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Immunoregulatory cytokines in mouse placental extracts inhibit in vitro osteoclast differentiation of murine macrophages

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ABSTRACT

Introduction: Previous studies showed that placental extracts (PE) alleviates arthritic symptoms in animal models of arthritis.
Methods: To evaluate whether murine PEs obtained at embryonic days 7.5 (PE7) and 17.5 (PE18) regulate RANKL-induced osteoclast differentiation, RAW 264.7 cells were cultured with RANKL and MCSF in presence or not of PEs. Tartrate-resistant acid phosphatase (TRAP) was stained and multinucleated TRAP positive cells were visualized under a light microscope. *Cathepsin K* and metalloprotease expression was assessed by RT-PCR and gelatin zymography respectively. NFATc1 expression was determined by immunoblot. To analyze NFAT-dependent transcription, macrophages were transfected with a luciferase reporter plasmid. Cytokines were determined in PEs by ELISA and immunoblot. Transforming growth factor (TGF)- beta and Interleukin (IL)-10 receptor were inhibited in cell cultures with specific antibodies.
Results: PE7 and PE18 inhibited RANKL-induced multinucleated TRAP positive cells, *Cathepsin K* expression and metalloprotease activity, as well as NFATc1 expression and activity, thereby inhibiting osteoclast differentiation of RAW cells. Inflammatory/Regulatory cytokine ratio was higher in PE7 than in PE18. Blocking TGF-beta abolished the effect of both, PE7 and PE18, on multinucleated TRAP positive cells and metalloprotease expression, whereas blocking IL-10 receptor reverted the effect of PE18 but not of PE7.
Discussion: Inhibition of osteoclast differentiation by PEs was not unexpected, since cytokines detected in extracts were previously found to regulate osteoclast differentiation.
Conclusions: PEs inhibited osteoclast differentiation of macrophages in vitro. Downregulation of NFATc1 might be involved in this effect. Regulatory/Th2 cytokines play a role in the effect of PEs on osteoclast differentiation.

1. Introduction

A beneficial effect of pregnancy on clinical symptoms has been observed in several Th1-mediated autoimmune diseases (reviewed in Ref. [1]).

Pregnancy is viewed as a Th2-predominant state, although several Th1-related cytokines are vital to early pregnancy [2]. Th2 cytokines are associated with the down-regulation of Th1 cytokines and may confer protection from Th1-mediated autoimmune diseases.

Placental derived-immunoregulatory factors have been described so far [3–6]. We have previously reported that rat placental culture supernatant improves clinical symptoms in an experimental model of arthritis in rats [7].

Placental extracts (PE) have been traditionally used to treat chronic inflammatory diseases in Oriental medicine. It was reported that PE alleviated the arthritic symptoms in adjuvant-induced arthritis [8]. Furthermore, it was described that PE inhibited IL-1β- induced bone resorption in mouse, as measured by ^{45}Ca release [9], and protected cartilage from degradation, in experimental osteoarthritis [10].

Bone resorption is performed by osteoclasts, multinucleated giant cells, originated mainly from monocytes. They undergo a series of differentiation steps: acquire specific markers such as tartrate-resistant acid phosphatase (TRAP), fuse to multinucleated giant cells, and polarize upon contact with bone [11]. Essential

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signals for osteoclast differentiation are macrophage colony-stimulating factor (M-CSF) and Receptor Activator of Nuclear factor (NF)-κB (RANK)- ligand (RANKL) [12]. The induction of the transcription factor Nuclear Factor of Activated T cells (NFAT) plays a major role [13,14].

Based on the protective role of pregnancy during arthritis, we ask whether protein extracts obtained from mouse placenta at different days of pregnancy could modulate osteoclast differentiation of monocyte precursors.

2. Methods

2.1. Animals

BALB/c mice, 5 wk of age, were maintained at our Institutional Animal Care Facility. All procedures were conducted under consent of the Committee on the Ethics of animal experiments of "Instituto de Estudios de la Inmunidad Humoral Prof. R. Margni", in accordance with guidelines of EU Directive 2010/63/EU.

2.2. Isolation of placental tissue

Conceptuses were collected from timed-pregnant mice on embryonic days E7.5 and E17.5 (vaginal plug on day 0.5). Five mice were employed per group, and tissue from each mouse was processed separately. Fetus and decidua basalis were discarded from conceptuses and the remaining placental tissue was isolated. To note is that the developing placenta is not readily separated from the decidual components at day E7.5.

2.3. Preparation of isogeneic placental extracts

The placental tissue was washed twice in Tris buffered saline, (TBS), pH 7.4 and homogenized manually in TBS containing protease inhibitor cocktail (Roche, Buenos Aires, Argentina). The homogenate was exposed to 4 freeze–thaw cycles and after centrifugation (6000 g, 20 min, 4 °C) the supernatant was collected and sterilized with 0.2 micron filters. Total protein concentration was determined by the Bradford Assay (Bio Rad Laboratories, CA, USA). The aqueous placental extracts (PEs) were aliquoted and stored at −80 °C.

2.4. Cell cultures

The murine monocyte cell line RAW 264.7 differentiates into osteoclast like cells in the presence of RANKL and M-CSF [15]. Cells were grown in α-minimum essential medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Natocor, Córdoba, Argentina), 1% (v/v) penicillin–streptomycin solution, 1 mM sodium pyruvate, 2 mM L-glutamine, and 0.1 mM non-essential amino acids (all from Gibco) at 37 °C in a humidified atmosphere of 5% CO_2. For osteoclastogenesis experiments, cells were seeded in either 100 mm^2 culture plates or 24 well plates at a density of 2.5×10^6/plate or 5×10^3/well respectively, and cultured during the indicated times in the presence or absence of 100 ng/ml of RANKL and 20 ng/ml of M-CSF (both from Chemicon, MA, USA). Concentrations of RANKL and M-CSF were as previously described [15].

To asses the effect of PE on osteoclast differentiation, cells were pre-cultured for 1 h in the presence or absence of 50 μg/ml of either, PE obtained at E7.5 (PE7) or at E17.5 (PE18), before RANKL and M-CSF treatment and were present throughout the experiments. The concentration of PE used was selected as the maximal concentration at which cell viability of RAW 264.7 was not affected (Fig. 1).

2.5. Tartrate-resistant acid phosphatase (TRAP) staining

Cells plated at $5 \times 10^3/cm^2$ were cultured in the presence or absence of RANKL, M-CSF, and PEs, during 5 days, with a medium change on the third day. TRAP staining was performed using a commercially available kit (Sigma Aldrich, St. Louis, MO, USA). Cells were visualized in a light microscope. The number of TRAP Positive Multinucleated Cells (TPMC, osteoclast like-cells) per microscopic field was determined, out of 25 fields for each condition.

2.6. Cell viability

The WST-1 tetrazolium salt was employed under manufacturer's instruction (Roche, Buenos Aires, Argentina). Optical density was measured at 450 nm in a microplate reader (Multiscan EX, Thermo Fischer Scientific, Rockford, IL, USA).

2.7. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Cells were cultured in the presence or absence of stimuli, for 48 h. Total RNA was isolated from the cells with TriZol isolation reagent (Invitrogen, Grand Island, NY, USA) and transcripts encoding mouse *Cathepsin K* and β-*actin* were analyzed by semiquantitative RT-PCR. Primers for PCR amplification were: *Ctpsk* forward, 5′-GAGGGCCAACTCAAGAAGAA -3′; reverse, 5′- GCCGTGGCGTTATACATACA -3′; *Actinb* forward, 5′-GTCGACAACGGCTCCGGCA-3′; reverse, 5′-GTCAGGTCCCGGCCAGCCA-3′. Amplified cDNAs were separated by agarose gel electrophoresis, and bands were visualized by ethidium bromide staining.

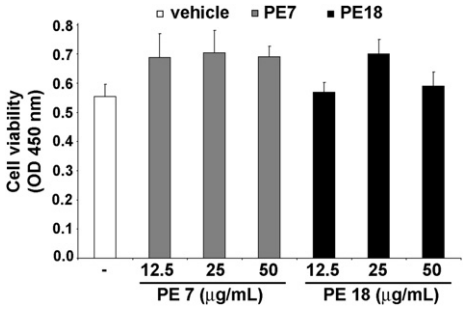

Fig. 1. Cell viability of RAW 264.7 cells cultured with extracts of placental tissue obtained from mouse at day 7.5 (PE7) and 17.5 (PE18) of pregnancy. Cells were seeded in a 96 well plate at $1 \times 10^4/cm^2$ and cultured during 48 h with 0, 12.5, 25, and 50 μg/ml of PE7 (open bars) or PE18 (closed bars). WST-1 tetrazolium salt reagent was added to cultures during the last 90 min. Optical density was measured at 450 nm. PEs showed no effect on the viability of the cells. Data are expressed as mean ± SD from triplicate cultures. Shown is a representative experiment of three performed.

2.8. Gelatin zymography

Cells were seeded in either 1% or 0% fetal bovine serum-containing culture medium and cultured for 48 h in the presence or absence of stimuli. Conditioned medium was analyzed for MMP-9 activity by gelatin substrate gel electrophoresis [16], and quantified by densitometric analysis using the NIH Image Program.

2.9. Cell lysis and NFATc1 immunoblot analysis

Macrophages were cultured in the presence or absence of RL + M, during 96 h. To asses the role of PEs on NFAT expression, cells were precultured with either PE7 or PE8 for 1 h before RANKL plus M-CSF (RL + M) stimuli.

Macrophages were lysed in a hypertonic buffer and NFATc1 was assessed in the total extracts by immunoblot as described [17].

2.10. Plasmid constructs and transient transfection assays

The luciferase reporter plasmid NFAT/AP1 luc was as described [18]. Null *Renilla* was from Promega (Madison, WI, USA). Cells were transfected by the FuGene HD method (Roche Applied Sciences, Penzberg, Germany) in accordance with the manufacturer's instructions. Forty eight hours later, the cells were exposed to vehicle or placental extracts (PE7, PE18) for 1 h and then treated with RL + M for 24 h. Cells were lysed according to the instructions of the Dual Glo Luciferase assay kit (Promega), and luciferase activity was measured in a luminometer (Victor X Plate Reader, PerkinElmer, Waltham, MA, USA). Results were normalized to a *Renilla* luciferase internal control.

2.11. Cytokine assays

Cytokine-specific ELISA (BD Biosciences, San Jose, CA) was used for detecting IL-4, IL-6, IL-10, IL-12 and IFN-γ concentrations in PE7 and PE8, according to the manufacturer's instructions.

PE7 and PE18 were assessed for TGF-β by immunoblot. Placental extracts were resolved by 12% SDS-PAGE under reducing conditions. Proteins transferred to nitrocellulose membranes were immunoblotted with goat polyclonal anti-TGF-β or goat polyclonal anti- β-actin antibodies, and then incubated with a peroxidase labeled secondary antibody (all from Santa Cruz Biotechnology, Delaware, CA, USA). Bound antibodies were detected by ECL (Pierce).

2.12. Inhibition studies

Anti-IL-10R neutralizing antibody (BD Biosciences) was added to cells 30 min before incubation with PEs, and RL + M. Anti-TGF-β blocking antibody was incubated for 30 min with PEs and then, antibody-treated PEs were added to macrophages and cultured for 30 min before stimulation with RL + M. Medium and stimuli were replaced at day 3. After 5 days of culture cells were stained for TRAP. Metalloprotease (MMP) activity was assessed by gelatin zymography after 2 days of culture.

2.13. Data analysis

Data are presented as means ± SD of several determinations. Differences between groups were tested for significance using one-way analysis of variance (ANOVA), and the Bonferroni multiple comparison post-test. Differences between means in ELISA were tested by Student *t* test. (***) $P < 0.001$, (**) $P < 0.01$, (*) $P < 0.05$. (Motulsky, HJ Prism 4 Statistics Guide. Graph-Pad Software Inc., San Diego CA 2003).

3. Results

3.1. Mouse placental extracts obtained at E7.5 (PE7) and E17.5 (PE18) inhibited the M-CSF/RANKL-induced differentiation of RAW 264.7 macrophages

We first investigated whether PE7 or PE18 affected cell viability of RAW 264.7 in culture. We found that neither PE7 or PE18 at concentrations ranging from 12.5 to 50 μg/ml affected the viability of the cells (Fig. 1).

RANKL stimulation increased the number of TPMC compared to control non-stimulated cells (ns). M-CSF increased the number of TPMC induced by RANKL stimulation. Treatment with M-CSF alone induced a mild increase in the number of TPMC, although it was not significantly different from ns cells. Pre-culture of the cells with PE7 or PE18 (50 μg/ml), diminished the increase of the number of TPMC induced by RANKL treatment (Fig. 2).

Placental extracts (PE7 or PE18) also inhibited the MCSF/RANKL-induced *Ctpsk* mRNA expression in the cells (Fig. 3A). Notably, PE18 diminished the basal expression levels of *Ctpsk* mRNA in absence of stimulation, while PE7 did not.

Metalloprotease activity in RANKL/M-CSF stimulated cells was diminished in PE7 or PE18 pretreated cells (Fig. 3B and C). PE18 also inhibited the basal levels of MMP in non stimulated cells. It is remarkable that in cultures performed in 1% FBS, RANKL induction of MMP was not observed compared to the basal levels found in non-stimulated cells (Fig. 3B). When FBS was absent, basal levels of MMP activity diminished, the effect of PE18 on basal MMP activity was not further observed, and increased MMP activity in RANKL/MCSF stimulated cells was observed, as well as the inhibition of this induction by PE7 and PE18 (Fig. 3C).

3.2. PE7 and PE18 inhibited the RANKL/M-CSF-induced NFAT-dependent transcription, and protein expression of NFATc1

We analyzed NFATc1 protein expression in macrophages stimulated or not with RL + M (Fig. 4A). Bands recognized by the antibody diminished in RL + M- stimulated cells when either PE7 or PE18 were present. Remarkably in absence of RL + M, PE7 stimulated NFATc1 expression in the cells, whereas the effect of PE18 was not significantly different from the control. NFAT-protein dephosphorylation was evident in the faster mobility of the protein bands recognized by the antibody in the protein samples of PE- treated, and RL + M-treated cells (Fig. 4B, lanes 2–4). Pretreatment of the cells with PE 7 or PE18 inhibited the RL + M-induced dephosphorylation of NFATc1.

Next, we analyzed NFAT-dependent transcription in RAW cells transfected with the AP1/NFAT-luc reporter plasmid. The treatment of the transfected cells with RL ± M during 24 h, increased 1.7 fold the luciferase activity respect to the untreated cells (Fig. 4B). Pre-incubation of the cells with both, PE7 and PE18 for 1 h before stimulation, inhibited the luciferase activity induced by RL + M. Accordingly with data from western blot, PE7 but not PE18, induced NFAT-dependent transcription in absence of RL + M.

3.3. Cytokine levels in PE7 and PE18 placental extracts

Higher levels of IL-6, IL-12, IFN-γ, and TNF-α levels were found in PE 7 than in PE18. On the other hand, PE18 showed higher levels of IL-10 than PE 7, while differences between PE7 and PE18 were not observed regarding levels of IL-4 (Fig. 5A). We compared the levels of expression of TGF-β between PE 7 and PE18 by western blot. PE18 showed an intense band recognized by specific anti-TGF-β antibodies, which faintly appeared in PE7 (Fig. 5B).

3.4. Blocking of interleukin-10 receptor and TGF-β abolished the inhibition of RANKL-induced- TPMC and MMP activity

Among cytokines described to be involved in the inhibition of osteoclastogenesis, we focused on IL-10 and TGF-β, regarding their role in Th1-mediated autoimmune diseases [19–21].

Blocking of IL-10R abolished the inhibitory effect of PE18 on the RL + M-induced TPMC in RAW cells. The effect of PE7 was not affected. Blocking TGF-β during cell cultures with PEs abolished the effect of both PE7 and PE18 on the RL + M-induced TPMC (Fig. 6A).

Consistently, inhibition by PE7 and PE18 of MMP activity induced by RL + M was abolished when PEs were pre-incubated with anti-TGF-β antibodies (Fig. 6B). It is notably that MMP activity diminished in supernatants of cells cultured in presence of anti-TGF-β antibodies compared to levels found in cells cultured with unrelated antibody. We found that cells cultured with anti-TGF-β antibodies showed lower viability than cells cultured with the unrelated antibody (Fig. 6C).

4. Discussion

We found that PE7 and PE18, when present throughout cell culture, inhibited the M-CSF/RANKL-induced osteoclast differentiation of RAW 264.7 macrophages, as measured by TRAP expression, multinucleation, and protease expression by the cells. PE18 but not PE7 inhibited the basal expression of both *CtpsK* mRNA and 92 kDa MMP by cells cultured in media supplemented with 1% FBS, suggesting a differential role. Nevertheless, when FBS was absent from culture media, basal level of MMP diminished clearly, and PE7 and PE18 equally inhibited M-CSF plus RANKL-elicited MMP activity.

In osteoclast precursors RANKL activates NF-κB transcriptional activity and induces NFATc1 and AP-1 expression, which in turn activate the transcription of osteoclast-related markers [15,22]. Activation of NFAT requires its dephosphorylation by calcineurin, after which it translocates to the nuclei and binds to specific binding sites in the promoter of target genes (reviewed in [23]). PEs might be acting on signaling pathways involved in NFATc1 protein expression. It has been reported that activation of the transcription factor STAT-1 downregulated c-Fos expression and thus abolished RL + M-induced NFATc1 expression and osteoclast differentiation [24]. We found that PE7 and PE18 inhibited the RL + M-elicited NFATc1 expression and activity.

Unexpectedly, PE7 but not PE18 induced NFATc1 expression and activity in absence of RL + M stimulation. RANKL-independent osteoclastogenesis was reported to be induced by cytokines, such as IL-6, IL-11, and TNF-α/IL-1 [25,26]. Nevertheless, in spite of increasing NFATc1 expression and activity, PE-treatment of macrophages did not induce osteoclast differentiation in absence of RL + M stimulation, as regards the levels of TRAP activity, multinucleation and protease expression by the cells.

In order to investigate whether the inhibition of differentiation of the cells was mediated by cytokines present in the extracts, we first analyzed cytokines concentration in PEs. We found higher Th2-regulatory/Th1- proinflammatory- cytokine ratio in PE18 compared to PE7. This is in accordance to the previously described immunobalance during pregnancy. Regulatory IL-10 and TGF-β are produced by several cell types which are present at the maternal–fetal interface, including mesenchymal stem cells [27], CD4/Foxp3+ regulatory T lymphocytes [28], γδ T cells [29], tissue-resident

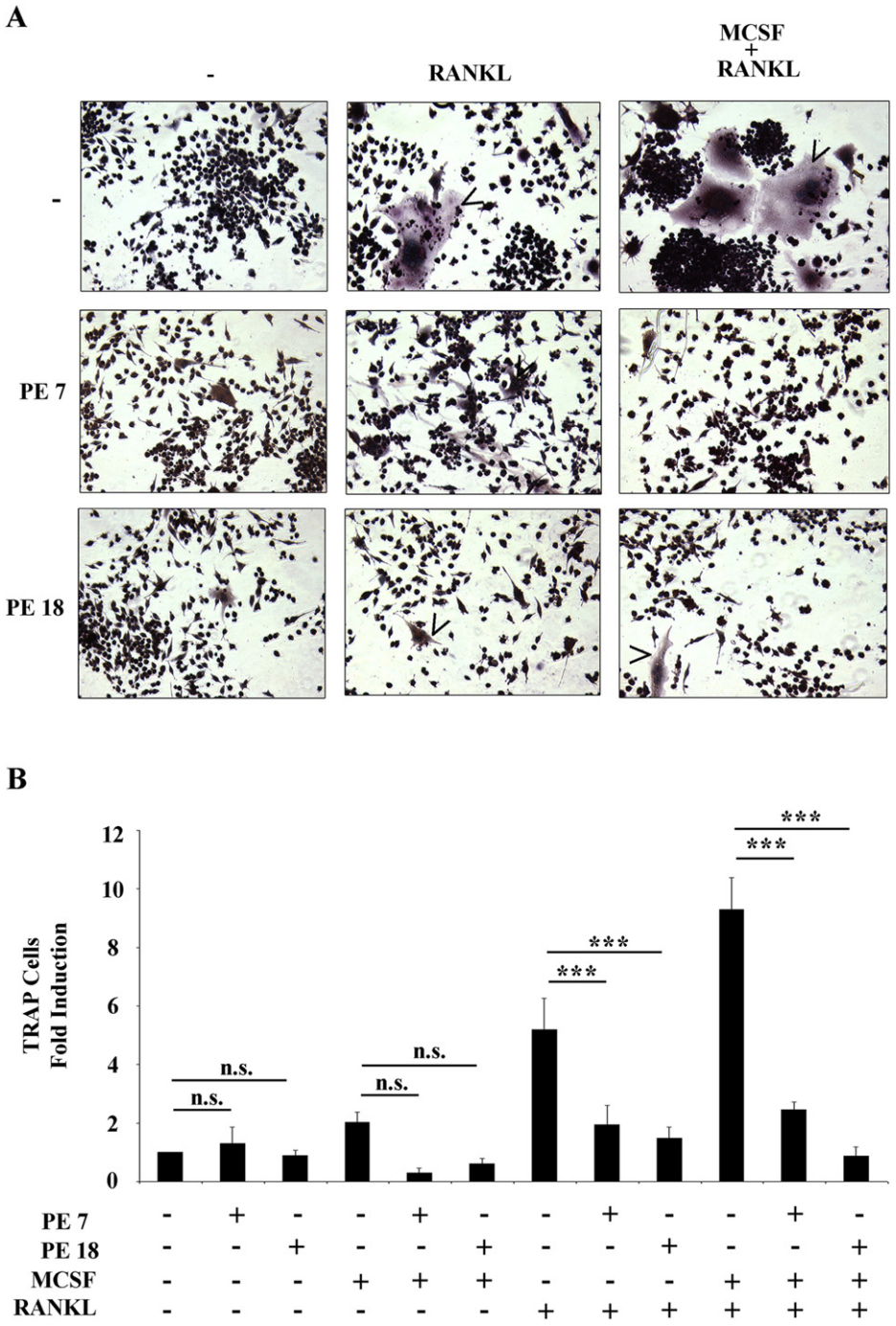

Fig. 2. Extracts of placental tissue (PE7 and PE18) inhibited the MCSF/RANKL-induced TRAP staining. RAW cells seeded on glass coverslips in a 24 well plate at $5 \times 10^3/cm^2$ were cultured in α-MEM supplemented with 10% FCS, in the absence (−) or presence (+) of 100 ng/ml RANKL, 20 ng/ml M-CSF, and 50 μg/ml PEs, during 5 days. Medium was replaced on the third day and cells were cultured for 2 more days. Cells were stained for TRAP expression. (A) Arrowheads indicate TRAP + multinucleated cells. Shown is a representative experiment which was repeated three times. Magnification 100×. (B) The number of multinucleated cells positive for TRAP in 10 microscopic fields at 100× magnification was determined and plotted as fold induction relative to the control, non stimulated cells (bar 1). Data are expressed as mean ± SD from triplicate cultures. n.s: not significant; $^{***}p < 0.001$ (ANOVA).

tolerogenic dendritic cells [30], and M2 macrophages [31]. In addition, TGF-β is abundantly expressed by syncytiotrophoblast cells of the placental villi [32].

Cytokines that have regulatory properties in inflammatory responses negatively regulate osteoclast formation. IL-4 reversibly inhibits osteoclastogenesis via inhibition of NF-κB and mitogen-activated protein kinase signaling [33]. IL-10 may down-regulate osteoclastogenesis in part through inhibition of the expression of NFATc1, c-Fos and c-Jun and by decreasing the RANKL-induced expression of NF-κB p50 and phosphorylation of JNK [34]. Although usually found to be stimulatory, TGF-β1 suppressed M-CSF + RANKL-driven osteoclast differentiation from bone marrow-derived monocytes when cytokine was present throughout differentiation time [35]. Also, Karsdal et al. [36] described that TGF-β down-regulated RANK expression on purified monocytes and modulated all of the osteoclastic phenotypes investigated.

We found that neutralizing IL-10R abolished the inhibition by PE18 of the TPMC induced by RL + M stimulation (Fig. 6A).

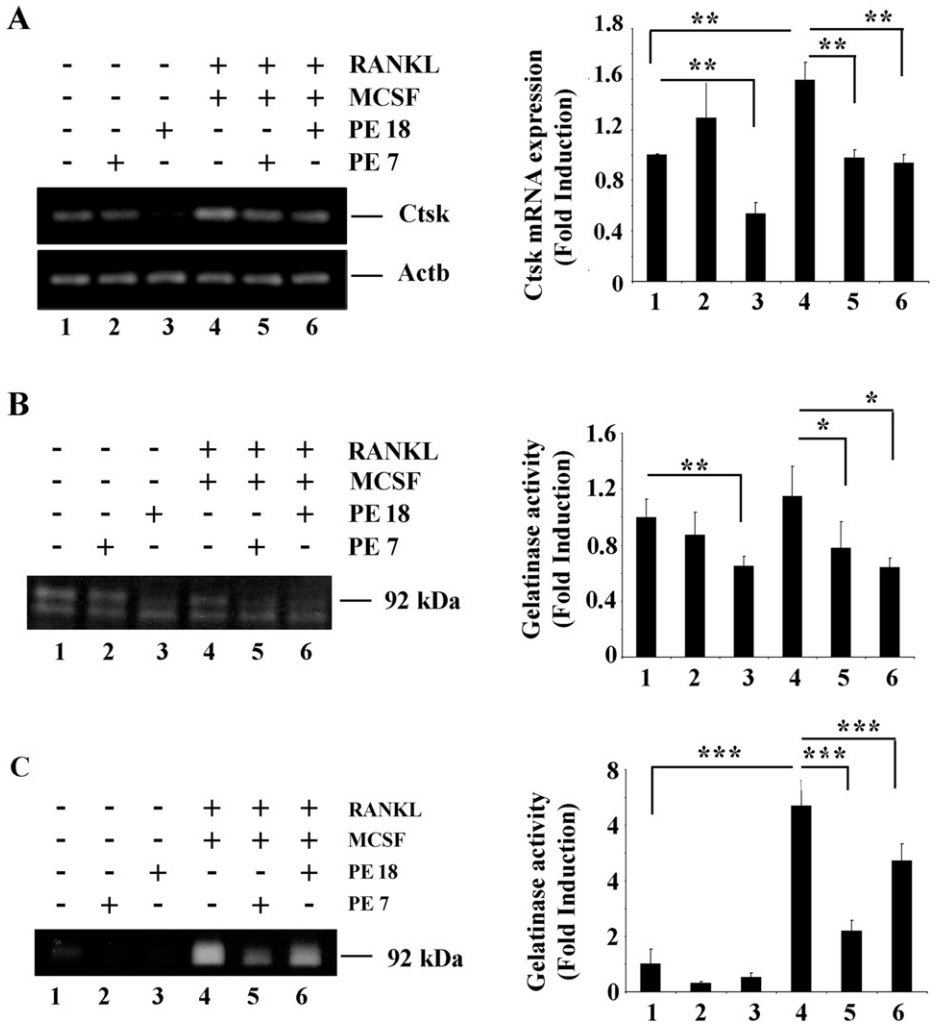

Fig. 3. Extracts of placental tissue (PE7 and PE18) inhibited the MCSF/RANKL-induced expression of *Cathepsin K* (*Ctpsk*) mRNA and Matrix-metalloprotease activity. (A) *Ctpsk* mRNA was amplified from total RNA by semi-quantitative RT-PCR. The transcript of *β-actin (Actb)* was used as internal control. RAW cells seeded in a 6 well plate at $1 \times 10^4/cm^2$ were cultured in α-MEM supplemented with 10% FCS in the absence (−) or presence (+) of 100 ng/ml RANKL, 20 ng/ml M-CSF, and 50 μg/ml PEs, during 4 days. Medium was replaced on the second day and cells were cultured for 2 more days. (B and C) Metalloprotease activity was assessed by gelatin zymography as described in M&M. The band with the electrophoretic mobility corresponding to MMP9 (92 kDa) is indicated. RAW cells seeded in a 24 well plate at $1 \times 10^4/cm^2$ were cultured in α-MEM supplemented with 10% FCS in the absence (−) or presence (+) of 100 ng/ml RANKL, 20 ng/ml M-CSF, and 50 μg/ml PEs, during 4 days. (B) Medium was replaced on the second day for 1% FCS supplemented α-MEM and cells were cultured for 2 more days. (C) Medium was replaced on the second day for α-MEM without FCS and cells were cultured for 2 more days. Right panels bar plots show the densitometric data analysis of the results shown in the left panels A, B, and C. The *Ctpsk*/*Actb* ratio was calculated and plotted against the values obtained with the control, non-stimulated cells (bar 1), which were assigned a value of 1. The values plotted are the means ± SD of the fold induction values obtained from three independent experiments. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ (ANOVA).

Nevertheless, the inhibition exerted by PE 7 was not affected. On the other hand, when TGF-β was neutralized in PEs, the inhibitory effect of PE7 and PE18 was not further observed. Blocking TGF-β also diminished the inhibition exerted by both, PE7 and PE18, of the RL + M-induced metalloprotease activity (Fig. 6B). In spite TGF-β was faintly visualized in PE7 by western blot, it was reported that TGF-β is expressed early at the fetal–maternal interface [28,32]. Accordingly, minor concentrations of TGF-β in PE7 could be responsible of the inhibitory effect observed on both TRAP and MMP expression by macrophages. Although blocking IL-10R was not sufficient to inhibit the effect of PE7 on TPMC induced by RL + M, anti-IL-10R inhibited the effect of both PE7 and PE18, on MMP activity by the cells (data not shown).

Therefore both, IL-10 and TGF-β appear to be cooperatively necessary to sustain the inhibition of osteoclast differentiation by PE18. This is not unexpectedly. Interleukin (IL)-10 producing- and TGF-β producing- regulatory T cells were found to be interdependent for protection against collagen-induced arthritis (CIA), although each subset of regulatory cells could protect against CIA separately [21].

However, PE7 inhibition of TPMC occurred even neutralizing IL-10R. Two scenarios could be drawn to explain this finding. First, no other cytokine but TGF-β mediated the inhibition exerted by PE7. Second, another cytokine in PE7 could be collaborating with TGF-β in the inhibition of TPMC, perhaps through same signaling pathways than IL-10, such as activation of STAT [37]. It was found that RANKL-induced activation of NF-kB, JNK and osteoclastogenesis, was markedly inhibited in IFN-γ-treated bone marrow-derived monocytes through activation of STAT-1 [38]. We found higher concentration of IFN-γ in PE7 compared to PE18, consistent with the reported role of IFN-γ in early pregnancy [39]. Despite this, neutralization of this cytokine should be made in order to confirm whether inhibition of osteoclast differentiation by PE7 also involves IFN-γ.

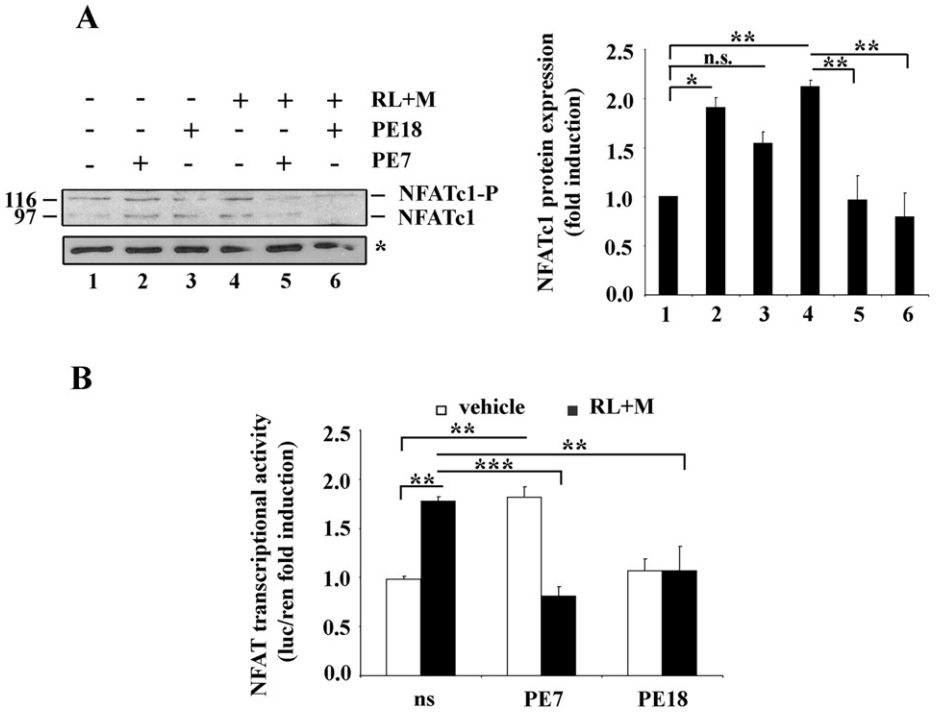

Fig. 4. PE7 and PE18 inhibited the MCSF/RANKL-induced protein expression of NFATc1 and NFAT-dependent transcription. (A) Immunoblot of whole extracts of RAW cells showing endogenous expression of phosphorylated (NFATc1-P) and dephosphorylated (NFATc1) protein. Cells were pretreated for 1 h with vehicle (lanes 1, 4), 50 μg/ml PE 7 (lanes 2, 7), 50 μg/ml PE 18 (lanes 3, 6), and then exposed for 96 h to 100 ng/mL RANKL plus 20 ng/ml M-CSF (RL + M, lanes 4–6), or were left unstimulated (lanes 1–3). Molecular weights (kDa) are indicated to the left of blot. (*) indicates unspecific staining shown as loading control. Shown is one representative experiment of a minimum of three performed. Bar plot in the right panel shows densitometric analysis of the results shown in the blot. Data are plotted as fold induction respect to the control (bar 1). The values plotted are the means ± SD of the fold induction values obtained from three independent experiments. (B) Inhibition of NFAT-mediated transcription. RAW cells were transiently transfected with an NFAT/AP1 luciferase reporter gene. Cells were co-transfected with the *Renilla* plasmid to normalize for transfection efficiency. Twenty four hours later, cells pretreated for 1 h with 50 μg/ml PE7 (*closed gray bars*), 50 μg/ml PE18 (*closed black bars*), or vehicle (*open bars*), followed by exposure for 24 h to 100 ng/ml RANKL plus 20 ng/ml M-CSF (RL + M), or left non stimulated (−). Transcriptional activity is expressed as fold induction respect to control, non stimulated cells. Values are the means ± S.D. of triplicate luciferase determinations for each condition in a representative experiment of a minimum of three performed. n.s. = not significant; $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ (ANOVA).

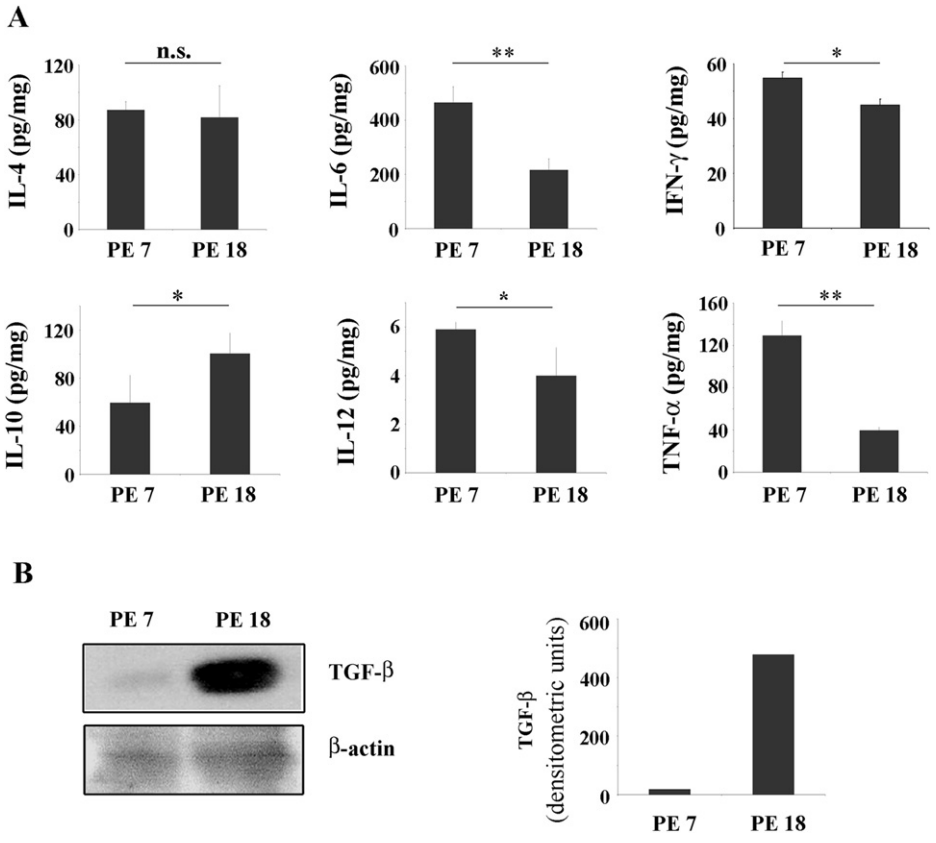

Fig. 5. Quantification of cytokines in PE7 and PE18. (A) Concentration (pg/mg) of IL-4, IL-6, IL-10, IL-12 and TNF-α assessed in PE7 and PE18 by ELISA. Values plotted are the means ± S.D of duplicate determinations from five experiments. n.s = not significant; $^{**}p < 0.01$; $^{*}p < 0.05$ (Untailed *t* test). (B) Immunoblots of PE7 and PE18 showing endogenous protein expression of TGF-β and β-actin as a loading control. TGF-β/β-actin ratios were calculated and showed in the bar plot on the right. Values plotted are from one representative determination of three performed.

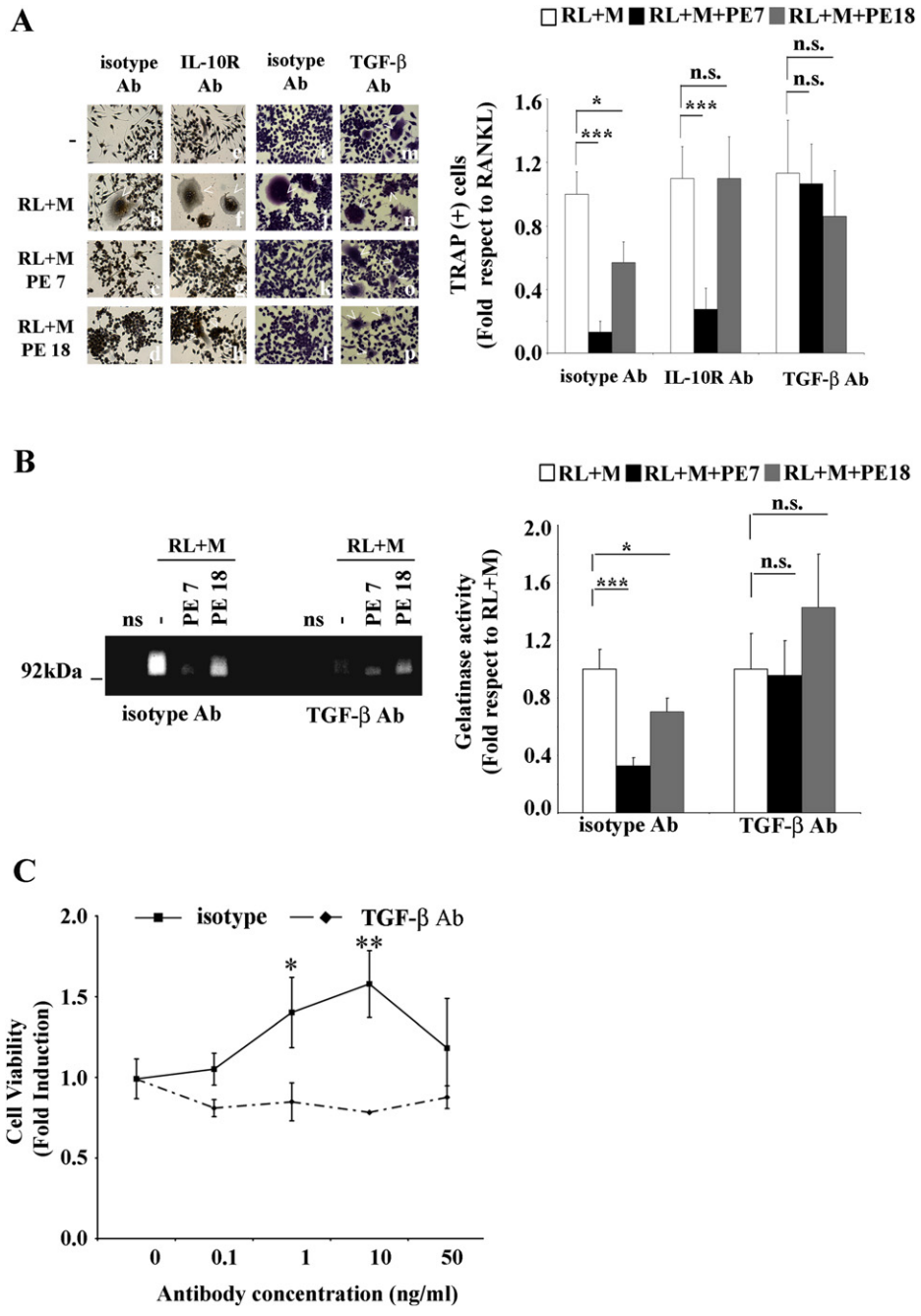

Fig. 6. Effect of neutralizing TGF-β or IL-10R on the PE-mediated inhibition of RANKL-induced- TRAP + cells and MMP activity. (A) RAW 264.7 cells stained for TRAP expression. In rows 1 and 2: RAW cells were incubated 30 min with neutralizing anti-IL-10R antibody (e–h) or the isotype control antibody (a–d) and then treated with 50 μg/ml of either PE7 (c, g) or PE18 (d, h), and 100 ng/mL RANKL plus 20 ng/ml M-CSF (RL + M, b, c, d, f, g, h) during 5 days, or left non treated (a, e). Medium was replaced on the third day and cells were cultured for 2 more days. In rows 3 and 4: neutralizing anti-TGF-β antibody (m–p), or the isotype control antibody (i–l) were incubated 30 min with 50 μg/ml PE 7 (k, l), PE18 (o, p), or with medium alone (i, m), and then added to cultures of RAW 264.7 cells, which were then incubated (j, k, l, n, o, p) or not (i, m) with 100 ng/ml RANKL plus 20 ng/ml M-CSF (RL + M), during 5 days. Medium was replaced on the third day and cells were cultured for 2 more days. Arrowheads indicate TRAP + cells. Shown is a representative experiment which was repeated three times. Magnification 100×. The number of multinucleated cells positive for TRAP in 10 microscopic fields at 100× magnification was determined and plotted as fold induction relative to isotype antibody-treated, RANKL- stimulated cells. (B) Metalloprotease activity assessed by gelatin zymography in RAW cell culture supernatants. The band with the electrophoretic mobility corresponding to MMP9 (92 kDa) is indicated. Neutralizing anti-TGF-β antibody (lanes 5–8), or the isotype control antibody (1–4) were incubated 30 min with 50 μg/ml PE 7 (3, 7), PE18 (4, 8), or medium alone (2, 6), and then added to cultures of RAW 264.7 cells, which were then incubated (2, 3, 4, 6, 7, 8) or not (1, 5) with 100 ng/ml RANKL plus 20 ng/ml M-CSF (RL + M) during 4 days. Medium was replaced on the second day for α-MEM without FCS and cells were cultured for 2 more days. Right panel bar plot shows the densitometric data analysis of the results shown in the left panel. The values plotted are the fold induction values respect to RL + M stimulated cells. (C) Viability of RAW cells assessed by WST-1 tetrazolium salt oxidation. Cells were cultured during 48 h with 0–50 ng/ml of neutralizing anti-TGF antibody (closed line) or with the isotype control antibody (doted line). WST-1 tetrazolium salt reagent was added to cultures during the last 2 h. Optical density was measured at 450 nm (A, B, and C) Data are expressed as mean ± SD from triplicate cultures. n.s: not significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (ANOVA).

Other factors could be involved instead. Also through STAT-1 activation, IL-27 abrogates RANKL mediated- c-Fos expression and osteoclastogenesis [24]. This cytokine was reported in trophoblasts [40]. It was reported that TGF-β-transduced mesenchymal stem cells inhibited osteoclastogenesis through induction of osteoprotegerin (OPG) expression [41]. OPG binds to RANKL, blocking RANK-RANKL signaling pathway. It is expressed in several tissues other than bone, including placenta [42].

In addition to cytokines, it was reported recently that estradiol plus relaxin diminished RANKL/OPG ratio in vivo, in a rat adjuvant-induced arthritis model of RA, and in vitro on osteoblasts cells, suggesting that it may contribute to the antiarthritic effects of estrogens and relaxin on bone during pregnancy [43].

In conclusion, we described that extracts of murine placenta obtained at E7.5 and E17.5 diminished multinucleated TRAP positive cells, *Cathepsin K* mRNA expression and metalloprotease activity in M-CSF plus RANKL stimulated RAW 267.4 macrophages, thereby inhibiting osteoclast differentiation of these cells. The effect of PEs could be explained by the inhibition of the RANKL-induced NFATc1 expression observed in PE treated cells. We demonstrated that TGF-β and IL-10 play a role in the inhibition of osteoclast differentiation exerted by PE18. Nevertheless another cytokine or growth factor could be taking part, together with TGF-β, in the inhibitory effect of PE7. All together these results provide new insights into the mechanisms by which regulatory proteins of placental origin improve symptoms in osteoarthritic related-diseases.

Contributions

Conceived and designed the experiments: A. Canellada, T. Gentile. Performed the experiments: A. Canellada, A. Custidiano, F. Abraham, E. Rey. Analyzed the data: A. Canellada, E. Rey, T. Gentile. Contributed reagents/materials/analysis tools: A. Canellada, T. Gentile. Wrote the paper: A. Canellada.

Role of the funding source

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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