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Supplementary Information for

Glioblastoma ablates pericytes anti-tumor immune function through aberrant upregulation of chaperone-mediated autophagy.

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This PDF file includes:

Supplementary materials and methods
Figures S1 to S7
SI References

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Supplementary materials and methods

qPCR: To assess levels of cytokine expression, the following primers (Sigma-Aldrich) were used: mouse *Lamp2a* 5′-AGGTGCTTTCTGTGTCTAGAGCGT-3′ and 5′-AGAATAAGTACTCCTCCCAGAGCTGC-3′, mouse *Tgf*-β 5′-CTCCCGTGGCTTCTAGTGC-3′ and 5′-GCCTTAGTTTGGACAGGATCTG-3′; mouse *Il10*, 5′-GCTCTTACTGACTGGCATGAG-3′ and 5′-CGCAGCTCTAGGAGCATGTG-3′; mouse *Tnfa* 5′-CCCTCACACTCAGATCATCTTCT-3′ and 5′-GCTACGACGTGGGCTACAG-3′; Mouse (5'-AAGGACTCCTATGTGGGTGACGA-3'; 5'-ATCTTCTCCATGTCGTCCCAGTTG-3') and human (5'-GTCTGCCTTGGTAGTGGATAATG-3'; 5'-TCGAGGACGCCCTATCATGG-3') β-Actin expression were measured as reference housekeeping controls.

Plasmids transfections and reporter assays: The firefly luciferase reporter plasmid under the control of a murine *Lamp2* proximal promoter element (1) was transfected in PC using lipofectamine 2000 (Thermofisher) following the manufacturer's recommendations. A Renilla luciferase expression vector regulated by the pRL-TK promoter (ratio 20:1) was used as an internal control. PC were conditioned by GB or treated with H_2O_2 (Sigma Aldrich) for 24 h after transfection. Cells were lysed and assayed for luciferase activity using the Promega Dual-Luciferase Reporter Assay System.

To measure CMA using the photoactivable CMA reporter, PC were transfected with vectors expressing KFERQ-PA-mCherry-1 (2). Twenty-four hours post-transfection, cells were exposed to a 405 nm light immediately following co-cultured with GB cells to photoactivate PA-mCherry. PC were collected at different times after activation, fixed and changes in the numbers of lysosomes highlighted by the reporter were analyzed by fluorescence microscopy. Acquisition of images was performed with an Axiovert 2000 fluorescence microscope with apotome and quantification of images was done using ImageJ software (National Institutes of Health). Activation of CMA was measured as increase in the number of fluorescent puncta per cell.

Lentiviral infection of pericytes: Knock down of *Lamp2a* was achieved as described previously (1) by transducing PC with lentivirus expressing hairpin versions of one of three sequences targeting exon 8a of the *Lamp2* gene: 5′-GACTGCAGTGCAGATGAAG-3′, 5′-CTGCAATCTGATTGATTA-3′ or 5′-TAAACACTGCTTGACCACC-3′).

Microvesicles preparation, quantification and function: Following the protocol described by Gaceb et al. (3), vesicles were isolated from the cell supernatant of WT PC and KO PC with or without GB, from supernatant of GB control and from cell culture media after 72 hours of culture. Supernatants free from debris after centrifugation at 1500g for 5 minutes, were centrifuged at 16,000 g for 45 minutes. Pellets were washed three times in 1 ml of 0.9% NaCl at 16,000 g for 45 minutes. Then the pellet was resuspended in 100 μl of 0.9% NaCl and stored at 4ºC until quantification by flow cytometry using flow count beads (Biocytex/Stago). Vesicles size was determined according to the diameter of Megamix-Plus fluorescent beads (Biocytex/Stago), analyzing 20,000 events and identifying pericyte-derived vesicles from the total using an antibody against mouse CD63. Unspecific fluorescence was evaluated using a control isotype antibody. 10 μl of isolated vesicles or controls, were added to 1 ml of GB cultures in 24 well plate to measure GB proliferation.

Immunofluorescence, immunohistochemistry and microscopy: PC were fixed with 4% paraformaldehyde in PBS, permeabilized, blocked with BSA and incubated with antibodies against mouse LAMP-2A (Invitrogen), human LAMP2 (Abcam), alpha-Smooth Muscle Actin (α-SMA; Abcam), mouse monoclonal antibody specific for human cytoplasmic marker (STEM121; Cellartis), mouse occludin, GBA (Thermofisher). AlexaFluor 595 Phalloidin (Invitrogen) was used to detect F-actin.

GB samples from patients included in this study were provided by the BioBank "Biobanco en Red de la Región de Murcia" (PT17/0015/0038), integrated in the Spanish National Biobank Network (B.000859). GB samples from patients and some xenografts selected for immunohistochemistry, were fixed in 4% buffered formalin (Panreac Quimica) and paraffin embedded. Three-micrometers thick serial sections were obtained from paraffin embedded samples using an automatic rotary microtome (Thermo Scientific). For colorimetric single immunolabelling, sections were incubated overnight (4ºC) with the primary antibody (rabbit anti-CD3 (Dako-Agilent), rabbit anti-CD4 (Abcam), rabbit anti-CD8 (Abcam), rat anti-FOXP3 (EBioscience), rabbit anti-mouse CD44 (Abcam), rabbit anti-mouse CD62L (Abcam), rabbit anti-LAMP2A (Abcam), mouse anti-NG2 (Merck Millipore) or mouse anti-human STEM121 (Cellartis)). Sections were finally incubated with 3-3´Diaminobencidine (DAB) (Vector Labs) and hematoxylin counterstained. Positive immunoreaction was identified as a dark-brown precipitated.

For cryostat sections, we used the following antibodies: rabbit anti-RFP (MBL), rat anti-RFP (Chromoteck), chicken anti-GFP (AVES), rabbit anti-Ki67 (Thermo Scientific), rabbit anti-mouse PD-1 (Novus), anti-mouse CD3 (Ebioscence), rabbit anti-LAMP-2A (Invitrogen), STEM121

(Cellartis). Single and double labelling were visualized by fluorescence microscopy using the corresponding secondary antibodies conjugated to AlexaFluor488, AlexaFluor594 or Cy5 (Invitrogen/Molecular Probes, respectively), or biotinylated secondary antibodies (VectorLabs), followed by incubation with Cy3-streptavidin (Amershan-GEHealthcare). All fluorescence samples were counterstained with DAPI (Invitrogen/Molecular Probes) prior to mounting with Mowiol.
A TCS-SP2-AOBS laser scanning spectral inverted Confocal Microscope (Leica Microsystems) was used for analysis of histological sections and live PC/GB cultures. A fluorescence automated DM6000B microscope and a MZ16FA Fluorescence Stereomicroscope (for wide-field microscopy), running Leica Application Suite (LAS) AF6000 Software (version 2.0.2), equipped with a DFC350-FX (monochrome) or DC500 (color) digital cameras (all purchased from Leica Microsystems) were used to analyze fixed cells and histological sections from xenografts and GB human biopsies.

For scanning electron microscopy images, PC and GB were growth on coverslides Thermanox D13MM (Thermo Scientific, Nunc). Samples were fixed overnight by immersion fixation in 2.5% glutaraldehyde (at 4ºC). Afterwards, cells were washed three times in 0.1M cacodilate buffer pH 7.2, dehydrated through a graded ethanol series to 100% ethanol and then critically point dried. Samples were processed by the Microscopy Core facility of University of Murcia and analyzed by a scanning electron microscope JEOL-6100 with Microanalysis system by X rays INCA and with detection and captation systems of digital images (Oxford Instruments).
Morphometric measurements and quantification of cells were performed using ImageJ (NIH, USA) software and Adobe Photoshop. Pictures for illustrations and quantitative analysis were uploaded from direct microscopic images and were not manipulated in subsequent steps of figures preparation, except for framing and scaling.

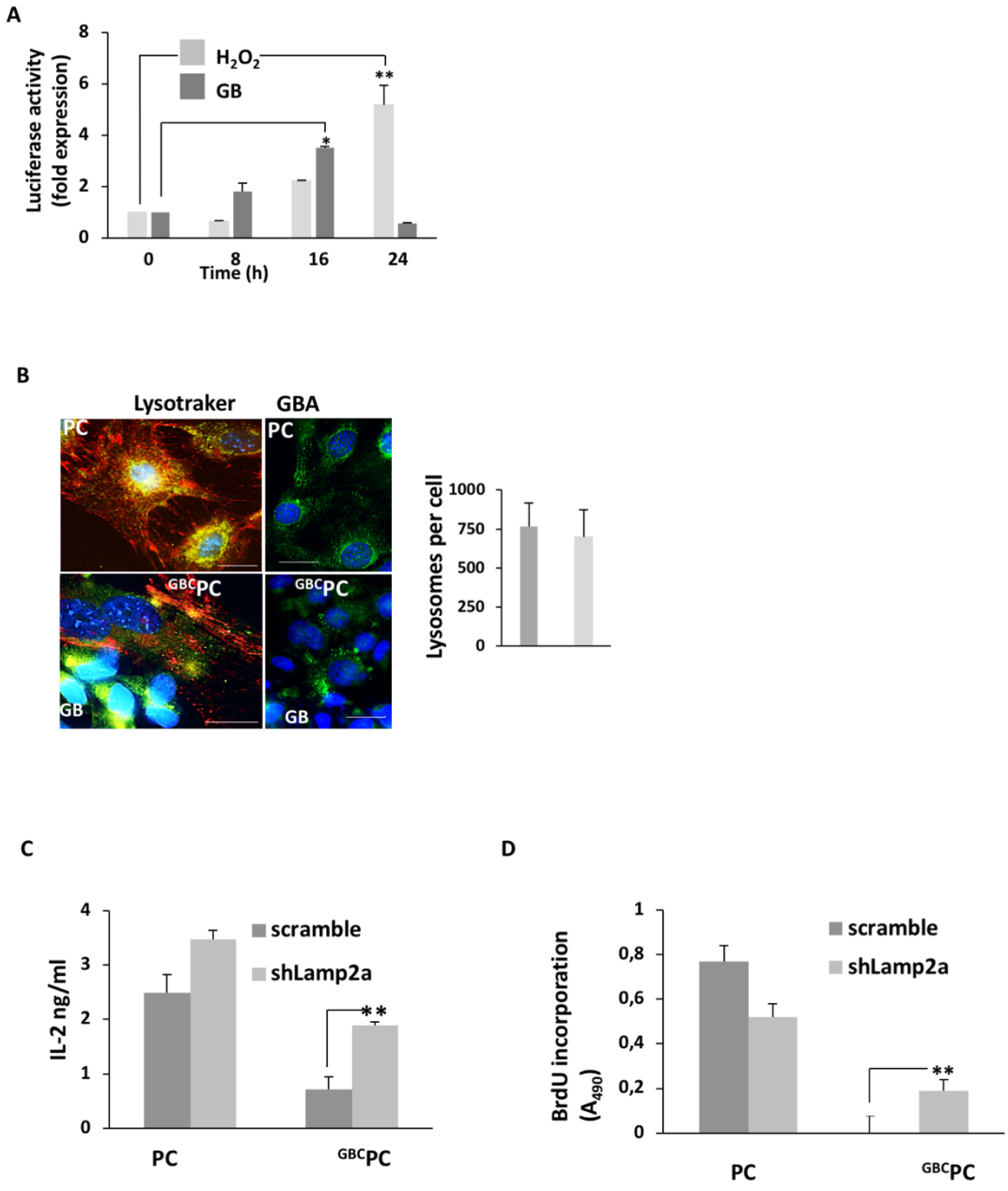


Fig. S1. (**A**) Relative luciferase activity (to basal levels in PC) in PC transiently transfected with a vector containing a luciferase reporter under the control of the *Lamp2* proximal promoter and co-cultured with GB or treated with 250 μM H_2O_2 for 8, 16 or 24 h. Results are mean+S.D from 3 different experiments. (**B**) Immunofluorescence of the lysosomal compartment (green) with lysotraker (left panel) and GBA (right panel) in PC and ^{GBC}PC. α-SMA (in red) was used to identify PC (Scale bars: 50 μm). Quantification of numbers of lysosomes per cell from those images (right). **(C, D)** IL-2 production and T cell proliferation was measured by ELISA and BrdU incorporation

respectively, in naïve $CD4^+$ T cells activated for 72 hours with OVA323-339 peptide presented by PC conditioned or not by GB (^{GBC}PC). PC had been previously transduced with lentivirus expressing a shRNA specific for *Lamp2a* to suppress its expression (shLamp2a), or with lentivirus expressing a scramble non-targeting shRNA. Values are normalized to basal levels of IL-2 in resting T cells. Results are mean + S.D from five different experiments, **$p<0.01$.

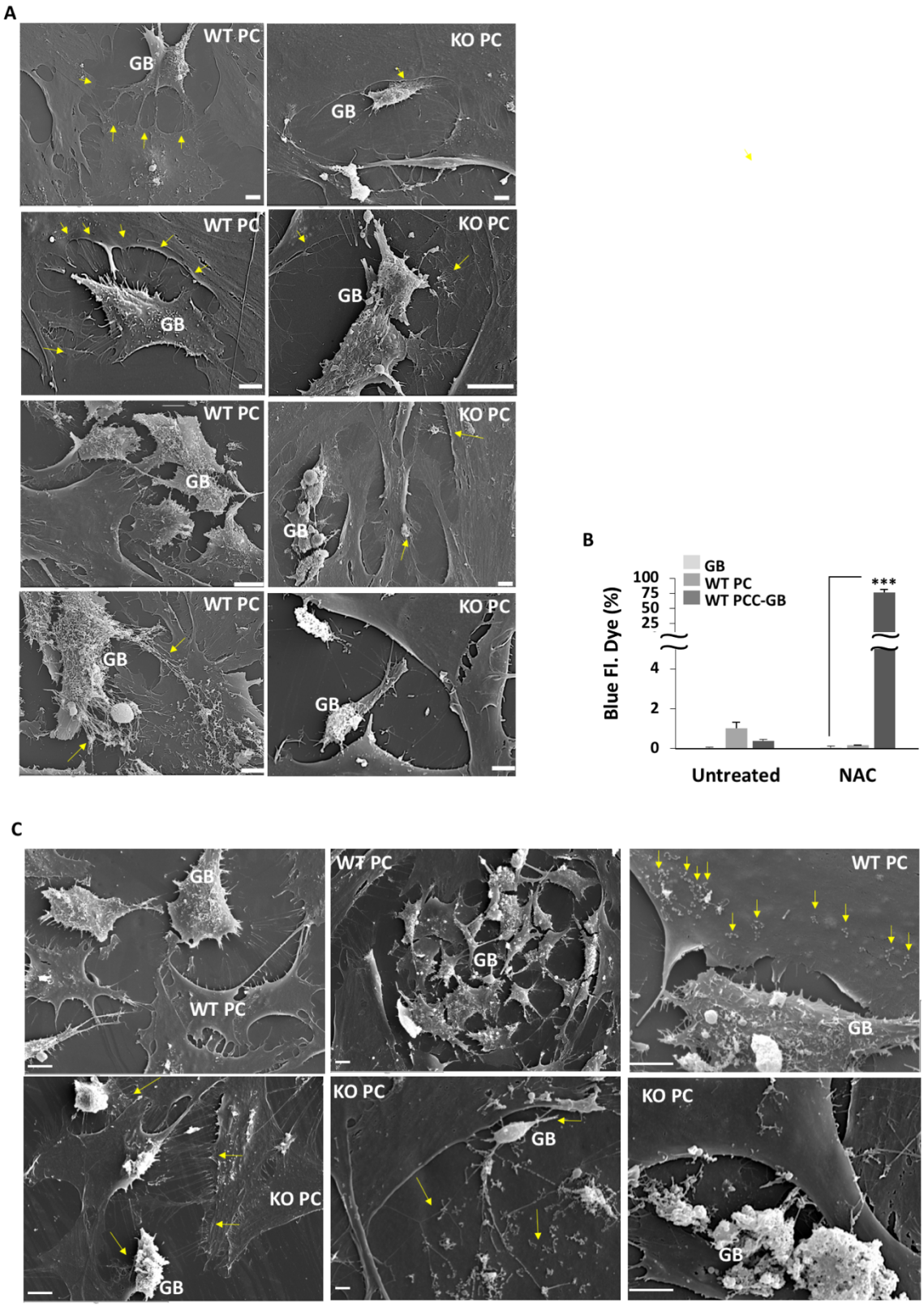

A
WT PC
KO PC
GB
B
GB
WT PC
WT PCC-GB
Blue Fl. Dye (%)
100
75
50
25
4
2
0
***
Untreated
NAC
C
WT PC
KO PC
GB

Fig. S2. **(A)** Images of GB and PC cells after 72 hours of co-culture. Images were obtained by scanning electron microscopy and are representative of at least three independent experiments. The four upper panels show PC-GB interactions through organized nanotubes formation in WT PC and unorganized or absent nanotubes in KO PC (arrows); The four lower panels show proliferation and propagation of microtubes (arrows) in GB coculture with WT PC; and defective proliferation microtube propagation and GB cell death (arrows) in the presence of KO PC. Scales bars: 25 μm. (**B**) Detection of cell death by blue-fluorescent reactive dye in GB after 72 hours co-culture with WT PC pretreated or not with N-Acetyl-cysteyne (NAc). Graph shows percentage of cell death of GB cells mean+S.D from 3 different experiments, ***$p<0.005$ using U373 and U87 GB lines independently. Gating strategy shown in Fig. 1E was used to separate populations of PC and GB cells in all flow cytometry analyses. (**C**) Images of GB and PC cells after 72 hours of co-culture obtained by scanning electron microscopy. Upper panels show nanotube in WT PC interacting with GB, GB proliferation and detailed WT PC and GB interaction. Arrows indicate vesicles in the WT PC. Arrows in lower panels show unorganized nanotubes in KO PC interactions, GB cell death, vesicles secreted in the intercellular space and absence of intracellular vesicles in KO PC. Scale bar: 10 μm.

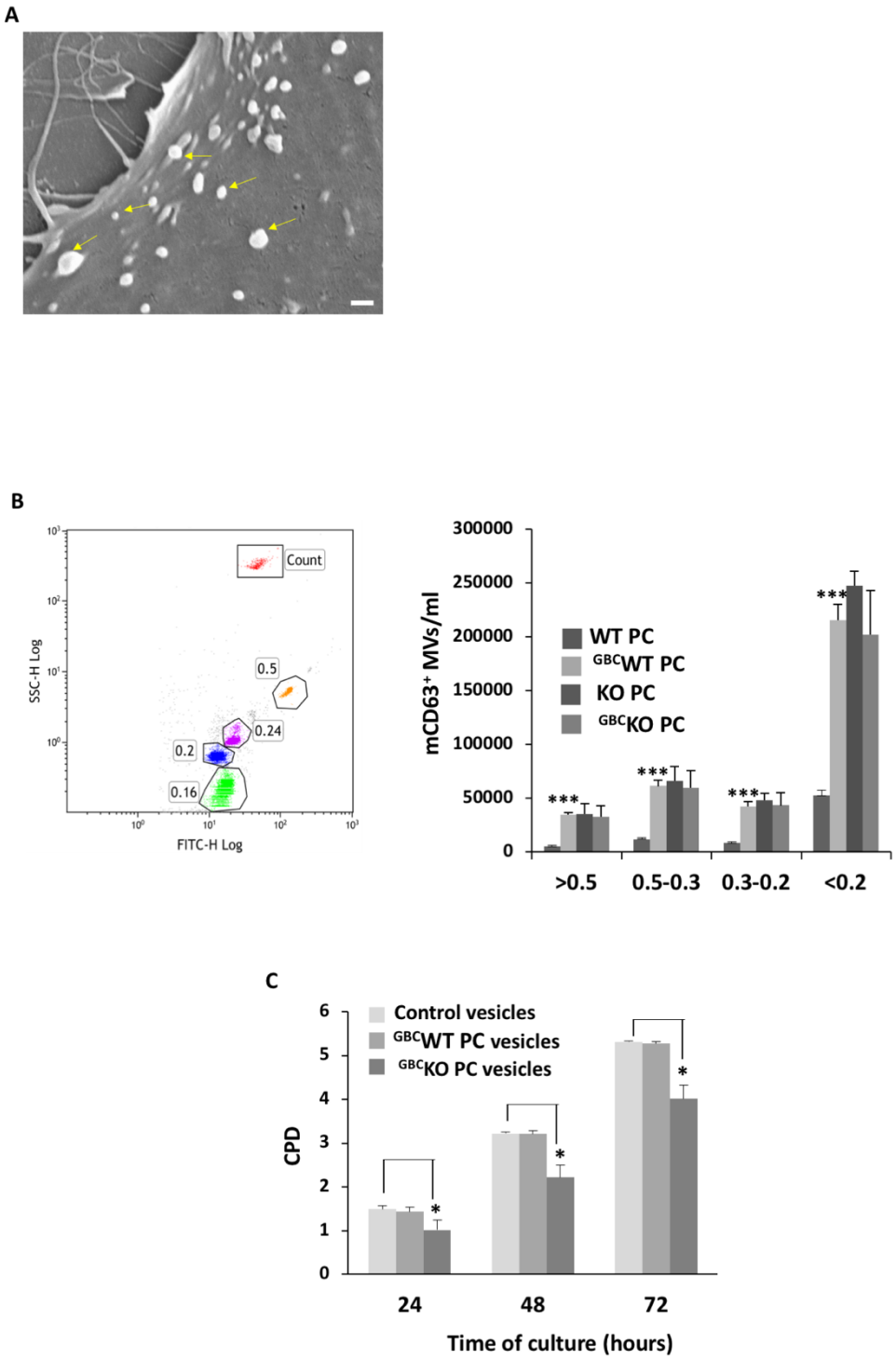


Fig. S3. (A) Representative scanning microscopy image of PC with secreted vesicles of different sizes (arrows). Scale bar: 1 μm. **(B)** Flow cytometry analysis of the number and size of mouse

$CD63^+$ vesicles in the collected media from WT PC and KO PC and compared to the conditioned by GB (^{GBC}WT PC;^{GBC}KO PC) respectively, after 72 hours of coculture, ***p<0.001. Dot plot shows the different sizes of the fluorescence counting beads from less than 0.2 μm (<0.2) up to more than 0.5 μm (>0.5). **(C)** Proliferation of GB cells (measured as cumulative population doubling: CPD)s cultured in the presence of 10 μl of isolated vesicles isolated from media of ^{GBC}WT PC and ^{GBC}KO PC. Vesicles from cultured of WT PC, KO PC, GB cells or culture media, and the vesicles vehicle (sodium chloride), were used as GB proliferation controls, *p<0.05; **p<0.01; ***p<0.001.

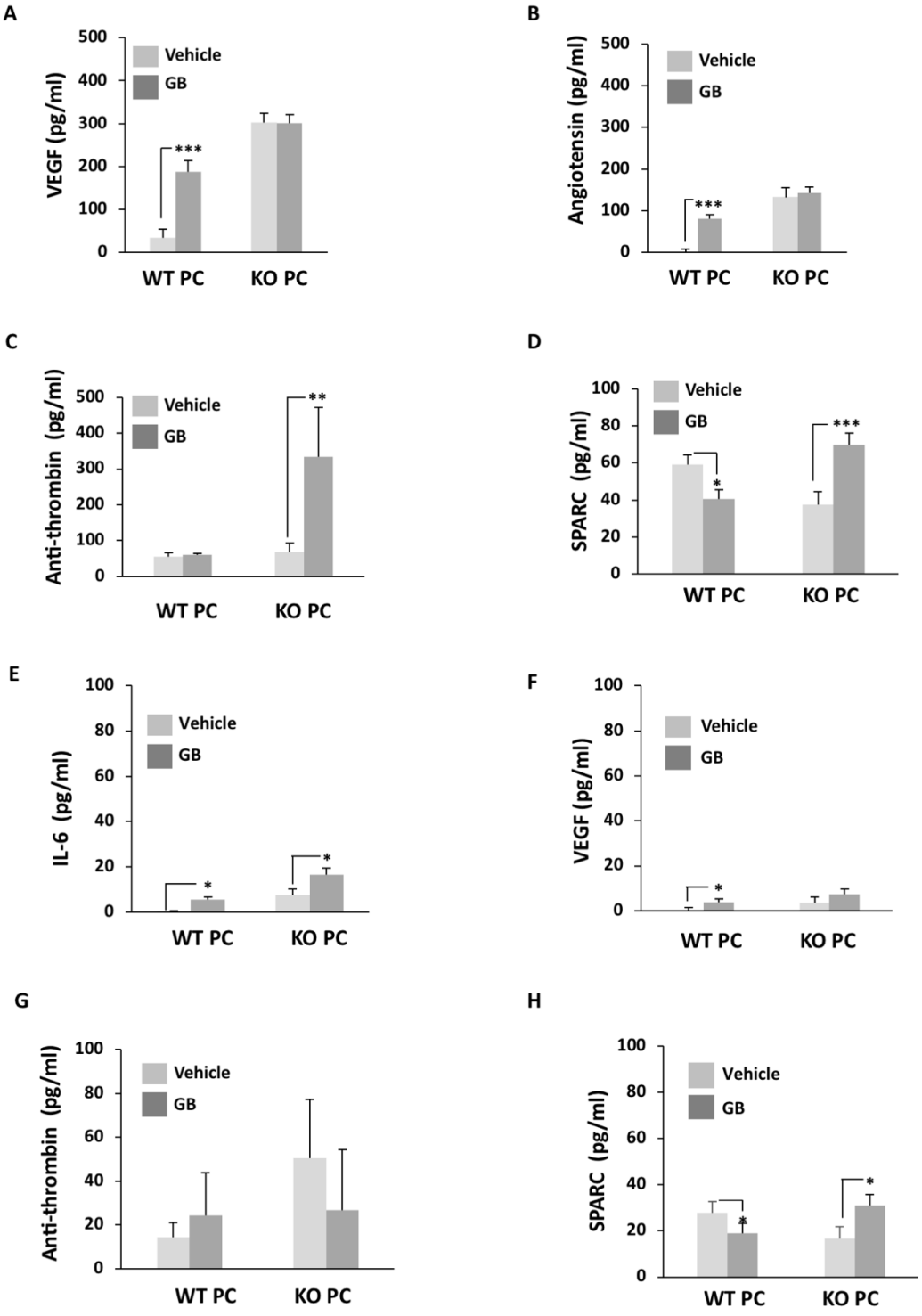
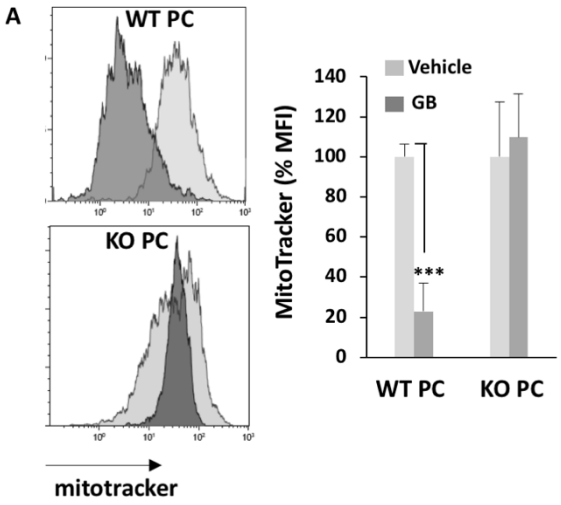


Fig. S4. (A-D) ELISA measuring mouse VEGF, Angiotensin, Anti-thrombin and SPARC levels produced by WT PC or KO PC cultured in presence or absence (vehicle) of GB for 72 hours. **(E-G)** ELISA measuring mouse IL-6, VEGF, Angiotensin and SPARC levels detected in the vesicles

collected from media of WT PC or KO PC cultured for 72 h in presence or absence(vehicle) of GB . *$p<0.05$.


A
WT PC
KO PC
mitotracker
Vehicle
GB
MitoTracker (% MFI)
140
120
100
80
60
40
20
0
***
WT PC
KO PC

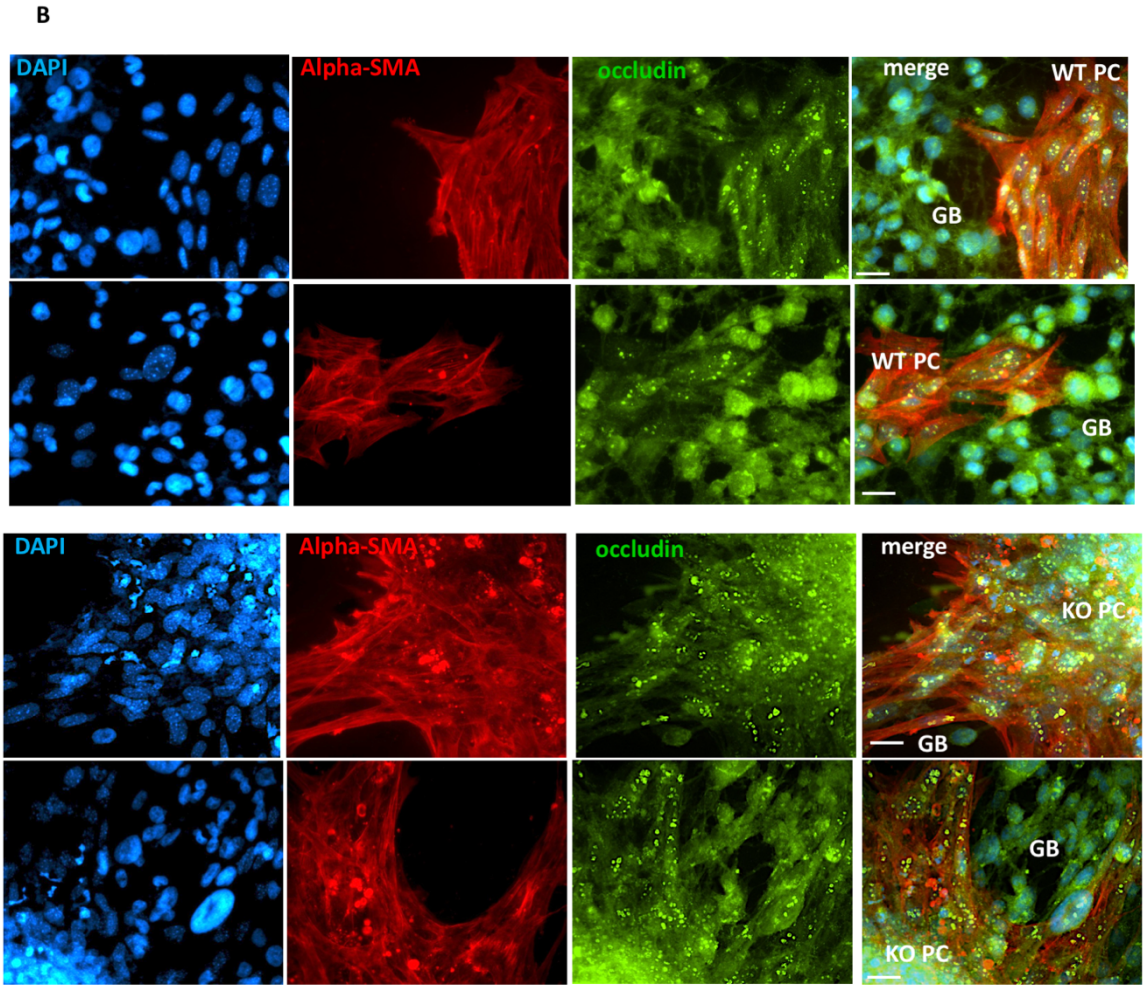

B
DAPI
Alpha-SMA
occludin
merge
WT PC
GB
WT PC
GB
DAPI
Alpha-SMA
occludin
merge
KO PC
GB
GB
KO PC

Fig. S5. **(A)** Flow cytometry analysis of mitochondria content using mitotracker staining in WT PC and KO PC after 12 hours of co-culture with GB cells. Representative histograms and quantification of mean fluorescence intensity (normalized to values obtained with PC cultured alone) are shown. **(B)** Occludin expression (in green) in WT PC and KO PC cultured in presence of GB cells for 48 hours. Alpha-smooth muscle actin (SMA) was used as PC marker (in red). Nuclei of GB and PC were stained with DAPI (in blue). Scale bar: 50 μm.

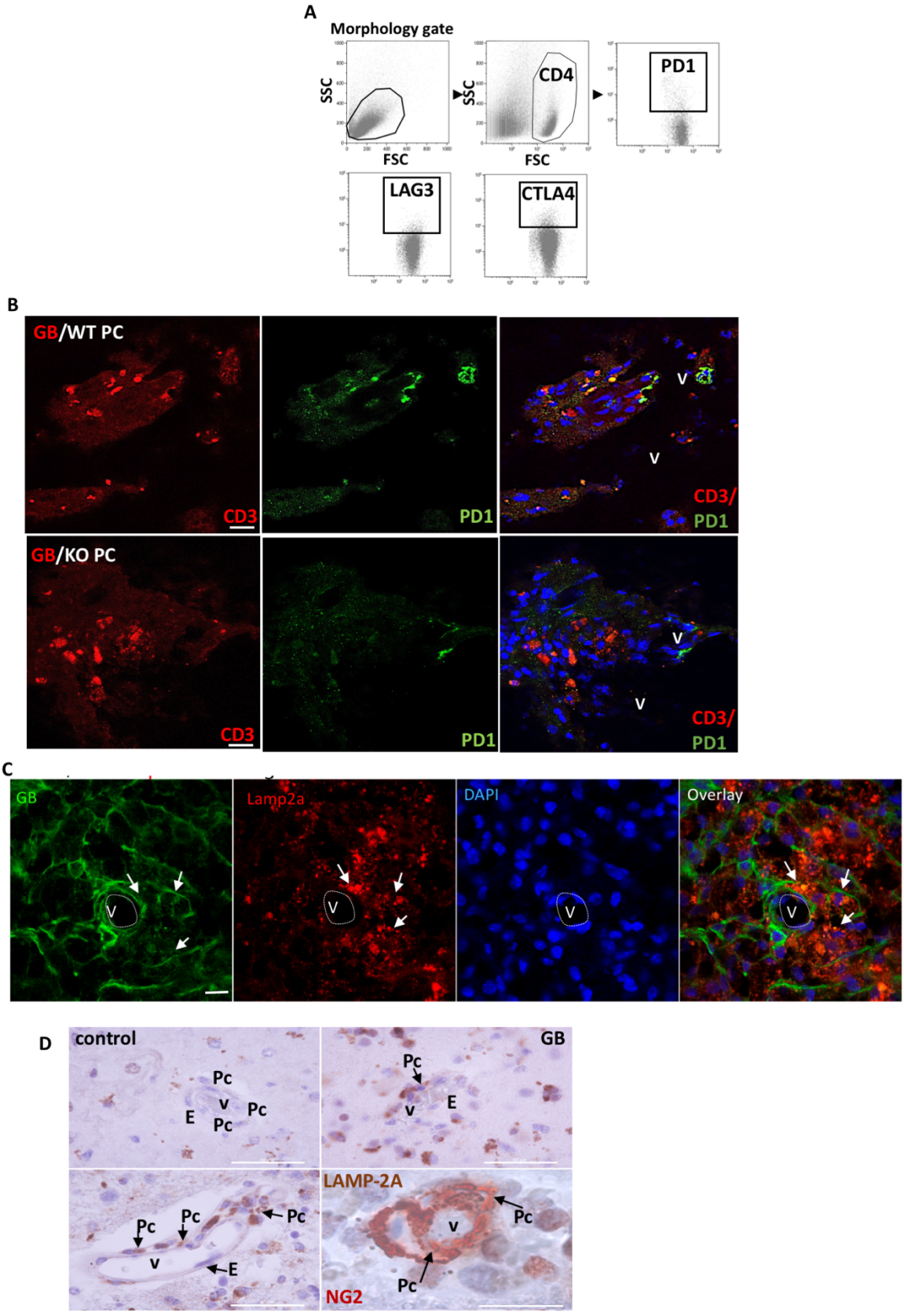

A
Morphology gate
SSC
FSC
SSC
FSC
CD4
PD1
LAG3
CTLA4
B
GB/WT PC
CD3
PD1
CD3/
PD1
V
V
GB/KO PC
CD3
PD1
CD3/
PD1
V
V
C
GB
Lamp2a
DAPI
Overlay
V
V
V
V
D
control
GB
Pc
v
Pc
E
Pc
Pc
v
E
Pc
Pc
Pc
v
E
LAMP-2A
Pc
v
Pc
NG2

Fig. S6. (A) Gating strategy of $CD4^+$ T cells and flow cytometry analysis of the expression of PD-1, LAG-3 and CTLA-4. **(B)** T cell infiltration ($CD3^+$) and PD-1 expression around blood vessels (V) of areas of GB infiltration in mice brains grafted with WT PC + GB cells (GB/WT PC) compared to mice grafted with KO PC+GB cells (GB/KO PC). Scale bar: 20 μm. **(C)** Expression of LAMP-2A in GB cells and perivascular cells in the tumor. Xenografts of co-cultured WT PC and GB cells (GB+PC). Confocal microphotographs (1-μm thick section) of the tumor detected by anti-STEM121, that stains human cytosolic regions (GB in green). Scale bar: 45 μm. LAMP-2A immunostaining inside the tumor was heterogeneous and stronger in tumor cells close to the blood vessels where PC are located (arrows). Overlay, showing the co-localization of LAMP-2A expression in GB cells. **(D)** Perivascular expression of LAMP2-A (brown) in GB infiltration areas of patient samples (GB) versus not infiltrated GB cortex (control), stained with cresyl violet. Arrows in blood vessels (v) show LAMP-2A expression in PC and endothelial cells (E). PC are identified by NG2 expression, a pericyte marker (red), and show LAMP-2A expression in their cytoplasm (brown dots labeled by arrows). Scale bars are 50 μm in all images except for the right, lower image, where the scale bar is 20 μm.

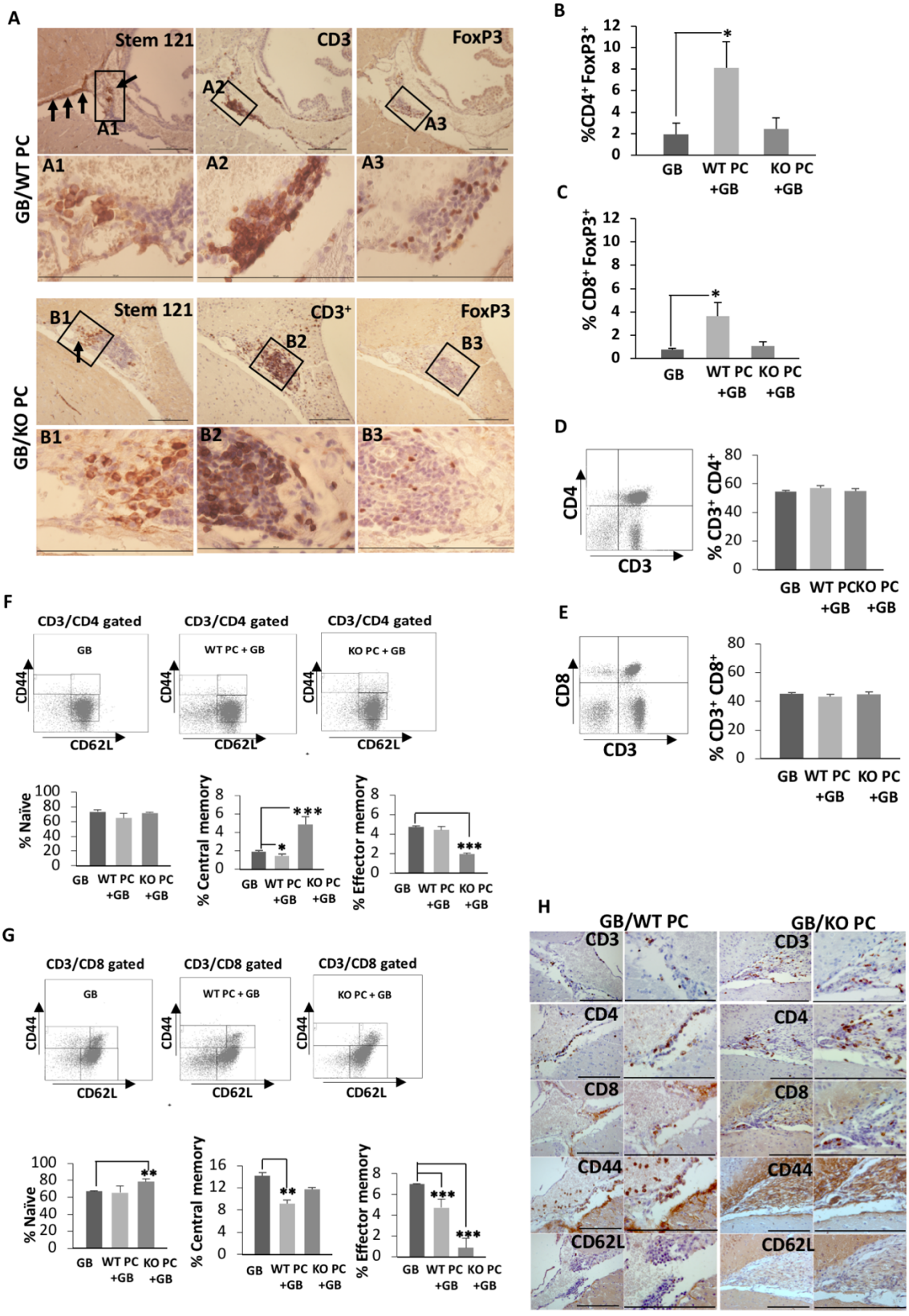

A
Stem 121
CD3
FoxP3
GB/WT PC
A1
A2
A3
GB/KO PC
B1
B2
B3
CD3+
B
%CD4+ FoxP3+
GB
WT PC +GB
KO PC +GB
C
% CD8+ FoxP3+
D
CD4
CD3
% CD3+ CD4+
E
CD8
% CD3+ CD8+
F
CD3/CD4 gated
GB
WT PC + GB
KO PC + GB
CD44
CD62L
% Naïve
% Central memory
% Effector memory
G
CD3/CD8 gated
H
GB/WT PC
GB/KO PC
CD3
CD4
CD8
CD44
CD62L

Fig. S7. (A) Expression of FoxP3 in T cells in xenografts. Upper panel shows meningeal and perivascular tumor cell infiltration, with perivascular infiltration expressing high FoxP3 in GB/WT PC xenografts. GB cells were stained with Stem 121. Three arrows indicate meningeal tumor and the box shows a perivascular tumor, which is shown in A1 in detail. High perivascular lymphocytic infiltration (CD3) is shown in detail in A2. T cells with high expression of FoxP3+ cells are shown in detail in A3. Lower panels show remaining perivascular tumor cells (arrow, B1) close to high lymphocytic infiltration (B2) and with low levels of $FoxP3^+$ T cells (B3) in GB/KO PC xenografts. (Scale bars: 100 μm). **(B, C)** FACS analysis of the percentage of $CD4^+$ **(B)** and CD8+ **(C)** T cells from cervical and maxillary lymph nodes of xenografted mice expressing FoxP3. *p<0.05. **(D)** FACS analysis of percentage of $CD4^+$ T cells and **(E)** and $CD8^+$ T cells from the total $CD3^+$ T cells of lymph nodes from xenografted mice**. (F)** FACS analysis of the expression of CD44 and CD62L in $CD4^+$ **(G)** and $CD8^+$T cells in lymph nodes of xenografted mice, *p<0.05; **p<0.01; ***p<0.001. **(H)** Characterization of infiltrating T cells in GB xenografts. Left panel shows representative immunohistochemistry images in different magnifications of the peritumoral area, in the meningeal space of GB+WT PC xenografts. Images show high perivascular infiltration of $CD4^+$ T cells and very few $CD8^+$ cells, all positive for CD44 and negative for CD62L. Right panel shows representative images in different magnification of areas where the few remaining tumor cells where found in GB+KO PC xenografts, with accumulation of $CD4^+$ and $CD8^+$ T cells. High expression of CD44 in all the area co-localizes with both types of T cells that are negative for CD62L (Scale bars: 100 μm).

References

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