GM-CSF and GM-CSF-induced dendritic cells enhance fracture repair by stimulating the proliferation of pluripotent stem cells

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INTRODUCTION:

Fracture is among the most prevalent public health concerns. Despite optimal treatment, nonunion remains a challenging problem, occurring in 10.9% and 4.7% of patients with open and closed fractures, respectively[1]. Fracture heal begins with mesenchymal stem cells (MSCs) differentiate into chondrocytes and osteoblasts, forming cartilage and bone matrix to bridge the fracture. Despite decades of research, clinical application of MSCs for treating fracture nonunion has shown limited success. One possible issue is that the MSCs currently used in therapy are often partially committed or terminally differentiated, limiting their osteogenic potential. In addition, MSCs require support from accessory cells to effectively regenerate bone, but the identity and role of these supporting cell populations remain poorly defined. Identifying naturally occurring pluripotent stem cells (PSCs) with strong bone-healing potential, along with the factors that activate them, is critical for developing new therapeutic strategies for fracture nonunion. Macrophages are considered the most important immune cells contributing to fracture healing. However, transplanted Macs fail to improve bone defect repair [2]. Dendritic cells (DCs) and Macs are derived from same macrophage/dendritic progenitor cells. DCs are present in hematoma after fracture [3,4]. However, their role in fracture healing remains unknown. The aim of this project was to test the role of DCs on fracture healing and the mechanism involved.

METHODS:

(**1**) The effects of GM-CSF (GM) and GM-induced DCs (GM-IDCs) on osteoblast (OB) differentiation were tested using three culture systems: **a)** Bone marrow (BM) cells from adult male or female C57BL/6 mice were first stimulated with GM vs M-CSF, TNF and Flt3L to expand stromal cell populations enriched with myeloid cells. This was followed by induction of OB differentiation; **b**) purified bone-derived mesenchymal progenitor cells (BdMPCs) from adult male or female mice; and **c**) Co-culture of BdMPCs and GM-IDCs cultured adult male or female mouse PBMCs. (**2**) Cellular components of BM stromal cells, BdMPCs and GM-IDCs were tested by flow cytometry. (**3**) The effect of GM on the proliferation of MSCs in BM stromal cells enriched with myeloid cells and purified BdMDPs was assessed through cell cycle analysis. (**4**) Evaluate the effect of locally delivered GM-IDCs and GM via fibrin sealant on fracture healing in a critical-sized bone defect (CSBD) model and a large bone defect window model, respectively, in adult male C57Bl6 mice. (**5**) Flow cytometry was used to test the expression of DC and PSC markers by chondrocytes whin fracture callus in adult male or female mice. (**6**) Tibial fractures were created in adult *CX3CR1^{creER}:R26$^{lsl\text{-}YFP}$* male or female mice followed by tamoxifen treatment to trace the fate of Cx3Cr1 labelled cells during fracture healing. All statistical analyses will be performed at the two tailed 0.05 significance level using independent sample *t* tests for 2 groups, ANOVA for 3 or more groups, Chi-square test for comparisons of frequency. All experimental protocols were approved by the University of Rochester Committee for Animal Resources.

RESULTS SECTION:

GM-CSF, but not M-CSF or Flt3L, stimulated OB differentiation significantly (ALP^+ OB area 0.58±0.01 vs. 0.04±0.01 cm^2, n=4, p=0.000) from BM stromal cells initially containing 80% $CD45^+F4/80^+$ macrophages. Flow cytometry indicates that GM-IDCs cultured from mouse PBMCs contain $CD11b^+F4/80^-$ and $CD11b^+F4/80^-$ subsets, 54% and 42% of which are $CD11c^+$ cells, respectively, and few of them express XCR1. These suggest that GM-IDCs are monocytic DCs (moDCs). M-CSF-induced macrophages from PBMCs did not express CD11c. Notably, GM did not promote OB differentiation from pure BdMPCs, most of which are $CD45^-Sca\text{-}1^+$ cells. In contrast, co-culture with GM-IDCs—but not M-CSF-induced macrophages—significantly enhanced OB differentiation of BdMPCs. Interestingly, GM stimulated the proliferation of $CD45^-$ MSCs, shown by increased % of S phase cells (20.4 ± 2.3 % vs. 5.6 ± 1.8%, n=3, p=0.00), in mouse BM stromal cells containing myeloid cells. Consistent with this, GM increased the % of $CD45^-$ MSCs in BM stromal cells (37.5 ± 4.3% vs. 7.9 ± 2.6%, n=3, p=0.00). However, GM did not stimulate the proliferation of pure BdMPCs.

Interestingly, transplantation of BdMPCs alone into the CSBD model did not result in bony union after 6 weeks, and the gaps were filled with cartilage and fibrous tissue, suggesting that these non-unions were both hypertrophic and atrophic. In contrast, all three CSBDs treated with local GM-IDCs plus BdMPCs achieved bony union without the use of any scaffold (p=0.0143 vs. BdMPCs alone, X^2 test, n=3 male each group) and the gap was bridged with new compact bone partially. In the large cortical bone defect window model, the defect remained visibly open when fibrin sealant was applied alone while delivery of 10 ng GM using 10 µL fibrin sealant led to complete closure of the bone defect, as observed by X-ray two weeks post-surgery (p=0.000 vs. vehicle, X^2 test, n=7 male each group). The µCT analysis indicate that the cortical defect holes were completely filled with cancellous bone in GM-treated group, whereas the holes remained open in the vehicle group.

Flow cytometry indicates that 2-5% of callus' cells were Aggrecan $(Acan)^+$ chondrocytes at day 7 to 11. About 60% and 20% of $Acan^+$ chondrocytes express surface $CX3CR1^+$ and $CD11c^+$, respectively, and 20% of $Acan^+CX3CR1^+$ chondrocytes express GM receptor α subunit (GMRα) in the callus at day 7 while DC marker expression by the chondrocytes were markedly reduced at day 11 post-fracture. Consistent with this, reanalysis of published scRNA-seq dataset shows higher % and level of CX3CR1, CD11c & GMRα expression by $Col2^+Acan^+$ chondrocytes in the callus at day 5 and their expressions were reduced at day 10 after fractures. Importantly, tamoxifen inducible CX3CR1 cre-driven lineage cell tracing shows that most $Sox9^{high}$ chondrocytes express high level of CX3CR1 at day 11 fracture callus. In addition, flow cytometry shows that 60% and 95% chondrocytes express Sca-1 and noggin, respectively, whin the callus at day 7 post-fracture.

DISCUSSION: (1) GM-CSF strongly stimulates OB differentiation by stimulating the proliferation of MSCs through it-induced DCs. (2) Chondrocytes within the fracture callus, which contribute to fracture healing, are derived from PSCs that transiently express DC markers such as Cx3CR1, GMRα, and CD11c, and kept the prosperities of, PSCs. DC markers are conserved in the pluripotent chondrocytes. These PSCs can be rapidly expanded by GM-CSFs and subsequent GM-IDCs during the early phase of callus formation. (3) Locally delivery of GM-CSF and pre-generated GM-IDCs significantly accelerate fracture healing to prevent nonunion.

SIGNIFICANCE/CLINICAL RELEVANCE: Naturally occurring pluripotent stem cells (PSCs) are present within the fracture callus during the early phase of healing. These PSCs express DC markers and can be rapidly expanded in response to GM-CSF and GM-IDCs. Therefore, local delivery of GM-CSF or pre-generated GM-IDCs represents a promising strategy to accelerate fracture healing and prevent nonunion.

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