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(71) Applicant (for all designated States except US): **THE MACFARLANE BURNET INSTITUTE FOR MEDICAL RESEARCH AND PUBLIC HEALTH LIMITED** [AU/AU]; Alfred Medical Research and Education Precinct, 85 Commercial Road, Melbourne, Victoria 3004 (AU).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **GOWANS, Eric James** [AU/AU]; 22 Erica Street, Prahran, Victoria 3181 (AU). **MARTYN, John Charles** [AU/AU]; Unit 2, 26 Hopetoun Avenue, West Brunswick, Victoria 3055 (AU).

(74) Agents: **HUGHES, E., John, L.** et al.; Davies Collison Cave, 1 Nicholson Street, Victoria, Melbourne 3000 (AU).

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(54) Title: A HEPATITIS C VACCINE DELIVERY VEHICLE

(57) Abstract: The present invention relates generally to a delivery vehicle useful in vaccination and gene therapy. More particularly, the delivery vehicle induces a humoral and cell mediated immune response protective against hepatitis C Virus (HCV) or other pathogens or may be used to facilitate gene therapy. Methods for vaccinating against HCV or other pathogens or for facilitating gene therapy and compositions comprising the delivery vehicle are also contemplated.

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A HEPATITIS C VACCINE DELIVERY VEHICLE

FIELD

5 The present invention relates generally to a delivery vehicle useful in vaccination and gene therapy. More particularly, the delivery vehicle induces a humoral and cell mediated immune response protective against hepatitis C virus (HCV) or other pathogens or may be used to facilitate gene therapy. Methods for vaccinating against HCV or other pathogens or for facilitating gene therapy and compositions comprising the delivery vehicle are also
10 contemplated.

BACKGROUND

Bibliographic details of references in the subject specification are also listed at the end of
15 the specification.

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

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The threat posed to the Australian and other health systems in developed and developing countries of HCV infection continues to grow at an alarming rate. HCV is currently one of the most common notifiable infectious diseases in Australia with 16,000 new cases per annum (Dore *et al*, *J Clin Virol* 26:171-84, 2003). There are approximately 210,000 HCV-
25 positive individuals in the country, a figure which continues to grow, and approximately 200 million carriers in the world (Lavanchy & McMahon, *In: Liang, Hoofnagle, eds. Hepatitis C. San Diego, CA. Acad Press*:185-201, 2001). Despite the introduction of needle and syringe exchanges, it has been very difficult, if not impossible, to change human behavior and most new cases in Australia are derived from 15-18 year old
30 individuals who share injecting equipment. This trend is not expected to alter without the

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introduction of new, effective intervention protocols. The development of an effective vaccine for HCV will considerably reduce the number of newly acquired infections, as the use of such a vaccine has been estimated to be the most cost-efficient means to prevent virus infections in the community.

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Impediments to the development of an HCV vaccine include i) the quasispecies nature of HCV; ii) six HCV genotypes; and iii) lack of a laboratory animal model, as HCV only infects humans and chimpanzees. This has resulted in an inconsistent empirical approach to vaccine development. For example, the core protein has been reported to inhibit the
10 immune response (Yao *et al*, *J. Immunol* 167:5264-5272, 2001), yet has been used as a potential vaccine (e.g. Polakos *et al*, *J. Immunol* 166:3589-3598, 2001) since it contains a number of highly conserved, cytotoxic T lymphocyte (CTL) epitopes.

HCV structural proteins are encoded at the 5' end of the single long open reading frame in
15 the genome and comprise the core (C) or capsid protein and two envelope glycoproteins, E1 and E2 (Major and Feinstone, *Hepatology* 25:1527-1538, 1997). The mature polypeptides are cotranslationally cleaved from the polyprotein precursor by a cellular protease. The E1 and E2 proteins are typical type 1 transmembrane proteins with a large ectodomain and a COOH-terminal hydrophobic domain that anchors the proteins to the endoplasmic
20 reticulum (ER) [Cocquerel *et al*, *J Virol* 72:2189-2191, 1998, Cocquerel *et al*, *J Virol* 73:2641-2649, 1999]. Studies of E1/E2 expression are generally performed using recombinant plasmid or viral systems (Brazzoli *et al*, *Virology* 332:438-453, 2005, Martire *et al*, *Virology* 280:176-182, 2001). These studies show that a proportion of E1/E2 expressed in cell lines forms a non-covalently bound heterodimer that is thought to represent the form
25 of E1/E2 on the surface of virus particles (Deleersnyder *et al*, *J Virol* 71:697-704, 1997) and this is recognised by conformation-dependent monoclonal antibodies. It is thought that the hydrophobic transmembrane anchors are important for the heterodimer formation, as it is abrogated by substitution with transmembrane domains from other proteins (Op De Beeck *et al*, *J Biol Chem* 275:31428-31437, 2000). However, the heterodimer comprises
30 only a small proportion of the E1/E2 which is expressed and the bulk of the proteins form

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heterogeneous disulphide-linked aggregates, which, although localised in the ER, are thought to be misfolded artefacts of the system.

Although the bulk of the E1/E2 is retained in the ER, a small proportion can be detected on the plasma membrane, the likely result of over-expression and leakage, and this characteristic has been exploited to generate HCV pseudotype viruses which contain the E1/E2 proteins (Bartosch *et al*, *J Exp Med* 197:633-642, 2003, Drummer *et al*, *FEBS Lett* 546:385-390, 2003, Hsu *et al*, *Proc Natl Acad Sci, USA* 100:7271-7276, 2003).

The E1/E2 proteins are also contained in HCV-like particles (HCV-LP) which are produced in either insect or human cell lines infected with a recombinant baculovirus (RecBV) (Baumert *et al*, *J Virol* 72:3827-3836, 1998, Matsuo *et al*, *Biochem Biophys Res Commun* 340:200-208, 2006). HCV-LP purified from insect cell lysates are recognised by conformation-dependent monoclonal antibodies and HCV-LP purified from human cell culture supernatants bind the HCV co-receptor CD81, indicating that the E1/E2 proteins form the authentic heterodimer in HCV-LP derived from both cell types.

Vaccination of chimpanzees with recombinant E1/E2 envelope glycoproteins protected against challenge with homotypic virus (Houghton, *Curr Top Microbiol Immunol* 242:327-339, 2000) but was ineffective against heterologous virus. Although neutralizing antibodies have been described (Farci *et al*, *PNAS* 93:15394-15399, 1996), the commonly recognized epitopes are located in hypervariable region 1 (HVR1) at the N-terminus of the E2 protein. The HVR1 sequence is unique in individual isolates of the virus and mutations in this region result in the appearance and selection of neutralizing antibody escape mutants (Kato *et al*, *J Virol* 68:1117-1120, 1994). However, a study using HCV pseudotypes (Hsu *et al*, 2003 *supra*) identified additional neutralizing antibody epitopes downstream of HVR1 in E2 and a monoclonal antibody to the N-terminus of the E1 protein was reported to block virus attachment and infectivity (Keck *et al*, *J Virol* 78:7257-7263, 2004). Furthermore, three peptide mimotopes of HVR1 were recognized by serum antibodies from 88% of HCV-positive patients (Puntoriero *et al*, *EMBO J* 17:3521-3533, 1998) and two monoclonal antibodies induced by immunization with peptides containing

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the conserved G - -Q motif at the C-terminus of HVR1 were able to capture HCV from 25/31 (81%) patients tested (Li *et al*, *J Virol* 75:12412-12420, 2001). Passive immunization with pooled, inactivated immunoglobulin from HCV carriers modulated infection in experimentally-infected chimpanzees (Krawczynski *et al*, *J Infect Dis* 5 173:822-8228, 1996) and *in vitro* neutralization of challenge virus with a similar product [Gammagard HCV immune globulin (HCIGIV)] failed to result in infection of a chimpanzee (Yu *et al*, *PNAS* 101:7705-7710, 2004). Collectively, these reports suggest that antibody to the HCV envelope proteins may have a role in protection against HCV infection and at the very least, neutralize a proportion of the challenge dose. It is clear that 10 the problem is to induce neutralizing antibody not only against the parental strain, but also against the whole range of quasispecies, as the HCIGIV with neutralization activity was derived from 198 anti-HCV positive individuals (Yu *et al*, 2004 *supra*).

Studies of the immune response to HCV infection have identified many MHC-restricted T- 15 cell epitopes. Most were identified in patients with persistent infection, and may differ to those targeted during recovery from acute infection and in protection. Individuals with specific MHC class II haplotypes are more likely to clear the virus (Donaldson, *Eur J Clin Invest* 29:280-283, 1999) indicating the importance of CD4⁺ T-cell responses. Recovery is also associated with a Th0/Th1 cytokine profile (Tsai *et al*, *Clin Exp Immunol* 128:195- 20 203, 1997), whereas those who develop persistent infection show a Th2 response. Initially, it was reported that convalescent individuals and chimpanzees could be re-infected (Lai *et al*, *Lancet* 343:388-390, 1994; Prince *et al*, *J Infect Dis* 189:1846-1855, 1992). Challenge of convalescent chimpanzees showed that one animal had sterilizing immunity, while others showed accelerated clearance of homologous or heterologous virus (Bassett, 25 *Hepatol* 33:1479-1487, 2001; Weiner *et al*, *J Virol* 75:7142-7148, 2001; Lanford *et al*, *J Virol* 78:1575-1581, 2004). A cohort of multiply-exposed IDU was reported to be protected against infection, showing that protective immunity can be induced in humans (Mehta *et al*, *Lancet* 359:1478-1483, 2002). Moreover, women who were infected from a single source infection around 20 years ago and cleared the acute infection, had CMI 30 memory and not residual antibody (Takaki *et al*, *Nat Med* 6:578-582, 2000). Thus, the evidence suggests that CMI is more likely to correlate with recovery and/or protection

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from HCV infection than humoral immunity (Racanelli & Rehermann, *Trends Immunol* 24:456-464, 2003). Indeed, studies have showed that some individuals who ordinarily might be expected to be infected with HCV, were not viraemic and appeared to be protected in the absence of anti-HCV antibodies (Post *et al*, *J Infect Dis* 189:1846-1855, 5 2004).

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SUMMARY

A recombinant baculovirus (RecBV) is provided which acts as a delivery vehicle for a surface (envelope) antigen from HCV to a subject's immune system in order to generate a humoral response and/or a cell mediated response. In particular, the RecBV comprises at least one HCV protein which is incorporated into its envelope and at least one HCV protein which is expressed in a cell of the target subject to which the vaccine delivery vehicle is introduced. Alternatively, the RecBV comprises a protein antigen from another pathogen in order to generate an immune response against that pathogen. The RecBV delivery vehicle may also be used in gene therapy applications. The RecBV delivery vehicle may also be targeted to particular cells *via* binding to a ligand such as but not limited to *inter alia* an Fc portion on the delivery vehicle binding to a cell carrying a Fc receptor or an immunoglobulin receptor.

Accordingly, a delivery vehicle is provided comprising a recombinant baculovirus (RecBV) having an envelope which contains a surface antigen from Hepatitis C Virus (HCV) or an immunologically cross-reactive homolog thereof and which baculovirus comprises a nucleic acid molecule which encodes at least one protein from HCV and which is expressed in a cell of a target subject.

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A delivery vehicle is also provided comprising a RecBV having an envelope which contains an antigen from a pathogen or an immunologically cross-reactive homolog thereof which RecBV induces a cell mediated response by cross presentation.

A delivery vehicle is further contemplated comprising a RecBV having an envelope comprising a ligand for a complementary molecule on a cell surface.

A delivery vehicle is also provided comprising a RecBV carrying genetic material for gene therapy or genetic-based vaccination.

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Hence, a delivery vehicle is provided herein comprising a RecBV having an envelope comprising an agent selected from the list consisting of a surface antigen from HCV, an antigen from a pathogen and a ligand capable of binding to a cell surface molecule which RecBV further comprises a nucleic acid molecule encoding a protein or RNA species and
5 which is expressed in a cell of a target subject.

The delivery vehicle described herein may be described as a RecBV delivery vehicle, a virus-like particle (VLP) or a gene therapy facilitating vehicle as well as a vaccine delivery vehicle.

10

In general, the delivery vehicle comprises a RecBV having an envelope which contains a heterologous protein and a nucleic acid molecule encoding a heterologous expression product wherein the heterologous protein is an antigen from a pathogen and/or a ligand to a complementary molecule on a cell surface and wherein the heterologous expression
15 product is a peptide, polypeptide or protein or RNA species produced by a cell of a target subject.

The vaccine delivery vehicle aspect is based on the supposition that optimal protection against HCV challenge is a combination of a humoral and cell mediated response and that
20 the immune responses are directed against highly conserved epitopes and hence will protect against infection by any genotype of HCV.

In a particular embodiment, a vaccine delivery vehicle is provided comprising a RecBV having an envelope which contains HCV envelope glycoproteins E1/E2 in dimeric form
25 and which RecBV comprises a nucleic acid molecule which encodes at least one HCV protein selected from the list consisting of E1, E2, core protein, NS3/4A and NS3m/4A and which protein is expressed in a target mammalian cell wherein said vaccine delivery vehicle when introduced to a subject generates a protective humoral and cell mediated immune response to HCV or an immunologically cross-reactive homolog thereof.

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In another embodiment, the delivery vehicle comprises a RecBV having an envelope containing a portion of Fc which is capable of binding to an FcR receptor (FcR) on the surface of a cell. This aspect includes RecBV's having antigens from pathogens such as HCV in its envelope and/or containing a nucleic acid molecule encoding a heterologous peptide, polypeptide or protein or RNA species.

The baculovirus vector is preferably but not necessarily derived from the vector described by Condreay *et al*, *Proc. Natl. Acad. Sci USA* 96:127-132, 1999. In this donor vector the HCV genes required for expression in the cells (e.g. core, E1, E2, NS3 and/or NS4A) are cloned into ECOR1/XbaI sites of the multiple cloning site and HCV genes for expression in insect cells (e.g. E1 and/or E2) replace the neo gene as a ScxA1/Csp451 fragment. The donor plasmid is reproduced in Figure 11. The vaccine delivery vector may be targeted to a particular cell *via* a Toll-like receptor (TLR) such as TLR2, TLR3, TLR4, TLR7, TLR8 and/or TLR9. Proteins which bind to and hence target TLRs are referred to herein as TLR binding proteins. Hence, the vaccine delivery vector may further comprise a TLR binding protein from HCV or another virus which facilitates targeting to a TLR and hence to a particular cell or group or family of cells.

Examples of or suitable sources for TLR binding proteins include those listed in Table 2.

In addition, the baculovirus envelope may contain other targeting moieties such as an IgG constant region to target any antigen-presenting cell with IgG receptors, decay accelerating protein (DAF) which increases resistance to complement, CD40L to target CD40 on DC and/or an Fc portion which binds to Fc receptor (FcR).

The expression of the HCV protein in, for example, mammalian cells is conveniently controlled *via* a suitable promoter such as but not limited to a CMV promoter. The CMV promoter is useful for inducing expression in mammalian cells.

Methods of vaccinating subjects against HCV infection or infection by other pathogens or for facilitating gene therapy are also contemplated.

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Hence, another aspect of contemplates a method for vaccinating a subject against HCV infection said method comprising administering to said subject a vaccine delivery vehicle comprising a recombinant baculovirus having an envelope which contains a surface antigen from HCV or an immunologically cross-reactive homolog thereof and which
5 baculovirus comprises a nucleic acid molecule which encodes at least one protein from HCV and which is expressed by a cell of a target subject, wherein said vaccine delivery vehicle induces a protective humoral and cell mediated immune response to HCV or an immunologically cross-reactive homolog thereof.

10

Yet a further aspect provides a method for vaccinating a subject against a pathogen said method comprising administering to said subject a vaccine delivery vehicle comprising a recombinant baculovirus having an envelope which contains a surface antigen from the pathogen or an immunologically cross-reactive homolog thereof and which baculovirus
15 comprises a nucleic acid molecule which encodes at least one protein from the pathogen and which is expressed by a cell of a target subject, wherein said vaccine delivery vehicle induces a protective humoral and cell mediated immune response to the pathogen or an immunologically cross-reactive homolog thereof.

20 Still another aspect contemplates a method for gene therapy in a subject said method comprising administering to said subject a delivery vehicle comprising a recombinant baculovirus having an envelope which contains a surface ligand capable of binding to a complementary ligand on a target cell and which baculovirus comprises a nucleic acid molecule which encodes a peptide, polypeptide or protein or RNA species and which is
25 expressed by a cell of a target subject, wherein said delivery vehicle facilitate peptide, polypeptide or protein replacement and/or gene regulation.

Compositions comprising the HCV vaccine or other pathogen vaccine and the use of the RecBV in the manufacture of a medicament to induce a protective immune response
30 against HCV or other pathogen are also described herein as is the use of a RecBV in gene therapy.

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Throughout this specification, unless the context requires otherwise, the word “comprise”, or variations such as “comprises” or “comprising”, will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the
5 exclusion of any other element or integer or group of elements or integers.

All scientific citations, patents, patent applications and manufacturer's technical specifications referred to hereinafter are incorporated herein by reference in their entirety.

10 Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 [SEQ ID NO: 1], <400>2 [SEQ ID NO: 2], etc.

A summary of sequence identifiers used throughout the subject specification is provided in
15 Table 1.

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TABLE 1
Summary of sequence identifiers

SEQUENCE ID NO:	DESCRIPTION
1	nucleotide primer for HCV E1 (forward)
2	nucleotide primer for HCV E2 (reverse)
3	nucleotide primer for generating cDNA fragment for HCV IRES, Core, E1 and E2 genes

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BRIEF DESCRIPTION OF THE FIGURES

Some figures contain color representations or entities. Color photographs are available from the Patentee upon request or from an appropriate Patent Office. A fee may be imposed if obtained from a Patent Office.

Figure 1 is a diagrammatic representation showing the construction of the RecBV-E. (A) Line diagram showing the organization of the HCV genome (upper) and the region subcloned into the pFBM transfer vector (lower). (B) Primers used to amplify the E1E2 cDNA with upstream signal sequence.

Figures 2A through C are photographic and tabular representations showing the RecBV-E transduction is more efficient than DNA transfection. Huh7 cells were transduced with RecBV-E or transfected with pFBM-E. (A) Expression of E2 after RecBV-E infection (upper) or transfection with pFBM-E (lower). The percentage of cells expressing high and low levels of E2 is indicated. (B) Immunoblot of duplicate samples. (C) Co-immunoprecipitation of radiolabeled E1 and E2 using polyclonal anti-HCV E2. The E1 and E2 bands are indicated by the arrows. M - molecular weight markers, pFBME - donor plasmid with E1E2 insert, pFBM - donor plasmid without insert, RecBV-E1E2 - recombinant baculovirus encoding E1/E2.

Figures 3A and B are photographic and tabular representations showing the efficiency of transduction of primary marmoset hepatocytes with RecBV-E compared to transfection with pFBM-E (A) Immunofluorescence using polyclonal anti-HCV E2. The percentage of cells expressing high and low levels of E2 is indicated. (B) Western blot using polyclonal anti-HCV E2. pFBM-E - donor plasmid with E1E2 insert, pFBM - donor plasmid without insert, RecBV-E1E2 - recombinant baculovirus encoding E1/E2, pFBM-FL - donor plasmid encoding full-length HCV polyprotein.

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Figures 4A through C are photographic representations showing generation of stable cell lines expressing HCV E1 and E2 following transduction of the HL1 cell line with RecBV-E. (A) Immunofluorescence of pools of G418-resistant colonies using polyclonal anti-HCV E2 serum. Cells in pools 1 and 2 were transduced at a moi of 10,000 and 1,000, respectively. (B) Immunofluorescence of clonal stable cell lines (P2C3, P3D1) expressing E1 and E2, and a clone (P2B6) negative for E1E2. Primary antibody was polyclonal anti-HCV E2. (C) Western blot of clonal stable cell lines expressing E1 and E2 compared to transient transduction of cured HL1 with RecBV-E. Equivalent amounts of total protein were loaded per lane. HL1 - parental cell line, 1-5, clones P3B4, P3D1, P2C3, P3C3 and P2C6, respectively, RecBVE - baculovirus expressing HCV E1 and E2, RecBV-wt - baculovirus without insert, M -molecular weight markers. The position of HCV E2 is indicated by the arrow.

Figures 5A and B are graphical representations of the characteristics of cell lines constitutively expressing E1/E2. (A) Growth kinetics of 5 stable cell lines (P3C3, P2C3, P3D1, P3B4, P2C6) compared to the parental HL1 cell line. (B) Flow cytometric analysis of apoptosis in cell line P2C6 compared to HL1. The gated area (M1) of the histogram includes the apoptotic cells in the total population (R1). The positive control was P2C6 cells treated with 20% ethanol prior to staining.

20

Figures 6A and B are photographic representations showing conformation and localisation of HCV E2 expressed by the stable cell line. (A) Binding of a conformation-dependent E2 monoclonal antibody, H53, to acetone-fixed cell lines P2C6 and the parental line, HL1 (negative control). (B) Flow cytometric analysis of P2C6 and HL1 cell lines following surface staining with polyclonal anti-E2 serum diluted 1:10, 1:20 and 1:40. Normal goat serum (Isotype C') was the negative control. Δ is the difference in net mean fluorescence intensity of the P2C6 cell line (grey fill) compared to the HL1 cell line (no fill).

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Figure 7 is a photographic representation showing the persistence of RecBV following transduction of the HL1 cell line as determined by a timecourse of GFP expression in HL1 cells transduced with RecBV-GFP. The HL1 cells were infected with RecBV-eGFP at moi 1,000 and split 1:2 every 5 days post-transduction. Cells were seeded from this flask onto coverslips, incubated for 3 or 4 days, then examined for fluorescence. Cells transduced with RecBV-wt represented the negative control.

Figure 8 is a diagrammatical representation of genes encoding expression in insect cells.

Figure 9 is a photographical representation of Huh7 cells infected with HCV and stained by immunofluorescence for core antigen expression. The JFH1 virus replication system in Huh7 cells was established, as described (Zhong *et al*, *Proc. Natl. Acad. Sci. USA* 102:9294-9299, 2005). JFH virus, derived from RNA-transfected Huh7 cells, can infect naïve Huh7 cells. For neutralization, the virus will be adjusted to 50TCID₅₀, incubated at 37°C with dilutions of the mouse serum for 1 hour then inoculated into uninfected Huh7 cells. Virus growth will be determined three days later by immunofluorescence of inoculated cells.

Figure 10 is a graphical representation showing the organisation of the BVDV genome and position of inserted HCV NS3m/4 gene.

Figure 11 is a diagrammatic representation of the baculovirus donor plasmid as described by Condreay *et al*, 1999 *supra*. The HCV genes for mammalian expression (core, E1, E2, NS3 and/or NS4A) are cloned into ECOR1/XbaI sites of the MCS. HCV genes for insect expression (E1 and/or E2) replace the neogene as a ScxA1/Csp451 fragment.

Figure 12 is a graphical representation showing the antibody responses after vaccination of Balb/C mice with RecBV-E1/E2. Groups of mice were injected by the IM route with WT or RecBV-E1/E2. The antibody titre was determined by ELISA against purified E2 protein or purified WT BV.

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Figure 13 is a diagrammatic representation of a map of the transfer vectors (A) pFBM-CE1E2-gp64+Fc and (B) pFBM-CE1E2-hTfR+Fc used to construct the corresponding recombinant baculoviruses RecBV:gp64+Fc and RecBV:hTfR+, respectively. Each transfer vector contains a dual expression cassette. Cassette 1 contains the coding sequence of human IgG Fc fused to either the transmembrane (TM) region of the baculovirus gp64 envelope protein (construct A) or the human transferrin receptor (construct B) downstream of the polyhedrin promoter for constitutive expression in insect cells. Cassette 2 is identical in both constructs and contains the coding sequence of the HCV structural proteins (core, E1, E2) downstream of the CMV-IE promoter for constitutive expression in mammalian cells.

Figures 14A and B are schematic diagrams illustrating the conformation of the Fc molecule in the envelope of the RecBV, gp64 Ig Fc and hTfR Ig Fc. NB. TM1 and TM2 represent any suitable transmembrane domain sequences which target proteins to the plasma membrane.

Figure 15 is a photographic representation showing that IgG Fc fusion proteins are expressed on the surface of RecBV-infected insect cells. IF of live (unfixed) Sf9 insect cell monolayers infected with RecBVs and stained at 48h pi with goat anti-human IgG Alexa 488 conjugate. Insect cell nuclei were stained red with Syto 61. (A) hTfR-Fc RecBV, (B) gp64-Fc RecBV, (C) parental (non-pseudotyped) RecBV, and (D) mock infection.

Figure 16 is a photographic representation showing that IgG Fc fusion proteins are incorporated into the envelope of RecBV particles. (A) Detection of human IgG Fc on RecBV by ELISA. Dilutions of the rabbit anti-human IgG HRP conjugate are shown on the x-axis, while the OD reading is shown on the y-axis. Dilutions were performed in duplicate, and the average represented by solid bars. (B) Immune EM. RecBV particles were dual labeled with 10nm protein A gold to detect IgG Fc, and anti-gp64 mAb + 5nm protein A gold (Panels A, B) hTfR-Fc RecBV particles, (Panels C, D) gp64-Fc RecBV particles, (Panel E) parental (non-pseudotyped), (Panel F) RecBV (negative control).

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Representative 10nm (arrows) and 5nm (arrowheads) protein A gold particles are indicated for each RecBV pseudotype. All magnification bars are 200nm.

Figure 17 is a photographic representation showing that IgG Fc fusion proteins are incorporated into the envelope of RecBV particles. (A) Detection of human IgG Fc on RecBV by ELISA. Dilutions of the rabbit anti-human IgG HRP conjugate are shown on the x-axis, while the OD reading is shown on the y-axis. Dilutions were performed in duplicate, and the average represented by solid bars. (B) Immune EM. RecBV particles were dual labeled with 10nm protein A gold to detect IgG Fc, and anti-gp64 mAb + 5nm protein A gold. (Panels A, B) hTfR-Fc RecBV particles, (Panels C,D) gp64-Fc RecBV particles, (Panel E) parental (non-pseudotyped), (Panel F) RecBV (negative control). Representative 10nm (arrows) and 5nm (arrowheads) protein A gold particles are indicated for each RecBV pseudotype. All magnification bars are 200nm.

Figure 18 is a graphical representation showing that IgG Fc fusion proteins displayed on the surface of RecBV-infected insect cells are functional according to FACS analysis. (Upper panels) Recombinant Fc molecules detected on the surface of insect cells infected with (A) the gp64-Fc and (B) the hTfR-Fc or (C) the parental BV by flow cytometry using anti-human IgG FITC. (Lower panels) Recombinant soluble FcγRIIa was incubated with the RecBV- infected cells at 4°C, washed and the cells examined by flow cytometry with anti-human FcγRIIa (MAb 8.2) followed by anti-mouse IgG FITC (D-gp64-Fc, E-hTfR-Fc and F-parental BV).

Figure 19 is a graphical representation showing that IgG Fc fusion proteins displayed on the surface of RecBV bind to the soluble receptor in a reaction that is inhibited by the anti FcγRIIa blocking MAb, IV-3. Dilutions of RecBV were added to the wells of a microtiter plate, coated with soluble FcγRIIa; BSA-coated wells constituted negative controls and human heat aggregated IgG represented a positive control. (A) Binding of RecBVs to soluble monomeric HSA-FcγRIIa receptor by ELISA. (B) The results of the ELISA after the FcγRIIa sites were blocked by specific antibody.

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Figure 20 is a graphical representation of RecBV displaying IgG Fc fusion proteins on their surface bind to cells expressing FcγRIIa. RecBV was added to the IIA1.6 cell line expressing the FcγRIIa receptor and the level of binding assessed by flow cytometry using anti-pg64. Before and after blocking the FcγRIIa with blocking MAb IV-3.

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Figure 21 is a representation showing that differential binding of RecBV by human monocyte-derived dendritic cells (Mo-DC). RecBV was added to cultures of human Mo-DC at 4°C, incubated for 1h, washed and the degree of RecBV binding assessed by flow cytometry using anti-pg64 PE.

10

Figure 22 is a representation showing that distinct cellular populations in PBMC bind different BV. Human PBMC were purified by Ficoll-Paque gradient centrifugation and examined for their ability to bind RecBV, as detected by flow cytometry using anti-gp64 PE. The monocyte population was labeled with anti-CD14 FITC. Different concentrations of the RecBV were added to the purified PBMC, incubated at 4°C for 1h, washed and binding examined.

15

Figure 23 is a graphical representation summarizing flow cytometric analyses.

Figure 24 is a diagrammatic representation of a RecBV delivery vehicle having different proteins fused to the transmembrane domain of transmembrane domains of pg64, CD4 or hTFR.

20

Figure 25 is a graphical representation of a complement assay showing levels of complement sensitivity of various fusions to IgG Fc. A gp64-Fc fusion particularly showed resistance to complement.

25

DETAILED DESCRIPTION

The present disclosure is not limited to specific formulation components, manufacturing methods, biological materials or reagents, dosage regimens and the like, as such may vary.

5 It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

As used in the subject specification, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a vaccine" includes a single vaccine, as well as two or more vaccines; reference to "an agent" or "a reagent" includes a single agent or reagent, as well as two or more agents or reagents; reference to "the invention" includes a single aspect or multiple aspects of an invention and so forth.

10

15 The terms "vaccine delivery vehicle", "delivery vehicle", "gene therapy facilitating vehicle", "RecBV delivery vehicle", "agent", "reagent", "compound", "pharmacologically active agent", "medicament", "therapeutic", "active" and "drug" are used herein to refer to a chemical or biological entity which induces or exhibits a desired effect such as inducing a protective humoral and cell mediated response to HCV or other pathogen and/or which facilitates gene therapy. When the terms "vaccine delivery vehicle", "delivery vehicle", "gene therapy facilitating vehicle", "RecBV delivery vehicle", "agent", "reagent", "compound", "pharmacologically active agent", "medicament", "therapeutic", active" and "drug" are used, then it is to be understood that this includes the active entity *per se* as well as pharmaceutically acceptable, precursor forms thereof.

20

25

Reference to a vaccine delivery vehicle in particular includes combinations of two or more vehicles or a vehicle and one or more anti-viral agents. A "combination" also includes multi-part such as a two-part composition where the agents are provided separately and given or dispensed separately or admixed together prior to dispensation. For example, a multi-part pharmaceutical pack may have two or more agents separately maintained.

30

Hence, this aspect of the present invention includes combination therapy. Combination

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therapy includes the co-administration of two or more delivery vehicles or a delivery vehicle and an anti-viral agent or immune potentiating agent or a nucleic acid molecule capable of being expressed to produce a peptide, polypeptide or protein or RNA species. Examples of RNA species include mRNA, single stranded short or long RNA molecules, RNA hairpins, double stranded short or long RNA molecules and the like.

The vaccine delivery vehicle is preferably but not necessarily based on or derived from the baculovirus donor plasmid of Condreay *et al*, 1999 *supra* (see Figure 11). The baculovirus donor plasmid may be further modified to express a protein, such as in or in its envelope which facilitates interaction with a TLR such as but not limited to TLR2, TLR3, TLR4, TLR7, TLR8 and/or TLR9. Hence, the baculovirus envelope may contain a non-HCV protein in order to target the virus to a particular TLR and therefore a particular cell or group of cells. Examples of suitable targeting proteins are described in Boehme and Compton, *J Virol* 78:7867-7873, 2004. Examples are provided in Table 2.

- 20 -

TABLE 2.
Viruses detected by Toll-like receptors (from Boehme & Compton, 2004)

Toll-like receptor	Virus (molecular target if known)
TLR2	MV ^a (hemagglutinin)
	HCMV ^b
	HSV-1 ^c
TLR3	MCMV ^d
TLR4	RSV ^e (F protein)
	MMTV ^f (envelope protein)
TLR7 and TLR8	Influenza A virus (genomic RNA)
	HIV-1 ^g (synthetic RNA oligonucleotide from U5 region of genome)
	VSV ^h
TLR9	HSV-1
	HSV-2 (genomic DNA)
	MCMV

^aMeasles virus.5 ^bHuman cytomegalovirus.^cHerpes simplex virus type 1.^dMurine cytomegalovirus.^eRespiratory syncytial virus.^fMouse mammary tumor virus.10 ^gHuman immunodeficiency virus type 1.^hVesicular stomatitis virus.

In addition, the baculovirus envelope may contain other targeting moieties such as an IgG constant region to target any antigen-presenting cell with IgG receptors, decay accelerating protein (DAF) which increases resistance to complement and/or CD40L to target CD40 on DC. Hence, the envelope may contain a portion of Fc which binds to FcR or antigen-presenting cells (APCs).

For expression in a mammalian cell of HCV or other proteins, a suitable promoter is required such as but not limited to the CMV promoter. Other promoters active in mammalian cells may also be used.

The terms "effective amount" and "therapeutically effective amount" of an agent as used herein mean a sufficient amount of the agent to provide the desired therapeutic or

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physiological or effect or outcome. Such an effect or outcome includes inducing a humoral and cell mediated response to HCV or other pathogen or for inducing an outcome following the generation of an expression product from the nucleic acid molecule within the RecBV. Outcomes include enabling production of a protein or inhibiting expression by
5 post transcriptional or post translational gene silencing, methylation and/or RNAi-mediated gene silencing. Undesirable effects, e.g. side effects, are sometimes manifested along with the desired therapeutic effect; hence, a practitioner balances the potential benefits against the potential risks in determining what is an appropriate "effective amount". The exact amount required will vary from subject to subject, depending on the
10 species, age and general condition of the subject, mode of administration and the like. Thus, it may not be possible to specify an exact "effective amount". However, an appropriate "effective amount" in any individual case may be determined by one of ordinary skill in the art using only routine experimentation.

15 The effective amount is deemed the amount required to induce an immune response against HCV or other pathogen which is protective.

By "pharmaceutically acceptable" carrier, excipient or diluent is meant a pharmaceutical vehicle comprized of a material that is not biologically or otherwise undesirable, i.e. the
20 material may be administered to a subject along with the selected active agent without causing any or a substantial adverse reaction. Carriers may include excipients and other additives such as diluents, detergents, coloring agents, wetting or emulsifying agents, pH buffering agents, preservatives, and the like.

25 Similarly, a "pharmacologically acceptable" salt, ester, amide, prodrug or derivative of a compound as provided herein is a salt, ester, amide, prodrug or derivative that this not biologically or otherwise undesirable.

I

"Treating" a subject may involve both active treatment and prophylaxis.

30

The "subject" as used herein refers to an animal, preferably a mammal and more preferably

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a primate including a lower primate and even more preferably a human who can benefit from the formulations and methods of the present invention. A subject regardless of whether a human or non-human animal may be referred to as an individual, patient, animal, host or recipient. The compounds and methods of the present invention have applications in human medicine, veterinary medicine as well as in general, domestic or wild animal husbandry. For convenience, an "animal" includes an avian species such as a poultry bird (including ducks, chicken, turkeys and geese), an aviary bird or game bird. The condition in a non-human animal may not be a naturally occurring but induced such as in an animal model.

10

In a particular embodiment, at least one HCV protein is expressed in a cell of the subject to which it is introduced. The subject includes a mammal, such as a human or test primate animal. Hence, at least one of the HCV or other heterologous peptides, polypeptides or proteins or RNA species encoded in the baculovirus is expressed in mammalian cells of the target subject.

15

Accordingly, a "target mammalian cell" is a cell in a mammalian subject to which the delivery vehicle has been administered. The HCV genes, for example, may be operably linked to a suitable promoter such as a CMV promoter to permit expression in mammalian cells.

20

Reference to "heterologous" in this context includes any peptide, polypeptide or protein or RNA species which is not naturally present in a baculovirus. Hence, an example of a heterologous peptide, polypeptide or protein is an antigen from a pathogen such as HCV. A heterologous expression product includes peptide, polypeptide or protein or RNA species from a pathogen or from a mammalian cell.

25

As indicated above, the animal subjects include humans, non-human primates such as chimpanzees marmosets, baboons, orangutangs, lower primates such as tupia, livestock animals, laboratory test animals, companion animals or captive wild animals. A human is a particularly important target. However, non-human animal models may be used. A "target

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cell", therefore, includes a human cell.

Examples of laboratory test animals include mice, rats, rabbits, guinea pigs and hamsters. Rabbits and rodent animals, such as rats and mice, provide a convenient test system or animal model as do primates and lower primates. Livestock animals include sheep, cows, pigs, goats, horses and donkeys. Non-mammalian animals such as avian species, zebrafish, amphibians (including cane toads) and *Drosophila* species such as *Drosophila melanogaster* are also contemplated. Instead of a live animal model, a test system may also comprise a tissue culture system.

10

One aspect is predicated in part on the supposition that a protective immune response against HCV requires both a humoral and a cell mediated immune response. Hence, in accordance with the present invention a recombinant baculovirus (RecBV) is used as a vaccine delivery vehicle to introduce to a subject being vaccinated a surface antigen from HCV to generate a humoral response against this antigen. In addition, the baculovirus carries nucleic acid which encodes at least one protein from HCV which generate at least a cell mediated immune response and which protein, in a preferred embodiment, is expressed in a mammalian cell of a target subject. One convenient vaccine delivery vehicle is derived from the baculovirus donor plasmid depicted in Figure 11 (Condreay *et al*, 1999 *supra*). Into this plasmid or a structurally similar or related plasmid is inserted HCV genes destined to be expressed in mammalian cells to induce a cell mediated response (e.g. core, E1, E2, NS3 and/or NS4A) or HCV genes to be expressed as part of the baculovirus envelope (e.g. E1 and/or E2). In addition, HCV or non-HCV proteins which target particular TLRs may also be incorporated into the baculovirus envelope. Examples of such proteins are listed in Table 2.

25

In addition, the baculovirus envelope may contain other targeting moieties such as an IgG constant region to target any antigen-presenting cell with IgG receptors, decay accelerating protein (DAF) which increases resistance to complement and/or CD40L to target CD40 on DC. An Fc portion may also be employed as a ligand which binds to FcR on a cell such as an antigen-presenting cell (APC). An example of an APC is a dendritic cell (DC).

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Hence, one aspect is directed to a vaccine delivery vehicle comprising a recombinant baculovirus having an envelope which contains a surface antigen from hepatitis C Virus (HCV) or an immunologically cross-reactive homolog thereof and which baculovirus
5 comprises a nucleic acid molecule which encodes at least one protein from HCV and is expressed in a cell of a target subject.

Reference to a "immunologically cross-reactive homolog" of HCV includes any virus which exhibits a structural relatedness to HCV such that an antibody to HCV will bind to
10 the other virus and/or where the other virus can induce a CTL positive response to HCV exposed or vaccinated subjects. Examples of immunologically cross-reactive homologs of HCV include natural or induced variants of HCV or natural or induced recombinant virus comprising parts or portions of HCV.

15 In a particular embodiment, the vaccine delivery vehicle is capable of inducing humoral and cell-mediated immune protection for any genotype of HCV or a particular strain or group of strains of HCV.

Hence, a vaccine delivery vehicle comprising a recombinant baculovirus is also provided
20 having an envelope which contains a surface antigen from HCV and which baculovirus comprises a nucleic acid molecule which encodes at least one protein from HCV which is expressed in a cell of a target subject.

Reference to the "surface antigen" from HCV includes any component of HCV which is
25 accessible to the external environment and to which an antibody can bind. In other words, the antigen is a surface exposed component of HCV. Particularly preferred examples of surface antigens include HCV envelope glycoproteins such as E1 or E2 or a translational precursor thereof.

30 Accordingly, another aspect is directed to a vaccine delivery vehicle comprising a recombinant baculovirus having an envelope which contains a HCV envelope glycoprotein

- 25 -

or an antigenic fragment thereof or a translational precursor thereof and which baculovirus comprises a nucleic acid molecule which encodes at least one protein from HCV and is expressed in a cell of a target subject.

- 5 Preferred HCV envelope glycoproteins are E1 or E2 or a homo- or hetero-dimeric form thereof. Reference to a "homo-dimer" includes E1/E1 forms or E2/E2 forms. A "hetero-dimer" includes E1/E2 or E2/E1.

10 The aim of the HCV envelope glycoprotein is to provide a stimulatory source for the generation of a humoral response. Hence, the HCV envelope glycoprotein may be an "antigenic fragment" thereof meaning that sufficient B-cell epitopes exist to induce a humoral response to HCV envelope glycoproteins.

15 Accordingly, another aspect is directed to a vaccine delivery vehicle comprising a recombinant baculovirus having an envelope which contains an HCV envelope glycoprotein selected from E1, E2, E1 homo-dimer, E2 homo-dimer, E1/E2 or E2/E1 hetero-dimer and a fragment of E1, E2, E1 homo-dimer, E2 homo-dimer or E1/E2 or E2/E1 hetero-dimer which is capable of inducing a humoral immune response in a subject said baculovirus comprising a nucleic acid molecule which encodes at least one protein
20 from HCV and which is expressed in a cell of the target subject.

As indicated above, the target subject is the subject in need of vaccination or treatment. Target subjects include mammals, such as primates and most particularly humans.

25 The protein encoded by the recombinant baculovirus may be the core or capsid protein, an NS protein such as but not limited to NS3/4A or a non-functional mutant thereof such as NS3m/4A or an HCV envelope glycoprotein such as E1 and/or E2. These proteins are produced by cells which are infected by the recombinant delivery vehicle.

30 Hence, another aspect is directed to a vaccine delivery vehicle comprising a recombinant baculovirus having an envelope containing a surface antigen from HCV or an

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immunologically cross-reactive homolog thereof and which baculovirus comprises a nucleic acid molecule which encodes at least one protein selected from the list consisting of E1, E2, core protein, an NS protein and a mutant of a NS protein from HCV that is not immunosuppressive and which protein is expressed in a cell of a target subject.

5

As indicated above, the envelope of the baculovirus may also carry an HCV protein and/or a non-HCV protein to target the baculovirus to a particular TLR and hence to a cell or group or family of cells carrying the TLR. Examples of such TLR-binding proteins are listed in Table 2.

10

The present invention is still further directed to the use of an HCV surface antigens and another HCV protein in the manufacture of a medicament for the induction of humoral and cell mediated protective immunity against HCV.

15 The vaccine delivery vehicle may be formulated in a range of excipients, diluents and/or carriers. A convenient description of a suitable formulation can be found in Remington's Pharmaceutical Sciences, 20th ed. Williams and Wilkins (2000) and The British National Formulary 43rd ed. (British Medical Association and Royal Pharmaceutical Society of Great Britain, 2002; <http://bnf.rhn.net>), the contents of which are hereby incorporated by
20 reference. The pH and exact concentration of the various components of the pharmaceutical composition are adjusted according to routine skills in the art. See Goodman and Gilman's The Pharmacological Basis for Therapeutics (7th ed., 1985).

The RecBV delivery vehicles of the present invention may also be used in gene therapy
25 and/or for inducing an immune response to non-HCV pathogens or HCV and non-HCV pathogens. Reference to "gene therapy" includes replacing a defective gene, protein replacement or augmentation or for inducing gene silencing *via* antisense, sense or RNAi suppression.

30 A delivery vehicle is also contemplated comprising a RecBV having an envelope comprising a ligand for a complementary molecule on a cell surface. Examples include Fc

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which binds to FcR, IgG or a portion thereof which binds to IgGR, CD40L to target CD40 and DAF. The HCV E1/E2 proteins in the RecBV envelope may also target DC through an interaction between E2 and DC-SIGN or CD81.

- 5 A delivery vehicle is further provided comprising a RecBV carrying genetic material for gene therapy or genetic-based vaccination.

Hence, a delivery vehicle is provided herein comprising a RecBV having an envelope comprising an agent selected from the list consisting of a surface antigen from HCV, an antigen from a pathogen and a ligand capable of binding to a cell surface molecule which
10 RecBV further comprising a nucleic acid molecule encoding a protein or RNA and which is expressed in a cell of a target subject.

The delivery vehicle described herein may be described as a RecBV delivery vehicle, a
15 virus-like particle (VLP) or a gene therapy facilitating vehicle.

In general, the delivery vehicle comprises a RecBV having an envelope which contains a heterologous protein and a nucleic acid molecule encoding a heterologous expression product wherein the heterologous protein is an antigen from a pathogen and/or a ligand to a
20 complementary molecule on a cell surface and wherein the heterologous expression product is a peptide, polypeptide or protein or RNA species produced by a cell of a target subject.

The present invention is now further described by reference to the following Examples.
25

In the Examples presented below, the following methods and materials were employed:

Cell Culture

30 Sf9 insect cells were grown as suspension cultures in polycarbonate Erlenmeyer flasks (Corning, Acton, MA) in Sf-900 II serum-free medium [SFM] (Invitrogen, Carlsbad, CA)

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containing 10µg/ml gentamicin. HL1 cells, an interferon-α cured derivative of the Huh8 HCV-replicon positive cell line (Blight *et al*, *Science* 290:1972-1973, 2000) and COS-1 cells were grown in DMEM supplemented with GlutaMAX (Invitrogen), 10% v/v fetal bovine serum (FBS) and penicillin/streptomycin (DMEM10). Primary marmoset
5 hepatocytes were isolated from liver following collagenase perfusion, cryopreserved in Universal Wisconsin solution/20% v/v FBS/0.92 mg ml⁻¹ glutathione-SH/10% w/v DMSO and cultured as described (Beames *et al*, *J Virol* 74:11764-11772, 2000).

The mouse B-cell line IIA1.6 and the stably transformed derivative IIA1.6-FcγRIIa LR
10 which constitutively expresses the FcγRIIa receptor were grown in RPMI medium 1640 supplemented with 300µg/ml L-glutamine (Invitrogen), 10% v/v fetal bovine serum (FBS), 7µM β-mercaptoethanol, 100units/ml penicillin and 100µg/ml streptomycin.

Construction of RecBV-E

15

A cDNA fragment encoding 36 amino acids at the COOH terminus of the HCV core protein and the complete coding regions of E1 and E2 were amplified from a full length cDNA clone using primers E1for and E2rev (Figure 1) and PfuTurbo DNA polymerase (Stratagene, La Jolla, CA). It should be noted that this clone is infectious in chimpanzees.
20 Infectious clones are generally used which are infectious in human cells or other mammalian cells. The 1.8 kb product was purified using a High Pure Purification Kit (Roche, Mannheim), digested with HindIII and XbaI, and then gel purified using the QIAEX II Gel Extraction Kit (Qiagen, Hilden). The fragment was then ligated into the HindIII/XbaI site of plasmid pFastBacMam (pFBM) [Condreay *et al*, 1999 *supra*]
25 downstream of the CMV-IE promoter, to create plasmid pFBM-E (Figure 1). Translation of E1/E2 initiates from an ATG codon inserted between the Kozak sequence and the E1 signal sequence. The E1/E2 insert in pFBM-E was sequenced using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA) and the plasmid used to construct RecBV-E using the Bacto-Bac system (Invitrogen). pFBM without the insert was used to construct
30 RecBV-wt. pFBM-GFP was used to construct the GFP expressing virus RecBV-GFP.

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Construction of recombinant baculovirus.

A cDNA fragment containing the HCV IRES, core, E1 and E2 genes (nt 1 to nt 2582) were
5 . amplified from a full length cDNA clone using primers
5'-ATATGAATTCGCCAGCCCCCGATTGGGG-3' [SEQ ID NO:3] and
5'-AATATCTAGATTAGGCCTCGGCCTGGGCTATCAG-3' [SEQ ID NO:2] and
*PfuUltra*TM high-fidelity DNA polymerase (Stratagene, La Jolla, CA). The 2.6-kb PCR
product was purified using a High Pure Purification Kit (Roche, Mannheim), digested with
10 *Eco*RI and *Xba*I, and then gel purified using the QIAEX II Gel Extraction Kit (Qiagen,
Hilden). The fragment was then ligated into the *Eco*RI/*Xba*I site of the baculovirus donor
plasmid pFastBacMam [pFBM] (Condreay *et al*, 1999 *supra*), downstream of the CMV-IE
(Figure 13). A cassette for expression of gp64 TM-IgG Fc or human transferrin receptor
(hTfR) TM-IgG Fc fusion proteins in insect cells was inserted into the *SexAI/Csp45I* site
15 of pFBM-S, replacing the Neo gene, to generate 2 donor plasmids, pFBM-S-gp64 Fc
(Figure 13A) and pFBM-S-hTfR Fc (Figure 13B), respectively, which were used to
construct the corresponding gp64 Fc and hTfR Fc RecBVs using the Bac-to-Bac
recombination system (Invitrogen).

20 *Preparation of RecBV-E*

Baculovirus stocks were purified from the supernatant of Sf9 cells infected 5 days
previously. The supernatant was clarified by centrifugation (3,000 rpm, 10min, 4°C) and
the virus filtered through a 0.45µm filter unit (Nalge Nunc), then concentrated by
25 ultracentrifugation in a Beckman 60Ti fixed angle rotor (30,000 rpm, 75 min, 18°C) and
the pellet resuspended in PBS. The virus titre was determined using the BacPAK
Baculovirus Rapid Titer Kit (BD Biosciences, Palo Alto, CA).

The titers of the RecBV preparations were determined by plaque immunoassay as
30 described previously (Martyn *et al*, *Arch Virol* 152(2):329-343, 2007) and expressed as
plaque forming units per millilitre (pfu/ml).

- 30 -

Transduction

Monolayer cultures of continuous cell lines or primary marmoset hepatocytes were prepared on glass or plastic (Thermanox, Nalge Nunc) coverslips, respectively, for immunofluorescence (IF), or on 6-well plates for immunoblot or immunoprecipitation (IP) analysis. The medium was replaced with RecBV diluted in DMEM10 (moi =1,000 for Huh7 cells and 10 for SF9 cells), adsorbed for 1h at 37°C and 28°C, respectively, the cells washed with PBS, and incubated in fresh medium for 48h at 37°C and 28°C, respectively.

10

Transfection

Huh7 and COS-1 cell lines, and primary marmoset hepatocytes were transfected at 60-70% confluency using 6µl FuGENE 6 (Roche, Indianapolis, IN) and 2µg plasmid/6-well.

15

Immunofluorescence (IF)

Acetone fixed coverslip cultures were blocked with 2% v/v FBS in PBS, then incubated in anti-HCV E2 (Virostat, Portland, ME) or monoclonal antibody H53 [9], followed by AlexaFluor 488 specific conjugate (Molecular Probes, Eugene, Oregon). All antibody incubations were for 1h at 37°C. The nuclei were stained with propidium iodide (1µg/µl) for 10 min prior to mounting, the samples were examined with a Bio-Rad 1024 confocal microscope and images obtained using LaserSharp 2000 software.

25 For immunofluorescence, the cells were either stained live at 4°C or fixed with acetone prior to staining. After immunostaining the nuclei were stained red with propidium iodide or Syto 61. For immunoblot, the proteins were transferred to a Hybond-C nitrocellulose membrane. Otherwise, the methods were as previously described (Martyn *et al*, 2007 *supra*).

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Immunoprecipitation (IP)

Twenty-four hours post-transfection, COS-1 cells were incubated in met- or cys-free DMEM10 (MP Biomedicals, Aurora, Ohio) for 30min, then pulsed with 150 μ Ci Trans
5 35S-label (MP Biomedicals) for 4h and chased in complete DMEM10 for 24h. The cells were lysed in 1ml RIP buffer (50mM Tris-HCl pH 7.4, 600mM KCl, 1mM EDTA, 1mM PMSF, 1% v/v Triton X-100) for 10min on ice, the debris pelleted, and the supernatant incubated overnight at 4°C with anti-HCV E2 antibody. The complex was precipitated by the addition of Protein G Sepharose 4 Fast Flow (Amersham Biosciences, Uppsala) then
10 resuspended in Laemmli loading buffer for analysis by SDS-PAGE.

The wells of microtitre tray (Supplier) were coated with 5×10^7 pfu of each baculovirus. IgG on the surface of RecBVs was detected using rabbit anti-human IgG/HRP conjugate (DAKO).
15

Immunoblot

Forty-eight hours after transduction or transfection, the cells were washed, then lysed on ice with Laemmli buffer. The lysates were denatured, separated by SDS-PAGE and the
20 proteins transferred to Hybond-C extra nitrocellulose membrane (Amersham Biosciences, Buckinghamshire). The membrane was blocked (3% w/v skim milk in PBST), then incubated with anti-HCV E2 followed by anti-goat Ig/HRP (DakoCytomation, Glostrup). Bands were visualised by ECL and exposure to Hyperfilm ECL (Amersham Biosciences).

25 Virus preparations were loaded on to a carbon and formvar coated grid, incubated with 10nm protein-A gold (Utrecht University, Utrecht, The Netherlands) to label Fc molecules, then fixed with 1% w/v glutaraldehyde (ProSci Tech) as a masking agent, followed by anti-gp64 and 5nm protein-A gold (Utrecht University, Utrecht, The Netherlands). The samples were stained with 1.8% methylcellulose/0.4% uranyl acetate and examined in a
30 JOEL 1010 transmission electron microscope. Images were captured on a MegaView III side-mounted CCD camera (Soft Imaging Systems, USA) and processed for publication in

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Adobe Photoshop (trade mark).

Binding of RecBVs to soluble monomeric FcγRIIa was assessed by ELISA. A 96-well plate (Costar) was coated with 5μg/ml HSA-FcγRIIa then blocked with PBS/1% w/v BSA.

- 5 The plate was washed 3x with PBS only then dilutions of RecBV added and incubated at 37°C for 1h. Control wells contained heat aggregated IgG (HAGG) starting at 50μg/ml. The plate was washed 6x with PBS only, then bound virus detect with an anti-human IgG-HRP conjugate followed by TMB substrate. The color development was stopped with 50μl 1M HCl and the plate read at 450nm.

10

Binding of RecBVs to cells expressing FcRs was assessed by flow cytometry, essentially as described previously (Martyn *et al*, 2007 *supra*). In this case, the bound virus was detected using PE-conjugated anti-pg64 (Expression Systems).

15 ***Generation of stable cell lines***

- The HL1 cell line was infected with RecBV-E at multiplicity of infection (moi) ranging from 10⁴ to 10⁶ as described above; 48h later the cells were selected with medium containing G418 (400μg/ml). Colonies of G418-resistant cells were pooled, and E1/E2-
20 positive cells detected by IF. Cloned stable lines were isolated by sorting single cells from each pool using a FACStar Plus flow cytometer (Becton Dickinson). Two weeks later, cells derived from a single colony were further expanded under G418 selection and confirmed as E1E2-positive by IF.

25 ***Doubling time of stable cell lines***

Stable cells were seeded into a 6-well plate and replicate wells washed and trypsinised at 24h, 48h, 72h and 96h. A viable cell count was performed and the doubling time determined in the logarithmic phase of growth.

30

Detection of E2 on the surface of stable cell lines

A single cell suspension of the stable line P2C6 was resuspended in FACS Wash (PBS containing 2% w/v BSA, 2mM EDTA and 0.1% w/v azide). The cells were stained with
5 anti-HCV E2, diluted from 1:10 to 1:40 in FACS Wash or the isotype control, followed by AlexaFluor IgG conjugate (Molecular Probes). The cells were washed, pelleted and resuspended in FACS Wash for analysis.

Annexin V staining for apoptosis of stable cell lines

10

A suspension of the stable line P2C6 was incubated in 20% v/v ethanol for 20min then resuspended in Annexin V-binding buffer (10mM HEPES pH 7.4, 140mM NaCl, 2.5mM CaCl₂) as a positive control. The parental cell line, HL1, represented the negative control. Annexin V-PE (BD Biosciences) was added to each sample and incubated for 15min at
15 room temp. 5µl of 50µg/ml 7-amino actinomycin D (7-AAD; Sigma) was added to each sample 10min prior to FACS analysis.

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EXAMPLE 1

Transduction of Huh7 cells and primary marmoset hepatocytes

5 Initially, the aim was to compare the efficiency of transfection and transduction in cell lines and primary marmoset hepatocytes. Two days post transfection/transduction, E2 expression was examined by IF and immunoblot. IF of RecBV-E transduced Huh7 cells (Figure 2A) demonstrated a transduction efficiency of 86% compared to a transfection efficiency of 35% with the corresponding plasmid, pFBM-E. It was also observed that the

10 E2-positive cells consisted of two populations viz. high (46%) and low (40%) levels of expression. IF of RecBV-E transduced primary marmoset hepatocytes (Figure 3A) demonstrated a transduction efficiency of 22% compared to a transfection efficiency with pFBM-E of 0.4%. Consistent with the data from the Huh7 cells, the E2-positive hepatocytes also comprised two populations of cells with high (12%) and low (10%) levels

15 of expression. Immunoblot analysis identified a specific band of the predicted electrophoretic mobility for E2 of ~60 kDa (Figure 2B) in RecBV-E transduced Huh7 cells, similar to the major species observed in pFBM-E transfected Huh7 cells. A similar analysis of the primary hepatocytes transduced with RecBV-E identified a broad specific band of ~65 kDa (Figure 3B). This emphasises the efficiency of transduction compared

20 with transfection of primary cells. The E2-specific band from the primary hepatocytes showed a noticeably higher molecular weight than that from Huh7 cells transfected with pFBM-E. This suggests that there are differences in the degree and/or nature of the glycosylation pattern in the primary hepatocytes. Expression of E1 and E2 was demonstrated by co-immunoprecipitation of radiolabeled E1 and E2 using polyclonal anti-

25 E2 antibody following transfection of COS-1 cells with pFBM-E (Figure 2C). Major specific bands of ~35 kDa and ~60 kDa corresponded to the predicted molecular weights for E1 and E2, respectively.

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EXAMPLE 2***Generation of stable cell lines***

It was proposed to generate stable cell lines using the Neo gene in the pFBM plasmid to permit selection by G418. Three weeks after transduction of HL1 cells with RecBV-E followed by G418 selection, colonies were obtained in the wells transduced with a moi of 10,000 and 1,000. G418-resistant colonies were pooled into Pool 1 and Pool 2, respectively and expanded. The percentage of E2-positive cells in these pools was estimated to be 13% and 24%, respectively by IF (Figure 4A). G418-resistant clonal lines derived from each pool after single cell sorting were screened for E2 expression by IF (Figure 4B). Eight E2-positive clones were obtained from 39 colonies resulting from pool 2 and E2 expression confirmed by Western blot (Figure 4C). For the 5 clones analysed, a broad specific band of ~65 kDa corresponded to the predicted molecular weight for E2. The level of expression was similar in all stable clones but slightly lower than that for transient transduction of the parental HL1 cell line with RecBV-E.

EXAMPLE 3***Doubling times of E1/E2-positive stable cell lines***

The doubling times of the 5 stable cell lines and the parental HL1 cell line were determined from the increase in viable cell numbers during the logarithmic phase of growth (Figure 5A). A lag phase of ~48h was observed before cell division. The doubling time of cell line HL1 was estimated to be 31.1h and for 4 of the stable lines ranged between 26.9h to 37.5h, with an average of 31.2h, almost identical to the parental line. One stable cell line, P2C3, considered to be an outlier, had a considerably slower doubling time of 47.2h. Thus, expression of the E1/E2 proteins generally had no effect on cell growth or doubling times.

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EXAMPLE 4

Apoptosis of E1/E2-positive stable cell lines

A representative stable cell line, P2C6, and the HL1 cell line were stained with Annexin V
5 to detect apoptotic cells and the relative percentage of apoptotic cells in the 2 cell lines was
determined by flow cytometry (Figure 5B). The percentage of cells in the gated population
(M1), which is enriched for apoptotic cells, relative to the total population of cells (R1),
was almost identical for the stable cell line P2C6 (7.0 %) and the HL1 parental line (6.9
%). Annexin V staining of ethanol treated P2C6 cells (positive control) showed 92.6 % of
10 apoptotic cells. Thus, the expression of E1/E2 did not result in an increased rate of
apoptosis.

EXAMPLE 5

Localization of E1/E2 in stable cell lines

15

E2 expressed in the stable cell line P2C6 was shown to be correctly folded by a specific,
though weak, reactivity with a conformation-dependent E2 mAb, H53 (Figure 6A). A
proportion of the expressed E2 was detected on the surface of the P2C6 cells by flow
cytometry using the polyclonal anti-E2 antibody (Figure 6B). Detection of E2 on P2C6
20 cells was detected using a high concentration (1:10) of the primary antibody and showed a
significant increase in the net mean fluorescence intensity compared to parental HL1 cells.
The level of specific binding progressively decreased using 1:20 and 1:40 dilutions of
primary antibody.

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EXAMPLE 6

Persistence of RecBV following transduction of HL1 cell line

To assess the duration of persistence of RecBV DNA in cultured cells following
5 transduction, GFP expression was monitored at various times following transduction of the
HL1 cell line with RecBV-GFP (Figure 7). It was estimated that ~90% of cells exhibited
varying levels of fluorescence above background (transduction with RecBV-wt) on Day 3,
~40% of cells were still fluorescent on Day 9, <1% of cells were fluorescent on Day 14,
while no fluorescent cells were detected on either Day 18 or Day 28. Thus, expression
10 from RecBV in mammalian cells can be considered transient and these data confirm an
earlier publication (Tjia *et al*, *Virology* 125:107-117, 1983).

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EXAMPLE 7

Vaccine Delivery Vehicle

The findings in the previous Examples demonstrate the delivery of genes by recombinant
5 baculovirus *in vitro*. At least *in vitro*, it suggests that RecBV DNA is lost from transduced
cells after 18 days of culture without selection.

The data demonstrate the utility of transduction with RecBV expressing the E1/E2
glycoproteins of HCV, characterized by these proteins being expressed transiently in
10 continuous cell lines and, more importantly, in primary hepatocyte cultures which are
difficult to transfect.

Replication studies of HCV in primary hepatocytes enables delivery of the infectious
replicon in a RecBV.

15

The results of this experiment showed inclusion of the human IgG Fc region in the
envelope of RecBV fused to the gp64 transmembrane domain induced resistance to
complement (Figure 25).

20 It has been noted in the past that BV are inactivated by human complement but inclusion
of the VSV G protein into the virus envelope was able to overcome this process (Tani *et al*,
J Virol 77:9799, 2003). As the half life of HCV in the circulation was previously
calculated to be approximately 6 hours, it is most likely that the inclusion of the HCV
E1/E2 proteins into the RecBV envelope will have the same effect. It is determined
25 whether the E1/E2 proteins protect against complement inactivation by incubation of the
RecBV with untreated or heat-inactivated human serum and test the residual infectivity by
inoculation of Huh7 cells, essentially as described (Tani *et al*, 2003 *supra*).

In addition, as HCVpp bind to and are internalized in DC through an interaction with DC-
30 SIGN (Lozach *et al*, *J Biol Chem* 279:3203, 2004), the RecBV with E1/E2 embedded in
the envelope can be expected to target dendritic cells and elicit a strong immune response

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as a result. In fact, mammalian cell-derived West Nile Virus normally binds to DC-SIGNR, whereas insect cell-derived virus binds to DC-SIGN, as a result of the differential glycosylation patterns (Davis *et al*, *J Virol* 80:1290, 2006) suggesting that the insect cell-derived RecBV will also bind strongly to DC-SIGN. Furthermore, the RecBV with HCV
5 E1/E2 in the envelope is likely to bind to DC through CD81, known to be a cellular receptor for HCV.

Hence, three recombinant baculoviruses which contain the HCV envelope glycoproteins embedded in the RecBV envelope and also encode the virus structural proteins
10 (core/E1/E2) and a mutant NS3/4A protein (Ns3m/4) (Figure 8) are constructs. RecBV-E also elicited an antibody response against the HCV E2 protein, proving that the virus transduced cells in the mouse, resulting in expression of the E1/E2 in the cells (driven by the CMV promoter) that in turn resulted in the development of a humoral immune response. The rationale for the design of the RecBV is based on the fact that the RecBV-
15 E1/E2 elicited a strong antibody reaction against the baculovirus envelope (Figure 12), which contains gp64. Baculoviruses are enveloped by budding from the plasma membrane, and although E1/E2 is normally localized to the endoplasmic reticulum. The proteins are directed to the plasma membrane by inserting the E1/E2 genes downstream of the leader sequence of the gp64. Inclusion of the HCV E1/E2 proteins into the RecBV
20 envelope is achieved by expressing the E1/E2 in a stable insect cell line which can be infected with the virus, or by encoding E1/E2 in the baculovirus genome, in this case, controlled by the polyhedrin promoter. The latter approach is chosen since the expression of constitutively-expressed proteins in stable cell lines is often reduced with time in passage. In addition, this strategy has been used successfully in the past to incorporate
25 foreign proteins into the baculovirus envelope (see Kost *et al*, *J Virol* 68:1117-1120, 1994). Thus, the E1/E2 in the virus envelope is expected to elicit a neutralizing antibody response, while the core/E1/E2 and NS3m/4, expressed as endogenous protein in the transduced mammalian cells, are expected to generate an effective cell mediated immune response. However, it is possible that the structural proteins will form virus-like particles
30 (VLPs) that can also be expected to elicit neutralizing antibody responses. In addition, as HCVpp bind to- and are internalized in-dendritic cells through an interaction with DC-

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SIGN (Barth *et al*, *Blood* 105:3605-3614, 2005), the recombinant baculoviruses with E1/E2 inserted into the envelope can be expected to target dendritic cells and elicit a strong immune response as a result. NS3/4 is chosen for use in the vaccine because the protein is highly conserved and contains a large number of CD4+ and CD8+ cell epitopes
5 (Wertheimer *et al*, *Hepatol* 37:577-589, 2003). Others have suggested that NS3 may also be used as an immunogen.

Construction and characterization of the recombinant baculoviruses

10 i) RecBV-J (Japan). The core-p7 region from JFH1 is subcloned into the pFastBacMam donor vector downstream of the CMV promoter. JFH1 is a genotype 2a isolate that replicates in continuous cell lines. An additional downstream region is inserted encoding the EMCV IRES upstream of the NS3m/4 region from the Australian genotype 1b isolate, to generate a bicistronic transfer vector. The E1/E2 gene from JFH1 will then be inserted
15 downstream of the polyhedrin promoter, and the the transfer vector is used to generate RecBV-J.

ii) RecBV-A (Australia). The RecBV viruses which has been constructed is based on the HCV genotype 1b (Trowbridge & Gowans, *Arch Virol* 143:501-511, 1998) and the
20 RecBV-core/E1/E2 virus is modified by inserting the EMCV-NS3m/4 region downstream of the existing HCV genes in the transfer vector. The transfer vector is further modified by cloning the genotype 1b E1/E2 genes downstream of the polyhedrin promoter and the RevBV-A generated as described above.

25 iii) RecBV-Am (Australia mutant). The RecBV-A encodes the wild-type HVR1 sequence. To construct a virus with a greater ability to induce anti-HVR1 antibodies which cross react with a wide range of HCV isolates, the wild-type HVR1 in RecBV-A is substituted with the sequence of the G31 mimotope (Puntoriero *et al*, 1998, *supra*) as this was the most cross reactive of the mimotopes. The G31-containing-RecBV-Am will express three
30 published neutralizing epitopes that can be expected to elicit antibodies with broad cross reactive, neutralizing activity.

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- In addition, the NS3/4 gene in each of the above RecBV encodes a mutant form of NS3 (NS3m/4) containing a C1125A mutation in the protease domain that inactivates NS3 protease function. This will ensure that Interferon Regulatory Factor-3 (IRF-3) function is not cleaved because the protease function of wild-type NS3/4 has been shown to block the effector action of IRF-3 (Foy *et al*, *Science* 300:1145-1148, 2003). The NS3m/4 gene downstream of the EMCV or the HCV IRES elements have been constructed and it has been shown that human Mo-DC, transfected with the specific mRNA matured the DC more effectively than did RNA encoding the wild-type protein.
- iv) Characterization of RecBV. Each of the above viruses is expected to express the E1/E2 proteins in insect cells (to result in incorporation of the HCV envelope glycoproteins in the RecBV envelope) and the core/E1/E2 and NS3m/4 proteins in mammalian cells. The E1/E2 genes encoded downstream of the CMV and polyhedrin promoters, respectively will be homologous. The Sf9 insect cell line is infected with the individual viruses and examine the expression of the E1 and E2 proteins (from the polyhedrin promoter) by immunofluorescence and immunoblot. The expression of E1/E2 is examined on the plasma membrane of the infected insect cell by flow cytometry as performed in the above Examples. To confirm that the E1/E2 proteins are present in the virus, the virus is purified by gradient ultracentrifugation and the infectious fractions tested for E1/E2 by immunoblot. Similarly, expression of the proteins controlled by the CMV promoter 48 hours after transduction of Huh7 cells is confirmed by immunofluorescence and immunoblot.
- v) Exclude RecBV DNA integration. Although the circular, ds, baculovirus DNA does not integrate during replication, it will be vital to exclude integration after intramuscular injection of the RecBV. A previous study (Tjia *et al*, 1983 *supra*) used Southern blot hybridization analysis to show that baculovirus DNA did not persist beyond 48 hours in mammalian cells and studies showed that proteins expressed from the RecBV in transduced cells did not persist beyond 18 days post infection (see Examples above). To exclude DNA integration *in vivo* the mice are killed at intervals after intramuscular

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injection of the RecBV and the muscle removed. Integrated viral DNA is usually only detected after clonal amplification of a cell which contains the inserted DNA, as infrequent integration events are undetected in the vast excess of normal cellular DNA prior to this. Assuming that all the plasmid DNA detected by PCR is integrated, it is calculated that the risk from mutation by insertional mutagenesis, is approximately 350 times lower than that of the spontaneous rate of mutation. Clearly, the calculated risk will be directly dependent on the amount of residual RecBV DNA, the amount of RecBV DNA is measured and then the relative risk calculated. The injection of RecBV with a ds circular DNA genome poses a lower risk than that after injection of plasmid DNA, some of which is likely to be linear and more likely to result in integration.

The baculovirus may also be engineered to contain a TLR-targeting protein either from HCV or a non-HCV source. Examples or suitable sources include those listed in Table 2. In addition, the baculovirus envelope may contain other targeting moieties such as an IgG constant region to target any antigen-presenting cell with IgG receptors, decay accelerating protein (DAF) which increases resistance to complement and/or CD40L to target CD40 as DC.

Vaccinate mice and examine the neutralizing antibody responses in an HCV neutralization assay

i) Vaccination with RecBV-J. Balb/C are vaccinated with the RecBV-J by the intramuscular route as described (Facciabene *et al*, *J Virol* 78:8663-8673, 2004) using two doses of 10^8 ffu, three weeks apart. The anti-E2 response will be measured at regular intervals by ELISA as described above. The anti-core response is detected using ELISA (Trowbridge *et al*, *J Hepatol* 24:532-538, 1996) to act as an internal standard for the efficacy of vaccination with RecBV-J, RecBV-A and RecBV-Am, possible because the protein is highly conserved. Similarly, the anti-NS3 response will be titrated by ELISA and immunoblot using purified NS3 protein. Most importantly, the neutralizing antibody response is tested against JFH1 (Figure 9).

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- ii) Vaccination with RecBV-A. In a similar series of experiments, mice are vaccinated with RecBV-A. In this case, neutralizing antibody is measured against the HCV pseudotyped virus (HCVpp) as described (Drummer *et al*, *Clin Exp Immunol* 142:362-369, 1996). In the event that a genotype 1 isolate is discovered that replicates in cell culture, the mouse sera is tested in an authentic neutralization assay, as described above for JFH1. The degree of cross neutralization resulting from vaccination with the homologous and heterologous virus is also determined.
- iii) Vaccination with RecBV-Am. To determine if it is possible to induce a greater degree of cross-reactive neutralizing antibodies, mice are vaccinated with RecBV-Am, and test serum samples for neutralization against Am and A pseudotypes and JFH1 virus.
- iv) Immune capture assay. The ability of all serum samples from mice vaccinated with RecBV-JH, RecBV-A or RecBV-Am to precipitate HCV virions in an immune capture assay is examined. This assay uses purified mouse IgG to coat 0.2ml PCR strip tubes which are then blocked prior to the addition of HCV-positive serum. The RNA in bound virus is released, purified and detected by nested RT-PCR. This assay determines the antibodies are capable of recognizing whole virus particles and is particularly relevant for samples from animals which were tested against the HCVpp, but will also extend the information related to the potential cross neutralizing ability of antibodies elicited by the different RecBV, because HCV-positive serum is used from patients infected with different isolates and genotypes.
- vi) Anti-E1/E2 antibody isotype. It has been shown that anti-HCV antibodies detected in persistent infection are almost entirely IgG1, whereas most viral antigens induce IgG1 and IgG3, with some IgG2 and IgG4. Consequently, it is determined which Ig subclass of the anti-E1/E2 antibodies is induced by the RecBV, as it is most likely that the lack of IgG3 during HCV infection is not related to the proteins but to HCV subversion of the immune response. In addition, the induction of IgG2 indicates a preference for the Th1 pathway.

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Examine CMI elicited by RecBV-E1/E2 and RecBV-NS3/4 in Balb/C mice

After vaccination, endogenous expression of the HCV proteins is expected to result in a cell mediated immune response. The CD4⁺ T-cell response will be evaluated by lymphocyte proliferation (³H-T incorporation) using recombinant HCV protein
5 restimulated spleen cells from the vaccinated mice. CD8⁺ CTL activity is detected by IFN- γ ELISpot using irradiated, recombinant vaccinia virus (recVV)-HCV- infected spleen cells from naïve mice as antigen-presenting, stimulatory cells, or with pools of HCV peptides (18mers overlapping by 11). An *in vivo* CTL assay is also performed spleen cells
10 from naïve syngeneic mice is pulsed with pooled HCV-specific peptides. The HCV-peptide-pulsed cells, and unrelated peptide-pulsed controls cells are labeled with high and low concentrations of CFSE, respectively as described (Coles *et al*, *J Immunol* 168:834, 2002) then irradiated. The cells are then mixed, injected into the mice by the intravenous route and the cell viability measured 16 hours later by flow cytometry of a spleen cell
15 suspension to identify the differential CFSE populations. These experiments detect CMI to peptides which are restricted by the mouse H-2² (Balb/C).

Challenge with recombinant vaccinia virus

20 To examine the protective efficacy of the CMI generated against the HCV proteins, the vaccinated female mice are challenged with recVV-HCV. Two weeks after immunization, the mice are injected by the intraperitoneal route with a standard dose of virus (10⁷ pfu) and the titre in the ovaries (the preferred site of VV replication) determined four days later by plaque assay.

25

Construct a chimeric BVDV encoding the HCV NS3/4 proteins

Ideally, one challenges a large animal model with an authentic virus, to prove vaccine efficacy. Although the chimpanzee model can be used to prove the efficacy of HCV
30 vaccines, the model is very expensive. To determine if the vaccinated sheep generate protective immunity, as a result of the cell mediated responses against the NS3/4 proteins

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recombinant BVDV is constructed which encodes the wild-type NS3/4 proteins. Although BVDV primarily infects cattle, it also infects sheep. The HCV IRES-NS3/4 (wild-type) gene is inserted downstream of the NS5B gene in the BVDV genome. The NS5A/5B cleavage site in the BVDV polyprotein recognized by the BVDV NS3 is substituted with
5 the HCV 5A/5B cleavage site that is recognized by the NS3/4A protease. This will ensure that continued expression of the HCV NS3/4A is required for replication of the mutant BVDV (Figure 10).

The virus is titrated in sheep to determine a 50% infectivity dose and vaccinated sheep will
10 be challenged with 10-100ID₅₀.

EXAMPLE 8

Construction of recombinant baculovirus vectors designed to display human IgG Fc

15 In this Example, RecBvs were constructed and characterized displaying the Fc region of human IgG in their envelope. It was proposed that the baculovirus vectors displaying Fc would more efficiently transduce cell lines expressing Fc receptors and antigen-presenting cells, in a process analogous to the uptake of immune complexes, compared to the wild-type (wt) baculovirus vector. Thus, the aim of the study was to assess the relative levels of
20 binding of the RecBVs and wtBV to cells expressing the Fc receptor (cell lines and APCs) and determine if the levels of expression of the HCV structural proteins (core, E1 and E2) following transduction of mammalian cells showed a corresponding increase.

It hypothesized that the relatively large cytoplasmic domain at the N-terminus of the
25 transferrin receptor (aa 1-67) may lead to exclusion of the hTfR-IgG Fc fusion protein from the BV envelope in the process of budding. For this reason, most of the cytoplasmic N-terminal domain of the TfR sequence from aa 1-53 was deleted in the construct, as this truncation was previously reported to have no effect on expression of the TfR in the type II orientation (Zerial *et al*, *EMBO J* 5:1543-1550, 1986).

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IgG Fc is expressed as a homodimer on the plasma membrane of RecBV-infected insect cells and on the envelope of RecBV particles.

Human IgG Fc was expressed as a fusion protein with either the gp64 TM or the hTfR TM in RecBV-infected, but not wtBV-infected, Sf9 insect cells, as determined by IF (Figure 15). Analysis of live (unfixed) Sf9 insect cells infected with RecBVs showed that the expressed Fc fusion proteins were localised to the plasma membrane (Figures 15A, 15B) while the wtBV-infected and mock-infected cells were negative (Figures 15C, 15D). Minor differences in the distribution of Ig Fc were noted between gp64 and hTfR RecBV (Fig 3A, 3B) but it was clear that the proteins were expressed on the plasma membrane. Analysis of the infected cells by flow cytometry, showed that the hTfR Fc RecBV-infected cells appeared to express approximately 2-fold levels of Ig Fc compared with gp64 RecBV-infected cells and this point is discussed more fully below (Figure 18). Immunoblot analysis of infected Sf9 cells and the RecBV samples detected a major band of the expected MW for the monomeric Fc fusion proteins (~35kDa) and another major band of ~70kDa, predicted to represent dimerised Fc fusion proteins that were resistant to reduction. The proportion of 35:70kDa proteins was approximately 1:1 in the gp64 Fc Ig RecBV-infected cells but was approx 100:1 in the hTfR FcIg RecBV-infected cells (Figure 16). Monomeric and dimeric Fc fusion proteins were also detected in RecBV, but not wtBV, particles (Figure 16) but in this case, the 35:70kDa proportion was similar in both virus preparations and was considered to be approximately 2:1. As the concentration of the two RecBV preparations was adjusted to contain 2×10^8 pfu, the data in the right panel (Figure 16), show that each virus preparation appeared to contain similar levels of Ig-Fc and similar ratios of the 35:70kDa fusion proteins. However, the ratio of the p35:p70 proteins may simply reflect the degree of denaturation achieved in this denaturing gel system and thus the level of the p70 dimer may actually be much higher.

In summary, the results of this part of the study show that the RecBV gp64 Ig Fc and RecBV hTfR Ig Fc both direct the expression of human Ig Fc on the plasma membrane of infected Sf9 insect cells and that semi-purified preparations of the viruses contain dimeric forms of the Ig Fc and may also contain monomeric forms.

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Ig Fc is expressed on the envelope of RecBV.

Although the above data indicated that the RecBV contained the human Ig Fc molecule, it was necessary to determine if the protein was expressed on the virus envelope. To address this question, an equivalent amount (pfu) of semi-purified RecBV was adsorbed to the wells of a microtitre plate and the expression of Ig Fc examined by ELISA using a rabbit anti-human IgG/HRP conjugate. The results of this experiment showed that Ig Fc was detected by the conjugate in a dose-dependent manner in both RecBV preparations (Figure 17A), but not the wtBV, although the gp64 Ig Fc RecBV generated a fourfold increase in the OD compared with hTfR Ig Fc RecBV. As the concentration of both virus preparations was previously equilibrated, this suggests that either the gp64-Ig Fc fusion protein on the virus particle may be more accessible to the antibody or that this virus contains a higher density of IgFc on the envelope.

15

To confirm this observation and investigate the localization of the Ig Fc molecule on the virus particles, an immunoelectron microscopy was performed. The results of this analysis showed that the Ig Fc proteins were localized to both poles of the particle (Figure 17B) of intact RecBV and confirmed that the proteins were present in the envelope of both virus preparations, although it was not possible to determine from this analysis if the density of the gp64 IgG Fc was higher.

20

IgG Fc displayed on the plasma membrane of RecBV-infected insect cells binds specifically to FcγRIIa.

25

The above data demonstrated that Ig Fc was expressed on the surface of RecBV-infected cells and on the envelope of the RecBV themselves. A series of experiments were performed to determine if the Ig Fc molecule was functional. To demonstrate this, soluble FcγRIIA protein was added to RecBV-infected Sf9 cells, 48h post infection, and the level of binding measured by flow cytometry (Figure 18). The mean fluorescence intensity (MFI) was then compared with the MFI of the Ig Fc detected by flow cytometry using anti-

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Ig FITC. The results (Figure 18A-C) show that although the MFI of the hTfR-expressed Ig Fc was twofold greater than that of gp64 Ig Fc (MFI=326 v 153), the MFI of bound FcγRIIA was approximately 4.5-fold greater on the gp64-infected cells (Figure 18D-F). Wild type BV-infected cells showed no FcγRIIA binding activity. Thus, although both Ig Fc fusion proteins expressed on the plasma membrane of RecBV-infected cells were able to bind FcγRIIA, it appears that a higher proportion of the gp64-Ig Fc was functional.

RecBV bind soluble FcγRIIA and bind to cells which express surface FcγRIIA.

The inclusion of Ig Fc into the envelope of RecBV should impart the particles with the ability to bind soluble FcγRIIA protein and also result in increased binding and uptake of the RecBV to cells which express the FcγRIIA. To investigate this, dilutions of RecBV or wtBV were added to the wells of a microtitre plate, previously coated with soluble FcγRIIA, and bound virus detected with an anti-human IgG HRP conjugate. BSA-coated plates were used as negative controls and heat aggregated IgG (HAGG) included as a positive control. The results of this study (Figure 19) showed that the RecBV showed a similar level of high affinity binding as HAGG to the FcγRIIA-coated wells but failed to bind to the BSA-coated wells, while wtBV showed no binding to either. RecBV binding to the FcγRIIA showed a typical dose response curve (Figure 19) that was inhibited by prior incubation of the coated wells with the blocking monoclonal antibody IV-3 by ~75% (gp64-Fc) ~100% (hTfR-Fc).

To determine if the RecBV show an increased level of binding to cells which express the FcγRIIA, the IIA1.6 cell line, which was constructed to express the FcγRIIA in a constitutive manner, was transduced and the MFI resulting from detection of bound virus was then determined by flow cytometry (Figure 20). The parental cell line failed to bind RecBV hTfR-Fc, whereas the IIA1.6 line bound the virus in a dose-dependent manner. To confirm the specificity of this reaction, the RecBV hTfR-Fc-IIA1.6 interaction was inhibited by the prior addition of MAAb IV-3 (Figure 20), in a similar manner to that described above to soluble RecBV hTfR-Fc. Thus, hTfR-Fc RecBV binding is FcγR dependent.

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The binding of gp64-Fc and hTfR-Fc RecBVs to primary cells which express FcγRs was also assessed (Figure 21). Immature human CD14⁺ monocyte-derived dendritic cells (Mo-DC) were prepared, mixed with virus as described (Martyn *et al*, 2007 *supra*) and bound virus detected by flow cytometry.

This experiment showed that, while the parental virus showed minimum binding to the Mo-DC, the gp64 Fc RecBV showed no binding, whereas the hTfR-Fc RecBV showed dose-dependent binding RecBVRecBVRecBV (Figure 21). It is likely that the parental virus binds through a different mechanism to that of the hTfR Fc RecBV which is likely to bind through the FcγR, while it is possible that steric hindrance, resulting from inclusion of the gp64 Fc into the RecBV envelope prevented a similar level of gp64 Fc RecBV binding as shown by the parental virus.

A similar binding experiment was performed to investigate if the RecBV were able to bind to other populations of cells within peripheral blood mononuclear cells (PBMC). Virus was incubated with PBMC as described above and binding assessed by flow cytometry (Figure 22) to two major cell populations viz. granulocytes, determined by FSC characteristics and monocytes, determined by staining with anti-CD14. The results of this experiment showed that the parental virus was able to bind to a proportion of cells, determined to be monocytes, in a dose dependent manner (R1 in panel A, Figure 22) and to a minor proportion of granulocytes (L1 in panel A)-compare these data with the no virus control (see inset). In contrast, the gp64 Fc RecBV was able to bind to a high proportion of granulocytes (L1 in panel B) and monocytes (R1 in panel B), but the level of binding was not increased with higher concentrations of virus, suggesting that the gp64 Fc RecBV receptors were already saturated at a moi of 20. In contrast, the hTfR Fc RecBV was not only able to bind to granulocytes and monocytes at low moi (Panel C) but showed increased levels of binding related to increased virus moi.

In summary (Figure 23), the parental virus was able to bind to monocytes (and granulocytes to a lesser degree) in a dose-dependent manner, that was clearly independent

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of an Fc- FcγRIIa interaction, whereas the gp64 Fc RecBV showed some binding to both cell types that was saturated at 20 moi. This result is similar to the binding pattern to Mo-DC described above. In contrast, the hTfR Ig RecBV was able to bind to a high proportion of granulocytes and monocytes, in a dose dependent manner, most likely through the
5 FcγRIIa.

RecBVs displaying IgG Fc exhibit enhanced expression of HCV structural proteins compared to wild-type baculovirus following transduction of cells expressing Fc receptors.

10

It was determined whether the level of expression of the HCV proteins encoded in the RecBV DNA and controlled by the CMV promoter was increased as a result of the increased level of RecBV binding to FcγRIIa-positive cells. Consequently, the levels of expression of the HCV proteins (core, E1 and E2) following transduction of the IIA1.6 cell
15 line with the gp64-Fc RecBV, hTfR-Fc RecBV and wtBV were compared. The cells were transduced with virus at a moi of x and at 48h post-transduction the relative levels of HCV protein expression were quantitated by flow cytometry and immunoblot. Flow cytometry indicated that a higher percentage of the IIA1.6 cells were transduced with the RecBVs compared to wtBV and that the level of expression in the RecBV-transduced cells was
20 generally higher than in cells transduced with wtBV. Thus, the increased binding efficiency of the gp64-Fc RecBV and hTfR-Fc RecBV also resulted in an increase in the levels of expression of the HCV proteins driven by the CMV promoter.

This Example provides a RecBVs displaying functional IgG Fc in the envelope in type I
25 and type II orientations. These RecBVs are improved baculoviral vectors able to enhance binding to cell lines and APCs expressing Fc receptors *in vitro*, thereby delivering encoded genes and targeting expression to these cells. These viral vectors have application for the delivery of genes *in vivo* for the purpose of vaccination or gene therapy.

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EXAMPLE 9

Use of RecBV as a virus-like particle to generate humoral and cell mediated immune responses

5 A RecBV is generated which contains foreign (heterologous) proteins inserted into the virus envelope which is a VLP that, in addition to inducing humoral immune responses, also induces cell mediated immune responses as a result of cross presentation. Cross presentation is described as the process of exogenous proteins being taken up by antigen presenting cells, processed and presented in the context of MHC class 1.

10

VLPs constructed in this way are used for vaccination purposes either with or without the addition of a suitable adjuvant which may help to induce MHC class I-restricted responses. This is useful as there are currently no adjuvants which are licensed to elicit cell mediated responses in humans.

15

In the example shown in Figure 24, different proteins, fused to the transmembrane domain of the transmembrane domains of pg64, CD4 or hTfR are shown as examples. These proteins represent any of the HCV structural proteins (core, E1 and E2) or the non-structural proteins (NS2, NS3, NS4, NS5) but the conserved highly immunogenic core protein might be preferred as might be the NS3 protein which is not only conserved, but
20 also because cellular immune responses to this protein are associated with recovery from acute phase HCV infection.

Those skilled in the art will appreciate that the invention described herein is susceptible to
25 variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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- 56 -

CLAIMS:

1. A vaccine delivery vehicle comprising a recombinant baculovirus having an envelope which contains an antigen from a pathogen or an immunologically cross-reactive homolog thereof and which baculovirus comprises a nucleic acid molecule which encodes at least one protein from the pathogen and which protein is expressed in a cell of a target subject.
2. The vaccine delivery vehicle of Claim 1 wherein the pathogen is Hepatitis C virus (HCV).
3. The vaccine delivery vehicle of Claim 1 wherein the envelope comprises is an HCV envelope glycoprotein.
4. The vaccine delivery vehicle of Claim 3 wherein the HCV envelope glycoprotein is E1 or a translational precursor thereof.
5. The vaccine delivery vehicle of Claim 3 wherein the HCV envelope glycoprotein is E2 or a translational precursor thereof.
6. The vaccine delivery vehicle of Claim 3 wherein the HCV envelope glycoprotein comprises an E1/E2 dimer.
7. The vaccine delivery vehicle of any one of Claims 2 to 6 wherein the protein encoded by the nucleic acid molecule in the baculovirus is the HCV core protein or a mutant thereof or a translational precursor thereof.
8. The vaccine delivery vehicle of any one of Claims 2 to 6 wherein the protein encoded by the nucleic acid molecule in the baculovirus is the HCV NS3/4A protein or a mutant thereof or a translational precursor thereof.

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9. The vaccine delivery vehicle of Claim 8 wherein the mutant of HCV NS3/4A is NS3m/4A.
10. The vaccine delivery vehicle of any one of Claims 2 to 6 wherein protein encoded by the nucleic acid molecule in the baculovirus is an HCV envelope glycoprotein.
11. The vaccine delivery vehicle of Claim 10 wherein the HCV envelope glycoprotein is E1 or a translational precursor thereof.
12. The vaccine delivery vehicle of Claim 10 wherein the HCV envelope glycoprotein is E2 or a translational precursor thereof.
13. The vaccine delivery vehicle of Claim 10 wherein the HCV envelope glycoprotein is an E1/E2 dimer.
14. The vaccine delivery vehicle of any one of Claims 2 to 6 wherein the nucleic acid molecule in the baculovirus encodes two or more of E1, E2, core protein, NS3/4A and/or NS3m/4A.
15. The vaccine delivery vehicle of Claim 1 wherein the target subject is a mammal.
16. The vaccine delivery vehicle of Claim 15 wherein the target mammal is a human.
17. The vaccine delivery vehicle of any one of Claims 1 to 16 further comprising a targeting moiety in the baculovirus envelope.
18. The vaccine delivery vehicle of Claim 17 wherein the targeting moiety is selected from the list consisting of a TLR-binding protein, an IgG constant region or IgG receptor binding portion thereof, CD40L, DAF protein and an Fc portion which binds to Fc receptor.

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19. The vaccine delivery vehicle of Claim 18 wherein the TLR-binding protein is selected from the list in Table 2.
20. A vaccine delivery vehicle comprising a recombinant baculovirus having an envelope which contains HCV envelope glycoproteins E1/E2 in dimeric form and which baculovirus comprises a nucleic acid molecule which encodes at least one protein selected from the list consisting of E1, E2, core protein, NS3/4A and NS3m/4A which protein is expressed in a cell of the mammalian subject wherein said vaccine delivery vehicle when introduced to a mammalian subject generates a protective humoral and cell mediated immune response to HCV or an immunologically cross-reactive homolog thereof.
21. A method for vaccinating a target subject against HCV infection said method comprising administering to said subject a vaccine delivery vehicle comprising a recombinant baculovirus having an envelope which contains a surface antigen from Hepatitis C Virus (HCV) or an immunologically cross-reactive homolog thereof and which baculovirus comprises a nucleic acid molecule which encodes at least one protein from HCV which is expressed in a cell of said subject, wherein said vaccine delivery vehicle induces a protective humoral and cell mediated immune response to HCV or an immunologically cross-reactive homolog thereof.
22. The method of Claim 21 wherein the surface antigen is an HCV envelope glycoprotein.
23. The method of Claim 22 wherein the HCV envelope glycoprotein is E1 or a translational precursor thereof.
24. The method of Claim 22 wherein the HCV envelope glycoprotein is E2 or a translational precursor thereof.

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25. The method of Claim 22 wherein the HCV envelope glycoprotein comprises an E1/E2 dimer.
26. The method of any one of Claims 21 to 25 wherein the protein encoded by the nucleic acid molecule in the baculovirus is the core protein or a mutant thereof or a translational precursor thereof.
27. The method of any one of Claims 21 to 25 wherein the protein encoded by the nucleic acid molecule in the baculovirus is the NS3/4A protein or a mutant thereof or a translational precursor thereof.
28. The method of Claim 27 wherein the mutant of NS3/4A is NS3m/4A.
29. The method of any one of Claims 21 to 25 wherein protein encoded by the nucleic acid molecule in the baculovirus is an HCV envelope glycoprotein.
30. The method of Claim 29 wherein the HCV envelope glycoprotein is E1 or a translational precursor thereof.
31. The method of Claim 29 wherein the HCV envelope glycoprotein is E2 or a translational precursor thereof.
32. The method of Claim 29 wherein the HCV envelope glycoprotein is an E1/E2 dimer.
33. The method of any one of Claims 21 to 25 wherein the nucleic acid molecule in the baculovirus encodes two or more of E1, E2, core protein, NS3/4A and/or NS3m/4A.
34. The method of Claim 21 wherein the target subject is a mammal.

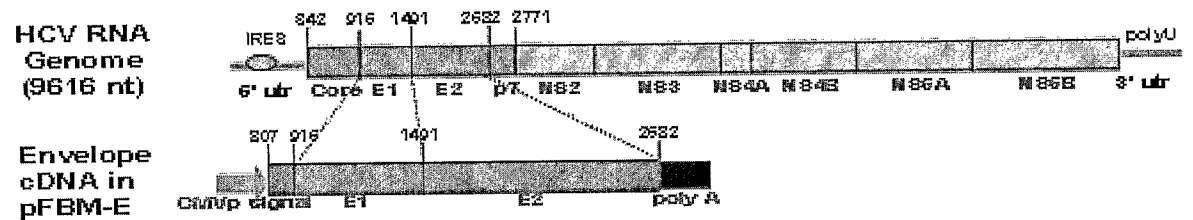
- 60 -

35. The method of Claim 34 wherein the target mammal is a human.
36. The method of Claim 21 to 35 further comprising a targeting moiety in the baculovirus envelope.
37. The method of Claim 36 wherein the targeting moiety is selected from a TLR-binding protein, an IgG constant region or IgG receptor binding portion thereof, CD40L, DAF protein and an Fc portion which binds to FcR.
38. The method of Claim 27 wherein the TLR-binding protein is selected from the list in Table 2.
39. A composition comprising a vaccine delivery vehicle of any one of Claims 1 to 20 and one or more pharmaceutically acceptable carriers, diluents and/or excipients.
40. Use of a vaccine delivery vehicle of any one of Claims 1 to 19 in the manufacture of a medicament for inducing a protective humoral and cell mediated immediate immune response to HCV or an immunologically cross-reactive relative thereof.
41. A method for vaccinating a subject against a pathogen said method comprising administering to said subject a vaccine delivery vehicle comprising a recombinant baculovirus having an envelope which contains a surface antigen from the pathogen or an immunologically cross-reactive homolog thereof and which baculovirus comprises a nucleic acid molecule which encodes at least one protein from the pathogen and which is expressed by a cell of a target subject, wherein said vaccine delivery vehicle induces a protective humoral and cell mediated immune response to the pathogen or an immunologically cross-reactive homolog thereof.

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42. A method for gene therapy in a subject said method comprising administering to said subject a delivery vehicle comprising a recombinant baculovirus having an envelope which contains a surface ligand capable of binding to a complementary ligand on a target cell and which baculovirus comprises a nucleic acid molecule which encodes a peptide, polypeptide or protein or RNA species and which is expressed by a cell of a target subject, wherein said delivery vehicle facilitate peptide, polypeptide or protein replacement and/or gene regulation.

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A:**B**

Primer E1for

5'- ATAATAAGCTTGCACCATGCGGGTTCTGGAGGACGGCGTG -3'

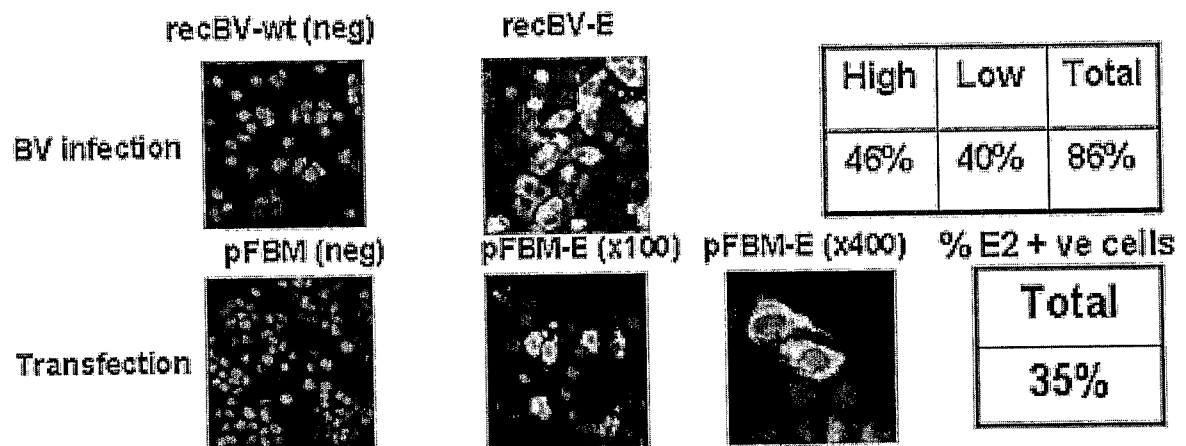
Primer E2rev

5'- AATATCTAGATTAGGCCTCGGCCTGGGCTATCAG -3'

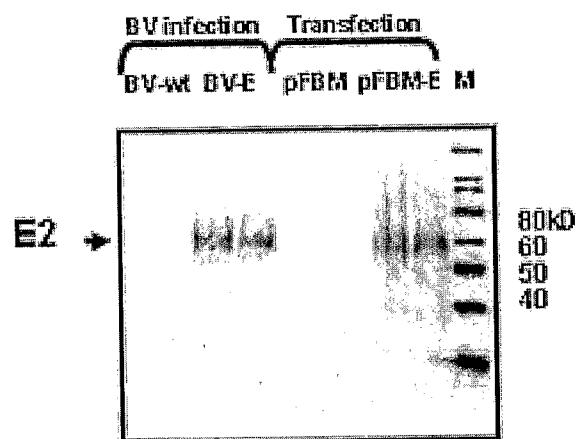
Figure 1

2/26

A:



B:



C:

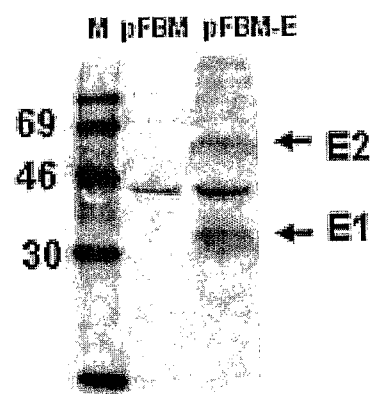


Figure 2

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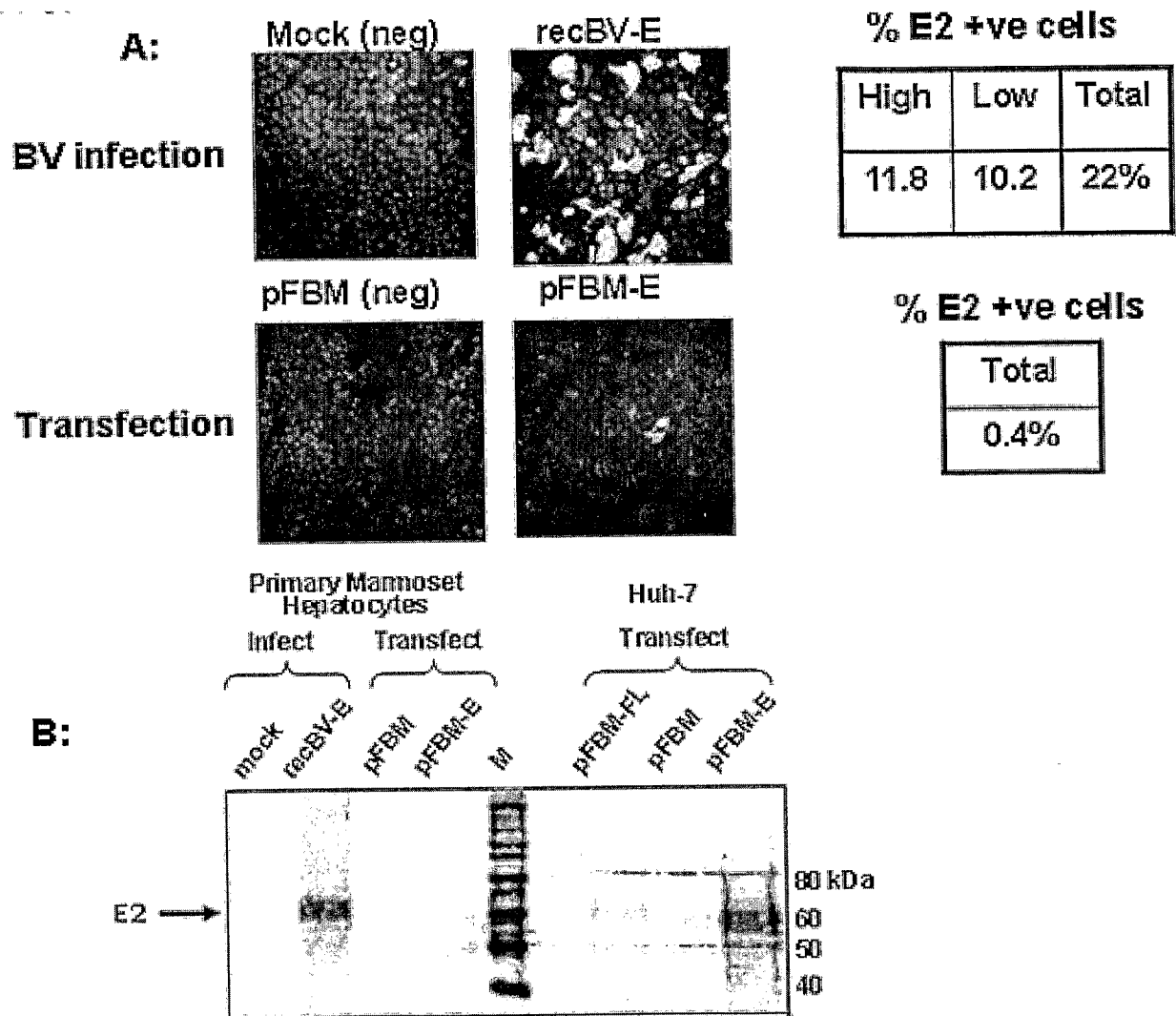
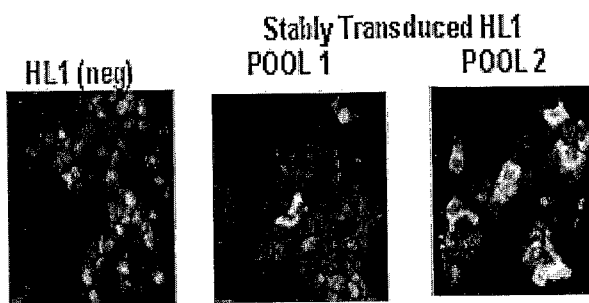


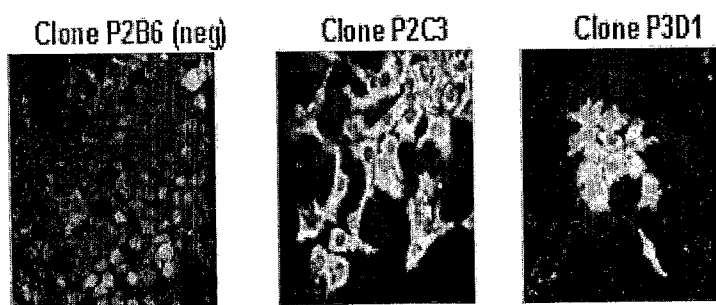
Figure 3

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A:



B:



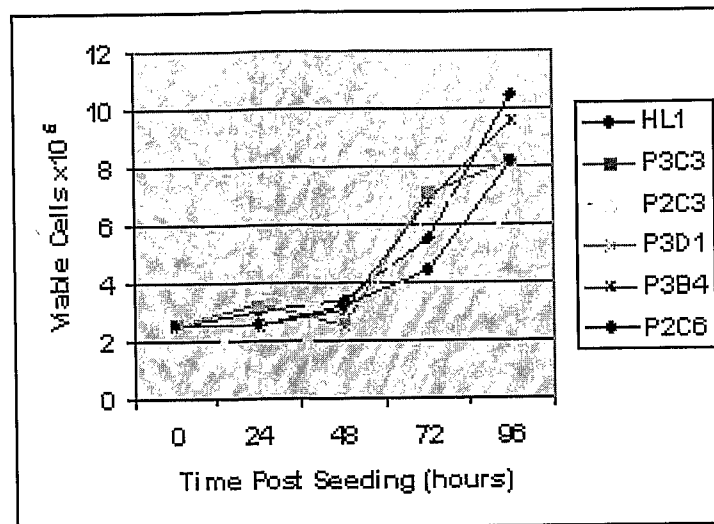
C:



Figure 4

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A:



B:

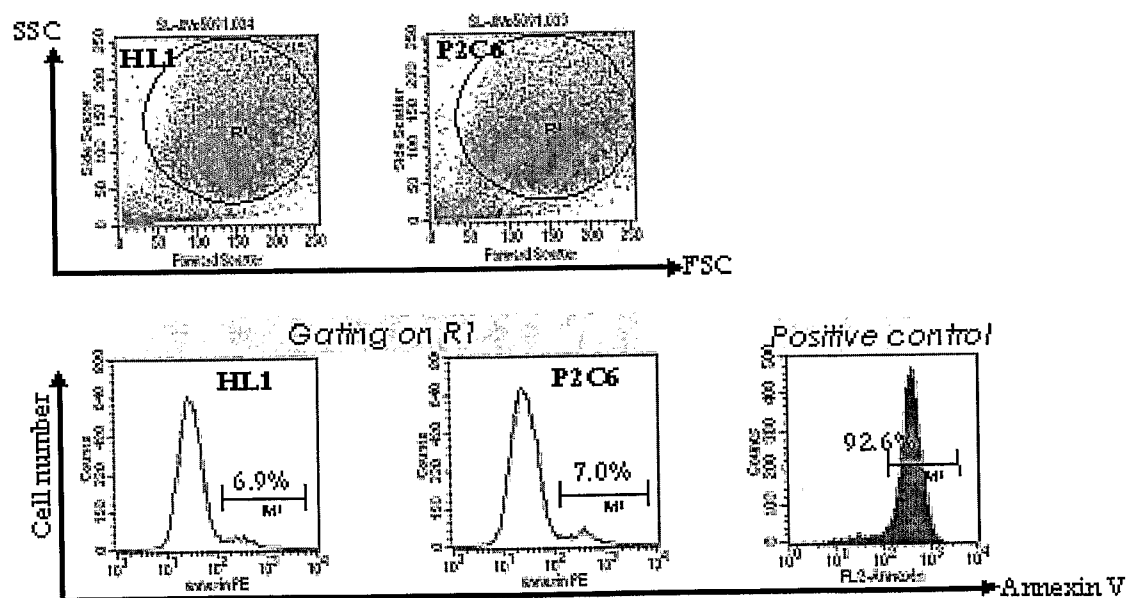
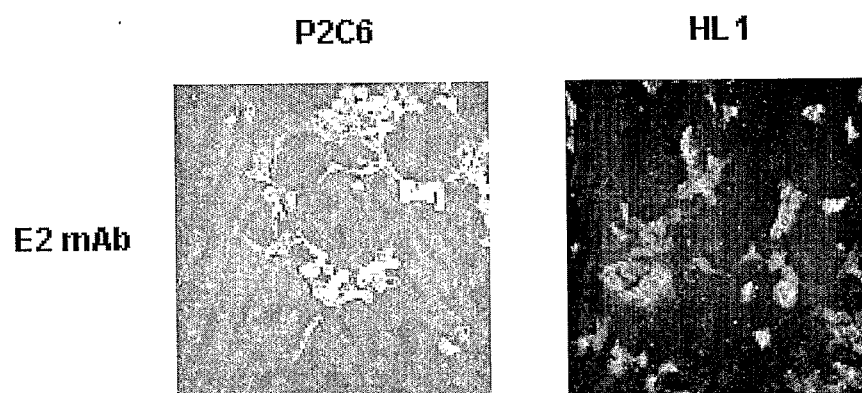


Figure 5

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A:



B:

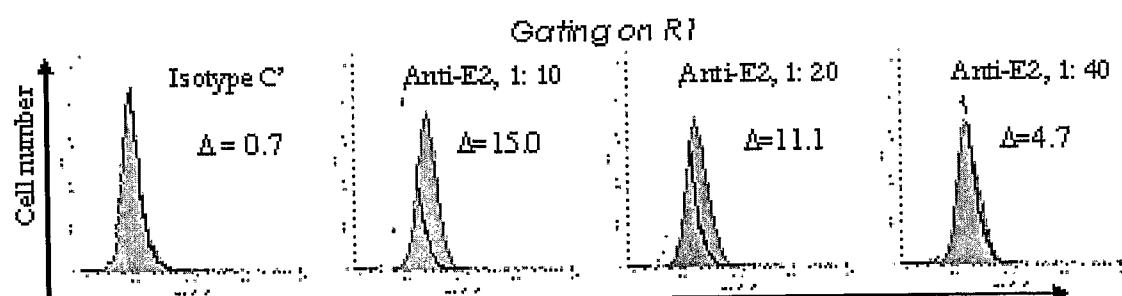


Figure 6

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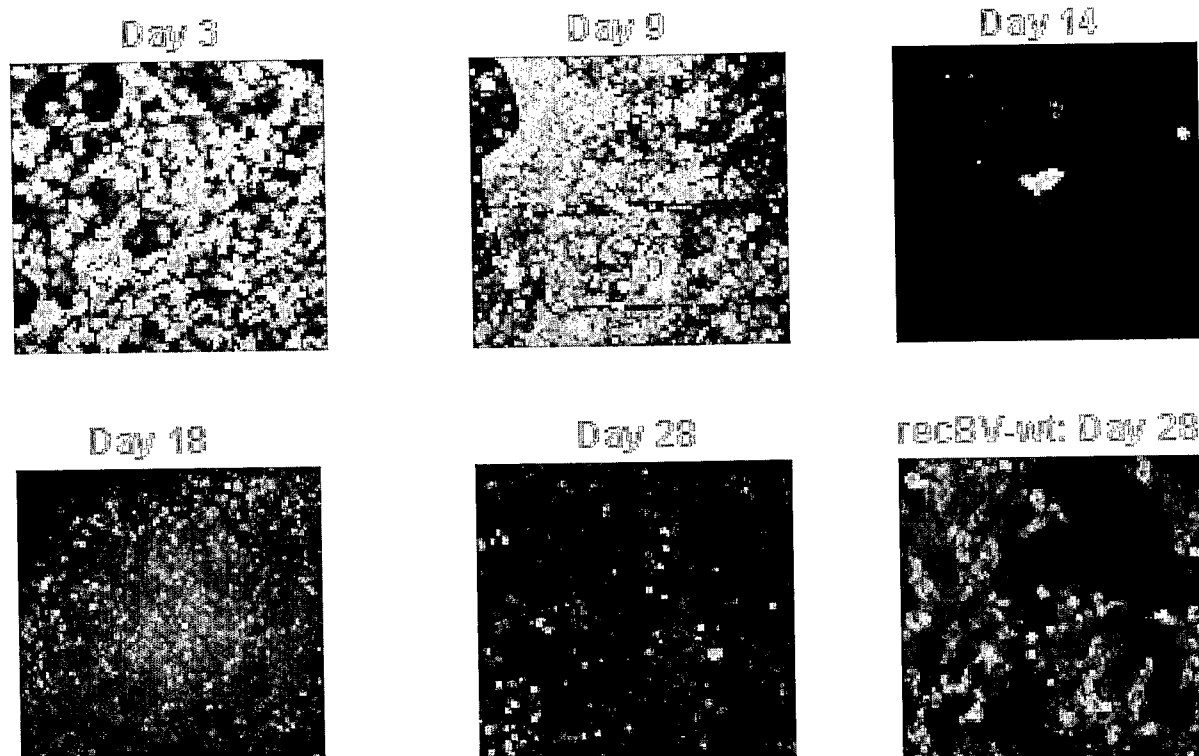


Figure 7

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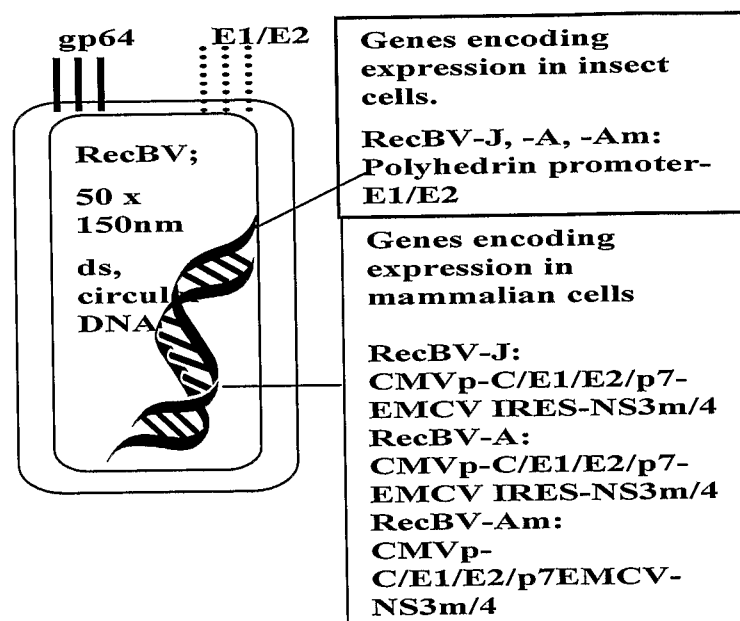


Figure 8

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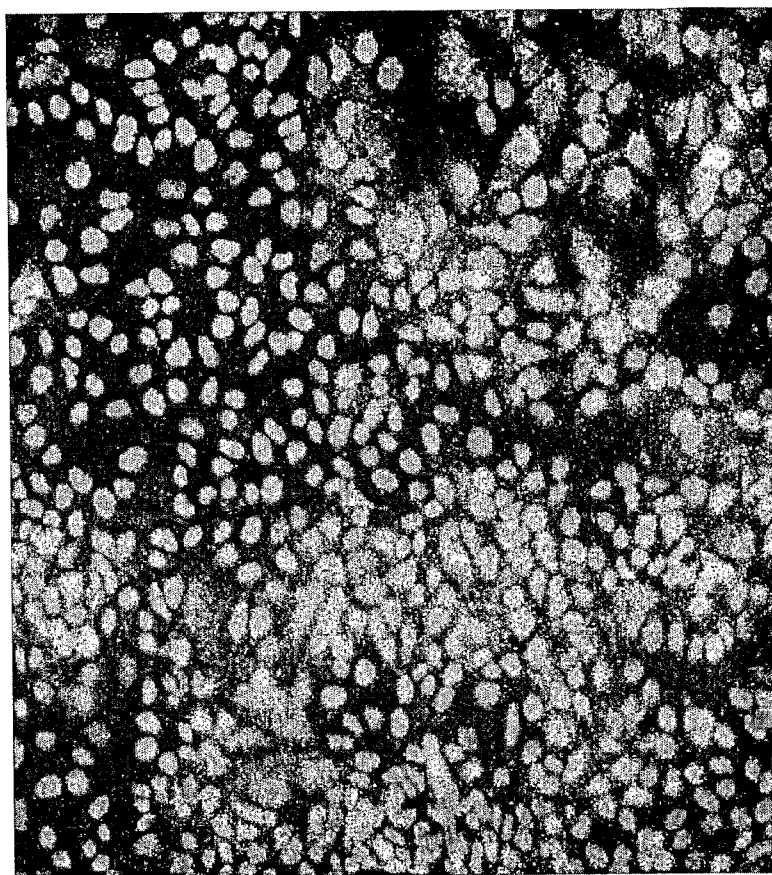


Figure 9

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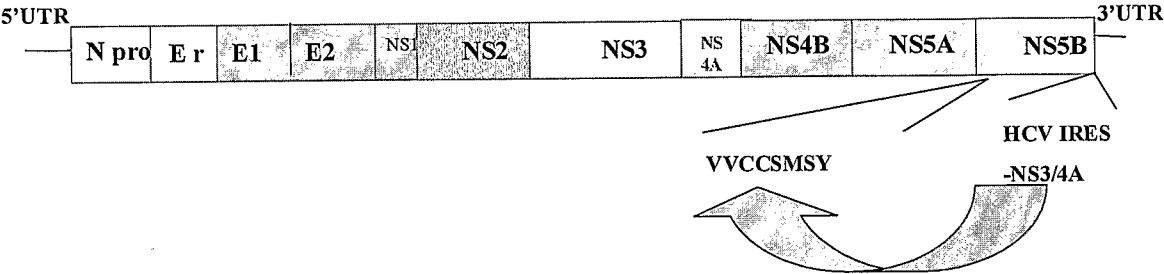


Figure 10

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Map of pFastBacMam - the Baculovirus Donor Plasmid

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(1999) PNAS 96,
127-132

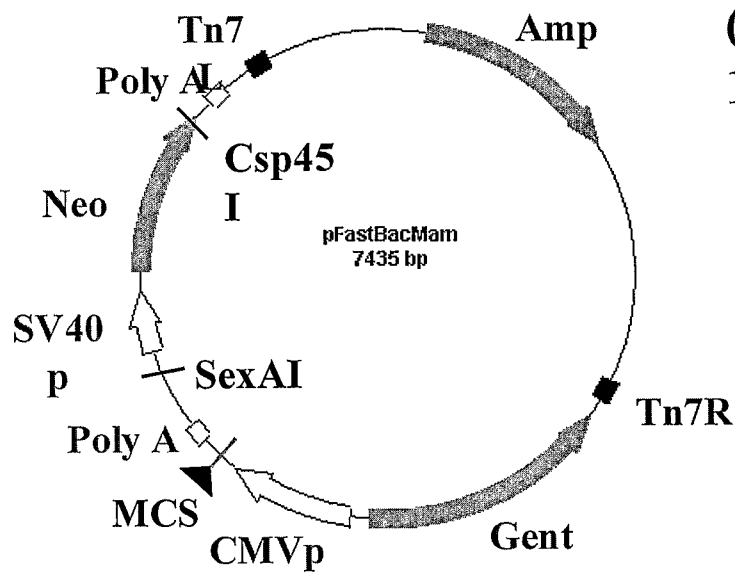


Figure 11

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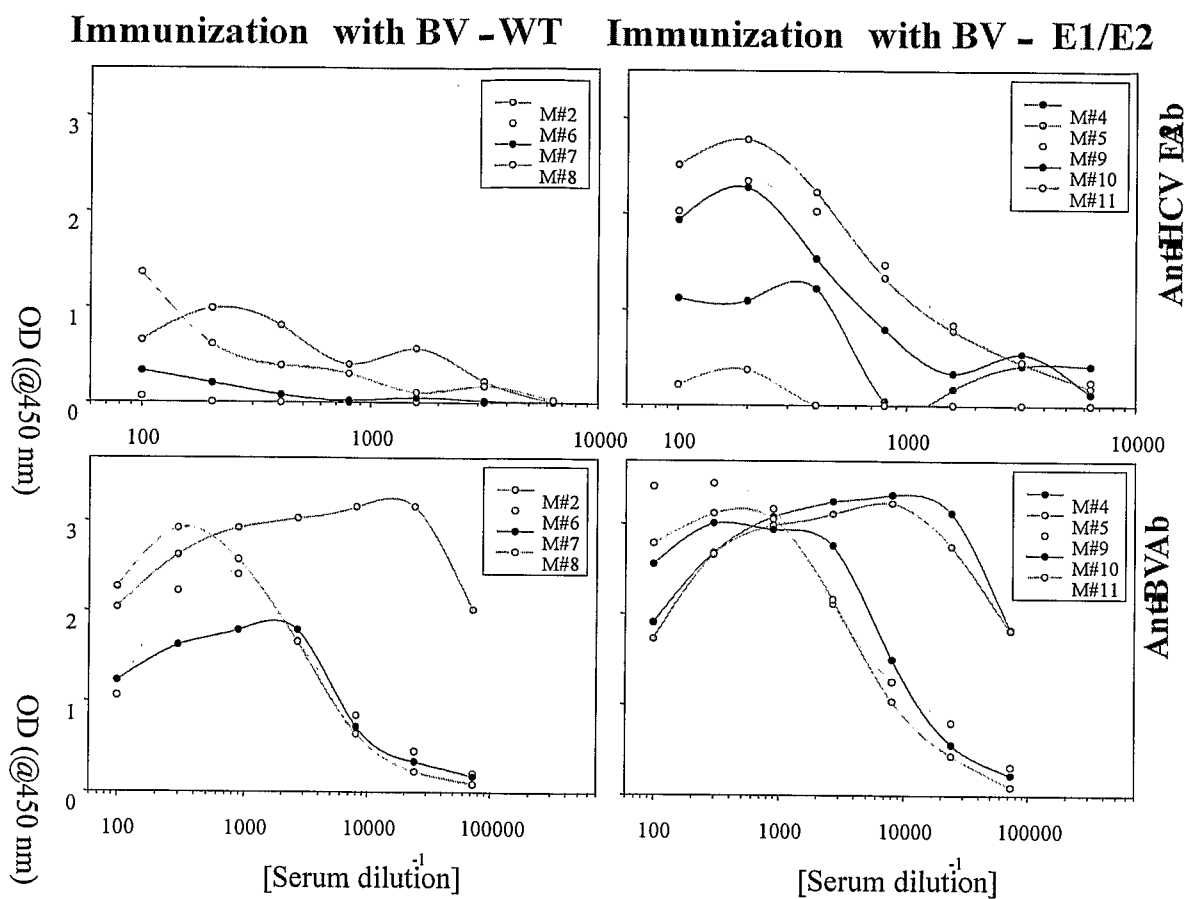


Figure 12

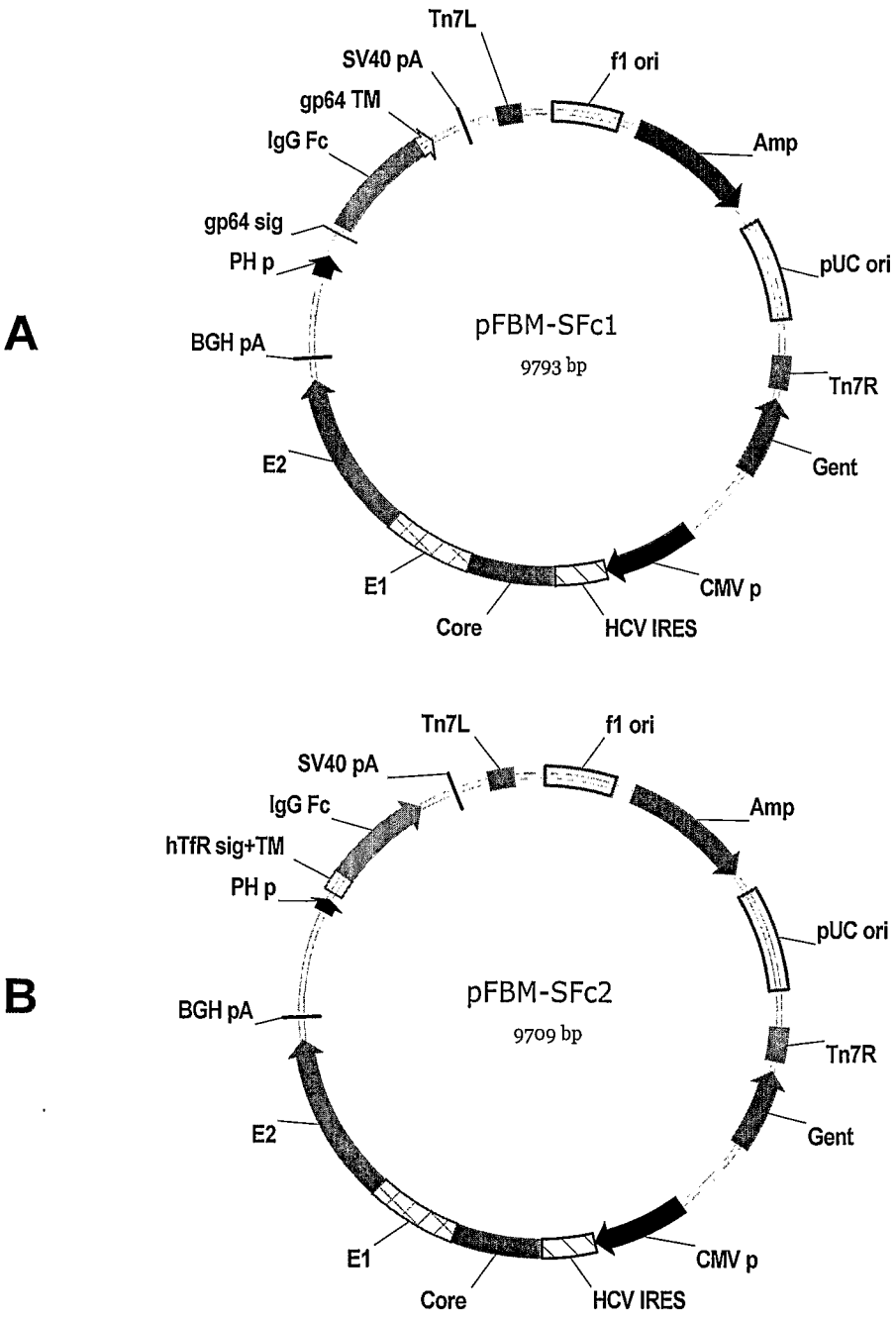


Figure 13

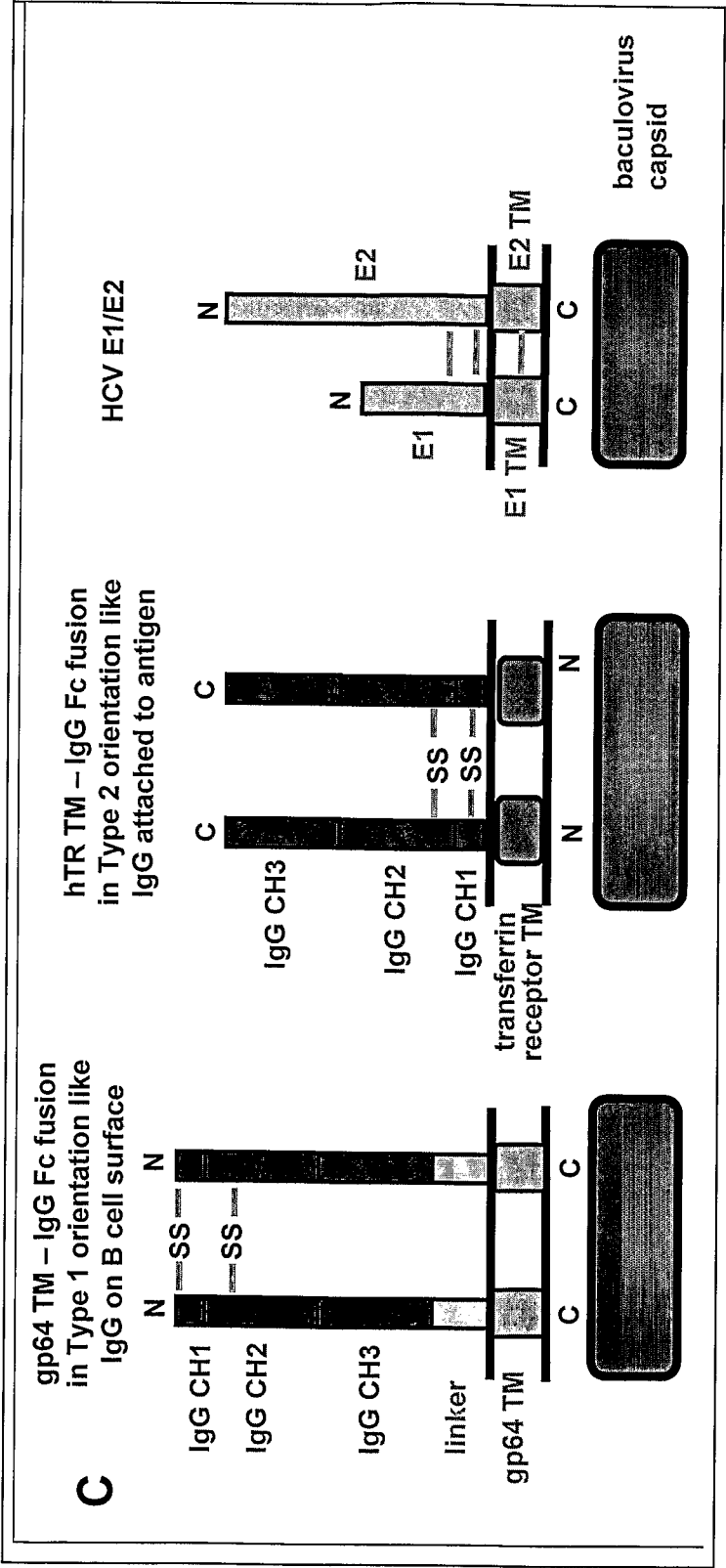


Figure 14A

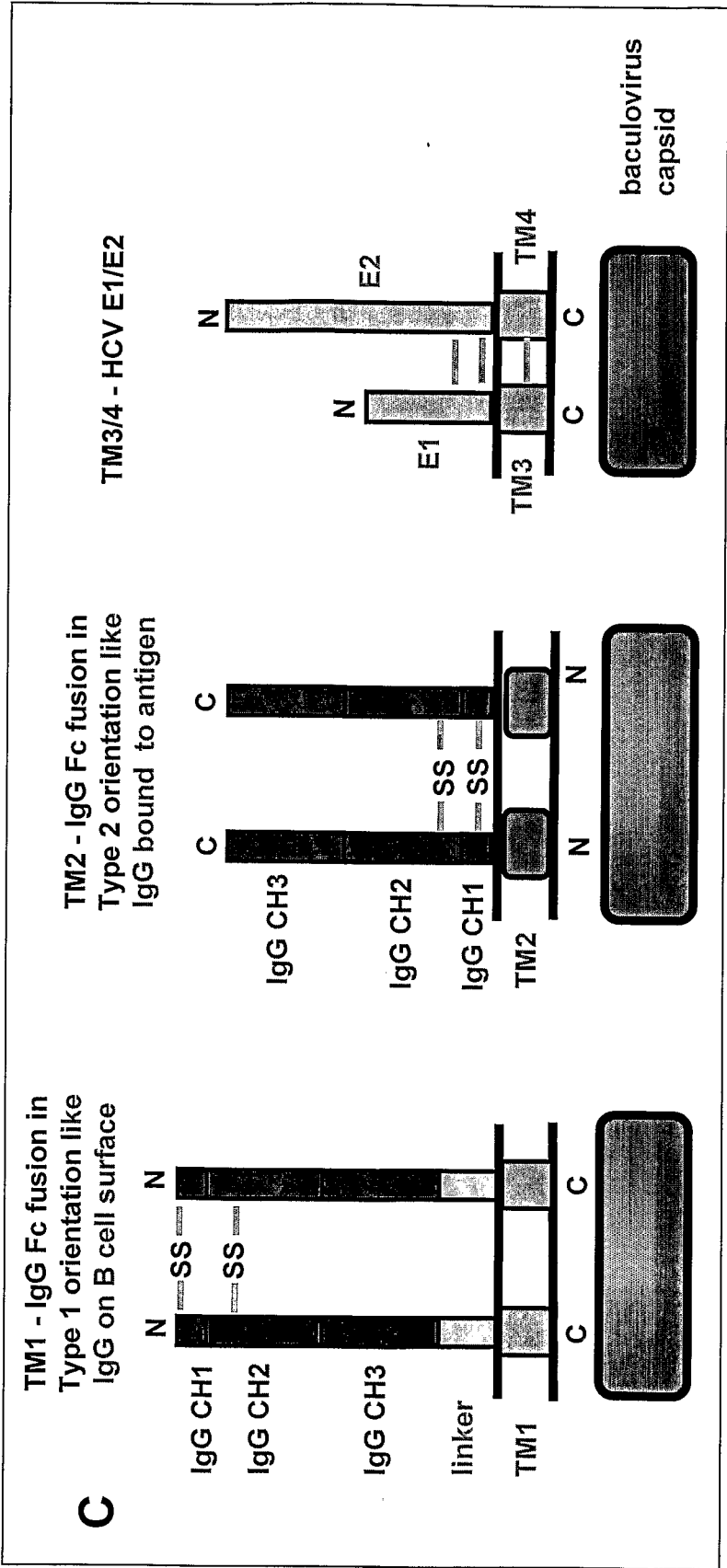


Figure 14B

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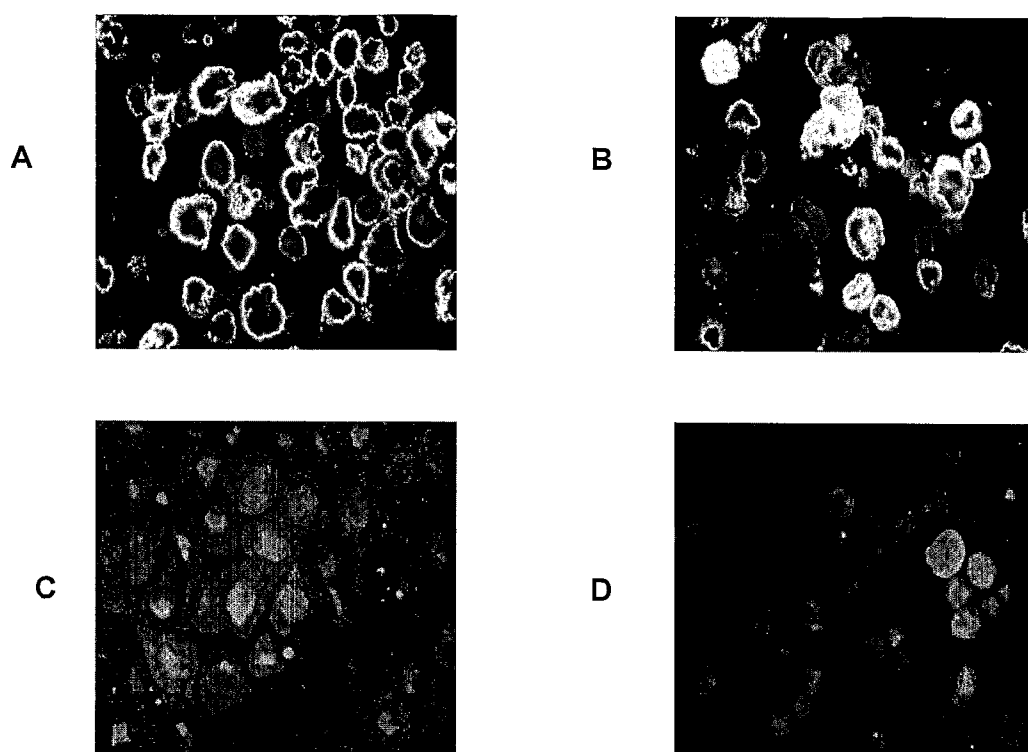


Figure 15

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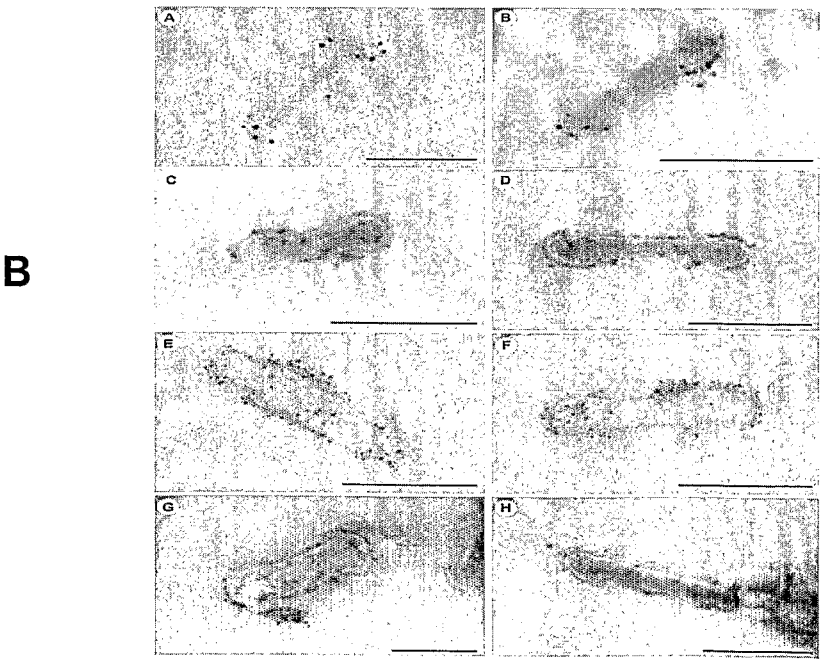
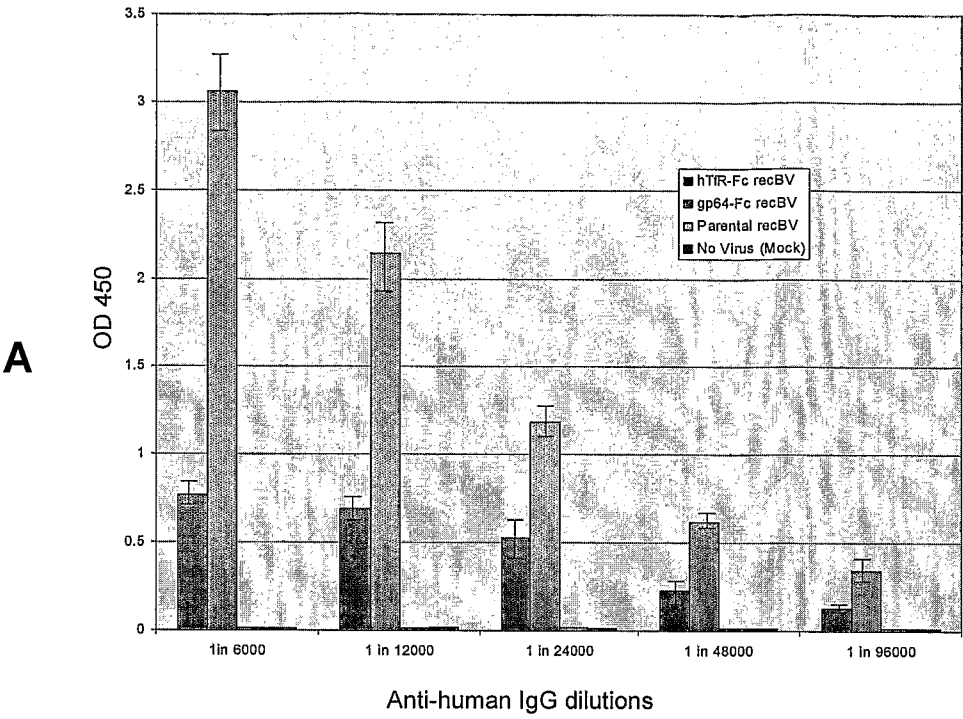


Figure 16

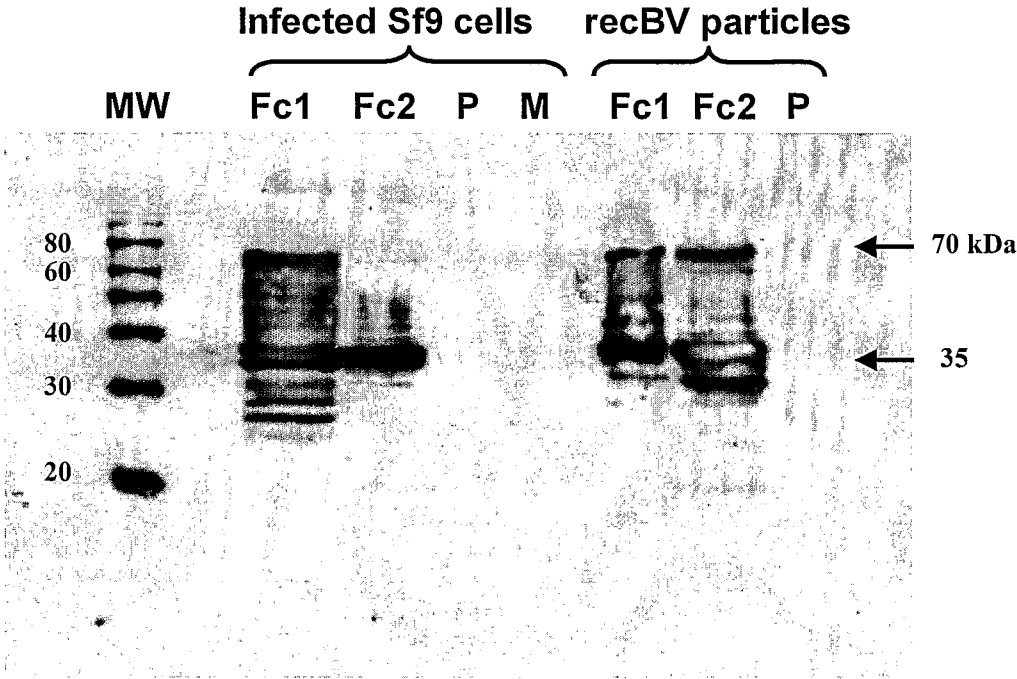


Figure 17

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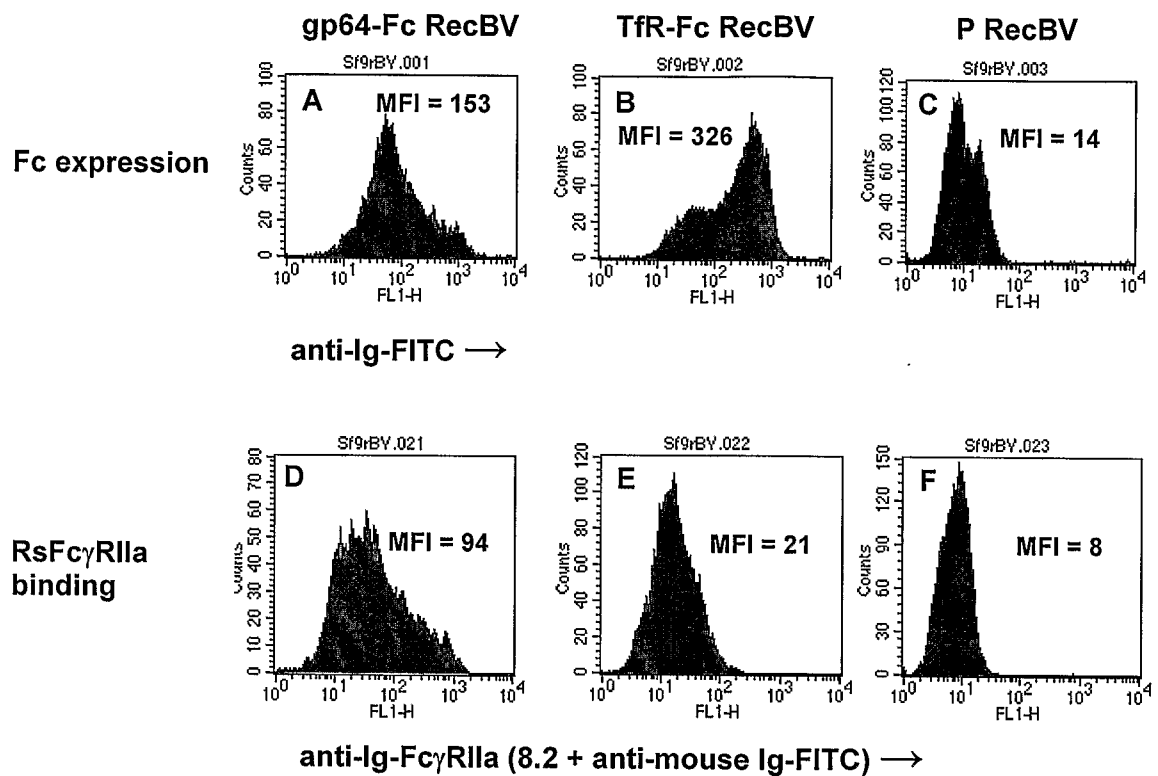


Figure 18

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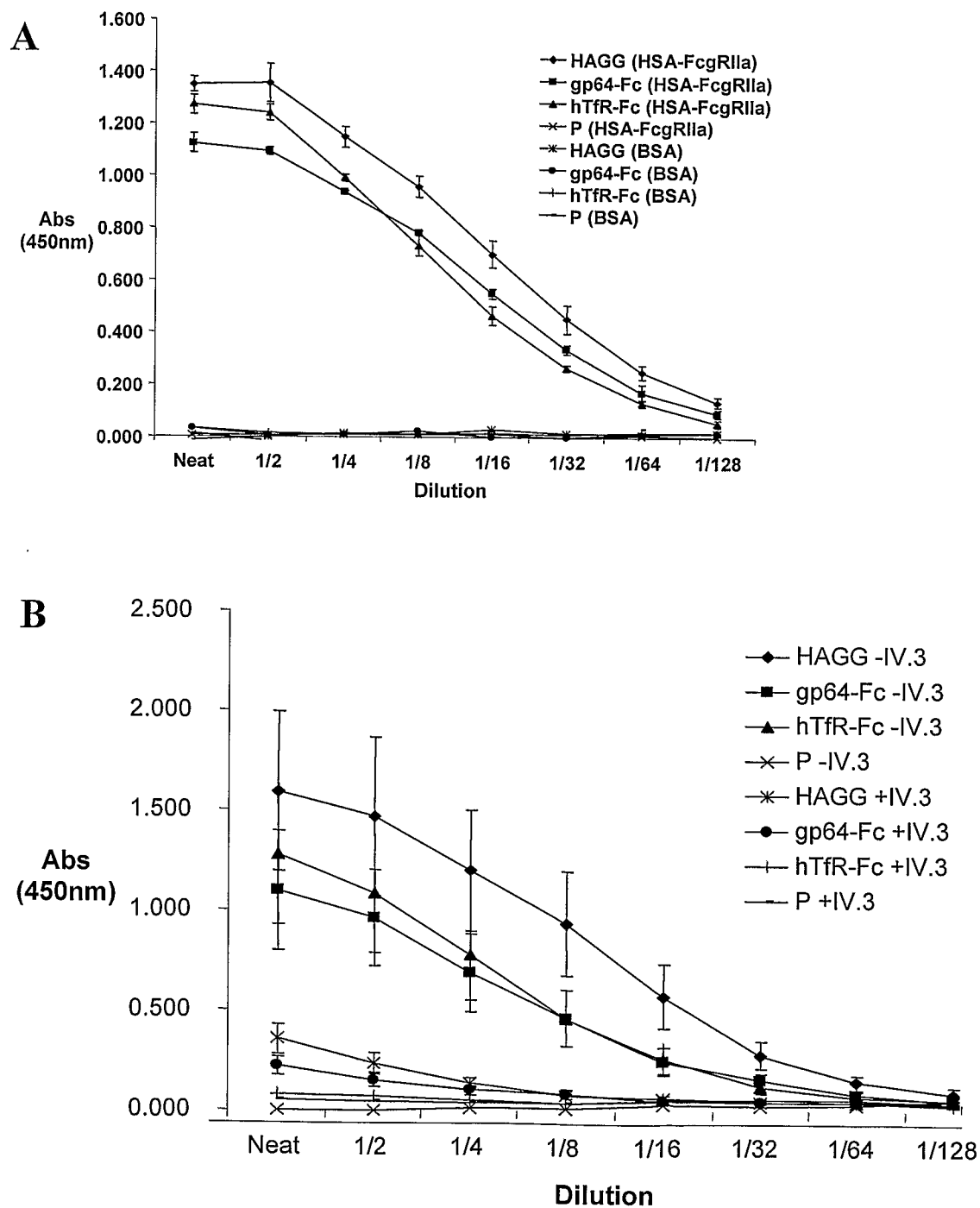


Figure 19

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A

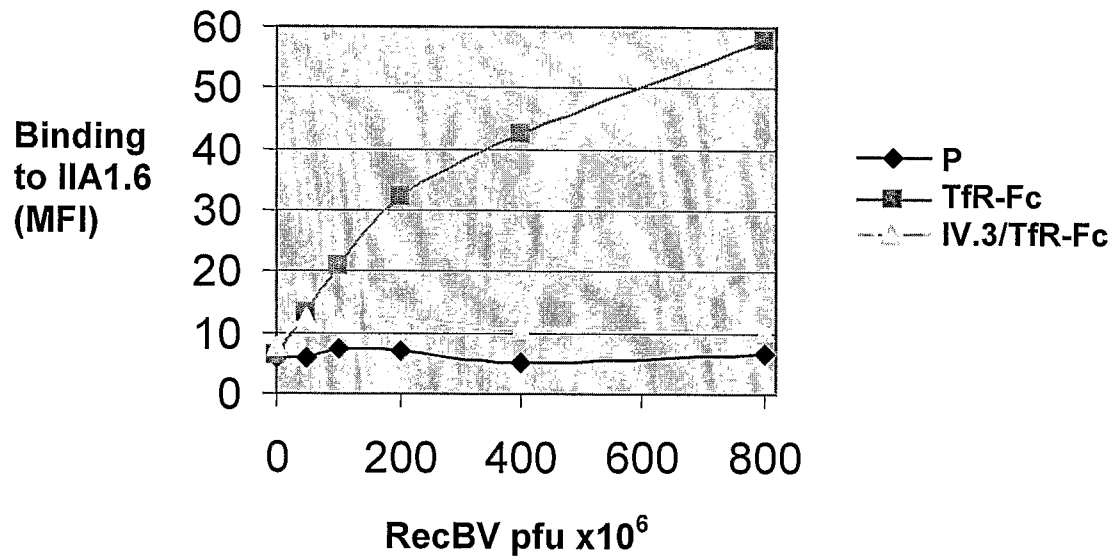


Figure 20

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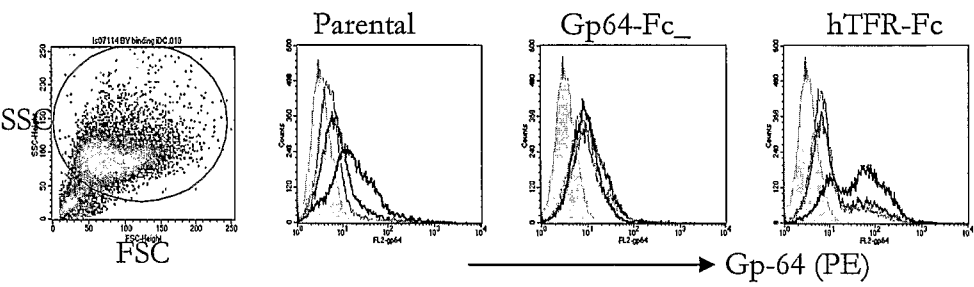


Figure 21

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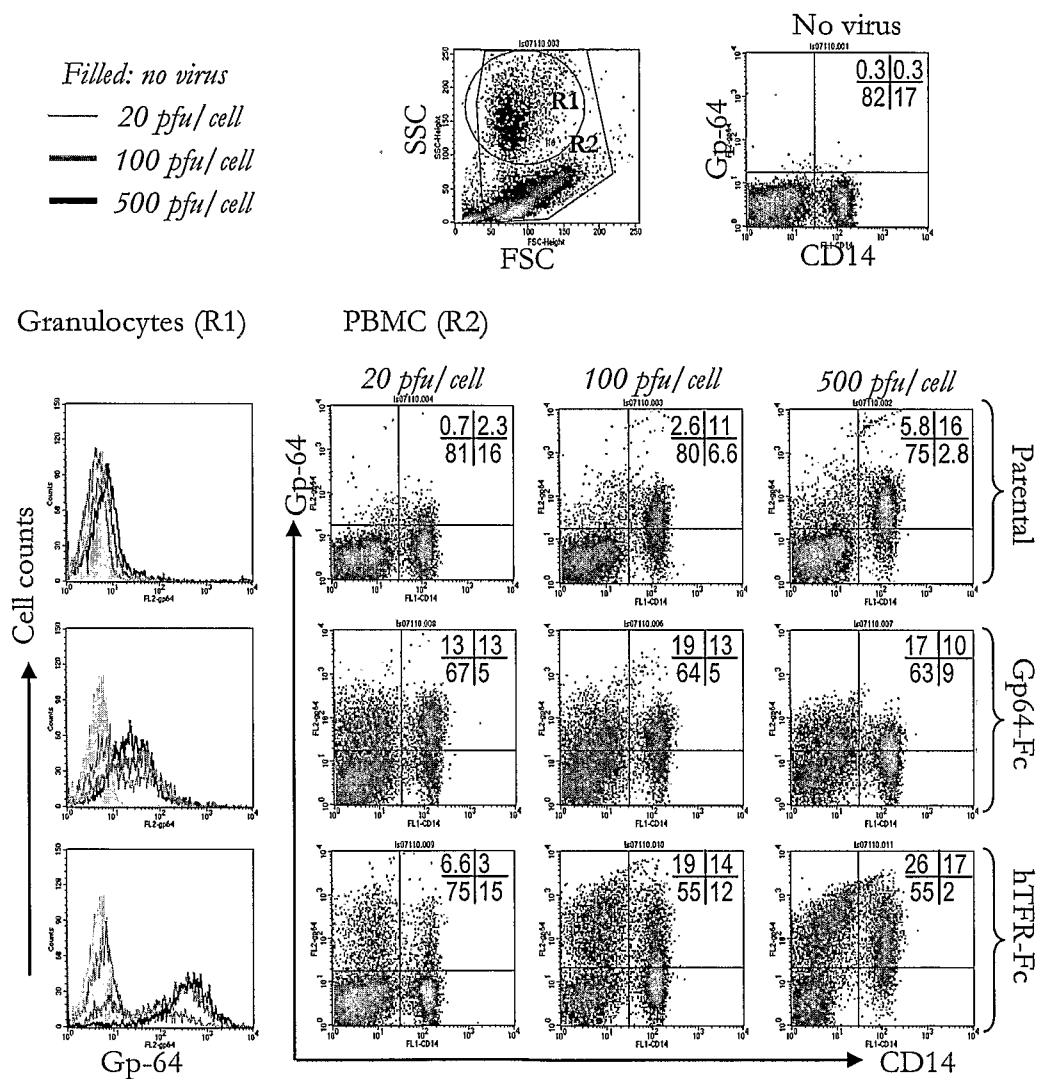


Figure 22

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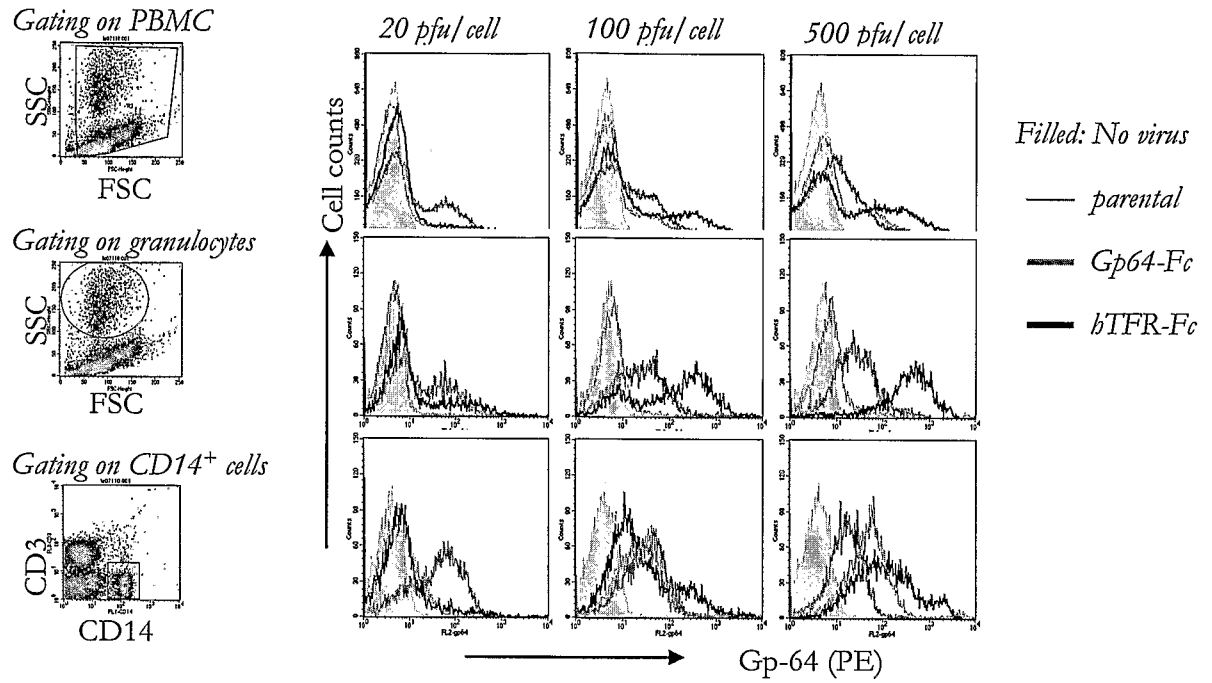


Figure 23

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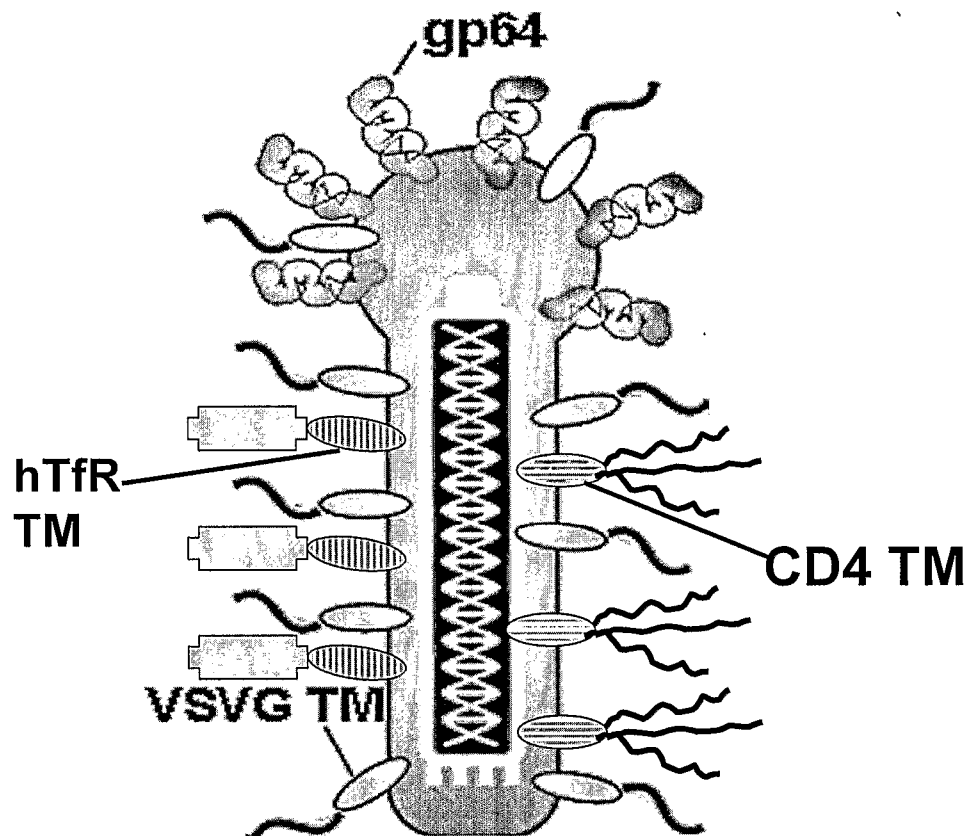
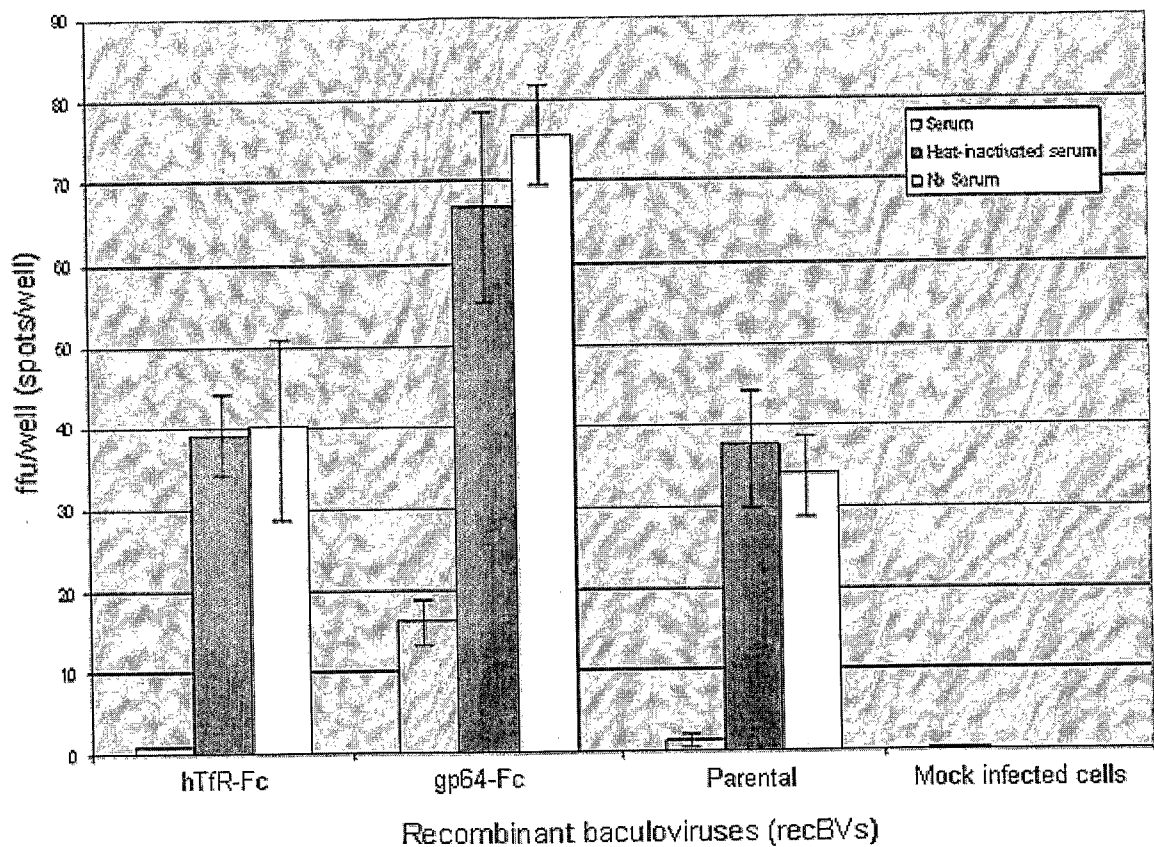


Figure 24

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Complement assay

**Figure 25**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2007/000578

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

A61K 39/29 (2006.01)

A61K 35/76 (2006.01)

A61K 39/12 (2006.01)

A61K 39/00 (2006.01)

C12N 7/04 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIDS and Medline. Keywords: Baculovirus, Antigen, Vaccine, Hepatitis C, HEPC, HCV, Envelope, Glycoprotein:

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2004/0001853 A1 (George et al) 01 January 2004. See Figs 4, 47-50 and 53-58, paragraphs [0008], [0018-0025], [0260-0262], Examples 9, 33-39, Claims 2 and 5.	1-42
X	US 2005/0013828 A1 (George et al) 20 January 2005. See Abstract, Claims 34 and 36, Figs 4, 45a-48b, 51a-56b, paragraphs [0009], [0037-0045], [0052], [0163], [0191], [0223], [0237].	1-42
X	WO 2002/022155 A (Hawaii Biotechnology Group Inc.) 21 March 2002. See Abstract, pages 2 to 5, 8 to 9 and 15, Fig 5.	1-6, 10-16, 20-25, 29-35, 39-42



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

14 June 2007

Date of mailing of the international search report

29 JUN 2007

Name and mailing address of the ISA/AU

AUSTRALIAN PATENT OFFICE
PO BOX 200, WODEN ACT 2606, AUSTRALIA
E-mail address: pct@ipaustalia.gov.au
Facsimile No. (02) 6285 3929

Authorized officer

S. CHEW

AUSTRALIAN PATENT OFFICE
(ISO 9001 Quality Certified Service)
Telephone No : (02) 6283 2248

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2007/000578

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 1998/021338 A (The Government Of The United States Of America) 22 May 1998. See whole document, particularly the Abstract, pages 2 to 5, 7, 10, 12, Example 1, Claim 9.	1-16, 20-35, 39-42
X	Jeong S.H. et al. Immunization with Hepatitis C Virus-Like Particles Induces Humoral and Cellular Immune Responses in Nonhuman Primates. Journal of Virology, July 2004, vol. 78, No. 13, pages 6995-7003. See page 6995 Abstract, left-hand column last paragraph and right-hand column paragraphs 1-3, Results on pages 6997-6998.	1-7, 10-16, 20-26, 29-35, 39-42
X	Baumert T.F. et al. Hepatitis C Virus Structural Proteins Assemble into Viruslike Particles in Insect Cells. Journal of Virology, May 1998, Vol. 72, No. 5, pages 3827-3836. See page 3827 Abstract, right-hand column paragraph 1, Fig 1, page 3829 Results, paragraph 1.	1-16, 20-35, 39-42
X	Overton H. et al. Recombinant baculovirus-expressed NS3 proteinase of hepatitis C virus shows activity in cell-based and in vitro assays. Journal of General Virology, 1995, Vol. 76, pages 3009-3019. See pages 3009-3011 and page 3016 left-hand column	1-2, 8-9, 14-16, 20-21, 27-28, 33-35, 39-42
Y	Aoubala M. et al. The inhibition of cAMP-dependent protein kinase by full-length hepatitis C virus NS3/4A complex is due to ATP hydrolysis. Journal of General Virology, 2001, Vol. 82, pages 1637-1646. See page 1637 Abstract, page 1638 right-hand column paragraph 4	1-2, 8-9, 14-16, 20, 21, 27-28, 33-35, 39-42
Y	Bartenschlager R. The NS3/4A proteinase of the hepatitis C virus: unravelling structure and function of an unusual enzyme and a prime target for antiviral therapy. Journal of Viral Hepatitis, May 1999, Vol. 6, pages 165-181. See whole document.	1, 2, 8-9, 14-16, 20, 21, 27-28, 33-35, 39-42

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU2007/000578

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
US	2004001853	US	2005013828	WO	2005087813		
US	2005013828	US	2004001853	WO	2005087813		
WO	0222155	AU	92667/01	EP	1326625	US	6682909
		US	2002119495				
WO	9821338	AU	23479/97	CA	2269097	EP	0941337
		US	6387662				
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.							
END OF ANNEX							