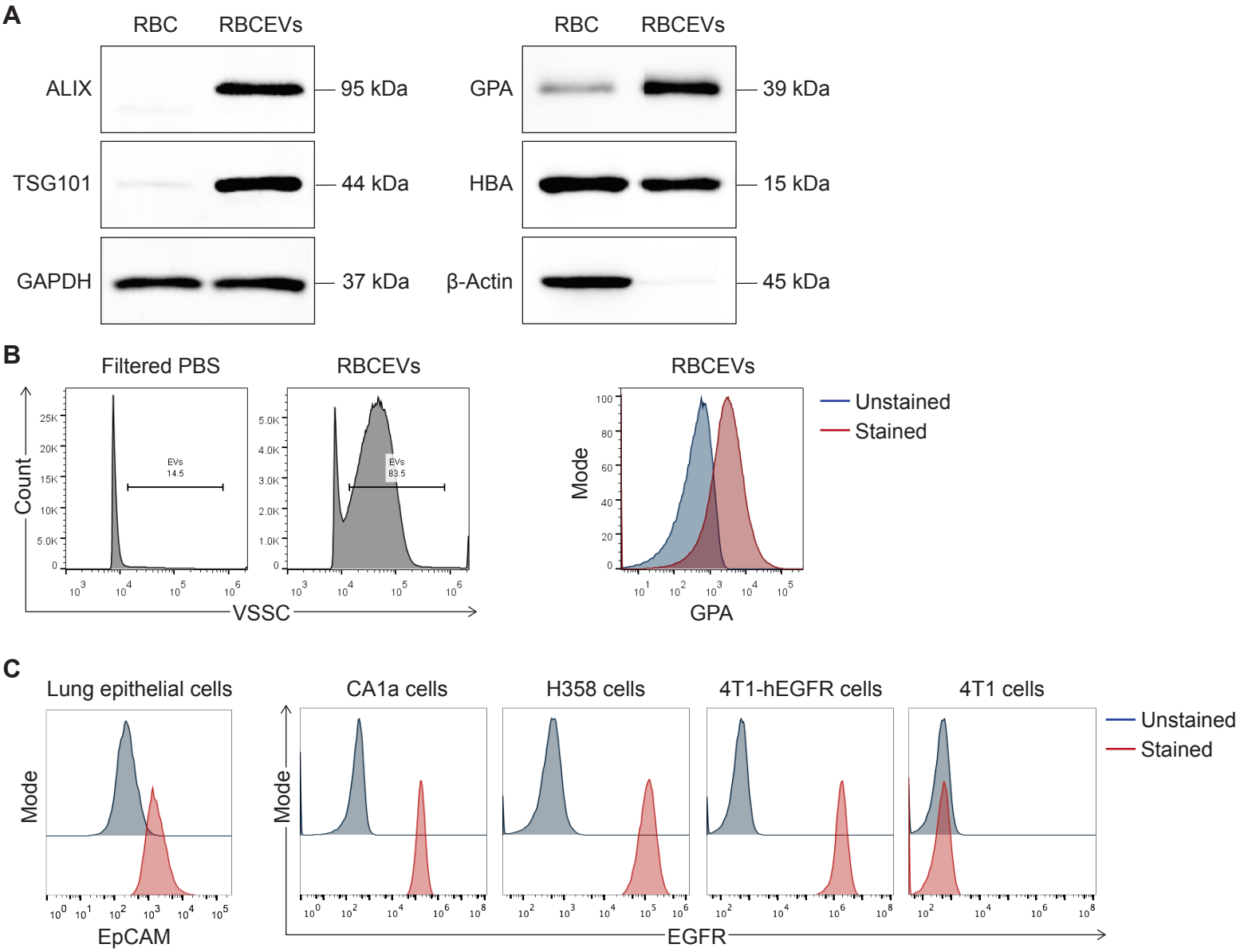


Figure S1| Characterization of RBCEVs, mouse lung epithelial cells and α-EGFR-VHH binding affinity
(A) Western blot of ALIX, TSG101, GPA, HBA, β-actin and GAPDH in RBCs and RBCEVs. **(B)** Single-EV flow cytometry analysis of GPA in RBCEVs with a gating strategy to obtain a distinct population of RBCEVs from the background noise. **(C)** Flow cytometry analysis of EpCAM expression in mouse lung epithelial cells and α-EGFR-VHH binding affinity to multiple cells.

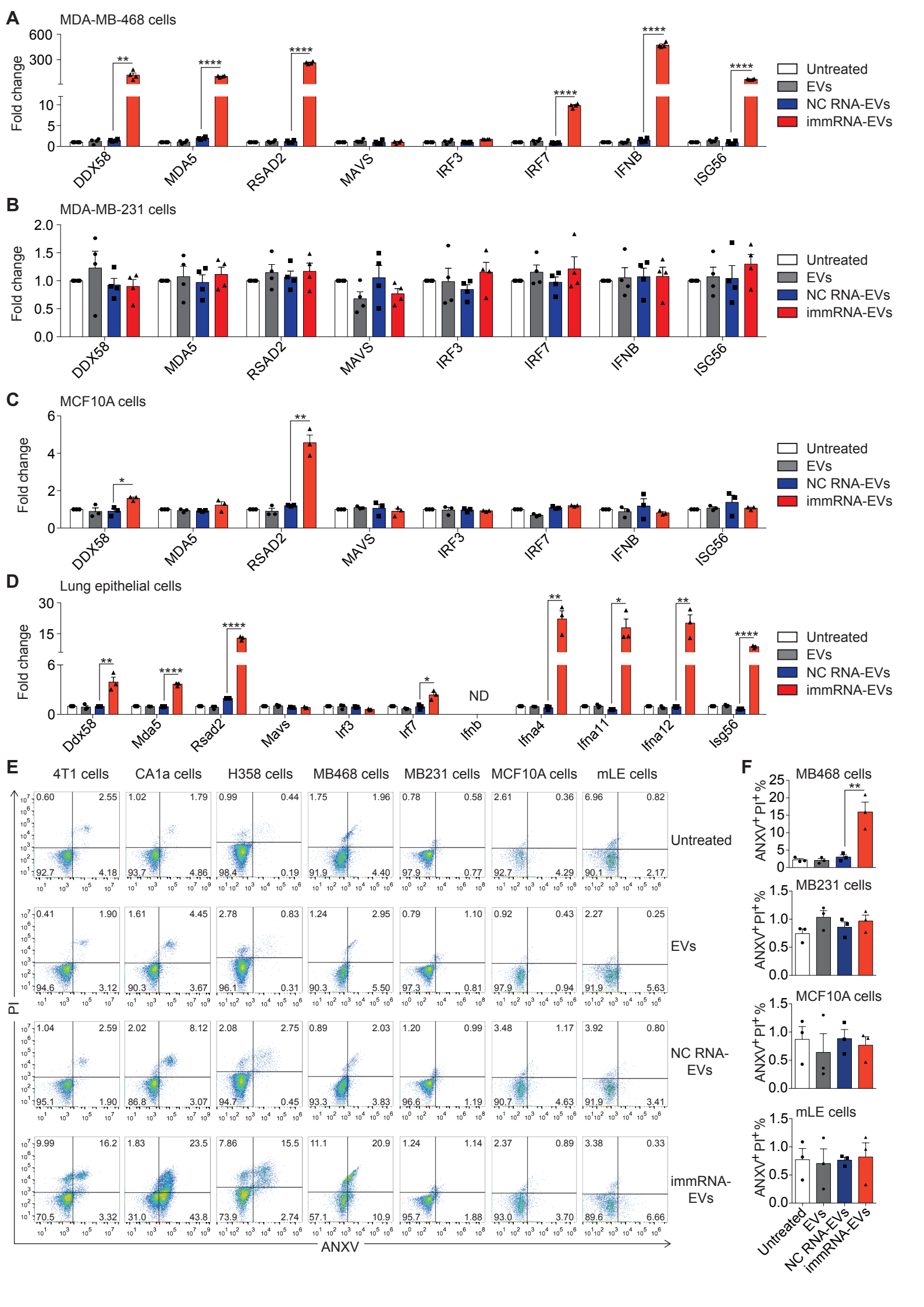

A
MDA-MB-468 cells
Fold change
600
300
10
5
0
DDX58
MDA5
RSAD2
MAVS
IRF3
IRF7
IFNB
ISG56
Untreated
EVs
NC RNA-EVs
immRNA-EVs
B
MDA-MB-231 cells
2.0
1.5
1.0
0.5
0.0
C
MCF10A cells
6
4
2
0
D
Lung epithelial cells
30
15
6
0
Ddx58
Mda5
Rsad2
Mavs
Irf3
Irf7
Ifnb
ND
Ifna4
Ifna11
Ifna12
Isg56
E
4T1 cells
CA1a cells
H358 cells
MB468 cells
MB231 cells
MCF10A cells
mLE cells
Untreated
EVs
NC RNA-EVs
immRNA-EVs
PI
ANXV
F
MB468 cells
MB231 cells
MCF10A cells
mLE cells
ANXV+PI+ %
Untreated
EVs
NC RNA-EVs
immRNA-EVs

Figure S2| The effects of immRNA-loaded RBCEVs on cancer cells and non-malignant cells

(A-D) qPCR analysis of *DDX58* and its downstream effectors relative to *GAPDH* in MDA-MB-468 (A), MDA-MB-231 (B), MCF10A (C) and mouse lung epithelial cells (D) treated with 0.1 μg/μL unloaded or NC-RNA-loaded, immRNA-loaded RBCEVs for 24 hours (n = 3-4, RNA loaded using REG1) ND, not detected. **(E)** Representative flow cytometric plots of ANXV/PI staining in 4T1, CA1a, H358, MDA-MB-468 (MB468), MDA-MB-231 (MB231), MCF10A and mouse lung epithelial (mLE) cells treated with 0.1 μg/μL unloaded RBCEVs, NC RNA-loaded RBCEVs and immRNA-loaded RBCEVs for 72 hours. **(F)** Average percentage of $ANXV^{+}PI^{+}$ population in MB468, MB231, MCF10A and mLE cells treated with RBCEVs as in (E) (n = 3). All bar graphs represent mean ± SEM. **P* < 0.05, ***P* < 0.01 and *****P* < 0.0001 determined by Student's two-tailed *t*-test.

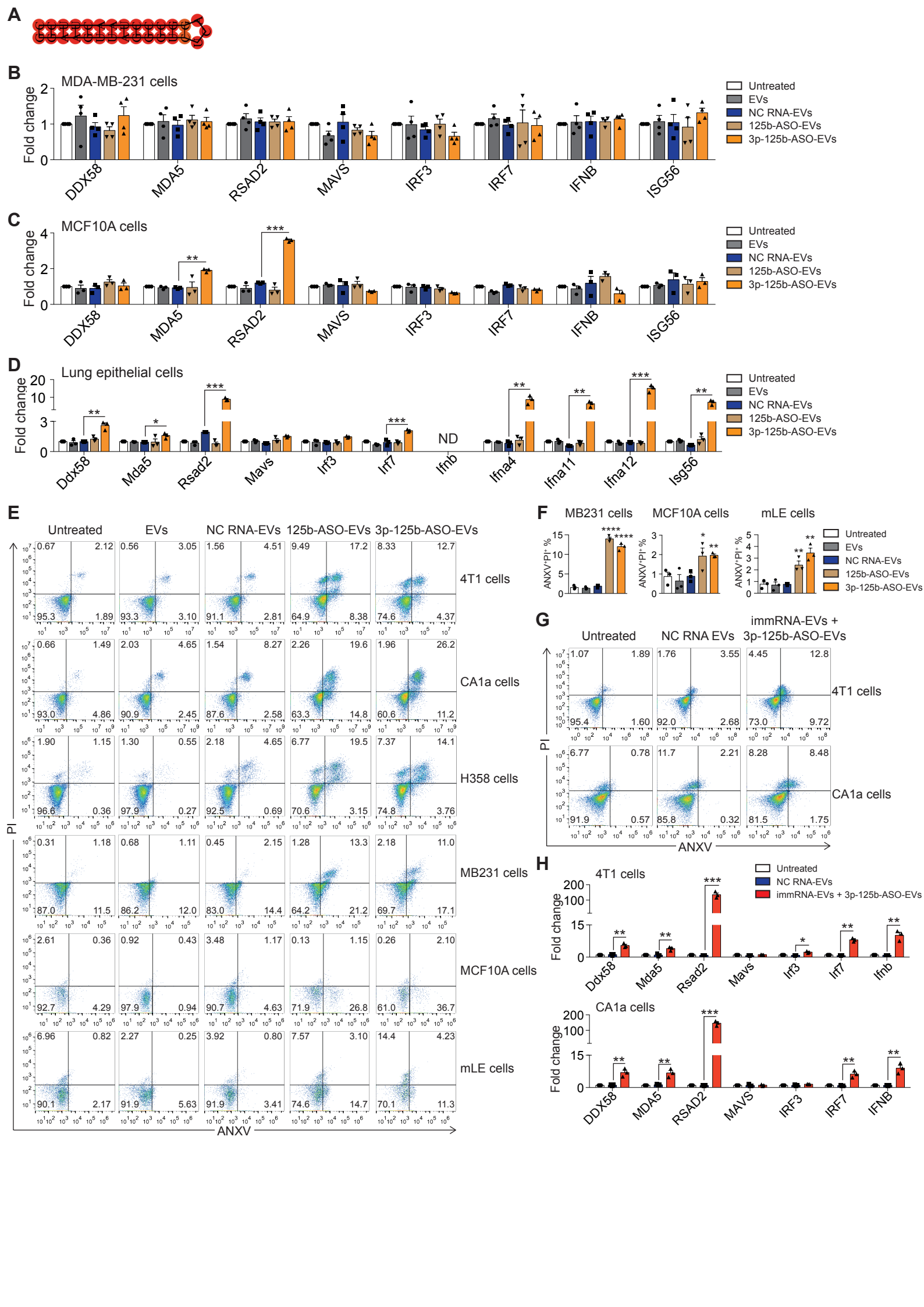

A
B
MDA-MB-231 cells
Fold change
Untreated
EVs
NC RNA-EVs
125b-ASO-EVs
3p-125b-ASO-EVs
DDX58
MDA5
RSAD2
MAVS
IRF3
IRF7
IFNB
ISG56
C
MCF10A cells
D
Lung epithelial cells
Ddx58
Mda5
Rsad2
Mavs
Irf3
Irf7
Ifnb
Ifna4
Ifna11
Ifna12
Isg56
ND
E
4T1 cells
CA1a cells
H358 cells
MB231 cells
MCF10A cells
mLE cells
PI
ANXV
F
MB231 cells
MCF10A cells
mLE cells
ANXV+PI+ %
G
immRNA-EVs +
3p-125b-ASO-EVs
NC RNA EVs
H
immRNA-EVs + 3p-125b-ASO-EVs

Figure S3| The effects of 3p-125b-ASO-loaded RBCEVs on cancer cells and non-malignant cells

(A) Secondary structure of 3p-125b-ASO. **(B-D)** qPCR analysis of *DDX58* and its downstream effectors relative to *GAPDH* in MDA-MB-231 (B), MCF10A (C) and mouse lung epithelial cells (D) treated with 0.1 μg/μL unloaded or NC-RNA-loaded, 125b-ASO-loaded and 3p-125b-ASO-loaded RBCEVs for 24 hours (n = 3-4, RNA loaded using REG1) ND, not detected. **(E)** Representative flow cytometric plots of ANXV/PI staining in 4T1, CA1a, H358, MDA-MB-231 (MB231), MCF10A and mouse lung epithelial (mLE) cells treated with 0.1 μg/μL unloaded or NC RNA-loaded, 125b-ASO-loaded and 3p-125b-ASO-loaded RBCEVs for 72 hours. **(F)** Average percentage of $ANXV^{+}PI^{+}$ population in MB231, MCF10A and mLE cells treated with RBCEVs as in (E) (n = 3). **(G)** Representative flow cytometric plots of ANXV/PI staining in 4T1 cells and CA1a cells after a treatment with combined 3p-125-ASO- and immRNA-loaded RBCEVs for 72 hours. **(H)** qPCR analysis of RIG-I pathway gene expression in 4T1 cells and CA1a cells after a treatment with combined 3p-125-ASO- and immRNA-loaded RBCEVs for 24 hours. All bar graphs represent mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001 determined by Student's two-tailed *t*-test.

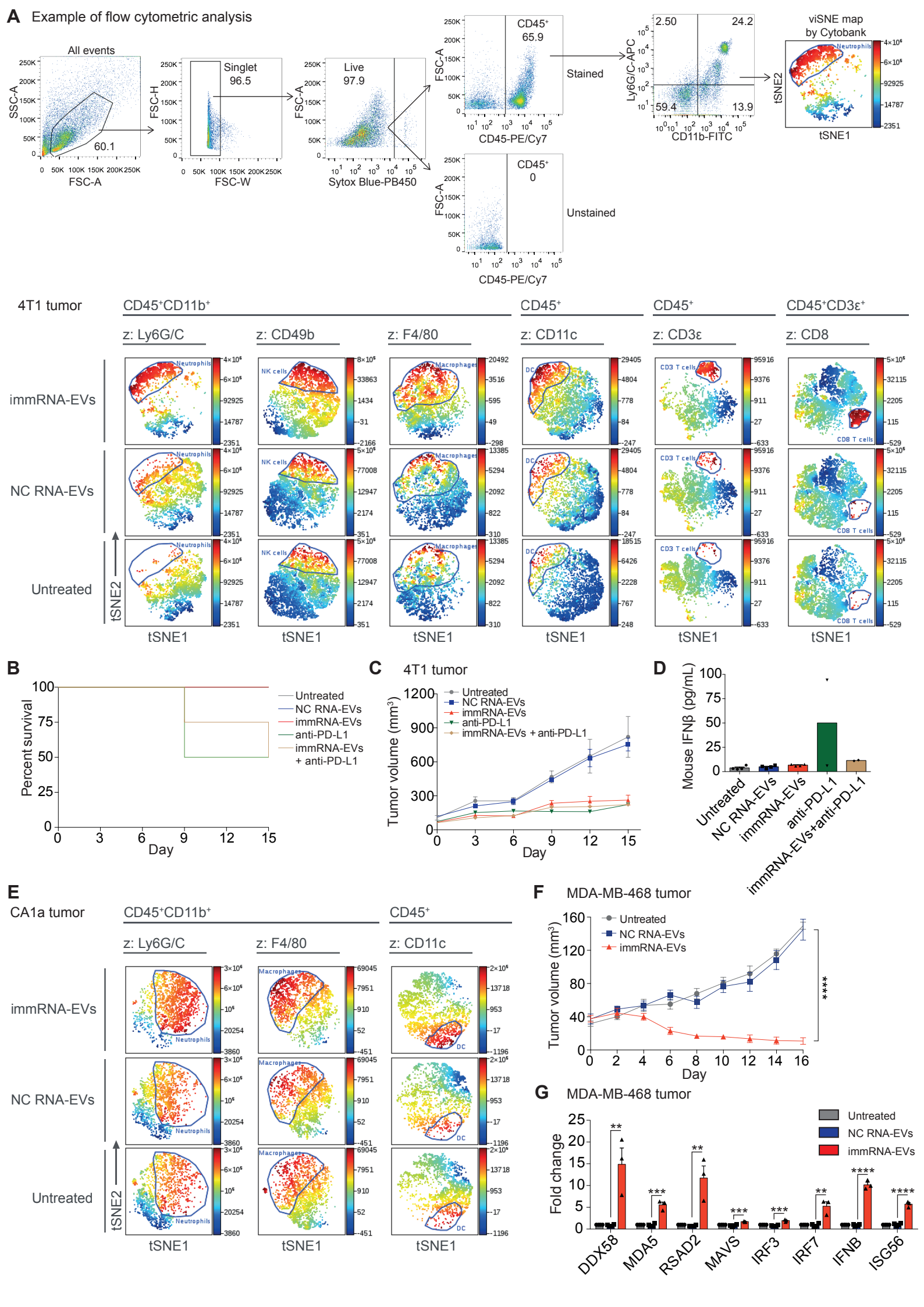

A
Example of flow cytometric analysis
All events
SSC-A
FSC-A
60.1
Singlet
96.5
FSC-H
FSC-W
Live
97.9
Sytox Blue-PB450
CD45+
65.9
CD45-PE/Cy7
Stained
2.50
24.2
59.4
13.9
Ly6G/C-APC
CD11b-FITC
viSNE map
by Cytobank
Neutrophils
tSNE2
tSNE1
CD45+
0
Unstained
4T1 tumor
CD45+CD11b+
CD45+
CD45+
CD45+CD3ε+
z: Ly6G/C
z: CD49b
z: F4/80
z: CD11c
z: CD3ε
z: CD8
immRNA-EVs
NC RNA-EVs
Untreated
Neutrophils
NK cells
Macrophages
DC
CD3 T cells
CD8 T cells
B
Percent survival
Day
Untreated
NC RNA-EVs
immRNA-EVs
anti-PD-L1
immRNA-EVs
+ anti-PD-L1
C
4T1 tumor
Tumor volume (mm3)
Day
Untreated
NC RNA-EVs
immRNA-EVs
anti-PD-L1
immRNA-EVs + anti-PD-L1
D
Mouse IFNβ (pg/mL)
Untreated
NC RNA-EVs
immRNA-EVs
anti-PD-L1
immRNA-EVs+anti-PD-L1
E
CA1a tumor
CD45+CD11b+
CD45+
z: Ly6G/C
z: F4/80
z: CD11c
immRNA-EVs
NC RNA-EVs
Untreated
tSNE2
tSNE1
Neutrophils
Macrophages
DC
F
MDA-MB-468 tumor
Tumor volume (mm3)
Day
Untreated
NC RNA-EVs
immRNA-EVs
****
G
MDA-MB-468 tumor
Fold change
Untreated
NC RNA-EVs
immRNA-EVs
DDX58
MDA5
RSAD2
MAVS
IRF3
IRF7
IFNB
ISG56

Figure S4| Therapeutic efficacy of immRNA-loaded RBCEVs in mammary tumors

(A) Flow cytometry analysis of immune cells in 4T1 mammary tumors treated with immRNA-loaded RBCEVs as in Figure 4. Cells were gated based on FSC-A and SSC-A to exclude the debris and dead cells. The single cells were further gated based on FSC-W and FSC-H to exclude doublets and aggregates. The live cells were gated from singlet population base on Sytox™ Blue negative. Subsequently, the fluorescence of phenotypic markers for immune cells were gated based on the fluorescent channels and subjected to viSNE analysis. **(B)** Survival of mice with orthotopic 4T1 tumors treated with 2.5 mg/kg NC-RNA-RBCEVs (i.t.), 2.5 mg/kg immRNA-RBCEVs (i.t.), 2 mg/kg anti-PD-L1 antibody (i.p.) as well as 2.5 mg/kg immRNA-RBCEVs (i.t.) in combination with 2 mg/kg anti-PD-L1 antibody (i.p.) (n = 2-4 mice). **(C)** Tumor growth of mice treated as in (B) (n = 2-4 mice). **(D)** ELISA quantification of IFNβ in the sera of mice bearing 4T1 tumors treated as in (B) (n = 2-4 mice). **(E)** Representative viSNE plots of immune cells in CA1a mammary tumors treated with immRNA-loaded RBCEVs as in Figure 4. **(F)** Volume of MDA-MB-468 tumors injected intratumorally with 2.5 mg/kg RBCEVs containing immRNA or NC RNA every three days (n = 3-4 mice). **(G)** qPCR analysis of the RIG-I pathway gene expression relative to *GAPDH* in untreated and treated MDA-MB-468 tumors (n = 3-4 mice). All bar graphs represent mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ determined by Student's two-tailed *t*-test.

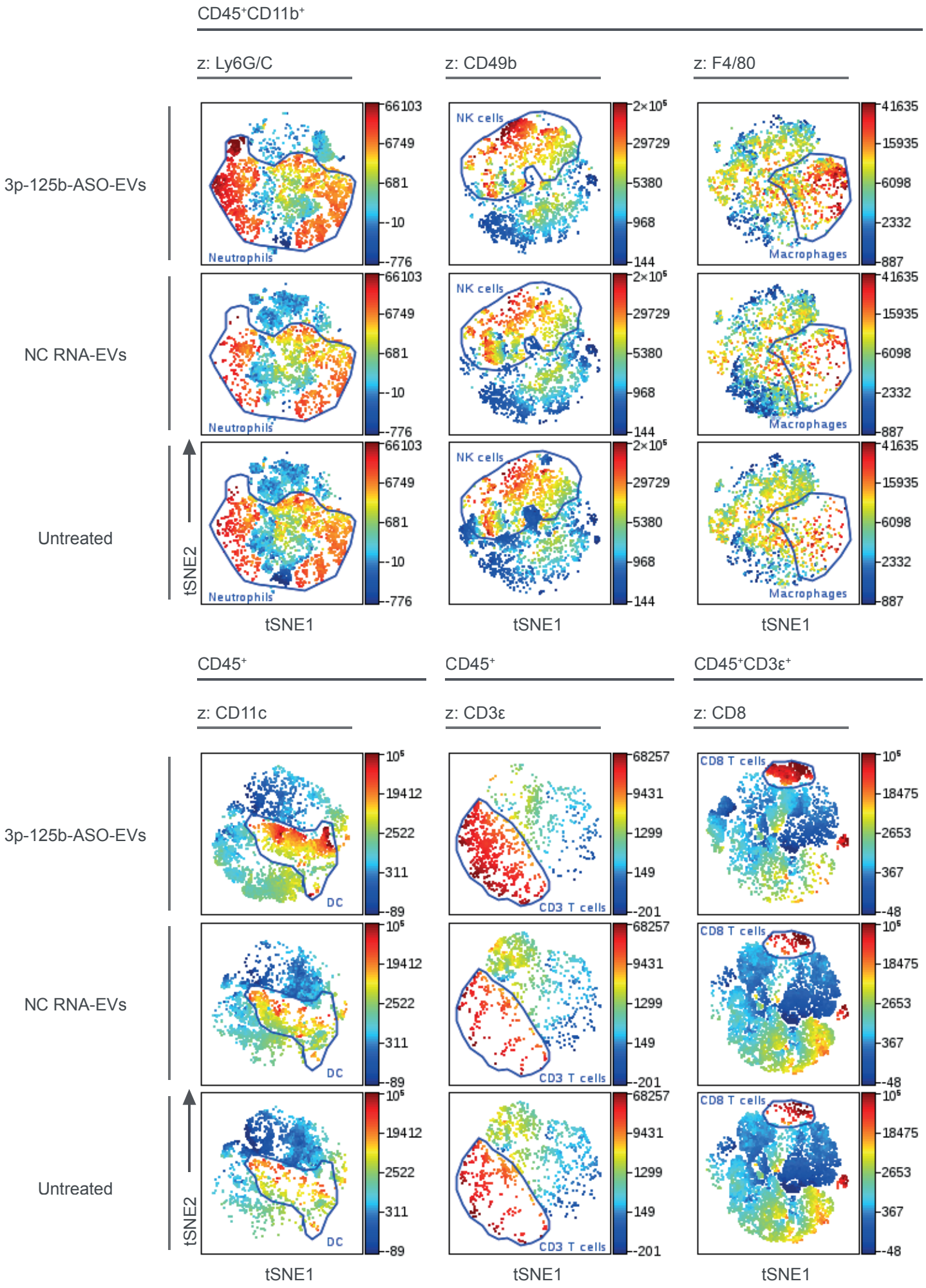


Figure S5| Flow cytometry analysis of immune cells in 4T1 mammary tumors treated with 3p-125b-ASO-loaded RBCEVs
Representative viSNE plots of immune cells in 4T1 mammary tumors treated with 3p-125b-ASO-loaded RBCEVs.

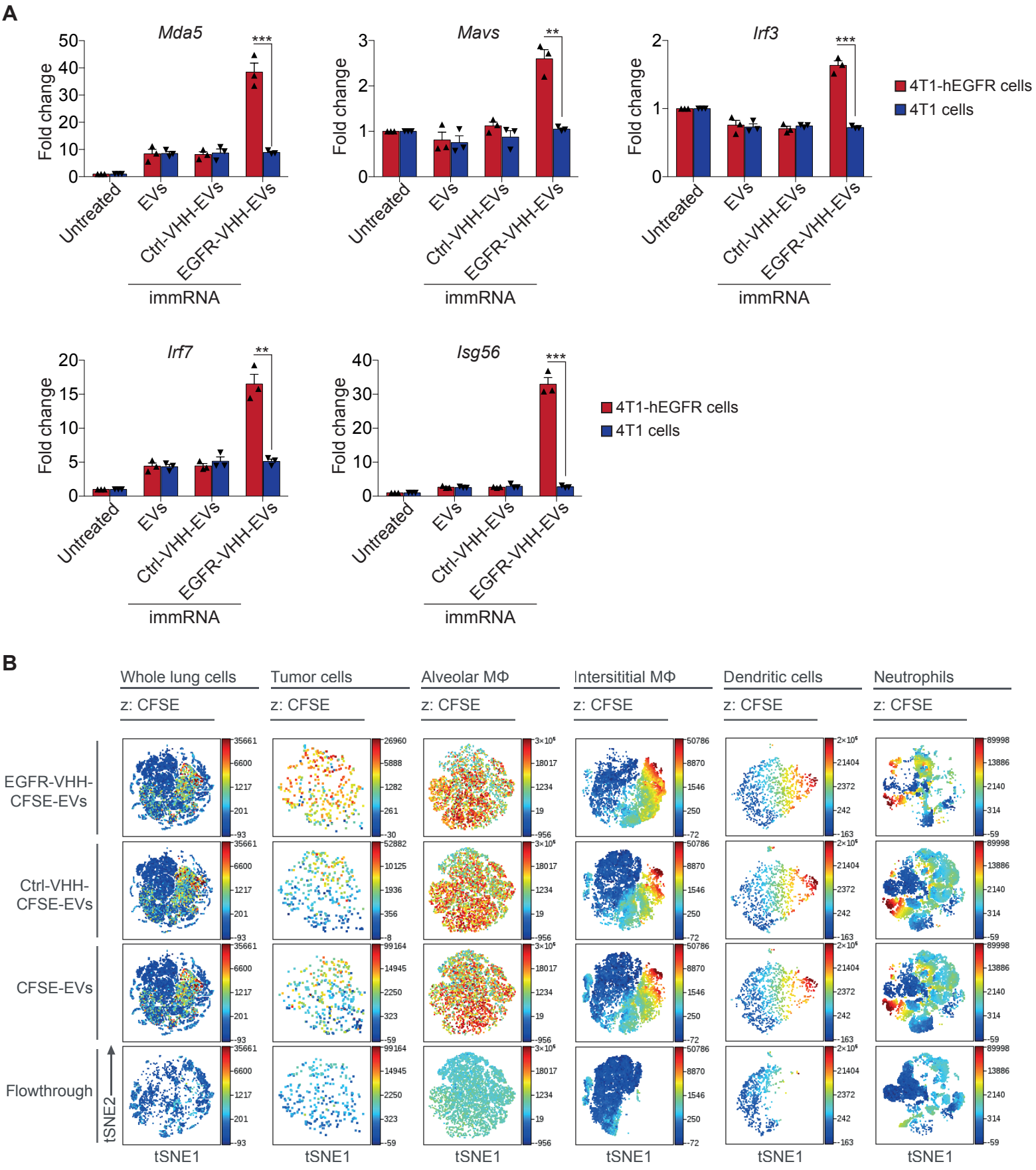
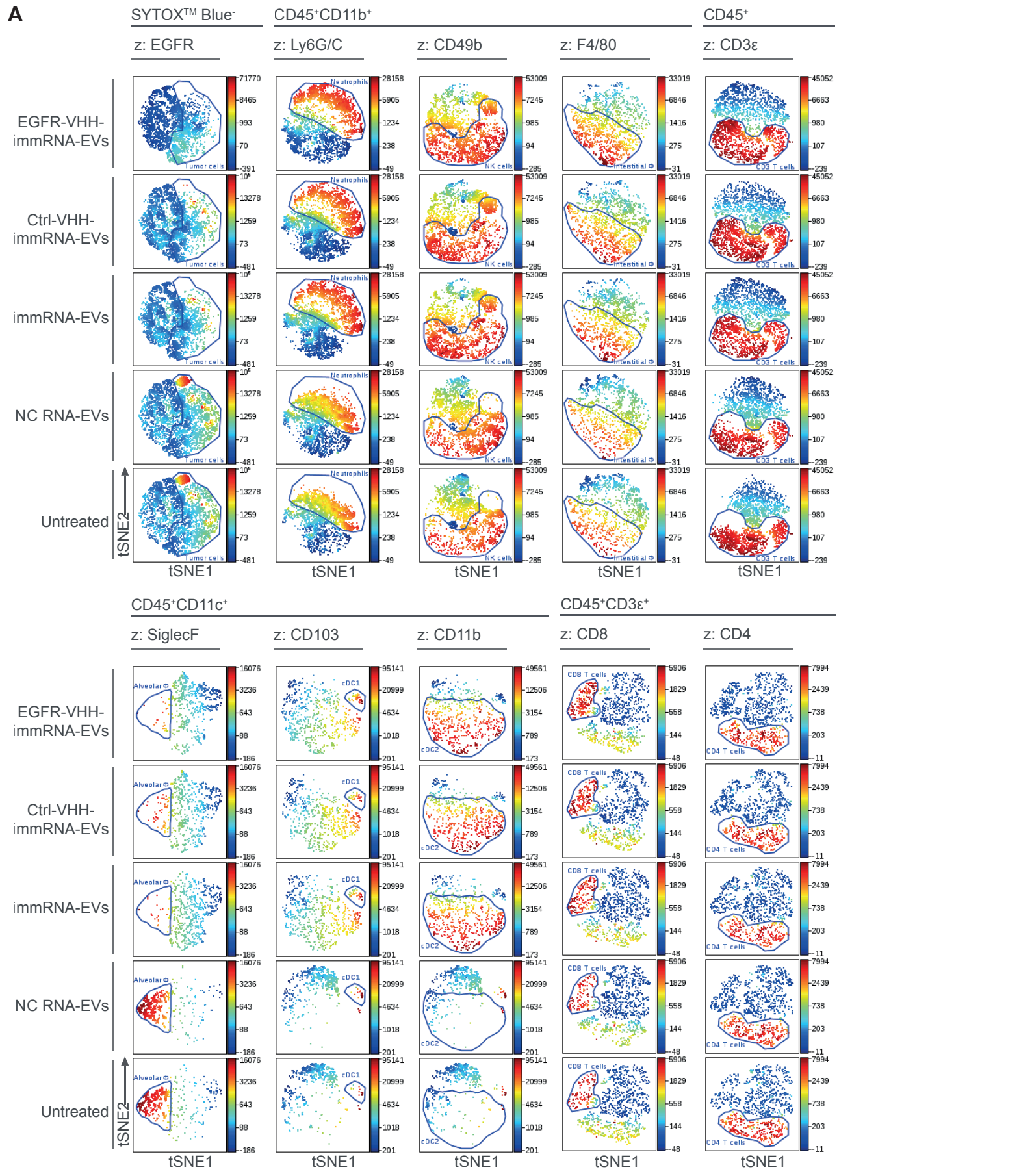


Figure S6| EGFR-VHH-RBCEVs target 4T1-hEGFR cells *in vitro* and 4T1-hEGFR lung metastatic tumors *in vivo* **(A)** qPCR analysis of the RIG-I pathway gene expression relative to *Gapdh* in 4T1-hEGFR cells and parental 4T1 cells treated with 0.1 μg/μL uncoated, Ctrl-VHH-coated and EGFR-VHH-coated RBCEVs containing immRNA (n = 3). All bar graphs represent mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ determined by Student's two-tailed *t*-test. **(B)** Representative viSNE plots of biodistribution of EGFR-VHH-coated CFSE-RBCEVs in respective cell type in the lungs with 4T1-hEGFR metastatic tumors.


A
SYTOX™ Blue-
CD45+CD11b+
CD45+
z: EGFR
z: Ly6G/C
z: CD49b
z: F4/80
z: CD3ε
EGFR-VHH-immRNA-EVs
Ctrl-VHH-immRNA-EVs
immRNA-EVs
NC RNA-EVs
Untreated
tSNE2
tSNE1
Tumor cells
Neutrophils
NK cells
Interstitial Φ
CD3 T cells
CD45+CD11c+
CD45+CD3ε+
z: SiglecF
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z: CD11b
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z: CD4
Alveolar Φ
cDC1
cDC2
CD8 T cells
CD4 T cells

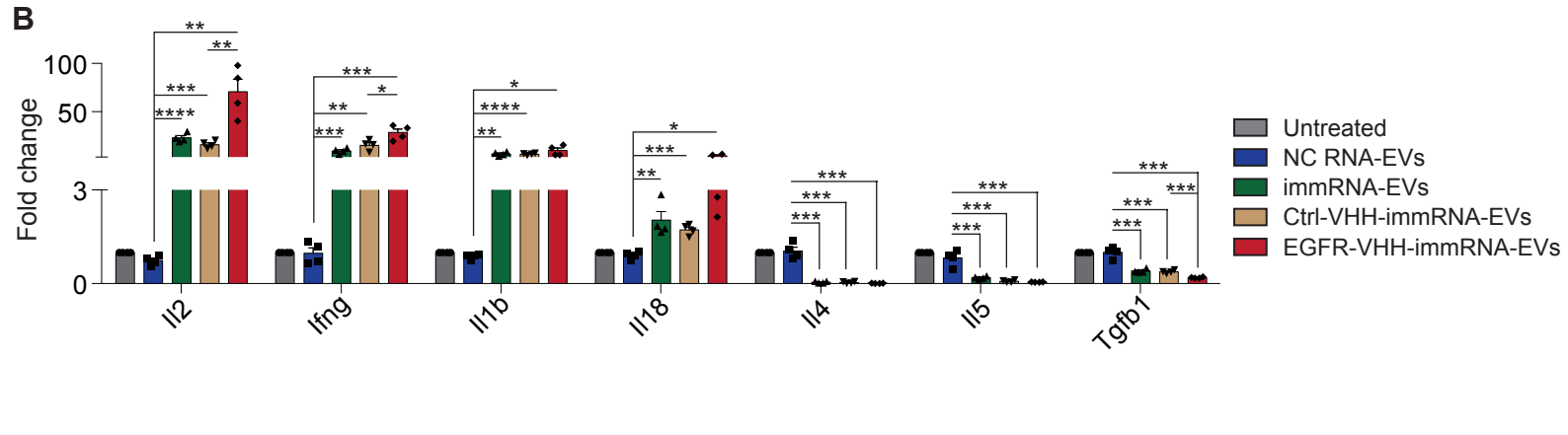
B
Fold change
100
50
3
0
Il2
Ifng
Il1b
Il18
Il4
Il5
Tgfb1
Untreated
NC RNA-EVs
immRNA-EVs
Ctrl-VHH-immRNA-EVs
EGFR-VHH-immRNA-EVs

Figure S7| Flow cytometry analysis of immune cells in the lungs with 4T1 metastatic tumors treated with EGFR-VHH-immRNA-RBCEVs

(A) Representative viSNE plots of respective cell type in the lungs with 4T1 metastatic tumors treated with EGFR-VHH-immRNA-RBCEVs. **(B)** qPCR analysis of cytokine gene expression relative to *Gapdh* in the lungs of mice with 4T1-hEGFR metastatic tumors (n = 4 mice). All bar graphs represent mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001 determined by Student's two-tailed *t*-test.

Table S1| List of primers

Genes	F/R	Mouse	Human
GAPDH	F	AGGTCGGTGTGAACGGATTTG	GGAGCGAGATCCCTCCAAAAT
	R	TGTAGACCATGTAGTTGAGGTCA	GGCTGTTGTCATACTTCTCATGG
DDX58	F	GAG AGT CAC GGG ACC CAC T	GCCATTACACTGTGCTTGGAGA
	R	CGG TCT TAG CAT CTC CAA CG	CCAGTTGCAATATCCTCCACCA
MDA5	F	TGATGCACTATTCCAAGAACTAAC	GAGCAACTTCTTTCAACCACAG
	R	TCTGTGAGACGAGTTAGCCAAG	CACTTCCTTCTGCCAAACTTG
RSAD2	F	ACAGCCAAGACATCCTTCGT	CACAAAGAAGTGTCCTGCTTGGT
	R	AAAAGTTGATCTTCTCCAAACCA	AAGCGCATATATTCATCCAGAAT
MAVS	F	CTGCCTCACAGCTAGTGACC	AGGAGACAGATGGAGACACA
	R	CCGGCGCTGGAGATTATTG	CAGAACTGGGCAGTACCC
IFNB	F	AACCTCACCTACAGGGCGGACTTC	CTCTCCTGTTGTGCTTCTCC
	R	TCCCACGTCAATCTTTCCTCTTGCT	GTCAAAGTTCATCCTGTCCTTG
IRF3	F	CGGAAAGAAGTGTTGCGGTT	ACCAGCCGTGGACCAAGAG
	R	TTTTCCTGGGAGTGAGGCAG	TACCAAGGCCCTGAGGCAC
IRF7	F	AGGGCGTTTTATCTTGCG	TGGTCCTGGTGAAGCTGGAA
	R	TGGAGCCCAGCATTTTCTCT	GATGTCGTCATAGAGGCTGTTGG
ISG56	F	TACAGGCTGGAGTGTGCTGAGA	TAGCCAACATGTCCTCACAGAC
	R	CTCCACTTTCAGAGCCTTCGCA	TCTTCTACCACTGGTTTCATGC
IL4	F	ATCATCGGCATTTTGAACGAGG	
	R	GCAGCTCCATGAGAACACTA	
IL5	F	CTCTGTTGACAAGCAATGAGACG	
	R	TCTTCAGTATGTCTAGCCCCTG	
IFNG	F	GCCACGGCACAGTCATTGA	
	R	TGCTGATGGCCTGATTGTCTT	
IL2	F	GTGCTCCTTGTCAACAGCG	
	R	GGGGAGTTTCAGGTTCCTGTA	
TGFB1	F	CTTCAATACGTCAGACATTCGGG	
	R	GTAACGCCAGGAATTGTTGCTA	
IL1B	F	GCAACTGTTCCTGAACTCAACT	
	R	ATCTTTTGGGGTCCGTCAACT	
IL18	F	GACTCTTGCGTCAACTTCAAGG	
	R	CAGGCTGTCTTTTGTCAACGA	
IL10	F	AGCCTTATCGGAAATGATCCAGT	
	R	GGCCTTGTAGACACCTTGGT	
IL6	F	TCTATACCACTTCACAAGTCGGA	
	R	TCTATACCACTTCACAAGTCGGA	
IL12B	F	TGGTTTGCCATCGTTTTGCTG	
	R	ACAGGTGAGGTTCACTGTTTCT	

TNF	F	CCTGTAGCCCACGTCGTAG
	R	GGGAGTAGACAAGGTACAACCC
IFNA4	F	CTGGTAATGATGAGCTACTACTG G
	R	CCTTCTCCAAGGGGAATCCAA
IFNA11	F	GGTCCTGGCACAAATGAGGA
	R	TCCAAGCAGCAGATGAGTCC
IFNA12	F	AAGACTGAGTGAGAAGGAGTGAG
	R	GAGATGCCAGAATTTGAGCAGTG