Extracellular vesicles from triple negative breast cancer promote pro-inflammatory macrophages associated with better clinical outcome

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Supplementary Information

Supplementary Materials and Methods

Cell culture and transfections

MDA-MB-231 stably expressing CD9-tdTomato (Supplementary Figure 1) were generated by transfection with the Amaxa kit V (Lonza), following manufacturer's instructions. tdTomato-CD9-10 was a gift from Michael Davidson (Addgene plasmid # 58076; http://n2t.net/addgene:58076; RRID: Addgene_58076). The antibiotic for selection (Geneticin, 2 mg/mL, Gibco) was added the day after transfection. Cells expressing the fluorescent molecules were sorted on a BD FACSARIA III (BD Biosciences) instrument and kept in culture under antibiotic selection.

MCF-7 cells overexpressing CSF-1 were generated by transduction with recombinant retroviruses. Briefly, the retroviral vector MSCV-(pbabe puro mcs)-human CSF-1 full length (CSF4)-IRES-GFP (a gift from Martine Roussel & Charles Sherr (Roussel and Sherr 1989); Addgene plasmid # 86796 ; http://n2t.net/addgene:86796 ; RRID:Addgene_86796), the packaging construct pCL-10A1 (Novus Biologicals) and the envelope pCMV-VSV-G were co-transfected into HEK 293 LTV cells using the TransIT®-293 Transfection Reagent (Mirus Bio) according to the manufacturer's instructions, and culture fluid was harvested 48-72 h post-transfection and concentrated by ultracentrifugation. Resuspended retroviruses were used to transduce MCF-7 cells. Transduced cells were selected with 2 µg/ml puromycin

(ThermoFischer Scientific) that was added the day after transduction. Cells expressing GFP were sorted on a BD FACSARIA III (BD Biosciences) instrument and kept in culture under antibiotic selection.

CRISPR/Cas9 edited cells

STING KO THP1 cells were established by Cas9/gRNA RNP electroporation. Predesigned Alt-R CRISPR-Cas9 crRNAs, Hs. Cas9.TMEM173.1. AA, was ordered from IDT technologies. crRNA (100uM) was mixed with tracrRNA (100uM) and annealed 5 min at 95°C. To form the RNP complex, the tracrRNA-crRNA duplex was incubated with 10ug of Cas9 Nuclease V3 protein (IDT technologies). After 10 min at room temperature, the RNP complex was electroporated in $2x10^6$ THP-1 cells using Lonza Electroporation Kit (SG Cell Line 4D-NucleofectorTM X Kit S, V4XC-3032) following manufacturer's instructions. After 24h at 37°C cells were expanded. The knockout efficiency was evaluated by western blotting.

cGAS KO, CSF-1 KO or Rab11 KO MDA-MB-231 cells were established by lentiviral-mediated CRISPR/Cas9 method. Briefly, lentiviruses were produced with the co-transfection in HEK 293-LTV cells using the TransIT® 293 Transfection Reagent (Mirus Bio) of the envelope pCMV VSV G, the packaging psPAX2 with plasmids pLentiCRISPRv2 for CSF-1KO (guide RNA sequences 1 and 2 respectively CACCGGCGAGCAGGAGTATCACCG and CAC-CGGTGTCCACTCCCAATCATG) and Rab11KO (guide RNA sequence CATATGAAAATGTAGAGCGA), or pLCRISPR-CMV for cGAS (pLCRISPR-CMV-cGAS3 described in [1], guide RNA sequence GGGGATCCCGACTTCCTGGC). Supernatants were recovered after 48h, concentrated by ultracentrifugation at 120,000xg during 1h 30 min in a SW32 Ti rotor. Resuspended pellets containing the lentiviruses were added to MDA-MB-231 cells. Following transduction, cells were selected with puromycin (2 µg/ml). The knockout efficiency was evaluated by RT-qPCR, western blotting and/or LegendPLEX/ELISA.

Transfer of CD9-tdTomato EVs to PBMCs

EVs from CD9-tdTomato-MDA-MB-231 cells (Supplementary Figure 1) were isolated by differential ultracentrifugation. Briefly, cells were incubated for 24 hours in DMEM-Glutamax (Gibco) without serum before collection of CM for EV isolation. CM was centrifuged 350xg for 10 min, at 4°C to pellet cells. Supernatant was centrifuged at 2,000xg for 20 min at 4°C, transferred to new tubes and centrifuged directly for 120 min at 200,000xg in a 45Ti rotor (Beckman). The pellet was washed in 40-50 ml of PBS and re-centrifuged at the same speed before being re-suspended in sterile PBS (1 µl per million of secreting MDA-MB-231 cells). For the transfer assay, EVs from 10 million secreting cells were incubated for 18 h with fresh PBMCs (5 million cells) in a round bottom 96 well plate. Cells were incubated with human Fc block reagent (Miltenyi) and LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (ThermoFischer) before the staining with the following anti-human antibodies: CD3 FITC (Clone UCHT1, BD), CD4 BV785 (clone OKT4, Biolegend), CD8 BUV395 (Clone RPA-T8, BD), CD14 BV605 (Clone M5E2, Biolegend), CD56 BV711 (Clone NCAM16.2, BD), CD11c PE-Cy7 (Clone Bu15, Biolegend), HLA-DR PE-Cy5 (Clone G46-6, BD), CD19 Alexa Fluor 700 (Clone HIB19, BD) and BDCA-2 BV421 (Clone 201A, Biolegend). Cell populations within PBMCs were identified as follows: Monocytes ($CD14^+$ $CD19^-$ $CD3^-$), CD4 T cells ($CD3^+$ $CD4^+$), CD8 T cells ($CD3^+$ $CD8^+$), NK cells ($CD56^+$ $CD3^-$), B cells ($CD19^+$ $CD3^-$), DCs ($CD3^-$ $CD56^-$ $CD19^-$ $CD11c^+$ HLA-DR^+), pDC ($CD3^-$ $CD56^-$ $CD19^-$ $CD11c^-$ HLA-DR^+ $CD123^+$).

CSF-1 gene expression quantification

Knockout efficiency of CSF-1 using CRISPR/Cas9 was evaluated by RT-qPCR. Briefly, RNA was extracted from one million MDA-MB-231 control cells or cells transduced with CSF-1 plentiCRISPRCas9 (or pLentiCRISPRv2-CSF-1) lentiviruses using RNeasy Mini Kit (Qiagen). cDNA was synthesized with random hexamer from 1 µg total RNA using SuperScript II Reverse Transcriptase (ThermoFischer Scientific). Real-time qPCR was performed using QuantiFast SYBR Green (Qiagen). Expression level of mRNA was evaluated by the cycle of quantitation thresholds (Cq) normalized to Cq of GAPDH and the expression level was

calculated as compared with the control cells value. The primers for hGAPDH and hCSF-1 are from Qiagen (QuantiTect Primer Assay).

Western blot

Cell lysates (CL) for western blot were obtained by incubating 1.10^6 cells in 25 µL of lysis buffer (50 mM Tris, pH 7.5, 0.3 M NaCl, 1% Triton X-100, 0.1% sodium azide) with 2% complete protease inhibitor (Roche) for 20 min on ice, followed by a 13,000 rpm centrifugation for 15 min at 4°C to recover the supernatant. 20 µL of the 500 µL unconcentrated SEC fractions from 400.10^6 cells or pooled EV-R and EV-P fractions obtained from 20.10^6 cells and CL from 2.10^5 cells were mixed with Laemmli sample buffer without reducing agent (BioRad). After boiling 5 min at 95°C, samples were loaded on a 4-15% Mini-protean TGX-stain free gels (BioRad). Transfer was performed on Immuno-Blot PVDF membranes (BioRad), with the Trans-blot turbo transfer system (BioRad) during 7min. Blocking was performed during 30 min with Roche blotting solution in TBS 0,1% Tween. Primary antibodies were incubated overnight at 4°C and secondary antibodies (HRP-conjugated goat anti-rabbit IgG (H+L), HRP conjugated goat anti-mouse IgG (H+L) and HRP-conjugated goat anti-rat IgG (H+L), Jackson Immuno-Research) during 1h at room temperature (RT). Development was performed using Clarity western ECL substrate (BioRad) and the Chemidoc Touch imager (BioRad).

Bead-Based Multiplex Flow Cytometry Assay

EV-R fractions were subjected to bead-based multiplex analysis by flow cytometry (MACSPlex Exosome Kit, human, Miltenyi). Samples were processed according to manufacturer instructions. EV-R fraction corresponding to EVs secreted by 40 million cells were diluted with MACSPlex buffer to a final volume of 120 µL and 15 µL of MACSPlex Exosome Capture Beads were added. Samples were incubated on an orbital shaker overnight at room temperature protected from light. After washing, detection antibodies (APC-conjugated anti-CD9/anti-CD63/ anti-CD81 mix at dilution recommended by provider, or anti-CSF1-APC (clone 26786, R&D systems, 1/50) were incubated for 1 h at RT. Flow cytometric analysis was performed

with a MACSQuant Analyzer 10 with the corresponding software (Miltenyi Biotec) following the acquisition recommendations for the MACSPlex Exosome kit (Miltenyi Biotec). FlowJo software (v10, FlowJo LLC) was used to analyze flow cytometric data. The 39 single bead populations were gated to allow the determination of the APC signal intensity on the respective bead population. Median fluorescence intensity (MFI) for each capture bead was background corrected by dividing respective MFI values from matched non-EV controls that were treated exactly like EV-containing samples. Values were log10 transformed. Only normalized log10 values higher than 0 were considered as positive.

Imaging Flow Cytometry

10^8 EVs were stained with lipid dye Membright 488 (Idylle, now MemGlow 488, Cytoskeleton, Inc) to reach a final concentration of 200 nM in filtered PBS (0.22 µm filters). After 30 min of incubation at room temperature in the dark, EVs were washed with 1 ml of filtered PBS and ultracentrifugation at 100,000 xg for 30 min in a TLA-45 rotor (Beckman Coulter). EVs were then stained with anti-CD81-APC (dilution 1/25) (Clone 5A6, Biolegend) and anti-CSF-1-PE (dilution 1/25) (Clone 26786, R&D Systems). Washing step was repeated before resuspending the EV pellet in 40 µl of filtered PBS ($2.5x10^6$ EVs/µl). Adequate PBS, unstained, Membright488, CD81, CSF-1 monocolors and Fluorescence Minus One (FMOs) EVs controls were performed in parallel. EVs were analyzed with a four lasers Image Stream X MKII (Amnis/Luminex) equipped with two cameras. The instrument was set to remove beads during acquisition and to collect Brightfields (channels 1 and 9), Side Scatter (channel 6 laser 785 nm 25 mW), Membright 488 (channel 2 laser 488 nm 100 mW), anti-CSF-1-PE (channel 3 laser 561 nm 110 mW) and anti-CD81-APC (channel 11 laser 642 nm 150 mW) with the 60x magnification. Data were analyzed with IDEAS software.

Monocyte isolation and culture

Peripheral Blood Mononuclear Cells (PBMC) were prepared by centrifugation on a Ficoll gradient (Lymphoprep, Greiner Bio-One). Blood CD14+ monocytes were isolated from healthy donors' PBMC by positive selection using magnetic beads (Miltenyi). Monocytes ($2x10^5$ cells) were cultured for 5 days in complete medium (RPMI-1640-GlutamaxTM supplemented with 10% FCS, 10mM Hepes (Gibco), 0.1 mM nonessential amino acids (Gibco), 1 mM Sodium Pyruvate (Gibco), 100 U/mL penicillin, 100µg/mL streptomycin) with treatments or left untreated. On day 5, supernatants and cells were recovered for analysis.

Flow cytometry

Cells were stained in PBS containing 0.5% BSA and 2mM EDTA with different combinations of the primary antibodies or isotype-matched control antibodies. Prior to the staining with the primary antibodies, cells were incubated with human FC Blocking reagent (Miltenyi) and LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (ThermoFischer). For CD68 staining, cells were permeabilized with BD Cytofix/Cytoperm™ Fixation/Permeablization Kit (BD Biosciences). For IRF7 staining, cells were permeabilized with Transcription Factor Staining Buffer (Thermo). Cells were analyzed on a FACSVerse (BD Biosciences) or MACSQuant (Miltenyi) instrument. Data was analyzed with FlowJo (FlowJo LLC)

RNA-seq library preparation and analysis

RNA from mo-macs cells (5.10^5-10^6 cells) was extracted by RNeasy Mini Kit (QIAGEN), including on-column DNase digestion as described by the manufacturer's protocol. The integrity of the RNA was confirmed in BioAnalyzer using RNA 6000 Pico kit (Agilent Technologies). Libraries were prepared according to Illumina's instructions accompanying the TruSeq Stranded mRNA library Prep Kit (Illumina). 500 ng of RNA was used for each sample. Library length profiles were controlled with the LabChip GXTouchHT system (Perkin Elmer). Sequencing was performed using NovaSeq (Illumina) (100-nt-length reads, paired end).

Genome assembly was based on the Genome Reference Consortium (hg38). Quality of RNA-seq data was assessed using *FastQC* (v0.11.5)[2]. Reads were aligned to the transcriptome using *STAR* (v2.5.3a)[3]. Differential gene expression analysis was performed using *DESeq2* (v1.22.2)[4]. Genes with low number of counts (<10 across all samples) were filtered out. Differentially expressed genes between each pair of conditions were calculated with the design 'donor + condition'. Only genes displaying an adjusted p-value <0.01 and log2FoldChange > 0.5 were kept. The union of these genes was used as input for k-means clustering of gene expression. The normalized count matrix was scaled and centered, and k-means clustering was performed using *stats* (v3.5.0) with k=7.

The gene ontology analysis was performed using Enrichr (https://maayanlab.cloud/Enrichr/) [5]. We evaluated results from Enrichr for Gene Ontology (GO) annotated sets of genes based on the biological processes in which they participate. For these analyses, enrichments were considered statistically significant if they had *q*-values (i.e., *p*-values adjusted for multiple testing) <.05. EV-R-genes involved in cytokine mediated signaling pathway (the most significant GO term) were queried in a database of IFN-regulated genes, Interferome (http://www.interferome.org/)[6] .

Gene signatures for the EV-R-mo-macs (Figure 6C, Supplementary Dataset 2) and the EV-P-mo-macs groups (Supplementary Dataset S2) were generated considering the differentially expressed genes displaying an adjusted p-value<0.01 and log2FoldChange>2 when compared among all the other RNAseq groups. A canonical IFN-gene signature was generated by curation of the literature (Supplementary Dataset S2, kindly provided by L. Niborski, INSERM U932).

scRNASeq analysis of myeloid cells of TNBC patients

Breast cancer patient cohorts and tissue specimens

Tumor (TUM) and adjacent-juxta (JT) tissue samples were obtained from early-stage, treatment-naïve triple-negative breast cancer (TNBC) (N=4). For single-cell RNAseq studies,

fresh TUM specimens from treatment-naïve TNBC (N=4) pts were obtained, of which 3 were paired with JT samples.

Tissue dissociation

Samples from prophylactic mastectomies, TUM and JT were gently dissociated and enzymatically digested by an incubation of 30-40 minutes, at 37°C in agitation, in CO_2-independent medium (Gibco) supplemented by Collagenase I (2mg/mL), (Sigma) Hyaluronidase (2mg/mL) (Sigma), and DNase (25ug/mL) (Roche). Afterwards, single cell suspensions were obtained by disrupting the fragments with a syringe plunger over a 40uM cell strainer (Fisher Scientific), washing with PBS (Gibco) supplemented by 2mM EDTA (Gibco) and 1% of human serum (BioWest). Cell suspension was obtained after 10' of centrifugation. After isolation of total tissue-infiltrating cells, cells were counted in CO_2-independent medium plus 5% of FBS. Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep solution (StemCell).

Myeloid cell sorting, Single-cell RNAseq and library preparation

HLA-DR^+CD11c^+ myeloid cells from TUM and JT-infiltrating cells were FACS sorted (ARIA-BD Bioscience). DAPI solution (BD- Bioscience) was used for dead cells exclusion. CD11c (PE), HLA-DR (PE-Cy7), CD45 (APC-Cy7), CD3-CD56-CD19 (Alexa Flour 700) were included for the staining. Single-cell suspension was loaded into a Chromium Single Cell Chip (10X Genomics) according to the manufacturer's instructions. Target capture rate correspond to 5000 -10.000 individual cells/sample. Single-cell RNA-seq libraries were prepared using Chromium Single Cell 3' v3 Reagent Kit (10X Genomics) according to manufacturer's protocol. Indexed libraries were equimolarly pooled and sequenced on an Illumina NovaSeq 6000 system using paired-end 28x91bp as sequencing mode. A coverage corresponding to 50,000 reads/cell was obtained.

Single cell-RNA-sequencing data processing

Data have been deposited in the zenodo repository: https://zenodo.org/record/5939839.

Single-cell expression was analyzed using the Cell Ranger single-cell Software Suite (v3.0.1 - 10X Genomics) to perform quality control, sample de-multiplexing, barcode processing and single-cell 3' gene counting. Sequencing reads were aligned to the GRCh38 human reference genome. Further analysis was performed in R (v3.5.1) using the Seurat package (v3.1.1) (https://pubmed.ncbi.nlm.nih.gov/31178118/). Cells were then filtered out when expressing less than 500 genes, or when expressing more than 10% mitochondrial genes. From the 7 samples, 11340 cells (10005 cells for TUM and 1335 for JT) were kept for statistical analysis. For each sample, the gene-cell-barcode matrix of the samples was then normalized to a total of 1e4 molecules. The top 5000 variable features were identified using the "vst" method from Seurat where both lowly and highly expressed genes are transformed onto a common scale. For the 7 samples altogether, we computed the integration anchors using the Seurat v3 integration method. This method is leveraging closely-related cells (termed anchors) between datasets to compute a batch-corrected matrix. Top 30 CCA components were used to find transfer anchors between datasets and used to generated the integrated matrix for the 7 samples. Top 30 Principal Components were computed and Uniform Manifold Approximation and Projection (UMAP) was performed using the top 30 PCs of the integrated matrix. Clusters were identified using the *FindNeighbors* and *FindClusters* function in Seurat with a resolution parameter of 0.6 and using the first 30 principal components. Unique cluster-specific genes were identified by running the Seurat *FindAllMarkers* function using Wilcoxon test on the uncorrected matrix. Then clusters containing contaminating cells were removed from the analysis. CD3, CD4, CD8 expression was used to remove T cell clusters. CD19, MS4A1 expression was used to remove B cell clusters, CD56 expression was used to remove NK cell

clusters. CD1c, LAMP3, AXL and CLEC9A were used to remove DCs. Signature scores were computed using the Seurat function *AddModuleScore* using the gene signature of interest and the integrated matrix. Briefly this function calculates for each individual cell the average expression of each gene signature, subtracted by the aggregated expression of control gene sets.

Migration assay

Migration assays were performed with the xCELLigence RTCA instrument according to Manufacturer's recommendations. Briefly in the lower chamber of the CIM-16 plate, CM from EV-R-mo-macs, EV-P-mo-macs and CSF-1-mo-macs was added. DMEM 10% FBS, 1%P/S without or with 100 ng/ml of CXCL10 was added as negative and positive control respectively. Once the upper chamber was placed on top of the lower chamber, 50 µl of medium with FBS were added to each well of the upper chamber to cover the membrane surface. Plates were left during 1h inside the 37°C incubator to reach equilibrium. After this incubation, background measurement was performed. 40000 CD3 T cells in 100 µl of medium with FBS were added to each well of the upper chamber. Plates were left at room temperature for 30 minutes to allow the cells to settle evenly. Plates were then loaded into the xCELLigence RTCA DP instrument inside a 37°C incubator. A run of 24 hours with readings every 15 minutes was programmed. Data was collected and analyzed by RTCA software.

References:

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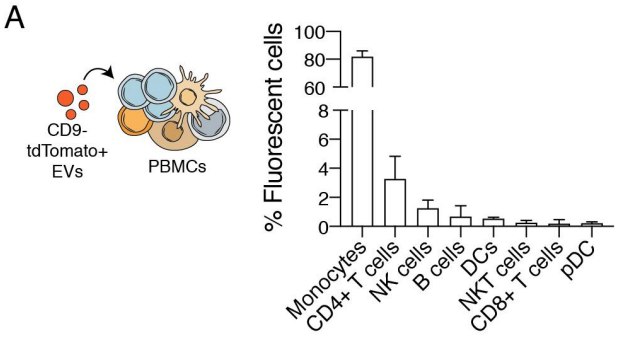
Supplementary Dataset S1: List of genes present in the K-means clusters.

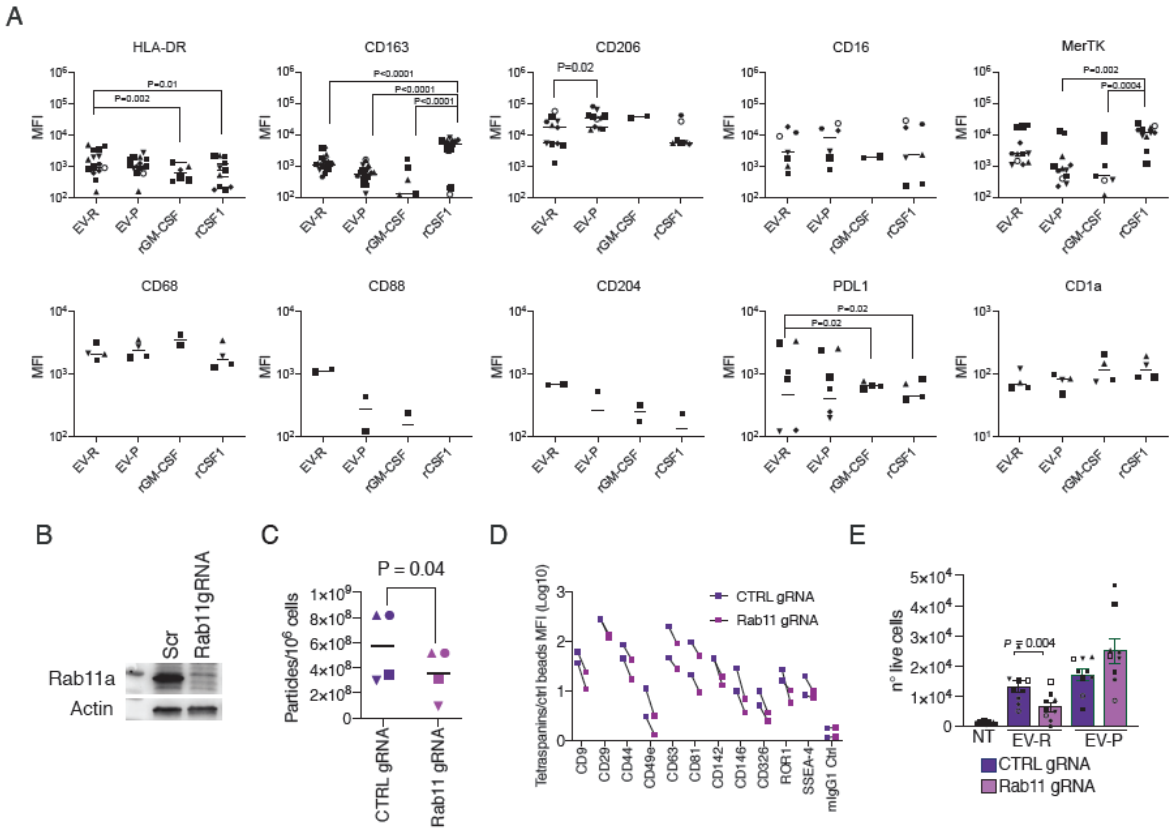
Supplementary Dataset S2: List of gene signatures calculated as the differentially expressed genes with an adjusted p-value <0.01 and log2FC > 2 when compared to all the other RNAseq groups, and of canonical IFN-genes curated from the literature.

Supplementary Figure 1



Supplementary Figure 1. PBMC uptake of tumor derived EVs
(A) EVs from MDA-MB-231-CD9-tdTomato cells were cultured ON with total PBMCs from healthy donors. Cells were stained with different markers in order to identify all cell populations present in peripheral blood mononucleated cells. We gated on the positive tdTomato cells and then calculated the percentage from the tdTomato+ cells that corresponded to each immune cell type. The mean + SD of five individual PBMC donors is shown.

Supplementary Figure 2



Supplementary Figure 2 (related to figure 2). Quantification of surface markers on d5 monocyte cultures, Rab11a-depleted MDA-MB-231 cells and EV characterization and effect on monocyte cultures.

(A) Expression of different markers in monocytes exposed to EV-R, EV-P, rGM-CSF and rCSF-1, calculated as Gmean of whole live cells. Each dot represents an individual biological replicate.

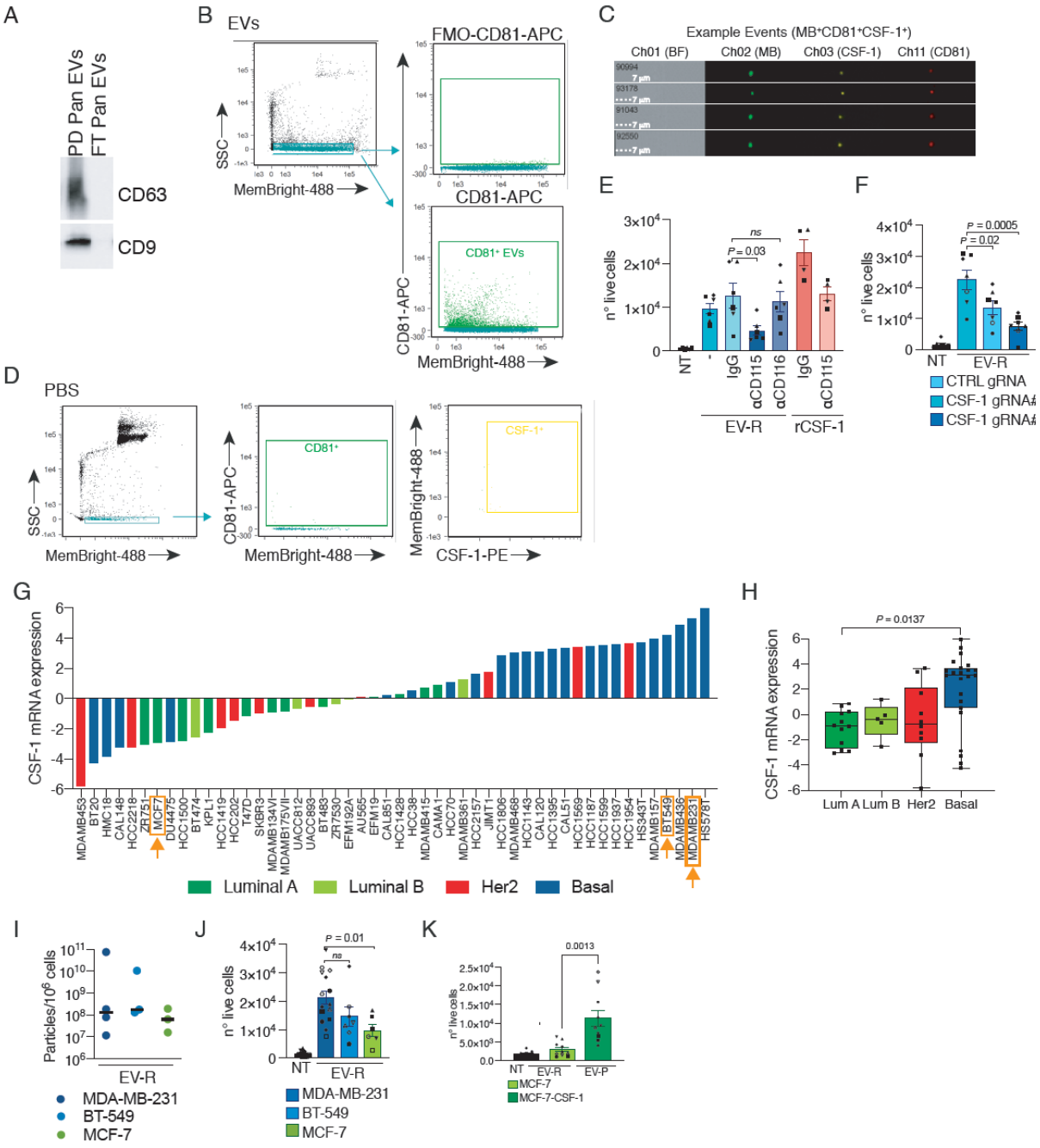
(B) Expression of Rab11a and actin in MDA-MB-231 transduced with a control gRNA or gRNA against Rab11a (n = 3, one representative experiment is shown).

(C) Quantification by NTA of EVs released by control or Rab11a-deleted MDA-MB-231 cells.

(D) MACSPlex Exosome analysis on EVs from equal numbers of CTRL or Rab11a-deleted MDA-MB-231 cells developed using a mix of antibodies against tetraspanins (CD9, CD63 and CD81).

(E) Total number of live cells recovered after monocyte exposure to EVs from CTRL or Rab11 gRNA MDA-MB-231. Comparison between groups was performed by two-tailed, Wilcoxon test. P values ≤0.05 were considered significant and are indicated for each comparison. Each individual donor is shown. Results shown represent mean ± SEM.

Supplementary Figure 3



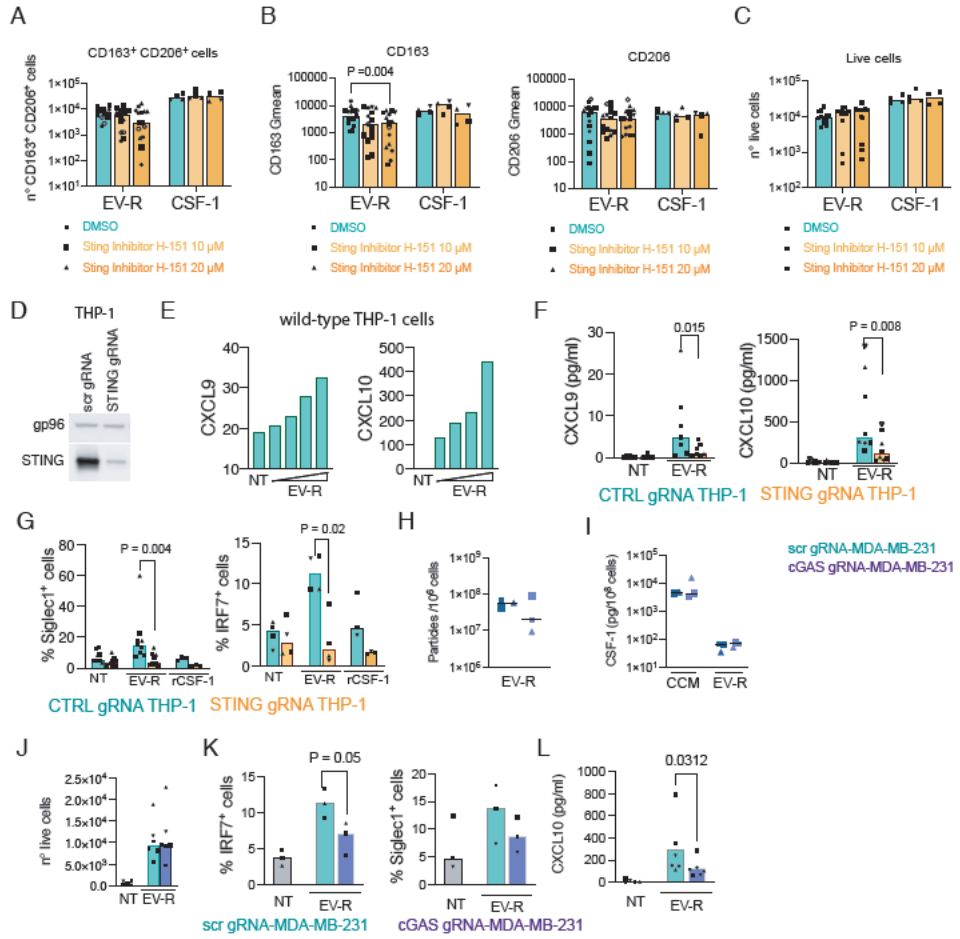
Supplementary Figure 3 (related to figure 3). CSF-1 expression on EVs and in breast cancer cell lines and role in monocyte survival

(A) EVs were subjected to immuno-isolation with beads coupled to antibodies against TSPs (CD9, CD63 and CD81) (Pan Exosome Isolation Kit Miltenyi). Bead-associated (Pull-down: PD) EVs and those left behind (flow-though: FT) were analyzed by western blot with specific antibodies for CD63 and CD9. Representative of three independent experiments.

(B) Gating strategy for the imaging flow cytometry analysis of single EVs. MDA-MB-231 EVs were stained with the lipid dye Membright488 with or without the addition of fluorochrome-coupled anti-CD81 antibody (CD81-APC). Washed samples were acquired with the ImageStream-X. Dot plots gated on low SSC membright488+ events were analyzed for CD81. Membright+ CD81+ events were gated for further analysis of CSF-1 on samples stained simultaneously for CD81 and CSF-1. Representative of two independent experiments.

(C) Example images for Membright(+) CD81-APC(+) CSF-1-PE(+) triple positive events. Representative of two independent experiments.
(D) Dot plots following the same gating strategy performed on non-EV containing samples labeled with the same combination of Membright488, CSF-1 and CD81 antibodies.
(E) Quantification of total live macrophages on day 5 of culture of monocytes with MDA-MB-231 EV-R in the presence of blocking antibodies against CSF-1 Receptor (CD115) or GM-CSF Receptor (CD116) molecules. Each individual donor is shown (n = 6). Results shown represent mean ± SEM.
(F) Total number of live cells recovered after monocyte exposure to EVs from CTRL or CSF1 gRNA MDA-MB-231.
(~~E~~ G) CSF-1 expression in breast cancer cell lines according to the Broad Institute Cancer Cell Line Encyclopedia (CCLE). Cells lines were categorized according to the subtype they have been described to belong to.
(~~F~~ H) CSF-1 expression values from CCLE breast cancer cell lines grouped according to cancer subtype.
(~~G~~ I) Number of particles released per 10^6 cells by MDA-MB-231, BT-549 and MCF-7.
(J) Total number of live cells recovered after monocyte exposure to EVs from MDA-MB-231, BT549 or MCF-7 cells.
(K) Total number of live cells recovered after monocyte exposure to EVs from MCF-7 control or overexpressing CSF-1.
Comparison to control group was performed by two-tailed, Wilcoxon test. P values ≤0.05 were considered significant and are indicated for each comparison. Each individual donor is shown. Results shown represent mean ± SEM.

Supplementary Figure 4



Supplementary Figure 4 (related to Figure 5). Characterization of Sting-inhibited EV-R-monocytes and STING-ko THP-1 response to EV-R, of EVs from cGAS ko tumor cells and response of THP1.

(A) CD14+ monocytes were incubated with the indicated concentrations of the Sting inhibitor (H-151) or DMSO prior to the treatment with MDA-MB-231 EV-R or rCSF-1. The number of CD163+CD206+ cells was evaluated by FACS on day 5 of macrophage differentiation.

(B) Expression of CD163 (left) and CD206 (right) on macrophages differentiated upon EV-R or rCSF-1 treatment in the presence or not of a Sting inhibitor (H-151).

(C) Number of live cells at day 5 of monocyte culture with EV-R or rCSF-1 in the presence of STING inhibitor.

(D) Expression of STING and gp96 on THP-1 wild-type cells (scr-gRNA) or STING-deleted (STING-gRNA) by CRISPR/Cas9 (n=1).

(E) CXCL9 and CXCL10 secretion was measured by CBA on THP-1 wild-type cells treated for 18 hours with increasing amounts of EV-R from MDA-MB-231 cells (0.25-2 µg of proteins).

(F) THP-1 wild-type control cells or CRISPR/Cas9-STING-deleted THP-1 cells were stimulated for 18 hours with EV-R from MDA-MB-231 cells and secretion of CXCL9 and CXCL10 into the supernatant was evaluated.

(G) Expression of Siglec1 (left) and IRF7 (right) in THP-1 wild-type control cells or CRISPR/Cas9-STING-deleted THP-1 cells stimulated for 18 hours with EV-R from MDA-MB-231 cells or rCSF-1.

(H) Particle count in EV-R from MDA-MB-231-SCR-gRNA and MDA-MB-231-cGAS-gRNA.

(I) CSF-1 levels in CCM or pooled EV-R fractions from MDA-MB-231 deleted for *cGAS* as indicated. Quantification done on independent EV isolations is shown.

(J) Number of live cells at day 5 of monocyte culture with EV-R from MDA-MB-231-SCR-gRNA and MDA-MB-231-cGAS-gRNA.

(K) THP-1 wild-type cells were stimulated for 18 hours with EV-R from MDA-MB-231-SCR-gRNA or MDA-MB-231-cGAS-gRNA and IRF7 (left) and siglec1 (right) expression was evaluated by flow cytometry.
(L) Secretion of CXCL10 into the supernatant was evaluated on THP-1 treated as described in K.
For (F), (G), (K) and (L), comparison between groups was performed by two-tailed, Wilcoxon test. P values ≤ 0.05 were considered significant and are indicated for each comparison.
Each individual donor is shown. Results shown represent mean ± SEM.