Biochemical Pharmacology 80 (2010) 1335–1342

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

The rGel/BLyS fusion toxin inhibits STAT3 signaling via down-regulation of interleukin-6 receptor in diffuse large B-cell lymphoma

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ARTICLE INFO

Article history:
Received 27 April 2010
Accepted 12 July 2010

Keywords:
Diffuse large B-cell lymphoma
Fusion toxin
Interleukin-6 receptor
STAT3
Apoptosis

ABSTRACT

Aberrant signal transducer and activator of transcription (STAT)3 signaling participates in the development and progress of human cancers. We previously generated a highly cytotoxic fusion toxin designated rGel/BLyS for receptor-mediated delivery of the rGel toxin to malignant B-cells. In this study, we examined this fusion toxin for its ability to impact STAT3 signaling in diffuse large B-cell lymphoma (DLBCL). The activated B cell-like DLBCL lines were found to express higher levels of interleukin-6 receptor (IL-6R) and STAT3 than did the germinal center B cell-like DLBCL lines. Treatment of DLBCL cells with rGel/BLyS resulted in down-regulation of IL-6R and inhibited STAT3 phosphorylation, STAT3-DNA binding activity, and IL-6-inducible STAT3 reporter gene activity. In agreement with these results, we additionally found that rGel/BLyS down-regulated levels of several STAT3 targets (c-Myc, p21, Mcl-1, and Bcl-x_L) and p-SYK, a positive regulator of STAT3. Inhibition of IL-6R-mediated STAT3 signaling by rGel/BLyS led to growth inhibition, triggered accumulation of cells in the sub-G_1 phase of the cell cycle, and induced apoptosis. Our results indicate that rGel/BLyS is an excellent candidate for the treatment of aggressive DLBCL which is resistant to conventional chemotherapeutic regimens and STAT3 signaling pathway may be an attractive therapeutic target for non-Hodgkin's lymphoma.

1. Introduction

Diffuse large B-cell lymphomas (DLBCLs) are highly aggressive B-cell non-Hodgkin lymphomas (NHLs). These tumors respond poorly to conventional therapy due to either rapid relapse after initial response or primary resistance to drugs [1–3]. Therefore, the development of novel therapies is essential for patients with relapsed and/or refractory DLBCLs.

Members of the signal transducer and activator of transcription (STAT) family of transcription factors regulate the expression of gene products involved in cell survival, proliferation, chemoresistance, and angiogenesis [4,5]. One STAT family member, STAT3, is constitutively active in many human cancer cells including multiple myeloma, leukemia, lymphoma, and solid tumors [6,7]. The activation of STAT3 results in expression of numerous gene products including Bcl-x_L, c-Myc, p21, cyclin D1, and Mcl-1 [8–10]. The Mcl-1, antiapoptotic protein, was shown to be regulated by STAT3 in leukemic large granular lymphocytes [11] and is essential both during early lymphoid development and later for maintenance of mature T and B lymphocytes [12]. Importantly, expression levels of Mcl-1 are inversely associated with in vitro response to chemotherapeutic agents [13]. Recent studies have examined STAT3 signaling in subtypes of DLBCL [14,15]. These results suggest that inhibitors of STAT3 signaling may have the potential to serve as excellent candidates for the treatment of malignant B lymphoma.

We previously generated a fusion toxin rGel/BLyS containing rGel at the N-terminus followed by a G_4S peptide tether to the B lymphocyte stimulator (BLyS) molecule for the specific delivery of rGel toxin to malignant B-cells expressing BLyS receptors. We showed that the rGel/BLyS fusion toxin demonstrated highly specific cytotoxic activity against mantle cell lymphoma cell lines and B chronic lymphocytic leukemia cells expressing the BLyS receptor BAFF-R [16,17].

In the current study, we examined this fusion toxin for its ability to suppress STAT3 signaling in DLBCL. Our results show that rGel/BLyS inhibits STAT3 signaling via down-regulation of

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doi:10.1016/j.bcp.2010.07.017

interleukin-6 receptor (IL-6R) and this is further evidence that this fusion toxin may be an excellent candidate for the treatment of aggressive DLBCL.

2. Materials and methods

2.1. Materials

The following monoclonal and polyclonal antibodies were used: IL-6R, TNF-receptor 1 (TNF-R1), STAT3, p-STAT3, c-Myc, p21, Mcl-1, Bcl-x_L, HSP-90, TRADD, p-STAT1, STAT-1, p-AKT, β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA) and p-SYK, and SYK (Cell Signaling, Danvers, MA). The IL-6 was purchased from Sigma (St. Louis, MO). Cell proliferation kit II (XTT) was purchased from Roche (Mannheim, Germany). Alexa Flour 488-conjugated Annexin V antibody was purchased from Molecular Probes (Eugene, OR).

2.2. Cell lines and cell culture

The human embryonic kidney cell line (A293) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The eight DLBCL cell lines (SUDHL-4, SUDHL-6, SUDHL-7, OCI-Ly1, OCI-Ly3, OCI-Ly4, OCI-Ly10, and OCI-Ly19) [18–20] used this study were obtained from Dr. Ricardo Aguiar (UT Health Science Center, San Antonio, TX). Five germinal center B cell-like (GBC)-DLBCL lines (SUDHL-4, SUDHL-6, OCI-Ly1, OCI-Ly4, and OCI-Ly19) and one unclassified DLBCL line (SUDHL-7) were grown in RPMI 1640 medium (ATCC) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals Inc., Lawrenceville, GA), 100 units/ml penicillin (Gibco, Grand Island, NY) and 100 μg/ml streptomycin (Gibco). Two activated B cell-like (ABC)-DLBCL lines (OCI-Ly3 and OCI-Ly10) were grown in 20% fetal bovine serum (Atlanta Biologicals). A293 cell line was grown in DMEM (Gibco) supplemented with 10% fetal bovine serum (Atlanta Biologicals).

2.3. Cytotoxic activity of rGel/BLyS against diffuse large B-cell lymphoma cells

To examine the cytotoxic activity of rGel/BLyS against DLBCL lines, SUDHL-4, SUDHL-6, OCI-Ly3 or OCI-Ly10 cells were seeded (1×10^4 cells/well) in flat-bottom 96-well microtiter plates (Becton Dickinson Labware, Franklin Lakes, NJ) and various concentrations of rGel/BLyS were added in quadruplicate wells. After 4 days, cell viability was assessed using the XTT assay kit (Roche) as described previously [16]. Absorbance was measured at 450 nm using an ELISA reader (Bio-Tek Instruments, Winooski, VT).

2.4. Electrophoretic mobility shift assay for STAT3-DNA binding

STAT3-DNA binding was analyzed by electrophoretic mobility shift assay using a ^{32}P-labeled high affinity sis-inducible element probe as previously described [21]. Briefly, nuclear extracts were prepared from rGel/BLyS-treated cells and incubated with a high affinity sis-inducible element probe (5′-CTTCATTTCCCGTAAATCCC-TAAAGCT-3′ and 5′-AGCTTTAGGGATTTACGGGAAATGA-3′). DNA–protein complex formed was separated from free oligonucleotide on 5% native polyacrylamide gels. The dried gels were visualized, and the radioactive bands were quantitated with a Stroma 820 and Imagequant software (Amersham, Piscataway, NJ).

2.5. STAT3–luciferase reporter assay

A293 cells (ATCC) were plated in 6-well plates (Becton Dickinson) with 5×10^5 cells per well in DMEM (Gibco) containing 10% FBS (Atlanta Biologicals). The STAT3-responsive elements linked to a luciferase reporter gene were transfected with wild-type or dominant-negative STAT3-Y705F (STAT3F). Transfections were done according to the manufacturer's protocols using Fugene-6 (Roche). At 24 h post-transfection, cells were pre-treated with rGel/BLyS for 24 h and then induced by IL-6 (Sigma) for additional 24 h before being washed and lysed in luciferase lysis buffer (Promega, Madison, WI). Luciferase activity was measured with a luminometer using a luciferase assay kit (Promega) and was normalized to β-galactosidase activity. All luciferase experiments were done in triplicate.

2.6. Cell cycle analysis

To determine the effect of rGel/BLyS on the cell cycle, OCI-Ly3 or OCI-Ly10 cells were plated in 12-well plates (Becton Dickinson) with 1×10^6 cells per well and treated with different concentrations of rGel/BLyS. After 24 h of incubation, the cells were washed, fixed with 70% ethanol (Fisher Scientific, Fair Lawn, NJ), and then incubated for 30 min at 37 °C with 0.1% RNase A (Sigma) in PBS (Gibco). Cells were then washed again, resuspended, and stained in PBS (Gibco) containing 25 μg/ml propidium iodide (Sigma) for 30 min at room temperature. Cell cycle was analyzed with a Coulter Epics XL flow cytometer (Beckman Coulter, Brea, CA).

2.7. Detection of apoptosis by flow cytometer

To determine the effect of rGel/BLyS on apoptosis, OCI-Ly3 or OCI-Ly10 cells were plated in 12-well plates (Becton Dickinson) with 1×10^6 cells per well and treated with different concentrations of rGel/BLyS. After 24 h of incubation, the cells were collected and apoptosis was measured with Alexa Fluor488-conjugated Annexin V antibody (Molecular Probes) and propidium iodide (Sigma) using to the instructions from the manufacturer (Molecular Probes). Annexin V positive cells were detected by a Coulter Epics XL flow cytometer (Beckman Coulter).

2.8. Western blot analysis

To check the status of IL-6R, STAT3, and Mcl-1, eight DLBCL cell lines (SUDHL-4, SUDHL-6, SUDHL-7, OCI-Ly1, OCI-Ly3, OCI-Ly4, OCI-Ly10, and OCI-Ly19) were washed with PBS (Gibco). To examine the effects of rGel/BLyS on the IL-6R, TNF-R1, STAT3, p-STAT3, c-Myc, p21, Mcl-1, and Bcl-x_L, OCI-Ly3, or OCI-Ly10 cells were seeded at 1×10^6 cells/12-well plate (Becton Dickinson), and then treated with different concentrations of rGel/BLyS for 24 h. Whole cell extracts or cytoplasmic extracts were prepared after wash with PBS (Gibco) and lysed on ice for 20 min in 0.2 ml of lysis buffer (Promega). Cell lysates (50 μg) were separated by SDS-PAGE (8–15%) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Bedford, MA) overnight at 4 °C in transfer buffer [25 mM Tris (Fisher)–HCl (pH 8.3), 190 mM glycine (Sigma), 20% methanol (Fisher)]. The PVDF membranes were blocked for 1 h in Tris-buffered saline (TBS) containing 5% non-fat dry milk (LabScientific Inc., Livingston, NJ) and then probed with different primary antibodies for 1 h. After three washes, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse/anti-rabbit (Bio-Rad Laboratories, Hercules, CA) or bovine anti-goat antibodies (Santa Cruz Biotechnology) for 1 h at RT. Detection of immunoreactive proteins was performed with ECL detection reagent (Amersham). β-Actin was used as a control for protein loading.

2.9. Statistical analysis

All statistical analyses were done with Microsoft Excel software (Microsoft, Redmond, WA). Data are presented as mean ± SD. *P* values were obtained using two-tailed *t* test with 95% confidence

interval for evaluation of the statistical significance compared with the controls. P value <0.05 was considered statistically significant.

3. Results

3.1. rGel/BLyS induces down-regulation of IL-6R

We initially determined the endogenous expression levels of IL-6R in DLBCL cell lines. Compared with GC-DLBCL lines (OCI-Ly1, OCI-Ly4, OCI-Ly19, SUDHL-4, and SUDHL-6), ABC-DLBCL lines (OCI-Ly3 and OCI-Ly10) constitutively expressed IL-6R and OCI-Ly3 cells were found to express the highest levels of IL-6R (Fig. 1A, *upper*). To assess the effect of rGel/BLyS on IL-6R expression, we then treated ABC-DLBCL lines (OCI-Ly3 and OCI-Ly10) with various doses of rGel/BLyS and found that rGel/BLyS induced down-regulation of IL-6R expression. In contrast, rGel/BLyS demonstrated no effect on TNF-R1 expression (Fig. 1A, *bottom*), suggesting that the effect of rGel/BLyS on IL-6R expression appears to be specific.

The biological effects of BLyS are mediated by three cell-surface receptors designated BAFF-R, TACI, and BCMA [22–24]. Therefore, we next examined pre-treatment with BLyS might block the binding of rGel/BLyS to BLyS receptors and the rGel/BLyS-mediated down-regulation of IL-6R expression. Addition of BLyS showed no impact on IL-6R basal expression whereas pre-treatment with BLyS for 2 h was able to block the rGel/BLyS-mediated down-regulation in IL-6R levels (Fig. 1B).

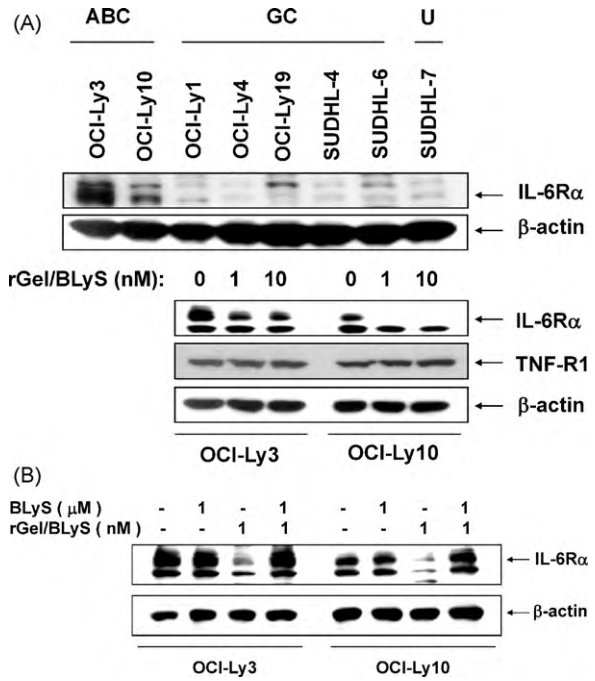

Fig. 1. rGel/BLyS induces down-regulation of IL-6 receptor. (A) Effect of rGel/BLyS on IL-6 receptor α and TNF-receptor 1 (TNF-R1) expression in DLBCL lines. *Upper*: To detect endogenous expression level of IL-6Rα, whole cell extracts from eight diffuse large B-cell lymphoma lines (OCI-Ly1, OCI-Ly3, OCI-Ly4, OCI-Ly10, OCI-Ly19, SUDHL-4, SUDHL-6, and SUDHL-7) were prepared. *Bottom*: To examine the effect of rGel/BLyS on IL-6Rα and TNF-R1 expression, OCI-Ly3 or OCI-Ly10 cells were seeded (1×10^6 cells/well) in 12-well plates and treated with different concentrations of rGel/BLyS for 24 h. (B) Competitive inhibition of rGel/BLyS binding by pre-treatment with BLyS. OCI-Ly3 or OCI-Ly10 cells were seeded (1×10^6 cells/well) in 12-well plates and pre-treated with 1 μM of BLyS for 2 h and then treated with 1 nM rGel/BLyS or media for 24 h. After 24 h, the cells were collected, washed, and lysed in 0.2 ml of lysis buffer. Whole cell extracts were fractionated by 8–15% SDS-PAGE and electrophoretically transferred to PVDF membranes. The membranes were blocked, and then probed with various antibodies. Secondary antibodies conjugated with horseradish peroxidase were used to visualize immunoreactive proteins using ECL detection reagent. β-Actin was used as a control for protein loading.

3.2. rGel/BLyS inhibits constitutive STAT3 phosphorylation

We determined the endogenous expression level of STAT3 in DLBCL lines. All DLBCL lines were found to express endogenous STAT3. Compared with GC-DLBCL cells (OCI-Ly1, OCI-Ly4, OCI-Ly19, SUDHL-4, and SUDHL-6), ABC-DLBCL lines (OCI-Ly3 and OCI-Ly10) expressed the highest levels of total STAT3 (Fig. 2A, *upper*). We next investigated whether rGel/BLyS could inhibit constitutive STAT3 phosphorylation in ABC-DLBCL lines. Treatment with rGel/BLyS was found to induce down-regulation of phospho STAT3 but had no impact on total STAT3 levels (Fig. 2A, *bottom*). We next examined whether pre-treatment with BLyS might block the rGel/BLyS-mediated down-regulation of STAT3 phosphorylation. Addition of BLyS showed no impact on STAT3 phosphorylation. However, pre-treatment with BLyS for 2 h was able to block the rGel/BLyS-mediated down-regulation in phospho STAT3 levels (Fig. 2B).

3.3. rGel/BLyS inhibits STAT3-DNA binding activity

The translocation of STAT3 to the nucleus leads to a specific DNA binding, which in turn regulates gene transcription [25]. We examined whether rGel/BLyS treatment could impact STAT3-DNA binding activity. We found that rGel/BLyS decreased STAT3-DNA binding activity in both a dose-dependent manner and a time-dependant manner (Fig. 2C).

3.4. rGel/BLyS suppresses IL-6-induced STAT3 reporter gene activity

We next examined whether rGel/BLyS can modulate IL-6-induced STAT3 reporter gene activity. As shown in Fig. 2D, the cells showed 7-fold induction of STAT3 reporter gene activity with IL-6 (Sigma) stimulation whereas dominant-negative STAT3 blocked this activity. Moreover, rGel/BLyS inhibited IL-6-induced STAT3 reporter gene activity in a dose-dependent manner ($P < 0.05$).

3.5. rGel/BLyS down-regulates STAT3-regulated gene products

STAT3 activation has been shown to regulate the expression of various gene products (Mcl-1, survivin, Bcl-x_L, c-Myc, cyclin D1, VEGF, etc.) involved in cell survival, cell cycle progression, and angiogenesis [21]. We determined the endogenous expression level of Mcl-1 in DLBCL lines. All DLBCL lines were found to express endogenous Mcl-1. Compared with GC-DLBCL cells (OCI-Ly1, OCI-Ly4, OCI-Ly19, SUDHL-4, and SUDHL-6), ABC-DLBCL lines (OCI-Ly3 and OCI-Ly10) expressed the highest levels of Mcl-1 (Fig. 3A, *upper*). We next investigated whether the expression of STAT3-regulated gene products such as c-Myc, p21, Mcl-1, and Bcl-x_L was modulated by rGel/BLyS. rGel/BLyS down-regulated these STAT3-target proteins (Fig. 3A, *bottom*). We next examined whether pre-treatment with BLyS might block the rGel/BLyS-mediated down-regulation of STAT3 targets. Addition of BLyS showed no impact on c-Myc, p21, Mcl-1, and Bcl-x_L expression whereas pre-treatment with BLyS for 2 h blocked the rGel/BLyS-mediated down-regulation in c-Myc, p21, Mcl-1, and Bcl-x_L levels (Fig. 3B).

3.6. rGel/BLyS has no impact on STAT3 negative targets

We next investigated whether rGel/BLyS is inactive against STAT3 negative targets such as HSP-90 and TRADD and observed that treatment with this fusion toxin had no impact on HSP-90 and TRADD expression (Fig. 3C).

3.7. rGel/BLyS has no impact on SHP-1 expression

SHP-1 is a non-transmembrane protein tyrosine phosphatase that has been linked with regulation of STAT3 activation [26].

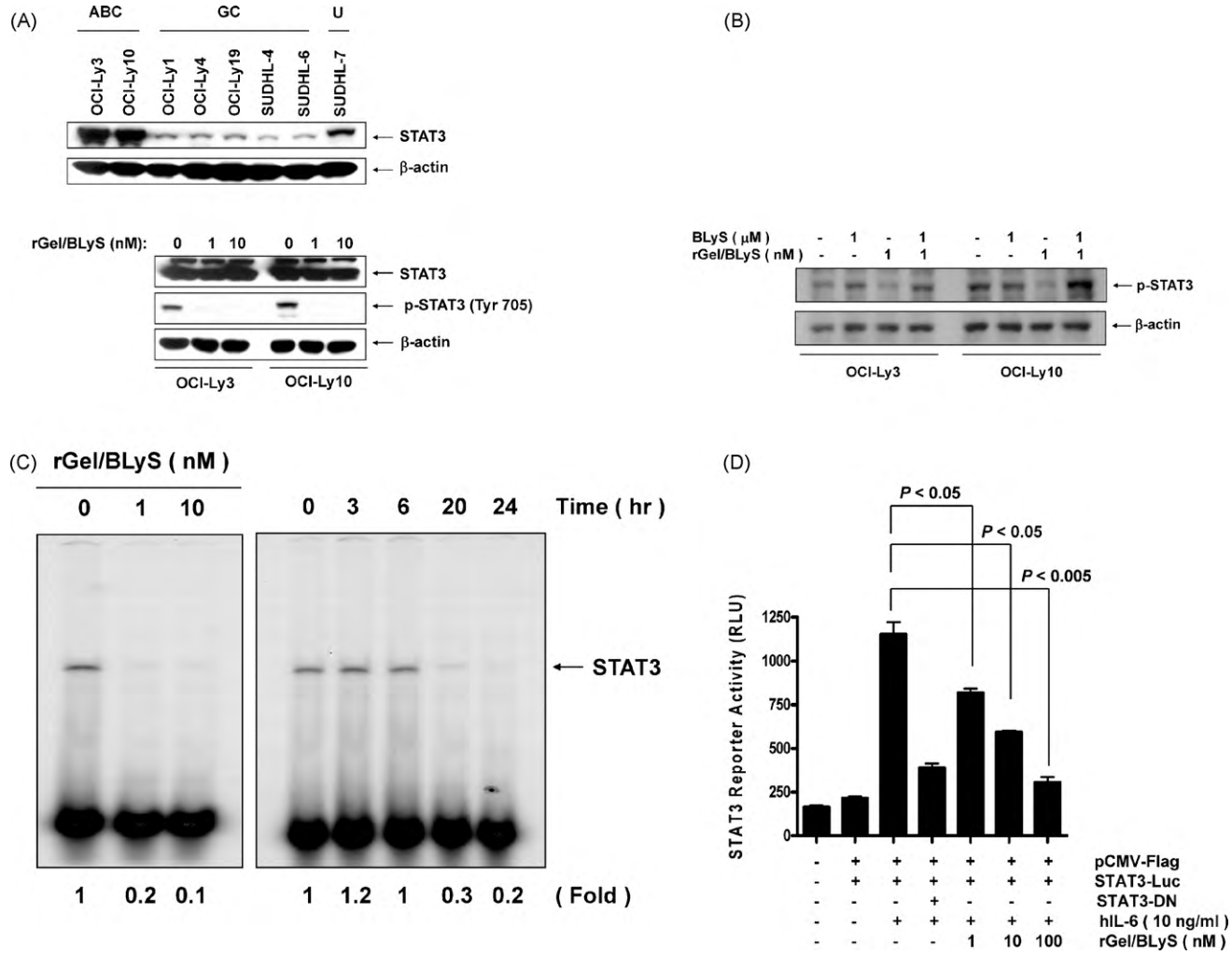

Fig. 2. rGel/BLyS inhibits constitutive STAT3 phosphorylation in DLBCL lines. (A) Effect of rGel/BLyS on STAT3 expression in DLBCL lines. *Upper*: To detect endogenous expression level of STAT3, whole cell extracts from eight diffuse large B-cell lymphoma lines (OCI-Ly1, OCI-Ly3, OCI-Ly4, OCI-Ly10, OCI-Ly19, SUDHL-4, SUDHL-6, and SUDHL-7) were prepared. *Bottom*: To examine the effect of rGel/BLyS on STAT3 and p-STAT3 expression, OCI-Ly3 or OCI-Ly10 cells were seeded (1×10^6 cells/well) in 12-well plates and treated with different concentrations of rGel/BLyS for 24 h. (B) Competitive inhibition of rGel/BLyS binding by pre-treatment with BLyS. OCI-Ly3 or OCI-Ly10 cells were seeded (1×10^6 cells/well) in 12-well plates and pre-treated with 1 μM of BLyS for 2 h and then treated with 1 nM rGel/BLyS or media for 24 h. After 24 h, the cells were collected, washed, and lysed in 0.2 ml of lysis buffer. Whole cell extracts were fractionated by 8–15% SDS-PAGE and electrophoretically transferred to PVDF membranes. The membranes were blocked, and then probed with various antibodies. Secondary antibodies conjugated with horseradish peroxidase were used to visualize immunoreactive proteins using ECL detection reagent. β-Actin was used as a control for protein loading. (C) rGel/BLyS inhibits STAT3-DNA binding activity in OCI-Ly10 cells. *Left*: OCI-Ly10 cells were seeded (1×10^6 cells/well) in 12-well plates and treated with different concentrations of rGel/BLyS for 24 h. *Right*: OCI-Ly10 cells were seeded (1×10^6 cells/well) in 12-well plates and treated with 10 nM of rGel/BLyS for the indicated durations. After treatment, nuclear extracts were prepared and analyzed for nuclear STAT3 levels by EMSA assay. (D) rGel/BLyS inhibits IL-6-induced STAT3 reporter gene activity. A293 cells (5×10^5 cells/ml) were transfected with STAT3–luciferase (STAT3–Luc) plasmid, incubated for 24 h, and then treated with different concentrations of rGel/BLyS for 24 h and then stimulated with IL-6 (10 ng/ml) for 24 h. Whole cell extracts were prepared and analyzed for luciferase activity. Cells were cotransfected with β-gal and the data were normalized with β-galactosidase assay (data not shown). $P < 0.05$ (Student's *t* test).

Whether inhibition of STAT3 phosphorylation by rGel/BLyS is due to induction of the expression of SHP-1 was examined. As shown in Fig. 3D, treatment with rGel/BLyS had no impact on SHP-1 expression.

3.8. rGel/BLyS down-regulates SYK phosphorylation

Spleen tyrosine kinase (SYK) is a cytoplasmic protein tyrosine kinase and DLBCL lines constitutively express SYK and p-SYK (Tyr525/526) [27]. STAT3 is a substrate of SYK in B-lineage leukemia/lymphoma cells exposed to oxidative stress [28]. Whether inhibition of STAT3 phosphorylation by rGel/BLyS is due to inhibition of SYK phosphorylation was examined. As shown in Fig. 3D, treatment with rGel/BLyS resulted in down-regulation of p-SYK, suggesting that modulation of p-SYK by this fusion toxin also triggers down-regulation of p-STAT3.

3.9. rGel/BLyS has no impact on STAT1 and AKT phosphorylation

We next investigated whether rGel/BLyS is inactive against STAT1 and AKT pathways and observed that treatment with this fusion toxin had no impact on STAT1 and AKT phosphorylation (Fig. 3E).

3.10. rGel/BLyS is specifically toxic for ABC-DLBCL cells

Whether down-regulation of IL-6R, phospho STAT3, STAT3-DNA binding activity, and several STAT3 targets led to inhibition of DLBCL growth, we examined the cytotoxic activity of rGel/BLyS against four DLBCL cell lines. Compared with two GC-DLBCL lines (SUDHL-4 and SUDHL-6) expressing low levels of IL-6R and STAT3 (IC_{50} = 5–50 nM), two ABC-DLBCL lines (OCI-Ly3 and OCI-Ly10) expressing high levels of IL-6R and constitutively active STAT3

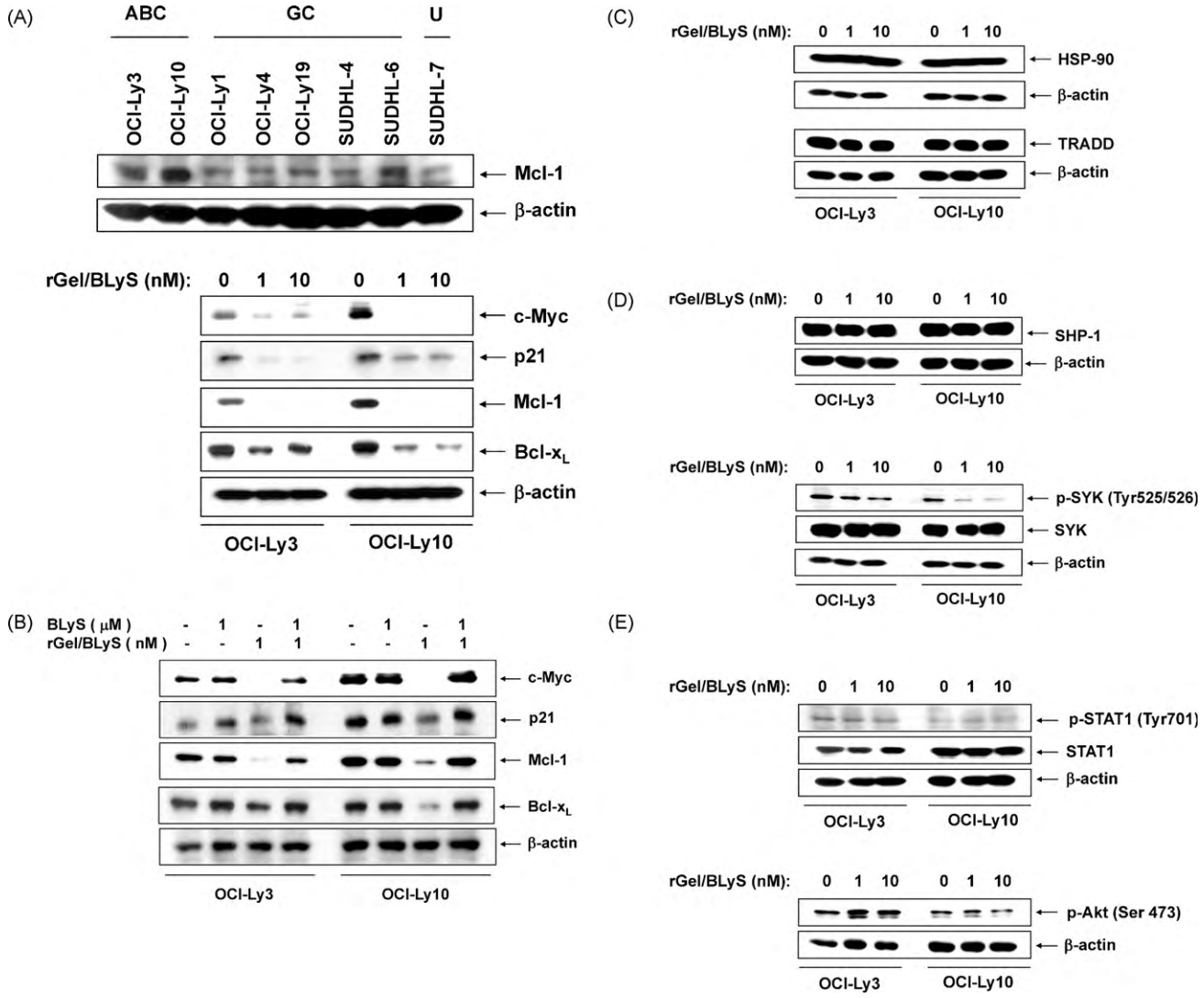

Fig. 3. rGel/BLyS down-regulates STAT3-regulated targets. (A) Effects of rGel/BLyS on STAT3-regulated proteins. *Upper*: To detect endogenous expression level of Mcl-1, whole cell extracts from eight diffuse large B-cell lymphoma lines (OCI-Ly1, OCI-Ly3, OCI-Ly4, OCI-Ly10, OCI-Ly19, SUDHL-4, SUDHL-6, and SUDHL-7) were prepared. *Bottom*: To examine the effect of rGel/BLyS on STAT3-regulated proteins, OCI-Ly3 or OCI-Ly10 cells were seeded (1×10^6 cells/well) in 12-well plates and treated with different concentrations of rGel/BLyS for 24 h. (B) Competitive inhibition of rGel/BLyS binding by pre-treatment with BLyS. OCI-Ly3 or OCI-Ly10 cells were seeded (1×10^6 cells/well) in 12-well plates and pre-treated with 1 μM of BLyS for 2 h and then treated with 1 nM rGel/BLyS or media for 24 h. (C) rGel/BLyS has no impact on STAT3 negative targets. (D) Effects of rGel/BLyS on SHP-1 and SYK expression. (E) rGel/BLyS has no impact on STAT1 and AKT phosphorylation. OCI-Ly3 or OCI-Ly10 cells were seeded (1×10^6 cells/well) in 12-well plates and treated with different concentrations of rGel/BLyS for 24 h. After treatment, the cells were collected, washed, and lysed in 0.2 ml of lysis buffer. Whole cell extracts were fractionated by 8–15% SDS-PAGE and electrophoretically transferred to PVDF membranes. The membranes were blocked, and then probed with various antibodies. Secondary antibodies conjugated with horseradish peroxidase were used to visualize immunoreactive proteins using ECL detection reagent. β-Actin was used as a control for protein loading.

were found to be the most sensitive to the rGel/BLyS fusion toxin (IC_{50} = 1–3 pM) (Fig. 4).

3.11. rGel/BLyS causes the accumulation of the cells in the sub-G_1 phase of the cell cycle

The transcriptional targets (e.g., cyclin D1, c-Myc, and p21) play roles in cell cycle progression. We investigated whether rGel/BLyS modulated the cell cycle and found that rGel/BLyS-treated OCI-Ly3 and OCI-Ly10 cells showed 47% and 51% of cell arrest at sub-G_1 phase, respectively whereas non-treated OCI-Ly3 and OCI-Ly10 cells showed 5% and 4% of cell arrest at sub-G_1 phase, respectively (Fig. 5).

3.12. rGel/BLyS induces apoptosis

To determine whether the cytotoxic effect of rGel/BLyS was associated with apoptosis, two ABC-DLBCL lines (OCI-Ly3 and OCI-Ly10) were examined for apoptosis by Annexin V (Molecular Probes) and propidium iodide (Sigma) staining. As shown in Fig. 6, rGel/BLyS-treated OCI-Ly3 and OCI-Ly10 cells showed 62% and 53% of Annexin V positive apoptotic cells, respectively whereas non-treated OCI-Ly3 and OCI-Ly10 cells showed 16% and 2% of Annexin V positive cells, respectively.

4. Discussion

In lymphoid malignancies, the role of STAT3 has been studied in multiple myeloma, where IL-6 autocrine/paracrine action is well known to provide the pivotal survival signal via STAT3 activation [29]. IL-6 is a pleiotropic immunomodulatory cytokine produced by a variety of cell types, including fibroblasts, endothelial cells, monocytes, and several tumors, in particular in multiple myeloma and NHL [30,31]. Serum IL-6 levels are correlated to a poor prognosis in multiple myeloma and diffuse large cell NHL [30,32].

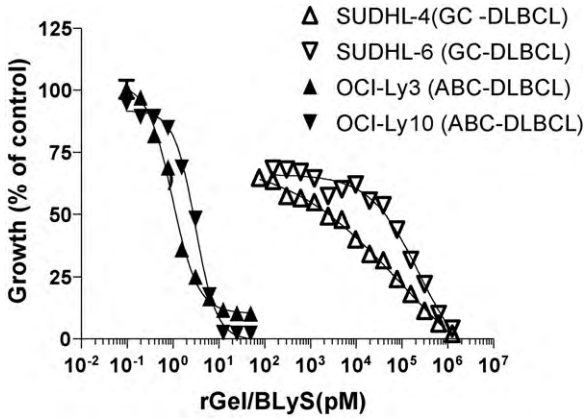

Fig. 4. rGel/BLyS inhibits DLBCL growth. To examine the cytotoxic activity of rGel/BLyS against diffuse large B-cell lymphoma cell lines, SUDHL-4, SUDHL-6, OCI-Ly3 or OCI-Ly10 cells were seeded (1×10^4 cells/well) in flat-bottom 96-well microtiter plates and various concentrations of rGel/BLyS were added in quadruplicate wells. After 4 days, cell viability was assessed using the XTT assay kit.

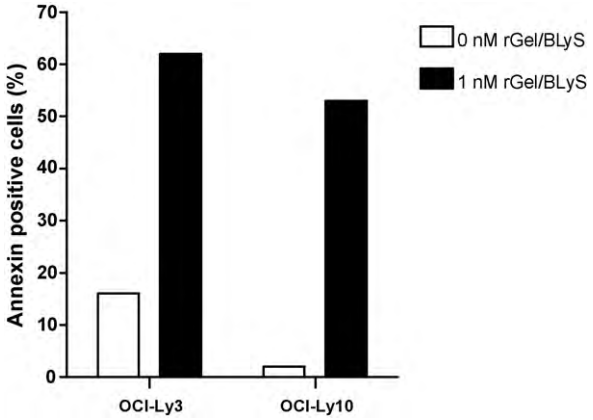

Fig. 6. rGel/BLyS induces apoptosis in DLBCL cells. OCI-Ly3 or OCI-Ly10 cells were treated with 1 nM rGel/BLyS or media. After 24 h, the cells were assayed for apoptosis by Annexin V and propidium iodide staining.

After the binding of IL-6 to its receptor, Janus activated kinase (JAK) working downstream of cytokine receptors or several other receptor and non-receptor tyrosine kinases is activated, which in turn phosphorylates and activates STAT3 [8]. On activation, tyrosine-phosphorylated STAT3 homodimerizes and translocates to the nucleus and then bind to specific DNA response elements in the promoters of target genes and thereby regulate gene expression [33–36].

In this study, we showed that rGel/BLyS fusion toxin was specifically toxic for ABC-DLBCL lines expressing IL-6R and constitutively active STAT3. Previous studies have shown IL-6 secretion by ABC-DLBCL lines [14,15,29]. STAT3 has been described in mediating IL-6 signaling through interaction with the IL-6R. Constitutive STAT3 activation was regulated by IL-6R in the majority of the head and neck squamous cell carcinoma cell lines [37]. Tocilizumab, a humanized anti-IL-6R antibody, significantly reduced in vivo growth of SAS cells with a drastic reduction of STAT3 phosphorylation in tumor cells in mice [38]. Our results also demonstrated that inhibition of IL-6R expression by rGel/BLyS led to inhibition of STAT3 signaling and STAT3 phosphorylation in ABC-DLBCL cells.

STAT3 activation has been shown to regulate the expression of various gene products (Mcl-1, survivin, Bcl-x_L, c-Myc, cyclin D1, p21, VEGF, etc.) involved in cell survival, cell cycle progression, and angiogenesis [9,10,21]. Mcl-1, antiapoptotic protein, is essential for development and maintenance of B lymphocytes and expression levels of Mcl-1 which was regulated by STAT3 are inversely associated with in vitro response to chemotherapeutic agents [11–13]. Another critical target gene of STAT3 implicated in oncogenesis is Bcl-x_L, which confers resistance to apoptosis [39,40]. Growth inhibition and induction of apoptosis correlates well with down-regulation of c-Myc, a known downstream of target gene of STAT3. Our results also demonstrated that inhibition of STAT3 signaling by rGel/BLyS led to inhibition of Mcl-1, Bcl-x_L, c-Myc, and p21.

SYK is a cytoplasmic protein tyrosine kinase and has recently emerged as a potential therapeutic target for the treatment of B-lineage leukemias and lymphomas [41,42]. Uckun et al. [28] have reported that STAT3 is a substrate of SYK in B-lineage leukemia/lymphoma cells exposed to oxidative stress and oxidative stress does not induce tyrosine phosphorylation of STAT3 in SYK-deficient human proB leukemia cells. Whether inhibition of STAT3 phosphorylation by rGel/BLyS is connected with suppression of p-SYK was examined. We observed that treatment with rGel/BLyS resulted in down-regulation of p-SYK in DLBCL, suggesting that modulation of p-SYK by this fusion toxin also triggers down-regulation of p-STAT3.

Our recent study has shown that NF-κB was found to be constitutively activated in ABC-DLBCL cells and treatment with

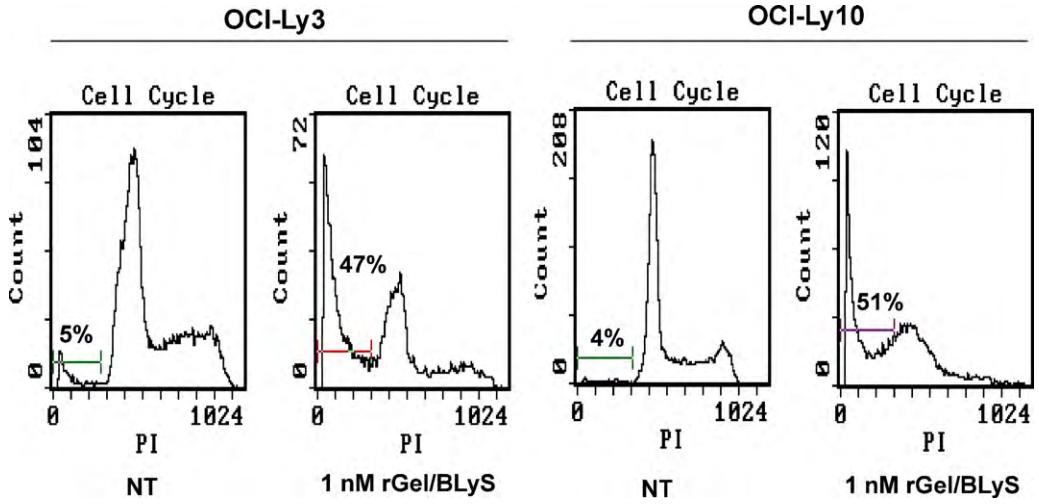

Fig. 5. rGel/BLyS causes the accumulation of the cells in the sub-G_1 phase of the cell cycles. OCI-Ly3 or OCI-Ly10 cells were seeded (1×10^6 cells/well) in 12-well plates and treated with 1 nM rGel/BLyS or media for 24 h. After treatment, the cells were washed, fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry.

rGel/BLyS down-regulated NF-κB binding activity and NF-κB targets. Lam et al. [15] have reported that STAT3-high ABC-DLBCLs had higher expression of NF-κB activity and combined treatment of ABC-DLBCL cells with IKKβ and JAK inhibitors induced additive toxicity for ABC-DLBCL. The p65 subunit of NF-κB has been shown to communicate with STAT3 [43] and JAK2 kinase needed for STAT3 activation has been shown to be required for erythropoietin-induced NF-κB activation [44]. These findings suggest that there is a cross talk between the STAT3 and NF-κB pathways in DLBCLs.

BLyS is crucial for B-cell survival and the biological effects of BLyS are mediated by three cell-surface receptors designated BAFF-R, TACI, and BCMA [22–24]. We determined whether a correlation existed between BAFF-R expression and sensitivity to rGel/BLyS using four cell lines. Expression of BAFF-R was similar between the two subgroups of DLBCL (data not shown). Compared with two GC-DLBCL lines (SUDHL-4 and SUDHL-6) expressing low levels of IL-6R and STAT3, two ABC-DLBCL lines (OCI-Ly3 and OCI-Ly10) expressing high levels of IL-6R and constitutively active STAT3 were 1600–50,000-fold more sensitive to rGel/BLyS (Fig. 4). This may suggest that constitutive activation of IL-6R-mediated STAT3 signaling is crucial for survival of ABC-DLBCL cells but not GC-DLBCL cells.

Taken together, our results suggest that rGel/BLyS may be an excellent candidate for the treatment of the ABC-subtypes of DLBCL expressing high levels of IL-6R and constitutively active STAT3 which are resistant to conventional chemotherapeutic regimens and STAT3 may be a new therapeutic target in these malignant B-cell lymphomas.

Acknowledgements

This research was conducted, in part, by the Clayton Foundation for Research and a Translational Research award (LLS 6234-07) by the Leukemia and Lymphoma Society (to MGR).

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