Human immunoglobulin selection associated with class switch and possible tolerogenic origins for Cδ class-switched B cells

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Current paradigms of peripheral B cell selection suggest that autoreactive B cells are controlled by clonal deletion, anergy, and developmental arrest. We report that changes to the human antibody repertoire likely resulting from these mechanisms both for a well-characterized autoreactivity from antibodies encoded by the V_H4-34 gene and for other hallmarks of an autoreactive repertoire are apparent mainly for class-switched B cells and not for IgM germinal center, IgM memory, or IgM plasma cells. Other possible indicators of autoreactivity found selected with immunoglobulin class include J_H6 gene segment usage, increased frequency of B cells with long third hypervariable regions, and distal J_κ gene segment bias. Of particular interest is the finding that B cells with these same characteristics are selected into the lineage of B cells that have undergone the unusual class switch from constant region Cμ to Cδ (Cδ-CS). The Cδ-CS population also displays an increased frequency of charged amino acids localized to the complementarity-determining regions, further suggesting autoreactivity, and evidence is presented that these B cells had undergone extensive receptor editing. Thus, the Cδ-CS lineage may be a "sink" for B cells harboring autoreactive specificities in normal humans. A model for a new tolerizing mechanism that could account for the Cδ-CS lineage is presented.

Introduction

One of the most prolific topics in immunology today is the mechanism and regulation of Ig class-switch recombination (CSR). In this report, we find that changes to the human Ig repertoire occur mainly in association with class switch. Naive B cells can be induced by antigen encounter and T cell help to form germinal centers (GCs), ultimately leading to the generation of memory and plasma cells (PCs). Antigen-activated GC precursors differentiate to centroblasts, which rapidly proliferate and initiate the somatic hypermutation process (1–3). Centroblasts differentiate to centrocytes that can undergo CSR from IgM/IgD to secondary classes such as IgG and IgA (4). CSR involves a genetic recombination that deletes all intervening sequence between switch regions, thus permanently juxtaposing the specific variable-diversity-joining (VDJ) region to the various secondary constant (C) regions. Of particular interest to this study is a lineage of B cells that we refer to as "Cδ-CS" for "Cδ class-switched." Unlike normal Cμ-versus-Cδ usage involving differential splicing of a single VDJ-Cμ-Cδ transcript, Cδ-CS B cells actually undergo class switch from Cμ to Cδ at the genetic level using cryptic switch regions between the Cμ and Cδ exons (5, 6). Cδ-CS GC cells can differentiate into IgD-secreting PCs (7) and memory cells (8) and have the unusual characteristics that they use more than 90% λ light chains (7) and accumulate more somatic mutations than any other B cell population (9), and we have shown this population has the highest frequency of receptor-edited heavy chain V regions (10).

V_H4-34 gene segments encode natural autoantibodies that recognize the i and/or I (iI) antigens, which are the glycan "scaffold" for a number of common molecules, including the A, B, O, H, and Lewis blood group antigens and keratan sulfate (11–13). Neoplastic transformation and infections causing proliferation of V_H4-34$^+$ B cells can cause pathological cold agglutinin disease. V_H4-34 also has an enhanced role in a number of autoimmune syndromes, including lupus (14–16). There is also a report of an expanded IgD$^-$CD27^{high} B cell population in patients with lupus in which 20% of the total cells utilized V_H4-34 and another 20% expressed antibodies encoded by the V_H4-61 gene segment (17). Despite the autoimmune potential of V_H4-34, it is significantly over-represented in developing and naive B cells because it has the most efficient recombination signal sequence of all human V_H genes (18), representing 6–8% of naive and developing B lymphocytes in normal people (14, 19, 20). However, there is a powerful counterselection against V_H4-34$^+$ B cells in GC cell, memory cell, and PC populations, and V_H4-34$^+$ antibodies are difficult to detect in most serum samples. There is no other naturally encoded antibody or V_H gene that is known to undergo such a profound change in repertoire representation, thus analysis of V_H4-34 usage provides an excellent model of B cell selection in normal humans.

B cells harboring receptors that bind to our own tissues are normally controlled by clonal deletion or by editing of their B cell receptor through further V gene recombination (21, 22). Various related processes also alter the normal immunogenesis of autoreactive B cells, including induction of anergy (23), exclusion from B cell follicles and developmental arrest (24, 25), and autoimmune "ignorance" (26). It has recently been found that 20% of B cells

Nonstandard abbreviations used: antinuclear antigen (ANA); class-switch recombination (CSR); complementarity-determining region (CDR); constant (C); Cδ class-switched (Cδ-CS); diversity (D); germinal center (GC); i and/or I (iI); Ig heavy chain joining region (gene) (J_H); Ig heavy chain variable region (gene) (V_H); Ig κ chain joining region (gene) (J_κ); Ig κ light chain variable region (gene) (V_κ); joining (J); plasma cell (PC); recombination signal sequence (RSS); third complementarity-determining region (CDR3); variable (V).

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emerging from the bone marrow still retain some degree of autospecificity that must be controlled by peripheral selective mechanisms (27). We initiated this study to characterize the dynamics of selection of peripheral B cells in normal humans. Our study includes a comprehensive characterization of peripheral B cell selection involving cytometric analysis of 25 normal donors, and 86 V gene libraries from 38 donors totaling over 4,200 V gene sequences from various peripheral B cell differentiations (Table 1). Various libraries isolated from particular donors were analyzed for selection against use of the V_H4-34 gene segment in addition to several other well-characterized hallmarks typical of an autoimmune or counterselected repertoire and for evidence that the cells were subjected to receptor editing during development. The most significant finding was that changes to the Ig V gene repertoire in the periphery occur mainly in association with the Ig CSR process rather than just with differentiation to GC cells, memory cells, and PCs. Because the various features of the Ig repertoire, and particularly those features associated with autoimmunity, were selected in association with B cell class rather than B cell compartment, we conclude that although avoidance of autoreactive IgM-utilizing B cells must certainly occur, the prevailing gross effect of peripheral selective mechanisms may be avoidance of secondary class-switched B cells with

Table 1
Donors, populations, sequences, and clones analyzed

Donor	Population	Sequences[A]	V_H4[B]	J_H[B]	CDR3[B]	Charged residues[B]	Donor	Population[B]	Sequences[A]	V_H4[B]	J_H[B]	CDR3[B]	Charged residues[B]
1	Naive	125	*	*	*	*	14	IgM GC	17	*	*	*	*
	IgM GC	128	*	*	*	*		IgG GC	25	*	*	*	*
	IgM Mem	137	*	*	*	*		Cδ-CS GC	19	*	*		
	IgG GC	217	*	*	*	*	15	IgM Mem	29	*	*	*	*
	IgG Mem	108	*	*	*	*		IgG Mem	23	*	*	*	*
2	Naive	127	*	*	*		16	IgG Mem	20	*	*	*	*
	Pre-GC	124	*	*	*		17	IgG Mem	24	*	*	*	*
	Pre-GC V_H1	19		*			18	Cδ-CS GC	63	*	*	*	*
	IgM GC	93	*	*	*			Cδ-CS GC (scPCR)	78	*	*		
	IgG GC	128	*	*	*			Cδ-CS V_H3	46		*		
	Cδ-CS GC	65	*	*	*	*	19	Cδ-CS GC	26	*	*	*	*
	Cδ-CS GC V_H1	26		*				Cδ-CS GC (scPCR)	35	*	*		
3	IgM PC	81	*	*	*	*		Cδ-CS V_H3	24		*		
	IgG PC	76	*	*	*	*	20	Cδ-CS GC	15	*	*	*	
	Cδ-CS PC	87	*	*	*	*	21	Naive	22	*	*	*	*
4	Cδ-CS GC	60	*	*	*	*	22	Naive	24	*	*	*	*
	Cδ-CS GC V_H1	34		*				IgM Mem	29	*	*	*	*
	Total B cell IgD	73		For comparison only			23	Naive	24	*	*	*	*
5	Naive	16	*	*	*	*	24	Naive	51	*	*	*	*
	Pre-GC	23	*	*	*	*		IgM Mem	30	*	*	*	*
	IgM GC	19	*	*	*	*	25	Naive	50	*	*	*	*
	IgG GC	18	*	*	*	*	26	Naive V_H1	24		*	*	*
	IgG Mem	22	*	*	*	*		IgG GC V_H1	29		*	*	*
	Cδ-CS GC	19	*	*	*	*		Cδ-CS V_H3	30		*	*	*
6	IgG Mem	16	*	*	*	*		Cδ-CS V_H1	55		*	*	*
7	IgM Mem	15	*	*	*	*	27	IgM Mem	36	*	*	*	*
	IgG Mem	15	*	*	*	*	28	IgM Mem	29	*	*	*	*
8	IgG GC	52	*	*	*	*	29	IgM Mem	15	*	*		
	IgG PC	65	*	*	*	*	30	IgM GC	31		*	*	
9	IgM Mem	22	*	*	*	*		Cδ-CS V_H1	48		*	*	*
	IgG Mem	23	*	*	*	*		Cδ-CS V_H3	27		*		
10	Naive	32	*	*	*	*	31	Naive κ	56	κ analysis			
	IgM GC	88	*	*	*	*		Cδ-CS κ	44				
	IgM Mem	60	*	*	*	*	32	Naive κ	55				
	IgM PC	26	*	*	*	*	33	Naive κ	50				
11	Pre-GC	69	*	*	*	*		Cδ-CS κ	59				
	Cδ-CS GC	257	*	*	*	*	34	IgG Mem κ	25				
12	IgM GC	58	*	*	*	*	35	IgG Mem κ	24				
	IgG GC	42	*	*	*	*	36	Mem κ	24				
	Cδ-CS GC	34	*	*	*	*		Cδ-CS κ	25				
	Cδ-CS GC V_H1	23		*			37	Mem κ	21				
13	IgM GC	14	*	*	*	*		Cδ-CS κ	24				
	Cδ-CS GC	21	*	*	*	*	38	Cδ-CS κ	59				
							Total		4221				

[A]To ensure accuracy, particular sequences were excluded from certain analyses (Methods). [B]Studies for which indicated populations were analyzed. Asterisks indicate the studies in which the various populations were included. sc, single-cell.

autoreactive specificity. In addition, the Cδ-CS lineage of B cells appears to be a "sink" for autoreactive B cells in normal humans. These findings provide an important step toward understanding the homeostasis between antibody-mediated immunity and dangerous autoimmunity in normal humans.

Methods

B lymphocyte isolation and cytometry. B lymphocytes were isolated from human tonsils obtained during routine tonsillectomy following established NIH guidelines with proper institutional board approval or from the buffy coat component from 500 ml of freshly donated peripheral blood purchased from the Oklahoma Blood Institute (Oklahoma City, Oklahoma, USA). B cell purification by magnetic beads, staining, and sorting were performed as described previously (3, 7, 28), except for use of anti-CD27 to sort or identify memory B cells as described (29). The 9G4 rat mAb was purified from hybridoma supernatants using protein G–Sepharose columns (Amersham Biosciences, Arlington Heights, Illinois, USA) and was used in conjunction with PharMingen FITC-conjugated anti-rat V_κ protein (PharMingen, San Diego, California, USA).

V_H4 library population *n* values are indicated in Table 1. The populations for which V gene libraries were generated included naive ($IgD^+IgM^+CD38^-$ [tonsil] or $CD27^-$ [blood]), pre-GC ($IgD^+IgM^+CD38^+$), GC (IgD^-CD38^+, also $CD27^-$ for donors 13 and 14), centroblast ($IgD^-CD38^+CD77^+$), centrocyte ($IgD^-CD38^+CD77^-$), memory ($IgD^-CD38^-CD27^+$), PC (low density, $CD38^{high}CD20^{low}$), and Cδ-CS GC ($IgD^+IgM^-CD38^+$). Detailed phenotypes and isolation procedures of all populations have been published previously (3, 4, 7, 9, 30–32). It is notable that the IgM GC (IgD^-CD38^+) fraction can contain a minor portion of recent bone marrow immigrants (33), and a potential counterpart to the murine marginal zone B cell has been recently described that would also fall into this category (34).

Immunohistochemistry. Sections 5 μm in thickness from "snap-frozen" tonsils were cut and fixed with acetone. For three-step IgM analysis (Figure 1C), incubation with primary antibody 9G4 (anti–V_H4-34) was followed by incubation with FITC-conjugated rabbit anti-rat IgG (Sigma-Aldrich, St. Louis, Missouri, USA), and then biotinylated goat anti-human IgM (Jackson ImmunoResearch, West Grove, Pennsylvania, USA) followed by Texas red streptavidin (Jackson ImmunoResearch) and finally aminomethylcoumarin acetate–conjugated donkey anti-human IgG (Jackson ImmunoResearch). Analyses were made with a Zeiss Axioplan fluorescent microscope in the Oklahoma Medical Research Foundation imaging facility.

Sequencing Ig VDJ and VJ gene transcripts. For analyses of V gene repertoires presented in Figures 2, 3, 4, and 5 and Table 2, all PCR primer sequences are listed in Supplemental Table 1 (supplemental material available at http://www.jci.org/cgi/content/full/113/8/1188/DC1) or as previously described (10, 28). Isolation of RNA, production of cDNA, and generation and screening of the various V gene libraries was done as described previously (10). An alternate V_H4 gene primer (VH4P2) was used for several libraries to ensure changes in the repertoire were not introduced by primer bias. PCR products were cloned using the TA Cloning Kit or the TOPO-TA Cloning Kit (Invitrogen, Carlsbad, California, USA). Individual colonies from the various V gene libraries were randomly picked for preparation of plasmid DNA using the miniprep kit (Qiagen, Valencia, California, USA) and sequencing at the Oklahoma Medical Research Foundation Sequencing Facility using either Applied Biosystems ABI-377 or ABI-3730 DNA sequencers. All V gene sequences were analyzed using "in-house" analysis software and the National Center for Biotechnology Information IgBlast server (http://www.ncbi.nlm.nih.gov/igblast/) or the Immunogenetics server (http://imgt.cines.fr/textes/vquest/) (35). Statistical analyses were performed using Microsoft Excel (Redmond, Washington, USA).

Background mutation frequencies were determined to average 0.79 base mismatches per 1,000 bp based on analysis of a section of C-region gene sequence isolated as part of each cDNA clone analyzed. Sequences of questionable quality for incorporated mutations were analyzed for excessive C-region mutations above background and if above background in the C region they were excluded from analyses of charged residues, somatic mutation, and iI antigen–binding residue analysis. Sequences in which hybrid V genes or other ambiguities were included for various analyses only if they were judged to have unique long third complementarity-determining region (CDR3) segments; that is, a sequence with an ambiguous J region identity but a clearly unique VDJ junction (CDR3) was included for analysis of V gene usage but not J gene usage. Populations isolated as naive were given greater scrutiny and only accepted as "naive" if one or zero mutations were detected in a V gene. Clonally related sequences were included if they differed by more than one mutation and thus could be considered differentially somatically mutated in order to represent the "true" repertoire. In order to determine if inclusion of clonal expansions would affect the outcome of this study, each analysis was also done in which only V genes with unique CDR3s were considered. These analyses generated the same conclusions as the analyses presented that included all clones. Clones with identical CDR3s and differing by only one or no mutations were not included because they may have represented dual cloning of a single PCR amplification product, although inclusion would have been unsubstantial (tested empirically).

Analysis of $V_\kappa4$-1 to $J_\kappa2$ for receptor editing. Genomic DNA was isolated using DNAzol reagent (Molecular Center Research Inc., Cincinnati, Ohio, USA) from naive ($IgM^+IgD^+CD38^-CD27^-$), Cδ-CS ($IgM^-IgD^+CD38^+$), and memory ($IgD^-CD38^-CD27^+$) B cells. PCR of $V_\kappa4$-to-$J_\kappa2$ rearrangements and recombination signal sequence (RSS) junctions were performed using the primer pairs (Supplemental Table 1) illustrated in Figure 5B. PCR reactions were done using Qiagen HotStar Taq polymerase with the following cycle conditions: 15 minutes at 95°C, then 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, with a final 10-minute extension at 72°C. Ratios of genomic DNA isolates were first normalized for the abundance of $V_\kappa4$-to-$J_\kappa2$ rearrangements. Normalization based on presence of the $V_\kappa4$-1–to–$J_\kappa2$ rearrangement frequency also accounts for any loss of the RSS junctions due to the κ-deleting element rearrangement (36) that often accompanies receptor editing, because both the rearrangement and the junction would be lost with the κ locus. Comparison with memory B cells as well as naive B cells controls for the possibility that the increased RSS junctions are from epigenic (recombination circle) DNA due to previous rearrangement that might be detectable in the naive cells after recent immigration from the bone marrow.

Antibody expression and testing for 9G4 reactivity and cold agglutination. Transcripts of antibody V_H genes were cloned into an expression vector containing human IgG and were cotransfected with an FS7-κ V gene into the 293A fibroblast cell line using Roche FuGene transfection reagent following the manufacturer's suggested protocol (Roche Diagnostics Corp., Indianapolis, Indiana, USA). The FS7 antibody is a classic cold agglutinin encoded by V_H4-34, and the FS7-κ

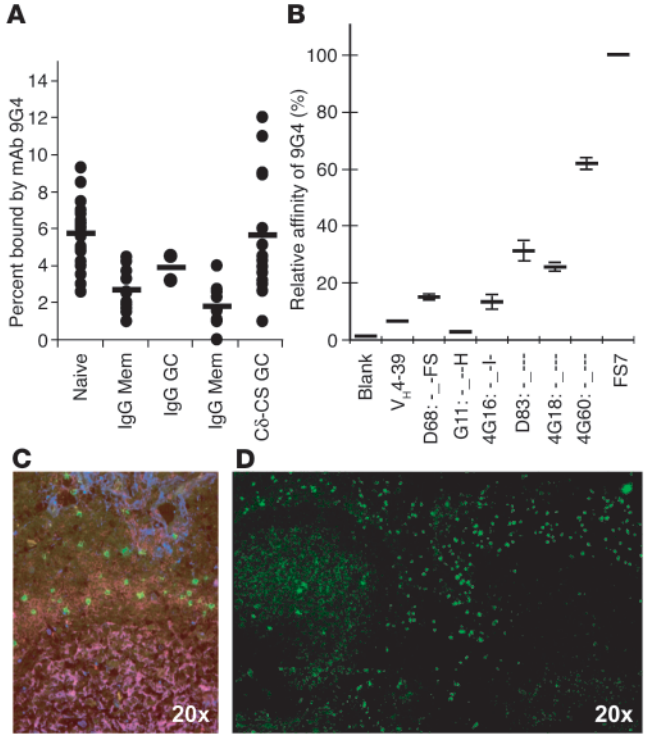

Figure 1
Immunohistochemical and cytometric analysis of V_H4-34$^+$ antibody expression. (**A**) Cytometric analysis to determine the percentage of naive (24 donors), IgM$^+$ GC (2 donors), IgM$^+$ memory (Mem) cells (11 donors), IgG$^+$ memory cells (12 donors), and Cδ-CS GC cells (14 donors) utilizing V_H4-34 (mAb 9G4$^+$). Bars indicate mean. (**B**) Detection of V_H4-34–encoded antibodies by 9G4 is inhibited by somatic hypermutation. Relative binding by 9G4 was compared by ELISA for an unmutated V_H4-34–encoded antibody (FS7) plus six V_H4-34–encoded heavy chains harboring somatic mutations and a non–V_H4-34 control antibody (V_H4-39). Dashes adjacent to the clone names indicate mutations to the 9G4 mAb epitope at amino acids W7, A23, V24, and Y25, and replacements are indicated by single-digit amino acid code. The mean and SD of three assays are indicated. (**C**) Micrograph of a human tonsil section stained with anti-IgM (red), anti-IgG (blue), and anti-V_H4-34 (9G4 mAb, green). The field is bisected by a follicular mantle (naive B cells) surrounding a GC at the bottom. Individual naive cells were scored for frequency of V_H4-34 usage from 4 donors (7% ± 1% SD were V_H4-34$^+$). (**D**) Micrograph of a tonsil section stained with 9G4 mAb (anti–V_H4-34, green). Two GCs are evident from the mantles of 9G4$^+$ stained naive cells. The GC on the left is made up of an oligoclonal expansion of V_H4-34-utilizing (9G4$^+$) cells with 9G4^{bright} plasmablasts differentiating within the GC proper, indicating that V_H4-34$^+$ B cells can contribute to immune reactions. The GC on the right is 9G4$^-$.

gene is permissive for expression of 9G4-reactive antibodies (37). Transfected 293A cells were allowed to secrete antibodies in serum-free DMEM supplemented with 1% Nutridoma-SP (Roche) for 5 days. Antibodies were purified using a protein G–Sepharose column (Amersham Biosciences). Antibody concentrations were determined by ELISA and Western blot and were adjusted to 1 μg/ml. Reactivity to the 9G4 antibody was determined by capture ELISA. ELISA plates were coated with anti-rat IgG (Jackson ImmunoResearch), followed by 9G4 mAb, and then were incubated with the various expressed $V_H/FS7_\kappa$ antibodies in serial threefold dilutions. Bound antibody was detected using biotin–anti-human IgG (Jackson ImmunoResearch) followed by steptavidin-HRP (Jackson ImmunoResearch) and development with HRP substrate (BioRad, Hercules, California, USA). Analysis of cold agglutination was performed by Coomb's assay using commercially available reagents following the manufacturer's protocol (Immucor Inc., Norcross, Georgia, USA).

Single-cell RT-PCR. IgD$^+$IgM$^-$CD38$^+$ Cδ-CS B cells were bulk sorted from the B cell fraction of isolated human tonsils using a Cytomation MoFlo cytometer as described above. The bulk-sorted Cδ-CS cells were then re-sorted into 96-well PCR plates. V_H4 gene libraries were then prepared from the unused bulk-sorted cells and single-cell analysis was performed on the plates for comparison. Single-cell RT-PCR was performed as described previously (27). Individual V genes were cloned and sequenced as described above.

Results

Selection of the naturally autoreactive V_H4-34 gene segment is evident by analysis with the 9G4 mAb. In order to gain insight into the dynamics of tolerance to autoantibody-producing B cells in humans, we analyzed V_H4-34 gene segment usage in various mature B cell subpopulations. The mAb 9G4 is highly specific for Ig encoded by the V_H4-34 gene and no other, but as described below it is highly sensitive to incorporated somatic mutations. The predicted outcome of this analysis was that B lymphocytes expressing the V_H4-34 gene would be excluded after the highly selective environment of the GC reaction. Evidence of this selection in GCs had been previously reported using the 9G4 mAb (14). Consistent with previous studies, analysis of B cells from 24 donors (Figure 1A) by flow cytometry found that 6% of all naive B cells utilize the V_H4-34 gene (6% ± 2% SD). Given that there are approximately 50 functional V_H genes, the V_H4-34 gene is used at threefold the random frequency. Also consistent with previous analyses, 3% ± 1% of IgM$^+$ memory cells (11 donors) and 3.9% ± 1% of IgM$^+$ GCs (2 donors) were 9G4$^+$. Analysis of 12 donor blood and tonsil specimens found that only 1.8% ± 1% of IgG$^+$ memory B cells were "encoded" by V_H4-34, which is significantly fewer than the IgM$^+$ cells by analysis of the variance of means between donors by Student's *t* test ($P < 0.05$). Interestingly, in results averaged from 14 tonsil specimens, 6% ± 3% of the unusual Cδ-CS GC B cells utilized V_H4-34. Because this population accumulates the most somatic hypermutations of all B cells (ref. 9 and Table 3), and, as discussed below, binding by the 9G4 antibody is highly sensitive to somatic mutations, detection of 6% of the cells as V_H4-34$^+$ likely indicated a much higher frequency of V_H4-34$^+$ utilization, which did indeed turn out to be the case (see below). Thus, this analysis gave the first indication that the Cδ-CS lineage might differentiate from B cells expressing natural autoreactivities in normal humans.

In conclusion, analysis with the 9G4 mAb demonstrated that V_H4-34 usage is powerfully counterselected from IgG-utilizing B cells and, unlike the results in previous reports, is significantly greater in IgM$^+$ memory or IgM$^+$ GC cells than the IgG$^+$ cells. In addition, analysis with the 9G4 mAb would indicate counterselection between naive and IgM$^+$ GC and memory cells. The 9G4 mAb does not bind to all V_H4-34–encoded antibodies (37), and there is evidence that it is inhibited by accumulation of somatic mutations. Therefore, we were concerned that analyses of V_H4-34 usage as detected by 9G4 binding both in our own studies reported above

and by other groups previously might underestimate the somatically mutated populations. Indeed, expression of six different mutated V_H4-34–encoded VDJ transcripts with a "permissive" light chain were significantly less reactive to 9G4 than to a germline V_H4-34 antibody (Figure 1B). The epitope of the 9G4 mAb includes a tryptophan residue (W) at position 7, and alanine, valine, and tyrosine residues (AVY) at positions 23–25 (37). Clones having mutations directly to the W and AVY positions are indicated in Figure 1B by dashes (identity to W AVY = "-_---"), and replacements are identified by replacement of the dashes with the amino acid single-letter code. Three of these antibodies harbored mutations directly to the 9G4-bound epitope, and three had no mutations to the epitope directly but were otherwise mutated, suggesting that 9G4 binding is sensitive to mutations both directly and outside of the defined epitope. Assuming that 9G4 affinity translates directly to detection by flow cytometry, this sensitivity could account for the reduced 9G4 binding in all somatically mutated populations reported both here and previously (14). In addition, a comparison of 9G4 detection of V_H4-34 usage with V_H4 gene repertoires generated by random sequencing of the same populations demonstrated that only one half to three quarters of somatically mutated V_H4-34 gene–utilizing B cells are detectable by 9G4 staining (data incorporated into Figures 1 and 2). In conclusion, there appears to be selection of the V_H4-34 gene in association with class switch more than with B cell compartment. However, because of the caveat that 9G4 antibody binding is highly sensitive to somatic mutations, we initiated a more accurate analysis of V_H4-34 selection using random sequencing.

Analysis of the V_H4 gene repertoire indicates that selection of the autoreactive V_H4-34 gene segment is associated mainly with class switch. V_H4-34 gene segment utilization was analyzed relative to that of the other nine V_H4 family gene segments by random sequencing to provide a more accurate and comprehensive analysis of the selection of this gene segment than the cytometric and histological studies provided. A V_H gene family is defined as all V_H genes (totaling 50) having greater than 90% similar nucleotides (38). Comparison of V_H4-34 usage only to other V_H4 family genes allowed a more manageable and quantitative analysis, as all V_H4 cDNA molecules can be similarly PCR amplified by a single nondegenerate priming site in the V_H4 leader region. Sixty-one libraries of V_H4 gene family VDJ rearrangements were generated by random cloning of RT-PCR–amplified transcripts from various B cell subpopulations sorted by cytometry from 30 subjects. As indicated in Table 1 and Supplemental Figure 1, one to six B cell populations were isolated from each donor. Figure 2A depicts the selection of V_H4-34 gene usage in various populations sorted from two donors typical of the selection of this gene. Similar analysis of all donors is provided in Supplemental Figure 1 and means with standard deviation (SD) are provided in Table 2. As depicted by the error bars (SD) of Figure 2, A and B, and by the comparison of all patients for V_H4-34 use in Supplemental Figure 1, there was great variation between individual donors for V_H4-34 gene segment utilization probably due to different immunological histories and different genetic backgrounds. This deviation demonstrates the need for sufficient *n* values to allow consideration of variance in analyses of human B cell repertoires. Despite the deviation in the overall frequency of V_H4-34 usage between donors, V_H4-34 was typically used consistently between naive and secondary IgM populations (pre-GC, GC, memory, and PC), but at double the frequency in the IgM B cell subsets over the IgG populations. On average, V_H4-34 was over-represented in naive B cells (37% ± 10% from nine donors, Figure 2B) and was used consistently in all other B cell populations that had not undergone class switch, including 40% ± 29% of V_H4 transcripts from IgM^+ pre-GC cells (three donors), 43% ± 26% of V_H4 transcripts from IgM^+ GC cells (six donors), 28% ± 24% of IgM^+ memory transcripts (ten donors), and 46% ± 21% of IgM^+ PC transcripts (two donors). In contrast, B cells utilizing V_H4-34 were stringently counterselected from IgG populations, including IgG GC (mean = 11% ± 8%; six donors), and IgG memory (12% ± 8%; eight donors) or IgG PC (20% ± 5%; two donors). Consistent with the flow cytometry analyses, V_H4-34–utilizing B cells were highly enriched in the unusual Cδ-CS populations, including GC (65% ± 23%; ten donors) and a single Cδ-CS PC population (58%). It is notable that Student's *t*-test comparisons did not demonstrate statistically significant differences between the various IgM naive, GC, memory, and PC populations, but showed they were all significantly different from the IgG populations with probabilities significant at least at the level of $P < 0.05$. IgG^+ GC, memory, and PC populations were also statistically similar. V_H4-34 frequency in the Cδ-CS lineage was different from that of all other B cell populations to at least the level of $P < 0.005$. It is notable that loss of V_H4-34 utilization in the IgG populations was compensated for mainly by increases in V_H4-39 and V_H4-59, which are the next two J-distal V_H4 family gene segments in the V_H locus (Table 2). Certain other V_H4 genes (V_H4-31, V_H4-39, V_H4-59, and V_H4-61) displayed significant increases or reductions in particular donor populations, but not in a consistent fashion.

Reorganization of V_H4-34 usage by Ig class demonstrates the highly significant association of V_H4-34 selection solely with class switch (see *P* values, Figure 2C). There is stringent selection against V_H4-34^+ B cells when naive or secondary IgM B cells undergo class switch to IgG (naive, 37% ± 10%; secondary IgM, 36% ± 25%; IgG, 13% ± 8%), and there is selection of V_H4-34–utilizing B cells into the Cδ-CS lineage (64% ± 22%). In conclusion, a more accurate and extensive analysis of V_H4-34 gene usage by cDNA sequencing indicates that peripheral differentiation or progression through a GC reaction of IgM^+ B cells to any other stage does not effect V_H4-34 usage. Selection of B cells using this naturally autoreactive V_H gene is only apparent in association with Ig class switch.

Correspondence between PCR/sequencing data, immunohistochemistry, cytometry, and single-cell PCR analysis. As described above, the 9G4 antibody binds with high affinity to unmutated V_H4-34 gene–encoded antibodies as found on naive B cells. In order to control for the quantitative capacity of the analyses performed with a different technology, we stained tonsil tissue sections from four donors to count naive cells expressing V_H4-34–encoded Ig usage ($9G4^+$) (two donors are presented in Figure 1, C and D). The tissue section in Figure 1C has a clearly defined B cell follicular mantle stained with anti-IgM (red) arcing through the center of the image, over a GC at the bottom that is purple due to staining of the follicular dendritic cell network coated with captured IgM (red) and IgG (blue) antibodies. It is notable that unlike a previous report in which no GCs could be found that were made up from expansions of V_H4-34^+ cell populations (14), we readily found $9G4^+$ GCs in tonsil specimens from all donors analyzed. An example of a V_H4-34^+ GC and a V_H4-34^- GC in the same microscopic field is presented in Figure 1D. There are also $9G4^{bright}$ plasmablasts (early PCs) that have differentiated within the GC proper. Note that this section was stained only with the 9G4 mAb (and FITC-conjugated anti-rat) as secreted antibody captured in the follicular dendritic cell network are brightly stained by anti-IgM and

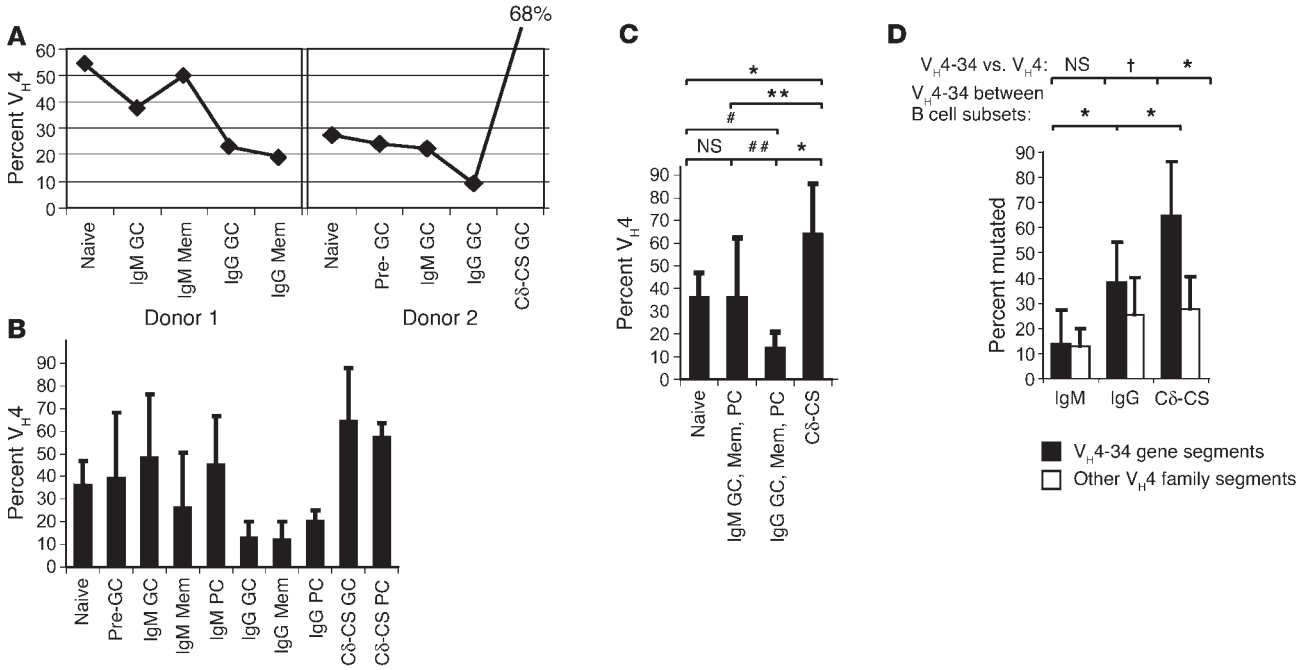

Figure 2

V_H4-34$^+$ B cell selection correlates with antibody isotype and is due to iI antigen autoreactivity. V_H4 family genes were randomly cloned and sequenced from various B cell subpopulations sorted by flow cytometry. B cell subpopulation phenotypes are in the text, and Table 1 lists populations isolated from each donor and number of V genes sequenced per population. (**A**) V_H4-34 usage in the various subpopulations of two donors. Similar analyses of all 29 donors are presented in Supplemental Figure 1. (**B**) Average use of V_H4-34 by the various B cell subpopulations between donors (means ± SD; Table 2 lists mean use of the remaining V_H4 genes). V_H4-34 encodes antibodies from the various IgM populations significantly more than the IgG populations (Student's *t* test, $P < 0.05$) and was used in the Cδ-CS populations more than all others ($P < 0.01$). (**C**) V_H4-34 usage organized by Ig class. Student's *t* test probabilities are indicated. $^*P < 0.0001$; $^{**}P = 0.004$; $^{\#}P = 0.002$; $^{\#\#}P = 0.0008$. (**D**) V_H4-34 is probably selected due to its autoreactivity to iI glycans. Bars indicate mean ± SD of V genes with mutations at amino acid positions 7, 23, 24, or 25. These amino acids are used for iI glycan binding by V_H4-34$^+$ antibodies (W7 and AVY23–25). Despite equivalent mutation frequencies between V_H4-34$^+$ and other V_H4 genes (Table 3), mutations at these amino acid positions are significantly selected only for V_H4-34 genes, and only in association with CSR (χ^2 probabilities are indicated). $^\dagger P < 0.05$.

anti-IgG, masking the GC cells that express only low levels of surface Ig (Figure 1C). Upon assigning scores for 9G4 staining, we found that 7% ± 1% (average ± SD) of the follicular mantle B cells from four donors are "encoded" by the V_H4-34 gene segment. Analysis by PCR determined that V_H4 encodes 37% of V_H4 transcripts sequenced (Figure 2). Because V_H4 genes represent approximately 20% of the total naive pool, based on the genetic frequency of V_H4 genes (38), our own analyses (Methods), and those of others (14, 27, 39), this 36% represents 7.4% of all V_H genes (0.2 multiplied by 36%), corresponding excellently to the immunohistochemical analysis. The sequencing data also corresponds well with the cytometric analysis presented in Figure 1A, where 9G4$^+$ cells averaged 6% ± 2% of the total from 24 donor samples. Previous analyses have demonstrated that analysis of V gene repertoires by bulk PCR and random sequencing is similarly quantitative to single-cell PCR (40). However, with the unique conditions of PCR primers used herein, we felt it necessary to verify the bulk PCR data by single-cell PCR. Cδ-CS B cells from two donors were bulk sorted, and then a portion of these cells was distributed as single cells into a 96-well PCR plate by the flow cytometer for single-cell RT-PCR. We found that as long as the single-cell PCR efficiency approached 90%, the bulk–versus–single-cell analysis of V_H4 and J_H gene use corresponded excellently for all genes (V_H4-34, J_H4, and J_H6 correspondence is presented in Table 4).

It is notable that bulk PCR analysis amplifying only V_H4 genes does not provide correspondence of gene expression relative to other V_H gene families unless it is known what proportion of the total cells use V_H4 gene segments. For example, 20% of all naive cells use V_H4 gene segments; thus reference to 50% of the naive cells expressing V_H4-encoded antibodies would represent 10% of all naive cells. However, relative abundance of V_H4 genes was not previously known for the Cδ-CS lineage. As indicated in Table 4, single-cell analysis of two donors indicates that 46% and 54% of total B cells use V_H4 family Ig genes, and V_H4-34 accounts for 30% and 42% of all Cδ-CS B cells, respectively, in these donors. Thus, V_H4-34 is even more over-represented in the Cδ-CS lineage than as determined by the analyses in Figure 2. As indicated in Table 3 and previously (9), the Cδ-CS lineage B cells harbor more somatic mutations than any other B cell lineage, and thus this analysis further demonstrates the sensitivity of the 9G4 mAb to incorporated mutations, as analysis with 9G4 appears to have detected only approximately 20% of the V_H4-34$^+$ Cδ-CS B cells (Figure 1A).

Selection of V_H4-34–utilizing B cells in association with class switch is dependent on their autospecificity to the iI antigen. Due to the well-characterized natural autoreactivity of the V_H4-34 gene to the iI glycan, it is predicted that loss of V_H4-34–utilizing B cell clones in the class-switched pools is due to negative selection to avoid autoreactivity in the secondary Ig repertoire. Alternatively, B cells expressing Ig

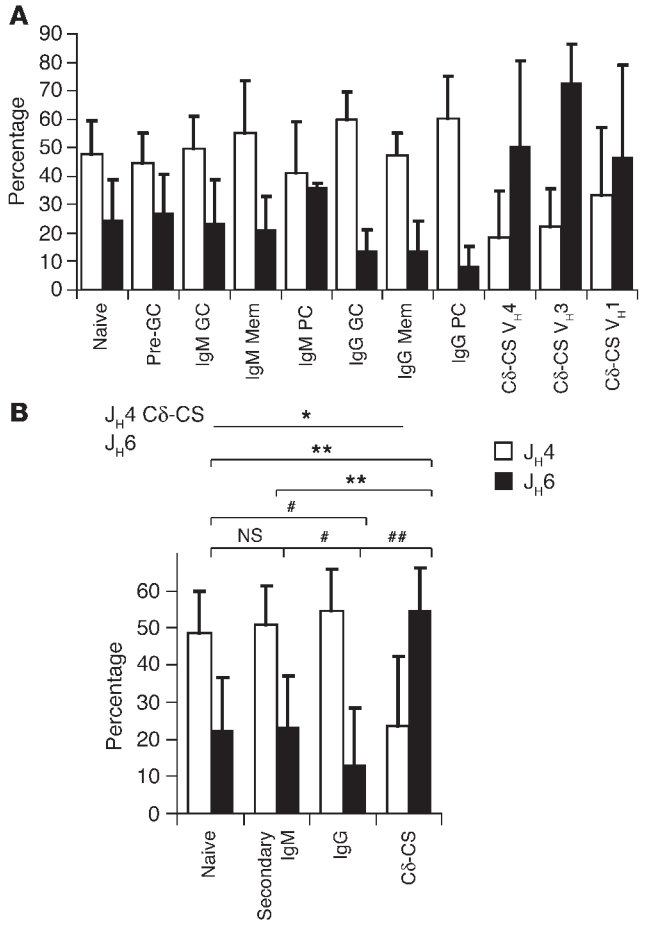

Figure 3
The autoimmune-associated J_H6 gene is counterselected from IgG populations and selected into the Cδ-CS lineage. (**A** and **B**) The J_H4 gene segment is normally the dominant J_H gene used in human V_H gene rearrangements. The J_H6 gene is upregulated in receptor-edited and autoimmune B cells, linking it to autoimmune repertoires. Only usage of J_H4 versus J_H6 gene segments is shown and compared, as all other J_H gene segment use is unchanged (other J_H representations are in Table 2). J_H analysis was based on naive B cells (ten donors), IgM/D$^+$ pre-GC cells (three donors), IgM$^+$ GC cells (eight donors), IgM$^+$ memory cells (ten donors), IgM$^+$ PCs (two donors), IgG$^+$ GC cells (seven donors), IgG$^+$ memory cells (eight donors), IgG$^+$ PCs (two donors), Cδ-CS PCs (one donor), and Cδ-CS GC cells (ten, five, and four donors for V_H4, V_H1, and V_H3 libraries, respectively). Bars indicate means of all donors and error bars, the SD. Student's *t* test *P* values are indicated. $^*P < 0.0005$ vs. all; $^{**}P < 0005$; $^{\#}P < 0.01$; $^{\#\#}P < 0.0001$.

encoded by V_H4-34 may simply not be positively selected to participate in IgG-mediated immune responses. In order to address this issue, we considered the specific binding of V_H4-34–encoded antibodies to the iI autoantigen. If indeed B cells utilizing V_H4-34 are negatively selected, it can be predicted that V_H4-34$^+$ B cells surviving to selected populations, and particularly the IgG populations in which V_H4-34 counterselection is so apparent, might do so because the well-defined amino acids involved in iI glycan binding were somatically mutated during GC reactions. In Figure 2D, V_H4-34$^+$ V genes from the various isotype pools are analyzed for loss of the iI antigen binding site due to accumulated somatic mutations. iI antigen specificity is generally independent of the CDR3 or the Ig light chain. Similar to the epitope of mAb 9G4 on V_H4-34–encoded antibodies, the specific amino acids important for binding of V_H4-34 to iI antigen are known through mutagenesis studies to be W at position 7 and AVY at positions 23–25 (41, 42). V genes using V_H4-34$^+$ segments from IgG$^+$ and Cδ-CS B cells have accumulated somatic mutations causing replacement of amino acid positions 7 and 23–25 at a significantly higher frequency than did V genes encoded by other V_H4 family gene segments (for Cδ-CS cells, 67% ± 18% of V_H4-34$^+$ vs. 28% ± 16% of other V_H4, χ^2, $P < 0.0001$; and for IgG$^+$ cells, 39% ± 12% vs. 26% ± 8%, χ^2, $P < 0.05$; Figure 2D). These mutations are not due to an overall greater frequency of mutations to V_H4-34 gene segments which are mutated at a frequency similar to that of V genes using other V_H4 family gene segments (Table 3). However, selection of iI binding site mutations is not evident for somatically mutated secondary IgM populations (14% ± 8% of V_H4-34 and 13% ± 9% of other V_H4; analysis only of IgM$^+$ clones with >1 mutation). Thus, there appears to be a profound selection against V_H4-34–utilizing B cells due to iI glycan binding, but in class-switched populations only (χ^2, $P < 0.0001$ for Cδ-CS vs. IgG or secondary IgM, and also χ^2, $P < 0.0001$ for IgG vs. secondary IgM). It is interesting to note that despite the unexpected selection of V_H4-34–utilizing B cells into the Cδ-CS lineage (Figures 1A and 2, A and B), the cells must still be tolerized against iI glycan binding to survive. In addition, the six recombinant V_H4-34–encoded antibodies from class-switched populations expressed for the analysis of Figure 1B were tested for their ability to agglutinate erythrocytes at low temperatures (cold agglutination). Cold agglutination is a hallmark of V_H4-34 gene–encoded antibodies and is indicative of their ability to bind the iI antigen (42, 43). Although three of these antibodies had mutations not directly at amino acid positions 7 and 23–25, only one of the six was able to agglutinate red blood cells in the cold at a low level, but not at a level comparable to that of control sera from a patient with cold agglutinin disease or the FS7 control antibody (data not shown). In conclusion, the selection for specific mutations of amino acids at the iI antigen binding site of V_H4-34 genes but not other V_H4 genes is highly suggestive that B cells using V_H4-34 are being counterselected due to their iI antigen autoreactivity.

As described in the previous section, consistent with this analysis of iI epitope mutations, mAb 9G4 only detects 20% of the Cδ-CS B cells and half of the already greatly reduced "IgG-encoded" B cell clones. However, based on the low frequency (14%) of iI glycan–binding mutations and thus the 9G4 epitope of mutated IgM clones, we would have expected higher detection of IgM clones by the 9G4 mAb. Because V_H4-34 is found at a similar frequency in IgM cells and naive cells, we would have predicted 9G4 binding to equal the frequency of naive cell detection by 9G4 multiplied by the frequency of clones retaining the 9G4 epitopes in the IgM GC and memory cells, or 6% × 0.86 = 5%. However, only 3% of IgM memory cells were actually detected (Figure 1A). Though seemingly minor, this 2% discrepancy is significant because binding of 9G4 mAb to IgM clones in Figure 1A is significantly different from that of naive clones by Student's *t* test ($P < 0.01$). Figure 1A is an analysis of CD27$^+$ IgM memory cells from blood. The autoantigen bound by V_H4-34 is the iI glycan that is found in abundance on erythrocytes. Both iI antigen binding and the 9G4 epitope found in the FW1 region are affected in some V_H4-34–encoded antibodies by the CDR3 and light chain sequences (37, 42), possibly explaining the discrepancy between the current sequencing analysis and the cyto-

Table 2
V_H4 and J_H gene segment utilization

	V_H4-04[A]	V_H4-28[A]	V_H4-30-2[A]	V_H4-30-4[A]	V_H4-31[A]	V_H4-34[A]	V_H4-39[A]	V_H4-59[A]	V_H4-61[A]	V_H4-b[A]
Naive	8 ± 7%	0 ± 0%	0 ± 1%	5 ± 8%	11 ± 9%	37 ± 10%	12 ± 6%	18 ± 11%	10 ± 11%	0 ± 0%
Pre-GC	3 ± 4%	0 ± 0%	2 ± 3%	7 ± 2%	19 ± 13%	40 ± 29%	10 ± 4%	16 ± 14%	2 ± 2%	1 ± 3%
IgM GC	5 ± 8%	1 ± 3%	1 ± 3%	4 ± 4%	18 ± 15%	43 ± 26%	12 ± 7%	13 ± 9%	2 ± 3%	1 ± 2%
IgG GC	5 ± 4%	0 ± 1%	2 ± 2%	2 ± 4%	22 ± 14%	11 ± 8%	22 ± 7%	29 ± 11%	6 ± 7%	1 ± 3%
IgM memory	3 ± 5%	1 ± 1%	1 ± 2%	3 ± 6%	8 ± 7%	28 ± 24%	19 ± 15%	21 ± 14%	15 ± 10%	1 ± 2%
IgG memory	4 ± 5%	0 ± 0%	3 ± 5%	8 ± 12%	16 ± 12%	12 ± 8%	23 ± 11%	26 ± 14%	6 ± 8%	1 ± 2%
IgM PC	9 ± 7%	0 ± 0%	0 ± 0%	4 ± 5%	0 ± 0%	46 ± 21%	1 ± 2%	11 ± 4%	30 ± 39%	0 ± 0%
IgG PC	13 ± 2%	1 ± 1%	1 ± 1%	9 ± 12%	14 ± 20%	20 ± 5%	19 ± 3%	21 ± 1%	3 ± 2%	0 ± 0%
Cδ-CS GC	0 ± 1%	0 ± 0%	2 ± 4%	4 ± 8%	10 ± 8%	65 ± 23%	4 ± 8%	7 ± 9%	4 ± 8%	3 ± 3%
Cδ-CS PC	1%	0%	18%	19%	0%	58%	0%	4%	1%	0%
	J_H-1[A]	J_H-2[A]	J_H-3[A]	J_H-4[A]	J_H-5[A]	J_H-6[A]				
Naive	3 ± 4%	3 ± 3%	10 ± 7%	48 ± 12%	12 ± 8%	25 ± 14%				
Pre-GC	1 ± 1%	4 ± 6%	10 ± 4%	45 ± 11%	13 ± 6%	27 ± 14%				
IgM GC	1 ± 2%	3 ± 3%	10 ± 7%	50 ± 11%	12 ± 6%	24 ± 15%				
IgG GC	2 ± 2%	3 ± 2%	10 ± 6%	60 ± 10%	13 ± 5%	14 ± 7%				
IgM memory	3 ± 3%	4 ± 4%	6 ± 6%	54 ± 19%	15 ± 6%	19 ± 12%				
IgG memory	3 ± 5%	5 ± 6%	11 ± 5%	47 ± 8%	20 ± 11%	14 ± 11%				
IgM PC	0 ± 0%	0 ± 0%	7 ± 4%	41 ± 18%	16 ± 12%	36 ± 2%				
IgG PC	2 ± 1%	5 ± 5%	6 ± 2%	60 ± 15%	19 ± 9%	8 ± 7%				
Cδ-CS[B]	1 ± 2%	3 ± 6%	13 ± 22%	23 ± 18%	9 ± 11%	52 ± 29%				

[A]Values represent the mean percent utilization of each gene for the various donors ± the standard deviation. [B]Combined total mean J_H usage of V_H1, V_H3, and V_H4 rearrangements.

metric analysis of this and a previous report from a different group (14). Thus, although the repertoire representation of V_H4-34–utilizing B cells in the secondary IgM pools may not be significantly reduced, there could be selection of IgM^+V_H4-34^+ B cell clones with light chains or CDR3 regions that effectively "edit" the ability to bind iI glycan, which would also affect the 9G4 epitope involving the same region and amino acids of V_H4-34–encoded Ig molecules. Similarly, there may be selection of IgM^+V_H4-34^+ B cell clones with somatic mutations of the more-mutable CDR-encoded amino acids also indirectly affecting iI-glycan binding and also with the ancillary effect on detection by 9G4. A search for other possible mutated sites in the IgM clones that could account for this discrepancy was fruitless. In conclusion, (a) there is selection against V_H4-34–utilizing B cells that retain iI glycan binding from all populations of B cells, including Cδ-CS cells, despite profound selection of V_H4-34–utilizing B cells toward this differentiation; and (b) although not significant at the gross level of repertoire analysis or sequence analysis for iI binding mutations, IgM memory cells may still be selected to avoid this autoreactivity. In the following sections, a number of other features of the human V gene repertoire associated with autoreactivity or autoimmunity are characterized to generalize the findings for V_H4-34 gene usage to the total Ig repertoire.

Peripheral selection of the autoimmune-associated J_H6 gene is also associated with class switch. J_H6 predominance has been detected in a population of receptor-editing human B cells (44–46) and is counterselected from the pre–B cell repertoire upon progression to naive mature B cells (27), and thus it is already known to be subjected to primary B cell–selective mechanisms. Increased J_H6 usage has also been associated with autoreactive lymphocytic leukemia (47) and Sjogren syndrome (48). Finally, direct antibody expression analyses have recently demonstrated that J_H6-encoded antibodies are more likely to "encode" antinuclear antigen (ANA) autoreactivity and polyreactivity (27, 46). As indicated in Figure 3, the J_H6 segment encodes about one-quarter of the Ig molecules of the naive and secondary IgM populations (naive = 25% ± 14%; IgM/D^+ pre-GC = 27% ± 14%; IgM^+ GC = 24% ± 15%; IgM^+ memory = 21% ± 12%; and IgM^+ PC = 36% ± 2%; these populations were statistically similar), but use of J_H6 is stringently counterselected from all IgG populations (IgG^+ GC = 14% ± 7%, IgG^+ memory = 14% ± 11%, and IgG^+ PC = 8% ± 7%; these populations are all similar, but each is statistically different from naive and the various IgM populations by Student's *t* tests at a level of $P < 0.05$). In addition, similar to V_H4-34 usage, cells utilizing J_H6 appear to preferentially class switch to Cδ, where 50% ± 30% of the cells have V genes encoded by J_H6 ($P < 0.001$ versus all other populations), with a significant reduction in the use of the normally dominant J_H4 gene segment ($P < 0.001$). Populations analyzed for the various donors are indicated in Table 1. Reorganization of J_H gene segment usage by Ig class in Figure 3B more pointedly demonstrates the class-related selection of the J_H gene repertoire. Compared with V genes from naive cells (22% ± 14% J_H6; 11 donors) and V genes from differentiated secondary IgM B cells (23% ± 13% J_H6; 20 donors), B cells with J_H6-utilizing V genes are significantly counterselected from the IgG populations (13% ± 9%; 18 donors; $P < 0.01$ by paired Student's *t* tests). B cells utilizing the normally dominant J_H4 gene are significantly selected into the IgG

Table 3
Mean mutations in various isotype pools

	V_H[A]	V_H4-34[B]	Sequences
IgM GC, memory, PC	5	5	512
IgG GC, memory, PC	14	14	419
Cδ-CS lineage	23	24	265

[A]Mean mutations per sequence for all V_H rearrangements. [B]Mean mutations per sequence for V_H4-34 rearrangements.

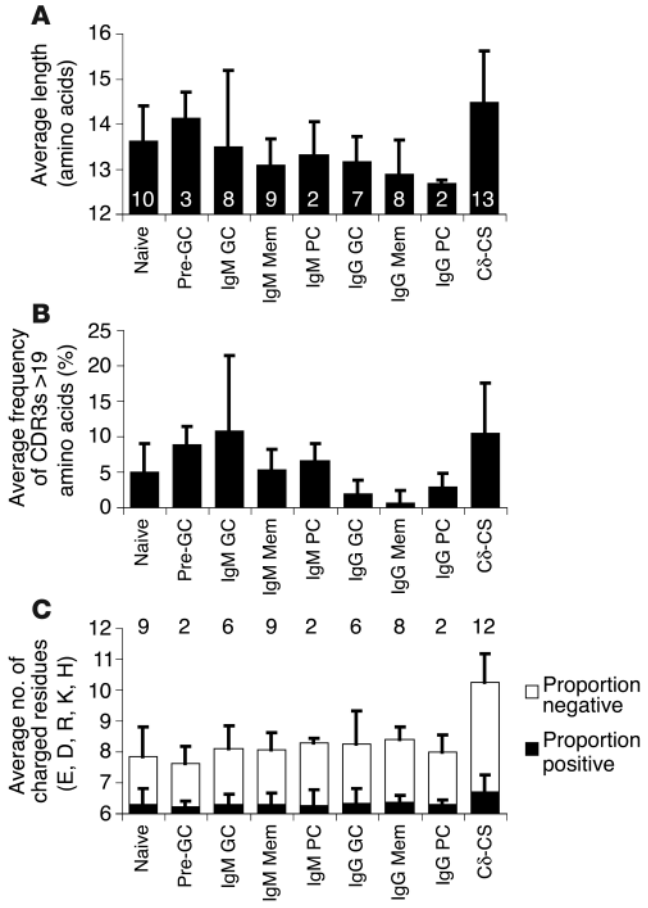

Figure 4
Analysis of CDR3 length and frequency of charged residues in various V gene libraries. V genes with long CDR3s and increased numbers of charged amino acids have been associated with increased autoreactivity and are typical in the repertoires of people and mice with autoimmune disease. The *n* values of donors analyzed per population are indicated on and above the bars, and the particular donors compared are in Table 1. (**A**) Average length of CDR3s in amino acids of the various donors for the various populations. The average lengths of the Cδ-CS and pre-GC populations are significantly greater then all IgG populations and the IgM memory population (*t* test, $P < 0.01$), although the pre-GC cells were from only three donors, one of whom had a particularly large average CDR3 length. (**B**) Average frequency of V gene clones between donors that are more than 19 amino acids in length. The Cδ-CS frequency of clones with CDR3s longer than 19 amino acids is similar to that of the naive and IgM populations. The IgG populations have on average significantly fewer clones with lengths greater than 19 amino acids than do the naive, IgM, and Cδ-CS populations (*t* test, $P < 0.05$). (**C**) The mean number of charged residues in the CDR regions averaged among donors for each population where positively charged residues (R, K, H) are the black portion of the bars, and negatively charged (acidic) residues (E and D) are represented by the white portion of the bars. Error bars indicate standard deviations between the various donors for all panels.

lineages ($P = 0.04$). Finally, J_H6 is highly over-represented in the Cδ-CS lineage in 50 ± 18% of the V_HDJ_H transcripts (17 donors, $P < 0.0005$ for Cδ-CS vs. naive cells and secondary IgM^+ B cells; and $P < 0.0001$ vs. IgG^+ B cells). Use of the remaining J_H genes is listed in Table 2. Selection of the J_H6 gene segment into the Cδ-CS lineage is also independent of the V_H gene used, as similar results were obtained for V genes formed by rearrangements of V_H1 family genes (46% ± 33%) and V_H3 family genes (73% ± 14%) (Figure 3). In conclusion, J_H6 gene usage is an independently selected feature indicative of an autoimmune antibody repertoire that is selected in the periphery solely associated with class switch.

Long CDR3s are a classic hallmark of an autoimmune repertoire that are also selected in mature B cells solely on the basis of Ig class. The correlation of long CDR3s to autoimmunity has been made both for humans (49) and in mouse models of lupus, such as mrl/lpr mice (50, 51). Longer CDR3s have also been demonstrated in receptor-editing human B cells (44–46) and are counterselected during normal human B cell development (27). Finally, direct antibody expression analyses have recently demonstrated that antibodies with long CDR3s are more likely to have ANA autoreactivity and polyreactivity (27, 46). Typically, a population is judged as having longer CDR3 regions if a higher frequency of B cells are found with excessively long CDR3 regions. Average CDR3 lengths are of limited utility because most V genes predominantly have CDR3 lengths between 13 and 14 amino acids with some proportion of outliers. If there is counterselection of these outliers, then there will still be little effect on the mean. However, as indicated in Figure 4A, V genes from B cells of the Cδ-CS lineage average the longest CDR3s, although only significantly greater than the average lengths of CDR3s of V genes from IgG (Student's *t* test, $P < 0.01$) and the IgM memory (Student's *t* test, $P < 0.01$) populations. In regards to difference in mean CDR3 length between IgM and IgG populations, although longer CDR3s are generally found in the V genes of IgM populations, only the means of naive and the IgM^+D^+ pre-GC populations were significantly increased over the IgG memory and IgG PC populations (not the IgG GC). However, when the mean numbers of clones between the various donors with excessively long CDR3 regions (>19 residues) were considered, the naive, all IgM populations (including IgM pre-GC, GC, memory, and PC), and the Cδ-CS lineage were statistically similar, and all had significantly more clones with CDR3 lengths greater than 19 amino acids than any of the IgG GC, memory, or PC populations at a level of $P < 0.05$ by Student's *t* test. It is interesting to note the immense variation between the eight donors from which IgM GC populations were analyzed for both average CDR3 lengths and regarding the number of clones with CDR3s longer than 19 amino acids (see Figure 4, A and B, error bars representing SD), suggesting a significant effect of anti-

Table 4
Single-cell RT-PCR (scRT-PCR) and bulk PCR analysis of Cδ-Cs cells

	V_H4^A	V_H4-34^B		J_H		Sequences
		V_H4	Total	J_H4	J_H6	V_H/J_H
Donor 19, scPCR:	46%	66%	30%	18%	75%	76/35[C]
Donor 19, bulk PCR:		69%		16%	76%	26
Donor 18, scPCR:	54%	79%	42%	14%	72%	78
Donor 18, bulk PCR:		81%		14%	76%	26

[A]Percent of "V_H4 family–encoded" cells from total cells. [B]Percent "V_H4-34–encoded" rearrangements of V_H4 genes and of total B cells. [C]J_H analysis was considered for V_H4 only for donor 1 because bulk analysis was only of V_H4 genes and there is a V_H3 clonal expansion utilizing JH6. For each donor, 84 cells were analyzed (efficiency: 90%, donor 1; 93%, donor 2).

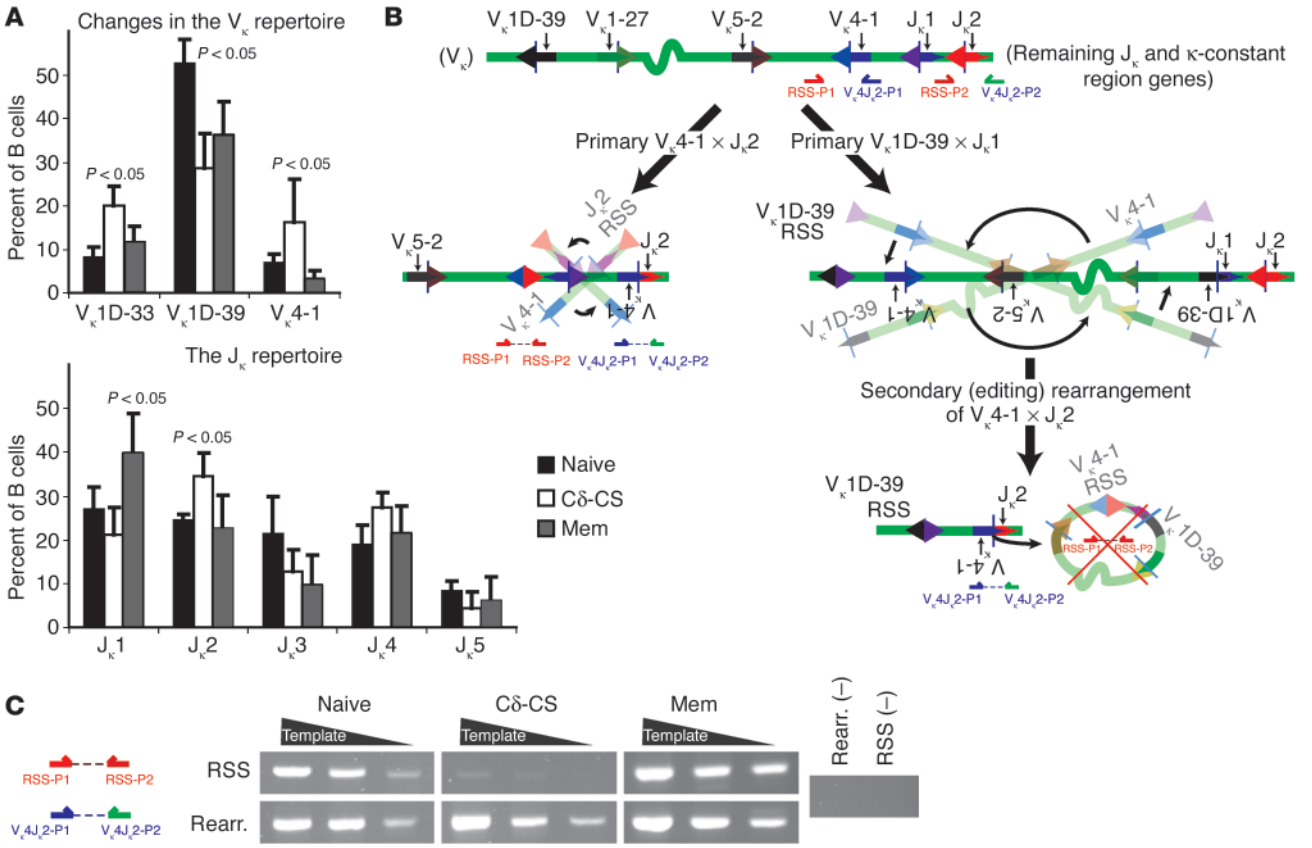

Figure 5

Analysis of the VJκ repertoire and configuration of the Vκ loci for evidence of receptor editing. (**A**) Mean ± SD of Vκ and Jκ gene segments used at significantly different frequencies between naive (four donors), Cδ-CS (five donors), and combined total memory (two donors) plus IgG+ memory (two donors). (**B**) Semi-quantitative PCR was used to determine if increased Vκ4-1 usage provides evidence of receptor editing for Cδ-CS cells. Vκ4-1 is the most Jκ-proximal Vκ gene and is inverted. Thus, primary Vκ4-1 rearrangements retain the RSS junction (left), but following any other primary inversional rearrangement, a secondary (editing) Vκ4-1 rearrangement will delete the RSS junction (right). Thus, analysis of the genetic configuration of Vκ4-1–to-Jκ2 rearrangements provides a measure of receptor editing for all Vκ genes. The relative proportion of primary versus editing rearrangements was determined by PCR using the primer sets indicated to get the ratio of Vκ4-1–to-Jκ2 rearrangements (primers: VκJκ2-P1, blue arrows; and VκJκ2-P2, green arrows) compared to RSS junctions (RSS-P1, red arrows; and RSS-P2, orange arrows). (**C**) Genomic DNA template from naive, Cδ-CS, and memory B cells were normalized for Vκ4-1–to-Jκ2 rearrangements (Rearr.); then the relative proportion of RSS junctions were determined by PCR at three dilutions of template (wedges). There was ten-fold less Vκ4-1–to-Jκ2 RSS junctions for the Cδ-CS population, indicating that the increased Vκ4-1 rearrangements for this population are due to receptor editing. (–), buffer controls.

gen selection on average CDR3 length. In summary, as with analyses of V_H4-34 and J_H gene usage, CDR3 length demonstrates another repertoire variation that is associated with autoreactive B cells and is counterselected from the IgG populations.

Additional distinctions analyzed for the various B cell subpopulations provided no further evidence of repertoire variation beyond that found in association with class switch. Separation of naive cells ($IgD^+IgM^+CD38^-$) from two of the donors (donors 2 and 24) into $CD23^-$ and $CD23^+$ subsets (or Bm1 and Bm2, as previously described) (3) found that these two naive B cell differentiations were the same for all analyses described above, and thus these groups were pooled. Similar differential analyses were performed comparing IgM versus IgD V gene libraries for the naive cells of one patient (donor 2, Bm1) and the pre-GC cells ($IgM^+IgD^+CD38^+$) of two patients (donors 2 and 11). IgM versus IgD libraries were also equivalent and the data pooled for the various analyses when from a single donor and population. In addition, two of the donor GC cell populations (from donors 2 and 5) were separated with anti-CD77 into centroblast ($CD77^+$) and centrocyte ($CD77^-$) pools and were found to be the same. Finally, inclusion of anti-CD27 (with anti-IgD and anti-CD38) for sorting cells of two tonsil specimens (donors 18 and 19) provided finer distinction of GC ($IgD^-CD38^-CD27^+$) and memory cells ($IgD^-CD38^+CD27^-$) but produced no significant differences from GC or memory populations analyzed from other donors. None of these finer distinctions produced differential V gene repertoires other than the association with Ig class. It is also notable that PCs can arise via GC-independent differentiation; however, there is no way to sort IgM^+ PCs into GC-dependent versus GC-independent fractions. However, because naive versus IgM^+ pre-GC, IgM^+ GC, and subdivisions of IgM^+ GC cells (centroblasts versus centrocytes), or IgM^+ memory cells from both blood and tonsil were all similar, we can predict that differentiation of IgM^+ PCs into GC-dependent or -independent fractions would not affect selection of V_H4-34, J_H, or CDR3 length.

B cells selected into the Cδ-CS population have an increased number of charged residues in their CDRs. Charged residues in autoantibody hypervariable regions are a common feature associated with polyre-

activity, ANA autoreactivity, and are particularly important in producing anti-DNA reactivity (52–54). Charged residues are increased in human immature and naive B cells that are ANA reactive or are polyreactive (27, 46). In addition, increased numbers of charged residues are found among the Ig's of editing human B cells (44–46). Finally, antibodies with excessive charged residues are counterselected during progression from pre-B and immature to naive B cells (27). As indicated in Figure 4C, Cδ-CS lineage populations from the various donors averaged significantly more charged residues in the CDRs than did all other B cell populations studied (*t* test, $P < 0.05$ or more significant for Cδ-CS versus any other population). The Cδ-CS lineage populations averaged 25–30% more positively charged residues (Figure 4C, black portion of bars) associated with anti-DNA reactivity, including arginine, lysine, and histidine, and 15–20% more acidic residues (Figure 4C, white portion of bars), including glutamic acid and aspartic acid. Unlike the comparisons of V_H4-34 and J_H6 gene segment usage or CDR3 length, the naive, various differentiated IgM, and various IgG populations all had a similar mean number of charged residues. The average number of charged residues was eight. It is notable that on average, around 35% of the clones of the naive populations and their most similar compartment, the pre-GC populations, had more than eight (average) charged residues. In comparison, approximately 45% (43–51%) of the clones of the various IgM or IgG GC, memory, and PC compartments had more than eight charged residues, although the variance between donors within each of these populations precluded statistical significance. Only the Cδ-CS lineage had significantly more than the others, averaging more than eight charged residues in 72% of the clones. However, this reduction of charged residues for naive and pre-GC cells and the remaining populations gives rise to the possibility that antigen encounter selects for B cells with charged residues in the antibody hypervariable region. Thus, the combination of selection of B cells that are autoreactive due to an excess of charged residues and simultaneous selection for antigenic reactivity may account for the particularly high frequency of charged residues in the Cδ-CS lineage. In conclusion, B cells with V_H genes encoding increased numbers of charged residues in the CDRs preferentially undergo class switch to Cδ.

Cδ-CS B cells are selected from a population of B cells that has been subjected to extensive receptor editing. Receptor editing is the most clearly established selective mechanism that permanently alters an autoreactive B cell, and therefore it can be assayed experimentally. The Cδ-CS lineage uses more than 90% λ light chains, in contrast to the normally expected 30–40% λ usage (7, 9). Light chain rearrangement is an ordered process (V_κ, then Vλ [ref. 55]), and increased λ usage has been associated with receptor editing (56). However, increased λ usage in the Cδ-CS lineage might also be due to selection of λ-utilizing cells. Although it is not a direct indication, increased evidence of V_κ editing would suggest that the increased λ usage might also have been induced by editing. Thus, the 10% of Cδ-CS cells that retained κ rearrangements were analyzed for evidence that they had been subjected to editing during primary B cell development.

One indicator of receptor editing commonly considered for analyses of the murine V_κ locus is the proximity of V_κ and J_κ genes used. A distal V–to–distal J gene-editing rearrangement can delete a more proximal V-to-J gene rearrangement in a "leapfrog" fashion. Thus, evidence of receptor editing is a VJ_κ repertoire skewed to the more distal V and J gene segments. However, due to the presence of many inverted V_κ genes in the human κ locus that rearrange by inversion rather than deletion of the intervening sequence, analysis of the V_κ repertoire for editing is not necessarily informative. As indicated in Figure 5A, V_κ4-1 (or B3), which is actually the most J-proximal of all V_κ genes, was significantly over-represented in the Cδ-CS cells compared with either memory or naive cells. This finding was not surprising, as V_κ4-1 was previously found to be over-represented in isolated human B cells that were in the process of receptor editing (44) and in the lupus V_κ repertoire (57). Therefore, we hypothesized that because V_κ4-1 is the segment most proximal to the J_κ genes, any primary rearrangement of an upstream inverted V_κ gene will result in its inversion and availability for secondary, editing rearrangements (Figure 5B). Thus, increased V_κ4-1 could be indicative of editing. Because V_κ4-1 is also inverted, as a primary rearrangement product, the V_κ4-1 RSS–to–J_κ RSS junction will be retained in the locus. However, as a secondary (editing) rearrangement occurs after it is first inverted, the V_κ4-1–to–J_κ RSS junction will be lost (Figure 5B). Thus, because of the position of V_κ4-1 and its orientation in the V_κ locus, analysis of the genetic configuration of V_κ4-1–to–J_κ2 rearrangements provides a measure of receptor editing for all V_κ genes. Analysis by semiquantitative PCR indicated that for samples normalized based on V_κ4-1–to–J_κ2 rearrangements, relative to that of naive and memory B cells, there was tenfold less of the V_κ4-1 RSS–to–J_κ2 RSS product in Cδ-CS cells and therefore tenfold more editing (Figure 5C). In conclusion, the increased frequency of V_κ4-1–to–Jk2 rearrangements in Cδ-CS B cells results from extensive receptor editing. If it is assumed that the more than 90% λ usage is also due to editing, than it would appear that nearly all B cells that undergo class switch to Cδ were selected for editing during primary B cell development. A second possibility is that the Cδ-CS lineage is proficient for receptor editing in peripheral lymphoid tissues, as we have previously reported that from this lineage, V gene clones can be isolated that have apparently edited their heavy chain V regions after the onset of somatic hypermutation (10). However, the origin of these recombinations is controversial because it is not clear if the RAG genes can be expressed during a GC reaction (58, 59). Thus, evidence of extensive receptor editing further demonstrates that B cells are probably selected into the Cδ-CS lineage due to encoded Ig autoreactivities.

Jκ gene usage demonstrates selection of cells that did not require receptor editing to the IgG populations. Because there are only five J_κ genes, all situated in the same orientation, unlike the V_κ repertoire, analysis of J_κ usage for distal bias provides evidence of receptor editing. There is a bias for distal J_κ utilization in the Cδ-CS population (Figure 5A), and distal J_κ bias was previously reported in B cells in the process of receptor editing (45). Consistent with other analyses herein, both the IgG memory ($IgD^-IgG^+CD27^+$) repertoire from two donors and total memory (IgD^-CD27^+) from two additional donors, which consist of more than 90% class-switched cells, were dominated by rearrangements to J_κ1 (Figure 5A). This is in contrast to the naive cells that utilize the J_κ1 through J_κ4 genes in an unbiased fashion. Thus, B cells harboring previously edited immune receptors and thus identified during primary selection as autoreactive are counterselected from the predominantly IgG memory populations.

Discussion

This report includes an extensive characterization of the expressed human V_HDJ_H gene repertoire involving simultaneous analysis of many stages of human peripheral B cell differentiation. We found significant changes in V gene repertoire features correlated with autoimmunity and receptor editing associated mainly with CSR,

rather than just differentiation to GC or memory B cells and PCs. The repertoire changes characterized included (a) profound selection of V_H4-34–utilizing B cells likely due to tolerization against the natural specificity to iI glycans on "self" tissues, as evidenced by selection for mutations of V_H4-34 to nucleotides encoding the residues involved in iI-glycan binding and hemagglutination; (b) class-related selection of J_H6 gene segment usage; (c) CDR3 length selection; (d) increased numbers of charged residues in the Cδ-CS lineage; and (e) selection of B cells with receptors that were edited during primary selection both into the Cδ-CS lineage and out of the IgG memory cell compartment. In fact, there were few significant differences in any of these categories in association with the B cell compartment of a particular isotype pool or between differentiated or somatically mutated versus unmutated IgM^+ B cell clones (see below). Thus, we conclude that selective mechanisms acting on naive and IgM^+ B cells in the periphery, including clonal deletion, anergy (23), follicular exclusion and developmental arrest (24–26), autoimmune "ignorance" (26, 60), and a possible novel mechanism proposed below, are predominantly associated with the avoidance of autoreactivities in class-switched B cell populations. This is not to suggest that peripheral selection is not active on IgM^+ B cells, but rather that the gross effects of selection on the V gene repertoire are evident mainly for class-switched B cells. Indeed, part of the observed changes could be due to the high attrition of B cells with IgM receptors (61), leading to a more frequent turnover of the IgM pool on the whole without sustained tolerizing influences, as occurs for longer-lived IgG^+ B cells. This possibility is supported by the finding of a higher level of variance observed for the IgM^+ GC and memory cells compared with IgG pools from various donors: the IgG cells are consistently reduced for the various measures of an autoimmune repertoire studied, whereas IgM populations are more variable. Alternative but not mutually exclusive hypotheses are also possible, given that B cells that undergo class switch to IgG may have particular sensitivities to tolerizing stimuli, as supported by a recent report of unique selection against B cells switched to IgG during primary development in a transgenic mouse model (62). In addition, IgG cells can reenter or continue GC reactions, as evidenced by the differential mutations of clonally expanded IgG^+ B cell clones. Thus, to control possible autoreactivites generated by mutations, the IgG^+ GC cells must also continue to be subjected to mechanisms of negative selection.

The reduced mutation frequency of the IgM^+ GC, IgM^+ memory, and IgM^+ PC populations (Table 3; refs. 3, 28) relative to that of IgG populations might suggest that these populations are heterogenous, derived from a combination of GC-dependent and GC-independent processes. Therefore, as a further control, the IgM^+ populations were separated into somatically mutated and unmutated fractions from each donor. Both fractions were unchanged for the various criteria studied. Assuming that most (but not all; refs. 60, 63) mutations are generated in GC-dependent processes, this analysis provides further evidence that the V gene repertoire is not significantly altered at the gross level except in association with Ig class. The high attrition rate of IgM^+ cells relative to class-switched cells (61) could account for the reduced mutation frequency, as the IgM^+ cells simply do not survive long enough to accumulate mutations. As described herein, the selective pressures on IgG^+ and Cδ-CS cells appear to be greater than those on IgM^+ cells, and thus cells from the class-switched pools may have accumulated a greater number of mutations to deal with the more stringent selective criteria. A final possibility that may contribute to the reduced mutation frequency of IgM^+ B cells is that a portion of the cells may continue to differentiate to the GC, memory, or PC phenotypes after abortive GC reactions (64, 65) and thus have less exposure to the mutation machinery. Several recent reports have suggested that inappropriate control of the GC reaction may lead to systemic lupus erythematosus (66, 67), and thus disruption of GC reactions may also represent a means of censoring autoreactive B cell clones and contribute to the counterselection from IgG^+ populations.

It should also be noted that further subdivisions of B cells commonly studied in mice, such as marginal zone B cells and B1 ($CD5^+$) versus B2 ($CD5^-$) cells, were not considered because these populations are not well characterized in humans. A marginal zone B cell counterpart in humans has been reported to be $IgM^+IgD^-CD38^{low}$ (68) and thus might be a minor contaminant of the IgM GC populations. However, marginal zone B cells in mice have recently been shown to have functional heterogeneity and may be formed of a mixture of plasmablasts and memory cells (69). It has not been ruled out that these marginal zone equivalent cells in humans are heterogenous as well. Use of the CD5 marker to sort B1 from B2 cells remains controversial for humans because unlike the situation in mice, CD5 is expressed on a portion of almost all known subdivisions of human B cells, including class-switched populations, and thus we felt this distinction was also beyond the scope of this report. However, it should be noted that there is an intriguing report of a differential repertoire in IgM^+CD5^+ versus IgM^+CD5^- B cells (70), although the differences noted were modest and that study was based only on two subjects.

Although the Cδ-CS lineage may be a specialized differentiation with specific antigenic reactivity giving rise to its unique V gene repertoire, the impressive selection of hallmarks of autoimmunity and evidence of receptor editing suggests that this population arose due to some mechanism of tolerance. We propose that a novel mechanism of tolerance causes the differentiation of these cells and contributes to class-related selection, as naturally occurring or mutationally generated autospecific B cells may become tolerized so that class switch is inhibited. If these autoreactive B cells are strongly activated by foreign antigen with T cell help or other accessory signals, they become competent to differentiate to GC cells and initiate class switch and somatic hypermutation, except that the class switch to IgG is blocked. However, these cross-reactive cells may be permitted to undergo class switch from Cμ to Cδ, possibly because of the cryptic nature of the Cδ switch regions. Alternatively, switch may be actively induced to the δ switch region as a specialized lineage. In any case, this lineage of cells may represent a specialization that can provide some level of immunity to the activating antigen despite their cross-reactive receptors, as evident by their differentiation to both PCs (7) and memory cells (8). It is notable that the Cδ-CS lineage typically represents only a small proportion of total B cells (1%) but is sometimes expanded to as many as 5% for some donors (ref. 7 and data not shown). Thus, certain immune responses may be highly dependent on the Cδ-CS response, particularly if the "best" B cell clone activated exhibits a cross-reactive specificity.

Selection of mutations to the amino acid positions involved in the binding of V_H4-34 gene–encoded antibodies to iI glycans for the Cδ-CS lineage (Figure 2D) suggests that despite the preferential differentiation of autoreactive B cells to this lineage, the cells must still adhere to some level of tolerance. Thus, we propose that induction of previously tolerized (switch-inhibited) B cells during GC differentiation leads to Cμ-to-Cδ class switch and accumulation of somatic mutations. The intense selection required to select B cells with reduced autoreactivity in addition to continued or increased foreign antigen affinity (specificity) may account for

both the excessive somatic hypermutation frequency of this population (ref. 9 and Table 3) and the high frequency of related clones (10) indicative of only limited survival or input to this lineage. Although it is controversial whether it is RAG mediated or possibly associated with somatic mutation, ongoing receptor editing and V_H or V_L chain replacement may also play a role in the tolerization of these clones. We have previously reported that clonally related but differentially mutated Cδ-CS–lineage B cells can have portions of their V_H genes replaced (10), in addition to the evidence of V_κ editing reported herein. However, the increased evidence of V_κ editing could just as well represent selection of cells edited during development, as proposed above.

The inhibition of class switch proposed as a new mechanism of IgM cell tolerization could simply be anergy (71) or some degree of anergy. It is known that T cell signals (CD40L and IL-4) can overcome anergy in autoreactive transgenic B cells to induce IgM differentiation and secretion (72). In addition, for a similar model system of anergy, but with the autoreactive V gene "knocked into" the Ig locus rather then as a nontargeted transgene, the same T cell factors will induce efficient class switch to IgG (73). Thus, classically defined anergy as described by the large body of work using the anti–hen egg lysozyme mouse model would not seem to account for a process of switch inhibition leading to the generation of Cμ-to-Cδ switch. Thus, the possibility exists that there is a mechanism separate from full-blown anergy that inhibits class switch directly for cells with low to moderate self-antigen interaction without disrupting the B cells' participation in an immune response. This leniency in nonswitched B cells is understandable, as the IgM response is much less prolific and shorter in duration in those cells than in switched cells (61) and the IgM antibodies themselves are generally not as damaging. In fact, the reduced survival of IgM-utilizing B cells after activation may have coevolved with a direct inhibition of class switch as a means of providing opportunity for differentially mutated IgM variants to overcome their natural autoreactivity and then undergo class switch, but a limited lifespan or specialization by Cμ-to-Cδ switch for those that retain autospecificities.

In conclusion, the demonstration of significant shifts in the V gene repertoire mainly associated with Ig class provides important insight into the overall dynamic of peripheral immune selection resulting from the many mechanisms of tolerance. Both as an additional basic mechanism of tolerance and because of the dangerous potential of a class-switched autoreactive repertoire in autoimmune disease, insight into the control of class switch or tolerizing inhibition of class switch could be of great clinical value for the generation of effective therapeutics. Therefore, we continue to pursue the mechanism leading to preferential switch from Cμ to Cδ of B cells that appear to harbor predominantly autoimmune repertoires. Finally, through antibody expression studies of reactivities "selected into" the Cδ-CS population in healthy people, we hope to gain further insight into the types of self antigens to which autoreactive antibodies are naturally "encoded." These analyses are of central importance to the study of autoimmunity, as they provide insight into the initiating autoantigens involved in the breakdown of tolerance that lead to autoimmune disease.

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