Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of tumor B-cells within the blood and tissues [1]. Patients with unmutated (U-CLL) or mutated (M-CLL) IGHV genes typically have a progressive or indolent disease, respectively. However, B-cell receptor (BCR) signaling adds a further complexity and is pivotal to CLL pathogenesis, promoting tumor survival, proliferation, and consequently tumor progression [2]. Indeed, inhibition of this pathway has revolutionized clinical responses in patients [3].

Macrophagy (herein Autophagy) maintains cellular homeostasis through bulk protein degradation. During the autophagy process LC3 (microtubule associated protein 1 light chain 3) and GABARAP (GABA type A receptor proteins) family proteins, ATG3 and ATG7 are essential for autophagosome formation, autophagosome-lysosome fusion, and cargo degradation (Supplementary Fig. S1) [4]. In CLL, high basal expression of autophagy genes, BECN1, PIK3C3, and PIK3R4, have been associated with a shorter time-to-first treatment (TTFT) and worse overall survival in patients [5]. Furthermore, BECN1 and ATG5 RNA expression have been shown to correlate with poorer clinical outcome [6] and autophagy was shown to reduce therapy-induced apoptosis [7]. However, how BCR activation regulates autophagy in CLL has not been previously evaluated.

Evaluation of basal autophagy-protein levels demonstrated significantly greater LC3B-II, GABARAPL2, ATG3, and ATG7 in CLL cases (Supplementary Table S1; for antibody information, see Supplementary Table S2) compared with healthy donor B-cells (HDB) (Fig. 1a and Supplementary Fig. S2A). The basal levels of LC3B-II, but not GABARAPL2, ATG3, or ATG7, were significantly greater still in U-CLL compared with M-CLL (Fig. 1b and Supplementary Fig. S2B). In addition, LC3B-II and ATG3 levels, but not GABARAPL2 or ATG7, associated with BCR signaling capacity (Fig. 1c and Supplementary Fig. S2C). With significantly greater levels of LC3B-II and ATG3 observed within U-CLL compared with MCLL-LS due to the contrasting extremes of anti-IgM-mediated signaling between these two subgroups. M-CLL-S samples that retained anti-IgM signaling capacity expressed LC3B-II at an intermediate level between U-CLL and M-CLL-LS cases (Fig. 1c), suggesting LC3B-II levels were influenced by both inherent BCR signaling and the cells’ origin. Indeed, greater LC3B-II levels were associated with a
Characterization of autophagy-marker protein levels at baseline and following BCR engagement. a Protein was extracted from snap-frozen PBMCs isolated from CLL patients or HDB purified by negative selection. The level of LC3B-II \((n=43)\), GABARAPL2 \((n=42)\), ATG3 \((n=42)\), and ATG7 \((n=40)\) was quantified by immunoblotting and normalized protein levels relative to the Hsc70 loading control are shown. Mean values are indicated. A Mann–Whitney test was used for statistical analysis. b Basal LC3B-II protein levels in CLL samples \((n=43)\) divided by IGHV mutational status into mutated (M-CLL) and unmutated (U-CLL) cases, and (c) BCR signaling capacity defined by U-CLL, M-CLL signallers (M-CLL-S) \((>5\% \text{ Ca}^{2+} \text{ flux})\), and M-CLL low signallers (M-CLL-LS) \((\leq 5\% \text{ Ca}^{2+} \text{ flux})\).

![Graphs](image)

Fig. 1 Characterization of autophagy-marker protein levels at baseline and following BCR engagement. a Protein was extracted from snap-frozen PBMCs isolated from CLL patients or HDB purified by negative selection. The level of LC3B-II \((n=43)\), GABARAPL2 \((n=42)\), ATG3 \((n=42)\), and ATG7 \((n=40)\) was quantified by immunoblotting and normalized protein levels relative to the Hsc70 loading control are shown. Mean values are indicated. A Mann–Whitney test was used for statistical analysis. b Basal LC3B-II protein levels in CLL samples \((n=43)\) divided by IGHV mutational status into mutated (M-CLL) and unmutated (U-CLL) cases, and (c) BCR signaling capacity defined by U-CLL, M-CLL signallers (M-CLL-S) \((>5\% \text{ Ca}^{2+} \text{ flux})\), and M-CLL low signallers (M-CLL-LS) \((\leq 5\% \text{ Ca}^{2+} \text{ flux})\).
overlap in the anti-IgM-mediated LC3B-II levels was identified between IGHV subsets that maybe a result of M-CLL cases with active BCR signaling. Compared with anti-IgM, anti-IgD-induced signaling is short-lived in CLL cases [8]. Therefore, although significant increases in LC3B-II were observed following bead-bound anti-IgD treatment at all time points, with trends for increased GABARAPL2, in most instances these increases were smaller than that observed with anti-IgM (Supplementary Fig. S5D). Similarly, a small but significant increase in ATG7 levels were observed at 24 h with bead-bound anti-IgD, with only a trend for increased ATG3 expression at 24 h (Supplementary Fig. S5D).

We subsequently characterized the signaling pathways involved in BCR-mediated autophagy (Supplementary Fig. S6). Phospho-ERK and -AKT levels increased 15 min post stimulation with bead-bound anti-IgM or anti-IgD, which is indicative of active BCR signaling. Interestingly, anti-IgM and anti-IgD increased phospho-p70 S6 kinase levels at 6 h, indicating active mammalian target of rapamycin signaling, despite an induction of LC3B-II expression, suggesting activation of non-canonical autophagy [9]. At 24 h, anti-IgM- and anti-IgD-dependent increases in LC3B-II, p62, and phospho-ATG13 suggested simultaneous activation of canonical and non-canonical autophagy [9, 10].

Next, we assessed autophagosome formation using LC3B puncta formation by immunofluorescence [11]. Basal autophagy was observed in cells treated with bead-bound control antibody (Supplementary Fig. S7A, B), whereas treatment with bead-bound anti-IgM increased LC3B puncta to levels above that seen in the control (Supplementary Fig. S7A, B).

To confirm that bead-bound anti-IgM-mediated autophagy was BCR-mediated and not a bead-dependent affect, CLL cells were treated with bead-bound or soluble anti-IgM in the presence or absence of the autophagosome inhibitor hydroxychloroquine (HCQ). Both soluble and bead-bound anti-IgM significantly increased LC3B-II and GABARAPL2 levels compared with the control (Supplementary Fig. S8A, B), confirming that the induction of autophagy proteins was mediated by BCR engagement and was not a bead-dependent effect. Treatment with soluble anti-IgM resulted in smaller autophagy protein increases in the same CLL samples compared with bead-bound anti-IgM, likely due to the relatively higher BCR signal strength and duration with bead-bound versus soluble anti-IgM [12].

To determine whether the anti-IgD-dependent regulation of autophagy was B-cell or tumor specific, we treated HDB with bead-bound or soluble anti-IgM or anti-IgD for 24 h. Bead-bound anti-IgM, and to a lesser extent anti-IgD, promoted significant increases in LC3B-II levels (Supplementary Fig. S9A, B), suggesting that this phenomenon was B-cell and not tumor-specific. However, in contrast to CLL, these changes were not consistently replicated in the other autophagy markers examined.

To determine whether BCR engagement blocked LC3B-II degradation or induced autophagosome formation, we assessed autophagic flux in response to bead-bound anti-IgM using immunoblotting in the presence or absence of HCQ as described in the autophagy guidelines [11]. CLL cells treated with HCQ accumulated LC3B-II in a concentration-dependent manner, which was further augmented with bead-bound anti-IgM (Fig. 2a). The LC3B-II increases were not a result of HCQ-mediated effects on BCR signaling (Supplementary Fig. S10) and also occurred in a time-dependent manner at the RNA level (Supplementary Fig. S11A). Comparatively, bead-bound anti-IgD stimulation produced similar but much smaller responses than anti-IgM (Supplementary Fig. S11B). These data confirm that BCR engagement does not block autosomal degradation but induces the expression of autophagy-associated genes leading to increased autophagic flux.

Next, we used interleukin (IL)-4 and BCR kinase inhibitors to confirm the role of BCR signaling in the regulation of BCR-mediated autophagy in CLL. We previously demonstrated that IL-4 induced surface IgM (sIgM) expression and subsequent downstream signaling [13]. IL-4 treatment significantly increased sIgM expression as previously demonstrated and augmented anti-IgM-dependent LC3B-II levels (Fig. 2b and Supplementary Fig. S12A, B). These effects appeared to be BCR-mediated, as IL-4 had no substantive effect on LC3B-II levels alone, even in the presence of HCQ. Next, we inhibited BCR signaling with tamatinib (SYK) and Ibrutinib (BTK), and observed the effect on LC3B-II levels. Both inhibitors significantly reduced LC3B-II to basal levels (Fig. 2c and Supplementary Fig. S12C). These data confirm the role of BCR signaling in the regulation of autophagy in CLL.

Previous studies have shown a role for autophagy in resistance to venetoclax-mediated killing in follicular lymphoma [14]. Therefore, we hypothesized that autophagy inhibitors may synergize with established therapies to inhibit basal, BCR-induced, or therapy-induced autophagy in CLL. To address this, we examined the effect on CLL cell viability of combining the autophagy inhibitor, VPS34-IN1, with venetoclax following bead-bound anti-IgM treatment. We observed significant synergy between VPS34-IN1 and venetoclax compared with single-agent treatment (Fig. 2d, e). Importantly, VPS34-IN1 inhibited BCR-mediated LC3B-II increases but had no effect on BCR signaling (Supplementary Fig. S13A, B), and both ABT-199 and VPS34-IN1-mediated cell death was largely caspase-dependent (Fig. 2d). These data indicate a protective cellular effect of BCR-mediated autophagy in CLL and highlight the therapeutic potential of inhibiting autophagy pathways to promote greater CLL cell killing.
Fig. 2 BCR-mediated autophagy is dependent on BCR signaling and provides a survival advantage to CLL cells. a CLL samples \((n = 11)\) were treated with bead-bound isotype control antibody (IC) or anti-IgM with or without HCQ at indicated concentrations for 24 h and LC3B-II levels evaluated by immunoblotting. A representative immunoblot is shown. Blots were quantified and the mean fold change \((\pm\text{SEM})\) in LC3B-II level with each treatment vs. IC without HCQ is shown in the accompanying graph. A Wilcoxon’s matched-pairs signed-rank test was used for statistical analysis. b CLL samples \((n = 6)\) were treated with or without IL-4 (10 ng/ml), in the presence or absence of HCQ, for 24 h before treatment with bead-bound IC or anti-IgM for 24 h. LC3B-II levels were evaluated by immunoblotting and the mean fold change \((\pm\text{SEM})\) in LC3B-II levels with each treatment vs. IC without HCQ is shown. A Wilcoxon’s matched-pairs signed-rank test was used for statistical analysis. c CLL samples \((n = 10)\) were treated with HCQ and a SYK (t amatinib; Tam) or BTK (ibrutinib; Ibr) inhibitor (both 5 \(\mu\text{M}\)) for 1 h before stimulation with bead-bound IC or anti-IgM for 24 h and the LC3B-II level evaluated by immunoblotting. Hsc70 was used as a loading control. A representative immunoblot is shown. Blots were quantified and the mean fold change \((\pm\text{SEM})\) in LC3B-II level with each treatment vs. IC DMSO is shown in the accompanying graph. A Wilcoxon’s matched-pairs signed-rank test was used for statistical analysis. d CLL samples were treated for 6 h with bead-bound IC or anti-IgM before treatment with autophagy inhibitor, VPS34-IN1 (3 \(\mu\text{M}\)), either alone or in combination with venetoclax (5 \(\text{nM}\) \(n = 6\), or 10 \(\text{nM}\) \(n = 8\) where shown) for 24 h. All conditions were carried out with or without Q-VD-Ph (10 \(\mu\text{M}\)) to identify caspase-dependent drug-mediated cell killing. Cell viability was assessed by the CellTiter-Glo Cell Viability Assay. The mean \((\pm\text{SEM})\) percentage of viable cells relative to IC is shown. A Wilcoxon’s matched-pairs signed-rank test was used for statistical analysis. e Synergy between VPS34-IN1 (3 \(\mu\text{M}\)) and venetoclax (5 nm, left and 10 nm, right) was evaluated as detailed in the Supplementary Materials and Methods. XY line, observed survival = expected survival. Points below the line, synergistic interactions; points above the line, additive interactions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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References