Supplemental Methods

T-cell clones

EBNA1-specific $CD8^+$ T-cell clones were generated from freshly isolated PBMCs which were depleted of $CD4^+$ T cells using anti-CD4 conjugated MACS microbeads and magnetic selection (Miltenyi Biotec). EBNA1-specific $CD4^+$ T-cell clones were generated without prior $CD8^+$ T cell depletion. PBMCs were stimulated with 5µM of EBNA1 peptide library including overlapping peptides of 12- to 22-aa length (average of 15-aa length) with 11-aa overlap, which were designed for the $EBNA1_{400-641}$ sequence of the B95.8 EBV strain (1) for four hours. IFNγ-secreting cells were enriched by IFNγ secretion assay (Miltenyi Biotec) and were cloned by limiting dilution at 3 and 30 cells per well on HLA-matched gamma-irradiated LCLs (10^4/well) loaded with the relevant peptide or peptide mix at 5µM and allogeneic gamma-irradiated, PHA-treated PBMCs (10^5/well) in IL-2-supplemented (150IU/mL) medium with 10% pooled human sera (T-cell medium). Wells with growing microcultures were screened for peptide-reactivity by IFNγ ELISA (Mabtech). Selected T-cell clones were expanded on autologous gamma-irradiated LCLs loaded with the relevant peptide at 5µM and allogeneic gamma-irradiated, PHA-treated PBMCs in T-cell medium. T-cell medium was changed two times per week. Every third week, T-cell clones were restimulated with gamma-irradiated peptide-pulsed autologous LCLs and allogeneic PHA-treated PBMCs. To control for continuous specificity, 5'000 T-cell clones were restimulated with 5 µM of relevant peptide. After overnight incubation, co-culture supernatants from single wells were tested by ELISA for IFNγ content. The resulting $CD8^+$ T-cell clones used in this study were specific for $EBNA1_{407-417}$ (**HPV**GEADYFEY, HLA-B*3501, clones from two donors). The $CD4^+$ T-cell clones were specific for $EBNA1_{519-532}$ (**NLR**RGTALAIPQCR, HLA-DRB1*01), $EBNA1_{482-496}$ (**AEG**LRALLARSHVER, HLA-DQB1*0302) and $EBNA1_{473-487}$ (**GSN**PKFENIAEGLRA, HLA-DRB5*01, clones from two donors).

Staining by flow cytometry (FACS)

At sacrifice spleens were processed by mechanical disruption through a 70µm nylon mesh, followed by a Ficoll-Paque (GE healthcare) gradient centrifugation. Mouse whole blood was collected by heart puncture into K2 EDTA tubes (BD), and lysed twice with ACK lysis buffer (Lonza) for 5min and washed in PBS. Total white blood cells in whole blood were counted using a Beckman cell counter according to the manufacturer's instructions. Mouse lymph nodes were harvested from the brachial, axillary, inguinal and the superficial cervical sites. Lymph nodes were processed by mechanical disruption through a 70µm nylon mesh and washed in PBS. Intracellular cytokine staining was performed from LN cells, blood cells and splenocytes. Cells were stimulated

with 2µg/mL of purified NA/LE hamster-α-mouse CD28 antibodies and either 1µM of EBNA1 peptide pool (aa400-641, 51 overlapping peptides) or 1µM of a peptide pool from human cytomegalovirus (HCMV) protein pp65 (51 overlapping peptides) as a control. As positive control αCD3ε was added instead of the EBNA1 or HCMV peptide pool. After one hour of incubation, Brefeldin A (Sigma) was added to a concentration of 10µg/ml. The incubation was continued four hours, before proceeding to the staining. After 20-30min of extracellular staining, cells were washed, permeabilized and stained intracellular with the Cytofix/Cytoperm Plus kit (BD) according to manufactures instructions.

The following purified α-mouse mAbs were used for staining CD4 Pacific Blue (BioLegend, GK1.5), CD4 PerCP/Cy5.5 (BioLegend, RM4-4), CD8a FITC (eBioscience, 53-6.7), CD19 Pacific Blue (BioLegend, 6D5), PD1 APC (BioLegend, 10F.9G2), IL-2 PE (Pharmngen, JES6-5H4), TNFα Brilliant Violet 650 (BioLegend, MP6-XT22). Live/Dead fixable Aqua Dead Cell Stain (Life technologies, AmCyan) was used for dead cell discrimination.

Acquisitions were performed on an LSR Fortessa or FACS Canto II (BD) flow cytometer, the data was analyzed with FlowJo (v9, Tree Star).

References

1. Heller KN, Upshaw J, Seyoum B, Zebroski H, and Münz C. Distinct memory $CD4^+$ T-cell subsets mediate immune recognition of Epstein Barr virus nuclear antigen 1 in healthy virus carriers. *Blood.* 2007;109(3):1138-46.

Supplemental Figure Legends

Supplemental Figure 1: Human $CD4^+$ and $CD8^+$ T-cell recognition of EBNA1 carrying or encoding vaccine formulations

A. The binding specificity of EBNA1 fusion antibodies (EBNA1-Abs) was tested using a competitive binding assay. Target cells (CD207-expressing HEK293T cells for testing αCD207-EBNA1 or PBMCs for all other EBNA1-Ab fusion proteins) were incubated for 20min in undiluted cell culture supernatant containing the respective EBNA1-Ab fusion proteins (purple). As positive controls the respective specific monoclonal purified Abs diluted to the same concentration as the EBNA1-Ab fusion proteins (dark grey) or diluted to 100fold of the concentration of the staining Abs (light grey) was used. As a negative control served an irrelevant Ab diluted to 100fold of the concentration of the staining Ab (black). After the incubation, the cells were washed and stained

with the corresponding original fluorochrome-labeled mouse Ab. The mean percentage of blocking efficiency for the different receptor stainings is depicted. **B.** Western blot analysis of human fusion antibodies under reducing conditions using rat anti-EBNA1 Ab (clone 1H4). **C.** Reducing condition EBNA1 Western Blot of human PBMC protein lysate 48 to 96 hours after infection with viral vectors. **D.** Quantification of EBNA1 Western Blot of viral vector infected PBMCs. Band strength was normalized to the corresponding actin band. Fold increase over uninfected PBMCs is depicted.

Supplemental Figure 2: Comprehensive priming of mouse $CD4^+$ and $CD8^+$ T-cell responses against EBNA1 by heterologous vaccination in huDEC205tg mice

A. Representative dot plots of intracellular cytokine staining (ICS) of re-stimulated splenocytes, gated for CD4 or CD8 expression and IFNγ. One dot plot is shown for the homologous vaccination groups of Lenti-IiE1, Adeno-E1&LMP, MVA-IiE1.

B. Broad analysis of immunodominant sites of the $EBNA1_{400-641}$ protein. At sacrifice bulk splenocytes of vaccinated mice were harvested and $2x10^5$ cells were re-stimulated with 1 µg/mL of each single peptide of the EBNA1 peptide pool (each number indicates a single of the 51 peptides). IFNγ secretion was measured by ELISPOT assays. Mean and SD of four mice are shown.

C. Cytokine profile of total $CD4^+$ or $CD8^+$ T cells in Adeno-E1&LMP, Lenti-IiE1 and MVA-IiE1. Pie charts show percentage of mean of each cytokine-secreting subset.

Supplemental Figure 3: Protection from EBNA1-expressing EL4 lymphoma challenge by heterologous prime-boost vaccination in huDEC205tg mice

A. EL4 cells were infected with Lenti-EBNA1-GFP. GFP-positive lymphoma cells were enriched by fluorescence-activated single cell sorting and controlled for EBNA1 expression by Western Blot. On the left EL4-E1 cells after sorting, on the right EL4-E1 cells from tumor-bearing untreated mice are shown. **B.** Untreated EL4-E1 tumors were fixed and embedded in paraffin for histology and stained with H&E, αCD4, αCD8 and αFoxP3 antibodies (original magnification, 20x, scale bar 20µm). **C.** HuDEC205tg mice were immunized with different combinations of vaccines for prime and boost set 10 days apart. Before prime and before boost mice were depleted with injections of αCD4 or αCD8 antibody on three consecutive days. Mice were challenged with $2x10^5$ EL4-E1

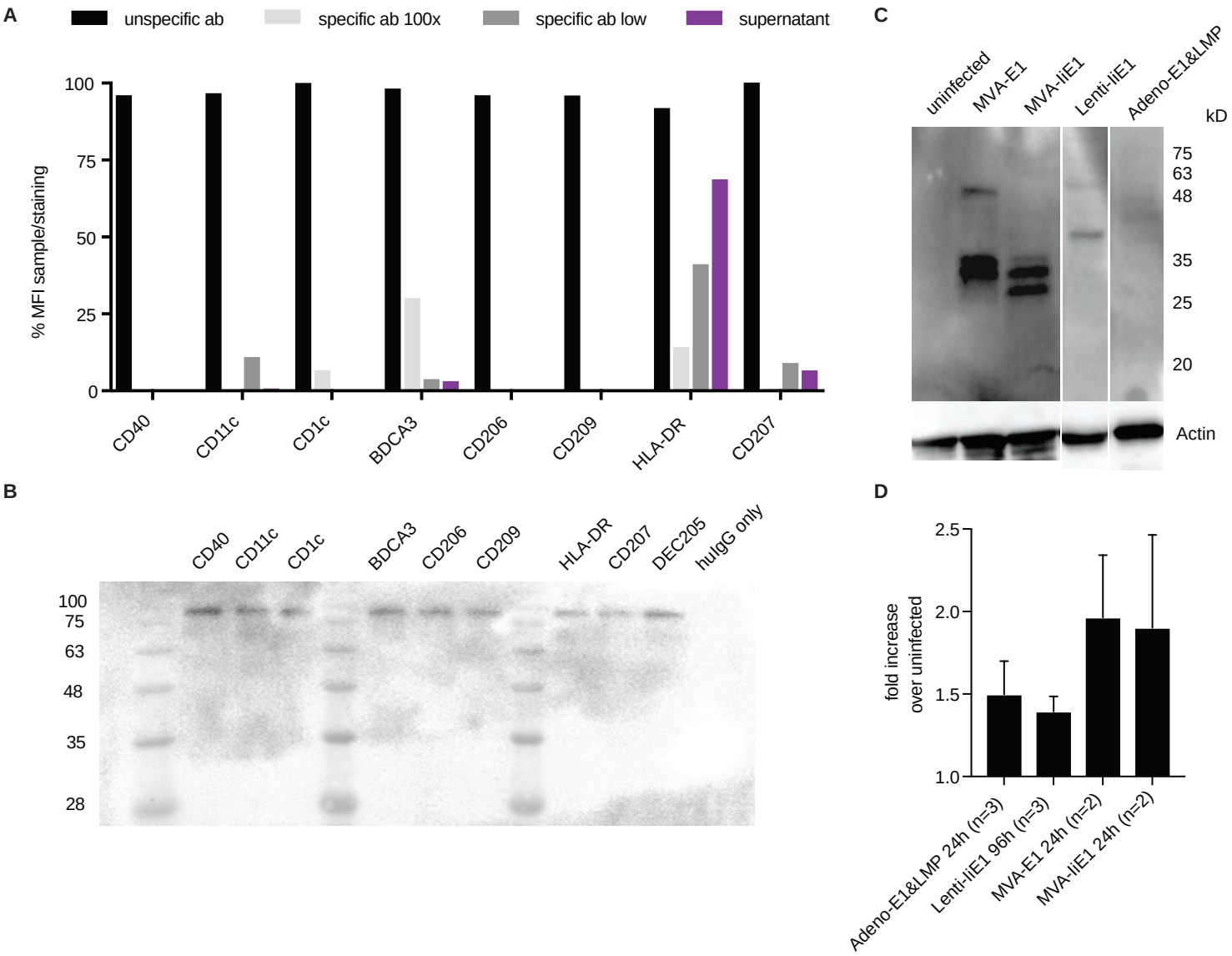
cells s.c. 14 days after the boost. Mice were monitored every second day, weight was measured and tumor size was analysed by caliper. At sacrifice bulk splenocytes were analysed by FACS. Percentage of $CD4^+$ and $CD8^+$ cells is displayed. **D.** Untreated EL4-E1 tumor was fixed and embedded in paraffin for histology and stained with EBNA1 antibody (clone 1H4) (original magnification, scale bar 40µm). **E.** Serum obtained from mice from depletion experiments with EL4-E1 challenged was analysed for anti-EBNA1 IgG by ELISA. Each data point represents one individually analysed mouse from one experiment. Error bars represent SEM. **F.** At sacrifice tumor were harvested and analysed by EBNA1 qPCR. Abundance of EBNA1 gene is normalized to UBC gene. Mean and SD from two independent experiments with at least five mice per group is shown.

Supplemental Figure 4: Characteristics of EBNA1-induced B-cell lymphomas

A. Representative Western blot for EBNA1 expression by splenocytes of a mice challenged by EµEBNA1 transgenic splenocyte (BL-E1) transfer that were PBS-treated or vaccinated with αDEC-E1+Adeno-E1&LMP or Adeno-E1&LMP+MVA-IiE1 compared to a healthy control mouse.

B. Bulk splenocytes were gated for $CD4^+$ and $CD8^+$ cells and those were quantified in 10^6 fold per spleen. Mean and SEM from two independent experiments with at least five mice per group are shown.

C. Liver immunohistochemistry for H&E, B ($CD19^+$) and T cells ($CD3^+$) of a healthy control (top row) and a mouse after BL-E1 transfer (bottom row). Representative images are shown, (original magnification, scale bar 20µm).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specific ab 100x
specific ab low
supernatant
% MFI sample/staining
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75
50
25
0
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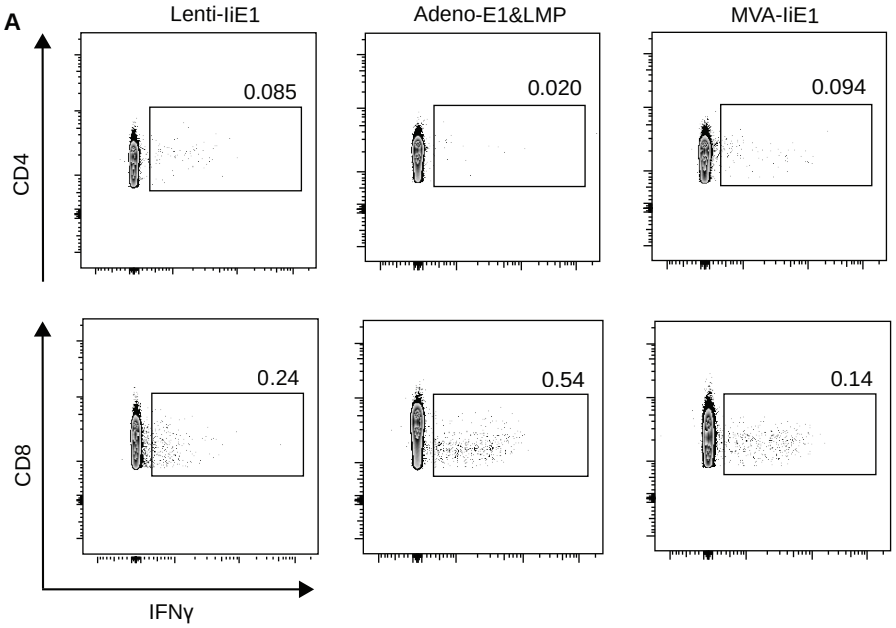
CD11c
CD1c
BDCA3
CD206
CD209
HLA-DR
CD207
C
uninfected
MVA-E1
MVA-liE1
Lenti-liE1
Adeno-E1&LMP
kD
75
63
48
35
25
20
Actin
B
CD40
CD11c
CD1c
BDCA3
CD206
CD209
HLA-DR
CD207
DEC205
huIgG only
100
75
63
48
35
28
D
fold increase over uninfected
2.5
2.0
1.5
1.0
Adeno-E1&LMP 24h (n=3)
Lenti-liE1 96h (n=3)
MVA-E1 24h (n=2)
MVA-liE1 24h (n=2)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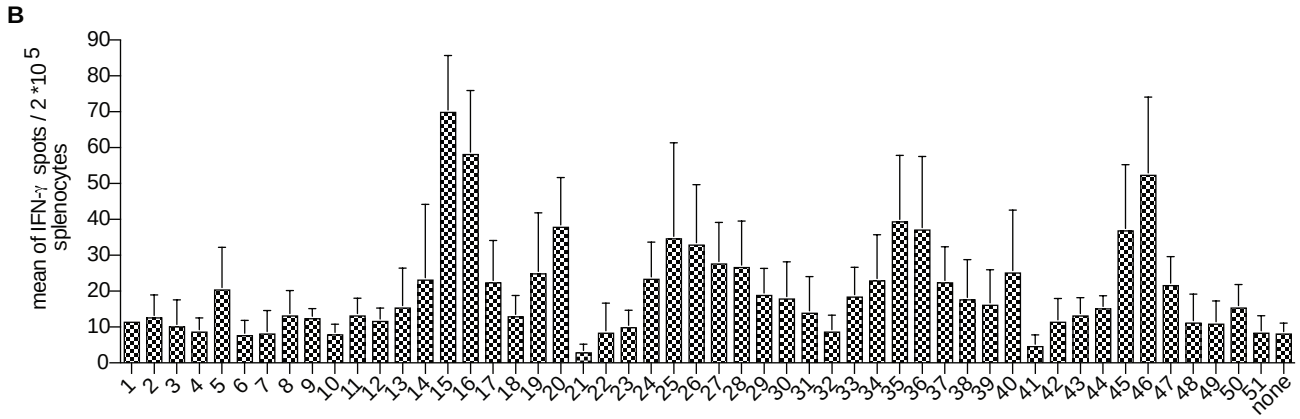
CD8
IFNγ


B
mean of IFN-γ spots / 2 *10 5 splenocytes
90
80
70
60
50
40
30
20
10
0
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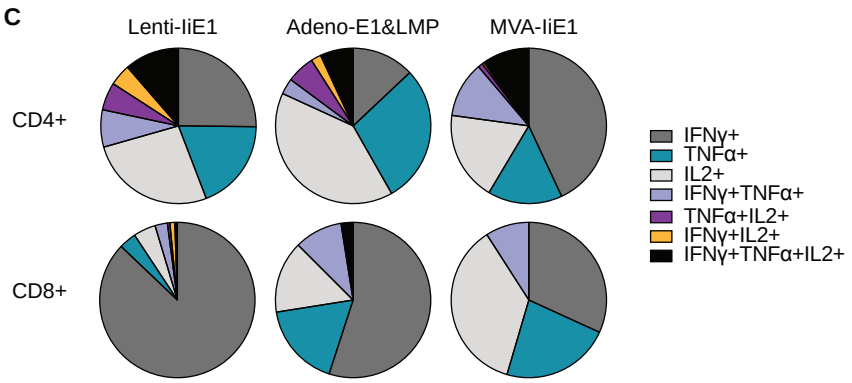
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Adeno-E1&LMP
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CD4+
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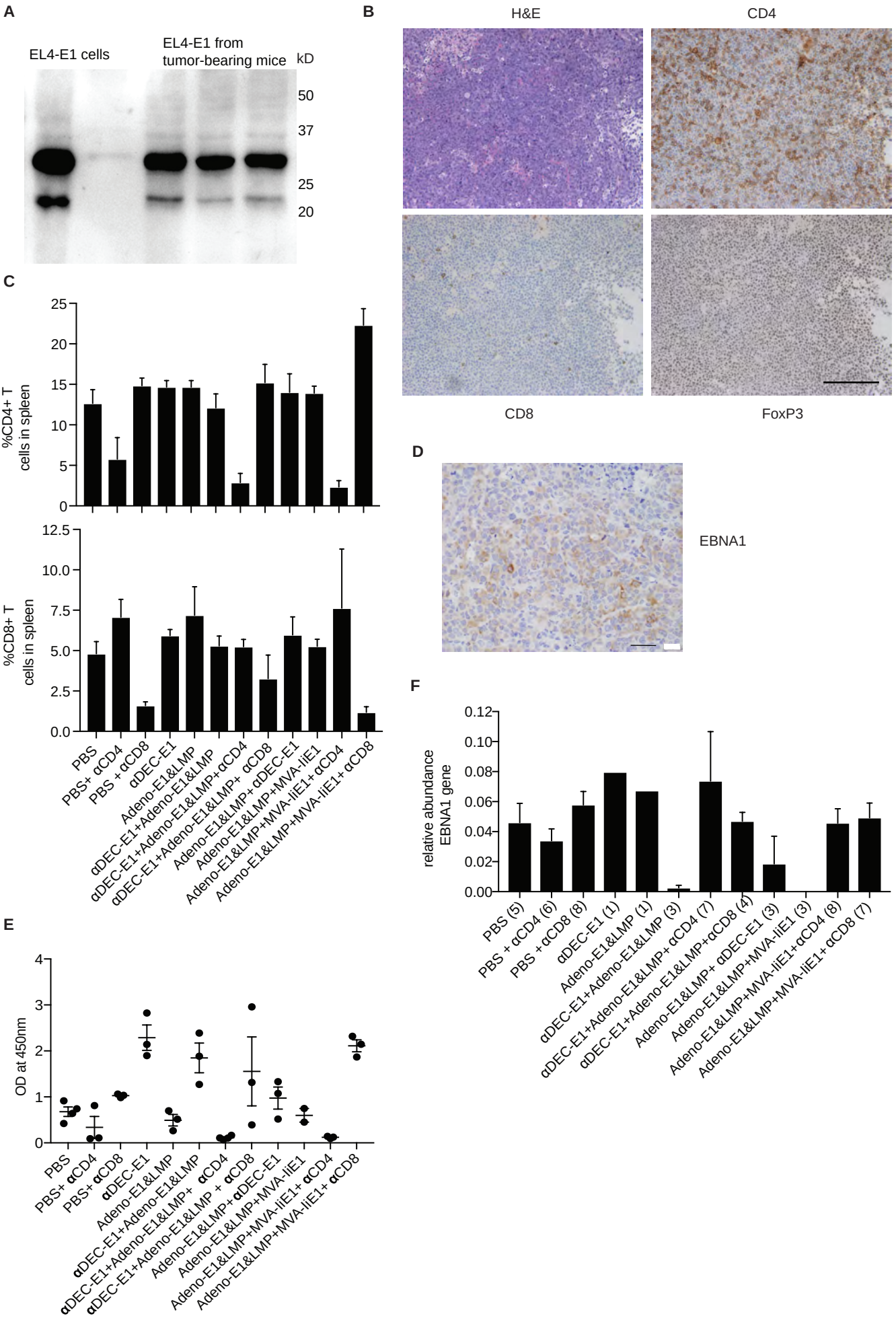
IFNγ+IL2+
IFNγ+TNFα+IL2+


A
EL4-E1 cells
EL4-E1 from tumor-bearing mice
kD
50
37
25
20
B
H&E
CD4
CD8
FoxP3
C
%CD4+ T cells in spleen
%CD8+ T cells in spleen
PBS
PBS+ αCD4
PBS + αCD8
αDEC-E1
Adeno-E1&LMP
αDEC-E1+Adeno-E1&LMP
αDEC-E1+Adeno-E1&LMP+αCD4
αDEC-E1+Adeno-E1&LMP+ αCD8
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Adeno-E1&LMP+MVA-liE1+αCD4
Adeno-E1&LMP+MVA-liE1+ αCD8
D
EBNA1
E
OD at 450nm
F
relative abundance EBNA1 gene
PBS (5)
PBS + αCD4 (6)
PBS + αCD8 (8)
αDEC-E1 (1)
Adeno-E1&LMP (1)
Adeno-E1&LMP (3)
αDEC-E1+Adeno-E1&LMP+ αCD4 (7)
αDEC-E1+Adeno-E1&LMP+αCD8 (4)
αDEC-E1+Adeno-E1&LMP+ αDEC-E1 (3)
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Adeno-E1&LMP+MVA-liE1 (3)
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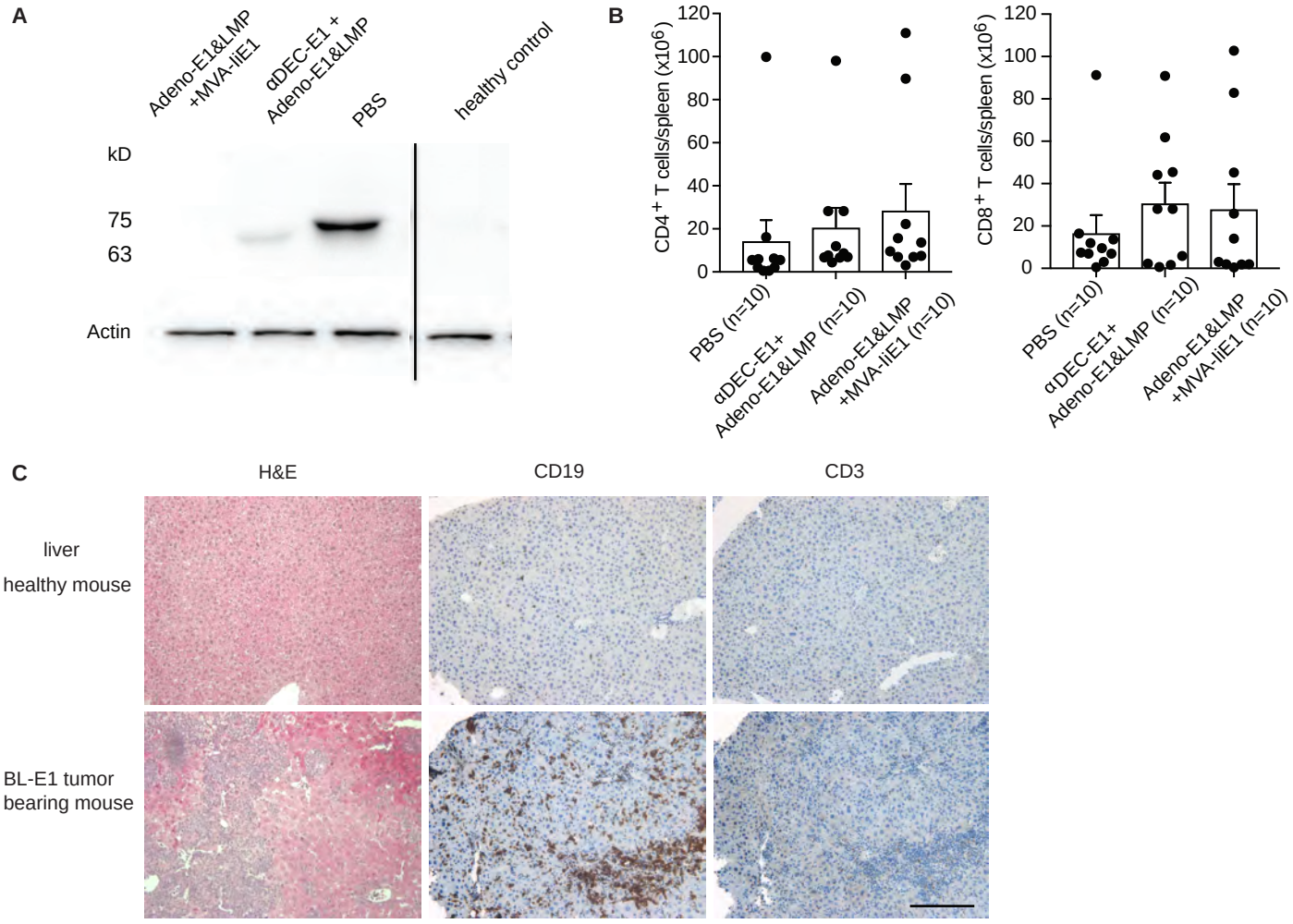
Adeno-E1&LMP+MVA-liE1+ αCD8 (7)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αDEC-E1 + Adeno-E1&LMP
PBS
healthy control
kD
75
63
Actin
B
CD4+ T cells/spleen (x10^6)
CD8+ T cells/spleen (x10^6)
120
100
80
60
40
20
0
PBS (n=10)
αDEC-E1+ Adeno-E1&LMP (n=10)
Adeno-E1&LMP +MVA-liE1 (n=10)
C
H&E
CD19
CD3
liver
healthy mouse
BL-E1 tumor bearing mouse