**Tmem178 Is A Negative Regulator Of Bone Homeostasis And Osteoclast Activation In Mice And Humans Via A Novel Negative Feedback Loop Targeting Endoplasmic Reticulum Ca2+ Mobilization**

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**Introduction:** Dysregulation of innate immune cells is thought to be the main driver of systemic Juvenile Idiopathic Arthritis (sJIA) (1). Differentiation of macrophages into bone resorbing osteoclasts (OCs) was responsible for persistent erosive arthritis in up to 50% of sJIA patients in the pre-biologic era, and remains an issue for a significant subset of patients (CARRAnet registry data, manuscript in preparation, Mellins and colleagues). Thus, macrophages appear to be central players in the pathogenesis of sJIA. Unfortunately, finding effective combined treatments of both inflammatory reactions and their osteolytic consequences, without inducing global immune suppressive effects, represents the major challenge of current sJIA therapies. An efficacious approach would be to selectively target the signaling molecules controlling the inflammatory and bone resorptive effects of macrophages.

Our previous studies demonstrated that Phospholipase C gamma 2 (PLCγ2) is required for macrophage activation and OC formation during inflammatory arthritis (2-4). PLCγ2 catalytic activity triggers Ca2+ fluxes leading to robust activation of NFATc1, the central transcription factor of osteoclastogenesis (OCG). Despite PLCγ2’s essential role in promoting OC formation, its therapeutic targeting is impeded by high homology with the ubiquitously expressed PLCγ1. This work identifies Tmem178, as a PLCγ2 dependent gene, controlling OC responses in sJIA.

**Methods:** To identify specific mediators downstream of PLCγ2, we performed a gene array comparing WT and PLCγ2-/- OC precursors and identified Tmem178 to be highly expressed in WT but not in PLCγ2-/- cells. To examine the effects of Tmem178 deficiency on bone homeostasis we performed micro-CT analysis on 16weeks old WT and TMEM178-/- mice, followed by histological analysis to count the number of TRAP+ OCs. To examine the in vivo OC response under pathological conditions, WT and Tmem178-/- mice were injected over the calvaria with LPS (100ug) or subjected to serum transfer arthritis. In vitro OCG was performed by culturing whole bone marrow cells from WT or Tmem178KO mice in the presence of RANKL (100ng/ml) and M-CSF (25ng/ml) for 5 days. When indicated, exogenous LPS or TNF were added to the cultures. OC markers were examined by RT-PCR and compared to their expression in macrophages. Activation of the osteoclastogenic pathways NFKB and NFATc1 was examined by western blot in cells stimulated with RANKL. To measure basal intracellular calcium levels, WT or Tmem178-/- OC precursors were loaded with Fura-2 and the ratio of fluorescence emission at 510nm with alternating excitation wavelengths of 340 and 380nm was measured. Increased 340 over 380 ratio indicates increase in calcium levels. Finally, co-immuneprecipitation assays were carried out in Hek293 cells expressing the two components of the CRAC channel Myc-conjugated STIM1 and Flag-tagged Orai1, in the presence or the absence of exogenous HA-tagged Tmem178.

**Results:** We identified Tmem178, a previously uncharacterized integral membrane protein, as a novel PLCγ2-dependent gene that is upregulated by RANKL in WT cells but not in PLCγ2-/- cells. In striking
contrast to the osteopetrotic phenotype of PLCγ2-/- mice, Tmem178 deficiency leads to a significant 35% decrease in trabecular bone accompanied by enhanced OC numbers (KO 25.7+/−4.4, WT 12.5+/−1.9). Tmem178-/- OC precursors are more sensitive to RANKL in vitro as shown by rapid induction of TRAP, DC-STAMP, and calcitonin receptor, suggesting that Tmem178 acts in a negative feedback loop downstream of RANKL-PLCγ2. As the addition of LPS or TNF to Tmem178-/- preOCs further exacerbates OCG, we tested Tmem178’s role in restraining pathological bone loss using 2 models of inflammatory osteolysis: supracalvarial LPS and serum-transfer arthritis. In both conditions Tmem178 null mice suffer profound bone loss due to significant increase in OCs compared to WT mice. Thus, Tmem178 regulates OCG and bone mass in basal and pathological states. Increased OC numbers and responsiveness to RANKL are often associated with augmented NF-kB and MAPK activation. Remarkably, we found no changes in these pathways in Tmem178-/- preOC. Nevertheless, NFATc1 total protein levels and nuclear translocation are strikingly amplified in Tmem178-/- cells. To determine how Tmem178 controls NFATc1 levels we measured cytosolic Ca2+ in preOCs stimulated with RANKL and found a higher immediate spike and sustained increase in [Ca2+] in null cells compared to WT. By immunofluorescence and co-immunoprecipitation, we detected Tmem178 in the endoplasmic reticulum (ER) where it interacts with Stim1, a known regulator of Ca2+ mobilization during OCG. Importantly, ectopic Tmem178 suppresses thapsigargin-induced ER Ca2+ release and likewise dampens RANKL-stimulated NFATc1 and OCG. These findings suggest that Tmem178 is a previously unknown fine-tune regulator of ER Ca2+ stores.

Finally, to examine the relevance of TMEM178 in sJIA, a disease mainly driven by aberrant activation of monocytes/macrophages, we examined Tmem178 mRNA in human monocytes treated with plasma from sJIA patients or healthy controls. Strikingly, we find that Tmem178 levels are downregulated in human monocytes stimulated with plasma from sJIA patients compared to controls. More importantly, downregulation of Tmem178 levels in sJIA samples correlates with increased number of OCs. In contrast to sJIA, Tmem178 expression did not change in monocytes from patients with rheumatoid, psoriatic and reactive arthritis compared to healthy controls.

Discussion: In sum, we define Tmem178 as a critical mediator of bone homeostasis via a novel negative feedback loop targeting the RANKL-Ca2+-NFATc1 axis. Our data also position TMEM178 as a possible, specific regulator of OC activation in children with sJIA.

Significance: This study is the first to identify any function for Tmem178. We demonstrate that Tmem178 is a negative regulator of calcium fluxes in the OCs. Furthermore our data suggest that Tmem178 levels are altered in the context of a human disease, sJIA. This finding represents the first disease association with Tmem178 and correlate reduced Tmem178 expression levels with increased osteoclastogenic potential. Future studies are needed to examine the therapeutic effects of targeting Tmem178 in sJIA. Conceptually, targeting TMEM178 and calcium signaling represents a novel therapeutic approach to re-establish the cellular homeostasis in sJIA, where dysregulation of macrophage responses play a dominant role, compared to current cytokine blockade.