Supplementary information

SUMOylation of Blimp-1 is critical for plasma cell differentiation

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Supplementary methods

Cell cultures and reagents
NCI-H929 (H929) human multiple myeloma cells, 18-81 murine pre-B cells, SKW6.4 (SKW) human lymphoblastoid cells, 293T, 3T3, HeLa and COS-1 cells were maintained as described [1-3]. Mouse splenic B cells were purified using anti-B220 microbeads (Miltenyi Biotec) from 6- to 8-week-old C57BL/6 (purchased from National Laboratory Animal center, Taiwan), Prdm1f/fCD19Cre+ (KO) or Prdm1f/fCD19Cre− (Ctrl) mice as described [4] and were stimulated with 2 μg/ml lipopolysaccharide (LPS) (Sigma). SUMO-isopeptidase inhibitor N-ethyl maleimide (NEM), cycloheximide and PKH26 were purchased from Sigma.

Plasmids
Details of plasmids, including FLAG-tagged Blimp-1 and its various deletion mutants expressed by the pCMV promoter in the pFLAG-CMV-5b vector, DBD–Blimp-1, pGC–Blimp-1–yellow fluorescent protein (YFP), enhanced green fluorescent protein (EGFP)-tagged SUMO1 were as described [3, 5]. Detailed information on the construction of plasmids expressing hemagglutinin (HA)-tagged PIAS family proteins (PIAS1, PIAS3, PIAS2-α, PIAS2-β and PIAS4); and various Blimp-1 mutants (Δ527–574, K235R, K245R, K574R, K767R, K816R and E818A) in either pFLAG-CMV-5b, pGEX-4T3 or pGC-YFP retroviral vectors [6]; DBD–K816R Blimp-1 are available upon request. Lentiviral vector producing Blimp-1 fused to GFP was as described [7]. The protocol for generating shRNA for PIAS1 or the negative control in a lentiviral vector was essentially as described [2, 8]. The PIAS1 shRNA contains the sense targeting sequences 5’-GGGTTTGTCCTGTCTGTGATA-3’, corresponding to nucleotides 1115–1135 of mouse and human PIAS1 mRNA. The detailed protocol for construction of these plasmids will be available upon request.

Immunoprecipitation and immunoblot analysis
NEM was added to total cell lysates or nuclear extracts to a final concentration of 10 mM, unless otherwise indicated. Immunoprecipitation performed with anti-Blimp-1 and anti-FLAG essentially was as previously described [5]. Anti-PIAS1 antibody used in immunoprecipitation was purchased from Santa Cruz. The primary antibodies for immunoblot analysis used in this study are mouse anti-SUMO1 (1:200, Santa Cruz), goat anti-GST (1:2,000, Amersham Biosciences), mouse anti-Blimp-1 (1:500; [9]), rabbit
anti-GFP (1:1,000; Santa Cruz), mouse anti-FLAG (1:2,000; Sigma), rabbit anti-HA (1:1,000; Sigma), goat anti-PIAS1 (1:200; Santa Cruz), mouse anti-HDAC2 (1:1,000; Upstate Biotech) and mouse anti-α-tubulin (1:5,000; Thermo). 2% of the input lysates was used in IB.

**In vitro SUMOylation assay**

FLAG-tagged WT, K235R, K245R, K574R, K767R and K816R Blimp-1 cDNAs were cloned into the pGEX-4T3 vector (Amersham Biosciences). Recombinant glutathione S-transferase (GST) fusion proteins were expressed and purified as described [5]. The *in vitro* SUMOylation assay was performed as described [10]. Briefly, GST- and FLAG-tagged Blimp-1 and its various mutant proteins were purified from *E. coli*. Purified Blimp-1 or its mutant proteins (1 µg) were incubated with 0.2 µg of GST-SAE1/2 (E1), 4 µg of His-UBC9 (E2) and 4 µg of His-SUMO-1, in the presence or absence of 0.2–0.5 µg of PIAS1, at 37°C for 90 min in 40 µl of reaction buffer containing 2 mM ATP, 20 mM HEPES (pH 7.5) and 5 mM MgCl₂. After incubation, the proteins were subjected to SDS-PAGE and immunoblot analysis with anti-FLAG.

**Nano-liquid chromatography–tandem mass spectrometry (Nano-LC-MS/MS)**

Nano-LC-MS/MS experiments were performed on a linear trap quadrupole–Fourier transform (LTQ-FT) mass spectrometer (Thermo) equipped with a nano-electrospray ion source (New Objective, Inc.) in positive ion mode. The liquid chromatography system consisted of an Agilent 1100 Series binary high-performance liquid chromatography pump (Agilent Technologies) with a Famos autosampler (LC Packings). The procedures and data analysis were performed as described [11, 12]. Briefly, the enzyme-digested protein samples were injected onto a self-packed precolumn (150 µm I.D. × 20 mm, 5 µm, 200 Å). Chromatographic separation was performed on a self-packed reversed-phase C18 nano-column (75 µm I.D. × 300 mm, 5 µm, 100 Å) by using 0.1 % formic acid in water (mobile phase A) and 0.1 % formic acid in 80 % acetonitrile (mobile phase B). A linear gradient was applied from 5 to 45 % mobile phase B for 40 min at a flow rate of 300 nl/min. Electrospray voltage was applied at 2 kV, and capillary temperature was set at 200°C. A scan cycle was initiated with a full-scan survey MS spectrum (m/z 300–2,000) performed on a Fourier transform ion cyclotron resonance mass spectrometer (Thermo) with a resolution of 100,000 at 400 Da. The ten most-abundant ions detected in this scan were subjected to a MS/MS experiment performed in the LTQ-FT mass spectrometer. Ion accumulation (Auto
Gain Control target number) and maximum ion accumulation time for the full scan and MS/MS were set at $1 \times 10^6$ ions, 1,000 ms and $5 \times 10^4$ ions, 200 ms, respectively. Ions were fragmented by use of collision-induced dissociation with the normalized collision energy set to 35%, activation Q set to 0.3 and an activation time of 30 ms. For data analysis, all MS/MS spectra were converted to the mxXML format from the RAW file format by MM File Conversion Tools; the database search was then carried out by MassMatrix (http://www.massmatrix.net). A custom protein database containing O75626 (Blimp-1_HUMAN) and P63165 (SUMO1_HUMAN) was used for MassMatrix. The search parameters in MassMatrix included the following: 1) the custom enzyme digestion sites were on the C-terminal side of Arg, Lys, Asp, Glu and Gly; 2) the enzyme miss cleavage number was nine; 3) the error tolerance of precursor ions and the MS/MS fragment ions in the spectra were 10 ppm and 0.6 Da, respectively, and 4) the custom cross-linking was Lys to Gly.

**Enzyme-linked immunosorbent spot (ELISPOT) assay and enzyme-linked immunosorbent assay (ELISA)**

ELISPOT analysis for detecting IgM-secreting mouse splenic YFP$^+$ B cells or SKW cells and ELISA were essentially as described [1, 5]. Photomicrographs of the spots were analyzed with the AID EliSpot Reader System (AID Autoimmun Diagnostika GmbH).

**Flow cytometry**

The procedure for flow cytometric analysis was as described [2]. Antibodies used in this study were phycoerythrin-conjugated anti–mouse CD138/syndecan-1 (clone 281-2; BD PharMingen) and allophycocyanin-conjugated anti–mouse CD45R/B220 (clone RA3-6B2). Cells were then analyzed by FACSCanto™ (Becton Dickinson) and FCS Express 3.0 software. Annexin V and 7-AAD staining of splenic B cells was performed as a previous report [7].

**RNA isolation and reverse transcription–quantitative PCR (RT-QPCR)**

Total RNA was isolated with the RNeasy mini kit (Qiagen). cDNA was synthesized with the High-Capacity cDNA Reverse Transcriptase kit (ABI), and the subsequent RT-QPCR was performed as described [5]. L32 mRNA was used for internal normalization in all experiments. The primer sequences are as follows: L32: 5’-AGAGGTGCTGGGAGCTGCTA-3’ and 5’-GATGGATGGTCTCTGGACGG-3’, Pax5:
5'-TGGCTGGAGGCCTGGAT-3’ and 5’-AACGCTGCTCCCGATGTC-3’, 
Ciita: 5’-AGAGACCCGGAGGCCCTAT-3’ and 5’-GGGTATCCTGGAACACGTACTGA-3’,
Bcl2a1: 5’-CAGAATTCTAAATGAATAACAGGAGAA-3’ and 5’-AGTCAGCCAGCCAGATTTGG-3’, and Xbp1: 5’-TCCCATGGACTCTGACACTGTTT-3’ and 5’-CCAGAATGCACCAAAAAGGATATC-3’.

Immunofluorescence staining
The procedure was essentially carried out as described [5]. Briefly, the COS-1 or HeLa transfectants were washed with PBS twice and incubated with detergent extraction buffer (10 mM PIPES [pH 7.0], 300 mM sucrose, 100 mM NaCl, 3 mM MgCl2, 0.1 % [w/v] Triton X-100) for 5 min, followed by fixation with 4 % paraformaldehyde for 10 min and three washes with PBS. After blocking with 2 % milk in PBS for 30 min, the cells were incubated with the following primary antibodies: mouse anti-FLAG (1:2,000; Sigma), rabbit anti-HA (1:1,000; Sigma) in blocking buffer for 1 h. Cells were washed with PBS three times and incubated with the following secondary antibodies: anti–mouse IgG conjugated with Alexa Fluor 555 dye (Invitrogen) or anti–rabbit IgG conjugated with Alexa Fluor 405 dye (Invitrogen) in blocking buffer for 1 h. Cells were washed with PBS three times and incubated with 4’-6-diamidino-2-phenylindole (DAPI; Sigma) in PBS for 1 min, followed by three more washes. The entire procedure was performed at room temperature. The fluorescent images were examined by either a confocal microscope (Leica SP5) or a fluorescence microscope (Leica DM6000B).

Chromatin immunoprecipitation (ChIP)
ChIP using anti–Blimp-1 was performed essentially as described [13]. Briefly, 2 × 10⁷ 18-81 cells were electroporated with vectors expressing WT or K816R Blimp-1. Cells harvested 1 day later were subjected to ChIP using anti–Blimp-1. Immunoprecipitated DNA and input DNA were used to amplify regions encompassing or adjacent to Blimp-1 binding sites by quantitative PCR using the CYBR green method on an ABI prism 7300 system. The values obtained from immunoprecipitated DNA were normalized to input DNA according to a described method [14]. The primer sequences used in this study were as follows: Myc promoter: 5’-TCCACCTGGCTGACTGA-3’ and 5’-AGCGCTCGGCTGAACTGT-3’, Pax5 promoter: 5’-CCAGAGCCACGTCTTCAAT-3’ and 5’-CCGGACATCGCAATTCCTCG-3’, Ciita promoter: 5’-CCCAATTTGGCCCACAAG-3’ and
5'-CCCTAGCCACCACACAGCTTCT-3'; *Ciita* 3' untranslated region (UTR): 5'-AAGTGGCTGCTGGGTGTGA-3' and 5'-TGCCCAGGAGCCTTTGTG-3'.
Supplementary references:


**Supplementary Figure Legends**

**Supplementary Figure S1** Nuclear expression of various Blimp-1 mutants. HeLa cells were transfected with 100 ng of vector expressing FLAG-tagged Blimp-1 or its various mutants. Cells were fixed 1 day later for immunofluorescence staining with anti-FLAG followed by DAPI staining. (A) Nuclear localization of WT, K235R, K245R, K574R, K767R and K816R Blimp-1. (B) Nuclear localization of various forms of Blimp-1 and E818A Blimp-1. Results shown are representative of three independent experiments. Scale bar, 10 μm.

**Supplementary Figure S2** SUMO1, but not SUMO2 or SUMO3, modifies Blimp-1. FLAG–Blimp-1 expression plasmid was co-transfected with enhanced green fluorescent protein (EGFP) (−), EGFP-SUMO1, EGFP-SUMO2 or EGFP-SUMO3 expression plasmids in 293T cells, followed by immunoprecipitation (IP) using anti-FLAG and the subsequent immunoblot (IB) analysis. Arrowhead indicates the Blimp-1–EGFP-SUMO1 conjugate. Results shown are representative of three independent experiments.

**Supplementary Figure S3** Immunoblot (IB) analysis shows the similar expression of WT and mutant Blimp-1 proteins in sorted Prdm1 knockout splenic B cells that were YFP⁺. Tubulin was used as the loading control. Results are representative of two independent experiments.

**Supplementary Figure S4** Amino acid residues 527 to 574 of Blimp-1 mediate interaction with PIAS1. (A) Linear domain structure maps of full-length Blimp-1 and its various deletion mutants. Known motifs: acidic, acidic domain; PR, PR domain that resembles a SET domain; proline-rich, proline-rich domain; Zn, the five zinc-finger motifs. (B) The hemagglutinin (HA)-PIAS1 expression plasmid was co-transfected into 293T cells with the plasmid encoding FLAG (−) or the indicated FLAG-tagged Blimp-1 deletion mutants, followed by immunoprecipitation (IP) with anti-FLAG and immunoblot (IB) analysis. Results shown are
representative of three independent experiments.

Supplementary Figure S5 SKW cells expressing K816R Blimp-1 produced less IgM than do cells that express WT Blimp-1. SKW cells were transduced with retroviral vectors expressing WT or K816R Blimp-1. Yellow fluorescent protein+ (YFP+) cells were seeded at different densities for ELISPOT analysis of the number of IgM-secreting cells 3 days later. Results are the mean ± SD from triplicate samples. One representative result from three independent experiments is shown.

Supplementary Figure S6 The frequency of apoptotic cells in Prdm1 knockout (KO) splenic B cells reconstituted with either WT or mutated Blimp-1. Flow cytometric analysis of annexin V and 7-AAD levels was carried out on yellow fluorescent protein+ (YFP+)-gated lipopolysaccharide-stimulated splenic B cells from KO mice that had been transduced with the indicated retroviral vectors for 2 days. Results are representative of two independent experiments.

Supplementary Figure S7 SUMO modification of Blimp-1 does not globally alter subnuclear localization. The subnuclear localization of Blimp-1 occurs regardless of its SUMO modification. An expression plasmid encoding hemagglutinin-tagged promyelocytic leukemia (HA-PML) was co-transfected with plasmids encoding FLAG-tagged WT or K816R Blimp-1 into COS-1 cells. Confocal-plane images of immunofluorescence staining with anti-FLAG and anti-HA are shown. Scale bar, 10 μm.
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Ying et al. Fig. S2

**Diagram Description:**

- **EGFP-SUMO** and **FLAG-Blimp-1** conditions are represented with '+' and '-'.
- IP: FLAG and IB: GFP/FLAG are indicated for each condition.
- Lanes labeled as 'Input' are also visualized.

**Band Analysis:**

- Bands appear at 170, 130, and 100 kDa for IP: FLAG and IB: GFP/FLAG.
- Input bands are observed at 130 and 100 kDa.
Ying et al. Fig. S5

![Bar graph showing IgM-secreting cells with YFP, WT, K816R conditions at different cells per well.](image-url)
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WT PML Merge

K816R PML Merge