Modulation of macrophage and B cell function by glycosaminoglycans

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Abstract: There is increasing evidence that the behavior of antigen-presenting cells may be regulated, in part, by the surrounding microenvironment. Components of the microenvironment of solid tissues that might influence antigen-presenting cell functions include glycosaminoglycans. We previously showed that heparan sulfate glycosaminoglycans activate macrophages, leading to profound alterations in T cell responses. Here we demonstrate the functional changes that occur in murine antigen-presenting cells induced by heparan sulfate and other glycosaminoglycans, and postulate how these functional changes influence the nature of local immune responses. Heparan sulfate triggered up-regulation of ICAM-1 and I-A, caused the release by antigen-presenting cells of interleukin (IL)-1, IL-6, tumor necrosis factor, IL-12, transforming growth factor β, and prostaglandin E_2 (PGE_2), and (in macrophages) induced cytotoxic capability. Heparin induced IL-12 and interferon-γ production but did not promote the release of other cytokines. Chondroitin sulfate and dermatan sulfate, although not stimulating the production of cytokines or of PGE_2, elicited the production by macrophages of nitric oxide. These findings support a model in which the glycosaminoglycan composition of a given tissue, which may be altered by inflammatory processes, helps to regulate the behavior of antigen-presenting cells, which in turn determines the characteristics of the immune response that ensues. *J. Leukoc. Biol.* 66: 391–400; 1999.

Key Words: *macrophages · heparan sulfate · cytokines*

INTRODUCTION

Antigen-presenting cells (APC) such as macrophages and B cells play a central role in the development and manifestations of cell-mediated immune responses, through functions such as the processing and presentation of protein antigens, expression of cell surface and soluble mediators, and direct destruction of foreign cells through cytotoxic mechanisms [1]. Although these functional properties of APC have been characterized primarily *in vitro*, there is increasing evidence that the behavior of APC *in vivo* may be determined in part by the extracellular environment, which acts on APC through specific receptors present on the cell surface. For example, B cells and macrophages express CD44, a glycoprotein that binds hyaluronic acid (HA) and other ligands [2]. HA is a glycosaminoglycan that plays a significant role in embryogenesis [3] and tissue malleability [4], and is felt to influence B cell differentiation [5]. APC also express the integrin VLA-4, which binds fibronectin [6], another component of extracellular matrices. The binding of macrophages to fibronectin induces granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion and augments phagocytosis [7].

We previously showed that heparan sulfate (HS), a heterogeneous glycosaminoglycan with discrete influences on several different cell types, is released from cell surfaces and extracellular matrices in the course of immune reactions [8] and inflammation [9]. In soluble form HS regulates splenocyte proliferation [10] and T cell-mediated cytotoxicity [11] through an influence on APC function. HS does this by mediating specific signal transduction pathways in APC [12].

HA and heparin may also be released as soluble glycosaminoglycans. For example, elevated serum levels of HA fragments are detected in certain liver diseases (such as cirrhosis), in sepsis, in rheumatoid arthritis, and as a marker for impending rejection of liver transplants [13]. Heparin glycosaminoglycan is released from activated mast cells in the lung and intestine [14]. HA and heparin influence immune responses through specific signaling events [12, 15, 16], although the nature of these signals and their cellular consequences are incompletely understood.

The functional consequences of changes induced by the activation by glycosaminoglycans, and the influence these changes may have on immune responses, are incompletely understood. We investigated the functional properties of murine macrophages after exposure to soluble glycosaminoglycans. We report here that HS induces the production of costimulatory molecules, cytokines [especially by increasing interleukin-12 (IL-12) and decreasing IL-4 production], and, in macrophages, promotes cytotoxic capabilities; changes that would likely enhance cell-mediated immunity in general and Th1-type helper immune responses in particular.

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MATERIALS AND METHODS

Materials

Female BALB/c mice, age 6–8 weeks, were obtained from Harlan Laboratories (Indianapolis, IN). Dulbecco's minimal essential medium (DMEM) was from GIBCO (Grand Island, NY). BALB/c mouse serum was from Bioproducts for Science (Indianapolis, IN). Heparan sulfate (bovine kidney), chondroitin sulfate (whale cartilage), dermatan sulfate (pig skin), heparin (bovine lung), hyaluronic acid (bovine vitreous), ionomycin, and thioglycollate were from Sigma Chemical Corp. (St. Louis, MO). Anti-murine IL-1α and anti-murine IL-6 antibodies were obtained from R & D Systems (Minneapolis, MN). Anti-ICAM-1, anti-B220, anti-B7-2, anti-IA^d, and anti-K^b were from PharMingen (San Diego, CA). Prostaglandin E_2 (PGE_2) radioimmunoassay kit and goat anti-rat IgG conjugated to magnetic beads were from Advanced Magnetics (Cambridge, MA). Lipopolysaccharide (LPS) was from Difco (Detroit, MI). Murine IL-1α, IL-1β, IL-6, and tumor necrosis factor α (TNF-α) oligonucleotide probes were from Amgen (Thousand Oaks, CA). Rat anti-mouse Thy-1.2 (clone 30-H12), rat anti-mouse Mac-1 (clone M1/70.15, 11.5, HL), the T helper clone D10.G14, and L1210 murine lymphocytic leukemia cells were from American Type Culture Collection (Rockville, MD).

Isolation of macrophages

Peritoneal macrophages were elicited in BALB/c mice by intraperitoneal injection with 2 mL of 3% thioglycollate. The macrophages were harvested by peritoneal lavage 4 days after injection of thioglycollate, and were subsequently cultured in DMEM with 0.6% BALB/c mouse serum for 7–10 days to allow reversion to a quiescent state. FACS analysis of macrophages partially purified by adherence revealed the following composition: Mac-1^+ cells (macrophages) 80%, pan NK^+ cells (NK cells) 3%, $CD19^+$ cells (B cells) 6%, and $CD4^+$ cells 0.6%. Resting (unelicited) peritoneal macrophages were obtained in a similar fashion without prior thioglycollate injection.

B cell isolation

B lymphocytes were isolated from suspensions of murine splenocytes by the immunodepletion of T cells through the use of monoclonal rat anti-mouse Thy-1.2 antibodies and by the depletion of macrophages with rat anti-mouse Mac-1 antibodies. T cells and macrophages to which the monoclonal antibodies bound were removed from the cell suspension with goat anti-rat IgG linked to magnetic beads. This procedure was carried out according to the manufacturers' instructions. The B cell preparation was contaminated by 1% $CD4^+$ T cells after completion of the isolation.

Mixed lymphocyte culture (MLC)

Splenocytes isolated after mechanical disruption of mouse spleens were suspended in DMEM with 1% mouse serum. Responder (BALB/c; 5×10^6 cells/mL) and stimulator (C57BL/6; 5×10^6 cells/mL) cells were incubated together in 96-well plates (Costar, Cambridge, MA) at a final volume of 200 µL per well. After 3 days in culture, glycosaminoglycans were added to the MLC by removal of 50 µL of 200 µL medium/well and addition of 50 µL glycosaminoglycan at 4× the final concentration. Supernatants were collected after 24 h for assay of PGE_2 levels.

IL-6 production

IL-6 levels in macrophage supernatants were determined by bioassay based on proliferation of the IL-6-dependent cell line B9 [17]. B9 cells were adjusted to a concentration of 0.1×10^6 cells/mL and incubated for 3 days with serial dilutions of the macrophage supernatant. The B9 cells were labeled with 2 µCi/mL [^{3}H]thymidine for the last 6.5 h of the incubation period and subsequently harvested using a PHD cell harvester (Cambridge Technology, Watertown, MA) onto glass filter paper. Radioactivity was assayed in a β scintillation counter (Beckman Instruments, Palo Alto, CA). Results were converted to micrograms per milliliter using a standard curve generated by serial dilutions of murine IL-6. Anti-murine IL-6 antibodies (1 µg/mL) completely blocked the proliferation of B9 cells incubated with 0.01 µg/mL murine IL-6. The data shown were the mean ± SD of triplicate analyses and were representative of at least three experiments.

IL-1 production

The relative amount of IL-1 in culture supernatants was measured using the IL-1-dependent T helper clone D10.G14 [18]. The D10 helper cells were diluted to 4×10^5 cells/mL and incubated for 3 days with 2.5 µg/mL concanavalin A (ConA) and serial dilutions of the culture supernatant to be tested. For measurement of membrane IL-1, the D10 cells and ConA were incubated for 3 days with macrophages fixed in 1% paraformaldehyde. The D10 cells were subsequently labeled for the last 6.5 h of the incubation period with 2 µCi/mL [^{3}H]thymidine and then harvested as described above. The bioassay was standardized using serial dilutions of human IL-1β (Cistron, Pinebrook, NJ) beginning at a concentration of 0.05 U/mL. Results shown were the mean ± SD of triplicate analyses and were representative of at least three experiments.

Other cytokine production

Levels of all other cytokines [IL-4, IL-12, tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ), IL-4, and transforming growth factor β (TGF-β)] were determined by enzyme-linked immunosorbent assay (ELISA). The data shown were the mean ± SD of three separate experiments (TNF-α), the mean ± SD of duplicate analyses representative of two to five experiments (IL-4, IL-12, and IFN-γ), or single determinations (TGF-β) representative of five separate experiments.

PGE_2 production

PGE_2 levels in supernatants from macrophages were measured by radioimmunoassay (Advanced Magnetics). The data shown, from a single experiment, were the mean ± SD of duplicate analyses and were representative of results obtained in three to ten individual experiments.

Nitric oxide production

Production of nitric oxide was estimated by measuring nitrite levels with the Griess reaction [19]. Equal volumes (50 µL) of Griess reagent (1:1 of 0.1% *N*-1 naphthylethylenediamine in H_2O and 1% sulfanilamide in 5% phosphoric acid) and sample were incubated together at room temperature for 10 min. Absorbance at 550 nm was determined in an automated microplate reader (Bio-Tek Instruments, Winooski, VT). The data shown (mean ± SD of duplicate analyses) were representative of results obtained in four separate experiments.

Macrophage-mediated cytotoxicity

Macrophage-mediated cytostasis/cytolysis [20] was tested using L1210 target cells. The murine macrophages were incubated with L1210 cells for 3 days at an effector-to-target ratio of 100:1. The L1210 cells and macrophages were labeled with 2 µCi/mL [^{3}H]thymidine for the last 6.5 h of the incubation period and then harvested and counted as described above. The percent cytolysis/cytostasis was calculated as 100 − [100 × cpm (L1210 + macrophage) experimental/cpm (L1210 + macrophage) control]. Results shown were the mean ± SD of triplicate analyses and were representative of three separate experiments. Incubation of the L1210 cells with HS 100 µg/mL in the absence of cytotoxic macrophages did not influence their viability.

Extraction of mRNA

Messenger RNA was isolated as follows from resting (unelicited) murine macrophages and B cells by acid guanidium thiocyanate-phenol-chloroform extraction [21]. Approximately 5×10^6 APC were lysed in 0.5 mL of guanidium thiocyanate solution (4 M guanidium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol). Sequentially, 50 µL of 2 M sodium acetate (pH 4.0), 0.5 mL phenol, and 0.1 mL chloroform/isoamyl alcohol (49:1) were added to the lysate with thorough mixing after each addition. The mixture was then incubated on ice for 15 min and centrifuged for 20 min at 10,000 *g*. The aqueous layer (containing the RNA) was removed, mixed with an equal volume of isopropanol, and placed at −20°C for at least 1 h. The mixture was centrifuged for 20 min at 10,000 *g* and the pellet that contained cellular RNA was resuspended in 0.3 mL of guanidium thiocyanate solution. The RNA was reprecipitated in isopropanol as described above,

washed one time in 75% methanol, vacuum dried, then resuspended in approximately 25 µL water.

Analysis of mRNA

RNA samples of equal amounts were incubated with 300 µL of 6.15 M formaldehyde in 5× saline sodium citrate (SSC) for 15 min at 65°C. Four hundred microliters of 5× SSC were then added to each sample. Serial dilutions of the samples were applied under vacuum to a nylon membrane, using a slotted filtration manifold (Minifold II, Schleicher & Schuell, Keene, NH). The membrane was then baked at 80°C for 1 h. Oligonucleotide probes end-labeled with [^{32}P]ATP were added to the hybridization solution (50% formamide, 5× SSC, 2× Denhardt's solution, 15 mM $NaPO_4$, 0.1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 0.1 mg/mL denatured salmon sperm DNA) to achieve a final concentration of 10^6 cpm/mL. The hybridization solution was incubated with the nylon membranes at 42°C for 18–24 h. The membranes were washed twice in 6× SSC, 0.1% SDS at 45°C for 15 min, twice in 2× SSC, 0.1% SDS at 25°C for 10 min, and once in the latter at 45°C for 15 min. The membranes were exposed to X-ray film (Kodak XAR-2) for 12–24 h at −70°C using an intensifying screen, after which the X-ray film was scanned (OneScanner, Apple Computer, Cupertino, CA). The mean intensity of the bands ± SD was shown.

Detection of costimulatory molecules on macrophages

Cultured thioglycollate-elicited peritoneal macrophages were plated in 96-well plates at a concentration of 1×10^6/mL. Cell surface antigens were detected by ELISA as follows. After fixation in 1% paraformaldehyde, nonspecific binding was blocked by incubation with 1% bovine serum albumin (BSA) and 2 µg/mL Fc block (PharMingen) for 1 h. After washing with 0.5% BSA and 0.1% Na azide in PBS, the macrophages were incubated with the biotinylated primary antibody on ice for 1 h. The plates were again washed and a streptavidin-linked alkaline phosphatase (PharMingen) was added for 30 min. The plates were washed and developed for approximately 30 min, then read at a wavelength of 405 λ. Results shown were the mean optical density ± SE of triplicate analyses and were representative of four separate experiments.

RESULTS

Expression of co-stimulatory molecules

The ability of various glycosaminoglycans to alter the expression of costimulatory molecules by murine peritoneal macrophages was tested. Although the frequency of contaminating lymphocytes was not tested, extensive depletion experiments have shown that the influence of HS is on APCs and not on lymphocytes [10]. Thioglycollate-elicited macrophages were cultured for 7–10 days to attain a quiescent state, as measured by a low cell-surface expression of I-A^d (**Fig. 1**). Exposure to 100 µg/mL HS for a period of 24 h increased the expression of ICAM-1 and I-A in comparison to untreated controls. This level was comparable to that achieved in macrophages stimulated with LPS. Although chondroitin sulfate (CS) and dermatan sulfate (DS) may have a small effect on I-A^d expression in the experiment shown, this result was not consistently reproducible. Unlike LPS, HS only intermittently (approximately 20% of experiments performed) increased the expression of B7-2 and did not alter the expression of CD40 (not shown).

Four lines of evidence indicated that the effects of HS on macrophages were not due to contaminating substances such as endotoxin or proteins. First, the presence of endotoxin was undetectable in the glycosaminoglycans with the use of *Limulus* amebocyte assay (Biowhittaker, Walkersville, MD) with a sensitivity level of 0.10 EU/mL. Second, passage of HS through a filter that removes up to 1 µg endotoxin (Cape Cod Associates) did not abrogate the influence of HS. Third, HS-induced IL-1 production by macrophages or HS-induced splenocyte proliferation in the presence of low-dose ionomycin (which is primarily dependent on IL-1 and IL-6 production, data not shown) were abrogated by digestion of HS with nitrous acid, a treatment that does not degrade proteins or endotoxin. Fourth, production of IL-1 by macrophages in response to HS was sometimes greater

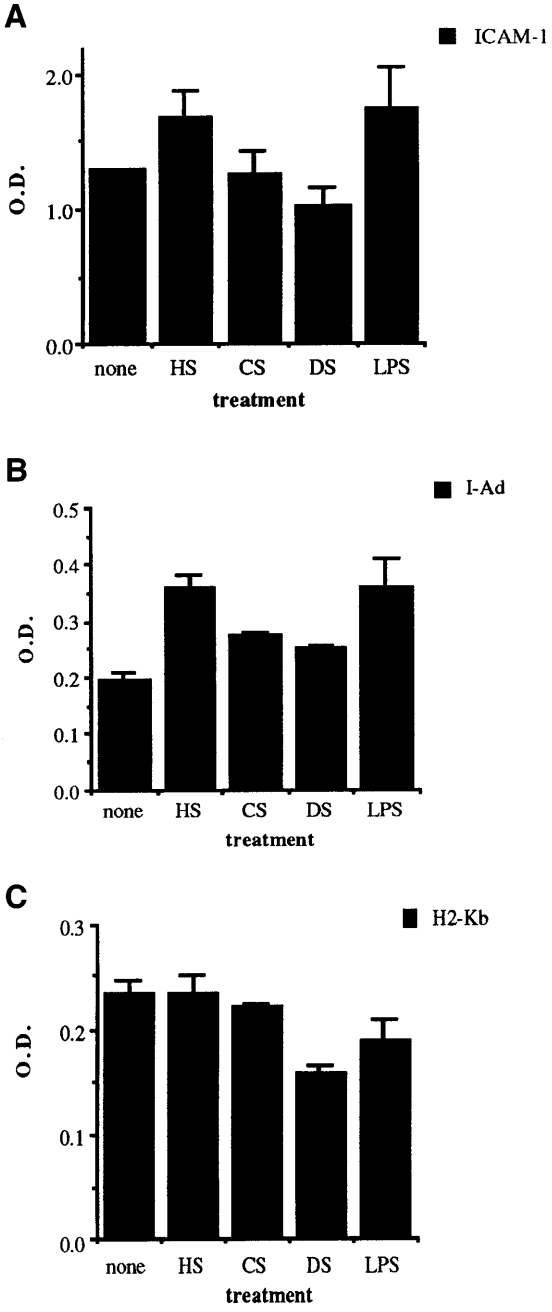

Fig. 1. Augmentation of costimulatory molecule expression by macrophages exposed to heparan sulfate. HS, CS, DS, or LPS (1 µg/mL) was incubated overnight with thioglycollate-elicited peritoneal macrophages from BALB/c mice that had been placed in culture 10 days before. All glycosaminoglycans were used at a concentration of 100 µg/mL. Cell surface expression of ICAM-1 (A), I-A^d (B), or H2-K^b (negative control) (C) was measured by ELISA (see Materials and Methods).

than that induced by optimal concentrations of LPS plus IFN-γ, suggesting that significant levels of endotoxin would need to be present to induce this response. Furthermore, LPS-dependent responses of splenocytes were not potentiated by HS over a wide range of concentrations, implying that HS did not add endotoxin to the system or augment the effect of low levels of endotoxin.

Effect of glycosaminoglycans on cytokine production by macrophages

Given the influence of HS on expression of costimulatory molecules, we asked whether production of cytokines might also be induced. At concentrations of 10–100 μg/mL, HS induced the production of cell membrane-associated IL-1 and soluble IL-1, IL-6, and TNF (**Fig. 2, A–D**). Heparin, DS, and CS had no effect on production of IL-1 or IL-6 by macrophages (data not shown).

The increase in cytokine production caused by HS was associated with increased levels of IL-1α, IL-1β, IL-6, and TNF mRNA (**Fig. 3**). Kinetic studies using HS to stimulate peritoneal macrophages revealed maximum expression of IL-6 and TNF at 4 h (Fig. 3, C and D). Maximum expression of IL-1α and IL-1β were not seen until 24 h after stimulation of macrophages was begun. Heparin, DS, and CS had little effect on mRNA expression of these cytokines (Fig. 3F). Consistent with the findings of Noble et al. [22], HA induced increases in expression of IL-1α, IL-1β, TNF, and IL-6 mRNA (as well as the corresponding proteins), however, the possibility that this effect might be caused by endotoxin contamination could not be completely ruled out in our studies.

Because IL-4 and IL-12 might drive T cell responses toward either Th2 or Th1 responses, the effect of glycosaminoglycans on IL-4 and IL-12 production was assessed. Because macrophages do not produce IL-4, but may indirectly alter the production of IL-4 by T cells, the influence of HS on IL-4 release in mixed lymphocyte cultures was assessed. HS was added to co-cultures consisting of irradiated splenocytes from C57BL/6 mice and splenocytes from BALB/c mice, and the level of IL-4 in the supernatants was determined. As **Figure 4A** shows, HS strongly inhibited IL-4 production in MLC. CS, DS, and heparin had little influence on IL-4 production (not shown). Because HS does not inhibit splenocyte proliferation when present throughout the entire culture period, and increases proliferation when present for the first 24 h of a MLC, the influence of HS on IL-4 is unlikely to be due to generalized toxicity [10].

In cultures of peritoneal macrophages, HS stimulated levels

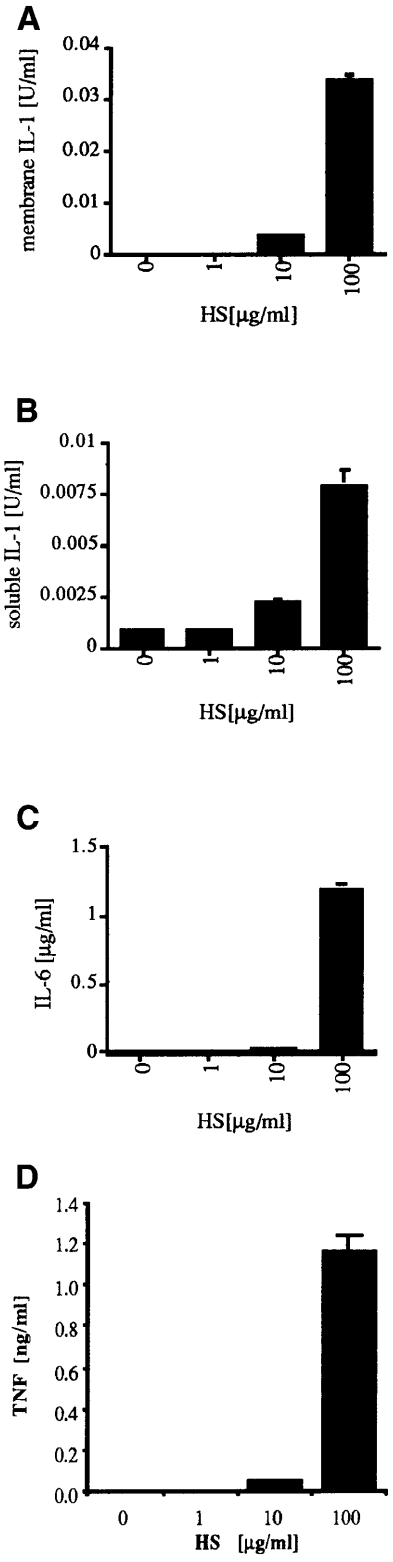

Fig. 2. Stimulation by heparan sulfate of the production of membrane IL-1, soluble IL-1, IL-6, and TNF-α by peritoneal macrophages. (A) Membrane IL-1: peritoneal macrophages obtained by thioglycollate injection were diluted to a concentration of 1×10^6 cells/mL, cultured (for 7 days before use to allow reversion to a quiescent state), incubated overnight with various concentrations of HS, then fixed in 1% paraformaldehyde. Membrane IL-1 activity was evaluated by bioassay based on proliferation of IL-1-dependent D10 T helper cells measured by [^{3}H]thymidine incorporation. Data were converted to U/mL of IL-1 activity based on a standard curve using recombinant human IL-1. (B) Soluble IL-1: IL-1 activity in the supernatants of 1×10^6 cultured, thioglycollate-elicited peritoneal macrophages incubated for 24 h with various concentrations of HS was determined by D10 bioassay. Proliferation of D10 cells plus ConA was 5355 cpm and proliferation of D10 cells plus ConA and HS 0.1 mg/mL was 5167 cpm. Data were converted to U/mL of IL-1 activity as in panel A. (C) IL-6: supernatants from cultured, thioglycollate-elicited peritoneal macrophages were incubated for 3 days with 0.1×10^6 B9 cells/mL. B9 proliferation was determined by [^{3}H]thymidine incorporation. Proliferation of B9 cells alone was 2040 cpm and proliferation of B9 cells plus HS 0.1 mg/mL was 3638 cpm. Data were converted to μg/mL of IL-6 based on a standard curve using recombinant murine IL-6. (D) TNF-α: TNF-α levels in the supernatants of thioglycollate-elicited peritoneal macrophages, cultured with heparan sulfate for 24 h, were measured by ELISA.

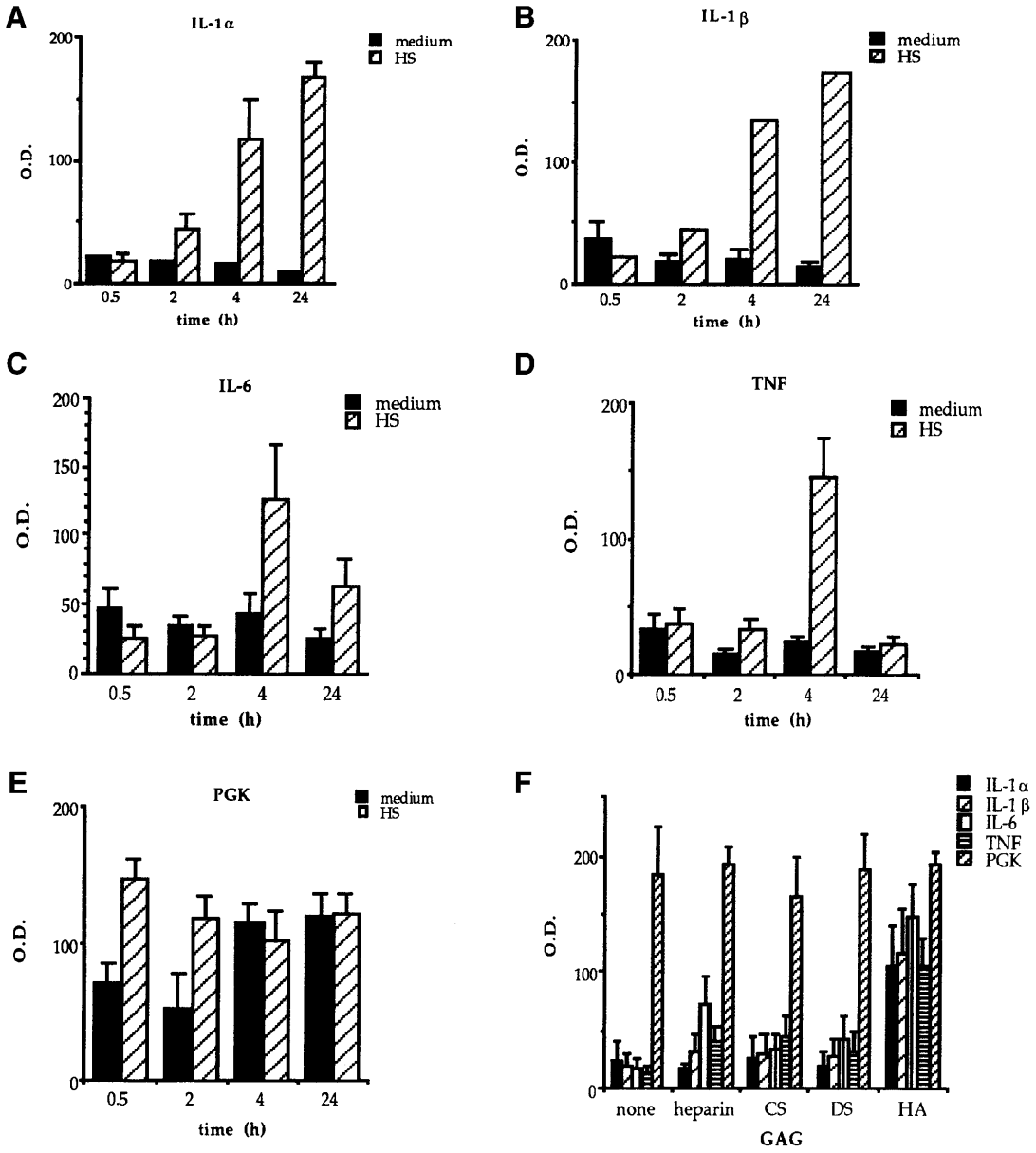

Fig. 3. Effect of heparan sulfate and other glycosaminoglycans on the expression of IL-1α, IL-1β, IL-6, and TNF mRNA by murine peritoneal macrophages. (A–E) Kinetics of the effect of HS on expression of IL-1α (A) , IL-1β (B), IL-6 (C), and TNF mRNA (D). Resident peritoneal macrophages were incubated with 100 μg/mL HS for the times indicated. Messenger RNA was isolated by acid guanidium thiocyanate-phenol-chloroform extraction and applied to a nylon membrane. Expression of phosphoglycerate kinase (PGK) (E) was measured as a standard. Blots were hybridized with radiolabeled oligonucleotide probes, exposed to X-ray film for 2 h, and scanned. The mean intensity of the bands ± the standard deviation is shown (F). Heparin, CS, or DS do not alter the expression of cytokine message. Resident peritoneal macrophages were incubated with 100 μg/mL of the indicated glycosaminoglycans for 3 h. Messenger RNA was extracted and blots were prepared as described in panel A. Expression of PGK was measured as a standard.

of IL-12 comparable to those stimulated by incubation with LPS (Fig. 4B). While having no influence on the release of cytokines previously tested, heparin induced the production of a significant amount of IL-12. The production of IL-12 in response to heparin did not result from contamination by endotoxin, because heparin was endotoxin-free based on a *Limulus* amebocyte lysate assay with a sensitivity of 0.1 EU/mL. Nor was this result an artifactual effect of heparin on the ELISA, because addition of heparin to the IL-12 standard did not alter results obtained with IL-12 alone. Thus, heparan sulfate and heparin may promote Th1-type responses.

In light of the potential effects of HS and heparin on Th differentiation, we asked whether these GAG might also induce the release of IFN-γ and TGF-β, which in turn might further influence T cell responses toward Th1 or Th2-type immunity. Thioglycollate-elicited macrophages, cultured for 7–10 days, were incubated for 24 h with HS, heparin, CS, or DS. HS caused a slight increase in IFN-γ levels in culture supernatants, whereas heparin induced a significant increase in IFN-γ production (Fig. 4C). Conversely, HS stimulated the production of TGF-β, whereas heparin had either a lesser effect or no effect on TGF-β production over a series of seven separate experiments (Fig. 4D). Given the magnitude of the influence of heparin on IL-12 and IFN-γ, and the combined effects of HS on

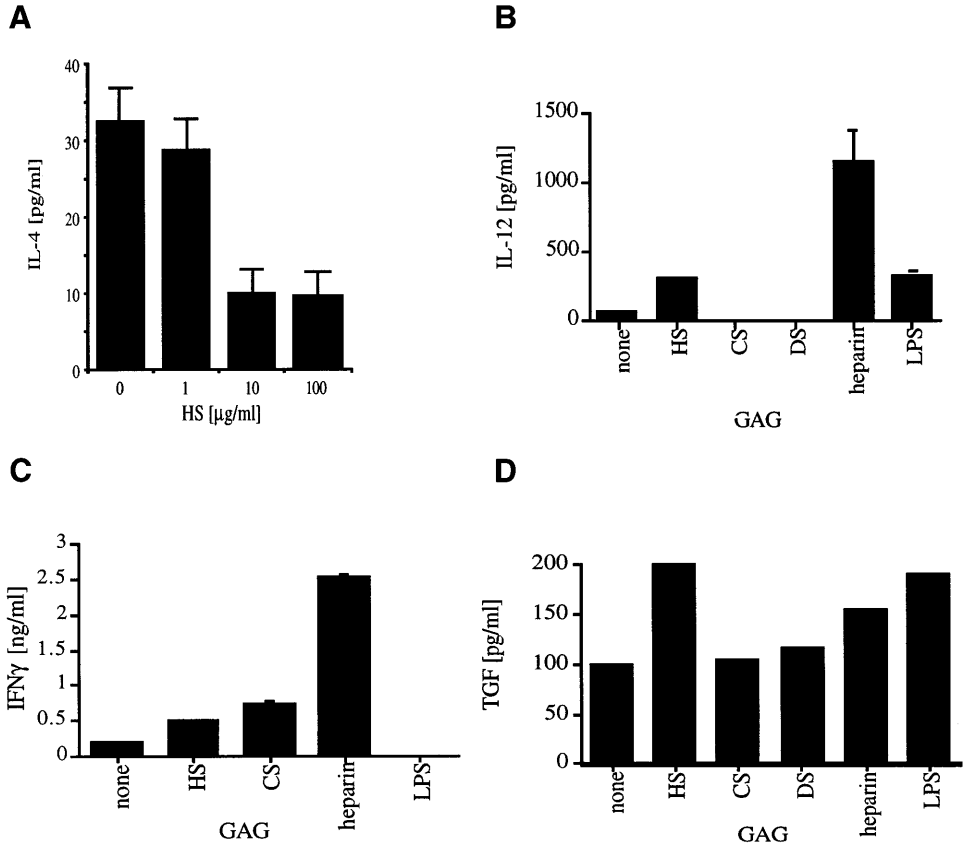

Fig. 4. Alteration by glycosaminoglycans of Th1- and Th2-type cytokine release by T lymphocytes and macrophages. (A) HS inhibits IL-4 production by activated T lymphocytes. HS (100 µg/mL) was added to MLCs consisting of irradiated H-2^b splenocytes as stimulator cells and H-2^d splenocytes as responder cells. IL-4 levels in supernatants removed 24 h later were measured by ELISA. Results are expressed as pg/mL and are representative of at least three experiments. (B) Heparin is a potent inducer of IL-12 production by macrophages. Cultured, thioglycollate-elicited peritoneal macrophages were incubated for 24 h with the indicated glycosaminoglycans at 100 µg/mL. IL-12 levels in culture supernatants were determined by ELISA. (C) Heparin stimulates IFN-γ production by macrophages. Thioglycollate-elicited peritoneal macrophages were incubated for 24 h with the indicated glycosaminoglycans at 100 µg/mL. IFN-γ levels were determined by ELISA. (D) HS elicits TGF-β production by macrophages. TGF-β levels in cultured, thioglycollate-elicited macrophages exposed to the indicated glycosaminoglycans for 24 h were measured by ELISA.

IL-12 and IL-4, these results suggest that the availability of HS or heparin to APC in inflamed tissue or tissue transplants might promote the development of Th1-type immune responses. On the other hand, because HS stimulates macrophages to produce IL-1, IL-6, and TGF-β, it is possible that the presence of HS in inflamed tissues could also promote Th2 differentiation, which may be stimulated by these cytokines [23, 24].

PGE_2 production in macrophages

Eicosanoids such as PGE_2 represent another class of immunoregulatory agents produced by macrophages [25]. PGE_2 inhibits production of IL-12 and promotes Th2-type immune responses *in vitro* [26]. We have demonstrated that HS elicits PGE_2 production by macrophages [12] and that it is the production of PGE_2 that explains the paradoxical properties of heparan sulfate illustrated by our previous data in MLC (the presence of HS during the early days of a MLC enhances proliferation, whereas addition later in the culture period inhibits proliferation). We now tested whether other glycosaminoglycans would evoke a similar response in MLC-activated APC. Exposure of MLC-activated APC to 100 µg/mL of CS, DS, or heparin did not induce production of PGE_2, whereas incubation with 100 µg/mL HS did, consistent with our previous work (**Fig. 5**, **A** and **B**). Thus, the presence of heparan sulfate early in immune responses may serve to enhance immunity or promote Th1 responses as shown above, yet later in the immune response heparan sulfate may serve to down-regulate Th1 responses and return cells to quiescence.

Nitric oxide (NO) metabolism by macrophages

NO, at high doses, inhibits cellular immune responses by impairing mitochondrial respiration and by causing DNA damage [27]. Because macrophages can be a significant source of NO, we asked whether glycosaminoglycans stimulate the release of NO by macrophages. NO production was tested by measuring levels of nitrite, a stable metabolite, in the supernatants of macrophages incubated with glycosaminoglycans for 24 h. All glycosaminoglycans tested induced production of NO by murine macrophages (**Fig. 6**), however, CS and DS were more effective at doing so than HS. Exposure to heparin caused the macrophages to release NO in quantities comparable to those induced by HS. It is interesting that the induction of NO production by HS and CS was much less dependent on extracellular arginine than NO production induced by conventional stimuli such as LPS (data not shown). Thus, glycosaminoglycans modify NO metabolism in macrophages and this process appears to be separate from the pathway(s) leading to production of cytokines and PGE_2 by APC.

Macrophage-mediated cytotoxicity

Secretion of TNF [28], IL-1 [29, 30], and NO [31] by macrophages exposed to glycosaminoglycans might cause damage to nearby cells. Whether glycosaminoglycans thus elicit or enhance macrophage-mediated cytotoxicity was tested using L1210 leukemia cells as targets. HS caused macrophages that had been primed by incubation for 24 h with IFN-γ [32] to induce cell death in the L1210 leukemia cells (**Fig. 7**). HS was

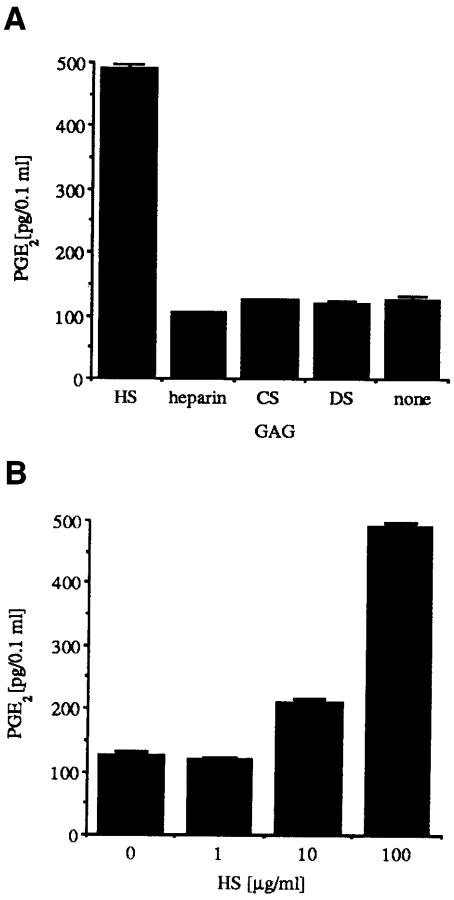

Fig. 5. Influence of heparan sulfate and other glycosaminoglycans on PGE_2 production by antigen-presenting cells. (A) Heparin, CS, and DS do not increase PGE_2 production in APC activated by alloantigen. Glycosaminoglycans at 100 μg/mL were added to MLCs of murine splenocytes 3 days after onset of the culture period. PGE_2 levels in the supernatants collected 24 h after addition of the glycosaminoglycans were measured by radioimmunoassay. (B) HS mediates PGE_2 production by APC in MLCs consisting of irradiated H-2^b splenocytes as stimulator cells and H-2^d splenocytes as responder cells. HS at 1–100 μg/mL was added to MLCs 3 days after initiation of the culture. PGE_2 levels in the supernatants collected 24 h after addition of HS were measured by radioimmunoassay.

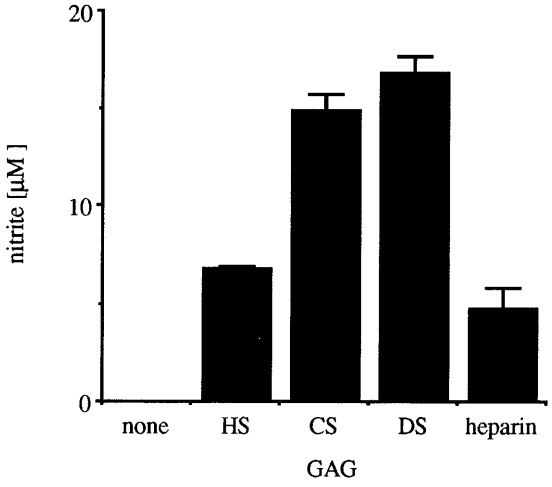

Fig. 6. Modulation by glycosaminoglycans of nitrite production by peritoneal macrophages. Thioglycollate-elicited peritoneal macrophages at a concentration of 1×10^6 cells/mL were incubated with 100 μg/mL glycosaminoglycan for 24 h. Nitrite levels in the supernatants were determined by the Griess reaction.

ineffective as a priming agent for cytolytic responses elicited by HS or by LPS (not shown). CS, DS, and heparin did not induce macrophage-mediated cytotoxicity (Fig. 7B), suggesting that the killing of target cells was not mediated by NO. Cytotoxicity induced by HS probably reflected in part the production of TNF because TNF-resistant P815 mastocytoma cells were not killed by HS-treated macrophages (not shown) and anti-TNF-αβ antibodies partially abrogated the cytotoxicity against TNF-sensitive targets (Fig. 7C).

B cell function

Because B cells may act as APC, experiments were conducted to determine whether HS altered B cell proliferation and/or cytokine production. At 100 μg/mL, HS augmented the proliferation of isolated B cells that were also stimulated with suboptimal concentrations of ionomycin (**Fig. 8A**). HS stimulated IL-1 production by isolated B cells (Fig. 8B), and by B cells separated by Percoll density gradient into low density (activated, $CD5^+$ cells) and high density (resting) populations (not shown). None of the other glycosaminoglycans studied (CS, DS, or heparin) stimulated the production of IL-1 by isolated B cells (data not shown). Exposure to HS increased expression of IL-1α, IL-1β, IL-6, and TNF mRNA by murine B cells (Fig. 8C). These results suggest that HS modulates B cell function *in vitro* and may influence the behavior of B cells *in vivo*.

DISCUSSION

Here we report that glycosaminoglycans, components of cell surfaces and extracellular matrices, induce changes in APC that would enhance the ability to stimulate T cell responses and mediate cytotoxicity. These findings support an overall concept that the extracellular environment in which activation of APC takes place influences the characteristics and magnitude of APC activation and thus may serve to condition immune responses that ensue. These findings are also of import in the analysis of APC function because the signals provided by HS and other GAG derive from the microenvironment of lymphoid organs and parenchymal tissues but are generally not considered in *in vitro* studies.

Our studies demonstrate that HS GAG activates macrophages and induces the production of several cytokines. Given that the macrophage preparation contained a few B cells and NK cells (6 and 3%, respectively), we cannot exclude the possibility that a small fraction of the cytokine production measured is coming from these cells. Because 5× more B cells are needed to produce amounts of IL-1 equivalent to that produced by macrophages (see Figs. 2B vs. 8B), any contribution from the B cell population is likely to be minimal.

Glycosaminoglycans usually exist *in vivo* as proteoglycans in which the glycosaminoglycan is covalently linked to a protein core (HS proteoglycan). In this form, the glycosaminoglycans of proteoglycans are probably not readily accessible to inflammatory cells. However, under conditions of acute or chronic inflammation glycosaminoglycans are severed from the protein core and thus solubilized by various means.

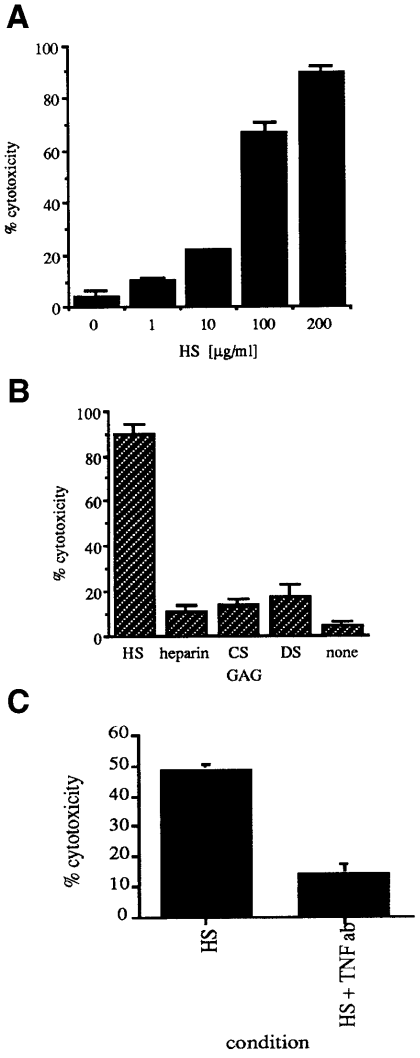

Fig. 7. Induction of cytotoxicity in peritoneal macrophages by heparan sulfate. (A) HS stimulates a dose-dependent increase in macrophage-mediated cytotoxicity. Cultured, thioglycollate-elicited peritoneal macrophages were incubated with IFN-γ 100 U/mL for 24 h, washed, then incubated with HS 100 μg/mL and L1210 leukemia cells at 0.01×10^6 cells/mL for 3 days. The amount of cytolysis/cytostasis of the L1210 cells was based on a decrease in incorporation of [^{3}H]thymidine. (B) Heparin, CS, or DS do not stimulate increases in macrophage-mediated cytotoxicity. Macrophage-mediated cytotoxicity was determined as described in Figure 6A. Glycosaminoglycans were used at 100 μg/mL. (C) Blockade of TNF partially abrogates HS-mediated induction of macrophage cytotoxicity. Macrophage-mediated cytotoxicity was determined as described above. A blocking anti-TNF-αβ antibody was added with HS to the L1210 leukemia cells.

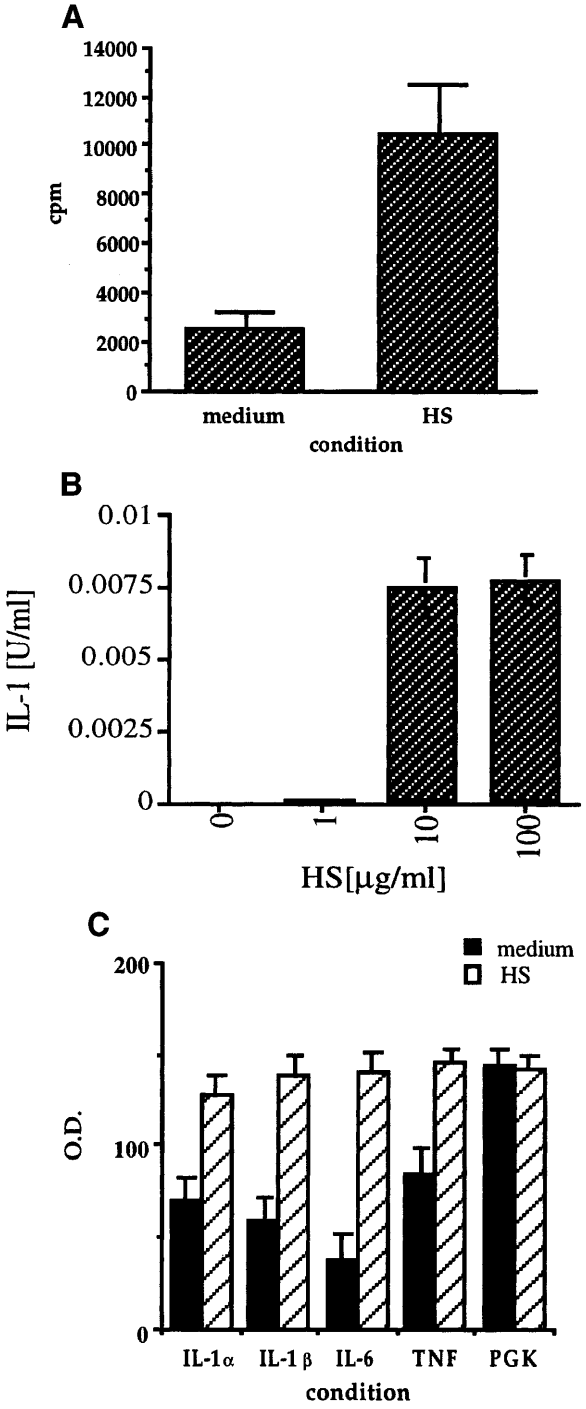

Fig. 8. Influence of heparan sulfate on B cell proliferation and cytokine production. (A) HS, in the presence of a suboptimal concentration of ionomycin, stimulates B cell proliferation. B cells isolated by immunodepletion from a murine spleen cell suspension were incubated with ionomycin 0.3 μg/mL in the presence or absence of HS 100 μg/mL. B cell proliferation was determined after 2 days by incorporation of [^{3}H]thymidine. (B) HS induces production of IL-1 by B cells. IL-1 activity in culture supernatants of 5×10^6 B cells/mL incubated for 24 h in the presence or absence of HS 1–100 μg/mL was based on D10 proliferation measured by incorporation of [^{3}H]thymidine. The data was converted to U/mL of IL-1 activity based on a standard curve using recombinant human IL-1. (C) HS induces the expression of IL-1α, IL-1β, IL-6, and TNF mRNA by B cells. B cells at a concentration of 3×10^6 cells/mL were incubated with medium or HS 100 μg/mL for 3 h. Slot blot analysis of mRNA levels of the indicated cytokines was performed as described in Figure 3. Expression of PGK, which should not be affected by activation of B cells, was measured as a standard. The mean intensity of the bands ± the standard deviation is shown.

Mechanisms underlying the solubilization of glycosaminoglycans primarily involve the action of proteases [33] and endoglycosidases [34], although other mechanisms such as the deaminative cleavage of HS by nitric oxide may be effective *in vivo* as well [35]. Many cells such as macrophages [36], liver cells [37], and activated T cells [38] express endoglucuronidases that might cleave HS glycosaminoglycans to yield fragments of sufficient size to mediate biological effects [33]. Activation of endothelial cells by exposure to anti-endothelial cell antibodies and complement [39] (which occurs in xenotransplantation), or interaction of activated T cells with endothelial cells [8], in fact causes an enzyme-induced release of HS. Manipulations inhib-

iting this loss, which based on our findings may diminish APC activation, prolong xenograft survival [40].

Serine proteases and metalloproteases, which participate in the normal turnover of the matrix but may be elevated in inflammatory states [41], cleave proteoglycans into soluble glycosaminoglycan-peptide fragments. In rheumatoid arthritis and degenerative joint disease, elevated levels of proteinases [42], proteoglycan fragments [43, 44], and IL-1 [45] have been found in the synovial fluid of these patients. Based on our findings, HS glycosaminoglycan may quite possibly contribute to these increased levels of IL-1. It is interesting that administration of metalloprotease inhibitors has been shown to diminish symptoms in patients with this disease process [46]. Another mechanism that may increase the availability of glycosaminoglycans is an increase in glycosaminoglycan synthesis [47, 48] or composition stimulated by local cytokines [49, 50].

The alterations in APC function were induced by relatively high concentrations of HS glycosaminoglycan. Although these concentrations might seem supraphysiological, they may in fact represent local concentrations occurring *in vivo*. The concentration of heparan sulfate in rabbit synovial fluid, for example, is 80 μg/mL [51]. We have previously shown that relatively large amounts of HS are needed to recapitulate HS-mediated biochemical changes seen in whole organs and tissues [52, 53], presumably because smaller amounts of soluble glycosaminoglycans in tissues are restricted to relatively confined spaces (such as intercellular). For example, we have found that 0.1–1 μg/mL HS are needed to reverse the developmental effects induced by only a 20% decrease in HS synthesis in the thymus [52]. A difference of even greater magnitude has been seen in evaluating the role of HS in kidney development [53].

Tissue-specific influences on immunity have been described [54, 55]; however, underlying mechanisms are largely unknown. The glycosaminoglycan composition of a given tissue, and distinct biochemical mechanisms that release these glycosaminoglycans, could underlie some tissue-specific aspects of immune responses. For example, tissues rich in CS or DS (such as cartilage and skin) might tend to increase NO production by local APC; tissues rich in HS, such as the kidney or endothelial cell surfaces, would tend to stimulate upregulation of I-A and ICAM-1 and increase production of IL-1, IL-6, IL-12, TGF-β, and PGE_2, but not NO or IL-4. Because activated mast cells release heparin, tissues rich in mast cells may have increased concentrations of IL-12 and IFN-γ. It is interesting that connective tissue mast cells contain primarily heparin, whereas pulmonary mast cell granules contain only approximately 60% heparin, and intestinal mast cells release mainly chondroitin sulfate [14]. This heterogeneity may contribute to the differences in cytokine responses obtained after administration of antigen at mucosal vs. nonmucosal sites [56].

The potent stimulation of IFN-γ release in peritoneal macrophages by heparin is of particular interest because direct evidence of IFN-γ production by macrophages has only recently been described. Because IL-12 has been shown to induce IFN-γ production in murine peritoneal macrophages, it is possible that the effect of heparin is an indirect one mediated by IL-12 [57].

Our findings thus support a model wherein glycosaminoglycan metabolism contributes to the control of immune responses engendered at sites of tissue injury. Local glycosaminoglycan concentrations may be heightened during inflammation due to increased production and/or increased degradation. The elevated levels of free glycosaminoglycan chains contribute to the activation of APC, which in turn propagate inflammatory responses through induction of cytokine production, release of NO, costimulatory molecule expression, and increased numbers of cytotoxic macrophages. Immune responses to an antigenic stimulus delivered *in vivo* may therefore reflect a complex interaction involving cellular components, inflammatory mediators, and the local environment.

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