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(54) **Title:** COMPOSITIONS AND METHODS FOR MAKING HUMAN ANTIBODIES

(57) **Abstract:** The present invention provides compositions and methods for culturing B-cells to generate libraries of stable hybridomas expressing antigen-specific human antibodies. The methods include culturing B cells in the presence of at least one of CD40L, HCV E2, and CD 14.



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TITLE OF THE INVENTION

Compositions and methods for making human antibodies

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims priority to U.S. Provisional Application Serial No. 61/775,201, filed March 8, 2013, the content of each of which is incorporated by reference herein in its entirety.

10 STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR
DEVELOPMENT

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15 BACKGROUND OF THE INVENTION

 Problems associated with the use of antisera were circumvented with the discovery of mouse hybridomas capable of secreting monoclonal antibodies (MAbs) that bind to specific antigens by Kohler and Milstein (Kohler and Milstein, 1975 Nature 256: 495). Since the report of Kohler and Milstein, the production of
20 mouse monoclonal antibodies has become routine.

 Monoclonal antibodies are produced by hybrid cells that result from a fusion between normal B-lymphocytes and myeloma cells. The myeloma cell lines used for fusion are B-lymphocyte tumor cell lines that grow well in vitro and can propagate indefinitely, in contrast to normal B-lymphocytes that cannot replicate or
25 produce antibody in vitro for more than a few days. Cells derived from a fusion of the two types of cells combine the in vitro growth characteristics of the myeloma cell line with the production of an antibody derived from the B-lymphocyte.

 Hybrid cells (hybridomas) are generally produced from mass fusions between murine splenocytes, which are highly enriched for B-lymphocytes, and
30 myeloma "fusion partner cells" (Alberts et al., Molecular Biology of the Cell (Garland Publishing, Inc. 1994); Harlow et al., Antibodies. A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, 1988). The cells in the fusion are subsequently distributed into pools that can be analyzed for the production of antibodies with the desired specificity. Pools that test positive can be further

subdivided until single cell clones are identified that produce antibodies of the desired specificity. Antibodies produced by such clones are called monoclonal antibodies.

Many investigators have attempted to generate human monoclonal antibodies by generating hybridomas with human B-lymphocytes (Chiorazzi et al., J. Exp. Med. 156:930 (1982); Croce et al., Nature 288:488 (1980); Edwards et al., Eur. J. Immunol. 12:641 (1982); Nowinski et al., Science 210:537 (1980); Olsson et al., Proc. Natl. Acad. Sci. USA 77:5429; Pickering et al., J. Immunol. 129:406 (1982)). Unfortunately, these hybrid cells exhibited poor growth in vitro, low levels of antibody expression, instability of antibody expression, and a poor ability to be cloned by limiting dilution.

Consequently, diverse and cumbersome approaches have been used to produce human monoclonal antibodies. These include “humanizing” mouse antibodies by creating hybrid murine/hybrid immunoglobulin genes and generating antibodies in transgenic mice that bear human immunoglobulin gene loci. However, these methods are only able to produce antibodies that have been generated in mice by the murine immune system. They do not allow the isolation, production, and use of the naturally-occurring antibodies, the immunological memory that the human immune system produces in response to infections and other antigen exposures. The ability to make monoclonal antibodies directly from human B-lymphocytes is therefore needed and would be of considerable value.

Recently, there has been progress in generating human monoclonal antibodies by generating hybridomas using the SP2/0 cell line as a fusion partner. The SP2/0 cell line is an immortal murine myeloma cell line (a malignant B-lineage cell) that expresses an endogenous murine telomerase gene. U.S. Patent Application Publication No. 20030224490 discloses the genetic modification of the SP2/0 cell line to ectopically express interleukin-6 (IL-6) and human telomerase catalytic subunit (hTERT).

However, progress in making fully human monoclonal antibodies has been hampered by the absence of human myelomas suitable for use as fusion partners with the desirable attributes of mouse myeloma cells such as stability, and high antibody production. The use of Epstein-Barr virus (EBV) has proved to be quite efficient for human lymphocyte immortalization (Kozbor and Roder, J. Immunology 1981; 127:1275; Casual, Science 1986; 234:476), but has certain limitations such as

low antibody secretion rate, poor clonogenicity of antibody-secreting lines and frequent loss of antibody expression.

Immortalized human B cells have been employed for monoclonal antibody production. This approach involves the steps of: (a) isolation of peripheral
5 blood lymphocytes enriched in B cells; (b) transformation of the B cells with EBV-viruses or fusion with immortalized human lymphoblastoid cells, and (c) massive screening for the B cell transformants or hybridomas exhibiting the desired antigen-binding specificity. B cell transformation itself is an inefficient process yielding at best 0.1-10% stable transformants. Thus, most B cells with the desired specificity are
10 lost in the pool used for subsequent selection process. Whereas researchers have attempted to enrich the population of B cells expressing the desired immunoglobulin by in vitro immunization/activation with the antigen of interest, the activation is again inefficient in the sense that non-specific B cells also proliferate during this process. The identification of specific antibody producing B cells thus largely depends on the
15 final stage of screening, during which tens and thousands of transformed B cell clones are tested for their abilities to bind the antigen. This approach is time consuming and labor intensive.

Traditionally, the production of high-affinity antibodies was dependent on B cells expressing antibodies that have undergone the process of somatic
20 hypermutation, which is the result of a complex set of events that mostly occur within germinal centers (GC). A post-germinal center B cell is a cell that has undergone somatic hypermutation of its immunoglobulin genes. After completing the germinal center maturation response, B cells can become either memory B-cells, which circulate in the blood and form the foundation of a future immune response against
25 the original antigen, or plasma cells, which home to the bone marrow, terminally differentiate, and secrete immunoglobulins. The development of memory B cells and plasma cells takes place in germinal centers of lymphoid follicles where antigen-driven lymphocytes undergo somatic hypermutation and affinity selection, presumably under the influence of helper T cells.

30 Typically, to generate hybridomas that secrete human antibodies, human peripheral blood mononuclear cell populations (PBMCs) are fused with a fusion partner cells because human splenic mononuclear cells, which contain about 40% B cells, are not readily available. PBMCs are readily accessible by routine phlebotomy, but contain only about 5% B cells (Klein et al., 1997 Blood 89: 1288;

Dessain et al., 2004 J. Immunol. Methods 291: 109; Tian et al., 2007 Mol Immunol 44: 2173). However, only about 15% of the B-cells available in peripheral blood express class-switched, post-GC antibodies (Klein et al., 1997 Blood 89: 1288; Tian et al., 2007 Mol Immunol 44: 2173). Accordingly, fusions with unselected PBMCs commonly yield hybridomas that express IgM antibodies with germline sequences, which are not as desirable as class switched, post-GC antibodies. A disadvantage of prior art methods is the high background of IgM secreting hybridomas. The present invention serves to address the low yield and success rate in generating desirable IgG class switched, post GC antibodies.

There is thus a need in the art for high affinity human antibodies that are specific to a target antigen. The present invention addresses this need in the art.

SUMMARY OF THE INVENTION

The invention provides a method of making a hybridoma. In one embodiment, the method comprises culturing B cells in the presence of HCV E2 for a period of time in vitro, and fusing the cultured B cells with a fusion partner cell line, thereby producing a hybridoma.

In one embodiment, the B cells are CD27+. In one embodiment, the B cells are CD27-.

In one embodiment, the B cells are also cultured in the presence of at least one selected from the group consisting of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG oligonucleotides.

In one embodiment, the CD40L is provided in a soluble form.

In one embodiment, the HCV E2 is provided in a soluble form.

In one embodiment, the CD40L is provided in the form of CD40L displayed on the surface of a CD40L-expressing cell during the B cell culturing.

In one embodiment, the HCV E2 is provided in the form of HCV E2 displayed on the surface of HCV E2-expressing cell during the B cell culturing.

In one embodiment, the CD14 is provided in the form of CD14 displayed on the surface of CD14-expressing cell during the B cell culturing.

In one embodiment, the fusion partner cell line ectopically expresses mIL-6 and hTERT.

In one embodiment, the B cells are isolated from an immunized subject.

In one embodiment, the B cells are isolated from an immunized human.

The invention also provides a mammalian cell line that co-expresses CD40L and HCV E2.

5 The invention also provides a hybridoma produced by culturing B cells in the presence of HCV E2 for a period of time in vitro, and fusing the cultured B cells with a fusion partner cell line, thereby producing a hybridoma.

The invention also provides a monoclonal antibody produced by the hybridoma of the invention.

10 The invention also provides a method of producing a monoclonal antibody. In one embodiment, the method comprises culturing B cells in the presence of HCV E2 for a period of time in vitro, fusing the cultured B cells with a fusion partner cell line to produce a hybridoma, selecting a hybridoma that produces a monoclonal antibody, and culturing the hybridoma to produce the monoclonal
15 antibody. In one embodiment, the invention includes a monoclonal antibody or an antigen-binding fragment thereof produced by the method of the invention.

In one embodiment, the antigen-binding fragment is one selected from the group consisting of a single chain Fv (scFv) fragment, a Fab fragment, a (Fab')₂ fragment or a (scFv')₂ fragment.

20 The invention also provides a method of neutralizing antigen in a subject in need thereof. In one embodiment, the method comprises administering to the subject a therapeutically effective amount of the antibody or antigen-binding fragment of the invention.

The invention also includes an isolated nucleic acid molecule encoding
25 the monoclonal antibody, or antigen-binding fragment thereof of the invention. In one embodiment, the invention includes an isolated nucleic acid molecule comprising a first nucleic acid segment encoding the antibody heavy chain polypeptide and a second nucleic acid segment encoding the antibody light chain polypeptide.

The invention also provides a method of making a library of
30 hybridomas. In one embodiment, the method comprises culturing B cells in the presence of HCV E2 for a period of time in vitro, and fusing the cultured B cells with a fusion partner cell line, thereby producing a hybridoma. In one embodiment, the invention provides a library of monoclonal antibodies produced by the method of the invention.

DETAILED DESCRIPTION

The present invention provides a novel in vitro culture strategy for using B-cells to generate libraries of stable hybridomas expressing antigen-specific human antibodies. In various embodiments, the method includes culturing B cells in the presence of at least one of CD40L, Hepatitis C Virus (HCV) E2, and CD14 before, during, or after fusion of the B cell with a fusion partner for the generation of a hybridoma library. In other embodiments, the method includes culturing B cells in the presence of at least one of CD40L, HCV E2, and CD14, as well as in the presence of at least one of IL-4, IL-10, IL-15, IL-21, and IFN γ before, during, or after fusion of the B cell with a fusion partner for the generation of a hybridoma library. In one embodiment, the invention allows for the high yield generation of antibodies. Preferably, the antibodies generated using the methods of the invention exhibit high affinity against a desired target antigen.

In one embodiment, the method includes culturing B cells in the presence of HCV E2 before, during, or after fusion of the B cell with a fusion partner for the generation of a hybridoma library. In other embodiments, the method includes culturing B cells in the presence of HCV E2, as well as in the presence of at least one of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG oligonucleotides before, during, or after fusion of the B cell with a fusion partner for the generation of a hybridoma library. CpG oligonucleotides as used in the present invention are an example of a type of B-cell stimulating agent.

In one embodiment, the invention allows for the high yield generation of antibodies. Preferably, the antibodies generated using the methods of the invention exhibit high affinity against a desired target antigen.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

As used herein, each of the following terms has the meaning associated with it in this section.

The articles “a” and “an” are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

“About” as used herein when referring to a measurable value such as
5 an amount, a temporal duration, and the like, is meant to encompass variations of $\pm 20\%$ or $\pm 10\%$, more preferably $\pm 5\%$, even more preferably $\pm 1\%$, and still more preferably $\pm 0.1\%$ from the specified value, as such variations are appropriate to perform the disclosed methods.

The term “abnormal” when used in the context of organisms, tissues,
10 cells or components thereof, refers to those organisms, tissues, cells or components thereof that differ in at least one observable or detectable characteristic (e.g., age, treatment, time of day, etc.) from those organisms, tissues, cells or components thereof that display the “normal” (expected) respective characteristic. Characteristics which are normal or expected for one cell or tissue type, might be abnormal for a
15 different cell or tissue type.

A disease or disorder is “alleviated” if the severity of a symptom of the disease or disorder, the frequency with which such a symptom is experienced by a patient, or both, is reduced.

The term “antibody” as used herein refers to an immunoglobulin
20 molecule that contains an antigen binding site which specifically binds an antigen. Structurally, the antibody comprises four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. The term also encompasses polyclonal and monoclonal antibodies, animal, human, hybrid, and humanized antibodies.

25 An “antibody heavy chain,” as used herein, refers to the larger of the two types of polypeptide chains present in all antibody molecules in their naturally occurring conformations.

An “antibody light chain,” as used herein, refers to the smaller of the two types of polypeptide chains present in all antibody molecules in their naturally
30 occurring conformations.

As used herein, “antigen-binding fragment” with respect to any antibody are fragments of the antibody, such as Fab, $F(ab')_2$, Fv fragments, and single chain variable fragments (scFv), which are capable of binding an epitopic determinant. Antibody fragments can refer to antigen-binding immunoglobulin

peptides which are at least about 5 to about 15 amino acids or more in length, and which retain some biological activity or immunological activity of an immunoglobulin. Examples of antigen-binding fragments encompassed within the term “antigen-binding fragments” include but are not limited to (i) an Fab fragment consisting of the VL, VH, CL and CH1 domains; (ii) an Fd fragment consisting of the VH and CH1 domains; (iii) an Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (iv) a dAb fragment (Ward et al., (1989) Nature 341:544-546) which consists of a VH domain; (v) an isolated complementarity determining region (CDR); and (vi) an F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region. Furthermore, although the two domains of the Fv fragment are generally coded for by separate genes, a synthetic linker can be made that enables them to be made as a single protein chain (known as single chain Fv (scFv); Bird et al. (1988) Science 242:423-426; and Huston et al. (1988) PNAS 85:5879-5883) by recombinant methods. Such single chain antibodies are also encompassed within the term “antigen-binding fragments.” Preferred antibody fragments are those which are capable of crosslinking their target antigen, e.g., bivalent fragments such as F(ab')₂ fragments. Alternatively, an antibody fragment which does not itself crosslink its target antigen (e.g., a Fab fragment) can be used in conjunction with a secondary antibody which serves to crosslink the antibody fragment, thereby crosslinking the target antigen.

The term “amino acid” is used interchangeably with “amino acid residue,” and may refer to a free amino acid and to an amino acid residue of a peptide. It will be apparent from the context in which the term is used whether it refers to a free amino acid or a residue of a peptide.

An “antigen” is any agent, e.g., a protein (or immunogenic fragments), a peptide or peptide conjugate, immunogen, vaccine, or a polysaccharide, that elicits an immune response.

“Biologically active,” as used herein with respect to antibodies, fragments, derivatives, homologs, and analogs means that the antibodies, fragments, derivatives, homologs or analogs have the ability to bind an antigen.

As used herein, “class switching” or “isotype switching” means a change in the phenotype of an Ig-producing cell. Ig class switching is a critical step in the generation of the diversified biological effector functions of the antibody response. For example, B cells initially produce primarily IgM, a phenotype change

into the production of IgG, IgE or IgA is an “isotype switch” or “class switch.” Class switching, as used herein, includes two steps: the first step is the provision of trans-spliced transcripts to act as bridging templates for conforming genomic immunoglobulin DNA, and the second step is switch recombination that results in the
5 production of switch circles and rearrangement of genomic Ig DNA to allow production of a different Ig (antibody). In particular, Ig class switching involves DNA recombination between two IgH switch (S) regions through a non-homologous recombination, a process known as class switch recombination (CSR). This process leads to the rearrangement of the S region of the upstream Ig locus to a downstream
10 targeted S region and results in the expression of the downstream isotype. The intervening DNA is looped-out, and excised as the switch circular DNA.

As used herein, the terms “conservative variation” or “conservative substitution” as used herein refers to the replacement of an amino acid residue by another, biologically similar residue. Conservative variations or substitutions are not
15 likely to change the shape of the peptide chain. Examples of conservative variations, or substitutions, include the replacement of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine, and the like.

20 A “disease” is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal’s health continues to deteriorate.

In contrast, a “disorder” in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal’s state of health is
25 less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal’s state of health.

An “effective amount” or “therapeutically effective amount” of a compound is that amount of compound which is sufficient to provide a beneficial effect to the subject to which the compound is administered. An “effective amount” of
30 an antibody, is an amount sufficient to detectably bind to an antigen.

The term “expression,” as used with respect to an antibody mRNA, refers to transcription of a nucleic acid sequence encoding a heavy or light chain, resulting in synthesis of an antibody mRNA, and/or to translation of an antibody mRNA, resulting in synthesis of an antibody.

As used herein, the term “fragment” or “segment” as applied to a nucleic acid, refers to a subsequence of a larger nucleic acid. A “fragment” or “segment” of a nucleic acid can be at least about 20 nucleotides in length; for example, at least about 50 nucleotides to about 100 nucleotides; preferably at least about 100 to about 500 nucleotides, more preferably at least about 500 to about 1000 nucleotides, even more preferably at least about 1000 nucleotides to about 1500 nucleotides; particularly, preferably at least about 1500 nucleotides to about 2500 nucleotides; most preferably at least about 2500 nucleotides.

As used herein, the term “fragment” or “segment” as applied to a protein or peptide, refers to a subsequence of a larger protein or peptide. A “fragment” or “segment” of a protein or peptide can be at least about 20 amino acids in length; for example at least about 50 amino acids in length; more preferably at least about 100 amino acids in length, even more preferably at least about 200 amino acids in length, particularly preferably at least about 300 amino acids in length, and most preferably at least about 400 amino acids in length.

As used herein, the term “gene” refers to an element or combination of elements that are capable of being expressed in a cell, either alone or in combination with other elements. In general, a gene comprises (from the 5' to the 3' end): (1) a promoter region, which includes a 5' nontranslated leader sequence capable of functioning in any cell such as a prokaryotic cell, a virus, or a eukaryotic cell (including transgenic animals); (2) a structural gene or polynucleotide sequence, which codes for the desired protein; and (3) a 3' nontranslated region, which typically causes the termination of transcription and the polyadenylation of the 3' region of the RNA sequence. Each of these elements is operably linked by sequential attachment to the adjacent element. A gene comprising the above elements is inserted by standard recombinant DNA methods into a plant expression vector.

As used herein, “gene products” include any product that is produced in the course of the transcription, reverse-transcription, polymerization, translation, post-translation and/or expression of a gene. Gene products include, but are not limited to, proteins, polypeptides, peptides, peptide fragments, mRNAs, cDNAs, and other polynucleotide molecules.

“hTERT” means human telomerase catalytic subunit.

As used herein, “homology” is used synonymously with “identity.”

The term “hybridoma,” as used herein refers to a cell resulting from the fusion of a B-lymphocyte and a fusion partner such as a myeloma cell. A hybridoma can be cloned and maintained indefinitely in cell culture and is able to produce monoclonal antibodies. A hybridoma can also be considered to be a hybrid
5 cell.

The term “inhibit,” as used herein, means to suppress or block an activity or function by at least about ten percent relative to a control value. Preferably, the activity is suppressed or blocked by 50% compared to a control value, more preferably by 75%, and even more preferably by 95%.

10 As used herein, an “instructional material” includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of a compound, composition, vector, or delivery system of the invention in the kit for effecting alleviation of the various diseases or disorders recited herein. Optionally, or alternately, the instructional material can describe one or
15 more methods of alleviating the diseases or disorders in a cell or a tissue of a mammal. The instructional material of the kit of the invention can, for example, be affixed to a container which contains the identified compound, composition, vector, or delivery system of the invention or be shipped together with a container which contains the identified compound, composition, vector, or delivery system.
20 Alternatively, the instructional material can be shipped separately from the container with the intention that the instructional material and the compound be used cooperatively by the recipient.

“Isolated” means altered or removed from the natural state through the direct or indirect actions of a human being. For example, a nucleic acid or a peptide
25 naturally present in a living animal is not “isolated,” but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is “isolated.” An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell. An “isolated” cell is a cell that has been purified from the other cellular
30 components of a tissue. Cells can be isolated by mechanical and/or enzymatic methods. In several embodiments, an isolated population of cells includes greater than about 80%, about 85%, about 90%, about 95%, or greater than about 99% of the cells of interest. In another embodiment, an isolated population of cells is one in which no other cells of a different phenotype can be detected. In a further embodiment, an

isolated population of cells is a population of cells that includes less than about 20%, about 15%, about 10%, about 5%, or less than about 1% of a cells of a different phenotype than the cells of interest.

As used herein, the term “library” refers to a polyclonal population of hybridoma cells or to the antibodies secreted by the cells. A library exists in a form that can be screened to identify members of the library (either cells or antibodies) that possess specific characteristics.

The terms “medium,” “cell culture medium” and “culture medium” are used interchangeably. The terms refer to the aqueous environment in which eukaryotic or prokaryotic cells are grown in culture. The medium comprises the chemical, nutritional, and hormonal environment. The cell culture medium is “serum-free”, when the medium is essentially free of serum from any mammalian source, (e.g. sera from fetal bovine, horse, human, rabbit). By “essentially free” is meant that the cell culture medium comprises between about 0-5% serum, preferably between about 0-1% serum and most preferably between about 0-0.1% serum.

The term “microarray” refers broadly to both “DNA microarrays” and “DNA chip(s),” and encompasses all art-recognized solid supports, and all art-recognized methods for affixing nucleic acid molecules thereto or for synthesis of nucleic acids thereon.

As used herein, the term “monoclonal antibody” includes antibodies which display a single binding specificity and affinity for a particular epitope. These antibodies are mammalian-derived antibodies, including murine, human and humanized antibodies. The term “human monoclonal antibody” as used herein, refers to antibodies displaying a single binding specificity which have variable and constant regions derived from human germ-line immunoglobulin sequences.

A “mutation,” as used herein, refers to a change in nucleic acid or polypeptide sequence relative to a reference sequence (which is preferably a naturally-occurring normal or “wild-type” sequence), and includes translocations, deletions, insertions, and substitutions/point mutations. A “mutant,” as used herein, refers to either a nucleic acid or protein comprising a mutation.

“Neutralize,” as used herein, means to inhibit the biological activity of an antigen. Preferably, “neutralize,” as used herein with respect to an, means to reduce or inhibit progression of antigen exposure in a subject or to reduce or prevent progression in a subject at risk of exposure to an antigen. In some embodiments,

antibodies of this invention act to neutralize (reduce or eliminate) an activity of an antigen.

A “nucleic acid” refers to a polynucleotide and includes poly-ribonucleotides and poly-deoxyribonucleotides.

5 The term “oligonucleotide” typically refers to short polynucleotides of about 50 nucleotides or less in length. It will be understood that when a nucleotide sequence is represented herein by a DNA sequence (e.g., A, T, G, and C), this also includes the corresponding RNA sequence (e.g., a, u, g, c) in which “u” replaces “T”.

 The terms “patient,” “subject,” “individual,” and the like are used
10 interchangeably herein, and refer to any animal, or cells thereof whether *in vitro* or *in situ*, amenable to the methods described herein. In certain non-limiting embodiments, the patient, subject or individual is a human.

 As used herein, the terms “peptide,” “polypeptide,” and “protein” are used interchangeably, and refer to a compound comprised of amino acid residues
15 covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids which can comprise a protein's or peptide's sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also
20 commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. “Polypeptides” include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptide, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs,
25 fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

 “Pharmaceutically acceptable” means physiologically tolerable, for either human or veterinary applications.

 As used herein, “pharmaceutical compositions” include formulations
30 for human and veterinary use.

 As used herein, “polynucleotide” includes cDNA, RNA, DNA/RNA hybrid, anti-sense RNA, ribozyme, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified to contain non-natural or derivatized, synthetic, or semi-synthetic nucleotide

bases. Also, included within the scope of the invention are alterations of a wild type or synthetic gene, including but not limited to deletion, insertion, substitution of one or more nucleotides, or fusion to other polynucleotide sequences, provided that such changes in the primary sequence of the gene do not alter the expressed peptide ability
5 to elicit passive immunity.

A “preventive” or “prophylactic” treatment is a treatment administered to a subject who does not exhibit signs or symptoms, or exhibits only early signs or symptoms, of a disease or disorder. A prophylactic or preventative treatment is administered for the purpose of decreasing the risk of developing pathology
10 associated with a disease or disorder.

A “sample,” as used herein, refers to a biological sample from a subject, including normal tissue samples, blood, saliva, feces, or urine. A sample can also be any other source of material obtained from a subject which contains a compound or cells of interest.

15 By the term “specifically binds,” as used herein with respect to an antibody, is meant an antibody which recognizes a specific antigen, but does not substantially recognize or bind other molecules in a sample.

A “subject,” as used herein, can be a human or non-human animal. Non-human animals include, for example, livestock and pets, such as ovine, bovine,
20 porcine, canine, feline and murine mammals, as well as reptiles, birds and fish. Preferably, the subject is a human.

“Substantially purified” refers to a peptide or nucleic acid which is substantially homogenous in character due to the removal of other compounds (e.g., other peptides, nucleic acids, carbohydrates, lipids) or other cells originally present.
25 “Substantially purified” is not meant to exclude artificial or synthetic mixtures with other compounds, or the presence of impurities which do not interfere with biological activity, and which may be present, for example, due to incomplete purification, addition of stabilizers, or formulation into a pharmaceutically acceptable preparation.

“Synthetic mutant” includes any purposefully generated mutant or
30 variant protein or nucleic acid. Such mutants can be generated by, for example, chemical mutagenesis, polymerase chain reaction (PCR) based approaches, or primer-based mutagenesis strategies well known to those skilled in the art.

A “therapeutic” treatment is a treatment administered to a subject who exhibits signs of pathology, for the purpose of diminishing or eliminating those signs.

The terms to “treat” or “treatment,” as used herein, refer to administering antibodies or compounds to reduce the frequency with which the signs or symptoms of a disease or disorder are experienced by a subject, to reduce the severity of the signs or symptoms experienced by a subject, or to prevent the signs or symptoms from occurring.

The term “vaccine” as used herein is defined as a material used to provoke an immune response after administration of the material to an animal.

“Variant” as the term is used herein, is a nucleic acid sequence or a peptide sequence that differs in sequence from a reference nucleic acid sequence or peptide sequence respectively, but retains essential properties of the reference molecule. Changes in the sequence of a nucleic acid variant may not alter the amino acid sequence of a peptide encoded by the reference nucleic acid, or may result in amino acid substitutions, additions, deletions, fusions and truncations. Changes in the sequence of peptide variants are typically limited or conservative, so that the sequences of the reference peptide and the variant are closely similar overall and, in many regions, identical. A variant and reference peptide can differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A variant of a nucleic acid or peptide can be a naturally occurring such as an allelic variant, or can be a variant that is not known to occur naturally. Non-naturally occurring variants of nucleic acids and peptides may be made by mutagenesis techniques or by direct synthesis.

Ranges: throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

Abbreviations, Acronyms and Short Forms - The following abbreviations, acronyms and short forms are used in this specification.

“CD40L” means CD40 ligand.

“ELISA” means enzyme-linked immunosorbent assay.

“GC” means germinal center.

“huMAb” means human monoclonal antibody.

“Ig” means immunoglobulin.

5 “Ig H” means immunoglobulin heavy chain.

“Ig L” means immunoglobulin light chain.

“MAb” means monoclonal antibody.

“PCR” means polymerase chain reaction.

“RT-PCR” means reverse transcription PCR.

10 “scFv” means single chain variable fragment.

“IL” means interleukin.

Description

The present invention provides a novel in vitro culture strategy for
15 using B-cells, preferably human B cells, to generate libraries of stable hybridomas
expressing antigen-specific human antibodies. As such, the invention provides an
enriched population of B-cells that secrete antibodies for generating a hybridoma
library. In various embodiments, the method includes culturing B cells in the presence
of HCV E2, before, during, or after fusion of the B cell with a fusion partner for the
20 generation of a hybridoma library. In some embodiments, the method includes
culturing B cells in the presence of HCV E2, as well as in the presence of at least one
of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG oligonucleotides before,
during, or after fusion of the B cell with a fusion partner for the generation of a
hybridoma library. B cells useful in the methods of the invention include primary B
25 cells expressing at least one B cell marker, such as CD19 or CD20.

In some embodiments, the methods of the invention include the use of
CD27+ B cells. CD27 is a marker of post germinal center B-cells that have undergone
somatic hypermutation and class switch recombination. The culturing of CD27+ B
cells with HCV E2, as well as with at least one of CD14, CD40L, IL-4, IL-10, IL-15,
30 IL-21, IFN γ , and CpG oligonucleotides, further induces somatic hypermutation and
class switch recombination. Therefore, CD27+ B cells cultured with HCV E2, as well
as with at least one of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG
oligonucleotides, provide for a B cell population that is enriched for cells that secrete
a diverse repertoire of IgG antibodies. The fusion of CD27+ B cells cultured with

HCV E2, as well as with at least one of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG oligonucleotides, before, during or after fusion of the B cell with a fusion partner results in the generation of hybridomas that secrete IgG antibodies at an increased frequency.

5 In some embodiments, the methods of the invention include the use of CD27- B cells. CD27- B cells are naïve B cells that have not undergone somatic hypermutation or class switch recombination. The culturing of CD27- B cells with HCV E2, as well as with at least one of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG oligonucleotides, induces class switch recombination and somatic
10 hypermutation among CD27- B cells. Therefore, CD27- B cells cultured with HCV E2, as well as with at least one of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG oligonucleotides, provide for a B cell population that has not been negatively selected in vivo according to its antibody binding specificities. Thus, CD27- B cells
15 cultured with HCV E2, as well as with at least one of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG oligonucleotides, provides a B cell population that is enriched for cells that secrete a diverse repertoire of antibodies having diverse functional characteristics and binding specificities, including antibodies that specifically bind to self antigens. The fusion of CD27- B cells cultured with HCV E2, as well as with at least one of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and
20 CpG oligonucleotides, before, during or after fusion of the B cell with a fusion partner results in the generation of hybridomas that secrete antibodies at an increased frequency.

 Also provided are hybridoma libraries generated from the fusion of B cells cultured with HCV E2, as well as with at least one of CD14, CD40L, IL-4, IL-
25 10, IL-15, IL-21, IFN γ , and CpG oligonucleotides, with a fusion partner. In some embodiments, the libraries provide a larger percentage of cells that secrete IgG antibodies, as compared with known human antibody hybridoma libraries known in the art. In other embodiments, the libraries provide a larger percentage of cells that secrete antibodies that specifically bind to self antigens, as compared with human
30 antibody hybridoma libraries known in the art. In some embodiments, the libraries are also enriched for IgG antibodies relative to IgM antibodies, in part because culturing the CD27+ B cells in the presence of HCV E2, as well as with at least one of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG oligonucleotides, induces class switch recombination CD27+ B cells. In other embodiments, the libraries are also

enriched for antibodies that specifically bind to self antigens, in part because culturing CD27- B cells, which have not been subject to negative selection in vivo, in the presence of HCV E2, as well as with at least one of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG oligonucleotides, induces somatic hypermutation and class switch recombination in CD27- B cells.

In some embodiments, the libraries are advantageous compared to the prior art because of the in vitro class switch recombination step that facilitates cloning of antibody variable domains from post-germinal center IgM antibodies in the more useful IgG isotype. In other embodiments, the libraries of the present invention differ from libraries of the prior art that are made from B-cells selected for the expression of IgG antibodies prior to fusion, because they express IgG antibodies that originated in B-cells that had expressed IgM antibodies prior to cell culture and fusion and would therefore have been lost by a selection step that selects only for IgG-expressing B-cells.

In some embodiments, the invention provides a method of creating a library of hybridomas that consist essentially of cells derived from post-germinal center B-cells. Such an embodiment can serve to streamline the cloning of antigen-specific hybridomas by reducing the background of hybridomas that express pre-germinal center cell antibodies, such as IgM antibodies. The libraries created using CD27+ B cells cultured in the presence of at least one of HCV E2, as well as with at least one of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG oligonucleotides, are enriched for IgG antibodies relative to IgM antibodies, because the culture conditions of the invention serve to induce class switch in IgM+IgD+CD27+ B cells and because the CD27 selection step prevents the incorporation of IgM+IgD+CD27- cells, which express low-affinity un-mutated (natural) IgM antibodies, into the libraries.

In other embodiments, the invention provides a method of creating a library of hybridomas that consist essentially of cells derived from pre-germinal center B-cells, which have not yet undergone class switch recombination or somatic hypermutation. Such an embodiment can serve to provide a B cell population that is enriched for cells that secrete a diverse repertoire of antibodies having diverse functional characteristics and binding specificities, including antibodies that specifically bind to self antigens.

B Cells

B cells useful in the compositions and methods of the invention include both pre-germinal center B cells and post-germinal center B cells. CD27 is a marker for post-GC B cells. A cell that is “CD27+” or that “expresses CD27” is
5 contrasted herein to a cell that is CD27- or does not express a detectable level of CD27. The present invention encompasses methods and kits for the isolation and expansion of both CD27- and CD27+ B cells. The term “enriched”, as used herein refers to at least 20%, preferably at least 30%, more preferably at least 40%, even more preferably at least 50%, even more preferably at least 60%, even more
10 preferably at least 70%, even more preferably at least 80%, even more preferably at least 90%, and even more preferably 100% more than a sample that is not enriched with respect to B cells expression of CD27. B cells can be isolated from any tissue, fluid or organ of an immunized animal where B cells are present, including peripheral blood, spleen, lymph node, or bone marrow.

15 B cells can be isolated from any tissue, fluid or organ of an immunized animal where B cells are present by any known method in the art. For example, an antibody specific to a B cell antigen (e.g., CD19, CD20, CD27, etc.) can be used to separate B cells, or subpopulations of B cells (e.g., CD27+, CD27-, etc.) from the sample.

20 In one embodiment, the method comprises contacting a cell population believed to include B cells with B cells specific antibody and substantially separating the B cell-antibody complex from a population of cells in the sample.

The anti-B cell antibody may be attached to a solid support to allow for separation. Procedures for separation may include magnetic separation, using
25 antibody-coated magnetic beads or dynal beads, affinity chromatography, cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, e.g., complement and cytotoxins, and “panning” with antibody attached to a solid matrix, e.g., plate, or other convenient technique. Techniques providing accurate cell separation include fluorescence activated cell sorters, which can have varying
30 degrees of sophistication, e.g., a plurality of color channels, low angle and obtuse light scattering detecting channels, impedance channels, etc., as well as magnetic activated cell sorters.

The separation techniques employed should maximize the retention of viability of the fraction of the cells to be collected. The particular technique employed

will, of course, depend upon the efficiency of separation, cytotoxicity of the method, the ease and speed of separation, and what equipment and/or technical skill is required.

Cells that are bound by the antibody can be removed from the cell
5 suspension by simply physically separating the solid support from the cell suspension. The exact conditions and duration of incubation of the cells with the solid phase-linked antibodies will depend upon several factors specific to the system employed. The selection of appropriate conditions, however, is well within the skill in the art.

Anti-B cell antibodies can be conjugated to biotin, which then can be
10 removed with avidin or streptavidin bound to a support, or fluorochromes, which can be used with a fluorescence activated cell sorter (FACS), to enable cell separation. For example, cells expressing B cell specific antigen are separated from other cells by the cell-surface expression of the B cell antigen. Conveniently, the anti-B cell
15 antibodies may be conjugated with markers, such as magnetic beads, which allow for direct separation, biotin, which can be removed with avidin or streptavidin bound to a support, fluorochromes, such as FITC, which can be used with a fluorescence activated cell sorter, or the like, to allow for ease of separation of the particular cell type. Any cell separation technique discussed herein may be employed which is not unduly detrimental to the viability of the remaining cells. Other cell separation
20 techniques include, but are not limited to, dense particles for density centrifugation, an adsorption column, an adsorption membrane, and the like.

Where the anti-B cell antibody is conjugated to a magnetic bead, a population of peripheral blood derived mononuclear cells is contacted with the magnetic bead-antibody conjugate under conditions suitable for binding of the
25 antibody conjugate to the B cell antigen displayed on the surface of the B cells. After incubation under conditions suitable for binding, such as, but not limited to, an incubation at 4°C for 20 minutes, the population of B-cells positive for the B cell antigen are selected by passing the entire sample through a magnetic-based separation apparatus. Upon evacuation or elution of free solution from the apparatus, only the
30 magnetically-retained marker-containing cells remain. The B cells are then eluted from the apparatus, resulting in an enriched, isolated or purified population of B cells.

One advantage of using CD27+ B cells over the standard unenriched population of human B cells is that cultured CD27+ B cells have undergone somatic hypermutation and therefore express affinity-matured antibodies. Thus, in some

embodiments, removing the CD27- B cell population from a fusion experiment used to generate a hybridoma library reduces the background of hybridoma cells that express un-mutated IgM antibodies that have low potential for value as diagnostic reagents or therapeutics. This improves the likelihood that a hybridoma expressing a
5 useful antibody can be identified and expanded as a monoclonal cell.

One advantage of using CD27- B cells over the standard unenriched population of human B cells is that cultured CD27- B cells have not yet undergone class switch recombination or somatic hypermutation. Therefore, CD27- B cells can express a diverse repertoire of antibodies having diverse functional characteristics and
10 binding specificities, including antibodies that specifically bind to self antigens.

Culturing

B cells can be cultured according to standard culturing procedures. For example, following their isolation, B cells are incubated in cell medium in a culture apparatus for a period of time or until the cells reach confluency before passing the
15 cells to another culture apparatus. The culturing apparatus can be of any culture apparatus commonly used in culturing cells in vitro. Preferably, the level of confluence is greater than 70% before passing the cells to another culture apparatus. More preferably, the level of confluence is greater than 90%. A period of time for incubation can be any time suitable for the culture of cells in vitro. B cell medium
20 may be replaced during the culture of the B cell at any time. Preferably, the B cell medium is replaced every 3 to 4 days. B cells are then harvested from the culture apparatus whereupon the B cells can be used immediately or cryopreserved to be stored for use at a later time.

“Cell culture” refers generally to cells taken from a living organism
25 and grown under controlled conditions. Cells are expanded in culture when they are placed in a growth medium under conditions that facilitate cell growth and/or division, resulting in a larger population of the cells. When cells are expanded in culture, the rate of cell proliferation is typically measured by the amount of time required for the cells to double in number, otherwise known as the “doubling time.”

30 In preparation for fusion with a fusion partner for hybridoma generation, B cells are cultured in the presence of HCV E2. In some embodiments, the B cells cultured in the presence of HCV E2, are also cultured in the presence of at least one of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG

oligonucleotides. The presence of one or more of these factors in the culture modulates the ability of the B cells to undergo isotype switching, class switch recombination and/or somatic hypermutation.

The B cells cultured in the presence of at least one of at least one of
5 CD40L, HCV E2, and CD14 can be fused with a fusion partner to generate human B cell hybridomas. One advantage of using the treated CD27⁺ B cells in the generation of hybridomas is that a higher percentage of cells in the hybridoma library secrete IgG antibodies. One advantage of using the treated CD27⁻ B cells in the generation of hybridomas is that the cells have not yet undergone class switch recombination or
10 somatic hypermutation, and thereof possess the ability to secrete a diverse repertoire of antibodies having diverse functional characteristics and binding specificities, including antibodies that specifically bind to self antigens.

The CD40L used in the method of the invention can be isolated from cells which naturally express CD40L, or can be purified from cells which have been
15 altered to express CD40L. Soluble CD40L can be added to the culture medium for culturing B cells. Alternatively, the B cells can be cultured in the presence of feeder cells, where the feeder cells secrete CD40L or express CD40L on their cell surface. Preferably, the B cells are cultured in the presence CD40L-expressing feeder cells. A CD40 stimulating activity can also be provided in the form of a peptide or antibody
20 molecule that binds to CD40 and has a stimulatory capability similar to that of CD40L. The peptide or antibody molecule may be provided in solution or bound to a feeder cell layer.

The CD14 used in the method of the invention can be isolated from cells which naturally express CD14, or can be purified from cells which have been
25 altered to express CD14. Soluble CD14 can be added to the culture medium for culturing B cells. Alternatively, the B cells can be cultured in the presence of feeder cells, where the feeder cells secrete CD14 or express CD14 on their cell surface. Preferably, the B cells are cultured in the presence CD14-expressing feeder cells. A CD14 stimulating activity can also be provided in the form of a peptide or antibody
30 molecule that binds to a ligand of CD14 and has a stimulatory capability similar to that of CD14. The peptide or antibody molecule may be provided in solution or bound to a feeder cell layer.

The HCV E2 used in the method of the invention can be isolated from cells which naturally express HCV E2, or can be purified from cells which have been

altered to express HCV E2. Soluble HCV E2 can be added to the culture medium for culturing B cells. Alternatively, the B cells can be cultured in the presence of feeder cells, where the feeder cells secrete HCV E2 or express HCV E2 on their cell surface. Preferably, the B cells are cultured in the presence HCV E2-expressing feeder cells. A
 5 HCV E2 stimulating activity can also be provided in the form of a peptide or antibody molecule that binds to a ligand of HCV E2 (e.g., CD81) and has a stimulatory capability similar to that of HCV E2. The peptide or antibody molecule may be provided in solution or bound to a feeder cell layer.

The cytokines useful in the methods of the invention (e.g., IL-4, IL-10,
 10 IL-15, IL-21, and IFN γ) can be obtained by any known method in the art. Sources of IL-4, IL-10, IL-15, IL-21, and IFN γ have been described and the DNA sequences encoding the molecules are also known. Therefore, natural or recombinant forms of IL-4, IL-10, IL-15, IL-21, and IFN γ can be used for the culturing of B cells.

The percentage of B cells that produce an antibody selective for a
 15 specific antigen can be increased by adding to the cell media an antigen which was used to immunize the host which was the source of the B cells. The combination of antigen, HCV E2, and in some embodiments, at least one of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG oligonucleotides, serves to modulate class switching and/or somatic hypermutation of B cells in vitro in an antigen-specific manner. The
 20 antigen can be a compound that has been shown, or can be shown, to stimulate an antibody response when administered to a mammalian host. The antigen can be added to the culture medium prior to, concomitantly, or after adding HCV E2, and in some embodiments, at least one of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG oligonucleotides, to the culture. Further, the skilled artisan can employ
 25 additional procedures to increase the antigenicity of an antigen as well as to insure that the antigen comes in contact with the cultured cells. Such procedures include coupling the antigen to a carrier to increase solubility or antigenicity.

After culturing the B cells according to the methods of the invention, the B cells can be fused with a fusion partner to generate a library of hybridomas.

30 Hybridomas

B cells prepared by the methods of the invention can be fused with any fusion partner to generate a hybridoma. A preferred fusion partner cell line is one that ectopically expresses hTERT and mIL-6. Preferably, a retroviral expression system is

used to express mIL-6 and hTERT in the fusion partner cell line. A preferred fusion partner cell line is the B5-6T cell line which was deposited under the terms of the Budapest Treaty with the American Type Culture Collection, 10801 University Blvd, Manassas, Va. 20110-2209, USA, on Jan. 15, 2007 and assigned ATCC Accession
5 No. PTA-8869. Other fusion partner cell lines may also be used, such as the SP2/mIL-6 MPT cell line, a murine cell line that also ectopically expresses mIL-6 and hTERT.

Generally, the B cells are fused with a fusion partner and treated with 40-50% polyethylene glycol of MW 1000-4000, at about 37°C for about 5-10 minutes to allow for the fusion. Alternatively, cell fusions can be induced by the standard
10 method of electrofusion, in which an electrical charge is used to cause fusion of cell plasma membranes. Following cell fusion, nascent hybrid cells are separated from the fusion mixture and propagated in media selective for the desired hybrids. When the hybrid cell is resistant to 8-azaguanine, the cell is conveniently selected by successive passaging of the cell on HAT or AH medium. Other selective procedures can be used
15 depending on the nature of the cells used in fusion. Clones secreting antibodies having the required binding specificity are identified by assaying the antibody secreted into the culture medium for the ability to bind to a particular target antigen or an epitope thereof. The antibody producing cells having the desired specificity are subcloned by a limiting dilution technique and grown in vitro in culture medium, or are injected
20 into selected host animals and grown in vivo.

The B5-6T fusion partner cell line stably expresses hTERT. It is further characterized in that it is capable of fusing with B cells and generating hybridomas at a highly efficient rate. An advantage of using the B5-6T fusion partner cell line is that the B5-6T fusion partner cell line allows for the generation of
25 hybridomas at an increased frequency. A characteristic of the B5-6T fusion partner cell line is the ability for the cell line to fuse with B-lymphocytes and produce hybridomas capable of surviving HAT selection at a highly efficient rate.

The B5-6T fusion partner cell line has the ability to produce hybridomas that are stable (i.e., hybridomas that maintain the ability to produce a
30 particular antibody for an extended period of time, such as, for example, at least three rounds of culturing in vitro). A monoclonal-antibody producing hybridoma cell line produced using the B5-6T fusion partner cell line can be subcloned and subcultured for many passages, until sufficient numbers of cells are obtained to produce antibodies in gram quantities or greater.

Antibodies

In one embodiment, the invention is directed to a human antibody that specifically binds to a particular target antigen or epitope thereof. The antibody comprises a heavy chain polypeptide comprising an antibody heavy chain variable domain and an antibody light chain polypeptide comprising an antibody light chain variable domain.

The antibodies produced may be tested for the ability to specifically bind to a particular target antigen or an epitope thereof. Antibodies may also be tested for the capacity to neutralize a particular antigen. Antigen toxicity can be determined in vivo. For example, one can measure the toxicity of a particular antigen in a test animal (e.g. mouse) in the presence of one or more putative neutralizing antibodies. A neutralizing antibody can be combined with the antigen prior to administration, or the antibody can be administered to the animal prior to, simultaneous with, or after administration of the antigen.

Preferred antibodies of this invention act to neutralize (reduce or eliminate) the toxicity of an antigen. In some embodiments, in vivo neutralization measurements involve measuring changes in the lethality (e.g. LD₅₀ or other standard metric) due to antigen administration due to the presence of one or more antibodies being tested for its neutralizing activity. The antigen can be directly administered to the test organism (e.g. mouse) or the organism can harbor a disease (e.g., cancer, autoimmunity, infection, etc.) involving the antigen. The antibody can be administered before, during, or after introducing the antigen or disease to the test animal. A decrease in the frequency or severity of a sign or symptom, the rate of progression, or mortality rate indicates that the antibody has neutralizing activity.

The antibodies of the invention are useful in the treatment of pathologies associated with a disease (e.g., cancer, autoimmunity, infection, etc.) associated with the antigen to which the antibody binds. The treatments essentially comprise administering to the diseased animal (e.g. human or non-human mammal) a quantity of neutralizing antibody sufficient to neutralize (e.g. mitigate, reduce or eliminate) the antigen and reduce or eliminate the severity or frequency of at least one sign or symptom of the disease. Treatment with a neutralizing antibody can be provided alone or as an adjunct to another therapy (e.g., antibiotic treatment).

Modification of Antibodies

The invention includes antibodies that specifically bind to a particular antigen. The invention also includes functional equivalents of the antibodies produced by the methods described herein. Functional equivalents have binding characteristics comparable to those of the antibodies described herein, and include, for example, hybridized and single chain antibodies, as well as fragments thereof. Methods of producing such functional equivalents are disclosed in PCT Application WO 93/21319 and PCT Application WO 89/09622.

Functional equivalents of the antibodies described herein further include antibodies or fragments thereof that have the same, or substantially the same, binding characteristics to those of the whole antibody. Such fragments may contain one or both Fab fragments or the $F(ab')_2$ fragment. In some embodiments, the antibody fragments contain all six complement determining regions (CDRs) of the whole antibody. In other embodiments, the antibody fragments contain fewer than all six CDRs, such as three, four or five complement determining regions. In some embodiments, the functional equivalents are members of the IgG immunoglobulin class and subclasses thereof, but may be or may combine any one of the following immunoglobulin classes: IgM, IgA, IgD, or IgE, and subclasses thereof. Heavy chains of various subclasses, such as the IgG subclasses, are responsible for different effector functions and thus, by choosing the desired heavy chain constant region, hybrid antibodies with desired effector function are produced. Non-limiting examples of constant regions useful in the antibodies of the invention include gamma 1 (IgG1), gamma 2 (IgG2a and IgG2b), gamma 3 (IgG3) and gamma 4 (IgG4). In various embodiments, the light chain constant region can be of the kappa or lambda type.

In various embodiments, the immunoglobulins of the present invention can be monovalent, divalent or polyvalent. Monovalent immunoglobulins are dimers (HL) formed of a hybrid heavy chain associated through disulfide bridges with a hybrid light chain. Divalent immunoglobulins are tetramers (H_2L_2) formed of two dimers associated through at least one disulfide bridge.

A) Phage Display

A phage display can be used to increase antibody affinity. To create antibodies of higher affinity for a particular antigen, mutant single chain variable fragment (scFv) gene repertoires, can be created and expressed on the surface of phage. Display

of antibody fragments on the surface of viruses that infect bacteria (e.g., bacteriophage or phage) makes it possible to produce human or other mammalian antibodies having a wide range of affinities and kinetic characteristics. To display antibody fragments on the surface of phage (phage display), an antibody fragment
5 gene is inserted into the gene encoding a phage surface protein and the antibody fragment-fusion protein is expressed on the phage surface (McCafferty et al., 1990, Nature 348: 552-554; Hoogenboom et al., 1991, Nucleic Acids Res. 19:4133-4137).

Since the antibody fragments on the surface of the phage are functional, those phage bearing antigen binding antibody fragments can be separated
10 from non-binding or lower affinity phage by antigen affinity chromatography (McCafferty et al., 1990, Nature 348:552-554). Mixtures of phage are allowed to bind to the affinity matrix, non-binding or lower affinity phage are removed by washing, and bound phage are eluted by treatment with acid or alkali. Depending on the affinity of the antibody fragment, enrichment factors of 20 fold-1,000,000 fold are obtained
15 by single round of affinity selection.

One approach for creating mutant scFv gene repertoires involves replacing either the V_H or V_L gene from a binding scFv with a repertoire of V_H or V_L genes (otherwise known as chain shuffling) (Clackson et al., 1991, Nature 352:624-628). Such gene repertoires contain numerous variable genes derived from the same
20 germline gene as the binding scFv, but with point mutations (Marks et al., 1992, Biotechnology 10:779-783). Using light or heavy chain shuffling and phage display, the binding avidities of the antigen-binding antibody fragment can be dramatically increased.

In order to generate an antibody having an increased affinity, during
25 the screening for the antibody, the antigen concentration is decreased in each round of selection, reaching a concentration less than the desired K_d by the final rounds of selection. This results in the selection of a desired antibody on the basis of affinity (Hawkins et al., 2002, J. Mol. Biol. 226: 889-896).

30 B) Site Directed Mutagenesis

It is known in the art that mutating amino acids, through for example, site-directed mutagenesis, that contact ligand has been shown to be an effective means of increasing the affinity of one protein molecule for its binding partner (Lowman et al., 1993, J Mol. Biol. 234:564-578; Wells, 1990, Biochemistry 29:8509-8516). The

majority of antigen-contacting, amino acid side chains in an antibody are located in the CDRs. Three of the CDRs occur in the V_H and three in the V_L (Chothia et al., 1987, J. Mol. Biol. 196:901-917; Chothia et al., 1986, Science 233:755-8; Nhan et al., 1991, J. Mol. Biol. 217:133-151). These residues contribute the majority of binding
5 energetics responsible for antibody affinity for antigen.

The CDRs are separated by framework regions. The framework regions spatially orient the CDR regions to shape the antigen-binding structure. Mutations to residues in either CDR regions or framework regions may alter and/or improve the binding characteristics of an antibody. Due to their structural role,
10 changes to residues in framework regions may result in improperly folded antibody structures that may be inactive (Shlomchik et al, 1989, Prog Immunol. 7:415-423). Consequently, changes to framework region residues should be conservative changes and should preserve hydrophobic packing interactions and buried salt bridges. The determination of which amino acids in an immunoglobulin protein sequence
15 contribute to which domains is well understood in the art. See Lefranc et al., (2005, Nucleic Acids Res 33:D593-D597).

CDR and FR residues are determined according to a standard sequence definition (Kabat et al., Sequences of Proteins of Immunological Interest, National Institutes of Health, Bethesda Md. (1987), and a structural definition (as in Chothia
20 and Lesk, J. Mol. Biol. 196:901-917 (1987). Where these two methods result in slightly different identifications of a CDR, the structural definition is preferred, but the residues identified by the sequence definition method are considered important FR residues for determination of which framework residues to import into a consensus sequence.

25 Accordingly, mutation of the CDRs and screening of the resulting mutants against an antigen, or the epitopes thereof, may be used to generate antigen-binding antibodies having improved binding affinity to an epitope and/or bind with higher affinity to specific antigens.

In a preferred embodiment, each CDR is randomized in a separate
30 library. To simplify affinity measurements, existing antibodies or other lower affinity antigen-binding antibodies, are used as a template, rather than a higher affinity scFv. The CDR sequences of the highest affinity mutants from each CDR library are combined to obtain an additive increase in affinity. A similar approach has been used to increase the affinity of human growth hormone (hGH) for the growth hormone

receptor over 1500 fold from 3.4×10^{-10} to 9.0×10^{-13} M (Lowman et al., 1993, J. Mol. Biol., 234:564-578).

To increase the affinity of antigen-binding antibodies, amino acid residues located in one or more CDRs are partially randomized by synthesizing a
5 “doped” oligonucleotide in which the wild type nucleotide occurred with a frequency of about, for example, 49%. The oligonucleotide is used to amplify the remainder of the antigen-binding scFv gene(s) using PCR.

For example, in one embodiment, to create a library in which V_H CDR3 is randomized, an oligonucleotide is synthesized which anneals to the antigen-
10 binding antibody V_H framework 3 and encodes V_H CDR3 and a portion of framework 4. At the four positions to be randomized, the sequence “NNS” can be used, where N is any of the 4 nucleotides, and S is “C” or “T”. The oligonucleotide is used to amplify the antigen-binding antibody V_H gene using PCR, creating a mutant antigen-binding antibody V_H gene repertoire. PCR is used to splice the V_H gene repertoire
15 with the antigen-binding antibody light chain gene, and the resulting scFv gene repertoire is cloned into a phage display vector. Ligated vector DNA is used to transform electrocompetent E. coli to produce a phage antibody library.

To select higher affinity mutant scFv, each round of selection of the phage antibody libraries is conducted on decreasing amounts of antigen. Typically, 96
20 clones from the third and fourth round of selection are screened for binding to the antigen by ELISA on 96 well plates.

Other methods known in the art and used for mutagenizing antibodies include error-prone PCR, over-expression of dominant-negative mismatch repair proteins (WO 2004/046330), parsimonius mutagenesis (Razai et al., 2005, J Mol Biol.
25 351:158-169) and chemical mutagenesis. See also: Chowdhury et al. (2005, Methods 36:11-27) and Carter (2006, Nat Rev Immunol. 6:343-357). Identification of antibodies with desirable properties can be achieved using a variety of common screening methods (Hoogenboom, 2005, Nat Biotechnol. 23:1105-1116).

30 C) Creation of Antigen-Binding scFv', Homodimers

To create antigen-binding (scFv')₂ antibodies, two antigen-binding scFvs are joined, either through a linker (e.g., a carbon linker, a peptide, etc.) or through a disulfide bond between, for example, two cysteines. Thus, for example, to

create disulfide linked antigen-binding scFv, a cysteine residue can be introduced by site directed mutagenesis.

In a particularly preferred embodiment, the (scFv')₂ dimer is created by joining the scFv fragments through a linker, more preferably through a peptide linker.

- 5 This can be accomplished by a wide variety of means well known to those of skill in the art. For example, one preferred approach is described by Holliger et al., 1993, Proc. Natl. Acad. Sci. USA 90:6444-6448 (see also WO 94/13804).

- Typically, linkers are introduced by PCR cloning. For example, synthetic oligonucleotides encoding the linker can be used to PCR amplify the antigen-binding antibody V_H and V_L genes which are then spliced together to create the antigen-binding diabody gene. The gene is then cloned into an appropriate vector, expressed, and purified according to standard methods well known to those of skill in the art.

- 15 D) Preparation of Antigen-Binding (scFv)₂, Fab, and (Fab')₂ molecules

- Antigen-binding antibodies, such as an antigen-binding scFv, or variant with higher affinity, are suitable templates for creating size and valency variants. For example, an antigen-binding (scFv')₂ is created from a parent scFv derived from the variable domains of an antibody. An scFv gene can be excised using appropriate restriction enzymes and cloned into another vector.

- An antibody-binding Fab is expressed in E. coli using an expression vector similar to the one described by Better et al., 1988, Science 240:1041-1043. To create an antigen-binding Fab, the V_H and V_L genes are amplified from the scFv using PCR. The V_H gene is cloned into an expression vector (e.g., a PUC119 based bacterial expression vector) that provides an IgG C_{H1} domain downstream from, and in frame with, the V_H gene. The vector also contains a leader sequence to direct expressed V_H-C_{H1} domain into the periplasm, a leader sequence to direct expressed light chain into the periplasm, and cloning sites for the light chain gene. Clones containing the correct V_H gene are identified, e.g., by PCR fingerprinting. The V_L gene is spliced to the C_L gene using PCR and cloned into the vector containing the V_HC_{H1} gene.

Genetic Modification

In addition to obtaining antigen-binding antibodies from a hybridoma, the antibodies can also be generated by cloning antibody genes into one or more

expression vector, and transforming the vector into a cell line such as the cell lines typically used for expression of recombinant or humanized immunoglobulins.

The genes encoding the heavy and light chains of immunoglobulins secreted by the cell lines are cloned according to methods, including but not limited to, the polymerase chain reaction (PCR), known in the art (see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, N.Y., 1989; Berger & Kimmel, Methods in Enzymology, Vol. 152: Guide to Molecular Cloning Techniques, Academic Press, Inc., San Diego, Calif., 1987; Co et al., 1992, J. Immunol. 148:1149). For example, genes encoding heavy and light chains are cloned from the antibody secreting cell's genomic DNA or cDNA is produced by reverse transcription of the cell's RNA. Cloning is accomplished by conventional techniques including the use of PCR primers that hybridize to the sequences flanking or overlapping the genes, or segments of genes, to be cloned.

Nucleic acids encoding the heavy and light chains of the antibodies or portions thereof can be obtained and used in accordance with recombinant DNA techniques for the production of the specific immunoglobulin, immunoglobulin chain, or variants thereof, in a variety of host cells or in an in vitro translation system. For example, the nucleic acids, including cDNAs, or derivatives thereof encoding variants such as a the heavy and light chains, can be placed into suitable prokaryotic or eukaryotic vectors, e.g., expression vectors, and introduced into a suitable host cell by an appropriate method, e.g., transformation, transfection, electroporation, infection, such that the nucleic acid is operably linked to one or more expression control elements, e.g., in the vector or integrated into the host cell genome.

The heavy and light chains, or portions thereof, can be assembled in two different expression vectors that can be used to cotransfect a recipient cell. Each vector can contain two selectable genes, one for selection in a bacterial system and one for selection in a eukaryotic system. These vectors allow for the production and amplification of the genes in bacterial systems, and subsequent cotransfection of eukaryotic cells and selection of the cotransfected cells. The selection procedure can be used to select for the expression of immunoglobulin chain genes introduced on two different DNA vectors into a eukaryotic cell.

Alternatively, the genes encoding a heavy chain and light chain may be expressed from one vector. Although the light and heavy chains are coded for by separate genes, they can be joined, using recombinant methods. For example, the two

polypeptides can be joined by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al., 1988, Science 242: 423-426; and Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883).

- 5 The invention provides for an isolated nucleic acid molecule comprising a nucleic acid sequence encoding at least a heavy and a light chain variable region. A nucleic acid molecule comprising sequences encoding both the light and heavy chain variable regions can be engineered to contain a synthetic signal sequences for secretion of the immunoglobulin chains when produced in a cell.
- 10 Furthermore, the nucleic acid molecule comprising both the heavy and light chain variable regions can contain specific DNA links which allow for the insertion of other immunoglobulin sequences and maintain the translational reading frame so to not alter the amino acids normally found in immunoglobulin chains.

- In accordance with the present invention, nucleotide sequences coding
- 15 for heavy and light chains may be inserted into an appropriate expression vector. In various embodiments, the vector comprises the necessary elements for transcription and translation of the inserted protein-coding sequence so as to generate recombinant DNA molecules that direct the expression of heavy and light chain immunoglobulins for the formation of an antibody.

- 20 In addition to the DNA segments encoding antigen-binding immunoglobulins or fragments thereof, other substantially homologous modified immunoglobulins can be readily designed and manufactured utilizing various recombinant DNA techniques known to those skilled in the art such as site-directed mutagenesis. Such modified segments will usually retain antigen binding capacity
- 25 and/or effector function. Moreover, the modified segments are usually not so far changed from the original genomic sequences of the antibody producing cell to prevent hybridization to these sequences under stringent conditions. Because, like many genes, immunoglobulin genes contain separate functional regions, each having one or more distinct biological activities, the genes may be fused to functional regions
- 30 from other genes to produce fusion proteins (e.g., immunotoxins) having novel properties or novel combinations of properties.

 A variety of methods can be used to express genes in a cell. Nucleic acids can be cloned into a number of types of vectors. However, the present invention should not be construed to be limited to any particular vector. Instead, the present

invention should be construed to encompass a wide variety of vectors which are readily available and/or known in the art. For example, the nucleic acid of the invention can be cloned into a vector including, but not limited to a plasmid, a phagemid, a phage derivative, an animal virus, and a cosmid. Vectors of particular
5 interest include expression vectors, replication vectors, probe generation vectors, and sequencing vectors.

In specific embodiments, the expression vector is selected from the group consisting of a viral vector, a bacterial vector and a mammalian cell vector. Numerous expression vector systems exist that comprise at least a part or all of the
10 compositions discussed above. Prokaryote- and/or eukaryote-vector based systems can be employed for use with the present invention to produce polynucleotides, or their cognate polypeptides. Many such systems are commercially and widely available.

Viral vector technology is well known in the art and is described, for
15 example, in Sambrook et al. (2012), and in Ausubel et al. (1999), and in other virology and molecular biology manuals. Viruses, which are useful as vectors include, but are not limited to, retroviruses, adenoviruses, adeno-associated viruses, herpes viruses, and lentiviruses. Preferably, a murine stem cell virus (MSCV) vector is used to express a desired nucleic acid. MSCV vectors have been demonstrated to
20 efficiently express desired nucleic acids in cells. However, the invention should not be limited to only using a MSCV vector, rather any retroviral expression method is included in the invention. Other examples of viral vectors are those based upon Moloney Murine Leukemia Virus (MoMuLV) and HIV. In general, a suitable vector contains an origin of replication functional in at least one organism, a promoter
25 sequence, convenient restriction endonuclease sites, and one or more selectable markers. (See, e.g., WO 01/96584; WO 01/29058; and U.S. Pat. No. 6,326,193).

Retroviral and lentiviral vectors have been used extensively to deliver genes into a host cell or animal. Retroviral integration can take place at many locations. Retroviral insertion biases have been estimated by a variety of methods
30 reviewed in Uren et al., 2005 *Oncogene* 24: 7656-7672. There is evidence that there is a preference for integration close to DNaseI sensitive and/or hypomethylated regions suggesting that retroviral integration has a tendency to insert within actively transcribed regions of the genome. Other evidence suggests that retroviral integration preferentially occurs near gene promoters. Generally, the evidence suggests that

retroviral integration is correlated with the target DNA's local characteristics, including conformation and methylation status, gene density, chromatin conformation, host DNA associated proteins and local transcriptional activity. Accordingly, retroviral integration is partially affected by nucleosome structure rather than any particular sequence specificity. Thus, the integration site is unpredictable.

For expression of the desired gene, at least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements, e.g., enhancers, modulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 by upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the thymidine kinase (tk) promoter, the spacing between promoter elements can be increased to 50 by apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

A promoter may be one naturally associated with a gene or polynucleotide sequence, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as "endogenous." Similarly, an enhancer may be one naturally associated with a polynucleotide sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding polynucleotide segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a polynucleotide sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a polynucleotide sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other prokaryotic, viral, or eukaryotic cell, and promoters or enhancers not "naturally

occurring,” e.g., containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including
5 PCR, in connection with the compositions disclosed herein (U.S. Pat. No. 4,683,202, U.S. Pat. No. 5,928,906). Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

Naturally, it will be important to employ a promoter and/or enhancer
10 that effectively directs the expression of the DNA segment in the cell type, organelle, and organism chosen for expression. Those of skill in the art of molecular biology generally know how to use promoters, enhancers, and cell type combinations for protein expression, for example, see Sambrook et al. (2012). The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate
15 conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or endogenous.

An example of a promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter
20 sequence capable of driving high levels of expression of any polynucleotide sequence operatively linked thereto. However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, Moloney virus promoter, the avian leukemia virus
25 promoter, Epstein-Barr virus immediate early promoter, Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the hemoglobin promoter, and the muscle creatine promoter. Further, the invention should not be limited to the use of constitutive promoters. Inducible promoters are also contemplated as part of the invention. The
30 use of an inducible promoter in the invention provides a molecular switch capable of turning on expression of the polynucleotide sequence which it is operatively linked when such expression is desired, or turning off the expression when expression is not desired. Examples of inducible promoters include, but are not limited to a metallothionein promoter, a glucocorticoid promoter, a progesterone promoter, and a

tetracycline promoter. Further, the invention includes the use of a tissue specific promoter, which promoter is active only in a desired tissue. Tissue specific promoters are well known in the art and include, but are not limited to, the HER-2 promoter and the PSA associated promoter sequences.

5 In order to assess the expression of the desired gene(s), the expression vector to be introduced into a cell can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors. In other embodiments, the selectable marker may be carried on a separate piece of DNA
10 and used in a co-transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers are known in the art and include, for example, antibiotic-resistance genes, such as neo and the like.

 Reporter genes are used for identifying potentially transfected cells and
15 for evaluating the functionality of regulatory sequences. Reporter genes that encode for easily assayable proteins are well known in the art. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a protein whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Expression of the reporter gene is assayed at a suitable time
20 after the DNA has been introduced into the recipient cells.

 Suitable reporter genes may include genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (see, e.g., Ui-Tei et al., 2000 FEBS Lett. 479:79-82). Suitable expression systems are well known and may be prepared using well
25 known techniques or obtained commercially. In general, the construct with the minimal 5' flanking region showing the highest level of expression of reporter gene is identified as the promoter. Such promoter regions may be linked to a reporter gene and used to evaluate agents for the ability to modulate promoter-driven transcription.

 Methods of introducing and expressing genes into a cell are known in
30 the art. In the context of an expression vector, the vector can be readily introduced into a host cell, e.g., mammalian, bacterial, yeast or insect cell by any method in the art. For example, the expression vector can be transferred into a host cell by physical, chemical or biological means.

Physical methods for introducing a polynucleotide into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. See, for example, 5 Sambrook et al. (2012) and Ausubel et al. (1999).

Biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, e.g., human cells. Other viral vectors can be derived from lentivirus, 10 poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. See, for example, U.S. Pat. Nos. 5,350,674 and 5,585,362.

Chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water 15 emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system for use as a delivery vehicle in vitro and in vivo is a liposome (e.g., an artificial membrane vesicle). The preparation and use of such systems is well known in the art.

Regardless of the method used to introduce exogenous nucleic acids into a host cell or otherwise expose a cell to the nucleic acid of the present invention, 20 in order to confirm the presence of the recombinant DNA sequence in the host cell, a variety of assays may be performed. Such assays include, for example, “molecular biological” assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; “biochemical” assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs 25 and Western blots) or by assays described herein to identify agents falling within the scope of the invention.

Cells that have undergone alteration of their DNA in order to cause such ectopic expression can be identified by single cell cloning and analyzing genomic DNA of the cloned cells for the presence of the altered DNA sequences 30 using PCR with primers specific for the altered DNA sequences.

Cells that have integrated an ectopic gene into the genome of a cell can be identified by single cell cloning and analyzing genomic DNA of the cloned cells for the presence of the ectopic telomerase gene using PCR with primers specific for the altered DNA sequences. Expression of the ectopic gene can be confirmed with

RT-PCR.

Therapeutic Use and Pharmaceutical Compositions

One skilled in the art can readily determine an effective amount of antibody to be administered to a given subject, by taking into account factors such as the size and weight of the subject; the extent of disease penetration; the age, health and sex of the subject; the route of administration; and whether the administration is regional or systemic. Generally, the amount of antibody administered to a subject depends upon the amount of antigen that needs to be neutralized and the amount of antigen-neutralizing activity exhibited by the antibodies. Those skilled in the art may derive appropriate dosages and schedules of administration to suit the specific circumstances and needs of the subject. For example, suitable doses of each antibody to be administered can be estimated from the amount of antigen to which a subject has been exposed, or the amount of antigen to which the subject is in risk of being exposed. Typically, dosages of antibody are between about 0.001 mg/kg and about 100 mg/kg body weight. In some embodiments, dosages are between about 0.01 mg/kg and about 60 mg/kg body weight.

It is understood that the effective dosage will depend on the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The most preferred dosage will be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation.

In some embodiments, a mixture of antigen-neutralizing human antibodies can be administered in equimolar concentrations to a subject in need of such treatment. In other embodiments, the human antibodies are administered in concentrations that are not equimolar. In some instances, the antibodies are administered as equal amounts of protein, by weight, per kilogram of body weight. For example, the antibodies can be administered in equal amounts, based on the weight of the subject. In other instances, the antibodies are administered in unequal amounts. In yet other instances, the amount of each antibody to be administered is based on its neutralizing activity. For example, a mixture with between about 1 IU/kg body weight and about 50 IU/kg body weight of antigen-neutralizing activity can be administered. In general, the schedule or timing of administration of a mixture of

antigen-neutralizing human antibodies is according to the accepted practice for the procedure being performed.

When used in vivo, the antibodies, either in their native form and/or in a recombinant form, are preferably administered as a pharmaceutical composition, comprising a mixture, and a pharmaceutically acceptable carrier. The antibodies may be present in a pharmaceutical composition in an amount from 0.001 to 99.9 wt %, more preferably from about 0.01 to 99.0 wt %, and even more preferably from 0.1 to 50 wt %. To achieve good plasma concentrations, an antibody, or a combination of antibodies, may be administered, for example, by intravenous injection, as a solution comprising 0.1 to 1.0% of the active agent.

The antibodies of the invention are useful for prophylactic and/or therapeutic treatment. The antibodies can be a component of a pharmaceutical composition. The pharmaceutical compositions of this invention are particularly useful for parenteral administration, such as intravenous administration or administration into a body cavity or lumen of an organ. The compositions for administration will commonly comprise a solution of antibody dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of antibodies in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the subject's needs.

Thus, a typical pharmaceutical composition for intravenous administration would be about 0.1 to 10 mg per subject per day. Dosages from 0.1 up to about 100 mg per subject per day may be used, particularly when the drug is administered to a secluded site and not into the blood stream, such as into a body cavity or into a lumen of an organ. Methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in

more detail in such publications as Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pa. (1980).

The compositions containing the antibody of the present invention can be administered for therapeutic treatments. In therapeutic applications, preferred
5 pharmaceutical compositions are administered in a dosage sufficient to neutralize (mitigate or eliminate) antigen (e.g., reduce or eliminate a sign or symptom of a pathology associated with an antigen). An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the disease and the general state of the subject's health.

10 Single or multiple administrations of the compositions may be administered depending on the dosage and frequency as required and tolerated by the subject. In any event, the composition should provide a sufficient quantity of the antibodies of the invention to effectively treat the subject.

As used herein, "parenteral administration" of a pharmaceutical
15 composition includes any route of administration characterized by physical breaching of a tissue of a subject and administration of the pharmaceutical composition through the breach in the tissue. Parenteral administration thus includes, but is not limited to, administration of a pharmaceutical composition by injection of the composition, by application of the composition through a surgical incision, by application of the
20 composition through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration is contemplated to include, but is not limited to, subcutaneous, intraperitoneal, intramuscular, intrasternal injection, and kidney dialytic infusion techniques.

Formulations of a pharmaceutical composition suitable for parenteral
25 administration comprise the active ingredient combined with a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. Such formulations may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold in unit dosage form, such as in ampules or in multi-dose containers containing a
30 preservative. Formulations for parenteral administration include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In one embodiment of a formulation for

parenteral administration, the active ingredient is provided in dry (e.g., powder or granular) form for reconstitution with a suitable vehicle (e.g., sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition.

The pharmaceutical compositions may be prepared, packaged, or sold
5 in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution may be formulated according to the known art, and may comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents, wetting agents, or suspending agents described herein. Such sterile injectable formulations may be prepared using a non-toxic parenterally-acceptable
10 diluent or solvent, such as water or 1,3-butane diol, for example. Other acceptable diluents and solvents include, but are not limited to, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono- or di-glycerides. Other parentally-administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form, in a liposomal preparation, or as a
15 component of a biodegradable polymer systems. Compositions for sustained release or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

As used herein, "additional ingredients" include, but are not limited to,
20 one or more of the following: excipients; surface active agents; dispersing agents; inert diluents; granulating and disintegrating agents; binding agents; lubricating agents; sweetening agents; flavoring agents; coloring agents; preservatives; physiologically degradable compositions such as gelatin; aqueous vehicles and solvents; oily vehicles and solvents; suspending agents; dispersing or wetting agents;
25 emulsifying agents, demulcents; buffers; salts; thickening agents; fillers; emulsifying agents; antioxidants; antibiotics; antifungal agents; stabilizing agents; and pharmaceutically acceptable polymeric or hydrophobic materials. Other "additional ingredients" which may be included in the pharmaceutical compositions of the invention are known in the art and described, for example in Remington's
30 Pharmaceutical Sciences (1985, Genaro, ed., Mack Publishing Co., Easton, Pa.), which is incorporated herein by reference.

EXPERIMENTAL EXAMPLES

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following
 5 examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed
 10 methods. The following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

Example 1: Creation of the cell line expressing the Hepatitis C E2 protein.

15 DNA was synthesized that contained sequences derived from the Hepatitis C virus (isolate H77) that included the E2 coding region. This sequence was derived from the sequence Genbank accession number AAB67038 and constructed to contain 5' BamHI and 3' EcoRI sites. The DNA fragment was inserted into the retroviral transfer vector pBabePuro at the BamHI and EcoRI sites in the vector
 20 polylinker domain, to create the plasmid pBabePuro E2; Nucleic Acids Research, vol 18, issue 4, page 1068, year 1990. The vector was used to create replication-defective viral particles by co-transfection of 293T cells with 0.9 µg gag/pol expression vector, 0.1 µg VSV-G expression vector, and 1 µg pBabePuro E2. Retroviral particles expressed in the cell culture supernatant were isolated by passage through a 45 µm
 25 syringe filter and then incubated with the tCD40L cell line (Urashima et al., 1995 Blood 85(7): 1903-12) in 45% F12, 45% DMEM, 10% IFS, L-glutamine and penicillin/streptomycin, in the presence of polybrene 4 µg/ml, for 24 hours. 24 hours later the cells were selected with 2 µg/ml puromycin in the same culture medium. A resistant cell population was obtained (tCD40L-E2) and expression of the HCV E2
 30 protein was verified by immunoblotting.

SEQUENCE of HCV_H77_E2_AAB67038 (SEQ ID NO: 1)

GGATCCGCCGCCACCATGGACATGATCGCTGGGGCCCATTTGGGGCGTCCT
 GGCAGGAATTGCTTACTTCAGCATGGTTCGGGAATTGGGCCAAGGTCCTGG

TGGTGCTGCTGCTGTTTGCTGGTGTGGATGCCGAAACCCATGTCACAGGG
 GGAAATGCTGGTAGAACTACCGCAGGCCTGGTGGGACTGCTGACACCCGG
 AGCTAAACAGAACATTCAACTGATTAATACTAACGGCTCCTGGCACATCA
 ATTCTACTGCTCTGAATTGCAATGAATCACTGAACACAGGTTGGCTGGCA
 5 GGACTGTTCTATCAGCACAAGTTTAATAGTTCCGGTTGCCCAGAGAGGCT
 GACTTCCTGCCGACGACTGACCGACTTCGCTCAGGGCTGGGGACCTATCT
 CCTACGCTAACGGGTCAGGACTGGACGAGCGCCCCTATTGCTGGCATTAC
 CCCCCAAGACCCTGCGGCATCGTCCCTGCCAAAAGTGTCTGTGGTCCTGTC
 TACTGTTTTACACCCTCACCAGTGGTGGTCCGTACCACTGATAGGTCCGGA
 10 GCACCTACATATTCTTGGGGCGCCAATGATACTGACGTGTTTGTCTGAAT
 AACACACGGCCCCCTCTGGGTAACTGGTTTGGATGCACCTGGATGAATAG
 TACCGGATTCACTAAGGTCTGTGGAGCCCCACCCTGTGTCATTGGTGGAGT
 CGGGAATAACACTCTGCTGTGCCCAACTGACTGTTTTTCGCAAACACCCCG
 AAGCAACTTATAGTCGATGCGGCAGCGGTCCCTGGATCACACCTAGATGT
 15 ATGGTCGATTATCCTTATAGGCTGTGGCACTATCCTTGCACCATTA ACTAT
 ACTATCTTCAAAGTGAGGATGTACGTCGGGGGAGTGGAACACCGGCTGGA
 GGCCGCTTGTA ACTGGACAAGAGGCGAGAGATGTGACCTGGAGGACCGG
 GATCGATCCGAACTGTCTCCTCTGCTGCTGTCAACCACTCAATGGCAAGTC
 CTGCCATGTAGTTTTACAACCCTGCCAGCCCTGTCAACTGGTCTGATT CAT
 20 CTGCATCAAAATATCGTGGACGTGCAATATCTGTACGGCGTGGGTT CATCT
 ATTGCAAGCTGGGCTATCAAATGGGAGTATGTCGTCCTGCTGTTCTCTGCTG
 CTGGCTGACGCACGCGTGTGCTCATGTCTGTGGATGATGCTGCTGATTAGT
 CAGGCTGAAGCCGAATTC

25 Example 2: Creation of hybridomas expressing monoclonal antibodies specific for
 rabies virus antigens using the tCD40L-E2 cell line

Human monoclonal antibodies were cloned following methods
 previously described in Adekar et al. (2008 J Immunol Methods, vol 333, pages 156-
 166). Peripheral blood mononuclear cells (PBMCs) were obtained from healthy
 30 volunteers who had received multiple doses of rabies vaccine because they were at
 risk of occupational exposure to the virus. PBMCs were stored frozen in 90%
 Hyclone Defined FBS (Invitrogen, Carlsbad, CA) and 10% DMSO (Sigma-Aldrich)
 under liquid nitrogen. Prior to cell fusion, CD27-positive cells were isolated with anti-
 CD27 magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's

instructions, and were cultured for 8 days on a monolayer of tCD40L-E2 cells in IMDM supplemented with 10% human serum, IL-4 (2 ng/ml), IL-10 (10 ng/ml), transferrin (50 micrograms/ml), cyclosporine A (5.5×10^{-4} M), L-glutamine (2 mM), and penicillin/streptomycin (Sigma-Aldrich). To prepare the tCD40L-E2 monolayer, tCD40L-E2 cells were irradiated with 96 Gy from a Cs source and plated at a density of 5×10^4 /well in 12-well tissue culture plates (Corning, Corning, NY). The cultured cells were fused to the B5-6T heteromyeloma cell line and plated at ~1000 B-cells/well in 96 well plates, and the nascent hybrid cells were selected with HAT (Sigma-Aldrich) in Advanced RPMI + 1% fetal calf serum by known methods (see, Adekar et al., 2008 J Immunol Methods, vol 333, pages 156-166). Hybridomas were tested for expression of IgG reactive with rabies purified protein extract. Positive clones were stabilized by limiting dilution cloning, after which they were adapted to medium with 5% Ultra Low IgG fetal bovine serum (Life Technologies, Grand Island, NY), incubated for 5 days in a 500-ml roller bottle. Filtered supernatants were purified over protein G-Sepharose (Life Technologies). Antibody concentrations were determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Using this method, 6 human monoclonal IgG antibodies were isolated that are immunoreactive with rabies purified protein.

Example 3: Creation of hybridomas expressing monoclonal antibodies specific for polio virus antigens using the tCD40L-E2 cell line

Using the methods described elsewhere herein, PBMCs isolated from volunteer donors that have a history of poliovirus vaccination were processed. Three cell fusions were performed with CD27+ PBMCs isolated from three different subjects. Nine stable hybridomas were obtained that expressed distinct human monoclonal antibodies that have neutralizing activity against one or more Sabin PV strains.

Example 4: Creation of hybridomas

Another embodiment of the present invention results in the creation of a library creation of hybridomas expressing human monoclonal antibodies specific for binding to cancer cell antigens using the tCD40L-E2 cell line described elsewhere herein. It is well-established that some patients with cancer make antibodies that are specific for cancer antigens Cancer Chemother Pharmacol, 2000, vol 46, suppl

volume pages S3-7. Mononuclear cells are obtained from patients with cancer either by phlebotomy, surgery, or biopsy from peripheral blood, tumor tissue, spleen, lymph nodes, granulomas, or other tissue. The CD27+ mononuclear cell population is enriched using the methods described elsewhere herein and the resultant population is expanded in the presence of E2 and/or CD14, additionally in the presence of molecules selected from the group consisting of CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG oligonucleotides. An alternate strategy includes specifically isolating B-cells from these sources, on the basis of selection for CD27+ combined with negative selection against any cells with antigens specific for cells that are not of the B-cell lineage. The selected and expanded cells are fused to the B5-6T or alternate fusion partner cell line using an electrofusion or PEG fusion method. The cells are plated at a density of 300-2000 B-cells per well in 96-well plates in the presence of cell culture medium that contains HAT. Following 5-7 days of HAT treatment, the cells are cultured in medium that contains HT supplementation. Approximately 14 days after the cell fusion, the wells contain healthy hybridoma cells that secrete human monoclonal antibodies into the cell culture supernatants. Each well contains 3-10 hybridoma clones. Therefore, each 96-well plate (with a starting number of 3000-20,000 B-cells per plate) contains a library 300-1000 hybridoma clones that each express a human mAb derived from the immune response of a patient with cancer. Cancer-specific human mAbs can be identified by assays to detect binding of the mAbs to materials containing cancer-specific molecules and/or cells, using methods well-known to the art, including ELISA and flow cytometry. Cancer-specific human mAbs identified by this approach have utility for the creation of cancer therapeutics and/or diagnostics.

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The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

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CLAIMS

What is claimed is:

1. A method of making a hybridoma, the method comprising culturing B cells in the presence of HCV E2 for a period of time in vitro, and fusing the cultured B cells with a fusion partner cell line, thereby producing a hybridoma.
2. The method of claim 1, wherein the B cells are CD27+.
3. The method of claim 1, wherein the B cells are CD27-.
4. The method of claim 1, wherein the B cells are also cultured in the presence of at least one selected from the group consisting of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG oligonucleotides.
5. The method of claim 4, wherein the CD40L is provided in a soluble form.
6. The method of claim 1, wherein the HCV E2 is provided in a soluble form.
7. The method of claim 4, wherein CD40L is provided in the form of CD40L displayed on the surface of a CD40L-expressing cell during the B cell culturing.
8. The method of claim 1, wherein HCV E2 is provided in the form of HCV E2 displayed on the surface of HCV E2-expressing cell during the B cell culturing.
9. The method of claim 4, wherein CD14 is provided in the form of CD14 displayed on the surface of CD14-expressing cell during the B cell culturing.

10. The method of claim 1, wherein the fusion partner cell line ectopically expresses mIL-6 and hTERT.
11. The method of claim 1, wherein the B cells are isolated from an immunized subject.
12. The method of claim 1, wherein the B cells are isolated from an immunized human.
13. A mammalian cell line that co-expresses CD40L and HCV E2.
14. A hybridoma produced by the method of claim 1.
15. A monoclonal antibody produced by the hybridoma of claim 14.
16. A method of producing a monoclonal antibody, the method comprising culturing B cells in the presence of HCV E2 for a period of time in vitro, fusing the cultured B cells with a fusion partner cell line to produce a hybridoma, selecting a hybridoma that produces a monoclonal antibody, and culturing the hybridoma to produce the monoclonal antibody.
17. The method of claim 16, wherein the B cells are CD27+.
18. The method of claim 16, wherein the B cells are CD27-.
19. The method of claim 16, wherein the B cells are also cultured in the presences of at least one selected from the group consisting of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG oligonucleotides.
20. The method of claim 19, wherein the CD40L is provided in a soluble form.

21. The method of claim 16, wherein the HCV E2 is provided in a soluble form.
22. The method of claim 19, wherein the CD14 is provided in a soluble form.
23. The method of claim 19, wherein CD40L is provided in the form of CD40L displayed on the surface of a CD40L-expressing cell during the B cell culturing.
24. The method of claim 16, wherein HCV E2 is provided in the form of HCV E2 displayed on the surface of HCV E2-expressing cell during the B cell culturing.
25. The method of claim 19, wherein CD14 is provided in the form of CD14 displayed on the surface of CD14-expressing cell during the B cell culturing.
26. The method of claim 16, wherein the fusion partner cell line ectopically expresses mIL-6 and hTERT.
27. The method of claim 16, wherein the B cells are isolated from an immunized subject.
28. The method of claim 16, wherein the B cells are isolated from an immunized human.
29. A monoclonal antibody produced by the method of claim 16, or an antigen-binding fragment of the antibody.
30. The antigen-binding fragment of the antibody of claim 29, wherein the fragment is one selected from the group consisting of a single chain Fv (scFv) fragment, a Fab fragment, a (Fab')₂ fragment or a (scFv')₂ fragment.

31. A method of neutralizing antigen in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of the antibody or antigen-binding fragment of claim 29.

32. An isolated nucleic acid molecule encoding the monoclonal antibody, or antigen-binding fragment thereof, of claim 29.

33. The isolated nucleic acid molecule of claim 32, comprising a first nucleic acid segment encoding the antibody heavy chain polypeptide and a second nucleic acid segment encoding the antibody light chain polypeptide.

34. A method of making a library of hybridomas, the method comprising culturing B cells in the presence of HCV E2 for a period of time in vitro, and fusing the cultured B cells with a fusion partner cell line, thereby producing a hybridoma.

35. The method of claim 34, wherein the B cells are CD27+.

36. The method of claim 34, wherein the B cells are CD27-.

37. The method of claim 34, wherein the B cells are also cultured in the presences of at least one selected from the group consisting of CD14, CD40L, IL-4, IL-10, IL-15, IL-21, IFN γ , and CpG oligonucleotides

38. The method of claim 37, wherein the CD40L is provided in a soluble form.

39. The method of claim 34, wherein the HCV E2 is provided in a soluble form.

40. The method of claim 37, wherein CD40L is provided in the form of CD40L displayed on the surface of a CD40L-expressing cell during the B cell culturing.

41. The method of claim 34, wherein HCV E2 is provided in the form of HCV E2 displayed on the surface of HCV E2-expressing cell during the B cell culturing.

42. The method of claim 40, wherein HCV E2 and CD40L are provided by the mammalian cell line of claim 13.

43. The method of claim 34, wherein the fusion partner cell line ectopically expresses mIL-6 and hTERT.

44. The method of claim 34, wherein the B cells are isolated from an immunized subject.

45. The method of claim 34, wherein the B cells are isolated from an immunized human.

46. A library of monoclonal antibodies produced by the method of making a library of hybridomas of claim 34.