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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/allogenic 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 ($K_D$, ~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 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: 2177–2185.
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-α-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-migratory 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 15% (v/v) DMSO for 120 h (34). The murine pro-B cell line 300-19 (35) and stable transfectants expressing CXCR1 (clone IB4) 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 × 10^5 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 in the absence/presence of rhTSG-6 or Link_TSG6 (WT or mutant) at the concentrations indicated.

For transmigration assays, differentiated HL-60 cells (as a model of human neutrophils) (34) or human neutrophils isolated fromuffy coats (36) were washed, resuspended (3.75 × 10^6 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 Pruenster 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 nM, circular 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% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 5 mM EDTA). All samples were subject to SDS-PAGE and CXCL8 was quantified by western blot analysis with 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 6.1]) 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-hydroxysuccinimide (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 6 (SPR 6.0) or pH 7.2 (SPR 7.2); the resulting sensograms were analyzed using the 1:1 Langmuir interaction model with BiAevaluation software (GE Healthcare).

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

Mictroplate 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 (0–500 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 as described above. 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 unlabeled Link_TSG6 (0–1000 nM). Binding was detected as described above.

CXCL8/heparin binding assays

Mictroplate 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 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 (b-heparin) was added along with 0–100 nM heparin (molecular weight) or (at 25 ng/well) in combination with TSG-6 proteins (0–1000 nM) for 4 h at room temperature. Bound b-heparin was detected by addition of extravidin–alkaline phosphatase (1:100,000; 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 × 10^5 in PBS) were incubated at 4˚C for 1 h with b-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 was used to compare each condition with controls (GraphPad Prism, version 5.0). A two-tailed Student’s 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](https://example.com/figure1.png)

**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 primary human neutrophils across a monolayer of HUVECs was determined in response to WT CXCL8 (1 nM) and Link_TSG6 (0–10 nM) in a 1:1, 2:1, 5:1, and 10:1 molar ratios (n = 3–7) or . (E) CXCL8 (3.6 nM [+] alone or in combination with Link_TSG6 or rhTSG-6 (n = 3). Migration of primary human neutrophils across a monolayer of HUVECs was determined in response to WT CXCL8 (3.6 nM [+] alone or in combination with Link_TSG6 (at 1:1, 2:1, 5:1, and 10:1 molar ratios) (n = 3). Data are plotted as mean values (±SEM) relative to nonstimulated controls (−). *p < 0.05, **p < 0.01, ***p < 0.001 compared with non stimulated controls (A, B) or to CXCL8 alone (C–E), as determined using repeated measures ANOVA analysis with a Bonferroni post hoc test. In this and subsequent figures, the dotted line indicates baseline neutrophil migration in the absence of CXCL8 stimulus.
Table I. 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) ((\text{nM})^a)</th>
<th>(\chi^2)</th>
<th>(k_{on} ) ((\text{M}^{-1} \text{s}^{-1})^b)</th>
<th>(k_{off} ) ((\text{s}^{-1})^c)</th>
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<tr>
<td>rhTSG-6</td>
<td>CXCL8 (pH 6)</td>
<td>26</td>
<td>6.5</td>
<td>(2.9 \times 10^9)</td>
<td>(7.5 \times 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 \times 10^9)</td>
<td>(6.5 \times 10^{-4})</td>
</tr>
<tr>
<td>Link_TSG6</td>
<td>CXCL8 (pH 6)</td>
<td>6</td>
<td>44.3</td>
<td>(5.3 \times 10^4)</td>
<td>(3.2 \times 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 \times 10^4)</td>
<td>(1.1 \times 10^{-3})</td>
</tr>
<tr>
<td>rhTSG-6</td>
<td>CXCL8_S (pH 6)</td>
<td>581</td>
<td>1.9</td>
<td>(3.1 \times 10^3)</td>
<td>(1.8 \times 10^{-3})</td>
</tr>
<tr>
<td>rhTSG-6</td>
<td>CXCL8_T (pH 6)</td>
<td>2,109</td>
<td>1.4</td>
<td>640</td>
<td>(1.4 \times 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 \times 10^3)</td>
<td>(1.7 \times 10^{-3})</td>
</tr>
<tr>
<td>CXCL8</td>
<td>rhTSG-6 (pH 6)</td>
<td>74</td>
<td>8.9</td>
<td>(5.7 \times 10^3)</td>
<td>(4.2 \times 10^{-4})</td>
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<tr>
<td>CXCL8</td>
<td>rhTSG-6 (pH 7.2)</td>
<td>20</td>
<td>15.3</td>
<td>(2.3 \times 10^4)</td>
<td>(1.1 \times 10^{-3})</td>
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<tr>
<td>Link_TSG6</td>
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<td>2</td>
<td>7.3</td>
<td>(5.7 \times 10^4)</td>
<td>(1.1 \times 10^{-4})</td>
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<tr>
<td>rhTSG-6</td>
<td>CXCL11 (pH 6)</td>
<td>16</td>
<td>7.2</td>
<td>(6.4 \times 10^3)</td>
<td>(1.1 \times 10^{-4})</td>
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<tr>
<td>rhTSG-6</td>
<td>CCL3 (pH 6)</td>
<td>15,100</td>
<td>7.4</td>
<td>(4.4 \times 10^3)</td>
<td>(6.6 \times 10^{-3})</td>
</tr>
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</table>

\(^a\) \(K_d\) values determined by full kinetic analysis of SPR data.
\(^b\) 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 7.5.

\(^c\) D values determined by full kinetic analysis of SPR data.

\(^d\) SEM using OriginPro version 8. In (B) an IC\(_{50}\) of 15 ± 3 nM was obtained for the inhibition of biotinylated Link_TSG6 binding to immobilized CXCL8 by unlabeled Link_TSG6.

( Supplementary 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 IC\(_{50}\) 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.

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 (IC\(_{50}\)s of ~70 and ~80 nM, respectively); CXCL8/heparin binding was completely abolished in the presence of 0.5–1 \(\mu M\) 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/K43A/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 \(\mu M\) (Fig. 3C).

FIGURE 2. TSG-6 binds specifically to CXCL8 via its Link module domain. (A) rhTSG-6 or Link_TSG6 (50 pmol/well) was immobilized overnight onto a MaxiSorp microtiter plate. CXCL8 (0–500 nM) was added in the fluid phase and binding was detected using a biotinylated anti-CXCL8 Ab (n = 4). (B) CXCL8 (50 pmol/well) was immobilized onto a MaxiSorp plate overnight and biotinylated Link_TSG6 (10 nM) was added in fluid phase in combination with unlabeled Link_TSG6 (0–1000 nM) (n = 4). Binding was detected using extravidin–alkaline phosphatase followed by diosidum p-nitrophenyl phosphate; absorbance at 405 nm was determined after 10 min. Data were plotted as mean values ± SEM using OriginPro version 8. In (B) an IC\(_{50}\) of 15 ± 3 nM was obtained for the inhibition of biotinylated Link_TSG6 binding to immobilized CXCL8 by unlabeled Link_TSG6.
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 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. 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.
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 IC50 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 venular 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 the dimerization 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 trancytosis (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.** Link_TSG6 can inhibit the chemotaxis of human neutrophils and the interaction of CXCL8 with its receptor CXCR2. (A) Purified human neutrophils were added to the upper chambers of Chemotx plates, where the lower chambers contained CXCL8 (1 nM) alone or in combination with increasing concentrations of Link_TSG6 (0–20 μM). Migrated neutrophils were counted after 2 h; data were plotted as mean values (n = 3) ± SEM. (B) Biotinylated CXCL8 (120 nM), alone or following preincubation with Link_TSG6 (at 10:1 to 200:1 molar excess), was added to cells expressing CXCR2. Cell-associated CXCL8 was detected by flow cytometry following addition of avidin-conjugated fluorescein, with gating applied to select live cells. An overlay of histograms (incidents of absorbance against fluorescence), each representative of three independent experiments, is shown as an inset. Data, as a percentage of the maximal signal (i.e., CXCL8 alone), were plotted as mean values (n = 3) ± SEM. *p < 0.05, **p < 0.001 relative to cells incubated with CXCL8 alone. Graphs were generated and data fitted using OriginPro version 8 giving rise to an IC50 of 2.4 ± 0.3 μM for the inhibition of neutrophil chemotaxis by Link_TSG6 (A), and an IC50 of 4.9 μM (i.e., ~40-fold molar excess) for the inhibition of CXCL8 binding to CXCR2 by Link_TSG6 (B).

**FIGURE 7.** Potential mechanisms for the modulation of CXCL8 function by TSG-6. Inhibition by TSG-6 of CXCL8/GAG interactions could antagonize (a) binding of CXCL8 to HSPGs on the luminal surface of the endothelium, thereby preventing the formation of haptotactic gradients and/or (b) binding of CXCL8 to GAGs on the abluminal surface, thus impairing transcytosis of CXCL8. Very high local concentrations of TSG-6 might inhibit the CXCL8/CXCR2 interaction (c, d), thereby limiting the movement of neutrophils in response to a chemotactic gradient of CXCL8. •, CXCL8; ○, TSG-6.
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). Evasion-3, in the saliva of ticks, binds selectively to CXCL8 (K_D 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/limit 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**

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**References**


