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O7

GM-CSF and IL-4 enhance dendritic cell differentiation and inhibit osteoclastogenesis by ectodomain shedding of M-CSF receptor in monocytes

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Monocytes are a common precursor for both osteoclasts (OC) and myeloid dendritic cells (DC). We found that GM-CSF and IL-4 potently induce myeloid DC and completely suppress OC formation in the presence of excess M-CSF and soluble RANK ligand. However, the precise mechanism for regulating such deflected differentiation from monocytes is unclear. The present study was undertaken to clarify the mechanism of triggering deflection of OC and myeloid DC differentiation from human monocytes, by focusing on the effect

of GM-CSF and IL-4 on M-CSF signaling. GM-CSF and IL-4 potently down-regulated surface expression of M-CSFR on monocytes and concomitantly up-regulated soluble form of M-CSFR in their culture supernatants, suggesting ectodomain shedding of membrane-bound M-CSFR. Interestingly, GM-CSF and IL-4 potently enhanced the activity as well as mRNA expression of TNF-alpha converting enzyme (TACE), a sheddase for M-CSFR. TAPI-0, a TACE inhibitor, restored cell-surface M-CSFR and decreased soluble M-CSFR in monocytes in the presence of GM-CSF and IL-4. Importantly, TAPI-0 restored OC formation induced by M-CSF and RANK ligand even in the presence of GM-CSF and IL-4 in a soluble M-CSFR-inhibitable fashion. These observations demonstrate that GM-CSF and IL-4 inhibit M-CSF and RANK ligand-induced OC formation by enhancing TACE expression and activity, and that TACE mediates M-CSFR ectodomain shedding to disrupt M-CSF signaling by down-regulating M-CSFR and up-regulating soluble M-CSFR as a decoy receptor. These results unveil a novel inhibitory mechanism of M-CSF signaling by GM-CSF and IL-4 in deflection of OC and DC differentiation from monocytes.

O8

Disrupted Wnt signaling in LRP6 mutant ringelschwanz caused by impaired interaction with Mesd leads to up-regulation of RANKL and increased bone resorption

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Loss- and gain-of-function mutations in low-density lipoprotein receptor-related protein 5 (LRP5) result in low and high bone mass, respectively. Although both LRP5 and LRP6 function as co-receptors for Wnt ligands, the roles of LRP6 in bone metabolism have not been fully elucidated. We previously identified a spontaneous point mutation ringelschwanz (rs) in murine Lrp6 leading to an amino acid substitution R886W. To address the roles of Lrp6 in bone metabolism, we analyzed the bone phenotype in rs mutant mice. Bone histomorphometry showed decreased bone volume and, unexpectedly, increased eroded surface in rs/rs mice. In consistent with this observation, the RANKL mRNA expression was increased in primary osteoblasts derived from calvariae of rs/rs mice. Treatment with Wnt3a as well as over-expression of Lrp6 suppressed the RANKL expression induced by $1,25(OH)_2D$ in ST2 cells, a marrow-derived stromal cell line. As the next step, we performed functional analyses to determine how R886W mutant Lrp6 (Lrp6[rs]) influenced Wnt signaling and its localization at cell surface. When introduced into ST2 cells, mLrp6[rs] failed to increase T-cell Factor (TCF) reporter activity efficiently compared with wild-type Lrp6 (Lrp6[WT]). Co-immunoprecipitation studies demonstrated that mLrp6[rs] exhibited impaired interaction with Mesd (mesoderm development), a molecular chaperone for Lrp6. Immunofluorescent studies showed that mLrp6[WT] were localized to cell surface when transfected along together with Mesd, whereas mLrp6[rs] accumulated in perinuclear regions. Taken together, our results indicate that disrupted Wnt signaling in Lrp6 mutant ringelschwanz caused by impaired interaction with Mesd leads to up-regulation of Rankl and increased bone resorption.

O9

Oncostatin M critically regulates both bone formation and bone resorption

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The gp130 receptor is common to many cytokines, including IL-6, IL-11, LIF and CT-1. In mice, oncostatin M (OsM) signals through gp130 and a ligand-specific receptor (OsMR). Osteoblasts express OsMR, and OsM increases osteoclast formation in the presence of osteoblasts. The effects of OsM on bone formation however, are not clearly defined.

To determine the effects of OsM on bone formation, the 4B10 stromal cell line was treated with OsM (1.25 - 10ng/ml). Under mineralising conditions, OsM treated cells mineralised more rapidly, and at higher levels than untreated controls. Under adipogenic conditions, adipocyte formation was dramatically reduced.

To determine whether OsM critically regulates bone formation we studied the bone phenotype of 10 week old OsMR null mice. Male and female OsMR null mice demonstrated a 75% increase in BV/TV in the distal tibia. Femoral trabecular BMD was elevated by 40% in both males and females. Cortical dimensions and density were not significantly altered. This mild osteopetrosis was associated with a significant (~30%) reduction in osteoclast surface and reduced resorption. Bone formation was also significantly reduced, evidenced by low osteoid thickness and osteoblast surface. In contrast the number of marrow adipocytes was elevated approximately 4 fold. This reveals a critical role for OsMR in maintaining normal levels of bone resorption and formation in vivo, as well as an inhibitory role in adipocyte differentiation.

O10

Cytoplasmic terminus of a V-ATPase accessory subunit Ac45 is necessary for interaction with V_o domain subunits and play a critical role in osteoclastic bone resorption

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Solubilization of bone mineral by osteoclasts is dependent on the acidification of the extracellular resorption lacuna by means of a multimeric vacuolar type proton pump (V-ATPases). Besides such specialized function the V-ATPases is also essential for acidification of diverse intracellular compartments that includes the Golgi apparatus, endosomes, lysosomes and secretoy granules. The core structure of V-ATPases comprises of two functionally and structurally distinct domains, V_1 and V_0. The peripheral cytoplasmically oriented V_1 domain is responsible for ATP hydrolysis which provides the energy for the translocation of protons across the integral membrane bound V_0 domain. Here, we have identified an accessory subunit, Ac45 that interacts with the V_0 domain and is involved in V-ATPase-mediated function in osteoclasts. Ac45 was localized to the ruffled border region of polarized resorbing osteoclasts and partially colocalized with pH-dependent transferrin receptor and lysotracker, marker of lysosomal structures. Furthermore using bioluminescence resonance energy transfer (BRET) assays, we showed that Ac45 interacts with subunits a_3, c, c" and the newly identified e, but not d of the V_0 domain. Deletion of the 26 residue-cytoplasmic tail (aa437-463) of Ac45 (Ac45ΔC) disrupted the interaction of Ac45 with subunits of V_0 domain. Strikingly, over-expression of Ac45ΔC in osteoclasts by a retroviral system impaired bone resorption in vitro compared to Ac45 wild type and GFP control. In all, our data indicate that cytoplasmic terminus of Ac45 is essential for interaction with V-ATPase V_0 domain and plays a critical role in osteoclastic bone resorption.

O11

Expression profile of RhoGTPases and RhoGEFs during RANKL-stimulated osteoclastogenesis: identification of essential genes in osteoclasts

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The formation of osteoclasts requires significant actin cytoskeletal rearrangements as mononuclear haemopoietic cells fuse to form large flat multinuclear cells capable of resorbing bone. The importance of actin cytoskeletal reorganisation not only limits itself to differentiation of the osteoclast but also adhesion to bone, the formation of a sealing zone and osteoclast movement. Key regulators of actin dynamics as found in many other cellular systems are the RhoGTPases. These highly conserved enzymes control cell adhesion, migration and morphology through their action on actin cytoskeleton. In mouse, there are 18 RhoGTPases which are activated by guanine nucleotide exchange factors (RhoGEFs), which compose 76 RhoGEFs in total. In order to investigate whether RhoGTPases and RhoGEFs could play an important role in osteoclasts, mRNA levels of

the complete families of RhoGTPases and RhoGEFs during RANKL-stimulated osteoclastogenesis were investigated while small hairpin RNAs were used to knock down genes of interest. Of the 18 RhoGTPases and 76 RhoGEFs, only three genes were up-regulated by RANKL: Wrch1 (RhoGTPAse) and Net1 and Dock5 (RhoGEFs). Silencing of Wrch1 and Arhgef8 expression severely inhibited differentiation and affected osteoclast morphology. Dock5 suppression was lethal in osteoclast precursors while having no effect in fibroblasts. In conclusion, three genes were identified amongst RhoGTPase signalling pathways that are up-regulated during RANKL-induced osteoclastogenesis. These genes are novel essential actors in osteoclasts, most likely through the control of actin cytoskeleton dynamics.

O12

GP130 STAT1/3 signals are required for osteoblast support of osteoclastogenesis

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In co-cultures of osteoblasts and bone marrow haemopoietic cells, osteolytic factors such as IL-11, and 1,25$(OH)_2$-D_3 stimulate osteoclast (OC) formation by inducing RANKL expression by osteoblasts. Osteoclastogenic action of PTH, PGE_2 and 1,25$(OH)_2$-D_3 depends on their stimulation of autocrine osteoblast IL-11 production. gp130 mediates IL-11, IL-6, OSM and CT-1 action through STAT1/3 and SHP2/ras/ERK signals which are ablated in, respectively, $gp130^{\Delta STAT}/gp130^{\Delta STAT}$ (Δ/Δ) and $gp130^{Y757F}/gp130^{Y757F}$ knock-in mice. Employing osteoblasts from these mice we found IL-11-stimulated osteoclastogenesis requires STAT1/3 but not SHP2/ras/ERK signals. However, the role of these signals in RANKL induction is unknown.

Unlike wild type (WT) osteoblasts, Δ/Δ mouse osteoblasts did not support OC formation from WT bone marrow cells in response to IL-11, IL-6, CT-1 or OSM. Consistent with this, IL-11 caused a sustained 30-fold increase in RANKL mRNA levels in WT osteoblasts but only a transient 3-fold increase in Δ/Δ osteoblasts and little effect on OPG mRNA levels. Δ/Δ osteoblasts also formed very few OC in response to 1,25$(OH)_2$-D_3 or 1,25$(OH)_2$-D_3/PGE_2 compared to WT osteoblasts, however 1,25$(OH)_2$-D_3/PGE_2 treatment robustly induced RANKL and suppressed OPG mRNA levels in both Δ/Δ and WT osteoblasts. Furthermore, addition of exogenous RANKL did not rescue 1,25$(OH)_2$-D_3/PGE_2 dependent osteoclastogenesis in Δ/Δ osteoblasts, although dexamethasone addition did.

Combined, these data suggest osteoblast gp130 STAT1/3 are required for maximal OC generation in co-cultures, is not due to a lack of RANKL production. This may point to suppression of an osteoclastogenesis inhibitor by STAT1/3 signals and perhaps also by dexamethasone treatment.

013

A role for TWEAK in human bone remodelling

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TNF-like weak inducer of apoptosis (TWEAK), a member of the TNF superfamily is pro-angiogenic, pro-inflammatory and proliferative for endothelial and other cell types. We recently showed that TWEAK is a novel mediator of mouse collagen-induced arthritis(1) and have identified TWEAK expressing cells in human rheumatoid arthritis (RA) tissues. The aim of this work was to investigate a potential role for TWEAK in human bone remodelling.

Human primary osteoblasts (OB) and were found to express high levels of the TWEAK receptor, Fn14, by flow cytometry. OB also expressed abundant TWEAK mRNA. Treatment of OB with exogenous TWEAK was highly proliferative. TWEAK dose-dependently suppressed OCN mRNA expression and increased both the RANKL:GAPDH and the RANKL:OPG mRNA ratios. Time-course studies under mineralising conditions showed that TWEAK suppressed the osteogenesis-related genes OCN and BSP-1 early in cultures, corresponding with the period in which RANKL expression was increased. Whereas RANKL induction was

transient (up to day 9), OCN, BSP-1 and type I collagen mRNA expression were inhibited throughout the mineralisation period. Consistent with this, continuous TWEAK exposure inhibited *in vitro* mineralisation by OB and induced the expression of sclerostin, which inhibits bone formation *via* BMP/Wnt signalling. TWEAK also had direct effects on osteoclastogenesis, enhancing RANKL-mediated osteoclast formation from human PBMC precursors. These data, showing that TWEAK can negatively regulate osteogenesis and also act pro-resorptively, provide the first evidence for TWEAK as a novel regulator of bone turnover and are consistent with a role for TWEAK in inflammatory bone loss pathologies, such as RA.

1. Perper S *et al.* 2006 TWEAK is a novel arthritogenic mediator. J Immunol (In Press)

O14

Calcium/calmodulin-dependent kinase activity is required for efficient induction of osteoclast differentiation and bone resorption by receptor activator of nuclear factor Kappa B ligand (RANKL)

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Many bone lytic disorders associated with inflammatory arthritis and immobility and post-menopausal osteoporosis are mediated by enhanced osteoclast formation and activation; therefore, identification of novel molecular targets and mechanisms which capable of controlling osteoclasts activities and functions are essential. The multifunctional calcium/calmodulin-dependent protein kinase (CaMK) is a major down stream mediator of Ca^{2+} signaling in a wide range of cellular functions, including ion channel and cell cycle regulation and neurotransmitter synthesis and release. In here, we have investigated the role of the CaMK signaling pathway in osteoclast differentiation and bone resorption. First of all, our result showed that the CaMKI, CaMKII γ isoforms were present in both bone-marrow derived macrophages and $RAW_{264.7}$ murine macrophage cell line, and that expression persisted during osteoclast differentiation in the presence of Receptor Activator of Nuclear factor Kappa B (NF-κB) Ligand (RANKL). RANKL-induced differentiation was accompanied by increased cyclic AMP response element transcriptional activity, and ERK phosphorylation, which are both downstream targets of CaMK. In an attempt to determine the role of CAMKs in RANKL-mediated osteoclastogenesis, two selective inhibitors of CaMKs, KN-93 and KN-62 were used. We found that both inhibitors suppressed osteoclastogenesis in a concentration, timing-dependent and reversible manner. In addition, this inhibition was accompanied by suppression of cathepsin K expression and osteoclastic bone resorption, which are markers for differentiated osteoclast function; as well as RANKL-induced ERK phosphorylation and CREB transcriptional activity. In summary, results generated from this study for the first time imply an important role for CaMK in osteoclast differentiation and bone resorption.