Supplemental Information for

Catch-and-release of cytokines mediated by tumor phosphatidylserine converts transient exposure into long-lived inflammation.

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Contents

1 Supplemental Figures

Figure S1: Related to Figure 1. Transient IFNγ drives persistent T cell activation caused by up-regulation of antigen processing and presentation. (A) The k-means algorithm was applied to our RNAseq data (Figure 1E) for increasing number of clusters (n=1-50). The sum of distance represents the total Euclidean distance between cluster centroid and the data. (B) B16 cells were pulsed with IFNγ, washed, and cultured in fresh media. At indicated time-points, cells were harvested and MHC-I (H2-D^b and H2-K^b) were quantified by flow cytometry. Alternatively, at each time-point, RNA was harvested, purified, and *h2db* and *h2kb* transcripts were quantified by RT-qPCR. For both mRNA and protein, data are representative of at least 3 independent experiments.

Figure S2: Related to Figure 2. Jak-STAT signaling drives persistent transcription. (A) B16 cells were exposed to IFNγ for 20 minutes, then JAKi was added. Cells were harvested at indicated time-points, fixed and permeabilized, and stained for pSTAT1. pSTAT1 was quantified by flow cytometry and the resultant time kinetics were fitted with a double exponential decay function to highlight the rapid dephosphorylation of pSTAT1 (τ <10min). Data plotted is representative of 3 independent experiments. (B) B16 cells were stimulated with IFNγ for 5h, washed, then cultured in fresh media. At the time of wash, one cohort of cells received neutralizing antibodies directed against both the common receptor for IFN$\alpha\beta$ and the cytokine IFNβ. The other cohort received IgG control antibodies. MHC-I (H2-K^b) was quantified by flow cytometry. Data plotted is representative of 3 independent experiments. (C) B16 cells were pulsed for 5h with IFNγ then washed. Immediately after the last wash, conditioned media was harvested. A fresh cohort of B16 cells were cultured in either conditioned media, 10nM IFNγ or media alone for 1 day. MHC-I (H2-K^b) was quantified by flow cytometry.

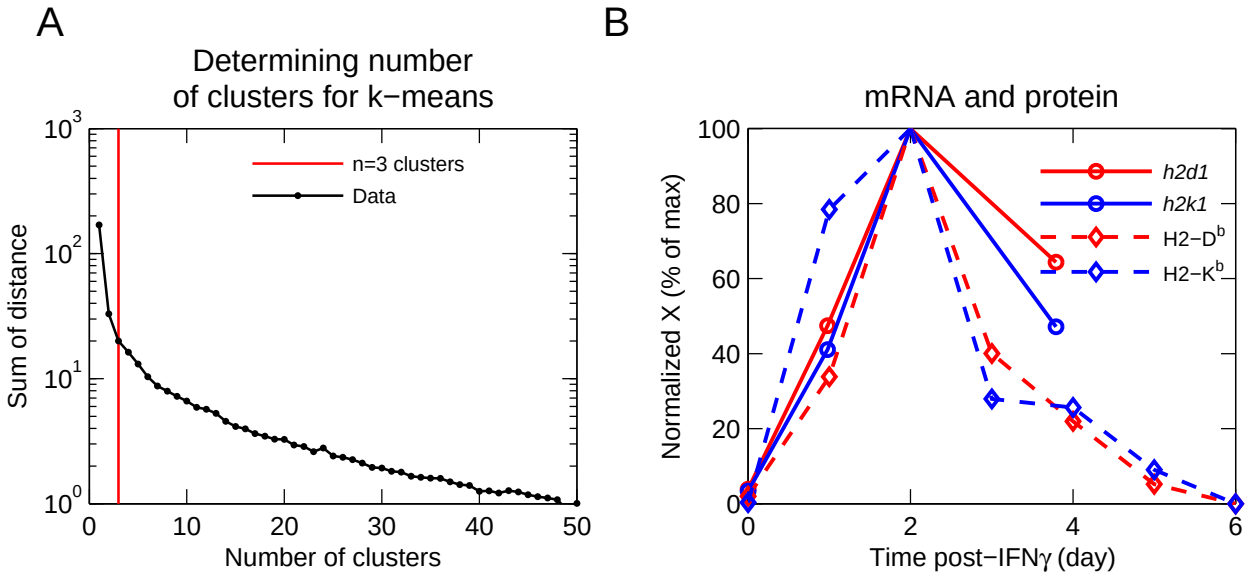
Figure S3: Related to Figure 3. Cytokine-pulsed cells slowly release IFNγ. (A) One group of B16 cells was pulsed with IFNγ for 5h, then washed. Pulsed cells were then co-cultured in the bottom of a transwell with fluorescent-labeled unstimulated sensor cells. An additional group of sensor cells were cultured on top of the transwell insert. Neutralizing antibodies against IFNγ were added where indicated as controls. MHC-I (H2-K^b) was measured at indicated time-points. (B) T cell-derived IFNγ was harvested from the supernatant of PMA/Ionomycin activated C57Bl/6 IFNγR KO splenocytes. B16 IFNγR KO cells were pulsed with the indicated concentration of either T cell-derived or recombinant IFNγ in a well-mixed setting for 5 hours. Culture media was then harvested from cells and a bead-based ELISA was used to quantify the depletion of IFNγ from the media. (C) For cartoon, see Figure 5G. B16 IFNγR KO cells were incubated with the glycolipid inhibiting drugs Fumonisin B, PDMP, or DMSO as a vehicle control for 3 days before performing the IFNγ cell-capture assay. Treated cells were cultured with 50pM IFNγ for 5 hours, then the culture supernatant was harvested and depletion of IFNγ from the media was quantified by bead-based ELISA. Alternatively, cells were incubated with dynasore, or treated with the enzymes heparinase I (hepI), heparinase III (hepIII), chondroitin ABC lyase (chABC), or pronase prior to performing the IFNγ cell-capture assay. All plots are representative of at least 2 independent experiments. In the case of enzymatic treatments (hepI, hepIII, chABC, and pronase), cells were incubated for only 2 hours before harvesting culture supernatant. (D) B16 IFNγR KO cells were pulsed with IFNγ-A647 while tumbling in RPMI, then washed (black). To pre-treat cells with unlabeled IFNγ, cells were pulsed with unlabeled, recombinant IFNγ, then an equimolar concentration of IFNγ-A647 was spiked in and incubated for an equivalent amount of time. Cells were then washed (cyan). To check whether dye particles left in the protein prep could bind cells non-specifically, we prepared a protein-free sample of Alexa647 dye treated exactly as the IFNγ-A647 was treated and exposed an equal quantity to cells before washing. Cell fluorescence was quantified by flow cytometry. Data is representative of 2 independent experiments. (E) B16 IFNγR KO cells were pulsed with 10nM either IFNγ-A488 or unlabeled IFNγ for 5 hours, then washed and co-cultured with CTV-labeled B16 IFNγR competent B16 cells. To relevant wells, αIFNγ was added. MHC-I (H2-K^b) was measured by flow cytometry after 1 day of co-culture. (F) B16 IFNγR KO cells were adhered to glass bottom dishes, incubated with IFNγ-A647, washed, and imaged with confocal microscopy. Image is representative of at least 3 independent experiments.

Figure S4: Related to Figure 4. A mathematical model, including a slow catch and release process, recapitulates experimental results. (A) B16 cells were stimulated with different doses of IFNγ for 20 minutes. Cells were fixed, permeabilized, and stained for pSTAT1. Fluorescence was quantified by flow cytometry and the resultant curve was fitted with a Hill function with a coefficient of 1. Data is representative of at least 3 independent experiments. (B) B16 cells were exposed to a constant, saturating dose of IFNγ. RNA was harvested periodically and the kinetics of *h2kb* transcript accumulation were quantified by RT-qPCR. The data are representative of at least 3 independent experiments. (C) The transcription kinetics in B were converted into the transcription rate using and the adaptation timescale was computed from the fit. (D) The model generates a concentration of free IFNγ that decreases depending on consumption. The pSTAT1 profile is generated from the free IFNγ concentration and the experimentally-determined EC_{50} of signaling. (E) The model was run by simulating the kinetics of mRNA for cells pulsed for 5h with 10nM IFNγ, then washed. The model was setup to capture and release IFNγ based on the rates acquired from our catch-and-release mechanism, published rates for IL10 binding to heparan sulfate **?**, or with no capture and release. (F) B16 cells were stimulated with a constant dose of 1nM IFNγ or media alone. At the indicated time-points, cells were fixed, permeabilized, and stained for pSTAT1. Fluorescence was quantified by flow cytometry. (G) The model was run by calculating the H2-K^b protein that accumulates based on the concentration of free IFNγ generated by the model and the experimentally-determined EC_{50} of signaling. Sharing cells were simulated by assuming that they were exposed to 10nM IFNγ for 5 hours prior to removal of the exogenous signal. Sensor cells were simulated by assuming that their first exposure to IFNγ was after removal of exogenous IFNγ (i.e. they were only exposed to the free IFNγ originating from sharing cells). (H) This plot shows some data originally plotted in Figure S3A, with one additional condition not shown in that graph. One group of B16 cells was pulsed with IFNγ for 5h, then washed. Pulsed cells were then co-cultured in the bottom of a transwell with fluorescent-labeled unstimulated sensor cells. MHC-I (H2-K^b) was quantified on both IFNγ-pulsed cells (sharing cells), and mixed sensor cells at the indicated timepoints.

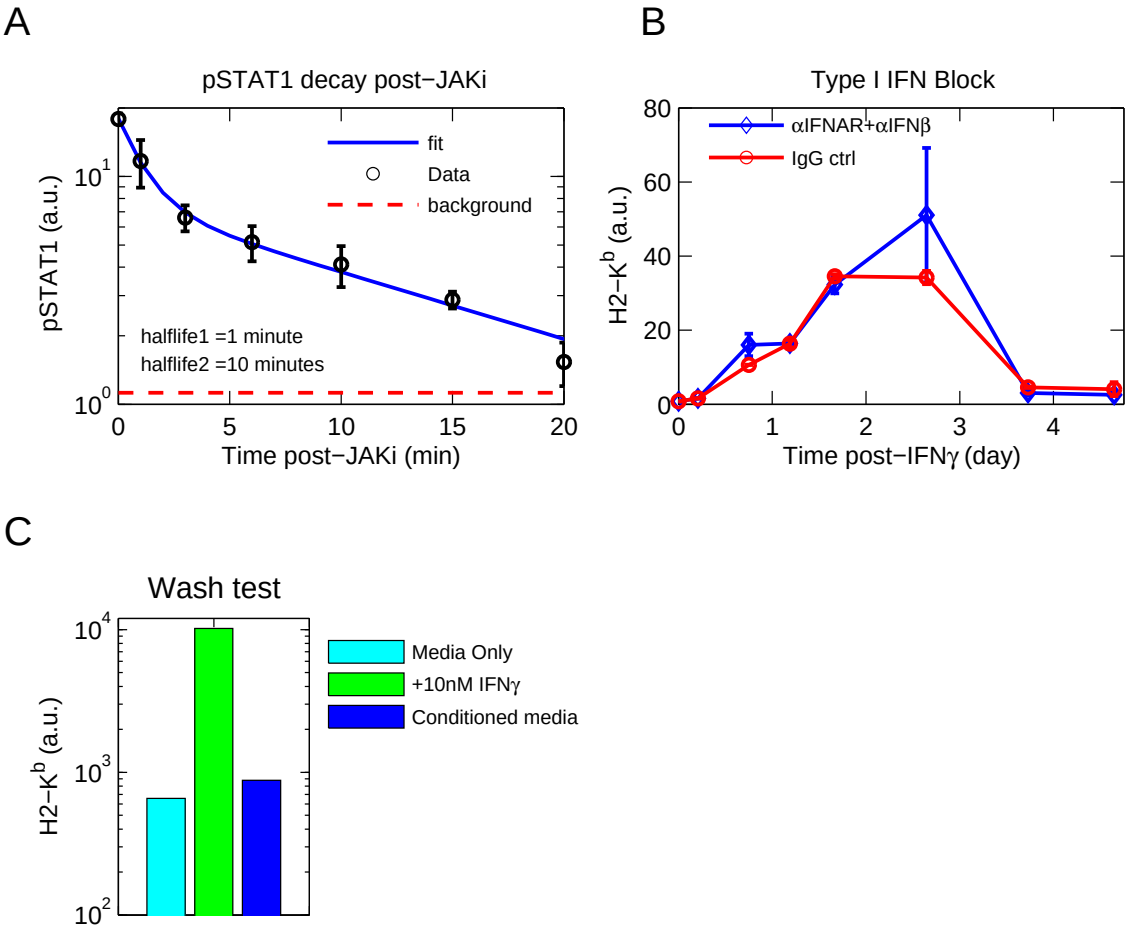
Figure S5: Related to Figure 5. Phosphatidylserine is necessary for IFNγ cell binding. (A-B) B16 IFNγR KO cells were either stained live, or fixed and permeabilized then stained for cardiolipin (A) or PI(4)P (B). As a negative control, cells were stained with the secondary antibody only. Fluorescence was quantified with flow cytometry. (C) B16 IFNγR KO cells were stained with Annexin V. Dead cells were identified as rounded, detached from the glass surface, and stained brightly in a uniform pattern on the plasma membrane. (D) All of the indicated cell lines were stained with Annexin V. Cells of interest were gated as DAPI-. (E) All of the indicated mouse cell lines were stained live or fixed and permeabilized then stained with MFG-E8 and antibodies directed against MFG-E8. As a negative control, MFG-E8 was omitted and some cells were stained with only with the biotinylated secondary antibody and fluorescent streptavidin. Cells of interest were gated as DAPI-. (F) B16 IFNγR KO cells were incubated with either MFG-E8 or Annexin V to block PS, then the IFNγ cell capture assay was performed.

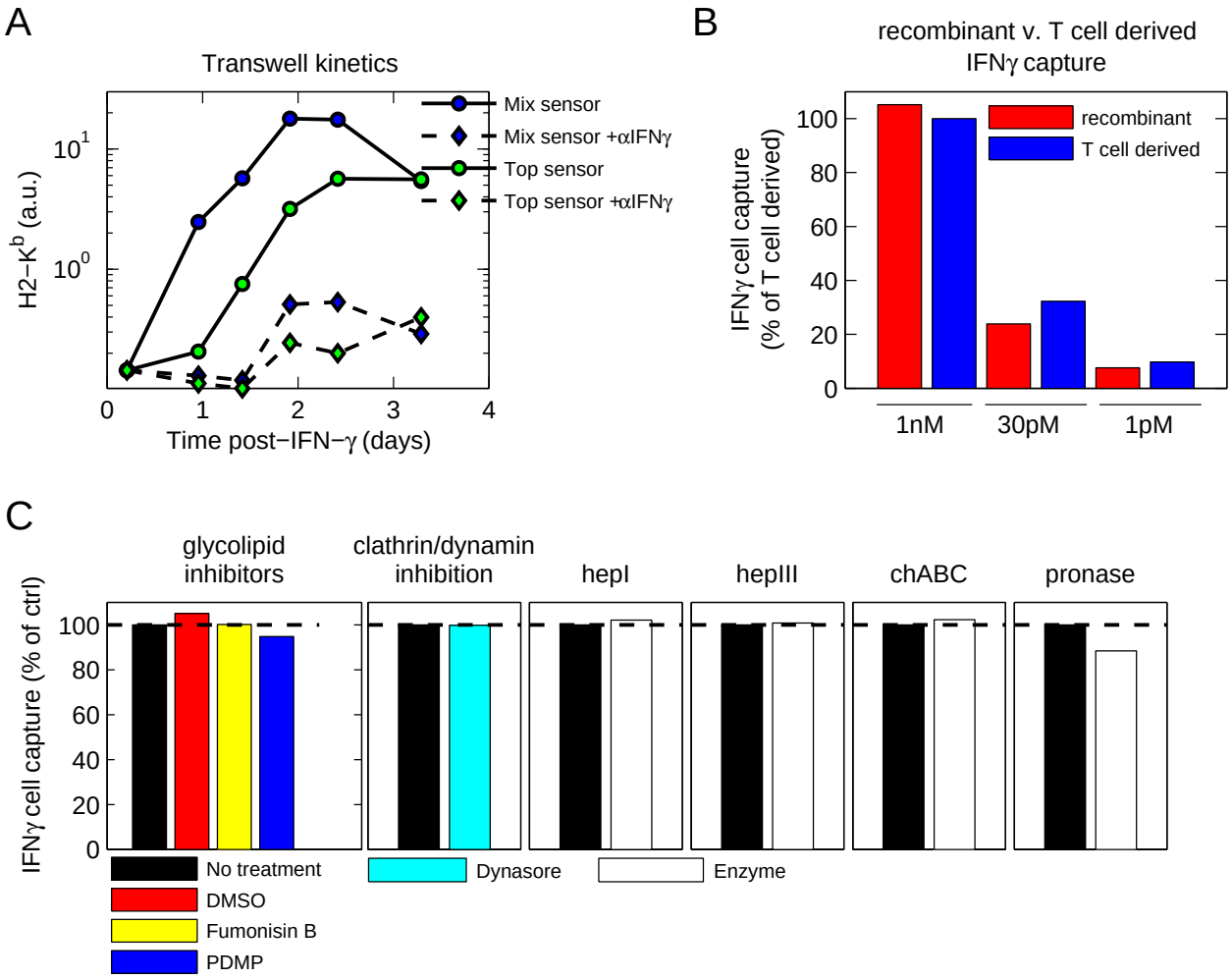
Figure S6: Related to Figure 5. Cytokine catch-and-release could enable communication between spatio- temporally separate cells and is also observed for IL23. (A) **CL**: cardiolipin, **PG**: phosphatidylglycerol, **PC**: phosphatidylcholine, **PE**: phosphatidylethanolamine, **PS**: phosphatidylserine, **PA**: phosphatidic acid, **DAG**: diacylglycerol, **TG**: triglyceride, **ST**: sulfatide, **SM**: sphingomyelin, **Ch**: cholesterol, **PI(3,4,5)P_3**: phosphatidylinositol (3,4,5) triphosphate, **PI(4,5)P_2**: phosphatidylinositol (4,5) bisphosphate, **PI(4)P**: phosphatidylinositol (4) phosphate, **PI**: phosphatidylinositol (B) Naive or activated C57Bl/6 IFNγR KO splenocytes were stained with fluorescent-labeled Annexin V and antibodies directed against B220, CD4 and CD8. Live cells were identified as DAPI-. (C) The tissues noted were isolated from C57Bl/6 IFNγR KO mice and homogenized into a single cell suspension. The IFNγ cell capture assay was then performed. B16 IFNγR KO cells were used as a positive control. N.D. stands for not detected. (D) T cells were isolated from a C57Bl/6 IFNγR KO mouse and activated, or maintained naive in culture in IL7. The IFNγ cell capture assay was performed after 2 days of activation. B16 IFNγR KO cells were used as a positive control. (E) RNA was purified from thyroid tumors, naive T cells, activated T cells, and activated T cells incubated with drug inhibitors. RT-qPCR was performed on each sample for *Ifng* and *Gapdh* mRNA. The quantity of *Ifng* transcripts was computed using the $\Delta\Delta C_T$ method. All samples were normalized to the negative control - naive T cells. (F) Splenocytes were isolated and homogenized from B10.A mice and activated for 48h. T cells were then cultured with a combination of inhibitors for 1h before performing a cytokine secretion assay to detect IFNγ secretion.

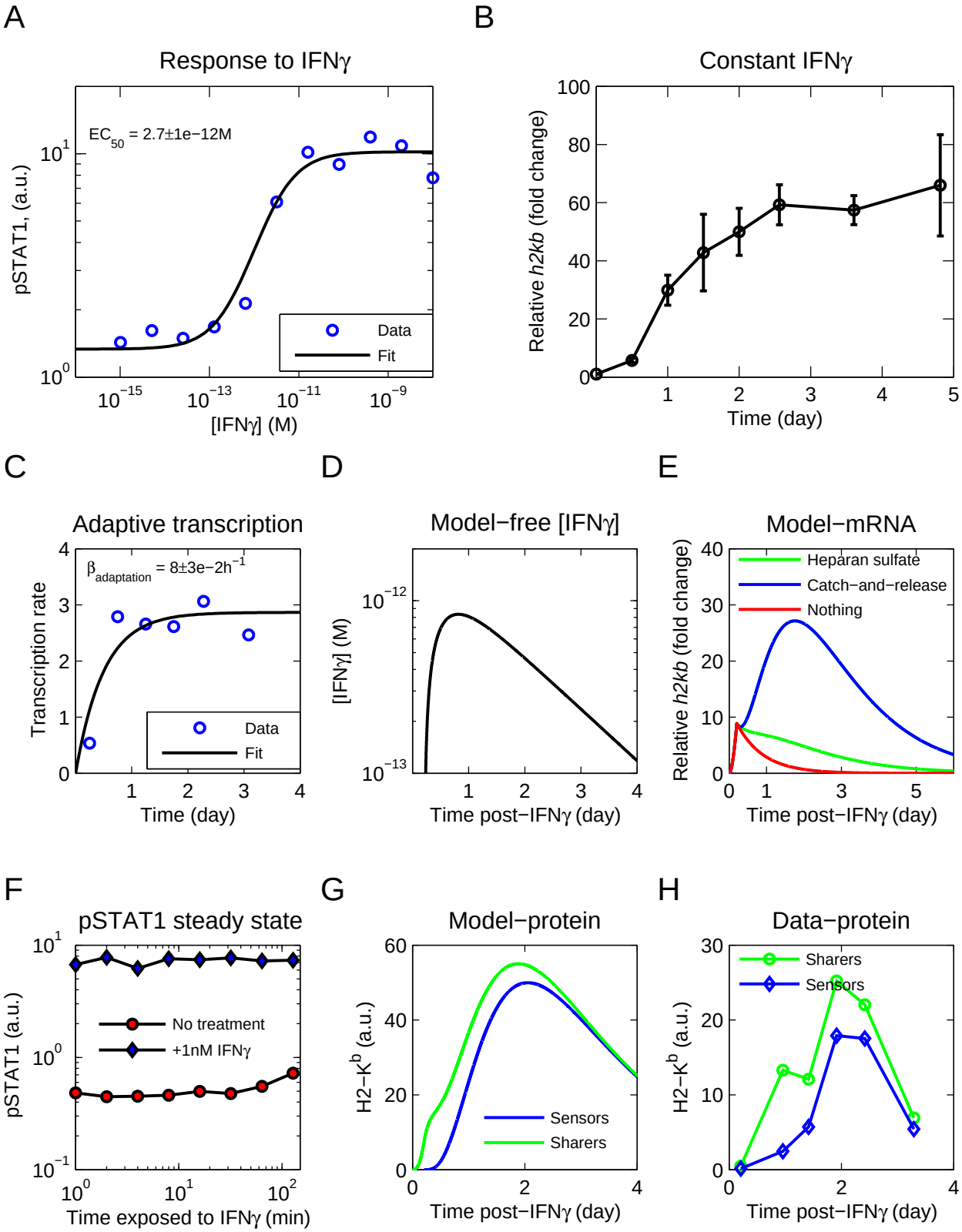
Oyler-Yaniv et al. Figure S1

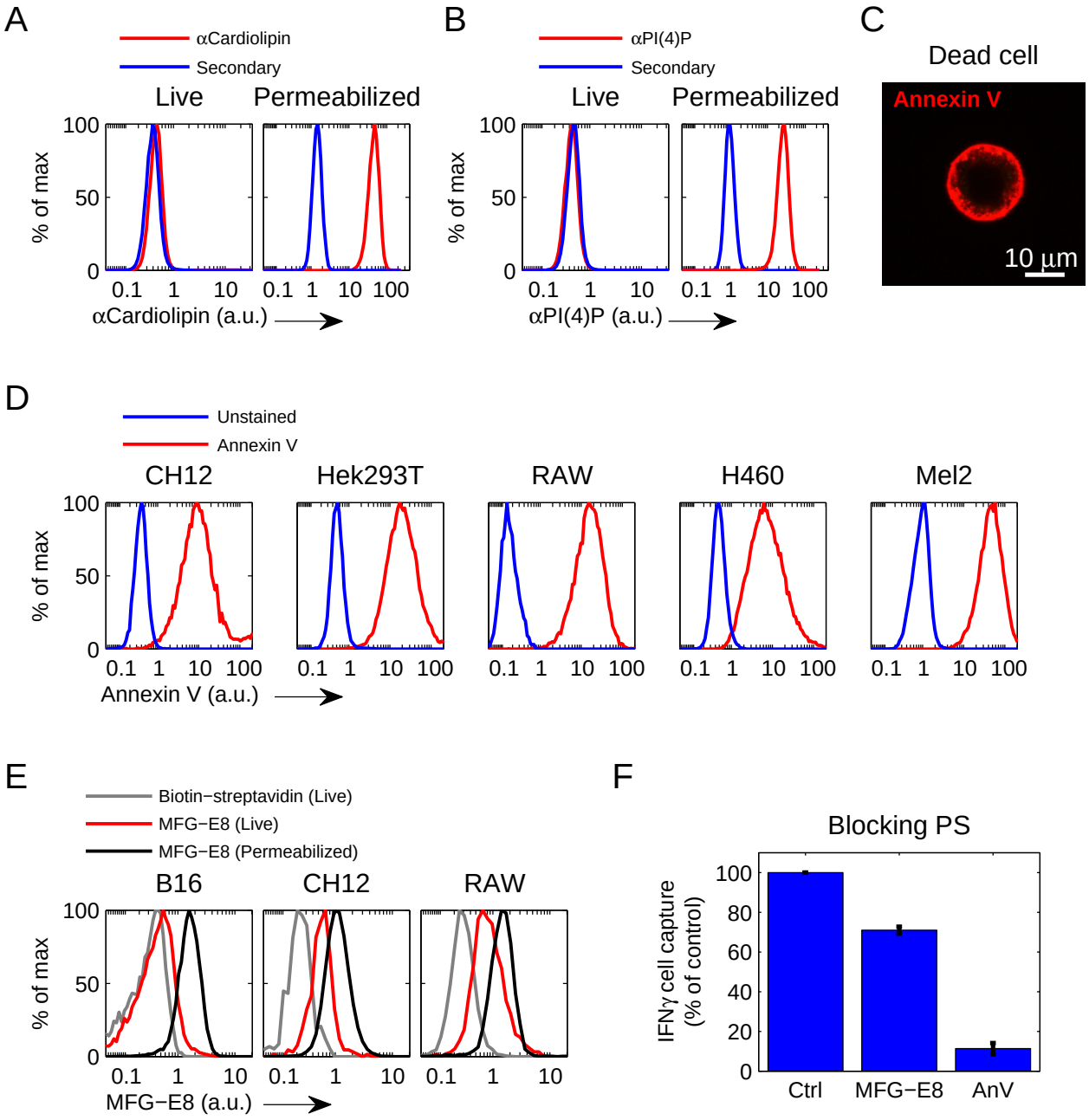


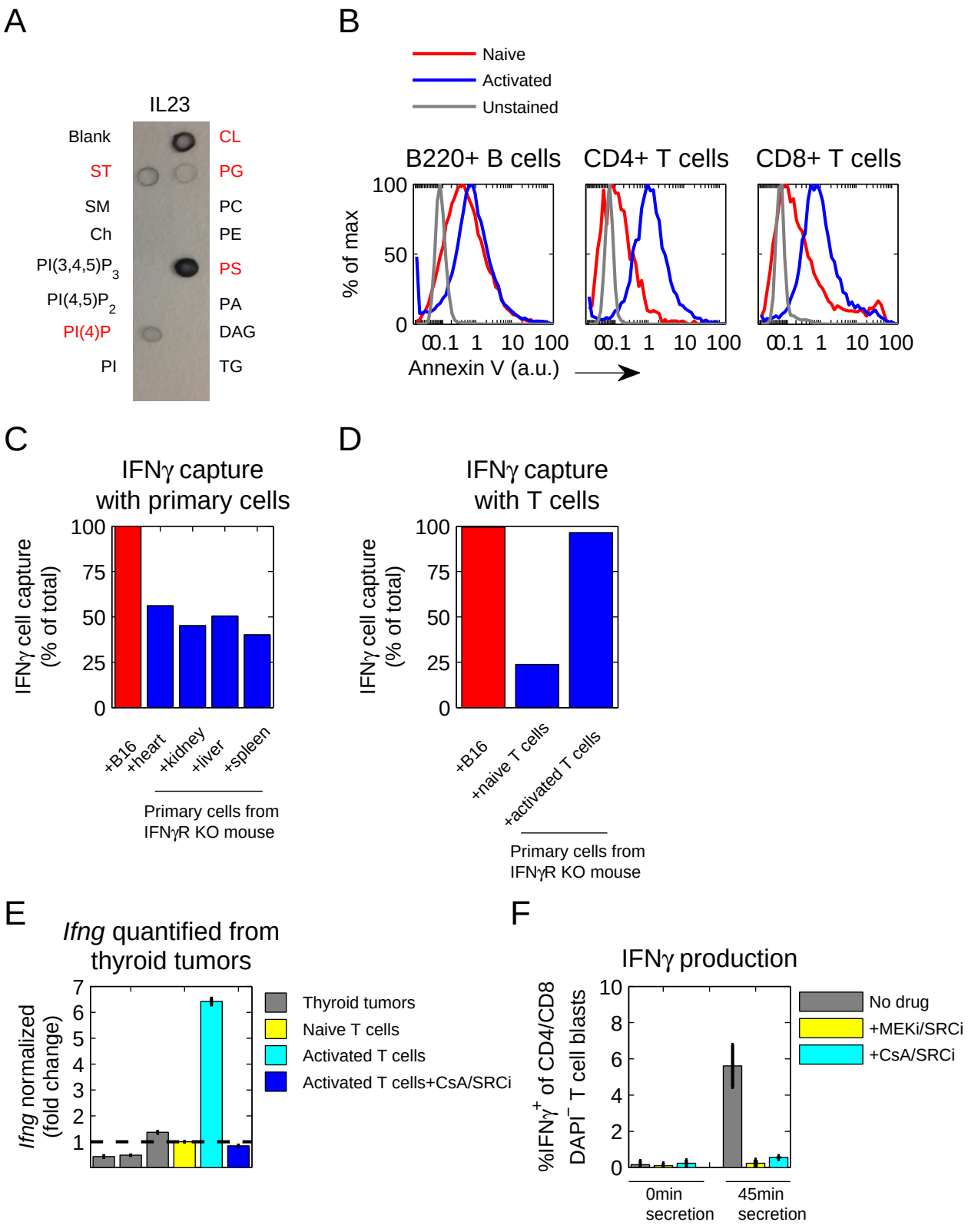
Oyler-Yaniv et al. Figure S2




A
Transwell kinetics
Mix sensor
Mix sensor +αIFNγ
Top sensor
Top sensor +αIFNγ
H2−K^b (a.u.)
Time post−IFN−γ (days)
B
recombinant v. T cell derived IFNγ capture
recombinant
T cell derived
IFNγ cell capture (% of T cell derived)
1nM
30pM
1pM
C
glycolipid inhibitors
clathrin/dynamin inhibition
hepI
hepIII
chABC
pronase
IFNγ cell capture (% of ctrl)
No treatment
DMSO
Fumonisin B
PDMP
Dynasore
Enzyme
D
Wash only
Unlabeled IFNγ
Dye only
Unpulsed
IFNγ−A647 Controls
% of max
IFNγ (a.u.)
E
Alone
+IFNγ pulsed sharer
+IFNγ pulsed sharer+αIFNγ
+IFNγ−488 pulsed sharer
+IFNγ−488 pulsed sharer+αIFNγ
Fluorescent IFNγ share test
Sensor H2−K^b (a.u.)
F
IFNγ
10 μm


A
Response to IFNγ
EC50 = 2.7±1e−12M
pSTAT1, (a.u.)
[IFNγ] (M)
Data
Fit
B
Constant IFNγ
Relative h2kb (fold change)
Time (day)
C
Adaptive transcription
βadaptation = 8±3e−2h−1
Transcription rate
Time (day)
Data
Fit
D
Model−free [IFNγ]
[IFNγ] (M)
Time post−IFNγ (day)
E
Model−mRNA
Heparan sulfate
Catch−and−release
Nothing
Relative h2kb (fold change)
Time post−IFNγ (day)
F
pSTAT1 steady state
No treatment
+1nM IFNγ
pSTAT1 (a.u.)
Time exposed to IFNγ (min)
G
Model−protein
Sensors
Sharers
H2−Kb (a.u.)
Time post−IFNγ (day)
H
Data−protein
Sharers
Sensors
H2−Kb (a.u.)
Time post−IFNγ (day)


A
αCardiolipin
Secondary
Live
Permeabilized
% of max
100
50
0
0.1 1 10 0.1 1 10 100
αCardiolipin (a.u.)
B
αPI(4)P
Secondary
Live
Permeabilized
% of max
100
50
0
0.1 1 10 0.1 1 10 100
αPI(4)P (a.u.)
C
Dead cell
Annexin V
10 µm
D
Unstained
Annexin V
CH12
Hek293T
RAW
H460
Mel2
% of max
100
50
0
0.1 1 10 100
Annexin V (a.u.)
E
Biotin−streptavidin (Live)
MFG−E8 (Live)
MFG−E8 (Permeabilized)
B16
CH12
RAW
% of max
100
50
0
0.1 1 10
MFG−E8 (a.u.)
F
Blocking PS
IFNγ cell capture (% of control)
100
80
60
40
20
0
Ctrl
MFG−E8
AnV


A
IL23
Blank
ST
SM
Ch
PI(3,4,5)P3
PI(4,5)P2
PI(4)P
PI
CL
PG
PC
PE
PS
PA
DAG
TG
B
Naive
Activated
Unstained
B220+ B cells
CD4+ T cells
CD8+ T cells
% of max
100
50
0
0 0.1 1 10 100
Annexin V (a.u.)
C
IFNγ capture
with primary cells
IFNγ cell capture
(% of total)
100
75
50
25
0
+B16
+heart
+kidney
+liver
+spleen
Primary cells from
IFNγR KO mouse
D
IFNγ capture
with T cells
IFNγ cell capture
(% of total)
100
75
50
25
0
+B16
+naive T cells
+activated T cells
Primary cells from
IFNγR KO mouse
E
Ifng quantified from
thyroid tumors
Ifng normalized
(fold change)
7
6
5
4
3
2
1
Thyroid tumors
Naive T cells
Activated T cells
Activated T cells+CsA/SRCi
F
IFNγ production
%IFNγ+ of CD4/CD8
DAPI- T cell blasts
10
8
6
4
2
0
No drug
+MEKi/SRCi
+CsA/SRCi
0min
secretion
45min
secretion

2 Confocal microscopy analysis

Image type	# independent experiments	# cells analyzed
Fluorescent IFNγ alone	4	87
Annexin V alone	3	57
Fluorescent IFNγ and Annexin V	2	66
Membrane stain and IFNγ	3	63
BODIPY-cholesterol and IFNγ	4	132

Table S1: Image analysis quantification - Related to Figure 5