Title: Immunodominant surface epitopes power immune evasion in the African trypanosome

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Short title: Immunodominance can power immune evasion

Abstract

The African trypanosome survives the immune response of its mammalian host by antigenic variation of its major surface antigen (the Variable Surface Glycoprotein, or VSG). Here we describe the antibody repertoires elicited by different VSGs. We show that the repertoires are highly restricted, directed predominantly to epitopes on the surface of the VSGs. They are also highly discriminatory: minor alterations within these exposed epitopes confer antigenically-distinct properties to these VSGs and elicit different repertoires. We propose that the patterned and repetitive nature of the VSG coat focuses host immunity to a restricted set of immunodominant epitopes per VSG, eliciting a highly stereotyped response, minimizing cross reactivity between different VSGs and facilitating prolonged immune evasion through epitope variation.

One Sentence Summary: Hyper-focused antibody responses to variable antigenic coats allow pathogens to escape immune recognition.

Main Text:

The African trypanosome (*Trypanosoma brucei spp*) is a strictly extracellular parasite, infection with which elicits a robust antibody response against its Variant Surface Glycoprotein (VSG) coat (1, 2). This response mediates parasite clearance and also enables immune escape of variants that have switched surface coat composition(3). The dynamic interplay between VSG-coat-switching by trypanosomes and the B cell response of the host that they evade is central to the maintenance of chronic infection (4).

Very little is known about the nature of the antibody response against the VSG coat. A small number of papers beginning in the 1990s revealed that acute infection with *T. brucei* leads to extraordinarily high levels of IgM in humans and in mice (1). The antibody response has often been characterized as polyclonal and many of those antibodies are autoreactive (1, 5, 6). However, these data are conceptualized in the context of a prolonged infection that itself is polyclonal in VSG-archetype. An understanding of the response to individual VSG variants in a controlled setting is therefore lacking, since the culmination of multiple separate responses to different VSGs would produce a “polyclonal” B cell output in the greater infection context. Importantly, immunization or exposure to specific VSG surface coats prevents later infection by cells expressing that same coat, indicating that coat-specific antibodies must be produced during infection (3, 7).

To begin to explore the parameters of the antibody response to *T. brucei*, we examined the antibody repertoires elicited by two specific VSGs. These were chosen to broadly represent the two primary VSG “classes”: VSG2 (a class-A VSG) and VSG3 (a class-B VSG) (8). To do this we infected animals with each specific VSG-expressing parasite, and either “cleared” the parasite with the anti-protozoan compound diminazene four days after infection (as often infection is

rapidly lethal) (9) or allowed the animals to clear the parasite naturally (whenever feasible) after the first peak of parasitemia (at which point the parasites that have switched VSG coats remain and seed the next peak of parasitemia). Initial analyses of infected C57BL/6 mice showed a robust expansion of plasma cells (Fig. 1A, Fig. S1A). This expansion was common to all infections regardless of whether parasites were cleared by the animals (usually by day 6 or day 7 post infection) or whether they were pharmacologically cleared earlier with diminazene (days 4 and 5), allowing for the maturation of the antibody response until collection (which was uniformly at day 8 post infection – Fig. 1B). This is also consistent with known parameters of disease progression (3, 10, 11).

Typically, the characterization of an antigen-specific antibody repertoire benefits from the “baiting” of antigen specific B cells using the antigen itself conjugated to a fluorophore (PE or APC) (12, 13). However, plasma cells are notoriously hard to bait as they downregulate surface expression of Ig and rather secrete the vast majority of the antibody they produce, so that very little antibody remains surface-bound and accessible. We therefore proceeded to sort all plasma cells according to surface marker expression (CD138⁺/CD19^{lo}) (14) into 384-well plates. The antibody repertoire of plasma cells was then assembled after two rounds of single cell PCR and paired heavy (IgH) and light (Igκ/λ) gene sequence analysis (12).

We first focused on the repertoire elicited by VSG2-coated parasites. Surprisingly for a non-baited repertoire, plasma cells elicited from mice infected with VSG2 were dominated by cells carrying only four VH+VL chain pairings (VH10.1 or VH10.3 with VL19-20 or VL19-14, Fig. 1C). These combinations, which are absent in naïve mice (Fig. S1B), formed the plurality of the repertoire when mice were pharmacologically treated to remove trypanosomes four days after infection, but they became increasingly prevalent in repertoires of animals that cleared

trypanosomes naturally a day before collection, and even more dominant in repertoires of animals that cleared the infection the day we collected plasma cells (Fig. 1C). This suggests a rapid and robust response to the parasite, stereotyped between mice, that becomes increasingly diversified as more antigens become available after parasite lysis.

We reasoned that the repertoire we collected closest to natural clearance would contain the B cells likely expressing the most potent anti-coat-specific antibodies and proceeded to analyze it further. While dominated by cells expressing two distinct immunoglobulin (Ig) heavy and Ig light chain gene pairs, the B cells elicited by VSG2-coated *T. brucei* were not monoclonal: each VH10 heavy and 19-20 or 19-14 light chain V region was joined to a variety of DJ or J gene segments representing a wide swath of CDR3 (Fig. 1D and Supplemental file 1). This lack of clonal expansion is also evident from the Shannon entropy index on a sequence level (Fig. 1C, bottom of each circos plot).

We therefore picked a small number of heavy and light chain pairs from the overrepresented V gene segments identified and proceeded to express them recombinantly as soluble antibodies in 293T cells. Indeed, 7 out of 11 of these antibodies are capable of binding live, VSG2-coated *T. brucei*, but not parasite cells coated with a different VSG protein (Fig. 1D, Fig. 2F). Antibodies that did not use these V segments were not able to bind VSG2-coated parasites (Fig. S2). We conclude that the VSG2-elicited plasma cell repertoire represents an oligoclonal expansion primarily of four variable region defined pairs, which are mostly unmutated (Fig. 1D and Supplemental file 1). The V gene signature that defines this repertoire is stereotyped between mice (Fig. 1C), suggesting both that the VSG2-specific paratope is likely located within highly specific variable regions of the antibody protein, and that these antibodies are likely generated against a common immunodominant epitope.

Epitope identification is often achieved by structural analyses of repertoire-defined antibodies together with the antigen that elicited them (15). We therefore sought to crystallize VSG2 (specifically, the larger, surface-exposed N-terminal domain of the VSG, or “NTD”) together with VSG2-specific Fab-fragments. All crystals obtained were of VSG2 alone, however (Fig. S3A, Table S2), likely because most antibodies elicited by *T. brucei* infection are of the IgM isotype (Fig. S4) (1) and are likely of low affinity (but high avidity); therefore, they do not bind with high-affinity when produced recombinantly as an IgG (or Fab) fragment (as current methods for the recombinant production of pentameric IgM are poorly optimized). Serendipitously, the crystals did allow us to re-solve the structure of VSG2 to much higher resolution compared to previous work (16, 17), with unexpected findings. The protein structure of VSG2 from these new crystals is nearly identical to that from the 1990 crystal form, with a root-mean-square deviation of 0.87Å over 1420 atoms of a monomer in the dimer (Fig. 2A, Fig. S5). However, in later stages of model refinement, unusually large electron density around solvent molecules was modeled effectively with calcium (Fig. 2, B and C), an ion not present in older structures likely due to the use of chelating agents in the purification from animal blood (18, 19). Calcium is octahedrally coordinated in VSG2 through a network of oxygen bonds: four from three consecutive amino acid side-chains at the end of a β -strand (bidentate-D208, N209, and D210, henceforth the “DND motif”), the carbonyl-oxygen from G155 in an opposing loop, and three water molecules in the solvent shell above the protein (each water coordinated by other side-chain and main-chain contacts from the protein, Fig. 2C).

There is little change in the fold or amino acid side-chain positioning between the calcium bound and free structures of VSG2 (Fig. 2, A-C), indicating that the pocket does not undergo any significant conformational changes upon calcium binding. The presence of calcium was verified

both by mass spectrometry as well as isothermal titration calorimetry (Fig. S6). The calcium binding pocket in VSG2 appears to be unique, not closely resembling any published structures in the PDB (e.g., DALI homology search) (20). The pocket occurs near the “top” of the NTD of VSG2, slightly sheltered from the uppermost surface (Fig. 2A). A triple mutation of the DND motif to alanine (VSG2_{AAA}) abrogates calcium binding (Fig. S3B and S5) without any significant conformational changes (Figure 1, A and C), although electrostatic surfaces generated from the three structures show an expected change to a more neutral charge distribution in the pocket region of the mutant (Fig. 2D).

Unexpectedly, despite the high similarity between the wild type and mutant structures, antisera elicited by infection with VSG2_{WT} coated trypanosomes, do not bind trypanosomes expressing the minor variant represented by the VSG2_{AAA} mutant (Fig. 2E). Furthermore, our recombinantly expressed antibodies specific to the VSG2_{WT} coat were unable to bind the VSG2_{AAA} mutant coat (Fig. 2F, Fig. S2), despite the fact that these mutations did not cause conformational changes in VSG2 (Fig. 2, A and C, Fig. S6). This suggests that the VSG2 epitope recognized by both antisera and monoclonal antibodies is located within the region defined by the DND motif and conversely, that antibodies to VSG2-coated trypanosomes are capable of a high degree of epitope discrimination.

To assess whether this combination of epitope immunodominance and antibody discrimination characterizes the epitope space of other VSGs, we analyzed antibody repertoires from plasma cells collected from animals infected with VSG3-coated trypanosomes. VSG3 is a member of a different VSG class with significant structural and post-translational modifications distinguishing it from VSG2 (class A vs class B VSGs, respectively) (8). Previously, we had solved the crystal structure of the VSG3 NTD, leading to the unexpected identification of an *O*-linked

glycosylation of a serine residue (S317, heterogeneously modified with 0-3 hexose residues) located in the center of the top surface of the protein (21). Mutation of that serine to an alanine residue ablated glycosylation, reduced parasite virulence, and enhanced the effectiveness of the host immune response (21). At the time we hypothesized that this was due to the tendency of *O*-linked sugars to adopt multiple conformational states (22), thus further diversifying the epitope space of the VSG3 coat. Therefore, to assess whether VSG3 coated trypanosomes lacking this post-translational modification could also elicit a stereotyped immune response toward an immunodominant epitope (such as we have seen for VSG2-coated parasites), we first infected mice with S317A mutant parasites (VSG3_{S317A}), and analyzed the resulting plasma cell antibody responses either after natural clearance or after treatment with diminazene. As was the case for VSG2 coated parasites, VSG3_{S317A} coated parasites were also capable of reproducibly eliciting a restricted B cell repertoire in multiple distinct infections (Fig. 3A). However, this repertoire was now defined by a major light chain V gene (VL_gn33) pairing with a larger number of heavy chains (Fig. 3A). Again, recombinant expression of a number of these heavy and light chain pairs as secreted IgG1s in 293T cells show that many of the antibodies elicited by VSG3_{S317A} coats are capable of binding their cognate parasites (Fig. 3, C and D, Fig. S7). Although the common light chain variable gene (gn33) is clearly important for binding, the proper heavy chain is also necessary, since pairing of the gn33 light chain with a different VH can result in a loss of binding (Fig. S8). Whether the common light chain plays a role in increasing antibody binding to the repetitive coat remains to be investigated.

We also analyzed the resulting plasma cell response after infection with VSG3_{WT} followed by treatment with diminazene (the high virulence of the strain is incompatible with natural clearance). We found that the light-chain restriction seen with VSG3_{S317A} elicited repertoires is now entirely

abolished, strongly suggesting that the addition of up to 3 *O*-linked hexoses per VSG3 molecule functions to diversify the presumed immunodominant epitope presented by the VSG3_{S317A} coated parasite (Fig. 3B). Furthermore, the addition of sugars entirely prevented the major VL (gn33) carrying antibody pairs from binding VSG3_{WT} coated parasites (Fig. 3, C and D), suggesting that antibodies elicited to the parasite coat are focused near-exclusively on the epitope that contains the *O*-linked hexoses. Conversely, of the four antibodies we reconstituted and tested that were elicited by the wildtype (VSG3_{WT}) infection, three were able to bind the sugarless coat, and one was selective to the sugar on S317 (capable only of binding the wildtype) (Fig. 3, C and D).

A comparison of mutant and wild type structures verified that the protein surface between the two VSG3 variants was identical (Fig. 4A) but yielded a surprise in the form of an additional sugar in a neighboring serine (S319). We confirmed this in the VSG3_{WT} structure by re-solving it (Fig. 4, A and B, Fig. S9A, Fig. S10, Table S3). S319 is part of the surface-exposed loop harboring S317, and in a previous study (21), both by crystallography and mass spectrometry, glycosylation of S319 was not clearly present (likely due to intrinsic lability of the moiety). However, here we were able to observe strong density for the sugar on S319 in the VSG3_{WT} and VSG3_{S317A} mutant crystal structures, as well as to confirm the presence of hexose groups on S317 and S319 by mass spectrometry (Fig. S11). Subsequent determination of the crystal structures of the S319A mutant as well as the double mutant (combining S317A and S319A, or “SSAA”) did not reveal any significant structural differences when compared to VSG3_{WT} or VSG3_{S317A} (Fig. 4, A and B, Fig. S9, B-D, Fig. S10).

Following the experimental protocol that we had used to assess the quality of the repertoires elicited by VSG3_{WT} and VSG3_{S317A}, we also generated plasma cell antibody repertoires elicited by VSG3_{S319A} and VSG3_{SSAA}, (Fig. 4C) and tested those for their ability to bind either their cognate

parasites or to cross-react with the wild type and other mutants. We found that VSG3_{SSAA} parasites elicited repertoires very similar to those elicited by the VSG3_{S317A} mutant alone (e.g. dominant presence of the gn33 light chain V gene and 30-60.6.70 or J558.26.116 heavy chain gene), whereas VSG3_{S319A} single mutants elicited broader repertoires that were very similar to those elicited by VSG3_{WT} (no gn33 dominance for example) (Fig. 4C). In turn, this suggests that the VSG3_{WT} repertoire is strongly influenced by the one-to-three hexoses linked to S317 (21) but negligibly by the glycans linked to S319; similarly, that the S317A determined repertoire is indifferent to the presence of the sugar on S319, as the repertoire does not change in the double mutant.

These results underscore the immunodominance of the relevant epitopes and the exquisite discrimination of the resulting antibodies. Specifically, they suggest that the immunodominant epitope that characterizes the VSG3_{WT} coat is limited to the immediate amino-acids surrounding the S317-*O*-glucose (including that sugar but excluding its companion on S319). Consequently, when we tested recombinant antibodies that were elicited after infection with each of these variants for the ability to cross-react with each of the other variants, we identified four classes of binders:

(a) Antibodies that could bind all four variants (like ab239 or ab222, Fig. 4, D and E); these were elicited by both VSG3_{WT} and VSG3_{SSAA} suggesting that they bind a common peptide epitope at a distance from the epitope defining sugar.

(b) Antibodies that bound only the epitope-defining sugar-containing variants VSG3_{WT} and VSG3_{S319A} (like ab250, Fig. 4, D and E) but not the other two variants; suggesting that they bind the sugar containing epitope but not the same epitope without the carbohydrate (and are therefore potentially sugar-selective);

(c) Antibodies that bound only the variants that lacked the epitope-defining sugar at S317 (VSG3_{S317A} and VSG3_{SSAA}, like ab021); suggesting that they bind the same peptide epitope defined by the sugar but are inhibited by the presence of the sugar in the other two variants.

(d) Finally, antibodies that only bound the VSG3_{S317A} variant (e.g. ab234), potentially suggestive of an additional minor epitope partly defined by the S319 sugar.

Overall, by examining two distinct VSGs and variants thereof, we demonstrate that the antibody response that mediates clearance to a given *T. brucei* coat can be hyperfocused to a restricted set of immunodominant epitopes that are surface exposed. Consequently, the immune response elicited by each *T. brucei* variant coat has the potential to be highly restricted and likely mediated by paratopes within the germline V regions of antibody genes. How *T. brucei* achieves this “immunofocusing” is likely related to the highly dense, repetitive and patterned nature of its surface coat. We hypothesize that this extreme focusing allows the parasite to use short-tract gene conversion or even point mutations (collectively referred to as mosaic formation (3, 23)) to expand its antigenicity well beyond what is encoded in its considerable genomic archive. By ensuring that cross reactivity between coat-defined epitopes will be exceedingly rare, even within similar variants, immunofocusing helps the parasite perpetually evade the immune response.

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Supplementary Materials:

Materials and Methods

Figures S1-S12

Tables S1-S4

References (24-35)

Fig.1: VSG₂^{WT} parasites elicit a highly restricted antibody repertoire, defined by a small number of heavy and light chain pairs. (A) Percentage of CD138⁺ plasma cells (PC) within the live lymphocyte population in a non-infected naïve mouse and in infected mice that were treated with diminazene or that naturally cleared the first peak of parasitemia. Plasma cells from this gate were sorted for antibody repertoire analysis. Representative gateings to determine live lymphocytes are shown in Supplementary Figure 1A. **(B)** Parasitemia infection curves from the 2 mice that naturally cleared the first peak of parasitemia (panel A) at day 6 (black) and day 7 (grey). Both mice were sacrificed at day 7 post-infection. A representative value of parasitemia at day 4 for mice treated with diminazene is indicated in red. **(C)** Circos plots generated for the VSG₂^{WT} V gene signatures of two independent mice whose infections were treated with diminazene (left - n=89 pairs) and of two additional mice that naturally cleared the first peak of parasitemia at days 6 (center - n=53 pairs) and 7 (right - n=50 pairs) post infection. Different colors depict each heavy

chain variable gene (bottom half of the plot), and each light chain variable gene (top half of the plot). The heavy and light chain variable gene pairings that form the antibodies are illustrated as connector lines starting from the heavy chain genes, providing also information about the frequency of the pairings. Genes from both chains that appeared only once and resulted in single heavy-light pairings, were considered background and were removed from the plot. Shannon entropy shows clonal diversity on a sequence level with a value of 1.0 representing 100% clonal diversity (no clones), while a value of 0.0 corresponding to 0% clonal diversity (only clones). **(D)** Table with representative VSG2_{WT} antibodies elicited in the context of diminazene-treated infections, which are mostly composed of VH10 heavy chains paired with 19-14 and 19-20 V_κ light chains. The CDR3 from each antibody is described, indicating that the repertoire is not monoclonal. The original antibody isotype, as well as somatic hypermutations, if any, are also shown.

Fig.2: Residues surrounding the calcium binding pocket of VSG2_{WT} define an immunodominant epitope. (A) Left - Crystal structure of VSG2_{WT}. The homodimer is shown as a ribbon diagram colored in gold, N-linked glycans and calcium atoms are displayed as red and green space-filling atoms, respectively. Right – Structural alignment of monomers of VSG2_{WT} (from left panel, in gold), VSG2_{WT} (from (16), in blue) and VSG2_{AAA} (in pink), highlighting the coordination of the calcium atom. (B) Close-up of the coordination of calcium binding pocket with corresponding electron density maps. Water molecules and calcium atoms are displayed as red and green space-filling atoms, respectively. (C) Superposition of the calcium binding pockets of the 3 structures presented in (A). Water molecules and calcium atoms are displayed as red and green space-filling atoms, respectively. (D) Molecular surfaces of VSG2_{WT} + calcium (this study), VSG2_{WT} - calcium (from (16)) and VSG2_{AAA}. The VSGs are oriented looking ‘down’ on the top lobe of the protein, the orientation rotated 90° about a horizontal axis in comparison to upper

panels. The surfaces are colored by relative electrostatic potential (blue is basic/positively charged, red is acidic/negatively charged, and white is neutral). **(E)** Histograms reflecting binding intensities of antisera collected from mice infected with VSG2_{WT} and VSG2_{AAA} parasites (top two panels, respectively) with VSG2_{WT} and VSG2_{AAA} parasites (blue and red correspond to 2 independent clones of VSG2_{WT} parasites; green and orange correspond to 2 independent clones of VSG2_{AAA} parasites). Bottom panel shows quantification of binding intensities of antisera collected from mice infected with VSG2_{WT} (black dots) and VSG2_{AAA} (grey dots) parasites with VSG2_{WT} and VSG2_{AAA} parasites (median from n=12-18). **(F)** Histograms reflecting binding intensities of the antibodies presented in Figure 1C with wild type parasites (colors) and AAA-mutant parasites (black). Different colors are meant to distinguish differential binding intensity: red – no binding; purple – poor binding; blue – good binding. The gating strategy is shown in Fig. S12.

Fig.3: VSG3_{S317A} parasites elicit a restricted B cell response, with preferential utilization of a light chain V κ gene (gn33). (A) Circos diagrams generated for the VSG3_{S317A} V gene signatures of four mice infections, two naturally cleared (left - n=114 pairs) and two diminazene treated (right - n=48 pairs). Different colors depict each heavy chain variable gene (bottom half of the plot), and each light chain variable gene (top half of the plot). The heavy and light chain variable gene pairings that form the antibodies are illustrated as connector lines starting from the heavy chain genes, providing also information about the frequency of the pairings. Genes from both chains that appeared only once and resulted in single heavy-light pairings, were considered background and were removed from the plot. Colored-asterisks depict individual B cells that correspond to clones (ie share the same V heavy and V light gene segments but also the same DJ or J genes and the same CDR3 length and sequence). Shannon entropy shows clonal diversity on a sequence level, a number of 1.0 shows 100% clonal diversity (no clones), while a value of 0.0 corresponds to 0%

clonal diversity (only clones). **(B)** Circos plots generated for the VSG3_{WT} V gene signatures from two mice whose infections were treated with diminazene (n=53 pairs), as described in (A). **(C)** Left: FACS analysis of heavy and light chain pairs selected from the VSG3_{WT} repertoire, produced recombinantly as IgG1 antibodies. The plots depict antibody binding to VSG3_{WT} (red) or VSG3_{S317A} (blue) *T. brucei* cells. Parasites were first stained with each antibody supernatant, and counter stained with (mouse anti-human) AlexaFluor488-IgG1 secondary antibody. The gating strategy is shown in Fig. S12. Staining with supernatants from untransfected cells (no plasmid) serves as a negative control. All data are normalized to mode. Right: FACS showing the same experimental setup but now for binding of recombinant antibodies selected from the VSG3_{S317A} repertoire. **(D)** Table of all the VSG3_{S317A} antibodies with gn33 as a light chain that bound to the cognate and WT cells, as well as of all the VSG3_{WT} antibodies and their binding to cognate and mutant cells. The (+) symbol indicates binding while the (-) lack of binding. V and J segments as well as the CDR3 of both heavy and light chain genes are shown, along with the binding to the respective parasites. The original antibody isotype, as well as somatic hypermutations, if any, are also shown.

Fig.4: Nearly identical VSG3 variant coats elicit distinct V gene signatures. (A) Overlaid crystal structures of all four VSG3 monomers, shown as ribbon diagrams and colored in blue (WT), gold (S317A), salmon (S319A) and purple (SSAA). The N-glycans are represented as red spheres on the bottom lobe, while the O-linked sugars as blue (S317-sugar) and green (S319-sugar) spheres on the top lobe. **(B)** Electron density maps focused on the presence or absence of the S317 and S319 O-Glucose molecules for all individual proteins as indicated in the labeling. **(C)** Circos plots of the diminazene-treated plasma cell antibody repertoires (V signatures shown only) of WT (n=53 pairs), S317A (n=48 pairs), S319A (n=109 pairs) and SSAA (n=102 pairs). Different colors depict each heavy chain variable gene (bottom half of the plot), and each light chain variable gene (top half of the plot). The heavy and light chain variable gene pairings that form the antibodies are illustrated as connector lines starting from the heavy chain genes, providing also information about

the frequency of the pairings. Genes from both chains that appeared only once and resulted in single heavy-light pairings, were considered background and were removed from the plot. The antibody pairs picked for validation are presented in color, while all other genes are in gray. Shannon entropy shows clonal diversity on a sequence level (a value of 1.0 shows 100% clonal diversity (no clones), while a value of 0.0 corresponds to 0% clonal diversity (only clones)). **(D)** FACS data showing the binding of the validated antibodies, secreted as IgG1s, to VSG3_{WT} (red), VSG3_{S317A} (blue), VSG3_{S319A} (green) and VSG3_{SSAA} (purple) -covered trypanosomes. Parasites were first stained with each antibody supernatant, followed by counter staining with a (mouse anti-human) AlexaFluor488-IgG1 secondary antibody. The gating strategy is shown in Fig. S12. Staining with supernatants from untransfected cells (no plasmid) is used as a negative control. All data were normalized to mode. **(E)** Table of representative binding patterns of the produced antibodies (also in 4D) to different trypanosome cell lines. The (+) symbol indicates binding while the (-) non-binding. V and J segments as well as the CDR3 of both heavy and light are shown, along with the binding to the individual cell lines. The original isotypes and somatic hypermutations, if any, are also listed.