***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 strain was obtained through homogenisation of a nasal polyp isolated at the time of functional endoscopic sinus surgery. The supernatant was streaked, with bacteria grown and isolated (37°C, 5% CO2). The strain was confirmed to be *S aureus* through DNase agar and blood agar plating, colonial morphological examination, Baird-Parker agar supplemented with egg yolk tellurite plating, as well as slide coagulase testing.

The isolate was grown to mid-log phase, under static conditions, at 37°C in Roswell Park Memorial Institute (RPMI) medium. The optical density (OD600nm) 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% CO2) 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% CO2.

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

LAD2 mast cells (2 x 106) 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% CO2), 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% CO2). *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 (OD600nm) 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 x106 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 106 cells; no IgE without *S aureus*, no IgE with *S aureus*, IgE without *S aureus* and IgE 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 106 cells and 5 x 106 *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 106 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% CO2). 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% CO2). 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 37oC (5% CO2). 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% CO2).

**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.

**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 106 cells per condition within T25 flasks (Fisher Scientific limited, Loughborough, UK). The culture medium was adjusted accordingly to 10ml per flask. After overnight IgE sensitisation, cells were infected with *S aureus* over 4 hours.

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

**Statistics**

Statistical analysis was performed using Graph Pad Prism 7.0 software (Graph Pad Software Inc., San Diego, CA, USA). Paired t-tests were selected to study statistical differences between the original and internalised strains (C1-5) due to the parametric and paired nature of the data. One-way ANOVA, with multi-variate analysis, was used for parametric data in degranulation experiments and cytokine/protein data. Two-way ANOVA was used for phosphorylation experiments. This was to avoid statistical inaccuracy linked to performing multiple t-tests and following formal statistical advice. Methods used are 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 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. One-way ANOVA was undertaken, p≤0.05 (\*).

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

**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