

Evidence for Selective Transformation of Autoreactive Immature Plasma Cells in Mice Deficient in *Fasl*

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Abstract

Germline mutations in *Fas* and *Fasl* induce nonmalignant T cell hyperplasia and systemic autoimmunity and also greatly increase the risk of B cell neoplasms. B lymphomas occurring in *Fasl* mutant (*gld*) mice usually are immunoglobulin (Ig) isotype switched, secrete Ig, and are plasmacytoid in appearance but lack *Myc* translocations characteristic of other plasma cell (PC) neoplasms. Here, we explore the relationship between B cell autoreactivity and transformation and use gene expression profiling to further classify *gld* plasmacytoid lymphomas (PLs) and to identify genes of potential importance in transformation. We found that the majority of PLs derive from antigen-experienced autoreactive B cells producing antinuclear antibody or rheumatoid factor and exhibit the skewed Ig V gene repertoire and Ig gene rearrangement patterns associated with these specificities. Gene expression profiling revealed that both primary and transplanted PLs share a transcriptional profile that places them at an early stage in PC differentiation and distinguishes them from other B cell neoplasms. In addition, genes were identified whose altered expression might be relevant in lymphomagenesis. Our findings provide a strong case for targeted transformation of autoreactive B cells in *gld* mice and establish a valuable model for understanding the relationship between systemic autoimmunity and B cell neoplasia.

Key words: autoimmunity • B cell lymphoma

Introduction

Mice and humans with germline (GL) mutations in *Fas* or *Fasl* develop early onset, progressive autoimmune lymphoproliferative syndromes, termed ALPS in humans, characterized by nonmalignant T cell hyperplasia, massive lymphadenopathy and splenomegaly, hypergammaglobulinemia, and systemic autoimmunity. As the disease progresses, mice

mutant in *Fas* (*lpr*) or *Fasl* (*gld*) also accumulate memory-like B cells and plasma cells (PCs), and produce high titers of IgG antinuclear antibody (ANA; references 1 and 2). These anti-

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Abbreviations used in this paper: ANA, antinuclear antibody; BCR, B cell receptor; BL, Burkitt lymphoma; BLL, Burkitt-like lymphoma; CBL, centroblastic; CDR, complementarity-determining region; CL, cardiopilin; dsDNA, double-stranded DNA; FBL, follicular B cell lymphoma; FWR, framework region; GC, germinal center; GL, germline; IBL, immunoblastic; MALT, mucosal-associated lymphoid tissue; MZL, splenic marginal zone lymphoma; PC, plasma cell; PCA, principal component analysis; PCT, plasmacytoma; PL, plasmacytoid lymphoma; PPC, phosphorylcholine; R mutation, replacement mutation; RF, rheumatoid factor; S mutation, silent mutation; SBL, small B cell lymphoma; SJL, SJL- $\beta 2M^{-/-}$ lymphoma; ssDNA, single-stranded DNA.

bodies include specificities for double-stranded DNA (ds-DNA) and histones and, in large part, are encoded by a restricted set of IgH and IgL genes with members of the V_H J558 and 7183 families as a prominent feature (3). V_L gene use by antibodies specific for DNA–histone complexes, although also restricted, is more diverse than V_H use and involves members of at least 10 V_κ gene groups (3). In spite of this biased repertoire, the autoimmune responses to nuclear antigen by *lpr/gld* and other autoimmune mice resemble normal, secondary antibody responses to foreign antigen, and affinity maturation of the autoimmune ANA responses appears to be the result of antigen drive (4, 5). Thus, within the B cell compartment of *lpr* and *gld* mice, there is evidence for loss of tolerance to and chronic stimulation by DNA–histone complexes.

GL mutations in *Fas/Fasl* also greatly increase the risk of B cell neoplasia in humans and mice. B cell lymphomas in autoimmune lymphoproliferative syndrome patients are predominantly follicular and phenotypically diverse (6). In mice, lymphomas develop between 6 and 12 mo of age, and tumor incidence is strain related. By 1 yr of age, ~30% of C3H-*gld* and 60% of BALB-*gld* mice have monoclonal outgrowths of B cells in the spleen and LN that metastasize to nonlymphoid organs (2). These tumors can be passaged to purity in immunodeficient *scid* mice and are lethal for their hosts (2). The *scid*-passaged *lpr* and *gld* B cell lymphomas are predominantly plasmacytoid in morphology, are κ^{dull+} CD23⁻ CD21⁻ Mac-1⁺, have undergone Ig isotype switching, and spontaneously secrete Ig (2), suggesting that they derive from antigen-experienced, possibly germinal center (GC)-selected B cells.

Lymphomagenesis is a multistep process involving both genetic and environmental factors. There is mounting evidence that chronic antigen stimulation might be an important environmental factor. In support of this, there are reports that within the Ig V_H and V_L genes of many B cell tumors and clonal progeny of nontransformed cells there is a counterselection for replacement mutations (R mutations) in the framework regions (FWRs) of the B cell receptor (BCR) and generally higher ratios of R versus silent mutations (S mutations) in the complementarity-determining regions (CDRs; references 7–11). The persistence of these mutational patterns in the face of ongoing somatic mutation suggests that there is strong selective pressure to preserve the structural integrity of the BCR. Moreover, analyses of intraclonal variation in human follicular lymphomas revealed genealogic relations between subclones, indicative of antigen-driven clonal evolution (12, 13). Other compelling evidence that chronic antigen stimulation might be a factor in B cell lymphomagenesis comes from studies of mucosal-associated lymphoid tissue (MALT) B cell lymphomas. These tumors are thought to derive from marginal zone B cells and develop at sites of chronic inflammation caused by autoimmune disease or infection (14–19). As examples, patients with Sjögren's syndrome or Hashimoto's thyroiditis have an increased risk of developing MALT lymphomas of the salivary and thyroid glands, respectively (15–17). Notably, in both patient populations, MALT lymphomas

have been reported that produce rheumatoid factor (RF) and exhibit nonrandom use of IgV_H and V_L genes, suggesting that autoreactive B cells chronically stimulated by nominal self-antigen might be selectively targeted for transformation (14, 17, 18). Of interest, C57BL/6-*lpr* mice deficient in all T lineage cells also develop B lymphomas that produce RF (20).

The goals of this study were twofold. First, we sought to determine whether B cell transformation in *gld* mice is a random event or is skewed toward chronically stimulated, autoreactive B cells. Second, we undertook gene expression profiling using oligonucleotide-based microarrays to determine whether the *gld* plasmacytoid tumors exhibit a transcriptional profile that might be useful for diagnosis and relevant to pathogenesis.

Materials and Methods

Mice. BALB/*c-gld/gld* (BALB-*gld*) mice were bred and maintained in the Holland Laboratory vivarium. C.B-17-*scid/scid* (*scid*) mice were purchased from Taconic. SJL/J mice with a null mutation of the β-2 microglobulin gene [SJL/J-β2M^{-/-}/Dcr (N11)] were bred and raised at The Jackson Laboratory. Animal research protocols were approved by the American Red Cross Institutional and Animal Care Use Committee.

Tumors. Early passage number (P1 or P2) plasmacytoid lymphomas (PLs), including 13 BALB-*gld* and 6 C3H-*gld*, were transplanted i.p. into 4–6-wk-old *scid* mice. Sera, spleens, and mesenteric LNs were harvested from tumor-bearing mice. Single cell suspensions were made for FACS analysis and isolation of DNA and RNA. Southern hybridization analysis for IgH and IgL gene rearrangements was used to establish clonality (2). The proportion of tumor cells in spleen and LN samples was determined by FACS analysis (BD Biosciences). The majority of the tumor cell preparations contained >90% CD19⁺ B cells with high forward and side scatter. Primary tumor cells were obtained from four mice with advanced tumors and were enriched by MACS sorting (Miltenyi Biotec) of CD19⁺ cells. These cells were predominantly CD23⁻ CD21⁻ Mac-1⁺ with high forward and side scatter. Non-*gld* lymphomas used in array analyses were mostly from NFS.V⁺ congenic mice (21), but included plasmacytoma (PCT) cell lines provided by M. Potter (National Cancer Institute, Bethesda, MD) and primary lymphomas of SJL/J-β2M^{-/-} mice.

Cultures. Sorted CD19⁺ CD23⁻ tumor cells were cultured at 10⁷/ml in supplemented RPMI medium for 48 h in the presence or absence of 25 μg/ml LPS (Sigma-Aldrich).

ELISA for Ig Isotypes, ANA, Single-Stranded DNA (ssDNA), and RF. Ig isotypes and ANA, anti-ssDNA, and RF titers in sera from tumor-bearing mice and tumor tissue culture supernatants were determined using a mouse Ig isotyping ELISA kit (BD Biosciences) and ANA, ssDNA, and RF ELISA kits (Alpha Diagnostic International). Antibodies to dsDNA were detected by ELISA as described previously (2). Nuclear staining was detected using a kit (Antibodies Inc.).

Cloning and Characterization of Ig Genes. Total RNA was isolated from tumor cells using TRIzol reagent (Invitrogen). First-strand cDNA was generated using oligo (dT) or random primers and SuperScript (Invitrogen). PCR amplification of tumor V_H and V_L genes was performed using a set of degenerate primers and conditions listed in Table S1 (see Online Supplemental Material section below). Selected PCR products were cloned using a TOPO

TA cloning kit (Invitrogen). Multiple independent plasmid isolates for V_H and V_L were sequenced for each tumor. Ig gene sequences were searched for their closest GL sequence match against a DNA and protein database (<http://www.ncbi.nlm.nih.gov/BLAST>), a database for Ig genes (<http://www.ncbi.nlm.nih.gov/igblast>), and the Celera database. The probability that an excess of R mutations in CDR1 and CDR2 regions or a scarcity of R mutations in FWRs occurred by chance was calculated using a multinomial distribution model and a published program (<http://www.stat.stanford.edu/immunoglobulin>; reference 22).

Oligonucleotide Microarrays. Microarray chips, printed by the Microarray Research Facility at NIAID (<http://madb.niaid.nih.gov>), comprised ~6,700 mouse gene targets represented by 70-mer oligonucleotides purchased from Compugen. Total tumor cell RNA was extracted by the TRIzol protocol and further purified using the RNeasy column-based protocol (QIAGEN). A reference sample was created by extracting total RNA from 10 well-characterized hematopoietic cell lines and making a pool comprising equal amounts of RNA from each line (Table S2; see Online Supplemental Material section below). The labeling of cDNA, chip hybridization, and chip scanning were performed as described previously (23). The microarray data accession number is GSE1908.

Microarray Data Analysis. The microarray dataset was organized and analyzed using SAS software (SAS Institute). From a total of 6,800 genes, 5,770 genes had <30% of missing values across all 134 samples. For these genes, the missing values were replaced with the overall mean of each individual gene, and this no-missing-value dataset was used in the subsequent principal component

analysis (PCA). Student's *t* test and the nonparametric Kruskal-Wallis test were used to compare the expression levels of genes among different lymphoma groups. Hierarchical clustering (average linkage method) of selected significant genes against lymphoma samples was performed using a program available in the NIAID microarray database application (<http://madb.niaid.nih.gov>).

Quantitative PCR. Primers and conditions for quantitative PCR are given in Table S3 (see Online Supplemental Material section below).

Online Supplemental Material. The supplemental materials include the following: (a) primers and conditions for PCR amplification of V_H and V_L genes (Table S1); (b) cell lines used to generate the standard for microarrays (Table S2); (c) primers for quantitative PCR (Table S3); (d) translated amino acid sequences of V_H and V_L for *gld* tumors (Fig. S1); (e) V_H-D_H-J_H rearrangement of tumor V_H CDR3 regions (Fig. S2); (f) hierarchical clustering of 415 genes that discriminate *gld* PL from other mouse lymphomas (Fig. S3); (g) the same data as shown in Fig. S3 with all genes identified (Fig. S4); (h) hierarchical clustering of gene expression data for 99 genes that distinguish *gld* PLs from other PC neoplasms (Fig. S5); and (i) the same data as shown in Fig. S5 with all genes identified (Fig. S6). Tables S1–S3 and Figs. S1–S6 are available at <http://www.jem.org/cgi/content/full/jem.20041575/DC1>.

Results

Phenotype and Antibody Specificity of *gld* PLs. PLs, 12 from BALB-*gld* and 6 from C3H-*gld* mice, were analyzed for sur-

Table I. *Isotypes and Nuclear Antigen Reactivity of Antibodies Secreted by *gld* Tumors*

Tumor	Isotype	ssDNA	dsDNA	ANA	Nuclear staining	RF	CL	PPC
BALB- <i>gld</i>								
208	IgG1, Igκ	— ^a	—	—	—	+ ^b	—	—
329	IgM, Igκ	—	—	+	+	—	—	—
421	IgM, Igκ	+	—	—	—	—	—	—
424	Multiple ^c , Igκ	—	+	+	+	—	+	+
425	IgG2a, Igκ	(+) ^d	—	—	(+)	+	—	—
426	IgA, Igκ	+	—	+	+	(+)	—	—
531	IgM, Igκ	—	—	+	+	—	—	—
593	IgG2a, Igκ	—	—	+	+	—	—	—
600	IgG2a, IgG3, Igκ	—	—	+	+	—	—	—
2685	IgG2a, Igκ	—	+	+	+	—	—	—
3294	IgM, Igκ	—	—	+	+	—	—	—
8850	ND	+	—	ND	—	+	ND	ND
C3H- <i>gld</i>								
205	IgG2a, Igκ	—	—	—	—	(+)	—	—
217	IgG3, Igκ	(+)	—	—	—	—	—	—
221	IgA, Igκ	—	+	+	+	—	—	—
223	IgG3, Igκ	—	—	—	—	—	—	—
355	IgG2a, Igκ	—	—	—	—	—	—	—

^a—, no detectable reactivity.

^b+, strongly reactive.

^cMultiple, more than two Ig isotypes secreted.

^d(+), weakly reactive.

face phenotype, Ig isotype, and Ig specificity. The panel included 14 previously characterized tumors (2) and 4 new BALB-*gld* tumors (593, 600, 2685, and 3294). One partially characterized BALB-*gld* tumor, 8850, also was included. Early *scid* passages (P1 or P2) of tumors were used to maximize the closeness of genotype and phenotype to the primary tumors. The majority of tumors was CD19⁺ Mac-1^{dull} Igκ^{dull} CD23⁻ CD21⁻ CD5^{dull} or CD5⁻, a phenotype remarkably similar to that of the nontransformed B cells that selectively accumulate in old *lpr* and *gld* mice (2, 24, 25). The Ig isotype produced by the tumors was determined by testing sera from tumor-bearing *scid* mice and supernatants from cultures of sorted tumor cells. All 16 sera tested contained significantly elevated levels of Ig, and isotype switching had occurred in 11 (Table I). One tumor, 424, produced polyreactive antibodies of different isotypes that were reactive in ELISA assays for dsDNA, phosphorylcholine (PPC), and cardiolipin (CL). Tumor 600 consistently secreted both IgG2a and IgG3 in several *scid* passages (Table I). Ig was not detected in sera from control *scid* mice (not depicted). The sera also were assayed for ANA, RF, and antibodies to dsDNA, ssDNA, PPC, and CL. 9 of 16 (56%) sera tested

positive for ANA by ELISA and for nuclear staining detected by immunohistochemistry (Table I). Within this group, Ig from tumor 426 also reacted strongly with ssDNA and was weakly positive for RF. Only antibodies from 221, 424, and 2685 reacted with dsDNA. antibodies from two ANA⁻ tumors, 217 and 421, also reacted with ssDNA. Although negative for ANA activity by ELISA, antibodies from 425 showed weak reactivity with nuclei, weak reactivity with ssDNA, and had high titers of RF. Tumor 8850 produced high titers of RF and anti-ssDNA antibodies, and tumors 205 and 208 produced RF only. Cross-reactivity between RF and ssDNA has been reported previously (26). With the exception of 424, no tumors produced antibodies reactive with PPC or CL. In all, 15 of 17 Ig-secreting tumors produced RF and/or antibodies reactive with nuclear antigens. 4 of 17 tumors produced antibodies that appeared to be polyreactive. Results obtained with tumor culture supernatants conformed to those from sera of *scid* mice bearing the same tumor (not depicted).

Selected Use of the V_HJ558 Family and Individual Subfamily Members in gld Tumors. To determine the Ig gene repertoire of the tumors, we sequenced the V_H and V_L genes us-

Table II. Use of V, D, and J Segments of Tumor IgV_H and IgV_L Sequences

Tumor	Heavy chain variable region						Light chain variable region				
	DNA ^a	AA ^a	GL ^b	V _H ^c	D _H ^d	J _H	DNA ^a	AA ^a	GL ^b	V _L ^e	J _L
<i>BALB-gld</i>											
208	99.3	97.8	<i>VGK1A</i>	VH9	P2.4/6, P2.7/9	J _H 4	99.6	98.9	<i>21-7</i>	vk21	J _k 2
329	96.4	96.7	<i>J558.52</i>	J558	P2.12	J _H 4	99.6	100	<i>cy9</i>	vk9/10	J _k 2
421	96.3	94.6	<i>J558.17</i>	J558	D16.1, P2.9	J _H 4	97.8	95.7	<i>8-27</i>	vk8	J _k 5
424	97.3	94.9	<i>J558.35</i>	J558	P2.5/7	J _H 1	99.2	97.6	<i>ai4</i>	vk4/5	J _k 5
425	95.7	89.1	<i>J558.32</i>	J558	P2.5/7, P2.13	J _H 2	98.5	95.7	<i>cr1</i>	vk1	J _k 1
426	96.8	91.5	<i>V-BK</i>	7183	P2.4/6, P2.10	J _H 2	98.9	98.9	<i>12-44</i>	vk12/13	J _k 5
531	98.2	95.7	<i>J558.52</i>	J558	D16.1, D16.2	J _H 2	97.3	94.3	<i>19-13</i>	vk19/28	J _k 2
540	91.3	85.9	<i>J558.2</i>	J558	D16.2	J _H 2	100	100	<i>21-2</i>	vk21	J _k 4
593	98.3	96.9	<i>J558.52</i>	J558	P2.4/6	J _H 4	98.9	96.6	<i>ba9</i>	vk9/10	J _k 1
600	97.8	96.7	<i>J558.22</i>	J558	P2.9, P2.12	J _H 4	99.2	97.6	<i>aq4</i>	vk4/5	J _k 5
2685	99.6	98.9	<i>J558.18</i>	J558	P2.4/6	J _H 4	97.3	95.4	<i>21-7</i>	vk21	J _k 2
3294	98.9	96.7	<i>VOx-1</i>	Q52	D16.2	J _H 4	97.5	94.7	<i>bd2</i>	vk2	J _k 1
<i>C3H-gld</i>											
142	90.5	81.0	<i>V130</i>	J558	P2.x	J _H 3	97.4	94.5	<i>VL1</i>	VL1	J _k 1
205	88.7	87.6	<i>J558.18</i>	J558	P2.9	J _H 4	92.3	88.5	<i>19-13</i>	vk19/28	J _k 4
217	97.1	94.4	<i>J558.47</i>	J558	P2.9	J _H 2	98.6	96.8	<i>gj38c</i>	vk38c	J _k 2
221	96.8	95.8	<i>VH7183.3b</i>	7183	P2.5	J _H 2	99.6	98.9	<i>23-39</i>	vk23	J _k 4
223	94.4	91.7	<i>J558.40</i>	J558	P2.12	J _H 4	99.2	97.7	<i>at4</i>	vk4/5	J _k 5
355	99.3	97.8	<i>VH7183.1j</i>	7183	P2.12	J _H 3	99.3	99.0	<i>12-44</i>	vk12/13	J _k 1

^aPercent homology between tumor DNA and amino acid (AA) sequences and GL.

^bClosest matched GL genes.

^cV_H family.

^dD_H segment. DH family abbreviations: P, DSP; D, DFL; T, DST.

^eV_L family.

ing multiple plasmid isolates from each of the 18 tumors in the panel. As shown in Table II, 13 of the tumor V_H gene sequences (72.2%) aligned with a member of the J558 (V_H1) family, 3 (16.7%) belonged to the 7183 (V_H5) family, and 1 each to the Q52 and VGAM3.8 (V_H9) families. The V_H J558 family GL gene assignments were made on the basis of homology with BALB/c J558 GL sequences (27) and C3H GL sequences in the Celera database. The overall DNA and predicted amino acid sequence homology between each tumor DNA and the assigned GL V_H gene was >94% in the majority of cases. For the five tumors with <95% homology, the degree of homology exceeded 80%, the considered lower limit of homology required for accurate assignment to a particular V_H family (28). Although we are confident that the family assignments are correct, the subfamily assignments for tumors 142, 205, and 540 may need revision as more GL J558 sequences become available. The frequency of J558 family use among the BALB-*gld* tumors (75%, 9 of 12 sequences) differed significantly (χ^2 , $P < 0.05$) from that reported for normal BALB/c B cells (51%, 30 of 59 sequences; reference 29). A similar trend was observed in the C3H-*gld* tumors, where 66.7% (four of six) used J558 genes.

Analysis of J558 subfamily gene use revealed some bias in the BALB-*gld* tumors, particularly those with nuclear reactivity. Three ANA-producing tumors, 329, 531, and 593, were highly homologous (>96% similarity) with *J558.52*, a gene found only once in 128 randomly selected J558 cDNA from normal BALB/c B cells (Table II; reference 27). The alignment of the predicted V_H amino acid sequences for these tumors with the GL *J558.52* sequence from FWR1 to FWR3 is shown in Fig. S1 A, available at <http://www.jem.org/cgi/content/full/jem.20041575/DC1>. Another J558 gene, *J558.18*, was used by two tumors, 205 and 2685. Consistent with these findings, anti-DNA antibodies derived from MRL-*lpr* and (NZBxNZW) F_1 mice frequently are encoded by *J558* genes with a high degree of homology with *J558.52* and *J558.18* (3, 5, 30). Similarly, tumor 221, which secretes antibodies reactive with nuclear antigen and ds-DNA, used *7183.3b*, a gene frequently used by anti-dsDNA antibodies (Table II; references 5 and 30). Tumor 221 and ANA-producing tumor 426, which also is encoded by a 7183 family member, retained the GL putative DNA-contacting RN motif at positions 76 and 77 of FWR3 (Fig. S1 A; reference 3). In all cases, tumor cDNA Ig sequences were verified by genomic sequencing.

Evidence for Nonrandom Use of D_H and J_H Genes in PLs. Analysis of 18 tumor CDR3 D_H sequences revealed evidence of D-D fusions and significant skewing in D_H family use (Table II and Fig. S2). Fusion of two D segments, rare in normal B cells, was observed in six tumors. Altogether, 15 of 18 tumors (83.3%) used a D segment from the DSP2 family, whereas 4 tumors used D genes from the DFL family. In contrast, in a previous study, 50% to 55% of BALB/c follicular B cells used DSP family members (29). Notably, many tumor V_H CDR3 sequences contained one or more R and N residues, a frequent feature of ANA (3). In many cases, the R and N residues were generated by N region nucleotides (Fig. S2). One motif, [(W)LR(R) $_n$], found re-

currently in anti-DNA antibodies (3, 5, 30), was present in the V_H CDR3 region of tumors 426, 593, 600, and 2685, a finding consistent with the production of ANA and anti-DNA antibodies by these tumors (Table I).

Biased usage of J_H segments was also observed in the tumors, particularly in the BALB-*gld* group (Table II and Fig. S2). Overall, the J_H distribution was 5.6% J_H1 , 33.3% J_H2 , 11.1% J_H3 , and 50% J_H4 . By comparison, the J_H family usage in normal BALB/c B2 cells was reported to be 9% J_H1 , 25% J_H2 , 32% J_H3 , and 34% J_H4 (29). The tumor and B2 cell J_H distributions differ significantly (χ^2 , $P < 0.0001$). The J_H4 bias was even more prominent (57%) if only the 14 tumors with demonstrated autoreactivity were considered (Tables I and II).

Biased Use of V_L Gene Families and J_K Genes in PLs. In normal B cells, the pattern of V_L family usage generally corresponds to the estimated family size (31). Two exceptions are the V_{K1} and V_{K4} families, which each account for ~13% of the expressed V_L genes (31, 32). Analyses of PL V_L sequences revealed a pattern different from that in normal B cells (Table II). Although 10 V_K families were represented among the PLs, the following three families were over-represented: $V_{K4/5}$ (15.8%), V_{K21} (15.8%), and $V_{K12/13}$ (10.5%). Although the sample size is small, preferential subfamily use within these families also was observed. Tumors 355 and 426 used V_{K12-44} , and tumors 208 and 2685 used *21-7* (Table II). Notably, both of these V_K subfamilies are represented frequently in anti-DNA antibodies (3, 5).

Similar to the V_H genes, the V_L genes also showed a trend toward increased rearrangement to distal J genes (Table II). J_{K1} , J_{K2} , and J_{K5} each were used by 5 of 18 tumors (27.8%), and J_{K4} was used by 3 of 18 tumors (16.7%). In normal mouse B cells, the frequency of rearrangement to J_K loci is $J_{K1} > J_{K2} > J_{K5} \gg J_{K4}$, with ~35% rearranging to J_{K4} (13%) or J_{K5} (22%; reference 31). The frequency of J_{K4} or J_{K5} usage by the tumors (45%) differed significantly from that of normal B cells (χ^2 , $P = 0.036$).

Evidence for Antigen Selection in PLs. Patterns of DNA mutation in Ig genes can provide valuable insights into the maturational history of the tumor precursors and the importance of BCR integrity, specificity, and affinity in tumorigenesis (7–11). To investigate the frequency and nature of the mutations in tumor Ig genes, the distribution of R versus S mutations in the FWR, CDR1, and CDR2 of V genes was analyzed (22). Only data for V_L genes is shown because most of the mouse V_L GL genes have been sequenced and the GL assignments for the tumors can be made with greater confidence. In this analysis, a significantly higher frequency of R mutations in CDRs than expected by chance ($P_{CDR} < 0.05$) is considered evidence for positive, antigen-mediated selection of cells with the mutated Ig. Conversely, a frequency of R mutations in FWRs that is statistically lower than expected by chance ($P_{FWR} < 0.05$) implies that there is pressure to maintain the structural integrity of Ig, possibly because antigen is providing important growth/survival signals to the tumor cells (7).

As shown in Table III, two tumors had GL V_L sequences and the rest had 1–10 R mutations. CDR hypermutation

Table III. Mutational Analysis of Tumor V_L Sequences

Tumor	Light chain variable region					
	FWR1, 2, and 3			CDR1 and 2		
	R ^a	S ^b	P ^c	R ^a	S ^b	P ^c
<i>BALB-gld</i>						
208	1	0	0.715	0	0	0.595
329	1	0	0.696	0	0	0.577
421	3	2	0.389	1	0	0.549
424	0	0	0.086	2	0	0.014
425	0	1	0.004	4	1	0.009
426	1	1	0.199	0	1	0.226
531	3	0	0.472	2	0	0.096
540	0	0	NA ^d	0	0	1.0
593	1	0	0.197	2	0	0.038
600	1	0	0.385	1	0	0.145
2685	1	4	0.004	3	1	0.154
3294	3	1	0.254	2	1	0.264
<i>C3H-gld</i>						
142	1	1	0.016	4	1	0.010
205	7	6	0.032	3	2	0.401
217	1	0	0.206	1	1	0.238
221	0	0	NA	1	0	0.075
223	2	0	0.82	0	0	0.64
355	1	1	0.390	0	0	0.641

^aNumber of R mutations.^bNumber of S mutations.^cP value was calculated according to Lossos et al. (reference 22). P values <0.05 are in bold.^dNA, not applicable.

was observed in 4 of 18 tumors and FWR conservation was observed in 4 of 18 tumors. Tumors 142 and 425 exhibited both V_L CDR hypermutation and V_L FWR preservation. Notably, six V_L sequences had R mutations in CDR1 and/or CDR2 that gave rise to arginine (R) or asparagine (N) residues important for DNA binding (3). Overall, 6 of 18 tumors had mutated CDR and/or conserved FWR in V_L sequences, suggesting that at least one third of the B cells undergoing transformation were antigen selected. All six tumors in this group were Ig isotype switched and those producing antibodies (five out of six) were reactive with nuclear antigens or RF. To test for evidence of ongoing antigen-mediated selection, sequences from up to 15 clones from each tumor were examined for intraclonal variation. This was observed only in tumor 593 and at low frequency. 2 of 15 clones had a single amino acid change at the same position in V_H CDR1 (not depicted).

PLs Exhibit a Gene Expression Profile Distinct from That of Other B Cell Lineage Neoplasms. Recently, the opportunity to understand the characteristics of different murine lymphoma types has been expanded through the use of gene

expression profiling (33). This work included lymphomas classified on histologic grounds as small B cell lymphoma (SBL), splenic marginal zone lymphoma (MZL), Burkitt-like lymphoma (BL), Burkitt lymphoma (BL), follicular B cell lymphoma (FBL), and diffuse large B cell lymphomas of centroblastic (CBL) or immunoblastic (IBL) origin, as well as PCT cell lines. The results uncovered class-specific expression signatures for SBL, BL, PCT, CBL, and MZL, whereas the other types exhibited less distinctive profiles (33).

To determine how PLs relate molecularly to these lymphoma types and to PCT, we compared their gene expression profiles using a 6,680 gene chip. We also studied a series of primary PC-related neoplasms from *SJL- β 2M^{-/-}* mice that have not been studied previously at the molecular level and whose place in the scheme of terminal B cell differentiation is not known. These tumors resemble those that arise in aging SJL mice, but they develop at an accelerated rate (unpublished data).

PCA of the microarray data revealed that the PL tumors were remarkably similar to each other in terms of gene expression. In the majority of cases, they also were readily distinguished from lymphoma classes that originate from cells that have not yet entered the terminal progression program of PCs (Fig. 1). Further analyses of these data identified 415 genes whose expression levels by *t* test distinguished PLs from BLLs, BLs, SBLs, CBLs, FBLs, MZLs, and IBLs (Figs. S3 and S4). Most of these genes were expressed at significantly lower levels in the PL than the other lymphoma types, consistent with the well-established down-regulation of many genes that occurs as B cells mature toward secretory stages of development. Fewer than 20 genes

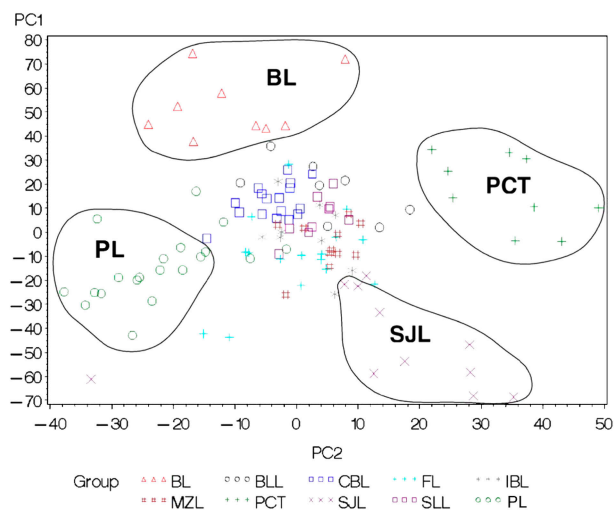


Figure 1. PCA of lymphoma gene expression measured by oligonucleotide microarray. Expression levels of 6,680 genes were measured for these 10 types of mouse lymphomas: BL, BLL, CBL, FBL, IBL, MZL, PCT, SJL, SBL, and *scid*-passaged *gld* PL. Approximately 4,800 genes (no missing value) were used for the analysis. PC1 and PC2 represent the principal component 1 and principal component 2, respectively. Most samples belonging to the same group are enclosed within a circle.

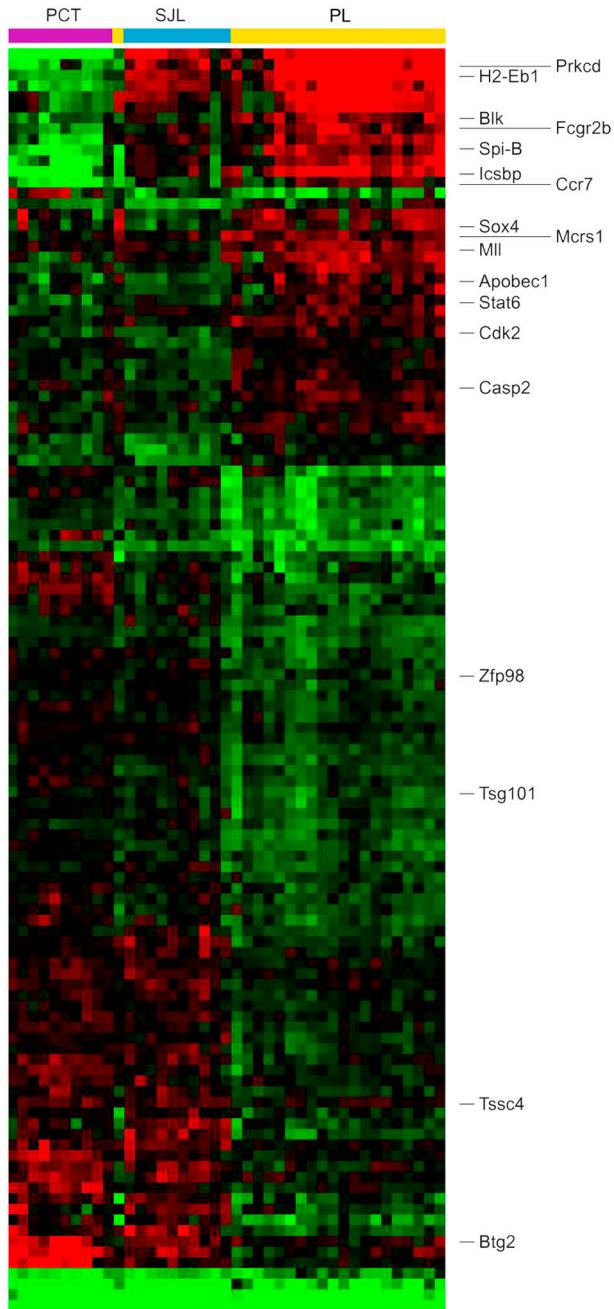


Figure 2. Hierarchical clustering of gene expression data for 99 genes that distinguish *gld* PLs from other PC neoplasms as determined by *t* test at $P < 0.0000001$. Only genes with high quality spots in each of the arrays were evaluated. Genes of particular interest are listed on the right and discussed in Results. Tumor populations include PCT lines (PCT), primary SJLs (SJL), and *scid*-passaged *gld* PLs (PL). PCT, SJL, and PL tumors are identified by purple, blue, and yellow bars, respectively.

were selectively expressed at high levels in PL. One gene of particular interest in this group was *Apobec1*, which encodes an RNA editing protein that also may function as a DNA mutator (34, 35).

Notably, the PL tumors also were distinct by PCA from the PCT and primary SJL- $\beta 2M^{-/-}$ lymphomas (Fig. 1). To further investigate the developmental relationships among

the PC-related neoplasms, we determined the genes that best distinguish PLs from primary SJL- $\beta 2M^{-/-}$ tumors and PCT cell lines using a *t* test. The expression patterns of the 99 genes identified in this manner showed that the SJL neoplasms had features of both PCTs and PLs (Fig. 2). 13 genes were expressed at high to moderate levels in *gld* tumors, at lower levels in the SJL tumors, and at very low levels in PCTs. Among these were several genes—MHC class II, *Blk*, *Ccr7*, and *Icsbp*—known to be down-regulated as B cells mature to PCs (36, 37). This pattern of gene expression suggested that the SJL tumors were intermediate in maturity between the PCTs and PLs.

Another set of 25 genes was expressed at higher levels in PL than either the SJL or PCT series. These included *Casp2*, another gene known to be down-regulated during the maturation of B cells to PCs (37), and several genes that were found to distinguish the *gld* tumors from all the non-PC lymphomas examined—*Mll*, *Apobec1*, and *Mcrc1*. Of interest, four genes with established or suspected tumor suppressor function were among the larger number of genes expressed at lower levels in the PL—*Zfp98* (38), *Tsg101* (39), *Tssc4* (40), and *Btg2* (41).

To further examine the developmental relationships among the three sets of PC neoplasms, we interrogated a series of 30 genes modulated in expression late in B cell differentia-

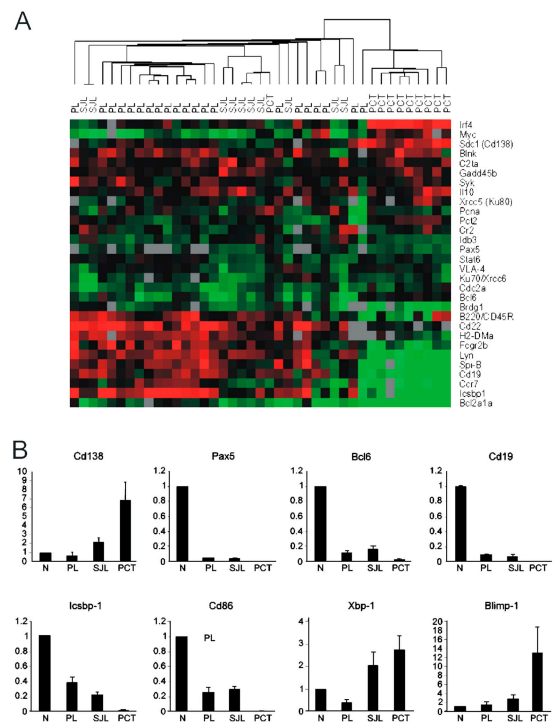


Figure 3. Changes in gene expression associated with PC differentiation. (A) Hierarchical clustering of genes and arrays for 30 selected genes altered in expression during late B cell differentiation. Gray spots indicate missing or excluded data. (B) Quantitative PCR of eight selected genes. Values represent mean \pm SEM for five separate samples normalized to a value of 1 for BALB/c B cells. N, normal BALB/c splenic B cells; PL, *scid*-passaged *gld* PL; SJL, primary SJL; PCT, PCT lines.

tion (36, 37). The matrix generated by hierarchical clustering of both the genes and the arrays is shown in Fig. 3 A. All PCTs but one fell on one branch of the tree generated by the array samples, whereas PL and SJL neoplasms again occupied the second branch. 13 of the 21 PL (61%) and 5 of the 10 SJL tumors were tightly grouped, with the remainder being interspersed outside these immediate alignments. The genes also fell into several groups. In one major group, 10 genes generally were expressed at the highest levels by PL, at somewhat lower levels by many of the SJL tumors, and at very low levels by the PCT. Among these were the genes encoding CD45, CD22, CD19, CCR7, ICSBP1, and FCGR2B (Fig. 3 A). A smaller group of genes, including *If4*, *Sdc1*, and *Xrac5*, were expressed at highest levels in PCT and at similarly lower levels in PL and SJL tumors (Fig. 3 A).

As an independent approach to comparing the maturational state of these neoplasms and at the same time validating the array results, we used quantitative PCR to determine expression levels of eight genes that are differentially expressed during differentiation to PCs (Fig. 3 B; references 36 and 37). Relative to BALB/c splenic B cells, the levels of *Sdc1* (CD138), *Xbp1*, and *Prdm1* (Blimp) were generally found to increase from PL to SJL to PCT, whereas those for *Pax5*, *Bcl6*, *Cd19*, *Icsbp*, and *Cd86* decreased. These patterns of gene expression were consistent

with those seen on the microarrays. We conclude that lymphomas that develop in aging *gld* mice are readily distinguished from all the non-PC B cell lineage lymphomas currently defined histologically and molecularly (33) and, in the majority of cases, are less mature than pristane-induced PCT and primary SJL- $\beta 2M^{-/-}$ PC neoplasms.

Primary and scid-passaged PLs Exhibit Similar Patterns of Gene Expression. To ensure that the remarkably similar gene expression profiles observed in the *scid*-passaged PLs were not a reflection of tumor selection in the *scid* environment, we compared gene expression in *scid*-passaged BALB-*gld* PLs, isolated primary BALB-*gld* PL cells, and BALB/c splenic B cells by quantitative PCR. The genes selected for this comparison were chosen from those that distinguish the *scid*-passaged PLs from other B cell lineage tumors and were either significantly increased (Fig. 4 A) or decreased (Fig. 4 B) in expression relative to purified BALB/c splenic B cells. Notably, these genes included candidates of potential importance in tumorigenesis (*Apobec-1* [34, 35], *Tssc4* [40], *Btg2* [41], *Bin1* [42], *Prkcb* [43], and *Mad111* [44]), lymphoma survival (*BAFF* [45–47] and *IL-10* [48, 49]), and autoimmunity (*IL-10* [50, 51] and *Ifi202/203* [52]). *Il6st*, which encodes the gp130 component of the IL-6 receptor (53), also was significantly decreased in expression in primary and passaged tumors (Fig. 4 B). For some genes, such as *Apobec1*, there were quantitative differences between levels expressed by primary and passaged tumors, but in general, the distinguishing patterns of gene expression for PLs were in evidence before transplantation (Fig. 4, A and B).

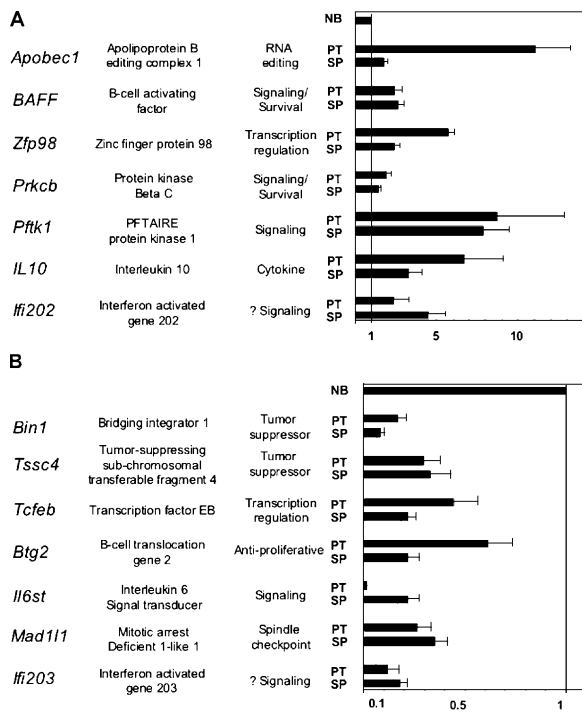


Figure 4. Primary and *scid*-passaged *gld* PLs have similar gene expression profiles. Quantitative PCR of selected genes expressed at significantly higher levels (A) or significantly lower levels (B) in primary and *scid*-passaged PLs relative to normal BALB/c B cells. Values represent mean \pm SEM for four primary BALB-*gld* PLs and six *scid*-passaged BALB-*gld* PLs normalized to a value of 1 for BALB/c splenic B cells. NB, normal B cells; PT, primary tumor; SP, *scid*-passaged tumor.

Discussion

The antibody specificities, characteristics of Ig genes, and gene expression patterns presented here define the B cell lineage lymphomas of *Fasl*-deficient mice as a novel subset of PC neoplasms with an unusually restricted pattern of V_H , V_L , D_H , J_H , and J_L gene use and BCR specificities skewed toward nuclear antigen and RF. Among 17 monoclonal Ig-secreting PLs studied, 15 were autoreactive, with nuclear antigen being the predominant specificity. 4 tumors were reactive with both nuclear antigen and RF, and 6 of 12 BALB-*gld* PLs exhibited D–D fusions in V_H CDR3. In addition, there was preferential use of J_H4 and J_K4/J_K5 in the tumor BCR. Gene expression profiling revealed that PLs were readily distinguished from pre-GC or post-GC lymphomas of NFS.V⁺ mice that have not reached the PC state of differentiation and that they have many features in common with normal human and mouse B cells on their way to becoming mature PCs (36, 37). A number of genes implicated in tumorigenesis, B lymphoma growth, and autoimmunity were differentially expressed in both primary and transplanted PLs.

The biased pattern of V_H and V_L gene use observed in the PL is reminiscent of the distinctive repertoires of autoreactive B cells specific for nuclear antigen. Like the PL, anti-dsDNA antibodies and ANA from MRL-*lpr*, MRL- $+/+$, (NZB \times NZW) F_1 , and (SWR \times NZW) F_1 mice also

disproportionately used the J558 and 7183 families and the J558.18, J558.33, J558.40, J558.47, and J558.52 subfamilies (3, 5). These V_H genes also tended to pair preferentially with a restricted set of V_L genes including members of the V_κ8, V_κ2, V_κ4/5, V_κ9/10, V_κ21, and V_κ23 groups (3, 5). These similarities and the autospecificities of the PL strongly imply that they derive from the peripheral autoreactive B cell pool characteristic of *lpr/gld* mice.

D–D fusions and preferential use of downstream J_H and J_L genes are common features of anti-DNA and antinucleosome antibodies in humans and mice with systemic lupus erythematosus (3, 54–59). The preferential use of upstream D genes and J_H4 is suggestive of secondary D–J_H rearrangements (54, 55, 59), whereas preferential use of downstream J_L genes is indicative of secondary rearrangement of IgL loci or receptor editing (56–58). The high proportion of PLs using J_H4 (55%) and the increased incidence of rearrangement of V_L to J_κ4 or J_κ5 strongly imply that the majority of PLs derive from autoreactive B cells that have undergone repeated D–J_H and/or V–J_L rearrangements. Secondary V_H rearrangements using D–J_H4 also may occur in PLs by a mechanism proposed previously by Klonowski and Monestier (55) and Monestier and Zouali (59). Although chimeric V_H chains indicative of secondary rearrangement were not observed in the PL, it is possible that they preferentially used the embedded heptamer at the 3' end of FWR3 that results in virtually seamless rearrangements (60–62).

Why predominantly Ig isotype-switched, autoreactive B cells with the hallmarks of receptor revision appear to be selectively targeted for transformation in *gld* mice is intriguing. One explanation is that transformation occurs randomly in a B cell population that is remarkably skewed toward receptor-edited autoreactive B cells. Although hybridomas derived from *lpr* splenic B cells are enriched for autoreactive specificities (3–5), the actual representation of autoreactive B cells within the unmanipulated B cell pool in tumor-free *gld* mice is unknown. Another possible reason for selected transformation of autoreactive B cells is that repeated activation of *Aid* during somatic hypermutation or class switch recombination increases the risk of transforming genetic mutations (63, 64). BCR specificity also might be a factor in the transformation process. Autoreactive B cells in *lpr* or *gld* mice in all likelihood are chronically activated and have an extended lifespan. These two factors are conducive to the accumulation of transforming genetic defects and the clonal expansion of mutated cells. Indeed, there is compelling evidence that chronic stimulation of B cells by infectious agents such as hepatitis C virus or *Helicobacter pylori* can culminate in lymphoma development (65–67). Notably, these lymphomas often produce RF and other auto antibodies (11, 14, 17–19, 68). This might be explained by molecular mimicry or, alternatively, RF-producing tumors may develop from RF B cells driven to clonally expand by immune complexes (14, 17, 18, 67). With the caveat that the assignments of GL genes may not be absolutely correct, the Ig V_H and V_L regions of most PLs exhibited multiple nucleotide substitutions in FWRs and CDRs. This observation, together with Ig isotype switching in most tumors and a gene expression

profile consistent with early PC differentiation, strongly implies that PLs derive from antigen-experienced B cells that have encountered a GC-like environment. Somatic mutation may occur in follicular GCs or extrafollicularly at the T zone red pulp border as reported for RF B cells (69). ANA-producing B cells and immune complex-binding RF B cells might be particularly prone to chronic stimulation because they can present multiple nucleosomal epitopes to T helper cells and thereby generate polyclonal T cell help (70).

The relative importance of antigen drive in lymphomagenesis and lymphoma survival can be implied from analysis of R and S mutations in the predicted amino acid sequences of IgH and IgL FWRs and CDRs (22). Using this type of analysis on IgL genes, 33% of PLs showed evidence of selection for FWR sequence conservation or diversification of CDR sequence, suggesting selection by antigen. The proportion of tumors with the hallmarks of antigen selection increased to 61% if the R/S mutation analysis of Ig V_H genes also was included (not depicted). The lack of evidence for intraclonal variation among individual PLs may indicate a lack of pressure to enhance binding affinity, antigen independence after transformation, or a tumor microenvironment not conducive to further mutation. Although chronic stimulation by auto antigen might be an etiologic factor in lymphomagenesis in *gld* mice, the importance of continued antigen stimulation for proliferation and survival of PLs is unclear. The reduced Ig expression on PLs may preclude antigen-driven proliferation but still facilitate the delivery of survival signals.

Earlier histologic and phenotypic analyses of PL tumors propagated in immunodeficient mice indicated that the majority of the tumors resembled activated B cells in phenotype and had a plasmacytoid morphology, although a proportion of cells differentiated into typical PCs (2). This phenotype suggested that most of the tumor cells were arrested at an early stage in PC differentiation. Gene expression profiling and quantitative PCR established that the PLs indeed are less differentiated than PCs or PCTs and readily distinguishable from less mature B cell lineage tumors. The similarities in gene expression patterns between the *scid*-passaged PLs and primary PLs provides assurance that the transplanted PLs are not undergoing appreciable changes in gene expression as a result of selection or a change from the *gld* to the *scid* microenvironment. Given the rarity of murine immature PC tumors, the PLs will be valuable tools for studying gene expression during PC differentiation and possibly for developing diagnostic transcriptional profiles.

Microarray analyses also led to the identification of genes differentially expressed in PLs, including some that might contribute to the transformation process. Genes that were significantly up-regulated relative to BALB/c follicular B cells included *Apobec1*, *Prkcb*, *BAFF* (*Tnfrsf13b*), *Ifi202*, and *IL-10*. *Apobec1* is of particular interest because it was selectively overexpressed in PLs and levels were particularly high in primary tumors. APOBEC1, a cytidine deaminase that constitutes the catalytic component of an RNA editing complex, has homology to AID, an activation-induced cy-

tidine deaminase required for somatic hypermutation, and class switch recombination in B cells (34, 35). Constitutive expression of *Aid* in transgenic mice leads to hypermutation of TCR and *Myc* genes and development of T cell lymphomas (71). Although the function of APOBEC1 in B cells is unknown, it may have oncogenic potential, as overexpression in liver leads to hepatocellular carcinomas (72). High expression of *Prkcb*, although not unique to PLs, might be significant in view of the role of protein kinase C β in survival signaling via NF- κ B and the finding that inhibitors of protein kinase C β induce cell death in lymphomas with dysregulated expression of the gene (73). Both BAFF and IL-10 can be important autocrine growth and survival factors for autoreactive B cells and B cell lineage lymphomas (45–51). IL-10 also may protect lymphomas from immune attack through its immunomodulatory effects on T cells and macrophages (50). Overexpression of *Iff202* in PLs relative to normal BALB/c B cells warrants further exploration considering the association of this gene with susceptibility to systemic lupus erythematosus and the survival of autoreactive B cells in NZB mice (52). *Iff202* expression might be regulated by IFN or IL-6 (74); however, the consistently low level of *Il6st* transcripts in PLs suggests that *Iff202* regulation in PLs might be IL-6 independent. In agreement with the down-regulation of *Il6st*, IL-6 treatment of PL cell lines failed to induce phosphorylation of relevant JAKs or STATs (unpublished data). The block in IL-6 signaling might be a factor in the arrest of PLs at an early stage in PC development.

Genes of interest that were selectively down-regulated in PLs and may have a role in tumorigenesis included *Bin1*, *Mad111*, and *Btg2*. Although defective expression of these genes has been described in a variety of nonlymphoid cancers, this is the first report of their down-regulation in B lymphomas (41, 42, 44). *Bin1*, which was greatly reduced in expression in both primary and transplanted PLs, encodes a Myc-interacting protein with tumor suppressor properties (42, 75). BIN1 frequently is greatly reduced or absent in malignant cells, and this deficit correlates with resistance to cell death (42, 75). Defective stimulation of Myc-induced apoptosis might be an important factor in progression to malignancy in PLs.

In summary, we have demonstrated that the PLs that develop spontaneously in *gld* mice derive predominantly from antigen-driven autoreactive B cells, are arrested at an early stage of PC differentiation, and exhibit a transcriptional profile that distinguishes them from other murine B cell neoplasms and also might be relevant to pathogenesis. This model will be invaluable for further investigation of the relationship between autoimmunity and B lymphomagenesis and for elucidating changes in gene expression relevant to early events in PC differentiation.

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