

The Remarkable Flexibility of the Human Antibody Repertoire; Isolation of Over One Thousand Different Antibodies to a Single Protein, BLyS

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It is well established that the humoral immune response can generate antibodies to many different antigens. The antibody diversity required to achieve this is believed to be substantial. However, the extent to which the immune repertoire can generate structural diversity against a single target antigen has never been addressed. Here, we have used phage display to demonstrate the extraordinary capacity of the human antibody repertoire. Over 1000 antibodies, all different in amino acid sequence, were generated to a single protein, B-lymphocyte stimulator (BLySTM protein). This is a highly diverse panel of antibodies as exemplified by the extensive heavy and light chain germline usage: 42/49 functional heavy chain germlines and 19/33 V_λ and 13/35 V_κ light chain germlines were all represented in the panel of antibodies. Moreover, a high level of sequence diversity was observed in the V_H CDR3 domains of these antibodies, with 568 different amino acid sequences identified. Thus we have demonstrated that specific recognition of a single antigen can be achieved from many different VDJ combinations, illustrating the remarkable problem-solving ability of the human immune repertoire. When studied in a biochemical assay, around 500 (40%) of these antibodies inhibited the binding of BLyS to its receptors on B-cell lines. The most potent antibodies inhibited BLyS binding with sub-nanomolar IC₅₀ values and with sub-nanomolar affinities. Such antibodies provide excellent choices as candidates for the treatment of BLyS-associated autoimmune diseases.

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Introduction

Monoclonal antibodies (mAbs) are fundamental to many areas of biological science as laboratory tools for both research and diagnostic purposes.¹ Human mAbs are of particular interest, as these minimize the risk of immune reactions in patients and hold significant promise as therapeutic entities. Although difficult to generate in the past, human mAbs have recently enjoyed a renaissance as a consequence of technology advances in phage

display and transgenic mice.² Transgenic technologies generate between one and six different human mAbs^{3–6} per target protein and phage display up to 20,^{7–10} although both approaches can potentially generate more mAbs than actually reported.

Phage display enables the rapid selection of antibodies from large single-chain fragment variable (scFv) libraries by virtue of their binding to the target antigen. After two or three rounds of selection, the resulting phage population is markedly enriched for scFvs that bind the antigen specifically.² We have applied this technology to isolate antibodies to a recently identified member of the tumor necrosis family (TNF) superfamily, B-lymphocyte stimulator (BLySTM protein).¹¹ BLyS

Abbreviations used: mAb, monoclonal antibody;
IMAC, immobilized metal affinity chromatography.

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plays a key role in stimulating B-cell proliferation, differentiation and survival,^{11–13} through binding to specific receptors expressed exclusively on B-cells.^{14,15} Administration of BLyS *in vivo* results in splenic B-cell hyperplasia and elevated immunoglobulin secretion,¹¹ and mice over-expressing BLyS have been shown to develop symptoms characteristic of autoimmune conditions such as systemic lupus erythematosus.^{14–17} Antibody antagonism of the effects of BLyS therefore has potentially important therapeutic value in the treatment of B-cell-associated autoimmune disease.

Selection of phage antibody libraries on proteins like BLyS typically produces populations ranging from a few thousand to several million antibodies. However, only a tiny fraction, typically 10–100 (<1%), of all the potential antibodies in these populations are sampled routinely. This may preclude the identification of mAbs with the desired or optimal attributes, especially if clinical candidates are being sought. To address this issue, we have combined antibody isolation by phage display with a high-throughput DNA sequencing approach to characterize a panel of antibodies on a scale never previously reported. Using this approach, we demonstrate that the human antibody repertoire is extraordinarily diverse and has the potential to generate thousands of antibodies to a single antigen.

Results

Antibody isolation

Purified recombinant BLyS (51 kDa, homotrimer) was generated in insect and bacterial systems, and used as the sole target protein for antibody isolation. Large, non-immunized, human scFv phage display libraries,¹⁰ expanded recently from a total of 10^{10} to 10^{11} clones, were used for all selections. These libraries contain *in vivo* rearranged V genes derived from over 80 people. Selections were performed on BLyS immobilized on plastic, on biotinylated BLyS bound to streptavidin-coated plates, or on biotinylated BLyS in solution.¹⁸ Up to five rounds of selection were performed and phage antibodies from the second round of selection onwards were screened by ELISA¹⁹ for specific binding to BLyS and not to an unrelated (BSA) or related (TNF α) protein (Figure 1). Antibodies that recognized BLyS with an ELISA signal at least threefold higher than that observed on the control antigens were deemed to be specific for BLyS. Over 7000 antibodies on 81 (96 well) plates were screened in this way and 2875 (39%) were demonstrated to bind human BLyS specifically (Table 1).

Antibody diversity

The nucleotide sequence of all 2875 anti-BLyS antibodies was determined and validated scFv sequence generated for 2508 (87%). To identify duplicates, we first organized the antibodies into

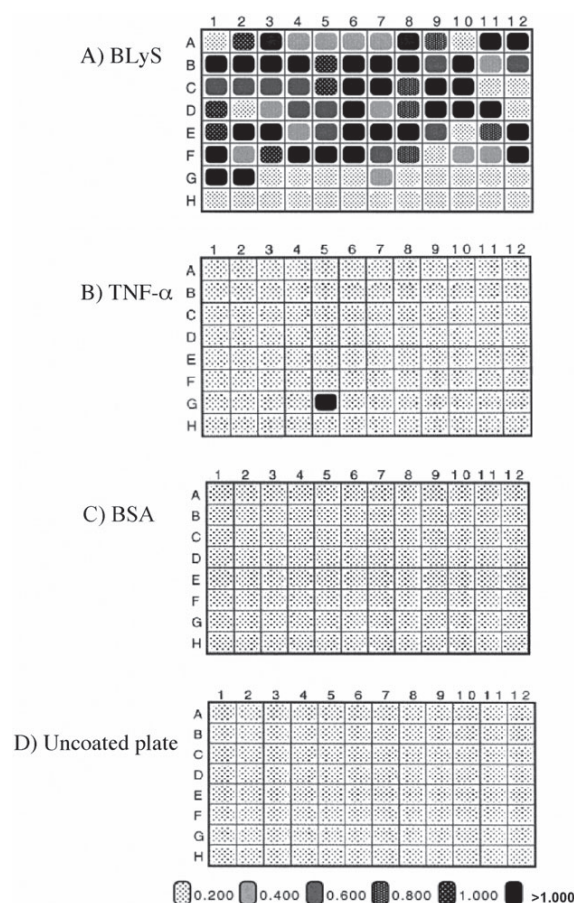


Figure 1. ELISA demonstrating specificity of antibodies for BLyS. Anti-BLyS antibodies were tested by phage ELISA for binding to (A) BLyS, (B) TNF- α , (C) BSA, or (D) an uncoated plate. Data are presented as a grey-scale scan representing different intensities of ELISA signal (A_{450} scale indicated). Antibodies that recognized BLyS with an ELISA signal at least threefold higher than that observed on TNF- α and BSA were deemed to be specific for BLyS. Using these criteria, this plate (derived from panning selections on baculovirus derived BLyS) was shown to contain 67 antibodies that recognize BLyS specifically and which do not cross-react with TNF- α , BSA or an uncoated plate. Two control “irrelevant” antibodies were included for reference (well G4, anti-eotaxin antibody; well G5, anti-TNF- α antibody) and wells G8 to G12 and H1 to H12 were left blank (no antibody).

groups with identical variable heavy (V_H) and light (V_L) chain amino acid sequences. This analysis identified a remarkable total of 1287 different antibodies to BLyS (Figure 2(A)). The antibodies within this panel were comprised of 1098 different V_H sequences combined with any one of 705 different V_L sequences (Figure 2(B)).

The majority of the antibodies were isolated just once, with only a handful of the antibodies being isolated several times (Figure 2). Thus, by analyzing the full sequences of the scFv produced in our selections on BLyS we were able to demonstrate

Table 1. Summary of BLyS selection strategies, ELISA screening and DNA sequencing results

Selection method	Source of BLyS protein (expression host)	Chemical modification	Selection round	BLyS ELISA positives (%)	Number of different antibodies
Panning	Baculovirus	None	2	307/1920 (16)	492
			3	1071/3072 (35)	
Panning	Baculovirus	Biotinylated	2	103/288 (36)	110
			3	92/96 (96)	
Panning	<i>E. coli</i>	None	2	265/288 (92)	237
			3	285/288 (99)	
Soluble	Baculovirus	Biotinylated	2	177/384 (46)	448
			3	214/384 (55)	
			4	204/480 (43)	
			5	157/192 (82)	
Total				2875/7392 (39)	1287

Selections were performed either by panning (BLyS immobilized on plastic or biotinylated BLyS immobilized on streptavidin plates) or in solution (biotinylated BLyS captured using streptavidin-coated magnetic beads). ELISA analysis of selections was conducted from round 2 onwards. For each round of selection, the number of ELISA positives/the total number of clones screened are given (with percentage in parentheses). The nucleotide sequence of all ELISA positives was determined and the number of different antibodies from each selection strategy is shown.

that the panel is comprised mainly of distinct scFv but also of scFv that have been enriched as a result of the selection process. Each selection strategy contributed a significant number of antibodies to the panel of 1287, indicating that antibody diversity can be influenced by both the source of antigen and the manner in which it is presented to the phage library (Table 1).

V_H and V_L germline usage

To illustrate the diversity within the antibody panel, the closest human germline for each anti-BLyS antibody was identified by aligning its nucleotide sequence to germline V_H, V_L, D (diversity) and J (joining) databases^{20–24} using BLASTN (version 2.0.9). The results are presented as dendograms that illustrate the familial relationships between the different germlines (Figure 3(a)–(d)). The heavy chain germline usage for a panel of antibodies to a single protein antigen is very extensive, with 42 of the possible 49 functional V_H germlines, 24/27 D and all six J_H germlines represented (Figure 3(a) and (b)). The light chain usage, although less diverse than that seen for the heavy chain, is still considerable with 19 of 33 known functional V_κ germlines, 13 of 35 functional V_λ germlines, three of seven J_κ and four of five J_λ germlines exemplified (Figure 3(c) and (d)).

Detailed analysis of the dendograms reveals that the most frequently used heavy chain germline family is V_H1 (68%), with V_H1-69 (21%), V_H1-e (12%), V_H1-03 (12%) and V_H1-18 (9%) most prevalent. Since these four V_H1 germlines appear to be structurally preferred for BLyS binding, it is no surprise to find that they are closely related and are predicted to arise from a common ancestor (Figure 3(a)). Light chain germline usage was restricted predominantly to the V_λ family (92%) in preference to V_κ (8%), with the V_λ 2a2 (33%), V_λ 3l

(24%), V_λ 1c (12%) and V_λ 1e (9%) most prevalent (Figure 3(c)).

V_H CDR3 sequence diversity

A further indication of antibody diversity can be derived from an analysis of V_H CDR3 (complementarity-determining region) sequences. This region of an antibody is highly diverse and there is evidence to suggest that it is the key determinant of specificity in antigen recognition.^{25,26} In the panel of 1287 anti-BLyS antibodies, we identified a total of 568 distinct V_H CDR3 s, ranging in length from five to 25 amino acid residues (Figure 4). Again, this represents a level of diversity previously unreported for a single protein and suggests that antibodies may have been generated to many epitopes.

The V_H CDR3 domain of an antibody is generated by recombination of three sets of germline heavy chain segments, the variable (V_H), diversity (D) and joining (J_H) genes.²⁷ As would be predicted for a panel of antibodies utilizing 568 different V_H CDR3 sequences, the V_H, J_H and D germline gene usage is highly diverse (Figure 3(a) and (b)). However, there are preferences in both V_H germline usage (described above) and also in D gene usage (Figure 3(b)). Nearly 35% of antibodies in the panel possessed V_H CDR3 sequences related to D gene segment D3-9 (YYDILTGYYN), indicating a strong preference for this motif in BLyS binding (see Table 2 for examples).

A selection of 33 different V_H CDR3 domains is presented in Table 2. These V_H CDR3 domains were all derived from antibodies that inhibit BLyS binding to its receptors on B-cell lines (see also Antibody characterization section below). The majority of the 33 V_H CDR3 domains presented were observed between one and 20 times in the total panel of 1287. Two exceptions were the V_H CDR3 sequences

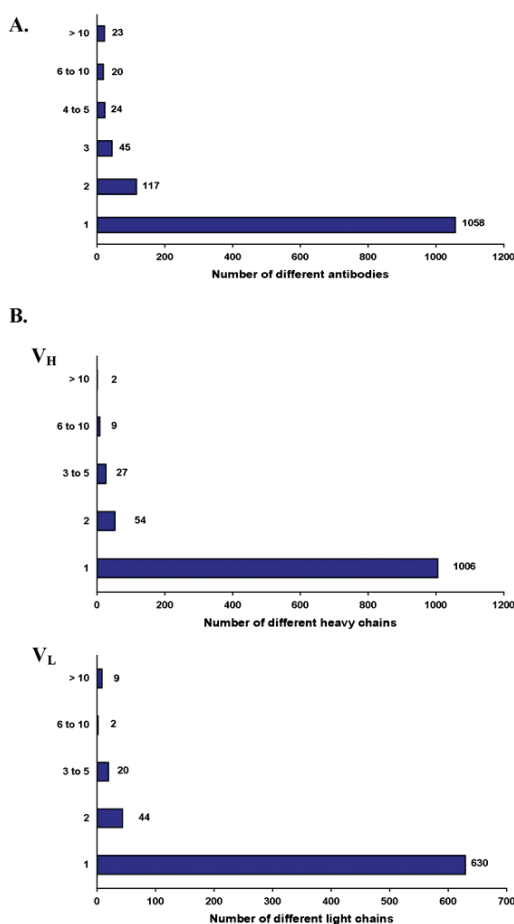


Figure 2. (A) Frequency of isolation of different anti-BLyS antibodies. Antibodies were grouped according to whether they were isolated on one (1058 members), two (117 members), three (45 members), four or five (24 members), six to ten (20 members) or more than ten (23 members) occasions. The large majority (82%) of the 1287 different antibodies were isolated just once in the panel of 2508. (B) Frequency of isolation of different V_H and V_L in the final panel of 1287 antibodies. In all, 1098 different V_H (distinct number of VDJ) and 705 different V_L (distinct number of VJ) were identified in the final panel and these were grouped according to the frequency at which they were isolated. A total of 1006 different V_H sequences and 630 different V_L sequences were utilized just once in the panel of 1287 antibodies, indicating that sequence diversity was greatest in the V_H region.

ELGLSIVGATTGALDM and ATYDPLTGYSFDGFDI that were observed in 65 and 167 different antibodies, respectively. Therefore, some V_H CDR3 domains were observed in more than one antibody in the final panel of 1287. The remaining diversity was generated through unique V_H-V_L pairings and by the presence of additional point mutations.

Unique V_H-V_L pairings

Of the 568 different V_H CDR3 domains that were

observed, several were observed in the context of different V_H germline sequences. Moreover, a high degree of light chain diversity was observed, with a single heavy chain sequence observed to pair with multiple light chain variants.

In Table 2, it can be seen that five of the V_H CDR3 sequences were observed in more than one V_H germline sequence, and that these tended to be restricted to particular families. For example, V_H CDR3 sequence EGSYDILTGYVGVGRMDV was observed with five different V_H germlines of the V_H3 family (3-30.3, 3-30.5, 3-23, 3-53, 3-66). Similarly, V_H CDR3 sequence DGSYDILTGYID NYMDV was observed with three different V_H germlines of the V_H1 family (1-08, 1-18, 1-69). However, there were exceptions to this rule; for example, V_H CDR3 ATYDPLTGYSFDGFDI, which was observed predominantly in a V_H1 backbone (1-24, 1-03, 1-46, 1-e, 1-02, 1-69) but was observed also in the context of V_H4 (4-39) (Table 2).

Some preference in V_L pairing was observed (for example, V_λ 2a2 and 3l) but, in general, these were highly diverse. Again, focusing on the 33 V_H CDR3 domains in Table 2, it can be seen that light chains from multiple families can pair with a single V_H. Moreover, in several cases either a lambda or kappa V_L can combine with the same V_H (or VDJ combination) with no loss in antigen specificity. For example, V_H CDR3 ELGLSIVGATTGALDM was observed in a single V_H framework (1-e) but was paired with any one of 11 different V_λ germlines from multiple families (1a, 1b, 1c, 1e, 1g, 2a2, 3h, 3l, 3r, 5c, 10a) or with a single V_κ germline (A27). In another example, V_H CDR3 SHYDILTGLNYWYFDL was observed in the context of three different V_H germlines (3-30.3, 3-30.5, 3-33) that were paired with any one of three different V_λ germlines (1b, 2a2, 3l). Ten antibodies possessed this V_H CDR3 sequence and their respective V_H and V_L germline pairings are detailed in Table 3.

Point mutations between antibody siblings

In addition to the 568 different V_H CDR3 domains and the wide range of V_H-V_L pairings that were observed, other amino acid differences were observed between otherwise identical antibodies (siblings) that enabled us to define an antibody as “unique”. Such amino acid differences may have arisen as a result of somatic hypermutation²⁸ or may have been introduced by PCR error during library construction.¹⁰

To illustrate an example of the mutations that were observed between siblings, Figure 5 shows an alignment of seven antibodies all with the same V_H germline 1-e, V_L germline 1b and V_H CDR3 ELGLSIVGATTGALDM. These antibodies differ from each other by between 1 and 26 amino acid residues, illustrating the considerable diversity that can exist even between siblings. Interestingly, the amino acid differences that are observed are distributed throughout the entire antibody

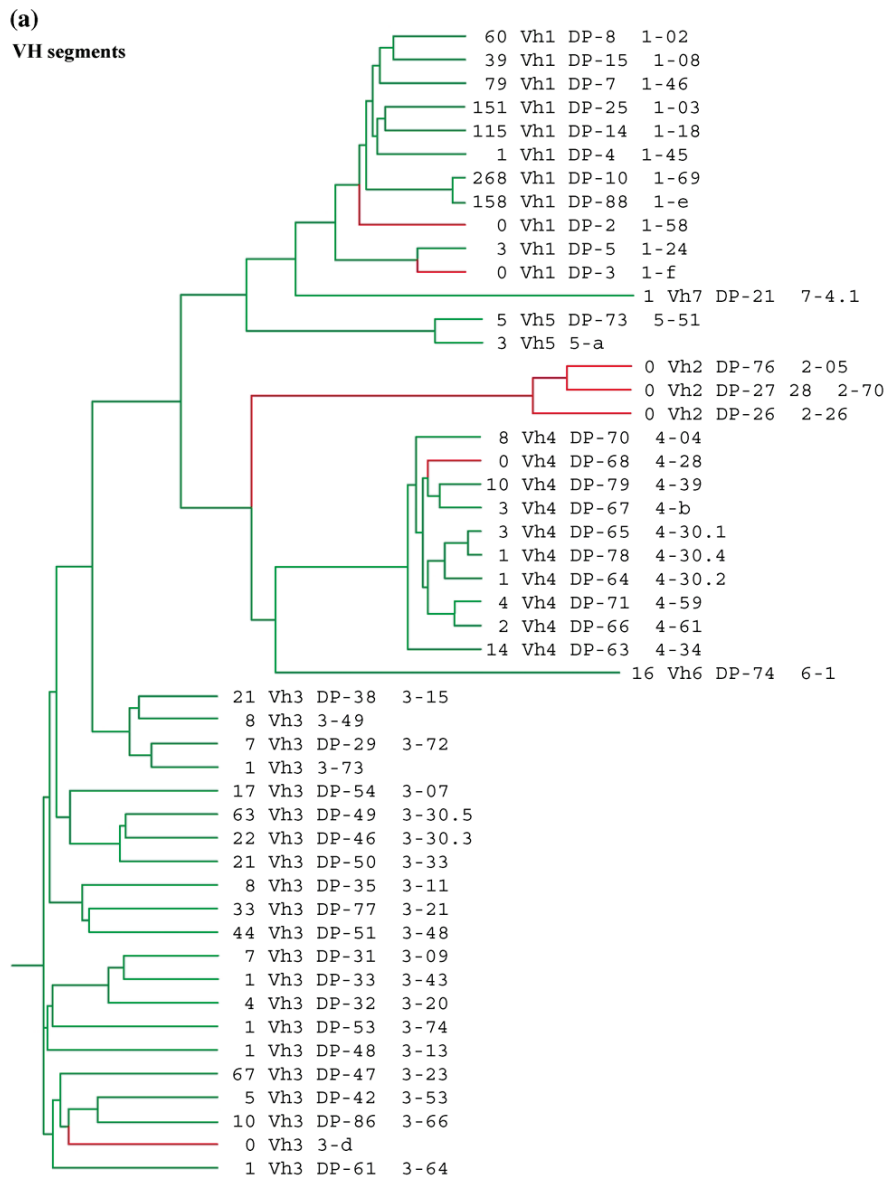


Figure 3 (legend on p.110)

sequence, with changes seen in both framework and CDR regions.

Identification of BLyS antagonists

We have described here a panel of 1287 different anti-BLyS antibodies that recognize BLyS by ELISA. In addition, we developed a biochemical assay to determine whether any of these antibodies were able to antagonize the binding of BLyS to its receptors expressed on B-cell lines. The assay that was developed measured the specific binding of biotinylated BLyS to its receptor on IM9 myeloma B cells using europium-labelled streptavidin

followed by dissociation enhancement time-resolved fluorometry for detection.²⁹

All of the 1287 anti-BLyS antibodies were prepared as phage supernatants and screened in this assay to identify potential inhibitors of BLyS binding. Although the active concentrations of each antibody could not be normalized as a consequence of using phage supernatants, the assay still proved a useful preliminary screen for activity with around 500 (40%) of the antibodies demonstrating an ability to inhibit BLyS binding to IM9 B cells (for example data, see Figure 6). Moreover, when assessed in detail, the assay results enabled us to rank the anti-BLyS antibodies into groups with potentially different potencies. Approximately

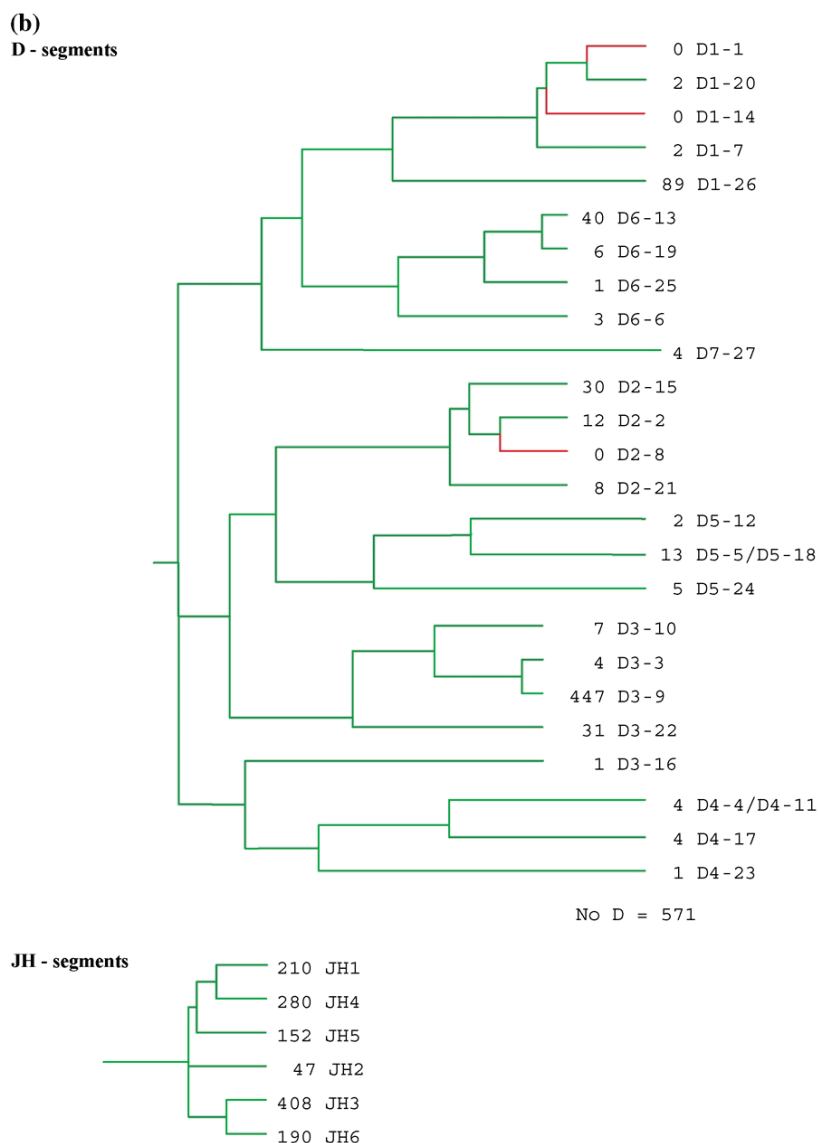


Figure 3 (legend on p.110)

100 antibodies inhibited BLYS binding by over 80%, a further 200 antibodies inhibited binding by 60–80% and another 200 inhibited binding by 40–60% (data not shown). Antibodies that did not inhibit BLYS binding by at least 40% were deemed non-inhibitory.

Thirty-three anti-BLYS antibodies that blocked BLYS binding by over 80% in the preliminary screen were prepared as immobilized metal affinity chromatography (IMAC)-purified scFv and re-tested in the inhibition assay to determine IC_{50} values. These antibodies are highly diverse by sequence (see Table 2) and, as purified scFv, inhibited BLYS binding with IC_{50} values ranging from 9 nM to 420 nM (Table 4). The most potent antibodies were derived from panning selection strategies with nine of the top ten antibodies being

derived from selections on BLYS immobilized on plastic or on biotinylated BLYS immobilized on streptavidin plates. Inhibitory antibodies were isolated also from the soluble selection strategies, the most potent being AB-6 (18 nM) but, in general, these were of lower potency than those isolated from panning strategies. No inhibitory antibodies were isolated from selections performed on BLYS produced in *Escherichia coli* expression systems. Example IC_{50} data, including that of the most potent scFv identified in the panel, is presented for ten antibodies in Figure 7(A).

Five anti-BLYS scFv were converted to IgG format and then re-tested in the BLYS inhibition assay. Following conversion to IgG format, three of the antibodies (AB-3, AB-7 and AB-12) showed improved potencies in the assay (Figure 7(B);

(c)

VL segments

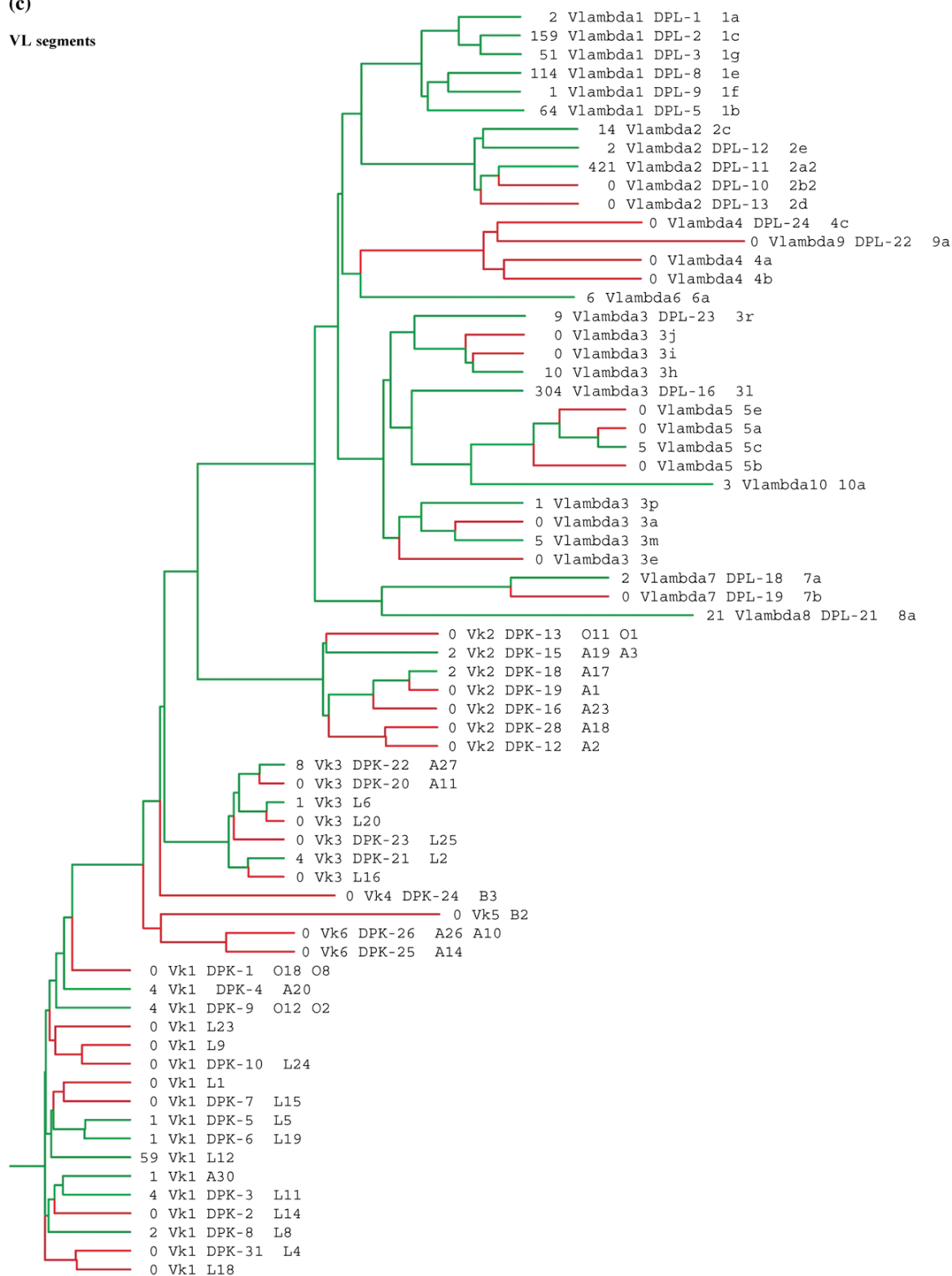


Figure 3 (legend on p.110)

(d)

JL segments

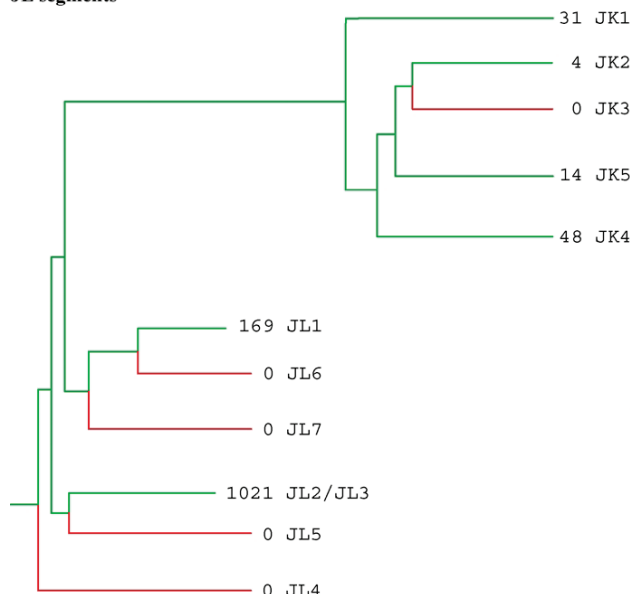


Figure 3. Anti-BLyS antibody germline usage. (a) The human immunoglobulin $V_{H\alpha}$, (b) D and $J_{H\alpha}$, (c) V_L (combined V_{κ} and V_{λ}) and (d) J_L (combined V_{κ} and V_{λ}) germline family gene usage. The total number of anti-BLyS antibodies (out of 1287) utilizing the different germline gene segments is listed together with the germline locus name.^{20–24} Results are shown as dendograms illustrating familial relationships. Leaf nodes in green indicate that the given germline was observed. Note that for 571 antibodies in the panel the V_H CDR3 domains were either too short or too novel to confidently assign a D gene segment (No D).

Table 5). This increase in potency was particularly striking for AB-3 which improved 130-fold following conversion to IgG. Consequently, AB-3 IgG had an IC_{50} value in the sub-nanomolar range and compared very favourably with BLyS itself in the same inhibition assay (Figure 7(B)). These increases in potency are most likely due to avidity effects following conversion of these antibodies from a monomeric scFv format to dimeric IgG. Since BLyS, like other members of the TNF family, is a homotrimeric protein,³⁰ it is feasible that some of the anti-BLyS antibodies, once converted to IgG, will be able to bind to two binding sites on BLyS

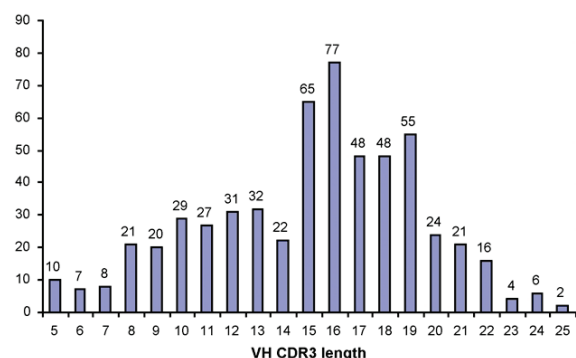


Figure 4. Distribution of V_H CDR3 length. V_H CDR3s ranged between 5 and 25 amino acid residues in length. V_H CDR3 regions were defined according to Chothia & Lesk.⁵¹ Between 15 and 19 amino acid residues in length were used most frequently. The mean V_H CDR3 length, 16.4, is higher than reported previously,⁵² suggesting that longer V_H CDR3 domains are preferred for BLyS binding.

simultaneously, thereby increasing the effective potency of the antibody. However, this bonus effect is most likely to be epitope-dependent due to the structural constraints of an IgG molecule. Consequently, not all of the anti-BLyS antibodies have increased in potency following conversion to IgG format, e.g. AB-1 and AB-2 (Table 5).

The binding affinities of the five antibodies converted to IgG format were determined by BIAcore analysis. Affinity measurements (IgG) and IC_{50} data generated from the binding assay (scFv and IgG) for these five antibodies are presented in Table 5. These antibodies bind to BLyS with affinities ranging from 400 pM to 12 nM and further support our findings¹⁰ and those made by others,^{7–9} that potent, high-affinity antibodies can be isolated directly from large non-immune phage antibody libraries. Interestingly, despite two of these antibodies being of sub-nanomolar binding affinity, they did not dominate the selection process, indicating that antigen binding affinity is not the only factor influencing phage selections. Moreover, the most potent antibody identified in the BLyS inhibition assay (AB-3) was not the highest-affinity antibody as determined by BIAcore. This would suggest that factors other than affinity, e.g. epitope recognition and avidity, play a significant role in determining how well an antibody inhibits ligand binding.

Discussion

Here, we have described the integration of two technologies, antibody phage display and high-throughput DNA sequencing, to isolate and

Table 2. Combinatorial diversity of inhibitory anti-BLyS antibodies

V _H CDR3	N	V _H germlines utilized	V _L germlines utilized	
			V _λ	V _κ
DRYDILTGYYYGMDV	1	6-1	2a2	
VQMDSEYYDLLTGINVGPYYFDY	5	1-18	2a2, 3l	
PFYDILTYSYVFQYFDH	2	1-18	2c	A27
DGIDILLVPAALMDV	4	1-18	2a2	
GRWDYDLLTGEHLGYFDY	1	4-61	3l	
ELGLSIVGATTGALDM	65	1-e	1a, 1b, 1c, 1e, 1g, 2a2, 3h, 3l, 3r, 5c, 10a	A27
APYDLLTHYFHYFDY	1	1-69	3l	
QDNDPLTGKLGFDY	1	1-03	1e	
AATTSQKHNKYAYFYGMDV	4	1-69	1c, 2a2	A19
HDDDVLTGYFES	1	5-51	2a2	
FNPTYDILTGYYIGGYFQH	3	3-15	2a2	
DGYDILTGSYYGMDV	1	3-30.5	3l	
DQGRYLDL	23	1-69	1b, 1c, 1e, 1g, 3h, 3l, 3p, 7a	L2
GMGDHYGMDV	21	1-69, 1-46	1c, 2a2, 3l	
DEGRDLLTGYYWPNFFDS	2	1-18	1c	
ATYDPLTGYSFDGFDI	167	1-24, 1-03, 1-46, 1-e, 1-02, 1-69, 4-39	1c, 2a2, 3l, 6a	
DRGAPNYDILTGYYAPAQGVAFDI	1	1-03	1b	
SHYDILTGLNYWYFDL	10	3-30.3, 3-30.5, 3-33	1b, 2a2, 3l	
DGRLSYDILTGYYARDYYGMDD	1	1-18	2a2	
GRETDKVKPWDRYYHYMDV	13	1-69	1b, 1c, 1e, 2a2, 2c, 3l, 5c	
GGDYDILTGLYYGMDV	1	3-23	1c	
SLATRPLGMDV		1-69	2a2	
GYHDLTSYNNWFDP	1	1-03	3l	
SEGTIFGVD	1	1-69		L11
EKSAAGYFDY	1	1-e		L12
DGSYDILTGYYIDNYMDV	10	1-08, 1-18, 1-69	1c, 2a2, 3l	
GYDSSAFRAFDI	19	1-69	1b, 1c, 1e, 1g, 3l	L2, A27
SSPPKWYDALTGDSYHSAMDV	7	1-69	1b, 1c, 1e, 3l	O12
EGGNYDILTGYYIGNGAFDI	15	3-21	2a2	
EGSYDILTGYYVGVGRMDV	9	3-30.3, 3-30.5, 3-23, 3-53, 3-66	2a2, 3l	
DREAYDILTGYYLYYYMDV	1	3-30.3	2a2	
GGEYDILTGYYFGLGVYDY	2	1-46	1b	
TDYDILTGYPMGYFDP	2	1-18	2c, 3l	

In all, 33 different V_H CDR3 sequences are listed according to the frequency (N) at which they were observed in the panel of 1287 anti-BLyS antibodies and the different V_H/V_L germline sequences in which they were used. For example, V_H CDR3 GYDSSAFRAFDI was observed 19 times in the panel of 1287 antibodies. All 19 antibodies with this V_H CDR3 possessed a V_H derived from the germline sequence V_H1 1-69. However, the 19 antibodies with this V_H CDR3 sequence were also observed to be paired with a wide range of different light chains, including five different V_λ families and 2 V_κ families. The closest human germlines for the V_H and V_L are given as locus names.^{20–24} V_H CDR3 is defined according to Chothia & Lesk.⁵¹

characterize a large panel of human monoclonal antibodies to BLyS. Using a large (10¹¹) phage antibody library prepared from human B-cells, selections were performed on BLyS and over 7000

Table 3. V_H–V_L pairings observed for a single V_H CDR3 family

		V _H		
		3-30.5	3-33	3-30.5
V _L (λ)	3l	4	1	1
	2a2	1	2	0
	1b	0	1	0

The V_H CDR3 sequence SHYDILTGLNYWYFDL was observed in ten antibodies out of the final panel of 1287. This V_H CDR3 was utilized in three different V_H germline families that, in turn, were paired with three different V_L germlines. The proportion of the ten antibodies with a particular V_H–V_L pairing is shown. For example, four of ten antibodies with this V_H CDR3 were V_H 3-30.5 and V_L λ3l. The closest human germlines for the V_H and V_L are given as locus names.^{20–24}

antibodies picked for further study. By ELISA, 2875 (39%) of these antibodies recognized human BLyS specifically and did not cross-react with other irrelevant antigens. This figure is consistent with previous studies using phage display, since antibodies are enriched and amplified through successive rounds of selection and high (30–90%) numbers of binders by ELISA are typically observed.^{7,8,31}

We analyzed the sequences of over 2500 scFv produced against BLyS and identified 1287 distinct antibodies utilizing a wide array of germline families. The V_H diversity is particularly striking in its almost unqualified germline gene usage, with 42 of the possible 49 functional heavy chain germline families represented in the clone panel. Only the V_H2 subfamily, which is used rarely during an immune response, is not represented in the selected antibody panel. This may well represent a structural constraint for this particular antigen, since antibodies with V_H2 germlines have been isolated from this library.³¹ Of the V_H

VH_{V_H11e}

1	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	121
1	QVHLVQSGAEVKKPGSSVKVSCRASGGSFN	RHAIS	WVRQAPGRGLEWNG	GILPMYGTANYAQKFQG	RVTITADKLINTVYMDLSRLRYEDTAVYYCAR	ELGLSIVGATTGALDM	WGKGTILVTSS	
2	..Q.	..	..	..	..	..	..	..
3	..Q.	..	..	..	..	..	..	..
4	E.Q.	..	..	..	..	..	..	..
5	D.Q.L.	..	L..R.	..Y.	..	..	..	..
6	..Q.	..	..	..	..	..	..M.	..
7	..Q.	..	..	..	..	..	..Q..M.	..

VL_{V_L11b}

1	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	110
1	QSVLTQPPSVSAAPGQKVTISC	SGSSNIGNNNYS	WYQQIPGTAPKLLIY	ENNKRP	GIPDRFSGSKGASATLIDITGLQTGEADYYC	GTWHSSQV...V	FGGGTKLTVLGA	
2	..	..	..	..	..	..	..	..
3	..V..A.	..	..L.	D.D.	..V...T.S.A.	..D..LSAG.	..V.	..
4	..V..A.	..SY.	..L.	D.D.	..V...T.S.A.	..D..LSAG.	..V.	..
5	..	..SY.	..VF.	DSHR.	..A.T.DTA..A.	..A.D.KLNAY.	..T.Q.	..S.
6	..	..Q.	..HF.	DK.Q.	..T...T...V.	..D..LSHSA.	..	..
7	..	..I.	..	..	..E.	..	..	..

Figure 5. Point mutations that exist between antibody siblings. Seven antibodies (numbered 1–7), all utilizing V_H germline 1–e, V_L germline 1b and V_H-CDR3 ELGLSIVGA TTGALDM, were aligned to illustrate the additional amino acid differences that exist between antibody siblings. Amino acid changes between the antibodies are shown; dots indicate areas of amino acid homology and the CDR regions are boxed. In this example the antibodies differ from each other by between 1 and 26 amino acid residues, with changes observed in both the framework and CDR regions of the antibody. Other differences are noticeable between these antibodies, such as amino acid insertions in the V_L CDR3 domains.

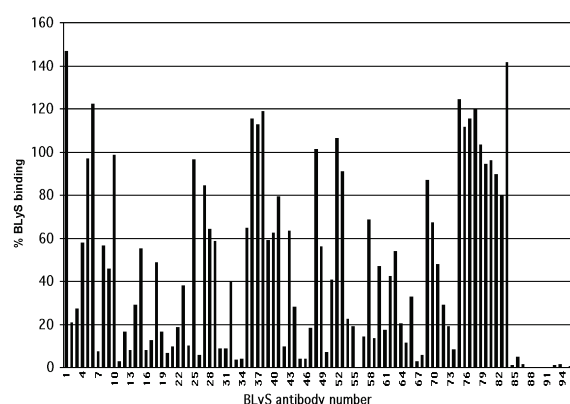


Figure 6. Inhibition of BlyS binding to its receptor on IM9 B cells by anti-BlyS antibodies. A panel of 74 different anti-BlyS antibodies were analyzed for their ability to inhibit the binding of biotinylated BlyS to IM9 cells. Percentage BlyS binding was determined using the fluorescence values obtained for “total binding”, obtained in the absence of antibody (nos 79–84), and “no binding”, obtained when no biotinylated BlyS was present (nos 85–96). Of the anti-BlyS antibodies tested here (nos 1–74), several candidates were identified that potentially antagonized BlyS binding to IM9 cells. The ability of each antibody to inhibit the binding of BlyS was compared with an irrelevant non-blocking antibody (nos 75–78).

germline families represented in the clone panel, the V_H1 germline is represented most often (68%) followed by V_H3 (27%) and V_H4 (4%). This level of V_H1 representation is much higher than that reported for B-cells isolated from peripheral blood, where V_H3 (56%) and V_H4 (20%) predominate over V_H1 (13%).³² Whilst the high representation of the V_H1 germline in the BlyS panel may be related directly to the numbers of each germline family in the starting library, it may have arisen because the *in vivo* antigenic constraints of the immune repertoire are not applicable to the phage *in vitro* system. Therefore, the germline usage *in vitro* is likely to be antigen-dependent and it is essential to maintain and develop antibody libraries with the highest possible level of diversity to expedite efficient selection on any antigen.

We have demonstrated here that the population of antibodies produced by phage display is influenced strongly by both the source of the antigen (expression host) and how it is presented to the library. It has been reported elsewhere that differences in panning strategies can influence antibody diversity,³³ and we observed similar results in our panning strategies, depending on whether we immobilized BlyS onto plastic or biotinylated BlyS onto streptavidin plates. In addition, selections on BlyS in solution and on BlyS derived from *E. coli* expression systems (which will lack any post-translational modifications) produced unique antibodies that were not seen in our other

selection strategies. These observations illustrate that antibody sequence diversity can be maximized by not building any restrictions into either the starting antibody library or the antibody isolation strategies.

Looking at the antibody sequences in more detail, the overall diversity could be attributed to several factors. First, nearly half (44%) of the anti-BlyS antibodies in the panel possessed different V_H CDR3 sequences. Since the V_H CDR3 domain is generated by V(D)J recombination, this result, coupled with the extensive V_H germline diversity, would suggest that there is considerable diversity to BlyS even at the level of the primary antibody repertoire.

In addition to the many V_H CDR3 sequences that were observed, diversity was also generated through unique V_H – V_L pairings. A total of 1098 different heavy chains and 705 different light chains were identified in the final panel of 1287 antibodies. V_L usage was particularly promiscuous, with several V_L from different germline families (both $V_{L\alpha}$ and $V_{L\kappa}$) observed to pair with a single heavy chain. Such light chain promiscuity has been observed before in phage display,^{10,34} and in antibodies derived by conventional hybridoma technology,³⁵ and in quasi-monoclonal mice.³⁶ These findings lend support to the hypothesis that the V_H contributes most to the binding specificity of an antibody.^{26,37}

The remaining diversity within the panel of 1287 antibodies was generated by additional mutations outside the V_H CDR3 domain. These additional point mutations may have arisen from either somatic hypermutation (up to 4% in V-genes) through untemplated, single-base substitutions²⁸ and, to a lesser extent through PCR error during library construction.¹⁰ Many such changes were observed in framework residues and in other CDRs, and these can often have significant effects on both antibody affinity and specificity.^{28,38} For example, the specificity of several anti-hapten antibodies has been altered by the addition of point mutations.^{39,40} Similarly, the specificity of a monoclonal antibody for 11-deoxycortisol was changed to cortisol by the introduction of mutations in both the CDRs and framework regions.⁴¹ Moreover, studies in quasi-monoclonal mice, whose primary antibody repertoire is virtually monoclonal, have shown that specificity can be altered through somatic hypermutation and secondary rearrangements.³⁶ Such changes in specificity were not observed in our panel of antibodies, but this was expected, since all the antibodies were selected and identified based upon their specific recognition of BlyS. However, in the BlyS inhibition assay, potency differences of up to tenfold were observed in certain groups of related BlyS antibodies (data not shown), indicating that even small numbers of amino acid changes can have dramatic effects on antibody potency. This is well documented, since amino acid mutations occur naturally in the process of somatic hypermutation

Table 4. Properties of 33 antibodies that inhibit BLYS binding to IM9 cells

Antibody ID	Selection strategy	V _H germline	V _H CDR3	V _L germline	Potency in BLYS inhibition assay (IC ₅₀ , nM)
AB-1	Pan (bio)	6-1	DRYDILTGYYYYGMDV	λ 2a2	9
AB-2	Panning	1-18	VQMDSEYYDLLTGINVGPYYFDY	λ3l	9
AB-3	Panning	1-18	PFYDTLTSYVFQYFDH	κ A27	13
AB-4	Pan (bio)	1-18	DGIDILLVPAALMDV	λ 2a2	15
AB-5	Panning	4-61	GRWDYDLLTGEHLGYFDY	λ3l	17
AB-6	Soluble	1-e	ELGLSIVGATTGALDM	λ1b	18
AB-7	Panning	1-69	APYDLLTHYFHYFDY	λ3l	21
AB-8	Panning	1-03	QDNDPLTGYKLGFDY	λ1e	21
AB-9	Panning	1-69	AATTSQKHNKYAYFYGMVDV	λ 2a2	22
AB-10	Panning	5-51	HDDVDLTGYFES	λ 2a2	23
AB-11	Pan (bio)	3-15	FNPTYDILTGYIIGGYFQH	λ 2a2	37
AB-12	Panning	3-30.5	DGYDILTGYSYGMVDV	λ3l	54
AB-13	Soluble	1-69	DQGRYLDL	λ1e	59
AB-14	Panning	1-69	GMGDHYGMVDV	λ 2a2	62
AB-15	Panning	1-18	DEGRDLLTGYWPNFFDS	λ1c	63
AB-16	Panning	1-24	ATYDPLTGYSFDFDI	λ 2a2	78
AB-17	Soluble	1-03	DRGAPNYDILTGYAPAQGVAFDI	λ1b	106
AB-18	Panning	3-30.5	SHYDILTGLNYWYFDL	λ3l	120
AB-19	Soluble	1-18	DGRLSYDILTGYARDYYGMDD	λ 2a2	130
AB-20	Soluble	1-69	GREDTDKVKPWDRYYHYGMVDV	λ 2a2	135
AB-21	Panning	3-23	GGDYDILTGLYYGMVDV	λ1c	135
AB-22	Soluble	1-69	SLATRPLGMDV	λ 2a2	144
AB-23	Panning	1-03	GYHDPLTSYNNWFDP	λ3l	155
AB-24	Soluble	1-69	SEGTIFGVD	κ L11	158
AB-25	Soluble	1-e	EKSAAGYFDY	κ L12	160
AB-26	Pan (bio)	1-08	DGSYDILTGYIDNYMDV	λ 2a2	160
AB-27	Panning	1-69	GYDSSAFRAFDI	λ1e	194
AB-28	Panning	1-69	SSPPKWDALTGDSYHSAMDV	λ1b	205
AB-29	Panning	3-21	EGGNYDILTGYIGNGAFDI	λ 2a2	300
AB-30	Pan (bio)	3-66	EGSYDILTGYVGVGRMDV	λ3l	350
AB-31	Pan (bio)	3-30.3	DREAYDILTGYLYYYMDV	λ 2a2	380
AB-32	Panning	1-46	GGEYDILTGYFGLGVYDY	λ1b	400
AB-33	Panning	1-18	TDYDILTGYPMGYFDP	λ3l	420

The selection strategy from which each antibody was derived is shown: Panning, selection on unmodified BLYS immobilized on plastic; Pan (bio), selection on biotinylated-BLYS immobilized on streptavidin plates; soluble, liquid phase selection on biotinylated-BLYS. The closest human germlines for the V_H and V_L are given as locus names. V_H CDR3 is defined according to Chothia & Lesk.⁵¹ For each antibody, inhibition constants (IC₅₀ values) were determined using IMAC-purified scFv in the BLYS inhibition assay.

to improve antibody affinity and to promote repertoire shift,^{42,43} and research groups often introduce mutations into antibody sequences to further optimize their antibody candidates *in vitro*.^{38,44}

In summary, several factors have accounted for the observed antibody diversity, from a pool of 568 different V_H CDR3 sequences up towards the final panel of 1287 different anti-BLYS antibodies through unique V_H-V_L pairings and the presence of additional point mutations. Even if we had critically defined an antibody “lineage” as a group of antibodies that all possessed the same V_H CDR3, V_L CDR3 and were derived from the same V_H and V_L germline, then 910 distinct lineages remained identifiable. This indicates that the majority (71%) of the observed diversity was generated through unique V_H-V_L pairings. While the original *in vivo* V-gene pairings are perhaps unlikely to have been recreated in the phage antibody library, our findings here have given a significant insight into the immense combinatorial flexibility and capacity of the human antibody repertoire to generate binding sites to individual protein antigens. Moreover, when one considers

that the diversity we have reported is to a self-antigen, and that in humans these same antibodies are unlikely to be produced *in vivo*, conclusions can be drawn on the excellent efficiency with which anti-self antibodies are edited from the human immune repertoire.

Whether the level of antibody diversity we have reported here for BLYS can be attained to other antigens remains to be elucidated. Other studies using phage libraries have demonstrated that antibodies utilizing different germline sequences can be isolated to a single antigen,^{8,10,34,45,46} although not on the scale reported here. However, it has been postulated that the primary antibody repertoire does possess a degree of degeneracy that enables broad antigen specificity and recognition,⁴⁷ and the present study does support this view. Potentially, the process we have described here, of high-throughput antibody isolation by phage display and DNA sequencing, could be utilized in future to define the diversity of the human antibody repertoire to many different antigens, including other self-antigens like BLYS.

The existence of an extensive panel of antibodies

Table 5. Binding kinetics and potencies of 5 anti-BLyS antibodies converted to whole IgG format

Antibody	Potency in BLyS inhibition assay (IC ₅₀ , nM)		Binding affinity (IgG)		
	scFv	IgG	k _{on} (1/Ms)	k _{off} (1/s)	K _D (M)
AB-1	9	46	3.0 × 10 ⁺⁰⁵	3.5 × 10 ⁻⁰³	1.2 × 10 ⁻⁰⁸
AB-2	9	95	1.5 × 10 ⁺⁰⁶	2.0 × 10 ⁻⁰³	1.3 × 10 ⁻⁰⁹
AB-3	13	0.1	7.5 × 10 ⁺⁰⁵	1.0 × 10 ⁻⁰³	1.3 × 10 ⁻⁰⁹
AB-7	21	6	2.2 × 10 ⁺⁰⁶	8.7 × 10 ⁻⁰⁴	4.0 × 10 ⁻¹⁰
AB-12	54	4	5.3 × 10 ⁺⁰⁵	2.5 × 10 ⁻⁰⁴	4.7 × 10 ⁻¹⁰

For each antibody, inhibition constants (IC₅₀ values) were generated using both purified scFv and IgG in the BLyS inhibition assay. Association (k_{on}) and dissociation (k_{off}) constants were determined by BIAcore for each antibody as purified IgG and K_D calculated as k_{off}/k_{on}.

to a biologically significant protein, such as BLyS, will both facilitate understanding of the molecule’s function as well as provide potential therapeutic agents. Indeed, by producing an antibody panel of

this size and diversity it is likely that antibodies to many different epitopes of BLyS have been isolated. Of the 1287 antibodies described here, several candidates were identified that were of very high affinity and were able to potently antagonize the binding of BLyS to its receptor. Such high-affinity, inhibitory, human antibodies could be utilized for the therapy of BLyS-associated autoimmune diseases and one candidate is presently being developed by Human Genome Sciences, Inc. as a novel therapy for the treatment of autoimmune disease.⁴⁸

Materials and Methods

Selections

Purified recombinant BLyS (Human Genome Sciences, Inc.), derived in baculovirus and *E. coli* systems and diluted to 10 μg/ml in PBS, was immobilized onto immunotubes (Nunc) overnight at 4 °C. Baculovirus-derived BLyS was biotinylated using NHS-biotin (Pierce) at a molar ratio of 20:1 biotin to BLyS. Biotinylated BLyS was immobilized on streptavidin-coated plates (Pierce) at 1 μg/ml in PBS for 30 minutes at room temperature. Selections were then performed as described.^{10,18}

Phage ELISA

BLyS, TNF-α and BSA, all at 1 μg/ml in PBS, were immobilized onto 96 well polystyrene plates (Falcon) overnight at 4 °C. Anti-BLyS antibodies were prepared as phage supernatants and screened by phage ELISA as described.^{19,33}

DNA sequencing

Each scFv was subjected to four or more sequencing reactions, at least two in each direction.¹⁰ For each scFv, the resulting trace files were analyzed and assembled using the Phred and Phrap software.^{49,50} The assembled sequence of each scFv was translated in all frames and the longest open reading frame (ORF) was parsed for expected elements. The translated ORF was assumed to be well-formed if it contained the pattern:

PAMA-{heavy chain}-GGGSGGGSGGGGS-{light chain}-AAAH

Here, PAMA is the translation of the 5’ cloning site, GGGSGGGSGGGGS is the linker, and AAAH is the

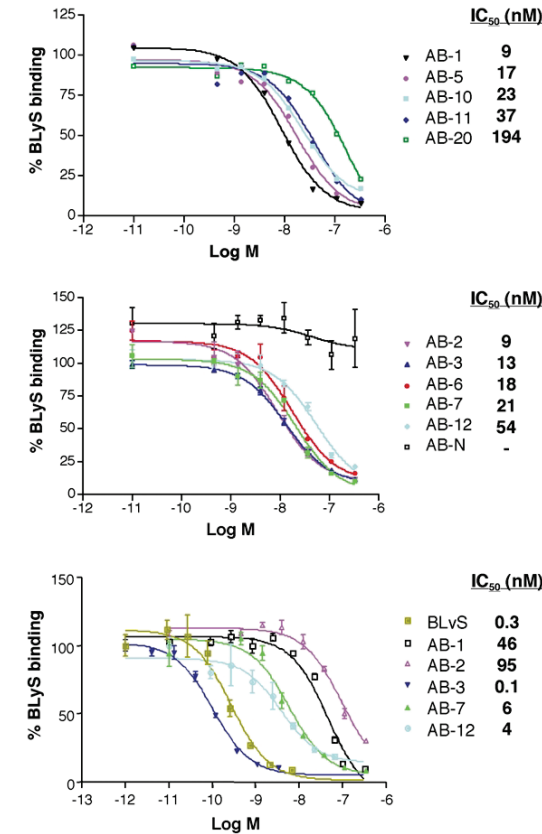


Figure 7. Inhibition of BLyS binding to its receptor on IM9 B cells by anti-BLyS antibodies in purified scFv and IgG formats. (A) The ten IMAC-purified scFv (see also Table 4) were titrated from 10 pM to 300 nM in the assay to observe dose-dependent inhibition of biotinylated BLyS binding to IM9 cells. IC₅₀ values were determined for each scFv and compared to a non-inhibiting anti-BLyS scFv (AB-N). (B) Five anti-BLyS antibodies, as purified IgG, were titrated from 1 pM to 300 nM in the assay to observe dose-dependent inhibition of biotinylated BLyS binding to IM9 cells. IC₅₀ values were determined for each IgG and compared to that observed for unmodified BLyS in the same experiment.

translation of the 3' cloning site. In some cases where the N-terminal pattern PAMA was not present, the first three amino acid residues of the heavy chain, QVQ, if present with at most one mismatch, served as an indicator of the N terminus of the heavy chain.

BLyS inhibition assay

Anti-BLyS antibodies were prepared as phage supernatants as described,¹⁹ or as IMAC-purified scFv⁴⁸ or as purified IgG1 molecules.⁴⁸ IM9 B-cells (ATCC) were centrifuged onto poly-L-lysine coated, 96 well plates at a cell density of 1×10^5 cells/well and then blocked in 3% (w/v) Marvel/PBS for one hour at room temperature. For each anti-BLyS antibody, either 50 μ l of overnight phage supernatant or 50 μ l of purified scFv/IgG (titred from 300 nM down to 1 pM) was pre-mixed with biotinylated BLyS (final concentration of 25 ng/ml), transferred to the IM9-coated plates and then incubated for one hour at room temperature. Plates were washed using three consecutive PBS-Tween washes followed by three washes in PBS. Detection of bound biotinylated BLyS was detected using streptavidin-Delfia technology (Wallac) according to the manufacturer's instructions.²⁹ Plates were read at 340 nm (excitation)/615 nm (emission) on the Wallac Victor counter.

Kinetic analysis

A CM-5 sensorchip was derivatized with protein A using an amine coupling kit (BIAcore). Then 150–300 response units of each anti-BLyS IgG was coupled to this surface for kinetic studies. Serial dilutions of BLyS were prepared in HBS-EP buffer (BIAcore) in a concentration range of 19.6–0.6 nM and injected over the surface at a flow-rate of 30 μ l/min. The dissociation phase was analyzed for 15 minutes by injection of HBS-EP buffer at the same flow-rate. The resultant sensorgrams were corrected for background by subtracting a blank flow-cell control and binding kinetics calculated using the Global Fit option in the BIAevaluation software 3.1. All calculations were done for 1:1 binding models assuming that BLyS exists in solution as a 51 kDa homotrimer.

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