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 aureus* species are responsible for 75% of all osteomyelitis cases1, with *Staphylococcus aureus* as the primary pathogen2,3, and methicillin-resistant *S. aureus* (MRSA) causing 50% of all implant-associated infections2,4. 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 cytokine–cytostatic 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 cells5. 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* infection.

**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 transistbil L-shaped pin model of implant-associated osteomyelitis using bioluminescent MRSA (USA300 LAC::lacZ) strain as previously described5. 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 C57BL6 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. 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 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 *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 signaling could play an important role in modulating host response to *S. aureus* osteomyelitis.


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