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Crosstalk among interleukin 23 and DNAX activating protein 12-dependent pathways promotes osteoclastogenesis

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Abstract

Interleukin 23 (IL-23) has been well studied in the context of T cell differentiation, however, its role in the differentiation of myeloid progenitors is less clear. In this paper we describe a novel role of IL-23 in myeloid cell differentiation. Specifically, we have identified that in human peripheral blood mononuclear cells IL-23 induces the expression of MDL-1 a PU.1 transcriptional target during myeloid differentiation, which orchestrates osteoclast differentiation through activation of DAP12 and its immunoreceptor tyrosine activation motifs (ITAM's). The molecular events that lead to the differentiation of human macrophages to terminally differentiated osteoclasts are dependent on SYK and PLCγ2 phosphorylation for the induction of intracellular calcium flux and the subsequent activation of master regulator osteoclast transcription factor nuclear factor of activated T cells cytoplasmic 1 (NFATc1). IL-23 elicited osteoclastogenesis is independent of the RANKL pathway and utilizes a unique MDL-1^{+}/$DAP12^{+}$ cell subset. Our data define a novel pathway that is utilized by IL-23 in myeloid cells and identify a major mechanism for the stimulation of osteoclastogenesis in inflammatory arthritis.

INTRODUCTION

The mononuclear phagocyte system (MPS) consists of a population of cells derived from progenitor cells in the bone marrow, which differentiate to form neutrophils and monocytes, and contribute to immunosuppression, disease resolution, and tissue repair (1). Macrophage-colony stimulating factor (M-CSF) signalling through its receptor (CSF-1R) promotes the differentiation of myeloid progenitors into heterogeneous populations of monocytes,

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macrophages, dendritic cells, and bone-resorbing osteoclasts (2). On the contrary to disease resolution myeloid populations elicited by MCSF are also associated with exacerbation of a broad spectrum of pathologies, including cancer, inflammation, and bone disease (3).

MCSF and receptor activator of nuclear factor κ B ligand (RANKL) are essential for the differentiation of osteoclasts from human bone marrow and circulating monocyte precursors (4–6). Pro-inflammatory mediators such interleukin 17 (IL-17) have also been observed to contribute to the proliferation and differentiation of myeloid progenitors (7–9). IL-17 is mainly secreted by Th17 cells and the differentiation of these Th17 cells is largely regulated by interleukin 23 (IL-23) (10). We have previously shown that gene transfer of IL-23 in rodents induces myelopoiesis, which also results in severe bone destruction (11). IL-23 is predominantly expressed by monocytes and dendritic cells and acts via IL-23R, which is expressed at low levels on monocytes (12–14). As IL-23R is also expressed on $CD4^+$ T cells the actions of IL-23 in osteoclast differentiation from myeloid precursors have been largely overshadowed by the ability of Th17 cells to produce RANKL and hence the interactions of IL-23 with $IL\text{-}23R^+$ myeloid cells are only partly known (15). In this paper we sought to examine the cellular and molecular mechanisms that regulate IL-23-induced osteoclast differentiation in myeloid cells.

T-cells and myeloid cells share a requirement for costimulatory signals that are mediated by immunoreceptor tyrosine-based activation motifs (ITAMs). The ITAM is a conserved signalling motif contained in the cytoplasmic domain of transmembrane adaptor molecules that associate with and transmit signals from various immunoreceptors. In myeloid cells, immunoreceptors signal through two main ITAM-containing adaptors, the DNAX activating protein of 12 kDa (DAP12) and FcRγ, to regulate osteoclastogenesis. Double deletion of DAP12 and FcRγ in mice leads to impaired osteoclast differentiation and osteopetrosis (16). Deletions in the DAP12 gene in humans, causes Nasu-Hakola disease, which is characterized by bone fractures and presenile dementia (17). DAP12 associates with multiple immunoreceptors in myeloid precursors including Myeloid DAP12 associated Lectin (MDL)-1. MDL-1 is a type II transmembrane protein that belongs to the C-type lectin superfamily. It is exclusively expressed in monocytes, macrophages and dendritic cells and contains a charged residue in the transmembrane region that enables it to pair with DAP12 (18). The ligation of ITAM-coupled receptors in myeloid cells leads to the phosphorylation of ITAM tyrosine residues by SRC family kinases, followed by the recruitment and activation of the spleen tyrosine kinase (SYK) (19). ITAM-coupled receptors and cytokine receptors were shown to be linked by calcium-mediated signaling pathways, and the ITAM-dependent activity of calcium-dependent calmodulin kinase (CaMK) and protein tyrosine kinase 2 (PYK2) were found to augment IFN-induced JAK (and STAT1) activation (20).

In this manuscript we describe a novel interaction of IL-23 signalling with ITAM-coupled receptors in human $CD16^+/MDL\text{-}1^+/DAP12^+$ cell subsets. These interactions lead to the phosphorylation of SRC, recruitment of SYK, and activation of NFATc1 to induce the terminal differentiation of these progenitor cells to osteoclasts (16, 21–26). Our data define a novel pathway that is utilized by IL-23 in myeloid cells and identify a major mechanism for the stimulation of osteoclastogenesis in inflammatory arthritis.

MATERIALS AND METHODS

Reagents and Antibodies

Soluble RANKL, OPG, IL-23, TNF, RANKL ELISA and anti-MDL-1/CLEC5A antibody (283834) were purchased from R&D Systems (USA). Anti-phospho-SYK (Y525/526, Y323, Y352), anti-PLCγ2 (polyclonal), and anti-phospho-PLCγ2 (Y759) antibodies were purchased from Cell Signaling (USA). Anti-SYK antibody (SYK-01) was purchased from Abcam (UK). Anti-GAPDH, (6C5) anti-SRC (GD11), anti-phospho-SRC (Y418), anti-phosphotyrosine (4G10) antibodies were purchased from Millipore (USA). Anti-human CD16 PE-cy7 (clone CB16), isotype Mouse IgG1κ and anti-human CD16 PE-cy5 (clone 3G8) and isotype mouse IgG1 PE-cy5 were from Ebioscience USA and Biolegend USA respectively. Anti-human CD14 FITC and isotype Mouse IgG1 FITC were from Biolegend, USA. Anti-human DAP-12 PE and isotype control Rat IgG1 PE were purchased from Beckman Coulter and Ebioscience, USA. Anti-NFATc1 antibody (7A6) was purchased from Thermo Scientific (USA). Anti-DAP12 antibody (406288) was purchased from Exalpaha (USA). Anti-IL-23R (H-300) was purchased from Santa Cruz (USA).

Surface and intracellular staining

For surface staining, 5×10^6 PBMCs were blocked with 2% FBS in PBS and then stained with CD14-FITC, CD16 PE-cy7 or MDL-1-APC with appropriate isotype controls on ice for 30 min. Cells were then washed with FACS buffer. Cells were acquired on a DAKO Cyan or BD Aria and analysed using FlowJo software (TreeStar Ashland, OR). For intracellular cytokine staining, cells were fixed with 4% paraformaldehyde fixation and then permeabilized with 0.5% saponin and then, were stained with anti-DAP12-PE mAb along with proper isotype control.

Human osteoclast cultures and functional assessment of osteoclast formation

UC Davis Institutional Review Board committee approved all protocols. Peripheral blood mononuclear cells were isolated from healthy volunteers by gradient density centrifugation with Histopaque-1077 (Sigma-Aldrich, St Louis, USA) as previously described(27). $CD14^+$ cells were isolated with the MACS monocyte isolation Kit (Miltenyi Biotech). PBMC or $CD14^+$ cells were added to 96-well tissue culture plates containing dentine slices (prepared in house) or coverslips (Electron Microscopy Sciences) as previously described(27). PBMC cultures were maintained in the presence of 30 ng/ml sRANKL and 25 ng/ml M-CSF as positive control or only M-CSF as a negative control. Functional assessment of osteoclast formation was performed using TRAP, F-actin, bone resorption assays, qPCR and scanning electron microscopy as previously described (28), (27).

Lentivirus transduction of DAP12 ITAM mutant forms

DAP12 ITAM mutant forms Y91F, Y102F and Y91F/102F (2YF) were generated by site directed mutagenesis (Stratagene) and clones were confirmed by sequencing. The majority of hematopoietic cells are non-dividing or slowly self-renewing; thus refractory to most non-viral or retroviral delivery methods. Since lentiviral vectors are capable of transducing non-dividing cells and maintaining long-term and sustained expression of the transgenes we used

HIV-1 derived vector system with minor modifications(29). Human wild-type DAP12 and DAP12 mutants containing the Xpress peptide epitope (DLYDDDDK), were subcloned into the pCCLsin.PPT.hEF1a.pre, vector (Sanjeev Gupta, Albert Einstein College of Medicine, NY, USA). To generate the retrovirus HEK293T cells were cotransfected with pCCL constructs and packaging plasmids pMDLg/pRRE, pRSV-Rev and pMD2.VSV-G using polyfect, (QIAGEN). Viral titers of 5×10^6 viral particles measured by QuickTiter™ Lentivirus Quantitation Kit (Cell biolabs, USA) were used for optimal infection. This viral titer had no cytotoxic effects on target cells. The virus-containing supernatant was collected 72 hrs after transfection and added to PBMC plated on 10 cm culture dishes. After 48 hrs, the viral supernatant was replaced with fresh medium. Cells were stimulated with IL-23 as described above.

Western blotting and Immunoprecipitation

Cytokine treated PBMC were washed two times with ice-cold D-PBS, and lysed with mammalian cell lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride] by incubation on ice for 15 min. The cell lysates were clarified by centrifugation at 15,000 rpm for 10 min at 4°C before analysed by Western blotting. For immunoprecipitation experiments, protein G-Dynabeads were incubated with the appropriate specific antibody for 1 hr at 4°C. Cell lysates were pre-cleared with Protein G-Dyna beads for 30 mins at 4°C, and then incubated with the Protein G-Dynabeads-appropriate specific antibody complex for over 2 hr at 4°C. After washing three times with wash buffer, immunoprecipitated protein lysates and whole cell lysates were separated by SDS–PAGE and transferred onto a PVDF membrane. The membrane was probed with the appropriate primary antibodies, followed by detection with horseradish peroxidase (HRP)-conjugated secondary antibody and detected by ECL reagent or Odyssey (Li-Cor, USA).

Intracellular calcium measurements

PBMC treated with MCSF for 8 days, and serum starved for 18 hours were labelled with 3 μM Fluo-4 AM for 30 min at 37°C prior to imaging. 100ng/ml of IL-23 or 100ng/ml of RANKL was added after 5 minutes of baseline imaging and calcium activity was tracked in the same cells over the next 10 minutes. For inhibition experiments, PBMC expanded with M-CSF for 8 days were pretreated with 1μM PLC inhibitor U73122 for 15 minutes prior to calcium imaging and stimulation with 100ng/ml of IL-23 or 100ng/ml of RANKL as before. Fluo-4 AM intensity per cell was measured and tracked over time using the Nikon NIS elements Br software

Statistical analysis

Data were analysed using Mann-Whitney test. One-way or two-way ANOVA with Bonferroni post-test were used where appropriate. $p < 0.05$ was considered to be statistically significant (minimum n=3).

RESULTS

IL-23 induces osteoclast formation in human PBMC in a RANKL-independent manner

To study the direct effect of IL-23 in myeloid cells human PBMC adherent cells were cultured for 8 days in the presence of MCSF. Exogenous IL-23 addition to MCSF expanded PBMC, stimulated the formation of large, $TRAP^{+}$, multi-nucleated cells in the absence of exogenous RANKL stimulation (Figure 1a). These cells were capable of F-actin ring formation and bone resorption when placed on dentine slices (Figure 1b,c). Although the percentage surface area of dentine resorption induced by IL-23 (16.12 ± 3%) was not significantly different than that of RANKL (22.29 ±7 %) (Figure 1d), the resorption pits induced by IL-23 were shallower with no distinct edges and not as deep and defined as in RANKL-induced cultures. This observation is consistent with the resorption pits typically induced by tumor necrosis factor (TNF), IL-1, IL-6, IL-11 and other pro-inflammatory cytokines (27, 30). IL-23 effects were independent of RANKL as addition of OPG was not as able to block the IL-23-induced osteoclastogenesis (Figure 1d). However, in agreement with the synergistic effect of pro-inflammatory cytokines with RANKL, IL-23 induced an increase in bone resorption synergistically with RANKL (Figure 1e).

Multi-analyte analysis of conditioned medium from M-CSF expanded PBMC stimulated with increasing levels of IL-23 was performed to address the role of known IL-23-induced factors on the osteoclast precursors. Since IL-23 induces IL-17 from a number of cell types (31) and IL-17 influences osteoclast formation (32) we performed a dose response of IL-23 alone and in conjunction with RANKL in 4 individual donors and measured the amount of IL-17 in the conditioned medium (Supplementary 1). We also checked for the expression of the osteoclastogenic factors RANKL and TNF in PBMC-CM as they are also released from Th17 cells. The amount of the osteoclastogenic cytokines in the CM was at least 1000-fold below what is required to be biologically active (32) in our in vitro osteoclastogenesis assays and furthermore neutralizing antibodies to IL-17 and TNF were not as able to block the IL-23-induced osteoclastogenesis. Taken together these data confirm that IL-23 induces human osteoclast formation in human PBMC in a Th17 and RANKL-independent manner.

IL-23 induces the upregulation of MDL-1 and NFATc1

PBMC were isolated from healthy donors and expanded for 8 days in the presence of M-CSF, (negative control), M-CSF+RANKL (positive control), or M-CSF+IL-23. IL-23 induced the upregulation of both NFATc1 message (Figure 2a) and protein (Figure 2b). Similarly, MDL-1 message (Figure 2c) and protein levels (Figure 2d) were also elevated upon stimulation by either IL-23 or RANKL. It is noteworthy that in certain donors the upregulation of MDL-1 and NFATc1 by IL-23 equalled or even exceeded the induction by RANKL. Since both MDL-1 and NFATc1 have direct roles in osteoclastogenesis (33, 34), we investigated whether MDL-1 may lead to osteoclast activation following IL-23 signalling.

IL-23 induces the formation of an IL-23R/MDL-1/DAP12 complex

In osteoclast precursors multiple immunoreceptors associate with DAP12 to stimulate NFATc1 induction and osteoclastogenesis (16, 23–26). Therefore we investigated the

interaction of MDL-1 with DAP12 in NFATc1 activation. DAP12 was immunoprecipitated from M-CSF expanded human PBMC stimulated with and without 100 ng/ml IL-23. Upon IL-23 stimulation, DAP12 formed a complex with MDL-1, DAP10, the IL-23 receptor, and SYK (Figure 3a). We expressed various N-terminal Xpress tagged DAP12 mutants using a lentiviral system to investigate the role of DAP12's ITAM in the immunocomplex. Specifically the Y91F, Y102F, (single mutants) and the Y91F/Y102F (2YF-double mutant) DAP12 mutants contained tyrosine to phenylalanine mutations to address the reduced or complete inhibition of DAP12 ITAM phosphorylation. IL-23 stimulation of DAP12 mutant transduced PBMC resulted in the absence of IL-23R and SYK recruitment (Figure 3b). The association of MDL-1 with DAP12 was not so dramatically affected, confirming previous observations that DAP12 pairs with MDL-1 in unstimulated cells.

IL-23 activates SRC, SYK and PLCγ2 phosphorylation and induces calcium transients

Next, we stimulated osteoclast precursors with 100 ng/ml IL-23 to investigate the time-dependent activation of NFATc1 by IL-23. IL-23 induced a rapid (within 5 minutes) phosphorylation of SRC (Y418), SYK (Y525/526) and PLCγ2 (Y759) (Figure 4a,b,c). We next compared IL-23's signal strength to RANKL at the concentrations used in the osteoclastogenesis cultures (i.e. 10 and 30 ng/ml, respectively). At 5 minutes, IL-23 induced SRC and SYK phosphorylation was comparable to RANKL's activity; however, PLCγ2 phosphorylation was slightly reduced in comparison to RANKL's effect (Figure 4a,b,c). We next investigated whether IL-23-induced phosphorylation lead to an increase in cytosolic calcium. IL-23 stimulation of M-CSF expanded PBMC showed an immediate increase in calcium flux within 10 seconds as detected by Fluo-4 AM (Figure 4d) with the calcium flux intensity being comparable to RANKL's calcium flux (Figure 4e,f). Addition of 1 μM PLCγ inhibitor U73122, 15 minutes prior to calcium measurements and stimulation with 100 ng/μl IL-23 or RANKL completely inhibited IL-23 or RANKL-induced calcium flux (Figure 4g,h).

IL-23 activates nuclear NFATc1 and initiates transcription of osteoclast specific genes

IL-23 induced the translocation of NFATc1 to the nucleus and induced NFATc1 activity to similar levels as RANKL (Figure 5a,b). We next investigated the IL-23 induction of a number of osteoclast-associated genes that were regulated by NFATc1 at 8 and 18 days of culture. IL-23 induced the expression of TRAP, matrix metallopeptidase 9 (MMP9), and calcitonin receptor (CALCR) in the absence of exogenous RANKL (Figure 5c,d,e). Cathepsin K, however, was not modulated by IL-23. Furthermore, NFATc1 and other osteoclast-related genes were also modulated by IL-23 in a dose response manner in synergy with RANKL (Supplementary 2).

MDL1 expressing cells are enriched in the promyelocytes

Next we sorted the $CD14^+$ fraction of the PBMC and we stimulated with MCSF+IL-23. Although IL-23 in the presence of MCSF induced the formation of multinucleated $TRAP^+$ cells these cells were not capable of forming F-actin rings or resorb dentine therefore could not be characterized as functional osteoclasts (Figure 6ab). Suprisingly, IL-23 did not induce osteoclast formation in the $CD14^+$ fraction of PBMC suggesting that this novel pathway may utilize a different cell precursor. We also observed that the expression of MDL-1 was

almost absent in the $CD14^+$ fraction of PBMC. Flow cytometric analyis of PBMC by gating on MDL-$1^{+/}$DAP12^+ cells revealed that the expression of MDL-$1^{+/}$DAP12^+ was largely confined within the $CD16^+$ myeloid population (Figure 6c).

DISCUSSION

In this paper we describe a direct mechanism of IL-23 in macrophage activation. Specifically our data have highlighted that human macrophages respond to IL-23 with a strong upregulation of MDL-1, a PU.1 transcriptional target during myeloid differentiation (35). MDL-1 was recently identified to play a major role in myeloid progenitors, capable of osteoclast formation in inflammatory arthritis (33, 36). As DAP12 interacts with MDL-1, we explored the IL-23 elicited responses within the MDL-1/DAP12 pathway. We found that DAP12 associates with MDL-1 and DAP10 following IL-23 stimulation. Furthermore this interaction induces SRC phosphorylation, which recruits SYK and phosphorylates effectors and transducers of calcium signalling including PLCγ2, which regulates osteoclastogenesis (37). IL-12, which shares IL-12/23p40 and IL-12Rβ1 with IL-23, regulates many classes of ITAM-bearing receptors in NK and T cells through synergy with DAP10 resulting in potent costimulatory signals (38, 39). Although DAP10 was previously shown to form an MDL-1-DAP12/DAP10 trimolecular complex, which triggers osteoclastogenesis the interactions with IL-23 have not been described previously (40).

Our data revealed that ITAM's are critical for the recruitment of SYK, PLCγ2 phosphorylation and generation of intracellular calcium after IL-23 signaling. A link between pro-inflammatory cytokines and Ca^{2+} signalling has previously been described through TNF, which induces Ca^{2+} osscilations in human macrophages, and leads to activation of NFATc1 (41). Although IL-23 did not induce Ca^{2+} osscilations, but Ca^{2+} trasnients (irregular osscilations) it was still able to induce NFATc1 suggesting that Ca^{2+} osscilations are not required in IL-23-induced dephosphorylation of the Ca^{2+}-dependent phosphatase Calcineurin and subsequent induction of NFATc1. In myeloid cells, where basal NFAT expression is low the initiation of osteoclast differentiation requires the induction of NFATc1 expression. IL-23 induces the molecular machinery to orchestrate upregulation of cytoplasmic NFATc1. Furthermore, we show that IL-23 induces the subsequent translocation of NFATc1 to the nucleus and the initiation of transcription of NFATc1 target genes such as TRAP, MMP9, CTSK, ITGB3 and CLCN7 further amplifies osteoclastogenesis (42). It is also noteworthy that the effects of IL-23 were directly compared to RANKL, which is the most potent osteoclastogenic factor *in vivo* and *in vitro* and in most cases the effects of IL-23 were greater than RANKL. However, IL-23 did not induce the upregulation of CTSK and this may account for the impaired dentine degradation observed in dentine discs compared to RANKL. This may be due to the fact that IL-23 regulates IFN, which is known to down-regulate Cathepsin K gene expression (43).

Another striking difference between RANKL and IL-23 signalling derived from the phenotypic analysis of the myeloid cells involved in NFATc1 activation. RANK receptor is predominantly expressed in the $CD14^+$ fraction of PBMC and much of the published literature regarding osteoclastogenesis has used $CD14^+$ as osteoclast precursors in vitro assays (44). Suprisingly sorted $CD14^+$ cells that were stimulated with IL-23 did not produce

any functional osteoclasts although they did induce cell fusion. Similar observations where IL-23 failed to induce osteoclast formation in $CD14^+$ have previously been reported but no mechanism was proposed (45). Other studies have also shown that IL-23 induces osteoclast formation but again no mechanism has been proposed (46). Phenotypic analysis of various PBMC fractions by flow cytometry revealed that the double possitive $MDL\text{-}1^+/DAP12^+$ cells are within the $CD16^+$ fraction of PBMC. Therefore IL-23 activates a different subset of myeloid progenitor cells than RANKL and induces their differentiation to osteoclasts. This is of particular importance as in inflammatory arthritis a combination of IL-23 and RANKL will have an additive and more bone destructive effect since it utilizes different osteoclast precursors whilst at the same time induces a costimulatory pathway. This explains why we see an increaced induction of RANKL induced osteoclastogenesis and further increase bone resorption *in vitro*, which correlated with an increase in osteoclast associated mRNA for NFATc1, TRAF6, TRAP, CTNN and ITGB3 in a dose dependent manner. It also points to the possibility that different cell subsets of monocytes and macrophages may get activated by pro-inflammatory cytokines to differentiate to osteoclasts in inflammatory arthitis which are completely different to those regulated by RANKL during physiological bone remodelling.

The data described in this paper are independent of the Th17 effects on osteoclast differentiation (15). IL-17 was barely detectable in IL-23-stimulated PBMC supernatants and the IL-17 concentrations were much lower than are needed to directly promote osteoclastogenesis using this assay system (32). A report by Chen et al, showed that IL-23 upregulated RANK in RAW 264.7 cells (47) however, in our studies with human PBMC there was no detectable elevation of RANK protein by flow cytometry even after 18 days of IL-23-stimulation. Our data show for the first time that IL-23 acts on human $CD16^+$ $MDL\text{-}1^{+/}DAP12^+$ osteoclast precursor population to induce osteoclast differentiation. This novel pathway utilizes the DAP12 ITAM's to recruit SYK and induce Ca^{2+} dependent activation of NFATc1. Collectively our data define a novel pathway that is utilized by IL-23 in myeloid cells and identify a major mechanism for the stimulation of osteoclastogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Glossary

IL-23	Interleukin-23
RANKL	receptor activator of NF-κB ligand
OPG	osteoprotegerin
M-CSF	macrophage-colony stimulating factor

TRAP tartrate-resistant acid phosphatase

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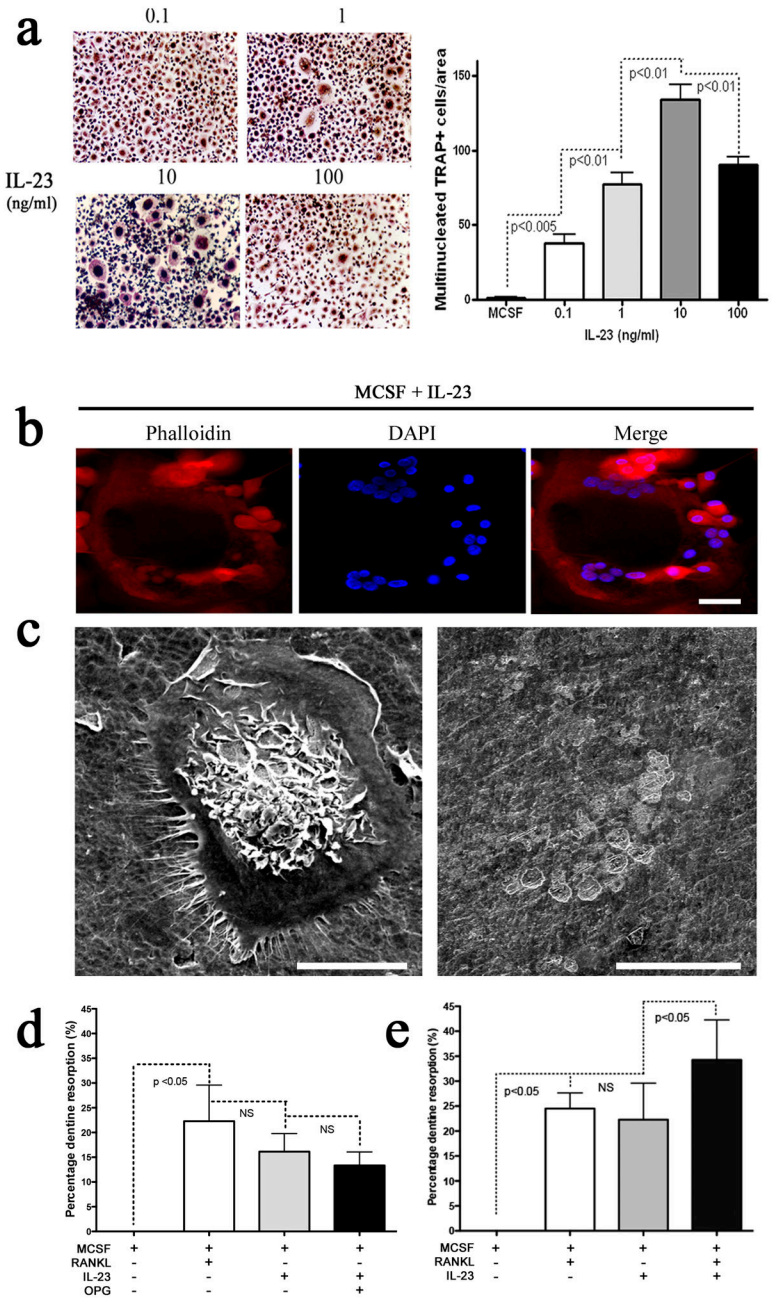

Figure 1. IL-23 induces human osteoclast formation in PBMC *in vitro*

a) TRAP staining of human PBMC isolated from healthy volunteers cultured for 14 days in the presence of MCSF and increasing IL-23 concentrations. (Data pooled from 4 individual experiments done in triplicates). **b)** Phalloidin, DAPI staining, and merged image of both stains of PBMC cultured on coverslips for 18 days in the presence of MCSF and IL-23 showing F-actin ring formation (Bar represents 25μm, representative of 20 independent experiments). **c)** PBMC cultured on dentine slices for 18 days in the presence of MCSF and IL-23 showing dentine erosion (bar represents 25μm and 100μm left and right respectively, representative of 20 independent experiments). **d)** Mean percentage lacunar resorption in

PBMC after treatment with M-CSF and soluble RANKL or M-CSF with IL-23 (+/− OPG) or **e)** M-CSF and soluble RANKL or M-CSF with RANKL and IL-23. Representative of 6 independent experiments Error bars represent the standard deviation of the mean ($p<0.05$). One-way ANOVA and t-test was used where appropriate.

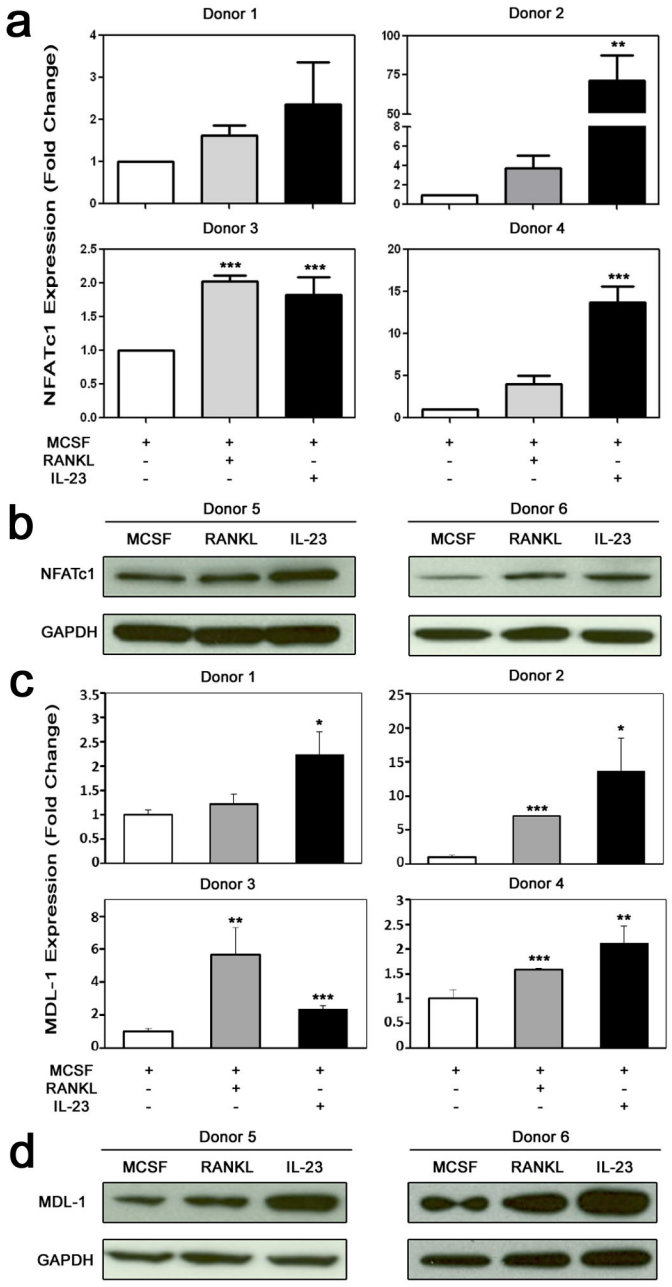

Figure 2. IL-23 induces expression of NFATc1 and MDL-1

a) qRT-PCR showing NFATc1 gene expression relative to 18S and **b)** Western blotting showing NFATc1 protein expression relative to GAPDH in PBMC 8 day cultures treated with MCSF and/or RANKL and/or IL-23. **c)** qRT-PCR showing MDL-1 gene expression relative to 18S in PBMC 8 day cultures treated with MCSF and/or RANKL and/or IL-23 **d)** Western blotting showing MDL-1 protein expression relative to GAPDH in PBMC 8 day cultures treated with MCSF and/or RANKL and/or IL-23. Error bars represent the standard deviation of the mean (t-test * < 0.05, ** < 0.01, *** < 0.001).

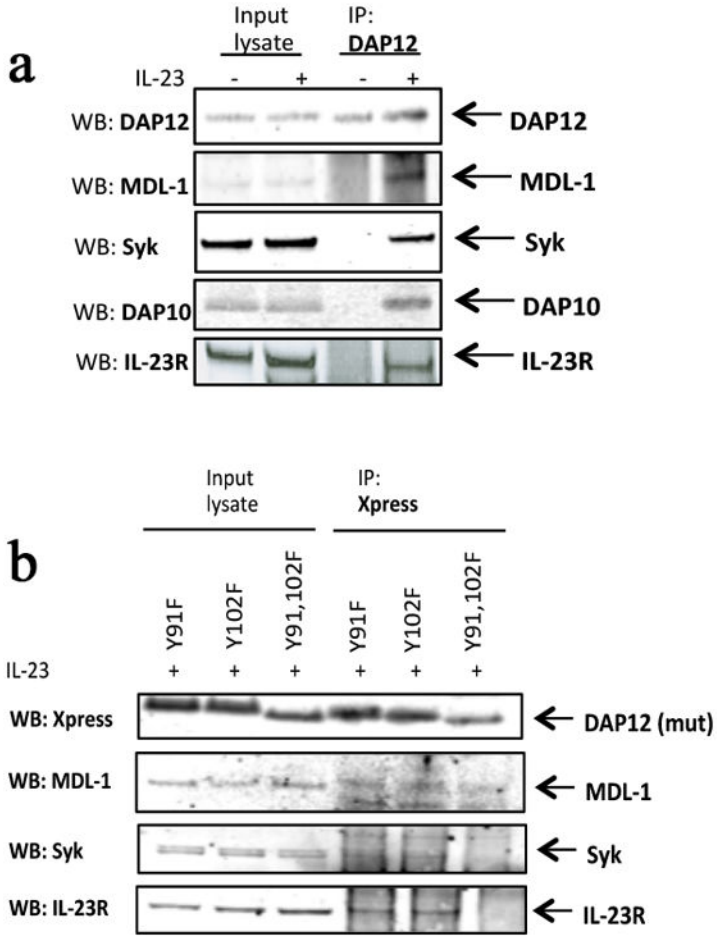

Figure 3. IL-23 elicits ITAM signaling through DAP12

a) Total cell lysates of human PBMC expanded for 8 days with MCSF serum starved, and stimulated with 100ng/ml of IL-23 (+) or control medium (−) were immunoprecipitated with DAP12 antibody and immunoblotted with DAP12, MDL-1, Syk, DAP10, or IL-23R antibodies. Representative of 2 independent experiments. **b)** Total cell lysates of lentivirally transduced mutant forms of DAP12, Y91F, Y102F and Y91, 102F (Y2F) expanded for 8 days with MCSF serum starved and stimulated with 100ng/ml of IL-23 (+) or control medium (−) were immunoprecipitated with DAP12 antibody and immunoblotted with DAP12, MDL-1, Syk, or IL-23R antibodies. Representative of 2 independent experiments.

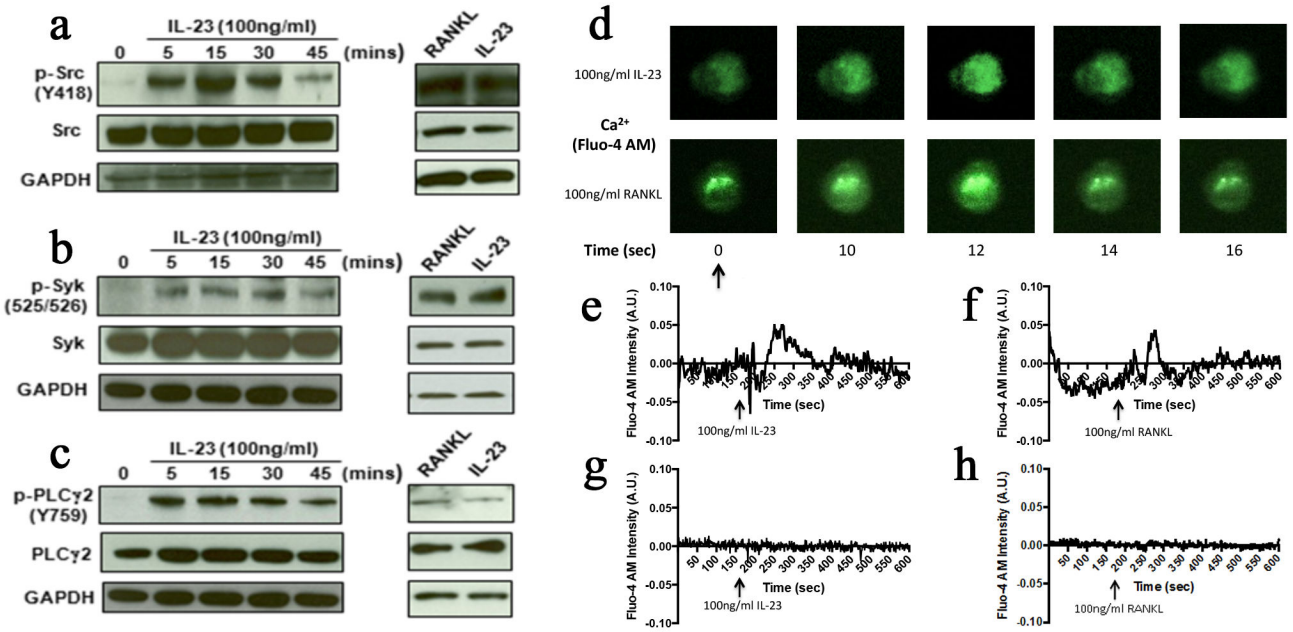

Figure 4. IL-23 activates PLCγ mediated calcium flux in osteoclast precursors

Western blotting of human PBMC expanded with MCSF for 8 days serum starved for 18 hours and stimulated with 100 ng/ml of MCSF, RANKL or IL-23 and IL-23 for indicated time, probed with **a)** anti-phosphotyrosine Src (Y418), and total Src, **b)** anti-phosphotyrosine Syk (Y525/526), and total Syk, and **c)** anti-phosphotyrosine PLCγ2 (Y759) and total PLCγ2. Right panels show comparative phosphorylation using 10ng/ml of IL-23 and 30ng/ml of RANKL at 5 min time-point. **d)** Calcium imaging intensity in human PBMC expanded with MCSF for 8 days, serum starved for 18 hours, and labeled with 3uM Fluo-4 AM, and stimulated with 100 ng/ml of **e)** IL-23 or **f)** RANKL for indicated times. **g)** and **h)** Same conditions as **e** and **f** but with prior incubation with PKC inhibitor before IL-23 or RANKL stimulation. Fluo-4 AM intensity was measured and normalized per cell. Representative images and curves of cells exhibiting calcium flux are shown. Representative of 4 independent experiments.

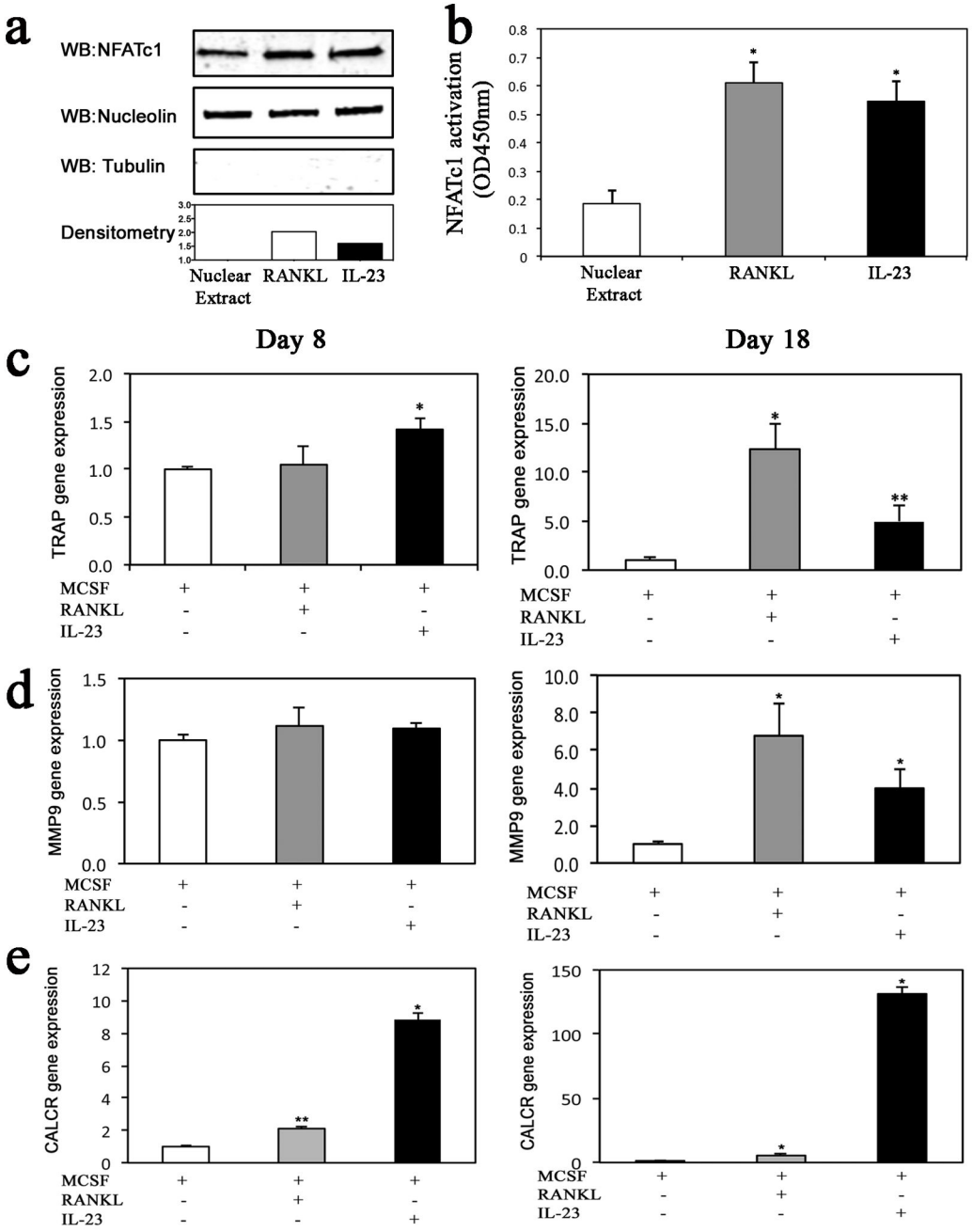

Figure 5. IL-23 induces NFATc1 dependent activation of osteoclast-associated genes

a) Nuclear extracts of human PBMC treated with MCSF for 8 days, serum starved for 18 hours, and stimulated with 100 ng/ml of IL-23 or RANKL. Extracts were probed with NFATc1, nucleolin and tubulin antibodies and densitometry measurements quantified NFATc1 nuclear translocation. **b)** NFATc1 activation was measured using a TransAM NFATc1 assay kit. **c)** qRT-PCR analysis showing TRAP, MMP9 and CALCR gene expression relative to 18S after 8 or 18 days treatment with MCSF, or MCSF+RANKL or MCSF+IL-23. Representative of 4 independent experiments.

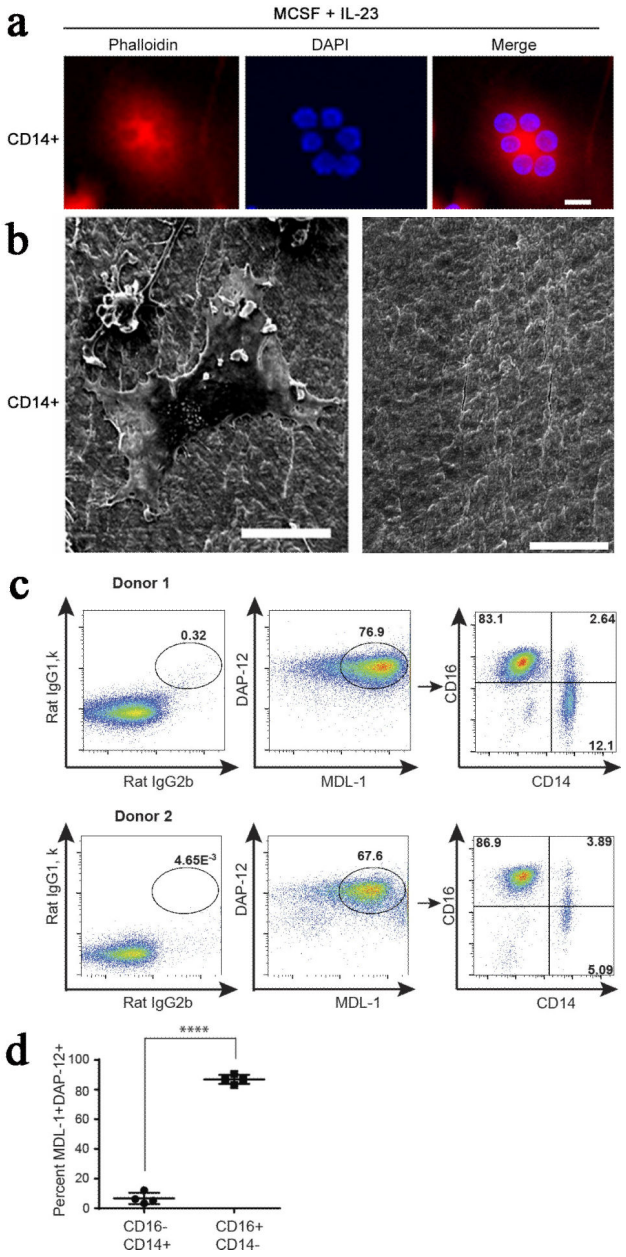

Figure 6. IL-23 does not induce osteoclast differentiation in the CD14$^+$ fraction of PBMC

a) Phalloidin, DAPI staining, and merged image of both stains of the CD14$^+$ fraction of human PBMC cultured on coverslips for 18 days in the presence of MCSF and IL-23 showing multi-nucleation but no F-actin ring formation (bar represents 25μm). **b)** CD14$^+$ fraction of human PBMC cultured on dentine slices for 18 days in the presence of MCSF and IL-23 showing absence of dentine erosion (Bars represent 25μm and 100μm left and right respectively). **c)** Representative data from flow cytometric analysis of 2 donors showing the expression of CD16 and CD14 in MDL-1$^+$/DAP12$^+$ double positive gated populations of the PBMC fraction and **d)** Graphical representation showing the mean

percentage + SEM of MDL+DAP12+ cells in CD14 and CD16 subsets of healthy donors (n = 4, solid line represents the mean. ****$p < 0.0001$)