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Experimental *in vitro* infection of rat osteoblasts with measles virus stimulates osteogenic differentiation

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ABSTRACT

In this work we characterized the infection of a primary culture of rat osteoblastic lineage cells (OBCs) with measles virus (MeV) and the effect of infection on cell differentiation and maturation. Infection of OBCs with MeV led to high titers of infectivity released early after infection. Also, analysis of mRNAs corresponding to osteogenic differentiation markers like alkaline phosphatase (ALP), bone sialo-protein (BSP) and bone morphogenetic proteins (BMPs) 1-4-5-7 in OBCs revealed higher values (2–75-fold of increment) for infected cells in comparison with uninfected controls. Differentiation of OBCs in osteogenic medium prior to infection influenced the level of stimulation induced by MeV. Furthermore, treatment of OBCs with Ly294002, a PI3K/AKT inhibitor, increased viral titers, whereas treatment with 10 μM or 100 μM ATPγS diminished MeV multiplication. In addition, increments of osteogenic differentiation markers induced by MeV infection were not modified either by treatment with Ly294002 or ATPγS. These data provide the first evidence demonstrating that MeV can infect osteoblasts *in vitro* leading to osteoblastic differentiation, a key feature in bone pathogenic processes like otosclerosis.

1. Introduction

Skeletal system provides a supportive framework for the body of vertebrates. It also functions as a reservoir of minerals, mainly calcium and phosphorus, which are distributed to the rest of the organism through the blood. Homeostasis of the skeletal system involves constant remodeling of bones. Balance between bone resorption mediated by osteoclasts and bone deposition mediated by osteoblasts underlies the process of remodeling. On this line, maturation and activity of mature osteoblasts control normal skeletal homeostasis. Maturation of osteoblasts involves the process of differentiation from mesenchymal cells of bone marrow and has been associated, at least in part, with an increment in the expression of bone morphogenetic proteins (BMPs) and alkaline phosphatase (ALP) activity [1–7]. BMPs function as growth factors belonging to the transforming growth factor β (TGF-β) superfamily, and exert a pivotal regulatory role in bone formation [8].

We have previously reported that ALP activity and ALP and BMPs gene expression are upregulated by treatment of OBCs with ATP and UTP (purinergic stimulus) via PI3K/AKT signaling pathway [9]. Furthermore, BMPs have been recently implicated in the pathogenesis of otosclerosis [10,11]. Otosclerosis is a primary disorder of bone remodeling unique to the human otic capsule and is characterized by disturbed equilibrium of bone resorption and new bone formation [12,13]. During the last 20 years, various hypothesis have been put forward to explain the etiology of otosclerosis: genetic, autoimmunity and inflammatory mechanisms, hormonal and metabolic factors as well as viral infection may contribute to the pathogenesis of the disease [12,14,15]. In particular, measles virus (MeV) has been associated to the disease [16,17].

In this study, we present evidence which demonstrates for the first time the capacity of MeV to infect osteoblasts *in vitro* and to increase the expression levels of osteogenic differentiation markers like ALP and BSP and BMPs. We also investigated the effect of MeV infection in OBCs stimulated with ATPγS and the role of the PI3K/AKT pathway in this process.

2. Materials and methods

2.1. Drugs

ATPγS and α-MEM were from Sigma Chemical Co. (St. Louis, MO, USA). Ly294002 was from Calbiochem (San Diego, CA, USA). Fetal bovine serum (FBS) was from Natocor (Córdoba, Argentina). Quick-RNA™ Miniprep for RNA isolation and the mix KAPPA SYBR®

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FAST qPCR Kit, for *Real Time Quantitative-PCR* (RQ-PCR) were from Biosystems S.A (Buenos Aires, Argentina). High-Capacity cDNA Reverse Transcription Kit and primers for RQ-PCR were from Invitrogen S.A (Buenos Aires, Argentina). All other reagents used were of analytical grade.

2.2. Virus and infections

MeV, strain Brazil/001/91, was kindly provided by Dr. Monica Wachsman (FCEyN, Universidad de Buenos Aires). Stock of MeV was prepared by infecting Vero cells and harvesting supernatants at two days p.i. Supernatants were frozen and subjected to three freeze–thaw cycles and sonicated. MeV was prepared by infecting Vero cells. At two days p.i., the supernatant was collected and frozen followed by three freeze–thaw cycles and sonicated. Stock was fractionated and stored at −80 °C. Virus titrations were performed by a plaque forming unit (PFU) assay on Vero cells as previously described [18].

Infection of OBCs was performed by inoculation with MeV at an input multiplicity (MOI) of 0.5–0.005 PFU/cell. After one hour of adsorption at 37 °C, inocula were removed and the treatment medium was either added or refurbished. At different times post-infection (p.i.) supernatants were collected and infectivity was assayed by the PFU technique. Cultures were observed by light microscopy and the cytopathic effect (CPE) was scored based on an arbitrary scale (1: several rounded cells in the monolayer, 2: more than 50% of cells rounded, 3: more than 50% of cells rounded and cells detached from substrate, 4: almost all cells rounded, 5: all cells detached).

2.3. Osteoblasts isolation, culture and treatment

Primary cultures of rat calvarial osteoblasts (OBCs) were obtained as previously described [9]. Cells were cultured at 37 °C in α-MEM supplemented with 15% FBS, 1% penicillin and streptomycin under humidified air (5.5% CO_2). After 24 h, the medium was replaced by α-MEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin and the cells further cultured until ~80% of confluence (2–3 days). Then the cells were trypsinized and frozen in liquid nitrogen until their use. For each experiment, cells were thawed and cultured at a density of $5{\cdot}10^3$ cells/cm^2 for 2 days in α-MEM supplemented with 10% FBS, in a humidified atmosphere (5.5% CO_2) at 37 °C. Before the onset of treatments cells were starved in 1% FBS medium for 18–21 h and then cultured in treatment medium for different times as indicated in figures. OBCs were infected with MeV at different times during the treatments.

All treatments were performed in osteogenic medium (α-MEM supplemented with 1% FBS, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid) containing vehicle or the indicated agonist amounts (ATPγS in the presence or absence of 10 μM Ly294002). If necessary, osteogenic medium with or without agonists and/or Ly294002 was renewed every 2–3 days.

2.4. Expression of osteogenic differentiation markers

The level of expression of osteogenic differentiation markers in OBCs subjected to infection or a specific treatment was monitored through the quantification of the corresponding mRNA. Total RNA was isolated using Quick-RNA™ Miniprep. The amount of RNA was measured by spectrophotometry. Reverse transcription of total RNA was performed using the High-Capacity cDNA Reverse Transcription Kit. Quantitative PCR was done by SYBR Green real-time PCR methods. PCR was performed according to manufacturer's instructions, with denaturation cycles at 95 °C for 3 s, and annealing/extend cycles at 60 °C for 30 s. The relative mRNA expression was calculated using the comparative threshold method (Ct-method) with GAPDH for normalization. The average value of expression level of different genes in control cells was defined as 1. All experimental conditions were processed in triplicate.

RQ-PCR primers were specifically designed to amplify the following cDNAs (Table 1).

2.5. Statistical analysis

Statistical significance of data was evaluated using Student's *t*-test and probability values below 0.05 ($p < 0.05$) were considered significantly different. Quantitative data are expressed as mean ± standard deviation (SD) from the indicated set of experiments.

3. Results

3.1. Infection of rat osteoblasts with measles virus

In an initial series of experiments, we investigated the capacity of measles virus (MeV) to infect osteoblasts. A primary culture of rat calvarial cells was used as a source of osteoblastic lineage cells (OBCs). OBCs were infected with ten-fold dilutions of the virus that covered multiplicities of infection ranging 0.5–0.005 PFU/cell. At different times post-infection (p.i.) supernatants were collected in order to determine the level of infectivity produced by the infected culture. At the same time, the cytopathic effect (CPE) that appeared in the culture as a consequence of infection was

Table 1
Primers for RT-PCR.

Target*	Forward	Reverse
ALP	5′ AGAACTACATCCCCCACG 3′	5′ CAGGCACAGTGGTCAAGGT 3′
BSP	5′ TCCCCACCAGCCAGAAAG 3′	5′ TCGTTGCCTATTTGTTCGTATTCTT 3′
Col 1α1	5′ CCCTACCCAGCACCTTCAAA 3′	5′ ATGGAGATGCCAGATGGT 3′
Col 1α2	5′ ACCCAGAGTGGAAGAGCGATTA 3′	5′ GCATCCATAGTGCATCCT 3′
GAPDH	5′ GGCAAGTTCAACGGCACAGT 3′	5′ TGGTGAAGACGCCAGTAGACTC 3′
OPG	5′ AGCTGGCACACGAGTGATGAA 3′	5′ CACATTCGCACACTCGGTTGT 3′
rBMP-1	5′ CCCCCACCCAGTACCGTATC 3′	5′ ATCATTGCCCTCAGTCTCGAA 3′
rBMP-2	5′ GGCCACGACGGTAAAGGA 3′	5′ GCTTCCGCTGTTTGTGTTTG 3′
rBMP-3	5′ GGACCCTCCAATCCAACCA 3′	5′ AGGTTTGGCCGTATCTACTGACA 3′
rBMP-4	5′ CCAAGCGTAGTCCCAAGCAT 3′	5′ CGACGGCAGTTCTTATTCTTCTTC 3′
rBMP-5	5′ TCCCTTTGATGGCGTTGGT 3′	5′ AATCTGCCGGTCAGAAGCAA 3′
rBMP-6	5′ CGCTCCGCTCTTCATGCT 3′	5′ TGACACCCCATCCTCTTCGT 3′
rBMP-7	5′ CCGCTCCAAGACTCCAAAGA 3′	5′ GCTGCTGTTTTCTGCCACACT 3′

* Primers for: ALP (alkaline phosphatase), BSP (bone sialo-protein), Col 1α1 (collagen 1 type α1), Col 1α2 (collagen 1 type α2), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), OPG (osteoprotegerin), rBMP (rat bone morphogenetic protein).

monitored by observing the cells under light microscopy. Results were recorded based on an arbitrary scale that assigns a value from 0 (no CPE) to 5 (maximal severity) for each observation. Fig. 1(A and B) shows that CPE could be readily observed at 24 h p.i. in the case of the cultures infected at an MOI of 0.5 and 0.05 PFU/cell whereas the onset of CPE was delayed by 24 h for the cells infected at an MOI of 0.005 PFU/cell. Taken into account that cultures infected at the highest MOI were destroyed soon afterwards infection, infectivity was determined in samples collected from cultures infected at an MOI of 0.05 UFP/cell. As can be seen in Fig. 1(C), a peak of infectivity, reaching 1.3×10^7 PFU/ml, was detected in the supernatants of MEV-infected cells during the second day of infection. Level of infectivity dropped by the third day p.i. reaching a titer two log units lower than previous day. Undetectable levels of infectivity were obtained by the fifth day p.i. (data not shown). Onset of CPE and production of infectivity demonstrate that MeV was able to productively infect OBCs.

3.2. Measles virus stimulates osteogenic differentiation in osteoblasts

In order to study the effect of MeV infection on the differentiation process of rat OBCs *in vitro*, cells were infected at an MOI of 0.05 PFU/cell and at 12 h p.i. total RNA was extracted from cell lysates and bone morphogenetic proteins (BMPs) mRNA was quantified by RQ-PCR analysis as described in Section 2. As shown in Fig. 2, the levels of BMPs 1, 4, 5, 6 and 7 mRNAs, as well as those corresponding to the differentiation markers: ALP, Col 1α1, BSP and OPG were significantly higher in MeV infected cells in comparison with those of uninfected controls. Highest increments achieved by BMP-4, BMP-6, ALP and BSP mRNAs were 4-, 17-, 1.8- and 75-fold of increment. These proteins were selected to continue with the study.

In order to investigate if MeV effect was dependent on the stage of differentiation of OBCs, cells were infected at different times after contact with osteogenic medium. As can be seen in Fig. 3, MeV infection increased the transcriptional activities of all tested osteogenic markers in a time-dependent manner, showing a peak of osteogenic markers mRNA levels in MeV infected cells that had been cultured in the osteogenic medium from 2 to 4 days prior to infection depending on the analyzed marker. BMP-4 increments due to MeV infection were evident from day 1 onwards and decreased at day 7 of treatment with the osteogenic medium. A similar panorama was observed for BMP-6 with a peak of activation at day 4. BSP stimulation was maximal at day 1 and 2 and decreased onwards. ALP increments occurred in a time dependent manner up to day 3 of treatment and decreased when OBCs were infected after 4 days of culture in osteogenic medium. These results indicate that the degree of differentiation of OBCs at the moment of MeV infection determines the activation profile of the osteogenic markers analyzed.

3.3. Stimulation of osteoblasts by measles virus infection is not dependent on the PI3K/AKT pathway

The phosphatidyl inositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway is involved in cell survival by promoting cell growth and inhibiting apoptosis. We have previously demonstrated that ATP stimulation of $P2Y_2$ receptors activates the PI3K/AKT pathway in rat OBCs [9]. In view of the results obtained when OBCs were infected with MeV, we decided to investigate the effect of ATPγS on virus production and osteogenic markers stimulation in MeV infected OBCs. On that purpose starved OBCs were infected and maintained in osteogenic medium supplemented or not with ATPγS. Results shown in Fig. 4(A) indicate that the ATPγS

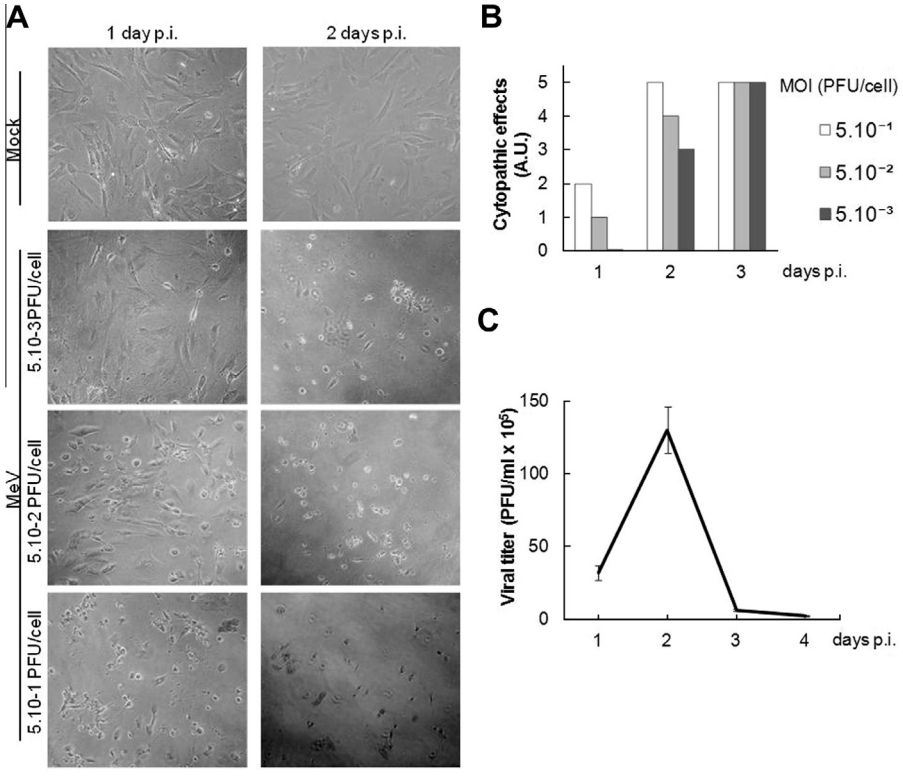

Fig. 1. Multiplication of MeV in OBCs. OBCs were infected (MOI 0.5–0.005 PFU/cell) and CPE was observed by light microscopy (A) and quantified in arbitrary units (A.U.) in accordance to severity (see Section 2) (B). At the same time supernatants were collected and viral infectivity was quantified by a plaque assay. Results shown in panel (C) represent the viral titers obtained from OBCs infected at an MOI of 0.05 PFU/cell and are expressed as the mean ± SD of data from at least three separate experiments, each sample processed in duplicate.

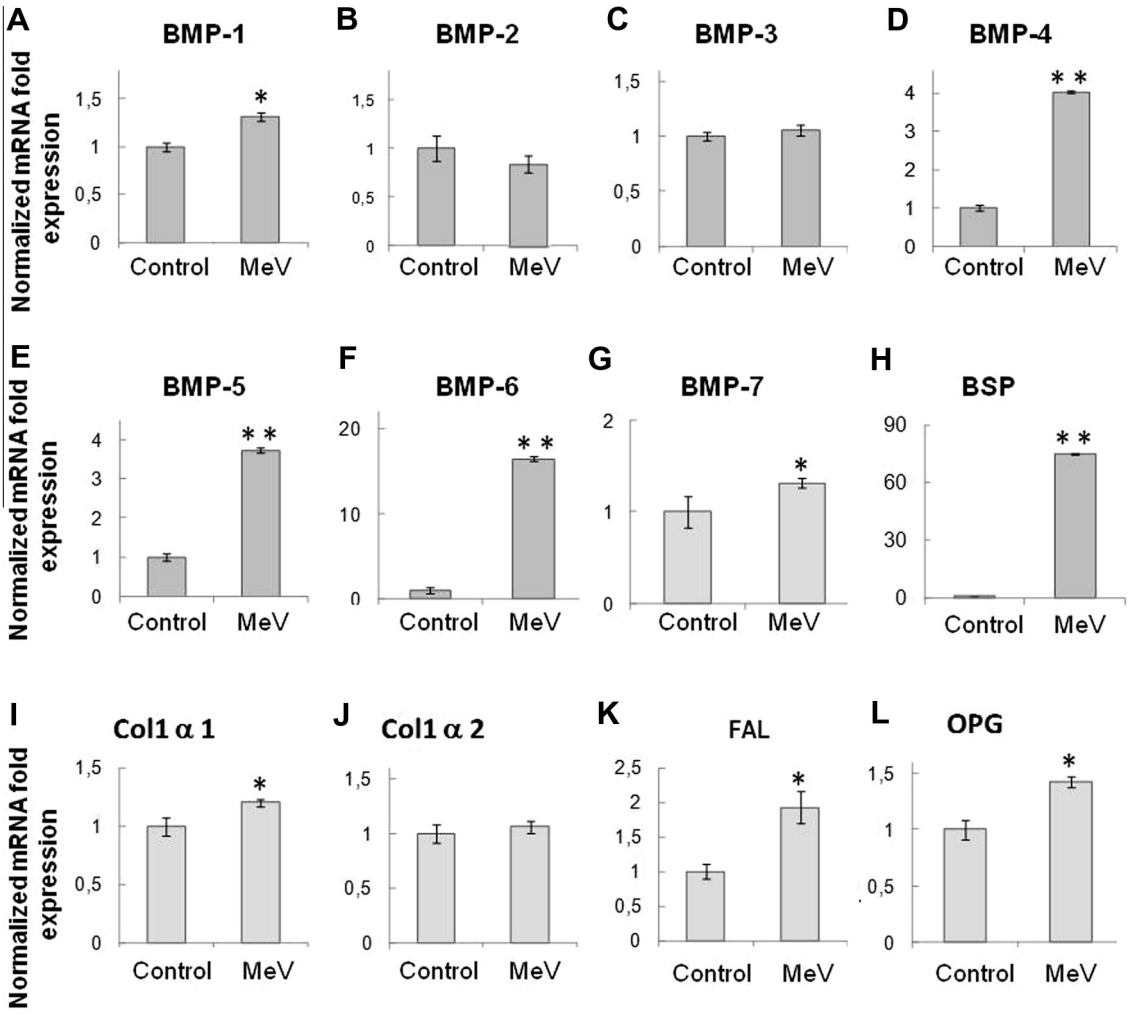

Fig. 2. MeV infection of OBCs induces osteogenic differentiation. OBCs were cultured for 36 h and mock or MeV infected (MOI 0.05 PFU/cell). At 12 h p.i. total RNA was extracted and quantification of mRNA levels of indicated proteins was performed by real-time PCR analysis as described in Section 2. The expression levels of target genes were standardized by GAPDH level in each sample and normalized respect to day 1 of mock infected cells. Results are shown as the mean ± SD of data from at least three separate experiments, each sample processed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.02$ vs mock infected.

treatment was able to diminish viral titer in comparison with untreated infected controls. Moreover, inhibition of PI3K/AKT pathway by treatment of cells with Ly294002 increased viral replication independently if they had been treated with ATPγS or not (Fig. 4B).

To determine whether PI3K/AKT stimulation was able to modify the expression of genes related with OBCs differentiation and function, cells were treated with 100 μM ATPγS in osteogenic medium, and then infected with MeV. At 12 h p.i., BMP-4 and ALP mRNA levels were quantified by RQ-PCR. As can be seen in Fig. 4(C and D) stimulation with 100 μM ATPγS increased BMP-4 and ALP mRNA levels in mock OBCs whereas ATPγS treatment of infected OBCs did not exert an additive effect respect to MeV stimulation. On the other hand, Ly294002 treatment did not affect the BMP-4 and ALP mRNA levels in MeV infected osteoblasts. These results suggest that stimulation of ALP and BMP-4 expression by MeV involves a signaling pathway different from PI3K/AKT.

4. Discussion

The present study demonstrates the capacity of MeV to infect rat OBCs *in vitro* and to induce differentiation of these cells through the augment of BMPs. Previous reports indicate that infection of primary cultures of mouse OBCs with Venezuelan and Eastern Equine Encephalitis viruses (VEEV and EEEV) leads to the production of high levels of infectivity [19] although no investigation was done about the physiology of the infected cell. Infected OBCs were also detected in mice inoculated with EEEV [20]. Recent findings indicate that the arthritogenic alphavirus, Ross River virus, is able to grow in primary cultures of human OBCs and to induce the production of IL-6, an inflammatory cytokine [21]. In our model, infection of OBCs with MeV leads to incremented levels of BMP-1, 4, 5, 6 and 7. A similar panorama was reported during early active stages of otosclerotic stapes footplates [10,11,22]. BMPs belong to TGF-β superfamily of proteins which has been described to exert a dual role in apoptosis [23]. In view that CPE was readily observed in MeV infected OBCs, augmented levels of BMPs would contribute to apoptosis or, at least, would not be enough to prevent apoptotic cellular response to infection. We also demonstrated that MeV infection of OBCs stimulated a variety of osteoblastic differentiation markers (increased BSP, Col 1α1, ALP and OPG gene expression). In agreement with these results, there have been reported mutations in Col 1α1 gene [24] and increments in ALP [25] and OPG expression [13,26], in cases of otosclerosis.

We have previously demonstrated that stimulation of $P2Y_2$ receptors, responsive to ATP, ATPγS and UTP) enhance osteoblastic differentiation via activation of the PI3K/AKT signaling pathway

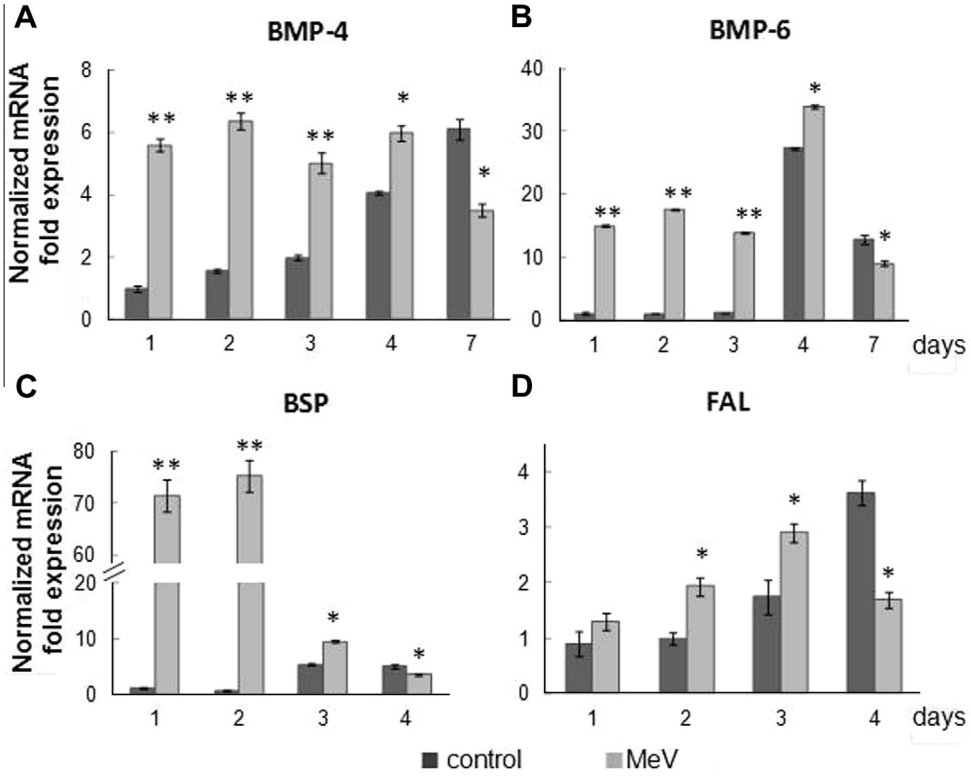

Fig. 3. MeV induction of osteogenic markers depends on the maturation of OBCs. OBCs were cultured for the indicated times and 12 h before the end of treatment were mock (dark gray) or MeV (light gray) infected (MOI 0.05 PFU/cell). At indicated times total RNA was extracted and quantification of mRNA levels of indicated proteins was performed by real-time PCR analysis as described in Section 2. The expression levels of target genes were standardized by GAPDH level in each sample and normalized respect to day 1 of mock infected cells. Results are shown as the mean ± SD of data from at least three separate experiments, each sample processed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.02$ vs mock infected.

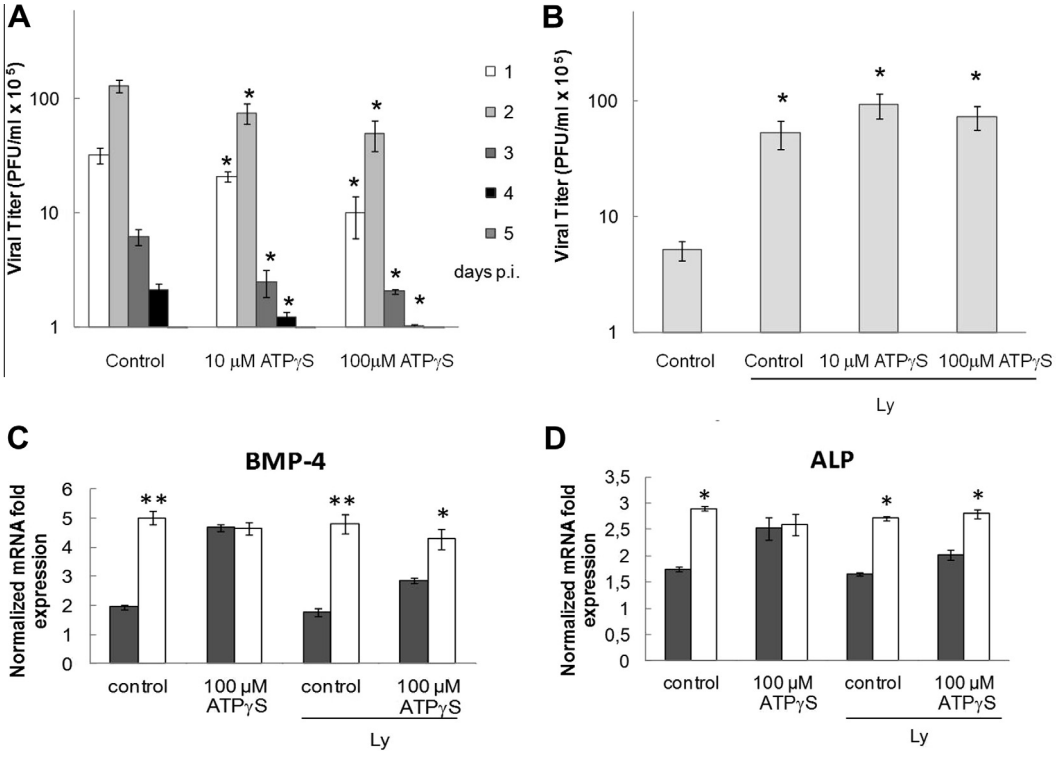

Fig. 4. Effect of purinergic treatment and PI3K/AKT pathway inhibition on the infection of OBCs by MeV. OBCs were infected with MeV (MOI 0.05 PFU/cell) and treated with 10 or 100 μM ATPγS or the vehicle (control). At indicated times p.i., supernatants were collected and viral infectivity was quantified by a plaque assay (A). Alternatively, OBCs were treated with 10 or 100 μM ATPγS or the vehicle (control), in the presence or absence of 10 μM Ly294002 (Ly) during three days and infected with MeV at 12 h before harvesting of supernatants. Viral infectivity in supernatants was quantified by a plaque assay (B). OBCs treated with 100 μM ATPγS were mock (gray bars) or MeV (white bars) infected. Total RNA was extracted and quantification of BMP-4 and ALP mRNA levels was performed by real-time PCR analysis as described in Section 2 (C) and (D). The expression levels of target genes were standardized by GAPDH level in each sample and normalized respect to day 1 of mock infected cells. Results are shown as the mean ± SD of data from at least three separate experiments, each sample processed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.02$ vs control.

and gene expression induction of ALP, and BMP-4 [9]. Here we found that increments of BMP-4 and ALP due to MeV infection are independent of this pathway. Carsillo et al. [27] reported that MeV infection reduces the level of phosphorylated (active) AKT in T cells while Avota et al. [28] showed that activation of AKT kinase was impaired after cell-MeV contact reducing proliferation of T cells after infection with the virus or contact with viral glycoproteins *in vitro* and *in vivo* [29,30]. These findings may explain the fact that the combination of ATPγS treatment together infection did not exert an additive effect on PI3K/AKT pathway in OBCs. If this is the case, blocking of the PI3K/AKT pathway would not be crucial for the stimulation of ALP and BMP-4 in infected OBCs.

Altogether our results point out the system OBCs-MeV as a useful experimental model to elucidate the mechanisms underlying otosclerosis.

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