***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). Activation of mast cells via localised anti-*S aureus* IgE would normally enable them to contribute towards clearance of some of the subepithelial bacterial colonies through phagocytosis (6). However, it is postulated that modulation of the local host immune response by *S aureus* may blunt this response, resulting in enhanced bacterial survival and persistence. In the present study we investigated the mast cell immune response to repeated *S aureus* internalisation and release cycles and further explored the role of IgE in its modulation, determining whether these mechanisms could confer a bacterial survival advantage.

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**). To study potential virulent phenotypic mutations resulting from sequential uptake and release of *S aureus* from mast cells, and their downstream effects on bacterial immune responses, additional *S aureus* populations designated C1 (internalised once) through to C5 (internalised five times) were used. *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 expression (Figure 1G) and TNFα protein excretion (Figure 1H). Exposure to experienced *S aureus* (C5 population) resulted in downregulated TNFα gene expression (Figure 1G) and TNFα protein excretion (Figure 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 (Figure 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, 7). 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 (8). 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 *S 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-derived *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 (7), although crucially these are non-pathogenic strains. This could further limit migration of phagocytic cells, thus compromising the ability to clear ongoing infection. A study by Rocha-de-Souza et al (2008) who previously demonstrated the interplay between human cord blood-derived mast cells and *S aureus*, showed that these mast cells can be infected by *S aureus* which in turn survived and triggered TNFα and IL8 release by binding to TLR2 and CD48 (9).Our findings of host immune modulation following *S aureus* uptake and release could be mediated through similar TLR mechanisms, although further work would be required to examine these associations in greater depth.

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) (10). Downregulation of GSK-3β phosphorylation has also been shown to reduce the pro-inflammatory response, and thereby favour survival of *S aureus* (11). 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(12) and atopic eczema (13), where *S aureus* and the generation of *S aureus*-IgE play a prominent role.

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**Conflict of interests**

* Dr. Biggs has nothing to disclose.
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**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≤0.05 (\*) and p≤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≤0.05), \*\*(p≤0.01), \*\*\*(p≤0.001), \*\*\*\*(p < 0.0001).