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Monocyte Cell Surface Glycosaminoglycans Positively Modulate IL-4-Induced Differentiation toward Dendritic Cells

Els den Dekker,* Sander Greffe,* Tonnie Huijs,* Gerdy B. ten Dam,† Elly M. M. Versteeg,† Lieke C. J. van den Berk,* Bellinda A. Bladergroen,* Toin H. van Kuppevelt,† Carl G. Figdor,* and Ruurd Torensma2*

IL-4 induces the differentiation of monocytes toward dendritic cells (DCs). The activity of many cytokines is modulated by glycosaminoglycans (GAGs). In this study, we explored the effect of GAGs on the IL-4-induced differentiation of monocytes toward DCs. IL-4 dose-dependently up-regulated the expression of DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN), CD80, CD206, and CD1a. Monocytes stained positive with Abs against heparan sulfate (HS) and chondroitin sulfate (CS) B (CSB; dermatan sulfate), but not with Abs that recognize CSA, CSC, and CSE. Inhibition of sialylation of monocyte/DC cell surface GAGs by sodium chlorate reduced the reactivity of sulfate-recognizing single-chain Abs. This correlated with hampered IL-4-induced DC differentiation as evidenced by lower expression of DC-SIGN and CD1a and a decreased DC-induced PBL proliferation, suggesting that sulfated monocyte cell surface GAGs support IL-4 activity. Furthermore, removal of cell surface chondroitin sulfates by chondroitinase ABC strongly impaired IL-4-induced STAT6 phosphorylation, whereas removal of HS by heparinase III had only a weak inhibitory effect. IL-4 bound to heparin and CSB, but not to HS, CSA, CSC, CSD, and CSE. Binding of IL-4 required iduronic acid, an N-sulfate group (heparin) and specific O sulfates (CSB and heparin). Together, these data demonstrate that monocyte cell surface chondroitin sulfates play an important role in the IL-4-driven differentiation of monocytes into DCs. The Journal of Immunology, 2008, 180: 3680–3688.

Interleukin-4 (IL-4) is a pleiotropic cytokine that plays a major role in immune and inflammatory responses. It is produced by Th-type 2 cells in response to Ag receptor engagement and by basophils and mast cells upon cross-linking of the high-affinity receptor for IgE (1–3). IL-4 exerts its effects via the IL-4R of which two types have been identified that are composed of a common α-chain (γ; type I IL-4R) or an IL-13Rα1 chain (type II IL-4R) (4, 5). Activation of the IL-4R leads to signaling cascades through activation of the Janus family tyrosine kinases and phosphorylation of STAT6 (6, 7).

A wide variety of cells express the IL-4R which allows IL-4 to regulate many biological processes (7). IL-4 is involved in the immune response where it is abundant for the development of Th2 cells, suppresses the appearance of Th-type 1 (Th1) cells, and controls the Ig class switching of B cells to IgE (1, 8, 9). IL-4 is also able to stimulate Th1 responses when it is present during the initial period of activation of APCs (10). In hematopoiesis, IL-4 enhances stromal cell-dependent proliferation of myeloid cells and adherence of hematopoietic progenitor cells to stroma cells (11). IL-4 is involved in tissue adhesion and inflammation as IL-4 up-regulates the expression of VCAM-1 and E-selectin on vascular endothelial cells and increases the adhesion of inflammatory cells to vascular endothelial cells (12). IL-4 acts on monocytes to differentiate them toward dendritic cells (DCs),3 and is used in combination with GM-CSF to generate monocyte-derived DCs in vitro (13, 14).

The activity of cytokines can be regulated on many levels. One of the regulatory mechanisms involves binding to glycosaminoglycans (GAGs). GAGs are linear polysaccharides consisting of repeating disaccharide units in which one residue is an amino sugar (D-glucosamine or D-galactosamine) and the other residue is either a hexuronic acid (D-glucuronic acid or L-iduronic acid) or galactose. GAGs can be divided into four groups which differ in the basic disaccharide composition: heparan sulfate (HS) and heparin, keratan sulfate, chondroitin sulfate, and hyaluronic acid. Differences in chain length, linkage position of saccharides, and modification by (de)acetylation, N- and O-sulfation, and epimerization creates a large heterogeneity within each group of GAGs (15). GAGs are ubiquitously expressed and can be soluble, surface bound, or shed as soluble ectodomains. With the exception of hyaluronic acid, GAGs are usually covalently attached to a protein core, forming proteoglycans. Activated leukocytes such as monocytes/macrophages, NK cells, T cells, mast cells, and basophils secrete proteoglycans which are released as a consequence of extracellular matrix degradation (16). At inflammatory sites, GAG structure and location are altered, which serves to modify the activity of GAG-dependent soluble and cell surface effectors of the inflammatory process (16).

Many cytokines bind to GAGs and this has been shown to 1) protect stromal cell-derived factor 1 (SDF), fibroblast growth...
factor (FGF), and IFN-γ from proteolysis (17–19), 2) promote oligomerization of MIP-1α, MIP-1β, and IFN-γ (20, 21), 3) create gradients of FGF (22), and 4) promote presentation of RANTES, MCP-1, IL-8, MIP-1β, and IFN-γ to their receptors (23–25). Also, IL-4 has been shown to bind to GAGs (26). However, no detailed information is available on the effect of GAG binding on IL-4 activity. Therefore, we set out to investigate the putative cooperative effect of GAGs in the function of IL-4.

Materials and Methods
Isolation of CD14+ monocytes
Buffy coats were obtained after informed consent from healthy donors from Sanquin Bloodbank Rivierenland (Nijmegen, The Netherlands). PBMC were isolated by lymphoprep centrifugation (1.077 g/ml; Axis Shield), followed by selection with anti-CD14 beads according to the manufacturer’s instructions using the autoMACS separator (Miltenyi Biotec). Isolated monocytes were >90% CD14+.

Cell culture
Monocytes were cultured at a density of 150,000 cells/cm² in RPMI 1640 medium containing 10% (v/v) heat-inactivated FCS, 10,000 U/ml penicillin, 10,000 U/ml streptomycin, 25 μg/ml amphotericin, 450 U/ml GM-CSF (Schering-Plough), and different concentrations of IL-4 (Schering-Plough) at 37°C in a humidified atmosphere with 5% CO2 for 6 days. GM-CSF and IL-4 were added as a single dose at the start of the culture. When indicated, different concentrations of sodium chloride (Aldrich Chemicals) were added at the start of the culture (27).

Analysis of cell surface markers and cell surface GAG expression
The expression of cell surface markers on monocytes cultured for 6 days was determined by flow cytometry on a FACSCalibur (BD Biosciences). Expression of CD1a was determined with a directly FITC-conjugated mAb (IgG1a, 5 μg/ml; BD Biosciences) using IgG1-FITC (5 μg/ml; BD Biosciences). Expression of CD1a was determined with a directly FITC-conjugated mAb (IgG1a, 5 μg/ml; BD Biosciences) using IgG1-FITC (5 μg/ml; BD Biosciences) as isotype control (5 μg/ml; BD Biosciences). Expression of DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) and CD1a was determined by flow cytometry on a FACSCalibur (BD Biosciences).

Digestion of monocyte cell surface GAGs
Freshly isolated monocytes were cultured overnight in an Ultra Low Cluster Plate (Corning) in culture medium with 1% (v/v) FCS. The next day, the cells were harvested and treated for 3 h at 37°C with 2 U/ml chondroitinase ABC (Sigma-Aldrich), which digests the linkage between N-acetylgalactosamine and glucuronic acid or iduronic acid, or with 0.4 U/ml heparinase III (Grampian Enzymes), which digests heparan sulfate at the 1–4 linkage between hexosamine and glucuronic acid, in RPMI 1640 medium containing 10% FCS.

Table I. Characteristics of GAGs used to define structures involved in binding of IL-4

<table>
<thead>
<tr>
<th>GAG</th>
<th>Characteristics</th>
<th>Obtained from</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>Porcine intestinal mucosa</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>HS</td>
<td>Bovine kidney</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>CSA</td>
<td>Chondroitin-4-sulfate from bovine trachea</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>CSB (dermatan sulfate)</td>
<td>Porcine intestinal mucosa</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>CSC</td>
<td>Chondroitin-6-sulfate from shark cartilage</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>CSD</td>
<td>2,6-di-O-sulfated chondroitin sulfate from shark cartilage</td>
<td>Seikagaku</td>
</tr>
<tr>
<td>CSE</td>
<td>4,6-di-O-sulfated chondroitin sulfate from squid cartilage</td>
<td>Seikagaku</td>
</tr>
<tr>
<td>4/2,4-CSB</td>
<td>4/2,4-di-O-sulfated CSF from Styela plicata (59) 28%</td>
<td>Dr. M. S. G. Pavao (Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil)</td>
</tr>
<tr>
<td>2,6-CSB</td>
<td>2,6-di-O-sulfated CSF from Ascidia nigra (60) 11.3% NAc, 88.7% NS, 69% 2OS, 79% 6OS</td>
<td>Dr. M. S. G. Pavao (Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil)</td>
</tr>
<tr>
<td>Heparin*</td>
<td>Completely desulfated/N-acetylated heparin</td>
<td>Seikagaku</td>
</tr>
<tr>
<td>CdSNSAc heparin</td>
<td>Completely desulfated/N-acetylated heparin</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>NdSNSAc heparin</td>
<td>N-desulfated/N-acetylated heparin 100% NAc, 0% NS, 69% 2OS, 79% 6OS</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>2OdS heparin</td>
<td>2-O-desulfated heparin 13% NAc, 87% NS, 0% 2OS, 79% 6OS</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>6OdS heparin</td>
<td>6-O-desulfated heparin 13% NAc, 87% NS, 67% 2OS, 23% 6OS</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ods</td>
<td>O-desulfated heparin</td>
<td>Neoparin</td>
</tr>
<tr>
<td>Carboxyl-reduced heparin</td>
<td>Uronic acid COOH-reduced heparin</td>
<td>Neoparin</td>
</tr>
</tbody>
</table>

* Heparin and its modifications are from porcine intestinal mucosa.
IL-4-induced STAT6 phosphorylation

Cells that were treated with chondroitinase ABC, heparinase III, or were left untreated, were resuspended in RPMI 1640 at a density of $7.5 \times 10^7$ cells/ml and were stimulated with different concentrations of IL-4 for 5 min at 37°C. Ice-cold lysis buffer was added (10 mM Tris (pH 7.8), 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, 10 mM pyrophosphate, 50 mM NaF) and cells were lysed for 45 min on ice. Insoluble material was removed by centrifugation and Laemmli sample buffer was added. After heating for 5 min at 95°C, samples were subjected to SDS-PAGE and Western blotting. Membranes were blocked with TBS (50 mM Tris-HCl (pH 7.5), 150 mM NaCl) supplemented with 0.2% Tween 20, 2% nonfat dry milk and 2% BSA. STAT6-PY641 was detected with a mAb (clone 18, 1:250; BD Biosciences;) and a peroxidase-labeled rabbit secondary Ab (1:3000; DakoCytomation). Bands were visualized by ECL (Pierce). After stripping of the blots, total STAT6 was detected with a rabbit polyclonal Ab (1:500; Santa Cruz Biotechnology) and a peroxidase-labeled secondary swine anti-rabbit Ab (1:3000; DakoCytomation). Pixel densities of the bands were quantified with Image J (NIH) on films with unsaturated pixel densities.

Binding of IL-4 to GAGs

Binding of IL-4 to GAGs was assessed by ELISA. Wells of a 96-well Microlon ELISA plate (Greiner Bio-one) were coated with GAGs (Table I) by overnight incubation with 100 μl of a 10 μg/ml solution. Coating of these GAGs has been analyzed in previous studies (32, 33). After coating, the wells were rinsed with PBS supplemented with 0.1% (v/v) Tween 20 (Sigma-Aldrich) (PBST) and blocked for 1 h with PBST supplemented with 2% BSA. Subsequently, wells were washed and incubated overnight with 460 U (50 ng) IL-4 in 100 μl of PBST supplemented with 2% BSA per well. Bound IL-4 was detected with sheep anti-hIL-4 (Endogen) followed by incubation with alkaline phosphatase-conjugated donkey anti-sheep (Sigma-Aldrich). Enzyme activity was detected using para-nitrophenyl phosphate (1 mg/ml; MP Biomedicals) in 1 M diethanolamine supplemented with 0.5 mM MgCl2. The absorbance at 405 nm was determined on a Benchmark Plus spectrophotometer (Bio-Rad).

Statistics

Statistical analysis was performed using SPSS software. Multiple comparisons were performed with ANOVA. For single comparisons, a two-tailed paired Student’s t test was used. Differences were considered statistically significant at $p < 0.05$.

Results

IL-4 regulates DC-SIGN, CD80, CD206, and CD1a expression in a dose-dependent manner

Monocytes can be differentiated toward DCs by IL-4 and GM-CSF, which results in up-regulation of markers such as DC-SIGN, CD80, CD1a, and CD206 (mannose receptor) and the ability to present Ags and stimulate T cell proliferation (13). The induction of these markers has been shown to depend on IL-4 (34–36). To determine the optimal concentration of IL-4 required for the induction of these markers, monocytes were cultured with sheep anti-hIL-4 (Endogen) followed by incubation with alkaline phosphatase-conjugated donkey anti-sheep (Sigma-Aldrich). Enzyme activity was detected using para-nitrophenyl phosphate (1 mg/ml; MP Biomedicals) in 1 M diethanolamine supplemented with 0.5 mM MgCl2. The absorbance at 405 nm was determined on a Benchmark Plus spectrophotometer (Bio-Rad).
from bovine trachea (containing CSA), shark cartilage (containing CSC), and squid cartilage (containing CSE) (32), did not react with GAGs on the cell surface of monocytes. The Abs LKN1 and GD3A12 that specifically recognize CSB (33) reacted with surface-expressed GAGs on monocytes. These results demonstrate that monocytes express HS and CSB on their cell surface.

**Sodium chlorate reduces Ab recognition of sulfated GAGs, suppresses expression of IL-4-induced DC markers, and suppresses DC-induced T cell proliferation**

The role of GAG sulfation was elucidated by experiments with sodium chlorate, which competitively inhibits the formation of 3′-phosphoadenosine 5′-phosphosulfate, the high-energy sulfate donor in cellular sulfation reactions. Sodium chlorate reduces GAG sulfation when added to Madin-Darby canine kidney cells at a concentration between 5 and 20 mM (39). To test whether sodium chlorate also reduces GAG sulfation of monocyte-derived DCs, different concentrations (0–20 mM) of sodium chlorate were added to the monocyte/DC culture medium. After culturing for 6 days, the cells were stained for several markers and staining was analyzed by flow cytometry. Only cells that were in the live gate on the FACS were analyzed, based on their forward-sideward scatter profile. The percentage of cells in the live gate was the same for chlorate-treated cells as compared with control (untreated) cells. Cell viability was also determined with trypan blue, and showed the same percentage of trypan blue-positive cells for chlorate-treated cells as compared with control (untreated) cells. This indicates that sodium chlorate was not toxic for the cells at the concentrations used. The reactivity of HS4C3 which recognizes sulfated HS, with monocyte cell surface HS, showed a dose-dependent decrease (Fig. 3A). However, due to large variations in staining between different donors, the differences between chlorate-treated cells and control cells were not statistically significant. Sodium chlorate dose-dependently reduced the binding of GD3A12 which recognizes sulfated CSB (G. B. ten Dam, unpublished data) (Fig. 3A). These data demonstrated that the level of sulfation of monocyte/DC cell surface GAGs is decreased by sodium chlorate. Sodium chlorate also dose-dependently reduced the IL-4-induced DC-SIGN and CD1a expression, but did not affect the expression of CD11b and MHC-I (Fig. 3A). Although the uptake of dextran-Alexa-647 by DCs was not affected by sodium chlorate (data not shown), the potency of DCs to stimulate PBL proliferation did decrease with increasing sodium chlorate concentrations (Fig. 3B). Interestingly, the decreased ability of IL-4 to induce expression of DC-SIGN and CD1a and to confer PBL stimulatory properties strongly correlated with a decrease in the recognition of sulfated cell surface GAGs. Together, these results underline the role of sulfated monocyte cell surface GAGs in IL-4-mediated effects.

**Digestion of monocyte cell surface chondroitin sulfates decreases IL-4-induced STAT6 phosphorylation**

To further establish the role of cell surface GAGs on IL-4 activity, monocytes were treated with heparinase III or with chondroitinase ABC. Subsequently, signaling to STAT6 by IL-4 was assessed by determining the phosphorylation of STAT6 on tyrosine 641 upon stimulation with different concentrations of IL-4. After digestion with heparinase III and chondroitinase ABC, staining of cell surface GAGs was performed to evaluate enzyme activity. As expected, heparinase III did remove HS from the cell surface of monocytes, as indicated by immunostaining with the anti-HS Ab AO4F12, whereas it did not significantly decrease CSB recognized by the Abs GD3A12 and LKN1 (Fig. 4A). Although
AO4F12-recognizable HS epitopes were removed by heparinase III. HS4C3-recognizable HS epitopes were not completely digested by heparinase III. Treatment with chondroitinase ABC did not significantly reduce the staining of monocyte cell surface HS by AO4F12 and HS4C3, but did strongly decrease the staining of CSB by GD3A12 (Fig. 4A). However, staining of CSB by LKN1 was not reduced after treatment with chondroitinase ABC, suggesting that some specific chondroitin sulfate epitopes remain present after enzyme treatment. This indicates that both heparinase III and chondroitinase ABC specifically digested the cell surface GAGs according to their specificity, but that some residual epitopes remain present on the cell surface of monocytes.

IL-4-induced STAT6 phosphorylation on tyrosine 641 was studied in heparinase III- or chondroitinase ABC-treated monocytes from four different donors. Treatment of monocytes with heparinase III seemed to result in a slightly decreased IL-4-induced STAT6 phosphorylation, although these differences were not statistically significant (Fig. 4B). Treatment of monocytes with chondroitinase ABC significantly inhibited the phosphorylation of STAT6 induced by IL-4. In monocytes from one donor, chondroitinase ABC treatment had a smaller inhibitory effect whereas heparinase III treatment had also an inhibitory effect on IL-4-induced STAT6 phosphorylation (data not shown). The inhibitory effect of chondroitinase ABC and heparinase III treatment on the IL-4-induced STAT6 phosphorylation became weaker at higher IL-4 concentrations and was lost at 512 U/ml IL-4 (data not shown). Together, these results indicate that IL-4 signaling to STAT6 is strongly impaired in the absence of monocyte cell surface chondroitin sulfates.
The binding to CSB was studied in more detail by performing an ELISA with two different CSB sulfation variants. Binding of IL-4 to 4/2,4- and 6,2-6-sulfated wells was 20- and 6-fold higher, respectively, as compared with binding of IL-4 to CSB-coated wells (Fig. 5B). This indicates that an additional 2-O-sulfate dramatically increases the binding to IL-4 to CSB and that IL-4 prefers binding to 4/2,4-di-O-sulfated CSB over binding to 2,6-di-O-sulfated CSB. The fact that IL-4 does not significantly bind to CSA (which is primarily 4-O-sulfated but does not contain iduronic acid) but does bind to CSB (which is primarily 4-O-sulfated and does contain iduronic acid) indicates that IL-4 needs iduronic acid for binding to chondroitin sulfates. This finding is further strengthened by the fact that IL-4 does not bind to CSD (which is primarily 2-O- and 6-O-sulfated but does not contain iduronic acid) but does bind to 2,6-CSB (which does contain iduronic acid).

The binding of IL-4 to heparin was studied in more detail by performing an ELISA with modified heparins (Fig. 5C). IL-4 bound much less to completely desulfated/N-acetylated heparin (CdSNAc heparin) than to heparin, indicating that sulfate groups are important for IL-4 binding. Binding improved when N-acetyl groups were substituted by N-sulfate groups (CdSNS heparin), which points to a role of N sulfates. Also the reduced binding to N-desulfated/N-acetylated heparin (NdSNAc heparin) compared with binding to heparin hints toward a role of N sulfates in IL-4 binding to heparin. Although completely 2-O-desulfated heparin and 6-O-desulfated heparin (which still contains 23% 6-O-sulfate) bind IL-4 as well as heparin does, the importance of O sulfates is clearly demonstrated by the fact that completely O-desulfated heparin (OdS heparin) binds IL-4 much less than heparin does. As binding of IL-4 to carboxyl-reduced heparin was lower than binding to heparin, the carboxyl group of uronic acid also seems to play a role in IL-4 binding. Together these data suggest that the presence of an N-sulfate combined with a 2-O-sulfate or a 6-O-sulfate is required for optimal binding of IL-4 to heparin. Also binding to CSB is strongly enhanced by the presence of additional 2-O- and 6-O-sulfates.

**Discussion**

GAGs are ubiquitously expressed throughout the body and can bind diverse molecules such as viral coat proteins, chemokines, and cytokines (24, 41, 42). GAGs can modulate the activity of cytokines and chemokines in either a positive or a negative way (for a review, see Ref. 40). Binding of FGF2 to cell surface GAGs promotes signaling via FGFR1 (43) whereas binding of IFN-γ to soluble heparin antagonizes its activating effect on endothelial cells (44). Although IL-4 has been shown to bind to GAGs, no detailed information is available on the effect of GAGs on IL-4 activity (26). In this study, the differentiation of monocytes toward DCs which has been shown to be induced by IL-4 (13) was investigated. The expression of the DC-differentiation markers DC-SIGN, CD80, CD206, and CD1a was induced by IL-4 showing a sigmoidal concentration dependency. The expression of CD11b and MHC-I did not increase with increasing IL-4 concentrations, demonstrating the specificity of the IL-4-mediated effects. Although GAGs were originally thought to have a space-filling function, necessary for the organization and orientation of the extracellular matrix, it has become clear that GAGs are also expressed intracellularly and on the surface of cells (42, 45). Using Abs that were selected against heparin/HS, CSB, and CSA/C/E we observed that monocytes mainly express HS and CSB on their cell surface. These findings are in correspondence with a recent study that demonstrates that monocytes and DCs express proteoglycans.

### FIGURE 4.

Effect of chondroitinase ABC and heparinase III digestion on IL-4-induced STAT6 phosphorylation. Monocytes were treated with chondroitinase ABC, heparinase III or were left untreated (control or “-”).

**A**. Cells were stained for HS with AO4F12 and HS4C3 and for CSB with GD3A12 and LKN1. Stainings were analyzed with FACS. Data are presented as the mean fluorescence intensity normalized to the control ± SD (n = 3). * Significant difference compared with the control (p < 0.05).

**B**. Cells were stimulated for 5 min at 37°C with different IL-4 concentrations and cell lysates were analyzed for phosphorylated STAT6 and total STAT6 by Western blot. Bands were visualized with ECL. The Western blots are representative for three experiments with cells from different donors. Pixel densities were normalized to ImageJ and the ratio of STAT6-pY641 divided by STAT6 was calculated and normalized to the ratio of the control stimulated with 128 U/ml IL-4. Data are presented as the mean normalized ratio ± SEM (n = 3). * Significant difference compared with the control (p < 0.05).

**IL-4 binds to heparin and CSB**

Many cytokines have been shown to interact with GAGs and this modulates their activity (for a review, see Ref. 40). To further prove whether IL-4 interacts with GAGs, an ELISA was performed in which IL-4 was allowed to bind to wells coated with different GAGs. Only binding of IL-4 to heparin and CSB was observed, whereas binding to HS, CSA, CSC, CSD, and CSE was not significant above the background (Fig. 5A). The same ELISA was performed for GM-CSF but binding of this growth factor to none of the GAGs was detectable at neutral pH (data not shown).
on their surface, the majority of which contain chondroitin/dermatan sulfate GAG chains (46). These GAGs can be present on GPI-anchored proteoglycans (glypicans) and integral membrane proteoglycans (syndecans). The great functional versatility of GAGs is largely defined by the numbers and positions of \(N\) - and \(O\)-sulfate groups in the monosaccharides (47). The spacing of negative groups along the GAGs endows these with unique properties to bind evenly spaced positive groups on chemokines and cytokines. A positive correlation has been found between an increasing 6-\(O\)-sulfate content and the ability to activate FGF2 (48). Moreover, the removal of sulfate groups can result in the complete loss of GAG functionality. To study the function of monocyte/DC cell surface GAGs, the effect of a reduced sulfation grade of these GAGs on the IL-4-induced expression of DC-SIGN and CD1a and on the DC-induced PBL proliferation was determined. Sodium chlorate was used to reduce monocyte/DC GAG sulfation, as this compound has been shown to reduce the 2-\(O\)- and 6-\(O\)-sulfation of GAGs (39).

The presence of sodium chlorate in the monocyte/DC culture medium resulted in a concentration-dependent decrease of the reactivity of Abs that recognize sulfated HS (HS4C3) and sulfated CSB (GD3A12) (29, 30) demonstrating that sodium chlorate indeed reduced the sulfation of monocyte cell surface GAGs and destroyed the spacing of the negative charges. Moreover, the decreased reactivity of the Abs against sulfated GAGs correlated with a decrease in IL-4-induced DC-SIGN and CD1a expression and a decrease in DC-induced PBL proliferation. These findings indicate that sulfated GAGs on the monocyte cell surface positively modulate the activity of IL-4.

It might be speculated that the modulatory effect of GAGs on the expression of DC-associated markers and DC-induced PBL proliferation are caused by diminished activity of GM-CSF, which is also present in the culture medium, instead of by an effect on IL-4. However, an interaction of GM-CSF with GAGs is only present at a low pH because of protonation of His\(^{83}\) and His\(^{87}\) in helix C of GM-CSF, which act as a pH-dependent molecular switch (49). Interaction between GM-CSF and GAGs is absent at a pH of 7 or higher. As dendritic cells are cultured in RPMI 1640 with a pH of 7.4, it is highly unlikely that GM-CSF binds to GAGs in this culture medium. Therefore, effects of sodium chlorate are expected to be exerted solely on the level of IL-4 signaling.

**FIGURE 5.** Binding of IL-4 to GAGs. Wells of a microtiter plate were coated with GAGs and subsequently incubated with IL-4. Bound IL-4 was detected with sheep anti-IL-4 and alkaline phosphatase-conjugated donkey anti-sheep. Phosphatase activity was detected using paranitrophenyl phosphate.

A, Binding of IL-4 to different GAGs. B, Binding of IL-4 to different sulfated CSB preparates. Predominant disaccharide structures of the indicated GAGs are shown at the left. C, Binding of IL-4 to modified heparins. Data are presented as the mean absorbance at 405 nm after subtraction of the blank ± SD (\(n = 3–5\)). *, Significant difference compared with the negative control (\(p < 0.05\)). †, Significant difference compared with CSB (\(p < 0.05\)). ‡, Significant difference between the indicated samples (\(p < 0.05\)).
To further prove the hypothesis that sulfated GAGs on the monocyte cell surface positively modulate the activity of IL-4, cell surface GAGs were enzymatically removed from monocytes by digestion with chondroitinase ABC or heparinase III. Digestion of HS with heparinase III slightly reduced the IL-4-induced STAT6 phosphorylation. Chondroitinase ABC treatment strongly impaired STAT6 phosphorylation induced by IL-4, even though after digestion with chondroitinase ABC, some CSB epitopes (recognized by Abs against CSB) remained present on the cell surface. At an IL-4 concentration of \(< 10 \text{ U/ml} (\sim 1 \text{ ng/ml})\), which is sufficient for maximal induction of DC-SIGN expression but is much higher than physiological IL-4 plasma levels \((\sim 10 \text{ pg/ml})\), the IL-4-induced STAT6 phosphorylation is completely blocked by chondroitinase ABC treatment. These data suggest that cell surface chondroitin sulfates act as a cofactor for IL-4 by positively modulating its activity. The inhibitory effect of chondroitinase ABC treatment gradually decreased with increasing IL-4 concentrations, which suggests an effect of cell surface chondroitin sulfates in sensitization of monocytes for low concentrations of IL-4. The finding that Abs against CSB react with monocyte cell surface GAGs, but Abs against CSA/C/E do not, suggests that CSB is the major monocyte cell surface GAG that is involved in modulation of IL-4 activity. This is supported by the finding that IL-4 bound to CSB, but not to CSA, CSC, CSD, and CSE. These results correspond with the report of Lortat-Jacob et al. (26) who demonstrated that CSB binds IL-4 with a much higher affinity than CSA. However, in this report the affinity of CSB and CSC for IL-4 was shown to be similar. Although binding of IL-4 to heparin was observed no binding to the structurally related HS was observed. These results are in contrast with a previous report showing that IL-4 does bind to HS (26). The discrepancies may be caused by the use of HS and CSC from a different source, as different sources may contain GAGs with different sulfation-profiles and therefore different properties.

The presence of iduronic acid is a prerequisite for binding of IL-4 to GAGs as IL-4 only bound to the iduronic acid-containing GAGs, heparin, and CSB. This is further strengthened by the fact that IL-4 does not bind to CSA (4-O-sulfated without iduronic acid) but does bind to CSB (4-O-sulfated with iduronic acid). Detailed analysis of binding of IL-4 to modified heparins showed that optimal interaction required the presence of N sulfates together with either a 2-O-sulfate or a 6-O-sulfate. This corresponds with data from a previous study showing that IL-4 binding to HS is decreased after digestion at N-sulfated glucosamines (26). Also, binding of IL-4 to CSB largely improved in the presence of additional 2-O- or 6-O-sulfates. The finding that sodium chloride, which reduces O-sulfation of GAGs at the tested concentrations (39), reduces the IL-4-induced DC-SIGN expression suggests the involvement of O sulfates in IL-4 function. This fits with the data from the ELISA which show that optimal binding of IL-4 to GAGs requires O sulfates. The exact IL-4-binding sequence in monocyte chondroitin sulfates and the amino acids of IL-4 that interact with these GAGs awaits resolving the crystal structure of the IL-4-CSB complex.

The binding of proteins to GAGs involves basic amino acids (histidine, lysine, and arginine) which form electrostatic interactions with the negatively charged sulfate groups of GAGs. Several consensus sequences have been postulated such as XBBBXBX, XBBBXXBBXBBX, and TXBBXXTBBX XTBB (where X represents hydrophobic or uncharged amino acids, B represents basic amino acids, and T defines a turn) (50–52). The finding that IL-4 binds to GAGs suggests that it contains a GAG-binding consensus sequence. Although IL-4 contains 26 basic amino acids, which are structural components of GAG-binding consensus sequences, it does not contain any of the postulated GAG-binding consensus sequences. Therefore, IL-4 contains a different yet unrecognized three dimensional basic amino acid domain that binds to GAGs, analog to the GAG-binding domain of basic FGF (53, 54). One of the mechanisms by which cell surface GAGs affect cytokine activity is to act as a cofactor for cytokine receptors by sequestering cytokines on the cell surface and stabilizing the cytokine-receptor complex, as has been demonstrated for FGF (25). Analogous to this, cell surface chondroitin sulfates on monocytes may bind IL-4 and thereby present it to the IL-4R and facilitate signaling. For the FGF:FGFR-heparin ternary complex crystal structures have been determined with a resolution of 3 Å. Although two different models have been proposed (55, 56) they both show that heparin not only interacts with FGF but also with the FGFR. As the IL-4R forms heterodimers upon binding of IL-4, stabilization of the ternary IL-4:IL-4R:γc or the complex IL-4:IL-4R:IL13r1 would be a likely mechanism by which CSB may facilitate IL-4 signaling (7).

IL-4 is important in immunity as in the absence of IL-4, the Th2 response is strongly impaired (57). Lack of IL-4 also has been shown to hamper the development of a protective Th1 response against Candida albicans and against mammary adenocarcinoma and colon carcinoma tumors, effects that are mediated by APCs such as DCs (58–60). As we observed that chondroitin sulfates on monocyte/DCs are necessary for optimal IL-4 activity, these GAGs may be important for the generation of Th1 and Th2 responses in vivo.

Taken together, our results show that chondroitin sulfates on the cell surface of monocytes positively regulate IL-4 activity. As IL-4 binds to CSB, but not to other chondroitin sulfates, a model is proposed that involves binding of IL-4 to monocyte cell surface CSB which facilitates IL-4 signaling via its receptor. This interaction may play an important role in the regulation of immune responses.

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Disclosures
R.T. and C.G.F. are inventors of a patent on DC-SIGN.

References
IL-4 activity modulated by cell surface GAGs


