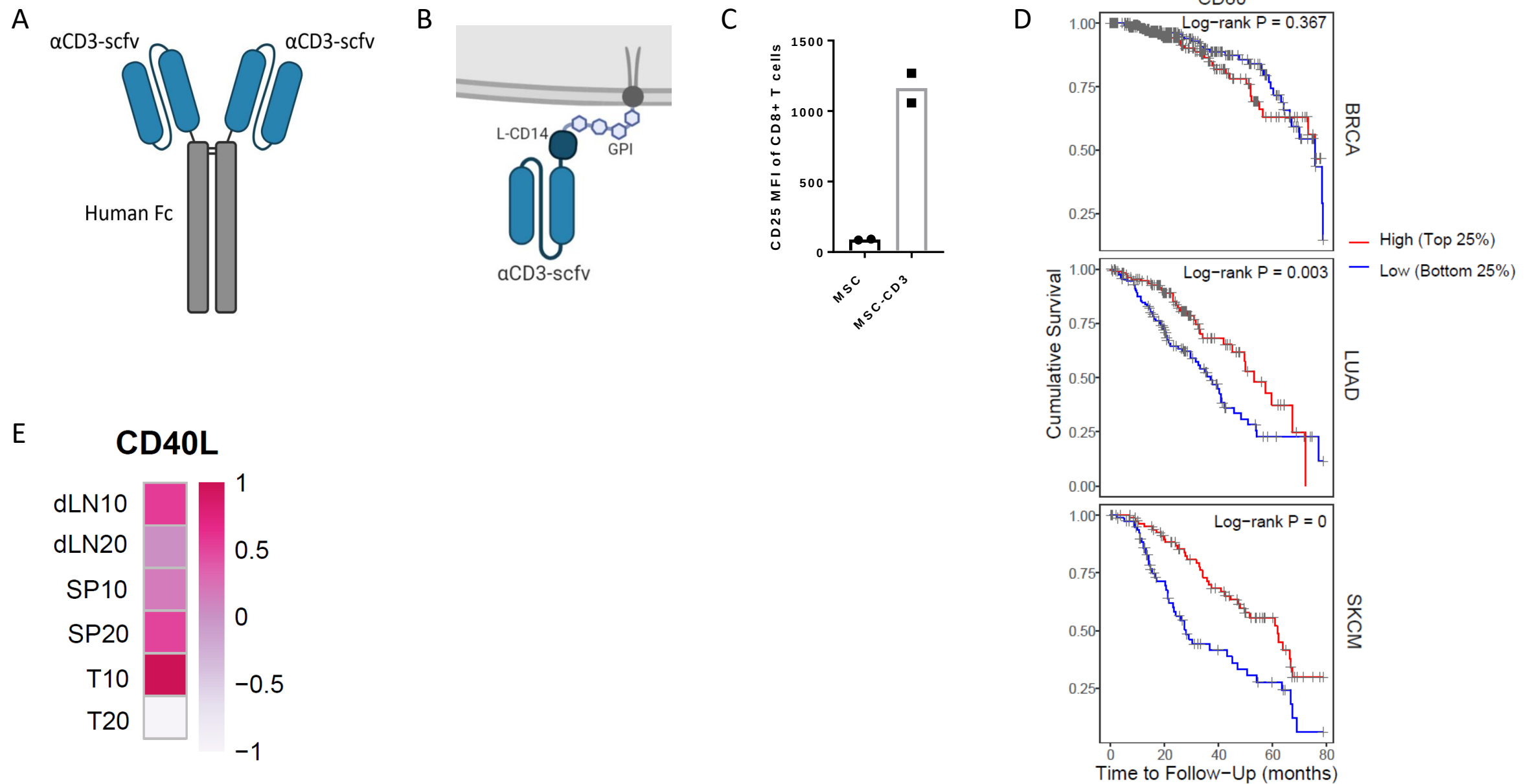
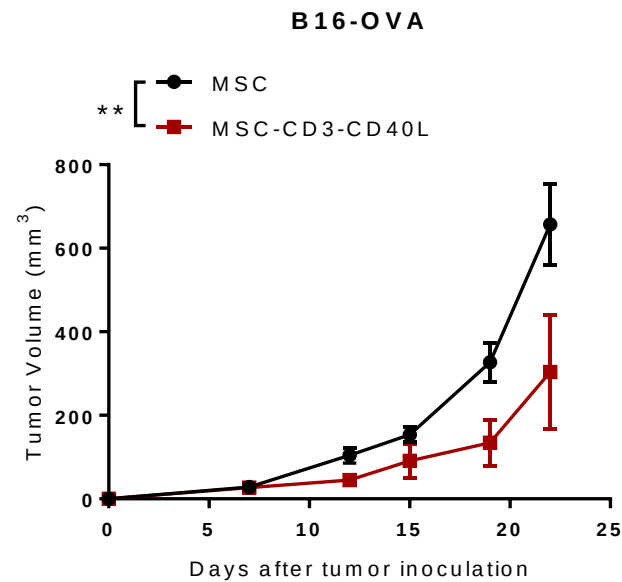
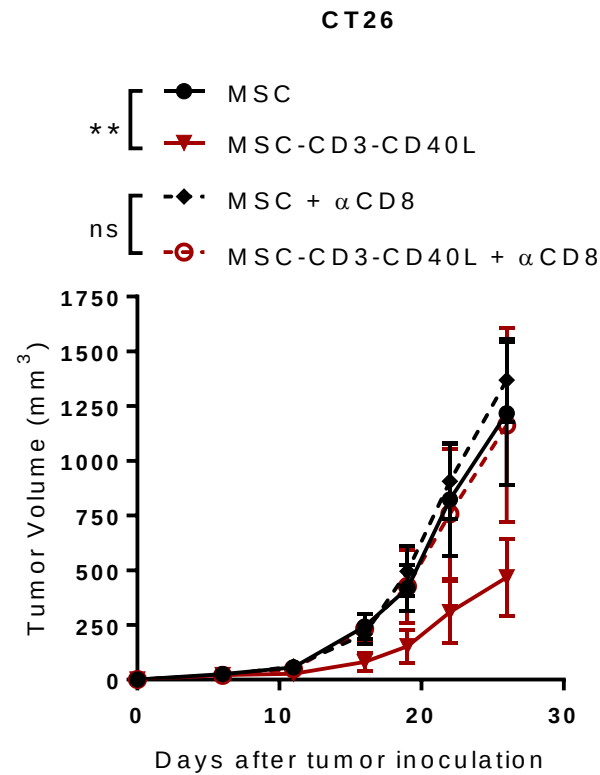
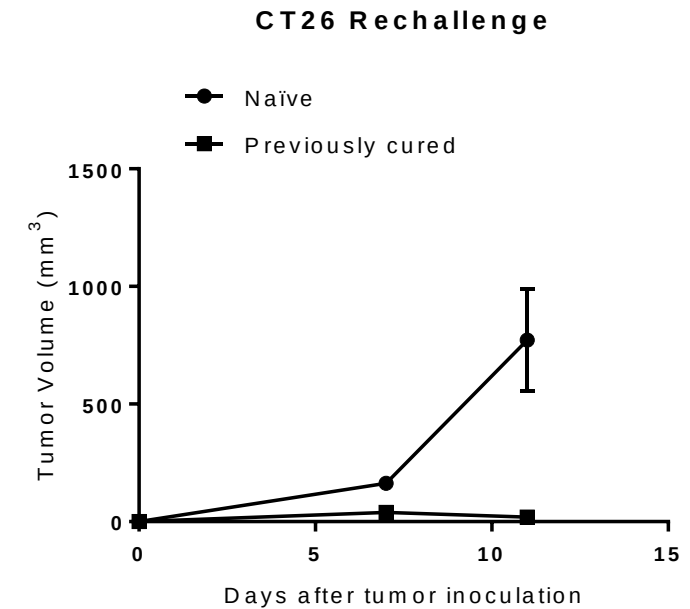


S1



Supplementary Figure 1. Design of CD3 stimulation and impact of co-stimulation in the TME.

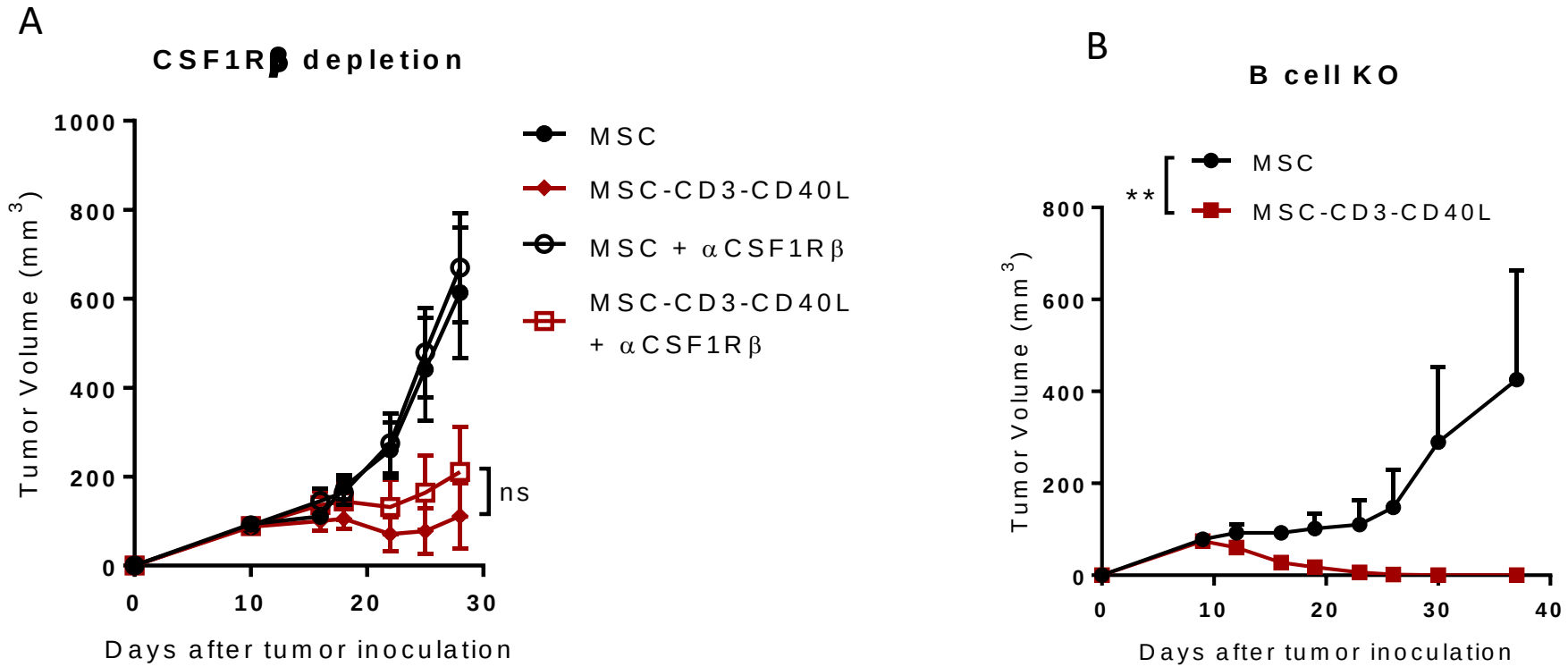
A Schematic of soluble α CD3 secreted by MSC-sCD3 in Figure 1A-B. **B** Schematic of membrane-bound α CD3 expressed by MSC-CD3 referenced in Figure 1D and throughout the manuscript. It is GPI anchored using the leading sequence of CD14. **C** CD25 expression on CD8+ T cells when co-cultured with MSC vs. MSC-CD3. **D** Cumulative survival in Breast Invasive Carcinoma (BRCA), Lung Adenocarcinoma (LUAD) and Skin Cutaneous Melanoma (SKCM) patients according to CD80 expression (top 25% vs. bottom 25%). **E** Relative expression of CD40 ligand on CD4+ T cells in draining lymph node (dLN) spleen (SP) and tumor (T) from day 10 and day 20 after subcutaneous MC38 tumor inoculation.



Supplementary Figure 2. Efficacy of MSC-CD3-CD40L in pre-clinical models.

A-B Balb/c mice were inoculated with 5×10^5 CT26 tumor cells (**A**) or C56BL/6J mice were inoculated with 3×10^5 B16-OVA tumor cells (**B**) and treated (p.t.) on days 8, 11, 14 with 1×10^6 of MSC or MSC-CD3-CD40L. Tumor growth was measured twice per week. (**C**) Two months after being cured of CT26, Balb/c mice were re-challenged with 5 million CT26 tumor cells on the opposite flank and tumor growth was monitored compared to naïve Balb/c mice. Statistical analysis was performed using two-way ANOVA. ** $P \leq 0.01$, ns not significant.

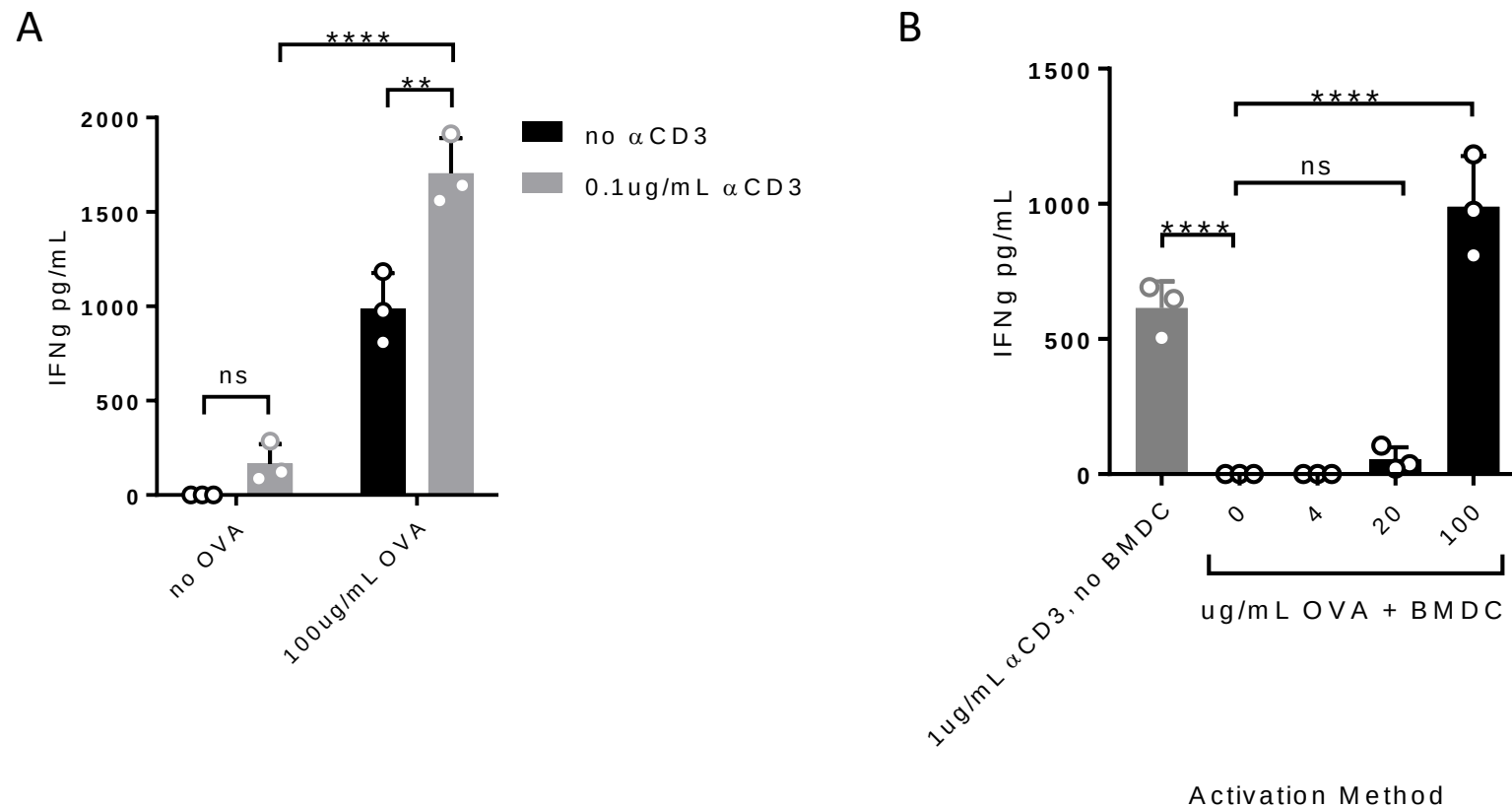
S3



Supplementary Figure 3. Macrophage depletion does not impact MSC-CD3-CD40L efficacy.

A C56BL/6J mice were inoculated with 1×10^6 MC38 tumor cells and treated (p.t.) on days 11, 14, 17 with 1×10^6 of MSC or MSC-CD3-CD40L and macrophages were depleted using 200ug/dose α -CSF1R β beginning 1 day before MSC treatment and continuing twice per week for 2 weeks. **B** MC38 bearing MuMt⁻ mature B cell deficient mice were treated with MSC or MSC-CD3-CD40L beginning on day 11. Tumor growth was measured twice per week. Data are presented as mean $\pm$ s.e.m. Statistical analysis was performed using two-way ANOVA. ** $P \leq 0.01$, ns not significant.

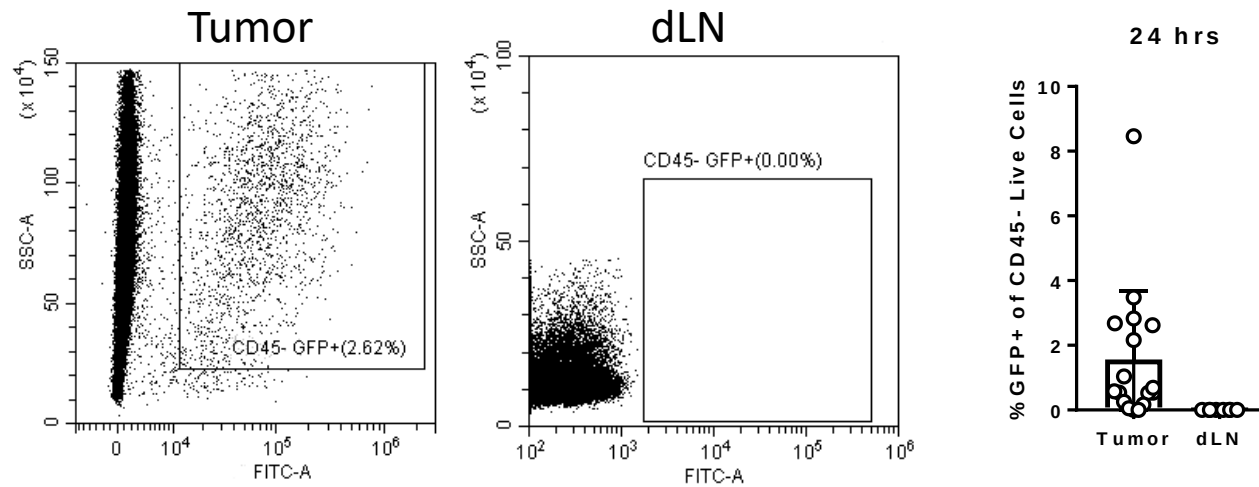
S4



Supplementary Figure 4. Antigen specific T cell activation by BMDC priming vs. CD3 ligation.

A BMDC (10k) and splenic OT-I CD8⁺ T cells (100k) were co-cultured with or without 100ug/mL OVA protein on plates with or without pre-coating of 0.1ug/mL α CD3. After 48hrs of co-culture, IFN γ in the supernatant was measured by CBA. **B** BMDC (10k) and splenic OT-I CD8⁺ T cells (100k) were co-cultured with 0, 4, 20 or 100ug/mL OVA protein (see Figure 3c for schematic). High dose 1ug/mL coated anti-CD3 is provided for perspective. After 48hrs co-culture supernatant IFN γ was measured by CBA. Data are presented as mean $\pm$ SD from a representative experiment (n=3) of 2 independent experiments. Statistical analysis was performed using two-way ANOVA with Sidak's multiple comparisons test (A) and one-way ANOVA with Dunnett's multiple comparisons test (B). ****P $\leq$ 0.0001, **P $\leq$ 0.01, ns not significant.

S5

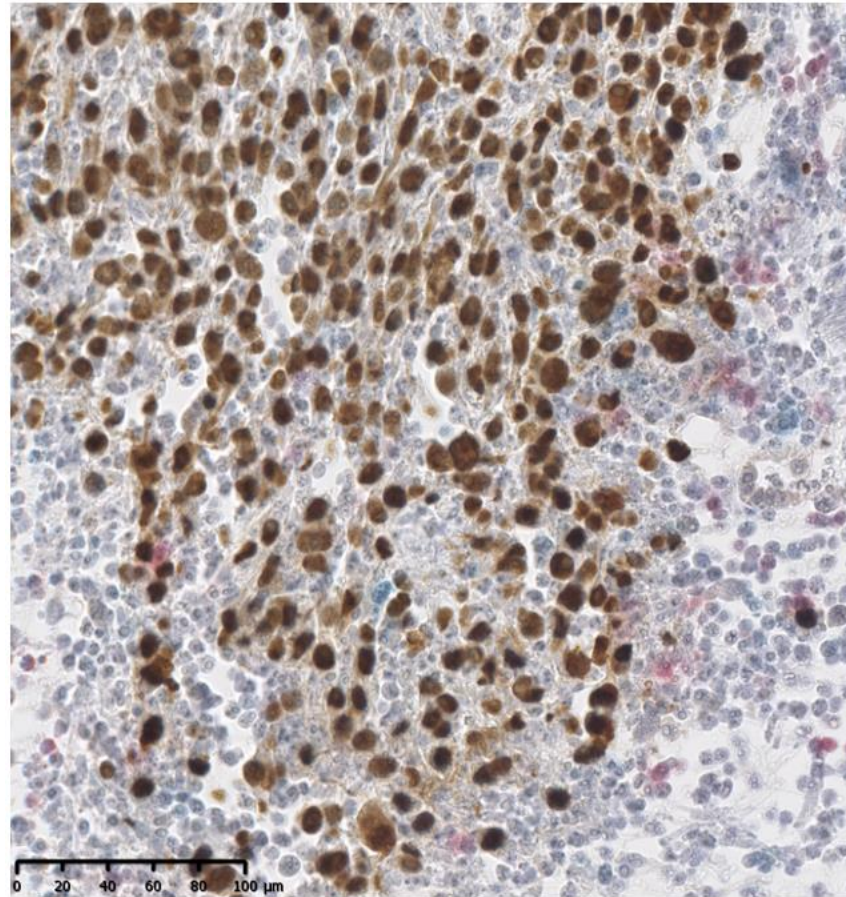


Supplementary Figure 5. MSC-CD3-CD40L migration into the TME vs. dLN.

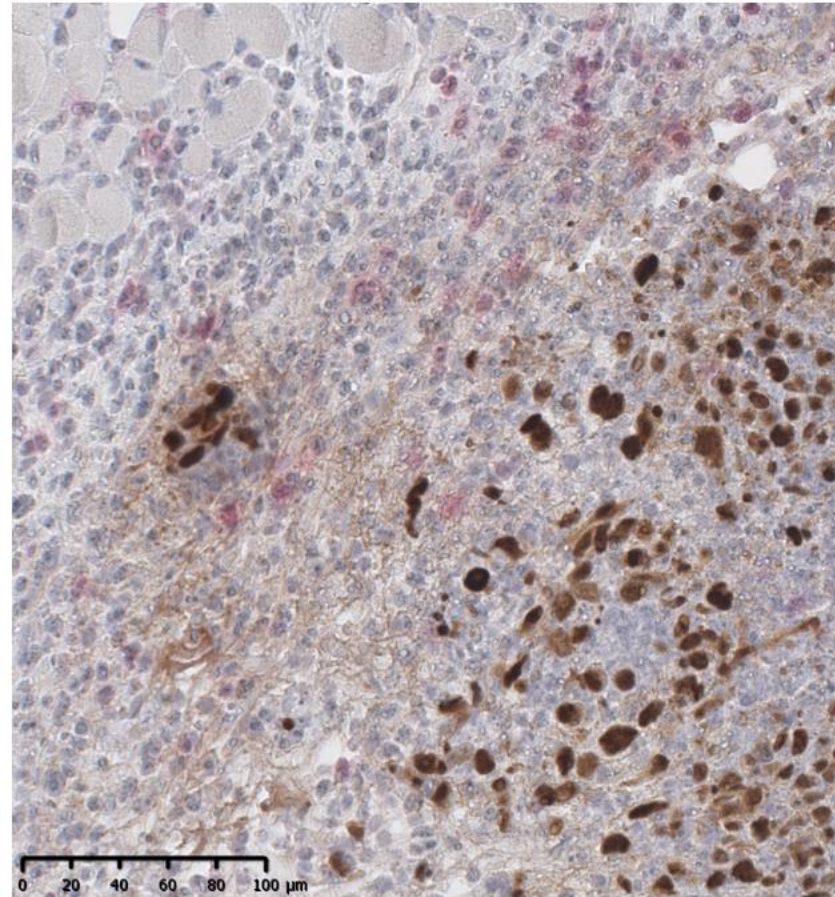
C57BL/6J mice were inoculated with 1×10^6 MC38 tumor cells and treated (p.t.) with 1×10^6 MSC or MSC-CD3-CD40L on day 11. Tumors and draining lymph node (dLN) were removed 1 day after the first treatment, isolated into a single cell suspension and analyzed by flow cytometry. Left is representative flow cytometry plots showing GFP on X axis in Tumor vs. dLN of live cells. Right shows quantitation of CD45- GFP+ cell percentages.

S6

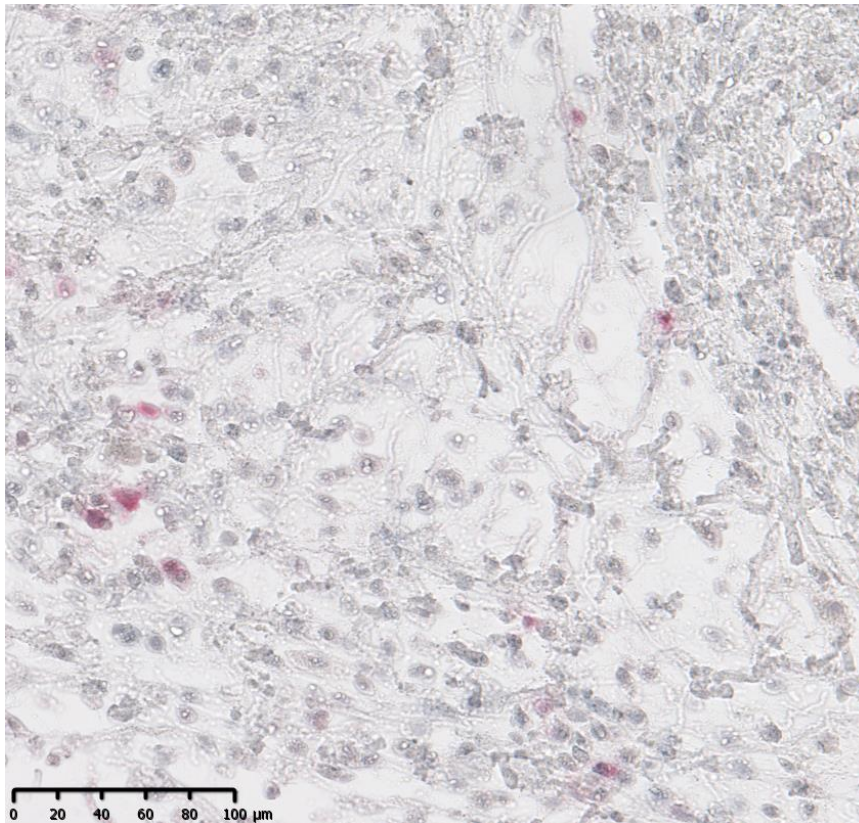
A Day 4 MSC



B Day 4 MSC-CD3-CD40L



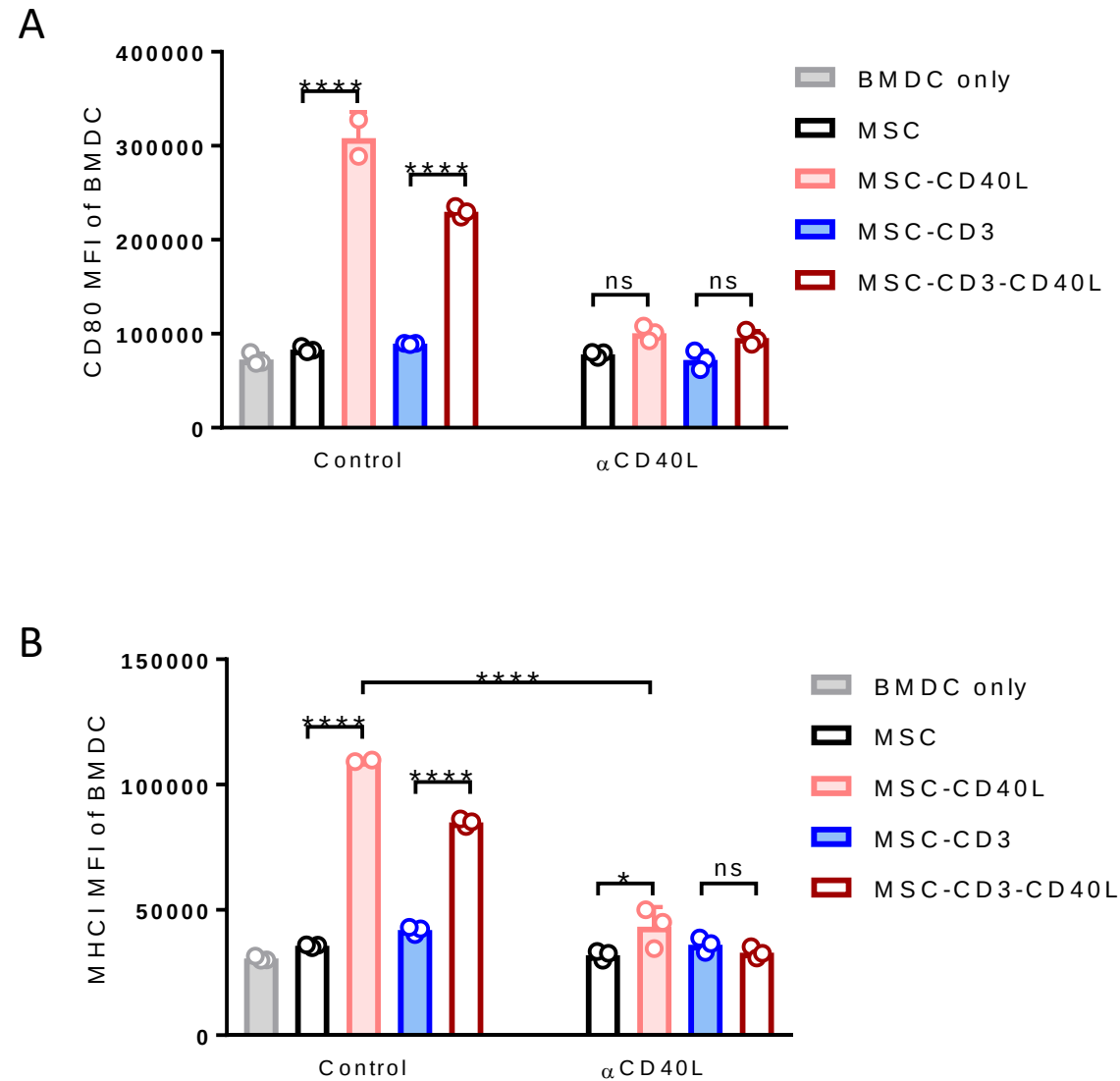
C No MSC injection



Supplementary Figure 6. Co-localization of MSC, CD8+ and CD11c+ cells in the TME.

A-C Representative images of formalin fixed and stained tumor collected 4 days after peri-tumor injection of MSC stained for SV40 (brown), CD8 (red) and CD11c (blue) treated with MSC (**A**), MSC-CD3-CD40L (**B**) or no MSC (**C**). Scale bar shown in lower left corner.

S7



Supplementary Figure 7. MSC co-activator expression increases BMDC activation markers.

A BMDC (50k) and MSC (10k) were co-cultured for 24 hours with or without α CD40L blocking antibody. Cells were trypsinized, stained with antibodies then analyzed by flow cytometry for CD80 expression (**A**) and MHC I expression (**B**). Data are presented as mean $\pm$ SD from a representative experiment ($n = 3$) of 2 independent experiments. Statistical analysis was performed using two-way ANOVA (A) and two-tailed unpaired Student's t-test with Sidak's multiple comparisons test. **** $P \leq 0.0001$, * $P \leq 0.05$, ns not significant.

Supplemental Table 1. Key reagents table

Reagent or resource	Source	Identifier
Antibodies		
Anti-mouse CD45 (flow cytometry, 30-F11)	BioLegend	catalog 103126
Anti-mouse CD3 (flow cytometry, 145-2C11)	BD Biosciences	catalog 564379
Anti-mouse CD8 (flow cytometry, 53-6.7)	BioLegend	catalog 100730
Anti-mouse CD4 (flow cytometry, RM4-5)	BD Biosciences	catalog 550954
Anti-mouse PD-1 (flow cytometry, 29F.1A12)	BioLegend	catalog 135224
Anti-mouse TIM-3 (flow cytometry, RMT3-23)	eBioscience	catalog 25587008
Anti-mouse TOX (flow cytometry, TXRX10)	eBioscience	catalog 50-6502-82
Anti-mouse Foxp3 (flow cytometry, MF-14)	BioLegend	catalog 126408
Anti-mouse CD25 (flow cytometry, PC61)	BD Biosciences	catalog 564021
Anti-mouse CD11b (flow cytometry, M1/70)	eBioscience	catalog 12-0112-82
Anti-mouse CD11c (flow cytometry, HL3)	BD Biosciences	catalog 550261
Anti-mouse F4/80 (flow cytometry, BM8)	BioLegend	catalog 123116
Anti-mouse CD103 (flow cytometry, 2E7)	BioLegend	catalog 121406
Anti-mouse CD19 (flow cytometry, 6D5)	BioLegend	catalog 115508
Anti-mouse I-A/I-E (flow cytometry, M5.114.15.2)	eBioscience	catalog 56-5321-82
Anti-CD16/32 (clone 2.4G2)	In house	N/A
Purified anti-mouse CD3 (17A2)	BioLegend	catalog 100202
Fixable Viability Dye eFluor 780	Thermo Fisher Scientific	catalog 65-0866-18
H-2Kb MuLV p15E Tetramer-KSPWFTTL-PE	MBL	catalog TB-M507-1
7-AAD Viability Staining Solution (flow cytometry)	BioLegend	catalog 420404
InVivoMAb anti-mouse CSF-1R β (CD115) (AFS98)	BioXCell	catalog BE0213
InVivoMAb anti-mouse CD8 (53-5.8)	BioXCell	catalog BE0223
InVivoMAb anti-mouse CD4 (GK1.5)	BioXCell	catalog BE0003-1
InVivoMAb anti-mouse CD80 (B7-1) (1G10)	BioXCell	catalog BE0024
InVivoMAb anti-mouse CD86 (B7-2) (GL-1)	BioXCell	catalog BE0025
InVivoMAb anti-mouse CD40L (CD154) (MR-1)	BioXCell	catalog BE0017-1
InVivoMAb anti-mouse IL-12p75 (R29A5)	BioXCell	catalog BE0233
Bacterial and virus strains		
N/A		
Biological samples		
N/A		
Chemicals, peptides, and recombinant proteins		
FTY720 (hydrochloride)	SelleckChem	catalog S5002
DT from corynebacterium diphtheriae	MilliporeSigma	catalog D0564
DMEM	Sigma-Aldrich	catalog D6429
RPMI-1640	Sigma-Aldrich	catalog R8758-500ML
MesenCult Expansion Media	Stemcell Technologies	catalog 05513
Collagenase type I	MilliporeSigma	catalog C0130
OVALBUMIN ENDOFIT	Invivogen	catalog vac-pova
DNase I	Roche	catalog 11284932001
GW2580	Stemcell Technologies	catalog 72472

continued on next page

Reagent or resource	Source	Identifier
Critical commercial assays		
BD Mouse IFN- γ ELISPOT Sets	BD Biosciences	catalog 551083
True-Nuclear Transcription Factor Buffer Set	BioLegend	catalog 424401
CD45 (TIL) MicroBeads, mouse	Miltenyi Biotec	catalog 130-110-618
BD Cytometric Bead Array (CBA) Mouse Inflammation Cytokine Kit	BD Biosciences	catalog 560485
Deposited data		
N/A		
Experimental models: Cell lines		
MC38	ATCC	N/A
CT26	ATCC	catalog CRL-2683
B16-OVA	Gao Lab	N/A
4T1	ATCC	catalog CRL-2539
293T	ATCC	catalog CRL-3216
Experimental models: Organisms/strains		
C57BL/6J	The Jackson Laboratory	Strain 000664
B6.129S4-Irfng ^{tm3.1Lky} /J	The Jackson Laboratory	Strain 017581
B6.129S(C)-Batf3 ^{tm1Kmm} /J	The Jackson Laboratory	Strain 013755
B6(Cg)-Zbtb46 ^{tm1(HBEGF)Mnz} /J	The Jackson Laboratory	Strain 019506
C57BL/6-Tg(TcraTcrb)1100Mjb/J	The Jackson Laboratory	Strain 003831
B6;129S1-Il12rb2 ^{tm1jm} /J	The Jackson Laboratory	Strain 003248
B6.129S2-Ighm ^{tm1Cgn} /J	The Jackson Laboratory	Strain 002288
Oligonucleotides		
N/A		
Recombinant DNA		
SSR#69 Immortalization Retroviral Vector	Gift from T.C. He	N/A
Plasmid: pMD2.G		
Plasmid: psPAX2		
Plasmid: pLVX-sCD3-GFP	This paper	N/A
Plasmid: pLVX-CD3-GFP	This paper	N/A
Plasmid: pLVX-CD40L-GFP	This paper	N/A
Plasmid: pLVX-CD80-GFP	This paper	N/A
Software and algorithms		
GraphPad Prism software 7.0	GraphPad Software Inc.	https://www.graphpad.com/scientific-software/prism/
CTL-ImmunoSpot S6 Analyzer	Cellular Technology Limited	https://immunospot.com/products/analyzers
CytExpert	Beckman Coulter	https://www.beckman.com/flow-cytometry/research-flow-cytometers/cytoflex/software
BD FACSCorus Software	BD Biosciences	https://www.bdbiosciences.com/en-us/products/software/instrument-software/bd-facschorus-software
Flowjo	Tree Star Inc.	https://www.flowjo.com/solutions/flowjo
TIMER	Ref. 24	https://cistrome.shinyapps.io/timer/
Other		
N/A		