A catalytic-independent role for the LUBAC in NF-κB activation
upon antigen receptor engagement and in lymphoma cells

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Running head: LUBAC function in lymphocytes and DLBCL
Scientific category: Lymphoid Neoplasia
Key points

- LUBAC elements HOIP and SHARPIN participate to T-cell receptor-mediated NF-κB activation independently of HOIP catalytic activity.
- LUBAC silencing compromises constitutive NF-κB activation and cell survival in ABC DLBCL lines.

Abstract

Antigen receptor-mediated nuclear factor κB (NF-κB) activation relies on the formation of a large multi-protein complex that contains CARMA1, BCL10 and MALT1 (CBM complex). This signaling cascade is hijacked in the activated B-cell like subgroup of diffuse large B cell lymphoma (ABC DLBCL) to drive aberrant NF-κB activation, thereby promoting cell survival and propagation. Using an unbiased proteomic approach, we screened for additional components of the CBM in lymphocytes. We found that the linear ubiquitin chain assembly complex (LUBAC), which was previously linked to cytokine-mediated NF-κB activation, dynamically integrates the CBM and marshals NF-κB optimal activation following antigen receptor ligation independently of its catalytic activity. The LUBAC also participates to preassembled CBM complex in cells derived from ABC DLBCL. Silencing the LUBAC reduced NF-κB activation and was toxic in ABC DLBCL cell lines. Thus, our findings reveal a role for the LUBAC during lymphocyte activation and in B cell malignancy.
Introduction
The Activated B-cell like (ABC) subtype of diffuse large B-Cell lymphoma (DLBCL) constitutes the most aggressive DLBCL entity. In contrast to germinal center B-cell like (GCB) subtype of DLBCL, ABC DLBCL survival and proliferation requires the constitutive activation of NF-κB transcription factors, which often results from somatic mutations in CD79B, CARD11 (also called CARMA1), MYD88, and TNFAIP3 genes. New perspectives for treatments restricted to the lymphoid compartment came from genomic-scale RNA interference screens, which unveiled that ABC DLBCL exploited a multi-protein complex that contains CARMA1, BCL10 and MALT1 (CBM complex) normally engaged in conveying NF-κB following antigen receptors engagement. Within the CBM, Lys-63 (K63)-linked ubiquitylation of BCL10 and MALT1 ensures the recruitment and the activation of inhibitor of NF-κB kinase (IKK, composed of IKKα, IKKβ and NEMO) through IKKβ phosphorylation and NEMO poly-ubiquitylation. IKK subsequently authorizes NF-κB to shuttle in the nucleus and exert its transcriptional activity by phosphorylating its cognate inhibitors (IkBs), which further undergo proteasomal degradation.

Here, we screened for additional CBM partners and identified the linear ubiquitin chain assembly complex (LUBAC), which comprises two E3 ligases HOIL-1 and HOIP, and SHARPIN. Although this tripartite complex was previously linked to cytokine-, bacteria-, and genotoxic stress-mediated NF-κB signaling, its involvement in adaptive immunity remains unknown. We now show that the LUBAC binds to the CBM and governs NF-κB activation upon antigen receptor engagement independently of HOIP catalytic activity. In ABC DLBCL cells, LUBAC is integral to preassembled CBM and its knockdown is lethal as it hampers aberrant NF-κB activity.

Methods
Cells and Reagents are described in supplemental Methods. Knockdown was achieved by transfecting siRNA, or via retroviral infection of shRNA (supplemental Methods). Confocal microscopy, luciferase assays, ELISA, immunoblots and immunoprecipitations were performed as previously described. Fraction of GFP-positive cells over time following retroviral infection and DiOC6 staining were determined by flow cytometry. This study involved in vitro experiments with primary human T lymphocytes from healthy volunteers donors. Buffy coat from healthy
volunteers were obtained from the “Etablissement Français du Sang” (EFS-Ile de France) according to an agreement between EFS and INSERM (convention 09EFS024, code 990029, SAP19DIV0336). The relative documents to patient free and inform consent, as well as the respect of confidentiality and privacy protection are handled by EFS. This study was conducted in accordance with the Declaration of Helsinki.

**Results and Discussion**

Promptly following antigen receptor engagement, the kinase CK1α binds the CBM and participates in NF-κB signaling. To uncover new NF-κB modulators, we performed a proteomic screen by mass spectrometry of CK1α partners in Jurkat T lymphoblastoid cells stimulated with PMA plus ionomycin (P/I) to mimic T-cell receptor (TCR) ligation. In addition to known interactants, two peptides covering HOIP were isolated (Table S1 and Figure S1). As expected, HOIP was constitutively tethered to SHARPIN and HOIL-1, regardless of stimulation (Figure S2). Coimmunoprecipitation experiments showed that LUBAC and CBM components bound to CK1α following TCR engagement (Figure 1A, S3 and S4). CARMA1 and HOIP also precipitated with BCL10/MALT1 heterodimers in stimulated cells. Likewise, SHARPIN dynamically recruited BCL10, MALT1, CK1α and NEMO. Corroborating these findings, LUBAC and CBM components co-precipitated with NEMO upon stimulation (Figure 1A). Similar results were obtained in P/I-stimulated BJAB B cells (Figure S5). Hence, LUBAC components integrate a signalosome containing both CBM and IKK complexes following antigen receptor engagement.

We next investigated whether the LUBAC participates in antigen receptor-mediated NF-κB activation. First, HOIP knockdown with three individual siRNA sequences curtailed both TCR- and TNFα-mediated NF-κB activation in a luciferase gene reporter assay (Figure 1B). TCR-driven redistribution of NF-κB p65 into the nucleus and downstream IL-2 production were blunted in HOIP-silenced human primary peripheral blood mononuclear cells and in Jurkat lymphocytes (Figures 1 C-F, S6 and S7). In addition, IKK and IκBα phosphorylation, which reflects NF-κB activation, was diminished (Figure 1G). Similarly, IκBα phosphorylation and degradation were reduced in HOIP-silenced BJAB cells stimulated with P/I (Figure 1H). Even though CK1α normally bound the CBM without HOIP (Figure S8), NEMO interaction with
BCL10 or MALT1, and NEMO poly-ubiquitylation were markedly decreased (Figure 1I and S9). LUBAC stability is compromised in HOIL-1- or SHARPIN-deficient cells, however this was partly the case when siRNA were used (Figure S10). We found that silencing of SHARPIN or HOIP diminished TCR- and TNFα-mediated NF-κB activation (Figure S10). In contrast to fibroblasts in which it participates to TNFα-mediated NF-κB activation, HOIL-1 impact in Jurkat cells was only modest, suggesting a cell type-dependent modus operandi. Collectively, our data indicate that silencing HOIP perturbs the interaction between CBM and IKK complexes, thus diminishing IKK modifications and ensuing NF-κB activation.

LUBAC catalyzes linear Met-1 (M1)-ubiquitin chains that accumulate within cytokines receptors-driven signalosomes to favor IKK/NF-κB signaling. While TNFα treatment efficiently assembled M1-ubiquitin chains that bound to TNFR and SHARPIN, little signal was detected following TCR stimulation (Figure 1J and S11). Moreover, TNFα- but not TCR-mediated NF-κB activation was boosted when the negative regulator of LUBAC catalytic activity OTULIN20,21,24 was silenced (Figure S12). Last, plasmids that contain an shRNA against human HOIP followed by a RNAi-resistant HOIP wild type (HOIP-WT) or a catalytically inactive mutant (C699S/C702S, HOIP-CS) allowed us to simultaneously silence and reconstitute HOIP in cells. Although important for TNFα signaling, HOIP catalytic activity was dispensable for NF-κB activation upon TCR engagement (Figure 1K and S13). Hence, HOIP operates independently of its catalytic activity to convey NF-κB activation upon TCR engagement.

To evaluate the LUBAC contribution to ABC DLBCL piracy of antigen receptor-mediated NF-κB signaling pathway, we first examined its participation to constitutively preassembled CBM complex. SHARPIN co-precipitated with the CBM in ABC DLBCL but not in GCB DLBCL cell lines (Figure 2A and S14). In addition, CBM and LUBAC elements were also found in CK1α pull-downs in ABC DLBCL lines (Figure S14). Next, DLBCL lines were retrovirally infected to express shRNA against HOIP together with GFP. As expected, BJAB cells stably expressing HOIP shRNA displayed reduced IκBα phosphorylation upon P/I treatment (Figure S15). The expression of HOIP shRNA decreased the fraction of GFP-positive cells in OCI-Ly10 cells, but not in the BJAB cells over time (Figure 2B), suggesting that HOIP knockdown is
selectively toxic in ABC DLBCL. Similar results were obtained with the ABC DLBCL U2932 line (Figure S15). Knocking down with siRNA the LUBAC components, individually or collectively significantly, albeit modestly, increased apoptosis in OCI-Ly3 and OCI-Ly10, as measured by mitochondrial transmembrane potential dissipation, phosphatidylserines exposure and propidium iodide incorporation (Figure 2C-E and S16). The same was true when CARMA1 was silenced (Figure S16). Consonant with an aberrant NF-κB activity in ABC DLBCL cells, IκBα was constitutively degraded, and this was reversed when LUBAC components were silenced (Figure 2F). By contrast, no overt changes in IκBα levels were observed in GCB DLBCL cells. Furthermore, p65 no longer accumulated in the nucleus of LUBAC-silenced ABC DLBCL lines (Figure 2G, H and S17). Altogether, our data suggest that the LUBAC is part of constitutively preassembled NF-κB activating signalosome in ABC DLBCL lines, and contributes to NF-κB-dependent cell survival.

In summary, we provide evidence that the LUBAC favors the association of CBM and IKK complexes and participates in NF-κB activation following TCR stimulation. This function we ascribe to the LUBAC expands its pivotal role in fine-tuning NF-κB to adaptive immunity. Although it deserves further investigation, our data suggests that HOIP rather functions as an adaptor upon TCR engagement since its catalytic activity is dispensable for NF-κB signaling. In line with this, B-cell receptor-mediated NF-κB activation occurs in splenocytes that lack HOIP catalytic activity. We also show that the LUBAC is integral to preassembled CBM complex in ABC DLBCL lines, and guarantees cell survival by maintaining constitutive NF-κB activity. Hence, strategies aimed at targeting the LUBAC might be relevant in the context of ABC DLBCL.
Acknowledgments

We thank H. Walczak and S. Kupka (UCL Cancer Institute, London) for providing reagents, N. Cordeiro and A. Le Moignic for helpful assistance. This work was supported by grants from the French National Research Agency (ANR-10-JCJC-1306), Fondation ARC, Ligue Nationale contre le Cancer, Fondation pour la Recherche Médicale, and Institut National du Cancer (INCA_5608). SMD is supported by a fellowship from Université Paris Sud.

Authorship Contributions

SMD designed the research, conducted experiments, analyzed the data, and wrote the manuscript; CA, HML, CL and ES designed and performed experiments, and analyzed the data; YW, TF, KT and ZJC provided essential tools; JG analyzed the data; and NB conceived the project, analyzed the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
References

Figures Legend

Figure 1. The LUBAC binds to CBM and IKK complexes and participates to antigen receptor-mediated NF-κB activation. (A) Jurkat T lymphocytes were stimulated with 20 ng.ml⁻¹ PMA plus 300 ng.ml⁻¹ ionomycin (P/I) or with 1 μg.ml⁻¹ anti-CD3 and anti-CD28 (CD3/28) for 0, 10 and 20 min. Cell extracts were prepared and immunoprecipitated (IP) with antibodies to CK1α, BCL10, SHARPIN or NEMO, and immunoblots (IB) were performed as indicated. Closed circle and asterisk indicate the protein of interest and nonspecific bands, respectively; Ub, ubiquitin. Molecular weight markers (kDa) are indicated. (B) Jurkat cells were transfected with a nonspecific control siRNA (NS) or with three individual siRNA against HOIP (HOIP.1-3). Three days later, cells were transfected with p-NF-κB-Luc reporter and pTKRL control plasmids for an additional 24 hours, prior stimulation with 0.5 μg.ml⁻¹ anti-CD3 and anti-CD28 (3/28), or with P/I as in (A), or with 10 ng.ml⁻¹ TNFα for 6 hours. Histograms represent the mean ± SEM of three independent experiments. **P < 0.001; ****P < 0.0001 compared to cells transfected with NS siRNA [analysis of variance (ANOVA)]. ns, non significant; RLU, Relative Light Units. Inset IB show the level of protein knockdown. (C) NS- and HOIP-silenced Jurkat T cells were stimulated with 1 μg.ml⁻¹ anti-CD3 and anti-CD28 (CD3/28) for 0, 30, and 60 min. Nuclear and cytosolic fractions were purified and IB were performed as indicated. Tubulin and PARP served as loading controls. (D, E) Confocal microscopy micrographs of NF-κB p65 (green) in Jurkat T cells transfected with a control nonspecific (NS) or with HOIP siRNA and stimulated with 1 μg.ml⁻¹ anti-CD3 and anti-CD28 (CD3/28) for 1 hour. Nuclei were also stained with 4’,6-diamidino-2-phenylindole (DAPI in blue). In (E), the percentage of cells with nuclear p65 was calculated by scoring >150 cells for each sample. Shown is the mean ± SEM from three independent experiments (ns, non significant; **** P<0.0001 by ANOVA). (F) NS- or RNF31-silenced human peripheral blood mononuclear cells (PBMC) were stimulated with 1 and 100 ng.ml⁻¹ anti-CD3 and anti-CD28 for 16h. IL-2 secretion in the culture supernatants was determined by ELISA. Histograms represent the mean ± SEM of three independent experiments (ns, non significant; **** P<0.0001 by ANOVA). Inset immunoblots show the knockdown efficiency. (G) Cell extracts from NS- and HOIP-silenced Jurkat cells stimulated as in (C) for 0, 10, 20 and 30 min were analyzed by IB. (H) NS- and HOIP-silenced BJAB B cells were stimulated with 10 ng.ml⁻¹ PMA plus 300 ng.ml⁻¹ ionomycin (P/I) for 0, 10, 20 and 30 min. Cell lysates were prepared and analyzed by IB as indicated. (I) NEMO binding to HOIP, BCL10 and
MALT1 was assessed by IP/IB in NS- and HOIP-silenced Jurkat cells stimulated with P/I as in (A). Lys., Lysate. (J) Cell extracts from Jurkat cells stimulated with (P/I) as in (A) or with 10 ng.ml⁻¹ TNFα for 0, 10 and 20 min were IP with an antibody against M1-linked ubiquitin. IB were performed as indicated. Lys., lysates. The asterisk shows the anti-M1-ubiquitin. (K) Jurkat cells were retrovirally infected to express GFP, GFP plus an shRNA against human HOIP (HOIPsh), RNAi-resistant HOIP-WT plus HOIPsh, and catalytically inactive HOIP (HOIP-CS) plus HOIPsh. Cells were stimulated as in (J) for 30 min and analyzed as in (E). Shown is the mean ± SEM from three independent experiments (ns, non significant; *** P<0.001; **** P<0.0001 by ANOVA). Cell lysates were prepared and IB performed as indicated. Molecular weight markers (kDa) are shown. Data are representative of two (I) or at least three independent experiments (A-H, J and K).

Figure 2. The LUBAC contributes to aberrant activation of NF-κB and survival of ABC DLBCL lines. (A) Cell lysates from ABC DLBCL lines (OCI-Ly3 and OCI-Ly10) and from GCB DLBCL lines (OCI-Ly7 and OCI-Ly19) were immunoprecipitated (IP) with antibodies to SHARPIN and immunoblots (IB) were performed as indicated. The asterisk indicates nonspecific bands. Lys., lysates. (B) DLBCL lines were infected with a retrovirus that expressed a control nonspecific (NSsh) or a HOIP (HOIPsh) shRNA together with GFP. Shown is the fraction of GFP-positive cells over time relative to day 4 post-infection. (C) Lysates from OCI-Ly3, OCI-Ly10, OCI-Ly19, and BJAB cells transfected with control nonspecific (NS) siRNA or with siRNA against the LUBAC (HOIP plus HOIL-1 plus SHARPIN) for 48h, were analyzed by IB as indicated. (D, E) DLBCL lines were transfected as in (C) for 72h, and stained with DiOC₆ and analyzed by flow cytometry. Histograms in (D) represent the fold of NS-treated sample. Shown is the mean ± SEM from three independent experiments (ns, non significant; **P < 0.001; ****P < 0.0001 by ANOVA). (F) IκBα levels were assessed by IB 72 and 96h post-transfection in lysates as in (C). (G, H) ABC DLBCL lines (OCI-Ly3 and OCI-Ly10) and GCB DLBCL lines (OCI-Ly19 and BJAB) were transfected as in (C) for 72h. NF-κB p65 subcellular location was examined by confocal microscopy. Nuclei were illuminated with 4',6-diamidino-2-phenylindole (DAPI). Histograms in (H) show the mean ± SEM from three independent experiments (ns, non significant; **** P<0.0001 by ANOVA). Data are representative of three independent experiments.
Figure 1
Figure 2
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