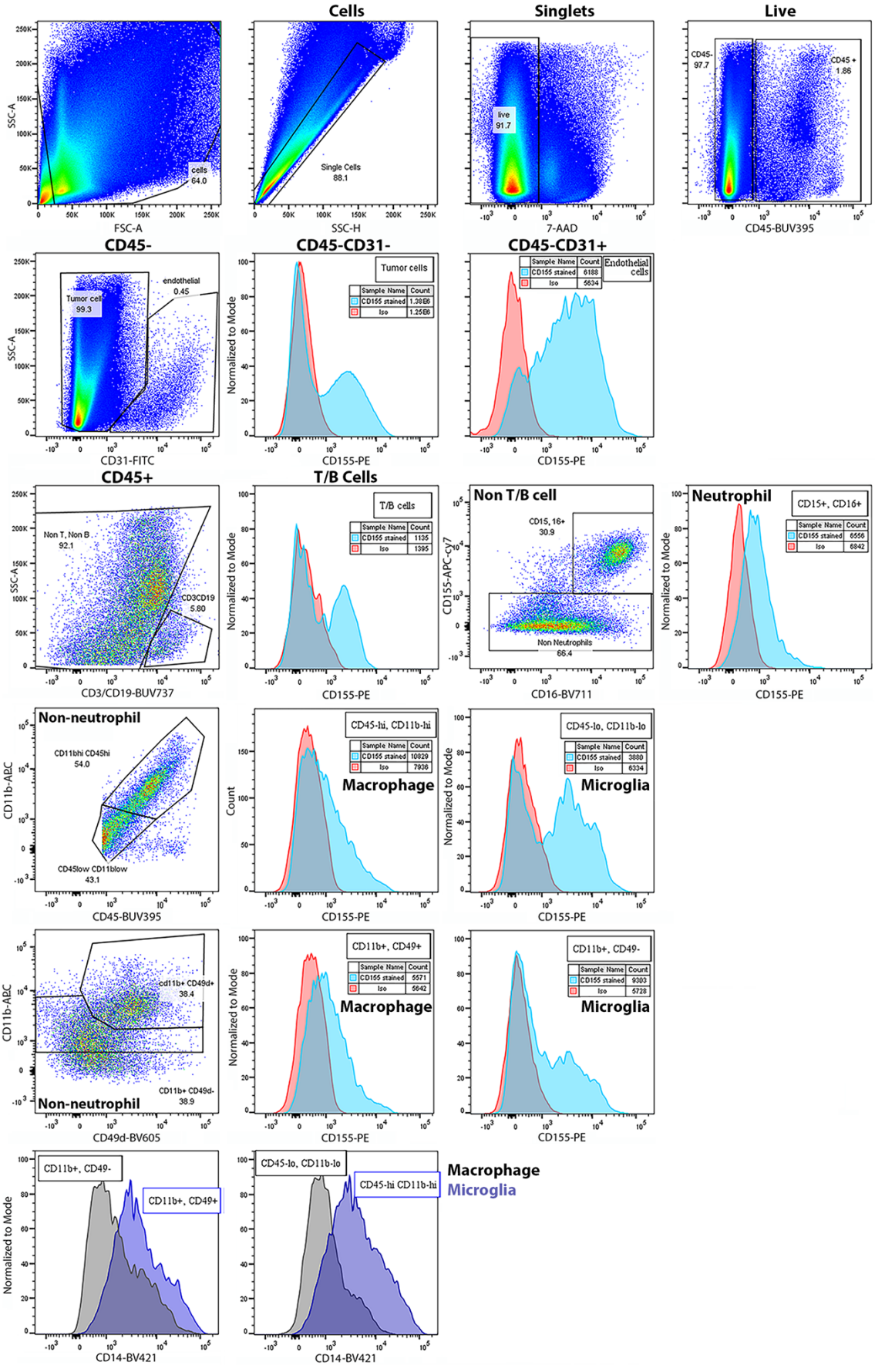
Supplementary Information

Viral Infection of the Tumor Microenvironment Mediates Antitumor Immunotherapy via Selective TBK1-IRF3 Signaling

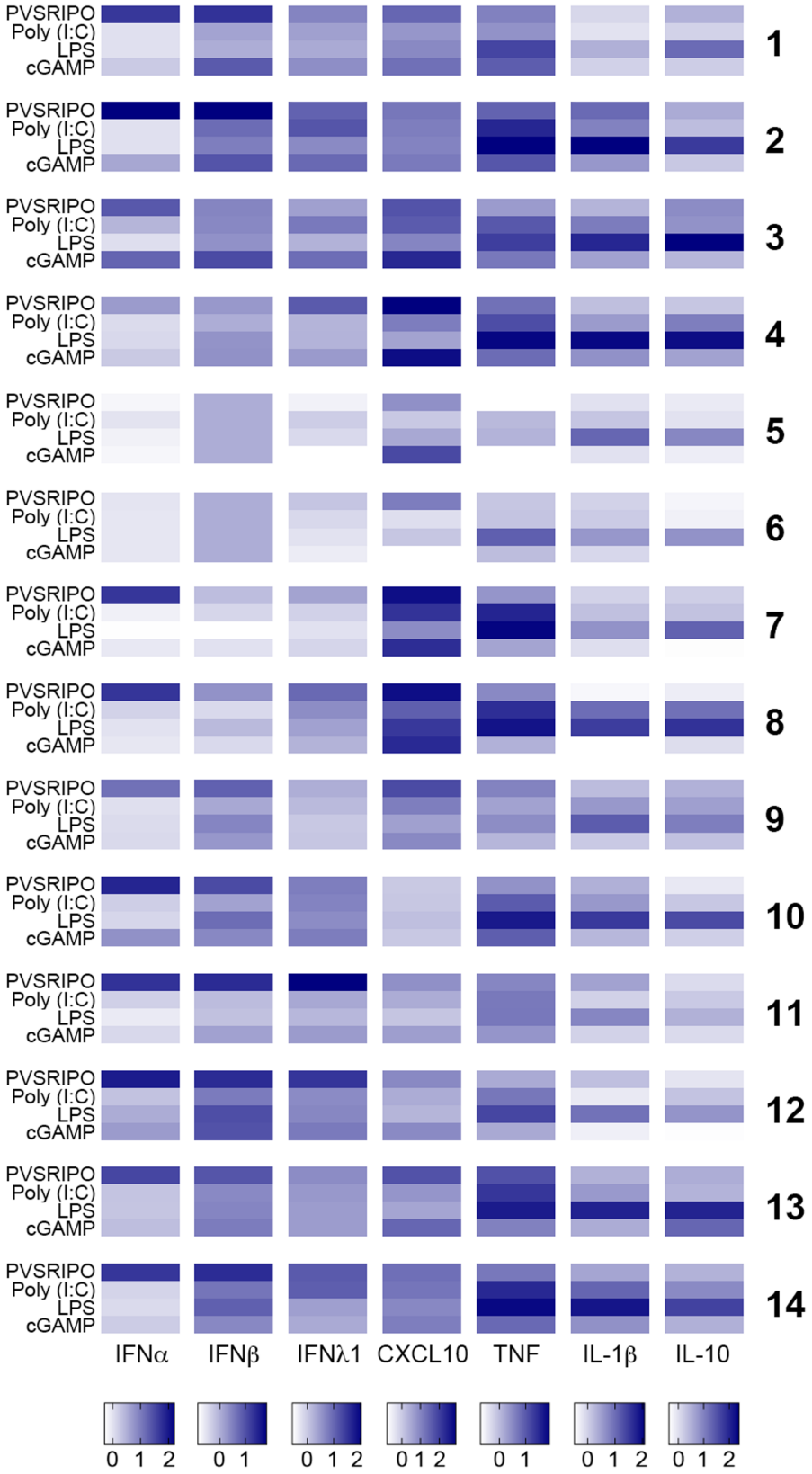
Brown *et al.*


Cells
Singlets
Live
cells
64.0
Single Cells
88.1
live
91.7
CD45-
97.7
CD45 +
1.86
FSC-A
SSC-A
SSC-H
7-AAD
CD45-BUV395
CD45-
CD45-CD31-
CD45-CD31+
Tumor cells
endothelial
0.45
Tumor cells
99.3
Sample Name | Count
CD155 stained | 1.38E6
Iso | 1.25E6
Endothelial cells
CD155 stained | 6188
Iso | 5634
CD31-FITC
Normalized to Mode
CD155-PE
CD45+
T/B Cells
Non T/B cell
Neutrophil
Non T, Non B
92.1
CD3CD19
5.80
T/B cells
CD155 stained | 1135
Iso | 1395
CD15, 16+
30.9
Non Neutrophils
66.4
CD15+, CD16+
CD155 stained | 6556
Iso | 6842
CD3/CD19-BUV737
CD155-APC-cy7
CD16-BV711
Non-neutrophil
CD11bhi CD45hi
54.0
CD45low CD11blow
43.1
CD11b-APC
CD45-hi, CD11b-hi
CD155 stained | 10829
Iso | 7936
Macrophage
CD45-lo, CD11b-lo
CD155 stained | 3880
Iso | 6334
Microglia
Count
cd11b+ CD49d+
38.4
CD11b+ CD49d-
38.9
CD11b+, CD49+
CD155 stained | 5571
Iso | 5642
CD11b+, CD49-
CD155 stained | 9303
Iso | 5728
CD49d-BV605
CD14-BV421
Macrophage
Microglia

Supplementary Figure 1, related to Figure 1b. Representative gating strategy for analysis of CD155 expression distribution in GBM tumor specimens.

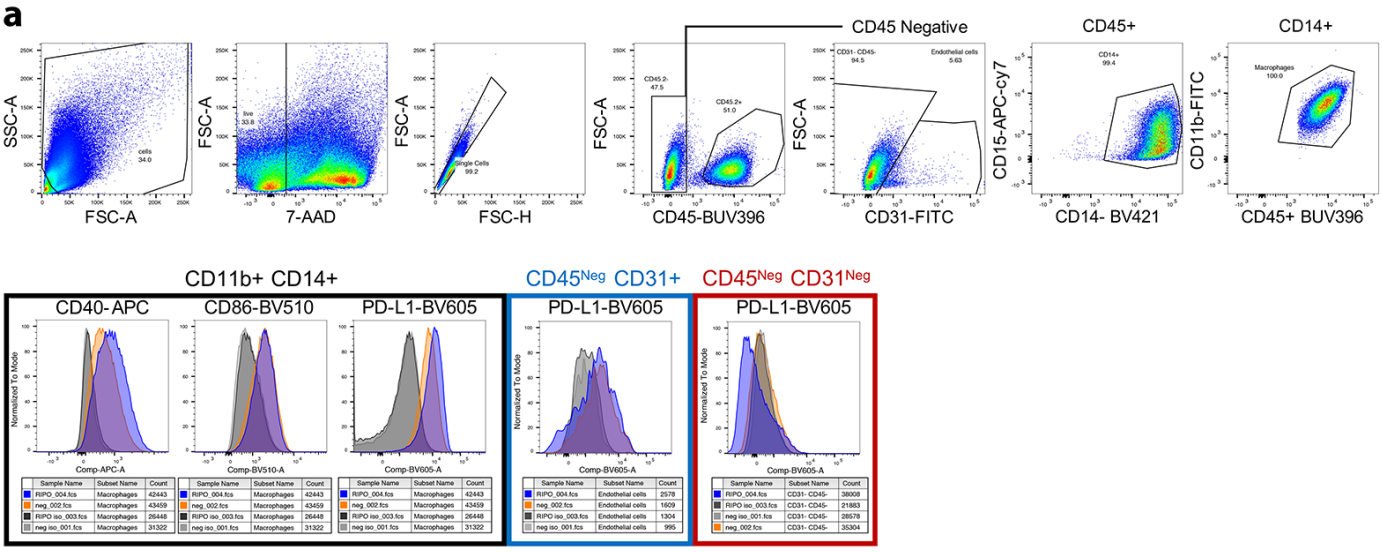
Red histograms are fluorescence minus one isotype control-PE antibody stained, light blue histograms are CD155-PE stained. Bottom 2 panels show comparison of CD14 expression in microglia and macrophages.

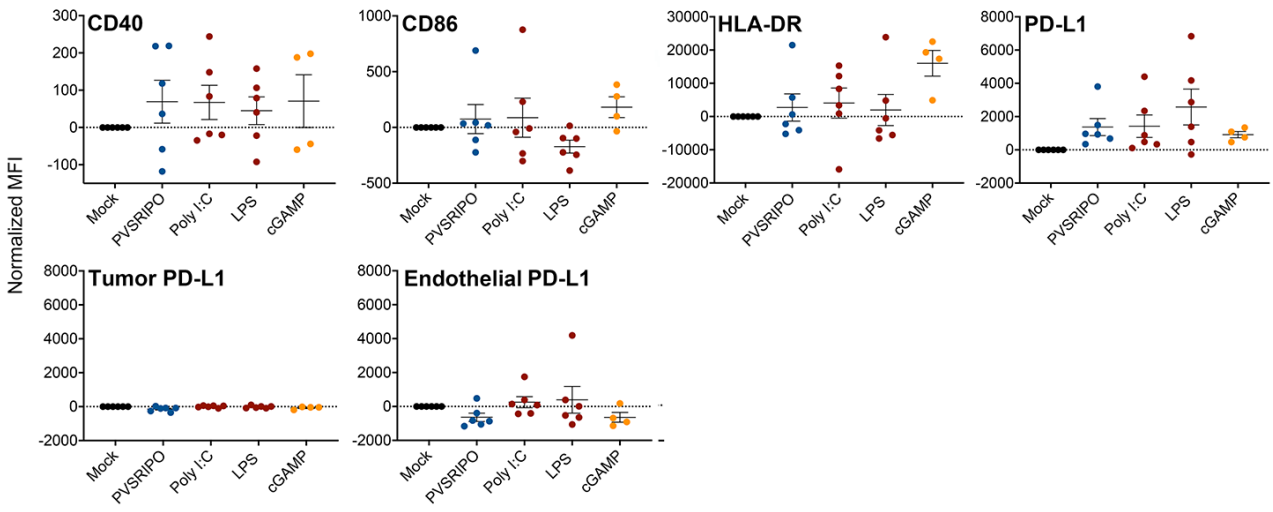
GBM tissue-matched cytokine induction

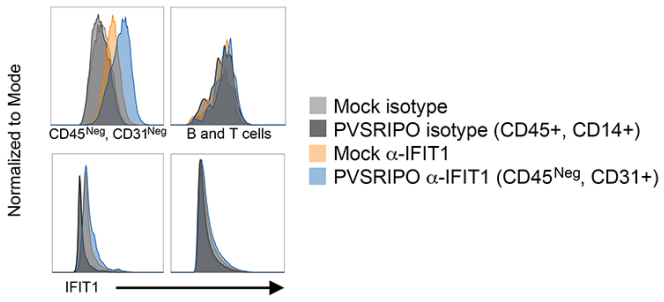


Supplementary Figure 2, related to Figure 1d. Patient-specific heatmaps depicting fold-change induction from mock control for each cytokine.

Only patients for which all 4 treatments were tested are shown (n=14); concentrations and experimental design are described in Figure 1.

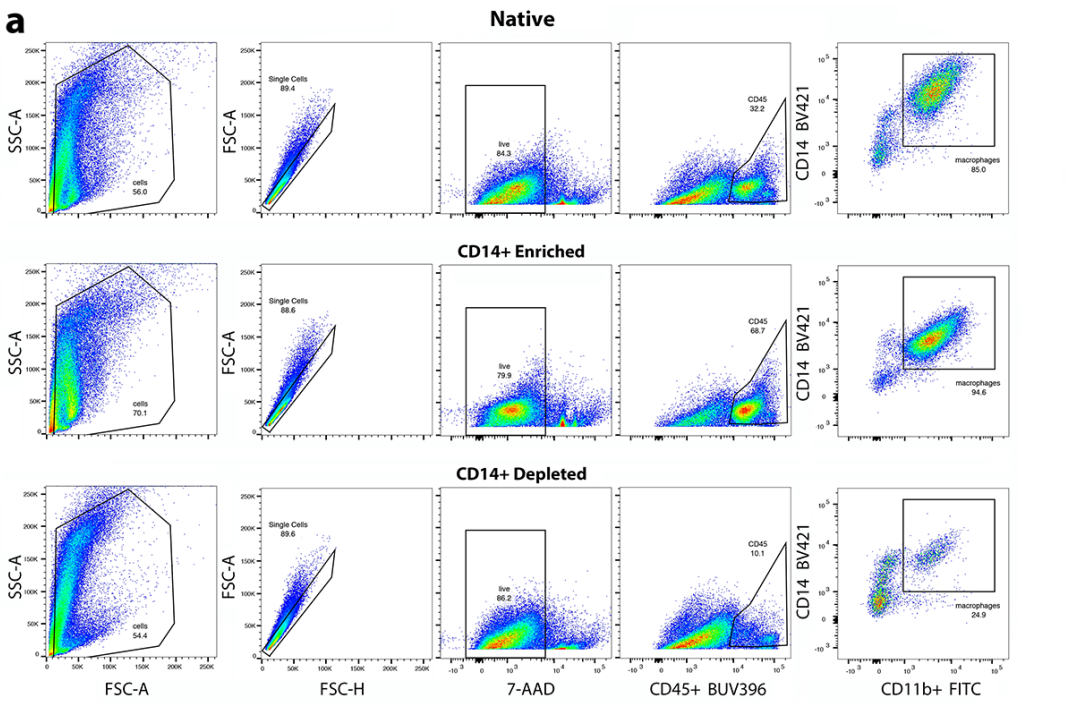

a
SSC-A
FSC-A
FSC-A
7-AAD
FSC-A
FSC-H
CD45 Negative
CD45-BUV396
CD31-FITC
CD45+
CD15-APC-cy7
CD14- BV421
CD14+
CD11b-FITC
CD45+ BUV396
CD11b+ CD14+
CD40- APC
CD86-BV510
PD-L1-BV605
CD45Neg CD31+
PD-L1-BV605
CD45Neg CD31Neg
PD-L1-BV605

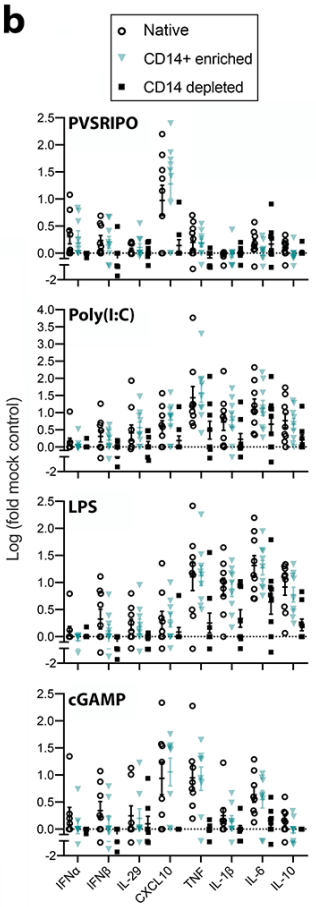

b
Macrophage (CD45+, CD14+, CD11b+)
Normalized MFI
CD40
CD86
HLA-DR
PD-L1
Tumor PD-L1
Endothelial PD-L1
Mock
PVSRIPO
Poly I:C
LPS
cGAMP

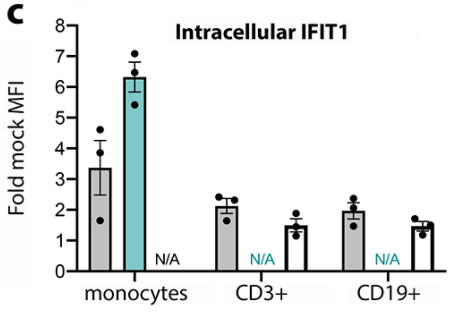

c
Normalized to Mode
CD45Neg, CD31Neg
B and T cells
IFIT1
Mock isotype
PVSRIPO isotype (CD45+, CD14+)
Mock α-IFIT1
PVSRIPO α-IFIT1 (CD45Neg, CD31+)

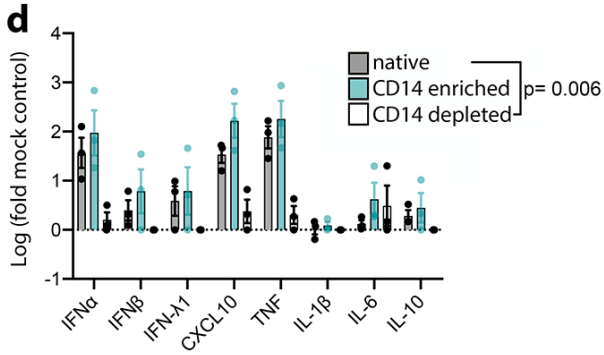
Supplementary Figure S3, related to Figure 1f and g. Gating strategy for tumor-associated macrophage activation and IFIT1 status after in vitro slice culture assay.

(**a**) Representative gating strategy for macrophage activation status in GBM and breast cancer specimens after *ex vivo* treatment (n=6 GBM, n= 2 breast cancer were tested); MFI (median florescence intensity) is shown on the x axis for histograms (bottom). (**b**) Post-treatment slice culture-derived tumor associated macrophages, endothelial cells, and tumor cells analyzed for PD-L1 in Figure 1f were also tested for CD40, CD86, HLA-DR; individual values are shown for each patient specimen and data brackets indicate mean -/+ SEM (n=6 GBM). (**c**) Representative histograms for intracellular IFIT1 staining after gating performed as in (a); data from all tested samples (n=4 GBM, n=2 breast cancer) presented in Fig. 1g.


a
Native
CD14+ Enriched
CD14+ Depleted
SSC-A
FSC-A
Single Cells
live
CD45
CD14 BV421
macrophages
cells
FSC-A
FSC-H
7-AAD
CD45+ BUV396
CD11b+ FITC

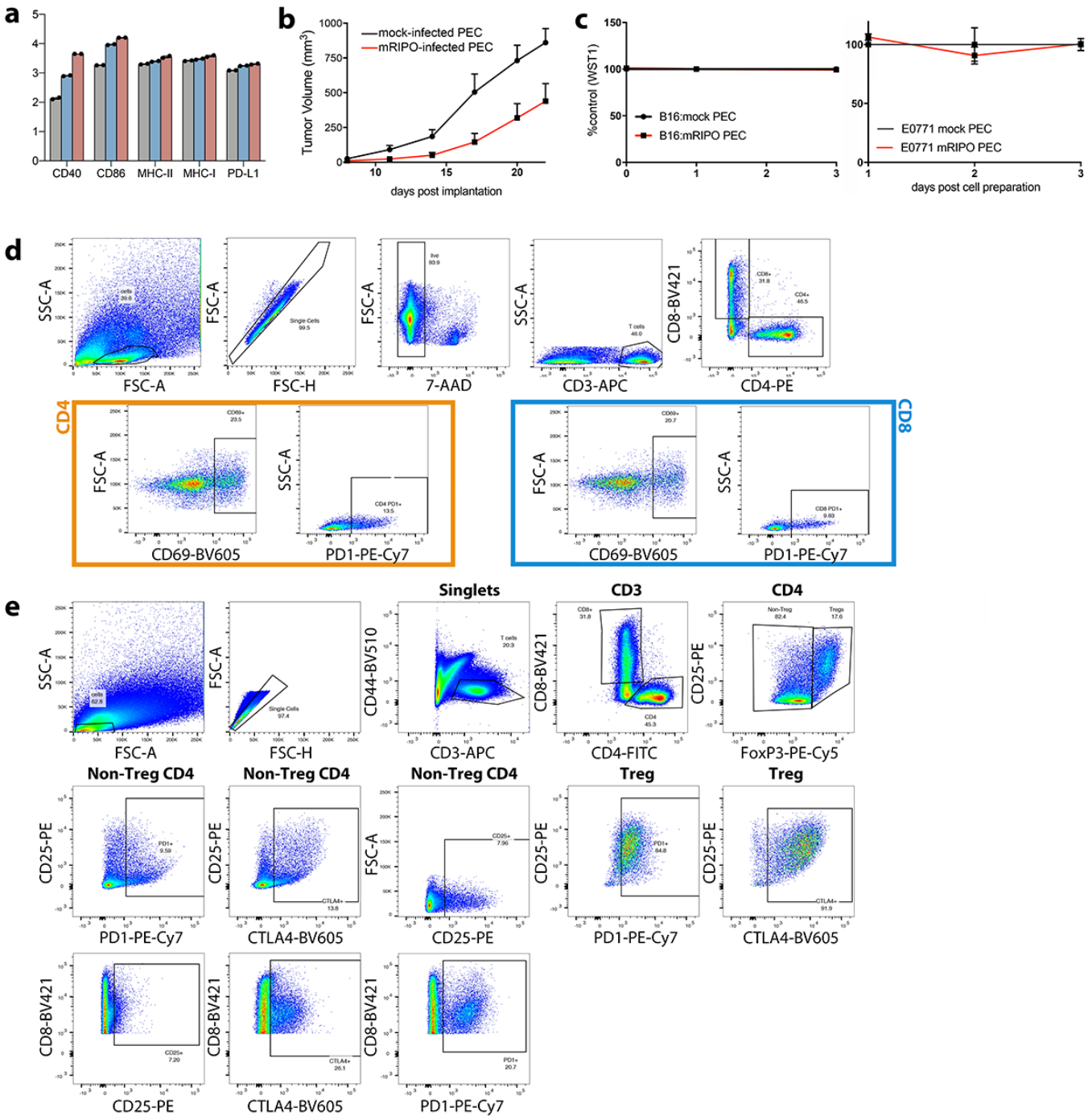

b
Native
CD14+ enriched
CD14 depleted
PVSRIPO
Poly(I:C)
LPS
cGAMP
Log (fold mock control)
IFNα
IFNβ
IL-29
CXCL10
TNF
IL-1β
IL-6
IL-10


c
Intracellular IFIT1
Fold mock MFI
N/A
monocytes
CD3+
CD19+


d
native
CD14 enriched
CD14 depleted
p= 0.006
Log (fold mock control)
IFNα
IFNβ
IFN-λ1
CXCL10
TNF
IL-1β
IL-6
IL-10

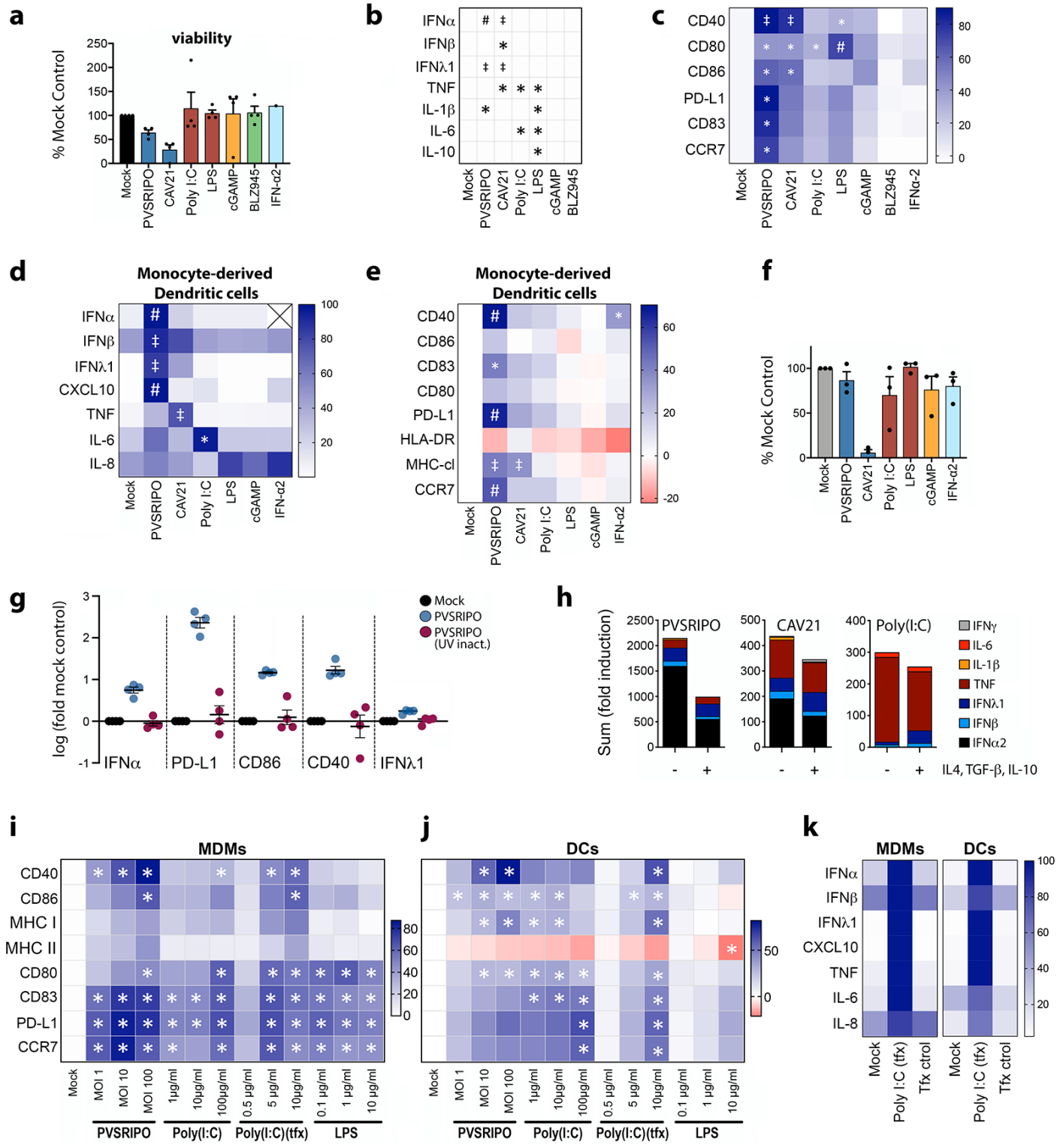
Supplementary Figure S4, related to Figure 1g and h. Cytokine responses of tumor cell suspensions and PBMCs after PVSRIPO/PRR agonist treatment depend upon CD14+ myeloid cells.

(**a**) Representative data assessing CD14+ cell enrichment/depletion for GBM single cell suspensions. (**b**) Individual data points for cytokine data presented in Figure 1h, including cytokine secretion from CD14+ cells (from n= 9 tumors). (**c**, **d**) Human PBMCs (n= 3 donors) were treated with mock or PVSRIPO after CD14+ pre-depletion, post-depletion, or CD14+ purified fractions, and tested for IFIT1 staining (**c**) as in Figure 1g, or supernatant cytokine analysis (**d**) as in Figure 1h; p-value is from paired t test (two-tailed) comparing sum fold-mock induction. (**b-d**) All data bars/brackets indicate mean -/+ SEM.


a
CD40
CD86
MHC-II
MHC-I
PD-L1
b
mock-infected PEC
mRIPO-infected PEC
Tumor Volume (mm3)
days post implantation
c
%control (WST1)
B16:mock PEC
B16:mRIPO PEC
E0771 mock PEC
E0771 mRIPO PEC
days post cell preparation
d
SSC-A
FSC-A
FSC-H
7-AAD
CD3-APC
CD8-BV421
CD4-PE
CD4
CD8
CD69-BV605
PD1-PE-Cy7
e
Singlets
CD3
CD4
CD44-BV510
CD4-FITC
FoxP3-PE-Cy5
Non-Treg CD4
Treg
CD25-PE
CTLA4-BV605
f
WT mice (CD155-)
CD155-tg mice
E0771 + Mock
E0771 + PVSRIPO
Days post implant

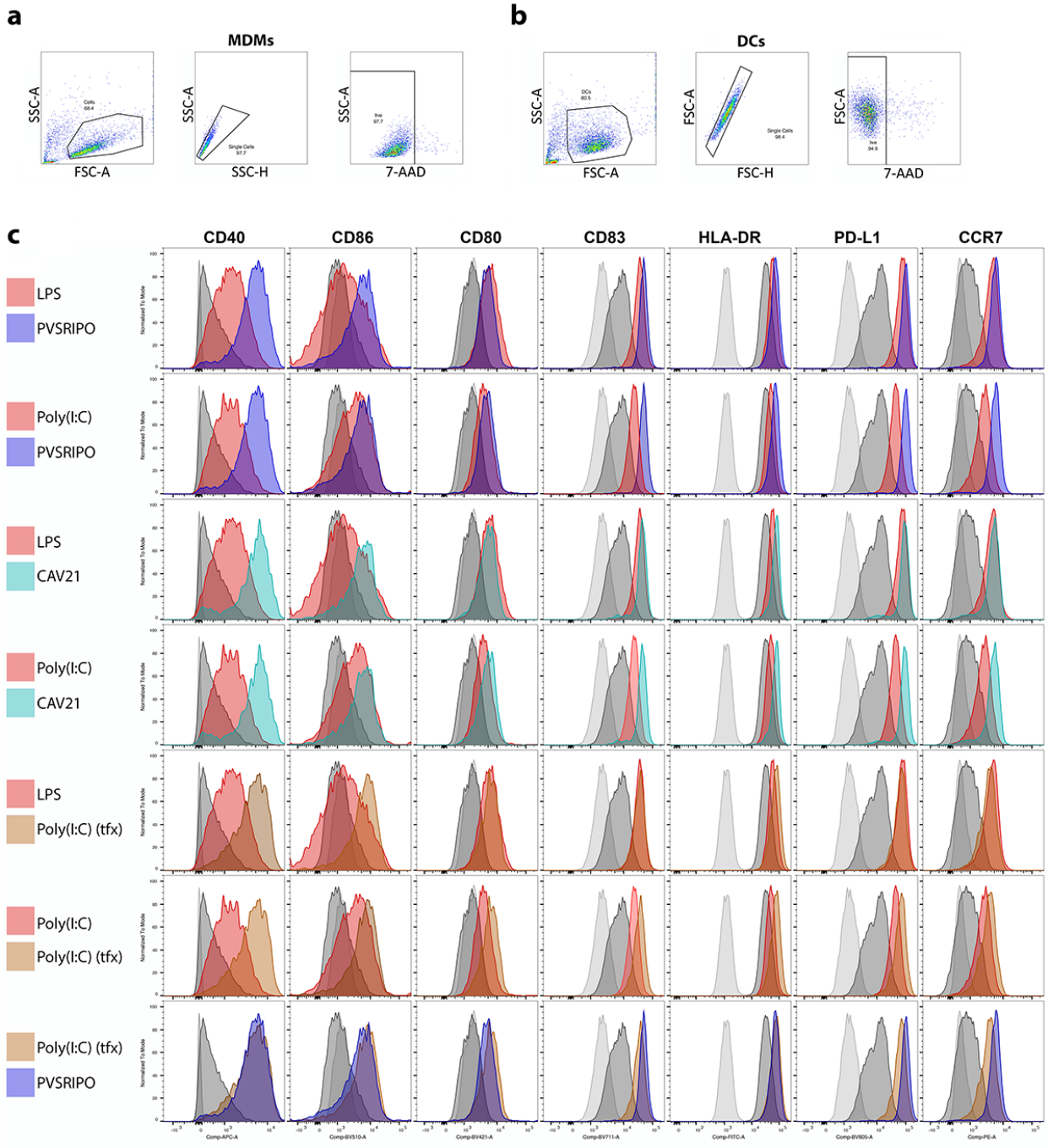
Supplementary Figure 5, related to Figures 2 and 3. Murine myeloid cells and the non-malignant TME mediate antitumor efficacy in response to mRIPO infection.

(**a**) Flow cytometry analysis of PEC treated with Mock (gray bars), mRIPO (MOI 10, blue bars) or LPS (100ng/ml, red bars) (24h), expressed as log MFI for each cell surface marker (n=2 experiments). (**b**) Repeat experiment for series presented in Figure 2b, left panel (B16, n= 10 mice/group); mean tumor volume + SEM is shown. (**c**) Tumor cell preparations used for implantation into mice in Figure 2b were plated in 96 well plates (1000 cells per well) and analyzed by WST1 assay for viability for 3 days; B16 (left panel) and E0771 (right panel), a representative series is shown from n=2 cell preparations per model; mean -/+ SEM is plotted from technical replicates within one experiment. (**d**) Representative gating strategy for T cell panel testing CD69 and PD1. (**e**) Representative gating strategy used to determine Treg and non-Treg CD4 phenotypes in Figure 2c. (**f**) WT E0771 cells were co-implanted into the fat pad of WT (left panel) or CD155-tg (right panel) mice with or without $1x10^8$ pfu mRIPO; mean tumor volume and SEM is shown (left panel: n=4/group; right panel: n=10/group).


a
viability
% Mock Control
Mock
PVSRIPO
CAV21
Poly I:C
LPS
cGAMP
BLZ945
IFN-α2
b
IFNα
IFNβ
IFNλ1
TNF
IL-1β
IL-6
IL-10
c
CD40
CD80
CD86
PD-L1
CD83
CCR7
d
Monocyte-derived Dendritic cells
IFNα
IFNβ
IFNλ1
CXCL10
TNF
IL-6
IL-8
e
Monocyte-derived Dendritic cells
CD40
CD86
CD83
CD80
PD-L1
HLA-DR
MHC-cl
CCR7
f
% Mock Control
g
log (fold mock control)
Mock
PVSRIPO
PVSRIPO (UV inact.)
IFNα
PD-L1
CD86
CD40
IFNλ1
h
Sum (fold induction)
PVSRIPO
CAV21
Poly(I:C)
IFNγ
IL-6
IL-1β
TNF
IFNλ1
IFNβ
IFNα2
IL4, TGF-β, IL-10
i
MDMs
CD40
CD86
MHC I
MHC II
CD80
CD83
PD-L1
CCR7
Mock
MOI 1
MOI 10
MOI 100
1μg/ml
10μg/ml
100μg/ml
0.5 μg/ml
5 μg/ml
10μg/ml
0.1 μg/ml
1 μg/ml
10 μg/ml
PVSRIPO
Poly(I:C)
Poly(I:C)(tfx)
LPS
j
DCs
k
MDMs
DCs
IFNα
IFNβ
IFNλ1
CXCL10
TNF
IL-6
IL-8
Mock
Poly I:C (tfx)
Tfx ctrol

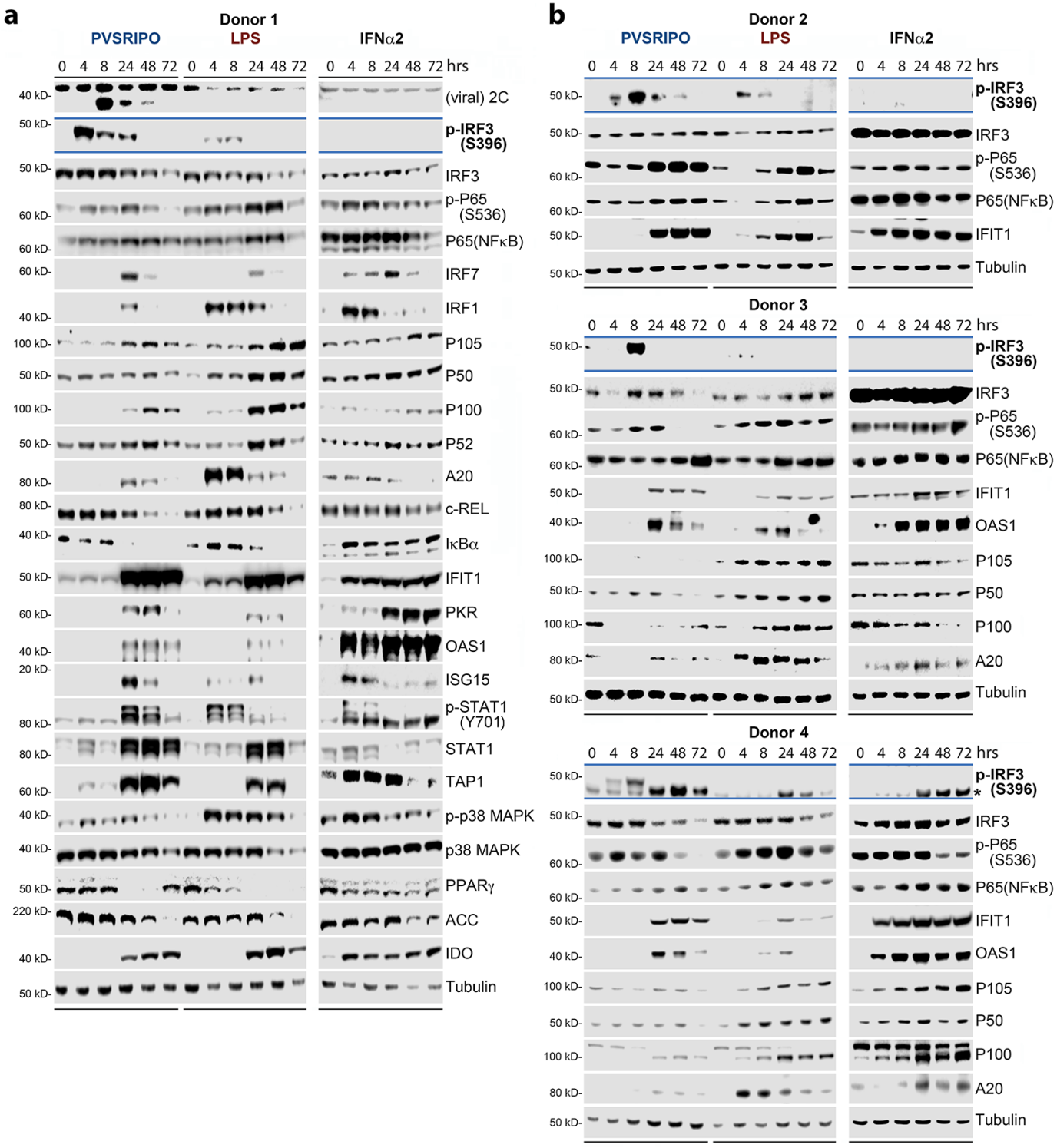
Supplementary Figure 6, related to Figure 4. Enteroviral replication/cytoplasmic dsRNA induce a distinct type-I/III IFN dominant phenotype in human macrophages and DCs.

(**a**) Viability determined by WST1 assay for MDMs after various treatments as shown in Figure 4a (n=4 experiments, except IFNα2 which was tested in this assay once); mean + SEM is shown. (**b**) Tukey's post hoc test $p<0.05$ (two-tailed) comparing each individual treatment in Figure 4a to mock (*), all other groups except PVSRIPO or CAV21 (‡), or (#) all other groups. (**c**) Flow cytometry analysis of activation markers on macrophages from Figure 4a (n=4 experiments/group except for cGAMP, BLZ945, and IFNα2 where n=2); symbols indicate same statistical information as in (b), comparisons were not performed for cGAMP, BLZ495, or IFNα2 due to n=2. (**d**-**f**) Cytokine (**d**), flow cytometry analysis (**e**), and viability (**f**, WST1 assay, data bars represent mean -/+ SEM) of monocyte-derived DCs treated as in Figure 4a (d and e n=4, f n=3 experiments); symbols indicate same as in (b). (**g**) MDMs were treated with Mock, PVSRIPO, or UV-inactivated PVSRIPO and supernatant was assayed for cytokine release. Cell surface staining (PD-L1, CD86, CD40) was also performed; relevant cytokines and cell surface markers are shown (n=4 experiments from 2 different donors) normalized between experiments by determining log-fold mock control. Brackets indicate mean values and SEM. (**h**) Macrophages were differentiated with M-CSF alone or in combination with IL-4, TGF-β, and IL-10 (all 20pg/ml) and treated with mock, PVSRIPO (MOI 10), CAV21 (MOI 10), or Poly(I:C) (10µg/ml). Cytokine release was measured and fold-mock control values were plotted together in each data bar to depict relative contribution to total cytokine induction (n=2 experiments). (**i**, **j**) Accompanying flow cytometry data for cells form experiments in Figure 4c (**i**) and 4d (**j**); %maximum MFI was determined followed by subtracting mock values for each activation marker; (*) indicates Tukey's post hoc test $p<0.05$ (two-tailed) vs mock treatment. (**k**) Comparison of Poly(I:C)-TFX vs transfection reagent (lyovec) alone in MDMs and DCs as indicated; %maximum concentration is shown.



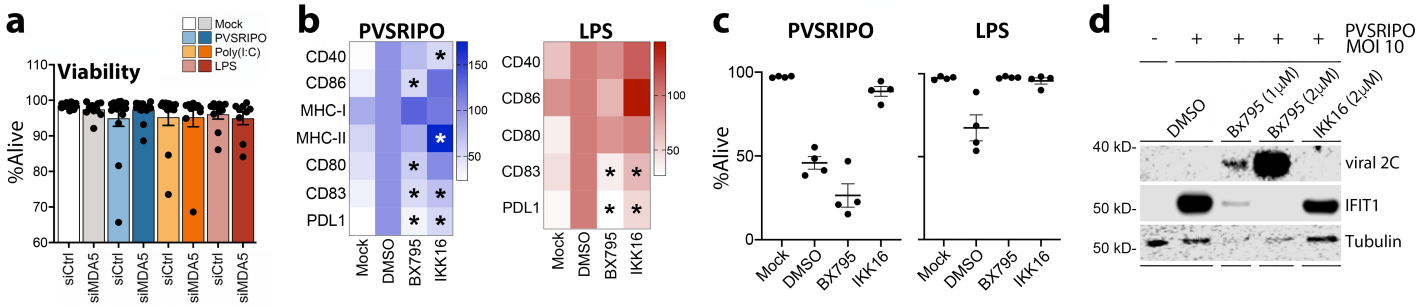
Supplementary Figure 7, related to Figure 4. Analysis of surface activation marker phenotype after enterovirus/cytoplasmic dsRNA compared to TLR agonists.

Gating strategy for MDMs (**a**) and DCs (**b**); histograms for surface activation markers on MDMs from a representative experiment (**c**).


a
Donor 1
PVSRIPO
LPS
IFNα2
0 4 8 24 48 72 0 4 8 24 48 72 0 4 8 24 48 72 hrs
40 kD-
(viral) 2C
50 kD-
p-IRF3 (S396)
50 kD-
IRF3
60 kD-
p-P65 (S536)
60 kD-
P65(NFκB)
60 kD-
IRF7
40 kD-
IRF1
100 kD-
P105
50 kD-
P50
100 kD-
P100
50 kD-
P52
80 kD-
A20
80 kD-
c-REL
40 kD-
IκBα
50 kD-
IFIT1
60 kD-
PKR
40 kD-
OAS1
20 kD-
ISG15
80 kD-
p-STAT1 (Y701)
80 kD-
STAT1
60 kD-
TAP1
40 kD-
p-p38 MAPK
40 kD-
p38 MAPK
50 kD-
PPARγ
220 kD-
ACC
40 kD-
IDO
50 kD-
Tubulin
b
Donor 2
PVSRIPO
LPS
IFNα2
0 4 8 24 48 72 0 4 8 24 48 72 0 4 8 24 48 72 hrs
50 kD-
p-IRF3 (S396)
50 kD-
IRF3
60 kD-
p-P65 (S536)
60 kD-
P65(NFκB)
50 kD-
IFIT1
50 kD-
Tubulin
Donor 3
0 4 8 24 48 72 0 4 8 24 48 72 0 4 8 24 48 72 hrs
50 kD-
p-IRF3 (S396)
50 kD-
IRF3
60 kD-
p-P65 (S536)
60 kD-
P65(NFκB)
50 kD-
IFIT1
40 kD-
OAS1
100 kD-
P105
50 kD-
P50
100 kD-
P100
80 kD-
A20
50 kD-
Tubulin
Donor 4
0 4 8 24 48 72 0 4 8 24 48 72 0 4 8 24 48 72 hrs
50 kD-
p-IRF3 * (S396)
50 kD-
IRF3
60 kD-
p-P65 (S536)
60 kD-
P65(NFκB)
50 kD-
IFIT1
40 kD-
OAS1
100 kD-
P105
50 kD-
P50
100 kD-
P100
80 kD-
A20
50 kD-
Tubulin

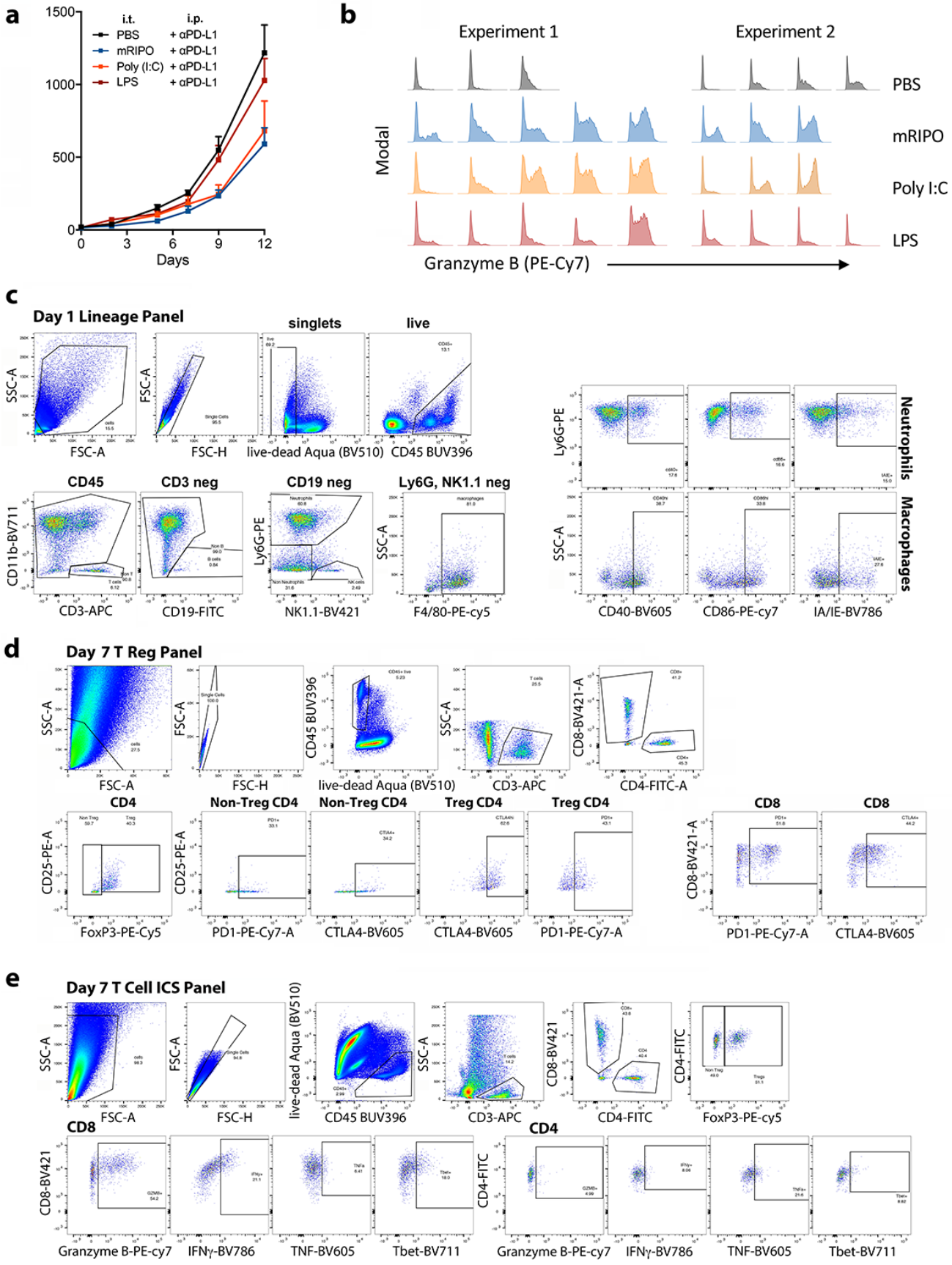
Supplementary Figure 8, related to Figure 5. **Immunoblots from 4 different donors, including all blots quantitated in Figure 5b.**

Each immunoblot series was performed once for each donor, with extended analysis performed for donor 1 (**a**). (**b**) Asterisk below p-IRF3 blot for donor 4 indicates a background band appearing below the true p-IRF3(S396) band, which was defined by ladder and detection of total IRF3 on a companion blot for all donors. N=4 experiments using 4 donors.



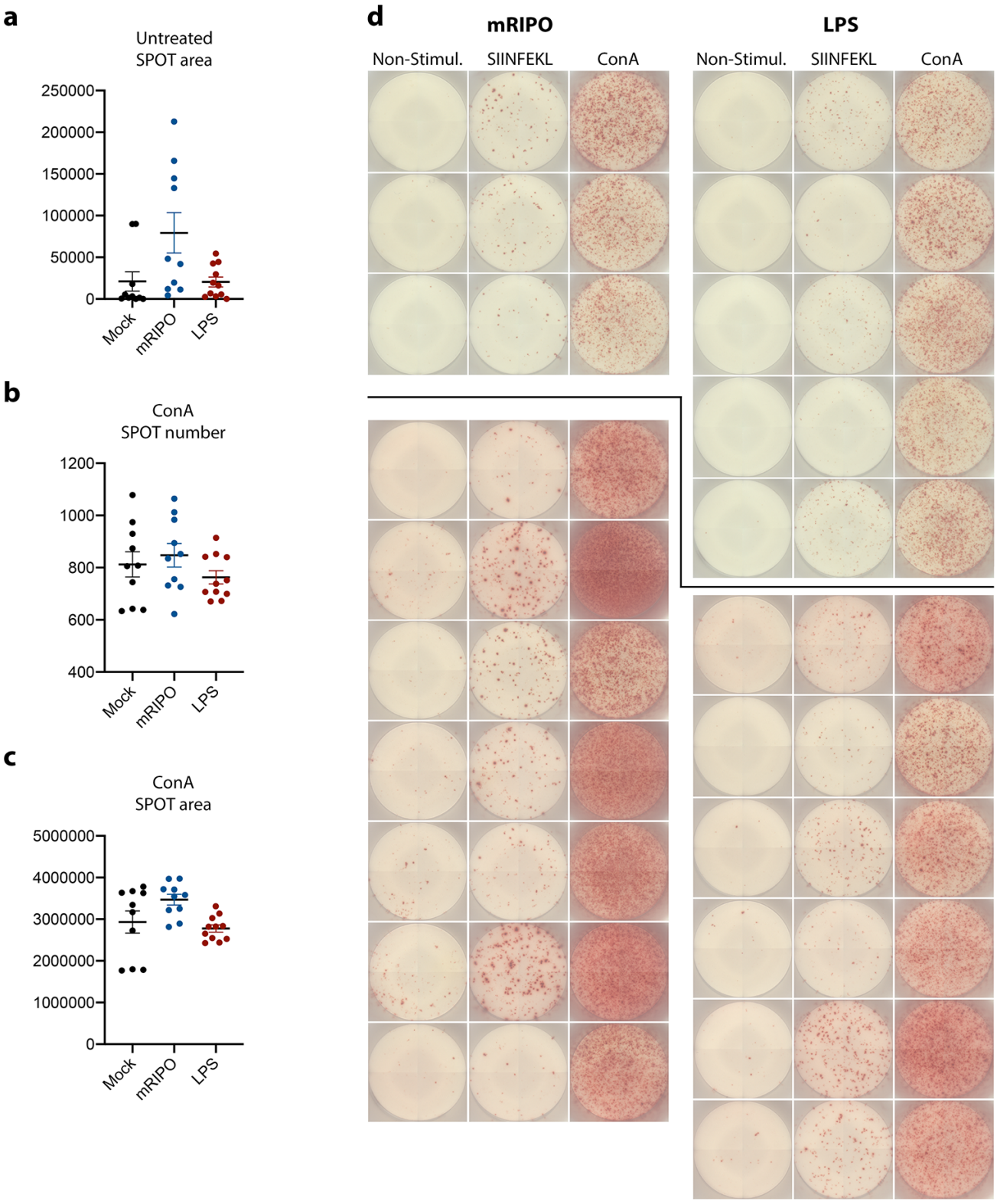
Supplementary Figure 9, related to Figure 6. Macrophage activation phenotype after PVSRIPO infection depends upon MDA5-TBK1 signaling.

(**a**) Cell viability by 7-AAD after control vs MDA5-targeting siRNA transfection, and stimulation with PVSRIPO/TLR agonists as shown in Fig. 6a-b (n=16 experiments PVSRIPO, n=10 LPS, n=12 all others; data bars represent mean -/+ SEM). (**b**) Heatmaps depicting levels of cell surface activation markers, and (**c**) viability (7-AAD) on cells from experiments in Fig. 6c (n=4 experiments; data brackets indicate mean -/+ SEM; (*) indicates Tukey's post-hoc $p<0.05$ vs DMSO treatment control. (**d**) Immunoblot analysis of viral protein (2C) and IFIT1 in the presence and absence of Bx795 or IKK16 during PVSRIPO infection, a representative series from n=2 experiments is shown.


a
1500
1000
500
0
0
3
6
9
12
Days
i.t.
i.p.
PBS
+ αPD-L1
mRIPO
+ αPD-L1
Poly (I:C)
+ αPD-L1
LPS
+ αPD-L1
b
Experiment 1
Experiment 2
Modal
PBS
mRIPO
Poly I:C
LPS
Granzyme B (PE-Cy7)
c
Day 1 Lineage Panel
singlets
live
SSC-A
FSC-A
FSC-H
live-dead Aqua (BV510)
CD45 BUV396
CD45
CD3 neg
CD19 neg
Ly6G, NK1.1 neg
CD11b-BV711
CD3-APC
CD19-FITC
Ly6G-PE
NK1.1-BV421
F4/80-PE-cy5
Neutrophils
Macrophages
CD40-BV605
CD86-PE-cy7
IA/IE-BV786
d
Day 7 T Reg Panel
CD45 BUV396
CD3-APC
CD8-BV421-A
CD4-FITC-A
CD4
Non-Treg CD4
Non-Treg CD4
Treg CD4
Treg CD4
CD8
CD8
CD25-PE-A
FoxP3-PE-Cy5
PD1-PE-Cy7-A
CTLA4-BV605
CTLA4-BV605
PD1-PE-Cy7-A
CD8-BV421-A
PD1-PE-Cy7-A
CTLA4-BV605
e
Day 7 T Cell ICS Panel
live-dead Aqua (BV510)
CD45 BUV396
CD3-APC
CD8-BV421
CD4-FITC
FoxP3-PE-cy5
CD8
CD4
CD8-BV421
CD4-FITC
Granzyme B-PE-cy7
IFNγ-BV786
TNF-BV605
Tbet-BV711

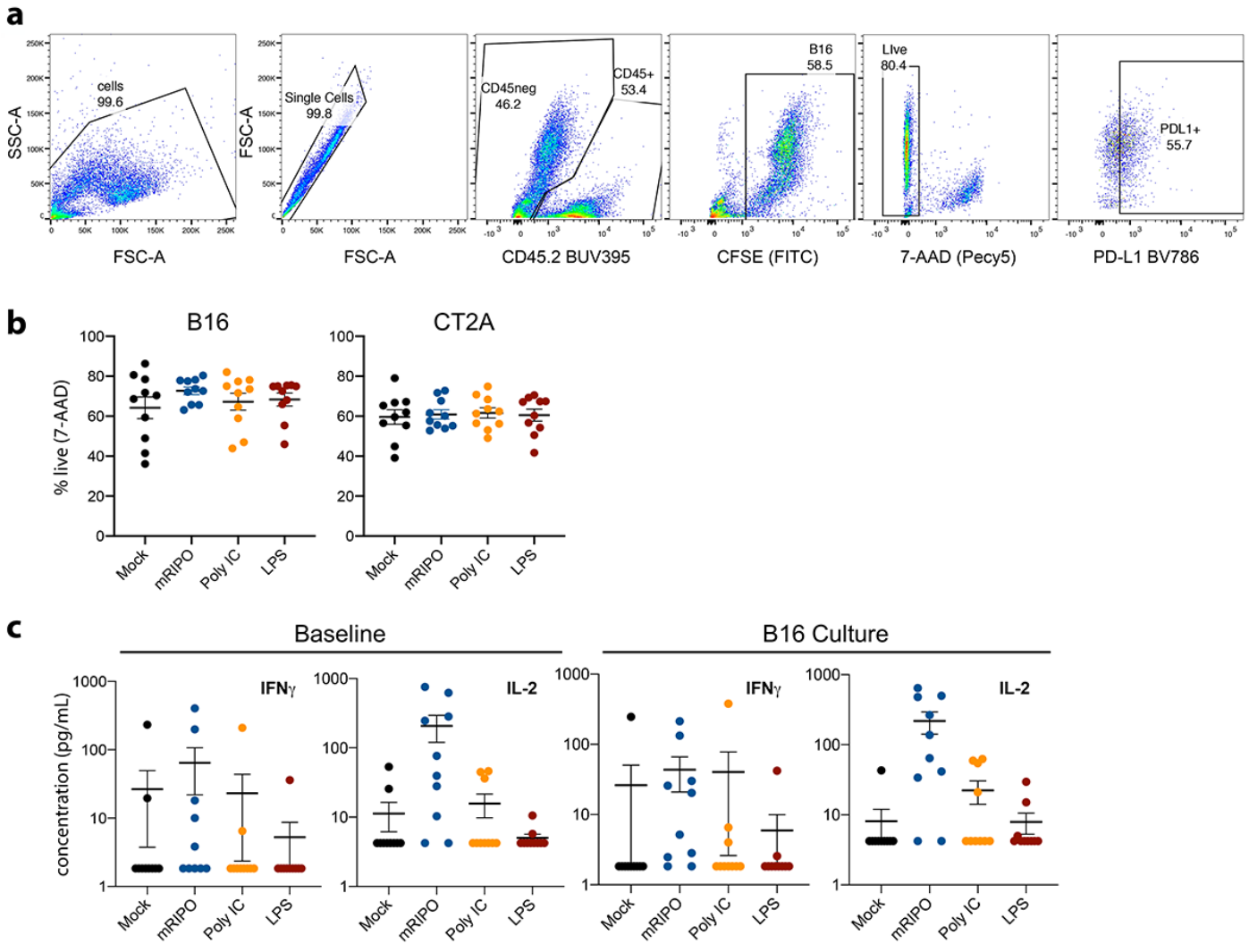
Supplementary Figure 10, related to Figure 7. mRIPO therapy is associated with polyfunctional tumor infiltrating T cell phenotypes.

(**a**) Repeat experiment for Figure 7a [n=5 for mRIPO/LPS, n=6 for PBS/Poly(I:C)]; mean tumor volume + SEM is plotted by treatment group. (**b**) Histograms of 2 experimental cohorts testing Granzyme B expression on CD8+ T cells after PBS, mRIPO, Poly(I:C) or LPS treatment, corresponding to data in Figure 7e. (**c**) Representative gating for macrophages, neutrophils, and NK cells along with relevant activation markers presented in Figure 7e; isotype control staining was used to define CD40, CD86, and IA/IE positivity. (**d**) Representative gating for Treg panel testing Treg, non-Treg, and CD8+ T cell expression of CTLA4 (intracellular stain), and PD1 in Figure 7e. (**e**) Representative gating for T cell panel assessing intracellular Granzyme B, IFNγ, TNF-α and T-bet expression by T cells as shown in Figure 7e; isotype control stained fluorescence minus one samples were used to determine positive intracellular staining (Granzyme B, IFNγ, TNF-α, and T-bet) populations.


a
Untreated
SPOT area
250000
200000
150000
100000
50000
0
Mock
mRIPO
LPS
b
ConA
SPOT number
1200
1000
800
600
400
Mock
mRIPO
LPS
c
ConA
SPOT area
5000000
4000000
3000000
2000000
1000000
0
Mock
mRIPO
LPS
d
mRIPO
LPS
Non-Stimul.
SIINFEKL
ConA
Non-Stimul.
SIINFEKL
ConA

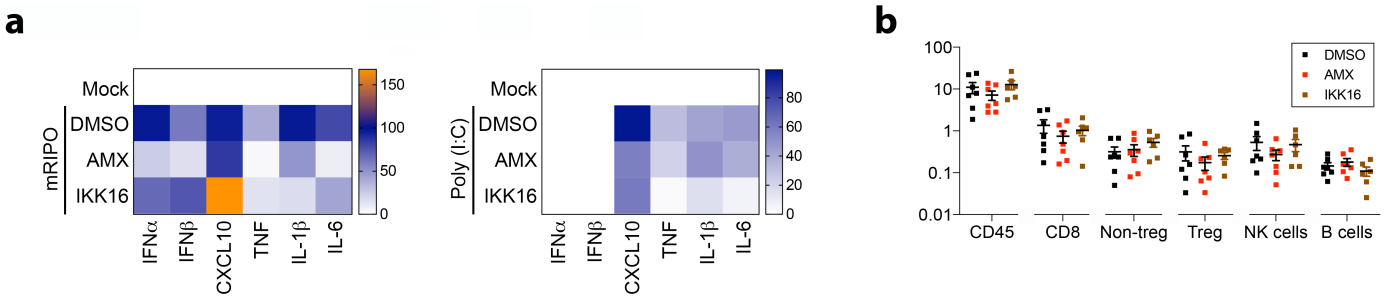
Supplementary Figure 11, related to Figure 8b. **Additional analyses for ELISpot data presented in Figure 8b.**

(**a**) Baseline total spot area for non-stimulated splenocytes. (**b**) Spot numbers for positive control (concanavalin A) treated splenocytes for ELISpot data presented in Figure 8b. (**c**) Total IFNγ spot area after Concanavalin A stimulation of splenocytes. (**a-c**) n=10 mice for PBS/mRIPO, n=11 mice LPS pooled from 2 experiments; data brackets indicate mean -/+ SEM. (**d**) Scans of ELISpot plates using splenocytes from tumor-bearing mice treated with mRIPO or LPS; the panels above and below the bar represent two independent experiments. In all ELISpot graphs/pictures all values for the mice with the 2 highest and 2 lowest IFNγ spot counts after SIINFEKL stimulation were excluded from each group to eliminate outliers; thus, the same samples (from the same mice) were excluded from all panels.



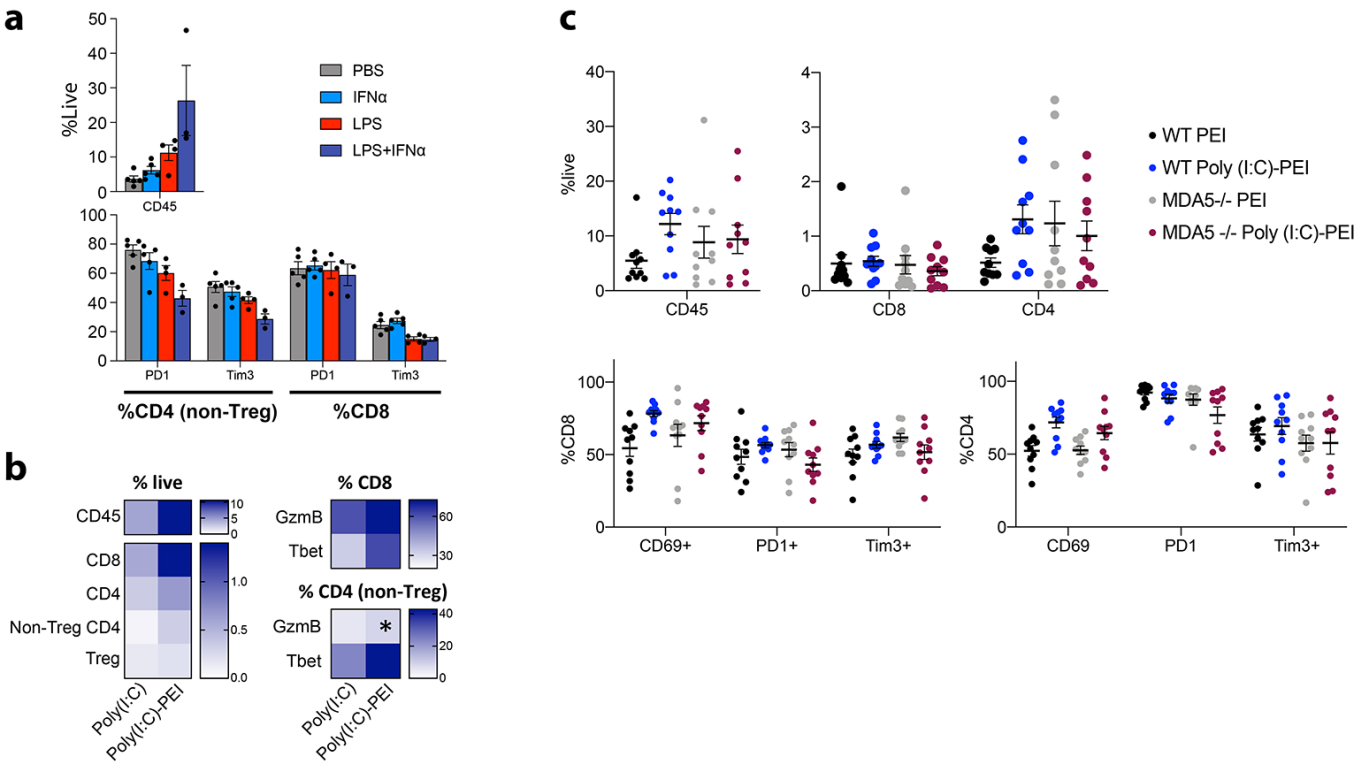
Supplementary Figure 12, related to Figure 8d. Analysis of TDLN:B16 co-cultures seven days after mRIPO vs TLR agonist therapy.

(**a**) Gating strategy for CFSE and PD-L1 analysis in Figure 8d. (**b**) %Live B16 and CT2A cells after TDLN cell co-culture presented in Figure 8d as measured by 7-AAD. (**c**) Supernatant cytokines were measured after 48h TDLN cell:B16 or CT2A co-culture from Figure 8d. (**b, c**) Data brackets represent mean -/+ SEM; n=10 mice per group.



Supplementary Figure 13, related to Figure 9a, b. Confirmation of AMX inhibitor activity in murine cells and extended analysis of immunological changes after TBK1 inhibition.

(**a**) Heatmaps presenting cytokine release (24h) of DMSO, Amlexanox (2.5μM), or IKK16 (50nM) treatment of FLT3L-derived BMDCs in the presence of mRIPO (left, MOI 10) or Poly(I:C) (right, 10μg/ml). Percent maximum cytokine concentration (pg/ml) was determined, mock values were subtracted; mean normalized values are plotted [n=2 experiments]. (**b**) Extended analyses for tumor infiltrating cell type density (%live cells) for each indicated cell type for the experiment presented in Figure 9a. Data brackets represent mean -/+ SEM; n= 7/group mRIPO + DMSO and AMX, n=6 for mRIPO + IKK16.



Supplementary Figure 14, related to Figure 9e, g, and i. Type-I IFN and cytoplasmic Poly(I:C) potentiate intratumor inflammation and T cell activation.

(**a**) Extended associated flow cytometry analyses for experiment performed in Figure 9e (n=3 mock/LPS + IFNα, n=5 IFNα, n=4 LPS only). (**b**) Tumors were harvested from surviving mice 16 days after treatment in the experiment described in Figure 9g and analyzed for immune infiltrates/T cell phenotypes [n=3 Poly(I:C), n=7 Poly(I:C)-PEI]; (*) t-test (two tailed) comparing Poly(I:C) vs Poly(I:C)-PEI, p=0.047. (**c**) Extended flow cytometry analyses for Figure 9i (n=10 mice/group). (**a, c**) All brackets/data bars represent mean -/+ SEM.