Supplementary information for

The HDAC inhibitor zabadinostat is a systemic regulator of adaptive immunity

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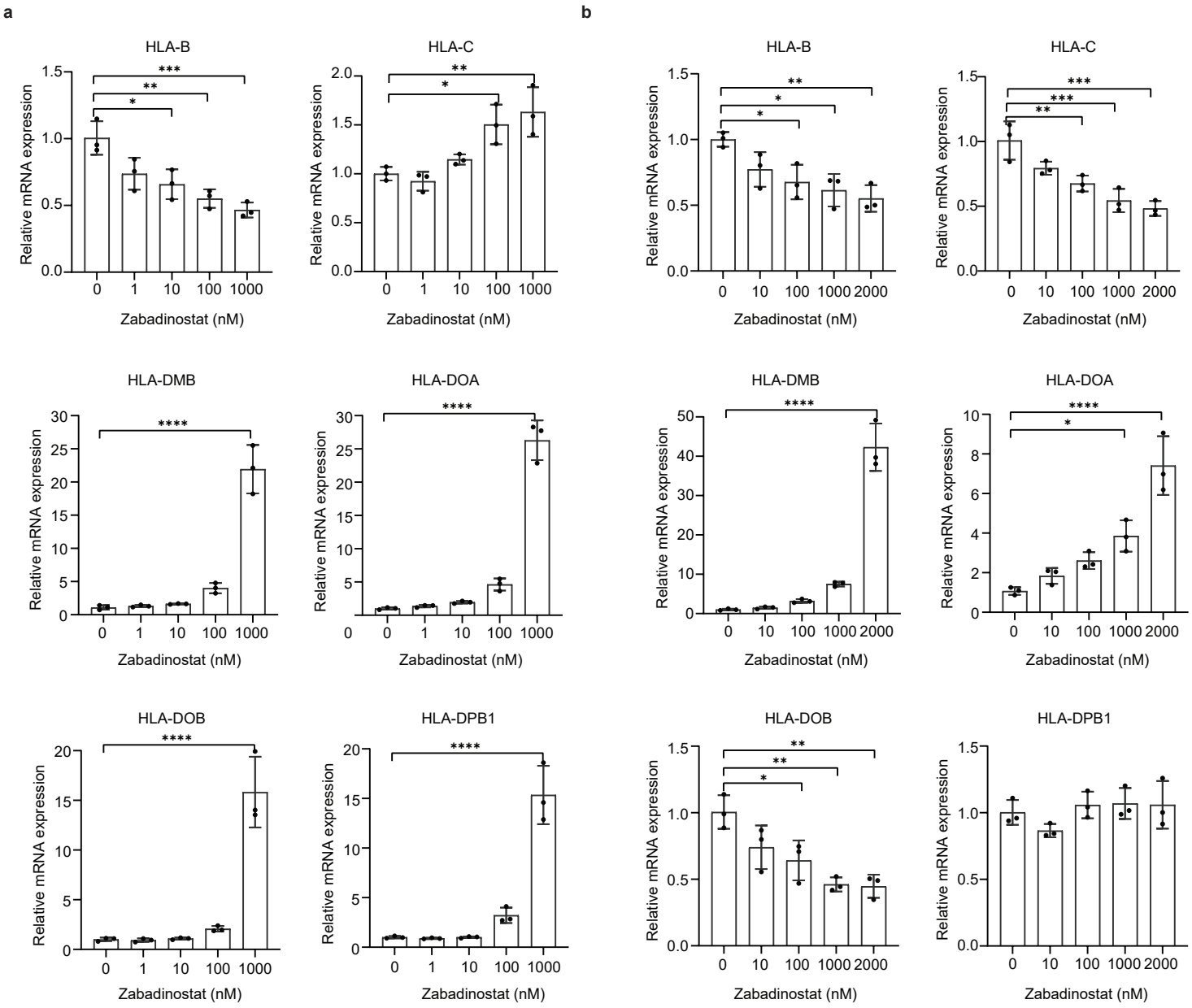
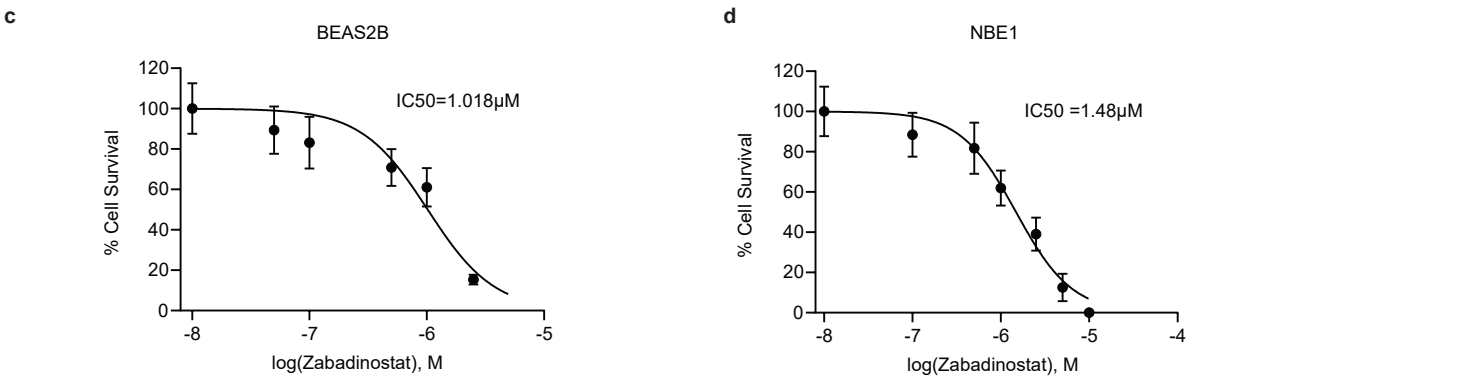
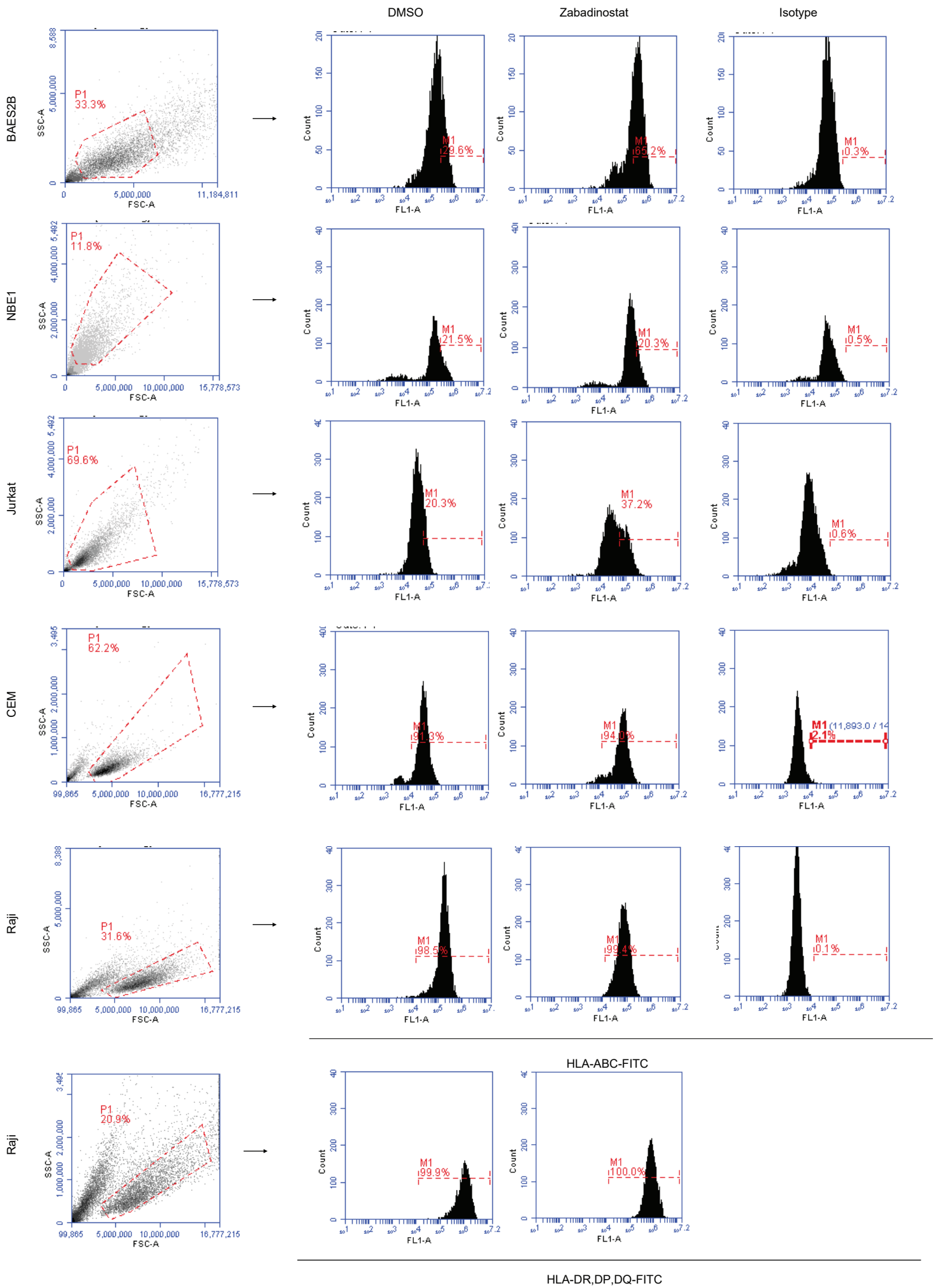
- Supplementary figures and legends S1-S18
- Supplementary dataset 1

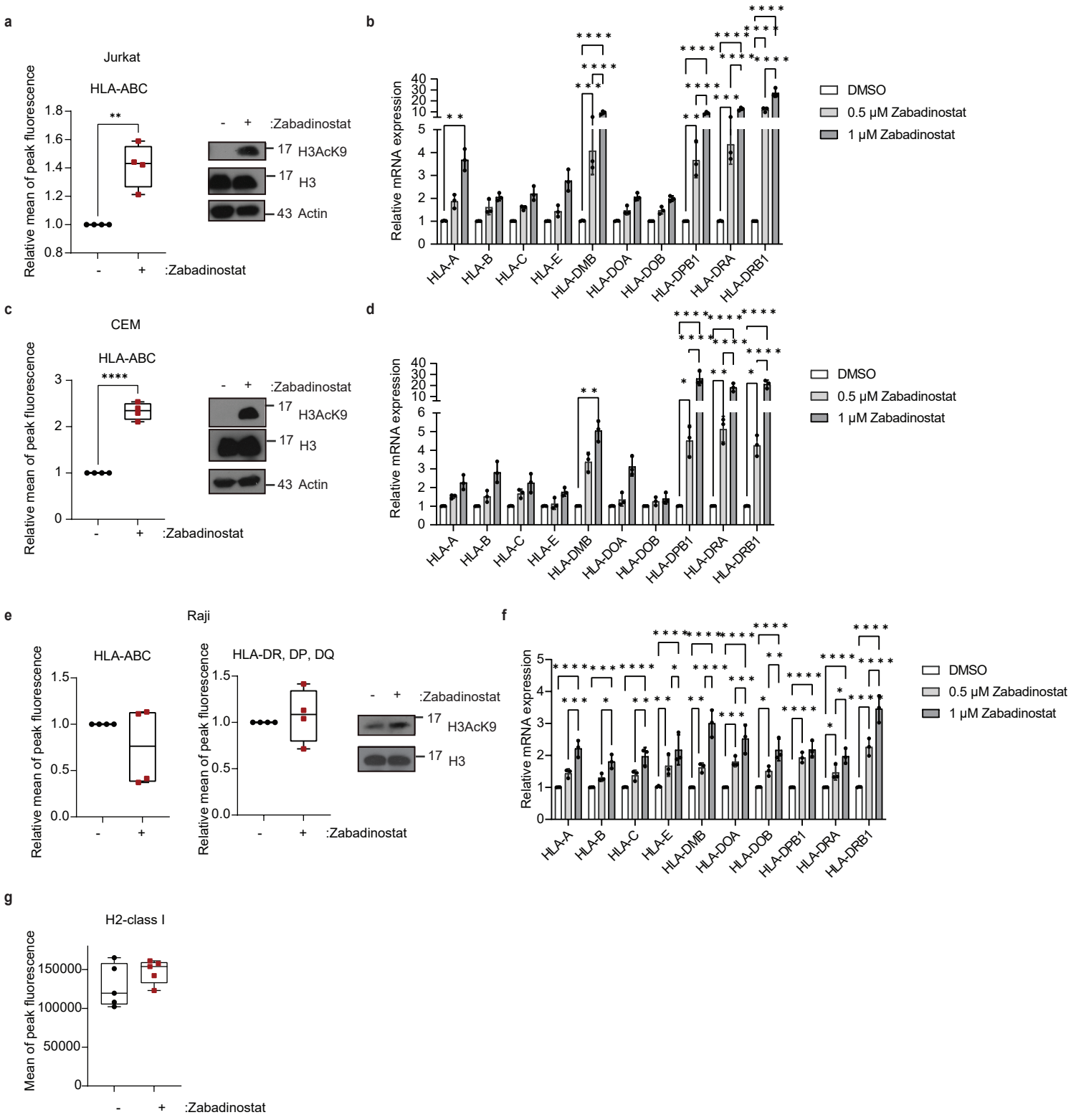

a
HLA-B
HLA-C
HLA-DMB
HLA-DOA
HLA-DOB
HLA-DPB1
Relative mRNA expression
Zabadinostat (nM)
0
1
10
100
1000
b
HLA-B
HLA-C
HLA-DMB
HLA-DOA
HLA-DOB
HLA-DPB1
Relative mRNA expression
Zabadinostat (nM)
0
10
100
1000
2000


c
BEAS2B
IC50=1.018μM
% Cell Survival
log(Zabadinostat), M
d
NBE1
IC50 =1.48μM
% Cell Survival
log(Zabadinostat), M

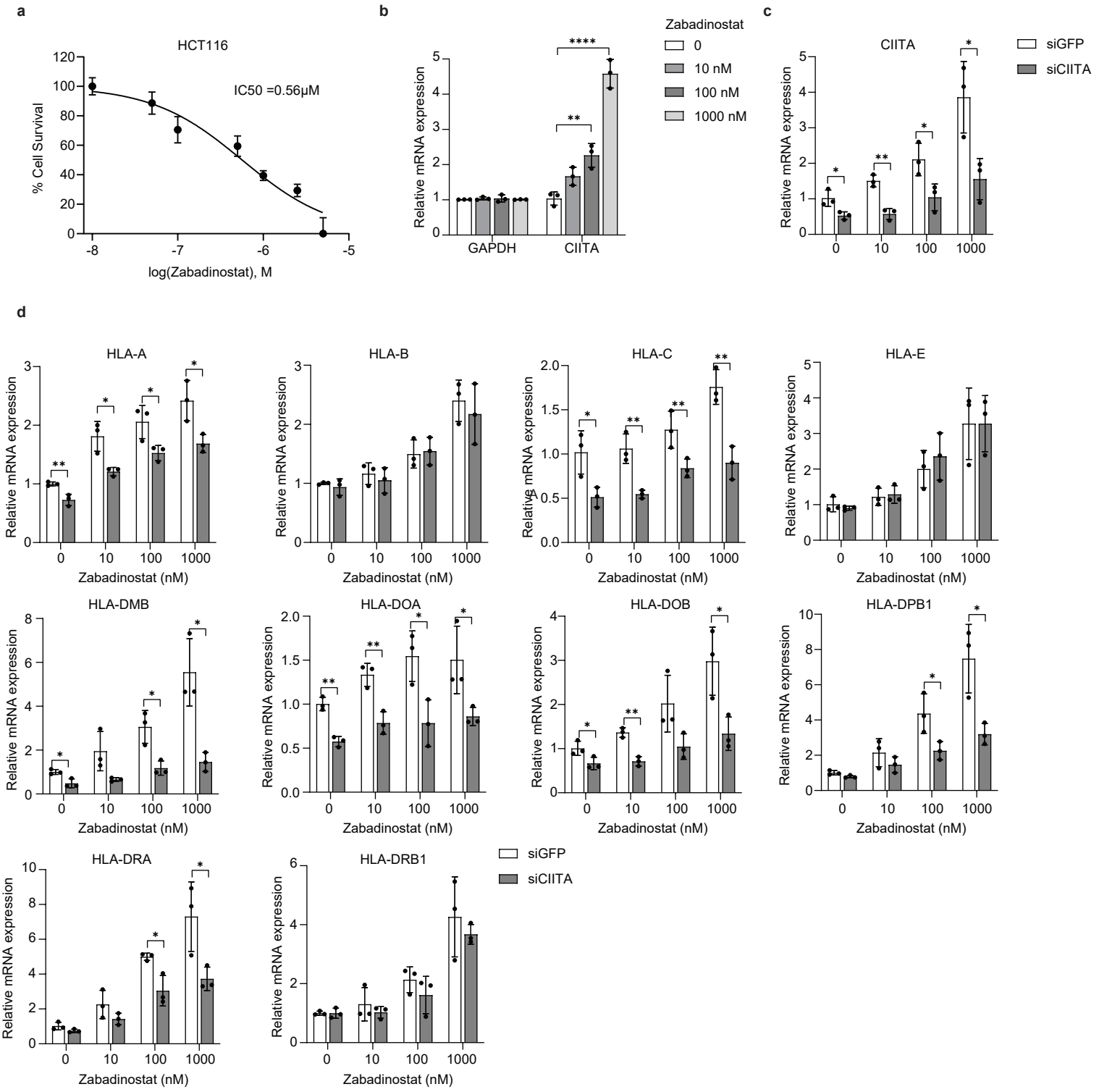
Supplementary Figure 1. Expression of MHC class I and class II genes upon zabadinostat treatment. a) Quantitative reverse transcription PCR (qRT-PCR) of MHC class I and class II genes in BEAS2B cells treated for 3 days with 1, 10, 100, 1000 nM zabadinostat or DMSO control; n=3; results presented as mean values +/-SD; one-way ANOVA; **b)** Quantitative reverse transcription PCR (qRT-PCR) of MHC class I and class II genes in NBE1 cells treated for 3 days with 10, 100, 1000, 2000 nM zabadinostat or DMSO control; n=3; results presented as mean values +/-SD; one-way ANOVA; **c)** An IC50 graph from MTT assays performed after treating BEAS2B cells for 3 days with increasing concentrations of zabadinostat; n=3, results presented as mean values +/-SD; **d)** An IC50 graph from MTT assays performed after treating NBE1 cells for 3 days with increasing concentrations of zabadinostat; n=3, results presented as mean values +/-SD.


DMSO
Zabadinostat
Isotype
BAES2B
P1
33.3%
M1
29.6%
65.2%
0.3%
NBE1
11.8%
21.5%
20.3%
0.5%
Jurkat
69.6%
20.3%
37.2%
0.6%
CEM
62.2%
91.3%
94.0%
2.1%
Raji
31.6%
98.5%
99.4%
0.1%
20.9%
99.9%
100.0%
HLA-ABC-FITC
HLA-DR,DP,DQ-FITC
SSC-A
FSC-A
FL1-A
Count

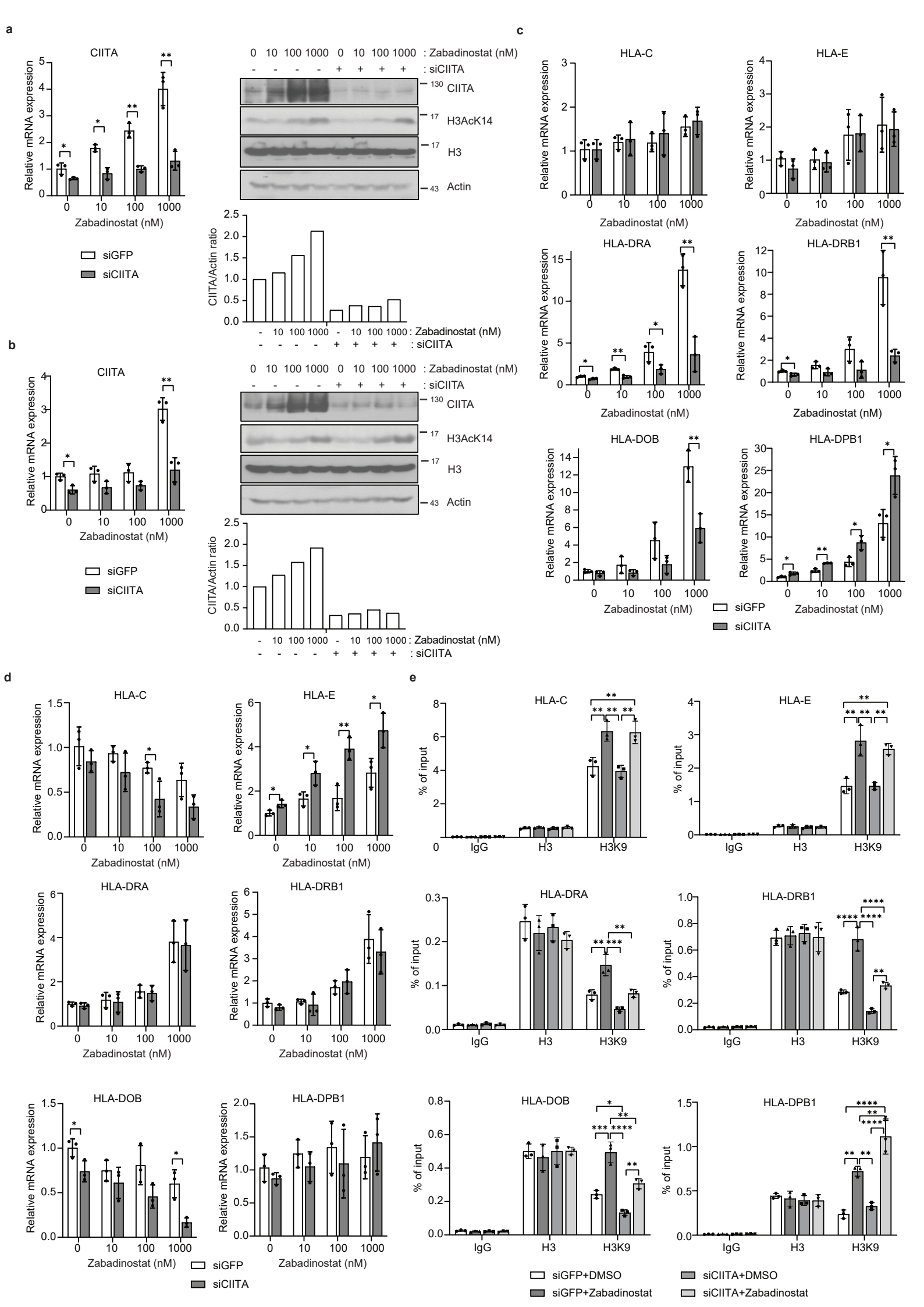
Supplementary Figure 2. Representative flow cytometry plots and gating strategy for the results presented in the Figure 1c-d and Supplementary Figure 3a-c.


a
Jurkat
HLA-ABC
Relative mean of peak fluorescence
:Zabadinostat
17 H3AcK9
17 H3
43 Actin
b
Relative mRNA expression
HLA-A
HLA-B
HLA-C
HLA-E
HLA-DMB
HLA-DOA
HLA-DOB
HLA-DPB1
HLA-DRA
HLA-DRB1
DMSO
0.5 μM Zabadinostat
1 μM Zabadinostat
c
CEM
HLA-ABC
d
e
Raji
HLA-ABC
HLA-DR, DP, DQ
f
g
H2-class I
Mean of peak fluorescence

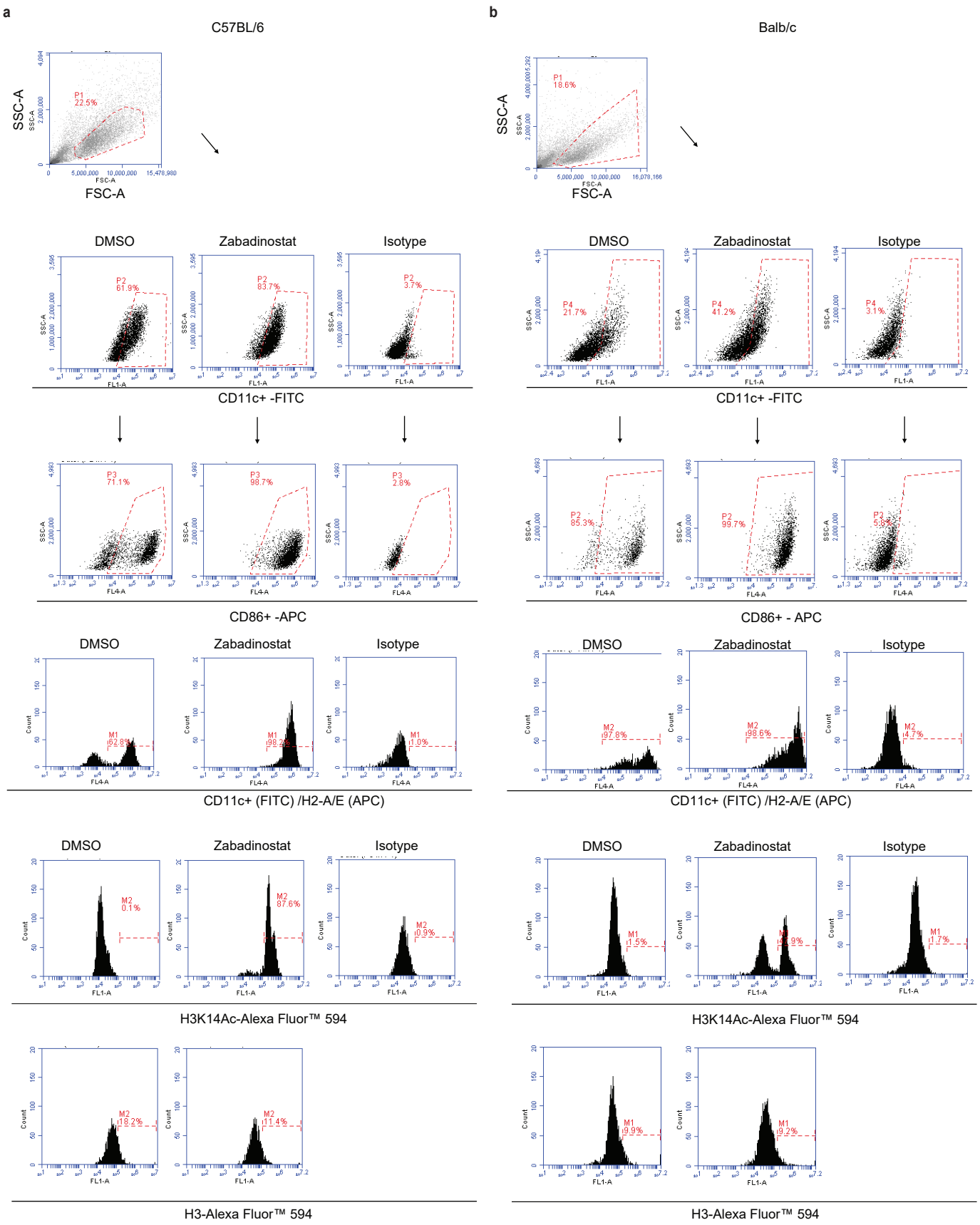
Supplementary Figure 3. Expression of MHC class I and class II genes upon zabadinostat treatment. a) flow cytometry analysis of extracellular MHC class I proteins in Jurkat cells treated for 2 days with 1 µM zabadinostat or DMSO control, n=4, results presented as mean values +/-SD, Student's t test; The acetylation mark (H3AcK9) was detected by immunoblotting; **b)** Quantitative reverse transcription PCR (qRT-PCR) of MHC class I and class II genes in Jurkat cells treated for 2 days with 0.5 and 1 µM zabadinostat or DMSO control; n=3; results presented as mean values +/-SD; one-way ANOVA; **c)** flow cytometry analysis of extracellular MHC class I proteins in CEM cells treated for 2 days with 1 µM zabadinostat or DMSO control, n=4, results presented as mean values +/-SD, Student's t test; The acetylation mark (H3AcK9) was detected by immunoblotting; **d)** Quantitative reverse transcription PCR (qRT-PCR) of MHC class I and class II genes in CEM cells treated for 2 days with 0.5 and 1 µM zabadinostat or DMSO control; n=3; results presented as mean values +/-SD; one-way ANOVA; **e)** flow cytometry analysis of extracellular MHC class I and II proteins in Raji cells treated for 2 days with 1 µM zabadinostat or DMSO control, n=4; The acetylation mark (H3AcK9) was detected by immunoblotting; **f)** Quantitative reverse transcription PCR (qRT-PCR) of MHC class I and class II genes in Raji cells treated for 2 days with 0.5 and 1 µM zabadinostat or DMSO control; n=3; results presented as mean values +/-SD; one-way ANOVA; **g)** flow cytometry analysis of extracellular MHC class I in bone marrow (collected from C57BL/6)-derived dendritic cells (CD11c+/CD86+) (from Figure 3a) treated with 1 µM zabadinostat or DMSO control for 48 hours; n=5.


a
HCT116
IC50 =0.56μM
% Cell Survival
log(Zabadinostat), M
b
Zabadinostat
0
10 nM
100 nM
1000 nM
Relative mRNA expression
GAPDH
CIITA
c
siGFP
siCIITA
CIITA
Relative mRNA expression
d
HLA-A
HLA-B
HLA-C
HLA-E
HLA-DMB
HLA-DOA
HLA-DOB
HLA-DPB1
HLA-DRA
HLA-DRB1
Relative mRNA expression
Zabadinostat (nM)

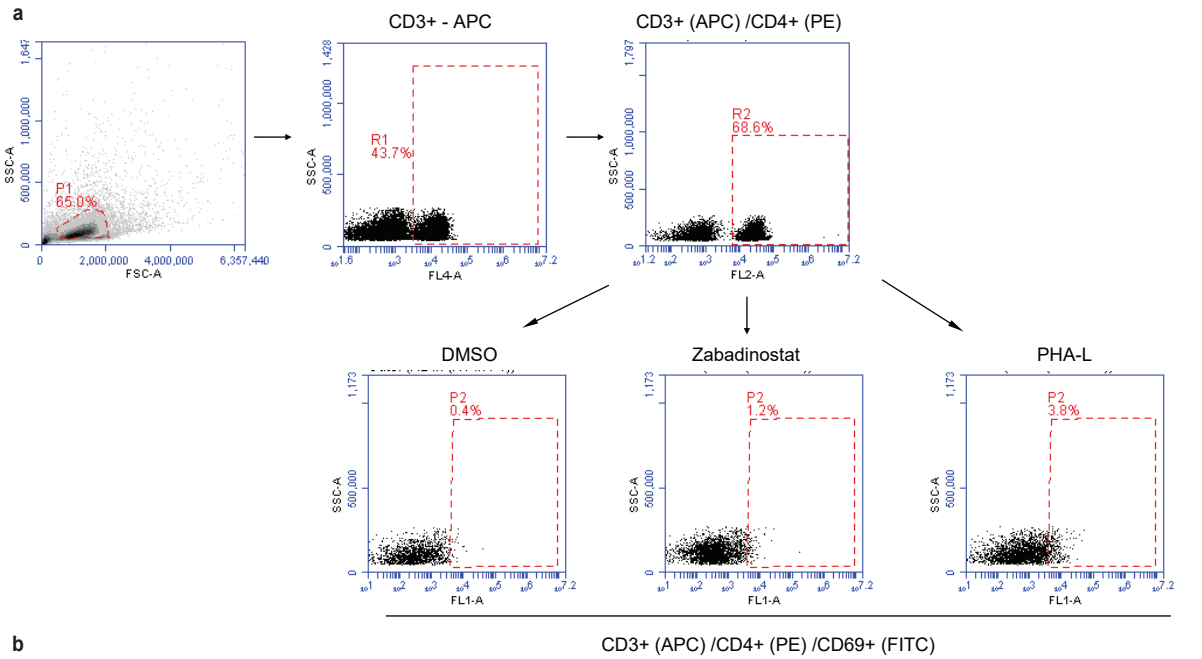
Supplementary Figure 4. CIITA is a positive regulator of MHC class I and class II gene expression in HCT116 cells. a) An IC50 graph from MTT assays performed after treating HCT116 cells for 3 days with increasing concentrations of zabadinostat; n=3, results presented as mean values +/-SD; **b)** Quantitative reverse transcription PCR (qRT-PCR) of CIITA in HCT116 cells treated for 3 days with 10, 100, 1000 nM zabadinostat or DMSO control; n=3; results presented as mean values +/-SD; one-way ANOVA; **c)** The expression of CIITA after siRNA treatment were detected by qRT-PCR; results presented as mean values +/-SD; one-way ANOVA; **d)** Quantitative reverse transcription PCR (qRT-PCR) of MHC class I and class II genes in HCT116 cells treated for 2 days with 50nM siCIITA and for 3 days with 10, 100, 1000 nM zabadinostat or DMSO control; n=3; results presented as mean values +/-SD; one-way ANOVA;.

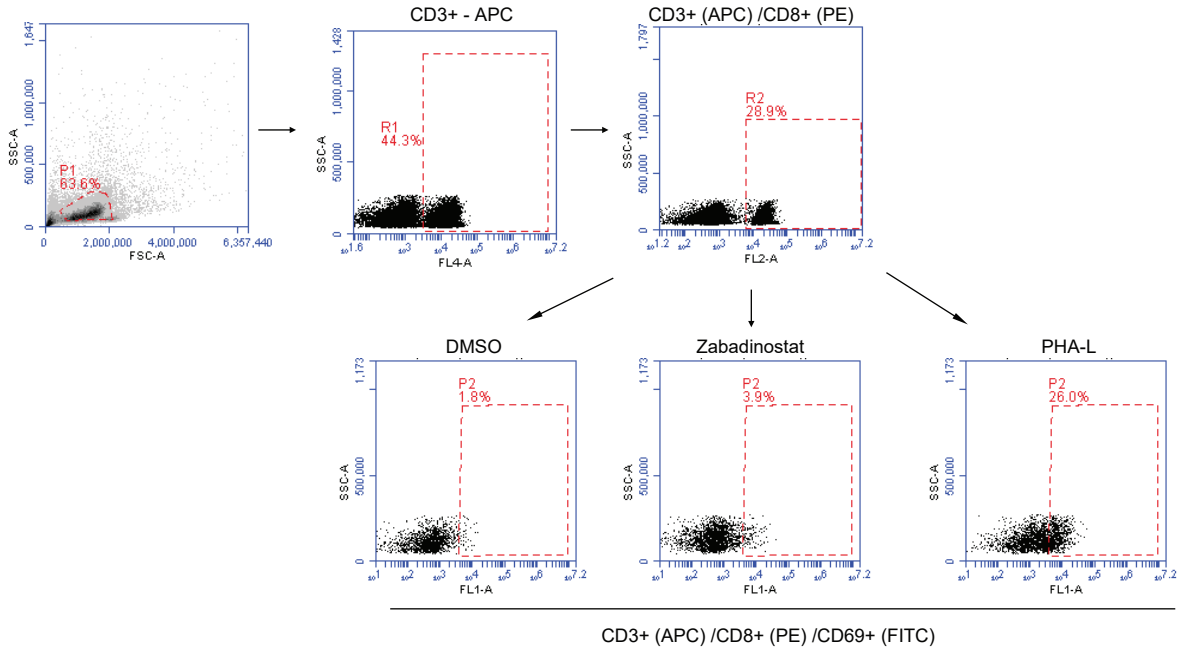

a
CIITA
Relative mRNA expression
Zabadinostat (nM)
siGFP
siCIITA
0 10 100 1000 0 10 100 1000 : Zabadinostat (nM)
- - - - + + + + : siCIITA
130 CIITA
17 H3AcK14
17 H3
43 Actin
CIITA/Actin ratio
b
CIITA
Relative mRNA expression
Zabadinostat (nM)
siGFP
siCIITA
0 10 100 1000 0 10 100 1000 : Zabadinostat (nM)
- - - - + + + + : siCIITA
130 CIITA
17 H3AcK14
17 H3
43 Actin
CIITA/Actin ratio
c
HLA-C
HLA-E
HLA-DRA
HLA-DRB1
HLA-DOB
HLA-DPB1
Relative mRNA expression
Zabadinostat (nM)
siGFP
siCIITA
d
HLA-C
HLA-E
HLA-DRA
HLA-DRB1
HLA-DOB
HLA-DPB1
Relative mRNA expression
Zabadinostat (nM)
siGFP
siCIITA
e
HLA-C
HLA-E
HLA-DRA
HLA-DRB1
HLA-DOB
HLA-DPB1
% of input
IgG
H3
H3K9
siGFP+DMSO
siGFP+Zabadinostat
siCIITA+DMSO
siCIITA+Zabadinostat

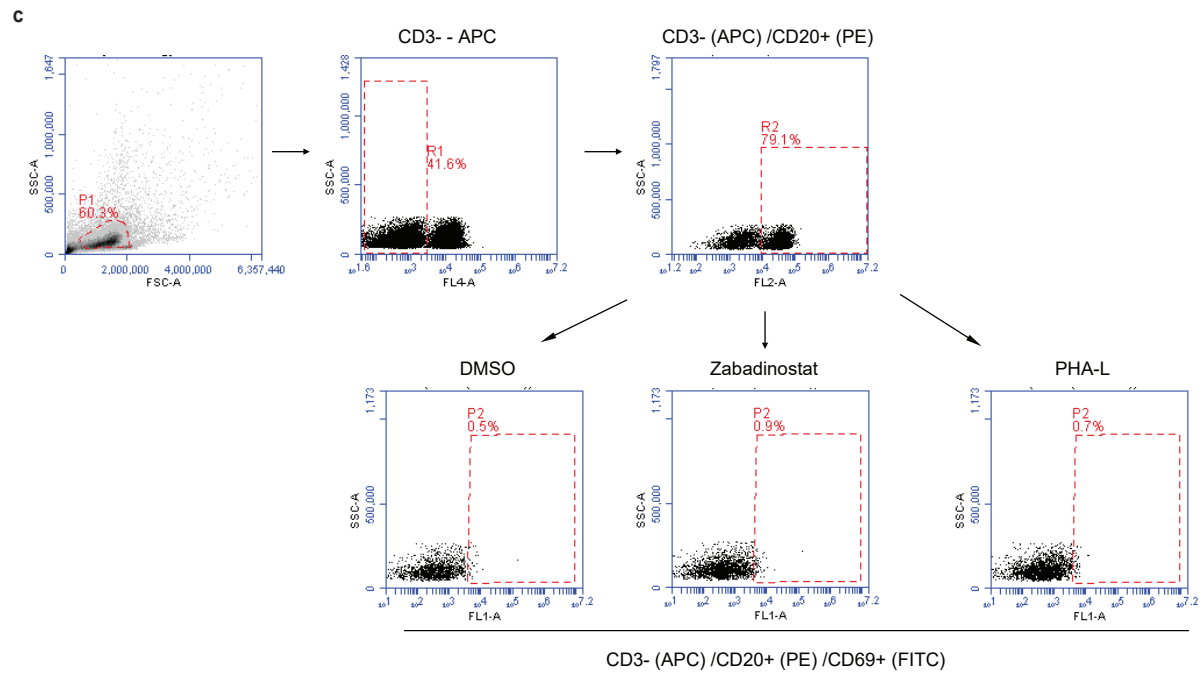
Supplementary Figure 5. CIITA is a positive regulator of MHC class I and class II genes in BEAS2B and NBE1 cells. a) The expression of CIITA after siRNA treatment were detected by qRT-PCR, n=3; results presented as mean values +/-SD; one-way ANOVA; The CIITA protein level and acetylation mark (H3AcK14) were detected by immunoblotting and quantified; **b)** The expression of CIITA after siRNA treatment was detected by qRT-PCR, n=3; results presented as mean values +/-SD; one-way ANOVA; The CIITA protein level and acetylation mark (H3AcK14) were detected by immunoblotting and quantified; **c)** Quantitative reverse transcription PCR (qRT-PCR) of MHC class I and class II genes in BEAS2B cells treated for 2 days with 50nM siCIITA and for 3 days with 10, 100, 1000 nM zabadinostat or DMSO control; n=3; results presented as mean values +/-SD; one-way ANOVA; **d)** Quantitative reverse transcription PCR (qRT-PCR) of MHC class I and class II genes in NBE1 cells treated for 2 days with 50nM siCIITA and for 3 days with 10, 100, 1000 nM zabadinostat or DMSO control; n=3; results presented as mean values +/-SD; one-way ANOVA; **e)** Histone H3 and H3AcK9 ChIP on MHC gene promoters in BEAS2B cells treated for 2 days with 50nM siCIITA and for 3 days with 1000 nM zabadinostat or DMSO control; n=3; results presented as mean values +/-SD; one-way ANOVA.


a
C57BL/6
b
Balb/c
SSC-A
FSC-A
P1
22.5%
P1
18.6%
DMSO
Zabadinostat
Isotype
P2
61.9%
P2
83.7%
P2
3.7%
P4
21.7%
P4
41.2%
P4
3.1%
CD11c+ -FITC
P3
71.1%
P3
98.7%
P3
2.8%
P2
85.3%
P2
99.7%
P2
5.8%
CD86+ -APC
CD86+ - APC
M1
62.8%
M1
98.2%
M1
1.0%
M2
97.8%
M2
98.6%
M2
4.7%
CD11c+ (FITC) /H2-A/E (APC)
M2
0.1%
M2
87.6%
M2
0.9%
M1
1.5%
M1
1.7%
H3K14Ac-Alexa Fluor™ 594
M2
18.2%
M2
11.4%
M1
9.9%
M1
9.2%
H3-Alexa Fluor™ 594

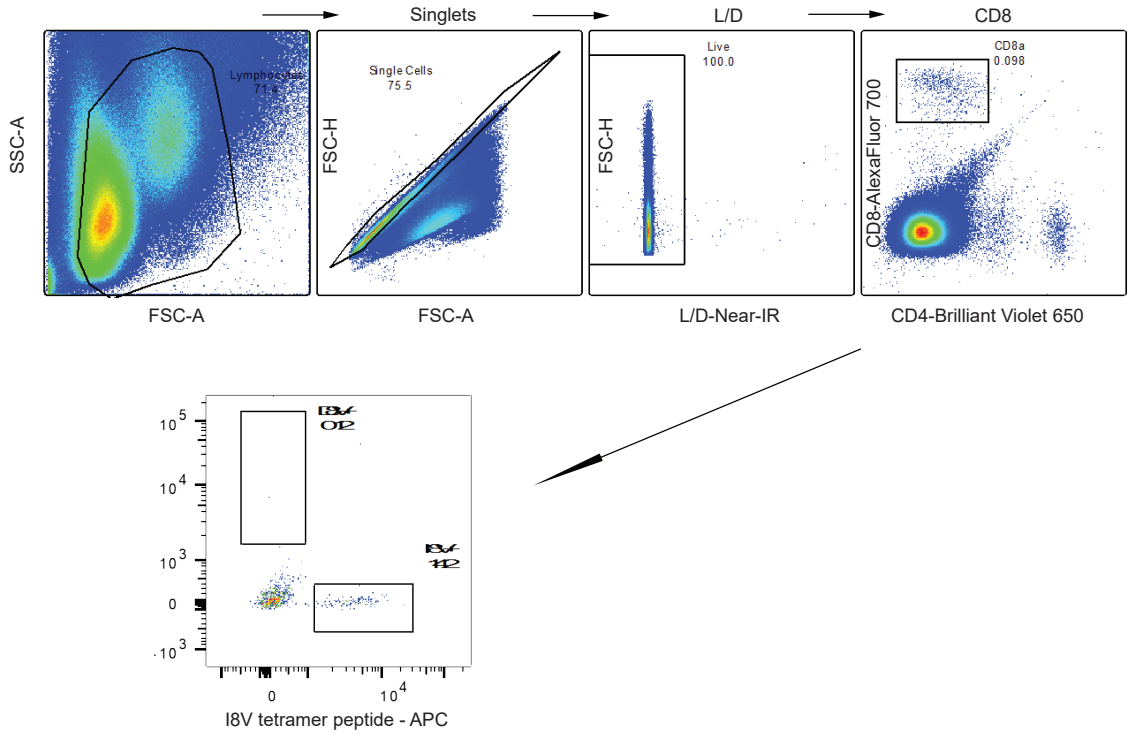
Supplementary Figure 6. Representative flow cytometry plots and gating strategy (a-b) for the results presented in the Figure 3a and 3c.


a
CD3+ - APC
CD3+ (APC) /CD4+ (PE)
P1
65.0%
R1
43.7%
R2
68.6%
SSC-A
FSC-A
FL4-A
FL2-A
DMSO
Zabadinostat
PHA-L
P2
0.4%
P2
1.2%
P2
3.8%
FL1-A
CD3+ (APC) /CD4+ (PE) /CD69+ (FITC)


b
CD3+ - APC
CD3+ (APC) /CD8+ (PE)
P1
63.6%
R1
44.3%
R2
28.9%
SSC-A
FSC-A
FL4-A
FL2-A
DMSO
Zabadinostat
PHA-L
P2
1.8%
P2
3.9%
P2
26.0%
FL1-A
CD3+ (APC) /CD8+ (PE) /CD69+ (FITC)

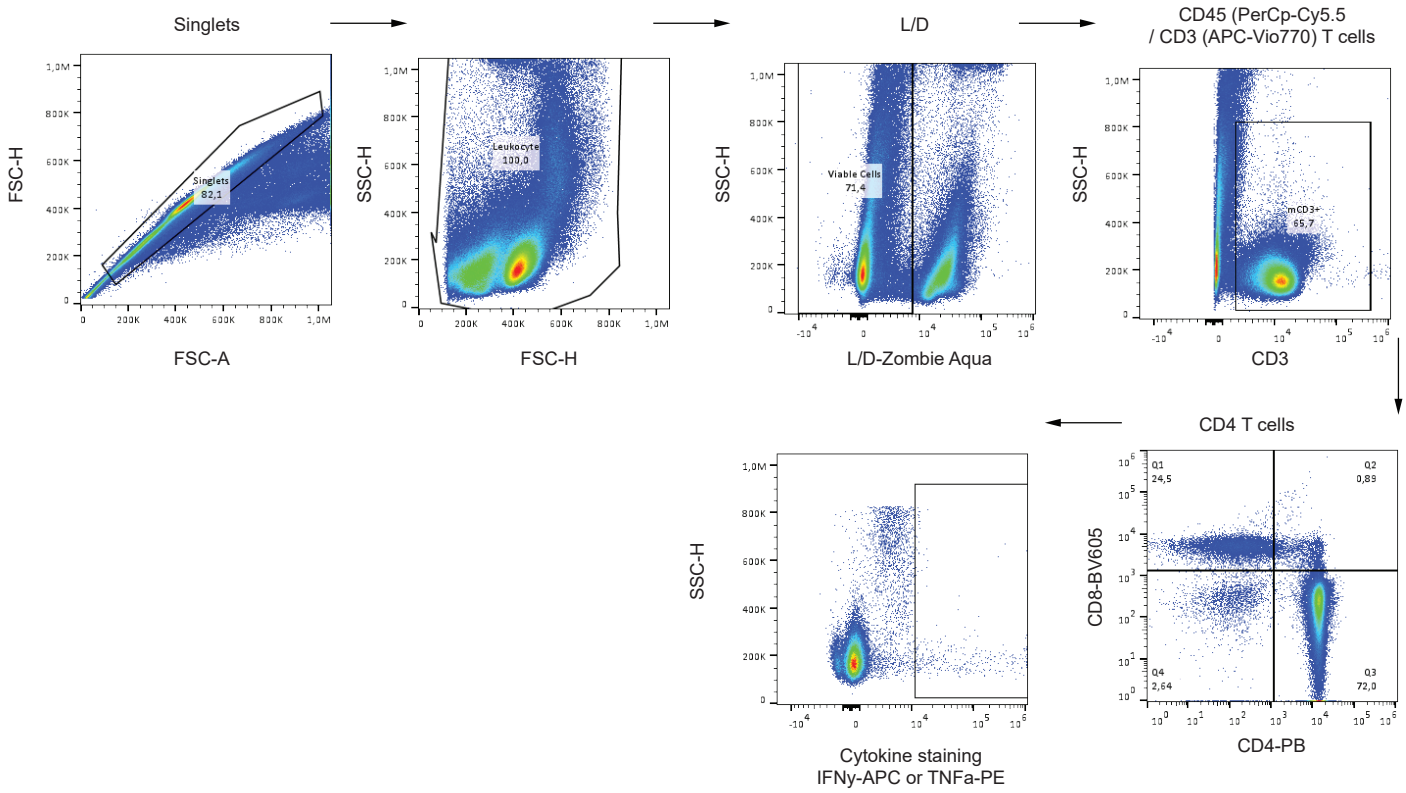

c
CD3- - APC
CD3- (APC) /CD20+ (PE)
P1
60.3%
R1
41.6%
R2
79.1%
SSC-A
FSC-A
FL4-A
FL2-A
DMSO
Zabadinostat
PHA-L
P2
0.5%
P2
0.9%
P2
0.7%
FL1-A
CD3- (APC) /CD20+ (PE) /CD69+ (FITC)

Supplementary Figure 7. Representative flow cytometry plots and gating strategy (a-c) for the results presented in the Figure 3d.

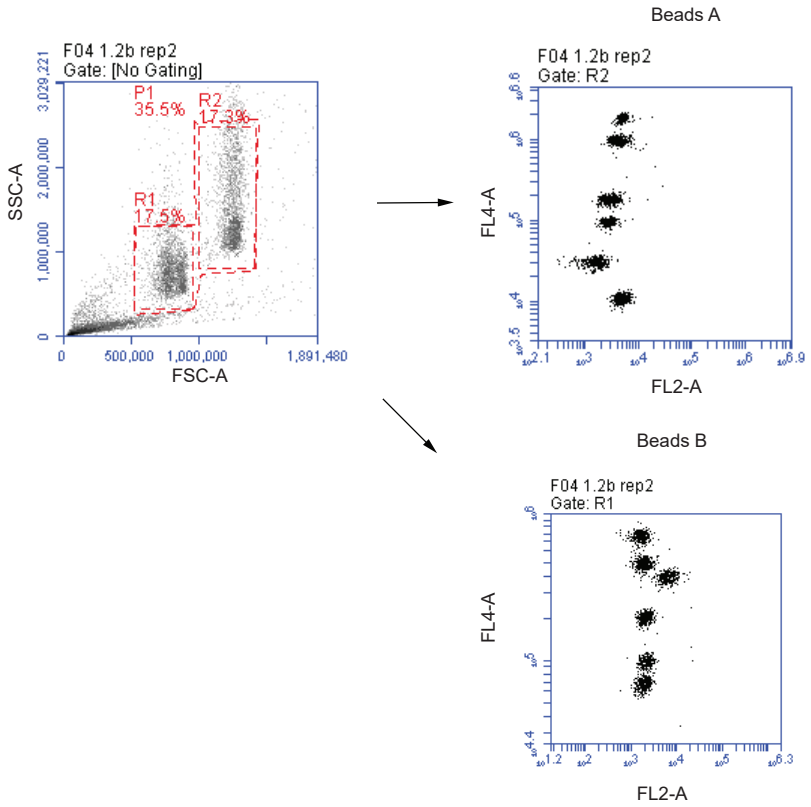

Singlets
L/D
CD8
SSC-A
Lymphocytes
71.4
FSC-A
FSC-H
Single Cells
75.5
FSC-A
FSC-H
Live
100.0
L/D-Near-IR
CD8-AlexaFluor 700
CD8a
0.098
CD4-Brilliant Violet 650

I8V tetramer peptide - APC

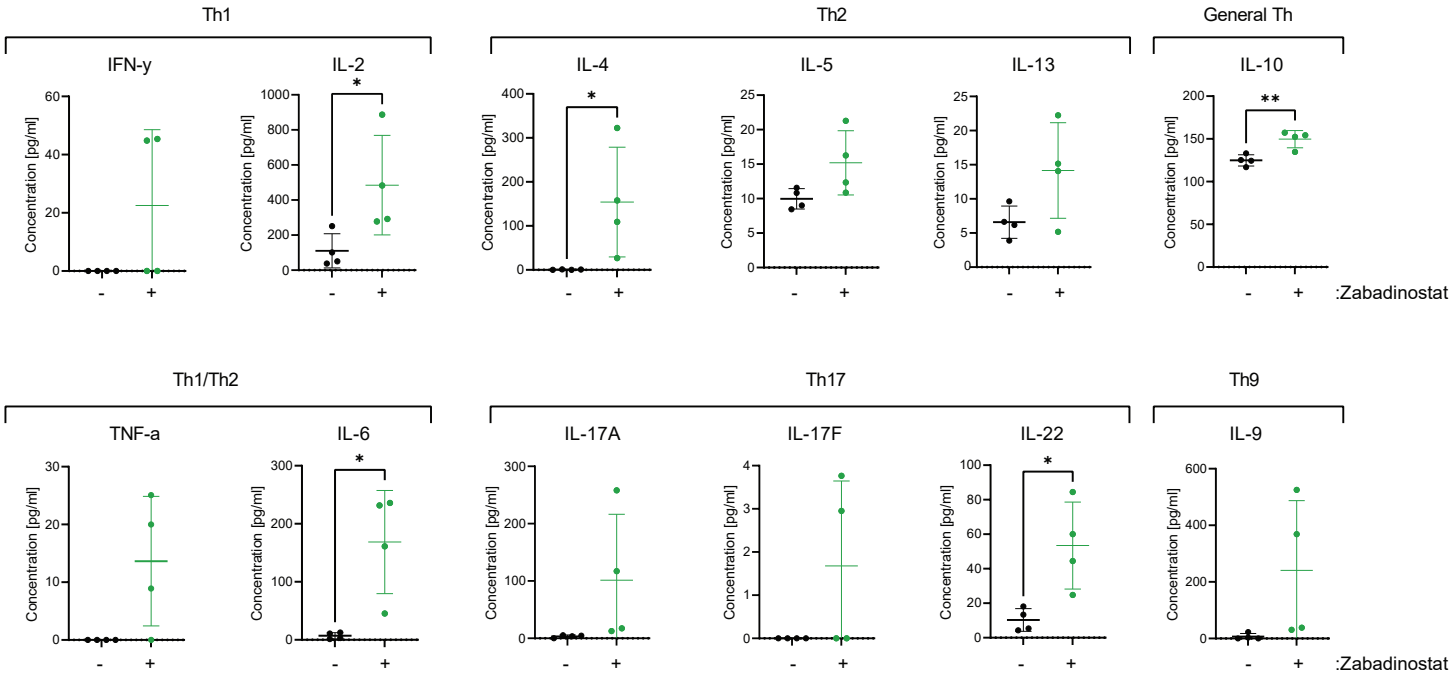
Supplementary Figure 8. Representative flow cytometry plots and gating strategy for the results presented in the Figure 4c-d.


Singlets
FSC-H
FSC-A
Singlets
82,1
SSC-H
FSC-H
Leukocyte
100,0
L/D
SSC-H
L/D-Zombie Aqua
Viable Cells
71,4
CD45 (PerCp-Cy5.5
/ CD3 (APC-Vio770) T cells
SSC-H
CD3
mCD3+
65,7
CD4 T cells
CD8-BV605
CD4-PB
Q1
24,5
Q2
0,89
Q4
2,64
Q3
72,0
SSC-H
Cytokine staining
IFNy-APC or TNFa-PE

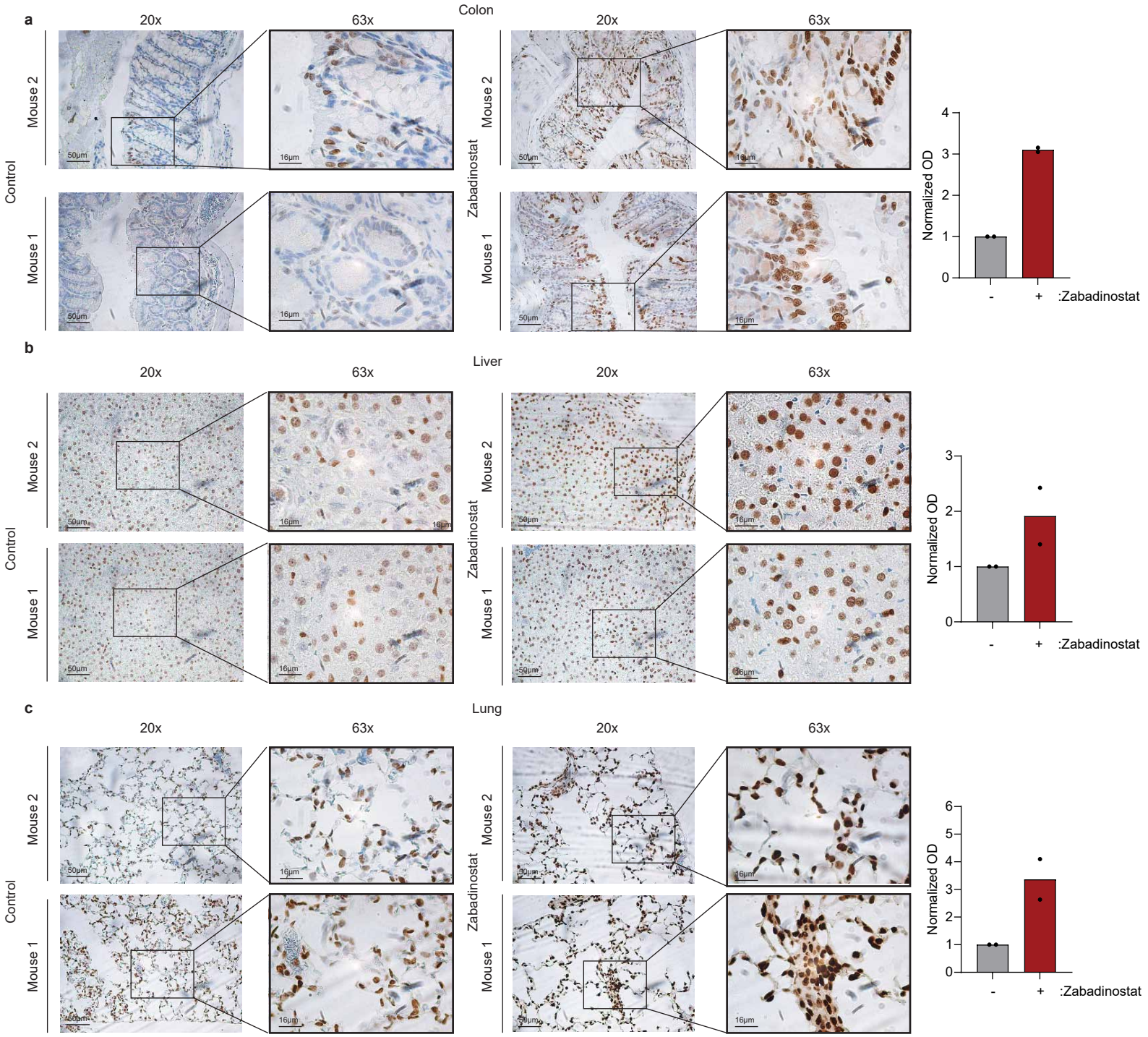
Supplementary Figure 9. Representative flow cytometry plots and gating strategy for the results presented in the Figure 5c and Supplementary Figure 15.


F04 1.2b rep2
Gate: [No Gating]
P1
35.5%
R2
17.3%
R1
17.5%
SSC-A
FSC-A
0
1,000,000
2,000,000
3,029,221
500,000
1,891,480
Beads A
F04 1.2b rep2
Gate: R2
FL4-A
FL2-A
Beads B
F04 1.2b rep2
Gate: R1
FL4-A
FL2-A

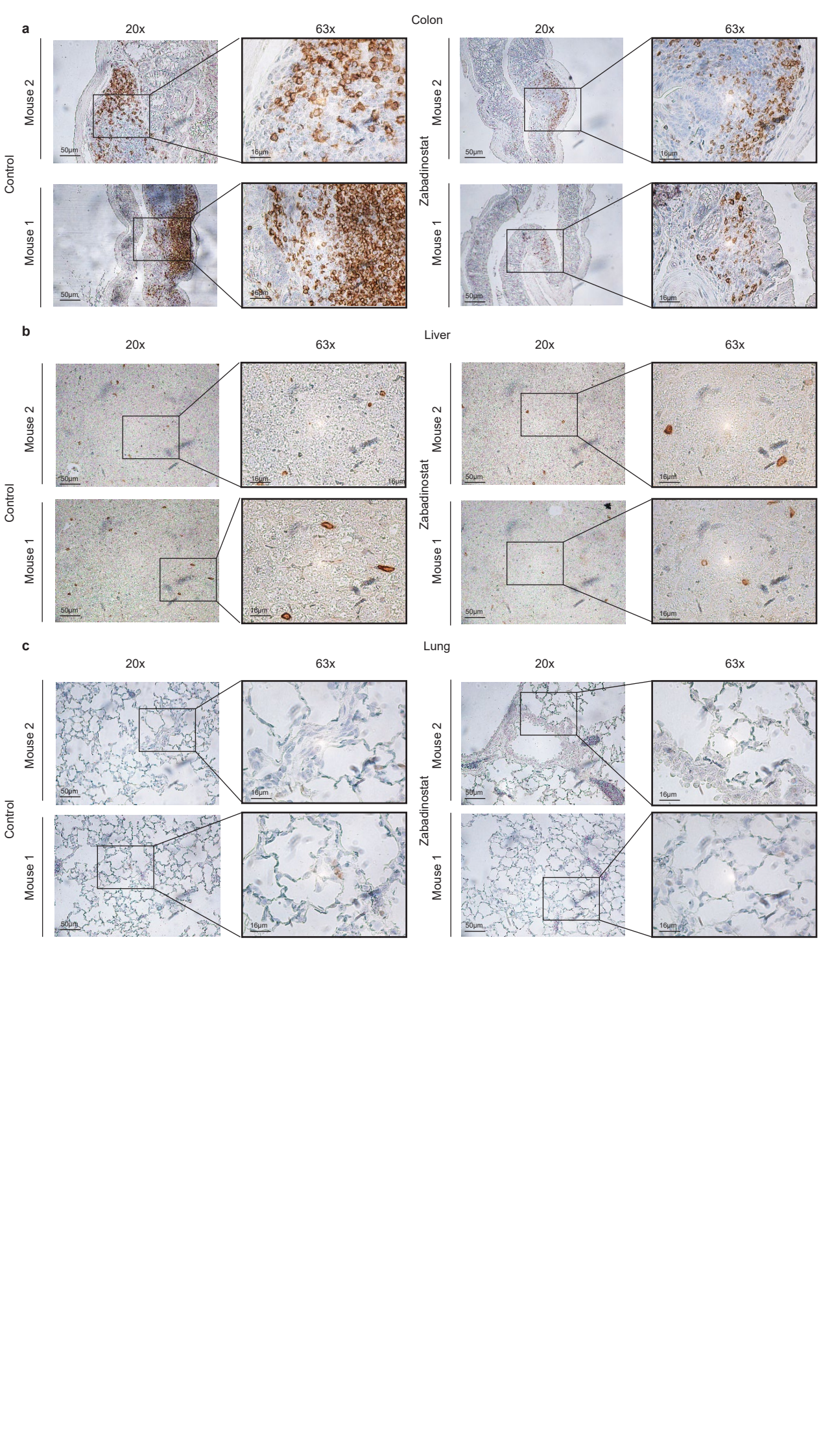
Supplementary Figure 10. Representative flow cytometry plots and gating strategy for the results presented in the Figure 5d and Supplementary Figure 11.


Th1
IFN-y
IL-2
Th2
IL-4
IL-5
IL-13
General Th
IL-10
Concentration [pg/ml]
:Zabadinostat
Th1/Th2
TNF-a
IL-6
Th17
IL-17A
IL-17F
IL-22
Th9
IL-9

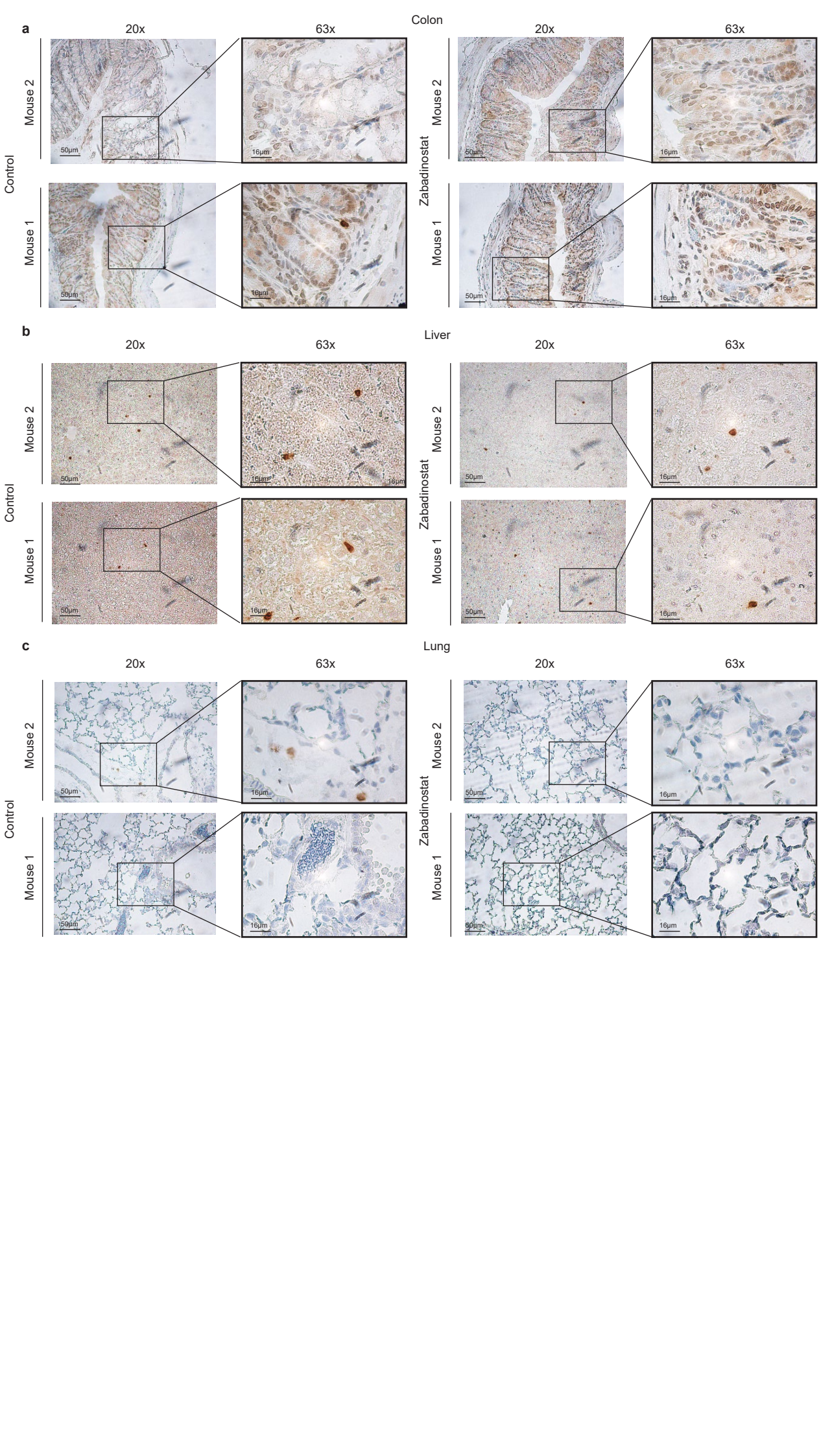
Supplementary Figure 11. Zabadinostat increases cytokine release. Analysis of panel of Th1, Th2, Th1/Th2, General Th, Th17, and Th9 cytokines in serum collected from mice treated with zabadinostat and the control group; Balb/c mice were treated with orally administrated zabadinostat at 25 mg/kg for 14 days (5 days on/2 days off) or vehicle only; n=4 per group; results presented as mean values +/-SD; Student's t test.


a
Colon
20x
63x
Mouse 2
Mouse 1
Control
Zabadinostat
50µm
16µm
Normalized OD
:Zabadinostat
b
Liver
c
Lung

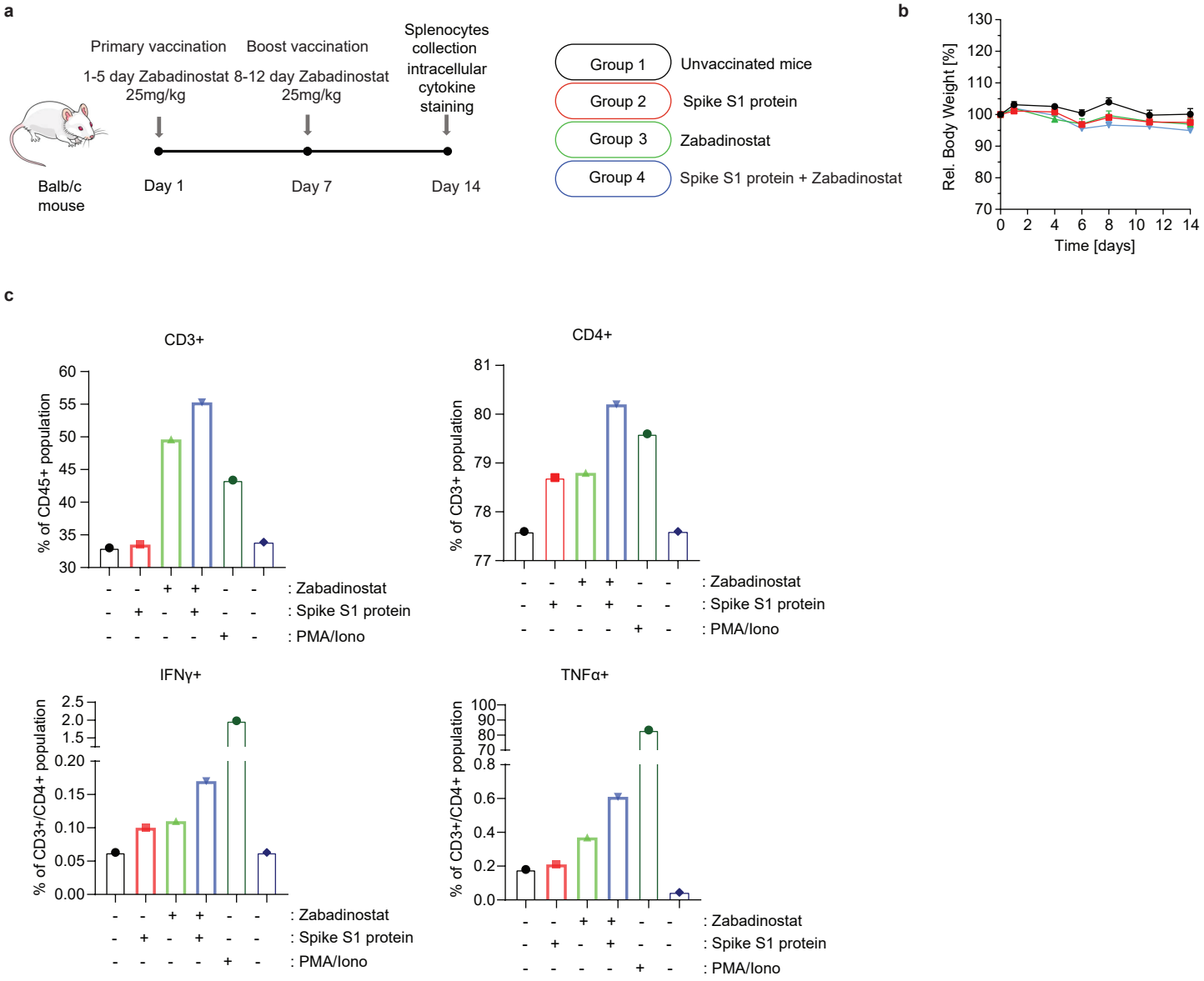
Supplementary Figure 12. Zabadinostat efficacy in different tissues. Representative examples of immunohistochemical staining of H3AcK9 in colon **(a)**, liver **(b)**, and lung **(c)** collected from Balb/c mice at 14 days treated with 25 mg/kg zabadinostat and non-treated control (5 days on/2 days off). Original magnification: 20x, scale bar, 50 µm; and 63x; scale bar, 16 µm. n=2; results were quantified by ImageJ Fiji software and normalised optical density was presented as a mean +/- SD. Statistical analysis was performed using two-tailed, unpaired Student's t-test with GraphPad Prism 8 software, n=2.


a
Colon
20x
63x
20x
63x
Mouse 2
Mouse 1
Control
Zabadinostat
50µm
16µm
b
Liver
20x
63x
20x
63x
Mouse 2
Mouse 1
Control
Zabadinostat
50µm
16µm
c
Lung
20x
63x
20x
63x
Mouse 2
Mouse 1
Control
Zabadinostat
50µm
16µm

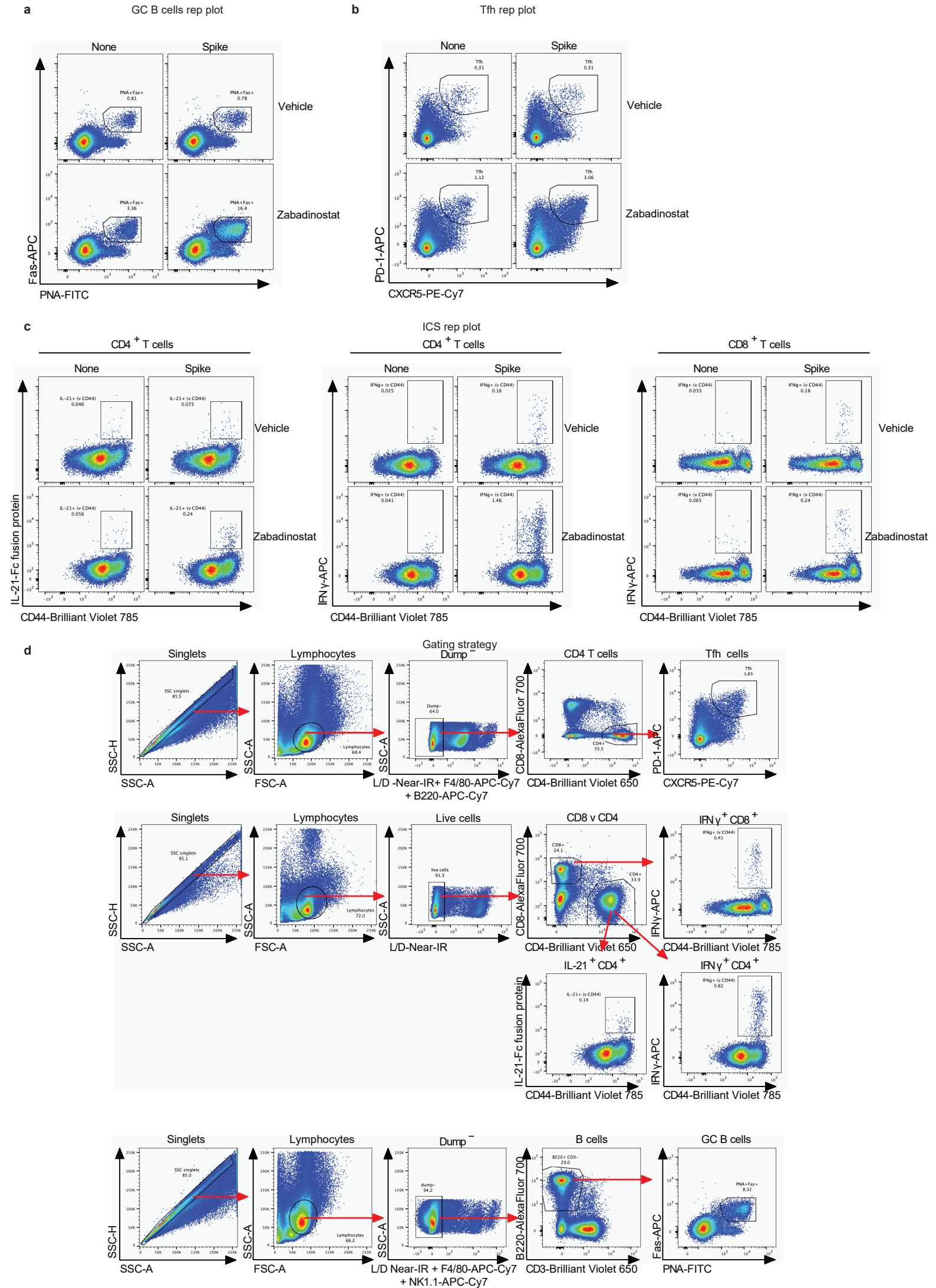
Supplementary Figure 13. CD4 T cell infiltration in different tissues upon zabadinostat treatment. Representative examples of immunohistochemical staining of CD4 T cells in colon **(a)**, liver **(b)**, and lung **(c)** collected from Balb/c mice at 14 days treated with 25 mg/kg zabadinostat and non-treated control (5 days on/2 days off). Original magnification: 20x, scale bar, 50 μm; and 63x; scale bar, 16 μm. n=2.


a
Colon
20x
63x
20x
63x
Mouse 2
Mouse 2
Control
Zabadinostat
Mouse 1
Mouse 1
50µm
16µm
b
Liver
20x
63x
20x
63x
Mouse 2
Mouse 2
Control
Zabadinostat
Mouse 1
Mouse 1
50µm
16µm
c
Lung
20x
63x
20x
63x
Mouse 2
Mouse 2
Control
Zabadinostat
Mouse 1
Mouse 1
50µm
16µm

Supplementary Figure 14. CD8 T cell infiltration in different tissues upon zabadinostat treatment. Representative examples of immunohistochemical staining of CD8 T cells in colon **(a)**, liver **(b)**, and lung **(c)** collected from Balb/c mice at 14 days treated with 25 mg/kg zabadinostat and non-treated control (5 days on/2 days off). Original magnification: 20x, scale bar, 50 μm; and 63x; scale bar, 16 μm. n=2.


a
Primary vaccination
Boost vaccination
Splenocytes collection intracellular cytokine staining
1-5 day Zabadinostat 25mg/kg
8-12 day Zabadinostat 25mg/kg
Balb/c mouse
Day 1
Day 7
Day 14
Group 1
Unvaccinated mice
Group 2
Spike S1 protein
Group 3
Zabadinostat
Group 4
Spike S1 protein + Zabadinostat
b
Rel. Body Weight [%]
Time [days]
c
CD3+
% of CD45+ population
CD4+
% of CD3+ population
IFNγ+
% of CD3+/CD4+ population
TNFα+
% of CD3+/CD4+ population
: Zabadinostat
: Spike S1 protein
: PMA/Iono

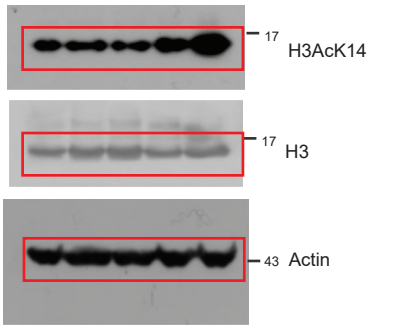
Supplementary Figure 15. **Zabadinostat enhances the T cell response to covid spike S1 protein. a)** Schematic representation of the experiment. Balb/c mice were treated with orally administrated zabadinostat at 25 mg/kg for 14 days (5 days on/2 days off) and intravenous spike S1 protein (days 1 and 7) with respect to vehicle only control; n=4 per group; **b)** relative body weight representation of treated and non-treated mice; results presented as mean values +/-SD; **c)** General CD3 positive T cell activation, CD4 positive T cells, and IFNγ, TNFα intracellular cytokine staining (within CD4 positive cell population) measurements by flow cytometry; measurements were performed in pooled splenocytes from 4 mice; also, we noticed similar percentage of CD8 positive T cells in control and zabadinostat treated groups (18.4% and 16%). Noteworthy, viability of the splenocytes was similar in zabadinostat treatment compare to untreated control (67.3% and 74.2% viable cells, respectively).

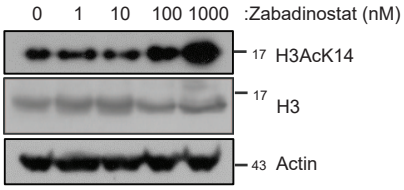

a
GC B cells rep plot
None
Spike
Vehicle
Zabadinostat
PNA+Fas+
0.81
PNA+Fas+
0.78
PNA+Fas+
3.36
PNA+Fas+
16.4
Fas-APC
PNA-FITC
b
Tfh rep plot
None
Spike
Vehicle
Zabadinostat
Tfh
0.31
Tfh
0.31
Tfh
1.12
Tfh
3.06
PD-1-APC
CXCR5-PE-Cy7
c
ICS rep plot
CD4 + T cells
CD4 + T cells
CD8 + T cells
None
Spike
Vehicle
Zabadinostat
IL-21+ (v CD44)
0.046
IL-21+ (v CD44)
0.073
IL-21+ (v CD44)
0.056
IL-21+ (v CD44)
0.24
IFNg+ (v CD44)
0.025
IFNg+ (v CD44)
0.16
IFNg+ (v CD44)
0.041
IFNg+ (v CD44)
1.46
IFNg+ (v CD44)
0.033
IFNg+ (v CD44)
0.18
IFNg+ (v CD44)
0.065
IFNg+ (v CD44)
0.24
IL-21-Fc fusion protein
IFNγ-APC
CD44-Brilliant Violet 785
d
Gating strategy
Singlets
Lymphocytes
Dump −
CD4 T cells
Tfh cells
SSC singlets
85.5
Lymphocytes
68.4
Dump−
64.0
Tfh
1.65
CD4+
55.5
SSC-H
SSC-A
FSC-A
SSC-A
L/D -Near-IR+ F4/80-APC-Cy7
+ B220-APC-Cy7
CD8-AlexaFluor 700
CD4-Brilliant Violet 650
PD-1-APC
CXCR5-PE-Cy7
Singlets
Lymphocytes
Live cells
CD8 v CD4
IFNγ + CD8 +
SSC singlets
95.1
Lymphocytes
72.0
live cells
91.3
CD8+
24.1
CD4+
33.9
IFNg+ (v CD44)
0.41
L/D-Near-IR
CD4-Brilliant Violet 650
CD44-Brilliant Violet 785
IL-21 + CD4 +
IFNγ + CD4 +
IL-21+ (v CD44)
0.14
IFNg+ (v CD44)
0.82
IL-21-Fc fusion protein
IFNγ-APC
CD44-Brilliant Violet 785
CD44-Brilliant Violet 785
Singlets
Lymphocytes
Dump −
B cells
GC B cells
SSC singlets
85.0
Lymphocytes
66.2
dump−
94.2
B220+ CD3−
29.0
PNA+Fas+
8.32
L/D Near-IR + F4/80-APC-Cy7
+ NK1.1-APC-Cy7
B220-AlexaFluor 700
CD3-Brilliant Violet 650
Fas-APC
PNA-FITC

Supplementary Figure 16. Representative flow cytometry plots and gating strategy (a-d) for the results presented in the Figure 7c.

a

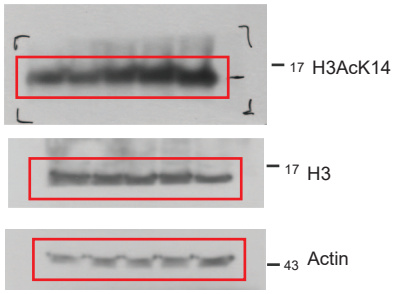
Figure 1a

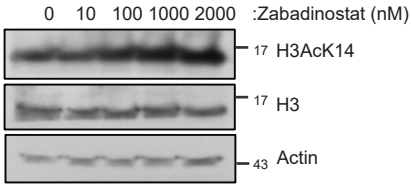




b

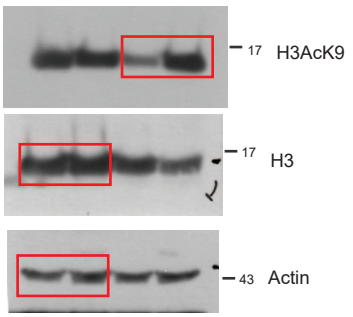
Figure 1b

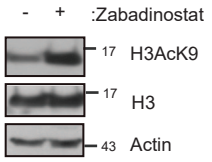




c

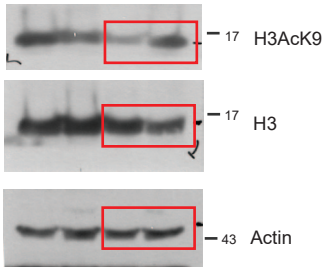
Figure 1e

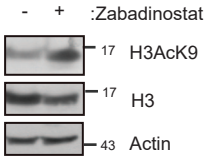




d

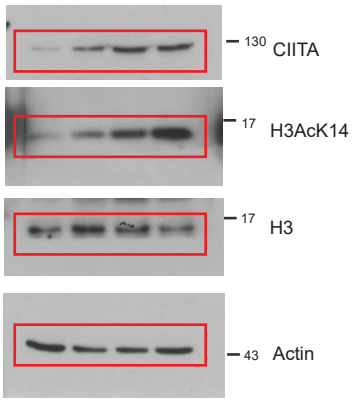
Figure 1f

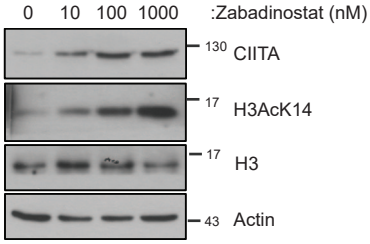




e

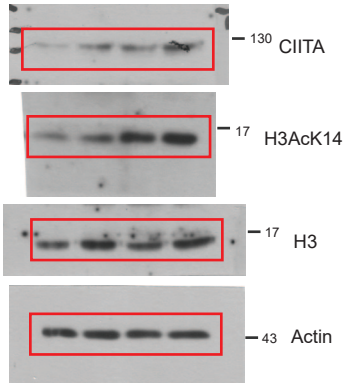
Figure 2a

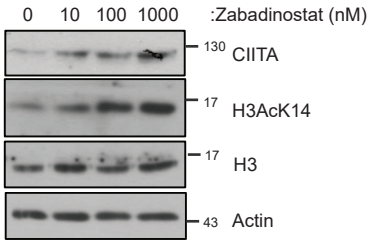




f

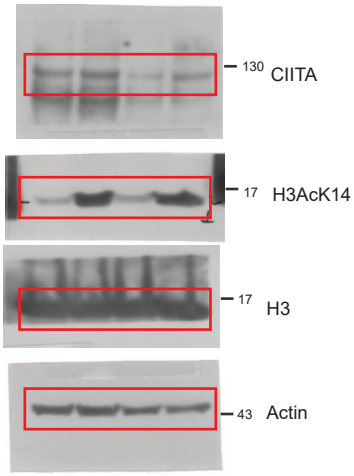
Figure 2b

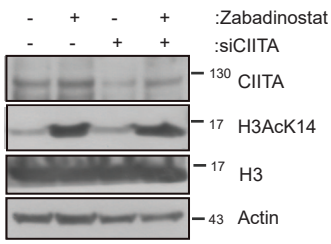




g

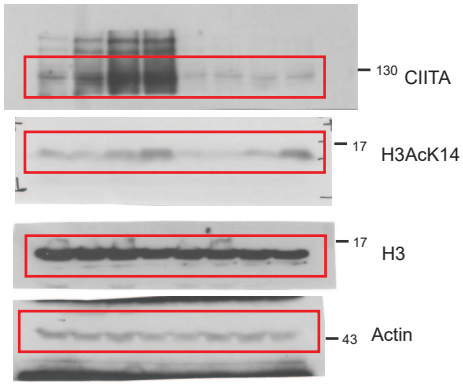
Figure 2e

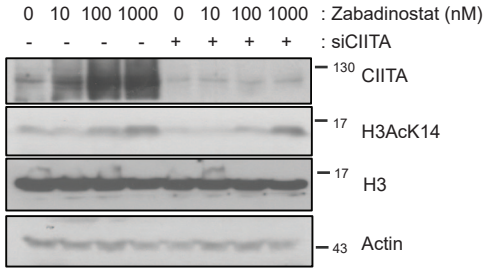




h

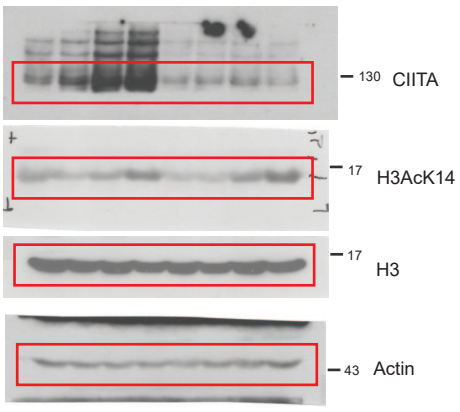
Supplementary Figure 5a

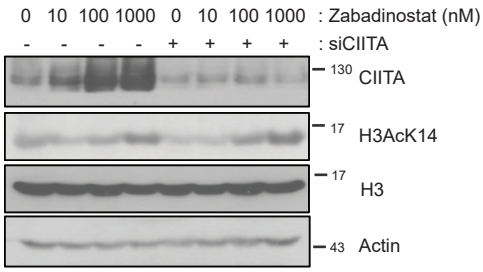




i

Supplementary Figure 5b

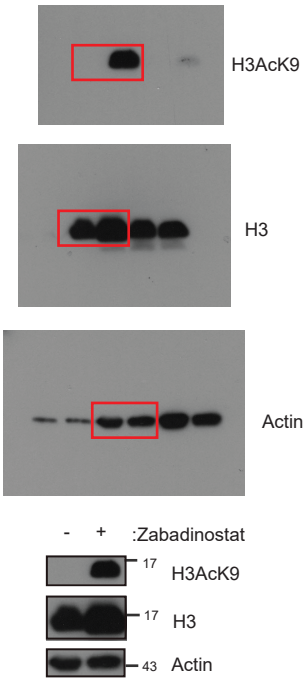




Supplementary Figure 17. Uncropped versions of immunoblots used in the Figures 1a, 1b, 1e, 1f, 2a, 2b, 2e, Supplementary Figure 5a, and 5b.

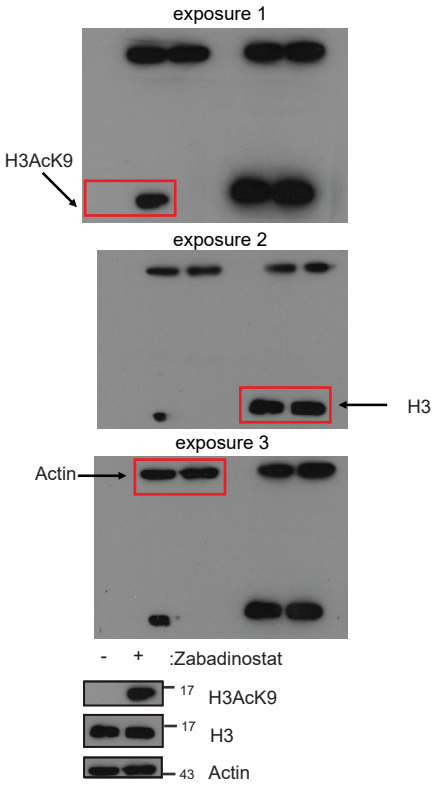
a Figure 1c

BAES2B



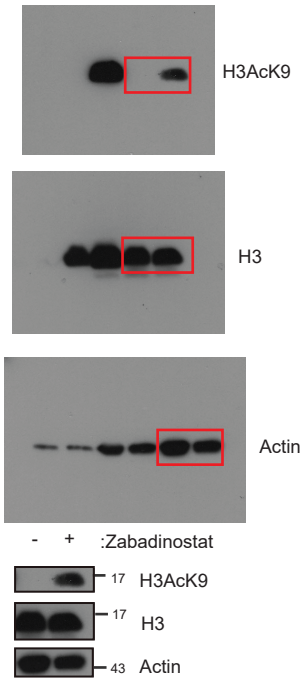
b Figure 1d

NBE1



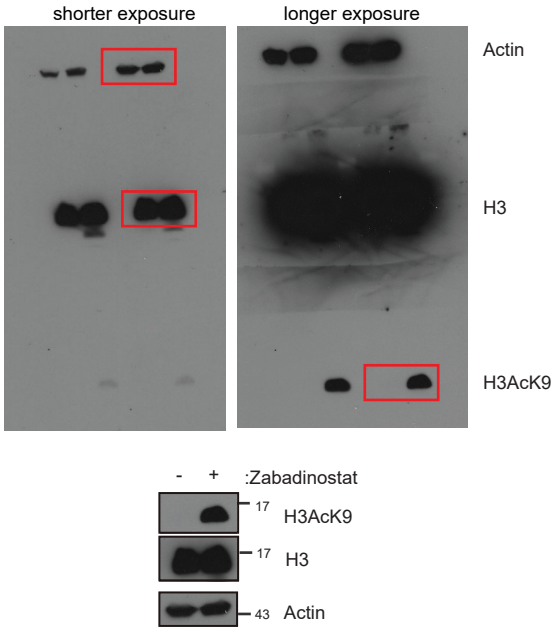
c Supplementary Figure 3a

Jurkat



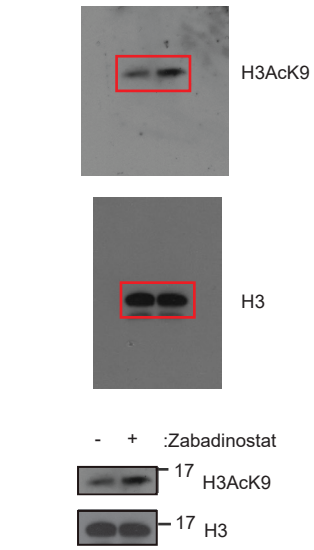
d Supplementary Figure 3c

CEM



e Supplementary Figure 3e

Raji



Supplementary Figure 18. Uncropped versions of immunoblots used in the Figures 1c, 1d, Supplementary Figure 3a, 3c, and 3e.