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Immunological Activity of Chondroitin Sulfate

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I. Chapter Overview

The use of chondroitin sulfate (CS) for the symptomatic treatment of osteoarthritis (OA) has become very popular; however, it has also been the subject of controversy for several reasons. First, the nutraceutical industry is less regulated than the pharmaceutical industry and thus, the nutraceutical CS often suffers from poor quality control. Second, the bioavailability of orally administered CS is not generally accepted. Third, the mechanism of the effect of CS for treatment of OA remains unclear. There is abundant *in vitro* and *in vivo* evidence from animal and human clinical studies demonstrating the efficacy and safety of CS. This chapter focuses on the immunological activity of structurally regulated CSs. The mechanism of this immunological activity appears to be through CS binding to receptors related to cytokine production in lymphocytes such as splenocytes.

II. Introduction

Most important pharmaceuticals have their origin in natural products, such as herbs and antibiotics, however, many physicians are deeply skeptical about the use of natural remedies. This skepticism is based on the concerns about the lack of scientific evidences of their efficacy. A new class has emerged called nutraceuticals, which are nutritional supplements with presumed pharmaceutical properties and efficacy. Because these substances are relatively unregulated, there is no requirement for rigorous scientific evidences before marketing. This lack of regulation also poses severe problems with purity and quality control. Glucosamine and CS sales alone in Japan are estimated at several billion JPY (several hundred million US dollars) in retail sales. Furthermore, the combination of glucosamine and CS is a very popular nutraceutical in the USA. While there is no scientific evidence on the efficacy of glucosamine and CS in the treatment of joint disease, the market of this nutraceutical product continues to grow. Self-medicating patients represent the driving force making nutraceutical products bestsellers throughout the world. Glucosamine and CS have been widely studied in tissue culture, animal models of arthritis, veterinary clinical trials, and human comparative or placebo controlled trials. All published studies suggest a positive effect, and no trial has shown significant side effects. Based on the absence of conclusive data, the National Institute of Health has started "*NIH Glucosamine/Chondroitin Arthritis Intervention Trial (GAIT)*" (http://www.niams.nih.gov/ne/press/2000/

gait_qa.htm#what) to obtain definitive scientific evidence for the efficacy of glucosamine and CS in the treatment of arthritis.

Glucosamine and CS are integral components of articular cartilage and are important to the physiologic and mechanical properties of this tissue. Glucosamine is involved in cartilage formation by acting as the precursor of the disaccharide unit in glycosaminoglycans (GAGs) (Baker and Ferguson, 2005; De los Reyes *et al*., 2000; Scott *et al*., 2005). Chondroitin sulfate is a GAG that is a component of the aggrecan structure that makes up articular cartilage (Freeman, 1979). It binds collagen fibrils and limits water content by cooperating with hyaluronan, which is also a GAG. Chondroitin sulfate plays a role in allowing the cartilage to resist tensile stresses during various loading conditions by giving the cartilage resistance and elasticity (Muir, 1986). Exogenously administered glucosamine and CS have been shown *in vitro* to have other physiological effects. Glucosamine stimulates chondrocytes to increase secretion of GAGs and proteoglycans (PGs) *in vitro* (Jimenez, 1996). There is also evidence of CS-based anti-inflammatory activity not related to prostaglandin metabolism, probably through a free radical scavenging effect (Raiss, 1985). Osteoarthritis is clinically characterized as the decomposition of cartilage by degradative enzymes. These enzymes are competitively inhibited by CS *in vitro* (Bartolucci *et al*., 1991; Bassleer *et al*., 1992). Moreover, laboratory studies have demonstrated a synergistic effect when glucosamine and CS are administered together. Lippiello *et al.* (2000) noted that the coadministration of CS and glucosamine resulted in a greater increase of $^{35}SO_4$ incorporation into GAGs (97%) than demonstrated by either agent alone (glucosamine, 32%; CS, 32%). This synergistic effect was also observed in experiments on CS's antiprotease activity *in vitro* (Arner, 2002). However, the orally administered CS has to be absorbed through gastric/intestinal system into blood flow to show these effects in its intact form.

There are many arguments regarding whether or not orally administered CS is absorbed through gastric/intestinal system (Owens, 2004). We have found only very small amounts of relatively low-molecular weight CS chains (average molecular weight 15,000) in the blood over 24 h following oral administration to mice. The failure to observe significant bioavailability suggests a novel concept that CS might act in the absence of absorption, on the humoral immunosystem by stimulating the intestinal intraepithelial lymphocytes (IEL) through cytokine production (Akiyama *et al*., 2004; Sakai *et al*., 2002a). This chapter describes the effects of CS on immunosystem *in vivo* and *in vitro.*

III. Clinical Experience (David and Lynne, 2003)

In an artificially induced cartilage injury model, Uebelhart *et al.* (1998) noted that treatment with CS resulted in a marked reduction in the loss of PSs as compared with controls. Lippiello *et al.* (1999) reported that the effect of CS given to normal dogs was an increase in the serum GAG levels. Using indirect assessments of cartilage metabolism, they found that serum from treated dogs increased biosynthetic activity (incorporation of radioactively labeled glucosamine) and decreased proteolytic degradation (release of ^{35}S) from prelabeled normal calf cartilage segments. Using a rabbit instability model created by transecting the anterior cruciate, Lippiello *et al.* (2000) found that the articular matrix was severely

degraded in the untreated group while remaining essentially intact in the treated group. In a canine model of unilateral carpal synovitis, although no effect was observed if the treatment was started after the synovitis occurred, dogs pretreated with the combination of glucosamine and CS have shown less evidence of bone remodeling and lower lameness scores (Canapp *et al*., 1999).

Glucosamine and CS are often used either separately or in combination for the treatment of arthritic ailments (Dechant *et al*., 2005). The safety profile of these nutraceuticals has been reviewed (Hungerford and Valaik, 2003). When recommending a supplement to patients, the physicians should take into account the purity of the ingredients, reputation of the manufacturer, and the molecular weight of chondroitin supplied. An analysis of Immunological Activity of CS 405 marketed products indicated that the amounts of glucosamine and CS present in the products sold often fell short of the declared values on the label (Adebowale *et al*., 2000). Most of the commercially available supplements sold in Japan analyzed in our laboratory contained less CS than indicated on their label, and significant amounts of carrageenan was found in many of these products (data not published). These discrepancies may introduce the confusion underlying the potential benefits of these nutraceuticals in treating arthritic disease.

Several clinical trials exploring the efficacy of both glucosamine and CS in the treatment of OA have been performed over the past 30 years as indicated in an earlier section; the outcomes of these studies have also been reviewed (Leeb *et al*., 2000; McAlindon *et al*., 2000; Richy *et al*., 2003). The goal of these reviews was to assess both the potential symptom-modifying (e.g., pain and functional efficacy) and structure-modifying (e.g., changes in joint space narrowing) activities of glucosamine and CS in alleviating symptoms of OA of the knee using outcome-oriented metaanalysis of these randomized clinical trials. The general conclusion from these reviews is that glucosamine ingestion shows efficacy in both narrowing joint space and some symptom-modifying parameters. However, although CS ingestion showed similar symptom-modifying effects, the structure-modifying benefits still need to be confirmed. Given this clinical evidence, there is clearly a need for more basic research aimed at elucidating the cellular and molecular mechanisms involved with these two interesting nutraceuticals.

IV. Metabolic Fate of Orally Administered Chondroitin Sulfates

The metabolic fate of orally administered CS is ambiguous (Ronca and Conte, 1993). Baici *et al.* (1993) investigated the ability of an oral dose of CS to impact the concentration of GAGs in humans. In this study, CS samples were administered to six healthy volunteers, six patients with rheumatoid arthritis, and six patients with OA. The concentration of GAGs in serum was reportedly unchanged following ingestion (Baici *et al.*, 1993). Morrison (1977) has indicated that the intact absorption of CS was extremely low, estimating the absorption rate to be between 0 and 8%. The complexity of this issue is based on the fact that CS is found in a wide range of molecular weights, chain lengths, charge distributions, with positional isomers of sulfo groups, and containing variable percentages of similar disaccharide residues comprised of sulfated glucuronic acid (GlcA) and *N*-acetylgalactosamine (GalNAc) as shown in Fig. 1. A further complication occurs because

lowmolecular weight derivatives of CS have also been pharmacologically produced and utilized in some of the pharmacokinetic and therapeutic studies and trials (Conte *et al*., 1991; Ronca *et al*., 1998). It is quite possible that the contrasting metabolic fates of orally administered CSs are a direct reflection of this dissimilarity in the primary structure and physical properties of these CS samples. The pharmacokinetic properties of a proprietary CS were investigated by Conte *et al*. (1995). Significant extraction procedures were utilized to generate a low-molecular weight product that could be characterized for structure, physiochemical properties, and purity. Only a fraction with a relative molecular weight of about 14 kDa was used in their experiments. This fraction showed a sulfate-to-carboxyl ratio of 0.95 due to the high percentage of monosulfated disaccharide sequences [55% CS-A (4-*O*-sulfonated Gal-NAc) and 38% CS-C (6-*O*-sulfonated GalNAc)], and a low amount of disulfated disaccharide sequences (1.1%). The purity of the preparation was greater than 97% CS. This sample was radioactively labeled and orally administered to the rats and dogs. Althoughmore than 70%of the radioactivity was absorbed and was subsequently found in urine and tissues, the radioactivity associated with an intact molecule of CS corresponding to the molecular mass of the administered CS was relatively small (approximately 8.5%), and this percentage decreased rapidly over time. The majority of the radioactivity absorbed was actually associated with molecules with a molecular mass of less than or equal in size to GalNAc residues. This radioactivity increased over time and remained elevated. Radioactivity after 24 h was highest in the small intestine, liver, and kidneys (tissues responsible for the absorption, metabolism, degradation, and elimination of the compound); however, relatively high amounts of radioactivity were also found in tissues, which utilize amino sugars such as joint cartilage, synovial fluid, and trachea. This group also orally administered CS to healthy volunteers in either a single daily dose of 0.8 g or in two daily doses of 0.4 g. Although both dosing schedules increased plasma concentration of exogenous molecules associated with CS, the results have indicated that the oral administration of one dose of 0.8 g CS was the more effective regimen. Some physiological parameters associated with GAGs, such as hyaluronan, were also analyzed to investigate whether orally administered exogenous CS affects synovial fluid in patients with OA. The results indicated that the treatment with CS might also modify these parameters. Thus, despite all of these studies, the oral bioavailability and efficacy of CS remains controversial. However, the majority of physiological benefits subsequent to administration of CS appears to be a direct result of increased availability of the monosaccharide/disaccharide residues of CS produced by the action of enzymes found in the intestine (Hong *et al*., 2002).

V. A New Concept for Explaining the Effect of Chondroitin Sulfate in Arthritis Treatment

Polysaccharides, such as CS, are clearly poorly absorbed through the digestive system. Moreover, we have shown that the half-life of CS in the circulatory system is 3–15 min, based on the pharmacokinetic study of intravenously administered CS (Sakai *et al*., 2002b). Accordingly, it appears unlikely that orally administered CS is systemically distributed to connective tissues, such as cartilage and skin, and that exogenously administered CS directly stimulates chondrocyte synthesis of extracellular matrix components. This suggests that the mechanism of action of exogenously administered CS might be mediated from within the

intestinal tract by other systems, such as the immunological system (Wrenshall *et al*., 1999). Our laboratory has already shown that CS affects and upregulates the *in vitro* antigen-specific Th1 immune response on murine splenocytes sensitized with ovalbumin (OVA) and that CS suppresses the antigen-specific IgE responses (Sakai *et al*., 2002a). These findings also suggest a therapeutic use of CS to control the IgE mediated allergic response. The number and position of O-sulfo groups varies among CS samples obtained from different sources (Alves *et al*., 1997; Farias *et al*., 2000; Santos *et al.*, 1992).We hypothesized that the immunological activity of orally administered CS might also be different among the several types of CS. Thus, it is important to determine the structure– activity relationship (SAR) of CS, particularly with respect to the number and position ofO-sulfo groups in CS. Knowledge of the SAR of CS will be necessary to further explore its effective use as a therapeutic agent.

It is generally accepted that CD4+ T cells are subpopulations containing 2 cell types (Th1 and Th2), based on their different patterns of cytokine secretion (Mossmann and Coffman, 1989a,b). Th1 cells secrete IFN-γ, IL-2, and IL-12. Th2 cells produce IL-4, IL-5, and IL-10. IFN-γ and IL-12 induce the differentiation of Th0 cells to Th1 cells, whereas IL-4 induces the differentiation to Th2 cells (Fig. 2). Therefore, it is believed that an increase in IFN-γ and IL-12 shifts the Th1/Th2 cell balance to predominantly Th1, while an increase in IL-5 and IL-10 shifts the balance to predominantly Th2 (Akiyama *et al*., 1999; Nagafuchi *et al*., 2000).We have previously reported that CS induced Th1-type cytokine (IFN-γ, IL-2, and IL-12) secretion but suppressed Th2-type cytokine (IL-5 and IL-10) secretion by the OVA-sensitized splenocytes (Sakai *et al*., 2002a).We have also already shown that both O-sulfo group content and position in CS is important for the Th1-promoted activity of murine splenocytes, in terms of the cytokine production and Th1/Th2 balance (Akiyama *et al*., 2004). We first examined whether the activity was associated with the O-sulfo groups in CS and confirmed that the sulfation of a polysaccharide has played an important role in the activity.We have reported that CS induced the Th1-promoted activity while dextran, a neutral polysaccharide used as a control, did not (data not shown). In contrast, dextran sulfate also did not show significant effects on cytokine production by murine splenocytes (Fig. 3). These results indicate that the polysaccharide type and sulfation is critical for the Th1-promoted activity (Akiyama *et al*., 2004). We subsequently showed the effect of the level of sulfation number and position of CS on the Th1-promoted activity (Akiyama *et al*., 2004). While fully sulfonated CS exhibits Th1-promoted activity, intact CS-A and the partially *O*-sulfonated CS demonstrate higher activity CS (Fig. 1). These results strongly suggested that excess sulfo groups in CS could decrease the Th1-promoted and Th2-inhibitory activities of CS. Among the monosulfated CS, CS-A, -C, and -B, the CS-A sample showed highest activity (Figs. 1 and 3). This result suggested that the [4)GlcA(β1–3)GalNAc4S(β1-]$_n$ sequence is more important for activity than the [4)GlcA(β1–3)GalNAc6S (β1-]$_n$ or [4)IdoA(β1–3)GalNAc4S(β1-]$_n$ sequences characteristic of CS-C and -B, respectively (Fig. 1). Chondroitin sulfate-B [dermatan sulfate (DS)], while nearly structurally identical to CS-A (it contains IdoA instead of GlcA), shows lower activity. This is surprising as the greater flexibility of the IdoA residue in CS-B is commonly used to explain the propensity of IdoA-containing GAGs to interact with proteins and display a large number of different biological activities (Kawashima *et al*., 2002). Examination of the disulfated CS samples shows that the effects of CS-E on the Th2-inhibitory activity were

higher than those of CS-D or -A (Fig. 3, also see structures shown in Fig. 1). These results suggested that the [4)GlcA(β1–3) GalNAc4S6S(β1-]$_n$ sequence in CS-E is more important for high activity than the [4)GlcA2S(β1–3)GalNAc6S(β1-]$_n$ sequence characteristically found in CS-D. Furthermore, these experiments demonstrate that the [4)GlcA(β1–3) GalNAc4S(β1-]$_n$ and [4)GlcA(β1–3)GalNAc4S6S(β1-]$_n$ sequences in CS are more critical for higher activity. Researchers have reported many biological activities for sulfated polysaccharides (Chaidedgumjorn *et al*., 2002; Koyanagi *et al*., 2003; Linhardt and Toida, 1997; Toida *et al*., 1999). In most cases the number of sulfo groups in the polysaccharide directly correlates with the level of bioactivity (Chaidedgumjorn *et al*., 2002; Koyanagi *et al*., 2003; Toida *et al*., 1999). Koyanagi *et al.* (2003), have shown that by increasing the number of sulfo groups in fucoidans (sulfonated fucans), its antiangiogenic and antitumor activities can be potentiated. Our laboratory has also reported the many biological activities of the chemically fully sulfated poly-and oligosaccharides (Chaidedgumjorn *et al*., 2002; Suzuki *et al*., 2001; Toida *et al*., 1999, 2000). Chondroitin sulfate has been found in many tissues (Suzuki *et al*., 1968) and cells (Ohhashi *et al*., 1984; Petersen *et al*., 1999; Stevens *et al*., 1988), and has been reported to interact with various biologically important molecules and regulate their functions. We have demonstrated the importance of the content, position, and number of O-sulfo groups in CS for immunological activity of the OVA-stimulated murine splenocytes *in vitro* (Akiyama *et al*., 2004; Sakai *et al*., 2002a). It was also shown that Th1-promoted and Th2-inhibitory activity of CS on murine splenocytes could be associated with binding to L-selectin. It has been reported that a large CS/DSPG interacts through its CS/DS chains with the adhesion molecules L-and P-selectin, CD44, and chemokines. Kawashima *et al*. (1999, 2000) and others (Capila and Linhardt, 2002; Hirose *et al*., 2001) reported that oversulfated CS/DS, containing [4)GlcA(β1-3)GalNAc4S6S (β1-]$_n$ sequences, interacts with L-selectin, P-selectin, and chemokines. Our findings may indicate that these same [4)GlcA(β1-3)GalNAc4S6S(β1-]$_n$ sequences in CS would be associated with the strongest effects on the promotion of the Th1-type cytokine production and the inhibition of the Th2-type cytokine production. The present structural characterization of CS to Th1-promoted, and Th2-inhibitory activity is consistent with the high-affinity binding of CS, containing the [4)GlcA(β1-3)-GalNAc4S(β1-]$_n$ and [4) GlcA(β1-3)GalNAc4S6S(β1-]$_n$ sequences, to L-selectin (Kawashima *et al*., 2002). These findings also may support our hypothesis that such an immunological activity could be associated with the binding of CS-A to L-selectin on T cell surface. These results also may indicate that differences in the content and position of O-sulfo groups in CS could markedly influence Th1-promoted and Th2-inhibitory activities, as do differences between GlcA and IdoA residues in CS. In these observations, however, the inhibition of CS binding to L-selectin could not be shown by FACS analysis using labeled antiL-selectin monoclonal antibody (Akiyama *et al*., 2004). This result may suggest that the epitope region on L-selectin to antiL-selectin monoclonal antibody might not be located in the lectin region that binds to CS. We have not yet established the relationship between the various CS samples from natural products and the immunological activities. Thus, further studies are required on the relative L-selectin binding affinity of different types of CS to fully elucidate the importance of L-selectin-CS binding. The effect of heparin was also examined on Th1/Th2 balance and found to demonstrate the same level of activity as CS at identical doses (results

not published). We are considering future studies to assess the effects of heparin and its derivatives on these activities to fully elucidate the SAR of GAG.

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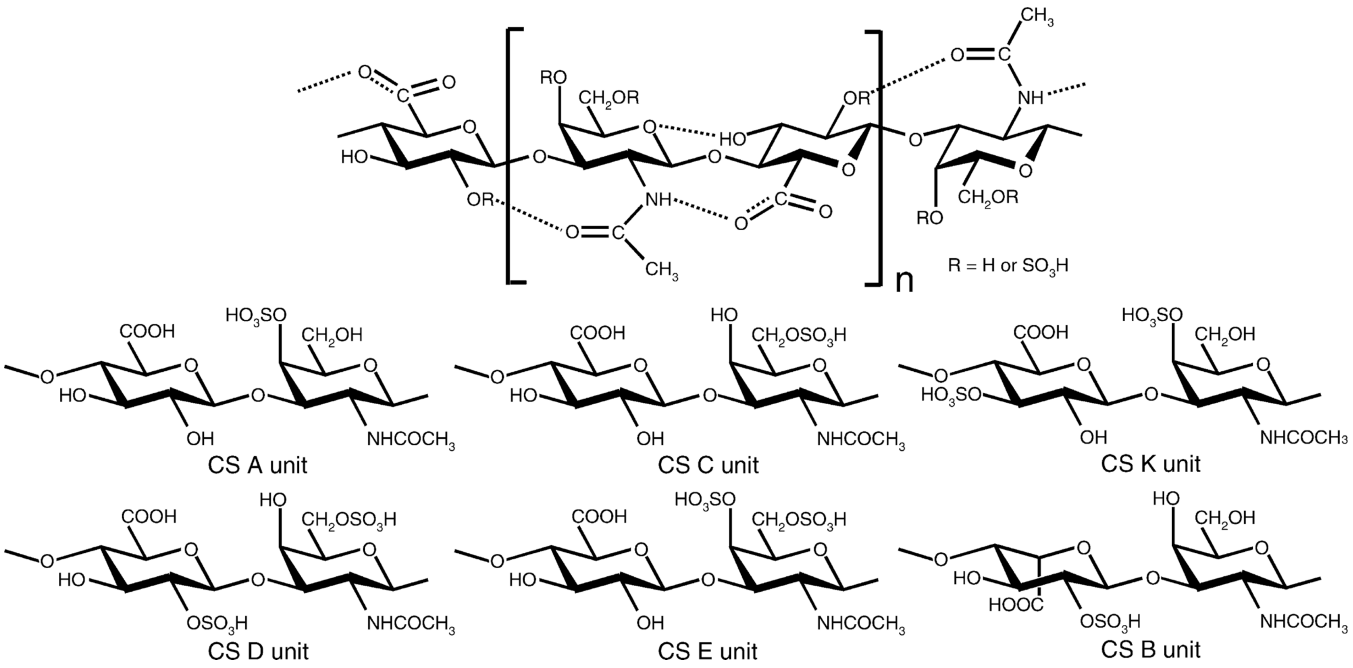

Figure 1.
Disaccharide structures found in chondroitin sulfate.

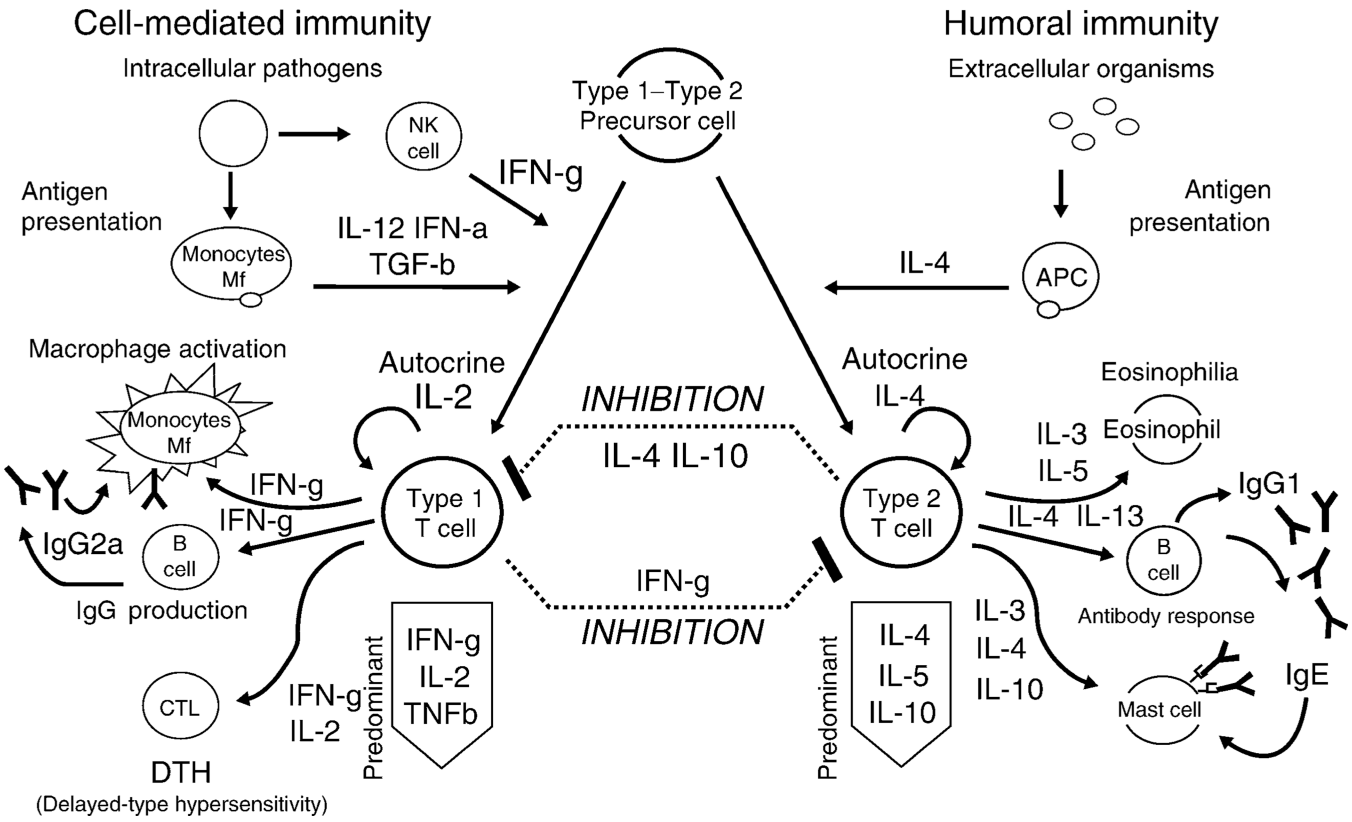

Figure 2.
Overview of Th1/Th2 balance.

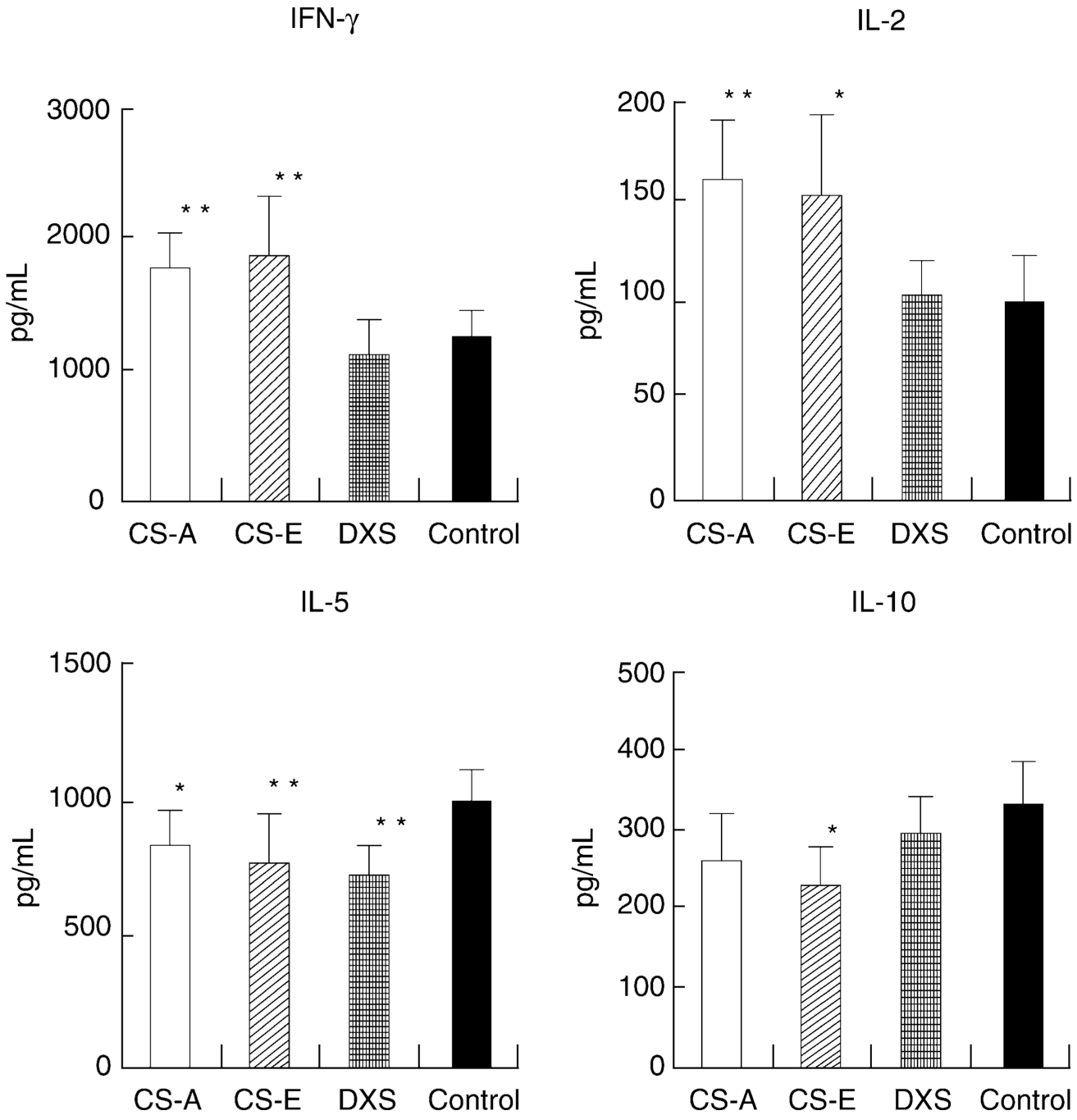

Figure 3.

Effect of CS on the cytokine production of murine splenocyte *in vitro*. BALB/c mice ($n = 5$) were intraperitoneally injected on day 0 and 13 with 20 μg of ovalbumin (OVA) and 2 mg of $Al(OH)^2$ at a total volume of 400 μl. Spleen cells (5.0×10^6 cells/ml) were collected on day 14 and were cocultured with OVA (final 100 μg/ml). The amounts of cytokines in the supernatant were measured by ELISA. Asterisk indicates significance of difference from control value (*$p < 0.05$, **$p < 0.01$). Bars represent mean values (±S.D.) for six wells.