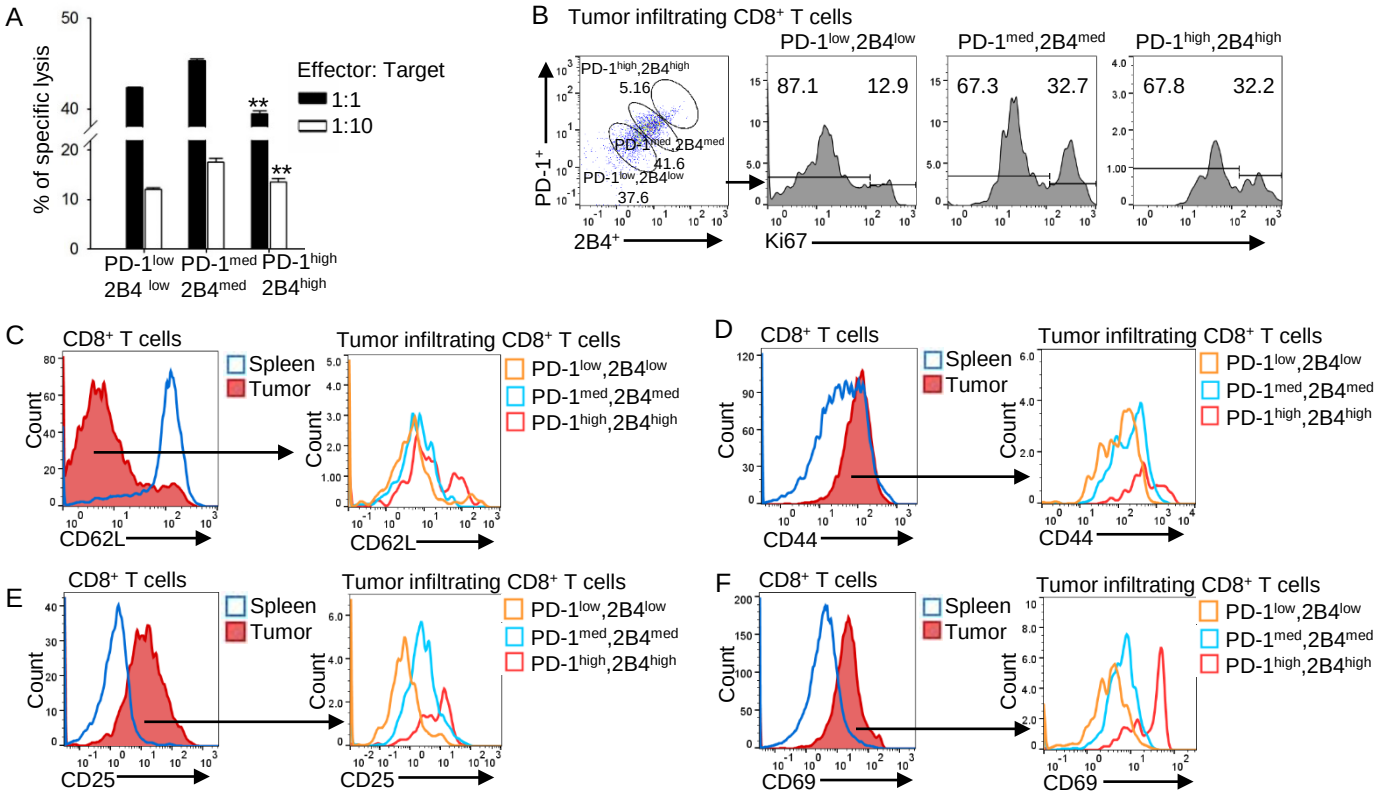
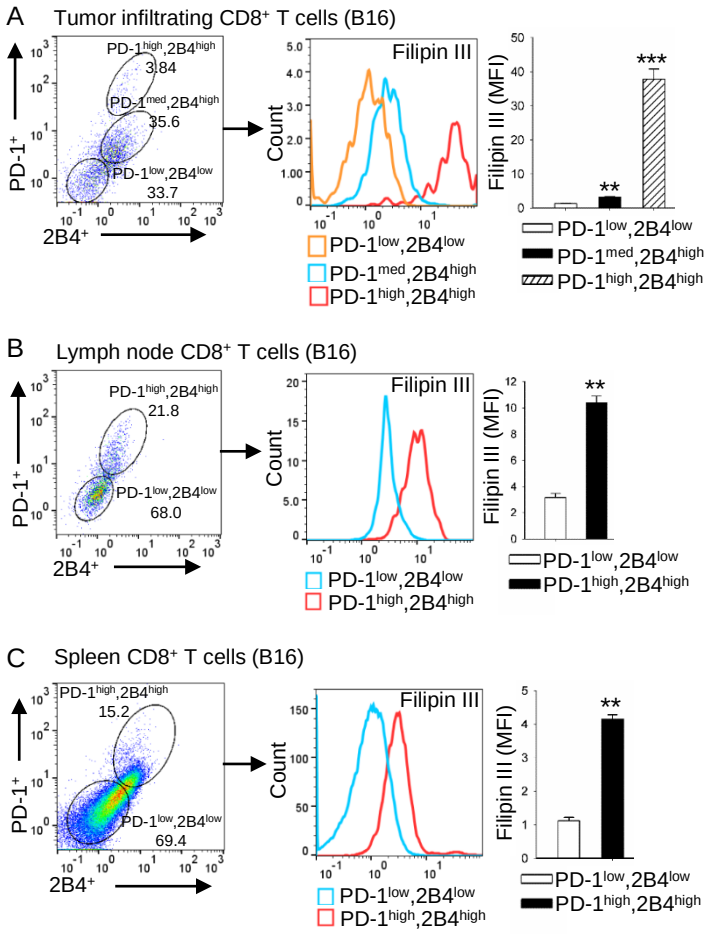

A
% of specific lysis
Effector: Target
1:1
1:10
PD-1low 2B4 low
PD-1med 2B4med
PD-1high 2B4high
B
Tumor infiltrating CD8+ T cells
PD-1high,2B4high
5.16
PD-1med,2B4med
41.6
PD-1low,2B4low
37.6
PD-1+
2B4+
PD-1low,2B4low
87.1
12.9
PD-1med,2B4med
67.3
32.7
PD-1high,2B4high
67.8
32.2
Ki67
C
CD8+ T cells
Spleen
Tumor
Count
CD62L
Tumor infiltrating CD8+ T cells
PD-1low,2B4low
PD-1med,2B4med
PD-1high,2B4high
D
CD44
E
CD25
F
CD69

Supplementary Figure 1. Cytotoxicity, proliferation, and cell surface marker expression of $CD8^+$ T-cell populations that express different levels of inhibitory checkpoints, Related to Figure 1.

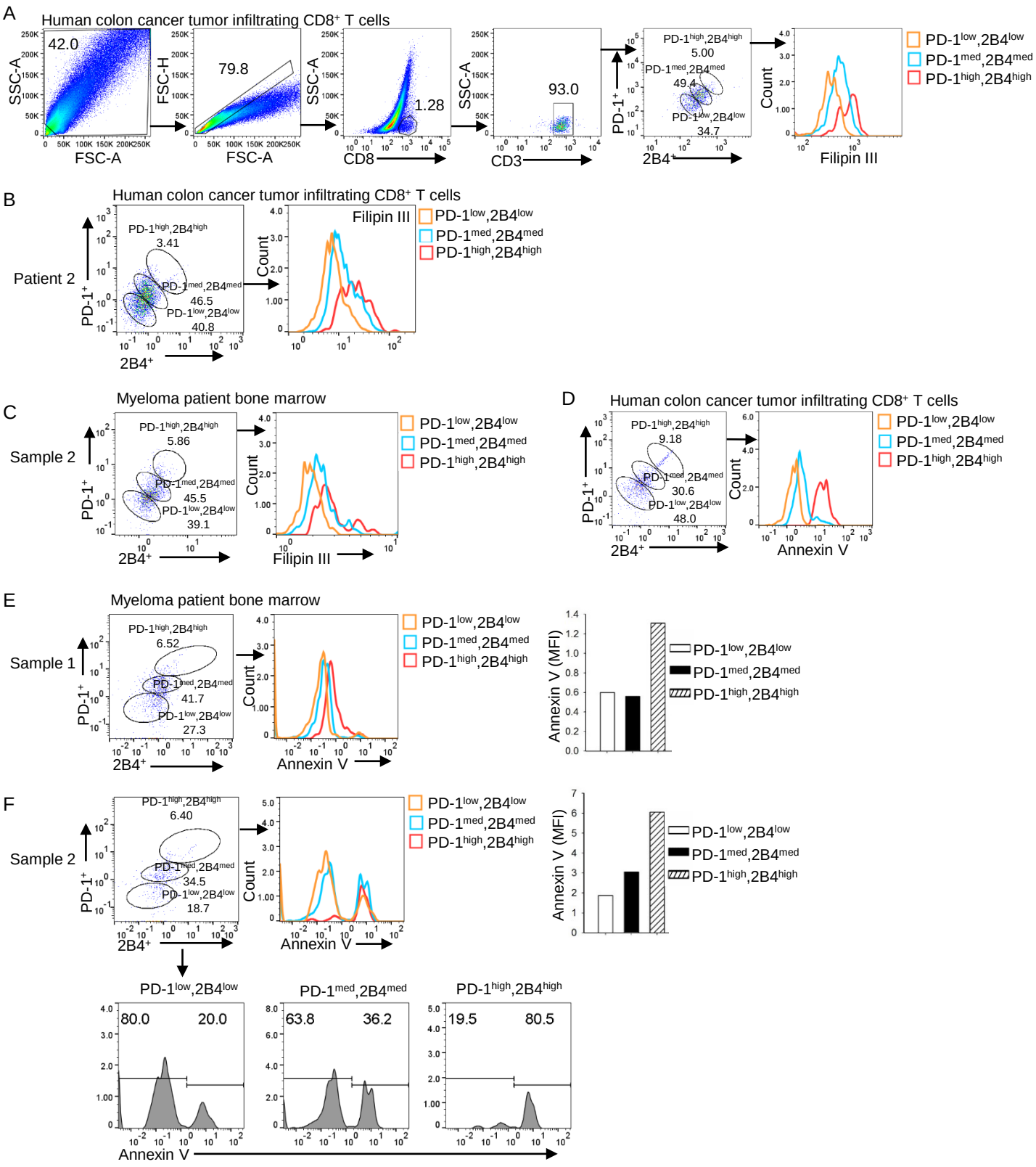
(A-F) B6 mice were injected intravenously with 1×10^5 B16 cells. Tumor-infiltrating $CD8^+$ T cells were sorted out based on the levels of PD-1 and 2B4 expression on day 16 after tumor transfer and analyzed for their cytotoxicity (A), proliferation (B), and expression of CD62L (C), CD44 (D), CD25 (E), and CD69 (F). Experiments were performed with at least three biological replicates, and data shown are representative of at least two independent experiments. Data are presented as mean ± SEM. $^{**}p < 0.01$.


A
Tumor infiltrating CD8+ T cells (B16)
PD-1high,2B4high
3.84
PD-1med,2B4high
35.6
PD-1low,2B4low
33.7
PD-1+
2B4+
Filipin III
Count
Filipin III (MFI)
**
***
PD-1low,2B4low
PD-1med,2B4high
PD-1high,2B4high
B
Lymph node CD8+ T cells (B16)
PD-1high,2B4high
21.8
PD-1low,2B4low
68.0
PD-1+
2B4+
Filipin III
Count
Filipin III (MFI)
**
PD-1low,2B4low
PD-1high,2B4high
C
Spleen CD8+ T cells (B16)
PD-1high,2B4high
15.2
PD-1low,2B4low
69.4
PD-1+
2B4+
Filipin III
Count
Filipin III (MFI)
**
PD-1low,2B4low
PD-1high,2B4high

Supplementary Figure 2. $CD8^+$ T-cell inhibitory checkpoint expression is progressively associated with increased cholesterol accumulation in T cells, Related to Figure 1.

(A-C) B6 mice were injected subcutaneously with 1×10^6 B16 cells. Tumor-infiltrating (A), lymph node (B), and spleen (C) $CD8^+$ T cells were analyzed for the expression of PD-1 or 2B4 and cholesterol level on day 10 after tumor inoculation. MFI = Mean fluorescence intensity Experiments were performed with at least three biological replicates, and data shown are representative of at least three independent experiments. Data are presented as mean ± SEM. **p < 0.01; ***p < 0.001.

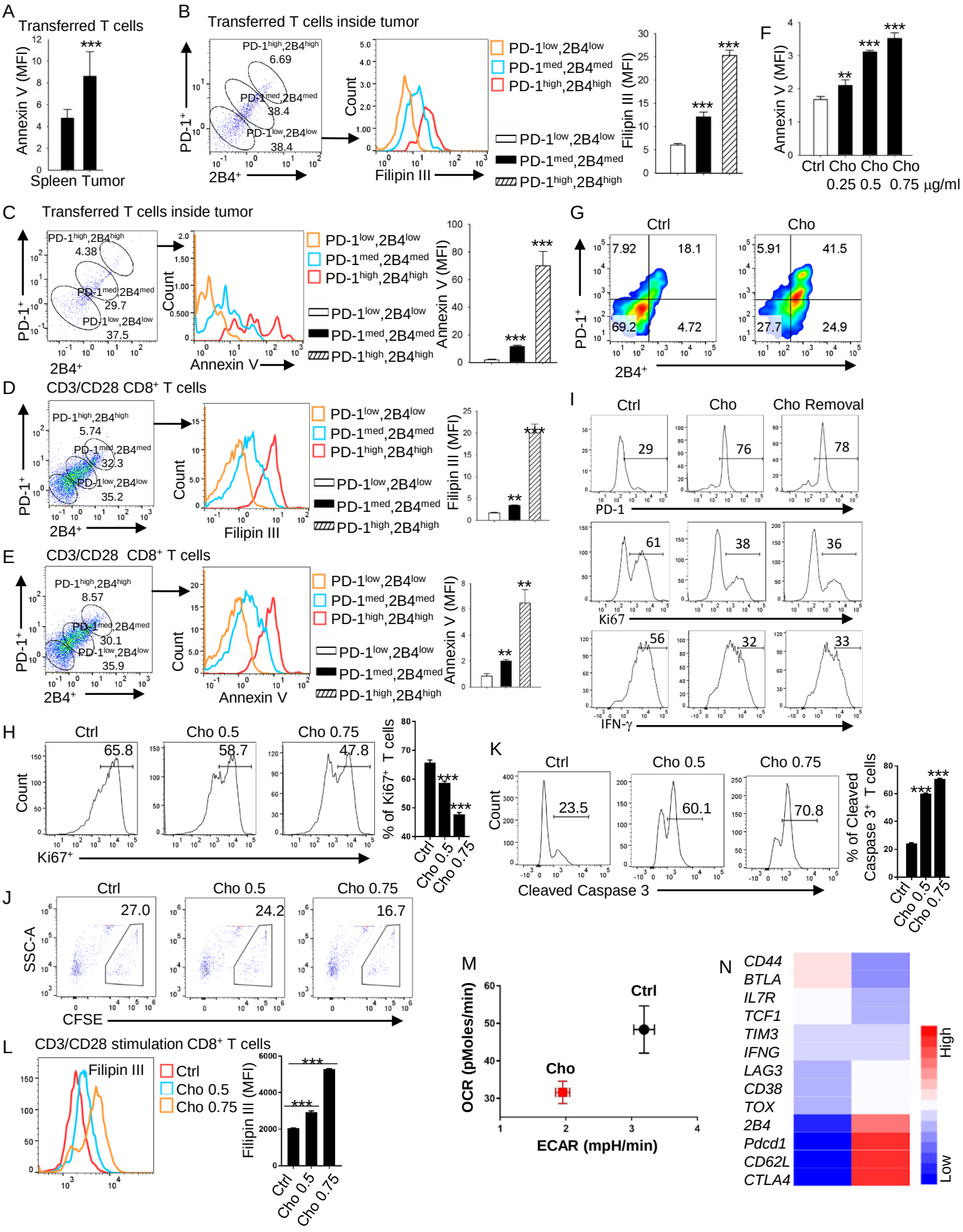
Supplementary 3



Supplementary Figure 3

Supplementary Figure 3. In human patient T cells, $CD8^+$ T-cell inhibitory checkpoint expression is associated with progressively increasing cholesterol accumulation and apoptosis, Related to Figure 1.

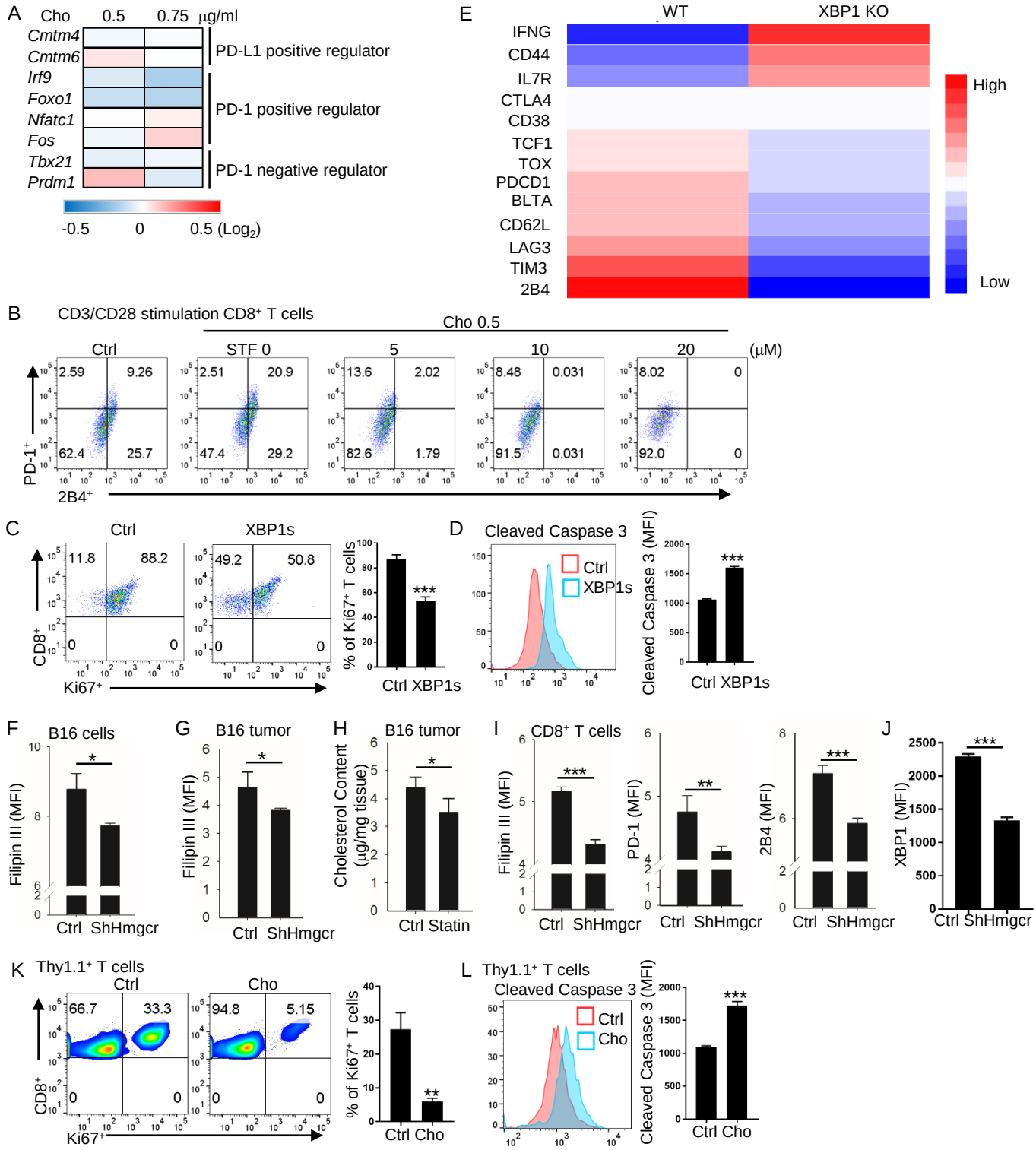
(A-C) Human colon cancer (A and B) and myeloma (C) patient tumor-infiltrating $CD8^+$ T cells were analyzed for the expression of PD-1 or 2B4 and cholesterol level. **(D-F)** Human colon cancer (D) and myeloma (E and F) patient tumor-infiltrating $CD8^+$ T cells were analyzed for the expression of PD-1 or 2B4 and annexin V. Data are representative of 2 of 4 colon cancer patients and 2 of 5 myeloma patients. Data are presented as mean ± SEM.


A
Transferred T cells
Annexin V (MFI)
Spleen Tumor
B
Transferred T cells inside tumor
PD-1high,2B4high
6.69
PD-1med,2B4med
38.4
PD-1low,2B4low
38.4
PD-1+
2B4+
Count
Filipin III
PD-1low,2B4low
PD-1med,2B4med
PD-1high,2B4high
Filipin III (MFI)
F
Annexin V (MFI)
Ctrl Cho Cho Cho
0.25 0.5 0.75 μg/ml
C
Transferred T cells inside tumor
PD-1high,2B4high
4.38
PD-1med,2B4med
29.7
PD-1low,2B4low
37.5
Annexin V
Annexin V (MFI)
G
Ctrl
Cho
7.92
18.1
69.2
4.72
5.91
41.5
27.7
24.9
D
CD3/CD28 CD8+ T cells
PD-1high,2B4high
5.74
PD-1med,2B4med
32.3
PD-1low,2B4low
35.2
Filipin III
I
Ctrl
Cho
Cho Removal
29
76
78
PD-1
61
38
36
Ki67
56
32
33
IFN-γ
E
CD3/CD28 CD8+ T cells
PD-1high,2B4high
8.57
PD-1med,2B4med
30.1
PD-1low,2B4low
35.9
H
Ctrl
Cho 0.5
Cho 0.75
65.8
58.7
47.8
Count
Ki67+
% of Ki67+ T cells
K
Ctrl
Cho 0.5
Cho 0.75
23.5
60.1
70.8
Count
Cleaved Caspase 3
% of Cleaved Caspase 3+ T cells
J
Ctrl
Cho 0.5
Cho 0.75
27.0
24.2
16.7
SSC-A
CFSE
M
Ctrl
Cho
OCR (pMoles/min)
ECAR (mpH/min)
N
CD44
BTLA
IL7R
TCF1
TIM3
IFNG
LAG3
CD38
TOX
2B4
Pdcd1
CD62L
CTLA4
High
Low
L
CD3/CD28 stimulation CD8+ T cells
Filipin III
Ctrl
Cho 0.5
Cho 0.75
Filipin III (MFI)

Supplementary Figure 4. $CD8^+$ T-cell inhibitory checkpoint expression is progressively associated with cholesterol accumulation and apoptosis, Related to Figure 2 and Figure 3.

Splenocytes from Pmel-1 mice were in vitro-differentiated for 5 days in the presence of $hgp100_{25-33}$ (1 mg/ml) and IL-2 (10 ng/ml). **(A)** B6 mice were injected intravenously (i.v.) with 1×10^5 B16 cells. At day 12 after tumor inoculation, 2×10^6 $CD8^+$ T cells were i.v. transferred into the tumor-bearing mice. Four days later, mice were sacrificed, and Pmel-1 $CD8^+$ T cells in tumor and spleen were analyzed for annexin V expression. **(B and C)** Transferred Pmel-1 T cells within the tumor were analyzed for expression of PD-1, 2B4, and cholesterol content (B), and annexin V (C). Experiments were performed with at least three biological replicates and are representative of at least two independent experiments. **(D and E)** $CD8^+$ T cells were isolated from Pmel-1 mice and stimulated in vitro with CD3/CD28 antibodies in the presence of IL-2. The cultured $CD8^+$ T cells were then analyzed for the expression of PD-1 or 2B4, cholesterol level (D), and annexin V (E). **(F)** $CD8^+$ T cells were isolated from Pmel-1 mice and stimulated in vitro with CD3/CD28 antibodies in the presence of IL-2 and cholesterol (Cho) at the concentrations indicated. The cultured $CD8^+$ T cells were then analyzed for annexin V expression. **(G)** $CD8^+$ T cells were stimulated and differentiated in vitro with plated-CD3/CD28 antibodies with (Cho) or without (Ctrl) cholesterol for 3 days, followed by culturing with soluble CD3 and CD28 antibodies for 7 days with (Cho) or without (Ctrl) cholesterol. T-cell expression of PD-1 and 2B4 was determined by flow. **(H)** Pmel-1 $CD8^+$ T cells were in vitro stimulated with plate-coated anti-CD3 and soluble CD28 antibodies in the presence of IL-2 for 3 days and then cultured with soluble CD3/CD28 antibodies for 5 days. Cholesterol was added to the cultures at the indicated concentrations. T-cell expression of Ki67 was examined on day 8 by flow. **(I)** $CD8^+$ T cells were stimulated with anti-CD3 and anti-CD28 with or without cholesterol

for 5 days. Cholesterol-treated $CD8^+$ T cells were washed and continued to be cultured with or without cholesterol in the presence of soluble anti-CD3 and anti-CD28 for an additional 5 days. Flow cytometry was used to examine related phenotype. **(J)** $CD8^+$ T cells were simulated with anti-CD3 and anti-CD28 antibodies with or without cholesterol (0, 0.5 or 0.75 μg/ml) for 5 days and then labeled with 2 μM CFSE. B16 cells (1×10^4) were seeded into 96-well plates overnight. CFSE labeled $CD8^+$ T cells (1×10^4) were seeded into the transwells. Infiltrating $CD8^+$ T cells to tumor cells were examined 24 hours later by flow cytometry. **(K and L)** Pmel-1 $CD8^+$ T cells were in vitro stimulated with plate-coated anti-CD3 and soluble CD28 antibodies in the presence of IL-2 for 5 days and continued to be cultured with soluble CD3/CD28 antibodies. Cholesterol was added to the cultures at the indicated concentrations. T-cell expression of Ki67, cleaved caspase 3 and Filipin III were examined on day 8 by flow. **(M)** Seahorse examination of control or cholesterol-treated T cell oxygen consumption rate (OCR) and glycolysis rate (ECAR). **(N** Microarray analysis of T cell exhaustion gene expression (GSE111033). Experiments were performed with at least three biological replicates, and data shown are representative of at least three independent experiments. Data are presented as mean ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$.


A
Cho 0.5 0.75 μg/ml
Cmtm4
Cmtm6
Irf9
Foxo1
Nfatc1
Fos
Tbx21
Prdm1
PD-L1 positive regulator
PD-1 positive regulator
PD-1 negative regulator
-0.5 0 0.5 (Log2)
E
WT
XBP1 KO
IFNG
CD44
IL7R
CTLA4
CD38
TCF1
TOX
PDCD1
BLTA
CD62L
LAG3
TIM3
2B4
High
Low
B
CD3/CD28 stimulation CD8+ T cells
Cho 0.5
Ctrl
STF 0
5
10
20
(μM)
PD-1+
2B4+
2.59 9.26 62.4 25.7
2.51 20.9 47.4 29.2
13.6 2.02 82.6 1.79
8.48 0.031 91.5 0.031
8.02 0 92.0 0
C
Ctrl
XBP1s
CD8+
Ki67+
11.8 88.2 0 0
49.2 50.8 0 0
% of Ki67+ T cells
Ctrl XBP1s
D
Cleaved Caspase 3
Ctrl
XBP1s
Cleaved Caspase 3 (MFI)
Ctrl XBP1s
F
B16 cells
Filipin III (MFI)
Ctrl ShHmgcr
G
B16 tumor
Filipin III (MFI)
Ctrl ShHmgcr
H
B16 tumor
Cholesterol Content (μg/mg tissue)
Ctrl Statin
I
CD8+ T cells
Filipin III (MFI)
PD-1 (MFI)
2B4 (MFI)
Ctrl ShHmgcr
J
XBP1 (MFI)
Ctrl ShHmgcr
K
Thy1.1+ T cells
Ctrl
Cho
CD8+
Ki67+
66.7 33.3 0 0
94.8 5.15 0 0
% of Ki67+ T cells
Ctrl Cho
L
Thy1.1+ T cells
Cleaved Caspase 3
Ctrl
Cho
Cleaved Caspase 3 (MFI)
Ctrl Cho

Supplementary Figure 5. Cholesterol has a minor effect on the expression of some PD-1 regulators, and the ER-stress inhibitor STF-083010 abrogates cholesterol-induced expression of the inhibitory checkpoints on CD8$^+$ T cells, Related to Figure 4 and Figure 5.

(A) CD8$^+$ T cells were isolated from Pmel-1 mice and in vitro-stimulated with CD3/CD28 antibodies in the presence of IL-2 and cholesterol (Cho) at different concentrations as indicated at the top of the heat map. The cultured CD8$^+$ T cells were then analyzed by microarray analysis for PD-1- and PD-L1-associated gene expression. Data are representative of at least two independent experiments. **(B)** Flow cytometry analysis of PD-1 and 2B4 expression on in vitro-differentiated CD8$^+$ T cells treated without or with cholesterol and/or STF-083010 (STF). Experiments were performed with at least three biological replicates, and data shown are representative of at least two independent experiments. **(C and D)** In vitro-differentiated Pmel-1-derived CD8$^+$ T cells were transfected with XBP1s virus to overexpress XBP1s. Cells were cultured in the presence of soluble CD3/CD28 antibodies and IL-2. T-cell expression of Ki67 and cleaved caspase 3 were examined on day 8 by flow. **(E)** Heatmap showing the exhaustion-related genes of tumor-infiltrating WT and XBP1-knockout CD4$^+$ T cells (data analyzed from GSE118430). **(F and G)** B16 cells were transfected with ctrl or Hmgcr shRNA and then injected s.c. into B6 mice. Cholesterol levels in B16 cells before injection (F) and after tumor formation (G) were examined by filipin III staining. **(H)** One dose of simvastatin (1 mg; 50 mg/kg body weight) was directly injected into 10-day large established B16 tumor. Cholesterol content in the tumors was examined 3 days later. **(I and J)** In vitro-differentiated Pmel-1-derived CD8$^+$ T cells were transfected with Hmgcr shRNA. PD-1, 2B4 and XBP1 expression and cholesterol content of the T cells were examined before transfer by flow. **(K and L)** In vitro-differentiated Pmel-1-derived CD8$^+$ T cells were cultured in standard T-cell culture medium or treated with 0.5 μg/ml

cholesterol for 5 days. On day 5, T cells were transferred into 10-day subcutaneous B16 tumor-bearing mice. Transferred, tumor-infiltrating T cells were examined for the expression of Ki67 and cleaved caspase 3 10 days later by flow. Data are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.