High Mobility Group Box 1 drives bone formation via osteoblastic Toll-like Receptor 4 following mechanical loading

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INTRODUCTION: Strain adaptive bone remodeling is mediated in part by the neurotrophin nerve growth factor (NGF), which is produced in osteoblasts following mechanical loading. Specifically, NGF expressed at the periosteal bone surface can bind to its high affinity receptor TrkA on peripheral sensory nerves to promote new bone formation[1,2]. Recent studies have uncovered a novel mechanism of NGF production in mature osteoblasts following mechanical loading[3]. Specifically, *Ngf* transcription can be driven by activation of toll-like receptor 4 (TLR4), a key receptor upstream of the NF-κB pathway, that is increasingly produced in periosteal osteoblasts following the application of a compressive load[3]. Furthermore, mice lacking this receptor in osteoblasts were found to have significant deficits in periosteal bone formation rate following axial forelimb compression[3]. However, the mechanism by which TLR4 is activated in osteoblasts in response to load remains unclear. In this study, we hypothesized that the damage-associated molecular pattern (DAMP) High Mobility Group Box 1 (HMBG1) protein – an endogenous ligand of TLR4 – can increase NGF expression in osteoblasts to support load-induced bone formation via TLR4. Here, we have directly tested this hypothesis using both *in vivo* and *in vitro* models.

METHODS: Animal procedures were performed in accordance with the IACUC of Thomas Jefferson University (Protocol #02204). Adult Hmgb1$^{fl/fl}$; osteocalcin (OC)-Cre+ mice (CKO) were generated on a C57BL/6 background, and Hmgb1$^{fl/fl}$ (WT) littermates were included as controls. To determine the response of Hmgb1 CKO mice to mechanical load, the right forelimb of each mouse was compressed using a 2 Hz sinusoidal rest-inserted waveform with a peak force of 3N for 100 cycles for three consecutive days (D0-2). Calcein (10 mg/kg) and alizarin red (30 mg/kg) fluorescent labels were administered by IP injection on D3 and D8, and forelimbs were harvested on D10 for imaging with confocal microscopy. Baseline skeletal phenotype of this novel mouse model was analyzed with microCT. To assess the effects of exogenous HMGB1 on osteoblasts *in vitro***,** both primary calvarial osteoblasts and MC3T3-E1 osteoblasts were treated with 40nM rhHMGB1 protein for 24 hours prior to 0, 7, or 14 days of exposure to osteogenic differentiation media. Total RNA was isolated at each time point for gene expression analysis of *Ngf, Tlr4, Tlr2* and *Nfkb1*. Finally, forelimbs from WT and CKO mice were harvested ~3 hours after the third bout of loading (D2), fixed, decalcified, and embedded in paraffin to perform immunohistochemistry. Transverse sections were stained with antibodies against TLR4 and OC by sequential overnight incubations, then analyzed using confocal microscopy. Statistical analysis of *in vivo* outcomes was performed using two-tailed, unpaired Student's t-tests. Differences for *in vitro* outcomes were assessed using one-way ANOVA ($p \leq 0.05$ was considered significant).

RESULTS: *HMGB1 is required for normal load-induced bone formation in female but not male mice.* As expected, both male and female WT mice displayed robust load-induced bone formation following axial forelimb compression, as demonstrated by new bone acquisition observed in transverse sections of the ulna on the medial periosteal surface (n=6-7 per group) (**Fig. 1A, E**). In female CKO mice, the loss of HMGB1 significantly reduced periosteal bone formation rate (Ps.BFR) as compared to WT mice following loading (-80%, p = 0.014) (**Fig. 1G**). Surprisingly, male CKO mice were not affected by the loss of osteoblastic HMGB1, with the bone formation rate on both periosteal (Ps.BFR) and endosteal (Es.BFR) surfaces remaining unchanged (**Fig. 1C, D**). Importantly, there were no significant differences in the baseline skeletal phenotype in female mice, and both cortical and trabecular bone parameters remained unchanged following Hmgb1 knockout (n=5). *HMGB1 promotes the TLR4-NGF signaling axis in mature osteoblasts in vitro*. In MC3T3-E1 cells subjected to osteoblastic differentiation for 7 days, rhHMGB1 significantly increased expression of *Ngf* (+130%), *Tlr4* (+70%), and *Nfkb1* (+140%). Similarly, undifferentiated primary calvarial osteoblasts also responded to rhHMGB1 by significantly increasing expression of *Ngf* (+130%) and *Tlr2* (+100%). However, MC3T3-E1 and primary osteoblasts were less responsive to HMGB1 treatment at other differentiation time points. Nonetheless, osteoblasts harvested from mice lacking TLR4 in the osteoblast lineage (Tlr4$^{fl/fl}$;OC-Cre+) did not respond to exogenous HMGB1 treatment at any differentiation time point. *HMGB1 is required for increased TLR4 expression in the periosteum of loaded ulnae*. Following three bouts of axial forelimb compression, we observed a significantly greater number of TLR4+ cells on the periosteal surface of the loaded ulna in female WT (**Fig. 2A,C**) but not CKO (**Fig. 2B,C**) mice as compared to the non-loaded contralateral limb, consistent with our observation that HMGB1 increases *Tlr4* gene transcription *in vitro*. Furthermore, nearly all (>80%) of TLR4+ periosteal cells were OC-expressing mature osteoblasts (n=4).

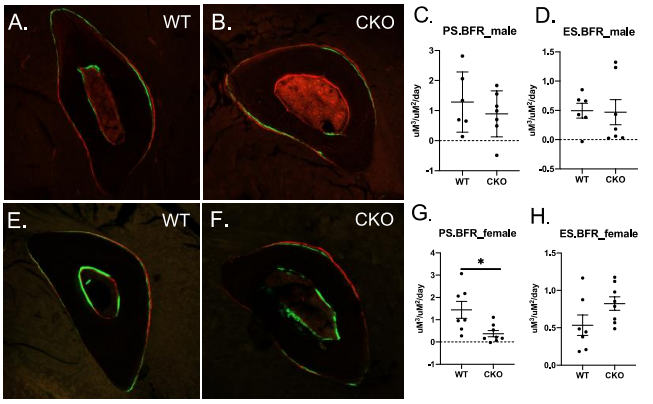

Fig 1. Bone formation following axial forelimb compression in adult WT and CKO mice

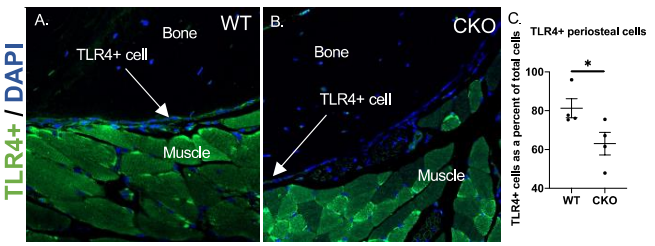

Fig 2. TLR4 expression in periosteal cells following axial forelimb compression in adult mice

DISCUSSION: In this study, we demonstrate that osteoblastic HMGB1 is required for load-induced bone formation in female mice. Moreover, we provide evidence that osteoblastic HMGB1 activates TLR4-NGF signaling *in vitro* as well as TLR4 expression *in vivo* following mechanical loading. In previous work, we have observed that loss of either *Tlr4* or *Ngf* in the mature osteoblast lineage significantly reduced the skeletal adaptation of bone to load in both male and female mice[3,4]. Surprisingly, male mice were unresponsive to the loss of osteoblastic HMGB1 in this study. Therefore, we propose that TLR4 may be activated by an additional, alternative endogenous ligand in male mice, which will be explored in future studies. Our work also suggests that differentiation status may play a role in the responsiveness of osteoblasts to HMGB1. In total, our results support a model in which HMGB1 can activate TLR4 expressed by mature osteoblasts to upregulate NGF transcription that supports a normal response to osteogenic mechanical loading in female mice.

SIGNIFICANCE / CLINICAL RELEVANCE: Our study advances the understanding of NGF transcriptional activation in the osteoblast, which may inform the development of anti-inflammatory drugs and non-opioid pain medications for bone and joint indications.

REFERENCES: 1- Tomlinson et al. 2017 PNAS, 2- Rajpar & Tomlinson 2022 Semin Cell Dev Biol, 3- Rajpar et al. 2023 iScience 4- Rajpar, McLaughlin et al. *in prep.*