TOSO interacts with SYK and enhances BCR pathway activation in chronic lymphocytic leukemia

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Abstract

Background: TOSO, also named Fas inhibitory molecule 3 (FAIM3), has recently been identified as an immunoglobulin M (IgM) Fc receptor (FcγR). Previous studies have shown that TOSO is specifically over-expressed in chronic lymphocytic leukemia (CLL). However, the functions of TOSO in CLL remain unknown. The B-cell receptor (BCR) signaling pathway has been reported to be constitutively activated in CLL. Here, we aimed to investigate the functions of TOSO in the BCR signaling pathway and the pathogenesis of CLL.

Methods: We over-expressed TOSO in B-cell lymphoma cell lines (Granta-519 and Z138) by lentiviral transduction and knocked down TOSO by siRNA in primary CLL cells. The over-expression and knockdown of TOSO were confirmed at the RNA level by polymerase chain reaction and protein level by Western blotting. Co-immunoprecipitation with TOSO antibody followed by liquid chromatography coupled with tandem mass spectrometry (IP/LCMS) was used to identify TOSO interacting proteins. Western blotting was performed to detect the activation status of BCR signaling pathways as well as B-cell lymphoma 2 (BCL-2). Flow cytometry was used to examine the apoptosis of TOSO-over-expressing B lymphoma cell lines and TOSO-down-regulated CLL cells via the staining of Annexin V and 7-AAD. One-way analyses of variance were used for intergroup comparisons, while independent samples t tests were used for two-sample comparisons.

Results: From IP/LCMS, we identified spleen tyrosine kinase (SYK) as a crucial candidate of TOSO-interacting protein and confirmed it by co-immunoprecipitation. After stimulation with anti-IgM, TOSO over-expression increased the phosphorylation of SYK, and subsequently activated the BCR signaling pathway, which could be reversed by a SYK inhibitor. TOSO knockdown in primary CLL cells resulted in reduced SYK phosphorylation as well as attenuated BCR signaling pathway. The apoptosis rates of the Granta-519 and Z138 cells expressing TOSO were (8.46 ± 2.90)% and (4.20 ± 1.10)%, respectively, significantly lower than the rates of the control groups, which were (25.20 ± 4.60)% and (19.72 ± 1.10)%, respectively (P < 0.05 for both). The apoptosis rate was reduced after knocking down TOSO in the primary CLL cells. In addition, we also found that TOSO down-regulation in primary cells from CLL patients led to decreased expression of BCL-2 as well as lower apoptosis, and vice versa in the cell line.

Conclusions: TOSO might be involved in the pathogenesis of CLL by interacting with SYK, enhancing the BCR signaling pathway, and inducing apoptosis resistance.

Keywords: Chronic lymphocytic leukemia; TOSO; B-cell receptor signaling pathway; SYK; Apoptosis

Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia disorder in the western hemisphere and is characterized by the accumulation of mature B cells in the blood, bone marrow, and lymphoid organs.[1] CLL is considered as a prototypic antigen-driven leukemia/lymphoma, as chronic antigen stimulation is thought to play an important role in its pathogenesis.[1-3] B-cell receptor (BCR) is the main receptor to transmit the extrinsic stimulation, and the BCR signaling pathway has been reported to be constitutively activated in CLL.[4] Strong evidence indicates that signaling via BCR plays a major role in the development
of CLL and determines variable clinical behaviors. Patients with immunoglobulin heavy variable (IGHV) somatic mutations exhibit more indolent disease and longer overall survival than patients with IGHV germline mutation or “unmutated” configurations. CLL cells display many characteristics of Ag-experienced B cells, including the skewed IGHV gene repertoire and the expression of BCRs with a structurally similar Ag-binding pocket, defined as a conserved “stereotypic” pattern of an immunoglobulin (IIG) variable region. The antigen-antibody reaction mediated by immunoglobulin is the main defense against intruding pathogens or autoantigens, which is achieved by binding of immunoglobulins to the antigen via their variable amino terminal regions and to the effector molecules, such as Fc receptors (FcRs), via their constant carboxyl-terminal regions. The interactions between FcRs and antibodies initiate a broad spectrum of effector functions that are important in host defense. Distinct FcRs have been identified for immunoglobulin G (IgG; FcγRI, FcγRII, and FcγRIII), IgE (FcεR), and IgA (FcaR). IgM is the first antibody isotype to appear in the immune response to pathogens and self-antigens. However, the IgM Fc receptor (FcμR) was not identified until recently and was found to be identical to TOSO, also known as the Fas inhibitory molecule 3 (FAIM3). We and others previously reported that TOSO/FcμR was selectively and highly expressed in CLL cells, compared with the expression of normal B cells and other B-cell lymphomas. Nevertheless, its function in CLL has not been well defined. The structural analysis of TOSO/FcμR indicates that it has a long cytoplasmic tail (118 amino acid residues) containing conserved residues, which makes it different from other FcRs. The binding of the IgM to TOSO/FcμR on natural killer (NK) cells can initiate intracellular signaling, inducing the phosphorylation of PLCγ and Erk1/2. We hypothesized that TOSO interacts with some proteins involved in important pathways up-regulated in B-cell hematologic malignancies. This study aimed to identify new partners of TOSO and uncover the role of TOSO in the oncogenesis of CLL.

Methods

Ethical approval

Written informed consent was obtained from all patients in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Institute of Hematology and Blood Disease Hospital (No. 81200395).

Cell culture and the generation of primary human cells from chronic lymphocytic leukemia patients

Human non-Hodgkin lymphoma B-cell lines (Granta-519 and Z138) and primary B lymphocytes isolated from nine CLL patients were used in this study. Freshly isolated peripheral blood mononuclear cells were subject to CD19+ B-cell enrichment (>98%) by standard positive selection using magnetic beads conjugated with a specific anti-CD19 antibody (Miltenyi Biotec, Auburn, CA, USA).

The human non-Hodgkin lymphoma B-cell line Z138 was propagated and maintained in Iscove modified Dulbecco medium supplemented with 10% fetal bovine serum. The human non-Hodgkin lymphoma B-cell line Granta-519 and primary B lymphocytes isolated from CLL patients were propagated and maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. All cells were cultured at 37°C with 5% CO2 in a humidified atmosphere. BCR stimulation was performed by adding goat F(ab’)2 anti-human IgM (μ-chain-specific; Sigma-Aldrich, Munich, Germany) at a final concentration of 10 μg/mL for 6 or 24 h, as described previously.

Vector construction and transfection

Full-length human TOSO cDNA was amplified with the following primers: forward, 5’-ATCAGTGGTGGCGCCGCTCCGG-3’; and reverse, 5’-ATCAGTGGTGGCGCCGCTCCGG-3’. The structural analysis of TOSO/FcR indicates that it has a long cytoplasmic tail (118 amino acid residues) containing conserved residues, which makes it different from other FcRs. The binding of the IgM to TOSO/FcμR on natural killer (NK) cells can initiate intracellular signaling, inducing the phosphorylation of PLCγ and Erk1/2. We hypothesized that TOSO interacts with some proteins involved in important pathways up-regulated in B-cell hematologic malignancies. This study aimed to identify new partners of TOSO and uncover the role of TOSO in the oncogenesis of CLL.

RNA extraction and quantitative real-time quantitative polymerase chain reaction

Total RNA from transfected and non-transfected Granta-519 and Z138 cells was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s instructions. Total RNA (2 μg) was reverse transcribed to cDNA with the PrimeScript1 RT reagent kit (TaKaRa, Shanghai, China). Real-time quantitative polymerase chain reaction (PCR) was performed using SYBR Green PCR MasterMix according to the manufacturer’s instructions. The following forward and reverse primers used: 5’-CCACCCCACTGGACATCCGGG-3’ and 5’-TTGATCAGTCATGATGATGG-3’. Relative expression levels were analyzed by the 2-ΔΔCt method.
Western blotting

The whole-cell lysate was generated with radio-immunoprecipitation assay (RIPA) lysis buffer, and the protein concentration was measured by Bio-Rad (Hercules, CA, USA) protein assay following the manufacturer’s instructions. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred onto polyvinylidene fluoride (PVDF) membranes, and the blots were blocked and incubated overnight at 4°C with the appropriate primary antibody. The following antibodies which obtained from Cell Signaling Technologies (Beverly, MA, USA) were used in this study: anti-SYK antibody (CST#80460), anti-NF-κB p65 antibody (CST#8242), anti-IκBα antibody (#4814), anti-ERK1/2 antibody (#4696), anti-p38 antibody (#8690), anti-p-SYK antibody (#2710), anti-p-NF-κB-p65 antibody (#3033), anti-p-IκBα antibody (#2859), anti-p-ERK1/2 antibody (#4370), and anti-p-p38 antibody (#4511). Both the anti-TOSO antibody (#ab56487) and anti-BCL-2 antibody (#ab13-8800) were obtained from Abcam, Cambridge, UK. The blots were then incubated with a specific HRP-conjugated secondary antibody (CST#7074 and 7076, Beverly). Immunodetection was performed by enhanced chemiluminescence.

Mass spectrometry studies and protein identification

Protein pellets were solubilized and digested by trypsin. Protein constituents were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Inspection of the LC-MS/MS data was undertaken to assess the exclusive presence of mass peaks belonging to candidate partner proteins in the samples from cells transfected with the TOSO plasmid. Peptides were eluted directly off the column into an LTQ system (ThermoFisher, Bremen, Germany) using a gradient of 2% to 80% B over 45 min, with a flow rate of 300 nL/min. The total run time was 65 min. The MS/MS was acquired according to standard conditions established in the laboratory.

The RAW data files collected on the mass spectrometer were converted to mzXML and MGF files by use of MassMatrix data conversion tools (Version 1.3). The resulting MGF files were searched using Mascot Daemon by Matrix Science, Version 2.2.2, and the data files were searched against the full SwissProt Version 57.5 database or NCBI database Version 20091013. Protein identifications were checked manually, and proteins with a Mascot score of 50 or higher with a minimum of two unique peptides from one protein having a -b or –y ion sequence tag of five residues or better were accepted.

Apoptosis assay

Apoptosis was assessed by Annexin V reagents (BD Biosciences, Shanghai, China) according to the manufacturer’s instructions. Samples were analyzed on a FACScalibur flow cytometer (BD Biosciences, Piscataway, NJ, USA) with CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA).

Co-immunoprecipitation

A total of 500 μg of extracted cellular proteins was incubated with anti-Toso (Santa Cruz-101253)/anti-SYK (CST#80460, Beverly)/anti-phosphor-SYK (CST#2710, Beverly) and anti-IgG (Santa Cruz, Heidelberg, Germany) at 4°C overnight. The protein-antibody complexes were captured by protein A/G plus-agarose (Santa Cruz) and pelleted with the agarose beads after the final wash. Western blotting of each protein was then performed.

Statistical analysis

GraphPad Prism 6 software (Graphpad Software, San Diego, CA, USA) was used for all statistical analyses. Each experiment was repeated at least three times, and the average value was analyzed. Statistical significance was analyzed by t test or one-way analysis of variance. The data presented are the mean ± standard deviation, with significant differences determined by P < 0.05.

Results

TOSO interacted with spleen tyrosine kinase in B cells

To identify TOSO-interacting proteins, we constructed a plasmid that expressed the TOSO gene and then transfected into Granta-519 and Z138 cell lines. TOSO was successfully over-expressed in the transfected cells at both RNA and protein levels [Figure 1A]. By TOSO co-immunoprecipitation (Co-IP) and subsequent mass spectrometric analysis, we identified TOSO-binding candidates, among which was spleen tyrosine kinase (SYK), a core target because its well-established role in BCR signaling transduction and the previously identified correlation between increased expression of TOSO and enhanced autoreactive BCR signaling pathways as well as unmaturated IGHV gene status. [13, 15]

As shown in Figure 2A and 2B, we confirmed the interaction between TOSO and SYK by Co-IP in the TOSO-over-expressing Granta-519 and Z138 cells and the primary B lymphocytes from three CLL patients.

Using a siRNA strategy, primary CD19+ B lymphocytes with TOSO over-expression isolated from CLL patients were successfully down-regulated at both RNA and protein levels [Figure 1B].

After 1 h treatment with the SYK inhibitor fostamatinib disodium (R788) at a concentration of 10 μmol/L, the TOSO protein in the over-expressed Granta-519 and Z138 cells was collected by Co-IP and was found to have a low-level interaction with SYK, while the B lymphocytes isolated from CLL patients had a notable reduction in the interaction of TOSO and SYK after SYK inhibitor treatment [Figure 2C]. These observations suggested that TOSO and SYK interacted directly in CLL cells.

TOSO increased the phosphorylation of SYK and regulated downstream B-cell receptor signaling pathways

To elucidate the molecular mechanism of the interaction between TOSO and SYK, the phosphorylation of SYK (p-SYK) was analyzed by Western blotting. High levels of p-SYK were detected in the TOSO-over-expressing Granta-519 and Z138 cells, and lower p-SYK status was observed in
the TOSO-down-regulated primary CLL cells [Figure 3A], which indicated that TOSO increased the phosphorylation of SYK.

To study the regulation by TOSO in a functional context, we analyzed the possible pathways influenced by TOSO expression. After BCR stimulation, we screened the activated downstream components of the BCR signaling pathway and found lower phosphorylation levels of IkBa, p65, ERK, and p38 in the primary CLL cells with down-regulated TOSO [Figure 3B] and higher phosphorylation levels of IkBa, p65, ERK, p38 in the Granta-519 and Z138 cells over-expressing TOSO [Figure 3C].

After treating with the SYK inhibitor fostamatinib disodium (R788), the levels of p-SYK, p-p65, p-IkBα, p-ERK, and p-p38 were reduced in the TOSO-over-expressing Granta-519 and Z138 cells [Figure 3D].

These evidences demonstrated that TOSO regulated the components downstream of the BCR signaling pathway, including the NF-κB and MAPK pathways, in CLL.

**TOSO induced an anti-apoptosis effect and up-regulated BCL-2 expression**

TOSO has been observed to play a role in anti-apoptosis. We sought to verify this finding in CLL cells, as apoptosis resistance is the main characteristic of CLL cells. After stimulation with anti-IgM, the percentage of apoptotic cells of TOSO-over-expressing Granta-519 and Z138 was (8.46 ± 2.90)% and (4.20 ± 1.21)%, respectively, which was significantly lower than that of the control groups, (25.20 ± 4.60)% and (19.72 ± 1.10)% (P < 0.05 in both) [Figure 4A and 4B]. Besides, TOSO siRNA treatment in primary CLL cells from three patients significantly increased the apoptosis [Figure 4C].

B-cell lymphoma 2 (BCL-2) over-expression is a known mechanism for the intrinsic apoptosis resistance of CLL, and there was a positive correction between TOSO and BCL-2 expression.[14] Here we found that the BCL-2 expression level was decreased in the primary cells from the three CLL patients after TOSO knockdown [Figure 4D] and that it was increased when TOSO was over-expressed in the two cell lines (Granta-519 and Z138 cells) [Figure 4E]. This finding indicated that TOSO might induce apoptosis resistance by up-regulating BCL-2 expression.

**Discussion**

Compared to the level in other B-cell lymphomas, TOSO is over-expressed in CLL cells,[13] but its role in the pathogenesis of CLL has not been determined. Our group and others have reported that TOSO over-expression is associated with poor survival and progressive CLL.[13,15,18] However, the mechanism of TOSO over-expression in CLL cells remains unknown. Vire et al[19] reported that TOSO/FcζR localized to the cell membrane and could be internalized upon IgM binding and shuttled to the lysosome for degradation. TOSO over-expression could also be down-regulated in response to TLR activation. Nguyen et al[20] demonstrated that TOSO mediates the balance between apoptotic and non-apoptotic death receptor signaling by facilitating RIP1 ubiquitination. In this study, we showed that TOSO interacted with the SYK protein and activated the BCR signaling pathway [Figure 5].

Previous clinical observations discovered that TOSO was over-expressed in patients with unmutated IGHV,[13,15]
indicating an association between TOSO over-expression and the BCR signaling pathway. Ouchida previously found that TOSO contributed to events downstream of the BCR signaling pathway when triggered by BCR cross-linking in mice. BCR is a trans-membrane complex located on the outer surface of B cells and composed of a heterodimer with heavy chain and light-chain Igs, Igα/CD79A, and Igβ/CD79B. The interaction between antigens and the BCR antigen-binding site triggers intrinsic downstream signaling, involving proteins such as LYN, SYK, BLNK, BTK, PLC-γ, and PI3K, and determines the fate of a BCR-bearing B cell. Because the BCR signaling pathway plays an important role in the pathogenesis of CLL and mediates the communication between CLL cells and the environment, we hypothesized that TOSO might contribute to the pathogenesis of CLL through the BCR pathway. Indeed, TOSO was found to interact with SYK. Moreover, the SYK phosphorylation and subsequent activation of the BCR signaling pathway were enhanced when TOSO was over-expressed and could be blocked by the SYK inhibitor or by siRNA knockdown of TOSO in primary CLL cells. These results showed that the TOSO interacted with SYK enhanced its phosphorylation and downstream BCR signaling pathway.

Our results provide evidence to explain why the BCR signaling pathway is constitutively activated in CLL. Cytogenetic aberrations of the components in the BCR signaling pathway are rare in CLL cells compared with those in other B-cell malignancies, such as the mutation of CD79A/B and CRAD11 in diffuse large B-cell lymphoma and the mutation of TCF3 and ID3 in Burkitt lymphoma, which contribute to the activation of the BCR signaling pathway in these lymphomas. Additional elements cooperate with the BCR complex to trigger or enhance this pathway, which may explain its constitutive activation in CLL. In the lymph node and/or bone marrow environment, where the IgM level is higher than it is in the serum, antigens stimulate CLL cells through the BCR, while IgM interacts with TOSO/FcγR to enhance the activation of the downstream BCR signaling pathway. When pathogens are involved, IgM recognizes them and produces IgM-antigen immune complexes, which may

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**Figure 2:** TOSO interacted with SYK in both cell lines and primary chronic lymphocytic leukemia cells. By Co-IP, the anti-TOSO antibody precipitates the SYK protein (A), and anti-SYK precipitates TOSO (B) in TOSO-over-expressing Granta-519 and Z138 cell lines and three TOSO-over-expressing CLL primary cells. (C) The SYK inhibitor R788 reduced the interaction of TOSO and SYK. CLL: Chronic lymphocytic leukemia; TOSO: Fas inhibitory molecule 3; SYK: Spleen tyrosine kinase; CO-IP: Co-immunoprecipitation; DMSO: Dimethyl sulfoxide; R788: Fostamatinib disodium.
crosslink the BCR complex (antigens) and TOSO (IgM Fc fragment) together, leading to the constitutive activation of BCR.

Binding of IgM to TOSO to initiate intracellular signaling in NK cells has also been reported by other groups. Murakami et al.\(^{[16]}\) reported that anti-Toso mAb can pull down PLC-\(\gamma\)2 and activate ERK signaling after IL-2 stimulation. Therefore, Toso can generate intracellular signals and regulate cellular processes. In liver cells, TOSO can promote the activation of MAPK and NF-\(\kappa\)B signaling pathways in response to CD95L and TNF\(\alpha\) stimulation.\(^{[20]}\) These results are in accordance with our conclusion that TOSO can sense outer stimulation and trigger the intracellular responses.

The evasion of apoptosis is a hallmark of CLL. Both the intrinsic and extrinsic apoptosis pathways were dysregulated in CLL, which led to apoptosis resistance.\(^{[27]}\) BCL-2 is the critical protein of the intrinsic apoptosis pathway, the level of which is increased in more than 80% of CLL cases. Although there is controversy about the role of TOSO in the Fas-mediated apoptosis pathway (extrinsic pathway),\(^{[12,20,28]}\) TOSO might be involved in the anti-apoptotic activity. Based on the positive correlation observed between TOSO and BCL-2 expression,\(^{[14]}\) TOSO may induce apoptosis resistance through the BCL-2 pathway. In the study, TOSO over-expression led to apoptosis resistance in B-cell lymphoma cell lines and down-regulating the expression of TOSO increased the apoptosis sensitivity of primary CLL cells, suggesting that TOSO is involved in apoptosis resistance.

TOSO is a good candidate for target therapy because it is highly expressed in CLL cells and not expressed in normal blood components.\(^{[13]}\) Besides, blocking its function induces cell apoptosis, as shown in this study. Faitschuk et al.\(^{[29]}\) demonstrated that TOSO was a good target for
chimeric antigen receptor T cell therapy in experiments conducted in vitro and in vivo.

Although we have identified that TOSO can interact with SYK and activate BCR downstream components after stimulation and induce apoptosis resistance, the mechanism by which TOSO interacts with SYK remains unclear and it has not been discovered how TOSO influences BCR signaling downstream. In addition, the mechanism by which TOSO up-regulated BCL-2 expression still requires further investigation.

In summary, the over-expression of TOSO is involved in the pathogenesis of CLL by enhancing the BCR signaling.
pathway through interactions with SYK and elevation of its phosphorylation. TOSO up-regulates BCL-2 expression and participates in the apoptosis resistance of CLL.

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Conflicts of interest
None.

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