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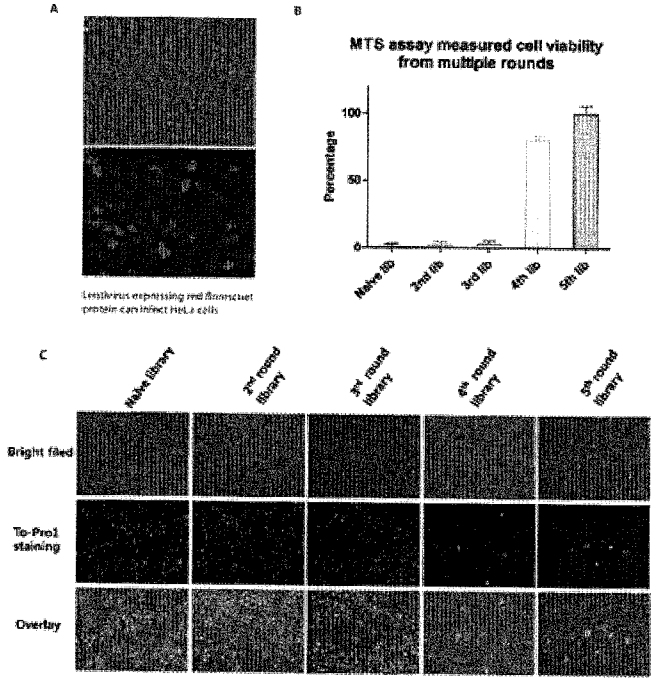
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(54) Title: FUNCTIONAL ANTIBODIES THAT MODULATE CELL DEATH AND RELATED METHODS

(57) Abstract: The invention provides methods for identifying antibody modulators of cell death from an intracellularly expressed library of intrabodies or membrane-tethered antibodies. The antibody modulators can promote or inhibit death of various target cells (e.g., tumor cells or neurons). Also provided in the invention are specific functional antibodies which can prevent death of cells caused by viral infections, as well as therapeutic applications of the antibodies. Further provided in the invention are polynucleotide sequences and vectors for expressing the functional antibodies disclosed herein.

Figure 1



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Functional Antibodies That Modulate Cell Death and Related Methods

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The subject patent application claims the benefit of priority to U.S. Provisional Patent Application Number 61/963,995 (filed December 20, 2013). The full disclosure of the priority application is incorporated herein by reference in its entirety and for all purposes.

BACKGROUND OF THE INVENTION

[0002] One of the most important phenotypes in biology is cell death. The mechanism(s) of cell death are poorly understood. There is an unmet need in the art for controlling and possibly interfering with processes that lead to cellular pathology including death. The present invention is directed to this and other needs.

SUMMARY OF THE INVENTION

[0003] In one aspect, the invention provides method for identifying antibodies that can modulate death of a eukaryotic cell. The methods involve (a) expressing in a population of the eukaryotic cells a library of intrabodies or membrane-tethered antibodies, or antigen-binding fragments thereof, to produce a heterogeneous population of antibody-expressing cells, and (b) selecting a specific antibody-expressing cell with a phenotype indicating an altered state of cell death. These allow identification of the antibody expressed in the specific antibody-expressing cell as one that modulates death of the cell. Preferably, the eukaryotic cell employed in the methods is of a mammalian cell type. In some preferred embodiments, the intracellularly expressed antibodies are intrabodies. In some preferred embodiments, the antibody library is expressed in the cell via a lentiviral vector or a retroviral vector. In some embodiments, each cell of the population of antibody-expressing cells expresses only one different member of the library of antibodies.

[0004] Some of the methods are directed to identifying antibodies that can delay cell death or enhance cell survival. For example, the methods can be employed for selecting antibodies that inhibit or prevent death of neurons. In some of these methods, the

heterogeneous population of antibody-expressing cells can be contacted with a cytotoxic or pathogenic agent prior to the selection. Some other methods of the invention are directed to identifying antibodies that can promote or enhance cell death. For example, some of the methods can be used for identifying antibodies that promote death of tumor cells. Some selection methods of the invention can additionally contain a step of determining amino acid sequences of heavy chain and light chain variable regions of the identified antibody.

[0005] In another aspect, the invention provides functional antibodies or antigen-binding fragments thereof which bind to human Rhinovirus B 3C protease with the same binding specificity as that of a reference antibody. The reference antibody can be one that comprises (1) heavy chain CDR1, CDR2 and CDR3 sequences respectively shown in SEQ ID NO:9, SEQ ID NO:10 and SEQ ID NO:11; and light chain CDR1, CDR2 and CDR3 sequences respectively shown in SEQ ID NO:12, SEQ ID NO:13 and SEQ ID NO:14; or (2) heavy chain CDR1, CDR2 and CDR3 sequences respectively shown in SEQ ID NO:15, SEQ ID NO:16 and SEQ ID NO:17; and light chain CDR1, CDR2 and CDR3 sequences respectively shown in SEQ ID NO:18, SEQ ID NO:19 and SEQ ID NO:20. Some of the antibodies of the invention comprise (1) heavy chain CDR1, CDR2 and CDR3 sequences that are substantially identical to SEQ ID NO:9, SEQ ID NO:10 and SEQ ID NO:11, respectively; and light chain CDR1, CDR2 and CDR3 sequences that are substantially identical to SEQ ID NO:12, SEQ ID NO:13 and SEQ ID NO:14, respectively; or (2) heavy chain CDR1, CDR2 and CDR3 sequences that are substantially identical to SEQ ID NO:15, SEQ ID NO:16 and SEQ ID NO:17, respectively; and light chain CDR1, CDR2 and CDR3 sequences that are substantially identical to SEQ ID NO:18, SEQ ID NO:19 and SEQ ID NO:20, respectively. Some other antibodies of the invention comprise (1) heavy chain CDR1, CDR2 and CDR3 sequences that are respectively identical to SEQ ID NO:9, SEQ ID NO:10 and SEQ ID NO:11; and light chain CDR1, CDR2 and CDR3 sequences that are respectively identical to SEQ ID NO:12, SEQ ID NO:13 and SEQ ID NO:14; or (2) heavy chain CDR1, CDR2 and CDR3 sequences that are respectively identical to SEQ ID NO:15, SEQ ID NO:16 and SEQ ID NO:17; and light chain CDR1, CDR2 and CDR3 sequences that are respectively identical to SEQ ID NO:18, SEQ ID NO:19 and SEQ ID NO:20. Some functional antibodies of the invention comprise (1) heavy chain and light chain variable region sequences show in SEQ ID NO:5 and SEQ ID NO:6, respectively; or (2) heavy chain and light chain variable region sequences show in SEQ ID NO:7 and SEQ ID NO:8, respectively. In some preferred

embodiments, the functional antibody of the invention is a scFv antibody fragment, e.g., a scFv molecule that comprises an amino acid sequence shown in SEQ ID NO:3 or SEQ ID NO:4.

[0006] In some related embodiments, the invention provides polynucleotides encoding the variable region of the heavy chain or light chain of the functional antibodies disclosed herein. Vectors harboring one or more of these polynucleotides sequences are also encompassed by the invention. Further provided by the invention are methods for preventing cell death caused by a Rhinoviral infection or for treating a common cold in a subject. These therapeutic applications of the invention typically entail administering to the subject a pharmaceutical composition that contains a therapeutically effective amount of a functional antibody disclosed herein.

[0007] Additional aspects and embodiments of the invention, as well as the specific properties and advantages of the present invention, are described in the remaining portions of the specification and claims.

DESCRIPTION OF THE DRAWINGS

[0008] Figures 1A-1C show that protection from HRV HeLa cell killing by Lentiviral antibody libraries is selectable. (A) Lentivirus carrying a monomeric version of the td-Tomato fragment was used to infect HeLa cells in order to confirm the capability of lentivirus to infect these cells. When adjusted to M.O.I=2, about 80% of cells show florescence. (B) An MTS assay was used to measure the ability of intra-body libraries to protect cells from killing by HRV. Nearly all the cells die in the naïve library group, Survival is not significantly improved after the 2^{nd} and 3^{rd} rounds of selection. However, the survival rate markedly increases in the 4^{th} round and by the 5^{th} round almost 100% of cells survive HRV infection. The error bars represent standard deviation. (C) To-Pro-1 dye as a marker of apoptotic cells was used to stain cultured cells. The dye uniquely enters apoptotic cells because their plasma membranes are leaky. When excited at 488nm, the dead cells show bright dots in their center. The percentage of dead cells is shown in the bottom overlay panel.

[0009] Figures 2A-2C show analysis of the isolated functional clones: (A) Sequence of the CDR regions of their heavy chains. Note that the first clone contains a sequence of "GGVV" in the middle of its CDR-H3 while the second clone has a sequence of "YDF",

therefore they are named as such respectively. (B) A MTS assay was used to measure protection from HRV killing by the Lentiviral clones containing functional antibodies. Both of the two clones completely blocked HRV induced HeLa cell death. The error bars represent standard deviation. (C) To-Pro-1 staining shows HRV induced apoptosis was minimal when the cells are protected by the two lentiviral clones encoding protective antibodies.

[0010] Figures 3A-3B show enrichment of functional clones studied by RT-PCR during the rounds of selection. Real time PCR using Taqman primers matching the unique regions between CDR-H2 and CDR-H3 was used to monitor the copy number change of GGVV (A) and YDF (B) in the plasmids from different selection rounds. The change was normalized to the number of promoter regions.

[0011] Figure 4 shows snapshots of functional enrichment using deep sequencing. For each library, the distribution of antibody sequences is plotted as a function of CDRH3 length (*x* axis) and sequence identity to a target antibody (y axis). Given a library, the sequences above 90% identical to the target antibody are considered to be functionally related and used to calculate the percent enrichment.

[0012] Figure 5 shows identification of the target antigen. The nature of the target antigen was determined by a Mass Spectrometry analysis using samples isolated by antibody affinity columns. This analysis showed that the target for both antibodies was the viral encoded 3C protease that is an enzyme necessary for viral maturation because it cleaves the viral encoded poly-protein. To confirm that the target antigen was the Rhinovirus encoded 3C protease, purified 3C protease was incubated with GGVV-Fc and YDF-Fc antibodies and the mixtures were collected using protein G and assayed by SDS-page (lane 1/2). An irrelevant antibody was used as a control (lane 3). An irrelevant protein with a His tag was used to control for non-specific binding to the His tag (lane 4/5). The 3C protease alone was incubated with protein G to control for non-specific interactions (lane 6).

[0013] Figures 6A-6B show selection using a pre-enriched library. (A). After three rounds of phage panning against the purified 3C protease, the output was converted into a Lentiviral library and was used for selection using the scheme outlined in Figure 1. In this case, the phenotype of protection from death was observed after only one round of selection. The output from the selected Lentiviral library was used to compare the degree of protection relative to pure virus expressing GGVV and YDF antibodies. The error bars represent

standard deviation. (B). GGVV and YDF Taqman primer-probe sets were used to detect the enrichment of these two sequences in the selection from the pre-enriched library. DNA plasmids were used as templates. As before, the fold of increase was normalized to the copy number of promoter region. Note that the GGVV sequence enriches faster than YDF in the pre-selected case.

[0014] Figure 7 is a gross representation of the Lentiviral library mediated protection from HRV induced cell death. HeLa cells in T225 flasks were infected with the combinatorial antibody library in lentivirus obtained after 5 rounds of selection and HRV was added 2 days later. These cells were compared to control unprotected cells. After 36 hours, massive cell death was observed in the control flask. However, the cells were nearly completely protected by the selected lentiviruses. In the control flask all the cells died and detached from the bottom while the cells protected by the antibody library formed a nearly confluent monolayer. The growth of the protected cells caused the flask to be less transparent as illustrated when one tries to view a picture thru the flask.

[0015] Figures 8A-8C show surface plasmon resonance (SPR) analysis of the interaction between the Rhinovirus encoded 3C protease and the purified functional antibodies. (A,B). Binding curves of the protease interaction with immobilized antibodies. Eight different concentrations were tested for each antibody. (C). The two antibodies bind two different epitope on the 3C protein. The YDF antibody was immobilized on the chip, perfused with solutions in the order shown in the picture. The results indicate the GGVV and the YDF antibodies bind additively to the 3C protease.

[0016] Figures 9A-9B show peptide digestion assay of 3C protease inhibiting antibodies. (A) Substrate peptide was designed with a biotin tag and 3C protease recognition/digestion sequence followed by a FLAG fragment. The peptide was immobilized on the bottom of a dish coated with streptavidin and incubated with 3C protease in the presence of the different antibodies. After washing, HRP conjugated secondary antibody was added to determine the efficiency of digestion which was determined by using a 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) substrate. (B) The dose response curve of antibody inhibition of 3C protease activity. Only YDF inhibits the protease. The GGVV has no inhibitory effect in the concentration region tested.

DETAILED DESCRIPTION OF THE INVENTION

I. Overview

[0017] The invention is predicated in part on the studies of the present inventors to probe the mechanism(s) of cell death by selecting molecules that prevent it. As detailed herein, the inventors employed intracellular combinatorial antibody libraries to select antibodies that protected cells from killing by Rhinovirus infection. The selection from unbiased combinatorial antibody libraries allowed discovery of antibodies that, when expressed in the cytoplasm as a pure population, completely protect cells from Rhinovirus-induced death. The power of the selection process was shown by the fact that only 2 out 10^8 antibodies were protective. These rare antibodies functioned by inhibiting the virus-encoded protease that is necessary for viral maturation. The studies revealed how selection systems operate when a replicating viral killing system is pitted against replicating cells where, initially, only a tiny minority are protected. These studies should pave the way for study of other systems where one wants to learn how to prevent cell death such as occurs in cellular senescence. Once one learns the mechanism by which the antibodies prevent cell death, other molecules can be designed to perturb the identified target antigens or the antibodies, themselves, may become therapeutic agents.

[0018] The selection process also had unusual parameters in that, until the final rounds there is, at any given time, the simultaneous presence of virus producing as well as resistant cells. This brings a population-based parameter into the selection that is different from static systems where one simply selects for binding. It is likely that a critical ratio of protected/susceptible cells must be reached such that the output virus titer from unprotected cells does not reach a level that overwhelms the protective effects of the antibodies. Thus, In terms of observable parameters, the early rounds of selection are essentially an all or none process that operates at the population level. The protective effect of the antibodies is only observable because cell survival is such a highly selectable phenotype and one can take advantage of kinetic parameters. This population effect is likely to only be pertinent in viral systems that pit two replicating against each other and should not be seen in other systems designed to inhibit cell death such as senescence.

[0019] It cannot be presumed that antibodies that inhibit the virus encoded protease could be discovered based on what we know about the molecular basis of Rhinovirus morphogenesis. This is because, as replicating agents, viruses present special problems and

disabling their encoded targets may be more difficult than inhibition of the more static proteins encoded by the host genome. The main problem is that functional inhibition by antibodies must be virtually perfect because any escape will be amplified by viral replication. Further, since, in this case, the target is an enzyme, any uninhibited molecules can likely facilitate replication of multiple virions. Some other questions that are associated with the inhibition process concern whether the cells can make enough antibodies to achieve the necessary stoichiometry to inhibit all the protease on a molar basis, and whether appearance of viral escape mutants would evade the antibodies, as happens in so many viral systems. These collective difficulties are underscored by our observation that when pre-selected libraries were used, we found that only two of the 10^5 binding antibodies that when converted to the intracellular format prevented viral killing. In the case of Rhinoviruses, host cell protein synthesis is shut off after infection and the cell is turned into a "factory" whose sole function is production of virus. Thus, it is likely that in this system the only proteins available for antibody inhibition are those encoded by the viral genome. Nevertheless, one should be able to generalize the method to discover host factors in other systems that are permissive for viral replication and/or pathogenesis.

[0020] The nature of the selected anti-protease antibodies is also interesting. There appear to be at least two mechanisms. In one the catalytic function of the protease is simply inhibited. In the other inhibition of catalysis is not required, likely because antibody binding inhibits entry of the protease into the morphogenic pathway required for virus assembly. In this respect, it is known that virus formation is associated with the formation of highly organized replication complexes that include tightly packed membranes and vesicles. Thus, simple steric factors associated with binding of some rare antibodies may prevent the integration of the protease into these highly organized replication complexes.

[0021] In accordance with these studies, the invention provides methods for selecting functional antibodies that can present or inhibit death of various cell types. Further provided in the invention are specific functional antibodies that are capable of preventing cell death.

II. Definitions

[0022] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention pertains. The following references provide one of skill with a general definition of

many of the terms used in this invention: *Academic Press Dictionary of Science and Technology*, Morris (Ed.), Academic Press (1st ed., 1992); *Oxford Dictionary of Biochemistry and Molecular Biology*, Smith et al. (Eds.), Oxford University Press (revised ed., 2000); *Encyclopaedic Dictionary of Chemistry*, Kumar (Ed.), Anmol Publications Pvt. Ltd. (2002); *Dictionary of Microbiology and Molecular Biology*, Singleton et al. (Eds.), John Wiley & Sons (3rd ed., 2002); *Dictionary of Chemistry*, Hunt (Ed.), Routledge (1st ed., 1999); *Dictionary of Pharmaceutical Medicine*, Nahler (Ed.), Springer-Verlag Telos (1994); *Dictionary of Organic Chemistry*, Kumar and Anandand (Eds.), Anmol Publications Pvt. Ltd. (2002); and *A Dictionary of Biology (Oxford Paperback Reference)*, Martin and Hine (Eds.), Oxford University Press (4th ed., 2000). In addition, the following definitions are provided to assist the reader in the practice of the invention.

[0023] The term "antibody" or "antigen-binding fragment" refers to polypeptide chain(s) which exhibit a strong monovalent, bivalent or polyvalent binding to a given antigen, epitope or epitopes. Unless otherwise noted, antibodies or antigen-binding fragments used in the invention can have sequences derived from any vertebrate, camelid, avian or pisces species. They can be generated using any suitable technology, e.g., hybridoma technology, ribosome display, phage display, gene shuffling libraries, semi-synthetic or fully synthetic libraries or combinations thereof. Unless otherwise noted, the term "antibody" as used in the present invention includes intact antibodies, antigen-binding polypeptide fragments and other designer antibodies that are described below or well known in the art (see, e.g., Serafini, J Nucl. Med. 34:533-6, 1993).

[0024] An intact "antibody" typically comprises at least two heavy (H) chains (about 50-70 kD) and two light (L) chains (about 25 kD) inter-connected by disulfide bonds. The recognized immunoglobulin genes encoding antibody chains include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

[0025] Each heavy chain of an antibody is comprised of a heavy chain variable region (V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, C_{H1}, C_{H2} and C_{H3}. Each light chain is comprised of a light chain variable region (V_L) and a light chain constant region. The light chain constant region is comprised

of one domain, C_L. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system and the first component (Clq) of the classical complement system.

[0026] The V_H and V_L regions of an antibody can be further subdivided into regions of hypervariability, also termed complementarity determining regions (CDRs), which are interspersed with the more conserved framework regions (FRs). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxyl-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The locations of CDR and FR regions and a numbering system have been defined by, e.g., Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, U.S. Department of Health and Human Services, U.S. Government Printing Office (1987 and 1991).

[0027] Antibodies to be used in the invention also include antibody fragments or antigen-binding fragments which contain the antigen-binding portions of an intact antibody that retain capacity to bind the cognate antigen. Examples of such antibody fragments include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and C_{H1} domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an intact antibody; (v) disulfide stabilized Fvs (dsFvs) which have an interchain disulfide bond engineered between structurally conserved framework regions; (vi) a single domain antibody (dAb) which consists of a V_H domain (see, e.g., Ward et al., Nature 341:544-546, 1989); and (vii) an isolated complementarity determining region (CDR).

[0028] Antibodies suitable for practicing the present invention also encompass single chain antibodies. The term "single chain antibody" refers to a polypeptide comprising a V_H domain and a V_L domain in polypeptide linkage, generally linked via a spacer peptide, and which may comprise additional domains or amino acid sequences at the amino- and/or carboxyl-termini. For example, a single-chain antibody may comprise a tether segment for linking to the encoding polynucleotide. As an example, a single chain variable region fragment (scFv) is a single-chain antibody. Compared to the V_L and V_H domains of the Fv fragment which are coded for by separate genes, a scFv has the two domains joined (e.g., via

recombinant methods) by a synthetic linker. This enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules.

[0029] Antibodies that can be used in the practice of the present invention also encompass single domain antigen-binding units which have a camelid scaffold. Animals in the camelid family include camels, llamas, and alpacas. Camelids produce functional antibodies devoid of light chains. The heavy chain variable (V_H) domain folds autonomously and functions independently as an antigen-binding unit. Its binding surface involves only three CDRs as compared to the six CDRs in classical antigen-binding molecules (Fabs) or single chain variable fragments (scFvs). Camelid antibodies are capable of attaining binding affinities comparable to those of conventional antibodies.

[0030] The various antibodies or antigen-binding fragments described herein can be produced by enzymatic or chemical modification of the intact antibodies, or synthesized de novo using recombinant DNA methodologies, or identified using phage display libraries. Methods for generating these antibodies or antigen-binding molecules are all well known in the art. For example, single chain antibodies can be identified using phage display libraries or ribosome display libraries, gene shuffled libraries (see, e.g., McCafferty et al., Nature 348:552-554, 1990; and U.S. Pat. No. 4,946,778). In particular, scFv antibodies can be obtained using methods described in, e.g., Bird et al., Science 242:423-426, 1988; and Huston et al., Proc. Natl. Acad. Sci. USA 85:5879-5883, 1988. Fv antibody fragments can be generated as described in Skerra and Plückthun, Science 240:1038-41, 1988. Disulfide-stabilized Fv fragments (dsFvs) can be made using methods described in, e.g., Reiter et al., Int. J. Cancer 67:113-23, 1996. Similarly, single domain antibodies (dAbs) can be produced by a variety of methods described in, e.g., Ward et al., Nature 341:544-546, 1989; and Cai and Garen, Proc. Natl. Acad. Sci. USA 93:6280-85, 1996. Camelid single domain antibodies can be produced using methods well known in the art, e.g., Dumoulin et al., Nature Struct. Biol. 11:500–515, 2002; Ghahroudi et al., FEBS Letters 414:521–526, 1997; and Bond et al., J Mol Biol. 332:643-55, 2003. Other types of antigen-binding fragments (e.g., Fab, $F(ab')_2$ or Fd fragments) can also be readily produced with routinely practiced immunology methods. See, e.g., Harlow & Lane, *Using Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1998.

[0031] An intrabody is an antibody that works within the cell to bind to an intracellular protein. Due to the lack of a reliable mechanism for bringing antibodies into the cell from

the extracellular environment, this typically requires the expression of the antibody within the target cell. Because antibodies ordinarily are designed to be secreted from the cell, intrabodies require special alterations, including the use of single-chain antibodies (scFvs), modification of immunoglobulin V_L domains for hyperstability, selection of antibodies resistant to the more reducing intracellular environment, or expression as a fusion protein with maltose binding protein or other stable intracellular proteins.

[0032] Binding affinity is generally expressed in terms of equilibrium association or dissociation constants (K_a or K_d, respectively), which are in turn reciprocal ratios of dissociation and association rate constants (k_d and k_a, respectively). Thus, equivalent affinities may correspond to different rate constants, so long as the ratio of the rate constants remains the same.

[0033] The term "contacting" has its normal meaning and refers to combining two or more agents (e.g., polypeptides or phage), combining agents and cells, or combining two populations of different cells. Contacting can occur in vitro, e.g., mixing two polypeptides or mixing a population of antibodies with a population of cells in a test tube or growth medium. Contacting can also occur in a cell or in situ, e.g., contacting two polypeptides in a cell by coexpression in the cell of recombinant polynucleotides encoding the two polypeptides, or in a cell lysate.

[0034] A "fusion" protein or polypeptide refers to a polypeptide comprised of at least two polypeptides and a linking sequence or a linkage to operatively link the two polypeptides into one continuous polypeptide. The two polypeptides linked in a fusion polypeptide are typically derived from two independent sources, and therefore a fusion polypeptide comprises two linked polypeptides not normally found linked in nature.

[0035] "Heterologous", when used with reference to two polypeptides, indicates that the two are not found in the same cell or microorganism in nature. Allelic variations or naturally-occurring mutational events do not give rise to a heterologous biomolecule or sequence as defined herein. A "heterologous" region of a vector construct is an identifiable segment of polynucleotide within a larger polynucleotide molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by polynucleotide that does not flank the mammalian genomic polynucleotide in the genome of the source organism.

[0036] "Linkage" refers to means of operably or functionally connecting two biomolecules (e.g., polypeptides or polynucleotides encoding two polypeptides), including, without limitation, recombinant fusion, covalent bonding, disulfide bonding, ionic bonding, hydrogen bonding, and electrostatic bonding. "Fused" refers to linkage by covalent bonding. A "linker" or "spacer" refers to a molecule or group of molecules that connects two biomolecules, and serves to place the two molecules in a preferred configuration with minimal steric hindrance.

[0037] Multiplicity of infection or MOI refers to the ratio of infectious agents (e.g. phage or virus) to infection targets (e.g., cell). For example, when referring to a group of cells inoculated with infectious virus particles, the multiplicity of infection or MOI is the ratio of the number of infectious virus particles to the number of target cells present in a defined space.

[0038] The term "operably linked" when referring to a nucleic acid, refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame.

[0039] As used herein, an altered state of cell death refers to a change in a cell indicating that death or the cell has been modulated (inhibited or delayed). This can be an improved survival of the cell, an accelerated rate of death of the cell, a morphological change indicating accelerated or slowed cell death, or presence of a biochemical marker evidencing a different physical attribute related to cell death.

[0040] The term "polynucleotide" or "nucleic acid" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides, that comprise purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. Polynucleotides of the embodiments of the invention include sequences of deoxyribopolynucleotide (DNA), ribopolynucleotide (RNA), or DNA copies of ribopolynucleotide (cDNA) which may be isolated from natural sources, recombinantly produced, or artificially synthesized. A further example of a polynucleotide of the embodiments of the invention may be polyamide polynucleotide

(PNA). The polynucleotides and nucleic acids may exist as single-stranded or double-stranded. The backbone of the polynucleotide can comprise sugars and phosphate groups, as may typically be found in RNA or DNA, or modified or substituted sugar or phosphate groups. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. The sequence of nucleotides may be interrupted by non-nucleotide components. The polymers made of nucleotides such as nucleic acids, polynucleotides and polynucleotides may also be referred to herein as "nucleotide polymers.

[0041] Polypeptides are polymer chains comprised of amino acid residue monomers which are joined together through amide bonds (peptide bonds). The amino acids may be the L-optical isomer or the D-optical isomer. In general, polypeptides refer to long polymers of amino acid residues, e.g., those consisting of at least more than 10, 20, 50, 100, 200, 500, or more amino acid residue monomers. However, unless otherwise noted, the term polypeptide as used herein also encompass short peptides which typically contain two or more amino acid monomers, but usually not more than 10, 15, or 20 amino acid monomers.

[0042] Proteins are long polymers of amino acids linked via peptide bonds and which may be composed of two or more polypeptide chains. More specifically, the term "protein" refers to a molecule composed of one or more chains of amino acids in a specific order; for example, the order as determined by the base sequence of nucleotides in the gene coding for the protein. Proteins are essential for the structure, function, and regulation of the body's cells, tissues, and organs, and each protein has unique functions. Examples are hormones, enzymes, and antibodies. In some embodiments, the terms polypeptide and protein may be used interchangeably.

[0043] Unless otherwise noted, the term "receptor" broadly refers to a molecule that has an affinity for a given ligand. Receptors may-be naturally-occurring or manmade molecules. Also, they can be employed in their unaltered state or as aggregates with other species. Receptors may be attached, covalently or noncovalently, to a binding member, either directly or via a specific binding substance. A typical example of receptors which can be employed in the practice of the invention is cell surface signaling receptor.

[0044] Rhinoviruses are the most common viral infective agents in humans and are the predominant cause of the common cold. Rhinovirus infection proliferates in temperatures between 33-35 °C (91-95 °F), and this may be why it occurs primarily in the nose. Rhinovirus is a species in the genus Enterovirus of the Picornaviridae family of viruses.

Human rhinoviruses occur worldwide and are the primary cause of common colds. Symptoms include sore throat, runny nose, nasal congestion, sneezing and cough; sometimes accompanied by muscle aches, fatigue, malaise, headache, muscle weakness, or loss of appetite.

[0045] The term "subject" refers to human and non-human animals (especially non-human mammals). In addition to human, it also encompasses other non-human animals such as cows, horses, sheep, pigs, cats, dogs, mice, rats, rabbits, guinea pigs, monkeys.

[0046] The term "target," "target molecule," or "target cell" refers to a molecule or biological cell of interest that is to be analyzed or detected, e.g., a eukaryotic cell the death of which is to be modulated.

[0047] A cell has been "transformed" by exogenous or heterologous polynucleotide when such polynucleotide has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming polynucleotide may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming polynucleotide has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming polynucleotide. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth in vitro for many generations.

[0048] Transmembrane domain usually denotes a single transmembrane alpha helix of a transmembrane protein. It is called a "domain" because an alpha-helix in a membrane can fold independently from the rest of the protein, similar to domains of water-soluble proteins. More broadly, a transmembrane domain is any three-dimensional protein structure which is thermodynamically stable in a membrane. This may be a single alpha helix, a stable complex of several transmembrane alpha helices, a transmembrane beta barrel, a beta-helix of gramicidin A, or any other structure. Various eukaryotic transmembrane domain polypeptides can be used in the practice of the present invention. As exemplified herein, the transmembrane domain of platelet-derived growth factor receptor (PDGFR) is suitable for the invention.

[0049] A "vector" is a replicon, such as plasmid, phage or cosmid, to which another polynucleotide segment may be attached so as to bring about the replication of the attached segment. Vectors capable of directing the expression of genes encoding for one or more polypeptides are referred to as "expression vectors".

III. Expressing combinatorial antibody library inside eukaryotic cells

[0050] The invention provides methods that allow one to select directly for functional antibodies in eukaryotic cells that modulate the death of the cells. The methods rely on construction of a combinatorial antibody library (e.g., intrabodies expressed via lentiviral vectors) which, upon infection, lead to efficient expression of antibodies inside the eukaryotic host cells or localized to the cell membrane. In some embodiments, the antibodies expressed inside the cells remain inside the cell as intrabodies. Because antibodies ordinarily are designed to be secreted from the producer cell, intrabodies require special alterations, including the use of single-chain antibodies (scFvs), modification of immunoglobulin V_L domains for hyperstability, selection of antibodies resistant to the more reducing intracellular environment, or expression as a fusion protein with maltose binding protein or other stable intracellular proteins. Such optimizations can improve the stability and structure of intrabodies. A library expressing intrabodies can be used in selecting for modulators of cell death through intracellular target molecules. For example, a combinatorial library of intrabodies can be used to select for modulators of cell death in a healthy or diseased cell. Identification of such antibody modulators could in turn lead to discovery of novel drug targets against which smaller, cell-penetrable compounds can be designed or screened (e.g., siRNA or small organic agents).

[0051] In some other embodiments, the library of candidate antibodies are expressed as molecules tethered to the cell membrane. For expressing membrane tethered library of candidate antibodies, the antibody sequence (e.g., a scFv sequence) is operably liked at the N-terminus or the C-terminus to a transmembrane domain. Any transmembrane protein domain known in the art may be used these embodiments of the invention, e.g., the PDGFR transmembrane domain. See , e.g., Remm et al., Genome Res. 10: 1679-1689, 2000; and Hubert et al., Cell Adh. Migr. 4: 313–324, 2010. In some embodiments, the antibody sequence can be connected to the transmembrane domain via a short linker peptide or linker

sequence. For example, a linker peptide comprising tandem repeats of GGGGS (SEQ ID NO:21) can be used for connecting the transmembrane domain.

[0052] Typically, to directly correlate an observed phenotype indicating an altered state of cell death with a specific antibody molecule or antibody-encoding sequence, the antibody library is introduced into and expressed in the cells under conditions each cell expresses no more than about 2 or 3 different antibodies (e.g., intrabodies or intrabody-encoding sequences) (e.g., scFv sequences). In some preferred embodiments, each individual cell of the heterogeneous population of recombinantly produced cells expresses no more than one different member of the intrabody library or membrane-tethered antibody library. With a lentiviral or retroviral based vector system as exemplified herein, this can be accomplished by infecting the producer or indicator cells the antibody-expressing viruses at a relatively low multiplicity of infection (MOI), e.g., not higher than 2 or 3. For selection of homodimeric antibodies only, infection of the cells by the viruses can be performed at a lower MOI, e.g., less than about 1. To allow for selection of bispecific heterodimeric antibody modulators, the antibody-expressing sequences can be transduced into the cells at a higher MOI, e.g., about 2 or 3. Under these conditions, an antibody modulator can be directly identified from an observed phenotype alteration with little or no further test of the antibodies that are isolated from positive clones in the phenotype assay.

[0053] While the intrabody library or membrane-tethered antibody can be expressed in different forms, they are preferably single chain molecules. As exemplified herein, sequences of the intrabody library or membrane-tethered antibody library do not contain any secretion leader sequence to allow for intracellular expression. Single chain antibody libraries can comprise the heavy or light chain of an antibody alone or the variable domain thereof. More typically, members of single-chain antibody libraries are generated by a fusion of heavy and light chain variable domains separated by a suitable spacer within a single contiguous protein. See e.g., Ladner et al., WO 88/06630; McCafferty et al., WO 92/01047. Various known libraries of antibodies can be utilized and modified as necessary in the practice of the selection methods of the invention. As exemplified, the antibody (e.g., intrabody) library can comprise unrelated antibodies from a naïve antibody library. For example, libraries of naïve antibodies (e.g., scFv libraries from human spleen cells) can be prepared as described in Feldhaus et al., Nat. Biotechnol. 21:163-170, 2003; and Lee et al., Biochem. Biophys. Res. Commun. 346:896-903, 2006. Park et al. (Antiviral Res. 68:109-

15, 2005) also described a large non-immunized human phage antibody library in single-chain variable region fragment (scFv) format. A library of antibodies (e.g., intrabodies) derived from a subject with a specific disease can be prepared from RNA extracted from peripheral blood lymphocytes of the subject, using methods as described in Kausmally et al. (J. Gen. Virol. 85:3493-500, 2004). Alternatively, the intrabody or membrane-tethered antibody library can comprise synthetic antibodies or antibodies derived from a specific antibody, e.g., by DNA shuffling or mutagenesis. For example, Griffiths et al. (EMBO J 13:3245–3260, 1994) described a library of human antibodies generated from large synthetic repertoires (lox library). Some embodiments of the invention can employ libraries of intrabodies or membrane-tethered antibodies that are derived from a specific scaffold antibody. Such antibody libraries can be produced by recombinant manipulation of the reference antibody using methods described herein or otherwise well known in the art. For example, Persson et al. (J. Mol. Biol. 357:607-20, 2006) described the construction of a focused antibody library for improved hapten recognition based on a known hapten-specific scFv.

[0054] In some preferred embodiments of the invention, the intrabody or membrane-tethered antibody library expresses single chain antibodies such as single chain variable region fragments (scFv). A specific scFv library suitable for the present invention is described in the Examples below and also in the art, e.g., Zhang et al., Chemistry & biology 20, 734-741, 2013; Yea et al., Proc. Natl. Acad. Sci. 110, 14966-71, 2013; and Gao et al., Proc. Natl. Acad. Sci. 99:12612-6, 2002. Such an antibody library can be generated with and expressed from various vectors well known in the art. Preferably, the antibody library used in the invention is constructed via a lentiviral or retroviral based vector. Construction of such antibody library for expression inside a eukaryotic host cell can be performed in accordance with the techniques exemplified herein and other methods well known in the art. In some embodiments, the antibody library is constructed with a lentiviral vector. Lentiviral vectors are retroviral vectors that are able to transduce or infect both dividing and non-dividing cells and typically produce high viral titers.

[0055] Examples of lentiviral based vectors suitable for the invention include, e.g., lentiviral vector pLV2 exemplified herein. For example, as detailed in the Examples below, a lentiviral based combinatorial scFv antibody library can be generated by cloning SfiI digested genes encoding the scFv into SfiI digested pLV2 vector to express scFv in the same

frame as the Fc portion of human IgG1 (from hinge to C_H3). Other lentiviral vectors that may be employed and modified for practicing the invention include, e.g., pLVX-Puro, pLVX-IRES-Neo, pLVX-IRES-Hyg, and pLVX-IRES-Puro. The various lentiviral vectors with cloned antibody sequences can be introduced into an appropriate host cell for expressing the antibody library. For example, the HEK293T cell line exemplified herein, as well as other packaging cell lines well known in the art (e.g., TF-1 or Lenti-X 293T cell line), may be employed for expressing the antibody library in the invention. In addition to lentiviral based vectors and host cells, other retroviral based vectors and expression systems may also be employed in the practice of the methods of the invention. These include MMLV based vectors pQCXIN, pQCXIQ and pQCXIH, and compatible producer cell lines such as HEK 293 based packaging cell lines GP2-293, EcoPack 2-293 and AmphoPack 293, as well as NIH/3T3-based packaging cell line RetroPack PT67.

IV. Selecting antibodies that modulate cell death

[0056] The invention provides methods for identifying antibody modulators (agonists or antagonists) of death of eukaryotic cells, especially mammalian cells. The selection methods can be applied to identifying antibody modulators that inhibits or promotes death of various cells. In some methods, antibodies can be identified that enhance or promotes death of cells in many circumstances. For example, antibodies can be selected for promoting death of various tumor cells. In some other methods, antibodies that promote survival or prevent death of cells are also desirable in many circumstances. For example, modulator antibodies can be selected for suppressing or delaying senescence or cell death in neurons. Methods that are exemplified herein and/or well known in the art can be used and adapted for expressing the intrabody or membrane-tethered antibody library (e.g., via a lentiviral vector), producing viral particles, infecting the target cell with the viruses, and selecting the antibody modulators.

[0057] To select for modulator antibodies of cell death, a library of candidate intrabodies are first introduced and expressed in a population of the target cells. Typically, the intrabodies or membrane-tethered antibodies are expressed under conditions that each member of the cell will harbor a unique sequence that encodes a member of the antibody library. Cells are then selected for a phenotype indicating an altered state of cell death. For selecting inhibitors of cell death, the cells can be contacted with a cytotoxic or pathogenic

agent that will normally induce death of the cell. For example, the cells can be contacted with a virus which is known to infect the cell type. Alternatively, the cells can be subject to a physical or chemical cidal agent such as heat, gamma radiation, incineration, ultraviolet light, and chemicals such as ethylene oxide, glutaraldehyde and ozone. Specific cells that are resistant to killing of the agent are then selected. Antibodies can be isolated from the death-resisting cells before subject to additional analysis to confirm their ability to prevent death of the cell.

[0058] For selecting antibodies that promote cell death (e.g., cancer cells), the intrabodies or membrane-tethered antibodies can be inducibly expressed in the target cells. Antibodies that promote death of the cells can be discovered by constructing colony assays and selecting for growth arrest only when gene expression is activated. Using inducible antibody library expression to identify modulators can be performed as described in the art. See, e.g., Melidoni et al., Proc. Natl. Acad. Sci. 110(44): 17802-17807, 2013. Death (e.g., apoptosis) or altered growth of the target cell can also be readily assessed via various other methods well known in the art. For example, apoptosis of the target cell can be monitored via an ethidium homodimer (EthD-1) assay which is a routinely practiced assay used to detect dead or dying cells.

[0059] In other embodiments, the antibody (e.g., intrabody) expressing cells can be selected for presence of early markers of cell death. Many cell death markers well known in the art can be readily employed in the selection methods of the invention, including an array of biochemical markers apoptotic cell death and autophagic cell death, and necrotic cell death, e.g., internucleosomal cleavage of DNA for apoptotic death. In addition, other cell death markers (e.g., morphological change) may also be used. For example, apoptosis (type I cell death) is characterized by internucleosomal cleavage of DNA and a sequence of specific morphological changes in the dying cell: cellular shrinkage with condensation of the cytoplasm, sharply delineated of chromatin masses lying against the nuclear membrane, nuclear fragmentation (karyorrhexis), and the subsequent formation of membrane-confined apoptotic bodies containing a variety of cytoplasmic organelles and nuclear fragments. In apoptosis, mitochondria appear to be normal or shrunken rather than dilated or swollen. Apoptotic bodies are engulfed by nonprofessional and professional phagocytes. Autophagy is characterized by the presence of autophagic structures with a double membrane. It is important to note that autophagy is foremost a survival mechanism activated in cells

undergoing different forms of cellular stress. If cellular stress continues, cell death may continue by autophagy alone, or else it may develop apoptotic or necrotic features. Necrosis (type III cell death) is characterized by swelling of the organelles (endoplasmic reticulum, mitochondria) and the cytoplasm, followed by collapse of the plasma membrane and lysis of the cells. As a consequence of the prominent swelling of the cytoplasm, this type of cell death was also designed as oncosis. Necrotic cell death is often considered a passive process lacking underlying signaling events and occurring under extreme physicochemical conditions, such as abrupt anoxia, sudden shortage of nutrients, and exposure to heat or detergents. It has become evident that necrotic cell death is as well controlled and programmed as apoptotic cell death and that it results from extensive cross talk between several biochemical and molecular events at different cellular levels.

[0060] Modulation of the phenotype in the examined cells is typically determined by comparing to the same phenotype of control cells which are not subject to interaction with the antibodies or cells expressing other antibodies. A significant departure or change of the phenotype in the cell contacted with a specific antibody relative to that of the control cell or other antibody-expressing cells would identify the specific antibody as a modulator of the cell. Many assays can be modified and adapted for use in the selection methods of the invention. Exemplary methods for evaluating phenotypes of cells include microscopy (e.g., light, confocal, fluorescence, scanning electron, and transmission electron), fluorescence based cell sorting, differential centrifugation, differential binding, immunoassays, enzymatic assays, growth assays, and in vivo assays. Fluorescence based cell sorting can be used to select antibody modulators of a signaling cascade in cells wherein expression of a fluorescent marker gene is linked to activation of that signaling pathway. In some embodiments, phenotypic behaviors of the cell such as chemotaxis, morphological changes, or apoptosis can be monitored via visual inspection or microscope examination. Optionally, computer software programs can be used to automatically detect cells with altered phenotype. To this end, various high-content screens ("HCS") have been developed to address the need for more detailed information about the temporal-spatial dynamics of cell constituents and processes. High-content screens automate the extraction of multicolor fluorescence information derived from specific fluorescence-based reagents incorporated into cells (see, e.g., Giuliano and Taylor, Curr. Op. Cell Biol. 7:4, 1995). Cells are analyzed using an optical system that can measure spatial, as well as temporal dynamics. In addition,

many fluorescent physiological indicators and "biosensors" are available to monitor changes in biochemical and molecular activities within cells (see, e.g., Giuliano et al., Ann. Rev. Biophys. Biomol. Struct. 24:405, 1995).

[0061] Various cell types can be employed in the selection methods of the invention. These include established cell lines as well as primary cells isolated from a eukaryotic organism (e.g., a mammal such as human). For example, primary cells such as neurons isolated from a subject can be readily used to select for antibodies (e.g., intrabodies) that inhibit or delay cell death. Similarly, established mammalian cells lines such as TF-1 and HEK293T can also be used for selecting antibody modulators of cell death. Other well-known mammalian cell lines that can be used and modified for practicing the methods of the invention include, e.g., CHO, HeLa, D10S, COS, MDCK, 293, WI38 and Jurkat E6 cells. In some other methods, primary cells can be tumor cells isolated from a subject, e.g., a human subject, to select for antibodies that stimulate apoptosis of the cells.

V. Functional antibodies that prevent cell death

[0062] As exemplification, the invention provides antibodies or antigen-binding molecules that can prevent death of cells caused by viral infection. As described in the Examples below, these functional antibodies are capable of suppressing Rhinoviral replication by binding to and inhibiting a key component of the viral replication pathway. General methods for preparation of monoclonal or polyclonal antibodies are well known in the art. See, e.g., Harlow & Lane, *Using Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1998; Kohler & Milstein, *Nature* 256:495-497, 1975; Kozbor et al., Immunology Today 4:72, 1983; and Cole et al., pp. 77-96 in Monoclonal Antibodies and Cancer Therapy, 1985. The specific functional antibodies disclosed herein were identified by selecting antibodies from a lentiviral vector based intrabody library for ability to prevent death of Hela cells caused by Rhinoviral infection.

[0063] Functional antibody agonists of the invention are preferably monoclonal antibodies like the antibodies exemplified in the Examples below. Preferably, they have the same binding specificities as that of the exemplified functional antibodies (e.g., GGVV antibody or YDF antibody). These antibodies typically harbor variable region sequences that are the same or substantially identical to that of the exemplified antibodies. In addition to containing variable regions sequences derived from the exemplified antibodies, some

functional antibodies of the invention can also contain other antibody sequences fused to the variable region sequences. For example, the antibodies can contain the Fc portion of human IgG1 sequence (from hinge to C_H3). Further, various modifications can be introduced into the antibody sequences for desired properties. For example, to enhance formation of heterodimeric antibodies, the scFv-Fc fusions can harbor "Knobs-Into-Hole" C_H3 mutations (e.g., T366Y and/or Y407T mutations).

[0064] Some of the antibodies are derived from the specific homodimer scFv antibody (GGVV) which comprises the heavy chain and light chain variable region sequence shown in SEQ ID NO:5 and SEQ ID NO:6, respectively. The CDR sequences of the heavy chain variable region of this antibody are GDIFSTYG (HCDR1; SEQ ID NO:9), IAPVFDTL(HCDR2; SEQ ID NO:10), and ARAGQGGVVGNYLDY (HCDR3; SEQ ID NO:11). The CDR sequences of its light chain variable region are QGISNY (LCDR1; SEQ ID NO:12), AAS (LCDR2; SEQ ID NO:13), and QKYNSAPLT (LCDR3; SEQ ID NO:14). Some other antibodies of the invention are derived from the specific scFv antibody YDF which comprises the heavy chain and light chain variable region sequence shown in SEQ ID NO:7 and SEQ ID NO:8, respectively. The CDR sequences of the heavy chain variable region of this antibody are GFTFSSYA (HCDR1; SEQ ID NO:15), ISYDGSNK (HCDR2; SEQ ID NO:16), and ARVGKGGYDFWSGSGYMDV (HCDR3; SEQ ID NO:17). The CDR sequences of its light chain variable region are KLGDKY (LCDR1; SEQ ID NO:18), QDS (LCDR2; SEQ ID NO:19), and QAWDSSTVV (LCDR3; SEQ ID NO:20).

[0065] DNA sequence of the GGVV scFv antibody (SEQ ID NO:1)

ATGGCACAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGCAGCCTGGGTCC
TCGGTGAAGGTCTCCTGCAAGACCTCTGGAGACATTTTCAGCACTTATGGTTTCA
ACTGGGTGCGACAGGCCCCTGGACAAGGGCTTGAGTGGATGGGAGGGATCGCC
CCTGTCTTTGATACATTGAAATACGCACAGAGGTTCCAGGGCAGATTACTAATA
ACCGCGGACGAGTCCGCGACCTCAGTGTACATGGAACTGAGCAGCCTAAGATCT
GACGACACGGCCGTCTACTACTGTGCGAGGGCCGGCCAGGGTGGAGTGGTCGGC
AACTACCTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGGCGGC
GGCGGCTCTGGCGGAGGTGGCAGCGGCGGTGGCGGATCCGACATCCAGATGAC
CCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACCATCACTTGC
CGGGCGAGTCAGGGCATTAGCAATTATTTAGCCTGGTATCAGCAGAAACCAGGG
AAAGTTCCTAAGCTCCTGATCTATGCTGCATCCACTTTGCAATCAGGGGTCCCAT
CTCGGTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGCCT
GCAGCCTGAAGATGTTGCAACTTATTACTGTCAAAAGTATAACAGTGCCCCGCT
CACTTTCGGCCAAGGGACCAAAGTGGATATCAAACGT

[0066] DNA sequence of the YDF scFv antibody (SEQ ID NO:2)

ATGGCACAGGTGCAGCTGTTGCAGTCTGGGGGAGGCGTGGTCCAGCCTGGGAGG
TCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTCAGTAGCTATGCTATGC
ACTGGGTCCGCCAGGCTCCAGGCAAGGGGCTGGAGTGGGTGGCAGTTATATCAT
ATGATGGAAGCAATAAATACTACGCAGACTCCGTGAAGGGCCGATTCACCATCT
CCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCTG
AGGACACGGCTGTGTATTACTGTGCGAGAGTAGGAAAGGGGGGTTACGATTTTT
GGAGTGGTTCAGGCTACATGGACGTCTGGGGCAAAGGGACCACGGTCACCGTCT
CCTCAGGCGGCGGCGGCTCTGGCGGAGGTGGCAGCGGCGGTGGCGGATCCTCCT
ATGTGCTGACTCAGCCACCCTCAGTGTCCGTGTCCCCAGGACAGACAGCCAGCA
TCACCTGCTCTGGAGATAAATTGGGGGATAAATATGCTTGCTGGTATCAGCAGA
AGCCAGGCCAGTCCCCTGTGCTGGTCATCTATCAAGATAGCAAGCGGCCCTCAG
GGATCCCTGAGCGATTCTCTGGCTCCAACTCTGGGAACACAGCCACTCTGACCA
TCAGCGGGACCCAGGCTATGGATGAGGCTGACTATTACTGTCAGGCGTGGGACA
GCAGCACTGTGGTATTCGGCGGAGGGACCAAGCTGACCGTCCTAGGT

[0067] Amino acid sequence of the GGVV antibody (SEQ ID NO:3)

MAQVQLVQSGAEVKQPGSSVKVSCKTSGDIFSTYGFNWVRQAPGQGLEWMGGIAP
VFDTLKYAQRFQGRLLITADESATSVYMELSSLRSDDTAVYYCARAGQGGVVGNY
LDYWGQGTLVTVSSGGGGSGGGGSGGGGSDIQMTQSPSSLSASVGDRVTITCRASQ
GISNYLAWYQQKPGKVPKLLIYAASTLQSGVPSRFSGSGSGTDFTLTISSLQPEDVAT
YYCQKYNSAPLTFGQGTKVDIKR

[0068] Amino acid sequence of the YDF antibody (SEQ ID NO:4)

MAQVQLLQSGGGVVQPGRSLRLSCAASGFTFSSYAMHWVRQAPGKGLEWVAVISY
DGSNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARVGKGGYDFW
SGSGYMDVWGKGTTVTVSSGGGGSGGGGSGGGGSSYVLTQPPSVSVSPGQTASITC
SGDKLGDKYACWYQQKPGQSPVLVIYQDSKRPSGIPERFSGSNSGNTATLTISGTQA
MDEADYYCQAWDSSTVVFGGGTKLTVLG

[0069] GGVV antibody heavy chain amino acid sequence (SEQ ID NO:5)

QVQLVQSGAEVKQPGSSVKVSCKTSGDIFSTYGFNWVRQAPGQGLEWMGGIAPVF
DTLKYAQRFQGRLLITADESATSVYMELSSLRSDDTAVYYCARAGQGGVVGNYLD
YWGQGTLVTVSS

[0070] GGVV antibody light chain amino acid sequence (SEQ ID NO:6)

DIQMTQSPSSLSASVGDRVTITCRASQGISNYLAWYQQKPGKVPKLLIYAASTLQSG

VPSRFSGSGSGTDFTLTISSLQPEDVATYYCQKYNSAPLTFGQGTKVDIK

[0071] YDF antibody heavy chain amino acid sequence (SEQ ID NO:7)

QVQLLQSGGGVVQPGRSLRLSCAASGFTFSSYAMHWVRQAPGKGLEWVAVISYDG
SNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARVGKGGYDFWSGS
GYMDVWGKGTTVTVSS

[0072] YDF antibody light chain amino acid sequence (SEQ ID NO:8)

SYVLTQPPSVSVSPGQTASITCSGDKLGDKYACWYQQKPGQSPVLVIYQDSKRPSGI
PERFSGSNSGNTATLTISGTQAMDEADYYCQAWDSSTVVFGGGTKLTVL

[0073] A typical intact antibody interacts with target antigen predominantly through amino acid residues that are located in the six heavy and light chain complementarity determining regions (CDR's). The functional antibodies of the invention encompass antibodies or antigen-binding fragments having at least one of their heavy chain CDR sequences and light chain CDR sequences that is the same as or substantially identical to the corresponding CDR sequence of exemplified GGCC or YDF antibody. Some of the functional antibodies of the invention have the same binding specificity as that of the exemplified antibodies disclosed in the Examples below. These antibodies can compete with the exemplified antibodies for binding to Rhinovirus encoded 3C protease.

[0074] In addition to having CDR sequences respectively identical to the corresponding CDR sequences of an exemplified antibody (e.g., the GGCC or YDF antibody), some of the functional antibodies of the invention have their entire heavy chain and light chain variable region sequences respectively identical to the corresponding variable region sequences of the exemplified antibodies. In some other embodiments, other than the identical CDR sequences, the antibodies contain amino acid residues in the framework portions of the variable regions that are different from the corresponding amino acid residues of the exemplified antibodies. Relative to the exemplified antibodies, the functional antibodies of the invention can undergo non-critical amino-acid substitutions, additions or deletions in the variable region without loss of binding specificity or effector functions, or intolerable reduction of binding affinity or receptor agonizing activities. Usually, antibodies incorporating such alterations exhibit substantial sequence identity to a reference antibody (e.g., the GGCC or YDF antibody) from which they were derived. For example, the mature light chain variable regions of some of the functional antibodies of the invention have at least 75% or at least 85% sequence identity to the sequence of the mature light chain variable region of the exemplified antibodies. Similarly, the mature heavy chain variable regions of the antibodies typically show at least 75% or at least 85% sequence identity to the sequence of the mature heavy chain variable region of the exemplified antibodies. In various embodiments, the antibodies typically have their entire variable region sequences that are substantial identical (e.g., 75%, 85%, 90%, 95%, or 99%) to the corresponding variable region sequences of the exemplified antibodies. Some functional antibodies of the invention

have the same specificity but improved affinity if compared with the exemplified antibodies (e.g., the GGCC or YDF antibody).

[0075] The functional antibodies disclosed herein can have many therapeutic applications. For example, they can be used for preventing cell death caused by infection of Rhinoviruses in a subject, e.g., a human patient. Rhinoviral infection is the primary cause of common colds. By preventing cell death caused by Rhinoviral infections, these antibodies can also be employed for treating or alleviating symptoms associated with common cold. Typically, an antibody of the invention can be administered to a subject suffering from Rhinoviral infection or common cold in a pharmaceutical composition that contains a therapeutically effective of the antibody. Preparation of such pharmaceutical compositions and their various routes of administration can be carried out in accordance with methods well known in the art. See, e.g., Remington: *The Science and Practice of Pharmacy*, Mack Publishing Co., 20th ed., 2000; and *Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

VI. Polynucleotides, vectors and host cells for producing functional antibodies

[0076] The invention provides substantially purified polynucleotides (DNA or RNA) which encode polypeptides comprising segments or domains of the functional antibody chains or antigen-binding molecules described herein. Some of the polynucleotides of the invention comprise the nucleotide sequence encoding the heavy chain variable region as shown in SEQ ID NO:5 or 7 and/or the light chain variable region sequence as shown in SEQ ID NO:6 or 8. Some other polynucleotides of the invention comprise nucleotide sequences that are substantially identical (e.g., at least 65, 80%, 95%, or 99%) to SEQ ID NO:1 or 2. When expressed from appropriate expression vectors, polypeptides encoded by these polynucleotides are capable of exhibiting antigen binding capacity.

[0077] Also provided in the invention are polynucleotides which encode at least one CDR region and usually all three CDR regions from the heavy or light chain of the functional antibodies described in the Examples below. Some other polynucleotides encode all or substantially all of the variable region sequence of the heavy chain and/or the light chain of the exemplified antibodies. For example, some of these polynucleotides encode the amino acid sequence of the heavy chain variable region shown in SEQ ID NO: 5 or 7, and/or the amino acid sequence of the light chain variable region shown in SEQ ID NO:6 or 8.

Because of the degeneracy of the code, a variety of nucleic acid sequences will encode each of the immunoglobulin amino acid sequences.

[0078] The polynucleotides of the invention can encode only the variable region sequence of a functional antibody. They can also encode both a variable region and a constant region of the antibody. Some of polynucleotide sequences of the invention nucleic acids encode a mature heavy chain variable region sequence that is substantially identical (e.g., at least 80%, 90%, or 99%) to the mature heavy chain variable region sequence shown in SEQ ID NO:5 or 7. Some other polynucleotide sequences encode a mature light chain variable region sequence that is substantially identical to the mature light chain variable region sequence shown in SEQ ID NO:6 or 8. Some of the polynucleotide sequences encode a polypeptide that comprises variable regions of both the heavy chain and the light chain of one of the exemplified antibody. Some other polynucleotides encode two polypeptide segments that respectively are substantially identical to the variable regions of the heavy chain and the light chain of one of the exemplified antibodies (e.g., the GGCC or YDF antibody).

[0079] The polynucleotide sequences can be produced by *de novo* solid-phase DNA synthesis or by PCR mutagenesis of an existing sequence (e.g., sequences as described in the Examples below) encoding an exemplified functional antibody. Direct chemical synthesis of nucleic acids can be accomplished by methods known in the art, such as the phosphotriester method of Narang et al., Meth. Enzymol. 68:90, 1979; the phosphodiester method of Brown et al., Meth. Enzymol. 68:109, 1979; the diethylphosphoramidite method of Beaucage et al., Tetra. Lett., 22:1859, 1981; and the solid support method of U.S. Patent No. 4,458,066. Introducing mutations to a polynucleotide sequence by PCR can be performed as described in, e.g., *PCR Technology: Principles and Applications for DNA Amplification*, H.A. Erlich (Ed.), Freeman Press, NY, NY, 1992; *PCR Protocols: A Guide to Methods and Applications*, Innis *et al.* (Ed.), Academic Press, San Diego, CA, 1990; Mattila et al., Nucleic Acids Res. 19:967, 1991; and Eckert *et al.,* PCR Methods and Applications 1:17, 1991.

[0080] Also provided in the invention are expression vectors and host cells for producing the functional antibodies described herein. Specific examples of lentiviral based vectors for expressing the antibodies are described in the Examples below. Various other expression vectors can also be employed to express the polynucleotides encoding the functional antibody chains or binding fragments. Both viral-based and nonviral expression vectors can

be used to produce the antibodies in a mammalian host cell. Nonviral vectors and systems include plasmids, episomal vectors, typically with an expression cassette for expressing a protein or RNA, and human artificial chromosomes (see, e.g., Harrington et al., Nat. Genet. 15:345, 1997). For example, nonviral vectors useful for expression of the antibody polynucleotides and polypeptides in mammalian (e.g., human) cells include pThioHis A, B & C, pcDNA3.1/His, pEBVHis A, B & C (Invitrogen, San Diego, CA), MPSV vectors, and numerous other vectors known in the art for expressing other proteins. Useful viral vectors include vectors based on lentiviruses or other retroviruses, adenoviruses, adenoassociated viruses, herpes viruses, vectors based on SV40, papilloma virus, HBP Epstein Barr virus, vaccinia virus vectors and Semliki Forest virus (SFV). See, Brent et al., supra; Smith, Annu. Rev. Microbiol. 49:807, 1995; and Rosenfeld et al., Cell 68:143, 1992.

[0081] The choice of expression vector depends on the intended host cells in which the vector is to be expressed. Typically, the expression vectors contain a promoter and other regulatory sequences (e.g., enhancers) that are operably linked to the polynucleotides encoding a functional antibody chain or fragment. In some embodiments, an inducible promoter is employed to prevent expression of inserted sequences except under inducing conditions. Inducible promoters include, e.g., arabinose, lacZ, metallothionein promoter or a heat shock promoter. Cultures of transformed organisms can be expanded under noninducing conditions without biasing the population for coding sequences whose expression products are better tolerated by the host cells. In addition to promoters, other regulatory elements may also be required or desired for efficient expression of a functional antibody chain or fragment. These elements typically include an ATG initiation codon and adjacent ribosome binding site or other sequences. In addition, the efficiency of expression may be enhanced by the inclusion of enhancers appropriate to the cell system in use (see, e.g., Scharf et al., Results Probl. Cell Differ. 20:125, 1994; and Bittner et al., Meth. Enzymol., 153:516, 1987). For example, the SV40 enhancer or CMV enhancer may be used to increase expression in mammalian host cells.

[0082] The expression vectors may also provide a secretion signal sequence position to form a fusion protein with polypeptides encoded by inserted functional antibody sequences. More often, the inserted functional antibody sequences are linked to a signal sequences before inclusion in the vector. Vectors to be used to receive sequences encoding the functional antibody light and heavy chain variable domains sometimes also encode constant

regions or parts thereof. Such vectors allow expression of the variable regions as fusion proteins with the constant regions thereby leading to production of intact antibodies or fragments thereof. Typically, such constant regions are human.

[0083] The host cells for harboring and expressing the functional antibody chains can be either prokaryotic or eukaryotic. In some preferred embodiments, mammalian host cells are used to express and produce the antibody polypeptides of the present invention. For example, they can be either a hybridoma cell line expressing endogenous immunoglobulin genes or a mammalian cell line harboring an exogenous expression vector (e.g., the TF-1 cells or HEK293T cells exemplified below). These include any normal mortal or normal or abnormal immortal animal or human cell. In addition to the cell lines exemplified herein, a number of other suitable host cell lines capable of secreting intact immunoglobulins are also known in the art. These include, e.g., the CHO cell lines, various Cos cell lines, HeLa cells, myeloma cell lines, transformed B-cells and hybridomas. The use of mammalian tissue cell culture to express polypeptides is discussed generally in, e.g., Winnacker, *From Genes to Clones*, VCH Publishers, N.Y., N.Y., 1987. Expression vectors for mammalian host cells can include expression control sequences, such as an origin of replication, a promoter, and an enhancer, and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. These expression vectors usually contain promoters derived from mammalian genes or from mammalian viruses. Suitable promoters may be constitutive, cell type-specific, stage-specific, and/or modulatable or regulatable. Useful promoters include, but are not limited to, EF1α and human UbC promoters exemplified herein, the metallothionein promoter, the constitutive adenovirus major late promoter, the dexamethasone-inducible MMTV promoter, the SV40 promoter, the MRP polIII promoter, the constitutive MPSV promoter, the tetracycline-inducible CMV promoter (such as the human immediate-early CMV promoter), the constitutive CMV promoter, and promoter-enhancer combinations known in the art.

[0084] Methods for introducing expression vectors containing the polynucleotide sequences of interest vary depending on the type of cellular host. For example, calcium chloride transformation is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts (see generally Sambrook et al., supra). Other methods include, e.g., electroporation, calcium phosphate treatment, liposome-mediated transformation, injection and microinjection, ballistic

methods, virosomes, immunoliposomes, polycation:nucleic acid conjugates, naked DNA, artificial virions, fusion to the herpes virus structural protein VP22 (Elliot and O'Hare, Cell 88:223, 1997), agent-enhanced uptake of DNA, and *ex vivo* transduction. For long-term, high-yield production of recombinant proteins, stable expression will often be desired. For example, cell lines which stably express the antibody chains or binding fragments can be prepared using expression vectors of the invention which contain viral origins of replication or endogenous expression elements and a selectable marker gene. Following introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth of cells which successfully express the introduced sequences in selective media. Resistant, stably transfected cells can be proliferated using tissue culture techniques appropriate for the cell type.

EXAMPLES

[0085] The following examples are provided to further illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims.

Example 1 Materials and methods for selection of functional antibodies

[0086] This Example describes some materials and methods employed in selecting functional antibodies that modulate cell death.

[0087] Intrabody Lentiviral library construction: Single-Chain Fvs (scFvs) from a naïve combinatorial antibody library in phage were excised by SfiI digestion. The excised fragments were inserted into a Lentiviral vector with compatible asymmetrical SfiI sites to form the naïve antibody library in lentivirus. The scFv region is under an EF1a promoter, followed by a FLAG tag, No constant region or secretion leader sequence is attached since we desired intracellular expression.

[0088] Recovery of scFv from the Cell Genome by PCR: Genomic DNA from the surviving HeLa cells was recovered using a Qiagen kit (69504) according to the manufacture's protocol. 100ng of the genomic DNA was used as PCR template. A pair of primers matching the regions in the front and after the scFv fragment were used to amplify

the integrated antibody fragment from the genomic DNA. The PCR product was digested by SfiI and inserted back into the intrabody Lentiviral vector for next round of selection.

[0089] Rhinovirus Amplification and Titer: The Human Rhinovirus serotype 14 (HRV-14) was initially purchased from Virapur. The virus was amplified in HeLa cells of the H1 strain. For infection, HeLa cells in a T225 flask at 90% confluence were inoculated with HRV-14 (MOI=0.5). Three days later, both cells and culture medium was harvested and sonicated. The mixture was clarified by centrifugation and filtration, aliquoted, and frozen at -80°C. The virus titer was determined by the standard TCID50 method. The final infectivity titer reached $3x10^8$/ml.

[0090] Preparation of Lentivirus: The intrabody lentiviral vectors with the pCMVD8.9 and pVSVg viral packaging vectors at ratio of 1:1:1 were co-transfected into HEK 239T cells to produce virus. 60 hours post transfection, the virus containing supernatant was collected. Cell debris were removed by filtration through a 0.22-μm membrane filter unit (Millipore). The p24 level of lentivirus prep was determined using Lenti-X p24 ELISAs (Clontech) to normalize the amount used for infection. The Infectious particle concentration was normalized by monitoring the infectivity of simultaneously prepared "Tomato" virus relative to its p24 level. The virus preparations were aliquoted and frozen at −20 °C.

[0091] Purification of the scFv-Fc Fusion protein: The DNA fragment encoding scFv was cloned into a pFUSE protein expression vector modified with the Fc portion of human IgG1 (CH2 and CH3). The pFuse-Fc-scFv constructs were transfected to FreeStyle™ 293F (R790-07) suspension cells according to the manufacturer's instructions. After3 days, the culture supernatant was collected, passed through a protein G column and the bound antibody was eluted with glycine buffer (pH 2.7). The purified protein was concentrated to 1mg/ml in PBS buffer and kept at 4°C.

[0092] MTS assay: Lentivirus Infected HeLa cells plated in a 96-well microplate at 2×10^4 cells per well in DMEM with 10% FBS. The wells were brought to a volume of 100 μL and incubated for 36 h at 30 °C with Rhinovirus. A total of 20 μL of MTS solution (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega) was added to each well. After 2 h, the absorbance at 490 nm was measured.

[0093] *In vitro* co-precipitation assay: Two micrograms of antibody was incubated with 1ug of 3C protease (Millipore 71493-3) for 30mins at room temperature, after which 50μl of protein G magnetic beads (NEB S1430S) were added to the tube. The mixture was incubated

for another 30min after which the beads were harvested using a magnet and washed with PBS 5 times. 50µl of loading buffer was added to the pellet the mixture was boiled for 5 minutes at 90°C before SDS-PAGE.

[0094] RT-PCR detection of antibody copy number change during selections: Taqman RT-PCR primers and probes were designed specific to the GGVV and YDF heavy chain sequences. Another set of Taqman primers were designed specific to the EF1α promoter region. The PCR reagent is TaqMan® Fast Advanced Master Mix (Life Technologies 4440040). 50ng DNA template was used for each PCR reaction, in a 50µl system according to the manufacturer's instruction. The copy number change of each sequence was normalized to the copy number of the EF1α promoter region which co-exists in the plasmids with each antibody sequence at 1:1 ration.

[0095] Surface Plasmon Resonance (SPR): All binding experiments were performed on a Biacore T200 (GE Healthcare) at 25°C using HBS-EP+(GE Healthcare) as running buffer. A CM4 sensor chip (GE Healthcare) was prepared by standard amine coupling (GE Healthcare) of a mouse anti-human antibody using the Human Antibody Capture Kit (GE Healthcare) following the manufacturer's guidelines. GGVV or YDF antibody were captured at approximately 400 response units followed by injection of the 3C protein at a flow rate of 30µl/min. For GGVV, a 2-fold serial dilution of 3C starting at 100nM was injected in duplicates with an association time of 3min followed by a dissociation time of 10min. For YDF, a 2-fold serial dilution of 3C starting at 2000nM was injected in duplicates with an association time of 4min followed by a dissociation time of 30min. The surface was regenerated by injection of 3 M magnesium chloride for 30s at 10µl/min. Sensorgrams were globally fitted to the 1:1 Langmuir binding model after background subtraction (blank injections and injections of 3C on a reference flow cell without any captured antibody). All data processing and analysis were performed using the Biacore T200 evaluation software (GE Healthcare).

[0096] For the double binding experiment, YDF antibody was directly amine coupled to a CM4 sensor chip at approximately 1000 response units. The sensorgram was generated by 3 subsequent injections at 30µl/min: (i) GGVV antibody for 1min (ii) 3C protein for 2min and (iiI) GGVV antibody for 1min. The GGVV antibody and 3C protein samples were at a concentration of 1000nM and each injection was followed by an approximate 3min wait period. Signal from an empty reference channel was subtracted as background.

[0097] Protease inhibition assay: A 96 well plate was filled with 100µl 1x digestion buffer/ well, 0.5 µl (1u) of the 3C protease (Millipore 71493-3) and 0.05µl of the synthetic substrate peptide (400µM DMSO stock). Different amounts of the antibodies were added to the wells, mixed well, and incubated at room temperature for 2 hours. After incubation, the wells were washed by PBST and a HRP conjugated anti-FLAG secondary antibody was added, incubated for 1 hour, washed by PBST again after which the samples were subjected to ABTS colorimetric detection. The absorption was read at 405nm on a plate reader.

[0098] Deep Sequencing: A primer matching the conserved region of the J_H chains and another primer matching the promoter region were used together to amplify all the V_H fragments. Each of the five libraries was assigned a unique barcode to facilitate the sequencing analysis and the barcoded libraries were mixed with a ratio of 1:1:10:50:100 based on the estimated enrichment rate during selection. In order to achieve a balance between read length and throughput, we used the Ion Torrent Personal Genome Sequencing Machine (PGM) with 400bp kits and a 318 chip. A single PGM run yielded a total of 7,030,852 raw reads with a median length of 489bp. Note that the 3'-triming option in base calling was turned off in initial data processing to obtain the maximum read length as all the raw reads would be filtered later with the Antibodyomics 1.0 pipeline. Antibodyomics 1.0, developed originally for the analysis of broadly neutralizing antibody (bnAb) repertoires from HIV-1-infected patients, was used here to process and analyze the sequencing data obtained from antibody libraries.

Example 2 Selecting antibodies that prevent cell death

[0099] Cell survival is, arguably, the most selectable of all possible phenotypes. We began our studies on the selection of antibodies that inhibit cell death by a study of cell survival after virus infection. We examined how unbiased combinatorial antibody libraries expressed inside cells can be used to identify molecules that, when inactivated, prevent virus induced cell death. While we focused on virus induced cell death as an exemplar, one should be able to extend this method to other systems where there is a selection for survival such as occurs when tumors evade killing by immune cells or small molecules or when cells escape senescence.

[00100] The selection system is predicated on the assumption that cell survival is a highly selectable phenotype. An iterative selection was devised where antibody genes recovered

from surviving cells were used for each new round. A naïve antibody library in lentiviruses containing approximately 10^8 different members was used to induce cytoplasmic expression of antibodies in HeLa cells prior to infecting them with Rhinovirus. When a MOI=2 was used, about 80% of 10^8 cells are infected with lentivirus (Figure 1A). A single chain scFv format linked to a flag tag was used to aid in antibody purification and minimize bias due to the improper folding of antibodies containing C_H1, C_L, and Fc domains in the reducing environment of the cytoplasm. Since not all antibodies may fold properly in the reducing environment of cytoplasmic, the size of effective library may be somewhat less than 10^8. Two days after infection with the lentiviral vector containing the antibody genes, the cells were infected with a human Rhinovirus type 14 (HRV-14) virus at a M.O.I=0.3, after which the cells were grown for 36 hours at 340 C. After 36 hours in culture, massive cell death was observed and most of the cells had detached from the flask and were in suspension. At this point, no difference was observed between cells pre-infected with the combinatorial antibody library in lentiviruses and control cells. Thus, the selection protocol was adjusted. The new selection scheme was predicated on the concept that in the previous selection some cells contained protective antibodies but they were ultimately overwhelmed by massive production of virus from unprotected cells. Thus, until the protected cells became a majority, the expressed antibodies could delay but not abolish their killing. In the adjusted protocol, cells were harvested by trypsinization at a point where 5-10% of them were still attached. Genomic DNA was extracted from these adherent cells and the integrated antibody sequences were recovered by PCR using primers specific to their flanking regions on the lentiviral vector. The recovered antibody genes were re-inserted back into lentiviral vectors that were used for the next round of selection. To distinguish subsequent rounds of selection from that of the naïve library, the "sub-libraries" obtained after each round are termed "2^{nd} library", 3^{rd} library, etc. Again, no difference in cell viability was observed in the second round of selection and only a very minor difference was observed after the 3^{rd} round of selection. However, after the 4^{th} round of selection, a dramatic selection for viability was observed for the cells infected with the antibody library. Nearly 95% of cells pre-infected with the antibody library obtained from the 4^{th} round of selection remained attached to the bottom of the flask after 36 hours following HRV-14 infection (M.O.I. =0.3), whereas, again, the control cells were virtually all killed. The difference in the cultures was obvious to visual inspection (Figure 7). The protected cells formed a confluent monolayer whereas

no attached cells were present in the cultures unprotected by antibodies. To quantitate the effect, the percentage of living cells was determined by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Figure 1B). Cell death was also confirmed by staining for To-Pro1 that is a marker of apoptosis (Figure 1C). There was a dramatic increase in the number of surviving cells after the third round and after the 4^{th} round almost all cells survived.

[00101] The recovered DNA encoding antibody fragments obtained from the protected cells after the last selection round was inserted into lentivirus plasmids that were used to transform bacteria. The plasmids containing the antibody genes were isolated from 200 individual bacterial clones after which each was incorporated into lentiviruses. HeLa cells in a 96 well plate format were infected with the now clonal lentiviruses isolated from these bacterial clones. After two days, HRV-14 was added to the wells and the cells were transferred to 34^0 C for 36 hours after which cell viability was determined using the MTS based assay. About 30% of the individual cultures were protected from cell death. Sequence analysis of the DNA from the clones showed that they contained only two different sequences, indicating that the selection was powerful (Figure 2A). These lentiviral clones were named GGVV and YDF in accord with the antibody CDRH3 sequences that they contained (Figure 2A). The two antibody heavy chains are from different germlines (IGHV3-30 for GGVV, IGHV1-69 for YDF). The two lentiviral clones were tested for their protective effect against HRV-14 induced cell death (Figures 2B and 2C). Both antibodies were very potent in that they protected almost 100% of cells from death even when cells were exposed to a high Rhinovirus MOI.

Example 3 Qualitative and quantitative analysis of the selection process

[00102] The kinetics of the selection of the protective antibodies was interesting in that a substantial effect was observed only after the 4^{th} round. To better understand how this selection proceeded, we designed Taqman Realtime PCR primers for the CDRH3 regions of both antibodies in order to quantitate their enrichment parameters after each round of selection. Another pair of primers targeting the promoter region was used for normalization. Plasmid DNA pools prepared after each round of selection were used as templates for the comparative analysis. The data showed that both of the antibody sequences enriched during the multiple rounds of selection. Eventually the abundances of GGVV and YDF antibodies

were enriched more than 60,000 and 6,000,000 fold respectively after the last round of selections relative to their abundances in the starting naïve library (Figure 3A and 3B).

[00103] To analyze this enrichment effect in action, we employed the next-generation sequencing (NGS) technology to characterize the antibody library generated in each round, creating a series of snapshots of the antibody population under selective pressure. In short, after the five selection rounds 1,136,809; 954,385; 75,797; 14,182 and 4,501 full-length V_H domains were recovered after pipeline processing and multiple quality-filtering steps. A detailed bioinformatics analysis indicated that, as the diversity of the libraries continued to decrease in selection rounds, the clonal population of GGVV and YDF increased significantly during the process. Using CDR-H3 length (13aa for GGVV and 17aa for YDF) and CDR-H3 sequence identity (≥98%) as criteria, we confirmed that GGVV and YDF represent the most prevalent lineages in library #5 (Figure 4), accounting for 27.0% and 28.5%, respectively. However, this population accounted for only 0.1% or less of the library in round #3, highlighting a rapid convergence towards the end of selection. If a herd immunity effect applies, it is likely that the selection process will reach the herd threshold (e.g. 80~90%) in round #6 and the entire cell population will be effectively protected. A focusing effect can be observed from the antibody population with 90% identity or greater, as the high-density (red) area on the 2D plot shifted towards GGVV and YDF over the last three rounds. We also carried out a lineage analysis for library #5 without using GGVV and YDF as reference. Among the top 5 lineages identified, the two largest correspond precisely to YDF and GGVV, indicating that the phenotype-based functional selection coupled with deep sequencing analysis may offer a direct and efficient approach to antibody discovery.

Example 4 Characterization of the selected antibodies

[00104] Target Identification: To understand the mechanism by which these antibodies protected cells, it was necessary to identify their target antigen. The two antibodies were expressed extra-cellularly with appended Fc fragments and purified using protein G affinity columns. The purified antibodies were coupled to agarose beads that were used to purify their target antigens from Rhinovirus infected HeLa cell lysates. Both of the antibodies reacted with small proteins that had apparent molecular weights of about 20kd as determined by SDS-PAGE analysis. To determine the identity of the target antigens, the gel bands were excised and analyzed by Mass Spectrometry. Search of the entire protein sequence database

for the peptide sequences generated from the gel bands led to the suggestion that the protein to which the antibodies bound was the human Rhinovirus B 3C protease. To confirm the interaction with the target, an antigen-binding assay using his-tagged 3C protease was carried out. Both of the antibodies selectively bind to the purified 3C protease (Figure 5). The antibodies did not bind to irrelevant His-tagged proteins (Figure 5, Lane 4,5), Additional control experiments showed that the purified 3C protease did not bind nonspecifically to control antibodies (Figure 5, Lane 3) or protein A alone (Figure 5, Lane 6).

[00105] Mechanism of Action: We tested the binding affinity of the two antibodies for the Rhinovirus 3C protease by Surface Plasmon Resonance (Figures 8A-8C). Both antibodies bound very tightly to the protease with a 1 to 1 stoichiometry. Antibodies GGVV and YDF had K_Ds of 1.5nM and 6.14nM respectively. A sequential binding analysis showed that the two antibodies bound to different epitopes on the protease.

[00106] Since the 3C protease is a critical intracellular component of the Rhinovirus replication pathway, one can imagine that its function would be perturbed by antibodies against it that are also present in the cytoplasm. To further understand the mechanism by which these antibodies inhibit the protease function, we used a peptide-cleavage assay to determine whether they interfere with its enzymatic activity (Schünemann et al., Bioorganic & Medicinal Chem. Letters 22, 5018-5024, 2012). The results are shown in Figures 9A-9B. Interestingly, although both antibodies bound tightly to the protease, only the YDF antibody inhibited the enzymatic activity of the protease.

[00107] Functional Antibodies are Rare: Since one antibody inhibited protease function in vivo by simply binding to it, we wondered whether this was general and any antibody that bound would inhibit its ability to engage in the viral maturation cascade. As mentioned in the introduction, we were now in a position to study this, because once an unbiased selection identifies a target, a much larger number of antibodies that simply bind to it can be harvested from phage selections that have an input diversity three orders of magnitude larger than that that can be used in lentivirus based selections. This enriched library can then be transferred to lentivirus such that now all the library members at least bind to the target. Thus, a phage library that was enriched by panning and now contained about 105 members all of whom bound to the 3C protease was transferred to lentivirus and the selection was repeated. In these experiments only one round of selection was needed to achieve nearly complete protection (Figure 6A). To address the diversity of the library selected on the basis of

binding, we sequenced 70 randomly picked clones, and found that at least 64 of them were different. Remarkably, only the same two antibodies were recovered, indicating that binding to the protease is a necessary but not generally sufficient parameter for inhibition of viral killing of cells. Relative to the naïve library, the previously identified antibodies were already significantly enriched by pre-selection in phage (Figure 6B, phage output). Importantly, none of the clones that were selected on the basis of prevention of cell death were present in the randomly picked 70 clones. After the first round of cell infection using the enriched gene pool in lentivirus, the selected antibodies seemed to be fully enriched (Figure 6). In total, these findings underscore the value of large antibody libraries because when one adds functional constraints to the discovery process, antibodies that fulfill the criteria may be rare and, thus, only be revealed after search through large numbers.

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[00108] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

[00109] All publications, databases, GenBank sequences, patents, and patent applications cited in this specification are herein incorporated by reference as if each was specifically and individually indicated to be incorporated by reference.

WE CLAIM:

1. A method for identifying an antibody that modulates death of a eukaryotic cell, comprising (a) expressing in a population of the eukaryotic cells a library of antibodies or antigen-binding fragments thereof to produce a heterogeneous population of antibody-expressing cells, wherein the antibodies are intrabodies or membrane-tethered antibodies; and (b) selecting a specific antibody-expressing cell with a phenotype indicating an altered state of cell death; thereby identifying the antibody expressed in the specific intrabody-expressing cell as one that modulates death of the cell.

2. The method of claim 1, wherein the library of intrabodies are intrabodies.

3. The method of claim 1, wherein the eukaryotic cell is of a mammalian cell type.

4. The method of claim 1, wherein the antibody library is expressed in the cell via a lentiviral vector or a retroviral vector.

5. The method of claim 1, wherein each cell of the population of antibody-expressing cells expresses only one different member of the library of intrabodies.

6. The method of claim 1, wherein the altered state of cell death is delayed cell death or enhanced survival.

7. The method of claim 6, wherein the identified antibody inhibits or prevents death of the cell.

8. The method of claim 6, wherein the cell is a neuron.

9. The method of claim 6, wherein the heterogeneous population of antibody-expressing cells are contacted with a cytotoxic or pathogenic agent prior to the selection.

10. The method of claim 1, wherein the altered state of cell death is enhanced cell death.

11. The method of claim 10, wherein the identified antibody promotes death of the cell.

12. The method of claim 10, wherein the cell is a tumor cell.

13. The method of claim 1, further comprising determining amino acid sequences of heavy chain and light chain variable regions of the identified antibody.

14. An antibody or antigen-binding fragment thereof which binds to human Rhinovirus B 3C protease with the same binding specificity as that of a reference antibody, wherein the reference antibody comprises (1) heavy chain CDR1, CDR2 and CDR3 sequences respectively shown in SEQ ID NO:9, SEQ ID NO:10 and SEQ ID NO:11; and light chain CDR1, CDR2 and CDR3 sequences respectively shown in SEQ ID NO:12, SEQ ID NO:13 and SEQ ID NO:14; or (2) heavy chain CDR1, CDR2 and CDR3 sequences respectively shown in SEQ ID NO:15, SEQ ID NO:16 and SEQ ID NO:17; and light chain CDR1, CDR2 and CDR3 sequences respectively shown in SEQ ID NO:18, SEQ ID NO:19 and SEQ ID NO:20.

15. The antibody or antigen-binding fragment thereof of claim 14, comprising (1) heavy chain CDR1, CDR2 and CDR3 sequences that are substantially identical to SEQ ID NO:9, SEQ ID NO:10 and SEQ ID NO:11, respectively; and light chain CDR1, CDR2 and CDR3 sequences that are substantially identical to SEQ ID NO:12, SEQ ID NO:13 and SEQ ID NO:14, respectively; or (2) heavy chain CDR1, CDR2 and CDR3 sequences that are substantially identical to SEQ ID NO:15, SEQ ID NO:16 and SEQ ID NO:17, respectively; and light chain CDR1, CDR2 and CDR3 sequences that are substantially identical to SEQ ID NO:18, SEQ ID NO:19 and SEQ ID NO:20, respectively.

16. The antibody or antigen-binding fragment of claim 14, comprising (1) heavy chain CDR1, CDR2 and CDR3 sequences that are respectively identical to SEQ ID NO:9, SEQ ID NO:10 and SEQ ID NO:11; and light chain CDR1, CDR2 and CDR3 sequences that are respectively identical to SEQ ID NO:12, SEQ ID NO:13 and SEQ ID NO:14; or (2) heavy chain CDR1, CDR2 and CDR3 sequences that are respectively identical to SEQ ID NO:15, SEQ ID NO:16 and SEQ ID NO:17; and light chain CDR1, CDR2 and

CDR3 sequences that are respectively identical to SEQ ID NO:18, SEQ ID NO:19 and SEQ ID NO:20.

17. The antibody or antigen-binding fragment of claim 14, comprising (1) heavy chain and light chain variable region sequences show in SEQ ID NO:5 and SEQ ID NO:6, respectively; or (2) heavy chain and light chain variable region sequences show in SEQ ID NO:7 and SEQ ID NO:8, respectively.

18. The antibody or antigen-binding fragment of claim 14, which is a scFv antibody fragment.

19. The antibody or antigen-binding fragment of claim 18, comprising a sequence shown in SEQ ID NO:3 or SEQ ID NO:4.

20. A polynucleotide encoding the variable region of the heavy chain or light chain of the antibody of claim 14.

21. A vector harboring the polynucleotide of claim 20.

22. A method for preventing cell death caused by a Rhinoviral infection or treating a common cold in a subject, comprising administering to the subject a pharmaceutical composition that comprises a therapeutically effective amount of an antibody of claim 13.

Figure 1

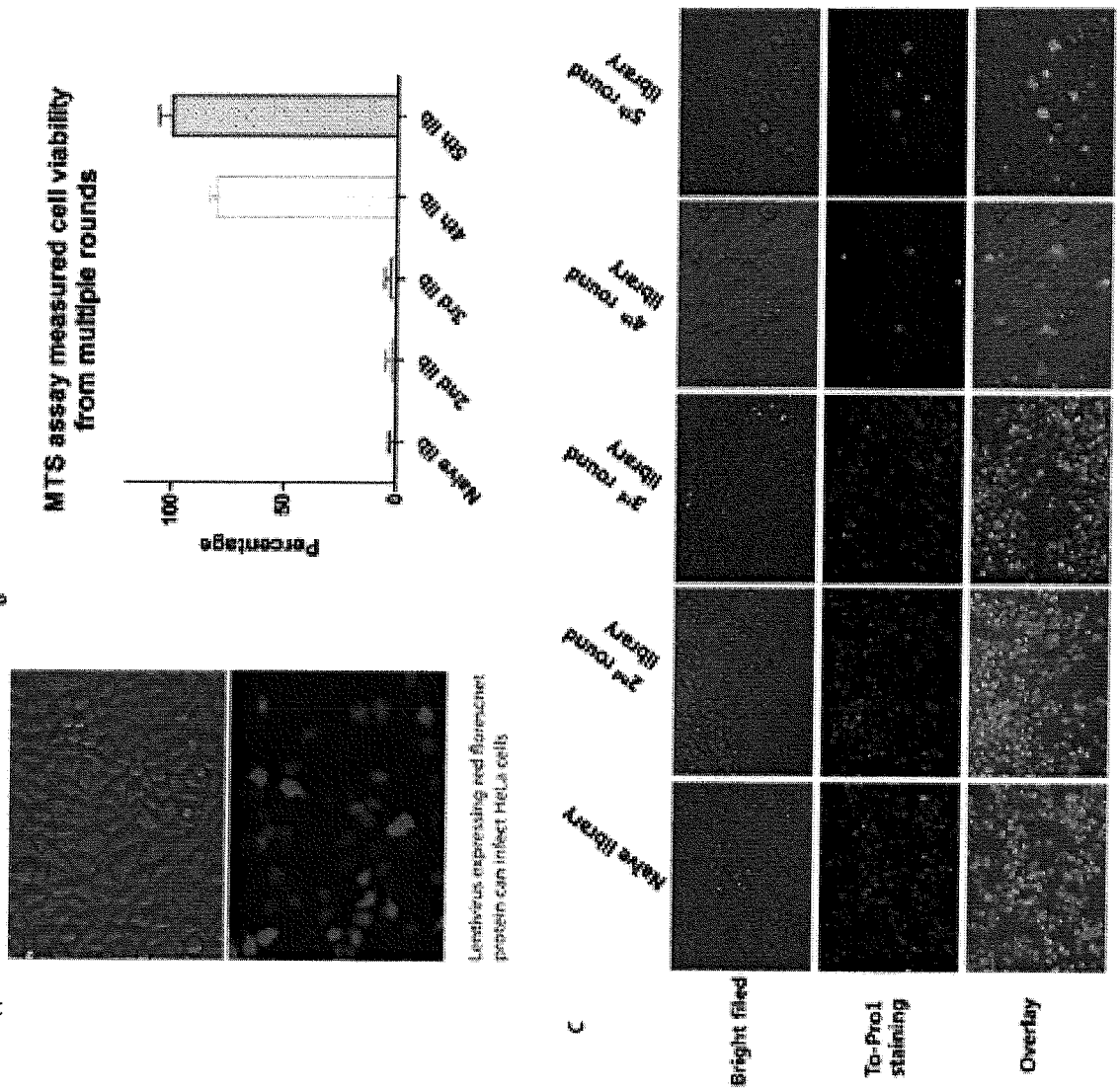

A
Lentivirus expressing red fluorescnet protein can infect HeLa cells
B
MTS assay measured cell viability from multiple rounds
Percentage
100
50
0
Naïve lib
2nd lib
3rd lib
4th lib
5th lib
C
Naïve library
2nd round library
3rd round library
4th round library
5th round library
Bright filed
To-Pro1 staining
Overlay

Figure 2

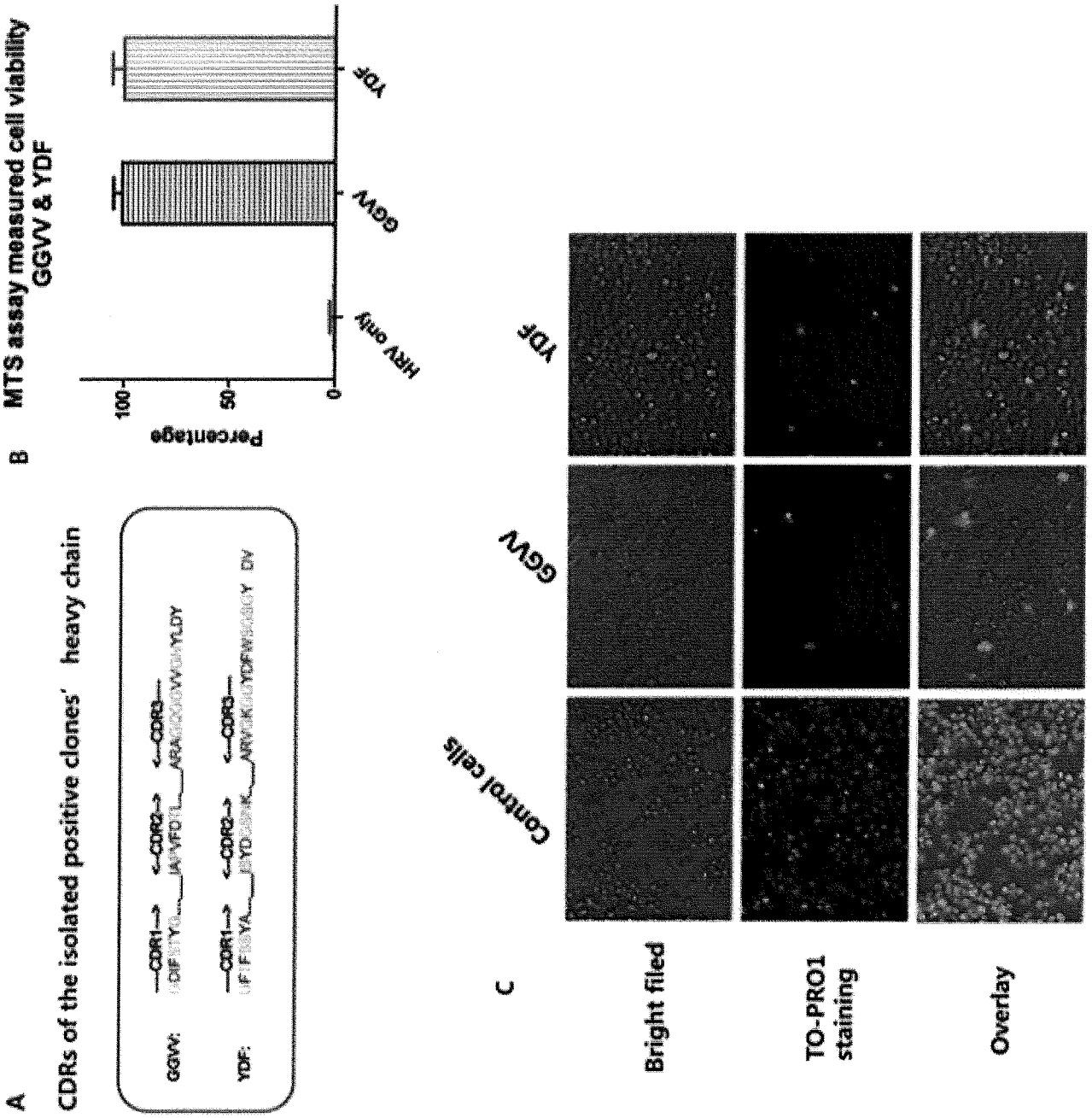

A
CDRs of the isolated positive clones' heavy chain
GGVV:
YDF:
CDR1
CDR2
CDR3
B
MTS assay measured cell viability
GGVV & YDF
Percentage
100
50
0
HRV only
GGVV
YDF
C
Control cells
GGVV
YDF
Bright filed
TO-PRO1 staining
Overlay

Figure 3

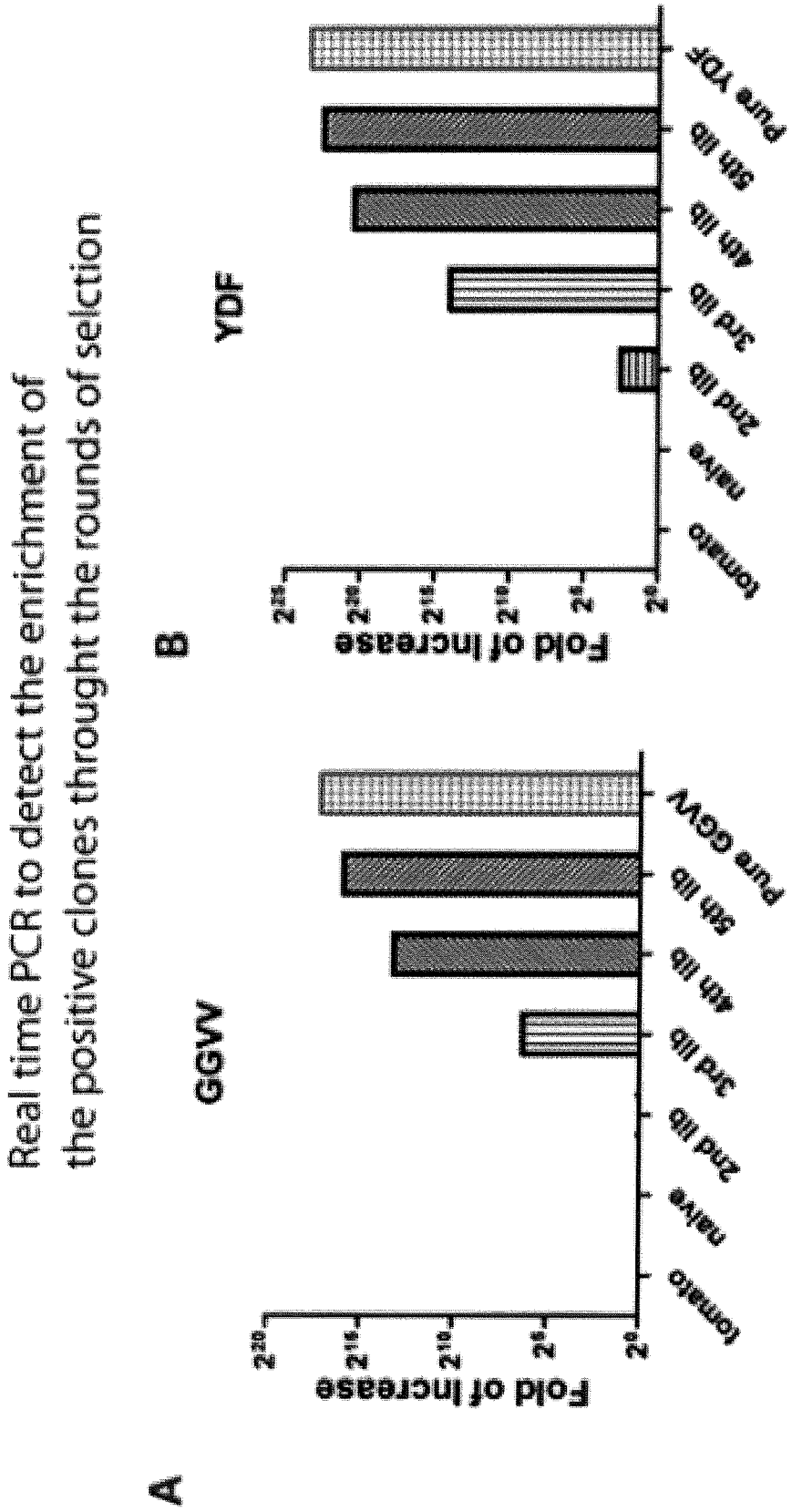

Real time PCR to detect the enrichment of the positive clones throught the rounds of selction
A
GGVV
Fold of Increase
tomato
naive
2nd lib
3rd lib
4th lib
5th lib
pure GGVV
B
YDF
Fold of Increase
tomato
naive
2nd lib
3rd lib
4th lib
5th lib
pure YDF

Figure 4

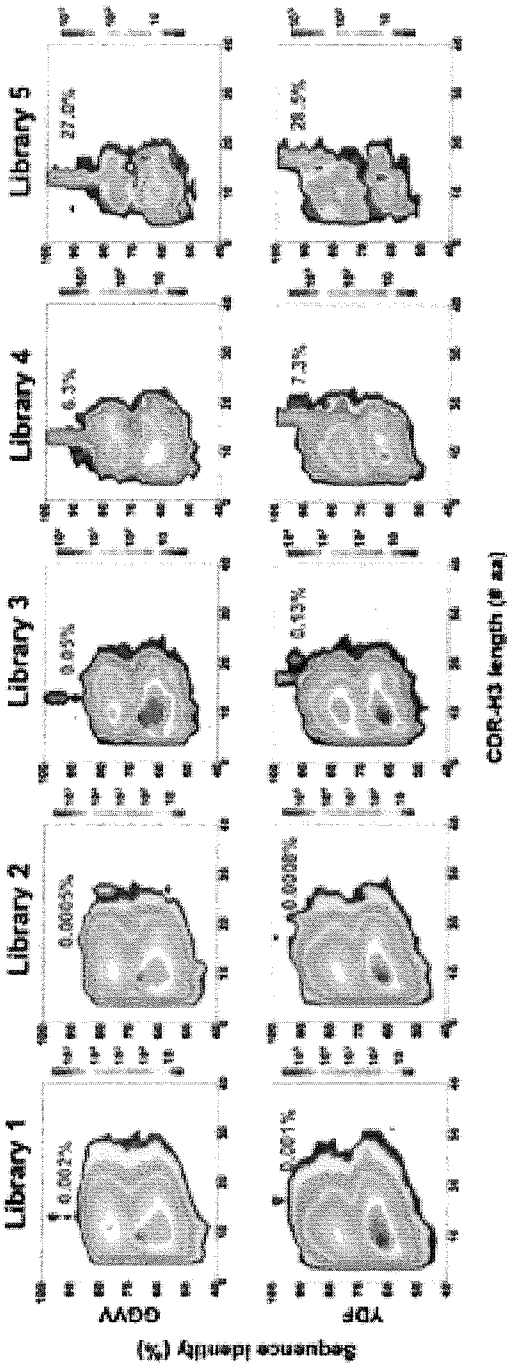

Library 5
Library 4
Library 3
Library 2
Library 1
GGVV
YDF
Sequence identity (%)
CDR-H3 length (# aa)

Figure 5

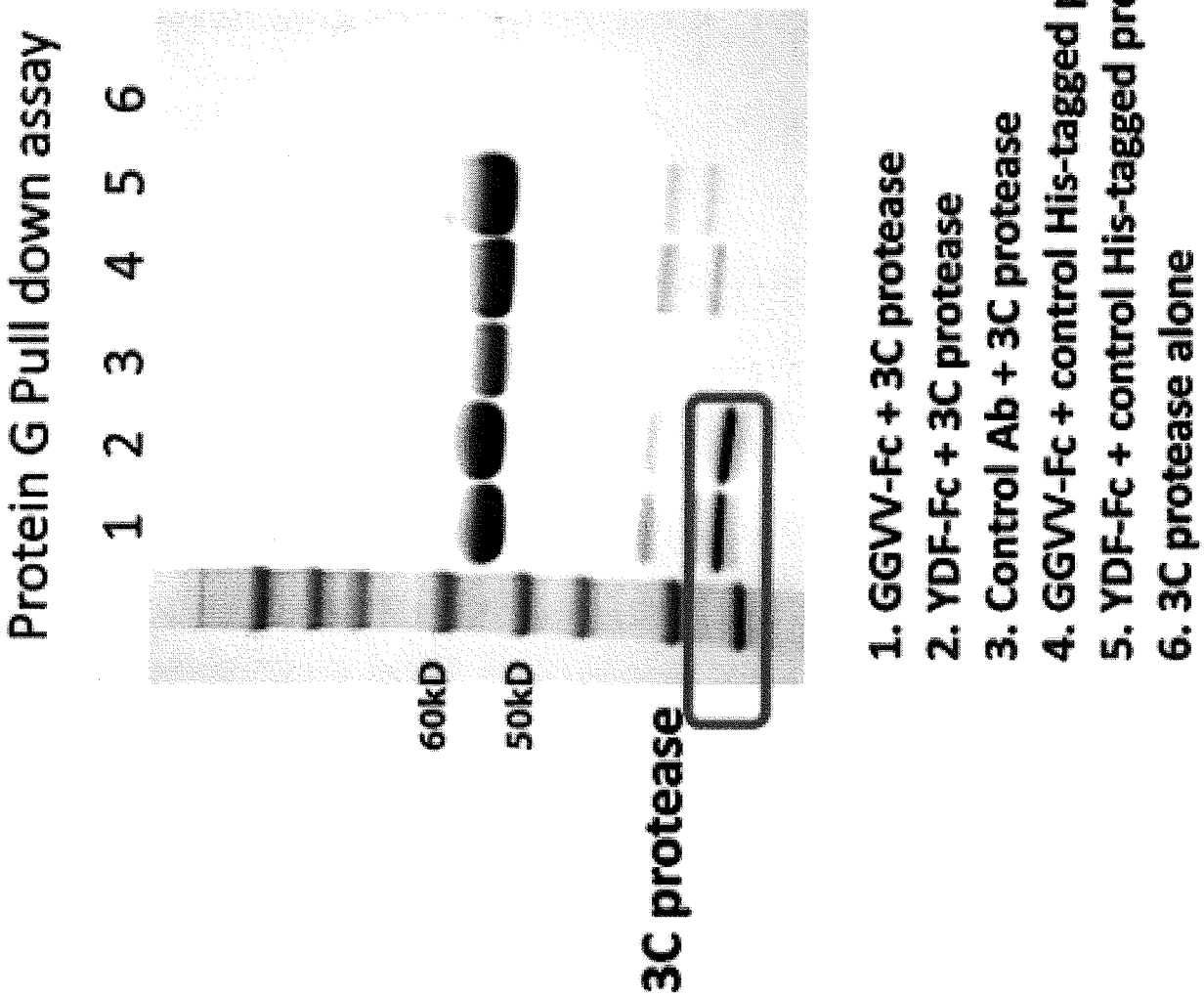

Protein G Pull down assay
1 2 3 4 5 6
60kD
50kD
3C protease
1. GGVV-Fc + 3C protease
2. YDF-Fc + 3C protease
3. Control Ab + 3C protease
4. GGVV-Fc + control His-tagged protein
5. YDF-Fc + control His-tagged protein
6. 3C protease alone

Figure 6

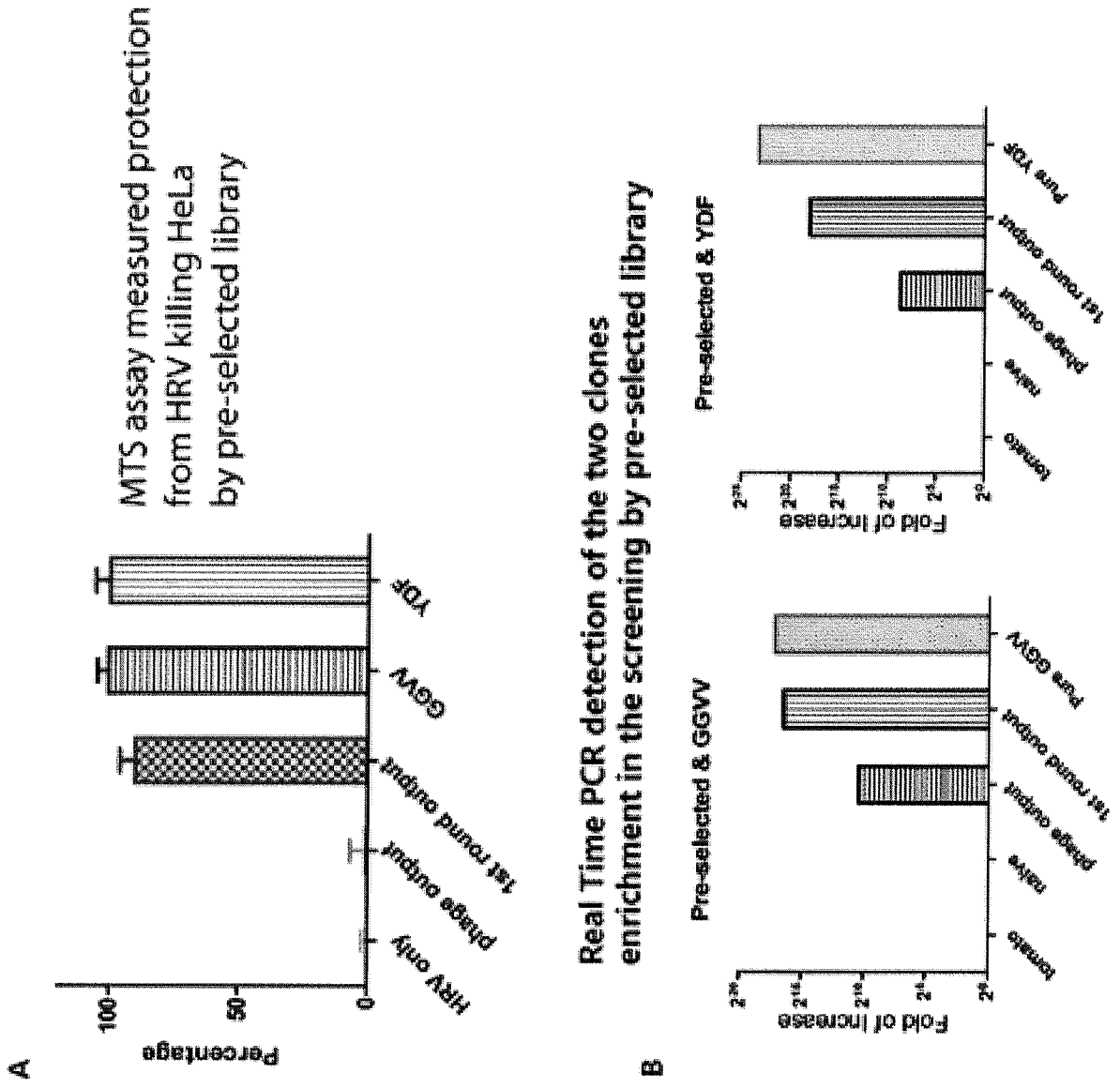
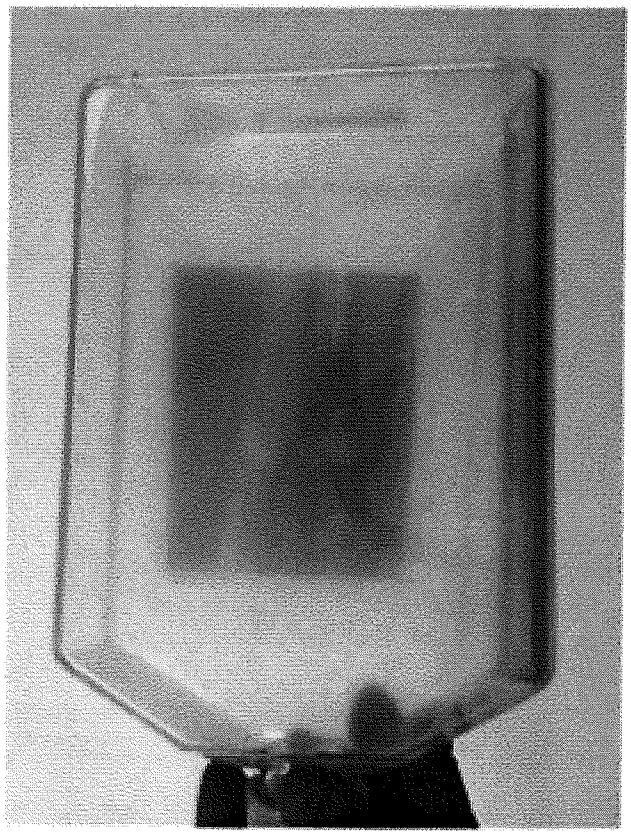

A
MTS assay measured protection
from HRV killing HeLa
by pre-selected library
Percentage
100
50
0
HRV only
phage output
1st round output
GGVV
YDF
B
Real Time PCR detection of the two clones
enrichment in the screening by pre-selected library
Pre-selected & GGVV
Pre-selected & YDF
Fold of Increase
Isotype
naive
phage output
1st round output
Pure GGVV
Pure YDF

Figure 7



Culture infected by HRV and control Lentivirus
Flask is transparent because no cells are attached.



Culture infected by HRV and 5th library Lentivirus
Flask is only semi-transparent because a monolayer of cells is present

Figure 8

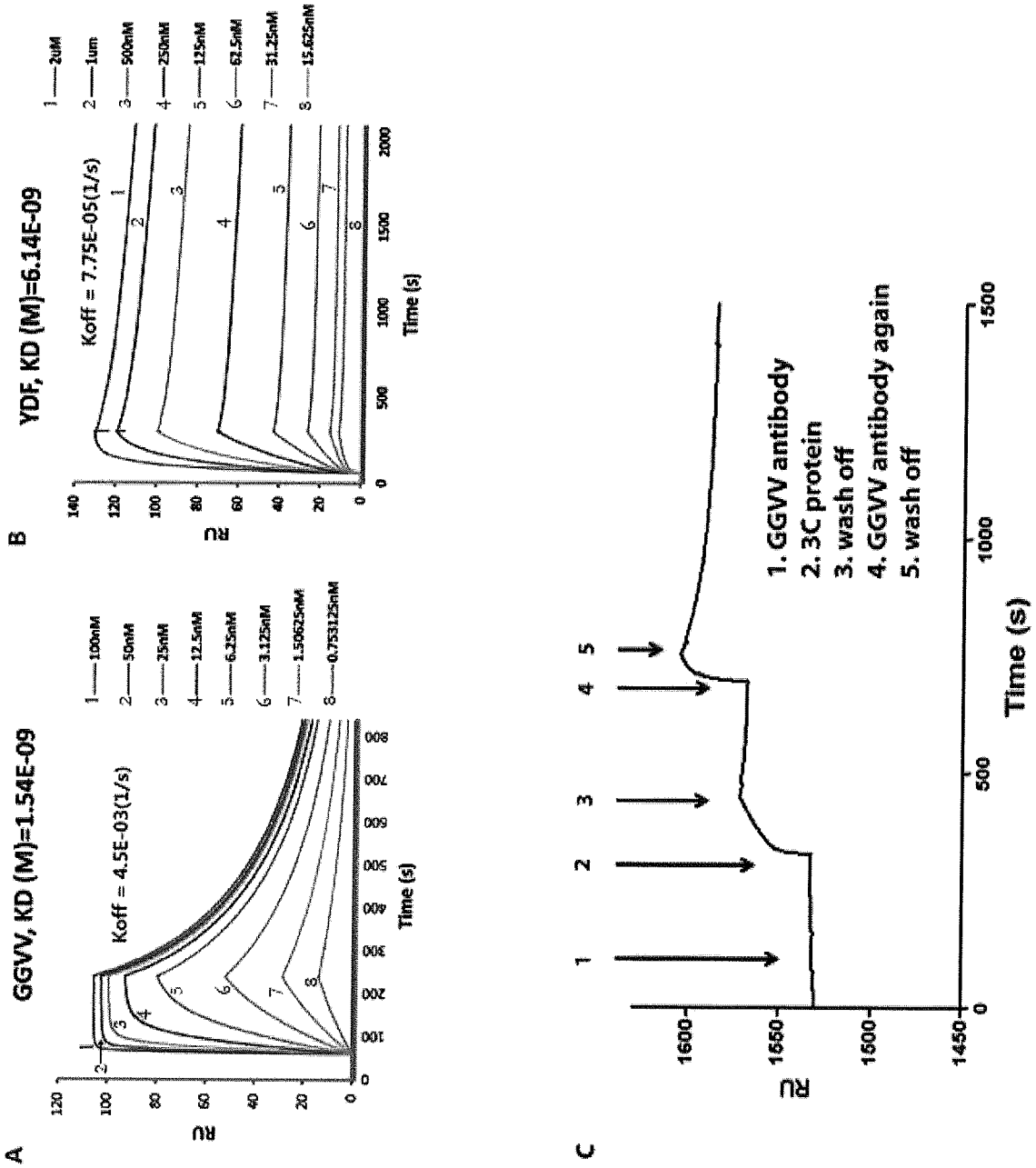
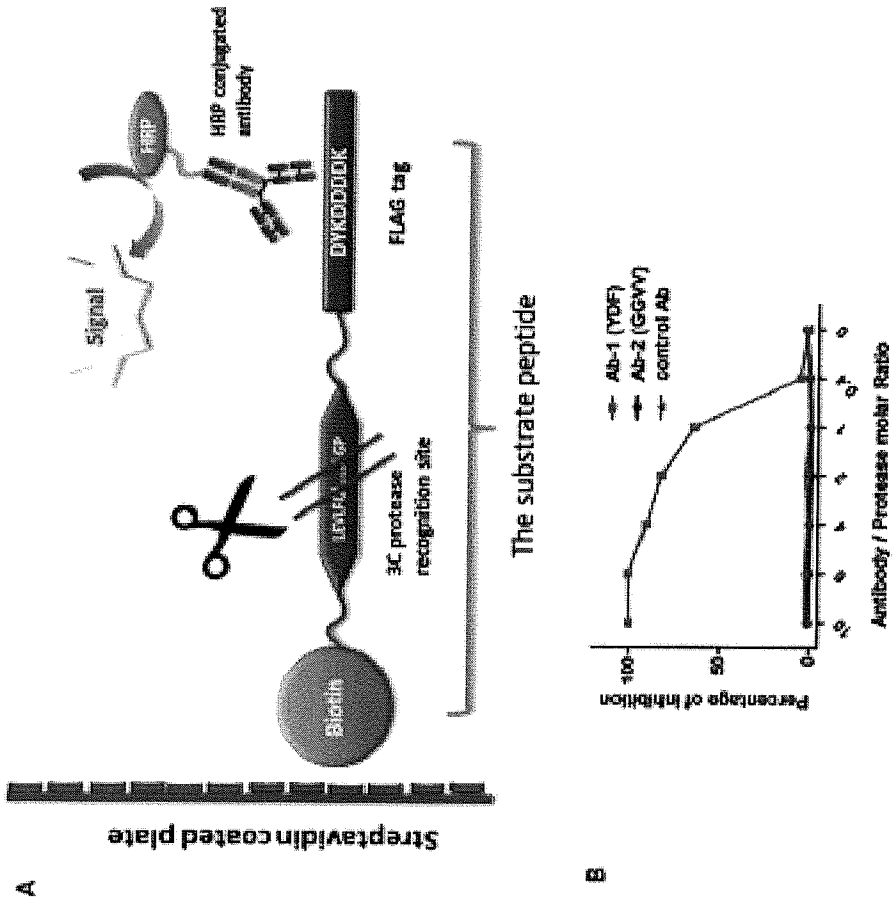

A
GGVV, KD (M)=1.54E-09
Koff = 4.5E-03(1/s)
1—100nM
2—50nM
3—25nM
4—12.5nM
5—6.25nM
6—3.125nM
7—1.50625nM
8—0.753125nM
RU
Time (s)
B
YDF, KD (M)=6.14E-09
Koff = 7.75E-05(1/s)
1—2uM
2—1um
3—500nM
4—250nM
5—125nM
6—62.5nM
7—31.25nM
8—15.625nM
RU
Time (s)
C
1. GGVV antibody
2. 3C protein
3. wash off
4. GGVV antibody again
5. wash off
RU
Time (s)

Figure 9


A
Streptavidin coated plate
Biotin
3C protease recognition site
FLAG tag
HRP conjugated antibody
Signal
The substrate peptide
B
Ab-1 (YDF)
Ab-2 (GGVV)
control Ab
Percentage of inhibition
100
50
0
Antibody / Protease molar Ratio