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Dysregulation of autophagy in human follicular lymphoma is independent of overexpression of BCL-2

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ABSTRACT

Overexpression of the anti-apoptotic protein BCL-2 is characteristic of human follicular lymphoma (FL) and some cases of diffuse large B cell lymphoma (DLBCL). We aimed to determine autophagy status in primary FL and DLBCL samples and the BCL-2⁺/BCL-2⁻ lymphoma cell lines using both autophagy PCR array and tissue microarray (TMA). A greater number of autophagy machinery genes were up-regulated in the BCL-2⁺ Su-DHL4 cell line compared with BCL-2⁻ Su-DHL8 cells, at both the basal level and in response to autophagic stress. The autophagy-related gene expression profiles were determined in purified and unpurified malignant human lymph node biopsies. Seven autophagy machinery genes were up-regulated in purified FL B-cells compared with reactive B-cells. Only 2 autophagy machinery genes were up-regulated in DLBCL B-cells. In unpurified tissue biopsies, 20 of 46 genes in FL and 2 of 5 genes in DLBCL with increased expression were autophagy machinery genes. Expression of autophagy substrates p62 and LC3 were determined by TMAs. FL samples showed significantly decreased levels of both p62 and LC3 compared with reactive and DLBCL, indicative of an increased autophagy activity in FL. In summary, these results demonstrate that FL showed increased basal autophagy activity, regardless of overexpression of BCL-2 in this disease.

INTRODUCTION

Macroautophagy (hereafter referred to as autophagy) is a physical and pathological process that allows eukaryotic cells sequester portions of cytoplasm to form autophagosomes and target them for degradation through the fusion of autophagosomes with lysosomes where they are degraded and recycled [1-4]. Growing evidence demonstrates that autophagy plays important and paradoxical roles in tumorigenesis and in the treatment of cancer [4-7]. Autophagy can suppress tumorigenesis by removing damaged organelles/proteins and limiting cell growth and genomic instability [6]. In contrast, induction of autophagy by metabolic stress in apoptosis deficient tumor cells can support tumor cell survival [8]. Abundant preclinical evidence indicates that stress-induced autophagy in tumor cells is predominantly cytoprotective

and inhibition of autophagy can enhance tumor cell death by diverse anticancer therapies [9-11].

Follicular lymphoma (FL) is the second most common lymphoma diagnosed in the United States and Western Europe, accounting for approximately 20% of all non-Hodgkin lymphomas (NHLs) and 70% of indolent lymphomas and is generally considered incurable [12, 13]. The t(14;18)(q32;q21) translocation characterizes approximately 85% of FL and 20% of diffuse large B-cell lymphoma (DLBCL) and results in constitutive overexpression of the anti-apoptotic protein BCL-2 [14, 15]. Overexpression of BCL-2 in NHLs plays important roles in disease pathogenesis and resistance to apoptosis. It is currently unknown whether BCL-2 plays an important role in regulation of autophagy in indolent FL and more aggressive DLBCL.

The role of the anti-apoptotic protein BCL-2 in

autophagy is currently debated and remains unclear. BCL-2 has been proposed as a major binding partner of Beclin-1 which it binds in a nutrient-dependent manner, consequently down-regulating levels of starvation-mediated autophagy [16]. Recently, it was reported that the anti-apoptotic BCL-2 family members do not directly inhibit components of the autophagic pathway but instead affect autophagy indirectly, owing to their inhibition of Bax and Bak [17]. Autophagy is often activated as an adaptive response against ER stress [18, 19]. In cancer cells, metabolic stress strongly induces autophagy which is sustained when apoptosis is blocked [8, 20, 21]. Questions raised over the roles of BCL-2 in autophagy are: whether overexpression of BCL-2 in human tumors inhibits both apoptosis and autophagy; and whether inhibition of apoptosis by overexpression of BCL-2 could activate the autophagic pathway in favor of prolonged tumor cell survival.

The role of BCL-2 in autophagy in human NHLs has not previously been reported. We hypothesize that overexpression of BCL-2 in FL may cause an increased autophagy activity due to suppression of apoptosis. In this article, we aimed to determine whether overexpression of BCL-2 could alter autophagy status in BCL-2 positive (BCL-2⁺) and negative (BCL-2⁻) DLBCL cell lines, primary FL, DLBCL and reactive (RA) samples using both autophagy RT² Profiler PCR Array and tissue microarray (TMA). We demonstrate for the first time that overexpression of BCL-2 does not inhibit autophagy in human FL.

RESULTS

BCL-2⁺ DLBCL cells showed an increased basal autophagy activity

To evaluate the impact of BCL-2 overexpression on autophagy in human lymphoma, we first compared the autophagy status of the BCL-2⁺ Su-DHL4 with the BCL-2⁻ Su-DHL8 DLBCL cell lines using Western blotting and the RT² Profiler PCR array (Figure 1). Overexpression of BCL-2 in Su-DHL4 cells was confirmed by Western blotting and there was no differential expression of Beclin-1 or Bcl-xL, another binding partner of Beclin-1, between these two cell lines. SQSTM1/p62 (p62) serves as a link between LC3 and ubiquitinated substrates resulting in these two proteins being incorporated into the completed autophagosome and degraded in the autolysosome [22, 23]. Both p62 and LC3-II showed increased expression in the Su-DHL8 cell line (Figure 1 A), indicative of inhibited autophagic degradation [24], suggesting that the autophagy flux may be inactive in this BCL-2⁻ cell line compared with the BCL-2⁺ Su-DHL4 cells. Using autophagy PCR array, we detected that the Su-

DHL4 cell line was also distinguishable from Su-DHL8 cells based on its autophagy-related gene expression profile (GEP) (Figure 1 B and Suppl Figure 1). Four autophagy machinery and nine autophagy regulatory genes were up-regulated in the Su-DHL4 cell line compared to Su-DHL8 cells (Figure 1 C and D). These data support that BCL-2⁺ Su-DHL4 cells may have higher basal level autophagy activity compared with BCL-2⁻ Su-DHL8 cells.

We next sought to determine the capacity of the autophagic flux in both BCL-2⁺ and BCL-2⁻ cells. Inhibition of the autophagic flux by treatment of cells with CQ [7, 25] or induction of autophagy by nutrient deprivation led to increased or decreased p62 and LC3B-I/LC3B-II protein expression respectively in both cell lines (Figure 2 A and B), demonstrating that autophagy activity was not suppressed in the BCL-2⁺ Su-DHL4 cells. Beclin-1 expression did not change in response to either treatment, indicating that its levels are not governed by the autophagy flux. Both Su-DHL4 (Figure 2 C and E and Suppl Figure 2) and Su-DHL8 (Figure 2 D and F and Suppl Figure 3) cell lines responded to nutrient deprivation by up-regulating and down-regulating autophagy-related genes. Among them, *SQSTM1* (p62) and *CDKN1B* genes showed significantly increased expression in both cell lines (Figure 2 E and F). Between 2 and 6 hours starvation, we also noted increased p62 protein expression despite increased autophagic degradation (Figure 2 B). A similar phenomenon has also been observed in mouse embryonic fibroblasts [26]. Increased expression of *CDKN1B* in response to starvation may cause cell cycle arrest in the G1 phase [27]. In addition, the Su-DHL4 cell line also showed significantly increased expression of key autophagy machinery genes including *GABARAPL1*, *GABARAPL2*, *MAP1LC3B* (LC3B) and *CTSS* (Figure 2 C and E). These results suggest that BCL-2⁺ cells may have increased autophagy activity in response to autophagy stress compared with BCL-2⁻ cells.

FL B-cells showed an increased expression of autophagy-related genes

We next evaluated autophagy gene expression levels in primary FL and DLBCL samples and compared them to RA controls. In order to differentiate autophagy activity in lymphoma B-cells from surrounding stromal cells, tumor-infiltrating T-cells and macrophages, B-cell subsets were isolated by flow cytometry. CD3⁺ T-cells were excluded and CD10⁺/CD19⁺ B-cells (FL) and CD20⁺ B-cells (DLBCL and RA) were purified from primary single cell suspensions. B-cell receptor (BCR) isotype restriction is a hallmark of FL cells, and purified CD19⁺/CD10⁺ FL B-cells were found to be either κ or λ light-chain restricted (Figure 3 A). After flow sorting, mean purities of B-cells were $\geq 95\%$ for all samples.

We analyzed the autophagy-related GEP of highly

Table 1: Aberrantly expressed autophagy-related genes in purified and unpurified FL and DLBCL samples.

	Gene name and function	FL				DLBCL			
		F.C. (UP)	P value	F.C. (P)	P value	F.C. (UP)	P value	F.C. (P)	P value
A U T O P H A G Y M A C H I N E R Y G E N E S	BECN1 (1)	2.07	<0.005						
	RGS19 (1)	2.14	<0.05						
	AMBRA1 (1)	2.23	<0.005						
	ATG16L1 (1,3)	2.42	<0.05	3.00	0.24				
	MAP1LC3A (1)	2.60	<0.005	5.38	0.2			5.22	0.33
	GABARAPL1 (1,3)			7.98	0.65				
	GABARAPL2 (1,3)	2.64	<0.005						
	GABARAP (1,2,4)	2.06	<0.005						
	ATG9A (1,3)	3.26	<0.005	2.56	<0.05				
	ATG4D (1,2,3,6)	3.75	<0.005						
	ATG4B (1,2,3,6)	4.66	<0.005						
	ULK1 (1)			3.29	0.06				
	WIPI1 (1)	3.97	<0.005						
	ATG16L2 (3)	2.047	<0.05						
	RAB24 (3)	2.38	<0.05						
	ATG7 (3,5)	2.39	<0.005						
	ATG10 (3)	7.21	<0.005						
	DRAM1 (4)	2.38	<0.05			4.24	<0.05	10.77	0.27
	CTSD (4,7,10)	2.41	<0.005			4.14	<0.05		
	LAMP1 (4,9)	2.65	<0.05	2.02	<0.05				
HDAC6 (5)	3.91	<0.005	2.23	<0.05					
A U T O P H A G Y R E G U L A T O R Y	BNIP3 (7)			-2.8	0.05			-15.37	0.12
	FADD (7)	2.07	<0.005						
	BAD (7)	2.12	<0.005						
	BID (7)							3.96	0.11
	FAS (7)	2.16	<0.05	3.58	0.10			7.16	0.08
	MTOR (7)	2.18	<0.005						
	MAPK8 (7)	2.31	<0.05						
	BAK1 (7)	2.31	<0.05						
	CLN3 (7)	2.33	<0.005						
	CXCR4 (7)	2.25	<0.05						
	AKT1 (7)	2.69	<0.005						
	BAX (7)	2.95	<0.05						
	IFNG (7)			3.04	0.34	6.54	0.1	-5	0.44
	HTT (7)	3.20	<0.005						
	HDAC1 (7)	3.23	<0.005					-2.0	<0.05
	PIK3CG (7)	3.33	<0.005					-8.07	<0.005
	BCL2L1	3.81	<0.005						
DAPK1 (7)	5.04	<0.05							

	BCL2 (7)	8.88	<0.005	7.03	0.1				
G	TGM2 (7)					2.99	<0.05	4.56	0.16
E	TP53 (7,8)	2.98	<0.005						
N	CDKN1B (7,8)	2.11	<0.05						
E	CDKN2A (7,8)	6.19	<0.05	4.22	0.11	3.27	0.12	4.12	<0.05
S	EIF4G1 (10)	2.16	<0.005						
	UVRAG (10)	2.33	<0.005						
	RPS6KB1 (10)	2.36	<0.005						
	MAPK14 (10)	2.50	<0.05						
	ESR1 (10)	2.52	<0.05						
	GAA (10)	2.95	<0.005						
	TMEM74 (10)	3.56	0.31	3.71	0.51			5.69	0.33
	HGS (10)	7.38	<0.05						

Fold changes (F.C.) in gene expression levels of unpurified (UP) FL or DLBCL samples were compared with unpurified RA-LNs and those of purified (P) FL or DLBCL samples were compared with purified reactive B cells. Genes listed in the table are genes with expression changes greater than or less than 3 fold and those greater than or less than 2 fold with a P value <0.05. Numbers of samples: purified RA n=3; FL n=5; DLBCL n=2; unpurified RA n=8; FL=8; DLBCL n=10. Significantly increased or decreased fold changes were identified using a Mann Whitney U test. Numbers in the first column indicate the functions of these genes, according to the classification of human autophagy PCR array (Sabiosciences): (1-6) Autophagy machinery genes: (1) Genes involved in autophagic vacuole formation; (2) Gene responsible for protein targeting to membrane/vacuole; (3) Genes responsible for protein transport; (4) Genes linking autophagosome to lysosome; (5) Genes involved in protein ubiquitination; (6) Genes with protease activity; (7-10) Autophagy regulatory genes: (7) Co-regulators of autophagy and apoptosis; (8) Co-regulators of autophagy and the cell cycle; (9) Autophagy induction by intracellular pathogens; (10) Autophagy in responsible to other intracellular signals.

purified and unpurified FL and DLBCL diagnostic tissue biopsies and compared them with non-malignant RA samples. The results of unsupervised hierarchical clustering are shown for purified reactive and malignant B-cells (Figure 3 B) and unpurified tissue biopsies (Figure 3 C). Seven and two autophagy machinery genes were up-regulated in purified FL and DLBCL samples, respectively (Table 1), one of which, *MAP1LC3A* was commonly up-regulated in both FL and DLBCL purified B-cells. Only one gene, *BNIP3*, showed significantly decreased expression in both FL and DLBCL B-cells. *BNIP3* is a hypoxia-dependent autophagy inducer and its expression is suppressed in many types of cancer [28] but overexpressed in lung and breast carcinomas [29]. Gene expression patterns in both FL and DLBCL were not associated with Ann Arbor stage or international prognostic index (IPI) scores (data not shown). Among the 46 genes which showed increased expression in these samples, 19 genes in FL and 2 genes in DLBCL were autophagy machinery genes (Figure 3 C and Table 1) and 27 genes in FL and 3 in DLBCL were autophagy regulatory genes. Both *BECN1* and *BCL2* genes were up-regulated in FL but not in DLBCL tissue biopsies. Expression of two lysosomal components *CTSD* (cathepsin D) and *DRAM1* (damage-regulated autophagy modulator 1) [30] was significantly up-regulated in both FL and DLBCL tissue biopsies, suggesting they may be expressed at higher levels in the tumor microenvironment. *CDKN2A* (p16), a tumor-suppressor gene, is up-regulated

in both purified and unpurified FL and DLBCL samples (Table 1). To consolidate these findings, increased expression of *BECN1*, *MAP1LC3A*, *ATG4B*, *DRAM1* and *CTSD* was validated in unpurified tissue using qRT-PCR. Results were comparable to those obtained from the PCR array (Suppl Table 8). These data demonstrate that both FL and DLBCL samples aberrantly express autophagy genes at the basal levels. In particular, FL samples which frequently overexpress *BCL-2* have increased expression of numerous autophagy machinery and regulatory genes.

In the cohort of purified samples, two FL patients (T1979 and T5728) with high global expression of autophagy genes subsequently underwent transformation to the more aggressive DLBCL later in their clinical course (Suppl Figure 4 A and Suppl Table 1). In unpurified tissue biopsies, 7 of 8 FL and 1 of 10 DLBCL samples showed increased global expression of autophagy genes compared to RA tissues (Suppl Figure 4 B). A larger number of purified DLBCL samples will be required to establish aberrant expression of autophagy genes in this disease.

FL showed significantly decreased expression of autophagy substrates p62 and LC3 proteins

To establish the autophagy status of FL and DLBCL, primary FL, DLBCL and RA LN tissue biopsies on TMAs were stained using IHC for the autophagy substrate

proteins p62 and LC3, and autophagy initiating protein Beclin-1 (Figure 4A). RA and FL samples were stained with CD10 antibody to distinguish follicular center B-cells from surrounding cells. CD10⁺ area were classified as intra-follicular areas; CD10⁻ areas were classified as inter-follicular areas [31] (Suppl Figure 5A).

First, protein expression in FL intra-follicular areas was compared with RA and DLBCL whole core samples. p62, LC3 and Beclin-1 showed significantly decreased expression in FL intra-follicular areas compared with DLBCL and RA controls. Only Beclin-1 displayed

significantly decreased expression in DLBCL (Figure 4 B, D and F). Most strikingly, all FL samples showed consistently lower levels of p62. Expression of p62 in DLBCL and LC3 and Beclin-1 in FL and DLBCL displayed a heterogeneous expression pattern. In FL, significantly decreased expression of p62, LC3 and Beclin-1 was observed in both intra-follicular and non-malignant inter-follicular areas (Figure 4 C, E and G), suggesting autophagy may be altered in both malignant FL cells and surrounding tumor infiltrating cells. Approximately 91% FL samples were BCL-2 positive

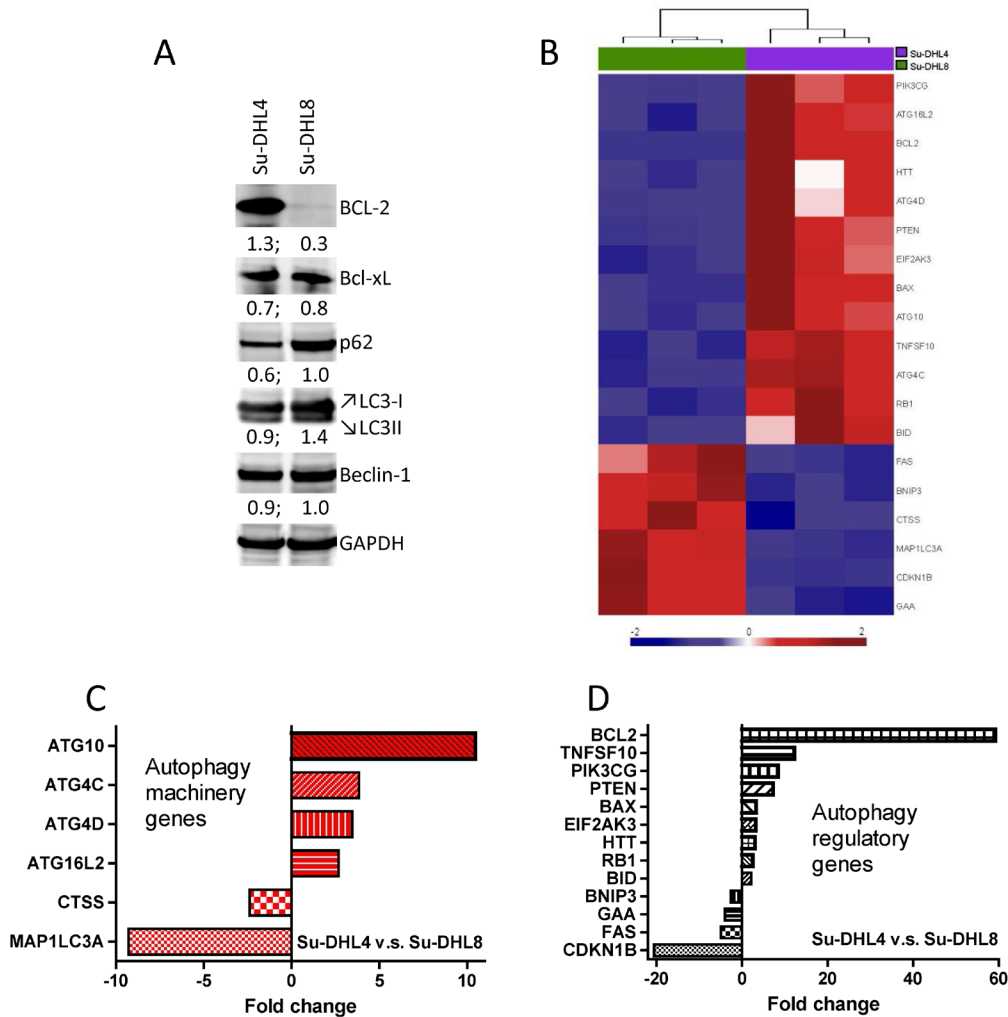


Figure 1: Determination of basal autophagy status in BCL-2⁺ Su-DHL4 and BCL-2⁻ Su-DHL8 cell lines. (A) Comparison of autophagy-related protein expression by Western blotting. 50 µg proteins were loaded onto each lane of a 12-well SDS-PAGE gel. Proteins were transferred to a PVDF membrane which was probed with primary antibody at 4°C overnight. Primary antibodies were used at a 1:2000 dilution for GAPDH and at 1:1000 for all other antibodies. Levels of protein expression were measured by densitometry. Numbers below panels of Western blots indicate the ratio of a specific protein to GAPDH. (B) Supervised hierarchical clustering of significantly differentially expressed autophagy machinery and autophagy regulation genes. Heat-map shows triplicate RQ values for Su-DHL4 and Su-DHL8 cell lines. Each column represents an mRNA/RQ value and each row a gene. Gene expression levels are represented as a gradient of blue to red color indicating low and high expression respectively. Side bars were removed for clarity. (C and D) RQ values of significantly increased or decreased autophagy machinery (C) and autophagy regulatory (D) genes differentially expressed in the Su-DHL4 cell line compared with the Su-DHL8 cell line, analyzed using student *t*-test ($P < 0.05$) and represented as fold changes.

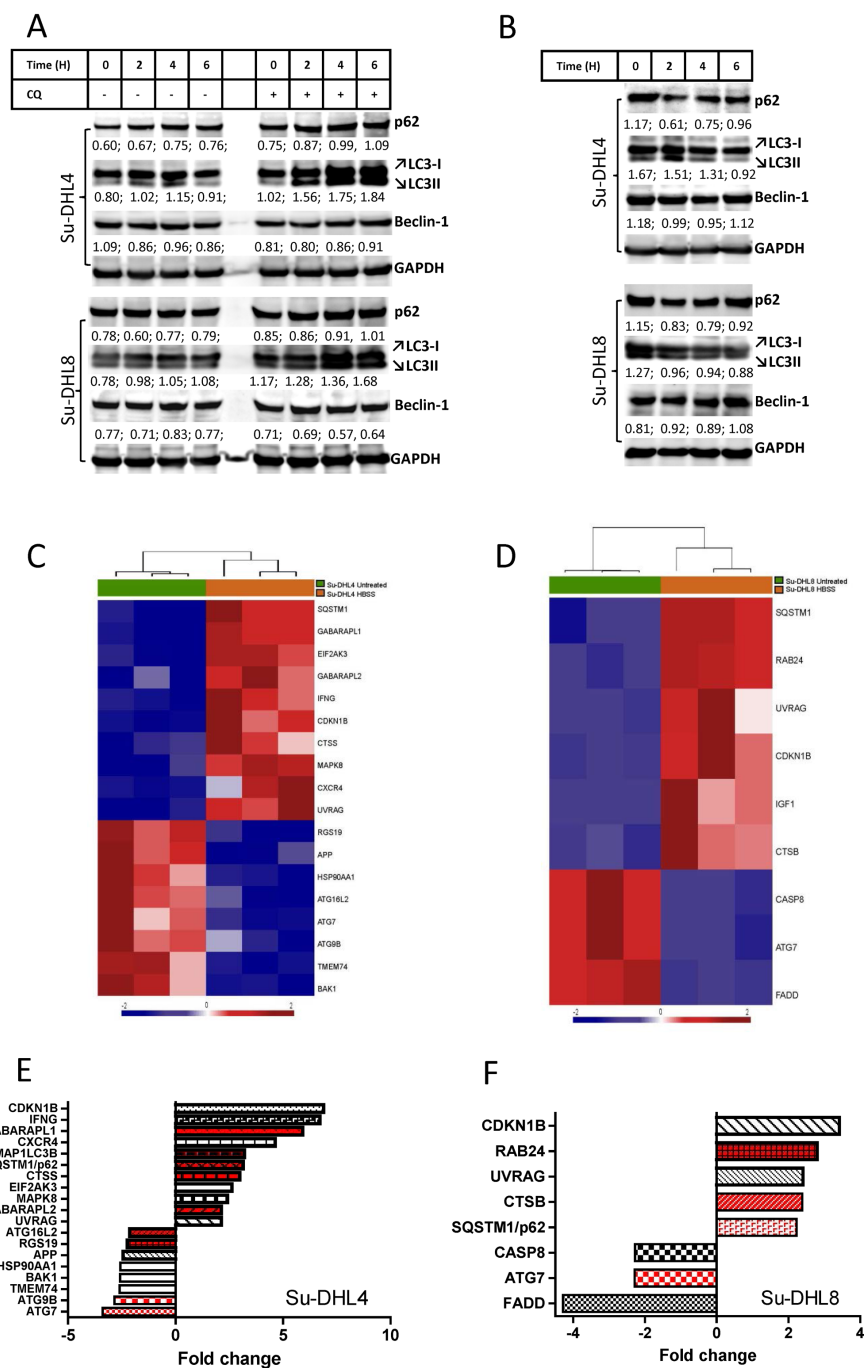


Figure 2: Inhibition or induction of the autophagic flux in Su-DHL4 and Su-DHL8 cell lines. (A) Blocking autophagic flux. Cells were incubated in normal culture medium in the presence or absence of 50 μ M CQ. (B) Induction of autophagy by starvation. Cells were incubated in HBSS for up to 6 hours. Cells were collected at each indicated time point for protein extraction and Western blotting. Numbers below each band indicate ratios of specific proteins to GAPDH which were determined by densitometry. (C and D) Supervised hierarchical clustering of significantly differentially expressed autophagy related genes in Su-DHL4 (C) and Su-DHL8 (D) cells after incubation in HBSS for 6 hours. Heat-map shows triplicate RQ values for normal and HBSS cultured Su-DHL4 and Su-DHL8 cell lines. Each column represents an mRNA/RQ value and each row a gene. Gene expression levels are represented as a gradient of blue to red color indicating low and high expression respectively. (E and F) RQ values of autophagy related genes significantly differentially expressed in Su-DHL4 (E) and Su-DHL8 (F) cells after incubation in HBSS for 6 hours were analyzed by the paired student *t*-test ($p < 0.05$) and represented as fold changes. Red bars indicate changes in autophagy machinery genes and black and white bars indicate changes in autophagy regulatory genes.

(>30% of stained area); DLBCL samples displayed a heterogeneous BCL-2 expression pattern (Suppl Figure 5 B and C). These results demonstrate that FL has an increased basal autophagy activity, regardless of overexpression of BCL-2.

In order to understand the association between BCL-2 and p62, LC3 or Beclin-1, correlations between these proteins in RA, FL and DLBCL were analyzed by Pearson product-moment correlation method (Figure 5).

BCL-2 levels showed a negative correlation with p62 in FL ($P < 0.05$) and positive correlation with p62, LC3 or Beclin-1 in DLBCL ($P < 0.01$), suggesting that BCL-2 does not play a dominant role in autophagy status in these lymphomas. Expression levels of p62 and LC3 showed strong positive correlation ($P < 0.0001$) in RA, FL and DLBCL samples, indicating that p62 or LC3, individually or in combination, can be used as a marker for evaluating autophagy activity. Interestingly, Beclin-1 expression

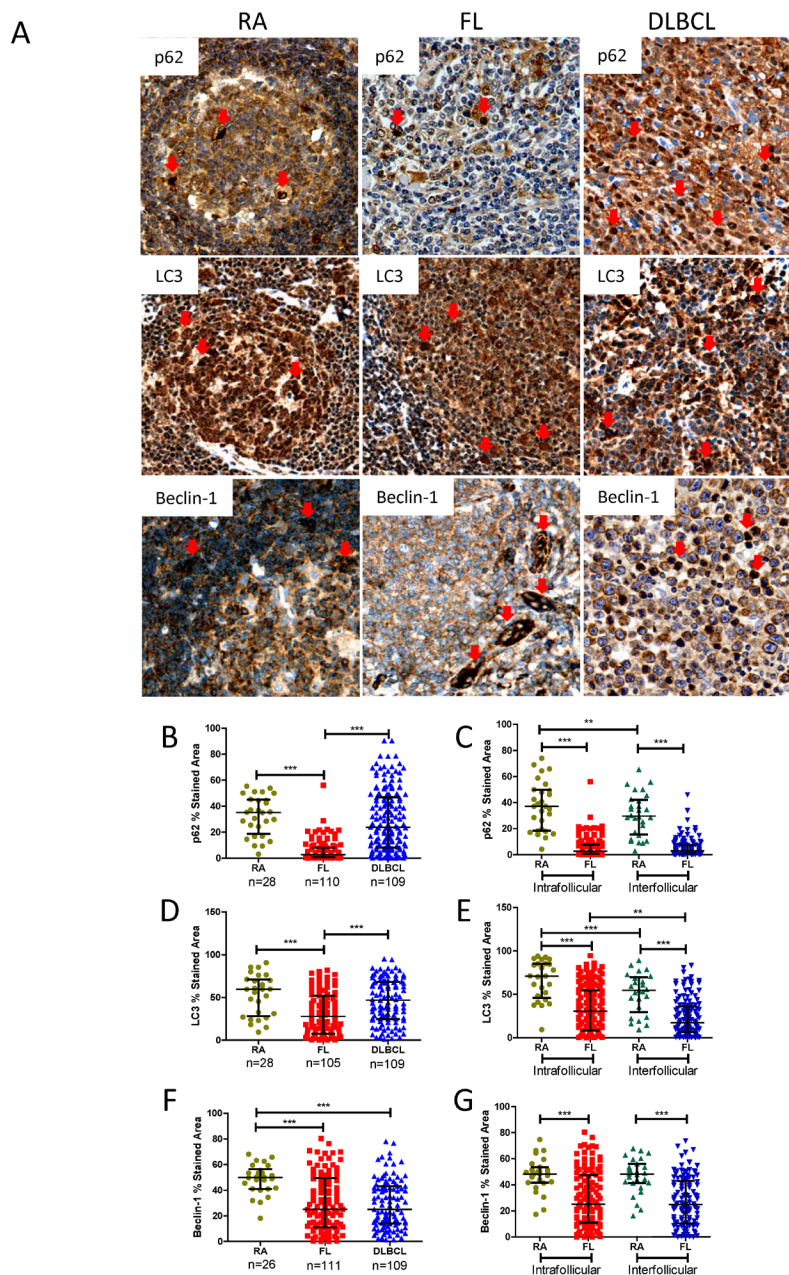


Figure 4: Comparison of p62, LC3 and Beclin-1 protein expression in FL and DLBCL with RA-LN. (A) Representative histochemical stained images of p62, LC3, and Beclin-1 in RA, FL and DLBCL. Proteins were stained with polyclonal anti-p62, polyclonal anti-LC3B antibody, or a mouse anti-Beclin-1 antibody respectively. Detailed information of antibodies and their dilution are listed in the Suppl Table 5. Red arrows indicate positive cells. (B-G) Statistical analysis p62 (B and C), LC3B (D and E) and Beclin-1 (F and G) expression. All data presented are medians with interquartile ranges. Sample numbers for RA and FL in C, E and G are as same as listed in B, D and F. Statistical difference between samples was analyzed by unpaired Mann-Whitney *U* test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

levels were positively correlated with levels of p62 and LC3 in both FL ($P < 0.01$) and DLBCL ($P < 0.0001$), suggesting that Beclin-1 levels is not positively associated with autophagy activity.

Cathepsin D and tissue transglutaminase-2 are highly expressed in the tissue macrophages

Gene expression profiling of DLBCL malignant B-cells may be affected by the presence of stromal cells [32]. *CTSD* showed significantly increased expression at the gene level in FL and DLBCL sample biopsies but not in purified B-cells. Increased expression of *TGM2* (Tissue transglutaminase-2) was detected in both purified and unpurified DLBCL samples (Table 1). To distinguish the origins of these components, we evaluated expression

of cathepsin D and TGM2 proteins in previously untreated FL, DLBCL and RA tissue biopsies and the results were reviewed by expert histopathologist (MC and RC). The expression pattern of cathepsin D and TGM2 suggested that they are not expressed by lymphoma cells, but rather macrophages (Figure 5 A). To confirm cells highly expressing cathepsin D and/or TGM2 are tumor infiltrating cells, DLBCL and RA TMAs were stained with CD68, a marker for tumor-associated macrophages (TAMs) [33, 34] (Figure 5 B). These data confirmed that cells expressing cathepsin D and/or TGM2 are indeed macrophages, although co-localization between these proteins and CD68 was not evaluated. Cathepsin D expression was significantly lower in FL but significantly higher in DLBCL samples compared with RA controls (Figure 5 C), while both FL and DLBCL samples showed decreased expression of TGM2 (Figure 5 D). Neither

RA					
A	Protein	Beclin-1	LC3	p62	Correlation parameters
	BCL-2	0.079	0.140	0.166	γ
		>0.05	>0.05	>0.05	<i>P</i> value
	Beclin-1	0.005	0.453		γ
		>0.05	<0.05		<i>P</i> value
	LC3		0.659		γ
			<0.0001		<i>P</i> value

FL					
B	Protein	Beclin-1	LC3	p62	Correlation parameters
	BCL-2	0.207	-0.087	-0.342	γ
		>0.05	>0.05	<0.05	<i>P</i> value
	Beclin-1	0.417	0.390		γ
		<0.01	<0.01		<i>P</i> value
	LC3		0.536		γ
			<0.0001		<i>P</i> value

DLBCL					
C	Protein	Beclin-1	LC3	p62	Correlation parameters
	BCL-2	0.273	0.305	0.243	γ
		<0.01	<0.001	<0.01	<i>P</i> value
	Beclin-1	0.564	0.318		γ
		<0.0001	<0.0001		<i>P</i> value
	LC3		0.553		γ
			<0.0001		<i>P</i> value

Figure 5: Multiple analysis of correlation between BCL-2, p62, LC3 and Beclin-1. (A) RA; (B) FL and (C) DLBCL. Correlation was analyzed by Pearson product-moment correlation coefficient test. ' γ ' indicates correlation coefficient. Sample numbers, RA=30; FL=50 and DLBCL=109.

cathepsin D nor TGM2 expression was correlated with autophagy status in FL or DLBCL (data not shown). Both cathepsin D and TGM2 showed strong positive correlation ($P < 0.0001$) with CD68 (Supp Figure 6 A and B). Expression of cathepsin D and TGM2 were also strongly correlated ($P < 0.0001$) (Supp Figure 6 C). These results suggest that higher expression of autophagy genes in TAMs may lead to misinterpretation of the gene

signature of malignant B-cells when analyzing autophagy in unpurified tissue biopsies by PCR array.

DISCUSSION

Here we report that FL, an indolent NHL which frequently overexpresses the anti-apoptotic protein BCL-2, showed significantly increased expression of

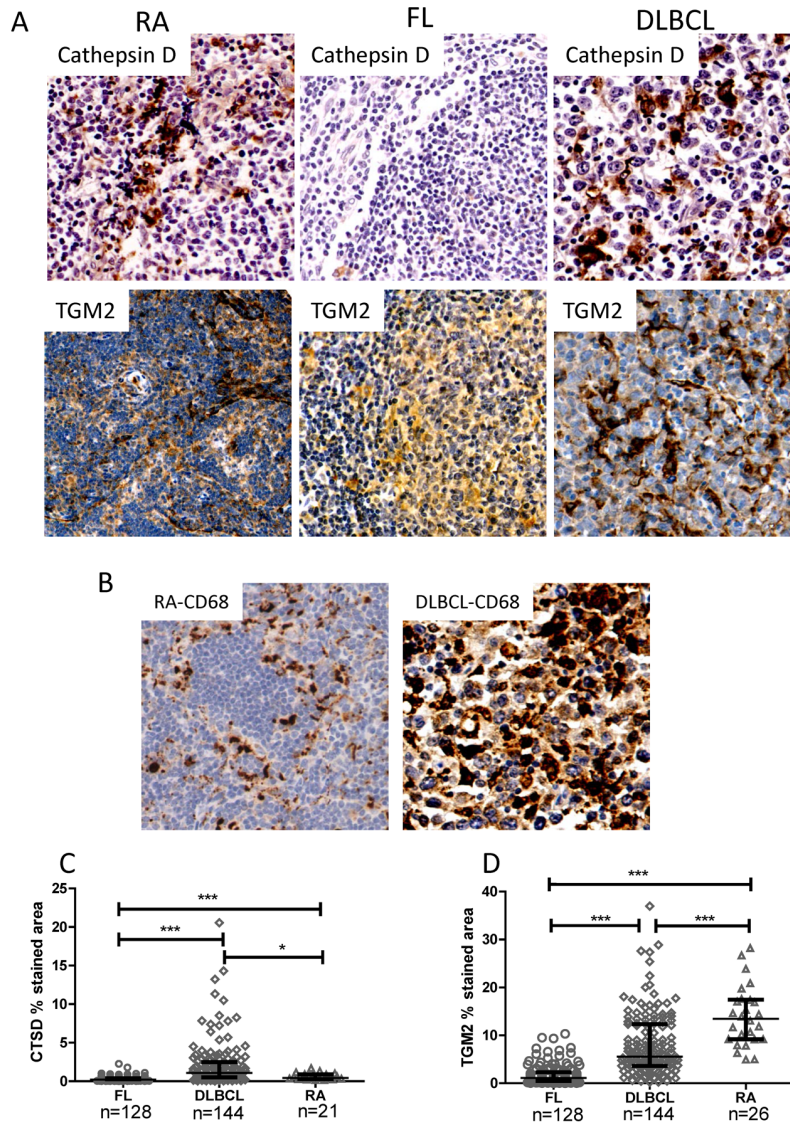


Figure 6: Immuno-histochemical staining of cathepsin D, TGM2 and CD68. (A) Representative images of cathepsin D and TGM2 expression in RA, FL and DLBCL. (B) Representative images of CD68 expression in RA and DLBCL. Antibody dilutions were 1:1000, 1:750, or 1:8000 for anti-cathepsin D, anti-TGM2, or anti-CD68 antibodies, respectively. Images were taken with a Leixa DM2500 microscope: original magnification X200. Cathepsin D, TGM2 and CD68 protein expression levels were defined as % stained viable tissue area. (C and D) Statistical analysis of protein levels of cathepsin D (B) and TGM2 (C) were calculated as the percent stained area of viable tissue. Data collected for RA and DLBCL were from whole cores and for FL were from the CD10⁺ intra-follicular area. Significantly increased or decreased expression between samples was analyzed by the Mann-Whitney *U* test. Numbers of samples used for analysis were indicated.

key autophagy genes and decreased levels of autophagy substrate protein p62 and LC3 compared with RA B-cell controls. Whereas, the autophagy-related GEP and levels of autophagy substrate p62 and LC3 proteins in DLBCL, an aggressive NHL, was more similar to those of RA B-cell controls. A constitutive basal level of autophagy in normal tissue provides an important homeostatic, housekeeping function to survive stress, such as nutrient deprivation [21, 35]. The role of autophagy in cancer is complex as it can prevent tumor initiation by suppressing chronic tissue damage, inflammation, and genome instability via its quality control function or it can sustain tumor metabolism, growth, and survival via nutrient recycling [36], suggesting a link between dysregulated autophagy and tumor progression.

Higher expression of the anti-apoptotic protein BCL-2 is more common in B-cell NHL than T-cell NHL and is heterogeneously expressed among the different histological subtypes [37]. Although BCL-2 is a well-established anti-apoptotic protein, it was also proposed to suppress autophagy by binding and inhibiting Beclin-1 in a cardiac BCL-2 transgenic mice model [16]. A recent finding demonstrated that BCL-2 does not bind directly to Beclin-1 but instead regulate autophagy by inhibiting Bax/Bak mediated apoptosis [17]. We therefore determined the role of BCL-2 on autophagy in BCL-2⁺ and BCL-2⁻ cell lines and human primary lymphoma samples. Higher levels of autophagy machinery genes were found in BCL-2⁺ cells compared with the BCL-2⁻ cell line. BCL-2⁺ and BCL-2⁻ DLBCL cell lines showed similar autophagy flux activity upon either autophagy inhibition by CQ or autophagy induction by nutrient-deprivation, evidenced by accumulation or degradation of p62 and LC3 proteins, respectively. Interestingly, the BCL-2⁺ cell line up-regulated more autophagy-related genes in response to starvation compared to BCL-2⁻ DLBCL cells. These results demonstrate that the autophagic flux is not altered by the BCL-2 protein in human malignant B-cells. In fact, BCL-2⁺ cells up-regulate more autophagy-related genes than BCL-2⁻ DLBCL cells in both the resting state and in response to autophagy induction.

As expected, the BCL-2 gene was highly expressed in both purified FL B-cells and unpurified tissue biopsies compared with RA and DLBCL primary samples. To identify expression signatures of autophagy-associated genes in the malignant population specifically, B-cells were purified from FL and DLBCL tissue biopsies by flow sorting. Normal B-cells were also purified from RA-LNs which served as controls. Seven autophagy machinery genes were up-regulated in purified FL B-cells, including *ATG9A*, *ATG16L1*, *MAP1LC3A*, *GABARAPL1* and *ULK1*, which are involved in autophagosome formation and protein transport; *LAMP1*, involved in autolysosome formation; and *HDAC6*, involved in protein ubiquitination. This result demonstrates that FL B-cells, which overexpress BCL-2 up-regulate the basal autophagy

activity at the gene level. We have also confirmed by decreased expression of autophagy substrate proteins p62 and LC3 in FL tissue specimens using TMAs and IHC, demonstrating an active autophagy at the protein levels in FL. Nevertheless, there is no strong and clear correlation between BCL-2 expression levels and autophagy activity. We therefore propose that autophagy activity in lymphoma samples may not be controlled by BCL-2.

FL frequently transforms to the more aggressive DLBCL. Our data showed that only one gene, *MAP1LC3A*, was up-regulated in DLBCL B-cells. It was recently reported that autophagy is positively regulated by *LITAF* which is silenced by promoter hypermethylation in germinal center-derived B-cell lymphomas, suggesting that autophagy may be inhibited in these lymphomas [38]. We found that levels of p62 and LC3 proteins showed a heterogeneous expression pattern in DLBCL and had no significant difference compared with RA controls. In addition, expression levels of p62 and LC3 did not show association with clinical outcomes of patients with FL (data not shown). This suggests that up-regulated autophagy in FL may not be associated with transformation to DLBCL. Instead, active autophagy in FL may even suppress tumor progression by eliminating damaged organelles and controlling genetic instability.

Increased expression of autophagy-related genes was more readily detected in FL bulk tissue biopsies. Similar to FL purified B-cells, FL unpurified tissue also showed up-regulation of autophagy machinery genes, including *ATG16L1*, *MAP1LC3A*, *ATG9*, *LAMP1* and *HDAC6*; many other autophagy machinery and regulatory genes were also significantly up-regulated. Among the up-regulated autophagy regulatory genes, *TP53*, *MAPK8*, *HDAC1*, *DAPK1*, *CDKN1B*, *CDKN2A*, *UVRAG*, and *RPS6KB1* are positive regulators of autophagy, whereas *AKT1*, *PIK3CG*, *BCL-2*, *BCL-2L1*, *mTOR*, *EIF4G1*, and *MAPK14* are negative autophagy regulators. It is obvious that more aberrantly expressed autophagy-related genes were detected in unpurified FL samples compared with purified B-cells. Our previous studies show that FL tissues have increased numbers of CD163⁺ infiltrating macrophages [39] and CD4⁺ T-cells [40] in the microenvironment. This suggests that autophagy activity might also be altered in FL tumor infiltrating cells and this is being actively explored. Notably, we did not find evidence of upregulation of autophagy related genes in our previous studies of GEP of the tumor infiltrating T-cells in FL [31].

Fewer autophagy-related genes had altered expression levels in DLBCL, regardless of sample purification. Significantly up-regulated genes in DLBCL samples included *CTSD*, *DRAM1* and *TGM2*. *CTSD* and *DRAM1* are lysosomal proteins which regulate the autophagic flux through the lysosome [30, 41], while *TGM2* is involved in autophagy-dependent clearance of ubiquitinated proteins [42]. We therefore tested the

origin of cells expressing high levels of cathepsin D and TGM2 using TMAs. Expression of both cathepsin D and TGM2 proteins was significantly lower in FL samples but significantly higher in DLBCL samples compared with RA controls. Morphological features of cells expressing higher cathepsin D or TGM2 were identical to CD68-expressing TAMs. Indeed, expression levels of both cathepsin D and TGM2 were strongly correlated to CD68 expression levels in DLBCL. We found that increased cathepsin D expression was associated with a shorter overall survival of DLBCL patients (data not shown), in agreement with a previous report by Nicotra et al [43]. TGM2 has been reported as a marker for progression and therapeutic intervention in colorectal cancer and non-small cell lung cancer.[44, 45] However, the role of TGM2 in DLBCL is unknown. We found that TGM2 expression is not associated with shorter overall survival or other prognostic markers in DLBCL (data not shown). Nevertheless, *CTSD* and *TGM2* expression levels in DLBCL did not reflect their expression in malignant B-cells. We therefore propose that determination of autophagy-related gene expression using unpurified lymphoma specimens could be distorted by high lysosome-containing TAMs.

In summary, the role of BCL-2 in autophagy is currently elusive. Using the RT² Profiler Human Autophagy PCR array and TMAs, we demonstrate that basal autophagy activity was up-regulated in primary BCL-2 overexpressing FL B-cells and their microenvironmental cells. A greater number of autophagy machinery genes were up-regulated in the BCL-2⁺ DLBCL cell line Su-DHL4 at the basal level and in response to stress, indicating that overexpression of BCL-2 does not inhibit the autophagic flux. Instead, inhibition of apoptosis by overexpression of BCL-2 may switch on autophagy in the cell in favor of eliminating aged organelles and damaged proteins. However, the mechanism by which FL cells up-regulate autophagy-related gene expression is not clear and may be due to multiple factors. We therefore propose that overexpression of the anti-apoptotic protein BCL-2 does not suppress autophagy activity in human FL.

MATERIALS AND METHODS

Cell lines and cell culture

Human DLBCL cell lines Su-DHL4 (BCL-2⁺) and Su-DHL8 (BCL-2⁻) [46] were used in this study. Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 25mM HEPES, and 2.0mM L-glutamine at 37°C in a 5% CO₂ humidified incubator. To induce autophagy, cells were cultured in HBSS for up to 6 hours.

Human samples and ethical considerations

Ethical approval for the human biological materials used in this study was obtained in accordance with the requirements of the East London and the City Health Authority Local Research Ethics Committee (Ref. No. 10/H0704/65). All samples were obtained from patients by informed consent. Patients selected presented at St. Bartholomew's hospital between the years 1970-2012. For RT² Profiler PCR array analysis, lymph node (LN) biopsies and cryopreserved single cell suspensions were obtained from diagnostic, previously un-treated FL (n=13) and DLBCL (n=11) patients (Suppl Table 1 and 2); RA-LNs (n=11) were used as controls. For tissue microarrays (TMAs), LN biopsies from 128 FL and 144 DLBCL patients at diagnosis for whom quality formalin fixed paraffin embedded tissue, clinical and follow-up data were available, as well as 28 reactive LN biopsies, were included on the TMAs (Suppl Table 3 and 4).

Reagents

RT² Profiler Human Autophagy PCR Array (PAHS084ZE), RNeasy mini kit, RT² First Strand Kit, primers for qPCR validation, including *BECN* (PPH05670B), *MAP1LC3A* (PPH19436A), *ATG4B* (PPH15916A), *DRAM1* (PPH19768F), *CTSD* (PPH00112F) and *RPLPO* (PPH21138F) were purchased from Qiagen-Sabiosciences. 3,3'-diaminobenzidine (DAB) was from BioGenex. TRIzol® reagent, 4-12% NuPAGE gels and Hanks balanced salt solution (HBSS) were from Invitrogen. Chloroquine (CQ), 4',6-diamidino-2-phenylindole (DAPI), and all other chemicals used were from Sigma. Antibodies used in this study are listed in Suppl Table 5.

Purification of B-cells by flow cytometry sorting

Primary single cell suspensions (10⁶cells/ml) were washed once with washing buffer containing 2% FCS in PBS (phosphate buffered saline). Non-specific bindings were blocked by incubating cells with 2% human anti-γ-globulin antibody for 30 min at 4°C. Cells were stained with conjugated anti-CD3/anti-CD20 or anti-CD3/anti-CD19/anti-CD10 antibodies for 30 min at 4°C and subsequently washed once with washing buffer. Cells were resuspended in blocking buffer containing DAPI (50ng/ml) and sorted on a BD FACSAria II Cell Sorter. DAPI was used to discriminate live and dead cells. Following selection of DAPI negative cells, T-cells were excluded by gating on CD3 negative (CD3⁻) cells. B-cells were isolated from the DAPI⁻/CD3⁻ population based on expression of B-cell markers. DLBCL and reactive B-cells were identified by CD20 expression while FL B-cells were

isolated based on dual expression of CD19 and CD10. FL B-cells were further confirmed as the malignant cell population by demonstrating kappa/lambda (κ/λ) light chain restriction [47].

RNA extraction and cDNA conversion

Total RNA was isolated from purified single B-cell suspensions or solid LN tissue biopsies with TRIzol® and/or the RNeasy mini kit. RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies) and a Nanodrop spectrophotometer (Thermo-Scientific); all samples had an RNA integrity number (RIN) greater than 6 and 260/280 ratios higher than 1.9. RNA (300ng) was converted to cDNA by RT-PCR using the RT² First Strand Kit.

RT² Profiler PCR array for detection of expression of autophagy related genes

The RT² Profiler PCR Array combines qRT-PCR technology with a microarray format to allow the simultaneous detection of multiple gene expression levels in a rapid manner. The RT² Profiler Human Autophagy PCR Array contains primers against 84 genes involved in different stages of the autophagy pathway (Suppl Table 6 and 7) as well as primers against five housekeeping genes which are used for data normalization. According to the manufacturer's protocol, qRT-PCR was performed by adding 2.8ng cDNA mixed with RT² SYBR Green Mastermix (Sabiosciences) to each well which already contained primers directed against the gene of interest. mRNA levels were analyzed using the ABI Prism 7900HT Fast *Real-Time* PCR System (Applied Biosystems) and a dissociation curve analysis step included to verify PCR specificity. Relative quantity (RQ) values were calculated using the formula $RQ = 2^{-\Delta\Delta C_T}$. Target cycle threshold (C_T) values were normalized to the housekeeping gene RPLPO generating a ΔC_T value. The average ΔC_T of reactive controls was used as the calibrator sample on a per gene basis and was subtracted from each ΔC_T generating a delta ΔC_T value which was linearized by raising to the power of 2 ($2^{-\Delta\Delta C_T}$). Genes with a fold change ≥ 3 or ≤ -3 or a FC ≥ 2 or ≤ -2 and a p value < 0.05 using a Mann-Whitney U test or student t -test were taken to be biologically meaningful. Hierarchical clustering was performed using Euclidean distance measure and an average agglomeration available within the R statistical computing environment.

qRT-PCR validation

Following conversion of RNA (500ng) to cDNA, samples were prepared for qRT-PCR as previously described and the qRT-PCR assay run under the previously

stipulated conditions. All genes validated, including the housekeeping gene RPLPO, were analyzed in triplicate and a no-template control (NTC) also included per gene. Primers used in validation experiments were identical to those present on the RT² Profiler Human Autophagy PCR Array.

Tissue microarray (TMAs) and IHC analysis

TMAs were constructed using a semi-automated arraying system (TMABooster-Alphelys). Sections of biopsy material were stained with hematoxylin and eosin and reviewed by an expert histopathologist (C.M.) who identified areas rich in malignant cells. Triplicate 1mm² cores were then taken from these areas, arrayed and stained as previously described [31, 33, 34]. Slides were digitalized and image analysis was performed using a digital pathology system (Ariol, Lieca Microsystems). The Ariol image analysis classifier was trained based on the hue, saturation and intensity of DAB staining, such that only areas stained above a pre-determined threshold representing the highest intensity staining were classed as positive, and lower level background expression was excluded. A further classifier was used to determine the total viable tissue area. Combining these two classifiers, the percent stained area was calculated. In FL cores, intra-follicular areas were selected based on expression of CD10 and clear follicle morphology. Results were manually and blindly reviewed and reported as an average of the triplicate cores. Protein levels were expressed as % stained area [33].

Western blotting

Proteins were extracted with lysis buffer and 50 μ g of proteins added to each lane of 4-12% NuPAGE gels and Western blotting was performed as previously described [33].

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 5.03). Data are shown as either mean \pm SD or median with interquartile range when variation was high. Significant differences between groups with unequal size were analyzed with the Mann-Whitney U test and those with equal size were analyzed using the student t -test. Pearson product-moment correlation method was used to analyze linear correlation between two groups. All P -values less than 0.05 were considered statistically significant.

Conflict of interest

The authors declare that they have no conflict of interest.

Editorial note

This paper has been accepted based in part on peer-review conducted by another journal and the authors' response and revisions as well as expedited peer-review in *Oncotarget*.

Authors' contribution

L.J and J.G.G contributed project design and manuscript writing. A.M, A.C, and R.C performed experiments. A.M, L.J, J.M, and R.D.P contributed data collection and analysis. J.M and A.W provided clinical information of CLL patients. M.C, R.C and A.C contributed histopathological identification of lymphoma samples. S.I provided excellent tissue bank service. All authors contributed manuscript writing.

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