Osteogenesis is improved by low Tumour Necrosis factor-α concentration through the modulation of Gs-coupled receptor signals

Running title: Regulation of A_{2B}AR-mediated osteogenesis by TNF-α

Simona Daniele[a], Letizia Natali[a*], Chiara Giacomelli[a], Pietro Campiglia[b], Ettore Novellino[b], Claudia Martini[a#], Maria Letizia Trincavelli[a].

[a]Department of Pharmacy, University of Pisa, 56126 Pisa, Italy.

[b]Department of Pharmacy, University of Naples Federico II, 80131 Naples, Italy.

* these authors equally contributed to this work

Corresponding author at: Department of Pharmacy, University of Pisa. Via Bonanno 6, 56126 Pisa, Italy. Phone: +39-050-2219509/522; Fax: +39-050-2219609. *E-mail address:* claudia.martini@unipi.it

Abstract

In the early phase of bone damage, low concentrations of the cytokine Tumour Necrosis factor-alpha (TNF-α) favour osteoblast differentiation. In contrast, chronic high doses of the same cytokine contribute to bone loss, demonstrating opposite effects in dependence on its concentration and on the time of exposure.

In the bone microenvironment, TNF-α modulates the expression/function of different G protein coupled receptors (GPCRs) and of their regulatory proteins, GPCR regulated kinases (GRKs), thus dictating their final biological outcome in controlling bone anabolic processes.

Herein, the effects of TNF-α were investigated on the expression/responsiveness of the A_{2B} adenosine receptor (A_{2B}AR), a Gs-coupled receptor that promotes mesenchymal stem cell (MSC) differentiation into osteoblasts.

Low TNF-α concentration exerted a pro-differentiating effect on MSCs, pushing on them towards an osteoblast phenotype. By regulating GRK2 turnover and expression, the cytokine impaired A_{2B}AR desensitisation, accelerating receptor-mediated osteoblast differentiation.

These data supported the anabolic effect of TNF-α sub-maximal concentration, and demonstrated that the cytokine regulates GPCR responses by interfering with the receptor desensitisation machinery, so enhancing the anabolic responses evoked by A_{2B}AR ligands. Overall, these results indicated that GPCR desensitisation plays a pivotal role in osteogenesis and that its manipulation may be an effective strategy to favour bone remodelling.

Bone diseases, characterized by a progressive reduction of bone strength and susceptibility to fracture, are increasingly frequent pathologies, especially due to the remarkable increase in the average age of the population. Currently, no Food and Drug Administration-approved therapy is available to effectively promote bone regeneration (1,2). For this reason, understanding molecular pathways involved in fracture reparative processes is pivotal to develop new, simple and cost-effective therapeutic agents and strategies.

In a healthy body, injury is usually followed by both inflammation and immunologic reaction that is caused by local necrosis and bacterial infection. Inflammatory cells and factors released in the injured site influence the microenvironment of bone tissue in order to favour osteogenic reparative processes (3-5). Among the inflammatory players, Tumour Necrosis Factor-alpha (TNF-α) assumes an important role in bone healing affecting mesenchymal stem cell (MSC) behaviour in a dose and time-dependent manner. In the early inflammatory phase, immediately after fracture, TNF-α is released at low levels and favours MSC migration, survival and differentiation, thus promoting bone repair (6). In contrast, in the late and chronic uncontrolled phase of inflammation, higher doses of the same cytokine have destructive effects on bone and contributed to bone loss. However, the intracellular pathways and mechanisms involved in bone remodelling and responsible for the "paradoxical effects" of TNF-α remain unclear (7-9). For that matter, it is ascertain that TNF-α dialogues with different G protein coupled receptors (GPCRs), and through the modulation of the expression and/or functional activity of these receptors, may interfere with the biological responses evoked by receptor stimulation under physiological and pathological conditions.

Several GPCRs, such as the parathyroid hormone receptor (PTH1R), E- series prostaglandins receptors (EP2 and EP4), P1 and P2Y purinergic receptors and much others are directly involved in bone remodeling (10,11). Clinical trials with PTH R-targeted molecules demonstrated that receptor desensitisation, subsequent to the continuous administration of receptor agonists, may explain the switch from anabolic to catabolic effects of PTH on bone (12-14). Based on these evidences, the

investigation of the molecular mechanisms underlying desensitisation processes of GPCRs may be crucial to understand the final biological outcome of these receptors to control bone remodelling and to propose new and alternative therapeutic strategies for bone diseases.

Among GPCRs, the adenosine A_{2B} receptors (A_{2B}ARs) have been demonstrated to promote MSC differentiation and increase osteogenesis both *in vitro* and *in vivo*, suggesting this receptor as an innovative target for bone diseases (15-19). A_{2B}ARs are functionally activated by endogenous adenosine only in damaged and inflamed tissues, in which adenosine is massively released following ATP degradation. Moreover, several studies have demonstrated that the responses evoked by A_{2B}ARs are selectively regulated by pro-inflammatory cytokines, in both chronic and acute inflammation (20,21). In particular, TNF-α has been shown to modulate the A_2AR desensitisation process through a direct effect on GPCR receptor kinase-2 (GRK2) in human astroglial cells, thus modifying the receptor-mediated responses (22,23). Indeed, GPCR desensitisation is largely mediated by direct phosphorylation of receptor serine and threonine residues by a family of kinases, termed GRKs. Among the different isoforms, GRK2 and GRK3 are the most common kinases involved in the regulation of GPCR responsiveness in bone (24). The expression of GRK2 is temporally regulated during osteoblast development, and this kinase has been shown to modulate GPCR responsiveness during the osteogenic process (25). Noteworthy, the activity of GRKs is regulated by the inflammatory microenvironment. Several studies have demonstrated that TNF-α decreases the cellular levels of GRK2 in *in vitro* and *in vivo* models of inflammatory diseases, and may contribute to regulate the desensitisation processes of different GPCRs, so prolonging agonist-mediated receptor activation over time (26-28).

To date, no data are available on the regulation of A_{2B}AR desensitisation in bone under inflammatory conditions. Based on this evidence, the aim of this work was to investigate the effect of TNF-α on the kinetics of A_{2B}AR desensitisation (taken as a model of Gs protein coupled receptors), in the regulation of osteoblast differentiation from MSCs. We demonstrated that a low

93 TNF-α concentration, miming a sub-maximal inflammatory state, induced a significant inhibition of
94 GRK2 activity, so impairing agonist-induced A_{2B}AR desensitisation. Therefore, the inflammatory
95 cytokine increased the osteogenic effects elicited by the purinergic system, contributing to
96 osteoblast differentiation. These data indicate that the local inflammatory environment manipulation
97 may be a simple and effective way to enhance bone formation and accelerate fracture repair.
98

MATERIALS AND METHODS

Materials. The 2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-ylsulfanyl]acetamide (BAY60-6583), and N-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide (MRS1706) were purchased by Tocris Bioscience (Bristol, UK). RNeasy® Mini Kit was obtained from Qiagen S.p.A. The Script cDNA synthesis kit was furnished by Bio-rad s.r.l. Fluocycle® II SYBR® was from Euroclone s.p.a. (Milan, Italy). TNF-α were purchased by Sigma Aldrich.

Cell cultures. Human bone marrow MSCs and cell culture medium were purchased by Lonza (Milan, Italy). The cell line was monitored for DNA profiling by HLA-DP beta, and confirmed as human by PCR. The cells were used until passage 5, for fewer than 6 months after resuscitation.
Cells sub-cultured in normal growth medium (MSCGM, Lonza) and plated ($5x10^3$ cells/cm^2). The medium was changed to remove non-adherent cells every 3 to 4 days, and the cells were used at passages 0 to 3. For osteoblast differentiation studies, cells were seeded ($9x10^3$ cells/cm^2) and cultured as previously described (15).

Cell viability assay. MSCs were seeded in 96-well microplates ($5x10^3$ cells/well) and cultured in proliferation or osteogenic medium for the indicated days, in the absence (control) or in the presence of TNF-α (0.1 ng/ml-10 ng/ml). To evaluate cell viability, MTS assay was used following the manufacturer's instruction (Promega, Milan, Italy).

Real-time RT-PCR analysis. MSCs were cultured in normal growth medium and were treated with osteogenic medium in presence or absence of TNF-α (1 ng/ml) and BAY 60-6583 (5 nM), alone or in combination. The mentioned treatments were repeated every three days, and the expression levels of osteogenic markers (Runx2, Osterix, ALP and Osteocalcin) were quantified after 0, 5, 15 days of treatment. Moreover, MSCs were cultured in the presence or absence of TNF-α (1 ng/ml) in normal growth medium for 48 hours or in osteogenic medium for 5 days. Then, gene expression was assessed by real-time RT-PCR, as previously described (29).

Mineralization assay. MSCs were seeded ($9x10^3$ cells/cm^2) and then treated in the absence (control) or in the presence of TNF-α (1 ng/ml), BAY 60-6583 (5 nM), MRS1706 (1 μM), KRX 29 (1 μM) alone or in combination. Treatments were repeated every three days, and the mineralization was quantified after 15 or 21 days of treatment. The rate of mineralization was quantified using alizarin red staining (19).

Western blot analysis. MSCs were differentiated in osteogenic condition for 48 h or 5 days in absence (control) or presence of TNF-α (1 ng/mL). When indicated, MSCs were pre-treated 10 μM with the proteasome/calpain inhibitor MG-132 (30) for 3 h, and then incubated in osteogenic medium for 48 h in absence (control) or presence of TNF-α (1 ng/mL).

Thereafter, cells were lysed and 30 μg of protein added with Laemmli solution) were loaded in a SDS-PAGE (7.5%). Protein were electrotransferred into PVDF membranes and incubate overnight at 4°C using the following primary antibodies anti-A_{2B}AR (sc-28996, Santa Cruz Biotechnology; 1:150); anti-GRK2 (sc-562, Santa Cruz Biotechnology; 1:200); and anti-GAPDH (G9545, Sigma Aldrich; 1:5000) and by the appropriate peroxidase-conjugated secondary antibodies. Signal were detected using a chemioluminescent substrate (ECL, Perkin Elmer, Waltham, MA, USA). ImageJ Software was used to perform the densitometric analysis of the immunoreactive bands (version 1.41; Bethesda, MD, USA).

Measurement of cAMP levels during BAY60-6583 desensitisation induction in MSCs. MSCs ($2x10^4$ cells/well) were plated in 24-well plates and after 24 h, the osteogenic differentiation is induced in presence or absence of TNF-α (1 ng/mL) for 0, 5 or 15 days. At each time of differentiation, cells were stimulated with BAY60-6583 (5 nM) for 15 min, and cAMP levels were quantified. In the desensitisation assays, MSCs (differentiated for 0, 5 or 15 days in the absence or presence of TNF-α) were pre-treated with BAY60-6583 (5 nM) for different times (5–120 min), then washed and stimulated with BAY60-6583 (5 nM) for 15 min, in the presence of 1 unit/ml of Adenosine deaminase (ADA) and the phosphodiesterase inhibitor Ro 20-1724 (20 μM) (31). At the

end of treatments, intracellular cyclic AMP (cAMP) levels were quantified using a competitive protein binding method (32).

In order to investigate the effect of the GRK2 inhibition on A_{2B}AR functional response during MSC differentiation, the cells were cultured in osteogenic medium for 0, 5 or 15 days. At each time point, the cells were challenged with 5 nM BAY60-6583 for different times (5–120 min), in the absence or presence of the GRK2 inhibitor KRX 29 (1 μM).

GRK2 association to A_{2B}AR or Mouse Double Minute 2 (Mdm2). To test the A_{2B}AR-mediated recruitment/association with GRK2, as well as Mdm2 involvement in GRK2 regulation, a quantitative immune-enzymatic assay on MSC lysates on crude was used (33,34,35). MSCs were differentiated for 0, 5, or 15 days in the absence or in the presence of TNF-α (1 ng/mL), and then stimulated for 5 min with 5 nM BAY60-6583. Conversely, for Mdm2-GRK2 association, MSCs were incubated in osteogenic medium for 48 h or 5 days in the absence or in the presence of TNF-α (1 ng/mL). At the end of treatments, cells were suspended in lysis buffer containing 1% of the Protease inhibitor Cocktail (Sigma Aldrich, Milan , Italy). Cell lysates (30 μg/well) were incubated for 60 min into wells pre-coated with a rabbit anti-A_{2B}AR or anti-Mdm2 antibody; after washes, each well was incubated for 15 min with 1% BSA, to block non-specific sites, and then for 2 h at room temperature with a mouse primary anti-GRK2 antibody. Then, wells were washed and incubated for 1 h with an anti-mouse HRP-conjugate antibody, and washed again (33). The colorimetric substrate kit allowed a colorimetric quantification of the receptor-GRK2 association.

GRK2 overexpression. GRK2 overexpression was obtained by transfecting MSCs using the PEI method (33) MSCs were cultured in osteogenic medium for 48 h, in the absence or presence of TNF-α. The, cells were incubated with 1 μg either of GRK2 plasmid (Origene, MD, USA) or the corresponding empty vector (OriGene, MD, USA) for 30 h. Transfected MSCs were rapidly seeded in 24 well plate and subjected to the cAMP assay, as described above. GRK2 overepression were confirmed by western blot analysis.

Statistical analysis. A non-linear multipurpose curve-fitting program, Graph-Pad Prism (Version 5.00), was used for data analysis and graphic presentation. Data are reported as the mean ± SEM of 3-4 different experiments. Statistical analyses were performed using a one-way ANOVA study followed by the Bonferroni test for repeated measurements. Differences were considered statistically significant when $P < 0.05$.

RESULTS

MSC proliferation and differentiation to osteoblasts: effects of TNF-α. MSCs were cultured in complete growth medium. To induce differentiation into osteoblasts, a specific osteogenic medium was used (15). The time course and complete cell osteogenic process was monitored as described previously (15).

The effects of different concentrations of TNF-α (0.1 ng/ml-10 ng/ml) on MSC proliferation were evaluated. TNF-α did not induced any significant effect on the proliferation rate of both undifferentiated MSCs (Fig 1A) and cells differentiated for 5 days in osteogenic medium (Fig. 1B). These data demonstrated that the cytokine, in our culture experimental medium, did not has toxic effect up to 10 ng/ml concentration.

Then, we investigated the effects of TNF-α on MSC differentiation to osteoblasts. MSCs were subjected to the pro-inflammatory stimulus during their differentiation in osteogenic medium. The effects of TNF-α (1 ng/ml) on the expression of Runx2, a pivotal transcription factor that drives cells towards osteoblast phenotype, and ALP, an early marker of osteoblast differentiation, were evaluated at different stages of differentiation. TNF-α induced a time-dependent increase in the expression levels of both Runx2 (19.69 ± 2.69 vs 12.44 ± 0.91 in control, after 15 day treatment, Fig. 1C) and ALP (20.37±1.34 vs 13.26±1.09 fold in control, after 15 days of treatment, Fig. 1D).

In addition to the induction of osteogenic markers, calcium deposition after 15 days of cell incubation with the cytokine in osteogenic medium was measured, as a late indicator of osteogenic differentiation. TNF-α significantly increased calcium deposition, confirming it favoured matrix mineralization (Fig. 1E and F). In contrast, any significant effects were observed in MSCs cultured in growth medium (data not shown). These results suggested the pro-inflammatory cytokine may contribute to the early osteogenic differentiation of MSCs, potentiating the effects of pro-differentiating factors.

Osteoblast differentiation: interplay between TNF-α and A_{2B}AR. It is known that pro-inflammatory cytokines regulate the expression and activity of several intracellular proteins so contributing to modulate the responses evoked by GPCRs (22,36). Among these, TNF-α has been shown to modulate expression and functionality of the A_{2B}AR (21,23), a GPCR involved in MSC differentiation (17).

Based on this evidence, the modulation of TNF-α on A_{2B}AR-induced MSC differentiation was evaluated. Consistent with literature data, the selective A_{2B}AR agonist, BAY60-6583 caused a time-dependent increase in both ALP and Runx2 expression, after 5 and 15 days of cell treatment (Fig. 2A and B). These results confirmed that A_{2B}AR stimulation induced a significant enhancement of osteogenic process. Moreover, the treatment with BAY60-6583 for 15 or 21 days produced an evident increase of mineralization (Fig. 2C and D), as previously reported (15).

When BAY60-6583 was used in combination with TNF-α, the pro-osteogenic effects of A_{2B}AR agonist was significantly increased. Indeed, the expression levels of Runx2 and ALP induced by BAY60-6583 in the pro-inflammatory medium were 1.28 and 1.25 fold higher than those obtained with the agonist alone (Fig. 2A and B).

These results were confirmed by the mineralization assay (Fig. 2C and D). Indeed, a significant increase in matrix mineralization was detected when TNF-α and BAY60-6583 were used in combination. The percentage of calcium deposit induced by the A_{2B}AR stimulation in the presence of cytokine accounted to 144.6 ± 5.1 % and 155.3 ± 4.8 % respectively after 15 and 21 days of differentiation, with respect to 127.1 ± 6.0 % and 136.5 ± 7.6 % obtained in the absence of TNF-α.

The effect of TNF-α on the responses evoked by BAY60-6583 was completely abrogated by the selective A_{2B}AR antagonist MRS1706, confirming it is selectively ascribed to the activation of A_{2B}AR subtype. Notably, the effect of TNF-α alone was slightly decreased by the treatment with MRS1706 pointing out that the A_{2B}AR activation could be almost one of the several mechanism at the basis of the TNF-α osteoblastogenesis induction.

These data taken together suggested that the MSC inflammatory microenvironment may modulate the mineralization process favouring the responses evoked by A_{2B}AR stimulation.

Agonist-induced A_{2B}AR desensitisation at different stages of MSC differentiation: effect of TNF-α. The putative molecular mechanisms involved in the effects of TNF-α on A_{2B} AR responses were investigated. First, the modulation ofA_{2B} AR gene and protein expression evoked by the cytokine was investigated. As shown in Fig. 3, a significant up-regulation of A_{2B}AR gene (2.03 ± 0.17 fold vs control) and protein (147 ± 8 % vs control) expression was detected after 48 h of MSC treatment with the cytokine (Fig. 3A, C and D). In contrast, challenging MSCs for 5 days in osteogenic medium did not affected A_{2B} AR content, at both gene and protein levels (Figure 3B, C and D).

Then, the effects of TNF-α on A_{2B}AR functional responsiveness were investigated. A_{2B}ARs are mainly coupled to Gs proteins and activated adenylyl cyclase so increasing intracellular cAMP levels. The desensitisation is one of the main process that regulate GPCR functionality. On this basis, TNF-α modulation on agonist-mediated A_{2B}AR desensitisation were evaluated, at different time stages of MSC differentiation program (0-5 and 15 days). For this purpose, control and TNF-α-treated cells were pre-incubated with A_{2B}AR agonist (BAY60-6583) for different times (5-120 min), then washed and stimulated with the same agonist for 15 min. As depicted in Fig. 4, A_{2B}AR functional responses were quickly impaired by cell pre-challenge with the agonist BAY60-6583 (Fig. 4) (37,38). The desensitisation kinetics appeared to be faster in undifferentiated MSCs (t ½ 1.46 ± 0.008 min^{-1}) and in the early phase of differentiation (t ½ 4.17 ± 0.021 min^{-1}, Fig. 4A and B). In contrast, in the late phase of differentiation program, A_{2B}AR functional responses remained preserved for a longer time, showing a slower desensitisation kinetics (t ½ 16.32 ± 1.12 min^{-1}, Fig 4C). These differences may be ascribed to the different expression of A_{2B}ARs along with the MSC maturation.

TNF-α did not significantly alter the basal response of A_{2B}AR to agonist, but substantially impaired the degree and the rate of receptor desensitisation. These effects appeared already evident in un-differentiated cells (t ½ 17.61 ± 1.22 min^{-1}) and became more significant at the different stages of differentiation process (5 days: t ½ 36.75 ± 2.15 min^{-1}; 15 days: t ½ 50.77 ± 3.81 min^{-1}). The diverse effect of TNF-α during the differentiation process could reflect the presence of different cell phenotypes that are characterized by the expression of peculiar intracellular proteins involved in the receptor regulatory machinery.

Effects of TNF-α on GRK2 expression. GRKs, and in particular the isoform 2, are the most relevant kinases involved in the phosphorylation and desensitisation of GPCRs, including A_{2B}ARs (39,40). To elucidate the mechanism through which TNF-α impaired A_{2B}AR functionality, we investigated the effects of the inflammatory cytokine on GRK2 expression and on its association to A_{2B}ARs upon agonist-mediated receptor stimulation. Notably, the cytokine caused a significant and a time dependent inhibition of GRK2 protein levels (Fig. 3A-C).

In order to dissect if TNF-α-mediated decrease in GRK2 levels could involve a transcriptional mechanism, a real-time PCR analysis was performed. The results showed that the cytokine did not affect the expression of GRK2 mRNA after 48 h or after 5 days of differentiation (Fig. 3D). Based on such data, post-transcriptional regulation of GRK2 by TNF-α was investigated, considering, in particular, the involvement of the proteasome pathway, which has been identified as a major mechanism for modulating GRK2 expression levels (41,42,43). To this purpose, MSCs were challenged with the proteasome inhibitor MG-132 (30), in the absence or presence of the inflammatory cytokine for 48 h. As depicted in Figure 3E and F, MG-132 significantly, but not completely, prevented TNF-α-mediated decrease of GRK2 expression.

Because several proteins targeted to the proteasome pathway go through polyubiquitination (33,41,44), the potential role of ubiquitination in GRK2 degradation was examined by assessing the kinase association to the Mdm2 ubiquitin ligase (33,45). The results showed that the cytokine

induced a significant GRK2 association to Mdm2 following both 48 h and 5 days of treatment (Fig. 3G). Altogether, our data indicate that TNF-α regulation of GRK2 involves, at least partially, the ubiquitin-dependent proteasome pathway.

Effects of TNF-α on GRK2 association to A_{2B}ARs. The stimulation with BAY60-6583 caused a significant increase of A_{2B}AR-GRK2 association (148.6 ± 4.8 %, P<0.001 vs. basal; Fig. 4D). Moreover, A_{2B}AR-GRK2 association decreased during the different time point of MSC differentiation to osteoblast. The degree of this association paralleled with the rate of desensitisation induced by the agonist (Fig. 4A-C). In addition, a reduction in GRK2 association to A_{2B}ARs was detected when the MSC were maintained in the presence of TNF-α, particularly in the early phase of the MSC differentiation process (Fig. 4D). These data suggest the cytokine may modulate the desensitisation machinery of different Gs-coupled receptors in MSCs so favouring the functional activity of these receptors in response to agonist stimulation, such as demonstrated for the A_{2B}AR.

Effect of GRK2 inhibition/overexpression on the kinetics of A_{2B} AR desensitisation. In order to corroborate the hypothesis that TNF-α affected the GRK2 activity leading to the impairment of Gs coupled receptor desensitisation, the effects of a selective GRK2 inhibition and overexpression on A_{2B}AR desensitisation were evaluated. Challenging MSCs with the synthetic GRK2 inhibitor KRX29 (40,46) completely prevented A_{2B}AR desensitisation at all the osteoblast differentiation stages (Fig. 5A-C). The data are in accordance with the effect of KRX29 on mineralization (Fig. 2C and D). In fact, the inhibitor slightly increased the mineralization induced by the agonist BAY60-6583 producing an effect comparable to that exerted by TNF-α. The similar effect exerted by the cytokine and the kinase inhibitor highlighted the involvement of the GRK2 in agonist-mediated A_{2B}AR desensitisation.

304 In parallel, we also evaluated the effect of GRK2 over-expression on TNF-α-mediated impairment
305 of A_{2B} AR functional responses. MSC transfection with GRK2 plasmid induced a significant
306 increase in protein expression (2.5 fold vs empty vector transfected cells, $P<0.001$), as demonstrated
307 by Western blot analysis (Fig. 6A and B) (33). GRK2 over-expression induced a significant
308 increase in the degree of A_{2B}AR desensitisation, as shown in Fig. 6C. Furthermore, the high levels
309 of GRK2 protein were able to overcome almost completely the inhibitory effect of TNF-α on
310 A_{2B}AR functionality (Fig. 6D): the receptor reduced functional responses to its selective agonist in
311 a time dependent manner even in the presence of the cytokine. These data support our hypothesis
312 that TNF-α impaired Gs protein coupled receptor desensitisation by affecting, at least in part, the
313 activity of intracellular GRK2 proteins, as demonstrated for the A_{2B}AR.
314

DISCUSSION

TNF-α have been widely reported to exert opposite and conflicting effects on MSCs (6-9), primarily depending on cytokine concentration, time of exposure, and stage of cell differentiation (7,9). In this study, a low concentration of TNF-α was demonstrated to enhance and accelerate the A_{2B} AR-mediated differentiation of MSCs towards an osteoblast phenotype, according to the consolidated evidence that low, sub-maximal concentration (1-10 ng/ml) of TNF-α, in particular in the early phase of tissue injury, has an anabolic effect (47,48). The mechanisms involved in the cytokine-elicited action on osteogenesis remain to be elucidated. Dissecting such molecular aspects is a crucial goal for a therapeutic intervention aimed at controlling bone remodelling, both in physiological and pathological conditions. Herein, the interplay between TNF-α and the A_{2B} AR was investigated in MSCs, examining in particular the GPCR desensitisation, a process primarily mediated by intracellular GRK proteins (39,49,50). The induction of osteogenesis mediated by low TNF-α concentrations was primary related to a reduction of GRK2 expression, leading to an enhancement of A_{2B} AR functionality.

Several pharmacological strategies targeting GPCRs for promoting osteogenesis (10,14,15,18) have been limited by the loss of receptor functionality that reduces the response to agonists over-time. Notably, the occurrence of desensitisation processes has been linked to the switch from anabolic to catabolic events in bone remodelling, suggesting that manipulating the GPCR desensitisation machinery may be a useful strategy for the treatment of bone diseases (12,13,51).

TNF-α has been demonstrated to play an important role in bone healing by affecting MSC behaviour (7). Consistent with recent literature (47,48), herein a low TNF-α concentration was confirmed to stimulate MSC differentiation to osteoblasts within 15 days of cell treatment. The presence of the cytokine in osteogenic medium significantly increased Runx2 and ALP expression levels, so favouring MSC mineralization. Of note, this effect was not associated with significant changes in cell proliferation. TNF-α affects not only the differentiation processes, but also

positively or negatively influence the rate of proliferation (52,53). In our experimental model of induced-MSC differentiation, TNF-α caused a complete switch to differentiation process blocking the proliferative machinery.

Among the different GPCR involved in bone remodelling and osteoblastogenesis, the purinergic receptor A_{2B}AR has arisen recently (15-19). Of note, this receptor responds to micromolar concentrations of adenosine, which is released under pathological conditions, such as stress or inflammation (54).

A functional interplay between A_{2B}ARs and TNF-α has been shown in different cell models, including glioma (23), intestinal epithelial (21), and vascular smooth muscle (20) cells. In particular, the cytokine has been shown to enhance the adenosine-mediated responses under inflammatory conditions, by regulating A_{2B}AR responses at different levels, including an up-regulation of protein expression and/or an increase of receptor functional responsiveness by inhibiting receptor phosphorylation (21,23).

To date, no evidence are available on the regulation of A_{2B} ARs by TNF-α in MSCs, as well as on the role of this dialogue in controlling the bone remodelling process. Herein, TNF-α was shown to induce a significant increase in the A_{2B} AR-mediated osteogenic effects. The cytokine treatment increased the receptor mRNA and protein levels only in undifferentiated cells. Thus, the enhancement of the GPCR responses elicited by TNF-α was primary ascribed to a direct effect on GRK2, which is the most important kinase involved in GPCR desensitisation in response to agonist stimulation. Spurney et al., have demonstrated that GRK2 and β–arrestin, are temporally regulated during osteoblast differentiation, in a pattern that would tend to enhance GPCR responsiveness and favour MSC differentiation (13). When an injury occurs, the release of cytokines and other soluble factors in cellular microenvironment may regulate the activity of these kinases and in turn affect GPCR final biological outcome (55,56). In particular, the increase of Gs-coupled receptor responsiveness and the consequent accumulation of higher levels of cAMP are crucial events in pushing osteoblast precursors to a differentiated phenotype. In this respect, Sinha and collaborators

366 have recently demonstrated that cAMP signalling downstream to Gs-coupled receptors plays a
367 critical role in determining the commitment of osteoblast precursors to bone with respect to
368 adipocyte (57).
369 TNF-α has been shown to prevent Gs-coupled receptor desensitisation by regulating GRK2
370 association with plasma membranes, so inhibiting receptor phosphorylation and desensitisation in
371 different cell lines (22). These data may support a beneficial role of low cytokine concentrations in
372 potentiating GPCR functional responses. In MSCs, we demonstrated that TNF-α caused a
373 significant down-regulation of GRK2 protein level without affecting its gene expression. The kinase
374 regulation was shown to partially involve the Mdm2-ubiquitin-dependent proteasome pathway,
375 consistent with previous data (30,41,43,45). Because TNF-α– mediated GRK2 downregulation was
376 not completely counteracted by the use of a proteasome inhibitor, we speculate that additional
377 mechanisms could intertwine in such outcome. In this respect, GRK2 turnover has been linked to its
378 phosphorylation by c-Src (58) or by MAPK (59); these effects, together with Mdm2 recruitment,
379 can be favoured by the β-arrestin machinery (42), which has been demonstrated to play different
380 scaffold functions to coordinate both Mdm2-dependent and -independent processes implicated in
381 GRK2 stability (42,45,58,59).
382 The decrease in GRK2 expression was accompanied by a reduction of GRK2-A_{2B}AR association in
383 response to agonist stimulation. Globally, these events caused an impairment of receptor
384 desensitisation producing a time-prolonged accumulation of intracellular cAMP upon A_{2B}AR
385 stimulation (Fig. 7). In this scenario, it is likely to suggest that pro-inflammatory soluble factors
386 may regulate the commitment of MSC towards osteoblasts by modulating the activity of
387 desensitising proteins and consequently increasing Gs-coupled receptors activity.
388 The unequivocal involvement of GRK2 in TNF-α-mediated regulation of A_{2B}AR was demonstrated
389 by using a specific GRK2 inhibitor and by overexpressing GRK2 in MSCs. The chemical inhibition
390 of GRK2 by the synthetic peptide KRX29 enhanced the osteogenic effects evoked by the A_{2B}AR
391 agonist, similarly to the TNF-α evoked effect. Otherwise, the over-expression of the GRK2 isoform

in MSCs almost completely overcame the inhibitory effect of TNF-α on A_{2B}AR desensitisation, thus confirming GRK2 as a target for the cytokine. Because GRK2 overexpression did not show a complete reversal of A_{2B} AR desensitisation kinetics, additional factors, such as TNF-α–induced A_{2B} AR up-regulation or the involvement of other GRK subtypes, could be speculated.

These data indicate that the release of cytokines in inflammatory environment may dictate MSC differentiation, and may represent a useful target to enhance bone formation, favouring the response evoked by different Gs coupled receptors endowed with anabolic properties on bone.

FUNDING INFORMATION

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Conflict of interest

The authors declare no conflict of interest.

406 **Figure legends**

407 **Fig. 1.** Effect of TNF-α on MSCs and osteoblast viability and differentiation. MSCs were cultured
408 in proliferation medium for 72h (A) or in osteogenic medium for 5 (B) days in the presence of TNF-
409 α (0.1 ng/ml – 10 ng/ml). After treatments, cell viability was detected using the MTS assay. The
410 data were expressed as percentage of cell viability with respect to the untreated cells (control, OM),
411 which was set to 100%, and they were presented as the mean values ± SEM of three independent
412 experiments, each performed in triplicate. C, D) MSCs were cultured in osteogenic medium for
413 different time (0-15 days), in the absence (control, OM) or in the presence of 1 ng/ml TNF-α. At
414 each time, mRNA expression levels of transcription factors Runx2 (C) and ALP (D) were
415 quantified by real time RT-PCR. The data were expressed as fold of changes with respect to basal
416 value, set to 1 (mean values ± SEM, N=3). ***P < 0.001 vs. basal; ##P < 0.01 vs. untreated cells. E,
417 F) MSC mineralization were evaluated after 15 days of differentiation in the absence (control) or
418 presence of TNF-α (1 ng/ml). After treatments, cells were stained with alizarin red S, representative
419 images were taken (E) and absorbance was counted using a plate reader (530 nm) (F). The data
420 were expressed as percentage of MSCs mineralization with respect to the untreated cells (control),
421 which was set to 100% (mean values ± SEM, N=3). *P < 0.05 vs. control.

422

423 **Fig.2.** TNF-α modulation of A_{2B}AR-mediated MSC mineralization. MSCs were cultured in
424 osteogenic medium for different time (0-15 days), in the absence (control) or in the presence of 5
425 nM BAY60-6583 or 1 ng/ml TNF-α, alone or in combination. At each time, mRNA expression
426 levels of transcription factors Runx2 (A) and ALP (B) were quantified by real time RT-PCR. The
427 data were expressed as fold of changes with respect to basal value (set to 1) and they were presented
428 as the mean values ± SEM of three independent experiments. The significance of the differences
429 was determined by one-way ANOVA, followed by Bonferroni's post hoc test. ***P < 0.001 vs.

basal; ##P < 0.01, ###P < 0.001 vs. untreated cells; §P < 0.05 vs. BAY60-6583 alone. The MSCs mineralization were evaluated after 15 (B) or 21 (C) days of differentiation in the absence (control, OM) or presence of BAY60-6583 (5 nM), TNF-α (1 ng/ml), MRS1706 (1 μM) and KRX 29 (1 μM), alone or in combination. After treatments, cells were stained with alizarin red S and absorbance was counted using a plate reader (530 nm). The data were expressed as percentage of MSC mineralization with respect to the untreated cells (control), which was set to 100% (mean values ± SEM, N=3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. control; #P < 0.05, ##P < 0.01, ###P < 0.001.

Fig. 3. Effect of TNF-α on A_{2B}AR and GRK2 expression. A-D) MSCs were cultured in osteogenic medium for 48 h (A, C, D) or for 5 days (B, C, D) in the absence (control) or in the presence of TNF-α (1 ng/ml). A-C) At the end of the treatment cells were lysates and the A_{2B}AR and GRK2 protein (A-C) or mRNA (D) levels were evaluated using western blot or real time RT-PCR analyses, respectively. A, B) Representative western blots; GAPDH was the loading control. C) Densitometric analysis of the immunoreactive bands, performed using the ImageJ program. The data were expressed as OD percentage with respect to the untreated cells (control, set to 100%), and are as the mean values ± SEM (N=3). D) Real time PCR data, expressed as fold of changes with respect to control value, set to 1 (mean values ± SEM, N=3). E, F) MSCs were pre-treated with saline or 10 μM MG-132, and then incubated in osteogenic medium for 48 h in the absence (control) or in the presence of TNF-α (1 ng/ml). At the end of the treatment, GRK2 protein levels were evaluated using western blot analysis as reported in panels A-C. G) MSCs were treated as in A. At the end of the treatment period, the interaction between Mdm2 and GRK2 was quantified by an ELISA method as reported in the Materials and methods section. The data were expressed as percentage of GRK2 association with respect to the untreated cells (mean values ± SEM, N=3). *P<0.05, **P < 0.01, ***P < 0.001 vs. CTRL; # P<0.05 vs cells treated with TNF-α.

Fig. 4. Effect of TNF-α on A_{2B}AR functional response during MSC differentiation. MSCs were cultured in osteogenic medium in the absence or presence of 1 ng/ml TNF-α for 0 (A), 5 (B) or 15 (C) days. At each time point, MSCs were incubated with 5 nM BAY60-6583 for different times (5–120 min). After extensive washing, cells were treated for 15 min with 5 nM BAY60-6583. Intracellular cAMP levels were evaluated as reported in the Materials and methods section. The data were expressed as cAMP percentage with respect to the untreated cells (basal), which was set to 100% (mean values ± SEM, N=3). **P < 0.01, ***P < 0.001 vs. respective BAY60-65383; #P < 0.05, ##P < 0.01 vs. – TNF-α. D) MSCs were differentiated for 0, 5, or 15 days in the absence or in the presence of TNF-α (1 ng/mL), and then stimulated for 5 min with 5 nM BAY60-6583. At the end of the treatment period, cells were collected, and the interaction between A_{2B}AR and GRK2 was quantified by an ELISA method as reported in the Materials and methods section. The data were expressed as percentage of GRK2 association with respect to the untreated cells (basal), which was set to 100% (mean values ± SEM, N=3). *P < 0.05, ***P < 0.001 vs. basal; #P < 0.05, ###P < 0.001 vs. respective control.

Fig. 5. Effect of the GRK2 inhibitor on A_{2B}AR functional response during MSC differentiation. MSCs, cultured in osteogenic medium for 0 (A), 5 (B) or 15 (C) days, were treated with 5 nM BAY60-6583 for different times (5–120 min) in the absence or presence of 1 μM KRX 29. After extensive washing, cells were treated for 15 min with 5 nM BAY60-6583. Intracellular cAMP levels were evaluated as reported in the Materials and methods section. The data were expressed as cAMP percentage with respect to the untreated cells (basal), which was set to 100% (mean values ± SEM, N=3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. respective BAY60-65383; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. – KRX 29.

Fig. 6. Effect of GRK2 overexpression on A_{2B}AR functional response during MSC differentiation. A,B) MSCs were cultured in osteogenic medium and then transfected with a GRK2 plasmid as reported in the Materials and methods section. At the end of the treatment period, the cells were lysates and the GRK2 protein levels was evaluated using western blot analysis. The GAPDH were used as the loading control. A) Representative western blots. B) Densitometric analysis of the immunoreactive bands performed using the ImageJ program. The data were expressed as OD percentage with respect to the untreated cells (empty vector), which was set to 100% (mean values ± SEM, N=3). ***P < 0.001 vs. CTRL. C,D) MSCs were cultured in osteogenic medium in the absence (C) or presence (D) of TNF-α and then transfected with a GRK2 plasmid as reported in the Materials and methods section. The 5th day of differentiation, cells were incubated with 5 nM BAY60-6583 for different times (5–120 min) in the absence or presence of 1 ng/ml TNF-α. After extensive washing, cells were treated for 15 min with 5 nM BAY60-6583. Intracellular cAMP levels were evaluated as reported in the Materials and methods section. The data were expressed as cAMP percentage with respect to the untreated cells (basal), which was set to 100% (mean values ± SEM, N=3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. respective BAY60-65383; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. – TNF-α.

Fig. 7. A cartoon illustrating the modulatory effect of TNF-α on A_{2B}AR responses in MSCs. (A) Agonist-mediated A_{2B}AR stimulation induces GRK2 recruitment, with consequent receptor phosphorylation and reduction of the functional responsiveness. (B) TNF-α reduces GRK2 levels and its association to A_{2B}AR, by the recruitment of the Mdm2/ubiqutin proteasome pathway. The GPCR desensitisation process was impaired in the presence of the cytokine, thus enhancing the pro-differentiating effects elicited by an A_{2B}AR stimulation.

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Table 1. Human primers used for real time RT-PCR

Gene	Primer nucleotide sequences	Product size (base pairs)
Runx2	FOR: 5'-GGCCCTGGTGTTTAAATGGT -3'	178
	REV: 5'-AGGCTGTTTGACGCCATAGT-3'	
ALP	FOR: 5'-CTGCAAGGACATCGCCTATC-3'	101
	REV: 5'-CATCAGTTCTGTTCTTGGGGTA-3'	
$A_{2B}AR$	FOR: 5'- TCCATCTTCAGCCTTCTGGC -3'	128
	REV: 5'- AAAGGCAAGGACCCAGAGGA -3'	
β-actin	FOR: 5'-GCACTCTTCCAGCCTTCCTTCC-3'	254
	REV-5'-GAGCCGCCGATCCACACG-3'	

Figure 1

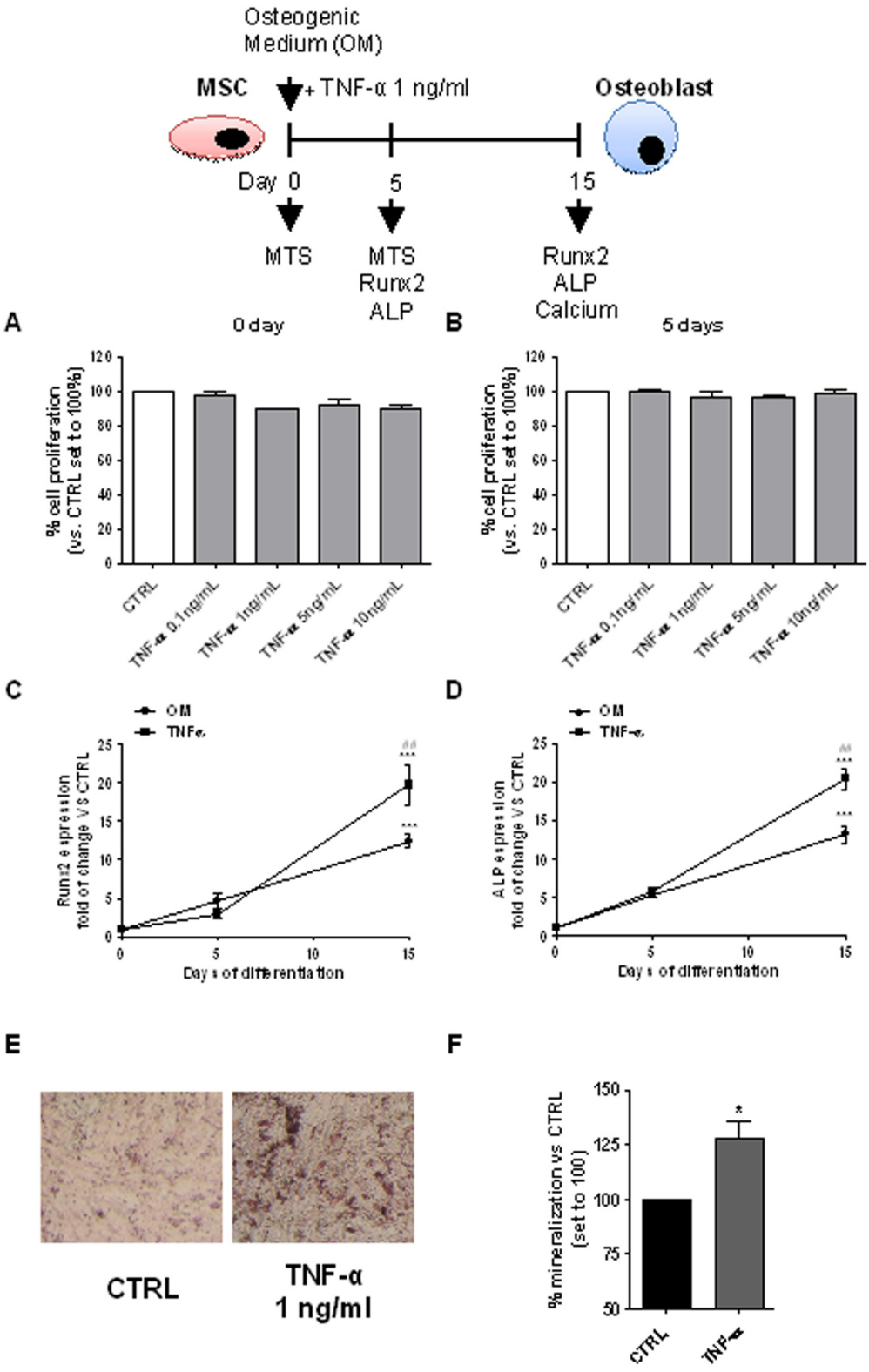

Fig. 1. Effect of TNF-α on MSCs and osteoblast viability and differentiation. MSCs were cultured in proliferation medium for 72h (A) or in osteogenic medium for 5 (B) days in the presence of TNF-α (0.1 ng/ml – 10 ng/ml). After treatments, cell viability was detected using the MTS assay. The data were expressed as percentage of cell viability with respect to the untreated cells (control, OM), which was set to 100%, and they were presented as the mean values ± SEM of three independent experiments, each performed in triplicate. C, D) MSCs were cultured in osteogenic medium for different time (0-15 days), in the absence (control, OM) or in the presence of 1 ng/ml TNF-α. At each time, mRNA expression levels of transcription factors Runx2 (C) and ALP (D) were quantified by real time RT-PCR. The data were expressed as fold of changes with respect to basal value, set to 1 (mean values ± SEM, N=3). ***P < 0.001 vs. basal; ##P < 0.01 vs. untreated cells. E, F) MSC mineralization were evaluated after 15 days of differentiation in the absence (control) or presence of TNF-α (1 ng/ml). After treatments, cells were stained with alizarin red S, representative images were taken (E) and absorbance was counted using a plate reader (530 nm) (F). The data were expressed as percentage of MSCs mineralization with respect to the untreated cells (control), which was set to 100% (mean values ± SEM, N=3). *P < 0.05 vs. control.

Figure 2

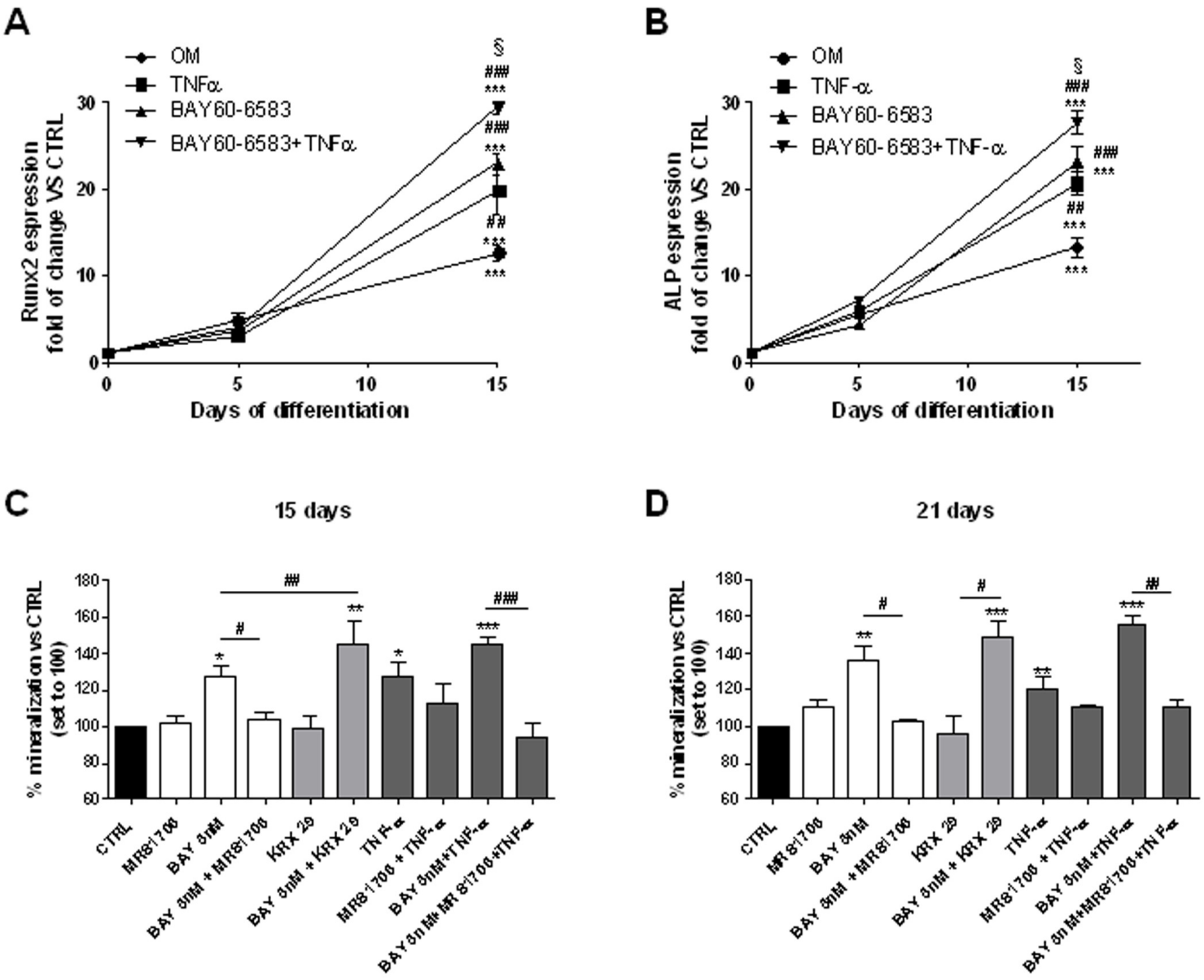

Fig.2. TNF-α modulation of A_{2B}AR-mediated MSC mineralization. MSCs were cultured in osteogenic medium for different time (0-15 days), in the absence (control) or in the presence of 5 nM BAY60-6583 or 1 ng/ml TNF-α, alone or in combination. At each time, mRNA expression levels of transcription factors Runx2 (A) and ALP (B) were quantified by real time RT-PCR. The data were expressed as fold of changes with respect to basal value (set to 1) and they were presented as the mean values ± SEM of three independent experiments. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's post hoc test. ***P < 0.001 vs. basal; ##P < 0.01, ###P < 0.001 vs. untreated cells; §P < 0.05 vs. BAY60-6583 alone. The MSCs mineralization were evaluated after 15 (B) or 21 (C) days of differentiation in the absence (control, OM) or presence of BAY60-6583 (5 nM), TNF-α (1 ng/ml), MRS1706 (1 μM) and KRX 29 (1 μM), alone or in combination. After treatments, cells were stained with alizarin red S and absorbance was counted using a plate reader (530 nm). The data were expressed as percentage of MSC mineralization with respect to the untreated cells (control), which was set to 100% (mean values ± SEM, N=3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. control; #P < 0.05, ##P < 0.01, ###P < 0.001.

Figure 3

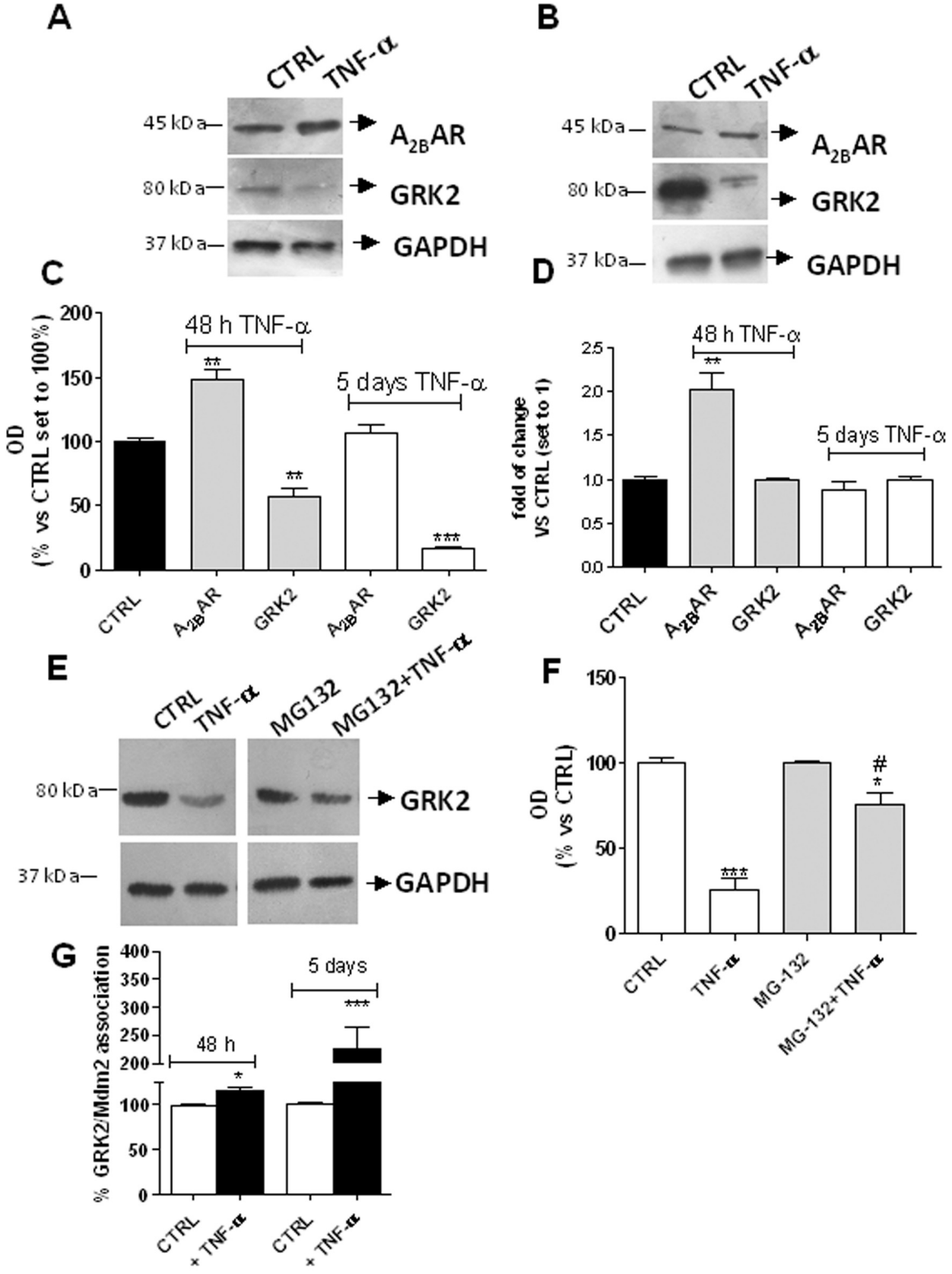

Fig. 3. Effect of TNF-α on A_{2B}AR and GRK2 expression. A-D) MSCs were cultured in osteogenic medium for 48 h (A, C, D) or for 5 days (B, C, D) in the absence (control) or in the presence of TNF-α (1 ng/ml). A-C) At the end of the treatment cells were lysates and the A_{2B}AR and GRK2 protein (A-C) or mRNA (D) levels were evaluated using western blot or real time RT-PCR analyses, respectively. A, B) Representative western blots; GAPDH was the loading control. C) Densitometric analysis of the immunoreactive bands, performed using the ImageJ program. The data were expressed as OD percentage with respect to the untreated cells (control, set to 100%), and are as the mean values ± SEM (N=3). D) Real time PCR data, expressed as fold of changes with respect to control value, set to 1 (mean values ± SEM, N=3). E, F) MSCs were pre-treated with saline or 10 μM MG-132, and then incubated in osteogenic medium for 48 h in the absence (control) or in the presence of TNF-α (1 ng/ml). At the end of the treatment, GRK2 protein levels were evaluated using western blot analysis as reported in panels A-C. G) MSCs were treated as in A. At the end of the treatment period, the interaction between Mdm2 and GRK2 was quantified by an ELISA method as reported in the Materials and methods section. The data were expressed as percentage of GRK2 association with respect to the untreated cells (mean values ± SEM, N=3). *P<0.05, **P < 0.01, ***P < 0.001 vs. CTRL; # P<0.05 vs cells treated with TNF-α.

Figure 4

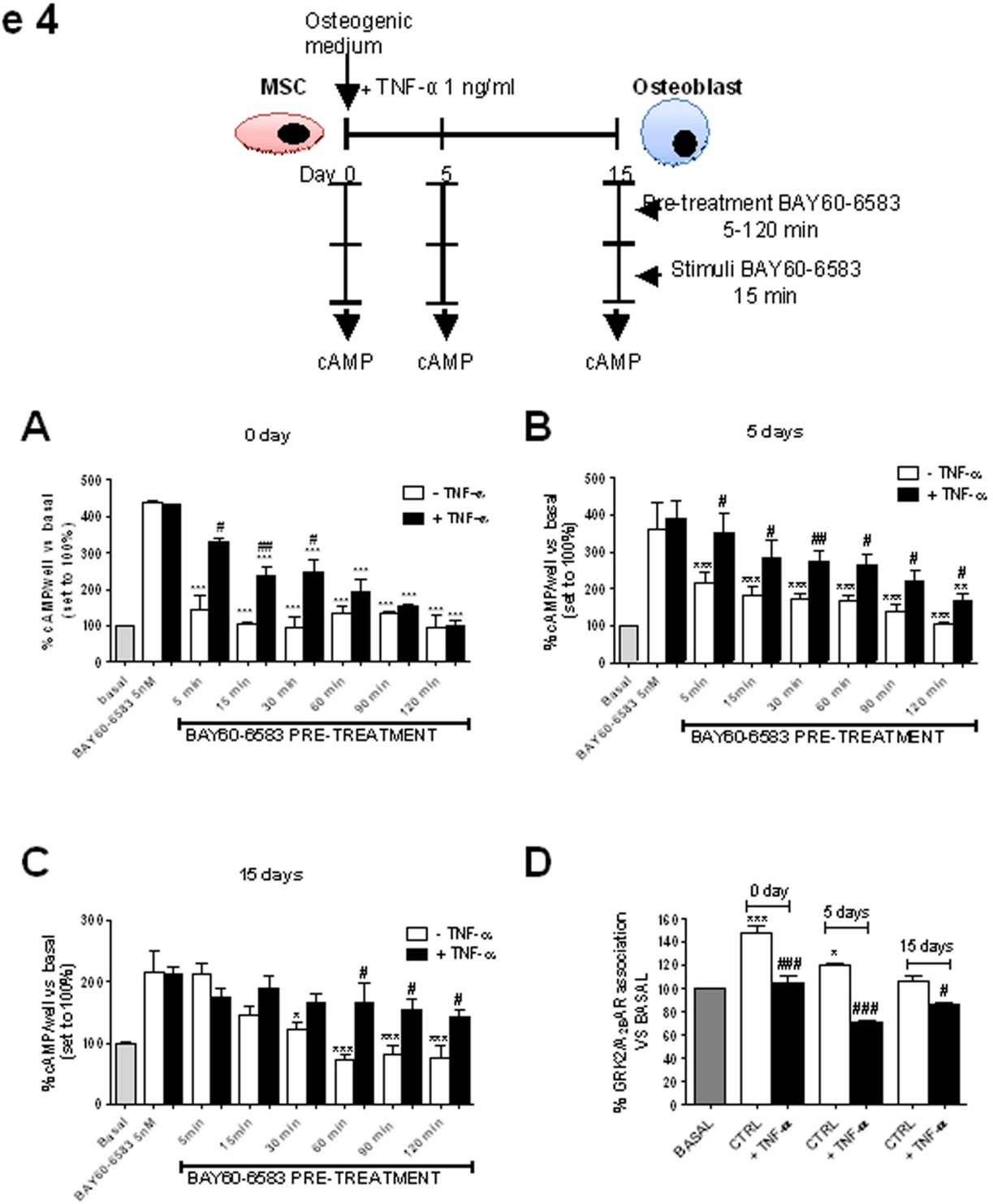

Fig. 4. Effect of TNF-α on A_{2B}AR functional response during MSC differentiation. MSCs were cultured in osteogenic medium in the absence or presence of 1 ng/ml TNF-α for 0 (A), 5 (B) or 15 (C) days. At each time point, MSCs were incubated with 5 nM BAY60-6583 for different times (5–120 min). After extensive washing, cells were treated for 15 min with 5 nM BAY60-6583. Intracellular cAMP levels were evaluated as reported in the Materials and methods section. The data were expressed as cAMP percentage with respect to the untreated cells (basal), which was set to 100% (mean values ± SEM, N=3). **P < 0.01, ***P < 0.001 vs. respective BAY60-65383; #P < 0.05, ##P < 0.01 vs. – TNF-α. D) MSCs were differentiated for 0, 5, or 15 days in the absence or in the presence of TNF-α (1 ng/mL), and then stimulated for 5 min with 5 nM BAY60-6583. At the end of the treatment period, cells were collected, and the interaction between A2BAR and GRK2 was quantified by an ELISA method as reported in the Materials and methods section. The data were expressed as percentage of GRK2 association with respect to the untreated cells (basal), which was set to 100% (mean values ± SEM, N=3). *P < 0.05, ***P < 0.001 vs. basal; #P < 0.05, ###P < 0.001 vs. respective control.

Figure 5

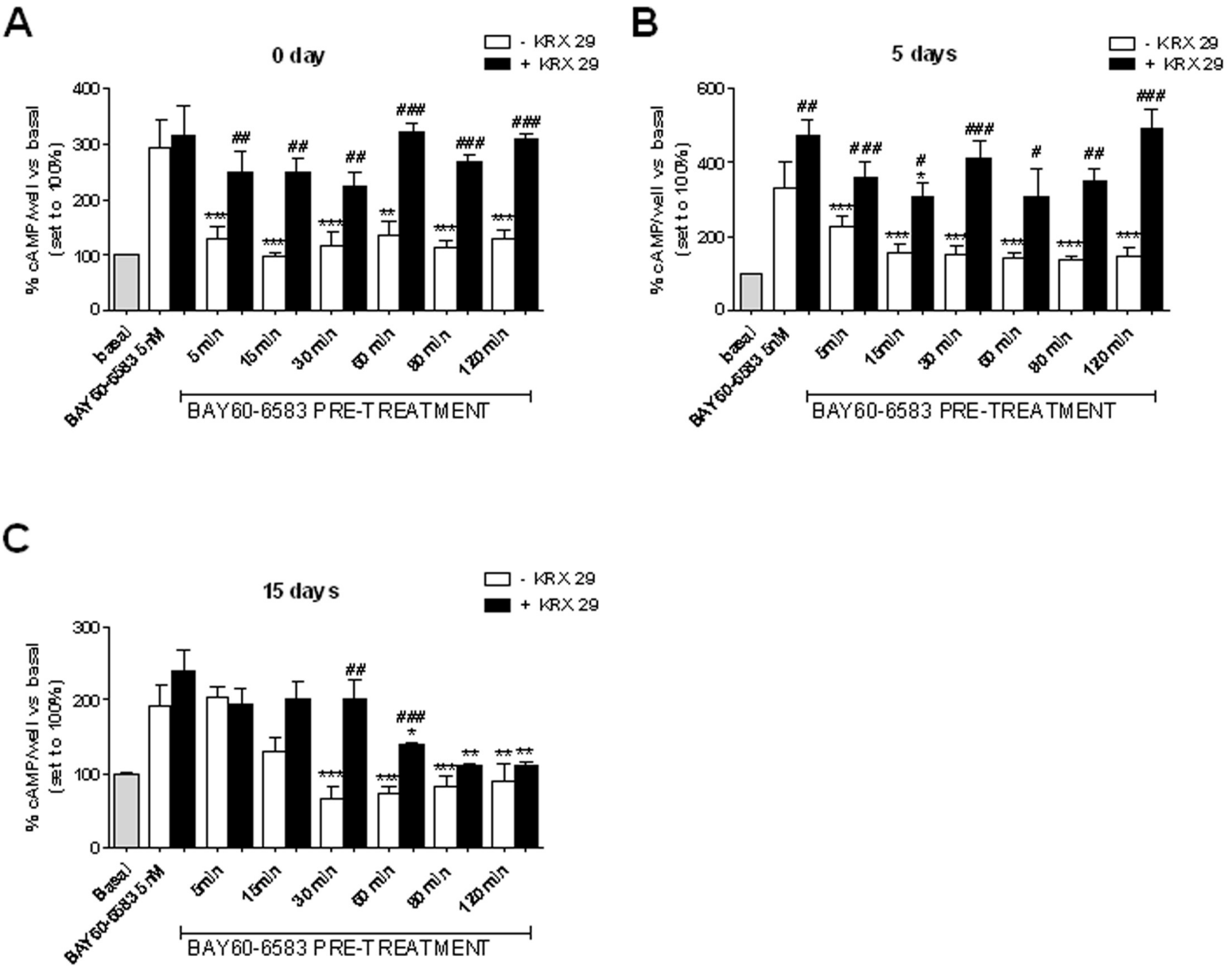

Fig. 5. Effect of the GRK2 inhibitor on A_{2B}AR functional response during MSC differentiation. MSCs, cultured in osteogenic medium for 0 (A), 5 (B) or 15 (C) days, were treated with 5 nM BAY60-6583 for different times (5–120 min) in the absence or presence of 1 μM KRX 29. After extensive washing, cells were treated for 15 min with 5 nM BAY60-6583. Intracellular cAMP levels were evaluated as reported in the Materials and methods section. The data were expressed as cAMP percentage with respect to the untreated cells (basal), which was set to 100% (mean values ± SEM, N=3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. respective BAY60-65383; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. – KRX 29.

Figure 6

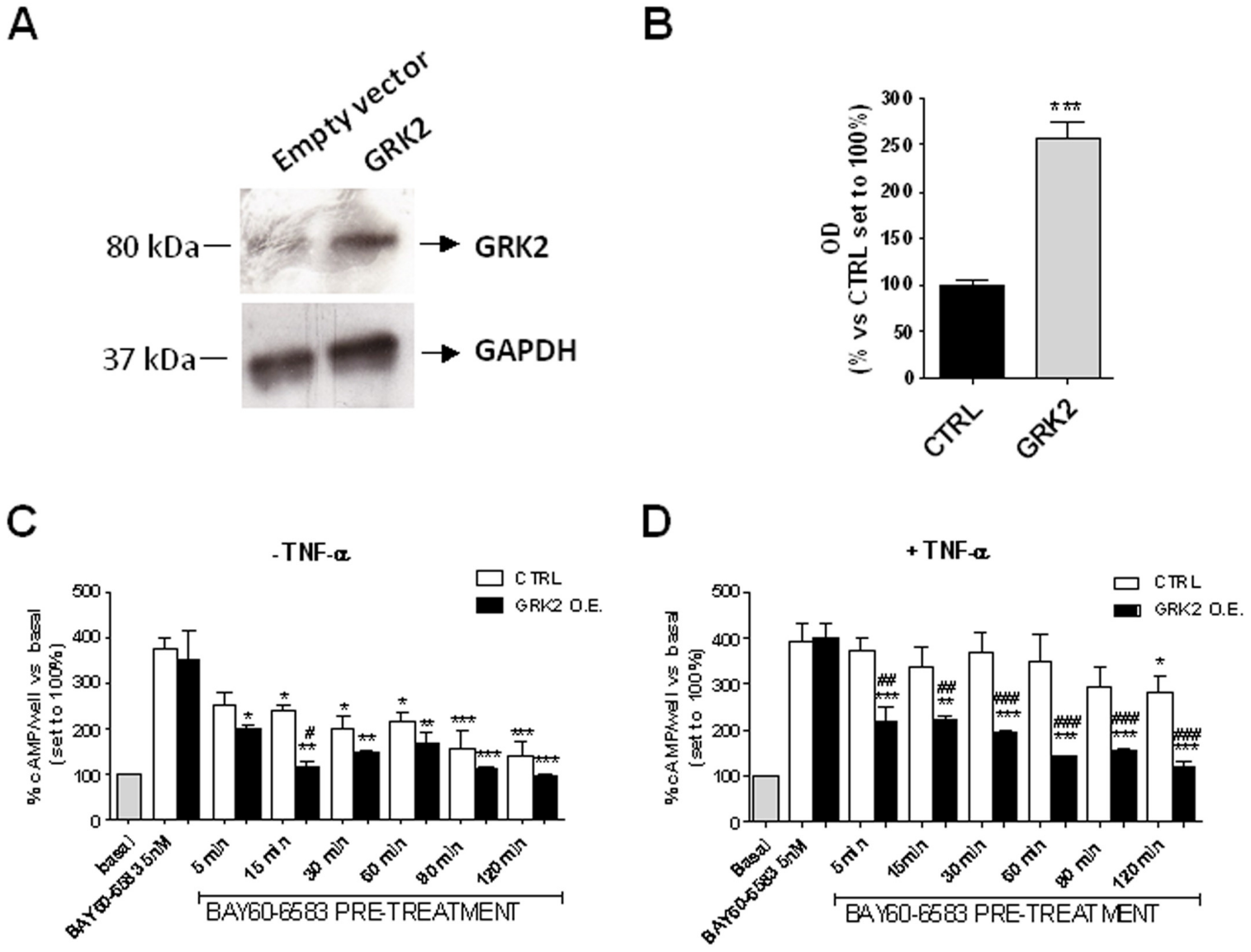

Fig. 6. Effect of GRK2 overexpression on A_{2B}AR functional response during MSC differentiation. A,B) MSCs were cultured in osteogenic medium and then transfected with a GRK2 plasmid as reported in the Materials and methods section. At the end of the treatment period, the cells were lysates and the GRK2 protein levels was evaluated using western blot analysis. The GAPDH were used as the loading control. A) Representative western blots. B) Densitometric analysis of the immunoreactive bands performed using the ImageJ program. The data were expressed as OD percentage with respect to the untreated cells (empty vector), which was set to 100% (mean values ± SEM, N=3). ***P < 0.001 vs. CTRL. C,D) MSCs were cultured in osteogenic medium in the absence (C) or presence (D) of TNF-α and then transfected with a GRK2 plasmid as reported in the Materials and methods section. The 5th day of differentiation, cells were incubated with 5 nM BAY60-6583 for different times (5–120 min) in the absence or presence of 1 ng/ml TNF-α. After extensive washing, cells were treated for 15 min with 5 nM BAY60-6583. Intracellular cAMP levels were evaluated as reported in the Materials and methods section. The data were expressed as cAMP percentage with respect to the untreated cells (basal), which was set to 100% (mean values ± SEM, N=3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. respective BAY60-65383; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. – TNF-α.

Figure 7

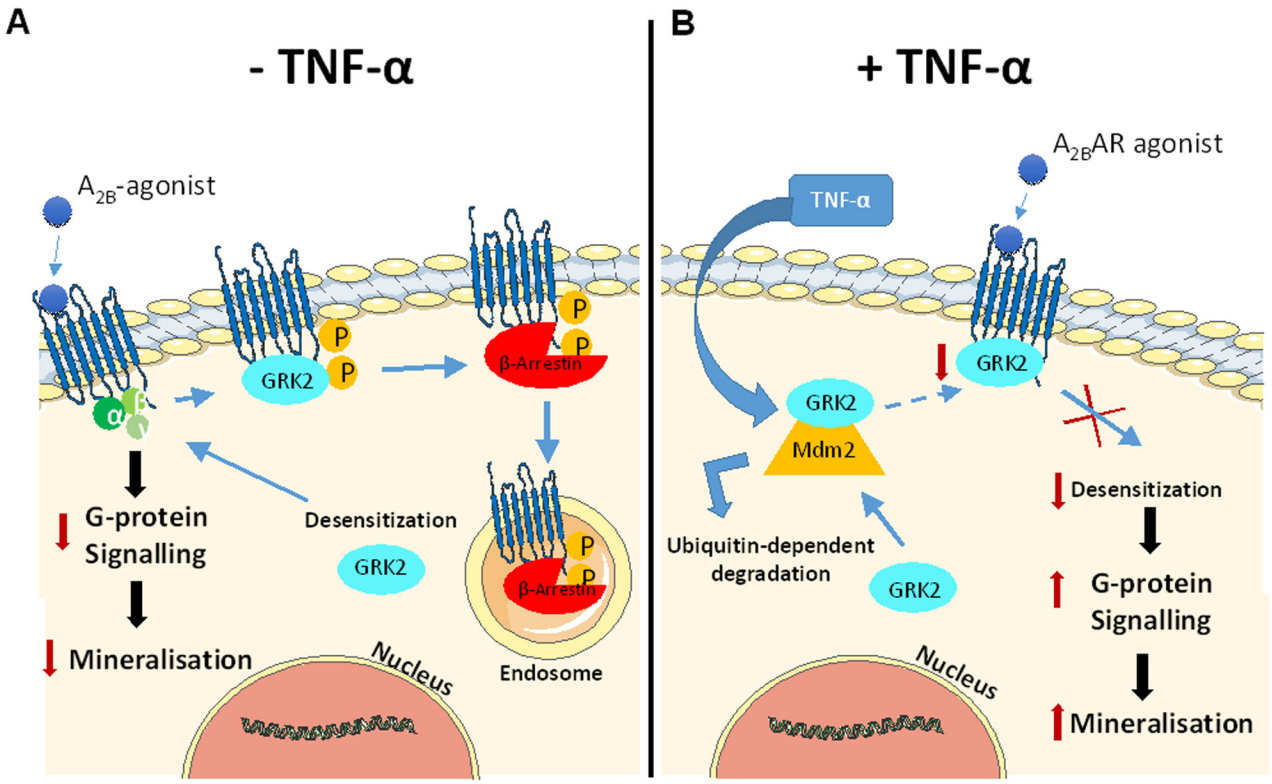

Fig. 7. A cartoon illustrating the modulatory effect of TNF-α on A_{2B}AR responses in MSCs. (A) Agonist-mediated A_{2B}AR stimulation induces GRK2 recruitment, with consequent receptor phosphorylation and reduction of the functional responsiveness. (B) TNF-α reduces GRK2 levels and its association to A_{2B}AR, by the recruitment of the Mdm2/ubiqutin proteasome pathway. The GPCR desensitisation process was impaired in the presence of the cytokine, thus enhancing the pro-differentiating effects elicited by an A_{2B}AR stimulation.