Heparin-disaccharide affects T cells: inhibition of NF-κB activation, cell migration, and modulation of intracellular signaling

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Abstract: We previously reported that disaccharides (DS), generated by enzymatic degradation of heparin or heparan sulfate, inhibit T cell-mediated immune reactions in rodents and regulate cytokine [tumor necrosis factor α (TNF-α), interleukin (IL)-8, and IL-1β] secretion by T cells, macrophages, or intestinal epithelial cells. Here, we investigated the effects of a trisulfated heparin DS (3S-DS) on two aspects of T cell function: secretion of proinflammatory cytokines and migration to an inflamed site. 3S-DS down-regulated nuclear factor-κB activity and reduced the secretion of TNF-α and interferon-γ (IFN-γ) by anti-CD3-activated T cells. In addition, 3S-DS inhibited CXC chemokine ligand 12 (CXCL12; stromal cell-derived factor-1α)-dependent migration in vitro and in vivo and decreased CXCL12-induced T cell adhesion to the extracellular matrix glycoprotein, fibronectin (FN). This inhibition was accompanied by attenuation of CXCL12-induced Pyk2 phosphorylation but did not involve internalization of the CXCL12 receptor, CXCR4, or phosphorylation of extracellular-regulated kinase. Despite inhibiting CXCL12-induced adhesion, 3S-DS, on its own, induced T cell adhesion to FN, which was accompanied by phosphorylation of Pyk2. A monosulfated DS showed no effect. Taken together, these data provide evidence that 3S-DS can regulate inflammation by inducing and modulating T cell-signaling events, desensitizing CXCR4, and modulating T cell receptor-induced responses. *J. Leukoc. Biol.* 75: 1139–1146; 2004.

Key Words: *chemokines · CXCL12 · extracellular matrix · TNF-α · IFN-γ*

INTRODUCTION

Heparin and heparin-like compounds possess anti-inflammatory activity, independent of anticoagulation [1–7]. Heparin has been reported to have a therapeutic effect in a wide range of inflammatory-related conditions in humans and in animal models, including rheumatoid arthritis, bronchial asthma, inflammatory bowel disease, colitis, skin burns, Lichen Planus, and experimental autoimmune diseases [6, 8–13]. We have demonstrated previously that a trisulfated disaccharide molecule (3S-DS) generated upon enzymatic cleavage of heparin, inhibits T cell-mediated immune reactions such as adjuvant arthritis and delayed-type hypersensitivity in rodents and that this is accompanied by down-regulation of proinflammatory cytokine secretion by macrophages [14]. In the present study, we investigated the effect of this DS on T cell activation and its mechanisms of action. The results shown in this study demonstrate that 3S-DS can indeed down-regulate T cell activation and migration following cell activation via the T cell receptor (TCR) anti-CD3 and chemokine receptor ligations. The notion that 3S-DS can, on its own, induce T cell activation and adhesion to extracellular matrix (ECM) proteins suggests that this compound has a potential therapeutic use in such instances in which the termination of the inflammatory reaction is required.

MATERIALS AND METHODS

Reagents and monoclonal antibodies (mAb)

The following reagents were used: CXC chemokine ligand 12 (CXCL12; PeproTech, Rocky Hill, NJ); fibronectin (FN; Chemicon, Temecula, CA); 3S-DS [o-(α-l-ido-4-enepyranosyluronic acid 2-sulfate)-(1-4)-2-sulfamio-2-deoxy-D-glucose 6-sulfate] and monosulfated DS [1S-DS; o-(α-l-ido-4-enepyranosyluronic acid 2-sulfate)-(1-4)-2-deoxy-n-acetyl-D-glucoseamin; Sigma-Aldrich, St. Louis, MO]; bovine serum albumin (BSA; Sigma-Aldrich); HEPES buffer, antibiotics, fetal calf serum (FCS), and RPMI 1640 (Kibbutz Beit Ha-Emek, Israel); Fluo-3 (Molecular Probes, Eugene, OR); $Na^{51}[Cr]$ (Amersham, High Wycombe, UK); and pertussis toxin (PTX) and GF109203X (Calbiochem, La Jolla, CA). The antibodies used were anti-human interferon-γ (IFN-γ) mAb (PharMingen, San Diego, CA) and anti-human tumor necrosis factor α (TNF-α; Endogen, Woburn, MA), antiphosphorylated Pyk2 mAb (p-Pyk2; clone py881, Biosource, Camarillo, CA), anti-Pyk2 mAb (t-Pyk2; clone N-19), anti-extracellular-regulated kinase (ERK; t-ERK; Sigma-Aldrich), antiphosphorylated ERK (p-ERK; clone E-4, Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD45–fluorescein isothiocyanate (FITC; Immuno Quality Products, Groningen, The Netherlands), anti-CXC chemokine receptor 4 (CXCR4)–phycoerythrin (PE; 12G5, BD PharMingen, San Diego, CA),

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anti-CXCR4 mAb (R&D Systems, Abingdon, UK), and secondary FITC-conjugated goat anti-mouse Ab (Dako, Glostrup, Denmark). Anti-CD3 was purified from ascites fluid of mice injected with OKT3 hybridoma cell (American Type Culture Collection, Manassas, VA).

Mice

Nonobese diabetic /LtSz-Pkdc^{scid} (NOD/SCID) mice were bred and maintained under defined flora conditions at the Weizmann Institute of Science (Rehovot, Israel) in sterile microisolator cages. The Animal Care Committee of the Weizmann Institute of Science approved all experiments. Mice (8–10 weeks old) were sublethally irradiated (375 cGy from a ^{60}Co source) and transplanted with human cells by an intravenous (i.v.) injection into the tail vein 24 h after irradiation.

Purification of human T cells

Human T cells were purified from the peripheral blood of healthy donors as described previously [15, 16]. Briefly, human peripheral blood was isolated on Ficoll gradients, washed, resuspended in phosphate-buffered saline (PBS) containing 3% heat-inactivated FCS, and incubated (45 min, 37°C, 7% CO_2-humidified atmosphere) on nylon-wool columns (NovaMed, Jerusalem, Israel). Nonadherent cells were eluted and washed, and platelets were removed by centrifugation (700 rpm, 15 min, 18°C). Residual monocytes were removed by incubation of the cells on tissue-culture plates (2 h, 37°C), after which nonadherent cells were collected. The $CD3^+$ content of these peripheral blood lymphocytes was >95%.

Cytokine secretion

T cells (2×10^6 cells/ml) were activated (2 h, 37°C) with the indicated concentrations of 3S-DS or 1S-DS in serum-free RPMI containing 0.1% BSA. The cells were then washed and replated on 24-well plates (nontissue-culture grade), precoated with anti-CD3 mAb (1 μg/ml, overnight). Supernatants were collected, and their cytokine content (TNF-α, IFN-γ) was determined by enzyme-linked immunosorbent assay (ELISA), using the appropriate mAb, according to the manufacturer's instructions.

Nuclear factor (NF)-κB activation

T cells (15×10^6) were activated as above (2 h with 3S-DS, washed, and transferred to plates precoated with anti-CD3 mAb). NF-κB activity was assayed using a NF-κB/p65-active ELISA kit (Imgenex, San Diego, CA) according to the manufacturer's instructions. Cytoplasmic and nuclear extracts were prepared, and the latter were subjected to ELISA using specific, anti-NF-κB antibodies.

T cell adhesion assay

Adhesion of T cells to FN was analyzed as described previously [15]. Briefly, flat-bottomed microtiter (96-well) plates were precoated with FN (0.5 μg/well), and the remaining binding sites were blocked with 0.1% BSA. The purified human T cells were labeled with ^{51}[Cr]. Some of the ^{51}[Cr]-labeled cells were preincubated (usually 30 min, 37°C, 7% CO_2-humidified atmosphere) with DS before being exposed (usually 30 min, 37°C, 7% CO_2-humidified atmosphere) to CXCL12. The ^{51}[Cr]-labeled T cells (10^5 cells in 100 μl RPMI containing 0.1% BSA) were then added to the FN-coated microtiter plates. The plates were incubated (30 min, 37°C, 7% CO_2-humidified atmosphere) and then washed gently to remove nonadherent cells. The remaining adherent cells were lysed with 0.1% Triton X-100 in 1 N NaOH, and the radioactivity associated with the resulting supernatants was measured with a γ-counter. For each experimental group of quadruplicate wells, the results were expressed as the mean percentage (±SD) of bound T cells. The percentage of cells that adhered was calculated as follows: [cpm of residual bound cells in the well/(total cpm of cells added to the well–spontaneous release of ^{51}Cr)] × 100.

Chemotaxis assays

Cell migration was measured in Transwells (6.5 mm diameter, Costar, Corning, NY) fitted with polycarbonate filters (5 μm pore size). The filters separating the upper and lower chambers were pretreated (1 h, 37°C) with FN (25 μg/ml). Aliquots (100 μl) of ^{51}[Cr]-labeled T cells (2×10^6/ml RPMI containing 0.1% BSA, 0.1% L-glutamine, and antibiotics) were added to the upper chambers. The bottom chambers contained 0.6 ml of the same medium with or without human CXCL12 (250 ng/ml). After a 3-h incubation (37°C, 7.5% CO_2-humidified atmosphere), T cell migration through the FN-coated filters was determined by collecting the transmigrated cells from the lower chambers. The cells were centrifuged and resuspended in 100 μl distilled water containing 1 M NaOH and 0.1% Triton X-100, and the radioactivity associated with the resulting supernatants was measured with a γ-counter. Cell migration was calculated as the number of migrating cells (counted in the lysates from the lower chamber) expressed as the percentage (±SD) of the total counts per 100 μl aliquot of the starting cell mixture, i.e., per 2×10^5cells.

In vivo homing assay

Homing of human T cells to the spleen and bone marrow of mice was assayed as described previously [17]. Human T cells (5×10^6 cells/mouse) from single donors were injected i.v. 24 h after irradiation. Prior to injection, the cells were incubated for 30 min at 37°C with or without 100 μg/ml 3S-DS or 1S-DS or with anti-CXCR4 mAb (10 μg/mouse) as a control. Cells were recovered from the bone marrow and spleens of transplanted mice 16 h after the injection and were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Mountain View, CA) for the presence of human T cells using human-specific anti-CD45–FITC and anti-CXCR4–PE antibodies, acquiring at least 10^6 cells per sample. Mouse immunoglobulin G and human plasma were used to block Fc receptors. Cells obtained from nontransplanted mice or labeled with mouse isotype-control antibodies were used as negative controls.

Surface expression of CXCR4

Purified human T cells (3×10^6 cells/ml) were incubated (60 min, 37°C, 7.5% CO_2-humidified atmosphere) with CXCL12 (250 ng/ml), with 3S-DS (100 ng/ml), or with various combinations of the two. The T cells were washed twice with PBS and were incubated (30 min, 4°C) with anti-human CXCR4 mAb. They were then washed and stained with a secondary FITC-conjugated goat anti-mouse Ab (30 min, 4°C). The cells were washed again, and their surface expression of CXCR4 was determined using a FACScan flow cytometer (Becton Dickinson) at 525 nm.

Phosphorylation of ERK and Pyk2

Human T cells were maintained (37°C, 7% CO_2, humidified atmosphere) for 48 h in RPMI medium containing only 1% HEPES, and antibiotics and were then resuspended (10^7 cells/ml) in RPMI medium containing 0.1% BSA. Where indicated, the cells were pretreated with PTX (100 ng/ml, overnight) or with the protein kinase C (PKC) inhibitor GF109203X (5 nM, 30 min). These serum-starved T cells (5×10^6 cells, 300 μl/sample) were then activated with CXCL12 (250 ng/ml), 3S-DS (100 ng/ml), or both and were seeded onto 24-well plates (Costar), precoated with FN (25 μg/ml, 300 μl/well). After 5, 10, or 15 min at 37°C, the reactions were terminated by keeping the plates at −70°C for at least 30 min. The cells were then thawed, incubated (30 min, 4°C) in lysis buffer (0.5 mM EDTA, 150 mM $NaCl_2$, 10 nM NaF, 25 mM Tris, pH 7.5, 1% Triton-X-100, 200 mg/ml phenylmethylsulfonyl fluoride, and 1% phosphatase-inhibiting cocktail), and then cleared by centrifugation (15 min, 10,000 *g*, 4°C), and the protein contents of the resulting supernatants were determined. Sample buffer was added to the samples, which were boiled, and equal amounts of proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% polyacrylamide) for protein separation. The separated proteins were transferred to nitrocellulose membranes, which were then blocked with Tris-buffered saline/Tween 20 (TBST; 20 mM Tris, 135 mM $NaCl_2$, 0.1 Tween 20, pH 7.5), containing 5% low-fat milk, and were probed with the appropriate mAb in TBST. The antibodies used were anti-p-Pyk2 (1.5 μg/ml), anti-t-Pyk2 (0.2 μg/ml), anti-p-ERK (0.2 μg/ml), and anti-t-ERK. Stripping and reblotting the membranes achieved sequential subjection of the nitrocellulose membrane to different antibodies. Immunoreactive protein bands were visualized using a horseradish peroxidase-conjugated secondary Ab and the enhanced chemiluminescence system.

Statistical analysis

Statistical analysis was performed using Student's *t*-test.

RESULTS

3S-DS treatment down-regulates NF-κB activation

NF-κB activity is governed by its translocation to the nucleus, where it controls the transcription of genes responsible for regulation of proliferation, cell survival, and inflammation [18]. We examined the ability of 3S-DS and 1S-DS to regulate NF-κB activity induced via TCR activation by mAb anti-CD3. We found that 3S-DS (10 ng/ml) decreased NF-κB activity to background levels (**Fig. 1**). 1S-DS, however, did not affect the NF-κB activity.

3S-DS treatment decreases IFN-γ and TNF-α secretion by activated T cells

As NF-κB mediates the gene transcription of TNF-α and of IFN-γ, we examined the ability of the heparin-derived DS molecules 3S-DS and 1S-DS to regulate cytokine secretion by anti-CD3-activated human T cells. **Figure 2** shows that 3S-DS (10 ng/ml) markedly decreased the secretion of IFN-γ and TNF-α by the activated T cells. 1S-DS did not affect the ability of anti-CD3-activated T cells to secrete TNF-α or IFN-γ. Thus, 3S-DS appears to inhibit NF-κB and its resultant, proinflammatory cytokines IFN-γ and TNF-α.

The decrease in cytokine secretion by T cells treated with 3S-DS was not accompanied by increased cell death as indicated by their ability to incorporate thymidine in response to mitogen or antigen (data not shown).

3S-DS inhibits T cell chemotaxis to CXCL12

Progression of inflammation usually involves the migration of T cells to the inflammatory site. Therefore, we tested the effect of 3S-DS and 1S-DS on chemotaxis. The DS molecules themselves did not exhibit chemotactic activity (data not shown). To test whether they interfere with CXCL12-induced T cell chemotaxis, we studied the effects of uniform (nongradient) fields of 3S-DS and 1S-DS on the migration of ^{51}[Cr]-labeled T cells toward CXCL12. Uniform fields of DS were obtained by adding equal concentrations of DS to the upper and lower chambers of the Transwell apparatus [19]. We found that T cell migration

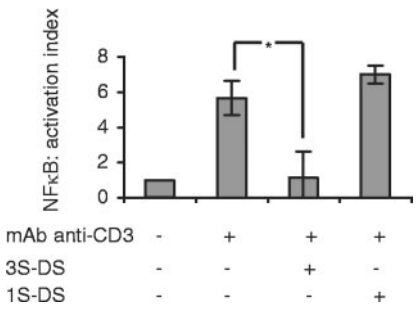

Fig. 1. 3S-DS, but not 1S-DS, inhibits the activation of NF-κB in anti-CD3-activated T cells, which were incubated (2 h, 37°C) with 10 ng/ml 3S-DS or 1S-DS, washed, and replated on mAb anti-human CD3-precoated plates. The cells were collected, nuclear lysates were prepared, and translocation of NF-κB to the nucleus was determined by ELISA. The data shown are means (±SD) of one representative experiment out of the three performed. *, $P < 0.05$.

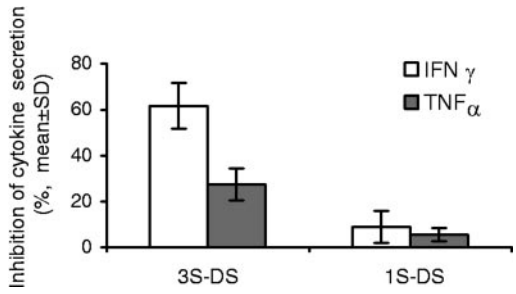

Fig. 2. 3S-DS, but not 1S-DS, inhibits the secretion of IFN-γ and TNF-α from anti-CD3-activated T cells, which were incubated (2 h, 37°C) with 10 ng/ml 3S-DS or 1S-DS, washed, and replated on mAb anti-human CD3-precoated plates. Supernatants were collected after 18 h, and the amounts of secreted IFN-γ and TNF-α were determined by ELISA. The data shown are means (±SD) of three experiments.

was reduced by 50% in the presence of nanogram amounts of 3S-DS, whereas 1S-DS had no significant effect (**Fig. 3**).

3S-DS inhibits the homing of human T cells to the spleens and bone marrow of mice

We also studied the effect of 3S-DS on T cell migration in vivo. It was recently reported that treatment of CD34+ progenitor cells with anti-CXCR4 antibody abolishes their homing to the spleen and bone marrow of irradiated NOD/SCID mice [17]. We used this system to study T cell homing. **Figure 4** shows that human T cells treated with anti-CXCR4 antibody were inhibited in their ability to home to the spleen and bone marrow of NOD/SCID mice. We then examined the effects of 3S-DS on T cell homing in this model. Pretreatment (30 min, 37°C) of T cells with 3S-DS (100 ng/ml) partially, but significantly, inhibited the homing of T cells to the spleens and bone marrow of mice (Fig. 4, C and E). Similar pretreatment of T

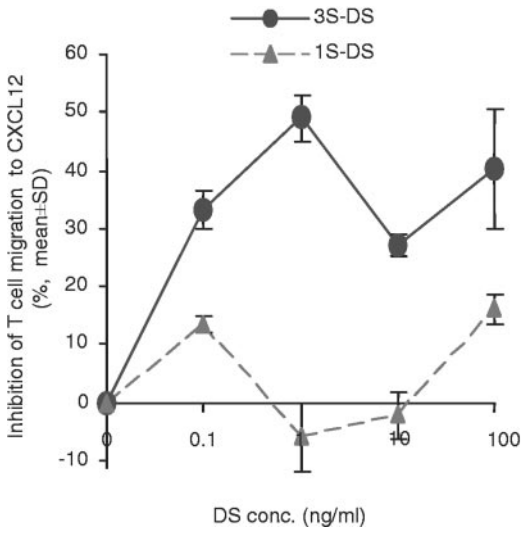

Fig. 3. 3S-DS inhibits CXCL12-induced migration of T cells, which were placed in the upper chambers of a Transwell apparatus, CXCL12 was introduced into the lower chambers, and 3S-DS or 1S-DS was added to both chambers. Migration of T cells through FN-coated filters into the lower chambers was assayed after 3 h. Values (means±SD) are expressed as percentages of control. The results of one representative experiment out of three are shown.

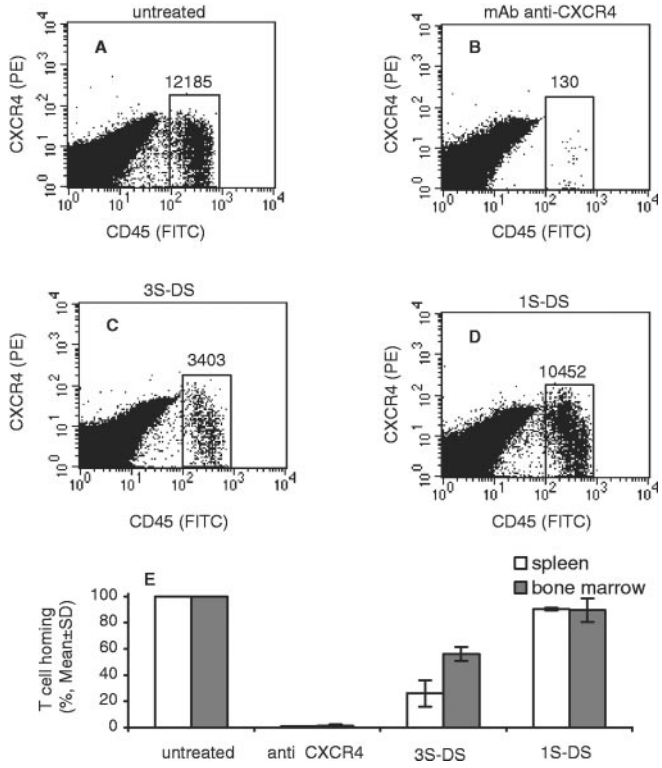

Fig. 4. 3S-DS inhibits the in vivo homing of T cells to lymphoid organs. Freshly purified human T cells were left untreated (A and E), were pretreated (30 min, 37°C) with mAb anti-CXCR4 (10 μg/mouse; B and E), 3S-DS (100 ng/ml, C and E), or 1S-DS (100 ng/ml, D and E), and were injected i.v. into irradiated NOD/SCID mice. After 16 h, the cells were isolated from the spleens (A–E) and bone marrow (E) of the injected mice, stained with mAb anti-CXCR4 and mAb anti-CD45, and analyzed by FACSCalibur. (A–D) One experiment representative of three is shown. (E) Means ± SD of three independent experiments are shown.

cells with 1S-DS, however, did not affect their homing (Fig. 4, D and E). Thus, 3S-DS can down-regulate CXCR4-dependent migration in vivo.

3S-DS induces T cell adhesion to FN but inhibits T cell adhesion induced by CXCL12

CXCL12 is a potent inducer of cell adhesion, which is a first step in T cell migration. We therefore tested whether 3S-DS could affect the adhesion of ^{51}[Cr]-labeled human T cells to immobilized FN. It is surprising that 3S-DS (1 ng/ml) induced T cell adhesion to FN to an extent comparable with that induced by the proadhesive chemokine CXCL12 (**Fig. 5**). However, when introduced to the cells before their exposure to CXCL12, 3S-DS strongly inhibited CXCL12-induced adhesion. This inhibitory effect on the chemokine's proadhesive activity was exerted only when the cells were exposed to 3S-DS before or together with the chemokine. In contrast to 3S-DS, 1S-DS failed to induce T cell adhesion to FN and did not interfere with CXCL12-induced adhesion of T cells to FN. Thus, 3S-DS has proadhesive activity when introduced alone onto T cells, but like other proadhesive factors, such as CCL5 and CCL4 (regulated on activation, normal T expressed and secreted and macrophage-inflammatory protein-1β, respectively) [20], 3S-DS is also capable of inhibiting the proadhesive effect of CXCL12.

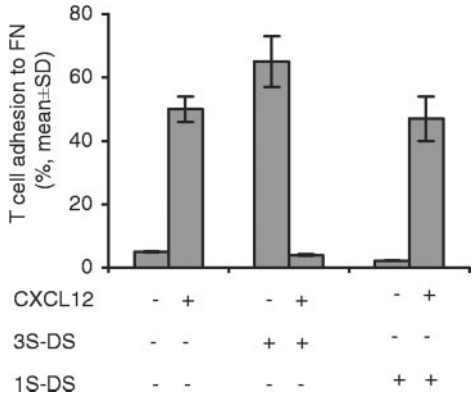

Fig. 5. 3S-DS, but not 1S-DS, induces adhesion of T cells to FN and inhibits CXCL12-induced adhesion. ^{51}[Cr]-labeled human T cells were pretreated (30 min, 37°C) with the indicated DS (1 ng/ml each). CXCL12 (20 ng/ml) was then added, and the cells were seeded onto FN-coated microtiter plates and further incubated (30 min, 37°C). Nonadherent cells were washed off, and the remaining bound cells were lysed. Radioactivity of lysates, reflecting the quantity of adherent cells, was determined with a γ-counter. Values are means ± SD of triplicate wells. The results of one representative experiment out of four are shown.

3S-DS does not affect CXCR4 surface expression

Adhesive and migratory responses of T cells can be inhibited by desensitization of chemokine receptors, a process that often involves receptor internalization. Thus, we examined the effects of 3S-DS on the surface expression of the CXCL12 receptor CXCR4 on human T cells.

Exposure (30 min, 37°C) of human T cells to 3S-DS (1 ng/ml or 100 ng/ml) did not affect their surface expression of CXCR4, as determined by fluorescein-activated cell sorter analysis (**Fig. 6A**). As expected, treatment of T cells with CXCL12 (250 ng/ml) markedly decreased their surface expression of CXCR4 (Fig. 6B), and this internalization was not affected by pretreatment of the cells with 3S-DS (Fig. 6B) or by their simultaneous exposure to 3S-DS and CXCL12 (data not shown). Thus, the inhibition of T cell adhesion and migration toward CXCL12 by

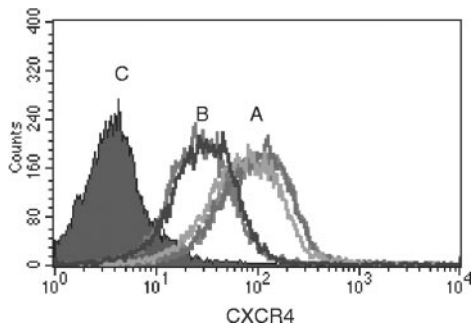

Fig. 6. 3S-DS does not interfere with CXCR4 expression in T cells, which were treated with PBS or with 3S-DS (100 ng/ml) alone (A), with CXCL12 (250 ng/ml) alone, or together with 3S-DS (B) and were analyzed by FACScan for CXCR4 expression. (C) Isotype control is shown.

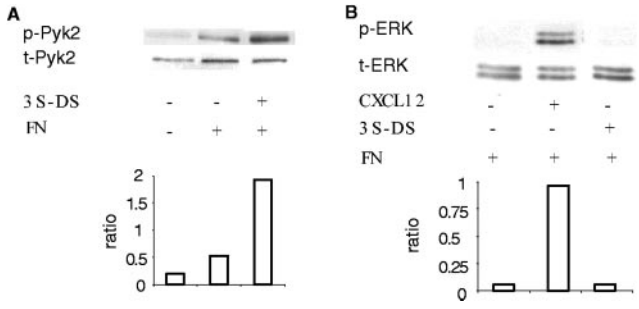

Fig. 7. 3S-DS induces phosphorylation of Pyk2, but not of ERK, in T cells. Serum-starved T cells were stimulated with 3S-DS (100 ng/ml) or CXCL12 (250 ng/ml) as indicated on FN. Cell lysates were subjected to Western blotting, and phosphorylation was assayed using Ab specific to p-Pyk2 or nonphosphorylated (t-Pyk2, A) and ERK (B). Densitometric analysis of the blots disclosed the extent of phosphorylation, which was determined as the ratio between phosphorylated and total Pyk2 or ERK. The results of one representative experiment out of three are shown.

3S-DS is not mediated via interference with the level of CXCR4 surface expression.

3S-DS modulates the phosphorylation of Pyk2 but not of ERK

Activation of ERK and of the focal adhesion kinase, Pyk-2, was reported to be important for T cell adhesion and migration [21–25]. We therefore examined the effects of 3S-DS on Pyk2 and ERK phosphorylation. Pyk2 in resting (nonactivated) T cells was hardly activated, but significant phosphorylation occurred during a short (5 min) incubation of the cells on immobilized FN, and this activation was further increased upon addition of 3S-DS to the cells (**Fig. 7A**). In contrast, FN or 3S-DS did not activate ERK, which was significantly activated upon stimulation of T cells with CXCL12 (Fig. 7B).

We also examined whether 3S-DS might interfere with the phosphorylation of Pyk2 and ERK induced by CXCL12 in T cells plated on immobilized FN. T cell exposure to 3S-DS and CXCL12 resulted in significantly lower Pyk2 phosphorylation than in the presence of CXCL12 alone (**Fig. 8A**). The inhibitory effect of 3S-DS became evident as early as 5 min after exposure and decreased after 15 min. To further explore the involvement of the G protein-coupled receptor (GPCR) in 3S-DS, we examined whether the phosphorylation of Pyk2 induced by 3S-DS was affected by preincubation of the T cells with the signaling inhibitors PTX or GF109203X. Preincubation of T cells with the signaling inhibitors resulted in partial inhibition of 3S-DS-induced phosphorylation of Pyk2 (Fig. 8B). Notably, Pyk2 phosphorylation induced by CXCL12 was inhibited to the same extent. The remaining phosphorylation could be explained by signaling via integrins [26]. In contrast to the phosphorylation of Pyk2, the phosphorylation of ERK induced by CXCL12 in T cells plated on immobilized FN was not affected by the presence of 3S-DS (Fig. 8C). These results indicate that 3S-DS can interfere with specific signaling pathways related to T cell adhesion and migration.

DISCUSSION

Our aim in the present study was to explore the effect of heparin DS on T cell NF-κB activation, proinflammatory cytokine secretion, and migration. We found that preincubation of human T cells with 3S-DS resulted in inhibition of NF-κB activation, as manifested in reduced translocation of the p65 subunit to the nucleus in activated T cells. NF-κB plays a critical role in the regulation of immunity and inflammation by stimulating the transcription of many cytokine genes including TNF-α and IFN-γ [27]. Indeed, pretreatment of T cells with

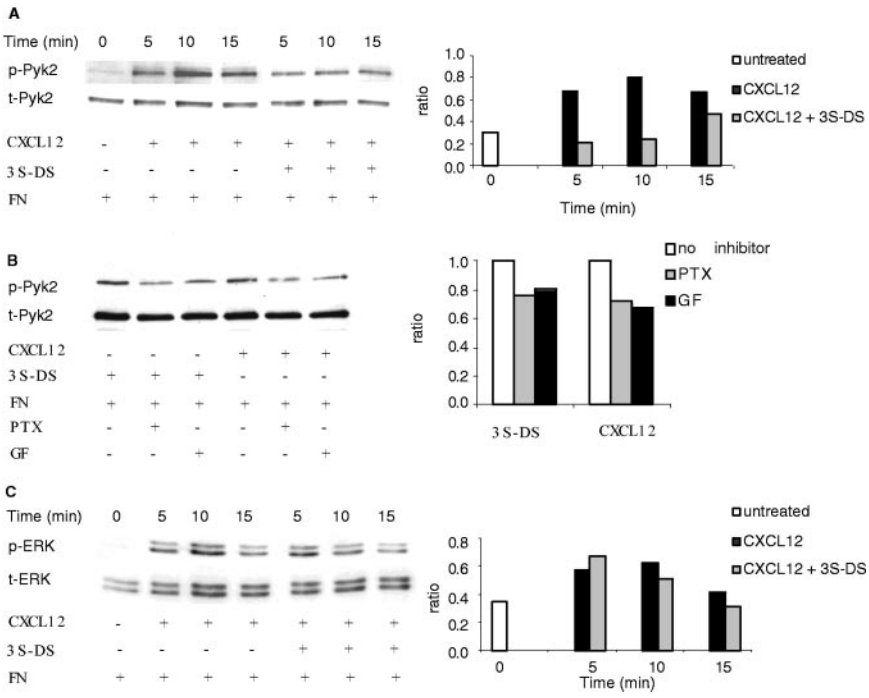

Fig. 8. 3S-DS interferes with the activation of Pyk2, but not of ERK, via CXCR4 and is susceptible to PTX and GF109203X. Serum-starved T cells were stimulated with CXCL12 (250 ng/ml), alone or in combination with 3S-DS (100 ng/ml), on FN after being treated with PTX (100 ng/ml, overnight) or GF109203X (GF; 5 nM, 30 min). Cell lysates were subjected to Western blotting, and phosphorylation was assayed using Ab specific to p-Pyk2 or t-Pyk2 (A and B) and ERK (C). Densitometric analysis of the blots disclosed the extent of phosphorylation, which was determined as the ratio between phosphorylated and total Pyk2 or ERK. The results of one representative experiment out of three are shown.

3S-DS resulted in inhibition of TNF-α and IFN-γ secretion induced by mAb anti-CD3. By itself, TNF-α, as well as other cytokines, activate NF-κB and thus, might initiate an autoregulatory feedback loop [28]. In addition to its ability to regulate cytokine production, NF-κB is also involved in the regulation of the acute-phase response of inflammation, which provides systemic defense and restores homeostasis after infection or injury [29, 30]. Anti-inflammatory drugs such as salicylates and corticosteroids have been reported to inhibit NF-κB [29, 31–33]. Although the mechanism by which 3S-DS inhibits NF-κB activity needs to be studied further, the anti-inflammatory effect of 3S-DS on arthritis and hypersensitivity, which was reported earlier [14], could be explained by down-regulation of NF-κB, IFN-γ, and TNF-α.

An additional mechanism by which the heparin DS could exert anti-inflammatory activity operates through inhibition of the chemokine-mediated homing of T cells to inflamed sites. CXCL12 and its receptor CXCR4 were recently shown to participate in the pathogenesis of inflammatory diseases, such as rheumatoid arthritis [34–36], and in T cell-induced diseases in animal models [37]. Studies from our laboratory demonstrated that 3S-DS arrests joint swelling in adjuvant arthritis [14]. The ability of 3S-DS to control the progression of inflammatory diseases and the reported presence of CXCL12 at these sites prompted us to examine the ability of 3S-DS to regulate CXCL12-induced responses. We found that nanomolar amounts of 3S-DS down-regulated the CXCL12-induced adhesion of T cells to FN and their CXCL12-induced migration through FN. Furthermore, pretreatment of T cells with micromolar amounts of 3S-DS inhibited their homing to the spleens and bone marrow of irradiated NOD/SCID mice, a feature known to depend on the intact functioning of CXCR4 [17]. The inhibition of T cell homing mediated by 3S-DS in vivo reinforces the validity of the antimigratory effect of DS observed in vitro. Hence, the antiadhesive and antimigratory activities of 3S-DS might well be central to its anti-inflammatory activity.

A number of explanations have been put forward to elucidate the mechanism by which the DS molecules inhibit chemokine-induced adhesion and migration. It was suggested that the ability of DS molecules to inhibit CXCL12-induced responses, as shown here and in previous studies [38], might result from their direct interaction with the chemokine, thus affecting the chemokine's function. Indeed, function-altering interactions between glycosaminoglycans and chemokines have been reported previously [39–42]. We found, however, that the inhibitory effect on adhesion persisted even when 3S-DS was removed from the cells before adding the chemokine (data not shown), suggesting that direct interaction between the two molecules was not required. As an alternative possibility, we suggest that 3S-DS might affect the ability of chemokines to bind their specific receptors, perhaps through their direct interaction with the chemokine receptor, in this case, CXCR4, or with other GPCR(s). Such interaction(s) might explain the ability of the DS to induce PTX-sensitive Pyk2 signaling on its own and to down-regulate chemokine-induced responses.

Inhibition of activities mediated by chemokine receptors is often the result of receptor desensitization, a phenomenon in which there is interference with multiple signaling events, such as receptor internalization, $[Ca]^{2+}$ mobilization, and PKC activation [43]. We found that 3S-DS did not change the surface expression of the CXCL12 receptor CXCR4; however, it attenuated the CXCL12-induced $[Ca]^{2+}$ mobilization in RBL-2H3 cells, a mucosal mast cell line (data not shown). In addition, we found that the ability of 3S-DS to induce Pyk2 phosphorylation was inhibited by GF109203X, indicating that 3S-DS transmits its signal via the PKC pathway. In contrast, 3S-DS did not induce ERK phosphorylation nor did it affect the phosphorylation of ERK induced by CXCL12. We therefore suggest that the signaling cascade activated by 3S-DS is triggered via activation of the Gαi protein, which regulates the mobilization of $[Ca]^{2+}$ and affects the activation of the downstream signaling molecules PKC and Pyk2 [26]. Further studies are needed to determine whether 3S-DS needs to be bound to a surface receptor to exert its activity or whether 3S-DS penetrates into the cell and directly interacts with the G protein [44].

The present findings suggest that 3S-DS down-regulates CXCL12-induced responses by cross-desensitizing CXCR4. Chemokine responses can be cross-desensitized by a wide range of agents [20, 21, 45–49]. However, this is the first report of a heparin DS that can activate signal transduction machinery on the one hand and down-regulate chemokine-induced signaling events on the other. Cross-desensitization, which leads to reduced cell responsiveness to chemokines, is of major importance in designing anti-inflammatory, anticancer, and in the case of CXCR4, also anti-human immunodeficiency virus drugs [50–54].

It is interesting that structurally similar DS can be generated by the degradation of heparan sulfate by heparanase [14, 55, 56]. Thus, we postulate that the degradation of ECM glycoproteins by enzymes secreted by migrating T cells might provide the cells with regulatory signals that are unique to the inflammatory environment and do not require de novo synthesis. In such a case, accumulation of these degradation products might alert the cells to down-regulate their proinflammatory activity.

We have shown that 3S-DS affects the regulation of inflammation via the short-term (migration) and the long-term (cytokine secretion) proinflammatory activities of the cells. Although we have to further investigate the mechanisms underlying the inhibition of NF-κB activation and to identify the receptor that binds 3S-DS, our findings indicate that the use of 3S-DS as a therapeutic agent should be considered.

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