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Article

Prevention of Cell Death by Antibodies Selected from Intracellular Combinatorial Libraries

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SUMMARY

One of the most important phenotypes in biology is cell death. One way to probe the mechanism(s) of cell death is to select molecules that prevent it and learn how this was accomplished. Here, intracellular combinatorial antibody libraries were used to select antibodies that protected cells from killing by rhinovirus infection. These rare antibodies functioned by inhibiting the virus-encoded protease that is necessary for viral maturation. Snapshots of the selection process after each round could be obtained by deep sequencing the ever-enriching populations. This detailed analysis of the enrichment process allowed an interesting look at a "test tube" selection process that pitted two replicating systems against each other. Thus, initially a minority of cells containing protective antibodies must compete against a majority of unprotected cells that continue to produce large amounts of virus.

INTRODUCTION

Recently we developed a method in which large antibody libraries are rendered infectious for eukaryotic cells, thereby directly linking the antibody genotype to the phenotype of individual cells (Zhang et al., 2013; Yea et al., 2013). The power of the method derives from the fact that the potential agonists are selected from a large naive or preselected diversity system that contains ~10^8 unique members. In the best case, when autocrine screening systems are used, one can analyze about two million phenotypic events per hour (Sklar et al., 2007). Using these methods, a variety of agonist antibodies have been discovered that regulate cell fates, including those that are phenocopies of important cytokines as well as those that regulate the lineage specification and transdifferentiation of stem cells (Xie et al., 2013; Zhang et al., 2013). The method only seems to be limited by the availability of selection systems.

One of the most important cellular fates yet to be explored by this method is that of survival from death. 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. One can imagine three selection formats for such studies. In the first, one uses prior knowledge to hypothesize which proteins, if perturbed, would inhibit the pathogenic effects of viral infection. In this case, an intracellular library that was enriched by phage panning or binding to the target protein would be used. In this format, one simply asks whether the selection of the target was correct and if antibodies expressed inside cells can disable the target. In the second scenario, one does not presume any prior knowledge of the target (Yea et al., 2013). Unbiased libraries are used and the selection becomes a forward proteomics discovery tool for finding molecules that are critical to pathogenic pathways. Finally, when the unbiased format is used, one can follow the discovery rounds by subsequent selection rounds that enrich the libraries against the newly identified protein to validate the discovery and find more antibodies that interact with the target. Here, we demonstrate these principles by showing 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 being killed by immune cells or small molecules or when cells escape senescence.

Selection Scheme

The system is predicated on the assumption that cell survival is a highly selectable phenotype (Figure 1). An iterative selection was devised in which antibody genes recovered from surviving cells were used for each new round. A naive antibody library in lentiviruses containing ~10^8 different members was used to induce cytoplasmic expression of antibodies in HeLa cells prior to infecting them with rhinovirus. When a multiplicity of infection (MOI) of 2 was used, ~80% of 10^8 cells were infected with lentivirus (Figure 2A). 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 (Chames et al., 2009; Stocks, 2006). Because not all antibodies may fold properly in the reducing environment of the cytoplasm, the size of the effective library may be somewhat less than 10^8 (Shaki-Loewenstein et al., 2005). 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 MOI = 0.3, after which the cells were grown for 36 hr at 34°C. After 36 hr 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

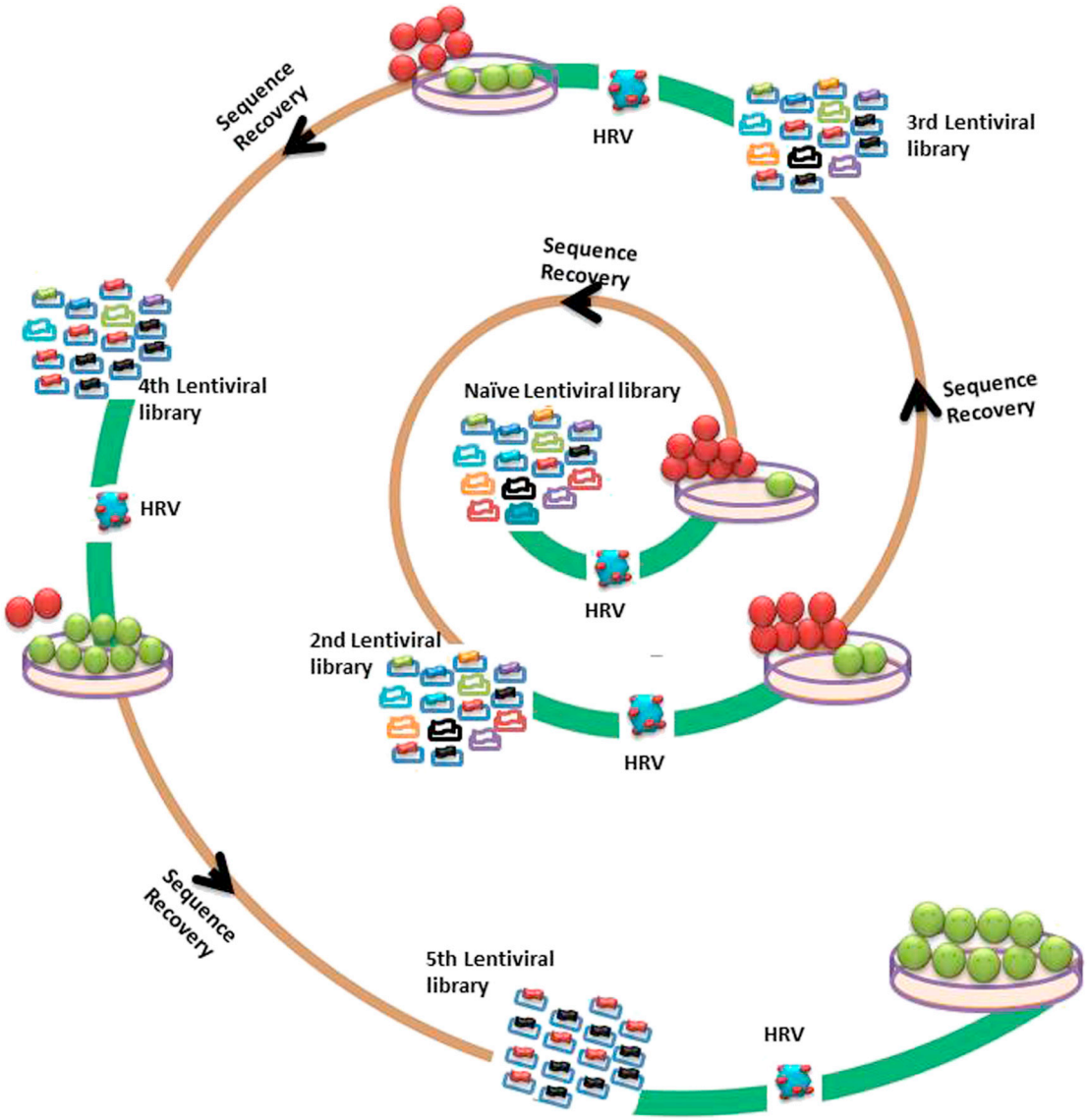

Figure 1. Scheme of the Phenotype Selection-Based Information Enrichment

The selection starts with a naive intrabody lentiviral library. HeLa cells were sequentially infected with the antibody library followed by infection with human rhinovirus. Cells expressing functional antibodies are protected from death while other cells are killed. The integrated antibody fragments (the information) from the surviving cells were recovered and converted into a secondary lentiviral library that is the starting point for the next round of selection. The selection was carried out for five rounds. The number of surviving cells (green circles) relative to killed cells (red circle) increases in each round. The percentage of functional antibody sequences (red and black patches) also enrich after each round of selection.

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 naive library, the "sublibraries" obtained after each round are termed second library, third 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 third round of selection. However, after the fourth 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 fourth round of selection remained attached to the

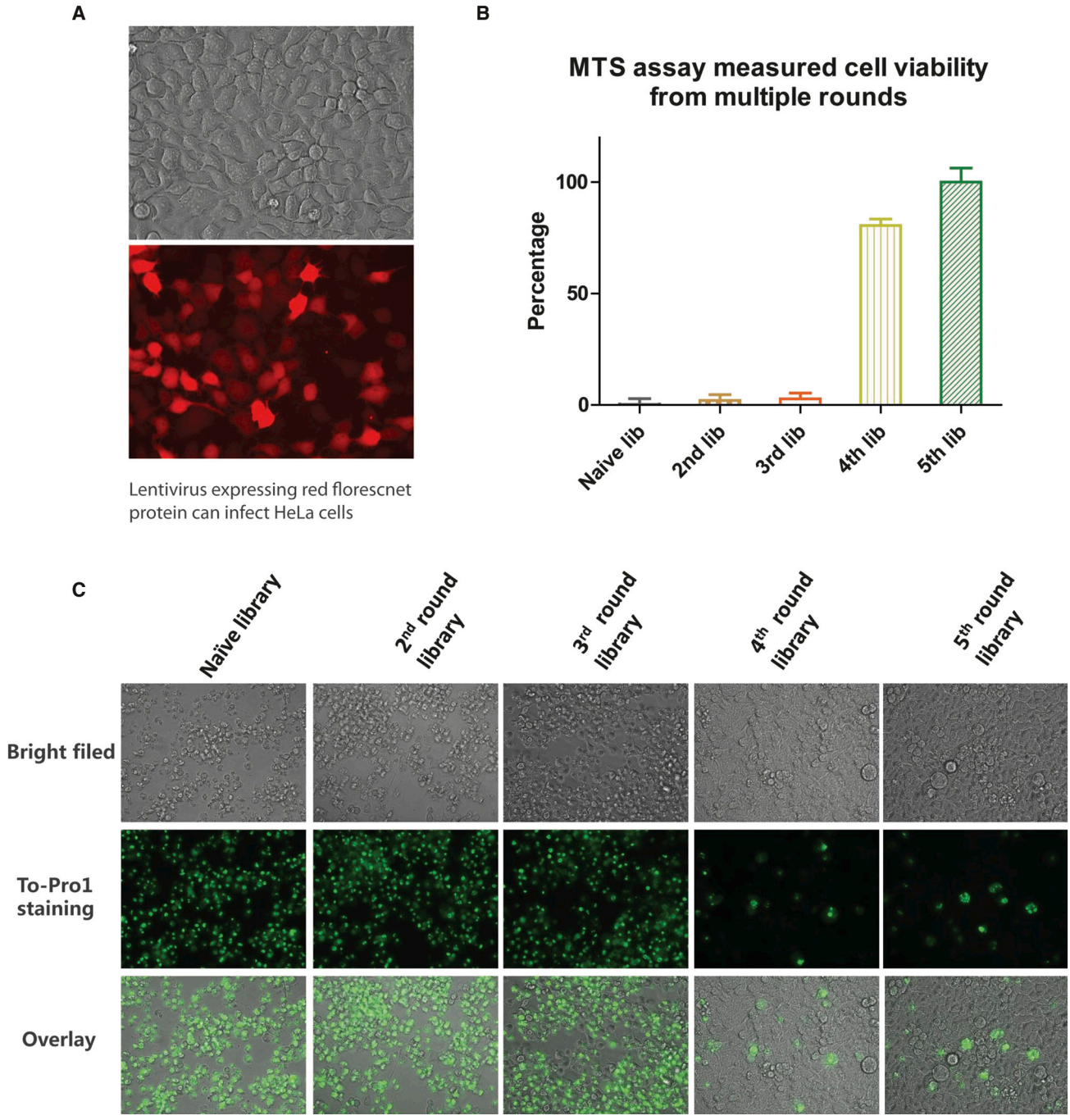

Figure 2. Protection from HRV HeLa Cell Killing by Lentiviral Antibody Libraries Is Selectable

(A) Lentivirus carrying a monomeric version of the td-Tomato fragment was used to infect HeLa cells to confirm the capability of lentivirus to infect these cells. When adjusted to MOI = 2, ~80% of cells show red florescence.

(B) An MTS assay was used to measure the ability of intrabody libraries to protect cells from killing by HRV. Nearly all the cells die in the naive library group. Survival is not significantly improved after the second and third rounds of selection. However, the survival rate markedly increases in the fourth round and by the fifth round, almost 100% of cells survive HRV infection. The error bars represent SD.

(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 488 nm, the dead cells show bright green dots in their center. The percentage of dead cells is shown in the bottom overlay panel.

bottom of the flask after 36 hr following HRV-14 infection (MOI = 0.3), whereas, again, the control cells were virtually all killed. The difference in the cultures was obvious on visual inspection (Figure S1 available online). The protected cells formed a confluent monolayer, whereas no attached cells were present in the cultures unprotected by antibodies (Figure S1). To quantitate the

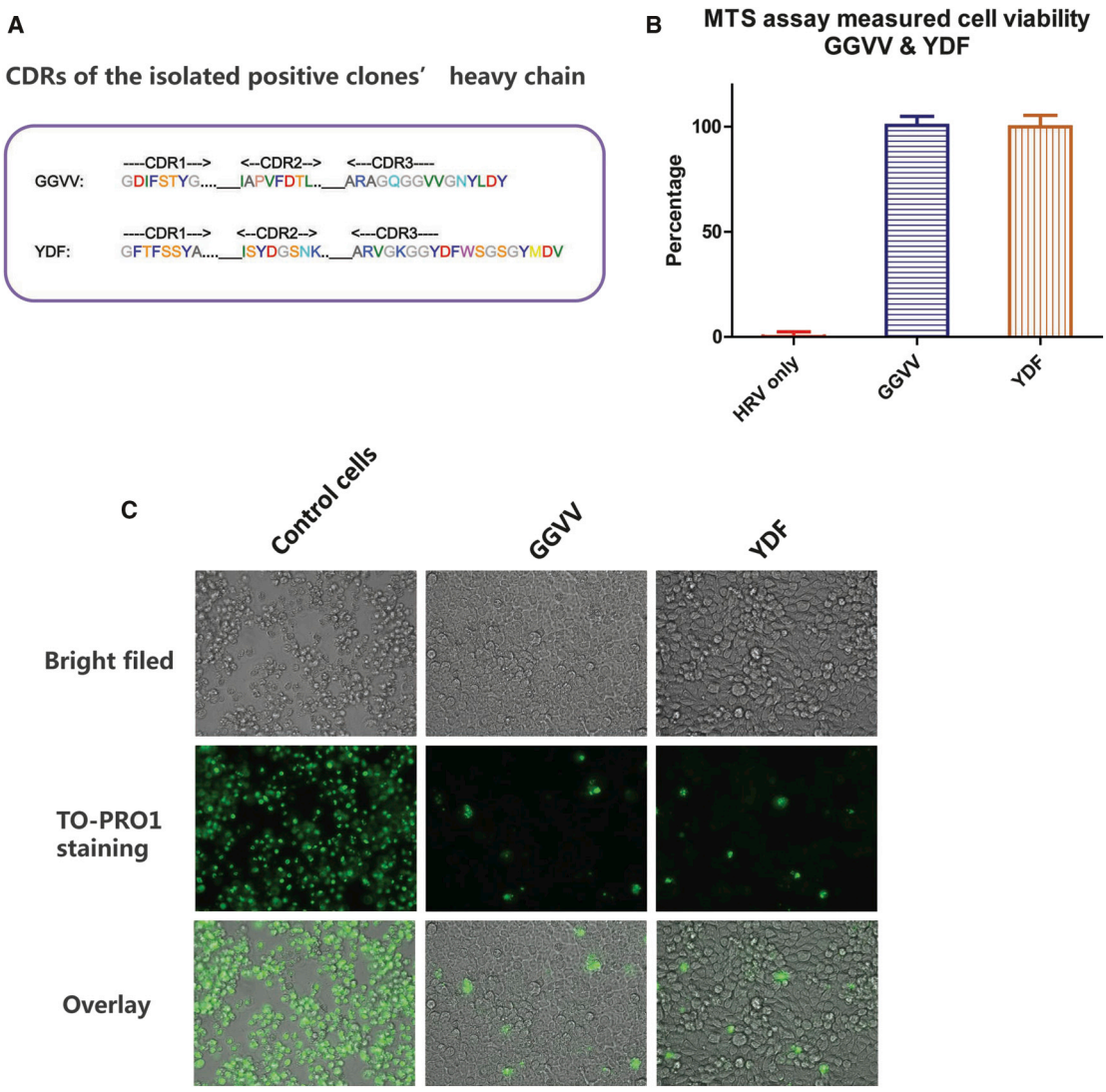

Figure 3. Analysis of the Isolated Functional Clones

(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) An 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 SD.

(C) To-Pro-1 staining shows HRV-induced apoptosis was minimal when the cells are protected by the two lentiviral clones encoding protective antibodies.

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 2B). Cell death was also confirmed by staining for To-Pro1, which is a marker of apoptosis (Figure 2C). There was a dramatic increase in the number of surviving cells after the third round and, after the fourth round, almost all cells survived.

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 2 days, HRV-14 was added to the wells and the cells were transferred to 34°C for 36 hr 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 3A). These lentiviral clones were named GGVV and YDF in accord with the antibody CDRH3 sequences that they contained (Figure 3A). 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 3B and 3C). Both antibodies were very potent in that they protected almost 100%

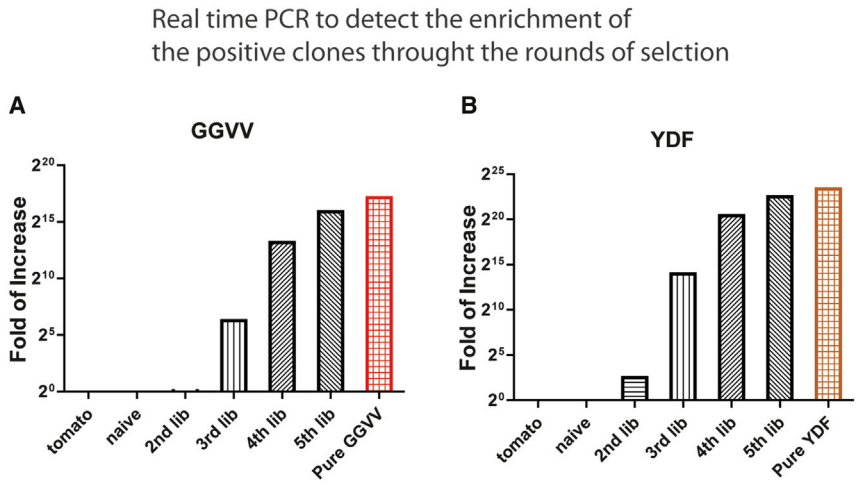

Figure 4. Enrichment of Functional Clones Studied by RT-PCR during the Rounds of Selection

(A and B) 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.

of cells from death even when cells were exposed to a high rhinovirus MOI.

Qualitative and Quantitative Analysis of the Selection Process

The kinetics of the selection of the protective antibodies was interesting in that a substantial effect was observed only after the fourth round. To better understand how this selection proceeded, we designed Taqman Realtime PCR primers for the CDRH3 regions of both antibodies 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 were 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 naive library (Figures 4A and 4B).

To analyze this enrichment effect in action, we used 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 ($\geq$98%) as criteria, we confirmed that GGVV and YDF represent the most prevalent lineages in library 5 (Figure 5), 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 toward 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% to ~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 two-dimensional (2D) plot shifted toward 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 five 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.

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 extracellularly 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 ~20 kd as determined by SDS-PAGE analysis (Figure S2A). 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 (Figure S2B). 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 6). The antibodies did not bind to irrelevant His-tagged proteins (Figure 6, lanes 4 and 5), Additional control experiments showed that the purified 3C protease did not bind nonspecifically to control antibodies (Figure 6, lane 3) or protein A alone (Figure 6, lane 6).

Mechanism of Action

We tested the binding affinity of the two antibodies for the rhinovirus 3C protease by surface plasmon resonance (Figure S3). Both antibodies bound very tightly to the protease with a 1-to-1 stoichiometry. Antibodies GGVV and YDF had K_Ds of 1.5 nM and 6.14 nM, respectively. A sequential binding analysis showed that the two antibodies bound to different epitopes on the protease.

Because 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

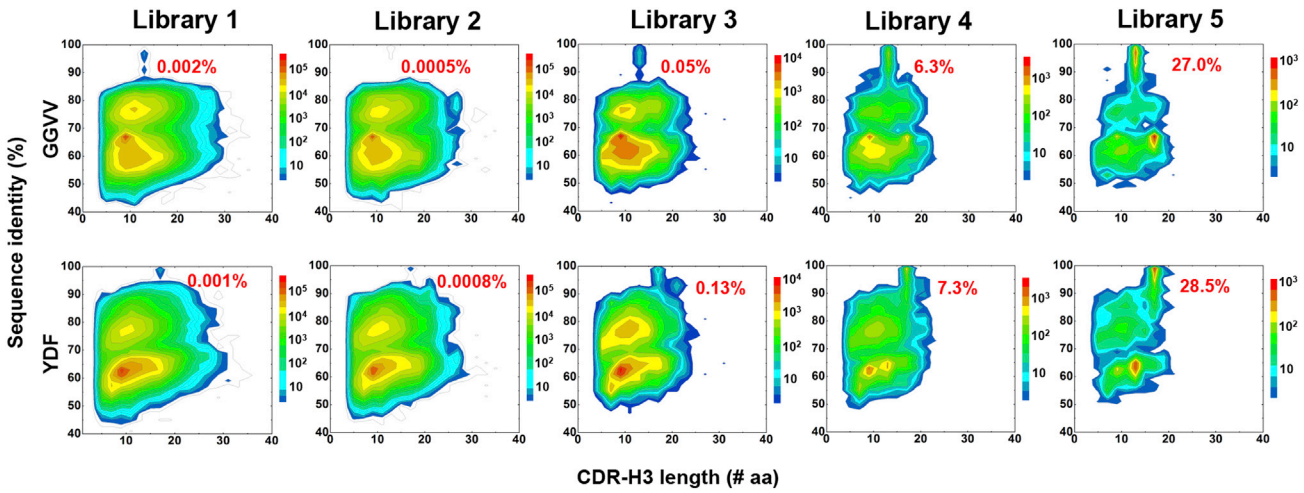

Figure 5. Snapshots of Functional Enrichment Using Deep Sequencing

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). The density of antibody population is color-coded on the 2D contour plot, with red denoting the highest density and blue the lowest. Given a library, the sequences above 90% identical to the target antibody are considered to be functionally related and are used to calculate the percent enrichment, which is labeled in red on the 2D plot.

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., 2012; Figure S4). Interestingly, although both antibodies bound tightly to the protease, only the YDF antibody inhibited the enzymatic activity of the protease.

Functional Antibodies Are Rare

Because 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 so 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 10^5 members, all of which 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 7A). To address the diversity of the library selected based on 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 naive library, the previously identified antibodies were already significantly enriched by preselection in phage (Figure 7B, phage output). Importantly, none of the clones that were selected based on 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 7). 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.

DISCUSSION

Although intracellular combinatorial libraries have been used to select antibodies that control cell differentiation, the studies reported here are very different in that the "phenotype" studied was prevention of cell death. 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 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 so that the output virus titer from unprotected cells does not reach a level that overwhelms the protective effects of the antibodies. Essentially, this is the in vitro analog of herd immunity or prey-predator models (Abrams, 2000; John and Samuel, 2000; Rashid et al., 2012) 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 systems against each other and should not be seen in other systems designed to inhibit cell death such as senescence.

While one might argue that the discovery of antibodies that inhibit the virus-encoded protease could have been anticipated from what we know about the molecular basis of rhinovirus

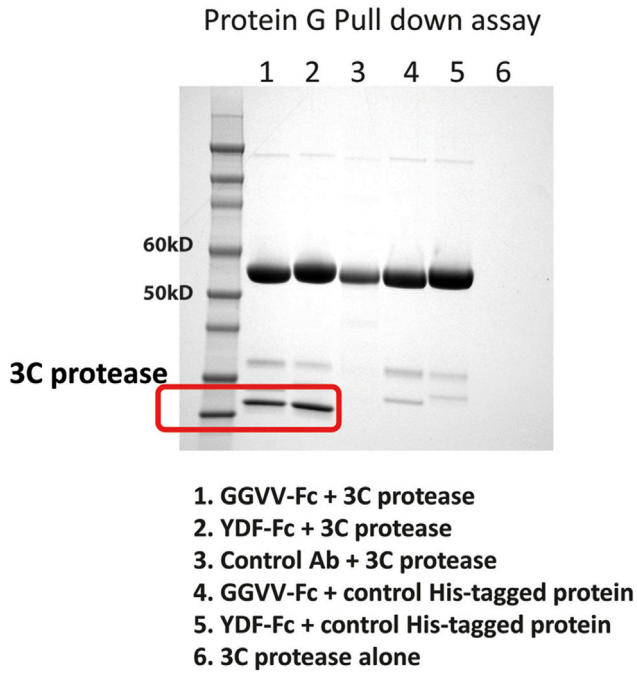

Figure 6. Identification of the Target Antigen

The nature of the target antigen was determined by a mass spectrometry analysis using samples isolated by antibody affinity columns (Figures 2A and 2B). This analysis showed that the target for both antibodies was the viral-encoded 3C protease, which 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 (lanes 1/2). An irrelevant antibody was used as a control (lane 3). An irrelevant protein with a His tag was used to control for nonspecific binding to the His tag (lanes 4/5). The 3C protease alone was incubated with protein G to control for nonspecific interactions (lane 6).

morphogenesis, such presumptions would have been far from certain. 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. Furthermore, because 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 preselected 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.

The nature of the selected antiprotease 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 (Jacobs et al., 2013; Suomalainen and Greber, 2013; Tapparel et al., 2013). Thus, simple steric factors associated with binding of some rare antibodies may prevent the integration of the protease into these highly organized replication complexes.

There is increasing appreciation of the role of deep sequencing in the discovery of functional antibodies in combinatorial libraries. The diversity of antibody libraries is usually superior to most of chemical compound libraries and they are extraordinarily information-rich. It would be ideal if one can monitor the change of every sequence across the selection process. In recent years, NGS technologies have proved to be a powerful tool in the analysis of phage-derived antibody libraries and natural antibody repertoires obtained from animal models and human donors. Thus, our previous work introduced a bioinformatics-guided method to identify the binding antibodies obtained from phage panning (Zhang et al., 2011). The Sidhu group also reported "coevolution" of the PDZ domain and its phage peptide ligands aided by 454 pyrosequencing (Ernst et al., 2010). A bioinformatics analysis revealed that the two functional clones selected here were enriched rapidly through the selection process, providing quantitative evidence that the functional selection was highly effective. Furthermore, the correct prediction of functional clones from deep sequencing of an end-point library suggests a new paradigm for antibody discovery. Empowered by deep sequencing, this approach is likely to be more cost-effective than the conventional, large-scale test of individual clones. One question is whether this paradigm can be applied to those scenarios where the phenotype has a lesser effect and the functional clones could still be minor even after several rounds of enrichment. The present study suggests that a lineage analysis of the antibody library, as demonstrated for library 5, will predict a small list of clones for functional validation. Such bioinformatics-aided profiling of antibody populations (Wu et al., 2011; Zhou et al., 2013; Zhu et al., 2012, 2013a, 2013b) will significantly reduce the pool of antibody candidates to be tested, especially when the phenotype is less dramatic even in the final rounds. The backward tracing of potentially "functional clones" in early selection rounds (Figure 5) will establish the enrichment pattern that can further facilitate antibody discovery before wet-bench validation.

Ultimately, one would like to perform experiments that are the inverse of those reported here to select antibodies that selectively kill cancer cells or make them more susceptible to natural killing mechanisms. It could be argued that selection for survival is unrelated to killing because survival is such a selectable phenotype and it is difficult to recover the antibody genes from dead cells. However, if the design of the selection is changed

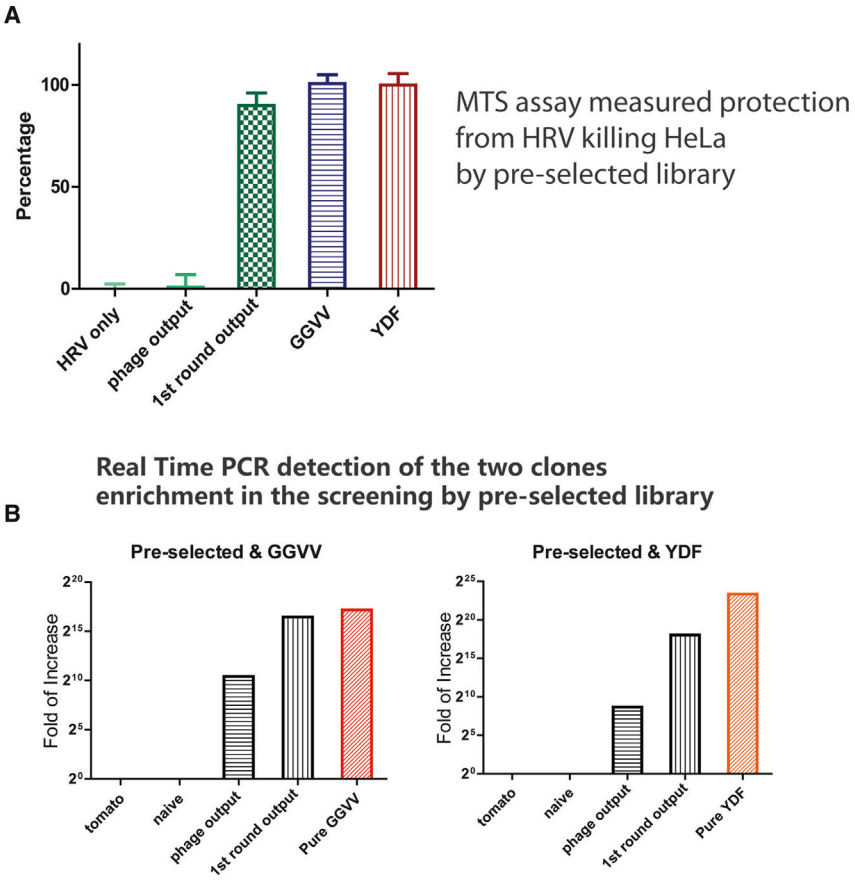

Figure 7. Selection Using a Pre-Enriched Library

(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 SD.

(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 preselected case.

using early markers of cell death or inducible antibody expression (Melidoni et al., 2013), one should be able to discover antibodies that influence the death of cancer cells. For instance, one can construct colony assays using inducible antibody genes to select for growth arrest only when gene expression is activated.

SIGNIFICANCE

Ultimately, scientists would like to learn how to control and perhaps interfere with processes that lead to cellular pathology including death. Recently, several reports have shown that intracellular expression of combinatorial antibody libraries can influence cell fates that include differentiation and transdifferentiation. In these systems, $\sim$1.0 $\times$ 10^8 different antibodies are tested simultaneously, and success seems only limited by the availability of a selection system. Here we used survival, which is arguably the most selectable cell event, to select antibodies that prevent viral killing. The power of the selection process was shown by the fact that only two of 10^8 antibodies were protective. The experiments 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.

EXPERIMENTAL PROCEDURES

Intrabody Lentiviral Library Construction

Single-chain Fvs (scFvs) from a naive 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 naive antibody library in lentivirus. The scFv region is under a EF1a promoter, followed by a FLAG tag, No constant region or secretion leader sequence is attached because we desired intracellular expression.

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. One hundred nanograms of the genomic DNA was used as a PCR template. A pair of primers matching the regions in the front and after the scFv fragment was 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.

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 were 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 3 $\times$ 10^8/ml.

Preparation of Lentivirus

The intrabody lentiviral vectors with the pCMVD8.9 and pVSVg viral packaging vectors at ratio of 1:1:1 were cotransfected into HEK 239T cells to produce virus. Sixty hours after transfection, the virus containing supernatant was collected. Cell debris was 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.

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. After 3 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 1 mg/ml in PBS buffer and kept at 4°C.

MTS Assay

Lentivirus-infected HeLa cells plated in a 96-well microplate at 2×10^4 cells per well in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The wells were brought to a volume of 100 μl and incubated for 36 hr at 30°C with rhinovirus. A total of 20 μl of MTS solution (CellTiter 96 aqueous nonradioactive cell proliferation assay; Promega) was added to each well. After 2 hr, the absorbance at 490 nm was measured.

In Vitro Coprecipitation Assay

Two micrograms of antibody was incubated with 1 ug of 3C protease (Millipore 71493-3) for 30 min at room temperature, after which 50 μl of protein G magnetic beads (NEB S1430S) were added to the tube. The mixture was incubated for another 30 min, after which the beads were harvested using a magnet and washed with PBS five times. Fifty microliters of loading buffer was added to the pellet, and the mixture was boiled for 5 min at 90°C before SDS-PAGE.

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). A 50 ng DNA template was used for each PCR reaction, in a 50 μl system according to the manufacturer's instructions. The copy number change of each sequence was normalized to the copy number of the EF1α promoter region that coexists in the plasmids with each antibody sequence at a 1:1 ratio.

Surface Plasmon Resonance

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 was captured at approximately 400 response units followed by injection of the 3C protein at a flow rate of 30 μl/min. For GGVV, a 2-fold serial dilution of 3C starting at 100 nM was injected in duplicates with an association time of 3 min followed by a dissociation time of 10 min. For YDF, a 2-fold serial dilution of 3C starting at 2,000 nM was injected in duplicates with an association time of 4 min followed by a dissociation time of 30 min. The surface was regenerated by injection of 3 M magnesium chloride for 30 s at 10 μl/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).

For the double binding experiment, YDF antibody was directly amine-coupled to a CM4 sensor chip at approximately 1,000 response units. The sensorgram was generated by three subsequent injections at 30 μl/min: (1) GGVV antibody for 1min, (2) 3C protein for 2 min, and (3) GGVV antibody for 1 min. The GGVV antibody and 3C protein samples were at a concentration of 1,000 nM and each injection was followed by an approximate 3 min wait period. Signal from an empty reference channel was subtracted as background.

Protease Inhibition Assay

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

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 at a ratio of 1:1:10:50:100 based on the estimated enrichment rate during selection. To achieve a balance between read length and throughput, we used the ion torrent personal genome sequencing machine (PGM) with 400 bp kits and a 318 chip. A single PGM run yielded a total of 7,030,852 raw reads with a median length of 489 bp. Note that the 3′-trimming option in base calling was turned off in initial data processing to obtain the maximum read length because 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 repertoires from HIV-1-infected patients, was used to process and analyze the sequencing data obtained from antibody libraries.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2013.12.006.

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