Chondroitin sulfate activates B cells in vitro, expands CD138$^+$ cells in vivo, and interferes with established humoral immune responses

Hilke Brühl,* Josef Cihak,† Nicole Goebel,‡ Yvonne Talke,‡ Kerstin Renner,‡ Fabian Hermann,‡ Manuel Rodriguez-Gomez,‡ Barbara Reich,‡ Jiří Plachý,§ Manfred Stangassinger,† and Matthias Mack‡,1

Departments of *Internal Medicine I and ‡II, University Hospital Regensburg, Regensburg, Germany; †Institute for Animal Physiology, University of Munich, Munich, Germany; and §Institute of Molecular Genetics, Czech Academy of Sciences, Prague, Czech Republic

ABSTRACT

Glycosaminoglycans have anti-inflammatory properties and interact with a variety of soluble and membrane-bound molecules. Little is known about their effects on B cells and humoral immune responses. We show that CS but not dextran or other glycosaminoglycans induces a pronounced proliferation of B cells in vitro compared with TLR4 or TLR9 ligands. With the use of inhibitors and KO mice, we demonstrate that this proliferation is mediated by the tyrosine kinases BTK and Syk but independent of CD44. Antibodies against Ig-α or Ig-β completely block CS-induced B cell proliferation. Injection of CS in mice for 4–5 days expands B cells in the spleen and results in a marked increase of CD138$^+$ cells in the spleen that is dependent on BTK but independent of CD4$^+$ T cells. Long-term treatment with CS for 14 days also increases CD138$^+$ cells in the bone marrow. When mice were immunized with APC or collagen and treated with CS for up to 14 days during primary or after secondary immune responses, antigen-specific humoral immune responses and antigen-specific CD138$^+$ plasma cells in the bone marrow were reduced significantly. These data show that CD138$^+$ cells, induced by treatment with CS, migrate into the bone marrow and may displace other antigen-specific plasma cells. Overall, CS is able to interfere markedly with primary and fully established humoral immune responses in mice. *J. Leukoc. Biol.* 96: 65–72; 2014.

Introduction

Glycosaminoglycans are a family of highly anionic polysaccharides with repeating disaccharide units, including hyaluronic acid, CSA and CSC, dermatan sulfate, heparan, heparin sulfate, and keratin sulfate. CS consists of glucuronic acid and N-acetylgalactosamine, which is sulfated on carbon 4 (CSA) or on carbon 6 (CSC) [1]. Glycosaminoglycans interact with a variety of cytokines, chemokines, growth factors, and membrane proteins, such as integrins and selectins [2–6]. CS binds directly to chemokines, such as RANTES, MCP-1, MIP-1α, MIP-1β, and IL-8 [7–9]. We have shown that CSC and CSA interfere with presentation of RANTES to cell surfaces and thereby, block RANTES-induced adhesion of monocytes to endothelial cells [10, 11].

Little is known about the effects of CS on B cells. It was shown in vitro that CSB induces proliferation of B cells, which could be blocked with an antibody against CD44, suggesting that binding of CS to CD44 is involved in this process [12]. In addition, it was shown in vitro that CSB induces secretion of IgM and up-regulates CD138 on B cells, a marker also expressed on plasma cells [13, 14]. B cell proliferation could be blocked with an inhibitor of PKC and PI3K, whereas IgM production was not dependent on PI3K. Apart from CS, dextran sulfate also has been described to induce B cell proliferation in vitro [14].

A variety of different stimuli is known to induce activation and proliferation of B cells. Antigen-specific activation of B cells occurs via the BCR and results in preferential expansion of antigen-specific B cells and further differentiation into plasma cells. Except for polyvalent antigens (such as dextran) antigen-specific activation and production of antigen-specific Igs usually require the help of CD4$^+$ T cells. However, B cells can also be activated in an antigen-independent manner, e.g.,

by ligands for TLR4 or TLR9, which results in proliferation of B cells with a lot of different and mostly unknown specificities.

Here, we have compared the effects of CS and dextran sulfate on B cell proliferation/activation in vitro and in vivo and analyzed how treatment of mice with both reagents affects the humoral immune response against defined antigens (APC or collagen). In addition, we investigated in vitro and in vivo how CS induces B cell proliferation. We show that CS but not dextran sulfate induces B cell proliferation in a BTK- and Syk-dependent but CD44-independent manner. Long-term treatment with CS expands CD138$^+$ cells in the spleen and bone marrow. It also interferes with newly generated and established antigen-specific humoral immune responses by reducing the number of CD138$^+$ antigen-specific plasma cells in the bone marrow.

MATERIALS AND METHODS

Reagents and glycosaminoglycans

CS from shark cartilage, consisting mainly of CSC and balanced with CSA (C4384; Sigma, Munich, Germany) [15], CSA from bovine trachea (C9819; Sigma), CSB from porcine intestinal mucosa (C3788; Sigma), dextran (31392; Sigma), heparan sulfate (H7640; Sigma), heparin (H3149; Sigma), hyaluronic acid (H7630; Sigma), LPS (Sigma), and CpG-DNA (TCCAT-GACGTTCCTGATGCT; TIB MolBiol, Berlin, Germany), was dissolved in 0.9% sodium chloride and injected i.p. or used at concentrations given in the text. The BTK inhibitor LFM-A13 [α-cyano-β-hydroxy-β-methyl-N-(2,5-dibromophenyl)propenamide], the Syk inhibitor II [2-(2-aminoethyl-amino)-4-(3-trifluoromethylanilino)-pyrimidine-5-carboxamide], and the Syk inhibitor IV (2-{7-(3,4-dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-yl-amino}-nicotinamide) were obtained from Calbiochem (Darmstadt, Germany) and dissolved in DMSO. Cross-linked APC was obtained from ProZyme (Hayward, CA, USA). The CD44 antibody (clone KM81), described to block interaction with hyaluronate, was obtained from Acris Antibodies (Herford, Germany) [16]. Antibodies against Ig-α (CD79a, clone F11-172) and Ig-β (CD79b, clone HM79b) were obtained from BioLegend (San Diego, CA, USA) and BD Biosciences (Heidelberg, Germany), respectively.

Flow cytometry

The following directly labeled antibodies were used for flow cytometry: anti-CD45 (leukocyte common antigen; 30-F11), anti-CD11b (M1/70), anti-CD138 (281-2), anti-CD19 (1D3), anti-GR-1 (RB6-8C5), anti-CD3 (145-2C11), and anti-CD8 (53-6.7; all BD Biosciences). Unfixed cells were preincubated for 15 min on ice with Fc-block (2.4G2, 5 μg/ml) and then with combinations of directly labeled antibodies for 45 min on ice. After three washing steps, red blood cells were lysed with FACS lysing solution (BD Biosciences), and samples were analyzed on a FACSCalibur (BD Biosciences). Mononuclear cells were first gated by light-scatter properties. Monocytes were identified by expression of CD11b. B cells and CD138$^+$ B cells were identified by expression of CD19 and CD138. Neutrophils were identified by light-scatter properties and expression of CD11b and GR-1. Absolute cell numbers in spleen or femurs were determined by addition of counting beads (Coulter, Krefeld, Germany) into the samples.

APC-specific plasma cells were detected as described previously [17, 18]. Briefly, splenocytes or bone marrow cells were first stained with anti-CD138, treated with Fix-Perm and Perm-Wash solutions (BD Biosciences) and then incubated with APC (0.1 μg/ml) for 30 min to detect intracellular antibodies directed against APC.

ELISA

IL-6 was measured with an ELISA kit from BD Biosciences and IL-1β and MIP-2 with ELISA kits from R&D Systems (Minneapolis, MN, USA). To measure antibodies against collagen, ELISA plates were coated overnight with collagen (20 μg/ml). Plasma samples were diluted in PBS/3% BSA (1:5000, if not otherwise indicated) and applied to the plate. Igs bound to collagen were detected with HRP-labeled antibodies against murine IgG1 (clone LO-MG1-2; Serotec, Duesseldorf, Germany) or murine IgG2a (clone R19-15; BD Biosciences). For detection of antibodies against APC, ELISA plates were coated with 10 μg/ml APC, and plasma samples were applied in a dilution of 1:5000, followed by biotinylated antibodies against IgG1 (A85-1; BD Biosciences) and IgG2a (R19-15; BD Biosciences) and streptavidin-linked HRP. 2,2′-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (Roche, Penzberg, Germany) was used to reveal bound Igs.

Isolation and culture of cells

B cells were isolated from splenocytes with magnetic microbeads against CD19 (Miltenyi Biotec, Bergisch Gladbach, Germany) and labeled for 15 min at 37°C with 5 μM CFSE. B cells (10^5 cells/well) were cultured for 5 days in round-bottom 96-well plates in a total volume of 200 μl medium (RPMI 1640 with 10% FCS, penicillin-streptomycin, nonessential amino acids, 1 mM sodium pyruvate, and 50 μM β-ME). The number of proliferated B cells was quantified by flow cytometry and costaining with CD19.

Mice and immunization with APC

C57BL/6 and BALB/c mice were obtained from Charles River (Sulzfeld, Germany). CBA/CaHN-Btk^{xid}/J and CBA/CaJ mice were purchased from The Jackson Laboratory (Bar Harbor, MA, USA). MyD88$^{-/-}$ and TLR3$^{-/-}$ mice on a C57BL/6 background and TLR4$^{-/-}$ mice on a BALB/c background were kindly provided by Bernhard Holzmann (Department of Surgery, University of Munich, Germany). CD44$^{-/-}$ mice on a C57BL/6 background were kindly provided by Dr. Véronique Orian-Rousseau (Karlsruher Institute for Technology, Baden-Württemberg, Germany). C57BL/6 mice were immunized twice at a 4-week interval with i.p. injection of 10 μg APC, precipitated in alum.

Immunization of mice with collagen

Male DBA/1 mice were immunized by a single intracutaneous injection of 125 μg bovine collagen type II (MD Biosciences, Egg, Switzerland) in CFA at the tail base. The clinical score of arthritis was evaluated in a blinded manner as follows: 0, normal; 1, swelling in one joint; 2, swelling in more than one joint; 3, swelling of the entire paw; and 4, deformity and/or ankylosis. Animal experiments were performed in accordance with the legal requirements of the Government of Bavaria.

Preparation of synovial tissue

The skin was removed from the front paws, and the synovial tissue was recovered with a scalpel in a volume of 500 μl PBS [19]. Samples were centrifuged for 10 min at 400 g. The supernatant was frozen immediately and used for quantification of cytokines by ELISA. The synovial tissue was digested with collagenase I (Sigma) for 20 min at 37°C to obtain a single-cell suspension and used for FACS analysis.

Histological analysis

Hind paws were fixed in 3.7% formalin for 24 h, decalcified with a RDO rapid decalcifier (Medite GmbH, Burgdorf, Germany) and embedded in paraffin. At least 10, 5 μm-thick sections of the tarsometatarsal joints were stained with H&E and evaluated in a blinded fashion on a scale from 0 (normal) to 2 for all categories: synovial inflammation (1, focal inflammatory infiltrates; 2, inflammatory infiltrate dominating the cellular histology); synovial hyperplasia (1, continuous, at least three layer-thick synovial lining in one joint; 2, in several joints); pannus formation and cartilage loss (1, cartilage partially covered by pannus, no cartilage loss; 2, with cartilage loss); and bone destruction (1, small areas of bone destruction; 2, widespread bone destruction).

Statistics

Error bars indicate the SEM. Significance were calculated with a one-sided Student's t-test, *P < 0.05, **P < 0.01, and ***P < 0.001.

RESULTS

Effects of glycosaminoglycans on B cells

To analyze the effects of CS and other glycosaminoglycans on B cells, we purified CD19$^+$ splenic B cells and incubated them for 5 days with various concentrations of the reagents. B cells were labeled with CFSE to measure cell proliferation. Incubation with CS at concentrations between 10 and 1000 μg/ml induced a strong proliferation of B cells. Up to 90% of the B cells proliferated, as demonstrated by a reduced cellular CFSE content. Other glycosaminoglycans (heparan sulfate, heparin, dextran, and hyaluronic acid) induced no or only a marginal proliferation of B cells in culture (**Fig. 1A**).

To investigate the effects of CS and dextran in vivo, we injected BALB/c mice for 4 consecutive days with 10 mg CS, 10 mg CSA, and 10 mg dextran or sodium chloride as a control (Fig. 1B). It was described previously that CS up-regulates expression of CD138 in vitro [14]. We therefore also analyzed the number of CD138$^+$ B cells in vivo. Analysis on Day 5 showed that the number of total B cells and especially the number of CD138$^+$ B cells were markedly increased in the spleen of mice treated with CS. Treatment with dextran did not increase the number of B cells. In the bone marrow, no significant changes were observed with CS or with dextran (Fig. 1B).

To get some insight into the cellular mechanisms of how CS induces B cell proliferation, we analyzed various TLR$^{-/-}$ mice (**Fig. 2A**). B cells from MyD88$^{-/-}$, TLR3$^{-/-}$, and TLR4$^{-/-}$ mice were cultured for 5 days with CS, CSA, CSB, CpG-DNA, or LPS. As expected, MyD88$^{-/-}$ mice showed a pronounced reduction in CpG-DNA and a mild reduction in LPS-induced B cell proliferation [20, 21]. TLR4$^{-/-}$ mice showed only a reduction in LPS-induced B cell proliferation, whereas TLR3$^{-/-}$ mice were comparable with WT mice [22, 23]. None of the KO mice showed a convincing reduction in CS-induced B cell proliferation, indicating that the stimulatory effects of CS are not mediated by TLRs (Fig. 2A).

The tyrosine kinases BTK and Syk play an important role in B cell activation [24, 25]. Inhibition of BTK with the cell-permeable inhibitor LFM-A13 almost completely prevented CS-induced B cell proliferation with clearly weaker effects on LPS- and CpG-DNA-induced proliferation (Fig. 2B). Similarly, inhibition of Syk markedly reduced CS-induced B cell proliferation with negligible effects on LPS- and CpG-DNA-induced proliferation (Fig. 2C). The involvement of Syk and BTK in CS-induced B cell proliferation points to the involvement of signaling pathways that are closely related to the signaling pathway of the BCR. We therefore analyzed whether Ig-α and Ig-β, which can provide both activating and inhibitory signals to B cells [26], are involved in CS-induced B cell proliferation. Incubation of B cells with antibodies against Ig-α (clone F11-172) or Ig-β (clone HM79b) completely blocked CS-induced B cell proliferation but had only minor effects on B cell proliferation induced with LPS or CpG-DNA (**Fig. 3**). As it was described previously that CD44 is required for CS-induced B cell proliferation [12], we analyzed B cells from CD44$^{-/-}$ mice and used a mAb against CD44 (KM81), which was described to block the interaction of CD44 and hyaluronic acid [16]. As shown in Supplemental Fig. 1, proliferation of purified
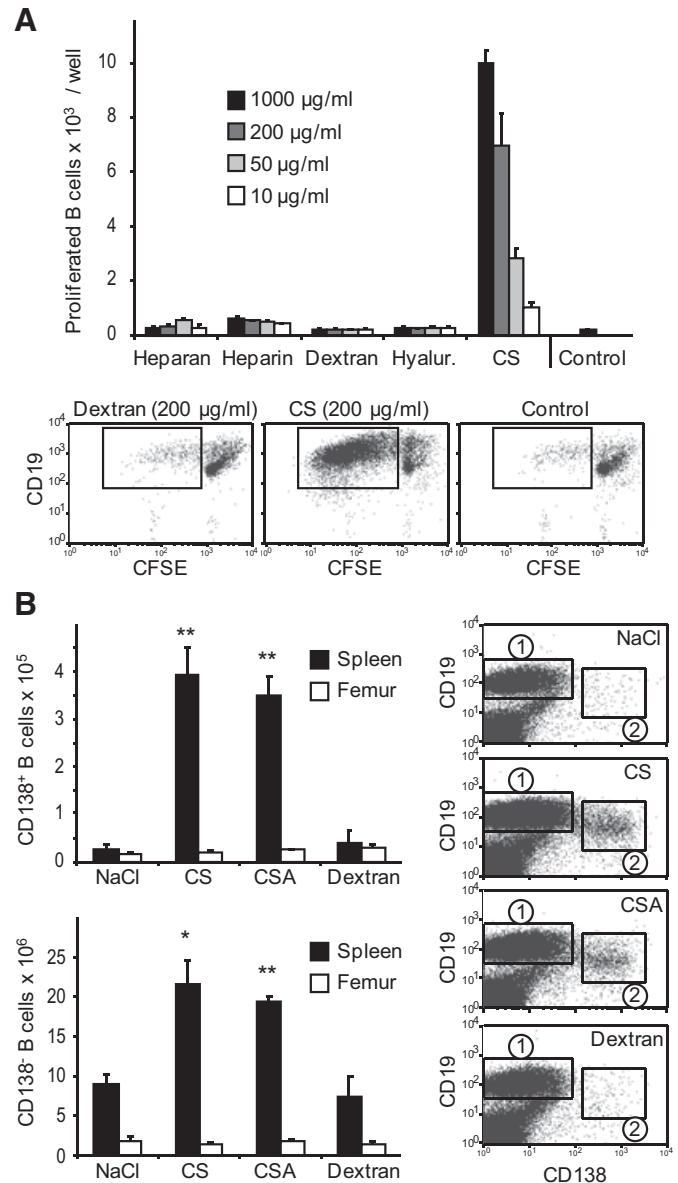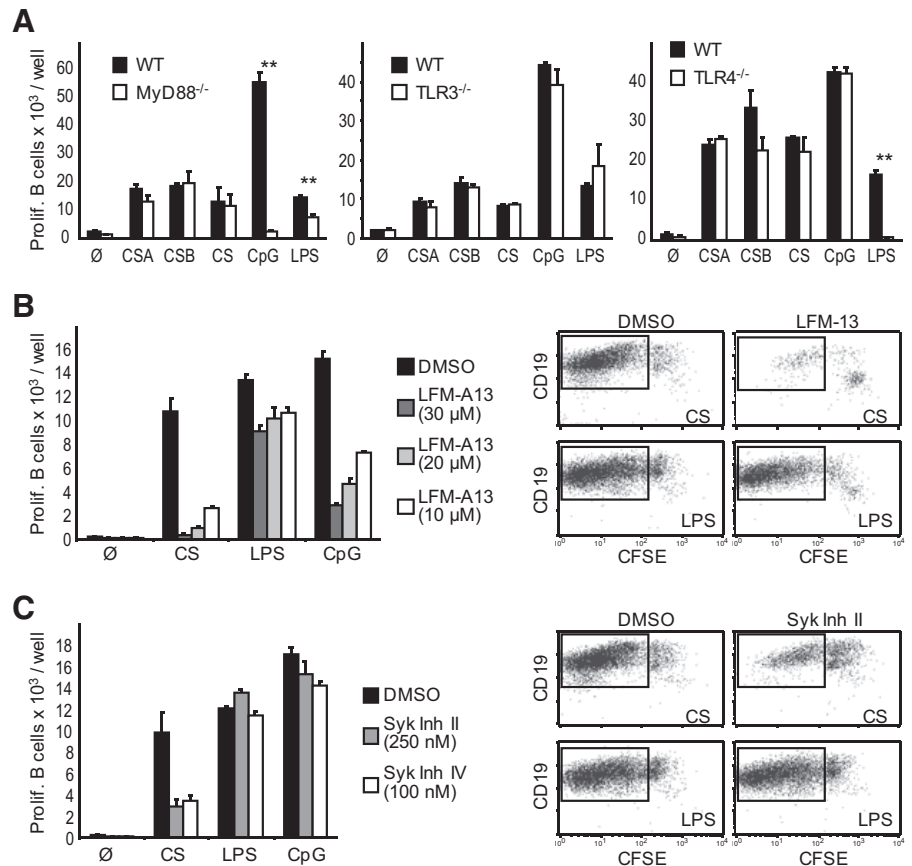

Figure 1. CS induces proliferation of B cells in vitro and expansion of B cells in the spleen. (A) Purified CFSE-stained B cells from C57BL/6 mice (10^5 cells/well) were cultured in triplicates for 5 days with various concentrations of glycosaminoglycans or sodium chloride (NaCl) as control. The number of proliferated (CFSE^{low}) CD19$^+$ B cells (gated cells) was quantified by flow cytometry and is shown in representative FACS plots. Hyalur., Hyaluronic acid. (B) BALB/c mice ($n=3$/group) were injected i.p. for 4 consecutive days with 10 mg CS, CSA, dextran, or sodium chloride. On Day 5, CD19$^+$ B cells and CD138$^+$ B cells were quantified in the spleen and bone marrow (Femur) by flow cytometry. CD138$^-$ B cells (Gate 1) and CD138$^+$ B cells (Gate 2) are depicted in representative FACS plots.

CD44$^{-/-}$ B cells in response to CS was not reduced. Also, addition of the blocking CD44 antibody KM81 did not reduce CS-induced B cell proliferation.

To demonstrate further the involvement of BTK, we used BTK$^{-/-}$ CBA/CaHN-*Btk^{xid}* mice compared with CBA/CaJ WT

Figure 2. Mechanisms of CS-induced B cell proliferation. (A) Purified CFSE-stained B cells (10^5 cells/well) were cultured in triplicates for 5 days with CSA, CSB, and CS (200 μg/ml each); CpG-DNA (1 nmol/ml); LPS (10 μg/ml); or sodium chloride as control (Ø). B cells were isolated from the spleen of MyD88$^{-/-}$ and WT C57BL/6 mice, from TLR3$^{-/-}$ and WT C57BL/6 mice, or from TLR4$^{-/-}$ and WT BALB/c mice. The number of proliferated (Prolif.; CFSE^{low}) CD19$^+$ B cells was quantified by flow cytometry. (B and C) Purified CFSE-stained B cells from C57BL/6 mice (10^5 cells/well) were cultured in triplicates for 5 days with CS, CpG-DNA (1 nmol/ml), LPS (10 μg/ml), or sodium chloride as control (Ø). Inhibitors of the tyrosine kinases BTK (LFM-A13) or Syk (Syk Inh II and Syk Inh IV), dissolved in DMSO or DMSO alone, were added at various concentrations. The number of proliferated (Prolif.; CFSE^{low}) CD19$^+$ B cells was quantified by flow cytometry. Representative FACS plots are depicted with proliferated B cells in the gate.

controls [27]. B cells from WT controls responded toward stimulation with CS, LPS, and CpG-DNA, whereas BTK$^{-/-}$ B cells only responded to LPS and CpG-DNA but not to CS (**Fig. 4A**). The same was observed in vivo, when WT and BTK$^{-/-}$ mice were injected for 4 consecutive days with CS (10 mg/injection i.p.). The number of CD138$^-$ and CD138$^+$ B cells increased significantly in the spleen of WT but not BTK$^{-/-}$ mice after injection of CS. No significant changes were observed in the bone marrow (Fig. 4B). In addition, CD44 was not required for CS-mediated expansion of CD138$^+$ cells in the spleen, as shown with CD44$^{-/-}$ mice (Supplemental Fig. 2).

We also analyzed whether the expansion of splenic B cells by CS is dependent on CD4$^+$ T cells (**Fig. 5**). CD4$^+$ T cells were completely depleted in C57BL/6 mice by injection of 300 μg of the anti-CD4 antibody (GK1.5) for 3 consecutive days. Control mice were treated with the same amount of an isotype control antibody. One day after the last injection of the antibodies, mice were i.p.-injected daily for 5 days with 10 mg CS or sodium chloride. One day later, the numbers of CD19$^+$ B cells, CD4$^+$ T cells (CD3$^+$ CD8$^-$), CD11b$^+$ monocytes, and B cells were quantified in the spleen by flow cytometry. Depletion of CD4$^+$ T cells did not prevent the expansion of CD138$^-$ or CD138$^+$ B cells in the spleen. Taken together, these data show that CS directly induces proliferation of B cells and increases the numbers of CD138$^+$ B cells in the spleen in a BTK-dependent and CD44- and T cell-independent manner.

Inhibition of humoral immune responses by CS

As shown above, treatment of mice with CS for 4 days markedly increased the number of CD138$^+$ cells in the spleen but not in the bone marrow. However, CD138$^+$ cells may also migrate into the bone marrow at later time-points. We therefore treated naive C57BL/6 mice with CS for 14 days and quantified B cells and CD138$^+$ cells at Day 15. As shown in Supplemental Fig. 3, long-term treatment with CS expanded the CD138$^+$ cells, not only in the spleen but also in the bone marrow. B cell numbers were increased in the spleen and peripheral blood but not in the bone marrow.

To analyze whether treatment with CS affects antigen-specific humoral immune responses, we analyzed the effects of CS on the immune response against the fluorescent protein APC. C57BL/6 mice were immunized twice at a 4-week interval with 10 μg APC precipitated in alum. From Day 21 to 34, after the second immunization, mice were treated with daily i.p. injections of 10 mg CS ($n=10$) or sodium chloride ($n=10$). Blood was drawn 1 day before CS treatment (Day 20) and on Days 29 and 35. On Day 20, both groups had the same level of APC-specific IgG1. In control mice, this level did not change until Day 35. However, in CS-treated mice, the level of APC-specific IgG1 markedly decreased over time and was significantly lower on Days 29 and 35 (**Fig. 6A**). Immunization with APC enabled us to quantify the numbers of total and APC-specific plasma cells in the spleen and bone marrow by flow cytometry [17,
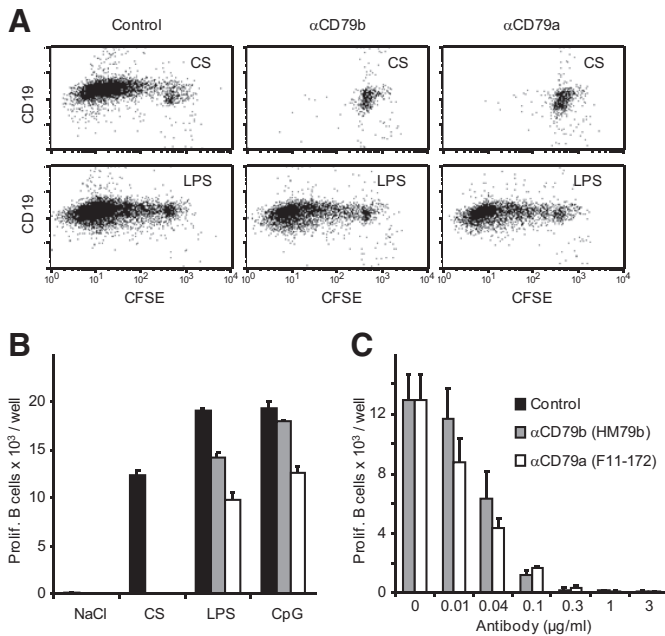
Figure 3. CS-induced proliferation of B cells is blocked completely by antibodies against Ig-α or Ig-β. Purified CFSE-stained B cells (10^5 cells/well) from C57BL/6 mice were cultured in triplicates for 5 days with sodium chloride as control, CS (200 μg/ml), LPS (10 μg/ml), or CpG-DNA (1 nmol/ml). As indicated, antibodies against Ig-α (HM79b) or Ig-β (F11-172) were present throughout the culture. The number of proliferated B cells was measured by flow cytometry. (A and B) Representative FACS plots and statistical analysis of B cell proliferation in the presence of 3 μg/ml control IgG, anti-Ig-α (clone HM79b), or anti-Ig-β (clone F11-172). (C) B cell proliferation in the presence of various concentrations of antibodies against Ig-α (HM79b) or Ig-β (F11-172).

18]. Treatment with CS markedly increased the number of $CD138^+$ cells in the spleen but did not alter the number of $CD138^+$ cells in the bone marrow (Fig. 6B). However, consistent with the reduction in APC-specific IgG1, APC-specific plasma cells were reduced by ~50% in the bone marrow. In the spleen, only very few APC-specific plasma cells were found and remained unchanged after treatment with CS (Fig. 6B). These data indicate that treatment with CS is able to suppress a fully established antigen-specific humoral immune response.

In addition, we analyzed in DBA/1 mice, whether treatment with CS or dextran is able to suppress the humoral immune response against collagen. Mice were immunized on Day 0 by a single intracutaneous injection of 125 μg bovine collagen type II in CFA. Beginning at 21 days after immunization, mice were treated until Day 35 with daily i.p. injections of 10 mg CS or sodium chloride as a control. On Day 36, we quantified the plasma levels of collagen-specific and total Igs in both groups. Collagen-specific IgG1 and IgG2a were reduced significantly in CS-treated mice by ~50%, whereas total IgG1 and IgG2a levels remained unchanged (**Fig. 7A**). To exclude that CS interferes with detection of collagen-specific Igs by ELISA, we added CS (10 or 100 μg/ml) to the plasma of mice immunized with collagen or nonimmunized controls and measured collagen-spe-

cific IgG1 by ELISA (data not shown). By flow cytometry, we quantified the number of B cells and $CD138^+$ B cells in the spleen and bone marrow (Fig. 7B). As observed in other strains of mice, we detected an almost fivefold increase in the number of $CD138^+$ B cells in the spleen of CS-treated mice. In the bone marrow, there were no significant differences (Fig. 6B). Consistent with published data [28–31], treatment with CS reduced development of arthritis, as demonstrated by clinical and histological analysis of the paws and quantification of synovial cytokines and paw-infiltrating leukocytes (Supplemental Fig. 4).

We also treated mice immediately after collagen immunization with CS or dextran. Mice were immunized with collagen on Day 0 and treated from Day 0 to Day 21 by daily i.p. injection of 10 mg CS or dextran (Fig. 7C). The humoral immune response against collagen was quantified on Day 22. Also, in a primary immune response, treatment with CS significantly reduced collagen-specific IgG1 and IgG2a plasma levels, whereas treatment with dextran had no effect (Fig. 7C).
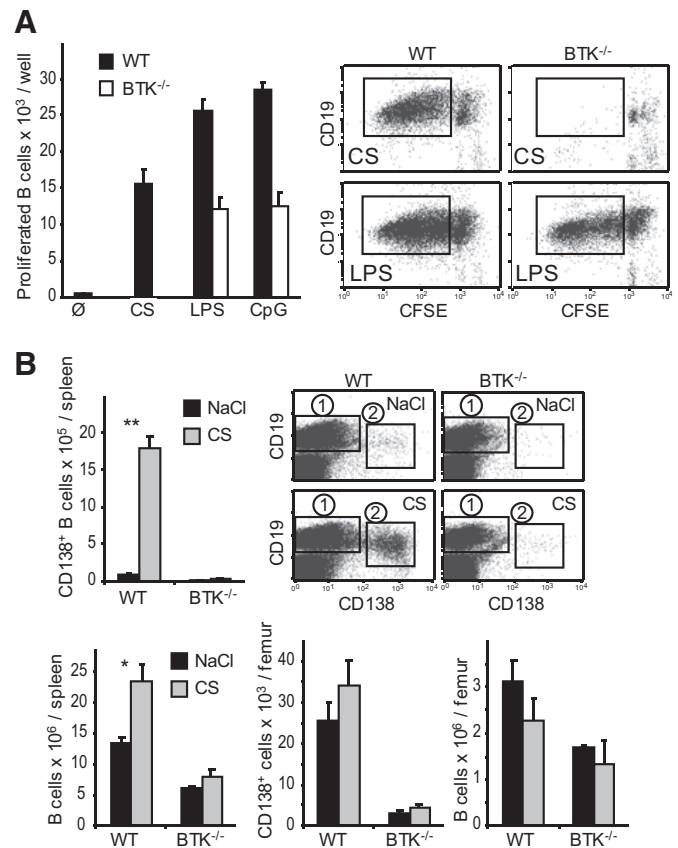

Figure 4. Analysis of $BTK^{-/-}$ mice. (A) Purified CFSE-stained B cells (10^5 cells/well) from WT CBA/CaJ or $BTK^{-/-}$ CBA/CaHN-*Btk^{xid}* were cultured in triplicates for 5 days with sodium chloride as control (Ø), CS (200 μg/ml), LPS (10 μg/ml), or CpG-DNA (1 nmol/ml). The number of proliferated B cells was measured by flow cytometry. B cell proliferation (gate) is shown in representative FACS plots. (B) WT or $BTK^{-/-}$ mice ($n=3$/group) were injected i.p. for 4 consecutive days with 10 mg CS or sodium chloride. On Day 5, $CD19^+$ B cells and $CD138^+$ B cells were quantified in the spleen and bone marrow (femur) by flow cytometry. Representative FACS plots of splenic B cells (Gate 1) and $CD138^+$ B cells (Gate 2) are shown.
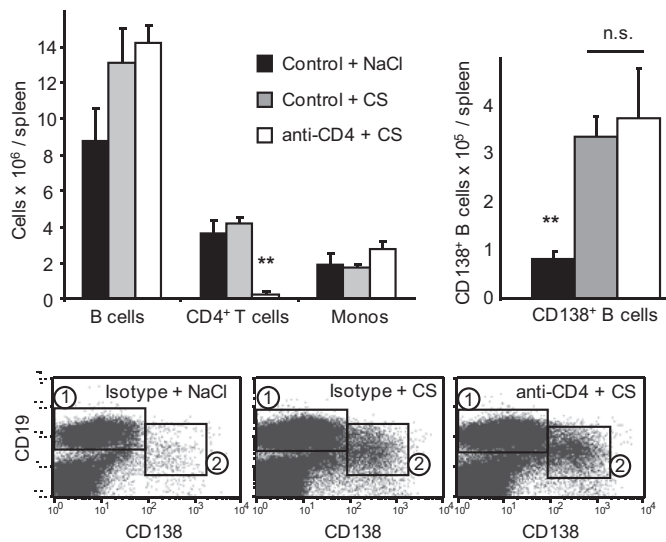
Figure 5. CD4$^+$ T cells are not required for CS-induced expansion of splenic B cells. C57BL/6 mice (n=3/group) were treated for 3 days by daily injection of 300 μg anti-CD4 antibody (GK1.5) or isotype control antibody (Control) to deplete CD4$^+$ T cells. Subsequently, mice were i.p.-injected daily for 5 days with 10 mg CS or sodium chloride. One day later, the numbers of CD19$^+$ B cells, CD3$^+$ CD8$^-$ T cells (CD4$^+$ T cells), CD11b$^+$ monocytes, and CD138$^+$ B cells were quantified in the spleen by flow cytometry. Representative FACS plots show the CD4$^+$ T cell-independent expansion of CD19$^+$ B cells (Gate 1) and CD138$^+$ B cells (Gate 2) in the spleen.

DISCUSSION

Negatively charged glycosaminoglycans have been implicated in several pathways of an immune response. They interact with a variety of soluble and membrane-bound molecules that play important roles during autoimmunity and inflammation (e.g., cytokines, chemokines, and growth factors). Although CS has been described as an anti-inflammatory agent [4, 32, 33], its effects on B cells have only been studied in vitro, and little is known about the interaction of CS with antigen-specific humoral immune responses. We therefore evaluated more closely the effects of CS on B cell responses in vitro and in vivo and found that CSA and CSC, as well as CSB (dermatan sulfate) but not other glycosaminoglycans, such as heparan, heparin, dextran, and hyaluronic acid, induce a profound proliferation of purified B cells in culture. Cultured B cells were highly purified, excluding an indirect effect of CS on B cells, e.g., via T cells. A previous report described B cell proliferation with CSB and dextran but only marginal effects with CSA and CSC [13].

We analyzed which signal-transducing molecules are involved in the stimulatory effects of CS on B cells. With the use of various KO mice, we demonstrate that TLRs or CD44 are not involved, whereas inhibitors of the tyrosine kinases BTK and Syk markedly reduced CS-induced proliferation of B cells. The effects of LPS and CpG-DNA were not, or to a much lower degree, influenced by inhibition of BTK and Syk. B cells from BTK$^{-/-}$ mice did not proliferate after ex-

posure to CS but responded well to LPS and CpG. In a previous report, B cell proliferation by dermatan sulfate was found to be dependent on PKC and PI3K [13], signal transducing molecules that are known to be downstream of BTK and Syk. We also analyzed signal-transducing molecules that are upstream of BTK and Syk, i.e., Ig-α and Ig-β. Both molecules can provide activating and inhibitory signals to B cells [26]. Antibodies against Ig-α or Ig-β completely abrogated CS-induced B cell proliferation but had much less effects on B cell proliferation induced by LPS or CpG-DNA. These data indicate that the action of CS is closely related to the activation of B cells via the BCR.

Our in vitro data suggest that CS induces a direct and antigen- and T cell-independent proliferation of B cells. We therefore in-
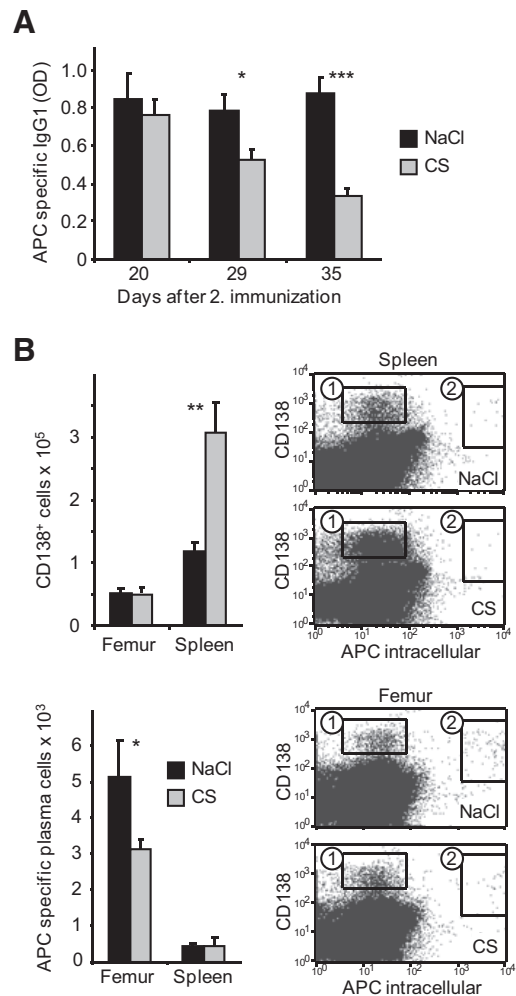

Figure 6. CS interferes with an established immune response against APC. C57BL/6 mice were immunized twice with 10 μg APC in alum at a 4 weeks interval. From Day 21 until Day 34, after the second (2.) immunization, mice were treated with daily i.p. injections of 10 mg CS (n=10) or sodium chloride (n=10). (A) APC-specific IgG1 antibodies were quantified by ELISA immediately before CS treatment (Day 20) and on Days 29 and 35. (B) CD138$^+$ and APC-specific plasma cells were analyzed by flow cytometry in the spleen and bone marrow (Femur) on Day 35. Representative FACS plots show CD19$^+$ B cells (Gate 1) and CD138$^+$ cells (Gate 2) in the spleen and bone marrow.
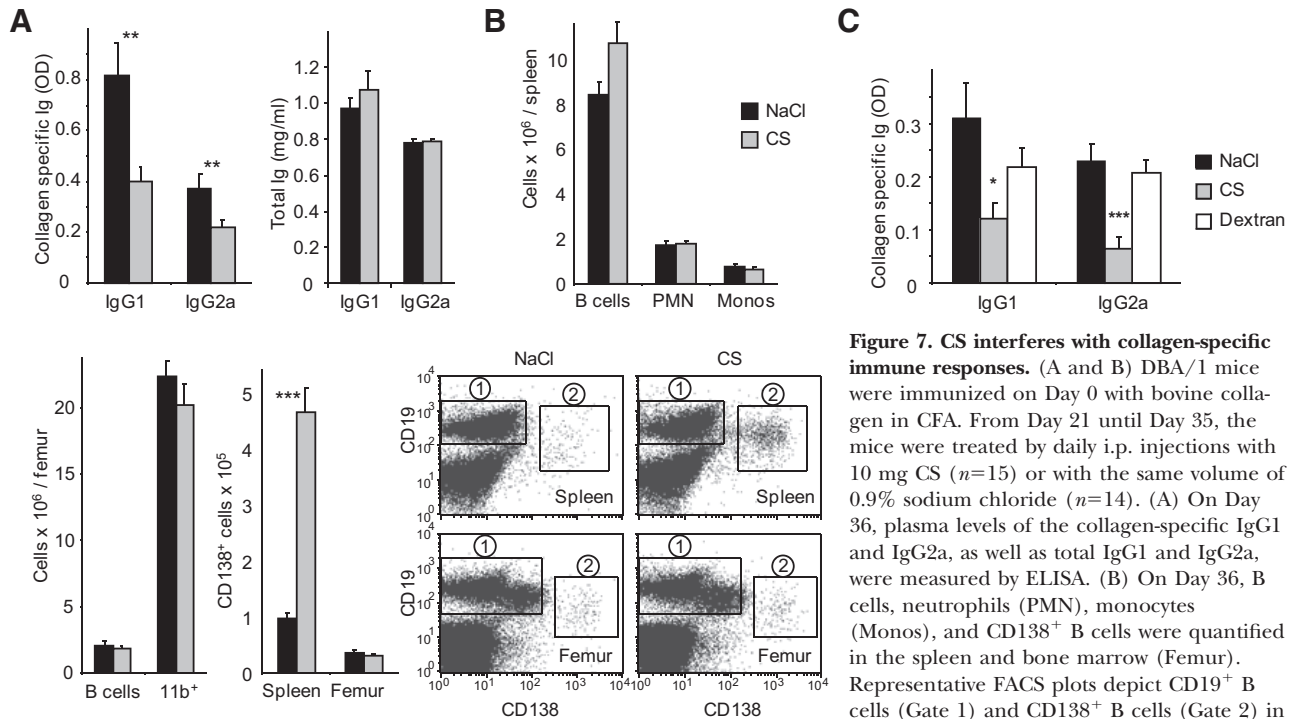
Figure 7. CS interferes with collagen-specific immune responses. (A and B) DBA/1 mice were immunized on Day 0 with bovine collagen in CFA. From Day 21 until Day 35, the mice were treated by daily i.p. injections with 10 mg CS ($n=15$) or with the same volume of 0.9% sodium chloride ($n=14$). (A) On Day 36, plasma levels of the collagen-specific IgG1 and IgG2a, as well as total IgG1 and IgG2a, were measured by ELISA. (B) On Day 36, B cells, neutrophils (PMN), monocytes (Monos), and CD138$^+$ B cells were quantified in the spleen and bone marrow (Femur). Representative FACS plots depict CD19$^+$ B cells (Gate 1) and CD138$^+$ B cells (Gate 2) in the spleen and bone marrow. (C) In a separate experiment, DBA/1 mice were immunized on Day 0 with collagen and treated daily from Day 0 until Day 21, with 10 mg CS ($n=11$), 10 mg dextran ($n=11$), or 0.9% sodium chloride ($n=11$). On Day 22, the collagen-specific IgG1 and IgG2a antibodies were measured in the plasma by ELISA and were only reduced in CS-treated mice.

vestigated the effects CS on a humoral immune response in vivo. CS could act as a costimulatory molecule and enhance antigen-specific immune responses, or, e.g., by expansion of nonantigen-specific B cells and CD138$^+$ plasma cells, it could down-regulate humoral immune responses in vivo. Treatment of naive mice with CS significantly expanded the number of B cells in the spleen and also resulted in a pronounced increase in the number of CD138$^+$ B cells in the spleen, which was completely dependent on BTK and independent on CD4$^+$ T cells. Short-term treatment of mice with CS (4 days) increased CD138$^+$ cells only in the spleen but not in the bone marrow. In contrast, long-term treatment with CS also increased CD138$^+$ cells in the bone marrow from 16,000–29,000/femur.

To investigate the effects of CS on antigen-specific immune response, we immunized mice with protein antigens (e.g., APC or collagen) and treated the mice with CS during the primary immune response (from Day 0 until Day 21), after the primary immune response (from Day 21 until Day 35) or after the secondary immune response (from Day 21 until Day 34 after the second immunization). In all of these settings, treatment with CS significantly reduced the plasma levels of antigen-specific Igs. In addition, antigen-specific plasma cells in the bone marrow but not in the spleen were reduced significantly by CS. These data show that CS is able to interfere with primary and also fully established humoral immune responses. Immunized mice have higher numbers of CD138$^+$ cells/femur (40,000–50,000) than naive mice (16,000). Long-term treatment of immunized mice with CS did not increase the total number of CD138$^+$ cells but reduced the number of antigen-specific CD138$^+$ plasma cells. As CS-induced CD138$^+$ cells migrate into the bone marrow (see

Supplemental Fig. 3), these cells most likely displace other CD138$^+$ cells and thereby, reduce the frequency of antigen-specific plasma cells in the bone marrow. It is unlikely that CS interferes with chemokines directing plasma cells to migrate into the bone marrow, as treatment with CS was started only 21 days after the second immunization with APC. At this time-point, antigen-specific plasma cells have already migrated into the bone marrow. Consistent with our in vitro data, dextran was unable to expand the number of total or CD138$^+$ B cells in vivo or to suppress antigen-specific immune responses.

CS has been studied in models of arthritis with somewhat controversial results. Repeated i.d. injections of CSA and CSC into BALB/c mice resulted in induction of arthritis with swelling, edema, and erythema of the paws [34, 35]. In contrast, other studies showed a beneficial effect of CS for collagen-induced arthritis in mice [28–31] and rats [36, 37], as well as adjuvant or antigen-induced arthritis in rats [38–40]. Consistent with the later data, we found that prophylactic treatment with CS (from Day 0 until Day 21; data not shown) or therapeutic treatment (from Day 21 until Day 35) significantly reduced clinical and histological signs of arthritis and reduced the expression of cytokines in the paws and the numbers of infiltrating cells.

Taken together, our data show that CS has a direct BTK- and Syk-dependent stimulatory effect on B cells and markedly expands CD138$^+$ cells in the spleen. After prolonged treatment, some of these cells migrate into the bone marrow. Long-term treatment with CS reduces antigen-specific plasma cells in the bone marrow and antigen-specific IgG in the plasma, most likely by displacement antigen-specific plasma cells from bone marrow niches. CS is able to interfere with

primary and fully established humoral immune responses against specific antigens and has a beneficial effect on the development of collagen-induced arthritis.

AUTHORSHIP

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DISCLOSURES

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