SUMOylation of Blimp-1 is critical for plasma cell differentiation

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Transcriptional repressor B lymphocyte-induced maturation protein-1 (Blimp-1) is a master regulator of plasma cell differentiation. Here we show that Blimp-1 is covalently modified by SUMO1 at lysine 816, a modification mediated by SUMO E3 ligase PIAS1. Mutation of Blimp-1 lysine 816 reduces transcriptional repression—correlating with a reduced interaction with a histone deacetylase, HDAC2—and impairs differentiation of antibody-secreting cells. Thus, the SUMO pathway critically regulates Blimp-1 function during plasma cell differentiation.

Keywords: differentiation; plasma cell; SUMO; transcriptional repression

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INTRODUCTION

B lymphocyte-induced maturation protein-1 (Blimp-1), a transcriptional repressor, has a central role in the differentiation of B cells into antibody-secreting plasma cells [1] as well as many other cell lineages of multiple organisms [2]. Although plasma cell fate specification can be initiated independently of Blimp-1, Blimp-1 is required for complete plasma cell differentiation [3]. Blimp-1 is present in virtually all plasma cells induced by T cell-dependent or -independent antigen immunization [4], and gene-targeted deletion of the Blimp-1 gene, *Prdm1*, in mouse B cells blocks their ability to mount an antibody response [1].

By interacting with several chromatin-modifying enzymes including HDAC1/2 [5], Blimp-1 suppresses genes implicated in

proliferation and in the mature B-cell transcription programme [6–9], leading to the specification of plasma cell fate. Blimp-1 was recently shown to be modified by SUMO1 [10], but the underlying molecular and biological consequences are not known.

SUMOylation, a post-translational modification that involves the covalent conjugation of the SUMO protein to a target substrate, regulates many important molecular and cellular processes, including transcriptional control [11]. The conjugation of SUMO with its substrate involves the E3 ligases, which enhance substrate selectivity *in vivo* [12]. The PIAS family, one of the three types of mammalian E3 ligases, consists of four proteins: PIAS1, PIAS2 (PIAS2- α and PIAS2- β), PIAS3 and PIAS4 [13]. The interactions of transcription factors with PIAS proteins can either positively or negatively affect transcriptional activities [14].

In this report, we demonstrate the significance of PIAS1-dependent SUMOylation of Blimp-1 in plasma cell differentiation.

RESULTS

Blimp-1 is modified by SUMO1 at Lys816

We first verified by co-immunoprecipitation (co-IP) that endogenous Blimp-1 is sumoylated in plasma cells NCI-H929 (H929) that constitutively express Blimp-1, and in lipopolysaccharide (LPS)-stimulated mouse splenic B cells that express induced Blimp-1. Indeed, SUMO1 is present in anti-Blimp-1 immunoprecipitates in both cultured cells, and the slower-migrating form shows a molecular-weight shift of SUMO1 conjugate, which suggests the sumoylated Blimp-1 (Fig 1A). Protein SUMOylation occurs at the lysine (K) residue of the consensus SUMOylation motif, ψ KXE, where ψ represents hydrophobic residues and X represents any amino acid [15]. Using the computational SUMOylation prediction programme SUMOsp [16], we found that there are five putative SUMOylation sites in human Blimp-1, which are located at amino-acid residues K235, K245, K574, K767 and K816 (accession number NP_001189 Fig 1B) and are conserved in mouse Blimp-1. Five individual Blimp-1 mutants, K235R, K245R,

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also not modified by SUMO1 (Fig 1D). An *in vitro* SUMOylation assay further demonstrated that WT, K235R, K245R, K574R and K767R Blimp-1 were conjugated to SUMO *in vitro*, whereas K816R Blimp-1 failed to be sumoylated (Fig 1E), showing again that K816 is the major SUMO acceptor site of Blimp-1. Indeed, liquid chromatography–tandem mass spectrometry revealed a SUMO peptide branch at K816 of Blimp-1. Lysates prepared from 293T cells that transiently expressed FLAG–Blimp-1 and EGFP-SUMO1 and that had been processed for anti-FLAG IP and trypsin digestion were used for liquid chromatography–tandem mass spectrometry identification (Fig 1F).

Along this line, we wondered whether SUMO2 or SUMO3 can, like SUMO1, modify Blimp-1. In spite of the similar expression levels of all three EGFP-SUMOs, EGFP-SUMO2 and EGFP-SUMO3 failed to conjugate with Blimp-1 (supplementary Fig S2 online).

SUMOylation of Blimp-1 is of functional importance

Prdm1-deficient (knockout (KO)) splenic B cells that are stimulated *in vitro* by LPS fail to cease cell cycling and to commit to the plasma cell fate [1]. To address the biological importance of SUMO modification of Blimp-1, we examined the proliferation rate of LPS-stimulated KO B cells that were retrovirally reconstituted with WT or K816R Blimp-1. Proliferation rate was monitored by dilution of the fluorescent tracking dye PKH26. Notably, after stimulation, cell proliferation was halted by WT Blimp-1, but K816R-expressing cells divided as effectively as cells that had been transduced with control vector (Fig 2A). Consistently, transduction of WT or K245R Blimp-1 in stimulated KO B cells restored the formation of plasmacytic cells that express the CD138 plasma cell surface marker (Fig 2B). In contrast, despite the similar expression of the reconstituted Blimp-1 proteins (supplementary Fig S3 online), the frequency of CD138⁺ cells generated by K816R reconstitution was reduced (Fig 2B). Similarly, K816R, unlike WT and K245R Blimp-1, was much less able to compensate for IgM production in KO B cells (Fig 2C). The suggestion that disruption of SUMOylation of Blimp-1 impairs its ability to trigger the plasma cell fate was further supported by another reconstitution assay in which E818A Blimp-1 was expressed in LPS-stimulated KO B cells and led to results that were similar to K816R expression (Fig 2B,C).

Re-introduction of WT Blimp-1 in KO B cells resulted in downregulation of *Pax5* and upregulation of *Xbp1* (Fig 2D), a crucial gene for antibody secretion [17]. The induction of *Xbp1* is due to the de-repression effect from Blimp-1-mediated suppression of the gene encoding *Pax5*, which suppresses *Xbp1* [8]. The mRNA level for *Pax5* was repressed less, and the induction of *Xbp1* mRNA was hampered in cells that expressed K816R Blimp-1 (Fig 2D). Similarly, the other two Blimp-1 target genes, *Ciita* and *Bcl2a1* [7,18], were repressed less by K816R than by WT Blimp-1 (Fig 2D), in agreement with the less-differentiated phenotype in K816R-expressing cells.

Together, these data show that Blimp-1, in the absence of SUMOylation, is not as effective at blocking cell proliferation and is defective in driving plasma cell formation.

Involvement of PIAS1 in Blimp-1 SUMOylation

We next dissected the SUMO pathways that contribute to Blimp-1 SUMOylation. Various haemagglutinin-tagged human E3 ligases,

including PIAS1, PIAS2- α , PIAS2- β , PIAS3 and PIAS4, were used to test their effects on Blimp-1 SUMOylation. Interestingly, PIAS1 dramatically enhanced Blimp-1 SUMOylation, even though the expression of PIAS1 was lower than that of the other PIAS proteins (Fig 3A). In contrast, PIAS3 was much less capable of promoting Blimp-1 SUMOylation, and PIAS2- α , PIAS2- β and PIAS4 were nearly inert in the co-transfection assay (Fig 3A). The direct effect of PIAS1 on promoting Blimp-1 SUMOylation was confirmed by an *in vitro* SUMOylation assay in which bacterially expressed PIAS1 was able to further increase the SUMO1-modified Blimp-1 in a dose-dependent manner (Fig 3B). We also detected the interaction between Blimp-1 and PIAS1 at endogenous levels by co-IP in lysates from H929 plasma cells (Fig 3C). The RING-finger-like zinc-binding domain (RLD) of PIAS1 has the SUMO E3 ligase enzymatic activity [13]. We found that PIAS1 without the RLD (Δ RLD) had decreased ability to promote SUMO1 conjugation with Blimp-1 (Fig 3D), indicating that the RLD domain of PIAS1 is required in this context. Similarly, depletion of PIAS1 by a lentiviral vector that expresses the short hairpin RNA against PIAS1, PIAS1i, led to reduced SUMO1 conjugation as compared with the effect of the control short hairpin RNA, Ctrli (Fig 3E).

We mapped the Blimp-1 region involved in the PIAS1 interaction by using various plasmids encoding deleted forms of FLAG–Blimp-1 (supplementary Fig S4A online). These deleted forms of FLAG–Blimp-1 were expressed in the nucleus (supplementary Fig S1B online). Analysis indicated that amino-acid residues 527–574 of Blimp-1 were crucial for the PIAS1 interaction (supplementary Fig S4B online). As expected, a Blimp-1 deletion lacking this region (Δ 527–574) failed to interact with PIAS1 (Fig 3F) and was less able to be modified by EGFP-SUMO1 (Fig 3G). LPS-stimulated KO B cells expressing the Δ 527–574 Blimp-1 mutant showed decreased formation of CD138⁺ plasmacytic cells (Fig 3H) and IgM production (Fig 3I) as compared with cells that expressed WT Blimp-1, even though both proteins were expressed at similar levels (supplementary Fig S3 online).

As the interaction with PIAS1 appears to be important for the effect of Blimp-1, we wondered whether PIAS1 contributes to plasma cell differentiation. Indeed, knockdown of PIAS1 in LPS-triggered differentiating splenic B cells caused reduced production of antibody (Fig 3J). However, it remains possible that knocking down PIAS1 during plasma cell differentiation may also affect the Blimp-1. We thus ectopically expressed Blimp-1 in a human lymphoblastoid cell line SKW 6.4 (SKW) possessing the ability to produce IgM [9], and found that knockdown of PIAS1 reduced Blimp-1-driven IgM production (Fig 3K). This result is consistent with another observation that SKW cells that were transduced with K816R Blimp-1 produced less IgM when compared with WT Blimp-1-transduced SKW cells (supplementary Fig S5 online).

SUMO affects transcriptional repression by Blimp-1

Lastly, we examined the molecular mode of action underlying the consequence of Blimp-1 SUMOylation. Cycloheximide-based protein chase experiment showed that K816R Blimp-1 is not markedly different with respect to its protein stability, in comparison with WT Blimp-1 (Fig 4A). We next examined if SUMO-modified Blimp-1 has changed transcriptional repression activity. We previously showed that Blimp-1 fused with the Gal-4 DNA-binding domain (DBD–Blimp-1) effectively suppresses

Fig 2 | SUMO modification of Blimp-1 is required for plasma cell differentiation. (A) Histogram of flow-cytometric analysis of PKH26 intensity. *Prdm1*^{fl/fl} CD19^{Cre+} (KO) B cells were labelled with PKH26, followed by LPS stimulation and retroviral vector transduction. Flow-cytometric analysis of PKH26 levels using the YFP⁺ gate. The numbers indicate the PKH26 MFI $\pm$ s.d. from three experiments. (B) Flow-cytometric analysis of surface expression of B220 and CD138 on the stimulated and retrovirally transduced (YFP⁺) splenic B cells from *Prdm1*^{fl/fl} CD19^{Cre-} (Ctrl) or KO mice. Results are representative of three independent experiments. (C) ELISPOT analysis of the number of IgM-secreting cells in retrovirally transduced (YFP⁺) Ctrl or KO B cells stimulated with LPS. (D) RT-QPCR shows the relative mRNA levels of *Ciita*, *Bcl2a1*, *Pax5* and *Xbp1* regulated by WT and K816R Blimp-1 in YFP⁺ cells sorted as described in C. Blimp-1, B lymphocyte-induced maturation protein-1; Ctrl, control; KO, knockout; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; WT, wild type; YFP⁺, yellow fluorescent protein⁺.

luciferase reporter activity driven by a thymidine kinase (tk) promoter composed of four Gal4-binding sites, (Gal4)₄-tk-Luc [19]. Here the (Gal4)₄-tk-driven luciferase activity was suppressed by DBD-Blimp-1, but was suppressed to a lesser extent by DBD-K816R Blimp-1 (Fig 4B). Similar results were observed in Blimp-1-dependent repression of the activities of the *CIITA* promoter III (Fig 4C). These data suggest that a SUMO conjugation-defective Blimp-1 is a less potent transcriptional repressor.

We next determined whether the effect of SUMOylation on the transcriptional repression ability of Blimp-1 results from an alteration in the DNA-binding ability of Blimp-1. WT Blimp-1 and K816R bound similarly with the endogenous target gene loci, as demonstrated by the anti-Blimp-1 chromatin IP assay, which examined the binding of WT Blimp-1 and K816R with endogenous target sites, including *Myc*, *Pax5* and *Ciita*, in B cells (Fig 4D). HDAC1/2 is involved in Blimp-1-mediated gene suppression [5]. We found that HDAC2 was co-immunoprecipitated with WT Blimp-1, as expected (Fig 4E). Notably, the level of HDAC2 that interacted with K816R Blimp-1 was statistically decreased (Fig 4E).

DISCUSSION

Here we provide evidence that, among SUMO family members, SUMO1 selectively mediated Blimp-1 SUMOylation and that Blimp-1 is subjected to PIAS1-mediated SUMOylation at lysine 816, which has a crucial role in plasma cell differentiation. We here show that the expression of Blimp-1 downstream genes, including *Pax5*, *Ciita*, *Bcl2a1* and *Xbp1*, was altered in K816R-reconstituted KO B cells when compared with that in WT Blimp-1-reconstituted cells. The changes in the expression of these genes in K816R- versus WT Blimp-1-rescued cells are marginal but statistically significant, which could be due to the heterogeneity of splenic B cells that leads to unsynchronized differentiation. Additionally, the reason that SUMOylation-defective forms of Blimp-1 cannot drive plasma cell fate is not due to their influence on cell viability, because we observed a similar apoptotic rate in cells reconstituted with mutated or WT Blimp-1 (supplementary Fig S6 online).

Although there are five members of the PIAS family and all are expressed in mouse splenic B cells (unpublished observations), PIAS1 functioned most effectively as the E3 SUMO ligase of Blimp-1. The interaction between Blimp-1 and PIAS1 appears to

Fig 3 | PIAS1 regulates Blimp-1 SUMOylation. (A) PIAS1 enhances SUMO1 modification of Blimp-1 in a co-transfection experiment in 293T cells. (B) IB analysis with anti-FLAG to show that *in vitro* SUMOylation of purified GST- and FLAG-tagged WT Blimp-1 is enhanced by bacterially expressed PIAS1. The asterisks and arrow are as described for Fig 1E. (C) Endogenous Blimp-1 interacts with endogenous PIAS1 in H929 plasma cells. (D) The RLD of PIAS1 is required for Blimp-1 SUMOylation as demonstrated by a co-transfection assay in 293T cells. (E) Depletion of PIAS1 reduced SUMOylation of Blimp-1. 293T cells transduced with vectors expressing shRNA for PIAS1 (PIAS1i) or control shRNA (Ctrl) for 2 days were then co-transfected with vectors encoding the indicated proteins for 2 days. The relative ratios of the quantification results for Blimp-1-EGFP-SUMO1 (arrowhead; modified) versus Blimp-1 (arrow; unmodified) band intensities are shown. (F,G) Plasmids encoding indicated proteins were co-transfected into 293T cells, followed by IP and IB analysis in F and IB analysis in G. (H) Flow-cytometric analysis of surface expression of B220 and CD138 on LPS-stimulated KO splenic B cells transduced with indicated retroviral vectors. (I) The number of IgM-secreting cells was determined by ELISPOT assay for cells expressing the indicated proteins. Sorted YFP⁺ cells from H were used here. (J) Knockdown of PIAS1 reduces the formation of antibody-secreting cells. Stimulated splenic B cells were transduced with lentiviral construct encoding GFP and expressing either PIASi or Ctrl, followed by ELISA analysis of the amounts of IgM from supernatants of sorted GFP⁺ cells. Results are from triplicate data from two independent experiments. (K) PIAS1 knockdown reduced the IgM production in SKW cells ectopically expressing GFP or GFP-Blimp-1 fusion protein by lentiviral vectors. IB analysis in J and K showed knockdown of PIAS1 in sorted GFP⁺ PIAS1i-expressing cells. Arrowheads in A,D and G indicate the SUMO1-conjugated form. Blimp-1, B lymphocyte-induced maturation protein-1; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; HA, haemagglutinin; IB, immunoblot; KO, knockout; LPS, lipopolysaccharide; RLD, RING-finger-like zinc-binding domain; shRNA, short hairpin RNA; WT, wild type; YFP⁺, yellow fluorescent protein⁺.

be of biological importance, as a Blimp-1 mutant that lacks the region required for PIAS1 interaction was much less capable of promoting plasma cell differentiation. Also, depletion of PIAS1 attenuated the Blimp-1-mediated production of IgM. In addition to serving as a SUMO E3 ligase, PIAS1 is a transcriptional

co-repressor for some transcription factors, whereby it prevents their binding to target genes without the involvement of SUMO [20]. Our result that lack of RLD in PIAS1 shows reduced ability to promote Blimp-1 SUMOylation suggests that PIAS1 relies on its E3 ligase domain to regulate Blimp-1.

Fig 4 | SUMO-modified Blimp-1 is a more potent transcriptional repressor. (A) Stability of Blimp-1 and K816R. 293T cells transfected with vectors encoding WT or K816R Blimp-1 were subjected to CHX (100 µg/ml) chase experiment and lysed at the indicated time points for IB analysis. The relative ratios of protein levels are quantified by normalization of the band intensity of Blimp-1 to that of tubulin for each time point, and the data are presented as fold changes as compared with the sample at 0 h. Results are representative of two experiments. (B) Representation of the Gal4 DBD construct. Relative luciferase activity of mouse 18–81 pre-B cells transfected with plasmids encoding indicated proteins together with (Gal4)₄-tk-Luc. (C) Relative luciferase activity of 18–81 cells transfected with plasmids encoding WT or K816R Blimp-1 and a luciferase reporter driven by the *CIITA* promoter III. In B and C, total cell lysates were analysed by IB. Tubulin was used as a loading control. (D) A chromatin IP assay with anti-Blimp-1 shows that ectopically expressed WT and K816R Blimp-1 bound as effectively to the indicated endogenous loci in 18–81 cells. Regions encompassing the Blimp-1 binding site in the indicated loci and a negative control region located in the 3' UTR of *Ciita* were amplified by QPCR. Input DNA was used to normalize the data. Results are the mean ± s.e.m. from triplicate samples of two independent experiments. (E) K816R is less capable of interacting with HDAC2 than is WT Blimp-1. 18–81 cells electroporated as described in (D) were used for co-IP using anti-FLAG, followed by IB analysis. The number indicates the quantitative ratios of band intensities obtained from IP versus its corresponding input. Blimp-1, B lymphocyte-induced maturation protein-1; CHX, cycloheximide; DBD, DNA-binding domain; IB, immunoblot; IP, immunoprecipitation; tk, thymidine kinase; UTR, untranslated region; WT, wild type.

In many cases, SUMO modification converts the function of transcription factors from positive to negative regulators [21]. It appears that SUMO-modified Blimp-1 is a more potent transcriptional repressor. In the case of K816R Blimp-1, its reduced transcriptional repression may not result from globally changing subcellular localization, as immunofluorescence staining illustrated that the majority of WT and K816R Blimp-1 had a speckled nuclear distribution pattern and that WT and K816R Blimp-1 did not co-localize with promyelocytic leukaemia protein (supplementary Fig S7 online). It is more likely that the level of HDAC2 that interacted with K816R Blimp-1 was decreased. Although a recent paper suggested that SUMOylation results in proteasomal degradation of Blimp-1 [10], we did not observe that an absence of the SUMOylation site affected Blimp-1 expression. It is possible that the fusion of SUMO1 to the amino terminus of Blimp-1, the approach used in that study, caused the conflicting observation.

In summary, our study here provides a mechanistic insight into the modulation of cell proliferation and plasma cell fate commitment through a SUMO site in the master regulator

Blimp-1, which may provide new strategies for regulating humoral immune responses.

METHODS

Detailed experimental procedures are described in the supplementary information online.

IP and IB analyses. IP and IB analyses were performed as described [19]. Antibodies used in this study are described in the supplementary information online.

Transfection and luciferase reporter assay. The luciferase reporter fused to the *CIITA* promoter III was described previously [7]. *Renilla* luciferase reporter driven by the tk promoter (RL-tk) was used for normalization. Mouse 18–81 pre-B cells were transfected by electroporation; the amounts of DNA were chosen and the procedure performed as described previously [5]. Cells were lysed 24 h after transfection and were used for measurement of firefly luciferase and *Renilla* luciferase activities using the Dual-Luciferase Reporter[®] Assay kit (Promega). The luminescence was detected by TopCount NXT[™] (Perkin Elmer). Fold repression was calculated as described [19].

Generation of retrovirus or lentivirus and virus transduction. The procedure for preparing viral vectors was as described [19]. B cells were transduced with virus, at a multiplicity of infection of 5–10 for lentiviral transduction or 1–5 for retroviral transduction, in the presence of 5 µg/ml of polybrene. Transduced B cells were analysed or sorted by flow cytometry 2–3 days after infection.

Statistics. Statistical significance was determined by two-tailed Student's *t*-tests. $P < 0.05$ was considered significant. Data shown here are the mean $\pm$ s.d. from at least three experiments unless otherwise indicated.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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