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RESEARCH ARTICLE

# Osteogenesis Is Improved by Low Tumor Necrosis Factor Alpha Concentration through the Modulation of Gs-Coupled Receptor Signals

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

Department of Pharmacy, University of Pisa, Pisa, Italy[a]; Department of Pharmacy, University of Naples Federico II, Naples, Italy[b]

**ABSTRACT** In the early phase of bone damage, low concentrations of the cytokine tumor necrosis factor alpha (TNF-$\alpha$) favor osteoblast differentiation. In contrast, chronic high doses of the same cytokine contribute to bone loss, demonstrating opposite effects depending on its concentration and on the time of exposure. In the bone microenvironment, TNF-$\alpha$ 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. Here, the effects of TNF-$\alpha$ 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-$\alpha$ concentrations exerted a prodifferentiating effect on MSCs, pushing them toward an osteoblast phenotype. By regulating GRK2 turnover and expression, the cytokine impaired $A_{2B}$AR desensitization, accelerating receptor-mediated osteoblast differentiation. These data supported the anabolic effect of TNF-$\alpha$ submaximal concentration and demonstrated that the cytokine regulates GPCR responses by interfering with the receptor desensitization machinery, thereby enhancing the anabolic responses evoked by $A_{2B}$AR ligands. Overall, these results indicated that GPCR desensitization plays a pivotal role in osteogenesis and that its manipulation is an effective strategy to favor bone remodeling.

**KEYWORDS** A2B adenosine receptor, G protein-coupled receptor kinases, tumor necrosis factor alpha, mesenchymal stem cells, osteoblasts, proteasome

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Address correspondence to Claudia Martini, claudia.martini@unipi.it.

S.D. and L.N. contributed equally to this work.

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 favor osteogenic reparative processes (3–5). Among the inflammatory players, tumor necrosis factor alpha (TNF-$\alpha$) assumes an important role in bone healing, affecting mesenchymal stem cell (MSC) behavior in a dose- and time-dependent manner. In the early inflammatory phase, immediately after fracture, TNF-$\alpha$ is released at low levels and favors 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 remodeling and responsible for the paradoxical effects of TNF-$\alpha$ remain unclear (7–9). For that matter, it is uncertain whether or not TNF-$\alpha$ 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 prostaglandin receptors (EP2 and EP4), P1 and P2Y purinergic receptors, and many others, are directly involved in bone remodeling (10, 11). Clinical trials with PTH R-targeted molecules demonstrated that receptor desensitization, 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 pieces of evidence, the investigation of the molecular mechanisms underlying desensitization processes of GPCRs may be crucial to understanding the final biological outcome of these receptors to controlling bone remodeling 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 is 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 proinflammatory cytokines in both chronic and acute inflammation (20, 21). In particular, TNF-$\alpha$ has been shown to modulate the $A_2$AR desensitization 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 desensitization 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). It is worth noting that the activity of GRKs is regulated by the inflammatory microenvironment. Several studies have demonstrated that TNF-$\alpha$ decreases the cellular levels of GRK2 in *in vitro* and *in vivo* models of inflammatory diseases and may contribute to the regulation of the desensitization processes of different GPCRs, thereby prolonging agonist-mediated receptor activation over time (26–28).

To date, no data are available on the regulation of $A_{2B}$AR desensitization in bone under inflammatory conditions. Based on this evidence, the aim of this work was to investigate the effect of TNF-$\alpha$ on the kinetics of $A_{2B}$AR desensitization (taken as a model of Gs protein-coupled receptors) in the regulation of osteoblast differentiation from MSCs. We demonstrated that a low TNF-$\alpha$ concentration, mimicking a submaximal inflammatory state, induced a significant inhibition of GRK2 activity, thereby impairing agonist-induced $A_{2B}$AR desensitization. Therefore, the inflammatory cytokine increased the osteogenic effects elicited by the purinergic system, contributing to osteoblast differentiation. These data indicate that local inflammatory environment manipulation is a simple and effective way to enhance bone formation and accelerate fracture repair.

## RESULTS

**MSC proliferation and differentiation to osteoblasts: effects of TNF-$\alpha$.** 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 were monitored as described previously (15).

The effects of different concentrations of TNF-$\alpha$ (0.1 ng/ml to 10 ng/ml) on MSC proliferation were evaluated. TNF-$\alpha$ did not induce 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 have a toxic effect up to a 10 ng/ml concentration.

We then investigated the effects of TNF-$\alpha$ on MSC differentiation to osteoblasts. MSCs were subjected to the proinflammatory stimulus during their differentiation in osteogenic medium. The effects of TNF-$\alpha$ (1 ng/ml) on the expression of Runx2, a pivotal transcription factor that drives cells toward an osteoblast phenotype, and ALP, an early marker of osteoblast differentiation, were evaluated at different stages of differentiation. TNF-$\alpha$ induced a time-dependent increase in the expression levels of both Runx2 (19.69- $\pm$ 2.69-fold versus 12.44- $\pm$ 0.91-fold in the control after 15 days of treatment) (Fig. 1C) and ALP (20.37- $\pm$ 1.34-fold versus 13.26- $\pm$ 1.09-fold in the 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-$\alpha$ significantly increased calcium deposition, confirming it favored 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 proinflammatory cytokine contributes to the early osteogenic differentiation of MSCs, potentiating the effects of prodifferentiating factors.

**Osteoblast differentiation: interplay between TNF-$\alpha$ and $A_{2B}$AR.** It is known that proinflammatory cytokines regulate the expression and activity of several intracellular proteins; therefore, it contributes to the modulation of the responses evoked by GPCRs (22, 29). Among these, TNF-$\alpha$ 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-$\alpha$ 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 processes. 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-$\alpha$, the pro-osteogenic effects of $A_{2B}$AR agonist were significantly increased. Indeed, the expression levels of Runx2 and ALP induced by BAY60-6583 in the proinflammatory 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-$\alpha$ and BAY60-6583 were used in combination. The percentage of calcium deposit induced by the $A_{2B}$AR stimulation in the presence of cytokine was 144.6% $\pm$ 5.1% and 155.3% $\pm$ 4.8% after 15 and 21 days of differentiation, respectively, and it was 127.1% $\pm$ 6.0% and 136.5% $\pm$ 7.6% in the absence of TNF-$\alpha$.

The effect of TNF-$\alpha$ 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 the $A_{2B}$AR subtype. Notably, the effect of TNF-$\alpha$ alone was slightly decreased by the treatment with MRS1706, pointing out that the $A_{2B}$AR activation could be one of several mechanisms at the basis of TNF-$\alpha$ osteoblastogenesis induction.

These data, taken together, suggested that the MSC inflammatory microenvironment modulates the mineralization process, favoring the responses evoked by $A_{2B}$AR stimulation.

**Agonist-induced $A_{2B}$AR desensitization at different stages of MSC differentiation: effect of TNF-$\alpha$.** The putative molecular mechanisms involved in the effects of

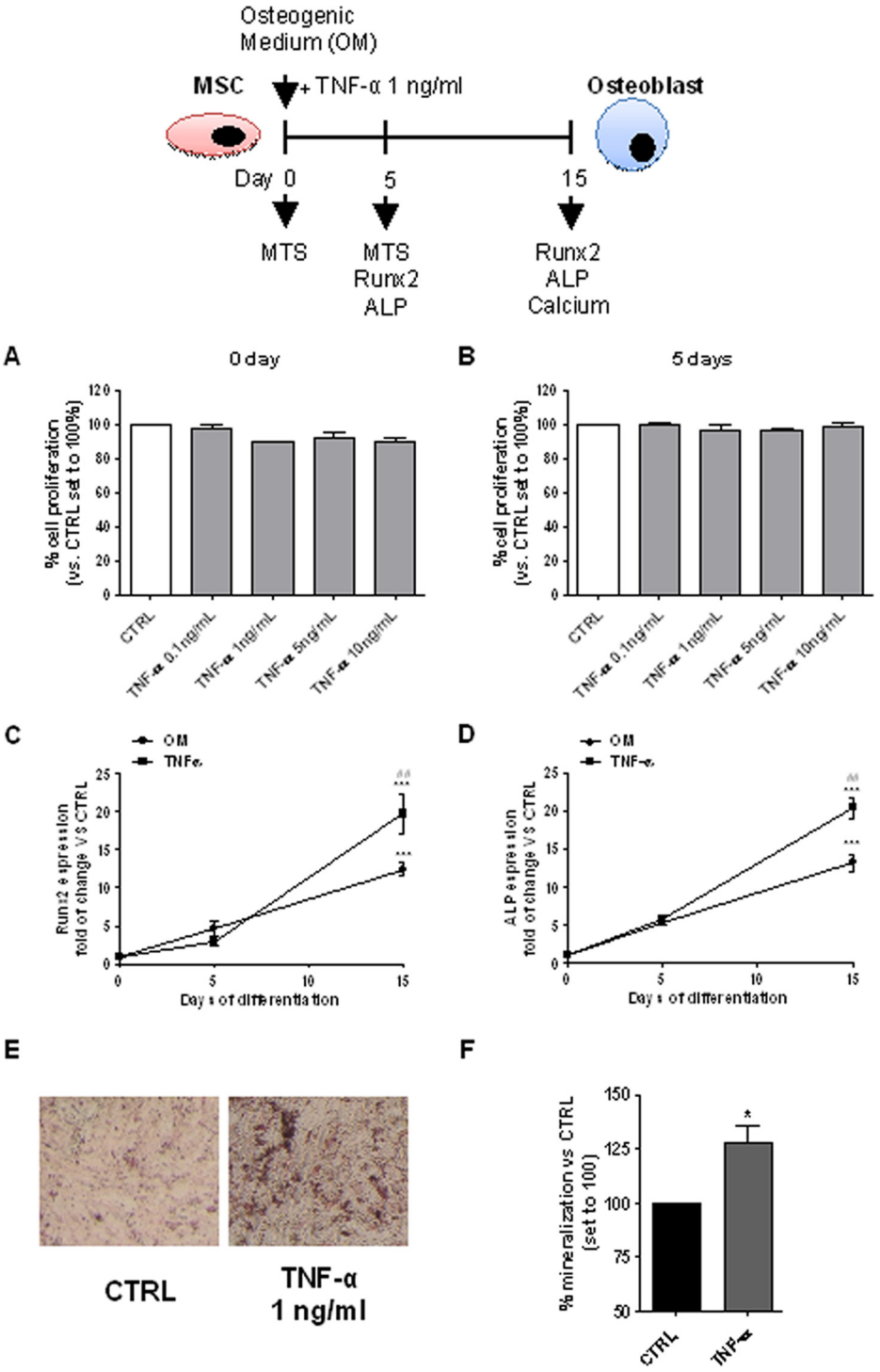


**FIG 1** Effect of TNF-$\alpha$ on MSCs and osteoblast viability and differentiation. (A and B) MSCs were cultured in proliferation medium for 72 h (A) or in osteogenic medium for 5 days (B) in the presence of TNF-$\alpha$ (0.1 ng/ml to 10 ng/ml). After treatments, cell viability was detected using the MTS assay. The data were expressed as percentages of cell viability with respect to the untreated cells (control, OM), which was set to 100%, and they were presented as the mean values $\pm$ SEM from three independent experiments, each performed in triplicate. (C and D) MSCs were cultured in osteogenic medium for different times (0 to 15 days), in the absence (control, OM) or in the presence of 1 ng/ml TNF-$\alpha$. 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 changes with respect to the basal value, which was set to 1 (mean values $\pm$ SEM; $n = 3$). ***, $P < 0.001$ versus basal; ##, $P < 0.01$ versus untreated cells. (E and F) MSC mineralization was evaluated after 15 days of differentiation in the absence (control) or presence of TNF-$\alpha$ (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 a percentage of MSC mineralization with respect to that for the untreated cells (control), which was set to 100% (mean values $\pm$ SEM; $n = 3$). *, $P < 0.05$ versus the control.

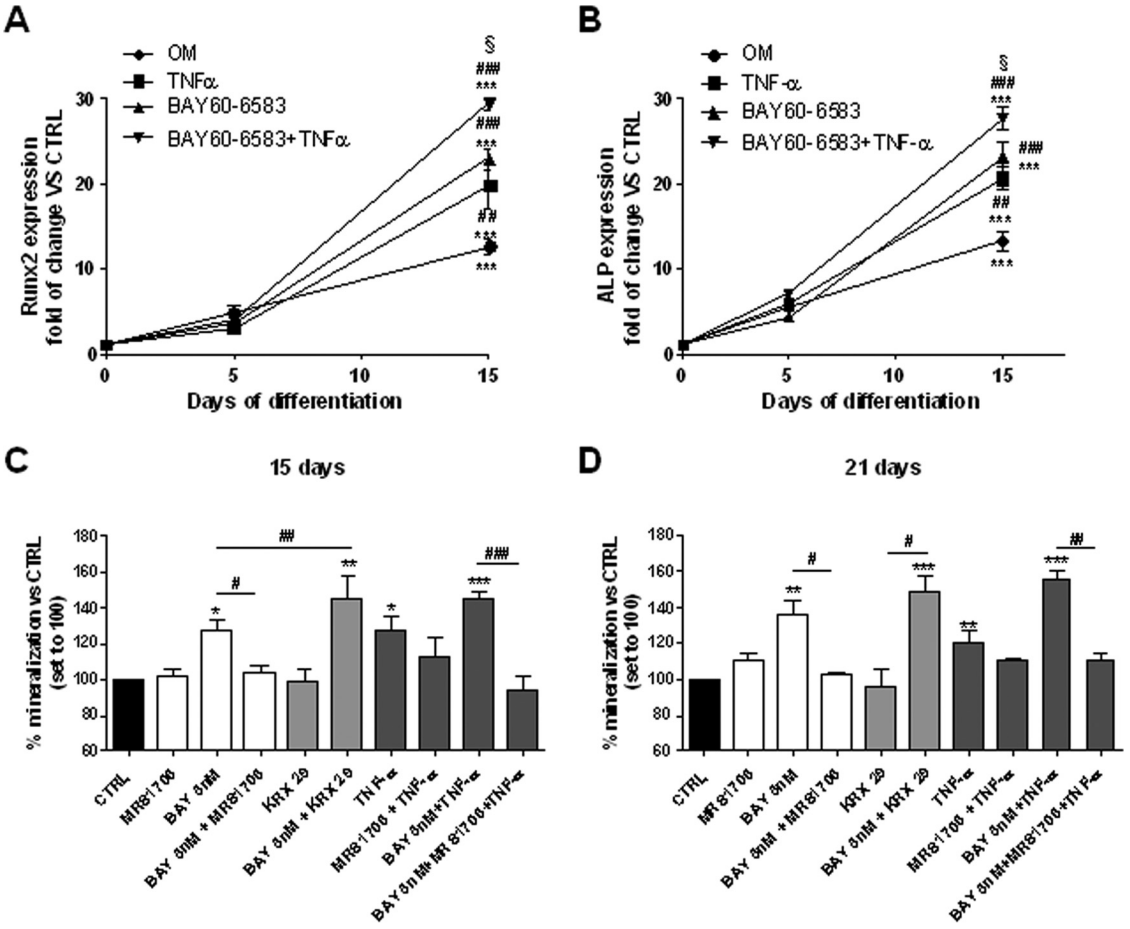


**FIG 2** TNF-$\alpha$ modulation of $A_{2B}$AR-mediated MSC mineralization. (A and B) MSCs were cultured in osteogenic medium for different times (0 to 15 days), in the absence (control) or in the presence of 5 nM BAY60-6583 or 1 ng/ml TNF-$\alpha$, alone or in combination. At each time, mRNA expression levels of transcription factors Runx2 (A) and ALP (B) were quantified by real-time RT-PCR. The data were expressed as fold changes with respect to the basal value (set to 1), and they were presented as the mean values ± SEM from three independent experiments. The significance of the differences was determined by one-way ANOVA, followed by Bonferroni's *post hoc* test. ***, $P < 0.001$ versus basal; ##, $P < 0.01$; ###, $P < 0.001$ versus untreated cells; §, $P < 0.05$ versus BAY60-6583 alone. (C and D) The MSC mineralizations were evaluated after 15 (C) or 21 (D) days of differentiation in the absence (control, OM) or presence of BAY60-6583 (5 nM), TNF-$\alpha$ (1 ng/ml), MRS1706 (1 $\mu$M), and KRX29 (1 $\mu$M), 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 a percentage of MSC mineralization with respect to that for the untreated cells (control), which was set to 100% (mean values ± SEM; $n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus the control; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$.

TNF-$\alpha$ on $A_{2B}$AR responses were investigated. First, the modulation of $A_{2B}$AR gene and protein expression evoked by the cytokine was investigated. As shown in Fig. 3, a significant upregulation of $A_{2B}$AR gene (2.03- ± 0.17-fold versus the control) and protein (147% ± 8% versus the 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 affect $A_{2B}$AR content at either gene or protein levels (Fig. 3B, C, and D).

The effects of TNF-$\alpha$ on $A_{2B}$AR functional responsiveness then were investigated. $A_{2B}$ARs are mainly coupled to Gs proteins and activated adenylyl cyclase, thereby increasing intracellular cyclic AMP (cAMP) levels. Desensitization is one of the main processes that regulate GPCR functionality. On this basis, TNF-$\alpha$ modulation on agonist-mediated $A_{2B}$AR desensitization was evaluated at different stages of MSC differentiation (0 to 5 and 15 days). For this purpose, control and TNF-$\alpha$-treated cells were preincubated with $A_{2B}$AR agonist (BAY60-6583) for different times (5 to 120 min), 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 prechallenge with the agonist BAY60-6583 (Fig. 4) (30, 31). The desensitization kinetics appeared to be faster in

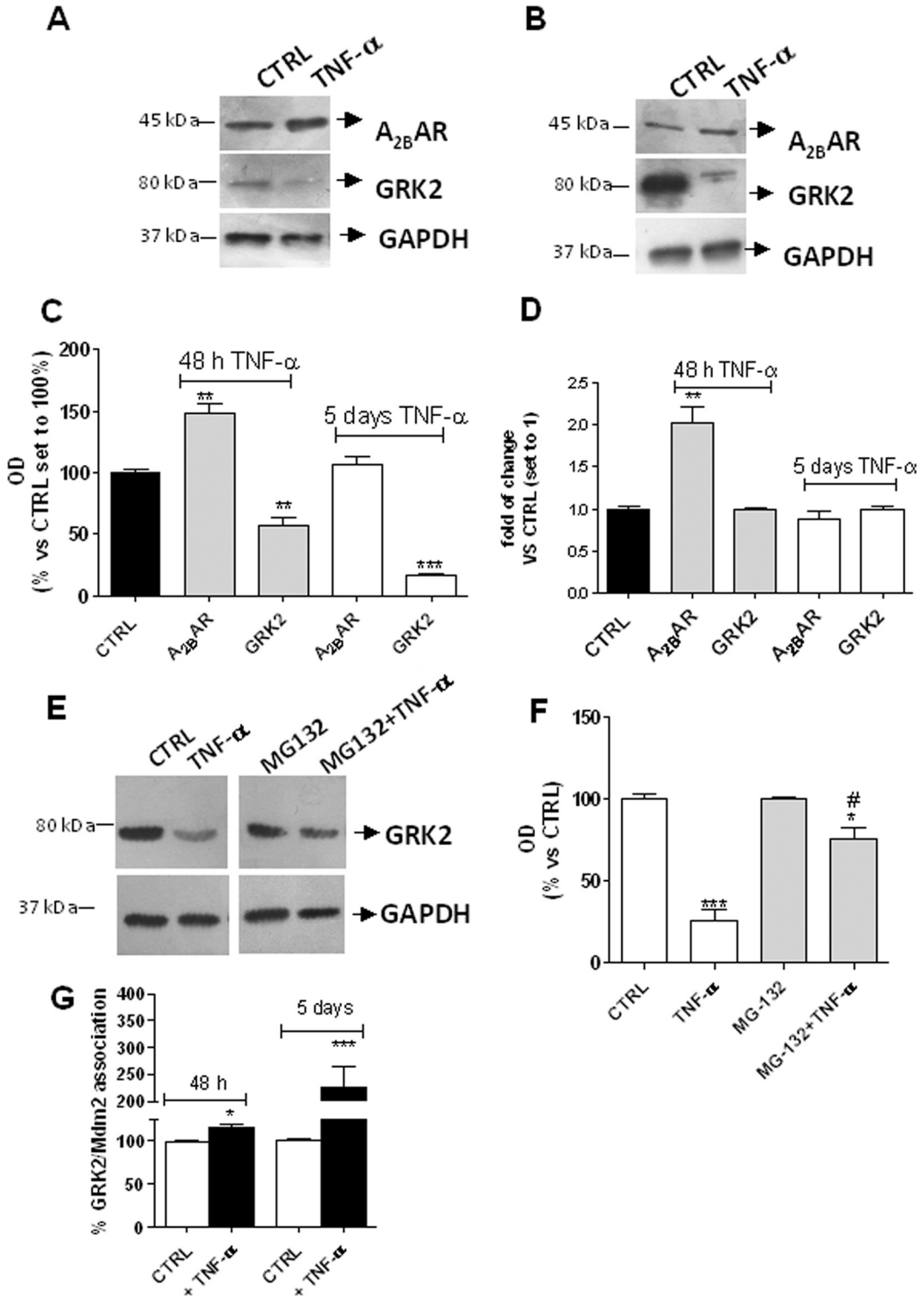


**FIG 3** Effect of TNF-$\alpha$ on $A_{2B}$AR and GRK2 expression. (A to D) MSCs were cultured in osteogenic medium for 48 h (A, C, and D) or for 5 days (B, C, and D) in the absence (control) or in the presence of TNF-$\alpha$ (1 ng/ml). (A to C) At the end of the treatment, cells were lysed and the $A_{2B}$AR and GRK2 protein (A to C) or mRNA (D) levels were evaluated using Western blotting or real-time RT-PCR analyses, respectively. (A and 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 optical density (OD) percentage with respect to the untreated cells (control; set to 100%) and are mean values $\pm$ SEM ($n$ = 3). (D) Real-time PCR data, expressed as fold changes with respect to the control value, set to 1 (mean values $\pm$ SEM; $n$ = 3). (E and F) MSCs were pretreated with saline or 10 $\mu$M MG-132 and then incubated in osteogenic medium for 48 h in the absence (control) or in the presence of TNF-$\alpha$ (1 ng/ml). At the end of the treatment, GRK2 protein levels were evaluated using Western blot analysis as reported for panels A to C. (G) MSCs were treated as described for panel A. At the end of the treatment

(Continued on next page)

undifferentiated MSCs (half-life [$t_{1/2}$], 1.46 $\pm$ 0.008 $min^{-1}$) and in the early phase of differentiation ($t_{1/2}$, 4.17 $\pm$ 0.021 $min^{-1}$) (Fig. 4A and B). In contrast, in the late phase of the differentiation program, $A_{2B}$AR functional responses remained preserved for a longer time, showing slower desensitization kinetics ($t_{1/2}$, 16.32 $\pm$ 1.12 $min^{-1}$) (Fig. 4C). These differences may be ascribed to the different expression of $A_{2B}$ARs along with MSC maturation.

TNF-$\alpha$ did not significantly alter the basal response of $A_{2B}$AR to agonist but substantially impaired the degree and the rate of receptor desensitization. These effects were already evident in undifferentiated cells ($t_{1/2}$, 17.61 $\pm$ 1.22 $min^{-1}$) and became more significant at different stages of the differentiation process (5 days, $t_{1/2}$ of 36.75 $\pm$ 2.15 $min^{-1}$; 15 days, $t_{1/2}$ of 50.77 $\pm$ 3.81 $min^{-1}$). The diverse effect of TNF-$\alpha$ 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-$\alpha$ on GRK2 expression.** GRKs, particularly the isoform 2, are the most relevant kinases involved in the phosphorylation and desensitization of GPCRs, including $A_{2B}$ARs (32, 33). To elucidate the mechanism through which TNF-$\alpha$ impaired $A_{2B}$AR functionality, we investigated the effects of the inflammatory cytokine on GRK2 expression and on its association with $A_{2B}$ARs upon agonist-mediated receptor stimulation. Notably, the cytokine caused a significant and time-dependent inhibition of GRK2 protein levels (Fig. 3A to C).

In order to dissect if a TNF-$\alpha$-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, posttranscriptional regulation of GRK2 by TNF-$\alpha$ was investigated, considering, in particular, the involvement of the proteasome pathway, which has been identified as a major mechanism for modulating GRK2 expression levels (34–36). For this purpose, MSCs were challenged with the proteasome inhibitor MG-132 (37) in the absence or presence of the inflammatory cytokine for 48 h. As depicted in Fig. 3E and F, MG-132 significantly, but not completely, prevented TNF-$\alpha$-mediated decrease of GRK2 expression.

Because several proteins are targeted to the proteasome pathway go through polyubiquitination (34, 38, 39), the potential role of ubiquitination in GRK2 degradation was examined by assessing the kinase association with the mouse double minute 2 (Mdm2) ubiquitin ligase (38, 40). The results showed that the cytokine induced a significant GRK2 association with Mdm2 following both 48 h and 5 days of treatment (Fig. 3G). Altogether, our data indicate that TNF-$\alpha$ regulation of GRK2 involves, at least partially, the ubiquitin-dependent proteasome pathway.

**Effects of TNF-$\alpha$ on GRK2 association with $A_{2B}$ARs.** The stimulation with BAY60-6583 caused a significant increase of $A_{2B}$AR-GRK2 association (148.6% $\pm$ 4.8%; *P* value of $<$0.001 versus basal expression) (Fig. 4D). Moreover, $A_{2B}$AR-GRK2 association decreased during different time points of MSC differentiation to osteoblasts. The degree of this association paralleled the rate of desensitization induced by the agonist (Fig. 4A to C). In addition, a reduction in GRK2 association with $A_{2B}$ARs was detected when the MSCs were maintained in the presence of TNF-$\alpha$, particularly in the early phase of the MSC differentiation process (Fig. 4D). These data suggest the cytokine modulates the desensitization machinery of different Gs-coupled receptors in MSCs, thereby favoring the functional activity of these receptors in response to agonist stimulation, as was demonstrated for the $A_{2B}$AR.

**Effect of GRK2 inhibition/overexpression on the kinetics of $A_{2B}$AR desensitization.** In order to corroborate the hypothesis that TNF-$\alpha$ affected the GRK2 activity

**FIG 3** Legend (Continued)
period, the interaction between Mdm2 and GRK2 was quantified by an ELISA method as reported in Materials and Methods. The data were expressed as percentages of GRK2 association with respect to the untreated cells (mean values $\pm$ SEM; $n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus the control; #, $P < 0.05$ versus cells treated with TNF-$\alpha$.

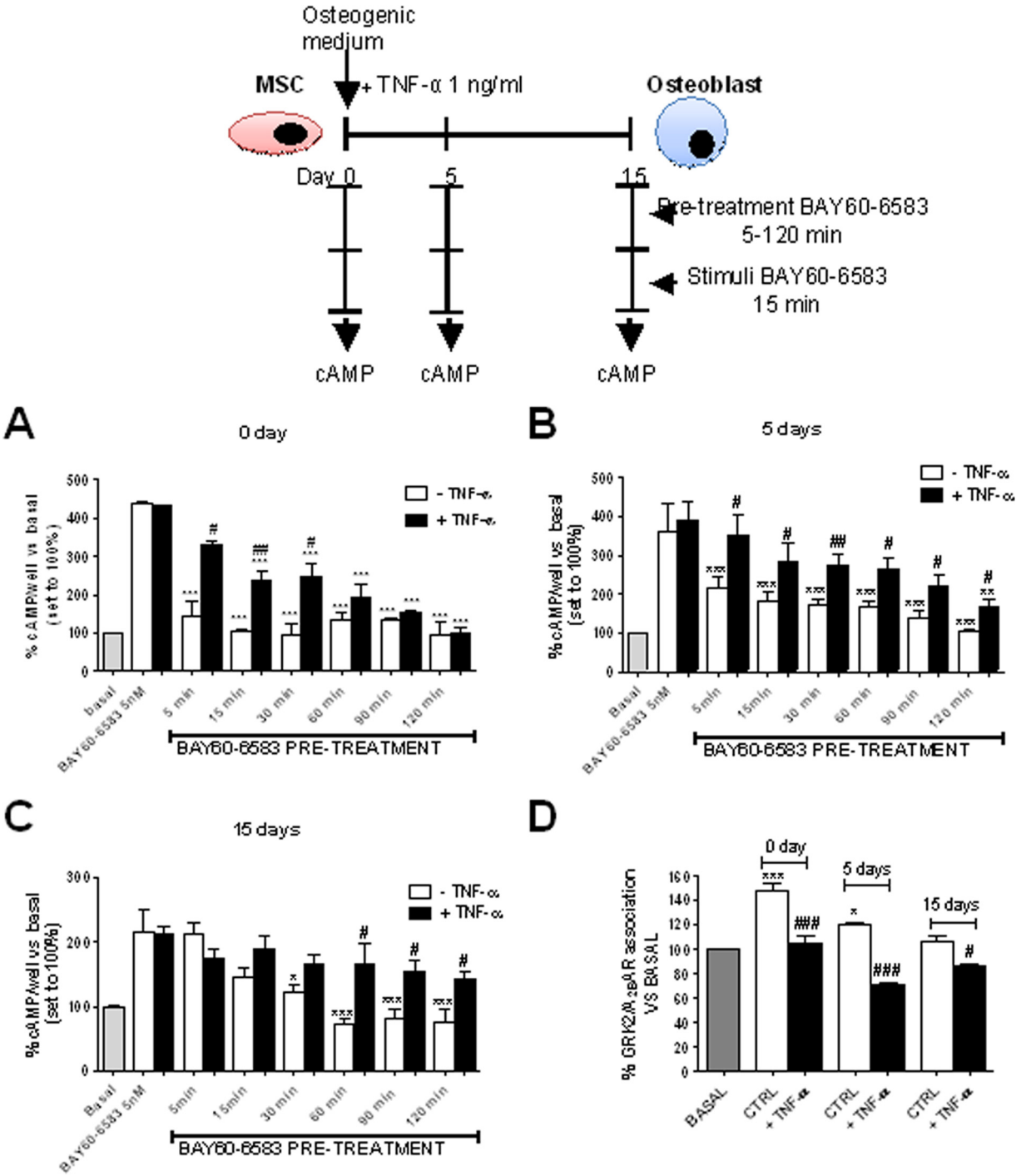


**FIG 4** Effect of TNF-$\alpha$ on $A_{2B}$AR functional response during MSC differentiation. (A to C) MSCs were cultured in osteogenic medium in the absence or presence of 1 ng/ml TNF-$\alpha$ for 0 (A), 5 (B), or 15 (C) days. At each time point, MSCs were incubated with 5 nM BAY60-6583 for different times (5 to 120 min). After extensive washing, cells were treated for 15 min with 5 nM BAY60-6583. Intracellular cAMP levels were evaluated as reported in Materials and Methods. The data were expressed as cAMP percentages with respect to the level for untreated cells (basal), which was set to 100% (mean values $\pm$ SEM; $n = 3$). **, $P < 0.01$; ***, $P < 0.001$ versus respective BAY60-65383 treatments; #, $P < 0.05$; ##, $P < 0.01$ versus TNF-$\alpha$. (D) MSCs were differentiated for 0, 5, or 15 days in the absence or in the presence of TNF-$\alpha$ (1 ng/ml) and then were 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 Materials and Methods. The data were expressed as a percentage of GRK2 association with respect to that for the untreated cells (basal), which was set to 100% (mean values $\pm$ SEM; $n = 3$). *, $P < 0.05$; ***, $P < 0.001$ versus the basal level; #, $P < 0.05$; ###, $P < 0.001$ versus the respective control.

leading to the impairment of Gs-coupled receptor desensitization, the effects of selective GRK2 inhibition and overexpression on $A_{2B}$AR desensitization were evaluated. Challenging MSCs with the synthetic GRK2 inhibitor KRX29 (33, 41) completely prevented $A_{2B}$AR desensitization at all of the osteoblast differentiation stages (Fig. 5A to C).

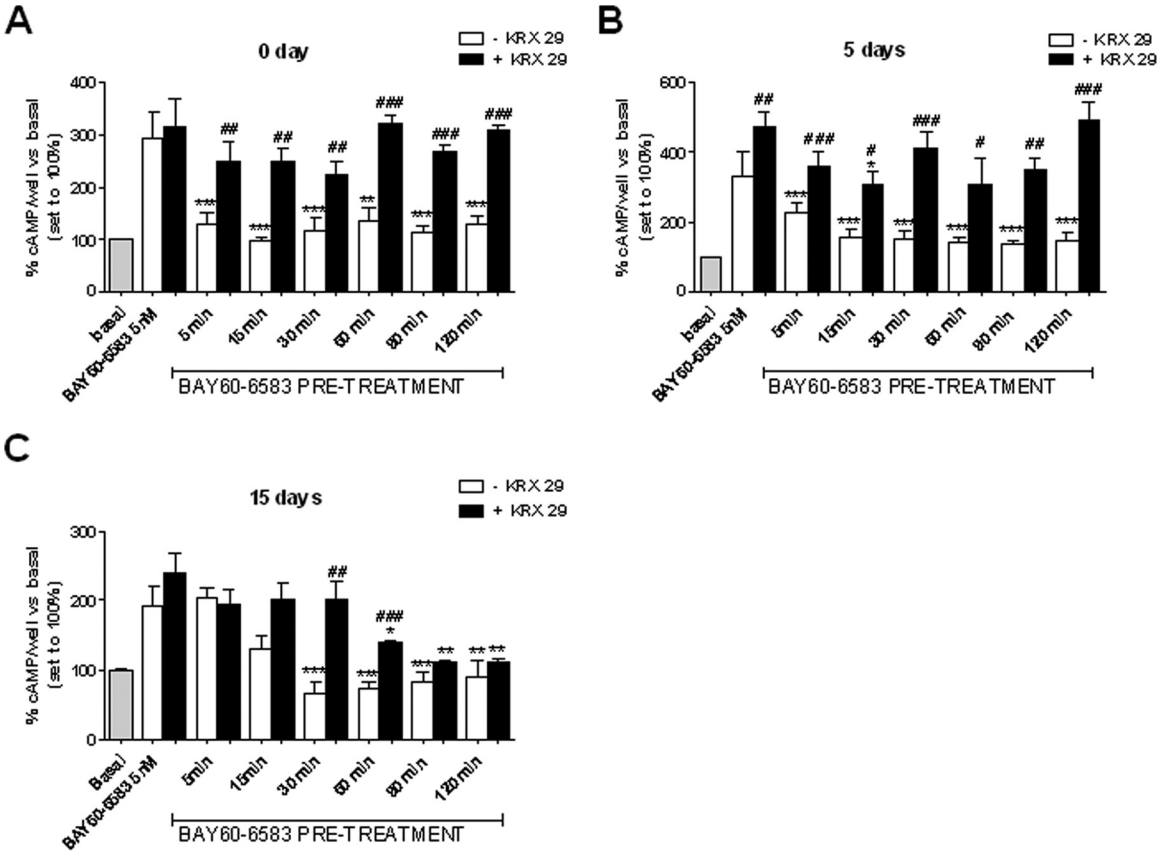


**FIG 5** Effect of the GRK2 inhibitor on $A_{2B}$AR functional response during MSC differentiation. MSCs, cultured in osteogenic medium for 0 (A), 5 (B), or 15 (C) days, were treated with 5 nM BAY60-6583 for different times (5 to 120 min) in the absence or presence of 1 $\mu$M KRX29. After extensive washing, cells were treated for 15 min with 5 nM BAY60-6583. Intracellular cAMP levels were evaluated as reported in Materials and Methods. The data were expressed as cAMP percentages with respect to the level for the untreated cells (basal), which was set to 100% (mean values $\pm$ SEM; $n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus respective BAY60-65383 treatments; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ versus KRX29.

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-$\alpha$. The similar effect exerted by the cytokine and the kinase inhibitor highlighted the involvement of GRK2 in agonist-mediated $A_{2B}$AR desensitization.

In parallel, we also evaluated the effect of GRK2 overexpression on TNF-$\alpha$-mediated impairment of $A_{2B}$AR functional responses. MSC transfection with GRK2 plasmid induced a significant increase in protein expression (2.5-fold versus empty vector-transfected cells; $P < 0.001$), as demonstrated by Western blotting (Fig. 6A and B) (38). GRK2 overexpression induced a significant increase in the degree of $A_{2B}$AR desensitization, as shown in Fig. 6C. Furthermore, the high levels of GRK2 protein were able to overcome almost completely the inhibitory effect of TNF-$\alpha$ on $A_{2B}$AR functionality (Fig. 6D). The receptor reduced functional responses to its selective agonist in a time-dependent manner even in the presence of the cytokine. These data support our hypothesis that TNF-$\alpha$ impaired Gs protein-coupled receptor desensitization by affecting, at least in part, the activity of intracellular GRK2 proteins, as demonstrated for the $A_{2B}$AR.

## DISCUSSION

TNF-$\alpha$ has 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-$\alpha$ was demonstrated to enhance and accelerate the $A_{2B}$AR-mediated differentiation of MSCs toward an

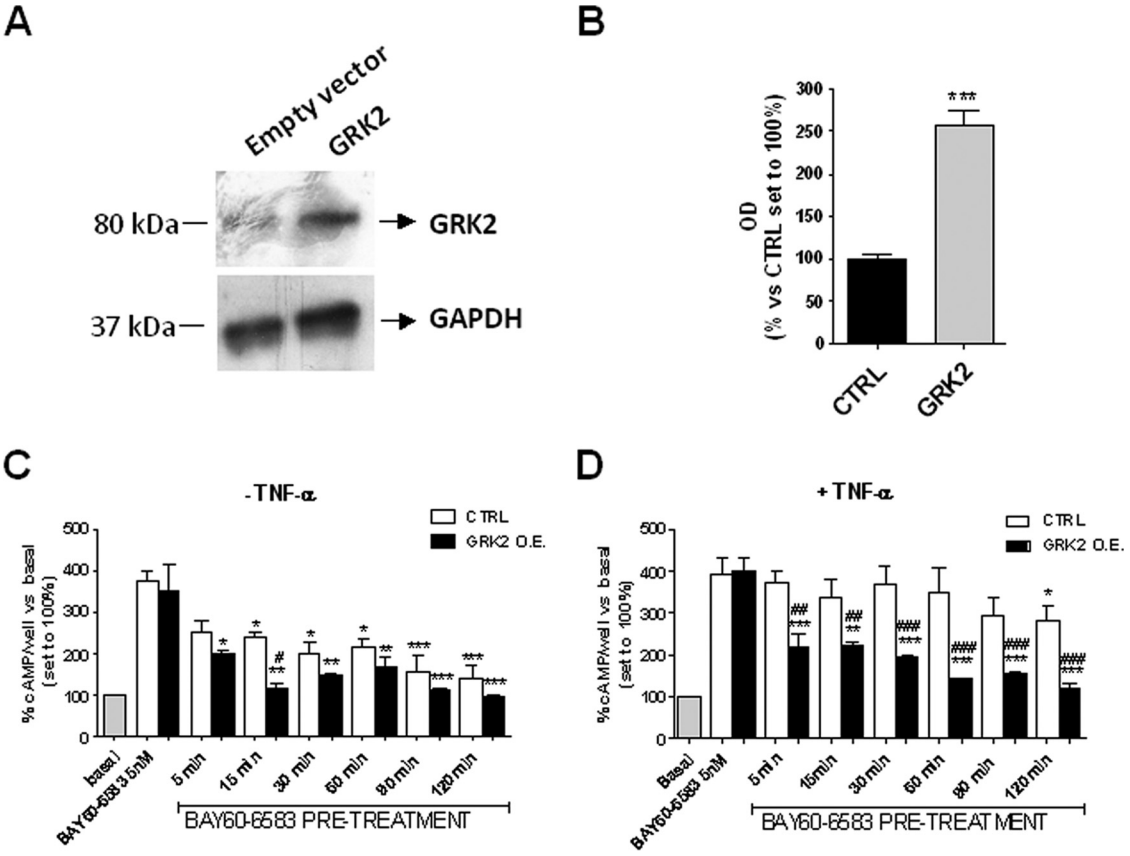


**FIG 6** Effect of GRK2 overexpression on $A_{2B}$AR functional response during MSC differentiation. (A and B) MSCs were cultured in osteogenic medium and then transfected with a GRK2 plasmid as reported in Materials and Methods. At the end of the treatment period, the cells were lysed and the GRK2 protein levels were evaluated using Western blot analysis. GAPDH was 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 percent optical density (OD) with respect to the level for untreated cells (empty vector), which was set to 100% (mean values ± SEM; $n = 3$). ***, $P < 0.001$ versus the control. (C and D) MSCs were cultured in osteogenic medium in the absence (C) or presence (D) of TNF-$\alpha$ and then transfected with a GRK2 plasmid as reported in Materials and Methods. On the 5th day of differentiation, cells were incubated with 5 nM BAY60-6583 for different times (5 to 120 min) in the absence or presence of 1 ng/ml TNF-$\alpha$. After extensive washing, cells were treated for 15 min with 5 nM BAY60-6583. Intracellular cAMP levels were evaluated as reported in Materials and Methods. The data were expressed as cAMP percentages with respect to the level for untreated cells (basal), which was set to 100% (mean values ± SEM; $n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus respective BAY60-65383 treatments; #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ versus TNF-$\alpha$.

osteoblast phenotype according to the consolidated evidence that low, submaximal concentrations (1 to 10 ng/ml) of TNF-$\alpha$, particularly in the early phase of tissue injury, have an anabolic effect (42, 43). 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 remodeling, under both physiological and pathological conditions. Here, the interplay between TNF-$\alpha$ and the $A_{2B}$AR was investigated in MSCs, examining in particular GPCR desensitization, a process primarily mediated by intracellular GRK proteins (32, 44, 45). The induction of osteogenesis mediated by low TNF-$\alpha$ concentrations was primarily 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 desensitization processes has been linked to the switch from anabolic to catabolic events in bone remodeling, suggesting that manipulating the GPCR desensitization machinery is a useful strategy for the treatment of bone diseases (12, 13, 46).

TNF-$\alpha$ has been demonstrated to play an important role in bone healing by affecting MSC behavior (7). Consistent with recent literature (42, 43), here a low TNF-$\alpha$ concen-

tration 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, thereby favoring MSC mineralization. Of note, this effect was not associated with significant changes in cell proliferation. TNF-$\alpha$ not only affects the differentiation processes, it also positively or negatively influences the rate of proliferation (47, 48). In our experimental model of induced MSC differentiation, TNF-$\alpha$ caused a complete switch to a differentiation process blocking the proliferative machinery.

Among the different GPCRs involved in bone remodeling 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 (49).

A functional interplay between $A_{2B}$ARs and TNF-$\alpha$ 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 upregulation of protein expression and/or an increase of receptor functional responsiveness by inhibiting receptor phosphorylation (21, 23).

To date, no evidence is available on the regulation of $A_{2B}$ARs by TNF-$\alpha$ in MSCs or the role of this dialogue in controlling the bone remodelling process. Here, TNF-$\alpha$ 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-$\alpha$ was primarily ascribed to a direct effect on GRK2, which is the most important kinase involved in GPCR desensitization in response to agonist stimulation. Spurney et al. have demonstrated that GRK2 and $\beta$-arrestin are temporally regulated during osteoblast differentiation in a pattern that would tend to enhance GPCR responsiveness and favor MSC differentiation (13). When an injury occurs, the release of cytokines and other soluble factors in the cellular microenvironment may regulate the activity of these kinases and in turn affect the GPCR final biological outcome (50, 51). 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 have recently demonstrated that cAMP signaling downstream of Gs-coupled receptors plays a critical role in determining the commitment of osteoblast precursors to bone with respect to adipocyte (52).

TNF-$\alpha$ has been shown to prevent Gs-coupled receptor desensitization by regulating GRK2 association with plasma membranes, thereby inhibiting receptor phosphorylation and desensitization in different cell lines (22). These data may support a beneficial role of low cytokine concentrations in potentiating GPCR functional responses. In MSCs, we demonstrated that TNF-$\alpha$ caused a significant downregulation of GRK2 protein level without affecting its gene expression. Kinase regulation was shown to partially involve the Mdm2-ubiquitin-dependent proteasome pathway, consistent with previous data (34, 36, 37, 40). Because TNF-$\alpha$-mediated GRK2 downregulation was not completely counteracted by the use of a proteasome inhibitor, we speculate that additional mechanisms are involved in such an outcome. In this respect, GRK2 turnover has been linked to its phosphorylation by c-Src (53) or by mitogen-activated protein kinase (54); these effects, together with Mdm2 recruitment, can be favored by the $\beta$-arrestin machinery (35), which has been demonstrated to play different scaffold functions to coordinate both Mdm2-dependent and -independent processes implicated in GRK2 stability (35, 40, 53, 54).

The decrease in GRK2 expression was accompanied by a reduction of GRK2-$A_{2B}$AR association in response to agonist stimulation. Globally, these events caused an impairment of receptor desensitization, producing a time-prolonged accumulation of intracellular cAMP upon $A_{2B}$AR stimulation (Fig. 7). In this scenario, it is likely that proinflammatory soluble factors regulate the commitment of MSC toward osteoblasts

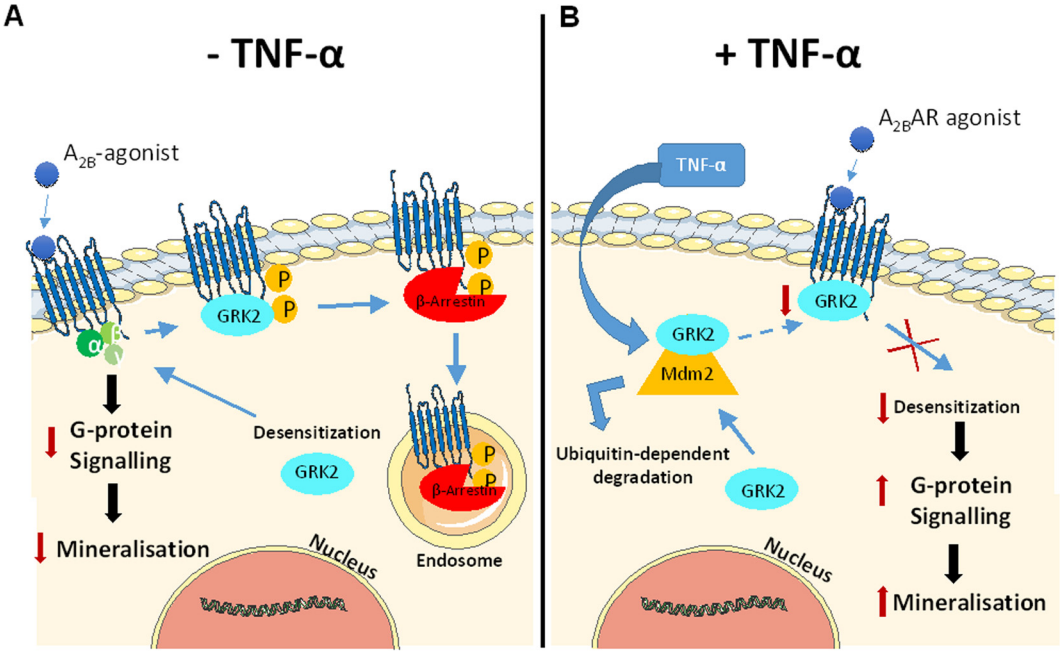


**FIG 7** Cartoon illustrating the modulatory effect of TNF-$\alpha$ on $A_{2B}$AR responses in MSCs. (A) Agonist-mediated $A_{2B}$AR stimulation induces GRK2 recruitment, with consequent receptor phosphorylation and reduction of the functional responsiveness. (B) TNF-$\alpha$ reduces GRK2 levels and its association with $A_{2B}$AR by recruitment of the Mdm2/ubiquitin proteasome pathway. The GPCR desensitization process was impaired in the presence of the cytokine, thus enhancing the prodifferentiating effects elicited by $A_{2B}$AR stimulation.

by modulating the activity of desensitizing proteins and consequently increasing Gs-coupled receptor activity.

The unequivocal involvement of GRK2 in TNF-$\alpha$-mediated regulation of $A_{2B}$AR was demonstrated by using a specific GRK2 inhibitor and by overexpressing GRK2 in MSCs. The chemical inhibition of GRK2 by the synthetic peptide KRX29 enhanced the osteogenic effects evoked by the $A_{2B}$AR agonist, similarly to the TNF-$\alpha$-evoked effect. Otherwise, the overexpression of the GRK2 isoform in MSCs almost completely overcame the inhibitory effect of TNF-$\alpha$ on $A_{2B}$AR desensitization, thus confirming GRK2 as a target for the cytokine. Because GRK2 overexpression did not show a complete reversal of $A_{2B}$AR desensitization kinetics, additional factors, such as TNF-$\alpha$-induced $A_{2B}$AR upregulation or the involvement of other GRK subtypes, can be speculated.

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

## MATERIALS AND METHODS

**Materials.** The chemicals 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 from Tocris Bioscience (Bristol, United Kingdom). An RNeasy minikit was obtained from Qiagen. The Script cDNA synthesis kit was furnished by Bio-Rad s.r.l. Fluocycle II SYBR was from Euroclone (Milan, Italy). TNF-$\alpha$ was purchased from Sigma-Aldrich.

**Cell cultures.** Human bone marrow MSCs and cell culture medium were purchased from 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 were subcultured in normal growth medium (MSCGM; Lonza) and plated ($5 \times 10^3$ cells/cm$^2$). The medium was changed to remove nonadherent cells every 3 to 4 days, and the cells were used at passages 0 to 3. For osteoblast differentiation studies, cells were seeded ($9 \times 10^3$ cells/cm$^2$) and cultured as previously described (15).

**Cell viability assay.** MSCs were seeded in 96-well microplates ($5 \times 10^3$ cells/well) and cultured in proliferation or osteogenic medium for the indicated days in the absence (control) or in the presence of TNF-$\alpha$ (0.1 ng/ml to 10 ng/ml). To evaluate cell viability, a 3-(4,5-dimethylthiazol-2-yl)-5-(3-

**TABLE 1** Human primers used for real time RT-PCR

| Protein | Primer nucleotide sequences[a] | Product size (bp) |
|---|---|---|
| Runx2 | FOR, 5′-GGCCCTGGTGTTTAAATGGT-3′; REV, 5′-AGGCTGTTTGACGCCATAGT-3′ | 178 |
| ALP | FOR, 5′-CTGCAAGGACATCGCCTATC-3′; REV, 5′-CATCAGTTCTGTTCTTGGGGTA-3′ | 101 |
| $A_{2B}$AR | FOR, 5′-TCCATCTTCAGCCTTCTGGC-3′; REV, 5′-AAAGGCAAGGACCCAGAGGA-3′ | 128 |
| $\beta$-Actin | FOR, 5′-GCACTCTTCCAGCCTTCCTTCC-3′; REV, 5′-GAGCCGCCGATCCACACG-3′ | 254 |

[a]FOR, forward; REV, reverse.

carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium (MTS) assay was used by following the manufacturer's instructions (Promega, Milan, Italy).

**Real-time RT-PCR analysis.** MSCs were cultured in normal growth medium and were treated with osteogenic medium in the presence or absence of TNF-$\alpha$ (1 ng/ml) and BAY60-6583 (5 nM), alone or in combination. The treatments were repeated every 3 days, and the expression levels of osteogenic markers (Runx2, osterix, ALP, and osteocalcin) were quantified after 0, 5, and 15 days of treatment. Moreover, MSCs were cultured in the presence or absence of TNF-$\alpha$ (1 ng/ml) in normal growth medium for 48 h or in osteogenic medium for 5 days. Gene expression then was assessed by real-time reverse transcription-PCR (RT-PCR) as previously described (55), and the primers used for the analysis are indicated in Table 1.

**Mineralization assay.** MSCs were seeded ($9 \times 10^3$ cells/cm$^2$) and then treated in the absence (control) or in the presence of TNF-$\alpha$ (1 ng/ml), BAY60-6583 (5 nM), MRS1706 (1 $\mu$M), and KRX29 (1 $\mu$M), either alone or in combination. Treatments were repeated every 3 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 under osteogenic conditions for 48 h or 5 days in the absence (control) or presence of TNF-$\alpha$ (1 ng/ml). When indicated, MSCs were pretreated with 10 $\mu$M proteasome/calpain inhibitor MG-132 (37) for 3 h and then incubated in osteogenic medium for 48 h in the absence (control) or presence of TNF-$\alpha$ (1 ng/ml).

Thereafter, cells were lysed (and 30 $\mu$g of protein added with Laemmli solution) and loaded for SDS-PAGE (7.5%). Proteins were electrotransferred into polyvinylidene difluoride (PVDF) membranes and incubated overnight at 4°C using the primary antibodies anti-$A_{2B}$AR (sc-28996; 1:150; Santa Cruz Biotechnology), anti-GRK2 (sc-562; 1:200; Santa Cruz Biotechnology), and anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (G9545; 1:5,000; Sigma-Aldrich) and by the appropriate peroxidase-conjugated secondary antibodies. Signals were detected using a chemiluminescent substrate (ECL; PerkinElmer, Waltham, MA). ImageJ software was used to perform the densitometric analysis of the immunoreactive bands (version 1.41; NIH, Bethesda, MD).

**Measurement of cAMP levels during BAY60-6583 desensitization induction in MSCs.** MSCs ($2 \times 10^4$ cells/well) were plated in 24-well plates, and after 24 h, osteogenic differentiation was induced in the presence or absence of TNF-$\alpha$ (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 desensitization assays, MSCs (differentiated for 0, 5, or 15 days in the absence or presence of TNF-$\alpha$) were pretreated with BAY60-6583 (5 nM) for different times (5 to 120 min) and then washed and stimulated with BAY60-6583 (5 nM) for 15 min in the presence of 1 U/ml of adenosine deaminase (ADA) and the phosphodiesterase inhibitor Ro 20-1724 (20 $\mu$M) (56). At the end of treatments, intracellular cAMP levels were quantified using a competitive protein binding method (57).

In order to investigate the effect of GRK2 inhibition on $A_{2B}$AR functional responses 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 to 120 min) in the absence or presence of the GRK2 inhibitor KRX29 (1 $\mu$M).

**GRK2 association with $A_{2B}$AR or 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 crude MSC lysates was used (38, 58, 59). MSCs were differentiated for 0, 5, or 15 days in the absence or presence of TNF-$\alpha$ (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 presence of TNF-$\alpha$ (1 ng/ml). At the end of treatments, cells were suspended in lysis buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich, Milan, Italy). Cell lysates (30 $\mu$g/well) were incubated for 60 min into wells precoated with a rabbit anti-$A_{2B}$AR or anti-Mdm2 antibody; after washes, each well was incubated for 15 min with 1% bovine serum albumin (BSA) to block nonspecific sites and then for 2 h at room temperature with a mouse primary anti-GRK2 antibody. Wells then were washed and incubated for 1 h with an anti-mouse horseradish peroxidase (HRP)-conjugated antibody and washed again (38). The colorimetric substrate kit allowed a colorimetric quantification of the receptor-GRK2 association.

**GRK2 overexpression.** GRK2 overexpression was obtained by transfecting MSCs using the polyethyleneimine method (38). MSCs were cultured in osteogenic medium for 48 h in the absence or presence of TNF-$\alpha$. The cells were incubated with 1 $\mu$g of either GRK2 plasmid (OriGene, MD) or the corresponding empty vector (OriGene, MD) for 30 h. Transfected MSCs were rapidly seeded in 24-well plates and subjected to the cAMP assay as described above. GRK2 overexpression was confirmed by Western blot analysis.

**Statistical analysis.** A nonlinear multipurpose curve-fitting program, GraphPad Prism (version 5.00), was used for data analysis and graphic presentation. Data are reported as the means ± standard errors

of the means (SEM) from 3 or 4 different experiments. Statistical analyses were performed using a one-way analysis of variance (ANOVA) study followed by the Bonferroni test for repeated measurements. Differences were considered statistically significant for $P$ values of $<0.05$.

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We have no conflicts of interest to declare.

## REFERENCES

1. Office of the Surgeon General. 2004. The burden of bone disease. Bone health and osteoporosis: a report of the surgeon general. Department of Health and Human Services, Rockville, MD. http://www.ncbi.nlm.nih.gov/books/NBK45502/.
2. Marie PJ, Kassem M. 2011. Osteoblasts in osteoporosis: past, emerging, and future anabolic targets. Eur J Endocrinol 165:1–10. https://doi.org/10.1530/EJE-11-0132.
3. Ai-Aql ZS, Alagl AS, Graves DT, Gerstenfeld LC, Einhorn TA. 2008. Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis. J Dent Res 87:107–118. https://doi.org/10.1177/154405910808700215.
4. Gibon E, Lu L, Goodman SB. 2016. Aging, inflammation, stem cells, and bone healing. Stem Cell Res Ther 22:7–44.
5. Pape HC, Marcucio R, Humphrey C, Colnot C, Knobe M, Harvey EJ. 2010. Trauma-induced inflammation and fracture healing. J Orthop Trauma 24:522–525. https://doi.org/10.1097/BOT.0b013e3181ed1361.
6. Gerstenfeld LC, Cho TJ, Kon T, Aizawa T, Cruceta J, Graves BD, Einhorn TA. 2001. Impaired intramembranous bone formation during bone repair in the absence of tumor necrosis factor-alpha signaling. Cells Tissues Organs 169:285–294. https://doi.org/10.1159/000047893.
7. Kotake S, Nanke Y. 2014. Effect of TNF$\alpha$ on osteoblastogenesis from mesenchymal stem cells. Biochim Biophys Acta 1840:1209–1213. https://doi.org/10.1016/j.bbagen.2013.12.013.
8. Karnes JM, Daffner SD, Watkins CM. 2015. Multiple roles of tumor necrosis factor-alpha in fracture healing. Bone 78:87–93. https://doi.org/10.1016/j.bone.2015.05.001.
9. Osta B, Benedetti G, Miossec P. 2014. Classical and paradoxical effects of TNF-$\alpha$ on bone homeostasis. Front Immunol 5:48.
10. Keinan D, Yang S, Cohen RE, Yuan X, Liu T, Li YP. 2014. Role of regulator of G protein signaling proteins in bone. Front Biosci 19:634–648. https://doi.org/10.2741/4232.
11. Graham S, Gamie Z, Polyzois I, Narvani AA, Tzafetta K, Tsiridis E, Helioti M, Mantalaris A, Tsiridis E. 2009. Prostaglandin EP2 and EP4 receptor agonists in bone formation and bone healing: In vivo and in vitro evidence. Expert Opin Investig Drugs 18:746–766.
12. Ma YL, Cain RL, Halladay DL, Yang X, Zeng Q, Miles RR, Chandrasekhar S, Martin TJ, Onyia JE. 2001. Catabolic effects of continuous human PTH (1-38) in vivo is associated with sustained stimulation of RANKL and inhibition of osteoprotegerin and gene-associated bone formation. Endocrinology 142:4047–4054. https://doi.org/10.1210/endo.142.9.8356.
13. Spurney RF, Flannery PJ, Garner SC, Athirakul K, Liu S, Guilak F, Quarles LD. 2002. Anabolic effects of a G protein-coupled receptor kinase inhibitor expressed in osteoblasts. J Clin Investig 109:1361–1371. https://doi.org/10.1172/JCI0214663.
14. Esbrit P, Alcaraz MJ. 2013. Current perspectives on parathyroid hormone (PTH) and PTH-related protein (PTHrP) as bone anabolic therapies. Biochem Pharmacol 85:1417–1423. https://doi.org/10.1016/j.bcp.2013.03.002.
15. Trincavelli ML, Daniele S, Giacomelli C, Taliani S, Da Settimo F, Cosimelli B, Greco G, Novellino E, Martini C. 2014. Osteoblast differentiation and survival: a role for A2B adenosine receptor allosteric modulators. Biochim Biophys Acta 1843:2957–2966. https://doi.org/10.1016/j.bbamcr.2014.09.013.
16. Corciulo C, Wilder T, Cronstein BN. 2016. Adenosine A2B receptors play an important role in bone homeostasis. Purinergic Signal 12:537–547.
17. Carroll SH, Wigner NA, Kulkarni N, Johnston-Cox H, Gerstenfeld LC, Ravid K. 2012. A2B adenosine receptor promotes mesenchymal stem cell differentiation to osteoblasts and bone formation in vivo. J Biol Chem 287:15718–15727. https://doi.org/10.1074/jbc.M112.344994.
18. Mediero A, Cronstein BN. 2013. Adenosine and bone metabolism. Trends Endocrinol Metab 24:290–300. https://doi.org/10.1016/j.tem.2013.02.001.
19. Gharibi B, Abraham AA, Ham J, Evans BA. 2011. Adenosine receptor subtype expression and activation influence the differentiation of mesenchymal stem cells to osteoblasts and adipocytes. J Bone Miner Res 26:2112–2124. https://doi.org/10.1002/jbmr.424.
20. St Hilaire C, Koupenova M, Carroll SH, Smith BD, Ravid K. 2008. TNF-alpha upregulates the A2B adenosine receptor gene: The role of NAD(P)H oxidase 4. Biochem Biophys Res Commun 375:292–296. https://doi.org/10.1016/j.bbrc.2008.07.059.
21. Kolachala V, Asamoah V, Wang L, Obertone TS, Ziegler TR, Merlin D, Sitaraman SV. 2005. TNF-alpha upregulates adenosine 2b (A2b) receptor expression and signaling in intestinal epithelial cells: a basis for A2bR overexpression in colitis. Cell Mol Life Sci 62:2647–2657. https://doi.org/10.1007/s00018-005-5328-4.
22. Khoa ND, Postow M, Danielsson J, Cronstein BN. 2006. Tumor necrosis factor-alpha prevents desensitization of Galphas-coupled receptors by regulating GRK2 association with the plasma membrane. Mol Pharmacol 69:1311–1319. https://doi.org/10.1124/mol.105.016857.
23. Trincavelli ML, Marroni M, Tuscano D, Ceruti S, Mazzola A, Mitro N, Abbracchio MP, Martini C. 2004. Regulation of A2B adenosine receptor functioning by tumour necrosis factor a in human astroglial cells. J Neurochem 91:1180–1190. https://doi.org/10.1111/j.1471-4159.2004.02793.x.
24. Wang L, Quarles LD, Spurney RF. 2004. Unmasking the osteoinductive effects of a G protein-coupled receptor (GPCR) kinase (GRK) inhibitor by treatment with PTH(1-34). J Bone Miner Res 19:1661–1670. https://doi.org/10.1359/JBMR.040708.
25. Spurney RF. 2003. Regulated expression of G protein-coupled receptor kinases (GRK's) and beta-arrestins in osteoblasts. Calcif Tissue Int 73:153–160. https://doi.org/10.1007/s00223-002-1018-5.
26. Eijkelkamp N, Heijnen CJ, Willemen HL, Deumens R, Joosten EA, Kleibeuker W, den Hartog IJ, van Velthoven CT, Nijboer C, Nassar MA, Dorn GW, Wood JN, Kavelaars A. 2010. GRK2: a novel cell-specific regulator of severity and duration of inflammatory pain. J Neurosci 30:2138–2149. https://doi.org/10.1523/JNEUROSCI.5752-09.2010.
27. Nijboer CH, Heijnen CJ, Willemen HL, Groenendaal F, Dorn GW, van Bel F, Kavelaars A. 2010. Cell-specific roles of GRK2 in onset and severity of hypoxic-ischemic brain damage in neonatal mice. Brain Behav Immun 24:420–426. https://doi.org/10.1016/j.bbi.2009.11.009.
28. Kleibeuker W, Ledeboer A, Eijkelkamp N, Watkins LR, Maier SF, Zijlstra J, Heijnen CJ, Kavelaars A. 2007. A role for G protein-coupled receptor kinase 2 in mechanical allodynia. Eur J Neurosci 25:1696–1704. https://doi.org/10.1111/j.1460-9568.2007.05423.x.
29. Ohba Y, Nakaya M, Watari K, Nagasaka A, Kurose H. 2015. GRK6 phosphorylates I$\kappa$B$\alpha$ at Ser(32)/Ser(36) and enhances TNF-$\alpha$-induced inflammation. Biochem Biophys Res Commun 461:307–313. https://doi.org/10.1016/j.bbrc.2015.04.027.
30. Peters DM, Gies EK, Gelb CR, Peterfreund RA. 1998. Agonist-induced desensitization of A2B adenosine receptors. Biochem Pharmacol 55:873–882. https://doi.org/10.1016/S0006-2952(97)00560-1.
31. Matharu AL, Mundell SJ, Benovic JL, Kelly E. 2001. Rapid agonist-induced desensitization and internalization of the A(2B) adenosine receptor is mediated by a serine residue close to the COOH terminus. J Biol Chem 276:30199–30207. https://doi.org/10.1074/jbc.M010650200.
32. Ribas C, Penela P, Murga C, Salcedo A, García-Hoz C, Jurado-Pueyo M, Aymerich I, Mayor F, Jr. 2007. The G protein-coupled receptor kinase (GRK) interactome: role of GRKs in GPCR regulation and signalling. Biochim Biophys Acta 1768:913–922. https://doi.org/10.1016/j.bbamem.2006.09.019.
33. Daniele S, Trincavelli ML, Fumagalli M, Zappelli E, Lecca D, Bonfanti E, Campiglia P, Abbracchio MP, Martini C. 2014. Does GRK-$\beta$ arrestin

machinery work as a "switch on" for GPR17-mediated activation of intracellular signaling pathways? Cell Signal 26:1310–1325. https://doi.org/10.1016/j.cellsig.2014.02.016.
34. Penela P, Ruiz-Gómez A, Castaño JG, Mayor F, Jr. 1998. Degradation of the G protein-coupled receptor kinase 2 by the proteasome pathway. J Biol Chem 273:35238–35244. https://doi.org/10.1074/jbc.273.52.35238.
35. Nogués L, Salcedo A, Mayor F, Jr, Penela P. 2011. Multiple scaffolding functions of {beta}-arrestins in the degradation of G protein-coupled receptor kinase 2. J Biol Chem 286:1165–1173. https://doi.org/10.1074/jbc.M110.203406.
36. Penela P. 2016. Chapter three–ubiquitination and protein turnover of G-protein-coupled receptor kinases in GPCR signaling and cellular regulation. Prog Mol Biol Transl Sci 141:85–140. https://doi.org/10.1016/bs.pmbts.2016.04.002.
37. Li JG, Benovic JL, Liu-Chen LY. 2000. Mechanisms of agonist-induced down-regulation of the human kappa-opioid receptor: internalization is required for down-regulation. Mol Pharmacol 58:795–801.
38. Fumagalli M, Bonfanti E, Daniele S, Zappelli E, Lecca D, Martini C, Trincavelli ML, Abbracchio MP. 2015. The ubiquitin ligase Mdm2 controls oligodendrocyte maturation by intertwining mTOR with G protein-coupled receptor kinase 2 in the regulation of GPR17 receptor desensitization. Glia 63:2327–2339. https://doi.org/10.1002/glia.22896.
39. Varshavsky A. 1997. The ubiquitin system. Trends Biochem Sci 22:383–387. https://doi.org/10.1016/S0968-0004(97)01122-5.
40. Salcedo A, Mayor F, Jr, Penela P. 2006. Mdm2 is involved in the ubiquitination and degradation of G-protein-coupled receptor kinase 2. EMBO J 25:4752–4762. https://doi.org/10.1038/sj.emboj.7601351.
41. Carotenuto A, Cipolletta E, Gomez-Monterrey I, Sala M, Vernieri E, Limatola A, Bertamino A, Musella S, Sorriento D, Grieco P, Trimarco B, Novellino E, Iaccarino G, Campiglia P. 2013. Design, synthesis and efficacy of novel G protein-coupled receptor kinase 2 inhibitors. Eur J Med Chem 69:384–392. https://doi.org/10.1016/j.ejmech.2013.08.039.
42. Hess K, Ushmorov A, Fiedler J, Brenner RE, Wirth T. 2009. TNFalpha promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-kappaB signaling pathway. Bone 45:367–376. https://doi.org/10.1016/j.bone.2009.04.252.
43. Briolay A, Lencel P, Bessueille L, Caverzasio J, Buchet R, Magne D. 2013. Autocrine stimulation of osteoblast activity by Wnt5a in response to TNF-$\alpha$ in human mesenchymal stem cells. Biochem Biophys Res Commun 430:1072–1077. https://doi.org/10.1016/j.bbrc.2012.12.036.
44. Ferguson SS, Barak LS, Zhang J, Caron MG. 1996. G-protein-coupled receptor regulation: role of G-protein-coupled receptor kinases and arrestins. Can J Physiol Pharmacol 74:1095–1110. https://doi.org/10.1139/y96-124.
45. Kohout TA, Lefkowitz RJ. 2003. Regulation of G protein-coupled receptor kinases and arrestins during receptor desensitization. Mol Pharmacol 63:9–18. https://doi.org/10.1124/mol.63.1.9.
46. Hock JM, Gera I. 1992. Effects of continuous and intermittent administration and inhibition of resorption on the anabolic response of bone to parathyroid hormone. J Bone Miner Res 7:65–72.
47. Radeff-Huang J, Seasholtz TM, Chang JW, Smith JM, Walsh CT, Brown JH. 2007. Tumor necrosis factor-alpha-stimulated cell proliferation is mediated through sphingosine kinase-dependent Akt activation and cyclin D expression. J Biol Chem 282:863–870. https://doi.org/10.1074/jbc.M601698200.
48. Ghali O, Chauveau C, Hardouin P, Broux O, Devedjian JC. 2010. TNF-alpha's effects on proliferation and apoptosis in human mesenchymal stem cells depend on RUNX2 expression. J Bone Miner Res 25:1616–1626. https://doi.org/10.1002/jbmr.52.
49. Trincavelli ML, Daniele S, Martini C. 2010. Adenosine receptors: what we know and what we are learning. Curr Top Med Chem 10:860–877. https://doi.org/10.2174/156802610791268756.
50. Wang L, Liu S, Quarles LD, Spurney RF. 2005. Targeted overexpression of G protein-coupled receptor kinase-2 in osteoblasts promotes bone loss. Am J Physiol Endocrinol Metab 288:E826–E834.
51. Vroon A, Heijnen CJ, Kavelaars A. 2006. GRKs and arrestins: regulators of migration and inflammation. J Leukoc Biol 80:1214–1221. https://doi.org/10.1189/jlb.0606373.
52. Sinha P, Aarnisalo P, Chubb R, Ono N, Fulzele K, Selig M, Saeed H, Chen M, Weinstein LS, Pajevic PD, Kronenberg HM, Wu JY. 2014. Loss of Gs$\alpha$ early in the osteoblast lineage favors adipogenic differentiation of mesenchymal progenitors and committed osteoblast precursors. J Bone Miner Res 29:2414–2426. https://doi.org/10.1002/jbmr.2270.
53. Penela P, Elorza A, Sarnago S, Mayor F, Jr. 2001. Beta-arrestin- and c-Src-dependent degradation of G protein-coupled receptor kinase 2. EMBO J 20:5129–5138. https://doi.org/10.1093/emboj/20.18.5129.
54. Elorza A, Penela P, Sarnago S, Mayor F, Jr. 2003. MAPK-dependent degradation of G protein-coupled receptor kinase 2. J Biol Chem 278:29164–29173. https://doi.org/10.1074/jbc.M304314200.
55. Vandesompele J, De Paepe A, Speleman F. 2002. Elimination of primer-dimer artifacts and genomic coamplification using a two-step SYBR green I real-time RT-PCR. Anal Biochem 303:95–98. https://doi.org/10.1006/abio.2001.5564.
56. Trincavelli ML, Giacomelli C, Daniele S, Taliani S, Cosimelli B, Laneri S, Severi E, Barresi E, Pugliesi I, Greco G, Novellino E, Da Settimo F, Martini C. 2014. Allosteric modulators of human A2B adenosine receptor. Biochim Biophys Acta 1840:1194–1203. https://doi.org/10.1016/j.bbagen.2013.12.021.
57. Nordstedt C, Fredholm BB. 1990. A modification of a protein-binding method for rapid quantification of cAMP in cell-culture supernatants and body fluid. Anal Biochem 189:231–234. https://doi.org/10.1016/0003-2697(90)90113-N.
58. Zappelli E, Daniele S, Abbracchio MP, Martini C, Trincavelli ML. 2014. A rapid and efficient immunoenzymatic assay to detect receptor protein interactions: G protein-coupled receptors. Int J Mol Sci 15:6252–6264. https://doi.org/10.3390/ijms15046252.
59. Daniele S, Taliani S, Da Pozzo E, Giacomelli C, Costa B, Trincavelli ML, Rossi L, La Pietra V, Barresi E, Carotenuto A, Limatola A, Lamberti A, Marinelli L, Novellino E, Da Settimo F, Martini C. 2014. Apoptosis therapy in cancer: the first single-molecule co-activating p53 and the translocator protein in glioblastoma. Sci Rep 4:4749.