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Interleukin 17 inhibits myogenic and promotes osteogenic differentiation of C2C12 myoblasts by activating ERK1,2

Jelena Kocić [a,*], Juan F. Santibañez [a], Aleksandra Krstić [a], Slavko Mojsilović [a], Ivana Okić Đorđević [a], Drenka Trivanović [a], Vesna Ilić [b], Diana Bugarski [a]

[a] Laboratory for Experimental Hematology and Stem Cells, Institute for Medical Research, University of Belgrade, Dr Subotića 4, 11129 Belgrade, Serbia
[b] Laboratory for Immunology, Institute for Medical Research, University of Belgrade, Dr Subotića 4, 11129 Belgrade, Serbia

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ABSTRACT

The present study evaluated the role of interleukin (IL) 17 in multilineage commitment of C2C12 myoblastic cells and investigated associated signaling pathways. The results concerning the effects on cell function showed that IL-17 inhibits the migration of C2C12 cells, while not affecting their proliferation. The data regarding the influence on differentiation demonstrated that IL-17 inhibits myogenic differentiation of C2C12 cells by down-regulating the myogenin mRNA level, myosin heavy chain expression and myotube formation, but promotes their osteogenic differentiation by up-regulating the Runt-related transcription factor 2 mRNA level, cyclooxygenase-2 expression and alkaline phosphatase activity. IL-17 exerted these effects by activating ERK1,2 mitogen activated protein kinase signaling pathway, which in turn regulated the expression of relevant genes and proteins to inhibit myogenic differentiation and induce osteogenic differentiation. Additional analysis showed that the induction of osteogenic differentiation by IL-17 is independent of BMP signaling. The results obtained demonstrate the potential of IL-17 not only to inhibit the myogenic differentiation of C2C12 myoblasts but also to convert their differentiation pathway into that of osteoblast lineage providing new insight into the capacities of IL-17 to modulate the differentiation commitment.

1. Introduction

Interleukin (IL) 17A is the prototype member of a newly defined and intensively investigated family of proinflammatory cytokines mainly produced by Th17 cells. Signature roles of IL-17 in inflammation and immune response are mediated by the release of CXC chemokines, proinflammatory and hematopoietic cytokines and antimicrobial peptides [1–3]. IL-17 induces neutrophil and granulocyte expansion, as well as neutrophil recruitment, leading to tissue inflammation [4–7]. Moreover, increasing evidence indicates that IL-17, within a complex network of interactive cytokines, contributes to the pathogenesis of various autoimmune and inflammatory diseases [1–3].

IL-17 family members signalize through a family of unique cognate receptors, which belong to type I transmembrane proteins [8]. The signaling downstream of IL-17 receptor (IL-17R) implicates activation of protein kinase A (PKA), mitogen-activated protein kinase (MAPK), janus kinase/signal transducer and activator of transcription (JAK/STAT) and nuclear factor-kappaB (NF-kB) cascades [8]. Even though IL-17 is considered to be secreted exclusively by Th17 cells, many cell types in the body express IL-17R, thus becoming potential targets of IL-17.

Previously, our group postulated IL-17 as an important regulator of hematopoietic progenitor cell activity which affects granulopoiesis and erythropoiesis, suggesting its role in cell proliferation and differentiation [5,6,9,10]. As different cell types appear to respond differently to IL-17, novel functions concerning cell differentiation have been recently discovered for this cytokine. IL-17 induces osteogenic differentiation of human mesenchymal stem cells (MSCs) [11], implying its involvement in bone turnover; while, it inhibits adipogenic differentiation of human bone marrow MSCs and enhances lipolysis in differentiated adipocytes [12]. Also, some reports implicate IL-17 in cell proliferation, as a growth factor for both human and mouse MSCs [11,13]. IL-17's involvement in cell differentiation is an issue that has just started to be explored, with diverse implications. In respect to this, some pathological states, such as inflammatory muscle and bone diseases, involve infiltration of Th17 cells and elevated levels of IL-17 in the tissue [2,14,15]. Although higher expression of IL-17 has been observed in muscle samples in inflammatory myopathies [14], the effect of IL-17 on myogenic differentiation is yet unknown.

This study was undertaken to investigate the potential of IL-17 to modulate the differentiation of myoblastic cell line C2C12 and to elucidate the signaling pathways involved. Namely, C2C12 myoblasts under myogenic culture conditions differentiate into myotubes rapidly, producing characteristic muscle proteins, but are also capable to shift differentiation pathway from myoblastic to osteoblastic within the appropriate osteogenic culture conditions [16–19]. Moreover, C2C12

* Corresponding author at: Institute for Medical Research, University of Belgrade, Dr Subotića 4, 11129 Belgrade, Serbia. Tel.: +381 11 2685 788; fax: +381 11 2643 691.
E-mail address: jelena.kocic@imi.bg.ac.rs (J. Kocić).

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cells can be differentiated into adipocytes, and have therefore been proposed as 'multiblasts' (multiple tissue blasts) by Wada et al. [18]. Hence, this cell line is widely used for studying mechanisms of specific lineage differentiation and commitment [18,20]. In this work we examined the ability of IL-17 to modulate the expression of specific genes and proteins in C2C12 cells related to myogenic and osteogenic differentiation as well as whether IL-17 affects the cell proliferation and migration. To explore the signaling pathways underlying IL-17-induced events, we analyzed the involvement of MAPKs, since numerous reports indicate that these signaling molecules, specifically ERK1,2 and p38, are implicated in the signaling elicited by IL-17 in various cell types [1,3,19,21]. Given that bone morphogenetic proteins (BMPs) are strong osteogenic inductors in C2C12 cells [16,20], we further aimed to elucidate whether IL-17 may interact with BMP-Smad signaling during osteogenic differentiation.

Results obtained demonstrate that IL-17 is capable of modulating the multilineage commitment of C2C12 cells by inhibiting myogenic and promoting, in a BMP-independent fashion, their osteogenic differentiation through the activation of ERK1,2 MAPK.

2. Materials and methods

2.1. Cell culture and differentiation

C2C12 cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in growth medium (GM) consisting of DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories GmbH, Pasching, Austria) in humidified environment at 37 °C and 5% CO_2 in air. Myogenic differentiation was induced with myogenic differentiation medium (MDM) consisting of DMEM with 2% HS (PAA), while osteogenic differentiation was induced with osteogenic differentiation medium (ODM) containing 50 μM ascorbic acid (Sigma-Aldrich), 10 nM dexamethasone, 10 mM beta-glycerophosphate, both from Applichem (Darmstadt, Germany), and 10% FBS in DMEM. Both myogenic and osteogenic differentiation of cells was induced during a period of six days. Selective signal transduction inhibitors of MEK1,2-ERK1,2 (PD98059) and p38 (SB203580), obtained from Calbiochem (Darmstadt, Germany), were used at 25 μM and 10 μM, respectively.

2.2. Proliferation assay

Proliferation rate of C2C12 cells was analyzed by MTT test. 5×10^3 cells/well were seeded in 96-well plates in GM. The following day GM was replaced with fresh GM, MDM or ODM and the cultivation was continued for 48 h in the presence of 0, 25, 50 and 100 ng/ml recombinant mouse (rm) IL-17 (R&D Systems, Minneapolis, MN, USA). After this period, MTT (Sigma-Aldrich) was added to cells at 0.5 mg/ml, and after 2 h the formazan crystals were dissolved in 0.1 N HCl in isopropanol. The absorbance was read at 530 nm.

2.3. Scratch assay

The motility of C2C12 cells was analyzed by *in vitro* scratch assay. Cells were seeded in a 24-well plate in GM. When cells reached confluence, a scratch in the monolayer over the total diameter of each well was made using a sterile pipette tip, and cell cultures were allowed to grow for 24 h in GM with 0, 25, 50 and 100 ng/ml rmIL-17. The cells were then fixed with ice-cold methanol and stained with 0.1% crystal violet. Migration of the cells into the scratch area was documented by light microscopy and quantified by TScratch software (Computational Science and Engineering Laboratory, Swiss Federal Institute of Technology, ETH Zurich, Zurich, Switzerland).

2.4. Immunofluorescence assay

Immunofluorescent labeling was used to detect IL-17R in C2C12 cells. Cells were seeded over rounded cover slips in GM and grown over night. After being fixed with 4% formaldehyde in PBS, cell monolayers were incubated with anti-IL-17R antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with anti-rabbit-FITC secondary antibody and 1 μg/ml DAPI (both from Sigma-Aldrich). The samples were examined and photographed using an epi-fluorescence microscope.

2.5. Myosin heavy chain (MyHC) detection

To confirm myogenic differentiation of the cells, we examined the presence of MyHC by immunocytochemistry. Following myogenic differentiation cells were fixed with ice-cold methanol and blocked with 4% bovine serum albumin (BSA) (Sigma-Aldrich) in PBS for 30 min at 37 °C. The cells were then incubated for 1 h at room temperature with primary antibody against MyHC, α-MF-20, from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA, USA), followed by incubation for 1 h at room temperature with secondary, HRP-conjugated anti-mouse antibody (Sigma-Aldrich). Finally, cells were incubated with a developer (0.6 mg/ml DAB from Sigma-Aldrich and 0.1% H_2O_2 in PBS) and examined under a light microscope to detect bound HRP.

2.6. Alkaline phosphatase (ALP) activity

ALP activity was used to demonstrate the level of osteogenic differentiation of C2C12 cells. Following differentiation the cells were fixed with formalin/ethanol (1:9) for 30 s at room temperature, and stained for ALP activity with alkaline phosphatase chromogen BICP/NBT (5-bromo-4-chloro-3-indolyl phosphate/p-nitroblue tetrazolium chloride) from Sigma-Aldrich. The reaction was stopped with 10 mM NaF in PBS. The cells were then examined using a light microscope.

2.7. Western blot assay

Antibodies against pERK1,2, ERK1,2, pp38, p38, pJNK and JNK were purchased from R&D Systems and antibodies against IL-17R, cyclooxygenase-2 (COX-2), phospho-Smad1 and Smad1 were obtained from Santa Cruz Biotechnology. Mouse monoclonal anti-HA antibody was kindly provided by Dr Carmelo Bernabeu (CIB, Spain). Cells were collected after cultivation and adequate treatment, as indicated in Results, and total protein extracts were isolated using lysis buffer. Protein samples from cell lysates were separated by SDS-PAGE and electrotransferred onto a nitrocellulose membrane. Membranes were blocked with 4% BSA in 0.5% Tween-20 in TBS and then incubated with primary antibodies against IL-17R, ERK1,2, p38, JNK, MyHC or COX-2, as specified in Results. Secondary antibodies conjugated with HRP (Sigma-Aldrich) were used to detect the immune complexes by enhanced chemiluminescence. Labeled proteins were visualized with enhanced chemiluminescence reagent system from Applichem (Darmstadt, Germany). Protein bands were quantified by densitometric scanning, using ImageMaster TotalLab Version 1.11 software (Amersham Biotech).

2.8. RT-PCR

After corresponding cell treatments, total RNA was obtained using TRIzol (Applichem) and complementary DNA was generated by the SuperScript First-Strand Synthesis System for RT-PCR from Invitrogen (Carlsbad, CA, USA), using oligo (dT) as a primer. PCR products were obtained after 30 and 35 cycles of amplification with an annealing temperature of 52 °C. Beta-actin primer was from R&D Systems and GAPDH, myogenin and IL-17R primers were from Invitrogen. Beta-actin or GAPDH was amplified as a control for the amount of cDNA

present in each sample. The primer sets for mouse IL-17R and myogenin were: forward 5′-GGTGGAGAGCAACTCCAAAA-3′, reverse 5′-AAACAACGTAGGTGCCGAAG-3′ and forward 5′-TTTCTACCAG-GAGCCCCACTT-3′, reverse 5′-TGATGGCTTTTGACACCAAC-3′ [19], respectively. The intensity of the bands was quantified using NIH-Image J software.

2.9. Transient transfections and reporter gene measurements

The MAPK/ERK reporter (SRE-luc) was provided by Dr. Angel Corbí (CIB-CSIC, Spain); pSRE-luc contains two copies of the c-fos SRE (nucleotides −357 to −275, containing both an SRF binding site and an adjacent Ets motif) upstream of a minimal Tk promoter and the luciferase gene. Myogenin promoter (G133-luc) was kindly provided by Dr. Zhenguo Wu (Hong Kong University of Science & Technology, Hong Kong, China); Runt-related transcription factor 2 (Runx2/Cbfa1) reporter (p6OSE2-luc, containing 6 response elements followed by the luciferase gene) [22] was kindly provided by Dr. Gerard Karsenty (Columbia University Medical Center, NY, USA). Constitutive active MEK1-HA tagged construct (pECE/HA MAPKK S218D/S222D) was kindly provided by Dr. Jacques Pouyssegur (University of Nice-Sophia Antipolis, France). pBRE-luc construct was kindly provided by Dr. P. ten Dijke (Leiden University Medical Center, RC Leiden, The Netherlands). The construct contains regions of the mouse Id1 promoter, important for the induction of Id1 transcription factor by BMPs, fused to a luciferase reporter gene [23]. Constitutively active (ca, Q207D) and kinase-dead (kd, K233R) ALK2 cloned into pCMV5 were kindly provided by Dr. C. Vary (Northwestern University, Chicago, USA) and described by Romero et al. [24]. For Smad1 transactivation assay, plasmids encoding Gal4-Smad1, kindly provided by Dr. K. Nakayama (University of Tokyo School of Medicine, Tokyo, Japan), were co-transfected with the Gal4-luciferase reporter pFr5-Luc (Stratagene, La Jolla, CA).

C2C12 cells were grown in a 24-well plate (~2×10^5 cells/well) in DMEM supplemented with 10% FBS. After reaching approximately 70% confluence, cells were transfected using Superfect transfection reagent (Qiagen, Hilden, Germany) with 500 ng/well of each specific luciferase construction and 25 ng/well of SV40-β-Gal as an internal control for transfection efficiency. Six hours after transfection, the cells were fed with fresh medium (DMEM with 10% FBS) and incubated overnight. Transfected cells were then treated for 48 h, as indicated in specific experiments. Cell extracts were then prepared and luciferase assays were done using the Luciferase Assay System (Promega, Madison, WI, USA). Luciferase activities were normalized with respect to parallel beta-galactosidase activities, to correct for differences in transfection efficiency. Beta-galactosidase assays were performed using the Galacto-Light Plus System from Tropix (Bedford, MA, USA).

2.10. Statistical analysis

The results shown are representative of at least three independent experiments. Data are given as means ± SEM. Statistical significance was evaluated using the Student's t-test. Differences were considered to be significant at a value of $p<0.05$.

3. Results

3.1. IL-17 inhibits migration without affecting the proliferation of C2C12 cells

Initially we elucidated whether C2C12 cells express IL-17 receptor. When labeled with anti-IL-17R antibody, a discrete positive punctuated pattern, typical for membrane cell receptors, was noticed at the surface of C2C12 cells (Fig. 1Aa), in comparison to the cells labeled with the control antibody (Fig. 1Ab). The expression of IL-17R was further confirmed by Western blot assay (Fig. 1B), as well as by RT-PCR (Fig. 1C).

Next, we determined the effect of IL-17 on the proliferation and migration of C2C12 cells. As determined by MTT test, IL-17 treatment did not alter the proliferation of C2C12 cells, neither when the cells were cultured in plain growth medium, nor in myogenic or osteogenic differentiation medium (Fig. 2A). As expected, due to the induction of differentiation, the change from GM to differentiation media itself produced a reduction in C2C12 cell proliferation, with stronger reduction noticed in cells grown in MDM.

The motility of C2C12 cells was also tested, using the *in vitro* scratch assay. Cells grown in GM displayed high migration capacity, since after a period of 24 h of incubation they almost completely colonized the scratch. During the same period of time, treatment with IL-17 inhibited C2C12 cell migration in a dose dependent manner (Fig. 2B).

3.2. IL-17 inhibits myogenic differentiation of C2C12 cells

To analyze whether IL-17 affects C2C12 myogenic differentiation, cells were induced to differentiate by cultivation in MDM for six days, after which the formation of myotubes immunostained for MyHC, a marker protein involved in terminal myogenic differentiation [17–19], was evaluated. As shown in Fig. 3A, the number of MyHC positive myotubes after IL-17 treatment was significantly lower, indicating that myogenic differentiation was inhibited by IL-17 in a dose-dependent manner. This result was further supported by Western blot analysis for MyHC, as in IL-17 treated cells grown in MDM, the expression of MyHC was reduced and its level was comparable to the level detected in C2C12 cells prior to myogenic differentiation (Fig. 3B). Moreover, RT-PCR analysis of myogenin, a transcription factor required for myogenic differentiation [17–19], demonstrated that the expression of myogenin mRNA transcript markedly decreased in differentiated C2C12 cells after IL-17 treatment, being at approximately the same level as in the cells grown in GM before the induction of differentiation (Fig. 3C). This was further confirmed after the transfection of C2C12 cells with a myogenin reporter (G133-luc), when the addition of IL-17 to the medium induced significant inhibition of myogenin promoter transactivation (Fig. 3D).

3.3. IL-17 induces osteogenic differentiation of C2C12 cells

Since C2C12 cells were inhibited from differentiating to myotubes by IL-17, our next goal was to determine whether the cells were directed to differentiate into another cell lineage. Considering recent report which demonstrated that IL-17 induces osteogenic differentiation of MSCs [11], we analyzed the involvement of IL-17 in osteogenic differentiation of C2C12 cell line. Cells were cultured in ODM, with or without IL-17, and after 6 days subjected to ALP determination. As shown in Fig. 4A, osteogenic differentiation induced by specific ODM was additionally stimulated by IL-17. We next aimed to elucidate the degree at which the effect of IL-17 on osteogenic differentiation was dependent of the osteogenic factors present in the culture medium. For this purpose, C2C12 cells were cultured in MDM, since this medium is supplemented with only 2% HS and deprived of any other culture supplements, including osteogenic ones. After six days in these culture conditions, as expected, the cells cultured in the absence of IL-17 were completely negative for ALP cytostain and the myotubes could be observed. However, after the IL-17 treatment, ALP positive colonies were detected, without visible myotubes (Fig. 4A). The appearance of this early phenotypic marker for osteogenic differentiation implied that the effect of IL-17 on the osteogenic commitment of C2C12 cells was independent of the induction media used. Therefore, we additionally evaluated the expression of COX-2, protein involved in osteogenic differentiation [25], in C2C12 cells grown in MDM, and detected a dose-dependent increase of its expression after IL-17 treatment, thus, confirming the stimulatory effect of IL-17 on osteogenic differentiation (Fig. 4B). Furthermore, when C2C12 cells were transfected with an osteogenic reporter

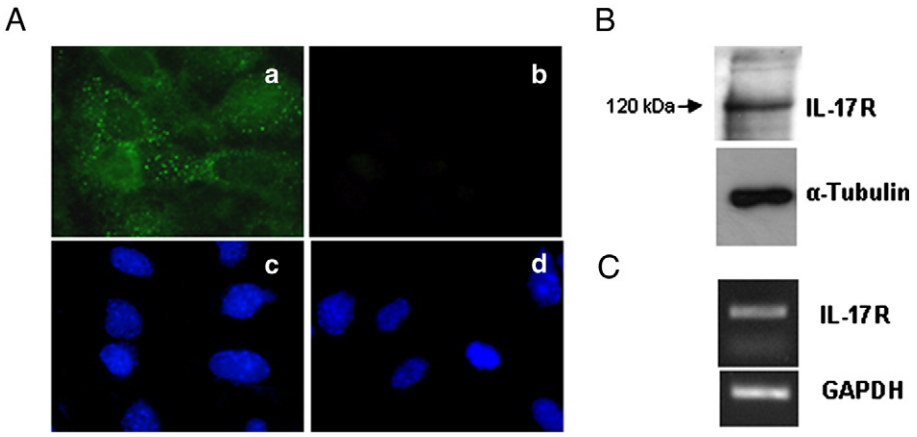

Fig. 1. IL-17 receptor is expressed in C2C12 cells.(A) C2C12 cells immunostained with IL-17R antibody and anti-rabbit FITC (a), isotype control antibody (b) and DAPI (c and d); cells were examined using an immunofluorescence microscope (400× magnification). (B) IL-17R expression detected in protein samples from C2C12 cell lysates after Western blot assay; α-tubulin was used as a loading control. (C) The expression of IL-17R determined by RT-PCR analysis; GAPDH was used as a gel loading control.

(p6SE2-luc reporter) for Runx2/Cbfa1, an essential transcription factor in osteogenic differentiation [26], significant elevation in the Runx2/Cbfa1 reporter transactivation was observed in transfected cells treated with IL-17 (Fig. 4C).

3.4. IL-17 activates MAPKs signaling in C2C12 cells

Next, we aimed to elucidate the signaling events triggered by IL-17 in C2C12 cells. To determine whether IL-17 activates MAPKs, growth-arrested cells were incubated with IL-17 for various time points and cell lysates were then analyzed for the expression of phosphorylated p38, JNK and ERK1,2 MAPKs. The results shown in Fig. 5A demonstrate significantly increased phosphorylation of ERK1,2 MAPK after treatment with IL-17 from 30 min after stimulation onwards. Additionally, increased levels of phosphorylated p38 were observed, with peak activation at 30 and 60 min after IL-17 treatment. In contrast, no expression of phosphorylated JNK was observable for both untreated and IL-17-treated C2C12 cells.

Our next goal was to determine whether MAPK signaling pathways mediate the IL-17-induced differentiation commitment of the C2C12 cells. To reveal the involvement of MAPKs, C2C12 cells were initially treated with specific pharmacological inhibitors of p38 and MEK1,2-ERK1,2 pathways, prior to their exposure to IL-17 and subsequent induction of myogenic or osteogenic differentiation. The usage of p38 specific inhibitor, SB203580, either alone or in combination with IL-17, blocked both the myogenic and the osteogenic differentiation of C2C12 cells (Fig. 5B), suggesting that p38 is involved as a basal regulator of C2C12 differentiation to both mesenchymal lineages, disregarding the presence of IL-17. Therefore, we focused our detailed experiments on ERK1,2 MAPK signaling. To confirm the stimulating effect of IL-17 on ERK1,2 MAPK phosphorylation, a transfection of C2C12 cells with MEK1,2 MAPK responsive reporter (SRE-luc) was carried out. Cells were then treated with IL-17 for 48 h and the transactivation of the reporter was measured by luminometry. In accordance with the IL-17-induced enhanced phosphorylation of ERK1,2 MAPK, IL-17 significantly enhanced the transactivity of the MAPK reporter, too (Fig. 5C).

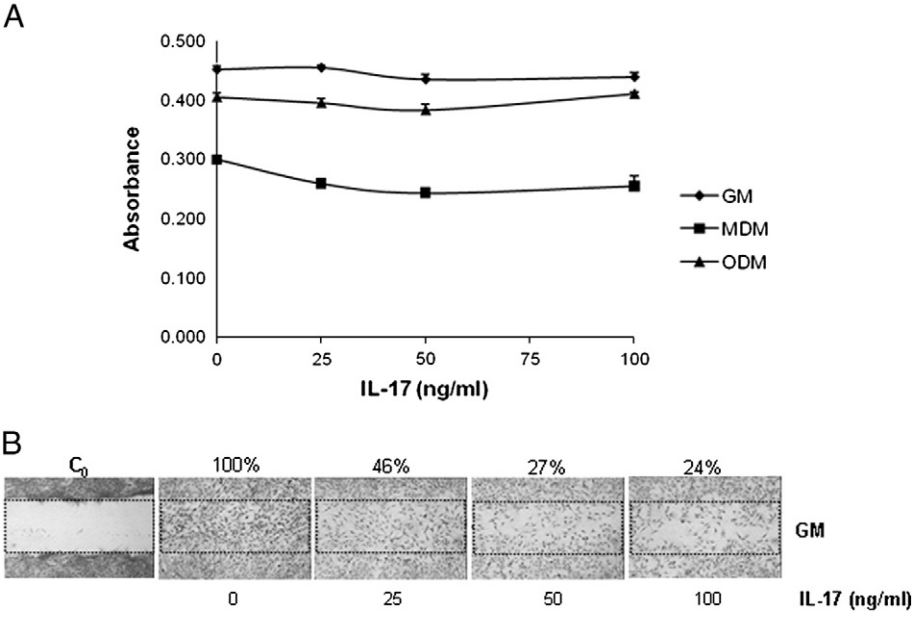

Fig. 2. IL-17 inhibits migration without affecting the proliferation of C2C12 cells.(A) Proliferation of C2C12 cells determined by MTT test after the cells were cultured in GM, MDM and ODM in the presence of 0, 25, 50 and 100 ng/ml IL-17 for 48 h. (B) Migration of C2C12 cells analyzed by scratch assay: a scratch was made in the confluent monolayer of C2C12 cells, and cells cultured in the presence of 0, 25, 50 and 100 ng/ml IL-17 in GM. The recolonization of the scratch by adjoining cells was documented after 24 h; C_0, scratch made in the monolayer at zero time. Numbers shown represent the percentage of the scratch area covered with migrating cells.

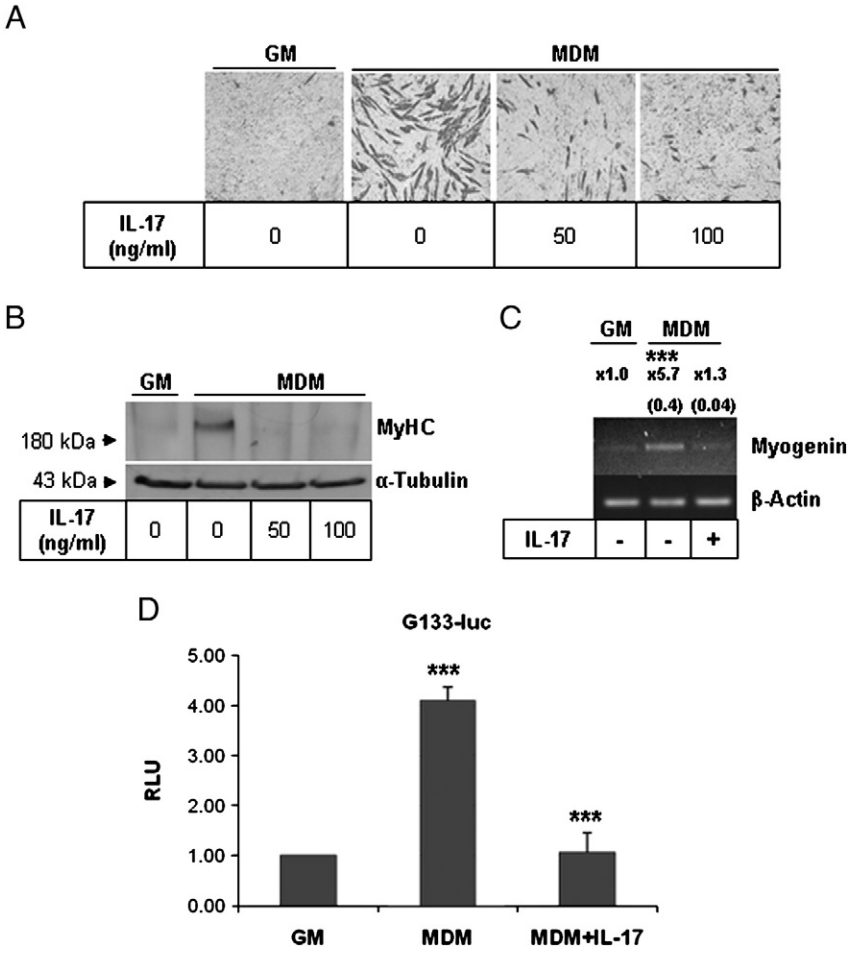

Fig. 3. IL-17 inhibits myogenic differentiation of C2C12 cells.MyHC expression in C2C12 cells cultivated in GM or MDM in the presence of different concentrations of IL-17 determined by (A) immunocytochemistry (dark colored cells) and (B) Western blot assay; α-tubulin was used as a loading control. (C) Myogenin expression determined by RT-PCR in cells grown in GM or MDM with or without 100 ng/ml IL-17; β-actin was used as a gel loading control. Densitometric quantification of the PCR bands is indicated at the top of images, expressed relative to untreated cells, to which an arbitrary value of 1 was given, ±SEM given in brackets. Representative results from three independent experiments are shown. Significant difference from the control (untreated cells) by t-test: ***$p<0.001$. (D) The transactivation of G133-luc myogenin reporter measured by luminometry in transiently transfected C2C12 cells grown in GM or MDM with or without IL-17 (100 ng/ml). The values were normalized to those found in cells grown in control GM; RLU, relative luciferase units. Significant difference from the control (GM) by t-test: ***$p<0.001$.

3.5. ERK1,2 signaling pathway mediates both IL-17-induced inhibition of myogenesis and IL-17-induced stimulation of osteogenesis

The effect of MEK1,2 pharmacological inhibitor, PD98059, was then examined in the IL-17-induced inhibition of C2C12 myogenic differentiation. As shown in Fig. 6A treatment of C2C12 cells with PD98059, in the presence of IL-17, partially recovered the capacity of the cells to form myotubes, visible after MyHC immunostaining. This result was further confirmed by Western blot analysis for MyHC expression, as C2C12 cells treated with PD98059, either in the presence or absence of IL-17, expressed MyHC at a level comparable to the control cells grown in MDM alone (Fig. 6B). In addition, after the cells were treated with PD98059, the expression of myogenin transcription factor was reverted to the level observed in C2C12 cells cultured in MDM before the addition of IL-17 (Fig. 6C). Further on, the MEK1,2 inhibitor reverted the suppressive effect of IL-17 on G133-luc myogenin promoter transactivation in C2C12 transfected cells (Fig. 6D).

The same PD98059 inhibitor was used to analyze the involvement of ERK1,2 MAPK signaling in IL-17 induced stimulation of C2C12 osteogenic differentiation. In order to exclude the influence of the osteogenic media and to determine the signaling pathways elicited solely by IL-17, the cells were cultured in serum-deprived MDM. The data obtained showed that IL-17-stimulated osteogenic differentiation was reverted after the treatment of C2C12 with PD98059, since no ALP positive colonies were observed when the cells were co-treated with IL-17 (Fig. 7A). This result was further confirmed when PD98059 inhibited the enhancing effect of IL-17 on the transactivation of Runx2/Cbfa1 reporter in C2C12 cells (Fig. 7B).

To confirm that ERK1,2 MAPK is directly involved in the induction of osteogenic differentiation by IL-17, a constitutively active mutant form of MEK1 (caMEK1) was used. First, as a positive control of MEK1-ERK1,2 signaling, detection of SRE-luc reporter activation by caMEK1 was performed (Fig. 7Ca) as well as Western blot analysis of HA tagged MEK1 protein expression in transfected cells (Fig. 7Ca, insert). Cells were then co transfected with caMEK1 (pECE/HA MAPKK), or empty vector, and Runx2/Cbfa1 reporter (p6OSE2-luc) and cultured in GM and MDM for 24 h. Ectopic expression of caMEK1 highly increased Runx2/Cbfa1 activation, in both conditions, suggesting that activation of ERK1,2 is the key event involved in the activation of the Runx2/Cbfa1 transcription factor which, consequently, initiates osteogenic differentiation (Fig. 7Cb).

3.6. IL-17 induced osteogenic differentiation of C2C12 myoblasts is BMP independent

BMP-Smad signaling pathway is known to be involved in the osteogenic differentiation of myogenic progenitor cells [16,20,27]. As previously demonstrated, BMP9-induced osteogenic differentiation in

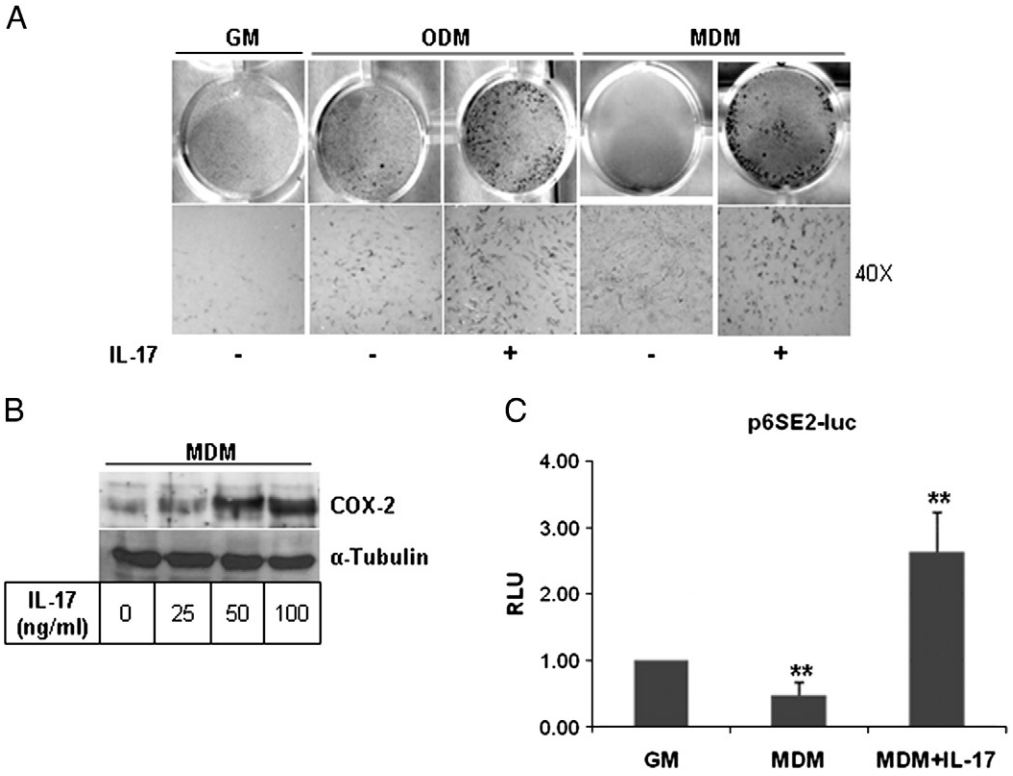

Fig. 4. IL-17 induces osteogenic differentiation of C2C12 cells.(A) ALP activity in C2C12 cells cultured in GM, ODM and MDM in the absence or presence of 100 ng/ml IL-17. (B) COX-2 expression in cells cultured in MDM with different concentrations of IL-17; α-tubulin was used as a loading control. (C) The transactivation of p6SE2-luc reporter for Runx2/Cbfa1 measured by luminometry after the cells were grown in GM or MDM with or without IL-17 (100 ng/ml). The values were normalized to those found in cells grown in control GM; RLU, relative luciferase units. Significant difference from the control (GM) by t-test: $^{**}p<0.01$.

C2C12 cells involves the activation of ALK2, a BMP type one receptor [28]. Therefore, we further analyzed whether BMP-Smad signaling is implicated in IL-17-induced osteogenic differentiation of C2C12 cells by using plasmids containing a kinase-dead ALK2, acting as dominant negative form [24], and constitutively active ALK2 mutant genes.

To validate the plasmids, a Western blot analysis of HA-tagged ALK2 protein expression in transfected cells was conducted. Also, a phosphorylated form of Smad1 was observed only in cells transfected with caALK2 indicating its functionality (Fig. 8A).

To examine whether IL-17 interacts with BMP-Smad signaling, C2C12 cells were co-transfected with either BRE-luc reporter plasmid or Gal4-Smad1 system and two mutant ALK2 expressing plasmids. The cells were then cultured in MDM with or without IL-17.

As shown in Fig. 8B and C, IL-17 did not activate Smads signaling, as neither pBRE-luc nor Gal4-Smad1 activation was observed when compared to basal levels. Only in cells transfected with caALK2 a strong BMP-dependent Smads activation was achieved. Furthermore, to establish whether the activation of Runx2/Cbfa1 transcription factor by IL-17 is independent of BMP, cells were co-transfected with Runx2/Cbfa reporter and kdALK2 or caALK2 plasmids. Activation of Runx2/Cbfa1 reporter was noted after IL-17 treatment, even in cells transfected with kdALK2, confirming that IL-17 induced osteogenic differentiation of C2C12 cells was not dependent on BMP activation (Fig. 8D). As expected, cells transfected with caALK2 showed high transactivation of Runx2/Cbfa1 reporter plasmid (Fig. 8D).

4. Discussion

Although IL-17, the signature Th17 cell cytokine, is known to regulate diverse biological activities in many somatic tissues, its functional role in cell differentiation is poorly studied. In the current study, we analyzed the involvement of IL-17 in C2C12 cell differentiation into myogenic and osteogenic lineage, as well as the signaling pathways implicated.

The inhibitory effect of IL-17 on myogenesis, associated with the attenuated expression of proteins and genes relevant for muscle differentiation is a novel regulatory role of IL-17, demonstrated for the first time in our study. Considering the fact that the differentiation of skeletal muscle cells is critically regulated by a MyoD family-belonging myogenin transcription factor [17–19], the observed inhibition of its mRNA expression, as well as its promoter transactivity, induced by IL-17, confirms a negative action of IL-17 on myogenic differentiation. Additionally, our results show that the expression of myogenic marker protein, MyHC, was severely attenuated by IL-17.

It is known that the skeletal muscle is able to undergo extensive regeneration and repair after injury through the activation of satellite cells present *in situ* leading them to proliferate, migrate to sites of injury and finally, differentiate into adult muscle cells [29,30]. As IL-17 did not alter cell proliferation, but inhibited the migration of C2C12 cells, it is possible that, in addition to lowering myogenin and MyHC expression, IL-17 achieves its inhibitory effect on myogenic differentiation by reducing the cell migration capacity, this way preventing myoblast alignment and fusion, and, consequently the formation of multinucleated myotubes [29]. The low number of short myotubes detected when cells were cultured in MDM in the presence of IL-17 is in line with such observation. Nevertheless, further studies are needed to investigate the role of IL-17 in the cell fusion involved in myogenic differentiation.

The results concerning the IL-17-dependent induction of osteogenesis are consistent with a recent study in which the ability of IL-17 to promote osteogenic differentiation of human mesenchymal cells was shown, although the effect on muscle differentiation was not studied [11]. Additionally, similar was the observation that IL-17 stimulates osteoblastic differentiation independently of its effect on cell proliferation. As the differentiation process is a sequential event

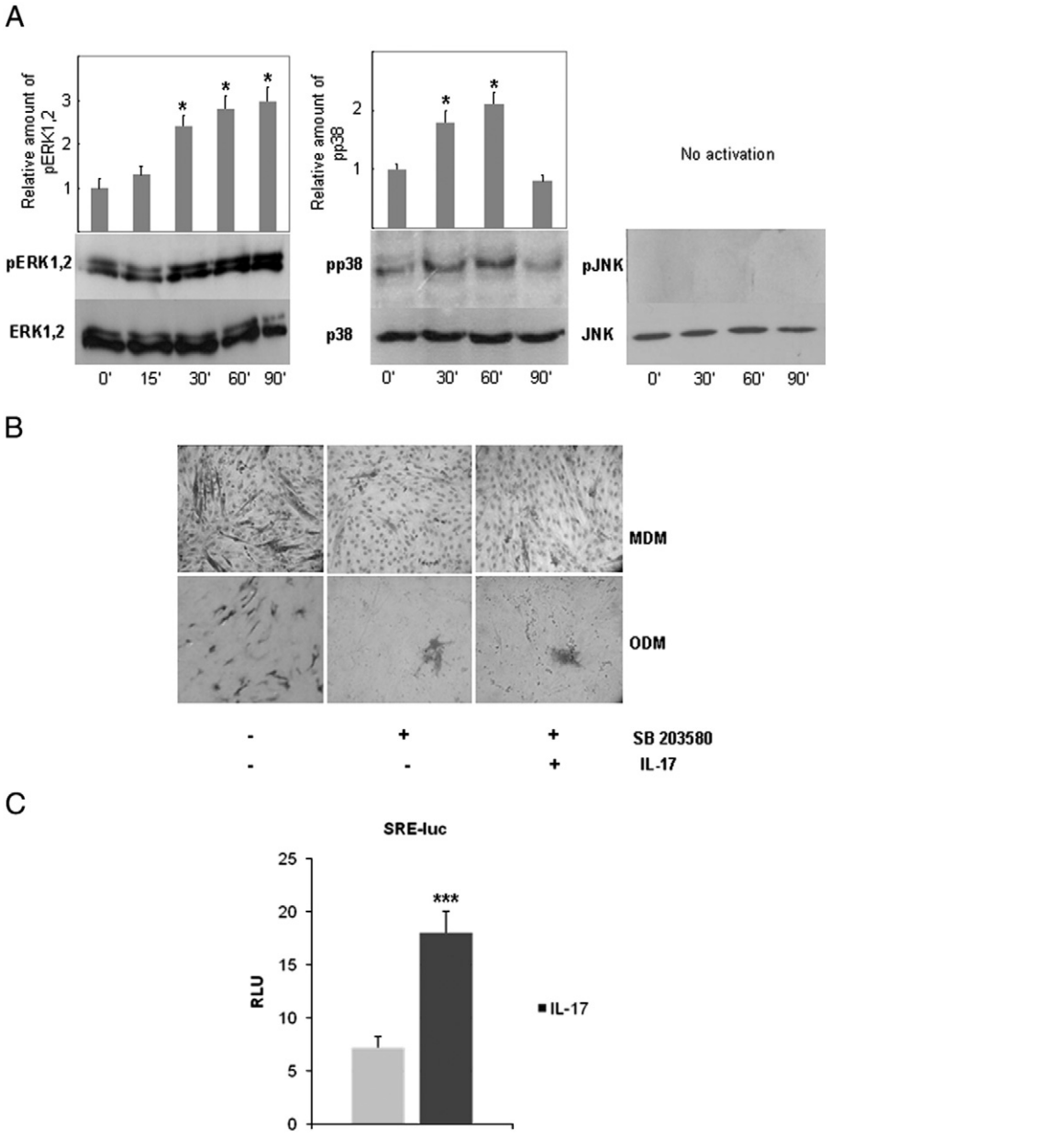

Fig. 5. IL-17 activates MAPK signaling in C2C12 cells.(A) Activation of ERK1,2, p38 and JNK MAPKs in C2C12 cells cultured in serum-free medium and treated with 100 ng/ml of IL-17 during indicated time intervals, determined by Western blot. Blots are from the representative experiment, while time course changes of ERK1,2 and p38 MAPK phosphorylation (in each time point normalized to the control level) are presented as means ± SEM from three experiments. Significant difference from the control by t-test: *$p<0.05$. (B) MyHC expression and ALP activity in C2C12 cells grown in MDM and ODM, respectively, in the absence or presence of p38 MAPK inhibitor, SB203580 (10 μM) and IL-17 (100 ng/ml). (C) The transactivation of MAPK/ERK reporter (SRE-luc) in transiently transfected C2C12 cells treated with 100 ng/ml IL-17; RLU, relative luciferase units. Significant difference from the control by t-test: ***$p<0.001$.

that follows attenuated cell proliferation, it is possible that a decrease in the rate of cell growth may result in the increasing level of differentiation. However, since our results did not reveal any effect of IL-17 on C2C12 proliferation, we can suppose that the cellular effects of IL-17 on the differentiation of C2C12 cells were not due to alterations in the proliferation rate. Moreover, as IL-17 was shown both by us and in other reports [11] to be a potent inducer of osteoblastic differentiation in the absence of any additional osteogenic signals, even in the medium known to promote myogenesis of C2C12 cells, we can postulate that the induction of osteogenic differentiation by IL-17 occurs concurrently with the inhibition of myogenic differentiation. Interestingly, a recent study by Lee et al. [31] demonstrated that IL-17 enhances adipogenic differentiation of C2C12 cells under specific conditions supporting adipogenesis.

Cell commitment to a particular lineage with suppression of alternative phenotypes, is governed by specific cell signaling and activation of specific transcription factors [32]. As for Runx2/Cbfa1, it is well established that this is an essential transcription factor that determines the osteogenic commitment in different cell lineages [33] and is also known to induce the transdifferentiation of primary skeletal myoblasts into an osteoblastic phenotype [34]. Therefore, our finding that IL-17 increases Runx2/Cbfa1 expression proposes one mechanism by which IL-17 may promote osteogenic differentiation in C2C12 cells. COX-2, an immediate-early response gene induced mainly at sites of inflammation in response to inflammatory stimuli [35], is also required for osteogenic differentiation [36]. The results presented here, demonstrating that IL-17 induces COX-2 expression in C2C12 cells, are in line with previously reported data showing

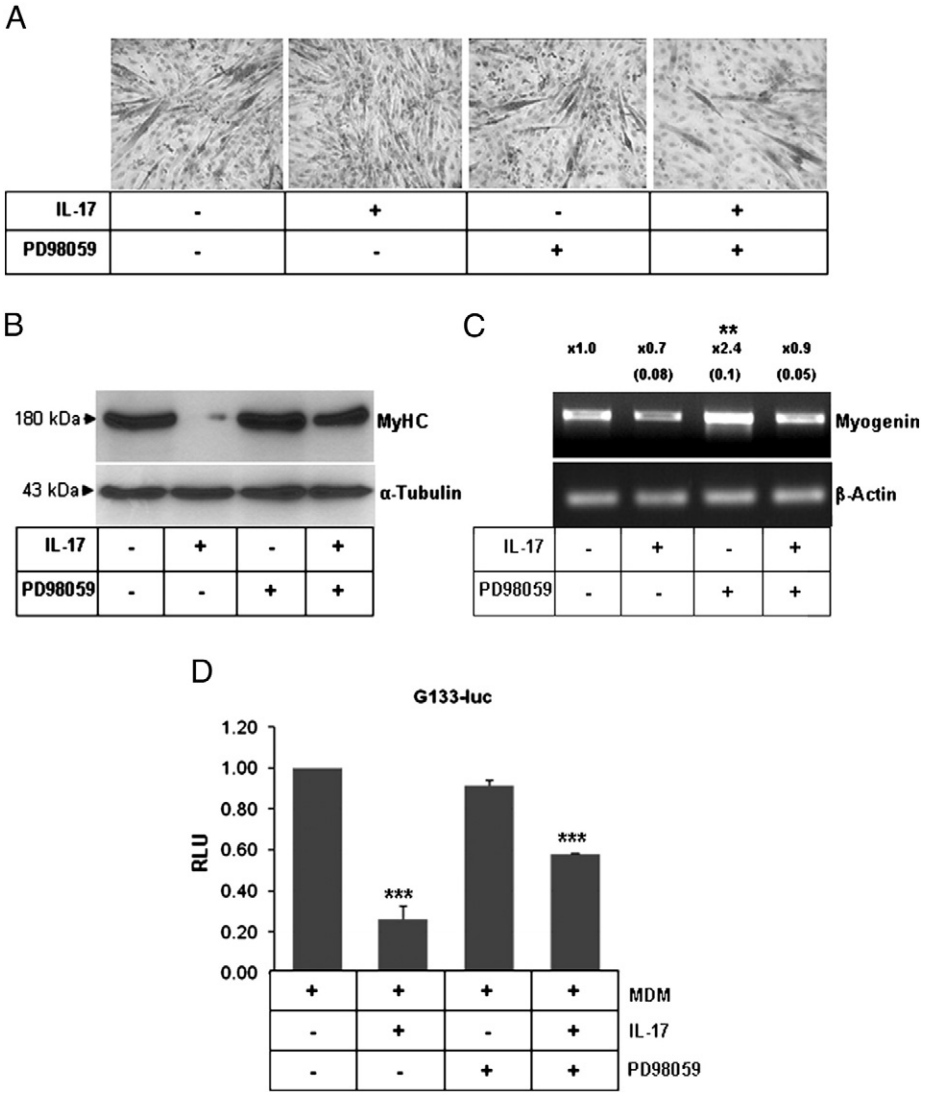

Fig. 6. ERK1,2 signaling pathway mediates IL-17-induced inhibition of myogenesis.C2C12 cells were cultured in MDM in the presence or absence of IL-17 (100 ng/ml) and PD 98059 (25 μM). Levels of MyHC in C2C12 cells determined by (A) immunostaining and (B) Western blot assay; α-tubulin was used as a loading control. (C) Myogenin expression analyzed by RT-PCR; β-actin was used as a gel loading control. Densitometric quantification of the PCR bands ± SEM is indicated at the top of images, expressed relative to untreated cells, to which an arbitrary value of 1 was given, ± SEM given in brackets. Representative results from three independent experiments are shown. Significant difference from the control (untreated cells) by t-test: $^{**}p<0.01$ (D) The transactivation of G133-luc reporter for myogenin measured by luminometry in transiently transfected C2C12 cells cultured in the absence or presence of IL-17 (100 ng/ml) and PD 98059 (25 μM) in MDM. The values were normalized to those found in cells grown in control MDM; RLU, relative luciferase units. Significant difference from the control (MDM) by t-test: $^{***}p<0.001$.

that the increment in IL-17-induced osteogenesis of human MSCs goes in parallel with COX-2 increment [11]. However, further studies are necessary to determine the exact role of COX-2 on the IL-17-modulated osteogenic differentiation of C2C12 myoblasts.

The interplay between extracellular signals and transcriptional regulation is a crucial nexus of control for cell lineage determination. Some of the most commonly studied pathways are the MAPKs [8,21]. MAPKs were shown to be critical for muscle development [37], as well as for the induction of osteogenic differentiation [29]. In this context, ERK1,2 MAPK has been reported as an inhibitor of myogenic differentiation induced by bFGF and TGF-β, and has also been proposed as an essential pathway involved in the decision of mesenchymal cells to differentiate into osteogenic and adipogenic lineage [21,38–40]. As for IL-17, MAPKs belonging to all three subgroups of MAPK family were shown to be activated by this cytokine, in various cell types and in a cell-specific manner [1–3,41]. In addition, we have detected the activation of MAPKs, including ERK1,2, by IL-17 in L6E9 myoblast rat cells as well as in primary human MSCs (data not shown).

In this study we demonstrated that both ERK1,2 and p38 MAPK pathways were activated by IL-17 in C2C12 cells, while no expression of phosphorylated JNK was observed in both the untreated or IL-17-treated cells, despite previous reports on the participation of JNK signaling in the myostatin-dependent inhibition of C2C12 cell myogenic differentiation [42]. By using the p38- and ERK1,2-specific pharmacological inhibitors in our further studies we demonstrated that ERK1,2 pathway is more critical for the differentiation commitment elicited by IL-17 in C2C12 cells. Namely, although IL-17 enhanced the phosphorylation of p38 MAPK in C2C12 cells, the inhibition of p38 blocked both the myogenic and the osteogenic differentiation of these cells, regardless of the IL-17 presence. This result confirmed that p38 is an indispensable regulator of the differentiation process towards both lineages, as demonstrated by previous reports [28,37]. On the other hand, inhibition of ERK1,2 phosphorylation reverted the inhibition of myogenic differentiation caused by IL-17 by eliminating the reduction in the number of myotubes, as well as the reduced expression of MyHC and myogenin. The inhibition of ERK1,2 MAPK reverted IL-17-induced osteogenic differentiation as well, by abolishing the

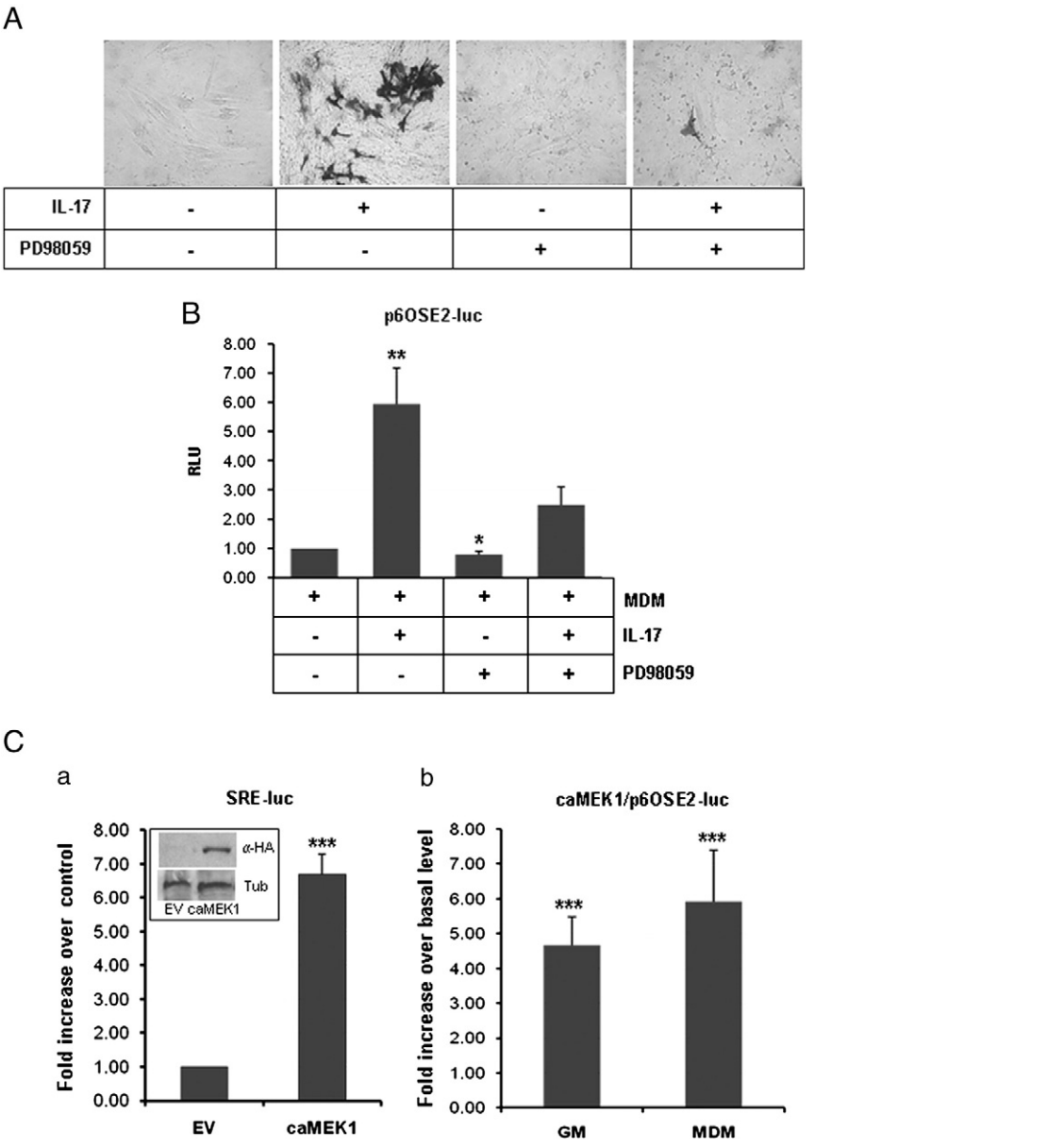

Fig. 7. ERK1,2 signaling pathway mediates IL-17-induced stimulation of osteogenesis.(A) ALP activity detected in C2C12 cells cultured in MDM with different combinations of IL-17 (100 ng/ml) and PD 98059 (25 μM). (B) The transactivation of p6OSE2-luc reporter for Runx2/Cbfa1 measured by luminometry in transiently transfected C2C12 cells cultured in the presence or absence of IL-17 (100 ng/ml) and PD 98059 (25 μM) in MDM. The values were normalized to those found in cells grown in control MDM. RLU, relative luciferase units. Significant difference from the control (MDM) by t-test: $^{**}p<0.01$, $^{*}p<0.05$. (C) a: Expression of SRE-luc in cells transfected with empty vector (EV) and constitutive active MEK1 (caMEK1), pECE/HA MAPKK, measured by luminometry. Significant difference from the control (EV) by t-test: $^{***}p<0.001$; insert: HA tagged-MEK1 expression in cells transfected with EV and caMEK1, determined by Western blot. b: The transactivation of p6OSE2-luc measured by luminometry in C2C12 cells transiently transfected with caMEK1 plasmid, cultured in GM and MDM. The values were normalized to those found at basal level, in cells transfected with EV. Significant difference from the basal level by t-test: $^{***}p<0.001$.

expression of Runx2/Cbfa1 and ALP activity. Furthermore, ectopic expression of caMEK1 resulted in a significant increase of Runx2/Cbfa1 expression, confirming ERK1,2 involvement in osteogenic differentiation induced by IL-17. Consistent with our results, there are previous studies demonstrating that Runx2/Cbfa1 is controlled by ERK MAPK, suggesting that this pathway has an important role in the control of osteoblast-specific gene expression [43,44]. In terms of differentiation, ERK1,2 has been shown to be important for osteogenesis [45], chondrogenesis [46], and myogenesis [40], but whether ERK1,2 is stimulatory or inhibitory to these processes remains unclear due to the amount of conflicting results in the field [40]. At the moment we can speculate that IL-17 might induce osteogenesis by activating simultaneously both ERK1,2 and p38 intracellular pathways in C2C12 cells, maintaining a strict balance between these MAPKs, but when the selective switch in the commitment, either to myogenic or osteogenic lineage, is needed, the activation of ERK1,2 becomes the key signal responsible. Therefore, the results obtained indicate that IL-17 through ERK1,2 opens a new signaling mechanism responsible for shifting the commitment of C2C12 cells.

It was previously shown that C2C12 myoblasts can be transdifferentiated into osteoblastic lineage upon BMP stimulation [28]. Canonical BMP signaling is mediated via the ALK1/2/3/6 type I receptors that phosphorylate Smad1, Smad5, and Smad8 [47]. C2C12 cells express mainly ALK2, while low expression of ALK3 and ALK6 was observed. Furthermore, it was recently reported that BMP9 can induce osteogenesis through ALK1 and ALK2 receptors in C2C12 cells [28]. Additionally, BMPRII expression and no BMP-2, BMP-6 and BMP-7 activity was detected in C2C12 cells grown in basal culture conditions [48]. Having this data in mind, our further experiments using ALK2 mutant constructs were aimed to elucidate whether BMP-Smad

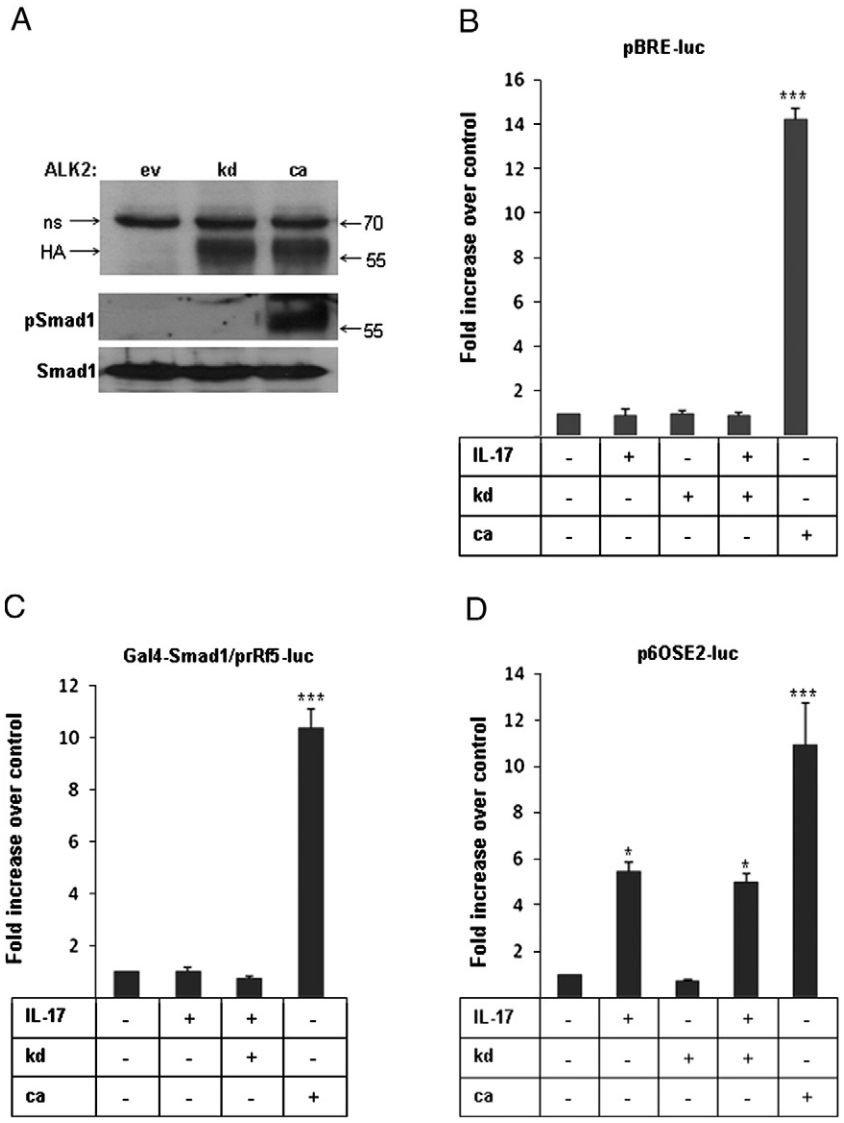

Fig. 8. IL-17 induced osteogenic differentiation of C2C12 myoblasts is BMP independent.(A) Ectopic expression of HA-tagged ALK2 in cells transfected with kinase dead (kd) ALK2, constitutively active (ca) ALK2 or empty vector (ev), and Smad1 phosphorylation determined by Western blot. ns, nonspecific bands serving as a control for equal loading. (B) The transactivation of pBRE-luc, reporter for BMP-Smad signaling, in C2C12 cells transfected with empty vector, kd ALK2 and ca ALK2, with and without IL-17 treatment (100 ng/ml). (C) Cells were transfected with the Gal4-Smad1/pRf5-luc and kd or ca ALK2 plasmids and treated as described in B. (D) The transactivation of Runx2/Cbfa1 reporter in C2C12 cells transfected and treated as described above. RLU, relative luciferase units. Significant difference from the control by t-test: $^{*}p<0.05$, $^{***}p<0.001$. The data presented is representative of three independent experiments.

signaling is involved in the differentiation shift induced by IL-17. Results obtained demonstrated that there is no activation of BMP-Smad pathway during IL-17 treatment of C2C12 cells. Moreover, data obtained after turning the ALK2 receptor, responsible for BMP signaling initiation, "on" and "off" showed that IL-17 activates Runx2/Cbfa1 transcription factor regardless from the activity of ALK2 receptor. Therefore, we were able to conclude that IL-17-induced osteogenic differentiation of C2C12 cells is mediated by ERK1,2 independently of BMP signaling. This conclusion is in line with other reports demonstrating the existence of BMP-independent mechanisms involved in the osteogenic differentiation of C2C12 cells [20].

The investigation of IL-17 regulation of the cell commitment into myogenic or osteogenic lineage might be of importance in the pathogenesis of inflammatory diseases in muscle and bone which involve the infiltration of Th17 cells and IL-17 production, such as polymyositis, dermatomyositis and rheumatoid arthritis [14,15,49,50]. The inflammation occurring in muscle [51,52] can also induce the formation of ectopic bone, and in all conditions, the presence of multipotent, not fully committed cells in the tissue is essential [52]. However, there is yet no evidence of IL-17's presence in the inflamed muscle during ectopic bone formation, and we believe that this is a question worth investigating in the future. According to numerous reports demonstrating that a major biological function of IL-17 is to act as a 'fine tuning' cytokine to enhance or even dampen immune and hematopoietic responses [2,6,49], IL-17 can be a part of a regulatory network, which functions to alter the balance of myoblast cell commitment to the osteogenic or myogenic lineages. However, IL-17 can also be one of the candidates that might be involved in muscle destruction in myopathies or in the pathological development of bone in the muscle tissue. Even though our data, as well as other reports, propose that IL-17 is an osteoblastic induction agent [11,53], further examinations, both *in vivo* and *in vitro* are needed to support this hypothesis.

Several lines of evidence presented here support the notion that IL-17 can regulate both the myogenic and osteogenic differentiation of C2C12 cells, as IL-17-dependent inhibition of myogenesis was associated with the down-regulation of the myogenin mRNA levels, MyHC expression and myotube formation, while IL-17-dependent induction of osteogenesis was associated with the up-regulation of the Runx2/

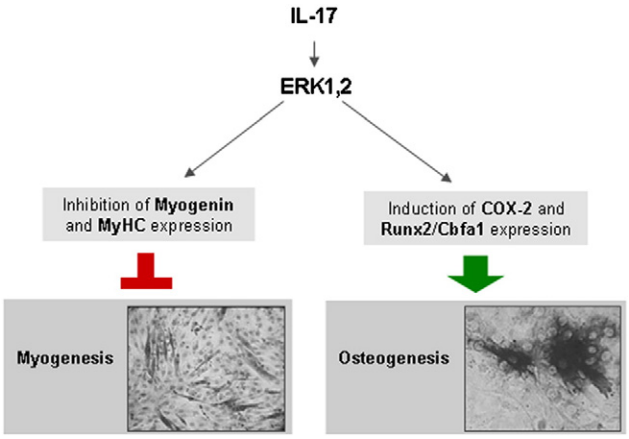

Fig. 9. Summary.IL-17 modulates the multilineage commitment of C2C12 cells by inhibiting their myogenic differentiation through myogenin and MyHC expression down-regulation and promoting their differentiation to osteogenic lineage, by up-regulating Runx2/Cbfa1 and COX-2 expression. The differentiation switch elicited by IL-17 is mediated through the activation of ERK1,2 MAPK.

Cbfa1 mRNA level, COX-2 expression and ALP activity (Fig. 9). The data also reveal that IL-17 exerts these effects by activating ERK1,2 MAPK signaling pathway, independently from BMP-Smad signaling. In conclusion, these data suggest that IL-17 modulates myoblast cell commitment *in vitro* by inducing osteogenic while inhibiting myogenic differentiation.

Acknowledgements

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