Supporting Information

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SI Materials and Methods

Murine Model. The murine-fracture model was performed as described previously (1). Sham fracture included all dissection but without surgical osteotomy. Animal procedures were approved by the institutional ethics committee and the United Kingdom Home Office (PPL 70/6502).

Obtaining Cell Populations from Muscle, Fasciocutaneous Tissue, and Bone Marrow. Tissue was harvested from C57/Bl6 mice and human subjects following reconstructive lower-limb surgery (COREC No: 07/Q0411/30). Five grams of muscle (human) or 0.1 g (mouse) were finely chopped, digested in 20 mL (human) or 5 mL (mouse) digestive medium [50 mg Collagenase II (Worthington Biochemical Corp.) and 100 mg Dispase (Invitrogen) in 20 mL Hanks BSS]. The suspension was gently agitated for 30 min at 37 °C, centrifuged, and cells resuspended in 12 mL culture medium [GIBCO DMEM containing 10% GIBCO FBS and 1% penicillin/streptomycin (PAA Laboratories GmbH)] then added to a 10-cm culture plate with 5% CO_2 at 37 °C. Populations of skin and adipose-derived stromal cells were obtained using the same method. Bone marrow samples were agitated in 12-mL culture medium and added to a 10-cm culture plate.

Staining of Muscle-Derived Stromal Cells for Alkaline Phosphatase. Cells were fixed with 1:1 100% acetone: 100% ethanol for 15 min, then rinsed three times. One SIGMAFAST tab (Sigma-Aldrich Corp.) was dissolved in 10 mL dH_2O and 0.5 mL added to each well. The plate was incubated at 37 °C for 30 min. Positive cells appeared purple.

Staining of Muscle-Derived Stromal Cells for Bone Nodules. Muscle-derived stromal cells (MDSCs) (1×10^4) were added to 24-well plates in triplicate. The cells were cultured for 28 to 35 d in culture medium (DMEM with 10% FBS, 1% penicillin/streptomycin) (Fig. 1); osteogenic medium [DMEM with 10% FBS and 1% P/S, also containing 100 nM dexamethasone (Sigma), 1 mM β-glycerophosphate, and 0.05 nM ascorbic acid] (Fig. 3), or 10% human serum media (HSM) with added recombinant human cytokine. Cells were fixed with 2 mL 10% neutral buffered formalin for 15 min, and washed twice with excess PBS. One milliliter filtered 40-mM Alizarin red solution (pH 4.1) was added to each well at room temperature for 30 min. The excess dye was aspirated and the plate washed four times in distilled water with gentle agitation. Bone nodules appeared red.

Alkaline Phosphatase Quantification Assay. Human MDSC (1×10^4) were added to 96-well plates in triplicate. At 24 h the media replaced by 200 μL test media and the media was changed on day 4. At day 7 the media was removed and the cells lysed in 20 μL Nonidet P-40 lysis buffer. An alkaline phosphatase (ALP) quantification assay (WAKO Pure Chemical Ltd.) was used according to the manufacturer's instructions.

Flow Cytometry. MDSCs were divided into aliquots of 1×10^5 cells for each antibody and fluorochrome-conjugated antibody was added according to manufacturer's instructions. Mouse anti-human PE, FITC, and APC isotype controls were used. The samples were analyzed using a BD-LSR bench-top flow cytometer (BD Biosciences). Typically, 10,000 events were measured and fluorescence detection expressed as a percentage of total events. The data were analyzed using Flowjo software (Tree Star Inc.).

Supernatants. Human bone fragments were collected during debridement of lower extremity trauma (Ethical approval No: 07/Q0411/30). Below-knee amputations for severe foot/ankle injury provided a source of surgically cut (nonfractured bone) and segments were cut using a surgical saw (Stryker) cooled with saline. These samples produced "fractured" and "surgically cut" supernatants, respectively. Serum-free media (DMEM + 1% P/S) was added to bone fragments/segments at 5 mL/g and incubated for 12 h. The supernatant was filter-sterilized and stored at −80 °C before use.

MDSC Migration Through a Transwell Membrane. Five-hundred microliters of serum-free media was added to each well of a 24-well plate. Next, 8-μm pore transwell membranes (VWR International Ltd.) were added to each well, dividing the well into "upper" and "lower" chambers. MDSCs (1×10^4) were seeded onto the membrane by adding the cells in serum-free media. At 12 h the serum-free media in the lower chamber was replaced with test or control media and the transwell was incubated at 37 °C for 36 h. The membrane in the upper chamber was then cleared of cells and those on the underside of the membrane were washed, fixed in 10% neutral buffered formalin, and stained with 1% crystal violet solution for 15 min. The membranes were washed and counted (three random fields at 20× magnification) for each triplicate experiment. Cell migration was calculated against the supernatant or HSM (controls) and expressed as a percentage of the control.

Histology. Limbs were fixed in 10% neutral buffered formalin for 24 h then underwent decalcification for 7 d in 10% formic acid. The limbs were bisected longitudinally and paraffin-embedded. Next, 4-μm sections were cut and stained using Masson's trichrome stain with a standard histological staining protocol.

MicroCT. Mouse tibiae were harvested at 28 d postfracture. Each bone was scanned using a SkyScan 1172 scanner (SkyScan, Kontich, Belgium) by Kevin MacKenzie at The University of Aberdeen, Scotland. The density range was calibrated to bone mineral density and a threshold density of 350 gm/cm^3 was selected to distinguish mineralized from unmineralized tissue, as previously described (1). The scans were analyzed using SkyScan CT Analyzer software, version 1.9.3.0 (SkyScan 2003–2009). Bones which exhibited angulation, rotation or hypertrophic nonunion were excluded. A volume of interest was selected for each bone. A region of 120 slices (1 mm in height), commencing at the fracture site and proceeding proximally was selected for analysis. This narrow region was chosen to avoid potential confounding effects proximally and distally.

Adipogenic Differentiation. Human MDSCs (1×10^4) were added to wells of a 24-well plate (in triplicate). At 24 h, the media was replaced with 1 mL adipogenic induction media (h-insulin, L-glutamine, dexamethasone, indomethacin, and 3-isobutyl 1-methylxanthine) added to DMEM +10% FCS +1% P/S. At day 3, the media was removed and replaced with 1 mL adipogenic maintenance media (culture media containing h-insulin and L-glutamine (Lonza). Induction and maintenance media were used alternately for 21 d, with media changes every 3 d. The cells were then fixed in 10% formalin and stained with oil red O, as described below.

Oil Red O Staining. Three parts filtered oil red O stock solution (containing 300 mg oil red O powder and 100 mL 99% isopropanol) were mixed with two parts deionized water and left at room temperature for 10 min. The fixed monolayer was rinsed in tap

water. One milliliter 60% isopropanol was pipetted into each well and left for 5 min. The isopropanol was removed and 1 mL of oil red O solution was added to each well for 5 min. The wells were then thoroughly rinsed with tap water. Images were taken at 20× magnification, as described above.

Chondrogenic Differentiation. Human MDSCs (1×10^5) were added to a 15-mL corning tube (in triplicate) and the tubes centrifuged at $0.15 \times g$ for 5 min. The media was removed, leaving the cell pellet. To two tubes, 1 mL chondrogenic media (ChondroDiff medium; Miltenyi Biotec) was added. To the third, standard (control) media was added. The medium was changed every 3 d. At 35 to 40 d, the cell pellet was fixed by immersion in 10% neutral buffered formalin for at least 1 h, then embedded in paraffin before sectioning. The sections were stained using Alcian blue. The slides were viewed under 10× magnification using an Olympus BX51 microscope (Olympus Optical). Images were taken using an Olympus DP71 camera and DP controller and manager, Olympus software.

Cell Proliferation Assay. MDSCs (2.5×10^3) (equivalent to 50 μL 5×10^4/mL MDSC) in serum-free media were added to wells of an opaque-bottomed 96-well plate (Perkin-Elmer) in triplicate. Each triplicate was set up to correspond to each condition tested. Plates were set up for days 1, 3, and 5. A further plate, containing one triplicate for day 0 was included, to act a baseline measurement for all cells. The next day (day 0), the serum-free media was aspirated from the plate and replaced by 200 μL test media and the plates were incubated at 37 °C. Plates were harvested at days 1, 3, and 5 by aspirating the test media and replacing with 100 μL serum-free media, followed by 100 μL CellTiter-Glo luminescence assay media (Promega Corp.). The plate was then agitated gently for 10 min before being read using a Perkin-Elmer fluorescence spectrometer and "FL WinLab" software on the "Luciferase" setting. The mean luminescence counts were then calculated from the absolute values. The luminescent signal was directly proportional to the concentration of ATP from viable cells.

ELISA Methodology. Commercially available ELISA kits were obtained to measure the concentrations of protein (antigen) in fracture and surgically cut supernatants. The standard, two-antibody quantitative "sandwich" enzyme immunoassay technique was used in all cases.

The sandwich technique uses a first purified antibody specific for the antigen under investigation, which is precoated onto a 96-well polystyrene microplate. The supernatant, containing the protein (antigen) is added to the well and the antigen is permitted time to complex with the bound antibody. A standard curve is generated by adding a standard protein of known concentration, in stepwise dilutions. After washing, an enzyme-linked polyclonal antibody specific for the antigen is added to the wells. Again, the antibody is permitted time to bind to the antigen. After washing to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. This process determines a color change proportional to the amount of bound antigen present. The color development is then stopped and the color change quantified spectrophotometrically, with the absorbance equated to the amount of antigen bound, and calculated using the standard curve. The specific procedure for each assay was followed as per the manufacturer's instructions.

Luminex Methodology. The multiplex bead immunoassay is a solid phase sandwich immunoassay, designed to be read using a Luminex xMAP system (Luminex Corp.). Polystyrene microspheres measuring 5.6 μm in diameter are precoated with antibodies specific to the proteins (antigens) being tested. The coated beads were added to each well of a prewetted filter-bottom 96-well micro plate with the sample or standard and incubated for 2 h to permit binding of the analytes to the beads. After washing, a solution containing a mixture of biotinylated, analyte-specific antibodies was added and incubated for a further 1 h. This stage permits binding of the biotinylated antibodies to the bound analytes on the polystyrene beads. After further washing, streptavidin, conjugated to R-Phycoerythrin (a flurorescent protein) was added and incubated for a further 30 min. During this stage, the fluorescent protein-bound streptavidin binds to the biotinylated antibody forming a solid-phase sandwich. After repeated washing the plate is read using a Luminex 100 instrument. The fluidics system of the Luminex 100 permits a stream of suspended microspheres to line up in single file before passing through the detection chamber. As each microsphere passes through the detection chamber, a red laser excites both the red and infrared dyes, permitting a unique signature of fluorescence corresponding to a "set" on the bead, which in turn represents the site of residence of a named analyte antibody. Simultaneously, a green laser excites the bound fluorescent protein (associated with binding of the analyte). Precision is ensured as each particle is read individually, and a mean value is generated using the Luminex software.

1. Harry LE, et al. (2008) Comparison of the healing of open tibial fractures covered with either muscle or fasciocutaneous tissue in a murine model. *J Orthop Res* 26:1238–1244.

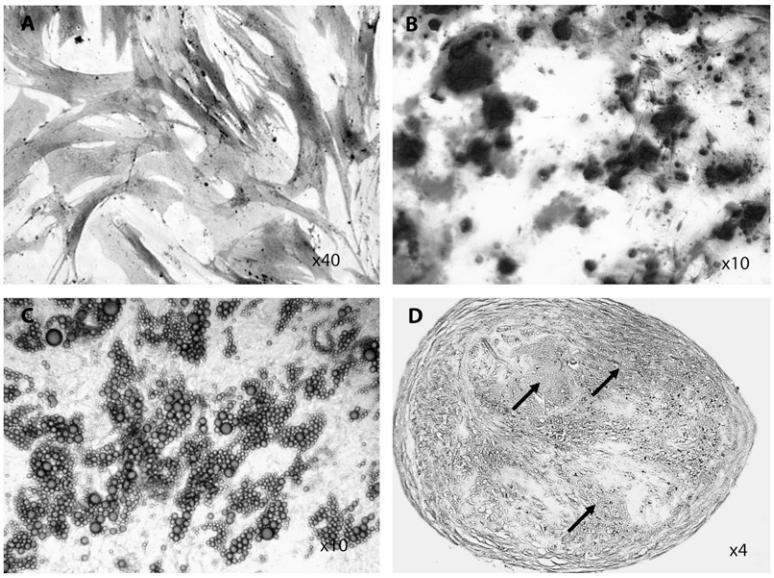

Fig. S1. MDSCs exhibit trilineage differentiation potential. (*A*) MDSCs stained with Crystal violet. MDSCs demonstrate (*B*) osteogenic differentiation, (*C*) adipogenic differentiation, and (*D*) chondrogenic differentiation.

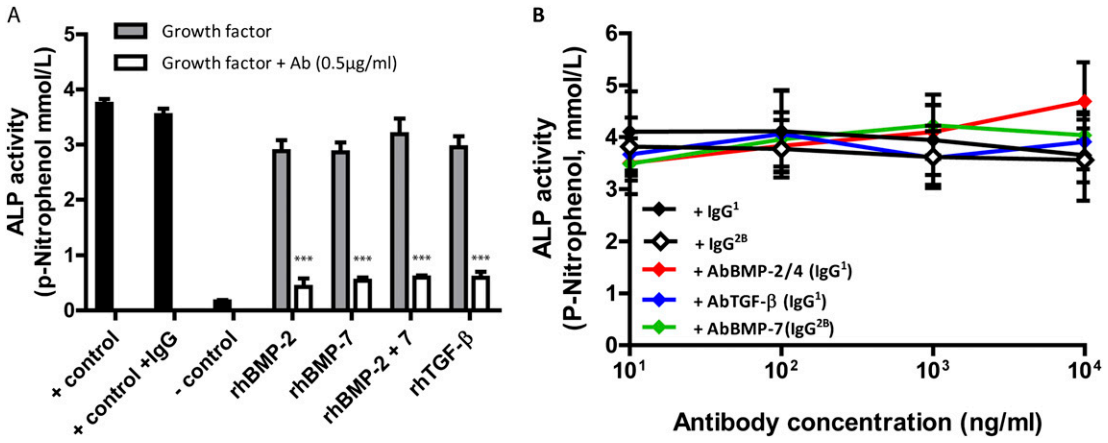

Fig. S2. Recombinant human bone morphogenetic proteins (rhBMPs) stimulate osteogenic differentiation of MDSC but antibody inhibition of BMPs in supernatant does not diminish the osteogenic effect. (*A*) ALP production by MDSCs with recombinant BMP-2 (0.5 μg/mL), BMP-7 (0.5 μg/mL), or TGF-β (1 ng/mL) ± the addition of AbBMP2/4, AbBMP-7, and AbTGF-β (all at 0.5 μg/mL). IgG^1 and IgG^{2B} as positive control at 0.5 μg/mL separately (not shown) and together (shown) to demonstrate lack of nonspecific inhibition. (*B*) ALP production by MDSC in fracture supernatant plus AbBMP-2/4, AbBMP-7, and AbTGF-β in dose-response. IgG^1 as control for AbBMP-2/4 and AbTGF-β, and IgG^{2B} for AbBMP-7. Results represent means of three experiments using MDSC from three donors (and fracture supernatant from three donors in *B*; ± SEM, $n = 3$). (***$P < 0.001$; one-way ANOVA with Bonferroni's multiple comparison test.)

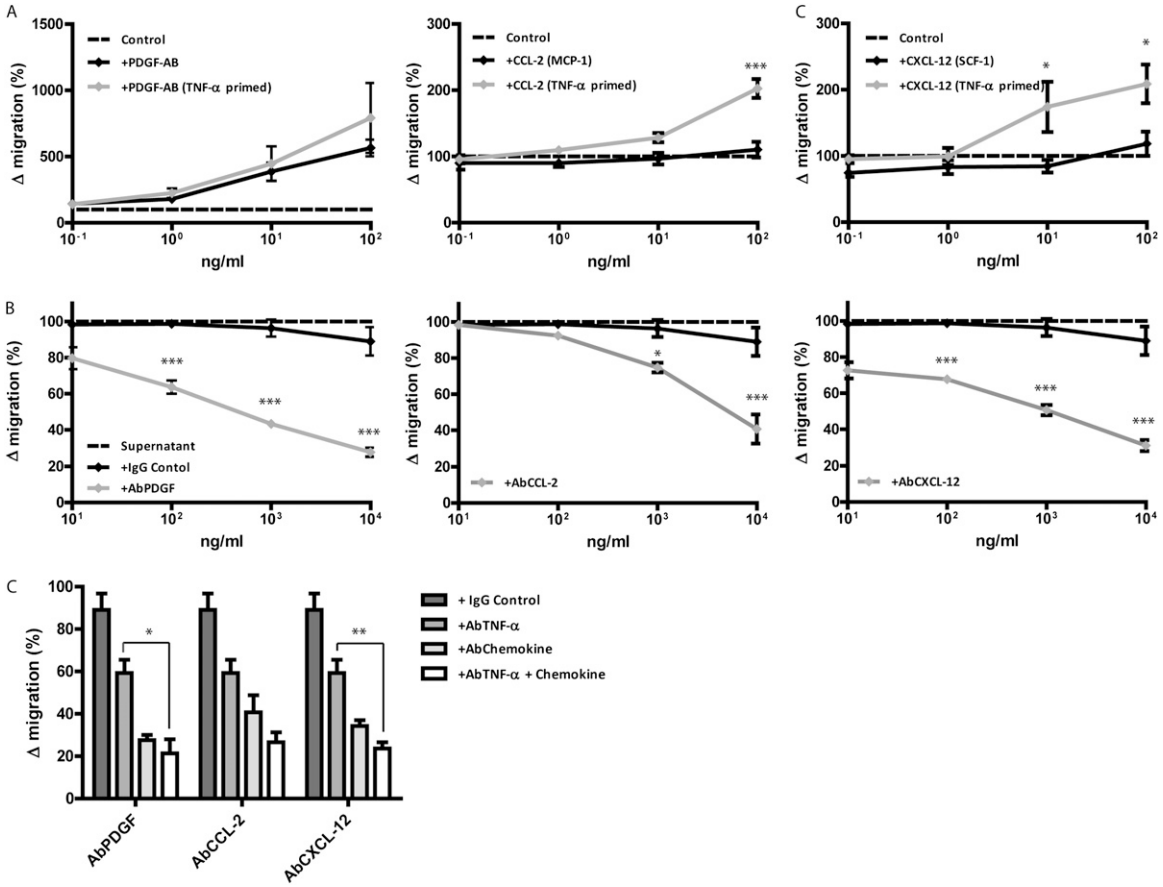

Fig. S3. PDGF, CCL-2, and CXCL-12 induce MDSC migration and cell responsiveness to CCL-2 and CXCL-12 is enhanced by TNF-α. (*A*) MDSC migration in response to HSM + rhPDGF, rhCCL-2, or rhCXCL-12 in dose-response, expressed relative to migration in media only. Cells were either naive or primed with rhTNF-α at 1 pg/mL for 72 h. (*B*) MDSC migration in response to fracture supernatant + AbPDGF, AbCCL-2, or AbCXCL-12 (polyclonal IgG as antibody control). (*C*) Comparison of MDSC migration in response to fracture supernatant with or without exogenous AbTNF-α ± AbPDGF, AbCCL-2, or AbCXCL-12 at 10 μg/mL. Results represent means of three experiments using three MDSC donors ± SEM in *A* and three supernatants in *B* and *C* (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). One-way ANOVA with Dunnett's multiple comparison test in *A* and Bonferroni's multiple comparison test in *B* and *C*.

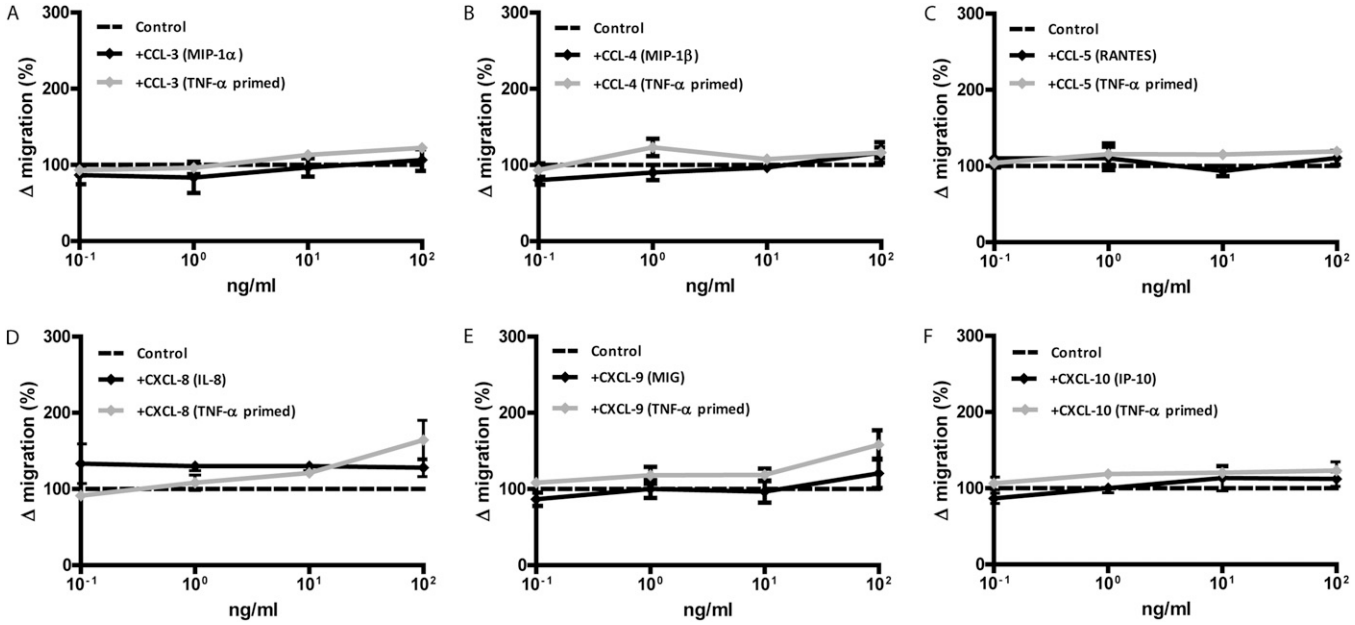

Fig. S4. Naive and primed MDSC do not migrate in response to other chemokines. MDSC migration in response to HSM with the addition of rhCCL-3 (*A*), rhCCL-4 (*B*), rhCCL-5 (*C*), rhCXCL-8 (*D*), rhCXCL-9 (*E*), or rhCXCL-10 (*F*) in dose-response, expressed relative to migration in the HSM-only control. Cells were either naive or primed with rhTNF-α. Results represent means of three experiments using MDSC from three donors (±SEM, $n = 3$). One-way ANOVA with Bonferroni's multiple comparison test revealed no significant difference in migration between the naive and primed cells.

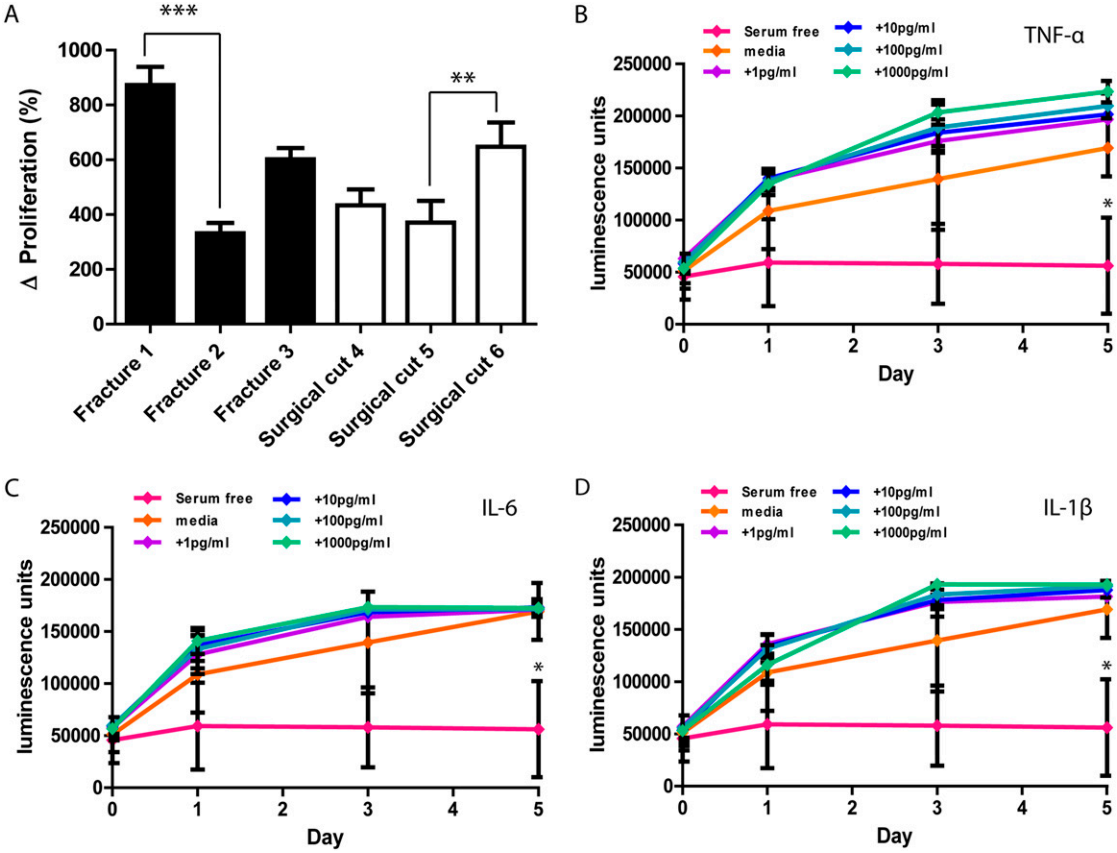

Fig. S5. Proinflammatory cytokines TNF-α, IL-6, and IL-1β do not promote MDSC proliferation. (*A*) MDSC proliferation in response to fracture and surgically cut supernatants. (*B–D*) CellTiter-Glo luminescent cell-viability assay for MDSCs in culture with (*B*) TNF-α, (*C*) IL-6, and (*D*) IL-1β. Results represent means of three experiments using MDSC from three donors (±SEM, $n = 3$) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). One-way ANOVA with Bonferroni's multiple comparison test in *A* and Dunnett's multiple comparison test in *B* to *D*.

Table S1. BMP-2, BMP-4, BMP-7, and TGF-β concentrations were measured in fractured and surgically cut bone supernatants

	Number	BMP-2 (pg/mL)	BMP-4 (pg/mL)	BMP-7 (pg/mL)	TGF-β (pg/mL)
Fracture supernatant	1	21.9 ± 2.3	5.7 ± 0.3	<5	1,092 ± 6
	2	23.2 ± 0.5	4.8 ± 0.7	<5	486 ± 39
	3	17.8 ± 2.3	8.2 ± 1.3	<5	3,644 ± 298
	4	16.5 ± 2.1	5.3 ± 1.1	<5	3,693 ± 182
	5	18.8 ± 2.8	8.9 ± 2.2	<5	654 ± 49
Surgically cut supernatant	9	5.9 ±1.8	2.3 ± 0.1	<5	1,224 ± 42
	10	4.4 ± 0.3	2.7 ± 0.1	<5	313 ± 27
	11	17.3 ± 12.6	3.5 ± 0.1	<5	783 ± 65

Values represent means of triplicate ±1 SD.

Table S2. Proinflammatory cytokine concentrations were measured in fracture and surgically cut bone supernatants

	Number	TNF-α (pg/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)
Fracture supernatant	6	<10	<10	1,841 ± 92
	7	<10	<10	9,522 ± 476
	8	<10	<10	17,671 ± 283
Surgically cut supernatant	12	<10	<10	11,414 ± 171
	13	<10	<10	580 ± 52
	14	<10	<10	764 $^{+}$± 82

Values represent means of triplicate ±1 SD.

Table S3. Chemokines concentrations were measured in fractured and surgically cut bone supernatants

	Supernatant number	CCL2 (μg/mL)	CCL4 (pg/mL)	CCL5 (pg/mL)	CXCL8 (μg/mL)	CXCL10 (pg/mL)	CXCL12 (pg/mL)
Fracture supernatant	6	0.8 ± 0.1	38 ± 1	108 ± 1	2.7 ± 0.2	<5	14 ± 2
	7	2.9 ± 0.2	46 ± 3	34 ± 1	87.7 ± 5	<5	34 ± 4
	8	3.5 ± 0.1	38 ± 3	28 ± 1	>800	<5	7 ± 1
	1	1.3 ± 0.3	39 ± 1	31 ± 4	10.4 ± 4	<5	28 ± 14
	2	1.0 ± 0.1	38 ± 2	107 ± 34	13.1 ± 4	<5	6 ± 2
Surgically cut supernatant	12	3.8 ± 0.7	<5	34 ± 4	46.2 ± 33	<5	<5
	13	0.6 ± 0.01	38 ± 2	85 ± 20	4.1 ± 0.1	<5	<5
	14	0.5 ± 0.01	<5	407 ± 44	1.5 ± 0.2	<5	<5
	9	1.2 ± 0.4	42 ± 5	87 ± 12	14.7 ± 0.7	<5	<5

Values represent means of triplicate ±1 SD.