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The paracaspase MALT1 cleaves the LUBAC subunit HOIL1 during antigen receptor signaling

Tiphaine Douanne¹,²,³,⁴, Julie Gavard¹,²,³,⁴ and Nicolas Bidère¹,²,³,⁴,*

ABSTRACT
Antigen-receptor-mediated activation of lymphocytes relies on a signalosome comprising CARMA1 (also known as CARD11), BCL10 and MALT1 (the CBM complex). The CBM activates nuclear factor κB (NF-κB) transcription factors by recruiting the ‘linear ubiquitin assembly complex’ (LUBAC), and unleashes MALT1 paracaspase activity. Although MALT1 enzyme shapes NF-κB signaling, lymphocyte activation and contributes to lymphoma growth, the identity of its substrates continues to be elucidated. Here, we report that the LUBAC subunit HOIL1 (also known as RBCK1) is cleaved by MALT1 following antigen receptor engagement. HOIL1 is also constitutively processed in the ‘activated B-cell-like’ (ABC) subtype of diffuse large B-cell lymphoma (DLBCL), which exhibits aberrant MALT1 activity. We further show that the overexpression of MALT1-insensitive HOIL1 mitigates T-cell-receptor-mediated NF-κB activation and subsequent cytokine production in lymphocytes. Thus, our results unveil HOIL1 as a negative regulator of lymphocyte activation and contributes to lymphoma growth, the exact role of HOIL1 remains elusive (Dubois et al., 2014). Additional MALT1 substrates, we performed an in silico analysis of known partners of the CBM complex in the literature. Examination of HOIL1 sequence revealed a putative MALT1 cleavage site at LQR165G (Fig. 1B). The transfection of HEK293T cells with a FLAG-tagged HOIL1 plasmid together with BCL10 and MALT1 resulted in the generation of a COOH-terminal HOIL1 cleavage fragment (HOIL1(C)) of >35 kDa (Fig. 1C). However, replacement of R165 with an alanine or with a glycine residue abolished this cleavage (Fig. 1C). Hence, MALT1 drives HOIL1 processing at R165 when overexpressed.

Because MALT1 catalytic activity is unleashed in the vicinity of the CBM complex (Pelzer et al., 2013; Rebeaud et al., 2008), where the LUBAC is dynamically recruited (Dubois et al., 2014), we next investigated the status of HOIL1 in Jurkat T lymphocytes. Stimulation with either antibodies to CD3 and CD28 or with phorbol 12-myristate 13-acetate (PMA) plus ionomycin, which both
mimic TCR ligation, led to a robust cleavage of HOIL1 (Fig. 2A). Of note, the known MALT1 substrate CYLD (Staal et al., 2011) is processed with similar kinetics. HOIL1 and CYLD, however, remained intact in response to tumor necrosis factor-α (TNFα, also known as TNF), which operates independently of MALT1 (Fig. 2A). Importantly, similar results were obtained with mouse primary T lymphocytes (Fig. 2B). We further observed that FLAG-tagged wild-type (WT) HOIL1 (HOIL1WT–FLAG) and not HOIL1R165A–FLAG was efficiently processed upon TCR stimulation, further confirming R165 as MALT1 cleavage site (Fig. 2C). The silencing of MAL1 and of CARMA1 with small interfering RNA (siRNA) abolished TCR-mediated cleavage of HOIL1 and CYLD (Fig. 2D). This was also true when MALT1 enzyme activity was blocked with the tetrapeptide protease inhibitor zVRPR.fmk or with Mepazine (Nagel et al., 2012; Rebeaud et al., 2014), suggesting that HOIL1 cleavage results from MALT1 processing alone. Our data suggest that HOIL1 impedes NF-κB independently of the LUBAC, as evidenced by co-immunoprecipitation experiments of endogenous SHARPIN in lysates from lymphocytes or from ABC DLBCL cells (Elton et al., 2015). Whereas HOIL1Nter promotes LUBAC-mediated NF-κB signaling, HOIL1Cter thrwarts it (Elton et al., 2015). We therefore assessed their effect on NF-κB activation following TCR engagement. In contrast to full-length HOIL1, its cleaved products had no overt impact on NF-κB signaling (Fig. 3F). Supporting previous reports (Klein et al., 2015; Tokunaga et al., 2009), HOIL1Cter was not part of the LUBAC, as evidenced by co-immunoprecipitation experiments of endogenous SHARPIN in lysates from lymphocytes or from ABC DLBCL cells (Fig. 3G; Fig. S2). In stimulated Jurkat, HOIL1, but not HOIL1Cter, bound to CK1α (also known as CSNK1A1), a kinase that dynamically interacts with the CBM and the LUBAC (Dubois et al., 2014), reinforcing the idea that HOIL1Cter is not part of these complexes (Fig. 3H). Taken together, our results suggest that HOIL1 negatively regulates NF-κB independently of the LUBAC and IKK complex, and is inactivated once cleaved by MALT1 upon TCR engagement.

MALT1 paracaspase activity exerts a central function in optimally orchestrating an immune response (Bornancin et al., 2015; Gewies et al., 2014; Jaworski et al., 2014). Yet, the full spectrum of its substrates continues to be elucidated (Demeyer et al., 2016). We now report that the LUBAC subunit HOIL1 mitigates TCR-mediated NF-κB signaling and is cleaved by MALT1 after the residue R165A, as well as in MALT1-dependent ABC DLBCL cells. Our data suggest that HOIL1 impedes NF-κB independently of IKK, although the exact mechanism remains unclear. In addition to
inactivating the NF-κB negative regulators A20 and RelB (Coomaert et al., 2008; Hailfinger et al., 2011), MALT1 initiates an IKK-independent NF-κB pathway through its own autoproteolysis (Baens et al., 2014) and participates in c-Rel activation (Ferch et al., 2009, 2007). Nevertheless, our data suggests that HOIL1 belongs, together with A20 and RelB (Coornaert et al., 2008; Hailfinger et al., 2011), to a group of proteins that curtail NF-κB when not cleaved by MALT1. In that
sense, defining whether HOIL1 is harmful when left intact in lymphocytes and contributes to the striking phenotype of MALT1 protease-dead mice would be of interest (Bornancin et al., 2015; Gewies et al., 2014; Jaworski et al., 2014). We also provide evidence that exacerbated MALT1 activity in ABC DLBCL cells results in the constitutive cleavage of HOIL1. In addition to interfering with the LUBAC stability (Yang et al., 2014), targeting HOIL1 cleavage might therefore offer a new angle for therapeutic targeting in ABC DLBCL.

Fig. 3. HOIL1 cleavage participates in the optimal activation of NF-κB. (A) NF-κB reporter luciferase assay (mean±s.e.m.; n=3) of cells transfected with 10 µg of plasmids encoding for HOIL1WT, HOIL1R165G or with an empty vector (EV). Cells were stimulated with 0.5 µg ml−1 anti-CD3 plus anti-CD28 (CD3/28), or with 20 ng ml−1 PMA plus 300 ng ml−1 ionomycin (PI or P/I). The inset panel shows the expression of the plasmids when overexpressed in HEK293T cells. Unst, unstimulated. **P<0.01; ***P<0.001; ****P<0.0001 (ANOVA). (B) NF-κB luciferase assay (mean±s.e.m. of triplicate experiments) of Jurkat cells stably expressing HOIL1R165G or an empty vector (EV), and stimulated with 0-20 ng ml−1 PMA plus 300 ng ml−1 ionomycin (PI). **P<0.01; ***P<0.001 (ANOVA). (C) Cells as in B were stimulated for 16 h. IL-2 secretion in the culture supernatants was determined by ELISA. Shown is the mean±s.e.m. of triplicate experiments. **P<0.01 (ANOVA). (D,E) Immunoblots of cells as in B. In E, cells were pretreated with 25 µM of the proteasome inhibitor MG132 for 30 min prior to stimulation. (F) NF-κB luciferase assay (mean±s.e.m.; n=5; ***P<0.001 by ANOVA) of Jurkat cells transfected with the indicated FLAG-tagged plasmids. Cells were stimulated as in A. The inset panel shows the expression of the constructs when overexpressed in HEK293T cells. (G,H) Jurkat lymphocytes were stimulated as in A. Cell lysates were immunoprecipitated (IP) with antibodies to SHARPIN (G) or to CK1α (H), and immunoblotting was performed as indicated. Lys., lysates; Ig, light chain immunoglobulin. The positions of molecular mass markers (kDa) are shown. Data are representative of three independent experiments.
architecture and allow NF-xB signaling (Elton et al., 2015; Klein et al., 2015; Tokunaga et al., 2009). This N-terminal fragment likely maintains the LUBAC activity and mediates aberrant NF-xB in ABC DLBCL (Dubois et al., 2014; Yang et al., 2014). HOIL1\textsuperscript{\textalpha} essentially bears the E3 ligase catalytic activity of HOIL1. Although HOIL1\textsuperscript{\textalpha} restrains the ability of the LUBAC to activate NF-xB when overexpressed in HEK293T (Elton et al., 2015), it had little impact on TCR-mediated NF-xB activation. In keeping with this, we observed that endogenous HOIL1\textsuperscript{\textalpha} is not retained in the LUBAC or the CBM. It also massively accumulates in ABC DLBCL cells, which exhibit aberrant NF-xB activation. Because HOIL1 has been shown to catalyze degradative K48-linked ubiquitylation (Elton et al., 2015), it is tempting to speculate that intact HOIL1 promotes the proteasomal degradation of substrates involved in NF-xB signaling, and that MALT1 cleavage counteracts HOIL1 enzyme activity. Our future work will therefore be aimed at defining the nature of HOIL1 substrates when uncleaved.

**MATERIALS AND METHODS**

**Cell culture and reagents**

Jurkat E6.1, BJ/AB and HEK293T were purchased from ATCC, U2932, RIVA, OCI-Ly3 and SUDHL4 were from DSMZ. OCI-Ly10 and OCI-Ly19, and HBL1 cell lines were kindly given by Karin Tarte (INSERM U917, France) and Martin Dyer (University of Leicester, UK), respectively. Mouse primary T lymphocytes were purified with a pan T cell isolation kit (Miltenyi Biotec) from spleens of C57bl/6 (Janvier). Cells were stimulated with 5 µg ml\(^{-1}\) anti-CD3 plus 1 µg ml\(^{-1}\) anti-CD28 antibodies (both from BD Biosciences), or with 10 ng ml\(^{-1}\) of TNF-α (R&D Systems). MALT1 protease activity was blocked with 75 µM zVRPR.fmk (Enzo Life Sciences), or with 20 µM Mepazine (Chembridge). siRNA against CARMA1 (HS1130975), and MALT1 (HS116800) were from Life Technologies.

**Expression plasmids, transfections and antibodies**

pCMV3flag8HOIL1 was a gift from Martin Dorf (Department of Microbiology and Immunobiology, Harvard Medical School, USA) (Addgene plasmid no. 50016; Fu et al., 2014). HOIL1 was further cloned into a pCDH1-MSCV-EF1α-GreenPuro vector (SB). MALT1-resistant expression mutants (R165A and R165G) were generated by site-directed mutagenesis, and were verified by sequencing (Genomics and Microbiology and Immunobiology, Harvard Medical School, USA) (Elton et al., 2015). HEK293T cells were transfected according to previously described (Bide, N., Ngo, V. N., Lee, J., Collins, C., Zheng, L., Wan, F., Davis, R. E., Lenz, G., Anderson, D. E., Arnout, D. et al. (2009). Casein kinase 1alpha governs antigen-receptor-induced NF-kappaB activation and human lymphoma cell survival. Nature 458, 92-96.

**Immunoblotting and immunoprecipitation**

Stimuli were washed away with ice-cold PBS prior to cell lysis with TNT buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% Igepal, 2 mM EDTA, protease inhibitors (Thermo Fisher Scientific)] for 30 min on ice. Samples were cleared by centrifugation at 9000 \( \times \) g for proteins concentration was determined by a BCA assay (Thermo Fisher Scientific). 5–10 µg proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare). Immunoprecipitation experiments were performed as previously described (Bide, N. et al., 2009; Dubois et al., 2014). Briefly, samples lysed with TNT buffer were precleared with Protein-G–agarose (Sigma) for 30 min and then incubated with 5 µg antibodies and Protein-G–agarose for 1–2 h at 4°C. After four washes, proteins were denaturated and resolved by SDS-PAGE.

**Statistical analysis**

Statistical significance was assessed with two-way ANOVA tests with post hoc Tukey’s analysis (Prism GraphPad Software), and P values are indicated in the figure legends.

**Competition interests**

The authors declare no competing financial interests.

**Authors contribution**

T.D. designed the research, conducted experiments, analyzed the data; J.G. analyzed the data; and N.B. conceived the project, designed and performed experiments, analyzed the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

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**Supplementary information**

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