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Original Article

A20 promoted the phagocytosis of lymphoma cells by dendritic cells from activated B-cell-like non-Hodgkin lymphoma

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Abstract

Non-Hodgkin lymphoma (NHL) is a lymphoproliferative disorder derived from either B or T lymphocytes. Among NHL, activated B-cell-like (ABC) diffuse large B-cell lymphoma (DLBCL) and T cell non-Hodgkin lymphomas (T-NHL) are poor prognosis and aggressive subtypes. Macrophages are professional phagocytic cells and dendritic cells (DCs) are professional antigen-presenting cells in immune system. Doxorubicin (Dox) and Etoposide (ET) are the most effective anti-cancer drugs. *A20* and *CYLD* are negative regulators of NF-κB-dependent functions in many cell types. Little is known about the roles of *A20* and *CYLD* in regulating functions of DCs and macrophages from NHL. The present study, therefore, explored whether *A20*/*CYLD* expression contributes to functions of DCs and macrophages from NHL. To this end, blood samples of seventy-nine patients with ABC DLBCL and T-NHL were examined. Gene expression profile was determined by quantitative RT-PCR and immunophenotype, cell apoptosis and phagocytosis by flow cytometry. As a result, immunophenotypic analysis showed that the numbers of $CD13^+CD117^-$, $CD56^+CD40^+$ and $CD23^+CD40^+$ expressing cells were significantly elevated in ABC DLBCL cases compared to healthy individuals and T-NHL patients. Interestingly, upon treatment of Dox and ET, the phagocytosis of lymphoma cells was significantly reduced by $CD11c^+CD123^-$ DCs and the percentage of $CD56^+$ mature DCs was significantly enhanced in ABC DLBCL patients only in the presence of *A20* siRNA, but not *CYLD* siRNA. In conclusion, ABC DLBCL patients with low *A20* expression were defective in elimination of lymphoma cells by DCs and linked to killer DC expansion in circulation

Keywords: A20, ABC DLBCL, Dendritic cells, Phagocytosis, T cell non-Hodgkin lymphomas

1. Introduction

Non-Hodgkin lymphoma (NHL) is a lymphoproliferative disorder derived from either B or T lymphocyte or natural killer (NK) cell type [1]. Globally, NHL represented 2.8% of all neoplasms and ranked as the 11th for cancer mortality for both sexes in 2020 [1]. Among NHL, diffuse large B-cell lymphoma (DLBCL) is a heterogeneous group of mature B lymphocytes and accounts for 30–40% of NHL [2]. According to Hans' criteria, DLBCLs were subclassified into germinal centre B cell like (GCB), activated B-cell-like (ABC) and primary mediastinal large B-cell lymphoma (PMBL) subtypes. Patients with the GCB subtype have better prognosis and survival than those with the ABC subtype, which are at high risk for relapse [2]. Similar to DLBCL, T cell non-Hodgkin lymphomas (T-NHL) is also a subtype of uncommon malignancy with poor prognosis, rare and aggressive, originating from mature T cells and accounting for approximately 10% of all new NHL [3]. Immunophenotyping investigation of NHL indicated that CD23 and CD40 expressions seem to be prognostically favorable in DLBCL [4]. Besides, CD13, a myeloid-associated antigen, is absent in follicular lymphoma and presents more often in lymphoplasmacytic lymphoma [5].

Dendritic cells (DCs) are professional antigen-presenting cells to take up and process extracellular and intracellular antigens and induce either active immunity or tolerance by presenting these antigens to T cells [6]. The number of DCs is significantly reduced in NHL and their maturation is associated with the course of hematologic diseases [6]. In humans, the administration of Fms-like tyrosine kinase 3 ligand (Flt3L) increases the number of $CD11c^+$DCs and plasmacytoid DCs [7]. Human pDCs originated from lymphoid precursors are $CD11c^{low}$ $CD123^{high}$ expressing cells and induce B cells to differentiate into plasma cells [8]. Macrophages are professional phagocytic cells in the innate immune response and have a central role in the defense against foreign substances and circulating cancer

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cells [9]. Macrophage phagocytosis is not only to engulf pathogens but also to present their antigens for stimulating the anti-tumor immune responses [9]. Macrophage differentiation is induced by macrophage colony-stimulating factor (M-CSF). Maturation of DCs and macrophages leads to the presence of costimulatory molecules and the release of pro-inflammatory cytokines. When activated, DCs and macrophages lose their phagocytic efficiency. Defects in maturation of DCs and macrophages can result in non-responsiveness of the immune system to tumors [10].

A20 and *CYLD* are considered as negative regulators of nuclear factor (NF)-κB-dependent functions in many different cell types. *A20*-deficient mice display severe inflammation, cachexia and premature mortality [7]. An inactivated expression of *A20* is frequently found in B cell lymphoma and its genetic alterations are involved in the pathogenesis of DLBCL [11]. Similar to *A20*, attenuated expression of *CYLD* is correlated with a poor prognosis in Hodgkin lymphoma [12]. The presence of *CYLD* is known to promote cell death in lymphocytic leukemia [13]. *CYLD*-knockout mice exhibit abnormalities in the activation and development of T cells and B cells [14]. The roles of *A20* and *CYLD* in negatively modulating functions of mouse DCs and macrophages have been well documented [7, 15].

Doxorubicin (Dox) and Etoposide (ET) are the most effective anti-cancer drugs and are widely used as essential parts of combination chemotherapies. The molecular mechanism of Dox and ET is inhibition of DNA replication and disrupts the function of topoisomerase II, thereby resulting in DNA disruption and cell death [16, 17]. The Dox-induced apoptosis of cancer cells elicits an effective antitumor immune response, while ET does not induce immunogenic cell death [17].

Investigations on the regulatory effects of signalling molecules in NHL revealed that abnormal expression of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway is linked to NHL, as *STAT-5* overexpression inhibits apoptosis [18] and promotes the expansion of T regulatory (T reg) cells [19] in NHL. Patients with follicular lymphoma carrying *STAT-6* mutation have an enhanced expression level [20]. Unlike STATs, the SH2 domain-containing protein tyrosine phosphatases (SHPs) are considered as negative regulators of inflammation and tumour suppressors [21, 22]. Mice lacking *SHP-1* display myelin deficiency, which is caused by increased inflammatory mediators [21]. The expression of *SHP-1* is inactivated in leukemia and lymphoma, resulting from DNA methylation of the its promoter [22]. Activation of SHP-1 signalling suppresses tumor growth through inducing cell apoptosis in NHL [23]. In contrast, the level of *SHP-2* is expressed higher in diffuse large B-cell lymphoma [24]. In solid cancer, SHP-2 is the negative regulator of interferon production to elicit cytotoxic effects in fibroblast cells [25]. SHP-2 promotes ERK activation through its catalytic activity, leading to improved cell viability [26].

Little is known about the regulatory roles of *A20* and *CYLD* on functions of DCs and macrophages from ABC DLBCL and T-NHL patients. To this end, DCs and macrophages were exposed to Dox or ET in the presence or absence of *A20/CYLD* siRNA and expression of maturation/differentiation markers, phagocytic capacity and survival were examined. Expressions of STATs and SHPs were assessed to determine the involvement of *A20/ CYLD* and the activation of these signalling molecules in ABC DLBCL and T-NHL patients.

2. Materials and methods

2.1. Patients and control subjects

Fresh peripheral blood samples were collected from untreated 79 NHL patients, including ABC subtype DLBCL (39 cases, 49.36%) and T-cell NHL (T-NHL) (40 cases, 50.64%) based on cytomorphology and cytochemistry according to the WHO [27] classifications, at the National Institute of Haematology and Blood Transfusion, Ha Noi, Vietnam. The control group comprised 58 healthy individuals. No individuals in the control population took any medication or suffered from any known acute or chronic disease.

2.2. Drugs and cell culture

Peripheral blood mononuclear cells (PBMCs) from whole blood samples of healthy donors and patients with ABC DLBCL and T-NHL were collected and transferred to sterile tubes containing EDTA as anticoagulant. The cells were isolated via density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare Life Sciences). Freshly isolated PBMCs were obtained by centrifuging at 400g for 30 min at room temperature. The cells were cultured for 6 days in RPMI-1640 (GIBCO) containing: 10% FCS, 1% penicillin/streptomycin, 1% glutamine and 1% non-essential amino acids (NEAA) at a density of 5×10^6 cells/ml. Cultures were supplemented with M-CSF (50ng/mL, Peprotech) or FLT3L (100ng/mL, Peprotech) on days 0 and 4 to attain macrophages or DCs, respectively. Experiments were performed on days 8-10. Doxorubicin hydrochloride and Etoposide were purchased from Sigma Aldrich.

2.3. Immunostaining and flow cytometry

Immunophenotypic features of ABC subtype DLBCL and T-NHL cells were determined by flow cytometry (FACSAria Fusion, BD Biosciences) as previously described [7]. Cells (2×10^5) were incubated in 100 µl FACS buffer (PBS plus 0.1% FCS) containing fluorochrome-coupled antibodies to CD45, CD3, CD4, CD13, CD23, CD25, CD40, CD44, CD56, CD86, CD117 and FoxP3 (all from eBioscience) at a concentration of 10 µg/ml. After incubating with the antibodies for 60 min at 4°C, the cells were washed twice and resuspended in FACS buffer for flow cytometry analysis.

2.4. Cytokine quantification

Sera were isolated from the blood samples of NHL patients and healthy subjects and stored at -20˚C until used for ELISA. TNF-α, IL-6 and IL-1β concentrations were determined by using ELISA kits (Thermo Scientific) according to the manufacturer's protocol.

2.5. Transfection of DCs with siRNA

Control- or *A20/CYLD*-targeted siRNA (pre-designed siRNA, Thermo Scientific) was transfected into DCs or macrophages (2×10^5 cells/1ml) with the help of Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific) according to the manufacturer's recommendations. Cells were incubated for 48 h at 37°C, 5% CO_2. After washing three times with PBS, cells were used for experiments.

2.6. RNA extraction and real-time PCR

Total mRNA was isolated using the Qiashredder and RNeasy Mini Kit from Qiagen according to the manufacturer's instructions. For cDNA first-strand synthesis, 1 µg of total RNA in 12.5 µl DEPC-H_2O was mixed with 1 µl of oligo-dT primer (500 µg/ml, Invitrogen) and heated for 2 min at 70°C. To determine transcript levels of *A20, CYLD, STAT-1, STAT-3, STAT-5, STAT-6, SHP-1* and *SHP-2*, the quantitative real-time PCR with the LightCycler System (Roche Diagnostics) was applied. The following primers were used: *A20* primers: 5'-TCCTCAGGCTTTGTATTTGA-3' (forward) and 5'- TGTGTATCGGTGCATGGTTTT-3' (reverse); *CYLD* primers: 5'-TGCCTTCCAACTCTCGTCTTG-3' (forward) and 5'-AATCCGCTCTTCCCAGTAGG-3' (reverse); *STAT-1* primers: 5'-CCCTTCTGGCTTTGGATTGAA-3' (forward) and 5'-CTTCCCGGGAGCTCTCACTGA-3' (reverse); *STAT-3* primers: 5'-GGA GGA GTT GCA GCA AAA AG-3' (forward) and 5'-TGT GTT TGT GCC CAG AAT GT-3' (reverse); *STAT-5* primers: 5'-CAGACCAAGTTTGCAGCCAC-3' (forward) and 5'-CACAGCACTTTGTCAGGCAC-3' (reverse); *STAT-6* primers: 5'-GCCCACTCACTCCAGAGGACCT-3' (forward) and 5'-GGTGTTGGGGAAAGTCGACAT-3' (reverse); *SHP-1* primers: 5'- GCC CAG TTC ATT GAA ACC AC-3' (forward) and 5'- GAG GGA ACC CTT GCT CTT CT-3' (reverse); *SHP-2* primers: 5'- GAGAGCAATGACGGCAAGTCT-3' (forward) and 5'- CCTCCACCAACGTCGTATTTC-3' (reverse) and *GAPDH* primers: 5'-GGAGCGAGATCCCTCCAAA-3' (forward) and 5'-GGCTGTTGTCATACTTCTCAT-3' (reverse). PCR reactions were performed in a final volume of 20 µl containing 2 µl cDNA, 2.4 µl $MgCl_2$ (3 µM), 1 µl primer mix (0.5 µM of both primers), 2 µl cDNA Master SybrGreen I mix (Roche Molecular Biochemicals), and 12.6 µl DEPC-treated water. The target DNA was amplified during 40 cycles of 95ºC for 10 s, 62ºC for 10 s, and 72ºC for 16 s, each with a temperature transition rate of 20ºC/s, a secondary target temperature of 50ºC, and a step size of 0.5ºC. Melting curve analysis was performed at 95ºC, 0 s; 60ºC, 10 s; 95ºC, 0 s to determine the melting temperature of primer dimers and the specific PCR products. The ratio between the respective gene and corresponding *GAPDH* was calculated per sample according to the ΔΔ cycle threshold method [28].

2.7. Phagocytosis assay

FITC-dextran uptake assay: Cells (2 x 10^5 cells/ml) were suspended in prewarmed serum-free RPMI 1640 medium, pulsed with FITC-conjugated dextran (Sigma-Aldrich) at a final concentration of 1 mg/ml and incubated for 3h at 37ºC. Uptake was stopped by adding ice-cold PBS. Then the cells were washed three times with FACS buffer containing 0.01% sodium azide. The cells were analysed for the uptake of FITC-dextran with flow cytometry (FACSAria Fusion, BD Biosciences).

Phagocytosis of lymphoma cell assay: DCs and macrophages were cultured for 3h with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled lymphoma cells at the ratio of 1:2 and then washed three times with PBS. Subsequently, the cells were stained with anti-CD11c and anti-CD123 for DCs or with anti-CD68 for macrophages at 4ºC, washed twice and resuspended in FACS buffer for flow cytometry analysis. Phagocytosing cells were defined as the percentage of $CD11c^+$ $CD123^-CFSE^+$ (myeloid DCs) or $CD11c^-CD123^+CFSE^+$ (plasmacytoid DCs) or $CD68^+CFSE^+$ (macrophages) expressing cells.

2.8. Phosphatidylserine translocation and propidium iodide incorporation

The presence of phosphatidylserine (PS) on the outer surface of apoptotic cells was detected with fluorescein isothiocyanate (FITC)-conjugated annexin V binding to PS at the cell surface and necrotic cells were assessed from the amount of propidium iodide (PI)-positive cells. In brief, 10^5 cells were harvested and washed twice with Annexin washing buffer (AWB). The cell pellet was resuspended in 100 µl of Annexin V/PI labelling solution (eBioscience), and incubated for 15 min at room temperature. After washing with AWB, the cells were analysed by flow cytometry.

2.9. Statistical analysis

Statistical analysis was performed with the SPSS and GraphPad Prism 8 softwares. The statistical significance of the differences was determined by Mann–Whitney U test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Correlation among A20 and CYLD expressions and clinical outcomes in NHL

Seventy-nine patients with ABC DLBCL and T-NHL were enrolled and clinical features regarding these patients are shown in Table 1. A comprehensive correlation analysis showed significant increases in glucose, ferritin, AST, ALT, LDH and β2 microglobulin concentrations in the patient group. Among clinical outcomes, significant positive correlations among creatinine and uric acid, AST and ferritin, ALT and ferritin, AST and LDH, AST and ALT levels were observed in these patients (Figure 1A). In addition, no changes in other clinical indicators in the patient group were found (Table 1).

Furthermore, levels of signalling molecules including STATs and SHPs are evaluated by quantitative real time-PCR. Results indicated that expressions of *STAT-1, STAT-5* and *SHP-2* were significantly higher and levels of *STAT-6* and *SHP-1* were significantly lower in the patient group compared to healthy controls (Figure 1B). In addition, no difference in the expression of *STAT-3* was found between the patient and control groups (data not shown).

Since *A20* and *CYLD* are known as negative regulators of the inflammatory response in leukemia and lymphoma. Therefore, we asked whether there are associations between *A20* and *CYLD* expression levels, clinical outcomes and activation of signalling genes including *STATs* and *SHPs*. Accordingly, the expression of *A20* and *CYLD* was examined by quantitative real time-PCR and divided into two groups based on the median *A20* and *CYLD* expression values in healthy controls (high vs. low). The high *A20* expression group was detected in 29 samples (36.7%) and the low *A20* expression group was detected in 50 samples (63.3%). The

high *CYLD* expression group was detected in 37 samples (46.8%) and the low *CYLD* expression group was detected in 42 samples (53.2%) (Table 1). Our data showed that patients with the low *A20* expression had higher levels of *STAT-5* and lower levels of *STAT-1, CYLD* and *SHP-2* compared to those with the high *A20* expression, while patients with the low *CYLD* expression had lower levels

Table 1. Association among *A20* and *CYLD* expressions and clinical parameters in ABC DLBCL and T-NHL patients.

Characteristic	Normal	Total	A20			CYLD		
Number of patients	range	n=79	Low (n=50)	High (n=29)	*p* value	Low (n=42)	High (n=37)	*p* value
Age (years)		56.6 (17–86)	57.5 (24–75)	55.5 (17–86)		55.9 (17–79)	57.6 (22–86)	
Sex, Female (n, %)		48 (0.61)	29 (0.58)	19 (0.66)		23(0.55)	25 (0.68)	
Urea (mmol/l)	3.3-6.6	5.93±2.91	6 ± 2.98	5.8 ± 1.85	0.75	6.12 ± 3.14	5.7 ± 1.84	0.48
Glucose (mmol/l)	3.9-5.6	**6.64 ± 2.87**	6.7 ± 3.36	6.53 ± 0.1.78	0.803	6.86 ± 3.63	6.39 ± 1.64	0.474
Creatinine (µmol/l)	50-110	80.56 ± 21.13	79.73 ± 24.27	81.98 ± 14.5	0.651	79.9 ± 24.7	81.32 ± 16.5	0.766
Uric acid (µmol/l)	< 420	328 ± 122	316 ± 136	348 ± 89.9	0.26	319 ± 134	337 ± 108	0.53
Total bilirubin (µmol/l)	0-21	12.87 ± 10.9	11.77 ± 7.6	14.76 ± 14.9	0.241	12.43 ± 8.07	13.37 ± 13.53	0.705
Indirect bilirubin (µmol/l)	0-17	3.39 ± 7.69	2.74 ± 3.69	4.49 ± 11.7	0.33	2.96 ± 3.99	3.86 ± 10.4	0.61
Total protein (g/l)	60-80	71.08 ±8.89	69.86 ± 8.98	73.18 ± 8.47	0.11	69.5± 9.42	72.9 ± 8	0.089
Albumin (g/l)	35-50	36.8 ± 5.37	36 ± 5.24	38.2 ± 5.39	0.07	36.1 ± 5.78	37.6 ± 4.84	0.24
Globulin (g/l)	20-35	34.3 ± 7.5	33.9 ± 7.51	34.94 ± 7.56	0.56	33.38± 7.55	35.32 ± 7.4	0.253
Ferritin (ng/ml)	10-300	**583.51 ± 516.15**	605.06 ± 516.67	566.34 ± 522.24	0.629	599.2 ± 545.8	556.4 ± 391	0.693
AST (GOT) (U/l)	5-40	**47.65 ± 78.04**	43.68 ± 47.6	56.24 ± 113.5	0.46	39.97 ± 48.19	56.37 ± 102	0.355
ALT (GPT) (U/l)	7-55	**58.71 ± 109.1**	51.93 ± 63.46	70.41 ± 160.8	0.471	42.21 ± 46.21	77.44 ± 150.5	0.153
GGT (U/L)	<66	58.9 ±85.5	66.4 ± 101	46.1± 47.7	0.31	50.4 ± 68.6	68.6± 102	0.35
LDH (U/l)	140-280	**579.2 ± 477.1**	562.2 ± 500.4	608.4± 441	0.681	599.2 ± 545.8	556.4 ± 391	0.693
β2 microglobulin level (mg/L)	0.8-2.2	**2.8 ±1.48**	2.87 ± 1.57	2.69 ± 1.33	0.608	2.909 ± 1.67	2.693 ± 1.24	0.521
Hemoglobin (g/L)	120-180	124.22 ± 19.83	122.58 ± 16.77	127.03 ± 24.29	0.34	124.05 ± 16.85	124.41 ± 22.9	0.938
Erythrocytes (10*12 cells/L)	3.8-5.9	4.12 ± 0.62	4.07 ± 0.53	4.2 ± 0.75	0.394	4.12 ± 0.545	4.12 ± 0.7	0.962

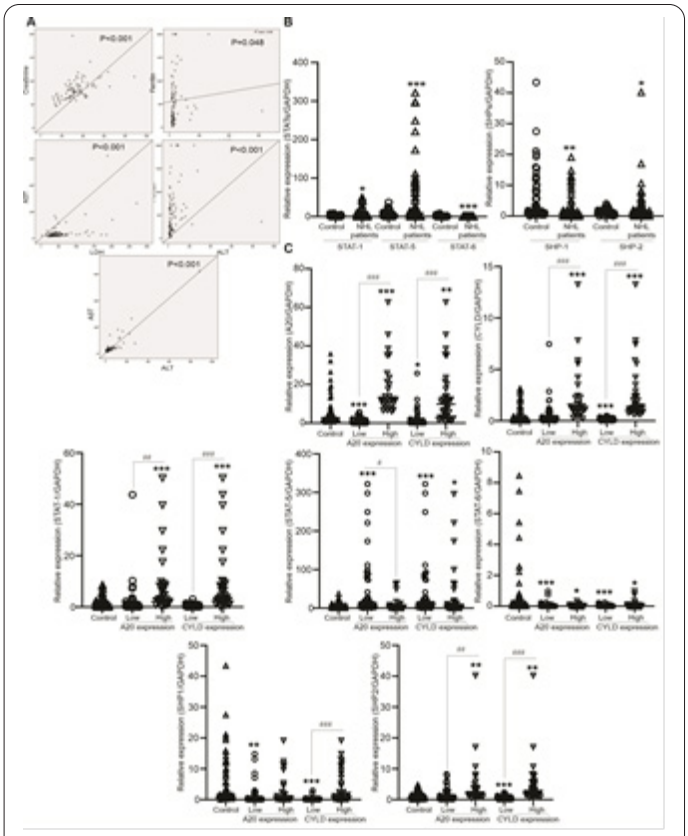

Fig. 1. Correlation among A20 and CYLD expression and clinical outcomes in ABC DLBCL and T-NHL patients. **A.** The Spearman correlation analysis shows the expression levels of Creatinine and Uric acid; AST and Ferritin; ALT and Ferritin; AST and LDH; AST and ALT (n=79) in ABC DLBCL and T-NHL patients. **B.** Graphs indicate the mRNA levels of *STAT-1, STAT-5, STAT-6, SHP-1* and *SHP-2* in control individuals (n=58) and NHL patients (n =79); *GAPDH* was used as a reference gene for relative quantification; each dot represents a single sample. * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) show significant differences from healthy individuals (Mann–Whitney U test). **C.** Graphs indicate the mRNA levels of *A20, CYLD, STAT-1, STAT-5, SHP-1* and *SHP-2* in control individuals (n=58) and NHL patients (n=29-50); ** ($p<0.01$) and *** ($p<0.001$) show significant differences from healthy individuals; $^{\neq}$($p<0.05$), $^{\neq\neq}$($p<0.01$) and $^{\neq\neq\neq}$($p<0.001$) show significant differences from the low *A20/CYLD* expression group (Mann–Whitney U test).

of *A20, STAT-1, SHP-1* and *SHP-2* compared to those with the high *CYLD* expression (Figure 1C). In addition, *A20* and *CYLD* expression levels were not significantly associated with clinical parameters, although levels of AST and ALT tended to increase in the low *A20*/*CYLD* expression group compared to the high *A20*/*CYLD* expression group, however, not reaching to the statistical significance (Table 1).

3.2. Immunophenotyping of ABC DLBCL and T-NHL patients

Immunophenotypic analysis showed that the number of $CD3^+CD4^+CD25^+$ $FoxP3^+$ (Treg) cells was more prominent in the circulation in both subtypes, whereas the number of $CD13^+CD117^-$, $CD56^+CD40^+$ (activated natural killer-NK) and $CD23^+CD40^+$ expressing cells were significantly elevated in ABC subtype DLBCL cases compared to healthy individuals and T-NHL patients. In contrast, the frequency of $CD4^+CD25^+$ cells was significantly enhanced in T-NHL than in healthy individuals and ABC DLBCL cases (Figure 2A-B). The results suggested that the high percentages of $CD13^+CD117^-$, $CD56^+$ $CD40^+$ and $CD23^+$ $CD40^+$ expressing cells might be prognostically favorable in ABC DLBCL subtype. In addition, the numbers of $CD23^+$, $CD13^-CD117^+$, $CD13^+CD117^+$ and $CD56^+CD25^+$ cells present in both subtypes were unaltered compared to controls (data not shown).

Next, cytokine production in sera of NHL patients was examined. Similar to a recent study [29], we also observed that levels of IL-6 and TNF-α in patient group were found higher than in control individuals, however, these patients showed no change in the serum level of IL-1β (Figure 2C).

3.3. Promoting effect of A20 on phagocytosis of lymphoma cells by DCs from ABC DLBCL patients

As indicated above, the low *A20* expression was found in 63.3% and the low *CYLD* expression was found in 53.2% of both NHL subtypes, therefore, we asked whether *A20* and *CYLD* expression affect functions of DCs and macrophages from both NHL subtypes. Firstly, DCs and macrophages are capable of phagocytosing foreign particles and cancer cells, which are assessed by FITC-dextran uptake and phagocytosis of CFSE-treated lymphoma cells. We observed that DC and macrophage counts obtained from both NHL subtypes were comparable to those from healthy controls. The percentages of DCs expressing $CD11c^+$ and percentages of macrophages expressing $CD68^+$ all were detected ≥80% in both NHL subtypes and healthy donors (data not shown).

Subsequently, the phagocytosis of lymphoma cells by

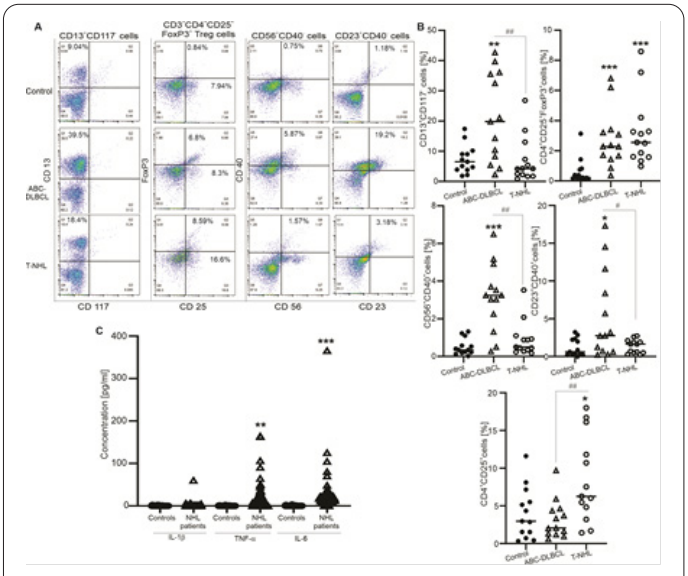

Fig. 2. Immunophenotyping of ABC DLBCL and T-NHL patients. **A**. Representative dot plots of $CD13^+CD117^-$, $CD3^+CD4^+CD25^+$ $FoxP3^+$ (Treg, gated on $CD3^+CD4^+$ cells), $CD56^+CD40^+$ and $CD23^+CD40^+$ expressing cells in control individuals, ABC DLBCL and T-NHL patients. **B**. Graphs indicate the percentages of $CD13^+CD117^-$, $CD3^+CD4^+CD25^+$ $FoxP3^+$, $CD56^+CD40^+$, $CD23^+CD40$ and $CD4^+CD25^+$ expressing cells in control individuals, ABC DLBCL and T-NHL patients. * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) show significant differences from healthy individuals; $^{\neq}$($p<0.05$) and $^{\neq\neq}$($p<0.01$) show significant differences from ABC DLBCL cases (Mann–Whitney U test). **C**. Graph indicates the serum concentrations of IL-1β, IL-6 and TNF-α in control individuals and NHL patients. ** ($p<0.01$) and *** ($p<0.001$) show significant differences from healthy individuals (Mann–Whitney U test).

DCs and macrophages from ABC DLBCL and T-NHL patients and healthy donors was determined. As shown in Figure 3A-B, phagocytosis of lymphoma cells by DCs from ABC DLBCL and T-NHL subgroups was significantly lower than that from healthy donors, while phagocytosis of lymphoma cells by macrophages was comparable among the three groups (data not shown). In addition, FITC-dextran uptake by DCs and macrophages was also similar to each other among the three groups (data not shown). The results demonstrated that DCs from ABC DLBCL and T-NHL subgroups were defective in phagocytosis of lymphoma cells.

To ask whether *A20* and *CYLD* influence in phagocytosis of DCs and macrophages from ABC DLBCL and T-NHL patients, the cells were transfected with control or *A20/CYLD* siRNA and followed by Dox and ET treatment. Interestingly, upon treatment of Dox and ET, the phagocytosis of lymphoma cells was significantly reduced by $CD11c^{+}CD123^{-}$ DCs from ABC DLBCL subtype in the presence of *A20* siRNA, but not *CYLD* siRNA (Figure 3C-D). In addition, *A20*/*CYLD* unaffected the phagocytosis of lymphoma cells by $CD11c^{+}CD123^{-}$ DCs from T-NHL subtype and $CD11c^{-}CD123^{+}$ DCs (plasmacytoid DCs) and $CD68^{+}$ macrophages from both NHL subtypes (data not shown) in the exposure with Dox and ET. The evidences indicated that the phagocytosis of lymphoma cells by $CD11c^{+}CD123^{-}$ DCs from ABC DLBCL subtype was decreased in the absence of *A20*.

Unlike the phagocytosis of lymphoma cells, FITC-dextran uptake by DCs and macrophages from both ABC DLBCL and T-NHL patients was unaltered upon silencing of *A20*/*CYLD* in the exposure with Dox and ET (data not shown).

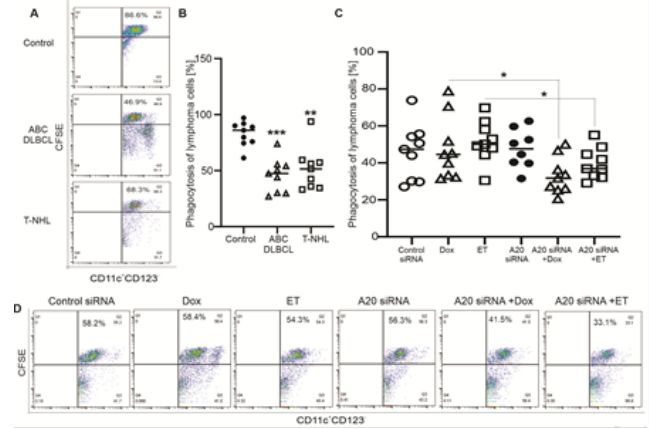

Fig. 3. Effect of A20 on phagocytosis of lymphoma cells by DCs from ABC DLBCL patients. **A.** Representative dot plots of CFSE positive $CD11c^{+}CD123^{-}$ DCs in control individuals, ABC DLBCL and T-NHL patients. **B**. Graph indicates the percentages of CFSE positive $CD11c^{+}CD123^{-}$ DCs (n=9) in control individuals, ABC DLBCL and T-NHL patients. ** ($p<0.01$) and *** ($p<0.001$) show significant differences from healthy individuals (Mann–Whitney U test). **C**. Graph indicates the percentages of CFSE positive $CD11c^{+}CD123^{-}$ DCs (n=9) from ABC DLBCL patients, which are transfected with control siRNA or *A20* siRNA and followed by Dox or ET treatment. * ($p<0.05$) shows significant difference from A20-silenced DCs (Mann–Whitney U test). **D.** Representative dot plots of CFSE positive $CD11c^{+}CD123^{-}$ DCs from ABC DLBCL patients, which are transfected with control siRNA or *A20* siRNA and followed by Dox or ET treatment.

3.4. Effect of A20 on maturation/differentiation and survival of DCs and macrophages from ABC DLBCL and T-NHL patients

As indicated above, the number of $CD13^{+}CD117^{-}$, $CD56^{+}CD40^{+}$ and $CD23^{+}CD40^{+}$ expressing cells was significantly elevated in ABC DLBCL cases compared to healthy individuals and T-NHL patients, for this reason, we asked whether the expression of these markers on DCs and macrophages are related to the presence of *A20*/*CYLD*. Similar to the phagocytosis of lymphoma cells, only $CD56^{+}CD40^{+}$ expressing DCs ($CD56^{+}$ mature DCs) from ABC DLBCL patients was dependent on the regulation of *A20,* as the percentage of $CD56^{+}CD40^{+}$, but not $CD13^{+}CD117^{-}$ and $CD23^{+}CD40$ expressing DCs from ABC DLBCL patients only was significantly enhanced in the presence of *A20* siRNA upon treatment of Dox and ET (Figure 4A-B). In addition, the percentages of $CD13^{+}CD117^{-}$, $CD56^{+}CD40^{+}$ and $CD23^{+}CD40^{+}$ expressing macrophages were unaltered in the absence of *A20* in both NHL subtypes (data not shown). Also, *CYLD* did not influence the expression of the markers on DCs and macrophages both NHL subtypes (data not shown). The results pointed out that $CD56^{+}$ mature DCs from ABC DLBCL cases was sensitive to *A20* expression.

Next, the expression of maturation markers including CD40, CD86 and CD44 on DCs and macrophages from both subtypes was examined. As shown in Figure 4C-D, treatment with Dox and ET significantly increased the number of $CD86^{+}CD40^{+}$, but not $CD44^{+}$ on DCs from both NHL subtypes in the presence of *A20* siRNA. However, the expression of the maturation markers on macrophages was unaffected by the absence of *A20*/*CYLD* and on DCs by the absence of *CYLD* (data not shown).

Dox and ET are known to exert cancer cell death via inhibiting DNA replication and disrupting the function of topoisomerase II [16, 17], therefore, we conducted experiments to ask whether the influence of Dox and ET on apoptosis of DCs and macrophages from both subtypes by the absence of *A20*/*CYLD*. Accordingly, treatment of DCs (Figure 4E) and macrophages (Figure 4F) with Dox and ET significantly increased the percentage of Annexin $V^{+}PI^{+}$ cells. However, the survival of DCs and macrophages in the two NHL subgroups was uninfluenced by the presence of *A20*/*CYLD* siRNA (data not shown).

4. Discussion

In this study, the role of *A20*-dependent DCs was significantly related to the elimination of lymphoma cells in ABC DLBCL patients, which was documented for the first time. The regulatory roles of *A20* on functions of DCs from ABC DLBCL and T-NHL patients were different from each other. A20 promoted the phagocytosis of lymphoma cells by $CD11c^{+}CD123^{-}$DCs and inhibited the number of $CD56^{+}$mature DCs from ABC DLBCL, but not T-NHL subtype when exposed to Dox and ET. In contrast, the phagocytosis of lymphoma cells by $CD11c^{-}CD123^{+}$ DCs was not sensitive to the presence of A20 in both patient groups. Differently, A20 inhibited the expression of CD40 and CD86 on DCs in both ABC DLBCL and NHL subtypes in the exposure with Dox and ET. The results suggested that ABC DLBCL patients with low *A20* expression lost the phagocytosis of lymphoma cells and had an enhanced number of $CD56^{+}$ mature DCs, which are identified as killer DCs [30]. CD56 expressing IFN-

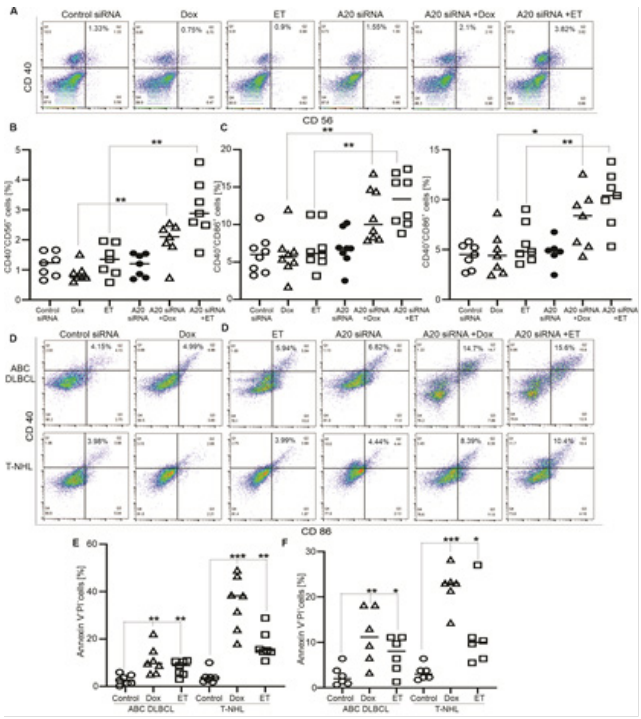

Fig. 4. Effect of A20 on maturation/differentiation and survival of DCs and macrophages from ABC DLBCL and T-NHL patients. **A**. Representative dot plots of $CD56^+CD40^+$ DCs from ABC DLBCL patients, which are transfected with control siRNA or *A20* siRNA and followed by Dox or ET treatment. **B**. Graph indicates the percentages of $CD56^+CD40^+$ DCs (n=7) from ABC DLBCL patients, which are transfected with control siRNA or *A20* siRNA and followed by Dox or ET treatment; ** ($p<0.01$) shows significant difference from *A20*-silenced DCs (Mann–Whitney U test). **C**. Graphs indicate the percentages of $CD86^+CD40^+$ DCs from ABC DLBCL (n=8, left graph) and from T-NHL (n=7, right graph) patients, which are transfected with control siRNA or *A20* siRNA and followed by Dox or ET treatment. * ($p<0.05$) and ** ($p<0.01$) show significant differences from *A20*-silenced DCs (Mann–Whitney U test). **D**. Representative dot plots of the percentages of $CD86^+CD40^+$ DCs from ABC DLBCL and from T-NHL patients, which are transfected with control siRNA or *A20* siRNA and followed by Dox or ET treatment. **E-F**. Graphs indicate the percentages of Annexin V^+PI^+ DCs (n=7, left graph) and Annexin V^+PI^+ macrophages (n=6, right graph) from ABC DLBCL and T-NHL patients, which are untreated (control) or treated with Dox or ET treatment. * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) show significant differences from control (Mann–Whitney U test).

stimulated DCs is known to exhibit tumoricidal activity [31]. In contrast to the effect of A20 in negatively regulating mouse macrophages [15], the regulatory effects of *A20* on functions of human macrophages in both ABC DLBCL and T-NHL subtypes were not found in this finding.

Unlike *A20, CYLD* did not participate in regulating functions of human DCs and macrophages from both ABC DLBCL and T-NHL subtypes. Differently, *CYLD* is considered as a negative regulator of hyperresponsive inflammation in macrophages in mice [9]. Attenuated expression of *CYLD* is correlated with a poor prognosis in Hodgkin lymphoma [12]. In contrast, increased CYLD phosphorylation is a frequent event in adult T-cell lymphoma [13]. Our study on *CYLD* gene polymorphisms in NHL patients even observed that several *CYLD* variants were at reduced risk of NHL (data not published).

In contrast to down-regulation of *A20/CYLD* resulting in inactivation of STAT-1, recent studies indicated that A20/CYLD inhibits STAT-1 expression [7, 9], suggesting that activation of STAT-1 in *A20/CYLD*- sensitive non-Hodgkin lymphoma cells was different from other cell types. Aberrant expression and mutations in *A20* gene have been reported in DLBCL patients [11]. In addition, *A20* and *CYLD* in DCs and macrophages from both ABC DLBCL and NHL subtypes were not associated with the regulation cell apoptosis and the release of TNF- α and IL-6. Differently, the promoting effect of *A20* on cell death is indicated in several cell types including lymphoma [32], while *CYLD* is reported as a positive regulator of cell apoptosis in lymphocytic leukemia [13].

In this finding, the expressions of *STAT-1, STAT-5* and *SHP-2* were significantly enhanced, whereas the levels of *STAT-6* and *SHP-1* were significantly reduced in both ABC DLBCL and T-NHL subtypes compared to healthy individuals. Recently, level of *STAT-1* is lowly expressed in ALK-positive anaplastic large-cell lymphoma [33], while strong intensity of STAT-1 staining is correlated with improved overall survival in Hodgkin lymphoma [34]. STAT-5 activation has anti-apoptotic functions [18] and induces the expansion of T reg cells [19] in lymphomas. In contrast to the expression of *STAT-6* in this study, constitutive activation of STAT-6 is found in Hodgkin lymphoma [35]. This finding suggested that STAT-6 distinctly regulated cell functions in NHL and Hodgkin lymphoma.

Similarly, activation of SHP-1 signalling is known to induce cell apoptosis in NHL [23]. Inactivation of *SHP-1* is reported in chronic myelogenous leukemia [22]. The lack of *SHP-1* leads to defective apoptosis and poor response to treatment [36]. Overexpression of *SHP-2* is also detected in patients with leukemia [24].

Concerning immunophenotype, the proportion of $CD13^+CD117^-$, $CD23^+CD40^+$ and $CD56^+CD40^+$ expressing cells were significantly elevated in ABC DLBCL cases compared to healthy individuals and T-NHL patients. In consistent, CD13 expression is present more often in ABC DLBCL than T cell lymphoma subtypes and absent in cases of follicular lymphoma [5]. CD23 and CD40 expressions are prognostically favorable in DLBCL [4] and the number of $CD56^+CD40^+$ NK cells, which are functionally involved in induction of the cytotoxic pathway [37]. Recently, NK cell activation is shown by $CD25^+CD56^+$ and $CD69^+CD56^+$ expressing cells in follicular lymphoma patients [38]. Similar to this finding, the expansion of $CD4^+CD25^+$ cells is found in T cell-NHL subtype [39] and Treg cells are recruited to circulatory system to NHL patients [39] and promote immune evasion and tumor dissemination [40].

5. Conclusion

The present study discloses that A20 promoted the phagocytosis of lymphoma cells by $CD11c^+CD123^-$ DCs and expansion of $CD56^-$positive mature DCs from ABC DLBCL patients in the exposure with Dox and ET. The events might contribute to anticancer effects of Dox and ET in *A20*-sensitive ABC DLBCL patients. Inactivation of A20 in ABC DLBCL cases might result in defect of the phagocytosis of lymphoma cells by DCs as well as infiltration of killer DCs into the circulation.

Conflict of Interests

The authors of this paper declare that they have no finan-

cial/commercial conflicts of interest.

Consent for publications
The authors read and approved the final manuscript for publication.

Ethics approval and consent to participate
All patients and volunteers gave written consent to participate in the study. Person care and experimental procedures were performed according to Vietnamese law for the welfare of humans and were approved by the Ethical Committee of the Institute of Genome Research, Vietnam Academy of Science and Technology.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
Conception and design: Ha NT, Trang DT, Nhat PV, Huong PT and Xuan NT.
Acquisition of data: Ha NT, Trang DT, Nhat PV, Huong PT, Giang NH, Mao CV.
Analysis and interpretation of data: Ha NT, Mao CV, Binh VD, Vuong NB and Xuan NT.
Writing of the manuscript: Vuong NB and Xuan NT.

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