

**PRDM1/BLIMP-1 expression in multiple B and T-cell lymphoma**

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Background and Objectives. The positive regulatory domain I (PRDM1) protein or BLIMP-1, belonging to the PRDM gene family of transcriptional repressors, is a key regulator of terminal differentiation in B-lymphocytes and is critical for plasma cell differentiation.

Design and Methods. Here we document the expression of PRDM1 in normal and neoplastic lymphoid cells, through the use of a monoclonal antibody that recognizes the molecule in paraffin-embedded tissue sections. A large series of B and T-cell lymphomas (679 cases) was studied, using tissue microarrays.

Results. Multiple myeloma, plasmacytoma and lymphoplasmacytic lymphoma cases (n=19) were positive. Plasmablastic lymphoma, oral mucosa-type (n=15), were also found to be positive. PRDM1 protein was expressed in some cases of B-cell neoplasia, i.e. chronic lymphocytic leukemia/small lymphocytic lymphoma (15%), diffuse large B-cell lymphoma (43%), classical Hodgkin's lymphoma (41%) and also in T-cell lymphoma (23%).

Interpretation and Conclusions. Most B-neoplastic cells showing plasmablastic differentiation were PRDM1-positive. Unexpectedly, a subset of diffuse large B-cell lymphoma expressed PRDM1, lacked detectable plasmablastic or immunoblastic changes and displayed more aggressive behavior, with a shorter failure-free survival. In contrast to normal B-cells, diffuse large B-cell lymphoma cases with increased PRDM1 expression co-expressed BCL-6 and MUM1/IRF4, confirming that PRDM1 expression in these tumors is insufficient to drive the full genetic program associated with plasmacytic differentiation.

Key words: PRDM1, BLIMP-1, lymphoma, plasma cell, monoclonal antibody.

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The positive regulatory domain I (PRDM1) protein, also named PRD1-BF1 or BLIMP-1 (B lymphocyte-induced maturation protein 1) in mice, belongs to the PRDM gene family of transcriptional repressors. It is a key regulator of terminal B-cell differentiation^{1,2} and has the ability to drive activated or germinal center B cells to become antibody-secreting cells with a plasma cell phenotype^{1,3} and activate an apoptotic response in immature B cells and pre-B cells.² The study of normal tissues shows that *in vivo* expression of PRDM1/BLIMP-1 in the adult mouse is restricted to plasma cells, including (a) those formed in a primary response to either T-cell-dependent or independent antigen, (b) those formed from memory cells in a secondary response, and (c) long-lived plasma cells in the bone marrow.⁴ PRDM1/BLIMP-1 is not found in memory B-cells but is expressed in a subset (5-15%) of germinal center B-cells with a plasma cell phenotype.⁴ These findings suggest that this subset of germinal center B-cells might be committed to a plasma cell fate and that the level of PRDM1/BLIMP-1 expression may determine whether they differentiate to become immunoglobulin-secreting plasma cells or enter the memory B-cell pool.⁴ Five genes that are direct targets for PRDM1/BLIMP-1 (all of them associated with

B-cell phenotype and proliferation), have been characterized to date: *c-Myc*,⁵ *MHC2TA* promoter III, responsible for regulating *CIITA*,^{3,6} B-cell lineage-specific activator protein (BSAP)/ *PAX5*,⁷ *Spi-B*, and *Id3*.⁸ Repression of *c-Myc*, *CIITA* and *PAX5* alone is unlikely to explain the entire program of plasma cell development activated by PRDM1/BLIMP-1.⁸ Previous immunohistological studies of normal human lymphoid tissue have shown PRDM1 expression mainly in a small population of large B cells in the germinal center and plasma cells.⁹ PRDM1 was absent in activated extrafollicular B cells, in the majority of resting T cells, in tissue histiocytes, and in antigen-presenting dendritic cells. PRDM1 expression was also found in human B-cell lymphomas with plasma cell differentiation and in a minority of diffuse large-B cell lymphomas (DLBCL). Marginal zone lymphomas, chronic lymphocytic leukemias (B-CLL) and Hodgkin's lymphomas were reported to be largely negative.⁹ Interestingly PRDM1 expression has been found in a small population of reactive non-neoplastic T cells (CD3-positive) in B-cell lymphomas,⁹ however PRDM1 positivity in T-cell lymphomas has not been described to date. In the current study, using tissue-microarrays, we investigated the expression of PRDM1 protein in a large series of both

normal and neoplastic lymphoid tissues, using a new anti-PRDM1 monoclonal antibody that recognizes its target molecule in paraffin-embedded tissue sections. Our results reveal the value of this anti-PRDM1 monoclonal antibody for the identification and study of reactive plasma cells and plasma cell-derived neoplasms. Immunostaining for PRDM1 in paraffin-embedded tissue showed positivity not only in myeloma/plasmacytoma cases, but also in subgroups of DLBCL and in a small proportion of B-CLL/small lymphocytic lymphoma (B-CLL/SLL), classical Hodgkin's lymphoma (cHL) and T-cell lymphomas. Due to the high proportion of PRDM1-positive DLBCL, we investigated the correlation between PRDM1 expression and clinical outcome. Our observations highlight the potential value of detecting PRDM1 protein for the experimental analysis and classification of human lymphomas.

Design and Methods

Production of PRDM1 monoclonal antibody

A cDNA encoding the full-length human PRDM1 protein was kindly provided by Dr. Tom Maniatis (Harvard University, Cambridge, USA). The human *PRDM1* gene was amplified by polymerase chain reaction (PCR) and introduced into the pDEST15 expression vector (Invitrogen, Carlsbad, CA, USA) by means of Gateway® technology. The GST-PRDM1 fusion protein was then expressed in *Escherichia coli* strain BL21 (DE3), purified by affinity chromatography on a GSTrap™ column (GE Healthcare, Little Chalfont, UK) and used as an immunogen. PRDM1 monoclonal antibody (clone ROS, isotype IgG1/κ) was produced in BALB/c mice, maintained in the Animal Facility Unit of the CNIO, with methods described previously.^{10,11}

Tissue samples and preparation of tissue microarrays

All tissues were obtained from the tissue archives of the CNIO Tumor Bank. We used a Tissue Arrayer device (Beecher Instruments, Sun Prairie, WI, USA) to construct tissue microarray blocks, according to conventional protocols.^{10,12} All cases were histologically reviewed and representative areas were selected. Tissue microarrays were produced comprising samples of normal tissue (tonsil, lymph node with follicular hyperplasia, spleen, bone marrow, fetal liver, fetal thymus, thymus, brain, larynx, parotid gland, thyroid, gall bladder, liver, lung, skin, skeletal muscle, kidney, pancreas, stomach, duodenum, appendix, small and large intestine, bladder, ovary, uterus, breast, placenta, prostate and testis). Tissue microarrays were also prepared containing a total of 679 lymphomas, for all of which the diagnosis had been confirmed by central review of standard tissue sections. All biopsies had been taken prior to treatment. The histological criteria used for diagnoses of cases were those described in the World Health Organization classification.¹³ Some of these cases have been included in previous studies.^{10,14-18}

The B-cell tumors included were DLBCL (n=250), cHL (n=22), follicular lymphoma (n=115), B-CLL/SLL (n=54), mantle cell lymphoma cases (n=52), Burkitt's lymphoma (n=21), marginal zone lymphoma (splenic, extranodal and

nodal) (n=14), B-cell lymphoblastic lymphoma (3), lymphoplasmacytic lymphoma (n=3), plasmacytoma and plasma cell myeloma (n=16). The DLBCL tumors included 15 cases of the plasmablastic variant (i.e. tumors of oral mucosa type (n=19) that typically present in the oral cavity in HIV-positive and EBV-positive patients) and eight cases with distinct plasmablastic morphology (>90% of the cells were immunoblasts with plasmacytoid differentiation).

T/NK-cell neoplasms comprised peripheral T-cell lymphoma (PTCL), unspecified (n=41), anaplastic large cell lymphoma (ALCL) (n=30), mycosis fungoides/Sézary syndrome (n=8), T-cell lymphoblastic lymphoma/leukemia (n=16), enteropathy T-cell lymphoma (n=4), angioimmunoblastic T-cell lymphoma (n=18) and T/NK nasal type lymphoma (n=12).

Cell lines

Myeloma cell lines (RPMI-8226, L-363, SK-MM-2, KARPAS-640, NCI-H929, OPM-2 and LP-1) and the Burkitt's lymphoma cell line (RAJI) were obtained from the German Collection of Micro-organisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were grown at 37°C in 5% CO₂ in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal calf serum (Sigma Chemical St. Louis, MO, USA). KARPAS-620 and NCI-H929 medium were also supplemented with interleukin-6 (20 ng/mL, PeproTech, London, UK).

Antibodies

Polyclonal anti-PRDM1 was kindly provided by Dr. Mark M. Davis, Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA, USA.¹ Other antibodies used were: BCL-6 (clone GI191E/A8, dilution 1:120,¹⁰ MUM-1/IRF4 (polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA dilution 1:200), CD10 (clone 56C6, Novocastra, Newcastle, UK, 1:10), CD138 (clone MI15, Dako-Cytomation, Glostrup, Denmark, dilution 1:50), p63 (clone VS38c, provided by Prof. David Y. Mason, dilution 1:1), CD30 (clone CON6D/B5, provided by Giovanna Roncador, dilution 1:1), CD3 (polyclonal antibody, Dako, dilution 1:100) and ALK (clone ALK1, provided by Prof. David Y. Mason, dilution 1:1). Goat anti-species-, isotype, or subclass-specific secondary antibodies, conjugated to Alexa Fluor 488 (green) and 594 (red) were obtained from Molecular Probes (Leiden, The Netherlands).

Western blotting and immunoprecipitation

Western blotting, immunoprecipitation and preparation of cytocentrifuge slides were carried out as previously described.^{10,20,21}

Immunostaining techniques

Immunohistochemical staining for PRDM1 was performed as follows: 2-4-μm-thick paraffin-embedded tissue microarray sections were cut onto Dako slides (Dako), and subsequently dewaxed, re-hydrated, and then subjected to antigen retrieval by heating in 50 mM Tris (Trizma base)-2 mM EDTA (Sigma-Aldrich, St. Louis, MO, USA) (pH 9) in a microwave pressure cooker (A. Menarini Diagnostics, Workingham, UK) at 900W for 2 minutes. The slides were cooled and treated with per-

oxidase-blocking solution (Dako) for 5 minutes. Sections were then immunostained with PRDM1 monoclonal antibody (clone ROS) by the two-stage peroxidase-based EnVision technique (Dako)²² and then counterstained with hematoxylin and mounted. Incubations omitting the specific antibody or containing unrelated antibodies were used as controls. Immunostaining on cytocentrifuge preparations was carried out as previously described.¹⁰

Double immunoenzymatic labeling

Double immunoenzymatic labeling of paraffin sections was carried out using the EnVision peroxidase and alkaline phosphatase kits (Dako)²³ on normal tonsillar tissue. PRDM1 was immunostained in brown with the DAB substrate (Dako) and CD138 or CD3 in blue with the Vector blue alkaline phosphatase substrate kit (Vector Laboratories, Peterborough, UK).

Immunoperoxidase technique combined with immunofluorescence

Immunoperoxidase staining, combined with single or double immunofluorescence labeling, was performed on paraffin sections of normal tonsil, ALK-positive ALCL, PTCL and cHL cases, following a previously described method.^{23,24} Briefly, slides were immunostained for PRDM1 by the two-stage peroxidase-based EnVision technique, washed in phosphate-buffered saline (PBS) for up to 5 minutes and then incubated in normal human serum for up to 10 minutes before immunofluorescence labeling with antibodies p63/VS38c (red) and CD20 (green) in normal tonsil; ALK (red) and CD138 (green) in ALK-positive ALCL cases; CD3 (red) in PTCL or CD30 (green) in cHL cases. The immunofluorescence labeling was performed as previously described.^{24,25} Sections were incubated for 45 minutes at room temperature with a pair of antibodies that were either from different species or of differing immunoglobulin isotype/subclass. Sections were washed in PBS for up to 5 minutes and then incubated in the dark for 45 minutes with secondary antibodies (specific for species, isotypes, or subclass) and labeled with contrasting green and red fluorochromes (Alexa Fluor 488 and Alexa Fluor 594, dilution 1:100). The slides were washed in PBS for up to 5 minutes, and then mounted in fluorescent mounting medium (Dako) containing 1 µg/mL DAPI (4,6-diamidino-2-phenylindole). Slides were examined on a Nikon E800 Eclipse fluorescence microscope (Nikon, Kingston-upon-Thames, UK) equipped for epifluorescence. Fluorescence images were captured with an Axiocam charge-coupled device (CCD) camera (Zeiss, Jena, Germany) and Axiovision software (Imaging Associates, Bicester, UK), and adjusted using Photoshop software (Adobe, San Jose, CA, USA).²⁴ The immunoperoxidase image was viewed by transmitting light, and corresponding gray-scale images were inverted and pasted into the blue (or red in cHL) channel of the red-green-blue (RGB) Photoshop image. Using this technique, cells expressing PRDM1 were visualized as blue (excepted in cHL case visualized in red), whereas the immunofluorescence labeling marker was detected as green or red. In all immunostained paraffin sections, positive plasma cells were present, providing an internal positive control.

Scoring of PRDM1 expression

PRDM1 protein expression was assessed by immunohistochemistry on normal and neoplastic human tissue microarrays and on complete tissue sections when necessary. Each case was scored on a semi-quantitative basis, depending on the number of positive cells, as negative (no positive tumor cells or < 10%); weak (10% to ≤50% positive cells); and strong (>50% to 100% positive cells). The threshold selected for biological correlation and clinical analysis was 10%, on the basis that this gave good inter-observer reproducibility. Scoring was performed by four independent observers (Juan-FG, SM-M, MAP and TM).

Clinical series

Clinical data and follow-up were available for 140 DLBCL cases, including HIV-negative cases diagnosed between 1990 and 1999. Patients were treated with regimes including multi-agent chemotherapy (mainly adriamycin-based) with or without adjuvant radiotherapy and/or surgery. Data on gender, response to treatment, International Prognostic Index, and follow-up disease were collected according to standard protocols. A subgroup of these DLBCL cases had been used previously in an earlier study.¹⁴ Data on Rai score, Binet classification and follow up were available for 36 B-CLL/SLL cases, for which the first line of treatment had been chlorambucil and CHOP.¹⁵

Statistical analysis

Survival analyses, failure-free survival, disease-free survival and overall survival were analyzed by curves plotted using the Kaplan-Meier method. The statistical significance of associations between PRDM1 and survival was determined using the log rank test and taking a *p* value of < 0.05 as significant. The SPSS software (SPSS Inc. Chicago IL 1999) was used for the analyses.

Results

Validation of the PRDM1 monoclonal antibody

The specificity of the anti-PRDM1 monoclonal antibody (clone ROS, isotype IgG1/κ) was demonstrated by western blotting analysis of lysates of tonsil, thymus and myeloma cell lines. A specific band of 97 kDa was observed in tonsil, thymus and all myeloma cell lines (Figure 1A). In five of the seven myeloma lines, SK-MM-2, KARPAS-620, NCI-H929, OPM-2 and LP-1, it was possible to detect a second band of 80 kDa corresponding to an alternative protein product, PRDM1 (Figure 1A).²⁶ Nuclei stains cytocentrifuge preparations of the cell lines analyzed were all concordant with the protein expression detected by western blotting (Figure 1C). We confirmed the specificity of clone ROS for human PRDM1 protein by using this antibody in western blotting of material that had been immunoprecipitated from tonsil lysates with the PRDM1-specific polyclonal antibody¹ or with the monoclonal antibody ROS. Both antibodies immunoprecipitated a protein with a molecular weight of 97 kDa that gave a positive western blotting reaction with the ROS antibody (Figure 1B).

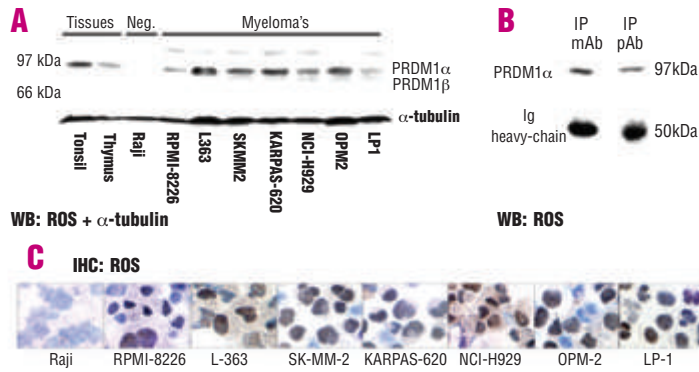


Figure 1. Biochemical characterization of the ROS monoclonal antibody. (A) Western blot analysis of PRDM1 expression in total protein extracts from lymphoid tissue, multiple myeloma and RAJI cell lines. Tonsil, thymus and all myeloma cell lines show a ~97 kDa band, corresponding to PRDM1 α protein. Myeloma cell lines, SK-MM-2, KARPAS-620, NCI-H929, OPM-2 and LP-1, also express an 80 kDa protein. The Burkitt's lymphoma cell line, RAJI, contains neither PRDM1 α nor PRDM1 β proteins, and was included as a negative control. (B) Immunoprecipitation of protein extracts from normal tonsil with the anti-PRDM1 monoclonal antibody (clone ROS, line IP mAb) and the specific polyclonal antibody (line IP pAb) followed by western blotting with the ROS monoclonal antibody, shows a ~97 kDa band. (C) Immunocytochemical staining for PRDM1 on cytocentrifuge preparations of cell lines using the ROS monoclonal antibody. Nuclei staining correlates with the pattern of protein expression detected by western blotting. Original magnification: 200X.

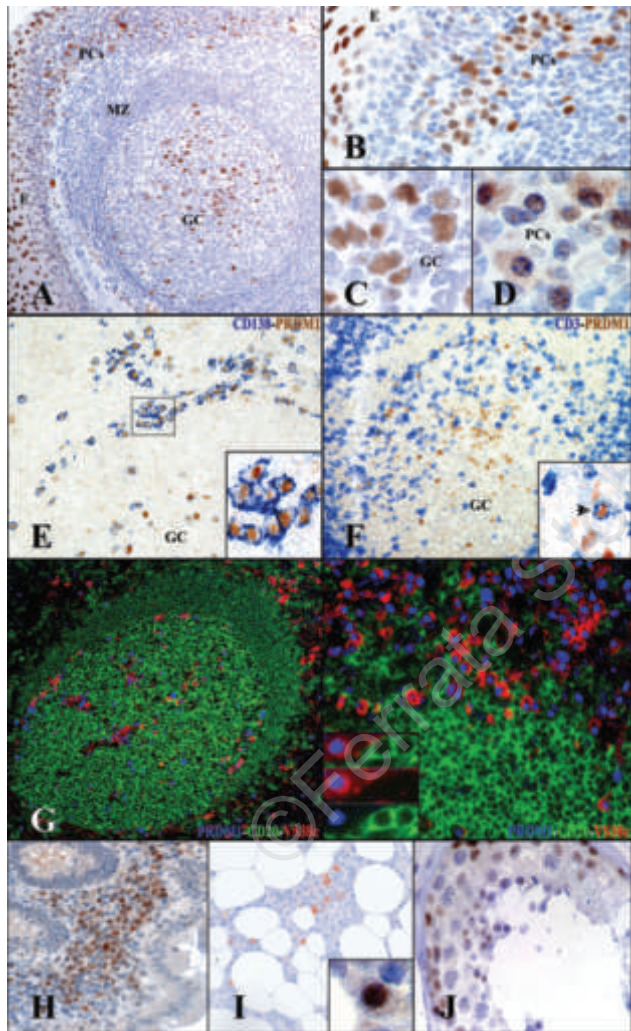


Figure 2. Immunostaining of PRDM1 protein in normal tissues. In tonsil PRDM1 protein is strongly expressed by plasma cells present in germinal centers, and in subepithelial areas (A, B, C and D). The subepithelial cells (B and D) show plasmacytoid morphology, but many positive cells in the germinal center (C) have a *centrocyte-like* morphology. Strong PRDM1 expression is also seen in the superficial layer of the epithelium (A and B). Double immunoenzymatic staining in normal tonsil reveals cells (mainly lying outside germinal centers) co-expressing PRDM1 (brown) and the plasma cell marker CD138 (blue). Furthermore a small population of PRDM1-positive cells that lack CD138 is seen in the germinal center (E). Within the germinal center, rare CD3-positive cells (blue staining) co-express PRDM1 (F and arrow). Triple staining of normal tonsil shows that PRDM1 protein (blue) is co-expressed by cells carrying the plasma cell-related antigen p63/VS38c (red), while CD20-positive cells (green) are mainly PRDM1-negative (G). PRDM1 protein is also detected in plasma cells located in other tissues, i.e. appendix (H) and bone marrow (I). Spermatogonia and Sertoli cells in the testis express PRDM1 strongly (J).

PRDM1 protein expression in normal human tissues

PRDM1 protein expression in normal human tissues was analyzed on paraffin-embedded tissue sections using the anti-PRDM1 monoclonal antibody (clone ROS). As previously reported,⁹ PRDM1 protein was detected in the nucleus of multiple cell types, with the strongest intensity in B-lymphocytes and squamous epithelium (Figure 2). Single and double immunostaining of normal tonsillar tissue showed that PRDM1 was expressed in (a) plasma cells in germinal centers (CD138-positive) lying in a band

between the light and dark zones, (b) plasma cells at the periphery of the germinal center and (c) plasma cells located within and below the crypt epithelium (Figure 2A-E). PRDM1 was also expressed in some CD138-negative cells in the germinal center, mainly located in the light zone (Figure 2C and E). Triple immunostaining showed co-expression of PRDM1 protein (blue) and the plasma cell-related antigen p63/VS38c (red), while CD20-positive cells were mainly PRDM1-negative (green) (Figure 2G). PRDM1 was also expressed in plasma cells in

Table 1. Immunohistological analysis of PRDM1 protein expression in lymphoma subtypes.

Diagnosis/lymphoma subtypes	Number of cases	Reactivity neoplastic cells			Total of positive cases (%)
		Negative	Weak	Strong	
B-cell neoplasms					
B-Lymphoblastic lymphoma	3	3	0	0	0 (0%)
Chronic lymphocytic leukemia/ small lymphocytic lymphoma	54	46	8	0	8 (15%)
Mantle cell lymphoma	52	52	0	0	0 (0%)
Follicular lymphoma	115	109	4	2	6 (5%)
Burkitt's lymphoma	21	21	0	0	0 (0%)
Marginal zone lymphoma (splenic, extranodal and nodal)	14	14	0	0	0 (0%)
Diffuse large B-cell lymphoma (DLBCL) ^a	235	134	38	63	101 (43%)
Plasmablastic DLBCL (EBER+ and HIV+) of oral mucosa type	15	0	0	15	15 (100%)
Myeloma/plasmacytoma	16	0	0	16	16 (100%)
Lymphoplasmacytic lymphoma	3	0	0	3	3 (100%)
Hodgkin's disease					
Classical Hodgkin's lymphoma	22	13	8	1	9 (41%)
T-cell neoplasms					
Peripheral T-cell lymphoma	41	30	7	4	11 (27%)
Anaplastic large cell lymphoma					
ALK protein +	16	5	5	6	11 (69%)
ALK protein -	14	8	3	3	6 (43%)
T-angioimmunoblastic lymphoma	18	17	1	0	1 (6%)
T/NK nasal type lymphoma	12	11	1	0	1 (8%)
Mycosis fungoides/Sézary	8	8	0	0	0 (0%)
T-lymphoblastic lymphoma/ leukemia	16	16	0	0	0 (0%)
Enteropathy-type T-cell Lymphoma	4	4	0	0	0 (0%)
Total	679	491	75	116	188 (28%)

^aIncluding eight selected DLBCL cases with distinct immunoblastic morphology: all eight cases (100%) showed >50% PRDM1 strong positive expression.

other organs, such as spleen, bone marrow and in the intestinal lamina propria (Figure 2H, I). Furthermore we also found a small number of T cells (CD3-positive) that expressed PRDM1 both within and outside germinal centers (Figure 2F). PRDM1 protein expression was not found in non-hematologic tissues, with the exception of squamous epithelium in different tissues. In the testis, we found PRDM1 expression in undifferentiated germ cells (spermatogonia) present in the basal compartment of the seminiferous tubule and in Sertoli cells (Figure 2J).^{27,28}

PRDM1 expression in human lymphomas

The immunostaining results on paraffin sections from 679 cases of human lymphoma are summarized in Table 1.

Non-Hodgkin's B-cell lymphoma

As expected, all plasma cell-derived neoplasms and small B-cell lymphomas with plasma cell differentiation (multiple myelomas [n=11], plasmacytomas [n=5], lymphoplasmacytic lymphomas [n=3]) showed strong PRDM1 expression (Figure 3).

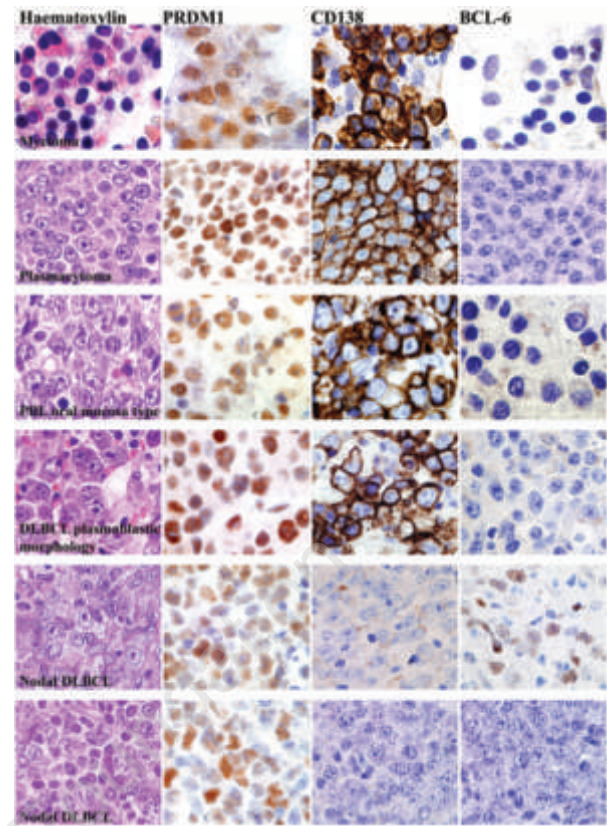


Figure 3. Immunostaining of lymphoid neoplasms for PRDM1. Representative examples of PRDM1-positive plasma cell-derived neoplasms, plasmablastic lymphoma of oral mucosa type and diffuse large B-cell lymphoma (DLBCL). Strong nuclear PRDM1 expression in multiple myeloma in bone marrow, in extramedullary plasmacytoma, in plasmablastic lymphoma of oral mucosa type and DLBCL with plasmablastic/immunoblastic cytological features. Note tumor cells express PRDM1 and CD138, but are BCL6-negative. A case of conventional nodal DLBCL shows strong nuclear positivity for the PRDM1 protein (tumor cells are CD138-negative and BCL6-positive). Tumor cells in a second PRDM1-positive DLBCL nodal case show strong nuclear positivity for the PRDM1 protein (and absence of CD138 and BCL-6). All stains were performed on paraffin sections using the immunoperoxidase technique.

Plasmablastic DLBCL of oral mucosa type

All 15 cases showing the characteristic clinical presentation, HIV-infection and EBER expression, considered to represent a clear-cut clinicopathologic entity, were PRDM1-positive (Figure 3).

Diffuse large B-cell lymphoma

Almost half of all cases of DLBCL (101/235) were PRDM1-positive. PRDM1 was strongly positive in 63 of the 235 cases analyzed (Figure 3), while a further 38 cases showed weaker PRDM1 expression. Eight cases of DLBCL with an immunoblastic morphology, distinct from the plasmablastic oral mucosa-type, were PRDM1-positive (Figure 3). We found MUM1/IRF4 expression in 68% of PRDM1-positive DLBCL. Among PRDM1-negative DLBCL cases, 48% expressed MUM1/IRF4. We also observed that many PRDM1-positive DLBCL cases also expressed BCL-6 protein (84%). As described by Hans *et al.*, it is possible to distinguish the germinal center-B and non-germinal center B subtypes of DLBCL using a panel of three immunostains (BCL-6, CD10 and MUM1/

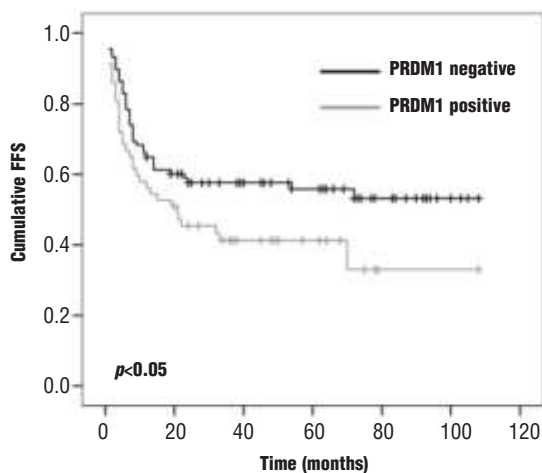


Figure 4. Kaplan-Meier analysis of failure-free survival (FFS) in DLBCL patients, grouped according to the expression of PRDM1 protein. FFS was shorter in the PRDM1-positive group (gray curve) than in the PRDM1-negative group (black curve).

IRF4).²⁹ However, we were unable to demonstrate any difference in expression of PRDM1 between germinal and non-germinal center B tumors, since PRDM1 expression was detected in just under half of the cases in both subgroups (36/81 and 42/97, respectively). We also investigated the prognostic value of PRDM1 protein expression in 146 patients with DLBCL, excluding plasmablastic DLBCL of oral mucosa type. Kaplan-Meier plots showed that the overall survival and disease-free survival of PRDM1-positive patients were not significantly different from those of PRDM1-negative cases; however when treatment failure was considered, PRDM1-positive patients had a shorter failure-free survival. This difference in the survival rate proved significant using the log rank test ($\chi^2 = 3.809$, $p < 0.05$) (Figure 4).

B-chronic lymphocytic leukemias/small lymphocytic lymphomas

A minority of cases of B-CLL/SLL (8/54) were PRDM1-positive. Most of these positive cases showed weak PRDM1 staining compared with the strong positivity of normal plasma cells or plasma cell-derived lymphomas (Figure 5). Expression of PRDM1 in B-CLL/SLL cases was not correlated with survival or IgVH mutational index.

Other non-Hodgkin's B-cell lymphomas

A small minority (6/115) of follicular lymphoma cases showed weak PRDM1 expression. In other B-cell lymphomas, i.e. B-lymphoblastic lymphoma (n=3), mantle cell lymphoma (n=52), Burkitt's lymphoma (n=21) and marginal zone lymphoma (n=14) (splenic, extranodal and nodal), PRDM1 expression was detected only in the non-neoplastic plasma cell component.

Classical Hodgkin's lymphoma

PRDM1 expression was found in a fraction (around 10%) of the nuclei of Hodgkin's and Reed Sternberg (H-RS) cells in 9 of the 22 cases of cHL analyzed (Figure 5).

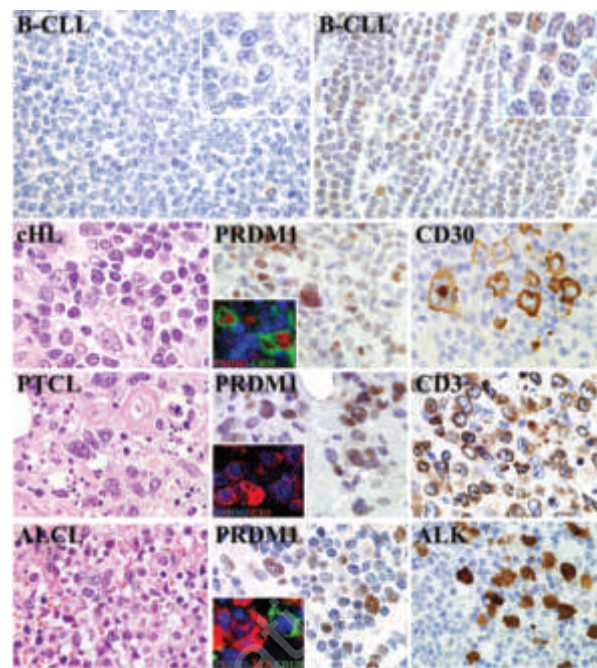


Figure 5. Immunostaining of lymphoid neoplasms for PRDM1. Representative examples of PRDM1-negative and PRDM1-positive B-chronic lymphocytic leukemia/small lymphocytic lymphoma (B-CLL) cases. Note weak nuclear PRDM1 staining in neoplastic cells in the positive B-CLL case (seen more clearly at higher power in the inset) compared with strong staining in normal plasma cells shown in Figure 1. Representative example of classical Hodgkin's lymphoma (cHL) case with high numbers of PRDM1-positive cells. Double immunofluorescent labeling of the cHL case shows CD30-positive neoplastic cells (green) expressing PRDM1 protein (red) (inset). Representative example of PRDM1-positive peripheral T-cell lymphoma (PTCL). Double immunofluorescent labeling shows that neoplastic cells (CD3-positive, red) also express PRDM1 (green) (inset). Representative example of ALK-positive anaplastic large T-cell lymphoma (ALCL) with high numbers of PRDM1-positive cells. Triple labeling shows ALK-positive neoplastic cells (red) and also plasma cells (CD138-positive) (green) which express PRDM1 (blue) (inset).

All PRDM1-positive cHL cases were PAX5-positive and CD138-negative. Furthermore, no correlation was seen between PRDM1 expression and Epstein-Barr virus infection. The presence of PRDM1 in tumor cells was confirmed by double immunofluorescence analysis, showing PRDM1-positive nuclei in a majority of atypical CD30-positive cells. PRDM1 protein was also detected by western blotting analysis in different cHL-derived cell lines, L-540 and L428 (*data not shown*), supporting the immunohistological data on PRDM1 expression in cHL biopsies.

T/NK-cell neoplasms

As described above, nuclear PRDM1 expression in reactive lymphoid tissue was observed in less than 1% of T cells. In contrast, approximately a quarter of the T-cell neoplasms analyzed (30/129) showed PRDM1 expression (Table 1). PRDM1 expression was mainly found in PTCL (11/41, 27%) and ALCL (17/30, 57%) (Figure 5). In ALCL cases PRDM1 staining could be detected in both ALK-positive (11/16, 69%) and ALK-negative cases (6/14, 43%). The presence of PRDM1 protein in neoplastic cells was confirmed by double immunofluorescence analysis, which showed that PRDM1-positive nuclei were found in CD3-positive cells in PTCL and in the majority of

ALK-positive cells in ALCL (shown at higher power in the insets of Figure 5).

Other types of T-cell neoplasia were negative (with the exception of one out of 18 cases of T-angioimmunoblastic lymphoma and one out of 12 cases of T/NK nasal type lymphoma).

Discussion

PRDM1 protein is a *master* regulator of B-cell terminal differentiation, with crucial roles in plasma cell differentiation.³⁰ This study provides a detailed description of the distribution of PRDM1 protein expression in a wide variety of normal and malignant human tissues. The results were obtained using a novel anti-PRDM1 monoclonal antibody (clone ROS), suitable for immunohistochemical staining of formalin-fixed paraffin-embedded tissue sections and western blotting analysis. PRDM1 expression was mainly restricted to lymphohematopoietic tissues, although nuclear labeling was also observed in spermatogonia, Sertoli cells and epithelial cells. In agreement with previous data, PRDM1 protein was strongly expressed in tonsil in the nuclei of mature plasma cells in germinal centres as well as in interfollicular areas, in a subset of B cells in the germinal center and in rare T cells.⁹ The strong staining for PRDM1 protein in normal plasma cells in tonsil and in other organs, such as spleen, bone marrow or intestinal lamina propria, shows that this anti-PRDM1 monoclonal antibody represents an important tool for the identification and classification of reactive plasma cells (in combination with other plasma cell markers such as CD138, p63/VS38c, CD38 and MUM1/IRF4).^{31,32}

Using tumor tissue microarrays, we also investigated PRDM1 expression in a large series of B and T cell lymphomas. PRDM1 was present in all lymphomas characterized by plasmacytic differentiation such as myeloma/plasmacytoma, lymphoplasmacytic lymphoma, plasmablastic lymphoma of oral mucosa type and DLBCL with immunoblastic morphology. This finding agrees with the consistent expression of PRDM1 protein in plasma cells and during plasma cell differentiation. However, we also identified aberrant PRDM1 expression in B-cell lymphomas that lack morphological features of plasmacytic differentiation (e.g. it was found to a varying degree in almost half of all cases of conventional DLBCL).

The existence of a subgroup of PRDM1-positive DLBCL cases confirms the heterogeneity of this category of lymphoma. The identification of clinical, histopathological and biological features is indispensable for the detection of different risk groups and for the therapeutic management of these lymphomas. With this in mind we investigated the possible correlation between PRDM1 and other markers commonly used for the classification of this lymphoma and outcome. Previous reports have shown that PRDM1-positive cells in normal germinal centers co-express MUM1/IRF4 in the absence of BCL-6 and that PRDM1 is required to induce plasma cell differentiation.³⁰ In our DLBCL study, we found that there are PRDM1-positive MUM1/IRF4-negative and PRDM1-negative MUM1/IRF4 positive cases. Interestingly, we also observed that many PRDM1-positive tumors also co-expressed BCL-6 protein.

These results indicate that the distribution of BCL6, MUM1/IRF4 and PRDM1 seen in normal germinal center cells is lost in DLBCL and that the regulation of these proteins is more complex than previously known.

We also investigated the relevance of PRDM1 expression in DLBCL to survival. In univariate analysis, PRDM1 expression was significantly correlated with shorter failure-free survival ($p < 0.05$). PRDM1-positive patients also showed a tendency towards decreased overall and disease-free survival. These findings suggest that tumors with high PRDM1 expression have a more aggressive behavior; however, the study of a larger series of DLBCL is necessary to confirm the statistical significance of this finding and to throw further light on the role of PRDM1 in DLBCL tumorigenesis. In all of the above studies we found no difference in terms of clinical behavior or phenotype between strongly and weakly PRDM1-positive cases.

An unexpected observation was the presence of PRDM1 protein in H-RS cells in almost half of the cases of cHL. This finding is consistent with the observation that H-RS cells could express plasma cell-associated markers and may well indicate that they arise from post-germinal center cells that are beginning to progress towards the stage of terminal B-cell differentiation, a process that is accompanied by downregulation of most of the genes comprising the B-cell transcriptional program.³³ Weak PRDM1 expression was also found in a small number of SLL/CLL (15%). Although rare cases of SLL/CLL show plasmacytic differentiation,³⁴ we were unable to show that PRDM1 expression was associated with this feature. Furthermore, no correlation with CD38, Epstein-Barr virus infection, mutation index or survival was found in this group. Finally, although PRDM1 expression is predominantly associated with late-stage B-cell differentiation, we observed that PRDM1 is not B-cell specific. PRDM1 was expressed in a small percentage of non-neoplastic T cells and also in some T-cell lymphomas. Among all the T-cell lymphomas tested we found that more than half of all ALCL and about one quarter of PTCL expressed PRDM1. At this moment the significance of PRDM1 expression in T cells is unknown and further studies are needed. The results obtained in our study are generally consistent with those previously described for normal lymphoid tissue and for some lymphoma types.⁹ However, we detected PRDM1 expression in other, not previously reported lymphomas. We believe that the high affinity and specificity of our monoclonal antibody allow more accurate detection of the protein, even in those cases expressing a low level of the protein. In summary, this article describes a new monoclonal antibody, clone ROS, suitable for the detection of PRDM1 on routine samples. The analysis of a large series of lymphomas revealed that PRDM1 expression, in addition to identifying plasma cell disorders, is also present in subsets of DLBCL, cHL, B-CLL/SLL and T-cell lymphomas. The PRDM1-monoclonal antibody could, therefore, become a valuable tool for research and diagnosis of lymphomas.

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References

- Turner CA, Jr., Mack DH, Davis MM. Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. *Cell* 1994;77:297-306.
- Messika EJ, Lu PS, Sung YJ, Yao T, Chi JT, Chien YH, et al. Differential effect of B lymphocyte-induced maturation protein (Blimp-1) expression on cell fate during B cell development. *J Exp Med* 1998; 188:515-25.
- Piskurich JF, Lin KI, Lin Y, Wang Y, Ting JP, Calame K. BLIMP-1 mediates extinction of major histocompatibility class II transactivator expression in plasma cells. *Nat Immunol* 2000;1:526-32.
- Angelin-Duclos C, Cattoretti G, Lin KI, Calame K. Commitment of B lymphocytes to a plasma cell fate is associated with Blimp-1 expression in vivo. *J Immunol* 2000;165:5462-71.
- Lin Y, Wong K, Calame K. Repression of c-myc transcription by Blimp-1, an inducer of terminal B cell differentiation. *Science* 1997;276:596-9.
- Ghosh N, Gyory I, Wright G, Wood J, Wright KL. Positive regulatory domain I binding factor 1 silences class II transactivator expression in multiple myeloma cells. *J Biol Chem* 2001;276:15264-8.
- Lin KI, Angelin-Duclos C, Kuo TC, Calame K. Blimp-1-dependent repression of Pax-5 is required for differentiation of B cells to immunoglobulin M-secreting plasma cells. *Mol Cell Biol* 2002;22:4771-80.
- Shaffer AL, Lin KI, Kuo TC, Yu X, Hurt EM, Rosenwald A, et al. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity* 2002; 17:51-62.
- Cattoretti G, Angelin-Duclos C, Shakhovich R, Zhou H, Wang D, Alobeid B. PRDM1/Blimp-1 is expressed in human B-lymphocytes committed to the plasma cell lineage. *J Pathol* 2005; 206:76-86.
- García JF, Maestre L, Lucas E, Sanchez-Verde L, Romero-Chala S, Piris MA, et al. Genetic immunization: a new monoclonal antibody for the detection of BCL-6 protein in paraffin sections. *J Histochem Cytochem* 2006;54:31-8.
- Roncador G, Brown PJ, Maestre L, Hue S, Martínez-Torrecedrera JL, Ling KL, et al. Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level. *Eur J Immunol* 2005;35:1681-91.
- Kallioniemi OP, Wagner U, Kononen J, Sauter G. Tissue microarray technology for high-throughput molecular profiling of cancer. *Hum Mol Genet* 2001; 10:657-62.
- Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, et al. The World Health Organization classification of neoplasms of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November, 1997. *Hematol J* 2000;1:53-66.
- Saez AI, Saez AJ, Artiga MJ, Perez-Rosado A, Camacho FI, Diez A, et al. Building an outcome predictor model for diffuse large B-cell lymphoma. *Am J Pathol* 2004;164:613-22.
- Rodríguez A, Martínez N, Camacho FI, Ruiz-Ballesteros E, Algara P, García JF, et al. Variability in the degree of expression of phosphorylated I κ B α in chronic lymphocytic leukemia cases with nodal involvement. *Clin Cancer Res* 2004;10:6796-806.
- García JF, Camacho FI, Morente M, Fraga M, Montalbán C, Alvaro T, et al. Hodgkin and Reed-Sternberg cells harbor alterations in the major tumor suppressor pathways and cell-cycle checkpoints: analyses using tissue microarrays. *Blood* 2003;101:681-9.
- Camacho FI, García JF, Cigudosa JC, Mollejo M, Algara P, Ruiz-Ballesteros E, et al. Aberrant Bcl6 protein expression in mantle cell lymphoma. *Am J Surg Pathol* 2004;28:1051-6.
- Martínez-Delgado B, Cuadros M, Honrado E, Ruiz de la Parte A, Roncador G, Alves J, et al. Differential expression of NF- κ B pathway genes among peripheral T-cell lymphomas. *Leukemia* 2005;19:2254-63.
- Colomo L, Loong F, Rives S, Pittaluga S, Martínez A, Lopez-Guillermo A, et al. Diffuse large B-cell lymphomas with plasmablastic differentiation represent a heterogeneous group of disease entities. *Am J Surg Pathol* 2004;28:736-47.
- García JF, Villuendas R, Sanchez-Beato M, Sanchez-Aguilera A, Sanchez L, Prieto I, et al. Nucleolar p14(ARF) overexpression in Reed-Sternberg cells in Hodgkin's lymphoma: absence of p14(ARF)/Hdm2 complexes is associated with expression of alternatively spliced Hdm2 transcripts. *Am J Pathol* 2002; 160:569-78.
- Erber WN, Pinching AJ, Mason DY. Immunocytochemical detection of T and B cell populations in routine blood smears. *Lancet* 1984;1:1042-6.
- Sabattini E, Bisgaard K, Ascani S, Poggi S, Piccioli M, Ceccarelli C, et al. The EnVision++ system: a new immunohistochemical method for diagnostics and research. Critical comparison with the APAAP, ChemMate, CSA, LABC, and SABC techniques. *J Clin Pathol* 1998; 51:506-11.
- Marafioti T, Jones M, Facchetti F, Diss TC, Du MQ, Isaacson PG, et al. Phenotype and genotype of interfollicular large B cells, a subpopulation of lymphocytes often with dendritic morphology. *Blood* 2003;102:2868-76.
- Gellrich S, Ventura R, Jones M, Tan SY, Mason DY. Immunofluorescent and FISH analysis of skin biopsies. *Am J Dermatopathol* 2004;26:242-7.
- Mason DY, Micklem K, Jones M. Double immunofluorescence labelling of routinely processed paraffin sections. *J Pathol* 2000;191:452-61.
- Gyory I, Fejer G, Ghosh N, Seto E, Wright KL. Identification of a functionally impaired positive regulatory domain I binding factor 1 transcription repressor in myeloma cell lines. *J Immunol* 2003;170:3125-33.
- Saitou M, Payer B, O'Carroll D, Ohinata Y, Surani MA. Blimp1 and the emergence of the germ line during development in the mouse. *Cell Cycle* 2005;4:1736-40.
- Ohinata Y, Payer B, O'Carroll D, Ancelin K, Ono Y, Sano M, et al. Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature* 2005;436:207-13.
- Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. *Blood* 2004; 103:275-82.
- Shapiro-Shelef M, Calame K. Regulation of plasma-cell development. *Nat Rev Immunol* 2005;5:230-42.
- Medina F, Segundo C, Campos-Caro A, Gonzalez-García I, Brieve JA. The heterogeneity shown by human plasma cells from tonsil, blood, and bone marrow reveals graded stages of increasing maturity, but local profiles of adhesion molecule expression. *Blood* 2002; 99: 2154-61.
- Tedoldi S, Paterson JC, Hansmann ML, Natkunam Y, Rudiger T, Angelisova P, et al. Transmembrane adaptor molecules: a new category of lymphoid cell markers. *Blood* 2005;107:213-21.
- Re D, Kuppers R, Diehl V. Molecular pathogenesis of Hodgkin's lymphoma. *J Clin Oncol* 2005;23:6379-86.
- Evans HL, Polski JM, Deshpande V, Dunphy CH. CD5+ true SLL/CLL with plasmacytic differentiation and an unusual 1p36 translocation: case report and review of the literature. *Leuk Lymphoma* 2000;39:625-32.