PRDM1/BLIMP-1 is involved in chemoresistance of T-cell lymphoma and
downregulated by the proteasome inhibitor

Running title: PRDM1/BLIMP-1 in T-cell lymphoma

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Key words: PRDM1, T-cell lymphoma, NF-κB
Abstract

The positive regulatory domain I (PRDM1) is a master regulator of terminal B-cell differentiation. However, PRDM1 is not B-cell specific. To determine its role in T-cell lymphoma, PRDM1 expression was investigated in 60 patients. PRDM1α and PRDM1β transcripts were detected in laser-microdissected T-lymphoma cells respectively in 27 and 14 patients, mostly in cases with IRF4 expression. PRDM1β was associated with increased c-MYC expression. PRDM1β-positive patients displayed advanced Ann Arbor stage, high-risk International Prognostic Index and were linked to short survival times. In vitro, PRDM1β was related to resistance to chemotherapeutic agents and could be downregulated by the proteasome inhibitor bortezomib. Kinetic studies showed that bortezomib downregulation of PRDM1β preceded decreased IRF4 and c-MYC expression. An earlier retaining of cytoplasmic IκBα in bortezomib-treated cells was revealed, concomitant with blockade of NF-κB nuclear translocation. These results demonstrate the involvement of PRDM1β in T-cell lymphoma, with possible therapeutic interference by the proteasome inhibitor.
Introduction

The positive regulatory domain I (PRDM1) belongs to the PRDM gene family of transcriptional repressors and is required for terminal B-cell differentiation into plasma cells. However, the involvement of PRDM1 in hematopoietic development is not restricted to the B-cell lineage. Recent studies have implicated PRDM1 in the regulation of T-cell activation and homeostasis. PRDM1 was also detected in a subset of T-cell lymphomas, while the significance of PRDM1 expression in this disease remains to be investigated.

PRDM1 has two isoforms: PRDM1α and PRDM1β. Generated from the same gene by alternative transcription initiation, PRDM1β differs from PRDM1α by the lack of 101 NH2-terminal amino acids, resulting in a disrupted PR domain and loss of repressive function on multiple target genes. Recently, we have shown that PRDM1β is associated with chemoresistance in B-cell lymphoma, which could be overcome by non-chemotherapeutic agent rituximab through NF-κB inactivation. Here we identify the role of PRDM1β in T-cell lymphoma and its modulation in vitro by the proteasome inhibitor bortezomib.

Study design

Patients

Sixty de novo T-cell lymphoma patients [32 male and 28 female, age 18 to 70 (median, 43) years] were included. Their main characteristics are summarized in Table 1. This study was approved by the Shanghai Institute of Hematology-Rui Jin Hospital.
Institutional Review Board. All patients gave accordingly their informed consent.

**Tissue samples**

Lymphoma tissue samples were immediately cut into 2 parts: one part fixed in formaldehyde and processed for paraffin embedding, and the other snap frozen and stored at -80°C. Systematic microscopic control of the lymphoma lesion was performed for each block.

**Cell culture**

T-lymphoma cell line HUT78, HUT102 and MOLT4 (American Type Culture Collection, Bethesda, MD) were treated with chemotherapeutic agents, bortezomib (Millennium Pharmaceuticals, Cambridge, MA, 25nM), or pyrrolidine dithiocarbamate (PDTC, Sigma, St. Louis, MO, 20µM) that specifically inhibits NF-κB activation. Methotrexate-resistant subclone was selected from the original MOLT-4 by exposure to increasing concentrations of methotrexate, as described by Fotoohi K et al.8

**Immunohistochemistry and immunofluorescence assay**

Immunohistochemical analysis was performed on 5-µm-paraffin sections with an indirect immunoperoxidase method, using antibodies against PRDM1,6 IRF4 (Dako, Danmark, 1:40), and c-MYC (Santa Cruz Biotechnology, Santa Cruz, CA, 1:100). Immunofluorescence assay was performed as previously reported.9
Laser microdissection

Seven-μm-frozen sections were incubated in RNAse-free conditions with anti-CD3 antibody (Dako, 1:50), fluorescein-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, 1:50) each for 5 minutes. Laser microdissection of fluorescent cells was immediately performed (Leica Microsystem, Wetzlar, Germany) for RNA extraction.

Semi-quantitative reverse-transcription PCR, sequence analysis and Western blot

As previously reported.\(^6\) The NF-κB (P65) and IκBα antibodies were obtained from Santa Cruz Biotechnology. The phosphorylated form of IκBα (p-IκBα) antibody was purchased from Cell Signaling (Beverly, MA).

Statistical Analysis

Patient characteristics were compared using \(\chi^2\) and Fisher exact test. Kaplan-Meier and log-rank test were performed to estimate the survival functions. Overall survival was measured from the time of diagnosis to the date of death or last contact. Event-free survival was calculated from the time of diagnosis to the date of progression, death, or last contact. \(P<0.05\) was considered significant. All statistical analyses were performed on SAS 8.2 software (SAS Institute Inc., Cary, NC).
Results and discussion

T-lymphoma cells expressed PRDM1

By immunohistochemistry, nuclear PRDM1 expression (Figure 1A) was observed in 19 of 60 (31.7%) patients. According to histological type, PRDM1 was found in peripheral T-cell lymphoma (10/23, 43.5%), anaplastic large-cell lymphoma (5/9, 55.6%), T/NK nasal-type lymphoma (3/12, 25.0%) and T-angioimmunoblastic lymphoma (1/5, 20.0%) (Table 1). T-lymphoblastic lymphoma did not express PRDM1, as shown by Garcia et al.4

Using semi-quantitative PCR, \( PRDM1\alpha \) and \( PRDM1\beta \) transcripts were identified in laser-microdissected lymphoma cells (Figure 1B) of 27 and 14 patients, respectively. \( PRDM1\alpha \) mRNA was detected in a higher number of patients (27/60) than PRDM1 protein (19/60). A similar discrepancy was also reported in B-cell lymphoma.10 Although systematic sequencing of PRDM1 in 30 patients with adequate frozen tissue samples revealed no mutation, other genetic/epigenetic inactivation as well as defects in protein translation or stability might contribute to the lack of PRDM1 protein expression. Instead, \( PRDM1\beta \) mRNA was detected in 14 of 19 cases positive for PRDM1 protein. The constant association between \( PRDM1\beta \) mRNA and PRDM1 protein led us to further consider the role of this altered isoform in T-cell lymphoma.

IRF4 was significantly related to \( PRDM1 \) transcripts: it was higher in \( PRDM1\alpha \) \( PRDM1\beta \) \( \times \) \( PRDM1\beta \) (14/14, 100%) and \( PRDM1\alpha \) \( PRDM1\beta \) cases (11/13, 84.6%), than in \( PRDM1\alpha \) \( PRDM1\beta \) cases (8/33, 24.2%, \( P=0.0076 \) and 0.0243, respectively).
Laser-microdissected lymphoma cells revealed IRF4 co-expressing with \textit{PRDM1\textsubscript{α}} and \textit{PRDM1\textsubscript{β}} (Figure 1C). Responsible for B-cell differentiation, IRF4 is expressed in neoplastic activated T cells and regulates T-cell function and transformation.\textsuperscript{11-14} This IFR4-PRDM1 co-expression confirmed that PRDM1 happens with IRF4 in T-cell lymphoma.

\textit{c-MYC} is a PRDM1-targeted gene and critical for cell proliferation.\textsuperscript{15,16} \textit{c-MYC} was documented more frequently in \textit{PRDM1\textsubscript{α}PRDM1\textsubscript{β}} cases (13/14, 92.9%), comparing to \textit{PRDM1\textsubscript{α}PRDM1\textsubscript{β}} cases (2/13, 15.4%, \textit{P}=0.0241). Therefore, \textit{PRDM1\textsubscript{β}} was unable to repress \textit{c-MYC}, which may cause T-cell proliferation.

**PRDM1\textsubscript{β} reflected poor disease outcome**

\textit{PRDM1\textsubscript{β}}, but not \textit{PRDM1\textsubscript{α}}, was significantly related to advanced Ann Arbor stage (\textit{P}=0.0326), high-risk International Prognostic Index (\textit{P}=0.0346, Table 1) and shorter survival times. The 3-year event-free survival and overall survival rates (± SE percentage) were significantly shorter for \textit{PRDM1\textsubscript{α}PRDM1\textsubscript{β}} patients [23.1% (±11.7%) and 55.6% (±14.9%)], than for \textit{PRDM1\textsubscript{α}PRDM1\textsubscript{β}} cases [48.5% (±16.6%) and 87.5% (±11.7%)] (\textit{P}=0.0400 and \textit{P}=0.0179, respectively). Therefore, like its analogue \textit{PRDM2} (RIZ) and \textit{PRDM3} (MDS1-EVI1) genes, each expressing a truncated protein missing the PR domain and critical for oncogenesis,\textsuperscript{17,18} \textit{PRDM1\textsubscript{β}} indicated poor disease outcome in T-cell lymphoma.

**Proteasome inhibitor bortezomib downregulated PRDM1\textsubscript{β} through NF-κB**
inactivation

HUT78 cells were resistant to doxorubicin, but sensitive to bortezomib. Since 6-hour incubation with bortezomib, either alone or with doxorubicin, \textit{PRDM1}\textsubscript{\beta} but not \textit{PRDM1}\textsubscript{\alpha}, was downregulated. \textit{IRF4} and \textit{c-MYC} were also decreased from 12-hour treatment. Their protein levels followed a similar reduction, observed later from 12 and 24 hours post-treatment (Figure 1D). Additionally, such alternation was also revealed in 6-mercaptopurine-resistant HUT102 cells and methotrexate-resistant MOLT4 subclone (Figure 1E). Bortezomib, the first proteasome inhibitor entering the clinic, has recently reported effective in T-cell lymphoma.\textsuperscript{19-21} \textit{PRDM1}\textsubscript{\beta} downregulation, with the subsequent inhibition of \textit{c-MYC} and \textit{IRF4}, could be one of the possible molecular mechanisms of its action.

\textit{PRDM1} is a downstream target of NF-\textit{κB},\textsuperscript{22} whose activation induces T-lymphoma cells resistance to chemotherapy.\textsuperscript{23,24} Nuclear localization of NF-\textit{κB}, required for target genes transactivation, was studied by immunofluorescence assay and showed an inhibition after 12-hour treatment of bortezomib (Figure 1F). This was confirmed on Western blot, with decrease of nuclear NF-\textit{κB} protein (Figure 1G). Further analysis of cytoplasmic I\textsubscript{κB}\textit{α} revealed striking differences: it decreased under doxorubicin, while increased under bortezomib treatment. Together, increasing cytoplasmic I\textsubscript{κB}\textit{α}, concomitant with blockade of NF-\textit{κB} nuclear translocation, suggested NF-\textit{κB} inactivation in T-lymphoma cells treated with bortezomib.\textsuperscript{25}

To further test this hypothesis, we associated PDTC, a NF-\textit{κB} inhibitor, with doxorubicin in T-lymphoma cell cultures. Growth inhibition (Figure 1H) and the
parallel decrease of PRDM1β (Figure 1I) could be reproduced as treated with bortezomib. We thus confirmed that NF-κB activity was responsible for PRDM1β downregulation by bortezomib.

In conclusion, abnormal PRDM1β expression was correlated with poor disease outcome in T-cell lymphoma patients. Bortezomib could downregulate PRDM1β through NF-κB inactivation. The value of proteasome inhibitor as a therapeutic strategy should be further assessed in T-cell lymphoma, especially those refractory to conventional chemotherapy.

Acknowledgments
This work was supported, in part, by the Chinese National Key Program for Basic Research (973:2004CB518600), the Chinese National High Tech Program (863:2006AA02A301 and 863:2006AA02A405), the National Natural Science Foundation of China (30750335), the Key Discipline Program of Shanghai Municipal Education Commission (Y0201), the Shanghai Commission of Science and Technology (44107025), the Shanghai Rising Star Program (05QMX1429), the Program for New Century Excellent Talents in University, the Scientific Research Foundation for the Returned Overseas Chinese Scholars, the Programme de Recherches Avancées (PRA B 06-01), and by the Samuel Waxman Cancer Research Foundation Laboratory, the Co-PI Program of Shanghai Rui Jin Hospital/Medical School of Shanghai Jiao Tong University.

Author contributions

Conflict of Interest Disclosure: The authors declare no competing financial interests.
References


### Table 1. PRDM1 expression in T-cell lymphoma

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<td>Low risk to intermediate low risk</td>
<td>23</td>
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<td>13 (56.5%)</td>
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<tr>
<td>Intermediate high risk to high risk</td>
<td>37</td>
<td>14 (37.8%)</td>
<td>20 (54.1%)</td>
<td>18 (48.6%)</td>
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Figure legends

Figure 1. PRDM1 was expressed in T-cell lymphoma and could be downregulated by bortezomib through NF-κB inactivation.

(A) Nuclear PRDM1 protein expression was revealed by immunohistochemistry on lymphoma tissue.

(B) PRDM1 mRNA and protein expression were detected, respectively, by semi-quantitative PCR on lymphoma cells and Western blot on lymphoma tissue. PRDM1 isoforms were expressed in laser-microdissected lymphoma cells, T and B-lymphoma cell lines (NA, namalwa, and SU, SU-DHL-4), but not in peripheral blood lymphocytes (PBL).

(C) Laser-microdissected lymphoma cells showed co-expression of PRDM1 and IRF4.

(D) In HUT78 cells resistant to doxorubicin (Dox), PRDM1β, IRF4 and c-MYC expression were reduced by bortizomib (Bor) both at transcriptional (upper panel) and protein level (lower panel).

(E) In HUT102 cells resistant to 6-mercaptopurine (6MP, 50µM), PRDM1β, IRF4 and c-MYC expression were also reduced by bortizomib (Bor, 25nM) (upper panel). In MOLT4 cells, when methotrexate-resistant was developed (MTX, 150nM), PRDM1β was expressed, and subsequently reduced by bortizomib (Bor, 25nM) (lower panel).

(F) Immunofluorescence study revealed that NF-κB (P65) was mainly located in nucleus in doxorubicin (Dox)-treated HUT78 cells, while in cytoplasm in those treated with bortezomib (Bor).
(G) Bortezomib (Bor) blocked NF-κB nuclear translocation and retained the phosphorylated form of IκBα (p-IκBα) and IκBα in the cytoplasm of HUT78 cells. Lamin B was used as a nuclear protein control.

(H) Addition of NF-κB inhibitor PDTC increased the anti-proliferative effect of doxorubicin (Dox) on HUT78 cells (left panel) and induced PRDM1β downregulation (right panel).
PRDM1/BLIMP-1 is involved in chemoresistance of T-cell lymphoma and downregulated by the proteasome inhibitor

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