LYMPHOID NEOPLASIA

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

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**Key Points**

- LUBAC elements HOIP and SHARPIN participate in 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.

**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 signalosome is pirated 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 in 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. (Blood. 2014;123(14):2199-2203)**

**Introduction**

The activated B-cell like (ABC) subtype of diffuse large B-cell lymphoma (DLBCL) constitutes the most aggressive DLBCL entity.1 In contrast to germinal center B-cell like (GCB) subtype of DLBCL, ABC DLBCL survival and proliferation require the constitutive activation of nuclear factor κB (NF-κB) transcription factors, which often results from somatic mutations in CD79B, CARD11 (also called CARMA1), MYD88, and TNFAIP3 genes.2 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 receptor engagement.3–5 Within the CBM, Lys-63 (K63)-linked ubiquitylation of BCL10 and MALT1 ensures the recruitment and activation of the inhibitor of NF-κB kinase (IKK, composed of IKKα, IKKβ, and NEMO) through IKKβ phosphorylation and NEMO poly-ubiquitylation.6–9 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.10 Here, we screened for additional CBM partners and identified the linear ubiquitin chain assembly complex (LUBAC), which comprises 2 E3 ligases, HOIL-1 and HOIP, and SHARPIN.11 Although this tripartite complex was previously linked to cytokine-, bacteria-, and genotoxic stress-mediated NF-κB signaling,12–17 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, the LUBAC is integral to preassembled CBM and its knockdown is lethal, as it hampers aberrant NF-κB activity.

**Study design**

Cells and reagents are described in the supplemental Methods (available on the Blood Web site). Knockdown was achieved by transfecting small interfering RNA (siRNA), or via retroviral infection of small hairpin RNA (shRNA) (supplemental Methods). Confocal microscopy, luciferase assays, enzyme-linked immunosorbent assay, immunoblots, and immunoprecipitations were performed as previously described.1 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 volunteer donors. Buffy coats from healthy volunteers were obtained from the “Établissement Français du Sang” (EFS-Ile de France) according to an agreement between EFS and Institut National de la Santé et de la Recherche Médicale (INSERM) (convention 09EFS024, code 990029, SAP19DIV036). The documents relative to patient free and informed 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 CK1\(\alpha\) binds the CBM and participates in NF-\(\kappa\)B signaling. To uncover new NF-\(\kappa\)B modulators, we performed a proteomic screen by mass spectrometry of CK1\(\alpha\) partners in Jurkat T lymphoblastoid cells stimulated with phorbol 12 myristate 13-acetate plus ionomycin (P/I) to mimic T-cell receptor (TCR) ligation. In addition to known interactants, 2 peptides covering HOIP were isolated (supplemental Table 1; supplemental Figure 1). As expected,\textsuperscript{13,15,16} HOIP was constitutively tethered to SHARPIN and HOIL-1, regardless of stimulation (supplemental Figure 1). Coimmunoprecipitation experiments showed that LUBAC and CBM components bound to CK1\(\alpha\) following TCR engagement (Figure 1A; supplemental Figures 3 and 4). CARMA1 and HOIP also precipitated with BCL10/MALT1 heterodimers in stimulated cells. Likewise, SHARPIN dynamically recruited BCL10, MALT1, CK1\(\alpha\), and NEMO. Corroborating these findings, LUBAC and CBM components coprecipitated with NEMO upon stimulation (Figure 1A). Similar results were obtained in P/I-stimulated BJAB B cells (supplemental Figure 5). 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 3 individual siRNA sequences curtailed both TCR- and tumor necrosis factor (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 interleukin-2 production were blunted in HOIP-silenced human primary peripheral blood mononuclear cells and in Jurkat lymphocytes (Figure 1C-F; supplemental Figures 6 and 7). In addition, IKK and IkBα phosphorylation, which reflects NF-κB activation, was diminished (Figure 1G). Similarly, IkBα 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 (supplemental Figure 8), NEMO interaction with BCL10 or MALT1, and NEMO poly-ubiquitylation were markedly decreased (Figure 1I; supplemental Figure 9). LUBAC stability is compromised in HOIL-1- or SHARPIN-deficient cells; however, this was partly the case when siRNAs were used (supplemental Figure 10). We found that silencing of SHARPIN or HOIP diminished TCR- and TNFα-mediated NF-κB activation (supplemental Figure 10). In contrast to fibroblasts in which it participates in 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 cytokine receptors-driven signalosomes to favor IKK/NF-κB signaling. Although TNFα treatment efficiently assembled M1-ubiquitin chains that bound to TNFR and SHARPIN, little signal was detected following TCR stimulation (Figure 1J; supplemental Figure 11). Moreover, TNFα- but not TCR-mediated NF-κB activation was boosted when the negative regulator of LUBAC catalytic activity OTULIN was silenced (supplemental Figure 12). Last, plasmids that contain an shRNA against human HOIP followed by a RNAi-resistant HOIP wild type 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; supplemental Figure 13). 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 in the constitutively preassembled CBM complex. SHARPIN co-precipitated with the CBM in ABC DLBCL but not in GCB DLBCL cell lines (Figure 2A; supplemental Figure 14). In addition, CBM and LUBAC elements were also found in CK1α pull-downs in ABC DLBCL lines (supplemental Figure 14). Next, DLBCL lines were retrovirally infected to express shRNA against HOIP together with GFP. As expected, BJAB cells stably expressing HOIP shRNA displayed reduced IkBα phosphorylation upon P/I treatment (supplemental Figure 15). 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 (supplemental Figure 15). 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; supplemental Figure 16). The same was true when CARMA1 was silenced (supplemental Figure 16). Consonant with an aberrant NF-κB activity in ABC DLBCL cells, IkBα was constitutively degraded, and this was reversed when LUBAC components were silenced (Figure 2F). By contrast, no overt changes in IkBα levels were observed in GCB DLBCL cells. Furthermore, p65 no longer accumulated in the nucleus of LUBAC-silenced ABC DLBCL lines (Figure 2G-H; supplemental Figure 17). 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 suggest that HOIP rather functions as an adaptor upon TCR engagement, because 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.

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Authorship
Contribution: S.M.D. designed the research, conducted experiments, analyzed the data, and wrote the manuscript; C.A., H.M.L., C.L., and E.S. designed and performed experiments and analyzed the data; Y.W., T.F., K.T., and Z.J.C. provided essential tools; J.G. analyzed the data, and wrote the manuscript; C.A., H.M.L., C.L., and A. Le Moignic for helpful assistance.

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