IMMUNOLOGY

Germinal center antibody mutation trajectories are determined by rapid self/foreign discrimination

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Antibodies have the specificity to differentiate foreign antigens that mimic self antigens, but it remains unclear how such specificity is acquired. In a mouse model, we generated B cells displaying an antibody that cross-reacts with two related protein antigens expressed on self versus foreign cells. B cell anergy was imposed by self antigen but reversed upon challenge with high-density foreign antigen, leading to germinal center recruitment and antibody gene hypermutation. Single-cell analysis detected rapid selection for mutations that decrease self affinity and slower selection for epistatic mutations that specifically increase foreign affinity. Crystal structures revealed that these mutations exploited subtle topological differences to achieve 5000-fold preferential binding to foreign over self epitopes. Resolution of antigenic mimicry drove the optimal affinity maturation trajectory, highlighting the value of retaining self-reactive clones as substrates for protective antibody responses.

Antibodies often distinguish nearly identical foreign and self antigens, such as the glycolipids on *Campylobacter jejuni* cell walls and those on human nerve cells, with fewer than 0.1% of infected people producing cross-reactive antibodies that result in paralysis and Guillain-Barré syndrome (*1*). Apparent limits to antibody discrimination of self versus foreign antigens are exploited by HIV, lymphocytic choriomeningitis virus, and Lassa fever virus. These viruses establish persistent infections and evade antibodies by mimicking self glycoproteins and cloaking their foreign envelope proteins with self glycans (*2*–*5*). Although self-reactivity can be removed from antibodies by V(D)J recombination (*6*) or by V-region hypermutation (*7*–*9*), the cellular basis and mutational pathways for resolving foreign-self mimicry after infection or immunization remain undefined.

We engineered bone marrow chimeric mice (Fig. 1, A and B, and figs. S1 to S4) in which the majority of developing B cells reaching the spleen from the bone marrow were polyclonal and expressed CD45.2 (CD45.2$^+$). However, 1% of transitional B cells and 0.1% of mature follicular B cells were CD45.1$^+$ SW$_{HEL}$ cells, which carry HyHEL10 antibodies on their surfaces. HyHEL10 antibodies have a defined structure and low affinity for a self protein [hen egg lysozyme with three substitutions (HEL^{3X}) (*10*–*13*); $1/K_D$ (equilibrium dissociation constant) = 1.2×10^7 M^{-1}] and for a structurally similar foreign protein [duck egg lysozyme (DEL); $1/K_D = 2.5 \times 10^7$ M^{-1}). In one group of chimeric mice, the self protein was displayed on all cells as an integral membrane protein, mHEL^{3X}, encoded by a transgene with a ubiquitin promoter (*14*). When SW$_{HEL}$ B cells were self-reactive, they reached the spleen as short-lived anergic cells with decreased surface immunoglobulin M (IgM) but normal surface IgD (Fig. 1B and figs. S1 to S4), located primarily in the T cell zone (Fig. 1C) as in other anergic models (*15*–*17*). The frequency of anergic SW$_{HEL}$ cells was lower than the frequency of circulating anergic IgD$^+$ IgM^{lo} VH4-34$^+$ B cells, which recognize ubiquitous cell surface antigens and mutate away from self-reactivity in humans (*8*).

We first tested whether self-reactive SW$_{HEL}$ B cells could respond to a foreign antigen that perfectly mimicked self antigen. Sheep red blood cells (SRBCs) were covalently coupled with self antigen at surface densities equivalent to those on endogenous mouse red blood cells (MRBCs) or 30-fold higher (Fig. 1D). Despite equal levels of T cell help for germinal center (GC) responses by the diverse repertoire of other B cells (Fig. 1F), self-reactive SW$_{HEL}$ B cells entered GCs only when SRBCs carried high antigen density (Fig. 1G). SRBCs with low antigen density could nevertheless induce GC responses from SW$_{HEL}$ B cells that were not self-reactive. These results are consistent with previous evidence that helper T cells cooperate with anergic B cells only when B cell–receptor cross-linking by foreign antigen is greater than that induced by self antigen (*18*).

Next, we tested the response of self-reactive SW$_{HEL}$ B cells to DEL, which differs from self antigen at four residues that make contact with the HyHEL10 heavy chain (H chain) (figs. S5 and S6A). GC reactions were initiated with unconjugated SRBCs, and 11 days later, SW$_{HEL}$ B cells were recruited into the reactions synchronously by a booster immunization with DEL coupled at high density to SRBCs (Fig. 2A). Four days after immunization with DEL-conjugated SRBCs, SW$_{HEL}$ B cells constituted ~20% of all GC B cells and were present in comparable total numbers regardless of self-reactivity (Fig. 2B and figs. S5 and S6, B and C). When the SW$_{HEL}$ GC B cells were self-reactive, they had lower densities of surface IgG1 per cell (Fig. 2C and fig. S6D), likely caused by engagement with self antigen on neighboring cells. At this early time point, the frequencies and numbers of IgG1$^-$ and IgG1$^+$ SW$_{HEL}$ B cells with low binding to self antigen were increased when the cells were self-reactive (Fig. 2, C and D, and fig. S6C). These low-binding cells had increased frequencies of missense mutations (fig. S6, E and F), with 55% having acquired a Ser^{52}→Arg^{52} (S52R) or Ser^{52}→Asn^{52} (S52N) mutation in complementarity-determining region 2 (CDR2) (Fig. 2E). Both mutations greatly decreased affinities for both self and foreign proteins (fig. S7 and table S1).

To determine whether rapid selection for mutant GC B cells with decreased affinity for self protein was followed by maturation of affinity for foreign protein, we analyzed antibody mutations 4, 7, and 11 days after SW$_{HEL}$ B cells were challenged with DEL-conjugated SRBCs (Fig. 3A and fig. S8). On day 4, the frequencies of S52R and S52N mutations were again significantly increased (11.55 versus 3.55%; $P = 0.0093$) when SW$_{HEL}$ B cells were self-reactive. However, the frequencies decreased on days 7 and 11. An Ile^{29}→Phe^{29} (I29F) mutation in CDR1 became prevalent instead on day 7, occurring as a single substitution in 31% of SW$_{HEL}$ B cells when they were self-reactive compared with only 1.7% when they were not. I29F conferred the property of distinguishing foreign from self protein, causing a 10-fold decrease in self affinity and a 2.6-fold increase in foreign affinity (Fig. 3A, fig. S7, and table S1).

The I29F mutation became paired with Ser^{52}→Thr^{52} (S52T) and Tyr^{53}→Phe^{53} (Y53F) mutations in CDR2. This pattern emerged in a small subset of self-reactive cells on day 7, but these mutations became most prevalent as pairs or a trio by day 11. S52T and Y53F were rarely found individually, but combined with the I29F foundation mutation, they increased foreign-self discrimination. Cells with the combined mutations retained 1×10^6 M^{-1} affinity for self but showed progressively increasing foreign affinity, up to to 6×10^9 M^{-1}. Strong epistatic (nonadditive) effects were observed. For example, the I29F-S52T-Y53F trio increased the apparent differential binding energy ($\Delta\Delta G$) for binding foreign antigen by −3.3 kcal/mol, compared with −1.6 kcal/mol expected for additive effects of the individual mutations (table S1). This trio of mutations became even more prevalent when self-reactive SW$_{HEL}$ B cells were recruited at the outset of the GC reaction and analyzed 15 days later (Fig. 3B

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and fig. S9). Thus, an antibody that was initially unable to distinguish foreign from self antigen had evolved a 5000-fold differential binding to foreign antigen over self antigen by first mutating away from binding self antigen and subsequently mutating toward binding foreign antigen. SW_{HEL}-derived cells that had lost self-binding but retained foreign binding were also frequent among the $IgG1^+$ memory B cell compartment (fig. S10). Foreign antigen–specific IgG1 serum titers were increased in mice with initially self-reactive SW_{HEL} B cells (fig. S11).

A different, less optimal evolutionary trajectory prevailed when SW_{HEL} B cells were not self-reactive. This trajectory was dominated by acquisition of a CDR2 mutation (Y58F) alone, paired, or in trio with S52T and Y53F (Fig. 3). Y58F alone or with S52T and Y53F increased self-affinity by a factor of four, explaining why this trajectory was not taken by self-reactive SW_{HEL} cells. The Y58F-S52T-Y53F trio increased foreign affinity to $2 \times 10^9\ M^{-1}$, which was one-third of the affinity obtained with the I29F-S52T-Y53F trio selected through the self-reactive trajectory.

To understand how these three mutations conferred a 5000-fold differential binding to foreign protein over self, we used x-ray crystallography to analyze the structure of $HyHEL10^{I29F,S52T,Y53F}$ in complex with DEL (Fig. 4, table S2, and movie S1) compared to that of wild-type HyHEL10 ($HyHEL10^{WT}$) in complex with HEL (*19*). I29F resulted in a structural rearrangement of the CDR1 loop to accommodate the larger phenylalanine side chain. Displacement of this loop (arrow 1 in Fig. 4C) opened up additional structural adjustments of CDR2 (arrow 2 in Fig. 4C) and, in particular, repositioned Y53F to interact with a hydrophobic pocket formed on the surface of DEL by the short Ala75 (A75) side chain, which is in contrast to the much longer leucine in HEL. The CDR2 backbone adjustments also allowed replacement of the smaller S52 side chain with threonine. Thus, our structural analyses were in agreement with the observed mutational trajectory, whereby the I29F foundation mutation introduces structural rearrangements into CDR1 and CDR2. These rearrangements enable secondary mutations at positions 52 and 53, which selectively increase foreign affinity in an epistatic manner. Binding studies confirmed that I29F confers 50-fold–lower binding to self versus foreign antigen by exploiting the $Leu^{75} \rightarrow Ala^{75}$ (L75A) foreign pocket coupled with the adjacent $Glu^{73} \rightarrow Lys^{73}$ charge reversal (table S1). This

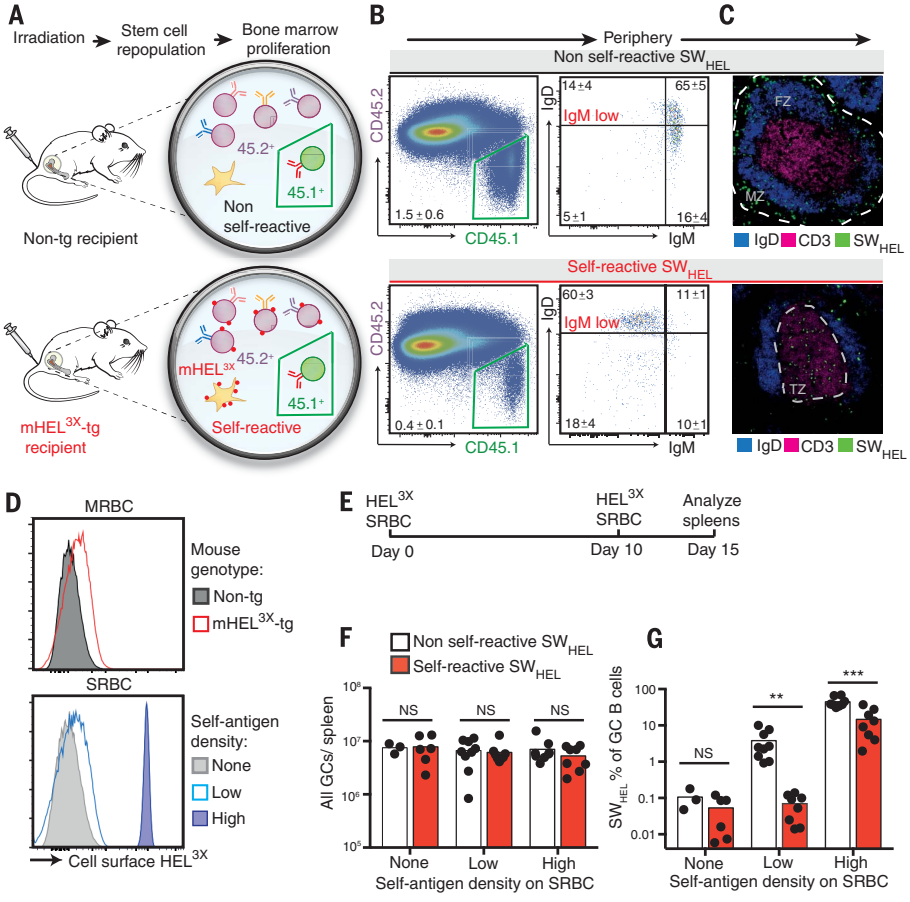

Fig. 1. Recruitment of anergic cells into GCs requires higher foreign antigen density. Construction of parallel groups of hematopoietic chimeras (**A**) and analysis of their spleens by (**B**) flow cytometry of all B cells (left) or $CD45.1^+$ SW_{HEL} B cells (right) (n = 14 spleens per group; values are mean percentages ± SEM) or (**C**) immunohistology showing localization of SW_{HEL} B cells (green), other B cells (blue), and T cells (pink). Dashed lines indicate borders between splenic compartments. FZ, follicular zone; MZ, marginal zone; TZ, T cell zone. (**D**) Relative abundances of self HEL^{3X} on MRBCs from $mHEL^{3X}$tg or nontransgenic mice and on foreign SRBCs conjugated with 0 (none), 0.1 (low), or 10 (high) μg/ml HEL^{3X}. (**E**) Timing of chimera immunizations. (**F**) Total GC cells per spleen. (**G**) Percentages of SW_{HEL} cells among GC B cells. NS, not significant ($P > 0.05$); $^{**}P < 0.01$; $^{***}P < 0.001$; Student's t test. Data points represent one chimera (two experiments, 16 to 26 chimeras in each).

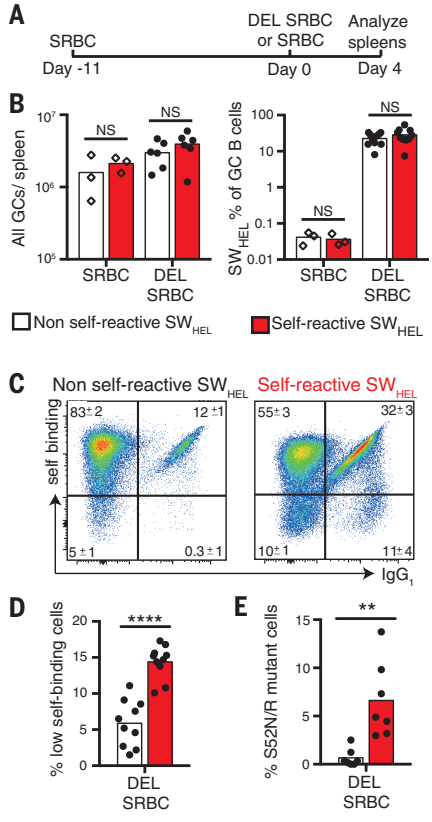

Fig. 2. Binding to similar foreign and self antigens triggers rapid mutation away from self affinity. (**A**) Timing of chimera immunizations. (**B**) Total GC cells per spleen and percentages of SW_{HEL} cells among GC cells in chimeras receiving DEL-coupled SRBCs or unconjugated SRBCs. (**C** and **D**) Analysis of SW_{HEL} GC B cells showing (C) the percentages (means ± SEM) that bind 0.14 μM HEL^{3X} or express cell surface IgG1 and (D) the percentages of nonbinding cells. (**E**) Percentages of sorted and individually sequenced SW_{HEL} GC cells with an S52N or S52R mutation. NS, $P > 0.05$; $^{**}P < 0.01$, $^{****}P < 0.0001$; Student's t test. Each data point represents one mouse. Data are from at least two independent experiments, each involving three to four mice in DEL-coupled SRBC groups.

effect was further confirmed by solving the structure of HyHEL10^{I29F} in complex with DEL (fig. S12).

We next identified anergic B cells in the mHEL^{3X} transgenic (mHEL^{3X}tg) mice within a polyclonal repertoire that displayed micromolar affinity for the same self antigen and tested whether these B cells too could resolve antigenic mimicry. HEL^{3X}-binding B cells constituted 2.7% of IgD^{+} IgM^{lo} anergic B cells and 0.5% of all splenic B cells (fig. S13A). These were sorted and added at 0.5% frequency to unselected CD45.1^{+} B cells, and the polyclonal mixture was injected together with T cells into mHEL^{3X}tg *Rag1*$^{-/-}$ mice immunized with DEL-conjugated SRBCs. In the recipients, 96% of the GC response was derived from the unselected CD45.1^{+} B cells, presumably recognizing mostly SRBC antigens. In contrast, 61% of the DEL-binding GC response was derived from the polyclonal HEL^{3X}-binding anergic CD45.2^{+} B cells (fig. S13B). Only 9.7% of these cells still bound self antigen, whereas 53% bound foreign DEL selectively (fig. S13C). Thus, in a normal repertoire, cells with micromolar affinity for self HEL^{3X} are dominant contributors to the GC response against the self mimic DEL and rapidly lose binding to self.

The findings here extend evidence for autoantibody redemption in human antibodies (*7*–*9*) by showing that mutation away from self-reactivity

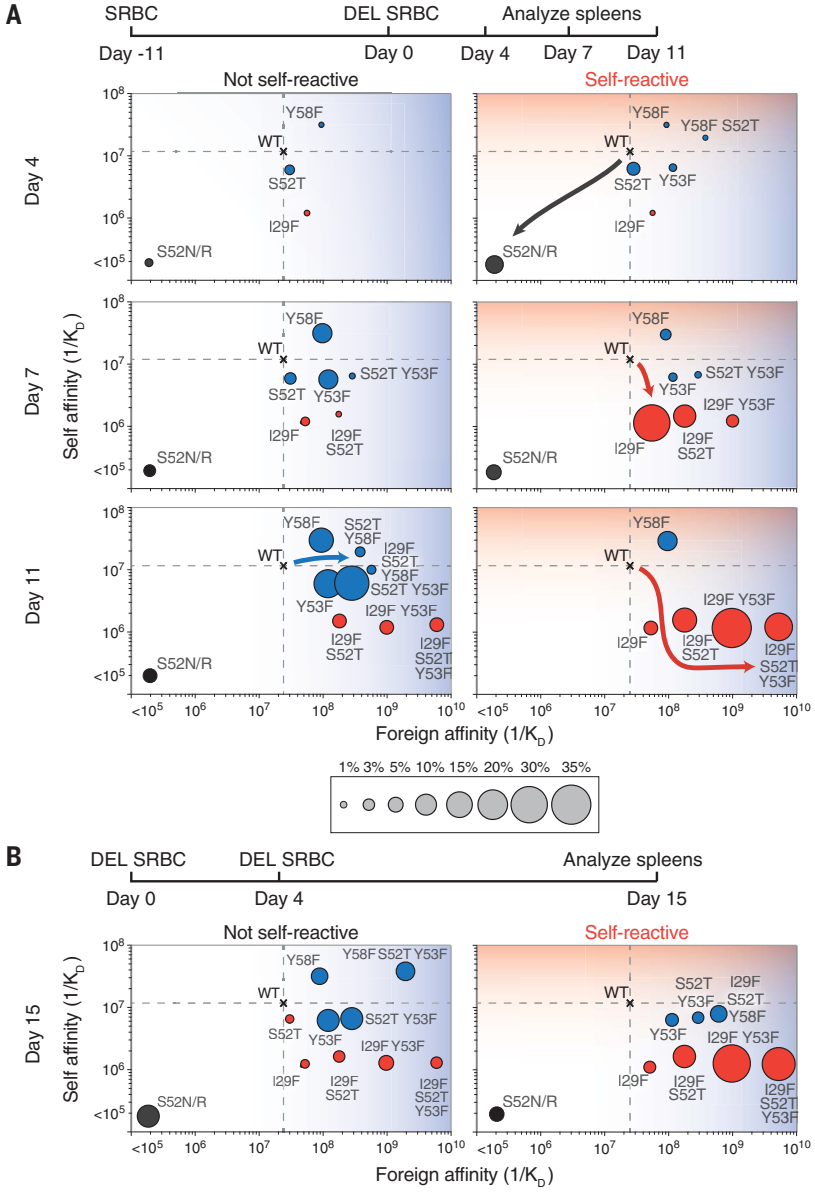

Fig. 3. Self-reactive cells follow distinct mutational trajectories to lose self-binding capacity, leading to optimal affinity for foreign antigen. Chimera immunization time points were selected to (**A**) synchronize recruitment of SW$_{HEL}$ B cells into established GCs or (**B**) recruit SW$_{HEL}$ B cells into GCs from the outset. SW$_{HEL}$ GC B cells were subjected to single-cell sequencing. Dashed lines show the affinities of unmutated (WT) antibody for self and foreign proteins. Circles show the affinities of recurring mutant antibodies for self and foreign proteins. Areas of the circles denote the percentages of SW$_{HEL}$ B cells with the indicated mutations. Red circles indicate mutations more frequent in self-reactive SW$_{HEL}$ B cells, and blue circles represent mutations more frequent in the non–self-reactive SW$_{HEL}$ B cells. Data are from one experiment and are representative of two experiments, each involving two to three mice per group at each time point. N/R, Asn or Arg.

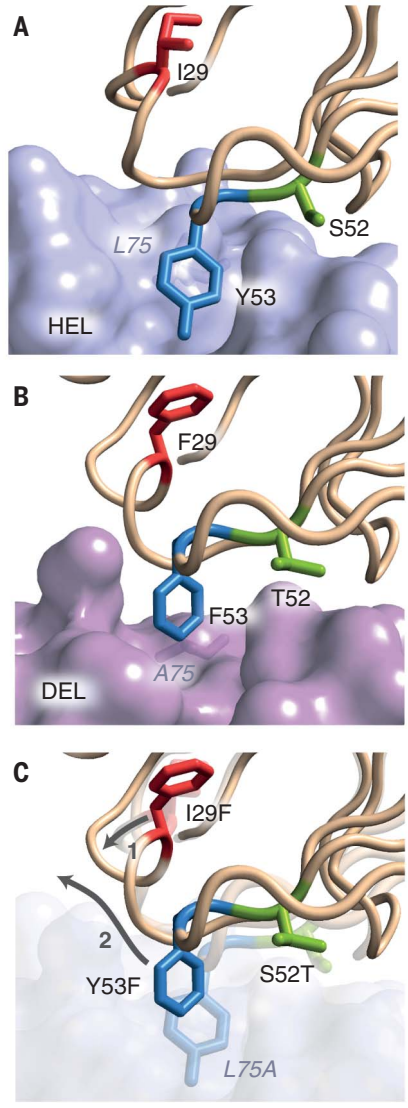

Fig. 4. Structural basis of mutation away from self. X-ray crystallographic structures of (**A**) unmutated HyHEL10 in complex with HEL and (**B**) HyHEL10^{I29F,S52T,Y53F} triple mutant antibody in complex with DEL. (**C**) Overlay of both structures showing the structural rearrangement of the CDR1 loop caused by the I29F mutation (arrow 1) and the complementary structural adjustments of positions 52 and 53 in the CDR2 loop to exploit the L75A pocket in the foreign antigen (arrow 2).

precedes mutation toward foreign affinity to create distinctive epistatic trajectories. Self-reactivity, rather than being a barrier to immunization, directed cells down an alternative trajectory, which produced a higher final affinity for the foreign immunogen. The higher threshold to activate anergic cells and recruit them to GC reactions is nevertheless an important constraint: for instance, a low density of Env molecules on HIV virions may fail to activate anergic B cells with moderate cross-reactive affinity for self glycans attached to foreign and self polypeptides, precluding mutation trajectories away from self-reactivity.

Antibody mutation away from self-reactivity in GC reactions defers the need to acquire stringent self-tolerance until after an infection. This process is complementary to the concept of purging self-reactive antibodies from the preimmune repertoire before they can be tested for binding foreign antigen (*1*, *6*, *20*–*22*) as well as to Jerne's hypothesis of mutation away from self in the bone marrow and bursa (*23*). Both concepts create a "holes in the repertoire" problem if applied too stringently (*24*, *25*). Crucially, autoantibody redemption minimizes the potential for microbes to evolve antigens that are "almost self," which could otherwise be recognized only by preimmune antibodies that had been deleted or edited in the bone marrow. Mutation away from self in response to one foreign antigen may allow progeny B cells to respond to an unrelated foreign antigen later. For example, intestinal microbes may induce polyspecific B cells to mutate away from self, providing a self-tolerant repertoire that would not be available in individuals treated with antibiotics or raised in a more hygienic environment. The evolution of an antibody along a limited set of mutation trajectories, driven by two selection pressures for higher affinity for one ligand and lower affinity for another, provides an example of deterministic molecular evolution. Our findings provide insights into the GC reaction and the evolution of specificity in antibody-antigen interactions.

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Author contributions: D.L.B. performed and analyzed mouse experiments; P.S. performed and analyzed binding affinity experiments; D.B.L. performed and analyzed crystallography experiments; J.J. and K.B. generated antibodies and antigens; T.D.C., J.H.R., and J.R.H. developed mHEL^{3X}tg mice; R.B. devised and developed the mHEL^{3X} × SW$_{HEL}$ system; C.C.G. supervised B cell biology aims; D.C. supervised structural and biophysical aims; D.L.B., P.S., D.B.L., R.B., D.C., and C.C.G. designed and interpreted experiments; D.L.B., D.B.L., P.S., K.P., R.B., D.C., and C.C.G. prepared figures; B.T.P. generated movie S1; and D.L.B. drafted and R.B., D.C., and C.C.G. revised the manuscript.
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Data and materials availability: Coordinates and structure factors have been deposited in the Protein Data Bank with accession codes 5VJO and 5VJQ. All other data needed to evaluate the conclusions in the paper are present in the paper or the supplementary materials.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/360/6385/223/suppl/DC1
Materials and Methods
Figs. S1 to S13
Tables S1 and S2
References (*26*–*35*)
Movie S1

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Autoantibody redemption through rapid mutations

Antibodies distinguish foreign epitopes from closely related self-antigens by poorly understood mechanisms. In mice, Burnett *et al.* found that a proportion of B cells could cross-react with similar foreign and self-antigens (see the Perspective by Kara and Nussenzweig). Challenge with self-antigen resulted in anergy (i.e., a lack of immune response), which was reversed by exposure to high-density foreign antigen. Mutations that decreased self-affinity were rapidly selected for, whereas selection for epistatic mutations that enhanced foreign reactivity took longer. Self-reactivity, rather than being an impediment to immunization, resulted in higher affinities against a foreign immunogen.

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