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Supplemental Information

BET inhibition triggers antitumor immunity by enhancing MHC class I expression in head and neck squamous cell carcinoma

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Figure S1

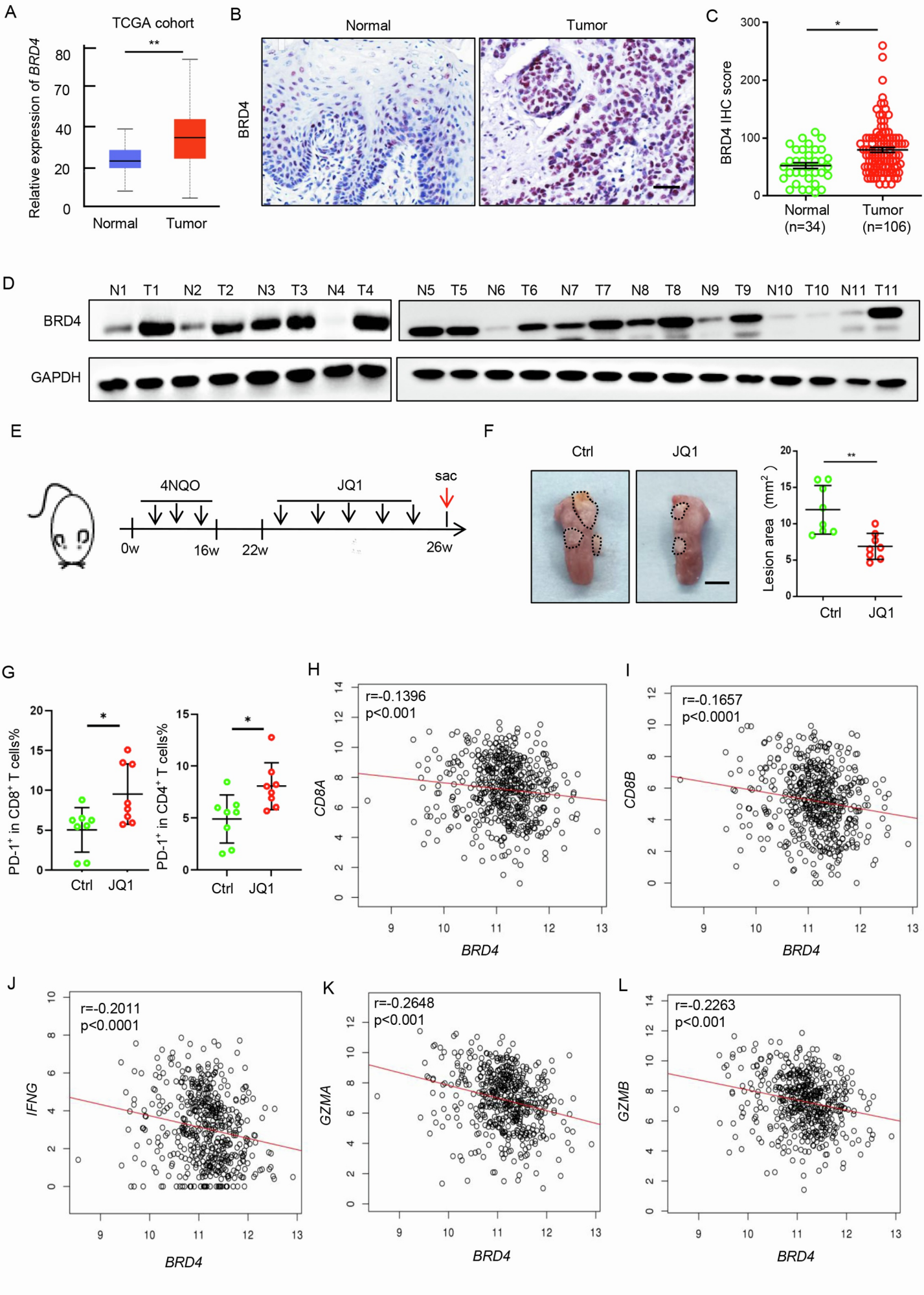

A
TCGA cohort
Relative expression of BRD4
Normal
Tumor
B
Normal
Tumor
BRD4
C
BRD4 IHC score
Normal (n=34)
Tumor (n=106)
D
N1 T1 N2 T2 N3 T3 N4 T4 N5 T5 N6 T6 N7 T7 N8 T8 N9 T9 N10 T10 N11 T11
BRD4
GAPDH
E
4NQO
JQ1
sac
0w
16w
22w
26w
F
Ctrl
JQ1
Lesion area (mm²)
G
PD-1⁺ in CD8⁺ T cells%
PD-1⁺ in CD4⁺ T cells%
H
r=-0.1396
p<0.001
CD8A
BRD4
I
r=-0.1657
p<0.0001
CD8B
BRD4
J
r=-0.2011
p<0.0001
IFNG
BRD4
K
r=-0.2648
p<0.001
GZMA
BRD4
L
r=-0.2263
p<0.001
GZMB
BRD4

Figure S1. BRD4 expression is increased and inversely correlated with *CD8A*, *CD8B*, *IFNG*, *GZMA* and *GZMB* expression in HNSCC, Related to Figure 1

(A) The expression level of *BRD4* mRNA in TCGA HNSCC database.

(B) Representative immunostaining of BRD4 in human HNSCC samples. Scale bar, 50 μm.

(C) The expression of BRD4 in human HNSCC samples. *p < 0.05 by Student's t test.

(D) The expression of BRD4 protein in human HNSCC samples and their corresponding normal tissue detected by western blot.

(E) Experimental design for JQ1 treatment in 4NQO-induced HNSCC mice model.

(F) JQ1 treatment attenuates growth of murine HNSCC tumors in 4NQO-induced HNSCC mice. Values are means ± SD from the pool of 3 independent experiments. n=8. **p < 0.01 by Student's t test. Scale bar, 3 mm.

(G) More $CD8^+$ T cells and $CD4^+$ T cells expressed PD-1 in TILs from 4NQO-induced HNSCC mice upon JQ1 treatment. *p < 0.05 by Student's t test.

(H-L) *BRD4* expression was inversely correlated with the expression of *CD8A*, *CD8B*, *IFNG*, *GZMA* and *GZMB* in human HNSCC from TCGA database.

Figure S2

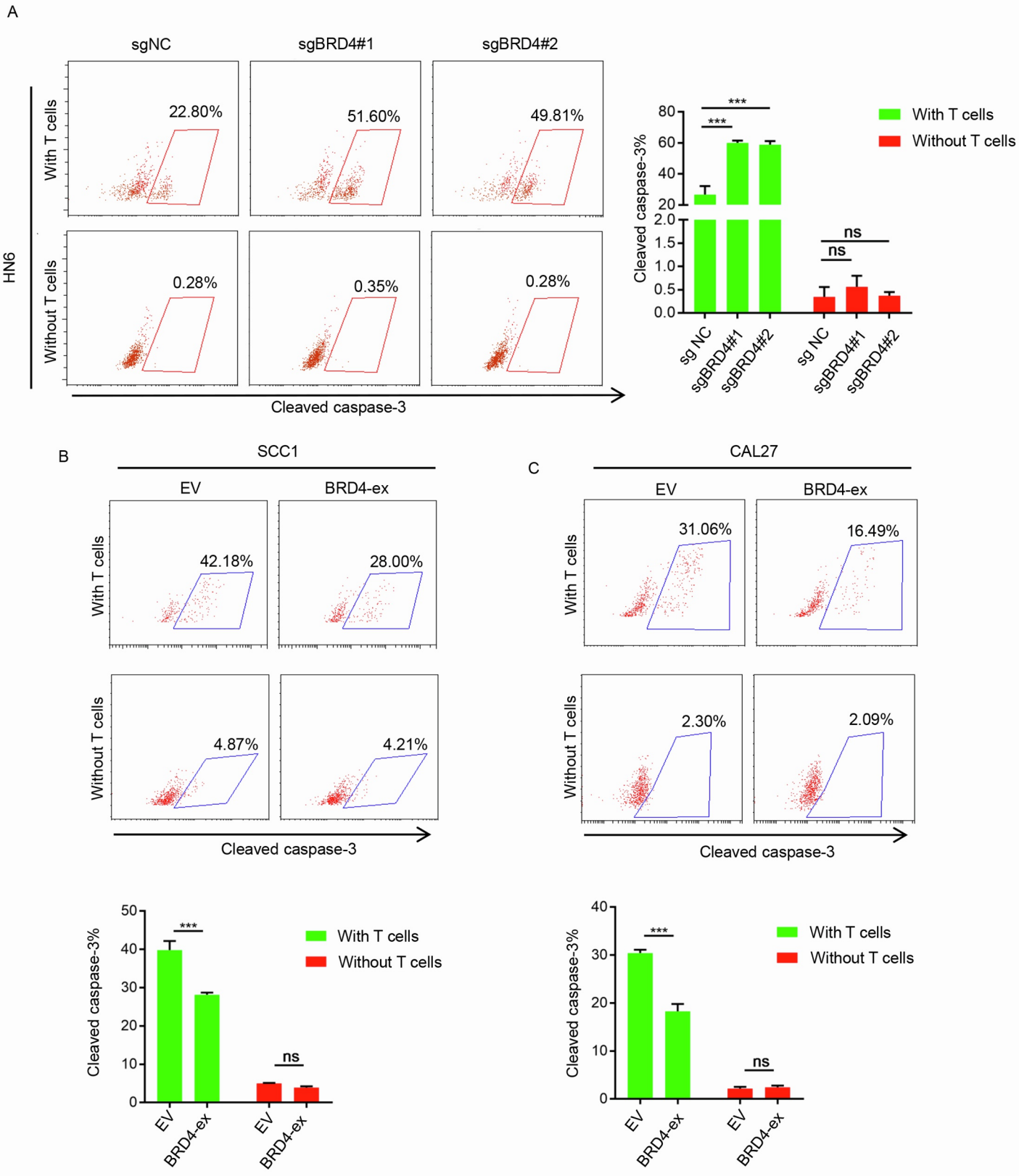

A
sgNC
sgBRD4#1
sgBRD4#2
With T cells
Without T cells
22.80%
51.60%
49.81%
0.28%
0.35%
0.28%
HN6
Cleaved caspase-3
Cleaved caspase-3%
***
***
ns
ns
With T cells
Without T cells
sg NC
sgBRD4#1
sgBRD4#2
B
SCC1
EV
BRD4-ex
42.18%
28.00%
4.87%
4.21%
Cleaved caspase-3
Cleaved caspase-3%
***
ns
With T cells
Without T cells
EV
BRD4-ex
C
CAL27
31.06%
16.49%
2.30%
2.09%
Cleaved caspase-3
Cleaved caspase-3%
***
ns
With T cells
Without T cells

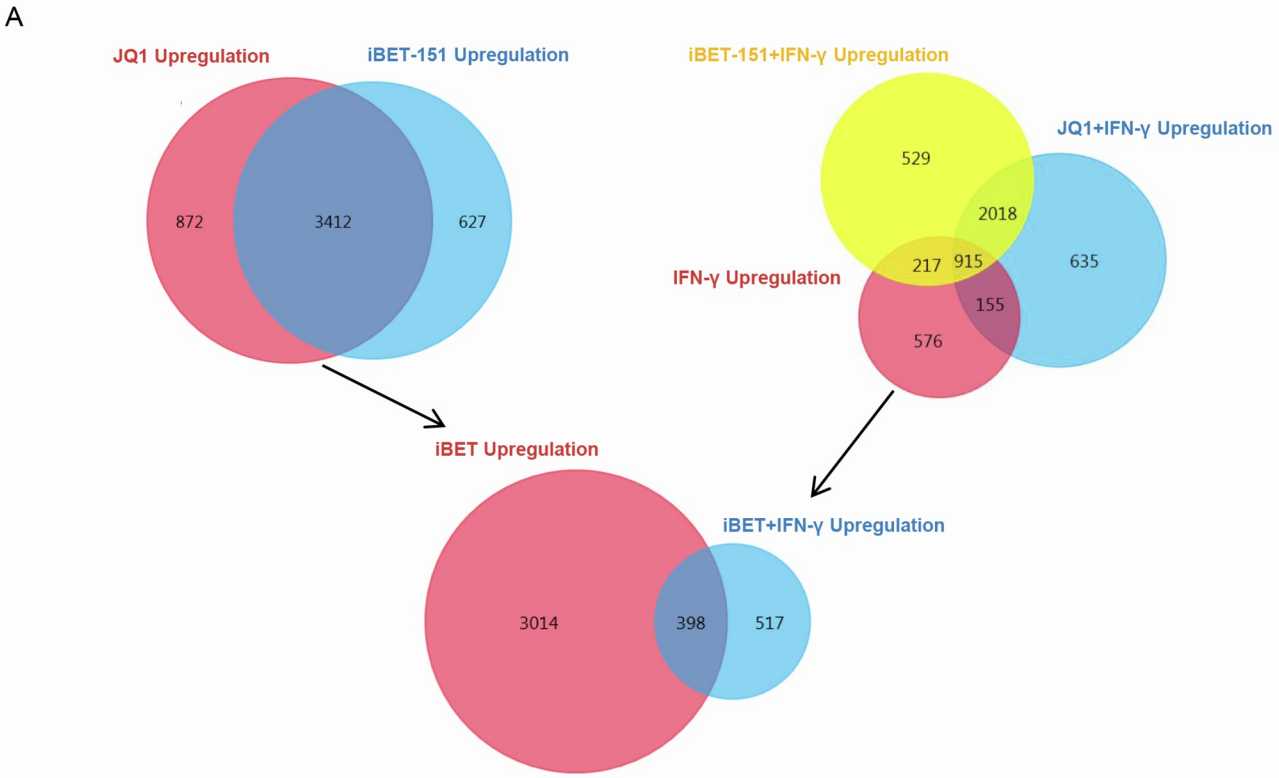
Figure S2. Modulation of BRD4 in HNSCC cells regulates the cytotoxicity of T cells, Related to Figure 3

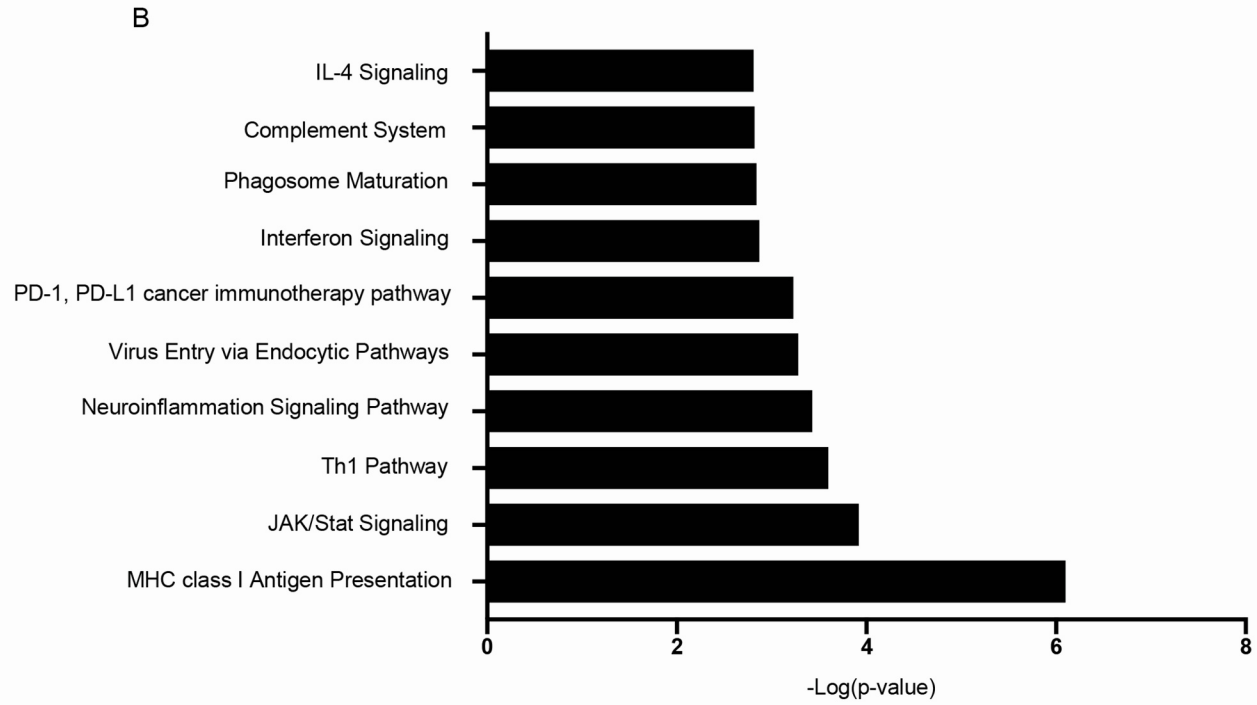
(A) Representative image and quantification of cleaved caspase-3 positive cells in HN6 cells with genetic depletion *BRD4*. ***p < 0.001 by 2-way ANOVA. ns, non-significant.

(B) Representative image and quantification of cleaved caspase-3 positive cells in SCC1 cells with ectopic overexpression of *BRD4*. ***p < 0.001 by 2-way ANOVA. ns, non-significant.

(C) Representative image and quantification of cleaved caspase-3 positive cells in CAL27 cells with ectopic overexpression of *BRD4*. ***p < 0.001 by 2-way ANOVA. ns, non-significant.

Figure S3


A
JQ1 Upregulation
iBET-151 Upregulation
872
3412
627
iBET-151+IFN-γ Upregulation
JQ1+IFN-γ Upregulation
IFN-γ Upregulation
529
2018
217
915
635
155
576
iBET Upregulation
iBET+IFN-γ Upregulation
3014
398
517


B
IL-4 Signaling
Complement System
Phagosome Maturation
Interferon Signaling
PD-1, PD-L1 cancer immunotherapy pathway
Virus Entry via Endocytic Pathways
Neuroinflammation Signaling Pathway
Th1 Pathway
JAK/Stat Signaling
MHC class I Antigen Presentation
0
2
4
6
8
-Log(p-value)

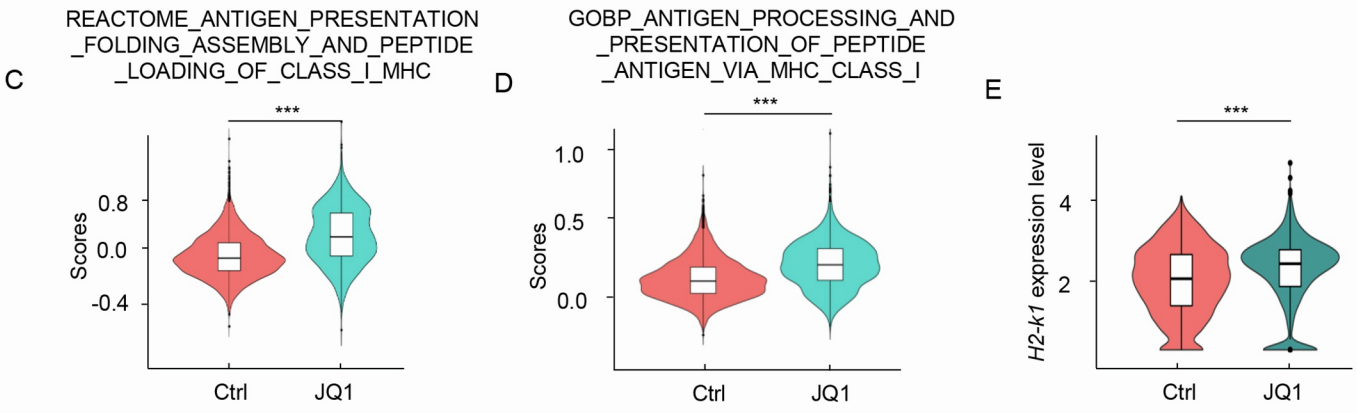

C
REACTOME_ANTIGEN_PRESENTATION
_FOLDING_ASSEMBLY_AND_PEPTIDE
_LOADING_OF_CLASS_I_MHC
***
Scores
0.8
0.0
-0.4
Ctrl
JQ1
D
GOBP_ANTIGEN_PROCESSING_AND
_PRESENTATION_OF_PEPTIDE
_ANTIGEN_VIA_MHC_CLASS_I
***
Scores
1.0
0.5
0.0
Ctrl
JQ1
E
***
H2-k1 expression level
4
2
Ctrl
JQ1

Figure S3. Targeting BRD4 promotes expression of MHC-I expression in HNSCC, Related to Figure 4

(A) RNA-seq analysis showed the genes commonly upregulated by JQ1 and iBET-151 in the presence or absence of IFN-γ in SCC1 cells.

(B) IPA analysis showed the canonical pathways which were correlated the genes commonly upregulated by JQ1 and iBET-151 in the presence or absence of IFN-γ in SCC1 cells.

(C and D) Violin plot of MHC class I antigen processing and presentation related gene signature scores in the two epithelial clusters. ***p < 0.001 by Wilcoxon rank-sun test.

(E) Violin plot showing the expression of *H2-K1* in the two epithelial clusters. ***p < 0.001 by Wilcoxon rank-sun test.

Figure S4

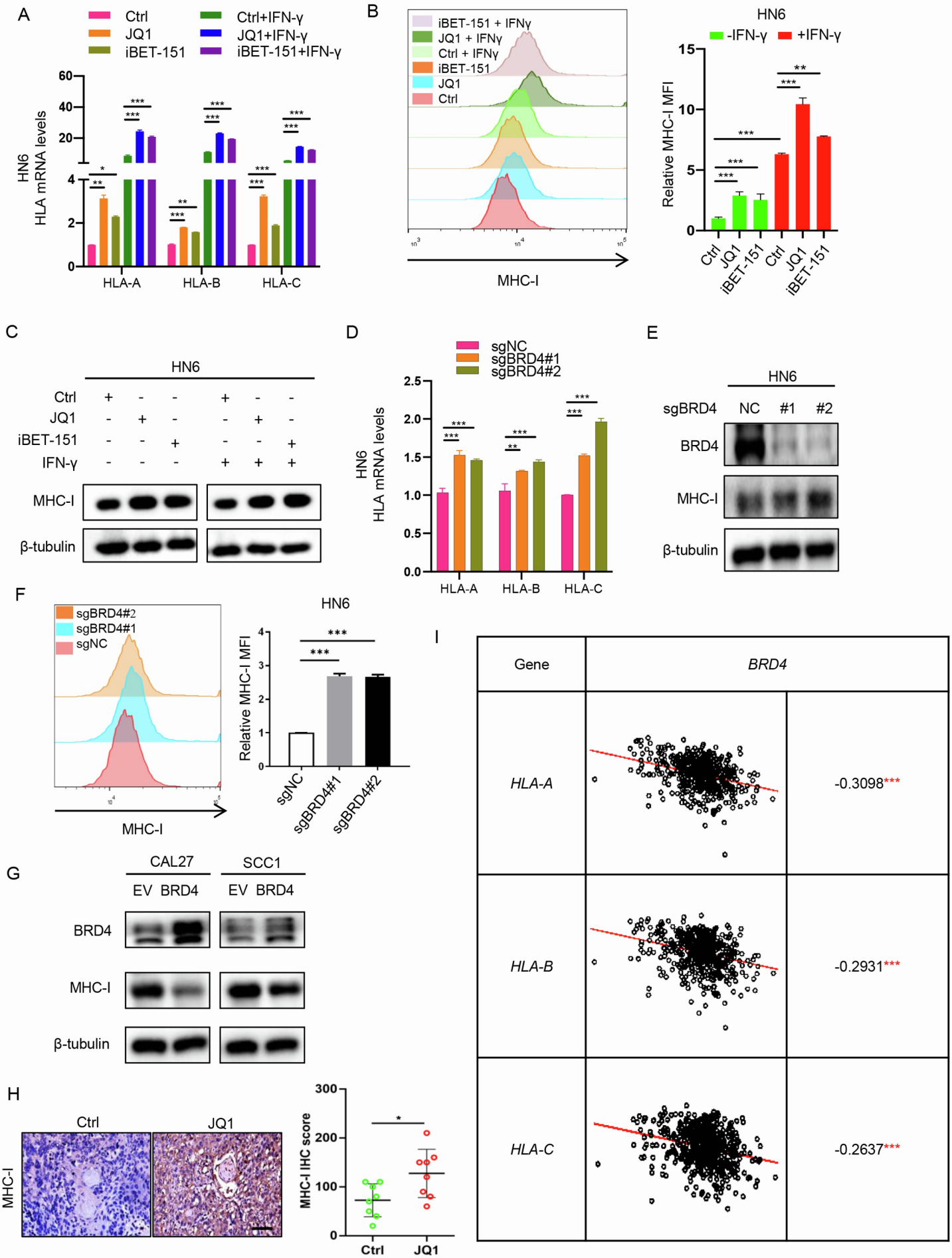

A
Ctrl
JQ1
iBET-151
Ctrl+IFN-γ
JQ1+IFN-γ
iBET-151+IFN-γ
HN6
HLA mRNA levels
HLA-A
HLA-B
HLA-C
B
iBET-151 + IFNγ
JQ1 + IFNγ
Ctrl + IFNγ
iBET-151
JQ1
Ctrl
MHC-I
HN6
-IFN-γ
+IFN-γ
Relative MHC-I MFI
C
HN6
Ctrl
JQ1
iBET-151
IFN-γ
MHC-I
β-tubulin
D
sgNC
sgBRD4#1
sgBRD4#2
HN6
HLA mRNA levels
HLA-A
HLA-B
HLA-C
E
HN6
sgBRD4
NC
#1
#2
BRD4
MHC-I
β-tubulin
F
sgBRD4#2
sgBRD4#1
sgNC
MHC-I
HN6
Relative MHC-I MFI
sgNC
sgBRD4#1
sgBRD4#2
G
CAL27
SCC1
EV
BRD4
EV
BRD4
BRD4
MHC-I
β-tubulin
H
Ctrl
JQ1
MHC-I
MHC-I IHC score
Ctrl
JQ1
I
Gene
BRD4
HLA-A
-0.3098***
HLA-B
-0.2931***
HLA-C
-0.2637***

Figure S4. BRD4 regulates the expression of MHC-I expression in HNSCC cells, Related to Figure 4

(A) RT-qPCR showing the expression of *HLA-A*, *HLA-B* and *HLA-C* in HN6 cells treated with JQ1 and iBET-151 in the presence or absence of IFN-γ. Means ± SDs were shown. *p < 0.05, **p < 0.01 and ***p < 0.001 by 1-way ANOVA.

(B) Flow Cytometry displayed cell surface MHC-I expression in HN6 cells treated with JQ1 and iBET-151 in the presence or absence of IFN-γ. Means ± SDs were shown. **p < 0.01 and ***p < 0.001 by 1-way ANOVA.

(C) Immunoblot showing the expression of MHC-I in HN6 cells treated with JQ1 and iBET-151 in the presence or absence of IFN-γ.

(D) RT-qPCR showed that the expression of *HLA-A, HLA-B* and *HLA-C* in HN6 cells transfected with sg*BRD4*. Means ± SDs were shown. **p < 0.01 and ***p < 0.001 by 1-way ANOVA.

(E) Immunoblot showed that the expression of MHC-I in HN6 cells transfected with sg*BRD4*.

(F) Flow Cytometry displayed cell surface MHC-I expression in HN6 cells transfected with sg*BRD4*. Means ± SDs were shown. ***p < 0.001 by 1-way ANOVA.

(G) Immunoblot showed that the expression of MHC-I in SCC1 and CAL27 cells transfected with *BRD4* overexpression vectors.

(H) Immunostaining for MHC-I in murine primary HNSCC from 4NQO-induecd mice treated with or without JQ1. *p < 0.05 by Student's t test.

(I) The expression of *BRD4* was inversely correlated with *HLA-A*, *HLA-B* and *HLA-C* expression based on TCGA HNSCC dataset.

Figure S5

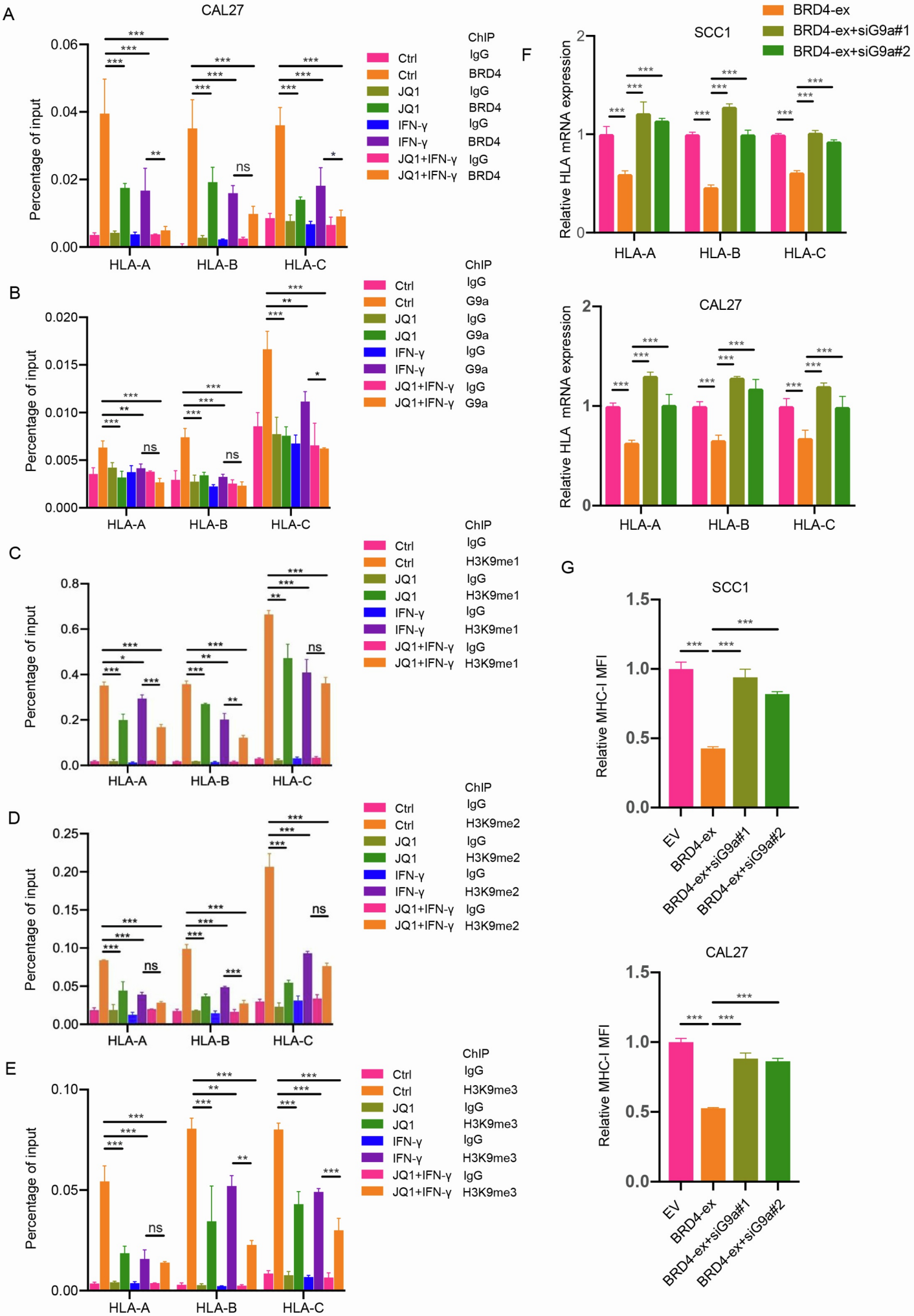

A
CAL27
Percentage of input
HLA-A
HLA-B
HLA-C
ChIP
Ctrl IgG
Ctrl BRD4
JQ1 IgG
JQ1 BRD4
IFN-γ IgG
IFN-γ BRD4
JQ1+IFN-γ IgG
JQ1+IFN-γ BRD4
B
Ctrl IgG
Ctrl G9a
JQ1 IgG
JQ1 G9a
IFN-γ IgG
IFN-γ G9a
JQ1+IFN-γ IgG
JQ1+IFN-γ G9a
C
Ctrl IgG
Ctrl H3K9me1
JQ1 IgG
JQ1 H3K9me1
IFN-γ IgG
IFN-γ H3K9me1
JQ1+IFN-γ IgG
JQ1+IFN-γ H3K9me1
D
Ctrl IgG
Ctrl H3K9me2
JQ1 IgG
JQ1 H3K9me2
IFN-γ IgG
IFN-γ H3K9me2
JQ1+IFN-γ IgG
JQ1+IFN-γ H3K9me2
E
Ctrl IgG
Ctrl H3K9me3
JQ1 IgG
JQ1 H3K9me3
IFN-γ IgG
IFN-γ H3K9me3
JQ1+IFN-γ IgG
JQ1+IFN-γ H3K9me3
F
Ctrl
BRD4-ex
BRD4-ex+siG9a#1
BRD4-ex+siG9a#2
SCC1
CAL27
Relative HLA mRNA expression
HLA-A
HLA-B
HLA-C
G
SCC1
CAL27
Relative MHC-I MFI
EV
BRD4-ex
BRD4-ex+siG9a#1
BRD4-ex+siG9a#2

Figure S5. BRD4 inhibition enhanced MHC-I expression via suppressing G9a in HNSCC, Related to Figure 5

(A-E) CAL27 cells treated with JQ1 and/or IFN-γ were subjected to ChIP assay with BRD4, G9a, H3K9me1, H3K9me2 and H3K9me3 antibodies. *p < 0.05, **p < 0.01 and ***p < 0.001 by 1-way ANOVA. ns, non-significant.

(F) RT-qPCR showed that the expression of *HLA-A*, *HLA-B* and *HLA-C* in SCC1 and CAL27 cells overexpressing *BRD4* and/or transfected with *G9a* siRNAs. ***p < 0.001 by1-way ANOVA.

(G) Flow Cytometry displayed cell surface MHC-I in SCC1 and CAL27 cells overexpressing *BRD4* and/or transfected with siRNA targeting *G9a*. ***p < 0.001 by 1-way ANOVA.

Figure S6

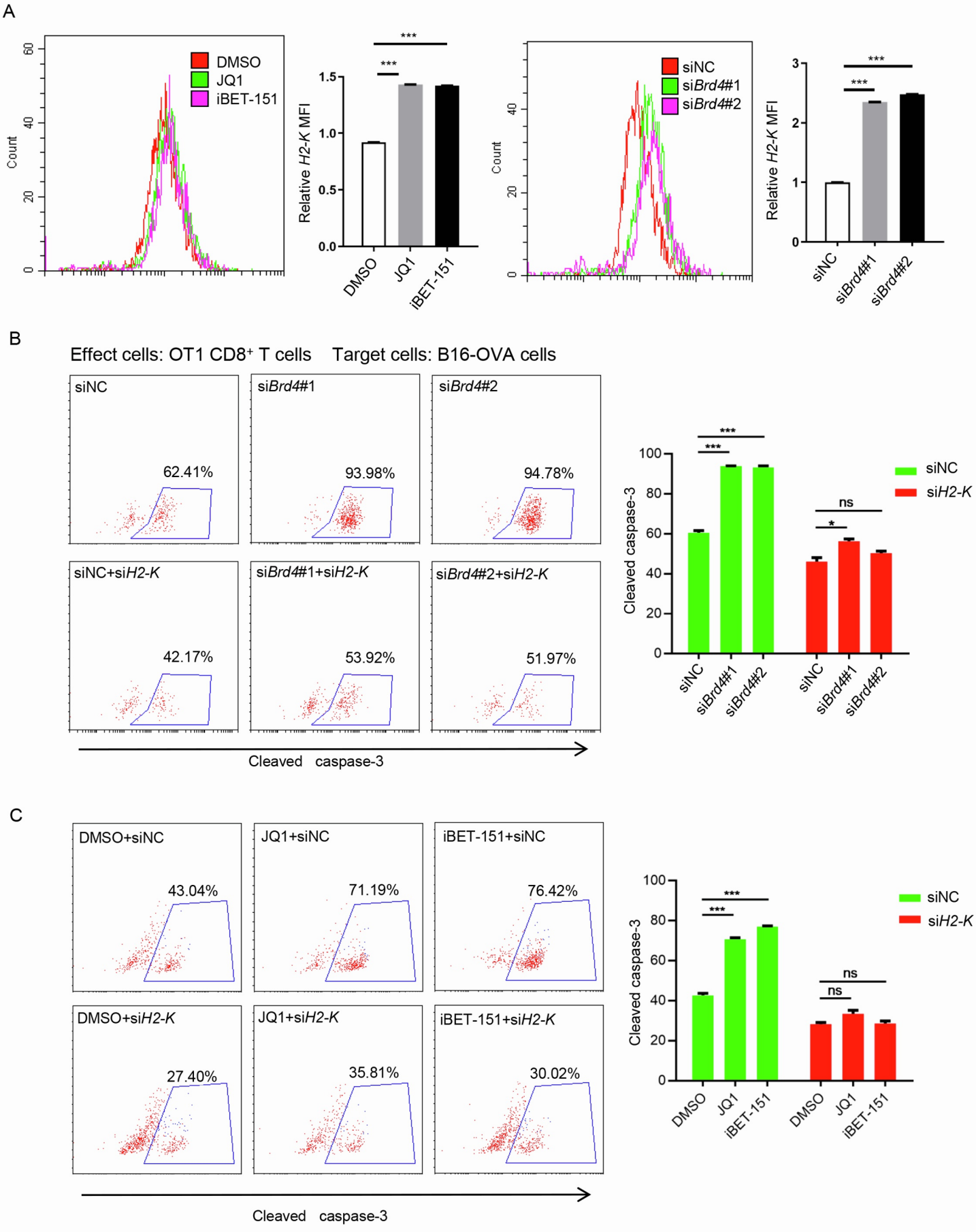

A
DMSO
JQ1
iBET-151
Count
Relative H2-K MFI
siNC
siBrd4#1
siBrd4#2
B
Effect cells: OT1 CD8+ T cells Target cells: B16-OVA cells
siNC
siBrd4#1
siBrd4#2
62.41%
93.98%
94.78%
siNC+siH2-K
siBrd4#1+siH2-K
siBrd4#2+siH2-K
42.17%
53.92%
51.97%
Cleaved caspase-3
Cleaved caspase-3
C
DMSO+siNC
JQ1+siNC
iBET-151+siNC
43.04%
71.19%
76.42%
DMSO+siH2-K
JQ1+siH2-K
iBET-151+siH2-K
27.40%
35.81%
30.02%
Cleaved caspase-3
Cleaved caspase-3

Figure S6. Pharmacological inhibition and knockdown of BRD4 in B16-OVA cells enhance cytotoxicity of OT-1 cells by modulating MHC-I *in vitro*, Related to Figure 6

(A) Flow Cytometry showed that the expression of MHC-I (*H2-K*) in B16/F10 cells upon JQ1 or iBET-151 treatment and transfected with *Brd4* siRNAs. ***p < 0.001 by 1-way ANOVA.

(B) Image and Quantification of cleaved caspase-3 positive cells in B16-OVA cells treated with si*Brd4* and si*H2-K* co-cultured with OT-1 cells. *p < 0.05 and ***p < 0.001, by 2-way ANOVA; ns, non-significant.

(C) Image and Quantification of cleaved caspase-3 positive cells in B16-OVA cells treated with JQ1 or iBET-151 and si*H2-K* co-cultured with OT-1 cells. ***p < 0.001 by 2-way ANOVA; ns, non-significant.

Figure S7

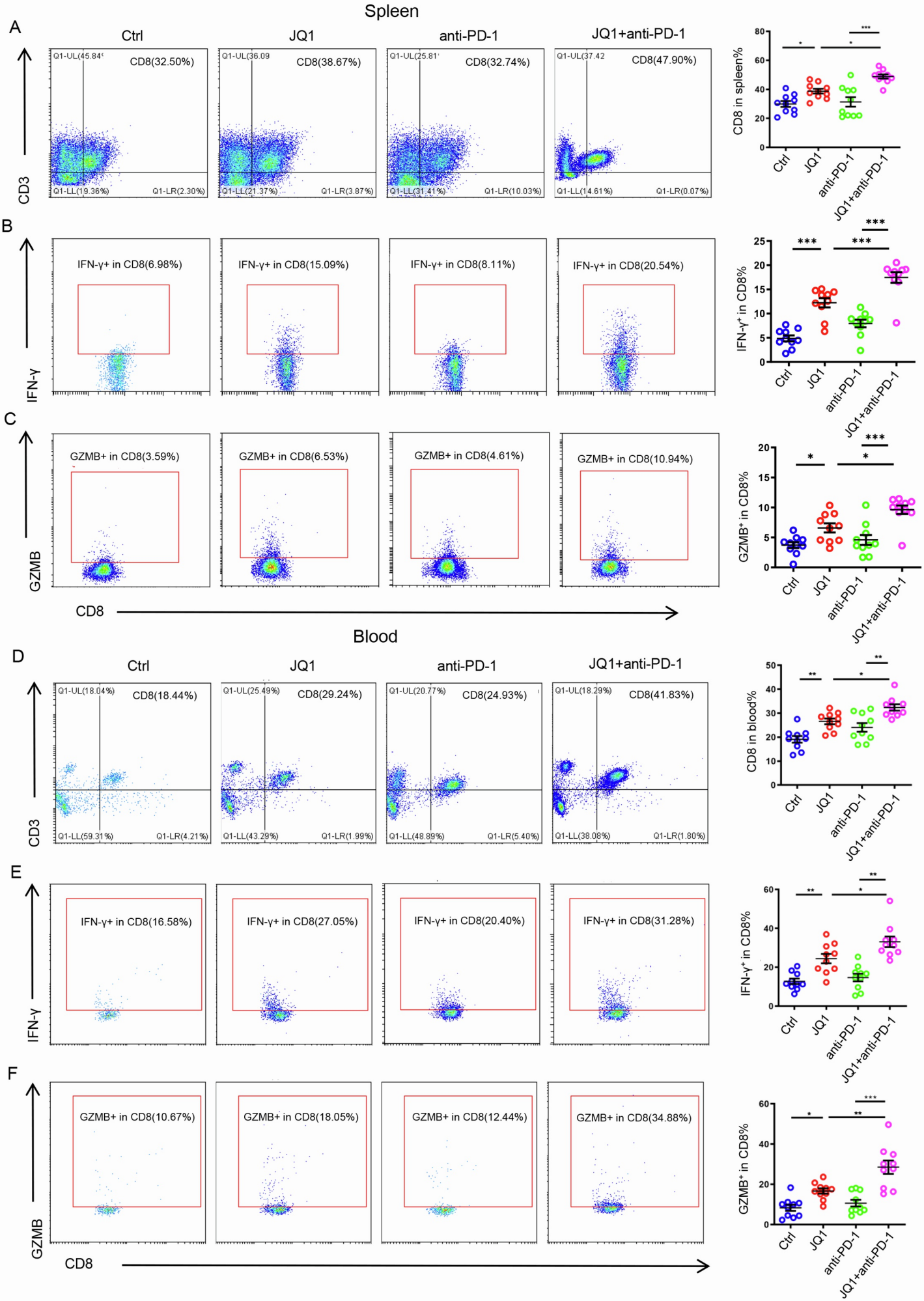

Spleen
A
Ctrl
JQ1
anti-PD-1
JQ1+anti-PD-1
CD8(32.50%)
CD8(38.67%)
CD8(32.74%)
CD8(47.90%)
CD3
CD8 in spleen%
B
IFN-γ+ in CD8(6.98%)
IFN-γ+ in CD8(15.09%)
IFN-γ+ in CD8(8.11%)
IFN-γ+ in CD8(20.54%)
IFN-γ
IFN-γ+ in CD8%
C
GZMB+ in CD8(3.59%)
GZMB+ in CD8(6.53%)
GZMB+ in CD8(4.61%)
GZMB+ in CD8(10.94%)
GZMB
CD8
GZMB+ in CD8%
Blood
D
CD8(18.44%)
CD8(29.24%)
CD8(24.93%)
CD8(41.83%)
CD8 in blood%
E
IFN-γ+ in CD8(16.58%)
IFN-γ+ in CD8(27.05%)
IFN-γ+ in CD8(20.40%)
IFN-γ+ in CD8(31.28%)
F
GZMB+ in CD8(10.67%)
GZMB+ in CD8(18.05%)
GZMB+ in CD8(12.44%)
GZMB+ in CD8(34.88%)

Figure S7. BET inhibition enhances the sensitivity of anti-PD-1 treatment in HNSCC with alterations of CD8$^+$ T cells in the spleen and peripheral blood, Related to Figure 7

(A) Percentage of CD8$^+$ T cells in the spleen from mice upon different treatment as indicated. n=10. *p < 0.05 and ***p < 0.001 by 1-way ANOVA.

(B) Percentage of intracellular staining of IFN-γ in CD8$^+$ T cells from spleen of mice upon different treatment as indicated. n=10. *p < 0.05 and ***p < 0.001 by 1-way ANOVA.

(C) Percentage of intracellular staining of Granzyme B in CD8$^+$ T cells from spleen of mice upon different treatment as indicated. n=10. *p < 0.05 and ***p < 0.001 by 1-way ANOVA.

(D) Percentage of CD8$^+$ T cells in the peripheral blood from mice upon different treatment as indicated. n=10. *p < 0.05 and **p < 0.01 by 1-way ANOVA.

(E) Percentage of intracellular staining of IFN-γ in CD8$^+$ T cells from peripheral blood of mice upon different treatment as indicated. n=10. *p < 0.05 and **p < 0.01 by 1-way ANOVA.

(F) Percentage of intracellular staining of Granzyme B in CD8$^+$ T cells from peripheral blood of mice upon different treatment as indicated. n=10. *p < 0.05, **p < 0.01 and ***p < 0.001 by 1-way ANOVA.

Tables

Table S1. Sequences for sgRNA and siRNA

Gene	Target sequences
Human sgRNA	
hBRD4 sgRNA1	5’-ACGCTCCTCTTTCTCCCGAG-3’
hBRD4 sgRNA2	5’-CTTTCTCCCGAGCGGCGCGG-3’
Human siRNA	
hMHC-I siRNA1	5’-CCUACGACGGCAAGGAUUAdTdT-3’
hMHC-I siRNA2	5’-GCUACUACAACCAGAGCGAdTdT-3’
hG9a siRNA1	5’-GGAUGAAUCUGAGAAUCUUdTdT-3’
hG9a siRNA2	5’-GAGUGAUGAUGUCCACUCAdTdT-3’
Mouse sgRNA	
mBrd4 sgRNA1	5’-TGGGATCACTAGCATGTCTA-3’
mBrd4 sgRNA1	5’-ACTAGCATGTCTACGGAGAG-3’
Mouse siRNA	
H2-K siRNA1	5’-UAUGAGAAGACAUUGUCUGUCdTdT-3’
H2-K siRNA2	5’-AACAAUCAAGGUUACAUUCAAdTdT-3’

Table S2. Primers for RT-qPCR and ChIP-PCR

Gene	Forward	Reverse
RT-qPCR primers		
HLA-A	AAAAGGAGGGAGTTACACTCAGG	GCTGTGAGGGACACATCAGAG
HLA-B	GGGATGGCGAGGACCAAAC	ACAGCTCCGATGACCACAAC
HLA-C	CTGTCCTGGTTGTCCTAGCTGT	TGAGAGCAGCTCCCTCCTTTT
BRD4	GTCCTATGAGGAGAAGCGGC	GACGGCTTCAGGGTCTCAAA
G9a	CAAGATTGACCGCATCAGCG	CAGTGGTGTTTGACCATGCG
TUBB4A	TGAGATCCGAAATGGCCCATA	TAGTGACCACGGGCATAGTTG
ChIP-PCR primers		
HLA-A promoter	GGACCCAGTTCTCACTCCCATT	GGGTGCGTGCGGACTTTAGAAC
HLA-B promoter	GCTTCATCTCAGTGGGCTACGT	TGTGTTCCGGTCCCAATACTCC
HLA-C promoter	GCGTCGGGTCCTTCTTCCTGAATA	GGAGACGCTGATTGGCTTCTCT