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TSG-6 Inhibits Neutrophil Migration via Direct Interaction with the Chemokine CXCL8

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TNF-stimulated gene/protein-6 (TSG-6) is expressed by many different cell types in response to proinflammatory cytokines and plays an important role in the protection of tissues from the damaging consequences of acute inflammation. Recently, TSG-6 was identified as being largely responsible for the beneficial effects of multipotent mesenchymal stem cells, for example in the treatment of animal models of myocardial infarction and corneal injury/allograft transplant. The protective effect of TSG-6 is due in part to its inhibition of neutrophil migration, but the mechanisms underlying this activity remain unknown. In this study, we have shown that TSG-6 inhibits chemokine-stimulated transendothelial migration of neutrophils via a direct interaction ($K_D \sim 25$ nM) between TSG-6 and the glycosaminoglycan binding site of CXCL8, which antagonizes the association of CXCL8 with heparin. Furthermore, we found that TSG-6 impairs the binding of CXCL8 to cell surface glycosaminoglycans and the transport of CXCL8 across an endothelial cell monolayer. In vivo this could limit the formation of haptotactic gradients on endothelial heparan sulfate proteoglycans and, hence, integrin-mediated tight adhesion and migration. We further observed that TSG-6 suppresses CXCL8-mediated chemotaxis of neutrophils; this lower potency effect might be important at sites where there is high local expression of TSG-6. Thus, we have identified TSG-6 as a CXCL8-binding protein, making it, to our knowledge, the first soluble proteoglycan and, hence, integrin-mediated tight adhesion and migration. We further observed that TSG-6 suppresses CXCL8-mediated chemotaxis of neutrophils; this lower potency effect might be important at sites where there is high local expression of TSG-6. Thus, we have identified TSG-6 as a CXCL8-binding protein, making it, to our knowledge, the first soluble mammalian chemokine-binding protein to be described to date. We have also revealed a potential mechanism whereby TSG-6 mediates its anti-inflammatory and protective effects. This could inform the development of new treatments for inflammation in the context of disease or following transplantation. The Journal of Immunology, 2014, 192: 000–000.

Neutrophils play an essential role in the inflammatory response to infection and injury, which is dependent on their rapid recruitment from the vasculature. This occurs via a multistep process that involves sequential tethering and rolling, activation by chemoattractants (e.g., chemokines), tight adhesion, and transendothelial migration (1). However, the destructive potential of neutrophils requires that their extravasation be tightly regulated, otherwise tissue damage occurs, as seen in conditions such as ischemia/reperfusion injury, rheumatoid arthritis, and asthma. The chemokine CXCL8 (IL-8) plays a key role in neutrophil activation and transmigration (2). It is produced in response to proinflammatory stimuli, for example, by endothelial cells (3), monocytes/macrophages, and mast cells (4), and it interacts with glycosaminoglycans (GAGs), such as heparan sulfate (HS) and chondroitin sulfate (5), on the vascular endothelium (6–8). GAG binding mediates presentation of CXCL8 to its G protein–coupled receptors on neutrophils (i.e., CXCR1 and CXCR2) (9), enables chemokine oligomerization (where the monomeric and dimeric forms of CXCL8 have differential functions in vivo) (10), and generates haptotactic gradients that guide neutrophil recruitment (11, 12). There is evidence that tissue-specific variations in the content, concentration, and distribution of GAGs can modulate the CXCL8 monomer/dimer equilibrium and, thereby, regulate neutrophil extravasation (13).

TNF-stimulated gene/protein-6 (TSG-6), an ~35-kDa secreted glycoprotein (14), is expressed at sites of inflammation and injury; for example, it is abundant in the synovial fluids of patients with inflammatory arthritis (15), and serum concentrations of TSG-6 correlate with the progression of murine proteoglycan-induced arthritis (16). There is growing evidence that TSG-6 acts to inhibit the extravasation of neutrophils in vivo (17–23), affecting both their rolling and transendothelial migration (17, 19). For example, TSG-6–deficient mice develop much more severe proteoglycan–induced arthritis than do controls, with elevated neutrophil infiltration into the paw joints (20). Furthermore, secretion of TSG-6 by multipotent mesenchymal stromal cells (MSCs) has been shown to reduce inflammatory damage in rodent models of myocardial infarction (21), corneal wounding (22), and corneal transplantation (24). These protective properties of TSG-6 and the fact that it is produced primarily in the context of inflammation suggest that it forms part of an endogenous pathway to limit tissue damage during acute inflammatory episodes: TSG-6 is released from the secretary granules of neutrophils (25) and mast cells (16) as well as...
as being expressed by macrophages (14, 26) and a wide variety of stromal cell types (18). Although it is evident that the inhibitory effect of TSG-6 on neutrophil transendothelial migration (17, 19) contributes to its protective effects in inflammatory models, the molecular basis of this activity has not yet been determined.

TSG-6 consists mainly of contiguous Link and CUB modules (14, 18). It interacts with protein ligands, including inter-con-inhibitor (17, 27) and thrombospondin-1 (28), as well as with various GAGs, for example, heparin, HS, chondroitin-4-sulfate, dermatan sulfate, and hyaluronan (HA) (27, 29, 30). All of these ligands bind to the Link module domain of TSG-6, which is also responsible for the inhibition of neutrophil migration (17, 19); however, it is not clear whether any of these interactions contributes to the anti-inflammatory activity of TSG-6 (17).

In this study, we have demonstrated that TSG-6 acts to inhibit CXCL8-induced transendothelial migration of human neutrophils via a direct interaction between the TSG-6 Link module and CXCL8, which antagonizes the binding of CXCL8 to heparin/HS. Our data indicate that TSG-6 can impair the transport of CXCL8 across the endothelium and its presentation by cell surface GAGs. At high concentrations TSG-6 was also seen to inhibit neutrophil chemotaxis. To our knowledge, this work identifies TSG-6 as the first soluble, mammalian chemokine-binding protein and reveals a molecular mechanism for its tissue-protective effects during inflammation.

Materials and Methods

Protein and GAG preparation

Full-length, wild-type (WT) recombinant human (rh)TSG-6, its isolated Link module (Link_TSG6), and biotinylated Link_TSG6 were produced as described previously (29, 31); the Link_TSG6_D (K34A/K54A) and Link_TSG6_T (K20A/K34A/K41A) mutants were prepared/characterized as in Mahoney et al. (27). WT CXCL8 and the CXCL8_S (R68A) and CXCL8_T (K64A/K67A/R68A) mutants, expressed and purified as described in Tanino et al. (32), and CCL3, CCL5, and CXCL11 were provided by the Pharmaceutical Research Laboratory (Amanda E. I. Proudfoot, Geneva, Switzerland). Heparin (Fourth International Standard) was biotinylated as described in Clark et al. (33).

Cell culture

All cell cultures were incubated at 37°C and 5% (v/v) CO2. EA.hy 926 cells and HUVECs were cultured in DMEM with 10% (v/v) FBS and endothelial basal medium (EBM-2; Lonza), respectively. HL-60 cells (American Type Culture Collection, Manassas, VA) were maintained in IMDM with 20% (v/v) FBS; differentiation to neutrophil-like cells, as assessed by CD11b upregulation and morphological changes, was induced by incubation with 1.5% (v/v) DMSO for 120 h (34). The murine pro-B cell line 300-19 (35) and stable transfectants expressing CXCR1 (clone 1B4) or CXCR2 (clone ID5) were cultured in RPMI 1640 with 10% (v/v) FBS, 1% (w/v) glutamine, and 5 × 10−5 M 2-ME, under puromycin (1.5 μg/ml) selection (35).

CXCL8 transport and chemokine-induced neutrophil transmigration across endothelial cell monolayers

EA.hy 926 cells were seeded on top of 6.5-mm Transwell filters in 24-well plates (Corning permeable supports, 3-μm polyester membrane), and HUVECs were seeded in the same way following coating of the filters with fibronectin (10 μg/ml in PBS for 1 h at 37°C). Cells (5 × 107 cells/well in 100 μl media) were incubated at 37°C overnight, with 600 μl media below the membrane. Monolayer formation was confirmed by eye using a light microscope, cells were washed with PBS, and Transwells were then transferred to fresh wells containing serum-free medium with or without chemokine. Cells were washed with PBS, and Transwells were then transferred to fresh wells containing serum-free medium with or without chemokine.

For transmigration assays, differentiated HL-60 cells (as a model of human neutrophils) (34) or human neutrophils isolated from buffy coats (36) were washed, resuspended (3.75 × 107 cells/ml) in fresh DMEM, and added (750,000 cells/well) to the top of endothelial monolayers. Transwells were then incubated at 37°C for 24 h (HL-60 cells) or 2 h (primary neutrophils) with CXCL8, without or with TSG-6, in the lower chamber. Migrated neutrophils were recovered from the media below each membrane by centrifugation (10 min, 400 × g) and counted.

The transport of CXCL8 across endothelial cell monolayers was investigated using a modification of the method described in Prounster et al. (37). Biotinylated (b-)CXCL8 (prepared as in Ref. 38 and shown to signal through CXCR2 with similar efficiency to unmodified CXCL8) was added (3 nM) below HUVEC monolayers, in the absence/presence of Link_TSG6 (50 pmol/well, 10% of total excess), and Transwells were incubated at 37°C for 2 h. Media were collected from the upper and lower chambers, followed by incubation with 10× PBS for 3 min to recover any CXCL8 bound to cell surface GAGs; cells were then lysed with RIPA buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1% (v/v) Triton X-100, 1% (v/v) sodium deoxycholate, 0.1% (w/v) SDS, 5 mM EDTA). All samples were subject to SDS-PAGE and Western blot analysis using streptavidin conjugated to IRDye 800CW (LI-COR Biosciences) using an Odyssey imaging system (LI-COR Biosciences).

Analysis of the TSG-6 interaction by surface plasmon resonance

Surface plasmon resonance (SPR) analysis was carried out using a Biacore 3000 (GE Healthcare), where ligands (i.e., Link_TSG6, rhTSG-6, or CXCL8) were immobilized onto a C1 Biacore chip (500 response units) as follows. The flow rate was set at 40 μl/min and the surface was equilibrated with SPR running buffer, that is, SPR 6.0 (10 mM NaOAc, 150 mM NaCl, 0.05% (v/v) Tween 20 [pH 6]) or SPR 7.2 (10 mM HEPES, 150 mM NaCl, 0.05% (v/v) Tween 20 [pH 7.2]), for 1 h (39). Paired cells (on the SPR chip) were then activated by injecting 70 μl of a 1:1 mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 0.1 M N-hydroxy-succinimide (39, 40). Protein ligands (rhTSG-6, Link_TSG6, or CXCL8 at 20 μg/ml) were immobilized by passing them over one of the activated cells, using buffer conditions found to be optimal in initial scouting experiments (rhTSG-6 in 10 mM sodium acetate [pH 6], Link_TSG6 and CXCL8 in 10 mM HEPES [pH 7.4]), until the signal increased by 500 response units. Any remaining active sites in the reference and ligand-containing cells were then blocked by injecting 1 M ethanolamine (70 μl) (39, 40). Analytes (rhTSG-6, CXCL8, or other chemokines), at a range of concentrations, were passed over the immobilized CXCL8, rhTSG-6, or Link_TSG6 in SPR running buffer at either pH 5 (SPR 6.0) or pH 7.2 (SPR 7.2); the resulting sensograms were analyzed using the 1:1 Langmuir interaction model with BIAXevaluation software (GE Healthcare).

Characterization of the CXCL8/TSG-6 interaction using plate-based binding assays

Mictrotiter plate assays to compare the interaction of CXCL8 with full-length TSG-6 and its isolated Link module were carried out essentially as described previously (27, 41). rhTSG-6 or Link_TSG6 (50 pmol/well) in coating buffer (20 mM NaOAc [pH 9.6]) was immobilized onto 96-well Nunc Maxisorp plates (Thermo Fisher Scientific) by incubation overnight at room temperature. Plates were washed three times and blocked with 1% (w/v) BSA for 90 min at 37°C. After further washing, CXCL8 (0–600 nM) was added to each well and plates were incubated for 2 h at room temperature and then for 90 min at room temperature with biotinylated anti-human CXCL8 Ab (PeproTech; 0.3 μg/ml). Bound CXCL8 was detected using extravidin–alkaline phosphatase (1:10,000; Sigma-Aldrich), followed by SigmaFast p-nitrophenyl phosphate solution (200 μl/well; Sigma-Aldrich), with absorbance measurements at 405 nm being taken after 10 min. To determine the specificity of the CXCL8-Link_TSG6 interaction, CXCL8 (50 pmol/well) was immobilized onto MaxiSorp plates overnight and biotinylated Link_TSG6 (10 nM) was added in the fluid phase, in combination with unlabelled CXCL8 (0–1000 nM). Binding was detected as described above.

CXCL8/heparin binding assays

Mictrotiter plate assays were carried out essentially as described previously (27, 33). Briefly, CXCL8, CXCL8_S, or CXCL8_T (50 pmol/well in 20 mM Na2CO3 [pH 9.6]) was immobilized onto 96-well Nunc Maxisorp plates (Thermo Fisher Scientific) by incubation at room temperature overnight. All subsequent washes (three times each after each incubation), dilutions, and incubations were performed in SAB6 (10 mM NaOAc, 150 mM NaCl, 2% [v/v] Tween 20 [pH 6]). After blocking with 5% (w/v) BSA, for 90 min at 37°C, biotinylated heparin (50 μg/ml) was added along with Link_TSG6 (50 pmol/well) or (at 25 ng/well) in combination with TSG-6 proteins (0–1000 nM) for 4 h at room temperature. Bound heparin was detected by addition of extravidin–alkaline phosphatase (1:10,000; Sigma-Aldrich) followed by...
SigmaFast p-nitrophenyl phosphate solution (200 μl/well; Sigma-Aldrich). Absorbance measurements at 405 nm were taken after 10 min and corrected against blank wells.

**Interaction of CXCL8 with cell surface receptors**

Binding of CXCL8 to murine 300-19 cells (35) stably expressing CXCR1 (clone 1B4) or CXCR2 (clone 1D5) was determined by flow cytometry using a biotinylated human CXCL8 Fluorokine kit (R&D Systems) according to the supplier’s instructions. Cells (1 × 10⁶ in PBS) were incubated at 4°C for 1 h with h-CXCL8 that had been preincubated (1 h at 37°C) in the absence or presence of Link_TSG6. Cell-associated CXCL8 was detected, following addition of avidin-fluorescein, using a CyAn ADP flow cytometer (Beckman Coulter) with excitation at 488 nm. Gating was applied to select live cells on the basis of forward scatter versus side scatter.

**CXCL8-mediated chemotaxis of human neutrophils**

Neutrophils were purified from fresh human blood and their chemotaxis through 3-μm pores was assayed as reported previously (36). CXCL8 (1 nM) and Link_TSG6 (0–10 μM) were placed in the lower chambers of 96-well ChemoTx plates (Neuroprobe, Cabin John, MD), neutrophils (in RPMI 1640 without red phenol, 2% [v/v] heat-inactivated FBS, 1% penicillin/streptomycin, 1% [w/v] l-glutamine) were added to the upper chambers, and migrated cells were counted (using a CyQUANT kit; Molecular Probes) after 45 min at 37°C.

**Statistical analysis**

Statistically significant differences between groups were identified by repeated measures ANOVA and a Bonferroni post hoc test (GraphPad Prism, version 5.0). A two-tailed Student t test was used for statistical analyses involving pairwise comparisons of data sets. A p value <0.05 was considered statistically significant. When data are presented as mean values ± SEM, the number of independent experiments (n) is indicated, with all conditions being set up at least in triplicate within each experiment.

**Results**

**TSG-6 inhibits CXCL8-mediated transendothelial migration of neutrophils via its Link module domain**

TSG-6 is a potent inhibitor of neutrophil extravasation in vivo (17, 20), and because CXCL8 is an important chemoattractant and activator for neutrophils, we chose to investigate the effects of TSG-6 on CXCL8 using a Transwell assay. In this system CXCL8 upregulated the transendothelial migration of both differentiated HL-60 cells (Fig. 1A) and primary human neutrophils (Fig. 1B) in a dose-dependent manner; 3.6 nM CXCL8 increased the numbers of migrated cells by ∼2- and ∼6-fold, respectively, which is consistent with previous studies (19, 42). Under these conditions, we showed that a 5-fold molar excess (i.e., 18 nM) of Link_TSG6 completely ablated the CXCL8-induced migration of differentiated HL-60 cells (Fig. 1C) and significantly reduced the migration of primary human neutrophils (Fig. 1D). Furthermore, Link_TSG6 and full-length rhTSG-6 had equivalent neutrophil inhibitory effects (Fig. 1E), confirming that this activity resides within the Link module domain of TSG-6. Given that the inhibition of neutrophil migration was seen when both the CXCL8 and TSG-6 proteins were added to Transwells below the endothelial cell layer, we hypothesized that this activity of TSG-6 was due to its direct interaction with CXCL8.

**TSG-6 binds to CXCL8 via its Link module**

We investigated the binding of TSG-6 to CXCL8 using SPR, which revealed that these proteins interact with high affinity (see Table I). For example, when CXCL8 was flowed over immobilized rhTSG-6, at pH 6 (Supplemental Fig. 1A) or pH 7.2

**FIGURE 1.** TSG-6 inhibits transendothelial migration of differentiated HL-60 cells via interaction of its Link module domain with CXCL8. Migration of differentiated HL-60 cells across a monolayer of EA.hy 926 cells (A) or primary human neutrophils across a monolayer of HUVECs (B) was measured in response to a range of concentrations of WT CXCL8 (1.2, 3.6, 6, 9, and 12 nM) (n = 3). Migration of differentiated HL-60 cells across a monolayer of EA.hy 926 cells was measured in response to a range of concentrations of WT CXCL8 (1.2, 3.6, 6, 9, and 12 nM) (n = 3). Migration of differentiated HL-60 cells across a monolayer of EA.hy 926 cells (Fig. 1A) and primary human neutrophils (Fig. 1B) in a dose-dependent manner; 3.6 nM CXCL8 increased the numbers of migrated cells by ∼2- and ∼6-fold, respectively, which is consistent with previous studies (19, 42). Under these conditions, we showed that a 5-fold molar excess (i.e., 18 nM) of Link_TSG6 completely ablated the CXCL8-induced migration of differentiated HL-60 cells (Fig. 1C) and significantly reduced the migration of primary human neutrophils (Fig. 1D). Furthermore, Link_TSG6 and full-length rhTSG-6 had equivalent neutrophil inhibitory effects (Fig. 1E), confirming that this activity resides within the Link module domain of TSG-6. Given that the inhibition of neutrophil migration was seen when both the CXCL8 and TSG-6 proteins were added to Transwells below the endothelial cell layer, we hypothesized that this activity of TSG-6 was due to its direct interaction with CXCL8.
The Link module of TSG-6 inhibits CXCL8/GAG interactions

Chemokine/GAG interactions play essential roles in neutrophil migration through enabling the sequestration of chemokines by HS proteoglycans (HSPGs), and hence the formation of haptotactic gradients, on the lumen of the endothelium (5, 11, 12); they have also been implicated in the transport of chemokines (produced in interstitial tissues) across the endothelium (11). For example, mutants of CXCL8 with impaired heparin-binding activities showed deficient transcytosis and reduced accumulation on the apical surface of the endothelium in skin (7). Therefore, we investigated whether binding of TSG-6 to CXCL8 could antagonize the interaction between CXCL8 and heparin, a GAG that is often used as a model for HS owing to its similar structure and greater availability. In plate-based assays, where immobilized WT CXCL8 bound to heparin in a dose-dependent manner (Fig. 3A), we found that Link_TSG6 and rhTSG-6 were similarly effective as competitors for this interaction (IC50s of ~70 and ~80 nM, respectively); CXCL8/heparin binding was completely abolished in the presence of 0.5–1 μM TSG-6 protein (Fig. 3B).

To determine whether this inhibitory effect was due to TSG-6 (a known heparin-binding protein) interacting with heparin, we used the Link_TSG6 mutants K34A/K54A (Link_TSG6_D) and K20A/K41A (Link_TSG6_T), which have ~50 and ~10% of WT heparin-binding activity, respectively (27). Both mutants antagonized the CXCL8/heparin interaction; Link_TSG6_D had similar activity to WT Link_TSG6, whereas Link_TSG6_T showed enhanced activity with maximal inhibition at 0.125 μM (Fig. 3C).

Table 1. Dissociation constants for the interactions of rhTSG-6 and Link_TSG6 with CXCL8 and other chemokines

<table>
<thead>
<tr>
<th>Immobilized Ligand</th>
<th>Fluid-Phase Analyte</th>
<th>K_D (nM)*</th>
<th>χ²</th>
<th>k_on (M⁻¹ s⁻¹)</th>
<th>k_off (s⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>rhTSG-6</td>
<td>CXCL8 (pH 6)</td>
<td>26</td>
<td>6.5</td>
<td>2.9 × 10⁶</td>
<td>7.5 × 10⁻⁴</td>
</tr>
<tr>
<td>rhTSG-6</td>
<td>CXCL8 (pH 7.2)</td>
<td>19</td>
<td>16.7</td>
<td>3.4 × 10⁴</td>
<td>6.5 × 10⁻⁴</td>
</tr>
<tr>
<td>Link_TSG6</td>
<td>CXCL8 (pH 6)</td>
<td>6</td>
<td>44.3</td>
<td>5.3 × 10⁴</td>
<td>3.2 × 10⁻⁴</td>
</tr>
<tr>
<td>Link_TSG6</td>
<td>CXCL8 (pH 7.2)</td>
<td>21</td>
<td>18.1</td>
<td>1.9 × 10⁴</td>
<td>1.1 × 10⁻³</td>
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<tr>
<td>rhTSG-6</td>
<td>CXCL8_S (pH 6)</td>
<td>581</td>
<td>0.2</td>
<td>5.1 × 10⁻¹</td>
<td>1.8 × 10⁻³</td>
</tr>
<tr>
<td>rhTSG-6</td>
<td>CXCL8_T (pH 6)</td>
<td>2,109</td>
<td>0.2</td>
<td>640</td>
<td>1.4 × 10⁻³</td>
</tr>
<tr>
<td>Link_TSG6</td>
<td>CXCL8_S (pH 6)</td>
<td>203</td>
<td>2.0</td>
<td>8.4 × 10³</td>
<td>1.7 × 10⁻³</td>
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<td>CXCL8</td>
<td>rhTSG-6 (pH 6)</td>
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<td>8.9</td>
<td>5.7 × 10⁴</td>
<td>4.2 × 10⁻⁴</td>
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<tr>
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<td>rhTSG-6 (pH 7.2)</td>
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<td>15.3</td>
<td>2.3 × 10⁴</td>
<td>1.1 × 10⁻³</td>
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<tr>
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<td>CCL5 (pH 7.2)</td>
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<td>3.3</td>
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<td>6.4 × 10⁴</td>
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<tr>
<td>rhTSG-6</td>
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<td>15,100</td>
<td>7.4</td>
<td>4.4 × 10³</td>
<td>6.0 × 10⁻³</td>
</tr>
</tbody>
</table>

*K_D values determined by full kinetic analysis of SPR data.

Table 1 (Supplemental Fig. 1D), analysis of the resultant sensograms revealed affinities (K_D) of 26 and 19 nM, respectively. When rhTSG-6 was used as the analyte (and CXCL8 immobilized), K_D values within the order of magnitude were obtained, that is, 74 nM at pH 6 (Supplemental Fig. 1C) and 20 nM at pH 7.2 (Supplemental Fig. 1F). Similar affinities were also observed with immobilized Link_TSG6 (6 nM at pH 6 [Supplemental Fig. 1B] and 21 nM at pH 7.2 [Supplemental Fig. 1E]), demonstrating that the interaction with CXCL8 is mediated via the Link module of TSG-6; this was further confirmed in plate-based assays where CXCL8 bound to immobilized rhTSG-6 and Link_TSG6 (Fig. 2A). Furthermore, unlabeled Link_TSG6 competed for the binding of biotinylated Link_TSG6 to immobilized CXCL8 with an IC50 of 15 ± 3 nM (Fig. 2B), which is comparable to the K_D value determined above by SPR.

Taken together, these results indicate that there is a specific, high-affinity interaction between the Link module of TSG-6 and CXCL8 (with K_D values in the range ~10–70 nM), which could contribute to the inhibition of chemokine-induced neutrophil migration. Overall, there was little difference between the binding affinities determined by SPR at pH 6 and pH 7.2; this is in contrast to other interactions of TSG-6, for example, with HA (43), heparin (27, 28), and thrombospondin-1 (28), which are all sensitive to pH in the range pH 6 to pH 7.5.

To determine whether this inhibitory effect was due to TSG-6 (a known heparin-binding protein) interacting with heparin, we used the Link_TSG6 mutants K34A/K54A (Link_TSG6_D) and K20A/K41A (Link_TSG6_T), which have ~50 and ~10% of WT heparin-binding activity, respectively (27). Both mutants antagonized the CXCL8/heparin interaction; Link_TSG6_D had similar activity to WT Link_TSG6, whereas Link_TSG6_T showed enhanced activity with maximal inhibition at 0.125 μM (Fig. 3C).
Furthermore, in the Transwell system, both Link_TSG6_D and Link_TSG6_T inhibited the transendothelial migration of differentiated HL-60 cells with potencies similar to WT Link_TSG6 (Fig. 3D). Overall, these data indicate that both the inhibitory effect of TSG-6 on CXCL8-induced neutrophil transmigration and its impairment of the CXCL8/heparin interaction are independent of the heparin-binding activity of TSG-6.

Using the Transwell system with b-CXCL8 and Link_TSG6 in the lower chamber, we went on to show that Link_TSG6 (at the same concentration seen to inhibit neutrophil transmigration) caused significant reductions in 1) the binding of b-CXCL8 to GAGs on the basolateral surface of HUVECs (∼4-fold), 2) the movement of b-CXCL8 out of the lower Transwell chamber, and 3) the subsequent association of chemokine with GAGs on the apical cell surface (∼5-fold) (Fig. 4). We observed no significant effect of Link_TSG6 on the amount of intracellular b-CXCL8. This could, at least in part, reflect that Link_TSG6 inhibits the GAG-mediated uptake of CXCL8 by endothelial cells, but has no effect on other mechanisms, for example, via the Duffy Ag receptor for chemokines. Additionally, Link_TSG6 did not alter the amount of b-CXCL8 in the media of the upper Transwell chamber. This is likely because most CXCL8 present here is due to nonspecific paracellular diffusion, as has been previously demonstrated (11), and is therefore independent of the transcytosis mechanism inhibited by Link_TSG6. Taken together, these data suggest that TSG-6 might inhibit both GAG-mediated transcytosis of CXCL8 and the presentation of CXCL8 on the lumen of the vascular endothelium.

TSG-6 inhibits CXCL8-mediated neutrophil transmigration by interacting with the GAG-binding surface of CXCL8 and blocking CXCL8/GAG binding

To further investigate the mechanism of the anti-migratory activity of TSG-6, we used the CXCL8 mutants R68A (CXCL8_S) and K64A/K67A/R68A (CXCL8_T) (5, 8, 32), which we found to have reduced heparin-binding activity (∼20–60% of WT) and no measurable activity, respectively (Fig. 3A). In SPR experiments TSG-6 showed weak binding to both mutants (Supplemental Fig. 1G–I, Table I); the affinities for the CXCL8_S and CXCL8_T interactions with rhTSG-6 were reduced by 20- and 80-fold, respectively, compared with WT CXCL8 (due to slower rates of association and faster rates of dissociation). These data suggest that the heparin- and TSG-6–binding sites on CXCL8 overlap.

Despite its somewhat impaired heparin-binding function, CXCL8_S induced transendothelial migration of differentiated HL-60 cells (Fig. 5A) with similar efficiency to WT protein; in contrast, CXCL8_T had no such activity (Fig. 5B), reflecting the essential role of GAG-binding in CXCL8-mediated neutrophil migration. However, Link_TSG6 had no inhibitory effect on the CXCL8_S-mediated migration of either differentiated HL-60 cells (Fig. 5C) or primary human neutrophils (Fig. 5D), even when present at 10- or 20-fold molar excess. Because the affinity of TSG-6 for CXCL8_S is ∼20-fold weaker than for WT CXCL8, these data indicate that a direct interaction between TSG-6 and CXCL8 is required to inhibit CXCL8-mediated transendothelial migration.

**FIGURE 3.** Interaction between the Link module domain of T6G-6 and the GAG-binding site of CXCL8 inhibits CXCL8/heparin binding and CXCL8-mediated neutrophil transmigration. WT or mutant CXCL8 (250 nM) was immobilized onto MaxiSorp plates and b-heparin (0–100 ng/well) (A) or b-heparin (25 ng/well) in combination with a range of concentrations of rhTSG-6 or Link_TSG6 (0-1000 nM) (B), or in combination with a range of concentrations of WT Link_TSG6, Link_TSG6_D, or Link_TSG6_T (0–2000 nM) (C), was added in the fluid phase. Binding of b-heparin was detected using extravidin–alkaline phosphatase followed by disodium p-nitrophenyl phosphate; absorbance at 405 nm was determined after 5 (A) or 10 min (B, C). Data are plotted as mean values (n = 4) ± SEM and fitted using OriginPro version 8. Kd values of 5 ± 12 and 26 ± 11 nM were determined for the binding of b-heparin to WT CXCL8 and CXCL8_D, respectively (A), IC50 values of 81 ± 18 and 71 ± 14 nM for the inhibition of b-heparin binding to CXCL8 by rhTSG-6 and Link_TSG6, respectively (B), and 105 ± 5, 172 ± 3, and 45 ± 5 nM for the inhibition of this interaction by WT Link_TSG6, Link_TSG6_D, and Link_TSG6_T, respectively (C), were determined. In (D), migration of differentiated HL-60 cells across an EA.hy 926 cell monolayer was determined in response to CXCL8 (3.6 nM [+] alone or in combination with 5:1 molar ratios of WT Link_TSG6, Link_TSG6_D, or Link_TSG6_T (n = 4). Data are plotted as mean values (± SEM) relative to nonstimulated controls (−). **p < 0.01, ***p < 0.001 compared with CXCL8 alone, as determined using repeated measures ANOVA analysis with a Bonferroni post hoc test.
migration. These data indicate that TSG-6 might inhibit neutrophil migration by impairment of CXCL8 binding to endothelial cell GAGs, and this is supported by our observation that Link_TSG6 and a heparin oligosaccharide (dp8) have essentially identical inhibitory effects on the interaction of b-CXCL8 with a HUVEC monolayer (data not shown).

**TSG-6 inhibits CXCL8-mediated neutrophil chemotaxis**

In the absence of an endothelial cell monolayer, we observed dose-dependent inhibition by Link_TSG6 of CXCL8-induced neutrophil chemotaxis with an IC50 of 2.4 ± 0.3 μM (Fig. 6A). To promote neutrophil migration, CXCL8 must bind to CXCR1 and/or CXCR2. We therefore used flow cytometry to directly test the effect of TSG-6 on the interactions of CXCL8 with its receptors. Preincubation with Link_TSG6 gave rise to a dose-dependent reduction in the binding of b-CXCL8 to cell surface CXCR2, with an IC50 of ~5 μM (Fig. 6B); however, we did not detect any effect on the CXCL8/CXCR1 interaction even at molar excesses of Link_TSG6 as high of 600:1 (not shown). The similar potencies with which Link_TSG6 inhibited CXCL8/CXCR2 binding and CXCL8-induced chemotaxis indicate that TSG-6 can operate via an alternative mechanism to modulate CXCL8 activity, whereby its binding to the chemokine weakly inhibits subsequent interaction with CXCR2 (Fig. 7). However, this activity is only seen with micromolar concentrations of TSG-6, in contrast to its inhibition of neutrophil transmigration (∼10 nM; Fig. 1C) and CXCL8/heparin binding (IC50 of ∼70 nM; Fig. 3B).

**Discussion**

In this study, we have determined that TSG-6 is a novel ligand for the neutrophil chemoattractant CXCL8. This high-affinity interaction (Kd of ∼25 nM; an average of the values obtained by SPR) occurs via the Link module domain of TSG-6 and directly inhibits CXCL8-induced neutrophil transendothelial migration and, to a lesser extent, chemotaxis. Both TSG-6 and CXCL8 are GAG-binding proteins, where associations with GAGs are important in regulating their functions (27, 44). We have demonstrated in the present study that binding of TSG-6 to CXCL8 inhibits the interaction of CXCL8 with heparin and, therefore, likely blocks binding to HS and other GAGs; consistent with this, TSG-6 was found to impair the presentation of CXCL8 on endothelial GAGs.

**FIGURE 4.** TSG-6 inhibits the binding of CXCL8 to endothelial surface GAGs and the transport of CXCL8 across an endothelial cell monolayer. HUVEC monolayers were incubated in Transwells with b-CXCL8 (3 nM), in the absence or presence of Link_TSG6 (15 nM), for 2 h. b-CXCL8, in the media from the upper and lower chambers, bound to GAGs on the apical and basolateral surfaces of the endothelial cells (recovered in 10× PBS) and in the HUVEC lysates (intracellular), was quantified by Western blot analysis using an Odyssey system. Data, represented as a percentage of the total b-CXCL8 recovered, are plotted as mean values ± SEM. *p < 0.05 as determined by pairwise comparison of samples with and without Link_TSG6 using the Student t test.

**FIGURE 5.** The mutant CXCL8_S, with impaired binding to heparin and TSG-6, mediates transendothelial migration of neutrophils, but TSG-6 does not inhibit this activity. Migration of differentiated HL-60 cells across EA.hy 926 monolayers was measured in response to WT CXCL8 (3.6 nM) (A and B), CXCL8_S (3.6, 7.2, 12, and 24 nM) (n = 4) (A), or CXCL8_T (3.6, 7.2, 12, and 24 nM) (n = 4) (B) alone, or in response to (C) CXCL8/S (3.6 nM [+]) alone or in combination with Link_TSG6 (at 1:1, 5:1, 10:1 and 20:1 molar ratios) (n = 6). Migration of primary human neutrophils across HUVEC monolayers (D) was measured in response to CXCL8_S (3.6 nM [+]) alone or in combination with Link_TSG6 (at 5:1 and 10:1 molar ratios) (n = 3). Data are plotted as mean values ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared with nonstimulated controls (A, B) or to CXCL8_S alone (C, D), as determined using repeated measures ANOVA analysis with a Bonferroni post hoc test.
The use of mutants revealed that suppression of CXCL8/GAG binding is independent of the heparin-binding properties of TSG-6 and that the TSG-6– and GAG–binding surfaces on CXCL8 are, at least, partially overlapping.

We have also shown that at high concentrations, TSG-6 antagonizes the binding of CXCL8 to its receptor CXCR2 and that this correlates with an inhibition of neutrophil chemotaxis. CXCL8 has distinct GAG- and receptor-binding sites, in contrast to some other chemokines such as CCL3 and CCL4 (36). However, the structure of CXCL8 (45) reveals that these are close enough together that by binding to a surface that primarily overlaps the GAG-binding site TSG-6 could partially occlude/perturb the receptor-binding site. This would be consistent with our observations that TSG-6 inhibits the CXCL8/heparin interaction more effectively than the CXCL8/CXCR2 interaction (i.e., with IC_{50} values of ∼70–80 nM and ∼5 μM, respectively).

The sites at which TSG-6 might act to regulate CXCL8-mediated neutrophil extravasation are summarized in Fig. 7. As noted above, the formation of haptotactic gradients, where CXCL8 is associated with proteoglycans on the luminal surface of the vascular endothelium, is critical for the presentation of CXCL8 to its receptors on neutrophils (5, 11, 12). Our data indicate that TSG-6 can inhibit the immobilization of CXCL8 on endothelial GAGs, for example, by blocking CXCL8/HS binding ((a) in Fig. 7). This would result in reduced concentrations of cell-associated versus fluid-phase chemokine (where the latter would then be washed away by vascular flow) and/or upset the equilibrium between monomeric and dimeric CXCL8, thereby reducing receptor activation and/or increasing receptor desensitization and internalization (see Ref. 46); GAG binding is directly coupled to theimerization of CXCL8 (8), where the dimer and monomer have distinct roles in the formation of chemokine gradients in vivo (10). In turn, this would limit integrin-mediated attachment of neutrophils to the endothelium and their subsequent transmigration.

CXCL8 that is produced (e.g., by macrophages) in inflamed or damaged extravascular tissue is transported to the luminal surface of the endothelium via pericellular and transcellular mechanisms (11, 37, 47). The Duffy Ag receptor for chemokines plays an important role in transcytosis (37, 48, 49), but this process is also dependent on the interaction of CXCL8 with HSPGs on the abluminal surface of endothelial cells (11). Our data indicate that TSG-6, expressed at an inflammatory site, could potentially limit the transport of CXCL8 across the endothelium by antagonizing CXCL8/HSPG interactions (b) in Fig. 7).

Human neutrophils carry the receptor CXCR1 that binds with high affinity to CXCL8 (and less tightly to CXCL6) and CXCR2, which binds to CXCL1–3 and CXCL5–8 (9). There is evidence that CXCR1 and CXCR2 act in a coordinated manner, with CXCR2 being most important for early stage CXCL8-induced neutrophil

![FIGURE 6.](Image)
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![FIGURE 7.](Image)
recruitment, whereas CXCR1 triggers cytotoxic effects, i.e., at sites of injury/infection (10, 46). Our observation that TSG-6 inhibits the interaction of CXCL8 with cell-associated CXCR2 and also CXCL8-induced chemotaxis, with similar potencies, suggests that TSG-6 obstructs the receptor-binding surface of CXCL8. Anagostion of the CXCL8/CXCR2 interaction in vivo could prevent tight adhesion/diapedesis of neutrophils (Fig. 7c) and/or their response to chemotactic gradients in the extravascular tissue (Fig. 7d). The effect of TSG-6 on chemotaxis (IC50 of ∼2.5 μM) is ∼250-fold less potent than its inhibition of neutrophil transmigration (IC50 of ∼10 μM), suggesting that TSG-6 may only regulate chemotaxis in situations where it is present at a high local concentration, for example, when secreted by neutrophils in response to inflammatory cytokines (25). This could reflect the greater affinity of the interaction between CXCL8 and CXCR2 (Kd of ∼1 nM) (50) than between CXCL8 and heparin (low micromolar IC50 value) (5, 32) and between CXCL8 and TSG-6 (Kd of ∼25 nM). We did not detect any effect of TSG-6 on CXCL8 binding to CXCR1; however, this does not exclude the possibility that the inhibition of neutrophil chemotaxis by TSG-6 might involve perturbation of both CXCR1- and CXCR2-mediated processes.

It is becoming well established that TSG-6, e.g., when expressed by tissue-resident cells in response to inflammation, represents an endogenous pathway that is anti-inflammatory and protects tissues from damage, for instance through the inhibition of neutrophil migration (17–23). Previously reported mechanisms that might contribute to its regulation of neutrophil recruitment in vivo include 1) the formation of TSG-6/HA complexes that can modulate CD44-mediated leukocyte attachment to the vascular endothelium (see Ref. 51), and 2) attenuation by TSG-6 of TLR2/NF-κB signaling in resident macrophages resulting in reduced production of proinflammatory mediators (23), again perhaps by regulating HA/CD44 engagement. The results described in the present study, where the binding of TSG-6 to CXCL8 directly suppresses the promigratory effects of CXCL8, are clearly independent of the HA-binding properties of TSG-6. Thus, we have identified a new mechanism whereby TSG-6 contributes to a negative feedback loop to limit excessive neutrophil recruitment at sites of inflammation. The CXCL8-binding activity of TSG-6 could largely explain its protective effects in vivo models of inflammatory disease, regardless of the source of the TSG-6, i.e., endogenous, exogenously administered (see Ref. 18), or MSC expressed (21–24). This could also provide the mechanism whereby MSCs can promote the survival of tissue transplants in animal models given the recent finding that TSG-6 can suppress rejection of corneal allografts in mice (24) and that transplantation can give rise to high levels of CXCL8 expression (52); TSG-6 might limit inflammation after surgery and thus prevent the onset of an adaptive immune response.

In this study we have identified TSG-6 as a soluble chemokine-binding protein (CKBP) that, to our knowledge, is the first to be described in mammals. In addition to CXCL8, we have shown (using SPR) that TSG-6 interacts with some, but not all, of the CC and CXC chemokines tested. For example, it binds with high affinity to CCL5 (Kd of 2 nM), CXCL11 (Kd of 16.4 nM) (see Table I), and CCL2 (not shown), but does not bind to CCL3 (Table I) or to the CXCR2 ligand CXCL1 and has no effect on CXCL1-mediated neutrophil transmigration (data not shown). Thus, TSG-6 binds to at least two members of each of the CC and CXC chemokine families. Soluble CKBPs are known to be produced by viruses (reviewed in Ref. 53) and also by parasites, such as the helminth *Schistosoma mansoni* (54) and blood-sucking ticks (55), enabling these organisms to evade the host immune response through neutralization of chemokine activities. The soluble CKBP from *S. mansoni* binds to various chemokines including CXCL8 and inhibits CXCL8-mediated neutrophil migration in models of inflammatory disease (54), whereas the viral CKBPs block chemokine functions through interacting with their GAG-binding and/or their receptor-binding domains (see Refs. 53, 56). Evasin-3, in the saliva of ticks, binds selectively to CXCL8 (Kd of ∼1 nM) and suppresses the CXCL8/CXCR1 interaction; it is a potent inhibitor of neutrophil recruitment (55, 57), reducing myocardial infarct size in a mouse model of ischemia/reperfusion injury and also decreases the local production of TNF in the synovial joints of mice with Ag-induced arthritis. Thus, there are many parallels between the effects of these various CKBPs and the activities of TSG-6 described in the present study and previously, for example, in murine models of inflammation (17), arthritis (20), and myocardial infarction (21). Although TSG-6 does not show any obvious relationship to the parasitic or viral CKBPs in either sequence or tertiary structure, there appear to be similarities in the mechanisms through which these proteins act to neutralize chemokine function; i.e., CKBPs from pathogenic organisms might mimic the activity of TSG-6.

In summary, this study has identified an endogenous mechanism whereby TSG-6 might regulate neutrophil extravasation in vivo and thus prevent/reduce tissue damage during acute inflammation. Our discovery of a soluble mammalian CKBP could inform the development of new anti-inflammatory therapeutics, for diseases of unresolved inflammation (e.g., rheumatoid arthritis and cardiovascular disease), where the regulation of chemokine activity is a potential target, or indeed lead to improved methods for transplantation.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


Figure S1. SPR analysis of the interaction of TSG-6 with CXCL8. rhTSG-6 (A, D, G, H) or Link_TSG6 (B, E, I) was immobilised on a C1 chip and WT CXCL8 (0.6, 1.2, 2.4, 4.8, 7.2, 9.6 and 12 μM) at pH 6 (A, B) or pH 7.2 (D, E) or CXCL8_S (4.79, 9.58, 12, 14, 18 and 23.8 μM) at pH 6 (G, I) or CXCL8_T (4, 6, 8, 10, 15, 20 and 25 μM) at pH 6 (H) was passed over the surface. Alternatively, CXCL8 was immobilised on a C1 chip and rhTSG-6 (0.6, 1.2, 2.4, 4.8, 7.2, 9.6 and 12 μM) was flowed over the surface at pH 6 (C) or pH 7.2 (F). Sensograms of relative responses (RU) are shown in red, with the fits determined using the 1:1 Langmuir model overlaid in black.