

Inactivation of the PRDM1/BLIMP1 gene in diffuse large B cell lymphoma

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PR domain containing 1 with zinc finger domain (PRDM1)/B lymphocyte-induced maturation protein 1 (BLIMP1) is a transcriptional repressor expressed in a subset of germinal center (GC) B cells and in all plasma cells, and required for terminal B cell differentiation. The BLIMP1 locus lies on chromosome 6q21–q22.1, a region frequently deleted in B cell lymphomas, suggesting that it may harbor a tumor suppressor gene. We report here that the BLIMP1 gene is inactivated by structural alterations in 24% (8 out of 34) activated B cell–like diffuse large cell lymphoma (ABC-DLBCL), but not in GC B cell–like ($n = 0/37$) or unclassified ($n = 0/21$) DLBCL. BLIMP1 alterations included gene truncations, nonsense mutations, frameshift deletions, and splice site mutations that generate aberrant transcripts encoding truncated BLIMP1 proteins. In all cases studied, both BLIMP1 alleles were inactivated by deletions or mutations. Furthermore, most non-GC type DLBCL cases ($n = 20/26$, 77%) lack BLIMP1 protein expression, despite the presence of BLIMP1 mRNA. These results indicate that a sizable fraction of ABC-DLBCL carry an inactive BLIMP1 gene, and suggest that the same gene is inactivated by epigenetic mechanisms in an additional large number of cases. These findings point to a role for BLIMP1 as a tumor suppressor gene, whose inactivation may contribute to lymphomagenesis by blocking post-GC differentiation of B cells toward plasma cells.

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Diffuse large B cell lymphoma (DLBCL) represents the most frequent type of B cell non-Hodgkin lymphoma (B-NHL) in the adult, accounting for ~40% of all diagnoses (1). Based on gene expression profile analysis, distinct subtypes of DLBCL have been identified, which reflect the origin from different stages of normal B cell differentiation (2, 3). These include the germinal center B cell–like (GCB) DLBCL, presumably derived from a GC centroblast, and the activated B cell–like (ABC) DLBCL, whose cell of origin is less clear but which resembles the expression pattern of a subset of GC cells undergoing plasmacytic differentiation or of mitogen-activated peripheral B cells (2, 3). A third group of DLBCL

is represented by primary mediastinal large B cell lymphomas, postulated to arise from thymic B cells (4, 5), whereas 15–30% of the cases remain unclassified (6). An additional classification, also based on gene expression profiling, identified three discrete subsets defined by the expression of genes involved in oxidative phosphorylation (OXP), BCR/proliferation (BCR), or tumor microenvironment/host inflammatory response (HR) (7).

Consistent with this heterogeneity, the genetic lesions associated with DLBCL are also diverse and include balanced reciprocal translocations deregulating the expression of BCL6, BCL2 and cMYC, gene amplifications, non-random chromosomal deletions, and aberrant somatic hypermutation (8–12). Nonetheless, a significant fraction of DLBCLs remains orphan

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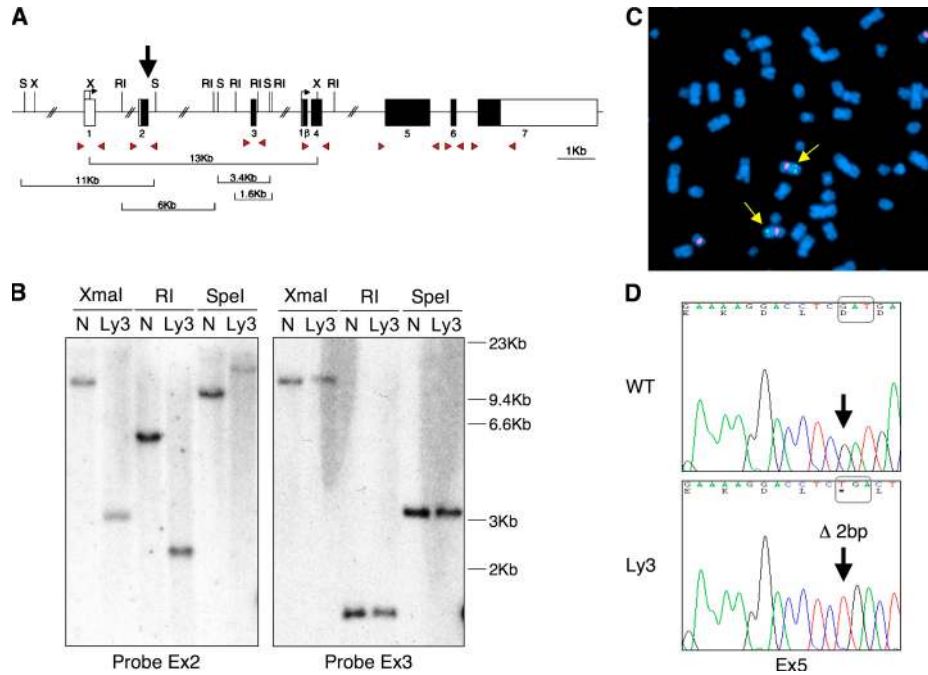


Figure 1. Biallelic inactivation of BLIMP1 by rearrangement and deletion in the Ly3 cell line. (A) BLIMP1 genomic locus: filled and empty boxes represent coding and noncoding exons, respectively. Arrows correspond to alternative transcription initiation sites, and triangles below the map depict the primers used for mutational analysis. Restriction sites for Southern blot analysis are also indicated (X, XmaI; RI, EcoRI; S, SpeI) and aligned to the predicted restriction fragments;

of any specific genetic changes and, in particular, no tumor suppressor genes have been identified, whose inactivation contributes to the pathogenesis of primary DLBCL.

One common alteration found in all B-NHL, including DLBCL, is represented by deletions affecting various portions of the long arm of chromosome 6 (10). Of these, 6q21 deletions are most frequently associated with high-grade lymphomas, such as DLBCL, where they may play a major pathogenetic role because they sometimes appear as the sole karyotypic abnormality present at diagnosis and correlate with poor prognosis (13, 14). Based on these observations, it has been proposed that this region may harbor a tumor suppressor locus. Among the genes mapped to band 6q21, PR domain containing 1 with zinc finger domain (PRDM1)/B lymphocyte-induced maturation protein 1 (BLIMP1) represents a good candidate because it encodes for a transcriptional repressor (15) that, in the B cell lineage, is expressed specifically in plasma cells and in a subset of GC centrocytes with plasmacytoid markers (16, 17). BLIMP1 is required for terminal differentiation of GC B cells into plasma cells, which it promotes by blocking the expression of genes implicated in B cell receptor signal and cell proliferation (18, 19).

In the present study, we investigated whether the structure and/or function of the BLIMP1 gene was altered in a panel of DLBCLs representative of the various phenotypic

subtypes. We report the frequent inactivation of BLIMP1 specifically in ABC-DLBCL, suggesting an important role for this gene in the pathogenesis of this lymphoma subtype.

RESULTS AND DISCUSSION

To test whether genetic alterations affecting BLIMP1 are involved in DLBCL pathogenesis, we performed mutational analysis of the BLIMP1 gene in 134 DLBCL cases, including 20 cell lines and 114 primary biopsies. 92 samples had been previously characterized by gene expression profiling and comprised 34 ABC, 37 GCB, and 21 unclassified DLBCL. Southern blot analysis was also performed in a subset of 30 cases (12 ABC, 10 GCB, and 8 unclassified) to examine the presence of gross gene rearrangements across an ~20-Kb region, spanning the promoter and exons 1–4 (Fig. 1 A).

Rearrangement of the BLIMP1 gene in the Ly3 cell line

BLIMP1 gene rearrangements were found in one of 30 DLBCL DNAs tested, represented by the ABC-DLBCL cell line Ly3. Fig. 1 B shows a Southern blot analysis of genomic DNA from Ly3 and a control sample; aberrant restriction fragments were detected in all Ly3 digests hybridized with an exon 2 probe, as well as in the XmaI digest hybridized with an exon 3 probe, consistent with a rearrangement within the EcoRI–SpeI region spanning exon 2 (Fig. 1 A). Moreover,

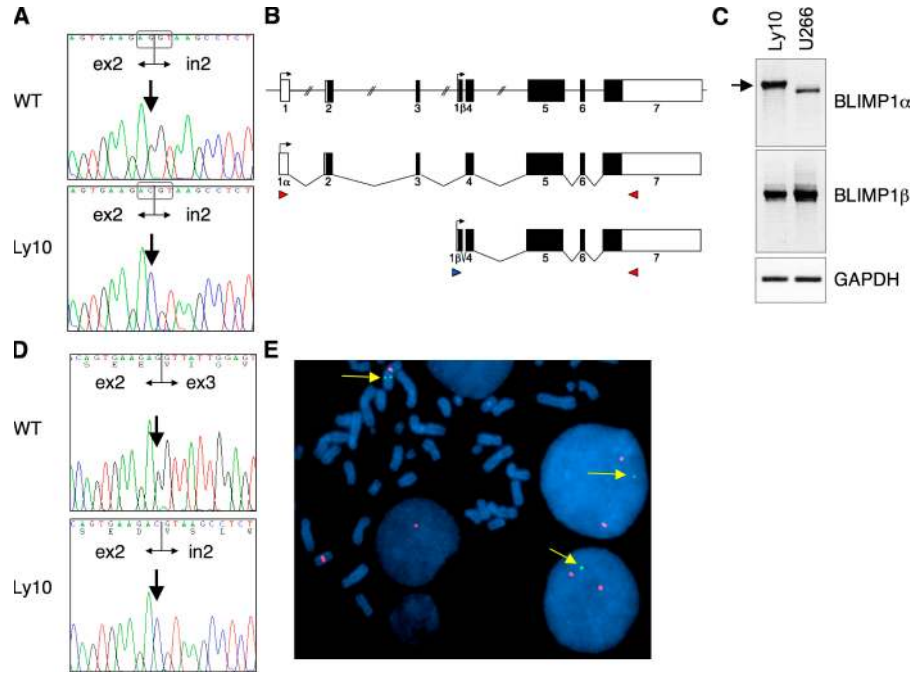


Figure 2. Splice donor site mutation and loss of the second allele in Ly10. (A) Sequencing analysis of genomic DNA from Ly10 and a control sample. The arrow indicates a single bp substitution affecting the splice donor site. (B) Schematic diagram of the BLIMP1 gene and its two transcripts; primers used for RT-PCR amplification (triangles) are positioned below the maps. (C) Aberrant BLIMP1 α transcripts in Ly10 (arrow),

as compared with the U266 multiple myeloma cell line. GAPDH controls for loading. (D) Sequencing of the RT-PCR-generated product reveals aberrant splicing as a result of retention of intronic sequences. (E) Dual-color FISH analysis was performed as described in Fig. 1 C. Arrows point to the BLIMP1 signal.

the WT allele could not be detected in Ly3, indicating its deletion. Dual-color fluorescence in situ hybridization (FISH) analysis confirmed the loss of one copy of the BLIMP1 locus in this tetraploid cell line (Fig. 1 C), whereas the remaining BLIMP1 signal maps to chromosome 6, suggesting that the aberrant fragment observed by Southern analysis is the result of an intrachromosomal rearrangement rather than of a translocation. As a consequence, the BLIMP1 α transcript cannot be produced, a result confirmed by RT-PCR and Northern blot analysis (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20052204/DC1>). In addition, a 2-bp deletion was found within the exon 5 sequences, leading to a premature stop codon and, therefore, abrogating the coding potential of the remaining BLIMP1 β transcript (Fig. 1 D). Consistently, no protein expression was detected by Western blot analysis (unpublished data).

Consensus splice site mutations

In three cases (Ly10, no. 289, and no. 359), a recurrent nucleotide change was found within the consensus splice donor site at the exon 2–intron 2 junction (see Fig. 2 A for a representative sample). RT-PCR and sequencing analysis using primers specific for the two known BLIMP1 transcripts could be performed in two out of the three cases and demonstrated the presence of an aberrant BLIMP1 α mRNA retaining 100 nucleotides from intron 2, leading to a pre-

mature translation termination signal (Fig. 2, B–D). In both tumors, inactivation of the second allele, a result of deletion (Ly10) or mutation (no. 359), was documented by FISH analysis and sequencing of cloned PCR products, respectively (Fig. 2 E and Table S1, available at <http://www.jem.org/cgi/content/full/jem.20052204/DC1>).

One additional primary biopsy (no. 438) displayed a G→A mutation affecting the consensus splice acceptor site at the intron 2–exon 3 junction (Fig. 3 A) and predicting an abnormal splicing. This sample expressed an aberrant transcript as a result of in-frame loss of the gene exon 3 (Fig. 3, B and C) that encodes for most of the PR domain, a region shown to contribute to the BLIMP1 activity as a transcriptional repressor (20, 21). RT-PCR and sequencing analysis also showed that the second allele was lost, consistent with a monosomy 6 detected by FISH analysis (Table S1).

Frameshift deletions and nonsense mutations

In four additional primary biopsies, frameshift deletions leading to premature stop codons were observed in exon 4 (no. 359), exon 5 (no. 295 and 284), and exon 7 (no. 340), whereas a nonsense mutation was detected in the cDNA sequence corresponding to exon 5 of no. 251 (no genomic DNA was available for this case). As a consequence, severely truncated polypeptides of 119–215 amino acids that have lost functionally relevant domains (including the PR, proline

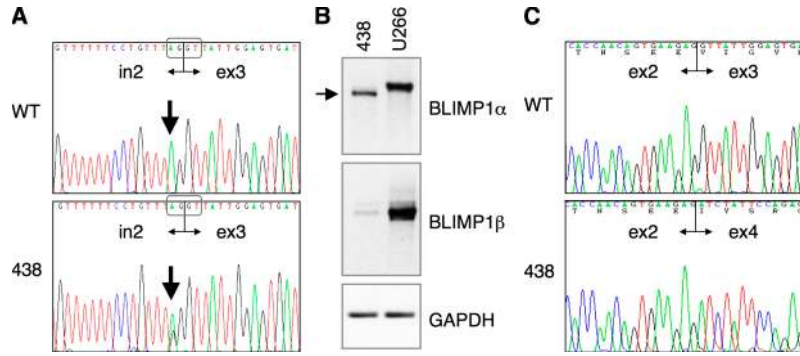


Figure 3. Mutation of the BLIMP1 intron 2 splice acceptor site and loss of WT allele in a primary DLBCL. (A) Chromatograms of genomic DNA from DLBCL no. 438 (and a control sample). The residual WT sequences are likely a result of normal cells contaminating the biopsy because this case carries a monosomy 6. (B) Gel electrophoresis of RT-PCR

products obtained from the indicated samples. A shorter BLIMP1 α transcript and absence of the WT allele are observed in case no. 438. (C) Sequencing analysis of the RT-PCR product from no. 438 reveals the in-frame loss of exon 3.

rich, and zinc finger DNA-binding domains) and are most likely inactive, are produced in no. 251, 284, 295, and 359 (Fig. 4). The remaining case (no. 340) is predicted to encode a truncated protein of 603 amino acids, lacking the three COOH-terminal zinc finger domains and the COOH-terminal acidic domain (Fig. 4). Although zinc fingers 1 and 2 have been shown to be sufficient for sequence specific DNA binding (22), artificial mutants carrying truncation of the COOH-terminal acidic domain display impaired repression activity and may therefore also interfere with the normal BLIMP1 function (21).

Notably, sequencing analysis of the BLIMP1 α transcript after amplification and cloning in no. 359, which carries a

second inactivating event in the exon 2 splice donor site (see previous paragraph section and Fig. 4 B), documented that the mutations were distributed in two separate alleles, thereby leading to biallelic inactivation of the gene. FISH analysis was performed on two of the remaining cases, for which material was available, and revealed deletion of one BLIMP1 allele, as a result of loss of the entire chromosome 6, in no. 284 (Table S1). Conversely, three copies of chromosome 6 were detected in no. 251; however, the mutant allele was overrepresented in the RT-PCR product amplified from this primary biopsy, suggesting that the WT allele is lost or silenced in the tumor population, and that the residual WT sequences derive from contaminating normal cells. In the former scenario, the

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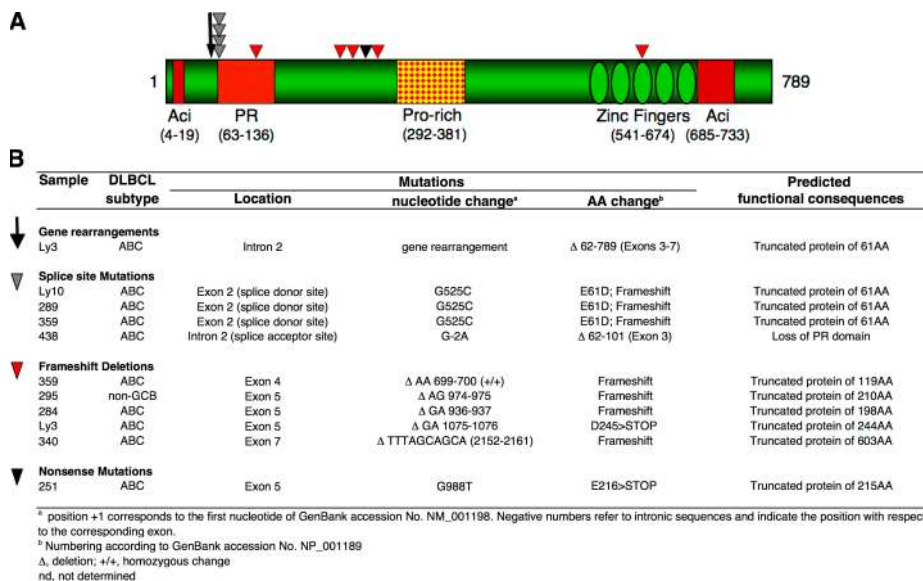


Figure 4. Distribution and features of BLIMP1 mutations in ABC-DLBCL. (A) Schematic representation of the human BLIMP1 protein with its functional domains. Color-coded symbols depict distinct

types of alterations leading to BLIMP1 inactivation as detailed in B. Aci, acidic domain; PR, positive regulatory domain; Pro-rich, proline rich domain.

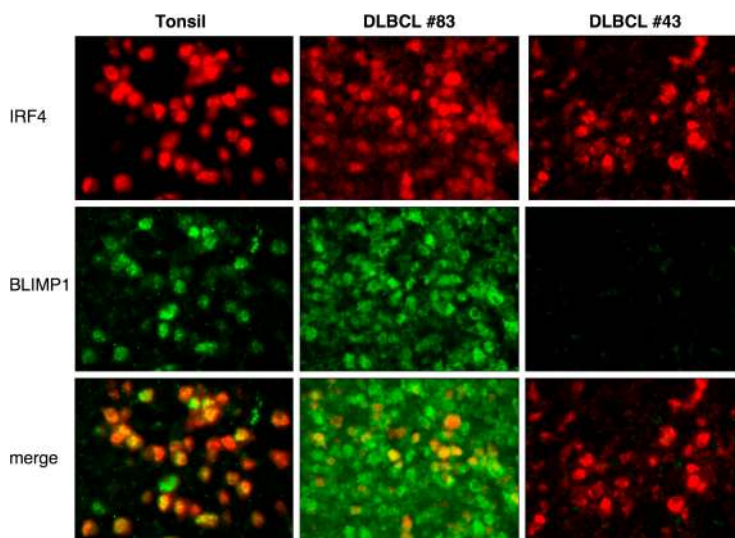


Figure 5. Lack of BLIMP1 protein expression in most non-GCB DLBCL. Double immunofluorescence staining for IRF4 (red) and BLIMP1

(green) in human tonsil (left) and two representative ABC-DLBCL cases (middle: IRF4⁺/BLIMP1⁺; right: IRF4⁺/BLIMP1⁻).

trisomy 6 may result from loss of the chromosome carrying the WT allele and triplication of the chromosome carrying the mutant allele.

Finally, five samples displayed single bp substitutions leading to amino acid changes in the gene exon 5 ($n = 3$) and exon 7 ($n = 2$) (unpublished data). Because no matched normal DNA was available from these samples, we cannot determine whether the changes represent rare germline polymorphisms or somatic mutations that may affect the BLIMP1 function.

In summary, BLIMP1 structural alterations clustered within the NH₂-terminal half of the protein in 8/9 mutated cases and predict severely truncated polypeptides that will not be functional or may act in a dominant-negative fashion. Moreover, biallelic inactivation of the gene as a clonally represented event was demonstrated in all cases studied, indicating that in these tumors BLIMP1 is inactivated by the classic “two-hit” mechanism previously described for several tumor suppressor genes (23). Accordingly, immunostaining with BLIMP1-specific antibodies documented the absence of protein expression in all mutated cases tested, despite the presence of BLIMP1 mRNA (Table S1 and not depicted).

BLIMP1 inactivation is restricted to the ABC type of DLBCL

To determine whether BLIMP1 alterations are associated with specific DLBCL subtypes, we examined their distribution in relation to both cell of origin (3, 6) and comprehensive consensus clustering-based classifications (7). No preferential distribution was observed with respect to the latter (alterations were found in 4 BCR, 3 HR, and 1 OXP case). However, all the mutated cases segregated with the ABC phenotype ($n = 8/34$, corresponding to 24%; the non-profiled case also displayed a non-GC phenotype, based on immunohistochemical analysis), whereas mutations were never observed in 37 GCB-type ($P < 0.01$, χ^2 test) and 21

“unclassified” DLBCLs ($P = 0.01$). Thus, BLIMP1 structural alterations represent a common and specific lesion in ABC-DLBCL.

Lack of BLIMP1 protein expression in ABC-DLBCL

Defective expression of a tumor suppressor protein can occur by gene deletion/mutation as well as epigenetic inactivation caused by promoter methylation, inefficient translation, or enhanced protein degradation (23). A recent study reported the lack of BLIMP1 protein in 95% of DLBCL, but did not distinguish between lymphoma subtypes (17). Thus, we performed immunohistochemical analysis of BLIMP1 expression in 58 primary biopsies, including 44 from the previous study, which were classified into GCB and non-GCB according to Hans et al. (24). BLIMP1 was completely absent in 29/32 (91%) GCB-DLBCL, as expected because they are thought to derive from GC B cells that do not normally express BLIMP1; however, 20/26 (77%) non-GCB DLBCLs, including 4/4 unmutated cases, were also negative, despite the fact that ABC-DLBCLs appear to derive from cells that normally express markers of plasmacytic differentiation, such as IRF4 and BLIMP1. Indeed, IRF4, the transcription factor consistently coexpressed with BLIMP1 in normal GC B cells (16, 17), was present in 16/20 (80%) BLIMP1⁻ cases (IRF4⁺BLIMP1⁺ cases: 6/26, 23%; IRF⁻BLIMP1⁻ cases: 4/26, 15%) (Fig. 5). The lack of BLIMP1 expression in most ABC-DLBCL was also surprising in view of the following facts: (a) BLIMP1 mRNA can be detected in a sizable fraction of ABC-DLBCL by gene expression profiling (~60% in the present cohort; also, see available databases from references 2 and 7) (Fig. S1 A); (b) the transcripts detected in tumor biopsies most likely derive from tumor cells rather than from contaminating normal T cells, which also express BLIMP1 (17) because they were present in DLBCL cases

purified by CD19 enrichment (Fig. S1 B); and (c) BLIMP1 mRNA is expressed in two out of two ABC-DLBCL lines carrying inactivating mutations (Fig. S1 C). These observations suggest that most ABC-DLBCLs express the BLIMP1 mRNA, but fail to express the BLIMP1 protein. Because the fraction of cases lacking BLIMP1 protein expression (77%) significantly exceeds that of cases with inactivating mutations (24%), it is likely that additional mechanisms, including defects in protein translation or stability, contribute to BLIMP1 inactivation in a higher fraction of ABC-DLBCL.

Conclusions

The results herein indicate that BLIMP1 is inactivated by a classic two-hit mechanism in ~25% of ABC-DLBCL and suggest that the BLIMP1 protein is abnormally missing in an additional sizable fraction of cases. These findings strongly suggest that BLIMP1 may act as a tumor suppressor gene, whose loss of function may contribute to the pathogenesis of ABC-DLBCL. Experiments aimed at directly demonstrating the tumor suppressor function of BLIMP1 by reintroduction of the BLIMP1 gene into BLIMP1-null DLBCL cell lines (Ly3, Ly10) were inconclusive because they showed a generalized apoptotic effect in both BLIMP1-null and control BLIMP1-positive B cell lines (unpublished data). These results are consistent with previous studies reporting the toxicity of exogenous BLIMP1 expression in multiple cell types (25, 26). Thus, ongoing studies monitoring lymphoma development in mice with conditional inactivation of BLIMP1 in GC B cells should provide definitive demonstration of the role of this gene as a tumor suppressor in the B lineage in vivo. Nonetheless, the substantial in vitro and in vivo evidence indicating that BLIMP1 is required for plasmacytic differentiation (18, 19) strongly suggests that BLIMP1 inactivation may contribute to lymphomagenesis by blocking post-GC B cell differentiation. Notably, translocations deregulating the BCL6 gene, a transcriptional repressor required for GC formation and down-regulated upon plasmacytoid differentiation, were never found in BLIMP1 mutated DLBCLs ($n = 0/7$), but were restricted to unmutated cases ($n = 8/62$, 13%). Because it has been proposed that BCL6 may block terminal B cell differentiation (27), these findings suggest that BCL6 deregulation and BLIMP1 inactivation may represent alternative pathogenetic mechanisms, both leading to a block in post-GC differentiation and, ultimately, to lymphomagenesis.

MATERIALS AND METHODS

Cell lines. The following DLBCL cell lines were used in the study: OCI-Ly1, OCI-Ly3, OCI-Ly4, OCI-Ly7, OCI-Ly8, OCI-Ly10, OCI-Ly18, RC-K8, VAL, SUDHL-4, SUDHL-5, SUDHL-6, SUDHL-7, SUDHL-8, SUDHL-10, HT, DB, TOLEDO, WSU, and FARAGE. Cells were maintained in IMDM supplemented with 10% FCS. The Ly10 line was cultured in IMDM with 20% heparinized human plasma and 55 μ M β -mercaptoethanol.

Tumor samples and classification. Primary biopsies from 114 newly diagnosed DLBCL patients were obtained from the archives of the Departments of Pathology at Columbia University and Memorial Sloan-Kettering

Cancer Center (MSKCC), and an additional recently described multi-institutional series (7). The study was approved by the Institutional Review Board Committee of Columbia University, the Dana Farber Cancer Institute, and MSKCC. 92 samples (including the 20 cell lines and 72 primary cases) had been previously characterized by gene expression profiling using the Affymetrix Gene Chip microarray system and were classified as GCB ($n = 37$), ABC ($n = 34$), and other (not otherwise specified; $n = 21$) as described previously (6, 7). Selection of the 92 cases was based on the availability of genomic DNA obtained from the same biopsy simultaneously with RNA extraction.

DNA extraction, amplification, and sequencing. Genomic DNA from 134 DLBCL samples was extracted according to standard methods. The oligonucleotides used for amplification of the BLIMP1 exons are reported in Table S2 (available at <http://www.jem.org/cgi/content/full/jem.20052204/DC1>). Purified amplicons were sequenced directly from both strands and compared with the corresponding germline sequences (NM_001198 for BLIMP1 α and NM_182907 for BLIMP1 β) using the Mutation Surveyor Version 2.41 software (Soft Genetics LLC). Where needed, additional internal primers were used and their sequences are available upon request. Mutations were confirmed on independent PCR products and changes due to germline polymorphisms, as verified by analysis of matched normal DNA, were excluded.

Southern blot analysis. High-molecular weight DNA (5 μ g) was digested with the appropriate restriction endonucleases, electrophoresed on a 0.8% agarose gel, and transferred overnight to Duralose filters (Stratagene) according to standard methods. Filters were sequentially hybridized with probes specific to the BLIMP1 exon 2 and exon 3.

RNA extraction, RT-PCR, and Northern blot analysis. Total RNA was isolated by TRIzol (Invitrogen) according to the manufacturer's instructions. For RT-PCR analysis, 1 μ g of total RNA was retrotranscribed using the Superscript Double Stranded cDNA Synthesis kit (Invitrogen) and 1/20 of the reaction served as template for amplification of the BLIMP1 α and BLIMP1 β cDNAs, using specific oligonucleotides (Table S2); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as internal control. Northern blot analysis was performed by standard methods, using the following radio-labeled probes: human BLIMP1 cDNA (exons 5–7), full-length IRF4 cDNA, BCL6 cDNA, and GAPDH.

FISH. Two PAC clones (RP1-101M23 and RP1-134E15) spanning the BLIMP1 gene were obtained from BACPAC Resources at <http://bacpac.chori.org>. DNA was labeled by nick-translation using spectrum green dUTP fluorochrome (Vysis). A Spectrum red-labeled centromeric probe (Vysis) was used to enumerate chromosome 6. Chromosome preparations were made from lymphoma cell lines using standard methods. 4–10- μ m-thick tissue sections were obtained from formalin-fixed paraffin or frozen archival tissues cut onto adhesive-coated slides. Paraffin-sections were baked overnight at 60°C and frozen sections were fixed in 3:1 methanol:acetic acid before hybridization. Both paraffin and frozen sections were subjected to protease treatment using paraffin pretreatment kit (Vysis). FISH was performed by standard methods and hybridization signals were scored on at least 20 metaphase spreads or 200 interphase nuclei on DAPI-stained slides. The sensitivity of the approach was determined by performing the same analysis on analogous sections from normal tonsils, which demonstrated loss of the 6q21 signal in 5–7% of the cells and monosomy 6 (i.e., loss of both 6q21 and a chromosome 6 centromeric probe) in 13–26% of the cells. Cases were diagnosed as deleted if the fraction of cells showing the deletion was >50%.

Tissue microarray, immunohistochemistry, and immunofluorescence staining. The construction of the DLBCL tissue microarray and the protocols for immunohistochemical and double-immunofluorescence staining are described in detail elsewhere (17). Cases were scored as positive if $\geq 30\%$ tumor cells were stained by the corresponding antibody and classified

into GCB and non-GCB DLBCL based on expression of CD10, BCL6, and IRF4 (24).

Online supplemental material. Fig. S1 shows expression of BLIMP1 mRNA in most ABC-DLBCL. Table S1 shows biallelic inactivation of BLIMP1 in mutated DLBCL cases. Table S2 reports the sequence of the primers used for amplification of BLIMP1 from genomic DNA and cDNA. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20052204/DC1>.

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