The Role of Tmem178 in Regulation of Osteo-Immune Activation and Inflammatory Bone Loss

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The Role of Tmem178 in Regulation of Osteo-Immune Activation and Inflammatory Bone Loss

by

Corinne Elaine Decker

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

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ABSTRACT OF THE DISSERTATION

The Role of Tmem178 in Regulation of Osteo-Immune Activation and Inflammatory Bone Loss

by

Corinne Elaine Decker

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(Immunology)

Washington University in St. Louis, 2015

Roberta Faccio, Ph.D., Chairperson

Pathological bone loss in human disease such as arthritis is largely due to excessive osteoclast recruitment as a consequence of localized inflammation. Innate immune cells, namely neutrophils and macrophages, infiltrate the joint space and release pro-inflammatory cytokines as well as proteases to drive local tissue damage and inflammation. Importantly, IL-1 and TNF-α in particular act on the synovial fibroblasts as well as directly on osteoclast precursors to potently augment osteoclast differentiation and thus bone resorption. Current therapeutics to treat pathological bone loss are widely unsuccessful at targeting both the resorptive and inflammatory components of disease. We have previously demonstrated that phospholipase C gamma-2 (PLCγ2) regulates both the osteoclast and innate immune cell functions. PLCγ2-/- mice are osteopetrotic resulting from defective OC formation and are protected from inflammatory bone loss due to impaired innate immune responses. While PLCγ2 function can be specifically repressed in the osteoclast via SH2 domain targeting, this approach does not block inflammation in vivo. Thus we sought to identify specific downstream targets of PLCγ2 that could regulate both the osteoclast and innate immune responses in arthritis.

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By a gene array approach, we identified the previously undescribed protein Tmem178 as a novel PLC\(\gamma\)2 effector whose expression is highly induced by RANKL stimulation, integrin-mediated adhesion, and TLR activation in WT but not PLC\(\gamma\)2-/-macrophages. Importantly, Tmem178 expression is confined to macrophages and osteoclasts and not in other bone marrow-derived cells or immune cell types such as T cells. In surprising contrast to the osteopetrotic phenotype of PLC\(\gamma\)2-/- mice, Tmem178-/- mice are osteopenic in basal conditions and are more susceptible to inflammatory bone loss, owing to enhanced osteoclast formation. We also find that Tmem178-null mice succumb faster to endotoxin-induced sepsis due to an increase in inflammatory cytokine production by macrophages. Mechanistically, Tmem178 localizes to the endoplasmic reticulum membrane where it interacts with the Ca\(^{2+}\)-sensing protein Stim1. In the ER, Tmem178 acts to restrain Ca\(^{2+}\) release, thus controlling activation of the transcription factor NFAT which is central to both osteoclast differentiation and pro-inflammatory cytokine production. Importantly, downregulation of Tmem178 is observed in human CD14\(^+\) monocytes exposed to plasma from systemic juvenile idiopathic arthritis patients. This defective Tmem178 expression correlates with augmented osteoclastogenesis in vitro and erosive disease in vivo, suggesting that Tmem178 may also play a fundamental role in controlling arthritic disease in humans. In sum, this dissertation identifies the first known function of Tmem178 in any cell type and positions Tmem178 as an essential regulatory component in both the inflammatory and erosive arms of pathological bone loss.
Chapter 1. Introduction
1.1 Background

Part I. The osteoclast and bone remodeling

The musculoskeletal system, comprised of bone, cartilage, and muscle, serves three essential functions in the organism. The mechanical function of the skeleton is required to mediate mobility and stability of the organism. Secondly, the skeleton serves to harbor and shield the bone marrow within the vertebrae and long bones, as well as protect the internal organs housed within the rib cage and skull. Lastly, the bone itself is a reservoir of mineral stores, namely calcium and phosphate, which are released from the skeleton during bone turnover and regulate whole-body metabolism. In these ways, the skeleton is a dynamic organ which exerts not only its obvious mechanical and structural functions, but also participates in cross-talk with the endocrine, immune, digestive, and nervous systems (1, 2).

The bone is comprised of an inorganic and organic matrix. The latter is made up primarily of type I collagen, as well as numerous growth factors including osteopontin, bone sialoprotein, osteonectin, osteocalcin, and glycosaminoglycans. The inorganic matrix of the bone, which is laid down on top of the organic matrix, is comprised of carbonated hydroxyapatite. Deposition of these organic and inorganic is carried out by osteoblasts (OB) derived from mesenchymal progenitors (MPs). OBs both form and mineralize the skeleton during embryonic and postnatal development, skeletal remodeling and bone turnover throughout life (3). In vivo, mature OBs are characterized by their cuboidal morphology on the bone surface which distinguishes them from flat, bone-lining cells. In vivo, it is believe that these mature OBs form the bone, while the more immature OBs produce RANKL, a factor required for osteoclast (OC) differentiation (4). The OC is the sole bone-resorbing cell that is required for proper shaping of the bone and bone turnover (5). The coupling of OC and OB activity is central to
skeletal formation and maintenance. As the body of this work focuses on the OC, OB differentiation and function will not be discussed in detail but are well-described in detail in references (6-8).

**Osteoclasts and skeletal resorption**

Osteoclasts (OCs) are the unique multinucleated, bone-resorbing cells that form from the fusing of mononuclear precursors (9). As depicted in Figure 1.1, OCs originate from hematopoietic precursors in the bone marrow which first encounter the cytokine macrophage colony stimulating factor (M-CSF) which pushes the precursors to the common myeloid progenitor (CMP). M-CSF supports the proliferation and survival of the myeloid precursors and also upregulates expression of receptor activator of NF-κB (RANK), the receptor for RANK ligand (RANKL), the key osteoclastogenic cytokine (10). Importantly, OBs present RANKL on their surface, and direct interaction of OB with OC precursors is the main driver of OC differentiation (11, 12). Mononuclear preOC begin expressing tartrate resistant acid phosphatase (TRAP), which is commonly used as a marker of OC both in vitro and in vivo. These mononuclear preOCs then fuse to form large, multi-nucleated OC which are fully mature when attached to the bone surface (13, 14). It is believed that in vivo OCs have a relatively short life span and undergo apoptosis following bone resorption, although the inability to perform lineage tracing experiments of OCs in vivo has limited the complete understanding of this cell type. OCs are mature and active on the bone surface where they polarize and adhere tightly to the bone via $\alpha v \beta 3$ integrin attachment. $\alpha v \beta 3$ integrin is required for proper bone resorption and specifically recognizes bone matrix proteins including fibronectin and osteopontin to form a tight sealing zone; it is within this sealing zone that the OC resorbs the bone (15-19).
After the mature OC attaches to the bone and establishes the sealing zone, the ruffled border structure is formed. The OC uses vesicle-mediated secretion of proteases and proteinases to resorb the bone. The fusion of these vesicles with the cell’s plasma membrane forms the appearance of a ruffled border made up of long projections of the plasma membrane into the bone matrix (20). Following the formation of the ruffled border, the OC first resorbs the inorganic matrix of the bone. OCs express protein pumps and chloride channels to form HCl in the resorptive lacunae and dissolve the hydroxyapatite of the bone (21-23). The organic matrix of the bone is then dissolved by proteolytic enzymes, namely matrix metalloproteinase 9 and cysteine proteases, which break down collagen (24). Cathepsin K is particularly required for resorption of the organic matrix, as the null mice present with severe osteopetrosis owing to defective resorption (25-27). Interestingly, Cathepsin K is released in discrete lysosomal vesicles independent of MMP9, although the exact regulatory mechanisms of Cathepsin K-containing vesicles are not yet understood (28). Following resorption, the OC must also remove the degradation products from the resorptive lacunae. Collagen fibers and calcium are taken up by the OC by vesicle-mediated endocytosis, and released from the top of the cell via transcytosis on the opposite side of the ruffled border.

**Bone remodeling**

The term “bone remodeling” is used to describe healthy skeletal turnover that occurs in adult organisms. This resorption of old bone and replacement with new bone is required to ensure skeletal integrity throughout life and is also required for bone healing in the instance of fracture or injury. Importantly, OC and OB functions are coupled during bone remodeling so that bone resorption and deposition are synchronized and equilibrated (Figure 1.2). Dysregulation of this
coupling underlies skeletal pathologies such as osteoporosis. Bone remodeling is broken down into 3 steps: initiation, transition, and termination (29, 30).

The initiation of bone remodeling can be spurred by numerous physiological changes including hormonal changes, deficits in serum calcium, mechanical load, or fractures in the bone. First, OC precursors must be recruited to the bone in response to chemoattractants including CCL2, also known as monocyte chemoattractant protein 1 (MCP-1) (31, 32). CCL2, which engages CCR2 or CCR4 on the OC precursor, is especially important and prevalent during inflammatory conditions, as its expression by OBs is induced by TNF-α and IL-1β. Additionally, vascular endothelial cells in the bone as well as OBs can produce stromal derived growth factor (SDF-1) which binds to CXCR4 on OC precursors (33, 34). After being recruited to the site of bone remodeling, OC precursors are mature and active on the bone surface, leading to the transition phase of remodeling.

During the transition phase of bone remodeling, OC activity is coupled to OB bone deposition. While resorbing the bone, the OC initiates the release of bone-derived growth factors to stimulate the OB. These factors include TFGβ, BMPs, and insulin-like growth factor II (IGF-II) (35, 36). Some molecules produced by the OCs themselves may also act on OBs. For example, sphingosine 1-phosphate (S1P) is secreted by OCs and can signal to OBs to enhance their recruitment and survival (37). The induction of OB activity also suppresses OC activity. Specifically, OBs express EphB4 which binds to ephrin B2 on the OC, inducing bi-directional signaling to promote OB proliferation while suppressing osteoclastogenesis (38). Ultimately, OCs undergo apoptosis during the transition phase while bone deposition predominates.

During the termination phase, OBs actively form new bone, a slow process which can last approximately 3 months in humans. During this phase, OC differentiation and activity must be
suppressed to allow for matrix deposition. For this purpose, OBs produce osteoprotegerin (OPG), the soluble decoy receptor for RANKL. OPG production by the OBs is largely turned on through both NOTCH and Wnt signaling pathways (39, 40). Mice lacking OPG present with osteoporosis due to excessive bone resorption (41). At the conclusion of the termination phase, OBs are believed to have 3 possible fates: undergo apoptosis, become inactive bone-lining cells, or become embedded within the bone matrix as osteocytes. The molecular mechanisms determining OB fate are not well understood, however. While all the physiological roles of osteocytes are still being elucidated, it is known that osteocytes secrete fibroblast growth factor-23 (FGF23), which acts on the kidney to regulate serum phosphorous levels and may also be important to bone formation induced by mechanical loading (42-44). The lack of a specific molecular marker to distinguish osteocytes from OBs, however, has precluded the successful generation of osteocyte-specific genetic models.

**Part II. Osteoclast signaling pathways**

As stated above, OCs arise from hematopoietic precursors in the bone marrow (45). The cytokines M-CSF and RANKL are required for precursor survival, proliferation, and commitment to the OC lineage. Exposure to M-CSF first pushes the cells to the myeloid lineage. While the exact OC precursor in vivo is unknown, several groups have characterized myeloid cells with high osteoclastogenic potential with the surface markers CD11b^-/loCD115^+CD117^+ (46, 47). Further stimulation of these cells with the cytokine RANKL commits them to the OC lineage. During the early phases of osteoclastogenesis, the cells exit the cell cycle and cease dividing, at which point they will become terminally differentiated OC (48).
**M-CSF signaling**

M-CSF, encoded by the gene *Csf1*, is produced by OBs in both a membrane and soluble form. The necessity of M-CSF specifically in the context of the OC and bone is underscored by the severe osteopetrotic phenotype of op/op mice which harbor a mutation in *Csf1*. Op/op mice fail to develop OC in vivo, leading to extremely high bone mass and impaired tooth eruption (49). Dai and colleagues also deleted the receptor for M-CSF, c-fms, and found a similar impairment in OC differentiation and bone resorption in vivo (50).

C-Fms is a receptor tyrosine kinase which undergoes dimerization and autophosphorylation following ligation to M-CSF. This autophosphorylation of tyrosine residues in the cytoplasmic tail provides a docking site for the SH2 motif of Src family kinases, namely c-Src. This signaling at the receptor activates 2 major intracellular signaling pathways: PI3K and MAPK (51, 52). PI3K is recruited directly to the receptor and activates downstream protein kinase B (Akt), which in turn stimulates gene transcription for cell proliferation via cyclin proteins. Akt also signals to mTOR to promote anti-apoptotic pathways and cell survival. The MAPK pathway induces extracellular related kinase (ERK) activation, which turns on the cascade of activation leading to p38, c-Jun, and JNK2 phosphorylation. This pathway is ultimately necessary to induce the transcription factor Mitf that turns on the anti-apoptotic protein Bcl-2 (53). In fact, overexpression of Bcl-2 is sufficient to rescue the high bone mass phenotype of the op/op mice (54). Through these pathways, M-CSF is indispensable to support the survival and proliferation of OC precursors, and importantly, also up-regulates their expression of RANK.
**RANKL signaling**

RANKL is a member of the TNF superfamily that is wholly necessary for OC differentiation. Mice lacking RANKL or its receptor RANK present with a complete lack of OCs and failure in tooth eruption (12, 55). RANKL exists as both membrane-bound form, such as it is found on OB and perhaps osteocytes, and also in secreted form following cleavage by ADAM (a disintegrin and metalloprotease domain) and matrix metalloproteases (56, 57). Secreted RANKL is produced by T cells in the inflamed synovium and appears to contribute significantly to joint destruction during rheumatoid arthritis (58). The majority of RANKL is present in the membrane-bound form, however, and thus osteoclastogenesis requires direct cell-to-cell contact.

The binding of RANKL monomers to the receptor RANK, a TNF superfamily member, induces receptor trimerization. Downstream signaling is dependent on protein scaffolding at the receptors’ cytoplasmic tail, which harbors no intrinsic enzymatic activity of its own. RANK can recruit several isoforms of the TNF-related factor (TRAF) family of adaptor proteins (59). Of these, TRAF6 is particularly important, as TRAF6-null mice present with high bone mass owing to defective OC differentiation (60). A second adaptor, Gab2, also appears to be critical in protein scaffolding at RANK, as Gab2-/- mice also suffer from osteopetrosis (61-63). The assembly of these molecules initiates downstream signaling of MAPK and NF-kB pathways that modulate both OC differentiation as well as bone resorption (Figure 1.3).

**NF-kB**

The family of NF-kB transcription factors, comprised of p50, p52, p65 (also known was RelA), RelB, and c-Rel, can be activated via 2 distinct pathways referred to as the classical and alternative pathways (64, 65). Both classical and alternative NF-kB are activated by RANKL signaling (Figure 1.4). In the classical pathway, the IKK kinase complex, composed of IKKα, β,
and γ, is activated by TRAF6, and in turn phosphorylates IKBα (66). In steady-state conditions, IKBα functions to retain the NF-κB family member p50 in the cytoplasm in complex with p65 or cRel. Phosphorylation of IKBα targets the protein for proteosomal degradation, allowing the release and nuclear translocation of the p50-p65/cRel dimer and activation of NF-κB responsive genes. On the other hand, the alternative pathway is dependent on the kinase NIK, which in steady state conditions is rapidly degraded via TRAF3-mediated ubiquitination. Recruitment of TRAF3 to RANK, however, allows for NIK stabilization and its activation of the IKK complex. IKK in turn activates the processing of p100 to the active form p52 (66, 67). RelB pairs with p52, and the dimer can then translocate to the nucleus and activate gene transcription.

Mice deficient in both p50 and p52 and thus totally lacking NF-κB signaling present with osteopetrosis due to lack of OC differentiation (68, 69). RelA knockout mice have significantly fewer OC in basal conditions due to increased apoptosis and are protected from inflammatory osteolysis (70). Similarly, RelB-/- mice fail to form OC when challenged with TNF-α in vivo, and RelB-/- precursors cannot undergo osteoclastogenesis in vitro (71). Thus, both the classical and alternative pathways are necessary for OC differentiation and function. While induction of the classical pathway occurs very rapidly and transiently within minutes of RANKL stimulation, the alternative pathway is turned on slowly over the course of hours and is sustained. Likewise, classical and alternative NF-κB signaling appear to regulate different processes during osteoclastogenesis. Classical NF-κB promotes OC precursor survival while alternative signaling contributes to differentiation.

**cFos/AP-1**

In addition to NF-κB, the transcriptional complex AP-1 is also essential to OC formation (Figure 1.5). During osteoclastogenesis, it appears that multiple signaling pathways synergize to
activate AP-1 induction, including NF-kB, calcium activation of CREB, and the MAP kinase component p38 (72, 73). Among the members of the AP-1 family, c-Fos is most important to the OC, as evidenced by the severe high bone mass and lack of OCs in the c-Fos -/- mouse (74). It is unclear why c-Fos is specifically important in the OC compared to other AP-1 proteins including Fra-1, JunB, or c-Jun, and which genes are uniquely targeted by c-Fos for osteoclastogenesis (75). Interestingly, it has recently been observed that c-Fos induction during osteoclastogenesis is more strongly dependent on M-CSF signaling compared to RANKL signaling cascades, and this pathway appears to be dependent on PLC\(\gamma\)-mediated generation of diacylglycerol (Zamani et al., in press).

**Co-stimulatory receptors in osteoclastogenesis**

The OC is wholly dependent on ongoing intracellular Ca\(^{2+}\) fluxes to induce and support differentiation. While Ca\(^{2+}\) fluxes during osteoclastogenesis are known to be compulsory to differentiation and function, these signals are not actually originated by RANK directly. It was previously known that in immune cells, phospholipase C gamma (PLC\(\gamma\)) generates Ca\(^{2+}\) fluxes downstream of co-stimulatory molecules (76). In the OC, co-stimulatory receptors are associated with adapter molecules, namely DAP12 and FcR\(\gamma\), containing an Immunoreceptor tyrosine-based activation motif (ITAM) which enables downstream signaling (77).

In the OC, DAP12 and FcR\(\gamma\) are ITAM-containing adaptors which appear to be essential to osteoclastogenesis. While the deletion of either of these genes alone does not result in a significant bone phenotype, double knock out mice present with severe osteopetrosis, suggesting that these co-stimulatory pathways may compensate for one another in vivo (78-81). ITAM-containing proteins do not bind ligand themselves but are coupled to receptors. In the OC, DAP12 couples to TREM2, TREM3, SIRb1, and MDL-1. Interestingly, patients with mutations
in either TREM2 or DAP12 suffer from recurring fractures and osteolytic lesions (82). Similarly, TREM2-/- mice show low bone mass due to accelerated OC formation in the face of dysregulated β-catenin (83). These studies suggest that TREM2 may be the most important co-stimulatory molecule in humans. The ligand for TREM2 is not yet known. On the other hand, FcRγ associates with the receptor OSCAR, which has recently been shown to bind collagen in the matrix (84).

During osteoclastogenesis, signaling at RANK cross-talks with the ITAM-containing adaptors to initiate downstream PLCγ-dependent signaling cascades (Figure 1.6). In the OC specifically, two Src family kinases, c-Src and Fyn, which are recruited to RANK, first phosphorylate the tyrosine residues of the ITAM domain. Deletion of c-Src results in cytoskeletal defects in the OC but not impaired differentiation, however, so it is currently unclear which Src-family kinases can activate ITAM signaling in the OC to modulate osteoclastogenesis. Following phosphorylation of the ITAM, the kinase Syk is recruited via its SH2 domain. Similarly to the phenotype of c-Src knock out cells, deletion of Syk disrupts the OC cytoskeleton but does not abrogate differentiation, suggesting that other kinases may be able carry out this step. Syk activation recruits the scaffolding proteins BLNK and SLP76, and this signalosome complex leads to eventual phosphorylation of PLCγ, turning on its catalytic activity.

As active lipases, PLCγ cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into the second messengers inositol-3-phosphate (IP3) and diacylglycerol (DAG). Through these pathways, PLCγ activates downstream Ca^{2+} fluxes and DAG-dependent signaling effectors including PKCs, respectively (85). There are two isoforms of PLCγ: PLCγ1 which is ubiquitously expressed and PLCγ2 which is expressed only in hematopoietic lineages. While PLCγ1-/- mice do not survive, PLCγ2-deficient mice are viable (86). These mice are
characterized by a severe osteopetrotic phenotype owing to defective OC differentiation. Surprisingly, although PLCγ1 is intact in these mice, PLCγ1 cannot compensate for the loss of PLCγ2. In vitro experiments revealed that impaired osteoclastogenesis in the absence of PLCγ2 is due to defective NFATc1 upregulation (87). Interestingly, overexpression of NFATc1 in PLCγ2-/- cells can rescue OC formation but not bone resorption (88). Further studies showed that DAG signaling to PKCd downstream of PLCg2 regulates Cathepsin K-containing vesicle secretion. DAG remains at the membrane and can activate multiple downstream effectors, including protein kinase C- delta (PKCδ) in the OC. This DAG-PKCδ pathway regulates secretion of Cathepsin K-containing vesicles in the mature OC (28).

**Ca^{2+} fluxes in osteoclastogenesis**

The OC is wholly dependent on sustained intracellular Ca^{2+} fluxes for differentiation (Figure 1.7). RANKL-induced activation of PLCγ2 leads to the generation of IP3, which diffuses through the cytosol and binds the IP3 receptor (IP3R) on the ER membrane. IP3Rs multimerize to form a Ca^{2+} channel, allowing release of Ca^{2+} from ER stores (89). In the OC, it appears that IP3R isoforms 1, 2, and 3 are expressed. The osteopetrotic phenotype of IP3R2/3 doubly deficient-mice suggest that these isoforms may be most important Ca^{2+} fluxes in the OC, however (90). Stim1 is an ER Ca^{2+} sensor that harbors an EF-hand Ca^{2+}-binding domain. When Ca^{2+} levels decline in the ER, Ca^{2+} is dissociated from Stim1, inducing a conformational change and its translocation within to ER-plasma membrane junctions (91-95). Here, Stim1 binds to and activates the protein Orai1 (96). Orai1 is the pore-forming subunit of the Ca^{2+} release activated Ca^{2+} (CRAC) channel. Extracellular Ca^{2+} enters the cell’s cytoplasm through the CRAC channel (97-99). ER Ca^{2+} stores are replenished via active transport of Ca^{2+} by the Sarco-endoplasmic reticulum Ca^{2+} ATPase (100, 101). Through these mechanisms, intracellular Ca^{2+} fluxes are
sustained in the OC. Genetic deletion of Orai1 or SERCA2, as well as pharmacological inhibition of Stim1, each abolish Ca^{2+} fluxes and OC differentiation (101-103).

*Nfatc1 as the OC master transcription factor*

Following RANKL stimulation, NF-kB, AP-1, and Ca^{2+} signaling pathways all contribute to the induction of NFATc1, a member of the family of NFAT transcription factors. NFAT proteins are sequestered in the cytosol in basal conditions due to masking of their nuclear localization sequence. De-phosphorylation by the calcium-dependent phosphatase calcineurin results in a conformational change to allow NFAT nuclear translocation (104). Within the nucleus, NFAT is also regulated by phosphorylation by a number of kinases including GSK3β (105, 106). This phosphorylation induces NFAT nuclear export to the cytosol. Interestingly, NFATc1 can also bind to its own promoter to auto-amplify its transcription and protein expression (107).

The importance of NFATs during osteoclastogenesis is evidenced by both pharmacological and genetic studies. Cyclosporin A, an inhibitor of calcineurin and therefore NFAT, strongly suppresses osteoclastogenesis in vitro. Although NFATc1-/- mice are embryonic lethal, NFATc1-null embryonic stem cells are unable to undergo osteoclastogenesis (108). More recently, conditional deletion of NFATc1 via Mx1-cre was demonstrated to increase bone mass in mice by inhibiting OC formation (109). NFATc1 has been deemed the “master transcription factor” of osteoclastogenesis, as it directly regulates OC-specific genes including TRAP, calcitonin receptor, Cathepsin K, and β3 integrin (107, 110, 111).

NFATc1 is regulated by several transcriptional repressors that control its activation. Interferon regulator factor-8 (IRF8) and Bcl6 are constitutively bound to the NFATc1 promoter, and their expression is down-regulated within 24 hours of RANKL stimulation (112-115).
Interestingly, Bcl6 is down-regulated via the RANKL-dependent induction of its repressor Blimp1 (114). Thus, RANKL-induced alleviation of transcriptional repression allows robust NFATc1 activation and auto-amplification.

**Part III. Osteo-immune activation in arthritis**

During pathological bone loss as occurs in arthritic conditions, both the bone and immune cells are activated. This osteo-immune cross-talk has become increasingly recognized as a driver of debilitating joint erosion. Both the innate and adaptive immune cells participate in this osteo-immune activation. In the state of an inflammatory trigger, innate immune cells, namely neutrophils, mast cells, and macrophages, are rapidly recruited to the joint. Importantly, multiple signaling pathways, including NF-kB, MAPK, and PLCγ2/Ca2+/NFAT, are shared in the response of both bone and immune cells and both to inflammatory cytokines (116).

**Inflammatory cytokines fuel bone loss**

Arthritic patients show high levels of inflammatory cytokines both in circulation and locally in the joint. TNF-α and IL-1 in particular upregulate RANKL expression by OBs and stromal cells, thereby increasing osteoclastogenesis (57, 117, 118). Moreover, these cytokines can also act directly on the OC precursor and synergize with RANKL to amplify OC differentiation (119-122). Both anti-TNF and IL-1 receptor antagonist are used as treatments in arthritic conditions, although not all patients are responsive to cytokine inhibition. The cytokine IL-17, produced by Th17 cells, is also believed to be pathogenic in psoriatic arthritis by increasing the production of RANKL by T cells and B cells (123).

The role of IL-6 and IFN-γ during pathological bone loss is less clear. IL-6 deficient mice show no OC phenotype in basal conditions but are protected from ovariectomy-induced bone loss (124, 125). Similarly, increased IL-6 levels have been observed in post-menoposal women,
suggesting that this cytokine may specifically contribute to estrogen-deficient bone loss (126). The role of IFN-γ in bone is also unclear. In mice but not humans, IFN-γ inhibits osteoclastogenesis in vivo in arthritic conditions (127). However, IFN-γ positively regulates macrophage activation and consequent inflammatory cytokine production (128), and therefore could indirectly increase osteoclastogenesis.

**Immune cells in bone**

Both adaptive and innate immune responses contribute to a pro-resorptive environment in the joint space. Adaptive immune responses by T cells and B cells through the recognition of specific auto-antigens and production of auto-antibodies can also contribute to uncontrolled immune responses. T cells produce IFN-γ which amplifies the innate pro-inflammatory response as well as antigen presentation (129). Additionally, T cells can secrete RANKL to activate OCs (130). B cells also have more recently been proposed to produce RANKL themselves (131, 132). Through these pathways, the immune response fuels OC over-activity and also amplifies the innate immune response, allowing progressive joint destruction.

Innate immune cells, including neutrophils, mast cells, and monocytes/macrophages, are the first cells to infiltrate the joint during arthritic conditions. Through the production of inflammatory cytokines, reactive oxygen species, and release of proteolytic granules, these cells induce local tissue damage, recruit more immune cells to the joint, and activate osteoclastogenesis (133). Unlike the adaptive immune cells, which edit their receptors to recognize specific antigens and can develop immunological memory, the innate response is dependent on fixed pattern recognition receptors (PRRs) which are fixed to identify discreet pathogen- associated molecular patterns (PAMPs) and elicit a rapid response, as well as Fc receptors which bind the Fc fragment of antibodies. In the context of inflammatory bone loss,
innate immune cells are activated via Toll-like receptor (TLR) ligands (including bacterial, viral, and fungal components as well as endogenous TLR ligands), complement deposition, and immune complex formation(134-136). In animal models of inflammatory arthritis, the depletion or inhibition of neutrophils or macrophages alone is sufficient to protect the joint from infiltrating leukocytes and bone loss, underscoring the importance of these cells types in arthritic disease (137-139). Natural killer (NK) cells are innate cytotoxic cells that are capable of directly killing infected cells by the release of granules containing perforin and proteases. NK cells are found in the synovial fluid of rheumatoid arthritis patients and also express both M-CSF and RANKL, thereby supporting osteoclastogenesis from monocytes in the joint (140). Lastly, dendritic cells (DCs) are the bridge between the innate and adaptive response. DCs take up antigen in the periphery and then migrate to the lymph nodes where they present antigen to T-cells, thus activating an antigen-specific response (141). DCs are present in the synovial fluid of RA patients where they appear to promote T cell-inflammatory responses, and DC migration and antigen presentation are wholly required in animal models of antigen-induced arthritis (142, 143). While much more work is ongoing to understand the contribution of each of these cell types to arthritic diseases, this thesis will focus on the monocyte/macrophage lineage for both its direct contribution to the OC lineage and pro-inflammatory functions.

**Macrophage development and function**

Tissue-resident macrophages are professional antigen presenting cells which maintain homeostasis in physiological conditions, rapidly respond to an immunological challenge, and mediate tissue repair and restoration. From the hematopoietic stem cell (HSC), there are 4 known intermediates leading to macrophage differentiation: common myeloid progenitors (CMP), granulocyte and macrophage progenitors (GMP), macrophage and dendritic cell progenitors
(MDP), and finally the common monocyte progenitors (CMoP). Bone marrow and splenic red pulp macrophages are believed to actually be established before birth and are independently maintained. On the other hand, splenic marginal zone macrophages are continually renewed by differentiating monocytes in circulation (144). In the context of arthritis, synovial macrophages and inflammatory monocytes are derived from the circulating population, whose numbers are increased in arthritic patients (145).

In tissues, macrophages are highly phagocytic cells that are on watch to engulf foreign material, cellular debris, as well as microbial and viral particles. The engulfed material is housed in a phagosome which fuses with a lysosome of low pH, allowing digestion in the phagolysosome. Through this pathway, macrophages are highly efficient at clearing unwanted matter from the tissue to prevent or control an infection before the adaptive immune response is mounted. As professional APCs, macrophages also express both MHC Class I and Class II molecules to present endocytosed and processed antigen to CD8+ and CD4+ T cells, respectively, thus stimulating a specific adaptive immune response (144, 146).

Macrophages can further dictate the immune response by producing a number of cytokines, both pro- and anti-inflammatory. In the past, macrophages were segregated into M1 and M2 subtypes, reflecting their contributing roles to T helper cell 1 and T-helper cell 2 responses, respectively. In these classifications, M1 macrophages are generated by exposure to IFN-γ in combination with the bacterial cell wall component lipopolysaccharide (LPS) or TNF. M1 macrophages are strongly pro-inflammatory and anti-tumorigenic through the production of IL-1, TNF, IL-6, and iNOS, as well as enhanced MHC Class II antigen presentation capacity. On the other hand, M2 macrophages result from stimulation with some combination of IL-4, IL-13, or IL-10. Stimulation with these cytokines upregulates production of arginase and IL-10 and
increases expression of the scavenger receptor CD206. These M2 macrophages participate in allergic and anti-parasitic responses, immunoregulation, and perhaps tumor promotion (146).

_Macrophages in rheumatic disease_

Monocyte and macrophage numbers are elevated in arthritic patients and can contribute to disease both through their inflammatory actions and their differentiation into bona fide bone-resorbing OCs. One model disease in which this monocyte/macrophage population is playing an essential yet enigmatic role is systemic juvenile idiopathic arthritis (sJIA). sJIA is an episodic auto-inflammatory disease that, unlike other arthritic subtypes, is primarily driven by innate immune cells. Expanded monocyte numbers, particularly during episodes of disease flare, are observed in sJIA patients. The trigger of this excessive monocyte response is unknown. These monocytes may be activated by an infectious stimuli or endogenous TLR ligands inducing a pro-inflammatory response. Intriguingly, M2-like monocytes appear during episodes of disease flare and persist into the transition to a quiescent state, suggesting that this M2 population arises in an attempt to ameliorate the systemic inflammatory environment (147-151). In fact, it is still unknown whether the excessive pro-inflammatory response or defective anti-inflammatory response truly underlies the pathologies of sJIA. Thus, a better understanding of the signaling pathways which modulate the plasticity of the monocyte/macrophage lineage and potentiate a switch to an anti-inflammatory response would be highly applicable to sJIA, in addition to numerous macrophage-mediated pathologies.

Approximately 10% of sJIA patients develop macrophage activation syndrome (MAS), which is responsible for the majority of the morbidities associated with sJIA. The onset of MAS is sudden, and patients present with high fever, hepatosplenomegaly, lymphadenopathy, cutaneous and mucosal bleeding, pancytopenia, as well as central nervous system, cardiac, and
renal involvement. Patients have very high levels of inflammatory cytokines, TNF-α, IL-1, IL-6, IL-18, as well as IFN-γ in the serum. Importantly, MAS is so-named due to a massive influx of activated macrophages in the bone marrow, liver and spleen. It is actually unknown if this increase in macrophages is driving pathogenesis or is an attempt to control a dysregulated adaptive immune response (152). The sJIA/MAS field is currently lacking a clear picture of how the activated monocyte/macrophage population functions at both a cellular and molecular level during the onset and course of disease.

**Part IV. PLCγ2 as an osteo-immune modulator**

**PLCγ structure and function**

PLCγ is a 150kDa protein containing both an active lipase domain as well as several scaffolding motifs to facilitate protein interactions. The catalytic domain is split into 2 hemi domains that are separated and thus non-functional in the resting state. PLCγ phosphorylation downstream of receptor activation induces a conformational change to bring together the split catalytic domain. The scaffolding domains of PLCγ include tandem SH2 domains, an N-terminal PH domain, a split PH domain, and an SH3 domain. The split PH domain comes together in PLCγ’s active conformation, but its exact role is unknown. The N-terminal PH domain is responsible for PLCγ membrane and substrate targeting by binding directly to PIP2. The tandem SH2 domains appear to be necessary for protein scaffolding at the receptor complex by binding to phosphorylated tyrosine motifs, while the SH3 domain recognizes the conserved PxxP motif on binding partners. Finally PLCγ also harbors a C2 calcium-binding domain at its C-terminus which also participates in membrane binding (85).
**PLC\(\gamma\)2 Signaling in Osteo-Immunology**

In order to better target both the overactive immune and OC responses underlying inflammatory osteolysis and arthritis, it is necessary to identify common signaling components shared by these systems. PLC\(\gamma\)2 is such a critical mediator of the osteoimmune system owing to its requirement to both innate immune response and OC formation and function. PLC\(\gamma\)2-/- mice are osteopetrotic due to a defect in both OC formation as well as function (28, 87). Interestingly, both the enzymatic and scaffolding functions of PLC\(\gamma\)2 play distinct yet essential roles in promoting osteoclastogenesis (87). While PLC\(\gamma\)2’s catalytic activity is necessary to stimulate Ca\(^{2+}\) fluxes leading to NFATc1 activation, its scaffolding domains are required for NF-kB and AP1 induction.

In addition to a pronounced bone phenotype, PLC\(\gamma\)2-/- mice exhibit defects in neutrophil, macrophage, dendritic cell, and NK cell responses. More specifically, PLC\(\gamma\)2 deletion prevents the induction of IL-1, TNF-\(\alpha\), and IL-6 by macrophages stimulated by TLR2 and TLR4 ligands (153, 154). Importantly, PLC\(\gamma\)2-/- mice are protected from inflammatory cytokine production in the K/BXN serum transfer model of arthritis, specifically due to impaired inflammatory cytokine expression (155). While PLC\(\gamma\)2 seems an appealing therapeutic target, its extensive homology with other PLC isoforms presents a challenge for specific targeting in vivo. Therefore, we must focus on developing novel methods to therapeutically target PLC\(\gamma\)2 and its downstream signaling pathways. In this thesis, I demonstrate a novel method of targeting PLC\(\gamma\)2 via its unique SH2 domains to protect from osteolysis. In chapters 3 and 4, I describe a novel PLC\(\gamma\)2-dependent protein, Tmem178, and its surprising role in the regulation of inflammatory bone loss and macrophage activation.
Figure 1.1 Osteoclast differentiation from myeloid precursors
Figure 1.2 Osteoclast-osteoblast coupling in bone remodeling
Figure 1.3 Protein scaffolding at RANK initiates NF-κB and MAPK cascades
Figure 1.4 Classical and alternative NF-κB pathways
Figure 1.5 RANK activates MAPK signaling to induce cFos and the AP1 transcriptional complex
Figure 1.6 RANK cross-talks with ITAM-coupled receptors to activate PLCγ2
Figure 1.7 PLCγ2 activates ER Ca\(^{2+}\) release coupled to Ca\(^{2+}\) entry, leading to cytosolic Ca\(^{2+}\) fluxes driving NFATc1 activation.
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Chapter 2. PLC\(\gamma\)2 Domain Targeting As a Novel Approach to Osteoclast Inhibition
2.1 Targeted inhibition of phospholipase C gamma 2 adaptor function blocks osteoclastogenesis and protects from pathological osteolysis

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2.2 Abstract

Phospholipase C gamma 2 (PLCγ2) is a critical regulator of innate immune cells and osteoclasts (OCs) during inflammatory arthritis. Both the catalytic domain and the adaptor motifs of PLCγ2 are required for OC formation and function. Due to the high homology between the catalytic domains of PLCγ2 and the ubiquitously expressed PLCγ1, molecules encompassing the adaptor motifs of PLCγ2 were designed to test the hypothesis that uncoupling the adaptor and catalytic functions of PLCγ2 could specifically inhibit osteoclastogenesis (OCG) and bone erosion. Wild-type (WT) bone-marrow macrophages (BMM) that over-express the tandem SH2 domains of PLCγ2 (SH2(N+C)) failed to form mature OCs and resorb bone in vitro. Activation of the receptor activator of NF-κB (RANK) signaling pathway, which is critical for OC development, was impaired in cells expressing SH2(N+C). Arrest in OC differentiation was evidenced by a reduction of p38 and Iκ-Bα phosphorylation as well as decreased NFATc1 and cFos/cJun levels. Consistent with our hypothesis, SH2(N+C) abrogated formation of the RANK/Gab2 complex which mediates NF-κB and AP-1 activation following RANK ligand (RANKL) stimulation. Furthermore, the ability of SH2(N+C) to prevent inflammatory osteolysis was examined in vivo following RANKL or LPS injections over the calvaria. Both models induced osteolysis in the control group, whereas the SH2(N+C)-treated cohort was largely protected from bone erosion. Collectively, these data indicate that inflammatory osteolysis can be abrogated by treatment with a molecule composed of the tandem SH2 domains of PLCγ2.
2.3 Introduction

Pathological bone loss is a debilitating complication associated with prosthetic implants, osteoarthritis, rheumatoid arthritis, and periodontal disease (1) and results from an increase in the number and/or function of the bone-resorbing OC (2). OCs are differentiated from BMM precursors in the presence of RANKL and macrophage colony-stimulating factor (M-CSF), which bind RANK and c-Fms, respectively (3). Pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, which are produced at inflamed joints, further promote OCG and resorptive activity (4). Such crosstalk between immune cells and OCs highlights the need for a therapeutic treatment capable of reducing inflammation and preventing bone erosion.

PLCγ1 and 2 molecules are critical regulators of innate and adaptive immune responses and are activated during OC differentiation. These enzymes convert phosphatidylinositol 4,5 bisphosphate (PIP2) into two second messengers, inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG), leading to activation of IP3/calcium and DAG-dependent pathways downstream of RANK, integrins, and a variety of immune receptors (5). For example, in vitro studies of T lymphocytes demonstrate that PLCγ1 is a critical modulator of T cell receptor responses (6-8). However, PLCγ1 is ubiquitously expressed, and its global deletion leads to early embryonic lethality in the mouse (9). Thus, an approach to inhibit PLCγ1 function is likely to have broad off-target effects. PLCγ2 expression is confined to cells of hematopoietic lineage, including B lymphocytes, natural killer cells, mast cells, neutrophils, dendritic cells, and OCs (10-14). Plcγ2-/- mice have increased bone mass (osteopetrosis) due to reduced differentiation of BMMs into OCs (15). Additionally, Plcγ2-/- mice are protected from serum-transfer induced arthritis and antigen-induced arthritis due to functional defects in neutrophils and dendritic cells, respectively (13,14). Therefore, PLCγ2 is a promising target for therapeutic
design in the context of inflammatory osteolysis. However, due to its high homology with the more ubiquitously expressed PLCγ1, it is necessary to find inhibitors which can selectively block PLCγ2 activation.

PLCγ1 and PLCγ2 are unique from other PLC molecules because they contain two tandem SH2 and an SH3 adaptor motifs. Initial studies suggested that these adaptor motifs harbor an intrinsic regulatory function of the protein’s catalytic activity (16), exposing the catalytic domain upon PLCγ1/β phosphorylation (17). However, it is now established that these SH2 and SH3 motifs can also mediate protein-protein binding via homodomain interactions (17). Considering that the catalytic domains of PLCγ1 and PLCγ2 are over 90% homologous, while their adaptor motifs are less than 60% homologous, the latter regions are a potential target for specific inhibition.

The scaffolding function of PLCγ2, in addition to its catalytic activity, is required for OC formation. Cells with a point mutation in the SH2 domain of PLCγ2 are incapable of in vitro OCG despite intact catalytic function (18). Thus, we hypothesized that the scaffolding function of endogenous PLCγ2 could be disrupted through a dominant negative effect by a molecule encompassing the adaptor domains of PLCγ2. We report that a molecule composed of the tandem SH2 motifs of PLCγ2 is able to abrogate OCG in vitro and in vivo by disrupting protein interactions between RANK and Gab2. This approach may represent a novel method of targeting PLCγ2 to prevent inflammatory bone loss.
2.4 Materials and Methods

Plasmids and retrovirus generation

The SH2 or SH3 domains of PLCγ2 were cloned into the blasticidin-resistant pMX retroviral vector and fused with HA. To generate retrovirus, PLAT-E cells were transfected with expression vector by using a TransIT transfection reagent (Mirus Bio). Viral supernatants were collected on day 2 and day 3 post-transfection and immediately used to transduce freshly isolated BMMs. After 24 h, medium containing 1 μg/ml blasticidin was added to cells for 48 h to select for expressing cells.

Primary cell culture

Bone marrow was isolated from long bones of 6- to 8-week-old C57BL/6 mice and cultured in alpha-MEM containing 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and glutamine (α-10 medium), with 1/10 vol CMG14-12 cell-conditioned medium as a source of macrophage colony-stimulating factor (M-CSF) (19) to obtain BMMs. To form OCs, BMMs were cultured in α-10 medium with 100 ng/mL glutathione S-transferase–receptor activator of NF-κB ligand (GST-RANKL) and 1/100 vol CMG14-12 for 5 days. Cells were fixed in 4% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRAP) using a commercial kit (Sigma 387-A).

Real-time PCR

BMMs were cultured with 100 ng/mL RANKL and 1/100 vol CMG14-12 for 0, 2, or 4 days. Adherent cells were harvested in Trizol. Total RNA was isolated by chloroform extraction followed by purification with the RNeasy Mini Kit (Qiagen). 1 μg of total RNA was reverse
transcribed to cDNA using SuperScript II according to the manufacturer’s instructions (Invitrogen). For real-time PCR, SYBR Green PCR Master Mix (Applied Biosystems) and primers specific for murine NFATc1, TRAP, cathepsin K, calcitonin receptor, and GAPDH, were used as follows: NFATc1, 5′-CCGTCACATTCTGGTCCAT-3′ and 5′-CAAGTAACCGTGTAGCTGCACAA-3′; TRAP, 5′-CAGCTCCCTAGAAGATGGATTCAT-3′ and 5′-GTCAGGAGTGAGCCATATG-3′; cathepsin K, 5′-ATGTGGGTGTTCAAGTCTGC-3′ and 5′-CCACAAGATTCTGGGACTC-3′; calcitonin receptor, 5′-CAAGAACCTTAGCTGCCAGAG-3′ and 5′-CAAGACCGCAGAATGTTG-3′; GAPDH 5′-TGTGTCCGTCGTGGATCTGA-3′ and 5′-CCTGCTTCACCACCTTCTTGA-3′). Ct values were normalized to GAPDH internal control. Data are expressed as the relative fold change compared to the expression in BMMs transduced with pMX empty vector control at day 0.

**Bone Resorption**

Analysis of bone resorption was completed as described previously (18). Briefly, BMMs were plated on bovine bone slices and cultured with 1/100 CMG14-12 and 100 ng/ml GST-RANKL for 10 days. Fresh medium was added every 2 days. Cells were removed from the bone surface by using mechanical force and 2 N NaOH. Bone slices were stained with 20 μg/ml peroxidase-conjugated wheat germ agglutinin for 30 min (Sigma) followed by 3,3′-diaminobenzidine (0.52 mg/ml in PBS containing 0.1% H2O2) for 15 min. Bone resorption pits were visualized with a light microscope and quantified using Image J software (NIH; http://rsbweb.nih.gov/ij).
**Immunoprecipitation**

Cells were harvested in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 10% glycerol) supplemented with protease inhibitors, and clarified by centrifugation. The protein concentration of each sample was determined using bicinchoninic acid protein assay (Biorad) and 1 mg of protein from each sample was used for IP. Samples were incubated with anti-PLCγ2 (Santa Cruz Biotechnology) or anti-Gab2 antibody (Millipore) overnight at 4°C and with protein G agarose beads (Amersham) for 3 h at 4°C. Beads were washed three times in lysis buffer and immunoprecipitates were used for western blotting.

**RANKL, M-CSF, and vitronectin stimulation**

For RANKL and M-CSF stimulation, pre-OCs were starved for 4 hours in α-MEM containing 2% FBS and then stimulated with RANKL (100 ng/ml) or M-CSF (100 ng/ml) in α-MEM for the indicated times. Cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with HALT protease and phosphatase inhibitor cocktail (Pierce). To obtain nuclear extracts from RANKL-treated cells, tissue-culture plates were washed with H2O and the adherent cells were lysed with hypotonic buffer (10 mM HEPES, 1.5 mM MgCl2, 1 mM KCl, 1 mM DTT, and protease and phosphatase inhibitors), followed by addition of 0.1% NP40. After centrifugation, the supernatants were collected (cytosolic fraction), while the pellets (nuclear fraction) were suspended in high-salt buffer (hypotonic buffer plus 400 mM NaCl). For vitronectin stimulation, pre-OCs were washed with PBS and lifted with 10% trypsin/EDTA in PBS. Cells were replated on tissue-culture plates coated with vitronectin for the indicated length of time and lysed in RIPA lysis buffer supplemented HALT protease and phosphatase inhibitor cocktail.
Western blot analysis and antibodies

The protein concentration of cell lysates or nuclear extracts was determined using bicinchoninic acid protein assay (Biorad). An equivalent amount of protein for each sample was mixed with 5x loading buffer (312.5mM Tris pH 6.8, 10% SDS, 50% glycerol, 0.05% bromophenol blue, 10% β-mercaptoethanol), separated by SDS-PAGE and transferred to PVDF membranes. Membranes were probed with antibodies as indicated. Antibodies specific to the phosphorylated forms of PLCγ1, PLCγ2, ERK, JNK, c-Jun, IκBα, and Src (Y416) were purchased from Cell Signaling Technology. Antibodies against p65, NFATc1, PLCγ2, TRAF6, RANK, Lamin B, and Sp1 were purchased from Santa Cruz Biotechnology. The monoclonal antibody against β-actin was purchased from Sigma-Aldrich. Polyclonal Gab2 Ab was purchased from Millipore.

In vivo osteolysis models

WT C57Bl/6 mice were treated with 100 μg of RANKL and 107 PFU of adenovirus containing LacZ (Ad-LacZ control) or SH2(N+C) (Ad-SH2(N+C)) by subcutaneous injections over the calvaria daily for 5 days. Mice were sacrificed on day 6 and the calvaria were collected for histological analysis. The calvaria were preserved in 10% buffered formalin overnight 24 h and decalcified with 14% EDTA for 4 days. Paraffin-embedded sections were stained for tartrate-resistant acid phosphatase (TRAP) to visualize OCs. WT C57Bl/6 mice were treated with 30 μg LPS and 107 PFU of adenovirus containing Ad-LacZ or Ad-SH2(N+C) by subcutaneous injections over the calvaria at day 0, followed by 107 PFU of Ad-LacZ or Ad-SH2(N+C) on day 1, 3, and 5. Mice were sacrificed on day 6, and the calvaria were processed for histological analysis as described above. Paraffin-embedded sections were stained for TRAP to visualize OCs (Sigma).
All mice used in these experiments were housed in the animal care unit of Washington University School of Medicine, where they were maintained according to guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experimentation was approved by the Animal Studies Committee of Washington University School of Medicine.
2.5 Results

**SH2(N+C) blocks OCG in vitro**

To design an efficient strategy to specifically oppose endogenous PLCγ2 activity in the context of pathological bone loss, we focused on the protein modular domains, as the catalytic motif is highly homologous to PLCγ1. Retroviral constructs harboring the N- and C-terminal SH2 domains alone or in combination (N+C), the SH3 domain, or encompassing both SH2 and SH3 regions were generated (Figure 2.1 A). These constructs were transduced into WT BMMs that also express endogenous PLCγ2. Expression of the indicated domains was confirmed by western blot (Figure 2.1 B). BMMs were treated with RANKL and M-CSF, and differentiation to mature OCs was evaluated by TRAP staining. RANKL and M-CSF induced differentiation of precursor cells into OCs in all groups, except in the presence of the SH2(N+C) and partially in SH2(N+C)+SH3 expressing cells. Ectopic expression of SH2(N+C) significantly impaired OC development (# of TRAP+ cells in SH2(N+C), 1.3±0.88 versus empty vector, 153.3±8.99, p < 0.0001). A 52% inhibition of OCG was observed in WT cells expressing SH2(N+C)+SH3, while the differentiation of cells expressing a single SH2 (either SH2-N or SH2-C) or the SH3 motif alone was similar to cells infected with the empty vector (pMX; Figure 2.1 C-D).

Consistent with its inhibitory effect on OC formation, BMMs that were transduced with SH2(N+C) and grown on cow bone slices in the presence of RANKL and M-CSF for 7 days, formed fewer resorptive pits compared to pMX-transduced controls (Figure 2.2).

To further understand which stage of OC differentiation was blocked by the expression of SH2(N+C), real time PCR was used to quantify expression of the early osteoclastogenic marker TRAP and the master transcription regulator of OCG NFATc1, the functional OC protease cathepsin K, and the calcitonin receptor expressed by mature OCs (Figure 2.3). WT cells
transduced with empty vector alone showed an upregulation for all conventional markers of OCG during four days of culture in the presence of RANKL and M-CSF. In contrast, expression of OC differentiation markers was dramatically reduced in WT cells expressing SH2(N+C), indicating that this molecule targets signaling pathways involved early in the OC differentiation process.

*RANK signaling cascade is inhibited by SH2(N+C) in vitro*

In order to understand how SH2(N+C) inhibits OCG in vitro, we analyzed activation of signaling pathways downstream of RANK/RANKL stimulation, M-CSF/c-Fms stimulation, or vitronectin/alpha-v beta-3 (αVβ3) integrin ligation. WT day 2 pre-OCs expressing SH2(N+C) or vector control were stimulated with 100ng/ml RANKL for 0 to 60 minutes, as indicated. Pre-OCs expressing SH2(N+C) failed to activate downstream RANK signals, as assayed by Western blot for phosphorylation of PLCγ2, IKBa and p38 and upregulation of cFos and NFATc1 (Figure 4A). Activation of JNK was unaffected (not shown). Furthermore, nuclear translocation of NFATc1 and phosphorylated c-Jun was inhibited by SH2(N+C) expression (Figure 4B). In contrast, when pre-OCs were stimulated with 100ng/ml of M-CSF over the indicated time course, cells expressing SH2(N+C) induced phosphorylation of the downstream molecules AKT and ERK equivalent to cells expressing the vector alone (Figure 2.4 C). Phosphorylation of Pyk2, c-Src, and IkBα was decreased in SH2(N+C)-expressing pre-OCs plated on vitronectin (Figure 4D). All together, these results indicate that SH2(N+C) primarily inhibits the RANKL- and integrin-mediated signaling pathways which are integral to OC differentiation.
**SH2(N+C) blocks association of Gab2 with PLCγ2 and RANK**

In order to understand the mechanism by which SH2(N+C) interferes with the RANKL signaling cascade, a co-immunoprecipitation assay of cell lysates from RANKL-stimulated pre-OCs was preformed. Following RANKL binding to its receptor RANK, the signaling adaptor Gab2 is recruited to the RANK complex (20). Gab2 associates with PLCγ2 to stimulate phosphorylation of Ik-Bα leading to OCG (15,20). SH2(N+C) dampened the association of PLCγ2 with Gab2 compared to pre-OC expressing vector only (Figure 2.5 A). Furthermore, SH2(N+C) also impaired the association of RANK with Gab2 but did not effect recruitment of TRAF6 to Gab2 (Figure 2.5 B). Thus, SH2(N+C) inhibits RANK signaling cascades by impeding protein scaffolding at the receptor.

**SH2(N+C)blocks OC formation in vivo**

Finally, the potential efficacy of targeting PLCγ2 adaptor motifs to inhibit bone erosion was examined in vivo, using two established models of OCG. To facilitate these studies, adenoviral vectors were generated to express SH2(N+C) or beta galactosidase (LacZ) as a control. LacZ expression after one intra-articular injection of 107 PFU was confirmed by X-gal staining (data not shown). In the first model, 100 μg of RANKL and 107 PFU of Ad-SH2(N+C) or Ad-LacZ were administered to WT mice by subcutaneous injections over the calvaria daily for 5 days. Mice were sacrificed on day 6, and calvaria were prepared for histological examination to assess the number of TRAP+ OCs. WT mice receiving SH2(N+C) adenovirus displayed significantly fewer OCs compared to LacZ-expressing controls (Figure 2.6 A). Furthermore, the effect of SH2(N+C) in the context of inflammatory bone loss was examined using an established model of OCG initiated by a single LPS injection over the calvaria. 30 μg of LPS and 107 PFU
of SH2(N+C) or control LacZ adenovirus were injected over the calvaria at day 0, followed by 107 PFU of SH2(N+C) or control LacZ adenovirus every other day for five days. Mice were sacrificed on day 6, and calvaria were examined by histology. WT mice receiving SH2(N+C) adenovirus developed half the number of TRAP-positive OC compared to LacZ-expressing controls (Figure 6B). These results indicate that PLCγ2 can be specifically targeted in vivo by a dominant-negative effect of the SH2(N+C), and that this approach can abrogate inflammatory-induced OC formation.
2.6 Discussion

Inhibitors that block the catalytic activity of PLC proteins, such as ET-18-OCH3 and U73122, have been used to establish the function of PLCs and the importance of PIP2 hydrolysis into IP3 and DAG in various cellular contexts. However, the major limitation of these inhibitors is lack of specificity. Using a genetic model consisting of Plcγ2-deficient mice, we previously showed that PLCγ2 is required for the maintenance of basal bone mass and for protection from inflammatory arthritis (13-15). Thus, the goal of the current study was to identify a specific approach to target endogenous PLCγ2 function. We report that ectopic expression of the tandem SH2 domains of PLCγ2 phenocopies the intrinsic OC deficit of Plcγ2-/- mice (15) by disrupting the protein association between RANK and Gab2. This approach is a novel method of targeting endogenous PLCγ2 function to negate pathological bone loss.

PLCγ2 is important for both OC differentiation and bone resorption. We previously proposed that in the context of OCG, PLCγ2 serves a dual purpose as both an active lipase, leading to expression of the master osteoclastogenic transcription factor NFATc1, and as a critical scaffolding molecule facilitating NF-κB and AP-1 induction. Point mutations in the SH2 domains of PLCγ2 (R564K/R672K) render BMMs unable to differentiate to OCs, despite intact catalytic activity (18). Here, we show that the overall effect of the SH2(N+C) was inhibition of the RANK signaling cascade and suppression of OCG. Our observations support an independent scaffolding function of PLCγ2 in which the SH2 motifs of PLCγ2 mediate the association between RANK and Gab2. Our results suggest that ectopic expression of SH2(N+C) exerts a dominant negative effect on formation of this complex by sequestering Gab2 and thus impairing its binding to the receptor RANK. These observations are consistent with modeling of intracellular RANK receptor interactions (21,22). During the first hour of RANKL stimulation
(early activation phase), PLCγ2 is recruited to RANK, and Gab2 binds weakly to the recruited PLCγ2 without interacting directly with RANK (21). Following prolonged RANKL stimulation (beyond 24 h) Gab2 may bind to the HCR region of RANK and recruit TRAF6 (21,22). Thus, it is hypothesized that the scaffolding function of PLCγ2 is critical in the early phase, whereas its catalytic activity may modulate signaling during the early and late phases of RANKL stimulation (21,22). Importantly, concurrent exposure to the SH2(N+C) and RANKL can limit OCG in vitro and in vivo.

Gab2 belongs to a family of adaptor molecules that mediate signals downstream of a diverse set of receptors for molecules such as growth factors, antigens, cytokines, TLR agonists, and the RANK receptor. Gab2-deficient mice exhibit impaired OCG and a net increase in bone mass. Gab2 is also necessary for the differentiation of human progenitor cells into OCs (20). In vitro, BMMs from Gab2-deficient mice do not form mature OCs due to an inherent defect that correlates with reduced RANKL-mediated activation of NF-kB, Akt, and JNK. Similarly, ectopic expression of SH2 (N+C) primarily affects RANKL signaling in OCs, without altering downstream signals from the MCSF receptor, thus implying a good level of specificity for this type of targeted approach. Since a Gab2/PLCγ2 complex is likely to form in response to various stimuli, inhibitory effects of the SH2(N+C) in other contexts are possible and warrant further investigation.

In contrast to the SH2(N+C), the anti-osteoclastic effects of molecules encompassing each single SH2 domain or the entire adaptor motif of PLCγ2, consisting of the two SH2 domains along with the SH3 motif, were very minimal. It is likely that targeting only one SH2 domain is not sufficient to sequester adaptor molecules from forming a complex with RANK. However, it was surprising to find that a larger fragment encompassing the entire adaptor region
of PLCγ2 had less effect than the SH2(N+C) molecule. Initial models of PLCγ1, and perhaps PLCγ2, function suggested that, at the 3D structural level, the entire adaptor region (SH2(N+C)+SH3) forms a cap that regulates exposure of the catalytic domain to its substrate (17). Indeed, overexpression of SH2(N+C)+SH3 was shown to block phospholipase activity of cell lysates in vitro (16). Similarly, this region exerted a dominant-negative effect to reduce PLCγ1 enzyme activity in prostate tumor cells and resulted in reduced tumor invasiveness (23). While previous reports clearly demonstrate that the entire adaptor region modulates catalytic activity, they do not clarify whether or not this region also mediates protein-protein binding. In the OCs, the incomplete inhibition of OCG by SH2(N+C)+SH3 supports the hypothesis that SH2(N+C)+SH3 is not fully capable of blocking PLCγ2 catalytic activity.

SH2(N+C) anti-osteoclastogenic effects are also observed in vivo using two models of bone loss, consisting of RANKL or LPS supracalvarial injections. While RANKL directly stimulates the differentiation of monocytes into OCs, LPS-induced bone loss is secondary to immune activation. In vivo immune phenotypes caused by genetic alterations of Plcγ2 have been described. We and others have shown that PLCγ2 regulates inflammation and autoimmunity, specifically within the bone microenvironment. Plcγ2/- mice are protected from immune activation in serum-transfer arthritis and antigen-induced arthritis (13,14). In humans, a dominantly-inherited disease called PLAID (PLCγ2-associated antibody deficiency and immune dysregulation) is caused by mutant alleles of PLCγ2 in which small, in-frame genomic deletions eliminate part or all of the C-terminal SH2 domain of PLCγ2. Immune cells from these patients displayed functional deregulation, with increased activation in response to some stimuli and decreased activation in other circumstances (12). Although we did not directly test the effects of SH2(N+C) on immune cell activation or function, it is possible that SH2(N+C) may also
modulate inflammatory responses involved in LPS-mediated bone loss in addition to directly targeting the OCs. Further studies are needed to examine potential anti-inflammatory effects of SH2(N+C). In conclusion, the results reported here provide strong evidence that PLCγ2 adaptor function can be targeted in vitro and in vivo to suppress OCG and bone erosion.
2.7 References


2.8 Figure Legends

FIGURE 2.1. SH2(N+C) blocks OC formation in vitro.

A, Schematic representation of the functional domains of the PLCγ2 protein and the regions of the protein present in retrovirus constructs. B, Western blot analysis was used to confirm expression of the indicated domains of PLCγ2 by detection of the HA tag. C-D, WT BMMs were transduced with retrovirus to express the indicated peptides and cultured under osteoclastogenic conditions. Cells were fixed and stained to detect TRAP+ cells. C, Quantification of the TRAP+ cells per well. D, Representative bright-field micrographs.

FIGURE 2.2 SH2(N+C) blocks bone resorption.

WT BMMs were transduced with pMX empty vector or -SH2(N+C) and cultured on tissue culture plastic, A, or cow bone slices, B, in the presence of 100 ng/mL RANKL and 10 ng/mL M-CSF. A, Representative images of cells stained with TRAP. B, Representative images of cow bone slices stained with wheat germ agglutinin to visualize areas of resorbed bone. C, The surface area of resorbed bone was quantified as percent of total bone surface area.

FIGURE 2.3. SH2(N+C) regulates the expression of osteoclastogenic genes.

WT BMMs expressing pMX empty vector control or SH2(N+C) were cultured in the presence of 100 ng/mL RANKL and 10 ng/mL M-CSF. Relative expression of the OC-specific markers TRAP, cathepsin K (CatK), calcitonin receptor (CTR), and NFATc1 were detected by real-time PCR. Ct values were normalized to GAPDH and are represented as fold change compared to pMX control at day 0.

FIGURE 2.4. SH2(N+C) suppresses RANK signaling.

WT BMMs were transduced with pMX empty vector or SH2(N+C) in the presence of 100 ng/mL RANKL and 10 ng/mL M-CSF prior to starvation and stimulation. A-B, Pre-OCs were stimulated with 100 ng/mL RANKL for 0 – 60 minutes, as indicated. Whole cell lysates (A) and nuclear extracts (B) were separated by SDS-PAGE and western blots were probed with the indicated antibodies. Total Pyk2, actin, or Lamin B are shown as loading controls. C, Pre-OCs were stimulated with 50 ng/mL M-CSF for 0 – 30 minutes, as indicated, and analyzed as in A. D, Pre-OCs were lifted and replated on plates coated with vitronectin for 0 – 60 minutes, as indicated, and analyzed as in A.

FIGURE 2.5. SH2(N+C) disrupts binding of Gab2 with PLCγ2 and RANK.

WT BMMs were transduced with pMX empty vector or SH2(N+C) and cultured in the presence of 100 ng/mL RANKL and 10 ng/mL M-CSF prior to starvation and stimulation. Pre-OCs were stimulated with 100 ng/mL RANKL for 0 – 60 minutes, as indicated. 1 mg of protein from
whole cell lysates were immunoprecipitated with anti-PLCγ2, A, or anti-Gab2 antibody, B, and then separated by SDS-PAGE. Western blots were probed with antibodies as indicated.

**FIGURE 2.6. SH2(N+C) inhibits OC formation in vivo.**

A, WT C57Bl/6 mice were treated with 100 μg RANKL and 107 PFU of Ad-LacZ or Ad-SH2(N+C) by supracalvarial injection daily for 5 days. Mice were sacrificed on day 6 and the calvaria were collected for histological analysis. Representative images of histological sections stained to detect TRAP+ OCs (red cells) and quantification of the percent quantification of OC surface per bone surface (Oc.S./B.S.) and OC number per bone surface (Oc.N./B.S.). B, WT C57Bl/6 mice were treated with γ0 μg LPS and 107 PFU of Ad-LacZ or Ad-SH2(N+C) by supracalvarial injection at day 0 followed by Ad-LacZ or Ad-SH2(N+C) every other day. Mice were sacrificed on day 6 and the calvaria were collected for histological analysis. Representative images of histological sections stained to detect TRAP+ OCs and quantification of OC surface per bone surface (Oc.S./B.S.) and OC number per bone surface (Oc.N./B.S.) are shown.
2.9 Figures

Figure 2.1
Figure 2.2
Figure 2.3
Figure 2.4
Figure 2.5
Figure 2.6
Chapter 3. The Role of Tmem178 in the Regulation of Osteoclastogenesis
3.1 Tmem178 modulates bone homeostasis in basal and pathological conditions by regulating Ca$^{2+}$ mobilization in osteoclasts

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3.2 Abstract

PLCγ2-dependent calcium (Ca$^{2+}$) oscillations are indispensable for NFATc1 activation and downstream gene transcription driving osteoclastogenesis during skeletal remodeling and pathological bone loss. Here we describe the first known function of transmembrane protein 178 (Tmem178), a PLCγ2 downstream target gene, as a critical modulator of the Ca$^{2+}$-NFATc1 axis. In surprising contrast to the osteopetrotic phenotype of PLCγ2-/- mice, Tmem178-/- mice are osteopenic in basal conditions and are more susceptible to inflammatory bone loss, owing to enhanced osteoclast formation. Mechanistically, Tmem178 localizes to the ER membrane and restrains ER Ca$^{2+}$ release, thus controlling NFATc1 induction. Importantly, downregulation of Tmem178 is observed in human CD14$^{+}$ monocytes exposed to plasma from systemic juvenile idiopathic arthritis patients. Similarly to the mouse model, reduced Tmem178 expression in human cells correlates with excessive osteoclastogenesis. In sum, these findings identify an essential role for Tmem178 to maintain skeletal mass and limit pathological bone loss.
3.3 Introduction

In recent years, the skeleton has been appreciated as a dynamic system which, in addition to serving its evident mechanical functions, impacts whole-body metabolism. The bone is a reservoir for mineral stores, namely calcium (Ca\(^{2+}\)) and phosphate, and also releases and responds to numerous growth factors and hormones. Through these pathways, skeletal turnover stimulates cross-talk with the nervous, endocrine, immune, reproductive, and digestive systems. Skeletal fragility is observed in inflammatory, endocrine, and metabolic disorders [1, 2]. Perhaps the most studied inflammatory condition associated with dysregulated bone homeostasis is rheumatoid arthritis (RA). Elevated levels of inflammatory cytokines acting in concert with the receptor activator of NF-κB ligand (RANKL) drive excessive osteoclast (OC) differentiation and lead to local joint erosion as well as systemic bone loss [3, 4]. Pathological bone loss was also observed in up to 50% of patients with systemic juvenile idiopathic arthritis (sJIA) before the pre-biological era. However, even in the face of optimal anti-inflammatory therapy, joint damage is still observed in up to 20% of children without active systemic features (Janow et al., manuscript submitted). Furthermore, low bone mass and high risk of fragility fracture are often seen in adults who suffered from sJIA during childhood [5]. Unfortunately, at the moment there are no biomarkers of specific factors that would help identify which subset of patients will develop active erosive disease or long term osteopenia.

Ca\(^{2+}\) is a ubiquitous signaling messenger directing diverse processes such as differentiation, proliferation, migration, and gene transcription. Ca\(^{2+}\) fluxes are particularly important during the OC differentiation process as a rise in cytoplasmic Ca\(^{2+}\) triggers the Ca\(^{2+}\)/calmodulin-dependent nuclear translocation of NFATc1, a master transcription factor for osteoclastogenesis [6-8]. Ca\(^{2+}\) signaling is initiated by binding of RANKL to its receptor RANK.
inducing the activation of PLCγ2-mediated release of ER Ca\(^{2+}\) stores through IP3 receptors (IP3R). Upon detecting depletion of ER Ca\(^{2+}\), Stim1, an ER Ca\(^{2+}\) sensor, binds to Orai1, a pore-forming unit of the CRAC (Ca\(^{2+}\) release activated Ca\(^{2+}\)) channel, and allows extracellular Ca\(^{2+}\) entry through the plasma membrane [9-19]. Ca\(^{2+}\) entry from the extracellular milieu in the OC can also occur via transient receptor potential V channels [20-22]. ER Ca\(^{2+}\) is re-filled by the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) [23]. Genetic or pharmacological interference of Ca\(^{2+}\) channel activity, as well as blockade of PLCγ2-mediated Ca\(^{2+}\) release, impedes osteoclastogenesis, cell motility, and bone resorption in vitro and in vivo [24-29].

Because of its importance to many cellular responses, Ca\(^{2+}\) mobilization is finely controlled by regulatory proteins that potentiate or suppress Ca\(^{2+}\) transport and dictate amplitude, duration, and/or frequency of Ca\(^{2+}\) fluxes. Several interacting partners of Stim1 and Orai1 have been identified as regulators of CRAC channel activity. Interestingly, emerging data also indicate that Stim1 and Orai1 can interact with regulatory proteins to exert non-CRAC channel-related functions [30, 31]. Such interacting proteins remain unknown in the context of the OC.

In this study we identify for the first time a previously uncharacterized protein, Tmem178, as a novel modulator of ER Ca\(^{2+}\) mobilization in OCs. Our data demonstrate that Tmem178 is a negative regulator of OC differentiation by limiting ER Ca\(^{2+}\) release and activation of the Ca\(^{2+}\)-dependent osteoclastogenic transcription factor NFATc1. In mice, Tmem178 deficiency leads to a significant osteopenic bone phenotype owing to increased OC numbers. Human monocytes treated with plasma from sJIA patients downregulate Tmem178 and undergo more robust osteoclastogenesis, suggesting that Tmem178 may be a modulator of disease-associated bone erosion.
3.4 Materials and Methods

Mice

PLCγ2-/- mice were kindly provided by Dr. JN Ihle (St. Jude Children’s Research Hospital, Memphis, Tennessee, USA). Tmem178-/- mice (Strain B6;129S5-Tmem178tm1Lex/Mmucd) were generated by the trans-NIH Knock-Out Mouse Project (KOMP) and purchased from the KOMP Repository at UC Davis (www.komp.org) (stock number 032664-UCD). Tmem178 bone phenotyping was performed with mice of the C57Bl/6-129 background maintained by heterozygous breeding. In vitro osteoclast experiments were performed with bone marrow from both mice of C57Bl/6-129 and C57Bl/6 background. All experiments were approved by the Washington University School of Medicine animal care and use committee.

Histology and μCT

Microcomputed tomography and histological analysis were performed as previously described [53]. For histology to detect OCs, bones were preserved in 10% buffered formalin overnight and decalcified using 14% EDTA, pH 7.0, for 10 days (long bones) or 3 days (calvaria) with gentle rocking and daily replacement of solution. Decalcified bones were then dehydrated in graded alcohol, cleared through xylene, and embedded in paraffin. Paraffin blocks were sectioned longitudinally. Five-micron sections were then stained with TRAP (Sigma) to detect OCs and counterstained with hematoxylin. For double-labeling, 4 week-old WT and Tmem178-/- mice were injected with calcein and alizarin red at a 4 day interval. Calvaria were harvested on day 5, stored in 70% ethanol and protected from light. Five-micron sections were analyzed from non-decalcified, methacrylamide embedded calvaria. Sections were analyzed with a Nikon Eclipse 80i microscope and a 10X objective. For μCT, 3D images from intact
mouse femurs or calvaria were obtained on a μCT40 scanner (Scanco Medical). For LPS-induced osteolysis on the calvaria, resorbed area was quantified using Image J Software.

**Inflammatory osteolysis models**

For serum-transfer arthritis, 200 µl of serum from K/BxN mice was injected i.p. into recipient mice on day 0, day 2 and day 6. 50 µg of Lipopolysaccharides (LPS) from Escherichia coli 0111:B4 (Sigma) was injected on day 6. Mice were sacrificed on day 14, and long bones were harvested for analysis by μCT and histology. For surpacalvarial LPS, 100 µg LPS was injected subcutaneously over the calvaria on day 0. Mice were sacrificed on day 5, and calvaria were harvested for analysis by μCT and histology.

**Primary cell culture**

BMMs were isolated from 6-8 week old mice as previously described [54]. Briefly, the marrow of long bones was flushed by centrifugation and isolated cells were grown in alpha medium (Sigma) in the presence of 100 ng/ml M-CSF for 3 days. To form OCs, BMMs were cultured with 50 ng/ml glutathione-S-transferase (GST)-RANKL and 10 ng/ml M-CSF for 3-5 days. Cells were fixed in 4% paraformaldehyde (Polysciences) and stained for TRAP using the leukocyte acid phosphatase kit (Sigma).

**Bone resorption**

5 × 10⁴ BMMs or preOCs were cultured on bovine bone slices the presence of 10ng/ml M-CSF and 50ng/ml GST-RANKL. Cells were then removed from the bone surface using sodium hydroxide and gentle agitation, and bone slices were stained with 20 µg/ml peroxidase-conjugated wheat-germ agglutinin (Sigma) for 30 min at room temperature, followed by incubation with 3,3′-diaminobenzidine (0.52 mg/ml in PBS containing 0.1% H₂O₂) for 30 min.
Bone resorption pits were analyzed using a light microscope (Nikon) and quantified using Image J software.

**Western blot and antibodies**

For RANKL and M-CSF signaling assays, BMMs were starved overnight in cytokine- and serum-free alpha medium; preOCs were starved for 4 hours in cytokine-free alpha medium containing 2% FBS. For total cell lysates, BMMs or preOCs were lysed in RIPA buffer supplemented with protease/phosphatase inhibitor cocktail (Pierce). For nuclear extracts, cells lysed with hypotonic buffer (10 mM HEPES, 1.5 mM MgCl₂, 1 mM KCl, 1 mM DTT, and protease and phosphatase inhibitors), followed by addition of 0.1% NP40. After centrifugation, the supernatants were collected (cytosolic fraction), while the pellets (nuclear fraction) were suspended in high-salt buffer (hypotonic buffer plus 400 mM NaCl). For immunoprecipitation, cells were harvested in TNE lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 10% glycerol). Protein concentration was determined by bicinchoninic acid protein assay (Biorad), resolved by SDS-page and subjected to western blot analysis.

For immunoblotting and immunoprecipitation, phospho-ERK (D13.14.4E), phospho-JNK (81E11), phospho-p38 (3D7), phospho-IkBα (14D4), phospho-AKT (587F11), Myc (9B11), Pyk2 (3292), and Lamin B1 (13435) antibodies were obtained from Cell Signaling Technology. NFATc1 (7A6) was purchased from Santa Cruz, as well as secondary anti-mouse and anti-rabbit HRP-conjugated antibodies. Mouse monoclonal HA.11 (16B12) was purchased from Covance. Flag-M2 and beta-actin were purchased from Sigma. Protein A/G beads were obtained from Santa Cruz.

**Real-time PCR**
Cells were lysed in TRIzol (Invitrogen), and whole tissues were snap frozen in liquid nitrogen then pulverized with a dismembrator (B. Braun Biotech International) in TRIzol. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed to cDNAs using EcoDry Premix (Oligo dT) (Clontech). For quantitative real-time PCR, NFATc1 was amplified using 5′-CCCGTCACATTCTGGTCCAT-3′ and 5′-CAAGTAACCGTGTAGCTGCACAA-3′. TRAP was amplified using 5′-CAGCTCCTAGAAGATGGATTCCAT-3′ and 5′-GTCAGGAGTGGGAGCCATATG-3′; cathepsin K using 5′-ATGTGGGTGTTCAAGTTTCTGC-3′ and 5′-CCACAAGATTCTGGGGACTC-3′; calcitonin receptor using 5′-CAAGAACCCTTAGCTGCCAGAG-3′ and 5′-CAAGCACGGCGACAATGTTG-3′; and cyclophilin using 5′-AGCATAACGGTCCTGGCATC-3′ and 5′-TTCACCTTCCAAAGACCAC-3′. Tmem178 in mouse cells was amplified using 5′-ATGACAGGGATATTTTGCACCAT-3′ and 5′-CCGGTTCAAGTCATAGGAGACC-3′. For measuring transcriptional repressors, Bcl6 was detected using 5′-TTAGAGCTTATAGATTGCATG-3′ and 5′-TCAAGTGAAGGATTGATTTGACC-3′; IRF8 using 5′-AAAGCTGACTAGTGAAGGATTG-3′ and 5′-GGAAGCTGACTAGTGAAGGATTG-3′. SYBR green dye was used for detection of the product using the SYBR Green PCR Master Mix assay (Applied Biosystems). The standard curve used a series of duplicate dilutions of plasmid for each gene and cyclophilin cDNA. The amplification reaction was performed for 40 cycles with denaturation at 95°C for 10 minutes, followed by annealing at 95°C for 15 seconds and extension and detection at 60°C for 1 minute. The relative abundance of each target was calculated as 1000×2^{-(Ct\ target-Ct\ cyclophilin)}.
gene−Ct cyclophilin), where Ct represents the threshold cycle for each transcript, and cyclophilin is the reference.

Plasmids and retrovirus generation

Human Tmem178 cDNA (Clone ID 528607- Open Biosystems) was cloned into the blasticidin-resistant pMX retroviral vector containing an HA tag at the C-terminus using BamHI and XhoI restriction sites. Stim1-Myc and Orai1-Flag were a gift from Dr. Monika Vig (Washington University).

To generate retrovirus, PLAT-E cells were transfected with expression vector using PolyJet transfection reagent (SignaGen). Viral supernatants were collected on day 2 post-transfection and immediately used to infect freshly isolated BMMs with the addition of 4 ug/ml polybrene. After 24 h, complete medium containing 1 μg/ml blasticidin was added to cells for 48 h to select for expressing cells. Selected cells were differentiated to OC with 10 ng/ml M-CSF and 50 ng/ml RANKL in the presence of 0.3 ug/ml blasticidin. For expression studies in HEK293T cells, Tmem178-HA, Stim1-Myc-His, or Orai1-Flag were co-transfected into HEK293T cells using PolyJet. Transfected cells were used for Ca\textsuperscript{2+} imaging or lysed for immunoprecipitation experiments 24 hours later.

Immunofluorescence and microscopy

OCs were differentiated on glass coverslips with 10 ng/ml M-CSF and 50 ng/ml RANKL. Cells were fixed with 4% paraformaldehyde (Polysciences) for 10 minutes then rinsed 3 times with PBS. Cells were permeabilized with 0.2% Triton for 10 minutes, and blocked in 3% BSA-1% FBS for 30 minutes. Primary antibody diluted in PBS supplemented with 3% BSA and 0.1% saponin was added for 2 hours at room temperature, followed by 2 hours incubation with
secondary antibody (1:500) together with phalloidin-Alexa Flour 488 (1:500) (Molecular Probes). The following antibodies and dilutions were used: Calnexin (C20) purchased from Santa Cruz (1:50), c-myc (9E10) from Santa Cruz (1:50), HA (HA-7) from Sigma (1:2000), HA-FITC (Ab1208) purchased from Abcam (1:100), anti-goat Alexa Fluor 594 (1:500), anti-mouse Alexa Fluor 594 (1:500), and anti-mouse Alexa Fluor 488 (1:100). Vectashield containing dapi nuclear stain was purchased from Vector Labs. Slides were analyzed using a Nikon Eclipse 80i microscope and a 63X Plan APO objective. Images were captured with a Nikon DS-Qi1MC camera (Nikon). For confocal images, slides were analyzed using a Zeiss Axiovert 200M microscope and Zeiss LSM 5 PASCAL system.

Single cell Ca$^{2+}$ measurements

For Ca$^{2+}$ measurements, cells were seeded in a black 96-well plate. BMMs were seeded at a concentration of 12,000 cells/well for 1 day with 10 ng/ml M-CSF and 50 ng/ml RANKL. Cells were loaded with 3 μM Fura-2-AM (Invitrogen) in phenol red-free DMEM (Invitrogen) containing 10 ng/ml M-CSF for 30 minutes at room temperature in the dark. Cells were washed twice with HBSS and imaged immediately in phenol red-free DMEM containing 10 ng/ml M-CSF. Cells were imaged for 1 minute before the addition of 100 ng/ml GST-RANKL. For preOC treated with thapsigargin or ionomycin, cells were imaged in Ca$^{2+}$-HBSS. For experiments using HEK293T cells, 24 hours post-transfection cells were plated at a concentration of 15,000 cells/well for 4 hours on poly lysine-coated wells. Cells were loaded with 3 μM Fura-2-AM in HBSS for 30 minutes at 37°C. Cells were washed twice in HBSS and imaged immediately in Ca$^{2+}$-free HBSS. Fura-2 ratios were measured by alternate excitation at 340 nm and 380 nm. An Olympus IX-71 inverted microscope with a Lamda-LS illuminator, Fura-2 (340/380) filter set, a 10X 0.3NA objective lens, and a Photometrics Coolsnap HQ2 CCD camera was used to capture
images at a frequency of 1 image pair every 2 seconds. Data were acquired and analyzed using MetaFluor. At least 40 cells per field were analyzed in each replicate.

*Human plasma preparation*

The study was approved by the Institutional Review Board at Stanford University. All subjects provided informed consent before participating in the study in accordance with the Declaration of Helsinki. Plasma was prepared from whole, anti-coagulated blood within 2 hours after blood draw. Samples were centrifuged at 25°C at 514 g for 5 minutes to remove cells, and were centrifuged for 2 additional rounds at 4°C at 1730 g for 5 minutes and 15 minutes to remove platelets. Plasma samples were stored at -80°C until use.

*Human osteoclastogenesis*

PBMCs were isolated from healthy donor by Ficoll gradient centrifugation. CD14⁺ cells were isolated by positive selection with anti-CD14 magnetic beads (Miltenyi Biotec). For osteoclastogenesis, 600,000 CD14⁺ PBMCs were seeded in a 96-well plate with 30 ng/ml human M-CSF. After 24 hours, media was added to a final concentration of 15 ng/ml human M-CSF and 100 ng/ml GST-RANKL. At 48 hours and 96 hours, the total media was changed. The complete media included 15 ng/ml hM-CSF, 100 ng/ml GST-RANKL, and 15% plasma by volume (25 μl in 200 μl total volume). For Tmem178 mRNA analysis, CD14⁺ PBMCs were cultured with hM-CSF and GST-RANKL for 48 hours. 15% control or patient plasma was added for 8 hours.

*Statistics*
All data represent mean ± SD. Data were analyzed using two-tailed Student’s $t$ test, with a $P$ value of <0.05 set as statistically significant. *$p<0.05$ **$p<0.01$ ***$p<0.001$
3.5 Results

*Tmem178 deletion decreases bone mass in basal conditions*

We have previously reported that ablation of PLCγ2 results in a blockade of osteoclastogenesis owing to defective NFATc1 induction in vitro and in vivo [32, 33]. PLCγ2-deficient mice are osteopetrotic and are also protected from inflammatory osteolysis [34, 35]. Because PLCγ2 shares significant homology with the more ubiquitously expressed isoform PLCγ1, however, attempts at pharmacological inhibition of PLCγ2 are likely to have broad off-target effects. Therefore, we sought to identify PLCγ2-dependent signaling mediators that could specifically direct therapeutic design for disorders caused by OC overactivity such as osteoporosis and rheumatoid arthritis. We performed a gene array comparing wild-type (WT) and PLCγ2-deficient OC precursors to identify novel genes downstream of PLCγ2 signaling. Transmembrane protein 178 (Tmem178) was highly expressed in WT cells, but Tmem178 expression was abrogated in cells lacking PLCγ2 (data not shown).

Tmem178 is a previously unstudied multi-pass integral membrane protein. Despite its name, Tmem178 does not share any structural domains or homology with other “Tmem” proteins. We began by assessing Tmem178 expression in whole tissues from WT and PLCγ2-/mice. Strikingly, we found that Tmem178 is highly expressed in WT whole bone, while transcript levels are low in other tissues including, spleen, liver, thymus, and testes (Figure 3.1 A). Confirming the gene array data, Tmem178 expression was strongly dependent on PLCγ2. Thus, we hypothesized that Tmem178 is a downstream target controlling PLCγ2’s effects on bone homeostasis.
To establish the physiological importance of Tmem178 we examined the bone phenotype of Tmem178-null mice in basal conditions. We expected Tmem178-/- mice to mirror the osteopetrotic phenotype of PLCγ2-/- mice. Surprisingly, 16 week-old female Tmem178-/- mice display a 35% decrease in trabecular bone volume with significant trabecular thinning compared to WT littermates (Figure 3.1 B-D). Tartrate-resistant acid phosphatase (TRAP) staining of long bone sections reveals a significant increase in OC surface normalized to bone surface in Tmem178-/- mice (Figure 3.1 E-F). Similarly, male Tmem178-/- mice show a 10% decrease in BV/TV compared to WT controls, with concomitant increase in OC surface/bone surface (n = 4-5) (data not shown). Because OC-mediated bone resorption is coupled to OB activity, we performed double labeling in 4 week-old mice by administering calcein and alizarin red at a 5 day interval. WT and Tmem178-/- mice show no significant differences in mineral apposition rate (MAR) and bone formation rate (BFR) (Figure 3.1 G-I). Moreover, quantitative real time PCR of whole bones flushed of marrow cells reveals equal expression of RANKL and OPG in WT and Tmem178-/- mice (Figure 3.1 J-K). Thus, the basal osteopenic phenotype of Tmem178-/- mice can be attributed to an OC-intrinsic effect. These in vivo data suggest that Tmem178 acts to suppress OC differentiation via an unexpected negative feedback loop downstream of PLCγ2.

Tmem178 expression is controlled by NFATc1

To determine whether Tmem178 exerts cell autonomous effects in the OC lineage, we measured its transcript levels during osteoclastogenesis in vitro. Tmem178 mRNA increases during osteoclastogenesis and is dependent on PLCγ2 (Figure 3.2 A-B). A similar extent of Tmem178 induction is observed in human CD14+ monocytes treated with RANKL compared to cells cultured with M-CSF alone (Figure 3.2 C). In contrast to the OC, Tmem178 is not
significantly expressed in the OB, confirming the observation that Tmem178 deletion does not affect bone formation (Figure 3.2 A).

PLCγ2’s catalytic activity is required for induction of NFATc1 early in osteoclastogenesis. To better understand how PLCγ2 controls Tmem178 expression, we utilized NFATc1-deficient (NFATc1Δ/Δ) precursors in which conditional deletion of NFATc1 is mediated by inducible Mx1-cre [36]. NFATc1fl/fl without Mx1-Cre served as control. Interestingly, RANKL-induced Tmem178 upregulation is blunted in NFATc1Δ/Δ cells compared to controls (Figure 3.2 D). These data position Tmem178 directly downstream of the RANKL-PLCγ2-NFATc1 axis in the OC.

Tmem178 deletion enhances osteoclastogenesis

To determine the role of Tmem178 in the OC, we cultured bone marrow macrophages (BMMs) with M-CSF and RANKL for 3-5 days. Tmem178-/- BMMs display accelerated differentiation and increased OC numbers compared to WT (Figure 3.2 E-F). This rapid formation of multi-nucleated polykaryons is paralleled by an earlier and greater magnitude of induction of the early OC marker TRAP (Acp5), the functional protease Cathepsin K (CtsK), and calcitonin receptor (Calcr), a marker of committed OCs (Figure 3.2 G). To determine if Tmem178 affects OC resorptive activity, we cultured BMMs on bovine bone slices in the presence of M-CSF and RANKL for 10 days. Resorptive pit area was significantly increased in Tmem178-/- cultures compared to WT (Figure 3.2 H-I). Because enhanced resorption could be a consequence of increased OC numbers, we analyzed the resorptive capacity of individual OCs by plating committed OCs (differentiated on plastic with M-CSF and RANKL for 3 days) on bone slices for 48 hours. Results show equivalent bone resorption by Tmem178-/- and WT OCs.
Tmem178 negatively regulates OC formation by suppressing NFATc1 activation

To understand how Tmem178 modulates OC differentiation, we assessed activation of RANKL signaling cascades including NF-κB and MAPKs, JNK, ERK and p38. We find no perceptible differences in NF-κB and MAPK activation between WT and Tmem178/-/- BMMs (top) or preOCs (bottom) stimulated with RANKL (Figure 3.3 A). Similarly, M-CSF induction of p-AKT and p-ERK is equivalent in both genotypes (Figure 3.3 B).

NFATc1 is another important pathway activated during osteoclastogenesis in response to RANKL. Despite similar changes in NF-kB or MAPK activation, Tmem178/-/- cells show heightened NFATc1 total protein and transcript levels throughout osteoclastogenesis (Figure 3.3 C-D). Immunofluorescence staining of NFATc1 in BMMs shows only 1% of cells positive for nuclear NFATc1 in both genotypes (Figure 3.3 E). However, after 24 and 48 hours of RANKL stimulation, we observed a higher percentage of Tmem178/-/- cells with NFATc1 nuclear staining compared to WT (Figure 3.3 E). Consistent with this finding, we also found a striking increase in NFATc1 nuclear translocation in Tmem178/-/- preOCs compared to WT cells in response to a RANKL time course (Figure 3.3 F). Notably, no differences in NFATc1 protein or transcript levels are observed prior to RANKL exposure, indicating that Tmem178 acts downstream of RANKL to regulate NFATc1 induction.

To confirm the proposed mechanism that Tmem178 suppresses osteoclastogenesis by limiting NFATc1 levels, we ectopically expressed Tmem178 in WT BMMs and analyzed their ability to induce NFATc1 and undergo OC differentiation. Tmem178-expressing cells show
attenuated NFATc1 levels compared to empty vector controls (Figure 3.3 G), with a resulting reduction in osteoclastogenesis (Figure 3.3 H, I). Altogether, these data indicate that Tmem178 modulates osteoclast differentiation by suppressing RANKL-induced NFATc1 activation.

*Tmem178 modulates RANKL-induced Ca^{2+} fluxes*

High NFATc1 levels in Tmem178-/- cells could result from increased transcriptional activity of known NFATc1 regulators. IRF8 and Bcl6 are transcriptional repressors constitutively bound to the NFATc1 promoter and down-regulated within 24 hours of RANKL-RANK engagement [37]. qRT-PCR analysis revealed that mRNA levels of Bcl6 and IRF8 are reduced to a similar extent and with equivalent kinetics in WT and Tmem178-/- cells during osteoclastogenesis (Figure 3.4 A, B). Thus, Tmem178 does not seem to modify NFATc1 levels by targeting its transcriptional regulators.

Alternatively, cytosolic Ca^{2+} fluxes stimulate the calmodulin/calcineurin pathway activating NFATc1 nuclear translocation. To test whether Tmem178 may restrain NFATc1 by affecting Ca^{2+} mobilization, we utilized ratiometric confocal imaging to measure Ca^{2+} in preOCs. Cells were loaded with Fura-2, a fluorescent Ca^{2+}-binding dye; the ratio of Fura-2 fluorescence intensity following excitation at 340 and 380nm reflects intracellular Ca^{2+} concentration ([Ca^{2+}_i]). The addition of exogenous RANKL induces the expected cytosolic Ca^{2+} oscillations in WT cells (Figure 3.4 C, average of 40-50 fluxing cells per genotype) while an immediate spike followed by sustained [Ca^{2+}_i] occurs in Tmem178-/- preOCs. Higher basal [Ca^{2+}_i] are already noted in Tmem178-/- preOC prior to the acute addition of RANKL. Quantification of the average Ca^{2+} measurements prior to any stimulation demonstrates that the majority of WT preOCs have a 340/380 ratio less than 1.0, while more than 60% of Tmem178-/- cells show a
340/380 ratio between 1.0-2.0 (Figure 3.4 D, more than 120 cells/genotype, data pooled from 3 independent experiments). These data suggest that Tmem178 is a novel negative regulator of 
\[\text{[Ca}^{2+}\text{]}_i\] in the OC.

To understand whether Tmem178 regulates cytosolic Ca\(^{2+}\) levels by affecting the ER pool or the extracellular exchange, we sought to first define Tmem178’s cellular localization. We expressed HA-tagged Tmem178 fusion protein (Tmem178-HA) in a puromycin-resistant retroviral vector in primary BMMs. Empty vector-expressing cells were used as a negative control. Cells surviving antibiotic selection were differentiated into OCs. Using phalloidin to stain polymerized actin at the cell membrane and calnexin as an ER marker, we found that Tmem178-HA localizes to the ER in mature OCs (Figure 3.4 E-F).

We next wanted to examine how Tmem178 functions in the ER to modulate Ca\(^{2+}\) mobilization. WT and Tmem178-/- preOCs were treated with thapsigargin (Tg) in Ca\(^{2+}\)-free buffer to measure ER Ca\(^{2+}\) release. Tmem178-/- preOCs show a greater increase in cytoplasmic [Ca\(^{2+}\)] compared to WT cells (Figure 3.4 G). Importantly, this is not due to an increase in ER Ca\(^{2+}\) content, as ionomycin-triggered ER Ca\(^{2+}\) depletion in the presence of the Ca\(^{2+}\) chelator EGTA is equivalent in WT and Tmem178-/- preOCs (Figure 3.4 H, average of at least 70 cells/condition and representative of 3 independent experiments).

To confirm the role of Tmem178 as a negative regulator of ER Ca\(^{2+}\) mobilization, we expressed ectopic Tmem178 or pMX empty vector control in HEK293T cells. Upregulation of Tmem178 causes a slower and reduced increase in cytosolic [Ca\(^{2+}\)] in response to Tg compared to cells transfected with pMX alone (Figure 3.4 I). Again, this effect is not due to a change in total ER Ca\(^{2+}\) concentration, as ionomycin-induced ER Ca\(^{2+}\) emptying is unchanged in
Tmem178-expressing cells versus controls (Figure 3.4 J). Suppression of ER Ca\textsuperscript{2+} release is in line with reduced NFATc1 expression and decreased osteoclastogenesis in primary OCs expressing exogenous Tmem178 (Figure 3.3 G-I). Taken together, these data establish the importance of Tmem178 as a negative regulator of ER Ca\textsuperscript{2+} mobilization.

*Tmem178 associates with Stim1*

Next, we wanted to know if Tmem178 interacts with known ER-resident proteins involved in Ca\textsuperscript{2+} mobilization. Inositol triphosphate receptors (IP3Rs) are ER-resident Ca\textsuperscript{2+} channels, which facilitate ER Ca\textsuperscript{2+} release and consequent intracellular Ca\textsuperscript{2+} fluxes in response to IP3 generated by PLC\(\gamma\) signaling. All IP3Rs have been shown to be expressed in the OC, and IP3R2 and IP3R3 are compulsory for the generation of RANKL-stimulated Ca\textsuperscript{2+} fluxes [24]. Thus, we hypothesized that Tmem178 may be modulating ER Ca\textsuperscript{2+} mobilization by interacting with IP3Rs. However, using co-immunoprecipitation and immunofluorescence approaches, we were unable to detect the interaction between Tmem178 and any IP3Rs in primary OCs (data not shown).

Stim1 is a transmembrane ER Ca\textsuperscript{2+} sensor which interacts with numerous Ca\textsuperscript{2+} channels and adaptor proteins to regulate Ca\textsuperscript{2+} fluxes. Stim1 is expressed by the OC and regulates store-operated Ca\textsuperscript{2+} entry (SOCE) through the association with Orai1. Targeting Stim1-mediated calcium entry suppresses osteoclastogenesis [25-27]. While Stim1 deletion abolishes SOCE, a modest defect in ER Ca\textsuperscript{2+} release was also previously noted in Stim1-deficient mast cells (Baba et al, 2008). Interestingly, new evidence suggests that Stim1 can potenti ate IP3R-mediated ER Ca\textsuperscript{2+} release [38]. To determine if Tmem178 can bind Stim1, we expressed Tmem178-HA alone and in combination with Stim1-Myc in HEK293T cells. By immunoprecipitation of Tmem178-
HA followed by Western blot for Stim1-Myc, we detected the association between Tmem178 and Stim1 (Figure 3.5 A, first lane). No bands were observed in cell lysates that did not express Stim1-Myc or in the IgG control (Figure 3.5 A second and third lane). Because Stim1 is best described for its coupling with Orai1, we also expressed Orai1-FLAG (Figure 3.5 B, C). We again found that Tmem178 and Stim1 interact, regardless of Orai1 expression (Figure 3.5 B, middle lane and Figure 3.5 C, third lane). In reciprocal co-IPs, we did not detect any interaction between Orai1 and Tmem178 (Figure 3.5 B, lane 1, Figure 3.5 C, bottom panel, lane 3), indicating that Tmem178 specifically interacts with Stim1. Additionally, the Stim1-Orai1 interaction is intact in the presence of Tmem178 (Figure 3.5 C, first and second lanes). These data indicate that Tmem178 binds to Stim1, but not Orai1.

To determine whether Tmem178 and Stim1 interact in primary cells, we transduced WT BMMs with Tmem178-HA and Stim1-Myc retroviral vectors and cultured the expressing cells with RANKL for 24 hours. By confocal microscopy, we found that Tmem178 and Stim1 co-localize in areas adjacent to the nuclei, presumably the ER (Figure 3.5 D, 4 representative fields).

Stim1 is most well-described in its role to activate store-operated Ca\(^{2+}\) entry (SOCE). To assess whether Tmem178 alters SOCE, we incubated Tmem178-/- preOCs and their WT counterpart with Tg in Ca\(^{2+}\)-free buffer to deplete ER Ca\(^{2+}\) stores, followed by addition of 1.8 mM Ca\(^{2+}\) to the medium. As shown in Figure 3.5 E, both genotypes achieve similar cytoplasmic Ca\(^{2+}\) concentrations in response to exogenous Ca\(^{2+}\) stimulation. A similar result was obtained in HEK293T cells expressing ectopic Tmem178 versus pMX vector control (Figure 3.5 F). Altogether, these findings indicate that Tmem178 is an ER protein that associates with Stim1. Tmem178 does not appear to participate directly in SOCE, however, but rather regulates Ca\(^{2+}\) mobilization out of the ER.
Having shown that Tmem178 restrains ER Ca$^{2+}$ mobilization to affect NFATc1 upregulation and RANKL-induced OC differentiation, we wondered if Tmem178 similarly regulates OC formation driven by inflammatory cytokines. First, we observed that Tmem178 mRNA expression is significantly increased in BMMs following exposure to inflammatory stimuli, including LPS (not shown). Second, we observed that the addition of TNF-α or LPS, potent activators of pathological osteolysis, further exacerbates Tmem178-/- OC differentiation in vitro (Figure 3.6 A). Moreover, Tmem178-/- mice injected with LPS over the calvaria develop twice the eroded area compared to WT controls (Figure 3.6 B-C). This striking increase in resorption corresponds to a significant increase in OC surface in Tmem178-/- mice as shown in TRAP-stained histological sections (Figure 3.6 D-E).

Next, we wanted to determine whether Tmem178 could modulate focal osteolysis in a model of inflammatory arthritis. Animals were injected with arthritogenic serum from K/BxN mice on days 0, 2, and 6 and sacrificed on day 14. Despite developing an equivalent inflammatory response to WT, measured by paw thickness and local production of inflammatory cytokines (data not shown), Tmem178-/- mice suffer significantly more bone loss, measured by remaining bone volume at the knee by μCT (Figure 3.6 F-G). Analysis of the TRAP-stained histological sections of arthritic bones revealed that the excessive erosion in Tmem178-/- mice is driven by a significant increase in OC differentiation (Figure 3.6 H). All together these data indicate that loss of Tmem178 drives robust OC formation and excessive bone loss in inflammatory conditions.
Based on the above observations, we wondered whether changes in Tmem178 levels would be detected in patients affected by inflammatory conditions accompanied by bone erosion and increased osteoclastogenesis. Systemic juvenile idiopathic arthritis (sJIA) is a chronic disease characterized by systemic inflammation and arthritis [39]. Importantly, sJIA patients present with joint inflammation and erosive disease despite blockade of inflammatory cytokines [40, 41]. Low bone mass and increased risk of fragility fractures often persist in adulthood [5]. Thus, we hypothesized that sJIA pathogenesis may be related to Tmem178 dysregulation. Due to the difficulty of obtaining sufficient cellular samples from pediatric patients, we utilized the plasma of sJIA patients, which contains circulating factors capable of activating healthy human CD14+ PBMCs [42, 43]. We cultured CD14+ PBMCs from healthy donors with M-CSF and RANKL for 2 days to generate preOCs, and then added the plasma collected from healthy controls (n = 10) or sJIA patients (n = 20). Interestingly, we observed a significant reduction in Tmem178 transcript in CD14+ PBMCs treated with sJIA plasma compared to healthy control plasma (Figure 3.6 I). Confirming the Tmem178-/- OC phenotype, the addition of sJIA plasma potently augments OC differentiation compared to healthy plasma (Figure 3.6 J-K). Tmem178 expression is further reduced, while OC numbers are significantly increased, following exposure to plasma from 5 patients with erosive disease compared to controls (Figure 3.6 I-K). Taken together, these data indicate that downregulation of Tmem178 levels may drive excessive osteoclastogenesis and contributes to erosive disease in sJIA.
3.6 Discussion

Basal and pathological OC differentiation is dependent on Ca\textsuperscript{2+} fluxes initiated by the cytokine RANKL to activate the transcription factor NFATc1 [6, 8, 44]. Here we identify for the first time a role for Tmem178 as a negative regulator of NFATc1 by specifically limiting Ca\textsuperscript{2+} release from the ER (Figure 3.7). Tmem178 is upregulated by RANKL to restrain excessive OC differentiation in mice and humans. Tmem178 deficiency induces osteopenia, owing to a significant increase in OC numbers, and this phenotype becomes even more pronounced during inflammatory conditions in both mice and sJIA patients. This work provides new evidence for the importance of stringent control of Ca\textsuperscript{2+} mobilization to maintain bone mass and protect from pathological osteolysis.

Amplitude and duration of dynamic Ca\textsuperscript{2+} signals have been shown to differentially modulate activation of various transcription factors. NFAT proteins have enhanced Ca\textsuperscript{2+} sensitivity compared to other transcription factors such as NF-kB or JNK, which enables NFAT transcriptional activation by low Ca\textsuperscript{2+} levels [45]. In the OC, a low but prolonged Ca\textsuperscript{2+} signal induces NFATc1 nuclear translocation and long-lasting nuclear localization throughout the osteoclastogenic process. We find that Tmem178-/- OC have higher basal intracellular Ca\textsuperscript{2+} levels than WT. Furthermore, in the OC, not only the amplitude of Ca\textsuperscript{2+} signaling but also the generation of continuous Ca\textsuperscript{2+} oscillations through the reduction and refilling of ER Ca\textsuperscript{2+} in response to RANKL is required for efficient NFATc1 transcriptional activation. Following RANKL exposure, Tmem178-/- cells reach higher intracellular Ca\textsuperscript{2+} levels compared to WT which correlate with greater NFATc1 induction and increased NFATc1 nuclear translocation. Consequently, Tmem178-/- cells undergo more rapid and robust osteoclastogenesis. We show that Tmem178 selectively modulates ER Ca\textsuperscript{2+} release, while not interfering with total ER Ca\textsuperscript{2+}
content or Ca\textsuperscript{2+} entry from the extracellular milieu. This result would suggest that Tmem178 prevents Ca\textsuperscript{2+} leakage from the ER. In support of this assumption, ectopic expression of Tmem178 reduces thapsigargin-induced ER Ca\textsuperscript{2+} release and consequently suppresses NFATc1 induction and osteoclastogenesis. It is important to note that ER Ca\textsuperscript{2+} leakage is only a fraction of the intracellular Ca\textsuperscript{2+} that can enter from the extracellular milieu. However, consistent with the differential functional consequences of Ca\textsuperscript{2+} signaling patterns, such as oscillations and spikes, this small but incremental difference in intracellular Ca\textsuperscript{2+} concentrations in KO cells exposed to RANKL can account for the heightened osteoclastogenic response that we observe in vitro and that persists in vivo.

Due to the importance of Ca\textsuperscript{2+} amplitude and duration in discriminating between different response pathways, Ca\textsuperscript{2+} levels in the cells are tightly regulated. Regulators of Ca\textsuperscript{2+} channel activity in the OC have only recently come under investigation. Ong et al described the importance of transient receptor potential cation channel 1 to enhance store-operated calcium entry (SOCE) from the extracellular milieu during OC formation [46]. Similarly, Tmem64 promotes RANKL-induced Ca\textsuperscript{2+} oscillations by bolstering the activity of Sarco-Endoplasmic Reticulum ATPase isoform 2, which actively re-fills ER Ca\textsuperscript{2+} stores, and is required for NFATC1 induction and osteoclastogenesis [28, 29, 47]. While these studies demonstrate that the loss of positive regulators of Ca\textsuperscript{2+} fluxes perturbs osteoclastogenesis and leads to increased bone mass, our work on Tmem178 is the first to identify a negative regulator of Ca\textsuperscript{2+} mobilization, whose ablation leads to an osteopenic phenotype. The relevance of our findings is bolstered by the significant reduction of Tmem178 expression levels in monocytes treated with plasma from sJIA patients with erosive disease.
In pursuing the mechanism by which Tmem178 modulates Ca\(^{2+}\) oscillations, we found that Tmem178 and Stim1 co-immunoprecipitate. Stim1 is an ER Ca\(^{2+}\) sensor expressed in numerous cell types, including OCs, lymphocytes, mast cells, B cells, and T cells. Pharmacological inhibition of Stim1 impairs SOCE, thereby blocking osteoclastogenesis [27]. In addition to associating with Orai1 to control SOCE, Stim1 also couples to numerous other Ca\(^{2+}\) channels, Ca\(^{2+}\) pumps, ER chaperone proteins, and regulatory adaptor proteins, thereby influencing multiple pathways of Ca\(^{2+}\) transport [48]. In kidney epithelial cells, Stim1 interacts with polycystin-1, diminishing IP3R-mediated ER Ca\(^{2+}\) release [49]. Most recently, Stim1 has been shown to potentiate IP3R-mediated ER Ca\(^{2+}\) release in aortic endothelial cells [38]. In muscle cells Stim1 inhibits Ca\(^{2+}\) discharge from the sarcoplasmic reticulum [50]. Differences in ER Ca\(^{2+}\) release were also noted in Stim1-/- mast cells in response to antigen or thapsigargin [51]. Thus, Stim1 has a critical role not only in sensing ER Ca\(^{2+}\) levels and activating SOCE, but also appears to regulate, at least in part, ER Ca\(^{2+}\) release in multiple cell types. In the OC, we find that Tmem178 localizes to the ER where it associates with Stim1. Interestingly, Tmem178 does not bind to Orai1 or inhibit Orai1-Stim1 coupling, and Orai1/Stim1-mediated Ca\(^{2+}\) entry is similar in both genotypes. Tmem178 also does not appear to associate with IP3Rs. While further studies are necessary to wholly understand the mechanism of Tmem178-mediated ER Ca\(^{2+}\) regulation and the significance of the Tmem178-Stim1 complex, we believe the Tmem178-Stim1 interaction is an important observation which expands the understanding of Ca\(^{2+}\) regulation in the OC.

Tmem178 interaction with Stim1 is particularly interesting in the context of arthritis. Stim1 single nucleotide polymorphisms (SNPs) were recently identified in patients with ankylosing spondylitis (AS), a chronic inflammatory disease of the spine and joints [52]. These
SNPs correlated with significantly higher inflammatory markers including C-reactive protein and in some cases higher circulating levels of TNF-α and IL-6. In unpublished data, we found that Tmem178 negatively regulates TNF-α, IL-1, and IL-6 production by macrophages. Although we did not have access to patient samples to measure Tmem178 levels or Tmem178/Stim1 binding in AS, we found that reduced Tmem178 expression is associated with augmented osteoclastogenesis in the context of sJIA. Further supporting a role for Tmem178 in inflammatory arthritis, Tmem178/- mice suffer profound osteolysis following LPS and in K/BxN arthritis and in vitro studies show increased responsiveness to TNF-induced osteoclastogenesis.

sJIA is characterized by arthritis and systemic inflammation. Bone erosion and systemic bone loss were observed in up to 50% of children with sJIA in the pre-biologic era. Even more recently, CARRAnet registry data (collected since 2010) on 435 children with sJIA show that joint damage remains a significant problem for at least 20% of these patients despite remission of the systemic inflammation (Janow et al., manuscript submitted). Low bone mass and high risks of fragility fractures are also seen in adult patients who suffered from sJIA during childhood and adolescence [5]. Unfortunately, there are no markers that identify the subset of sJIA patients who will develop erosive disease. Further, the diagnosis of sJIA is a clinical diagnosis of exclusion, and unfortunately a delayed diagnosis contributes to the development of erosive changes. We now show that Tmem178 expression is significantly reduced in human CD14⁺ PBMCs exposed to sJIA plasma, while OC differentiation is increased. We also observe a significant reduction in Tmem178 levels in samples treated with plasma from sJIA patients with erosive disease, positioning Tmem178 as a potential biomarker for the subset of sJIA patients who will develop...
erosive disease. These initial findings with a limited number of patient samples necessitate future investigations with larger cohorts of sJIA patients.

Mechanistically, we do not yet understand the complex upstream mechanism which regulates Tmem178 expression in response to sJIA plasma. Moreover, in unpublished studies we find that Tmem178 also participates in inflammatory cytokine production, raising the possibility that Tmem178 may be important not only to the erosive aspect of sJIA but could contribute to the autoinflammatory symptoms of disease. Further investigation into the role of Tmem178 in the pathogenesis of sJIA is warranted.

In conclusion, we have identified Tmem178 as a new PLCγ2-dependent gene. Despite its dependence on the RANKL-PLCγ2 pathway, however, Tmem178 acts in a negative feedback loop to restrain osteoclast responses in basal and pathological conditions. Moreover, we characterized the mechanism of Tmem178 to specifically modulate ER Ca^{2+} mobilization to repress NFATc1 induction.
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3.8 References


3.9 Figure Legends

Figure 3.1. Deletion of Tmem178 decreases bone mass in basal conditions

A. Quantitative real-time PCR analysis of Tmem178 mRNA in whole tissues harvested from WT or PLCγ2-/- mice. Expression levels of Tmem178 were normalized to cyclophilin.

B. Representative images of microcomputed tomography (μCT) of the proximal femurs of 16 week-old female WT and Tmem178-/- mice. WT n = 10, Tmem178-/- n = 11.

C. Trabecular bone volume per tissue volume (BV/TV) quantified from μCT shown in (B). Data represent mean ± SD. Two-tailed student’s T-test was used to assess statistical significance, * p < 0.05.

D. Trabecular thickness (Tb.Th.) quantified from μCT shown in (B). Data represent mean ± SD. Two-tailed student’s T-test was used to assess statistical significance, * p < 0.05.

E. Histological analysis of femurs analyzed in (B). Femurs were stained with TRAP to detect OCs and counterstained with hematoxylin. Arrows indicate OCs.

F. Quantification of OC surface per bone surface (Oc.S/B.S.) in (E). n = 5/genotype. Data represent mean ± SD. Two-tailed student’s T-test was used to assess statistical significance, * p < 0.05.

G. Dynamic histomorphometry of calvaria from 4 week-old WT and Tmem178-/- mice labeled with calcein and alizarin red.

H. Quantification of bone formation rate (BFR) of (G). n = 5/genotype. Data represent mean ± SD. Two-tailed student’s T-test was used to assess statistical significance, * p < 0.05.

I. Quantification of mineral apposition rate (MAR) of (G). n = 5/genotype. Data represent mean ± SD. Two-tailed student’s T-test was used to assess statistical significance, * p < 0.05.

Figure 3.2. Tmem178 deficiency enhances osteoclastogenesis

A. Quantitative real-time PCR analysis of Tmem178 mRNA in WT BMMs, mature OCs, and mature OBs cultured in vitro. Expression levels of Tmem178 were normalized to cyclophilin levels.

B. Quantitative real-time PCR analyses of Tmem178 mRNA during RANKL-induced osteoclastogenesis in vitro in WT and PLCγ2-/- cells. WT and PLCγ2-/- BMM were cultured with 10 ng/ml M-CSF and 50 ng/ml RANKL for the indicated days. Expression levels of Tmem178 were normalized to cyclophilin levels. Representative of 3 experiments.

C. Quantitative real-time PCR analysis of Tmem178 mRNA in human CD14+ PBMCs cultured in vitro with 50 ng/ml RANKL for indicated time points. Expression levels of Tmem178 were normalized to cyclophilin levels. Data represent mean ± SD. Two-tailed student’s T-tests was used to assess statistical significance, * p < 0.05.

D. Expression of Tmem178 mRNA during RANKL-induced osteoclastogenesis in vitro in control (NFATc1fl/fl) and NFATc1-deficient cells (NFATc1Δ/Δ). Controls and NFATc1Δ/Δ
BMMs were cultured with 10 ng/ml M-CSF and 50 ng/ml RANKL for the indicated days. Tmem178 expression levels were normalized to cyclophilin. Data represent mean ± SD. Two-tailed student’s T-test was used to assess statistical significance, * p < 0.05.

E. TRAP staining of WT and Tmem178-/- OCs in vitro after 4 days of culture with 10 ng/ml M-CSF and 50 ng/ml RANKL. Images are representative of more than 5 experiments, each performed in triplicate.

F. Quantification of TRAP+ OCs in WT and Tmem178-/- cultures after 3 and 4 days of culture with 10 ng/ml M-CSF and 50 ng/ml RANKL. Triplicate wells were counted for each condition, and data are representative of more than 5 experiments. Data represent mean ± SD, * p < 0.05, *** p < 0.001

G. Quantitative real-time PCR analysis of osteoclastogenic markers in WT and Tmem178-/- cells collected at the indicated days of culture with 10 ng/ml M-CSF and 50 ng/ml RANKL. Acp5, TRAP; Ctsk K, Cathepsin K. CalcR, calcitonin receptor. Expression levels of each marker were normalized to cyclophilin. Data represent the mean ± SD, ** p < 0.01, *** p < 0.0001, n = 3 independent experiments

H. Representative bright field images of resorptive pits in WT and Tmem178-/- cultures. WT and Tmem178-/- BMMs were cultured on bovine bone slices in the presence of 10 ng/ml M-CSF and 50 ng/ml RANKL for 10 days. Resorption pits were visualized by staining with peroxidase-conjugated wheat-germ agglutinin. Black lines delineate the resorbed areas.

I. Quantification of resorbed area in images from (H). n = 8-9/ genotype. Data represent mean ± SD, * p < 0.05.

J. Representative bright field images of resorptive pits in WT and Tmem178-/- cultures. WT and Tmem178 BMM were cultured on plastic dishes with 10 ng/ml M-CSF and 50 ng/ml RANKL for 3 days. Cells were then lifted and re-plated on bovine bone slices in the presence of 10 ng/ml M-CSF and 50 ng/ml RANKL for 48 hours. Resorption pits visualized by staining with peroxidase-conjugated wheat-germ agglutinin. Black lines delineate the resorbed areas.

K. Quantification of resorbed area in images from (J). n = 8-9/ genotype. Data represent mean ± SD, ns

Figure 3.3. Tmem178 negatively regulates the Ca^{2+}-NFATc1 signaling pathway

A. Western blot analysis of RANKL-induced MAPK and NF-κB activation. Top panels- WT and Tmem178-/- BMMs were serum- and cytokine- starved overnight, and stimulated with 50 ng/ml RANKL for the indicated time points. Total Pyk2 is shown as loading control. Bottom panels- WT and Tmem178-/- preOCs were cultured with 10 ng/ml M-CSF and 50 ng/ml RANKL for 2 days, serum- and cytokine-starved for 6 hours, and stimulated with 50 ng/ml RANKL for the indicated time points. Actin is shown as loading control. Representative of 3 experiments.
B. Western blot analysis of M-CSF-induced AKT and ERK phosphorylation. Top panels- WT and Tmem178-/- BMMs were serum- and cytokine-starved overnight, and stimulated with 100 ng/ml recombinant M-CSF for the indicated time points. Total Pyk2 is shown as loading control. Bottom panels- WT and Tmem178-/- preOCs were cultured with 10 ng/ml M-CSF and 50 ng/ml RANKL for 2 days, serum- and cytokine-starved for 6 hours, and stimulated with 100 ng/ml M-CSF for the indicated time points. Actin is shown as loading control. Representative of 3 experiments.

C. Western blot analysis of NFATc1 total protein levels at the indicated time points of osteoclastogenesis. WT and Tmem178-/- BMMs were cultured with 10 ng/ml M-CSF and 50 ng/ml RANKL for the indicated number of days. Total ERK levels are shown as loading control. Representative of 3 experiments.

D. Quantitative real-time PCR analysis of NFATc1 mRNA at the indicated days of osteoclastogenesis. WT and Tmem178-/- BMM were cultured with 10 ng/ml M-CSF and 50 ng/ml RANKL for the indicated number of days. NFATc1 expression levels were normalized to cyclophilin. Data represent the mean ± SD, ** p < 0.01, n = 3 independent experiments.

E. Quantification of NFATc1 nuclear localization by immunofluorescence. WT and Tmem178-/- BMMs were plated on glass coverslips and cultured with 10 ng/ml M-CSF and 50 ng/ml RANKL for the indicated number of days. Data are pooled from 2 independent experiments, 3-9 coverslips/condition/experiment. Cells were stained for NFATc1 and DAPI to detect nuclei. Data represent the mean ± SD, * p < 0.05.

F. Western blot analysis of NFATc1 nuclear translocation in response to RANKL. WT and Tmem178-/- BMMs were cultured with 10 ng/ml M-CSF and 50 ng/ml RANK for 2 days, serum- and cytokine-starved for 6 hours, and stimulated with 50 ng/ml RANKL for the indicated time points. Lamin B is shown as loading control. Representative of 3 experiments.

G. Quantitative real-time PCR analysis of NFATc1 transcript levels during osteoclastogenesis in WT cells expressing empty vector pMX or Tmem178-HA. BMMs expressing pMX or Tmem178-HA were cultured with 10 ng/ml M-CSF and 50 ng/ml RANKL for the indicated number of days. NFATc1 expression levels were normalized to cyclophilin. Data represent the mean ± SD, ** p < 0.01, *** p < 0.0001, n = 3 independent experiments.

H. Representative images of TRAP staining of OCs expressing pMX empty vector or Tmem178-HA in vitro after 4 days of culture with 10ng/ml M-CSF and 50ng/ml RANKL.

I. Quantification of OC area in (h). Data represent the mean ± SD, ** p < 0.01, n = 3/condition, representative of more than 3 experiments.
Figure 3.4. Tmem178 modulates ER Ca\textsuperscript{2+} mobilization

A. Quantitative real-time PCR analysis of Bcl6 transcript levels during osteoclastogenesis in WT and Tmem178-/- cells cultured with 10ng/ml M-CSF and 50ng/ml RANKL for the indicated days. Bcl6 expression levels were normalized to cyclophilin. Data represent the mean ± SD. n = 3 independent experiments.

B. Quantitative real-time PCR analysis of IRF8 transcript levels during osteoclastogenesis in WT and Tmem178-/- cells cultured with 10 ng/ml M-CSF and 50 ng/ml RANKL for the indicated days. IRF8 expression levels were normalized to cyclophilin. Data represent then mean ± SD. n = 3 independent experiments.

C. Cytosolic Ca\textsuperscript{2+} measurements in primary preOC by ratiometric confocal imaging. WT and Tmem178-/- BMMs were cultured with 10 ng/ml M-CSF and 50 ng/ml RANKL for 1-2 days to generate preOCs. Cells were loaded with 3µM Fura-2 in phenol red-free DMEM and stimulated with 100ng/ml RANKL in the presence of extracellular Ca\textsuperscript{2+}. 5 representative tracings of single cells are shown for WT and Tmem178-/- preOC.

D. Percent of WT and Tmem178-/- preOCs with low Ca\textsuperscript{2+} (Fura-2 ratio < 1) or high Ca\textsuperscript{2+} (Fura-2 ratio >1). The Fura-2 ratio of each cell was averaged over the first 60 seconds of imaging. Data was pooled from 3 experiments, combining more than 120 cells/genotype.

E-F. Localization of Tmem178 in mature OCs by immunofluorescence. BMMs expressing pMX or Tmem178-HA were cultured on coverslips with 10ng/ml M-CSF and 100ng/ml RANKL for 5 days. Phalloidin was used as a plasma membrane marker, calnexin was used as an ER marker and DAPI was used as a nuclear stain. Scale bar indicates 100 µm. Manually contoured white line denotes cell edge.

G. ER Ca\textsuperscript{2+} release measured by ratiometric confocal imaging of primary WT and Tmem178-/- preOCs, imaged in the presence of Ca\textsuperscript{2+}-free HBSS and treated with 1 µM Tg to measure ER Ca\textsuperscript{2+} release. Fura-2 340/380 ratios are the mean of at least 70 cells/condition, representative of 3 independent experiments.

H. Total ER Ca\textsuperscript{2+} content measured by ratiometric confocal imaging of primary WT and Tmem178-/- preOCs, imaged in the presence of Ca\textsuperscript{2+}-free HBSS containing 2mM EGTA. Cells were stimulated with 10µM ionomycin to trigger ER Ca\textsuperscript{2+} depletion. Fura-2 340/380 ratios are the mean of at least 70 cells/condition, representative of 3 independent experiments.

I. ER Ca\textsuperscript{2+} release detected by ratiometric imaging of cytosolic Ca\textsuperscript{2+} of HEK293T cells expressing empty vector or Tmem178-HA. Cells were treated with 1 µM Tg in Ca\textsuperscript{2+}-free HBSS to measure ER Ca\textsuperscript{2+} release. Fura-2 340/380 ratios represent the mean of at least 100 cells/condition, representative of 2 independent experiments.

J. Total ER Ca\textsuperscript{2+} content detected by ratiometric confocal imaging of HEK293T cells expressing empty vector or Tmem178-HA and treated with 10 µM ionomycin in Ca\textsuperscript{2+}
free HBSS containing 2mM EGTA. Fura-2 340/380 ratios represent the mean of at least 100 cells/condition. representative of 2 independent experiments.

**Figure 3.5. Tmem178 associates with Stim1 in the ER**

A. Co-immunoprecipitation of Tmem178 and Stim1. Top panel- HEK293T cells expressing Tmem178-HA alone or with Stim1-Myc were lysed, and subjected to immunoprecipitation with anti-HA antibody or control IgG. IP WB was probed with Myc tag antibody. Total cell lysates were blotted for Myc and HA.

B. Co-immunoprecipitation of Tmem178 and Stim1 in HEK293T cells expressing Tmem178-HA, Stim1-Myc, and Orai1-FLAG. Top panel-lysates were subjected to immunoprecipitation with anti-FLAG, anti-Myc, or IgG control antibodies, and Western blotted for HA to detect an association with Tmem178. Total cell lysates (bottom panel) were probed for FLAG, Myc, and HA to show co-expression of all proteins.

C. Co-immunoprecipitation of Stim1 with Tmem178 and Orai1. HEK293T cells expressing Tmem178-HA, Stim1-Myc, and Orai1-FLAG were immunoprecipitated with the indicated antibodies and Western blotted for Myc and FLAG (Top panel). Total cell lysates (bottom panel) were probed for FLAG, Myc, and HA to show co-expression of all proteins.

D. Representative confocal images of Tmem178 and Stim1 co-localization in primary cells. BMMs co-expressing Tmem178-HA and Stim1-Myc were plated on glass coverslips in the presence of 10 ng/ml M-CSF and 50 ng/ml RANKL for 24 hours. Cells were stained with a FITC-conjugated anti-HA antibody and anti-Myc tag antibody. Myc was detected using an Alexa Fluor 543 secondary antibody. Dapi was used for nuclear staining.

E.-F. SOCE was measured in WT and Tmem178-/- cells cultured with 10 ng/ml M-CSF and 50 ng/ml RANKL for 24 hours (E) and HEK293T cells expressing empty vector or ectopic Tmem178 (F). Cells were loaded with 3 μM Fura-2 in Ca^{2+}-free HBSS then treated with Thapsigargin (Tg) to release ER Ca^{2+}. 1.8 mM Ca^{2+} was then added back to the buffer to measure SOCE.

**Figure 3.6. Tmem178 deletion worsens inflammatory bone loss**

A. Quantification of TRAP+ OCs in WT and Tmem178-/- cells cultured with 10 ng/ml M-CSF and 100 ng/ml RANKL alone, or with the addition of 100 ng/ml LPS or 10 ng/ml TNF-α on day 2 of OC culture. Data represent the mean ± SD, *** p < 0.0001 ** p < 0.01, n = 3

B. Representative μCT 3D reconstructions of calvaria from WT and Tmem178-/- mice receiving 100 μg supracalvarial LPS on day 0. Calvaria were harvested on day 5 after LPS.

C. Quantification of the percent area resorbed on the calvaria from images shown in (B). Data represent the mean ± SD, ** p < 0.01, n = 10/genotype
D. Representative images of histological sections from calvaria shown in (B) stained with TRAP to detect OCs and counterstained with hematoxylin. Arrows indicate OCs.

E. Quantification of TRAP+ OC in (D). Data are expressed as OC surface per bone surface (Oc.S./B.S.). Data represent the mean ± SD, ** p < 0.01, n = 10/genotype.

F. Representative CT 3D reconstructions of knees from WT and Tmem178/-/- mice harvested on day 14 after 3 injections of K/BxN serum on days 0, 2 and 6 to induce inflammatory arthritis. n = 9/genotype.

G. Quantification of bone volume remaining at the knee in mice in (F). Data represent the mean ± SD, ** p < 0.01, n = 10/genotype.

H. Quantification of TRAP+ OCs on TRAP-stained histological sections from knees of WT and Tmem178/-/- mice in (F). Data are expressed as OC surface per bone surface (Oc.S./B.S.) Data represent the mean ± SD, ** p < 0.01, n = 10/genotype.

I. Quantitative real-time PCR analysis of Tmem178 mRNA expression in human CD14+ PBMCs from healthy donor were cultured with 30 ng/ml hM-CSF and 100 ng/ml RANKL for 2 days. Plasma from healthy controls or sJIA patients was added to the cultures for 8 hours. Tmem178 expression is normalized to cyclophilin expression. Data represent the mean ± SD, ** p < 0.01, * p < 0.05.

J. Quantification of area covered by TRAP+ OCs cultured with healthy control plasma or sJIA patient plasma. Human CD14+ PBMCs from healthy donor were cultured with 30 ng/ml hM-CSF and 100 ng/ml RANKL for 5 days. Plasma from healthy controls or sJIA patients was added on day 2 of osteoclastogenesis and replenished at media change every other day. Data represent the mean ± SD, * p < 0.05. Triplicate wells were counted for each plasma sample, and data are representative of 3 independent experiments.

K. Representative images of TRAP staining of OCs treated with healthy control plasma or sJIA patient plasma quantified in (j).

**Figure 3.7. Model of Tmem178 function.**

In the WT OC, Tmem178 represses ER Ca\(^{2+}\) release, thereby limiting cytosolic Ca\(^{2+}\), NFATc1 activation, and osteoclastogenesis. In Tmem178/-/- OCs, augmented ER Ca\(^{2+}\) release increases cytosolic Ca\(^{2+}\) levels, thereby amplifying NFATc1 activation leading to excessive osteoclastogenesis.
3.10 Figures

Figure 3.1
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Chapter 4. The Role of Tmem178 in the Regulation of the Macrophage Inflammatory Response
4.1 Up-regulation of Tmem178 in inflammatory macrophages is essential to the control of systemic inflammation

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This work presented in this chapter is a manuscript in preparation
4.2 Abstract

Pathological bone loss in human disease such as rheumatoid arthritis is largely due to excessive osteoclast recruitment and activation as a consequence of localized inflammation. PLC\(\gamma\)2-deficient mice are protected from inflammatory arthritis due to impaired neutrophil inflammatory responses. We now report that PLC\(\gamma\)2-/- bone marrow macrophages (BMM) display impaired NF-\(\kappa\)B activation leading to reduced IL-1, IL-6 and TNF\(\alpha\) mRNA levels following adhesion, suggesting that PLC\(\gamma\)2 controls the development of inflammation in innate immune cells. By a gene array approach, we identified Tmem178 as a novel PLC\(\gamma\)2 effector whose expression is highly induced by integrin-mediated adhesion and TLR activation in WT but not PLC\(\gamma\)2-/-macrophages. In contrast to PLC\(\gamma\)2-/- macrophages, we find that Tmem178-/-BMMs have a striking increase in inflammatory cytokine expression in response to adhesion and TLR stimulation. Mechanistically, Tmem178 targets Ca\(^{2+}\) fluxes downstream of TLR and integrin signaling to affect NFAT activation, thus dampening inflammatory cytokine transcription. Most importantly, Tmem178-null mice succumb faster to endotoxin-induced sepsis, indicating that Tmem178 functions in a novel negative feedback loop downstream of PLC\(\gamma\)2 to repress inflammation. These results are consistent with our previous observation reporting a reduction Tmem178 levels in the context of sJIA. Our data demonstrate a role for Tmem178 in controlling inflammation as well, positioning Tmem178 as an essential regulatory component in both the inflammatory and erosive arms of inflammatory arthritis.
4.3 Introduction

In arthritic conditions, chronic inflammation drives activation of the bone-resorbing osteoclast leading to bone resorption and progressive joint disease. An influx of leukocytes cells into the joint is a hallmark of inflammatory arthritis. Innate immune cells, namely neutrophils and macrophages, infiltrate the joint space and release pro-inflammatory cytokines including TNF-a, IL-1, and IL-6 as well as proteases to drive local tissue damage and inflammation. Importantly, IL-1 and TNF-α in particular potently augment osteoclast differentiation and thus bone resorption (1-7). Thus, suppressing the inflammatory response is fundamental to the efficacy of arthritis treatments. Current therapeutics such as disease-modifying anti-rheumatic drugs (DMARDs) and cytokine inhibitors, however, are not successful at controlling inflammation in all patients (8).

Patients suffering from systemic juvenile idiopathic arthritis (sJIA) exhibit dramatically elevated levels of inflammatory cytokines, specifically IL-1 and TNF-α, within the synovial fluid and in circulation (9, 10). sJIA is an episodic auto-inflammatory disease that, unlike other arthritic subtypes, is primarily driven by innate immune cells. Expanded monocyte numbers, particularly during episodes of disease flare, are observed in sJIA patients (9). Approximately 10% of sJIA patients develop MAS, which is responsible for the majority of the morbidities associated with sJIA. The onset of MAS is sudden, and patients present with high fever, hepatosplenomegaly, lymphadenopathy, cutaneous and mucosal bleeding, pancytopenia, as well as central nervous system, cardiac, and renal involvement. Importantly, MAS is so-named due to a massive influx of activated macrophages in the bone marrow, liver and spleen. (10, 11). It is actually unknown if this increase in macrophages is driving pathogenesis or is an attempt to control a dysregulated adaptive immune response (12). The sJIA/MAS field is currently lacking
a clear picture of how the activated monocyte/macrophage population functions at both a cellular and molecular level during the onset and course of disease.

We have previously reported that PLCγ2-deficient mice are osteopetrotic due to defective OC formation and are protected from joint inflammation and destruction during serum-transfer arthritis, a model strictly dependent on neutrophil and macrophage activation (13, 14). The aberrant inflammatory response observed in PLCγ2-/- mice was dependent on reduced production of inflammatory cytokines IL-1, IL-6 and TNF-α by local innate immune cells. We also have previously shown that specifically targeting PLCγ2 scaffolding domains in vivo can block the osteoclast and ameliorate the erosive pathology of inflammatory osteolysis, but this approach is not sufficient to dampen inflammation (15). Thus, to better understand PLCγ2-dependent genes which could modulate a pro-inflammatory immune response we performed a gene array comparing WT and PLCγ2-/- BMMs plated on integrin substrate. Herein we identify Tmem178 as a novel molecule that represses the pro-inflammatory response of macrophages, in vitro and in vivo. This observation in concert with our previous finding that Tmem178 levels are reduced in CD14 monocytes exposed to plasma from sJIA patients, raise the possibility that Tmem178 is a new gene of interest in the pathogenesis of sJIA and the associated disorder MAS.
4.4 Materials and Methods

Mice

PLCγ2-/- mice were kindly provided by Dr. JN Ihle (St. Jude Children’s Research Hospital, Memphis, Tennessee, USA). Tmem178-/- mice (Strain B6;129S5-Tmem178tm1Lex/Mmucd) were generated by the trans-NIH Knock-Out Mouse Project (KOMP) and purchased from the KOMP Repository at UC Davis (www.komp.org) (stock number 032664-UCD). Tmem178-/- C57Bl/6-SvEv129 mice were transferred to the pure C57Bl/6 background by speed congenics, and maintained on the C57Bl/6 background by homozygous breeding. Perforin-/- (prf-/-) breeding pairs were purchased from Jackson Labs (Strain Name:C57BL/6-Prf1tm1Sdz/J Stock Number: 002407) and maintained by homozygous breeding. C57Bl/6 Tmem178-perforin double knock out mice were generated in-house. All experiments were approved by the Washington University School of Medicine animal care and use committee.

Primary cell culture

Bone marrow was isolated from long bones of 6- to 8-week-old C57BL/6 mice and cultured in alpha-MEM containing 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and glutamine (α-10 medium), with 1/10 vol CMG14-12 cell-conditioned medium as a source of macrophage colony-stimulating factor (M-CSF) (16) to obtain bone marrow-derived macrophages (BMMs). Resident peritoneal macrophages were harvested from 6- to 8-week-old C56Bl/6 mice by lavage. Briefly, the peritoneum was washed with 4 ml of sterile PBS, exudate was collected, and recovered cells were washed in sterile PBS. Cells were cultured in vitro with DMEM containing 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and glutamine.
**Gene array**

Petri dishes were coated with Arg-Gly-Asp (pRGD) in PBS overnight at 4°C. WT and PLCg2-/- BMMs were serum- and cytokine- starved overnight, lifted with trypsin-EDTA, and 2 × 10^6 cells were plated on pRGD-coated dishes for 4 hours. Non-adherent cells were in suspension for 4 hours. Cells were lysed in TRIzol and RNA was extracted by RNeasy Mini kit (Qiagen). mRNA profiles were analyzed by Affymetrix Mouse Gene 430 2.0 array.

**Western blot and antibodies**

For signaling assays, BMMs were starved overnight in cytokine- and serum-free alpha medium. For adhesion assays, petri dishes were coated with Arg-Gly-Asp (pRGD) in PBS overnight at 4°C. Starved BMMs were lifted with trypsin-EDTA, and plated on pRGD-coated dishes for the indicated time points. For LPS stimulation, starved BMMs were treated with 1 ug/ml LPS from Escherichia coli 0111:B4 (Sigma) in cytokine- and serum free alpha medium for the indicated time points. For total cell lysates, BMMs or preOCs were lysed in RIPA buffer supplemented with protease/phosphatase inhibitor cocktail (Pierce). Protein concentration was determined by bicinchoninic acid protein assay (Biorad), resolved by SDS-page and subjected to western blot analysis. For immunoblotting phospho-ERK (D13.14.4E), phospho-JNK (81E11), phospho-p38 (3D7), phospho-IκBα (14D4), Pyk2 (3292), and ERK (9102) monoclonal antibodies were obtained from Cell Signaling Technology. Actin was purchased from Sigma. NFATc1 (7A6) was purchased from Santa Cruz, as well as secondary anti-mouse and anti-rabbit HRP-conjugated antibodies.
**Ratiometric Ca\textsuperscript{2+} imaging**

For Ca\textsuperscript{2+} flux assays, 500 000 BMMs were seeded in 29mm glass bottom dishes (In Vitro Scientific). Cells were cultured with 1 ug/ml LPS in alpha-mem media for 16 hours. Cells were loaded with 3 µM Fura-2-AM (Invitrogen) in phenol red-free DMEM (Invitrogen). Cells were washed twice with HBSS and imaged immediately in phenol red-free DMEM containing 1ug/ml LPS. Fura-2 ratios were measured by alternate excitation at 340 nm and 380 nm. A Till Photonics digital microscopes equipped with Polychrome V monochromators, Fura-2 (340/380) filter set, a 20X 0.3NA objective lens, and high resolution (1344×1200) cooled CCD camera was used to capture images at a frequency of 1 image pair every 2 seconds. Data were acquired and analyzed using Live Acquisition software.

**Real-time PCR**

Adherent cells were lysed in Trizol. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed to cDNAs using EcoDry Premix (Oligo dT) (Clontech). 1 µg of total RNA was reverse transcribed to cDNA using SuperScript II according to the manufacturer’s instructions (Invitrogen). For real-time PCR, SYBR Green PCR Master Mix (Applied Biosystems) and primers specific for murine Tmem178, IL-1, IL-6, TNF-a, NFATc1, NFATc3, and cyclophilin were used as follows for Tmem178, ATGACAGGGATATTTTGCACCAT (forward) and CCGGTTCAGGATCATAGGAGCACCT (reverse); IL-1, GCTTTCCTTGTGCAAGTGCTCTGA (forward) and TCAAAAGGTCAGTGCACACT (reverse); for IL-6, TTCTCTGGGAAATCGTGGAAA (forward) and TGCAAGTGCGCATCGTGTGT (reverse); for TNFα, CTGTAGGGAGCATCGGGATG (forward) and TTGAGATCCATGCGCAGT (reverse); for NFATc1,
CCCGTCACATTCTGGTCCAT (forward) and CAAGTAACCGTGTAGCTGCACAA (reverse); for NFATc3 GCTCGACTTCAAACTCGTCTT (forward) and GATGTGGTAAGCCAAGGGATG (reverse) for cyclophilin AGCATACAGGTCCCTGGCATC (forward) and TTCACCTTCCAAAGACCAC-3’ (reverse). SYBR green dye was used for detection of the product using the SYBR Green PCR Master Mix assay (Applied Biosystems). The standard curve used a series of duplicate dilutions of plasmid for each gene and cyclophilin cDNA. The amplification reaction was performed for 40 cycles with denaturation at 95°C for 10 minutes, followed by annealing at 95°C for 15 seconds and extension and detection at 60°C for 1 minute. The relative abundance of each target was calculated as 1000×2−(Ct target gene−Ct cyclophilin), where Ct represents the threshold cycle for each transcript, and cyclophilin is the reference.

*LPS-induced sepsis*

WT and Tmem178-/- C57Bl/6 mice were injected with 25 mg/kg Lipopolysaccharides (LPS) from Escherichia coli 0111:B4 (Sigma). Blood was collected by submandibular bleed 2 hours after LPS administration, and serum was harvested by centrifugation at 8000g for 5 minutes at 4 degrees. Serum cytokines were measured using the mouse inflammatory cytometric bead array (BD Biosciences).

*LCMV-induced MAS*

LCMV-WE viral stocks were kindly provided by Dr. Maria Salvato (University of Maryland). For LCMV infections, mice were administered 10⁴ PFU of LCMV-WE injected intravenously. PBS- and clodronate-loaded liposomes were purchased from www.clodronateliposomes.org. For macrophage depletion, mice were administered 200 ul of clodronate-loaded liposomes.
intravenously. Control mice received 200 ul of empty liposomes. Mice were monitored for survival, or sacrificed on the indicated days for macrophage population analysis. To measure serum cytokines, blood was collected via submandibular bleed and irradiated by exposure to UV for 30 minutes. Serum was isolated via centrifugation at 8000 g for 5 minutes at 4 degrees. Serum cytokines were measured using a mouse inflammatory Cytometric Bead Array (BD Biosciences).

Flow cytometry

Immediately upon sacrifice, single-cell suspensions were prepared from spleen and liver. Single cell suspensions were generated by physical disruption and passage through a .70 um cell filter followed by red blood cell lysis. Cell suspensions were then washed once and stained in PBS with 0.5% FBS with the following anti–mouse antibodies: FITC-conjugated anti-F4/80 (eBioscience) or PerCP-Cy5.5-conjugated F4/80 (eBioscience); phycoerythrin (PE)-conjugated antibodies to CD11b (BD), Alex Fluor 647-conjugated CD206 (Serotec), PE-Cy5- conjugated CD80 (B7-1) (eBioscience), and eFluor 450-conjugated MHC Class II (eBioscience). The respective isotype-matched conjugated controls were purchased from eBioscience, BD, and Serotec. Acquisition was performed on a FACSCalibur or Fortessa and the dedicated software CellQuest (BD). Data were analyzed with FlowJo 7.5.5 software (Tree Star).

Statistics

All data represent mean ± SD. Data were analyzed using two-tailed Student’s t test, with a P value of <0.05 set as statistically significant. *p<0.05 **p<0.01, ***p<0.001
4.5 Results

**PLCγ2 regulates pro-inflammatory gene transcription in macrophages**

We previously found that PLCγ2-/- mice are protected from inflammation in the K/BXN model of serum-transfer arthritis which is strongly dependent on the neutrophil pro-inflammatory response. Mechanistically, PLCγ2 is required for neutrophil degranulation in response to integrin-mediated adhesion (14). We hypothesized that PLCγ2 modulates a similar pro-inflammatory pathway downstream of integrin in macrophages. WT and PLCγ2-/- bone marrow macrophages (BMMs) were plated on the generic integrin ligand Arg-Gly-Asp (pRGD) for 2 and 4 hours. WT BMMs significantly upregulate transcript levels of TNF-α, IL-1, and IL-6 after 2 and 4 hours of integrin adhesion, compared to WT cells in suspension (Figure 4.1 A). This inflammatory response is abrogated in BMMs lacking PLCγ2-/- NF-κB is a transcription factor critical for induction of inflammatory responses and cytokine production. We hypothesized that PLCγ2 modulates inflammatory cytokine transcription via NF-κB activation. In WT cells, integrin ligation induces rapid phosphorylation of IκBα, an indicator of NF-κb pathway induction (Figure 4.1 B). IκBα phosphorylation is significantly blunted in PLCγ2-/- BMMs, however. Thus, downstream of integrin ligation, PLCγ2 induces NF-κB activation to turn on inflammatory cytokine production.

In the osteoclast, both PLCγ2’s catalytic and scaffolding motifs are necessary to generate downstream signal transduction (15, 17) To determine which domain(s) of PLCγ2 are mediating this NF-κB activation, we expressed full-length PLCγ2 or PLCγ2 constructs harboring mutations in the catalytic or SH2 scaffolding domain in PLCγ2-/- BMMs. Expression of full-length PLCγ2
rescued IKBα phosphorylation in PLCγ2-/- cells stimulated on pRGD. Cells expressing catalytically inactive PLCγ2, however, were still unable to activate NF-kB, while the SH2 domain appears to be dispensable in this pathway (Supplementary Figure 4.1). Collectively, these data suggest that PLCγ2 controls the induction of a pro-inflammatory pathway in activated macrophages.

Tmem178 is a PLCγ2-dependent gene in inflammatory macrophages

To identify specific downstream modulators of this PLCγ2-dependent pro-inflammatory pathway, we performed a gene array experiment comparing WT and PLCγ2-/- BMMs plated on pRGD. Transmembrane protein 178 (Tmem178) was highly induced in WT BMMs after 4 hours of adhesion (Figure 4.1 C). PLCγ2-/- BMMs, however, failed to upregulate Tmem178 transcript. We next asked what other inflammatory stimuli can induce Tmem178 expression. Confirming the gene array, Tmem178 transcript levels are significantly upregulated in WT BMMs plated on pRGD. LPS treatment also strongly upregulates Tmem178 (Figure 4.1 D). On the other hand, Tmem178 expression is low in neutrophils, dendritic cells (DCs), and is not detected in T cells. These results position Tmem178 as a specific modulator of the pro-inflammatory macrophage, acting downstream of PLCγ2.

Tmem178-/- mice are more susceptible to sepsis

We have previously described the impaired inflammatory response of PLCγ2-/- mice in the context of arthritis (14). PLCγ2 has previously been shown to mediate inflammatory cytokine production in LPS-treated macrophages (18). To evaluate the role of Tmem178 during an inflammatory response in vivo, we utilized the endotoxin model of sepsis. Strikingly, Tmem178-
/- mice rapidly succumbed to systemic LPS, with the majority of knock-out mice dying within 16 hours of challenge (Figure 4.2 A). This mortality was associated with a significant increase in TNF-α levels detected in the serum 2 hours post-LPS administration (Figure 4.2 B). Tmem178/- mice do not present with spontaneous inflammation in vivo, supporting that Tmem178 may be most highly expressed and physiologically important during an immune challenge (Supplemental Figure 4.2). These in vivo data indicate that Tmem178 is a negative regulator of inflammation, in contrast to the pro-inflammatory role played by PLCγ2.

*Tmem178 restrains inflammatory cytokine production by macrophages*

To confirm that Tmem178 affects the macrophage inflammatory response in vitro, we measured inflammatory cytokine production by WT and Tmem178/- BMMs in vitro in response to adhesion and LPS. Confirming our previous observations, WT BMMs plated on pRGD increase TNF-α, IL-6, and IL-1 mRNA expression (Figure 4.2 C). Each of these cytokines were each markedly increased in Tmem178/- BMMs however. Similarly, LPS treatment increases inflammatory cytokine transcription in WT BMMs as expected, but this response is even amplified in Tmem178-null BMMs (Figure 4.2 D). Together with our previous expression profiles and in vivo results, these data indicate that Tmem178 acts in a negative feedback loop to dampen inflammatory signaling in the macrophage.

*Tmem178 targets Ca²⁺-NFAT signaling to repress inflammation*

We next wanted to examine the mechanism by which Tmem178 restrains the pro-inflammatory response. Adhesion and TLR ligation both activate NF-κB and MAPK signaling cascades. We next asked if Tmem178 affects these pathways to modulate inflammation. We first plated WT and Tmem178/- BMMs on pRGD to up-regulate Tmem178 expression in WT cells,
and then stimulated the cells with LPS. We find that p-JNK, p-ERK, and p-IκBα induction occurs rapidly and equivalently in both genotypes (Figure 4.3 A). NFATc1, however, is higher in Tmem178-/- cells prior to LPS stimulation. Activation via integrin or LPS alone also shows a similar increase in NFATc1 (Supplementary Figure 4.3).

Multiple NFAT isoforms are expressed in macrophages and contribute to inflammatory response (19). In addition to NFATc1, NFATc3 is also important to the inflammatory response. Importantly, NFAT isoforms are regulated at the transcriptional level by binding to and auto-amplifying expression at its own promoter. We measured NFAT transcripts in peritoneal macrophages ex vivo, as well as in BMMs stimulated with LPS, and found that both NFATc1 and NFATc3 are higher in Tmem178-/- cells (Figure 4.3 B, C).

In basal conditions, NFAT is sequestered in the cytosol due to masking of its nuclear localization sequence. The Ca^{2+}/calmodulin-dependent phosphatase calcineurin de-phosphorylates NFAT, inducing a conformational change to allow nuclear translocation. Thus, cytosolic Ca^{2+} fluxes are essential to NFAT activation. We hypothesized that Ca^{2+} oscillations may be amplified in the Tmem178-/- inflammatory macrophage. WT and Tmem178-/- BMMs were stimulated with LPS for 16 hours, and then loaded with the Ca^{2+}-binding dye Fura-2. We used ratiometric confocal imaging to measure cytosolic Ca^{2+}, as measured by Fura-2 emission following alternating excitation at 340 and 380 nm. We found that Ca^{2+} fluxes were higher in Tmem178-/- cells, and a greater percentage of cells display robust Ca^{2+} fluxes compared to WT cells (Figure 4.3 D). Thus, Tmem178 appears to target intracellular Ca^{2+} signaling to regulate inflammation.
M1 and M2 macrophage populations participate in MAS

In order to evaluate the role of Tmem178 specifically in a clinically relevant, macrophage-driven disease, we turned to macrophage activation syndrome (MAS). MAS bears many clinical similarities to the inherited disease (fHLH), which is also characterized by excessive macrophage infiltration and systemic inflammatory disease with a similar inflammatory cytokine profile (20). Jordan et al previously established a mouse model of fHLH/MAS utilizing perforin (prf)-/- mice infected with lymphocytic choriomeningitis virus (LCMV-WE). Prf-/- mice infected with LCMV recapitulate the pathology of MAS and fHLH patients (21). Despite the expanded presence of macrophages in this disease, it is unknown if macrophages are the drivers of disease through excessive antigen presentation and inflammatory cytokine production, or if macrophages are futilely attempting to control the pathology. Thus, we first wanted to evaluate the role of macrophages in this model. We administered clodronate-loaded liposomes (CLL) to specifically deplete macrophages, and confirmed the depletion by FACS of F4/80+ cells (Supplemental Figure 4.4). We administered CLL to prf-/- mice 2 days prior to LCMV infection, and every 3 days thereafter for the course of infection. Because virally-infected WT mice depleted of macrophages die due to liver failure (22), we used liposomes containing PBS as controls in WT and prf-/- mice. As expected, 100% of LCMV-infected prf-/- mice died within 21 days (Figure 4.4 A). Prf-/- mice with macrophages depleted by CLL survived for more than 40 days post-infection, similarly to WT mice. Serum cytokines measured on day 10 of infection showed that macrophage depletion strongly reduces IFN-γ and MCP-1 levels (Figure 4.4 B). We also wanted to know if macrophage depletion later in the course of infection could cure prf-/- mice of HLH. We began CLL treatment on day 6 after LCMV inoculation, as this was the time point when mice showed obvious signs of disease.
including weight loss, hunched posture, and ruffled fur. Surprisingly, mice treated on day 6 died more rapidly, reaching 100% mortality by day 16 (Figure 4.4 C). From these data we hypothesized that both pro- and anti-inflammatory macrophages are present during HLH, with the latter population playing an essential role to limit the infection later in disease.

To examine this possibility, we characterized M1 and M2 macrophage populations during the course of infection in prf-/- mice. The livers and spleens of mice were harvested after the indicated days of infection. Both M1 (F4/80⁺MHC Class II⁺CD86⁺) and M2 (F4/80⁺CD206⁺) populations increase in liver (Figure 4.4 D) and spleen (Figure 4.4 E). There is a significant and transient increase in M2 macrophages by day 7 of infection in the liver and days 7-10 in the spleen. These data suggest that an infiltrating population of anti-inflammatory macrophages is important to control disease progression.
4.6 Discussion

Current therapeutics to treat pathological bone loss are widely unsuccessful at addressing both the resorptive and inflammatory components of disease. We have previously established the fundamental role of PLCγ2 in regulating both osteoclast differentiation and bone resorption as well as the immune cell compartment in arthritic disease (14, 17, 23). PLCγ2-specific targeting to treat inflammatory bone loss is impractical due to its high homology with the ubiquitously expressed isoform PLCγ1. Moreover, we know little about PLCγ2-dependent regulators of the innate inflammatory response. Here we characterized Tmem178 as a PLCγ2-dependent negative regulator of the pro-inflammatory macrophage which acts by repressing Ca^{2+} oscillations and downstream NFAT activation. Tmem178 is also required in vivo to control and repress a systemic inflammatory response. Ongoing studies will address the role of Tmem178 in MAS, a complicating disorder of arthritis.

To understand how Tmem178 restrains inflammation, we examined signaling pathways that are known to be activated by TLR signaling to promote inflammatory cytokine production. Similar to our findings in the osteoclast, Tmem178 deletion did not affect NF-kB or MAPK induction, but rather targets Ca^{2+} fluxes leading to NFAT activation. We found that both Nfatc1 and Nfatc3 isoforms are increased in Tmem178-/- BMMs. Ca^{2+} has long been recognized as an absolutely essential second messenger for both adaptive and innate responses. Mechanisms of calcium signaling in the context of immunity have largely been discovered and characterized in the context of the T cell and B cell receptors (24). While not as closely studied, PLCγ2-dependent Ca^{2+} pathways have previously been shown to regulate inflammatory cytokine production in macrophages (25-29). Here we show that Tmem178 functions in a novel negative
feedback loop downstream of PLCγ2 to repress inflammation, thus building on the understanding of how intracellular calcium is regulated during an inflammatory response. Importantly, calcineurin inhibitors are widely used as immunosuppressive drugs, and while effective, this approach lacks specificity. Here we have characterized a novel and specific intrinsic mechanism of targeting calcium signaling in macrophages.

Circulating and tissue-resident monocyte/macrophage populations can dictate the immune response by producing a number of cytokines, both pro- and anti-inflammatory. Macrophages have been segregated into “classically activated” M1 and “alternatively activated” M2 subtypes. M1 macrophages are strongly pro-inflammatory and anti-tumorigenic through the production of IL-1, TNF-α, IL-6, and iNOS, as well as enhanced MHC Class II antigen presentation capacity. On the other hand, M2 macrophages produce arginase and IL-10 to resolve inflammation and promote tissue repair (30). The relative contribution of M1 and M2 populations in systemic auto-inflammatory disorders including sJIA is not well understood. Although sJIA patients have an overall expansion in activated monocytes, they present a mixed M1 and M2 phenotype both on an overall and single cell level (31, 32). Thus, a better understanding of the intrinsic regulatory mechanisms that control macrophage inflammatory responses could be informative to understanding the molecular pathogenesis of sJIA. Here we identify a novel negative feedback loop mediated by Tmem178 that is enacted to control the pro-inflammatory macrophage response.

The large majority of mortalities associated with sJIA are due to the confounding disorder MAS. MAS patients present with a sudden onset of fever, pancytopenia, and very high levels of inflammatory cytokines, TNF-α, IL-1, IL-6, IL-18, as well as IFN-γ in the serum. Strikingly, there is a huge increase in macrophages in the spleen, liver, and bone marrow of these
patients. Importantly, MAS is clinically nearly identical to familial hemophagocytic lymphohistiocytosis (fHLH), an inherited condition owing to a genetic defect in cytotoxic killing by T-cells and NK cells. In particular, patients have been identified with mutations in perforin, granzyme B, and vesicular trafficking components required for cytotoxic granule release. While some sJIA patients also harbor such genetic mutations that predispose them to MAS, it is not the case for all patients. The cause of MAS is unknown, and other than inherited conditions, there are currently no predictive indicators of which subset of sJIA patients will develop MAS. We have previously shown that Tmem178 is dysregulated in the context of sJIA, and defective Tmem178 expression is associated with excessive osteoclast development. We now show a role for Tmem178 in controlling inflammation as well, positioning Tmem178 as an essential regulatory component in both the inflammatory and erosive arms of sJIA.
4.7 References


4.8 Figure Legends

Figure 4.1 PLCγ2 regulates pro-inflammatory gene transcription in macrophages

A. WT and PLCγ2-/- BMMs were lifted and re-plated on integrin ligand for 2 and 4 hours. Inflammatory cytokines IL-6, IL-1, and TNF-α were measured by quantitative RT-PCR, with gene expression normalized to the housekeeping gene cyclophilin.

B. WT and PLCγ2-/- BMMs were lifted and re-plated on integrin ligand for the indicated time points. Whole cell lysates were subjected to SDS-PAGE and Western blot to detect p-IKBα as a read-out of NF-kB activation.

C. Gene array of WT and PLCγ2-/- BMMs plated on integrin ligand pRGD for 2 and 4 hours. Tmem178 is upregulated in adherent WT BMMs but not highly detected in PLCg2-/- BMMs.

D. Quantitative RT-PCR of Tmem178 transcript in untreated WT BMMs or stimulated via integrin or TLR, as well as in bone marrow-derived neutrophils, bone marrow dendritic cells (DC) and T cells.

Figure 4.2. Tmem178 negatively regulates the pro-inflammatory response of macrophages

A. WT and Tmem178-/- mice were treated with 25 mg/kg LPS administered IP and monitored for survival. n = 12 WT, 17 Tmem178-/-

B. Serum TNF-a was measured 2 hours after LPS injection in (A).

C-D. Quantitative RT-PCR of inflammatory cytokine transcripts in WT and Tmem178-/- BMMs plated on integrin ligand (C) or treated with 1 ug/ml LPS. TNF-α, IL-6 and IL-1 expression was normalized to the housekeeping gene cyclophilin.

Figure 4.3. Tmem178 reduces Ca^{2+} fluxes leading to NFAT induction in pro-inflammatory macrophages

A. WT and Tmem178-/- BMMs were plated on pRGD for 8 hours, and then stimulated with 1 ug/ml LPS for the indicated time points. Whole cell lysates were separate by SDS-PAGE and immunoblotted with the indicated antibodies to detect MAPK and NF-kB pathway activation, as well as NFATc1 induction. Pyk2 was detected as a loading control.

B-C. Nfatc1 and Nfatc3 transcripts were measured by quantitative RT-PCR with values normalized to cyclophilin in resident peritoneal macrophages (B, n = 4/genotype) or BMMs stimulated with 1 ug/ml LPS for 4 hours (C).

D. Intracellular Ca^{2+} fluxes in single BMMs treated with 1 ug/ml LPS for 16 hours prior to imaging. Each line traces a single cell, representative of 3 independent experiments.

Figure 4.4. M1 and M2 macrophage populations participate in a mouse model of MAS/fHLH

A. WT and prf-/- mice were infected with LCMV-WE. Prf-/- mice were depleted of macrophages by systemic administration of clodronate-loaded liposomes (CLL) prior to infection. n = 5/group

B. Serum IFN-γ and MCP-1 were measured in mice in (A) on day 10 of infection.
C. WT and prf-/- mice were infected with LCMV-WE. Prf-/- mice were depleted of macrophages by systemic administration of CLL beginning on day 6 of infection. n = 5/group

D-E. Prf-/- mice were infected with LCMV-WE to induce MAS/fHLH. Liver and spleen were harvested after the indicated days of infection to measure surface expression of markers for M1 macrophage (F4/80⁺MHC Class II⁺CD86⁺) and M2 macrophages (F4/80⁺CD206⁺) n = 5/group

Supplemental Figure 4.1 PLCγ2 catalytic activity is required for adhesion-induced NF-kB activation

BMMs were plated on pRGD for the indicated time points. NF-kB induction in PLCγ2/-/- BMMs transfected with WT-PLCγ2 (left panels), PLCγ2 catalytic inactive mutant (middle panels), or PLCγ2 SH2 mutant (right panels).

Supplemental Figure 4.2. Tmem178-/- mice do not display an inflammatory phenotype in basal conditions

A. Serum cytokine levels in WT and Tmem178-/- mice in basal conditions. WT n = 5, Tmem178-/- n = 9

B-C. M1 and M2 macrophages detected in the peritoneum and spleen of un-manipulated WT and Tmem178-/- mice

Supplemental Figure 4.3. Tmem178 specifically affects NFATc1 in pro-inflammatory macrophages

A. WT and Tmem178-/- BMMs were stimulated on LPS for the indicated time points. Whole cell lysates were separate by SDS-PAGE and immunoblifted with the indicated antibodies to detect MAPK activation, as well as NFATc1 induction. Actin was detected as a loading control.

B. WT and Tmem178-/- BMMs were plated on pRGD for the indicated time points. NFATc1 was detected by Western blot of whole cell lysates. Total ERK was detected as loading control.

Supplemental Figure 4.4. Confirmation of macrophage depletion by clodronate loaded liposomes

Flow cytometry to detect F4/80⁺ cells in the spleen of PBS- or clodronate-treated mice.
4.9 Figures

Figure 4.1

[Graphs and figures showing gene expression and protein levels for different conditions]
Figure 4.2
Figure 4.3

A

B

Peritoneal MΦ ex vivo

C

BMM + LPS 4 hours

D

WT BMM

Tmem178-/- BMM

Figure 4.3
Figure 4.4
Supplemental Figure 4.1
Supplemental Figure 4.2

A  Basal serum cytokines

MFI

IFNγ  IL6  IL-10  IL-12p70  MCP-1  TNF

■ WT n = 5
■ Tmem178-/− n = 9

B  Basal Peritoneal Lavage

% of Peritoneal Cells


WT  Tmem178-/−

C  Basal spleen macrophages

% of Splenocytes


WT  Tmem178-/−
Supplemental Figure 4.3

A

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- NFATc1
- p-JNK
- p-p38
- actin

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- NFATc1
- Total ERK
Supplemental Figure 4.4
Chapter 5. Discussion
5.1 Conclusions and future directions

In this work, we have identified Tmem178 as a new PLCγ2-dependent gene and demonstrated the first known function of Tmem178 in any cell type. Tmem178 is not similar to any other “Tmem” molecules, or any other transmembrane proteins expressed in the OC or macrophage. Other than its Claudin-2 domain, which is not known to harbor any intrinsic function, Tmem178 does not contain any conserved structural domains or similarity to other Ca^{2+}-related proteins. Moreover, we found that Tmem178 expression is induced downstream of PLCγ2-dependent pro-osteoclastogenic and pro-inflammatory pathways, but Tmem178 actually acts in a negative feedback loop to restrain both osteoclastogenesis and inflammatory cytokine production. Thus, the findings presented in this thesis were neither obvious nor expected.

Current therapeutics to treat pathological bone loss are widely unsuccessful at addressing both the resorptive and inflammatory components of disease. Moreover, even patients who respond favorably to anti-inflammatory agents can suffer from progressive joint disease. Thus, an essential question in the field is how we could simultaneously target activation of both immune cells and osteoclasts. Our lab previously undertook studies to identify common signaling components shared in the osteo-immune system and successfully identified PLCγ2 as such a critical signaling molecule. PLCγ2/-/- mice are protected from inflammatory bone loss due to a blockade in both bone resorption as well as immune cell activation. While PLCγ2 seems an appealing therapeutic target, its extensive homology with other PLC isoforms presents a challenge for specific targeting in vivo.
We devised a strategy to specifically target PLCγ2 via its unique SH2 domains. By ectopically expressing a construct comprised of the tandem SH2 domains of PLCγ2, we were able to inhibit osteoclastogenesis in vitro by impeding protein scaffolding at RANK. Importantly, expression of this (N+C)-SH2 construct in vivo via an adenoviral vector also successfully repressed inflammatory osteolysis (1). This approach, however, did not block inflammation in vivo. Therefore, we must focus on characterizing unique downstream effectors of PLCγ2 which modulate osteo-immune activation.

Using a gene array approach to identify PLCγ2-dependent genes, we found that the uncharacterized protein Tmem178 was highly induced in stimulated WT cells but not in PLCγ2-/- cells. Tmem178 expression is upregulated in both osteoclasts in response to RANKL, as well as in macrophages in response to adhesion or TLR ligation. Importantly, Tmem178 expression appears to be limited to these compartments, and we do not detected Tmem178 transcript in osteoblasts, neutrophils, or T cells. Thus, Tmem178 may be the specific, PLCγ2 downstream effector we were seeking which could control osteo-immune activation. In striking contrast to the osteopetrotic PLCγ2-/- mice, Tmem178-null mice present with low bone mass in basal conditions and exaggerated osteoclast formation and bone loss during inflammatory arthritis. In vitro, Tmem178-/- cells display an enhanced ability to form osteoclasts. Thus, Tmem178 is a surprising negative regulator of osteoclastogenesis. Mechanistically, Tmem178 specifically targets NFATc1 induction in osteoclasts by controlling ER Ca^{2+} mobilization.

Ca^{2+} is a ubiquitous signaling mediator in nearly all cell types and thus calcium signaling must be regulated in all cell types, so why is Tmem178 so unique and its expression seemingly very limited? In primary cells, elegant studies have been performed
to characterize Ca$^{2+}$ signaling mechanisms in the context of the T and B cell receptor, or innate immune cells such as mast cells. In these cell types, receptor ligation induces an acute and transient spike in cytosolic Ca$^{2+}$, leading to an immediate signaling event. This is not the case in OCs, however, where intracellular Ca$^{2+}$ fluxes are sustained over a longer time course. Moreover, the amplitude and duration of dynamic Ca$^{2+}$ signals have been shown to differentially modulate activation of various transcription factors. The OC uses Ca$^{2+}$ to turn on NFATc1 activation. NFAT proteins have enhanced Ca$^{2+}$ sensitivity compared to other transcription factors such as NF-kB or JNK, which enables NFAT transcriptional activation by low Ca$^{2+}$ levels (2). In the OC, a low but prolonged Ca$^{2+}$ signal induces NFATc1 nuclear translocation and long-lasting nuclear localization throughout osteoclastogenesis. Therefore, it is conceivable that the OC requires additional regulatory mechanisms to carefully buffer ongoing and subtle changes in cytosolic Ca$^{2+}$.

Interestingly, Tmem178 couples to the ER Ca$^{2+}$ sensor Stim1, which is most well-characterized for its coupling to Orai1 to activate store-operated Ca$^{2+}$ entry (SOCE). Surprisingly, we did not detect a direct effect of Tmem178 expression on SOCE, nor did we find that Tmem178 interacts with Orai1 or modulates Orai1-Stim1 coupling. These findings that Tmem178 associates specifically with Stim1 independent of SOCE raise the possibility that Stim1 is also participating in ER Ca$^{2+}$ release in OCs. In recent studies, Stim1 has been shown to play a possible role in affecting ER Ca$^{2+}$ release through the IP3R channels (3, 4). IP3R activity is highly regulated by multiple adaptor proteins as well as kinases which phosphorylate IP3R to increase its sensitivity to IP3 (5). We have not been able to detect an association between Tmem178 and endogenous IP3Rs,
however. It is possible that Tmem178-Stim1 is affecting Ca$^{2+}$ release through the ryanodine receptor whose regulation is less well-understood than IP3Rs (6).

Further studies are underway to understand which functional domain(s) of Tmem178 are important to its function and coupling to Stim1, and vice versa. While very little information is available about Tmem178 structure, we know it contains 1 claudin-2 domain. Structure/function studies showed that the C-terminal tail of claudin-2 domain-containing proteins is required for their function, and phosphorylation of a Serine residue in the C-terminus was also shown to promote membrane localization and control ion transport (7-9). Therefore, we have generated a C-terminus truncation mutant of Tmem178 to evaluate its ability to bind to Stim1 and repress ER Ca$^{2+}$ release.

Extensive structure-function experiments delineated the mechanism of Stim1 activity during SOCE. In the ER luminal portion of the protein, Stim1 harbors a Ca$^{2+}$-binding EF hand as well as a SAM domain which facilitates protein-protein interactions. When ER Ca$^{2+}$ stores are depleted, Ca$^{2+}$ dissociates from the EF hand and causes EF-hand and SAM domains to interact between Stim1 molecules. This induces a conformational change in the cytoplasmic domain leading to the unfolding and extension of the C-terminus; this is the “activated” Stim1 dimer. Stim1 continues to oligomerize as it is re-localizing to the ER-plasma membrane junction. At the C-terminus of Stim1, the STIM-Orai1 activating region (SOAR) binds and activates Orai1, allowing CRAC channel activation (10). The domains of Stim1 which interact with IP3Rs have not been identified. By expressing the individual domains of Stim1, we can determine which regions are necessary and sufficient to interact with Tmem178. It is also necessary to understand if the same regions of Stim1 which modulate Orai1 activation, namely the
SAM and SOAR domains, are necessary to associate with Tmem178. Moreover, we have not assessed the temporal or spatial aspects of the Tmem178-Stim1 interaction. In future live cell imaging studies, we must understand when the Tmem178-Stim1 interaction occurs downstream of receptor signaling, and how this affects Stim1 translocation that is necessary for SOCE. We have observed that Tmem178 does not affect SOCE, suggesting that perhaps separate pools of Stim1 exist in the ER to regulate different intracellular Ca\(^{2+}\) events.

Having confirmed that Tmem178 is a negative regulator of osteoclastogenesis in vitro and in vivo, we sought a role for Tmem178 in human disease. We turned to systemic juvenile idiopathic arthritis (sJIA), as it is a predominantly monocyte-mediated auto-inflammatory disordered associated with progressive joint disease. Using the plasma from sJIA patients, we found that Tmem178 is strikingly down-regulated by sJIA plasma, permitting more exuberant osteoclastogenesis. Importantly, patients who presented with active erosive disease were each associated to significantly down-regulated Tmem178 levels in vitro. While our sample size at this point is too small to draw a correlation between defective Tmem178 expression and active joint erosion, it will be interesting in the future to see if Tmem178 levels could be a predictive biomarker of patients who will develop progressive joint disease.

sJIA is unique from other JIA subtypes in its dependence on the innate immune response, specifically monocytes, in disease pathogenesis with a limited role of the adaptive immune response. sJIA is not associated with MHC allele susceptibility or rheumatoid factor. In fact it is so distinct from other JIA subtypes that the categorization is considered by some to be a misnomer. Intriguingly, M2-like monocytes appear during
episodes of disease flare and persist into the transition to a quiescent state, suggesting that this M2 population arises in an attempt to ameliorate the systemic inflammatory environment (11, 12). In fact, it is still unknown whether the excessive pro-inflammatory response or defective anti-inflammatory response truly underlies the pathologies of sJIA.

We know that Tmem178 expression is specific to activated myeloid cells, including macrophages exposed to inflammatory stimuli such as TLR ligands. The trigger of sJIA is unknown, but endogenous TLR ligands are possible activators of the auto-inflammatory reaction. In studying the role of Tmem178 in the pro-inflammatory macrophage, we found that Tmem178 limits inflammatory cytokine release in response to both adhesion and LPS stimulation. Similarly, Tmem178-/- mice succumb faster to endotoxin-induced sepsis. These data position Tmem178 as a negative regulator of the pro-inflammatory macrophage response. While we have found that Tmem178 is down-regulated by sJIA serum during osteoclastogenesis, we have yet to characterize the role of Tmem178 in the inflammatory component of sJIA. It is possible that Tmem178 expression is also decreased in circulating inflammatory monocytes of sJIA patients. This reduction of Tmem178 would ramp up the pro-inflammatory response and hinder the anti-inflammatory attempts of M2-like monocytes. In these conditions, Tmem178 levels may also be a marker of the subset of sJIA patients who develop an acute, potentially fatal disorder known as macrophage activation syndrome (MAS).

While the cause of MAS is uncertain, it is possible that a dysregulated adaptive immune response to an infectious trigger stimulates the over-active macrophage population. It is actually unknown how the macrophage is participating in disease. It is possible that macrophages are attempting to control the adaptive immune response. On
the other hand, macrophages may be driving the pathology by facilitating excessive antigen presentation to T cells and producing inflammatory cytokines. The specific contribution of M1 and M2 macrophages during a course of MAS has not yet been studied.

MAS bears many clinical similarities to the inherited disease familial hemophagocytic lymphohistiocytosis (fHLH), which is also characterized by excessive macrophage infiltration and systemic inflammatory disease with a similar inflammatory cytokine profile (13). We used a mouse model of fHLH to define the requirement of macrophages in this disease (14). In preliminary studies, we have found that both M1 and M2 macrophage populations increase during the LCMV-induced model of MAS. In fact, limiting the M1 response early in disease is sufficient to protect mice from disease, while eliminating the infiltrating anti-inflammatory M2 population accelerates mortality. Could Tmem178 be participating in these distinct phases of macrophage activity? To this end, we have generated Tmem178-perforin double knock out mice in which we can induce MAS. If Tmem178 is important to the attempts to control MAS early in the course of disease, the double knock out mice will succumb faster than perforin-null mice alone. If so, it would be interesting to measure Tmem178 transcript levels in the monocytes of sJIA patients who go on to develop MAS.

In sum, this work defines the function of Tmem178 as novel regulator of osteo-immune activation. In contrast to our expectations, these studies identified a novel negative feedback loop acting via Ca\(^{2+}\) to control myeloid cell activation. As this is the first work describing Tmem178 in any cell type, there are many more studies to be done to fully understand the function of Tmem178 in homeostasis and disease, particularly in
sJIA. Most importantly, we identified a new pathway of Ca$^{2+}$ regulation in myeloid cells as a mechanism that can control both joint erosion and excessive inflammation.
5.2 References


