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Supplemental Information

Tissue nanotransfection causes tumor regression by its effect on nanovesicle cargo that alters microenvironmental macrophage state

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Supplementary Figures

Figure S1: **A**, EOMA cells have higher miR-126 abundance compared to MAE cells. *, $p<0.05$ (n=4), Mann Whitney test. **B,** Schematic representation of α-miR-126 delivery into HE tumor by tissue nanotransfection (TNT). **C,** Quantification of miR-126 in tumor after delivering α-126 into HE tumor by TNT, *$p<0.05$, n=7,9, Mann Whitney test. **D**, FITC labeled α-126 was delivered in 129P3/J mouse to check the efficiency of TNT delivery. 24h after delivery shows mostly in epidermis region. **E,** Abundance of miR-126 in different regions of tumor (d10) isolated using LCM after scramble and α-126 delivery for two doses (d3, d7) after EOMA injections, *$p<0.05$, n=4, Two-way ANOVA. **F,** Laser capture Micro dissection (LCM) evidences from different regions of tumor tissue (d10) sections before and after cut. **G,** Cutaneous perfusion analysis by using Perimed Laser speckle imaging at different timepoints post- TNT_{sham} and $TNT_{\alpha\text{-}126}$ and its analysis (**H**) in adult mice. n=6, Two-way ANOVA. **I**, Tumor volume of day-matched animals for the control and TNT_{sham} groups. (n=11, 5), Mann–Whitney test. **J**, Sequential evidences of tumor regression of an animal at different days after α-miR-126 delivery. **K,** H&E staining of tissue sections from animal treated with α-miR-126 in both tumor region and non-tumor region.

Figure S2: **A,** Schematic diagram of isolating extracellular vesicles from cell culture conditioned media by differential centrifugation. **B**, Nanosight distribution and quantification **(C)** of EVs isolated from MAE and EOMA cells. *, $p<0.05$ (n=5), Mann Whitney test. **D.** miR-126 is more abundant in EOMA EV compared to MAE EV. *, $p<0.05$ (n=6), Mann Whitney test. **E,** Nanosight characterization of EVs isolated from control and HE patient's urine samples. **F,** Schematic diagram of experimental plan for isolation of mouse urine samples after different days (d3 and d10) after EOMA cell injection. **G,** Transmission electron microscopy (TEM) images of negatively

stained EV with 2% uracyl acetate after removing the moisture. **H,** EV size distribution pattern of mouse urine samples collected at different days.

Figure S3: A, Plasmid vector map showing CDH5 promotor driven recombinant plasmids encoding CD81 with GFP. **B**, Confocal images shows expression of GFP tagged exosomes in EOMA cells (endothelial cells) but, not in keratinocytes, shows GFP tagged plasmids are specific for endothelial cells. 63× magnification. **C**, Optimization of puromycin concentration to generate transduced EOMA stable cell line. Representative day4 images after treating with 2.5µg/ml puromycin shows live transduced EOMA cells, not EOMA cells. **D,** Selection of stable transduced cells after treating with 2.5µg/ml puromycin.

Figure S4: A, Full image representation of F4/80 (Mϕ marker) and LYVE1 (endothelial marker) labelled tumor section and their colocalization **(B)** for Figure 3 H.10× magnification. **C,** Elaborated images for Figure 3G (63× magnification). Immuno labelling with F4/80, LYVE1 and GFP of tumor core, TAM_{LYVE1} and tumor perimeter. **D,** H&E image shows tumor regions 1, Tumor core, 2, TAM_{LYVE1} region, 3, tumor perimeter region**. E,** Graphical representation of tumor regions, LCM samples have been collected.

Figure S5: A, Expression of M1 and M2 markers in TAM_{LYVE1} region captured by LCM. M2 markers were more abundant in GW4869 treated HE tumor compared to control HE tumor. Tumors were collected on day10. *, $p<0.05$ (n=6), Mann Whitney test. **B,** Tumor tissue sections from HE patient's samples were stained with anti-LYVE (EOMA cell marker) and anti-CD68 (Mϕ marker) shows greater than 50% of EOMA cells (LYVE1) co-expressed with $CD68^+$ **(C).** *, $p<0.05$ (n=5), Mann Whitney test. **D**, Mouse tumor sections (d10) were stained with anti-LYVE1 and anti- $F4/80^+$ (Mϕ marker) shows about 50% of EOMA cells ($LYVE^+$) co-expressed $F4/80^+$ **(E).** *, $p<0.05$ (n=5), Mann Whitney test.

Figure S6: A, *in vitro* nanoelectroporation (NEP) delivery of nucleotides into cells based on the voltage gradient. **B**, Propidium Iodide (PI, 1μM/ml) immuno-staining of MAE and EOMA cells after delivering scrambled and antagomiR126 at 24, 48, 72h using NEP. *, $p<0.05$ (n=5), One way ANOVA. **C**, Evidence of successful knockdown (RT-PCR) of miR-126 level after 48h of NEP with control and α-126 *, $p<0.05$ (n=5,6), Mann Whitney test. **D**, Matrigel angiogenesis assay was used to evaluate the functional effects of miR-126 knockdown *in vitro*. Cells were stained with calcein-AM, and the area within the formed tubes was quantitated **(E)** by analyzing three high powered fields per well using AxioVision Rel 4.8 software. *, $p<0.05$ (n=5), Mann Whitney test. **F,** EV content was measured using nanosight detection and EV specific inhibition of miR-126 was established **(G)** in control and α-126 delivered cells. *, $p<0.05$ (n=4-6), Mann Whitney test. **H,** PI immuno-staining of control inhibitor (100nM) and α-126 (100nM) transfected (72h) EOMA cells. (n=5), Mann Whitney test. **I,** Significant change in miR-126 levels in EOMA cells after transfecting with α-126, *, $p<0.05$ (n=6), Mann Whitney test. **J,** Matrigel assay shows decrease in tube formation after mir-126 inhibition by transfection compared to control inhibition. **K,** Quantification of tube formation by analyzing three high powered fields per well using AxioVision Rel 4.8 software. *, $p<0.05$ (n=3,4), Mann Whitney test. **L,** EV isolated from these transfected cells showed less miR-126 abundance **(M)** in α-126 group. *, $p<0.05$ (n=6), Mann Whitney test. **N,** Transfection of α-126 to MAE cells resulted in miR-126 knock down in EV ($EV_{\alpha\text{-}126}$). EV isolated from MAE cells (EV) and MAE cells transfected with control inhibitor ($EV_{con\ inh}$) were used as controls. *, $p<0.05$ (n=6), One way ANOVA.

Figure S7. Quality check and identification of clusters markers.

(A) UMAP representing all cells before excluding low quality cells. **(B)** UMAP representing all cells with low quality cells colored by black and cells which passed quality check colored with grey. **(C)** Dotplot showing relative expression level of top 3 markers for each cluster. *Aif1*, more abundant in cluster 7, was used to sort the cluster 7 specific cells from Mϕ treated with EV_{EOMA} for tumorigenicity assay (Figures S10B – S10C). **(D)** Top transcription factors, transmembrane receptors and cytokines upregulated in cluster 7 when compared to the rest of the cells (using adjusted p value < 0.05 and logFC > 0.2). Red stars over x axes-es represents cluster 7.

Figure S8. Differential expression analysis and gene set enrichment analysis of Mϕ vs $M\phi_{EOMA\ EV}$. A, Heatmap representing relative expression level of top 10 upregulated and downregulated genes between Mϕ vs $M\phi_{EOMA\ EV}$. **B,** GSEA results with FDR q-val < 0.05 for DEG when comparing Mϕ vs $M\phi_{EOMA\ EV}$ using adjusted p value < 0.05 and logFC cutoff +-0.2. **C,** Heatmap representing relative expression level of top 10 upregulated and downregulated genes between cluster 7 and cluster 4. **D,** GSEA results with FDR q-val < 0.05 for DEG when comparing cluster 7 cells vs cluster 4 cells using adjusted p value < 0.05 and logFC cutoff +-0.2.

Figure S9. Expression level of upregulated vasculogenic genes in cluster 7 or in $M\phi_{EOMA\ EV}$.

A, Violin plots representing expression level of genes Mϕ, $M\phi_{miR\text{-}126\ null\ EV}$ and $M\phi_{EOMA\ EV.}$ Genes are found to be upregulated in $M\phi_{EOMA\ EV}$ compared to Mϕ and also upregulated in cluster 7 compared to cluster 4. **B,** Violin plots representing expression level of genes found to be upregulated in $M\phi_{EOMA\ EV}$ compared to Mϕ, but not in cluster 7 compared to 4. **C,** Violin plots representing expression level of genes found to be upregulated in cluster 7 compared to cluster 4, but not in $M\phi_{EOMA\ EV}$ compared to Mϕ. *, $p<0.05$ (n=6), student's t-test.

Figure S10. A, Time dependent increased expression of chemokines (*Ccl2, Ccl3, Ccl4, Ccl5*) in Mϕ treated with miR-126 null EV (Figure S6N) ($M\phi_{miR126\ null\ EV}$) and cluster 7 specific macrophages, sorted by using *Aif1* ($M\phi_{AIF1+}$) (Figure S7C). $p<0.05$ (n=3), Two-way ANOVA. **B,** Time dependent increase in colony formation in $M\phi_{AIF1+}$ as compared to $M\phi_{miR126\ null\ EV}$ displayed no colonies in soft gel agar plate. Representative images show colonies from each cell type. 10× magnification. **C,** Fluorescence based quantification of cell transformation assay at 485/520 filter. $p<0.05$ (n=5, 10), One-way ANOVA. **D,** Representative images show colony formation of EOMA cells, but not Mϕ. 10× magnification. **E**, Fluorescence based quantification of cell transformation assay of Mϕ and EOMA cells at 485/520 filter. $p<0.05$ (n=5), student's t-test.

Figure S11. Analysis of genes involved in macrophages activation process.

A, Heatmap representing relative expression of genes found in our data having role in macrophage activation based on the gene ontology term macrophage activation (GO:0042116). **B,** Pie chart representing the number of upregulated, downregulated and not altered genes identified to have a role in macrophage activation.

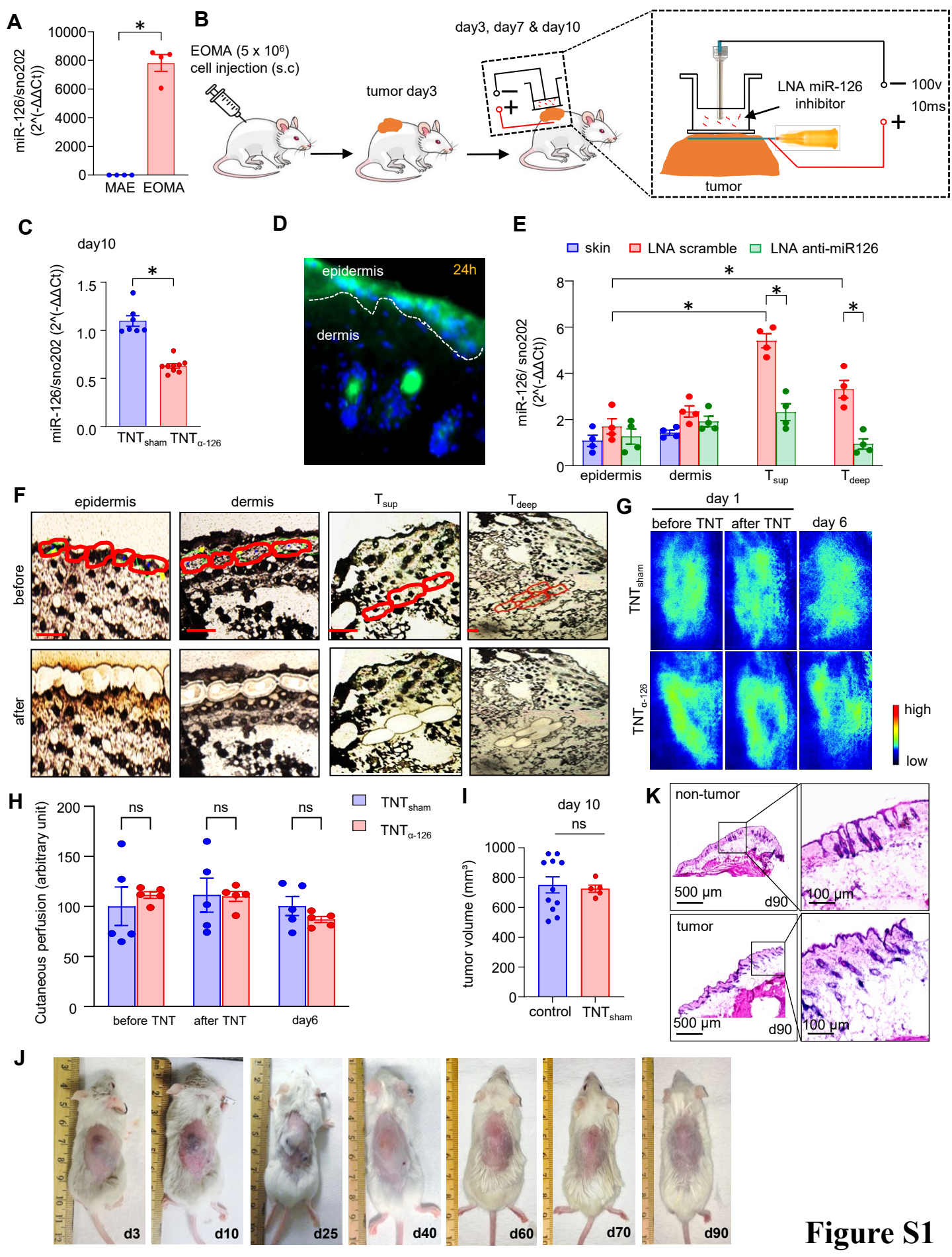

A
miR-126/sno202 (2^(-ΔΔCt))
MAE
EOMA
B
EOMA (5 x 10^6) cell injection (s.c)
tumor day3
day3, day7 & day10
LNA miR-126 inhibitor
100v
10ms
tumor
C
day10
miR-126/sno202 (2^(-ΔΔCt))
TNT sham
TNT α-126
D
epidermis
dermis
24h
E
skin
LNA scramble
LNA anti-miR126
miR-126/ sno202 (2^(-ΔΔCt))
epidermis
dermis
T sup
T deep
F
epidermis
dermis
T sup
T deep
before
after
G
day 1
before TNT
after TNT
day 6
TNT sham
TNT α-126
high
low
H
Cutaneous perfusion (arbitrary unit)
ns
TNT sham
TNT α-126
before TNT
after TNT
day6
I
day 10
ns
tumor volume (mm3)
control
TNT sham
K
non-tumor
tumor
500 µm
100 µm
d90
J
d3
d10
d25
d40
d60
d70
d90

Figure S1

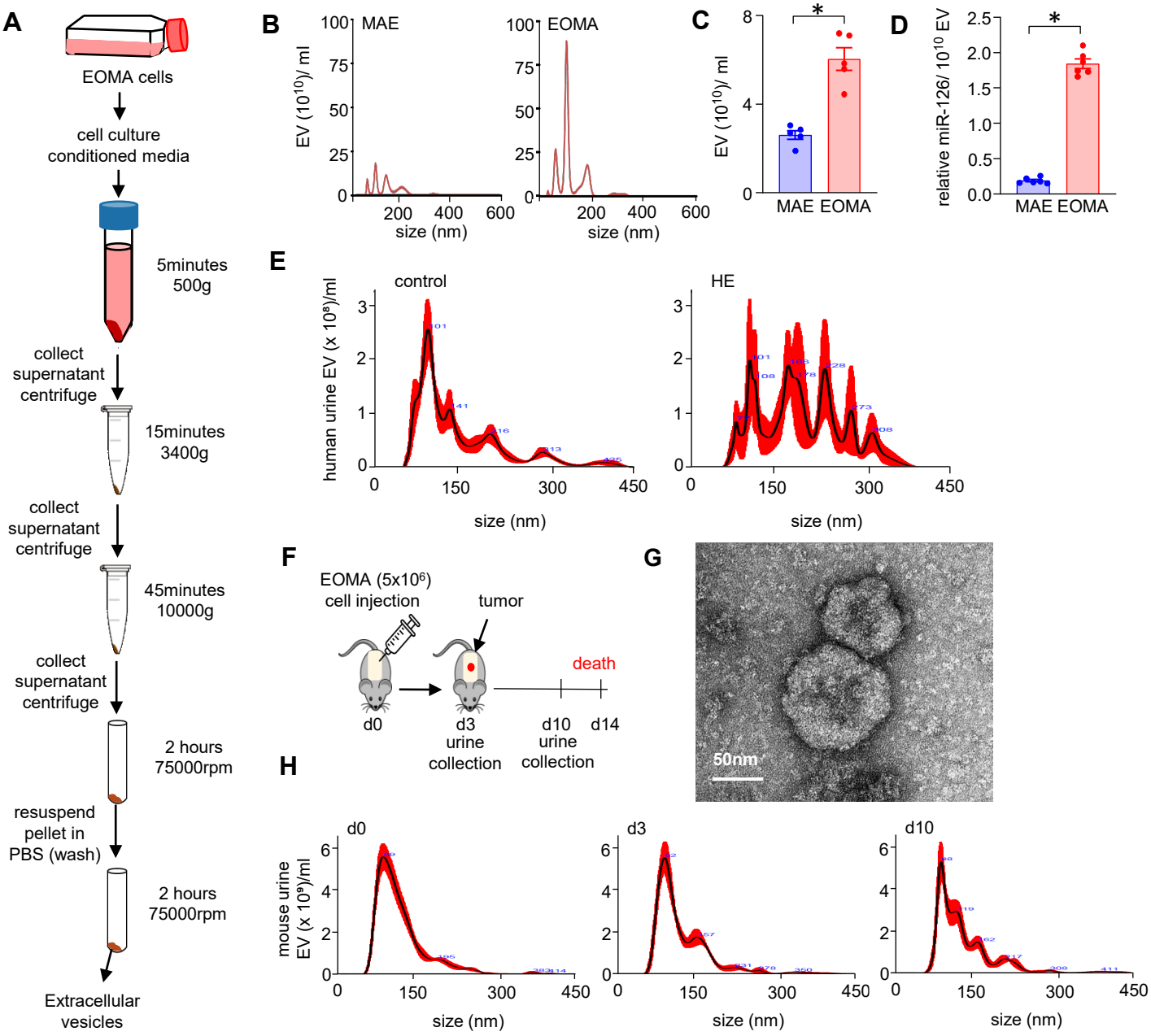

A
EOMA cells
cell culture conditioned media
5minutes 500g
collect supernatant centrifuge
15minutes 3400g
collect supernatant centrifuge
45minutes 10000g
collect supernatant centrifuge
2 hours 75000rpm
resuspend pellet in PBS (wash)
2 hours 75000rpm
Extracellular vesicles
B
MAE
EOMA
EV (10^10)/ ml
size (nm)
C
EV (10^10)/ ml
MAE EOMA
D
relative miR-126/ 10^10 EV
MAE EOMA
E
control
HE
human urine EV (x 10^8)/ml
size (nm)
F
EOMA (5x10^6) cell injection
tumor
death
d0
d3 urine collection
d10 urine collection
d14
G
50nm
H
d0
d3
d10
mouse urine EV (x 10^9)/ml
size (nm)

Figure S2

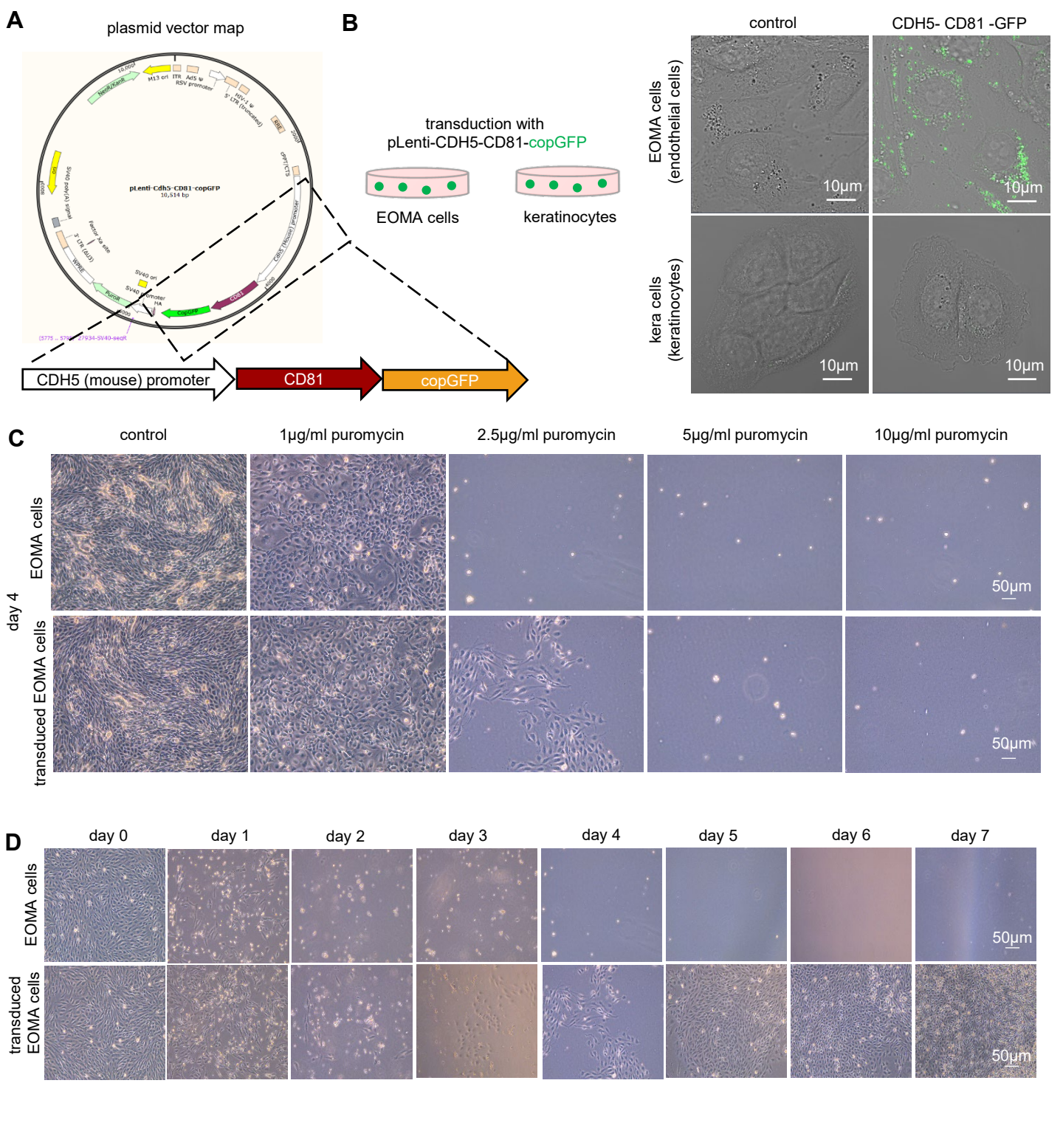
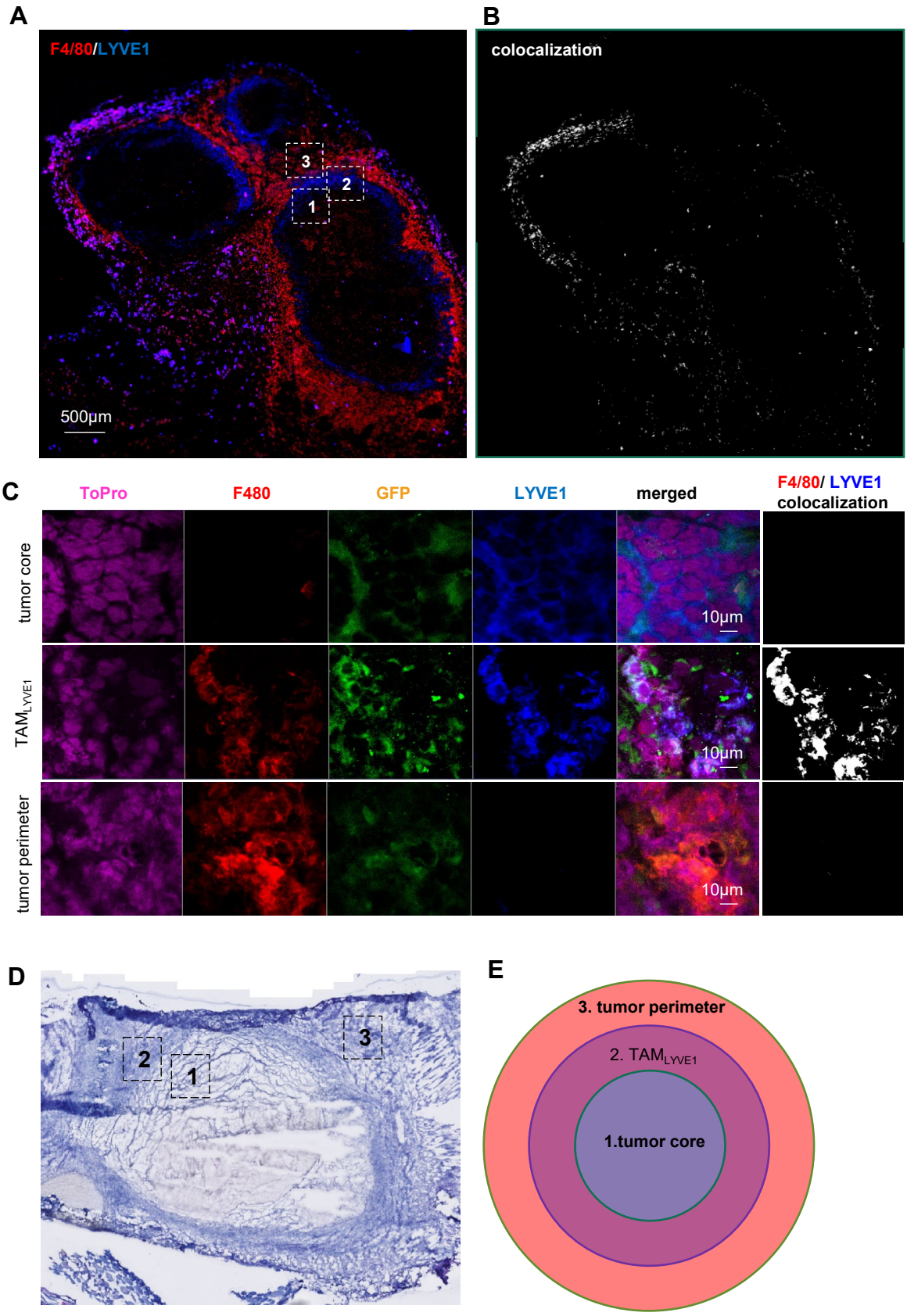

A
plasmid vector map
pLenti-Cdh5-CD81-copGFP
10,514 bp
B
transduction with
pLenti-CDH5-CD81-copGFP
EOMA cells
keratinocytes
CDH5 (mouse) promoter
CD81
copGFP
control
CDH5- CD81 -GFP
EOMA cells
(endothelial cells)
kera cells
(keratinocytes)
10μm
C
control
1μg/ml puromycin
2.5μg/ml puromycin
5μg/ml puromycin
10μg/ml puromycin
day 4
EOMA cells
transduced EOMA cells
50μm
D
day 0
day 1
day 2
day 3
day 4
day 5
day 6
day 7
EOMA cells
transduced
EOMA cells
50μm

Figure S3



(1) Mϕ_{EOMA}-deficent tumor core region (tumor core),
(2) Intermediary LYVE1^{+} tumor associated macrophage populated region (TAM$_{LYVE1}$)
(3) Mϕ_{EOMA}-deficent tumor perimeter region (tumor perimeter).

Figure S4

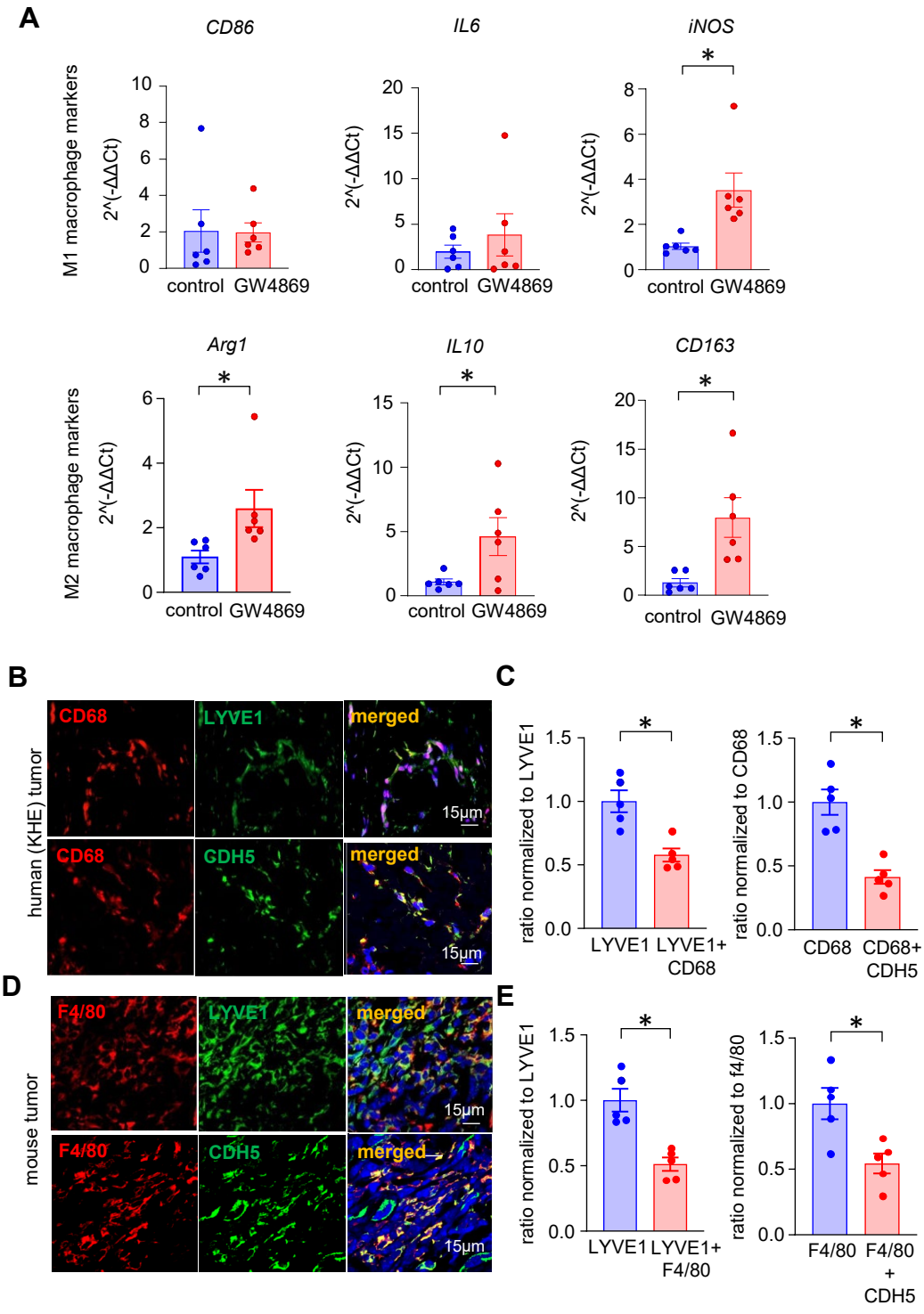

A
CD86
IL6
iNOS
M1 macrophage markers
2^(-ΔΔCt)
control
GW4869
Arg1
IL10
CD163
M2 macrophage markers
B
human (KHE) tumor
CD68
LYVE1
merged
GDH5
15μm
C
ratio normalized to LYVE1
LYVE1
LYVE1+ CD68
ratio normalized to CD68
CD68
CD68+ CDH5
D
mouse tumor
F4/80
CDH5
E
ratio normalized to f4/80
F4/80
F4/80 + CDH5

Figure S5

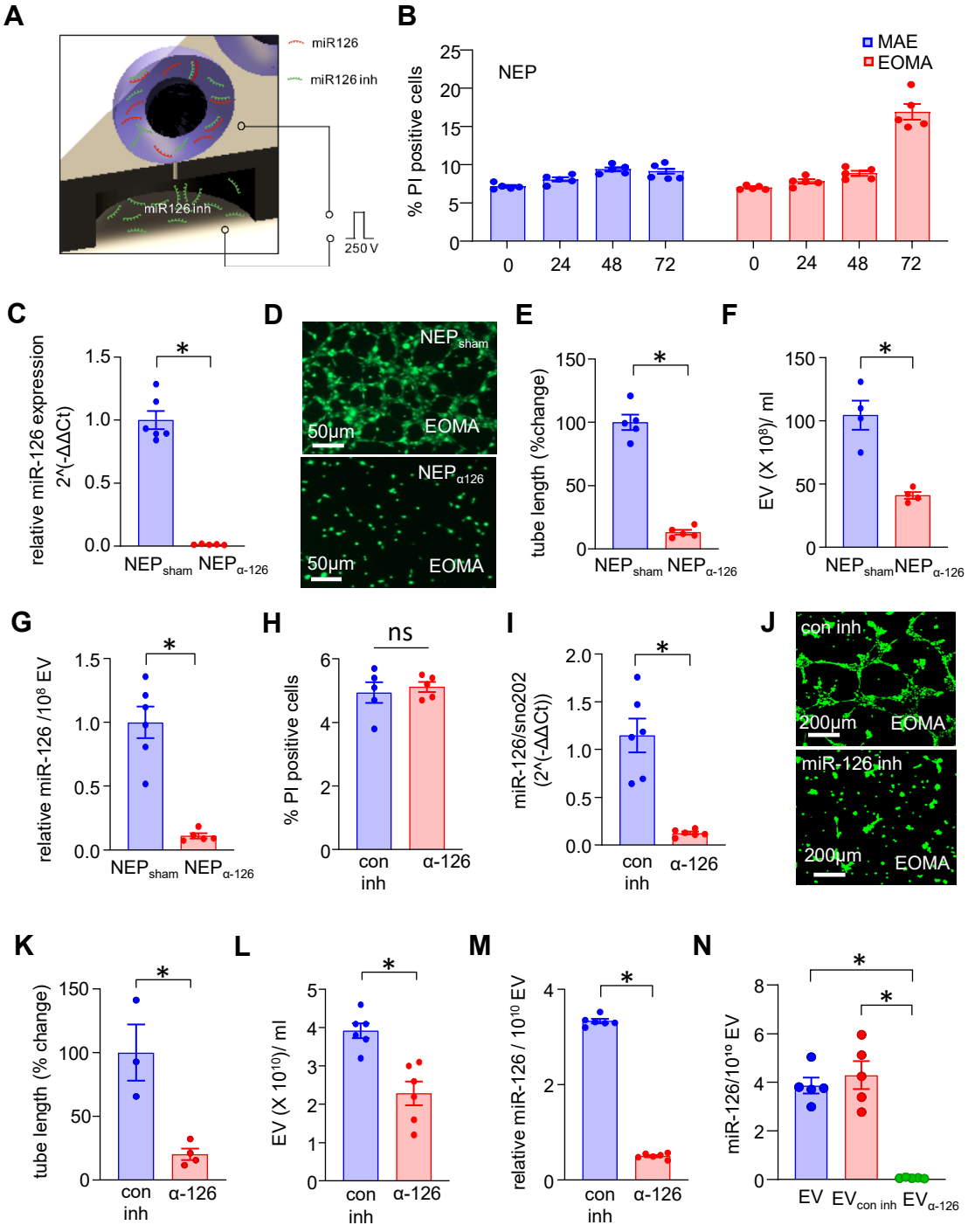

A
miR126
miR126 inh
miR126 inh
250 V
B
NEP
MAE
EOMA
% PI positive cells
25
20
15
10
5
0
0
24
48
72
0
24
48
72
C
relative miR-126 expression
2^(-ΔΔCt)
1.5
1.0
0.5
0.0
*
NEP sham
NEP α-126
D
NEP sham
EOMA
50μm
NEP α126
EOMA
50μm
E
tube length (%change)
150
100
50
0
*
NEP sham
NEP α-126
F
EV (X 10⁸)/ ml
150
100
50
0
*
NEP sham
NEP α-126
G
relative miR-126 /10⁸ EV
1.5
1.0
0.5
0.0
*
NEP sham
NEP α-126
H
ns
% PI positive cells
6
4
2
0
con inh
α-126
I
miR-126/sno202 (2^(-ΔΔCt))
2.0
1.5
1.0
0.5
0.0
*
con inh
α-126
J
con inh
200μm
EOMA
miR-126 inh
200μm
EOMA
K
tube length (% change)
150
100
50
0
*
con inh
α-126
L
EV (X 10¹⁰)/ ml
5
4
3
2
1
0
*
con inh
α-126
M
relative miR-126 / 10¹⁰ EV
4
2
0
*
con inh
α-126
N
miR-126/10¹⁰ EV
8
6
4
2
0
*
*
EV
EV con inh
EV α-126

Figure S6

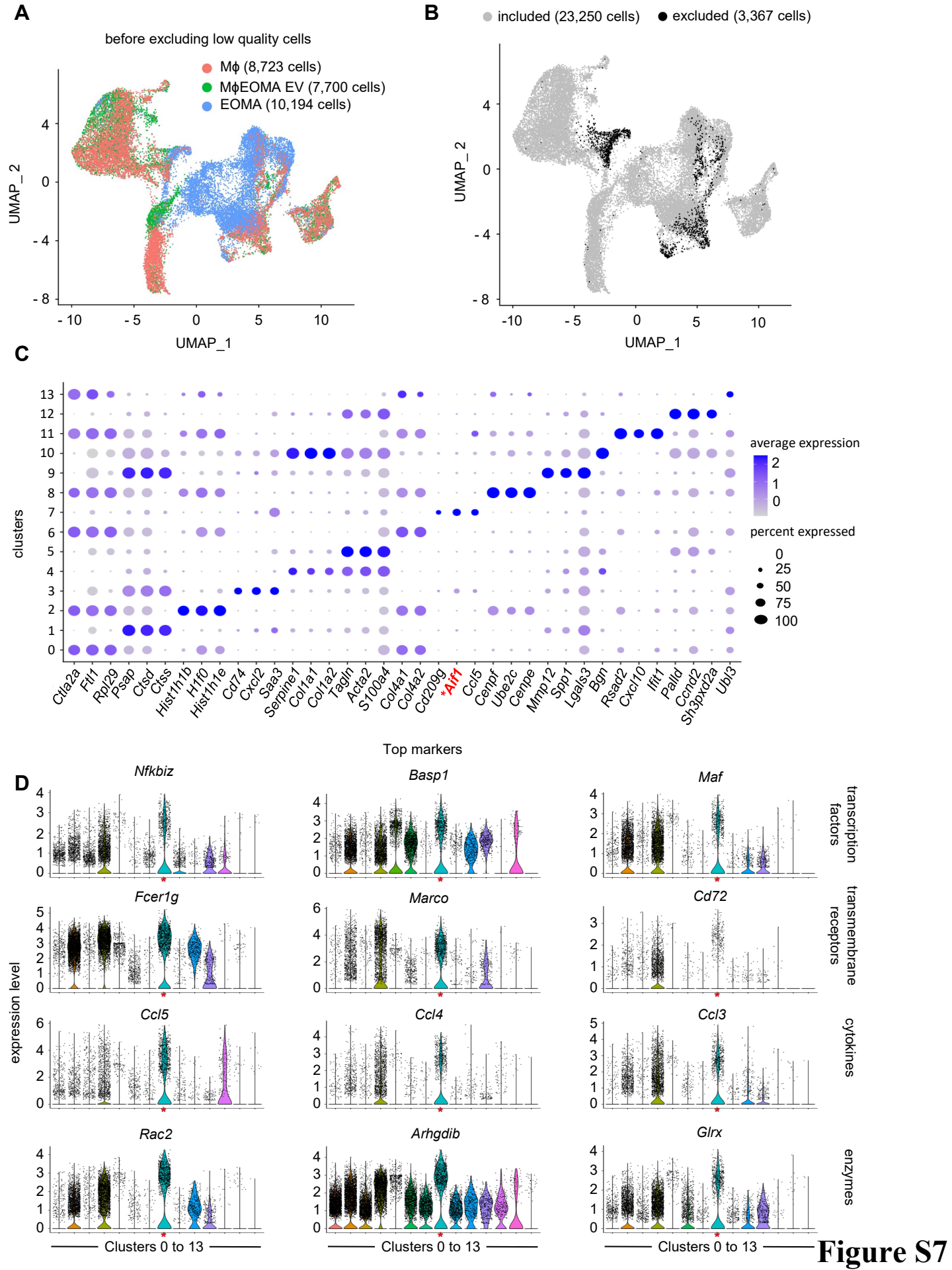

A
before excluding low quality cells
Mϕ (8,723 cells)
MϕEOMA EV (7,700 cells)
EOMA (10,194 cells)
UMAP_2
UMAP_1
B
included (23,250 cells)
excluded (3,367 cells)
UMAP_2
UMAP_1
C
clusters
average expression
percent expressed
Top markers
Ctla2a
Ftl1
Rpl29
Psap
Ctsd
Ctss
Hist1h1b
H1f0
Hist1h1e
Cd74
Cxcl2
Saa3
Serpine1
Col1a1
Col1a2
Tagln
Acta2
S100a4
Col4a1
Col4a2
Cd209g
*Aif1
Ccl5
Cenpf
Ube2c
Cenpe
Mmp12
Spp1
Lgals3
Bgn
Rsad2
Cxcl10
Ifit1
Palld
Ccnd2
Sh3pxd2a
Ubl3
D
Nfkbiz
Basp1
Maf
Fcer1g
Marco
Cd72
Ccl5
Ccl4
Ccl3
Rac2
Arhgdib
Glrx
transcription factors
transmembrane receptors
cytokines
enzymes
expression level
Clusters 0 to 13
Clusters 0 to 13
Clusters 0 to 13

Figure S7

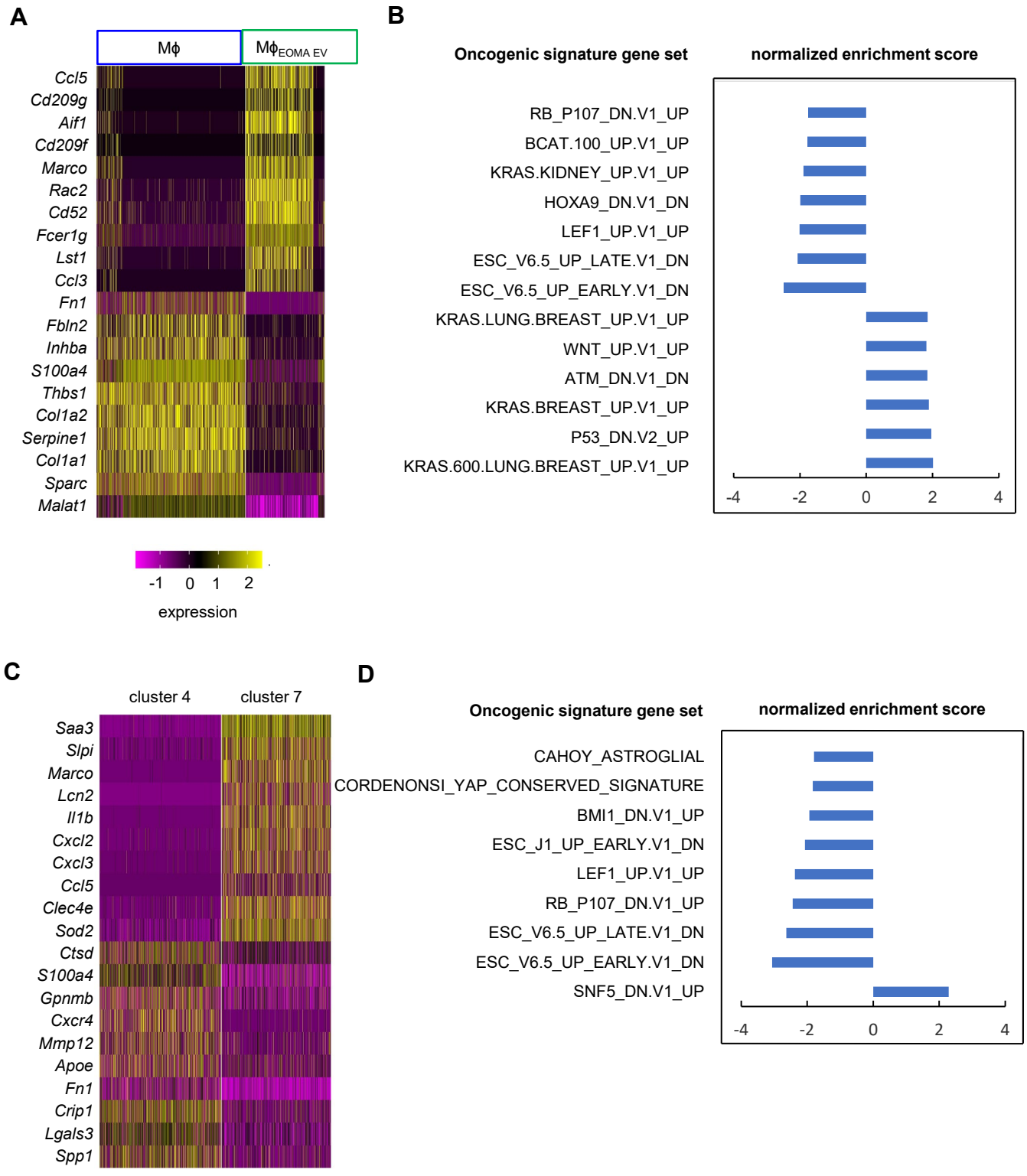

A
Mϕ
Mϕ EOMA EV
Ccl5
Cd209g
Aif1
Cd209f
Marco
Rac2
Cd52
Fcer1g
Lst1
Ccl3
Fn1
Fbln2
Inhba
S100a4
Thbs1
Col1a2
Serpine1
Col1a1
Sparc
Malat1
-1 0 1 2
expression
B
Oncogenic signature gene set
normalized enrichment score
RB_P107_DN.V1_UP
BCAT.100_UP.V1_UP
KRAS.KIDNEY_UP.V1_UP
HOXA9_DN.V1_DN
LEF1_UP.V1_UP
ESC_V6.5_UP_LATE.V1_DN
ESC_V6.5_UP_EARLY.V1_DN
KRAS.LUNG.BREAST_UP.V1_UP
WNT_UP.V1_UP
ATM_DN.V1_DN
KRAS.BREAST_UP.V1_UP
P53_DN.V2_UP
KRAS.600.LUNG.BREAST_UP.V1_UP
-4 -2 0 2 4
C
cluster 4
cluster 7
Saa3
Slpi
Marco
Lcn2
Il1b
Cxcl2
Cxcl3
Ccl5
Clec4e
Sod2
Ctsd
S100a4
Gpnmb
Cxcr4
Mmp12
Apoe
Fn1
Crip1
Lgals3
Spp1
D
Oncogenic signature gene set
normalized enrichment score
CAHOY_ASTROGLIAL
CORDENONSI_YAP_CONSERVED_SIGNATURE
BMI1_DN.V1_UP
ESC_J1_UP_EARLY.V1_DN
LEF1_UP.V1_UP
RB_P107_DN.V1_UP
ESC_V6.5_UP_LATE.V1_DN
ESC_V6.5_UP_EARLY.V1_DN
SNF5_DN.V1_UP
-4 -2 0 2 4

Figure S8

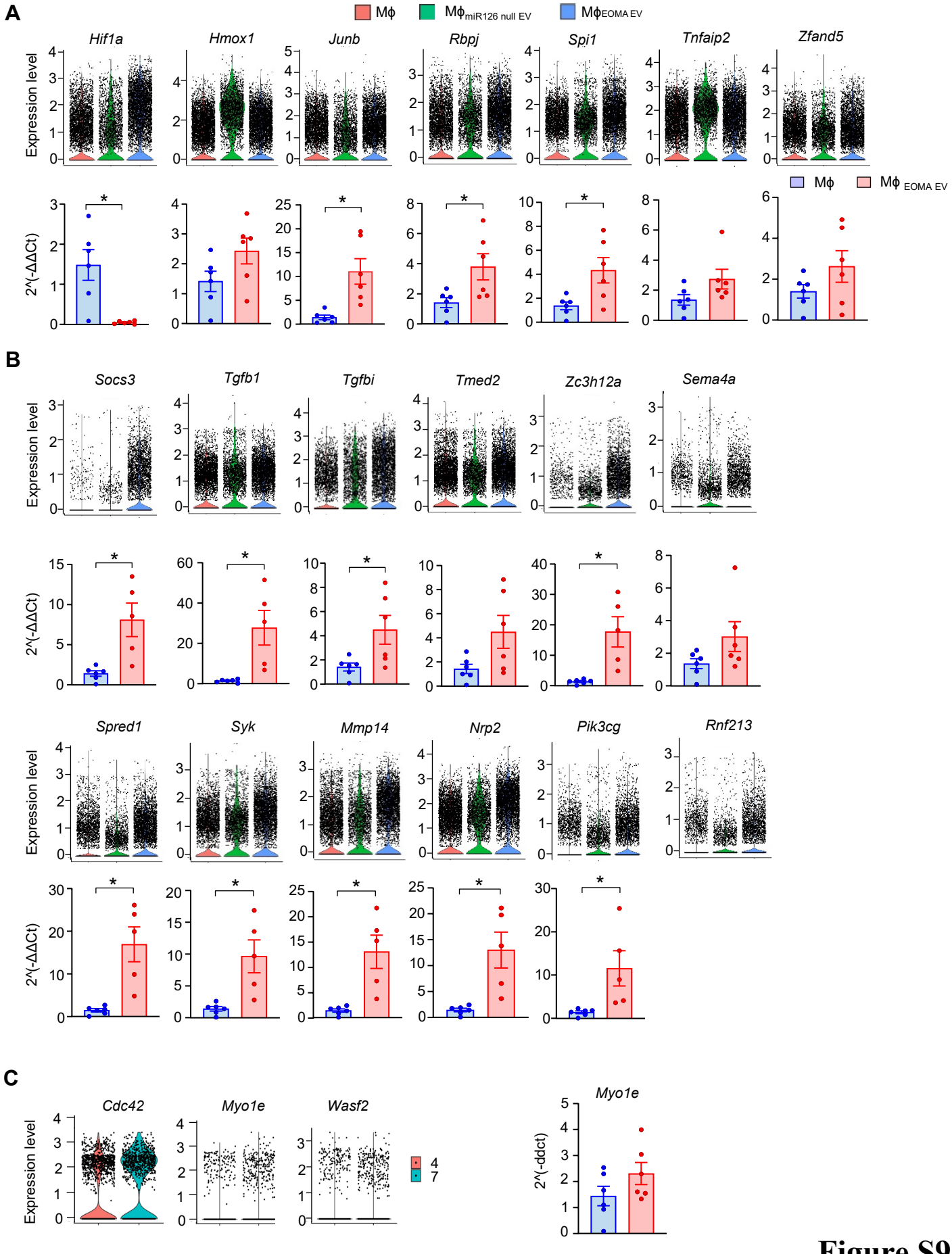

A
Mϕ
Mϕ miR126 null EV
Mϕ EOMA EV
Hif1a
Hmox1
Junb
Rbpj
Spi1
Tnfaip2
Zfand5
Expression level
2^(-ΔΔCt)
Mϕ
Mϕ EOMA EV
B
Socs3
Tgfb1
Tgfbi
Tmed2
Zc3h12a
Sema4a
Spred1
Syk
Mmp14
Nrp2
Pik3cg
Rnf213
C
Cdc42
Myo1e
Wasf2
4
7
Myo1e
2^(-ddct)

Figure S9

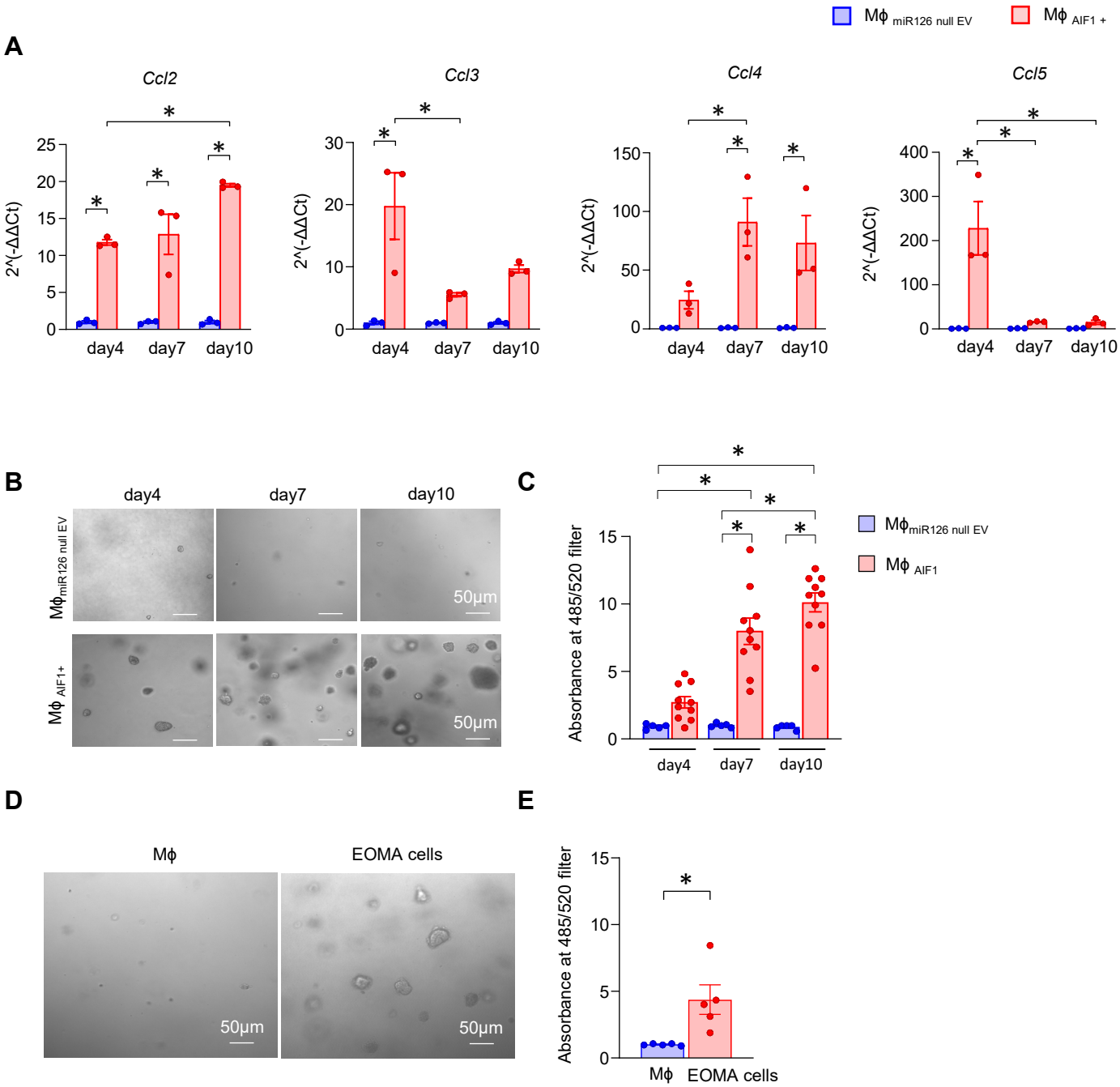

Mϕ miR126 null EV
Mϕ AIF1 +
A
Ccl2
Ccl3
Ccl4
Ccl5
2^(-ΔΔCt)
day4
day7
day10
B
day4
day7
day10
Mϕ miR126 null EV
Mϕ AIF1+
50μm
C
Absorbance at 485/520 filter
Mϕ miR126 null EV
Mϕ AIF1
D
Mϕ
EOMA cells
50μm
E
Absorbance at 485/520 filter
Mϕ
EOMA cells

Figure S10

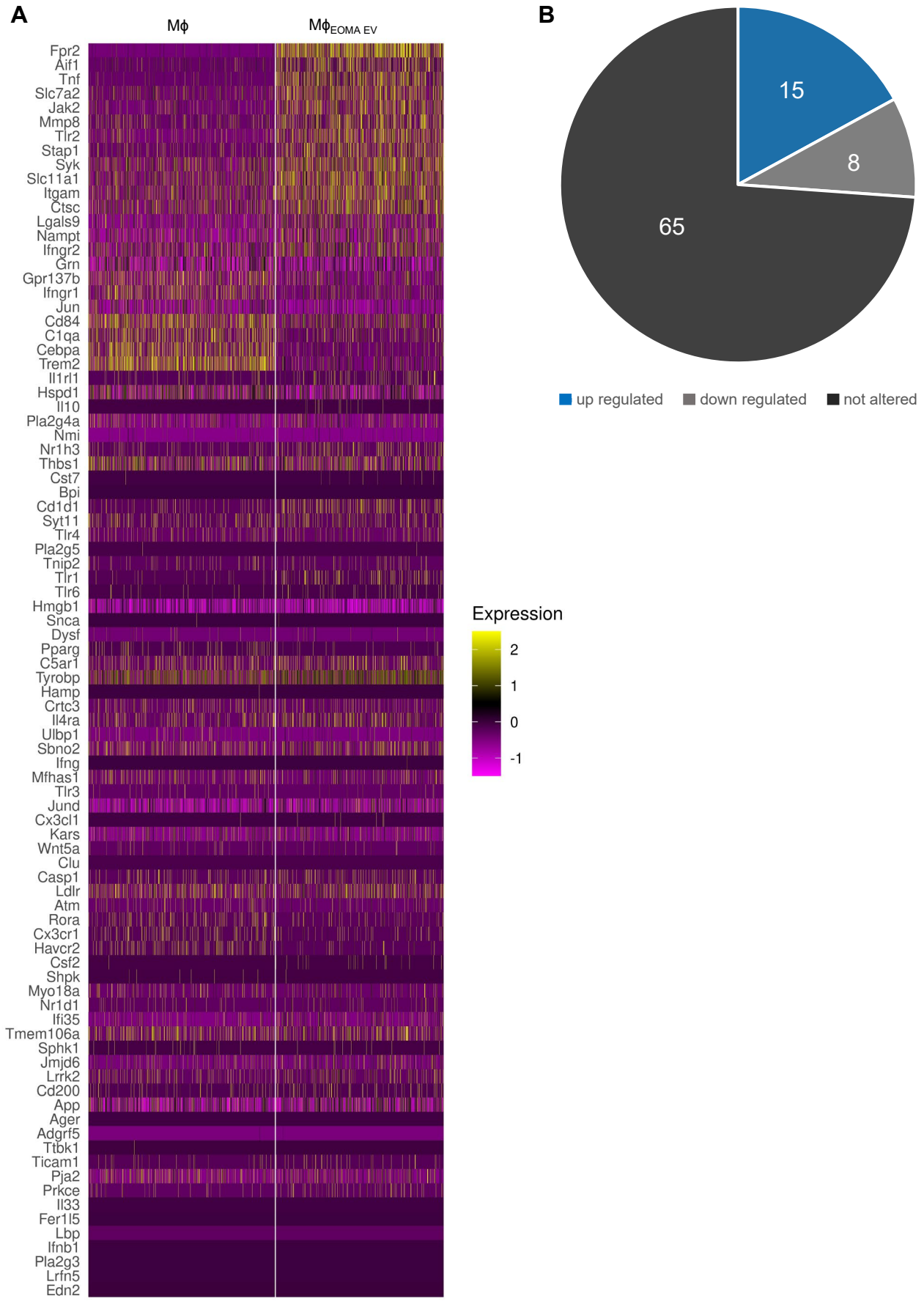

A
Mϕ
Mϕ EOMA EV
Fpr2
Aif1
Tnf
Slc7a2
Jak2
Mmp8
Tlr2
Stap1
Syk
Slc11a1
Itgam
Ctsc
Lgals9
Nampt
Ifngr2
Grn
Gpr137b
Ifngr1
Jun
Cd84
C1qa
Cebpa
Trem2
Il1rl1
Hspd1
Il10
Pla2g4a
Nmi
Nr1h3
Thbs1
Cst7
Bpi
Cd1d1
Syt11
Tlr4
Pla2g5
Tnip2
Tlr1
Tlr6
Hmgb1
Snca
Dysf
Pparg
C5ar1
Tyrobp
Hamp
Crtc3
Il4ra
Ulbp1
Sbno2
Ifng
Mfhas1
Tlr3
Jund
Cx3cl1
Kars
Wnt5a
Clu
Casp1
Ldlr
Atm
Rora
Cx3cr1
Havcr2
Csf2
Shpk
Myo18a
Nr1d1
Ifi35
Tmem106a
Sphk1
Jmjd6
Lrrk2
Cd200
App
Ager
Adgrf5
Ttbk1
Ticam1
Pja2
Prkce
Il33
Fer1l5
Lbp
Ifnb1
Pla2g3
Lrfn5
Edn2
Expression
2
1
0
-1
B
15
8
65
up regulated
down regulated
not altered

Figure S11

Supplementary Tables:

Sup Table 1: Number of cells in each cluster in the samples analyzed for single cell RNA sequencing

Sup Table 2: Top 10 markers for each cluster identified in single cell RNA seq data

Sup Table 3: Differentially expressed genes in MϕEOMA EV compared to Mϕ

Sup Table 4: Differentially expressed genes in Cluster 7 vs Cluster 4

Sup Table 5: Gene Set Enrichment Analysis (GSEA) between Mϕ and MϕEOMA EV

Sup Table 6: Vascular development genes analyzed between samples