CCL20/CCR6 limits the disease severity in *Staphylococcus aureus* osteomyelitis by increasing Th17 and macrophage recruitment at the site of inflammation

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INTRODUCTION: Implant-associated osteomyelitis remains a significant healthcare burden. *Staphylococcus* species are responsible for 75% of all osteomyelitis cases[1], with *Staphylococcus aureus* as the primary pathogen[1,2], and methicillin-resistant *S. aureus* (MRSA) causing 50% of all implant-associated infections[1,2]. The most devastating outcome of *S. aureus* osteomyelitis is multiple organ failure followed by death due to sepsis, and the underlying immune mechanisms are largely unknown. In a clinical pilot study, we found that serum cysteine–cysteine motif chemokine ligand 20 (CCL20) chemokine levels were significantly high **(~5 fold)** in patients with *S. aureus* osteomyelitis and even higher **(~100 fold)** in patients that died due to sepsis induced by *S. aureus* osteomyelitis (**Fig. 1**). CCL20 signals monogamously through its receptor CCR6, and the ligand-receptor pair is responsible for the chemotaxis of dendritic cells (DC), effector/memory T cells, and B cells[4]. It is involved in recruiting both the proinflammatory IL-17-producing helper T cells (Th17) and immunosuppressive regulatory T cells (Treg) to sites of inflammation. The role of CCL20/CCR6 axis in *S. aureus* osteomyelitis is currently unknown.

HYPOTHESIS: Osteoblast-derived CCL20/CCR6 axis is critical to mounting an immune response against *S. aureus*, and that lack of CCL20 or its receptor CCR6 will lead to increased susceptibility to *S. aureus* osteomyelitis.

METHODS: *In vitro studies:* Murine calvarial MC3T3-E1 cells, primary bone-marrow-derived osteoblasts, and macrophages harvested from C57BL6 mice were differentiated into osteoblasts and macrophage subsets (M0, M1 (IFN-γ (50ng/ml)), and M2 (IL-4 (20ng/ml)) and subjected to MRSA USA300 LAC infection (MOI 0, 1, 10, and 50) for 24 hours. Post-infection culture supernatants were harvested and assessed for CCL20 levels via ELISA. *In vivo osteomyelitis studies:* All animal experiments were performed using university approved IACUC protocols. The well-established transtibial L-shaped pin model of implant-associated osteomyelitis using bioluminescent MRSA (USA300 LAC::*lux*) strain as previously described[5]. We used CCL20$^{-/-}$ and CCR6$^{-/-}$ knockout mice (procured from Jax Labs) of age 8-12 weeks to evaluate the role of CCL20/CCR6 axis and compared in vivo data with wildtype (WT) age-matched C57BL/6 mice. We performed longitudinal assessments of disease severity as a measure of 1) body weight, 2) Bioluminescence assay (BLI), 3) ex vivo terminal assessment of CFUs of tibia and internal organs, and 4) histopathology (H& E and Brown-Brenn stain) and micro-CT (bone osteolysis and reactive bone formation). The immunofluorescence was performed to examine the influence of CCL20/CCR6 axis on immune cell (T-cell and macrophage) recruitment to the site of *S. aureus* infection.

RESULT: In vitro studies confirmed that osteoblasts and macrophages (M0 and M2 subtypes) secrete CCL20 following *S. aureus* infection (**Fig. 2A&B**). In vivo, we observed that CCL20$^{-/-}$ and CCR6$^{-/-}$ mice exhibited higher disease severity compared to WT C57BL6 mice as assessed by BLI, CFU, and micro-CT. Longitudinal BLI measurement of bacteria confirmed increased early planktonic *S. aureus* load in CCL20$^{-/-}$ and CCR6$^{-/-}$ compared to WT mice (**Fig 3A)**. Terminal ex vivo CFU assessments (14 days post-op) revealed a significant increase in soft tissue CFU in CCL20$^{-/-}$ mice and an increase in bone (**Fig 3B**) and implant/pin CFU in CCR6$^{-/-}$ mice compared to WT animals. (**Fig 3C&D**). Micro-CT analyses revealed increased bone loss in CCR6$^{-/-}$ mice (data not shown). Interestingly, we observed reduced staphylococcal abscess communities (SAC) formation in CCR6$^{-/-}$ mice compared to WT (**Fig 4A&B**). To decipher the mechanism behind the observed phenotype in knockout mice, we performed IHC, which showed increased recruitment of CCR6+ T cells (Th17 subtype) (**Fig 5**) adjacent to the SACs only in the wildtype mice and not in the CCR6$^{-/-}$ mice.

DISCUSSION: We are the first group to identify that osteoblasts produce CCL20 when challenged with MRSA. We also observed that CCL20/CCR6 axis is essential for the recruitment of CCR6+ T-cells and macrophages, with CCR6 potentially contributing to increased CCL20 production in a feed-forward manner. The lack of CCR6 receptor or CCL20 ligand increased disease severity due to defective immune response, resulting in diminished bone bacterial clearance. The increased levels of CCL20 in *S. aureus* osteomyelitis in humans and the increased disease severity in the absence of CCL20 or CCR6 suggest that the chemokine CCL20/CCR6 axis is essential to limit the disease severity during *S. aureus* osteomyelitis.

SIGNIFICANCE: It is of utmost importance to develop novel therapeutics which reduce the infection severity and boost the human immune response to infection. Our studies highlight that CCL20/CCR6 signaling could play an important role in modulating host response to *S. aureus* osteomyelitis.

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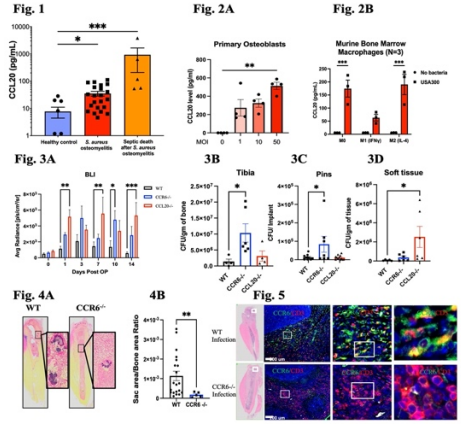

Fig. 1 Increased serum CCL20 in the patient diagnosed with osteomyelitis and patient died after septic death as compared to healthy patient. In a pilot clinical study of patients with *S. aureus* osteomyelitis, serum CCL20 levels were 5-fold and 100-fold higher as determined via Luminex assay.

Fig. 2 In-vitro experiment confirmed Osteoblast and macrophages are the source of CCL20 secretion. CCL20 is secreted by murine osteoblasts (**Fig. 2A**) and macrophages (**Fig. 2B**) as challenged with *S. aureus* in a dose dependent manner the levels were measured by ELISA on culture supernatants. (n=3-4)**.**

Fig. 3 Increase in disease severity in CCR6$^{-/-}$ and CCL20$^{-/-}$ mice as compared to WT mice. 14 days after infection as assessed by BLI measurements on days 0, 1, 3, 7, 10, and 14 (**Fig. 3A**) and terminal ex vivo CFU counts in tibiae, soft tissue, and implant (**Fig 3B-D**) (n=4-10).

Fig 4. Defective SAC formation in CCR6$^{-/-}$ mice. The Brown-Brenn stain showing bacterial SAC, reduced ratio of SAC area to bone area in CCR6$^{-/-}$ mice as compared to wild type mice was observed on quantification (**Fig. 4A&B**) (n=4-6).

Fig 5. The immunofluorescence showing reduced CCR6+ T-cell recruitment in CCR6$^{-/-}$ mice. The IHC was done using CCR6 (green) and CD3 (T-cell) markers, representative images showing H&E stain, and CCR6+ T-cell recruitment around abscess at the 20X magnification (scale bar =100 µm) (**Fig. 5**).

All data are presented as mean +/- SEM, statistical comparisons were performed with ANOVA/unpaired t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)