***Staphylococcus aureus* internalisation in mast cells in nasal polyps - Characterisation of interactions and potential mechanisms**

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**Abstract**

**Background** - Chronic rhinosinusitis with nasal polyps (CRSwNP) is a common chronic condition. The exact cause of nasal polyps remains unknown. Recently, we made the novel observation of intracellular localisation of *Staphylococcus aureus (S aureus)* within mast cells in nasal polyps.

**Objective** - This follow-up study aimed to further characterise interactions between *S aureus* and mast cells in this setting, and elucidate potential internalisation mechanisms with particular emphasis on the role of *S aureus* enterotoxin B (SEB).

**Methods** - A prospective study was performed using an explant tissue model with *ex vivo* inferior turbinate mucosa obtained from CRSwNP patients (n=7) and non-CRS (n=5) patients. Immunohistochemistry was used to characterise *S aureus* uptake into mast cells and investigate the effects of SEB on this process. An *in vitro* cell culture model was used to investigate mast cell-*S aureus* interactions using a combination of fluorescent *in situ* hybridisation, confocal laser scanning microscopy, scanning electron microscopy, transmission electron microscopy, and proliferation assays.

**Results** - *S aureus* were entrapped by extracellular traps and entered mast cells through phagocytosis. Proliferating intracellular *S aureus* led to the expansion and eventual rupture of mast cells, resulting in release of viable *S aureus* into the extracellular space. The presence of *SEB* appeared to promote internalisation of *S aureus* into mast cells.

**Conclusion** - This study provides new insights into the interactions between *S aureus* and mast cells, including the internalisation process, and demonstrates a prominent role for SEB in promoting uptake of the bacteria into these cells.

**Key Messages**

**-** New insights into the interactions between *S aureus* and mast cells in nasal polyps, including the internalisation process.

- Prominent role for SEB in promoting uptake of the bacteria into mast cells.

- Potential mechanism of contribution of intracellular *S aureus* towards nasal polyp pathogenesis presented.

**Capsule Summary**

This study sheds light on the potential contribution of intracellular *S aureus* to nasal polyp pathogenesis. Better understanding of this process will facilitate the development of novel future therapies specifically targeting these bacteria.

**KEY WORDS**

Chronic rhinosinusitis, chronic rhinosinusitis with nasal polyps, nasal polyps, intracellular bacteria, *Staphylococcus* *aureus*, mast cells, bacterial superantigens, SEB.

**Abbreviations**

**CLSM** Confocal Laser Scanning Microscopy

**CRS** Chronic rhinosinusitis

**CRSsNP** Chronic rhinosinusitis without nasal polyps

**CRSwNP** Chronic rhinosinusitis with nasal polyps

**CFU** Colony Forming Unit

**DAB** 3,3’-Diaminobenzidine

**DAPI** 4’,6’-diamidino-2-phenylindole

**GMA** Glycol methacrylate

**HBSS**  Hank’s Balanced Salt Solution

**HMC-1** Human mast cell culture line 1

**IBDR** Intracellular bacteria detection rate

**IFNγ** Interferon Gamma

**IgE** Immunoglobulin E

**IL** Interleukin

**MOI** Multiplicity of infection

**PBS** Phosphate Buffered Saline

**PIPES** Piperazine-N,N′-bis

**RANTES** **Regulated on Activation, Normal T Cell Expressed and Secreted**

**RPMI** Roswell Park Memorial Institute medium

***S aureus*** *Staphylococcus aureus*

**SEB** Staphylococcus enterotoxin B

**SPT** Skin Prick Test

**TEM**  Transmission Electron Microscopy

**TGFβ** Transforming Growth Factor Beta

**TH1** T helper 1

**TH2** T helper 2

**TNFα** Tumour Necrosis Factor Alpha

**Introduction**

Chronic rhinosinusitis is a chronic inflammatory condition of the nose and paranasal sinuses1. It is subdivided into two main disease categories - chronic rhinosinusitis with polyps (CRSwNP) and chronic rhinosinusitis without nasal polyps (CRSsNP)1. CRSwNP is a complex inflammatory disorder of the mucosal lining of the sinonasal cavity, characterised by the formation of benign oedematous out-growths (polyps), which obstruct the nasal cavity and sinus drainage pathways resulting in recurrent infections. It is characterised by a TH2-mediated cytokine profile, with eosinophilia and elevated IL-5, IgE, RANTES and eotaxin1. This is in contrast to CRSsNP which is characterised by the presence of pus and a TH1-mediated profile with neutrophilia and elevated TNF-α, IL-8 and IFN-ƴ 1. However, mixed TH1/TH2 patterns have been detected in some subtypes of CRSwNP and CRSsNP2-4.

CRSwNP affects up to 4% of the general population in Europe and the United States and presents with symptoms including nasal obstruction, facial pain, nasal discharge, post nasal drip and loss of smell, significantly impacting quality of life1. Furthermore, the disease has a profound socioeconomic impact placing a huge financial burden on already over stretched health systems1, 5-8. It has become apparent in recent years, that rather than a single causative agent, CRS is probably a manifestation of a degree of immunological dysfunction due to interactions between a number of host and environmental factors1. These include superantigens9-12, bacterial and fungal biofilms13-15, intracellular bacterial reservoirs16-20, environmental allergens21-22, genetic predisposition23, epithelial defects24-26, and changes in inflammatory cytokine cascades1.

Despite massive expenditure on medical and surgical therapies, a subset of CRS patients exhibits symptoms refractory to established treatments8, 14. Bacterial biofilms have been implicated as playing a crucial role in mediating the chronic inflammation in recalcitrant CRS8, 14, 27-29, with the commonest biofilm-forming microbe reported as *Staphylococcus aureus* (*S aureus*)13-15 30, 31. In 25% of the population, *S aureus* exists as a non-pathogenic commensal, harmlessly colonising the nasal cavity. However, under certain conditions, and in a bid for survival, *S aureus* has the ability to turn clinically pathogenic using several regulatory pathways to transition between commensal and pathogenic states32. This process of switching between different bacterial profiles leads to defects within the innate and adaptive immune systems at the host-environment interface, resulting in chronic inflammatory changes1 24, 33. In relation to nasal polyps, there is evidence that colonising *S aureus* has the ability to produce a variety of toxins including *Staphylococcus* enterotoxin B (SEB) with superantigenic properties which can directly influence both immunostimulatory and immunomodulatory inflammatory cytokines and effector cells10-11.

We recently made the novel observation of intracellular *S aureus* within mast cells in nasal polyps20. This follow-up study is aimed at further characterisation of the interactions between *S aureus* and mast cells in this setting, and elucidation of potential internalisation mechanisms with particular emphasis on the role of SEB.

**Methods**

Subjects

Seven patients with CRSwNP, meeting the diagnostic criteria defined in the latest European position paper on rhinosinusitis and nasal polyps 20121, and 5 control patients with no history of CRS, were included in the study. All CRSwNP patients underwent surgery by the senior authors (P.G.H. and R.J.S.) at University Hospital Southampton NHS Foundation Trust (UHSNFT). Non-CRS patients (control group, 3 patients with nasal blockage undergoing septoplasty, and 2 patients with sinonasal inverted papilloma undergoing endoscopic medial maxillectomy) were operated by Mr Huw Jones (Consultant Rhinologist, UHSNFT). Patients with CRSwNP were offered surgery after having failed an 8-week trial of maximal medical therapy, which included an 8 week course of topical corticosteroids, nasal saline irrigation and low-dose doxycycline. None of the patients had used either topical or systemic corticosteroid, antibacterial, or antihistamine therapies in the 8 weeks before surgery. Exclusion criteria for CRSwNP patients included being aged less than 18 years and suffering from cystic fibrosis or primary ciliary dyskinesia. For non-CRS patients, additional exclusion criteria included CRS, asthma, atopy and aspirin sensitivity. Radiological severity of sinus disease was graded on a computed tomography sinuses scan using the Lund-Mackay scoring system34. Preoperative demographical data were collected including age, sex, atopic status, previous sinonasal surgery, medical history, history of asthma, history of aspirin sensitivity, and smoking habits. Ethical approval was obtained from the local NHS Research Ethics Committee, United Kingdom (REC 09/H0501/74). All study participants provided informed written consent.

Tissue explant model

*Aims of tissue explant model*

To establish a model replicating the host environmental interface within the sinonasal cavity and attempt to reproduce the findings of intracellular *S aureus* within mast cells, by exposing the tissue to different treatments. Analysis using immunohistochemistry will allow microscopic assessment of intracellular *S aureus* as well as mast cell accumulation and degranulation. These observations will provide valuable information on how non-motile *S aureus* migrates into the sub-epithelial layer and how this relates to mast cell behaviour. It will also provide useful information on the effect SEB has on *S aureus* and the surrounding tissue.

*Tissue explant model*

Inferior turbinate specimens were collected in Hanks’ Balanced Salt Solution (HBSS) (Gibco, Paisley, United Kingdom) and transported on ice for processing. Inferior turbinate mucosa was dissected into 5mm x 5mm explants. Each sample was treated with Roswell Park Memorial Institute Medium (RPMI, Gibco, Paisley) supplemented with sterile penicillin/streptomycin (100ug/ml) for 30 minutes to eradicate exogenous bacteria. Samples were rinsed three times in sterile Phosphate Buffered Saline (PBS) and then immersed in 2mls of RPMI alone. Following this, each sample was used to an individual treatment group, as shown in Table I, and incubated for 24 hours at 37oC, 5% CO2. Explants were fixed with acetone, embedded in Glycol methacrylate (GMA), and processed for immunohistochemistry.

*Treatment groups*

The treatment groups were designed to investigate both the individual and combined effects of SEB and *S aureus* on each tissue sample. For each treatment group containing viable *S aureus*, a second group containing non-viable *S aureus* was used to demonstrate that microbial viability was essential in mediating observed effects (Table I).

*S aureus isolate*

*S aureus* was previously isolated from a patient with CRSwNP. The *S aureus* isolate was grown to mid-log phase at 37oC, 5% CO2 in RPMI medium, centrifuged (11,000 x*g* for 10 minutes) and washed with sterile PBS to remove toxins and diluted to the required concentration. Viable bacteria were quantified with colony forming unit (CFU) counts after serial dilution and plating on Columbia blood-agar containing 5% defibrinated horse blood (CBA) for 24 hours. Twenty microlitres of *S aureus* (containing 5.0 x 106 bacterial cells) was added to each treatment.

Non-viable *S aureus* were created using the same strain. *S aureus* was grown to mid-log phase at 37oC/5% CO2 in RPMI medium. The number of viable bacteria was determined through serial dilution and plating on CBA. The bacteria were centrifuged (11,000 x*g* for 10 minutes) then immersed in 16% paraformaldehyde for 1 hour.at room temperature. Viability was determined after serial dilution, plating on CBA and incubation for 48 hours at 37oC/5% CO2. Following confirmation of negative viability 5.0 x 106 non-viable *S aureus* were added to the appropriate samples.

*Immunohistochemistry*

The immunohistochemistry protocol was adapted from that of Britten et al35. Briefly, immunohistochemistry was undertaken in GMA-embedded tissue. Primary monoclonal antibodies were applied for 20 hours (at room temperature) at previously titrated optimal dilutions. Appropriate biotinylated secondary antibodies (Dako, Ely, United Kingdom) were then applied for 2 hours (at room temperature) followed by application of avidin biotin-peroxide complexes. Previously titrated chromogen 3,3′-diaminobenzidine (DAB) substrate was then applied, and finally the sections were counterstained with Mayer’s haematoxylin. All experiments included negative control slides with omission of primary antibodies and appropriate isotype-matched antibodies. A positive control model using an isolated CRSwNP *S aureus* strain was used to test the accuracy of the mouse anti–*S aureus* primary antibody (Abcam, Cambridge, United Kingdom) and to optimize the concentration of DAB substrate, before use on study sections. A primary monoclonal antibody specific for mast cell tryptase (AA1, Abcam, Cambridge, UK) was used to stain for mast cells.

*Degranulation*

Mast cell degranulation was assessed on sections, stained immunologically with the AA1 anti-mast cell tryptase monoclonal antibody. Degranulating mast cells were identified as mast cell tryptase ‘blushes’ with granule dissemination.

*Cell count*

The epithelial length and sub-epithelial area were measured with the aid of computerised image analysis (KS400 software with a Zeiss Axioskop 2 microscope and Axiocam, Zeiss, Bicester, UK). Cells were manually counted from 5 different tissue sections per patient for each treatment group. Epithelial cell rates were calculated using the equation: Epithelial cell rate (cells mm-1) = Total number of cells / Epithelial length (mm).Sub-epithelial cell rates were calculated using the equation: Sup-epithelial cell rate (cells mm-2) = Total number of cells / Sub-epithelial area (mm2).

Cell culture model

*Aims of the cell culture model*

To explore the mechanisms surrounding the internalisation of *S aureus* into mast cells at a cellular level. This involves combining human mast cells with a *S aureus* microbe isolated from a patient with CRSwNP. A proliferation assay is used to assess microbial viability. Changes in cellular morphology and mechanisms of bacterial internalisation is assessed using confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM).

*Human mast cell line*

The human mast cell (HMC-1) line was kindly donated by Dr J.H. Butterfield (Mayo Clinic, Rochester, Minn., USA).  HMC-1 cells were maintained in 75cm2 tissue culture flasks at 37°C/5% CO2.  Complete medium consisted of Iscove’s modified Dulbecco’s medium (IMDM) (Gibco, Paisley, UK) supplemented with 25mM HEPES, sodium bicarbonate and L-glutamine, 10% calf serum and 1.2 mM alphathioglycerol (Sigma).   The alpha thioglycerol and complete medium were freshly prepared weekly.  Cell cultures were split when cell density reached 1.0-1.5x106 cells/ml. Following this, 2.0 x106 cells were seeded into a 12-well plate in 2ml of culture medium.  The viability of cells was confirmed to be 99% before the start of the stimulation.

*Co-culture assay*

*S aureus* was grown in RPMI medium to mid-log phase. Periodical culture samples were taken, serially diluted and plated on CBA. Concurrent optical density (OD) measurements were taken and plotted against CFU counts. The number of bacteria was calculated per OD. The bacteria were centrifuged (11,000x*g* for 5 minutes), washed with sterile PBS and diluted to the required concentration. A final inoculum of 1.0 x 106 bacteria was added to 1.0 x106 HMC-1 cells per well for a multiplicity of infection (MOI) of 1:1. For non-viable *S aureus*, samples were prepared as before.

Seven treatment groups were used as outlined in Table II. Co-cultures were incubated at 37°C/5% CO2 for 2 hours then Lysostaphin (20 µg/ml) added to eradicate extracellular *S aureus*, leaving only viable intracellular reservoirs.

*Proliferation assay*

The Click-iT® EdU (5-ethynyl-2´-deoxyuridine) proliferation assay (Thermofisher, Loughborough, UK) was used to detect DNA synthesis and provides an accurate method of assessing cell proliferation. The Edu (5-ethynyl-2´-deoxyuridine) is a nucleoside analogue to thymidine and incorporates into DNA during active DNA synthesis. Detection is based on a click reaction, a copper catalyzed covalent reaction between picolyl azide (Alexa Fluor®488 dye) and an alkyne EdU. Demonstration of proliferating bacteria helps to confirm viability.

The assay was performed according to the manufacturer’s product protocol. Briefly, 10µM of EdU was added to each treatment group containing HMC-1 cells at 37oC, 5% CO2. After 24 hours, the HMC-1 cells were centrifuged (11,000 x*g*) for 10 minutes and the pellet fixed in 3.7% formaldehyde (prepared in PBS) for 15 minutes. The HMC-1 cells were rinsed twice with 3% bovine serum albumin (BSA) in PBS, followed by treatment with the permealisation buffer (0.5% Triton® X-100 in PBS) at room temperature for 20 minutes. Cells were further rinsed with BSA in PBS before the addition of the Click-iT® reaction cocktail (Click-iT® reaction buffer, CuSO4, Alexa Fluor®647 azide and reaction buffer additive) for 30 minutes. Cells were finally rinsed twice (with BSA in PBS) and suspended in PBS. The DAPI-488 (Sigma-aldrich, Dorset, UK) stain was added to the cells 30 minutes prior to imaging with an inverted Leica SP5 CLSM.

Five thousand cells were manually counted from each well (1000 from five separate randomly selected fields) and the proportion of intracellular *S aureus* was presented as a percentage of the total number of cells. Cell measurements were made using CLSM and the Leica Application Suite (LAS) AF software.

*Confocal Laser Scanning Microscopy*

CLSM imaging was performed as previously described26. Briefly, after staining, tissues were mounted within chamber slides with cover-slip bottoms and imaged with a Leica TCS SP5 inverted confocal system (Leica Microsystems, Milton Keynes, United Kingdom) using a 63× oil immersion lens. Sequential scanning was used to further eliminate cross-talk interference from multiple fluorophores. Images were collected and analysed using Leica LAS-AF software.

*Transmission electronic microscopy*

Cells were fixed at 2, 4 and 24 hours with 3% glutaraldehyde and 4% formaldehyde in 0.1M piperazine-N,N′-bis (PIPES) for TEM for 24 hours. Cells were rinsed in 0.1M PIPES and post fixed in 1% osmium tetroxide in 0.1M PIPES for 1 hour. After further rinsing in 0.1M PIPES, uranyl acetate was added to the cells. Cells were then serially dehydrated in ethanol (30%, 50%, 70%, 95%, 100%, 100%), and then placed in acetonitrile. Cells were then subjected to acetonitrile:resin (50:50) for 1 hour, then infiltrated in resin overnight. After 16 hours, the cells were embedded in new epoxy resin and polymerised at 60oC. Sections were each cut with an Ultra Cut E Ultramicrotome and were finally imaged using a **Hitachi H7000 TEM.**

*Colony forming unit enumeration assays*

As previously described, *S aureus* was grown to the mid-log phase and co-cultured with HMC-1 mast cells, with and without SEB (10µg/ml), at an MOI of 1:1, over various time points. Colony forming unit (CFU) enumeration of extracellular *S aureus* was undertaken by pelleting cells, serially diluting culture supernatants, then plating on CBA plates and incubating at 37°C, 5% CO2. Cells were treated with Lysostaphin (20µg/ml) for 30 minutes followed by serial washing, in order to eradicate extracellular *S aureus*. Cells were then treated with Triton X-100 and vortexed to disrupt the cell membrane and release intracellular *S aureus*, which were then assessed by CFU enumeration on CBA plates.

In order to examine *S aureus* infection over 24 hours, and determine the possible extracellular release of *S. aureus* from mast cells, HMC-1 mast cells were co-cultured with *S aureus* for 6 hours, following which cells were pelleted, washed and treated with Lysostaphin as detailed above. Following further washing, cells were re-suspended and the absence of extracellular bacteria confirmed through CFU enumeration of the culture supernatants, confirming lack of any *S aureus* growth. The co-culture was then continued to the 24-hour time point and intracellular and extracellular CFUs enumerated as previously described.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 7.0 software (Graph Pad Software, Inc, San Diego, USA). Comparisons between different treatment groups, were made using a paired (2-tailed) *t*-test. The level of significance was accepted as a *p* value of less than 0.05. Statistical significance was represented on graphs using the star rating: *\*p <0.05, \*\*p <0.001,\*\*\*p <0.0001.*

**Results**

Patient demographics

Patient demographics are presented in Table III. The control group had no clinical or radiological evidence of CRS, were non-atopic, non-asthmatic, with no history of aspirin sensitivity.

Explant tissue model

*Viable S aureus*

The presence of viable *S aureus* alone (without additional SEB) resulted in a significant increase in intracellular bacteria within both the epithelial and sub-epithelial layers compared to the no treatment group (*p*= 0.0009 and *p*= 0.0031, respectively). Within the epithelial layer, *S aureus* was identified within both mast cells and epithelial cells and in the sub-epithelial layer, within mast cells alone. A significant increase in the mast cell density was observed within the epithelial layer (*p*= 0.0031) in response to *S aureus* invasion (Figure I). The use of non-viable *S aureus* resulted in no significant increase in relative numbers of intracellular bacteriaor mast cell density, when compared to the no treatment group (Figure I).

Within the control tissue, no mast cells were seen within the epithelial layer and no significant increase in mast cell density was seen in the sub-epithelial layer, compared to all other treatment groups (Figure IE-H). Within the sub-epithelial layer, no intracellular *S aureus* was seen within any of the mast cells.

*S aureus* Enterotoxin B and viable *S aureus*

Addition of SEB alone to both the CRSwNP and control tissue explants did not significantly increase numbers of intracellular bacteria or mast cell density, compared to the no treatment group. However, the addition of SEB to viable *S aureus* resulted in an enhanced response within the CRSwNP tissue, significantly increasing both the number of mast cells containing intracellular *S aureus* and mast cell density, within both the epithelial and sub-epithelial layers, when compared to viable *S aureus* alone (Figure I). Within the control tissue, the addition of SEB to viable *S aureus* demonstrated no significant difference in either layer, compared to viable *S aureus* alone or the no treatment group (Figure 1E-H).

*Mast cell degranulation*

Within the CRSwNP tissue, mast cells degranulation occurred exclusively within the sub-epithelial layer. Addition of viable *S aureus* alone did not result in a significant increase in mast cell degranulation compared to the no treatmentgroup. However, in combination with SEB, *S aureus* induced a significant increase in mast cell degranulation compared to that in all other groups (Figure IIA&B). The effect of additional SEB to viable *S aureus* on mast cell degranulation was also demonstrated when looking at the proportional data (Figure IIC). The addition of SEB to viable *S aureus* caused 74% of mast cells to degranulate, more than twice that seen with viable *S aureus* alone (35%) and 5 times more than within the no treatment group (13%).

Within the control tissue, there was no significant difference in mast cell degranulation across all treatment groups (range 12-18%) and no significant difference when compared to the CRSwNP no treatment group.

Co-culture Assay

*Intracellular S aureus*

Intracellular *S aureus*within the HMC-1 mast cells was observed within both groups containing viable *S aureus*. Viability of the intracellular *S aureus* was confirmed using the Click-iT® EdU Edu proliferation assay. No intracellular *S aureus* was demonstrated in any of the other 5 treatment groups. The number of mast cells containing intracellular *S aureus* increased exponentially with time. Using TEM, 16% of mast cells contained *S aureus* at 2 hours and 76% at 24 hours. This was validated using CLSM and DAPI where 64% of mast cells were observed to contain *S aureus* at 24 hours.

*Mast cell size with S aureus internalisation*

Mast cell size differed significantly between treatment groups (Table IV). Both groups containing viable *S aureus* demonstrated a significant increase in mast cell size, compared to the no treatment group (Figures III A-C). The mean diameter of mast cells within the no treatment group was 10.95µm compared to 15.28µm in the *S aureus* alone group and 15.37µm in the *S aureus* and SEB group. Themast cells containing intracellular *S aureus* continued to expand, up to 20µm in diameter, where they eventually ruptured, seeding viable *S aureus* into the extracellular environment (Figure III D). This observation was supported by the detection of viable extracellular *S aureus* at 24 hours, despite initial treatment with antibiotics at 2 hours.

*Extracellular traps*

A number of HMC-1 mast cells were observed entrapping some *S aureus* through DNA arms projecting into the extracellular space (Figure IV).

*Internalisation of S aureus within mast cells*

*S aureus* was observed internalising within mast cells through phagocytosis. By 2 hours, *S aureus* had attached to the cell surface (Figures V A-B). By 4 hours, *S aureus* had entered the mast cells apparently through phagocytosis (Figure V C). Some bacteria appeared to be degraded, but other bacteria appeared to be within protective phagosomes where they began to proliferate. By 24 hours, both the intracellular and extracellular compartments contained viable *S* *aureus* (Figure V D).

These findings were validated using conventional culture, where CFU counts demonstrated viable intracellular *S aureus* within HMC-1 cells by 2 hours, increasing in numbers over 24 hours (Figure VI A). The addition of SEB significantly increased the numbers of intracellular *S aureus* within HMC-1 cells (Figure VI A). The seeding of *S aureus* into the extracellular space through cell rupture was confirmed in the CFU enumeration experiment, where viable extracellular *S aureus* were identified at 24 hours following complete extracellular *S aureus* eradication at 6 hours after antibacterial treatment (Figure VI B).

**Discussion**

CRSwNP and CRSsNP are complex conditions with as yet unclear pathophysiology8. Several aetiological factors have been proposed as playing a role in the ongoing inflammatory process, but strong evidence for a single agent remains lacking. In fact, it is becoming more evident that the pathogenesis of CRS and nasal polyp formation is multifactorial, including interactions between the environment and the host’s genetic profile1.

Bacteria were previously thought to primarily play a role in acute infective rhinosinusitis with little relevance to chronicity and the development of nasal polyps36. However, with the advancement of molecular detection techniques, a clearer understanding of the role and importance of bacteria and their different profiles in CRSwNP is emerging8. The most common bacteria to colonise the sinuses is *S aureus,* with rates of up to 60% in CRSwNP patients versus 33% in the general population37. The identification of *S aureus* colonisation in one third of the general population suggests that *S aureus* is unlikely to be pathogenic. However, in the presence of certain host and environmental factors, including changes in the local immune environment, these bacteria appear to transform into a more pathogenic phenotype, manifesting with more severe infections, development of chronicity, and recalcitrance. In a previous study we demonstrated that *S aureus* was present as surface-related bacterial biofilms on normal (non-polypoidal) CRS sinonasal mucosa samples, but not on the epithelial surface of nasal polyps or control tissue20. Instead, *S aureus* was observed as being sub-epithelial and residing in the cytoplasm of mast cells in nasal polyp samples20. In this study we aimed to extend these novel findings, and better elucidate the underlying mechanisms involved in the internalisation of *S aureus* within mast cells in nasal polyps.

The development of a tissue explant model allowed the study of *S aureus* and its most notable superantigen, SEB, at the host-environmental interface. Superantigens manipulate the local immune response, leading to increased tissue damage and remodelling10, 12, 37. Superantigens have been shown to exert effects on both host cells and the cytokines associated with local innate and adaptive immunity38. Host cells stimulated by the release of superantigens include epithelial cells, B and T cells, eosinophils, fibroblasts and mast cells10, 39. Superantigens also induce up-regulation of IL-4 and IL-5 and down-regulation of TGF-β and IL-1040-42. Other effects reported include manipulation of eicosanoid metabolism43-44, granulocyte augmentation45, and the induction of glucocorticoid insufficiency46. The net effect results in the conversion of a TH1 cytokine profile in sinonasal mucosa into a TH2 skewed cytokine profile, as seen in nasal polyps1.

The results presented in this study provide further evidence of the crucial role that superantigens play in the pathogenesis of nasal polyp formation. The addition of SEB appeared to amplify the effects that *S aureus* had on the host innate immune system, including a significant increase in the recruitment of mast cells into the epithelium and the internalisation of *S aureus* within these cells. This is a novel finding. These mast cells, laden with non-motile intracellular *S aureus* appear to migrate into the sub-epithelial layer, followed by release of their contents. This led to significantly increased numbers of *S aureus*-containing mast cells within the sub-epithelial layer. These findings, reproduced in our explant tissue model, suggest that this synergistic effect of combined SEB and *S aureus* is important in promoting and sustaining a chronic inflammatory milieu which downstream may contribute to nasal polyp pathogenesis. These findings were not evident in control tissue taken from patients with no history of CRS. The absence of mast cells within the epithelial layer of control tissue may be relevant due to the loss of a transport medium for the non-motile *S aureus* to passinto the sub-epithelial layer. This may suggest that patients with CRSwNP may have a defect in their local immune system, which is exploited by *S aureus* containing specific superantigens.

In addition to their well-established role in allergic inflammation, it is becoming more evident that mast cells play a crucial role in the innate response to pathogenic infections47. Described as sentinel cells, mast cells play an important role in the initial defence against pathogenic invasion47. Following incursion by environmental pathogens, mast cells initially respond by secreting a range of pro-inflammatory mediators, including histamine, IL-6, chemokines and TNF-α, resulting in the recruitment of further inflammatory cells, such as neutrophils, dendritic cells, and additional mast cells to the site of infection47-48. Furthermore, mast cells also appear to exert a direct effect on pathogens, through the secretion of preformed and *de novo* synthesised mediators, secretion of extracellular antimicrobial compounds, phagocytosis, and use of DNA extracellular traps47-49. The degree of mast cell reaction appears to be microbial-specific, likely through the recognition of pathogen-associated molecular patterns, facilitating a tailored innate response based on stored information from previous encounters1.

Direct interaction of mast cells with bacteria has been analysed through several *in vitro* studies47- 48. It is well established that one of the antimicrobial mechanisms of bacterial internalisation includes phagocytosis which was clearly shown within this study. Within 2 hours of *in vitro* co-culture of *S aureus* with HMC-1 mast cells, bacteria began to bind and adhere to the mast cells. By 4 hours, the mast cells had engulfed the *S aureus* within what appeared to be nascent phagosomes. At this stage, other *in vitro* studies have described the eventual incorporation of this phagosome into the mast cell endocytic pathway, fusing lysosomes with the net result of bacterial digestion49. In our study, this same observation was seen within the first 4 hours of co-culture. However, alongside phagosomes digesting *S aureus*, were also viable and proliferating bacteria. After 24 hours, the numbers of individual viable intracellular *S aureus*, had significantly increased, with no evidence at this stage of phagosomes digesting bacteria. Some bacteria appeared to be prone to degradation, whereas others were resistant to this process. This may be indicative of different bacterial phenotypes with varying degrees of susceptibility to the mast cell phagocytic activity. Within the co-culture assay, the *S aureus* phenotype with the ability to internalise and thrive within mast cells, proliferated and gradually replaced the other *S aureus* phenotypes being degraded, which may represent a basic survival mechanism for the bacteria. This may suggest that the ability of *S aureus* to survive within mast cells may be dependent on whether the invading pathogen is of a specific phenotype, and may help explain why intracellular internalisation is not a feature in all *S aureus*-related infections.

This study’s findings are consistent with another *in vitro* study that demonstrated *S aureus* within HMC-1 cells, which remained viable for up to 5 days47. After two hours, 20% of mast cells contained viable intracellular *S aureus*47. At two hours, our co-culture model demonstrated similar levels of intracellular *S aureus* in HMC-1 cells which increased significantly to 76% at 24 hours. Despite eradication of all extracellular bacteria at 2 hours with antibacterial therapy, by 24 hours the extracellular space was full of viable *S aureus*. Furthermore, the mast cells containing live, viable *S aureus* at this time point were significantly larger than cells with no intracellular bacteria with the addition of SEB further increasing cell size significantly. This may represent expansion of the mast cells secondary to the exponential growth of the proliferating intracellular bacteria. As the mast cells reach maximal capacity, the cell wall appears to rupture with release of its contents into the extracellular space. Through this mechanism, viable *S aureus* can exit the mast cells and enter into the extracellular space with further internalisation into newly recruited mast cells. This cycle is then repeated further amplifying these effects. Extracellular traps have been previously described as an antimicrobial mechanism adopted by mast cells to trap and ensnare extracellular bacteria47. This has also been demonstrated in eosinophils50. These traps composed of DNA and granule proteins have been shown *in vitro* to entrap extracellular *S aureus*, before killing them47.

Another anti-pathogenic mast cell defence mechanism includes the initial release of extracellular antimicrobial compounds, such as cathelicidin-related antimicrobial peptide, stored within the cell granules47. These granules are released through degranulation on exposure to certain pathogens. Abel et al (2011), demonstrated mast cell degranulation on exposure to *S aureus*47.Our study not only reproduced their findings, but demonstrated that addition of SEB to *S aureus* resulted in a two-fold increase in mast cell degranulation. Drake-Lee & Prince (1997) demonstrated significantly higher numbers of degranulating mast cells in nasal polyps, compared to inferior turbinate mucosa and non-CRS mucosa51. The internalisation of *S aureus* within mast cells may be a survival strategy to evade these extracellular compounds. The nature of degranulation related to *S aureus* exposure is a slow process, and differs from the rapid secretory events seen in anaphylactic reactions47. As well as antimicrobial compounds, mast cells also secrete tryptase, chymase, lysozymes, histamine, heparin, proteases, β-hexosaminidase and positively charged proteins, such as TNF-α47- 49, into the extracellular space. Slow mast cell degranulation, together with cell rupture due to proliferating intracellular *S aureus,* could result in a localised increase in the levels of extracellular pro-inflammatory cytokines and mediators, manifesting as oedema within the lamina propria, and potentially contributing to the formation of nasal polyps.

The pathogenesis of nasal polyps is undoubtedly complex and cannot be explained by a single aetiological factor or simple pathway. Nevertheless, we have used the findings from this study alongside other well-established evidence, to propose a potential mechanism for the contribution of intracellular *S aureus* towards the pathogenesis of nasal polyps. Clearly, this will require further work to validate, and whilst likely to represent a small piece of a complex jigsaw, we believe it is interesting and thought provoking.

*Proposed Mechanism (Figure VII)*

On exposure to an endotoxin-releasing strain of *S aureus*, mast cells are recruited into the epithelial layer by SEB, where non-motile *S aureus* are internalised within these mast cells through phagocytosis, and transported to the sub-epithelial layer for degrading. Some of the intracellular *S aureus* appear to possess the ability to remain viable and multiply within the mast cells. The combined effect of SEB and viable intracellular *S aureus* stimulates mast cell degranulation, releasing pro-inflammatory mediators and cytokines into the extracellular space. Intracellular *S aureus* proliferates within the mast cells, significantly increasing the cell size. Eventually, the mast cells rupture releasing their pro-inflammatory cytokines, mediators and viable *S aureus* into the extracellular space. Furthermore, SEB released from *S aureus* causes damage to the epithelial cells, leading to epithelial proliferation and remodelling, resulting in a disorganised and defective epithelial barrier. The end result of these processes is a build-up of pro-inflammatory cytokines and mediators within the lamina propria on a background of a defective epithelial barrier, leading to localised stromal oedema and downstream promotion of the formation and growth of nasal polyps.

**Limitations of study**

One of the potential limitations in this study was the use of inferior turbinate mucosa in the explants model, rather than actual polyp or non-polypoidal sinonasal tissue. A potential concern would be that inferior turbinates were not representative of nasal polyps, with differences particularly at the cellular level. The focus of this current study was to extend our original findings (reference 20) by undertaking mechanistic studies with an explant model using non-polypoidal CRSwNP tissue which has not yet progressed to frank nasal polyps. This was aimed at investigating the early mechanisms responsible for intracellular uptake of S aureus, and as such nasal polyp tissue would have been inappropriate to use as intracellular uptake was already established. The reliability of obtaining consistent unaffected non-polypoidal sinonasal mucosa from the same anatomical region in patients with severe polyposis, was felt to be too challenging and could have introduced significant variability. It was therefore decided to use the inferior turbinate tissue for the explant model.

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**DECLARATIONS OF INTEREST**

None

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**TABLES**

|  |  |  |
| --- | --- | --- |
| **TABLE I**. Tissue explant model treatment groups | | |
| Treatment Group (n=7) | | Treatment |
| A. | No treatment | RPMI alone |
| B. | SEB | SEB (f. conc. 10µg/ml) |
| C. | *S aureus* (non-viable) | With non-viable *S aureus* |
| D. | *S aureus* (viable) | With viable *S aureus* |
| E. | *S aureus* (non-viable)& SEB | With non-viable *S aureus* & SEB (f. conc. 10µg/ml) |
| F. | *S aureus* (viable) & SEB | With viable *S aureus* & SEB (f. conc. 10µg/ml) |

**Abbreviations**: **SEB** (*Staphylococcus* enterotoxin B), **RPMI** (Roswell Park Memorial Institute medium)

|  |  |  |
| --- | --- | --- |
| **TABLE II.** Co-culture assay treatment groups | | |
| **Treatment Group (n=7)** | | **Treatment** |
| A. | No treatment | RPMI alone |
| B | SEB | SEB (f. conc. 10µg/ml) |
| C. | *S aureus* (viable) | With viable *S aureus* |
| D. | *S aureus* (non-viable) | With non-viable *S aureus* |
| E. | *S aureus* (viable) & SEB | With viable *S aureus* & SEB (f. conc. 10µg/ml) |
| F. | *S aureus* (non-viable)& SEB | With non-viable *S aureus* & SEB (f. conc. 10µg/ml) |
| G. | Lysostaphin | Lysostaphin (f. conc. 10µg/ml) alone |

**Abbreviations**: **SEB** (*Staphylococcus* enterotoxin B), **RPMI** (Roswell Park Memorial Institute medium)

|  |  |  |
| --- | --- | --- |
| **TABLE III.** Study population demographics | |  |
| **Patient Groups** | **CRSwNP** | **Control** |
| Subject no. | 7 | 5 |
| Mean age (range) | 52 (33-78) | 50 (32-64) |
| Sex | 4M, 3F | 2M, 3F |
| Current smokers | 0 | 1 |
| Aspirin sensitivity | 0 | 0 |
| Asthma | 2 | 0 |
| Positive reaction on skin prick tests | 3\* | 0 |
| Mean Lund-Mackay Score (range) | 16.4 (11-21) | 2.2 (0-4) |
| \*Patient 1: tree, grass, house dust mite (HDM), cat & dog dander; Patient 2: feathers, HDM, cat & dog dander; Patient 3: tree, grass & HDM  **Abbreviations**: **CRSwNP** (Chronic rhinosinusitis with nasal polyps) | |  |

|  |  |  |  |
| --- | --- | --- | --- |
| **TABLE IV.** Diameter of mast cell size following culturing with viable and non-viable *S aureus* | | | |
| **Treatment Group** | **Mean diameter (mm)** | **Standard deviation** | ***P* value** |
| No treatment | 10.95 | 1.39 |  |
| *S aureus* (viable) | 15.28 | 2.02 | ***0.0001\**** |
| *S aureus* (non-viable) | 10.27 | 1.49 | *0.0715* |
| SEB | 10.99 | 1.42 | *0.9080* |
| *S aureus* (viable) & SEB | 15.37 | 2.98 | ***0.0001\**** |
| *S aureus* (non-viable) & SEB | 10.08 | 1.23 | *0.1170* |
| Lysostaphin | 10.56 | 0.94 | *0.2076* |

**Abbreviations**: **SEB** (*Staphylococcus* enterotoxin B). All *P* values calculated from the comparison of each treatment group with the ‘no treatment’ group.

**FIGURE LEGENDS**

**FIG I - CRSwNP Explant Tissue Model (A-D): (A)** Effect on the uptake of *S aureus* into epithelial host cells. **(B)** Recruitment of mast cells into the epithelial layer. **(C)** Uptake of *S aureus* into sub-epithelial host cells. **(D)** Recruitment of mast cells into the sub-epithelial layer. In all cases, the combination of viable *S aureus* with SEB significantly increased the number of mast cells containing *S aureus*and mast cell density within the epithelial and sub-epithelial layers compared to all other groups. **Control Explant Tissue Model (E-H):** No uptake of *S aureus* into mast cells was observed within either the epithelial layer **(E)** or the sub-epithelial layer **(G).** No mast cells were seen within the epithelial layer in any treatment groups **(F).** No increase in mast cell recruitment was seen in the sub-epithelial layer **(H)**. Error bars represent the means +/- 1SD. \**p*<0.05, \*\*\**p* <0.0001.

**FIG II - (A)** Mast cell degranulation. Photomicrograph of GMA-embedded CRSwNP inferior turbinate after 24-hour exposure to viable *S aureus* and SEB stained with AA1 anti-mast cell tryptase and AEC substrate. Released mast cell granules (yellow arrow). (**B)** The extent of mast cell degranulation was significantly increased after exposure to combined viable *S aureus* and SEB, compared to other treatment groups. **(C)** Proportion of degranulating mast cells within the sub-epithelial layer. Error bars represent the means +/- 1SD. \*\**p*<0.001, \*\*\**p*<0.0001.

**FIG III** - Morphological characteristics of mast cells within CRSwNP treatment groups. Representative CLSM images of mast cell (HMC-1 cell line) from the no Treatment group **(A).** The mast cellnucleushas stainedyellow indicating imminent or recent mitosis. This indicates a healthy nucleus and cell. (B) Representative CLSM images of mast cell from the combined viable *S aureus* and SEB group **(B)**. Mast cells within the combined viable *S aureus* and SEB group were significantly larger than those within the no treatment group. **(C)** Mean diameter of HMC-1 cells after 24-hour exposure to each treatment group. Treatment groups with viable *S aureus* contained significantly larger cells compared to the no treatment group. Error bars represent the means +/- 1SD. \*\*\**p*<0.0001. (**D)** Representative CLSM image of co-culture HMC-1 with *S aureus* and SEB after 24 hours, demonstrating mast cell rupture with seeding of intracellular *S aureus* into the extracellular space (yellow arrows). The blue arrow indicates the mast cell nucleus. The same effect was also observed in the *S aureus* without SEB group.

**FIG IV -** Mast cell extracellular DNA traps. **(A)** Representative TEM image showing clusters of *S aureus* attached to an extracellular trap (red arrow). **(B)** Higher magnification of extracellular traps.

**FIG V -** TEM Images demonstrating *S aureus* internalisation within HMC-1 mast cells (MC). (A) By 2 hours *S aureus* (red arrows) had adhered to the mast cell wall. **(B)** Higher magnification image of *S aureus* attaching to the mast cell wall. **(C)** At 4 hours, *S aureus* (red arrows) were engulfed and internalised. **(D)** At 24 hours, there was extensive internalisation of *S aureus* within the HMC-1 cells leading to cell wall rupture (blue arrow).

**FIG VI -** Colony forming unit (CFU) enumeration experiments. *S aureus* was grown to the mid-log phase and co-cultured with HMC-1 cells at an MOI 1:1 over set time points, following which CFUs were enumerated on CBA plates using serial dilution. (**A)** Intracellular *S aureus* enumerated with and without the presence of SEB (10µg/ml) over 24 hours**. (B)** Extracellular *S aureus* enumerated with HMC-1 mast cells.

**FIG VII -** Proposed illustrated mechanism for the contribution of intracellular *S aureus* towards the pathogenesis of nasal polyps.