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The effect of α2,6-linked sialic acid on anti-IgM antibody-induced apoptosis in Ramos cells

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Apoptosis in B cells is induced through the B cell antigen receptor (BCR) and affects the sialic acid recognition molecules on B cells. We investigated the effects of α_1-acid glycoprotein (AGP), which mainly contains α2,6-linked sialic acid, on anti-IgM antibody (Ab)-induced apoptosis in Ramos cells, which are derived from Burkitt's lymphoma. When Ramos cells were incubated with anti-IgM-Ab in plates coated with AGP, neuraminidase-digested AGP (asAGP) or α2,3-sialylated AGP (2,3AGP), apoptosis was suppressed only in those coated with AGP. We also studied the effects of CD22, which is expressed on the surface of mature B cells and binds to sugar chains containing α2,6-linked sialic acid, with anti-CD22 monoclonal antibody (mAb). Anti-CD22mAb enhanced anti-IgM Ab-induced apoptosis in Ramos cells. These contradictory results suggested that the recognition molecules for α2,6-linked sialic acid on AGP, which inhibits B-cell apoptosis, is distinct from CD22, or that different binding domains of CD22 between α2,6-linked sialic acid and anti-CD22 mAb exert opposite functions of suppression or enhancement to anti-IgM Ab-induced B cells.

Keywords: **α2,6-linked sialic acid, apoptosis, anti-IgM Ab, α_1-acid glycoprotein, CD22**

Introduction

The sugar chains on many cell surface glycoproteins and glycolipids play important roles in cell functions and cell-to-cell interaction. Among the various sugar chains on the cell surface, sialic acids are ubiquitously expressed on the non-reducing ends of sugar chains of glycoconjugates and are key determinants for a large variety of biological processes, including cell-to-cell communication and cell-to-matrix interactions [1,2].

Apoptosis, or programmed cell death, is a physiological process induced by various external and internal cellular signals. Suicide pathways of self-reactive B cells of the immune system are induced via stimulation of the B cell antigen receptor (BCR), surface IgM. B-cell apoptosis is also regulated by several factors, such as CD21 [3], CD22 [4] and CD40 [5,6]. Sugar chains on the cell surface are also thought to affect B-cell apoptosis. Human α_1-acid glycoprotein (AGP), contains five N-linked glycan chains per molecule [7–9], with mainly α2,6 linked sialic acids on the non-reducing ends of their sugar chains. Thus, we studied the effects of α2,6-linked sialyl lactosamine (Sia α2,6 Gal β1,4 GlcNAc) and α2,3-linked sialyl lactosamine (Sia α2,3 Gal β1,4 GlcNAc) on B cell apoptosis using plates coated with AGP, α2,3-sialylated AGP (2,3AGP) and sialidase treated AGP (asAGP).

The 130 kD glycoprotein, CD22, that is expressed on the surface of mature IgM^+IgD^+ B cells [10–15], is an adhesion molecule, belonging to a group of I type lectins that comprise the Siglec family [16–18]. CD22 recognizes sugar chains containing α2,6-linked sialyl lactosamine as ligands [19–23]. Some stages of cell-to-cell recognition or interactions are mediated by CD22 binding to α2,6-linked sialic acid. This binding may be important for B-cell biological functions such as differentiation and proliferation [24–29]. The present study also investigated the effects of CD22 stimulation by anti-CD22 monoclonal antibody (mAb), on anti-IgM Ab-induced B cell apoptosis. The findings provided new insight into the regulation of B-cell apoptosis.

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Materials and methods

Materials

Goat anti human IgM, $Fc_{5\mu}$-specific polyclonal Ab (anti-IgM Ab) was obtained from Jackson Immuno Research Laboratories (West Grove, PA). Mouse anti human CD22 monoclonal Ab (RFB4, IgG_1) was obtained from Harlan Sera-Lab (UK). Human α_1-acid glycoprotein, AGP (purified from Cohn fraction IV) was obtained from Sigma Chemical Co. (St. Louis, MO). Neuraminidase (*Streptococcus 6646K*, EC 3.2.1.18) was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). α1,3/4-L-Fucosidase (*Streptomyces* sp.142, EC 3.2.1.51) was obtained from Takara Shuzo Co. (Otsu, Japan). Peroxidase (POD)-conjugated anti-digoxigenin goat Ab and the digoxigenin-conjugated lectins, *Sambucus nigra bark* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA), as well as α2,3-(N)-sialyltransferase (rat liver, EC2.4.99.6) were obtained from Boehringer Mannheim Co. (Heidelberg, Germany). All other chemicals were purchased from Nacalai Tesque (Kyoto, Japan).

Cell lines and culture conditions

Ramos cells derived from Burkitt's lymphoma obtained from the Japanese Collection of Research Bioresources (JCRB, Japan) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS), 2.5% sodium bicarbonate, 1.0% glutamine and 0.2% antibiotics at 37°C in a humidified atmosphere containing 5% CO_2. Before stimulation with Ab, the cells (5×10^5 cells/ml) were incubated for $0 \sim 72$ h with various concentrations of anti-IgM Ab. Before stimulation on protein-coated plates, Ramos cells (1×10^6 cells/ml) were incubated with 50 mU/ml of neuraminidase in phosphate buffered saline (PBS) for 30 min at 37°C, then washed three times with PBS. Neuraminidase-treated Ramos cells (1×10^6 cells/ml) were incubated on protein-coated plates for 48 h with or without anti-IgM Ab, then cell viability was determined.

Preparation of plates

Twenty-four well plates were coated with 25 μg/well of AGP or BSA in 250 μl of PBS for 2 h at 37°C, washed three times with PBS and blocked with 0.1% bovine serum albumin (BSA) in PBS for 2 h at 37°C. The plates were washed again three times with PBS before use.

Plates were coated with asialo AGP (asAGP) by incubating AGP-coated plates with 200 mU/ml neuraminidase in 0.1 M phosphate buffer (pH 6.0) for 2 h at 37°C. Thereafter, the plates were washed three times with PBS and blocked with 0.1% BSA in PBS for 2 h at 37°C. The plates were washed three times with PBS before use.

Plates were coated with α2,3-sialylated AGP(2,3 AGP) as follows [30]. AsAGP-coated plates were incubated with 100 μU/ml α1,3/4-L-fucosidase in 0.1 M phosphate buffer (pH 6.0) containing 2 M ammonium sulfate for 48 h at 37°C. Thereafter, the plates were washed three times with PBS and incubated with 5 mU/ml α2,3-sialyltransferase in 0.1 M HEPES buffer (pH 7.0) containing 1 mM CMP-NeuAc and 10 mM $MgCl_2$ for 48 h at 37°C. Thereafter, the plates were washed three times with PBS before use.

Lectin-binding assay for analysis of sugar chains on coated plates

Digoxigenin-conjugated lectin (100 μl, 1 μg/ml; SNA and MAA) and 0.1% BSA in PBS containing 0.05% Tween 20 (PBS-T) was added to each well of the coated plates and incubated at room temperature for 1 h. After five washes with 0.1% BSA in PBS-T and five with PBS, 100 μl of POD-conjugated anti-digoxigenin Ab (1 μg/ml) was added to each well, and the plate was incubated at room temperature for 1 h. The wells were washed five times each with PBS-T and PBS, then incubated with 100 μl of 0.5% *o*-phenylenediamine and 0.009% H_2O_2 in PBS for 3 min at room temperature. The reaction was stopped with 100 μl of 1 M H_2SO_4. Color intensity was determined at 492 nm using a microtiter plate reader MPR-A4i II (TOSOH, Japan).

Cell viability and DNA fragmentation analysis

After stimulation, cell viability was estimated using the Trypan blue exclusion test. The DNA was extracted and resolved by electrophoresis as described [31]. Ramos cells were suspended in 200 μl of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and 10 μl of lysis buffer (20% NP-40: 1% SDS: 10 mM EDTA = 5:10:1, v/v), then incubated with 2 μl of proteinase K (10 mg/ml) for 1 h at 37°C. The DNA from Ramos cells was extracted with chloroform-phenol (1:1, v/v) and precipitated with cold ethanol at a final concentration of 70%. The DNA samples were dissolved in 10 μl of TE buffer and incubated with 2 μl of RNase A (10 mg/ml) for 1 h at 37°C. The DNA samples were analyzed by electrophoresis on 1.8% agarose gels and visualized by ethidium bromide staining.

Propidium iodide staining and flowcytometry

Cells (1×10^6) were stained with propidium iodide solution (50 μg/ml) in 0.1% sodium citrate as described by Krishan [32]. Samples were kept at 4°C in the dark, and the cell cycle population was analyzed using a CytoACE-300 flow cytometer (JASCO, Tokyo). A percent apoptotic cell was defined as the percentage of cells with hypodipoid DNA content (less DNA than those in G0/G1 phase) to total population.

Stimulation with anti-CD22mAb-coated plate

Twenty-four well plates were coated with 2.5 μg anti-CD22mAb/well in 1 ml of PBS for 2 h at 37°C, washed three times with PBS and blocked with 0.1% BSA in PBS for 2 h at 37°C. The plates were washed three times with PBS before use. Before stimulation on Ab-coated plates, Ramos cells were treated by neuraminidase as described above. Neuraminidase-treated Ramos cells (1×10^6 cells/ml) were incubated in anti-

CD22mAb-coated plates for 48 h with or without anti-IgM Ab and cell viability was determined.

Results

Effect of anti-IgM Ab on cell viability

Several investigations have shown that B cells stimulated with BCR underwent simultaneous contrary biological processes, namely cell proliferation and cell death. We therefore examined the effects of BCR stimulation with anti-IgM Ab on cell viability using Ramos cells incubated with 2.5 μg/ml of anti-IgM Ab for 72 h. Figure 1A shows that anti-IgM Ab induced a decrease in cell viability that was dependent on the length of the incubation period. After 48 h, cell viability decreased from 100% in the control to 61.0% in the presence of anti-IgM Ab. The ability of anti-IgM Abs to induce cell death was also estimated at concentrations of anti-IgM Ab ranging from 0.5 to 5.0 μg/ml for 48 h. In the presence of 5.0 μg/ml of anti-IgM Ab, cell viability decreased from 97.6% in controls to 48.9% after 48 h. Cell viability therefore decreased in a dose-dependent manner (Figure 1B). The DNA samples extracted from anti-IgM Ab-treated Ramos cells were examined by agarose gel electrophoresis (Figure 1C). DNA fragmentation with the ladder pattern indicating apoptosis increased dose-dependently.

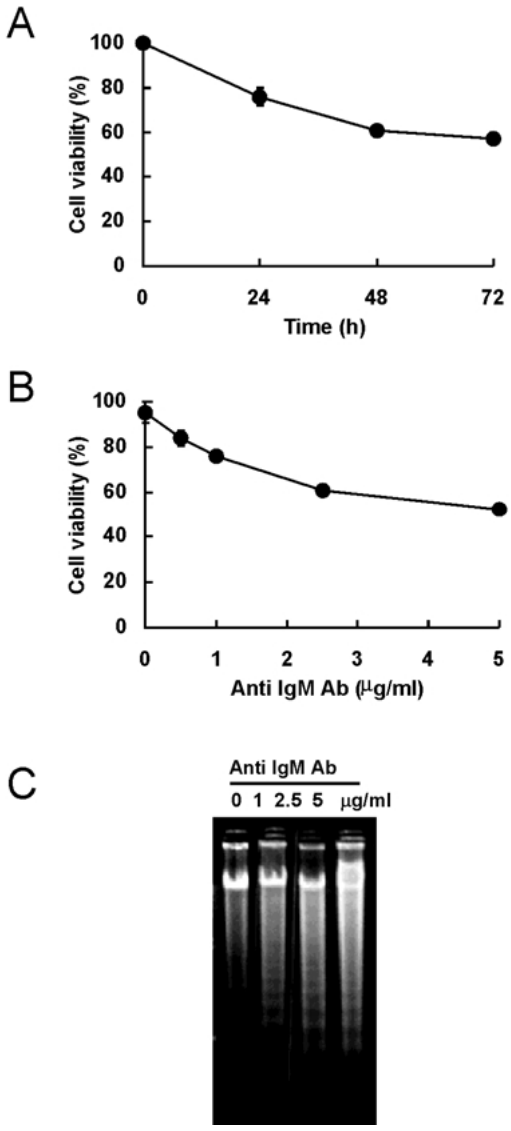

Figure 1. Anti-IgM Ab-induced cell death and DNA fragmentation in Ramos cells. (A) Time course of cell viability in anti-IgM Ab-treated Ramos cells. Ramos cells were incubated with anti-IgM Ab (2.5 μg/ml). Cell viability was determined at the indicated times. Data represent mean values for triplicate determinations ± SD. (B) Dose response for cell viability by incubation with anti-IgM Ab. Ramos cells were incubated with anti-IgM Ab for 48 h at the concentrations indicated in the Figure. Data represent mean values of triplicate determinations ± SD. (C) Agarose (1.8%) gel electrophoresis of DNA from Ramos cells stimulated with anti-IgM Ab and visualized with ethidium bromide as described in Materials and Methods.

Evaluation of sugar chains on coated plates

AGP consists of five glycosylation sites of tri- and tetra-antennary complex types, and the non-reducing ends of the sugar chains are mainly composed of α2,6-linked sialic acids. Thus, we stimulated Ramos cells with AGP, neuraminidase treated AGP (asAGP) and α2,3-sialylated AGP (2,3 AGP) to determine the effects of sialic acids on anti-IgM Ab-induced B cell apoptosis. Plates were coated with AGP, asAGP or 2,3AGP as described in Materials and Methods. We evaluated the sugar chain structures of the coated AGP, asAGP and 2,3 AGP by a lectin-binding assay using SNA and MAA, which bind to α2,6- and to α2,3-linked sialic acids, respectively. Figure 2 shows that SNA bound to AGP-coated plates at high levels and scarcely bound to MAA. On the other hand, SNA bound at negligible levels to asAGP-coated plates and did not bind at all to MAA, whereas MAA bound to 2,3 AGP-coated plates and SNA bound at negligible levels. Thus, we confirmed that the AGP-, 2,3AGP and asAGP used to coat the plates were α2,6-sialylated, α2,3-sialylated and asialo-α_1-acid glycoprotein, respectively.

Effect of AGP on anti-IgM Ab-induced apoptosis in Ramos cells

We investigated the effects of AGP-stimulation on apoptosis induced by anti-IgM Ab. We compared AsAGP, which does not contain sialic acids, 2,3 AGP, which does contain α2,3-sialic acids and BSA, which does not contain sugar chains, with AGP. The surface of Ramos cells contains numerous sialylated glycoconjugates. Thus, prior to stimulation, cells were digested with neuraminidase and their glycoconjugates were desialylated to promote binding efficiency between AGP and its recognition molecule on the cell surface. Figure 3 shows that the viability and the number of apoptotic cells of Ramos cells incubated in plates coated with AGP, asAGP, 2,3AGP or BSA in the absence of IgM Ab were not affected. When the cells were incubated in plates coated with asAGP,

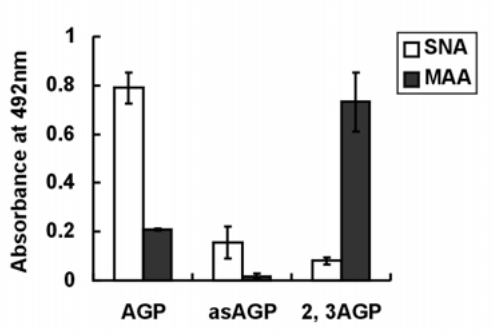

Figure 2. Lectin binding to plates coated with AGP, asAGP, or 2,3 AGP. AGP-, neuraminidase digested AGP (asAGP)- or α2,3 sialylated AGP (2,3AGP)-coated plates were reacted with SNA (open columns) or MAA (closed columns). Lectin binding assay was performed as described in Materials and Methods. Data represent mean values of triplicate determinations ± SD.

2,3AGP or BSA in the presence of 2.5 μg/ml anti-IgM Ab, apoptosis was induced at equivalent levels. On the other hand, incubation in plates coated with AGP rescued Ramos cells from apoptosis induced by anti-IgM Ab. Thus, glycoconjugates with α2,6-linked, but not α2,3-linked sialic acids inhibited anti-IgM Ab-induced apoptosis.

The effects of anti-CD22 mAb on anti-IgM Ab-induced apoptosis

The 130 kD glycoprotein, CD22, is expressed on the surface of mature $IgM^{+}IgD^{+}$ B cells [10–15]. CD22 is an adhesion molecule that recognizes sugar chains containing α2,6-linked sialyl lactosamine (Sia α2,6 Gal β1,4 GlcNAc) as a ligand [19–23]. Thus, we used anti-CD22 mAb to examine whether or not the interaction of CD22 with BCR affects anti-IgM Ab-induced apoptosis. Figure 4 shows that anti-CD22 mAb enhanced

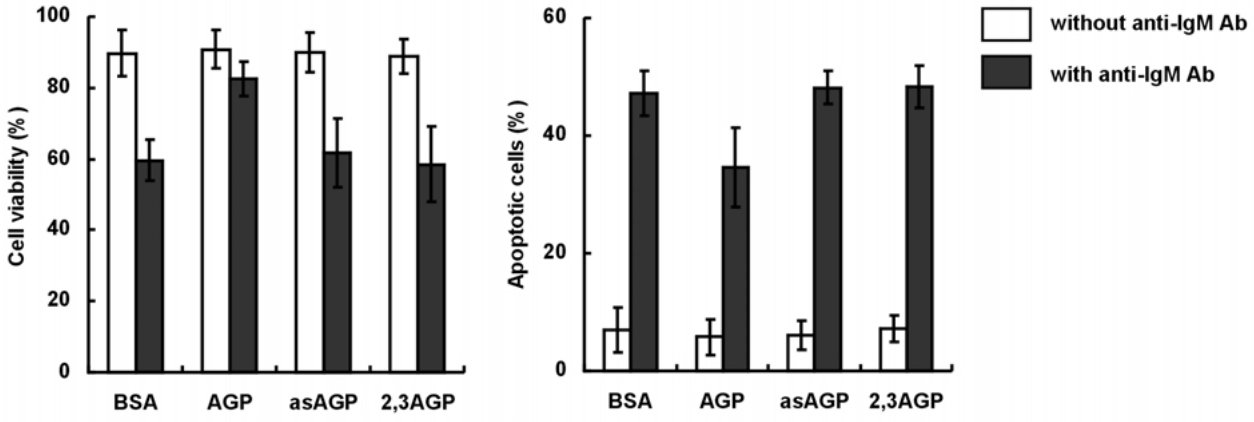

Figure 3. Effects of protein-coated plate on anti-IgM Ab-induced apoptosis. Ramos cells were incubated with (closed columns) or without (open columns) anti-IgM Ab (2.5 μg/ml) on plates coated with BSA, AGP, asAGP or 2,3 AGP. Cell viability (left panel) and apoptotic cells (right panel) were determined after 48 h as described in Materials and Methods. Data represent mean values of triplicate determinations ± SD.

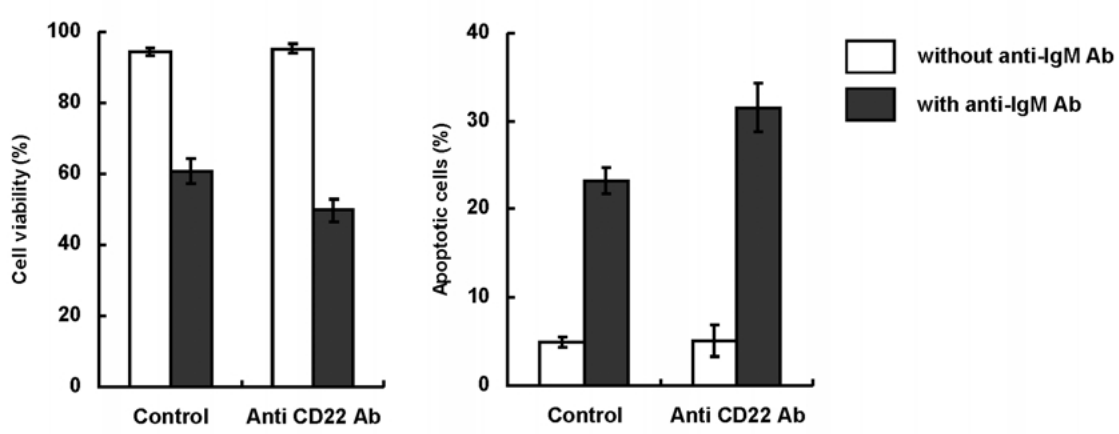

Figure 4. Effects of anti-CD22 mAb on anti-IgM Ab-induced apoptosis. Ramos cells were incubated on plates coated with or without anti-CD22 mAb. Closed and open columns indicate incubation with (2.5 μg/ml) or without anti-IgM Ab, respectively. Cell viability (left panel) and apoptotic cells (right panel) were determined after 48 h as described in Materials and Methods. Data represent mean values of triplicate determinations ± SD.

anti-IgM Ab-induced apoptosis although the cells did not undergo apoptosis when Ramos cells were incubated with anti-CD22 mAb alone. These findings suggested that CD22 stimulation with anti-CD22 mAb acts as an apoptosis enhancing regulatory factor in anti-IgM Ab-induced B cell apoptosis.

Discussion

The present study found that the α2,6- but not the α2,3-sialic acid on AGP inhibits anti-IgM Ab-induced B cell apoptosis, suggesting that the former is involved in B cell apoptosis. Stimulation of BCR induces apoptosis in mature B cells. Recent studies demonstrated that B cell apoptosis is regulated by cell surface antigens, such as CD21 [3], CD22 [4] and CD40 [5,6]. Among these surface antigens, CD22 recognizes sugar chains containing α2,6-linked sialyl lactosamine as a ligand [19–23]. AGP is a fraction of which had previously been demonstrated to exhibit high binding to CD22 [22] and used as a probe to study the lectin function of CD22 [33]. Thus, we studied the effects of stimulation with anti-CD22 mAb on anti-IgM Ab-induced apoptosis in Ramos cells, to clarify whether the binding of α2,6-linked sialic acid to CD22 is involved. Anti-IgM Ab-induced apoptosis was enhanced by co-stimulation with anti-CD22 mAb, whereas stimulation with anti-CD22 mAb alone did not affect apoptosis. These contradictory effects on anti-IgM Ab-induced apoptosis between α2,6-linked sialic acid and anti-CD22 mAb stimulation suggested that inhibitory effects of α2,6-linked sialic acid were not caused by CD22 stimulation.

Conflicting effects of anti-CD22 Abs on B-cell activation and proliferation through BCR-mediated signal have been reported [25,34–37], and the role of CD22 in B-cell apoptosis induced by anti-IgM Ab remains to be clarified. Chaouchi et al. [4] reported that anti-CD22 Ab and cross-linked Ab enhance anti-IgM Ab-induced B-cell apoptosis. During stimulation by anti-CD22 mAb, antibody cross-linking between CD22 and BCR may stimulate BCR and activate several tyrosine kinases. Tuscano et al. [34] showed using two anti-CD22 mAbs with different binding sites, that significant difference in CD22 tyrosine phosphorylation occurs. Thus, we supposed that the binding site of anti-CD22mAb (RFB4) in the present study was distinct from the α2,6-linked sialic acid binding site, and that stimulation by either of them induced phosphorylation at different sites in Ramos cells, and that the binding of α2,6-linked sialic acid to CD22 differs from that of anti-CD22mAb and induced opposite effects.

Hanasaki et al. [38] reported that interaction of CD22 with cell surface IgM via α2,6-sialylated sugar chains expressed on IgM molecules may regulate BCR signaling and that the final cellular effects through BCR-mediated signals are regulated by the intracellular balance of Syk (and other kinases) and SHP-1, tyrosine phosphatase recruitment. The interaction of CD22 with the α2,6-sialic acid of IgM on BCR down-regulates BCR-mediated signals to recruit SHP-1 in naive B cells [39,40]. In an α2,6-sialic acid-abundant environment, CD22 binds to other α2,6-sialylated glycoconjugates, thus reducing the amount of its binding to BCR, which results in enhanced BCR signals. In this study, α2,6-sialic acid expressed on AGP may bind to the lectin domain on CD22 and induce inhibitory effects on BCR-mediated apoptosis. However, studies in vivo have yet to establish that α2,6-sialylated glycoconjugates act as endogenous ligands for CD22.

With regard to the role of CD22 in B-cell function, the extracellular environment and its physiological ligands must be clarified. In mature B cells, cell surface glycoconjugates are highly sialylated and thus α2,6-linked sialic acid-mediated signals are usually masked. However, stimulating BCR likely induces a temporarily desialylated cell surface, which can contact other cells [41]. In particular, T-cell CD45, which abundantly expresses α2,6-linked sialic acids, is considered a physiologically important ligand of B-cells of the immune system. Thus, ligands on B cells during BCR-induced apoptosis bind to CD45 on T cells, and such interaction induces the suppression of B cell apoptosis. The present findings suggest that a series of these reactions constitute a rescue mechanism in B cell negative selection through apoptosis. Stimulation with α2,6-linked sialic acid is probably involved in the suppression of B cell apoptosis and in the regulation of plasma and memory cell differentiation through immune cell activation-mediated ingenious cross-talk.

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