Functional Analysis of the Chondroitin 6-Sulfotransferase Gene in Relation to Lymphocyte Subpopulations, Brain Development, and Oversulfated Chondroitin Sulfates

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Chondroitin 6-sulfotransferase (C6ST) catalyzes the transfer of sulfate to position 6 of the N-acetylgalactosamine residue of chondroitin. To obtain direct evidence regarding the function of C6ST and its product, chondroitin 6-sulfate, in vivo, we isolated the mouse C6ST gene (C6st) and generated mice deficient in this gene (C6st−/−) by embryonic stem cell technology. C6st−/− mice were born at approximately the expected frequency and were viable through adulthood. In the spleen of C6st−/− mice, the level of chondroitin 6-sulfate became almost undetectable. Analyses of these knockout mice provided insights into the biosynthesis of oversulfated chondroitin sulfates in mice; chondroitin sulfate D in the brain of null mice and the cartilage and telencephalon of null embryos disappeared, whereas the chondroitin sulfate E level in the spleen and brain of the null mice was unchanged. Despite the disappearance of chondroitin sulfate D structure, brain development was normal in the C6st−/− mice. Further analysis revealed that the number of CD62L+CD44low T lymphocytes corresponding to naive T lymphocytes in the spleen of 5–6-week-old C6st−/− mice was significantly decreased, whereas those in other secondary lymphoid organs were unchanged. This finding suggested that chondroitin 6-sulfate plays a role in the maintenance of naive T lymphocytes in the spleen of young mice.

Chondroitin sulfate is a family of glycosaminoglycans (GAGs) consisting of glucuronic acid (GlcA), N-acetylgalactosamine (GalNAc), and sulfate. The building block is a repeating disaccharide unit with GlcAβ1–3GalNAc, and its GalNAc residue is usually monosulfated. When sulfation occurs mainly at C-6 of the internal GalNAc residue, it is called chondroitin 6-sulfate, whereas the C-4 sulfated form is called chondroitin 4-sulfate. A variant of chondroitin 4-sulfate containing some iduronic acid residues instead of GlcA is known as dermatan sulfate, and its disaccharide unit is designated as the chondroitin sulfate B unit. Chondroitin sulfate D and E have oversulfated structures, GlcA(2S)β1–3GalNAc(6S) and GlcAβ1–3GalNAc(4S,6S), respectively (1).

The structural diversity including various pattern of sulfate group attachment in GAGs suggests the pathological and biological importance of proteoglycan molecules (2, 3). Chondroitin sulfate proteoglycans are the main components in the cartilage, and their sulfation profiles vary in relation to aging (4, 5). Chondroitin sulfates in chondroitin sulfate proteoglycans have been implicated in some aspects of neuronal functions such as modulation of neurite outgrowth (6–8) and axonal regeneration (9, 10). Chondroitin sulfate chains also play critical roles in lymphocyte-endothelial cell interactions (11) and enhancing stimulation of T cell responses (12). Furthermore, chondroitin sulfate proteoglycans bearing chondroitin sulfate D or E bind to a growth factor, midkine (13–16), and chemokines (17), and have been suggested to participate in signaling or modulation of the activities of these factors. However, the in vivo function of chondroitin sulfate has not been clarified directly.

Sulfate groups attached to GAG chains are transferred from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) by sulfotransferases with strict acceptor specificities (2). Chondroitin 6-sulfotransferase (C6ST) catalyzes the last step of synthesis of chondroitin 6-sulfate, and transfers sulfate to the C-6 position of the GalNAc residue of chondroitin. Recent studies have shown that C6ST can sulfate the Gal residues of keratan sulfate (18) as well as siylated lactosamine oligosaccharides (19). C6ST cDNAs have been cloned in the chicken (20) and subsequently in the mouse (21) and human (22). To determine the roles of C6ST and its product, chondroitin 6-sulfate, in vivo, we...
utilized gene knockout technology deleting the mouse C6ST gene, C6st. In the present study, we have shown that the number of naive T lymphocytes in the spleen of C6st−/− mice was significantly decreased.

**EXPERIMENTAL PROCEDURES**

**Molecular Cloning of the C6st Gene and Construction of Targeting Vector—**To obtain mouse C6ST genomic DNA, a 128SV/J mouse genomic library (Stratagene) was screened using mouse C6ST cDNA (m1; Ref. 21) as a probe. Plaque hybridization was carried out as described (21). Two positive clones (termed 8-6 and 3-1), containing a 15-kb DNA fragment that included the C6st coding exons, were isolated (Fig. 1A). After sequencing the insert DNA fragments, the localization of the exons was determined by comparison of the sequences between the inserted genomic DNA and several cDNAs obtained by 5′-rapid amplification of cDNA ends PCR as described previously (21) and Southern blot analysis using the cDNA as a probe (21). The C6st targeting vector was constructed from a basic targeting vector with MClneo (polyoma virus thymidine kinase gene promoter and neomycin resistance gene) and DTA (diphtheria toxin fragment A gene) (23) and C6st fragments. To delete a 1.2-kb portion of C6st exon II (KpnI-SmaI sites in Fig. 1B), a 4.7-kb SacII/KpnI fragment and 2.3-kb SmaI fragment were used as the 5′-arm and the 3′-arm, respectively (Fig. 1B). Generation of Targeted ES Cells and Mice—Aliquots of 15 μg of NolI-linearized targeting vector DNA were electroporated into 1 × 107 D3 ES cells. The cells were plated on mitomycin C-treated G418-resistant SL-10 cell feeder layers. G418 (250 μg/ml; Sigma) was added 24 h after plating. G418-resistant colonies were picked up after 7–8 days and then propagated to be stored and examined for homologous recombination by Southern blot analysis as described below. Approximately 15 ES cells of the targeted clones were injected into blastocyst-derived females from naturally mated C57BL/6J females. The injected embryos were transferred to the uteri of pseudopregnant ICR mice. To yield null mutants of 128SV/J background, chimeric male mice were mated with 128SV/J mice. Then, F1 progeny were back-crossed four times to 129SV/J mice and mated with each other.

**Southern and Northern Blot Analyses—**Southern blot analysis was performed as described previously (21) for DNA samples digested with EcoRI. The membrane was hybridized with a 0.7-kb HincII-NcoI fragment corresponding to the genomic DNA downstream of the 3′-arm of the targeting vector (Fig. 1B). The homologously recombined DNA gave a 7.8-kb band, whereas the wild-type DNA gave a 14.2-kb band (Fig. 1C). The DNA prepared from the 3-1 (Fig. 1C) and transferred onto a nylon membrane, and then hybridized with the radioactive probes as described previously (21). Hybridization was performed using a 400-bp DNA fragment corresponding to part of the C6st gene (PCR product described below) as a probe. PCR—Aliquots of 0.5 μg of DNA were mixed with 20 μl of AmpliTaq® buffer containing 0.2 mM each dNTP, 1.5 mM MgCl2, 10 pmol of each primer, and 0.5 unit of AmpliTaq® DNA polymerase (Applied Biosystems). PCR amplification was carried out at 94 °C for 3 min, with 35 cycles of 94 °C for 0.5 min, 56 °C for 0.5 min, and 72 °C for 1 min. To screen for homologously recombined DNA, C6st primers were used: 5′-ATGATCCTCTCTTGTGTCCTGCA-3′ (6ST-F) and 5′-CCATAC-GAGGCTGCGATACAA-3′ (6ST-R). The wild-type allele gave a 400-bp band, whereas the mutated allele gave no band. Neo primers were also used: 5′-CAGGCTGTTGTGATCCGGCA-3′ (Neo-F) and 5′-GCTTTCGTCCGATACCC-3′ (Neo-R). The wild-type allele gave no band, whereas the mutated allele gave a 600-bp band.

**Extraction of Chondroitin/Dermatan Sulfates from Mouse Tissues—**Mouse spleens (1 g) and brains (4.5 g) were removed from 8 mice (5-6-week-old males) and 20 mice (10-week-old males), respectively. Tissues were homogenized in 10 ml of ice-cold 10 mM Tris-HCl, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride for 3 min. The homogenate was mixed with 3 ml of 10 mM Tris-HCl, pH 7.5, containing 0.5 mM sucrose, 0.1 mM KCl, 10 mM MgCl2, and 2 mM CaCl2, and then centrifuged at 8,000 × g for 10 min. The pellet was suspended in 10 ml of 10 mM Tris-HCl, pH 7.5, containing 0.1 mM NaCl, 1 mM EDTA, 8 mM CHAPS, and 2.5 mM of protease K, and then incubated at 50 °C for 2 h. After further incubation with 2.5 mg of proteinase K at 50 °C for 12 h, 50 μg of RNAse A (Sigma) and 50 μg of DNase I (Takara, Tokyo) were added, and then the reaction mixture was stored at 37 °C for 1 h. The extract was centrifuged at 10,000 × g for 10 min. The supernatant was applied to a column containing 4 ml of DEAE-Sephadex following by adjusting its concentration of NaCl to 0.5 M. The column was washed with five volumes of 10 mM Tris-HCl, pH 7.5, containing 0.5 mM NaCl and 8 mM CHAPS. Chondroitin/dermatan sulfate fractions were then eluted with two column volumes of 10 mM Tris-HCl, pH 7.5, containing 1.5 mM NaCl and 8 mM CHAPS with monitoring at 210 nm. The collected fractions were dialyzed against distilled water.

**Disaccharide Analysis on HPLC—**Aliquots of 10 μg of chondroitin/dermatan sulfates extracted from the mouse spleen and brain were digested with chondroitinase ABC as described above. Samples before and after digestion were analyzed by high-performance liquid chromatography (HPLC) (YMCpack ODS-AM, 4.6 cm × 25 cm, Whatman) as described previously (20).

**Flow Cytometric Analysis—**After mincing the spleen, thymus, peripheral lymph nodes, mesenteric lymph nodes, and Peyer’s patches in Hanks’ balanced salt solution, single-cell suspensions were prepared in the same ice-cold solution. Cells were incubated at 4 °C for 15 min in 10 μg/ml Fc blocker (anti-CD32/16, 2.4G2; PharMingen) before staining with various antibodies. Cells were stained for 30 min at 4 °C in Hanks’ balanced salt solution containing 400-fold diluted antibody, and then washed with the same buffer. FACScanLibr flow cytometer and CellQuest soft ware (Becton Dickinson) were used to analyze the stained cells. The antibodies against the following antigens were from Pharmingen: CD3 (OKT3), CD4 (RM4-5), CD8 (53-1.7), CD43 (S7), CD44 (IM7), CD62L (MEL-14), mouse IgM (R6–60.2), mouse IgG (11–26c2a), Ly-6G (Gr-1), B220 (RA3–6B2), TER-119 (Ly-76), and IA-Aβ (Ab-120.1). Immunohistochemistry and Staining—Mouse tissues were frozen in Tissue-Tek (Sakura Finetek). Cryostat sections (5 μm thick) were fixed in acetone and reacted with Biocytin diacetate (CMFDA; Molecular Probes). After washing with Hanks’ balanced salt solution, sections were prepared as described above. Biotin-conjugated secondary antibody was used instead of secondary antibody. The antibodies used were from Polyclonal: CD8 (53–1.7), CD4 (RM4–5), CD3 (M1/69), CD11c (HL3), CD21 (7G6), CD22 (Cy34.1), CD23 (B3B4), CD24 (MH-1), and anti-keratan sulfate (5D4), all from Seikagaku Kogyo. Subsequently, spleen sections were counterstained by methyl green solution. For Nissl’s staining, brains were embedded in paraffin and serially sectioned in the coronal plane.

**Lymphocyte Trafficking—**Lymphocyte trafficking in vivo was carried out according to the published procedures with slight modifications (25, 26). Mesenteric and axillary lymphocytes from 5–6-week-old C6st−/− mice were isolated and then labeled with 5 μM 5-chloromethylfluorescein diacetate (CFDA; Molecular Probes). After washing with Hanks’ balanced salt solution, the cells (2.5 × 107 in 250 μl of the washing solution) were injected into the tail veins of age-matched C6st+/+ and C6st−/− mice. After 1.5 h, the animals were sacrificed and secondary lymphoid organs were removed. CFDA-positive lymphocytes in the organs were analyzed by flow cytometry. For detection of the trafficked cells in the spleen, the organ was immediately frozen and then cryostat sections were prepared as described above. Biotin-conjugated anti-CD3 antibody and PE-conjugated streptavidin (PharMingen) were used to detect the T cell zones.

**RESULTS**

**Genomic Organization of the Mouse C6st Gene—**Two overlapping clones covering the mouse C6st gene were isolated by screening a 128SV/J mouse genomic library (Stratagene) using mouse C6ST cDNA as a probe. Both clones, termed clone 8-6 and 3-1 (Fig. 1A), contained the whole protein-coding region of
The genomic organization and targeting strategy of the mouse C6st locus. The protein-coding region of the C6st is distributed in two exons (A). C6st transcripts are thought to be produced by alternative usage of multiple exons 1 encoding the 5'-UTR of C6st mRNA. Boxes denote exons. Solid and open boxes represent protein-coding and untranslated regions, respectively. The top shows isolated genomic clones, 8-6 and 3-1. The bottom shows the putative splicing patterns for each form of C6st mRNA and transcription initiation sites (arrows) (A). The targeting construct was designed to replace the exon encoding the C6ST catalytic domain with the neomycin resistance gene. Restriction enzyme sites indicated are: E, EcoRI; S, SacI; K, KpnI; Sm, SmaI; H, HincII; N, NcoI. Boxes denote exons (B). In homozygous mice, C6st−/−, Southern blot analysis confirmed homologous recombination (C). No expression of the mRNA was detected by Northern blot analysis. The bottom panel shows ribosomal RNAs, indicating the equal loading of the RNA samples (D). E and F, C6ST (E) and C4ST (F) activities in the organs of C6st−/− and C6st+/− mice. C6ST activities in various tissues of C6st−/− mice were less than 10 cpm/µg of protein/h (ND, not detected). PLN, peripheral lymph node; MLN, mesenteric lymph node; PP, Peyer’s patch.
the C6ST cDNA, and the region appeared to be encoded by two exons divided by a 1.2-kb intron (Fig. 1 A and F). Exon II contained the C6ST cDNA, and the region appeared to be encoded by two exons divided by a 1.2-kb intron (Fig. 1 A). Exon II contained the C6ST cDNA, and the region appeared to be encoded by two exons divided by a 1.2-kb intron (Fig. 1 A). Exon II contained the C6ST cDNA, and the region appeared to be encoded by two exons divided by a 1.2-kb intron (Fig. 1 A). Exon II contained the C6ST cDNA, and the region appeared to be encoded by two exons divided by a 1.2-kb intron (Fig. 1 A). Exon II contained the C6ST cDNA, and the region appeared to be encoded by two exons divided by a 1.2-kb intron (Fig. 1 A). Exon II contained the C6ST cDNA, and the region appeared to be encoded by two exons divided by a 1.2-kb intron (Fig. 1 A). Exon II contained the C6ST cDNA, and the region appeared to be encoded by two exons divided by a 1.2-kb intron (Fig. 1 A). Exon II contained the C6ST cDNA, and the region appeared to be encoded by two exons divided by a 1.2-kb intron (Fig. 1 A).

**Gene Targeting of Mouse C6st Locus**—We designed a targeting construct to delete part of the catalytic region in exon III. The deletion resulted in loss of the whole 5'-PAPS binding domain and a portion of the 3'-PAPS binding domain (28), so that any resulting translated enzyme would not have the ability to catalyze sulfation. By standard gene knockout technology, mice with deletion C6st (C6st<sup>−/−</sup>) were produced. By mating of C6st<sup>+/−</sup> mice, C6st<sup>−/−</sup> mice were born in the Mendelian ratio. There were no apparent differences between wild-type and the knockout mice in their gross morphology or results of histological examination. No significant differences were observed in body weight of C6st<sup>−/−</sup> mice as compared with C6st<sup>+/−</sup> mice 1, 2, 4, 6, 8, or 10 weeks after birth. The knockout mice reproduced normally and also survived for 2 years without any abnormalities as compared with wild-type controls. Southern blot analysis confirmed that the C6st gene was deleted in the knockout mice (Fig. 1C), and no C6st mRNA was detected in these mice (Fig. 1D). C6ST activity was not detected in the spleen, lung peripheral lymph nodes, mesenteric lymph nodes, and Peyer’s patches of C6st<sup>−/−</sup> mice (Fig. 1E). Chondroitin 6-sulfotransferase activity in such tissues was also assayed and appeared to be normal in C6st<sup>+/−</sup> mice (Fig. 1F).

**Participation of C6st in Formation of Chondroitin Sulfate Structures**—We chemically analyzed chondroitin/dermatan sulfates extracted from the spleen. After chondroitinase ABC digestion, the major disaccharides released from the sample of C6st<sup>+/−</sup> was ΔDi-4S (Fig. 2A, peak 3). In addition, a small peak appeared at the position of ΔDi-6S (peak 2) and ΔDi-diSe<sub>E</sub>. The presence of ΔDi-diSe<sub>E</sub> in these mouse samples was confirmed by additional chondroitinase ABC digestion. In the brain of over 10-week-old mice, ΔDi-6S (Fig. 2A, peak 3) was also present. Therefore, C6st<sup>−/−</sup> mice did not have any change in the disaccharides of the brain (Fig. 2B). C6st<sup>−/−</sup> mice did not have any change in the disaccharides of the brain (Fig. 2B). C6st<sup>−/−</sup> mice did not have any change in the disaccharides of the brain (Fig. 2B). C6st<sup>−/−</sup> mice did not have any change in the disaccharides of the brain (Fig. 2B). C6st<sup>−/−</sup> mice did not have any change in the disaccharides of the brain (Fig. 2B). C6st<sup>−/−</sup> mice did not have any change in the disaccharides of the brain (Fig. 2B). C6st<sup>−/−</sup> mice did not have any change in the disaccharides of the brain (Fig. 2B). C6st<sup>−/−</sup> mice did not have any change in the disaccharides of the brain (Fig. 2B).

**Function of Chondroitin 6-Sulfotransferase Gene**—We analyzed enzymatic activity in such tissues was also assayed and appeared to be normal in C6st<sup>+/−</sup> mice (Fig. 1F). Therefore, C6st is involved in formation of D unit,
GlcA(2S)β1-3GalNAc(6S), in the adult brain.

Expression of chondroitin sulfate was also examined by immunohistochemical staining. When cryostat sections of the spleen were stained with MC21C antibody, which reacts with chondroitin 6-sulfate, reticular fibers and trabeculae including arteries, respectively. Original magnifications in A and B are ×400, and in C–F are ×100.

**Fig. 3. Structural evaluation of chondroitin/dermatan sulfates and keratan sulfate by immunohistochemical study.** Cryostat sections of the spleen (A and B) and the day 13.5 (C and D) and 15.5 (E and F) embryos were prepared from C6st<sup><sup>−/−</sup></sup> (A, C, and E) and C6st<sup><sup>−/−</sup></sup> (B, D, and F) mice. They were stained with MC21C antibody that detects chondroitin 6-sulfate (A and B) or MO225 antibody that detects chondroitin sulfate D (C and D) or 5D4 antibody that detects keratan sulfate (E and F). Cartilage tissues (C and D) and dorsal thalamus (E and F) are shown. Arrowheads and arrows indicate reticular fibers and trabeculae including arteries, respectively. Original magnifications in A and B are ×400, and in C–F are ×100.

GlcA(2S)β1-3GalNAc(6S), in the adult brain.

Expression of chondroitin sulfate was also examined by immunohistochemical staining. When cryostat sections of the spleen were stained with MC21C antibody, which reacts with chondroitin 6-sulfate, reticular fibers and trabeculae including arteries were positive in the C6st<sup><sup>−/−</sup></sup> mice (Fig. 3A), whereas those from C6st<sup><sup>−/−</sup></sup> mice were negative except in the arteries (Fig. 3B). Cartilage tissues in C6st<sup><sup>−/−</sup></sup> day 13.5 embryos strongly expressed chondroitin sulfate D as determined by staining with MO225 antibody (Fig. 3C). MO225-positive signals completely disappeared in the C6st<sup><sup>−/−</sup></sup> embryos (Fig. 3D). Thus, C6st is also involved in formation of D unit in the embryo cartilage. C6ST also sulfates the C-6 position of Gal residues in keratan sulfate in the embryonic thalamus. C6ST also sulfates the C-6 position of GlcA residues in keratan sulfate, reticular fibers and trabeculae including arteries.

**Fig. 4. Normal organization of the cerebral cortex in the C6st<sup><sup>−/−</sup></sup> brain.** Whole brains of C6st<sup><sup>−/−</sup></sup> (A and C) and C6st<sup><sup>−/−</sup></sup> (B and D) mice were embedded in paraffin. Then, coronal sections were prepared and stained by Nissl’s method. Cerebral cortex (A and B) and pyramidal cells in the cortex (C and D) were stained with MC21C or MO225 antibody that detects chondroitin sulfate D. Neocortical neuropil in the telencephalon are shown (E and F). Original magnifications in A and B are ×400, and in C–F are ×400.

GlcA(2S)β1-3GalNAc(6S), in the adult brain.

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**Brain Development in C6st<sup><sup>−/−</sup></sup> Mice Was Normal—**Because the chondroitin sulfate D motif, which disappeared from the brain of C6st<sup><sup>−/−</sup></sup> mice, was shown to promote neurite outgrowth (7), we compared brain structure between C6st<sup><sup>+/+</sup></sup> and C6st<sup><sup>−/−</sup></sup> mice by histological analysis, and found no differences. In particular, normal organization of the cerebral cortex and orientation of processes for pyramidal cells in C6st<sup><sup>−/−</sup></sup> brain were revealed by Nissl’s staining method (Fig. 4, A–D). Then, we investigated whether the chondroitin sulfate D structure also disappeared from the brain of the null embryos. Using MO225 monoclonal antibody, chondroitin sulfate D structure was detected in the neocortical neuropil in the telencephalon of C6st<sup><sup>+/+</sup></sup> day 15.5 embryos, whereas the signals in that of C6st<sup><sup>−/−</sup></sup> day 15.5 embryos were not observed (Fig. 4, E and F).

**Decrease of Naïve T Lymphocytes in the Spleen of C6st<sup><sup>−/−</sup></sup> Mice—**Although gross morphology of lymphoid organs was not different between C6st<sup><sup>+/+</sup></sup> mice and C6st<sup><sup>−/−</sup></sup> mice, the relatively strong expression of C6st mRNA in the spleen and bone marrow of mice led us to examine leukocyte populations in the spleen. We determined the numbers of free cells from the spleen, peripheral lymph nodes, mesenteric lymph nodes and Peyer’s patches. The number of spleen cells in C6st<sup><sup>−/−</sup></sup> mice was similar to that of C6st<sup><sup>+/+</sup></sup> mice (Table I). Then, we analyzed lymphocyte subpopulations in C6st<sup><sup>−/−</sup></sup> and C6st<sup><sup>−/−</sup></sup> mice. Both the percentage and number of naïve T lymphocytes were significantly decreased in the spleen of C6st<sup><sup>−/−</sup></sup> mice at 5–6 weeks after birth (Fig. 5, Table I). No such difference was observed in other lymphoid organs. The absolute number of naïve T lymphocytes in the spleen of C6st<sup><sup>−/−</sup></sup> mice was 68% of that in C6st<sup><sup>+/+</sup></sup> mice 5–6 weeks after birth. This result was reproducible in three independent sets of experiments. Neither chondroitin 6-sulfate nor chondroitin sulfate D was expressed on the surface of naïve T lymphocytes as revealed by FACS using MC21C or MO225 antibody (data not shown). Thus, chondroitin 6-sulfate in the extracellular space (Fig. 3A) appears to be involved in the phenomenon.

We investigated the reason for the decrease in number of naïve T lymphocytes in the spleen of young C6st<sup><sup>−/−</sup></sup> mice. FACS analysis did not reveal significant differences in the populations of lymphoid cell progenitors such as Lin<sup><sup>−</sup></sup>IL-7Rα<sup><sup>−</sup></sup> c-Kit<sup><sup>−</sup></sup>Sca-1<sup><sup>−</sup></sup> cells (29) in bone marrow (data not shown). Moreover, differentiation and maturation of thymocytes were normal in C6st<sup><sup>−/−</sup></sup> mice as revealed by FACS using anti-CD4 and anti-CD8 antibodies (data not shown). Additional analyses of splenic B lymphocytes in C6st<sup><sup>−/−</sup></sup> mice indicated normal cell-surface expression of CD21, CD22, CD23, CD24, CD44, I-A<sup><sup>−</sup></sup>, IgM, IgD, and B220 (data not shown). With regard to the function of splenic lymphocytes, mitogenic responses to concanavalin A (for T cell response) and lipopolysaccharides (for B cell response) were not different between C6st<sup><sup>+/+</sup></sup> and C6st<sup><sup>−/−</sup></sup> (data not shown).
Lymphocyte Trafficking and Morphology of the Spleen in the C6st−/− Mice Were Normal—Because we could not exclude the possibility that the decrease of naive T lymphocytes in the spleen of C6st−/− mice was caused by the abnormal recruitment of cells to the spleen, a lymphocyte homing study was performed as described under “Experimental Procedures.” Lymphocyte trafficking of extrinsic labeled lymphocytes into secondary lymph nodes of C6st−/− mice was unaltered (Fig. 6A). Recruited cells were normally localized in the T cell area of the spleen of C6st−/− mice (Fig. 6B). The general architecture of the C6st−/− spleen was intact as revealed by hematoxylin and eosin staining (Fig. 6, C and D). Immunohistochemical analyses of spleen sections using anti-CD3, anti-B220, and anti-CD11c showed an overall normal architecture with a well organized T cell zone (Fig. 6B) and B cell zone (Fig. 6, E and F) in the white pulp and marginal zone (Fig. 6, E and F) in C6st−/− mice. These results indicated that C6st deficiency did not affect lymphocyte trafficking to secondary lymphoid organs, and the significant decrease in the number of naive T lymphocytes in the C6st−/− mice spleen might have been the result of abnormal maintenance of the cells in the spleen.

**DISCUSSION**

The C6st gene was knocked out in the mouse, and C6ST activity was abolished almost completely in the spleen, lung, and lymph nodes of the null mutant mice. This result indicated that the C6st gene is responsible for the synthesis of chondroitin 6-sulfate, at least in these organs. Analysis of chondroitin sulfate chains in the spleen of the knockout mice gave further insight into the biosynthesis of chondroitin sulfate in this organ. The amount of chondroitin 6-sulfate was far less than that of chondroitin 4-sulfate. As expected, the peak of chondroitin sulfate chains in the spleen of the knockout mice gave activity was abolished almost completely in the spleen, lung, and lymph nodes of the null mutant mice. The data from these experiments suggest the involvement of the C6st gene in the synthesis of chondroitin 6-sulfate, but the role of C6ST in other tissues remains to be elucidated.

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**Table I**

<table>
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<th>Lymphoid organs</th>
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<th>Percentage of positive cells</th>
<th>No. of positive cells (10⁶ cells/organ)</th>
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<td></td>
<td>C6st+/+</td>
<td>C6st−/−</td>
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<td>61.7 ± 22.8</td>
<td>59.9 ± 24.7</td>
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<td>29.0 ± 11.2</td>
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<td>Spleen*</td>
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<td>CD62L−/CD44b hom</td>
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<td>43.8 ± 5.7*</td>
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<td>Mesenteric lymph nodes*</td>
<td>24.1 ± 3.9</td>
<td>26.8 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>B220−/+</td>
<td>67.8 ± 5.3</td>
<td>65.8 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>CD3−/+</td>
<td>84.5 ± 3.5</td>
<td>84.7 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>CD62L−/CD44b hom</td>
<td>0.7 ± 0.1</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Peyer’s patches*</td>
<td>60.2 ± 6.1</td>
<td>57.6 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>B220−/+</td>
<td>26.7 ± 3.2</td>
<td>28.1 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>CD3−/+</td>
<td>44.2 ± 10.1</td>
<td>47.5 ± 10.8</td>
</tr>
<tr>
<td></td>
<td>CD62L−/CD44b hom</td>
<td>20.7 ± 0.4</td>
<td>17.6 ± 4.1</td>
</tr>
</tbody>
</table>

* The total number of free cells is shown (10⁶ cells/organ).

b Both the percentage to the total white blood cells and the number are shown.

* Both the percentage to the total CD3-positive cells and the number are shown.

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**Fig. 5. The number of naive T lymphocytes was decreased in the spleen of C6st−/− mice.** Cells were collected from the spleen (A and E), peripheral lymph nodes (B and F), mesenteric lymph nodes (C and G) and Peyer’s patches (D and H) of 5–6-week-old C6st+/+ (A–D) and C6st−/− (E–H) mice. The age of the C6st+/+ and C6st−/− mice was identical. The cells were stained with fluorescein isothiocyanate-conjugated anti-CD44, PE-conjugated anti-CD62L, and biotin-conjugated anti-CD3 antibodies followed by Cy-Chrome-streptavidin. Naive T lymphocytes (CD3−/CD62L−/CD44b hom) were determined by flow cytometry. The numbers indicate the percentages of CD62L−/CD44b hom in the total CD3− cell population. The results of one representative experiment among eight are shown.

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**Fig. 6.** Radiolabeled lymphocytes were injected into the spleen of C6st−/− mice. The spleen of C6st+/+ (A–D) and C6st−/− (E–H) mice was removed 5 h after injection. The spleen was fixed and serially sectioned. (A) Hematoxylin and eosin staining of spleen sections. The total number of free cells is shown (10⁶ cells/organ). (B) Labeled lymphocytes were injected into the spleen of C6st−/− mice. The spleen of C6st+/+ (A–D) and C6st−/− (E–H) mice was removed 5 h after injection. The spleen was fixed and serially sectioned. (A) Hematoxylin and eosin staining of spleen sections. The total number of free cells is shown (10⁶ cells/organ). (B) Labeled lymphocytes. The number of naive T lymphocytes was decreased in the spleen of C6st−/− mice. The significance of the trace amounts of Di-6S was decreased to less than 10% of the wild-type level in C6st−/− mice. The age of the C6st+/+ and C6st−/− mice was identical. The cells were stained with fluorescein isothiocyanate-conjugated anti-CD44, PE-conjugated anti-CD62L, and biotin-conjugated anti-CD3 antibodies followed by Cy-Chrome-streptavidin. Naive T lymphocytes (CD3−/CD62L−/CD44b hom) were determined by flow cytometry. The numbers indicate the percentages of CD62L−/CD44b hom in the total CD3− cell population.
peripheral lymph node; MLN normally recruited to T cell zones in the streptavidin (red). Injected CMFDA-positive lymphocytes (green) were C6st or were analyzed by flow cytometry (A) and mesenteric lymph nodes, and Peyer’s patches of each mouse (CMFDA; Molecular Probes) were injected into the tail vein of 6-week-old mice. CMFDA-positive lymphocytes in the spleen, peripheral lymphoid organs. In which nonprimed T lymphocytes, thymus of young mice migrate to the T cell areas of secondary lymphoid organs, in which nonprimed T lymphocytes, i.e. naive T lymphocytes, encounter antigen-presenting cells followed by recirculation into the blood. Because C6ST is most prominently expressed in the spleen and bone marrow (21), we performed extensive analysis on lymphocyte subpopulations of C6st−/− mice and found that naive T lymphocytes were selectively decreased in the spleen of the knockout mice 5–6 weeks after birth. Because growth monitored by the gain of body weight was not different between C6st+/+ and C6st−/− mice, this phenomenon is not because of retarded growth of C6st−/− mice. Most of peripheral naive T lymphocytes in young mice are composed of the cells emigrated from the thymus, and recirculated from peripheral lymphoid organs. C6st mRNA was not expressed in the thymus (21), and the population and number of CD4+CD8− and CD4−CD8+ cells in the thymus of 5–6-week-old C6st−/− mice were unchanged (data not shown), indicating that null mutation of C6st−/− may have not affected the differentiation, maturation, and emigration of thymocytes in C6st−/− mice. It is likely that the selective paucity of naive T lymphocytes in the spleen of C6st−/− mice was caused by the functional change in the maintenance of lymphocytes in the spleen. Because recruitment of peripheral lymphocytes to the spleen was not impaired in C6st−/− mice (Fig. 6A), we considered that survival, retention, and/or emigration of naive T lymphocytes was affected in the spleen of these mice. Chemo- kines as well as the growth and survival factor midkine have strong affinity for a subpopulation of chondroitin sulfate (13, 22)."

**Fig. 6. Lymphocyte trafficking and histological architecture of the spleen in C6st−/− mice were normal.** Lymphocytes extracted from online-b-week-old C6st+/+ mice and labeled with a fluorescent dye (CMFDA; Molecular Probes) were injected into the tail vein of C6st+/+ or C6st−/− mice. CMFDA-positive lymphocytes in the spleen, peripheral and mesenteric lymph nodes, and Peyer’s patches of each mouse were analyzed by flow cytometry (A). Cryostat sections of the spleen in C6st+/+ (upper panel) and C6st−/− (lower panel) mice were prepared 1.5 h after injection of the labeled cells and subsequently stained with biotin-conjugated anti-CD3 antibody followed by PE-conjugated streptavidin (red). Injected CMFDA-positive lymphocytes (green) were normally recruited to T cell zones in the C6st−/− spleen (B). PLN, peripheral lymph node; MLN, mesenteric lymph node; P.P, Peyer’s patch.
Furthermore, a genetic defect in the expression of secondary lymphoid organ chemokine leads to a decrease in number of naive T lymphocytes in the white pulp of the spleen as well as peripheral lymph nodes (36, 37). It is possible that secondary lymphoid organ chemokine or another factor involved in survival, retention, and emigration of naive T lymphocytes binds to chondroitin 6-sulfate, and this binding plays a role in naive T lymphocyte appropriate localization in the spleen. The decrease of naive T lymphocytes in the spleen of Cbs6−/− mice is expected to result in decreased immune response to bacteria and viruses in blood stream. One of the reasons of persistent existence of chondroitin 6-sulfate in the mouse might be their requirement for efficient defense to microorganisms.

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Functional Analysis of the Chondroitin 6-Sulfotransferase Gene in Relation to Lymphocyte Subpopulations, Brain Development, and Oversulfated Chondroitin Sulfates

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