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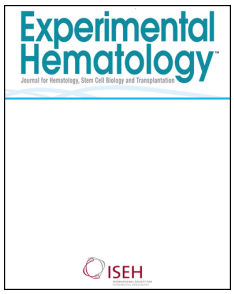

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Everolimus restrains the IL-17A-dependent osteoclast-like transdifferentiation of dendritic cells in multiple myeloma

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ABSTRACT

Interleukin-17A (IL-17A) promotes the osteoclast (OC)-like differentiation of dendritic cells (DCs) in multiple myeloma (MM), and contributes to the pathogenesis of myeloma bone disease (MBD). In our study, EVR significantly abrogated the in vitro OC-like activity of DCs from 12 MM patients. Exploring the EVR effects, we found that the inhibition of the osteo-erosive activity of OC-DCs was mostly due to the blockade of signals driven by the IL-17A receptor toward CEBPbeta/MAFB axis Therefore, MM patients with MBD would probably benefit from mTOR inhibition.

Introduction

The development of myeloma bone disease (MBD) stems from the osteoclast (OC) hyperactivity promoted by malignant plasma cells. In this context, immature DCs (iDCs) may transdifferentiate within myeloma bone marrow into functional OC-like cells according to their monocytoid derivation (1, 2) and the effect of the abundance of interleukin (IL)-1, IL-6, IL-11 as well as both IL-17A and RANK-L (receptor activator of nuclear factor kappa-B ligand) (3, 4).

In the bone marrow of multiple myeloma (MM) patients, T-helper (Th)-17 cells release variable amounts of IL-17A whose levels are of great effort for the formation of lytic lesions (5-8). In fact, this cytokine drives the differentiation of OC progenitors by enhancing the release of a number of osteoclastogenic factors such as monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1□, matrix metalloprotease-9 (MMP-9) and adhesion molecules (9, 10). Finally, IL-17A promotes the fusion of DCs, resulting in the multinucleated bone resorbing giant cells in Langherans histiocytosis while enhances the sensitivity of OC precursors to RANK-L (11, 12) that is also expressed by malignant plasma cells within MM milieu. Moreover, γc cytokines, namely IL-2 and IL-15, strongly regulate the IL-17A/IL-A receptor (IL-17RA) cascade promoting the Th17 polarization of T-cells while IL-21 shows the capability to up-regulate the IL-17RA, thus leading to the transcription of downstream signals (**13**).

IL-17A stimulates with high affinity the IL-17RA that drives the OC-like transdifferentiation of iDCs through the CEBPB (CCAAT/enhancer-binding protein beta) factor, thus leading the transcription of NFATc1 (nuclear factor of activated T-cells, cytoplasmic)-1, MITF (microphthalmia-associated transcription factor) and cFos while modulates MAFB (musculoaponeurotic fibrosarcoma oncogene homolog B) as the negative regulator of OC maturation (14, 15). In addition, IL-17A regulates the OC polarization and bone remodelling priming the CEBPB expression for the balance of Lip/Lap isoforms while restrains experimental and human osteoclastogenesis by MAFB up-regulation (14). CEBPB controls the proliferation and differentiation of OCs as well as of other cell types through the alternative translation-initiation at distinct in frame start sites of Lap and Lip isoforms (**16**). The production of Lap and Lip isoforms is also regulated by signals under the control of the mammalian

target of rapamycin (mTOR) pathway (**17**). In addition, rapamycin selectively inhibits the OC generation and proliferation in wild-type mice while those from CEBPB gene-deleted strains are apparently not affected and also show altered MAFB transcription (**18**).

Since the mTOR cascade is activated in bone resorbing OCs, its inhibition by rapamycin results, through the over-expression of Lap over Lip isoform, in defective osteoclastogenesis of giant cell tumor and osteoporotic murine model. (19, 20). It is noteworthy that MAFB is also deregulated in MM while IL-6 and other marrow growth factors activate the PI3K/AKT/mTOR pathway to promote MM cell survival, migration and drug resistance (21-23). In this context, the dual targeting of PI3K and mTOR by BEZ235 inhibitor in murine MM decreases the serum paraprotein production and the tumour burden with a parallel reduction of osteolytic lesions (24). In addition, targeting PI3K by buparlisib efficiently inhibits the growth of myeloma cells thus suggesting the central role of this pathway for the hyperactivation of ostoeclastogenesis in MM (**25**). Finally, also everolimus (EVR) may indirectly limit the OC differentiation by affecting the CEBPB/MAFB signalling downstream of the IL-17RA (14, 15, 22).

Based on the critical role of the CEBPB/MAFB cascade for the OC-like differentiation of iDCs driven by IL-17A stimulation, we explored the potential interference of EVR with marrow iDCs from MM patients, and its potential inhibitory activity on IL-17A/IL-17RA signalling.

Methods

MM patients and dendritic cell purification. Twelve patients with MM were enrolled in the study after obtaining written informed consent, as well as prior approval from the local Ethics Committee of the University of Bari 'Aldo Moro'. Both newly diagnosed and relapsed/refractory MM patients with skeleton involvement were included in the study. Bone marrow mononuclear cells (BMMCs) were derived from the iliac crest biopsy and stimulated in vitro to obtain enriched suspensions of iDCs (5).

Immature DCs were produced in vitro by culturing $1x10^6$ adherent BMMCs for 6 days in the presence of IL-4 (50 ng/ml) and GM-CSF (50 ng/ml) while their enrichment higher then 95% in flow-cytometry (FACScanto, Becton Dickinson, San Jose, CA) was investigated by gating the anti-Lyn$^-$

/CD11c$^+$/BDCA-1$^+$/BDCA3$^+$/HLA-DR$^+$ population with the relative monoclonal antibody (MoAb; Serotec, Raleigh, NC). After enrichment cells were stimulated up to 14 days with both M-CSF (30 ng/ml) and IL17A (10 ng/ml) to generate OC-DCs, as reported (8).

Everolimus treatment and functional assays. Both iDCs and OC-DCs were incubated with EVR at 0.1-50 nmole/ml for 24-72 hrs and analyzed by the MTT [3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymetho-xyohenil)-2-(4-sulfophenyl)-2H-tetrazolium] test to measure the cytotoxic effect, while viability was investigated by flow-cytometry through the propidium iodide (PI) uptake. Briefly, 5x10^4 cells were incubated with EVR for 24-72 hours, followed by treatment with 100 μg/ml of RNase A (Roche, Basel, Switzerland), and then with 100 μg/ml of PI. Parallel experiments explored the effect of EVR in restraining the adhesion of cells cultured on plastic dishes previously coated with 10 μg/ml vitronectin (Promega, Madison, WI, USA). Both the dose- (0.1-50 nmole/ml) and time-dependent (24-72 hrs) activity of EVR was evaluated by the MTS test after the removal of not-adherent cells. OC-DCs not exposed to EVR were used as control and each assay was performed in biological triplicate. Based on established sub-cytotoxic dose, iDCs were cultured in presence of EVR at 7.5 nmole/ml to explore its ability to restrain their transdifferentiation into OC-DCs. The cell morphology and multinuclearity were evaluated by May-Grünwald Giemsa staining whereas the effect on the bone erosive property of was assessed on calcium phosphate discs after Von-Kossa staining (5), and measured as the percentage of pits of erosions and resorbed area by counting 10 fields of 0.78 cm^2 with a light microscope equipped with dedicated software (Olympus, Milan Italy).

Real-time measurement of OC-related factors and IL17A pathway activity. Untreated or MM iDCs stimulated with EVR at 7.5 nmole/ml along the IL-17A-induced in vitro transdifferentiation in OC-DCs were measured by real-time PCR in RNA levels of *RANK, c-fms, IL-17RA, αVβ3, Cathepsin-K, TRAcP5b, Carbonic Anhydrase-2, TNF-α, ATPv0d2, cFos, NFATc1, DC-STAMP, MITF, NF-kB* and *MAFB* as functional OC genes. The mean threshold cycle (ct) value of each gene was subtracted from the *β-Actin* value to yield the Δct, whereas the ΔΔct was the result of the difference between EVR-treated OC-DCs and untreated cells. In addition, both Lip and Lap subunits of CEBPB (Euroclone, Milan, I), MAFB, AKT and mTOR (Santa Cruz Biotech, Heidelberg, D) were quantified as secreted

protein by western-blot (WB) using Image-J Software (NIH, USA) to measure total and phosphorylated proteins, employing the β-actin as housekeeping protein. Optical density (O.D.) was calculated as the ratio between EVR-treated and untreated cells; data are expressed as means from biological triplicates of each experiment.

Statistical analysis. The Mann-Whitney test was applied to calculate the differences in erosive pits, areas of erosion and RNA values. The one-way Anova test was used to calculate the effect of different concentrations of EVR on DC viability and adhesion to the bone substrates.

Results

The viability and adhesion of DCs from MM patients are mostly unaffected by EVR. Figure 1A shows the ability of both iDCs and OC-DCs to survive without significant differences (up; ≥80 % of viable cells) after the stimulation with for 24 hours EVR at variable concentrations as well as their apoptosis resistance at sub-cytotoxic (IC^{20}) drug concentrations of 7.5 nmole/ml. Cell cycle analysis by PI staining of iDCs and OC-DCs from MM patients exposed to EVR revealed a similar percentage of apoptotic cells (2.6±0.6 and 2.3±0.3%) as well as of cells in G0-G1 (82±2 and 85±4%), S (5.1±0.5 and 2.9±0.9) and G2 (8.1±1.7 and 6.9±0.8%) phases. In addition, the stimulation of both populations with EVR at 7.5 nmole/ml and different time points (48 and 72 hours) produced similar results (data not shown). As shown in panel B, in vitro adhesion of iDCs and OC-DCs was poorly affected by EVR treatment for 24 hours (77±3% and 81±4%, respectively) as well as 48 (75±5% and 78±6%, respectively) and 72 hours (76±4% and 77±7%, respectively; data not shown) with respect to untreated cells. Also, the morphology and the number of multinucleated OC-DCs exposed to EVR remained quite similar to that of untreated cells (13±1 and 14±6 cells/cm^2). On the contrary, the bone erosion behaviour of OC-DCs was greatly restrained in a dose-dependent fashion following the treatment with EVR. As shown in Figure 1C and in representative panels in Figure 1D, both the formation of pits (69.6±6% vs. 26.4±5.2%) and area of erosion (61.1±7.6% vs. 26.8±4.3%) induced by OC-DCs was significantly inhibited by the treatment with EVR as compared to untreated cells ($p<0.05$).

EVR down-regulates OC genes in OC-DCs. As shown in Figure 2A, RNA levels ($2^{-\Delta\Delta ct}$) of *αVβ3* (-18.3±1.8), *Cathepsin-K* (-16.2±0.4), *TRAcP5b* (-14.1±2.1), *Carbonic Anhydrase-2* (-13.2±1.7), *ATPv0d2* (-4.9±1.6), *NFATc1* (-8.2±2.1), *cFos* (-8.3±0.9), *c-fms* (-2.6±0.3) and *MITF* (-12-4±1.4) were variably inhibited in OC-DCs treated with EVR. By contrast, a concurrent increase was detected in *RANK* (5.3±1.1), *MAFB* (11.3±3.1), *DC-STAMP* (7.9±2.2), *NFKB* (1.5±1.1), *IL-17RA* (4.4±0.8) and *TNFα* (5.2±1.2). Since MAFB up-regulation restrains the OC polarization, we investigated the effect of EVR on kinases downstream the IL-17RA. Representative WB panels in Figure 2B and 2C show the phosphorylation levels of AKT in OC-DCs, that remained almost unchanged after EVR stimulation (O.D. ratio: 1.13) compared to untreated cells, thus confirming the poor interference of the rapamycin inhibitor with cell viability. By contrast, the phosphorylation of mTOR (O.D. ratio: 1.9) and expression of Lip-CEBPB (O.D. ratio: 1.99) were greatly suppressed, while Lap-CEBPB (O.D. ratio: 0.80) and MAFB (O.D. ratio: 0.67) were increased.

Discussion

The marrow microenvironment housing MM cells includes iDCs that contribute to the accelerated osteoclastogenesis according to their suitability for OC-like transdifferentiation by both RANK-L and IL-17A. Here, we have shown that EVR indirectly inhibits the signalling downstream of the IL-17RA and restrains the OC-like erosive activity of OC-DCs.

OC polarization is regulated by both RANK-L and c-fms, that phosphorylate independent and interconnected kinases for the functional transcription of cFos and NFATc1 nuclear factors, priming the bone resorbing activity (26, 27). High levels of IL-17A and Th17 polarization occur in bone marrow of MM patients suffering of severe skeleton colonization (6). The evidence that myeloma iDCs may undergo to OC-like transdifferentiation via the IL-17A pathway, however, is supported by the signals activated throughout the IL-17RA that converge on TRAF-6 resulting in stimulation of the NF-kB pathway or alternatively the CEBPB/MAFB axis whose activity is regulated, at least partly, by the phosphorylation levels of the mTOR kinase (20, 21). Moreover, the central role of marrow IL-17A over RANK-L for the OC differentiation of iDCs has been definitely proved in MM (8). Hyperactivity of

the mTOR signaling characterizes various cancer populations showing extensive osteotropic phenotype including MM cells whose proliferation is efficiently impaired by mTOR inhibitors, thus limiting the marrow availability of IL-1 and IL-6 as pro-osteoclastogenic cytokines. In contrast with the prevalent cytotoxic effect exerted by EVR, we explored the potential effect of mTOR inhibition on the OC-like properties of marrow MM iDCs. Interleukin-17RA drives the OC-like polarization of iDCs that, however, is restrained by EVR that indirectly limits the IL-17RA transcription through the over-expression of MAFB and, in parallel, of the Lap isoform of CEBPB (15).

Our experiments showed that EVR had practically no effect on viability of both iDCs and OC-DCs as well as on proliferation and adhesion to bone substrates, thus suggesting that osteoclastogenesis impairment mostly depends on the interference with the MAFB/CEBPB signaling. Moreover, osteoclast activity it is not correlated to the impaired production within microenvironment of pro-osteoclastogenic factors by tumor cells, as described in other cancer models (28, 29). Indeed, we showed that the effect of EVR on osteoclastogenesis by OC-DCs is mostly related to the Lip/CEBPB down-regulation in parallel to both the Lap isoform and MAFB activation. Therefore, the up-regulation by EVR of these transcription factors in OC-DCs definitely reflects the increased suppression of the OC-like activity through NFATc1 and cFos modulation (30).

Previous studies described that the OC-like properties of OC-DCs are coupled with an up-regulation of OC genes (27) and we found that the suppressive effect of EVR on the expression of *Cathepsin-K*, *TRAcP5b*, *MITF*, *Carbonic anhydrase-2* is dependent on their lower RNA levels. A parallel defect of those factors involved in bone resorbing activity as *NFATc1*, *Fos* and $\alpha_v\beta_3$ was also demonstrated, whereas the minimal *NFKB* activation was related to its potential subordinate role along the IL-17A/IL-17RA pathway (31). IL-17RA was found up-regulated by EVR treatment and a similar effect is apparently induced by other tyrosine kinase inhibitors such as lapatinib showing the ability to induce the accumulation of both EGFR and Her2-neu levels at the cell surface from breast as well as gastric and mesothelioma malignant populations (**32, 33**). A similar effect is also induced by the dual PI3K/mTOR blockade (**34**, **35**) in vitro as well as by other cytokines engulfing the tumor milieu in

vivo. However, the major activation of the heteromeric IL-17RA depends on IL-17A levels, both in vivo and in vitro (31).

Therefore, our data suggest that mTOR inhibition in OC-DCs suppress their bone resorbing functions mostly induced by marrow IL-17A while EVR could be considered an alternative therapeutic approach for the treatment of bone disease in MM.

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CONFLICT OF INTEREST

Authors have no conflict of interest.

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Legend to Figures

Figure 1. Effect of EVR on OC-DCs. A) The viability of immature DCs and OC-DCs from 12 MM patients was measured by MTT test (up) after incubation with different concentrations of EVR (0.1-50 nmole/ml) for 24 hours. Both iDCs and OC-DCs were variably resistant to EVR stimulation ($\geq$80% of viable cells in both instances). Squares are mean$\pm$SD of DC populations from MM patients and include triplets of experiments. Representative panels (down; pt. #3) show cell cycle variation by PI staining after stimulation with EVR at 7.5 nmole/ml of both iDCs and OC-DCs also revealing a low percentage of apoptotic cells in both instances. B) Both iDCs and OC-DCs from MM patients remained mostly adherent (left) at the highest EVR concentration ($\geq$75% of cells), whereas representative panels of May-Grünwald staining (right) of OC-DCs from pt. #4 and #7 revealed their apparently normal morphology and similar multinuclearity after EVR treatment. (C) EVR restrained the formation of pits of erosion and resorbed area by OC-DCs on calcium phosphate discs. Both the number of pits and the extension of the resorbed areas were evaluated by optical microscopy on at least 10 fields of approximately 0.78 cm^2 and found significantly reduced ($p<0.05$ in both instances). Bars are mean$\pm$SD of the percentage of pit number and resorbed area of untreated with respect to EVR-treated cells. D) Representative panels from pt. #3 showing the osteoerosive effect on calcium phosphate slices of untreated (left) and EVR-treated (right) OC-DCs.

Figure 2. Real-time PCR and western blot assays. A) A panel of genes related to osteoclastogenic functions was evaluated by real-time PCR (upper) in marrow iDC from MM patients after incubation with IL-17A. The RNA levels of major OC receptor genes (*RANK*, *$\alpha V\beta 3$,* and *c-fms*), enzymes (*Cathepsin-K*, *Carbonic anhydrase-2*, *TRAcP5b* and *ATP0d2*) and transcriptional factors (*NFATc1*, c*Fos* and *MITF*) enrolled along the IL-17RA pathway were significantly suppressed by EVR at 7.5 nM. Other genes, such as *MAFB*, were significantly up-regulated in parallel to *IL-17RA* and the cell fusion activator *DC-STAMP*, whereas *NFKB* resulted moderately upregulated. The mean threshold cycle (ct) value of the *β-Actin* housekeeping

gene was subtracted to yield Δct, whereas the ΔΔct value was the result of the difference between EVR-treated OC-DCs and untreated cells. Bars are mean+SD of OC-DCs triplicates from 12 MM patients. Grey box indicates arbitrary basal levels (from -1-0 to 1.0 $2^{-\Delta\Delta_{ct}}$) to calculate the statistical significance of up-regulated (right) and down-regulated (left) genes. Both the expression and phosphorylation of kinases belonging to the IL-17A/IL-17RA pathway were investigated in OC-DCs transdifferentiated by IL-17A; representative WB panels from pt. #3 (B), and relative values of optical density (O.D.) measured by Image-J Software (C), are illustrated. β-Actin was used as housekeeping protein. The expression of both the Lip/CEBPB isoform and phosphorylation of mTOR resulted decreased in the majority of OC-DCs treated with EVR at 7.5 nmole/ml in comparison with untreated cells, while Lap/CEBPB and MAFB were up-regulated ($p<0.05$ in all instances). By contrast, basal and phosphorylated AKT were little modified by EVR, thus confirming its modest effect on OC-DC proliferation.

n: patient number; (-): Untreated OC-DCs; (+): OC-DCs treated with 7.5 mM/ml EVR; S.D.: standard deviation; $p<0.05$ was considered significant at non-parametric Mann-Whitney test. Numbers are mean±S.D. calculated as the difference between the O.D. value from each kinase and the O.D. of β-Actin used as control. * Numbers are mean±S.D. of the O.D. ratio between untreated and treated OC-DCs. N.D.: not done

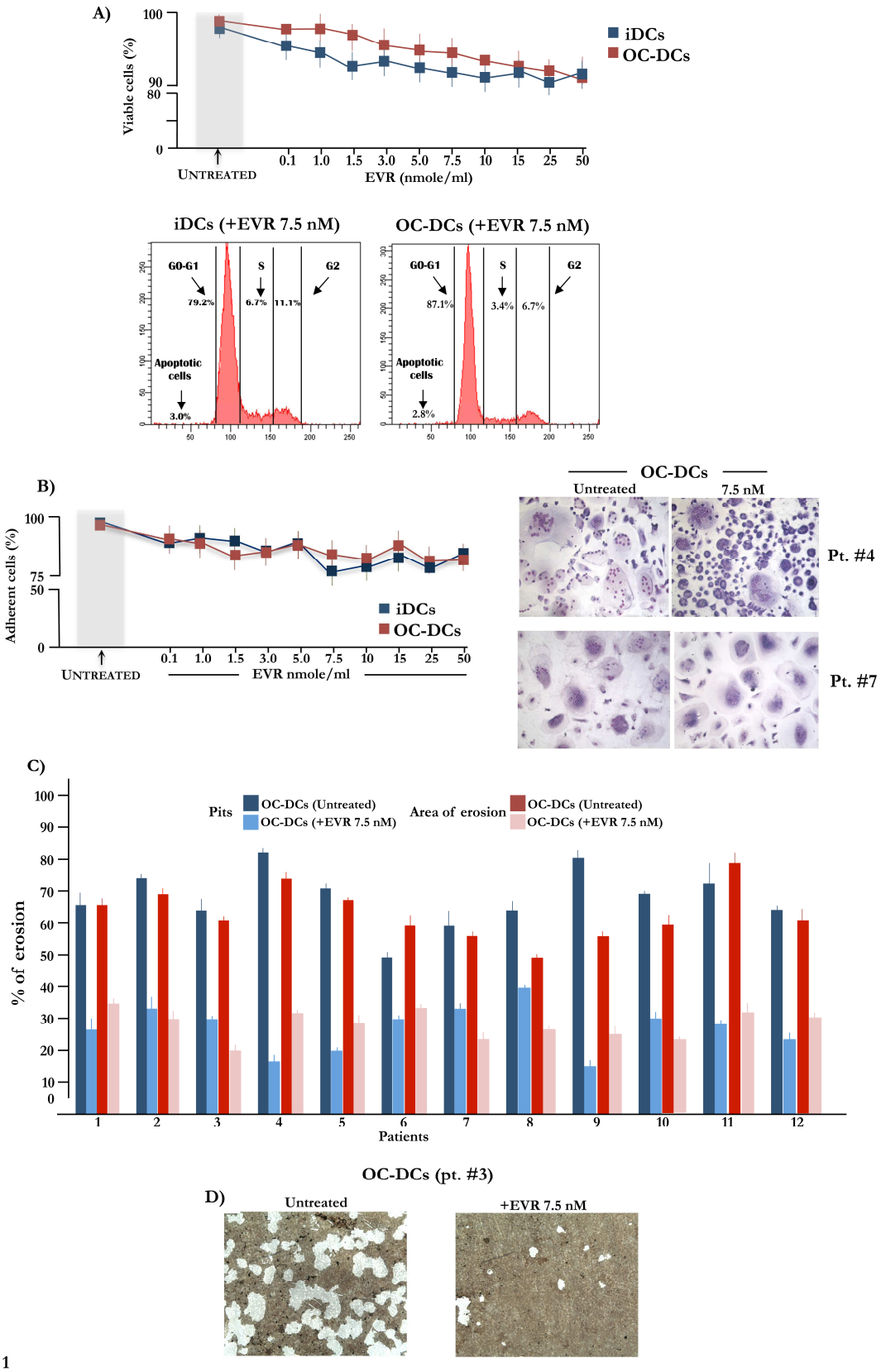
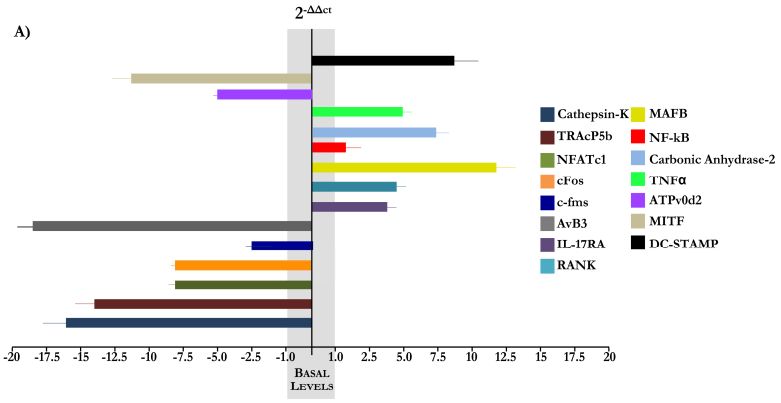
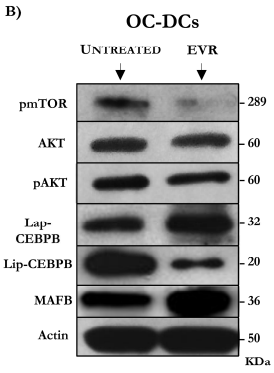

A)
Viable cells (%)
100
90
80
0
UNTREATED
0.1 1.0 1.5 3.0 5.0 7.5 10 15 25 50
EVR (nmole/ml)
iDCs
OC-DCs
iDCs (+EVR 7.5 nM)
OC-DCs (+EVR 7.5 nM)
G0-G1
S
G2
79.2%
6.7%
11.1%
Apoptotic cells
3.0%
87.1%
3.4%
6.7%
2.8%
B)
Adherent cells (%)
100
75
50
0
UNTREATED
0.1 1.0 1.5 3.0 5.0 7.5 10 15 25 50
EVR nmole/ml
iDCs
OC-DCs
OC-DCs
Untreated
7.5 nM
Pt. #4
Pt. #7
C)
% of erosion
Pits
OC-DCs (Untreated)
OC-DCs (+EVR 7.5 nM)
Area of erosion
OC-DCs (Untreated)
OC-DCs (+EVR 7.5 nM)
Patients
1 2 3 4 5 6 7 8 9 10 11 12
OC-DCs (pt. #3)
D)
Untreated
+EVR 7.5 nM

Figure 1





C)

Patients (n)	pmTOR		AKT		pAKT		Lap/CEBPB		LipCEBPB		MAFB	
	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)
1	7.3	2.3	N.D.	N.D.	N.D.	N.D.	8.7	12.3	19.3	6.3	9.1	13.2
2	5.5	1.4	N.D.	N.D.	N.D.	N.D.	15.4	17.7	29.7	11.4	15.2	21.3
3	3.1	0.9	5.3	5.0	6.7	6.2	11.4	18.7	32.3	7.5	13.4	20.4
4	9.3	5.5	8.8	7.3	7.9	6.3	9.4	19.7	16.7	11.4	16.9	18.9
5	11.2	7.0	7.2	7.8	5.4	4.7	16.3	18.3	14.5	9.7	10.1	16.8
6	8.8	3.2	9.2	5.1	11.9	8.5	13.0	11.2	19.5	17.5	8.5	12.4
7	2.7	1.1	5.5	5.5	4.8	3.9	7.6	18.1	N.D.	N.D.	15.2	14.0
8	6.9	6.0	N.D.	N.D.	N.D.	N.D.	17.2	23.9	11.2	10.9	9.0	17.9
9	9.1	8.7	10.2	8.7	13.9	10.4	11.8	18.8	N.D.	N.D.	10.2	28.3
10	8.1	8.0	6.1	7.2	7.9	9.8	16.2	26.2	15.4	4.7	17.2	31.9
11	11.7	2.2	7.7	7.2	11.9	12.7	9.2	13.5	17.8	10.1	14.4	17.3
12	10.0	1.3	11.7	10.6	13.1	11.2	9.5	9.9	26.7	12.6	16.2	24.0
O.D. ratio (-)/(+)*	1.9		1.11		1.13		0.8±0.16		1.99		0.65	
P	*0.004*		*0.37*		*0.40*		*0.0047*		*0.0012*		*0.0039*	

- Everolimus restrains the malignant osteoclastogenesis in multiple myeloma
- The IL17A/IL17RA cascade is partly blocked by Everolimus in OC-like DCs
- Everolimus should be considered for the treatment of cancer-related bone disease