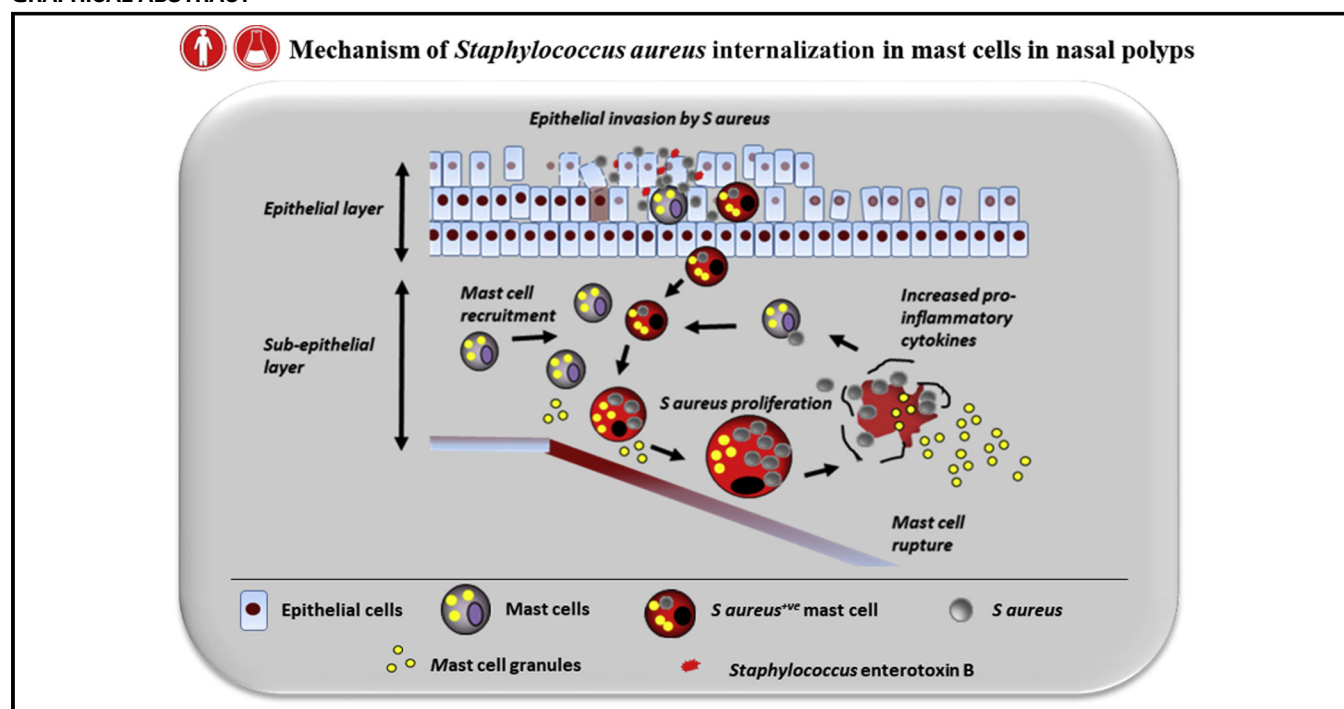


Staphylococcus aureus internalization in mast cells in nasal polyps: Characterization of interactions and potential mechanisms



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GRAPHICAL ABSTRACT



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

Objective: This follow-up study aimed to further characterize interactions between *S. aureus* and mast cells in this setting and

elucidate potential internalization mechanisms with particular emphasis on the role of staphylococcal enterotoxin B (SEB). **Methods:** A prospective study was performed using an explant tissue model with *ex vivo* inferior turbinate mucosa obtained from patients with chronic rhinosinusitis with nasal polyps (n = 7) and patients without CRS (n = 5).

Immunohistochemistry was used to characterize *S. aureus*

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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 by using a combination of fluorescent *in situ* hybridization, confocal laser scanning microscopy, scanning electron microscopy, transmission electron microscopy, and proliferation assays.

Results: *S aureus* was captured 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 internalization of *S aureus* into mast cells.

Conclusion: This study provides new insights into the interactions between *S aureus* and mast cells, including the internalization process, and demonstrates a prominent role for SEB in promoting uptake of the bacteria into these cells. (J Allergy Clin Immunol 2020;145:147–59.)

Key words: Chronic rhinosinusitis, chronic rhinosinusitis with nasal polyps, nasal polyps, intracellular bacteria, *Staphylococcus aureus*, mast cells, bacterial superantigens, staphylococcal enterotoxin B

Chronic rhinosinusitis (CRS) is a chronic inflammatory condition of the nose and paranasal sinuses.¹ It is subdivided into 2 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 characterized by formation of benign edematous outgrowths (polyps) that obstruct the nasal cavity and sinus drainage pathways, resulting in recurrent infections. It is characterized by a T_H2-mediated cytokine profile, with eosinophilia and increased IL-5, IgE, RANTES, and eotaxin levels.¹ This is in contrast to CRSsNP, which is characterized by the presence of pus and a T_H1-mediated profile with neutrophilia and increased TNF- α , IL-8, and IFN- γ levels.¹ However, mixed T_H1/T_H2 patterns have been detected in some subtypes of patients with CRSwNP and CRSsNP.^{2–4}

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, postnasal drip, and loss of smell, significantly affecting quality of life.¹ Furthermore, the disease has a profound socioeconomic effect, placing a huge financial burden on already overstretched health systems.^{1,5–8} It has become apparent in recent years that rather than a single causative agent, CRS is probably a manifestation of a degree of immunologic dysfunction caused by interactions between a number of host and environmental factors.¹ These include superantigens,^{9–12} bacterial and fungal biofilms,^{13–15} intracellular bacterial reservoirs,^{16–20} environmental allergens,^{21,22} genetic predisposition,²³ epithelial defects,^{24–26} and changes in inflammatory cytokine cascades.¹

Despite massive expenditure on medical and surgical therapies, a subset of patients with CRS exhibit symptoms refractory to established treatments.^{8,14} Bacterial biofilms have been implicated as playing a crucial role in mediating the chronic inflammation in patients with recalcitrant CRS,^{8,14,27–29} with the most common biofilm-forming microbe reported as *Staphylococcus aureus*.^{13–15,30,31} In 25% of the population, *S aureus* exists as a nonpathogenic commensal, harmlessly colonizing the nasal cavity. However, under certain conditions and in a bid for

Abbreviations used

CBA:	Columbia blood agar containing 5% defibrinated horse blood
CFU:	Colony-forming unit
CLSM:	Confocal laser scanning microscopy
CRS:	Chronic rhinosinusitis
CRSsNP:	Chronic rhinosinusitis without nasal polyps
CRSwNP:	Chronic rhinosinusitis with nasal polyps
5-Edu:	5-Ethynyl-2'-deoxyuridine
HMC-1:	Human mast cell culture line 1
MOI:	Multiplicity of infection
PIPES:	Piperazine-N,N'-bis(2-ethanesulfonic acid)
SEB:	Staphylococcal enterotoxin B
TEM:	Transmission electron microscopy

survival, *S aureus* has the ability to turn clinically pathogenic by using several regulatory pathways to transition between commensal and pathogenic states.³² 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 changes.^{1,24,33} In relation to nasal polyps, there is evidence that colonizing *S aureus* has the ability to produce a variety of toxins, including staphylococcal enterotoxin B (SEB) with superantigenic properties, which can directly influence both immunostimulatory and immunomodulatory inflammatory cytokines and effector cells.^{10,11}

Recently, we made the novel observation of intracellular *S aureus* within mast cells in nasal polyps.²⁰ This follow-up study is aimed at further characterization of the interactions between *S aureus* and mast cells in this setting and elucidation of potential internalization mechanisms with a 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 2012”¹ and 5 control subjects with no history of CRS were included in the study. All patients with CRSwNP underwent surgery by the senior authors (P.G.H. and R.J.S.) at the University Hospital Southampton NHS Foundation Trust. Patients without CRS (control group: 3 patients with nasal blockage undergoing septoplasty and 2 patients with sinonasal inverted papilloma undergoing endoscopic medial maxillectomy) were operated on by Mr Huw Jones (consultant rhinologist, University Hospital Southampton NHS Foundation Trust). Patients with CRSwNP were offered surgery after failure of 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 corticosteroids or antibacterial or antihistamine therapies in the 8 weeks before surgery. Exclusion criteria for patients with CRSwNP included age less than 18 years and with cystic fibrosis or primary ciliary dyskinesia. For patients without CRS, additional exclusion criteria included CRS, asthma, atopy, and aspirin sensitivity. Radiologic severity of sinus disease was graded on a computed tomographic sinus scan using the Lund-Mackay scoring system.³⁴ Preoperative demographic 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 National Health Service Research Ethics Committee, United Kingdom (REC 09/H0501/74). All study participants provided informed written consent.

TABLE I. Tissue explant model treatment groups

Treatment group (n = 7)		Treatment
A	No treatment	RPMI alone
B	SEB	SEB (f. conc. 10 µg/mL)
C	<i>S aureus</i> (nonviable)	With nonviable <i>S aureus</i>
D	<i>S aureus</i> (viable)	With viable <i>S aureus</i>
E	<i>S aureus</i> (nonviable) and SEB	With nonviable <i>S aureus</i> and SEB (f. conc. 10 µg/mL)
F	<i>S aureus</i> (viable) and SEB	With viable <i>S aureus</i> and SEB (f. conc. 10 µg/mL)

f. conc., Final concentration.

Tissue explant model

Aims of the tissue explant model. We sought to establish a model replicating the host-environment interface within the sinonasal cavity and to 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 nonmotile *S aureus* migrates into the subepithelial layer and how this relates to mast cell behavior. It will also provide useful information on the effect of SEB on *S aureus* and the surrounding tissue.

Tissue explant model. Inferior turbinate specimens were collected in HBSS (Gibco, Paisley, United Kingdom) and transported on ice for processing. Inferior turbinate mucosa was dissected into 5 × 5 mm explants. Each sample was treated with RPMI (Gibco) supplemented with sterile penicillin/streptomycin (100 µg/mL) for 30 minutes to eradicate exogenous bacteria. Samples were rinsed 3 times in sterile PBS and then immersed in 2 mL of RPMI alone. After this, each sample was used for an individual treatment group, as shown in Table I, and incubated for 24 hours at 37°C in a 5% CO₂ atmosphere. Explants were fixed with acetone, embedded in glycol methacrylate, and processed for immunohistochemistry.

Treatment groups. 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 nonviable *S aureus* was used to demonstrate that microbial viability was essential in mediating the 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 37°C in a 5% CO₂ atmosphere in RPMI, centrifuged (11,000g 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 microliters of *S aureus* (containing 5.0 × 10⁶ bacterial cells) was added to each treatment.

Nonviable *S aureus* was created by using the same strain. *S aureus* was grown to mid-log phase at 37°C in a 5% CO₂ atmosphere in RPMI medium. The amount of viable bacteria was determined through serial dilution and plating on CBA. The bacteria were centrifuged (11,000g for 10 minutes) and 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 37°C in a 5% CO₂ atmosphere. After confirmation of negative viability, 5.0 × 10⁶ unites of nonviable *S aureus* were added to the appropriate samples.

Immunohistochemistry. The immunohistochemistry protocol was adapted from that of Britten et al.³⁵ Briefly, immunohistochemistry was undertaken in glycol methacrylate-embedded tissue. Primary mAbs 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 substrate was then applied, and finally, sections were counterstained with Mayer hematoxylin. 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 3,3'-diaminobenzidine substrate before use on study sections. A primary mAb specific for mast cell tryptase (AA1; Abcam) was used to stain for mast cells.

Degranulation. Mast cell degranulation was assessed on sections stained immunologically with the AA1 anti-mast cell tryptase mAb. Degranulating mast cells were identified as mast cell tryptase “blushes” with granule dissemination.

Cell count. Epithelial length and subepithelial area were measured with the aid of computerized image analysis (KS400 software with a Zeiss Axioskop 2 microscope and AxioCam; Zeiss, Bicester, United Kingdom). Cells were manually counted from 5 different tissue sections per patient for each treatment group.

Epithelial cell rates were calculated by using the following equation:

$$\text{Epithelial cell rate (cells} \cdot \text{mm}^{-1}) = \frac{\text{Total number of cells}}{\text{Epithelial length (mm)}}$$

Subepithelial cell rates were calculated by using the following equation:

$$\text{Subepithelial cell rate (cells} \cdot \text{mm}^{-2}) = \frac{\text{Total number of cells}}{\text{Subepithelial area (mm}^2\text{)}}$$

Cell-culture model

Aims of the cell-culture model. We sought to explore the mechanisms surrounding internalization of *S aureus* into mast cells at the cellular level. This involves combining human mast cells with an *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 internalization are assessed by using confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM).

Human mast cell line. The human mast cell culture line (HMC-1) was kindly donated by Dr J. H. Butterfield (Mayo Clinic, Rochester, Minn). HMC-1 cells were maintained in 75-cm² tissue culture flasks at 37°C in a 5% CO₂ atmosphere. Complete medium consisted of Iscove modified Dulbecco medium (Gibco) supplemented with 25 mmol/L HEPES, sodium bicarbonate and L-glutamine, 10% calf serum, and 1.2 mmol/L α-thioglycerol (Sigma, St Louis, Mo). The α-thioglycerol and complete medium were prepared freshly weekly. Cell cultures were split when cell density reached 1.0 to 1.5 × 10⁶ cells/mL. After this, 2.0 × 10⁶ cells were seeded into a 12-well plate in 2 mL of culture medium. Cell viability was confirmed to be 99% before the start of stimulation.

Coculture assay. *S aureus* was grown in RPMI to the mid-log phase. Periodic culture samples were taken, serially diluted, and plated on CBA. Concurrent OD measurements were taken and plotted against CFU counts. Numbers of bacteria were calculated per OD. Bacteria were centrifuged (11,000g for 5 minutes), washed with sterile PBS, and diluted to the required concentration. A final inoculum of 1.0 × 10⁶ units of bacteria was added to 1.0 × 10⁶ HMC-1 cells per well for a multiplicity of infection (MOI) of 1:1. For nonviable *S aureus*, samples were prepared as before.

Seven treatment groups were used, as outlined in Table II. Cocultures were incubated at 37°C in a 5% CO₂ atmosphere for 2 hours, and then lysostaphin (20 µg/mL) was added to eradicate extracellular *S aureus*, leaving only viable intracellular reservoirs.

Proliferation assay. The Click-iT 5-ethynyl-2'-deoxyuridine (5-EdU) proliferation assay (Thermo Fisher, Loughborough, United Kingdom) was used to detect DNA synthesis and provides an accurate method of assessing cell proliferation. Edu 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

TABLE II. Coculture assay treatment groups

Treatment group (n = 7)		Treatment
A	No treatment	RPMI alone
B	SEB	SEB (f. conc. 10 µg/mL)
C	<i>S aureus</i> (viable)	With viable <i>S aureus</i>
D	<i>S aureus</i> (nonviable)	With nonviable <i>S aureus</i>
E	<i>S aureus</i> (viable) and SEB	With viable <i>S aureus</i> and SEB (f. conc. 10 µg/mL)
F	<i>S aureus</i> (nonviable) and SEB	With nonviable <i>S aureus</i> and SEB (f. conc. 10 µg/mL)
G	Lysostaphin	Lysostaphin (f. conc. 10 µg/mL) alone

f. conc., Final concentration.

(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, 10 µmol/L EdU was added to each treatment group containing HMC-1 cells at 37°C in a 5% CO₂ atmosphere. After 24 hours, HMC-1 cells were centrifuged (11,000g) for 10 minutes, and the cell pellet was fixed in 3.7% formaldehyde (prepared in PBS) for 15 minutes. HMC-1 cells were rinsed twice with 3% BSA in PBS, followed by treatment with permeabilization buffer (0.5% Triton X-100 in PBS) at room temperature for 20 minutes. Cells were further rinsed with BSA in PBS before 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. DAPI-488 (Sigma-Aldrich, Dorset, United Kingdom) stain was added to the cells 30 minutes before imaging with an inverted Leica SP5 CLSM (Leica Microsystems, Milton Keynes, United Kingdom).

Five thousand cells were manually counted from each well (1000 from 5 separate randomly selected fields), and the proportion of intracellular *S aureus* was presented as a percentage of total cell numbers. Cell measurements were made with CLSM and the Leica Application Suite AF software.

CLSM. CLSM imaging was performed, as previously described.²⁶ Briefly, after staining, tissues were mounted within chamber slides with coverslip bottoms and imaged with a Leica TCS SP5 inverted confocal system using a 63× oil immersion lens. Sequential scanning was used to further eliminate cross-talk interference from multiple fluorophores. Images were collected and analyzed with Leica Application Suite AF software.

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

CFU enumeration assays. As previously described, *S aureus* was grown to the mid-log phase and cocultured with HMC-1 mast cells with and without SEB (10 µg/mL) at an MOI of 1:1 over various time points. CFU enumeration of extracellular *S aureus* was undertaken by pelleting cells, serially diluting culture supernatants, and then plating on CBA plates and incubating at 37°C in a 5% CO₂ atmosphere. Cells were treated with lysostaphin (20 µg/mL) for 30 minutes, followed by serial washing, 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 means of CFU enumeration on CBA plates.

HMC-1 mast cells were cocultured with *S aureus* for 6 hours, after which cells were pelleted, washed, and treated with lysostaphin, as detailed above, to

TABLE III. Study population demographics

Patient groups	CRSwNP	Control
No. of subjects	7	5
Mean age (y [range])	52 (33-78)	50 (32-64)
Sex	4 M, 3 F	2 M, 3 F
Current smokers	0	1
Aspirin sensitivity	0	0
Asthma	2	0
Positive skin prick test response	3*	0
Mean Lund-Mackay score (range)	16.4 (11-21)	2.2 (0-4)

F, Female; M, male.

*Patient 1: Tree, grass, house dust mite, and cat and dog dander; patient 2: feathers, house dust mite, and cat and dog dander; and patient 3: tree, grass, and house dust mite.

examine *S aureus* infection over 24 hours and determine the possible extracellular release of *S aureus* from mast cells. After further washing, cells were re-suspended, and the absence of extracellular bacteria was confirmed through CFU enumeration of the culture supernatants, confirming lack of any *S aureus* growth. The coculture was then continued to the 24-hour time point, and intracellular and extracellular CFUs were enumerated, as previously described.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 7.0 software (GraphPad Software, San Diego, Calif). Comparisons between different treatment groups were made by using a paired (2-tailed) *t* test. The level of significance was accepted as a *P* value of less than .05.

RESULTS

Patients' demographics

Patients' demographics are presented in Table III. The control group had no clinical or radiologic evidence of CRS, were nonatopic and nonasthmatic, and had 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 subepithelial layers compared with the no-treatment group (*P* = .0009 and *P* = .0031, respectively). Within the epithelial layer, *S aureus* was identified within both mast cells and epithelial cells and in the subepithelial layer within mast cells alone. A significant increase in mast cell density was observed within the epithelial layer (*P* = .0031) in response to *S aureus* invasion (Fig 1). The use of nonviable *S aureus* resulted in no significant increase in relative numbers of intracellular bacteria or mast cell density when compared with the no-treatment group (Fig 1).

Within control tissue, no mast cells were seen within the epithelial layer, and no significant increase in mast cell density was seen in the subepithelial layer compared with all other treatment groups (Fig 1, E-H). Within the subepithelial layer, no intracellular *S aureus* was seen within any of the mast cells.

SEB 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 with 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

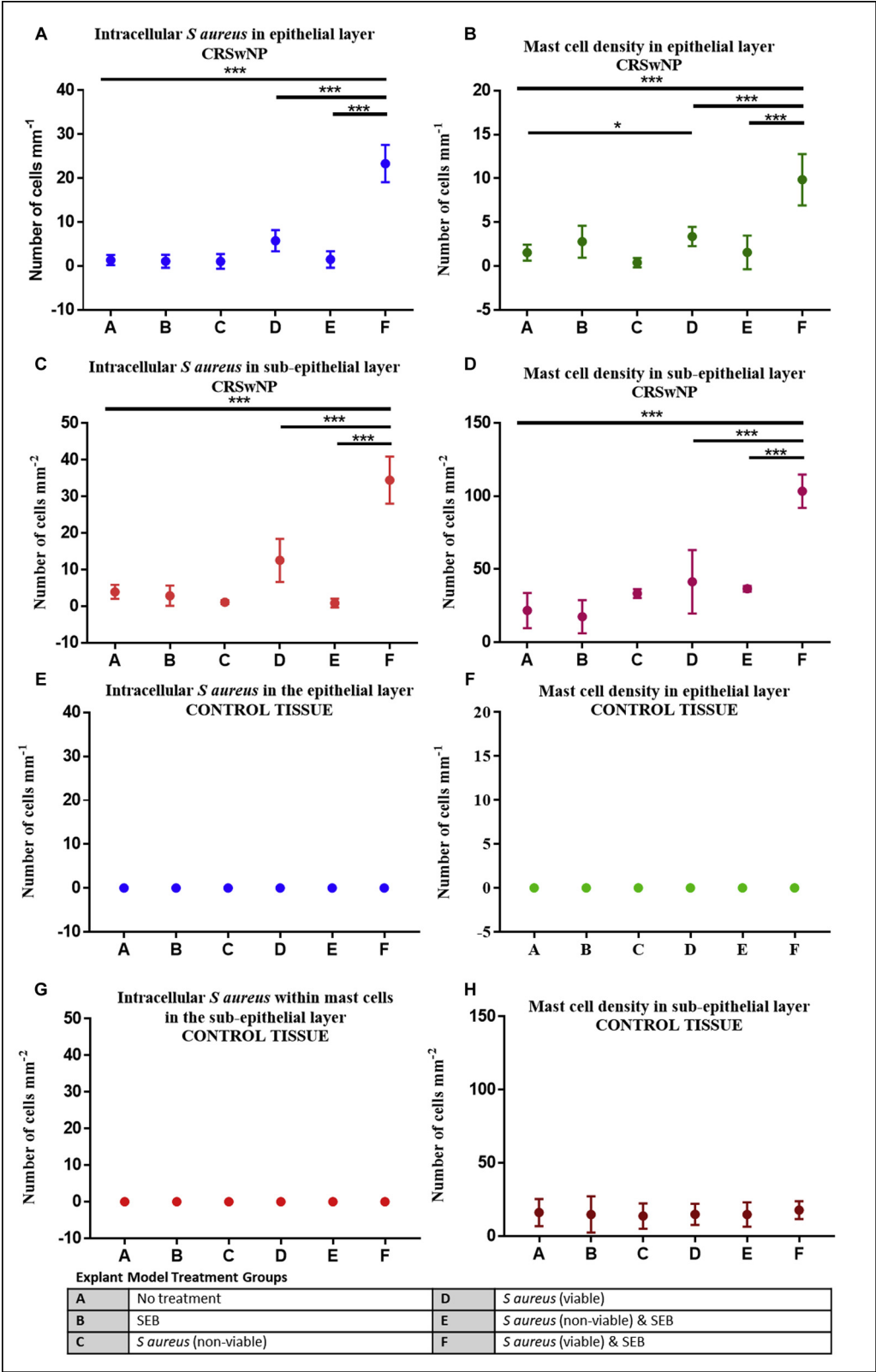


FIG 1. A-D, CRSwNP explant tissue model. Fig 1, A, Effect on uptake of *S aureus* into epithelial host cells. Fig 1, B, Recruitment of mast cells into the epithelial layer. Fig 1, C, Uptake of *S aureus* into subepithelial host cells. Fig 1, D, Recruitment of mast cells into the subepithelial layer. 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 subepithelial layers compared with all other groups. **E-H, Control explant tissue model.** No uptake of *S aureus* into mast cells was observed within either the epithelial layer (Fig 1, E) or the subepithelial layer (Fig 1, G). No mast cells were seen within the epithelial layer in any treatment group (Fig 1, F). No increase in mast cell recruitment was seen in the subepithelial layer (Fig 1, H). Error bars represent means $\pm$ 1 SD. * $P < .05$ and *** $P < .0001$.

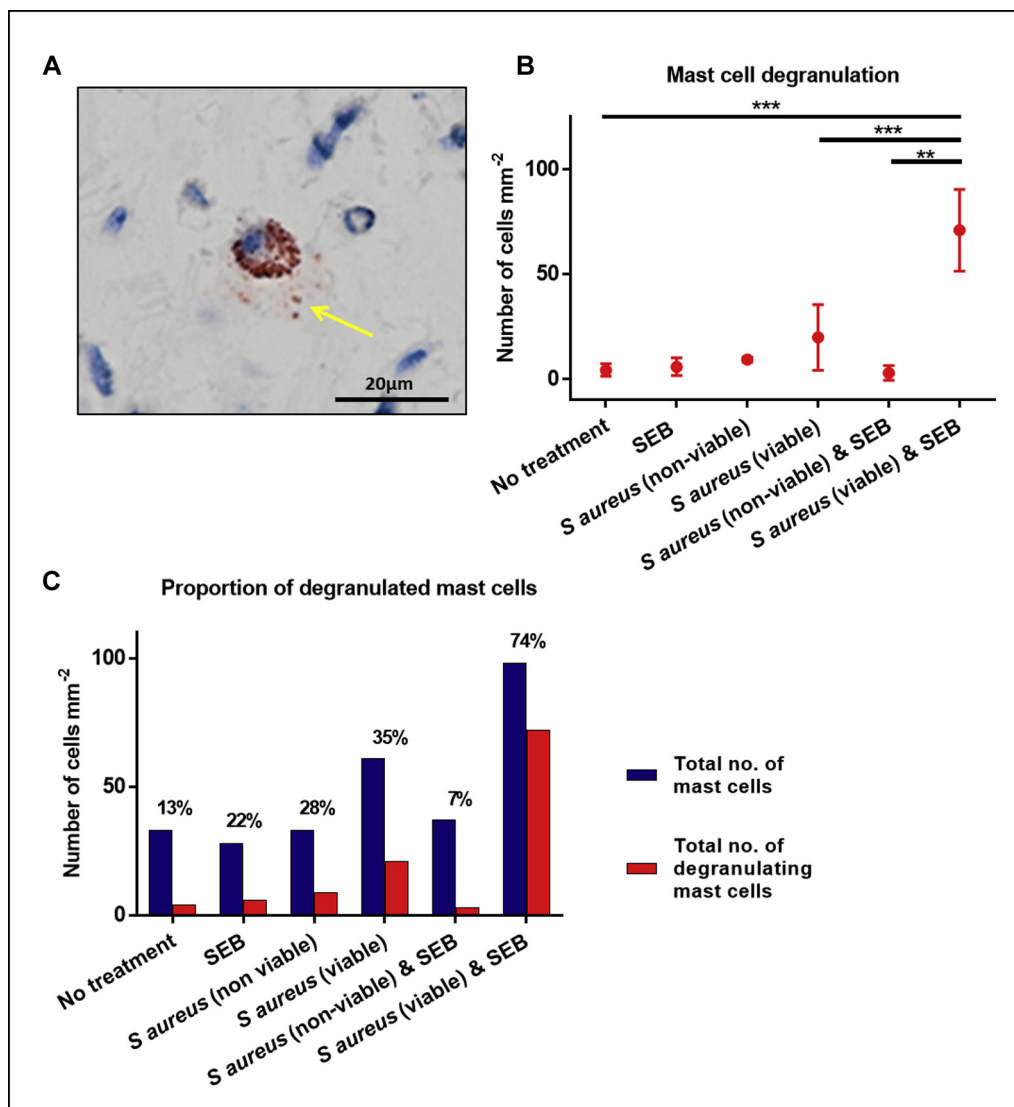


FIG 2. **A,** Mast cell degranulation. Photomicrograph of glycol methacrylate-embedded CRSwNP inferior turbinate after 24 hours of exposure to viable *S aureus* and SEB stained with AA1 anti-mast cell tryptase and AEC substrate. Released mast cell granules are indicated by the yellow arrow. **B,** Extent of mast cell degranulation was significantly increased after exposure to combined viable *S aureus* and SEB compared with other treatment groups. **C,** Proportion of degranulating mast cells within the subepithelial layer. Error bars represent means $\pm$ 1 SD. ** $P < .001$ and *** $P < .0001$.

TABLE IV. Diameter of mast cell size after culturing with viable and nonviable *S aureus*

Treatment group	Mean diameter (mm)	SD	P value
No treatment	10.95	1.39	
<i>S aureus</i> (viable)	15.28	2.02	.0001
<i>S aureus</i> (nonviable)	10.27	1.49	.0715
SEB	10.99	1.42	.9080
<i>S aureus</i> (viable) and SEB	15.37	2.98	.0001
<i>S aureus</i> (nonviable) and SEB	10.08	1.23	.1170
Lysostaphin	10.56	0.94	.2076

All P values were calculated from the comparison of each treatment group with the no-treatment group. Boldface P values indicate statistical significance.

within both the epithelial and subepithelial layers when compared with viable *S aureus* alone (Fig 1). Within control tissue, the addition of SEB to viable *S aureus* demonstrated no significant difference in either layer compared with viable *S aureus* alone or the no-treatment group (Fig 1, E-H).

Mast cell degranulation. Within the CRSwNP tissue, mast cell degranulation occurred exclusively within the subepithelial layer. Addition of viable *S aureus* alone did not result in a significant increase in mast cell degranulation compared with the no-treatment group. However, in combination with SEB, *S aureus* induced a significant increase in mast cell degranulation compared with that in all other groups (Fig 2, A and B). The effect of additional SEB to viable *S aureus* on mast cell degranulation was also demonstrated when looking at proportional data

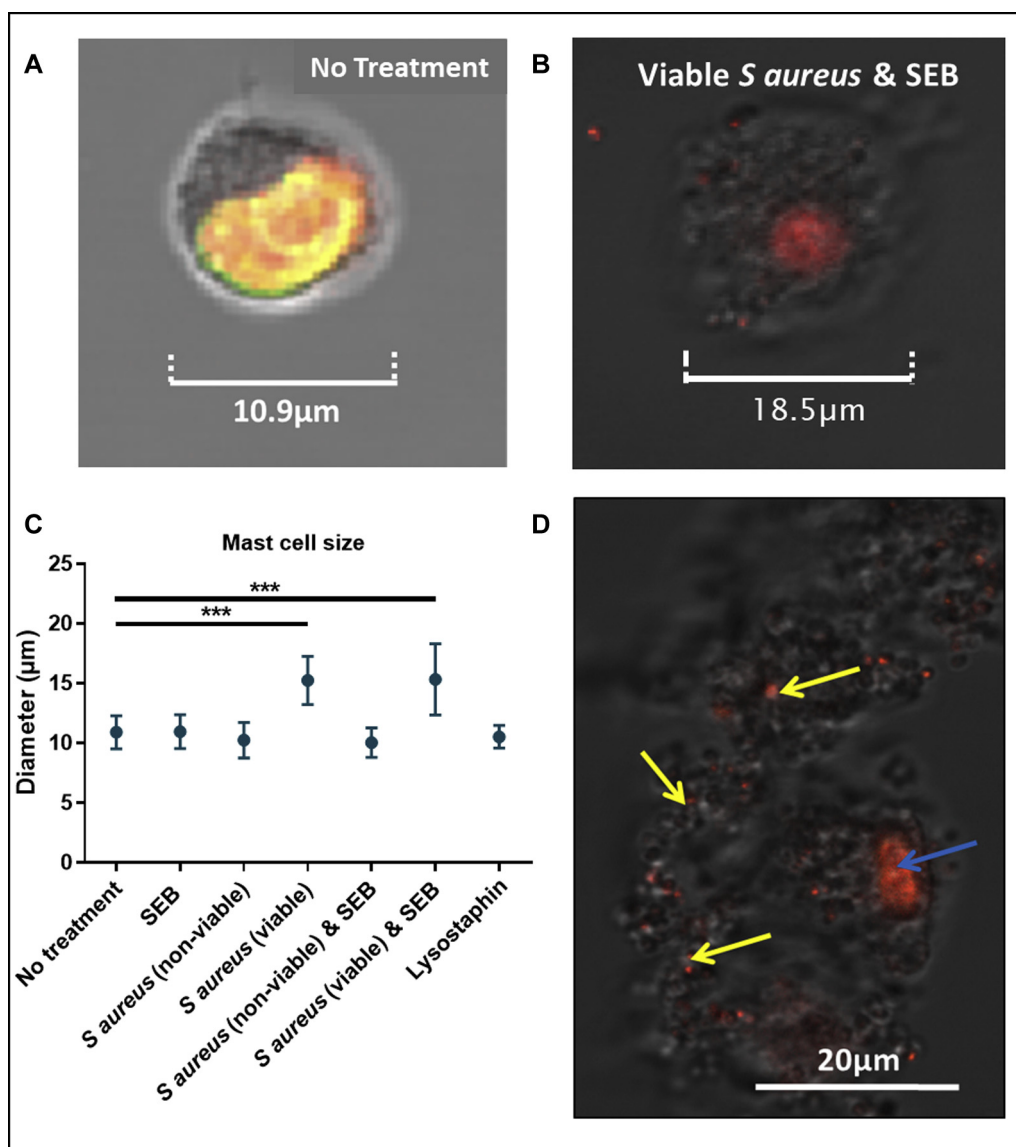


FIG 3. Morphologic characteristics of mast cells within CRSwNP treatment groups. **A**, Representative CLSM images of mast cells (HMC-1 cell line) from the no-treatment group. The mast cell nucleus has stained yellow, indicating imminent or recent mitosis. This indicates a healthy nucleus and cell. **B**, Representative CLSM images of mast cells from the combined viable *S aureus* and SEB group. 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 hours of exposure to each treatment group. Treatment groups with viable *S aureus* contained significantly larger cells compared with the no-treatment group. Error bars represent means $\pm$ 1 SD. *** $P < .0001$. **D**, Representative CLSM image of cocultured HMC-1 cells 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 2, C). 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% to 18%) and no significant difference when compared with the CRSwNP no-treatment group.

Coculture assay

Intracellular *S aureus*. Intracellular *S aureus* within HMC-1 mast cells was observed within both groups containing viable *S aureus*. Viability of intracellular *S aureus* was confirmed by using the Click-iT 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. S determined by using TEM, 16% of

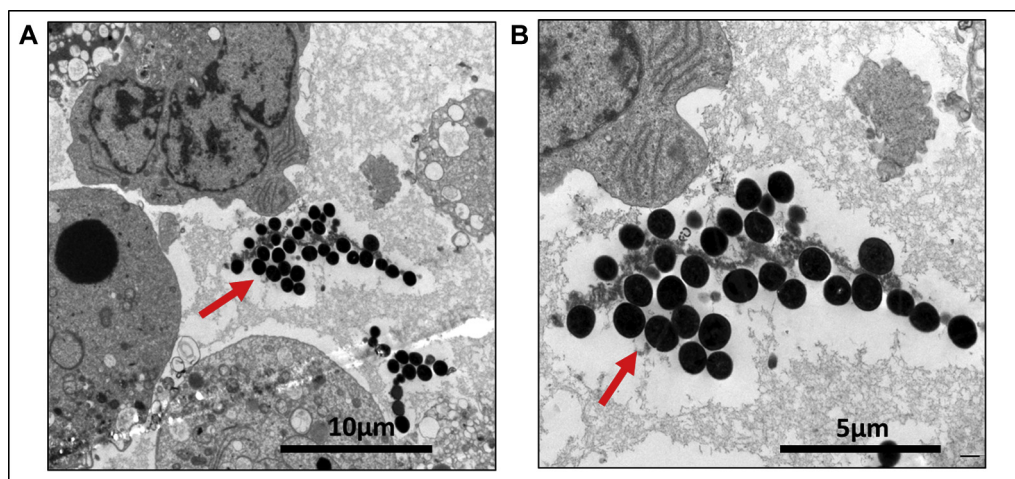


FIG 4. Mast cell extracellular DNA traps. **A**, Representative TEM image showing clusters of *S aureus* attached to an extracellular trap (red arrow). **B**, Greater magnification of extracellular traps.

mast cells contained *S aureus* at 2 hours, and 76% contained *S aureus* at 24 hours. This was validated by using CLSM and 4',6'-diamidino-2-phenylindole, where 64% of mast cells were observed to contain *S aureus* at 24 hours.

Mast cell size with *S aureus* internalization. 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 with the no-treatment group (Fig 3, A-C). The mean diameter of mast cells within the no-treatment group was 10.95 μm compared with 15.28 μm in the *S aureus*-only group and 15.37 μm in the *S aureus* and SEB group. Mast cells containing intracellular *S aureus* continued to expand, up to 20 μm in diameter, where they eventually ruptured, seeding viable *S aureus* into the extracellular environment (Fig 3, D). This observation was supported by 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 (Fig 4).

Internalization of *S aureus* within mast cells. *S aureus* was observed to be internalizing within mast cells through phagocytosis. By 2 hours, *S aureus* had attached to the cell surface (Fig 5, A and B). By 4 hours, *S aureus* had entered the mast cells, apparently through phagocytosis (Fig 5, 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* (Fig 5, D).

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

DISCUSSION

CRSwNP and CRSsNP are complex conditions with an as yet unclear pathophysiology.⁸ Several causative 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 profile.¹

Bacteria were previously thought to primarily play a role in patients with acute infective rhinosinusitis, with little relevance to chronicity and development of nasal polyps.³⁶ However, with advancement of molecular detection techniques, a clearer understanding of the role and importance of bacteria and their different profiles in patients with CRSwNP is emerging.⁸ The most common bacteria to colonize the sinuses is *S aureus*, with rates of up to 60% in patients with CRSwNP versus 33% in the general population.³⁷ Identification of *S aureus* colonization 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 (nonpolypoidal) CRS sinonasal mucosa samples but not on the epithelial surfaces of nasal polyps or control tissue.²⁰ Instead, *S aureus* was observed as being subepithelial and residing in the cytoplasm of mast cells in nasal polyp samples.²⁰ In this study we aimed to extend these novel findings and better elucidate the underlying mechanisms involved in internalization of *S aureus* within mast cells in nasal polyps.

Development of a tissue explant model allowed the study of *S aureus* and its most notable superantigen, SEB, at the host-environment interface. Superantigens manipulate the local immune response, leading to increased tissue damage and remodeling.^{10,12,37} Superantigens have been shown to exert effects on both host cells and the cytokines associated with local innate and adaptive immunity.³⁸ Host cells stimulated by release of superantigens include epithelial cells, B and T cells, eosinophils,

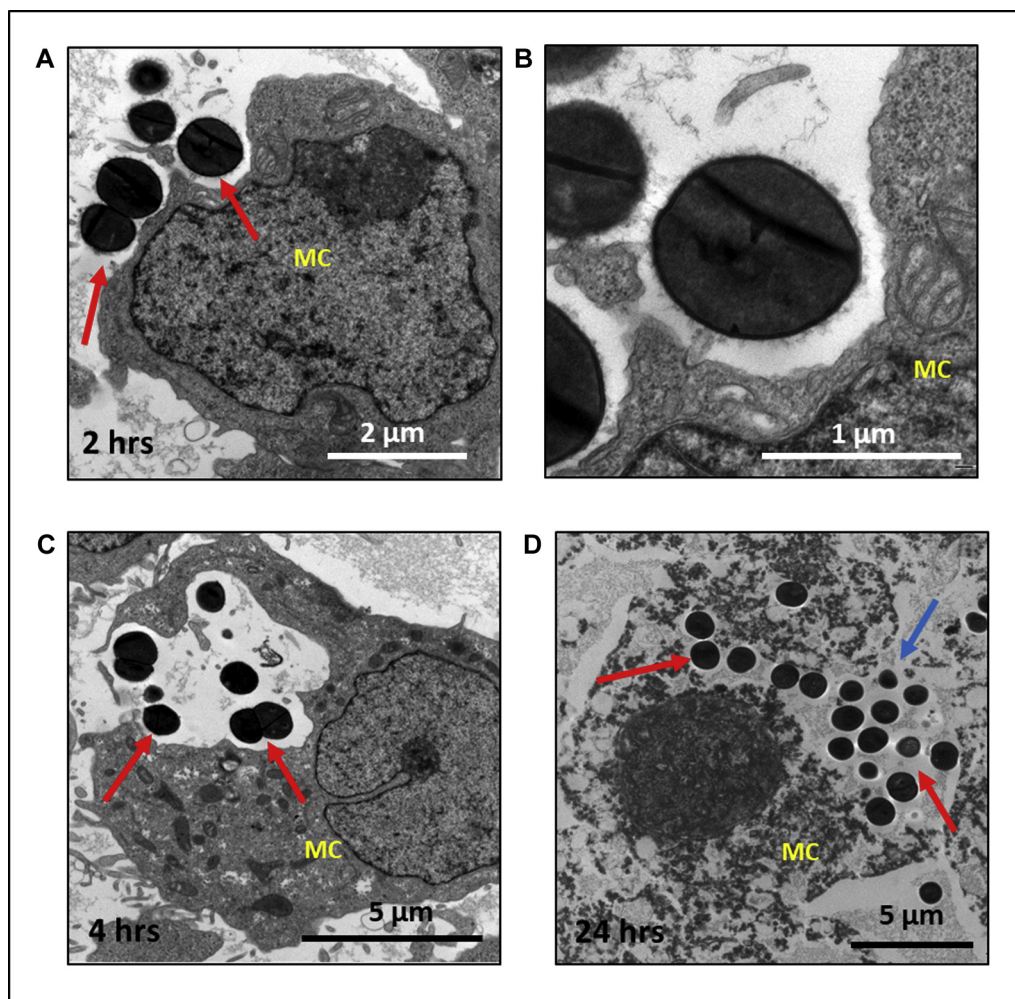


FIG 5. TEM images demonstrating *S aureus* internalization within HMC-1 mast cells (MC). **A**, By 2 hours, *S aureus* (red arrows) had adhered to the mast cell wall. **B**, Greater magnification image of *S aureus* attaching to the mast cell wall. **C**, At 4 hours, *S aureus* (red arrows) was engulfed and internalized. **D**, At 24 hours, there was extensive internalization of *S aureus* within the HMC-1 cells, leading to cell wall rupture (blue arrow).

fibroblasts, and mast cells.^{10,39} Superantigens also induce upregulation of IL-4 and IL-5 and downregulation of TGF- β and IL-10.⁴⁰⁻⁴² Other effects reported include manipulation of eicosanoid metabolism,^{43,44} granulocyte augmentation,⁴⁵ and induction of glucocorticoid insufficiency.⁴⁶ The net effect results in conversion of a T_H1 cytokine profile in sinonasal mucosa into a T_H2-skewed cytokine profile, as seen in nasal polyps.¹

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 internalization of *S aureus* within these cells. This is a novel finding. These mast cells, laden with nonmotile intracellular *S aureus*, appear to migrate into the subepithelial layer, followed by release of their contents. This led to significantly increased numbers of *S aureus*-containing mast cells within the subepithelial 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 might contribute to nasal

polyp pathogenesis downstream. 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 might be relevant because of the loss of a transport medium for the nonmotile *S aureus* to pass into the subepithelial layer. This might suggest that patients with CRSwNP can 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 infections.⁴⁷ Described as sentinel cells, mast cells play an important role in the initial defense against pathogenic invasion.⁴⁷ After incursion by environmental pathogens, mast cells initially respond by secreting a range of proinflammatory mediators, including histamine, IL-6, chemokines, and TNF- α , resulting in recruitment of further inflammatory cells, such as neutrophils, dendritic cells, and additional mast cells, to the site of infection.^{47,48} Furthermore, mast cells also appear to exert a direct effect on pathogens through secretion of preformed and *de novo*-synthesized mediators, secretion of extracellular

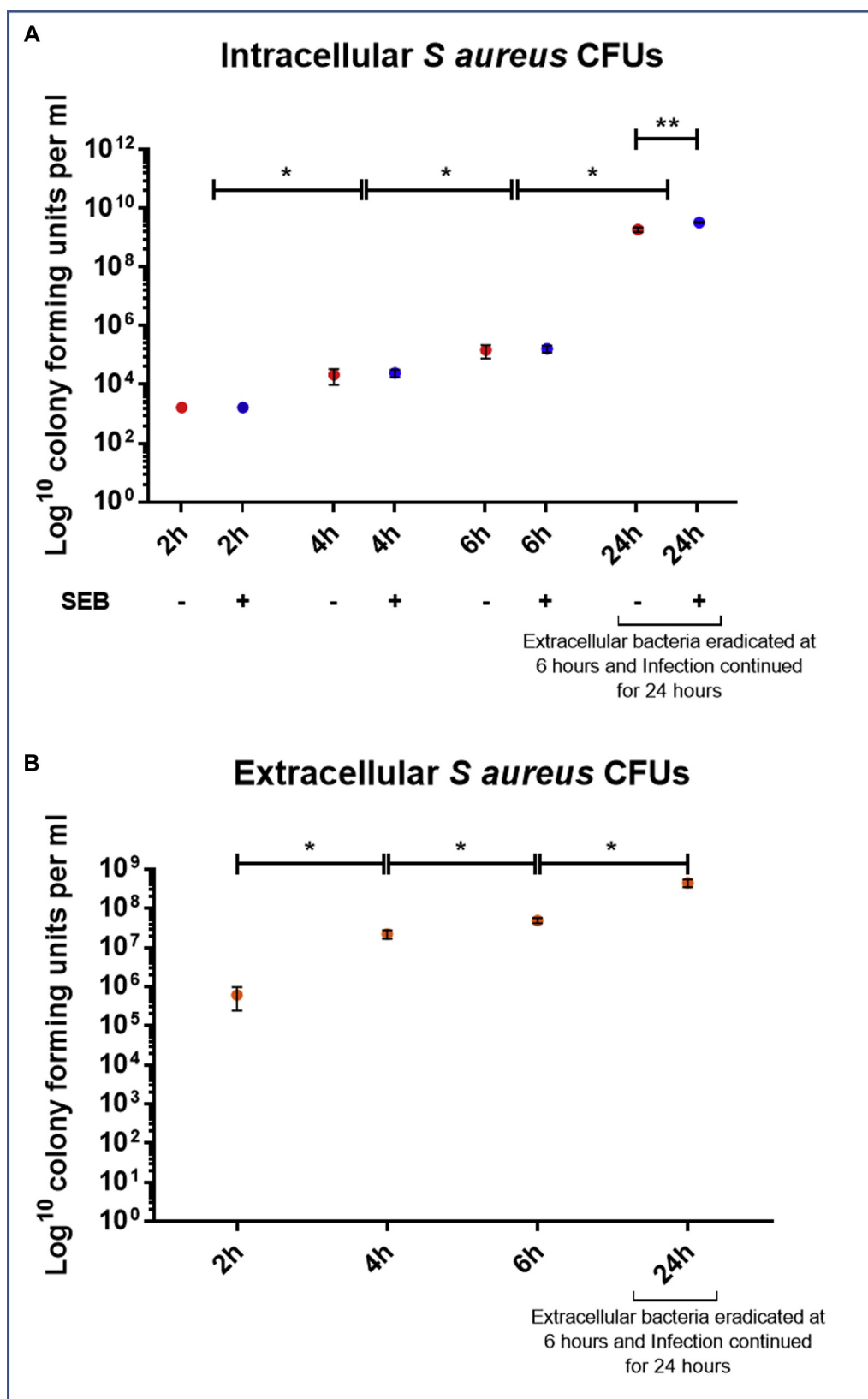


FIG 6. CFU enumeration experiments. *S aureus* was grown to the mid-log phase and cocultured with HMC-1 cells at an MOI of 1:1 over set time points, after which CFUs were enumerated on CBA plates by using serial dilution. **A**, Intracellular *S aureus* was enumerated with and without the presence of SEB (10 μ g/mL) over 24 hours. **B**, Extracellular *S aureus* enumerated with HMC-1 mast cells.

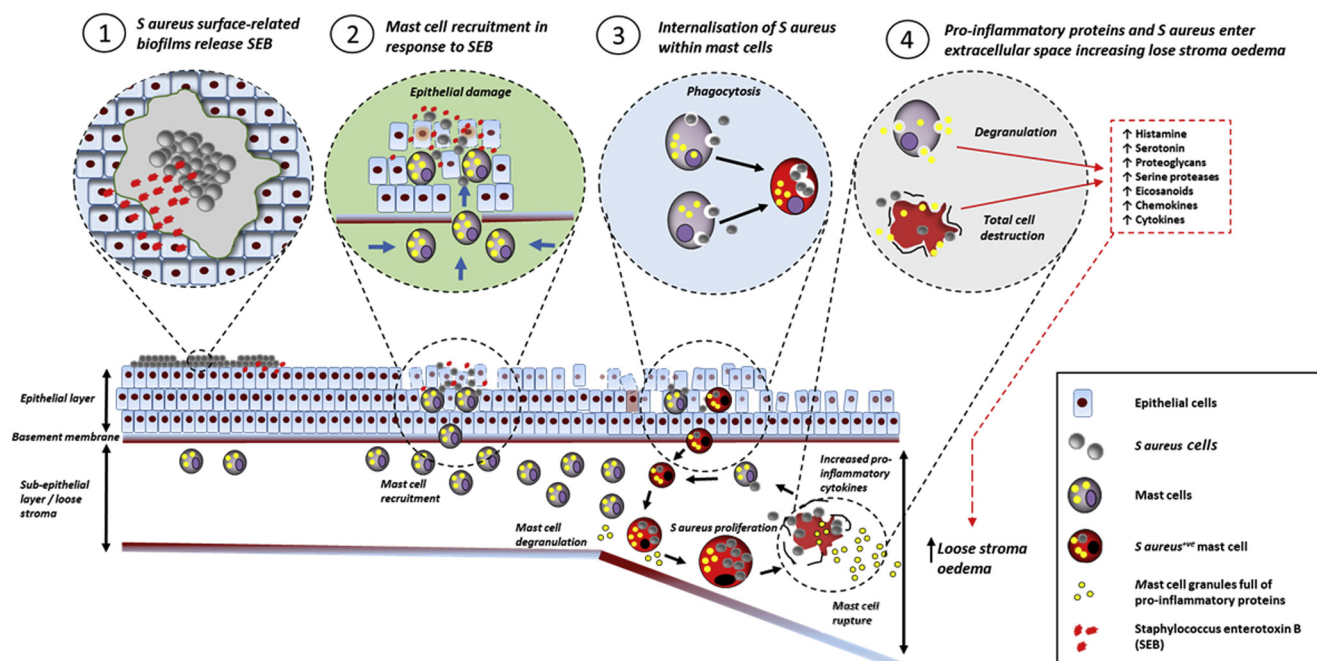


FIG 7. Proposed illustrated mechanism for the contribution of intracellular *S aureus* toward the pathogenesis of nasal polyps.

antimicrobial compounds, phagocytosis, and use of DNA extracellular traps.⁴⁷⁻⁴⁹ The degree of mast cell reaction appears to be microbe specific, likely through recognition of pathogen-associated molecular patterns, facilitating a tailored innate response based on stored information from previous encounters.¹

Direct interaction of mast cells with bacteria has been analyzed through several *in vitro* studies.^{47,48} It is well established that one of the antimicrobial mechanisms of bacterial internalization includes phagocytosis, which was clearly shown within this study. Within 2 hours of *in vitro* coculture of *S aureus* with HMC-1 mast cells, bacteria began to bind and adhere to 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 digestion.⁴⁹ In our study this same observation was seen within the first 4 hours of coculture. However, alongside phagosomes digesting *S aureus* were also viable and proliferating bacteria. After 24 hours, the amount 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 might be indicative of different bacterial phenotypes, with varying degrees of susceptibility to the mast cell phagocytic activity.

Within the coculture assay, the *S aureus* phenotype with the ability to internalize and thrive within mast cells proliferated and gradually replaced the other *S aureus* phenotypes being degraded, which might represent a basic survival mechanism for the bacteria. This could suggest that the ability of *S aureus* to survive within mast cells might be dependent on whether the invading pathogen is of a specific phenotype and might help explain why intracellular internalization is not a feature in all *S aureus*-related infections.

This study's findings are consistent with those of another *in vitro* study that demonstrated *S aureus* within HMC-1 cells, which remained viable for up to 5 days.⁴⁷ After 2 hours, 20% of mast cells contained viable intracellular *S aureus*.⁴⁷ At 2 hours, our coculture 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, 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 might represent expansion of 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 mast cells and enter the extracellular space, with further internalization 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 bacteria.⁴⁷ This has also been demonstrated in eosinophils.⁵⁰ These traps composed of DNA and granule proteins have been shown *in vitro* to entrap extracellular *S aureus* before killing it.⁴⁷

Another antipathogenic mast cell defense mechanism includes the initial release of extracellular antimicrobial compounds, such as cathelicidin-related antimicrobial peptide, stored within the cell granules.⁴⁷ These granules are released through degranulation on exposure to certain pathogens. Abel et al⁴⁷ demonstrated mast cell degranulation on exposure to *S aureus*. Our study not only reproduced their findings but also demonstrated that addition of SEB to *S aureus* resulted in a 2-fold increase in mast cell degranulation. Drake-Lee and Price⁵¹ demonstrated significantly

greater numbers of degranulating mast cells in nasal polyps compared with inferior turbinate mucosa and mucosa from patients without CRS. Internalization of *S aureus* within mast cells might 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 patients with anaphylactic reactions.⁴⁷

In addition to antimicrobial compounds, mast cells also secrete tryptase, chymase, lysozymes, histamine, heparin, proteases, β -hexosaminidase, and positively charged proteins, such as TNF- α ,⁴⁷⁻⁴⁹ into the extracellular space. Slow mast cell degranulation, together with cell rupture caused by proliferating intracellular *S aureus*, could result in a localized increase in the levels of extracellular proinflammatory cytokines and mediators, manifesting as edema within the lamina propria and potentially contributing to nasal polyp formation.

The pathogenesis of nasal polyps is undoubtedly complex and cannot be explained by a single causative 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* toward the pathogenesis of nasal polyps. Clearly, this will require further work to validate, and although likely to represent a small piece of a complex jigsaw, we believe it is interesting and thought provoking.

Proposed mechanism

On exposure to an endotoxin-releasing strain of *S aureus*, mast cells are recruited into the epithelial layer by SEB, where nonmotile *S aureus* is internalized within these mast cells through phagocytosis and transported to the subepithelial 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 proinflammatory mediators and cytokines into the extracellular space. Intracellular *S aureus* proliferates within the mast cells, significantly increasing the cell size. Eventually, mast cells rupture, releasing their proinflammatory cytokines, mediators, and viable *S aureus* into the extracellular space. Furthermore, SEB released from *S aureus* causes damage to epithelial cells, leading to epithelial proliferation and remodeling, resulting in a disorganized and defective epithelial barrier. The end result of these processes is a build-up of proinflammatory cytokines and mediators within the lamina propria on a background of a defective epithelial barrier, leading to localized stromal edema and downstream promotion of the formation and growth of nasal polyps (Fig 7).

Limitations of the study

One of the potential limitations in this study was the use of inferior turbinate mucosa in the explant model rather than actual polyp or nonpolypoidal 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 findings²⁰ by undertaking mechanistic studies with an explant model using nonpolypoidal CRSwNP tissue that 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 because intracellular uptake was already established. The reliability of obtaining consistent unaffected nonpolypoidal sinonasal mucosa from the same anatomic region in patients with severe polyposis was believed to be too challenging and could have introduced significant variability. Therefore we decided to use the inferior turbinate tissue for the explant model.

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Key messages

- We presented new insights into the interactions between *S aureus* and mast cells in nasal polyps, including the internalization process.
- We found a prominent role for SEB in promoting uptake of the bacteria into mast cells.
- We presented a potential mechanism of contribution of intracellular *S aureus* toward nasal polyp pathogenesis.

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