Supplemental Data

Myeloid cell-derived PROS1 inhibits tumor metastasis by regulating inflammatory and immune responses via IL-10

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Methods

Mice

Veterinary care was provided to all animals by the Hebrew University animal care facility staff in accordance with AAALAC standard procedures under specific pathogen-free conditions, and approved by the Hebrew University Ethics committee (approval MD-14025-5). $Pros1^{fl/fl}$ (1) , LysM-Cre (2); $CX3CR1^{GFP/WT}$ (3) ; and $Ai9^{LSL}$ (4) mice were previously described. $LysM\text{-}Cre^{+}$; *Pros1*$^{fl/fl}$; $Ai9^{TdT\text{-}LSL/+}$ experimental mice and $LysM\text{-}Cre^{+}$; *Pros1*$^{wt/wt}$; $Ai9^{TdT\text{-}LSL/+}$ controls were generated by crossing $LysM\text{-}Cre^{+}$; $Ai9^{TdT\text{-}LSL/TdT\text{-}LSL}$ and *Pros1*$^{fl/fl}$ or *Pros1*$^{+/+}$, respectively. Similarly, $LysM\text{-}Cre^{+}$; *Pros1*$^{fl/fl}$; $CX3CR1^{GFP/WT}$ and *Pros1*$^{fl/fl}$; $CX3CR1^{GFP/WT}$ mice were generated by crossing $LysM\text{-}Cre^{+}$; *Pros1*$^{fl/fl}$; $CX3CR1^{GFP/GFP}$ and *Pros1*$^{fl/fl}$ mice. Primer sequences for genotyping are given in Table 1.

Cells

Lewis Lung Carcinoma (LLC), LLC-GFP and the mouse mammary AT3-GFP tumor cell lines were previously described (5–8) and kindly provided by Dr. Zvi Granot. Cells were cultured in DMEM containing 10% FBS, 2mM L-Glutamine, 100 Units/ml of penicillin-streptomycin, and maintained in 5% CO_2 humidified incubator at 37°C, and confirmed to be mycoplasma-free. All tissue culture regents were purchased from Beit Haemek, Israel.

Experimental tumor models

To evaluate metastatic potency in vivo, LLC cells (5×10^5 in 100µl in sterile PBS $Ca^{2+}Mg^{+}$-free) were subcutaneously injected into the rear flank of 6-8 weeks old mice. Primary tumor growth was measured twice weekly using a caliper. Mammary AT3-GFP tumor cells (10^6 cells in 20µl medium containing 25% GFR

matrigel) were orthotopically injected into the two inguinal mammary glands of 6-8 weeks old female mice. 20-24 days post injection, mice were anesthetized, primary tumors and lungs removed for further analysis. For survival and metastatic seeding experiments, LLC-GFP cells (2×10^5 cells in 100µl of sterile PBS) were intravenously injected into the tail vein. Twenty days later mice were anesthetized and transcardially perfused with PBS/heparin solution after which the lungs were removed, documented and analyzed.

Cancer cell education

$0.25x10^6$ cancer cells were seeded in 6-well plates. The next day, cells were washed in PBS and incubated (24hrs for LLC; 96hrs for AT-3) in the presence of the designated BMDM-CM, filtered and prepared as described under BMDM and BMDC differentiation. For blocking experiments, 5µg/ml of IgG1 isotype (control) or anti IL-10 antibodies (MAB005 and MAB417 respectively, R&D Systems) was added 45 minutes prior to incubation with cells. Following education, the cells were washed in PBS, trypsinized, and subject to different assays, as described.

Analysis of Lung Metastasis

Mice were perfused with PBS/heparin for analysis of fluorescently-labeled metastasis, or further perfused with 4% paraformaldehyde and processed for Paraffin embedding. 8mM-thick sections were prepared according to standard procedures, stained with Hematoxylin and Eosin (H&E) (Sigma) and documented with an Olympus BX51 microscope equipped with a DP72 digital camera. To quantify lung metastatic efficiency and burden, 3-4 serial H&E stained lung sections were collected every 400 microns, spanning a total of 1200-1500 microns of lung tissue. Quantification was performed as in (9) using Olympus CellSens - XV Image processing software. The software calculated area (in microns squared) after marking the perimeter of the nodule using the polygonal measuring tool. Mets number represents the total number of metastatic nodules in each section. Fluorescently - labeled metastatic foci were documented under fluorescent illumination using an Olympus SZX10 stereoscope mounted with a DP72 cooled CCD followed by western blot or RT-qPCR analyses.

Quantitative real time PCR (q-PCR)

Total RNA was extracted with Tri reagent (Sigma) from cultured cells or from homogenized lung tissue. Mice were intracardially perfused with PBS/heparin, lungs isolated and manually chopped with a sterile blade, homogenized in 900µL TriReagent using 0.9-2mm stainless steel beads (NEXT ADVANCE) and incubated in a homogenizer device (Next Advance) for 5 min at 4°C, maximal speed. 0.5-1 µg of RNA was reverse-

transcribed using the QScript cDNA synthesis kit (Quanta). Quantitative RT-qPCR was performed using Perfecta SYBERgreen fast mix (Quanta) on a CFX96 thermocycler (Bio-Rad). A serial dilution of pooled cDNA from all samples was used to generate a linear equation curve with each primer set. Quantification was completed using the Bio-Rad Real-time software, normalizing to GAPDH and using a standard curve-generated formula.

Cq- Threshold cycle from each target gene was inserted into the standard curve formula and the final value was the ratio between the target gene divided by the standard gene. The PCR reaction is composed of 40 cycles. Primers used for RT-qPCR are listed in Table 2.

Western blot

Cells were lysed in Laemmli buffer and analyzed for total protein concentration by standard western blot techniques. Briefly, equal amounts of protein (15 to 30 μg) in Laemmli buffer were subject to PAGE on 8 or 10% gels, transferred to PVDF membranes (Millipore) and blocked with TBST blocking buffer (50 mM Tris-HCl pH 7.5, 0.15 M NaCl and 0.25% Tween-20) containing 5% skim milk (BD-Difco). Membranes were incubated overnight at 4 °C with primary antibodies dissolved in TBST buffer containing 5% BSA and 0.02% sodium azide. Blots were then washed in TBST and incubated for 1 hr at room temperature with secondary HRP-conjugated antibodies, diluted as per manufacturer's instructions in TBST/5% skimmed milk. Blots were washed and developed using the WesternBright ECL kit (Advansta). For quantification of phospho-specific and total band intensities, relevant bands were first normalized to β-actin levels. Primary and secondary antibodies used are listed in Table 3.

Immunoprecipitation

Immunoprecipitation was performed according to (10). Briefly, 3-4 minutes before cell harvesting, 0.12mM sodium vanadate (Na_3VO_4) diluted in 0.002% H_2O_2 was added to the culture to inhibit phosphatase activity. Then, cells were washed with cold PBS ($Ca_2^+MG_2^+$-free) and lysed with cold RIPA buffer (5mM β-glycerophosphate, 1% NP-40, 0.5% Sodium-deoxycholate, 0.1% SDS, 0.5mM EDTA in PBS) containing 1:100 protease and phosphatase cocktail inhibitors (P8340 and P5726 respectively, Sigma), and centrifuged (12,000g for 15 min at 4°C) or passed through a 0.45-mm filter. 500µg cell lysate was precleared with 30µl (packed volume) of protein G agarose beads (sc-2002, Santa Cruz) for 2hr, with continuous shaking at 4°C. Tubes were centrifuged (1000rpm/2mins) and the precleared supernatant was incubated with 1µg of phosphor-tyrosine antibody (05-321; clone 4G10, Millipore) overnight under the same conditions. The next day, 30µl

(packed volume) of protein G-agarose beads were added for another 2hr and immune-precipitates were washed 3X with IP washing buffer (150mM Nacl, 50mM Tris-HCl, 0.05% NP-40, 0.5mM EDTA, 0.1% SDS, pH 7.5). Immuno-precipitates were eluted in 2X Laemmli buffer, heated and analyzed by western blot using a MER-specific antibody (AF591, R&D).

Anchorage independent growth

Colony formation in soft agar was assayed as previously described (11). Briefly, $15x10^3$ cells were mixed with growth medium and 0.3% agarose and plated over a solidified later of 1% vol/vol agarose in 2 mL growth medium in a 6 well plate in duplicates. 30 mins later, the agarose was covered with 2mL growth medium as described above. 21 days later, colonies from were counted in ten different fields/well for each experimental condition. Colonies were documented using an Olympus CKX41 (x20 magnitude) mounted with an Olympus DP72 camera, converted to B&W. Colonies with a diameter greater than 25 µM were scored manually.

Colony Survival

Cells were seeded sparsely as indicated in the figure, grown for 7-10 days in 2 ml medium//6well plate grown, fixed in 2.5% Glutaraldehyde, rinsed 3-4 times with DDW and stained with 1% methylene blue (Sigma) dissolved in 1M boric acid buffer (pH=8.5). Colonies were documented using the GelDoc imaging system (Bio-Rad) with visible light and counted manually.

Invasion assay

Matrigel invasion assay was performed using 8 µm cell culture inserts (Millicell, #MCEP24H48) in 24 well plates coated with 100 µl containing 300 µg matrigel (Basement Membrane Extract, R&D systems; #343300501) diluted in cold serum-free medium. Matrigel was allowed to solidify for 12 hrs at 37°C, after which 7.5×10^5 cells were seeded into the upper chamber inserts in 100µl of serum-free medium. 600µl of growth medium (with 10% FBS) was added to the lower chamber. Cells were incubated in a humidified incubator at 37°C for 72hrs. Lower chamber wells with invaded cells were washed with PBS, fixed and stained as described for the colony survival assay. Cells were counted in 7-10 random-chosen fields using an Olympus CKX41 (x20 magnitude) and plotted as the average cell number per field.

BMDM and BMDC differentiation

Generation of BMDMs was performed as previously described (12). Briefly, bone marrow (BM) was flushed out of the femur and tibia of the indicated mice with PBS buffer using a 27G needle and cultured in DMEM/F12 medium supplemented with 10% FBS 2 mM L-Glutamine, 100 Units/ml of penicillin-streptomycin and 5% vol/vol filtered medium conditioned by L-929 cells (13). Fresh medium containing M-CSF (or L-929 medium) was added after 3 days. 5-7 days later, BMDM purity was evaluated by flow cytometry following immunostaining with CD11b, F4/80 and Ly6C. Cultures were >99% CD11b$^+$; F4/80$^+$; Ly6C$^-$ indicating a highly pure population of BMDMs. BMDM-conditioned medium used for education was collected after 7-10 days of growth, centrifuged at 1,500 rpm /4°C for 5 minutes to rid of debris, filtered (0.45μm; Millex), aliquoted and frozen at -80°C until used. For BMDC differentiation, bone marrow cells were cultured in RPMI medium supplemented with 10% FBS 2 mM L-Glutamine, 100 Units/ml of penicillin/streptomycin and 20 ng/ml GM-CSF (Peprotech, USA). Fresh medium containing 20 ng/ml GM-CSF was added after 3 days. BMDCs from the supernatant and loosely attached cells were collected at day 7 and their purity was evaluated by flow cytometry following immunostaining with CD11c (14). BMDC purity reached 86-88% as detected by CD11c$^+$ immunoreactivity.

In vitro stimulation of BMDMs

Differentiated BMDMs were washed with PBS containing 1mM EDTA and collected using a cell scraper. Cells were centrifuged at 1,100 rpm for 8 minutes and resuspended with DMEM/F12 medium supplemented with 10% FBS, 2 mM L-Glutamine, 100 Units/ml of penicillin-streptomycin, counted and seeded at 0.5×10^6 cells in 6-well plates. 12 hrs later, the cells were starved for 4-5 hrs in M-CSF free growth medium containing 0.5% FBS and stimulated with the indicated concentrations of LPS (SIGMA) for the specified times. For rescue experiments, purified hPROS1 (25 nM; Enzyme Research Laboratories, South Bend, IN) was added during macrophage differentiation on days 3 and 5 post seeding of BM cells, the medium collected and prepared as described above on day 7. To examine MERTK activation, BMDMs were starved as described above and stimulated for 15 mins with 25 nM purified hPROS1, followed by immunoprecipitation. UNC4241 (panTAM) or UNC4203 (MERTK specific) were added to the culture for 45 minutes prior to stimulation with PROS1 for 15 minutes, followed by IP as described. To evaluate cytokine secretion by BMDMs following TAM inhibition, BMDMs were starved and treated either with PROS1 alone or with 500 nM of the indicated UNC inhibitors followed by LPS (100 ng/ml) stimulation for 22 hours. Supernatants were analyzed by ELISA. BMDMs at steady state growth (10% FBS) were treated either with PROS1 alone or with of UNC4241 (500 nM) for 24 hours. Following incubation, CM was prepared from the indicated groups and used to educate LLC

cancer cells. For TNFα stimulation experiments, starved BMDMs were treated with recombinant mouse TNFα (PEPROTECH) at the indicated concentrations for 24 hrs. Supernatants were analyzed by ELISA. For blocking experiments, 5µg/ml of Rabbit IgG isotype (control) or anti TNFα antibodies (500-P00 and 500-P64 respectively, PEPROTECH) were added 1hr prior to LPS stimulation.

Cytokine expression

BMDM conditioned medium (100-200 µl) or plasma (Peripheral blood collected into heparin-coated tubes, centrifuged at 12,000 rpm/10 mins/4°C) were analyzed for mouse TNF-α and IL-6 by ELISA (ELISAMAX Deluxe Set, BioLegend) and IL-10 (900-TM53 mini TMB ELISA kit, PEPROTECH) according to the manufacturer's instructions. For cytokine expression, whole lungs were excised following perfusion with PBS/heparin, chopped and homogenized using a homogenizer device (Next Advance) equipped with 0.9-2 mm stainless steel beads for 5 min at 4°C in RIPA buffer containing 1:100 protease and phosphatase cocktail inhibitors. Pierce BCA Protein Assay kit (23227, Thermo Scientific) was used for quantification. 0.5mg of lysates were used for ELISA detection.

Tissue preparation for FACS analysis

Single cell suspensions of tumors and lungs were mechanically disrupted using a scalpel blade followed by enzymatic digestion (1mg/ml collagenase A; 15mg/ml Hyaluronidase and 10mg/ml DNase I (all from Sigma-Aldrich) in serum-free DMEM for 1-2 hrs at 37°C using constant gentile agitation produced by an orbital shaker. Enzymatic degradation was stopped by adding ELB solution (1M NH_4Cl_2; 1M $KHCO_3$; 0.5M EDTA) and a final wash with PBS.

Flow cytometry

Prior to antibody staining, cells were filtered through a 70µm cell strainer and suspended in FACS buffer (PBS with 2%FBS and 0.01% sodium azide). Fluorophore-conjugated monoclonal antibodies and their corresponding isotype controls were purchased from BioLegend (used at 1:2000 dilution): Pacific Blue-CD45.2(104), APC-F4/80(BM8), PE-Ly6G(1A8), PE-Cy7 Ly6C(HK1.4), FITC-CD11b(M1/70), FITC-CD3ε(145-2C11), PERCP-Cy5.5-CD8(53-5.8), PE-CTLA4(UC10-4B9), APC-PD-1(29F.1A12), PE-CD11c(N418), APC/Cy7-MHC-II(M5/114.15.2), FITC-CD86(GL-1), APC-CD40(3/23). $CD3^+$ cells that were CD8- are considered $CD4^+$. Cells were stained on ice, protected from light for 30 mins, centrifuged (1100

rpm/8 mins), washed with FACS buffer and analyzed using an LSR-II (BD Biosciences) flow cytometer. For TCRζ-chain intracellular staining, cells were fixed with 1% paraformaldehyde/PBS for 30 min at 4^{0}C, washed and permeabilized with 0.1% Saponin (47036, Sigma) for 10 min at room temperature, then stained for CD45.2, CD3ε and anti-ζ-chain (15) in the presence of anti-FcR (CD16/32(93) Biologend) in FACS buffer containing 0.1% saponin for 30 min on ice. Analysis was performed using FlowJo software.

Macrophage sorting from lungs

Lungs from the indicated mice were dissociated into single cell suspension, stained for F4/80 (30 minutes/ice) protected from light, washed with FACS buffer and filtered using a 40μm cell strainer. Cells from 6-8 mice/group were pooled and $F4/80^{+}/GFP^{-}$ cells were sorted using an Aria II sorter (BD). For RNA isolation, sorted cells were collected into PBS, spun at 1100rpm/8 mins, and the pellets resuspended with Tri reagent. For ex-vivo assays, cells were collected into DMEM/F12 containing 10% FBS, 2mM L-glutamine, 100Units/ml of penicillin-streptomycin.

Adoptive transfer of labeled BMDMs

Eight week old recipient *Pros1*$^{fl/fl}$ or LysM-Cre^{+};*Pros1*$^{fl/fl}$ (*Pros1*-cKO) mice were lethally γ-irradiated (900) rad. Twenty four hrs later the mice were injected intravenously with $4x10^{6}$ BM cells obtained from *Pros1*-WT and LysM-Cre;*Pros1*$^{fl/fl}$ mice bearing the tomato red reporter gene (Ai9-TdT-LSL). Donor mice carried the following genotypes: LysM-Cre^{+}; Pros1$^{+/+}$; Ai9$^{TdT-LSL}$ and LysM-Cre^{+}; Pros1$^{fl/fl}$; Ai9$^{TdT-LSL}$. Successful BM reconstitution was verified by flow cytometry for Ly6C^{+} and Ly6G^{+} positive cells, and quantitative real time PCR for the TdT reporter gene in blood cells.

Ex-vivo T-cell suppression assay

96-well plates were pre-coated with 3μg/ml of anti-CD3ε, Biolegend (145-2C11) and anti-CD28 (Biolegend 37.51) diluted in 100μl of 0.1M Borate buffer (pH=8.5) for 24h at 4°C. Plates were then blocked (1h at room temperature) with borate buffer containing 1% FBS and washed with PBS 3 times. T cells were isolated magetically from spleen of *Pros1*$^{fl/fl}$; *CX3CR1*$^{GFP/WT}$ mice (StemCell technologies, Cat#: 19851). Purified T cells (>98%) were evaluated by PE-anti-Thy1.2 (140307, Biolegend), and stained with CellTrace Violet (Thermo C34557) according to manufacturers instructions. 2×10^{5} labeled T-cells were seeded in RPMI (Gibco) supplemented with 10% FBS, 2mM L-glutamine and 1% pen-strep solution (Biological Industries). Upon

sedimentation of T cells (1 hour at 4^0C), sorted lung macrophages (F4/80$^+$;GFP$^-$) from *Pros1*$^{fl/fl}$; CX3CR1$^{GFP/WT}$ and LysM-Cre$^{+;}$ *Pros1*$^{fl/fl}$; CX3CR1$^{GFP/WT}$ tumor bearing mice were seeded with T-cells at 3:1 ratio in the presence of FcR (anti-CD16/32 (93), BioLegend). Co-cultures were treated with 5µg/ml of antibody (IL-10 nuetralizing MAB417 or IgG1 isotype MAB005; R&D systems), or with 50nM purified hPROS1. Following 72 hours of incubation (37^0C, 5% CO_2), the average number of T-cell divisions (division index-DI) was calculated as previously described (16) by dividing the total divisions to the total number of cells according to the following formula: DI= G1/2×1+G2/4×2+G3/8×3 (total divisions)/G0+G1/2+G2/4+G3/8 (total number of cells). G represents the number of cells in each generation, and G0 represents non dividing cells. All the analysis were performed using FCS-Express V6 proliferation analyzer (DeNovo software). Nitrite secretion in cells supernatant was measured using Griess reagent (abcam 234044), according to manufacturers protocol. Results were obtained following subtraction of absorbance in T cell supernatants. NaNO2 was used as internal standard, absorbance was measured at 540 nm in a TECAN microplate reader.

Statistics

Experiments were repeated at least 2-3 times unless otherwise mentioned. Analysis was performed by one or two-way ANOVA with multiple comparisons for multiple groups, or Student's t test, unless otherwise stated. P values (P) * $P \leq 0.05$; ** $P < 0.01$ and *** $P < 0.001$ were considered significant.

Table 1 – Primers used for genotyping

Gene	Forward primer	Reverse primer
Cre	5'ATTTGCCTGCATTACCGGTC 3'	5'ATCAACGTTTTCTTTTCGG 3'
Pros1 flox	5'CAATACAAGGCAAAGGGAATGAGG 3'	5'GAGCTCTCAGAGACTAAAC 3'
Ai9^{LSL}	5' CCGAAAATCTGTGGGAAGTC 3'	5'AAGGGAGCTGCAGTGGAGTA 3'

Table 2 – Primers used for RT-qPCR

Gene	Forward primer	Reverse primer
Tnfα	5' ATGAGCACAGAAAGCATGATC 3'	5' TACAGGCTTGTCACTCGA ATT 3'
IL-6	5' TCTCTGGGAAATCGTGGA 3'	5' CCAGTTTGGTAGCATCCATC 3'
NOS2	5' CCCTTCCGAAGTTTCTGGCAGCAGC 3'	5' GGCTGTCAGAGCCTCGTGGCTTTGG 3'

IL-12p35	5' ACGAGAGTTGCCTGGCTACTAG 3'	5’ CAGGTCTTCAATGTGCTGG 3’
Socs3	5’ TCCCATGCCGCTCACAG 3’	5’ ACAGGACCAGTTCCAGGTAATTG 3’
Pros1	5’ GCACAGTGCCCTTTGCCT 3’	5’ CAAATACCACAATATCCTGAGACGTT 3’
Gas6	5’ AGGTCTGCCACAACAAACCA 3’	5’ GCGTAGTCTAATCACGGGGG 3’
Mertk	5’ AATGCACAATGCCACCTGCACA 3’	5’ ATCCCGCAACATGCAATTCCGA 3’
Gfp	5’ ACAACAGACAATCGGCTGCTC 3’	5’ AGCAAGGTGAGATGACAGGAG 3’
Il-10	5’ GTGAAGACTTTCTTTCAAACAAAG 3’	5’ CTGCTCCACTGCCTTGCTCTTATT 3’
Gapdh	5’ TCCCACTCTTCCACCTTCGA 3’	5’ AGTTGGGATAGGGCCTCTCTT 3’
Luc	5’ CACCGTCGTA TTCGTGAGCA 3’	5’ AGTCGTACTCGTTGAAGCCG 3’

Table 3 – Antibodies used for western blot

Primary antibody	Antibody Dilution	Manufacturer	Cat. number
Ms anti pERK	1:10000	Sigma	M9692
Rb anti ERK	1:1000	Cell Signaling	#9102
Rb anti pAKT ser473	1:1000	Cell Signaling	#9271
Rb anti AKT	1:1000	Cell Signaling	#9272
Rb anti PROS1	1:1000	Millipore	AB15928
Gt anti MER	1:2000	R&D Systems	AF591
Rb anti pNF-kB (p65 ser 536)	1:1000	Cell Signaling	#3033
Rb anti NF-kB	1:1000	Cell Signaling	#4764
Gt anti GAPDH	1:2000	Cell Signaling	#2118

Rb anti β-actin	1:2000	Cell Signaling	#8457
Rb anti GFP	1:2000	Cell Signaling	#2956
Rb anti MMP9	1:1000	abcam	ab38898
Rb anti E-cadherin	1:1000	Cell Signaling	#3195
Rb anti N-cadherin	1:1000	Cell Signaling	#13116
Rb anti Vimentin	1:1000	Cell Signaling	#5741
Rb anti pSTAT1	1:1000	Cell Signaling	#9167
Rb anti STAT1	1:1000	Cell Signaling	#9172
Rb anti pSTAT3	1:1000	Cell Signaling	#9145
Rb anti STAT3	1:1000	Cell Signaling	#4904

Secondary antibodies	Antibody Dilution	Manufacturer	Cat. number
Dky anti goat	1:5000	Abcam	ab97120
Dky anti rabbit	1:5000	Abcam	ab7083
Horse anti mouse	1:5000	Cell Signaling	#7076S

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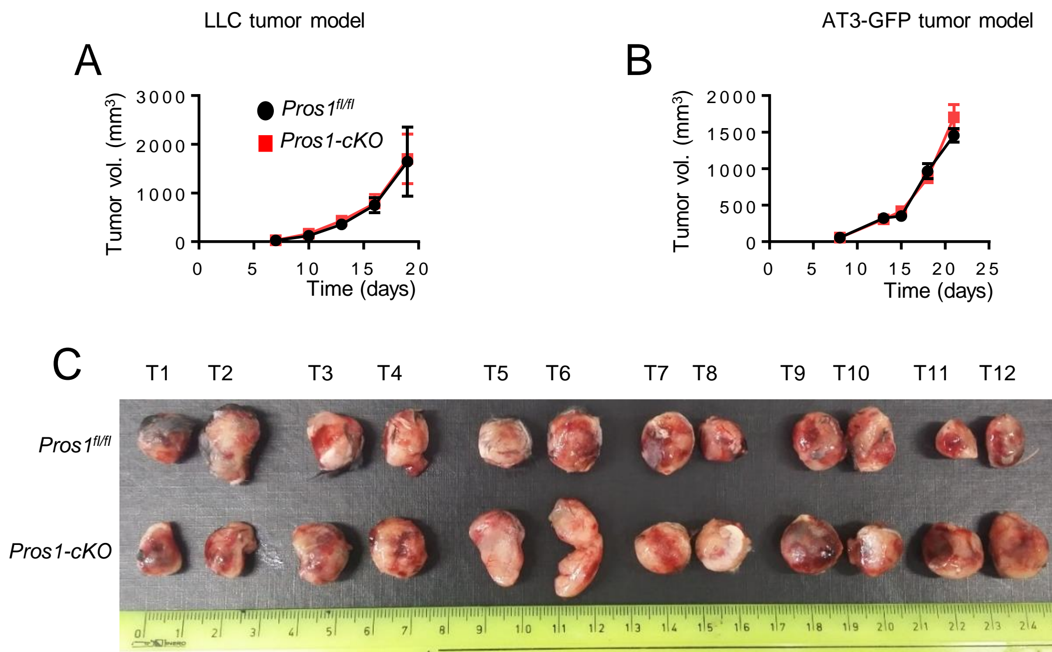
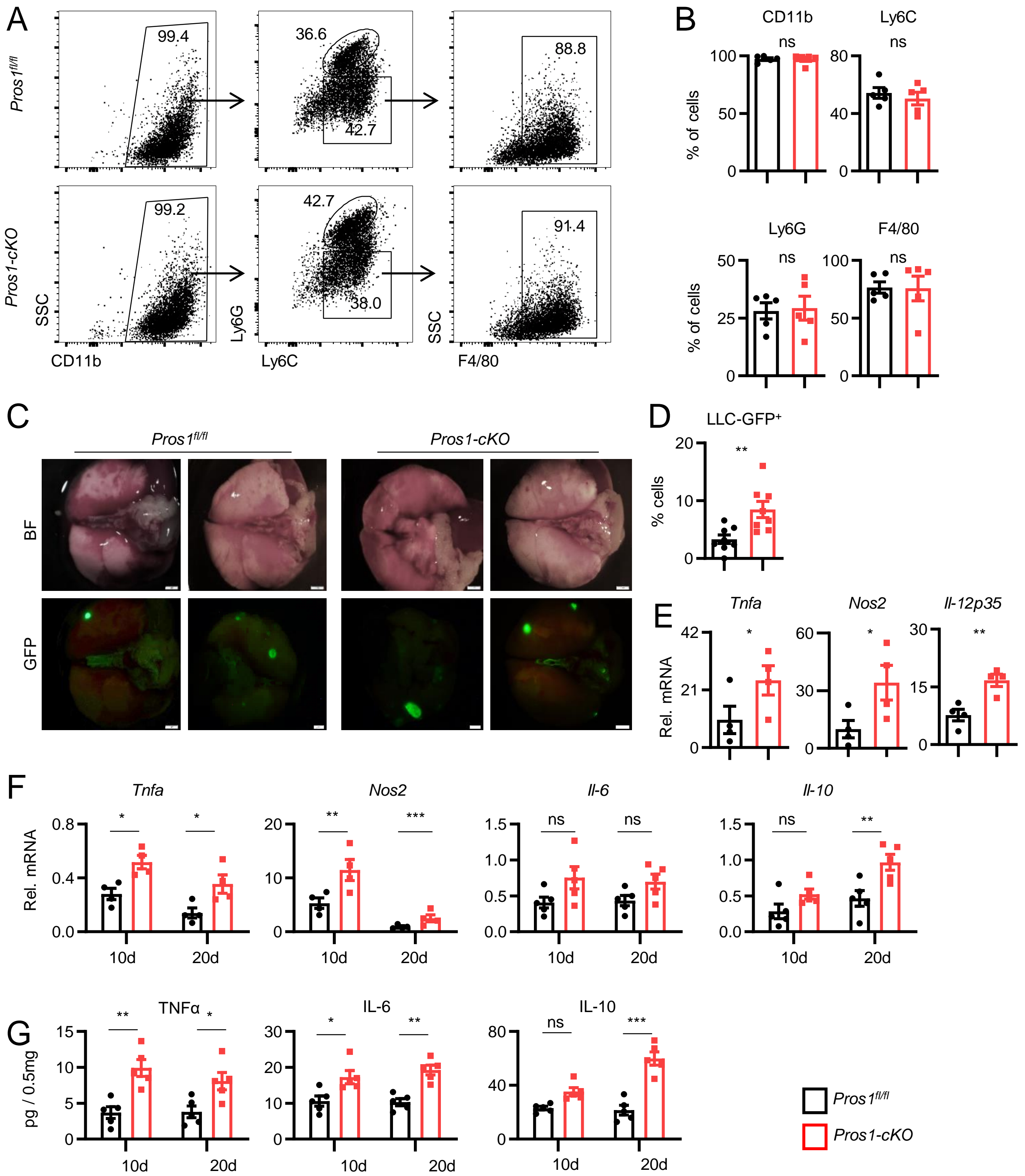


Supl. Fig.1: Primary tumor growth is not affected by genetic ablation of *Pros1* in host myeloid cells.

(A) Primary LLC tumor growth was measured at the indicated time points following subcutaneous injection of LLC cells; n=10, 15 mice/genotype. (B) Primary tumor growth dynamics in an orthotopic AT3-GFP mammary cancer model (n=16/group). (A, B) Not significant for all time points, t-test. (C) Representative image of end-point AT3-GFP primary tumors isolated from control (*Pros1*$^{fl/fl}$) and *Pros1*-cKO mice. AT3 cells were injected into the fat pads of two mammary glands/mouse.

Maimon et al – Sup. Figure 2



Supl. Fig.2: Elevated metastatic colonization is independent of the primary tumor.

(A) Analysis of primary tumors for immune cell infiltrates. Representative FACS plots of $CD45^+$ gated cells from the indicated mice stained for $CD11b^+$ (total myeloid cells), $Ly6C^+$/ $Ly6G^-$ (monocytes), $Ly6G^+$/ $Ly6C^-$ (granulocytes) and $Ly6G^-$/ $F4/80^+$ (macrophages). (B) Quantification of FACS plots described in (A). Mean ± SEM; n=5 mice/group. ns = non-significant; t-test. (C-E) LLC-GFP cells were injected intravenously (IV) into control and *Pros1*-cKO mice. Twenty-one days later, the lungs were isolated and assessed for metastatic colonization in-situ (C, D) and for cytokines expression (E). Acutely isolated lungs were documented using bright field (C, BF, top) and GFP (C, bottom) illumination. Scale= 1 mm. (D) Metastatic GFP^+ cells from lungs described in C were scored by flow cytometry (n=8/group; P=0.002). (E) Cytokine transcript levels from lungs described in (C) were measured by RT-qPCR. The relative mean ± SEM from one of two biological replicates is shown. P=0.04, 0.02, and 0.003 for *Tnfα, Nos2* and *Il-12p35*, respectively (t-test). (F-G) Dynamic expression of cytokines within the lungs of control and cKO mice. (F) RT-qPCR measurements of cytokine transcript levels at 10 and 20 days after subcutaneous injection of LLC cells. Mean ± SEM ; n=4-5 mice/group. 10 days; P=0.028 and 0.01 for *Tnfα* and *Nos2* respectively. 20 days; P=0.047, 0.0006 and 0.011 for *Tnfα, Nos2* and *Il-10* respectively, ns=non-significant; 2-way ANOVA. (G) Cytokine levels measured by ELISA from lungs described in F. Mean ± SEM; n=5 mice/group. 10 days; P=0.002, 0.02 and non-significant for TNFα, IL-6 and IL-10 respectively. 20 days; P=0.04, 0.002 and <0.0001 for TNFα, IL-6 and IL-10 respectively, 2-way ANOVA.

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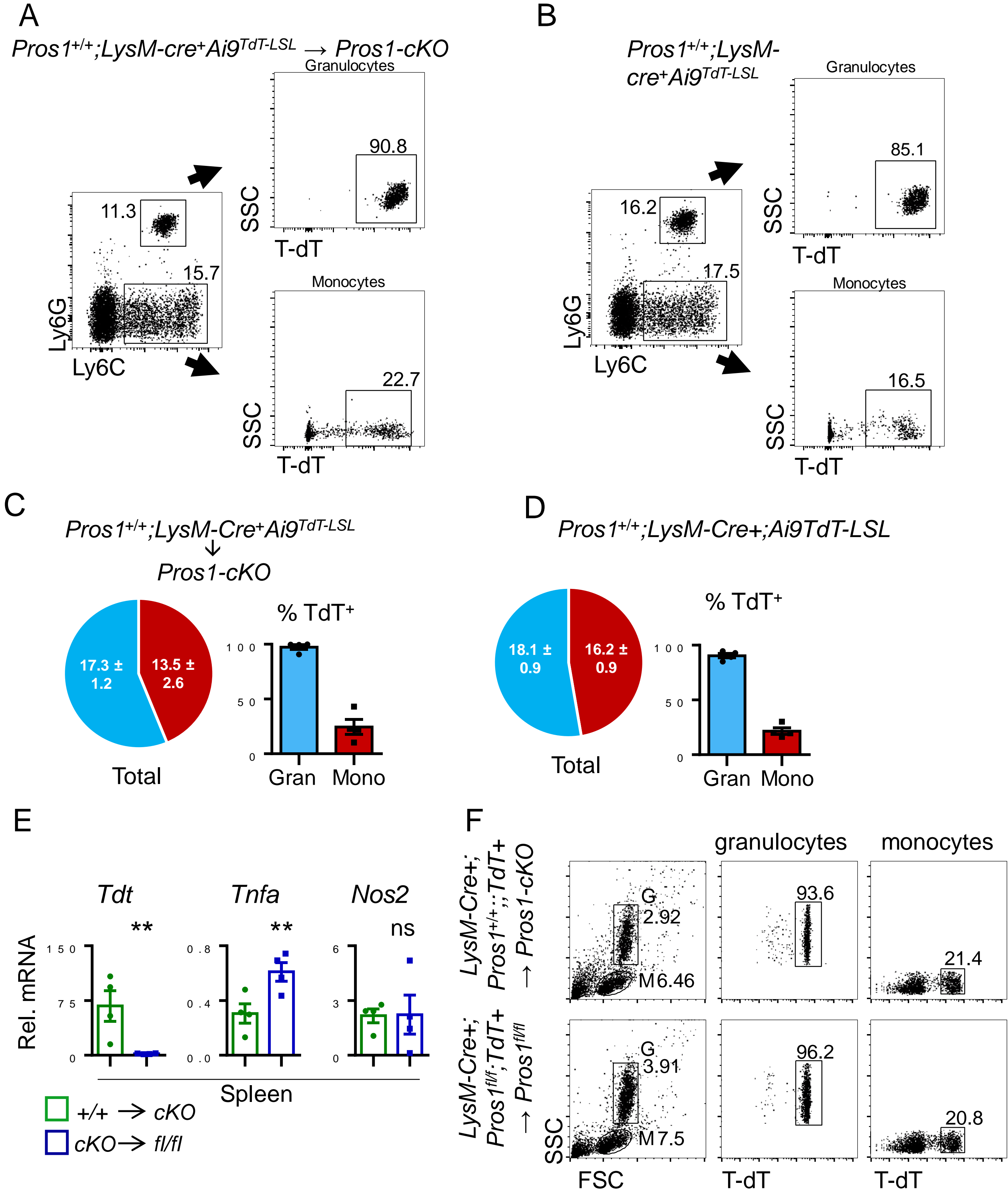
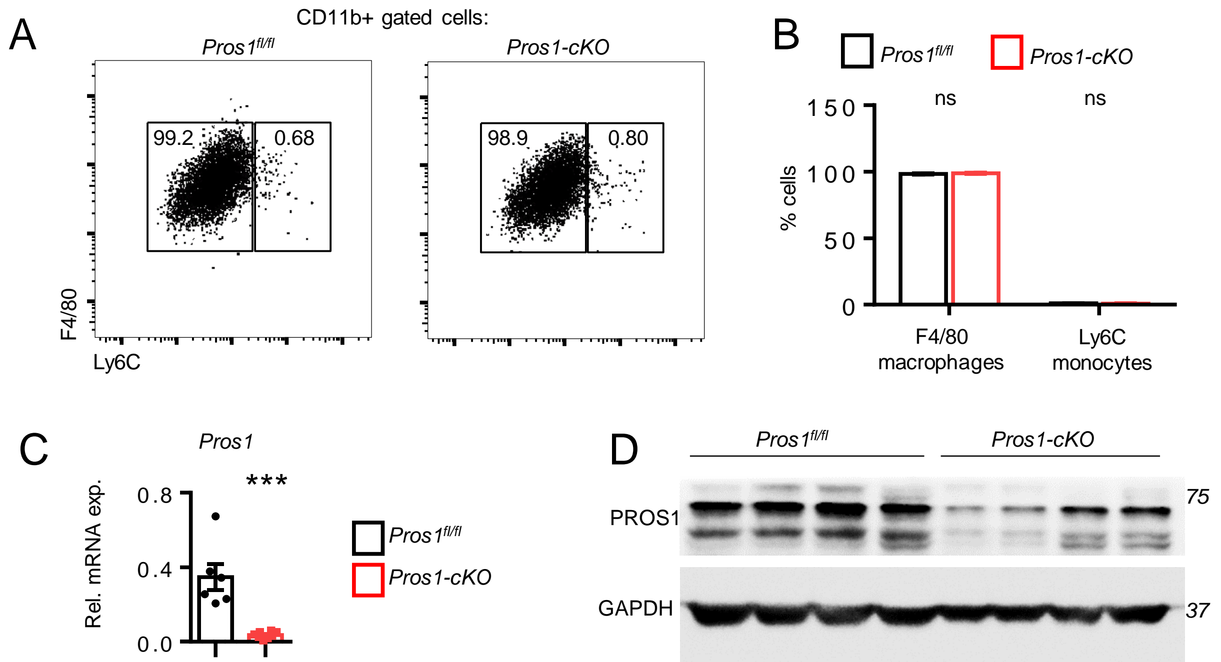


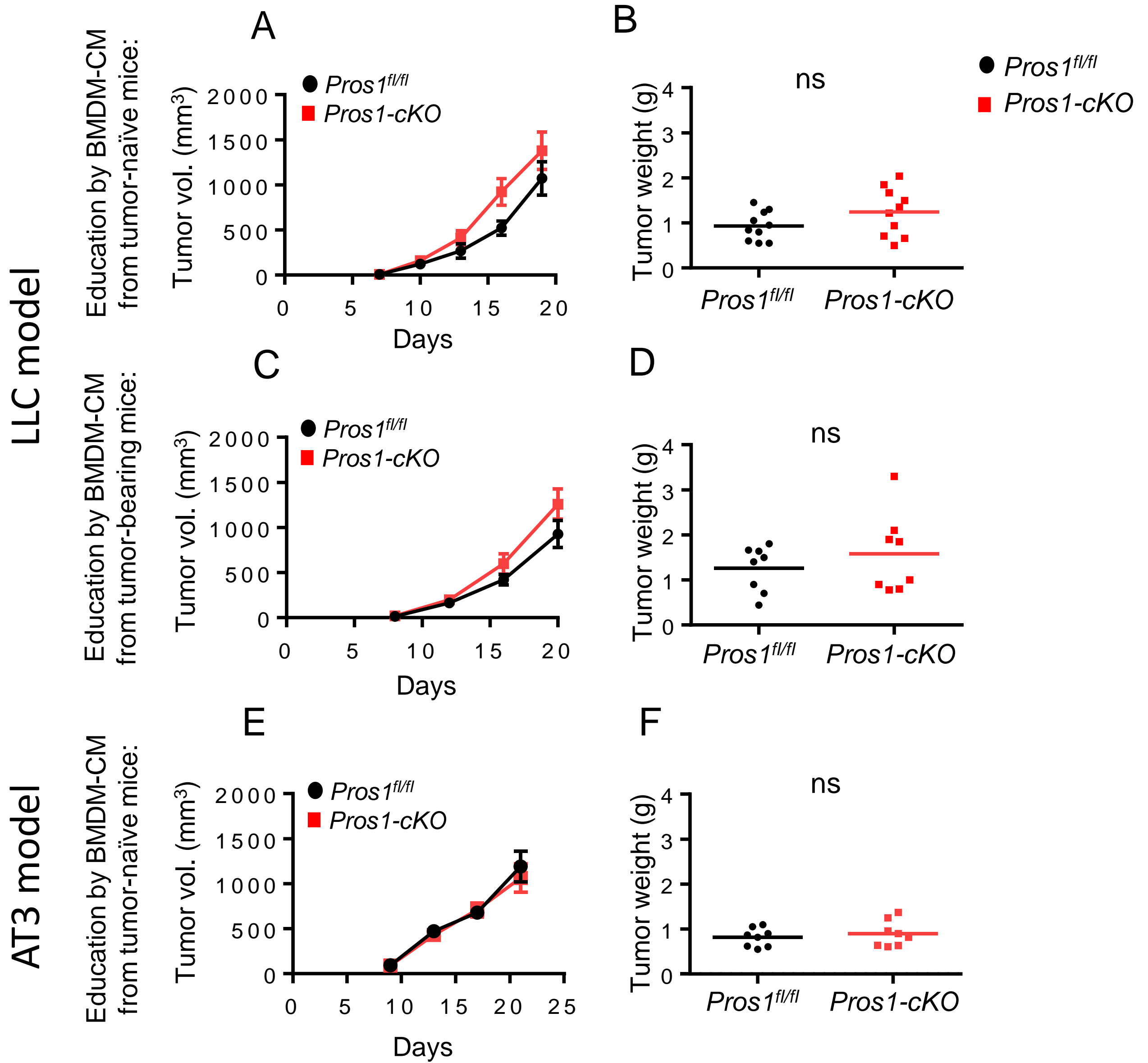
Suppl. Fig.3: Successful Bone Marrow chimerism in Pros1-cKO mice.

Representative FACS plots of TdT-positive myeloid cells from the blood of transplanted tumor naïve mice 8 weeks after BM transplantation (A) were found similar to naïve mice expressing TdT in myeloid cells (B) n=4/group. (C-D) The percent of total circulating granulocytes and monocytes (pie chart) and the fraction of TdT-positive cells (bars) from blood of mice described in A and found similar to naïve mice expressing TdT in myeloid cells (D). (E) Detection of TdT and cytokine transcripts by RT-PCR in spleens of transplanted mice following BM transfer from TdT^{+} cKO into TdT-deficient *Pros1*$^{+/+}$ (cKO$^{TdT-/-}$ → *Pros1*$^{fl/fl}$; blue bars), or the reciprocal group (*Pros1*$^{+/+}$;TdT^{+} into cKO, green bars) in tumor-free mice. Mean ± SEM; n=4; t-test. P=0.01 for *TdT* and *Tnfα.* ns = non-significant; t-test. (F) Representative FACS plots of TdT^{+} myeloid population and the fraction of monocytes and granulocytes found in the blood of the indicated transplanted mice 3 weeks after LLC-luc inoculation (7 weeks after BM transplantation).



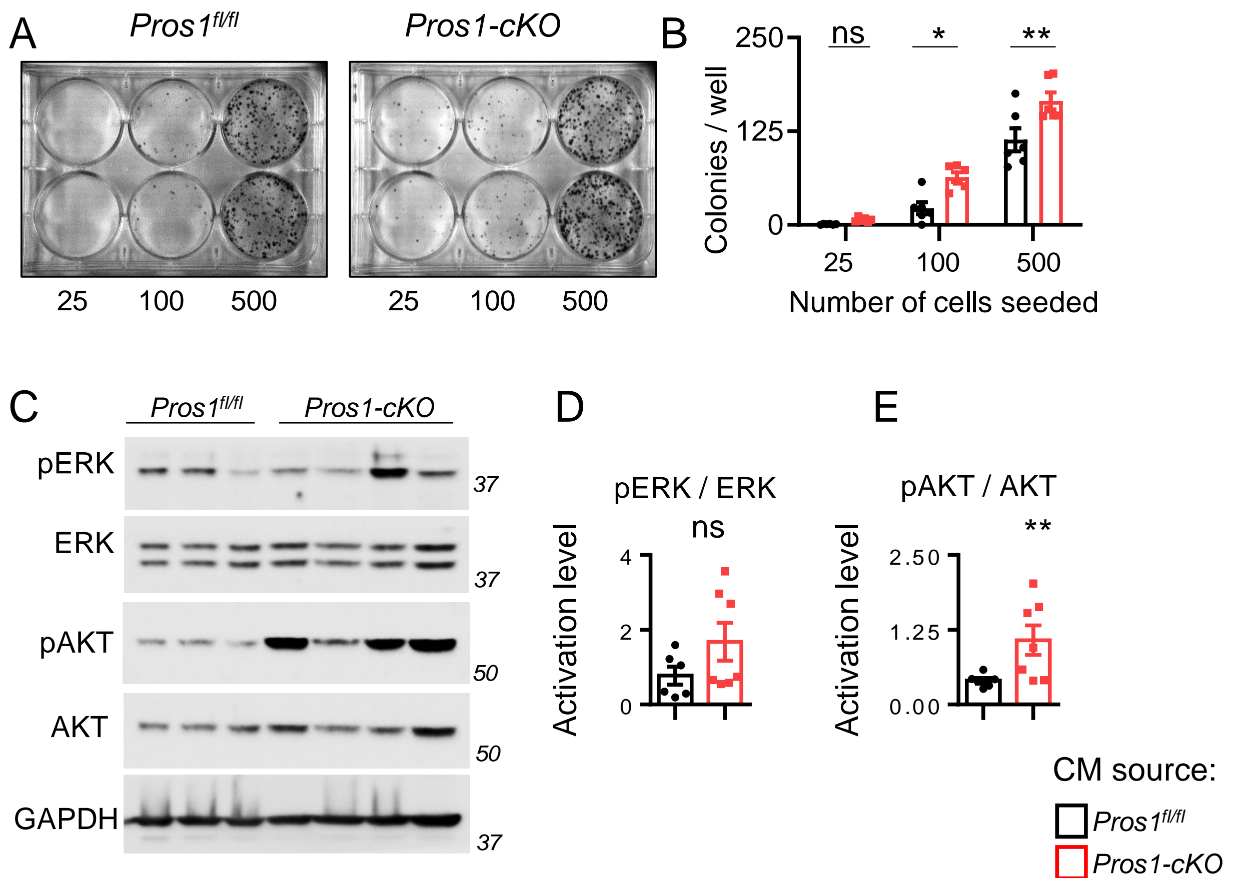
Supl. Figure 4: Effective LysM-Cre mediated excision of *Pros1* in BMDMs.

(A) Bone marrow cells were differentiated into BMDMs following stimulation with M-CSF for 7 days. Representative flow-cytometric analysis of $CD11b^+$ gated cells expressing F4/80 (macrophages) and Ly6C (monocytes), indicating a pure population of macrophages. (B) Average percent ± SEM; n=5 mice/group is shown for BMDMs and monocytes. ns=non significant, 2-way ANOVA. (C) Relative *Pros1* mRNA expression in BMDMs described in A. Data represent the mean relative values ± SEM; n=6-8 mice/group; P=0.0001; t-test. (D) A representative western blot indicating significantly reduced PROS1 protein levels in BMDMs generated from *Pros1*-cKO mice. Each lane represents BMDMs generated from an individual mouse. GAPDH was used as loading control.



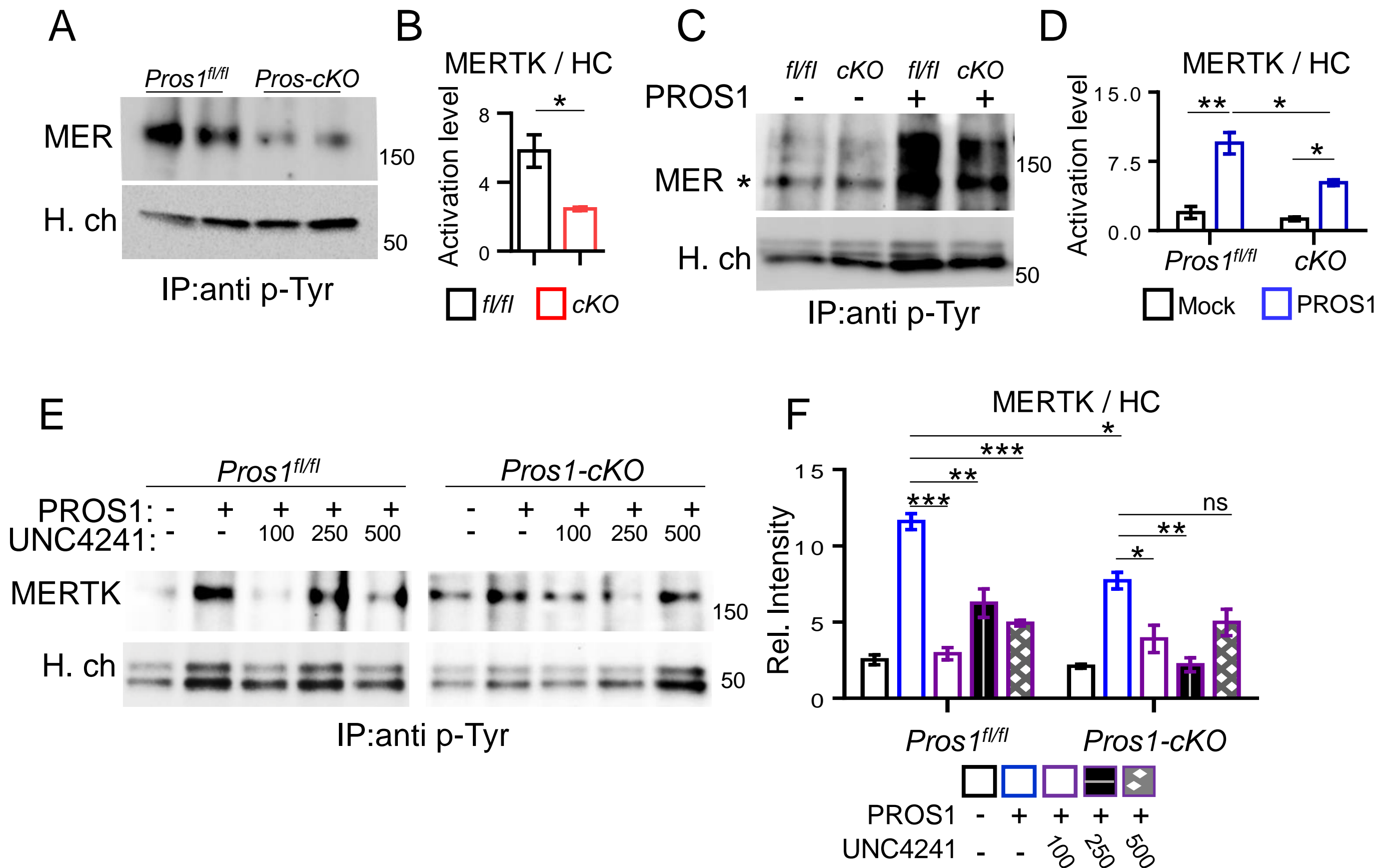
Supl. Figure 5: Education of tumor cells by BMDM-CM from cKO mice does not affect primary tumor growth.

(A-D) Growth parameters of LLC tumors generated by cells educated with BMDM-CM of cells derived from tumor-naïve (A, B) or tumor-bearing (C, D) control and *Pros1*-cKO mice. Average tumor volumes ± SEM at the indicated time points are presented (A, C) and the end-stage tumor weight was recorded (B, D), with the mean tumor weight represented by a horizontal line. (n=10 for A, B and n = 8 for C, D; ns = not significant, t-test). (E-F) Primary tumor growth (E) and end-point tumor weight of AT3-GFP tumors educated by CM from tumor-naïve *Pros1*-cKO and control BMDMs. N=8 mice/group, ns = non-significant, t-test.



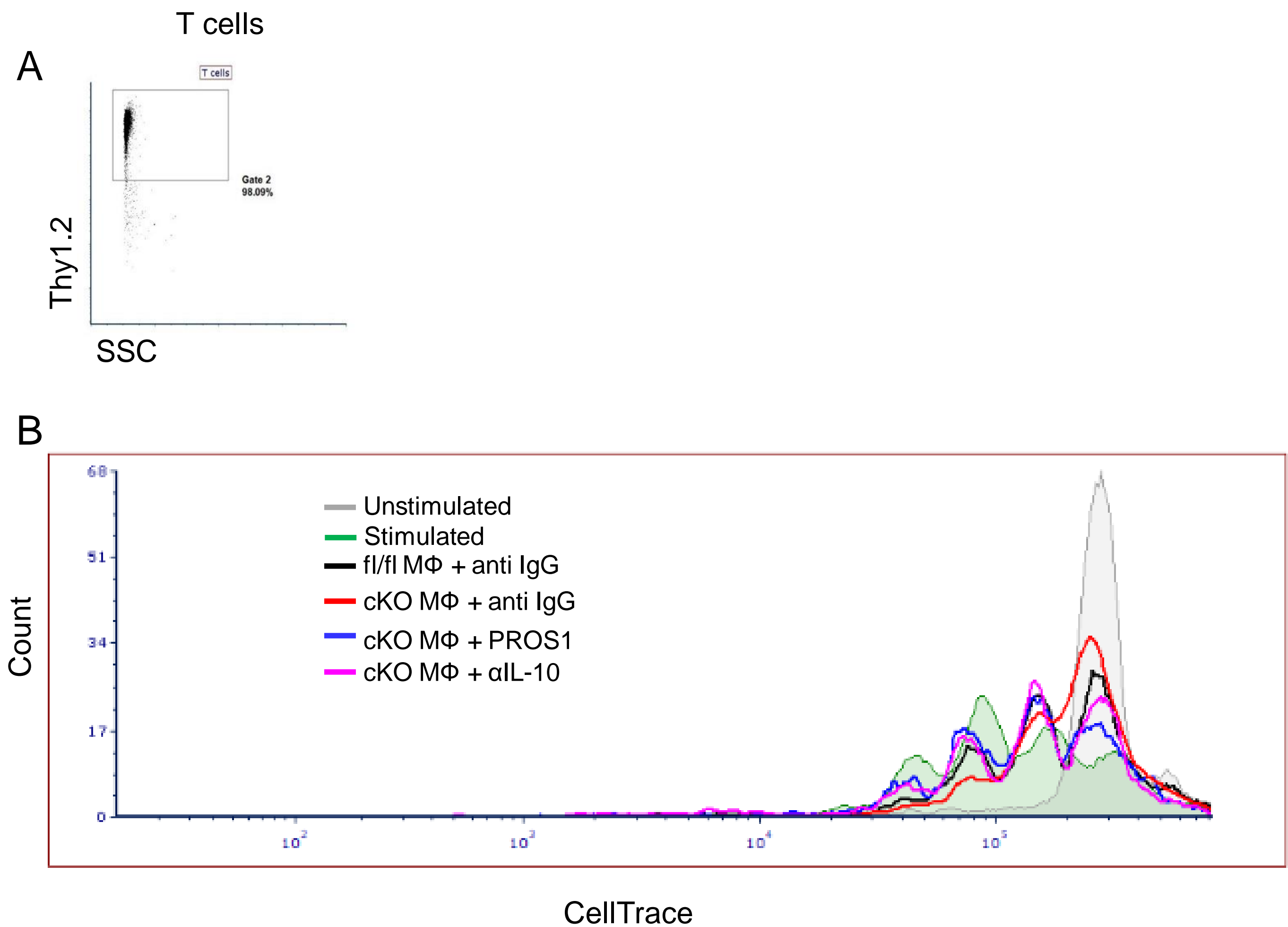
Supl. Figure. 6: Mouse mammary tumor cells acquire aggressiveness following education by Pros1-cKO BMDMs conditioned medium.

AT3 cells were incubated (96 hr) with medium conditioned by control (Pros1fl/fl) or Pros1-cKO BMDMs, and subject to a survival assay (A, B). Cells were plated at various dilutions, with the total cells/well indicated. Representative images (A) and quantification (B) of surviving colonies after 10 days. Mean ± SEM; n=6-7 mice/group; P=0.02, 0.003 for 100 and 500 cells/well respectively, ns= non-significant, by 2-way ANOVA. (C) A representative western blot and quantification (D, E) showing ERK and AKT phosphorylation in AT3 cells following education with BMDM-CM from control and Pros1-cKO mice. N=6-7 mice/group; 2 independent experiments. The average ratio ± SEM of pERK/ERK (P=0.053, ns=non-significant) and pAKT/AKT (P=0.01) is plotted; t-test.



Sup. Fig.7: Attenuated MERTK activation in *Pros1*-cKO BMDMs.

(A-B) Attenuated MERTK activation levels in *Pros1*-cKO BMDMs. (A) pMERTK levels in BMDMs grown at steady state (10% FBS, unstimulated) assayed by immune precipitation (IP) with pan-pTyrosine (p-Tyr) antibody, followed by immunoblot analysis with anti MERTK, and quantification (B; n=5-6 mice/group; P=0.03; t-test). The IgG heavy chain (H. ch) served as an internal control. (C, D) pMERTK in serum-starved BMDMs with (+) or without (-) PROS1 stimulation (25 nM). (D) Quantified band intensities of IPs shown in (C), n=4-6/group; **P=0.005, *P≤0.05; 2-way ANOVA. Relative band intensities ± SEM; 3 independent experiments. The IgG heavy chain (H. ch) served as an internal control (E-F) Pharmacological inhibition of TAM receptors. IP analysis of pMERTK in the presence of the pan-TAM inhibitor UNC4241 (100-500 nM) in serum starved BMDMs, stimulated with (+) or without (-) PROS1 (25 nM). IP:p-Tyr, WB: anti MERTK. Representative blots (E) and quantification of band intensities (F) of pMERTK and the heavy chain. Relative band intensities ± SEM; n=3 pooled samples/lane; 2 independent experiments; ***P≤ 0.0003, **P≤0.004, *P=0.02, and ns = non-significant 2-way ANOVA.



Supl. Figure. 8: PROS1 supports T cell proliferation.

(A) Purity of T cells isolated from the spleen, as determined by the Thy1.2 cell surface marker. (B) Representative histogram following 72 hrs of co-culture showing dilution of the intracellular marker CellTrace in unstimulated and stimulated T cells that were either left alone or incubated with the indicated macrophages (MΦ) and supplemented with control IgG (5μg/ml), αIL-10 (5μg/ml) or hPROS1 (50 nM).