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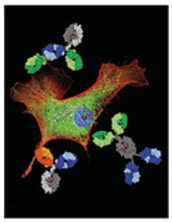

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REPORT

Discovery of high affinity anti-ricin antibodies by B cell receptor sequencing and by yeast display of combinatorial V_H:V_L libraries from immunized animals

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ABSTRACT

Ricin is a toxin that could potentially be used as a bioweapon. We identified anti-ricin A chain antibodies by sequencing the antibody repertoire from immunized mice and by selecting high affinity antibodies using yeast surface display. These methods led to the isolation of multiple antibodies with high (sub-nanomolar) affinity. Interestingly, the antibodies identified by the 2 independent approaches are from the same clonal lineages, indicating for the first time that yeast surface display can identify native antibodies. The new antibodies represent well-characterized reagents for biodefense diagnostics and therapeutics development.

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Antibody repertoire analysis; next-generation sequencing; ricin A chain; yeast surface display

Introduction

Ricin is a toxin derived from the castor bean *Ricinus communis*, and it is classified as a Category B Agent by the Centers for Disease Control and Prevention in part because of its high lethality (LD_{50} about 22 μg/kg body weight for human) and ease of production.[1] Ricin is composed of an enzymatic A chain, which deactivates eukaryotic ribosomes by depurinating adenine 4324 in the 28S rRNA of the 60S ribosomal subunit, and a lectin B chain, which binds carbohydrates on the cell surface.[1,2] As a result, ricin is a potent biological weapon, and past cases of malicious exposure to ricin highlight the need for both an increase in the sensitivity of diagnostics and for treatment after poisoning.[3,4] Antibodies to both the A chain and B chain of ricin have been produced using hybridoma-based technology,[5-8] and by phage display screening of immune libraries reconstituted from the bone marrow of immunized cynomolgus macaques,[9] resulting in antibodies with affinities ranging from 40 pM to 5 nM. However, there are currently no US. Food and Drug Administration-approved treatments for ricin poisoning, and the diagnostic potential of these reported antibodies has yet to be tested.

In vitro screening of large combinatorial libraries using display technologies that rely on phage, bacteria, yeast, mammalian cells or even in vitro transcription/translation systems are widely employed for antibody discovery.[10-12] Combinatorial antibody libraries are constructed either by mining the natural diversity of immunoglobulin genes in immunized or antigen-naïve animals,[13-15] or by diversifying the complementarity-determining regions (CDRs) within one or more "scaffold" antibodies.[16] Variable heavy (V_H) and variable light (V_L) chain genes are then joined combinatorially, yielding, at least in theory, libraries that contain combinations of heavy chains joined with all possible light chains.[13-16] Typically, immune libraries constructed from mRNA obtained from the spleen, bone marrow or from peripheral blood mononuclear cells (PBMCs, primarily in the case of human donors) are more likely to encode a significant fraction of antigen-specific antibodies and thus represent the most reliable route to high affinity antibodies (provided that the antigen is immunogenic).[13,17] Isolation of high affinity antibodies from immune and other libraries is most readily accomplished by taking advantage of the quantitative nature of fluorescence-activated cell sorting (FACS)-based library screening methods, and yeast display has been established as the dominant technology for screening libraries by FACS.[18]

The power of combinatorial library screening has been validated by the identification and development of therapeutic antibodies that are now entering clinical trials.[19] However, certain antibodies isolated from combinatorial libraries may express at low yields in mammalian cells and display poor biophysical properties in vitro,[20-24] which can hamper their development into therapeutics. The random pairing of heavy and light chains in combinatorial libraries results in antibodies with non-natively paired V_H and V_L genes, and this is potentially one cause of poor antibody expression and stability.[25] Moreover, a recent report has suggested that several mouse and human V_H germline genes exhibit strong preferential pairing with specific V_L chains,[26] and the random pairing of non-preferential V_H and V_L chains could lead to conformational incompatibilities, again affecting expression and stability. In the past decade, enormous efforts have been expended on advancing screening techniques

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to remove antibodies with mediocre biophysical properties and investigating methods to improve them.[21-23,27,28]

Our lab has pioneered methods for the discovery of high affinity antigen-specific antibodies directly via mining of the immunoglobulin repertoire by capitalizing on next-generation sequencing technologies without the need for screening.[29-32] Specifically, we developed methods for high-throughput determination of the natively paired V_H:V_L repertoire from single B cells.[30,31] More recently, we have shown that antibody secreting B cells ($CD138^+$ plasmablasts) within the draining lymph node are overwhelmingly antigen-specific, and that antibodies derived from these cells exhibit high binding affinity.[32]

In this report, we sought to compare the isolation of high-affinity anti-ricin antibodies via mining of the draining lymph node repertoire and via yeast display of immune combinatorial libraries constructed from antibody mRNAs obtained from spleen or bone marrow cells. Overall, both approaches yielded strong ricin A chain binders (the lowest K_d values were 0.97 and 0.58 nM, respectively, for these 2 methods). Interestingly, we found that antibodies isolated by yeast display from combinatorial libraries in which the V_H and V_L from spleen or bone marrow had been randomly paired were clonal relatives of antibodies identified via mining of the draining lymph node repertoire, and comprised authentic, natively paired, V_H and V_L sequences. Thus, in hyperimmune animals where antigen-specific antibodies comprise a significant portion of the repertoire,[29,32-35] flow cytometric screening of libraries constructed via the random pairing of V_H and V_L genes can nonetheless result in the isolation of native antibodies.

Results

Overview of the experimental approach

As we have previously shown, mouse footpad immunization results in a strong immune response in the popliteal draining lymph node (DLN), characterized by the robust expansion of antibody secreting B cells ($CD138^+$ plasmablasts).[32] Analysis of the DLN $CD138^+$ B cell antibody repertoire overwhelmingly revealed that the most highly represented sequences, i.e., those present in the largest number of reads, are antigen-specific.[32] Therefore, we sought to explore the antibody repertoire in DLN antibody-secreting B cells, and to compare antigen-specific antibodies with those selected from bone marrow and spleen combinatorial libraries, which are commonly used in antibody discovery efforts following intraperitoneal or subcutaneous immunization.[9,13,36] In contrast, samples from the DLN could only support high-throughput V_H:V_L sequencing and repertoire analysis, as we were limited by the number of cells. Four mice were immunized by footpad injection with ricin A chain in TiterMax Gold adjuvant (Fig. 1A), followed by 2 booster immunizations after 14 and 28 days, after which all 4 mice generated significant titers (>1 :10,000) against ricin A chain (Fig. S1A). One more booster immunization was performed, after which even higher titers were reached (Fig. S1B). Mice were sacrificed 6 d after the final booster immunization, at which point the draining lymph node was observed to be enlarged compared to the contralateral lymph node. Spleen and bone marrow were collected to screen for antigen-specific antibodies (Fig. 1B), and the draining lymph node was collected as well for V_H:V_L mining (Fig. 1C).

Isolation of high affinity ricin A chain antibodies by yeast display of combinatorial libraries

Single-chain variable fragment (scFv) libraries were constructed using mRNA from $\sim 5 \times 10^6$ total splenocytes, and separately from femur bone marrow cells (Fig. 1B). Briefly, V_H and V_L genes were amplified separately, paired combinatorially by overlap extension-polymerase chain reaction that also introduced a $(Gly_4Ser)_3$ linker,[13] and then subcloned into the yeast display vector pCTCON2 via homologous recombination.[37]

Two libraries of $\sim 2 \times 10^6$ transformants each (bone marrow-derived and splenocyte-derived) were obtained. Screening was performed by carrying out one round of magnetic-activated cell sorting (MACS), and then 3 rounds of FACS with progressively more stringent gates that collected cells positive both for expression (using Alexa Fluor 488-labeled anti-c-Myc) and for ricin A chain binding (using Alexa Fluor 633-labeled ricin A chain) (Fig. 2). For the first 2 rounds of selection, ricin A chain was used at a 1 μM concentration. Prior to selection, both libraries showed little binding to ricin A chain (Figs. 2A and B, before selection). Significant enrichment of cells that stained positively both with fluorescent anti-c-Myc antibody and ricin A chain was observed from both libraries after the second round (Figs. 2A and B, after first FACS). Subsequently, ricin A chain concentrations of 200 nM and 40 nM were used for the third and fourth rounds of sorting, respectively. After the fourth round, almost all of the cells that expressed scFv also showed binding to ricin A chain (Figs. 2A and B, after third FACS).

Twenty colonies from each library were sequenced, yielding a total of 5 unique clones (3 from the bone marrow-derived library and 2 from the splenocyte-derived library), indicating that screening had led to convergence of the libraries to a small number of scFv sequences (Table 1). Apparent equilibrium binding constants (K_d) for these 5 clones were estimated by incubating yeast cells displaying the respective antibodies with different concentrations of ricin A chain, ranging from 0.09 to 600 nM, and then measuring the mean fluorescence intensity of binding at each concentration by FACS. Binding constants were calculated using a Langmuir 1:1 binding model.[37] All 5 antibodies demonstrated low nanomolar affinities binding to ricin A chain (Table 2, Fig. S2). These 5 antibodies were then expressed in *E.coli* as single-chain antibodies (scAbs, scFv fused with human kappa light chain at the C-terminus[13]) and purified to >95% homogeneity by Ni-NTA chromatography (as determined by SDS-PAGE). The antibodies were further purified by size-exclusion chromatography, which also revealed that all antibodies were monomeric. Surface plasmon resonance (SPR) analyses indicated that, consistent with the flow cytometric analyses,[37] all 5 antibodies displayed high affinity binding to ricin A chain (Table 2, Fig. S3).

The question of whether in vitro screening technologies can identify native antibodies in a repertoire has generally not been addressed; the two methods often return different antigen-specific antibodies. Different screening platforms lead to the identification of different antibodies, but some platforms seem to

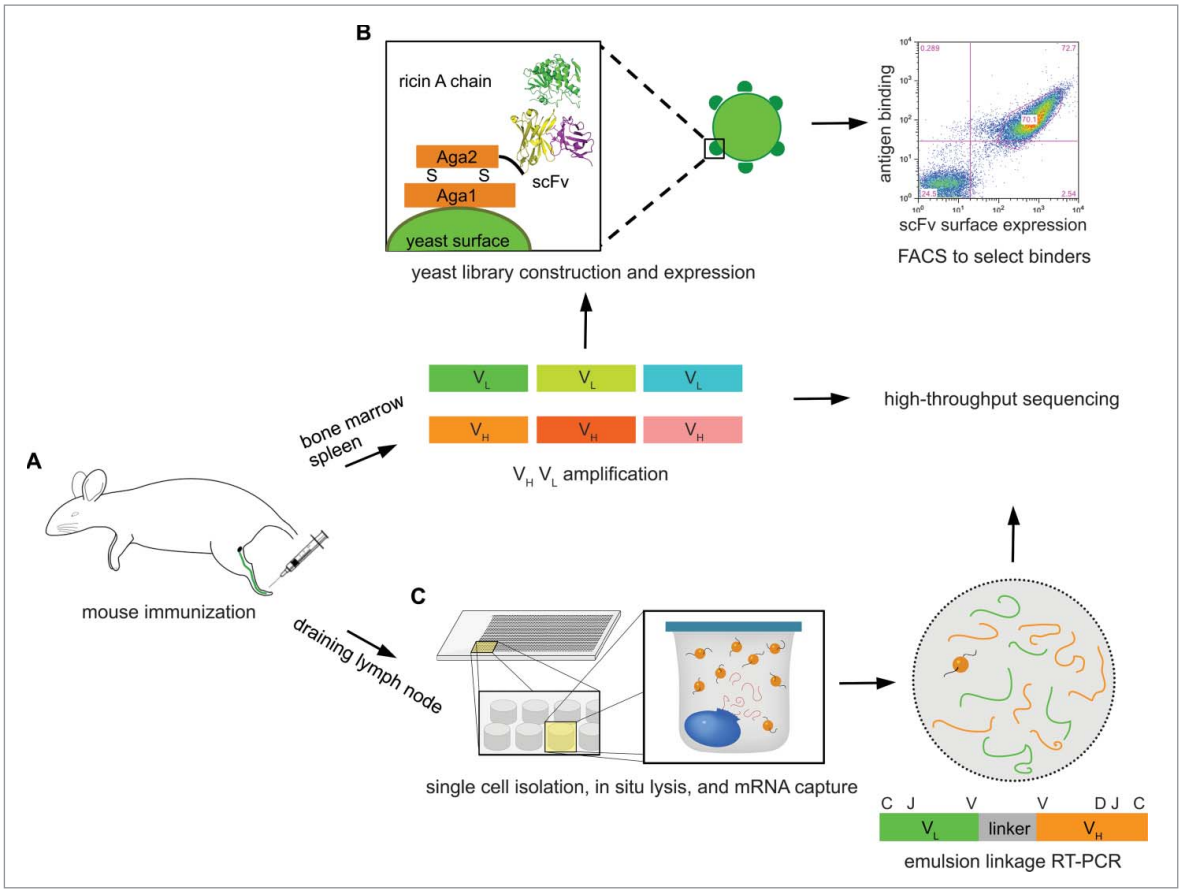

Figure 1. Overview of the experimental approach. (A) Mouse is immunized at footpad with ricin A chain, and peripheral lymphoid organs, including bone marrow, spleen, and draining lymph node are isolated after 3 booster immunizations. (B) Total cells in bone marrow and spleen are collected, and V_H and V_L mRNA are reverse transcribed and amplified, which are used to construct scFv libraries, respectively. These are selected against ricin A chain using yeast surface display to isolate high-affinity binders. V_H and V_L cDNAs are also sequenced on an Illumina platform. (C) $CD138^+$ antibody secreting cells are isolated from draining lymph node, and processed through our high-throughput V_H:V_L pairing platform.

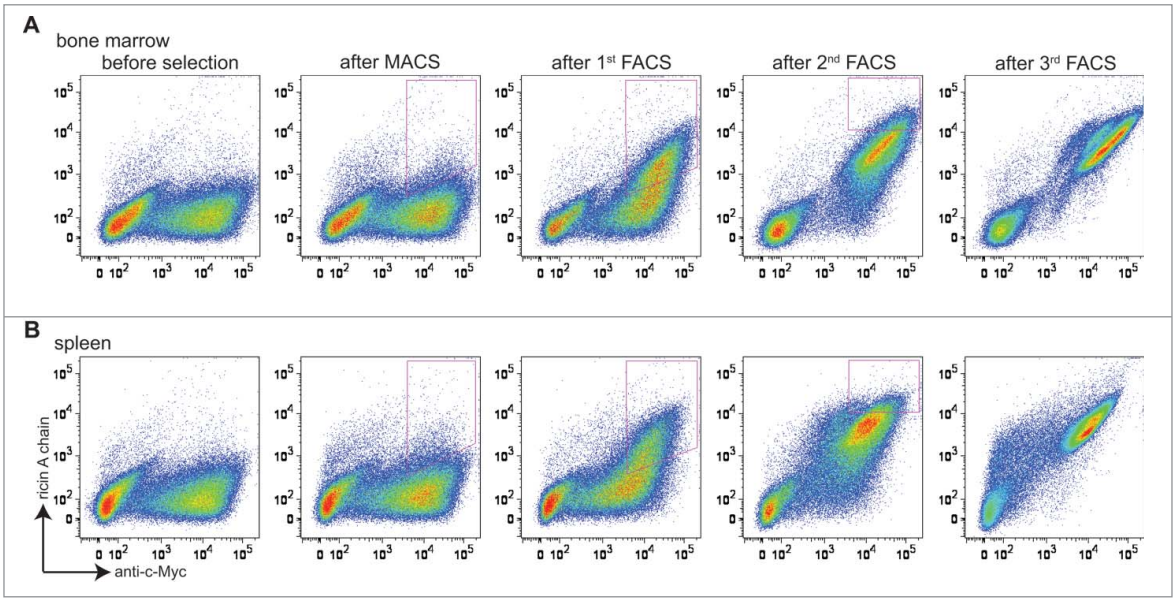

Figure 2. Selection of libraries constructed from bone marrow and spleen antibody repertoires. Cells are doubly stained with chicken anti-c-Myc IgY/GaC-488 for scFv surface expression and biotinylated ricin A chain/SA-633 for antigen binding. (A) Bivariate plots are shown for bone marrow antibody library stained with 100 nM ricin A chain before selection, after MACS, after 1st FACS, after 2nd FACS, and after 3rd FACS, with x axis being surface expression, and y axis being antigen binding. (B) Bivariate plots are shown for spleen antibody library stained with 100 nM ricin A chain before and after each round of selection. For both libraries, cells in the upper-right quadrant (both express scFv on the surface and bind ricin A chain) are sought. Cells falling within strict FACS sort gates designed to ensure enrichment of clones showing increased binding to ricin A chain are collected for the next round of selection.

Table 1. List of the antibodies isolated from the bone marrow and spleen combinatorial libraries by yeast display and abundance of their V_H and V_L sequences in the corresponding repertoires assessed by next-generation sequencing. Note that BM3 and BM17 have the same CDRH3 sequences, but differ in sequence outside of CDRH3 (Fig. 5).

Antibody	Source	CDRH3: CDRL3	Gene usage	V_H:V_L abundance (percentage and rank)
BM1	bone marrow	CTRSEFVNFGWFAYW: CQQYHNFPRTF	IGHV1-IGHD2-IGHJ3: IGKV4-IGKJ1	2.6%, 3rd: 3.1%, 3rd
BM3	bone marrow	CTRSEYVNFGWFAYW: CQQYHNFPRTF	IGHV1-IGHD2-IGHJ3: IGKV4-IGKJ1	5.1%, 2nd: 3.1%, 3rd
BM17	bone marrow	CTRSEYVNFGWFAYW: CQQYHNFPRTF	IGHV1-IGHD2-IGHJ3: IGKV4-IGKJ1	1.2%, 5th: 3.1%, 3rd
SP1	spleen	CSRDRTWYGTFYAMDYW: CHQYHRSPYTF	IGHV1-IGHD2-IGHJ4: IGKV4-IGKJ2	0.9%,5th: 2.8%,3rd
SP19	spleen	CTRSEFVNFGWFAYW: CHQYHNYPRTF	IGHV1-IGHD2-IGHJ3: IGKV4-IGKJ1	5.3%,2nd: 1.2%,4th

return antibodies that are of low abundance or apparently not present at all.[36,38] The identification of antibodies via yeast display presented an opportunity to compare the results with the natural repertoire, and thus we sequenced both the bone marrow and spleen V_H and V_L repertoires using an Illumina 2 × 250 Miseq platform. From the same 5×10^6 cells of bone marrow and spleen from a given mouse that were used for yeast library construction, about 10^6 and 7×10^5 reads, respectively, were obtained for V_H repertoires, from which 8735 and 7057 unique clonotypes (defined as the group of V_H sequences that share the same germ-line V and J segments, and have >90% amino acid identity in their CDRH3s[39]) were identified (Table 3). Similar to previous reports,[29,40] both repertoires were observed to have skewed germ-line V gene usage. For example, both repertoires showed biases for IGHV1, 5, 14, and IGKV1, 4, 6 families (Fig. 3A, B), with IGKV4 as the most commonly used light chain family in the bone marrow, and IGKV6 as the most commonly used light chain in the spleen. We noticed the preferential usage of the IGKV1 family in bone marrow relative to spleen, and of the IGKV6 family in spleen relative to bone marrow. It will be interesting to determine if similar repertoire biases toward specific germline light chain families are observed with other antigens and adjuvant combinations. Both repertoires showed a CDRH3 length distribution comparable to those previously reported from animals immunized with different antigens,[29] with a median CDRH3 length of 13 amino acids in the bone marrow repertoire and 11 amino acids in the spleen repertoire (Fig. 3C).

Importantly, we now find that the antibodies identified by yeast surface display are among the most abundant antibodies in the sequenced repertoire (Table 1). This indicates for the first time that yeast surface display of combinatorial libraries can identify native antibodies in the repertoire.

Table 2. The binding kinetics of the antibodies isolated from bone marrow and spleen as measured by yeast surface titration and surface plasmon resonance.

Antibody	K_d (nM, determined by yeast display titration)	Binding kinetics determined by SPR		
		k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_d (nM)
BM1	10.3	$(2.09 \pm 0.04) \times 10^5$	$(9.7 \pm 0.52) \times 10^{-4}$	4.64 ± 0.33
BM3	2.76	$(1.13 \pm 0.03) \times 10^5$	$(3.41 \pm 0.23) \times 10^{-4}$	3.03 ± 0.12
BM17	2.96	$(2.5 \pm 0.13) \times 10^5$	$(1.36 \pm 0.006) \times 10^{-3}$	5.44 ± 0.27
SP1	4.25	$(2.28 \pm 0.07) \times 10^5$	$(3.72 \pm 0.06) \times 10^{-4}$	1.64 ± 0.08
SP19	8.22	$(3.91 \pm 0.32) \times 10^5$	$(2.25 \pm 0.04) \times 10^{-4}$	0.58 ± 0.05

Isolation of high affinity anti-ricin A chain antibodies by next-generation sequencing of DLN B cells

$CD138^+$ antibody secreting B cells were isolated from the draining lymph node and the paired V_H:V_L repertoire was determined, as previously described.[32] Briefly, a total of 90,000 $CD138^+$ antibody secreting cells were isolated by magnetic sorting by first depleting $CD45R^+$, $CD49b^+$, and $CD19^+$ cells, and then enriching for $CD138^+$ cells. Approximately 40,000 antibody secreting cells were deposited into microfabricated nanowell plates such that >98% of wells contained a single cell, after which poly(dT) magnetic beads were added to capture mRNAs, the cells were lysed, and the poly(dT) beads were collected. The beads were emulsified, and overlap extension RT-PCR was performed to synthesize linked V_H:V_L amplicons (Fig. 1C).[30] The region of the linked amplicons comprising CDRH3 and CDRL3 was then sequenced in an Illumina MiSeq 2 × 250 run. Full-length V_H and V_L sequences were identified in separate MiSeq 2 × 250 runs. After bioinformatics analysis,[30] 212 unique V_H:V_L pairs were identified in the draining lymph node repertoire, and these showed great polarization, indicative of clonal expansion. Specifically, the top 10 most abundant V_H:V_L pairs constituted 65.8% of the total reads (Fig. 4A). The virtual absence of antibodies using IGLV families in the repertoire was expected, as only 5% of all mouse antibodies use the lambda light chain.[40]

Comparisons with mouse antibody repertoires generated by immunization with other antigens showed similar germ-line gene usage between the anti-ricin A chain antibody repertoire and other repertoires (Fig. 4B, compare to the repertoires in ref 29 and 32 generated against other antigens).[29,32] The CDRH3 length distribution in $CD138^+$ antibody secreting cells from the draining lymph node showed 3 peaks at 12, 13, and 15 amino acids (Fig. 4C).

Synthetic genes for the top 10 highest frequency paired V_H:V_L sequences from the DLN repertoire were constructed and the respective mouse-human chimeric antibodies (mouse V_H fused with the human IgG1 constant domain, and mouse V_L fused with the human kappa constant domain) were expressed in Expi293 cells and purified. Four/10 recombinant antibodies displayed binding by ELISA (Table 4, Fig. S4). SPR analysis was

Table 3. Next-generation sequencing statistics of bone marrow and spleen repertoires.

Tissue	Region	Total sequence reads	unique productive gene	unique clonotypes
bone marrow	V_H	1078639	849735	8735
	V_L	542905	385398	
spleen	V_H	690554	370357	7057
	V_L	696061	410954	

used to obtain antigen-binding kinetics for these 4 clones (Table 5, Fig. S3). All but one antibody showed high affinity binding to the ricin A chain. Interestingly, the affinities of the antibodies that bound ricin A chain correlated with their abundance in the V_H:V_L repertoire, with the antibody displaying the lowest K_d (RAM1.2) being the most abundant.

We compared the native antibodies recovered from the DLN to yeast display-selected antibodies from the bone marrow and spleen combinatorial libraries. Interestingly, BM1, BM3, BM17 and SP19 were found to share germ-line V and J segments with RAM1.5, and SP1 had the same germ-line V and J segments as RAM1.4. The two groups of antibodies also had the same V_H:V_L pairing. Furthermore, antibodies recovered from the DLN without selection had very similar CDRH3s with those isolated by yeast display from bone marrow and spleen (Table 1, 4), as well as additional amino acid substitutions, indicating that they correspond to somatic variants derived from the same antibody lineage during clonal expansion (Fig. 5).

Discussion

In vitro screening of antibody libraries constructed from different sources (synthetic, naïve, or immune) is one of the most commonly used techniques for antibody discovery.[11,12] In this work, we identified anti-ricin A chain antibodies by eliciting an immune response in mice and then exploring the resultant antibody repertoires via both yeast display of combinatorial libraries from the bone marrow and spleen and paired V_H:V_L sequencing from the draining lymph node.

While both approaches generated high affinity clones (K_d ranging from 0.55 nM to 6.12 nM), as has previously been observed,[38] unexpectedly the antibodies generated by these 2 very different approaches were found to be clonal relatives. Different amino acids encoded by different nucleotides in the isolated antibodies from different lymphoid organs indicated that BM1, BM3, BM17 (derived from bone marrow using yeast display), SP19 (derived from the spleen using yeast display) and RAM1.5 (derived from the draining lymph node using paired V_H:V_L sequencing), and separately SP1 and RAM1.4 were clonal variants (Fig. 5). A previous report has shown that while naïve mouse spleen and lymph node repertoires share some common CDRH3s, the scFvs isolated from these lymphoid organs following intraperitoneal and subcutaneous immunization were unique.[41] In contrast, when we probed different lymphoid organs, clonal variants were obtained. These differing conclusions may reflect differences in the immunization routes used, the numbers of boost immunizations, and significant differences introduced by the expression biases in phage compared to yeast display. The clonal variants we isolated from different lymphoid organs indicate that these antibodies were likely encoded by the same ancestral B cells that homed to different lymphoid organs and experienced clonal expansion there.

We have previously shown that by dissecting the draining lymph node antibody repertoire with paired V_H:V_L sequencing, the highly abundant clones were usually antigen specific as a result of clonal expansion.[32] Here, we again showed that the highly abundant clones in the draining lymph node probed with paired V_H:V_L sequencing encoded high-affinity, antigen-specific antibodies. Comparison of the antibodies isolated using these 2 approaches supports our hypothesis that yeast display can recover native antibodies in a repertoire, as antibodies isolated by yeast display are clonal variants of those isolated by paired V_H:V_L sequencing, which identifies native V_H:V_L pairings. Given the relative abundance of the V_H and V_L sequences of antibodies in the bone marrow and spleen repertoires, the calculated probability of obtaining these native antibodies by combinatorially random pairing would be about 0.2%. Thus, in

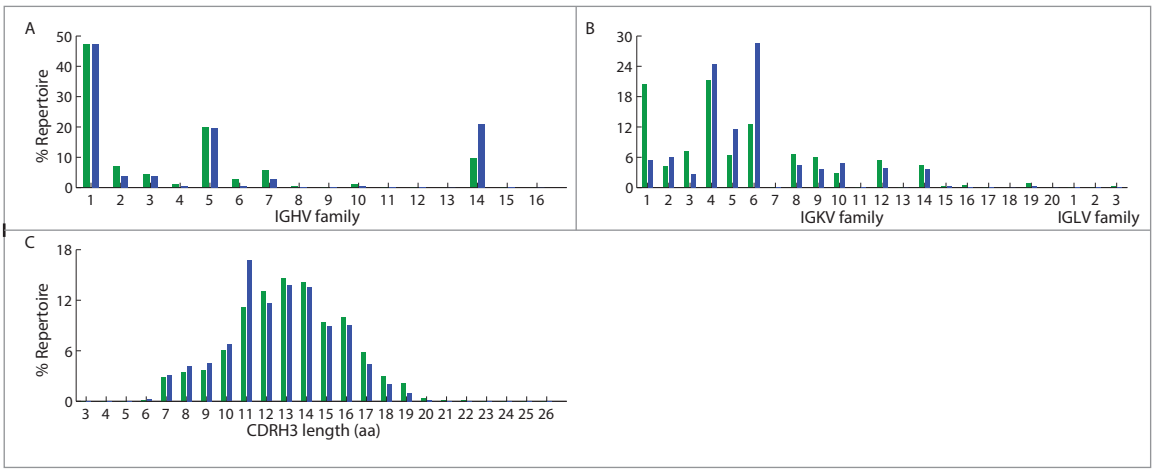

Figure 3. Characteristics of bone marrow and spleen antibody repertoires from the same mouse immunized with ricin A chain. (A) Germ-line V_H gene usage in bone marrow and spleen repertoires. (B) Germ-line V_L gene usage in the 2 repertoires. (C) CDRH3 length distribution in the 2 repertoires. Green denotes bone marrow repertoire, and blue denotes spleen repertoire.

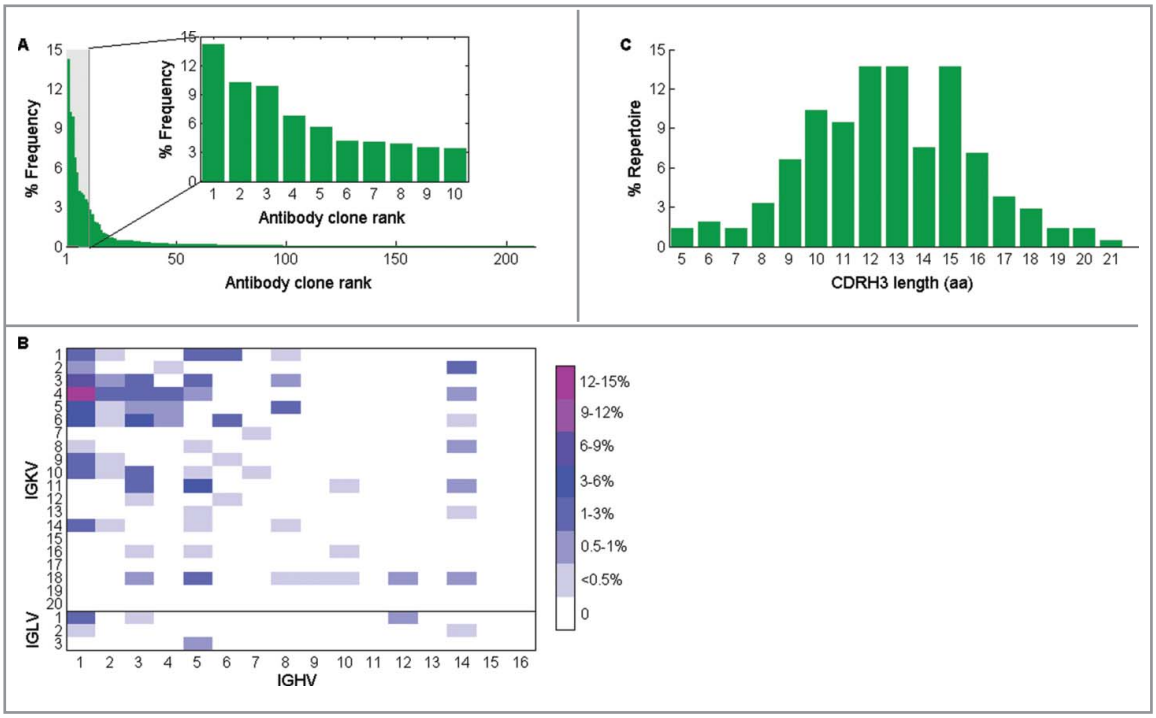

Figure 4. Characteristics of CD138^{+} antibody-secreting cells repertoire in draining lymph node from mouse immunized with ricin A chain. (A) The frequency of each unique antibody clone in the repertoire, which is calculated as the percentage of its read counts in the read counts of all clones, is shown with its rank, which is ordered by the number of read counts of each unique clone. Clones with only 1 read are removed, and CDRH3 nucleotide sequences are clustered to 96% identity. Inset shows the distribution of the top 10 most abundant clones in the repertoire. (B) Germ-line V gene family usage in the same repertoire is shown. (C) CDRH3 length (calculated as amino acid length) distribution of antibodies in the repertoire is shown as the percentage of antibodies that have the denoted CDRH3 length in the whole repertoire.

hyperimmunized animals the proportion of randomly paired V_H and V_L genes is sufficiently high that natively paired heavy and light chains occur. Moreover, high affinity native pairs can be isolated following yeast surface expression and FACS. To our knowledge this is the first report that yeast display selections can identify native antibodies from the immune repertoire.

The fact that an in vitro combinatorial library screening method can identify native V gene pairs is relevant for antibody discovery. Numerous successes in developing antibody drugs from combinatorial libraries generally validate this approach.[19] However, some high-affinity antibodies identified by library screening have mediocre biophysical properties, such as low yield and poor stability.[20-24] Such deficiencies are thought to emanate from random or non-native pairings of V_H and V_L that lead to unfavorable conformations that in turn promote poor biophysical properties.[25,26] Another contributor may be that expression in a non-native host (i.e., mouse antibody libraries screened by phage display) leads to selection at the expression level against certain clones.[38] In contrast, antibodies with natively paired heavy and light chains, such as those isolated from hybridomas, express better, and in general show much better stability.[25,26,42] Our methods can therefore potentially streamline the huge amount of effort put into the discovery process.[20-22,27,28]

Our results support the utility of next-generation sequencing in combinatorial library screening.[10,29,32] Hyperimmunization leads to a high degree of polarization of the B-cell repertoire in a particular compartment, such that the dominant antibodies are highly represented and combinatorial screening by a technique with low expression bias, such as yeast display, can further yield high-affinity native antibodies. The high-affinity anti-ricin A chain antibodies we identified may provide a greater diversity of reagents for both diagnostics and therapeutics.

Table 4. List of the antibodies isolated from CD138^{+} antibody-secreting cells in the draining lymph node.

Antibody	Rank in the repertoire	CDRH3:CDRL3	Gene usage
RAM1.2	2	CARPTLLYGSSPCFDYW: CQQWSSSPTF	IGHV1-IGHJ2:IGKV4-IGKJ4
RAM1.4	4	CTRERTWYGTFYAMDYW: CHQYHRSPYTF	IGHV1-IGHD2-IGHJ4:IGKV4-IGKJ2
RAM1.5	5	CTRSEYVDFGWFAYW: CQQYHSYPRTF	IGHV1-IGHD2-IGHJ3:IGKV4-IGKJ1
RAM1.10	10	CARSRDYDGYGDYW: CQQSNRWPLTF	IGHV1-IGHD2-IGHJ2:IGKV5-IGKJ5

Materials and methods

Cell line and media

The yeast strain EBY100 (MATa *AGA1::GAL1-AGA1::URA3 ura3-52 trp1 leu2Δ200 his3Δ200 pep4::HIS3 prb11.6R can1 GAL*) was used for library construction and screening. Yeast cells were maintained in YPD medium (20 g/l dextrose, 20 g/l peptone, and 10 g/l yeast extract); after library transformation, they were maintained in SDCAA medium (20 g/l dextrose, 6.7 g/l yeast nitrogen base, 5 g/l casamino acids, 8.56 g/l

Table 5. The binding kinetics of the antibodies isolated from the draining lymph node as measured by ELISA and surface plasmon resonance.

Antibody	EC_{50} (nM, determined by ELISA)	SPR binding kinetics		
		k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_d (nM)
RAM1.2	0.186	$(3.88 \pm 0.36)\times10^{5}$	$(3.73 \pm 0.13)\times10^{-4}$	0.97 ± 0.11
RAM1.4	0.284	$(1.28 \pm 0.12)\times10^{5}$	$(4.93 \pm 0.11)\times10^{-4}$	3.89 ± 0.35
RAM1.5	3.21	$(9.84 \pm 0.51)\times10^{4}$	$(6.01 \pm 0.12)\times10^{-4}$	6.12 ± 0.19
RAM1.10	20.9	$(5.46 \pm 2.4)\times10^{4}$	0.149 ± 0.01	$(3.12 \pm 1.36)\times10^{3}$

$NaH_2PO_4.H_2O$, and 10.2 g/l $Na_2HPO_4.7H_2O$). SGCAA medium (identical to SDCAA except 20 g/l galactose is used instead of dextrose) was used for library induction. *E. coli* strain DH10β was used for subcloning, and *E.coli* strain Jude-1 was used for soluble scAb expression. Expi293 cells (Invitrogen) were used for IgG expression, and were maintained in Expi293 expression medium (Invitrogen).

Antigen and antibodies

Ricin A chain was purchased from Sigma-Aldrich (cat# L9514). It was biotinylated using an EZ-Link Sulfo-NHS-LC-Biotin kit (Thermo Scientific). Chicken anti-c-Myc IgY, Alexa Fluor 488-goat anti-chicken IgG (GaC-488), and streptavidin-Alexa Fluor 633 (SA-633) were obtained from Invitrogen (cat# A-21281, A-11039, and S21375, respectively). Anti-biotin microbeads were purchased from Miltenyi Biotec (cat# 130-090-485).

Mouse immunizations

This study was approved by the University of Texas Institutional Animal Care and Use Committee under protocol# AUP-2013-00009. Ricin A chain was mixed with TiterMax Gold adjuvant (Sigma-Aldrich, cat# T2684) at a 1:1 ratio and pipetted several times to obtain stable emulsions for immunization. On day 1, 4 female BALB/c mice at 6 weeks of age (Jackson Laboratory) were injected subcutaneously at the left hind footpad with 5 μg of ricin A chain in 25 μl antigen adjuvant mixture. A booster immunization was performed on day 14. Seven days after the booster immunization, the serum antibody titers against ricin A chain were determined. About 30 μl of blood was collected from each mouse at a small tail vein incision made with a scalpel blade, and coagulated at room temperature (RT) for 30 min. Following centrifugation at 13000 rpm for 15 min, the supernatant (serum) was used for ELISAs. The serum was first serially diluted with phosphate-buffered saline (PBS) +2% milk (PBSB) at a 1:3 ratio from 1:100 to 1:218,700. The diluted serum was applied in triplicate onto ELISA plates (Corning) that had been coated with 50 μl of 4 μg/ml of ricin A chain overnight (O/N) at 4°C and then blocked with PBSB at RT for 2 hours. After incubation at RT for 1 hour, plates were washed with PBS+0.05% Tween-20 (PBST), followed by adding 50 μl of a 1:5000 diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, cat# 115-035-166). After 1 hr incubation, plates were washed again with PBST, after which 50 μl of TMB Ultra ELISA substrate (Thermo Scientific, cat# 34028) was added. The reaction was quenched after 10 min with 50 μl of 2 M H_2SO_4. Absorbance at 450 nm was determined using a Tecan M200 plate reader, and the serum titer was calculated as the dilution at which the absorbance was 3 times higher than background. The second booster was performed on day 28, after which significant titers (>1 :10,000) were generated in all mice. The final booster was performed on day 42, and 6 d later, mice were sacrificed for lymphoid organ collection.

Lymphoid organ collection

Bone marrow, spleen, and draining lymph node tissues were collected separately. Thirty min before sacrifice, 10 μl 2% Evans Blue in PBS (Sigma-Aldrich, w/v, cat# E2129) was injected into the left hind footpad. After CO_2 asphyxiation and cervical dislocation, the blue-stained popliteal lymph node behind the knee was collected. The spleen was also collected. For bone marrow collection, after clipping the ends of the tibias and femurs, bone marrow was flushed out with PBS+0.1% bovine serum albumin (BSA)+2 mM EDTA (PBSM) using a syringe. Each lymphoid organ tissue sample was first mechanically disrupted using a needle, then passed through a 70 μm cell strainer (Corning) to collect single cells. The single cell

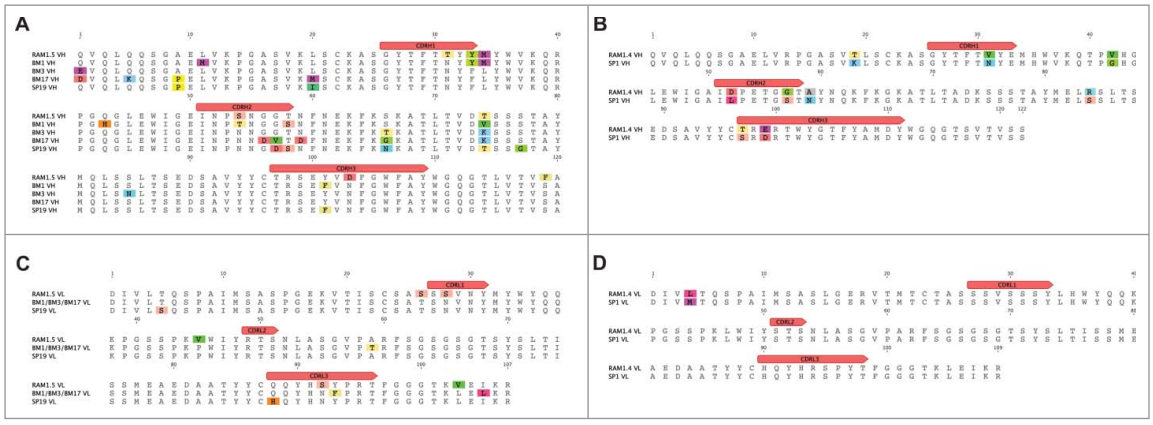

Figure 5. Sequence comparison between yeast surface display and next-generation sequencing discovered antibodies. (A), (B) V_H alignments of BM1, BM3, BM17, SP19 and RAM1.5 and of SP1 and RAM1.4 showed mutations through the complete V_H sequences, indicating they are somatic variants. (C), (D) V_L alignments of BM1/BM3/BM17, SP19 and RAM1.5 and of SP1 and RAM1.4 also showed mutations. Mutations are indicated by different colors.

suspension of each lymphoid organ was washed with 20 ml of PBSM buffer and resuspended in 2 ml red blood cell lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, and 0.1 mM EDTA). After incubation at RT for 5 min, cells were diluted with 20 ml of PBS and then spun down. Cells were washed twice more with PBSM buffer, then cells from the bone marrow and spleen were used for yeast library construction, and cells from draining lymph node were used for high-throughput V_H:V_L pairing.

Yeast library construction for bone marrow and spleen repertoires

Cells from the bone marrow and spleen were resuspended in TRI Reagent (Invitrogen), and RNA was extracted from each sample. cDNA was generated using 200 ng of RNA as a template with the SuperScript III First-Strand synthesis kit (Invitrogen). V_H and V_L sequences were amplified using mouse V gene specific primers (Table S1). For scFv library construction, 50 ng of V_H and V_L DNA were used as templates for overlap extension PCR, and amplified with primers that contained 50 nucleotides at each end that were in common with the display vector, which should be sufficient to promote homologous recombination upon yeast transformation. The display vector pCTCON2 was linearized by NheI and BamHI digestion[37] and the digested backbone was purified and co-transformed with 5-fold mass excess of scFv fragments into electrocompetent yeast cells.[43] The libraries were cultured in SDCAA medium at 30°C for 2 d.

Yeast library screening

Cells were induced in SGCAA medium at 20°C for 2 d. For the 1st round of selection, 2×10^7 cells (10-fold coverage relative to the number of transformants) were incubated with 1 μM biotinylated ricin A chain at RT for 1 hr and washed thoroughly with PBS+0.5% BSA+2 mM EDTA (PBSA) to remove any unbound ricin A chain protein. Cells were then mixed with 400 μl of anti-biotin microbeads and incubated at 4°C for 30 min, and washed again. Cells that bound ricin A chain were selected using MidiMACS system (Miltenyi Biotec), and recovered in SDCAA medium at 30°C. For the 2nd round of selection, 10^7 cells in 200 μl PBS+0.1% BSA (PBSF) were labeled with 1 μM biotinylated ricin A chain and 4 μg/ml chicken anti-c-Myc IgY at RT for 1hr. After washing with PBSF, cells were incubated with 5 μg/ml SA-633 and 5 μg/ml GaC-488 at 4°C for 30 min. After washing, cells were resuspended in PBSF buffer and sorted using FACS Aria (BD Bioscience). Gated cells were collected from the double positive quadrant as shown in Fig. 2. The subsequent 2 rounds of selection were performed in a similar way, except that the concentration of ricin A chain used was decreased to 200 nM and 40 nM, respectively. After 4 rounds of selection, 20 random colonies from each library were picked, and plasmids were isolated using Zymoprep Yeast Plasmid Miniprep II kit (Zymo Research). Plasmids were amplified in *E. coli* DH10β and sequenced. Three and 2 unique clones were thus isolated from the bone marrow and spleen libraries, respectively.

Plasmids encoding the 5 unique antibody clones above were re-transformed into strain EBY100. Cells were cultured and induced for scFv expression as described above. 10^6 cells were labeled with 1:3 serially diluted ricin A chain, ranging from 0.09 nM to 600 nM. The labeling process was the same as that used for library sorting. After labeling, cells were analyzed on BD FACS Aria and the mean fluorescence intensity (MFI) values were used to calculate apparent equilibrium binding constant (K_d), as described.[37]

scAb expression and characterization

For soluble antibody purification, antibody genes were subcloned into pMopac16 vector and expressed as scAb fragments in *E.coli* Jude-1 cells[13] and then purified by Ni-NTA chromatography according to the manufacturer's instructions (Thermo Scientific). After elution, proteins were dialyzed in PBS, then loaded onto a Hiload 16/600 Superdex 75 pg column (GE Healthcare) and antibody-containing fractions were pooled and concentrated to 2 mg/ml.

For affinity validation, SPR was performed for each antibody clone using a BIAcore 3000 biosenor (Biacore). About 400 RU (response units) of scAbs were immobilized on the CM5 sensor chip (GE Healthcare) using amine coupling chemistry. All binding experiments were done in HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% P20 surfactant) (GE Healthcare). Ricin A chain was injected at concentrations of 25, 50, 100, 200, and 400 nM with a flow rate of 30 μL/min for 2 min and a dissociation time of 10 min. The chip was regenerated after each injection by 100 mM citric acid, pH 3.0. The response generated by flowing ricin A chain over a BSA-coupled surface was used as control and consequently subtracted. Experiments were carried out in triplicates. All kinetic parameters were determined in BIAevaluation 3.0 software using a 1:1 Langmuir model and were reported as the average of the 3 technical replicates.

V gene repertoire sequencing and analysis

V_H and V_L cDNA for bone marrow and spleen samples (the same cDNAs used for yeast library construction) were prepared and sequenced using the Illumina 2 × 250 paired end Miseq platform as described previously.[30] Briefly, raw sequences were first filtered for sequences containing at least half of the nucleotides at a minimum Phred quality score of 20 to obtain high quality sequences, then assembled to obtain full length V_H and V_L sequences, respectively. Sequences represented by at least 2 reads were submitted to IMGT (the international immunogenetics information system, www.imgt.org) High V-Quest analysis[44] and those with productive V_H or V_L, and in-frame V(D)J junctions (CDRH3 or CDRL3) were analyzed further.

Paired V_H:V_L sequencing of antibody secreting cells from the draining lymph node

From single cell suspension of the draining lymph node, $CD138^+$ antibody secreting B cells were isolated using a mouse $CD138^+$ Plasma Cell Isolation kit (Miltenyi Biotec).[32] The paired V_H:V_L gene repertoire was determined as described.[30] Briefly, isolated single cells were deposited into 125 pL wells in PDMS slides together with poly(dT) magnetic beads (Invitrogen). After cell lysis *in situ*, beads with captured mRNA from

the same cell were collected, washed, and emulsified. Mouse V gene specific primers (Supplementary Table 2) were used to link endogenous V_H and V_L in the subsequent emulsion overlap extension RT-PCR. A second nested PCR was used to amplify the linked V_H:V_L pairs. Sequencing was performed by Illumina 2 × 250 paired end Miseq. The raw Miseq reads were filtered for reads where at least half of the nucleotides had a minimum Phred quality score of 20 to remove low-quality reads, and were then submitted to IMGT for V(D)J germ-line alignment.[44] Sequence data were again filtered for productive V_H or V_L, and for in-frame V(D)J junctions (CDRH3 or CDRL3), and were then matched using their Illumina read IDs. CDRH3 nucleotide sequences were clustered to 96% identity, and CDRH3:CDRL3 pairs represented by 2 or more reads were rank-ordered. In two separate sequencing runs the complete V_H and V_L sequences encoded within the linked V_H:V_L amplicons were determined. The sequences for the V_H:V_L junction and the separate V_H and V_L sequences were assembled to yield natively paired full length V_H and V_L sequences, as described.[30]

Antibody expression and characterization

Consensus V_H and V_L sequences of the top 10 most abundant antibodies from the draining lymph node were synthesized using gblocks (IDT) and subcloned into pcDNA3.4 vectors (Invitrogen) as fusions with human IgG1 and kappa constant domains, respectively. After sequence verification, the heavy and light chain plasmids were mixed at 1:3 ratio, and transfected into Expi293 cells for antibody expression. Six days after transfection, the supernatant was collected and antibodies were purified using protein A chromatography (Thermo Scientific).

To verify the specificity of these antibodies, ELISAs were carried out with both ricin A chain and BSA as a control. ELISA plates were coated with 50 μl of 4 μg/ml ricin A chain or BSA at 4°C O/N. On the next day, plates were blocked with PBSB at RT for 2 hr. After that, 1:5 serially diluted antibodies with concentrations ranging from 300 nM to 3.84 pM were added to the plates and the plates were incubated at RT for 1 hr. After washing with PBST, 50 μl of 1:5000 diluted HRP-conjugated donkey anti-human antibody (Jackson ImmunoResearch, cat# 709-035-149) was added and the plates were again incubated at RT for 1hr. After washing with PBST, 50 μl of TMB substrate was added and the reaction was stopped after 10 min by adding 50 μl of 2 M H_2SO_4. The absorbance at 450 nm was read and EC_{50} was calculated. For each antibody that showed binding to ricin A chain, affinity determinations were carried out via SPR analyses, as described above for scAbs.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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