CCL2, CCL5, and IGF-1 participate in the immunomodulation of osteogenesis during M1/M2 transition *in vitro*

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Abstract: The modulation of macrophage phenotype from pro-inflammatory (M1) to tissue healing (M2) via exogenous addition of interleukin-4 (IL-4) facilitates osteogenesis; however, the molecular mediators underlying this phenomenon remain unknown. This study characterizes the IL-4-dependent paracrine crosstalk between macrophages and osteoprogenitors and its effect on osteogenesis *in vitro*. Primary murine M1 were co-cultured with MC3T3 cells (M1-MC3T3) in both transwell plates and direct co-cultures. To modulate M1 to M2, M1-MC3T3 were treated with IL-4 (20 ng/mL) at day 3 after seeding (M1 + IL-4-MC3T3). Selected molecular targets were assessed at days 3 and 6 after seeding at protein and mRNA levels. Mineralization was assessed at day 21. Transwell M1 + IL-4-MC3T3 significantly enhanced the secretion of CCL2/MCP-1, IGF-1 and to a lesser degree, CCL5/RANTES at day 6. At day 3, alkaline phosphatase (*Alpl*) was upregulated in direct M1-MC3T3. At day 6, *Smurf2* and Insulin growth factor-1 (*IGF-1*) were downregulated and upregulated, respectively, in direct M1 + IL-4-MC3T3. Finally, M1 + IL-4-MC3T3 increased bone matrix mineralization compared with MC3T3 cells in transwell, but this was significantly less than M1-MC3T3. Taken together, macrophage subtypes enhanced the osteogenesis in transwell setting and the transition from M1 to M2 was associated with an increase in bone anabolic factors CCL2/MCP-1, CCL5/RANTES and IGF-1 *in vitro*. © 2017 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 105A: 3069–3076, 2017.

Key Words: CCL2/MCP-1, CCL5/RANTES, macrophages, insulin growth factor-1, osteogenesis

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INTRODUCTION

Tissue injury activates the characteristic host response featured by inflammation followed by tissue-specific repair.[1–4] Macrophages are multifunctional myelomonocytic derived cells that orchestrate inflammation and participate in tissue repair, leading either to full restitution of a morphological and functional tissue (regeneration)[2,4] or to incomplete repair via a dysmorphic and nonfunctional scar (fibrosis).[2–4] Thus, macrophages have been proposed as a "master switch" between inflammation, tissue regeneration, and scar formation.[2] In this line, modulation of the macrophage-driven inflammatory step after injury is a potential therapeutic approach for regenerative medicine.

Immediately after bone injury, naive macrophages (M0) are recruited into the injured site and subsequently activated by local environmental cues. At the injury site, the release of danger signal molecules induces classical activation of macrophages. This involves phenotypic and functional changes acquiring the proinflammatory phenotype (M1) by activating nuclear factor-kappa B (NF-κB), the master proinflammatory transcription factor. Activation of NF-kB leads to production of chemokines and cytokines, including macrophage-chemoattractant protein (MCP-1 also known as CCL2), regulated on activation normal T cell expressed and secreted (RANTES, CCL5), tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, and IL-8 among others. These factors mediate the acute inflammatory response.[5] With the disruption of local vasculature, bone injuries also represent an acute ischemic event for the tissue.[6] Hypoxia induces the homing of hematopoietic-,

mesenchymal-, and endothelial stem cells and progenitors from local and systemic reservoirs to the injured site.[6] Indeed, injured tissues activate the hypoxia-inducible factor (HIF)-1/stromal cell-derived factor (SDF)-1 axis, which also activates NF-κB, thus synergizing to the release of proin-flammatory mediators.[6]

Once proinflammatory signals are cleared out and the recruitment of inflammatory precursors subsequently aborted (after ~72 h), macrophages are reprogrammed to an anabolic phenotype, initiating the resolution phase of inflammation, a central step in re-establishing tissue homeostasis.[7] Accordingly, macrophages are alternatively activated to the anabolic macrophage phenotype (M2), characterized by the secretion of the anti-inflammatory cytokines IL-4, IL-10, IL-1ra, and growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor-BB (PDGF-BB), and insulin growth factor-1 (IGF-1).[8–10] M2 and recruited stromal stem cells contribute to the formation of a fibrovascular granulation tissue, ultimately resulting in local bone formation.[4]

The advantages of sequential transition from M1 to M2 to tissue regeneration, rather than complete abolition of the M1-driven inflammation were recently investigated.[8,11,12] We reported the improvement of osteogenesis by modeling the physiological transition from acute inflammation to tissue regeneration *in vitro* by modulating macrophage phenotype from M1- to M2-like cells via administration of M2 polarizing IL-4 at day 3 of direct co-culture.[12] However, the necessity of cell-to-cell contact and details of the paracrine crosstalk among cells of the macrophage and osteoprogenitor lineages underlying this transition remain poorly understood. In this line, we hypothesize that the exogenous addition of IL-4 to M1-preosteoblast co-cultures will promote the secretion of both inflammatory chemokines and cytokines (CCL2/MCP-1, CCL5/RANTES, IL-1β, and IL-10) and tissue anabolic growth factors (VEGF, PDGF-BB, and IGF-1). The aim of this study was to characterize the paracrine cross-talk established between these cell lineages during the resolution phase of inflammation *in vitro*. Also, this study aimed to correlate the secretion of these factors with bone matrix mineralization, the last event in osteogenesis. We demonstrate that M1-, M1 + IL-4-, and M2- macrophages promote MC3T3-osteogenesis also in a transwell co-culture by the paracrine cross-talk that involves at least CCL2/MCP-1, CCL5/RANTES, and IGF-1 *in vitro*.

MATERIALS AND METHODS
Experimental design
MC3T3 cells were co-cultured with M1 macrophages in both transwell [Fig. 1(a)] and standard culture plate systems [Fig. 1(b)]. Seventy-two hours after seeding, IL-4 was added to the co-cultures to polarize the M1 to the M2 phenotype according to an established protocol[12] [Fig. 1(a,b)].

Macrophage isolation and polarization
Institutional guidelines for the care and use of laboratory animals were carefully followed in all aspects of this study. Murine bone marrow macrophages were isolated as described by Pajarinen.[13] Stanford's Administrative Panel on Laboratory
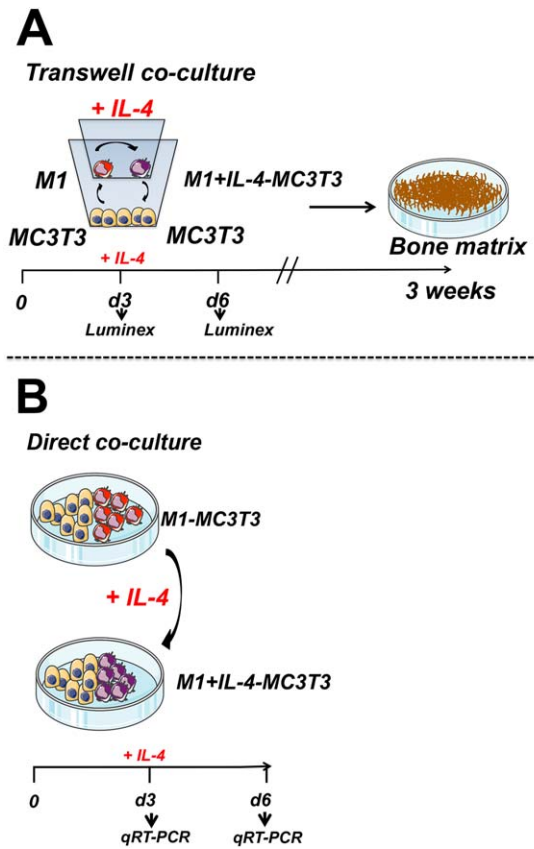

FIGURE 1. Approaches to assess the effect of induced transition from M1 to M2 phenotype via added IL-4 on osteogenesis by MC3T3 cells. (a) Transwell co-culture of M1 and MC3T3. IL-4 was administered at day 3 after seeding to modulate M1 into M1 + IL-4 macrophages. Then, mineralization of the bone matrix was evaluated at week 3 (endpoint). (b) Relative expression of osteogenic-, inflammatory-, and growth factors-related genes was assessed at days 3 and 6 after seeding from direct co-culture of M1-MC3T3 with or without IL-4 by qRT-PCR.

Animal Care (APLAC) approved this isolation protocol. Frozen macrophages were thawed in T-175 culture flasks (8×10^6 cells/flask). Confluent macrophages were polarized to M1 or M2 phenotype by 24 h exposure to basal medium (RPMI 1640, 10% heat inactivated fetal bovine serum (FBS), 1% antibiotic/antimycotic (all from Life Technologies, Carlsbad, CA), and 30% L929 conditioned medium (LCM)), with 100 ng/mL lipopolysaccharide (LPS) (Sigma Aldrich, St. Louis, MO), or with 20 ng/mL IL-4 (R&D Systems, Minneapolis, MN), respectively. Macrophage phenotype (TNFα for M1; CD206 for M2) was confirmed as established in our in our previous study.[12]

Co-cultures
Transwell co-culture. The optimal ratio 1:1 of M1 or M2 together with MC3T3 cells was defined in a pilot experiment (data not shown) and by our previous study.[12] Thus, 10^4 polarized M1 or M2 macrophages were seeded in the insert component of 24-well transwell co-culture system (0.4 μm) (Corning Inc., Lowell, MA) in mixed medium (1:1 ratio mix of minimum essential medium [MEMα; Life Technologies] and RPMI 1640 with 10% FBS, 5% LCM, 1%

antibiotic/antimycotic, 1% GlutaMAX, 50 μg/mL L-ascorbic acid [Sigma-Aldrich], 10 mM β-glycerophosphate [Sigma-Aldrich], and 10 nM dexamethasone [Sigma-Aldrich]) [Fig. 1(a)]. Then, 10^4 MC3T3-E1 subclone 4 cells (10^4/well; ATCC, Manassas, VA) were seeded in the bottom plate of 24-well transwell plate in mixed medium [Fig. 1(a)]. Seventy-two hours after seeding, IL-4 was added to the M1-MC3T3 transwell co-cultures to reach a final IL-4 concentration of 20 ng/mL [Fig. 1(a)]. For controls, single M1, M2, and MC3T3 cells (10^4/well) were plated in the transwell system in MC3T3 growth medium (MEMα, 10% FBS, and 1% antibiotic/antimycotic) or mixed medium, with or without IL-4 administration at 72 h. Media was changed twice a week for 3 weeks.

Direct co-culture. Macrophages were polarized prior to plating as described above. For untreated M1-MC3T3 co-cultures, M1 macrophages (10^4/well) were seeded simultaneously with MC3T3 cells in 24-well plates in mixed medium [Fig. 1(b)]. For IL-4 treated M1-MC3T3 co-culture (M1 + IL-4-MC3T3), M1 and MC3T3 co-cultures were set up as described above. Then, IL-4 was administered once at day 3 after seeding to reach the final concentration of 20 ng/mL [Fig. 1(b)]. For controls, MC3T3 cells (10^4/well) were plated in 24-well plates in MC3T3 growth medium or mixed medium, with or without IL-4 administration at 72 h. Media were changed twice a week for 3 weeks.

RNA isolation and qRT-PCR
After 3 and 6 days of co-cultures, RNA was extracted using Qiagen's RNeasy Mini Kit (Valencia, CA) and reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). qRT-PCR was performed using TaqMan Gene Expression Master Mix and *18 s*, *TNF-α*, *Cd206*, runt-related transcription factor 2 (*Runx2*), alkaline phosphatase, liver/bone/kidney (*Alpl*), SMAD-specific E3 ubiquitin protein ligase 2 (*Smurf2*), transcriptional co-activator with PDZ-binding motif (*Taz*), insulin growth factor-1 *(Igf-1)*, and vascular endothelial growth factor (*Vegfa*) probes on an ABI 7900HT Sequencing Detection System (all from Applied Biosystems). The GenBank accession numbers of the probe sequences are *18 s*: NR_003278, *Tnf-α*: NM_013693 and NM_001278601, *Cd206*: NM_008625 XM_001003164 XM_001003168, *Runx2*: NM_001146038, *Alpl*: NM_007431, *Smurf2*: NM_025481 XM_126673, *Taz* (*Wwtr1*): NM_001168281, *Igf-1*: NM_001111274, *Vegfa*: NM_001025250. *18 s* rRNA was the internal control. Relative gene expression was quantified with the comparative Ct method.[14]

Luminex
Secreted levels of inflammatory chemo and cytokines (CCL2/MCP-1, CCL5/RANTES, IL-1β, and IL-10) and growth factors (VEGF, PDGF-BB, and IGF-1) were assessed from day 3 and day 6 co-culture supernatants by Magnetic Luminex screening Assay (R&D Systems Inc.; Minneapolis, MN) following manufacturer's instructions.

Alizarin Red staining
Three weeks after seeding, co-cultures were stained with Alizarin Red (pH 4.1, Sigma-Aldrich) for analysis of bone matrix formation. Plates were scanned with Perfection 1640SU (Epson, Long Beach, CA). Staining was eluted with 10% cetylpyridinium chloride (Sigma-Aldrich) and absorbance was measured at 562 nm.

Statistical analysis
Analyses were performed with GraphPad Prism version 6.04 (San Diego, CA). Unpaired *t* test was used to analyze the difference between groups of two. Analyses for more than two groups were conducted with a one-way ANOVA followed by Tukey's multiple comparisons test to compare each group (untreated co-culture experiment) or Dunnett's multiple comparisons test to compare to the control group (IL-4 treated co-culture experiment). $p < 0.05$ was considered statistically significant. Data are the results of at least three independent experiments and are expressed as mean ± standard deviation of the mean (SD), where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

RESULTS
Added IL-4 modulates M1 into M2-like phenotype in direct co-cultures with MC3T3
To confirm the successful modulation of macrophage phenotype by added IL-4,[12] the expression of *TNF-α* and *Cd206* was assessed by qRT-PCR 3 days after the addition of IL-4 to the direct co-cultures at day 6. As expected, the addition of IL-4 to the cultures resulted to downregulation of M1-related *TNF-α* ($p < 0.05$) and upregulation of M2-related *Cd206* ($p < 0.01$) (Supporting Information, Fig. 1).

M0, M1, and M2 macrophages promote osteogenesis in transwell co-cultures
We previously determined that the M0, M1, and M2 macrophages at 1:1 ratio to MC3T3 significantly increased osteogenesis by MC3T3 in transwells ($p < 0.0001$) in a pilot experiment (Data not shown). Subsequently, to determine how the transition from M1 to M2 via administration of exogenous IL-4 influenced osteogenesis by MC3T3 in transwell system, we evaluated the mineralization of bone matrix, using the same methods. While either M1, M1 + IL-4, and M2 enhanced osteogenesis by MC3T3 compared with single cultured MC3T3 ($p < 0.00001$), M1 + IL-4 cells exhibited a significantly smaller increase in matrix mineralization than those exerted by M1 ($p < 0.00001$) and M2 ($p < 0.00001$) on MC3T3 cells (Fig. 2).

Macrophages and addition of IL-4 impacted the levels of CCL2, CCL4, IFG-1, and VEGF in transwell co-cultures
To characterize the cross-talk between M1 and MC3T3 and the effect of IL-4 on the co-cultures during the transition to M2-like (M1 + IL-4) phenotype, we assayed the secreted-levels of selected chemokines, cytokines, and growth factors from the transwell co-culture supernatant at day 3 (for M1-MC3T3) and day 6 (for M1 + IL-4-MC3T3) (Fig. 3). Although we assayed a total of 7 analytes, only 4 of them (CCL2/
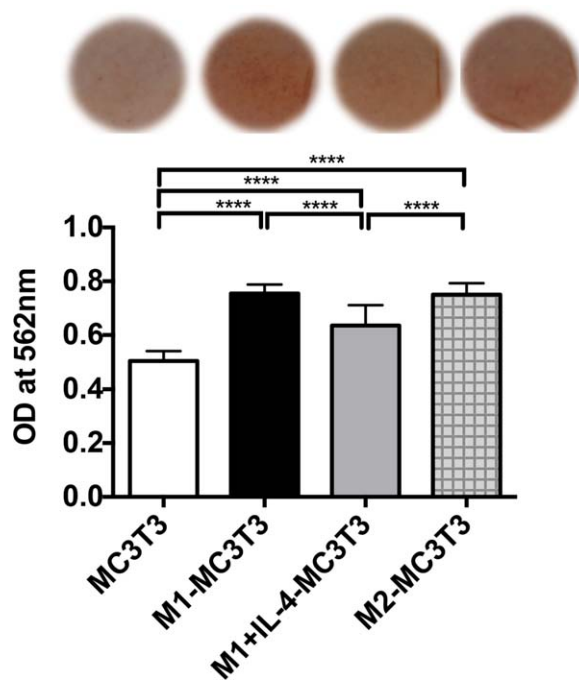
FIGURE 2. Mineralization of bone matrix by single MC3T3, M1-MC3T3 with or without IL-4, and M2-MC3T3 co-cultures was assessed in transwell by Alizarin Red assay at week 3 (OD) optical density, ****$p < 0.0001$.

MCP-1, IGF-1, CCL5/RANTES, and VEGF) were elevated in the culture supernatants.

CCL2/MCP-1. At day 3 [Fig. 3(b), left panel], we observed that M1-MC3T3 co-cultures secreted a significantly high level of CCL2/MCP-1 compared with single M1 ($p = 0.0054$) or MC3T3 cultures ($p < 0.00001$). The main source of CCL2/MCP-1 was M1 compared with MC3T3 ($p = 0.0001$). At day 6 [Fig. 3(b), right panel], the secreted levels of CCL2/MCP-1 were globally reduced compared with those observed at day 3. In fact, single M1 and MC3T3 cultures, and M1- and M2-MC3T3 co-cultures secreted similar levels of CCL2/MCP-1. However, in IL-4 treated M1-MC3T3 cells (M1 + IL-4-MC3T3), increased amounts of CCL2/MCP1 were detected and the concentrations were close to the ones observed at day 3 ($p < 0.0001$).

IGF-1. At day 3 [Fig. 3(c), left panel], the main source of IGF-1 was MC3T3 compared with low level secreted by M1 ($p < 0.00001$). IGF-1 secreted by M1–MC3T3 co-culture was significantly reduced compared to MC3T3 alone ($p < 0.00001$). At day 6, the main source of IGF-1 was MC3T3 compared with M1 or M2 ($p < 0.0001$) [Fig. 3(c), right panel]. The IL-4 treatment (M1 + IL-4-MC3T3) significantly enhanced the secretion of IGF-1 compared with untreated M1–MC3T3 ($p = 0.0004$). Finally, the highest levels of IGF-1 were secreted by M2–MC3T3 cells compared with MC3T3 ($p < 0001$), M1–MC3T3 ($p < 0.0001$), and M1 + IL-4-MC3T3 ($p < 0.005$).

CCL5/RANTES. At day 3, both M1 and M1–MC3T3 groups enhanced the secretion of CCL5/RANTES compared with single MC3T3 cultures ($p < 0.0001$) [Fig. 3(d), left panel]. However, day 6 CCL5/RANTES levels were globally undetectable. Only the IL-4 treated M1–MC3T3 group (M1 + IL-4-MC3T3) enhanced the secretion of CCL5/RANTES ($p < 0.0001$) [Fig. 3(d), right panel].

VEGF. At day 3, M1, MC3T3, and M1–MC3T3 groups secreted similar high levels of VEGF [Fig. 3(e), left panel]. At day 6, MC3T3 significantly decreased ($p < 0.0001$) the level of VEGF compared with M1, M2, and M1–MC3T3 [Fig. 3(e), right panel]. Moreover, the IL-4 treated M1–MC3T3 group (M1 + IL-4-MC3T3) significantly reduced the secretion of VEGF compared with M1–MC3T3 ($p < 0.0001$) and M2–MC3T3 ($p < 0.0001$) [Fig. 3(e), right panel].

Addition of IL-4 triggers pro-osteogenic signals by downregulating Smurf2 and upregulating IGF-1 in direct co-cultures

To further characterize the mechanisms of M1 macrophage and IL-4-induced increase in osteogenesis in direct co-cultures, we assessed the relative expression of both the classic osteogenic-related (*Runx2* and *Alpl*) and the NF-kB-related (*Smurf2* and *Taz*) genes from IL-4 untreated and treated (M1 + IL-4-MC3T3) M1–MC3T3 direct co-cultures at days 3 and 6 by qRT-PCR. Using the same methods, we characterized the mRNA relative expression of growth factors *IGF-1* and *Vegfa* at day 6 (Fig. 4). While *Runx2* was downregulated by M1–MC3T3 at day 3 compared with single MC3T3 culture ($p < 0.001$), no difference between M1–MC3T3 and M1 + IL-4-MC3T3 groups was detected at day 6 [Fig. 4(b)]. *Alpl* was upregulated at day 3 by M1–MC3T3 ($p < 0.001$), and downregulated by M1 + IL-4-MC3T3 at day 6 ($p < 0.05$) compared with MC3T3 [Fig. 4(c)]. *Smurf2* was upregulated by M1–MC3T3 at day 3 ($p = 0.09$) and significantly downregulated by M1 + IL-4-MC3T3 at day 6 ($p < 0.05$) [Fig. 4(d)]. No differences were registered in *Taz* expression at days 3 and 6 [Fig. 4(e)]. Interestingly, M1 + IL-4-MC3T3 upregulated *IGF-1* ($p < 0.001$) at day 6 compared with both single MC3T3 and M1–MC3T3 [Fig. 4(f)]. Finally, no difference was observed in *Vegfa* expression at day 6 [Fig. 4(g)].

DISCUSSION

Bone injuries represent tissue damaging and ischemic insults that trigger both inflammatory and hypoxic responses.[15] Inflammatory and bone cells establish highly coordinated temporal- and spatially-dependent cross-talk to fully reconstitute the oxygen level, eliminate cell and tissue byproducts, and facilitate bone repair leading to either bone regeneration or fibrosis.[4] Recently, we established the importance of a transient M1-proinflammatory period followed by IL-4-induced and M2-driven anti-inflammatory period to optimize osteogenesis by MC3T3 in direct co-cultures. Indeed, this study represents an *in vitro* model of the accelerated resolution phase of inflammation. As the
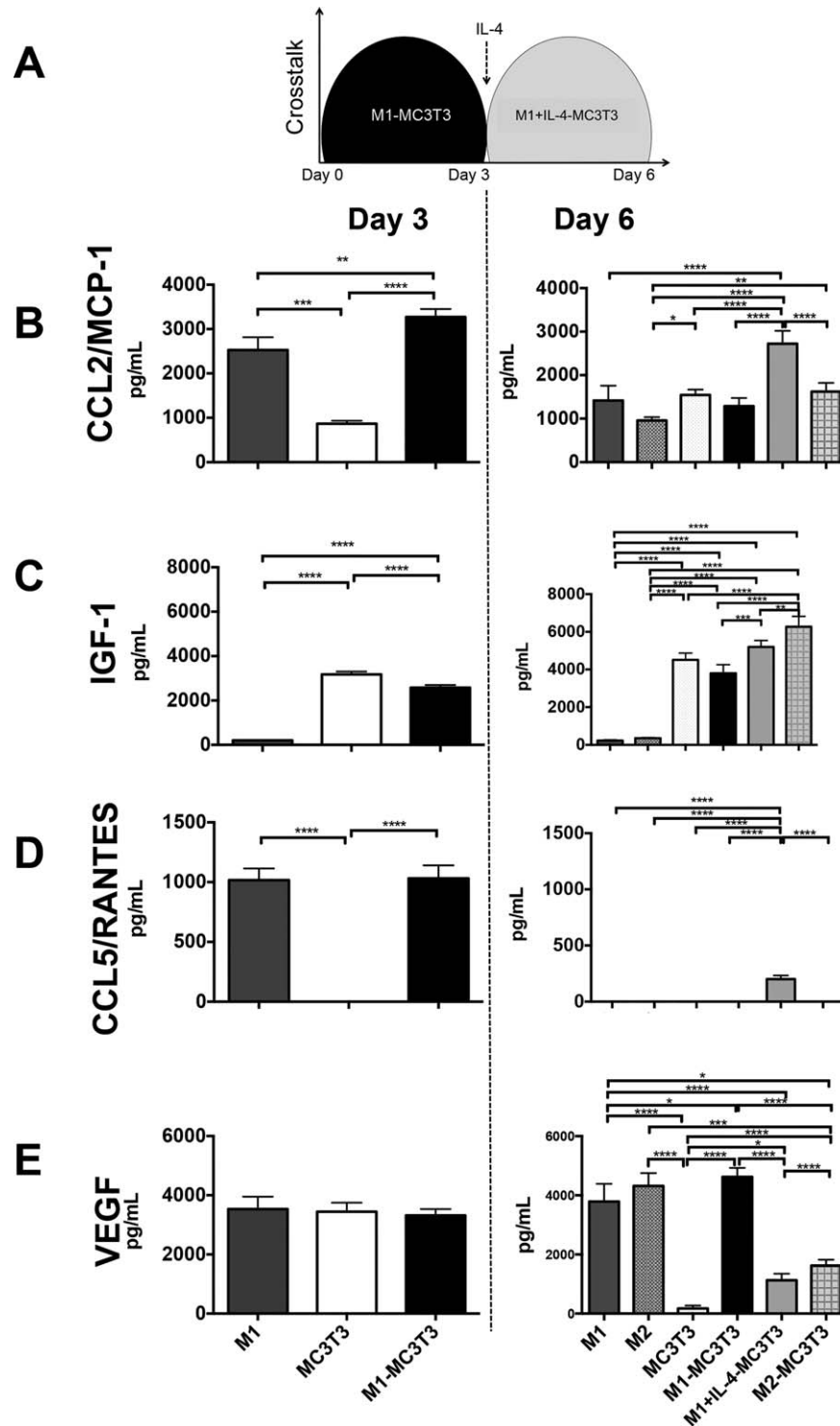
FIGURE 3. Added IL-4 promotes the secretion of CCL2/MCP-1, IGF-1, and CCL5/RANTES. (a) Protein levels from M1-MC3T3 and IL-4 treated M1-MC3T3 (M1 + IL-4-MC3T3) transwell co-cultures were assayed from transwell supernatants at days 3 and 6 after seeding respectively by Luminex. (b–e) Day 3 (left panels) and day 6 (right panels) CCL2/MCP-1, IGF-1, CCL5/RANTES, and VEGF protein secretions were quantified by Luminex, respectively. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; and $****p < 0.0001$.

mechanisms underlying this phenomenon remain unclear, we characterized the cross-talk between M1-, M1 + IL-4-, and M2–MC3T3 in an early stage of co-culture (days 3 and 6) using the transwell and direct cell–cell interaction culture systems. Moreover, we associated these findings with

osteogenesis at week 3 in the same co-culture conditions. In this study, we first determined that 10^4 macrophage subsets seeded in the insert and 10^4 MC3T3 seeded in the bottom well at a 1:1 ratio were able to improve osteogenesis at week 3 in the transwell system. These results suggest that
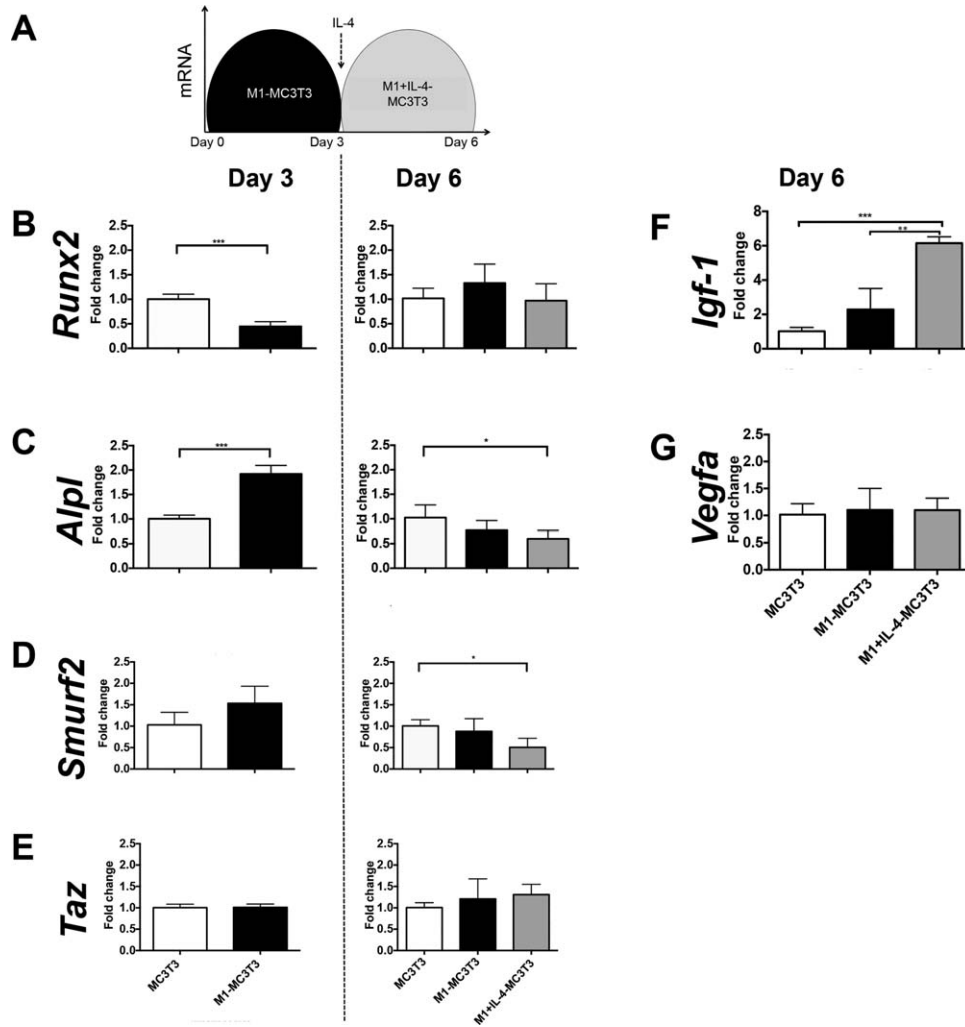
FIGURE 4. IL-4 triggers pro-osteogenic signals by downregulating *Smurf2* and upregulating *Igf-1*. (a) mRNA relative expression were determined from cell lysate by qRT-PCR at day 3 and 6 of direct co-culture. (b,c) Day 3 (left panels) and day 6 (right panels) osteogenic related genes (*Runx2* and *Alpl*). (d,e) Day 3 (left panels) and day 6 (right panels) NF-kB related genes (*Smurf2* and *Taz*). (f) Day 6 *Igf-1* and (g) day 6 *Vegfa*. $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$.

the impact of macrophages on MC3T3 osteogenic cell differentiation is mediated not only by direct cell contact as we demonstrated before but also by paracrine factors secreted by macrophages. Then, as previously reported, we confirmed the polarizing effect of 20 ng/mL of IL-4, by observing downregulation of *TNF-α* and upregulation of *Cd206* by direct M1 + IL-4-MC3T3 co-culture. We assessed the phenotype of co-cultured macrophages from cell lysates of direct macrophage–MC3T3 co-cultures as previously reported,[12] due to the technical difficulties to detach them from the thin membrane of the transwell insert using chemical and physical methods. Also, our group has utilized the methods to polarize naive macrophages into M1 and M2 phenotypes.[12,13]

MC3T3 is a clonal nontransformed cell line established from newborn mouse calvaria, which is a representative model of the preosteoblastic stage of differentiation.[16] MC3T3 cells exhibit alkaline phosphatase activity, synthesize collagen, and mineralize *in vitro*. However, the generation of subclones after extended passages (>P30) make them lose their ability to mineralize.[16–18] To prevent this reported issue, we considered MC3T3-E1 subclone 4 until passage 4 in our co-culture experiments.[17,18] Thus, the mineralization of the MC3T3-synthesized bone matrix was the most important endpoint outcome of this study. Here, we demonstrated that macrophage subsets regulate osteogenesis by MC3T3 cells by paracrine signals. In our previous study, we demonstrated that macrophage subsets regulate osteogenesis by juxtacrine signaling.[12] Somewhat unexpectedly, transwell co-cultured M1 + IL-4 macrophages were unable to optimize osteogenesis by MC3T3 cells at the same level of directly co-cultured M1 + IL-4-MC3T3. This controversial result suggests the potential role of adhesion molecules (e.g., E-selectin, VCAM-1) on osteogenesis.[19,20] While the control of early osteogenic programs during the transition from M1 to M2 involves both cell–cell contact and environmental cues, we propose that the tridimensional contact between macrophage subsets and MC3T3 cells is a crucial aspect.

To better understand the cross-talk between MC3T3 cells, macrophage subtypes, and added IL-4 in both transwell and direct co-cultures, we determined the levels of selected cytokines with multiplex assay and the expression of selected osteogenic factors with qRT PCR. Intriguingly, the proinflammatory environment (M1–MC3T3) activated both the antiosteogenic (downregulation of *Runx2* and upregulation of *Smurf2*) and the pro-osteogenic pathways at day 3 (upregulation of alkaline phosphatase). In this line, we previously reported that M1–MC3T3 secrets high levels of TNF-α and IL-1ra, the antagonist of IL-1β, at the same time point.[12] Taken together, these findings are consistent with the antiosteogenic effect of a proinflammatory environment created by M1 (TNF-α and IL-1β), by degrading *Runx2* and upregulating both *Smurf1* and *Smurf2*.[21,22] However, inflammation has also been reported as a promoter of osteogenesis. This paradoxical effect has been associated to the amount of TNF-α in the microenvironment.[22,23] Indeed, a low concentration of TNF-α promoted osteogenesis and mineralization and on the contrary, a high concentration decreases the same outcome.[22–24] IL-4 added to the cultures at day 3 induced generally a pro-osteogenic effect by inducing bone anabolic signals (downregulation of *Smurf2* and increased secretion of CCL2/MCP-1, CCL5/RANTES, and IGF-1).

As expected, M1-secreted CCL2/MCP-1 and CCL5/RANTES dominated the early inflammatory phase of co-culture (day 3); however, only CCL2/MCP-1 was significantly increased by M1 + IL-4-MC3T3 co-culture (day 6). Otherwise, CCL5/RANTES peaked transiently at day 3, with globally low or undetectable levels at day 6. While CCL-2/MCP-1 and CCL5/RANTES are leukocyte chemoattractants mainly secreted by M1,[5] CCL5/RANTES has been reported as responsible for the homing of endothelial stem cells during the initiation phase of angiogenesis in tissue engineered constructs.[8,25,26] Specifically for bone tissue, an increased level of CCL2/MCP-1 transcript was strongly associated with the administration of the bone anabolic treatment (parathyroid hormone) in rats.[27,28] Also, increased expression of CCL2/MCP-1 was reported in periosteal cells at the early stage of fracture healing.[29] On the other hand, the pro-osteogenic role of CCL5/RANTES was recently reported in human mesenchymal stem cells *in vitro*.[26] Besides, CCL5/RANTES is a key regulator of bone remodeling in immune-associated conditions such as rheumatoid arthritis or multiple myeloma.[29] Taken together, these findings suggest that the first three days are a critical period to gather the stock of both inflammatory and vascular cell precursors for the following steps of bone repair.

The peak of CCL2/MCP-1 in M1 + IL-4-MC3T3 cultures at day 6 can also be explained by a positive regulatory loop established between basal levels of CCL2/MCP-1 and the exogenous administration of IL-4. Gu et al. (2000) and Li et al. (2015) reported that CCL2/MCP1 promotes both the secretion of endogenous IL-4[30] *in vivo* and the M2 polarization of tumor-associated macrophages (TAM) *in vivo*.[31] Alternatively, the low levels of CCL2/MCP-1 secreted by MC3T3 at day 3 and at day 6 could be explained by the presence of proinflammatory cytokines in culture mixed media, which contains LCM.[32] Similarly, Bussard et al.

(2010) reported that the osteoblastic cell lines are important sources of inflammatory signals when they are in a tumor microenvironment.[33]

IGF-1 is an anabolic growth factor that regulates skeletal modeling,[34] bone remodeling,[3,34,35] and fracture healing.[36] IGF-1 is similarly expressed by both M1 and M2 macrophages with a transient upregulation during the resolution phase of inflammation.[11] Mechanistically, IGF-1 acts on MC3T3 cells by increasing protein synthesis via the autocrine loop of AKT/m-TOR pathway.[37] We observed that MC3T3 cells, the most important source of IGF-1, decrease IGF-1 levels when they were co-cultured with M1 and on the contrary, they raised IGF-1 levels when they were co-cultured with any IL-4 polarized M2 phenotype (M1 + IL-4 and M2). These findings confirmed that M1 and M2 regulate negatively and positively, respectively, the synthesis and secretion of IGF-1 by MC3T3 cells. We hypothesize that the exogenous administration of IL-4 to M1 and the endogenous secretion of IL-4 by M2[10] could be involved with this differential regulation of IGF-1 synthesis. In addition, Barret et al. (2015) reported that autocrine signalization of IGF-1 contributes to the adoption of the full IL-4-dependent M2 phenotype.[38]

VEGF secreted by both osteoblasts and macrophages is a known critical growth factor for fracture healing.[4,39] While VEGF has usually been associated to the tissue remodeling M2, recent studies showed that M1 is an important source of VEGF *in vitro*[10] and in tissue engineered constructs.[8] Our findings confirmed that M1 with and without MC3T3 cells was the most stable source of VEGF at both time points and, that the role of MC3T3-secreted VEGF is only transient at day 3. High levels of M1-secreted VEGF were also described by Spiller et al. (2014), using primary human blood-derived macrophage phenotypes seeded on a tissue-engineered scaffold.[8] These findings could be associated with the important chemoattractant role exerted by VEGF on circulating monocytes,[40] contributing to M1 functions. Further studies are needed to elucidate this relationship and the potential role of IL-4 on VEGF secretion.

In conclusion, we demonstrate that macrophage subtypes promote MC3T3-mediated bone formation also in a transwell setting. Adding IL-4 to the transwell co-cultures increased the secretion of bone anabolic factors including CCL2/MCP-1, CCL5/RANTES, and IGF-1, but it was not effective in enhancing the overall bone formation in the absence of cell-to-cell contact. While this study suggests that CCL2/MCP-1, CCL5/RANTES, and IGF-1 participate in the cross-talk between macrophages and MC3T3 cells during the resolution phase of inflammation *in vitro*, further functional studies, for example, with blocking antibodies are needed to elucidate the role of each of the above pathways on osteogenesis during the resolution of inflammation. The possible modulation of these detected molecules—or their receptors—may lead to developing a targeted therapy to optimize the bone healing in clinical settings.

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