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Summary

A two-step culture system was used to investigate the role of chondroitin sulfate(CS) B , which is mitogenic to B cells, in differentiation of B cells. Mouse spleen B cells were incubated for 3 days with CSB in the presence of interleukin (IL)-4 and IL-5. After washing, the cells were replated at 10^5 viable cells/well and recultured without CSB in the presence of IL-4 and IL-5. CSB dose-dependently increased IgM production, the greatest enhancement being 450%. Dextran sulfate had a similar effect, whereas other glycosaminoglycans, CSA, CSC, heparin and hyaluronic acid, were marginally effective. Treatment of B cells with CSB resulted in increases in the number of IgM-secreting cells and numbers of CD138-positive cells and CD45R/B220-negative cells. CSB-induced IgM production was inhibited by the protein kinase C (PKC) inhibitor GF109203X but not by the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin. These results demonstrated that CSB promoted differentiation of B cells in the presence of IL-4 and IL-5 and suggested that PKC but not PI3K is crucial for CSB-induced IgM production.

Keywords: Chondroitin sulfate B (CSB); Murine B cells; IgM; Differentiation; CD138; Protein kinase C

1. Introduction

Chondroitin sulfate is a sulfated glycosaminoglycan composed of a long unbranched polysaccharide chain with a repeating disaccharide structure of *N*-acetylgalactosamine and glucuronic acid [1,2]. Most of *N*-acetylgalactosamine residues are sulfated, particularly in the 4 or 6 position, making chondroitin sulfate a strongly charged polyanion with a high water-draining power. Chondroitin 4-sulfate and chondroitin 6-sulfate are often called chondroitin sulfate A (CSA) and chondroitin sulfate C (CSC), respectively. Chondroitin sulfate B (CSB), also known as dermatan sulfate, is a stereoisomeric form of chondroitin sulfate with varying proportions of iduronic acid in place of glucuronic acid [1-3]. *O*-Sulfo groups are found on the 4- or 6-position of *N*-acetylgalactosamine residues or the 2-position of iduronic acid residues of CSB. CSB is often found attached to a protein core, resulting in a macromolecule called a proteoglycan. CSB proteoglycans localize on cell surfaces and in the extracellular matrix and exist excessively in fibrous connective tissues such as skin, tendon, skeletal muscle, blood vessels, bone and cartilage. CSB has been the topic of limited studies in comparison to the more commonly studied glycosaminoglycans such as heparan sulfate, heparin and hyaluronic acid, but recent studies have shown that CSB has intriguing functions in central nervous system development, wound repair, infection, growth factor signaling, morphogenesis and cell division, in addition to its conventional structural roles [3-5].

CSB has also been implicated in the pathogenesis of rheumatoid arthritis, a chronic, systemic and inflammatory autoimmune disease of connective tissue. Several changes in glycosaminoglycan expression in synovial tissue and cartilage of rheumatoid arthritis and osteoarthritis patients have been described. In synovial tissue of rheumatoid arthritis patients, CSB has been shown to be the primary molecular species of chondroitin sulfate in inflammatory areas compared with fibrotic areas where CSA/C

expression dominates [6]. CSB is the dominant glycosaminoglycan in proteoglycans secreted by synovial cells [7]. Basic activity of the disease and proliferation of the synovial cells correlate with an increased percentage of CSB of the total glycosaminoglycan content in the synovium [8]. Furhtermore, rheumatoid arthritis chondrocytes are known to synthesize an increased proportion of proteoglycans, enriched in CSB [9]. A growing number of pathogens, including viruses, parasites and bacteria, have been shown to use cell-surface chondroitin sulfate and CSB, in addition to heparan sulfate, for their attachment to host cells and tissues [3,10]. CSB as well as hyaluronic acid has been shown to inhibit an activity of antithrombin, a plasma inhibitor of thrombin [11]. Thrombin plays a key role in enhancing synovial thickness and inflammation during the pathogenesis of rheumatoid arthritis through its cleavage of fibrinogen to form a fibrin clot and its mitogenic effect on synovial cells [12,13]. Moreover, in mice, injection of glycosaminoglycans such as CSB, CSA, CSC, and hyaluronic acid has been shown to induce the onset of arthritis, tendosynovitis and dermatitis [14].

Prompted by the growing evidence of involvement of CSB in the pathogenesis of rheumatoid arthritis, we initiated an examination of the effects of CSB on functions of immune cells infiltrating inflammatory areas. We have recently found that CSB but not CSA or CSC stimulated the polyclonal proliferation of murine B cells as markedly as did anti-IgM antibody and more markedly than did dextran sulfate [15]. Our data suggested that phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) are crucial but that extracellular signal-regulated kinase (ERK) is less important for the mitogenc activity of CSB, the signaling pathways of which may be at least partly distinct from those of anti-IgM and lipopolysaccharide (LPS). Rachmilewitz and Tykocinski reported that CSB stimulated the proliferation of B cells in human peripheral blood mononuclear cells in a monocyte-dependent manner: on depletion of monocytes by adherence to tissue culture flasks, the residual nonadherent cells no

longer proliferated when exposed to CSB [16]. Murine splenic B cells, however, responded to CSB independently of adherent cells [15]. Inflammation, infection or physical damage can lead to the release of soluble CSB via degradation of the extracellular matrix in various fibrous connective tissues. Increased soluble CSB may cause polyclonal activation of B cells that could play a role at sites of inflammation and participate in the regulation of autoimmune response. To the best of our knowledge, however, no studies on CSB-induced differentiation of B cells have been reported to date. Since some stimuli which induce proliferation of B cells do not promote or rather inhibit B-cell differentiation [17,18], this study was undertaken to examine the effect of CSB on polyclonal antibody production in B cells. Our data indicated that CSB promoted polyclonal differentiation of B cells to antibody-producing cells in the presence of IL-4 and IL-5, and we also obtained evidence that PKC but not PI3K is crucial for CSB-induced IgM production.

2. Materials and methods

2.1. Mice

Female BALB/c mice were purchased from Charles River Japan (Yokohama, Japan). They were maintained under specific pathogen-free conditions in the animal facility of Okayama University and used between 8 and 12 weeks of age.

2.2. Reagents and antibodies

CSB sodium salt (porcine intestinal mucosa), heparin sodium salt (porcine intestinal mucosa), hyaluronic acid (bovine vitreous humor), dextran sulfate sodium salt, LPS from *Escherichia coli* 055:B5, and propidium iodide were obtained from Sigma

Chemical Co. (St. Louis, MO). CSB sodium salt (porcine intestinal mucosa) was also purchased from Celsus Laboratories (Cincinnati, OH) and used for some experiments. CSA sodium salt (whale cartilage) and CSC sodium salt (shark cartilage) were obtained from Nacalai Tesque (Kyoto, Japan). Recombinant mouse IL-4 and IL-5 were purchased from R&D Systems (Minneapolis, MN). GF109203X (Gö6850) and PD98059 were obtained from Calbiochem (La Jolla, CA). Wortmannin was purchased from Wako Pure Chemical Industries (Osaka, Japan). Purified mouse IgM, goat anti-mouse IgM antibody, and horseradish peroxidase-conjugated goat anti-mouse IgM antibody were obtained from Zymed Laboratories Inc (San Francisco, CA), Organon Teknika Corporation (Durham, NC), and Kirkegaad & Perry Laboratories (Gaithersburg, MD), respectively. Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD45R/B220, phycoerythrin (PE)-conjugated rat anti-mouse CD138 (syndecan-1), FITC-conjugated hamster anti-mouse CD3 ε chain, anti-mouse CD16/CD32, anti-mouse IL-4, and biotinylated anti-mouse IL-4 monoclonal antibodies (mAbs) were purchased from BD Biosciences Pharmingen (San Jose, CA). Anti-mouse TNF-α antibody, biotinylated anti-mouse TNF-α antibody, anti-mouse IL-12 mAb, and biotinylated anti-mouse IL-12 antibody were obtained from R&D Systems (Minneapolis, MN). [6-^{3}H]Thymidine was obtained from GE Healthcare Bio-Sciences (Little Chalfont, UK).

2.3. Preparation of enriched B cells

Murine spleen resting conventional ($CD5^{-}$) B cells were enriched by negative selection using a mouse B lymphocyte enrichment set (BD Biosciences Pharmingen) as decribed previously [15]. Briefly, murine spleen cells depleted of erythrocytes by lysis of erythrocytes with ammonium chloride were suspended in Dulbecco's minimum essential medium supplemented with 4 mM L-glutamine, 100 U/ml penicillin and 100

μg/ml streptomycin and incubated on plastic dishes for 90 min at 37 °C. The nonadherent cells were then collected, suspended in the same medium, and incubated on ice for 60 min with biotin-conjugated anti-mouse CD4, CD43 and TER-119/erythroid cell mAbs. The cells bearing the biotinylated antibodies were bound to BD IMag streptavidin particles, and negative selection was then performed on a BD IMagnet according to the manufacturer's protocol to enrich the unlabeled B cells. The purity of recovered viable B cells was more than 97% when the cells were stained with FITC-conjugated anti-mouse CD45R/B220 mAb and propidium iodide after preincubation of the cells with anti-mouse CD16/CD32 mAb and analyzed by a flow cytometer (Epics XL, Beckman Coulter, Miami, FL). More than 95% cells in the enriched B cell preparations were small, resting B cells as judged by the cell size (forward scatter) in flow cytometry.

2.4. Antibody response of enriched B cells

Enriched B cells were first incubated with or without CSB in the basal culture medium (RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml of penicillin G and 100 μg/ml of streptomycin) in the presence of 50 μM 2-mercaptoethanol, IL-4 (200 U/ml) and IL-5 (150 U/ml) for 3 days in round-bottom 96-well plates (2.5×10^5 cells/200 μl/well) at 37 °C in an atmosphere containing 5% CO_2. The cells were washed twice with the basal culture medium to remove CSB and then recultured without CSB in the basal culture medium containing 50 μM 2-mercaptoethanol, IL-4 (200 U/ml) and IL-5 (150 U/ml) for the indicated days in triplicate in round-bottom 96-well plates at a density of 1×10^5 cells/200 μl/well. The culture supernatant was then collected and was frozen at –30˚C for an IgM enzyme-linked immunosorbent assay (ELISA). The IgM levels were assayed by a

sandwich ELISA as described previously [19]. The number of viable cells was determined by a Trypan Blue dye-exclusion test.

2.5. Enzyme-linked immunospot (ELISPOT) assay for detection of polyclonal IgM-secreting cells

Detection of IgM-secreting cells was carried out as decribed by Sedgwick and Holt [20] and by Hodgkin et al. [21]. Briefly, each well of 96-well microtiter plates was coated with 50 μl of goat anti-mouse IgM antibody (10 μg/ml) by being incubated overnight at 4 ˚C and then washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20. The plates were blocked with 200 μl of 1% bovine serum albumin in PBS for 2 h at room temperature and washed. To those plates, enriched B cells, which had been incubated with or without CSB for 2.5 days in the reculture period and washed with and suspended in the basal culture medium, were added (2.5×10^3 cells/200 μl/well) in triplicate and incubated for 2 h at 37 °C. After being washed three times with PBS containing 0.05% Tween-20, the plates was incubated with 50 μl of horseradish peroxidase-conjugated goat anti-mouse IgM antibody (0.2 μg/ml) for 1 h at room temperature. After washing, 100 μl of substrate solution consisting of 50 mM sodium phosphate buffer (pH 7.0), 1% agarose, 2 mg/ml of diaminobenzidine, 0.0018% H_2O_2, 0.0018% $NiCl_2$, and 0.0018% $CoCl_2$ was added. The plates were incubated overnight at 37 °C in the dark, and the number of spot-forming cells was counted under a stereomicroscope. The data are expressed as the number of spot-forming cells per 10^5 viable cells.

2.6. Flow cytometric analysis of cell surface antigen expression

Enriched B cells were incubated with or without CSB in the basal culture medium

containing 50 μM 2-mercaptoethanol, IL-4 (200 U/ml) and IL-5 (150 U/ml) for 3 days in 24-well plates (1.25×10^6 cells/1 ml/well). The cells were washed twice with the basal culture medium to remove CSB and then recultured without CSB in the basal culture medium containing 50 μM 2-mercaptoethanol, IL-4 (200 U/ml) and IL-5 (150 U/ml) for 2.5 days in 24-well plates at a density of 5×10^5 cells/1 ml/well. The cells were then pooled and washed with and suspended in PBS containing 0.5% bovine serum albumin and 0.1% sodium azide (~ 1×10^6 cells/100 μl). The cells were incubated with anti-mouse CD16/CD32 mAb (10 μg/ml) for 5 min on ice to block Fc-mediated binding of antibodies to Fcγ receptor of cells and stained with FITC-conjugated anti-mouse CD45R/B220 mAb (10 μg/ml) or PE-conjugated rat anti-mouse CD138 mAb (4 μg/ml) for 30 min on ice. After being washed with and suspended in PBS containing 0.5% bovine serum albumin and 0.1% sodium azide, the cells were stained with propidium iodide (2 μg/ml), and expression of CD45R/B220 and CD138 on viable cells was analyzed by a flow cytometer (Epics XL, Beckman Coulter).

2.7. Determination of DNA synthesis

Enriched B cells (2.5×10^5/200 μl/well) were incubated with or without CSB or LPS in the basal culture medium containing 50 μM 2-mercaptoethanol, IL-4 (200 U/ml) and IL-5 (150 U/ml) for 45 h in triplicate in round-bottom 96-well plates. The cells were then pulse-labeled with [^{3}H]thymidine (0.5 μCi/well, 2.5 Ci/mmol) for 3 h and harvested on glass fiber filters using a cell harvester as described previously [15]. The amount of [^{3}H]thymidine incorporated was measured in a liquid scintillation counter.

2.8. Determination of cytokine production

Enriched B cells (2.5×10^5/200 μl/well) were incubated with or without CSB (6.3,

25, 100 μg/ml) or LPS (10 μg/ml) in the basal culture medium containing 50 μM 2-mercaptoethanol for 1, 3 and 24 h. Levels of IL-4, IL-12 and TNF-α in cultured supernatants were measured by an ELISA.

2.9. Data analysis

Results are expressed as means and S.E.M. of three or four independent experiments. Data in two groups were analyzed by Student's *t*-test. Multiple comparison of the data was done by Dunnett's *t*-test or Dunnett's T3-test. *P* values less than 5% were regarded as significant.

3. Results

3.1. The mitogenic effect of CSB on IL-4- and IL-5-stimulated B cells

We have recently reported that CSB alone markedly stimulated proliferation of murine B cells in a basal culture medium [15]. The mitogenic effect of CSB on IL-4- and IL-5-stimulated B cells was examined. B cells, more than 95% cells of which were small, resting B cells, were incubated with or without varying doses of CSB in the presence of IL-4 and IL-5 for 45 h and pulse-labeled with [^{3}H]thymidine for the next 3 h. CSB stimulated DNA synthesis of B cells, and its dose-response curve was nearly linear up to 100 μg/ml (Fig. 1A). The number of viable cells in 72-h culture was dose-dependently increased by CSB, being a 50% increase over the control at 100 μg/ml of CSB (Fig. 1B).

3.2. Augmentation of IgM production in B cells by CSB and effects of other glycosaminoglycans

Those cells stimulated with or without varying doses of CSB for 72 h as described above were then washed and recultured without CSB in the presence of IL-4 and IL-5 for 4 days at an initial density of 1×10^5 cells/well. IgM produced was determined by an ELISA, and the results are shown in Fig. 2A. CSB dose-dependently increased IgM production, and its stimulatory effect reached a plateau at 6.3 μg/ml with maximal enhancement of 450%. The maximal effect of CSB was comparable to that of 0.1 μg/ml of LPS (Fig. 2A). Since CSB was omitted during the reculture period, the viable cell density in the treated cultures did not significantly differ from that of the control cultures at the end of the reculture period (Fig. 2B). CSB obtained from Celsus Laboratories was as effective as CSB from Sigma Chemical Company (data not shown). IgG production was also markedly increased by CSB, although the levels of IgG were less than one fourth of IgM levels in the same culture at any doses of CSB (data not shown). Figure 2C shows a time course for CSB-induced increase in IgM production as well as LPS-induced increase. IgM levels in the culture supernatant of B cells treated with 6.25 μg/ml of CSB lineally increased with incubation time in a manner similar to that in cultures treated with 0.1 μg/ml of LPS. The stimulating effect of LPS (0.1 μg/ml) but not that of CSB was potently inhibited by polymyxin B (Fig. 2D). The effects of other glycosaminoglycans and dextran sulfate on IgM production in B cells are shown in Fig. 3. Dextran sulfate was as effective as CSB, but CSA, CSC, heparin, and hyaluronic acid were marginally effective or ineffective.

3.3. CSB-induced differentiation of B cells

The number of antibody-secreting cells in cultures treated with or without CSB for 2.5 days in the reculture period was determined by the ELISPOT assay, and the results were expressed as the number of spots per 10^5 viable cells. Treatment with 6.3,

25, and 100 μg/ml of CSB in the presenc of IL-4 and IL-5 resulted in a more than 400% increase in the number of IgM-secreting cells, similar to the effect of 0.1 μg/ml of LPS (Fig. 4A). The number of antibody-secreting cells in cultures incubated without IL-4 and IL-5 was also significantly enhanced by CSB, although the absolute values were much lower than those in B cells incubated with IL-4 and IL-5 (Fig. 4B). B cells activated with CSB in the absence of IL-4 and IL-5 produced an increased amout of IL-12 but not IL-4 and, in contrast to B cells stimulated with LPS, TNF-α (data not shown). It is known that differentiation of naive B cells to plasma cells is accompanied by an increase in CD138 expression and by a decrease in CD45R/B220 expression [22,23]. B cells treated with or without CSB or LPS for 2.5 days in the second culture were subjected to analyses of expression of both cell-surface antigens by a flow cytometer. Both CSB and LPS significantly increased the percent of CD138-positive cells and the percent of CD45R/B220-negative cells (Fig. 5A–D).

3.4. Requirement of PKC but not PI3K in CSB-induced IgM production

We have recently shown that CSB caused translocation of PKC isoform β from cytosol to membrane fractions and increased phosphorylation of Akt but not phosphorylation of ERK of B cells and that PI3K and PKC are crucial but that ERK is less important for the B cell mitogenc activity of CSB [15]. In order to elucidate the role of those signaling pathways in CSB-induced differentiation of B cells, we conducted experiments using pharmacological inhibitors, and the results are shown in Fig. 6. When the inhibitors were included in the first culture period only, CSB-induced IgM production was potently inhibited by the PKC inhibitor GF109203X (1 μM) but not by the PI3K inhibitor wortmannin (100 nM) (Fig. 6A). In contrast, the mitogen-activated protein/ERK kinase (MEK) inhibitor PD98059 (12.5 μM) significantly enhanced IgM production promoted by CSB. On the other hand, LPS-induced IgM production was

partially inhibited by GF109203X but not by either wortmannin or PD98059. Similar results were obtained when each inhibitor was added to cultures during both the first culture and reculture periods (Fig. 6C), but none of the three inhibitors had any effect when they were included during the reculture period only (Fig. 6B).

4. Discussion

The results presented in this paper demonstrated that CSB markedly induced polyclonal differentiation of B cells to antibody-producing cells in the presence of IL-4 and IL-5. Since polymyxin B had no influence on IgM production in B cells treated with CSB, the differentiation-promoting effect of CSB is not due to, if any, a contaminating LPS in the preparation of CSB. In this study, we employed two-step cultures of B cells in order to separate the differentiation-inducing effect of CSB from its mitogenic effect; without separation of these effects, interpretation of antibody production data obtained may be difficult due to the presence of both mitogenic and differentiation-inducing effects. B cells were stimulated with CSB in the primary culture for 3 days, washed to remove CSB, and recultured at a density of 1×10^5 cells/well for 4 days to determine the effect of CSB on IgM production. Under these conditions, the viable cell number in the CSB-treated cultures did not significantly differ from that of the control cultures at the end of the reculture period, and thus the differentiation-promoting effect of CSB can be separately evaluated. The concentration for the maximal differentiation-inducing effect of CSB determined by this method was much less than the concentration required for the maximal mitogenic effect of CSB: the stimulatory effect of CSB on IgM production was maximal at 6.3 μg/ml, whereas its mitogenic effect was linealy increased up to 100 μg/ml (Fig. 1A and Fig. 2A). Since the addition of 6.3 μg/ml of CSB resulted in a 30% increase in the number of valiable cells during the primary culture for 3 days (Fig. 1B), overall IgM production in the

CSB-treated cultures was calculated to be seven-times greater than the IgM production in untreated cultures. Rafi et al. reported that stimulation of B cells with 0.1 and 0.5 mg/ml of hyaluronic acid resulted in 80% and 120% increases in IgM production, respectively [24]. However, in our two-step culture system hyaluronic acid was marginally effective but CSB markedly effective at 6.3 μg/ml. Thus CSB is a stronger differentiation-promoting agent than hyaluronic acid. Inflammation, infection or injury can lead to the release of soluble CSB via degradation of the extracellular matrix, and CSB comprises over half of the total soluble glycosaminoglycan content of wound fluid [25]. Increased soluble CSB may cause polyclonal activation and proliferation of B cells in the earlier phase, and in the later phase, increased B cells may produce a large amount of polyclonal antibody even at reduced or null concentrations of CSB.

Several lines of evidence suggest that CSB is involved in the pathogenesis of rheumatoid arthritis, an autoimmune disease of connective tissue. CSB is the dominant glycosaminoglycan in proteoglycans secreted by synovial cells [7]. Basic activity of the disease and proliferation of the synovial cells correlate with an increased percentage of CSB of the total glycosaminoglycan content in the synovium [8]. Furhtermore, rheumatoid arthritis chondrocytes are known to synthesize an increased proportion of proteoglycans, enriched in CSB [9]. A growing number of pathogens, including viruses, parasites and bacteria, have been shown to use cell-surface CS and CSB, in addition to heparan sulfate, for their attachment to host cells and tissues [3,10]. In mice, injection of glycosaminoglycans such as CSB, CSA, CSC, and hyaluronic acid has been shown to induce the onset of arthritis, tendosynovitis and dermatitis [14]. Our data showing CSB-induced polyclonal proliferation and differentiation of B cells further support the notion that CSB plays a role at sites of inflammation and participates in the regulation of autoimmune response.

Our previous results using specific pharmacological inhibitors of protein kinases suggest a role of PKC and PI3K in the proliferation of B cells induced by CSB [15].

Evidence for an essential role of PKC and PI3K in BCR signal transduction has been provided by various studies. B cells of mice deficient in PKCβ have been shown to exhibit a defective proliferative response to anti-IgM antibody, despite normal T-cell activation in response to T cell receptor stimulation [26]. B cells of mice deficient in the p85α or p85α-p55α-p50α adaptor proteins of PI3K had diminished proliferative responses to both anti-IgM antibody and LPS [27,28], although mice deficient in p110δ catalytic subunit had a poor B cell response to anti-IgM antibody but a normal response to LPS [29]. IgM production induced by CSB, however, was inhibited by the PKC inhibitor but not by the PI3K inhibitor, indicating that the proliferation and differentiation of B cells induced by CSB have different susceptibilities to signaling protein kinase inhibitors. Two key transcriptional regulators, B lymphocyte-induced maturation protein-1 (Blimp-1) and X-box-binding protein-1 (XBP-1), have been shown to be necessary for the terminal differentiation of B cells into antibody-secreting plasma cells [23]. Little is known about signals that induce Blimp-1 and XBP-1 although it has been shown that the expression of Blimp-1 is induced by IL-5 [30]. Hence it is not known at present whether or not PKC is involved in the regulation of expression of the both transcription factors. In contrast to the PKC inhibitor, the MEK inhibitor enhanced IgM production promoted by CSB. It has been shown that continuous BCR signaling through the Ras-MEK-ERK pathway inhibits LPS-induced plasma cell differentiation [31], but neither CSB nor IL-4 increased phosphorylation of ERK [15,32]. Thus, the mechanism responsible for augmentation of CSB-induced IgM production in B cells treated with the MEK inhibitor remains to be investigated.

CSB has been shown to interact with CD44, a broadly distributed cell surface protein, although the interaction of CD44 with CSB is more modest than that of CD44 with hyaluronic acid [33]. CD44 is an adhesion molecule with known importance in T cell activation, lymphocyte trafficking and tumor cell invasiveness [2,34]. Moreover, some studies have suggested that CD44 participates in the activation of monocytes and

B cells induced by hyaluronic acid, CSA, CSB or chondroitin sulfate proteoglycans [16,24,35]. The involvement of CD44 in the mitogenic and differentiation-inducing effects of CSB on murine B cells remains to be determined, although CD44 expression in murine B cells increases when B cells are activated with polyclonal stimuli, including LPS and interleukin 5 [36], and CD40 ligand-induced B cell proliferation and immunoglobulin production are inhibited by immobilized anti-CD44 mAbs [37].

In conclusion, the results of this study demonstrated that CSB promoted polyclonal differentiation of B cells to antibody-secreting cells in the presence of IL-4 and IL-5 at doses less than those required for the mitogenic effect, and the results suggest that PKC but not PI3K is crucial for CSB-induced IgM production. Our data further support the notion that CSB plays a role in the regulation of autoimmune response.

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Figure legends

Fig. 1. CSB-induced proliferation of IL-4- and IL-5-stimulated B cells. (A) Enriched B cells were incubated with the indicated doses of CSB or LPS for 45 h in the presence of IL-4 and IL-5 and then pulse-labeled with [^{3}H]thymidine for 3 h. The data are representative of two independent experiments and expressed as means ± S.E.M. of triplicate cultures. (B) Enriched B cells were incubated with the indicated doses of CSB or LPS for 3 days in the presence of IL-4 and IL-5. The number of viable cells was determined by a Trypan Blue dye-exclusion test. The data are means ± S.E.M. of three independent experiments. Values that are significantly different from those of control cultures are indicated by *$p < 0.05$ (Dunnett's *t*-test).

Fig. 2. Enhancement of IgM production in IL-4- and IL-5-stimulated B cells by CSB and no inhibitory effect of polymyxin B on CSB-stimulation. Enriched B cells were incubated with the indicated doses of CSB or LPS (A, B) or with CSB (6.25 μg/ml) or LPS (0.1 μg/ml) (C, D) for 3 days in the presence of IL-4 plus IL-5 and in the presence or absence of polymyxin B (PMB, 5 μg/ml) and then washed and recultured for 4 days (A, B, D) or for the indicated days (C) in the presence of IL-4 plus IL-5. IgM in the culture supernatants was measured by an ELISA (A, C, D). The number of viable cells was determined by a Trypan Blue dye-exclusion test (B). The data are means ± S.E.M. of three independent experiments. Values that are significantly different from those of the respective control cultures are indicated by *$p < 0.05$, ***$p < 0.001$ (Dunnett's *t*-test) (A, C) or (Student's *t*-test) (D).

Fig. 3. Effects of various GAGs on IgM production in IL-4- and IL-5-stimulated B cells. Enriched B cells were incubated with the indicated GAGs (6.25 μg/ml), dextran sulfate (6.25 μg/ml) or LPS (0.1 μg/ml) for 3 days in the presence of IL-4 and IL-5 and

then washed and recultured for 4 days in the presence of IL-4 and IL-5. IgM in the culture supernatants was measured by an ELISA. The data are means and S.E.M. of three independent experiments. Values that are significantly different from those of control cultures are indicated by ***$p < 0.001$ (Dunnett's *t*-test).

Fig. 4. Increase in the number of IgM-secreting cells in IL-4- and IL-5-stimulated or unstimulated cultures by CSB. Enriched B cells were incubated with the indicated doses of CSB or LPS for 3 days in the presence (A) and absence (B) of IL-4 and IL-5 and then washed and recultured for 2.5 days in the presence (A) and absence (B) of IL-4 and IL-5. The number of IgM-secreting cells was measured by an ELISPOT assay. The data are means ± S.E.M. of three (A) or four (B) independent experiments. Values that are significantly different from those of control cultures are indicated by *$p < 0.05$, ***$p < 0.001$ (Dunnett's *t*-test).

Fig. 5. Increases in CD138-positive cells and CD45R/B220-negative cells in cultures incubated with CSB. Enriched B cells were incubated with CSB (6.25 μg/ml) or LPS (0.1 μg/ml) for 3 days in the presence of IL-4 and IL-5 and then washed and recultured for 2.5 days in the presence of IL-4 and IL-5. The cells were then stained with PE-conjugated anti-CD138 mAb and propidum iodide (A and B) or FITC-conjugated anti-CD45R/B220 mAb and propidium iodide (C and D) and analyzed by a flow cytometer. Histograms (A and C) are representative of three independent experiments. The data (B and D) are means and S.E.M. of three independent experiments. The percent of CD138-positive cells and CD45R/B220-negative cells were less than 0.15% and less than 0.1%, respectively, as stained with isotype control IgGs, PE-conjugated rat IgG2a and FITC-conjugated rat IgG2a. *$p < 0.05$, ***$p < 0.001$, as compared with the values of control cultures (Dunnett's *t*-test).

Fig. 6. Effects of the MEK inhibitor PD98059, the PKC inhibitor GF109203X, and the PI3K inhibitor wortmannin on IgM production in CSB- and LPS-treated B cells. (A) Enriched B cells were treated with or without 12.5 μM PD98059, 1 μM GF109203X or 100 nM wortmannin for 60 min and then incubated with CSB (1.56 μg/ml) or LPS (0.1 μg/ml) for 3 days in the presence of IL-4 and IL-5. After washing, the cells were recultured for 4 days in the presence of IL-4 and IL-5. (B) Enriched B cells were incubated with CSB (1.56 μg/ml) or LPS (0.1 μg/ml) for 3 days in the presence of IL-4 and IL-5 and then washed and recultured with IL-4 and IL-5 for 4 days in the presence of 12.5 μM PD98059, 1 μM GF109203X or 100 nM wortmannin. (C) Enriched B cells were treated with or without 12.5 μM PD98059, 1 μM GF109203X or 100 nM wortmannin for 60 min and then incubated with CSB (1.56 μg/ml) or LPS (0.1 μg/ml) for 3 days in the presence of IL-4 and IL-5. After washing, the cells were recultured with IL-4 and IL-5 for 4 days in the presence of the same inhibitor. IgM in the culture supernatants was measured by an ELISA. The data are means ± S.E.M. of three independent experiments. Values that are significantly different from those of the respective control cultures are indicated by $*p < 0.05$ (Dunnett's *t*-test).