Supplemental Material and Methods

Flow cytometry

To analyze the cellular composition of blood, spleen, lymph node and bone marrow samples included in this study, the following fluorescently labeled antibodies were used: huCD45-PB, CD3-BV785, CD8-PerCp, CD4-APC-Cy7, BDCA-2-FITC, CD19-PE-Cy7, CD16-BV605, BDCA-1-PE-Cy7, CD16-APC-Cy7, HLA-DR-APC (BioLegend), NKp46-APC, CD123-PE, CD11c-A700, HLA-DR PECF594, (BD Biosciences), CD14-Qdots655 (Invitrogen), BDCA-3-PE (Miltenyi Biotec), CD3-PE-TxRed, CD19-PE-TxRed, CD56-PE-TxRed (Life Technologies). The amine-reactive fluorescent dyes Zombie Aqua Fixable Viability Kit or Zombie NIR Fixable Viability Kit (Biolegend) were used to exclude dead cells. Absolute numbers of different cell subsets were computed from the white blood cell count as measured by a Beckman Coulter A^{c}T diff Analyzer. To assess phosphorylation of STAT1 we used pSTAT1-PE (BD Biosciences) according to a previously described protocol [1]. Samples were acquired on a BD LSRFortessa or FACSCanto with the use of FACS Diva software (BD Biosciences) and analysis was performed with FlowJo software.

RT-qPCR for expression of IFNα subtypes and interferon stimulated genes (ISGs)

RNA was isolated using the RNeasy Micro Kit (QIAGEN) or Quick RNA Microprep (Zymo Research). Purified RNA was reverse transcribed using GoScript Reverse Transcriptase (Promega) following the recommendations of the manufacturer. Quantitative polymerase chain reactions were run on a CFX384 Touch Real-Time PCR Detection System (BIO RAD). A SYBR Green master mix (Applied Biosystems) and the following primers were used:

IFNα 1/13 FW 5’-CTT CAA CCT CTT TAC CAC AAA AGA TTC-3’

IFNα 1/13 REV 5’-TGC TGG TAG AGT TCG GTG CA-3’

IFNα2 FW 5’-CTT GAA GGA CAG ACA TGA CTT TGG A-3’

IFNα2 REV 5’- GGA TGG TTT CAG CCT TTT GGA-3’

IFNα4 FW 5’-ACT CCT GGA ACA AAT GGG AAG AAT CTC TCA-3’

IFNα4 REV 5’-GAG CCT TCT GGA ACT GGT GGC CA-3’

IFNα5 FW 5’- CCC TGG TGG TGC TCA ACT G-3’

IFNα5 Rev 5’-CTT CCC ATT TGT GCC ATT ATC-3’

IFNα6 FW 5’-TCC ATG AGG TGA TTC AGC AGA C-3’

IFNα6 REV 5’-GCT GCT GGT AAA GTT CAG TAT AGA GTT T-3’

IFNα7 FW 5’-GCC CGG TCC TTT TCT TTA CTG-3’

IFNα7 REV 5’-TTC ATG TCT GTC CTT CAA GC-3’

IFNα8 FW 5’-CCT TCT AGA TGA ATT CTA CAT CGA ACT TG-3’

IFNα8 REV 5’-ACT CTA TCA CCC CCA CTT CCT G-3’

IFNα10 FW 5’-TAG GAG GGC CTT GAT ACT CCT GGG-3’

IFNα10 REV 5’-TGC CAT CAA ACT CCT CCT GGG GGA T-3’

IFNα14 FW 5’-GTG GTG CTC AGC TGC AAG TC-3’

IFNα14 REV 5’-GGC TGT GGG TTT GAG ACA GAT T-3’

IFNα16 FW 5’-TAT GAT TTC GGA TTC CCC CAG GAG GTG-3’

IFNα16 REV 5’-GTC TCA TCC CAA GCA GCA GAT GAA TC-3’

IFNα17 FW 5’-CCG TGC TGG TGC TCA GCT A-3’

IFNα17 REV 5’-TGT GGG TCT GAG GCA GAT CA-3’

MXB FW 5’-CAGAGGCAGCGGAATCGTAA-3’

MXB REV 5’-TGAAGCTCTAGCTCGGTGTTC-3’

STAT1 FW 5’-GAAATACACCTACGAACATGACCCT-3’

STAT1 REV 5’-TTCACCAACAGTCTCAACTTCAC-3’

IFIT3 FW 5’-TCAGAAGTCTAGTCACTTGGGG-3’

IFIT3 REV 5’-ACACCTTCGCCCTTTCATTTC-3’

OAS2 FW 5’-ACGTGACATCCTCGATAAAACTG-3’

OAS2 REV 5’-GAACCCATCAAGGGACTTCTG-3’

SDHA FW 5’-CAGCATGTGTTACCAAGCTGT-3’

SDHA REV 5’-GGTGTCGTAGAAATGCCACCT-3’

GAPDH FW 5’-ATG GGG AAG GTG AAG GTC C-3’

GAPDH REV 5’-GGG TCA TTG ATG GCA ACA ATA TC-3’

Lysate Preparation and Immunoblotting

Raji cells were stimulated with interferon subtypes as indicated in the figures in RPMI containing 10% FCS for 8 min at 37 °C. After harvesting in ice-cold PBS and centrifugation, the cells were resuspended in ice-cold lysis buffer (10 mM Tris hydrochloride, 150 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 5 mM EDTA, 1% Trition X-100, cOmplete, EDTA-free Protease Inhibitor Cocktail

from Roche) and incubated on ice for 30 min. The cell lysates were centrifuged at 13'000 *g* for 10 min at 4 °C. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific) and lysates heated for 5 min at 95 °C in NuPage SDS-PAGE loading buffer (Life Technologies) with 1% β-mercaptoethanol. Equal total protein amounts were loaded on 12.5% SDS-PAGE gels and transferred to PVDF blotting membranes (GE Healthcare life sciences). The proteins of interest were detected using primary antibodies, secondary HRP-coupled antibodies and the Pierce ECL Western Blotting substrate detection system (Thermo Scientific). Densitometric quantification was performed on a Vilber Lourmat Fusion FX imaging system using the ImageJ software.

Histology, immunohistochemistry

Tissue was fixed in 10% saline buffered formalin and paraffin embedded. For immunohistochemistry 3 µm splenic sections were processed on a Leica BOND-MAX system. Stainings were performed with monoclonal mouse anti-EBNA2 (clone PE2, Abcam), mouse anti-CD20 (clone L26, Dako) and rabbit anti-huCD8 (clone SP16, Marque) antibodies. The number of labeled cells was determined per $1mm^2$ using the a Vectra3 automated quantitative pathology imaging system (PerkinElmer) using Vectra and InForm software analysis (PerkinElmer) as explained elsewhere [2].

References

1. Schulz KR, Danna EA, Krutzik PO, Nolan GP. Single-cell phospho-protein analysis by flow cytometry. Curr Protoc Immunol. 2012;Chapter 8:Unit 8 17 11-20.

2. Murer A, McHugh D, Caduff N, et al. EBV persistence without its EBNA3A and 3C oncogenes in vivo. PLoS Pathog. 2018;14(4):e1007039.

Supplemental Figure Legends

Figure S1. IFNα production in response to EBNA3 deficient viruses is pDC dependent and EBNA3A and 3C contribute to the low IL12p40 production in response to EBV.

(A) Total PBMCs or pDC depleted PBMCs were exposed to EBNA3A deficient EBV (3aKO) or EBNA3C deficient EBV (3cKO) virus at the indicated multiplicities of infection (MOI) for 18-24h. The amount of IFNα was measured in the culture supernatants by ELISA. MOI 0.005 n=2, MOI 0.05 n=4 and MOI 0.25 n=2 (B) IL12p40 was measured in the culture supernatants of PBMCs that were exposed to wild-type EBV (wt), EBNA3A deficient EBV (3aKO) or EBNA3C deficient EBV (3cKO) for 18-24h. MOI 0.005 n=3, MOI 0.05 n=3 and MOI 0.25 n=5. (C) IFNβ levels in PBS (n=4) or EBV treated cultures (MOI 0.5 n=2, MOI 0.25 n=5 and MOI 0.05 n=3) were detected by ELISA. Paired t tests were used. (D) The viability of pDCs 18-24h after EBV infection (MOI 0.25, n=9) was determined by flow cytometry. All single cells were analysed and the percentage viable cells from total pDCs (HLA-DR$^+$, CD3CD14CD16CD19CD56$^-$, CD11c$^-$, BDCA2$^+$) are shown.

Figure S2. IFNα subtypes can inhibit EBV entry into Ramos and primary human B cells.

(A) Ramos cells (EBV only n=7, IFNα6 n=3, IFNα14 n=5-7, IFNα16 n=3, IFNα17 n=1) or primary B cells (EBV only n=10, IFNα14 n=7-10) were exposed to EBV and the indicated concentrations of IFNα subtypes for 48h. GFP expression as a measure of EBV entry was determined by flow cytometry and ratios of EBV plus IFNα to EBV only are shown in the graph. Statistical analysis was done on raw data (percentage GFP positive cells) and paired t tests were applied. (B) B cell viability after 48h of culture (IFNα14 and IFNα17 n=4, IFNα6 and IFNα16 n=3) was determined using an amine-reactive fluorescent dye and is depicted as percentage viable cells, normalized to only EBV infection.

Figure S3. The biological activity of the tested IFNα subtypes was confirmed by STAT1 phosphorylation.

STAT1 phosphorylation was measured on Raji cells that were exposed to PBS or IFNα subtypes (100ng/ml) by intracellular phospho-protein staining for STAT1 using flow cytometry. One representative histogram and a graph that summarizes two experiments are shown. In

addition, pSTAT1 was detected by Western Blot after stimulation with the IFNα subtypes (50ng/ml). One representative blot is shown.

Figure S4. EBV infection of hu-NSGs

(A) IL12p40 production remains unchanged after EBNA3A and 3C deficient EBV infection in vivo. IL12p40 serum levels were measured by ELISA after 18h of PBS treatment (n=6) or $5x10^5$ RGU wild-type EBV (wt, n=6), EBNA3A deficient EBV (3aKO, n=6) or EBNA3C deficient EBV (3cKO, n=8) infection in huNSG mice. IL12p40 serum levels were measured at 72h post infection with $1x10^5$ RGU or $5x10^5$ RGU wt EBV (n=3 per group). The presence of IFNβ in the plasma was assessed after 18h (PBS n=7, EBV n=8). (B) HLA-A2 transgenic (hu-NSG-A2, n=13) and non- transgenic hu-NSGs (hu-NSG, n=19) were infected with $1x10^5$ RGU EBV and the CD8/CD4 T cell ratio was determined at different time points in the blood and at the termination of the experiment in blood, spleen and bone marrow. Total $CD4^+$ and $CD8^+$ T cell numbers were calculated in blood and spleen. (C) Splenic sections were stained for CD20, CD8 and EBNA2 in mice that were treated with PBS (n=4-5), EBV (n=6-7) or EBV plus IFNα14 administration (n=5). The graphs summarize the quantification of labeled cells per $1mm^2$.

Figure S5. DC subset gating in flow cytometry and pDC viability.

(A) Gating strategy in FloJo for DC subsets as used in Figure 6. (B) In huNSGs that were treated with PBS (n=10) or infected with $5x10^5$ RGU EBV wt (n=11) or EBNA3A deficient EBV (3aKO, n=6) or EBNA3C deficient EBV (3cKO, n=7) virus for 18h, the viability of pDCs ($huCD45^+$, $HLA\text{-}DR^+$, $lineage^-$, $CD11c^-$, $BDCA2^+$, $CD123^+$) in the blood was determined using an amine-reactive fluorescent dye. A representative FACS plot is shown to the right.

Figure S6

(A) EBV copy numbers per spleen were correlated (Spearman r) to percentage or total numbers of $CD8^+$ T cells (n=26), pDCs (n=21) and cDCs (n=21). EBV infected huNSGs were used from 7 independent experiments. (B) A potential linkage of tumor incidence with viral load or the cell percentages and total numbers of $CD8^+$ T cells, pDCs and cDCs was analysed. No tumor incidence (n=11), tumor incidence (n=6).

Figure S7. pDC depletion abolishes ISG induction after EBV infection, but does not alter the resulting T cell expansion.

(A) Flow cytometric gating strategy for DC subsets as used in Figure 7. (B) The effect of α-CD303 on ISG expression was determined by RT-qPCR of huNSG blood leukocytes 18h post infection with $5x10^5$ RGU EBV and unpaired t tests were applied. (PBS n=5, EBV+PBS n=6, EBV+α-CD303 n=6, EBV+isotype n=5). (C) After pretreatment with PBS, α-CD303 or isotype antibody, the mice were infected with $1x10^5$ RGU EBV (as schematically depicted in Fig. 7a). The ratio of CD8/CD4 T cells was measured at the endpoint of the experiment (week 5 or 6) in cells processed from blood, bone marrow and lymph nodes. (D) Total numbers of $CD8^+$ and $CD4^+$ T cells were determined per ml whole blood and per spleen. For (C) and (D), PBS n=5, PBS+α-CD303 n=5, EBV+PBS n=8, EBV+α-CD303 n=7, EBV+isotype n=3. In lymph nodes, T cell activation was measured in terms of HLA-DR upregulation (E), and the percentage of NKp46 expressing NK cells was assessed (F).

Figure S1

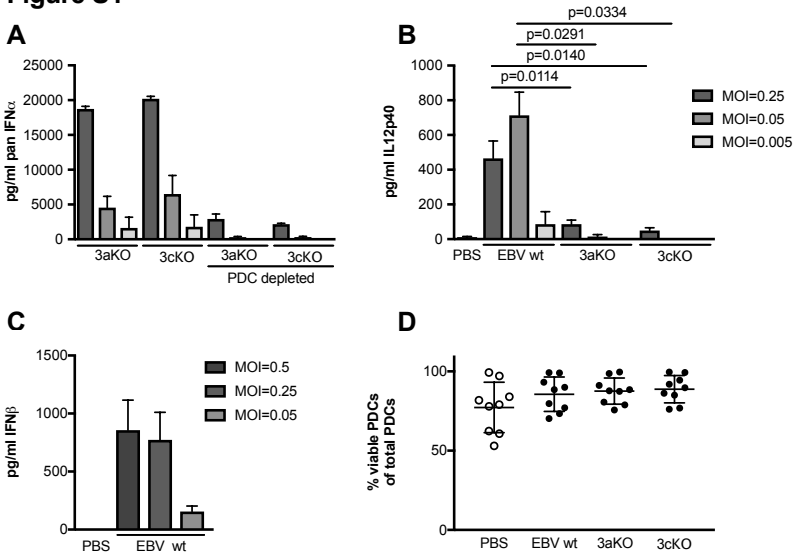

A
pg/ml pan IFNα
25000
20000
15000
10000
5000
0
3aKO
3cKO
3aKO
3cKO
PDC depleted
B
pg/ml IL12p40
1000
800
600
400
200
0
p=0.0334
p=0.0291
p=0.0140
p=0.0114
MOI=0.25
MOI=0.05
MOI=0.005
PBS
EBV wt
3aKO
3cKO
C
pg/ml IFNβ
1500
1000
500
0
MOI=0.5
MOI=0.25
MOI=0.05
PBS
EBV wt
D
% viable PDCs of total PDCs
100
50
0
PBS
EBV wt
3aKO
3cKO

Figure S2

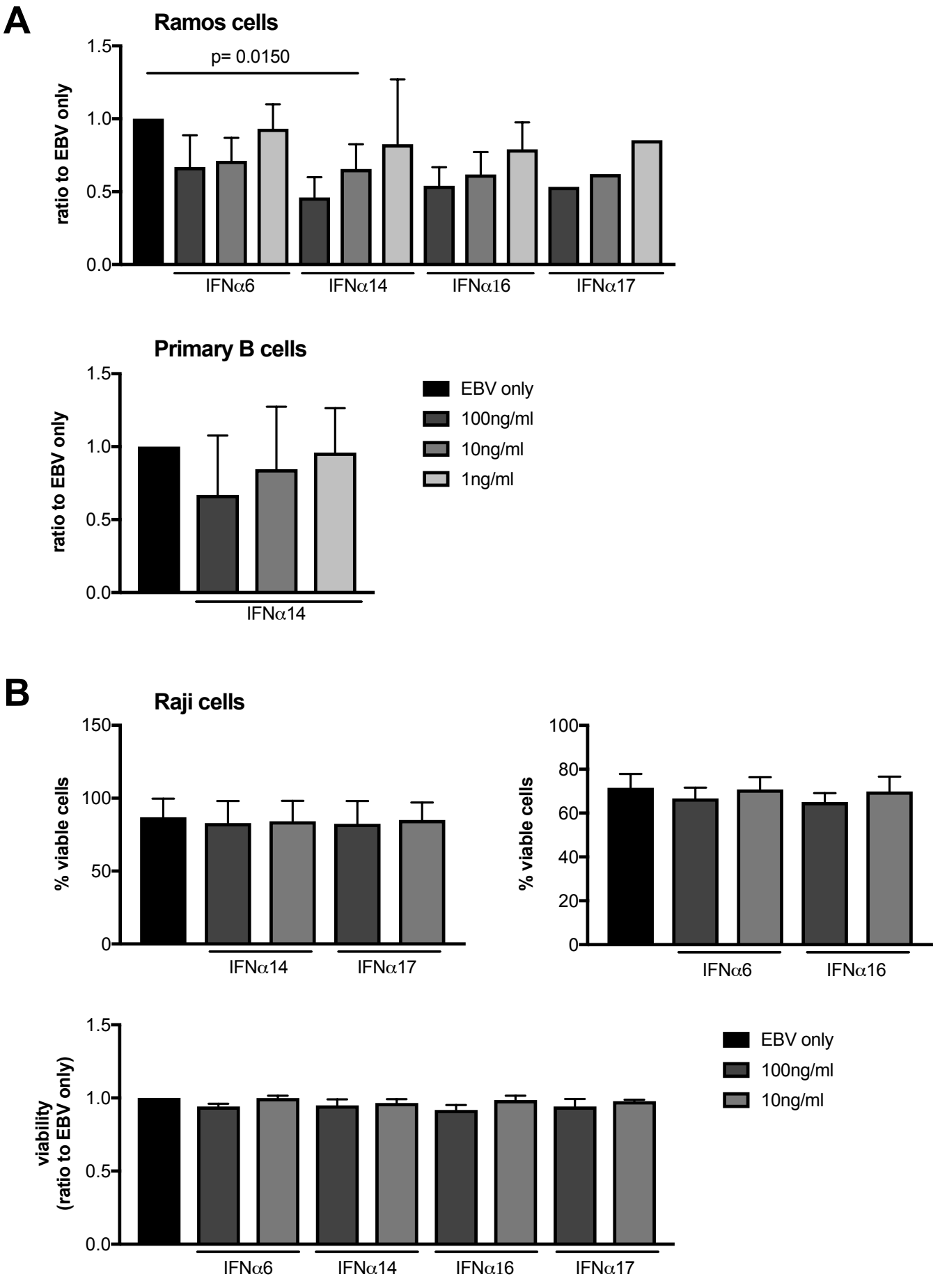
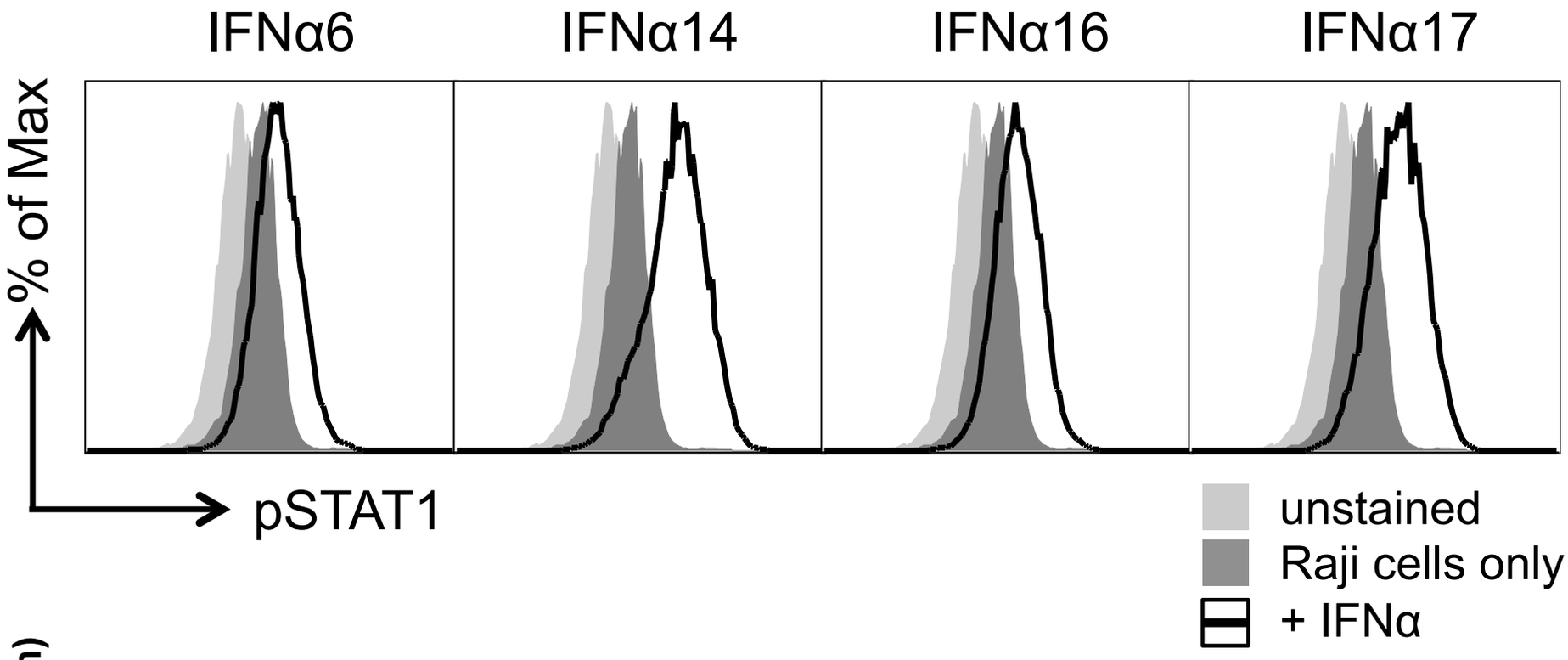
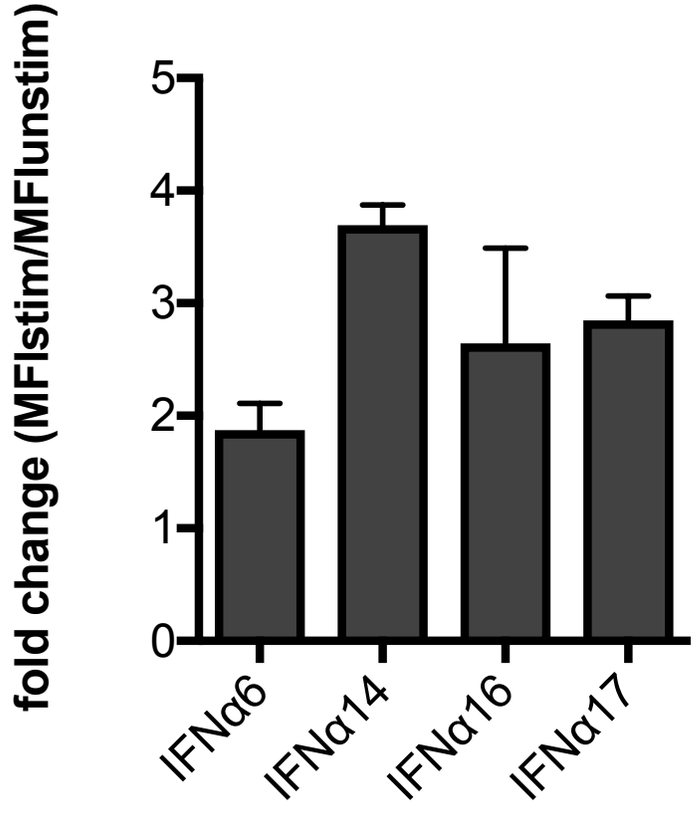

A
Ramos cells
p= 0.0150
ratio to EBV only
1.5
1.0
0.5
0.0
IFNα6
IFNα14
IFNα16
IFNα17
Primary B cells
ratio to EBV only
1.5
1.0
0.5
0.0
IFNα14
EBV only
100ng/ml
10ng/ml
1ng/ml
B
Raji cells
% viable cells
150
100
50
0
IFNα14
IFNα17
% viable cells
100
80
60
40
20
0
IFNα6
IFNα16
viability
(ratio to EBV only)
1.5
1.0
0.5
0.0
IFNα6
IFNα14
IFNα16
IFNα17
EBV only
100ng/ml
10ng/ml

Figure S3

STAT1 phosphorylation in Raji cells





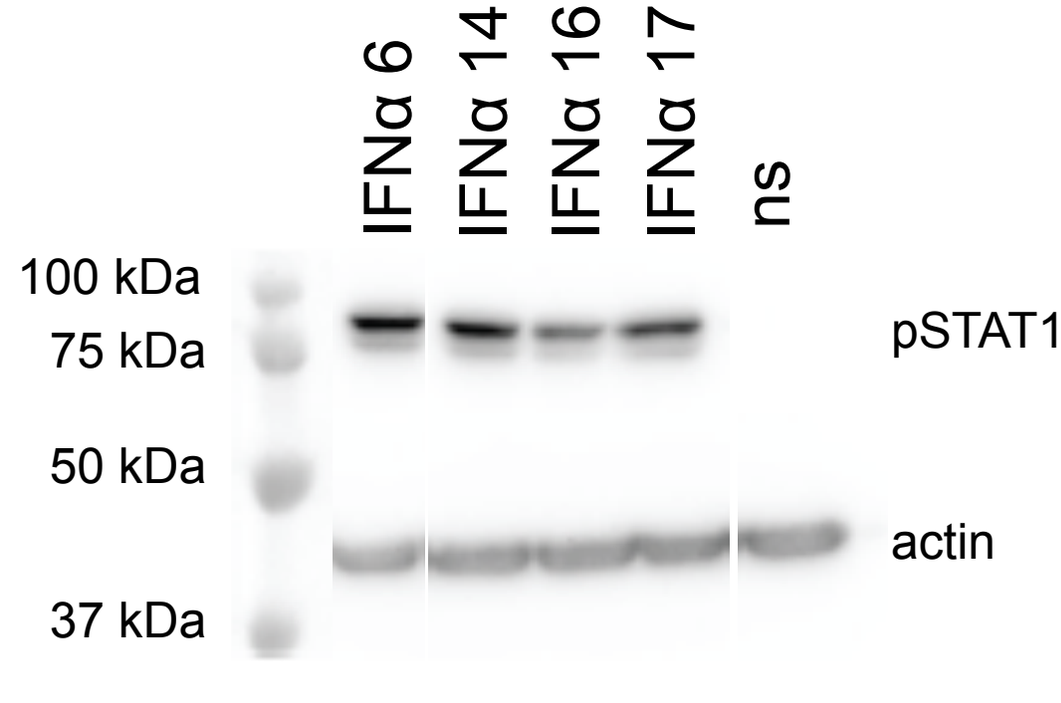


Figure S4

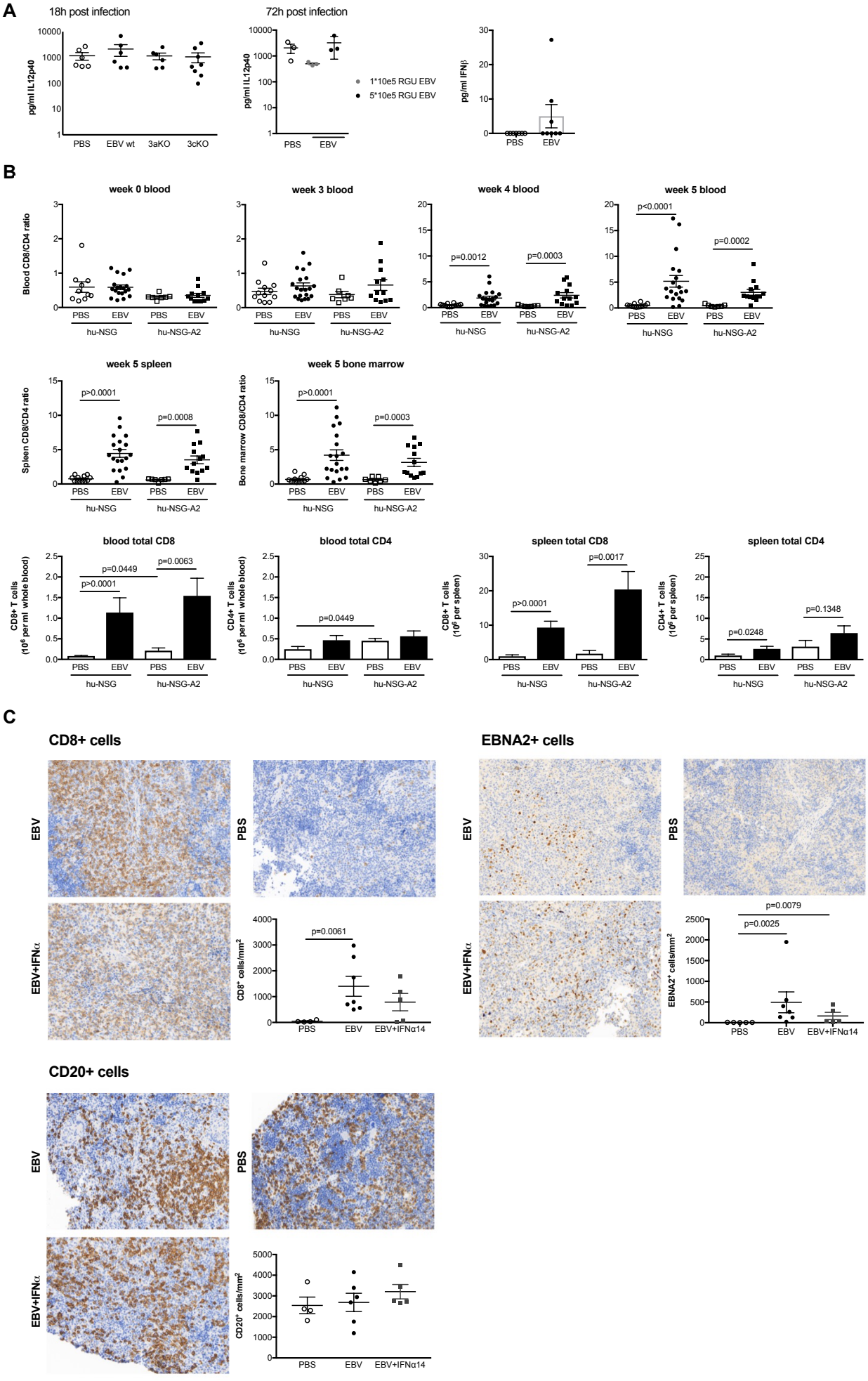
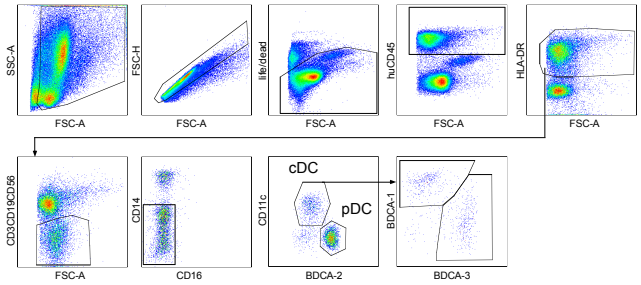
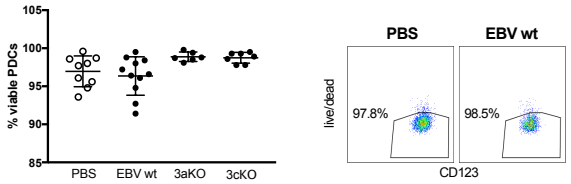

A
18h post infection
72h post infection
pg/ml IL12p40
pg/ml IFNβ
PBS
EBV wt
3aKO
3cKO
EBV
1*10e5 RGU EBV
5*10e5 RGU EBV
B
week 0 blood
week 3 blood
week 4 blood
week 5 blood
Blood CD8/CD4 ratio
p<0.0001
p=0.0002
p=0.0012
p=0.0003
hu-NSG
hu-NSG-A2
week 5 spleen
week 5 bone marrow
Spleen CD8/CD4 ratio
Bone marrow CD8/CD4 ratio
p>0.0001
p=0.0008
p=0.0003
blood total CD8
blood total CD4
spleen total CD8
spleen total CD4
CD8+ T cells (10⁶ per ml whole blood)
CD4+ T cells (10⁶ per ml whole blood)
CD8+ T cells (10⁶ per spleen)
CD4+ T cells (10⁶ per spleen)
p=0.0449
p=0.0063
p=0.0017
p=0.1348
p=0.0248
C
CD8+ cells
EBNA2+ cells
CD20+ cells
EBV
PBS
EBV+IFNα
CD8⁺ cells/mm²
EBNA2⁺ cells/mm²
CD20⁺ cells/mm²
p=0.0061
p=0.0079
p=0.0025
EBV+IFNα14

Figure S5

A


SSC-A
FSC-A
FSC-H
FSC-A
live/dead
FSC-A
huCD45
FSC-A
HLA-DR
FSC-A
CD3CD19CD56
FSC-A
CD14
CD16
CD11c
cDC
pDC
BDCA-2
BDCA-1
BDCA-3

B


% viable PDCs
105
100
95
90
85
PBS
EBV wt
3aKO
3cKO

PBS
EBV wt
live/dead
97.8%
98.5%
CD123

Figure S6

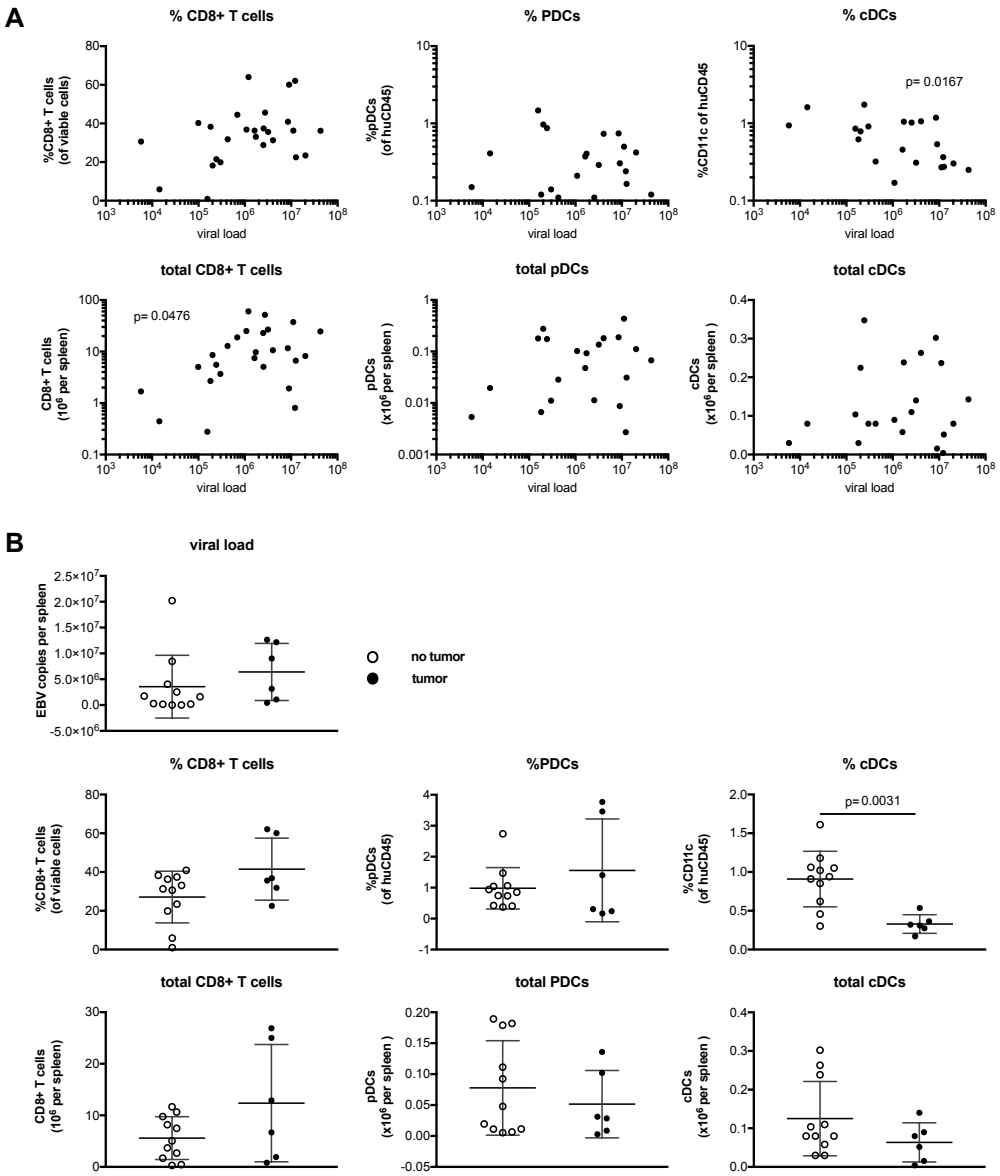
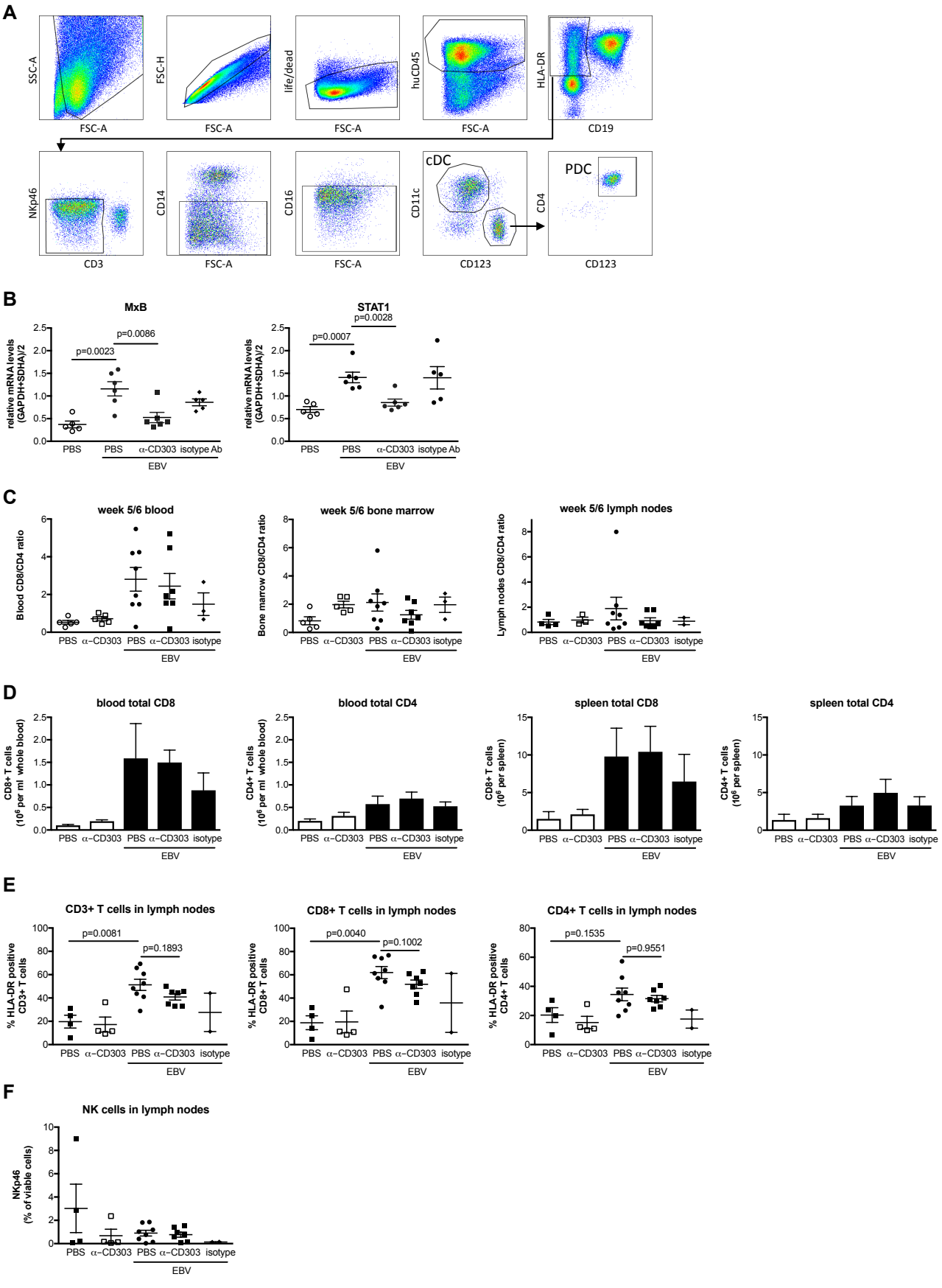

A
% CD8+ T cells
%CD8+ T cells (of viable cells)
viral load
% PDCs
%pDCs (of huCD45)
viral load
% cDCs
%CD11c of huCD45
p= 0.0167
viral load
total CD8+ T cells
CD8+ T cells (10^6 per spleen)
p= 0.0476
viral load
total pDCs
pDCs (x10^6 per spleen)
viral load
total cDCs
cDCs (x10^6 per spleen)
viral load
B
viral load
EBV copies per spleen
no tumor
tumor
% CD8+ T cells
%CD8+ T cells (of viable cells)
%PDCs
%pDCs (of huCD45)
% cDCs
%CD11c (of huCD45)
p= 0.0031
total CD8+ T cells
CD8+ T cells (10^6 per spleen)
total PDCs
pDCs (x10^6 per spleen)
total cDCs
cDCs (x10^6 per spleen)

Figure S7


A
SSC-A
FSC-A
FSC-H
FSC-A
life/dead
FSC-A
huCD45
FSC-A
HLA-DR
CD19
NKp46
CD3
CD14
FSC-A
CD16
FSC-A
cDC
CD11c
CD123
PDC
CD4
CD123
B
MxB
relative mRNA levels (GAPDH+SDHA)/2
p=0.0023
p=0.0086
PBS
PBS
α-CD303
isotype Ab
EBV
STAT1
p=0.0007
p=0.0028
C
week 5/6 blood
Blood CD8/CD4 ratio
week 5/6 bone marrow
Bone marrow CD8/CD4 ratio
week 5/6 lymph nodes
Lymph nodes CD8/CD4 ratio
PBS
α-CD303
isotype
EBV
D
blood total CD8
CD8+ T cells (10^6 per ml whole blood)
blood total CD4
CD4+ T cells (10^6 per ml whole blood)
spleen total CD8
CD8+ T cells (10^6 per spleen)
spleen total CD4
CD4+ T cells (10^6 per spleen)
E
CD3+ T cells in lymph nodes
% HLA-DR positive CD3+ T cells
p=0.0081
p=0.1893
CD8+ T cells in lymph nodes
% HLA-DR positive CD8+ T cells
p=0.0040
p=0.1002
CD4+ T cells in lymph nodes
% HLA-DR positive CD4+ T cells
p=0.1535
p=0.9551
F
NK cells in lymph nodes
NKp46 (% of viable cells)