Mast cells participate in regulation of lung-gut axis during *Staphylococcus aureus* pneumonia

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Abstract

**Objectives:** The lung-gut axis is known to be involved in the pathogenesis of *Staphylococcus aureus* pneumonia. However, the underlying mechanisms remain unclear. We examined the role of pulmonary mast cells (MCs) in the regulation of the lung-gut axis during *S. aureus* pneumonia.

**Materials and Methods:** We created a mouse model of *S. aureus* pneumonia using MC-deficient mice (*Kit*<sup>W-sh/W-sh</sup>) and examined the level of inflammation, bacterial burden, expression of cathelicidin-related antimicrobial peptide (CRAMP) and composition of the gut microbiota. We further evaluated anti-bacterial immunity by administering bone marrow MCs (BMMCs) or CRAMP into the lungs of *Kit*<sup>W-sh/W-sh</sup> mice.

**Results:** After *S. aureus* challenge, the MC-deficient mice, compared with wild-type (WT) mice, displayed attenuated lung inflammation, decreased expression of CRAMP, higher bacterial lung load and disturbance of the intestinal microbiota. Adoptive transfer of BMMCs into the lung effectively reconstituted the host defence against *S. aureus* in *Kit*<sup>W-sh/W-sh</sup> mice, thus resulting in recovery of *S. aureus* pneumonia-induced intestinal dysfunction. Similarly, exogenous administration of CRAMP significantly enhanced anti-bacterial immunity in the lungs of MC-deficient mice.

**Conclusions:** This study provides evidence for the involvement of MCs in the regulation of the lung-gut axis during *S. aureus* pneumonia.

1 | **INTRODUCTION**

*Staphylococcus aureus* is one of the common pathogens in hospital- and community-acquired pneumonia. In addition to the symptoms including headache, fever, dyspnoea and cough, patients with pneumonia often present with gastroenteritis-like discomforts such as vomiting and diarrhoea, especially in young children. Increasing evidence indicates that the intestinal microbiome and mucosal tissues broadly affect the progression of multiple diseases. The lung-gut axis, which represents the interaction between lung immunity and intestinal microbiota, is thus an area of interest, though the underlying mechanisms remain poorly understood.

Mast cells (MCs) are particularly abundant at host-environment interfaces, such as the skin and the respiratory and gastrointestinal tracts. Because of their location, MCs have been hypothesized to act as sentinel cells that sense pathogen attacks and initiate a protective immune response. However, knowledge of the role of MCs in the host defence against *S. aureus* is limited. Recent studies have demonstrated that MCs can be activated by *S. aureus* and exert antimicrobial activity. But, anti-*S. aureus* mechanisms of MCs have not yet been demonstrated in the lung. The lung is the major target tissue of diverse inhaled microbial pathogens, including *S. aureus*. To combat them, the respiratory system is armed with diverse mechanisms of innate mucosal immunity, including the secretion of antimicrobial peptides. Cathelicidins show anti-bacterial activity against both...
Gram-positive and Gram-negative bacteria through destruction of bacterial membrane. \(^{10-12}\) Cathelicidin expression is upregulated in the airways during bacterial infection \(^{13}\) and has been detected in alveolar macrophages, neutrophils and airway epithelial cells. \(^{10,14,15}\) There has been increasing interest in the role of cathelicidin-related antimicrobial peptide (CRAMP) during bacterial pneumonia.

C-kit receptor plays crucial roles in the differentiation, proliferation and inflammatory reaction of MCs. \(^{16,17}\) Our prior work has shown that MCs released inflammatory factors (eg, tumour necrosis factor (TNF)-α) and CRAMP in response to \(S. aureus\), through stimulation of c-kit receptor and its downstream molecules. \(^{18}\) In this study, using MC-deficient mice (Kit\(^{W-sh/W-sh}\)) with defective c-kit receptor, we evaluated the diversity of the intestinal microbial community in mice with \(S. aureus\) pneumonia and determined the role of MCs in regulation of the lung-gut axis. We showed that MC deficiency impaired lung inflammation and aggravated the imbalance in the gut microbiota upon \(S. aureus\) infection. Adoptive transfer of bone marrow mast cells (BMMCs) into the lung largely reconstituted the host defence against \(S. aureus\) in both the lung and the gut, thus demonstrating a critical role of MCs in the immune response through regulation of the lung-gut axis.

### 2 | MATERIALS AND METHODS

#### 2.1 | Animals

Kit\(^{W-sh/W-sh}\) mice on a C57BL/6 background were kindly provided by Harvard Medical School. \(^{19}\) Age- and sex-matched wild-type (WT) mice were used as controls. All animal experiments were approved by the Animal Care and Use Committee of The Second Affiliated Hospital, Zhejiang University School of Medicine.

#### 2.2 | Bacterial culture

A clinical isolate of \(S. aureus\) was grown aerobically overnight at 37°C in a shaking incubator. \(^{18,20}\) \(S. aureus\) in the mid-logarithmic phase was resuspended in phosphate-buffered saline (PBS). The numbers of bacteria was quantified according to the OD600-based bacterial growth curve and verified with colony-forming unit (CFU) assays. \(^{20}\)

#### 2.3 | BMMCs culture

Bone marrow mast cells were generated from the femurs of C57BL/6 mice and maintained in the presence of 10% pokeweed mitogen-stimulated spleen-conditioned medium, as described previously. \(^{18,21}\) After 4 weeks of culture, about 99% of the cells had developed into MCs. \(^{21}\)

#### 2.4 | A mouse model of \(S. aureus\) lung infection

Eight-week-old C57BL/6 and Kit\(^{W-sh/W-sh}\) mice were used to establish an \(S. aureus\) pneumonia model. \(^{22}\) In brief, 40 μL of \(S. aureus\) (5 \( \times \) 10\(^7\) CFUs) was inoculated intratracheally into anesthetized mice. Non-infected control mice were administered with an equal volume of PBS intratracheally. Lung specimens were weighed and homogenized in 1 mL PBS to determine bacterial load and production of cytokines and CRAMP.

#### 2.5 | Reconstitution of MCs in Kit\(^{W-sh/W-sh}\) mice

Kit\(^{W-sh/W-sh}\) mice (5-6 weeks old) were injected with \(1 \times 10^7\) BMMCs in 200 μL PBS via the tail vein. The mice treated with PBS were used as controls. After 3 weeks, the reconstituted mice were used to build \(S. aureus\) pneumonia model, and tissues were harvested for analysis after 24 hour post-infection. \(^{23}\)

#### 2.6 | CRAMP treatment

The effects of CRAMP on \(S. aureus\) pneumonia were examined through intratracheal administration of 20 mg/kg CRAMP (GL Biotech, Shanghai, China) in 40 μL PBS to mice for four consecutive days. \(^{24}\)

#### 2.7 | ELISA

The levels of cytokines including interleukin (IL)-1β, IL-6, keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2, TNF-α and CRAMP in lung homogenates were detected using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s instructions.

#### 2.8 | Histological analysis

Mouse lung specimens were fixed in 10% paraformaldehyde, embedded in paraffin, cut into 4 μm-thick sections and stained with haematoxylin-eosin (H&E). The stained sections were reviewed for morphology under a photomicroscope (Leica, Heidelberg, Germany). The neutrophil infiltration and inflammatory scores of the lung samples were determined as described previously. \(^{25,26}\) In brief, for evaluation of lung neutrophils, three lung sections from each mouse were analysed and three randomly selected high-power fields (HPF) were examined for each lung section, the average value of cells/HPF for each mouse was determined by summation of all numbers divided by 9. The inflammatory levels were semi-quantitatively determined as below: peribronchial/peribronchiolar inflammation 0-4, perivascular inflammation 0-4 and alveolar inflammation 0-2. Each lung section was given a total inflammatory score (maximum score of 10).

#### 2.9 | Immunofluorescence staining

Paraffin-wax sections of mouse lung tissues were placed on glass slides, and non-specific antibody binding was blocked by incubation with 1% bovine serum albumin for 1 hour. Alexa Fluor 488 anti-mouse CD117 (c-kit) antibody (1: 50, BioLegend, San Diego, CA, USA) and phycoerythrin anti-mouse CRAMP antibody (1: 50, BioLegend) were mixed and used for double staining. The sections were incubated with the above antibodies overnight at 4°C, then incubated at 37°C for 30 minutes. DAPI was used to stain the nuclei before viewing by confocal microscopy (Olympus, Tokyo, Japan).
2.10 | Analysis of the faecal microbiota

Faecal samples in ileocecal were collected from WT and Kit W‐sh/W‐sh mice 24 hours after S. aureus infection, then snap‐frozen in liquid nitrogen. Faecal DNA was extracted and used for 16S rRNA sequencing according to manufacturer’s instructions (Tianke, Hangzhou, China). Total genomic DNA was extracted using the PowerSoil® DNA Isolation Kit. 16S rRNA/ITS genes of distinct regions were amplified. PCRs were carried out with Phusion® High‐Fidelity PCR Master Mix. PCR products were mixed in equidensity ratios and purified with Qiagen Gel Extraction Kit. Sequencing libraries were generated using TruSeq® DNA PCR‐Free Sample Preparation Kit and examined on an Illumina MiSeq platform.

2.11 | Statistical analysis

Data are expressed as mean ± standard error of mean (SEM), unless otherwise stated. Differences between two or multiple groups were analysed with Student’s t test or one‐way ANOVA as appropriate. CFUs in lung tissue were compared using the Mann–Whitney U test. A P value <0.05 was considered statistically significant. All calculations were performed in GraphPad software (Version 5.01).

3 | RESULTS

3.1 | MC deficiency decreases the inflammatory response to S. aureus infection

We evaluated the effect of MCs on the inflammatory response to S. aureus infection. There were no differences in lung neutrophil counts between uninfected Kit W‐sh/W‐sh mice and WT animals; however, the inflammatory levels in Kit W‐sh/W‐sh mice were significantly lower than those in WT mice, based on H&E staining at 24 hours after infection (Figure 1A–C). We further detected the levels of inflammatory cytokines including IL‐1β, IL‐6, KC, MIP‐2, TNF‐α and CRAMP. The expression levels of all cytokines except IL‐1β were significantly lower in Kit W‐sh/W‐sh mice than those in WT mice (Figure 1D). The levels of CRAMP were also decreased in Kit W‐sh/W‐sh mice (Figure 1E). We also quantified the bacterial load in lungs, and found that the CFUs in lung tissue were lower in Kit W‐sh/W‐sh mice than in WT mice (Figure 1F).
lower in lung specimens from Kit\(^{W-sh/W-sh}\) mice than those from WT mice 24 hours post-infection (Figure 1D). Confocal microscopy examinations showed that \textit{S. aureus} stimulation upregulated the expression of lung MC-derived CRAMP in WT mice, but weak expression of CRAMP was found in Kit\(^{W-sh/W-sh}\) mice (Figure 1E). Meanwhile, Kit\(^{W-sh/W-sh}\) mice had significantly higher numbers of \textit{S. aureus} in their lungs after infection, compared to WT mice (Figure 1F).

### 3.2 Kit\(^{W-sh/W-sh}\) mice display impaired intestinal microbiota after \textit{S. aureus} lung infection

To explore the influence of MCs on the intestinal microbiota, we analysed the intestinal microbiota using 16S rRNA sequencing at 24 hours post-infection. The mouse intestinal microbiota consisted of three major bacterial phyla: \textit{Proteobacteria}, \textit{Bacteroidetes}, and \textit{Firmicutes}. The microbiota composition was shown at the phylum level (A) and genus level (B), respectively.

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**Figure 2** MCs alter the faecal microbiota composition after \textit{Staphylococcus aureus} infection. Faecal samples were collected from WT and Kit\(^{W-sh/W-sh}\) mice (n = 5 per group) at 24 h after infection. Analysis of faecal microbiota in WT and Kit\(^{W-sh/W-sh}\) mice was performed by 16S rRNA sequencing. Composition of the intestinal microbiota was showed at the phylum level (A) and genus level (B), respectively.

**Figure 3** BMMCs transfer reconstitutes host resistance to \textit{Staphylococcus aureus} infection in Kit\(^{W-sh/W-sh}\) mice. WT, Kit\(^{W-sh/W-sh}\) and reconstituted Kit\(^{W-sh/W-sh}\) mice (n = 6 per group) were injected intratracheally with \textit{S. aureus}. Lung tissues were collected at 24 h after infection. (A) Levels of IL-6, KC, MIP-2, TNF-\(\alpha\) and CRAMP in lung tissues. (B) \textit{S. aureus} counts in lung tissues. Individual values and medians are shown for each group. *\(P < 0.05\) and **\(P < 0.01\)
Bacteroidetes and Firmicutes. Abundant Proteobacteria were found in the intestinal microbiota in the infected mice. Kit W-sh/W-sh mice showed higher burden of Proteobacteria than WT mice after pulmonary S. aureus infection (Figure 2A). E. coli is an important component of the Enterobacteriaceae and a common cause of vomiting and diarrhoea in humans. Notably, the most striking change in the faecal microbial community after S. aureus infection was an increase in the abundance of the genus Escherichia (Figure 2B), which accounted for approximately 2% of the total faecal microbiota in uninfected WT and Kit W-sh/W-sh mice, but raised to approximately 6% and 15%, respectively, in infected mice.

3.3 | Adoptive transfer of BMMCs reconstitutes host defence against S. aureus infection in KitW-sh/W-sh mice

We performed an adoptive MC transfer experiment to determine whether the susceptibility of KitW-sh/W-sh mice to S. aureus was mainly attributed to MC deficiency. Treatment of WT-derived BMMCs led to significantly elevated levels of IL-6, TNF-α, KC, MIP-2 and CRAMP in KitW-sh/W-sh mice upon infection (Figure 3A). Correspondingly, MC-reconstituted KitW-sh/W-sh mice exhibited markedly improved bacterial clearance of S. aureus (Figure 3B). Furthermore, sequencing analysis of the microbiota also indicated that the imbalance in the intestinal flora after S. aureus infection was largely reversed in MC-reconstituted KitW-sh/W-sh mice (Figure 4A-B).

3.4 | Exogenous CRAMP treatment significantly ameliorates S. aureus pneumonia

Exogenous CRAMP was intratracheally administrated into the mice for 4 consecutive days. Although the lung-tissue levels of the pro-inflammatory cytokines IL-6, TNF-α, KC and MIP-2 were not significantly affected by CRAMP treatment (Figure 5A), the bacterial load was obviously decreased by CRAMP treatment in both WT and KitW-sh/W-sh mice (Figure 5B).

4 | DISCUSSION

Staphylococcus aureus is one of the most common causative pathogens of pneumonia. Although pneumonia is known to be a lung infection, many studies have shown that pneumonia is also associated with substantial extrapulmonary effects, including intestinal inflammation, apoptosis and injury. Mucosal tissues, including those in the respiratory and gastrointestinal tracts, act as the first line of host defence against invading pathogens. Gill et al. proposed that the mucosal immune system, which is associated with the commensal microbial community, is not isolated. Agwu et al. observed that respiratory S. aureus infection in mice caused immune injury not only in the lung but also in the gut, and resembled the symptoms exhibited by humans infected with S. aureus. Staphylococcus aureus-infected mice provide a good model for exploring the mechanisms of lung-gut axis during respiratory S. aureus infection. Furthermore, these observations give more evidence supporting the existence of a broad mucosal immune system.
Mast cells act as effector cells during infection and are increasingly recognized to have a major role in modulating the course of infection. Previous studies demonstrated that S. aureus infection causes a series of inflammatory responses in the lung, in line with infiltration of various inflammatory cells including neutrophils, macrophages and natural killer cells. Nevertheless, the role of MCs in host defence against S. aureus infection is largely unknown.

In this study, using KitW−/− mice, we revealed that MC-deficient mice were significantly more susceptible to S. aureus lung infection than WT animals, accompanied by a greater imbalance of the intestinal flora. We further carried out adoptive transfer of BMMCs into KitW−/− mice prior to S. aureus infection, to obtain additional supportive evidence for the role of MCs in keeping intestinal microflora homeostasis. Interestingly, the suppressed inflammatory response was largely reconstituted in KitW−/− mice after adoptive BMMCs administration. Moreover, the KitW−/− mice received WT-derived BMMCs had significantly fewer harmful bacteria in the gut than did controls. The present study thus indicates the critical role of MCs in keeping the host defence and maintaining gut microbial homeostasis against S. aureus infection.

In summary, our results demonstrated that lung MCs are involved in regulation of the lung-gut axis during S. aureus pneumonia. This study thus provides a potential clinical strategy focused on MCs for patients with S. aureus pneumonia accompanied by severe gut symptoms.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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