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Staphylococcus aureus internalisation enhances bacterial survival through modulation of host immune responses and mast cell activation

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12th October 2020

Resubmission of manuscript ALL-2020-00252 as 'Letter to Editor'

Dear Profs Wang & Akdis,

Many thanks indeed for allowing us the opportunity to resubmit our original '***Staphylococcus aureus* internalisation in chronic rhinosinusitis enhances bacterial survival through modulation of host immune responses and mast cell activation' manuscript (ALL-2020-00252) as a 'Letter to Editor'**. We apologise for the delay in resubmission due to the Covid pandemic. We have taken on board the editorial comments and reformatted the manuscript to comply with the instructions with the inclusion of online supplementary material. We have also removed the reference to chronic rhinosinusitis as recommended by the reviewers. We have adapted the title accordingly. We believe that this process has enhanced and improved the manuscript.

The majority of the data from the original manuscript has been retained. Please see the outline of this displayed below. Some of the minor gene and protein data from the original Figure 2 was removed. This was in response to the reviewer's comments and to improve the clarity and presentation of the results. We have retained the TNFα gene and protein data, but removed the other cytokines as there was no direct comparative gene and protein data available, again in accordance with the reviewers' comments.

- Original Figure 1 - All data retained, now displayed in Figure 1.
- Original Figure 2 - Gene and protein data for TNFα retained, displayed in Figure 1.
- Original Figure 3 - All data retained, now displayed in Figure 2.
- Original Figure 4 - All data retained, now displayed in supplementary Figure 1.
- Original Figure 5 - All data retained, now displayed in Figure 2.
- Original Figure 6 - This illustration is now displayed as supplementary Figure 2.

Thank you again for this opportunity, and look forward to hearing from you in the near future.

Kind Regards,

Rami Salib (on behalf of the authors)

Staphylococcus aureus internalisation enhances bacterial survival through modulation of host immune responses and mast cell activation

To the Editor,

Chronic rhinosinusitis (CRS) is a chronic inflammatory condition of the upper airways affecting up to 15% of the population, significantly impacting upon patients' quality of life and resultant healthcare resources (1). Whilst the pathophysiological mechanisms underlying CRS are not fully understood, *Staphylococcus aureus* (*S aureus*) has been shown to play a prominent role (2, 3, 4). In addition to its presence on the sinonasal mucosal surface, we previously demonstrated *S aureus* harbouring within mast cells in nasal polyps (2). We revealed that following *S aureus* intracellular uptake and proliferation, bacteria were released into the extracellular space following mast cell rupture, which potentially contributed to the repopulation of depleted surface colonies (3). Recent evidence suggests that patients with nasal polyps, and especially those with recalcitrant disease, not only have intracellular reservoirs of *S aureus* but also elevated levels of IgE specific to *S aureus* and their toxins (5). Accentuated through their activation via localised anti-*S aureus* IgE, mast cells should clear residual subepithelial bacterial colonies through phagocytosis. It is postulated, however, that *S aureus* may alter the local host immune response to favour its ongoing survival. In the present study we investigated mast cell immune response to repeated *S aureus* internalisation and release cycles and further explored the role of IgE in modulating the response of mast cells to *S aureus*.

The experimental model applied involved cells of the LAD2 mast cell line co-infected with a pathogenic CRS *S aureus* isolate (**see supplementary methods section for further details**). For the study of possible phenotypic mutations altering bacterial immune responses through the sequential uptake and release of *S aureus* from mast cells, additional *S aureus* populations were created; designated C1 (once internalised) through to C5 (five times internalised). *S aureus* was bound to mast cell walls at 2 hours (Figure 1B & C) and internalised between 2 to 4 hours (Figure 1D). Intracellular

S aureus were shown to be viable on agar plating (Figure 1E), with no significant difference in the number of viable internalised bacteria found between C1 and C5 populations (Figure 1F). Exposure of LAD2 mast cells to both the original and C1 population of *S aureus* was associated with significant upregulation of TNFα gene and protein expression (Figures 1G & 1H). Exposure to experienced *S aureus* (C5 population) resulted in downregulated TNFα gene and protein expression (Figures 1G& 1H). In IgE sensitised and anti-IgE exposed LAD2 cells, there was lower β-hexosaminidase release 4 hours after the addition of *S aureus* (Figure 2C). Infection of LAD2 cells with *S aureus* following IgE sensitisation was associated with reduced phosphorylation of AKT2 and glycogen synthase kinase 3 (GSK-3) α/β (Figures 2F & G), consistent with downregulation of phosphorylation pathways. Figure S1 reveals apparent downregulation of TNFα gene expression in *S aureus*-infected cells following IgE-dependent activation.

The key contributions of mast cells in allergic diseases are well established, but there is emerging evidence that they also play important roles in a number of chronic bacterial infections, including *Klebsiella pneumoniae*, *Mycoplasma pneumonia*, *Listeria monocytogenes*, and most recently *S aureus* in CRS (2, 6). Though mast cells may contribute to clearing infection, *S aureus* may actually use mast cells to evade detection and immune clearance. Using bone-derived murine mast cells and the HMC-1 line, Abel et al (2011) showed a mast cell response to eradicate *S aureus* infection through the release of pre-formed mediators and extracellular traps (7). However, *S aureus* was able to subvert these killing mechanisms through its internalisation. We have recently shown evidence of *S aureus* surviving within nasal polyp mast cells (2), with *S aureus* entering through phagocytosis, enhanced in the presence of *Staphylococcus aureus* enterotoxin B (SEB), followed by bacterial proliferation, cellular expansion and eventual rupture, with release of viable *S aureus* into the extracellular space propagating ongoing infection (3). This study has shown the ability of a CRS-specific *S aureus* strain, through its release and re-internalisation, to alter its immunogenic phenotype, resulting in a significant downregulation of pro-inflammatory cytokine gene expression and release. Modulation of the associated host immune response could confer a bacterial survival advantage. Furthermore, results also indicate inhibition of maximal degranulation of mast cells upon *S aureus* internalisation at 4

hours. These changes are not unique to *S aureus*, with mast cells infected with *Bifidobacteria*, *Lactobacillus* and *Escherichia coli* showing similar responses (6), although crucially these are non-pathogenic strains. This could further limit migration of phagocytic cells, thus compromising the ability to clear ongoing infection.

The observation of protein kinase phosphorylation gives insights into the possible role of IgE sensitisation in *S aureus* - mast cell interactions. There was reduced protein kinase phosphorylation following *S aureus* infection of IgE-sensitised LAD2 cells, reaching statistical significance for GSK-3α/β and AKT2. GSK exists in two isoforms (α and β) and regulates the function of over 50 substrates involved in various essential cellular functions. GSK-3β inhibition has been shown to suppress generation of pro-inflammatory cytokines whilst augmenting production of anti-inflammatory IL-10 in response to multiple TLR signalling pathways, through NF-κB and CREB interacting with the coactivator CREB-binding protein (CBP) (8). Downregulation of GSK-3β phosphorylation has also been shown to reduce the pro-inflammatory response, and thereby favour survival of *S aureus* (9). Downregulation of GSK-3β in response to IgE sensitisation and *S aureus* infection of LAD2 cells may constitute a possible route by which *S aureus* downregulates cytokine production within mast cells which have previously been sensitised with anti-*S aureus* IgE. GSK-3β is therefore potentially exploited by pathogenic bacteria as a means of evading the immune system, thus promoting their ongoing survival and persistence.

Our study provides additional insight into the pathophysiology of mast cell function and how *S aureus* can manipulate the cellular environment to favour its own survival. This could manifest clinically with treatment resistance and disease recalcitrance. These findings warrant further study, and may be relevant in a number of other chronic diseases, including asthma and atopic eczema, where *S aureus* and the generation of *S aureus*-IgE play a prominent role.

REFERENCES

1. Fokkens WJ, Lund VJ, Mullol J, et al. European Position Paper on Rhinosinusitis and Nasal Polyps 2012. *Rhinology Supplement*. 2012:1-298.
2. Hayes SM, Howlin R, Johnston DA, et al. Intracellular residency of Staphylococcus aureus within mast cells in nasal polyps: A novel observation. *J Allergy Clin Immunol*. 2015;135:1648-51.
3. Hayes SM, Biggs TC, Goldie SP, et al. Staphylococcus aureus internalisation in mast cells in nasal polyps - Characterisation of interactions and potential mechanisms. *J Allergy Clin Immunol*. 2020;145:147-159.
4. Tan NCW, Foreman A, Jardeleza C, Douglas R, Vreugde S, Wormald PJ. Intracellular Staphylococcus aureus: the Trojan horse of recalcitrant chronic rhinosinusitis? *Int Forum Allergy Rhinol*. 2013;3:261-6.
5. Ou J, Wang J, Xu Y, et al. Staphylococcus aureus superantigens are associated with chronic rhinosinusitis with nasal polyps: a meta-analysis. *Eur Arch Otorhinolaryngol*. 2014;271:2729-36.
6. Wesolowski J, Paumet F. The impact of bacterial infection on mast cell degranulation. *Immunol Res*. 2011;51:215-26.
7. Abel J, Goldmann O, Ziegler C, et al. Staphylococcus aureus evades the extracellular antimicrobial activity of mast cells by promoting its own uptake. *J Innate Immun*. 2011;3:495-507.
8. Martin M, Rehani K, Jope RS, Michalek SM. Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat Immunol*. 2005;6:777-84.
9. Silva-Garcia O, Rico-Mata R, Maldonado-Pichardo MC, Bravo-Patino A, Valdez-Alarcon JJ, Aguirre-Gonzalez J, et al. Glycogen Synthase Kinase 3alpha Is the Main Isoform That Regulates the Transcription Factors Nuclear Factor-Kappa B and cAMP Response Element Binding in Bovine Endothelial Cells Infected with Staphylococcus aureus. *Front Immunol*. 2018;9:92.

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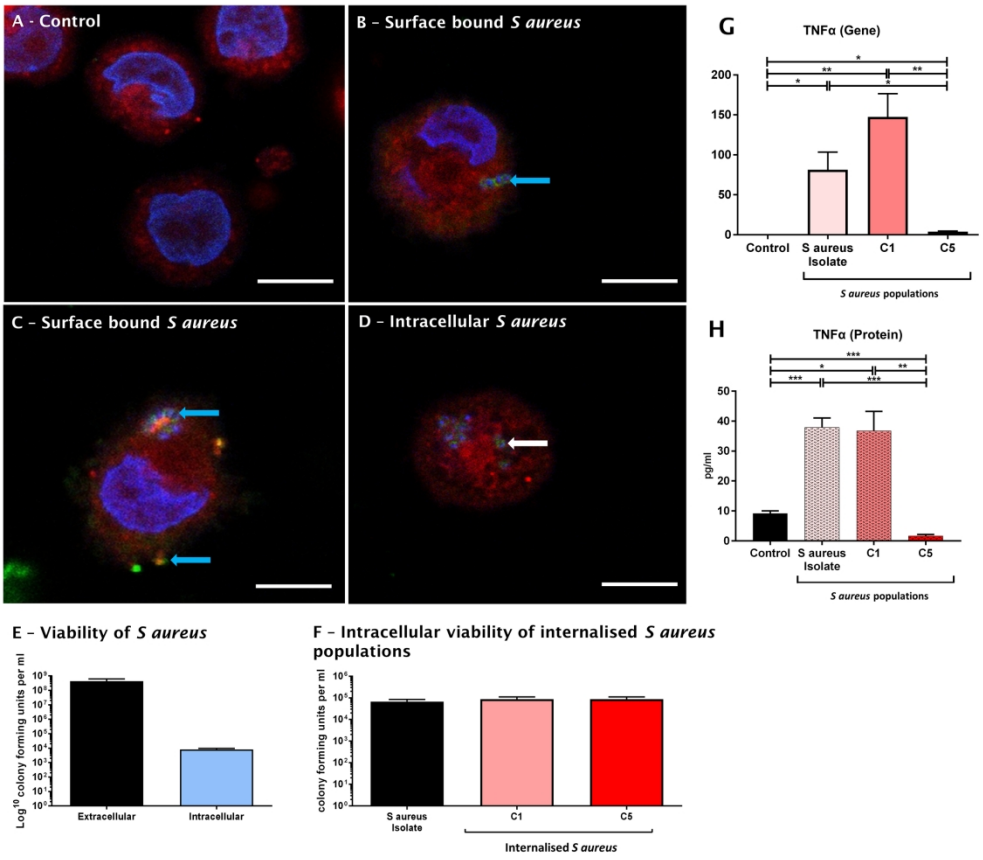
Conflict of interest: The authors have declared that no conflict of interest exists

FIGURE LEGENDS

Figure 1. *S aureus* is readily internalised by LAD2 mast cells, remaining viable and provoking a potent proinflammatory response, which was downregulated upon subsequent bacterial uptake and release from mast cells. (A-D) Representative CLSM images of LAD2 mast cells co-cultured with a CRS *S aureus* isolate, with the cell nucleus in blue and cell cytoplasm in red; surface binding *S aureus* (blue arrows) and intracellular *S aureus* (white arrow) shown. Scale bars in figures 1A-D represent 10µm. (**E&F**) *S aureus* viability experiments using CFUs. LAD2 mast cells were co-cultured with a CRS *S aureus*, C1 and C5 isolate over 4 hours, with TNFα gene expression and excreted protein concentration measured (**G&H**). Data is shown for the mean and SEM of three experimental repeats. Paired t-tests were performed, $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

Figure 2. *S aureus* inhibits IgE-mediated mast cell activation and inhibits GSK-3 α/β protein kinase phosphorylation. The percentage net release of β-hexosaminidase from LAD2 cells was measured in the culture supernatant prior to anti-IgE or calcium ionophore stimulation **(A)** and in response to anti-IgE or calcium ionophore stimulation **(B-E)**, with or without IgE sensitisation/*S aureus* infection. Paired t-tests were performed **(F)** Images for membranes showing phosphorylation of 11 main protein kinases in response to sensitisation and infection. **(G)** Corresponding analysis of differences in pixel density within the four groups of cells. Data shown were from a 76 second exposure. Images were analysed using Image Lab software. Statistical analysis was performed using two-way ANOVA. *($p \leq 0.05$), **($p \leq 0.01$), ***($p \leq 0.001$), ****($p < 0.0001$).



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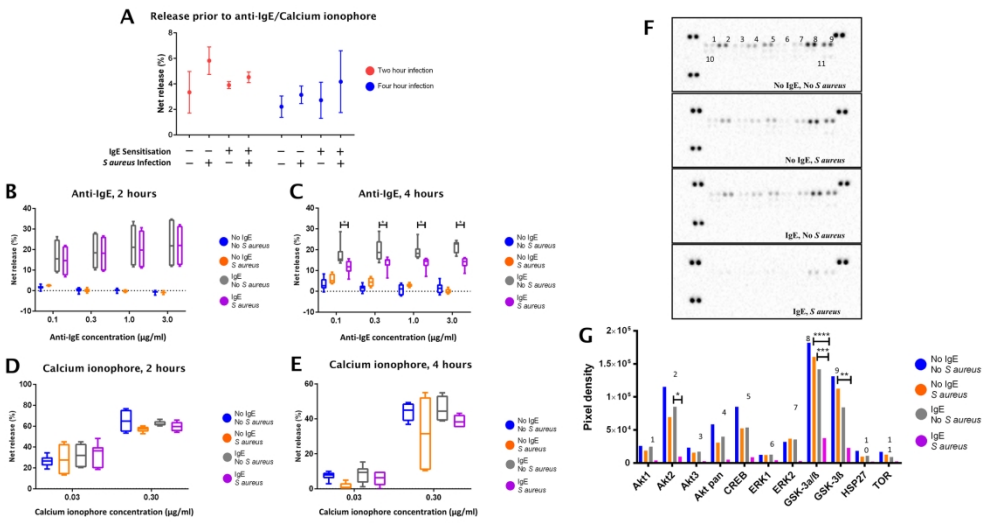

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SUPPLEMENTARY MATERIAL

Staphylococcus aureus internalisation enhances bacterial survival through modulation of host immune responses and mast cell activation

METHODS

Immortalised cell line (LAD2)

The LAD2 cell line, a kind gift from Dr Cem Akin and Dr Arnold Kirshenbaum, was developed at the National Institute of Allergy and Infectious Diseases (NIAID, Rockville, Maryland, USA) from a patient with mastocytosis, and represents a more mature mast cell line. LAD2 cells express FcεRI and contain granules that degranulate in response to antigen, making them more representative of tissue resident mast cells. LAD2 cells were cultured in StemPro-34 SFM media (Life Technologies Ltd, Carlsbad, CA, USA) supplemented with stem cell factor (SCF) and 0.2% Gentamycin, Penicillin and Streptomycin (Sigma-Aldrich Ltd, Gillingham, UK).

Bacterial strains and growth conditions

A well phenotyped *S aureus* isolate was obtained from a CRSwNP patient undergoing surgery at the University Hospital Southampton, with written informed consent (ethics approval 07/Q1702/64). The isolate was grown to mid-log phase, under static conditions, at 37°C in Roswell Park Memorial Institute (RPMI) medium. The optical density (OD_{600nm}) was measured and used to determine the total number of viable cells. Bacteria were centrifuged (12,000 g for 10 minutes), washed three times with sterile phosphate buffered saline (PBS), and diluted to the required cell number for co-culture, as detailed below.

Non-viable, but structurally intact (under microscopy), *S aureus* was used to investigate whether the viability of *S aureus* altered the immune response. The same strain of *S aureus* was used and grown to the mid-log phase (37°C, 5% CO_2) in RPMI medium. *S aureus* was centrifuged (12,000g for 10 minutes) and immersed in 16% paraformaldehyde solution for 1 hour. The non-viability of the culture was confirmed following lack of growth on colombia blood agar (CBA) plates after 48 hours at 37°C, 5% CO_2.

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Generation of internalised *S aureus* populations (C1-C5)

LAD2 mast cells (2 x 10^6) were co-cultured with *S aureus* at a multiplicity of infection (MOI) ratio of 1:1 for 4 hours. Co-cultured cells were then centrifuged at 250g for 5 minutes, and the supernatant discarded. Cells were washed three times in fresh media, then resuspended in media containing 20 µg/ml Lysostaphin, and incubated at 37°C for 30 minutes to kill all extracellular *S aureus*. Lysostaphin does not cross the mast cell membrane, thus preserving intracellular *S aureus*. Following treatment, cells were centrifuged at 250 g for 5 minutes, and a CBA plate inoculated with the supernatant incubated overnight (37°C, 5% CO_2), to confirm the absence of residual extracellular *S aureus*. Cells were then washed three times in fresh media, and vortexed for 10 minutes in 0.5% Triton X-100 to release intracellular *S aureus*. The bacterial suspension was streaked on to a CBA plate and incubated overnight (37°C, 5% CO_2). *S aureus* following internalisation by mast cells (and now designated C1) was grown to mid-log phase and stored at -80°C in 25% glycerol. This process was then repeated with the C1 population, followed by further mast cell internalisation (up to the 5th internalisation; C5). All glycerol stocks were stored at -80°C.

S aureus/LAD2 co-culture model

S aureus was grown to the mid-log phase, as detailed above, with the optical density (OD_{600nm}) measured, and the number of viable *S aureus* calculated. *S aureus* was washed twice and resuspended in Hanks' Balanced Salt solution. A known quantity of live and dead *S aureus* (5 x10^6 cells) was then added to an equal number of LAD2 mast cells (MOI 1:1) over 2 to 24 hours, with and without the addition of *S aureus* Enterotoxin B (SEB, 10µg/ml, Sigma-Aldrich Ltd). Cells were fixed in 2% paraformaldehyde solution for confocal imaging, centrifuged with the supernatant stored at -20°C for Luminex processing, and the cell pellet stored in RNAlater (Life Technologies Ltd, Carlsbad, CA, USA) for real-time quantitative polymerase chain reaction (RT-qPCR).

IgE sensitisation and mast cell activation

LAD2 cells were divided into four different experimental groups (three experimental repeats), each containing 5 x 10^6 cells; no IgE without *S aureus*, no IgE with *S aureus*, IgE without *S aureus* and IgE

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with *S aureus*. Sensitised LAD2 cells were exposed to 1.5µg Human Myeloma IgE (Merck Millipore, Hertfordshire, UK) over 15 hours, washed three times (250 g for 5 minutes) and resuspend in fresh media.

As previously detailed, *S aureus* was grown to mid-log phase and co-cultured with infected LAD2 cell groups at an MOI of 1:1, using 5 x 10^6 cells and 5 x 10^6 *S aureus* per well. Following culture, cells were centrifuged (250g for 5 minutes), and split into three groups; no stimulation, anti-IgE stimulation and calcium ionophore stimulation. Goat anti-human IgE (Merck Millipore, Hertfordshire, UK) at 3 µg/ml (from the study of the immune response) and 10, 3, 1, 0.3, and 0.1 µg/ml (for the β-hexosaminidase release assays) was added to cells over 2 hours, with calcium ionophore (Sigma-Aldrich Ltd, Gillingham, UK) groups exposed to 0.03 µg/ml and 0.3 µg/ml of calcium ionophore over 2 hours. All groups were then centrifuged and stored in RNAlater (Life Technologies Ltd, Carlsbad, CA, USA) at -20°C, with RT-qPCR carried out at a later date. Supernatants were snap frozen and stored at -80°C. RT-qPCR and Luminex was carried out on cells and supernatant samples as outlined below.

Colony forming unit experiments

Mast cells (2 x 10^6 LAD2 cells) were co-cultured with *S aureus* at an MOI of 20:1 for 4 hours in 1 ml of cell line specific media (37°C, 5% CO_2). A higher MOI was chosen for these experiments in order to accurately measure smaller volumes of bacteria using the CFU method. Co-culture suspensions were centrifuged at 250 g for 5 minutes to pellet the mast cells but retain non-internalised *S aureus* within the supernatant. The supernatant was serially diluted for colony forming unit (CFU) enumeration on CBA plates (overnight incubation at 37°C, 5% CO_2). Pelleted cells were resuspended in media containing 20 µg/ml of Lysostaphin, and incubated at 37°C for 30 minutes to eradicate extracellular *S aureus* (confirmed by plating wash supernatant on CBA plates with overnight incubation at 37°C (5% CO_2). Cells were then washed three times with fresh media and vortexed for 10 minutes in 0.5% Triton X-100 to release intracellular bacteria. Samples were then serially diluted and CFU enumeration undertaken on CBA plates following overnight incubation (37°C, 5% CO_2).

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Real time quantitative polymerase chain reaction

Cells were thawed on ice, diluted with chilled PBS (4°C) and centrifuged to pellet cells (500 g for 5 minutes). RT-qPCR was carried out as previously detailed (1), using a TNFα probe (Primerdesign Ltd, see Appendix 1) given this is the predominant pro-inflammatory cytokine of the mast cell.

Measurement of supernatant cytokines

Concentrations of tumour necrosis factor alpha (TNFα) were measured in tissue culture supernatants using Luminex® (R&D Systems Inc, Minneapolis, MN, USA), as per the manufacturer's instructions. The plate was analysed on the Luminex analyser (Bio-Plex® 200 System, Bio-Rad Laboratories Ltd, Hercules, CA, USA).

Confocal laser scanning microscopy

Following LAD2 and *S aureus* culture as detailed above, a 1/200 dilution of a mouse monoclonal anti-staphylococcus primary antibody (AB37644, Abcam, Cambridge, UK) in PBS with 3% bovine serum albumin (BSA) and 0.5% Triton X-100 was incubated overnight at 4°C. Cells were washed three times, then incubated at room temperature for 2 hours with a 1/500 dilution of a polyclonal donkey anti-mouse Alexa Fluor® 568 (AB175472, Abcam, Cambridge, UK) in PBS with 3% BSA and 0.5% Triton X-100. Following further washing steps, cells were incubated in a 25 µM solution of CellTracker™ Deep Red fluorescent dye (Life Technologies Ltd, Carlsbad, CA, USA) for 30 minutes. Cells were then washed three times with PBS and incubated with 0.5µg/ml of 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) with 3% BSA and 0.5% Triton X-100. Cells were then finally washed and stored in PBS for imaging. Cells were imaged with a Leica TCS SP8 inverted confocal microscope (Leica Microsystems, Milton Keynes, UK) using a 63x glycerol immersion lens. Sequential scanning was used to eliminate interference. Images were collected and analysed using Leica LAS-AF software.

SUPPLEMENTARY MATERIAL

β-hexosaminidase release assays

Following culture, with or without *S aureus* co-infection (for 2 and 4 hours as detailed in the figures), LAD2 cells were incubated with anti-IgE at 10, 3, 1, 0.3, and 0.1 µg/ml, calcium ionophore A23817 at 0.03 µg/ml and 0.3 µg/ml, or with buffer alone (for assessing spontaneous release) at 37°C, with 5% CO2 for 2 hours. Total cell-associated β-hexosaminidase content was determined by suspending cells in 1% Triton X-100 and agitating vigorously for 2 minutes. Cells were centrifuged (250g, 10 mins), and 150 µL of supernatant transferred to a microplate in triplicate. P-nitrophenyl-N-acetyl-β-D-glucosaminide, a substrate for β-hexosaminidase, was dissolved (130mg) in 0.1 M Na2HPO4, and adjusted to a pH of 4.5 with 0.4M citric acid solution, and 50 µl added to each well and incubated for 1 hour (at 37°C, 5% CO2). A stop solution (100µL) was added, and the plate read using a Thermo-max microplate reader (Molecular Devices, Wokingham, UK) at 410 nm wavelength. Net release of β-hexosaminidase with each stimulus (subtracting the level of spontaneous release) was expressed as a percentage of total cell-associated β-hexosaminidase.

Protein kinase phosphorylation assay

Phosphorylation of mitogen-activated protein kinases (MAPKs), the extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK1-3), and different p38 isoforms (α, β, δ, γ) was determined using a human Phospho-MAPK array kit (R&D systems Inc) according to the manufacturer's instructions.

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

Following incubation, cells were harvested and washed (PBS) and then resuspended with 500µl lysis buffer 6. The cell lysates were mixed gently by placing on a rocking platform (Stuart Scientific, Staffordshire, UK) for 30 minutes at 4°C. Cell supernatants were collected following centrifugation

SUPPLEMENTARY MATERIAL

(14,000g, 5 minutes) and transferred to new tubes, which were stored at -80°C until assay was completed.

Statistics

Statistical analysis was performed using Graph Pad Prism 7.0 software (Graph Pad Software Inc., San Diego, CA, USA). Paired and unpaired t-tests were undertaken, and where appropriate one-way and two-way ANOVA, with multi-parametric analysis, as detailed in individual figure legends.

Study approval

All methods were carried out in accordance with relevant guidelines and regulations. All experimental protocols were approved by the Southampton and South West Hampshire Research Ethics Committee (ref - 09/H0501/74). Informed written consent was obtained from all participants prior to inclusion in the study. Relevant non-infected control samples were used for all experimental techniques as a means of validating observed trends.

References

1. Biggs TC, Hayes SM, Harries PG, et al. Immunological profiling of key inflammatory drivers of nasal polyp formation and growth in chronic rhinosinusitis. *Rhinology*. 2019;57:336-342.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY FIGURE LEGENDS

Figure S1 - Upon initiation of the IgE signalling pathway, *S aureus* infected mast cells revealed significant downregulation of TNFα gene expression. Cells stimulated with or without IgE and/or co-infected with *S aureus*. IgE stimulation was performed overnight, with *S aureus* infection (MOI 1:1) undertaken over 4 hours. Following culture samples underwent 3µg/ml anti-IgE stimulation (**A & B**), no further stimulation **(C&D)**, or 0.03µM calcium ionophore stimulation **(E&F)**. Data is shown for three experimental repeats, with boxes representing the 25th to 75th percentile, and whiskers representing the 5th to 95th percentile. Two-way ANOVA was undertaken, $p \leq 0.05$ (*).

Figure S2 - Proposed illustrated model of Staphylococcus aureus survival in CRS.

SUPPLEMENTARY MATERIAL

Appendix 1

RT-qPCR primer sequences

Gene name	Manufacturer	Sense primer	Anti-sense primer
TNFα	Primerdesign	CCAGGGACCTCTCTCTAATCA	TGCTACAACATGGGCTACAG

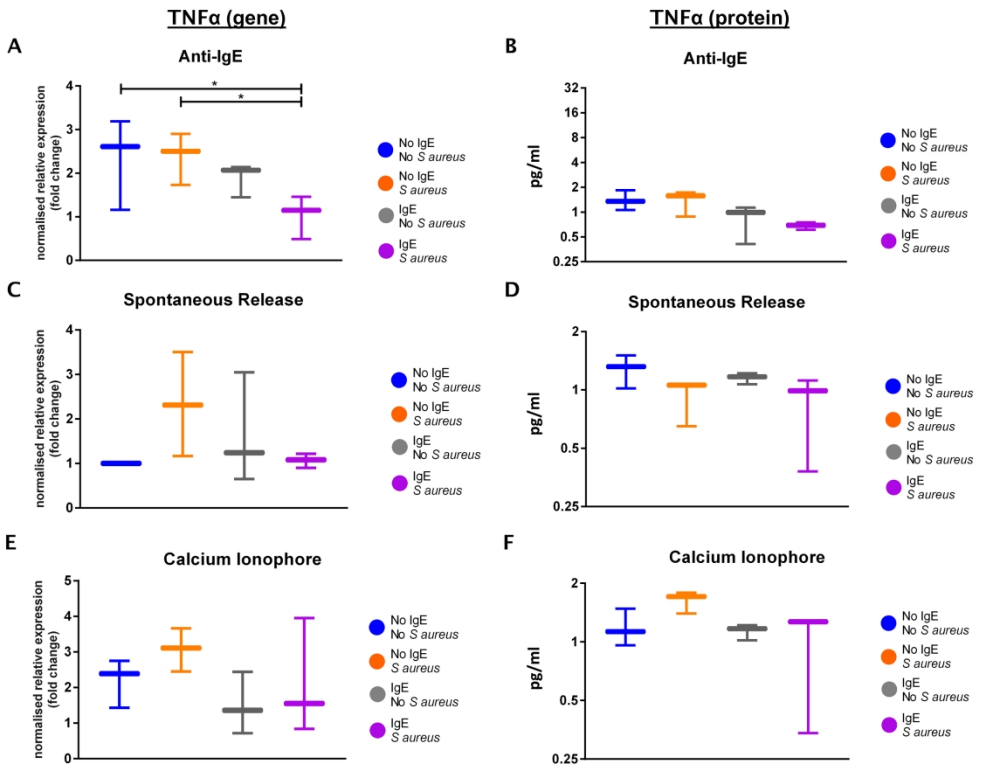
RT-qPCR Housekeeping gene amplicon context information

Human ACTB:

Accession number: NM_001101

Anchor Nucleotide position: 1195

Context sequence length: 106bp



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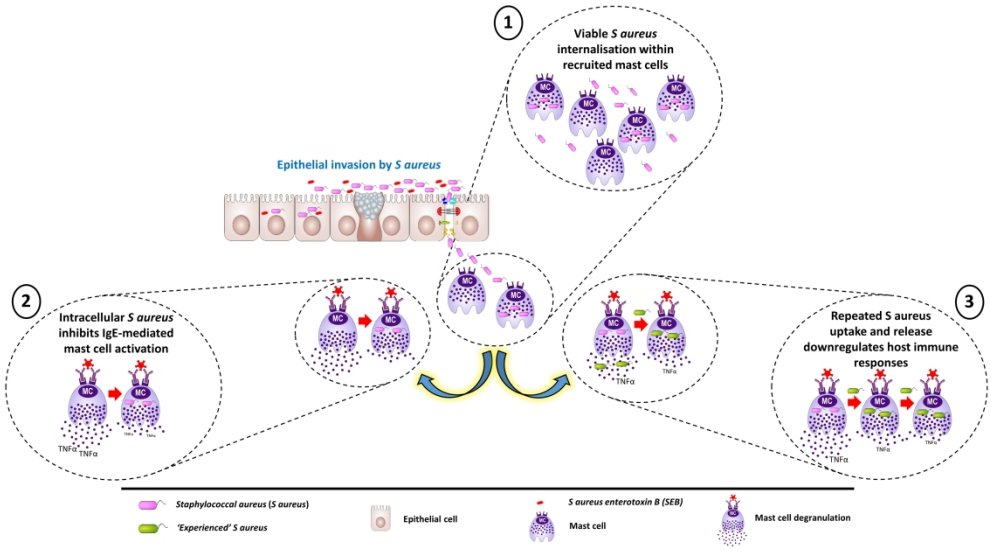

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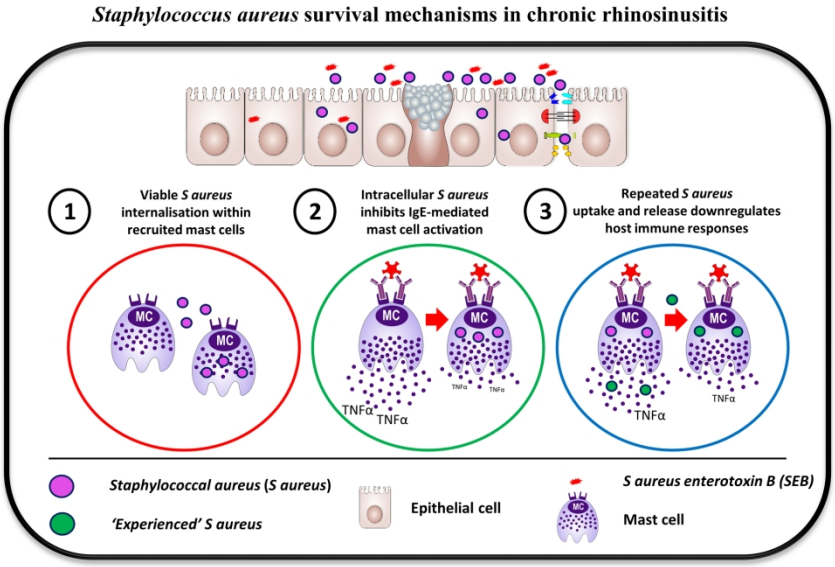

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