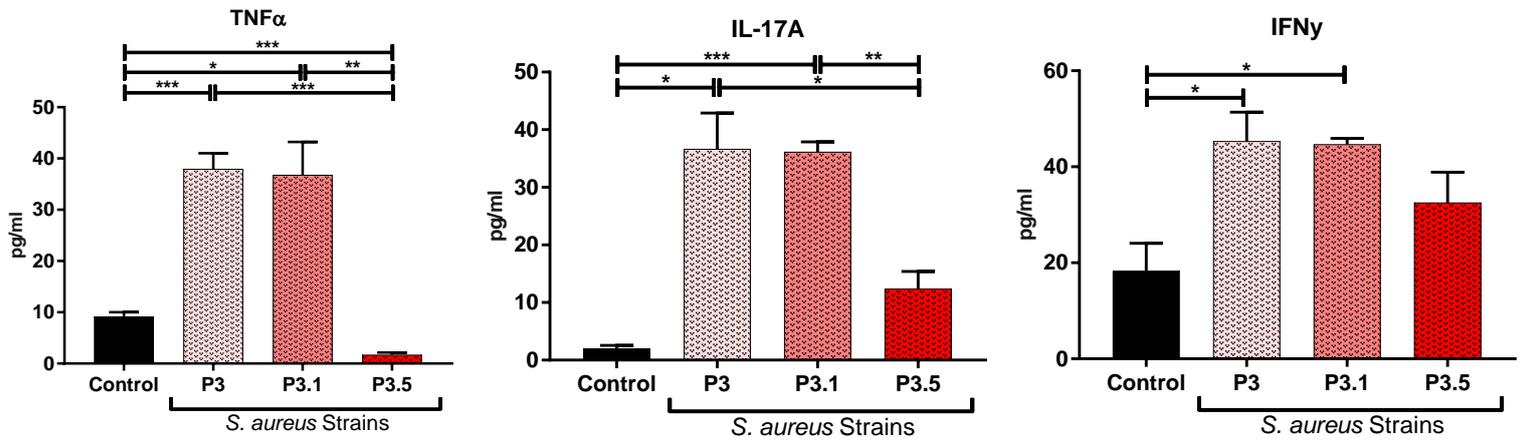
Figure 6.31 LAD2 mast cell culture supernatant cytokine concentrations of TNFα and IFNγ following co-infection using different internalised *S. aureus* strains. Luminex undertaken using a LAD2 mast cell line (5 million cells per ml) exposed to SEB (10µg/ml) and/or a live/dead CRS *S. aureus* isolate (MOI 20:1) over 4 hours. Three experimental repeats are displayed, with bars representing the mean and error bars representing SEM. Paired t-tests were performed, p≤0.05 (*), p≤0.01 (**) and p≤0.001 (***).

6.6.5 The inhibitory effect of IgE sensitisation in *S. aureus* infection

Experiments in sections 6.6.5.1, 6.6.5.2 and 6.6.5.4 were undertaken in collaboration with Rana Abadalkareem (PhD student), examining the effect of IgE sensitisation on the activation and immune response of *S. aureus* infected LAD2 cells.

6.6.5.1 β-hexosaminidase release

Exposure of LAD2 mast cells to P3 *S. aureus*, in the absence of IgE sensitisation, resulted in low levels of β-hexosaminidase release (**Figure 6.32**). In IgE sensitised and anti-IgE exposed LAD2 cells, there was a drop in β-hexosaminidase release with the addition of *S. aureus*. This was evident after 2 hours contact time with *S. aureus* (2-15% inhibition), with an enhanced effect (statistically significant, p≤0.05) following 4 hours of co-culture (25-37% inhibition).

An increase in calcium ionophore concentration was associated with increased rates of β-hexosaminidase release from all groups (**Figure 6.33**). After 2 hours of *S. aureus* exposure, there was minimal differences between studied groups, within the same calcium ionophore concentration. However, after 4 hours, there was an associated reduced level of β-hexosaminidase release from the *S. aureus* infected groups, irrespective of prior IgE sensitisation. This effect was more pronounced with the lower (0.3μM) calcium ionophore concentration, and with a longer *S. aureus* contact time. Indeed, the inhibition of β-hexosaminidase release was between 5-9% at 2 hours, and 15-79% at 4 hours.

There was an associated low net release of β-hexosaminidase (approx. 2-6%) following *S. aureus* infection of LAD2 cells (with and without IgE sensitisation), prior to anti-IgE or calcium ionophore stimulation (**Figure 6.34**).

A – 2 hours
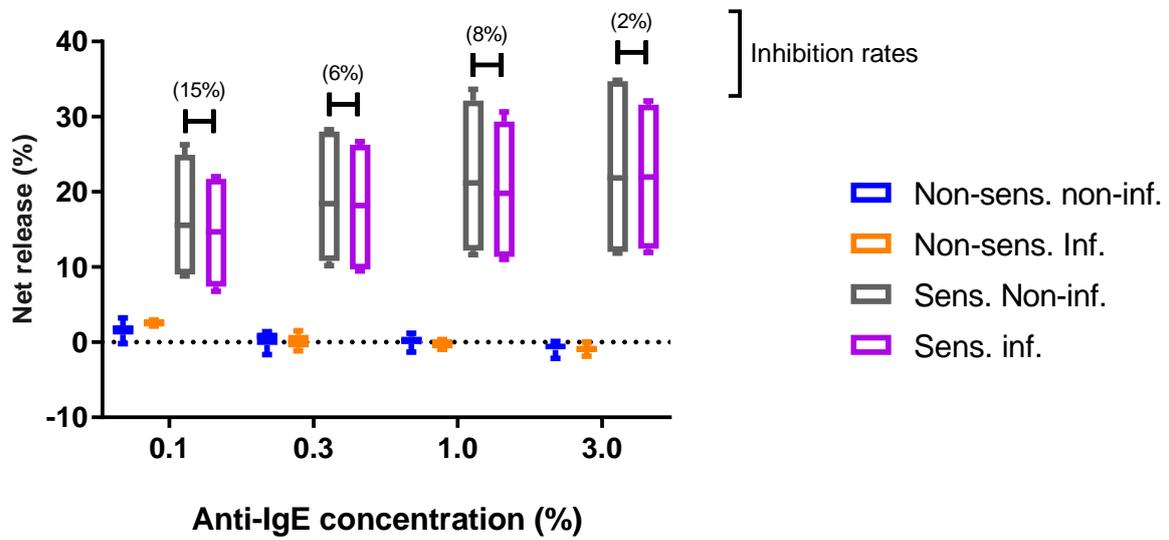
B – 4 hours
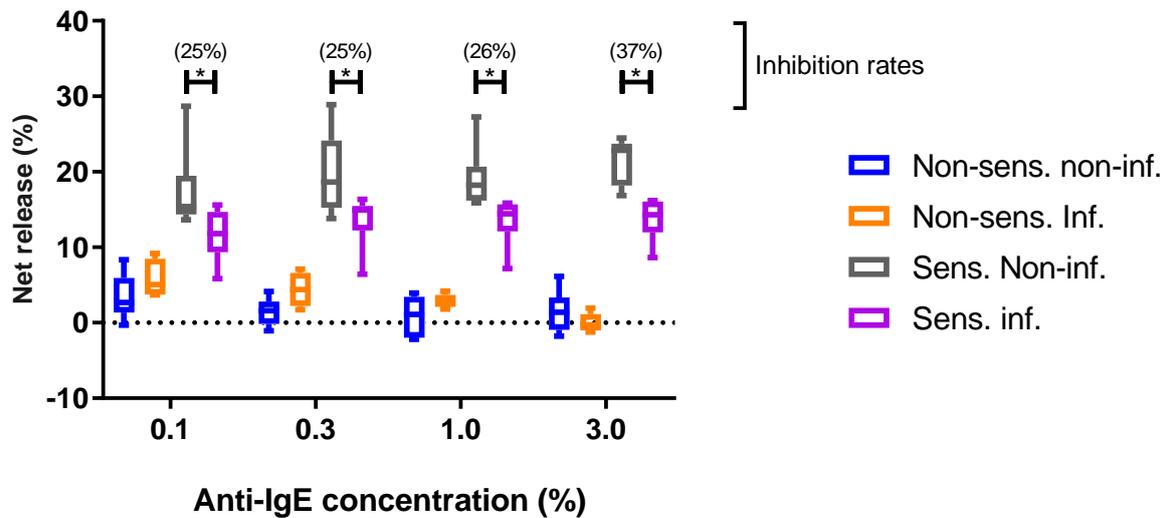
Figure 6.32 Percentage net release of β-hexosaminidase from LAD2 cells, with or without IgE sensitisation/*S. aureus* infection, followed by Anti-IgE treatment. IgE stimulation was performed overnight, with *S. aureus* infection (MOI 1:1) undertaken over 2 (**Graph A**) and 4 (**Graph B**) hours. Two experimental and three technical repeats are displayed. Boxes represent the 25th to 75th percentile, with whiskers representing the 5th to 95th percentile, with lines representing medians. Two-way ANOVA was undertaken, p≤0.05 (*). Assay undertaken by Rana Abadalkareem (PhD student).

A – 2 hours
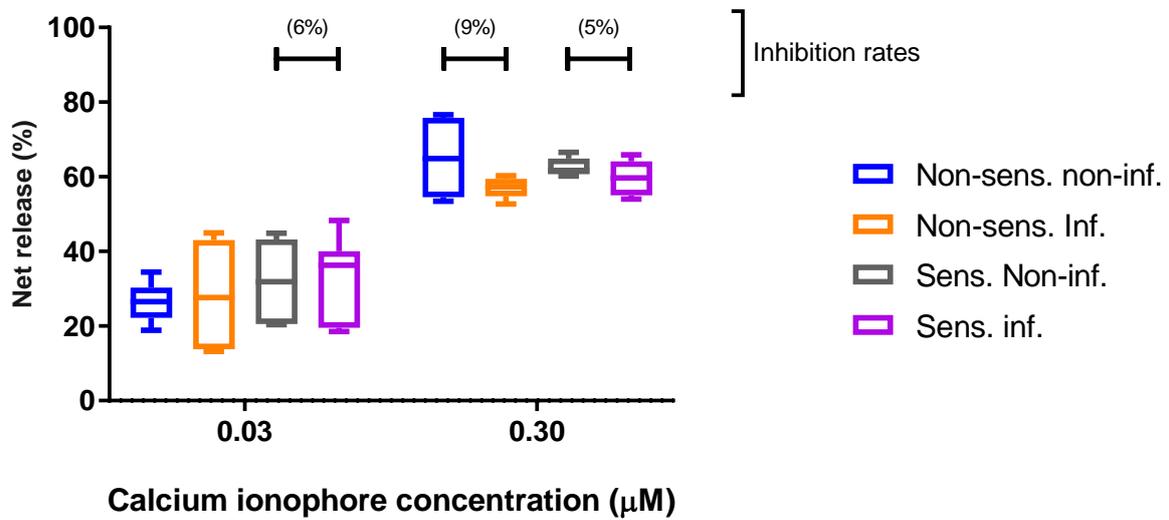
B – 4 hours
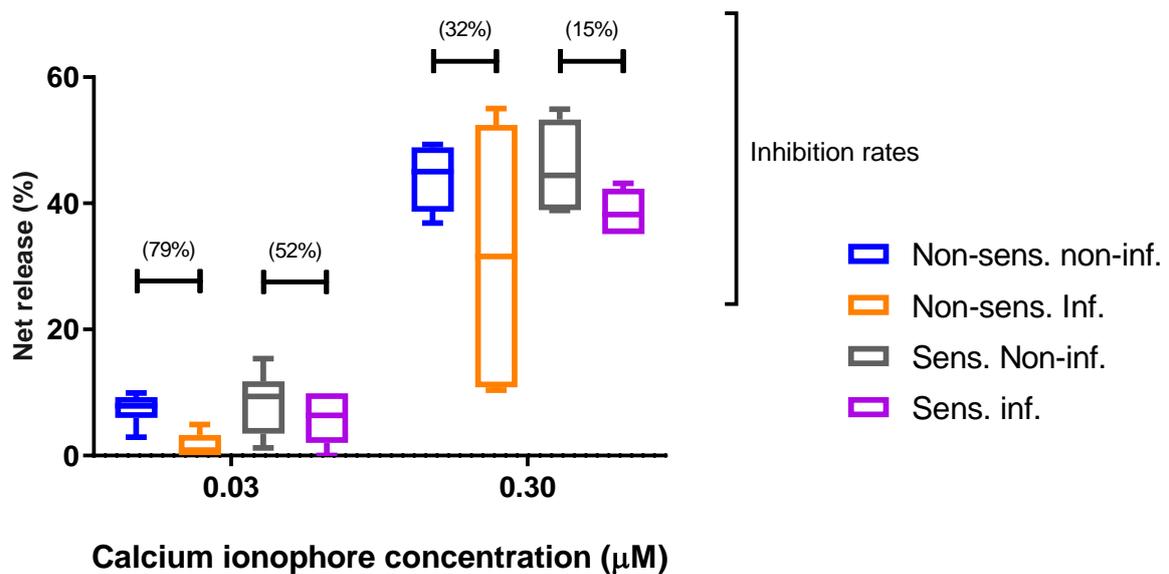
Figure 6.33 Percentage net release of β-hexosaminidase from LAD2 cells, with or without IgE sensitisation/*S. aureus* infection, followed by calcium ionophore treatment. IgE stimulation was performed overnight, with *S. aureus* infection (MOI 1:1) undertaken over 2 (**Graph A**) and 4 (**Graph B**) hours. Two experimental and three technical repeats are displayed. Boxes represent the 25th to 75th percentile, with whiskers representing the 5th to 95th percentile, with lines representing medians. Two-way ANOVA was undertaken. Assay undertaken by Rana Abadalkareem (PhD student).
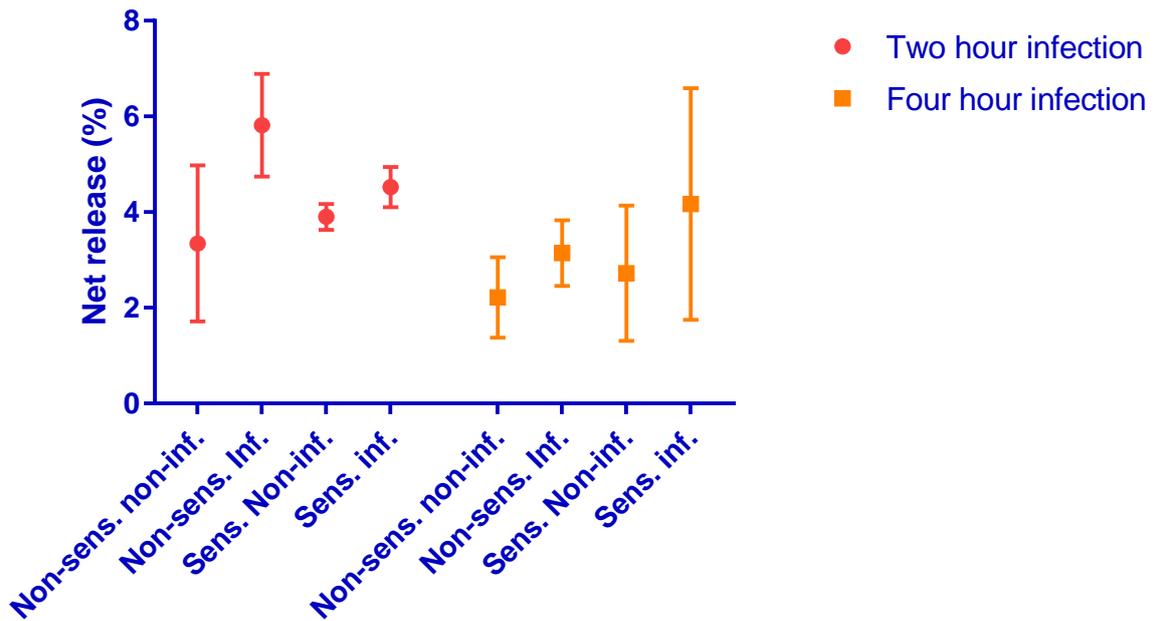
Figure 6.34 Percentage net release of β-hexosaminidase from LAD2 cells, with or without IgE sensitisation/*S. aureus* infection, measured in the culture supernatant prior to Anti-IgE or Calcium Ionophore stimulation. IgE stimulation was performed overnight, with *S. aureus* infection (MOI 1:1) undertaken over 2 and 4 hours. Two experimental and three technical repeats are displayed. Points represent mean values, with lines representing +/-SEM. Assay undertaken by Rana Abadalkareem (PhD student).

6.6.5.2   Mast cell viability (LDH production)

Results indicate a very low level of LDH release following 2 hours of LAD2 and *S. aureus* co-culture,

with and without IgE sensitisation (**Figure 6.35**). There was a significant elevation of LDH following 4

hours of *S. aureus* infection, which was not observed in the uninfected groups.
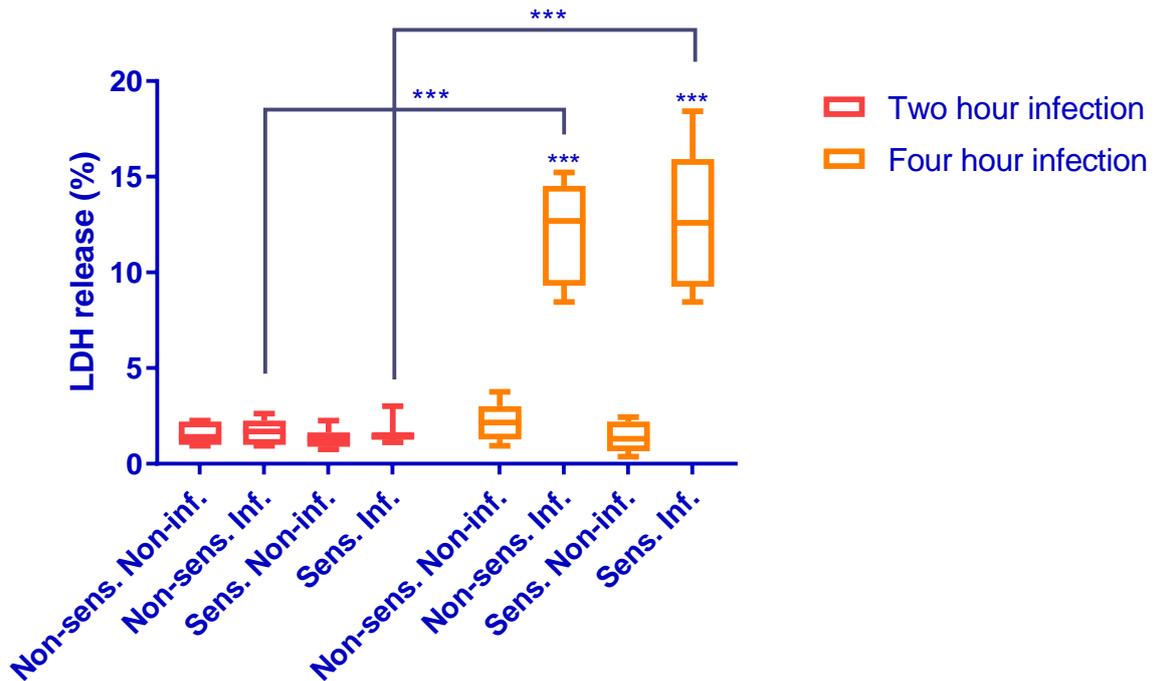
Figure 6.35 Percentage LDH release from LAD2 cells, with or without IgE sensitisation/*S. aureus* infection. IgE stimulation was performed overnight, with *S. aureus* infection (MOI 1:1) undertaken over 2 and 4 hours. Boxes represent the 25th to 75th percentile, with whiskers representing the 5th to 95th percentile, and lines representing medians. Two-way ANOVA was undertaken, p≤0.001 (***). Assay undertaken by Rana Abadalkareem (PhD student).

6.6.5.3 The immune response to IgE sensitisation and *S. aureus* infection

In keeping with previously presented results (**Figure 6.15**), non-IgE sensitised LAD2 cells revealed upregulation of pro-inflammatory cytokines (notably TNFα) following infection with *S. aureus* over 4 hours (**Figure 6.36A**). When examining *S. aureus* infected groups, there was an associated downregulation of TNFα (54%), IL-8 (58%) and IL-1β (7%) gene expression following IgE sensitisation, although this did not reach statistical significance. However, this rate of downregulation for TNFα became statistically significant with the further addition of 3µg/ml of anti-IgE (**Figure 6.36B**). Calcium Ionophore had no discernible effect on the gene expression of *S. aureus* infected or IgE sensitised LAD2 cells (**Figure 6.36C**).

Compared to the non-IgE sensitised *S. aureus* infected group, there was evidence of inhibited TNFα (10%) and IFNγ (25%) release when LAD2 cells were sensitised with IgE prior to *S. aureus* exposure (**Figure 6.37A**). This inhibitory effect was enhanced for TNFα in the presence of anti-IgE (**Figure 6.37B).** There was reduced IFNγ concentrations in *S. aureus* infected groups, irrespective of IgE sensitisation, when LAD2 cells were treated with calcium ionophore following co-culture (**Figure 6.37C**). There were inconsistent results when examining TNFα concentrations in *S. aureus* infected and sensitised groups, following calcium ionophore treatment.
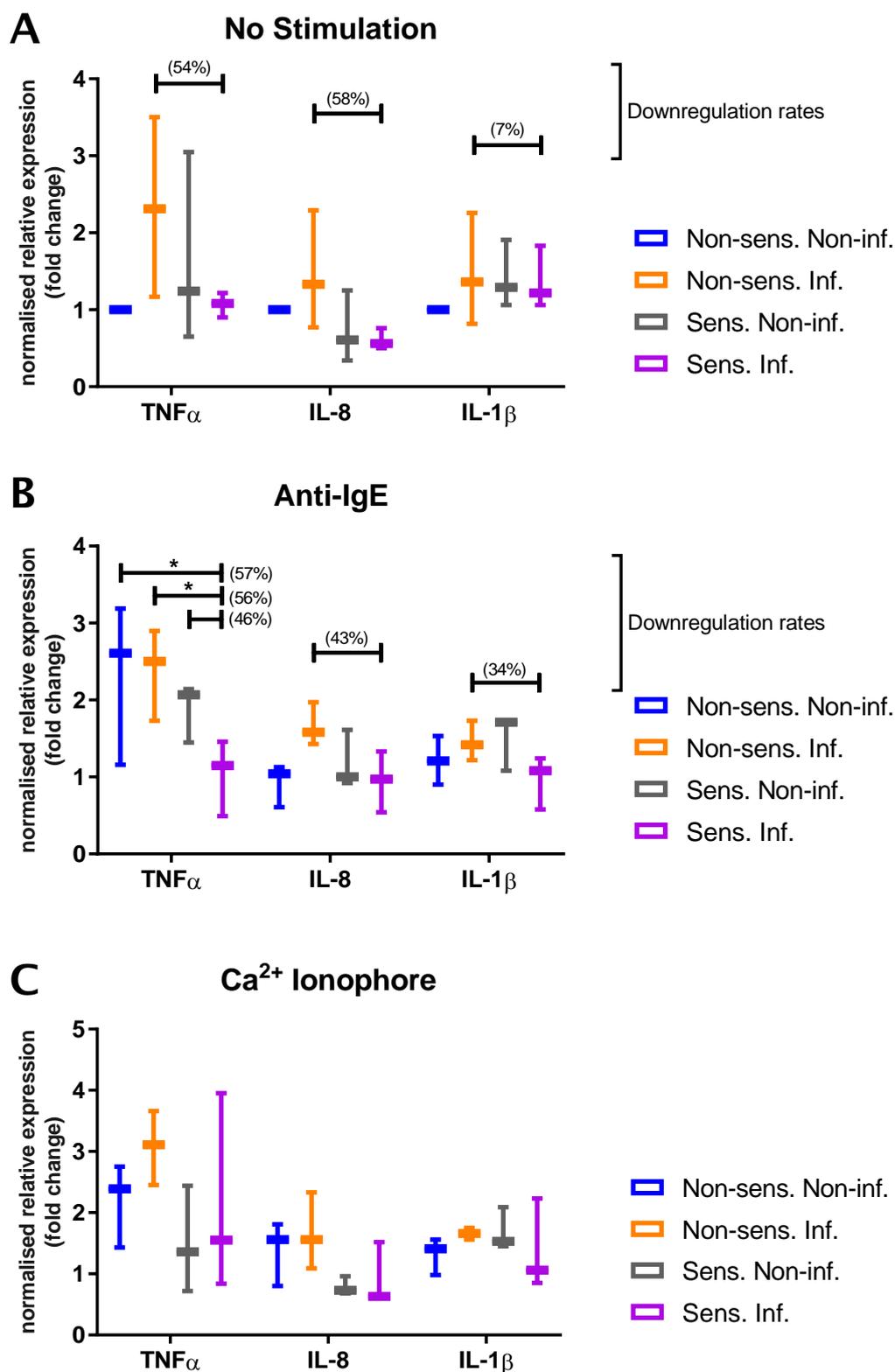
Figure 6.36 Normalised gene expression of TNFα, IL-8 and IL-1β in LAD2 cells stimulated with or without IgE and/or co-infected with _S. aureus_. IgE stimulation was performed overnight, with _S. aureus_ infection (MOI 1:1) undertaken over 4 hours. Following culture samples underwent, A) no further stimulation, B) 3µg/ml anti-IgE stimulation or C) 0.03µM calcium ionophore stimulation. Boxes represent the 25th to 75th percentile, with whiskers representing the 5th to 95th percentile. Two-way ANOVA was undertaken, p≤0.05 (*).
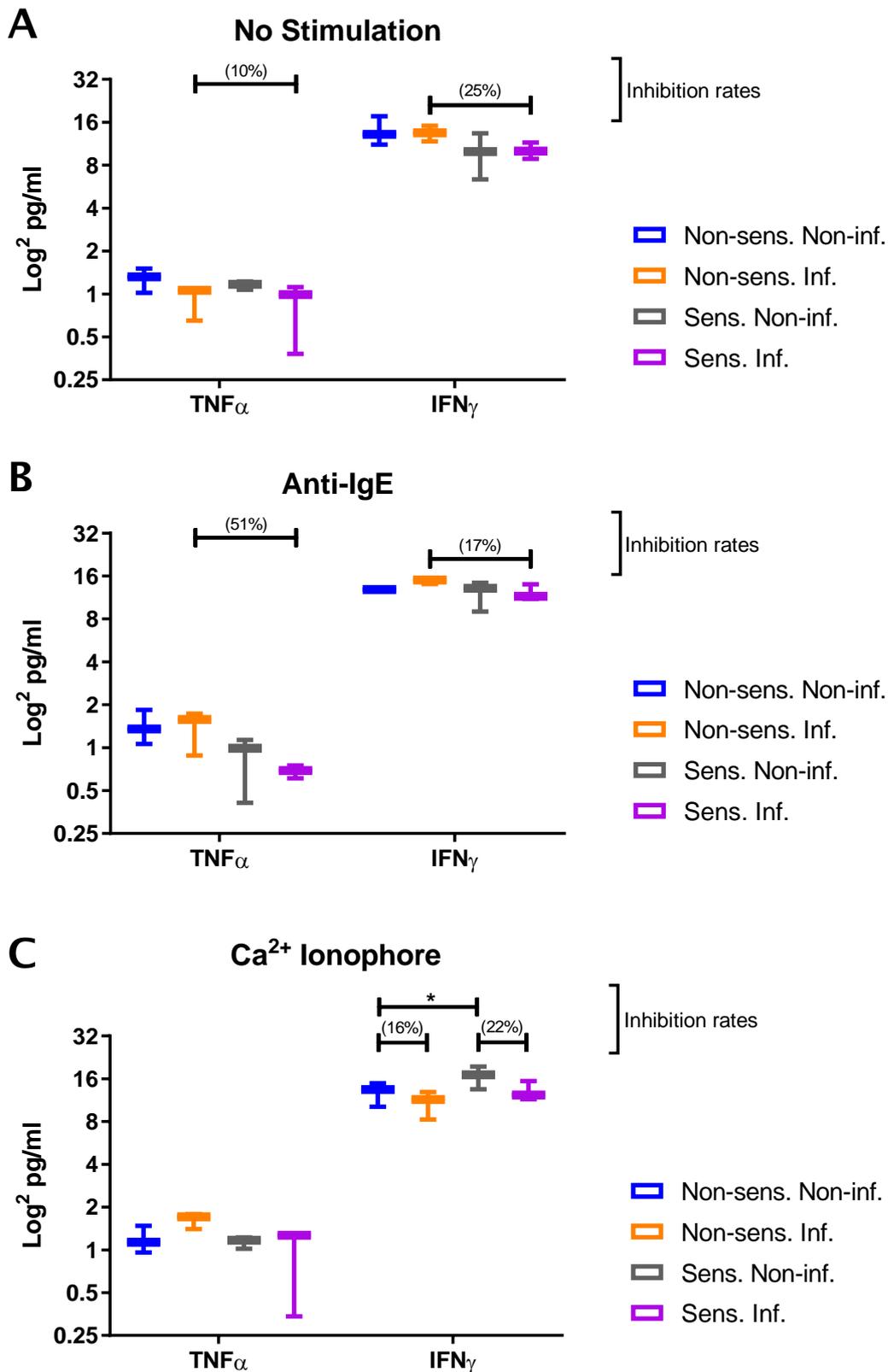
Figure 6.37 culture supernatant cytokine concentrations of TNFα and IFNγ in LAD2 cells stimulated with or without IgE and/or co-infected with *S. aureus*. IgE stimulation was performed overnight (1 million LAD2 cells per ml) with *S. aureus* infection (MOI 1:1) undertaken over 4 hours. Following culture samples underwent, A) no further stimulation, B) 3µg/ml anti-IgE stimulation or C) 0.03µM calcium ionophore stimulation. Boxes represent the 25th to 75th percentile, with whiskers representing the 5th to 95th percentile. Two-way ANOVA was undertaken, p≤0.05 (*).

6.6.5.4   Protein kinase phosphorylation

Sensitisation of LAD2 cells with IgE results in a small reduction in phosphorylation of measured protein kinases (**Figure 6.38, A & B**). This pattern was also observed when non-IgE sensitised LAD2 cells were exposed to *S. aureus* (**Figure 6.38, C & D**). These reductions in phosphorylation of protein kinases did not reach statistical significance.

When LAD2 cells were infected with *S. aureus*, in the presence of prior IgE sensitisation, there was significant reduction in the phosphorylation of Atk2 ($p \leq 0.05$) and GSK-3α/β ($p \leq 0.001$) (**Figure 6.39, A & B**). Thus, *S. aureus* infection, in the presence of prior IgE sensitisation, downregulates phosphorylation pathways.
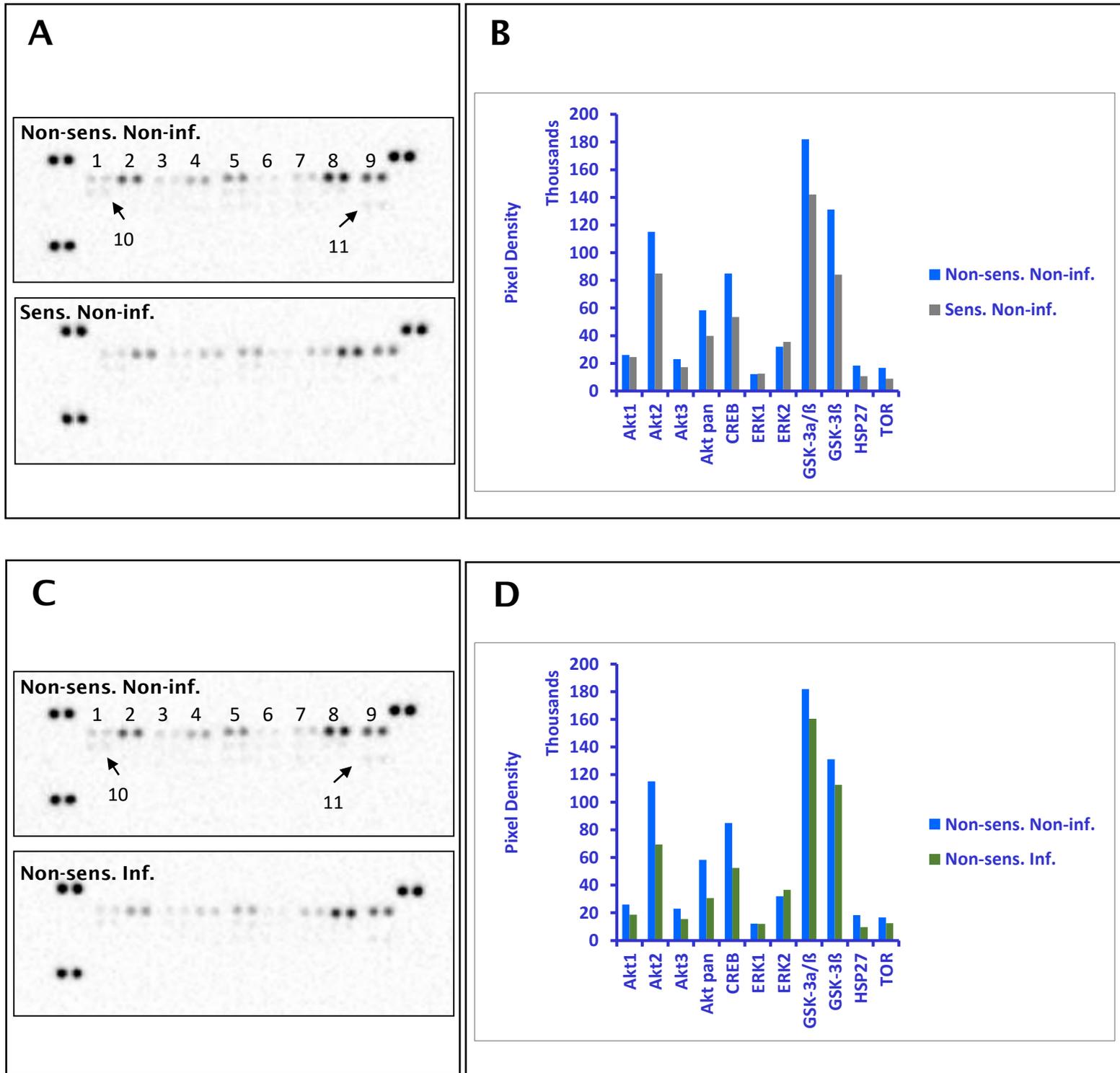
Figure 6.38 Pixel density of phospho-mitogen-activated protein kinases within LAD2 cell cultures, with or without IgE sensitisation/*S. aureus* infection. IgE stimulation was performed overnight, with infection undertaken using a CRS *S. aureus* isolate (MOI 1:1) over 4 hours. A,C) Pictures of membranes corresponding to graphs, B) Effect of IgE sensitisation on phosphorylation pathways in non-*S. aureus* infected LAD2 cells, D) Effect of *S. aureus* infection on non-IgE sensitised LAD2 cells. Two-way ANOVA was undertaken, p≤0.05 (*). Assay undertaken by Rana Abadalkareem (PhD student).
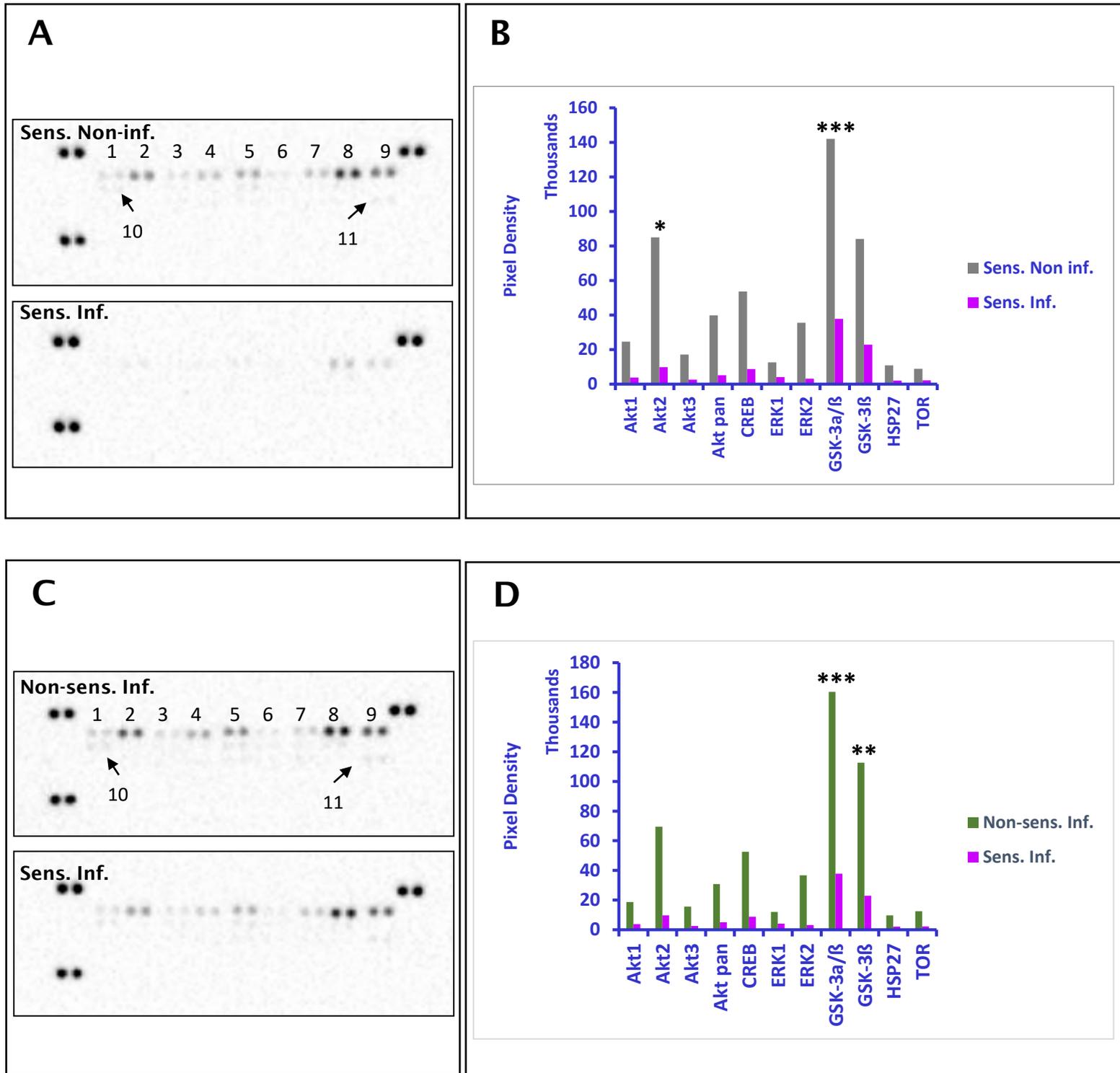
Figure 6.39 Pixel density of phospho-mitogen-activated protein kinases within LAD2 cell cultures, with or without IgE sensitisation/*S. aureus* infection. IgE stimulation was performed overnight, with infection undertaken using a CRS *S. aureus* isolate (MOI 1:1) over 4 hours. A,C) Pictures of membranes corresponding to graphs, B) Effect of *S. aureus* infection on IgE sensitised LAD2 cells, D) Effect of IgE sensitisation on *S. aureus* infected LAD2 cells. Two-way ANOVA was undertaken, p≤0.05 (*). Assay undertaken by Rana Abadalkareem (PhD student).

6.7 Discussion

S. aureus internalisation and viability within mast cell lines

Results have revealed that HMC-1 and LAD2 cell lines readily internalise a CRS-specific strain of *S. aureus*. This was found to bind to the surface of mast cells at 2 hours, with internalisation occurring from 2 hours onwards, and the number of viable intracellular *S. aureus* steadily rising up to 24 hours. Like in epithelial cells, internalised *S. aureus* remained viable within mast cells and capable of propagating extracellular infection.

When examining the ability of mast cells to internalise *S. aureus*, there was a significant reduction in the numbers of viable *S. aureus* found within LAD2 vs HMC-1 cells. This could be related to the differences in maturity, and immune profiles, of these two cell types. When culturing *S. aureus* infected HMC-1 cells in the presence of antibiotics (Lysostaphin) that do not cross the cell membrane, there was no significant increase in the number of *S. aureus* between 4 and 24 hours. This was not the case in earlier experiments where there was a large increase in intracellular *S. aureus* between 6 and 24 hours, even following extracellular *S. aureus* eradication at 6 hours. This effect is likely to be related to release of intracellular *S. aureus* into the extracellular space, followed by replication, re-infection and re-internalisation, as opposed to replication within the intracellular space. The absence of intracellular replication has also been observed by Abel et al (2011), using *S. aureus* infected HMC-1 mast cells, with no reported increase in the number of intracellular *S. aureus* after 5 days, following the eradication of extracellular *S. aureus* with antibiotics (143).

When SEB was added to *S. aureus* and mast cell co-cultures (HMC-1), there was a significant increase in the number of intracellular *S. aureus* at 24 hours, compared to *S. aureus* alone. As previously discussed, this observation was also made in the epithelial cell line (RPMI 2650), and could be related to the function of SEB acting as a bacterial virulence factor, promoting the uptake and internalisation of *S. aureus* into non-professional phagocytes (191).

Whilst mast cells are well established in allergic inflammation, their role in bacterial infections is not well understood. Mast cells are thought to play an important role in a number of serious bacterial

infections including *Klebsiella* pneumonia, Mycoplasma pneumonia and Listeria. Indeed, mast cell deficient mice are unable to clear *Klebsiella* pneumonia infection, which is reversed through the injection of mast cells into the peritoneal cavity (196). However, even though there is evidence of mast cells helping to effectively clear infections, there is recent evidence that *S. aureus* are able to use mast cells as a protective niche, to evade detection and clearance (25, 143, 194). Abel et al (2011), using bone-derived murine mast cells and the HMC-1 line, showed a mast cell response to kill *S. aureus* infection through the release of pre-formed mediators and extracellular traps (143). *S. aureus* was able to subvert these killing mechanisms through internalisation, with *S. aureus* remaining viable for up to 5 days. Furthermore, Hayes et al (2015) has shown intracellular localisation of *S. aureus* within mast cells in nasal polyps (25).

This work has revealed the ability of a CRS specific *S. aureus* strain to readily internalise within multiple mast cell lines, survive intracellularly and propagate ongoing extracellular infection. Furthermore, SEB has been shown to enhance the intracellular uptake of *S. aureus* into mast cells. Thus, it appears that *S. aureus* is parasitising mast cells, facilitated through the action of its own secreted toxins (SEB), to circumvent its own clearance, and aid its ongoing survival.

The immune response of mast cells to *S. aureus*

There was a noticeable difference in the immune response of HMC-1 mast cells compared to LAD2 mast cells against *S. aureus* infection. In HMC-1 cells exposed to live *S. aureus*, there was significant downregulation of IFNγ, IL-5, IL-6, IL-1β, TLR-2, MMP-7 and defensin 5 gene expression. In contrast, there was significant upregulation of TNFα and IL-5 gene expression in live *S. aureus* infected LAD2 cells. The widespread downregulation of pro-inflammatory cytokines in HMC-1 mast cells could explain the propensity of these cells to internalise significantly more *S. aureus* than the LAD2 cells, which display a more potent pro-inflammatory response. Research conducted by Swindle EJ et al (2015) has shown the ability of IFNγ to prime primary human mast cells for enhanced anti-bacterial

and anti-inflammatory responses to *S. aureus*. Downregulation of IFNγ gene expression could provide an environment which is more favourable for *S. aureus*, thus promoting its own survival.

Live *S. aureus* infection of HMC-1 cells caused significant downregulation of TLR-2 gene expression. TLR signalling is important in the cellular activation and recruitment of other immune cells to the site of infection, and its downregulation or inhibition could propagate ongoing *S. aureus* infection. Previous studies have associated TLR-2 and CD14 downregulation with worsening outcomes in sepsis (201), and the use of TLR-2 ligands have been used to reduce the incidence of *S. aureus* associated bacterial endophthalmitis in mice (202). Thus, TLR signalling is important in fighting bacterial infections, and its downregulation in mast cells treated with *S. aureus* could be significant.

In chapter 3, the significant downregulation of IL-1β gene expression in CRSwNP samples compared to CRSsNP and controls was shown, and chapter 4 provided evidence of significant downregulation of IL-1β gene expression in nasal polyps following stimulation with SEB. In this chapter, results show significant downregulation of IL-1β gene expression in *S. aureus* infected HMC-1 cells. This could provide further evidence of possible phagocytic dysfunction and a disordered immune response, compromising the ability to effectively clear *S. aureus*, with development of a chronic inflammatory state. On the whole, neither SEB nor dead *S. aureus* had a significant effect on the immune response of HMC-1 or LAD2 mast cells, highlighting the importance of live bacterial-cell interactions, including internalisation of live *S. aureus,* to propagate the immune modulated effects outlined here.

When examining cytokine concentrations in the culture supernatant, both mast cell lines were associated with release of cytokines into the extracellular space following *S. aureus* exposure, and these levels peaked following internalisation of live *S. aureus*. This release of cytokine will attract other immune cells to the site of infection, including further mast cells that may uptake further *S. aureus*, contributing to the development of a chronic inflammatory state.

Immunomodulatory effects of repeated *S. aureus* internalisation and release in mast cells

It is evident that *S. aureus* resides within nasal polyp mast cells, and previously presented results highlight the capacity of CRS-specific *S. aureus* strains to internalise within mast cell lines, propagating ongoing infection through its release into the extracellular space. Therefore, it is likely that in CRSwNP patients with *S. aureus* infection, there will be a continual cycle of bacterial uptake and release from mast cells. Further study of this reported uptake and release was important, particularly in relationship to whether this process resulted in a phenotypic change in the *S. aureus* following multiple internalisation cycles. This work is both unique and novel.

Findings show that the number of viable intracellular *S. aureus* do not change with more internalisations. Indeed, when *S. aureus* that had been internalised 5 times previously (P3.5) was compared to the original strain (P3), there was no significant difference in the number of intracellular *S. aureus* colonies. However, when examining different strains of *S. aureus* following increasing numbers of internalisation cycles, P3.1 to P3.5, there was notable differences in the immune response of these strains to newly exposed HMC-1 and LAD2 mast cells. In *S. aureus* infected HMC-1 cells there was a gradual downregulation of pro-inflammatory gene expression when *S. aureus* went through increasing numbers of internalisation cycles (P3 to P3.5). In LAD2 cells there was an initial upregulation of pro-inflammatory gene expression when comparing the P3 to P3.1 strain, but this was followed by a sharp downregulation of pro-inflammatory gene expression when comparing the P3.1 to P3.5 strain. Thus, it seems that when *S. aureus* is taken up to into mast cells, released (5 in this experiment) and re-exposed to naive HMC-1 or LAD2 cells there is a significantly downregulated immune response. Similar trends have also been observed for the cytokine concentrations in the culture supernatants, with reduced pro-inflammatory cytokine release when HMC-1 and LAD2 cells were exposed to P3.1 vs P3.5 strains. These changes suggest that with increased internalisations, the *S. aureus* becomes less immunogenic, possibly improving its chance of survival.

The importance of IgE mechanisms in the activation of *S. aureus* infected mast cells

When LAD2 cells were not sensitised with IgE prior to anti-IgE treatment, there was an associated low level of β-hexosaminidase release, as would be expected in this control situation. There was a slight increase in β-hexosaminidase release following *S. aureus* infection, in non-IgE sensitised LAD2 cells, but the rate of degranulation was still below 10%. Other authors, using primary mast cells as opposed to mast cell lines, have reported similar rates of β-hexosaminidase release (approx. 5-25%) in response to *S. aureus* infection (143, 170).

When LAD2 cells were pre-sensitised with IgE and treated with anti-IgE, there was an elevated level of β-hexosaminidase release, approx. 15-20%, which is expected as this is the classical way mast cells are activated, degranulating in response to allergic stimuli (203). However, when these same IgE sensitised, and anti-IgE exposed, LAD2 cells were infected with *S. aureus* there was a significant reduction (inhibition) in β-hexosaminidase release. This inhibition was more pronounced once the *S. aureus* had entered the intracellular compartment (at 4 hours).

There was also a reduction in β-hexosaminidase release from *S. aureus* infected LAD2 cells following calcium ionophore treatment, irrespective of IgE sensitisation. The lack of effect of IgE sensitisation in combination with calcium ionophore is expected, given that calcium ionophore works independently of IgE signalling. Thus, it is evident from these experiments that the reduction in β-hexosaminidase release following *S. aureus* infection is occurring through IgE mediated and non-IgE mediated pathways, enhanced by the intracellular uptake of *S. aureus*.

Previous research examining the effect of bacterial infection on degranulation of mast cells in the presence of IgE and anti-IgE, has identified diminished responses in the infected groups compared to non-infected groups. This was true for *Bifidobacteria* (204), *Lactobacillus* (205) and *E. coli* (206). Metcalfe et al (2006) found a downregulation of FCεR1 surface expression in *E. coli* infected primary mast cells (206). This was also observed in a study examining *Lactobacillus rhamnosus* infection of mast cells (207), which suggests the decrease in degranulation may be linked to reduced surface expression of FCεR1, and therefore reduced responsiveness to IgE signalling (194). However, as only a

limited number of FCεR1 receptors need to be activated to stimulate degranulation, and suppression of degranulation was also observed following recovery of FCεR1 receptors after longer exposure times to *Lactobacillus* (205), down-regulation of FcεRI may not be the only reason for the observed decrease in degranulation. This conclusion is further supported by the observation of downregulated degranulation using IgE/FcεRI receptor independent mechanisms, through calcium ionophore (208). These results support observations presented here using *S. aureus*, highlighting the likelihood of a shared pathophysiological mechanism amongst different bacterial strains.

When examining LDH production following stimulation and *S. aureus* infection of LAD2 cells, there was a significant increase in LDH production in *S. aureus* infected groups at 4 hours of co-culture. Although increased, these values were within acceptable limits, and revealed that the reduction of degranulation was not wholly attributable to cell toxicity. Furthermore, inhibition was evident at 2 hours, with no associated rise in LDH concentrations seen.

When examining the pro-inflammatory response, there was evidence of downregulated TNFα, IL-8 and IL-1β gene expression following IgE sensitisation of *S. aureus* infected LAD2 cells, which was statistically significant with the addition of anti-IgE. This drop was evident on the gene and protein levels (although not statistically significant). Research to date has identified elevated IgE in patients with CRS (24), and that this IgE can be directed against *S. aureus* and the toxins it produces (24). Furthermore, when IgE against SAE was present in nasal polyps, there were higher levels of recurrent disease and requirements for revision surgery (209). The rates of co-morbid asthma and aspirin sensitivity are also higher (80). Therefore, IgE is associated with nasal polyps and disease chronicity, and in mast cell IgE sensitisation lessened the associated pro-inflammatory response. Ultimately, this could result in reduced clearance of *S. aureus* from nasal polyps, facilitating its survival and promoting a persistent low-level chronic inflammatory state.

Finally, the study of protein kinase phosphorylation in LAD2 cells has given further mechanistic insight into possible role of IgE sensitisation in *S. aureus* - mast cell interactions. Results have revealed widespread downregulation of protein kinase phosphorylation following IgE sensitisation of *S. aureus*

infected LAD2 cells. This reached statistical significance for Glycogen synthase kinase 3 (GSK-3) α/β and GSK-3β. GSK exists in 2 isoforms (α and β) and regulates the function of over 50 substrates involved in various essential cellular functions, including glycogen metabolism, cell cycle control, apoptosis, embryonic development, cell differentiation, cell motility, microtubule function, cell adhesion and inflammation (210). GSK-3β inhibition has been shown to suppress proinflammatory cytokines whilst augmenting production of anti-inflammatory IL-10 in response to multiple TLR signalling pathways, through NF-κB and CREB interacting with the coactivator CBP (211). Downregulation of GSK-3β phosphorylation has also been shown to reduce the pro-inflammatory response of *S. aureus* infection (212), lipopolysaccharide (LPS)-stimulated macrophages and monocytes (211, 213, 214), and *Mycoplasma tuberculosis* infection (215). Therefore, downregulation of GSK-3β in response to IgE sensitisation and *S. aureus* infection of LAD2 cells highlights a possible mechanism used by *S. aureus* to downregulate cytokine production within mast cells. What is evident is that *S. aureus* uptake into mast cells results in multiple effects through many different pathways, and its therefore unlikely that a single mechanism will be identified that would explain how *S. aureus* alters so many cell functions to promote its own survival. Further work is required to fully elucidate these effects and pathways.

Clinical implications

These findings highlight the importance of *S. aureus* in promoting the development of a chronic inflammatory state. However, as highlighted, in atopic patients who have elevated IgE levels and associated worse post-operative outcomes (216-218), *S. aureus* is able to limit mast cell degranulation and release of proinflammatory cytokines. By limiting mast cell activation, and the subsequent influx of inflammatory cells, *S. aureus* may constitute a mechanism by which it evades the immune system, thus promoting its own survival. Controlling the allergic component may improve treatment outcomes in CRS patients, especially those with *S. aureus* colonisation.

6.8 Conclusion

This chapter has revealed the ability of *S. aureus* and IgE sensitisation to modulate the immune response of mast cells. *S. aureus* readily internalises within mast cells and has the ability, in the presence of IgE, to downregulate degranulation, pro-inflammatory responses, and protein kinase phosphorylation. These results not only highlight the ability of *S. aureus* to adapt and survive, but also the importance of IgE signalling, accentuated through previous *S. aureus* colonisation and co-morbid asthma/allergy. A better understanding of these survival strategies by *S. aureus* will enable the development of better targeted therapies for CRS in the future.

7 Conclusions

7.1 Summary of Findings

7.1.1 Upregulated cytokine levels within non-polypoidal mucosa in CRS

This study has revealed significant upregulation of pro-inflammatory cytokines in CRSwNP tissues compared to controls. Results revealed downregulation of cytokines associated with bacterial clearance (e.g. IL-1β) suggesting the possibility of a disordered immune response towards bacteria, which may facilitate their survival and the development of chronic inflammation. Furthermore, the analysis of mucosal samples has revealed upregulated pro-inflammatory cytokines within the non-polypoidal sinonasal mucosa of CRSwNP patients. This indicates the propensity of progression from macroscopically normal non-polypoidal mucosa to frank polyposis, and as such the importance of continued medical therapy in such patients to supress the inflammatory response, reducing new nasal polyp development, and their recurrence following surgery.

7.1.2 SEB acts as a driver of chronic inflammation

Findings outlining the effect of SEB on nasal polyps, have supported the role of bacteria in initiating a potent immune response. SEB stimulation of nasal polyps resulted in significant upregulation of pro-inflammatory gene expression, and cytokine release into the culture supernatant. These results highlight the importance of bacteria and their toxins in driving inflammation in CRSwNP patients and contributing to the development of chronicity. They also reveal the effect of SEB on downregulating IL-1β gene expression in nasal polyp samples, which may provide additional insight into bacterial toxins further compromising immune responses. It is likely that bacteria, and in particular *S. aureus*, play an important role in CRS and the development of recalcitrant disease and chronicity, facilitated through the secretion of bacterial toxins.

7.1.3 The epithelial barrier in response to *S. aureus*

S. aureus was able to survive intracellularly and re-populate extracellular infection through its own replication and release from mast cells. Furthermore, SEB amplified the ability of *S. aureus* to internalise and reside within epithelial cells, and infection resulted in a potent immune response on gene and protein levels. *S. aureus* infection of the epithelium is likely to contribute significantly towards the development of a chronic inflammatory state with increased levels of cytokines and proinflammatory mediators, resulting in localised tissue oedema. These changes may drive the formation and growth of nasal polyps downstream.

7.1.4 Mast cell – *S. aureus* interactions and the importance of IgE signalling

Similar to *S. aureus* infected epithelial cells, mast cells readily internalised *S. aureus* which was able to remain viable and repopulate eradicated extracellular populations. Internalisation of *S. aureus* was also amplified in the presence of SEB.

Immature mast cells (HMC-1) were found to release pro-inflammatory cytokines during *S. aureus* infection, but on the whole downregulated their pro-inflammatory gene expression, promoting the uptake of *S. aureus* in greater numbers within these cells. The more mature LAD2 cells displayed a greater immune response, through upregulation of pro-inflammatory gene expression and release of cytokine into the supernatant, but had less *S. aureus* residing within them. Likely as a result of their enhanced immune response, resisting the uptake and intracellular survival of *S. aureus*.

When examining mast cell degranulation, *S. aureus* infection of allergic sensitised LAD2 cells resulted in a drop in maximal degranulation, which could represent a strategy to limit influx of mast cells and other immune cells which would otherwise clear infection. When examining the associated immune responses, it was evident that IgE sensitisation was able to downregulate pro-inflammatory cytokine gene expression and inhibit cytokine release from *S. aureus* infected LAD2 cells. Furthermore, protein kinase phosphorylation, especially GSK-3β, was also downregulated in *S. aureus* infection and IgE sensitisation (in *S. aureus* infected LAD2 cells). Therefore, IgE appears to play an important role in

regulating the immune response to infection. This could provide an environment which is more favourable for *S. aureus* survival. This is relevant as the presence of allergic sensitisation is a common finding in CRS patients and is usually associated with a more resistant clinical course. The findings of downregulated immune responses in the presence of IgE sensitisation may facilitate the survival of *S. aureus* and development of low level chronic inflammation, thus tying in with these observations.

7.1.5 Intracellular *S. aureus* – a potential driver of nasal polyp formation and growth. Proposed mechanistic model.

My predecessor, Mr Stephen Hayes, made the observation of intracellular *S. aureus* residing in nasal polyp mast cells, a process accentuated through the presence of SEB. When intracellular uptake of *S. aureus* was studied in a mast cell line (HMC-1), there was cell rupture and degranulation, hypothesised to result in increased tissue oedema, and therefore nasal polyp development. My work as extended these observations, both in mast cells and epithelial cells. Data outlined during my PhD has been used to construct, and propose, an illustrated model of how intracellular *S. aureus* may act as a potential driver for nasal polyp formation and growth in chronic rhinosinusitis (**Figure 7.1**).

S. aureus initially breaches the epithelial barrier, aided through the action of SEB. Intracellular uptake of *S. aureus* further compromises the integrity of the epithelial barrier, but also acts as a reservoir of bacteria, which can re-populate eradicated surface colonies.

Live *S. aureus* exposure to the epithelium results in a potent immune response. This further compromises the integrity of the epithelium, but also recruits other immune cells, including mast cells, to the area. These processes are likely to contribute towards localised oedema which may begin to form nasal polyps.

Mast cells interact with *S. aureus* and uptake bacteria into the intracellular space. *S. aureus* is protected within this intracellular niche, and can be released to propagate ongoing infection. *S. aureus* results in pro-inflammatory cytokine release and pre-formed granule mediators, although this is limited through the sensitisation of IgE and multiple *S. aureus* internalisation and release cycles from mast cells. Through these mechanisms, *S. aureus* is able to continue to survive and produce ongoing low-level pro-inflammatory effects. The release of mediators contributes towards chronic inflammation, resultant tissue oedema, and the development and growth of nasal polyps.
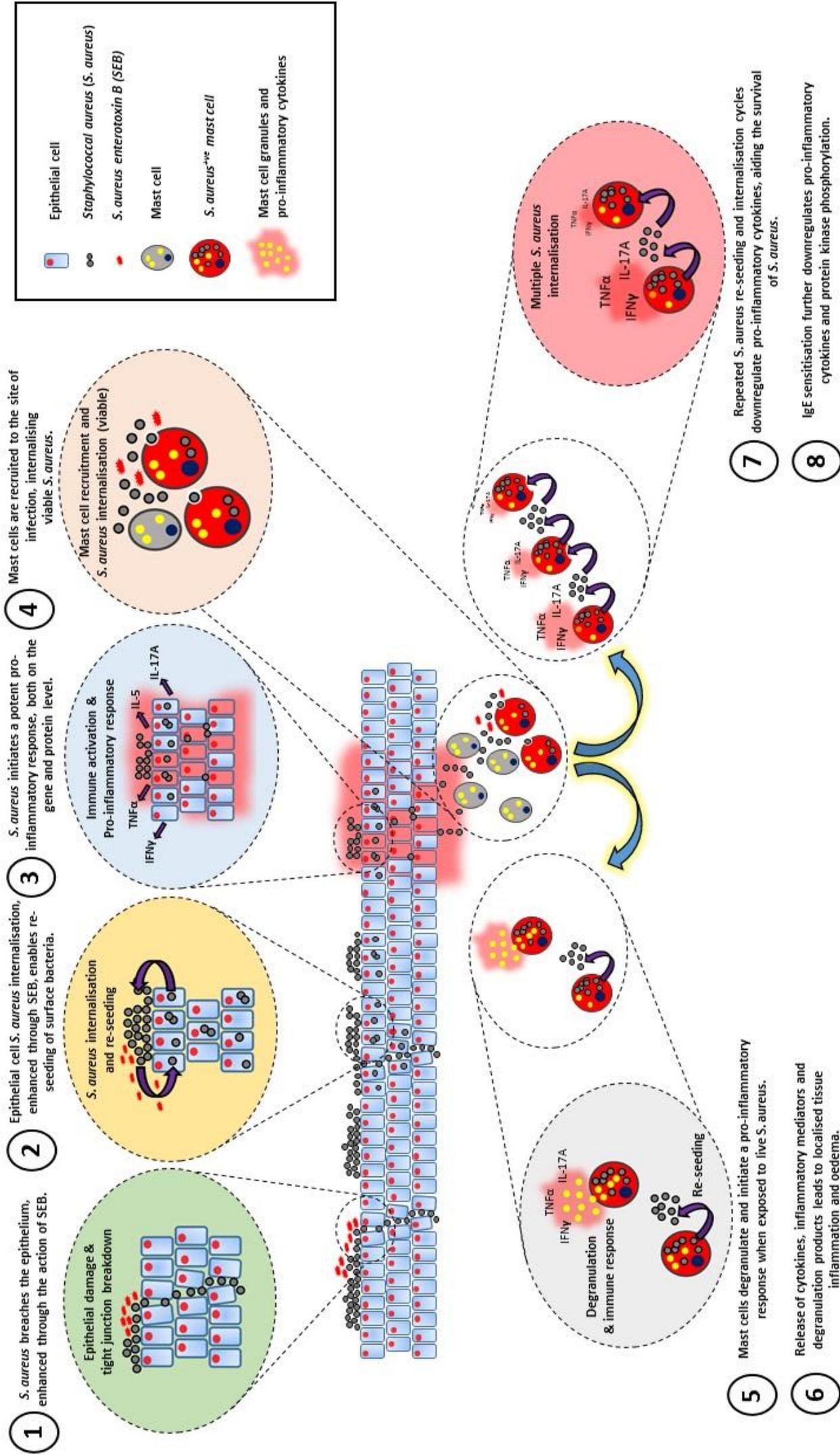
Figure 7.1 Proposed illustrated model of how intracellular *S. aureus* may promote a chronic inflammatory reaction

Legend:

- Epithelial cell
- *Staphylococcal aureus* (*S. aureus*)
- *S. aureus* enterotoxin B (SEB)
- Mast cell
- *S. aureus*⁺ᵛᵉ mast cell
- Mast cell granules and pro-inflammatory cytokines

1 *S. aureus* breaches the epithelium, enhanced through the action of SEB.

Epithelial damage & tight junction breakdown

2 Epithelial cell *S. aureus* internalisation, enhanced through SEB, enables re-seeding of surface bacteria.

S. aureus internalisation and re-seeding

3 *S. aureus* initiates a potent pro-inflammatory response, both on the gene and protein level.

Immune activation & Pro-inflammatory response
TNFα IFNγ IL-5 IL-17A

4 Mast cells are recruited to the site of infection, internalising viable *S. aureus*.

Mast cell recruitment and *S. aureus* internalisation (viable)

5 Mast cells degranulate and initiate a pro-inflammatory response when exposed to live *S. aureus*.

Degranulation & immune response
TNFα IFNγ IL-17A
Re-seeding

6 Release of cytokines, inflammatory mediators and degranulation products leads to localised tissue inflammation and oedema.

7 Repeated *S. aureus* re-seeding and internalisation cycles downregulate pro-inflammatory cytokines, aiding the survival of *S. aureus*.

Multiple *S. aureus* internalisation
TNFα IFNγ IL-17A

8 IgE sensitisation further downregulates pro-inflammatory cytokines and protein kinase phosphorylation.

7.2 Limitations

All possible precautions were taken to limit possible bias and errors in RT-qPCR experiments. RT-qPCR is a technique that measures gene expression ratios, normalised to controls. Due to the number of steps, complexity and the possibility of error, there are technical limitations inherent in any RT-qPCR assay. These possible limitations involve errors in template quality, operator variability, reverse transcription and amplification, and possible subjectivity in data analysis and reporting. These errors were minimised through operator training, validation of template quantity and quality (NanoDrop), followed by standardisation of techniques. Together with amplification and melt curve analysis, ensuring primers and template ran correctly, all samples were run in duplicate to ensure consistency and samples excluded if errors were detected. All measures were taken to reduce possible error and increase reproducibility. Furthermore, where possible both gene expression and protein production were measured, to further validate results.

Sample sizes were relatively small, due to limitations in tissue availability. This was an issue particularly during the winter months when bed pressures in the hospital were at their peak. Given the tertiary nature of patients undergoing surgery at Southampton and their severe nasal polyp disease, it was often difficult to obtain non-polypoidal mucosa for use in the combined analysis. In addition, due to complexity of disease many patients were on antibiotics or oral steroids prior to surgery, and/or had other significant associated diseases (primary ciliary dyskinesia, cystic fibrosis or chronic lung conditions) thus excluding them from the study. Larger sample sizes would have benefited the statistical analysis and generation of more robust results.

All patients within this study were selected from the same geographical region, and on the whole displayed similar ethnicity. Nasal polyps from Asian countries differ in their immunological profile (132), and as such the results presented here are applicable to the local population.

8 Future Work

8.1 Future work

Confirmation and extension of work using primary epithelial cells

- Undertaking primary epithelial culture models would validate results obtained using the RPMI 2650 cell line.

- This could be accomplished with a tissue explant model or air liquid interface model using nasal brushings in patients with CRS

- This work would confirm the intracellular uptake of *S. aureus* into primary epithelial cells, and further investigate the effect of infection on epithelial barrier function.

- Results in chapter 5 highlighted the downregulation of TLRs in response to live *S. aureus* infection, possibly as a bacterial survival mechanism. Further study using TLR agonists and antagonists would be of value in exploring these mechanisms in greater depth.

Confirmation of results using primary mast cells

- Extraction of tissue resident mast cells would provide validation of results conducted using representative cell lines.

- This work would confirm the intracellular uptake of *S. aureus* into primary mast cells, its survival and release into the extracellular space, and associated host immune response.

- This work may prove challenging, given the difficulty and complexity of harvesting terminally differentiated mast cells from mucosal tissues. However, given adequate time and resource including experimental optimisation, this should be feasible.

Extension of work using primary mast cells

- It is evident that *S. aureus* internalises and remains viable within mast cells, however it is unclear how bacterial viability is maintained.

- Results presented reveal the immunomodulatory effects of *S. aureus*, through prior allergic sensitisation. Experiments examining the survival of intracellular *S. aureus* following allergic sensitisation, will confirm whether this is a potential survival strategy for *S. aureus*.

- Proteomics or genome sequencing may be beneficial to further elucidate the pathways used by *S. aureus* to modulate the immune response and activation of mast cells.

- The study of pathway inhibitors may also shed light on the mechanisms utilised by *S. aureus*. Findings of chapter 6 identified downregulated phosphorylation of GSK-3α/β in IgE sensitised and infected LAD2 cells. Use of a GSK-3α/β inhibitor would be a first step in identifying whether this is a potential mechanism used by *S. aureus* to ensure its intracellular survival, and downregulation of cytokine gene expression and protein release.

- TLR inhibitors would also be of value, as this is one of the main ways *S. aureus* is likely to interact with mast cells, independent from IgE signalling.

Further studies of bacterial-related factors

- Phenotypic analysis of *S. aureus*, especially following its repeated uptake and release from mast cells, would be of value in identifying how *S. aureus* is able to adapt and manipulate the host immune response.

- Study of further bacterial strains would provide insight into possible shared mechanisms in other relevant diseases, such as *Pseudomonas aeruginosa* which is associated with CRS in CF patients.

Timothy C Biggs

Appendix I

Timothy C Biggs

PATIENT CONSENT FORM

Regulation of inflammation in the upper airways

Mr T Biggs, Mr S Hayes, Mr P Harries, Dr S Pender, Mr R Salib

Please initial box

1. I confirm that I have read and understand the information sheet dated 06/07/2012 (version 5) for the above study. I have had an opportunity to consider the information, ask questions and have had these questions answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

3. I agree to my nasal tissue, taken as part of my surgery, being stored for the duration of this study for use in this research project into   inflammatory processes.

4. I agree to my nasal tissue, taken as part of my surgery, to be transferred securely and anonymously to those centres to get the best information possible with respect to the assessing the presence of bacteria and fungi

5. I agree to genetic material being extracted from my nasal tissue to investigate genes which may be involved in inflammation.

_____   _____   _____

Name of Patient   Date   Signature

_____   _____   _____

Name of Person taking consent   Date   Signature

(if different from researcher)

_____   _____   _____

Name of Researcher   Date   Signature

When completed, 1 copy for patient; 1 copy for researcher site file; original to be kept in patient's medical notes

Appendix II

PATIENT INFORMATION SHEET

Regulation of inflammation in the upper airway

You are due to have nasal, sinus or skull base surgery (e.g. pituitary gland, repair of brain fluid leak, with access for the surgery through your nose and sinuses). We would like your help in our research investigating the causes of chronic sinusitis. Chronic infection of the sinuses (rhinosinusitis) with or without polyps is an extremely common clinical condition affecting up to 15% of the population within the United Kingdom. It is more common than arthritis or high blood pressure. The impact of the disease is often under-appreciated with afflicted patients having a significantly impaired quality of life. One of the unanswered and most pressing questions is whether the ongoing inflammation in chronic rhinosinusitis may be related to an underlying reaction to bacteria or fungus in the lining of the nose. In addition, the reason why polyps start is poorly understood but inflammation is known to be involved. Through a better understanding of the mechanisms of inflammation in chronic rhinosinusitis (with and without polyps), it is anticipated that improved treatments and better-targeted surgery will be possible in the future.

You are being invited to take part in a research study. Before you make your decision, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your doctors if you wish. Please ask us for further information if anything is unclear. Take time to decide whether or not you wish to take part.

Part one

What is the purpose of the study?

The purpose of this study is to help us understand the mechanisms that control inflammation in the nose and upper airways. The causes of chronic rhinosinusitis and nasal polyps are not completely understood. Therefore, current treatments tend to be very non-specific and may not be as effective as would be desirable. In this study we want to carry out experiments in the laboratory on the tissue removed as part of normal surgery, which we hope will help us to improve our understanding of how inflammation is controlled and what organisms (bacteria or fungi) are involved in driving the inflammatory reaction leading to chronicity of the disease process, with the ultimate aim of developing new drugs to treat polyps and/or rhinosinusitis.

Why have I been chosen?

You have been asked to take part in this study because your surgery involves removal of tissue from your nose and sinuses. The tissue which will be removed would usually be destroyed or thrown away. We are asking patients if we can use this tissue in laboratory experiments to help us understand the inflammation that causes nasal polyps and sinusitis.

Do I have to take part?

No, it is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You will still be free to withdraw from the study at any time without giving a reason. This will in no way affect the surgery you will be undertaking or the standard of care you receive as a patient.

What will happen to me if I take part?

Taking part in this study will in no way change what happens during the surgery or your subsequent medical care. We will only use tissue that has been removed as part of the normal course of your operation. No material will be removed specifically for the purpose of this study and your operation will be exactly the same whether you decide to participate in the study or not. We wish to ask you a few simple questions about your history of allergy or other disease to aid us with our studies, but this will be entirely optional.

Some of the samples will also be sent to those specialist centres to get the best information possible with respect to the assessing the presence of bacteria and fungi.

What are the possible side effects of taking part?

There are no side effects as a result of allowing us to take your nasal tissue that has already been removed by the surgeon as part of your routine surgical treatment.

What are the possible benefits of taking part?

Agreeing to participate in this study will be of no direct benefit to you. The information we gain from the study will hopefully lead to a better understanding of the inflammation that occurs in nasal polyps and sinusitis, and may lead to the development of new therapies.

What if there is a problem?

We do not anticipate any problems as entering into our study will not alter your normal course of care. However, any complaint about the way you have been dealt with during the study will be addressed; the detailed information on this is given in part two.

Timothy C Biggs

Will my taking part in the study be kept confidential?

Yes. All the information about your participation in the study will be kept confidential. Details are included in part two.

Contact Details

Mr Rami Salib

Chief Investigator

Telephone: 02380 796364

Email: R.J.Salib@soton.ac.uk

This completes part one of the information sheet. If the information in part one has interested you and you are considering participation, please continue to read the additional information in part two before making any decision.

Timothy C Biggs

Part two

What if there is a problem?

There are unlikely to be side effects from you allowing your nasal tissue to be used for research. This will not alter your surgery, or your treatment afterwards.

This study is covered by the indemnity of the University of Southampton. If you are harmed by taking part in this research study, there are no special compensation arrangements. However, if you are harmed due to someone's negligence then you may have grounds for legal action, but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way you have been approached or treated during this study, the normal National Health Service complaints process may be available to you.

Will my taking part in this study be kept confidential?

All tissue received during this study will be treated confidentially. The sample of tissue that we receive from you will be assigned a code on collection, by which it will be referred from that point onwards. It will not be possible for anyone to identify you from the results of this study. Any information about you which leaves the hospital, including samples transferred to other centres, will have your name and address removed so that you cannot be recognised by it.

Storage of samples and genetic material

Some of the tissue you donate may be stored in our laboratory for further research as part of this study. In addition, we may wish to store DNA so that we can look at genes that may be relevant to inflammation, but the DNA will not be used for any other purpose. All samples will be coded and will not carry your name or other identifiable information. Samples will be stored until the end of the study, after which any unused material will be destroyed. If you are happy for us to store your tissue but not your DNA, please make this clear to us and we will comply with your wishes. However, as

214

previously explained, the samples will not be identifiable as having come from you and will only be used for the purpose of research and not for any diagnostic purpose. Results and data from the study will be stored for up to 15 years, as required by law. No information about you will be revealed to third parties without your written consent.

What will happen to the results of the research study?

It is anticipated that the results of the study will be published in a scientific journal. It will not be possible for anyone to identify you in any report or publication.

Who is organising and funding the research?

This research is organised by investigators in the University of Southampton and is funded by research grants to the University. None of your doctors will receive extra payment as a result of you agreeing to be involved in this study.

Who has reviewed the study?

This study has been reviewed by the Southampton and South West Hampshire Research Ethics Committee and was given a favourable ethical opinion for conduct in the NHS.

Thank you for taking the time to read this information sheet. If you decide to participate in the study, please keep this sheet with your copy of the signed consent form.

Timothy C Biggs

Appendix III

RT-qPCR primer sequences

Gene name	Manufacturer	Sense primer	Anti-sense primer
T-bet	Primerdesign	CAGCACCAGACAGAGATGATC	GCCACAGTAAATGACAGGAATG
RORγT	Primerdesign	TGCCAGAATGACCAGATTGTG	GCCACCGTATTTGCCTTCA
TGF-β1	Primerdesign	CACTCCCACTCCCTCTCTC	GTCCCCTGTGCCTTGATG
IFNγ	Primerdesign	TCAACTTCTTTGGCTTAATTCTCTC	ATATGGGTCCTGGCAGTAACA
IL-1β	Primerdesign	TGGCAATGAGGATGACTTGTTC	CTGTAGTGGTGGTCGGAGATT
MMP-28	Primerdesign	CTCAATGAACAGGTCCCCAAAG	AACACGCCGCTGACAGGTA
FoxP3	Primerdesign	CAGCACATTCCCAGAGTTCCT	GCGTGTGAACCAGTGGTAGAT
IL-5	Primerdesign	CCCAGAGATAAGGTTCGTTCT	CAACCCCAGCATCTAGCATAG
GATA-3	Primerdesign	CTTCCCCTTCTTCTCTTTGCTAA	GGGTCGTTGAATGATTTGCTTT
TNFα	Eurofins	CCAGGGACCTCTCTCTAATCA	TGCTACAACATGGGCTACAG
IL-17A	Primerdesign	CCTCAGATTACTACAACCGATCC	CACTTTGCCTCCCAGATCAC
IL-23R	Sigma	ACAAGCCTACAGACTACAAGAAG	GGGGTTTATATCCAGTGTTGAGA
IL-23	Primerdesign	CAGGGCACCTTACTTCTGGAT	CAACATATAGGACCTGCTCACTG
MMP-7	Eurofins	GCTACACACAGAAGATGGAC	CAATAACTTGCCCAATGACAC
Defensin 5	Primerdesign	GACCATCGCCATCCTTGCTG	CAGACTGCTTCTGGGTTGTAG
IL-6	Primerdesign	GCAGAAAACAACCTGAACCTT	ACCTCAAACTCCAAAAGACCA
IL-8	Primerdesign	CAGAGACAGCAGAGCACAC	AGCTTGGAAGTCATGTTTACAC
TLR-2	Primerdesign	GCCGCAACTCAAAGAACTTTAT	GCATTCCTACTGATTTTCAATACTAG
TLR-4	Primerdesign	TGCCTTCACTACAGAGACTTTATTCC	GACACCACAACAATCACCTTTCG
MMP-9	Primerdesign	CTTCCAGTACCGAGAGAAAGC	CAGGATGTCATAGGTCACGTAG

RT-qPCR Housekeeping gene amplicon context information

Human ACTB:
Accession number: NM_001101
Anchor Nucleotide position: 1195
Context sequence length: 106bp

Timothy C Biggs

Appendix IV

Timothy C Biggs

SOP for Staphylococcus Aureus Isolation from CRS Tissue

The work in this appendix, the isolation of *S. aureus* from tissue samples, was undertaken by my predecessor Mr Stephen Hayes. These bacteria cultures were stored as a glycerol stock, which I used as part of my thesis.

S. aureus is a <u>facultatively anaerobic</u>, <u>Gram-positive</u> <u>coccus</u> and has large, round, golden-yellow colonies, often with <u>hemolysis</u>, when grown on <u>blood agar plates</u>.

S. aureus is <u>catalase</u>-positive (meaning that it can produce the enzyme "catalase") and able to convert <u>hydrogen peroxide</u> (H_2O_2) to water and oxygen, which makes the catalase test useful to distinguish staphylococci from <u>enterococci</u> and <u>streptococci</u>.

A small percentage of *S. aureus* can be differentiated from most other staphylococci by the <u>coagulase test</u>: *S. aureus* is primarily coagulase-positive (meaning that it can produce the enzyme "coagulase") that causes clot formation, whereas most other *Staphylococcus* species are coagulase-negative. However, while the majority of *S. aureus* are coagulase-positive, some may be atypical in that they do not produce coagulase.

1.1. Equipment & Chemicals

- o Blood agar (Oxoid)
- o Baird-Parker agar (Sigma)
- o Egg yolk tellurite as supplement for Baird-Parker agar (Oxoid/Sigma)
- o Trypticase soy broth (Oxoid/Sigma)
- o Brain Heart Infusion (Oxoid)
- o Hydrogen Peroxide (Sigma)
- o Hydrochloric acid (Sigma)
- o DNase agar (Sigma)
- o Coagulase slide test (Sigma)
- o Toluidine blue (Sigma)
- o Incubator set at 37 °C
- o Glass slides
- o Bunsen burner & lighter
- o Inoculation loops
- o Universal tubes

1.2. Culture and Identification of *S. aureus*

1.2.1. Primary isolation media

- Inoculate DNase agar and blood agar plates and grow at 37 °C for 18-24 h.
- Examine for colonial morphology (cream or golden colour up to 3mm in diameter)
- Baird-Parker agar (Sigma), supplemented with egg yolk tellurite (Sigma) can be used to identify *S. aureus* as shown in the Table 1 when incubated for 48 h at 37 °C.

Table 1.
Colonial morphology of *S. aureus* and other organisms on Baird-Parker agar.

Organisms	Colour of colony/comments
S. aureus	Grey-black, shiny colony due to reduction of tellurite. Surrounded by a zone of clearing
S. epidermidis	Not shiny black colony. Seldom produce clearing
S. saprophyticus	Irregular colony and may produce clearing/ Wide opaque zones may be produced in 24 h
Micrococcus	Very small colony in shades of brown and black. No clearing
Bacillus sp.	Dark matt brown colony with occasional clearing after 48 h
E. coli	Large brown-black colony
Proteus	Brown-black colony. No clearing

1.2.2. DNase production

This test is used to determine the ability of an organism to produce DNase. The test is used primarily to distinguish pathogenic Staphylcocci which produce large quantities of extracellular DNase. It reacts with medium containing DNA with the resulting hydrolysis of the DNA. The oligonucleotides liberated by the hydrolysis are soluble in acid and in a positive reaction, the addition of HCL results in a clear zone around the inoculum. A hazy zone is produced in a negative reaction, due the precipitation of DNA by HCL. In contrast to HCL, toluidine blue produces much more delineated zones of DNase activity.

Most strains of *S. aureus* hydrolyse DNA and give positive reactions in this test. However, some MRSA strains do not and some strains of coagulase-negative staphylocci may give weak reactions.

- Flood a DNase agar plate containing plated organism with Toluidine blue O solution (TBO/TBS @ 0.01-0.05 % [w/v] concentration) or 1M HCL.
- After 2 min, discard excess reagent
- TBO-positive reaction – TBO forms a complex with hydrolysed DNA to produce colonies surrounded by pink zone against a blue background
- HCL-positive reaction – colonies demonstrate a defined zone of clearing
- A negative result for both solutions equates to no zone of clearing.

1.2.3. Coagulase Production

Members of the genus Staphylococcus are differentiated by the ability to clot plasma by the action of the enzyme coagulase.

Coagulase exists in two forms: "bound coagulase" (or clumping factor) which is bound to the cell wall and "free coagulase" which is liberated by the cell wall. Bound coagulase is detected by the slide coagulase test, whereas free coagulase is detected by the tube coagulase test.

Bound coagulase absorbs fibrinogen from the plasma and alters it, so it precipitates on the Staphylococci causing them to clump resulting in cell agglutination. The tube coagulase test detects both bound and free coagulase. Free coagulase reacts with a substance in plasma to form a fibrin clot.

1.2.3.1. Slide Coagulase test

- Place a drop of distilled water on a slide
- Emulsify the test strain to obtain and homogenous thick suspension. False negative reactions will occur if the bacterial suspension is not heavy enough
- Observe for auto-agglutination
- Dip a loop in the plasma and mix gently with the homogenous suspension

- A positive result, will produce clumping within 10 seconds. Conversely, an negative control produces no visible clumping.
- Ensure you perform an auto-agglutination negative control test

1.2.3.2. Tube Coagulase Test

- Place approx. 1 ml of commercially available plasma suitable for tube coagulase in a sterile Eppendorf
- Emulsify test colonies in the plasma and incubate for 4 h at 37 °C
- Examine for a clot which gels the whole contents of the tube or forms a loose web of fibrin
- If negative, incubate overnight at 22-25 °C and re-examine at 24 h.
- A positive result will produce a formation of a clot at 4 h following 37 °C incubation or following overnight incubation at 22-25 °C. A negative result will produce no clot at either time point.

Table 2.
Tube and Slide Coagulase test results for a series of Staphylococci

Species	Tube Coagulase test	Slide Coagulase test
Staphylococcus aureus Subspecies aureus	+	+
Staphylococcus aureus Subspecies anerobius	+	-
Staphylococcus schleiferi Subspecies coagulans	+	-
Staphylococcus lugdunensis	-	(+)
Staphylococcus schleiferi Subspecies shleiferi	-	+
Staphylococcus delphini	+	-
Staphylococcus intermedius	+	d
Staphylococcus hyicus	d	-

*rare clinical isolates
d = 11-89 % of strains positive
(+) = delayed reaction

1.2.4. Catalase Test

This test is to detect the catalase enzyme present in most cytochrome-containing aerobic and facultatively anaerobic bacteria. Streptococcus and Enterococcus sp. are exceptions.

The catalase test is used to detect the presence of catalase enzymes by the decomposition of hydrogen peroxide to release oxygen and water. Hydrogen peroxide is formed by some bacteria as an oxidative end product of the aerobic breakdown of sugars. If allowed to accumulate it is highly toxic to bacteria and can result in cell death. Catalase either decomposes hydrogen peroxide or oxidises secondary substrates, but it has no effect on other peroxides.

Media containing whole red blood cells will contain catalase and could give a false positive. <u>Colonies taken from chocolate agar may be tested.</u> Hydrogen peroxide is unstable and should be stored in a spark proof fridge. Avoid undue exposure to light. Cultures of anaerobic bacteria should be exposed to air for 30 min prior to testing.

- Place approx. 0.2 ml of hydrogen peroxide solution in a test tube
- Carefully pick a colony and rub the colony on the inside wall of the test tube above the surface of the hydrogen peroxide solution
- Cap the tube or bottle and tilt it to allow the hydrogen peroxide solution to cover the colony.
- Look for vigorous bubbling occurring within 10 sec.

Table 3.
FINAL *S. aureus* CONFORMATION RESULTS

Test	Result
Culture	Grows on DNase and blood agar plates to produce cream/golden colonies Baird-Parker agar produces grey-black, shiny colonies surrounded by zone of clearing
DNase test	Positive reaction (identified by zone of clearing)
Coagulase test	Positive reaction (identified by agglutination)
Catalase test	Positive (identified by bubbling around colony)
Further test suggested: PCR	

1.3. *S. aureus* Biofilm Growth

S. aureus biofilms can be grown *in vitro* using brain heart infusion, Mueller-Hinton broth medium[3] or Trypticase Soy broth for 24 h at 37 °C.

Timothy C Biggs

Appendix V

General experimental set-up

As previously detailed in chapter 2.7.6, LAD2 cells were cultured and split into 4 different groups:

1. Non-sensitised, non-infected

2. Non-sensitised, infected

3. Sensitised, non-infected

4. Sensitised, infected

Sensitisation of LAD2 cells: LAD2 cells were cultured as previously outlined. The cells were harvested and centrifuged at 250g for 10 minutes. The medium was removed, and the cell pellet was resuspended with 10 ml PBS and centrifuged at the same mentioned conditions. The cells were counted and adjusted to 2×10^6 per treatment. The cells were cultured in a six-well plate using 5 ml StemPro-34 medium which had 50% less stem cell factor (50 ng/ml). Human Myeloma IgE (Merck Millipore, Hertfordshire, UK) was added to the cells at 1.5µg concentration and incubated overnight at 37 °C. A set of control cells (non-sensitised) were cultured under the same conditions.

Following incubation, samples were centrifuged (250g, 10 mins) with cells re-suspended in Tyrode's buffer for further stimulation (anti-IgE or Calcium Ionophore) followed by measurement of β - hexosaminidase release, gene expression (RT-qPCR) and phosphorylation of protein kinases; with supernatants saved for β-hexosaminidase release, cytokine release (Luminex) and lactate dehydrogenase.

Stimulation of LAD2 cells with anti-IgE and Calcium Ionophore: LAD2 cells were applied into sterile V-bottom 96-well plate by adding 180 µl/well. Serial dilutions of goat anti-human IgE (10, 3, 1, 0.3, and 0.1 µg/ml) and Calcium Ionophore (0.03µM and 0.3µM) were prepared and 20 µl/well were added to the cells. The spontaneous release of mediators was determined by adding 20 µl/well Tyrode's buffer to the cells, whereas the total release was determined following treatment with 20 µl/well 1% Triton X-100. The cells were incubated for one hour at 37 °C.

Beta-hexosaminidase assay

Following culture, with or without *S. aureus* co-infection, the following groups of samples were prepared:

1. Spontaneous release – Tyrode's buffer

2. Anti-IgE stimulation - 10, 3, 1, 0.3, and 0.1 µg/ml in Tyrode's buffer

3. Calcium ionophore stimulation - 0.03µM and 0.3µM in Tyrode's buffer

Samples were prepared, and incubated in the above groups (37°C, 5% CO_2) for 2 hours. Total release samples were also prepared by re-suspending cells in 1% triton X-100 and vortexing vigorously for 2 minutes. Following these preparations, LAD2 cells were centrifuged (250g, 10 mins), and 150µL of supernatant transferred to a V-bottomed plate in triplicate. 50 µl of β-hexosaminidase substrate (130mg of p-Nitrophenyl-N-acetyl-β-D-Glucosaminide) was added to 0.1 M Na2HPO4, adjusted to a pH of 4.5 with 0.4M citric acid solution) then added to each well and incubated for 1 hour (37°C, 5% CO_2). Following which 100µL of stop solution was added, and the plate read thermo-max microplate reader at 410 nm wavelength.

Net release of β-hexosaminidase in each sample was calculated by subtracting the absorbance of the spontaneous release from the absorbance of the sample, divided by the absorbance of the total release and then calculating the percentage:

$$\text{Net release (\%)} = \frac{(\text{sample} - \text{spontaneous})}{\text{total}} \times 100$$
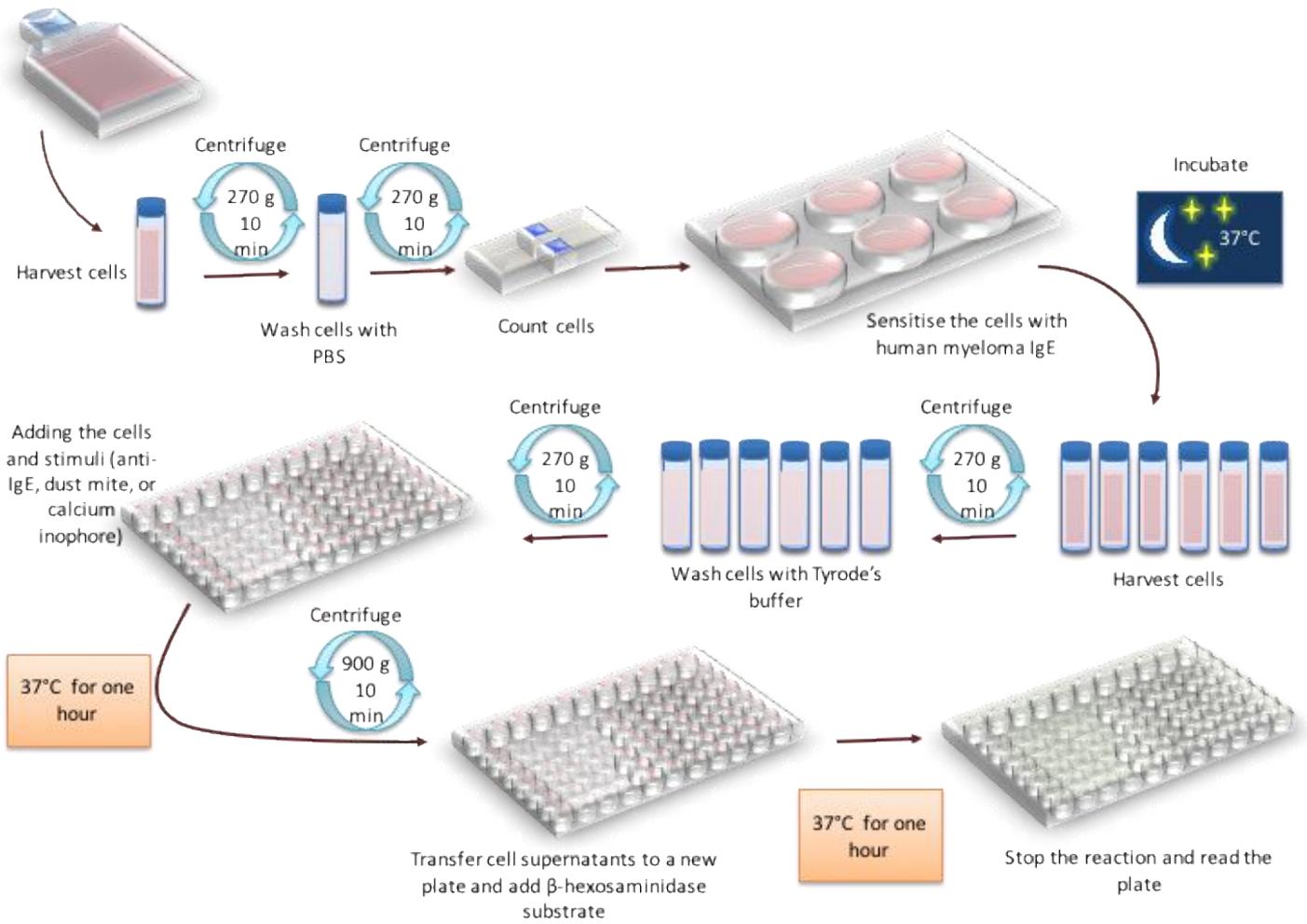
Figure 8.1 Illustration of sensitisation of LAD2 cells with human myeloma IgE antibody.

Testing cell viability by lactate dehydrogenase (LDH) assay

Lactate dehydrogenase (LDH) is an enzyme found in many cells including mast cells. When the plasma membrane breaks down, LDH is released and can be detected in culture supernatants. The detection is based on a colour reaction generated from conversion of lactate to pyruvate by LDH, catalysed by the reduction of NAD+ to NADH. The latter is used by diaphorase to reduce tetrazolium salt to a red-coloured formazan product (Figure 5.2) that can be detected at 490 nm.
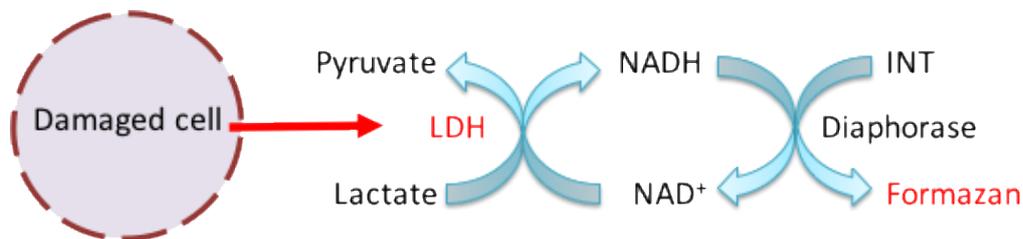
Figure 8.2 Principle of LDH assay

The release of LDH was determined using Pierce LDH assay kit (Life technology limited, Paisley, UK) according to the manufacturer's instructions. One hundred µl of cell culture supernatants collected after bacterial infection for each of the four sets of cells (non-sensitised non-infected, non-sensitised infected, sensitised non-infected and sensitised infected) were added to 96-well culture plate. Ten µl of lysis buffer (10x) were added next and incubated for 45 minutes at 37°C. The maximum release of LDH was determined by treating 4×10^4 cells with 10µl lysis buffer, whereas determination of the spontaneous release was performed by adding 10µl ultrapure water to the cells.

At the end of the incubation, 50µl of each sample were transferred to a new flat-bottom 96-well plate, where 50µl reaction mixture were added next and incubated for 30 minutes at room temperature. The reaction was then stopped by adding 50µl stopping solution and the absorbance was measured using thermo-max microplate reader at 450 nm with 595 nm correction wavelength.

Timothy C Biggs

Analysis of phosphorylation status of main protein kinases following bacterial infection of sensitised mast cells

Protein kinases play a key role in regulating cell function. They activate the target protein by adding phosphate groups and therefore altering the function of many proteins which are involved in a wide range of underlying cellular processes. Phosphorylation of mitogen-activated protein kinases (MAPKs), the extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK1-3), and different p38 isoforms (α, β, δ, γ) was determined using Human Phospho-MAPK array kit (R&D systems Inc, Minneapolis, MN, USA) according to the manufacturer's instructions.

The four sets of cells (non-sensitised non-infected, non-sensitised infected, sensitised non-infected and sensitised infected) were prepared as previously outlined, except for using 5×10^6 cells per condition within T25 flasks (Fisher Scientific limited, Loughborough, UK). The culture medium was adjusted accordingly to 10ml per flask. After overnight IgE sensitisation, cells were infected with *S. aureus* over 4 hours.

Following incubation, cells were harvested and washed (PBS) and then resuspended with 500µl lysis buffer 6. The cell lysates were mixed gently by placing on a rocking platform (Stuart scientific, Staffordshire, UK) for 30 minutes at 4°C. Cell supernatants were collected following centrifugation (14,000g, 5 minutes) and transferred to new tubes, which were stored at -80°C until assay.

On the day of the assay, the membranes supplied in the kit were transferred into 4-well multidish containing 2ml array buffer 5 to block non-specific binding sites. They were incubated for one hour at room temperature on a rocking platform. During this time, the samples were prepared by adding 400µl of each cell supernatant to a separate tube and adjusting the volume to 1.5 ml with array buffer 1. Twenty µl of detection antibody cocktail was then added to each tube and incubated for an hour at room temperature. After blocking the membranes, array buffer 5 was removed and sample-antibody mixtures were added into the membranes which were incubated overnight at 4°C on a rocking platform.

232

The membranes were washed three times with wash buffer for 10 minutes. This was followed by incubation with Streptavidin-HRP diluted in array buffer 5 to 1:2000 dilution for 30 minutes at room temperature on rocking platform. After three washing steps, the membranes were incubated with Chemi Reagent Mix for one minute and then covered with transparent film. Imaging was performed using ChemiDoc Imaging system (Bio-Rad laboratories Ltd., Hertfordshire, UK) with serial exposures up to 10 minutes.

References

1. Orhan M, Govsa F, Saylam C. A surgical view of the superior nasal turbinate: anatomical study. Eur Arch Otorhinolaryngol. 2010;267(6):909-16.

2. ENT for medical students. Nasal anatomy and physiology [Internet]. Department of Otolaryngology, University of Zagazig, Egypt. [updated 2012]. Available from: http://ent4students.blogspot.co.uk/2012/12/nose-anatomy.html.

3. American rhinologic society. Nasal physiology [Internet]. Jeremiah A, Cohen N, American rhinologic society [updated 2015]. Available from: http://care.american-rhinologic.org/nasal_physiology.

4. Medscape. Olfactory system anatomy [Internet]. Vokshoor A [updated 2013]. Available from: http://emedicine.medscape.com/article/835585-overview#a3.

5. Encyclopaedia Britannica. Olfactory epithelium [Internet]. [updated 2015]. Available from: https://www.britannica.com/science/olfactory-epithelium.

6. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. European Position Paper on Rhinosinusitis and Nasal Polyps 2012. Rhinol Suppl. 2012(23):1-298.

7. Ear, nose, throat allergy testing centre. Sinusitis [Interent]. Adelglass J [updated 2009]. Available from: http://www.entdocs.org/sinusitis.htm.

8. Ferguson B. Adult sinusitis, American rhinologic society (Internet). [updated 2015]. Available from: http://care.american-rhinologic.org/adult_sinusitis.

9. Ou J, Wang J, Xu Y, Tao ZZ, Kong YG, Chen SM, et al. Staphylococcus aureus superantigens are associated with chronic rhinosinusitis with nasal polyps: a meta-analysis. Eur Arch Otorhinolaryngol. 2014;271(10):2729-36.

10. Bachert C, Zhang N, Patou J, van Zele T, Gevaert P. Role of staphylococcal superantigens in upper airway disease. Curr Opin Allergy Clin Immunol. 2008;8(1):34-8.

11. 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. J Allergy Clin Immunol. 2008;121(1):110-5.

12. Derycke L, Eyerich S, Van Crombruggen K, Perez-Novo C, Holtappels G, Deruyck N, et al. Mixed T helper cell signatures in chronic rhinosinusitis with and without polyps. PLoS One. 2014;9(6):e97581.

13.   Lou H, Meng Y, Piao Y, Zhang N, Bachert C, Wang C, et al. Cellular phenotyping of chronic rhinosinusitis with nasal polyps. Rhinology. 2016;54(2):150-9.

14.   Kostamo K, Toskala E, Tervahartiala T, Sorsa T. Role of matrix metalloproteinases in chronic rhinosinusitis. Curr Opin Allergy Clin Immunol. 2008;8(1):21-7.

15.   Van Bruaene N, Bachert C. Tissue remodeling in chronic rhinosinusitis. Curr Opin Allergy Clin Immunol. 2011;11(1):8-11.

16.   Watelet JB, Bachert C, Claeys C, Van Cauwenberge P. Matrix metalloproteinases MMP-7, MMP-9 and their tissue inhibitor TIMP-1: expression in chronic sinusitis vs nasal polyposis. Allergy. 2004;59(1):54-60.

17.   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.

18.   Li YY, Li CW, Chao SS, Yu FG, Yu XM, Liu J, et al. Impairment of cilia architecture and ciliogenesis in hyperplastic nasal epithelium from nasal polyps. J Allergy Clin Immunol. 2014;134(6):1282-92.

19.   Lee JT, Kennedy DW, Palmer JN, Feldman M, Chiu AG. The incidence of concurrent osteitis in patients with chronic rhinosinusitis: a clinicopathological study. Am J Rhinol. 2006;20(3):278-82.

20.   Telmesani LM, Al-Shawarby M. Osteitis in chronic rhinosinusitis with nasal polyps: a comparative study between primary and recurrent cases. Eur Arch Otorhinolaryngol. 2010;267(5):721-4.

21.   Cho GS, Moon BJ, Lee BJ, Gong CH, Kim NH, Kim YS, et al. High rates of detection of respiratory viruses in the nasal washes and mucosae of patients with chronic rhinosinusitis. J Clin Microbiol. 2013;51(3):979-84.

22.   Tan NCW, Foreman A, Jardeleza C, Douglas R, Tran H, Wormald PJ. The multiplicity of Staphylococcus aureus in chronic rhinosinusitis: Correlating surface biofilm and intracellular residence. Laryngoscope. 2012;122(8):1655-60.

23.   Tan NCW, Foreman A, Jardeleza C, Douglas R, Vreugde S, Wormald P-J. Intracellular Staphylococcus aureus: the Trojan horse of recalcitrant chronic rhinosinusitis? Int Forum Allergy Rhinol. 2013;3(4):261-6.

24.   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.

25.   Hayes SM, Howlin R, Johnston DA, Webb JS, Clarke SC, Stoodley P, et al. Intracellular residency of Staphylococcus aureus within mast cells in nasal polyps: A novel observation. J Allergy Clin Immunol. 2015;135(6):1648-51.

26.   Sabirov A, Hamilton RG, Jacobs JB, Hillman DE, Lebowitz RA, Watts JD. Role of local immunoglobulin E specific for Alternaria alternata in the pathogenesis of nasal polyposis. Laryngoscope. 2008;118(1):4-9.

27.   Sproson EL, Thomas KM, Lau LC, Harries PG, Howarth PH, Salib RJ. Common airborne fungi induce species-specific effects on upper airway inflammatory and remodelling responses. Rhinology. 2016;54(1):51-5.

28.   Nasal Surgery Syndney. Nasal polyps [Internet]. Roth J, [updated 2017]. Available from: http://drjasonroth.com.au/nasal-polyps/.

29.   Van Zele T, Claeys S, Gevaert P, Van Maele G, Holtappels G, Van Cauwenberge P, et al. Differentiation of chronic sinus diseases by measurement of inflammatory mediators. Allergy. 2006;61(11):1280-9.

30.   Chen Y, Dales R, Lin M. The epidemiology of chronic rhinosinusitis in Canadians. Laryngoscope. 2003;113(7):1199-205.

31.   Pleis JR, Lethbridge-Cejku M. Summary health statistics for U.S. adults: National Health Interview Survey, 2006. Vital Health Stat. 2007(235):1-153.

32.   Min YG, Jung HW, Kim HS, Park SK, Yoo KY. Prevalence and risk factors of chronic sinusitis in Korea: results of a nationwide survey. Eur Arch Otorhinolaryngol. 1996;253(7):435-9.

33.   Hastan D, Fokkens WJ, Bachert C, Newson RB, Bislimovska J, Bockelbrink A, et al. Chronic rhinosinusitis in Europe--an underestimated disease. A GA(2)LEN study. Allergy. 2011;66(9):1216-23.

34.   Jarvis D, Newson R, Lotvall J, Hastan D, Tomassen P, Keil T, et al. Asthma in adults and its association with chronic rhinosinusitis: the GA2LEN survey in Europe. Allergy. 2012;67(1):91-8.

35.   Philpott C, Hopkins C, Erskine S, Kumar N, Robertson A, Farboud A, et al. The burden of revision sinonasal surgery in the UK-data from the Chronic Rhinosinusitis Epidemiology Study (CRES): a cross-sectional study. BMJ Open. 2015;5(4):e006680.

36.   Collins JG. Prevalence of selected chronic conditions: United States, 1990-1992. Vital Health Stat. 1997(194):1-89.

37.   Blackwell DL, Collins JG, Coles R. Summary health statistics for U.S. adults: National Health Interview Survey, 1997. Vital Health Stat. 2002(205):1-109.

38.   Papesch M, Watkins R. Epstein-Barr virus infectious mononucleosis. Clin Otolaryngol. 2001;26(1):3-8.

39.   Shashy RG, Moore EJ, Weaver A. Prevalence of the chronic sinusitis diagnosis in Olmsted County, Minnesota. Arch Otolaryngol Head Neck Surg. 2004;130(3):320-3.

40.   Pilan RR, Pinna FR, Bezerra TF, Mori RL, Padua FG, Bento RF, et al. Prevalence of chronic rhinosinusitis in Sao Paulo. Rhinology. 2012;50(2):129-38.

41.   Gao WX, Ou CQ, Fang SB, Sun YQ, Zhang H, Cheng L, et al. Occupational and environmental risk factors for chronic rhinosinusitis in China: a multicentre cross-sectional study. Respir Res. 2016;17(1):54.

42.   Hirsch AG, Stewart WF, Sundaresan AS, Young AJ, Kennedy TL, Scott Greene J, et al. Nasal and sinus symptoms and chronic rhinosinusitis in a population-based sample. Allergy. 2016;72(2):274-281.

43.   Settipane GA. Epidemiology of nasal polyps. Allergy Asthma Proc. 1996;17(5):231-6.

44.   Hedman J, Kaprio J, Poussa T, Nieminen MM. Prevalence of asthma, aspirin intolerance, nasal polyposis and chronic obstructive pulmonary disease in a population-based study. Int J Epidemiol. 1999;28(4):717-22.

45.   Johansson L, Akerlund A, Holmberg K, Melen I, Bende M. Prevalence of nasal polyps in adults: the Skovde population-based study. Ann Otol Rhinol Laryngol. 2003;112(7):625-9.

46.   Klossek JM, Neukirch F, Pribil C, Jankowski R, Serrano E, Chanal I, et al. Prevalence of nasal polyposis in France: a cross-sectional, case-control study. Allergy. 2005;60(2):233-7.

47.   Kim YS, Kim NH, Seong SY, Kim KR, Lee GB, Kim KS. Prevalence and risk factors of chronic rhinosinusitis in Korea. Am J Rhinol Allergy. 2011;25(3):117-21.

48.   Ahn JC, Kim JW, Lee CH, Rhee CS. Prevalence and Risk Factors of Chronic Rhinosinusitus, Allergic Rhinitis, and Nasal Septal Deviation: Results of the Korean National

Health and Nutrition Survey 2008-2012. JAMA Otolaryngol Head Neck Surg. 2016;142(2):162-7.

49.   Emanuel IA, Shah SB. Chronic rhinosinusitis: allergy and sinus computed tomography relationships. Otolaryngol Head Neck Surg. 2000;123(6):687-91.

50.   Lane AP, Pine HS, Pillsbury HC, 3rd. Allergy testing and immunotherapy in an academic otolaryngology practice: a 20-year review. Otolaryngol Head Neck Surg. 2001;124(1):9-15.

51.   Szczeklik A, Nizankowska E, Duplaga M. Natural history of aspirin-induced asthma. AIANE Investigators. European Network on Aspirin-Induced Asthma. Eur Respir J. 2000;16(3):432-6.

52.   Stevenson DD, Zuraw BL. Pathogenesis of aspirin-exacerbated respiratory disease. Clin Rev Allergy Immunol. 2003;24(2):169-88.

53.   Makowska J, Lewandowska-Polak A, Kowalski ML. Hypersensitivity to Aspirin and other NSAIDs: Diagnostic Approach in Patients with Chronic Rhinosinusitis. Curr Allergy Asthma Rep. 2015;15(8):47.

54.   Esmaeilzadeh H, Nabavi M, Aryan Z, Arshi S, Bemanian MH, Fallahpour M, et al. Aspirin desensitization for patients with aspirin-exacerbated respiratory disease: A randomized double-blind placebo-controlled trial. Clin Immunol. 2015;160(2):349-57.

55.   Jones NS. CT of the paranasal sinuses: a review of the correlation with clinical, surgical and histopathological findings. Clin Otolaryngol Allied Sci. 2002;27(1):11-7.

56.   Caughey RJ, Jameson MJ, Gross CW, Han JK. Anatomic risk factors for sinus disease: fact or fiction? Am J Rhinol. 2005;19(4):334-9.

57.   Holbrook EH, Brown CL, Lyden ER, Leopold DA. Lack of significant correlation between rhinosinusitis symptoms and specific regions of sinus computer tomography scans. Am J Rhinol. 2005;19(4):382-7.

58.   Bjarnsholt T, Hoiby N, Donelli G, Imbert C, Forsberg A. Understanding biofilms - are we there yet? FEMS Immunol Med Microbiol. 2012;65(2):125-6.

59.   Donlan RM, Costerton JW. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 2002;15(2):167-+.

60.   Otto M. Staphylococcal infections: mechanisms of biofilm maturation and detachment as critical determinants of pathogenicity. Annu Rev Med. 2013;64:175-88.

61.   Ferguson BJ, Stolz DB. Demonstration of biofilm in human bacterial chronic rhinosinusitis. Am J Rhinol. 2005;19(5):452-7.

62.   Post JC, Hiller NL, Nistico L, Stoodley P, Ehrlich GD. The role of biofilms in otolaryngologic infections: update 2007. Curr Opin Otolaryngol Head Neck Surg. 2007;15(5):347-51.

63.   Cryer J, Schipor I, Perloff JR, Palmer JN. Evidence of bacterial biofilms in human chronic sinusitis. ORL J Otorhinolaryngol Relat Spec. 2004;66(3):155-8.

64.   Psaltis AJ, Ha KR, Beule AG, Tan LW, Wormald P-J. Confocal scanning laser microscopy evidence of biofilms in patients with chronic rhinosinusitis. Laryngoscope. 2007;117(7):1302-6.

65.   Foreman A, Wormald P-J. Different Biofilms, Different Disease? A Clinical Outcomes Study. Laryngoscope. 2010;120(8):1701-6.

66.   Singhal D, Psaltis AJ, Foreman A, Wormald P-J. The impact of biofilms on outcomes after endoscopic sinus surgery. Am J Rhinol Allergy. 2010;24(3):169-74.

67.   Foreman A, Jervis-Bardy J, Wormald P-J. Do Biofilms Contribute to the Initiation and Recalcitrance of Chronic Rhinosinusitis? Laryngoscope. 2011;121(5):1085-91.

68.   Ramadan HH. Chronic rhinosinusitis and bacterial biofilms. Curr Opin Otolaryngol Head Neck Surg. 2006;14(3):183-6.

69.   Sanclement JA, Webster P, Thomas J, Ramadan HH. Bacterial biofilms in surgical specimens of patients with chronic rhinosinusitis. Laryngoscope. 2005;115(4):578-82.

70.   Sanderson AR, Leid JG, Hunsaker D. Bacterial Biofilms on the sinus mucosa of human subjects with chronic rhinosinusitis. Laryngoscope. 2006;116(7):1121-6.

71.   Healy DY, Leid JG, Sanderson AR, Hunsaker DH. Biofilms with fungi in chronic rhinosinusitis. Otolaryngol Head Neck Surg. 2008;138(5):641-7.

72.   Galli J, Calo L, Ardito F, Imperiali M, Bassotti E, Passali GC, et al. Damage to Ciliated Epithelium in Chronic Rhinosinusitis: What Is the Role of Bacterial Biofilms? Ann Otol Rhinol Laryngol. 2008;117(12):902-8.

73.   Hekiert AM, Kofonow JM, Doghramji L, Kennedy DW, Chiu AG, Palmer JN, et al. Biofilms correlate with T(H)1 inflammation in the sinonasal tissue of patients with chronic rhinosinusitis. Otolaryngol Head Neck Surg. 2009;141(4):448-53.

74.   Foreman A, Psaltis AJ, Tan LW, Wormald P-J. Characterization of bacterial and fungal biofilms in chronic rhinosinusitis. Am J Rhinol Allergy. 2009;23(6):556-61.

75.   Tan NCW, Tran HB, Foreman A, Jardeleza C, Vreugde S, Wormald PJ. Identifying intracellular Staphylococcus aureus in chronic rhinosinusitis: a direct comparison of techniques. Am J Rhinol Allergy. 2012;26(6):444-9.

76.   Krut O, Sommer H, Kronke M. Antibiotic-induced persistence of cytotoxic Staphylococcus aureus in non-phagocytic cells. J Antimicrob Chemother. 2004;53(2):167-73.

77.   Bachert C, Zhang N, van Zele T, Gevaert P, Patou J, van Cauwenberge P. Staphylococcus aureus enterotoxins as immune stimulants in chronic rhinosinusitis. Clin Allergy Immunol. 2007;20:163-75.

78.   Bachert C, Gevaert P, Zhang N, van Zele T, Perez-Novo C. Role of staphylococcal superantigens in airway disease. Chem Immunol Allergy. 2007;93:214-36.

79.   Van Crombruggen K, Van Bruaene N, Holtappels G, Bachert C. Chronic sinusitis and rhinitis: clinical terminology "Chronic Rhinosinusitis" further supported. Rhinology. 2010;48(1):54-8.

80.   Van Zele T, Gevaert P, Watelet JB, Claeys G, Holtappels G, Claeys C, et al. Staphylococcus aureus colonization and IgE antibody formation to enterotoxins is increased in nasal polyposis. J Allergy Clin Immunol. 2004;114(4):981-3.

81.   Tripathi A, Kern R, Conley DB, Seiberling K, Klemens JC, Harris KE, et al. Staphylococcal exotoxins and nasal polyposis: analysis of systemic and local responses. Am J Rhinol. 2005;19(4):327-33.

82.   Kim DW, Khalmuratova R, Hur DG, Jeon S-Y, Kim S-W, Shin H-W, et al. Staphylococcus aureus enterotoxin B contributes to induction of nasal polypoid lesions in an allergic rhinosinusitis murine model. Am J Rhinol Allergy. 2011;25(6):e255-61.

83.   Dong D, Yulin Z, Xiao W, Hongyan Z, Jia L, Yan X, et al. Correlation between bacterial biofilms and osteitisin patients with chronic rhinosinusitis. Laryngoscope. 2013.

84.   Singhal D, Foreman A, Bardy J-J, Wormald P-J. Staphylococcus aureus Biofilms: Nemesis of Endoscopic Sinus Surgery. Laryngoscope. 2011;121(7):1578-83.

85.   Pezato R, Bottura L, de Paula Santos R, Voegels RL, Bachi AL, Gregorio LC. Bone: the final frontier for Staphylococcus aureus penetration in chronic rhinosinusitis. J Otolaryngol Head Neck Surg. 2013;42(1):45.

86.   Wood AJ, Fraser J, Amirapu S, Douglas RG. Bacterial microcolonies exist within the sphenoid bone in chronic rhinosinusitis and healthy controls. Int Forum Allergy Rhinol. 2012;2(2):116-21.

87.   Georgalas C, Videler W, Freling N, Fokkens W. Global Osteitis Scoring Scale and chronic rhinosinusitis: a marker of revision surgery. Clin Otolaryngol. 2010;35(6):455-61.

88.   Radiopedia. Chronic sphenoidal sinusitis and osteomyelitis, Schubert R. [Internet]. [updated 2016]. Available from: https://radiopaedia.org/cases/chronic-sphenoidal-sinusitis-and-osteomyelitis.

89.   Kim HY, Dhong HJ, Lee HJ, Chung YJ, Yim YJ, Oh JW, et al. Hyperostosis may affect prognosis after primary endoscopic sinus surgery for chronic rhinosinusitis. Otolaryngol Head Neck Surg. 2006;135(1):94-9.

90.   Cho SH, Min HJ, Han HX, Paik SS, Kim KR. CT analysis and histopathology of bone remodeling in patients with chronic rhinosinusitis. Otolaryngol Head Neck Surg. 2006;135(3):404-8.

91.   Detwiller KY, Smith TL, Mace JC, Trune DR, Sautter NB. Steroid-independent upregulation of matrix metalloproteinase 9 in chronic rhinosinusitis patients with radiographic evidence of osteitis. Int Forum Allergy Rhinol. 2013;3(5):364-8.

92.   Snidvongs K, McLachlan R, Chin D, Pratt E, Sacks R, Earls P, et al. Osteitic bone: a surrogate marker of eosinophilia in chronic rhinosinusitis. Rhinology. 2012;50(3):299-305.

93.   Tuszynska A, Krzeski A, Postuba M, Paczek L, Wyczalkowska-Tomasik A, Gornicka B, et al. Inflammatory cytokines gene expression in bone tissue from patients with chronic rhinosinusitis- a preliminary study. Rhinology. 2010;48(4):415-9.

94.   The Histology Guide. Epithelium: Cell Junctions, Faculty of Biological Sciences, [Updated 2017]. Available from: http://www.histology.leeds.ac.uk/tissue_types/epithelia/epi_cell_junctions.php.

95.   Malik Z, Roscioli E, Murphy J, Ou J, Bassiouni A, Wormald PJ, et al. Staphylococcus aureus impairs the airway epithelial barrier in vitro. Int Forum Allergy Rhinol. 2015;5(6):551-6.

96.   Soyka MB, Wawrzyniak P, Eiwegger T, Holzmann D, Treis A, Wanke K, et al. Defective epithelial barrier in chronic rhinosinusitis: the regulation of tight junctions by IFN-gamma and IL-4. J Allergy Clin Immunol. 2012;130(5):1087-96.e10.

97.   Wise SK, Laury AM, Katz EH, Den Beste KA, Parkos CA, Nusrat A. Interleukin-4 and interleukin-13 compromise the sinonasal epithelial barrier and perturb intercellular junction protein expression. Int Forum Allergy Rhinol. 2014;4(5):361-70.

98.   Ramezanpour M, Moraitis S, Smith JL, Wormald PJ, Vreugde S. Th17 Cytokines Disrupt the Airway Mucosal Barrier in Chronic Rhinosinusitis. Mediators Inflamm. 2016;2016:9798206.

99.   Steelant B, Farre R, Wawrzyniak P, Belmans J, Dekimpe E, Vanheel H, et al. Impaired barrier function in patients with house dust mite-induced allergic rhinitis is accompanied by decreased occludin and zonula occludens-1 expression. J Allergy Clin Immunol. 2016;137(4):1043-53.e1-5.

100.   Chen X, Luo X, Han M, Wu X, Guo J, Zhang J, et al. Corticosteroid regulates epithelial occludin expression in nasal polyps through a MKP-1-dependent pathway. ORL J Otorhinolaryngol Relat Spec. 2014;76(5):248-56.

101.   Farhat K, Riekenberg S, Heine H, Debarry J, Lang R, Mages J, et al. Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. J Leukoc Biol. 2008;83(3):692-701.

102.   Hamilos DL. Host-microbial interactions in patients with chronic rhinosinusitis. J Allergy Clin Immunol. 2014;133(3):640-53.e4.

103.   Agnese DM, Calvano JE, Hahm SJ, Coyle SM, Corbett SA, Calvano SE, et al. Human Toll-Like Receptor 4 Mutations but Not CD14 Polymorphisms Are Associated with an Increased Risk of Gram-Negative Infections. J Infect Dis. 2002;186(10):1522-5.

104.   Hirschberg A, Kiss M, Kadocsa E, Polyanka H, Szabo K, Razga Z, et al. Different activations of toll-like receptors and antimicrobial peptides in chronic rhinosinusitis with or without nasal polyposis. Eur Arch Otorhinolaryngol. 2015:1-10.

105.   Salib RJ, Howarth PH. Transforming growth factor-beta in allergic inflammatory disease of the upper airways: friend or foe? Clin Exp Allergy. 2009;39(8):1128-35.

106.   Yang YC, Zhang N, Van Crombruggen K, Hu GH, Hong SL, Bachert C. Transforming growth factor-beta1 in inflammatory airway disease: a key for understanding inflammation and remodeling. Allergy. 2012;67(10):1193-202.

107.   Kostamo K, Tervahartiala T, Sorsa T, Richardson M, Toskala E. Metalloproteinase function in chronic rhinosinusitis with nasal polyposis. Laryngoscope. 2007;117(4):638-43.

108.   Parks WC, Wilson CL, Lopez-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol. 2004;4(8):617-29.

109.   Li X, Meng J, Qiao X, Liu Y, Liu F, Zhang N, et al. Expression of TGF, matrix metalloproteinases, and tissue inhibitors in Chinese chronic rhinosinusitis. J Allergy Clin Immunol. 2010;125(5):1061-8.

110.   Eisenberg G, Pradillo J, Plaza G, Lizasoain I, Moro MA. Increased expression and activity of MMP-9 in chronic rhinosinusitis with nasal polyposis. Acta Otorrinolaringol Esp. 2008;59(9):444-7.

111.   Kahveci OK, Derekoy FS, Yilmaz MD, Serteser M, Altuntas A. The role of MMP-9 and TIMP-1 in nasal polyp formation. Swiss Med Wkly. 2008;138(45-46):684-8.

112.   Chen YS, Langhammer T, Westhofen M, Lorenzen J. Relationship between matrix metalloproteinases MMP-2, MMP-9, tissue inhibitor of matrix metalloproteinases-1 and IL-5, IL-8 in nasal polyps. Allergy. 2007;62(1):66-72.

113.   Lu X, Liu Z, Cui Y. The protein expression difference of transforming growth factor beta1, matrix metalloproteinases 1,7,9 and tissue inhibitors of matrix metalloproteinases-1 between chronic rhinosinusitis, nasal polyps and normal mucosa tissues. Lin Chuang Er Bi Yan Hou Ke Za Zhi. 2005;19(14):633-5.

114.   Ma ZX, Tan X, Shen Y, Ke X, Yang YC, He XB, et al. MicroRNA expression profile of mature dendritic cell in chronic rhinosinusitis. Inflamm Res. 2015;64(11):885-93.

115.   Poposki JA, Peterson S, Welch K, Schleimer RP, Hulse KE, Peters AT, et al. Elevated presence of myeloid dendritic cells in nasal polyps of patients with chronic rhinosinusitis. Clin Exp Allergy. 2015;45(2):384-93.

116.   Pezato R, Perez-Novo CA, Holtappels G, De Ruyck N, Van Crombruggen K, De Vos G, et al. The expression of dendritic cell subsets in severe chronic rhinosinusitis with nasal polyps is altered. Immunobiology. 2014;219(9):729-36.

117.   Shi LL, Song J, Xiong P, Cao PP, Liao B, Ma J, et al. Disease-specific T-helper cell polarizing function of lesional dendritic cells in different types of chronic rhinosinusitis with nasal polyps. Am J Respir Crit Care Med. 2014;190(6):628-38.

118.   Ho J, Bailey M, Zaunders J, Mrad N, Sacks R, Sewell W, et al. Cellular comparison of sinus mucosa vs polyp tissue from a single sinus cavity in chronic rhinosinusitis. Int Forum Allergy Rhinol. 2015;5(1):14-27.

119.   Banks CA, Schlosser RJ, Wang EW, Casey SE, Mulligan RM, Mulligan JK. Macrophage Infiltrate Is Elevated in CRSwNP Sinonasal Tissue Regardless of Atopic Status. Otolaryngol Head Neck Surg. 2014;151(2):215-20.

120.   Krysko O, Holtappels G, Zhang N, Kubica M, Deswarte K, Derycke L, et al. Alternatively activated macrophages and impaired phagocytosis of *S. aureus* in chronic rhinosinusitis. Allergy. 2011;66(3):396-403.

121.   Zuniga LA, Jain R, Haines C, Cua DJ. Th17 cell development: from the cradle to the grave. Immunol Rev. 2013;252(1):78-88.

122.   Sanchez-Segura A, Brieva JA, Rodriguez C. T lymphocytes that infiltrate nasal polyps have a specialized phenotype and produce a mixed TH1/TH2 pattern of cytokines. J Allergy Clin Immunol. 1998;102(6 Pt 1):953-60.

123.   Hao J, Pang YT, Wang DY. Diffuse mucosal inflammation in nasal polyps and adjacent middle turbinate. Otolaryngol Head Neck Surg. 2006;134(2):267-75.

124.   Van Bruaene N, Perez-Novo CA, Basinski TM, Van Zele T, Holtappels G, De Ruyck N, et al. T-cell regulation in chronic paranasal sinus disease. J Allergy Clin Immunol. 2008;121(6):1435-41.

125.   Zhang N, Van Zele T, Perez-Novo C, Van Bruaene N, Holtappels G, DeRuyck N, et al. Different types of T-effector cells orchestrate mucosal inflammation in chronic sinus disease. J Allergy Clin Immunol. 2008;122(5):961-8.

126.   Jiang X-D, Li G-Y, Li L, Dong Z, Zhu D-D. The characterization of IL-17A expression in patients with chronic rhinosinusitis with nasal polyps. Am J Rhinol Allergy. 2011;25(5):E171-E5.

127.   Cao P-P, Li H-B, Wang B-F, Wang S-B, You X-J, Cui Y-H, et al. Distinct immunopathologic characteristics of various types of chronic rhinosinusitis in adult Chinese. J Allergy Clin Immunol. 2009;124(3):478-U135.

128.   Shen Y, Hu G-H, Yang Y-C, Ke X, Tang X-Y, Hong S-L. Allergen induced Th17 response in the peripheral blood mononuclear cells (PBMCs) of patients with nasal polyposis. Int Immunopharmacol. 2012;12(1):235-40.

129.   Shen Y, Tang XY, Yang YC, Ke X, Kou W, Pan CK, et al. Impaired Balance of Th17/Treg in Patients with Nasal Polyposis. Scand J Immunol. 2011;74(2):176-85.

130.   Derycke L, Zhang N, Holtappels G, Dutre T, Bachert C. IL-17A as a regulator of neutrophil survival in nasal polyp disease of patients with and without cystic fibrosis. J Cyst Fibros. 2012;11(3):193-200.

131.   Peters AT, Kato A, Carr T, Suh L, Norton J, Grammer LC, et al. Analysis of the Th17 Pathway in Chronic Rhinosinusitis. J Allergy Clin Immunol. 2009;123(2):S145-S.

132.   Wang X, Zhang N, Bo Ms M, Holtappels G, Zheng M, Lou H, et al. Diversity of TH cytokine profiles in patients with chronic rhinosinusitis: A multicenter study in Europe, Asia, and Oceania. J Allergy Clin Immunol. 2016;138(5):1344-1353.

133.   Konig K, Klemens C, Haack M, Nicolo MS, Becker S, Kramer MF, et al. Cytokine patterns in nasal secretion of non-atopic patients distinguish between chronic rhinosinusitis with or without nasal polys. Allergy Asthma Clin Immunol. 2016;12:19.

134.   Miljkovic D, Psaltis A, Wormald PJ, Vreugde S. T regulatory and Th17 cells in chronic rhinosinusitis with polyps. Int Forum Allergy Rhinol. 2016;6(8):826-34.

135.   Leung S, Liu X, Fang L, Chen X, Guo T, Zhang J. The cytokine milieu in the interplay of pathogenic Th1/Th17 cells and regulatory T cells in autoimmune disease. Cell Mol Immunol. 2010;7(3):182-9.

136.   Kaliner MA, Osguthorpe JD, Fireman P, Anon J, Georgitis J, Davis ML, et al. Sinusitis: bench to bedside. Current findings, future directions. Otolaryngol Head Neck Surg. 1997;116(6 Pt 2):S1-20.

137.   Matsuwaki Y, Ookushi T, Asaka D, Mori E, Nakajima T, Yoshida T, et al. Chronic rhinosinusitis: risk factors for the recurrence of chronic rhinosinusitis based on 5-year follow-up after endoscopic sinus surgery. Int Arch Allergy Immunol. 2008;146 Suppl 1:77-81.

138.   Soler ZM, Sauer D, Mace J, Smith TL. Impact of mucosal eosinophilia and nasal polyposis on quality-of-life outcomes after sinus surgery. Otolaryngol Head Neck Surg. 2010;142(1):64-71.

139.   Hulse KE, Stevens WW, Tan BK, Schleimer RP. Pathogenesis of nasal polyposis. Clin Exp Allergy. 2015;45(2):328-46.

140.   Van Crombruggen K, Zhang N, Gevaert P, Tomassen P, Bachert C. Pathogenesis of chronic rhinosinusitis: Inflammation. J Allergy Clin Immunol. 2011;128(4):728-32.

141.   Bachert C, Gevaert P, Holtappels G, Johansson SGO, van Cauwenberge P. Total and specific IgE in nasal polyps is related to local eosinophilic inflammation. J Allergy Clin Immunol. 2001;107(4):607-14.

142.   Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. Nat Rev Immunol. 2011;11(8):519-31.

143.   Abel J, Goldmann O, Ziegler C, Holtje C, Smeltzer MS, Cheung AL, et al. Staphylococcus aureus evades the extracellular antimicrobial activity of mast cells by promoting its own uptake. J Innate Immun. 2011;3(5):495-507.

144.   Bachert C, Gevaert P, Holtappels G, Johansson SG, van Cauwenberge P. Total and specific IgE in nasal polyps is related to local eosinophilic inflammation. J Allergy Clin Immunol. 2001;107(4):607-14.

145.   Carney AS, Tan LW, Adams D, Varelias A, Ooi EH, Wormald PJ. Th2 immunological inflammation in allergic fungal sinusitis, nonallergic eosinophilic fungal sinusitis, and chronic rhinosinusitis. Am J Rhinol. 2006;20(2):145-9.

146.   Pawankar R, Lee KH, Nonaka M, Takizawa R. Role of mast cells and basophils in chronic rhinosinusitis. Clin Allergy Immunol. 2007;20:93-101.

147.   Xue L, Gyles SL, Wettey FR, Gazi L, Townsend E, Hunter MG, et al. Prostaglandin D2 causes preferential induction of proinflammatory Th2 cytokine production through an action on chemoattractant receptor-like molecule expressed on Th2 cells. J Immunol. 2005;175(10):6531-6.

148.   Hua X, Naselsky WC, Jania CM, Chason KD, Huang JJ, Doerschuk CM, et al. Mast Cell Deficiency Limits the Development of Chronic Rhinosinusitis in Mice. Ann Otol Rhinol Laryngol. 2015.

149.   Lund VJ, Mackay IS. Staging in rhinosinusitus. Rhinology. 1993;31(4):183-4.

150.   Niedergang F, Hemar A, Hewitt CR, Owen MJ, Dautry-Varsat A, Alcover A. The Staphylococcus aureus enterotoxin B superantigen induces specific T cell receptor down-regulation by increasing its internalization. J Biol Chem. 1995;270(21):12839-45.

151.   Reis LO, Ferreira U, Billis A, Cagnon VH, Favaro WJ. Anti-angiogenic effects of the superantigen staphylococcal enterotoxin B and bacillus Calmette-Guerin immunotherapy for nonmuscle invasive bladder cancer. J Urol. 2012;187(2):438-45.

152.   Cho SN, Song CH, Jin J, Kim SH, Rha KS, Kim YM. Role of staphylococcal enterotoxin B on the differentiation of regulatory T cells in nasal polyposis. Am J Rhinol Allergy. 2014;28(1):e17-24.

153.   Jin J, Yoon YH, Kwak SY, Rha KS, Kim YM. Staphylococcal enterotoxin B induced expression of IL-17A in nasal epithelial cells and its association with pathogenesis of nasal polyposis. Eur Arch Otorhinolaryngol. 2014;271(3):525-34.

154.   Nilsson G, Blom T, Kusche-Gullberg M, Kjellen L, Butterfield JH, Sundstrom C, et al. Phenotypic characterization of the human mast-cell line HMC-1. Scand J Immunol. 1994;39(5):489-98.

155. Radinger M, Jensen BM, Kuehn HS, Kirshenbaum A, Gilfillan AM. Generation, isolation, and maintenance of human mast cells and mast cell lines derived from peripheral blood or cord blood. Curr Protoc Immunol. 2010;Chapter 7:Unit 7.37.

156. Kirshenbaum AS, Akin C, Wu Y, Rottem M, Goff JP, Beaven MA, et al. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. Leuk Res. 2003;27(8):677-82.

157. Moorhead PS. Human tumor cell line with a quasi-diploid karyotype (RPMI 2650). Exp Cell Res. 1965;39(1):190-6.

158. Bai S, Yang T, Abbruscato TJ, Ahsan F. Evaluation of human nasal RPMI 2650 cells grown at an air-liquid interface as a model for nasal drug transport studies. J Pharm Sci. 2008;97(3):1165-78.

159. Kreft ME, Jerman UD, Lasic E, Lanisnik Rizner T, Hevir-Kene N, Peternel L, et al. The characterization of the human nasal epithelial cell line RPMI 2650 under different culture conditions and their optimization for an appropriate in vitro nasal model. Pharm Res. 2015;32(2):665-79.

160. Perez-Novo CA, Claeys C, Speleman F, Van Cauwenberge P, Bachert C, Vandesompele J. Impact of RNA quality on reference gene expression stability. Biotechniques. 2005;39(1):56.

161. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101-8.

162. Morinaka S, Nakamura H. Inflammatory cells in nasal mucosa and nasal polyps. Auris Nasus Larynx. 2000;27(1):59-64.

163. Plouin-Gaudon I, Clement S, Huggler E, Chaponnier C, Francois P, Lew D, et al. Intracellular residency is frequently associated with recurrent Staphylococcus aureus rhinosinusitis. Rhinology. 2006;44(4):249-54.

164. Rugina M, Serrano E, Klossek JM, Crampette L, Stoll D, Bebear JP, et al. Epidemiological and clinical aspects of nasal polyposis in France; the ORLI group experience. Rhinology. 2002;40(2):75-9.

165. Browne JP, Hopkins C, Slack R, Topham J, Reeves B, Lund V, et al. Health-related quality of life after polypectomy with and without additional surgery. Laryngoscope. 2006;116(2):297-302.

166.   Farhood Z, Schlosser RJ, Pearse ME, Storck KA, Nguyen SA, Soler ZM. Twenty-two-item Sino-Nasal Outcome Test in a control population: a cross-sectional study and systematic review. Int Forum Allergy Rhinol. 2015.

167.   Van Bruaene N, Derycke L, Perez-Novo CA, Gevaert P, Holtappels G, De Ruyck N, et al. TGF-beta signaling and collagen deposition in chronic rhinosinusitis. J Allergy Clin Immunol. 2009;124(2):253-9.

168.   Baba S, Kagoya R, Kondo K, Suzukawa M, Ohta K, Yamasoba T. T-cell phenotypes in chronic rhinosinusitis with nasal polyps in Japanese patients. Allergy Asthma Clin Immunol. 2015;11:33.

169.   Van Bruaene N, Perez-Novo CA, Basinski TM, Van Zele T, Holtappels G, De Ruyck N, et al. T-cell regulation in chronic paranasal sinus disease. J Allergy Clin Immunol. 2008;121(6):1435-41, 41.e1-3.

170.   Swindle EJ, Brown JM, Radinger M, DeLeo FR, Metcalfe DD. Interferon-gamma enhances both the anti-bacterial and the pro-inflammatory response of human mast cells to Staphylococcus aureus. Immunology. 2015;146(3):470-85.

171.   Wang X, Dong Z, Zhu DD, Guan B. Expression profile of immune-associated genes in nasal polyps. Annals of Otology Rhinology and Laryngology. 2006;115(6):450-6.

172.   Tomassen P, Vandeplas G, Van Zele T, Cardell LO, Arebro J, Olze H, et al. Inflammatory endotypes of chronic rhinosinusitis based on cluster analysis of biomarkers. J Allergy Clin Immunol. 2016;137(5):1449-56.e4.

173.   Bachert C, Akdis CA. Phenotypes and Emerging Endotypes of Chronic Rhinosinusitis. J Allergy Clin Immunol Pract. 2016;4(4):621-8.

174.   Gevaert P, Van Bruaene N, Cattaert T, Van Steen K, Van Zele T, Acke F, et al. Mepolizumab, a humanized anti-IL-5 mAb, as a treatment option for severe nasal polyposis. J Allergy Clin Immunol. 2011;128(5):989-95.e1-8.

175.   Netea MG, Simon A, van de Veerdonk F, Kullberg BJ, Van der Meer JW, Joosten LA. IL-1beta processing in host defense: beyond the inflammasomes. PLoS Pathog. 2010;6(2):e1000661.

176.   LaRock CN, Todd J, LaRock DL, Olson J, O'Donoghue AJ, Robertson AA, et al. IL-1beta is an innate immune sensor of microbial proteolysis. Sci Immunol. 2016;1(2).

177.   Ali A, Na M, Svensson MN, Magnusson M, Welin A, Schwarze JC, et al. IL-1 Receptor Antagonist Treatment Aggravates Staphylococcal Septic Arthritis and Sepsis in Mice. PLoS One. 2015;10(7):e0131645.

178.   Hunter CA, Jones SA. IL-6 as a keystone cytokine in health and disease. Nat Immunol. 2015;16(5):448-57.

179.   Stach CS, Herrera A, Schlievert PM. Staphylococcal superantigens interact with multiple host receptors to cause serious diseases. Immunol Res. 2014;59(1-3):177-81.

180.   Proft T, Fraser JD. Bacterial superantigens. Clin Exp Immunol. 2003;133(3):299-306.

181.   Vanlaere I, Libert C. Matrix metalloproteinases as drug targets in infections caused by gram-negative bacteria and in septic shock. Clin Microbiol Rev. 2009;22(2):224-39, Table of Contents.

182.   Grigat J, Soruri A, Forssmann U, Riggert J, Zwirner J. Chemoattraction of macrophages, T lymphocytes, and mast cells is evolutionarily conserved within the human alpha-defensin family. J Immunol. 2007;179(6):3958-65.

183.   Frye M, Bargon J, Dauletbaev N, Weber A, Wagner TO, Gropp R. Expression of human alpha-defensin 5 (HD5) mRNA in nasal and bronchial epithelial cells. J Clin Pathol. 2000;53(10):770-3.

184.   Lopez-Castejon G, Brough D. Understanding the mechanism of IL-1beta secretion. Cytokine Growth Factor Rev. 2011;22(4):189-95.

185.   Chaaban MR, Kejner A, Rowe SM, Woodworth BA. Cystic fibrosis chronic rhinosinusitis: a comprehensive review. Am J Rhinol Allergy. 2013;27(5):387-95.

186.   Rogers GA, Den Beste K, Parkos CA, Nusrat A, Delgaudio JM, Wise SK. Epithelial tight junction alterations in nasal polyposis. Int Forum Allergy Rhinol. 2011;1(1):50-4.

187.   Murphy J, Ramezanpour M, Stach N, Dubin G, Psaltis AJ, Wormald PJ, et al. Staphylococcus Aureus V8 protease disrupts the integrity of the airway epithelial barrier and impairs IL-6 production in vitro. Laryngoscope. 2017.

188.   Lam K, Schleimer R, Kern RC. The Etiology and Pathogenesis of Chronic Rhinosinusitis: a Review of Current Hypotheses. Curr Allergy Asthma Rep. 2015;15(7):41.

189.   Ribet D, Cossart P. How bacterial pathogens colonize their hosts and invade deeper tissues. Microbes Infect. 2015;17(3):173-83.

190.   Ray K, Marteyn B, Sansonetti PJ, Tang CM. Life on the inside: the intracellular lifestyle of cytosolic bacteria. Nat Rev Microbiol. 2009;7(5):333-40.

191.   Kong C, Neoh H, Nathan S. Targeting Staphylococcus aureus Toxins: A Potential form of Anti-Virulence Therapy. Toxins (Basel). 2016;8(3).

192.   Takeuchi O, Hoshino K, Akira S. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. J Immunol. 2000;165(10):5392-6.

193.   Bax HJ, Keeble AH, Gould HJ. Cytokinergic IgE Action in Mast Cell Activation. Front Immunol. 2012;3:229.

194.   Wesolowski J, Paumet F. The impact of bacterial infection on mast cell degranulation. Immunol Res. 2011;51(2-3):215-26.

195.   Yu Y, Blokhuis BR, Garssen J, Redegeld FA. Non-IgE mediated mast cell activation. Eur J Pharmacol. 2016;778:33-43.

196.   Sutherland RE, Olsen JS, McKinstry A, Villalta SA, Wolters PJ. Mast cell IL-6 improves survival from Klebsiella pneumonia and sepsis by enhancing neutrophil killing. J Immunol. 2008;181(8):5598-605.

197.   Xu X, Zhang D, Lyubynska N, Wolters PJ, Killeen NP, Baluk P, et al. Mast cells protect mice from Mycoplasma pneumonia. Am J Respir Crit Care Med. 2006;173(2):219-25.

198.   Gekara NO, Weiss S. Mast cells initiate early anti-Listeria host defences. Cell Microbiol. 2008;10(1):225-36.

199.   Malaviya R, Ikeda T, Ross E, Abraham SN. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. Nature. 1996;381(6577):77-80.

200.   Dawicki W, Jawdat DW, Xu N, Marshall JS. Mast cells, histamine, and IL-6 regulate the selective influx of dendritic cell subsets into an inflamed lymph node. J Immunol. 2010;184(4):2116-23.

201.   Schaaf B, Luitjens K, Goldmann T, van Bremen T, Sayk F, Dodt C, et al. Mortality in human sepsis is associated with downregulation of Toll-like receptor 2 and CD14 expression on blood monocytes. Diagn Pathol. 2009;4:12.

202.   Kumar A, Singh CN, Glybina IV, Mahmoud TH, Yu FS. Toll-like receptor 2 ligand-induced protection against bacterial endophthalmitis. J Infect Dis. 2010;201(2):255-63.

203.   Wernersson S, Pejler G. Mast cell secretory granules: armed for battle. Nat Rev Immunol. 2014;14(7):478-94.

204.   Harata G, He F, Takahashi K, Hosono A, Kawase M, Kubota A, et al. Bifidobacterium suppresses IgE-mediated degranulation of rat basophilic leukemia (RBL-2H3) cells. Microbiol Immunol. 2010;54(1):54-7.

205.   Kawahara T. Inhibitory effect of heat-killed Lactobacillus strain on immunoglobulin E-mediated degranulation and late-phase immune reactions of mouse bone marrow-derived mast cells. Anim Sci J. 2010;81(6):714-21.

206.   Kulka M, Fukuishi N, Rottem M, Mekori YA, Metcalfe DD. Mast cells, which interact with Escherichia coli, up-regulate genes associated with innate immunity and become less responsive to Fc(epsilon)RI-mediated activation. J Leukoc Biol. 2006;79(2):339-50.

207.   Oksaharju A, Kankainen M, Kekkonen RA, Lindstedt KA, Kovanen PT, Korpela R, et al. Probiotic Lactobacillus rhamnosus downregulates FCER1 and HRH4 expression in human mast cells. World J Gastroenterol. 2011;17(6):750-9.

208.   Magerl M, Lammel V, Siebenhaar F, Zuberbier T, Metz M, Maurer M. Non-pathogenic commensal Escherichia coli bacteria can inhibit degranulation of mast cells. Exp Dermatol. 2008;17(5):427-35.

209.   Van Zele T, Holtappels G, Gevaert P, Bachert C. Differences in initial immunoprofiles between recurrent and nonrecurrent chronic rhinosinusitis with nasal polyps. Am J Rhinol Allergy. 2014;28(3):192-8.

210.   Cortés-Vieyra R, Bravo-Patiño A, Valdez-Alarcón JJ, Juárez MC, Finlay BB, Baizabal-Aguirre VM. Role of glycogen synthase kinase-3 beta in the inflammatory response caused by bacterial pathogens. J Inflamm (Lond). 2012;9(1):23.

211.   Martin M, Rehani K, Jope RS, Michalek SM. Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. Nat Immunol. 2005;6(8):777-84.

212.   Cheng YL, Wang CY, Huang WC, Tsai CC, Chen CL, Shen CF, et al. Staphylococcus aureus induces microglial inflammation via a glycogen synthase kinase 3beta-regulated pathway. Infect Immun. 2009;77(9):4002-8.

213.   Lin CF, Tsai CC, Huang WC, Wang CY, Tseng HC, Wang Y, et al. IFN-gamma synergizes with LPS to induce nitric oxide biosynthesis through glycogen synthase kinase-3-inhibited IL-10. J Cell Biochem. 2008;105(3):746-55.

214.   Rehani K, Wang H, Garcia CA, Kinane DF, Martin M. Toll-like receptor-mediated production of IL-1Ra is negatively regulated by GSK3 via the MAPK ERK1/2. J Immunol. 2009;182(1):547-53.

215.   Chan MM, Cheung BK, Li JC, Chan LL, Lau AS. A role for glycogen synthase kinase-3 in antagonizing mycobacterial immune evasion by negatively regulating IL-10 induction. J Leukoc Biol. 2009;86(2):283-91.

216.   Lin SY, Reh DD, Navas-Acien A. Allergic rhinitis, chronic rhinosinusitis, and symptom severity: a population-based study. Int Forum Allergy Rhinol. 2012;2(1):51-6.

217.   Alobid I, Benitez P, Valero A, Berenguer J, Bernal-Sprekelsen M, Picado C, et al. The impact of atopy, sinus opacification, and nasal patency on quality of life in patients with severe nasal polyposis. Otolaryngol Head Neck Surg. 2006;134(4):609-12.

218.   Krouse JH. Computed tomography stage, allergy testing, and quality of life in patients with sinusitis. Otolaryngol Head Neck Surg. 2000;123(4):389-92.

Timothy C Biggs

Bibliography

255

Janfaza P, Nadol J, Galla R, Fabian R, Montgomery W. Surgical anatomy of the head and neck. Lippincott Williams & Wilkins; 2001.

Flint P, Haughey B, Lund V, Niparko J, Richardson M, Robbins K, Thomas J. Cummings Otolaryngology Head and Neck Surgery. 5th Ed. Philadelphia: Mosby Elsevier; 2010.

Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002.

Bibliography