Clonal redemption of autoantibodies by somatic hypermutation away from self-reactivity during human immunization

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Clonal anergy is an enigmatic self-tolerance mechanism because no apparent purpose is served by retaining functionally silenced B cells bearing autoantibodies. Human autoantibodies with IGHV4-34*01 heavy chains bind to poly-N-acetyllactosamine carbohydrates (I/i antigen) on erythrocytes and B lymphocytes, cause cold agglutinin disease, and are carried by 5% of naive B cells that are anergic. We analyzed the specificity of three IGHV4-34*01 IgG antibodies isolated from healthy donors immunized against foreign rhesus D alloantigen or vaccinia virus. Each IgG was expressed and analyzed either in a hypermutated immune state or after reverting each antibody to its unmutated preimmune ancestor. In each case, the preimmune ancestor IgG bound intensely to normal human B cells bearing I/i antigen. Self-reactivity was removed by a single somatic mutation that paradoxically decreased binding to the foreign immunogen, whereas other mutations conferred increased foreign binding. These data demonstrate the existence of a mechanism for mutation away from self-reactivity in humans. Because 2.5% of switched memory B cells use IGHV4-34*01 and >43% of these have mutations that remove I/i binding, clonal redemption of anergic cells appears efficient during physiological human antibody responses.
antibody when transplanted into animals lacking the relevant self-antigen and provide a source of T cell help, we previously hypothesized that a physiological rationale for reversal of anergy might be following V-segment hypermutation and mutation away from self-reactivity in germinal centers (Goodnow et al., 1991; Goodnow, 1996, 1997). Diaz and Klinman (2000) considered a similar scenario as one theoretical explanation for their evidence that a CD24low subset of B cells contributed little antibody in a primary response but much of the secondary response antibody. Recently, we provided direct experimental evidence in transgenic mice for clonal redemption: anergic B cells with high affinity for self-lysozyme were induced to hypermutate their V-segments in germinal centers upon immunization with lysozyme coupled to foreign red cells, and mutant progeny were efficiently selected for loss of binding to self-lysozyme (Sabouri et al., 2014). It nevertheless remains an open question whether hypermutation away from self actually occurs, particularly during physiological human antibody responses.

The goal of the current study was to determine whether clonal redemption occurs in normal human immune responses. To address this question, we required a source of antibody from B cells that were (a) anergic, (b) reactive with a well defined, pathologically significant blood cell surface autoantigen, and (c) frequent in healthy humans. B cells carrying antibodies with the heavy chain (H chain) V-segment IGHV4–34*01 meet these criteria, accounting for 5–10% of circulating naive B cells in healthy individuals and displaying anergic characteristics of down-regulated surface IgM, but not surface IgD, and functional unresponsiveness (Cappione et al., 2005). Most unmutated IGHV4–34 IgM antibodies agglutinate human erythrocytes at low temperatures, causing cold agglutinin hemolytic disease if secreted at sufficient titer and glutinate human erythrocytes at low temperatures, causing cold agglutinin hemolytic disease if secreted at sufficient titer of antibody when transplanted into animals lacking the relevant self-antigen and provide a source of T cell help, we previously hypothesized that a physiological rationale for reversal of anergy might be following V-segment hypermutation and mutation away from self-reactivity in germinal centers (Goodnow et al., 1991; Goodnow, 1996, 1997). Diaz and Klinman (2000) considered a similar scenario as one theoretical explanation for their evidence that a CD24low subset of B cells contributed little antibody in a primary response but much of the secondary response antibody. Recently, we provided direct experimental evidence in transgenic mice for clonal redemption: anergic B cells with high affinity for self-lysozyme were induced to hypermutate their V-segments in germinal centers upon immunization with lysozyme coupled to foreign red cells, and mutant progeny were efficiently selected for loss of binding to self-lysozyme (Sabouri et al., 2014). It nevertheless remains an open question whether hypermutation away from self actually occurs, particularly during physiological human antibody responses.

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**RESULTS**

A previous Blastn search of deposited nucleotide sequences in the NCBI database identified foreign antigen-specific IgG antibodies with hypermutated IGHV4–34 H chains that had been elicited by immunization of healthy individuals (Sabouri et al., 2014). Here, we experimentally evaluated the self-and foreign reactivity of three of these antibodies, one against the erythrocyte alloantigen rhesus D (D) and two against vaccinia virus, for which the entire Ig H and L chain variable sequences were available and the foreign antigen readily obtained for binding studies. Each immune antibody was expressed as human IgG1. In parallel, the somatic mutations in the H and L chain of each antibody were reverted to the IMGT closest matched germ line V, D, and J sequences to express IgG1 corresponding to each antibody’s unmutated, preimmune ancestor (designated with the prefix “p”). IgG1 antibodies containing single forward or reversion mutations in the hydrophobic patch were also expressed and purified.

**Fog-1 RhD antibody**

Anti-RhD antibodies are routinely elicited by repeated allogeneic immunization of healthy RhD-negative men with foreign RhD-positive erythrocytes to provide Rho immunoglobulin for prevention of erythroblastosis fetalis in primiparous RhD-negative mothers and to isolate large panels of monoclonal anti-RhD antibodies for typing and a potential substitute to polyclonal gamma globulin fractions. A high-affinity anti-RhD IgG antibody isolated in this manner, Fog-1 (Bye et al., 1992), is highly somatically mutated using the IGHV4–34*01 V, IGHD2–21*02, and IGJH6*02 H chain elements with 19 amino acid substitutions and the IGKV1–17*01 and IGKJ2*01 L chain elements with 6 substitutions (Fig. 1, A and B). To test self-reactivity of Fog-1 and preimmune Fog-1 (pFog-1), we used flow cytometry to measure binding of the different expressed IgG antibodies to CD19+ IgD+ CD27– mature naive human B cells from normal blood donors, which display high densities of poly-N-acetyllactosamine I/i antigen O-linked carbohydrates on the B220 isoform of CD45 (Childs et al., 1983; Grillot-Courvalin et al., 1992). pFog-1 specifically and homogeneously bound CD19+ B cells compared with CD19+ lymphocytes in a dose-dependent fashion, whereas the hypermutated Fog-1 antibody had no measurable binding at any concentration tested (Fig. 1 C). pFog-1 binding to self-blood B cells occurred at 37°C or 4°C, although over a range of antibody concentra-
tions, binding was slightly reduced when performed at 37°C compared with 4°C (12% decreased; Fig. 1 E). One of the 19 H chain somatic mutations in Fog-1, Y26H (IMGT numbering), alters the hydrophobic patch alanine, valine, tyrosine (AVY) sequence (Fig. 1, A and F). When this single somatic mutation was introduced into the pFog-1 antibody, it was sufficient to completely abolish self-reactivity, comparable with Fog-1 with 18 additional H chain and 6 L chain mutations (pFog-1 Y26H; Fig. 1 C). Conversely, self-reactivity was restored when only this somatic mutation was reverted in Fog-1 (Fog-1 H26Y), albeit not to the level of pFog-1. Thus, a single Y26H somatic mutation acquired by pFog-1 was sufficient to remove all detectable self-reactivity, whereas all the other acquired mutations in Fog-1 were not sufficient to abolish binding to self-blood cells (Fig. 1 C).

Flow cytometry was also used to measure binding of Fog-1 and pFog-1 to foreign RhD⁺ and self-RhD⁺ erythrocytes from healthy donors. Fog-1 bound homogeneously and specifically to RhD⁺ erythrocytes compared with erythrocytes from an RhD⁻ donor (Fig. 1 D). The pFog-1 antibody showed minimal binding to RhD⁺ erythrocytes compared with RhD⁻ erythrocytes, consistent with the very low affinity of the preimmune antibody for the immunizing foreign blood cells. Note that there is too little binding of pFog-1 IgG to the low density of I/i antigens on RhD⁺ erythrocytes, even at 4°C, in contrast to the strong binding of pFog-1 to B cells displaying a high density of I/i antigens on B220. Interestingly, the Y26H somatic mutation that completely removed self-reactivity conferred no detectable increase in binding of pFog-1 Y26H to foreign RhD⁺ erythrocytes, nor did its reversion cause any measurable decrease in RhD⁺ binding of Fog-1 H26Y (Fig. 1 D). Although we cannot determine at which point Y26H was acquired in the evolution of Fog-1, whether it was acquired as the first or as the last mutation, it conferred no measurable benefit for binding foreign RhD but completely removed binding to self.

166 vaccinia antibody

Having shown evidence for autoantibody redemption in alloimmunization, we next evaluated this hypothesis in the context of a procedure that led to global eradication of small-pox: immunization of healthy people with vaccinia virus. The vaccinia-specific IgG antibodies 166 and 589 were isolated by single-cell PCR from peripheral blood plasmablasts 9–20 d after standard immunization with vaccinia virus Lister strain in healthy, previously vaccinated volunteers (Lantto et al., 2011). The 166 antibody employs an IGHV4-34*01, IGHD3-3*01, and IGKJ1*02 H chain with 13 nonsynonymous somatic mutations paired with an IGKV3-11*01 and IGKJ1*01 L chain carrying five somatic substitutions (Fig. 2, A and B). The hypermutated 166 antibody exhibited no detectable binding to normal naive B cells, whereas when all the somatic mutations were reverted, the preimmune 166 (p166) antibody demonstrated homogenous, dose-dependent self-reactivity (Fig. 2 C). p166 antibody binding to normal human B cells occurred at 37° or 4°C (Fig. 2 E). Acquisition of a single mutation within the hydrophobic patch Y26S was sufficient to completely remove self-reactivity from the p166 antibody (p166 Y26S; Fig. 2, C and F). However, in the context of the other somatic mutations in 166, the Y26S mutation was redundant for reducing auto-reactivity because when the S26 substitution was reverted to Y in the immune 166 antibody, this single back mutation alone did not restore self-binding (166 S26Y; Fig. 2, C and F).

Binding to vaccinia virus by the 166 antibody and its reverted preimmune ancestor was measured by ELISA (Fig. 2 D). Binding of p166 to the virus was less than the fully mutated 166, consistent with affinity maturation against the virus. However, acquisition of the Y26S mutation in the hydrophobic patch did not appear to contribute to affinity maturation because it decreased p166 binding to the virus (Fig. 2 D), providing further evidence for a mechanism that allows selection of mutations that reduce antibody affinity for self, independent of mutations that increase binding to foreign antigens.

589 vaccinia antibody

Both Fog-1 and 166 antibodies escaped self-reactivity by acquiring mutations in the Y26 residue, whereas the 589 vaccinia antibody maintained Y26 but acquired a mutation in another hydrophobic patch residue, Q6E (Fig. 3 F). The 589 antibody binds to a major membrane protein of the mature vaccinia virion A14 (Lantto et al., 2011) and employs an IGHV4-34*01, IGHJ2-2*02, and IGKJ4*02 H chain with 19 somatic substitutions paired with an IGKV1-39*01 and IGKJ1*01 L chain with 6 substitutions (Fig. 3, A and B). The hypermutated antibody lacked binding to self-B cells, but the preimmune 589 derivative was self-reactive, binding B cells at concentrations <4 µg/ml at 37°C or 4°C (Fig. 3 E). B cell binding was reduced but not eliminated by acquisition of the Q6E somatic mutation on its own (p589 Q6E; Fig. 3 C), and reversion of this somatic mutation alone was sufficient to restore an intermediate level of self-binding in the otherwise fully mutated antibody (589 E6Q). Although the single point mutation Q6E was necessary and sufficient to decrease self-reactivity, it also decreased binding to the virus: acquisition of this somatic mutation alone markedly decreased binding of p589 Q6E to vaccinia, and reversion of just this mutation enhanced binding of the otherwise fully hypermutated 589 E6Q (Fig. 3 D). The Q6E mutation provides a clear example of antibody mutation away from self-reactivity at the cost of decreased binding to the eliciting foreign antigen.

Acquisition of very low polyreactivity

Mutation away from self-reactivity with cell surface poly-N-acetyllactosamine, as observed for the IGHV4-34 antibodies studied here, contrasts to previous data demonstrating acquisition of polyreactivity with self-antigens through hypermutation in antibodies in the circulating IgG–memory B cell pool (Tiller et al., 2007). To determine whether Fog-1,
166, or 589 IGHV4-34 antibodies become polyreactive after somatic mutation, we evaluated the binding of preimmune and mutated antibodies to HEK293 cells, double-stranded DNA (dsDNA), and LPS compared with established polyreactive antibody controls. The positive control used was the weakly polyreactive eiJB40 antibody cloned from an early immature
bone marrow B cell with down-regulated surface IgM, and the negative control was the nonpolyreactive mGO53 antibody cloned from a mature naive B cell (Wardemann et al., 2003). None of the IGHV4-34 antibodies or mGO53 bound to surface or intracellular self-antigens expressed by HEK293 cells, whereas eiJB40 showed dose-dependent binding to both intact and permeabilized HEK293 (Fig. 4 A). ELISAs evaluating LPS and dsDNA reactivity showed dose-dependent binding of eiJB40. The reactivity of IGHV4-34 antibodies was equivalent to the negative control mGO53 with the exception of Fog-1 and 589, which showed low binding to dsDNA and LPS that was much less than eiJB40 and would be classed as negative on this basis (Tiller et al., 2007) but was, nevertheless, greater than mGO53 or the pFog-1 and p589 antibodies (Fig. 4 B). The rise in very low binding to these antigens that accompanies affinity maturation to foreign RhD and vaccinia in Fog-1 and 589 is consistent with the increased polyreactivity observed in randomly sampled switched memory B cells (Tiller et al., 2007), indicating that clonal selection to mutate away from specific binding to an abundant cell surface autoantigen, poly-N-acetyllactosamine, differs from selection acting upon polyreactive binding to DNA. The latter property may be ignored because of low affinity and limited B cell exposure to DNA and arise simply as

Figure 2. Discrete somatic mutations acquired by antivaccinia antibody 166 independently remove self-reactivity and increase virus reactivity. (A and B) Variable domain amino acid sequence of the p166 H chain (A) and L chain (B) and sequence substitutions acquired in the immune 166 antibody (shown below in red). (C) Number of amino acid substitutions (#aa subs) in each antibody and binding to mature naive B cells. Data points are the mean and standard deviation of four independent experiments using PBMCs from four individual donors. Statistical significance was assessed using two-way ANOVA (***, P ≤ 0.0001 for p166 vs. 166, p166 Y26S, and 166 S26Y). SHM, somatic hypermutation. (D) Binding of each antibody to vaccinia virus measured by ELISA. The data are representative of three independent experiments with each data point showing the mean of duplicates. Statistical significance was determined by two-way ANOVA (*, P ≤ 0.05 for p166 vs. p166 Y26S; ***, P ≤ 0.001 for 166 and 166 S26Y vs. p166 and p166 Y26S). (E) Binding of p166 to mature naive B cells at 4°C (continuous line) and 37°C (dashed line). Data are representative of two independent experiments, and statistical significance was determined by Student's paired t test (P = 0.05). (F) Three-dimensional structure of a hydrophobic patch for p166 (left) and 166 (right). Preimmune residues are depicted in blue with immune substitutions in dark red and oxygen in light red.
an accidental byproduct of mutations that enhance binding to the foreign antigens. Alternatively, weak polyreactivity could be positively selected to enhance persistence of memory B cells when insufficient foreign antigen remains (Mouquet et al., 2010; Wrammert et al., 2011).

**DISCUSSION**

All three of the human IgG antibodies, Fog-1, 166, and 589, are shown to have started out before somatic hypermutation as potentially pathogenic autoantibodies that bind under physiological conditions to an abundant human blood cell surface autoantigen, poly-N-acetyllactosamine. Rather than triggering clonal deletion or editing in the bone marrow, secretion of these potentially damaging autoantibodies was likely controlled by B cell clonal anergy because B cells expressing the IGHV4-34 segment without hydrophobic patch mutations exhibit the phenotypic and functional characteristics of anergic B cells in the naive B cell repertoire and are much less frequent in the germinal center and memory B cell subsets in healthy people (Cappione et al., 2005). B cells carrying each autoantibody were, nevertheless, recruited into physiological immunization responses and acquired numerous somatic mutations that had two effects: removal of self-reactivity and increased binding to the foreign antigen. These results are consistent with the Jerne (1971) concept of mutant-breeding organs seeded with self-reactive
precursors and with experimental evidence in transgenic mice that anergic B cells can be reactivated by foreign antigens and suitable T cell help to proliferate in germinal centers and undergo hypermutation away from self (Sabouri et al., 2014).

In each of the human antibodies analyzed here, we identified a single, acquired somatic mutation that was sufficient to decrease or eliminate self-reactivity. In the two vaccinia-elicited antibodies, this single mutation also decreased binding to the foreign antigen. In the case of the RhD antibody, binding of the unmutated preimmune antibody to foreign RhD+ erythrocytes was too weak to test whether the mutation that removed self-reactivity also decreased binding to the foreign antigen. Diminished binding to RhD is nevertheless likely because experimental mutagenesis of an IGHV4-34 IgM antibody to RhD revealed that substitution of Y26 or other hydrophobic patch residues diminished or abolished binding to RhD+ erythrocytes in parallel with abrogating I/i erythrocyte self-reactivity (Thorpe et al., 2008). Acquisition of these affinity-lowering hydrophobic patch mutations is difficult to explain by the conventional view of germinal center selection for increased affinity against the foreign antigen. These mutations can be explained, however, by positing existence in humans of a germinal center mechanism for selecting decreased binding to self, even at the expense of decreased affinity for foreign antigen (Sabouri et al., 2014). Two mechanisms could both account for these observations. First, lowered affinity for self-antigen would decrease occupancy and down-regulation of antigen receptors by poly-N-acetyllactosamine, increasing receptor availability to bind RhD or vaccinia and compensating for lower affinity to foreign antigens. Second, lowered affinity for self may decrease antigen receptor–induced apoptosis and migration away from the germinal center light zone (Shokat and Goodnow, 1995).

A recent study evaluating vaccination responses in patients with systemic lupus erythematosus reverted four influenza-specific antibodies (not IGHV4-34) to their predicted preimmune configuration and noted anti-dsDNA reactivity in two ancestral preimmune antibodies (Kaur et al., 2015).

Figure 4. Testing polyreactivity of IGHV4-34 antibodies. (A) Flow cytometric evaluation of binding preimmune and mutated IGHV4-34 and negative and low-positive control antibodies mGO53 and eJB40, respectively, to intact (extracellular) and permeabilized (intracellular) HEK293 cells. Data are representative of two independent experiments. (B) Binding of IGHV4-34 IgG and control antibodies to LPS and dsDNA by ELISA. Data points are the mean and standard deviation of triplicate data points, representative of two independent experiments.
However, it cannot be determined whether the loss of DNA recognition was a passive consequence of affinity maturation toward influenza or an active selection for mutations that reduced self-reactivity. Our study provides clear evidence for the latter in healthy immunized individuals because mutations that increased affinity to foreign antigens could be shown to be separate from mutations within the hydrophobic patch that reduced binding to both self- and foreign antigens.

Unlike the three IgG antibodies studied here, some antibodies may be trapped in an evolutionary cul-de-sac where it is difficult or impossible to mutate away from self without also decreasing binding to foreign antigens. This situation appears to exist for the majority of IgM antibodies initially elicited by RhD alloimmunization, which use the same IGHV4-34 segment as Fog-1 but paired with an IGLV3-3 lambda L chain (Thompson et al., 1991; Bye et al., 1992; Siegel et al., 2002). These do acquire somatic mutations but in regions other than the hydrophobic patch residues, presumably because mutating the latter also abrogates RhD binding (Thorpe et al., 2008), apparently trapping the IGHV4-34/IGLV3-3 combination to explain why it is not found among the high-affinity RhD antibodies may be trapped in an evolutionary cul-de-sac where it is difficult or impossible to mutate away from self without also decreasing binding to foreign antigens. This situation appears to exist for the majority of IgM antibodies initially elicited by RhD alloimmunization, which use the same IGHV4-34 segment as Fog-1 but paired with an IGLV3-3 lambda L chain (Thompson et al., 1991; Bye et al., 1992; Siegel et al., 2002).

Fog-1 is an interesting exception: pairing of IGHV4-34 with IGKV1-17 appears to open up a structure-evolving pathway to lose self-reactivity by acquiring a hydrophobic patch mutation without loss of RhD binding.

Does mutation of IGHV4-34 antibodies away from self-reactivity, as established in the three examples studied here, represent a rare salvage pathway or an efficient process for specificity maturation? Previous studies showed that in nonautoimmune humans, the frequency of B cells binding an IGHV4-34 antiidiotype antibody, 9G4, was 5–7% in naive B cells but only 0.4–1.2% of germinal center B cells and 0.6–1.6% of IgG memory B cells (Pugh-Bernard et al., 2001; Cappione et al., 2005). However, only a modest decrease in the use of the IGHV4-34*01 element was measured by deep sequencing IGH repertoire of normal human B cell subsets: from 5% of naive B cells to 2.5% of switched memory B cells (Wu et al., 2010, 2011). Of the switched memory IGHV4-34 sequences, 43% had acquired AVY somatic mutations (Soubouri et al., 2014), which typically inactivate 9G4 binding as well as I/i self-reactivity because 9G4 binding overlaps the hydrophobic patch and competes with I/i binding (Potter et al., 1993; Zheng et al., 2004). Similarly, Chapman et al. (1996) found AVY mutations in 4/14 IgG and 4/6 IgA sequences from tonsillectomy samples, and Zheng et al. (2004) found hydrophobic patch mutations in 38% of IGHV4-34 IgG-switched B cells in human tonsils. Scoring for mutations in the AVY residues alone will underestimate the frequency of mutations that remove self-reactivity, as illustrated here by the 589 antibody. Collectively, these data indicate that IGHV4-34 antibodies, despite being self-reactive and inducing anergy in naive B cells, efficiently mutate away from self so that their contribution to the switched memory antibody repertoire is 50% of their contribution to the naive repertoire. The loss of the 9G4 idiotype from most of the germinal center and memory B cells of healthy people (Pugh-Bernard et al., 2001; Cappione et al., 2005) may primarily reflect efficient selection in the germinal center for mutation away from self.

In contrast to healthy donors, patients with systemic lupus erythematosus have little decrease in the frequency of 9G4-reactive B cells in the germinal center, switched memory, and plasma cell compartments (Cappione et al., 2005; Richardson et al., 2013). In light of the data presented here, these people may not have a deficit in clonal anergy per se but instead a failure to select germinal center progeny for mutations that remove self-reactivity, e.g., because of over-abundant T follicular helper cells (Vinuesa et al., 2005; Simpson et al., 2010) or intrinsic abnormalities in the B cells (Butt et al., 2015). Cold agglutinin IGHV4-34 IgM antibodies are often produced during chronic EBV or mycoplasma infection, although rarely in sufficient titer to cause hemolytic anemia, and six EBV-induced antibodies that were cloned and sequenced all lacked somatic mutations (Chapman et al., 1993). In Wiskott-Aldrich syndrome, there is consistent secretion of IGHV4-34 IgM antibodies that bind polyclonal antibodies to simulate normal naive B cells, with little or no somatic mutation in the cloned antibodies (Grillot-Courvalin et al., 1992). Thus, certain infections and inherited defects allow anergic B cells to be reactivated and proceed into antibody secretion without hypermutation or selection for loss of self-reactivity.

Pathological formation of IGHV4-34 autoantibodies in infectious and autoimmune diseases raises the question: what might be the advantage of clonal redemption of anergic B cells as a tolerance mechanism when it has the inherent risk of autoimmunity? For T cells, holes in the naive repertoire produced by clonal deletion are difficult for microbes to exploit because MHC polymorphism ensures the holes are different in each individual of a species. For the naive B cell repertoire, however, holes produced by deletion or editing will be the same in all individuals and indeed in most species because self-antigen repertoire evolves slowly; providing steady targets for microbes to mimic and evade antibody formation. One evolutionary solution may lie in the high degree of inherited polymorphism in the IGHV repertoire (Kidd et al., 2012; Collins et al., 2015). The complementary solution is to retain autoreactive B cells in an anergic state within the naive repertoire, enabling somatic mutation away from self-reactivity when activated and receiving help from T follicular helper cells upon binding to a microbial antigen, as shown here for antibodies against vaccinia virus.

**MATERIALS AND METHODS**

**IGHV4-34 antibodies.** Deposited H and L chain sequences for each antibody were obtained from the NCBI database, and somatic mutations in the closest matching V, D and J elements were manually annotated using the IMGT V-quest junction analysis tool (Brochet et al., 2008; Giudicelli et al., 2011). Numbering follows the IMGT schema. Mutated and
preimmune-reverted H and L chain cDNA sequences were synthesized by Biomatik and cloned into pCDNA3.1+ expression vectors. IgG1 antibodies were transiently expressed in HEK293 cells using standard plate transfection and the Expi system (Thermo Fisher Scientific) and purified with protein G Sepharose (GE Healthcare) according to the manufacturers’ recommendations. After dialyzing against PBS and concentrating, antibodies were quantified by Nanodrop and ELISA.

**Assay for self-reactivity.** Whole blood from random healthy donors was obtained from the Australian Red Cross Blood Service. PBMCs were prepared by Ficoll Histopaque and frozen. Thawed PBMCs were washed twice in PBS/1% BSA and incubated with Fc Block (BD) for 30 min at 4°C. After washing, cells were incubated with IGHV4–34 antibodies diluted in PBS and incubated for 30 min at 4°C or 37°C. Cells were washed twice in PBS and stained with antibodies against IgG, CD19, IgD, CD27, Live Dead, CD3, and CD14. Mature naive B cells (CD19+, IgD−, and CD27−) were evaluated for IGHV4–34 antibody binding on an LSRII or CANTO II flow cytometer (BD). Each group of preimmune, hypermutated, and intermediate IGHV4–34 antibodies (e.g., pFog-1, Fog-1, pFog-1 Y26H, and Fog-1 H26Y) were always assessed within the same experiment for three to four independent experiments. To normalize data from the three to four experiments performed on different days and with different flow cytometers, antibody reactivity was expressed as relative mean fluorescence intensity (MFI) by dividing the MFI value by the MFI of the highest binding antibody within the experiment.

**Assay for RhD reactivity.** Erythrocytes were isolated from the whole blood of RhD+ and RhD− donors from the Red Cross Blood Service. Approximately 10 ml of whole blood was diluted 1:1 in PBS and centrifuged at 400 g for 15 min. Plasma and buffy coat layers were removed, and red cell pellets were washed twice in PBS before incubating with IGHV4–34 antibodies at room temperature for 30 min. After washing twice in PBS, erythrocytes were stained with anti–human IgG antibody for 30 min at room temperature and analyzed by flow cytometry.

**Assay for vaccinia virus reactivity.** IGHV4–34 antibody binding to vaccinia virus was evaluated by ELISA. MaxiSorp plates (Thermo Fisher Scientific) were coated with succrose cushion–purified stocks of Western Reserve vaccinia virus (provided by D. Tscharke, Australian National University, Canberra, ACT, Australia), inactivated with 1 µg/ml 4,5,8-trimethylpsoralen, and irradiated with UV-A for 20 min. ELISA plates were blocked with 2% BSA/PBS and then incubated with IGHV4–34 antibodies diluted in 1% BSA/PBS. After washing with 0.1% Tween 20/PBS, anti–human IgG alkaline phosphatase was applied, followed by para-nitrophenylphosphate substrate. All incubations were done for 1 h at room temperature. Binding of IGHV4–34 antibodies to vaccinia virus was quantified by measuring optical density at 405 nm.

**Assay for polyreactivity.** Polyreactivity was evaluated by flow cytometry and ELISA. For flow cytometry experiments, HEK293 cells were incubated with preimmune and mutated IGHV4–34 antibodies for 30 min at 4°C. After washing, anti–human IgG–BV605 was added for 30 min at 4°C and analyzed on a CANTO II flow cytometer. HEK293 cells were also permeabilized with Cytofix reagent (BD) to assess binding of antibodies to intracellular self-antigens. Polyreactivity ELISAs were performed as described previously using dsDNA and LPS (Tiller et al., 2008). For both flow cytometry and ELISA assays, established polyreactivity control antibodies were used: eiJB40, a low-positive control, and mGO53, a negative control antibody (Wardemann et al., 2003). These antibodies were produced as IgG1 as described in the IGHV4–34 antibodies section.

**Structural modeling.** Structural models depicting hydrophobic patch regions of IGHV4–34 were based on Protein Data Bank accession no. 1DNO (Cauerhff et al., 2000). All illustrations were generated using the PyMOL molecular graphics system (Schrödinger, LLC).

**Statistical analysis.** Dose-dependent binding of IGHV4–34 antibodies to either self- or foreign antigens was compared by two-way ANOVA with Tukey’s multiple comparison tests. Calculations were performed using Prism software (version 6; GraphPad Software). P-values <0.05 were considered significant.

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