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-

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, cardiopain; 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/J2M/H11002/H11408/H11002 lymphoma; ssDNA, single-stranded DNA.
bodies include specificities for double-stranded DNA (dsDNA) and histones and, in large part, are encoded by a restricted set of IgH and IgL genes with members of the V\textsubscript{H} J588 and 7183 families as a prominent feature (3). V\textsubscript{H} gene use by antibodies specific for DNA–histone complexes, although also restricted, is more diverse than V\textsubscript{H} use and involves members of at least 10 V\textsubscript{H} 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 \kappa^{null}\CD23\ CD21\ Mac-1\ 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\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\textsubscript{H} and V\textsubscript{L} genes of many B cell tumor 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 monocausal-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 Ig\textsubscript{H} and Ig\textsubscript{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 \beta-2 microglobulin gene [SJL/J-B2M\textsuperscript{−/−}/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 LN were harvested from tumor-bearing mice. Single cell suspensions were made for FACS analysis and isolation of DNA and RNA. Southern hybridization analysis for Ig\textsubscript{H} and Ig\textsubscript{L} 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\textsuperscript{+} congenic mice (21), but included plasmacytoma (PCT) cell lines provided by M. Potter (National Cancer Institute, Bethesda, MD) and primary lymphomas of SJL/J-B2M\textsuperscript{−/−} mice.

Cultures. Sorted CD19\ B cells were cultured at 10\textsuperscript{7}/ml in supplemented RPMI medium for 48 h in the presence or absence of 25 \mu g/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\textsubscript{H} and V\textsubscript{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...
Table I. Isotypes and Nuclear Antigen Reactivity of Antibodies Secreted by gld Tumors

| Tumor    | Isotype          | ssDNA | dsDNA | ANA   | Nuclear staining | RF | CL | PPC |
|----------|------------------|-------|-------|-------|------------------|----|----|-----|
| BALB-gld |                  |       |       |       |                  |    |    |     |
| 208      | IgG1, Igκ        | -     | -     | -     | -                | +  | -  | -   |
| 329      | IgM, Igκ         | -     | -     | +     | +                | -  | -  | -   |
| 421      | IgM, Igκ         | +     | -     | -     | -                | -  | -  | -   |
| 424      | Multiple Igκ, Igκ| -     | +     | +     | +                | -  | +  | +   |
| 425      | IgG2a, Igκ       | (+)   | -     | -     | (+)              | +  | -  | -   |
| 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-gld  |                  |       |       |       |                  |    |    |     |
| 205      | IgG2a, Igκ       | -     | -     | -     | -                | (+)| -  | -   |
| 217      | IgG3, Igκ        | (+)   | -     | -     | -                | -  | -  | -   |
| 221      | IgA, Igκ         | -     | +     | +     | +                | -  | -  | -   |
| 223      | IgG3, Igκ        | -     | -     | -     | -                | -  | -  | -   |
| 355      | IgG2a, Igκ       | -     | -     | -     | -                | -  | -  | -   |

Notes:
- "-" indicates no detectable reactivity.
- "+" indicates strongly reactive.
- "Multiple" refers to more than two Ig isotypes secreted.
- "(+)", 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⁺IgG2a⁺IgG3⁺CD21⁺CD23⁺ CD21⁺CD81⁺CD5⁺ 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 VH J558 Family and Individual Subfamily Members in gld Tumors. To determine the Ig gene repertoire of the tumors, we sequenced the VH and VL genes us-

| Tumor         | Heavy chain variable region | Light chain variable region |
|---------------|-----------------------------|-----------------------------|
|               | DNA⁺ | AA⁺ | GL⁺ | VH⁺ | D⁺ | J⁺ | DNA⁺ | AA⁺ | GL⁺ | V⁺ | J⁺ |
| BALB-gld      |      |     |     |     |     |     | 208 99.3 | 97.8 | V130 | J558 | 21-17 | 99.6 | 98.9 |
|               | 329 96.4 | 96.7 | J558 | 95.8 | 22-4/6, 2.7/9 | J4 99.6 | 98.9 |
|               | 421 96.3 | 94.6 | J558 | 95.8 | 21-17 | J4 97.8 | 95.7 |
|               | 424 97.3 | 94.9 | J558 | 95.8 | 22-4/6 | J4 99.2 | 97.6 |
|               | 425 95.7 | 89.1 | J558 | 95.8 | 22-4/6 | J4 98.9 | 95.7 |
|               | 426 96.8 | 91.5 | V-BK | 72-9 | J4 98.9 | 12-44 | J12/13 |
|               | 531 98.2 | 95.7 | J558 | 95.8 | 22-4/6 | J4 98.9 | 95.7 |
|               | 540 91.3 | 85.9 | J558 | 95.8 | 22-4/6 | J4 98.9 | 95.7 |
|               | 593 98.3 | 96.9 | J558 | 95.8 | 22-4/6 | J4 98.9 | 95.7 |
|               | 600 97.8 | 96.7 | J558 | 95.8 | 22-4/6 | J4 98.9 | 95.7 |
|               | 2685 99.6 | 98.9 | J558 | 95.8 | 22-4/6 | J4 98.9 | 95.7 |
|               | 3294 98.9 | 96.7 | V-Ox-1 | 95.8 | 22-4/6 | J4 98.9 | 95.7 |
| C3H-gld       |      |     |     |     |     |     | 205 97.1 | 95.4 | J13/14 |
|               | 217 96.8 | 97.1 | J558 | 95.8 | 22-4/6 | J4 98.9 | 95.7 |
|               | 221 96.8 | 95.8 | J558 | 95.8 | 22-4/6 | J4 98.9 | 95.7 |
|               | 225 94.4 | 91.7 | J558 | 95.8 | 22-4/6 | J4 98.9 | 95.7 |
|               | 355 99.3 | 97.8 | J558 | 95.8 | 22-4/6 | J4 98.9 | 95.7 |

⁺Percent homology between tumor DNA and amino acid (AA) sequences and GL.
⁺⁺Closest matched GL genes.
⁺⁺⁺VH family.
⁺⁺⁺⁺D family. DH family abbreviations: P, DSP; D, DFL; T, DST.
⁺⁺⁺⁺⁺V family.
ing multiple plasmid isolates from each of the 18 tumors in the panel. As shown in Table II, 13 of the tumor \( V_\text{H} \) gene sequences (72.2%) aligned with a member of the J558 (\( V_\text{H}1 \)) family, 3 (16.7%) belonged to the 7183 (\( V_\text{H}5 \)) family, and 1 each to the Q52 and VGAM3.8 (\( V_\text{H}9 \)) families. The \( V_\text{H} \) 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 \( V_\text{H} \) 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 \( V_\text{H} \) 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 (\( \chi^2, P < 0.05 \)) 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 sequences from 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.

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Selective Transformation of Autoreactive gld B Cells

was observed in 4 of 18 tumors and FWR conservation was observed in 4 of 18 tumors. Tumors 142 and 425 exhibited both V₅ L CDR hypermutation and V₅ L FWR preservation. Notably, six V₅ 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 V₅ 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 V₅ L CDR1 (not depicted).

**Table III. Mutational Analysis of Tumor V₅ L Sequences**

| Tumor | Rᵃ | Sᵇ | Pᶜ | Rᵃ | Sᵇ | Pᶜ |
|-------|----|----|----|----|----|----|
| BALB- gld | | | | | | |
| 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[A] | 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 |

| C3H- gld | | | | | | |
| 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.
[A]NA, not applicable.

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- B2M⁻/⁻ 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.
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-β2M<sup>−/−</sup> 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-β2M<sup>−/−</sup> 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 Isbp—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 Mcrs1. 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 differentiation.

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.

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 ± 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, ICOSBP1, and FCGR2B (Fig. 3 A). A smaller group of genes, including Irf4, Sdc1, and Xsc5, 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 maturation 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 expression levels of eight genes that are differentially expressed between levels expressed by 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).

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 and VL 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 VH CDR3. In addition, there was preferential use of J H4 and J H4/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-+/+, (NZBxNZW)F 1, and (SWRxNZW)F 1 mice also

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 ± 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.
disproportionately used the J558 and 7183 families and the J558, 18, J558, 33, J558, 40, J558, 47, and J558, 52 subfamilies (3, 5). These VH genes also tended to pair preferentially with a restricted set of Vh genes including members of the Vh23, Vh2, Vh4/5, Vh9/10, Vh21, and Vh23 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 JH and Jl 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 JlA is suggestive of secondary D-JH rearrangements (54, 55, 59), whereas preferential use of downstream Jl genes is indicative of secondary rearrangement of IgL loci or receptor editing (56–58). The high proportion of PLs using JlA (55%) and the increased incidence of rearrangement of Vl to J4 or J5 strongly imply that the majority of PLs derive from autoreactive B cells that have undergone repeated D-JH and/or V-Jl rearrangements. Secondary VH rearrangements using D-JlA also may occur in PLs by a mechanism proposed previously by Klonowski and Monester (55) and Monester and Zouali (59). Although chimeric VH 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 VH1 and VH1 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 VH 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 (Tnfsf 13b), 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-
ttidine deaminase required for somatic hypermutation, and
class switch recombination in B cells (34, 35). Constitutive
expression of Aid in transgenic mice leads to hypermuta-
tion 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 over-
expression in liver leads to hepatocellular carcinomas (72).
High expression of Pkcb, although not unique to PLs,
might be significant in view of the role of protein kinase C
in survival signaling via NF-κB and the finding that inhi-
bitors of protein kinase C β induce cell death in lympho-
mas 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 ef-
fects on T cells and macrophages (50). Overexpression of
Ifi202 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). Ifi202
expression might be regulated by IFN or IL-6 (74); how-
ever, the consistently low level of Ifi202 transcripts in PLs
suggests that Ifi202 regulation in PLs might be IL-6 inde-
pendent. In agreement with the down-regulation of Il6st,
IL-6 treatment of PL cell lines failed to induce phosphory-
lation 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,
Mad1l1, and Btg2. Although defective expression of these
genes has been described in a variety of nonlymphoid can-
cers, 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, en-
codes 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 re-
sistance 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 re-
lationship between autoimmunity and B lymphomagenesis
and for elucidating changes in gene expression relevant to
early events in PC differentiation.

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