Paracrine interactions between mesenchymal stem cells and macrophages are regulated by 1,25-dihydroxyvitamin D3

Laura Saldaña1,2, Gema Vallés1,2, Fátima Bensiamar1,2, Francisco José Mancebo2,1, Eduardo García-Rey1 & Nuria Vilaboa1,2

Mesenchymal stem cells (MSC) modulate the macrophage-mediated inflammatory response through the secretion of soluble factors. In addition to its classical effects on calcium homeostasis, 1,25-dihydroxyvitamin D3 (1,25D3) has emerged as an important regulator of the immune system. The present study investigates whether 1,25D3 modulates the paracrine interactions between MSC and macrophages. 1,25D3 stimulated MSC to produce PGE2 and VEGF and regulated the interplay between macrophages and MSC toward reduced pro-inflammatory cytokine production. Conditioned media (CM) from co-cultures of macrophages and MSC impaired MSC osteogenesis. However, MSC cultured in CM from 1,25D3-treated co-cultures showed increased matrix maturation and mineralization. Co-culturing MSC with macrophages prevented the 1,25D3-induced increase in RANKL levels, which correlated with up-regulation of OPG secretion. MSC seeding in three-dimensional (3D) substrates potentiated their immunomodulatory effects on macrophages. Exposure of 3D co-cultures to 1,25D3 further reduced the levels of soluble factors related to inflammation and chemotaxis. As a consequence of 1,25D3 treatment, the recruitment of monocytes toward CM of 3D co-cultures decreased, while the osteogenic maturation of MSC increased. These data add new insights into the pleiotropic effects of 1,25D3 on the crosstalk between MSC and macrophages and highlight the role of the hormone in bone regeneration.

Vitamin D plays an important role in maintaining bone integrity, as its deficiency has been related to impaired bone fracture healing and implant osseointegration1,2. Many biological actions of calcitriol (1,25-dihydroxyvitamin D3, 1,25D3) are mediated by the vitamin D receptor (VDR). Upon ligand binding, VDR heterodimerizes with the nuclear retinoid X receptor (RXR), and the resulting VDR-RXR complex can bind to specific DNA sequences, termed vitamin D-response elements (VDRE), located in the promoters of target genes3. Although VDR is expressed in bone tissue, direct effects of 1,25D3 on osteoblast-lineage cells are not yet fully defined. The actions of 1,25D3 on immature osteoblasts and osteoprogenitors are mainly directed toward maintaining serum calcium levels by increasing bone resorption through the regulation of receptor activator of NF-κB ligand (RANKL)4. On the other hand, 1,25D3 stimulates in vitro osteoblast differentiation and mineralization as well as mineral deposition in osteoid tissues, as evidenced in transgenic mice with osteoblast-specific overexpression of VDR5,6.

Beyond its well-documented role in regulating calcium and bone homeostasis, 1,25D3 has attracted considerable attention due to its immunoregulatory functions. Vitamin D deficiency has been associated with increased autoimmunity and susceptibility to infection7. Cells of the immune system, including promyelocytes, monocytes and dendritic cells express VDR and are capable of responding to 1,25D38. VDR-mediated signaling in immune cells results in the activation of downstream gene expression, which ultimately elicits anti-proliferative and immunomodulatory effects9. In cells of the monocyte/macrophage lineage, 1,25D3 decreases the production of inflammatory cytokines, chemokines and prostaglandins10,11. Macrophages are key mediators of osseous wound healing. They participate in the initial inflammatory phase following tissue injury and are essential for bone healing via secretion of inflammatory, osteotropic and pro-angiogenic factors. In bone tissue engineering

1Hospital Universitario La Paz-IdiPAZ, Paseo de la Castellana 261, 28046, Madrid, Spain. 2CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Madrid, Spain. Correspondence and requests for materials should be addressed to L.S. (email: laura.saldana@salud.madrid.org)
procedures, macrophages facilitate new bone formation around the implanted scaffolds, although excessive macrophage activation has been associated with the development of a fibrous capsule that confines the implant and impairs its physical connectivity with the host bone. Tissue engineering combines scaffolds and mesenchymal stem cells (MSC) to enhance regeneration of injured tissues. MSC modulate both the inflammatory reaction and the subsequent reparative phases of wound healing, mainly through the secretion of soluble factors. Based on in vitro and in vivo studies of inflammatory disorders, it has become clear that MSC modulate macrophage functions. Co-culturing with MSC drives macrophages toward a regulatory phenotype via increased expression of interleukin-10 (IL-10), IL-4 and reduced production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-6, IL-12, IL-1β and monocyte chemoattractant protein-1 (MCP-1) [13]. MSC cultured on three-dimensional (3D) polymeric substrates or encapsulated in synthetic hydrogels lessen in vitro macrophage activation [13, 14]. Moreover, MSC encapsulated within hydrogels reduce the thickness of the fibrous capsule upon subcutaneous implantation in mice, compared with acellular hydrogels. Several lines of evidence suggest that 1,25D3 might regulate cellular events during bone regeneration. After intravenous administration, 1,25D3 accumulates in the callus during rat femoral fracture healing and a vitamin D-rich diet improves peri-implant bone formation in ovariectomized rats [15, 16]. The present study investigated whether 1,25D3 modulates the paracrine interactions between MSC and macrophages. To this end, co-cultures of macrophages and MSC seeded on flat surfaces or 3D scaffolds were exposed to 1,25D3, and levels of soluble factors related to inflammation and bone remodeling were quantified. A comparative evaluation of 1,25D3 effects on single-cultured and co-cultured cells was performed. The influence of soluble factors contained in conditioned media (CM) from co-cultures treated with 1,25D3 on monocyte migration and functionality of MSC and osteoblasts was also evaluated.

Results

Cell responsiveness to 1,25D3.

Effect of 1,25D3 on levels of inflammation-related factors in MSC/dTHP-1 co-cultures.

We next investigated whether 1,25D3 modulates the inflammatory state of dTHP-1 when co-cultured with MSC. Co-culturing led to an increase in the levels of the anti-inflammatory cytokine IL-10 and a decrease in TNF-α next investigated whether 1,25D3 modulates the inflammatory state of dTHP-1 when co-cultured with MSC. Co-culturing led to an increase in the levels of the anti-inflammatory cytokine IL-10 and a decrease in TNF-α.
Effect of 1,25D3 on MSC osteogenesis and levels of bone-remodeling factors in MSC/dTHP-1 co-cultures. Inflammatory factors can interfere with the in vitro osteogenic capacity of MSC.\textsuperscript{2,23} In fact, the expression at the mRNA level of the osteogenic differentiation markers core binding factor alpha-1 (CBFA1), alkaline phosphatase (ALPL) and BGLAP in MSC decreased by 2-fold after co-culturing with dTHP-1 (Fig. 5A). Given 1,25D3 reduces the secretion of inflammatory factors in co-cultures, we examined whether the hormone could enhance the osteogenic potential of co-cultured MSC. CBFA1 expression in co-cultured MSC increased after 1,25D3 treatment, although this effect was lower than in isolated MSC (Fig. 5A). 1,25D3 did not affect ALPL mRNA levels in any condition (Fig. 5A). As observed for the BGLAP gene, exposure of MSC to 1,25D3 greatly increased transcript levels of the gene encoding for osteopontin (OPN) in a dose-dependent manner (Fig. 5A). 1,25D3-mediated increase of BGLAP mRNA levels was attenuated in co-cultures, whereas this effect was not observed for OPN (Fig. 5A). Although co-culturing did not affect the stromal expression of VDR, it prevented its dose-dependent increase in co-cultured MSC treated with 1,25D3 (Fig. 5A). To examine whether the reduction in CBFA1, ALPL and BGLAP expression in co-cultured MSC influences their osteogenesis ability, MSC were co-cultured with dTHP-1 for 72 h and then stimulated to undergo osteogenic differentiation in the absence of dTHP-1 for up to 14 days. Co-culturing MSC with dTHP-1 prior to osteogenesis induction did not influence
Figure 2. IL-10, TNF-α and VDR levels in co-cultures treated with 1,25D3. dTHP-1 cultured in isolation or co-cultured with MSC (Co-dTHP-1) were treated with 10 or 100 nM 1,25D3 or vehicle (−) for 72 h. IL-10 (A) and TNF-α (B) secretion (left panels) and mRNA fold changes (right panels). In these experiments, a relative secretion value of 100 corresponded to approximately 20 ± 0.8 pg of IL-10 and 307 ± 61 pg of TNF-α per ml of culture medium. VDR mRNA fold changes in dTHP-1 and Co-dTHP-1 (C). mRNA data are relative to those measured in untreated cells cultured in isolation, which were given the arbitrary value of 1. *p < 0.05 compared with untreated conditions; †p < 0.05 compared with 10 nM 1,25D3; ‡p < 0.05 compared with cells cultured in isolation under the same experimental conditions. N.D.: Not detected.
their ability to develop mineralized matrix, as assessed by quantification of ALP activity and alizarin red staining (Supplementary Fig. S1). MSC co-cultured or not with 1,25D3 exhibited similar responses (Supplementary Fig. S1). We next studied whether continuous exposure to CM from co-cultures affects MSC osteogenesis. MSC cultured under osteogenic conditions with CM from co-cultures showed decreased ALP activity (Fig. 5B) and alizarin red staining (Fig. 5C) compared with cells cultured in non-conditioned media. However, MSC that received CM from co-cultures treated with 1,25D3 increased matrix maturation and mineralization.

In addition to their ability to differentiate into functional osteoblasts, MSC secrete osteoprotegerin (OPG) and RANKL, important regulators of bone remodeling and targets in the skeletal action of 1,25D3. We next investigated the effect of 1,25D3 on these soluble mediators in MSC cultured in isolation or co-cultured. Treatment of MSC with 100 nM 1,25D3 decreased OPG secretion and mRNA levels, whereas no effects were detected when cells were treated with 10 nM 1,25D3 (Fig. 6A). Treatments with both doses similarly increased levels of free RANKL in media and the relative amount of RANKL transcripts (Fig. 6B). OPG secretion highly increased after co-culturing whereas the concentration of RANKL in media was similar in MSC cultured in isolation and in co-cultures (Fig. 6A, left panel). In contrast to that observed in single-cultured cells, both doses of 1,25D3 increased OPG secretion in co-cultures, whereas they did not have any effect on RANKL levels (Fig. 6A, left panels). Changes in OPG secretion induced by 1,25D3 treatment paralleled changes at the mRNA level (Fig. 6A, right panel). RANKL mRNA levels were higher in MSC co-cultured with dTHP-1 than in MSC cultured in isolation, and were further increased after 1,25D3 exposure (Fig. 6B, right panel). Treatments of MSC with both doses similarly increased IL-6 levels in media (Fig. 6C, left panel). In contrast, levels of this cytokine, highly induced by co-culturing MSC with dTHP-1, did not experience any significant change after exposure of co-cultures to 10 nM 1,25D3 and decreased after treatment with the higher dose. Regulation of IL6 mRNA levels by 1,25D3 correlated with the observed changes in IL-6 concentration in media (Fig. 6C, right panel).

**Effect of 1,25D3 and topographical cues on secreted factors by MSC/dTHP-1 co-cultures and MSC osteogenesis.** We recently reported that the architecture of the substrates that harbor MSC decisively
influences their crosstalk with macrophages. Using the same 3D substrates, we herein investigated whether 1,25D3 affected the secretion profile of soluble factors involved in inflammation, angiogenesis and bone remodeling in co-cultures of dTHP-1 and 3D-arranged MSC (3D co-cultures). Co-cultures with MSC seeded directly in flat transwells (conventional/2D co-cultures) were used for comparative purposes. TNF-α and IL-10 levels in co-cultures were not affected by seeding MSC in 3D substrates (Fig. 7A). The effect of 1,25D3 on these inflammatory factors in 3D co-cultures was similar to that observed in 2D co-cultures, decreasing the secretion of TNF-α while conversely stimulating the production of IL-10. Changes in TNF-α and IL-10 secretion induced by 1,25D3 treatment paralleled changes in mRNA levels in co-cultured dTHP-1 (Fig. 7B, left panel). Compared with 2D co-cultures, 3D disposition of MSC co-cultured with dTHP-1 induced an increase in PGE₂ levels while decreasing MCP-1 and MIP-1α secretion (Fig. 7A). PGE₂ production in 3D co-cultures was not affected by 1,25D3 treatment (Fig. 7A), although COX2 mRNA levels were subjected to differential modulation in each cell type, decreasing in co-cultured dTHP-1 and increasing in co-cultured MSC (Fig. 7B). Exposure of 3D co-cultures to 1,25D3 decreased MCP-1 and MIP-1α levels as well as their mRNA levels in co-cultured dTHP-1 (Fig. 7B, left panel). Treatment with 1,25D3 also decreased MCP1 mRNA levels in 3D-arranged MSC co-cultured with dTHP-1 (Fig. 7B, right panel). The levels of MCP-1 and MIP-1α in 1,25D3-treated 3D co-cultures were about 2-fold lower than in 1,25D3-treated 2D co-cultures (Figs 3 and 7). Given both chemokines are involved in the recruitment of monocytes, we investigated whether 1,25D3 treatment of co-cultures influenced THP-1 migration by using CM. Compared with untreated co-cultures, THP-1 recruitment was reduced when CM from co-cultures treated with 1,25D3 were employed as a stimulus (Fig. 7C). THP-1 migration induced by CM of 1,25D3-treated 3D co-cultures was lower than that induced by CM of 2D co-cultures exposed to the hormone.

Figure 8A shows that 3D arrangement of MSC co-cultured with dTHP-1 led to a decrease in the concentration of the pro-resorptive factors RANKL and IL-6. Treatment of 3D co-cultures with 1,25D3 further decreased IL-6 secretion. OPG and VEGF levels, which were similar in both 3D and 2D co-cultures, increased after 1,25D3 treatment. These changes in protein secretion induced by 1,25D3 paralleled changes in OPG and VEGF mRNA levels in co-cultured MSC (Fig. 8A, B). Finally, we studied whether topographical cues in combination with 1,25D3
Figure 5. Effect of 1,25D3 on osteogenic differentiation of MSC exposed to dTHP-1. MSC cultured in isolation or co-cultured with dTHP-1 (Co-MSC) were treated with 10 or 100 nM 1,25D3 or vehicle (−) for 72 h. mRNA fold changes of the indicated genes in MSC and Co-MSC (A). Data are relative to those measured in untreated cells cultured in isolation, which were given the arbitrary value of 1. ALP activity (B) and alizarin red staining and quantification (C) in layers of MSC cultured under osteogenic conditions for 7 days or 14 days, respectively, in non-conditioned media (NCM) or in conditioned media from co-cultures (CM) treated with 100 nM 1,25D3 (100D) or vehicle. As controls, cells were cultured in the absence of osteogenic inductors (Growth). Quantitative data in C are relative to those measured in cells cultured in growth medium, which were given the arbitrary value of 100. *p < 0.05 compared with untreated conditions; #p < 0.05 compared with 10 nM 1,25D3; $p < 0.05 compared with cells cultured in isolation under the same experimental conditions; &p < 0.05 compared with NCM under the same experimental conditions.
treatment could modulate osteogenic differentiation of MSC when co-cultured with dTHP-1. MSC seeded in 3D substrates and co-cultured with dTHP-1 expressed higher \textit{BGLAP} mRNA levels than MSC that had been co-cultured in 2D substrates (Fig. 8C). No changes were observed in \textit{CBFA1}, \textit{ALPL} and \textit{OPN} mRNA levels. When

\textbf{Figure 6.} OPG, RANKL and IL-6 levels in co-cultures treated with 1,25D3. MSC cultured in isolation or co-cultured with dTHP-1 (Co-MSC) were treated with 10 or 100 nM 1,25D3 or vehicle (−) for 72 h. OPG (A), RANKL (B) and IL-6 (C) secretion (left panels) and mRNA fold changes (right panels). In these experiments, a relative secretion value of 100 corresponded to approximately 1.5 ± 0.3 ng of OPG, 38 ± 8 pg of RANKL and 70 ± 17 ng of IL-6 per ml of culture medium. mRNA data are relative to those measured in untreated cells cultured in isolation, which were given the arbitrary value of 1. *p < 0.05 compared with untreated conditions; $p < 0.05$ compared with cells cultured in isolation under the same experimental conditions.
MSC co-cultured in 3D substrates were induced to undergo osteogenic differentiation in the absence of dTHP-1, matrix mineralization was similar to that of MSC that had been co-cultured in 2D substrates (Supplementary Fig. S2). However, experiments using CM revealed that continuous exposure to secreted factors from 3D co-cultures resulted in higher ALP activity and alizarin red staining than exposure to CM from 2D co-cultures (Fig. 8D,E). MSC incubated with CM from 1,25D3-treated co-cultures increased matrix maturation and mineralization, an effect more pronounced with CM from 3D than 2D co-cultures (Fig. 8D,E). We also conducted experiments to evaluate the effect of continuous exposure to CM from co-cultures on the functionality of primary osteoblasts isolated from trabecular bone. As observed for MSC, osteoblasts proliferated at the same rate when incubated in CM from co-cultures treated with 1,25D3 or vehicle (Supplementary Fig. S3). ALP activity and alizarin red staining increased when osteoblasts were incubated in CM from co-cultures treated with 1,25D3, but no differences were found between CM from 2D and 3D co-cultures (Fig. 8D,E).

Discussion

A positive correlation between vitamin D supplementation and bone healing has been observed in studies conducted with animal models, in which the hormone stimulated fracture repair and increased the mechanical strength of the callus24–26. Vitamin D supplementation during the early healing stage promoted new bone formation and increased bone density in mandibular sockets of dogs implanted with biphasic calcium phosphate ceramic27,28. The pro-osteogenic effect of vitamin D has been further substantiated by in vitro and in vivo studies using titanium implants coated with 1,25D3 precursor29,30. In addition to regulating osteoblast functionality, 1,25D3 has been shown to reduce the secretion of inflammatory factors by macrophages, which interact with osteoblast lineage cells during bone healing10,31. The present study provides insight into the mechanisms by which 1,25D3 might accelerate the bone regeneration process through the regulation of paracrine communications between MSC and macrophages. In addition, data herein highlight the importance of paracrine factors in the modulation of the biological actions of 1,25D3, given we have observed differential, and even opposing, effects of the hormone in single cultures of MSC or macrophages and co-cultures of both cell types.
Figure 8. Effect of 1,25D3 on bone-related factors in 3D co-cultures and on osteogenic differentiation. dTHP-1 co-cultured with MSC seeded in 3D substrates were treated with 100 nM 1,25D3 or vehicle (−) for 72 h. Secretion levels of the indicated soluble factors in 3D co-cultures (A). mRNA fold changes of the indicated genes in co-cultured MSC seeded in 3D substrates (B, C). Data are relative to the protein or mRNA values determined in untreated 2D co-cultures, which were given the arbitrary value of 1. ALP activity (D) and alizarin red staining and quantification (E) in layers of MSC or osteoblasts (OB) cultured under osteogenic conditions for 7 days or 14 days, respectively, in conditioned media from 2D co-cultures (CM) or 3D co-cultures (CM3D) treated with 100 nM 1,25D3 (100D) or vehicle. As controls, cells were cultured in the absence of osteogenic inductors (Growth). Quantitative data in E are relative to those measured in cells cultured in growth medium, which were given the arbitrary value of 100. *p < 0.05 compared with untreated 2D co-cultures or CM; †p < 0.05 compared with untreated 3D co-cultures or CM3D; ‡p < 0.05 compared with CM or CM 100D under the same experimental conditions.
Many studies have demonstrated that MSC can regulate the activation and function of macrophages through the secretion of soluble factors. We previously reported that MSC display immunomodulatory effects on dTHP-1, given co-culturing both cell types reduced TNF-α levels and increased IL-10 production. These changes in protein secretion correlated with increased levels of PGE2, MCP-1 and VEGF in the co-culture media. To our knowledge, the effect of 1,25D3 on the secretion of soluble mediators involved in MSC-mediated immunoregulation has not been explored to date. In this study, we detected that 1,25D3 activates MSC to produce PGE2, suggesting that this hormone might be potentiating their immunomodulatory actions. In addition, levels of the pro-angiogenic factor VEGF increase after treatment of MSC with the hormone, as previously observed in endothelial progenitor cells. Co-culture experiments demonstrated that 1,25D3 could regulate the paracrine interactions established between MSC and macrophages by modulating the production of some of these soluble factors. 1,25D3 promoted the switch of co-cultured macrophages toward an anti-inflammatory phenotype because it increased IL-10 expression while having the opposite effect on TNFA. VEGF levels in co-cultures were induced by treatment with the hormone, which likely reflects 1,25D3 enhancement of the angiogenic potential of MSC. As observed in isolated cultures of MSC and dTHP-1, 1,25D3 also decreased MCP-1 levels after co-culturing both cell types. However, 1,25D3 failed to modulate PGE2 levels in co-cultures, although cells cultured in isolation were sensitive to the hormone. These data highlight the importance of paracrine factors in understanding the effects of 1,25D3 on a particular cell type and emphasize the need for cautious extrapolation of data obtained in single-culture models. It is worth noting that dTHP-1 constitutively expresses VDR, but its mRNA and protein levels were not regulated by 1,25D3, as observed in human peripheral blood macrophages. VDR mRNA levels in dTHP-1 increased after co-culturing with MSC, suggesting that soluble factors produced in co-cultures might activate signaling cascades that lead to the regulation of the transcript levels. In this regard, the stress-activated protein kinases p38 and JNK can stimulate VDR promoter activity in human breast cancer cells independently of 1,25D3. Curiously, up-regulation of VDR mRNA levels in dTHP-1 co-cultured with MSC was prevented by 1,25D3, emphasizing the role of the inflammatory milieu in the adjustment of VDR expression by the hormone.

In contrast to dTHP-1, VDR expression in MSC was regulated positively by the hormone. 1,25D3 strongly regulated the stromal expression of the osteogenic markers BGLAP and OPN, which contain functional VDRE in their promoters. Cbfa1 expression was also stimulated by 1,25D3, an effect previously observed in human primary osteoblasts. In addition, TNF-α mRNA levels in dTHP-1 increased after co-culturing of MSC with the macrophages, suggesting that soluble factors produced in co-cultures might activate signaling cascades that lead to the regulation of the transcript levels. In this regard, the stress-activated protein kinases p38 and JNK can stimulate VDR promoter activity in human breast cancer cells independently of 1,25D3. Curiously, up-regulation of VDR mRNA levels in dTHP-1 co-cultured with MSC was prevented by 1,25D3, emphasizing the role of the inflammatory milieu in the adjustment of VDR expression by the hormone.

In contrast to dTHP-1, VDR expression in MSC was regulated positively by the hormone. 1,25D3 strongly regulated the stromal expression of the osteogenic markers BGLAP and OPN, which contain functional VDRE in their promoters. Cbfa1 expression was also stimulated by 1,25D3, an effect previously observed in human primary osteoblasts. In addition, TNF-α mRNA levels in dTHP-1 increased after co-culturing of MSC with the macrophages, suggesting that soluble factors produced in co-cultures might activate signaling cascades that lead to the regulation of the transcript levels. In this regard, the stress-activated protein kinases p38 and JNK can stimulate VDR promoter activity in human breast cancer cells independently of 1,25D3. Curiously, up-regulation of VDR mRNA levels in dTHP-1 co-cultured with MSC was prevented by 1,25D3, emphasizing the role of the inflammatory milieu in the adjustment of VDR expression by the hormone.

In contrast to dTHP-1, VDR expression in MSC was regulated positively by the hormone. 1,25D3 strongly regulated the stromal expression of the osteogenic markers BGLAP and OPN, which contain functional VDRE in their promoters. Cbfa1 expression was also stimulated by 1,25D3, an effect previously observed in human primary osteoblasts. In addition, TNF-α mRNA levels in dTHP-1 increased after co-culturing of MSC with the macrophages, suggesting that soluble factors produced in co-cultures might activate signaling cascades that lead to the regulation of the transcript levels. In this regard, the stress-activated protein kinases p38 and JNK can stimulate VDR promoter activity in human breast cancer cells independently of 1,25D3. Curiously, up-regulation of VDR mRNA levels in dTHP-1 co-cultured with MSC was prevented by 1,25D3, emphasizing the role of the inflammatory milieu in the adjustment of VDR expression by the hormone.
must be related to the lower concentration of pro-inflammatory factors, such as IL-6, MCP-1 and MIP-1α in 3D co-cultures. In fact, there are reports showing that the deleterious effects of inflammatory factors on osteoblast differentiation from MSC are dose-dependent. Induction of matrix mineralization was similar in primary osteoblasts incubated in CM from 2D or 3D co-cultures, suggesting that inflammatory factors modulate the osteogenic differentiation process of undifferentiated cells but do not affect the functionality of terminally differentiated osteoblasts. As expected, we observed that CM from co-cultures containing 1,25D3 stimulated ALP activity and matrix calcification. The increase in osteogenic differentiation of MSC associated with 1,25D3 treatment was higher after incubation with CM from 3D co-cultures than with CM from 2D co-cultures. These results suggest that topographical cues and 1,25D3 might act in concert to accelerate osteoprogenitor commitment by regulating paracrine interactions with macrophage lineage cells.

In summary, our results indicate that 1,25D3 reduces pro-inflammatory cytokine production in co-cultures of MSC and macrophages and promotes MSC osteogenic differentiation in an inflammatory milieu. Current efforts to minimize host reaction following implantation of a biomaterial device include tuning the biomaterial surface as well as delivery of anti-inflammatory drugs from the implanted device. Future studies will investigate whether manufacturing of 3D substrates that provide topographical cues and local delivery of 1,25D3 might promote an ideal balance of soluble mediators for the restoration of functional bone.

Methods

Cell culture. Purified human bone marrow-derived MSC were purchased from Lonza (Basel, Switzerland) and expanded in a defined medium (Lonza) consisting of basal medium and Single Quots growth supplements containing fetal bovine serum (FBS) and antibiotics. All the experiments were performed below the seventh cell passage using six different batches of MSC isolated from donors aged 18–31 years. Human acute monocytic leukemia THP-1 cells (ECACC, Salisbury, Wiltshire, UK) were grown in RPMI-1640 medium (Lonza) containing 10% (v/v) FBS and antibiotics. To induce macrophage differentiation, THP-1 cells were treated with 100 ng/ml TPA (Sigma, Madrid, Spain) for 12 h, washed with phosphate-buffered saline (PBS) and further incubated in fresh medium for 24 h. Human osteoblasts were obtained from trabecular bone explants aseptically collected from patients undergoing total knee replacement as previously described. Patients enrolled in this research signed an informed consent and all the procedures using human tissue designated "surgical waste" were approved by the Ethics Committee for Clinical Research at La Paz University Hospital (Date of Approval: 20/05/2016). All the experiments and methods were performed in accordance with relevant guidelines and regulations. Each bone sample was processed separately and experiments were performed using independent cultures obtained from six patients aged 62–70 years. Experiments were performed with osteoblasts cultured up to the second passage. Bone fragments were cultured in growth medium consisting of Dulbecco’s modified eagle’s medium (DMEM, Lonza) supplemented with 15% (v/v) FBS and antibiotics. Cells were maintained at 37 °C in a humidified 5% CO2 incubator.

Conventional 2D co-cultures of MSC and dTHP-1 were set up using a polyester membrane cell culture insert of 0.4 μm pore size (Corning, Lowell, MA) that allows humoral contact of both cell types in the absence of direct cell-to-cell contact. THP-1 cells were seeded at a density of 4 × 10⁵ cells/well into 6-well plates and treated with TPA, as described above. MSC were seeded at a density of 8 × 10⁴ cells in the inserts and cultured for 24 h. To set up the 3D co-cultures, MSC were seeded in highly porous polystyrene scaffolds (Alvetex®, Reinnervate, Durham, UK), which were placed into the inserts as previously described, and co-cultured with dTHP-1. As controls, only dTHP-1 or MSC were cultured in wells or inserts, respectively. The 1,25D3 (Sigma) was dissolved at 10 mM in 100% ethanol. Once assembled, the co-cultures were incubated in 3 ml of a mix of equal volumes of RPMI and DMEM containing 12.5% FBS, and immediately after, treated with 10 or 100 nM 1,25D3 or vehicle. Based on our previous study on the modulation of the secretory profile of inflammatory markers in co-cultures, the effect of 1,25D3 on paracrine interactions between MSC and dTHP-1 was examined after treatment for 72 h. A schematic illustration of the setup of co-cultures is shown in Supplementary Figure S4. Cell viability remained greater than 90% under all experimental conditions.

Gene expression. Total RNA was isolated using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA), according to the manufacturer’s instructions. Complementary DNAs were prepared from total RNA using the Transcripter First Strand cDNA Synthesis Kit using an anchored-oligo (dT)₁₈ primer (Roche Applied Science, Indianapolis, IN, USA). Real-time quantitative PCR was performed using LightCycler FastStart DNA Master SYBR Green I and LightCycler detector (both from Roche Applied Science). Quantitative expression values were extrapolated from standard curves, and normalized to β2-microglobulin (β2M) values. Specific oligonucleotide primers are shown in Supplementary Table S1.

Immunoenzymatic assays. Cell culture media were collected, filtered and centrifuged at 1200 g for 10 min. Media were supplemented with 2 μg/ml aprogin, 17.5 μg/ml phenyl-methylsulfonyl fluoride, 1 μg/ml pepstatin A and 50 μg/ml bacitracin (all from Sigma) and frozen at −80°C. Human specific ELISA kits were used to measure OPG (Bender MedSystems GmbH, Vienna, Austria), free soluble RANKL (Biomedica Gruppe, Vienna, Austria), MIP-1α (Biosensis, Thebarton, Australia) and PGE₂ (Cayman Chemical Company, Ann Arbor, MI), according to the manufacturer’s instructions. The detection limits of the kits were 2.5 pg/ml for OPG, 1.5 pg/ml for RANKL, 10 pg/ml for MIP-1α and 15 pg/ml for PGE₂. Levels of TNF-α, IL-6, IL-10 and MCP-1 and VEGF were determined using BD CBA Flex Sets (BD Biosciences, San Jose, CA), following the manufacturer’s instructions. The data were acquired using a FACScalibur flow cytometer and analyzed with the FCAP Array Software version 3.0 (BD Biosciences). The detection limits of the CBA Flex Sets were 3.7 pg/ml for TNF-α, 2.5 pg/ml for IL-6, 3.3 pg/ml for IL-10, 1.3 pg/ml for MCP-1 and 4.5 pg/ml for VEGF.
Immunofluorescence assays. Cells were seeded at a density of $2 \times 10^4$ cells/well in 8-well chamber slides and treated with 100 nM 1,25D3 or vehicle. After 3 days in culture, cells were fixed with 4% (w/v) formaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS. For immunostaining, cells were blocked in PBS containing 2% bovine serum albumin (BSA) and 0.05% Tween 20 and then stained with mouse anti-human Oct-4, Sox-2, Stro-1 Abs (all from Chemicon, Harrow, UK) and rabbit anti-human VDR Abs (Santa Cruz, Heidelberg, Germany) diluted 1:50 in 1% BSA in PBS. After washing with 0.05% Tween in PBS, cells were incubated with goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 594 secondary Abs (Molecular Probes, Leiden, Netherlands) diluted 1:1000 (v/v) in PBS containing 1% BSA. After washing with 0.05% Tween in PBS, cells were examined using a confocal microscope (Leica TCS SPE, Leica Microsystems, Heidelberg, Germany).

Flow cytometry assays. Immunofluorescence staining of cell surface antigens was performed by incubating cells for 30 min at 4°C in the dark with mouse anti-human CD44-FITC, CD34-FITC, human leukocyte antigen (HLA)-DR-FITC, CD105-PE, CD14-PE, CD11b-PE, CD29-APC and CD45-APC Abs (all from BD Bioscience, San Jose, CA). Cells incubated in the absence of antibodies were used as negative controls. Cells were then washed 3 times with PBS, fixed with 1% (w/v) formaldehyde in PBS and subjected to flow cytometry using a FACSCalibur analyzer and CellQuest software (BD Biosciences).

Migration assays. Migration assays were conducted using transwell inserts of 5 μm pore size (Corning) placed into 24-well plates. THP-1 cells were washed twice with PBS and resuspended in serum-free RPMI at a density of $1.5 \times 10^6$ cells/ml. Then, 200 μl of cell suspension was added to the upper chamber and 500 μl of CM of co-cultures treated with 100 nM 1,25D3 or vehicle were added to the lower chamber. After 5 h of incubation, cells that migrated to the lower chamber were collected and counted, employing the trypan blue dye exclusion method. Nonspecific migration was evaluated by using culture medium without serum.

ALP activity and cell layer calcification assays. Osteoblasts and MSC were seeded at a density of $1 \times 10^4$ cells/well in 12-well plates and incubated in a mix of equal volumes of osteogenic medium and CM from co-cultures treated with 100 nM 1,25D3 or vehicle. A parallel set of cells were cultured in a mix containing media not conditioned by co-cultures (NCM). To determine MSC osteogenic differentiation after co-culturing, MSC were co-cultured with dTHP-1 for 72 h and then cultured in osteogenic medium in the absence of dTHP-1. Osteogenic medium consisted of growth medium supplemented with $3 \times 10^{-4}$ M ascorbic acid, $10^{-3}$ M β-glycerophosphate and $10^{-3}$ M of dexamethasone (all from Sigma). Cells cultured in growth medium were used as control. Media were replaced every 3 days. After 7 days of culture, cell layers were extracted with $5 \times 10^{-3}$ M Tris–HCl pH 8.0, $5 \times 10^{-3}$ M NaCl and 1% Triton X-100 and supplemented with a mixture of protease inhibitors. ALP activity was assessed in cell layers by determining the release of p-nitrophenol from p-nitrophenyl phosphate (Sigma). The data were normalized to the total protein amounts determined by a Bradford-based protein assay (Bio-Rad Laboratories Inc., Hercules, CA), using BSA as protein standard. The degree of cell layer calcification was assessed in cells cultured for 14 days using alizarin red staining. Briefly, cells were fixed with ethanol and stained with 40 mM alizarin red in deionized water at pH 4.2. The bound stain was eluted with 10% (w/v) cetylpyridinium chloride and the absorbance at 562 nm was measured using a Synergy4 spectrofluorometer (BioTek Instruments, Winooski, VT).

Statistical analysis. The statistical analyses were performed using the Statistical Program for Social Sciences version 11.5 (SPSS Inc, Chicago, IL, USA). Data are presented as means ± SD of at least five independent experiments. Two-way ANOVAs for repeated measures were performed to analyze data from co-culture experiments, factoring for culture conditions and treatments. One-way ANOVAs were performed to analyze data from co-culture experiments. Post hoc comparisons were analyzed by a 95% confidence interval adjusted by the Bonferroni method. The p-values were considered < 0.05 were considered to be significant.

References
1. Kelly, J., Lin, A., Wang, C. J., Park, S. & Nishimura, J. Vitamin D and bone physiology: demonstration of vitamin D deficiency in an implant osseointegration rat model. J. Prosthodont. 18, 473–478 (2009).
2. Brinker, M. R., O’Connor, D. P., Monla, Y. T. & Earthman, T. P. Metabolic and endocrine abnormalities in patients with nonunions. J. Orthop. Trauma 21, 557–570 (2007).
3. Mangelsdorf, D. J. & Evans, R. M. The RXR heterodimers and orphan receptors. Cell 83, 841–850 (1995).
4. Christakos, S., Dhawan, P., Verstuyf, A., Verlinden, L. & Carmeliet, G. Vitamin D: metabolism, molecular mechanism of action, and pleiotropic effects. Physiol. Rev. 96, 365–408 (2016).
5. Gardiner, E. M. et al. Increased formation and decreased resorption of bone in mice with elevated vitamin D receptor in mature cells of the osteoblastic lineage. FASEB J. 14, 1908–1916 (2000).
6. van Driel, M. et al. Evidence that both 1alpha,25-dihydroxyvitamin D3 and 24-hydroxylated D3 enhance human osteoblast differentiation and mineralization. J. Cell. Biochem. 99, 922–935 (2006).
7. Aranow, C. Vitamin D and the immune system. J. Investig. Med. 59, 881–886 (2011).
8. Wang, X., Zhu, J. & Deluca, H. F. Where is the vitamin D receptor? Arch. Biochem. Biophys. 523, 123–133 (2012).
9. Dri Rosa, M., Malaguarnera, M., Nicoletti, F. & Malaguarnera, L. Vitamin D3: A helpful immuno–modulator. Immunology 134, 123–139 (2011).
10. Zhang, X. et al. Vitamin D inhibits monocyte/macrophage proinflammatory cytokine production by targeting MAPK phosphatase-1. J. Immunol. 188, 2127–2135 (2012).
11. Wang, Q. et al. Vitamin D inhibits cox-2 expression and inflammatory response by targeting thioredoxin superfamily member 4. J. Biol. Chem. 289, 11681–11694 (2014).
12. Miron, R. J. & Bosshardt, D. D. OsteoMacs: key players around bone biomaterials. Biomaterials 82, 1–19 (2016).
13. Lee, R. H., Oh, J. Y., Choi, H. & Bazhanov, N. Therapeutic factors secreted by mesenchymal stromal cells and tissue repair. J. Cell. Biochem. 112, 3073–3078 (2011).
14. Cho, D. I. et al. Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages. Exp. Mol. Med. 46, e70 (2014).
15. Swarttouw, M. D. et al. Immunomodulation by mesenchymal stem cells combats the foreign body response to cell-laden synthetic hydrogels. Biomaterials 41, 79–88 (2015).
16. Valles, G. et al. Topographical cues regulate the crosstalk between MSCs and macrophages. Biomaterials 37, 124–133 (2015).
17. Dvorak, G. et al. Impact of dietary vitamin D on osseointegration in the ovariectomized rat. Clin. Oral Implants Res. 23, 1308–1313 (2012).
18. Jingushi, S. et al. Serum 1α,25-Dihydroxyvitamin D 3 accumulates into the fracture callus during rat femoral fracture healing. Endocrinology 139, 1467–1473 (1998).
19. Hakim, I. & Bar-Shavit, Z. Modulation of TNF-alpha expression in bone narrow macrophages: Involvement of vitamin D response element. J. Cell. Biochem. 88, 986–998 (2003).
20. Kerner, S. A., Scott, R. A. & Pike, J. W. Sequence elements in the human osteocalcin gene confer basal activation and inducible response to hormonal vitamin D3. Proc. Natl. Acad. Sci. USA 86, 4455–4459 (1989).
21. Edderkaoui, B. Potential Role of Chemokines in Fracture Repair. Front. Endocrinol. 8, 39 (2017).
22. Lacey, D. C., Simmons, P. J., Graves, S. E. & Hamilton, J. A. Proinflammatory cytokines inhibit osteogenic differentiation from stem cells: implications for bone repair during inflammation. Osteoarthr. Cartil. 17, 735–742 (2009).
23. Mirsaidi, A., Tiaden, A. N. & Richards, P. J. Prostaglandin E2 inhibits matrix mineralization by human bone marrow stromal cell-derived osteoblasts via Epac-dependent cAMP signaling. Sci. Rep. 7, 2243 (2017).
24. Brumbaugh, P. F., Speer, D. P. & Pitt, M. J. 1 alpha,25-Dihydroxyvitamin D3 a metabolite of vitamin D that promotes bone repair. Am. J. Pathol. 106, 171–179 (1982).
25. Delgado-Martinez, A. D., Martinez, M. E., Carrascal, M. T., Rodriguez-Aval, M. & Munuera, L. Effect of 25-OH-vitamin D on fracture healing in elderly rats. J. Orthop. Res. 16, 650–653 (1998).
26. Fu, L., Tang, T., Miao, Y., Hao, Y. & Dai, K. Effect of 1,25-dihydroxy vitamin D3 on fracture healing and bone remodeling in ovariectomized rat femora. Bone 44, 893–898 (2009).
27. Hong, H. H., Chou, T. A., Yang, J. C. & Chang, C. J. The potential effects of cholecalciferol on bone regeneration in dogs. Clin. Oral Implants Res. 23, 1187–1192 (2012).
28. Hong, H. H., Yen, T. H., Hong, A. & Chou, T. A. Association of vitamin D3 with alveolar bone regeneration in dogs. J. Cell. Mol. Med. 19, 1208–1217 (2015).
29. Satue, M., Petzold, C., Córdoba, A., Ramis, J. M. & Monjo, M. UV photovativation of 7-dehydrocholesterol on titanium implants enhances osteoblast differentiation and decreases Rankl gene expression. Acta Biomater. 9, 5759–5770 (2013).
30. Satue, M., Monjo, M., Ronold, H. J., Lyngstadaas, S. P. & Ramis, J. M. Titanium implants coated with UV-irradiated vitamin D precursor and vitamin E: in vivo performance and coating stability. Clin. Oral Implants Res. 28, 424–431 (2017).
31. Takayanagi, H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat. Rev. Immunol. 7, 292–304 (2007).
32. Grundmann, M. et al. Vitamin D improves the angiogenic properties of endothelial progenitor cells. Am. J. Physiol. Cell Physiol. 303, 954–962 (2012).
33. Kreutz, M. et al. 1,25-dihydroxyvitamin D3 production and vitamin D receptor expression are developmentally regulated during differentiation of human monocytes into macrophages. Blood 82, 1300–1307 (1993).
34. Qi, X. et al. The p38 and JNK pathways cooperate to trans-activate vitamin D receptor via c-Jun/AP-1 and sensitize human breast cancer cells to vitamin D3-induced growth inhibition. J. Biol. Chem. 279, 25888–25892 (2002).
35. Noda, M. et al. Identification of a DNA sequence responsible for binding of the 1,25-dihydroxyvitamin D3 receptor and 1,25-dihydroxyvitamin D3 enhancement of mouse secreted phosphoprotein 1 (SPP-1 or osteopontin) gene expression. Proc. Natl. Acad. Sci. USA 87, 9995–9999 (1990).
36. Viereck, V. et al. Differential regulation of Cbfα1/Runx2 and osteocalcin gene expression by vitamin-D3, dexamethasone, and local growth factors in primary human osteoblasts. J. Cell. Biochem. 86, 348–356 (2002).
37. Gilbert, L. et al. Expression of the osteoblast differentiation factor RUNX2 (Cbfα1/AML3/Pebp2alpha A) is inhibited by tumor necrosis factor-alpha. J. Biol. Chem. 277, 2695–2701 (2002).
38. Gilbert, L. et al. Inhibition of osteoblast differentiation by tumor necrosis factor-alpha. Endocrinology 141, 3956–3964 (2000).
39. Kuno, H. et al. Inhibition of 1,25-dihydroxyvitamin D3 stimulated osteocalcin gene transcription by tumor necrosis factor-alpha: structural determinants within the vitamin D response element. Endocrinology 134, 2524–2531 (1994).
40. Piek, E. et al. Osteo-transcriptomics of human mesenchymal stem cells: Accelerated gene expression and osteoblast differentiation induced by vitamin D reveals c-MYC as an enhancer of BMP2-induced osteogenesis. Bone 46, 613–627 (2010).
41. Yamamoto, Y. et al. Vitamin D receptor in osteoblasts is a negative regulator of bone mass control. Endocrinology 154, 1008–1020 (2013).
42. Zhu, H. et al. Tumor necrosis factor-alpha alters the modulatory effects of mesenchymal stem cells on osteoclast formation and function. Stem Cells Dev. 18, 1473–1484 (2009).
43. Damanik, F. F., Rothuizen, T. C., van Bitterswijk, C., Rotmans, J. I. & Moroni, L. Towards an in vitro model mimicking the foreign body response: tailoring the surface properties of biomaterials to modulate extracellular matrix. Sci. Rep. 4, 6325 (2014).
44. Browne, S. & Pandit, A. Biomaterial-mediated modification of the local inflammatory environment. Front. Bioeng. Biotechnol. 3, 1–14 (2015).
45. Bensiamar, F. et al. Bioactivity of dexamethasone-releasing coatings on polymer/magnesium composites. Biomed. Mater. 11, 55011 (2016).
46. Saldaña, L., Bensiamar, F., Boré, A. & Vilaba, N. In search of representative models of human bone-forming cells for cytocompatibility studies. Acta Biomater. 7, 4210–4221 (2011).

Acknowledgements
This work was supported by grants from Instituto Salud Carlos III (ISCIII)-Fondo Europeo de Desarrollo Regional (FEDER), MINECO-AES (P11/00752 and P11/01118), Ministerio de Economía y Competitividad (MINECO) (MAT2012-37736-C05-05) and Comunidad Autónoma de Madrid (CAM) (S2013/MIT-2862). LS is supported by a Miguel Servet contract from ISCIII-MINECO-AES-FEDER-FSE. NV is supported by Program I2 from CAM.

Author Contributions
I.S. conceived the project, designed the experiments, interpreted the results and wrote the manuscript. G.V. was involved in the design of the experiments and analysis of data. F.B. and F.J.M. conducted the experiments. E.G.R. provided the biological samples. N.V. helped to write and critically review the manuscript. All the authors have reviewed the manuscript.
Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-15217-8.

Competing Interests: The authors declare that they have no competing interests.

Change History: A correction to this article has been published and is linked from the HTML version of this paper. The error has been fixed in the paper.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/. © The Author(s) 2017