Dendritic cells manipulating immune responses: Understanding the role of Flt3L and Flt3-dependent DCs in rheumatic diseases
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chapter SEVEN

Dendritic cell growth factor Flt3L drives osteoclastogenesis in rheumatoid arthritis

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* These authors contributed equally for this work
Abstract

Objective: Understanding the mechanisms regulating osteoclastogenesis in rheumatoid arthritis (RA) is fundamental for the prevention and treatment of bone loss. Here we studied the role of Fms-like tyrosine kinase 3 ligand (Flt3L) in driving osteoclastogenesis in RA patients and in mouse models of RA.

Methods: Osteoclasts (OCs) were generated from human RA peripheral blood mononuclear cells (PBMCs) and cultured for 21 days in the presence of M-CSF (25 ng/ml), Flt3L (25 ng/ml), RANKL (50 ng/ml), or CEP-701 (1 μM). TRAP and toluidine blue stainings were performed to access histological and functional properties of OCs. In mice, arthritis was induced in Flt3L-/- and WT littermates by injection of pooled K/B×N sera. For the treatment experiment mice were immunized to induce collagen-induced arthritis (CIA) and before showing clinical signs of disease they were treated with the Flt3L inhibitor CEP-701 or vehicle solution. Histological and bone parameters were analyzed for both animal experiments.

Results: Flt3L induced OC formation in RA PBMCs and this effect was blocked by CEP-701 treatment, however in vitro Flt3L-generated OCs did not display resorption capacity. CEP-701 treatment of M-CSF-induced OCs resulted in a significant reduction of their resorption capacity. Nevertheless no differences in histological and bone parameters between Flt3L-/- and WT mice in the K/B×N serum transfer model of RA. Despite histological differences in infiltration and cartilage damage in the CIA mouse model no differences in bone parameters were observed after CEP-701 treatment compared to vehicle-treated groups.

Conclusions: We have demonstrated that Flt3L can induce OC formation in RA patients. Importantly, in vitro disruption of FLT3 signaling by CEP-701 led to a reduction of OC resorption capacity suggesting that CEP-701 might have a potential therapeutic effect in preventing bone loss.
Introduction

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory autoimmune disease, affecting approximately 0.8 percent of adults worldwide. For many years, few treatment options were available for patients with RA, leading to extensive joint damage and increased mortality. During the past years, understanding the pathophysiology of RA has led to marked improvement in therapy. A common and characteristic feature of RA is focal articular bone loss, or erosion, that becomes apparent early in the disease (1). Current views of bone damage in RA suggest that pro-inflammatory cytokines produced by the inflamed synovium facilitate osteoclast (OC) activation and increase bone resorption at specific anatomical sites (2).

We have previously shown that there is an increase of dendritic cell (DC) numbers in RA synovial tissue (ST) and synovial fluid (SF) (3). DCs are heterogeneous and several subtypes with distinct features have been identified in lymphoid and non-lymphoid human and mouse tissues (4). DCs play several roles in autoimmunity, from priming of lymphocytes to production of pro-inflammatory mediators (5;6). DCs share common progenitors with OCs (7;8) and can dedifferentiate into OCs themselves (9); these cells also infiltrate bone adjacent tissues during inflammation, where their interactions with T cells constitute a key component of the inflammatory infiltrate at active disease sites in human and experimental RA (10). OCs are the bone resorbing cells and play an essential role not only in bone development and remodeling but also in calcium homeostasis. OCs are considered to be central players during inflammation-induced bone loss (11;12).

Fms-like tyrosine kinase 3 (FLT3), a receptor tyrosine kinase with homology to c-Kit (the receptor for stem cell factor) and c-fms (the receptor for macrophage-colony stimulating factor, M-CSF), has a non-redundant role in steady-state differentiation of immature progenitor cells and DCs (13). FLT3+ precursors can differentiate sequentially into OCs, DCs, and microglia (8), implying possible involvement of FLT3 in the development of OCs. In mouse studies it has been shown that Flt3 ligand (Flt3L) can induce OC differentiation by substitution for M-CSF, which plays a critical role in the formation and function of OCs (14). Supporting this notion, our recent data (15) revealed that abrogation of Flt3L signalling lead to reduced bone and cartilage destruction in a mouse model of RA. Another report demonstrated that FLT3 polymorphisms play a role in determination of bone mass density (BMD) and subsequent fractures in postmenopausal women (16). Moreover, we and others have shown that Flt3L levels are elevated in RA patients compared to disease controls (17;18). The aim of the present study was to investigate the involvement of FLT3/Flt3L axis in osteoclastogenesis and bone loss. We hypothesised that the DC growth factor Flt3L might be directly involved in osteoclastogenesis in RA and that blocking this pathway may prevent bone loss in animal models of RA.
Material and methods

Patients for osteoclast culture

Early untreated RA patients (fulfilling the 2010 ACR-EULAR classification criteria for RA (19;20)) followed up in the Rheumatology Department, Lisbon Academic Medical Centre, Portugal were recruited for this study. Patients were submitted to a clinical protocol as described for Reuma.pt (21). This study was approved by the local ethics committee and all participants gave written informed consent. Patient’s management was done in accordance with the standard practice and the study was conducted in accordance with the Declaration of Helsinki as amended in Seoul (2008). Early RA patients enrolled in the study are characterized in table 1. Serum and peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples. The collected serum was centrifuged at 300 g, 4°C and immediately frozen at -80°C.

Peripheral blood mononuclear cell isolation from human donor and osteoclast culture

PBMCs were isolated from 30 ml of heparinized blood following density gradient centrifugation with Histopaque®-1077 (Sigma-Aldrich, St. Louis, MO). Cellular count was performed with Trypan Blue (Sigma-Aldrich, St. Louis, MO). 7 x 10⁵ cells/200 μL in 96-well plates were seeded on bovine bone slices (Immuno Diagnostic Systems Ltd., London, UK). Isolated PBMC were cultured in Dulbecco’s Modified Eagle Medium

Table 1. Characterization of the RA patients enrolled in the study

<table>
<thead>
<tr>
<th></th>
<th>RA patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>9</td>
</tr>
<tr>
<td>Gender (% women)</td>
<td>100%</td>
</tr>
<tr>
<td>Age (years)</td>
<td>50 [31 - 58]</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>1 [0.6 - 1.3]</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>19 [17 - 90]</td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
<td>0.3 [0.2 - 2.3]</td>
</tr>
<tr>
<td>NSAIDs (% Yes)</td>
<td>33.3 %</td>
</tr>
<tr>
<td>DAS28</td>
<td>4.3 [3.8 - 5.4]</td>
</tr>
<tr>
<td>Tender joint count</td>
<td>2 [1 - 7]</td>
</tr>
<tr>
<td>Swollen joint count</td>
<td>2 [0 - 8]</td>
</tr>
<tr>
<td>HAQ</td>
<td>2.0 [1.2 - 2-3]</td>
</tr>
<tr>
<td>RF (% Pos)</td>
<td>66.7 %</td>
</tr>
<tr>
<td>ACPA (% Pos)</td>
<td>33.3 %</td>
</tr>
<tr>
<td>Erosive (% Yes)</td>
<td>44.4 %</td>
</tr>
</tbody>
</table>

ESR, erythrocyte sedimentation rate; CRP, C reactive protein, NSAIDs, non-steroidal anti-inflammatory drugs; DAS28, disease activity score; HAQ, health assessment questionnaire; RF, rheumatoid factor; ACPA, anti-citrullinated protein antibodies
(DMEM; Invitrogen, Paisley, UK) supplemented with 50000 units of penicillin/streptomycin (Invitrogen, Paisley, UK), 2 mM L-Glutamine (Invitrogen, Paisley, UK) and 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, Paisley, UK). The cultures were left overnight at 37°C with 5% CO$_2$ to allow monocytes to adhere. The next day, medium was replaced by an equal amount of DMEM as stated above, supplemented with sRANKL (50 ng/ml; Peprotech, Rocky Hill, NJ), M-CSF (25 ng/ml; Peprotech, Rocky Hill, NJ), Flt3L (25 ng/ml; R&D Systems, Bethesda, MD) and CEP-701 (1 μM; Sigma-Aldrich, St. Louis, MO) in different combinations as described below. PBMCs were cultured for 21 days in the presence of M-CSF, RANKL, Flt3L and CEP-701 at 37°C, 5% CO2 in multi-well culture plates and the media was changed every 3 days. OC differentiation was analyzed at day 21 of culture to assess cellular dynamics. At this time-point cells were stored for gene expression analysis and used in two functional assays.

**Functional assays: tartrate-resistant acid phosphatase staining and resorption assay**

Staining for cytoplasmic enzyme tartrate-resistant acid phosphatase (TRAP) was performed according to the manufacturer's instructions with Acid Phosphate, Leukocyte Kit (Sigma-Aldrich, St. Louis, MO). The resorptive cell (OC) is rich in TRAP, which, in the bone context, is a specific and sensitive OC marker (22). The cells were observed under an upright bright field microscope (model DM2500, Leica, Wetzlar, Germany) equipped with a color camera (Leica, Wetzlar, Germany). Images were captured with 10x magnification and all were analyzed with Image J (version 1.47g, Java 1.6.0_20, 64 bit, NIH, Bethesda, MD). TRAP positive cells with three or more nuclei were counted as OCs. TRAP positive cells with one or two nuclei were counted as pre-OCs.

Bone slices were incubated with 5% sodium hypochlorite (Sigma-Aldrich, St. Louis, MO), then stained with 0.1% toluidine blue (Sigma-Aldrich, St. Louis, MO). Toluidine blue stains acidic pH structures (in this case the resorption pits) with blue to purple color. Slides were washed with H$_2$O and observed under an upright brightfield microscope (model DM2500, Leica, Germany) equipped with a color camera (Leica, Germany). Images were captured with 10x magnification and all were analyzed with Image J (version 1.47g, Java 1.6.0_20, 64 bit, NIH, Bethesda, MD). The resorption pit number was counted and resorbed area was measured.

**Animals**

Male and female Flt3L$^{-/-}$ mice and WT littermates about 8–10 weeks old and 10 week-old DBA1 males mice (Harlan) were used for the experiments.

**Induction and assessment of K/BxN serum transfer arthritis model and CEP-701 treatment**

Arthritis was induced in Flt3L$^{-/-}$ and WT littermates (C57/B16 background) by i.p. injection of pooled K/B×N sera (150 µl) on day 0 (n=4 per group). Animals were
monitored for clinical signs of disease daily during 15 days. For CEP-701 treatment, DBA1 mice (CEP-701 treated group n=6 and vehicle treated group n=12) were immunized to induce CIA as previously described (23;24). Before showing clinical signs of arthritis mice were treated intraperitoneally (i.p.) with CEP-701 (20 mg/kg, LC laboratories, Woburn, MA) or vehicle solution every 12 hours for 15 consecutive days. Animals were monitored for clinical signs of disease daily by two independent observers who were not aware of the animal’s treatment group, during the whole course of the experiment. The severity of the arthritis was assessed using an established semiquantitative scoring system of 0–4 where 0=normal, 1=mild swelling, 2=moderate swelling, 3=swelling of all joints and 4=joint distortion and/or rigidity and dysfunction (25). The cumulative score for all four paws of each mouse (maximum possible score 16) was used as the arthritis score to represent overall disease severity and progression in an animal. Paws from both sets of experiments were collected for further examination.

Ex-vivo computed-tomography scans
Ex vivo scans were performed using Skyscan1172 ex-vivo micro computed-tomography (μCT) machine. Source settings were 50kV and 200 μM and a 0.5 mm aluminium filter was applied. Flatfield correction was applied before scans were started and used was continually used throughout all samples. Pictures were taken with an exposure of 590 ms set to a rotation step of 0.6’ and pixel size of 4.9 μm. Frame averaging of 2 was also used. Samples were submerged in PBS and positioned at a 90’ angle. Total scan time for each sample was approximately 18 minutes.

Reconstructions
Reconstructions were completed using the NRecon software (version: 1.6.9.4). Settings for smoothing (2), ring artefact reduction (7) and beam hardening correction (40%) were applied. Histogram for image conversion was set from zero to 0.121 for FLT3 inhibitor (CEP-701) experiment and 0.120 for the K/B×N serum transfer experiment. All other settings described were applied to both experiments.

mCT data analysis
Reconstructed images were analysed using CT Analyser software (version 1.13.5.1). For the general 3D analysis a region of interest (ROI) was specified from the lowest point of the tibia (left side) and then extended to include the entire width and length of the tarsals. The final ROI size was determined from the smallest available ROI size and applied to all others samples. The ROI was maintained at a constant total volume throughout. After selection of the ROI, this dataset was loaded for 3D analysis. Analysis was performed for bone volume (BV), bone surface (BS), total volume (TV), and ratios of bone surface to bone volume (BS/BV) and bone volume to total volume (BV/TV). Trabecular analysis was also performed (cortical bone analysis was not possible with the samples). Selection of the trabecular analysis ROI was constructed
from an anatomical landmark of the tibia whereby 100 images were selected 20 images above this point. After selection the trabecular bone was manually selected for the final ROI. These data were then subjected to trabecular analysis consisting of measures for trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp).

Analysis of results
For all experiments, the difference between three or more groups was calculated with the Kruskal-Wallis test. For comparison between two groups, the Mann-Whitney U test for unpaired data was used. All data was analyzed with GraphPad Prism (version 5.0; GraphPad, San Diego, CA) and p value under 0.05 was considered statistically significant.

Results
Flt3L induces osteoclast formation in RA
In order to assess the capacity of Flt3L to promote osteoclastogenesis in RA, PBMCs were isolated and incubated with RANKL in the presence of M-CSF or Flt3L or left unstimulated. The numbers of OCs were significantly higher in RANKL treated monocyte cultures supplemented with M-CSF and Flt3L compared to medium control (Figure 1A). OCs were identified as TRAP+ cells with more than three nuclei (Figure 1B). Morphologically, there were no differences between M-CSF and Flt3L generated OCs (Figure 1B). To evaluate whether the OCs generated with Flt3L were functional we assessed the capacity of cells to resorb mineralized bone. We observed a lower number of lacunar resorption pits and less resorbed area in Flt3L generated OCs compared to M-CSF generated OCs (positive control; Figure 1C). Gene expression analysis showed that the resorption inability of Flt3L-generated OCs was not due to lack of cathepsin K expression (Supplementary Figure 1A). In addition, no differences in ATP6v0d2, TRAF2 and TRAF6 mRNA expression were observed between M-CSF- and Flt3L-generated OCs (Supplementary Figure 1B, C and D respectively).

CEP-701 treatment blocks Flt3L-induced osteoclastogenesis
CEP-701 is a known inhibitor of FLT3 and is being investigated in clinical trials for patients with acute myeloid leukemia (AML) with FLT3-activating mutations (26). We used this compound, CEP-701, in our culture system to assess the therapeutic potential of this FLT3 signaling inhibitor in preventing Flt3L-dependent osteoclast generation. We found that CEP-701 treatment led to fewer OC numbers in cultures containing Flt3L compared to cultures with medium alone (Figure 2A and B). In addition, CEP-701 treatment also led to a significant reduction in Flt3L-generated pre-OC numbers (Supplementary Figure 2A). Morphologically, there were no differences between OCs generated in the presence or in the absence of CEP-701.
Figure 1. Flt3L induces osteoclastogenesis in RA patients. (A) Quantification of osteoclasts numbers, identified by TRAP expression and presence of more than 3 nuclei, at day 21 after monocyte culture with M-CSF (25 ng/ml) or Flt3L (25 ng/ml) in the presence of RANKL (50 ng/ml). (B) Representative TRAP staining for each culture condition. Osteoclasts are marked with open arrows and pre-OCs (defined as TRAP<sup>+</sup> cells with less than 3 nuclei) are marked with closed arrows. (C) Quantification of the number of resorption pits and resorbed area at day 21. (D) Representative toluidine-blue staining in bone slices for each culture condition. Resorption pits are marked by open arrows.
Figure 2. CEP-701 treatment blocks Flt3L induced osteoclastogenesis. (A) Quantification of osteoclasts numbers at day 21 after monocyte culture with M-CSF (25 ng/ml), Flt3L (25 ng/ml) or the combination of M-CSF + CEP-701 (1 μM) and Flt3L + CEP-701 in the presence of RANKL (50 ng/ml). Statistical differences were observed between Flt3L cultures with and without CEP-701 (p=0.01). (B) Representative TRAP staining for each culture condition. Osteoclasts are marked with open arrows and pre-OCs are marked with closed arrows. (C) Quantification of the number of resorption pits and resorbed area at day 21. (D) Representative toluidine-blue staining in bone slices for each culture condition. Resorption pits are marked by open arrows.
(Figure 2B). Although we did not find significant differences between the number of resorption pits in CEP-701-treated cultures compared to untreated cultures, a reduction in the resorbed area in M-CSF cultures was observed (Figure 2C and D). It has been shown previously that the resorption pit surface is linearly related to the nuclei number per OC, strongly suggesting that the functional advantage of OC multinucleation is to improve resorption efficiency (27). In this respect, we did not find differences in the number of nuclei per OC between the different culture conditions (Supplementary Figure 2B).

No differences in histological and bone parameters between Flt3L−/− and WT mice in the K/BxN serum transfer arthritis model

K/BxN mice spontaneously develop severe symmetrical polyarthritis, and serum from these mice induces a similar arthritis in a wide range of mouse strains (28). Serum transfer arthritis is rapid in onset and resembles histologically spontaneous arthritis in K/BxN mice. The serum transfer model provides an opportunity to investigate the role of Flt3L on osteoclastogenesis and bone erosion in inflammatory arthritis resembling RA but it is independent of cooperative T cell-DC interactions. No significant differences in histological parameters were found between Flt3L−/− and WT mice (Figure 3).

High-resolution CT allows for three-dimensional (3D) assessment of the bone microstructure and evaluation of the trabecular and cortical microstructure separately. Recent studies have suggested that µCT is a superior method for detecting bone erosions in RA (29). µCT evaluated by automated image analysis can be used to quantify bone loss and is commonly used in small animal models to detect morphological changes (30). Therefore we evaluated several bone parameters (Figure 4) in Flt3L−/− mice compared to WT mice after serum transfer. There were no significant differences in bone microstructure between Flt3L−/− mice and WT mice.

CEP-701 treatment reduces synovial infiltration and cartilage damage but does not change bone parameters compared to vehicle-treated animals

CEP-701 is a FLT3 inhibitor which has previously been used in an experimental autoimmune encephalomyelitis mouse model showing clinical efficacy associated with induction of apoptosis in DCs (31). We treated DBA1 mice immunized with type II collagen with CEP-701 before mice showed clinical signs of disease. CEP-701 treatment prevented the development of arthritis (M.I. Ramos et al., submitted) and these mice had a significant reduction in synovial infiltration and cartilage damage compared to vehicle-treated mice, as shown by histology (Figure 5). No differences were observed in bone parameters between CEP-701- and vehicle-treated mice (Figure 6).
Discussion

In pathological conditions such as RA, increased osteoclastic activity is responsible for bone loss and joint destruction. Understanding the fine tuning of OC activity is important to explain their deregulated functions in pathological conditions. Several lines of evidence in RA and in animal models of RA support a role for OCs in the pathogenesis of bone erosions (32). Here we show for the first time that Flt3L can induce OC formation \textit{in vitro} from human RA PBMCs in the absence of M-CSF. However the OCs generated with Flt3L were not functional as shown by reduced lacunar pit numbers and lack of resorption ability. We also found that using the FLT3 inhibitor CEP-701 Flt3L-induced osteoclastogenesis was blocked. In addition, CEP-701-treated M-CSF OC cultures lost the capacity to resorb bone suggesting that this inhibitor could be used to prevent bone loss. Although our \textit{in vitro} studies showed direct effects on OC function using CEP-701 suggesting a therapeutic potential in RA, \textit{in vivo} we mainly found an effect on synovial inflammation and cartilage...
Figure 4. Similar changes of bone parameters in Flt3L−/− mice compared to wild-type (WT) mice in K/BxN serum transfer arthritis model analyzed by μCT. Analysis was performed for bone volume (BV), bone surface (BS), total volume (TV), and ratios of bone surface to bone volume (BS/BV) and bone volume to total volume (BV/TV). Trabecular analysis was also performed for trabecular thickness (TH), separation (TS) and number (TN). Data are presented as means ± S.E.M.
chapter seven

degradation with little to no effect on OCs and bone damage. In contrast to c-fms, RANK lacks intrinsic kinase activity to phosphorylate and activate downstream signaling molecules. RANK recruits TRAFs, particularly TRAFs 1, 2, 3, 5 and 6, which acts as adapter proteins to recruit protein kinases (33). Of these, TRAF6 appears to have an essential function in osteoclastic cells. TRAF6 initiates a signaling cascade that is crucial for the maturation of monocyte precursors to fully differentiated OCs (34). TRAF2 positively and TRAF3 negatively regulates OC formation. There were no differences in TRAF2 and TRAF6 mRNA expression between M-CSF- and Flt3L-generated OCs. Nevertheless, despite similar OC numbers, Flt3L-generated OCs displayed a diminished lacunar pit number and were not able to resorb bone. These observations are in contrast with previous reports in mice showing that Flt3L and RANKL enable the differentiation of functional OCs in the absence of M-CSF (13). However, studies using human monocytes and mature OCs demonstrated

Figure 5. CEP-701 treatment reduces inflammation and cartilage degradation in the collagen-induced arthritis (CIA) model. Vehicle, n=12; CEP-701-treated, n=6. Representative hematoxylin/eosin (H&E) and toluidine blue stainings of the ankle joints of a vehicle and CEP-701 treated mice, and semi-quantitative scores for synovial infiltration, cartilage damage and proteoglycan (PG) depletion assessed by H&E, and toluidine blue stainings. A significant reduction in synovial infiltration (p=0.03) and cartilage degradation (p= 0.01) was observed in CEP-701 treated compared to vehicle treated mice. Magnification ×100. Data are presented as means ± S.E.M.
Figure 6. No differences in bone parameters in vehicle and CEP-701 treated mice after CIA model analyzed by μCT. Analysis was performed for bone volume (BV), bone surface (BS), total volume (TV), and ratios of bone surface to bone volume (BS/BV) and bone volume to total volume (BV/TV). Trabecular analysis was also performed for trabecular thickness (TH), separation (TS) and number (TN). Data are presented as means ± S.E.M.
that M-CSF is necessary for the resorptive activity and stimulated resorption in a concentration-dependent manner (35;36) indicating that Flt3L alone in humans might not be sufficient to generate functional osteoclasts. Cathepsin K is a cysteine proteinase that cleaves key bone matrix proteins and is believed to play an important role in degrading bone during bone resorption (37). Gene expression analysis on Flt3L-generated OCs showed that the resorption inability was not due to lack of cathepsin K expression. However, whether the protein is produced and cleaved to its functional form is unknown. Bone resorption relies on the extracellular acidification function of vacuolar (V-) ATPase proton pump(s) present in the plasma membrane of OCs. Atp6v0d2 was found to be an essential component of the OC-specific proton pump that mediates extracellular acidification in bone resorption (38). Here we did not find differences in the expression of Atp6v0d2 between M-CSF- and Flt3L-generated OCs. It has been shown that there is a positive correlation between the number of nuclei per OC and the volume of the pit made (21;27). Nonetheless, the lack of resorption capacity could not be explained by differences in the number of nuclei between the different culture conditions supporting the notion that in humans M-CSF seems to be required for the resorption capacity. Since in homeostatic and arthritic conditions both M-CSF and Flt3L are present simultaneously (16;17) it would be interesting to study whether the combination of these two factors could lead to an increase in OC number (higher than each condition alone) and whether the differentiated OCs would be functional. This is currently being investigated.

Here we demonstrated that using the FLT3 inhibitor CEP-701 Flt3L-induced osteoclastogenesis was significantly blocked/diminished. Notably, despite no differences in the number of lacunar pit numbers in CEP-701-treated cultures, CEP-701 treated M-CSF-generated OCs lost the capacity to resorb bone. Again, no differences were observed in mRNA expression of cathepsin K, ATP6v0d2, TRAF2, TRAF6 or number of nuclei per OC between CEP-701-treated and non-treated cultures. A number of studies indicate that the rate limiting proteinases for the solubilization of collagenous matrix belong to the groups of cysteine proteinases but also matrix metalloproteinases (MMPs) (28;29). The most direct evidence for a role of MMPs in the subosteoclastic resorption zone has been provided by experiments using cultured calvariae in the presence or absence of proteinase inhibitors and analyzed the ultrastructure of the subosteoclastic resorption compartment. It was shown that MMP inhibitors can block osteoclastic resorption (30). Nevertheless, it seems that the relative importance of cysteine proteinases and MMPs is dependent for instance on the bone type and/or on the stage in the resorption cycle. Studying the MMP expression profile in CEP-701-treated and non-treated M-CSF osteoclast cultures might shed some light on the mechanism by which CEP-701 could prevent bone resorption. The effect observed in resorption capacity on OCs after CEP-701 treatment might also result from direct or indirect targeting of additional molecules/pathways other than FLT3. Indeed, CEP-701 was also shown to inhibit JAK2 in an in vitro
kinase assay. At nanomolar concentrations, it markedly inhibits the phosphorylation of JAK2 and TrkA and components of the downstream signaling cascade, including STAT5, AKT, and ERK (39).

Despite the potential for Flt3L to promote osteoclastogenesis (13) the contribution for this process compared to M-CSF in vivo seems to be negligible since blocking this pathway was not sufficient to prevent bone loss in the mouse models of arthritis used in the present study. Indeed, Flt3L−/− mice have normal bone development (31). In contrast, mice with a defect in the production of functional M-CSF (op/op mice) have a severe deficiency in OCs, monocytes and macrophages (32) demonstrating that in homeostatic conditions the role of M-CSF is more important than Flt3L in osteoclastogenesis. Although OCs are severely reduced in op/op mice, they are still present and functional (33) suggesting that other molecules, namely Flt3L can substitute for the actions of M-CSF. FLT3 signaling blockade by the administration of FLT3-Fc dramatically reduced the number of OCs in the bones of op/op mice. This indicates that Flt3L accounts for the presence of OCs in these mice, in the absence of M-CSF (13). We did not observe differences in bone parameters in the serum transfer arthritis model using WT and Flt3L−/− mice. Our data supports the notion that Flt3L might have a more secondary role in osteoclastogenesis. We and others (16-17) have previously reported that Flt3L levels are increased in the serum of RA patients compared to healthy individuals and that synovial fluid from RA patients contained higher levels of Flt3L compared to paired serum. We propose that Flt3L alone might be insufficient to support normal osteoclastogenesis in vivo, however it might have an additive effect in combination with M-CSF. Taking into account that RA patients have higher Flt3L titters than healthy individuals, using an arthritic mouse model with increasing Flt3L titters or recombinant Flt3L administration would help to understand its role in osteoclastogenesis in vivo.

Since our in vitro studies showed direct effects on OC function using the FLT3 inhibitor CEP-701, we used an in vivo mouse model to study its therapeutic potential. To address this question we used the CIA model. We observed that CEP-701 treated mice did not develop clinical signs of arthritis (M.I. Ramos et al. manuscript submitted). In contrast to clinical scoring, which is mainly indicative of the degree of inflammation, histological examination provides additional information pertaining to cartilage and bone damage. In this respect, despite reduced cellular infiltration and cartilage damage in CEP-701-treated compared to vehicle treated mice we did not observe differences in several bone parameters. One explanation for the lack of effect of FLT3 inhibition (CEP-701 treatment) on bone loss compared to vehicle-treated group might be related to the arthritis model where CEP-701 was used prophylactically. As previously stated Flt3L levels are increased in the serum of RA patients compared to healthy individuals (16-17). Since homeostatic Flt3L levels in the circulation are low and the contribution to osteoclastogenesis in vivo is lower than M-CSF blocking the FLT3 signaling before the onset of disease might not be enough
to lead to a reduction in OC numbers. There is no evidence that other ligands for the FLT3 receptor exist, nor is there any evidence that Flt3L binds to any other protein besides FLT3. However since we are blocking the FLT3 receptor we cannot exclude the possibility that circulating Flt3L could bind to an unknown receptor maintaining normal osteoclastogenesis. From our _in vitro_ studies in humans we observed that M-CSF generated OCs in the presence of CEP-701 lost the capacity to resorb bone, however mice treated with CEP-701 had the same bone microstructure as the vehicletreated group suggesting that OCs from CEP-701-treated mice are fully functional.

The analysis of bone parameters in mice without arthritis might help to understand the lack of differences between the above mentioned groups. Another reason for the lack of differences in bone parameters might be due to low disease severity that as a consequence resulted in reduced bone loss. Importantly, in the present study the analysis were performed at day 43. It is tempting to speculate that the effects of CEP-701 on bone loss might occur at a later time points. In addition, the reduced effect of CEP-701 on bone loss might be due to the route of administration since differences in therapeutic responses are related to different routes of administration (40). Therefore, we propose that local administration of CEP-701 (intra-articular) might be more effective in reducing bone loss compared to systemic administration. Taken together, we have shown that Flt3L can induce OC formation in RA patients and that CEP-701 treatment _in vitro_ might have a potential therapeutic effect in preventing bone loss. Further studies are necessary to better understand the mechanism how Flt3L and its blockade might contribute to osteoclastogenesis in RA.

**Reference List**

9. Alnaeeli M, Park J, Mahamed D, Penninger JM, Teng YT. Dendritic cells at the osteo-


Supplementary Methods

Quantitative measurement of mRNA expression

RNA was isolated from OC samples at day 21 using trizol/chloroform extraction and the concentration was determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA was reverse transcribed using DyNAmo cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) to a final concentration of 0.6 ng/µL. Duplicate PCR reactions were performed using SYBR green (Applied Biosystems, Foster City, CA) with an ABI Prism® 7000 sequence detection system (Applied Biosystems, Foster City, CA). cDNA was amplified using specific primers (all from Invitrogen, Breda, The Netherlands): Cathepsin K forward GCCAGACAACAGATTTCCATC, Cathepsin K reverse CAGAGCAAAGCTCACCACAG; Atp6v0d2 forward CATTCTTGAGTTTGAGGCCG, Atp6v0d2 reverse CCGTAATGACCGCTACGTT; TRAF2 forward CGGCCACGATCAATCT, TRAF2 reverse GCTTTGAGGGTCCACATGA, TRAF6 forward GCACTAGAACGAGCAAGTGAT, TRAF6 reverse GCCAGTTCCACCCACACTAT and 18S forward GGACAACAAGCTCGTGAAAGA, 18S reverse CAGAAGTGACGCGCCCTCTA. All PCR data were normalized to the expression of 18S, used as an internal control. PCR data were obtained as Ct values and the mean of the duplicate Ct values of each sample was calculated. Relative levels of gene expression were normalised to 18S housekeeping gene (HK) using the comparative Ct method.
Supplementary Figure 1. No differences in mRNA gene expression of osteoclast genes. Quantification of Cathepsin K (A), Atp6v0d2 (B), TRAF2 (C) and TRAF6 (D) mRNA expression in OC at day 21 after monocyte culture with M-CSF (25 ng/ml), Flt3L (25 ng/ml) or the combination of M-CSF + CEP-701 (1 μM) and Flt3L + CEP-701 in the presence of RANKL (50 ng/ml). No significant differences were observed.
Supplementary Figure 2. CEP-701 reduces Flt3L induced pre-osteoclast formation but does not alter the number of nuclei per osteoclast. (A) Quantification of pre-osteoclasts numbers at day 21 after monocyte culture with M-CSF (25 ng/ml), Flt3L (25 ng/ml) or the combination of M-CSF + CEP-701 (1 μM) and Flt3L + CEP-701 in the presence of RANKL (50 ng/ml). Significant differences were observed between Flt3L cultures with and without CEP-701 (p=0.01) (B) Quantification of the number of nuclei per osteoclasts at day 21 after monocyte culture with M-CSF (25 ng/ml), Flt3L (25 ng/ml) or the combination of M-CSF + CEP-701 (1 μM) and Flt3L + CEP-701 in the presence of RANKL (50 ng/ml). No significant differences were observed.