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The Complement Inhibitor Low Molecular Weight Dextran Sulfate Prevents TLR4-Induced Phenotypic and Functional Maturation of Human Dendritic Cells

Rolf Spirig,* Cees van Kooten,† Carolina Obregon,† Laurent Nicod,† Mohamed Daha,‡ and Robert Rieben2*

Low molecular weight dextran sulfate (DXS), a sulfated polysaccharide of m.w. 5000, is known to inhibit alternative, classical, and lectin complement pathways (1) as well as the coagulation cascade (2). In addition, DXS has been shown to act as an endothelial cell protectant (3, 4). It prevents human complement- and NK cell-mediated cytotoxicity in vitro (3) and, in combination with cyclosporin A, induces long-term graft survival in a hamster-to-rat cardiac xenotransplantation model in vivo (5). Moreover, DXS is very effective in protecting vasculature and tissue from ischemia/reperfusion injury, as recently shown in a porcine model of acute myocardial infarction (6). Taken together DXS can be described as a substance that attenuates the proinflammatory effects of many mediators of innate immunity. We hypothesized, therefore, that DXS might also influence the function of APCs, which are crucially involved in graft rejection and tolerance induction.

Dendritic cells (DC) are the most potent APC and play an important role in bridging innate and adaptive immunity (7). APC are pivotal for the initiation of T cell-mediated immune responses, as seen for example in allograft rejection as well as in tolerance induction (8, 9). The properties of DC essentially depend on maturation and migration of the cells. DC residing in the peripheral tissue are normally in a phenotypically and functionally immature state. Immature DC do not induce primary immune responses because they do not express the costimulatory molecules CD80, CD86, and CD40, nor do they express antigenic peptides as stable complexes with MHC molecules (7). The balance between the induction of immune reactivity and immune modulation, including tolerance induction, seems to be determined by the subtype of the DC, their state of maturation, and the secretion of soluble mediators including cytokines. Furthermore, similar to macrophages, immature DC have been described as a rich source of the complement proteins C1q (10, 11) and C3 (12). C1q produced by immature DC is functionally active in complement activation and binding to apoptotic cells (13). The production of C1q is down-regulated upon DC maturation in vitro and in vivo (10). C3 synthesis by DC is reported to be essential for T cell activation (12). Interestingly, the binding of the C3 activation product iC3b to the complement receptor (CR3, CD11b/CD18), or αM on APC has been reported to be crucial for tolerance (14). DC also express TLR, which are pattern recognition receptors that distinguish conditions of well-being from conditions of disease (15). Activating signals such as pathogenic compounds derived from microorganisms, e.g., LPS, as well as endogenous molecules like heparan sulfates (HS) induce maturation of DC via TLR (16).

Healthy endothelial cell are covered by a layer of HS proteoglycans, which is crucial for the anticoagulant and anti-inflammatory properties of the endothelium. HS proteoglycans are rapidly released under conditions of inflammation and tissue damage (17–19). The release of HS is mediated by proteolytic cleavage of the protein core or by endoglycosidase cleavage of the HS chains.
It has been shown that soluble HS induces maturation of DC via TLR4 (15, 21). Mature DC are characterized by strongly reduced phagocytic activity, up-regulated Ag-presenting and T cell costimulatory molecules, as well as expression of other DC activation markers like CD83. Moreover, mature DC secrete predominantly proinflammatory cytokines such as IL-1β, IL-6, IL-12p70, and TNF-α. The

FIGURE 1. DXS prevents phenotypic maturation of human MoDC in response to HS in a dose dependent manner. A. Monocytes isolated from buffy coat were cultured in presence of GM-CSF and IL-4 for 6 days. DXS dose-dependently prevents HS-induced (10 μg/ml) DC maturation. Histograms show the typical expression profiles of CD80, CD86, and CD83. Data are representative of five (CD80, CD83) or eight (CD86) independent experiments with cells of different donors. To compare the levels of inhibition of up-regulation of the indicated surface molecules, the median fluorescence intensity ratios (MFIR) were calculated by dividing the median fluorescence of HS- and/or DXS-treated MoDC by the median fluorescence of immature MoDC. Mean ± SD data are shown. *, p < 0.05; **, p < 0.01; ***, p < 0.001 vs mature MoDC by unpaired Student’s t test. B. Histograms showing the effect of 5 mg/ml DXS on HS-induced expression of the indicated cell surface molecules. DXS was added to MoDC 30 min before maturation was induced by HS (10 μg/ml) for 24 h. Afterward, cells were harvested, stained, and analyzed by FACS. The typical expression profiles of the indicated surface molecules (gray filled histogram) are shown. The isotype control or second Ab alone are indicated (open histogram). Values shown indicate the median fluorescence intensity of the expression profiles (gray filled histogram). The data shown are from one donor and representative of four to eight independent experiments.
transcription factor NF-κB is crucial for the expression of surface marker molecules and cytokine production during DC maturation. In this study, we show that DXS interferes with activation of human DC at multiple levels by reducing immunostimulatory properties, secretion of proinflammatory cytokines, phagocytic activity, and production of the complement proteins C1q and C3. DXS prevents TLR signaling in response to endogenous and exogenous agonists by inhibiting phosphorylation of IkB-α and activation of NF-κB.

**Materials and Methods**

**Generation and stimulation of human monocyte-derived DC (MoDC)**

Human PBMC were isolated from buffy coats obtained from healthy blood donors (Regional Red Cross Blood Donation Center, Bern, Switzerland) by density gradient centrifugation over Ficoll-Paque (Amersham, Biosciences). Monocytes were isolated from PBMC as described recently (22, 23) by spontaneous aggregation and rosetting (24). In brief, purified PBMC were suspended in RPMI 1640 medium (Invitrogen) containing 2 μg/ml
polymyxin B sulfate (Sigma-Aldrich). Cells were incubated for 40 min at 4°C under rotation to allow aggregation followed by 10 min of incubation on ice. Monocyte-enriched pellets were further separated from nonaggregated PBMC by a gradient of FCS (Amimed/BioConcept) and another 10 min of incubation on ice. The monocyte-enriched fractions were incubated overnight with SRBC (bioMérieux) to deplete contaminant lymphocytes by rosetting. Monocyte fractions characterized by high expression of CD14 and low expression of CD83 and CD86 were then isolated by Ficoll-Paque density gradient centrifugation. The purified mononuclear cells, mainly monocytes, were incubated for 6 days in RPMI 1640 medium (Invitrogen) containing 10% FCS (Amimed/BioConcept), 1% L-glutamine (2 mM; Invitrogen), 1% penicillin/streptomycin (100 U/ml; Invitrogen), 10 ng/ml GM-CSF (R&D Systems), and 1 ng/ml IL-4 (R&D Systems) to generate MoDC as described initially by Sallusto and Lanzavecchia (25). The cells were kept at 37°C in a 5% CO2 humidified atmosphere. On day 3, the culture medium was replaced with fresh medium. For induction of maturation, 10 μg/ml HS (Seikagaku), 100 ng/ml LPS (Sigma-Aldrich), 5 μg/ml lipoteichoic acid (LTA; Sigma-Aldrich), 20 ng/ml TNF-α (R&D Systems), or 20 ng/ml IL-1β (R&D Systems) were added to the cultures for 24 or 48 h. Low molecular weight DXS (Sigma-Aldrich), used at the indicated concentrations, was given 30 min before HS, LPS, LTA, IL-1β, or TNF-α was added to the cells. LPS removal affinity resin (END-X B15) was purchased from Associates of Cape Cod to remove potential LPS contamination in HS.

Isolation of myeloid DC from peripheral human blood

Peripheral blood myeloid DC were isolated using a blood DC Ag-I DC isolation kit (Miltenyi Biotec) according the manufacturer’s protocol and cultured for 24 h in RPMI 1640 medium with 10% FCS, 1% L-glutamine and 1% penicillin/streptomycin. Stimulation procedures were the same as for MoDC.

**FIGURE 3.** Activation of peripheral myeloid DC is prevented by DXS. A, Peripheral myeloid DC were isolated using a blood DC Ag-I DC isolation kit. DXS was added to the DC 30 min before maturation was induced by HS (10 μg/ml) for 24 h. The typical expression profiles of the surface molecules CD80, CD86, and CD83 are shown (gray filled histogram). Values indicate the median fluorescence intensity of the expression profiles. Results are from one donor and representative of three independent experiments. 

**FIGURE 4.** Assessing the influence of the concentration of DXS used on the viability of MoDC. A, MoDC were treated with indicated concentrations of DXS. Thereafter, cells were harvested and stained with propidium iodide (5 μg/ml) and analyzed by flow cytometry. Data are mean ± SD of three independent experiments with cells of different donors. B, MoDC were treated with LPS (100 ng/ml) or HS alone (10 μg/ml), or together with DXS (5 mg/ml). Data represent mean ± SD of three independent experiments with cells of different donors.
FACS analysis and cell viability

Cells were incubated with FITC-labeled mAb against CD1a, CD80, CD83, CD86 (BD Biosciences), isotype control IgG1 (BD Biosciences), PE-Cy5-labeled mAb against HLA-DR (BD Biosciences), isotype control IgG2b (BD Biosciences), unlabeled mAb against CD40, ICAM-1 (Diaclone) CD14 and DC-SIGN (BD Biosciences) followed by a PE-labeled polyclonal goat anti-mouse IgG1 (Southern Biotechnology Associates) or FITC-labeled polyclonal goat anti-mouse IgG (Sigma-Aldrich).

For determination of viability, the cells were stained with 5 μg/ml propidium iodide (Invitrogen) and analyzed by flow cytometry. As positive control, cells were treated with PBS containing 0.1% BSA (Sigma-Aldrich) and 0.1% saponin (Sigma-Aldrich). Measurements were performed with a BD FACScan flow cytometer and the obtained data were analyzed using FlowJo (Tree Star).

Measurement of immunostimulatory activity

T cells purified from human PBMC using nylon wool columns were used as responder cells for MoDC, which had been stimulated with HS or LPS, with or without DXS. Tetanus toxoid (Calbiochem) was used as Ag. A total of 1.5 × 10⁶ autologous T cells were added to different numbers of MoDC (10,000, 5000, 1000) in 96-well tissue culture plates containing 0.2 ml of medium per well. T cells and MoDC were coincubated for 5 days and pulsed with 0.5 μCi of [³H]thymidine (Amersham Biosciences) for the last 18 h. The incorporated radioactivity was then measured using a liquid scintillation counter.

Cytokine assays

MoDC (10⁶ cells/ml) were treated with HS in the presence or absence of different concentrations of DXS for 24 h. The cell culture supernatants were analyzed using a Luminex multiplex array system (Bio-Rad) for IL-1β, IL-6, IL-12p70, IL-10 and TNF-α, according the manufacturer’s instructions.
Measurement was completed of C1q and C3 in supernatants of MoDC that were treated with different stimuli for 24 h, washed, and reincubated with fresh cell culture medium for 48 h. ELISA was used for measurement according to previous publications (10, 26, 27). In brief, the C1q-specific mAb 2204 was used as capture Ab and purified rabbit anti-human C1q, followed by HRP-conjugated goat anti-rabbit IgG (Jackson Immuno-Research Laboratory) was used for detection of bound C1q. Highly purified human serum C1q was used as a standard. For the detection of C3, wells were coated with a polyclonal anti-human C3 capture Ab followed by a digoxigenin-conjugated rabbit anti-human C3 Ab and HRP-conjugated sheep Fab anti-digoxigenin polyclonal Ab (Boehringer Mannheim Biochemica). The sensitivity of the ELISA for C1q was 0.125 ng/ml (10) and for C3 it was 0.03 ng/ml.

Measurement of phagocytic activity

For the analysis of phagocytic activity, 2 × 10^5 DC were incubated with FITC-dextran (m.w. 40,000; Invitrogen) for 1 h at 37°C. As negative control, cells were precooled before the incubation with FITC-dextran at 4°C for 1 h. The cells were washed four times and immediately analyzed by flow cytometry.

Measurement of phosphorylation of IκB-α

MoDC (2 × 10^6 cells/ml) were treated with HS or LPS in the presence or absence of DXS for 15 and 30 min. The cell lysate was analyzed using a Luminex multiplex array system from Bio-Rad for phosphorylation of IκB-α, according to the manufacturer’s instructions.

Detection of NF-κB activation by a transcription factor ELISA

The production of NF-κB p65 was measured with a NF-κB assay kit (Active Motif) according to the manufacturer’s instructions. In brief, cell extract of LPS- or HS-activated DC, with or without additional pretreatment by DXS (5 mg/ml), was added to each well coated with consensus-binding site oligonucleotides of NF-κB p65. A primary Ab specific for an epitope on the bound and active form of the transcription factor was then added, followed by subsequent incubation with secondary Ab.

Statistical analysis

Data are presented as mean ± SD representing experiments with up to eight different donors. Unpaired Student’s t tests were performed for evaluation of significance. Differences were considered as statistically significant at a value for p < 0.05. Data were analyzed using GraphPad Prism software.
Results

DXS inhibits phenotypic changes in MoDC as well as peripheral myeloid DC induced by HS, LPS, and LTA

Human immature DC were generated from monocytes isolated from buffy coat incubated for 6 days together with IL-4 and GM-CSF. Maturation was induced using HS, LPS, and LTA. To exclude that the activation through HS was due to endotoxin contamination, HS was absorbed on Limulus anti-LPS factor-coated silica beads. This treatment did not alter the ability of HS to induce maturation of DC, as already shown (21, 28). The same treatment of LPS eliminates its ability to induce maturation (data not shown).

To determine whether DXS affects the phenotypic maturation of MoDC induced by endogenous (HS) and exogenous TLR ligands (LPS, LTA), the cells were analyzed using flow cytometry and specific mAb against MHC class II, costimulatory, and adhesion molecules. Treatment with LPS (100 ng/ml), LTA (5 μg/ml), and HS (10 μg/ml) significantly increased the expression of CD80, CD86, CD83, CD40, and ICAM-1 (CD54) and the human MHC class II molecule HLA-DR. Incubation together with DXS significantly reduced the up-regulation of these mature DC surface markers in a dose-dependent manner, whereas DC-SIGN (CD209) expression was down-regulated (Fig. 1). The maximum inhibition by DXS was found at 5 mg/ml. Taken together, these findings indicate that DXS predominately prevents LPS- and HS-induced MoDC maturation, whereas there was less effect on LTA-induced maturation (Fig. 2).

We also examined the effect of DXS on HS-stimulated myeloid DC freshly isolated from peripheral blood. Preincubation or coinubcation of peripheral myeloid DC with DXS inhibited up-regulation of the costimulatory molecules CD80/CD86 and the maturation marker CD83 (Fig. 3). These data suggest that DXS is able to prevent the HS induced maturation of myeloid DC ex vivo.

To investigate how the concentration of DXS used influences the viability of MoDC, we monitored MoDC survival at different DXS concentrations. MoDC were incubated for 24 h with different concentrations of DXS and stained afterward with propidium iodiode and immediately analyzed by FACS. We found that DXS concentrations of up to 25 mg/ml, either alone or in combination with LPS or HS, did not affect the viability of MoDC within 24 h of exposure (Fig. 4).

Influence of DXS on maturation induced by TNF-α or IL-1β

MoDC were treated with TNF-α (20 ng/ml) or IL-1β (20 ng/ml), or a combination of both, for 24 or 48 h, and up-regulation of CD80, CD86, and CD83 was measured by flow cytometry. Only

![Diagram A](http://www.jimmunol.org/)

**A**. DXS prevents the secretion of proinflammatory cytokines, IL-10 and reduces the production of C1q, whereas C3 secretion is increased by MoDC. A, DXS prevents the release of the cytokines IL-1β, IL-6, IL-10, IL-12p70, and TNF-α by HS (10 μg/ml) treated MoDC. Immature MoDC were pretreated 30 min with the indicated concentrations of DXS before addition of HS. Supernatants after 24 h of incubation were analyzed by a multiplex suspension array. Cell-free medium was used to determine the background. Each point represents the value of one donor. Horizontal bar shows the mean of three different donors. Statistical significance between samples was indicated: * p < 0.05; ** p < 0.01; *** p < 0.001 vs mature MoDC by unpaired Student’s t test. B, DXS treatment of MoDC leads to a decreased C1q production, whereas C3 secretion is increased. Individual values and mean are shown from five different individuals. Immature MoDC were pretreated 30 min with the indicated concentrations of DXS before addition of HS. After 24 h the cells were washed and incubated for 48 h with new cell culture medium. Supernatants were analyzed by ELISA. Cell-free medium was used to determine the background. Data points represent the value of one donor. Horizontal bar represents the mean of five different donors. Statistical significance between samples was indicated. * p < 0.05; ** p < 0.01 vs mature MoDC by unpaired Student’s t test.
the highest concentration of DXS did significantly inhibit the up-regulation of CD80 at 24 or 48 h of stimulation (Fig. 5), whereas no inhibitory effect was observed for CD86 or CD83. The inhibitory effect of DXS seems to be more restricted to a TLR-mediated activation of MoDC.

DXS reduces HS-induced secretion of proinflammatory cytokines and IL-10 by MoDC

HS is known to induce the secretion of proinflammatory cytokines like IL-1β, IL-6, and TNF-α (28). As assessed by multiplex suspension array, DXS significantly inhibited the HS-induced production of IL-1β, IL-6, IL-12p70, and TNF-α by MoDC in a dose-dependent manner with a maximum effect at 5 mg/ml (Fig. 6A). In addition, DXS also reduced secretion of the immunosuppressive cytokine IL-10 (Fig. 6A). Incubation of MoDC with different concentrations of DXS without a TLR agonist had no effect on secretion of these cytokines.

Reduction of C1q but increase in C3 secretion by MoDC under DXS treatment

It was recently shown in vitro and in vivo that immature DC are a rich source of C1q (10). Furthermore, it has been demonstrated that DC are able to synthesize C3 (12). Treatment of MoDC with the complement inhibitor DXS with or without HS or LPS stimulation led to a reduced production of C1q as detected in our cell culture supernatants. Surprisingly, we observed no decrease of C1q production in the HS-treated MoDC in contrast to LPS-induced maturation. The production of C3 by MoDC treated with DXS and either HS or LPS was increased, whereas the incubation with DXS alone led to a reduced C3 secretion. The amount of C3 detected in the supernatants of DXS and TLR ligand treated cells were higher than in the MoDC treated only with a TLR agonist (data for LPS not shown) (Fig. 6B).

DXS does not prevent maturation-induced reduction of phagocytic activity of MoDC

Immature DC capture and process Ags as a consequence of their activity; during maturation this feature disappears (7). When immature MoDC were stimulated with LPS, a significant decrease of their phagocytic activities was observed. Treatment of DC with DXS alone or together with LPS reduced the phagocytic activity of MoDC as measured by uptake of FITC-Dextran (Fig. 7, B and D). As a next step, we evaluated the effect of DXS without additional stimulation with LPS on the phagocytic activity of MoDC. As shown in Fig. 7, A and C, already DXS alone led to a functionally impaired phagocytosis with significant effect at the highest used concentration.

DXS reduces MoDC-mediated proliferation of autologous T cells

To determine whether DXS influences Ag presentation by MoDC we performed a proliferation assay. Immature MoDC were pulsed with tetanus toxoid for 3 h. Thereafter they were incubated with HS (10 μg/ml) plus DXS (5 mg/ml) for up to 24 h. These MoDC
were then tested for their ability to present the Ag to T cells assessed by a standard [3H]thymidine incorporation assay. MoDC incubated together with DXS showed a markedly reduced capacity to induce proliferation of autologous T cells (Fig. 8A).

DXS inhibits phosphorylation of IκB-α and activation of the transcription factor NF-κB
To assess the effects of DXS-mediated inhibition of maturation of MoDC on intracellular signaling, we determined the phosphorylation of IκB-α and activation status of NF-κB, the essential transcription factor for DC maturation and function. The phosphorylation of IκB-α leads to its ubiquitylation and subsequent degradation, which results in a release of NF-κB (29). Treatment with HS or LPS led to phosphorylation IκB-α measured after 15 and 30 min and after 1 h to activation and translocation of NF-κB p65 into the nucleus. Pretreatment of MoDC with DXS caused a strong abrogation of HS- and LPS-induced phosphorylation of IκB-α and activation of NF-κB as shown in Fig. 8, B and C.

DXS partially affects the differentiation of monocytes into immature DC
To investigate the effect of DXS on the differentiation of monocytes into immature DC we added different concentrations of DXS during incubation of the cells with GM-CSF and IL-4 for MoDC generation. DXS did not influence the up-regulation of the DC marker CD1a, nor the down-regulation of the monocyte marker CD14. In addition, the up-regulation of CD40 and ICAM-1 was not influenced by DXS. These data strongly support our viability test that the cells under the treatment of DXS are still alive and metabolic active. However, as compared with control DC, we observed a significant difference in expression of HLA-DR and DC-SIGN (Fig. 9A). In addition, the capability was tested of MoDC generated in the presence of DXS to mature upon stimulation by LPS. The stimulation pattern observed with these cells, in particular the up-regulation of the maturation surface markers CD80 and CD86 was reduced, for CD86 significantly, compared with that of MoDC generated without DXS (Fig. 9B).

DXS binds specifically to immature MoDC but not via DC-SIGN or CR3
To further assess the mechanism of DXS on MoDC, we have incubated the cells with fluorescein-labeled DXS (DXS-fluo). As shown in Fig. 10A, DXS-fluo specifically binds to immature DC, whereas significantly less binding was observed to mature MoDC. The binding of DXS-fluo was inhibited by pretreatment of the cells

FIGURE 8. Immunostimulatory capacity of MoDC is reduced and activation of NF-κB is inhibited by DXS. A, T cell proliferation mediated by human MoDC is inhibited by DXS. MoDC were incubated for 24 h in the absence or presence of DXS (5 mg/ml). Cells were then harvested and incubated with autologous T cells in a 96-well plate. Stimulation experiments were conducted at several MoDC stimulator:responder T cell ratios (x-axis). Cells were pulsed with [3H]thymidine on day 5, and [3H]thymidine incorporation was assessed after 18 h. TT, Tetanus toxoid. Data represent mean ± SD of an experiment performed in triplicate and representative of four independent experiments with cells of different donors. B and C, IκB-α phosphorylation was measured in cell lysate 15 and 30 min after stimulation with LPS (100 ng/ml) (B) and HS (10 μg/ml) (C) by a Luminex multiplex array system. NF-κB activation was measured in cell extracts at 1 h after LPS (B) or HS (C) induced activation by a transcription factor ELISA. Cells were preincubated 30 min before TLR stimulation with DXS (5 mg/ml). Data represent mean ± SD of three independent experiments with cells of different donors. Statistical significance between samples was indicated. *, p < 0.05; **, p < 0.01; ***, p < 0.001 vs mature MoDC by unpaired Student’s t test.
with unlabeled DXS. It has been shown that heparin, another sulfated glycosaminoglycan, binds to CR3 (30). To examine whether DXS binds to CR3, we have incubated the cells with an anti-CR3 blocking Ab. The binding of DXS-fluo was not abolished by the pretreatment of the MoDC with the blocking, neither was the inhibitory effect of DXS reversed by the blocking Ab for CR3 measured by the up-regulation of CD86 by flow cytometry (Fig. 10B).

To assess whether DC-SIGN is involved, we have blocked DC-SIGN either with mannan (Fig. 10C) or with an anti-DC-SIGN blocking Ab described in the literature (31) (Fig. 10D). Blocking of DC-SIGN did not abolish the inhibitory effect of DXS either.

To assess whether DXS-mediated inhibition of MoDC maturation persists when the substance is removed from the cell culture medium, we thoroughly washed the cells after different preincubation periods and then stimulated them with HS or LPS. As shown in Fig. 10E, resistance against TLR induced activation increases depending on preincubation time with DXS, and after 24 h of preincubation the observed inhibition of CD86 expression is similar to the one observed with DXS coincubation (Figs. 1 and 2).

**Discussion**

In the present study we have investigated the effect of the complement inhibitor low molecular weight DXS on the maturation process of human DC. Several reports suggest that maintaining DC in an immature or semimature state (32), or activating them in an alternative manner (33), can be effective in preventing allograft rejection and favor the induction of tolerance.

Activation of vascular endothelial cell, as occurring in organ transplantation for example due to ischemia/reperfusion injury of the graft, leads to shedding of HS (18, 20). It has been shown that soluble HS serve as a danger signal, which is able to induce maturation of DC (28) via TLR4 (15). TLR4 therefore plays an important role in ischemia/reperfusion injury (34, 35) and in many stages of graft rejection (36).

The inhibitory effect of DXS is mainly observed for a TLR4 stimulation (HS, LPS), whereas the maturation induced via TLR2 (LTA) or proinflammatory cytokines like TNF-α or IL-1β seems to be less affected. Treatment of DC with DXS prevents HS- or LPS-induced up-regulation of the costimulatory molecules CD80/CD86, CD83 and CD40. Moreover, we observed a reduced expression of the human MHC class II molecule HLA-DR. Interestingly, we could also observe an inhibition of the up-regulation of the integrin ICAM-1 and a down-regulation of DC-SIGN, which is a member of the C-type lectin receptor family. The expression of the adhesion molecule ICAM-1 on DC has been reported not to influence the migration to the regional lymph nodes (37), whereas it seems to have an important role in DC-T cell interactions and induction of proliferation (38, 39). The inhibited up-regulation of
then stimulated with HS (10 μg/ml) for 24 h. Afterward, cells were evaluated for the expression of CD86 by flow cytometry. C and D, Immature MoDC were incubated with mannan (100 μg/ml) at 4°C or blocking anti-DC-SIGN (20 μg/ml) for 30 min before the exposure of DXS (5 mg/ml) for 30 min. The cells were then stimulated with HS (10 μg/ml) for 24 h and then analyzed for the expression of CD86 by flow cytometry. Data represent mean ± SD of three independent experiments with cells of different donors. Statistical significance is indicated. *p < 0.05 and **p < 0.01 vs immature MoDC by unpaired Student’s t test. E, immature MoDC were incubated for the indicated periods with DXS (5 or 25 mg/ml), washed two times and then stimulated with HS (10 μg/ml) or LPS (100 ng/ml) for 24 h. Afterward, cells were evaluated for the expression of CD86 by flow cytometry. The results shown are from one donor and representative of two independent experiments with cells from different donors.

ICAM-1 by DXS-treated MoDC might contribute to the observed effect of a reduced capacity of these cells to induce T cell proliferation. DC-SIGN mediates adhesion with T cells by stabilizing the DC/T cell contact and plays a crucial role in Ag capturing (40). Furthermore, there exist several reports suggesting a cross-talk between TLR and C-type lectin receptor to fine-tune the balance between immune activation and tolerance (41, 42). C-type lectin receptors are highly expressed on immature DC and our results indicate that DXS binds specifically to nonstimulated immature MoDC. Involvement of DC-SIGN was examined by receptor-blocking experiments. Our results indicate that DXS seems not to develop its inhibitory effect via DC-SIGN.

Analysis by multiplex suspension array revealed that secretion of the proinflammatory cytokines IL-1β, IL-6, and TNF-α as well as IL-12p70 in response to HS is inhibited in the presence of DXS. A reduction of the production of TNF-α and IL-1β, which are known to enhance the effect of maturation of DC induced by TLR ligands, could contribute to the inhibitory effect of DXS on DC maturation. However, we were not able to observe an up-regulation of the secretion of the immunoregulatory cytokine IL-10 after 24 h. Other known immunomodulating agents like corticosteroids (43) or vitamin D3 (44) are known to increase the IL-10 production by DC, whereas e.g., rapamycin or FK506 (tacrolimus), similar to what we observed with DXS, did not increase IL-10 production (43).

The biological importance of these findings was confirmed by T cell stimulation assays in which DXS-treated MoDC showed a reduced capacity to induce T cell proliferation. In contrast, we found that DXS does not prevent the LPS-induced reduction of the phagocytic activity of MoDC measured by the uptake of FITC-dextran.

Immature DC were recently shown to be a rich source of functional complement components like C1q (10) and C3 (12) in vitro and in vivo. We found that DXS alone as well as in combination with HS reduced the amount of secreted C1q by DC. In contrast, the secretion of C3 was enhanced by DXS in combination with HS or LPS. The activation products of C3, such as iC3b, have been implicated in the induction of tolerance (14). In contrast, Peng et al. (12) recently suggested, that C3 synthesis by DC is crucial for full T cell activation. Furthermore, recent studies showed that signaling through CR3 in DC provides a “non-dangerous” signal (45) and renders them tolerogenic (46). We found that DXS does not bind to CR3 and blocking of CR3 could not reverse the effect of DXS.

The presence of DXS during differentiation of monocytes into immature MoDC did not affect the expression of CD1a, CD14, CD40, and ICAM-1. In contrast, we observed a significantly impaired up-regulation of HLA-DR and DC-SIGN. Therefore, DXS seems to partially influence the differentiation of monocytes into immature MoDC.

Following the observation that DXS affects DC maturation, we evaluated potential effects on signaling pathways that regulate DC immune properties. Due to the highly negative charges of HS and LPS, a direct interaction of DXS with these activating ligands is unlikely. This hypothesis was confirmed by the observation that LPS-induced down-regulation of phagocytosis was not inhibited and C3 production was increased together with DXS. It might be possible that DXS binds the positively charged acute phase plasma protein lipid binding protein, which is known to enhance the activation of immune cells by mediating the transfer of LPS to the cell surface. Whether DXS directly binds lipid binding protein or other soluble members of the TLR signaling cluster, like soluble CD14 or MD-2, and thereby inhibits TLR complex formation needs to be evaluated.

Signaling pathways that trigger TLR are dependent on Toll-IL-1R domain-containing adaptor protein and MyD88. MyD88 recruits members of the IL-1R-associated kinase family and the TNFR-associated factor 6 to finally activate NF-κB and the MAPK.
pathways (29). Moreover, selective inhibition of NF-κB has been shown to be associated with tolerogenic properties of treated DC (47–49). In this study, we provide evidence that DXS significantly inhibited TLR-induced IκB-α phosphorylation and NF-κB activation, which could account for its major impact on MoDC maturation.

It should be noted that the used concentrations of DXS did not affect viability and metabolic activity of the cells, which is consistent with previously published in vitro and in vivo observations in which concentrations up to 25 mg/ml were used (5, 6). Recently, the maximum tolerated doses of DXS were determined in vivo in cynomolgus monkeys and i.v. or intraportal bolus injections of 3–6 mg/kg, followed by continuous infusion of 0.3–1.2 mg/kg/h for 6 days, and appeared to be safe (50). It should also be considered, however, that DXS binds to the surface of activated endothelial cells (3), which may limit the plasma concentration available for its inhibitory effect on DC. The in vivo concentrations of DXS required for complete or partial inhibition of DC maturation need to be elucidated in an appropriate animal model.

Our data suggest a new immunomodulatory function of the complement inhibitor and endothelial cell protein DXS. This compound might therefore be useful as a therapeutic reagent to impede the link between innate and adaptive immunity.

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Disclosures
The authors have no financial conflict of interest.

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