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BTK Inhibitor Downregulates IL-17 Secretion and Enhances CD20mb Sensitivity to ABC Type Diffuse Large B-cell Lymphoma via CYLD/NF-kB Signaling Pathway

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Abstract: AIM: To explore whether two novel Bruton's tyrosine kinase (BTK) inhibitors, acalabrutinib and zanubrutinib, in combination with Rituximab (RTX), enhance the cytotoxic effects on diffuse large B-cell lymphoma (DLBCL) cell lines and to elucidate the underlying mechanisms. **Method:** We selected the activated B-cell-like (ABC) DLBCL cell lines NU-DUL-1 and SU-DHL-2 as parental lines, and their corresponding Rituximab (CD20mb)-resistant lines NU-DUL-1-R as resistant strains. Using 20% fresh normal human serum as the source of complement, we employed 7-aminoactinomycin D (7-AAD) flow cytometric staining to assess the cytotoxic effects of BTK inhibitors combined with RTX via complement-dependent cytotoxicity (CDC) on tumor cells. Tumor cells were labeled with carboxyfluorescein diacetate N-succinimidyl ester (CFSE) and co-cultured with peripheral blood mononuclear cells (PBMCs). The cytotoxic effects of BTK inhibitors combined with RTX via antibody-dependent cell-mediated cytotoxicity (ADCC) were evaluated using 7-AAD and PE-Annexin V flow cytometric staining to determine whether sensitivity to RTX was increased. Flow cytometry was used to detect the expression of CD20 on DLBCL cell lines and the proportions of $CD4^+IL\text{-}17^+$ T cells in PBMNCs, granzyme B and TNF-α in $CD8^+$ T cells in the PBMCs-tumor cell co-culture system. ELISA was employed to measure the concentrations of related cytokines. qPCR was used to assess the effects of BTK inhibitors on the mRNA expression of retinoic acid-related orphan receptor (RORC) and interleukin-17 (IL-17). Western blotting was performed to detect the protein expression levels of p-NF-κB-p65 in $CD4^+$ T cells after treatment with BTK inhibitors. We also established a mouse model of BALB/c B-cell lymphoma using A20 cells overexpressing human CD20. Tumor volume was measured and recorded. Flow cytometry was used to detect the proportion of Th17 cells in peripheral blood and spleen, and ELISA was employed to measure the expression levels of IL-17 in serum. **Results: (1)** The novel BTK inhibitors had little effect on the expression of CD20 on the surface of NU-DUL-1, NU-DUL-1-R, and SU-DHL-2 cells. (2) When RTX exerted its CDC effect, compared with the monotherapy group, the combination of acatinib and RTX significantly enhanced the cytotoxic effects on NU-DUL-1, NU-DUL-1-R, and SU-DHL-2 cells (P<0.05), with a dose-dependent effect observed in NU-DUL-1 and NU-DUL-1-R cells (P<0.05). The combination of zanubrutinib and RTX also significantly enhanced the cytotoxic effects on NU-DUL-1 and NU-DUL-1-R cells (P<0.05), but the effect on SU-DHL-2 cells was not statistically significant at a zanubrutinib concentration of 10 μmol/L (P>0.05). (3) When RTX exerted its ADCC effect, compared with the monotherapy group, the combination of acalabrutinib and RTX significantly enhanced the cytotoxic effects on NU-DUL-1, NU-DUL-1-R, and SU-DHL-2 cells (P<0.05). The combination of zanubrutinib and RTX significantly enhanced the cytotoxic effects on NU-DUL-1 and SU-DHL-2 cells (P<0.05), but the effect on NU-DUL-1-R cells was not statistically significant at a zanubrutinib concentration of 3 μmol/L (P>0.05). (4) Flow cytometry results showed that both acalabrutinib and zanubrutinib significantly downregulated the proportion of Th17 cells and upregulated the secretion of granzyme B and TNF-α in $CD8^+$ T cells (P<0.05). (5) Compared with the control group, RTX alone significantly upregulated the proportion of Th17 cells and increased IL-17 secretion. In contrast, the combination of acalabrutinib or zanubrutinib with RTX significantly downregulated the proportion of Th17 cells and reduced IL-17 secretion (P<0.05). (6) Western blot results showed that both acalabrutinib and zanubrutinib inhibited the phosphorylation of NF-κB-p65 in $CD4^+$ T cells (P<0.05). (7) Both novel BTK inhibitors downregulated the expression of RORC and IL-17 mRNA in $CD4^+$ T cells and reduced the secretion of IL-17, significantly inhibiting the differentiation of Th17 cells (P<0.05). (8) In vivo experiments demonstrated that zanubrutinib significantly reduced tumor volume and downregulated the proportion of Th17 cells and IL-17 levels in the spleen and peripheral blood of mice (P<0.05). **Conclusions:** BTK inhibitors reduce IL-17 secretion via the CYLD/NF-κB pathway and enhance the sensitivity of ABC -DLBCL to CD20 mb The combination of novel BTK inhibitors with RTX may be an effective strategy to overcome RTX resistance in ABC -DLBCL.

Keywords: Rituximab; Novel BTK Inhibitors; Diffuse Large B-cell lymphoma; IL-17; Th17; NF-κB.

1. Introduction

In the past 30 years, the incidence rate of non Hodgkin lymphoma (NHL) has almost doubled, and DLBCL is the most common invasive NHL. So far, although the first-line R-CHOP treatment regimen based on CD20 monoclonal antibody (CD20mb, Rituximab) has greatly improved clinical efficacy, disease recurrence, but rituximab resistance has

always been a challenge in clinical work. The BCR signaling pathway plays a crucial role in the occurrence and development of DLBCL. Preliminary clinical trials have found that BTK inhibitors, represented by ibrutinib, greatly improve efficacy. The BTK inhibitor ibrutinib is an important new drug for treating NHL[1]. Ibrutinib inhibits BTK phosphorylation, blocks B cell activation, and exerts therapeutic effects in autoimmune diseases and B cell malignant tumor models[2]. Ibrutinib is a first generation selective, covalent, and irreversible BTK inhibitor with high activity in other BCR dependent lymphoid malignancies, such as CLL or mantle cell lymphoma (MCL)[3-5]. In phase II clinical trials of refractory DLBCL patients, ibrutinib was found to be more effective than GCB-DLBCL subtypes, possibly due to the sustained activation of the B cell receptor (BCR) signaling pathway in non GCB-DLBCL[5, 6]. The nuclear transcription factor kappa B (NF-κB) signaling pathway is a downstream pathway that activates BCR signaling through BTK, and is an important signaling pathway for regulating tumor apoptosis [7]. Ibrutinib can inhibit the NF-κB activity of DLBCL[7], especially the sustained activation of NF-κB signaling with non GCB DLBCL [8].

Cylindromatosis (CYLD) was first discovered in familial cylindrical tumors and demonstrated to be a tumor suppressor[9]. It inhibits the activation of NF-κB, plays an important role in inflammation, immune response, tumor development, and inhibition of tumor cell apoptosis, promoting tumor cell proliferation[10] . CYLD inhibits NF-κB activity through the activation of its deubiquitinase function. Phosphorylation is an important process for downregulating CYLD activity in many types of cells, such as skin cancer cells[11, 12]. The regulation of CYLD transcription and translation is highly variable, involving multiple mechanisms such as direct phosphorylation of kinases. At the protein level, CYLD is rapidly and transiently phosphorylated by TNF, LPS, and mitogens at multiple residues in the serine cluster between amino acids 418 and 444[13]. Our previous research has shown that CYLD phosphorylation is highly expressed in adult T-cell leukemia/lymphoma (ATLL), and increasing CYLD deubiquitinase activity by downregulating CYLD phosphorylation levels,and can promote apoptosis of ATLL tumor cells [14]. At present, only a few reports have shown good efficacy and safety of erlotinib, lenalidomide, and rituximab in the treatment of relapsed/refractory Non GCB type DLBCL[15]. However, some studies have suggested that the lack of improvement in the prognosis of DLBCL patients with R-CHOP regimen combined with erlotinib is related to the downregulation of CD20 expression in tumor cells by erlotinib. However, the role of other BTK inhibitors is still unclear[16].

The main characteristics of ABC type DLBCL are chronic active BCR signaling and BCR aggregation on the cell surface. Several genomic studies have shown that the activation of BCR signaling leads to the activation of downstream pathways such as NF-κB or PI3K/AKT signaling cascades, which play a critical role in the occurrence and development of ABC type DLBCL [8, 17-19]. Some clinical data of ibrutinib indicated that BTK inhibition in ABC type could play a greater role compared to GCB type DLBCL patients. However, due to the off-target inhibition of ITK by ibrutinib, it antagonizes NK cell-mediated anti-CD20 antibody dependent ADCC [20]. Meanwhile, a phase III clinical study also found that adding ibrutinib to R-CHOP did not improve efficacy in untreated non GCB type DLBCL patients [21]. Acalabrutinib (ACP-196) and zanubrutinib (BGB-3111) are novel, selective, and irreversible BTK inhibitors. The biggest difference from ibrutinib is their selectivity towards targets. The novel BTK inhibitors significantly reduce off-target effects beyond BTK, such as EGFR, TEC, and ITK[22-24]. A study has found that ibrutinib can also participate in improving T cell immune reconstitution in CLL patients[25]. CYLD is a tumor suppressor that inhibits the activation of NF-κB and plays an important role in inflammation, immune response, tumorigenesis, and inhibition of tumor cell apoptosis[10-12]. Our recent research has found that ibrutinib and acalabrutinib downregulate NF-κB activity by downregulating CYLD protein phosphorylation, promoting apoptosis in non GCB type DLBCL, including resistance to rituximab[26].

In summary, at present, research has elucidated that the CYLD/NF-κB pathway is also closely related to the immune regulation of T cells, and the pathogenesis of DLBCL is related to inflammation and immune imbalance.The combination of ibrutinib and R-CHOP regimen did not significantly improve the prognosis of ABC type DLBCL patients in clinical practice.This study aims to seek a new combination regimen of BTK inhibitors and rituximab. Therefore, in vitro and vivo,this study investigated the effects of novel BTK inhibitors ACP-196 and BGB-3111, via CYLD/NF-kB signaling pathway, combined with RTX on the killing of ABC type DLBCL cell lines, as well as the effects of novel BTK inhibitors combined or not combined with RTX on the functions of various T cell subsets, especially on Th17 cells.This study will provide therapeutic ideas and new theoretical basis for the combination of novel BTK inhibitors with RTX.

2. Materials and Methods

2.1. Cells

The non-GCB-type DLBCL cell lines NU-DUL-1 and SU-DHL-2 were purchased from Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd. The drug-resistant strain NU-DUL-1-R corresponding to NU-DUL-1 was constructed in-house; the construction method is described in our previous study[27]. All experiments were conducted using cells in the logarithmic growth phase. NU-DUL-1-R cells were used after they had been withdrawn from drug treatment for 1 week and had returned to the logarithmic growth phase.

2.2. Reagents

RTX was purchased from Shanghai Fosun Henlius Biopharmaceutical Co., Ltd. Acalabrutinib (ACP-196) and zanubrutinib (BGB-3111) were obtained from Selleck Chemicals (USA). The CCK8 assay kit was sourced from DOJINDO Laboratories. ELISA kits were purchased from Xibosheng Biotechnology Co., Ltd. qPCR primers were obtained from Sangon Biotech, and the qPCR kit was from Axygen Biosciences. CFSE and the following antibodies were purchased from Tonbo Biosciences (USA): anti-human CD3-PE-Cy7, anti-human CD4-FITC, and anti-human CD8-Percp-Cy5.5. The following antibodies were obtained from Invitrogen (USA): anti-human IL-17-APC-eFluor780 and anti-human TNF-α-FITC. The following antibodies were purchased from BioLegend (USA): anti-human CD20-FITC

and anti-human/mouse Granzyme B Recombinant-Brilliant Violet 421™. The mouse B-cell lymphoma cell line A20 was obtained from Tongpai (Shanghai) Biotechnology Co., Ltd. BALB/c mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. NF-κB p65 and pNF-κB p65 antibodies were sourced from Cell Signaling Technology (CST, USA).

3. Methods

3.1. Cell Culture

Cells were cultured and passaged in an incubator with 5% CO_2 at 37° C. NU-DUL-1, **NU-DUL-1-R**, and SU-DHL-2 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. The concentration of rituximab is 100 mg/L.

3.2. Assessment of the Cytotoxic Effects of Acalabrutinib and Zanubrutinib on DLBCL Cell Lines Using the CCK-8 Assay

NU-DUL-1 and SU-DHL-2 cells were seeded into 96-well plates at a density of 2×10^4 cells per well and divided into control and experimental groups. The experimental group was treated with various concentrations of BTK inhibitors, while the control group was treated with an equal volume of complete culture medium. After 72 h of incubation, 10 μL of CCK-8 solution was added to each well, followed by incubation in the dark for 4h. The optical density (OD) values were measured at 450 nm using a microplate reader.

3.3. Detection of CDC Effect by 7-AAD Staining Flow Cytometry

NU-DUL-1, NU-DUL-1-R, and SU-DHL-2 cells were resuspended in complete culture medium and seeded into 6-well plates at a density of 1×10^6 cells per well. After 72h of treatment with BTK inhibitors, the medium was replaced, and RTX was added according to different experimental groups. After 2-4 h of incubation, cells from each group were stained with 7-AAD and analyzed by flow cytometry.

3.4. Detction of ADCC Effect by CFSE/Annexin V/7-AAD Staining Flow Cytometry

PBMCs were isolated from peripheral blood and resuspended in complete culture medium. The PBMCs were added to wells coated with 10 μg/ml anti-CD3 monoclonal antibody and supplemented with 2 μg/ml anti-CD28 monoclonal antibody to activate lymphocytes overnight. On the second day, NU-DUL-1, NU-DUL-1-R, and SU-DHL-2 cells were labeled with CFSE and co-cultured with PBMCs at an effector-to-target ratio of 5:1. Each co-culture was resuspended in complete culture medium and seeded into 6-well plates at a density of 1×10^6 cells per well. After 72 hours of treatment with BTK inhibitors, RTX was added according to different experimental groups. After 24 hours of incubation, cells from each group were collected and stained with Annexin V and 7-AAD for apoptosis detection by flow cytometry.

3.5. Flow Cytometric Detection of Th17, Granzyme B, and TNF-α Proportions

Cells from the co-culture system were resuspended in complete culture medium and seeded into 6-well plates coated with anti-CD3 monoclonal antibody at a density of 2×10^6 cells per well. Anti-CD8 monoclonal antibody was added to the wells. Following the manufacturer's instructions, APC-eFluor780-conjugated anti-human IL-17 flow cytometry antibody, Brilliant Violet 421™-labeled anti-human/mouse Granzyme B Recombinant flow cytometry antibody, and FITC-labeled anti-human TNF-α flow cytometry antibody were added to the cell suspensions. After 72 hours of co-culture, the cells were treated with 3 μg/ml Brefeldin A (BFA) at 37° C for 3-6 hours to detect changes in the proportions of Treg and Th17 cells by flow cytometry.

3.6. qPCR for mRNA Detection

Cells from the co-culture system were treated for 72 hours according to different experimental conditions. Total RNA was extracted from the cells using Trizol reagent, and cDNA was synthesized using a reverse transcription kit. The qPCR system was then prepared. The relative expression levels of RORC and IL-17 mRNA in the cells were calculated using the $2^{-\Delta\Delta Ct}$ method, and statistical analysis was performed on the results. The primer sequences used are listed in Table 1.

3.7. ELISA Assay

Cells from the co-culture system were treated with BTK inhibitors for 72 hours, followed by the addition of RTX according to different experimental groups for 24 hours. The levels of IL-17 were detected according to the manufacturer's instructions for the ELISA kit.

3.8. Western Blot

$CD4^+$ T cells were isolated using magnetic beads and treated with acalabrutinib and zanubrutinib at a concentration of 10 μmol/L. After 48 hours of culture, the cell suspension was collected, centrifuged, and the supernatant was discarded. Total cellular protein was extracted, and protein samples were prepared. The proteins were then transferred to a membrane, blocked, incubated, and visualized.

3.9. Animal Experiments

Female BALB/c mice aged 5-6 weeks and the murine B-cell lymphoma cell line A20 were used to establish the A20 murine B-cell lymphoma model. A total of 2×10^7/L A20 tumor cells were inoculated into the posterior axillary region of the mice. The mice were observed daily for their condition and the size of the tumor mass. When the tumor volume reached approximately 50 mm^3, the mice were randomly divided into two groups: the control group (administered with an equivalent volume of 0.5% sodium carboxymethyl cellulose solution by gavage, with the same dosing frequency as the treatment group) and the zanubrutinib group (7.5 mg/kg, dissolved in 0.5% sodium carboxymethyl cellulose solution, administered by gavage twice daily) [74]. Tumor volume was measured every 3 days for a total of 21 days of continuous dosing. Each group consisted of five mice, which were individually labeled and numbered. At the end of the dosing period, peripheral blood was collected from the mice, and the spleen and tumor mass were isolated for analysis.

4. Statistical Analysis

All in vitro experiments were performed at least three times independently, and representative results are shown in the figures. The grayscale of the protein bands was semi-

quantified using Image J software, GraphPad Prism 8.0 was used for graphing, and SPSS 22.0 was employed for statistical analysis. Comparisons between two groups were conducted using the t-test, while comparisons among more than two groups were performed using one-way ANOVA. Data are presented as mean±SD. A *P* value of less than 0.05 was considered statistically significant.

5. Results

5.1. The Effects of Different Concentrations of BTK Inhibitors Acalabrutinib and zanubrutinib on the Viability of ABC Type Diffuse Large B-cell Lymphoma Cells

We first used the CCK-8 method to detect the changes in the viability of ABC type DLBCL cell lines treated with different concentrations of acalabrutinib and zanubrutinib, and estimated their half maximal inhibitory concentration (IC50) based on the change curve. The results showed that the viability of NU-DUL-1 and SU-DHL-2 cells was significantly affected and dose-dependent after treatment with different concentrations of acalabrutinib and zanubrutinib. At the same time, the IC50 values of NU-DUL-1 and SU-DHL-2 cells treated with acalabrutinib for 48 hours were 12.51 μ mol/L and 30.58 μ mol/L, respectively. The IC50 values of NU-DUL-1 and SU-DHL-2 cells treated with zanubrutinib for 48 hours were 5.90 μ mol/L and 22.64 μ mol/L, respectively, as shown in Figure 1. Therefore, in order to clarify whether different concentrations of acalabrutinib and zanubrutinib have sensitizing effects on rituximab, we selected two representative concentrations, IC50 and 1/2 IC50, for subsequent experiments. That is, for NU-DUL-1 cells, the treatment concentrations of acalabrutinib were 6 μ mol/L and 12 μ mol/L, and the concentrations of zanubrutinib were 3 μ mol/L and 6 μ mol/L. For SU-DHL-2 cells, the concentrations of acalabrutinib were 15 μ mol/L and 30 μ mol/L, while the concentrations of zanubrutinib were 10 μ mol/L and 20 μ mol/L.

5.2. The Effect of Novel BTK Inhibitors on CD20 Expression on the Surface of ABC Type Diffuse Large B-cell Lymphoma Cells

The flow cytometry results showed that the novel BTK inhibitor had little effect on the expression of CD20 on the surface of NU-DUL-1, NU-DUL-1-R, and SU-DHL-2 cells, as shown in Figure 2.

5.3. The CDC Effect of Novel BTK Inhibitors Acalabrutinib and zanubrutinib Combined with Rituximab on the Killing of ABC Type Diffuse Large B-cell Lymphoma Cell Lines

The results showed that for acalabrutinib, compared with the monotherapy group, the combination of acalabrutinib and RTX enhanced the killing effect on NU-DUL-1 cells, NU-DUL-1-R cells, and SU-DHL-2 cells, and induced an increase in cell death rate, with statistical significance ($P<0.05$). At the same time, for NU-DUL-1 cells and NU-DUL-1-R cells, the killing effect of the combination group also showed a dose-dependent effect, with an increase in the concentration of acalabrutinib, There were statistically diferent ($P<0.05$), as shown in Figure 3 (A, C). Similarly, for zanubrutinib, compared with the monotherapy group, the killing effect of zanubrutinib in combination with RTX on NU-DUL-1 cells, NU-DUL-1-R cells, and SU-DHL-2 cells was also enhanced. However, for SU-DHL-2 cells, although the cell mortality rate was also increased in the combination group of zanubrutinib at different concentrations compared to the monotherapy group, the difference was not statistically significant at a zanubrutinib concentration of 10 μ mol/L. For NU-DUL-1 cells and NU-DUL-1-R cells, compared with the monotherapy group, different concentrations of zanubrutinib enhanced the killing effect of RTX on tumor cells, and the difference was statistically significant ($P<0.05$), as shown in Figure 3 (B, D).

5.4. The Effect of ADCC of Novel BTK Inhibitors Acalabrutinib and zanubrutinib Combined with Rituximab on the Killing of ABC Type Diffuse Large B-cell Lymphoma Cells

The flow cytometry results showed that for acalabrutinib, compared with the monotherapy group, the killing effect of acalabrutinib combined with RTX on NU-DUL-1, NU-DUL-1-R, and SU-DHL-2 cells was enhanced, and the induced cell mortality rate was increased, with statistical significance ($P<0.05$), as shown in Figure4(A, C). At the same time, for zanubrutinib, compared with the monotherapy group, the killing effect of zanubrutinib combined with RTX on NU-DUL-1, NU-DUL-1-R, and SU-DHL-2 cells was also enhanced. However, unlike the CDC effect, for NU-DUL-1-R cells, although the cell mortality rate was also increased in the combination group of different concentrations of zanubrutinib compared to the monotherapy group, the difference was not statistically significant at a zanubrutinib concentration of 3 μ mol/L. For NU-DUL-1 and SU-DHL-2 cells, compared with the monotherapy group, different concentrations of zanubrutinib enhanced the killing effect of RTX on tumor cells, and the difference was statistically significant ($P<0.05$), as shown in Figure 4 (B, D).

5.5. The Effects of Novel BTK Inhibitor Acalabrutinib (ACP-196) on the Function of T Cells

The anti-tumor effect of BTK inhibitors on ABC type DLBCL is mainly achieved by inhibiting the BTK kinase to suppress the BCR signaling pathway. However, its anti-tumor effect on tumor microenvironment immune cells, especially its impact on T cells. Therefore, we conducted in vitro co-culture experiments of PBMCs and tumor cells to explore whether the novel BTK inhibitors acalabrutinib and zanubrutinib would affect the function of various T cell subsets in vitro. In the results, $CD3^+CD8^-IL\text{-}17^+$cells were used to label $CD4^+IL\text{-}17^+$cells, i.e. Th17 cells.

The flow cytometry results showed that compared with the control group, the expression of IL-17 in $CD4^+$T cells decreased in the monotherapy acalabrutinib group, while the secretion of granzyme B and TNF-α by $CD8^+$T cells significantly increased, with statistical significance, as shown in Figure4. The results of the zanubrutinib group were similar to those of the acabrutinib group, as it also promoted the secretion of granzyme B and TNF -α in $CD8^+$T cells and downregulated the proportion of Th17 cells. The results were statistically significant, as shown in Figure5.

5.6. The Effect of novel BTK Inhibitors Combined with Rituximab on Th17 and IL-17

Our study found that the novel BTK inhibitors acalabrutinib and zanubrutinib can downregulate the proportion of Th17 cells and decrease IL-17 secretion. Our previous research has shown that rituximab upregulates the proportion of Th17 cells and IL-17 secretion, which is related to rituximab resistance. Therefore, we will investigate whether the combination of novel BTK inhibitors acalabrutinib and zanubrutinib with rituximab can restore the Th17 cell proportion caused by rituximab.

The results showed that compared with the control group, RTX upregulated the proportion of Th17 cells. Compared with RTX alone treatment, the combination of acalabrutinib and RTX can downregulate the proportion of Th17 cells, as shown in Figure 7A. The effect of zanubrutinib in combination with RTX is similar, as shown in Figure 7B. ELISA detection of the effects of acalabrutinib and zanubrutinib combined with RTX on IL-17. The results showed that compared with the control group, the single drug RTX group secreted the most IL-17, while the combination of acalabrutinib and zanubrutinib with RTX group significantly reduced IL-17 secretion (P<0 05) (Figure 7C)

The aforementioned in vitro studies have found that the combination of novel BTK inhibitors and RTX can enhance the killing effect on ABC type DLBCL cells and increase the sensitivity of RTX resistant strains to RTX. And this may be related to the enhanced anti-tumor effect of BTK inhibitors on $CD8^+$T cells, as well as the downregulation of the proportion of Th17 cells in $CD4^+$T cells, thereby restoring the imbalance of Th17 proportion after RTX treatment on this basis, we explored the mechanism of downregulation of IL-17 secretion by a novel BTK inhibitor through in vitro experiments and validated it by animal models.

5.7. The Effects of Novel BTK Inhibitors Acalabrutinib and zanubrutinib on NF-κB-p65 in CD4+T Cells

Treat the sorted $CD4^+$T cells with novel BTK inhibitors (acalabrutinib or zanubrutinib, 10 μ mol/L), collect cells from each group after 48 hours of treatment, and extract proteins for Western Blot detection.The results showed that both acalabrutinib and zanubrutinib could inhibit NF-κB-p65 phosphorylation in $CD4^+$T cells, as shown in Figure 8.

5.8. The Effects of Novel BTK Inhibitors Acalabrutinib and zanubrutinib on IL-17 in CD4+T cells

Treat the sorted $CD4^+$T cells with novel BTK inhibitors (acalabrutinib or zanubrutinib, 10 μ mol/L) for 48 hours, then collect cells from each group for qPCR detection, and collect the supernatant from each group for ELISA detection.The qPCR results showed that both the novel BTK inhibitors acalabrutinib and zanubrutinib could downregulate the relative expression levels of *RORC* and *IL-17* mRNA in $CD4^+$T cells, with statistical significance (P<0 05), see Figure 9 A.The ELISA detection results also showed that the novel BTK inhibitor can downregulate the IL-17 levels secreted by $CD4^+$T cells, as shown in Figure 9B.

5.9. Animal Experiments

5.9.1. Construction of A20 Cells Expressing Human CD20

The A20 cell line is a BALB/c mouse B-cell lymphoma. We constructed a lentiviral vector overexpressing human B-cell antigen CD20 (hCD20) and screened to establish a stable mouse B-cell lymphoma A20 transgenic cell line A20-hCD20 expressing hCD20. The flow cytometry results showed that compared with the control group, the expression of CD20 on the surface of A20-hCD20 cells was significantly increased (P<0.05), as shown in Figure 6A. The qPCR results also showed a significant increase in human CD20 mRNA expression in A20-hCD20 cells (P<0.05), as shown in Figure10.

5.9.2. BTK Inhibitors Inhibit Tumor Growth

In vitro experiments have confirmed that the novel BTK inhibitorsAcalabrutinib and zanubrutinib can downregulate IL-17 secretion. Based on this, we established an animal model to verify whether the novel BTK inhibitor zanubrutinib can downregulate IL-17 secretion in vivo.We selected female BALB/c mice aged 6-8weeks to establish an A20 mouse B-cell lymphoma model(n=5). The administration method, dosage, and frequency were as described in the experimental method, and the tumor volume of the mice was measured and recorded. After continuous administration for 21 days, and peripheral blood was collected from the mice to isolate the tumor. As shown in the figure10, the tumor mass volume in the zanubrutinib group was significantly smaller than that in the control group,as shown in Figure11.

5.9.3. The Effect of zanubrutinib on the Proportion of Th17 Cells and IL-17 Secretion Level in Animal Models

We collected peripheral blood samples from the frontal and splenic regions of mice and tested for Th17 cells and IL-17.Flow cytometry results showed that compared to the control group, the proportion of Th17 in the spleen and peripheral blood of mice in the zanubrutinib group was downregulated. ELISA results also showed that zanubrutinib could downregulate IL-17 levels in the peripheral blood of mice(Fig12).

6. Discussion

Diffuse large B-cell lymphoma is the most common invasive NHL. So far, although first-line R-CHOP treatment based on Rituximab) has greatly improved clinical efficacy, Rituximab resistance greatly limits long-term efficacy. At present, there is an urgent need to seek new treatment options. The combination of small molecule targeted drugs and anti-CD20mb is an accessible and convenient treatment option. In this study, we firstly found that the new BTK inhibitor can enhance the CDC effect and ADCC effect of RTX on ABC type DLBCL cells, and enhance the sensitivity of RTX resistant strains to RTX. Secondly, we revealed that the novel BTK inhibitor enhanced the anti-tumor effect of $CD8^+$T cells and downregulated the proportion of Th17 cells in $CD4^+$T cells, reducing IL-17 secretion and restoring the imbalance of Th17 cell proportion after RTX action. Finally, we validated through in vivo and in vitro studies that the novel BTK inhibitor can suppress IL-17 secretion levels by inhibiting NF-κB-p65 phosphorylation levels in $CD4^+$T cells, and validated through animal models that the combination of BTK inhibitor and Anti-CD20mb enhances the sensitivity of Anti-CD20mb and overcomes its resistance.

So far, although the R-CHOP regimen based on RTX combined with cyclophosphamide, doxorubicin, vincristine, and prednisone has been effective for most DLBCL patients, a considerable proportion of patients still experience treatment failure or relapse after treatment, with ABC type DLBCL patients accounting for the majority[28]. In this study, we used human ABC type diffuse large B-cell lymphoma cell lines, NU-DUL-1 and SU-DHL-2 cell lines as research subjects. According to our previous research method, we constructed the Anti-C20mb resistant strain NU-DUL-1(NU-DUL-1R) using increasing concentration[26].

As novel, selective, and irreversible BTK inhibitors, acalabrutinib andzanubruinib, although also covalently bound to the Cys481 residue, have higher selectivity for BTK compared to ibrutinib, significantly reducing off target effects beyond BTK, such as EGFR, TEC, and ITK. Therefore, they may eliminate the shortcomings of ibrutinib in combination with rituximab, and improve safety due to reduced off target toxicity[22]. In this study, we conducted an in vitro study using Acalabrutinib and zanubruinib, in vivo study using zanubruinib.

To investigate the synergistic sensitization effect of BTK inhibitors on rituximab, we firstly used the CCK-8 method to detect the changes in the viability of ABC type DLBCL cells, and selected BTK concentrations with IC50 and 1/2 IC50 for the next experiment. Voso MT et al. found in vitro experiments that the ability to inhibit B-cell lymphoma cells was strongest when the concentration of rituximab was 100 μg/ml. There are also some reports that the average maximum plasma concentration of rituximab was 205 μg/ml and the average half-life was 76.3 hours when patients received a standard dose chemotherapy of 375mg/m^2 of rituximab. Therefore, we chose a rituximab concentration of 100 μg/ml which can be achieved in vivo[29, 30].

However, due to the off-target inhibition of ITK by ibrutinib, it antagonizes NK cell-mediated anti-CD20 antibody dependent ADCC[20]. Subsequently, scholars have continuously explored the efficacy of novel BTK inhibitors combined with Anti-CD20mb in the treatment of B-cell lymphoma. We focused on exploring the effects of novel BTK inhibitors zanubruinib and acalabrutinib on the CDC and ADCC effects of CD20mb. Regardless of the CDC or ADCC effects, compared to the monotherapy group, the killing effect of acalabrutinib or zanubrutinib combined with RTX on NU-DUL-1, SU-DHL-2 cells, and RTX resistant strain NU-DUL-1-R cells was enhanced. The induced cell mortality rate increased. The results revealed that the novel BTK inhibitor not only enhanced the sensitivity of RTX resistant strains to RTX, but also reversed RTX resistance.

As is well known, the resistance mechanism of rituximab includes reduced and ADCC, polymorphism of the FcγRIIIa on cytotoxic cells, loss and mutation of CD20, et al. Firstly, we treated lymphoma cells in vitro with a novel BTK inhibitor. Flow cytometry revealed that both BTK inhibitors had little effect on CD20 expression on the surface of NU-DUL-1, NU-DUL-1-R, and SU-DHL-2 cells.

The novel BTK inhibitor enhances the killing effect of RTX on ABC type DLBCL cell lines through multiple mechanisms. BTK inhibitors mainly block the BCR signaling pathway by inhibiting BTK kinase, killing tumor cells and remodeling the tumor microenvironment, including T cells. Research has shown that granzyme B and perforin are the two most important components for CD8$^+$ effector T cells to target and kill tumor cells, and have demonstrated anti-cancer properties in various cancers. Therefore, in order to explore whether the novel BTK inhibitors acalabrutinib and zanubrutinib affect T cell function in vitro, we conducted experiments in which PBMCs were co-cultured with tumor cells. The results showed that compared with the control group, monotherapy with acalabrutinib and zanubrutinibcould promote the secretion of granzyme B and TNF - α by CD8$^+$ T cells, along with downregulated Th17 differentiation. This indicated that the novel BTK inhibitor can activate the anti-tumor effect of T lymphocytes to enhance their killing ability against tumor cells, including RXT-resistant line NU-DUL-1R.

Our previous studies have shown that CYLD phosphorylation is highly expressed in adult T-cell leukemia/lymphoma (ATLL), and increasing CYLD deubiquitinase activity by downregulating CYLD phosphorylation can promote apoptosis of ATLL tumor cells [14]. Recently, our research has found that BTK inhibitors promote apoptosis of non GCB type DLBCL, including CD20mb resistant DLBCL, by downregulating CYLD protein phosphorylation and NF-KB activity[26]. In this study, we further investigated that BTK inhibitors not only directly act on lymphoma cells, but also act on T cells.

Th17 cells are a new type of CD4$^+$effector T cell discovered in recent years, mainly secreting IL-17, while IL-17R is present in various types of cells, including tumor cells. Our previous studies have found[31, 32] that radiotherapy and CD20mb promoted the secretion of IL-17 by Treg cells in the B-NHL tumor microenvironment, inhibited the expression of tumor suppressor gene p53, and thus suppressed the apoptosis of tumor cells triggered by radiotherapy and CD20mb. Our recent research has found that human bone marrow-derived mesenchymal stem cells (hBMMSCs) promoted the upregulation of IL-17 in the TME by secreting IL-6, activated the JAK2/STAT3 pathway or upregulate CyclinD2 through PI3K/Akt signaling, and synergistically promoted tumor growth with IL-17[33]. In summary, our preliminary results suggested that CD20mb and radiotherapy promote the secretion of IL-17, thereby inducing resistance and resistance to radiotherapy and CD20mb in DLBCL patients[31, 32, 34].

We used co-culture technolog of CD20mb, PBMNCs, BTK inhibitors acalabrutinib and zanubruinib, and tumor cells. We found that BTK inhibitors downregulate Th17 cell differentiation and IL-17 secretion induced by rituximab. We further sorted CD4$^+$ T cells and then adiminstrated with BTK inhibitors. In vitro and in vivo studies have confirmed that CYLD inhibits NF -κB activity through deubiquitination, but its regulatory mechanism may vary depending on cell type[35]. NF-κB family transcription factors regulate the expression of cytokines and chemokines involved in immune response through transcription, and NF-κB signaling is involved immune cell development and function, regulating the differentiation of Na ï ve CD4 (+) T cells into T cell subsets such as Th17 and regulatory T (Treg) cells[36]. Our results showed that BTK inhibitors downregulated NF- κB-p65 phosphorylation in CD4$^+$ T cells, with zanubruinib being more pronounced. Recent studies have found that in Th17 cells, RORC is a direct target of transcription factor P65. The binding of P65 to the RORC promoter site inhibits transcription factor p65 NF-κB activity and reduces RORγ t/IL-17 levels, indicating that NF-κB activity regulates transcription factor RORγ levels, regulates Th17 cell development, and IL-17 secretion[37].

Finally, we validated the effect of BTK inhibitors on lymphoma cells and their impact on Th17 differentiation

through animal models. We established a tumor bearing BALB/c mouse model inoculated with A20 cells. After continuous feeding of zanubruinib to mice for 21 days, the tumor mass significantly shrank compared to the control group. Compared with the control group, the proportion of Th17 in the spleen and peripheral blood of mice in the zanubruinib group was downregulated, and zanubruinib could downregulate the level of IL-17 in the peripheral blood of mice. Based on our previous research findings, we believe that the inhibition of lymphoma growth by BTK inhibitors is related to the downregulation of IL-17 by BTK inhibitors[31-34].

Although this article explains that BTK inhibitors regulate NFK-B signaling in $CD4^+$ T cells and downregulate IL-17 secretion, there are still few limitations to this article.For example, we were unable to validate it in DLBCL patients.. In summary, in this study, we elucidated that BTK inhibitors not only directly act on lymphoma, but also achieve dual pathway anti-tumor effects by regulating the immune status of the tumor microenvironment. Although BTK inhibitors did not regulate CD20 expression in tumor cells, their combination with rituximab enhanced the ADCC and CDC effects of rituximab. In addition, BTK inhibitors regulate the development of T cell subsets that it and enhance their anti-tumor effects. It inhibits the development of Th17, thereby downregulating IL-17 secretion. Based on our previous findings, our in vivo and in vitro studies have revealed that BTK inhibitors inhibited CYLD/NF-kB signaling. BTK inhibitors exert a dual effect, directly anti-tumor such as proapoptosis, and downregulating IL-17 secretion. Finally, we found that BTK inhibitors not only enhanced the killing effect of rituximab on parental lymphoma cells through the aforementioned mechanism, but also reversed the resistance of rituximab.

The R-CHOP like combined X regimen is currently an important strategy used in clinical practice to overcome Rituximab resistance and enhance sensitivity. This study reveal that CYLD phosphorylation is a potential beneficial clinical therapeutic target for non-GCB-DLBCL in the future.

7. Figures and Table

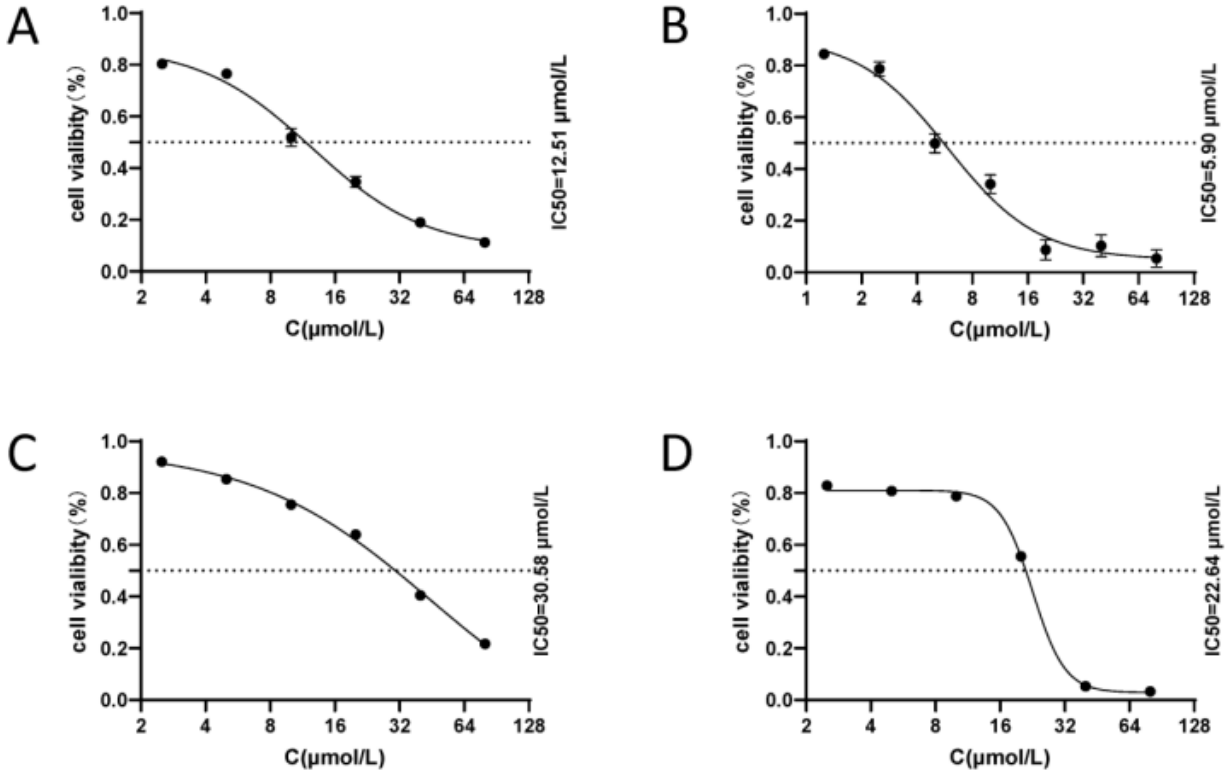

Figure 1. The viability of ABC-DLBCL cell lines after treated with different concentrations of BTK inhibitors detected by CCK-8 method. A: The viability of NU-DUL-1 cells after treated with different concentrations of acalabrutinib detected by CCK-8 method; B: The viability of NU-DUL-1 cells after treated with different concentrations of zanubrutinib detected by CCK-8 method; C: The viability of SU-DHL-2 cells after treated with different concentrations of acalabrutinib detected by CCK-8 method; D: The viability of SU-DHL-2 cells after treated with different concentrations of zanubrutinib detected by CCK-8 method.

Availability of Data and Materials

All the data can be found in this manuscript or received from the corresponding author upon reasonable request.

Contributions of the Authors

LQS conceived the study, obtained funding, and provided overall supervision. ZX designed and performed the experiments, analysed the data, and curated the supporting materials. GGL and WT performed additional experiments and provided technical support, SH prepared the figures and analyzed the data. ZX and SH drafted the initial manuscript; LQS critically revised it. All authors reviewed and approved the final version.

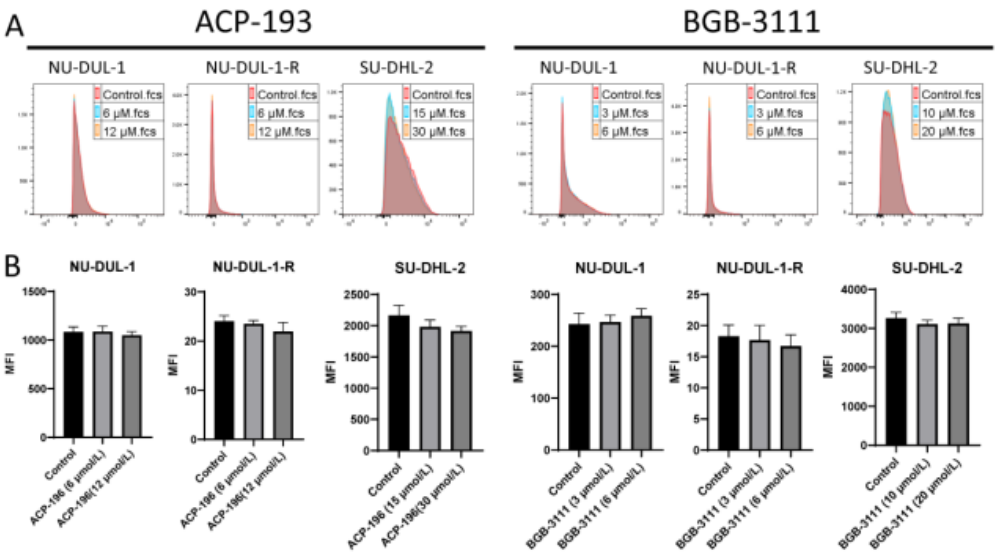

Figure 2. Effect of novel BTK inhibitors acalabrutinib (ACP-196) and zanubrutinib (BGB-3111) on the expression of CD20 antigen on the surface of ABC-DLBCL cell lines. A: The expression of CD20 antigen on the surface of ABC-DLBCL cell lines treated with acalabrutinib (ACP-196) and zanubrutinib detected by flow cytometry; B: Histogram of the expression of CD20 antigen on the surface of ABC-DLBCL cell lines in various groups. Mean±SD. n=3.

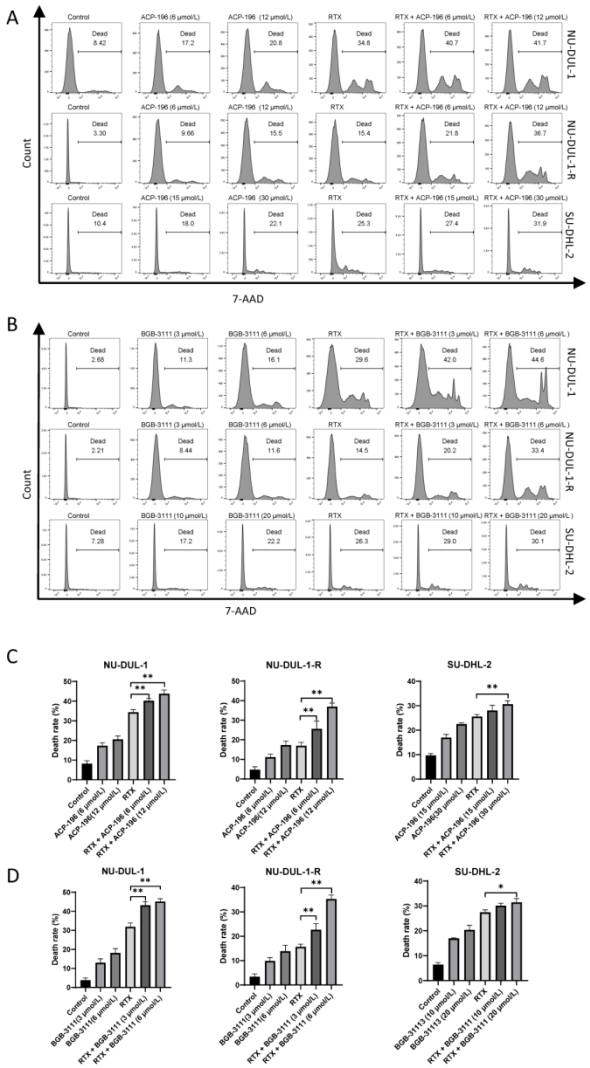

Figure 3. Death rates of ABC-DLBCL cell lines induced by the CDCof novel BTK inhibitors and RTX. A: Death rates of NU-DUL-1, NU-DUL-1-R and SU-DHL-2 cells induced by the CDC of acalabrutinib (ACP-196) and RTX detected by flow cytometry; B: Death rates of NU-DUL-1, NU-DUL-1-R and SU-DHL-2 cells induced by the CDC of zanubrutinib (BGB-3111) and RTX detected by flow cytometry; C,D: Histogram of death rates of ABC-DLBCL cell lines in various groups. Mean±SD. n=3. **P*<0. 05; ***P*<0. 01.

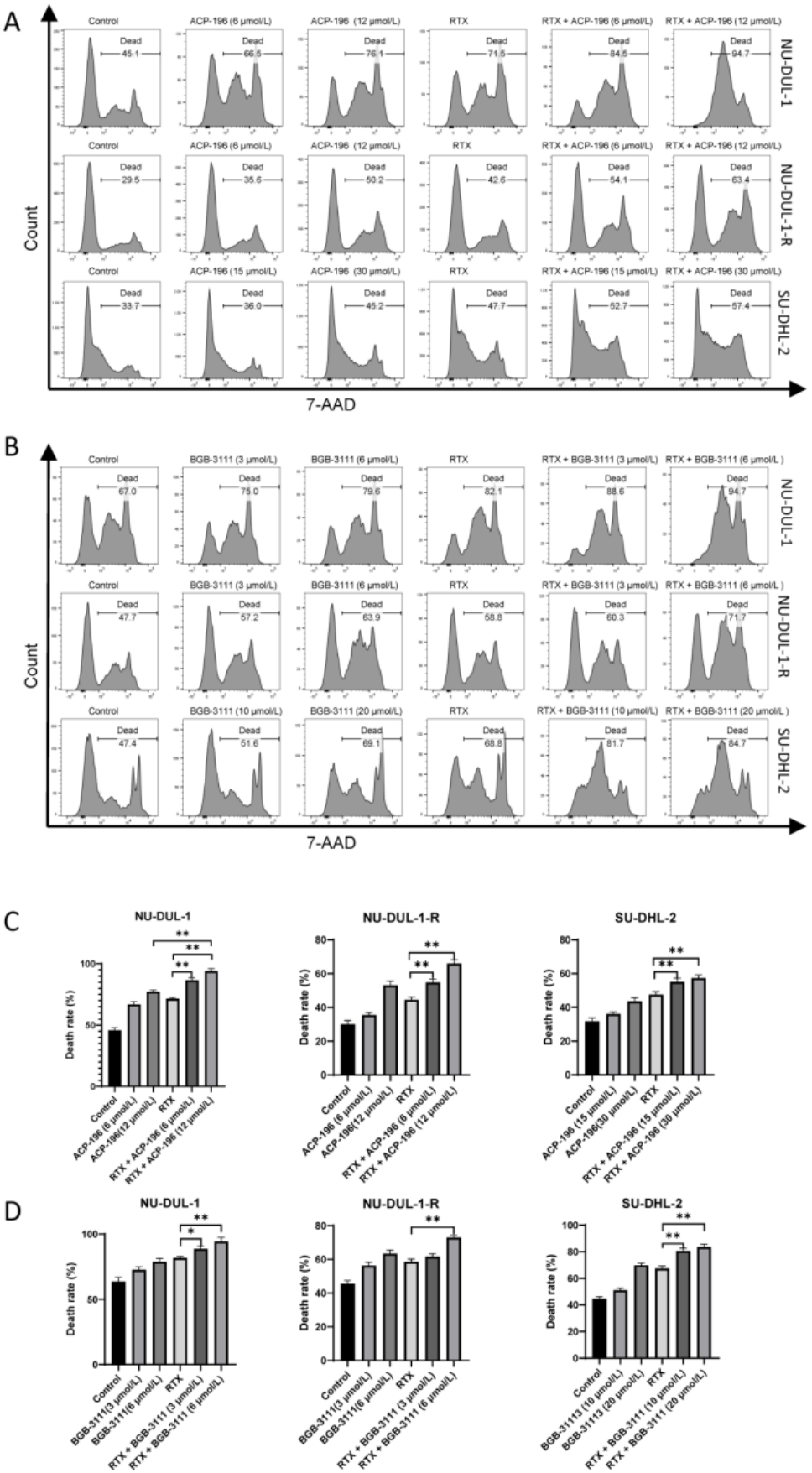

Figure 4. Death rates of ABC-DLBCL cell lines induced by the ADCC of novel BTK inhibitors and RTX. A: Death rates of NU-DUL-1, NU-DUL-1-R and SU-DHL-2 cells induced by the ADCC of acalabrutinib (ACP-196) and RTX detected by flow cytometry; B: Death rates of NU-DUL-1, NU-DUL-1-R and SU-DHL-2 cells induced by the ADCC of zanubrutinib (BGB-3111) and RTX detected by flow cytometry; C, D: Histogram of death rates of ABC-DLBCL cell lines in various groups. Mean ± SD. n=3. **P*<0. 05; ***P*<0. 01.

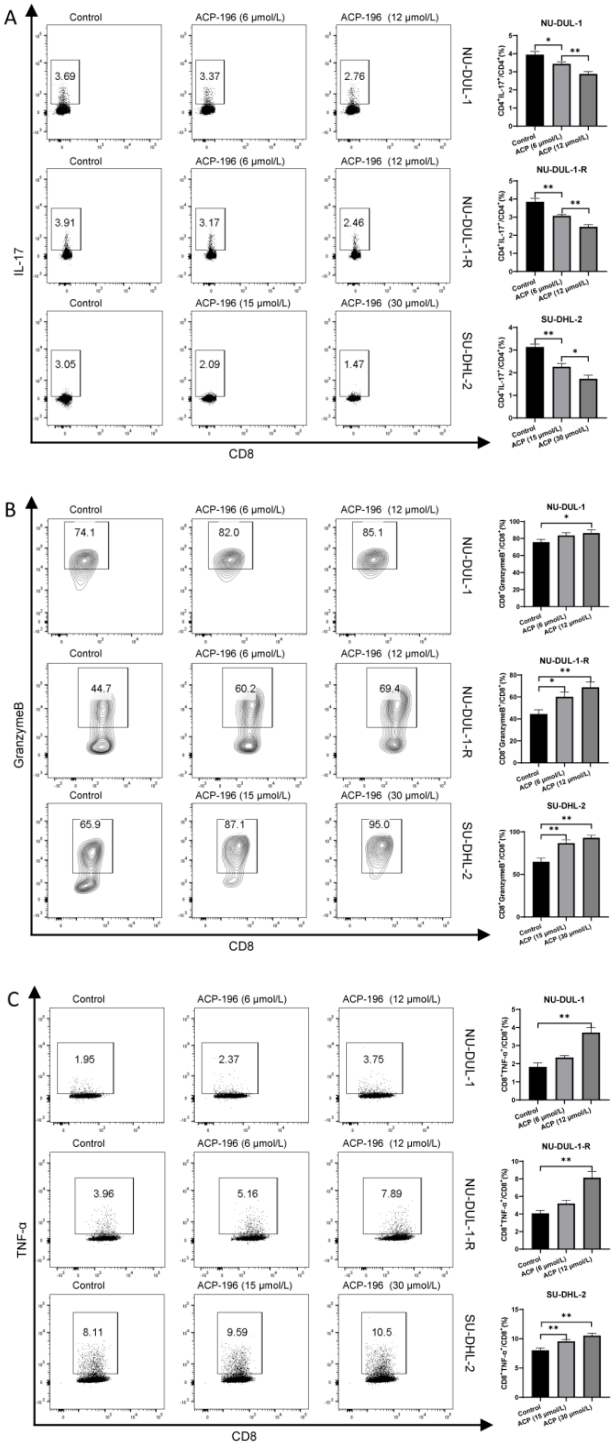

Figure 5. Effect of novel BTK inhibitor acalabrutinib (ACP-196) on the function of various T cell subsets in co-culture system. A: Effect of acalabrutinib on the percentages of $CD4^+IL\text{-}17^+$ T cells; B: Effect of acalabrutinib on the release of granzyme B by $CD8^+$ T cells; C: Effect of acalabrutinib on the release of TNF-α by $CD8^+$ T cells. Mean ± SD. n=3. **P*<0. 05; ***P*<0. 01.

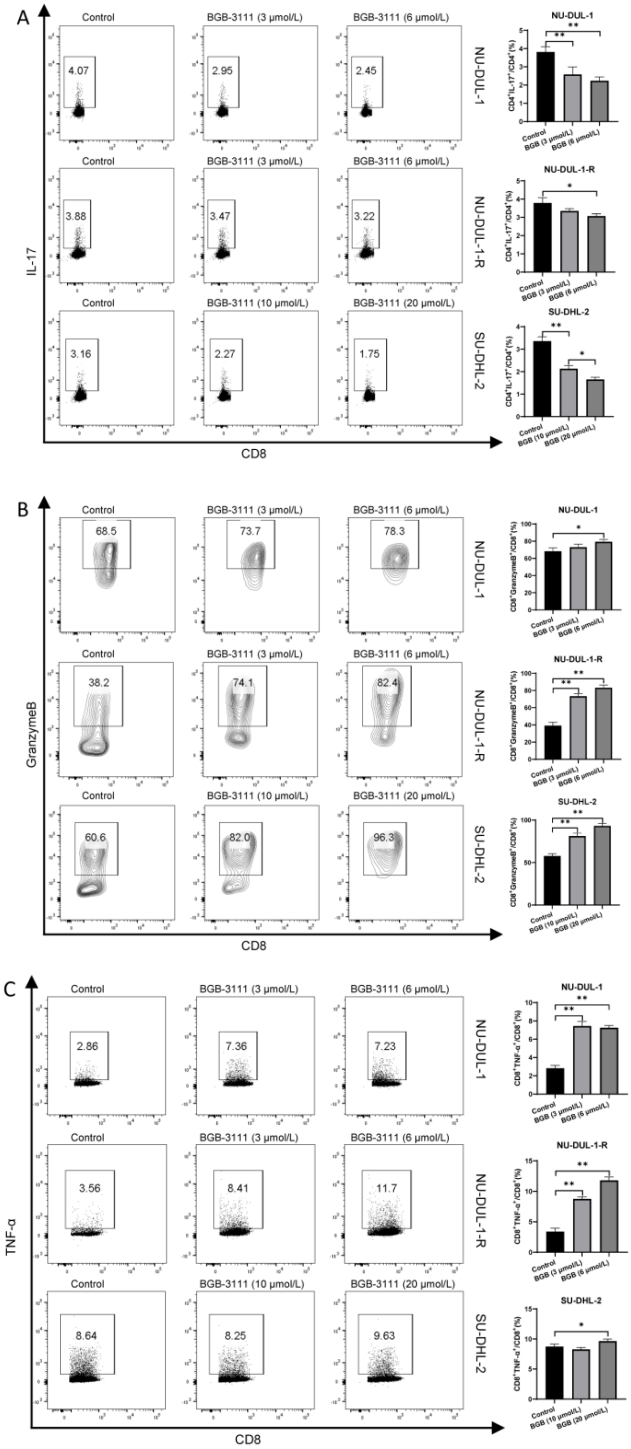

Figure 6. Effect of novel BTK inhibitor zanubrutinib (BGB-3111) on the function of various T cell subsets in co-culture system.A: Effect of zanubrutinib on the percentages of $CD4^+IL\text{-}17^+$ T cells; B: Effect of zanubrutinib on the release of granzyme B by $CD8^+$ T cells; C: Effect of zanubrutinib on the release of TNF-α by $CD8^+$ T cells. Mean ± SD. n=3. **P*<0. 05; ***P*<0. 01.

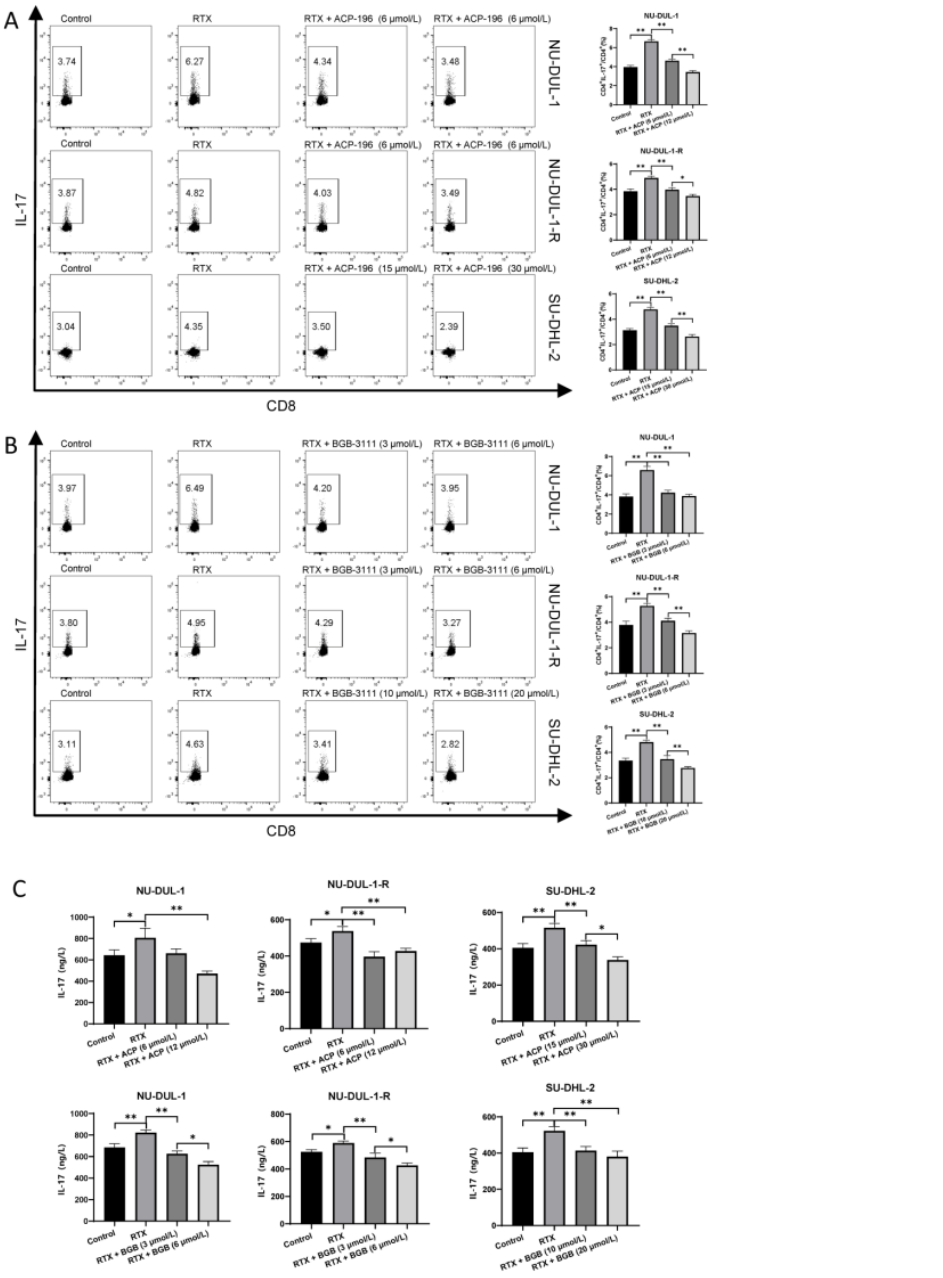

Figure 7. Effect of novel BTK inhibitors acalabrutinib (ACP-196) and zanubrutinib (BGB-3111) combined with RTX on the proportion of Th17 cells and the level of IL-17.A: Effect of acalabrutinib combined with RTX on the proportion of Th17 cells detected by flow cytometry; B: Effect of zanubrutinib combined with RTX on the proportion of Th17 cells detected by flow cytometry; C: Effect of novel BTK inhibitors combined with RTX on the levels of IL-17 detected by ELISA. Mean ± SD. n=3. **P*<0. 05; ***P*<0. 01.

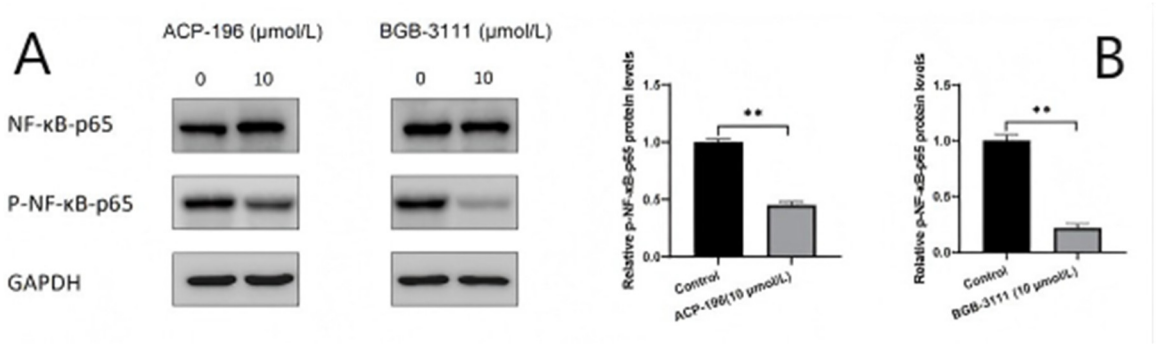

Figure 8. Effect of novel BTK inhibitors acalabrutinib (ACP-196) and zanubrutinib (BGB-3111) on the expression of p-NF-κB-p65 in CD4$^+$ T cells by western bolt. A: The expression of p-NF-κB-p65 in CD4+ T cells treated with acalabrutinib (ACP-196) and zanubrutinib (BGB-3111) detected by western bolt; B: Histogram of the protein level of p-NF-κB-p65 in CD4+ T cells treated with acalabrutinib (ACP-196) and zanubrutinib (BGB-3111). Mean ± SD. n=3. **P*<0. 05; ***P*<0. 01.

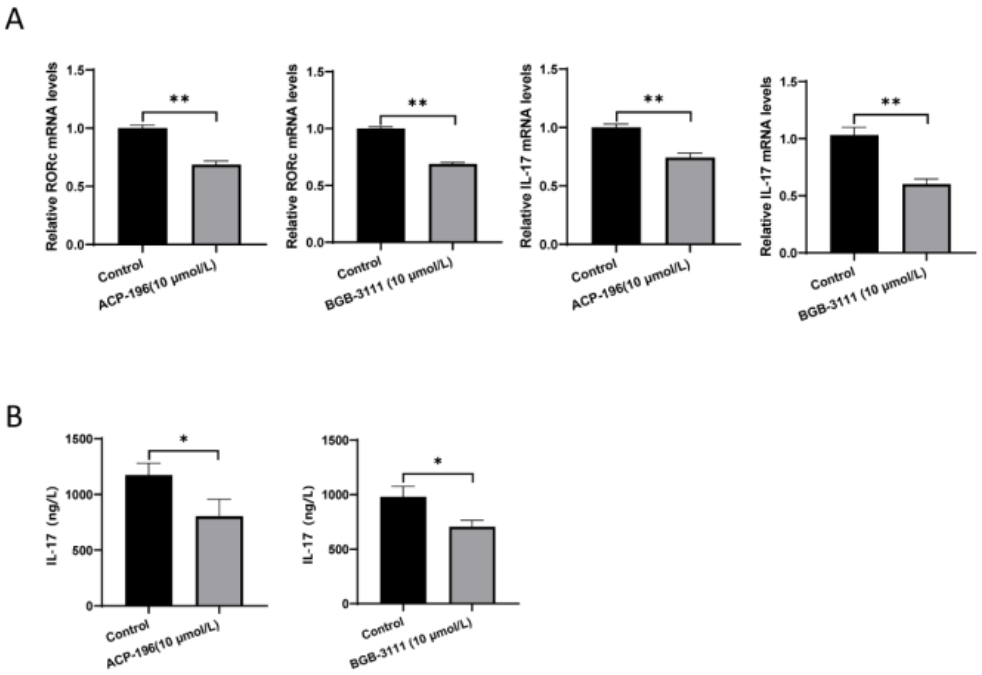

Figure 9. Effect of novel BTK inhibitors acalabrutinib (ACP-196) and zanubrutinib (BGB-3111) on the expression of RORC and IL-17 mRNA in CD4$^+$ T cells and the level of IL-17.A: Effect of novel BTK inhibitors acalabrutinib and zanubrutinib on the expression of RORC and IL-17 mRNA in CD4$^+$ T cells detected by qPCR;B: Effect of novel BTK inhibitors acalabrutinib and zanubrutinib on the level of IL-17 detected by ELISA. Mean ± SD. n=3. *P<0. 05; **P<0. 01.

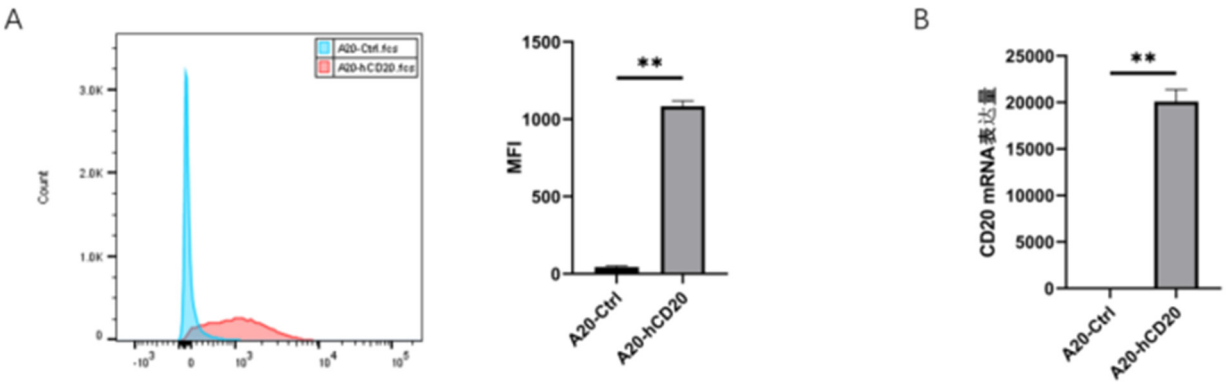

Figure 10. Expression of CD20 antigen (A) and CD20 mRNA (B) after lentiviral transfection of A20 cells.

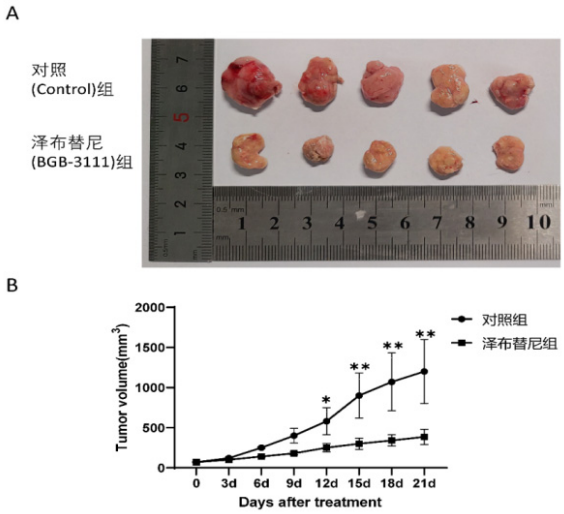

Figure 11. Zanubrutinib (BGB-3111) effectively inhibited tumor growth in animalmodels. A: The tumors in two groups after the end of treatment; B: Changes in the tumor volume in two groups during treatment. Mean ± SD. n=5. *P<0. 05; **P<0. 01.

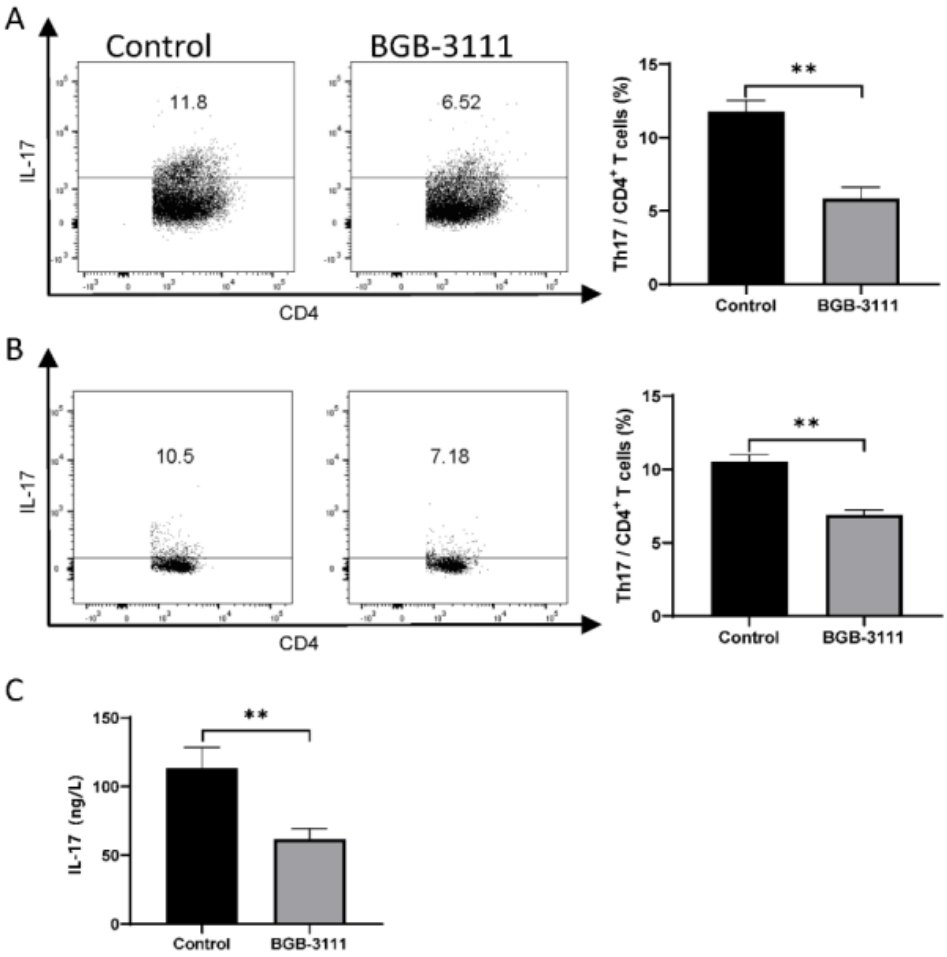

Figure 12. Effect of zanubrutinib (BGB-3111) on the proportion of Th17 cells and the level of IL-17 in animal models. A: Effect of zanubrutinib on the proportion of Th17 cells in the mouse splenic lymphocytes detected by flow cytometry; B: Effect of zanubrutinib on the proportion of Th17 cells in peripheral blood detected by flow cytometry; C: The level of IL-17 in peripheral blooddetected by ELISA. Mean ± SD. n=5. *P<0. 05; **P<0. 01.

Table 1. equences of the primers for RT-qPCR

Gene	Forward primer(5'-3')	Reverse primer(5'-3')
RORc	GAGAAGGACAGGGAGCCAAG	GGATCCCAGACGACTTGTCC
IL-17A	GAGGACAAGAACTTCCCCCG	CTCTCAGGGTCCTCATTGCG
GAPDH	CTTTGGTATCGTGGAAGGA	CACCCTGTTGCTGTAGCC

Conflict of Interest

No conflict of interest was disclosed by the authors.

Acknowledgments

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