Retraction

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Retraction for “Somatic hypermutation maintains antibody thermodynamic stability during affinity maturation,” by Feng Wang, Shiladitya Sen, Yong Zhang, Insha Ahmad, Xueyong Zhu, Ian A. Wilson, Vaughn V. Smider, Thomas J. Magliery, and Peter G. Schultz, which was first published February 25, 2013; 10.1073/pnas.1301810110 (Proc Natl Acad Sci USA 110:4261–4266).

The undersigned authors wish to note the following: “In the course of an examination of data unrelated to this publication, it came to our attention that some of the thermal melt data collected by a single researcher at The Ohio State University contains irregularities. We conducted an extensive review of the available primary thermal melt data, reprepared multiple key protein samples, and carried out differential scanning fluorimetry (DSF) thermal melts in replicate. Our reanalysis supports the basic conclusion of this manuscript that peripheral mutations accumulated during affinity maturation are stabilizing, whereas binding-site mutations are destabilizing. However, we do not have confidence in any of the aggregation/light scattering data that were intended to confirm the primary DSF thermal melt data because none of those primary data could be located, and we are concerned that the appearance of some of the DSF melting curves reported in the paper may have been altered, largely outside of the melting transition. Based on these concerns, we retract the entire report. These problems occurred despite blinding of the identities of all samples, which were prepared at the Scripps Research Institute and measured at The Ohio State University, as well as regular examination of the melting data by multiple authors throughout the course of this work. We apologize to the readers of this paper for not detecting these problems sooner and for any negative consequences that may have resulted.”

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Somatic hypermutation maintains antibody thermodynamic stability during affinity maturation

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Somatic hypermutation and clonal selection lead to B cells expressing high-affinity antibodies. Here we show that somatic mutations not only play a critical role in antigen binding, they also affect the thermodynamic stability of the antibody molecule. Somatic mutations directly involved in antigen recognition by antibody 93F3, which binds a relatively small hapten, reduce the melting temperature compared with its germ-line precursor by up to 9°C. The destabilizing effects of these mutations are compensated by additional somatic mutations located on surface loops distal to the antigen binding site. Similarly, somatic mutations enhance both the affinity and thermodynamic stability of antibody OKT3, which binds the large protein antigen CD3. Analysis of the crystal structures of 93F3 and OKT3 indicates that these somatic mutations modulate antibody stability primarily through the interface of the heavy and light chain variable domains. The historical view of antibody maturation has been that somatic hypermutation and subsequent clonal selection increase antigen–antibody specificity and binding energy. Our results suggest that this process also optimizes protein stability, and that many peripheral mutations that were considered to be neutral are required to offset deleterious effects of mutations that increase affinity. Thus, the immunological evolution of antibodies recapitulates on a much shorter timescale the natural evolution of enzymes in which function and thermodynamic stability are simultaneously enhanced through mutation and selection.

The generation of high-affinity, selective antibodies by the immune system involves the combinatorial assembly of V, D, and J gene segments followed by affinity maturation, during which somatic mutations in the antibody variable region are clonally selected on the basis of increased affinity for antigen (1, 2). Genetic, biochemical, and structural studies have revealed the molecular mechanisms that result in antibody variable region diversity and its role in antigen recognition. More recently, detailed structural and biophysical studies have shown that germ-line antibodies have significant combining-site conformational variability compared with affinity-matured antibodies, and this structural plasticity also plays a critical role in determining the enormous binding potential of the germ-line repertoire (3–6).

Somatic hypermutation and subsequent B-cell clonal selection further optimize antibody–antigen affinity and selectivity. In most cases, these somatic mutations occur throughout the antibody variable region, including sites quite remote from the antigen binding site. Structural studies have shown that these distal mutations can affect the combining-site structure and dynamics through a network of coupled side-chain hydrogen-bonding, electrostatic, and van der Waals interactions (3, 7). However, many of the somatic mutations that occur during affinity maturation appear to have little effect on antigen-binding affinity. A long-standing question has been what role if any such mutations play during the B-cell selection process.

The process of natural selection not only results in proteins with enhanced activity, it also affords soluble, thermodynamically stable polypeptides (8–11). Thus, one possible role for the apparent functionally silent somatic mutations in the antibody variable region may not be associated with antigen binding but rather with preserving the overall stability and solubility of the antibody molecule. Antigen binding fragments (Fab's) of antibodies generated from hybridomas exhibit a relatively small range of melting temperatures despite significant sequence variation (12). In contrast, phage display and other in vitro selection systems often afford high-affinity antibodies that are poorly expressed, aggregate, and/or have low stability (13). Thus, a subset of naturally occurring somatic mutations, especially those distal to the combining site, may compensate for destabilizing mutations that are selected on the basis of affinity alone. The antibody maturation process may act simultaneously to select for both enhanced binding affinity and protein stability—a process not recapitulated by most in vitro antibody display methods. To explore the possibility of an expanded role for somatic mutation during the immune response, we have expressed the germ-line and affinity-matured antibodies 93F3 (14) and OKT3 (15) and determined the effects of somatic mutations on both antibody affinity and overall thermodynamic stability.

Results and Discussion

Somatic Mutations in Antibody 93F3. Antibody 93F3, which was elicited to a small synthetic hapten (Fig. S1) and catalyzes aldol reactions, has been well-characterized structurally and biochemically (14, 16). Although the crystal structure of the 93F3–hapten complex is not available, the substrate binding site has been modeled on the basis of the structure of 33F12 (a related aldolase antibody) bound to a hapten analog (17). It is known that residue Lys89(L), which lies in a hydrophobic pocket and has a depressed pK_a, catalyzes the reaction by forming an enamine intermediate with substrate. (L and H refer to the light and heavy chains, respectively.) Bioinformatics analysis indicates that the V, D, and J germ-line origins of the heavy chain areIGHV2-6-5*01, IGHD2-14*01, and IGHM4*01, respectively. The light-chain germ-line V and J are IGKVY*21*01 and IGK2-1*01, respectively (International ImMunoGeneTics Information System; www.imgt.org), indicating that Lys89(L) is encoded in the germ-line sequence of the light-chain V region. A comparison of the mature and germ-line antibody sequences reveals that 13 and 9 amino acids are somatically mutated in the heavy-chain and light-chain variable regions, respectively (Fig. 1 A and B). Among them, mutations D98G(H), Y102S(H), K45T(L), L46K(L), N93D(L), and van der Waals interactions (3, 7). However, many of the somatic mutations that occur during affinity maturation appear to have little effect on antigen-binding affinity. A long-standing question has been what role if any such mutations play during the B-cell selection process.

The process of natural selection not only results in proteins with enhanced activity, it also affords soluble, thermodynamically stable polypeptides (8–11). Thus, one possible role for the apparent functionally silent somatic mutations in the antibody variable region may not be associated with antigen binding but rather with preserving the overall stability and solubility of the antibody molecule. Antigen binding fragments (Fab’s) of antibodies generated from hybridomas exhibit a relatively small range of melting temperatures despite significant sequence variation (12). In contrast, phage display and other in vitro selection systems often afford high-affinity antibodies that are poorly expressed, aggregate, and/or have low stability (13). Thus, a subset of naturally occurring somatic mutations, especially those distal to the combining site, may compensate for destabilizing mutations that are selected on the basis of affinity alone. The antibody maturation process may act simultaneously to select for both enhanced binding affinity and protein stability—a process not recapitulated by most in vitro antibody display methods. To explore the possibility of an expanded role for somatic mutation during the immune response, we have expressed the germ-line and affinity-matured antibodies 93F3 (14) and OKT3 (15) and determined the effects of somatic mutations on both antibody affinity and overall thermodynamic stability.
Effects of Somatic Mutations on Affinity and Thermodynamic Stability.

To determine the effects of these somatic mutations on antigen binding and stability, we first generated the Fab fragment derived from the germ-line heavy chain (H₅) and light chain (L₅) of 93F3 (Fig. 1 and Table S1). The six somatic mutations at the antigen binding site were then introduced into the germ-line sequence of the heavy and light chains to afford a heavy chain with D99G and Y102S substitutions (H₅) and a light chain with K45T, L46K, N93D, and N95P substitutions (L₅). The three Fabs H₅L₅ (germ-line precursor of 93F3), H₃M₅ (affinity-matured 93F3), and H₅L₅ (germ-line precursor with antigen binding site mutations) were fused to a polyhistidine tag at the C terminus of the heavy chain, expressed in HEK293 cells, and purified by Ni-NTA affinity chromatography.

We determined the binding affinity of these three Fabs to the hapten used to generate 93F3 by an ELISA as previously described (18). H₃M₅ (93F3) has the highest binding affinity (Kₐ = 0.6 μM); H₅L₅ has no detectable binding to the hapten; and H₅L₅ has a binding affinity (Kₐ = 1 μM) close to that of the affinity-matured Fab (Table S2). These results indicate that the six somatic mutations in the antigen binding site largely account for the increase in binding affinity. The thermodynamic stabilities (apparent melting temperature; Tₘ) of the Fabs were then determined with the environmentally sensitive extrinsic dye SYPRO Orange, as described (19) (Fig. 2). Similar methods have been used previously to measure the stabilities of affinity-matured antibodies, and the Tₘ values are in good agreement with those determined by standard calorimetric techniques, such as differential scanning calorimetry (20). Antibody aggregation was also assessed as a function of temperature by monitoring optical density at 600 nm. These methods gave apparent stability values in close agreement with each other (Fig. 2 and Tables S3 and S4). The affinity-matured antibody 93F3 (H₃M₅L₅) and its germ-line precursor H₅L₅ have similar thermal stabilities (melting temperatures of 69 °C and 68 °C, respectively; Fig. 2). In contrast, the variant H₅L₅ contains only the six somatic mutations in the antigen binding site, exhibits a significant decrease in stability (Tₘ = 60 °C) compared with H₃M₅L₅ and H₅L₅. Taken together, these results indicate that somatic mutations that increase binding affinity in antibody 93F3 significantly destabilize the Fab fragment relative to the germ-line starting point. Similar effects have been observed with mutational studies of enzymes in which there is a tradeoff between function and stability (21, 22).

Effects of Peripheral Mutations on Binding and Stability.

To determine the effects of the somatic mutations outside the binding site (the peripheral mutations) on binding and stability, we introduced all 16 peripheral mutations in the context of the germ-line heavy and light chains to afford the heavy chain (H₅) and light chain (L₅) (without any antigen binding site mutations). H₅L₅ has no detectable binding to antigen by ELISA, indicating that this set of somatic mutations is largely silent with respect to affinity maturation. However, the Tₘ of H₅L₅ increased by more than 8 °C compared with that of H₃M₅L₅ and 16 °C compared with that of H₅L₅. Next, we paired H₅ and L₅ with Lₐ and Hₐ, respectively, to generate H₅Lₐ and HₕLₐ. Both variants have higher stability than H₃M₅L₅ (Table S2). These results clearly show that these peripheral mutations contribute to “stability maturation,” resulting in an affinity-matured antibody with comparable stability to its germ-line precursor. We then introduced sets of peripheral mutations stepwise into the variable regions of the heavy and light chains of the Hₐ and Lₐ sequences to begin to determine their contributions to the stability maturation process. We grouped the peripheral mutations into subsets based on their proximity to each other to generate four additional heavy-chain (Hₐ; Q1E, Q3M, K5V; Hₐ; T30S, G54D, N73S; Hₐ; S65F, S68T, K81N; Hₐ; T87S) and two light-chain (Lₐ; V3Q, S5T, K24R; Lₐ; A15P, L27F) variants (Fig. 1). Twenty-four Fab fragments were generated by combination of these heavy- and light-chain variants with Hₐ, Hₐ, Lₐ, and Lₐ, and their thermal stabilities were determined (Fig. S2 and Table S3). In every case when Hₐ or Lₐ was paired with a subset of different peripheral mutations, the Tₘ was significantly greater than that of H₅L₅. For example, when the heavy chain is Hₐ (which has no peripheral mutations), the stabilities of the Fabs are HₐLₐ (60 °C) > HₐLₐ (65 °C) > HₐLₐ (67 °C) > HₐLₐ (69 °C). Furthermore, all combinations of sets of peripheral mutations
Stability of 93F3 variants. (A and B) The melting curves for HnLα, HnLγ, H3MnLα, and H3MnLnLα are shown as measured by thermal scanning (solid circles) and light scattering [aggregation (agg); open circles]. The melting temperatures are fit directly to the melting curves in A or to Gaussian function fits to the derivative plots in B (Fig. S3 and SI Materials and Methods). (C) Both thermal scanning and light scattering show that the germ-line and mature antibodies have similar stabilities, but the antigen-binding mutations alone destabilize whereas the peripheral mutations alone stabilize the germ-line scaffold. HTTS, high-throughput thermal scanning. (D) Heat map of Fab Tm from the combination of heavy-chain and light-chain mutation groups. Note that much of the stabilization by the peripheral mutations comes from the Hn group. The high stabilities of L2 Fabs indicate that antigen-binding light-chain mutations N93D and N95P are responsible for most of the destabilization upon maturation.

Fig. 2. Stability of 93F3 variants. (A and B) The melting curves for HnLα, HnLγ, H3MnLα, and H3MnLnLα are shown as measured by thermal scanning (solid circles) and light scattering [aggregation (agg); open circles]. The melting temperatures are fit directly to the melting curves in A or to Gaussian function fits to the derivative plots in B (Fig. S3 and SI Materials and Methods). (C) Both thermal scanning and light scattering show that the germ-line and mature antibodies have similar stabilities, but the antigen-binding mutations alone destabilize whereas the peripheral mutations alone stabilize the germ-line scaffold. HTTS, high-throughput thermal scanning. (D) Heat map of Fab Tm from the combination of heavy-chain and light-chain mutation groups. Note that much of the stabilization by the peripheral mutations comes from the Hn group. The high stabilities of L2 Fabs indicate that antigen-binding light-chain mutations N93D and N95P are responsible for most of the destabilization upon maturation.

were more stable than HnLα, and some had even higher stability than H3MnLα. For example, pairing of light-chain variant L1 or L2 with H2 (T30S, G54D, T73S) affords a Fab with equal or greater stability (up to 4 °C) than the affinity-matured antibody. To determine whether the enhanced stability of H2 variants results from any individual mutation in this subset or from a combined effect of the three mutations, we introduced each individual peripheral H2 somatic mutation into Hn to afford H2(30S), H2(54D), and H2(73S), and paired them with Lα (Table S4). It is interesting that each single mutation T30S or G54D is sufficient to make H2(30S)Lα (69 °C) or H2(54D)Lα (70 °C) comparable in stability to H3MnLα (69 °C). Analysis of a large database of antibody segments (Abysis; www.bioinf.org.uk/abisy) reveals that these mutations are not common; that is, they are likely not universal stabilizing mutations, but rather provide enhanced stability in the context of 93F3. Such individual stabilizing mutations likely occur in response to specific “affinity mutations” during the affinity maturation process, complicating an analysis of their overall additivity in the context of only the germ-line and affinity-matured antibody. Previous directed evolution experiments have shown that mutations in antibody variable regions, particularly those in turns at the surface or residues at the variable–constant interface (21, 22), can significantly increase the stability of antibodies or antibody fragments (23–26). Here we show that the clonal selection process is likely more sophisticated than previously realized—it efficiently selects such stabilizing mutations to compensate for somatic mutations that mature affinity but are deleterious to stability. Thus, the immunological evolution of antibodies recapitulates on a much shorter timescale the natural evolution of enzymes in which both function and thermodynamic stability are enhanced through mutation and selection (11).

Structural Analysis of Somatic Mutations. To understand the mechanism by which somatic mutations affect antibody stability, we compared the crystal structures of 93F3 (H3MnLα) and H3MnLα, the most unstable variant with only antigen binding site mutations; the latter structure was determined to 2.6-Å resolution (Table S5). The somatic mutations that enhance affinity are all located at the interface of the heavy and light chain variable domains, VH and VL, and likely adversely affect the interaction of VH and VL and, as a consequence, antibody stability. For example, somatic mutation Y102S(H) likely perturbs the hydrogen-bonding network between Asp100(H), Trp103(H), and Tyr36(L), which connects VH and VL. In contrast, all 16 peripheral mutations are on the surface of the antibody with their side chains extending toward the solvent. Although the overall structures are well-aligned, the superposition of H3MnLα and H3MnLα shows clear structural differences, with the most significant changes occurring in the loop regions connecting the β-strands (Fig. 3). For example, the stabilizing peripheral mutations T30S and G54D are located in the complementarity-determining region (CDRH1) and CDRH2 loops, respectively, which are connected to and likely control the conformation of strands βC, βC′, and βC″ at the interface of VH and VL. Indeed, we observe that the N-terminal region of βC in H3MnLα is shifted away from the interface of VH and VL in comparison with the
structure of $H_M L_M$ (Fig. 3C). By modulating the geometry of the $\beta$-strands at the interface of VH and VL, these peripheral mutations may serve to relieve the destabilizing effects caused by the somatic mutations in the antigen binding site. Another group of stabilizing peripheral mutations in $H_3$ and $H_4$ likely affect a hydrogen-bonding network formed by Arg66$(H)$, Asp36$(H)$, Arg38$(H)$, and Glu46$(H)$ (Fig. S4), which in turn modulates the interaction between the side chains of Gln39$(H)$ and Gln38$(L)$.

Somatic Mutations in Antibody OKT3 also Enhance both Affinity and Stability. To further explore the notion that somatic mutations are selected based on both affinity and stability, we analyzed a second murine antibody, OKT3, which binds the large protein CD3 (27). Again, bioinformatics analysis was used to determine the germ-line precursors of the heavy and light chains of OKT3, and revealed six and five somatic mutations in the heavy and light chains, respectively [$S31R(H)$, $S55R(H)$, $K9N(H)$, $A72T(H)$, $Y101D(H)$, $P96F(L)$, $H34N(L)$, $R61H(L)$, $S63R(L)$, $S77G(L)$] (Fig. 4 A and B). The affinity-matured Fab ($H_M L_M$) and its germ-line precursor ($H_L L_C$) were expressed and purified and their affinities and stabilities were determined (Fig. S5). The $T_m$s of OKT3 $H_L L_C$ and $H_M L_M$ are 70 °C and 71 °C, respectively, indicating again that the germ-line and affinity-matured antibodies have similar stabilities. As expected, the germ-line antibody $H_L L_C$ did not show any detectable binding affinity to antigen as determined by FACS (28) with Jurkat cells.

The crystal structure of the complex of OKT3 bound to the CD3ε chain shows that all six somatic mutations in the heavy chain and two somatic mutations in the light chain (Pro96Phe and His34Asn) are proximal to the antigen binding site, potentially interacting with antigen directly or indirectly (Fig. 4C). We made constructs to reverse these eight somatic mutations to the germ-line amino acids individually (Table S6), expressed the Fab variants, and measured the binding affinity. The results indicated that reversion of these somatic mutations with the exception of His34$(L)$ significantly decreased antigen binding (Table S7). We next expressed the Fab variant $H_M L_C$ and determined it to have lower stability ($T_m = 64 ^\circ C$) than $H_M L_M$ or $H_L L_C$, consistent with the notion that mutations that enhance affinity are destabilizing. We then introduced the Pro96Phe$(L)$ somatic mutation (which affected affinity when reverted) in the context of $L_C$ to afford light-chain variant $L_A$ and paired it with $H_M$, which contains all of the somatic mutations involved in antigen binding. The binding affinity of $H_M L_A$ increased to nearly the same level as $H_M L_M$; however, the $T_m$ remained 8 °C lower than that of $H_M L_M$. Finally, we introduced the somatic mutation His34Asn (which did not affect affinity when reverted) in the context of $L_A$ ($L_L$) and paired it with $H_M$. The $T_m$ of $H_M L_L$ (69 °C) increased to near that of $H_M L_M$ and its affinity was relatively unaffected, indicating that in this case the single His34Asn mutation can compensate the stability lost during affinity maturation. The other light-chain somatic mutations only increase the stability slightly and do not significantly affect binding affinity. However, it is likely that the effects of stability mutations are context-dependent, so the role of such mutations during the affinity maturation process is difficult to define. Interestingly, the key somatic mutation His34Asn that affects the stability of OKT3 is...
located at the interface of the heavy and light chains, in contrast to antibody 93F3, where the critical stabilizing mutations are in the loops connecting the two \(\beta\)-sheets. Nonetheless, OKT3 provides a second example of the dual role of somatic hypermutations in the affinity and stability of antibodies.

**Clonal Selection of Stabilizing Somatic Mutations.** The somatic hypermutation–clonal selection process has historically been viewed as a process for optimizing the binding affinity of a robust protein scaffold that was tacitly assumed to maintain stability, even with many binding-site mutations (29). Our results suggest that random mutations introduced by somatic hypermutation may be selected based on both affinity and stability. During clonal selection, B cells compete for antigen binding through B-cell antigen receptors. Surface-displayed immunoglobulins (Igs) are cross-linked upon binding antigen, triggering B-cell proliferation. As a result, cells with higher-affinity antibodies are selectively amplified (30, 31). Because protein expression levels often correlate with thermal stability (32), it is likely that antibody stability controls the surface density of Igs on B cells during clonal selection. Thus, higher-affinity antibodies with decreased T\(_{m}\)s may have decreased expression levels and lower surface densities, which adversely impacts antigen cross-linking and subsequent selection. This effect may be compensated by mutations that lead to higher stability and antibody expression levels, resulting in higher avidities and increased cross-linking by antigen, and as a result enhanced B-cell proliferation. To test this notion, we determined the expression levels of 12 Fab variants with significantly different thermal stabilities in mammalian cells. We transfected HEK293 cells with antibody Fabs and then measured the amount of protein secreted into the media by Western blot, as well as the yield of purified Fab. The stabilities of the Fabs correlate with their expression levels in mammalian cells (Fig. 5). Based on these results, we believe that more stable Igs may result in higher expression levels on the B-cell surface in vivo, whereas unstable Igs likely lead to misfolding and lower expression levels. B cells with either higher affinity or higher copy numbers of surface Igs have a greater avidity for antigen and are activated (until a threshold is reached at which antigen-dependent cross-linking no longer limits B-cell proliferation). This coselection mechanism during the affinity maturation process provides selective advantages for individual B cells producing antibodies with both enhanced affinity and stability. Alternatively, stabilizing mutations may simply accumulate in the variable region without positive selection. However, in the case of antibody 93F3, a surprising number of the peripheral mutations are

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**Fig. 4.** Somatic mutations during the maturation of OKT3. (A and B) The alignment of protein sequences of OKT3 variable domains with their germ-line precursors for (A) the heavy chain and (B) the light chain. Dashes indicate identity with the germ-line sequence. Residue numbering and CDRs are as defined by Wu and Kabat (37). (C) The crystal structure of OKT3 variable domains in complex with CD3e (rendered from PDB ID code 1SY6 with PyMOL). The ribbons of heavy chain and light chain are colored cyan and gray, respectively.

**Fig. 5.** Correlation between expression and stability. (A) Western blot of 93F3 variants secreted from HEK293. The blot was probed with an anti-His\(_6\) antibody that recognizes the 6×His tag on the heavy-chain variants. Although this is a nonreducing gel, some amount of the dissociated heavy chain can also be detected. The lanes are sorted by T\(_{m}\)(B) Plots of the protein yield from HEK293 expression versus the T\(_{m}\) of each Fab show that expression level is closely related to antibody stability. Data from three independent transfections of 20 mL of HEK293 culture of each Fab sample are shown (mean and standard error of the mean). H_\text{Lx}, H_\text{L10}, H_\text{L2}, H_\text{L2}, H_\text{L2}, and H_\text{L2} are colored in red, yellow, green, dark green, purple, and blue, respectively.
stabilizing relative to what one might expect on the basis of directed evolution experiments with other proteins (9, 33, 34). Indeed, most in vitro antibody-engineering techniques rely upon display systems that select for affinity alone (35, 36) and often produce high-affinity binders with poor biophysical characteristics (13).

**Conclusion**

The antibody molecule provides an excellent model system to investigate the evolution of protein-binding energy and catalysis (23). As the human and mouse antibody repertoires have been sequenced, somatic mutations in an affinity-matured antibody can often be identified by comparison with its germ-line precursor. In addition, many antibody sequences and structures are available for comparative analysis. Here we demonstrate the dual role of somatic mutations with antibodies 93F3 and OKT3. Some somatic mutations are selected based on their contributions to antigen binding but are thermodynamically destabilizing; these detrimental effects can be compensated by other somatic mutations that contribute to an increase in the stability of the antibody. Thus, this work has revealed another critical role for the somatic hypermutation process during the immune response, and may modify our historical view of complementarity-determining regions and their role in antigen recognition based on Wu and Kabat’s classic analysis of sequence variation in the antibody molecule (37).

**Materials and Methods**

93F3, OKT3, Germ-Line, and Mutant Variant Cloning. The heavy and light chains of 93F3 and OKT3 and their germ-line precursors were generated by gene synthesis (GenScript). All heavy and light chains of mutant variants were constructed by overlap PCR (Tables S1 and S6) and ligated into pFUSE plasmids (Invivogen).

**Antibody Expression and Purification.** Fab fragments were expressed by cotransfecting heavy chain- and light chain-encoding plasmids into HEK293 Freestyle cells using 293Fectin (Invitrogen). Briefly, HEK293 Freestyle cells were cultured in 293 Freestyle Media (Invitrogen) to 10^5 cells/mL. Heavy chain- and light chain-encoding plasmids (10 μg each) were added to 1 mL Opti-MEM (Invitrogen). Twenty microliters of 293Fectin was added to 1 mL Opti-MEM and incubated for 5 min, followed by gentle mixing with the plasmid DNA diluted and incubation for 45 min at room temperature. The mixture was transferred into 20 mL of HEK293 Freestyle cell culture. The culture supernatant was harvested after 48 h and cells were cultured in fresh media for another 48 h and then harvested a second time. The supernatant was loaded onto an Ni-NTA column bound to Sepharose with five column volumes of wash buffer (50 mM Tris-HCl, 20 mM imidazole, 300 mM NaCl, pH 8.0). The pure Fab fragment was eluted with five column volumes of elution buffer (50 mM Tris-HCl, 400 mM imidazole, 300 mM NaCl, pH 8.0) and then buffer-exchanged to low-salt buffer (25 mM Tris-HCl, 50 mM NaCl, pH 8.0).

Experimental methods for melting temperature, binding affinity, and structure determination are detailed in SI Materials and Methods.

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