SUPPLEMENTAL DATA

Defining the Role of M1 macrophage in Bone Repair via the Function of 1,25-Dihydroxyvitamin D in M1/M2 Differentiation

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Running title: $1,25(OH)_2D$ Blocks M1 Macrophage-MSC Crosstalk

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The authors have declared that no conflict of interest exists

Table S1. List of primers used in this study

Gene	Forward	Reverse
F4/80	CTTTGG CTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
IL-1α	AGGGAGTCAACTCATTGGCG	ACTTCTGCCTGACGAGCTTC
IL-1β	GTCGCTCAGGGTCACAAGAA	GTGCTGCCTAATGTCCCCTT
IL-6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
CD11b	CCATGACCTTCCAAGAGAATGC	ACCGGCTTGTGCTGTAGTC
TNF-α	GAACTCCAGGCGGTGCCTAT	TCGGCTGGCACCACTAGTTG
OSM	ATGCAGACACGGCTTCTAAGA	TTGGAGCAGCCACGATTGG
CD90	TGCTCTCAGTCTTGCAGGTG	TGGATGGAGTTATCCTTGGTGTT
CD105	TGCACTTGGCCTACGACTC	TGGAGGTAAGGGATGGTAGCA
CD73	AACCCCTTTCCTCTCAAATCCA	CAGGGCGATGATCTTATTCACAT
OCN	CTGACCTCACAGATCCCAAGC	TGGTCTGATAGCTCGTCACAAG
OSX	GGAAAGGAGGCACAAAGAAGC	CCCCTTAGGCACTAGGAGC
Runx2	TTCAACGATCTGAGATTTGTGGG	GGATGAGGAATGCGCCCTA
GAPDH	TGGCAAAGTGGAGATTGTTGCC	AAGATGGTGATGGGCTTCCCG

Notes: F4/80: adhesion G protein-coupled receptor E1, IL-1α: Interleukin-1α; IL-1β: Interleukin-1β;, CD11b: Cluster of differentiation 11b, IL-6: Interleukin-6;, TNF-α :Tumor Necrosis Factor -α ,OSM: Oncostatin M, CD90: Thy1, CD105: Endoglin, CD73: 5'-Nucleotidase, OCN:Osteocalcin, OSX-:Osterix, Runx2-:Runt related transcription factor 2, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Table S2. Mean fluorescence intensities (MFIs) of secreted proteins in $CD11b^{+}F4/80^{+}$ macrophages at fracture sites

Day 4				
Proteins\MFIs\Tx	Intact bones	Fractured bones		
		VC	100ng/kg VD	1000ng/kg VD
IL-1β	269.6±10.33****	994.3±51.26	712.3±6.6***	649.5±3.6***
IL-12	333.5±47.5***	540±25.5	500.3±44 ns	450.3±38 ns
IL-6	485.3±44.2****	815.4±5.9	479.2±37.8****	436.2±37.8****
TNF-α	435±6.6****	875.6±32.2	868±14.7 ns	805.5±12.6 ns
OSM-M	666±30.7***	1162±74.3	946±35.8 ns	667.3±67.2***

Day 7				
Proteins\MFIs\Tx	Intact bones	Fractured bones		
		VC	100ng/kg VD	1000ng/kg VD
IL-1β	1227±42.0 ns	1381±72.8	1661±31.8 ns	1585±97.2 ns
IL-12	1123±25.3***	1648.5±1.2	1798±1.6*	1579.5±20.0 ns
IL-6	1641±143.1 ns	1893±39.5	1791±25.5 ns	1769±72.1 ns
TNF-α	2268±177.6 ns	2349.6±57.5	2160±19.6 ns	2101±107.3 ns
OSM-M	1531.5±92.5 ns	1557±199.0	1489.667±9.7 ns	1725.5±60.5 ns

Note: "VD": $1,25(OH)_2D$. "VC": vehicle. "Tx": treatment. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P≤ 0.0001, "ns": not significant. ANOVA test: vehicle vs intact bones, 100ng/kg VD, and 1000ng/kg VD.

Table S3. Percent of MSC cell populations at days 4 and 7 post fracture surgery

Day 4				
MSC\%\Tx	Intact bones	Fractured bones		
		VC	100ng/kg VD	1000ng/kg VD
CD90	3.705±0.2***	8.1±0.03	4.2±0.4 **	3.9±0.3**
CD105	7.4±2.0*	14.9±0.5	9.8±0.7 ns	6.9±1.95*
CD29	9.6±2.5*	20.28±1.3	16.6±2.3 ns	15.3±3.7 ns
CD73	6.42±1.7**	14.9±0.6	11.8±1.2 ns	8.1±0.1*

Day 7				
MSC\%\Tx	Intact bones	Fractured bones		
		VC	100ng/kg VD	1000ng/kg VD
CD90	7.2±0.2 ns	10.5±0.9	16.9±0.7**	19±2.7**
CD105	2±0.5 ns	3.17±0.8	7.09±0.9*	6.5±0.9 ns
CD29	11.5±0.5 ns	11.47±0.8	21.6±2.5**	22.3±0.35**
CD73	9.0±1.3 ns	7.8±0.5	17.02±1.8*	16.0±3.7*

Note: "VD": 1,25$(OH)_2$D. "VC": vehicle. "Tx": treatment. *P≤ 0.05, **P≤ 0.01, ***P≤ 0.001, ****P≤ 0.0001, "ns": not significant. ANOVA test: vehicle vs intact bones, 100ng/kg VD, 1000ng/kg VD.

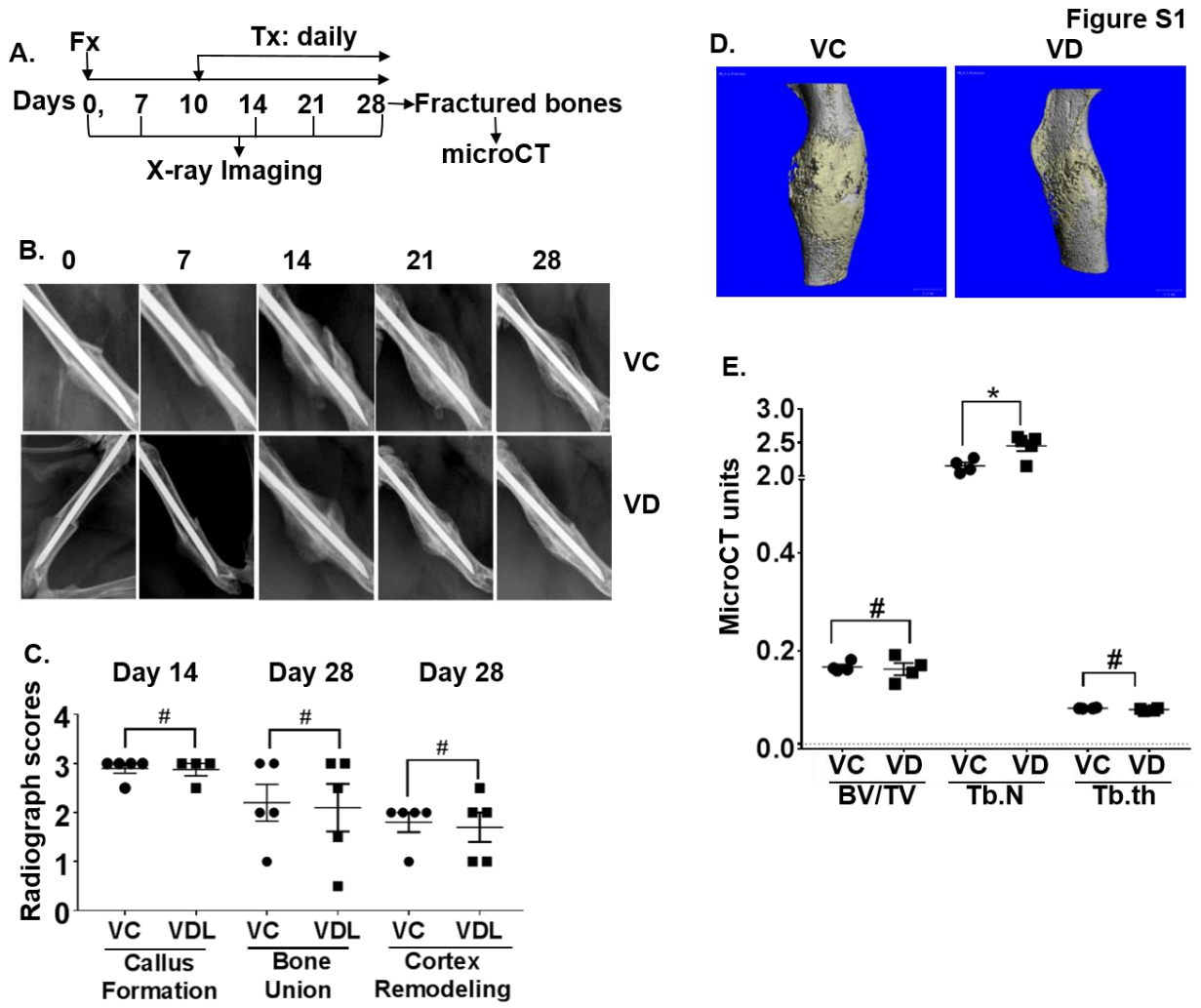

Figure S1. Local subcutaneous treatment with 1,25(OH)$_2$D during the regenerative stage did not impair fracture repair. ***A)*** C57BL/6 mice were subject to fracture surgery (Fx). Ten days later, the animals subcutaneously received at the fracture sites a daily dose of either vehicle (VC) or 100ng/kg/mouse 1,25(OH)$_2$D (VD). X-ray images of the fractured bones were taken at days 0, 7, 14, 21, and 28. At day 28, fractured bones were analyzed by microCT. ***B)*** Representative x-ray images of fracture sites are shown. ***C)*** X-ray images were scored at day 14 for callus formation and at day 28 for bone union and cortex remodeling. $^{\#}$P>0.05. t-test. N=5. ***D)*** Representative microCT 3D images are shown. ***E)*** Cumulative data show BV/TV (bone volume/total volume), Tb.N (trabecular number), and Tb.th (trabecular thickness) from the microCT analysis. *P<0.05; $^{\#}$P>0.05. t-test. N=5.

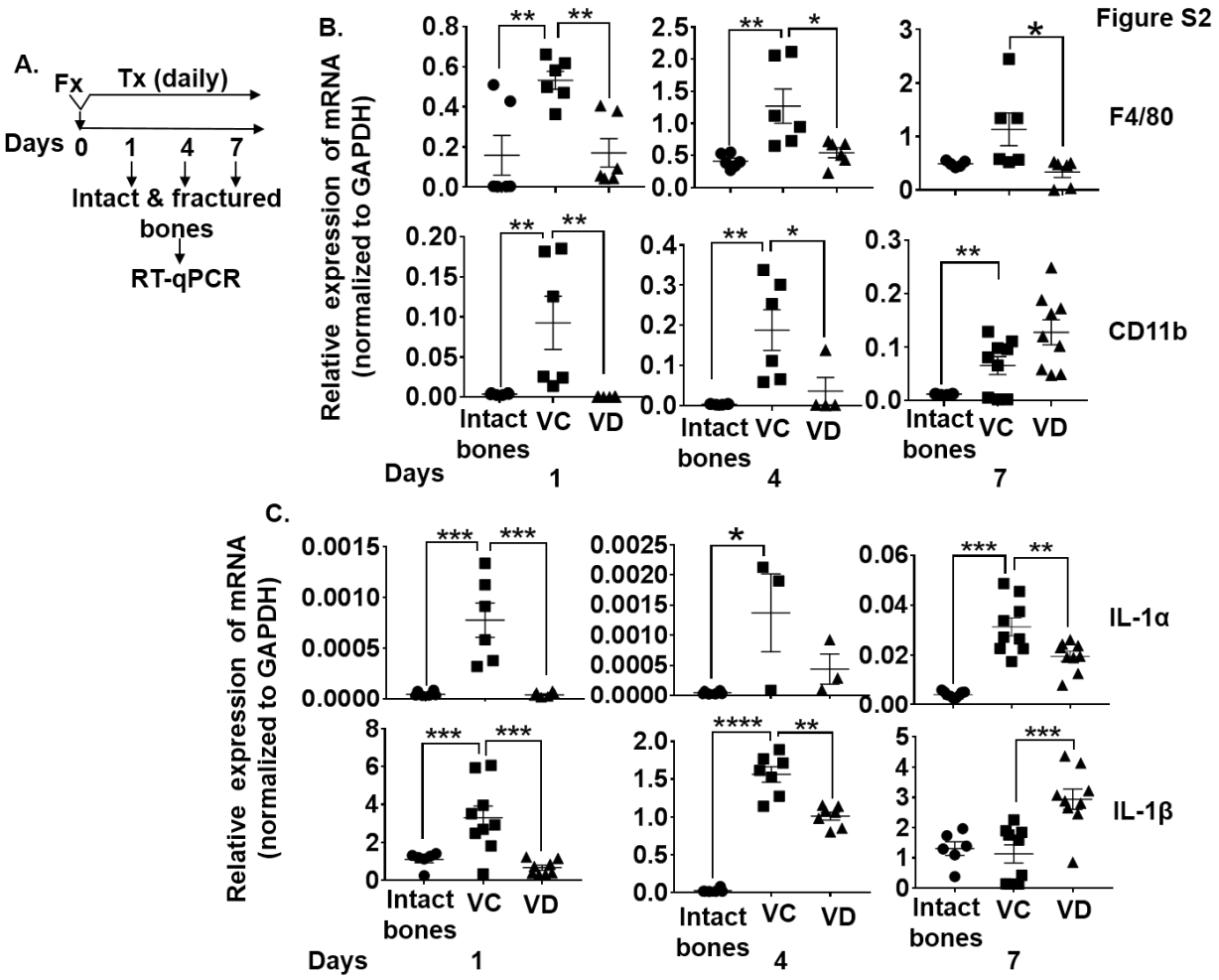

Figure S2. Local subcutaneous treatment with 1,25(OH)$_2$D during the pro-inflammatory stage decreased the expression of marker genes for M1 macrophages at fracture sites. ***A)*** C57BL/6 mice were subject to fracture surgery (Fx). Immediately after the fracture surgery, the animals subcutaneously received at the fracture sites a daily treatment (Tx) with vehicle (VC) or 100ng/kg/mouse 1,25(OH)$_2$D (VD). At days 1, 4, and 7, contralateral bones (Intact bones) and the bones at fracture sites (fractured bones) were examined by RT-qPCR analysis. ***B)*** Data show mRNA expressions of the marker genes for pan-macrophage (i.e. F4/80 and CD11b). ***C)*** Data show mRNA expressions of the marker genes for M1 macrophages (IL-1α and IL-1β). Data are means ± SE. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. ANOVA test. N=3.

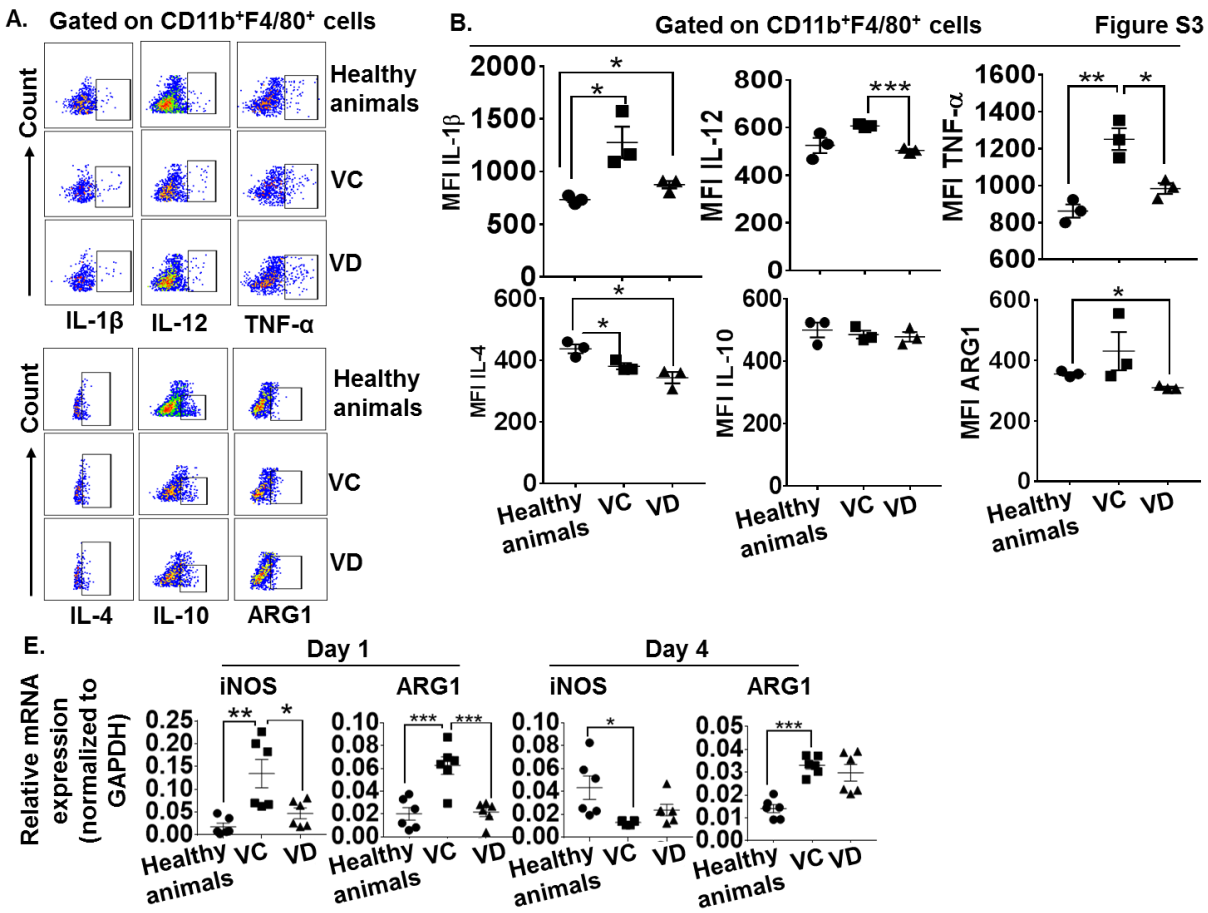

Figure S3. Bone fracture and 1,25(OH)$_2$D treatment had moderate effects on peripheral lymphoid systems. C57BL/6 mice were subjected to fracture surgery and immediately after the fracture surgery received a daily dose of either vehicle (VC) or 100ng/kg 1,25 (OH)$_2$D (VD) subcutaneously near fracture sites. Additionally, a group of healthy animals was also included as a control. At days 1, 4, and 7 post treatments, splenocytes were analyzed by FACS and RT-qPCR. ***A)*** Representative FACS dot plots show the expressions of IL-1β, IL-12, TNF-α, IL-4, IL-10 and arginase 1 (ARG1) among CD11b$^+$F4/80$^+$ monocytes/macrophages in splenocytes on day 1 after the treatments. ***B)*** Cumulative data of mean fluorescent intensities (MFIs) of IL-1β, IL-12, TNF-α, IL-4, IL-10, and ARG1 in CD11b$^+$F4/80$^+$ monocytes/macrophages in splenocytes on day 1 after the treatments. ***E)*** RT-qPCR analyses show the mRNA expressions of iNOS and ARG1 in the splenocytes at days 1 and 4 after the treatments. *P<0.05, **P<0.01, ***P<0.001. Two way ANOVA test. N=3.

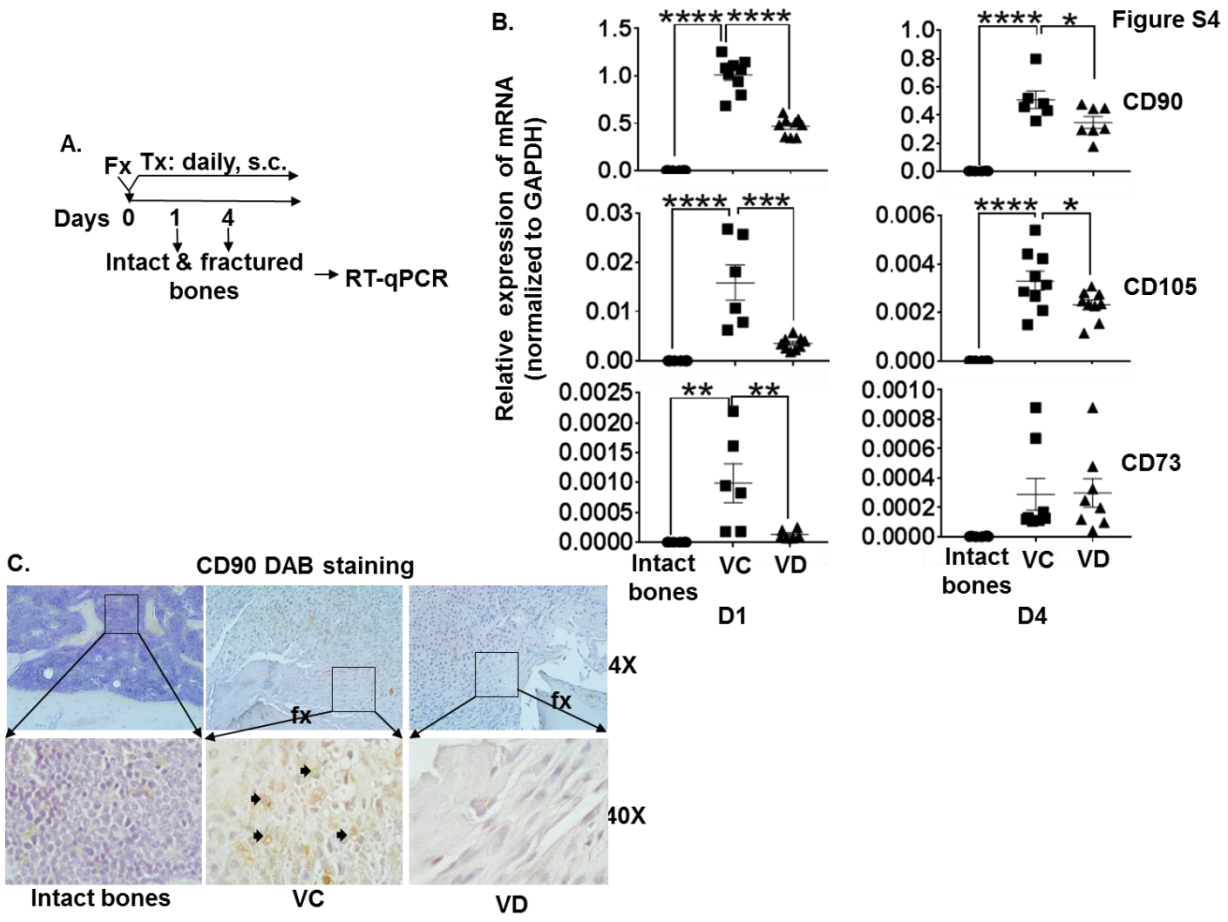

Figure S4. 1,25(OH)$_2$D, when locally administered at proinflammatory stage, decreased the expressions of MSC markers. ***A)*** C57BL/6 mice were subjected to fracture surgery (Fx). Immediately after the fracture surgery, the animals subcutaneously received a daily dose of either vehicle (VC) or 1,25(OH)$_2$D (100ng/kg) (VD) near fracture sites. Intact and fractured bones were collected from the animals at days 1, 4, and 7 for analysis by RT-qPCR. ***B)*** Data show the mRNA expressions of CD90, CD105, and CD73 at days 1 and 4. Data are means ± SEM. *P<0.05; **P<0.005; ***P<0.001; ****P<0.0001. ANOVA test. N=3. ***C)*** Paraffin sections of intact and fractured bones were stained for CD90 by DAB staining. Representative images are shown.

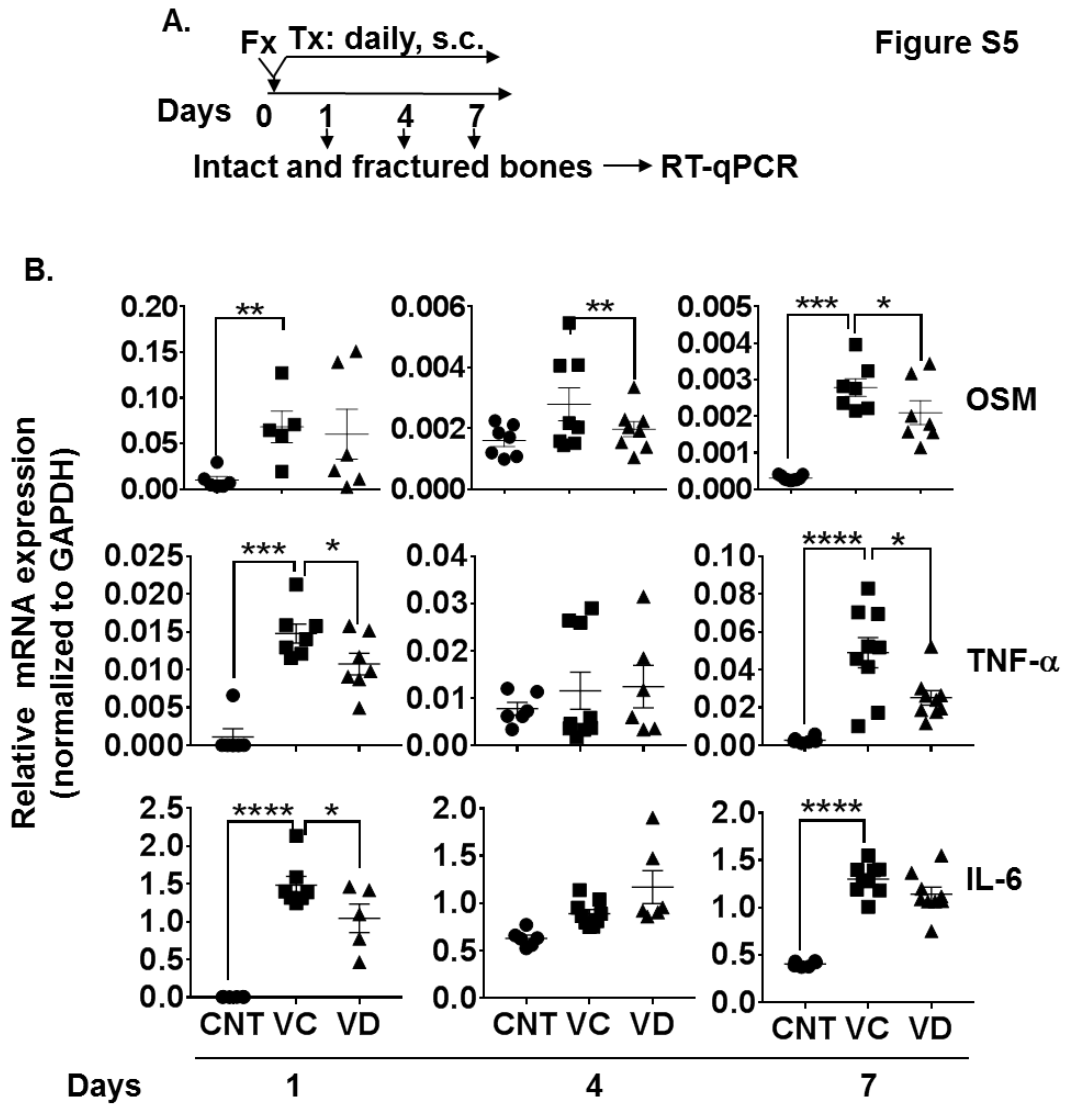

Figure S5. Local subcutaneous treatment with 1,25(OH)$_2$D during pro-inflammatory stage decreased the expression of marker genes of M1 macrophage-associated proteins important for osteogenic priming of MSCs and for bone repair. ***A)*** C57BL/6 mice were subjected to fracture surgery (Fx), treatments (Tx), and analyses as described in Figure S2. ***B)*** Data show the mRNA expressions of oncostatin-M (OSM), TNF-α, and IL-6 at days 1, 4, and 7 after the treatments.