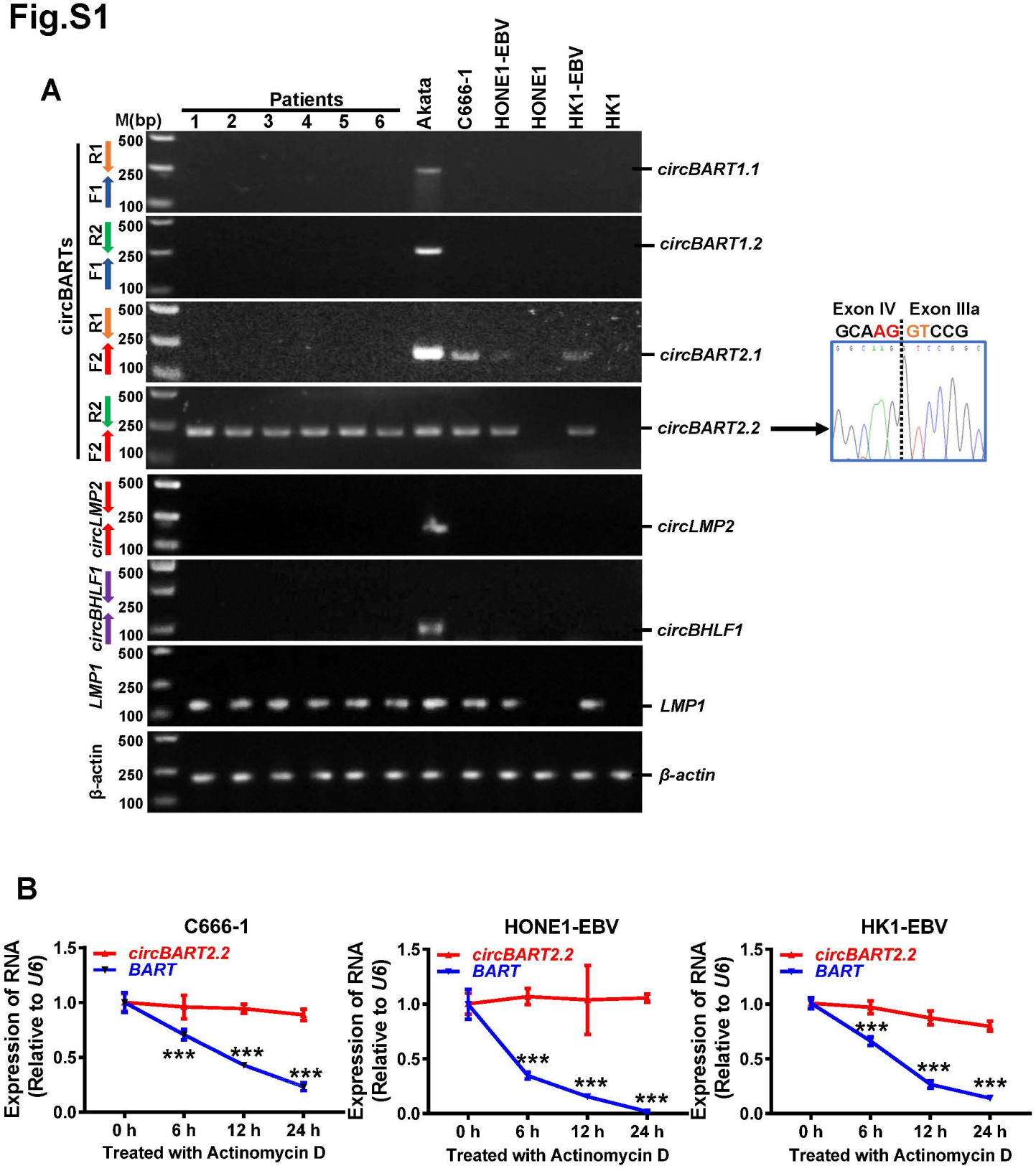
Fig.S1


A
Patients
M(bp)
1 2 3 4 5 6
Akata
C666-1
HONE1-EBV
HONE1
HK1-EBV
HK1
circBARTs
R1
F1
R2
F1
R1
F2
R2
F2
circLMP2
circBHLF1
LMP1
β-actin
500
250
100
circBART1.1
circBART1.2
circBART2.1
circBART2.2
circLMP2
circBHLF1
LMP1
β-actin
Exon IV Exon IIIa
GCAAG:GTCCG
B
C666-1
HONE1-EBV
HK1-EBV
Expression of RNA (Relative to U6)
circBART2.2
BART
***
0 h
6 h
12 h
24 h
Treated with Actinomycin D

Supplementary Data

Fig. S1. Detection of EBV-encoded circRNAs in EBV positive NPC cells

A. The expression of EBV-encoded circRNAs was examined in Akata, C666-1, HONE1-EBV, and HK1-EBV cells and six NPC clinical samples by real-time PCR (RT-PCR). EBV-negative HONE1 and HK1 cells were used as negative controls. Sanger sequencing was used to confirm the presence of *circBART2.2*. The linear *LMP1*mRNA and β-*actin* mRNA were used as negative controls.

B. The stability of *circBART2.2*, and the linear BART gene was examined in C666-1, HONE1-EBV, and HK1-EBV cells after actinomycin D (1μg/mL) treatment for 0,6,12, and 24 h. U6 RNA was used as an internal control, and BART mRNA was selected as a negative control. ***, $P< 0.001$.

Fig.S2

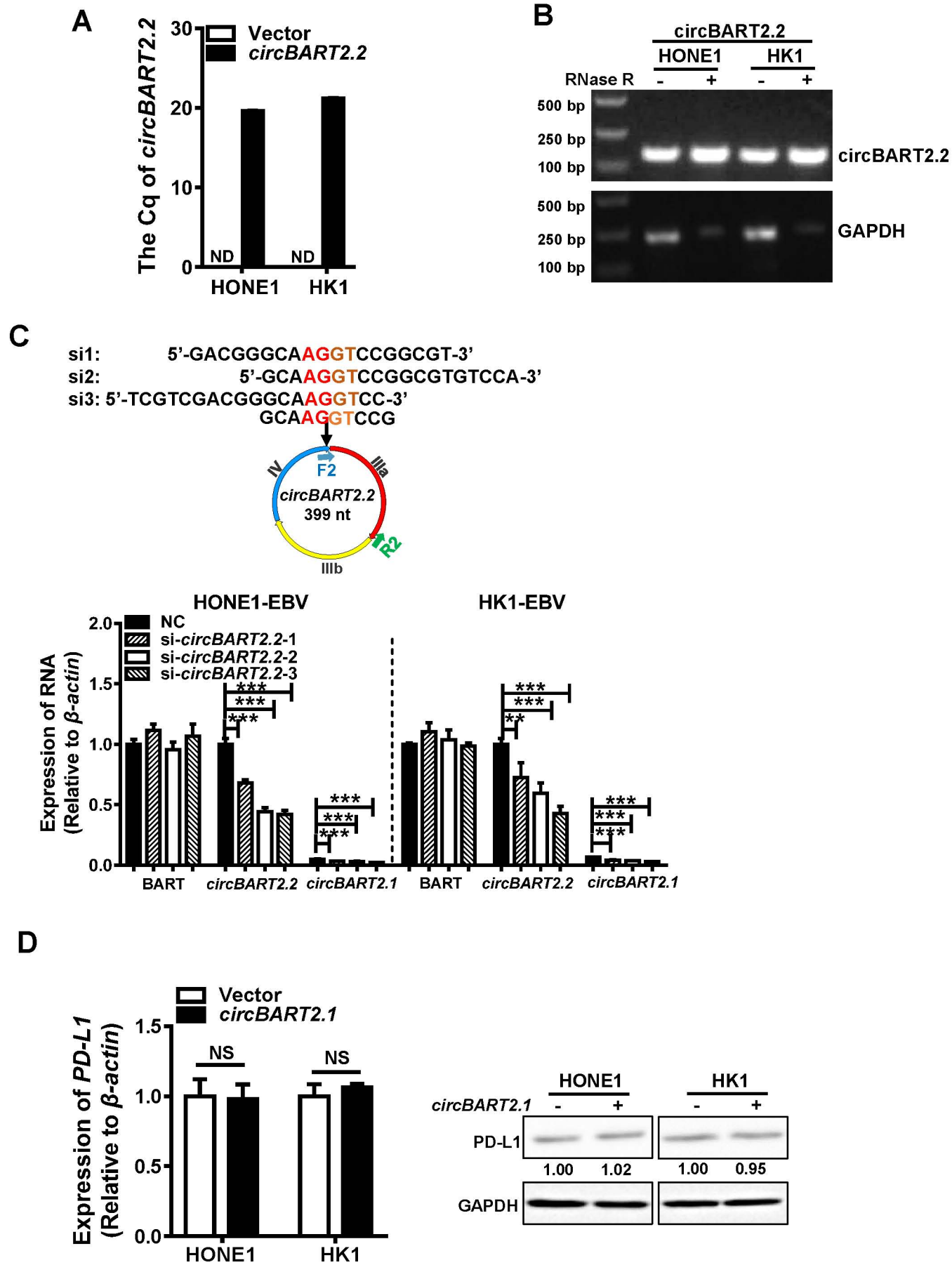

A
The Cq of circBART2.2
Vector
circBART2.2
ND
ND
HONE1
HK1
B
circBART2.2
HONE1
HK1
RNase R
500 bp
250 bp
100 bp
circBART2.2
GAPDH
C
si1: 5'-GACGGGCAAGGTCCGGCGT-3'
si2: 5'-GCAAGGTCCGGCGTGTCCA-3'
si3: 5'-TCGTCGACGGGCAAGGTCC-3'
GCAAGGTCCG
F2
circBART2.2
399 nt
IV
IIIa
IIIb
HONE1-EBV
HK1-EBV
NC
si-circBART2.2-1
si-circBART2.2-2
si-circBART2.2-3
Expression of RNA
(Relative to β-actin)
BART
circBART2.2
circBART2.1
D
Vector
circBART2.1
Expression of PD-L1
(Relative to β-actin)
NS
HONE1
HK1
circBART2.1
PD-L1
GAPDH
1.00
1.02
0.95

Fig. S2. Expression of *circBART2.2* in NPC cells after overexpression or knockdown

A. The overexpression efficiency of*circBART2.2* was examined in HONE1 and HK1 cells using the overexpression *circBART2.2* vector by RT-PCR. ND, no detection.

B. The stability of *circBART2.2* was detected in RNase R-treated HONE1-EBV and HK1-EBV cells after overexpression of *circBART2.2* by qPCR. GADPH mRNA was used as the negative control.

C. The knockdown efficiency of *si-circBART2.2* was examined in HONE1-EBV and HK1-EBV cells by RT-PCR using three *circBART2.2* siRNAs. All three siRNAs were specifically designed according to the circular splice site of *circBART2.2*. Linear *BART* RNA and *circBART2.1* were used as controls. **, $P < 0.01$; ***, $P < 0.001$.

D. The expression of PD-L1 protein was examined in EBV-negative HONE1 and HK1 cells after *circBART2.1* overexpression, as demonstrated by RT-PCR and western blotting.

Fig.S3

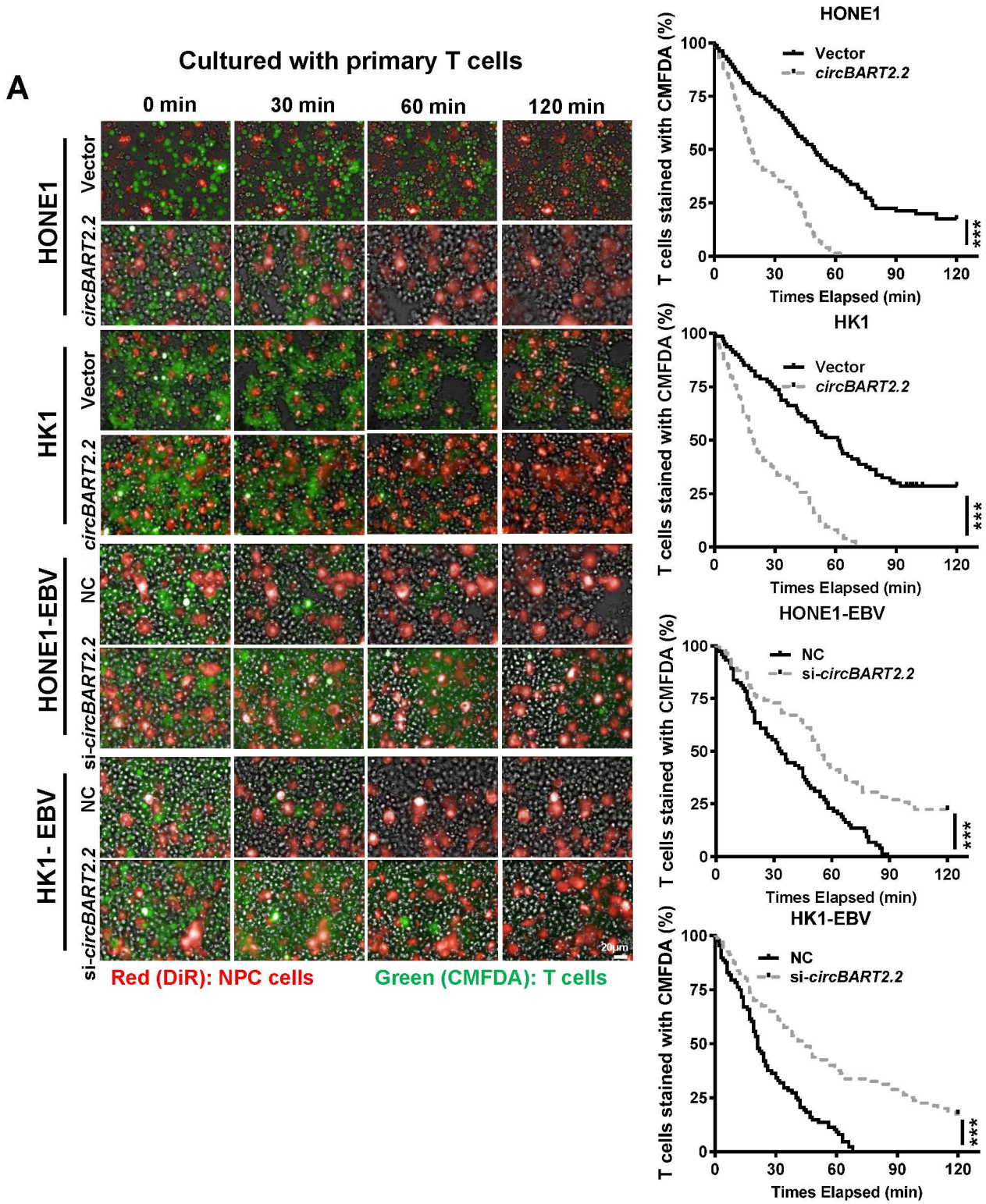

A
Cultured with primary T cells
0 min
30 min
60 min
120 min
HONE1
Vector
circBART2.2
HK1
Vector
circBART2.2
HONE1-EBV
NC
si-circBART2.2
HK1- EBV
NC
si-circBART2.2
20μm
Red (DiR): NPC cells
Green (CMFDA): T cells
HONE1
Vector
circBART2.2
T cells stained with CMFDA (%)
Times Elapsed (min)
***
HK1
Vector
circBART2.2
T cells stained with CMFDA (%)
Times Elapsed (min)
***
HONE1-EBV
NC
si-circBART2.2
T cells stained with CMFDA (%)
Times Elapsed (min)
***
HK1-EBV
NC
si-circBART2.2
T cells stained with CMFDA (%)
Times Elapsed (min)
***

Fig.S3

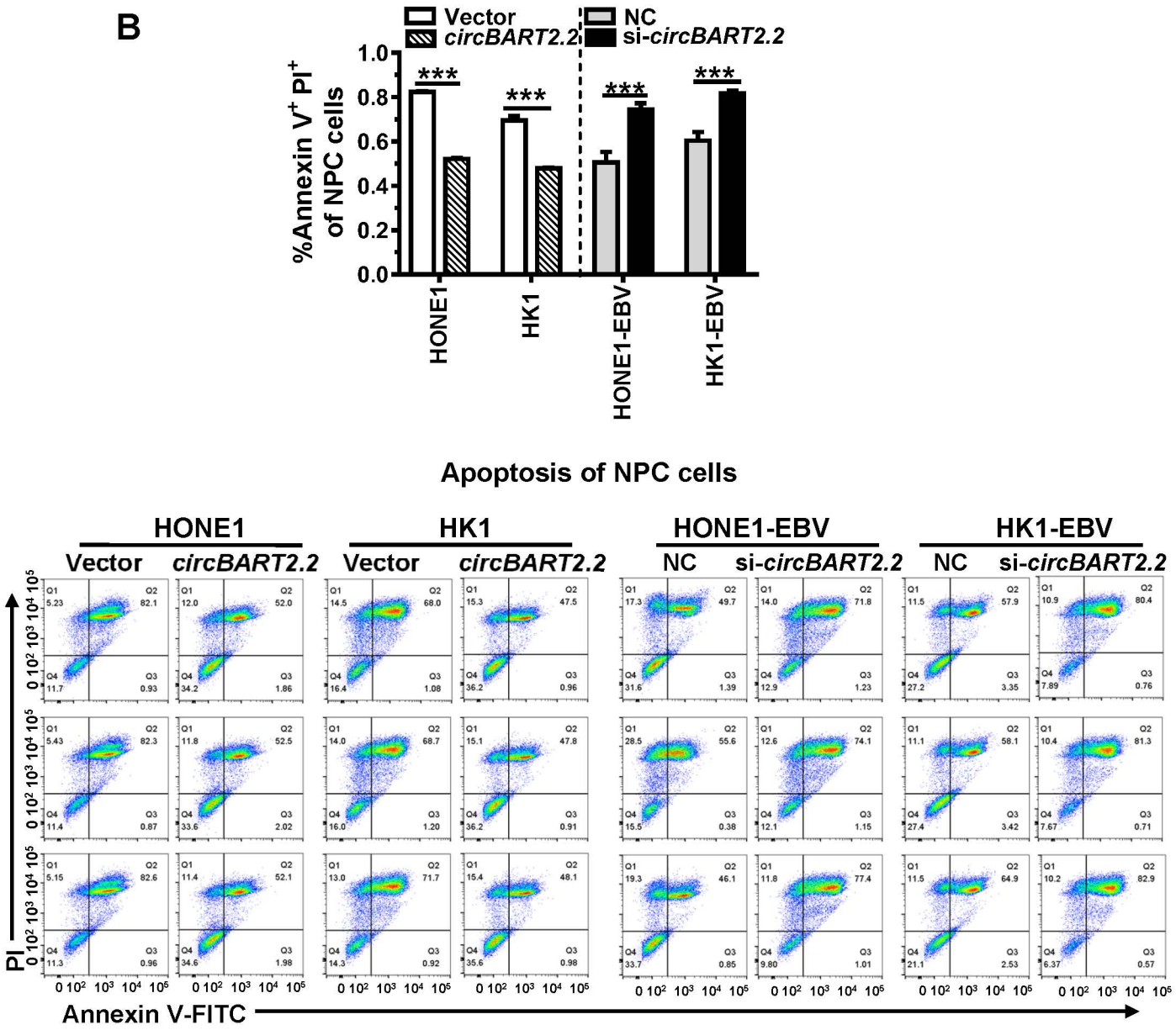

B
Vector
circBART2.2
NC
si-circBART2.2
%Annexin V+ PI+ of NPC cells
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
***
HONE1
HK1
HONE1-EBV
HK1-EBV
Apoptosis of NPC cells
HONE1
HK1
HONE1-EBV
HK1-EBV
Vector
circBART2.2
Vector
circBART2.2
NC
si-circBART2.2
NC
si-circBART2.2
PI
Annexin V-FITC

Fig.S3

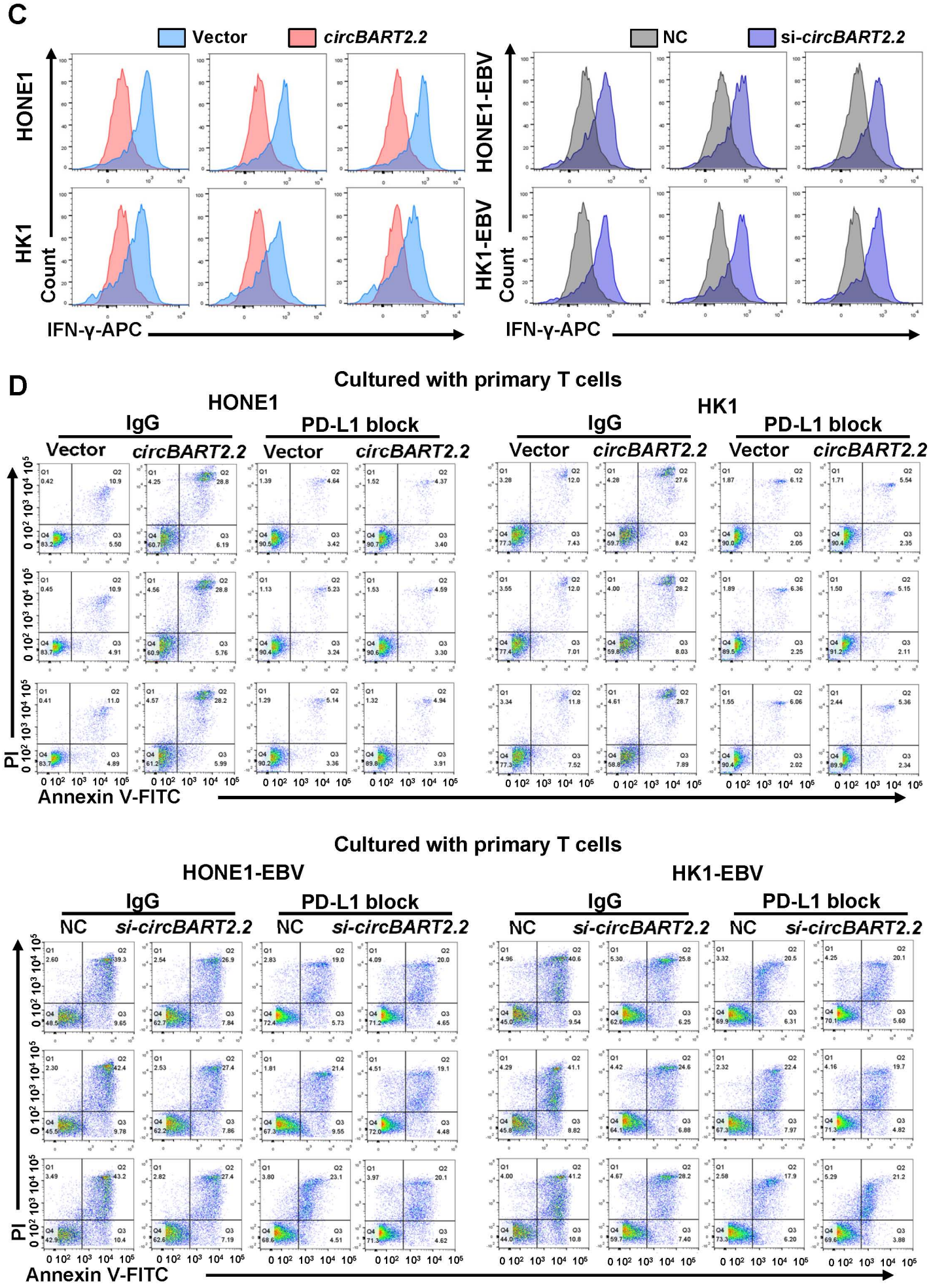

C
Vector
circBART2.2
NC
si-circBART2.2
HONE1
HK1
HONE1-EBV
HK1-EBV
Count
IFN-γ-APC
D
Cultured with primary T cells
HONE1
HK1
IgG
PD-L1 block
Vector
circBART2.2
PI
Annexin V-FITC
Cultured with primary T cells
HONE1-EBV
HK1-EBV
NC
si-circBART2.2

Fig. S3. *circBART2.2* affected the activity of T-cells through PD-L1

A. Real-time tracking of T-cell activity status was measured using a high-content cell imaging analysis system. Red: HONE1 or HK1cells after *circBART2.2* overexpression and simultaneous RIG-I knockdown. Red (DiR): NPC cells; green (CMFDA): activated human primary T-cells. Left, representative images for each time point. Right, statistical results according to the green CMFDA signal in T-cells. ***, $P < 0.001$.

B. The degree of NPC cell apoptosis in HONE1 and HK1 cells after circBART2.2 overexpression and co-culture with active primary T-cells was measured by flow cytometry using Annexin V-FITC and PI. Original flow cytometry results (left); statistical results (right).

C. The original flow cytometry results regarding the concentration of IFN-γ secretion by serum in HONE1 and HK1 cells after *circBART2.2* overexpression orHONE1-EBV and HK1-EBV cells after *circBART2.2* knockdown using APC-stained anti-IFN-γ antibody.

D. The original flow cytometry results regarding the degree of T-cell apoptosis in HONE1 and HK1 cells after *circBART2.2* overexpression or HONE1-EBV and HK1-EBV cells after *circBART2.2* knockdown when treated with anti-PD-L1 antibody using Alexa Fluor 488 Annexin V and propidium iodide (PI) staining.

Fig.S4

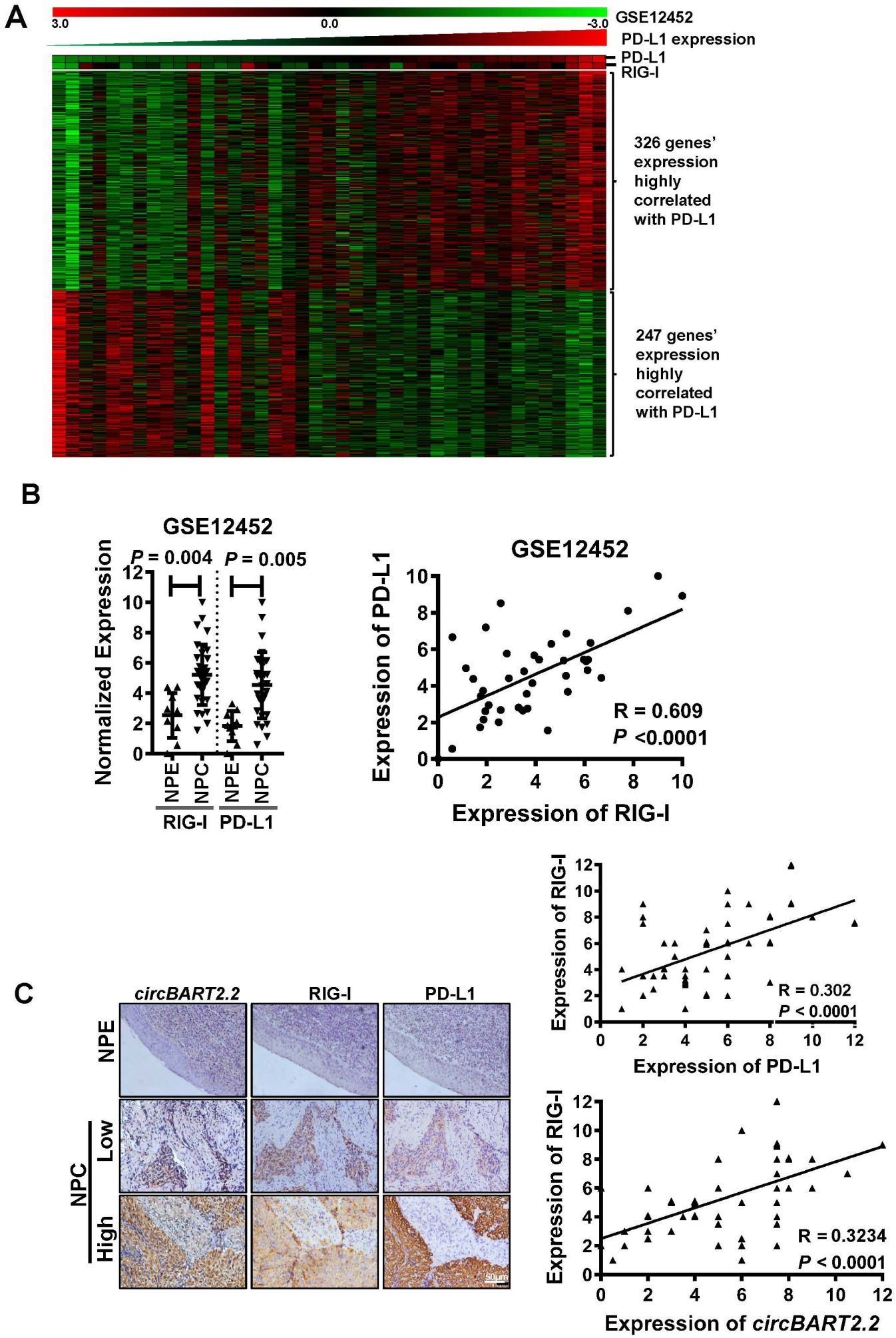

A
3.0
0.0
-3.0
GSE12452
PD-L1 expression
PD-L1
RIG-I
326 genes' expression highly correlated with PD-L1
247 genes' expression highly correlated with PD-L1
B
GSE12452
P = 0.004
P = 0.005
Normalized Expression
NPE
NPC
NPE
NPC
RIG-I
PD-L1
GSE12452
Expression of PD-L1
R = 0.609
P <0.0001
Expression of RIG-I
C
circBART2.2
RIG-I
PD-L1
NPE
NPC
Low
High
50 μm
Expression of RIG-I
R = 0.302
P < 0.0001
Expression of PD-L1
Expression of RIG-I
R = 0.3234
P < 0.0001
Expression of circBART2.2

Fig. S4. PD-L1 and RIG-I were highly expressed in NPC and correlated with each other

A. Heat-map of the 172 molecules' expression, which was screened from mass spectrometry, in the head and neck squamous cell carcinoma samples (HNSCC) TCGA database. The TCGA data were ranked by PD-L1 expression from low to high, and the 172 molecules were then analyzed for expression and correlation with PD-L1 expression in HNSCC samples. RIG-I was significantly correlated with PD-L1 expression in HNSCC samples from the TCGA database using heat-map analysis (P = 6.1e-4, R = 3.2).

B. The expression of PD-L1 and RIG-I was analyzed and correlated with each other in the public online NPC GSE12452 database. Both PD-L1 (P = 0.009) and RIG-I (P = 0.001) were highly expressed in 31 NPC clinical samples compared with 10 NPE samples NPE, n = 10; NPC, n = 31, P = 0.0124, R = 0.1499.

C. The correlation of PD-L1 (P < 0.0001, R = 0.3234) and RIG-I (P < 0.0001, R = 0.302) with *circBART2.2* was analyzed in 52 NPC tissues and 36 non-cancerous nasopharyngeal epithelial tissue samples by *in situ* hybridization or immunohistochemistry.

Fig.S5

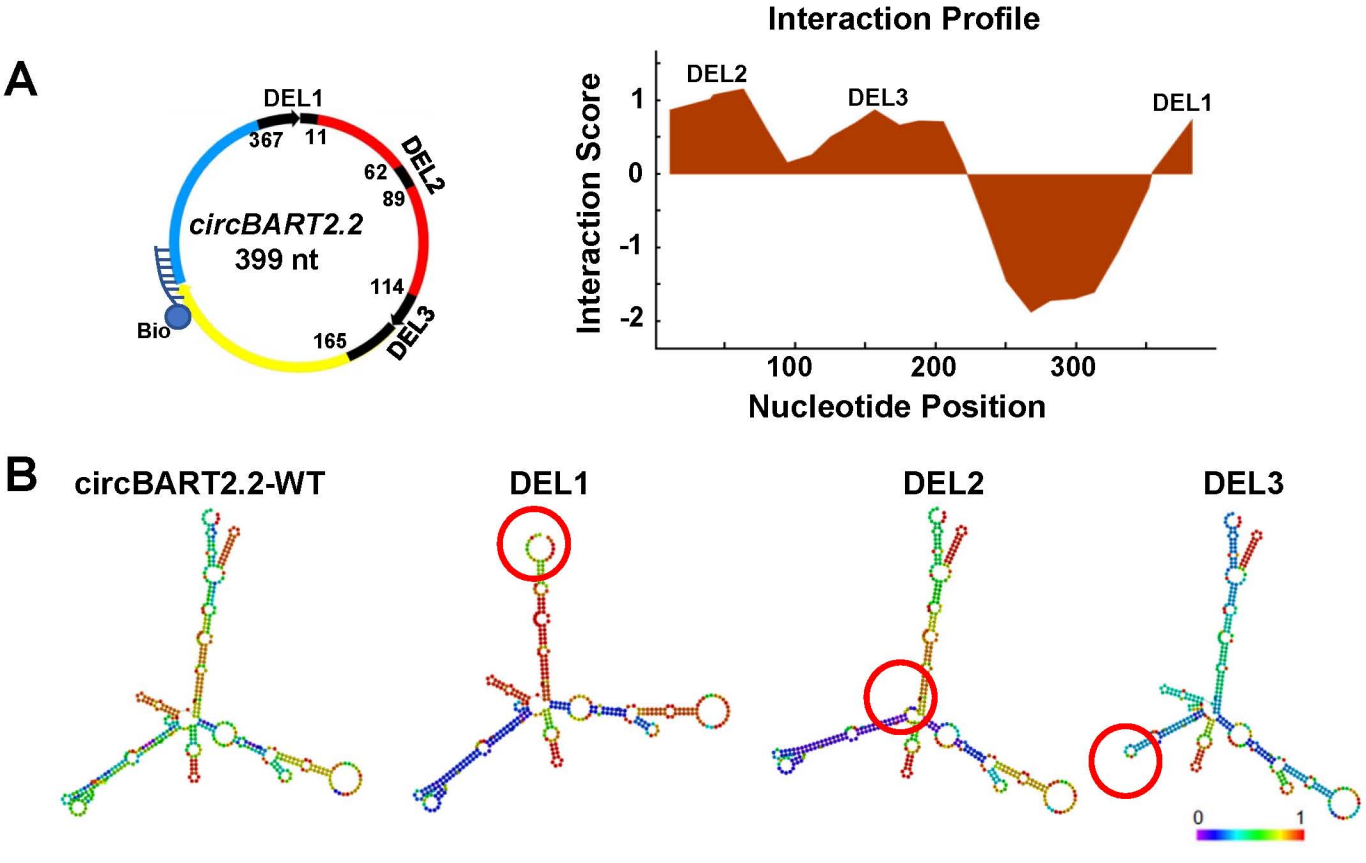

A
DEL1
367
11
DEL2
62
89
circBART2.2
399 nt
114
Bio
165
DEL3
Interaction Profile
DEL2
DEL3
DEL1
Interaction Score
1
0
-1
-2
100
200
300
Nucleotide Position
B
circBART2.2-WT
DEL1
DEL2
DEL3
0
1
C
circBART2.2
DEL1
DEL2
DEL3
RNase R
- + - + - +
500 bp
250 bp
100 bp
circBART2.2
fragments
500 bp
250 bp
100 bp
GAPDH

Fig. S5. *circBART2.2* interacted with RIG-I protein

A. Three well characterized stem loop structures (367–11 bp named Mut 1, 114–165 bp named Mut 2, and 62–89 bp named Mut 3) were predicted on the *circBART2.2* using the RNAfold database.

B. RIG-I was predicted to bind with the *circBART2.2* sequenceusing the catRAPID software.

C. Stability of three *circBART2.2* fragments was detected in RNase R-treated HONE1 cells after the overexpression of three *circBART2.2* fragments (Mut1, Mut2, and Mut3) by qPCR, respectively. The GADPH mRNA was used as a negative control.

Fig.S6

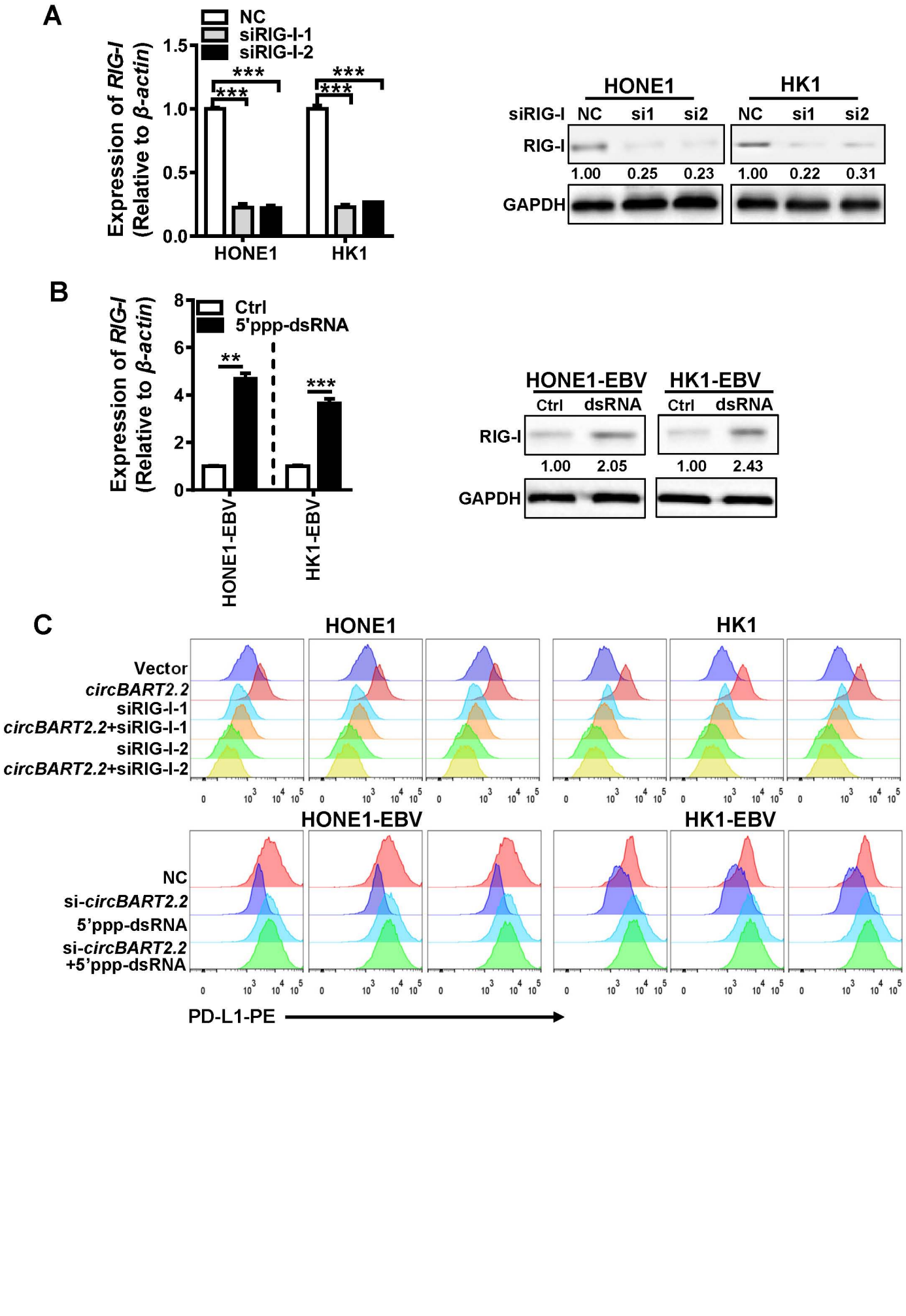

A
NC
siRIG-I-1
siRIG-I-2
Expression of RIG-I (Relative to β-actin)
1.5
1.0
0.5
0.0
***
***
***
***
HONE1
HK1
HONE1
HK1
siRIG-I NC si1 si2 NC si1 si2
RIG-I
1.00 0.25 0.23 1.00 0.22 0.31
GAPDH
B
Ctrl
5'ppp-dsRNA
Expression of RIG-I (Relative to β-actin)
8
6
4
2
0
**
***
HONE1-EBV
HK1-EBV
HONE1-EBV
HK1-EBV
Ctrl dsRNA Ctrl dsRNA
RIG-I
1.00 2.05 1.00 2.43
GAPDH
C
HONE1
HK1
Vector
circBART2.2
siRIG-I-1
circBART2.2+siRIG-I-1
siRIG-I-2
circBART2.2+siRIG-I-2
HONE1-EBV
HK1-EBV
NC
si-circBART2.2
5'ppp-dsRNA
si-circBART2.2 +5'ppp-dsRNA
PD-L1-PE

Fig.S6

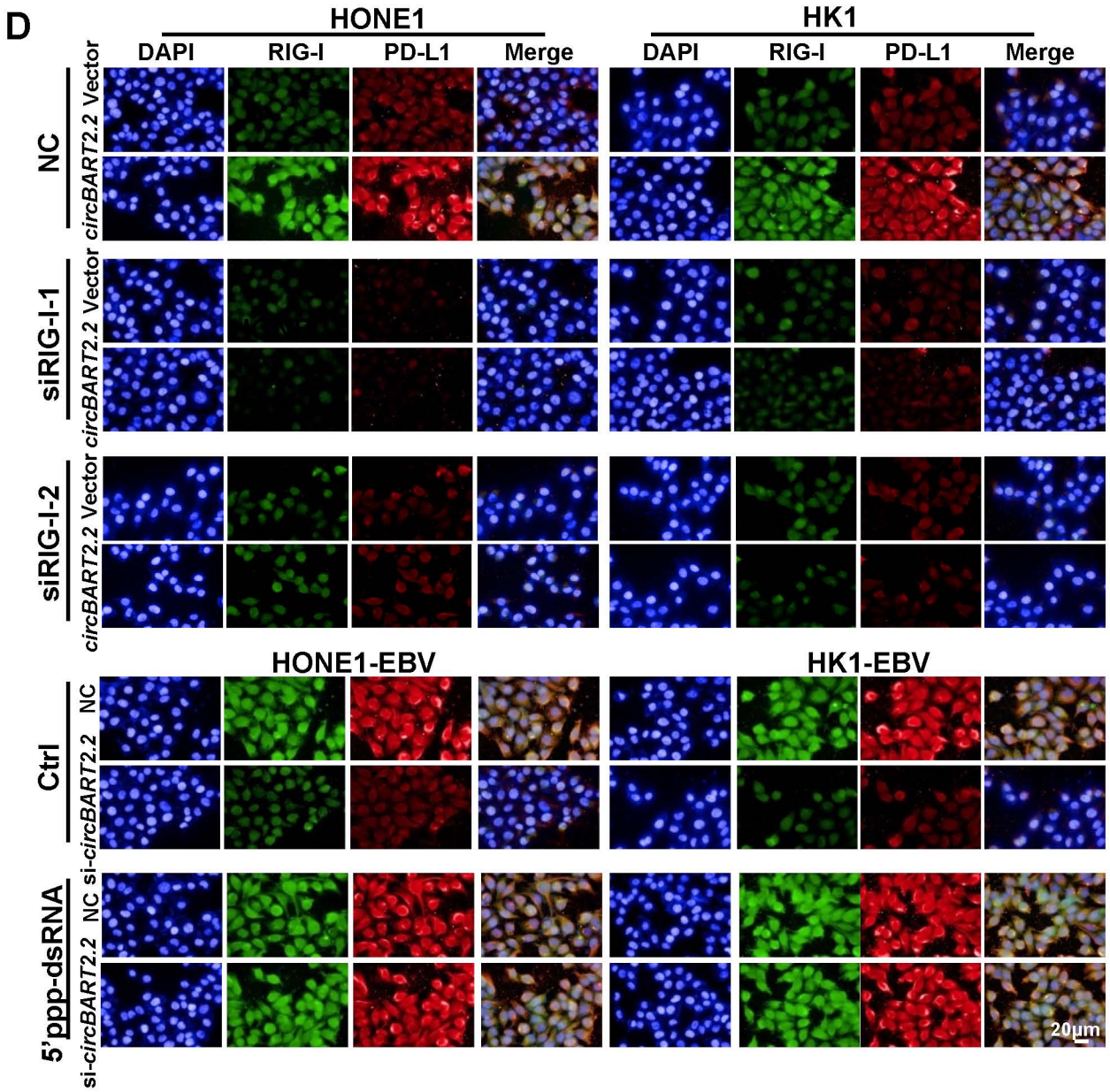

D
HONE1
HK1
DAPI
RIG-I
PD-L1
Merge
NC
siRIG-I-1
siRIG-I-2
Vector
circBART2.2
HONE1-EBV
HK1-EBV
Ctrl
5'ppp-dsRNA
si-circBART2.2
20μm

Fig. S6. *circBART2.2* regulated PD-L1 expression through RIG-I

A. The knockdown efficiency of *siRIG-I* was examined in HONE1 and HK1 cells using two RIG-I siRNAs by RT-PCR and western blotting. ***, $P < 0.001$.

B. The efficiency of RIG-I activator 5’ ppp-dsRNA was examined in HONE1-EBV and HK1-EBV cells by RT-PCR and western blotting. ***, $P < 0.001$.

C. The original flow cytometry results of PD-L1 expression in EBV-negative HONE1 and HK1cells after *circBART2.2* overexpression or knockdown of RIG-I simultaneously or in EBV-positive HONE1-EBV and HK1-EBV cells after *circBART2.2* knockdown and RIG-I activator treatment. Three independent replicates were evaluated for each group.

D. RIG-I (green) and PD-L1 (red) expression was measured in EBV-negative or -positive NPC cells after *circBART2.2* overexpression or knockdown by immunofluorescence using anti-RIG-I and anti-PD-L1antibodies. Nuclei were stained with DAPI (blue). 200 ×, scale bar = 50 μm.

Fig.S7

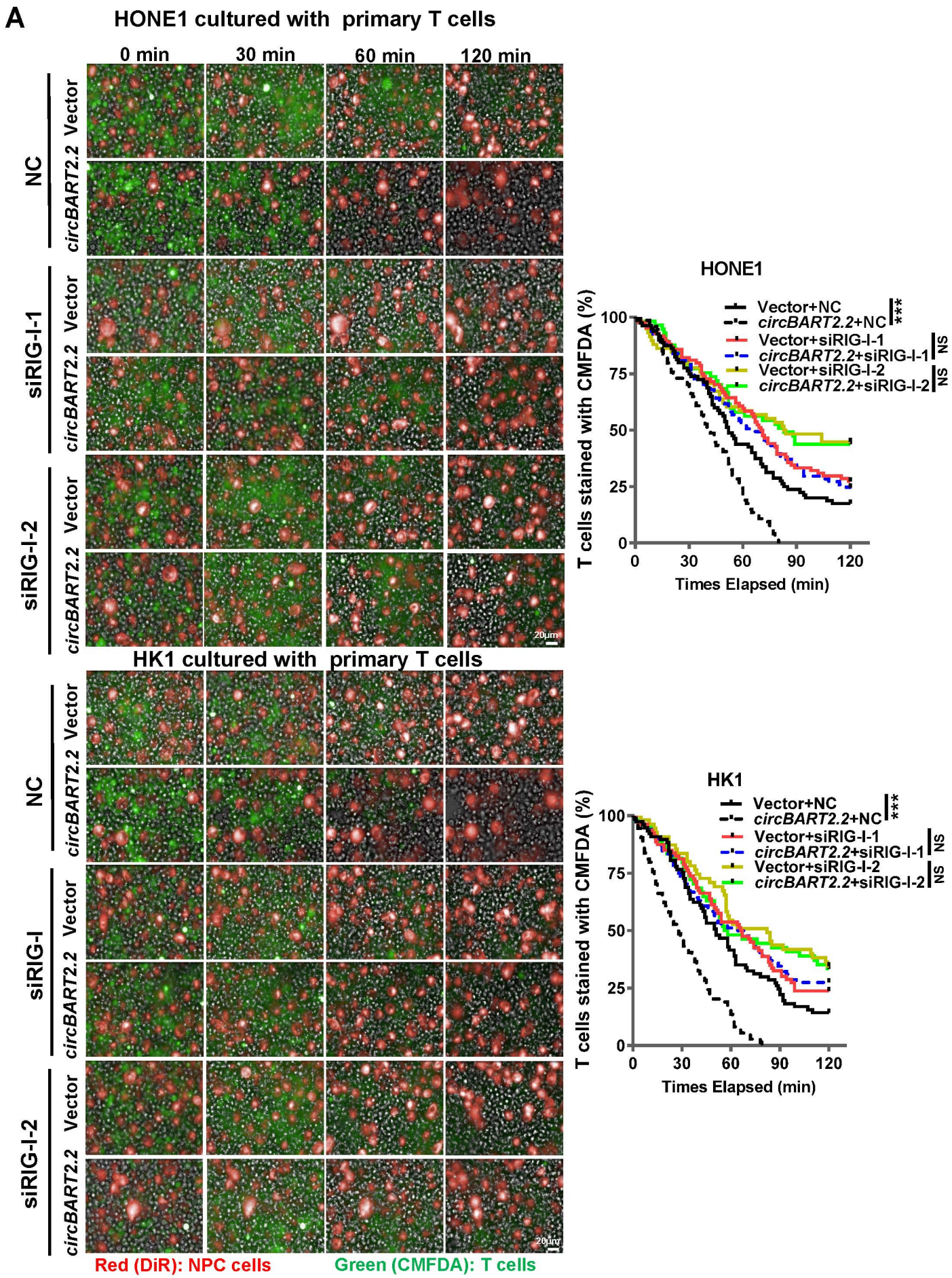

A
HONE1 cultured with primary T cells
0 min
30 min
60 min
120 min
NC
siRIG-I-1
siRIG-I-2
Vector
circBART2.2
20μm
HK1 cultured with primary T cells
siRIG-I
Red (DiR): NPC cells
Green (CMFDA): T cells
HONE1
T cells stained with CMFDA (%)
Times Elapsed (min)
Vector+NC
circBART2.2+NC
Vector+siRIG-I-1
circBART2.2+siRIG-I-1
Vector+siRIG-I-2
circBART2.2+siRIG-I-2
***
NS
HK1
**

Fig.S7

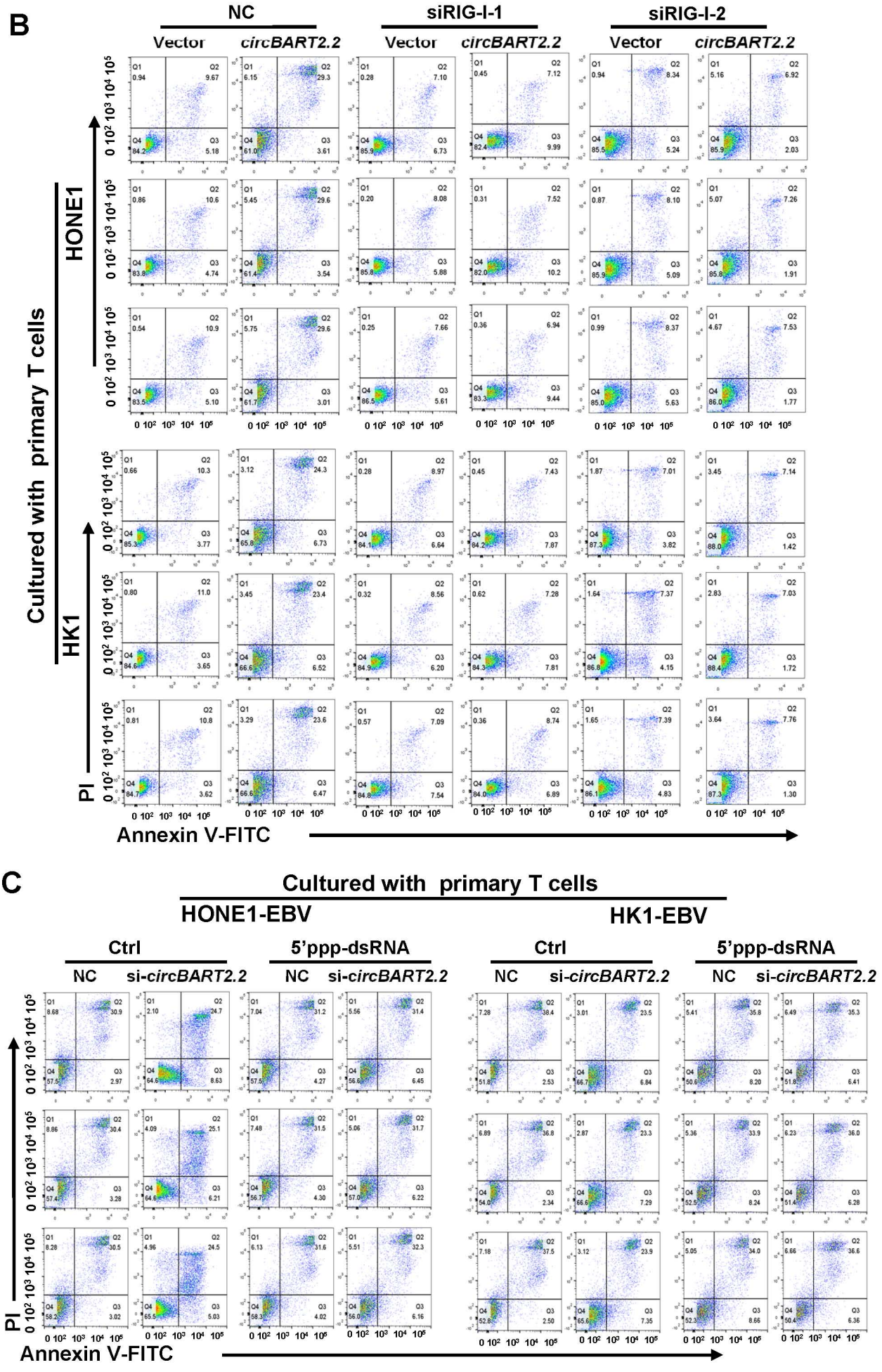

B
NC
siRIG-I-1
siRIG-I-2
Vector
circBART2.2
HONE1
HK1
Cultured with primary T cells
PI
Annexin V-FITC
C
Cultured with primary T cells
HONE1-EBV
HK1-EBV
Ctrl
5'ppp-dsRNA
NC
si-circBART2.2
PI
Annexin V-FITC

Fig. S7. *circBART2.2* affected the activity of T cells through RIG-I

A. Real-time tracking of T-cell activity status using high-content cell imaging analysis of co-culture of EBV-negative or -positive NPC cells after *circBART2.2* overexpression or knockdown. Red: NPC cell lines. Green: activated human primary T-cells Red (DiR): NPC cells; green (CMFDA): activated human primary T-cells. Left, representative images for each time point. Right, statistical results according to the green CMFDA signal in T-cells. ***, $P < 0.001$.

B. Apoptosis of primary human T-cells was examined by flow cytometric analysis in co-culture with HONE1 and HK1 cells after *circBART2.2* overexpression and simultaneous RIG-I knockdown. Each experiment was independently repeated three times. The statistical results are shown at the bottom. NS, not significant; *** $P < 0.001$.

C. The degree of T-cell apoptosis in HONE1-EBV and HK1-EBV cells after *circBART2.2* knockdown and simultaneous RIG-I activator 5'-ppp-dsRNA treatment using Alexa Fluor 488 Annexin V and propidium iodide (PI) staining. Each experiment was independently repeated three times; the statistical results are shown at the bottom. NS, not significant; *** $P < 0.001$.

Fig.S8

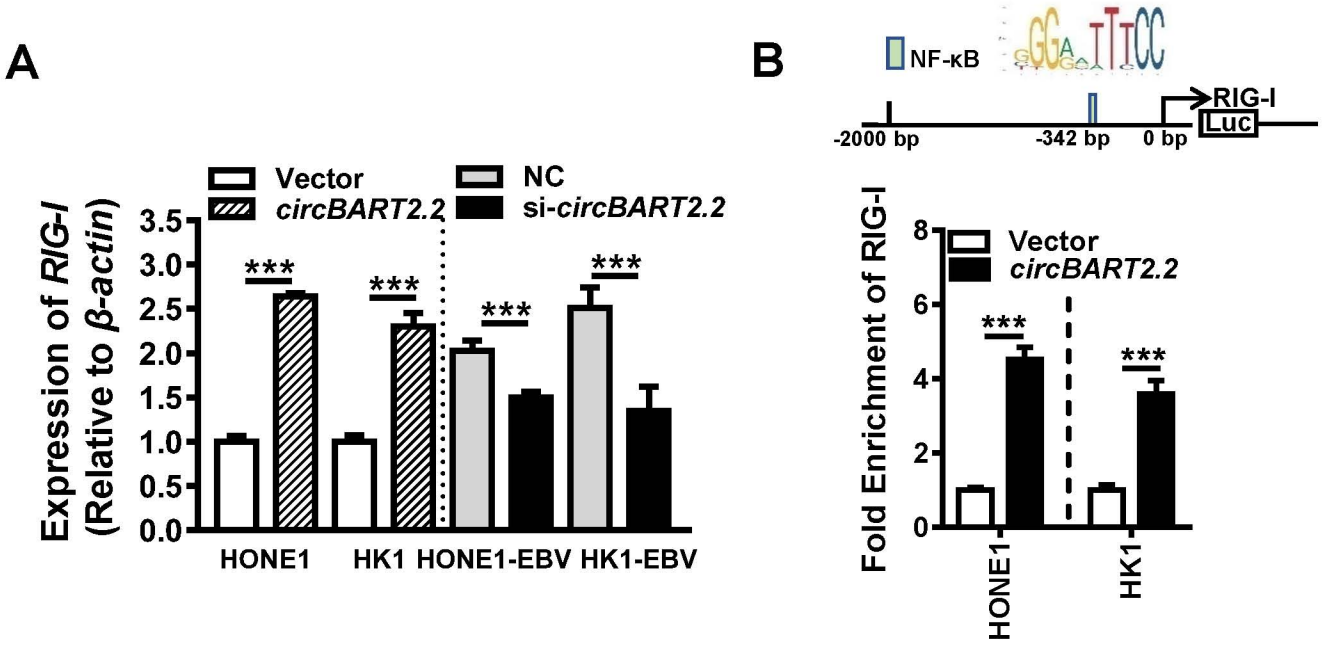
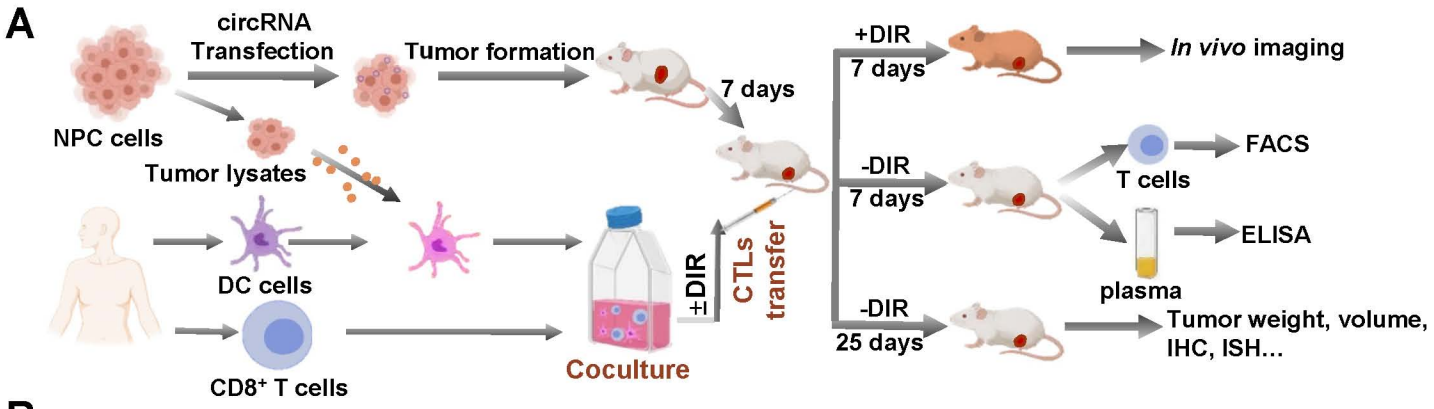

A
Vector
circBART2.2
NC
si-circBART2.2
Expression of RIG-I
(Relative to β-actin)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
***
***
HONE1
HK1
HONE1-EBV
HK1-EBV
B
NF-κB
RIG-I
Luc
-2000 bp
-342 bp
0 bp
Fold Enrichment of RIG-I
8
6
4
2
0
Vector
circBART2.2
***
***
HONE1
HK1

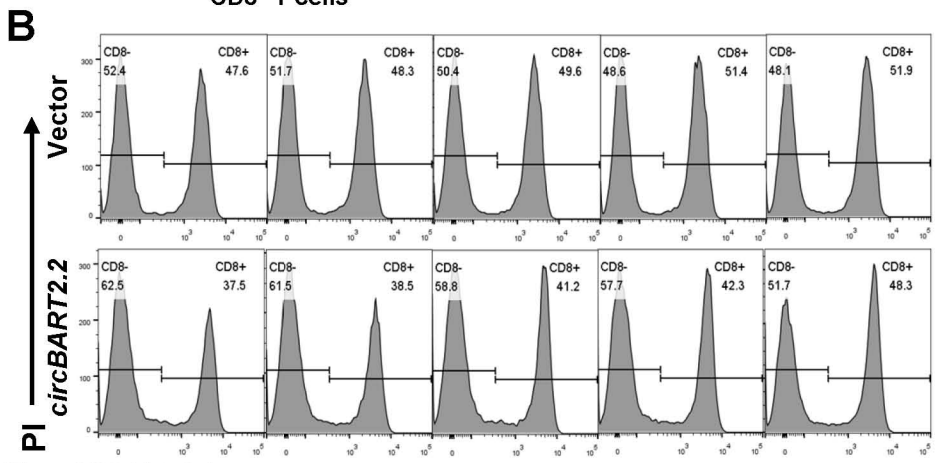
Fig. S8. *circBART2.2* promoted RIG-I activation

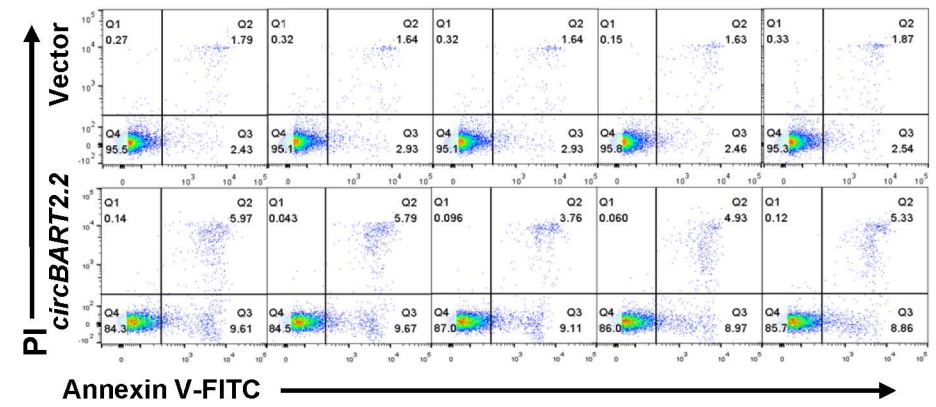
A. *RIG-I* mRNA expression was measured by RT-PCR in EBV-negative HONE1 and HK1 cells or EBV-positive HONE1-EBV and HK1-EBV cells after *circBART2.2* overexpression or knockdown.***, $P < 0.001$.

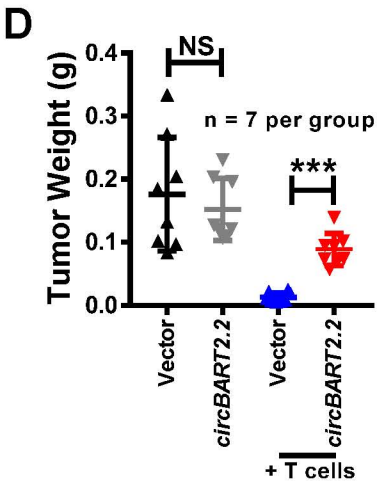
B. Binding of the transcription factor NF-κB near -342 bp on the RIG-I promoter region was predicted by the JASPAR software (up) and detected in HONE1 and HK1 cells after the overexpression of *circBART2.2* by ChIP assays according to the predicted NF-κB binding sites.

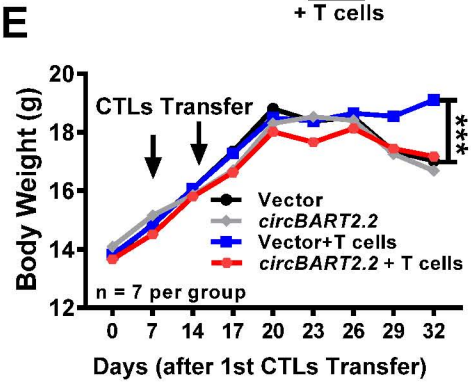
Fig.S9


A
circRNA Transfection
Tumor formation
7 days
NPC cells
Tumor lysates
DC cells
CD8+ T cells
Coculture
±DIR
CTLs transfer
+DIR
7 days
In vivo imaging
-DIR
7 days
T cells
FACS
ELISA
plasma
-DIR
25 days
Tumor weight, volume, IHC, ISH...


B
Vector
circBART2.2
PI
CD8-PE-Cy7


C
Vector
circBART2.2
PI
Annexin V-FITC


D
Tumor Weight (g)
NS
n = 7 per group
***
Vector
circBART2.2
Vector
circBART2.2
+ T cells


E
Body Weight (g)
CTLs Transfer
***
Vector
circBART2.2
Vector+T cells
circBART2.2 + T cells
n = 7 per group
Days (after 1st CTLs Transfer)

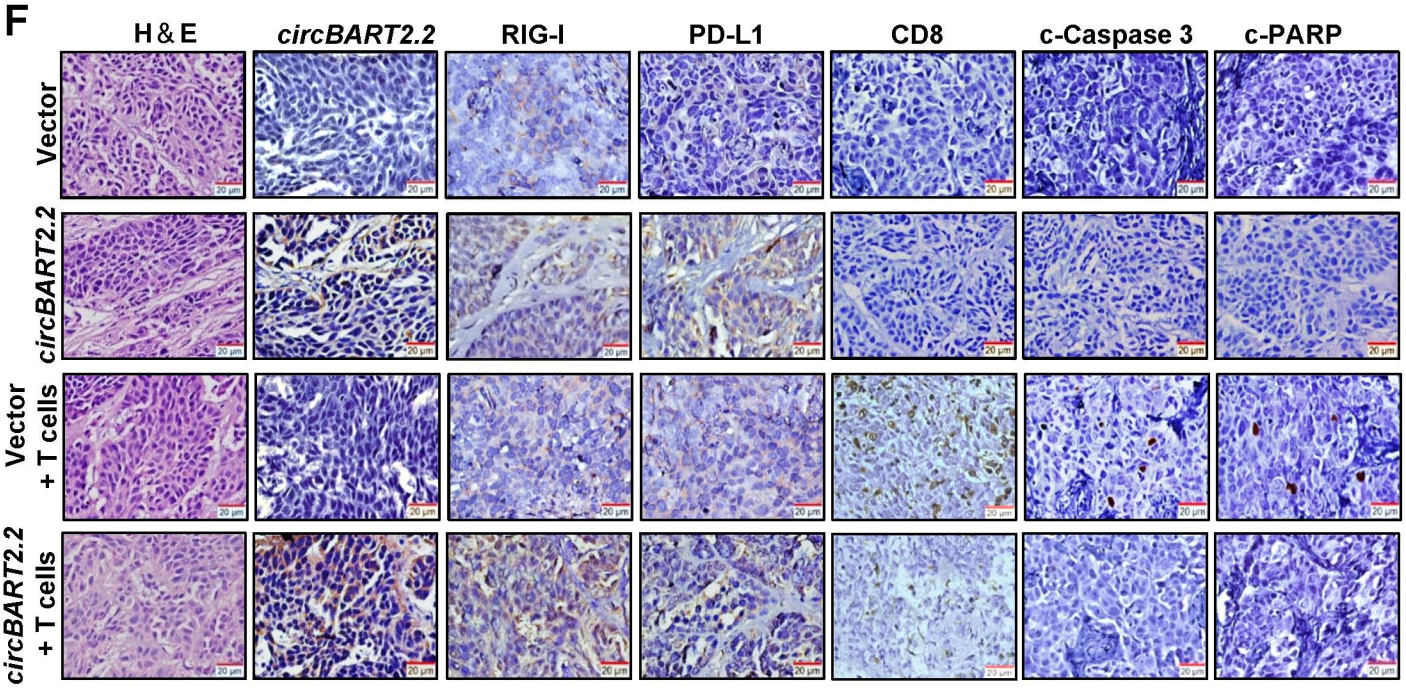

F
H & E
circBART2.2
RIG-I
PD-L1
CD8
c-Caspase 3
c-PARP
Vector
circBART2.2
Vector + T cells
circBART2.2 + T cells

Fig.S9. *circBART2.2* promoted NPC immune escape in xenograft mice models of NPC.

A. Schematic diagram of HONE1 cell-derived-xenograft mice models after injection of activated T cells. A density of 5×10^6 HONE1 cells that were transfected with the *circBART2.2* overexpression vector or the negative control (NC) were injected subcutaneously into the root of the right thigh of the mice (22 mice per group). To prepare dendritic cells and T cells, dendritic cells were first co-cultured with HONE1 cell lysate for 24 hours, and then co-cultured with T cells to present HONE1 specific tumor antigens to T cells to enable them to produce HONE1 specific T cells. Then 5×10^7 T cells with or without Deep Red live cell fluorescent dye (DiR) were infused into HONE1 cell-derived-xenograft mice through the tail vein when palpable tumors were formed after 7 days injection. For DiR-injected mice (3 mice per group), the small animal live imaging system was used to observe the accumulation of DiR+T cells at the tumor-forming site. For non-DIR-injected mice, activated T cells (5 mice per group) were injected into each tumor for another 7 days. Next, the peripheral blood of mice was extracted for flow cytometry, qRT-PCR, and ELISA. In another set, activated T cells were injected into each tumor for another 25 days and the tumor weight and volume were measured each day (7 mice per group).

B. The original flow cytometry results about CD8-positive T cells measured by flow cytometry using anti-CD8 antibody in HONE1 cell-derived-xenograft mice models, n = 5 per group.

C. The original flow cytometry results about the degree of T cell apoptosis measured by flow cytometry in HONE1 cell-derived-xenograft mice models. Mice peripheral blood was extracted for flow cytometry after incubation with Alexa Fluor 488 Annexin V and Propidium Iodide (PI), n = 5 per group.

D. The tumor weights for each group of nude mice, n = 7 per group. NS, no significance. ***, $P < 0.001$.

E. The body weight of the mice was measured in each group after 32 days of tumor cell administration, n = 7 per group. ***, $P < 0.001$.

F. Representative images of circBART2.2, RIG-I, PD-L1, CD8, c-Caspase 3, and c-PARP expression in nude mice using in situ hybridization or immunohistochemistry. The statistical results are shown in Fig. 8I, n = 7 per group.