Supplementary

Supplementary Material and Methods

Cells

The human lung carcinoma A549 cell line (ATCC, CCL-185) and the human embryonic kidney HEK 293 cell line (ATCC, CRL-1573) were used.

Western-blot analysis for HBV protein expression

Monolayers of A549 cells were mock-infected with AdTG15149 or infected at 250 Infectious Unit (IU) /cell with TG1050. MG132 (proteasome inhibitor, 10 µM) was added 18 h post-infection and 48 h post-infection, cells were lysed and analyzed by Western-blot using mouse monoclonal antibody anti-Pol Hep B Pol 2C8 (that recognizes aa 8-20 of the Polymerase) (Santa Cruz). Mouse monoclonal antibody anti-Pol Hep B 8D5 (that recognizes aa 225-250 of the Polymerase) and mouse monoclonal anti-Core HepB C1-5 (that recognizes aa 70-80 of the Core) (Santa Cruz) were also used (data not shown). A goat anti-mouse HRP conjugated antibody (DakoCytomation) was used as secondary antibody and immunocomplexes were detected using an enhanced HRP-luminol chemiluminescence system (Immune-Star Western-C, BIO-RAD).

2D gel electrophoresis and LC-MS/MS

2D gel electrophoresis samples were overall prepared as for western blot analysis experiments with few changes: HEK 293 cells were infected and lysed by 2D lysis buffer (9M Urea, 2M Thiourea, 4% CHAPS and 60 mM DTT). Each strip (Biorad *ReadStrip*^{TM} *IPG Strip* 17cm pH 3-10 L) was loaded with one mg of protein, isofocalized and then transferred on a 12% SDS-PAGE for a 2nd dimension separation.

Gels were either stained with Coomassie Instantblue™ overnight or blotted on PVDF membranes, incubated with 2C8 monoclonal antibody as described above and developed with colorimetric substrate 4CN (Biorad). Total protein spots on blots were developed using colloidal gold staining (Biorad). Areas on Coomassie stained gels corresponding to immunoreactive spots were located and 4 to 14 pieces of gel/area were excised for mass spectrometry analysis. Gel pieces were treated by either trypsin or pepsin after reduction and alkylation. Resulting peptides were directly analyzed by nanoLC-MS/MS on a nanoACQUITY Ultra-Performance-LC (UPLC, Waters, Milford, MA) equipped with C18 precolumn (Waters Corp.), and an analytical BEH130 C18 column (Waters Corp.), 75 µm x 250 mm, 1.7 µm particle size. The MS and MS/MS analyzes were performed on the SYNAPT™ (Waters, Milford, MA).[26] Data were interpreted using Mascot (version 2.4.1, Matrix science, London, England*)* and Scaffold 3 (version 3.6.5; Proteome Software Inc., Portland, OR, USA*)* software was used to identify proteins and hits corresponding to the TG1050 primary structures (deduced from the 6 possible reading frames).

Mice. All mice were used following the requirement of the CEE directive 86/6009 and French law. From 1st February 2013, the CEE directive 2010/63/UE of 22th September 2010 and the French décret n° 2013-118 of 1st February 2013 were applied. BALB/c, C57BL/6J and HLA-A*0201 transgenic, H-2 class I KO mice (HLA-A2 mice),[18] were housed for experiments at the Plateau de Biologie Expérimentale de la Souris (PBES, Lyon, France) while HLA-A*0201/DRB1*0101 transgenic, H-2 class I/class II KO mice (HLA-A2/DR1 mice),[19] were housed in the animal facility of Institut Pasteur (Paris, France).

Peptides. Synthetic peptide libraries were synthesized by ProImmune with Prospector™ LCMS Crude technique. Two pools of peptides covering the Core protein, 9 pools of peptides covering the Polymerase, 1 pool covering Env1 and 1 pool covering Env2 were composed (comprising from 9 to 25 peptides).

In vivo CTL assays

In vivo CTL assays were performed as described by Fournillier *et al*,[24]. Of note, for BALB/C and C57BL/6J mouse experiments pulsed and unpulsed cells were respectively stained with 2 and 16 µM of CFSE.

HBV-DNA qRT-PCR

Viral DNA from 50 µL mouse plasma samples has been purified using the MagMAX™-96 Viral RNA/DNA Isolation Kit (Ambion) according to manufacturer's specifications. Purified viral DNA has been resuspended in 50 µL of elution buffer. Five µL (diluted 10x in H_2O) were tested for HBV-specific DNA by qRT-PCR in a 20 µL reaction using the GeneSig HBV qPCR kit (PrimerDesign™Ltd) according to manufacturer's instructions on a 7500HT Real-Time PCR System (Applied Biosystems). Analysis was performed using SDS v2.0.6 software (Applied Biosystems).

Triple Intracellular Cytokine Staining Assays (IFNγ/TNFα/IL2)

Livers were collected 15 days after adenovirus injection. Red blood cells were lysed and liver infiltrating lymphocytes were incubated in supplemented αMEM culture medium in presence of 1µM of VSA (VSAAFYHLPL) peptide and GolgiPlug. After 5h at 37°C and overnight at 4°C cells were washed with 1 % FCS-PBS and incubated with anti-CD16/CD32 (clone

2.4G2) for 10min at 4°C. Then 1 % FCS-PBS containing Live/Dead Violet and monoclonal antibodies against CD4-V500 (RM4.5) and CD8a-APC-H7 (53-6.7) were incubated 30min at 4°C. After washes, cells were fixed and permeabilized for 20min at room temperature with Cytofix/Cytoperm and washed with Perm/Wash solution. Perm/Wash solution containing monoclonal antibodies against CD3-PerCP (145-2C11), IFNγ-A488 (XMG1.2), IL2-PE (JES6-5H4) and TNFα-APC (MP6-XT22) were incubated 30 min at 4°C. After washes, cells were resuspended in 1% FCS-PBS and analyzed by flow cytometry using a BD FACS Canto II cytometer. A technical cut-off value was determined as 25 x 100/average number of CD3e+, CD8α+ or CD4+ cells. Additionally an experimental cut-off value was calculated as 3 times the standard deviation (SD) of values obtained without stimulation (medium). A response was then considered as positive if the percentage of cytokine-positive cells was higher than both cut-off values.

Supplementary Figures and Tables

Figure S1 (-A, -B and -C): TG1050 *in vitro* and biochemical characterization

Expression of the HBV fusion protein by TG1050 was assessed by Western Blot **(S1-A)** and analysed using mouse monoclonal anti-Pol Hep B Pol 2C8 antibody. Arrow on the right indicates the position of the full length Core-Pol-Env protein. Proteomic analysis of 2C8 immunoreactive protein species was performed **(S1-B)**. Z1, Z2 and Z3 displayed respectively apparent MWs of 24 kDa and an isoelectric point (pI) of ~5 +/-0.5, 13 kDa and pI of ~5.5 +/-0.5 and 24 kDa and undetermined pI (no isofocalization). Sequences obtained after trypsin or pepsin digestions and identified by LC-MS/MS analysis are shown **(S1-C)**. Peptides corresponding to TG1050 polyprotein were reported on the partial sequence in green, orange and red for sequences respectively identified after pepsin treatment only, after pepsin and trypsin treatments and after trypsin treatment only. Epitope recognized by the 2C8 antibody is

underlined. All peptides matched the sequence of the expected reading frame but only covered the first 202 residues of the fusion protein. Peptides from Z1 and Z3 regions covered similar parts of the TG1050 HBV fusion protein (i.e. from F24 and D29, respectively for Z1 and Z3, to K202) potentially representing the same protein isofocalized and non isofocalized respectively. The difference of coverage observed between Z1 and Z3 could be explained by a difference of both quantity and purity of the same immunoreactive protein in the two zones. Z2-generated peptides covered a more restricted portion of the polyprotein than Z1/Z3 (from W71 to K202). It could be hypothesized that the main 2C8 immunoreactive products in Z1 Z2 and Z3 correspond to stable degradation products of the polyprotein that accumulate in the cell. The major immunoreactive 2C8 product in Z1 and Z3 regions could correspond to the first ~210 residues of the fusion protein containing a nearly intact core and the 50-60 first residues of Pol (calculated MW of 24 kDa and pI of 4.9). Z2 could be a more degraded product than Z1/Z3, lacking the first 50-60 residues of the polyprotein (calculated MW of 15 kDa and pI of 5.3). This N-terminal degradation could explain both the vertical (24 to 15 kDa) and horizontal (pI 4.9 to 5.3) migration shifts of Z2 versus Z1.

Figure S2: Analysis of induced T cell responses following single injection of TG1050 using IFNγ ELISPOT assay. HLA-A2 transgenic (2A), C57BL/6J (2B) and BALB/c (2C) mice were immunized once subcutaneously with TG1050 or an empty Ad (negative control). IFNγ ELISPOT assays were realized using spleen cells and pools of overlapping peptides covering respectively the HBV Core protein (PC1, PC2), the 2 Env domains (PE1, PE2) and the HBV polymerase protein (PP0 – PP8), or irrelevant peptides (IRR) or medium alone (MED). Each individual mouse is represented by a dot, median values are represented by the bar.

Figure S3: Analysis of induced *in vivo* cytolytic T cell responses following single injection of TG1050 in HLA-A2 transgenic, C57BL/6J and BALB/C mice. HLA-A2 transgenic mice or C57BL/6J or BALB/c mice were immunized once with TG1050 or an empty Adenovirus (negative control) and induced HBV-specific immune responses were monitored on spleen cells using *in vivo* CTL assays performed as described in Material and methods. An example of overlay histograms obtained in each mouse strain for one of the tested peptide (SLY for HLA-A2 mice **(S3A)**, N13F for C57BL/6J mice **(S3B)** and HYF for BALB/c mice **(S3C))** is shown, the plain grey histogram corresponding to an empty Ad immunized mouse injected with unloaded cells and HBV peptide loaded cells and the unfilled black line histogram corresponding to a TG1050 immunized mouse injected with unloaded cells and HBV peptide loaded cells. Percentages of specific *in vivo* cytolysis obtained for various HBV peptides in each mouse strain are represented after TG1050 or empty Ad immunization. Each empty or plain circle represents an individual mouse immunized respectively with empty Ad or TG1050. Black lines represent the mean values of each group for each tested HBV peptide. Dotted lines represent cut-off values for each peptide, being defined by mean value of the percentages of specific lysis obtained with mice immunized with empty Ad for each peptide + three times the standard deviation.

Figure S4: Induction of long term HBV-specific functional T cells in HLA-A2/HLA-DR1 AAV-HBV persistent mice following single TG1050 immunization. HLA-A2/HLA-DR1 transgenic mice were intravenously injected with PBS or AAV-HBV (day 0) and immunized one month later (day 32) with either TG1050 or empty Ad. Long term induced HBV-specific immune responses were then assessed 4 months post Adenovirus injection via IFNγ ELISPOT assay on spleen cells **(S4A)** and IFNγ/TNFα/IL2 ICS assays using spleen cells and liver infiltrating lymphocytes **(S4B and S4C)**. HLA-A2 specific peptides were used for *in vitro*

stimulation : SLY for polymerase, ILC and FLP for Core and FLG, VLQ and GLS for Env domains or an irrelevant peptide. Responses observed in the 3 tested groups are represented on 3 separate graphs. For ICS assays, cells were stimulated with a mix of the above mentioned peptides. Results are represented as the percentage of CD8+ T cells producing at least IFNγ or TNFα or IL2.

On each graph (S4B), observed percentages of IFNγ or TNFα or IL2 producing cells is represented by a box and whisker plot (encompassing the minimum value, the 25th percentile, the median, the 75th percentile and the maximum value) The percentage of mice displaying positive response is indicated at the top of the graph.. In addition mean percentage per group of HBV-specific induced CD8+ T cells producing 1, 2 or 3 of the tested cytokine among cells producing at least one cytokine was represented by pie charts for the 2 groups displaying positive responses ie PBS+TG1050 and AAV-HBV+TG1050 (S4C).

Figure S5: Monitoring of HBsAg in HLA-A2/HLA-DR1AAV-HBV mice. HBsAg levels in sera of mice were assessed at different time points post AAV-HBV injection. Data are represented as mean fold changes compared to their respective mean level before TG1050 (Plain circle curve) or empty Ad (empty circle curve) or buffer (cross curve) injection. Statistical differences are indicated by the star ($p<0.05$; two-way RM ANOVA/ Bonferonni).

Table S1: Peptide sequences used to recall specific T cells targeting epitopes from the Core Polymerase and Envelope proteins of HBV

Supplementary Table S1

HLA-A2 restricted peptides

Protein	Start Position	End Position	Sequence	Short name
Core	18	27	FLPSDFFPSV	FLP
	59	68	ILCWGELMTL	ILC
Env	14	22	VLQAGFFLL	VLQ
	20	28	FLL TRI LTI	FLL
	41	49	FLGGTTVCL	FLG
	185	194	GLSPTVWLSV	GLS
Pol	803	811	SLYADSPSV	SLY

H2b restricted peptides

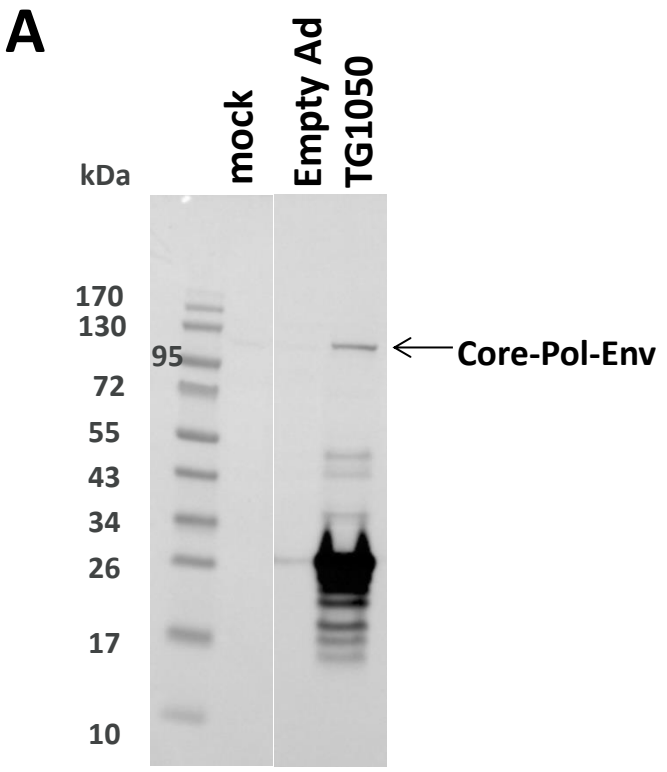
Protein	Start Position	End Position	Sequence	Short name
Core	93	100	MGLKFRQL	MGL
	129	143	PPAYRPPNAPILSTL	P13L
Env	179	186	FVQWFVGL	FVQ
	163	177	FLWEWASARFSWLSL	F13L
Pol	396	404	FAVPNLQSL	FAV
	419	428	VSAAFYHLPL	VSA
	44	58	NLNVSIPWTHKVGNF	N13F

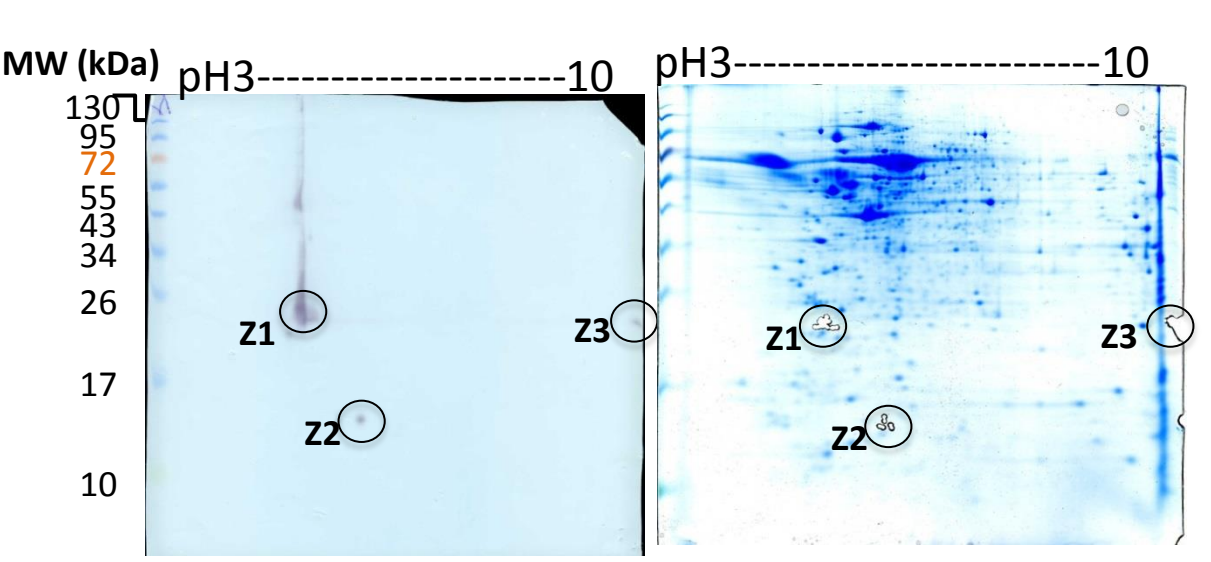
H2d restricted peptides

Protein	Start Position	End Position	Sequence	Short name
Core	87	85	SYVNTNMGL	SYV
Env	28	39	IPQSLDSWWTSL	IPQ
Pol	140	148	HYFQTRHYL	HYF

Supplementary

Figure S1





C

Z1

```
  1  MDIDPYKEFG ATVELLSFLP SDFFPSVRDL LDTASALYRE ALESPEHCSP
 51  HHTALRQAIL CWGELMTLAT WVGGNLEDPI SRDLVVSYVN TNMGLKFRQL
101  LWFHISCLTF GRETVIEYLV SFGVWIRTPP AYRPPNAPIL STLPETTVPL
151  SYQHFRRLLL LDDEAGPLEE ELPRLADEGL NRRVAEDLNL GNLNVSIPWT
201  HKVGNFTGLY SSTVPVFNPH ……
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Z2

```
  1  MDIDPYKEFG ATVELLSFLP SDFFPSVRDL LDTASALYRE ALESPEHCSP
 51  HHTALRQAIL CWGELMTLAT WVGGNLEDPI SRDLVVSYVN TNMGLKFRQL
101  LWFHISCLTF GRETVIEYLV SFGVWIRTPP AYRPPNAPIL STLPETTVPL
151  SYQHFRRLLL LDDEAGPLEE ELPRLADEGL NRRVAEDLNL GNLNVSIPWT
201  HKVGNFTGLY SSTVPVFNPH ……
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Z3

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  1  MDIDPYKEFG ATVELLSFLP SDFFPSVRDL LDTASALYRE ALESPEHCSP
 51  HHTALRQAIL CWGELMTLAT WVGGNLEDPI SRDLVVSYVN TNMGLKFRQL
101  LWFHISCLTF GRETVIEYLV SFGVWIRTPP AYRPPNAPIL STLPETTVPL
151  SYQHFRRLLL LDDEAGPLEE ELPRLADEGL NRRVAEDLNL GNLNVSIPWT
201  HKVGNFTGLY SSTVPVFNPH ……
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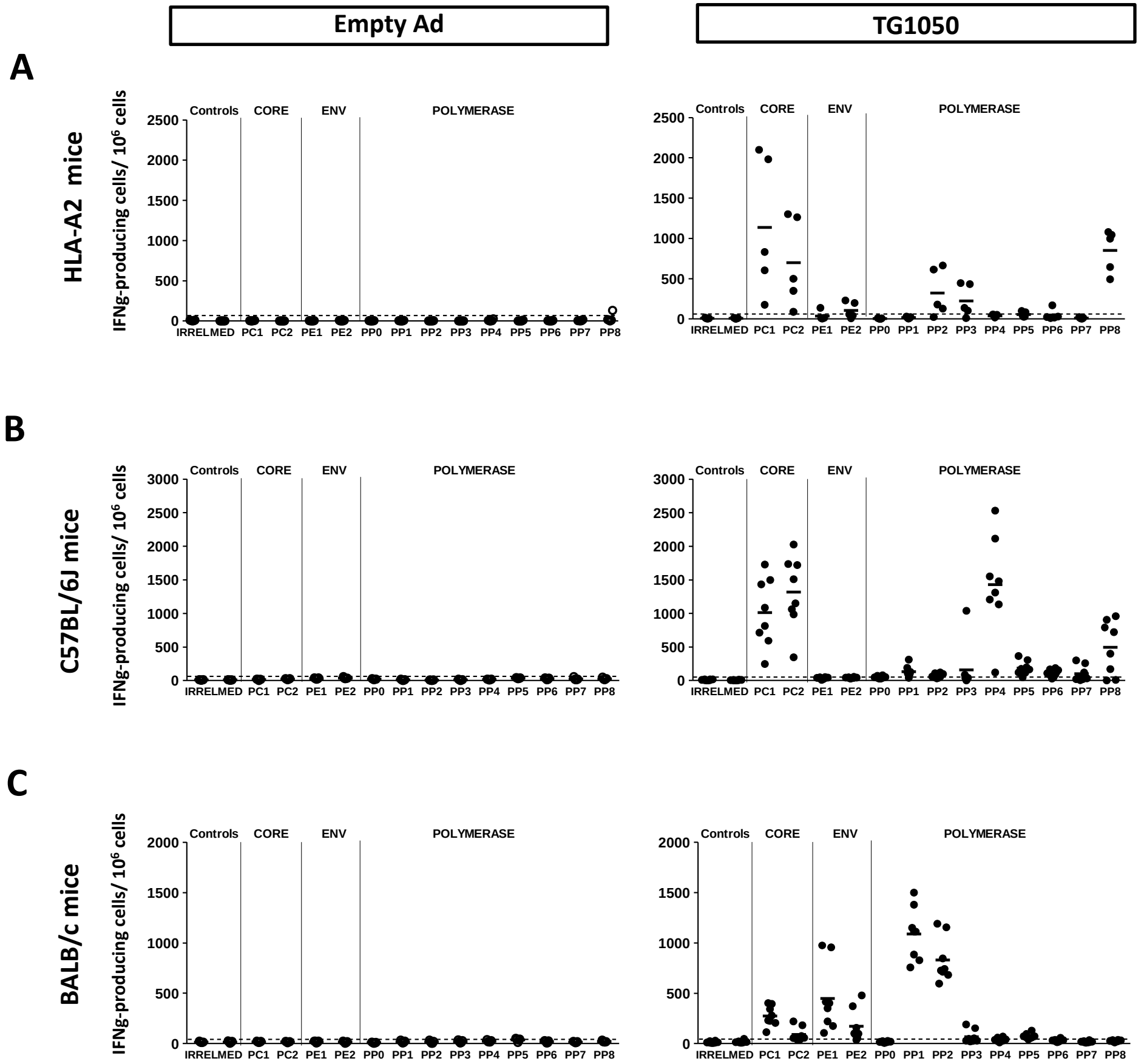
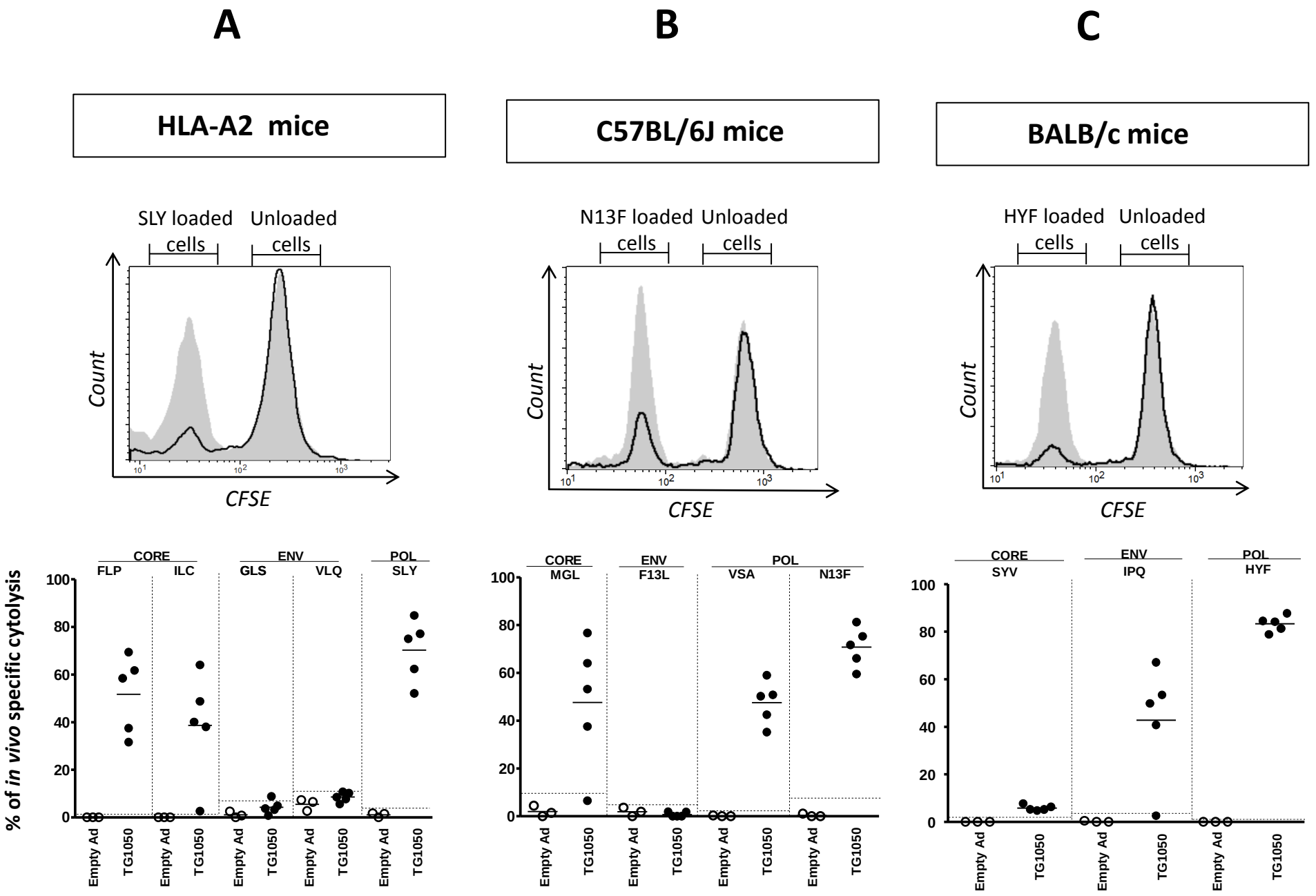
Supplementary Figure S2



Supplementary

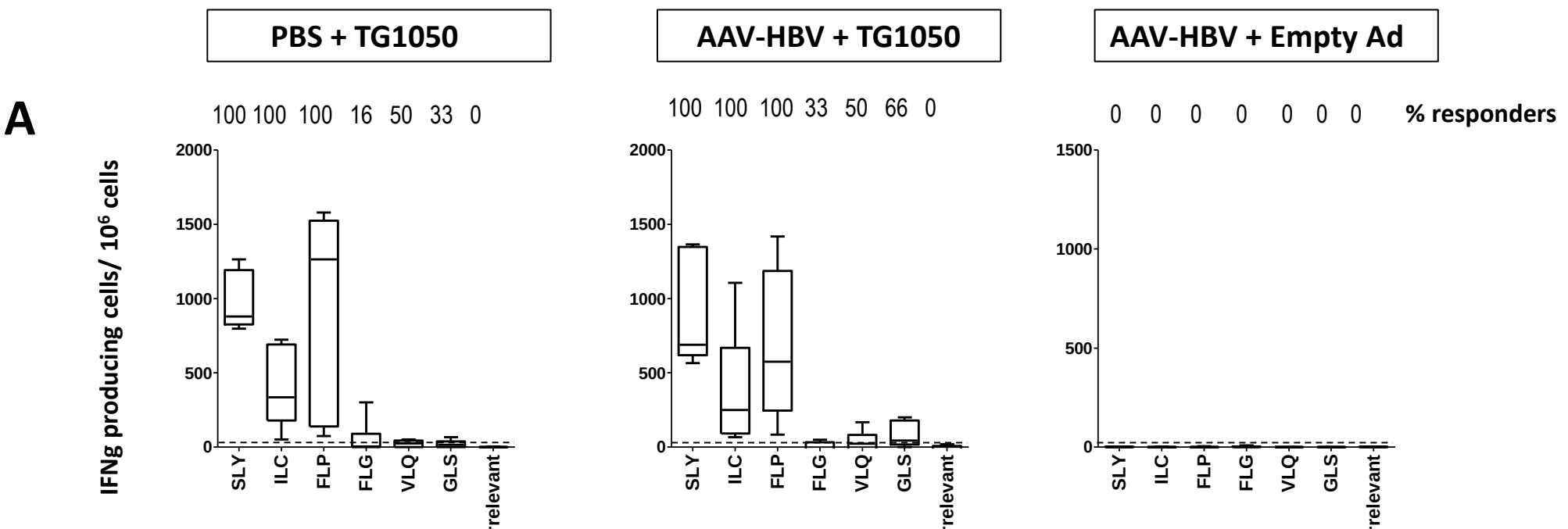
Figure S3



Supplementary

Figure S4

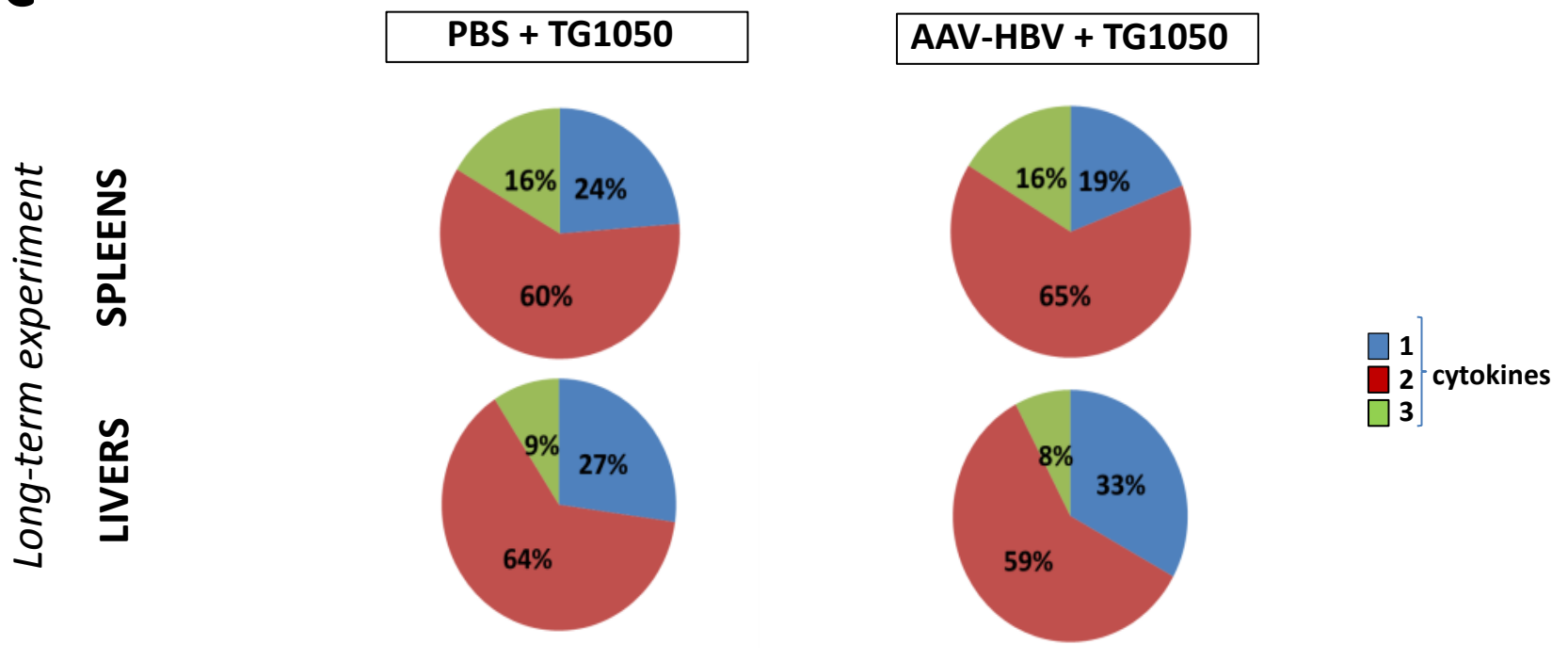
Average HBV DNA titers at D28 : 588 IU/mL
Average HBsAg titers at D28 : 7 µg/mL



Supplementary

Figure S4 (continuation)

C



Supplementary

Figure S5