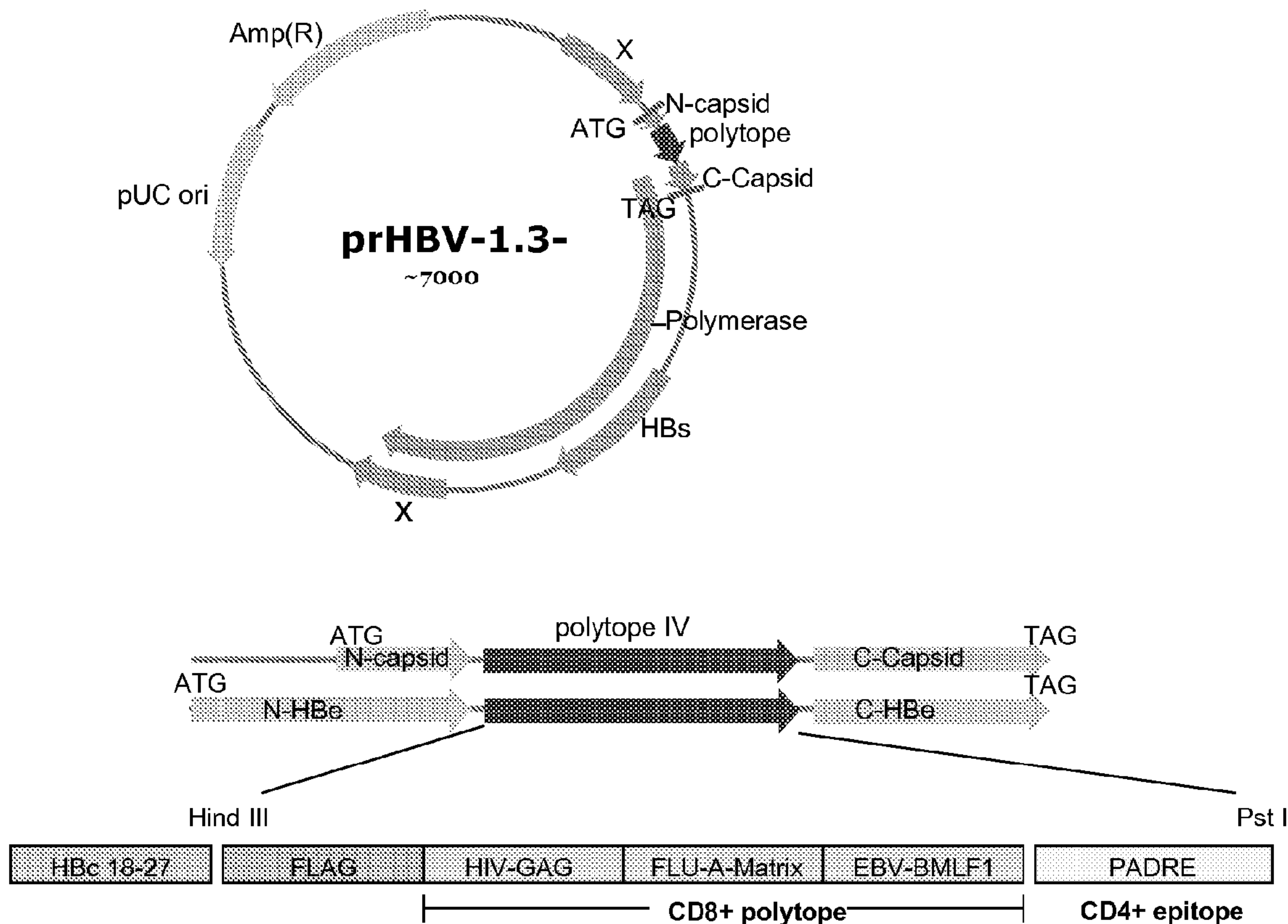




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(54) **Titre : POLYNUCLEOTIDES PERMETTANT L'EXPRESSION ET LA SECRETION D'UN PSEUDO-VIRUS RECOMBINE
 CONTENANT DES EPITOPES ETRANGERS, PRODUCTION ET UTILISATION DE CES POLYNUCLEOTIDES**
 (54) **Title: POLYNUCLEOTIDES ALLOWING THE EXPRESSION AND SECRETION OF RECOMBINANT PSEUDO-VIRUS
 CONTAINING FOREIGN EPITOPES, THEIR PRODUCTION, AND USE**



(57) **Abrégé/Abstract:**

The invention is directed to a recombinant replication defective hepatitis B virus comprising a hepatitis B virus genome defective for the expression of hepatitis B virus capsid protein (HBc), said virus containing a nucleotide sequence encoding a foreign peptide not

(57) Abrégé(suite)/Abstract(continued):

found in the wild type hepatitis B virus comprising at least one immuno-dominant epitope. The invention is also directed to the use of said recombinant replication defective hepatitis B virus as a medicament, preferably a vaccine, treating a patient chronically or persistently infected by a hepatitis B virus.

ABSTRACT

The invention is directed to a recombinant replication defective hepatitis B virus comprising a hepatitis B virus genome defective for the expression of hepatitis B virus capsid protein (HBc), said virus containing a nucleotide sequence encoding a foreign peptide not found in the wild type hepatitis B virus comprising at least one immuno-dominant epitope. The invention is also directed to the use of said recombinant replication defective hepatitis B virus as a medicament, preferably a vaccine, treating a patient chronically or persistently infected by a hepatitis B virus.

**POLYNUCLEOTIDES ALLOWING THE EXPRESSION AND SECRETION OF
RECOMBINANT PSEUDO-VIRUS CONTAINING FOREIGN EPITOPES,
THEIR PRODUCTION, AND USE**

5 This invention relates to polynucleotides for the expression of a recombinant, replication defective virus involved in a persistent infection, and to a recombinant, replication competent pseudo-virus, comprising the recombinant replication defective virus, and to the production of the viruses in host cells. The recombinant, replication defective virus and the replication competent pseudo-virus can contain a foreign epitope
10 or epitopes, such as foreign amino acid residues of a pathogen. The replication defective virus and the replication competent pseudo-virus are particularly useful in immunogenic compositions and as therapeutic vaccines. This invention also relates to T cell responses to viral infection and to recombinant viruses that deliver foreign antigenic epitopes to the liver and induce epitope-specific immune responses.

15

BACKGROUND OF THE INVENTION

An effective vaccine against hepatitis B virus (HBV) infection has been available for more than two decades, but 400 million people - more than 5% of the world's population - are chronically infected with HBV. More than 1 million people die
20 each year from HBV-related liver cirrhosis and hepatocellular carcinoma. (Ganem D., Prince, A.M. (2004) Hepatitis B virus infection--natural history and clinical consequences, N Engl J Med 350:1118-29).

HBV is mainly not directly cytopathic. The immune response to viral antigens is thought to be responsible for both liver disease and viral clearance following HBV
25 infection (Ganem et al., 2004). Immune responses with virus-specific CD8⁺ cytotoxic T lymphocytes (CTLs) and CD4⁺ T-helper (Th) cells play key effector and regulatory roles in both liver pathogenesis and viral clearance. HBV acute infection in immunocompetent adults usually results in a transient self-limited liver disease followed by viral clearance, and is characterized by vigorous polyclonal CTLs and type
30 1-Th responses specific for a number of epitopes within HBV viral proteins.

Patients with acute viral infection, who successfully clear the virus, display a multispecific polyclonal cytotoxic T-lymphocyte (CTL) response specific for a number

of epitopes within the core, polymerase, and envelope proteins. Viral specific, e.g., HBV-specific Th cells are also activated. Multispecific Th1-like responses have been detected in patients successfully clearing HBV after acute infection (Chisari et al., 1995, Hepatitis B virus immunopathogenesis, *Annu Rev Immunol* 13:29-60).

5 The HBV-specific T-cell response is weak or undetectable in patients who develop chronic infection and the mechanisms responsible for T cell hypo-responsiveness or tolerance in chronic infection are not completely understood. In chronically infected patients, the peripheral CD8⁺ T cell response is undetectable or weak and the CD4⁺ T cell response is much less vigorous than in patients who clear the
10 infection.

 Although functional effector T cells are initially generated during the early stages of infection, they gradually lose function during the course of a chronic infection by upregulating the programmed death 1 (PD-1) inhibitory receptor (Chisari et al., 1995). Accordingly, in chronic patients who spontaneously clear hepatitis B surface
15 antigen (HBsAg) and develop neutralizing anti-HBs antibodies, HBV-specific T-cell responses have been detected in the blood just before seroconversion. It has also been shown that effective therapeutic reduction of HBV viral load resulted in a transient restoration of HBV-specific CD4⁺ and CD8⁺ T-cell responses in the blood from patients with chronic hepatitis B.

20 The mechanisms responsible for T cell hypo-responsiveness and exhaustion during HBV persistent infection are still not completely understood. (Rehermann B., Nascimbeni M., 2005, *Immunology of hepatitis B virus and hepatitis C virus infection*, *Nat Rev Immunol* 5:215-29). Exhausted T cell responses observed during persistent viral infection reflect a balance between effector functions required to eliminate the
25 pathogen and the potential of T cells to cause immunopathology. Impaired dendritic cell functions and the presence of CD4⁺CD25⁺ regulatory T cells also contribute to the viral persistence. Moreover, the liver particularly biases the intrahepatic T cell response towards tolerance or anergy.

 Active immunotherapy based on specific viral-epitopes and hepatitis vaccine
30 injection provide promising approaches in inducing efficient cellular immune responses. A previous study of a phase I clinical trial suggested that HBV DNA vaccination could specifically restore T-cell responsiveness in chronic HBV carriers. However, the

activation of HBV-specific T-cells appeared to be transient and was followed by a progressive decline along the DNA injections (Mancini-Bourguine M., Fontaine H., Scott-Algara D., Pol S., Brechot C., Michel M.L., 2004, Induction or expansion of T-cell responses by a hepatitis B DNA vaccine administered to chronic HBV carriers, *Hepatology* 40:874-82). Bypassing the potential tolerance of T cells to HBV antigens, therefore, turns out to be a most crucial point in immunotherapy.

Collectively this suggests that, to treat chronic hepatitis, e.g., HBV, infection, the intrahepatic T cell responses should be switched from a state of exhaustion or anergy to a state in which the effector T cells are fully efficient. (Bertoletti A., Gehring A.J., 2006, The immune response during hepatitis B virus infection, *J Gen Virol* 87:1439-49; Rehermann et al., 2005).

Accordingly, there exists a need in the art for new therapies for the treatment of chronic hepatitis infection, for example, hepatitis B infection. These therapies should also be generally useful in the treatment of other viral persistent infections. The therapies should also be specific for the cells infected with the virus involved in the persistent infection. For example, in the case of a hepatitis viral infection, the therapies should be specific for hepatitis virus infected cells, especially human hepatocytes (Rehermann et al., 2005).

20 SUMMARY OF THE INVENTION

This invention aids in fulfilling these needs in the art. To achieve these results, this invention provides a recombinant, replication defective virus that co-maintains in vivo with wild type virus in cells infected with said virus, and that immunologically contributes, after complementation, to virus clearance by expressing foreign antigenic epitopes in virus infected cells. The invention provides a pseudo-virus to achieve these results. The recombinant defective virus of the invention comprises a genome defective for the expression of a protein essential for virus replication. Examples of such proteins are structural proteins and more particularly, capsid proteins.

The invention also provides a novel vaccine strategy modeled on the use of hepatitis virus as a vector to deliver foreign antigenic epitopes into the liver. Presentation of these epitopes by liver cells would, in turn, attract efficient (i.e., non-

exhausted) T cell responses to the target tissue, for example, the liver, and contribute to viral clearance.

In one embodiment, the recombinant, replication defective virus is hepatitis virus that co-maintains in vivo with wild type hepatitis virus in hepatitis virus-infected hepatocytes, and that immunologically contributes, after complementation, to hepatitis virus clearance by expressing foreign antigenic epitopes in hepatitis virus-infected hepatocytes. The invention provides a hepatitis pseudo-virus to achieve these results.

More particularly, the invention involves the design of polynucleotides and expression vectors for cloning and expressing foreign peptides or polypeptides, such as Flu polyepitopes, in the pseudo-virus. In one embodiment, the polynucleotides and expression vectors are designed for cloning and expressing foreign peptides or polypeptides, such as Flu polyepitopes, in the hepatitis pseudo-virus. Polynucleotides and expression vectors comprising these polynucleotides have been designed, all preserving recombinant hepatitis pseudo-virus formation.

In one embodiment, this invention provides a recombinant replication defective hepatitis virus comprising a hepatitis virus genome defective for the expression of hepatitis virus capsid protein (HBc). The virus contains a nucleotide sequence of up to about 195 nucleotides encoding at least one immuno-dominant epitope of a pathogen. The nucleotide sequence is located between nucleotide residue 1981 and nucleotide residue 2308 of the HBV ayw3 genome (numbering starts from the EcoRI site of the HBV genome, NCBI Accession No. V01460). Hepatitis B is a preferred virus for use in practicing the invention.

A vertebrate cell, transfected by the recombinant, replication defective virus of the invention is also provided. In one embodiment, the vertebrate cell is a hepatocyte cell and the virus is hepatitis virus. In one embodiment, the recombinant virus can be produced by co-transfection of a hepatocytic cell line such as Huh 7 (11) or Hep G2 (12) by one of the two constructs prHBV1.3-III or -IV with a plasmid encoding wild type capsid (pCMV-core or pMAS-core) or by transfection with a plasmid such as prHBV1.3/HB, which carries 1.3 copies of the rHBV genome and an additional expression cassette for HBV wild type core protein. The hepatocyte cell can further comprise a nucleotide sequence encoding HBc for complementation of the recombinant, replication defective, hepatitis virus to form the hepatitis pseudo-virus.

This invention thus provides a replication competent pseudo-virus comprising the recombinant, replication defective virus, complemented by the capsid protein of the virus, wherein the pseudo-virus replicates in vitro in human cells. In one embodiment, the replication competent pseudo-virus is a hepatitis pseudo-virus comprising the
5 recombinant, replication defective hepatitis virus complemented by HBc, wherein the pseudo-virus replicates in vitro in human hepatocytes.

Further, this invention provides a vertebrate cell, expressing the pseudo-virus. In one embodiment, the vertebrate cell is a hepatocyte cell expressing the hepatitis pseudo-virus.

10 In addition, this invention provides a method of forming a replication competent pseudo-virus, wherein the method comprises culturing the infected cells of the invention under conditions for expression of the nucleotide sequence encoding the capsid protein of the virus and complementation of the recombinant, replication defective virus to form the pseudo-virus, which can optionally be secreted from the host cell into extracellular
15 space. In one embodiment, the method can be used to form a replication competent hepatitis pseudo-virus by culturing the infected hepatocyte cells of the invention under conditions for expression of the nucleotide sequence encoding HBc and complementation of the recombinant, replication defective hepatitis virus to form the hepatitis pseudo-virus, which can optionally be secreted from the host cell into the
20 extracellular space.

A polynucleotide hybridizing under stringent conditions to the recombinant, replication defective virus or its complement is also provided. In one embodiment, the polynucleotide hybridizes under stringent conditions to the recombinant, replication defective hepatitis virus or its complement.

25 A cloning and/or expression vector comprising the recombinant, replication defective virus is further provided. In one embodiment, the cloning and/or expression vector comprises the recombinant, replication defective hepatitis virus.

Polypeptides encoded by the recombinant, replication defective virus and by the replication competent pseudo-virus are also provided. In one embodiment, the
30 polypeptides are encoded by the recombinant, replication defective hepatitis virus and by the replication competent hepatitis pseudo-virus.

Further, this invention provides a eukaryotic host cell comprising a vector of the invention. In one embodiment, the vector in the eukaryotic host cell comprises a eukaryotic promoter sequence operably linked to a nucleotide sequence for expression of hepatitis pseudo-virus. Optionally, the vector can comprise a nucleotide sequence
5 encoding a fusion protein comprising a foreign polypeptide and a viral protein, wherein the eukaryotic host cell produces pseudo-virus comprising the fusion protein. In one embodiment, the fusion protein comprises a hepatitis protein and the eukaryotic host cell produces hepatitis pseudo-virus comprising the fusion protein.

More particularly, in certain embodiments, this invention relates to a vector
10 derived from the HBV genome. The HBV genome is modified in order to express foreign antigenic epitope(s) fused within the N-terminal part of the HBV capsid protein yielding a recombinant HBV virus that will co-maintain in the liver of HBV-infected individuals through wild type capsid complementation. The HBV genome can be further modified to allow the expression and presentation of foreign epitope(s) at the
15 surface of hepatocytes after infection with this recombinant HBV virus. The foreign antigenic epitope(s) can be derived from common pathogens that are presented in association of MHC class 1 molecules and serve as targets of the host CD8⁺ T-cell immune response. The replication of the recombinant HBV vector in already HBV-infected hepatocytes can thereby immunologically contribute to HBV clearance by
20 expressing foreign antigenic epitope(s) in HBV-infected hepatocytes.

Only virus-infected cells that allow replication of the recombinant virus vector of the invention are the targets of the immune response. In one embodiment, the virus-infected cells are hepatitis virus-infected hepatocytes that allow replication of the recombinant hepatitis virus vector of the invention. The recombinant, replication
25 defective virus delivers foreign antigenic epitope(s) (for instance, polyepitopes) via the replication competent pseudo-virus of the invention in the cells of a vertebrate, and subsequently elicits strong (poly)epitope-specific immune responses. In one embodiment, the recombinant, replication defective virus is a recombinant, replication defective hepatitis virus and the vertebrate cells are hepatocytes. Hepatitis virus is
30 cleared through cytolytic or non-cytolytic mechanisms.

The pseudo-virus of the invention results in the induction of robust immune responses and the enhancement of the activation state of sequence-specific CD4⁺ and

CD8⁺ T lymphocytes so that the pseudo-virus can be employed in therapeutic applications. In one embodiment, the pseudo-virus of the invention is a hepatitis pseudo-virus.

The prHBV1.3 DNA may be used as a DNA vaccine for therapeutic intervention
5 in chronically HBV-infected patients. This construct expresses the three HBV envelope proteins, the polymerase, and the HBx protein. The vector is non-replicative when administrated as DNA through a systemic route. The pCMV-rHBe construct encodes a secreted form of HBeAg carrying foreign epitopes. It induces T cell responses specific for the foreign epitope and can be used as a vector for DNA immunization against
10 pathogens harboring those epitopes.

Accordingly, the invention provides a composition comprising the replication defective virus of the invention and a vaccine comprising said composition. The vaccines of the invention may be administered to a patient persistently infected with a virus in order to stimulate a T cell response against cells infected with the virus. Thus,
15 the invention also contemplates the use of the recombinant replication defective viruses of the invention for the preparation of a medicament for treating a patient persistently infected with the wild type virus. The invention also contemplates a method for targeting the expression of an epitope in a cell infected with a virus by providing to the cell the recombinant replication defective virus of the invention.

20 The invention provides antibodies to the chimeric antigenic fusion proteins produced by the pseudoviruses of the invention.

The invention also provides a mouse, for example, an HLA-A2/DR1 or an HbsAg/HLA-A2 double transgenic mouse, comprising a plasmid described herein. The plasmid may have entered the cells of the mouse using any method known in the art for
25 producing transgenic animals. In an embodiment, the animal is injected intramuscularly or hydrodynamically, e.g., through a tail vein.

In an embodiment, the animal comprises prHBV-1.3, e.g., prHBV-1.3-III or prHBV-1.3-IV. In an embodiment, the transgenic animal comprises a plasmid comprising a polynucleotide sequence encoding rHBe_III, rHBe_IV, polytope III, or
30 polytope IV.

In an embodiment, the percentage of CD8⁺ T cells of the transgenic animal increases in response to infection with a pseudo-virus of the invention. In an

embodiment, the transgenic animal mounts an epitope-specific T-cell response to a pseudo-virus of the invention. In an embodiment, a rHBV is expressed in liver cells, with an encoded foreign antigen processed into polypeptides for immune recognition.

5 The invention provides a method of vaccinating an animal chronically infected with a pathogenic virus by providing a recombinant replication-competent pseudo-virus comprising the recombinant, replication defective virus, complemented by the capsid protein of the virus. In an embodiment, the animal is a mammal, e.g., a human. In an embodiment, the plasmid is any of the plasmids of the invention, as described in greater detail herein.

10 **BRIEF DESCRIPTION OF THE DRAWINGS**

This invention will be described with reference to the drawings in which:

Figure 1 is a schematic diagram of a recombinant vector designated rHBV complemented by a plasmid expressing HBc to form a pseudo-virus of the invention containing foreign epitopes, which can stimulate CD4⁺ and CD8⁺ responses to hepatocytes
15 infected by the pseudo-virus.

Figure 2 depicts a construct designated prHBV-1.3-IV containing a polytope (polyepitope), as well as the construct designated polytope IV.

Figures 3A-3C depict the rescue of rHBV DNA from a human hepatocytic cell line co-transfected with prHBV 1.3-III and a plasmid expressing the capsid (PMAS core).

20 Figure 3A: HBV DNA was PCR amplified.

Figure 3B: depicts the production of HBsAg carrying particles in the supernatant from these cells.

Figure 3C: depicts LHBs-virion rescue. This is also depicted in Figure 12D.

25 Figure 4 shows the detection by Western blot of the recombinant antigen rHBe after transient transfection in HepG2 cell line (ATCC number HB-8065).

Figures 5A-5B show the detection by immunofluorescence staining with antibodies of the recombinant antigen rHBe after transient transfection in the HepG2 cell line. This is also shown in Figure 12B.

5 Figures 6A-6D show immunostaining of the foreign recombinant antigen in liver sections after hydrodynamic injection of prHBV1.3-III into mice (ATCC number HB-8065). This is also shown in Figure 12C.

Figure 7 shows cellular responses to the polyepitope in the rHBV genome of HLA-A2/DR1 Tg mice, detected by ELISPOT assay (left panel) and by a proliferation assay (right panel; this is also shown in Figure 14C).

10 Figure 8 shows the immune response of HLA-A2/DRB1 Tg mice immunized with pCMV-rHBe-IV. The left panel shows the immunodominant response to an epitope derived from influenza matrix protein. This is also shown in Figure 14A. The right panel shows the detection in spleen cells of Flu-specific CD8⁺ T cells labeled with HLA-A2 tetramers carrying the Flu epitope in HLA-A2/DRB1 non-immunized (upper
15 right) or immunized mice (lower right). This is also shown in Figure 14B.

Figure 9 shows the T cell response after hydrodynamic injection of prHBV-1.3III through the tail vein of the mouse (ELISPOT assay).

Figures 10A-10C show the T-cell responses to a polyepitope in vivo and the localization of Flu-specific T cells in the liver of mice after hydrodynamic injection of
20 prHBV1.3. This is also shown in Figures 15A-15D.

Figure 10A: Immunization time-line; prHBV1.3 (dotted square), pCMV- β Gal (control plasmid, empty square), pCMV-rHBe (grey square).

Figure 10B: Fluorescence Activated Cell Sorting (FACS) analysis of liver infiltrating lymphocytes; non-injected mice (panel B1), mice receiving pCMV- β Gal (panel B2),
25 mice receiving prHBV1.3 (panel B3); lower panels show Flu-specific T cells staining for each treatment.

Figure 10C: Localization of T cells and Flu-specific T cells in spleen (upper panel) and liver (lower panel); mice receiving hydrodynamic injection of prHBV1.3 (dotted bars), mice receiving pCMV- β Gal (empty bars), mice receiving pCMV-rHBe (grey bars).

30 Figures 11A-11D show a schematic representation of rHBV constructs and plasmids.

Figure 11 A: HBV pregenomic RNA (HBV/pgRNA) is represented by a thin line with a capping site (cap), encapsidation (ϵ), and polyadenylation (A_n) signals indicated. The distance between AUG codons of the core and polymerase (pol) open reading frames is 406 nucleotides (nts).

5 Figure 11B: rHBV pregenomic RNA is shown; a short DNA sequence encoding the foreign antigenic polyepitope was inserted in-frame within the core open reading frame, allowing the expression of a chimeric protein (rHBc).

Figure 11C: A schematic representation of the rHBc-encoding domain shows two in-frame ATG codons for the expression of HBe antigen and for core protein, respectively.

10 A polyepitope comprising a B cell epitope (FLAG) used as a detection marker; three HLA-A2 restricted CD8 T-cell epitopes derived respectively from HIV Gag, influenza matrix, and EBV BMLF-1 proteins; and a universal CD4- T cell epitope PADRE. The polyepitope sequences were inserted in-frame within the amino terminal portion of the core gene.

Figure 11D: A schematic representation of plasmids is shown. The pCMVrHBc plasmid allows expression of rHBc as well as the rHBV genome under the control of CMV early gene promoter (P-CMV). The prHBV1.3 plasmid carries 1.3 copies of the rHBV genome. The prHBV1.3/HBc plasmid carries, in addition to the 1.3 copies of rHBV genome, a cassette for the expression of wild type HBV core gene, under the control of an SV40 early gene promoter (P-SV40), and uses one or more bovine growth hormone gene-derived polyadenylation signal (BGH pA). Positions of core ORFs with the inserted polyepitope-encoding sequence is indicated by arrows. Nucleotide positions are indicated according to the sequence of the HBV genotype D ayw subtype. Position 0 corresponds to the *EcoRI* site and position 1981 corresponds to the 3'-end of the polyadenylation signal for mRNA in the HBV genome.

25 Figures 12A-12D show the expression of rHBV and chimeric antigenic protein. Figure 12A: Western blot analysis of cell lysate obtained after transfection of the HepG2 cell line (ATCC number HB-8065) with pFLAG-PCNA or prHBV1.3 plasmids. The molecular weights of proliferating cell nuclear antigen (PCNA) fused with Flag (lane 1; control) and rHBc (lane 2) are estimated according to molecular weight markers (kilodaltons (kDa)).

Figure 12B: Immunofluorescence staining of HepG2 cells transfected with prHBV1.3 plasmid using anti-HBs (upper panel) or anti-FLAG antibodies (lower panel).

Figure 12C: Antibody labeling (anti-HBs, left panel and anti-FLAG, right panel) and immunofluorescence staining on liver sections taken from mice (ATCC number HB- 8065) four days following hydrodynamic injection of prHBV1.3.

Figure 12D: Quantification of HBsAg particles containing HBV-L protein by sandwich ELISA. PreS1-specific monoclonal antibodies (5a91 and 18-7) were used as capture antibodies to detect L protein in culture supernatants of Huh-7 cells transfected with two different ratios of prHBV1.3 (rHBV) and pMAS-C (CORE) plasmids or with a control plasmid pIRES-EGFP (EGFP). Results are expressed as optical densities (OD) at 450 nm, by ELISA.

Figures 13A-13D show the encapsidation rHBV genome by wild type core protein.

Figure 13A: Viral DNA detected by Southern blot assay with an HBV-specific probe in the cell culture supernatants of Huh 7 cells transfected with two different concentrations of prHBV1.3 (lanes 1, 3) or prHBV1.3/HBc plasmids (lanes 2, 4). Wild type HBV DNA was extracted from the HepAD38 cell line as a control (lane 5). Bands corresponding to relaxed circular (RC), double-stranded linear (DSL) and single-stranded (SS) HBV DNA are indicated. M: molecular weight markers (Kb). HBc + indicates expression of capsid protein by the vector used in transfection experiments.

Figure 13B: ELISA assay results comparing HbsAg (left) and LHBsAg (right) production in culture medium (from day 3-5) of Huh 7 cells transfected with either prHBV1.3 (empty columns) or prHBV1.3/HBc (grey columns). HBsAg (ng/ml) was quantified by MonolisaTM detection kit (Bio-Rad, Hercules, CA). LHBsAg production was expressed as optical densities (OD) at 450 nm.

Figure 13C: Southern blot assay of the viral DNA in Huh 7 cells 3 days after cotransfection with payw 1.2 and prHBV 1.3. The concentrations of each are shown in the table below the blot.

Figure 13D: Detection of viral DNA by PCR in sera of C57/BL6 mice four days after hydrodynamic injection of prHBV1.3 with either pMAS-C (lanes 1-3) or pCMV-bGal (lanes 4-6). pFC80 plasmid was used as a positive control (lane 7).

Figures 14A-14C show polyepitope-specific T-cell responses in mice injected with pCMV-rHBe.

Figure 14A: ELISpot assay performed on splenocytes from ten HLA-A2/DR1 transgenic mice taken 15 days after one intramuscular injection of pCMV-rHBe. Each bar represents the number of IFN- γ -secreting T cells per million splenocytes for each individual mouse. Peptides used to stimulate splenocytes *ex vivo* are derived from HIV gag (HIV-G), Influenza matrix (Flu-M), EBV-BMLF1 (EBV-B), HBV capsid (HBc/18-27), and HBV envelope (HBs) proteins. PADRE is a promiscuous HLA-class II-binding peptide.

Figure 14B: FACS analysis of Flu-specific T cells from a non-immunized mouse (left panel) and from a representative HLA-A2/DR1 transgenic mouse (right panel) taken 15 days after one intramuscular injection of pCMV-rHBe. Spleen cells were stained with an APC-labeled anti-CD8 antibody and a HLA-A2-pentamer carrying the Flu peptide. Flu-specific T cells represent around 10% of CD8 T cells from the spleen (circle).

Figure 14C: Proliferative response of splenocytes from pCMV-rHBc-immunized HLA-A2/DR1 transgenic mice following *in vitro* stimulation with PADRE peptide. Responses are expressed as the proliferation index. The dotted line corresponds to the mean value of the stimulation index. SI>2 is considered positive.

Figures 15A-15D show T cell responses in the liver and spleen of HLA-A2/DR1 transgenic mice, following hydrodynamic injection of rHBV.

Figure 15A: The protocol for active immunization includes priming of T cell responses at day 0 (D0) by intramuscular injection of pCMV-rHBc. At day 15 (D15), mice were injected via the hydrodynamic route with either prHBV1.3 or control pCMV- β Gal plasmid. Lymphocytes from spleen and liver were collected at day 22 (D22) for FACS analysis.

Figure 15B: FACS analysis of intrahepatic lymphocytes stained with anti-CD3-PerCP and anti-CD8-APC antibodies (upper panels), and with PE-labeled Flu-specific tetramer and APC-labeled anti-CD8 (lower panels). Lymphocytes were prepared from non-immunized mice (B1, left panels), mice receiving pCMVrHBc priming/pCMV β Gal hydrodynamic injection (B2, middle panels), and mice receiving pCMVrHBc priming/prHBV1.3 hydrodynamic injection (B3, right panels). The percentages of CD8⁺ (circle) and CD4⁺ (square) T cells among the splenocytes are indicated.

Figure 15C: Analysis of intrahepatic lymphocytes from three groups of mice. The first group received pCMVrHBc priming/pCMV β Gal hydrodynamic injection (empty bars, n=5); the second group received pCMVrHBc priming/prHBV1.3 hydrodynamic injection (dotted bars, n=6); the third group were injected twice with pCMVrHBc via the intramuscular route (grey bars, n=3). Results are given as the mean \pm SEM percentage of CD8⁺, CD4⁺ and Flu-specific T cells in the total lymphocyte population.

Figure 15D: Analysis of the intrasplenic lymphocytes from the mice described in Figure 15C.

Figures 16A-16F show an analysis of liver-infiltrating lymphocytes.

Figure 16A: Histological analysis of liver sections taken four days after hydrodynamic injection. Hematoxylin/eosin staining of liver sections from a representative HLA-A2/DR1 mouse receiving pCMVrHBc priming/pCMV β Gal hydrodynamic injection (left panel, 100x); and from a representative mouse receiving pCMVrHBc priming/prHBV1.3 hydrodynamic injection (middle panel, 100x). The right panel shows an area from the middle panel at a magnification of 200x. Cell clusters of inflammatory foci are indicated with frames. Arrows indicate cells undergoing degeneration.

Figure 16B: Phenotype of intrahepatic lymphocytes taken from a representative mouse after pCMVrHBc priming/prHBV1.3 hydrodynamic injection. CD8⁺ T cells were gated for analysis of Flu⁺ or Flu negative cells following Flu-pentamer labeling (left panel). Quantification of CD69⁺ and CD62L⁺ cells was done on pentamer positive (middle panel) and pentamer negative (right panel) CD8 T cells.

Figure 16C: Functional profile of CD3⁺ CD8⁺ intrahepatic T cells. Cells were analyzed for CD107, a surface marker, intracellular INF γ , and TNF α , following *ex vivo* stimulation with polyepitope-derived peptides (mix of three) (lower panel) or without stimulation (upper panel).

Figure 16D: Immunostaining of HBsAg on liver sections taken four days after prHBV1.3 hydrodynamic injection from mice that were either primed by pCMVrHBc intramuscular injection (left panel) or unprimed (right panel) before hydrodynamic injection of prHBV1.3.

Figure 16E: Mean level of HBsAg (ng/ml) in the sera of the mice of Figure 16D with priming (empty columns) or without priming (grey columns), before hydrodynamic injection of prHBV1.3.

Figure 16F: Mean level of transaminase (ALT mU/ml) in the sera of the mice following pCMV-rHBc priming/prHBV1.3 hydrodynamic injection (grey column, n=11), and of mice with pCMV-rHBc primin/pCMV- β Gal hydrodynamic injection (empty column, n=4). Concanavalin A (ConA) injection was used as a positive control for ALT
5 increase.

Figures 17A-17C show the control of HBsAg expression in HBsAg/HLA-A2 transgenic mice.

Figure 17A: Protocol for active immunization in HBsAg/HLA-A2 transgenic mice.

Figure 17B: Decrease in HBsAg in the sera of individual HBsAg/HLA-A2 transgenic
10 mice after priming by intramuscular injection of pCMV-rHBc at week 0 (W0), followed by prHBV1.3 hydrodynamic injection two weeks later (W2). Mice were bled weekly and HBsAg (ng/ml) was quantified using a commercial ELISA.

Figure 17C: The percentage of HBsAg decrease over eight weeks in the sera of mice receiving prHBV1.3 (filled bars) or pCMV- β Gal (empty bars). HBsAg concentration at
15 week 0 was set at 100% and the results are expressed as mean \pm SEM.

Figure 18 shows sequence for prHBV-1.3-III (SEQ ID NO: 4) (polyepitope (polytope) sequence shadowed (SEQ ID NO: 5)).

Figure 19 shows sequence for rHBe_III_ (SEQ ID NO: 6) (the recombinant protein; polyepitope (polytope) sequence shadowed (SEQ ID NO: 7)).

20 Figure 20 shows sequence for polytope III (SEQ ID NO: 8) (amino acids in small letters are flanking residues).

Figure 21 shows sequence for polytope IV (SEQ ID NO: 9) (amino acids in small letters are flanking residues).

Figure 22 shows sequence for prHBV-1.3-IV (SEQ ID NO: 10) (polyepitope
25 (polytope) sequence shadowed (SEQ ID NO: 11)).

Figure 23 shows sequence for rHBe_IV_ (SEQ ID NO: 12) (the recombinant protein; polyepitope (polytope) sequence shadowed (SEQ ID NO: 13)).

Figure 24 shows NCBI Accession Number: V01460 Hepatitis B virus (strain ayw) genome, DNA sequence (ACCESSION V01460 J02203 VERSION V01460.1
30 GI:62276, VRL 28-JAN-2003) (SEQ ID NO: 14).

DETAILED DESCRIPTION OF THE INVENTION

Hepadnaviruses are small, enveloped hepatotropic DNA viruses. The prototype member of this family is the human hepatitis B virus (HBV). The hepadnaviral genome consists of a partially double-stranded, relaxed circular DNA, which has a compact
5 organization employing widely overlapping open reading frames and regulatory sequences. HBV genome is precisely-organized by various cis- or trans- elements that are overlapping each other.

By investigation of the viral genome, it was found that it might accommodate a piece of foreign sequence in the N-terminal part of the capsid-coding region to create a
10 pseudo-virus that would maintain in hepatocytes, while the interrupted protein would be complemented in *trans* by wild type HBV during natural infection. (Gunther S., Piwon N., Jung A., Iwanska A., Schmitz H., Will H., 2000, Enhanced replication contributes to enrichment of hepatitis B virus with a deletion in the core gene, *Virology* 273:286-99). Thus, recombinant HBV would act as a targeting vector with liver-tropism for gene
15 delivery (see Figure 1).

Thus, this invention provides a vector derived from the hepatitis virus genome. The hepatitis virus genome was modified in order to express foreign epitopes fused within the N-terminal part of the hepatitis virus capsid protein. The resulting virus is replication defective. This recombinant, replication defective, hepatitis virus co-
20 maintains in the liver of hepatitis virus-infected individuals through wild type capsid complementation. Infection with this recombinant virus leads to expression and presentation of foreign epitopes at the surface of hepatocytes. These epitopes presented in association with MHC molecules are the target of the host T-cell immune response. Therefore, only hepatitis virus-infected hepatocytes that allow replication of the
25 recombinant hepatitis virus vector will be the target of the immune response and cured. The invention exploits the pre-existing immunity against foreign epitopes, for example from common pathogens, such as Flu. Hepatitis virus is cleared through cytolytic or non-cytolytic mechanisms.

In patients, viral clearance can be quantified by measuring HBV DNA in sera
30 using commercially available kits. Clearance in the liver can be quantified by measuring DNA in biopsy. Sero-conversion from HBeAg, which is a marker of viral replication, to anti-HBe antibodies can be used as a marker for viral elimination. Elimination of

HBsAg from the sera and seroconversion to anti-HBs antibodies indicates the complete elimination of the virus.

This invention also provides a recombinant, replication competent hepatitis pseudo-virus based on the replication defective hepatitis virus of the invention. The
5 pseudo-virus comprises the recombinant, replication defective, hepatitis virus of the invention complemented by HBc, wherein the pseudo-virus replicates in vitro in human hepatocytes.

More particularly, this invention provides a recombinant, replication defective, hepatitis virus comprising a hepatitis virus genome defective for the expression of
10 hepatitis virus capsid protein (HBc), wherein the virus contains a nucleotide sequence of up to about 195 nucleotides encoding at least one immuno-dominant epitope of a pathogen, wherein the nucleotide sequence is located between nucleotide residue 1981 and nucleotide residue 2308 of the HBV ayw3 genome (numbering starts from the fourth nucleotide in the EcoRI site of the HBV genome, NCBI Accession No. V01460).
15 This virus is referred to herein as “the recombinant, replication defective, hepatitis virus” of the invention.

The recombinant, replication defective, hepatitis virus of the invention is complemented in the infected cell by expression of HBc by the hepatitis virus infecting cell. The genome of the replication incompetent virus is completely enclosed within the
20 capsid produced by the wild hepatitis virus infecting the cell. The complemented virus is referred to herein as “the hepatitis pseudo-virus”.

This invention thus provides polynucleotides and expression vectors for the production of proteins, which assemble into pseudo-virus, and which are efficiently produced in host cells. It is thus possible to make self-assembling, recombinant, pseudo-
25 virus with residues of a foreign peptide. This provides efficient monovalent, bivalent, and multivalent immunogenic compositions and therapeutic vaccines.

While the replication defective hepatitis virus and the hepatitis-pseudo-virus of the invention will be described in detail with reference to HBV, it will be understood that this invention is applicable to other hepatitis viruses, including Hepatitis A Virus
30 (HAV) (*Picornavirus*); Hepatitis Delta Virus (HDV) (*Deltavirus*); Hepatitis C Virus (HCV) (*Flavivirus*); and Hepatitis E Virus (HEV). Thus, as used herein, the term “hepatitis” includes hepatitis B and other hepatitis viruses. Hepatitis B is the preferred

virus for use in practicing this invention. More generally, the invention is applicable to other viruses.

In practicing the invention using other hepatotropic viruses, such as HCV, and more generally all viruses involved in persistent infection, the viral genome can be examined to identify an appropriate site for the insertion of foreign epitopes. For example, due to overlapping open reading frames encoding the structural and non-structural viral proteins of HBV, the only gene that can be targeted for epitope insertion is the nucleocapsid-encoding gene. However, other RNA or DNA viruses have different tolerances for genomic insertions. In addition, different viruses infect different tissues that can be targeted and destroyed by the induced T cell responses, without damaging other non-infected tissues.

The term "peptide" is generally understood in the art to refer to a small amino acid molecule, whereas the term "polypeptide" is generally understood to refer to a larger amino acid molecule. Both peptides and polypeptides are within the scope of this invention. Thus, for example, the foreign sequences can be either a peptide or a polypeptide. The terms are used interchangeably herein.

In one aspect, the invention provides hepatitis pseudo-viruses comprising epitope-bearing portions of foreign peptide(s) or polypeptide(s). As used herein, the terms foreign peptides and polypeptides or epitopes means a peptide or polypeptide or an epitope not found in wild-type hepatitis virus.

The epitopes are immunogenic or antigenic epitopes of the foreign peptides or polypeptides. An "immunogenic epitope" is defined as a part of a protein that elicits a humoral or cellular response *in vivo* when the whole polypeptide, or fragment thereof, is the immunogen. A region of a polypeptide to which an antibody can bind is defined as an "antigenic determinant" or "antigenic epitope". The antigenic epitope can also elicit a humoral or cellular response *in vivo* when employed in the hepatitis pseudo-virus. Thus, included in the present invention are hepatitis pseudo-viruses containing both immunogenic epitopes and antigenic epitopes, or either one of them. Foreign peptides or polypeptides comprising immunogenic or antigenic epitopes are at least 8 amino acids residues in length for epitopes binding to MHC class I molecules, and at least 12 amino acids in length for epitopes binding to MHC class II molecules (cellular response). B cell (humoral response) epitopes are at least four amino acids in length.

In some embodiments, the foreign peptide or polypeptide can contain from about 8 to about 140 amino acid residues, preferably from about 20 to about 140 amino acid residues, especially from about 60 to about 140 amino acid residues. In the case of HBV, it can contain up to about 68 amino acids. In one embodiment, in the case of
5 HBV, the foreign peptide or polypeptide contains 68 amino acids. In another embodiment, it contains about 65 amino acids. Flanking residues on either the N-terminal, C-terminal, or both N- and C-terminal ends may be added to the foreign peptide or polypeptide to generate the hepatitis pseudo-viruses.

The foreign peptide or polypeptide can also be derived from any number of
10 foreign proteins. The foreign peptide or polypeptide can be derived from any protein of any plant, animal, bacterial, viral, or parasitic organism.

In one embodiment, the foreign peptide or polypeptide can be derived from a polypeptide of a pathogen. The term "pathogen" as used herein, means a specific causative agent of disease, and may include, for example, any bacteria, virus, or
15 parasite.

The term "disease" as used herein, means an interruption, cessation, or disorder of body function, system, or organ. Typical diseases include infectious diseases. For example, the foreign peptide or polypeptide can be from the immunogenic proteins of an RNA virus, such as HIV-1, HIV-2, SIV, HCV, Ebola virus, Marburg virus, HTLV-I,
20 and HTLV-II. Specific examples are the structural or NS1 proteins of Dengue virus; the G1, G2, or N proteins of Hantaan virus; the HA proteins of Influenza A virus; the Env proteins of Friend murine leukemia virus; the Env proteins of HTLV-1 virus; the preM, E, NS1, or NS2A proteins of Japanese encephalitis virus; the N or G proteins of Lassa virus; the G or NP proteins of lymphocytic choriomeningitis virus; the HA or F proteins
25 of measles virus; the F or HN proteins of parainfluenza 3 virus; the F or HN proteins of parainfluenza SV5 virus; the G proteins of Rabies virus; the F or G proteins of respiratory syncytial virus; the HA or F proteins of Rinderpest; or the G proteins of vesicular stomatitis virus. These are just some of the possibilities and do not represent an exhaustive or restricted list.

30 The foreign peptide or polypeptide can also be derived from the immunogenic proteins of a DNA virus, such as gp89 of cytomegalovirus; gp340 of Epstein-Barr; gp13 or 14 of equine herpesvirus; gB of herpes simplex 1; gD of Herpes simplex 1; gD of

herpes simplex 2; or gp50 of pseudorabies. Once again, these are just some of the possibilities and do not represent an exhaustive or restricted list.

Further, the foreign peptide or polypeptide can be derived from the immunogenic proteins of bacteria, such as Streptococci A M6 antigens, or tumor
 5 antigens, such as human melanoma p97, rat Neu oncogene p185, human epithelial tumor ETA, or human papilloma virus antigens. Again, these do not comprise an exhaustive or restricted list.

In an embodiment of this invention, the foreign peptide or polypeptide is derived from a human immunodeficiency virus. Following are HIV-1 epitopes that can be
 10 employed in designing the foreign peptide or polypeptide.

	GAG	P17 (77-85)	SLYNTVATL (S9L)
		P24(19-27)	TLNAWVKW (T9V)
	POL	(79-88)	LLDTGADDTV (L10V)
		(263-273)	VLDVGDAYFSV (V11V)
15		(334-342)	VIYQYMDDL (V9L)
		(464-472)	ILKEPVHGV (I9V)
		(576-584)	PLVKLWYQL (P9L)
		(669-679)	ESELVNQIIEQ (E11Q)
		(671-680)	ELVNQIIEQL (E10L)
20		(956-964)	LLWKGEGAV (L9V)
	ENV	Gp41 (260-268)	RLRDLLLIV (R9V)
	NEF	(188-196)	AFHHVAREL (A9L)

Numbering is based on the amino acid sequence of the HIV-1 WEAU clone 1.60
 25 (Genbank accession No. U21135). The WEAU sequence may not be always identical to that of the reactive peptide and simply indicates its location in the viral proteins.

The foreign peptide or polypeptide can comprise one epitope or a multiplicity of epitopes linked to each other. In addition, it will be understood that the hepatitis pseudo-
 virus of the invention can contain multiple epitopes of one or more origins, such as
 30 epitopes from different immunogenic proteins of the same pathogen. It will also be understood that the hepatitis pseudo-virus can contain one or more epitopes from different origins, such as epitopes from different pathogens. In addition, mixtures of

hepatitis pseudo-viruses having different epitopes in different particles are contemplated by this invention.

The proteins containing the foreign sequence can be exposed on the surface of cells infected with the wild-type virus. The resulting exposed epitopes provide excellent
5 configurational mimics of the epitopes as they exist, for example, in pathogens, such as other infectious viruses. For these reasons, the virus-infected cells are suitable for exploitation as carriers for foreign peptides or polypeptides, such as protective determinants of etiologic agents, via the replication defective, hepatitis virus and the resulting hepatitis pseudo-virus of the invention.

10 Recombinant expression vectors containing a nucleic acid encoding proteins of the pseudo-viruses of the invention can be prepared using well known methods. The expression vectors include the sequence encoding the foreign peptide or polypeptide operably linked to suitable transcriptional or translational regulatory nucleotide sequences, such as those derived from a mammalian, viral, or insect gene. A
15 transcriptional or translational regulatory nucleotide sequence is operably linked if the nucleotide sequence controls the transcription or translation of another coding DNA sequence. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, an mRNA ribosomal binding site, tissue specific promoters and post-transcriptional regulatory elements (PRE), and appropriate sequences that control
20 transcription and translation initiation or termination. The ability to replicate in the desired host cells, usually conferred by an origin of replication, and a selection gene by which transformants are identified, may additionally be incorporated into the expression vector.

Among eukaryotic vectors for use in the preparation of vectors of the invention
25 are pWLNEO, pSV2CAT, pOG44, pXT1, and pSG available from Stratagene (La Jolla, CA); and pSVK3, pBPV, pMSG, and pSVL available from Pharmacia (Piscataway, NJ). Other suitable vectors will be readily apparent to the skilled artisan.

Among vectors for use in the preparation of vector of the invention, non-integrative eucaryotic vectors are not only useful, but integrative/transformant vectors
30 (i.e. vectors that integrate a part of their nucleic acid material in the genome of the eukaryotic host cell) can also be employed. Typical of these vectors are lentiviral vector Trips, adenovirus, and yeast integrative vectors.

In a preferred embodiment, the expression vectors of the invention include at least one selectable marker. Such markers include, for example, dihydrofolate reductase, G418, ampicillin or neomycin resistance for eukaryotic cell culture.

Any strong promoter known to those skilled in the art can be used for driving
5 expression. Suitable promoters include adenoviral promoters, such as the adenoviral major late promoter; heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase
10 promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs; the β -actin promoter; and human growth hormone promoters. The promoter also can be a native promoter from a hepatitis virus, such as HBV.

In vivo delivery of the recombinant viruses of the invention requires production of viral stocks. In the case of hepatitis virus, this can be achieved by using a hepatocytic
15 cell line expressing wild type hepatic virus capsid. A method for the production of viral stocks is described in Günther S. et al., *Virology*, 273:286-99 (2000).

Suitable host cells for expression of pseudo-virus include higher eukaryotic cells. For example, in the case of hepatitis virus, differentiated hepatocytes are required for HBV, HCV, and HDV replication. Appropriate cloning and expression vectors for
20 use with plant, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al. *Cloning Vectors: A Laboratory Manual*, Elsevier, New York, (1985). Representative examples of appropriate hosts include, but are not limited to, fungal cells, such as yeast cells; insect cells, such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells, such as CHO, COS, 293, and Bowes melanoma cells; and plant cells.
25 Appropriate culture mediums and conditions for the above-described host cells are known in the art.

Introduction of the vector of the invention into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection, or other methods. Such
30 methods are described in many standard laboratory manuals, such as Davis et al., *Basic Methods In Molecular Biology* (1986).

In another aspect, the invention is directed to an *in vitro* method for producing pseudo virus, comprising culturing *in vitro*, in a suitable culture medium, a cell incorporating an expression vector of the invention and collecting in the culture medium pseudo-virus produced by these cells.

5 Therefore, the invention is also concerned with cells, such as recombinant eucaryotic cells, infected, transformed, or transfected by a polynucleotide or vector of the invention for expressing the pseudo-virus. Methods for producing such cells and methods for using these cells in the production of recombinant viruses are well known in the art. The pseudo-virus can be recovered and purified from recombinant cell
10 cultures by well-known methods, including ammonium sulfate precipitation, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography, and lectin or heparin chromatography.

While this invention relates to hepatitis pseudo-virus carrying one or more
15 (poly)epitopes of foreign peptides or polypeptides, this invention contemplates the use of (poly)epitopes that have been optimized for incorporation in hepatitis pseudo-virus. The (poly)epitope nucleic and amino acid sequences can be modified in view of increasing the overall hydrophilicity of the (poly)epitope and ensuring a modified processing of epitopes. Epitopes in a polyepitope can be permuted in order to obtain
20 the best hydrophilic profile. Hydrophilic spacers can be added to counterbalance the generally hydrophobic epitopes.

The polypeptides or polynucleotides of this invention can be in isolated or purified form. The terms "isolated" or "purified", as used in the context of this specification to define purity, means that the protein, polypeptide, or polynucleotide is
25 substantially free of other proteins of natural or endogenous origin and contains less than about 1% by mass of protein or polynucleotide, of other contaminants residual of production processes.

In practicing the method of the invention, the replication defective virus is administered to a host using one of the modes of administration commonly employed
30 for administering drugs to humans and other animals. Thus, for example, the replication defective virus can be administered to the host by the oral route or parenterally, such as by intravenous or intramuscular injection. Other modes of administration can also be

employed, such as intrasplenic, intrahepatic, perfusion, intradermal, and mucosal routes. Preferably, the replication defective virus of the invention is administered according to the natural route of infection of the virus. For purposes of injection, the replication defective virus as described above can be prepared in the form of solutions, suspensions, or emulsions in vehicles conventionally employed for this purpose.

Accordingly, the invention contemplates compositions comprising the recombinant replication defective virus of the invention in combination with a pharmaceutically acceptable carrier. The invention also contemplates a vaccine comprising such compositions. The vaccines of the invention may be administered to a patient persistently infected with a virus in order to stimulate a T cell response against cells infected with the virus. Thus, the invention also contemplates the use of the recombinant replication defective viruses of the invention for the preparation of a medicament for treating a patient persistently infected with the wild type virus. The invention also contemplates a method for targeting the expression of an epitope in a cell infected with a virus by providing to the cell the recombinant replication defective virus of the invention.

It will be understood that the replication defective viruses of the invention can be used in combination with other microorganism antigens, antibodies, or mitogens or other prophylactic or therapeutic substances. For example, mixtures of different parasite antigens, antibodies, or mitogens or mixtures of different viral or bacterial antigens, antibodies, or mitogens can be employed in the method of the invention. Similarly, mixtures of different replication defective viruses can be employed in the same composition. The replication defective viruses can also be combined with other vaccinating agents, such as immunodominant, immunopathological, and immunoprotective epitope-based vaccines, or inactivated attenuated or subunit vaccines.

The replication defective viruses of the invention are employed in an effective amount sufficient to provide an adequate concentration to clear virus in infected cells. The amount of the replication defective hepatitis viruses thus depends upon absorption, distribution, and clearance by the host. Of course, the effectiveness of the replication defective hepatitis viruses is dose related. The dosage of the replication defective viruses should be sufficient to produce a minimal detectable effect, but the dosage

preferably should be less than the dose that activates a non-specific polyclonal lymphocyte response.

The dosage of the replication defective viruses of the invention administered to the host can be varied over wide limits. The viruses can be administered in the
5 minimum quantity, which is therapeutically effective, and the dosage can be increased as desired up the maximum dosage tolerated by the patient. The replication defective viruses can be administered as a relatively high amount, followed by lower maintenance dose, or the viruses can be administered in uniform dosages.

The dosage and the frequency of administration will vary with the replication
10 defective viruses employed in the method of the invention. The amount administered to a human can vary from about 50 ng per Kg of body weight to about 1 µg per Kg of body weight, preferably about 100 ng per Kg of body weight to about 500 ng per Kg of body weight. For chimpanzee infection, 2×10^7 to 5×10^7 HBV genome equivalents (which corresponds to about 35-90 pg DNA) are required (Guidotti L.G., et al., Science,
15 284:825-29 (1999)) This corresponds to 0.7 to 1.8 pg/Kg of body weight. Optimum amounts can be determined with a minimum of experimentation using conventional dose-response analytical techniques or by scaling up from studies based on animal models of disease.

The term "about" as used herein in describing dosage ranges means an amount
20 that has the same effect as the numerically stated amount as indicated by clearance of chronic viral infection in the host to which the replication defective viruses are administered, with an absence or reduction in the host of determinants of pathogenicity, including an absence or reduction in persistence of the infectious virus *in vivo*, and/or the absence of pathogenesis and clinical disease, or diminished severity thereof, as
25 compared to individuals not treated by the method of the invention.

The dose of the replication defective viruses of the invention is specified in relation to an adult of average size. Thus, it will be understood that the dosage can be adjusted by 20-25% for patients with a lighter or heavier build. Similarly, the dosage for a child can be adjusted using well known dosage calculation formulas.

30 The replication defective viruses of the invention can be used in therapy in the form of pills, tablets, lozenges, troches, capsules, suppositories, injectable in ingestible

solutions, and the like in the treatment of hepatitis infection in humans and susceptible non-human primates and other vertebrate animals and mammals.

Appropriate pharmaceutically acceptable carriers, diluents, and adjuvants can be combined with the replication defective viruses described herein in order to prepare the pharmaceutical compositions for use in the treatment of pathological conditions in animals. The pharmaceutical compositions of this invention contain the replication defective viruses together with a solid or liquid pharmaceutically acceptable nontoxic carrier. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable, or synthetic origin. Examples of suitable liquids are peanut oil, soybean oil, mineral oil, sesame oil, and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Physiological solutions can also be employed as liquid carriers, particularly for injectable solutions.

Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium carbonate, magnesium stearate, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol, and the like. These compositions can take the form of solutions, suspensions, tablets, pills, capsules, powders, sustained-release formulations and the like. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. The pharmaceutical compositions contain an effective therapeutic amount of the replication defective virus of the invention together with a suitable amount of carrier so as to provide the form for proper administration to the host.

The ability of the replication defective viruses of the invention to induce protection in a host can be enhanced by emulsification with an adjuvant, incorporation in a liposome, coupling to a suitable carrier, or by combinations of these techniques. For example, the replication defective viruses of the invention can be administered with a conventional adjuvant, such as aluminum phosphate and aluminum hydroxide gel. Similarly, the replication defective viruses can be bound to lipid membranes or incorporated in lipid membranes to form liposomes. The use of nonpyrogenic lipids free of nucleic acids and other extraneous matter can be employed for this purpose.

The host or patient can be an animal susceptible to infection by a virus, and is preferably a mammal. More preferably, the mammal is selected from the group consisting of a human, a dog, a cat, a bovine, a pig, and a horse. In an especially preferred embodiment, the mammal is a human.

5 Another aspect of the invention includes administering nucleic acids encoding the replication defective virus of the invention with or without carrier molecules to an individual. Those of skill in the art are cognizant of the concept, application, and effectiveness of nucleic acid vaccines (e.g., DNA vaccines) and nucleic acid vaccine technology as well as protein and polypeptide based technologies. The nucleic acid based technology allows the administration of
10 nucleic acids encoding replication defective virus of the invention, naked or encapsulated, directly to tissues and cells, especially muscle cells or keratinocytes, without the need for production of encoded proteins prior to administration. The technology is based on the ability of these nucleic acids to be taken up by cells of the recipient organism and expressed to produce a replication defective virus to which the recipient's immune system responds. Such nucleic acid vaccine
15 technology includes, but is not limited to, delivery of expression vectors encoding a replication defective virus of the invention. Although the technology is termed "vaccine" it is equally applicable to immunogenic compositions that do not result in a completely curative response. Such partial-protection-inducing compositions and methods are encompassed within the present invention.

20 The present invention also encompasses delivery of replication defective virus as part of larger or more complex compositions. Included among these delivery systems are viruses, virus-like particles, or bacteria containing the nucleic acids encoding the replication defective virus of the invention. Also, complexes of the invention's nucleic acids and carrier molecules with cell permeabilizing compounds, such as liposomes, are included within the scope of the invention.
25 Other compounds, such as molecular vectors (EP 696,191, Samain et al.) and delivery systems for nucleic acid vaccines are known to the skilled artisan and exemplified in, for example, WO 93/06223 and WO 90/11092, U.S. Patent No. 5,580,859, and U.S. Patent No. 5,589,466 (Vical patents) and can be made and used without undue or excessive experimentation.

During the replication cycle, HBV pregenomic RNA serves as the mRNA template for translation of the viral core and polymerase proteins. It is encapsidated together with the viral polymerase into a nucleocapsid consisting of around 200 subunits of the core protein. The viral envelope is densely packed with the large (L),
5 middle (M) and predominantly small (S) viral envelope proteins. In addition to envelope proteins, the virus encodes a regulatory protein (X), all translated from subgenomic RNAs.

HBV infects only human and chimpanzees. As alternative animal models, the invention provides HBV-or HBsAg transgenic mice, which replicate or express HBV
10 genes in the liver. Expression of the transgene from birth tolerizes dHBV-specific T cells responses in these animals.

The invention provides that hydrodynamic injection of rHBV to mimic gene expression in mouse livers circumvents the tolerization of dHBV-specific T cells response.

15 The invention also provides that, in HLA-transgenic mice, priming with a DNA vector encoding foreign HLA-A2 restricted epitopes activates T cell responses that subsequently localize to the liver of mice following hydrodynamic injection of recombinant HBV (rHBV). Since rHBV is expected to replicate only in hepatocytes bearing the wild type HBV and sharing the same mechanism in the viral cycle, the
20 strong immune responses elicited by the foreign polyepitope dominates over the exhausted T cell responses present during natural HBV persistent infection.

A key question in the development of immunotherapeutic strategies against hepatitis B chronic infection is whether HBV-specific T cells can be functionally restored using vaccination or other immunomodulatory approaches. It is also important
25 to assess whether vaccine or otherwise activated T cells can enter the liver and eliminate HBV-infected cells. In the current study, we used HBV as an immunotherapy vector to deliver a modified core protein fused with foreign immunogenic epitopes in liver cells. Following gene expression of this modified virus, functional epitope-specific T cells were attracted to liver in which they were able to control HBV gene expression in
30 hepatocytes.

An ideal vector for gene therapy can target abnormal cells without harming healthy neighboring cells. The invention provides an artificial rHBV, with the core gene

interrupted by insertion of a short sequence encoding immunodominant epitopes derived from common viruses. This modified virus was not competent for replication except in hepatocytes providing wild type viral capsids *in trans*. rHBV is, therefore, not expected to maintain in healthy hepatocytes, but only in cells from patients with chronic
5 HBV replication. Once the natural HBV infection is eliminated, the rHBV pseudo-viral life is subsequently interrupted.

The 3.2-Kb HBV genome is highly compacted, with overlapping open reading frames (ORF) for structural genes and various regulatory elements. It is technically difficult to engineer this virus, mainly because of space limitation. The present
10 invention provides that the foreign sequence in rHBV was inserted in a region located between polyadenylation signal for HBV mRNA and the start of the polymerase reading frame. No obvious *cis*-acting element has been found in this region that may participate to viral replication. However, a potential internal site for ribosome entry has been described. Thus, translation of the polymerase is a small probability event, with a
15 mechanism of ribosome shunting along the messenger RNA. HBV polymerase functions *in cis* to find the epsilon signal in pgRNA and to initiate the viral replication. Therefore, the size of foreign insertion should be compatible with the translation of HBV polymerase. Interestingly, the rHBV in our study is similar to a naturally occurring HBV variant (DC-144), identified by Will H et al during fulminant hepatitis.
20 This variant could produce 2- to 4.5-fold more progeny DNA than wild-type HBV when sufficiently complemented with wild-type core protein (Gunther S., Piwon N., Jung A., Iwanska A., Schmitz H., Will H., 2000, Enhanced replication contributes to enrichment of hepatitis B virus with a deletion in the core gene, *Virology* 273:286-99.) In addition, rHBV has a short viral genome that favors pgRNA packaging. It is thus expected that
25 rHBV could dominate the cccDNA pool in the cell nucleus, leading to the inhibition of wild type HBV replication.

Increasing evidence suggests that the host immune response plays a critical role in determining the various outcomes of HBV infection. In particular, HBV-specific CD8 T-cell responses are believed to be of considerable importance in viral control and
30 immune-mediated disease. However, during chronic infection, these responses are generally weak and narrowly focused. Virus-specific T cells from chronic patients rapidly become exhausted. T cell dysfunction has been attributed to high levels of

persisting viral antigens. But in chronic patients immune responses to other pathogens remain intact. We therefore thought to design a novel therapeutic approach based on activation of non-HBV specific T cells that were further redirected to liver following rHBV injection.

5 In summary, this invention provides a new approach to the design of a virus with a defective replication cycle, which can be rescued by wild type virus co-infection, and which expresses foreign antigenic epitopes that contribute to the elimination of virus infected cells and then to viral clearance. The vector of the invention, by expression of epitopes derived from common pathogens, by-passes existing tolerance of virus specific
10 T cell responses. The vector will only replicate in virus infected cells.

In a particular embodiment, this invention provides a new approach to the design of a hepatitis virus with a defective replication cycle, which can be rescued by wild type hepatitis virus co-infection, and which expresses foreign antigenic epitopes that contribute to the elimination of hepatitis virus infected hepatocytes and then to viral
15 clearance. The vector of the invention, by expression of epitopes derived from common pathogens, by-passes existing tolerance of hepatitis virus specific T cell responses. The vector will only replicate in hepatitis virus infected hepatocytes.

According to this invention, the foreign antigenic polyepitope can break or help to break the hepatitis immune tolerance. The induced T cell responses can silence,
20 specifically or nonspecifically, hepatitis genes through non-cytolytic mechanisms. The recombinant hepatitis virus carrying the polyepitope can be tested for replication in hepatocytes of UPA-SCID mice reconstituted with human liver cells.

E. coli strains carrying the following plasmids were deposited at the Collection Nationale de Cultures de Microorganismes (C.N.C.M.), 25, rue du Docteur Roux, F-
25 75724 Paris, Cedex 15, France, and assigned the following Accession Nos.:

	<u>Plasmid</u>	<u>Accession No.</u>
	prHVB1.3-IV	CNCM I-3833
	pCMVrHbc-IV	CNCM I-3834
	prHVB1.3-III	CNCM I-3832
30	pCMVrHBc	CNCM I-4077
	prHBV1.3	CNCM I-4078
	prHBV1.3/HBc	CNCM I-4079

With respect to ranges of values, the invention encompasses each intervening value between the upper and lower limits of the range to at least a tenth of the lower limit's unit, unless the context clearly indicates otherwise. Further, the invention encompasses any other stated intervening values. Moreover, the invention also
5 encompasses ranges including either or both of the upper and lower limits of the range, unless specifically excluded from the stated range.

Unless defined otherwise, the meanings of all technical and scientific terms used herein are those commonly understood by one of skill in the art to which this invention belongs. One of skill in the art will also appreciate that any methods and materials
10 similar or equivalent to those described herein can also be used to practice or test the invention.

It must be noted that, as used herein and in the appended claims, the singular forms "a", "or" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a subject polypeptide" includes a plurality
15 of such polypeptides and reference to "the agent" includes reference to one or more agents and equivalents thereof known to those skilled in the art, and so forth.

Further, all numbers expressing quantities of ingredients, reaction conditions, % purity, polypeptide and polynucleotide lengths, and so forth, used in the specification and claims, are modified by the term "about" unless otherwise indicated. Accordingly,
20 the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties of the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits, applying ordinary rounding techniques.
25 Nonetheless, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors from the standard deviation of its experimental measurement.

EXAMPLES

30 The examples, which are intended to be purely exemplary of the invention and should therefore not be considered to limit the invention in any way, also describe and detail aspects and embodiments of the invention discussed above. The examples are not

intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (for example, amounts, temperatures, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, 5 molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1: *In vitro* Assay for Recombinant HBV (rHBV) Replication

A polypeptide sequence was inserted into a 1.3 copy of HBV genome in the N- 10 terminal part of the original capsid-encoding fragment to create the recombinant HBV (prHBV-1.3 and its derivatives-III or -IV, see Figure 2). Co-transfection of prHBV1.3-III with the plasmid expressing capsid gene under a CMV promoter (pMAScore) to a liver cell line was carried out to initiate a replicating cycle mimicking the wild HBV replication *in vitro* (detection of cccDNA genome and other intermediate forms by 15 Southern blot assay). Results are presented in Figure 3A (detection of HBV genome in secreted viral particles by PCR).

The human hepatocytic cell line Huh7 (Nakabayashi H., Taketa K., Miyano K., Yamane T., Sato J., 1982, Growth of human hepatoma cell lines with differentiated functions in chemically defined medium, Cancer Res 42(9):3858-63) was transfected 20 with prHBV1.3-III with or without pMAS-core, with pMAS-core alone, or with pFC80 (positive control, plasmid containing two HBV genomes in tandem). Supernatants were collected and viral particles were precipitated using PEG 8000. After a DNase treatment to remove the residual plasmid DNA, particles were lysed and HBV genome was PCR amplified (PCR primers on N part of core gene and middle of HBs-encoding gene). 25 Alternatively, the primers may be on any part of the HBV genome, more particularly, before the S gene. The results are shown in Figure 3A. The expected fragment is detected only after cotransfection of prHBV1.3 and pMAS-core, but not after transfection of either pMAS-core or prHBV1.3 alone.

Hepatitis B surface antigen (HBsAg) was also detected in supernatant from cells 30 cotransfected with prHBV1.3 and pMAS-core or transfected with pFC80, but not after transfection of pMAScore alone (see Figure 3B).

To assess LHBs-virion rescue, two different ratios for co-transfection (1:1 and 1:2) and a control plasmid not expressing core but EGFP were used. After co-transfection of the prHBV1.3-III and pMAScore plasmids to the hepatocytic Huh 7 cell line, supernatants of transfected cells were collected. Viral particles containing the large
5 HBV envelope protein (LHBs) were quantified by a specific ELISA using two monoclonal antibodies specific for the large HBV envelope protein (MoAb 5a91 and MoAb 18-7) as capture antibodies and a labeled anti-HBs MoAb for detection. Results are shown in Figure 3C. This experiment shows that expression of core is required for efficient secretion of particles containing the LHBs in a dose-dependent way. LHBs is
10 known to be localized on the surface of 42 nm HBV Dane particles.

Example 2: *In vivo* Assay for rHBV Infection

An investigation is made to determine whether the *in vitro* produced rHBV virion could be infectious *in vivo*. Since there is no small animal model, a UPA
15 transgenic mouse with transplanted human liver tissue can be useful. (Morosan S., et al., 2006, Liver-stage development of *Plasmodium falciparum*, in a humanized mouse model, J Infect Dis, 193:996-1004). For infection, a small stock of infectious rHBV is required. This will be obtained by first creating a stable HepG2 (ATCC number HB-8065) cell line expressing the rHBV genome constitutively and second by transducing
20 this cell line with a lentiviral vector expressing the HBV core gene.

Example 3: Creation of a String of Immunodominant Epitopes

Based on the knowledge of HBV molecular biology, the space to accommodate a foreign sequence in HBV genome is limited to more or less 195 nucleotides. Due to
25 this space limitation, a short sequence encoding foreign immuno-dominant epitopes (polyepitope) is introduced into the recombinant HBV genome in order to induce a robust immune response. Regarding the polyepitope, it is arranged with 3 CD8⁺ T-cell epitopes combined with a promiscuous CD4⁺ T-cell epitope (PADRE), which could universally accommodate several prevalent MHC class II molecules. For the CD8⁺ T-
30 cell response, considering the clinical relevance, three well known HLA-A2-restricted epitopes derived from common human viruses (HIV gag, Influenza matrix, EBV BML-F1) are chosen. Additionally, a short B-cell epitope (FLAG) is introduced into the

foreign sequence as a convenient detection marker for molecular biology assays (see Figures 2 and 6A-6D). A HLA-A2-restricted capsid-derived epitope (core 18-27) is present in the N-terminal part of the capsid protein.

5 Example 4: Assessing Polyepitope Expression *In vitro*

The precore-core region of HBV encodes the hepatitis core antigen (HBcAg) that self assembles to form viral capsids, and a secreted polypeptide, the hepatitis e antigen (HBeAg). These two proteins are derived by alternative initiation of translation at two in frame codons (see Figure 2). The 16 Kd HBe protein is derived by proteolytic
10 cleavage from a precursor protein initiated at the first initiation codon. To assess expression of the polyepitope by the recombinant HBV, the hepatoma cell line HepG2 was transfected with prHBV1.3-IV and three days later cell lysate was collected for immunoblot analysis. Detection of recombinant rHBe protein using anti-Flag monoclonal antibody is shown in Figure 4.

15 Expression of the polyepitope carried by the recombinant virus was also detected after transfection of Huh7 cells with prHBV1.3 in immunofluorescence assays using anti-FLAG MoAb (see Figure 5, panel B). Expression of HBsAg was also detected on the Huh7 cells using anti-HBs MoAb (Figure 5, panel A). Localization of intracellular HBsAg was homogeneously dispersed in the cytoplasm, whereas the tagged
20 recombinant HBc antigen localized in the perinuclear area with some polarization.

Example 5: Assessing Polyepitope Expression *In vivo*

Expression of the polyepitope carried by the recombinant virus was also assessed after hydrodynamic injection of mice with prHBV1.3-III DNA (Yang P.L., et
25 al., 2002, Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection, Proc Natl Acad Sci, U.S.A, 99:13825-30; Pajot A., et al., 2004, A mouse model of human adaptive immune functions: HLA-A2.1/HLA-DR1-transgenic H-2 class I/class II-knockout mice, Eur J Immunol, 34(11):3060-9).

Three days after injection of prHBV1.3-III DNA, the liver was collected and
30 histochemical analysis was performed on liver sections. HBsAg expression was observed in liver cells using anti-HBs MoAb for detection (Figures 6A, 6C). The

recombinant HBeAg carrying the foreign epitopes was detected using an anti-FLAG MoAb (Figures 6B, 6D). Control mice received a hydrodynamic injection of PBS.

Example 6: Assessing T-Cell Response to Polyepitope *In vivo*

5 To evaluate the T-cell response against the foreign polyepitope, a plasmid with the polyepitope driven by CMV promoter (pCMV-rHBe) was tested by intramuscular DNA immunization (two injections) in HLA-A2/DRB1*01 transgenic mice (Pajot et al., 2004). Specific T cell responses against the foreign epitopes were analyzed by proliferation and ELISPOT assays one week after the second injection in mice. The
10 CD4 T-cell epitope PADRE activated IFN- γ -secreting cells (Figure 7, left panel) and was able to induce proliferation of lymphocytes from immunized mice (Figure 7, right panel).

The Flu matrix-derived epitope was the most frequently recognized among the CD8⁺ epitopes present in the polyepitope (4/6 responder mice). T cell responses to EBV
15 and core-derived epitopes were found in only one mouse. The immunodominant response to the Flu-derived epitope probably resulted from competition between peptides for fixation to the HLA-A2 molecule. This was confirmed in a second experiment with 8/10 immunized mice having T cell responses to the Flu-derived epitope and only 3/10 to the HBc 18-27-derived HLA-A2 epitope (Figure 8, left panel).
20 Nevertheless the Gag- and EBV-derived epitopes activated T cells were detectable after one week *in vitro* stimulation of splenocytes with the corresponding peptides before IFN- γ ELISPOT assays.

T cell responses to the Flu matrix epitope was also quantified using a HLA-A2-tetramer carrying the Flu epitope in splenocytes from DNA-immunized mice (two
25 injections of pCMV-rHBe). Flu-specific T cells represent around 10% of CD8⁺ T cells from the spleen (Figure 8, right panel).

The immune response to the polyepitope expressed by the recombinant virus was also assessed after one hydrodynamic injection of prHBV1.3-III into HLA-A2/-DRB1*01 transgenic mice. IFN- γ ELISPOT assays were performed on splenocytes
30 taken 16 days after hydrodynamic injection. T cells specific for the Flu matrix epitope were detected in 4 out of 5 mice (Figure 9). In this experiment, 2 mice were immunized by intramuscular injection with prHBV1.3-III as control (mouse 6 and 7). Intravenous

injection of recombinant HBV is less immunogenic than intramuscular injection. This could be related to the route of injection and to the expression of antigens in the liver, which is known to be a tolerogenic organ.

5 **Example 7: Assessing T-Cell Response to Polyepitope *In vivo* and Localization of Flu-Specific T-Cells**

Groups of HLA-A2/DRB1*01 mice (Pajot et al., 2004) were immunized intramuscularly with the plasmid pCMV-rHBe to prime T cell responses specific for foreign epitopes. Fifteen days after the priming, mice were injected by the
10 hydrodynamic route with either prHBV1.3 or pCMV- β Gal (control plasmid), or by intramuscular injection of pCMV-rHBe. Figure 10A presents a graphical depiction of the immunization time-line.

Liver infiltrating lymphocytes were prepared and stained with anti-CD8, anti-CD3 antibodies and with Flu-tetramers for FACS analysis. Non-immunized control
15 mice were used as a control. Quantification of CD8 T cells was performed after staining with anti-CD8, anti-CD3 antibodies. CD3⁺, CD8⁻ cells were considered as CD4⁺ T cells. FACS analysis showed that the number of CD8⁺ T cells infiltrating liver was much higher in mice receiving prHBV1.3 (Figure 10B, panel B3, 37.4%) compared to those receiving pCMV- β Gal (Figure 10B, panel B2, 7.31%) and non-injected mice
20 (Figure 10B, panel B1, 5.11%). Flu-specific T cells staining is shown on the lower panels for non-immunized mice, for mice receiving pCMV- β Gal or for mice receiving prHBV1.3 by hydrodynamic injection. For mice receiving prHBV1.3, 17% of T cells are Flu-specific. These cells represent 42% of CD8⁺ T cells. A comparable analysis was performed on spleen-derived lymphocytes.

25 T cells and Flu-specific T cells were localized in spleen and liver. Results are shown in Figure 10C. A strong increase of the % of CD8⁺ T cells infiltrating liver and to a lesser extent in spleen was shown after hydrodynamic injection of prHBV1.3, compared to mice receiving pCMV- β Gal or pCMV-rHBe. In contrast, the percentage of CD4 T cells in spleen or liver is comparable for the three groups of mice. In the liver,
30 the majority of the lymphocyte population consists of Flu-specific CD8⁺ T cells, as detected by tetramer-staining. These experiments indicate that after hydrodynamic

injection of prHBV1.3, the Flu-specific T lymphocytes re-localize from the spleen to the liver.

Example 8: Construction of rHBV Genome Bearing a Foreign Polypeptide

5 prHBV1.3 was constructed in the HBV ayw3 genotype background (Accession No. V01460, GenBank). The invention provides a plasmid of pCMV-Pol bearing a full length of polymerase gene and all the downstream viral elements in the HBV genome. Sequence adjacent to the start codon of the polymerase gene was modified as, 5'CCGAACATGGAG (SEQ ID NO: 1), consistent with the Kozak rule. Additionally,
10 two restriction enzyme sites (*Hind III* and *Pst I*) were arranged prior to an ATG start codon, in order to adopt a 180 nucleotide fragment coding for the foreign polypeptide (synthesized by Genscript Corp., Piscataway, NJ), that resulted in a new plasmid named pCMV-F-Pol. The embedded foreign sequence (F) shares the same reading frame with the remaining HBV core fragments (Figures 11A-11D).

15 To generate 1.3 copies of the rHBV genome, a DNA fragment covering nt1075 to nt1981 of the HBV genome was PCR amplified, and took the place of the CMV promoter in the parental plasmid by digestion with *Nru I* and *Hind III*. pCMV-rHBc encodes the recombinant foreign antigen (rHBc) driven by CMV promoter. pMAS-C comprises the HBV core gene, under a CMV promoter. The plasmid prHBV1.3HBc has
20 an additional expression cassette of HBV core protein. Briefly, a SV40 early promoter sequence (from pCDNA3, Invitrogen) was PCR amplified and inserted downstream of the rHBV genome in prHBV1.3, being separated by an *f1* origin. The HBV core gene, together with a BGH polyA processing site, was further subcloned under the SV40 early promoter (Figure 11D). Plasmids were purified using Qiagen DNA purification columns
25 (Endofree Plasmid KitTM; Qiagen, Hilden, Germany).

PCR amplification was performed by extracting viral DNA from mice sera with QIAamp DNA Blood KitTM (Qiagen). The extracted DNA was treated with *Pvu II* digestion, to linearize the residual prHBV1.3 DNA contaminant plasmid. DNA bands covering an area of 2.5-3.5 kb in the gel after electrophoresis were purified as a
30 template for PCR amplification, with the specific primers (3042F, 5'GTGGAGCCCTC AGGCTCAGGG (SEQ ID NO: 2); 459R, 5'GGACAAACGGGCAACATACC (SEQ ID NO: 3)).

rHBV was constructed to share most of its features with the wild type HBV genome (Figures 11A, 11B), with the exception of a 325-bp fragment within the HBV core gene, which was removed and substituted for by an in-frame 190-bp foreign sequence encoding a string of immunodominant T cell epitopes (Figure 11C). As a result of the deletion, the open reading frame of the polymerase gene was shifted forward by 135 bp, bringing the ATG of the pol ORF much closer to the 5' CAP in the HBV pregenomic RNA (Figures 11A, 11B). The ATG starting signal of the polymerase gene was optimized according to Kozak's rules, in order to facilitate ribosome entry for the translation.

The foreign polyepitope was engineered with three immunodominant CD8⁺ T-cell epitopes combined with a promiscuous CD4⁺ T-cell epitope (PADRE) which could universally match up most of prevalent MHC class II molecules. Considering the clinical relevance, three well-known HLA-A2-restricted epitopes derived from common human viruses (HIV gag, Influenza matrix, EBV BML-F1) were chosen, in order to elicit a vigorous immune response *in vivo*. In this construct, the well known HBc18-27 HLA-A2 restricted epitope present in the amino-terminal part of the core gene was preserved. Additionally, a short B-cell epitope (FLAG) was introduced at the N-terminal part of the foreign sequence as a convenient detection marker (Figure 11C). The core gene of HBV encodes two types of protein, the pre-core/HBeAg and the core proteins, which are translated from two distinct messenger RNA species. Two in-frame start codons are used for the translation of the two types of proteins. The core protein is the major constituent of the nucleocapsid, which carries HBcAg. HBeAg is a secreted protein produced by post-translational modifications of a precursor protein initiated at the first ATG of the core ORF. Therefore, a chimeric antigenic protein referred to as rHBc could be generated, with the foreign polyepitope fused in frame with the truncated HBe/capsid proteins (Figure 11C).

prHBV1.3/HBc is a plasmid with two expression cassettes, one for rHBV genome expression and the other for expression of the capsid protein (Figure 11D). The plasmid prHBV1.3 bears 1.3 copies of the rHBV genome only. Both plasmids were used for replication assays *in vitro* and *in vivo*. In pCMVrHBc, the expression of the rHBc is driven by the CMV early gene promoter. A polyadenylation signal for mRNA

is provided by HBV sequences (Figure 11D). This plasmid was among those used to immunize mice.

Example 9: Expression of the Recombinant Protein Carrying the Polyepitope

5 The expression of the chimeric rHBc protein was first studied in a cell culture system. The human hepatoma cell lines HepG2 and Huh 7 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Polyethylenimine (PEI) was purchased from Sigma-Aldrich (St. Louis, MO), and used for transient transfection assays. Cell culture supernatant was harvested
10 at day four post PEI transfection and used to prepare rHBV viral DNA.

 The immunofluorescence experiments were performed on HepG2 cells three days after DNA transfection, using published methods. Briefly, cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS). Cells or tissue sections were incubated with anti-Flag mAb (1084; 1:100; Sigma-Aldrich) or anti-HBs mAb (3E7;
15 1:100; Dako, Glostrup, Denmark) at 4°C overnight. After extensive washing, the bound primary antibody was detected by Alexa 488-labeled rabbit anti-mouse immunoglobulin G (Molecular Probes, Carlsbad, CA).

 Histological staining was performed on freshly prepared mouse liver, frozen in isopentane pre-cooled in liquid nitrogen, and embedded in OCT compound in
20 cryomolds. Five micron thick cryostat sections were mounted on superfrost plus slides and stored at -80°C. Before staining, slides were fixed in ice-cold acetone for 5-10 minutes. Liver sections were stained with hematoxylin and eosin, or immunostained with FITC labeled anti-HBs antibody (1:50; ab32914, Abcam, Cambridge, MA). After extensive washing with PBST and PBS, the liver sections were mounted with an anti-
25 fade reagent containing DAPI.

 After transient transfection of prHBV1.3 plasmid into hepatocyte-derived HepG2 cell line, a recombinant protein with the expected size (15 kD) was detected by Western blot in cell lysates using anti-FLAG antibody (Figure 12A). The rHBc protein was also detected by antibody staining and immunofluorescence in the cytoplasm of
30 HepG2 transfected cells, using anti-Flag antibody (Figure 12B, lower panel). Expression of HBV envelope proteins in cells transfected with the rHBV was detected using an antibody against HBsAg, which is the major antigenic determinant of the

envelope (Figure 12B, upper panel). Interestingly, labeling of intracellular HBsAg was homogeneously dispersed in the cytoplasm, whereas the rHBe localized in the perinuclear area, with polarization. In addition, HBsAg particles carrying the HBV large envelope protein (L) were also detected by ELISA in cell culture supernatant after
5 prHBV1.3 transfection (see below and Figure 12D).

Example 10: Rescue of rHBV Particles and Replication by *In Trans* Encapsidation

rHBV is a defective HBV virus owing to the disrupted core gene. However, it replicates and maintains in hepatocytes with the help of a wild type HBV capsid
10 produced *in trans* in infected hepatocytes. The invention provides replicative forms of rHBV viral DNA in cell culture supernatant, e.g., from the Huh-7 hepatic cell line, after transfection of prHBV1.3/HBc plasmid encoding both rHBV genome and capsid protein but not after transfection of prHBV1.3.

Viral DNA associated with rHBV virions was extracted from the cell culture
15 supernatants of HepG2- or Huh 7-transfected cells. Briefly, the virus particles in the medium were precipitated by incubation with 10% PEG 8000 overnight on ice. After centrifugation at 11,000 rpm for 30 minutes, the pellets were suspended in buffer (100 mM Tris/HCl (pH 8.0)), and further treated with DNase I (Invitrogen, Carlsbad, CA) in the presence of 10 mM MgCl₂. After proteinase K digestion (1 mg/ml), the viral
20 DNA was precipitated using ethanol and glycogen as carrier. A Southern blot assay was performed by methods known in the art, with a ³²P labeled probe specific to the HBV genome.

Following DNA extraction and HBV-specific probe hybridization in Southern blot assays, the viral DNA was detected, in both its typical relaxed circular (RC) and
25 double stranded linear (DSL) forms (Figure 13A), indicating normal packaging and maturation of rHBV nucleocapsid. In addition, the core-rescued rHBV exhibited a more efficient viral cycle than wild type HBV. Upon co-transfection with pwtHBV, the replicative intermediates of rHBV were expressed at significantly greater levels than the wild type replicative forms, indicating the rescue, packaging, and maturation of rHBV
30 virions in the presence of wild type capsid protein (Figure 13C).

Hydrodynamic injection technology (Liu F., 1999, Gene Therapy) was used to introduce the rHBV genome into mouse liver. Four days after hydrodynamic injection

of prHBV1.3 through the tail vein, both recombinant antigenic protein (rHBc) and HBV envelope proteins were detected in mouse liver by immunofluorescence staining of liver sections with anti-FLAG and anti-HBs antibodies respectively (Figure 12C). Taken together, these experiments suggest that HBV envelope proteins and rHBc protein
5 carrying the polyepitope are expressed after *in vitro* or *in vivo* transfection of the plasmid bearing 1.3 copies of rHBV genome.

In addition, recombinant rHBV virions were detected in the sera of mice receiving both prHBV1.3 and pMAS-C by hydrodynamic injection, using specific PCR amplification at day four after the injection. In contrast, in the absence of pMAS-C,
10 injection of prHBV1.3 alone or with co-injection of a plasmid encoding beta-galactosidase (pCMV- β Gal), no viral DNA was detected (Figure 13D). Therefore, the rHBV genome can be complemented *in trans* by capsid proteins *in vitro* and *in vivo* giving rise to complete viral particles containing replicative forms of viral DNA.

The large envelope protein (L) is known to be localized on the surface of 42 nm
15 HBV complete viral particles and on the filamentous sub-viral particles present in the sera of HBV-infected individuals. To demonstrate that complete viral particles can be produced from the rHBV genome, cotransfection experiments of prHBV1.3 and a plasmid encoding the core protein (pMAS-C) in Huh-7 cell line were performed. Cotransfection with the core-encoding plasmid resulted in an increase in the production
20 of L protein-carrying particles in cell culture supernatant, compared with transfection of prHBV1.3 alone or cotransfection with pIRES-GFP as control. The increase in L protein production was dose-dependent as shown in a specific ELISA using two different monoclonal antibodies recognizing the amino-terminal part of L protein (Figure 13B).

25

Example 11: Activation of Polyepitope-specific T-cell Responses

IFN- γ producing splenocytes were quantified by *ex vivo* Elispot assays after stimulation with peptide, as known in the art. Briefly, 96-well nitrocellulose HA plates (Millipore, Bedford, MA) were coated by incubation overnight at 4°C with capture
30 antibody against IFN- γ (551216; BD Pharmingen, San Diego, CA). Freshly isolated splenocytes (10^6 /well) were incubated with individual peptide at a concentration of 1 μ g/ml in supplemented α -MEM medium for 24 hours. Spots were developed by a

secondary biotin-conjugated antibody (554410; BD Pharmingen, San Diego, CA) and alkaline phosphatase conjugated streptavidin (Roche, Basel, Switzerland). A Zeiss Elispot automatic counter was used to score the number of spots. The response was considered positive if the median number of spot-forming cells (SFC) in triplicate wells was at least twice that in control wells containing medium alone.

For the proliferation assay, splenocytes (10^6 cells/well) were incubated with 20 μ g/ml of peptide for three days in supplemented HL1 serum-free medium (Biowhitaker, Walkersville, Maryland) (Pajot et al., 2004). Cells were pulsed for the final 16 h with 1 μ Ci of (3 H)-thymidine per well. The incorporated radioactivity was measured on a micro- β counter.

To evaluate T-cell responses against the foreign polypeptide, a DNA plasmid encoding rHBc (pCMV-rHBc, as described in Example 1) was used to immunize HLA-A2/DR1 transgenic mice (Pajot et al., 2004). Two weeks after intramuscular injection, nine of the ten mice tested mounted epitope-specific T cell responses, as detected using *ex vivo* IFN γ -ELISPOT assays (Figure 14A).

The Flu matrix-derived epitope is obviously the most frequently recognized and most powerful among the three foreign CD8 $^+$ T cell epitopes (9 out of 10 responder mice). Flu-specific T cell response even dominated over the response to the well-described capsid-derived HBc18-27 HLA-A2 epitope that is present in the N-terminal part of the protein. The immunodominant response to the Flu-derived epitope probably resulted from competition between peptides for fixation to the HLA-A2 molecule. Nevertheless, Gag- and EBV-specific T cells were detectable after one week *in vitro* stimulation of splenocytes with individual peptides. T cell responses to the Flu matrix epitope were also quantified using a HLA-A2-pentamer (ProImmune, Oxford, UK) carrying the Flu peptide to label splenocytes from DNA-immunized mice. Accordingly, Flu-specific T cells represent around 10% of CD8 $^+$ T cells from the spleen (Figure 14B, right panel).

Mice receiving pCMV-rHBe injection also developed T helper responses against the MHC class II-restricted epitope PADRE, as demonstrated by both I IFN γ -ELISPOT assay (Figure 14A) and specific proliferation observed in five out of six immunized mice, upon stimulation of splenocytes with PADRE peptide (Figure 14C).

Example 12: Retargeting Polyepitope-specific T cell Responses to Liver

Mice were perfused with 20 ml PBS via ventricle route. The liver was smashed with a syringe plunger in a 100 μ m cell strainer (100 μ m Nylon, BD, Franklin Lakes, NJ). Cell pellets were resuspended in 15 ml of 40% Purcell (Sigma, St. Louis, MO) and centrifuged at 2000 rpm for 20 minutes, to remove the hepatocyte clumps. The intrahepatic lymphocytes in the pellets were further purified through a Ficoll gradient centrifugation as for the separation of mouse splenocytes. Freshly isolated lymphocytes were stained by PerCP-labeled anti-CD3, APC-labeled anti-CD8 antibodies, or by PE-labeled HLA class I tetramer conjugated with Flu peptide.

For FACS analysis, at least 10000 events gated among the population of interest were analyzed on a FACSCalibur cytometer using CellQuest program (BD Biosciences, Franklin Lakes, NJ). To demonstrate that rHBV co-maintains with wild type HBV virus in liver, and in the absence of a mouse model of HBV infection and replication, the invention provides a protocol of rHBV-based active immunotherapy in HLA-A2/DR1 transgenic mice (Figure 15A).

HLA-A2/DR1 mice were immunized by intramuscular injection of plasmid pCMV-rHBc at day 0 to prime polyepitope-specific T cell responses in periphery. Two weeks later, prHBV1.3 was injected via a hydrodynamic route to bypass hepatocyte infection and mimic HBV replication in liver (Yang P., Althage L.A., Chung J., Chisari F.V., 2002, Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection, Proc Natl Acad Sci, U.S.A. 99:13825-30). Thus, rHBV can be expressed in liver cells with the encoded foreign antigen being processed into peptides and presented in situ providing, in turn, intrahepatic targets for a CD8⁺ T cell response. pCMV- β Gal was used as a control plasmid for hydrodynamic injection.

Following priming and hydrodynamic injection of prHBV1.3, mice mounted a vigorous intrahepatic T-cell response, with a large number of CD8⁺ T lymphocytes infiltrating the liver. CD8⁺ T lymphocytes accumulated in the mouse livers, as detected by FACS analysis of liver-infiltrating lymphocytes taken at days 3, 4 and 7. At day 7, the percentage of liver-infiltrating CD8⁺ T cells represented up to 37.4% of total lymphocytes in mice receiving prHBV1.3, compared to mice receiving pCMV- β Gal (7.31%) and to non immunized mice (5.11%) (Figure 15B). 17% of the T cells,

representing 42% of the total liver-infiltrating lymphocytes, in the mice that received prHBV1.3 were Flu-specific. In comparison, only 0.36% of the Flu-specific CD8⁺ T cells primed by intramuscular injection were present in the liver seven days after pCMV-βGal hydrodynamic injection.

5 Figures 15C and 15D demonstrate the relative distribution of CD8⁺ and CD4⁺ T cells in the livers and spleens from groups of primed mice receiving either prHBV1.3 or pCMV-βGal by hydrodynamic injection, or receiving two intramuscular injections of pCMV-rHBc. A strong increase in the percentage of CD8⁺ T cells was observed in the liver and, to a lesser extent, in the spleens of mice after hydrodynamic injection of
10 prHBV1.3, compared to mice receiving pCMV-βGal and to mice receiving pCMV-rHBc only.

Remarkably, a large increase in the percentage of Flu-specific CD8⁺ T cells was observed in the livers of mice receiving prHBV1.3. Following immunization, Flu-specific CD8⁺ T cells comprised a very high percentage of hepatic lymphocytes,
15 compared to the percentage of splenic lymphocytes ($p = 0.0002$). In contrast, the percentages of CD4⁺ T cells in the spleen and the liver were not significantly different in the three groups of mice. In the presence of the vigorous CD8⁺ T cell response, the CD4⁺ T cell reservoir was relatively reduced in the liver, but not in the spleen. These experiments demonstrate that the majority of Flu-specific peripheral CD8⁺ T
20 lymphocytes relocated to the liver following rHBV-based active immunization.

The increase in the percentage of total lymphocytes derived after immunization reached statistical significance in both the liver and the spleen. As shown in Figure 15C, the percentage of CD8⁺ lymphocytes observed in the livers of mice receiving pCMVrHBe priming followed by prHBV1.3 hydrodynamic injection was significantly
25 higher than in the livers of mice receiving pCMVrHBe priming followed by pCMVβGal hydrodynamic injection ($p = 0.0001$), and significantly higher than mice injected twice with pCMVrHBe via the intramuscular route ($p = 0.0009$).

A similar increase in the percentage of CD8⁺ lymphocytes observed in the spleens of mice receiving pCMVrHBe priming followed by prHBV1.3 hydrodynamic
30 injection was significantly higher than in the spleens of mice receiving pCMVrHBe

priming followed by pCMV β Gal hydrodynamic injection ($p = 0.0011$), and significantly higher than mice injected twice with pCMVrHBe via the intramuscular route ($p = 0.0114$).

5 **Example 13: Non-cytolytic Control of HBV Gene Expression in Liver Mediated by Polyepitope-specific T Cells**

CD8⁺ T cells are the major population in hepatic infiltrates on day seven after hydrodynamic injection, as described above. Further analysis of the liver infiltrates was performed by histochemical analysis of liver sections taken four days after prHBV1.3
10 injection (Figure 16A). A remarkable infiltration of inflammatory cells was observed in the liver, and was predominantly centered into clusters of various sizes, suggesting that they developed quickly to form inflammatory foci. The presence of these infiltrates was dependant on priming peripheral T cell responses, as few cell clusters were found in liver sections taken from mice receiving hydrodynamic injection of prHBV1.3 without
15 previous priming. Little or no clustered infiltrates were observed in mice receiving pCMV-rHBe priming followed by pCMV- β Gal hydrodynamic injection (Figure 16A).

Flu-specific T cells were further phenotyped as CD44⁺, CD62L^{low}, and CD69^{high} (Figure 16B), corresponding to activated or effector memory T cells undergoing an *in vivo* expansion. Upon an *ex vivo* stimulation by Flu peptide, these
20 cells, freshly isolated from liver, produced mostly INF γ but also produced TNF α , detected by intracellular staining (Figure 16C). Around 58% of CD8⁺ T cells were positive for surface staining with CD107a, a marker of cellular degranulation (Figure 16C). Taken together, these data demonstrate that the liver infiltrating cells were predominantly functional CD8⁺ effector T cells.

25 Knowing that HBV gene expression in liver is susceptible to a non-lytic control by IFN- γ -secreting T cells following antigen recognition, we monitored the expression of rHBV expression in the liver and sera of mice. Four days after prHBV1.3 hydrodynamic injection intrahepatic expression of HBsAg was undetectable in mice receiving pCMV-rHBe priming (Figure 16D, left panel) compared to mice receiving
30 prHBV1.3 only (Figure 16D, right panel). Accordingly a 100 fold decrease in HBsAg was observed in sera of mice in which T cells were primed before prHBV1.3 injection

(Figure 16E). In contrast, in the absence of peripherally primed T cells, mice exhibited a strong HBsAg expression after prHBV1.3 hydrodynamic injection. HbsAg expression was demonstrated by immunofluorescence staining of liver sections (Figure 16D, right panel) and HbsAg measurement in sera using a commercial detection kit (Monolisa
5 HBsAg ULTRA, Bio-Rad) (Figure 16 E, left panel). Taken together these experiments demonstrate a rapid non-cytolytic control of rHBV gene expression by polyepitope-activated CD8⁺ T cells.

These infiltrating T cells can hypothetically be responsible for liver injury. However, no significant increase in the injury marker ALT was observed in the sera of
10 mice receiving prHBV1.3 via hydrodynamic injection, as compared to those receiving pCMV- β gal as a control, four days following prHBV1.3 hydrodynamic injection (Figure 16F). At day four post hydrodynamic injection, the mean serum alanine transferase (ALT) level was 94.18 ± 30.33 mU/ml in the 11 mice receiving rHBV-based immunization. In comparison, the serum ATL levels remained normal in mice receiving
15 pCMV-rHBc priming followed by pCMV- β Gal hydrodynamic injection (mean = 38.00 ± 5.35), while a striking increase was observed in the sera of mice with Concanavalin A-induced acute hepatitis (Figure 16F) (Zhu R., et al., 2007, The Pro-Th1 cytokine IL-12 enhances IL-4 production by invariant NKT cells: relevance for T cell-mediated hepatitis, J. Immunol., 178:5435-42). It therefore suggests that, in the presence of the
20 peripherally built-up T cell response, expression of rHBV rapidly attracted T cell response in liver without raising a major liver injury.

Example 14: Active Immunotherapy in HBsAg Transgenic Mice

The invention further demonstrates organ-specific viral targeting and expression
25 of the pseudo-viruses of the invention, using a transgenic mouse lineage expressing HBV envelope proteins in liver and secreting HBsAg in sera. This lineage was previously back-crossed with HLA-A2 transgenic mice and is devoid of murine MHC class I molecules. The HLA-A2/DR1 (*HLA-A02.01/DR1*-transgenic, *H-2 class I/class II* KO) and HBsAg/HLA-A2 transgenic mice used in this study were bred in the animal
30 facilities of Institut Pasteur (Pajot et al., 2004).

The HBsAg/HLA-A2 double transgenic lineage (*H-2 class I* KO) is endowed with HLA-A2 background and produces HBsAg in mouse liver following transgene

expression. Intramuscular DNA immunization was carried out by injecting 100 μ g of plasmid DNA into regenerating (i.e. cardiotoxin-treated) tibialis anterior muscles. For hydrodynamic injection, female mice around 12-15 weeks old were used. Briefly, 25 μ g of plasmid DNA was injected through the tail vein in a volume of PBS equivalent to 8%
5 of the mouse's body weight. The total volume was delivered within five seconds. Mice were bled and sera assayed for HBsAg by specific ELISA at indicated times. All experiments involving mice were performed according to European guidelines.

Following priming and hydrodynamic injection, mice were bled weekly to monitor HBsAg concentration in sera. A decrease in HBsAg in sera was first observed
10 two weeks after priming and was followed by a second sharp decrease in all mice examined one to two weeks after prHBV1.3 hydrodynamic injection (Figure 17B). The decrease after priming corresponds to the influx of polypeptide specific T cells from the circulation to the liver. The decrease in HBsAg reached up to 90%, compared to the starting level in some of the mice tested (Figure 17B). In contrast, in mice receiving
15 pCMV- β Gal as control, no significant HBsAg decrease was observed following hydrodynamic injection (Figure 17C).

Clearance of HBsAg was not complete, and antigen level fluctuated around 25% of basal level during a two-month follow up. HbsAg clearance was strong and long-lasting, however, when compared to the pCMV- β Gal control animals eight weeks after
20 immunization ($p < 0.0001$).

We have previously shown that HBV mRNA in the liver is susceptible to down regulation by INF- γ secreted by HBsAg-specific vaccine-activated T cells (Mancini-Bourgine et al., 2004). Mice with HBsAg/HLA-A2 backgrounds transgenic for HbsAg also display an antiviral response to rHBV-based active immunization.

25 The clearance of HBsAg demonstrated by the methods of the invention is likely to be related to the non-HBV, polyepitope specific influx of Flu-specific T cells into the liver and to a bystander effect of INF γ -secreting T cells on HBsAg-expressing hepatocytes. This suggests that these functional effector T cells not only control rHBV expression, as shown in Figure 16E, but also demonstrates HBV transgene expression in
30 the liver.

In summary, the instant application presents a novel, efficient, and feasible strategy for the use of active immunization for the treatment of persistent viral infections, satisfying a long-felt need in the art.

Throughout this application, the terms “rHBe” and “rHBc” refer, without
5 distinction, to the translational products of the modified preCC open reading frame.

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0-1	Form PCT/RO/134 (SAFE) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared Using	PCT Online Filing Version 3.5.000.193 MT/FOP 20020701/0.20.5.9
0-2	International Application No.	
0-3	Applicant's or agent's file reference	353483D26925
1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	29
1-2	line	27
1-3	Identification of deposit	
1-3-1	Name of depositary institution	CNCM Collection nationale de cultures de micro-organismes
1-3-2	Address of depositary institution	Institut Pasteur, 28, rue du Dr Roux, 75724 Paris Cedex 15, France
1-3-3	Date of deposit	11 September 2007 (11.09.2007)
1-3-4	Accession Number	CNCM I-3833
1-5	Designated States for Which Indications are Made	all designations
2	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
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2-2	line	28
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3	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
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5-3-3	Date of deposit	11 September 2008 (11.09.2008)
5-3-4	Accession Number	CNCM I-4078
5-5	Designated States for Which Indications are Made	all designations

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6-3-3	Date of deposit	11 September 2008 (11.09.2008)
6-3-4	Accession Number	CNCM I-4079
6-5	Designated States for Which Indications are Made	all designations

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0-4-1	Authorized officer	Aulbers, Sabine

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CLAIMS:

1. A recombinant replication defective hepatitis B virus comprising:
a hepatitis B virus genome defective for the expression of hepatitis B virus capsid protein (HBc), wherein the virus contains a nucleic acid molecule of up to 195 nucleotides encoding a foreign peptide not found in the wild type hepatitis B virus comprising at least one immuno-dominant epitope, and wherein said nucleic acid molecule encoding said foreign peptide is inserted in a nucleocapsid-encoding gene.
2. The recombinant replication defective hepatitis B virus of claim 1, wherein said foreign peptide is expressed fused within the N-terminal part of HBV capsid protein.
3. The recombinant replication defective hepatitis B virus according to claim 1 or 2, wherein said nucleic acid molecule is located between nucleotide residue 1981 and nucleotide residue 2308 of the HBV ayw3 genome, wherein the numbering starts from the fourth nucleotide in the EcoRI site of the HBV genome, NCBI Accession No. V01460.
4. The recombinant replication defective hepatitis B virus according to any one of claims 1 to 3, wherein said foreign peptide comprises one or a multiplicity of epitopes of one or more different origins.
5. The recombinant replication defective hepatitis B virus according to any one of claims 1 to 4, wherein said foreign peptide comprises T-cell response eliciting epitope(s).
6. The recombinant replication defective hepatitis B virus according to any one of claims 1 to 5, wherein said foreign peptide is derived from a pathogen.
7. An isolated hepatocyte cell of a non-human vertebrate infected by the recombinant replication defective hepatitis virus as claimed in any one of claims 1 to 6.
8. The hepatocyte cell as claimed in claim 7, wherein the cell further comprises a nucleic acid molecule encoding HBc for complementation of the recombinant hepatitis virus to form a hepatitis pseudo-virus.

9. A recombinant hepatitis pseudo-virus comprising the recombinant replication defective hepatitis virus as claimed in any one of claims 1 to 6 complemented by HBc, wherein the pseudo-virus replicates *in vitro* in human hepatocytes.
10. An isolated hepatocyte cell of a vertebrate infected by the hepatitis pseudovirus as claimed in claim 9.
11. A method of forming a hepatitis pseudo-virus, wherein the method comprises culturing the hepatocyte cell as claimed in claim 8 under conditions for expression of the nucleic acid molecule encoding the HBc and complementation of the recombinant replication defective hepatitis B virus to form a hepatitis pseudo-virus.
12. A cloning and/or expression vector comprising a genome of a recombinant replication defective hepatitis B virus as claimed in any one of claims 1 to 6.
13. An isolated eukaryotic host cell comprising the vector as claimed in claim 12.
14. An *in vitro* method of producing hepatitis pseudo-virus, wherein the method comprises:
 - providing the host cell as claimed in claim 13;
 - expressing the hepatitis proteins under conditions in which the proteins assemble into hepatitis pseudo-virus, which are released from the host cell into extracellular space.
15. The method of claim 14, further comprising recovering hepatitis pseudovirus.
16. A composition comprising the recombinant replication defective virus of any one of claims 1 to 6, and a pharmaceutically acceptable carrier.
17. A vaccine comprising the composition of claim 16.
18. Use of the recombinant replication defective virus of any one of claims 1 to 6 for treating a patient chronically or persistently infected by a hepatitis B virus.

19. Use of the recombinant replication defective virus of any one of claims 1 to 6 for the preparation of a medicament for the treatment of a patient persistently infected with a wild type hepatitis B virus.
20. Use of the recombinant replication defective virus of any one of claims 1 to 6 for the preparation of a medicament for the treatment of a patient chronically infected by a hepatitis B virus.

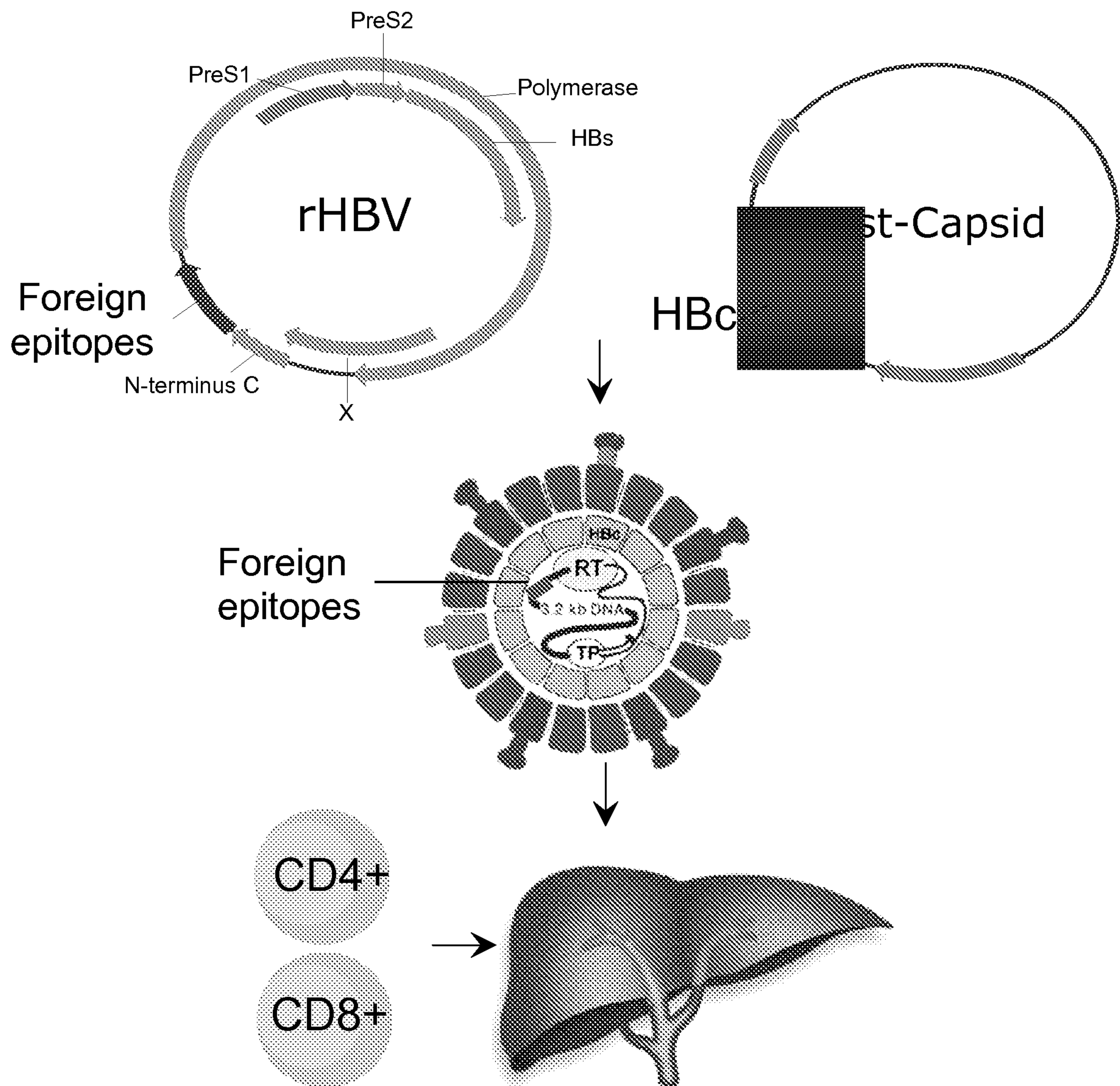


FIGURE 1

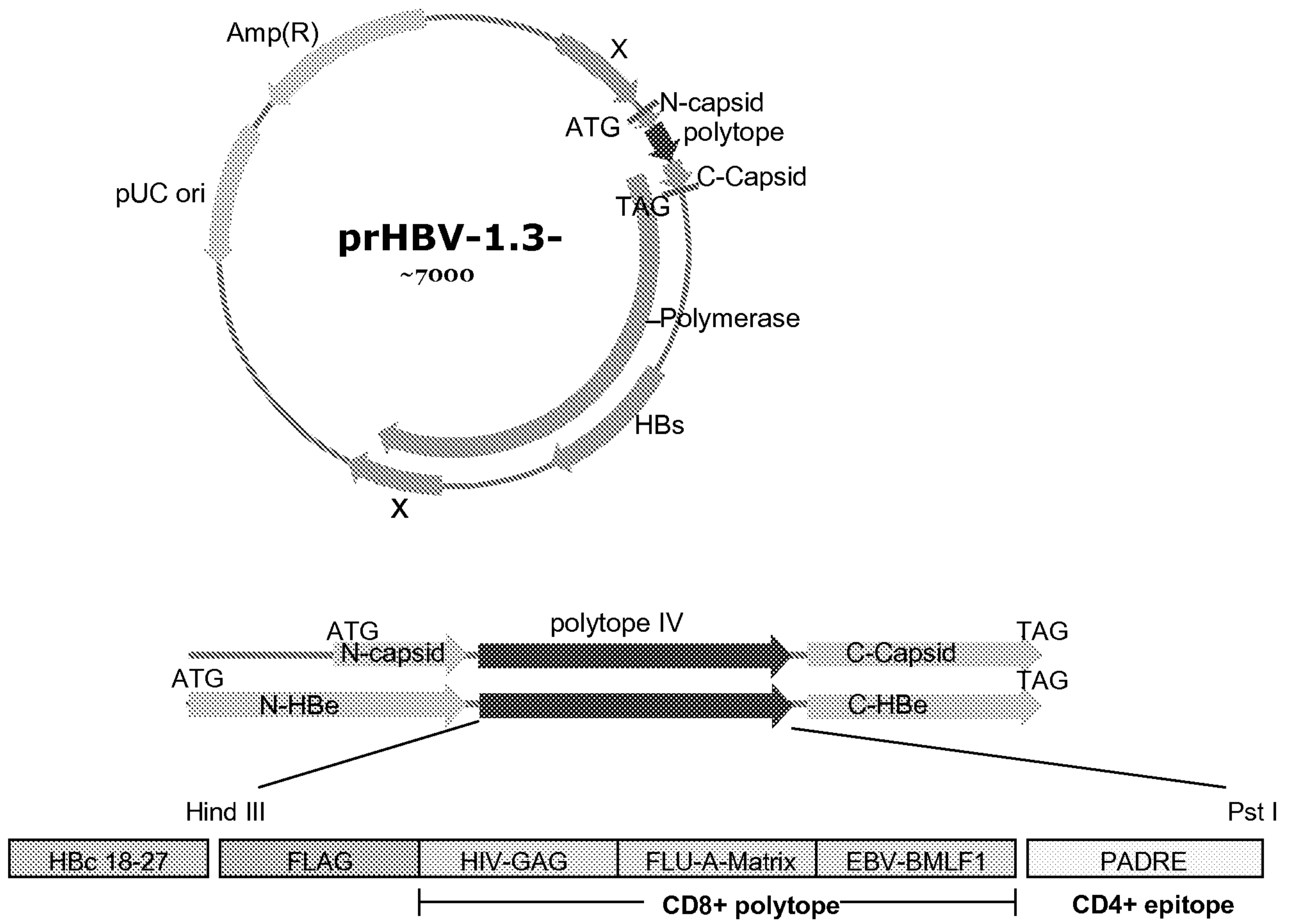


FIGURE 2

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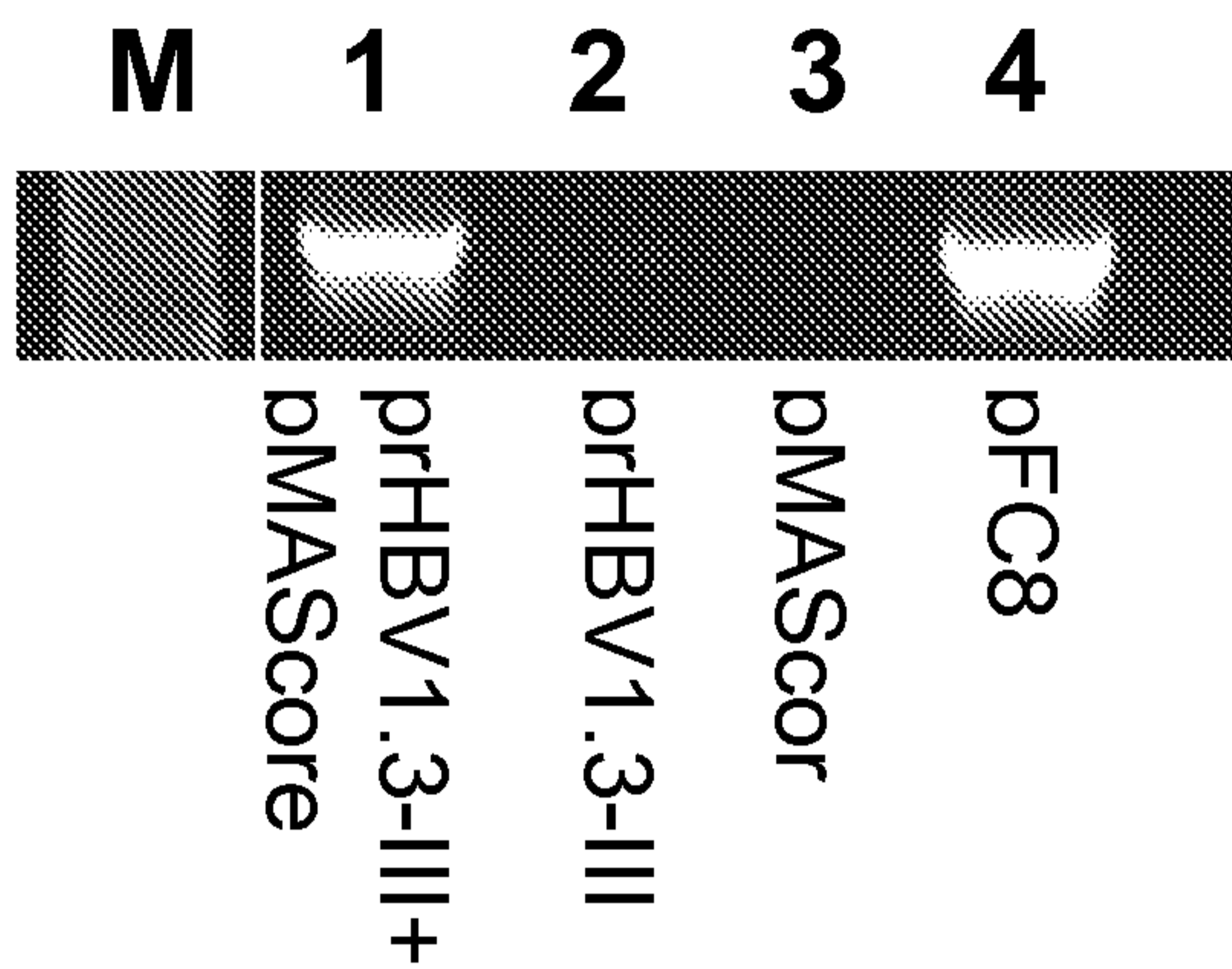


FIGURE 3A

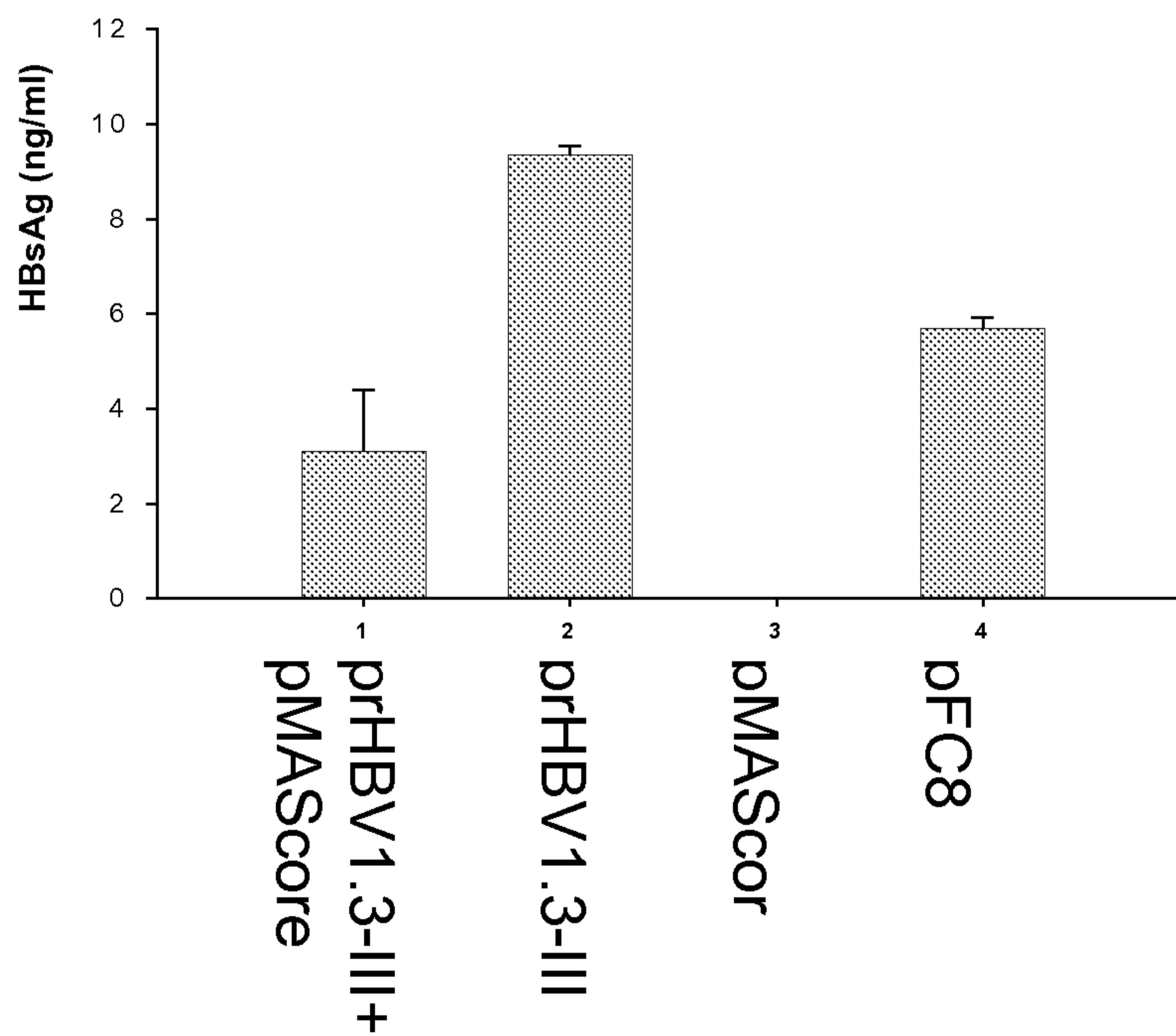


FIGURE 3B

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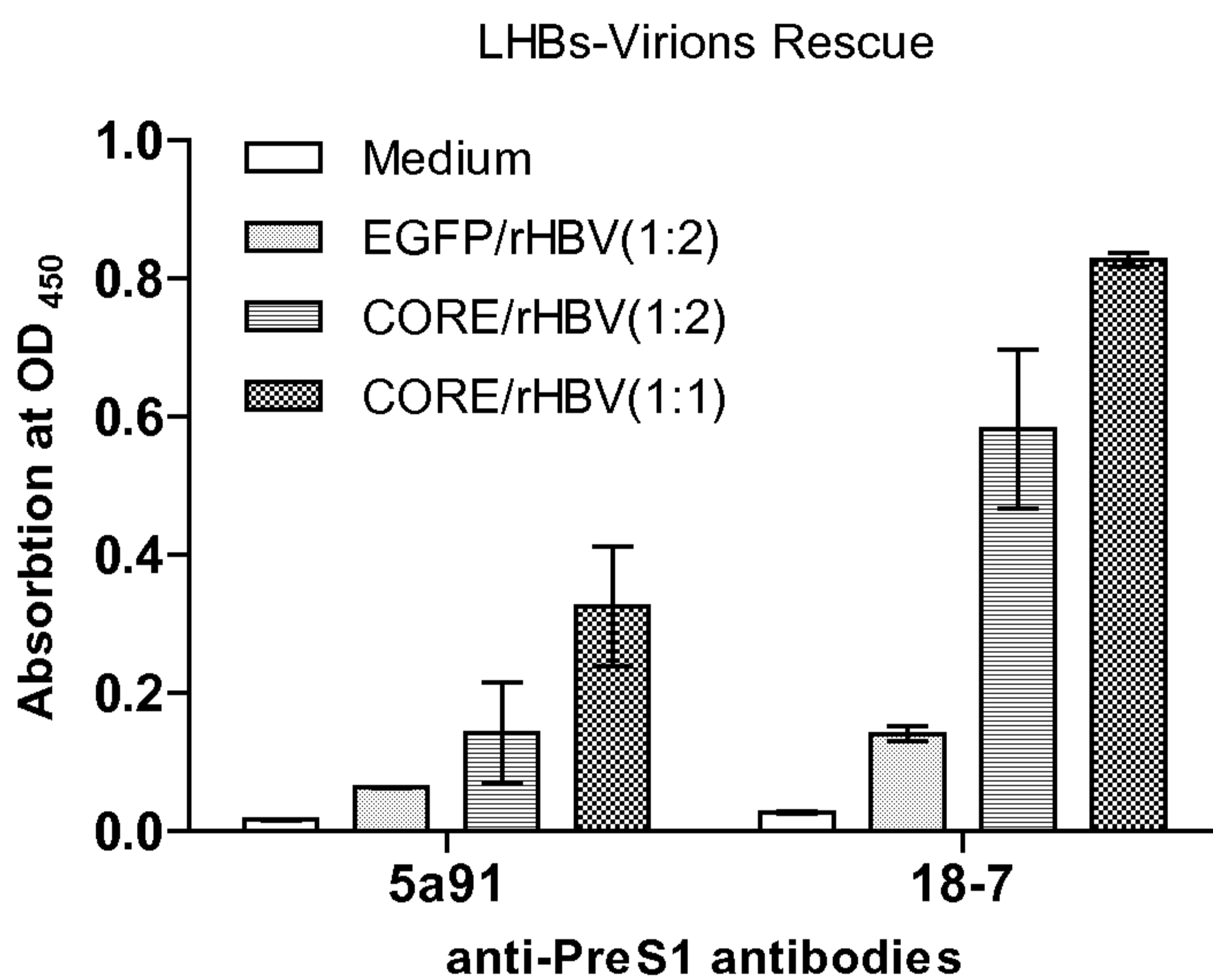


FIGURE 3C

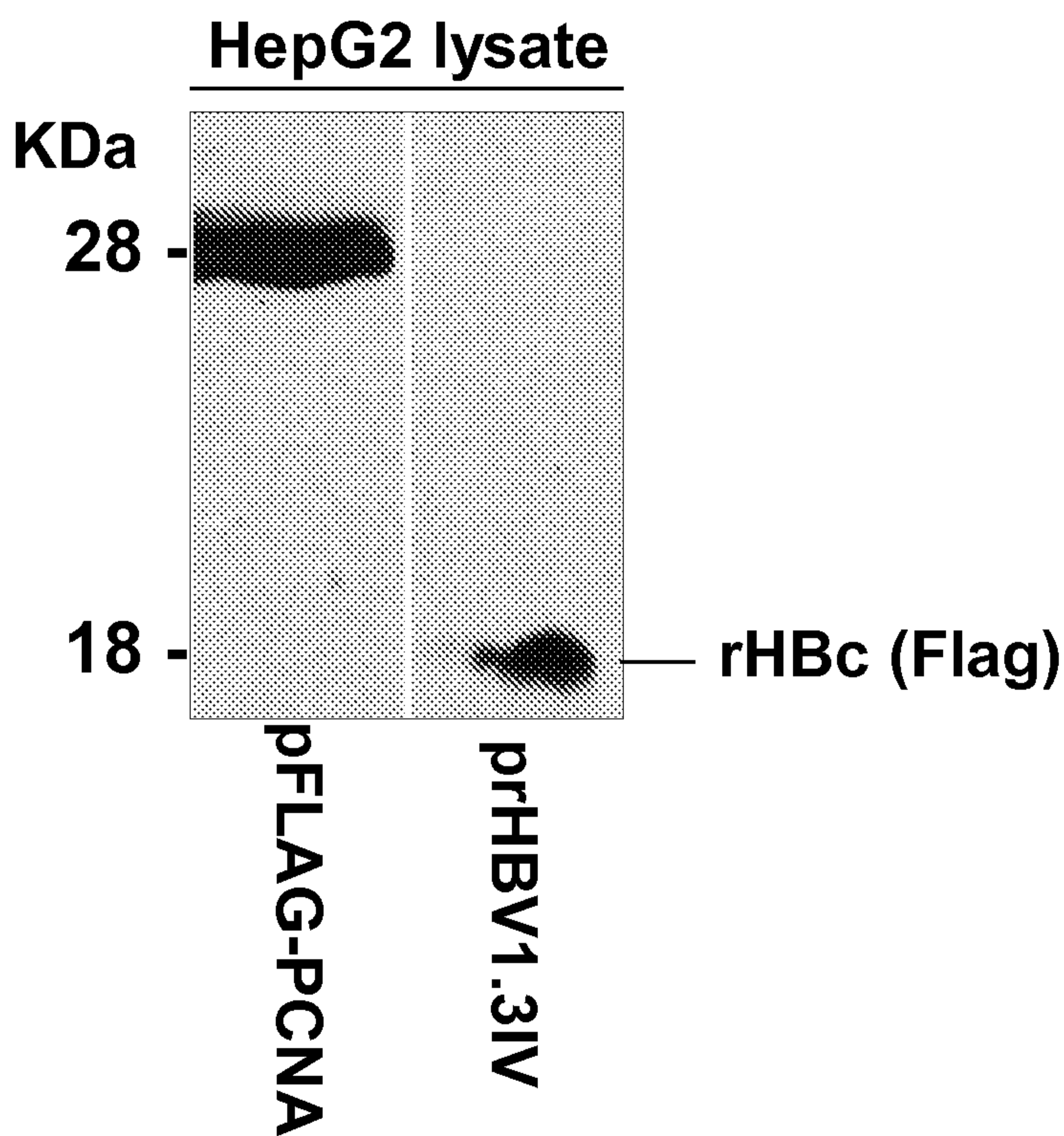


FIGURE 4

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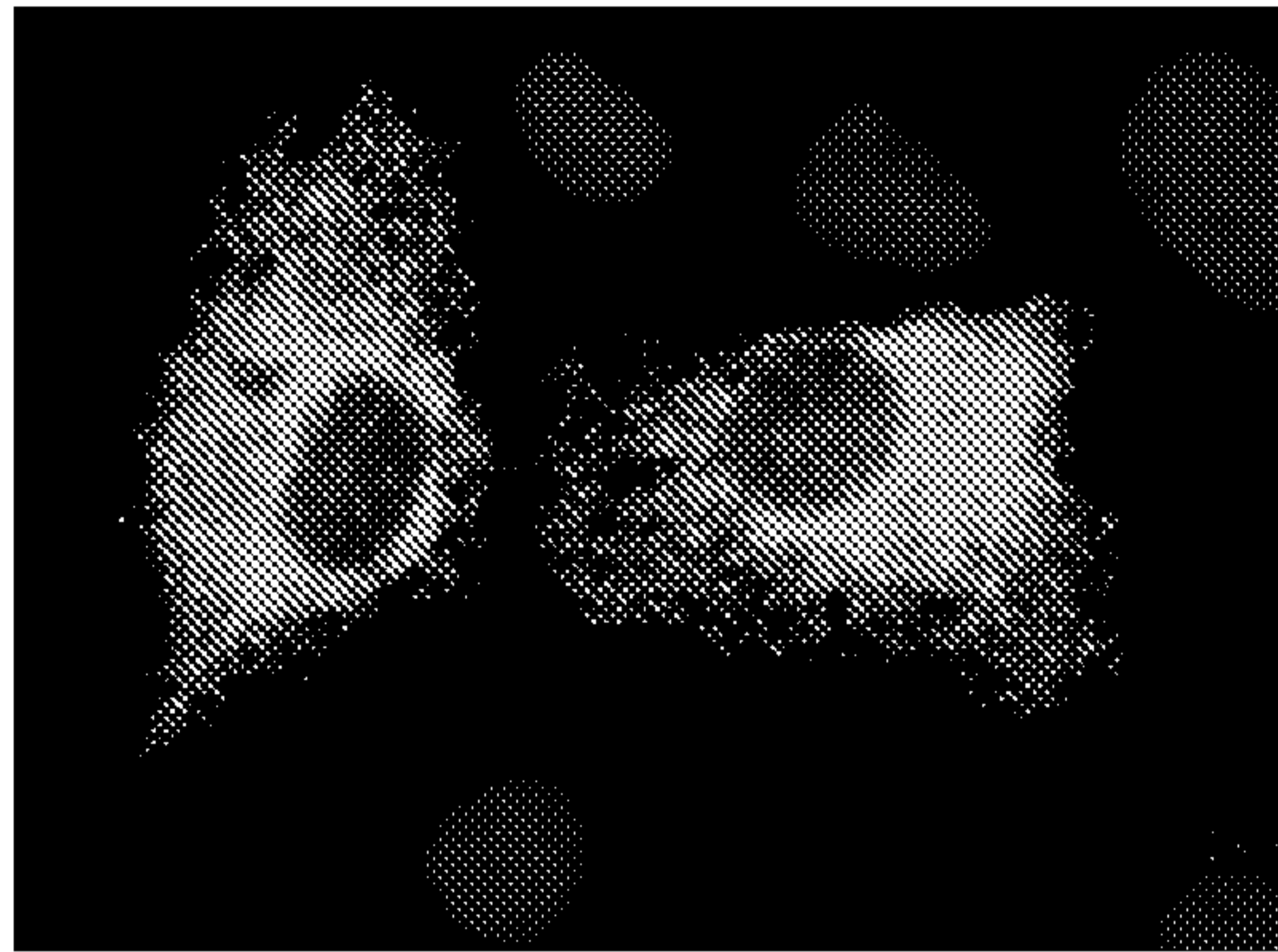


FIGURE 5A

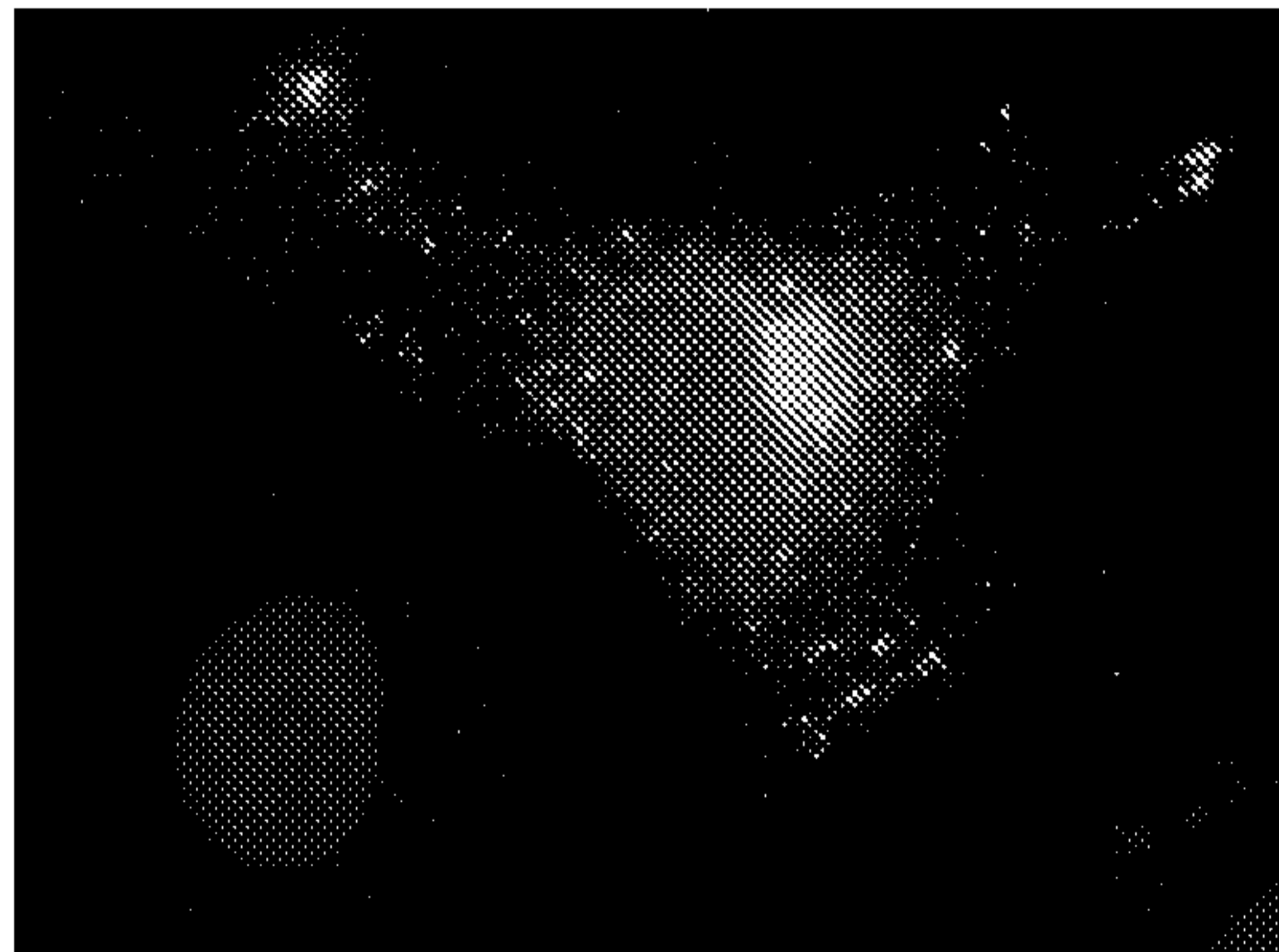
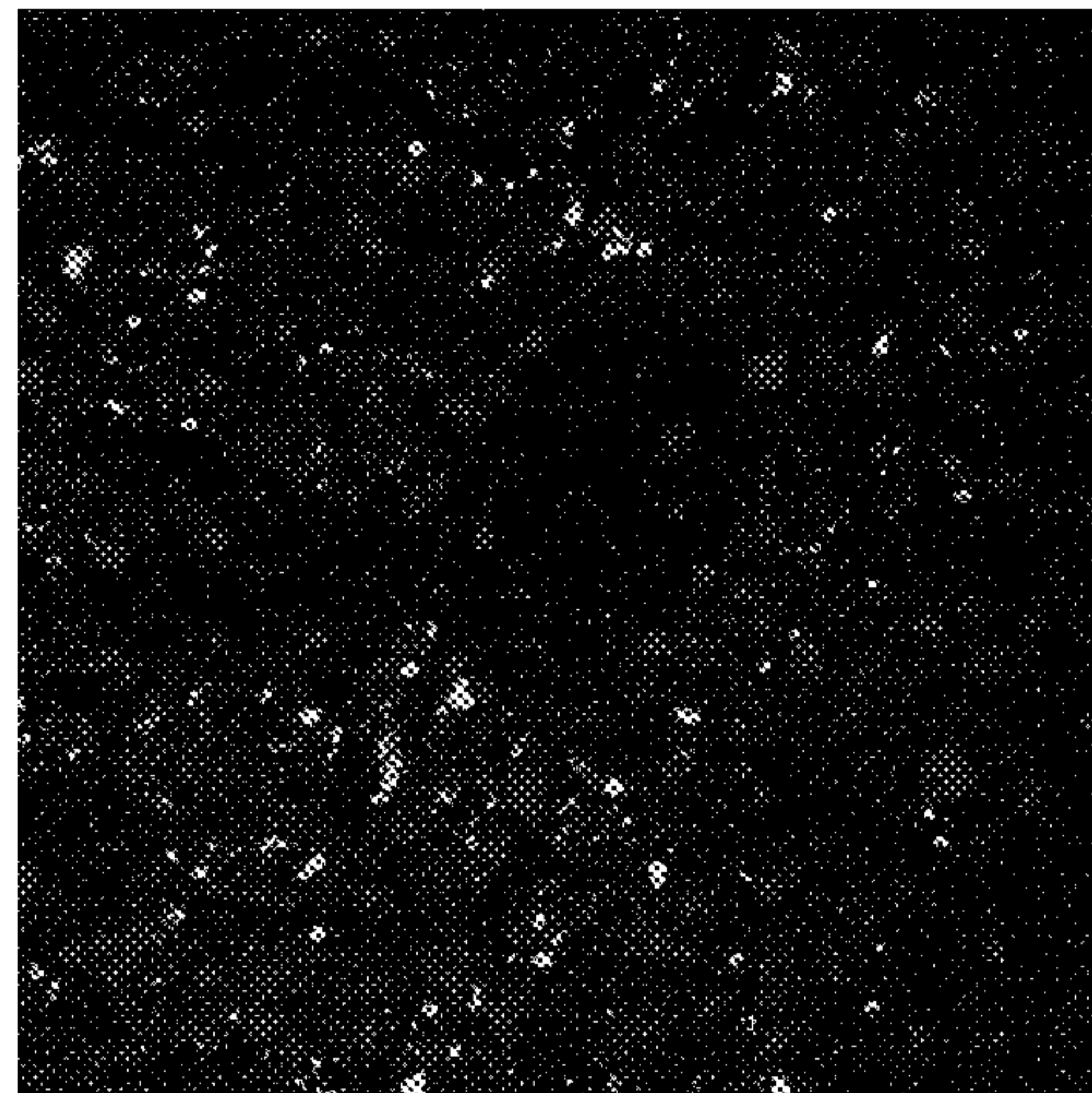
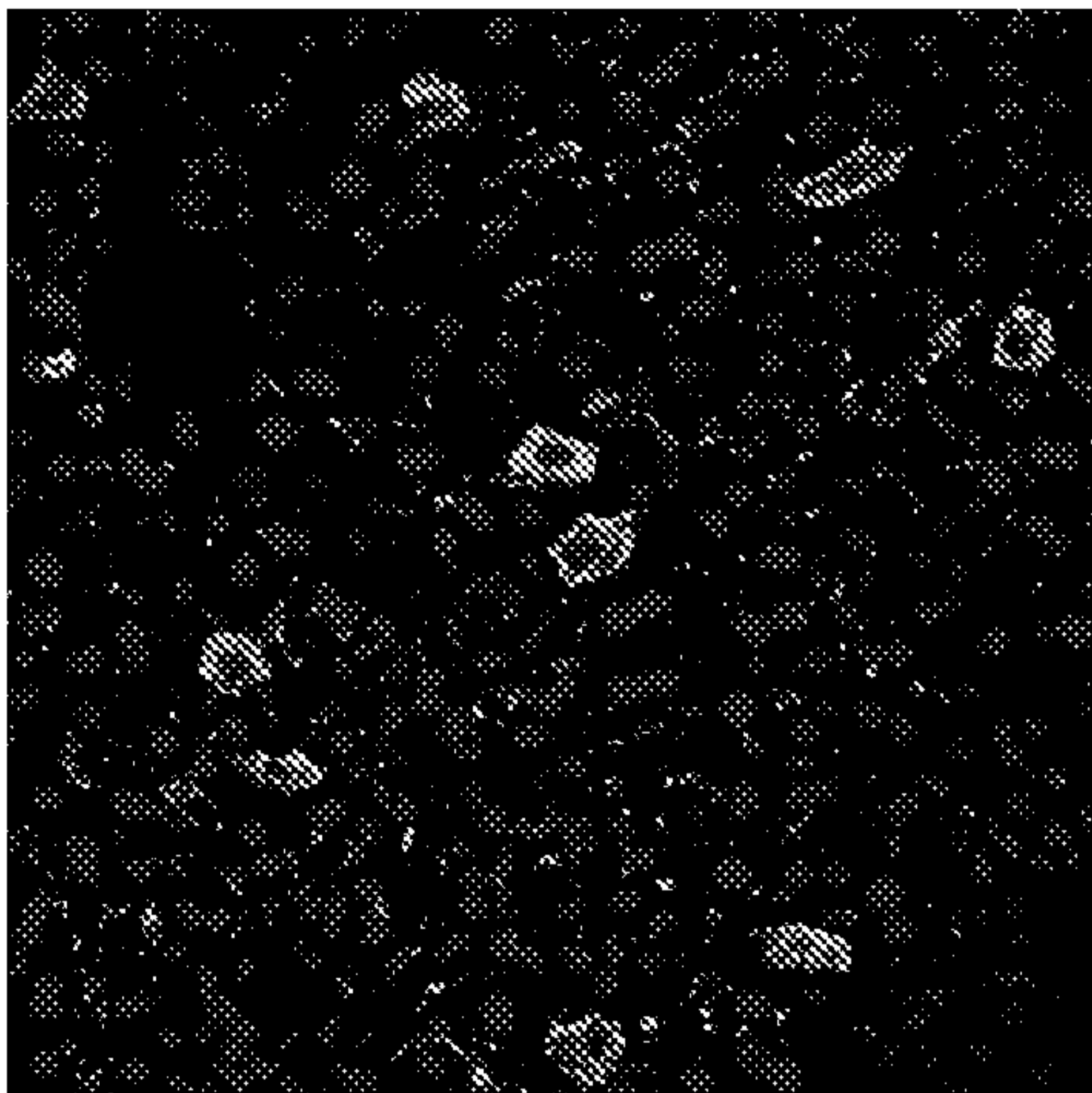


FIGURE 5B

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anti-HBs/HBsAg

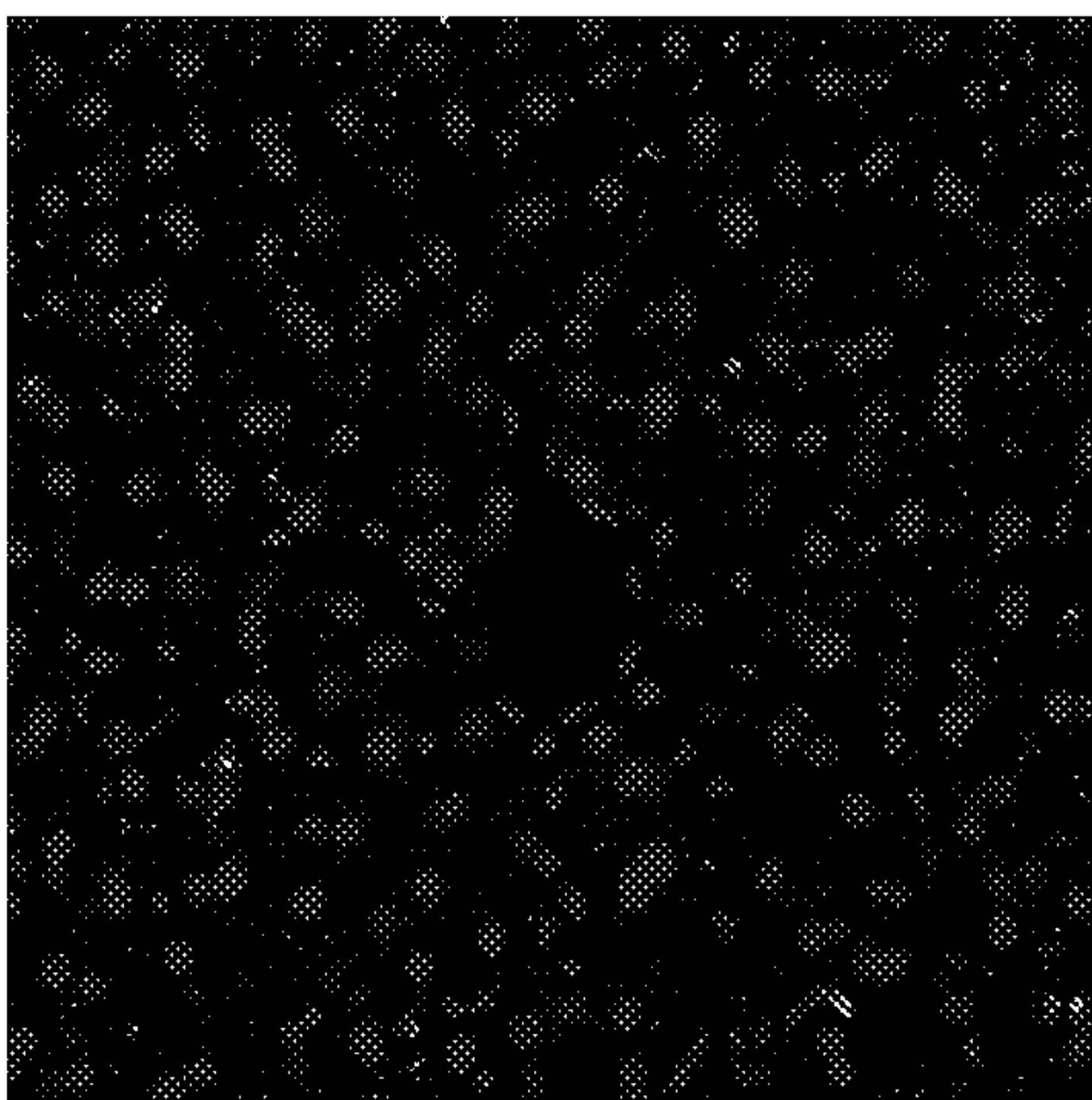
anti-FLAG/rHBc



prHBV1.3III

FIGURE 6A

FIGURE 6B



PBS

FIGURE 6C

FIGURE 6D

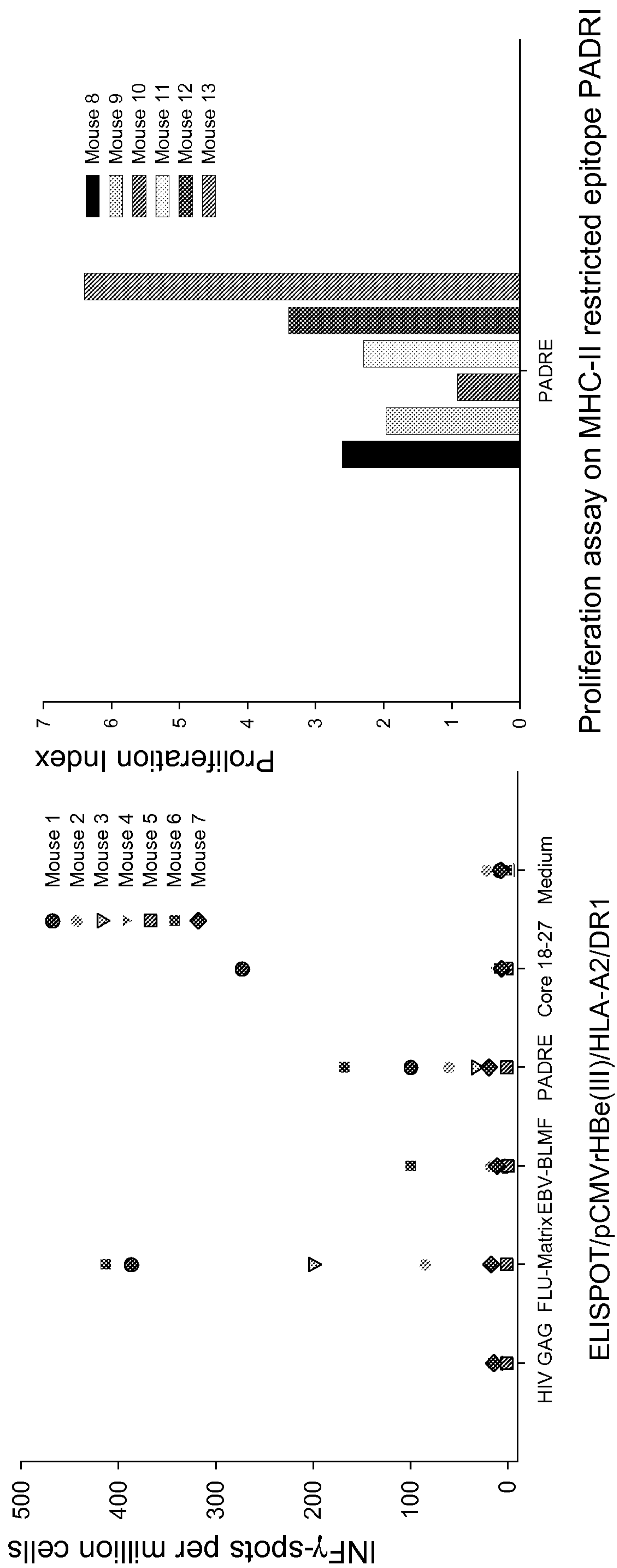


FIGURE 7

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HLA-A2/DRB1 Tg mice immunized with pCMV-rHBe-IV

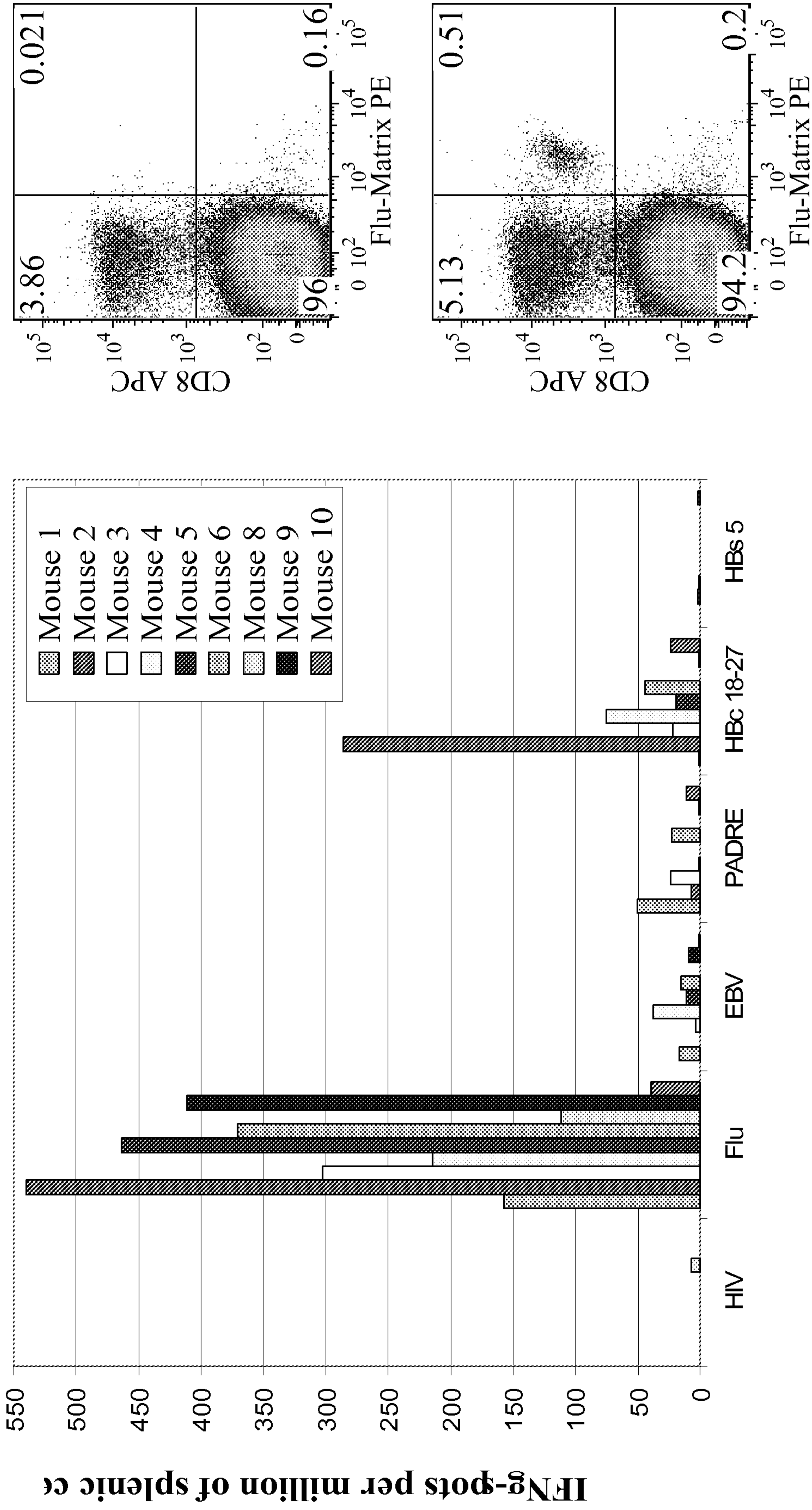


FIGURE 8

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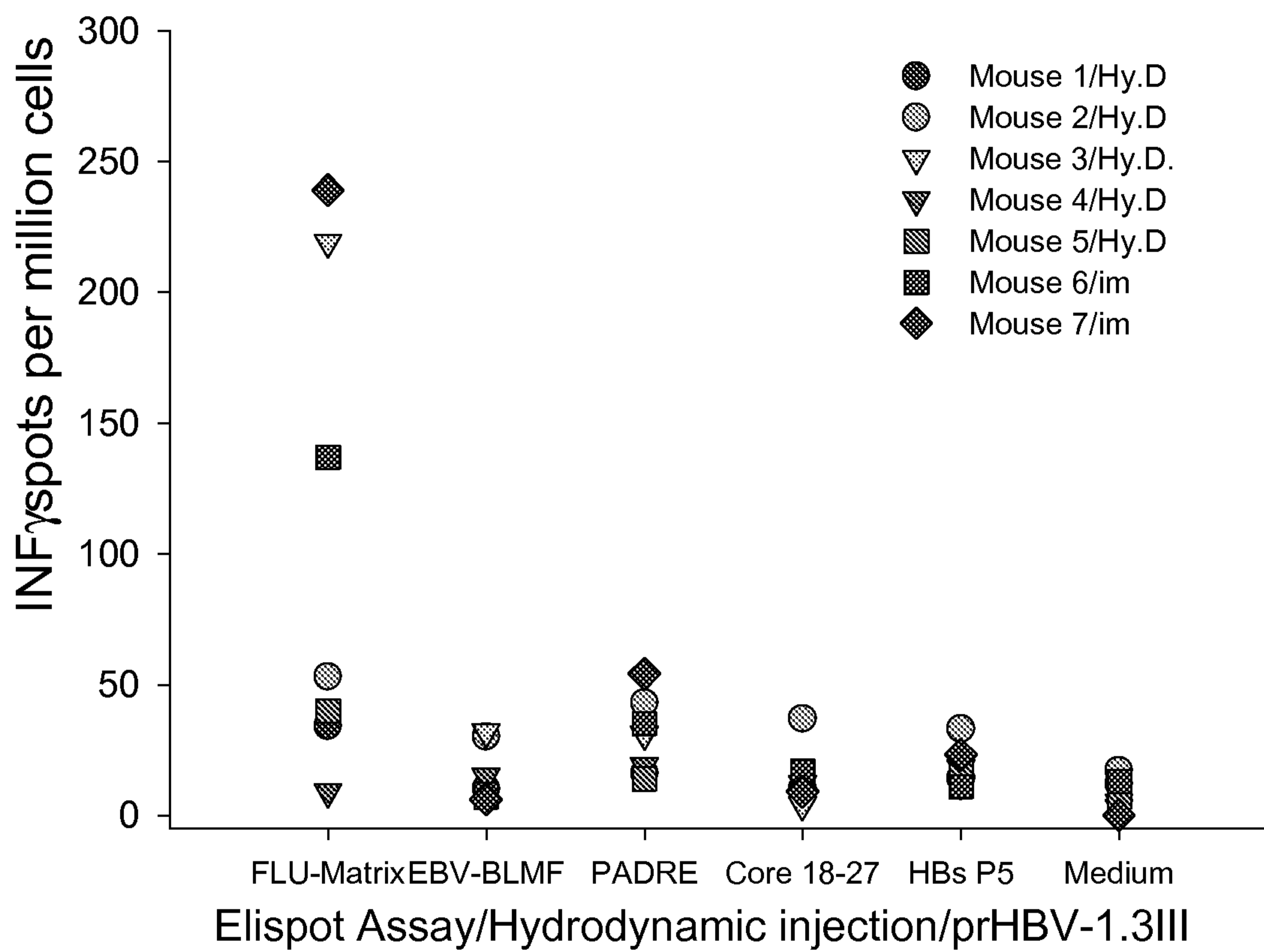


FIGURE 9

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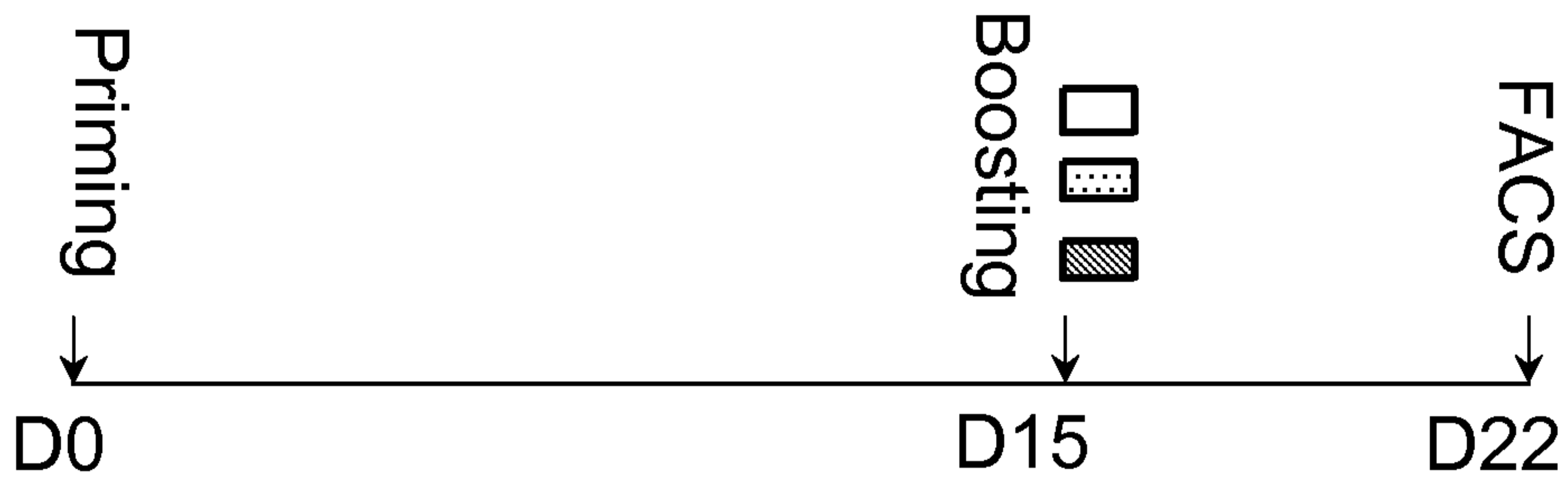


FIGURE 10A

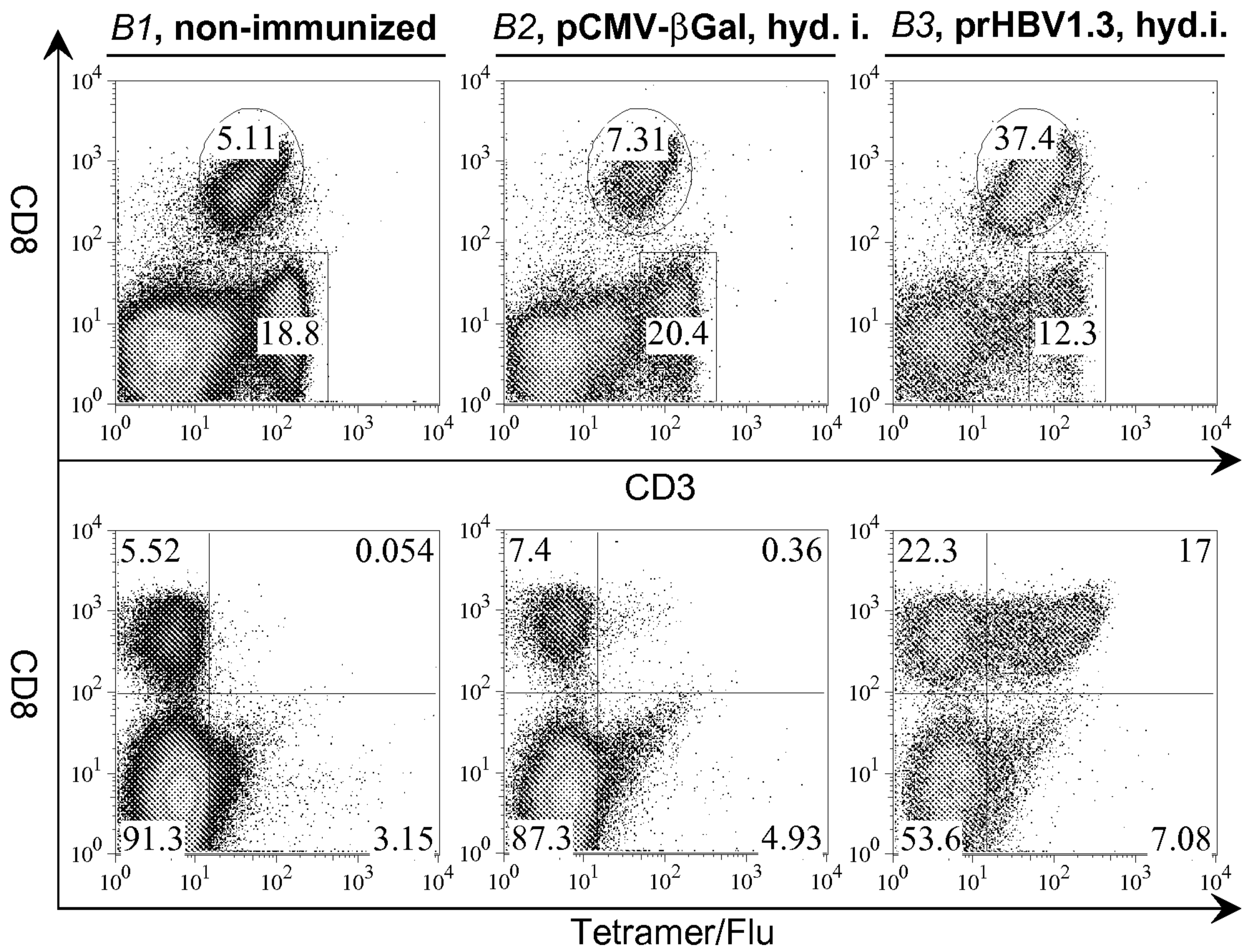


FIGURE 10B

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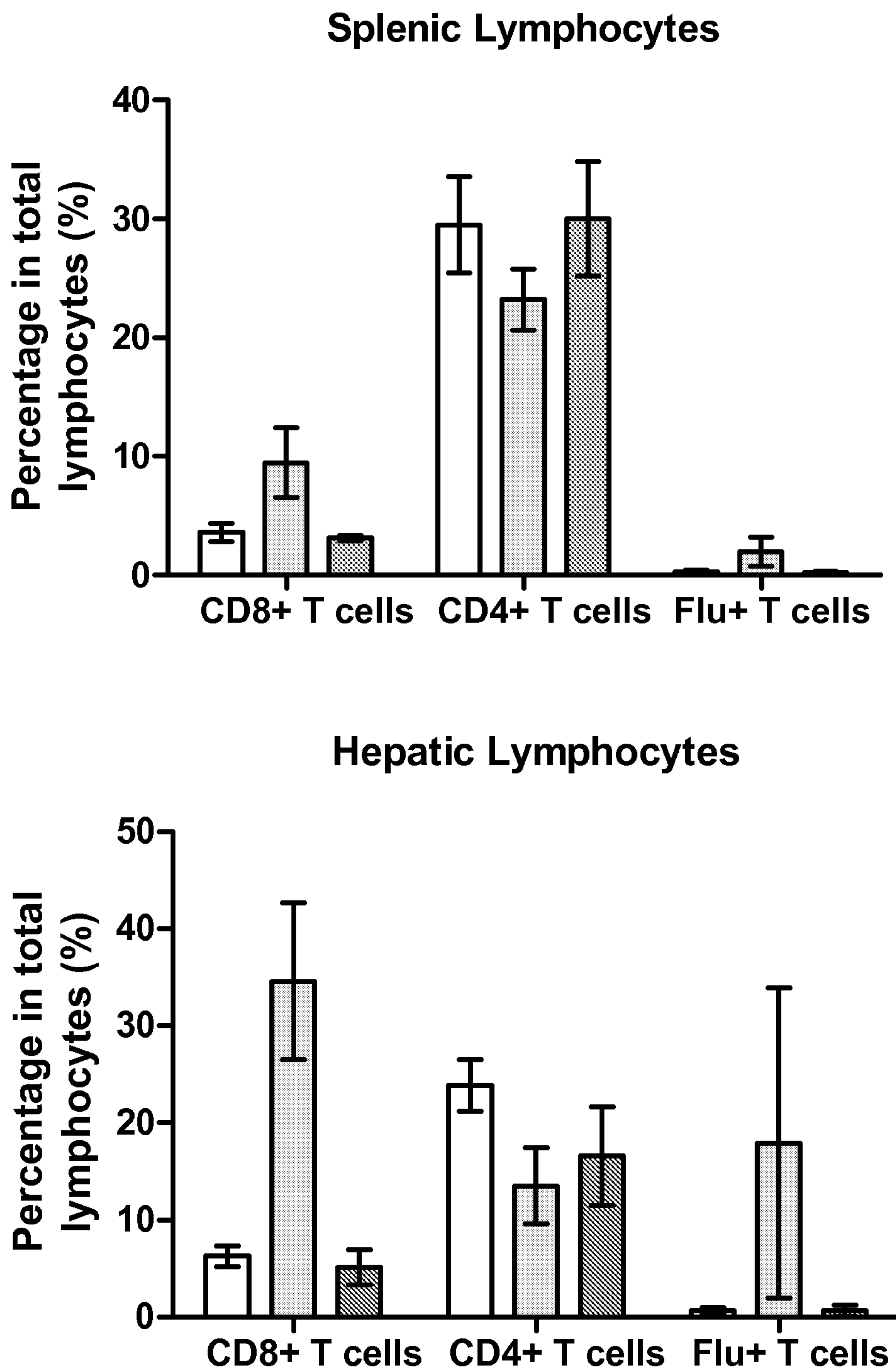


FIGURE 10C

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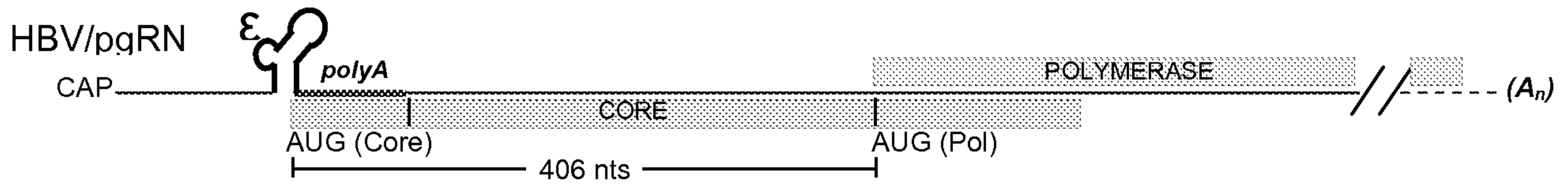


FIGURE 11A

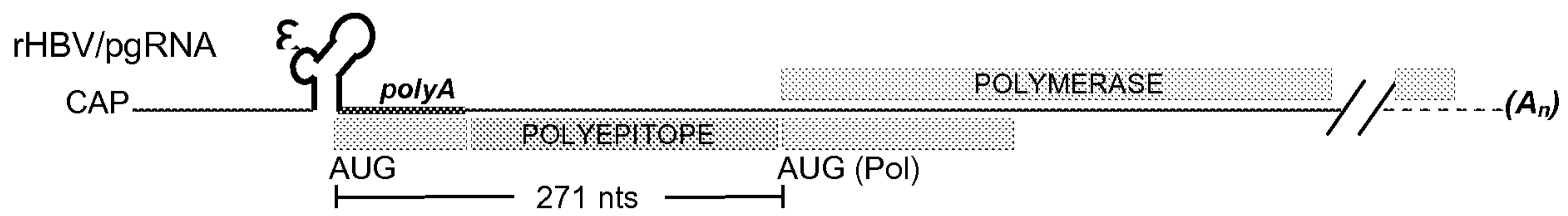


FIGURE 11B

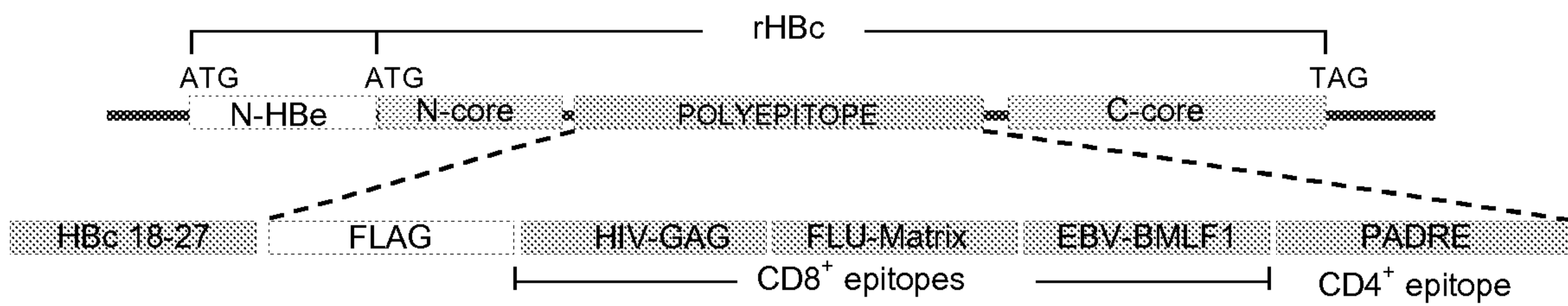


FIGURE 11C

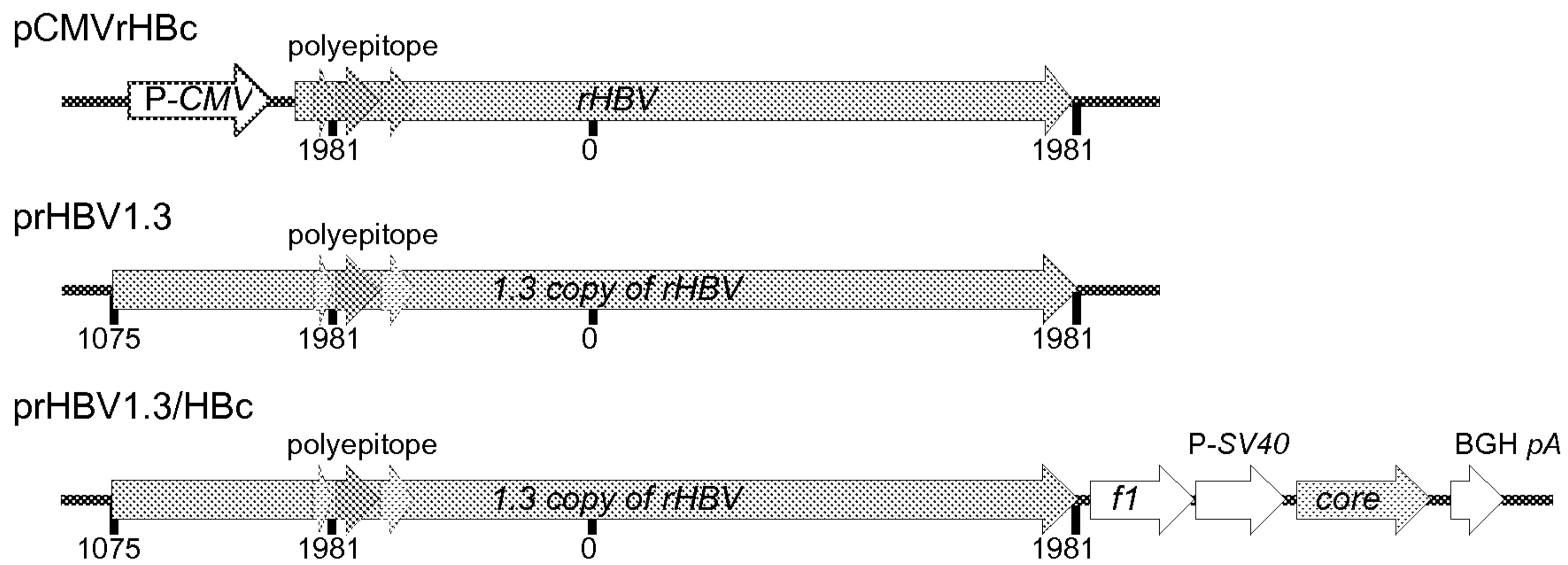


FIGURE 11D

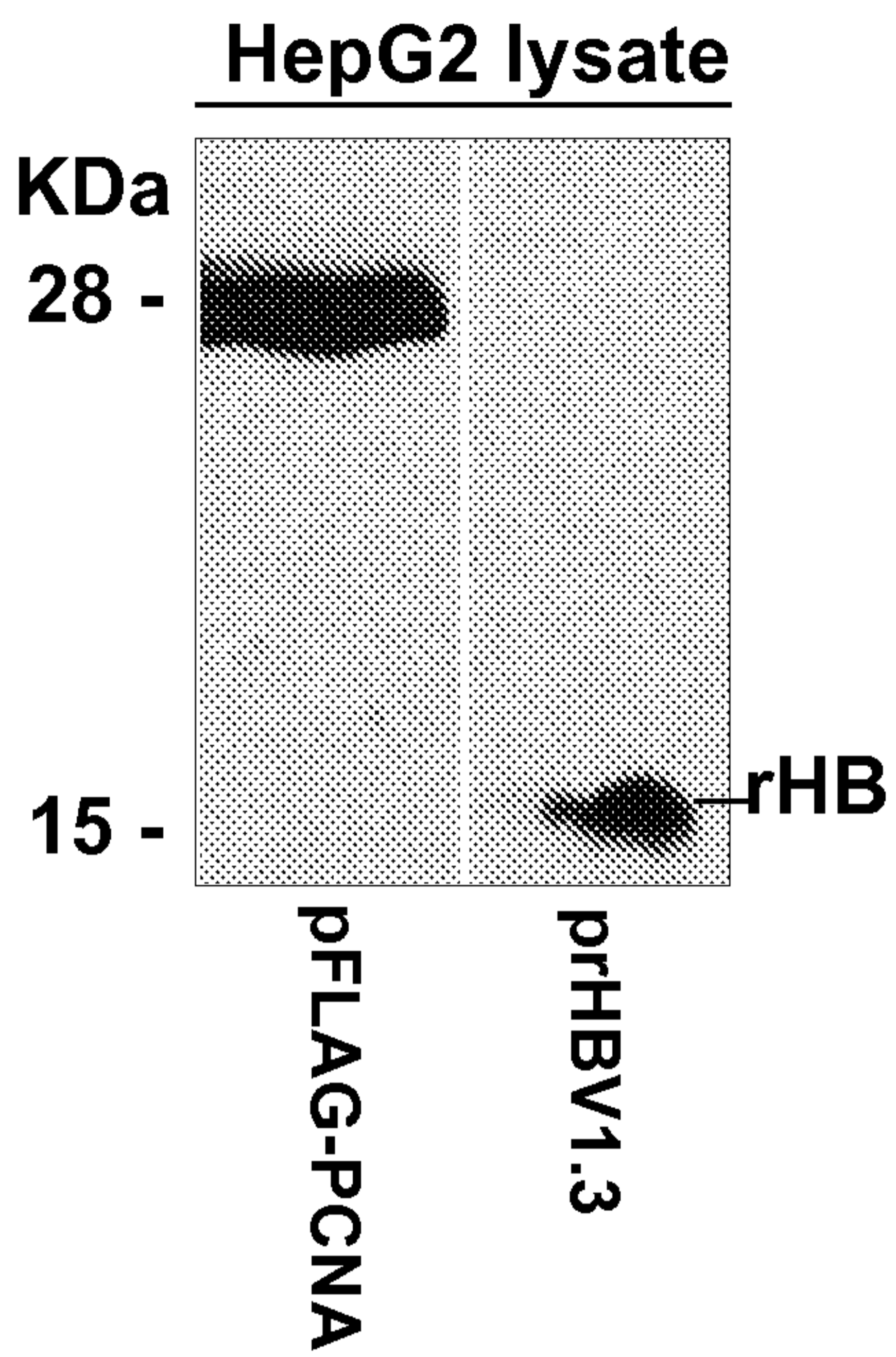


FIGURE 12A

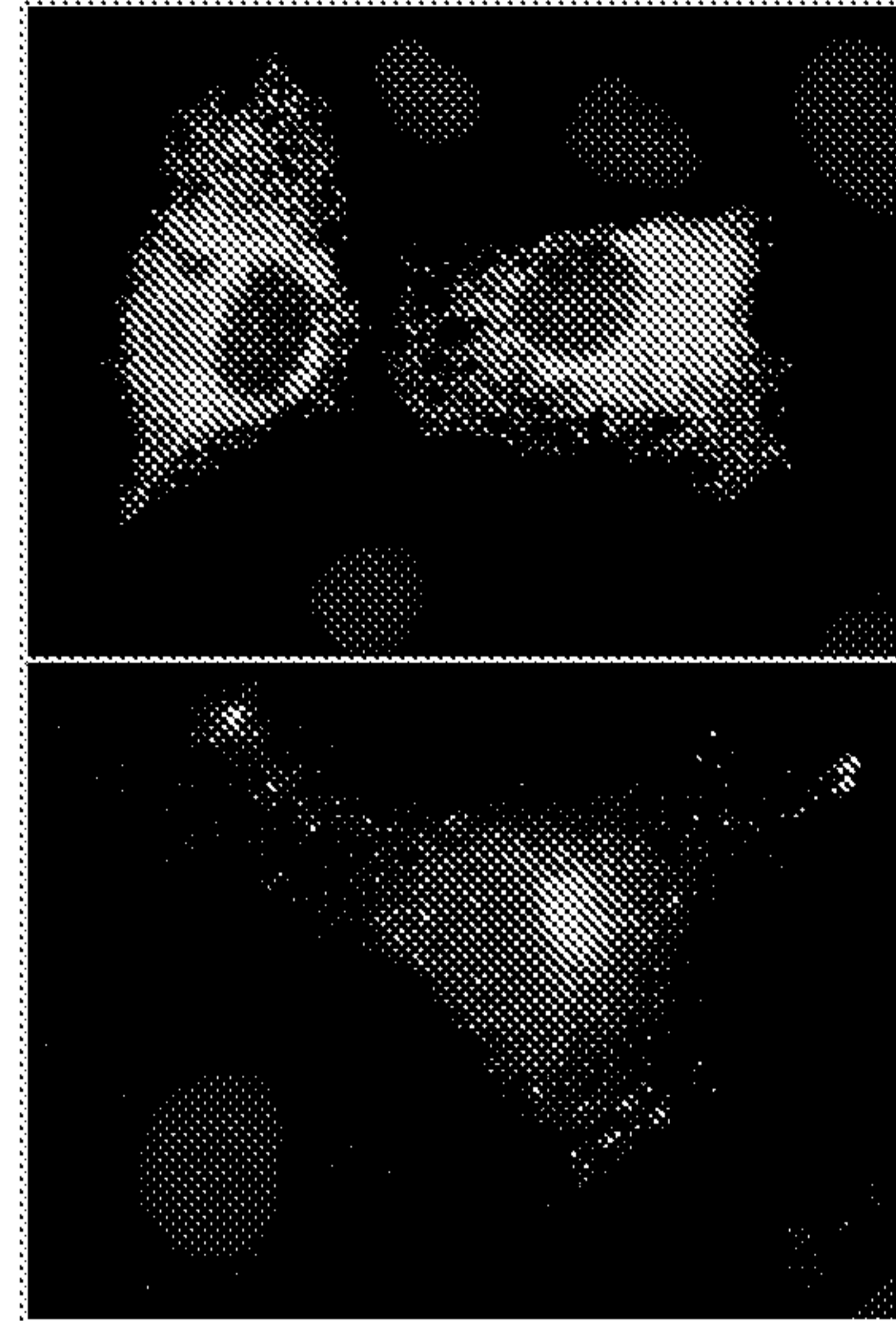


FIGURE 12B

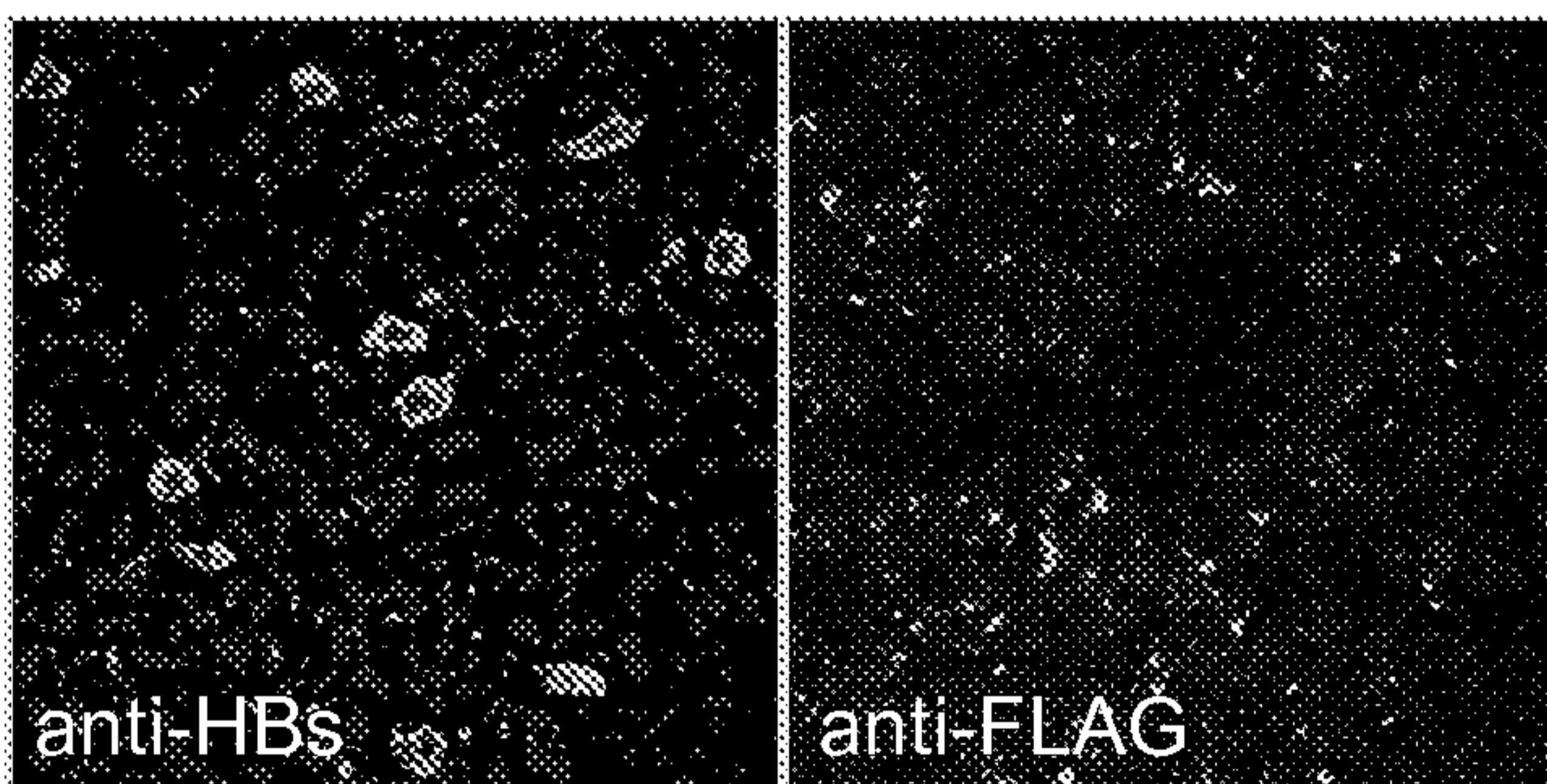


FIGURE 12C

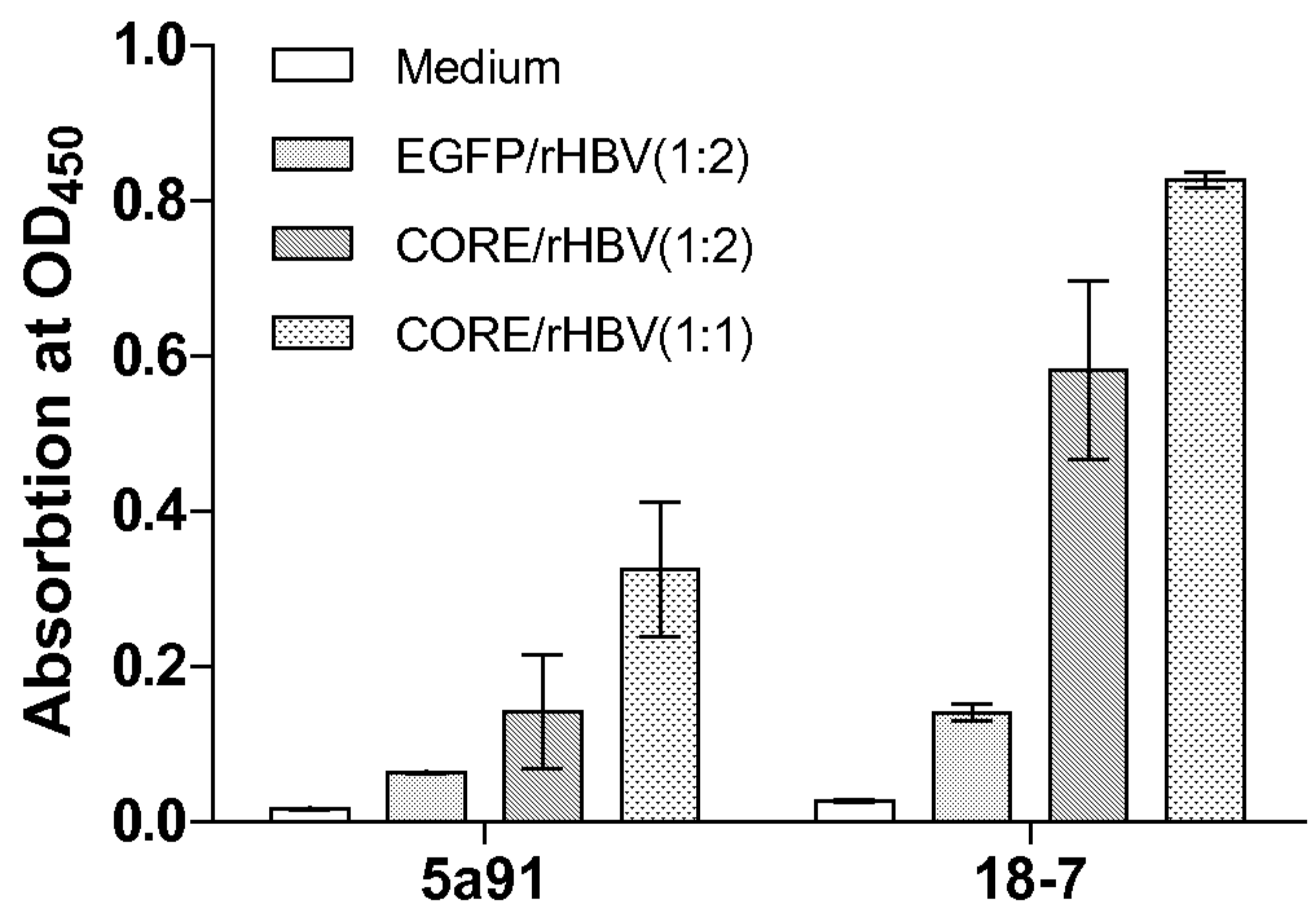


FIGURE 12D

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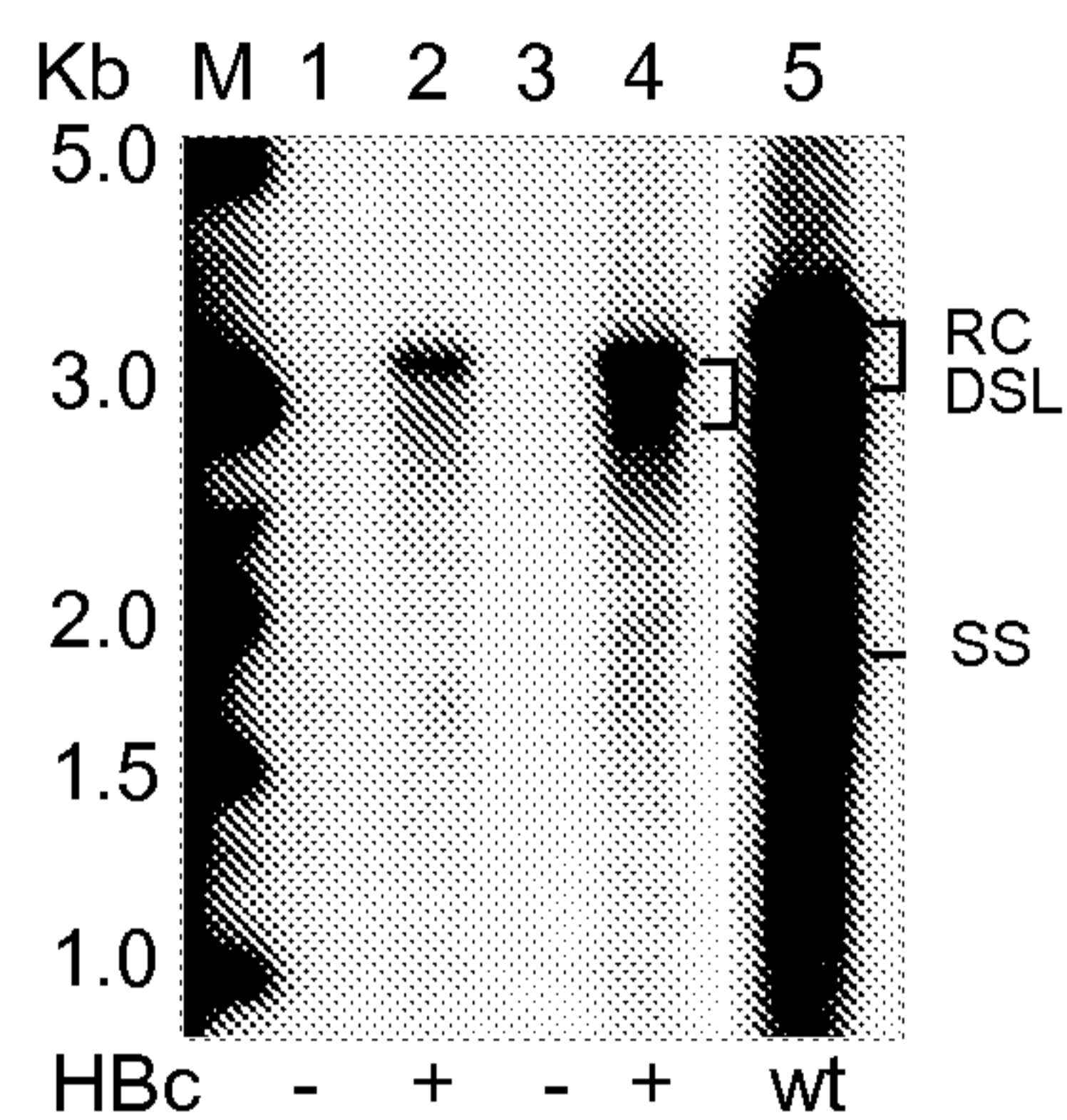


FIGURE 13A

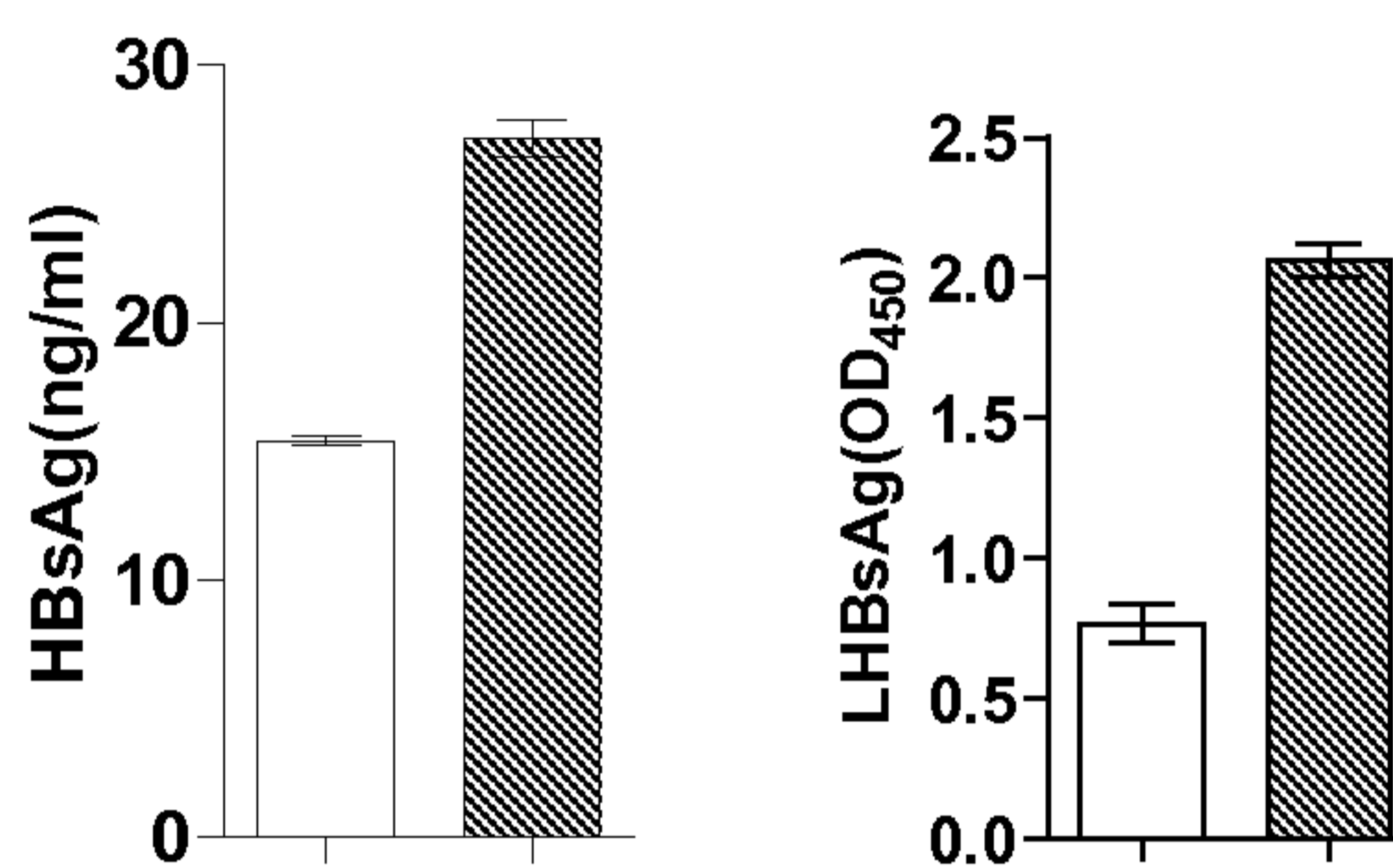


FIGURE 13B

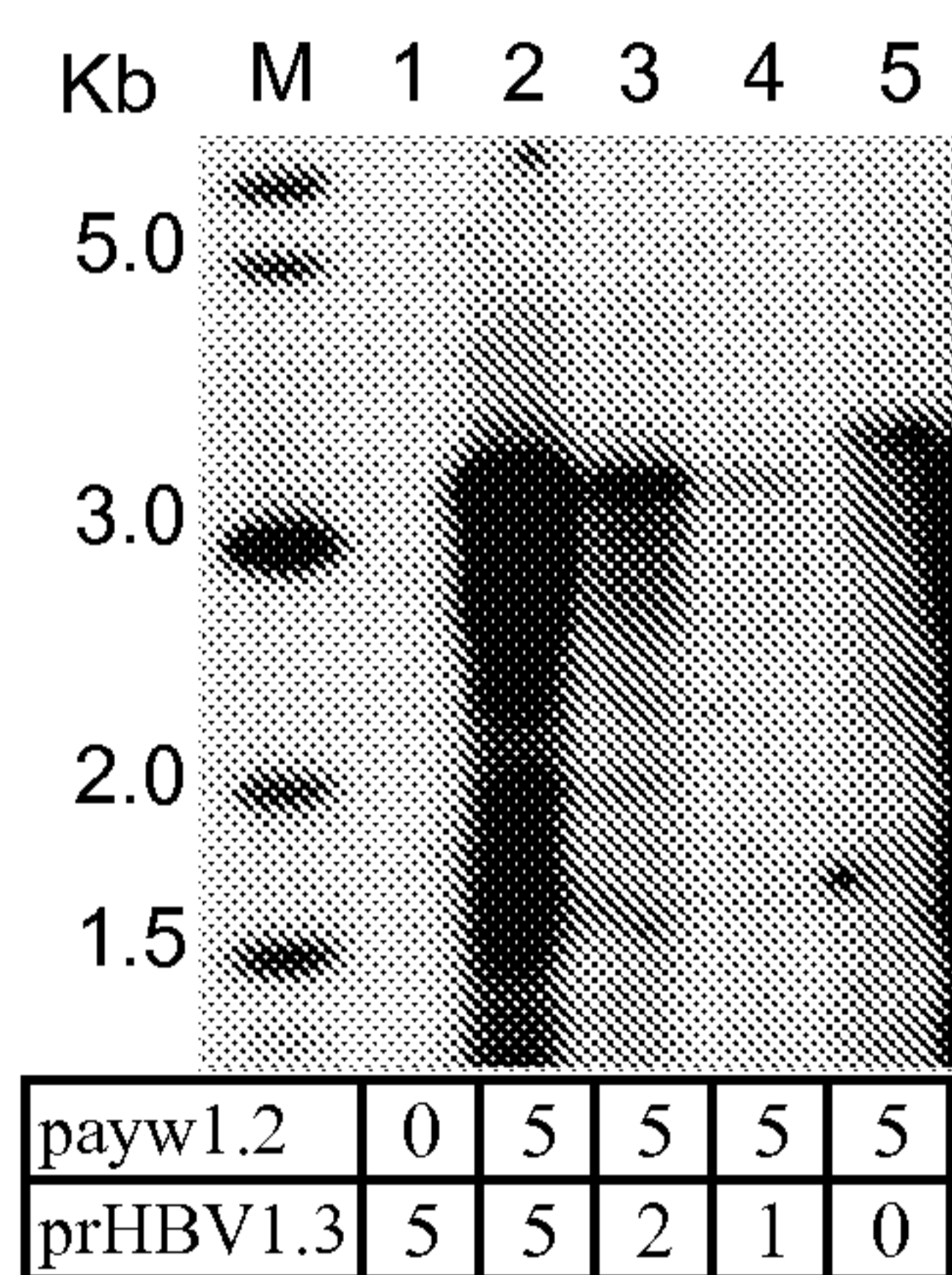


FIGURE 13C

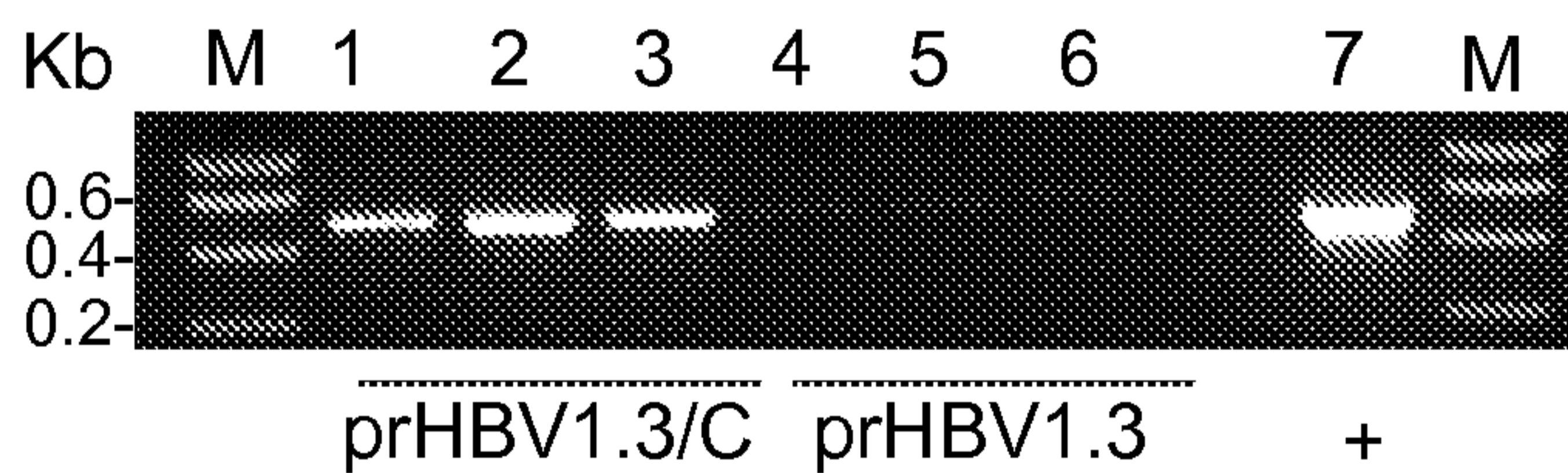


FIGURE 13D

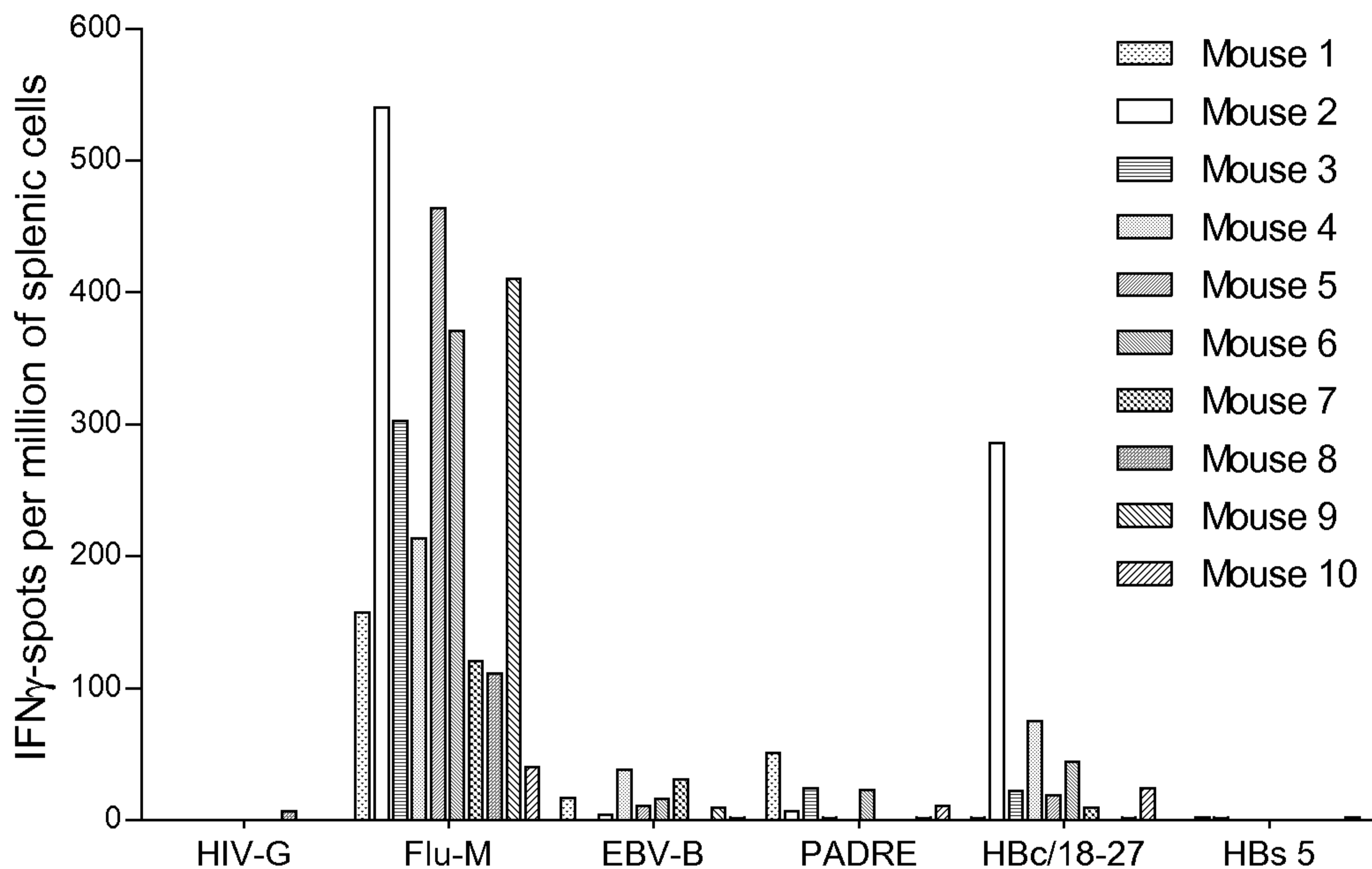


FIGURE 14A

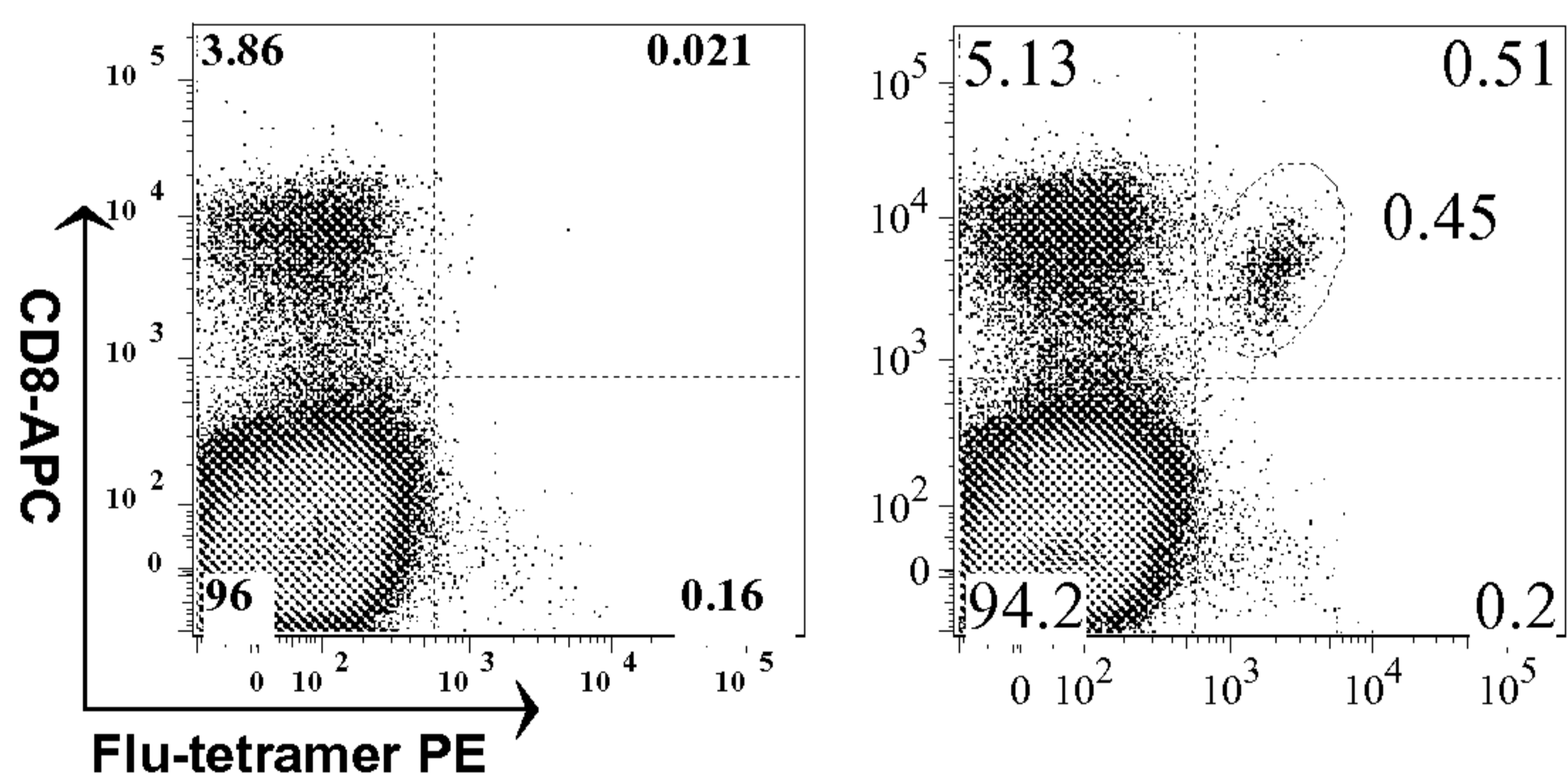


FIGURE 14B

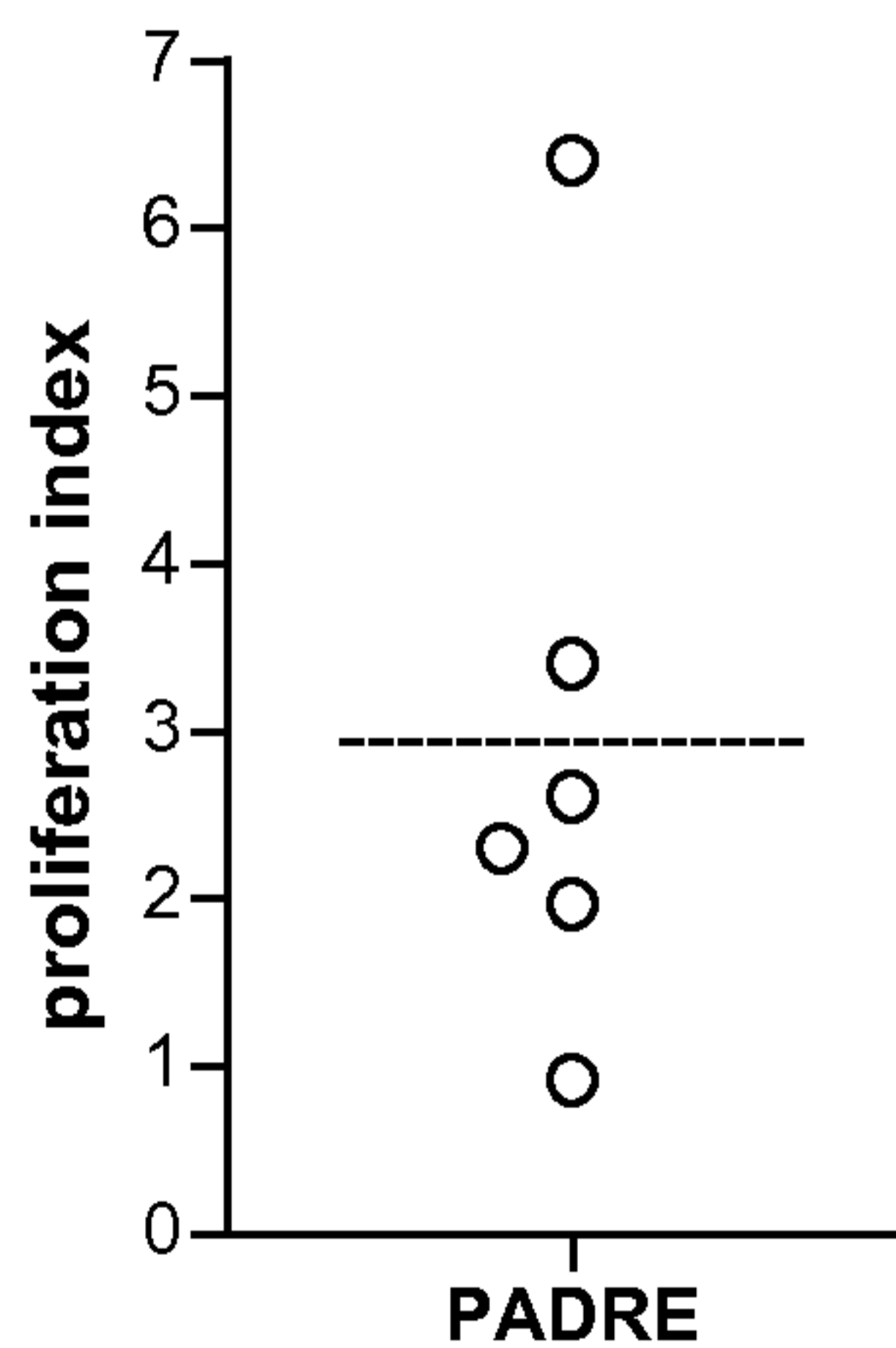


FIGURE 14C

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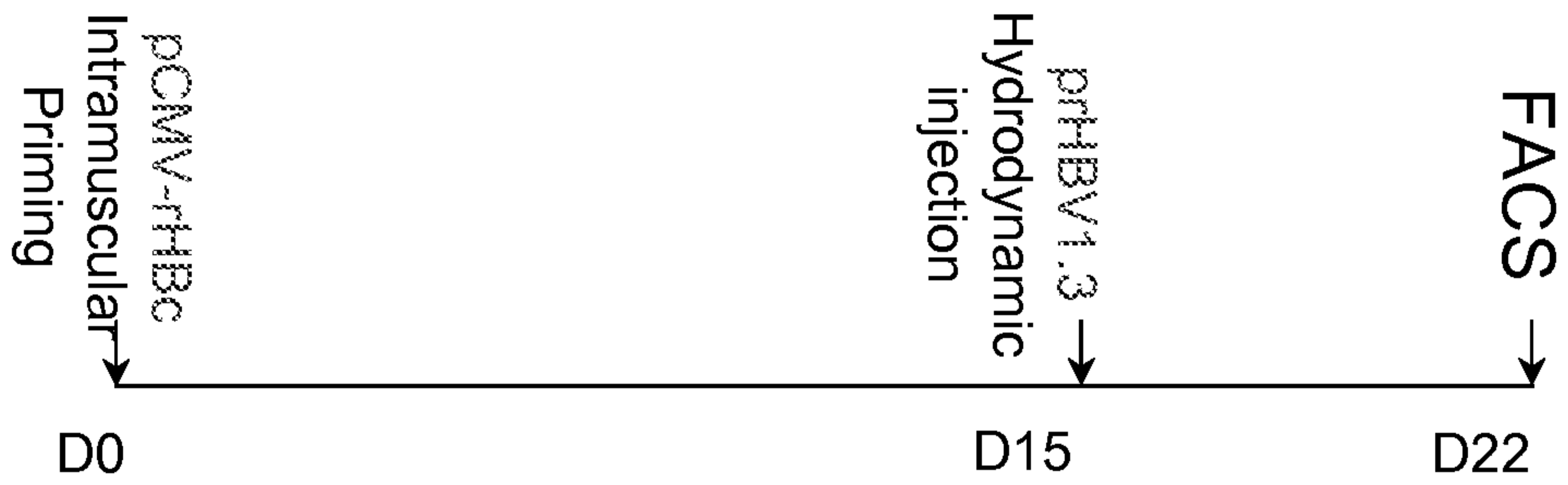


FIGURE 15A

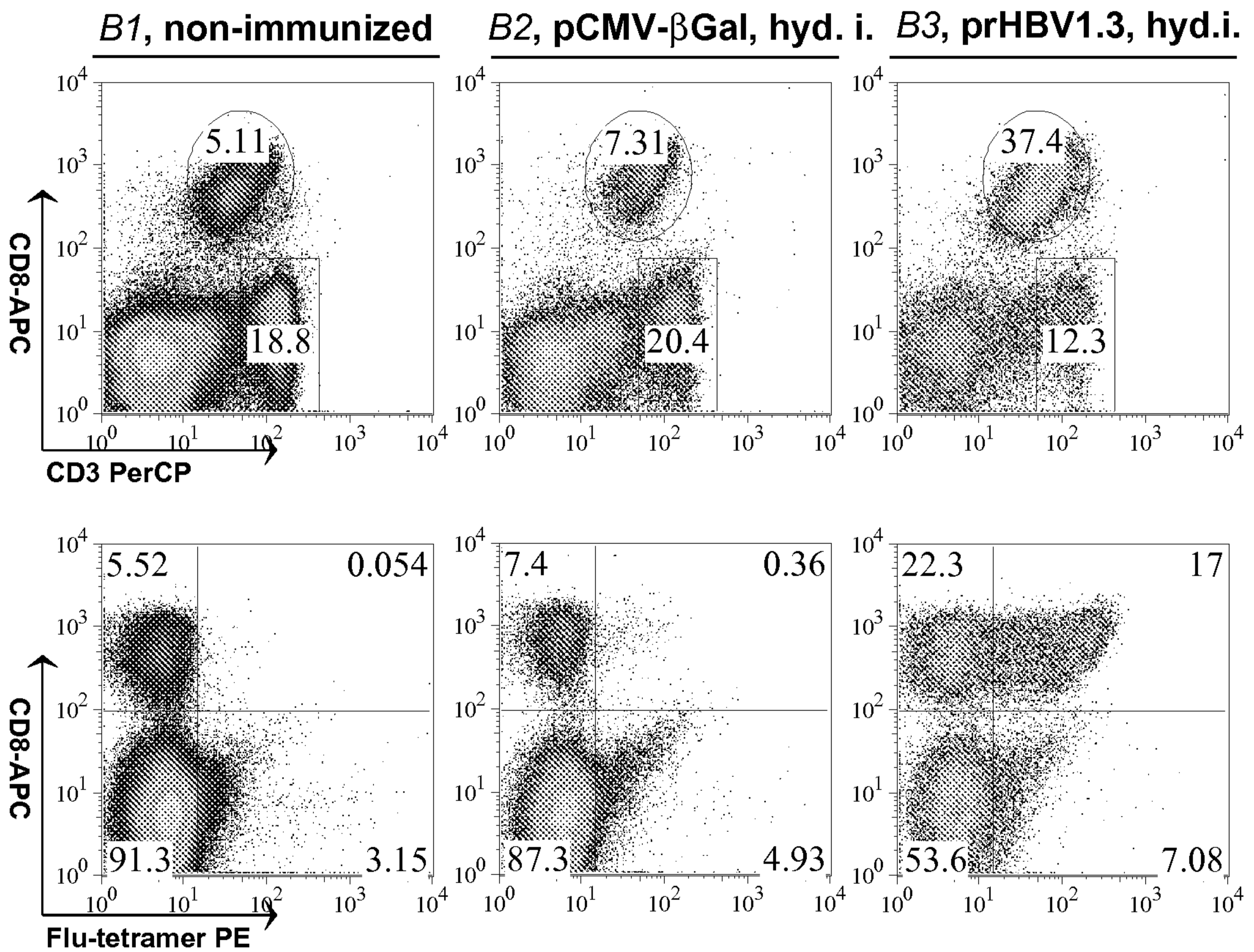


FIGURE 15B

Hepatic Lymphocytes

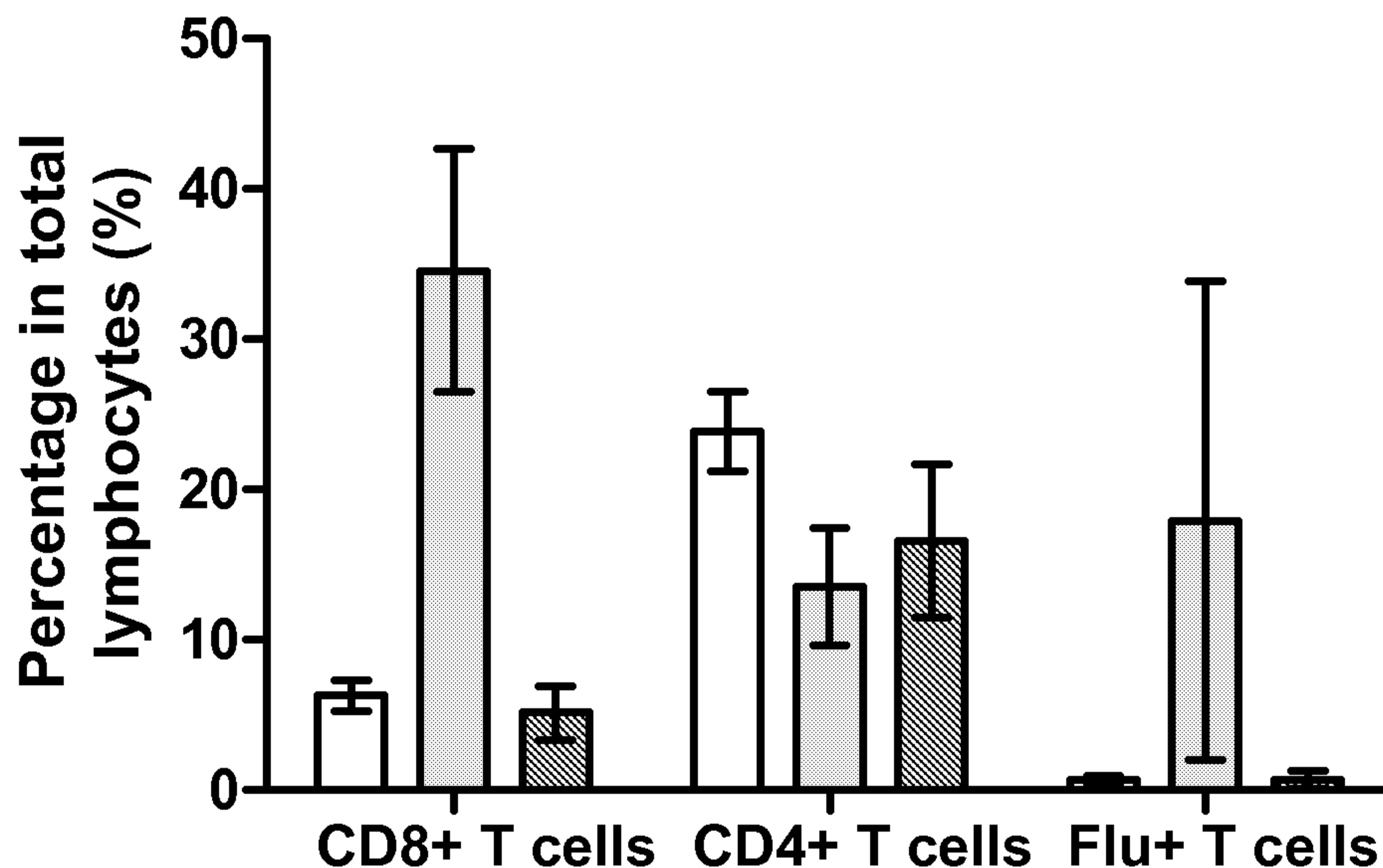


FIGURE 15C

Splenic Lymphocytes

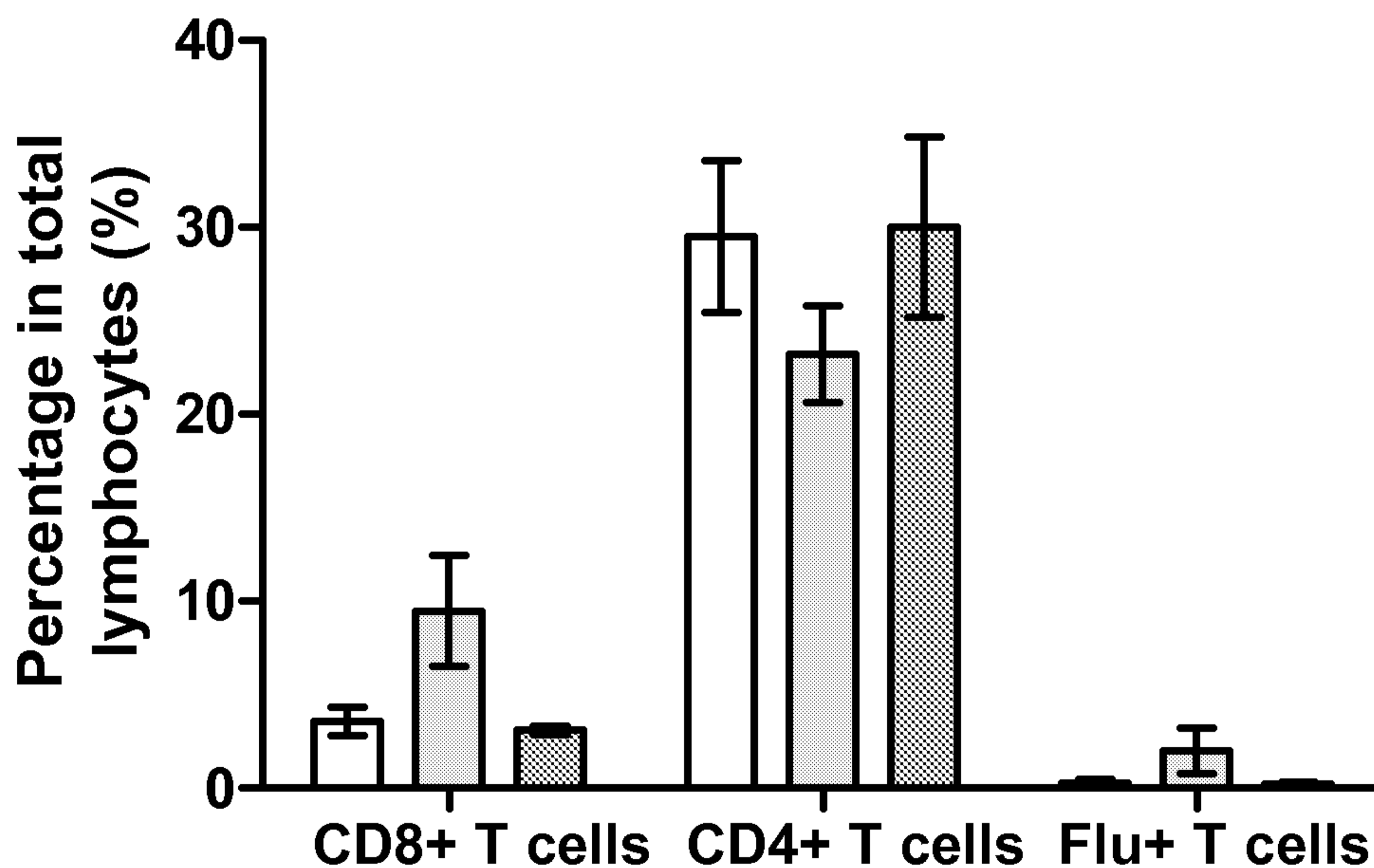


FIGURE 15D

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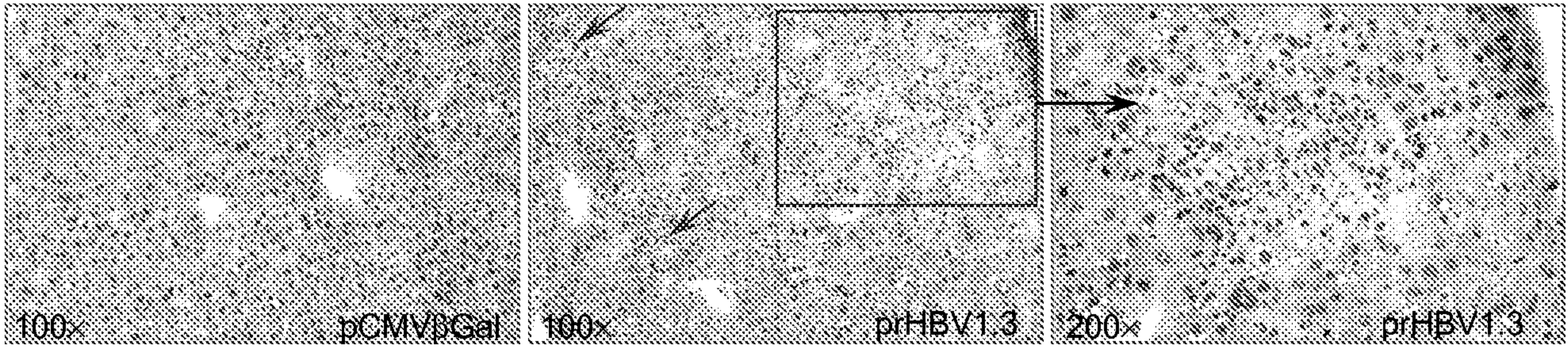


FIGURE 16A

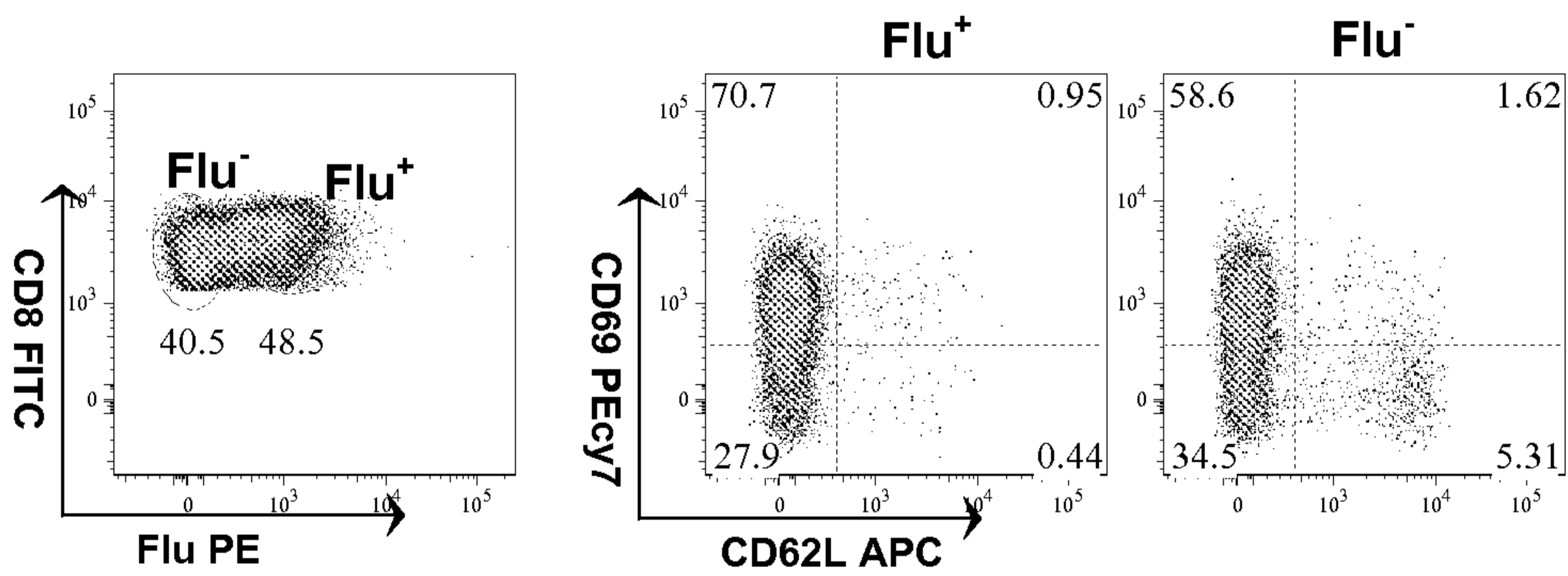


FIGURE 16B

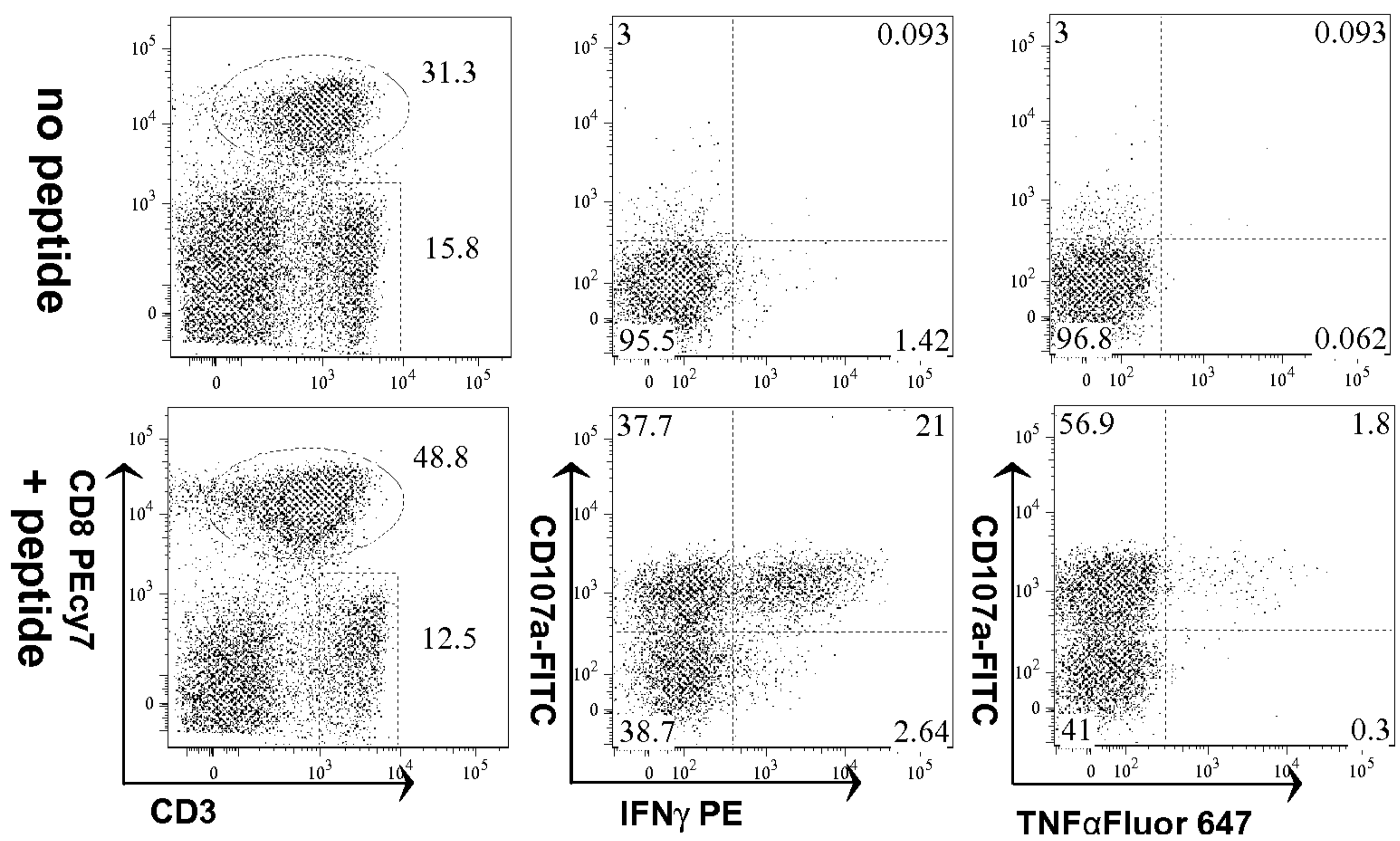


FIGURE 16C

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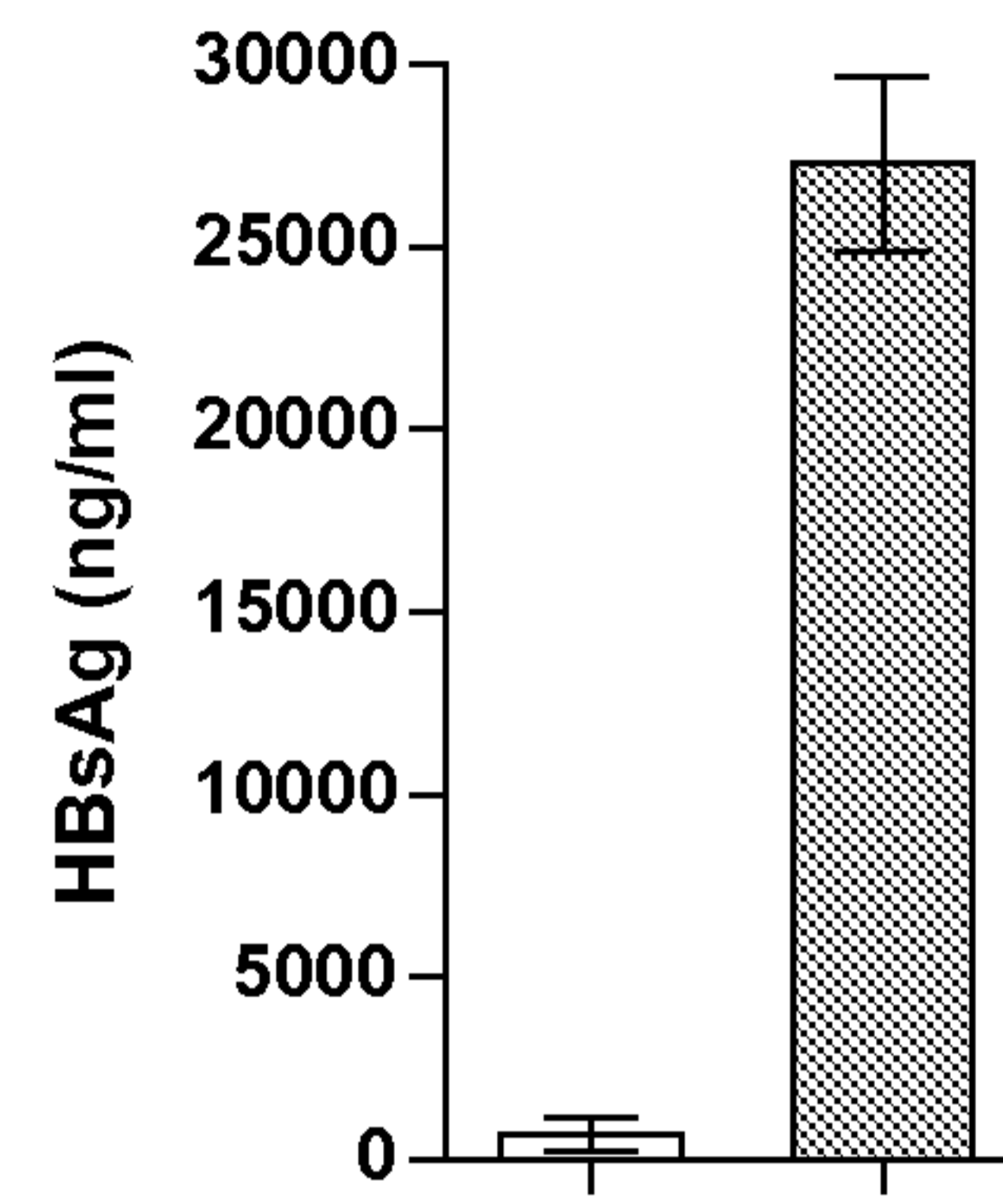
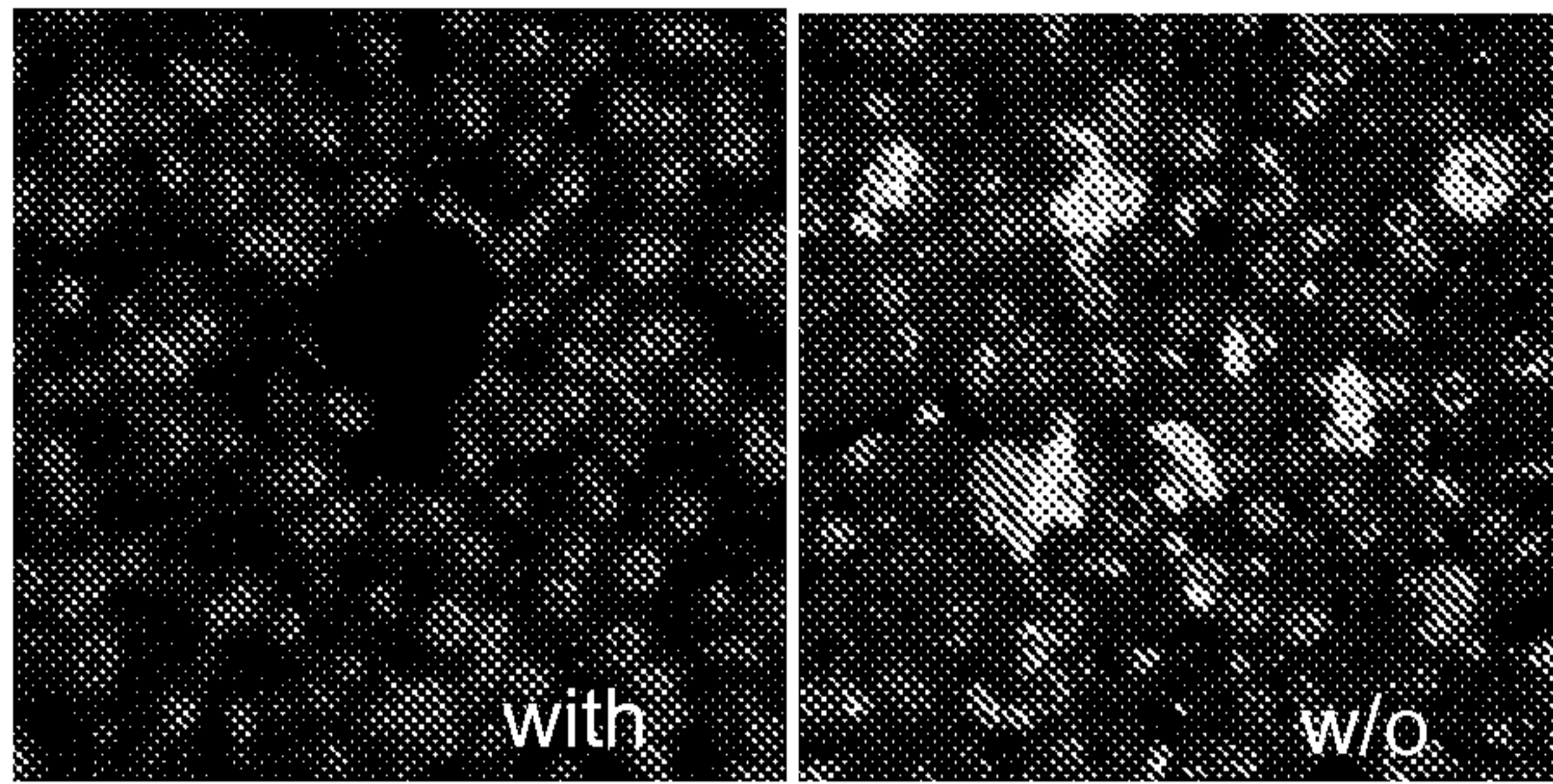


FIGURE 16D

FIGURE 16E

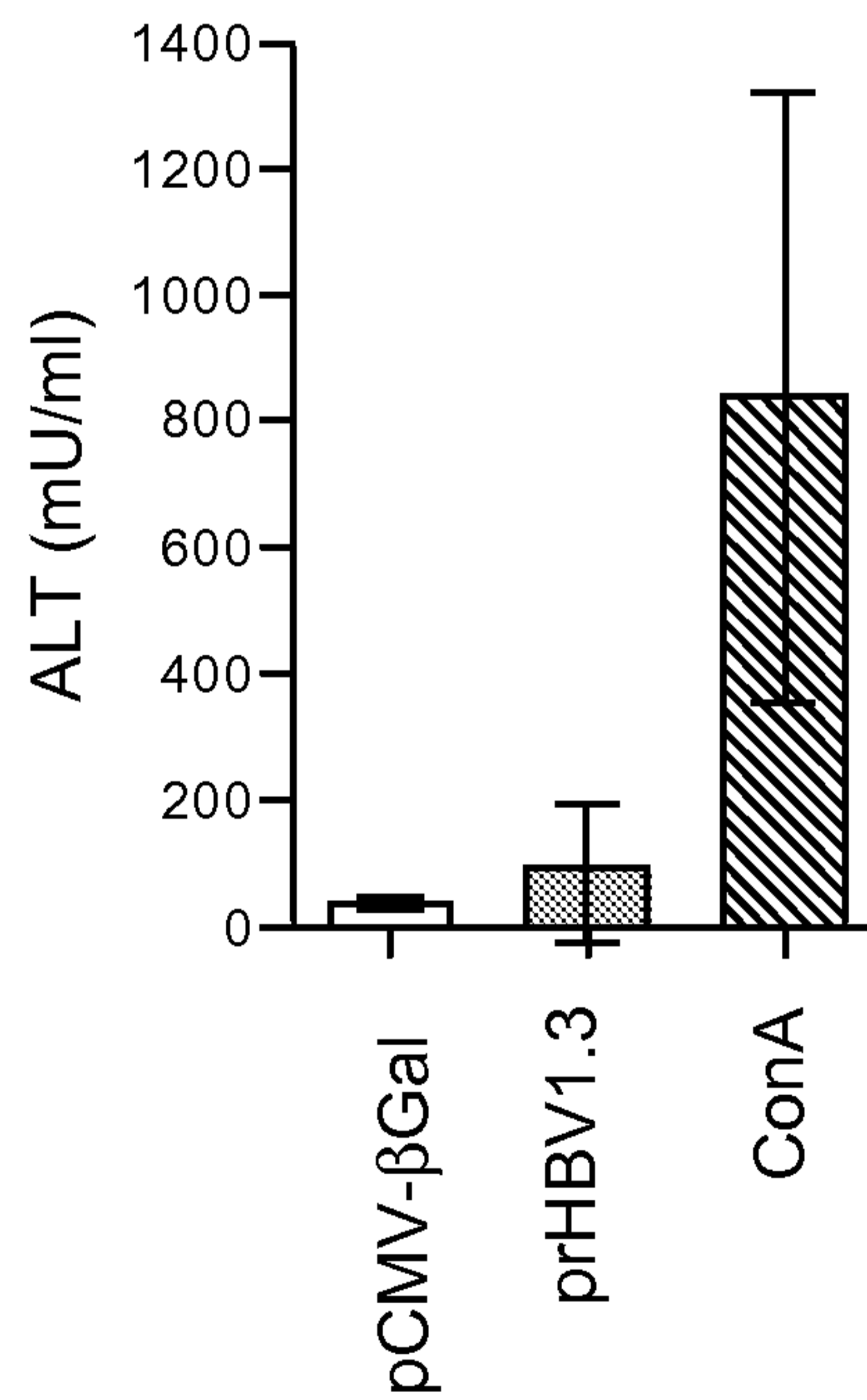


FIGURE 16F

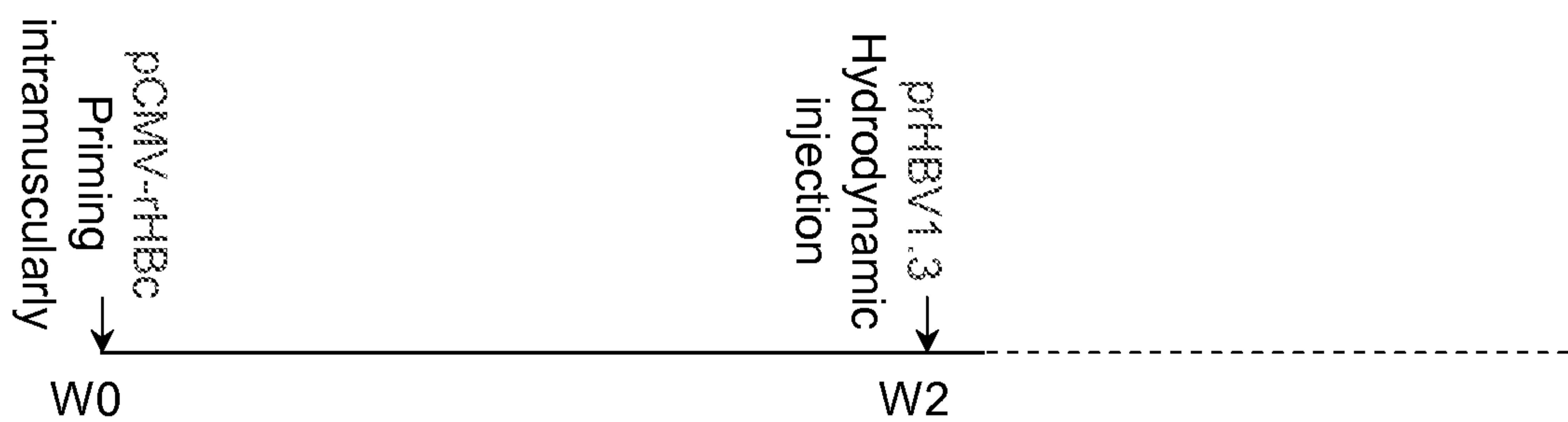


FIGURE 17A

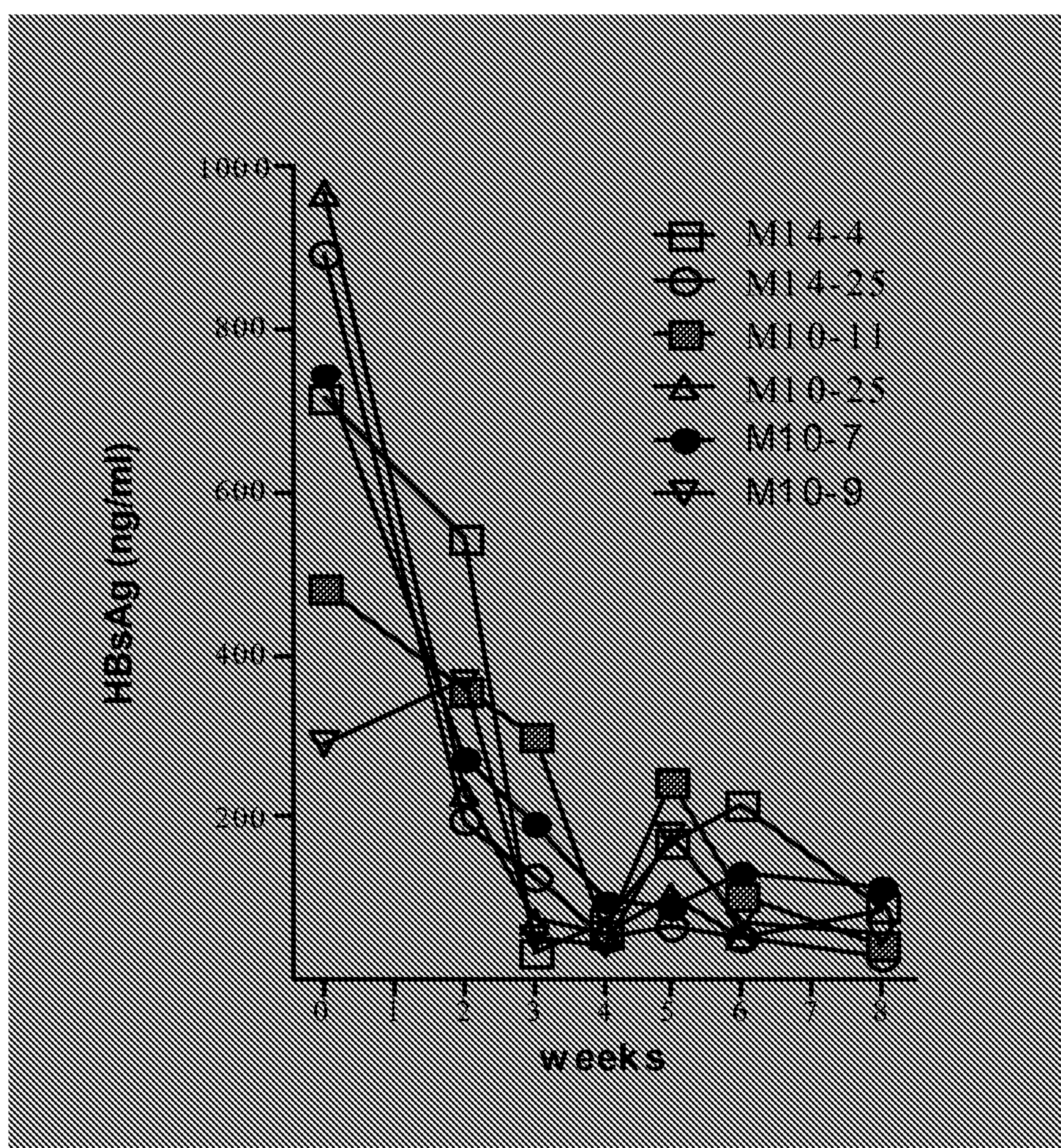


FIGURE 17B

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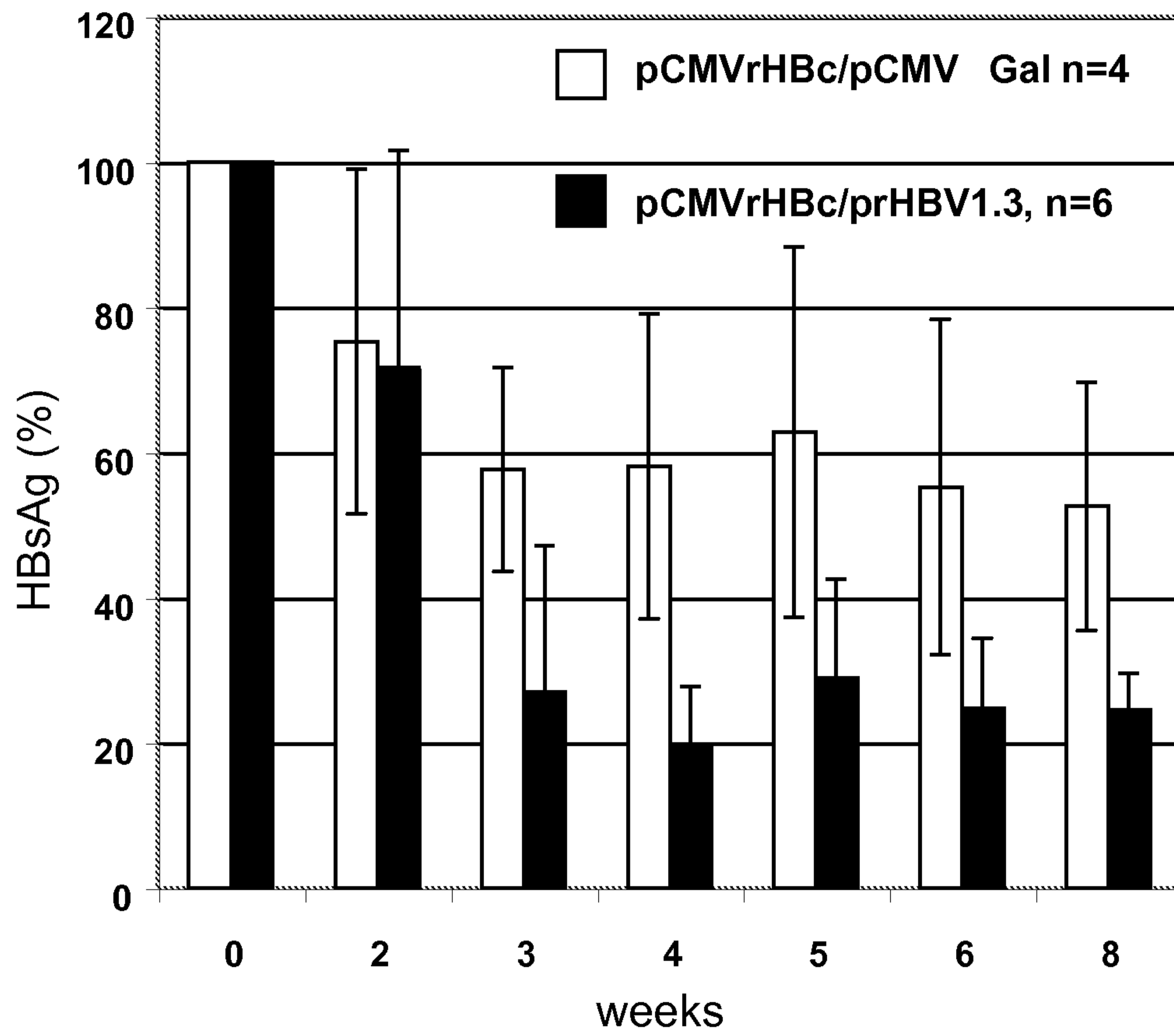


FIGURE 17C

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**Sequence for prHBV-1.3-III (SEQ ID NO: 4)
(polyepitope (polytope) sequence shadowed (SEQ ID NO: 5))**

GACGGATCGGGAGATCTCCCGATCCCCTATGGTTCGACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTAT
 CTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCGAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGA
 CAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCAATCTAAGCAGGCTTTCACCTTCTCGCCAAC
 TTACAAGGCCTTCTGTGTAAACAATACCTGAACCTTTACCCCGTTGCCCGGCAACGGCCAGGTCTGTGCCAAGTGTGTTG
 CTGACGCAACCCCACTGGCTGGGGCTTGGTCATGGGCCATCAGCGCATGCGTGGAACCTTTTCGGCTCCTCTGCCGATC
 CATACTGCGGAACTCCTAGC
 CGCTTGTGTTTGGCTCGCAGCAGGTCTGGAGCAAACATTATCGGGACTGATAACTCTGTTGTCTATCCCGCAAATATACAT
 CGTTTCCATGGCTGCTAGGCTGTGCTGCCAACTGGATCCTGCGCGGGACGTCTTTGTTTACGTCCCGTCGGCGCTGAAT
 CCTGCGGACGACCCTTCTCGGGGTGCTTGGGACTCTCTCGTCCCCTTCTCCGTCTGCCGTTCCGACCGACCACGGGGCG
 CACCTCTCTTACGCGGACTCCCCGTCTGTGCCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTACCTCTGCACGTCG
 CATGGAGACCACCGTGAACGCCACCAAATATTGCCCAAGGTCTTACATAAGAGGACTCTTGGACTCTCAGCAATGTCAA
 CGACCGACCTTGAGGCATACTTCAAAGACTGTTTGTGTTAAAGACTGGGAGGAGTTGGGGGAGGAGATTAGGTTAAAGGTC
 TTTGTACTAGGAGGCTGTAGGCATAAATTGGTCTGCGCACCAGCACCATGCAACTTTTTTACCTCTGCCTAATCATCTCT
 TGTTTATGTCCTACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGCATGGACATCGACCCTTATAAAGAATT
 TGGAGCTACTGTGGAGTTACTCTCGTTTTTGCCTTCTGACTTCTTTCTTTCAGTAAGCTTCGACTACAAGGACGACGACG
 ACAAGAGCCTGTTCAACACCGTGGCCACCCTGTACACCAAGGGCATCCTGGGGCTTCGTGTTTACCCCTGAAGAACGCGCGC
 CTGTGCACCCTGGTGGCCATGCTGGGCCCCGGCCCCGGCAAGGCCAAGTTCCGTGGCCGCTTGGACCCTGAAGGCTGCAGC
 CGAACATGGAGCCCTATCCTATCAACACTTCCGGAGACTACTGTTGTTAGACGACGAGGCAGGTCCCCTAGAAGAAGAA
 CTCCTCGCCTCGCAGACGAAGGTCTCAATCGCCGCGTCGCAGAAGATCTCAATCTCGGGAATCTCAATGTTAGTATTCC
 TTGGACTCATAAGGTGGGGAACCTTACTGGGCTTTATTCTTCTACTGTACCTGTCTTTAATCCTCATTGGAAAACACCAT
 CTTTTCTAATATACATTTACACCAAGACATTATCAAAAAATGTGAACAGTTTTGTAGGCCACTCACAGTTAATGAGAAA
 AGAAGATTGCAATTGATTATGCCTGCCAGGTTTTATCCAAAGGTTACCAAATATTTACCATTGGATAAGGGTATTAAACC
 TTATTATCCAGAACATCTAGTTAATCATTACTTCCAACTAGACACTATTTACACACTCTATGGAAGGCGGGTATATTAT
 ATAAGAGAGAAACAACACATAGCGCCTCATTTTTGTGGGTACCATATTTCTTGGGAACAAGATCTACAGCATGGGGCAGAA
 TCTTTCCACCAGCAATCCTCTGGGATTCTTTCCCGACCACCAGTTGGATCCAGCCTTCAGAGCAAACACCGCAAATCCAG
 ATTGGGACTTCAATCCCAACAAGGACACCTGGCCAGACGCCAACAAGGTAGGAGCTGGAGCATTCGGGCTGGGTTTCACC
 CCACCGCACGGAGGCCTTTTGGGGTGGAGCCCTCAGGCTCAGGGCATACTACAACTTTGCCAGCAAATCCGCCTCCTGC
 CTCACCAATCGCCAGTCAGGAAGGCAGCCTACCCCGCTGTCTCCACCTTTGAGAAACACTCATCCTCAGGCCATGCAGT
 GGAATTCCACAACCTTCCACCAAACCTCTGCAAGATCCAGAGTGAGAGGCCTGTATTTCCCTGCTGGTGGCTCCAGTTCA
 GGAACAGTAAACCCTGTTCTGACTACTGCCCTCTCCCTTATCGTCAATCTTCTCGAGGATTGGGGACCCTGCGCTGAACAT
 GGAGAACATCACATCAGGATTCTTAGGACCCCTTCTCGTGTTACAGGCGGGGTTTTTCTTGTGACAAGAATCCTCACAA
 TACCGCAGAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGAACCTACCGTGTGTCCTGGCCAAAATTCGCAG
 TCCCCAACCTCCAATCACTACCAACCTCTTGTCTCCAACCTTGTCTGGTTATCGCTGGATGTGTCTGCGGCGTTTTAT
 CATCTTCTCTTTCATCCTGCTGCTATGCCTCATCTTCTTGTGTTGTTCTTCTGGACTATCAAGGTATGTTGCCCGTTTTGTC
 CTCTAATTCCAGGATCCTCAACAACCAGCACGGGACCATGCCGGACCTGCATGACTACTGCTCAAGGAACCTCTATGTAT
 CCCTCCTGTTGCTGTACCAAACCTTCGGACGGAAATTGCACCTGTATTCCCATCCCATCATCCTGGGCTTTCGGAAAATT
 CCTATGGGAGTGGCCCTCAGCCCGTTTTCTCTGGCTCAGTTTACTAGTGCCATTTGTTTCAAGTGGTTTCGTAGGGCTTTCC
 CCACTGTTTTGGCTTTCAGTTATATGGATGATGTGGTATTGGGGGCCAAGTCTGTACAGCATCTTGAGTCCCTTTTTACCG
 CTGTTACCAATTTTCTTTTGTCTTTGGGTATACATTTAAACCCTAACAAAACAAGAGATGGGGTTACTCTCTAAATTTT
 ATGGGTTATGTCATTGGATGTTATGGGTCCCTGCCACAAGAACACATCATAAAAAAATCAAAGAATGTTTTAGAAAAT
 TCCTATTAACAGGCCTATTGATTGGAAAGTATGTCAACGAATTGTGGGTCTTTTGGGTTTTGCTGCCCTTTTACACAAT
 GTGGTTATCCTGCGTTGATGCCTTTGTATGCATGTATTCAATCTAAGCAGGCTTTCACCTTCTCGCCAACCTTACAAGGCC
 TTTCTGTGTAAACAATACCTGAACCTTTACCCCGTTGCCCGGCAACGGCCAGGTCTGTGCCAAGTGTGTTGCTGACGCAAC
 CCCACTGGCTGGGGCTTGGTCATGGGCCATCAGCGCATGCGTGGAACCTTTTCGGCTCCTCTGCCGATCCATACTGCCG
 AACTCCTAGCCGCTTGTGTTTGTCTCGCAGCAGGTCTGGAGCAAACATTATCGGGACTGATAACTCTGTTGTCTATCCCGC
 AAATATACATCGTTTTCCATGGCTGCTAGGCTGTGCTGCCAACTGGATCCTGCGCGGGACGTCTTTGTTTACGTCCCGTC
 GCGCTGAATCCTGCGGACGACCCTTCTCGGGGTGCTTGGGACTCTCTCGTCCCCTTCTCCGTCTGCCGTTCCGACCGA
 CCACGGGGCGCACCTCTCTTTACGCGGACTCCCCGTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTACCT
 CTGCACGTGCGATGGAGACCACCGTGAACGCCACCAAATATTGCCCAAGGTCTTACATAAGAGGACTCTTGGACTCTCA
 GCAATGTCAACGACCGACCTTGAGGCATACTTCAAAGACTGTTTGTGTTAAAGACTGGGAGGAGTTGGGGGAGGAGATTAG

FIGURE 18

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GTAAAGGCTTTGTACTAGGAGGCTGTAGGCATAAATTGGTCTGCGCACCAGCACCATGCAACTTTTTTCACCTCTGCCT
AATCATCTCTTGTTCATGTCTACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGCATGGACATCGACCCTT
ATAAAGAATTTGGAGCTACTGTGGAGTTACTCTCGTTTTTGCCTTCTGACTTCTTTCCTTCAGTACGAGATCCACTAGTT
CTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCTTTAGTGAGGGTTAATTGCGCGCATGCCCGACGGCGA
GGATCTCGTTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACT
GTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGGCGAA
TGGGCTGACCGCTTCTCGTGCTTTACGGTATCGCCGCTCCCGATTTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGA
GTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCAACCTGCCATCACGAGATTTTCGATTCCAC
CGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCA
TGCTGGAGTTCTTCGCCACCCCAACTTGTATTATGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTT
ACAAATAAAGCATTTTTTTTCCTGCTTCTAGTTGTGGTTTTGTCCAACTCATCAATGTATCTTATCATGTCTGTATAACC
GTCGACCTCTAGCTAGAGCTTGGCGTAATCATGGTCATAGCTGTTTCTGTGTGAAATTGTTATCCGCTCACAATTTCCAC
ACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGC
TCACTGCCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGGCGGTTT
GCGTATTGGGCGCTTCTCCGCTTCTCGCTCACTGACTCGCTGCGCTCGGTCGTTTCGGCTGCGGCGAGCGGTATCAGCTC
ACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAGGCCAGCAAAG
GCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAATCGACG
CTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATAACCAGGCGTTTTCCCTGGAAGCTCCCTCGTGCGCTCTC
CTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCTTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGC
TGTAGGTATCTCAGTTCGGTGTAGGTCGTTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCGTTTCAGCCCGACCGCTG
CGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACA
GGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACA
GTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCAC
CGCTGGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCT
TTTCTACGGGGTCTGACGCTCAGTGGAAACGAAAACCTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTC
ACCTAGATCCTTTTTAAATTAATAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCA
ATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGA
TAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGAT
TTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCTGCAACTTTATCCGCTCCATCCAGTCTAT
TAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTTCGCAACGTTGTTGCCATTGCTACAGGCATCG
TGGTGTACGCTCGTTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATG
TTGTGCAAAAAGCGGTTAGCTCCTTCGGTCTCCGATCGTTGTCAGAAGTAAGTTGGCCGAGTGTATCACTCATGGT
TATGGCAGCACTGCATAATTCTCTTACTGTTCATGCCATCCGTAAGATGCTTTTTCTGTGACTGGTGAGTACTCAACCAAGT
CATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGA
ACTTTAAAAGTGCTCATCATTTGGAAAACGTTCTTCGGGGCGAAAACCTCAAGGATCTTACCGCTGTTGAGATCCAGTTC
GATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAACAGGAA
GGCAAAATGCCGCAAAAAGGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGA
AGCATTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCG
CACATTTCCCCGAAAAGTGCCACCTGACGTC

FIGURE 18(continued)

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**Sequence for rHBe_III_ (SEQ ID NO: 6)
(the recombinant protein; polyepitope (polytope) sequence shadowed SEQ ID NO: 7))**

MDIDPYKEFGATVELLSFLPSDFPVSF**DYKDDDDKSLFNTVATLYTKGILGFVFTLKNAGLCTLVAMLGPGPGKAKFV**
AAWTLKAAAEHGAPILSTLPETTVVRRRGRSPRRRTPSPRRRRSQSPRRRRSQSRESQC

FIGURE 19

**Sequence for polytope III (SEQ ID NO: 8)
(amino acids in small letters are flanking residues)**

DYKDDDDKSLFNTVATLyt**kGILGFVFTL**knaGLCTLVAMlgpgpg**kAKFVAWTLKAAA**

FIGURE 20

**Sequence for polytope IV (SEQ ID NO: 9)
(amino acids in small letters are flanking residues)**

DYKDDDDKelrSLYNTVATLyt**kGILGFVFTL**knaGLCTLVAMlgpgpg**kAKFVAWTLKAAA**

FIGURE 21

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Sequence for prHBV-1.3-IV (SEQ ID NO: 10)
(polyepitope (polytope) sequence shadowed (SEQ ID NO: 11))

GACGGATCGGGAGATCTCCCGATCCCCTATGGTTCGACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTAT
 CTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGC GCGAGCAAATTTAAGCTACAACAAGGCAAGGCTTGACCGA
 CAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCAATCTAAGCAGGCTTTCACCTTCTCGCCAAC
 TTACAAGGCCCTTCTGTGTAAACAATACCTGAACCTTTACCCCGTTGCCCGGCAACGGCCAGGTCTGTGCCAAGTGTTTG
 CTGACGCAACCCCACTGGCTGGGGCTTGGTCATGGGCCATCAGCGCATGCGTGGAACCTTTTCGGCTCCTCTGCCGATC
 CATACTGCGGAACTCCTAGCCGCTTGTTTTGCTCGCAGCAGGTCTGGAGCAAACATTATCGGGACTGATAACTCTGTTGT
 CCTATCCCGCAAATATACATCGTTTTCCATGGCTGCTAGGCTGTGCTGCCAACTGGATCCTGCGCGGGACGTCCTTTGTTT
 ACGTCCCGTCGGCGCTGAATCCTGCGGACGACCCTTCTCGGGTTCGCTTGGGACTCTCTCGTCCCCTTCTCCGTCTGCCG
 TTCCGACCGACCACGGGGCGCACCTCTCTTTACGCGGACTCCCGTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTT
 CGCTTACCTCTGCACGTGCGATGGAGACCACCGTGAACGCCACCAAATATTGCCAAGGTCTTACATAAGAGGACTCT
 TGGACTCTCAGCAATGTCAACGACCGACCTTGAGGCATACTTCAAAGACTGTTTTGTTTAAAGACTGGGAGGAGTTGGGGG
 AGGAGATTAGGTTAAAGGTCTTTGTACTAGGAGGCTGTAGGCATAAATTGGTCTGCGCACCAGCACCATGCAACTTTTTTC
 ACCTCTGCCTAATCATCTCTTGTTCATGTCCTACTGTTCAAGCCTCCAAGCTGTGCCCTTGGGTGGCCTTGGGGCATGGAC
 ATCGACCCTTATAAAGAATTTGGAGCTACTGTGGAGTTACTCTCGTTTTTGCTTCTGACTTCTTTCCTTTCAGTAAGCTT
 CGACTACAAGGACGACGACGACAAGGAACCTAAGAAGCCTGTACAACACCGTGGCCACCCTGTACACCAAGGGCATCCTGG
 GCTTCGTGTTACCCCTGAAGAACGCGCGCCTGTGCACCCTGGTGGCCATGCTGGGCCCGGGCCCGGCAAGGCCAAGTTC
 GTGGCCGCTGGACCCTGAAGGCTGCAGCCGAACATGGAGCCCCTATCCTATCAACACTTCCGGAGACTACTGTTGTTAG
 ACGACGAGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAGGTCTCAATCGCCGCGTCGCAGAAGATCTC
 AATCTCGGGAATCTCAATGTTAGTATTCCCTTGGACTCATAAGGTGGGGAACTTTACTGGGCTTTATTTCTTCTACTGTACC
 TGTCTTTAATCCTCATTGGAAAACACCATCTTTTCCCTAATATACATTTACACCAAGACATTATCAAAAAATGTGAACAGT
 TTGTAGGCCCACTCACAGTTAATGAGAAAAGAAGATTGCAATTGATTATGCCTGCCAGGTTTTATCCAAAGGTTACCAA
 TATTTACCATTGGATAAGGGTATTAAACCTTATTATCCAGAACATCTAGTTAATCATTACTTCCAAACTAGACACTATTT
 ACACACTCTATGGAAGGCGGGTATATTATATAAGAGAGAAACAACACATAGCGCCTCATTTTGTGGGTCCACCATATTTCT
 GGAACAAGATCTACAGCATGGGGCAGAATCTTTCCACCAGCAATCCTCTGGGATTCTTTCCCGACCACCAGTTGGATCC
 AGCCTTCAGAGCAAACACCGCAAATCCAGATTGGGACTTCAATCCCAACAAGGACACCTGGCCAGACGCCAACAAGGTAG
 GAGCTGGAGCATTCCGGCTGGGTTTACCCACCGCACGGAGGCCTTTTGGGGTGGAGCCCTCAGGCTCAGGGCATACTA
 CAACTTTGCCAGCAAATCCGCCTCCTGCCTCCACCAATCGCCAGTCAGGAAGGCAGCCTACCCCGCTGTCTCCACCTTT
 GAGAAACTCATCCTCAGGCCATGCAGTGGAATTCACAACCTTCCACCAAACCTCTGCAAGATCCAGAGTGAGAGGCC
 TGTATTTCCCTGCTGGTGGCTCCAGTTCCAGGAACAGTAAACCCTGTTCTGACTACTGCCTCTCCCTTATCGTCAATCTTC
 TCGAGGATTGGGGACCCTGCGCTGAACATGGAGAACATCACATCAGGATTCCTAGGACCCCTTCTCGTGTTACAGGCGGG
 GTTTTTCTTGTGACAAGAATCCTCACAATACCGCAGAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGAA
 CTACCGTGTGTCTTGGCCAAAATTCGCAGTCCCCAACCTCCAATCACTACCAACCTTGTCTCCTCAACTTGTCTGTT
 TATCGCTGGATGTGTCTGCGGCGTTTTTATCATCTTCCCTTTCATCCTGCTGCTATGCCTCATCTTCTTGTGTTCTTCT
 GGACTATCAAGGTATGTTGCCCGTTTTGTCTTAATTCCAGGATCCTCAACAACCAGCACGGGACCATGCCGGACCTGCA
 TGACTACTGCTCAAGGAACCTCTATGTATCCCTCCTGTTGCTGTACCAAACCTTCCGGACGGAAATTGCACCTGTATTCCC
 ATCCCATCATCCTGGGCTTTCGGAAAATTCCTATGGGAGTGGGCCTCAGCCCGTTTTCTCCTGGCTCAGTTTACTAGTGCC
 ATTTGTTTCAAGTGGTTTCGTAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTATATGGATGATGTGGTATTGGGGGCCAAGTC
 TGTACAGCATCTTGAGTCCCTTTTTACCGCTGTTACCAATTTTCTTTTGTCTTTGGGTATACATTTAAACCCTAACAAAA
 CAAAGAGATGGGGTACTCTCTAAATTTTATGGGTTATGTCATTGGATGTTATGGGTCTTGGCACAAGAACACATCATA
 CAAAAAATCAAAGAATGTTTTAGAAAACCTTCTATTAACAGGCCTATTGATTGGAAAGTATGTCAACGAATTGTGGGTCT
 TTTGGGTTTTGCTGCCCTTTTACACAATGTGGTTATCCTGCGTTGATGCCTTTGTATGCATGTATTCAATCTAAGCAGG
 CTTTCACTTCTCGCCAACCTTACAAGGCCTTCTGTGTAAACAATACCTGAACCTTTACCCCGTTGCCCGGCAACGGCCA
 GGTCTGTGCCAAGTGTGTTGCTGACGCAACCCCACTGGCTGGGGCTTGGTCATGGGCCATCAGCGCATGCGTGGAACCTT
 TTCGGCTCCTCTGCCGATCCATACTGCGGAACTCCTAGCCGCTTGTTTTGCTCGCAGCAGGTCTGGAGCAAACATTATCG
 GGACTGATAACTCTGTTGTCTTATCCCGCAAATATACATCGTTTTCCATGGCTGCTAGGCTGTGCTGCCAACTGGATCCTG
 CGCGGGACGTCCTTTGTTTACGTCCCGTCGGCGCTGAATCCTGCGGACGACCCTTCTCGGGGTGCTTGGGACTCTCTCG

FIGURE 22

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TCCCCTTCTCCGTCTGCCGTTCCGACCGACCACGGGGCGCACCTCTCTTTACGCGGACTCCCCGTCTGTGCCTTCTCATC
 TGCCGGACCGTGTGCACTTCGCTTCACCTCTGCACGTCGCATGGAGACCACCGTGAACGCCACCAAATATTGCCCAAGG
 TCTTACATAAGAGGACTCTTGGACTCTCAGCAATGTCAACGACCGACCTTGAGGCATACTTCAAAGACTGTTTGTTTAAA
 GACTGGGAGGAGTTGGGGGAGGAGATTAGGTTAAAGGTCTTTGTACTAGGAGGCTGTAGGCATAAATTGGTCTGCGCACC
 AGCACCATGCAACTTTTTTACCTCTGCCTAATCATCTCTTGTTCATGTCTACTGTTCAAGCCTCCAAGCTGTGCCTTGG
 GTGGCTTTGGGGCATGGACATCGACCCTTATAAAGAATTTGGAGCTACTGTGGAGTTACTCTCGTTTTTGCCTTCTGACT
 TCTTTCCTTACAGTACGAGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCTTTAGTGA
 GGGTTAATTGCGCGCATGCCCACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGGA
 AATGGCCGCTTTTTCTGGATTTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCC
 GTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCCTCGTGCTTTACGGTATCGCCGCTCCCGATTTCGAG
 CGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCC
 AACCTGCCATCACGAGATTTTCGATTCCACCGCCGCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGG
 CTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCACCCCAACTTGTTTATTGCAGCTTATAATGGTT
 ACAAATAAAGCAATAGCATCACAAATTTACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTC
 ATCAATGTATCTTATCATGTCTGTATAACCGTCGACCTCTAGCTAGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGT
 GTGAAATTGTTATCCGCTCACAAATCCACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAG
 TGAGCTAACTCACATTAATTGCGTTCGCTCACTGCCCCGCTTTCCAGTCGGGAAACCTGTGCTGCCAGCTGCATTAATGA
 ATCGGCCAACGCGCGGGGAGAGGCGGTTTGGCTATTGGGCGCTCTTCCGCTTCCCTCGCTCACTGACTCGCTGCGCTCGGT
 CGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAA
 AGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGC
 CCCCCTGACGAGCATCACAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATAACCAGGCGTT
 TCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTCTCCCTTCGG
 GAAGCGTGGCGCTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCCGCTCCAAGCTGGGCTGTGTG
 CACGAACCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTT
 ATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGT
 GGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTT
 GGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTTGTTTGAAGCAGCAGATTACGCGCAGAAA
 AAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACCTCACGTTAAGGGATTT
 TGGTGATGAGATTATCAAAAAGGATCTTACCTAGATCCTTTTAAATTAATAAATGAAGTTTTAAATCAATCTAAAGTATA
 TATGAGTAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATC
 CATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATAC
 CGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCT
 GCAACTTTATCCGCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTTCG
 CAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTACGCTCGTCTGTTGGTATGGCTTCATTTCAGCTCCGGTTCCCAAC
 GATCAAGGCGAGTTACATGATCCCCATGTTGTGCAAAAAGCGGTTAGCTCCTTCGGTCCCTCCGATCGTTGTCAGAAGT
 AAGTTGGCCGAGTGTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTT
 TTCTGTGACTGGTGGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAA
 TACGGGATAATAACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACCTCTCA
 AGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTTAC
 CAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATAC
 TCATACTCTTCTTTTTCAATATTATTGAAGCATTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATT
 TAGAAAAATAAACAATAGGGGTTCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTC

FIGURE 22(Continued)**Sequence for rHBe_IV_ (SEQ ID NO: 12)****(the recombinant protein; polyepitope (polytope) sequence shadowed (SEQ ID NO: 13))**

MDIDPYKEFGATVELLSFLPSDFFPVSVDYKDDDDKELRSLYNTVATLYTKGILGFVFTLKNAGLCTLVAMLGPFPGKA
 KFVAAWTLKAAAEHGAPILSTLPETTVVRRRGRSPRRRTPSPRRRRSQSPRRRRSQSRESQC

FIGURE 23

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**NCBI Accession Number: V01460 Hepatitis B virus (strain ayw) genome, DNA sequence.
(ACCESSION V01460 J02203 VERSION V01460.1 GI:62276, VRL 28-JAN-2003) (SEQ ID NO: 14)**

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1 aattccactg catggcctga ggatgagtgt ttctcaaagg tggagacagc ggggtaggct
61 gccttcctga ctggcgattg gtggaggcag gaggcggatt tgctggcaaa gttttagta
121 tgccctgagc ctgagggctc caccccaaaa ggcctccgtg cgggtggggtg aaaccagcc
181 cgaatgctcc agctcctacc ttggtggcgt ctggccaggt gtccttggtg ggattgaagt
241 cccaatctgg atttgcgggtg tttgctctga aggctggatc caactggtgg tcgggaaaga
301 atcccagagg attgctgggtg gaaagattct gccccatgct gtagatcttg ttccaagaa
361 tatggtgacc cacaaaatga ggcgctatgt gttgtttctc tcttatataa tatacccgcc
421 ttccatagag tgtgtaaata gtgtctagtt tggaagtaat gattaactag atgttctgga
481 taataaggtt taataccctt atccaatggt aaatatgttg taacctttgg ataaaacctg
541 gcaggcataa tcaattgcaa tcttcttttc tcattaactg tgagtgggccc tacaactgt
601 tcacattttt tgataatgtc ttggtgtaaa tgtatattag gaaaagatgg tgttttccaa
661 tgaggattaa agacaggtac agtagaagaa taaagcccag taaagttccc caccttatga
721 gtccaaggaa tactaacatt gagattcccc agattgagat cttctgcgac gcggcgattg
781 agaccttcgt ctgcgaggcg agggagtctc tcttctaggg gacctgcctc gtcgtctaac
841 aacagtagtc tccggaagtg ttgataggat aggggcattt ggtggtctat aagctggagg
901 agtgcgaaac cacactccga aagacaccaa atactctata actgtttctc ttccaaaagt
961 gagacaagaa atgtgaaacc acaagagttg cctgaacttt aggcccatat tagtgttgac
1021 ataactgact actaggctct tagacgctgg atcttccaaa ttaacaccca cccaggtagc
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1141 acaatgctca ggagactcta aggcttcccc atacagagct gaggcgggat ctagaagatc
1201 tcgtactgaa ggaaagaagt cagaaggcaa aaacgagagt aactccacag tagctccaaa
1261 ttctttataa gggtcgatgt ccatgcccc aagccacca aggcacagct tggaggcttg
1321 aacagtagga catgaacaag agatgattag gcagaggatga aaaagttgca tgggtgctggt
1381 gcgcagacca atttatgcct acagcctcct agtacaaga cctttaacct aatctcctcc
1441 cccaactcct cccagtcttt aaacaaacag tctttgaagt atgcctcaag gtcggtcgtt
1501 gacattgctg agagtccaag agtcctctta tgtaagacct tgggcaatat ttggtgggcg
1561 ttcacgggtg tctccatgcg acgtgcagag gtgaagcgaa gtgcacacgg tccggcagat
1621 gagaaggcac agacggggag tccgcgtaaa gagaggtgcg ccccggtggtc ggtcggaacg
1681 gcagacggag aaggggacga gagagtccca agcgcacccg agaagggtcg tccgcaggat
1741 tcagcgcgga cgggacgtaa acaaaggacg tccgcgcgag gatccagttg gcagcacagc
1801 ctagcagcca tggaaacgat gtatatattgc gggataggac aacagagtta tcagtcccga
1861 taatgtttgc tccagacctg ctgcgagcaa aacaagcggc taggagttcc gcagtatgga
1921 tcggcagagg agccgaaaag gttccacgca tgcgctgatg gccatgacc aagccccagc
1981 cagtgggggt tgcgtcagca aacacttggc acagacctgg ccggtgccgg gcaacggggt
2041 aaaggttcag gtattgttta cacagaaagg ccttgtaagt tggcgagaaa gtgaaagcct
2101 gcttagattg aatacatgca tacaaggca tcaacgcagg ataaccacat tgtgtaaaag
2161 gggcagcaaa acccaaaaga cccacaattc gttgacatac tttccaatca ataggcctgt
2221 taataggaag ttttctaaaa cattctttga ttttttgat gatgtgttct tgtggcaagg
2281 acccataaca tccaatgaca taaccataa aatttagaga gtaaccccat ctctttgttt
2341 tgttaggggt taaatgtata ccaaagaca aaagaaaatt ggtaacagcg gtaaaaaggg
2401 actcaagatg ctgtacagac ttggccccc aataccacatc atccatataa ctgaaagcca
2461 aacagtgggg gaaagcccta cgaaccactg acaaatggc actagtaaac tgagccagga
2521 gaaacgggct gaggccact cccataggaa ttttccgaaa gcccaggatg atgggatggg
2581 aatacagggt caatttccgt ccgaaggttt ggtacagcaa caggagggat acatagagg
2641 tccttgagca gtagtcatgc aggtccggca tgggtcccgtg ctggttggtg aggatcctgg
2701 aattagagga caaacgggca acataccttg atagtccaga agaaccaaca agaagatgag
2761 gcatagcagc aggatgaaga ggaagatgat aaaacgccgc agacacatcc agcgataacc
2821 aggacaagtt ggaggacaag aggttgggtg gtgattggag gttggggact gcgaattttg
2881 gccaaagacac acggtagttc cccctagaaa attgagagaa gtccaccacg agtctagact
2941 ctgcggtatt gtgaggattc ttgtcaacaa gaaaaacccc gcctgtaaca cgagaagggg
3001 tcctaggaat cctgatgtga tgttctccat gttcagcgca gggccccca tcctcgagaa
3061 gattgacgat aagggagagg cagtagtcag aacagggttt actgttctctg aactggagcc
3121 accagcaggg aaatacaggc ctctcactct gggatcttgc agagtgtggt ggaaggttgt
3181 gg

```

FIGURE 24

