The Link Module from Human TSG-6 Inhibits Neutrophil Migration in a Hyaluronan- and Inter-α-inhibitor-independent Manner*

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TSG-6 protein (the secreted product of the tumor necrosis factor-stimulated gene-6), a hyaluronan-binding protein comprised mainly of a Link and CUB module arranged in a contiguous fashion, has been shown previously to be a potent inhibitor of neutrophil migration in an in vivo model of acute inflammation (Wisniewski, H. G., Hua, J. C., Poppers, D. M., Naime, D., Vilcek, J., and Cronstein, B. N. (1996) J. Immunol. 156, 1609–1615). It was hypothesized that this activity of TSG-6 was likely to be mediated by its potentiation of inter-α-inhibitor anti-plasmin activity (causing a down-regulation of the protease network), which was reliant on these proteins forming a stable, probably covalent 120-kDa complex. Here we have shown that the recombinant Link module from human TSG-6 (Link_TSG6; expressed in Escherichia coli) has an inhibitory effect on neutrophil influx into zymosan A-stimulated murine air pouches, equivalent to that of full-length protein (which we produced in a Drosophila expression system). The active dose of 1 μg of Link_TSG6 per mouse (administered intravenously) also resulted in a significant reduction in the concentrations of various inflammatory mediators (i.e. tumor necrosis factor-α, KC, and prostaglandin E2) in air pouch exudates. Link_TSG6, although unable to form a stable complex with inter-α-inhibitor (under conditions that promote maximum complex formation with the full-length protein), could potentiate its anti-plasmin activity. This demonstrates that formation of an ~120-kDa TSG-6-inter-α-inhibitor complex is not required for TSG-6 to enhance the serine protease inhibitory activity of inter-α-inhibitor. Six single-site Link_TSG6 mutants (with wild-type folds) were compared for their abilities to inhibit neutrophil migration in vivo, bind hyaluronan, and potentiate inter-α-inhibitor. These experiments indicate that all of the inhibitory activity of TSG-6 resides within the Link module domain, and that this anti-inflammatory property is not related to either its hyaluronan binding function or its potentiation of the anti-plasmin activity of inter-α-inhibitor.

The recruitment of leucocytes from the blood to sites of injury/inflammation is a vital component of host defense; this involves tethering to and rolling on the vascular endothelium, activation by chemokines, tight adhesion, and transendothelial migration (1). Differential expression and/or activation of adhesion molecules and triggering of the protease network (2) promote the migration of specific leucocyte subpopulations to a given site. These pro-migratory events need to be balanced by the production of endogenous inhibitors of extravasation (3, 4) such that the inflammatory response is generally localized and, after recovery, tissues resume their normal physiological function. This is particularly relevant to polymorphonuclear leucocytes (PMNs),1 which only migrate from the vasculature in inflammation; neutrophil accumulation into specific organs is a major cause of tissue damage associated with rheumatoid arthritis and cardiovascular pathologies such as ischemia/reperfusion injury and septic shock (5, 6).

TSG-6 (the secreted ~35-kDa product of tumor necrosis factor (TNF)-stimulated gene-6 (7)) may be one such endogenous regulator of PMN migration (8, 9). In this regard, it has been found that full-length recombinant human TSG-6 (expressed in insect cells (10)) is a potent inhibitor of neutrophil influx in a mouse air pouch model of acute inflammation (8). Furthermore, TSG-6 is not constitutively expressed in normal adult tissues but is rapidly induced in many different cell types (e.g. monocytes (11), fibroblasts (7), vascular smooth muscle cells (12), cervical smooth muscle cells (13), synovocytes (11), chondrocytes (14), and proximal tubular epithelial cells (15)) by inflammatory mediators such as interleukin-1 (IL-1), TNF-α, lipopolysaccharide, and prostaglandin E2 (PGE2). TSG-6 protein has been detected in synovial fluids and joint tissues from individuals with rheumatoid arthritis and osteoarthritis (11, 16), in the sera of patients with bacterial sepsis and systemic lupus erythematosus (9), and in rat blood vessel walls after injury (12). TSG-6 is also expressed during ovulation (17, 18) and cervical ripening (13), processes in which neutrophils have been implicated as having an important role (19, 20).

Recombinant full-length TSG-6 is able to form a stable (probably covalent) complex of ~120 kDa with inter-α-inhibitor (IaI) in vitro (10); IaI is a serine protease inhibitor found at high

1 The abbreviations used are: PMN, polymorphonuclear leucocyte; HA, hyaluronan; IaI, inter-α-inhibitor; ITC, isotothermal titration calorimetry; IL-1, interleukin-1; Link_TSG6, the recombinant Link module from human TSG-6; PGE2, prostaglandin E2; TNF, tumor necrosis factor; TSG-6, TNF-stimulated gene-6; WT, wild type; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; CMC, carboxymethylcellulose; DMPL, formaldehyde/xylylterephthalylalanine; MES, 4-morpholineethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydmethyl)ethyl]glycine.

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levels in serum (see Ref. 21). TSG-6-Iol complexes of this size have been detected in vivo, for instance in synovial fluids from rheumatoid arthritis patients (11) and in the extracellular matrix of expanded murine cumulus oocyte complexes (17, 18). TSG-6 has also been shown to potentiate the anti-plasmin activity of Iol (8); plasmin has an important role in the proteinase cascade activated during inflammation that results in extracellular matrix degradation and cellular infiltration (see Refs. 2 and 9). Wisniewski et al. suggest that the inhibition of neutrophil migration by TSG-6 in vivo is mediated by its potentiation of Iol anti-plasmin activity (8), i.e. via the down-regulation of the protease network. This hypothesis was based on the detection of a 120-kDa TSG-6-Iol complex in mouse air pouch exudates (after treatment with recombinant TSG-6) and the results from experiments with two mutants of the full-length protein (i.e. TSG-6^{R41K} and TSG-6^{K48E}) that had corresponding effects on both PMN infiltration and potentiation of Iol (8).

TSG-6 is comprised mainly of a Link module and a CUB module (residues 37–128 and 129–250, respectively, in the human preprotein (7)) that are arranged in a contiguous fashion (see Ref. 22). The Link module domain has been shown to mediate the binding of TSG-6 to the glycosaminoglycan hyaluronan (HA); a vital component of extracellular matrix that is implicated in immune cell adhesion/activation (see Refs. 23–25), whereas no function has yet been ascribed to the CUB domain of TSG-6. Previously, we have expressed the Link module from human TSG-6 in *Escherichia coli* (28) and used this material (denoted Link_TSG6) to solve its tertiary structure (29) and determine the position of the HA-binding site by NMR spectroscopy in solution (30). Site-directed mutagenesis of Link_TSG6 identified five amino acids clustered on one face of the Link module as having an important role in HA binding (26).

Recently, we identified a novel allele of the human TSG-6 gene (A^{41}I (27)) that encodes a glutamine at residue 144 in the preprotein rather than an arginine as described previously (7); the A^{41}I variant is the major allele in Caucasians. Molecular modeling of the CUB domain (where this amino acid polymorphism is located) indicated that this residue is likely to be solvent-exposed and could lead to functional differences in HA binding and/or formation of TSG-6-Iol complexes (27). Expression of the Arg-144 and Gln-144 allotypes (denoted here as TSG-6R and TSG-6Q, respectively) in a *Drosophila*-based system and functional characterization showed that there were no significant differences in the ability of these full-length proteins to bind HA or form a stable complex with Iol (27).

Here we found that Link_TSG6 is a potent inhibitor of neutrophil migration in vivo. Link_TSG6 has an equivalent effect to the full-length protein in a murine air pouch model, indicating that all of the inhibitory activity of TSG-6 is located within the Link module domain; the TSG-6R and TSG-6Q allotypes exhibit no significant difference in their inhibition of neutrophil migration. Link_TSG6, although unable to form a covalent complex with Iol (under conditions that promote macromolecule complex formation with full-length TSG-6), can potentiate the anti-plasmin activity of Iol. This demonstrates that formation of a ~120-kDa TSG-6-Iol complex is not required for TSG-6 to enhance the serine protease inhibitory activity of Iol. Six single-site mutants of Link_TSG6 (with wild-type folds) were compared for their abilities to inhibit PMN influx in vivo, bind HA, and potentiate Iol. Results from these experiments indicate that the inhibition of neutrophil migration by TSG-6 is not related to either its HA binding function or its potentiation of the anti-plasmin activity of Iol.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The Link module from human TSG-6 (Link_TSG6, residues 36–153 in the preprotein (7)) was prepared as described previously (28, 31). The mutants all had experimental masses within 1.9 Da of their theoretical masses and had spectra essentially identical to wild-type (WT) protein (data not shown). In the case of the E6K and K13E mutants, which have theoretical masses within 1 Da of the wild-type protein, the mutations were confirmed by N-terminal protein sequencing on an Applied Biosystems 494A Procise sequencer.

Full-length human TSG-6 proteins (with either an Arg (7) or Gln (27) at amino acid 144 in the preprotein, termed TSG-6R and TSG-6Q, respectively) were expressed in *Drosophila* Schneider 2 cells and purified by ion exchange chromatography and reverse-phase HPLC as described previously (27).

WT and mutant Link_TSG6, TSG-6R, and TSG-6Q were stored lyophilized at ~20 °C. Protein concentrations (i.e. for full-length TSG-6 and WT/mutant Link_TSG6) were determined by amino acid analysis as described previously (26). The recombinant proteins were tested for endotoxin with the *Limulus* amebocyte lysate QCL-100 kit with *E. coli* endotoxin as a standard (BioWhittaker); typically 1 ng of bacterial lipopolysaccharide corresponds to 1–10 endotoxin units. Link_TSG6, TSG-6R, and TSG-6Q had 84, 32, and 39 endotoxin units/mg of protein, respectively. For the experiments on animal models, the proteins were resuspended in sterile PBS. Iol was purified from human serum as in Bloem et al. (32), and the concentration was determined as described previously (33).

**Animals**—Male Swiss Albino (T.O. strain) or male BALB/c mice (20–22 g body weight, respectively) were purchased from Bantin and Kingman (Hull, UK) and maintained on a standard chow diet and tap water. Animals were housed 4 h after arrival. All animal work was carried out according to United Kingdom Government Home Office regulations (1986). T.O. mice were used in all experiments except where stated otherwise.

**Air Pouch Model of Inflammation**—Dorsal air pouches were formed by injection of air (2.5 ml subcutaneously) on day 0 and day 3. On day 6, vehicle or proteins (i.e. WT/mutant Link_TSG6, TSG-6R, or TSG-6Q) were administered intravenously into the tail vein (or subcutaneously in the right flank (i.e. a site remote from the air pouch) in the case of WT Link_TSG6) in a volume of 100 μl, 15 min before stimulation with proinflammatory stimulus (1 mg in 0.5 ml of PBS injected intraperitoneally into the pouch; see Ref. 34). Air pouches were washed 4 h after the inflammatory challenge with 2 ml of PBS containing 3 mM EDTA and 25 units/ml heparin. Lavage fluids were centrifuged at 400 × g for 10 min, and the resulting pellet was resuspended in 2 ml of wash buffer: supernatants were stored at ~20 °C before quantification of PGE$_2$, murine TNF-α, and KC with commercially available assays (BD Systems). An aliquot of the cell suspension was stained with Turk’s solution (0.01% (w/v) crystal violet in 3% (v/v) acetic acid), and the cells were counted; previously it has been shown that ≥90% of the cells in the exudate are PMNs (34, 35).

In separate experiments inflammation was initiated with 10 ng of recombinant murine IL-1β injected into the pouch in 0.5 ml 0.5% (w/v) carboxymethylcellulose (CMC) in PBS (35). Link_TSG6 (1 μg) was administered either intravenously into the tail vein or directly into the air pouch, and PMN infiltration was determined as described above.

**Skin Model of Inflammation**—The effect of Link_TSG6 on neutrophil accumulation in a mouse skin model was investigated by monitoring myeloperoxidase activity as described in Cao et al. (36). Briefly, animals were anesthetized, and the dorsal skin was shaved followed by intradermal injection of inflammatory mediator (5 mg of IL-1β or 1 μl FMLP) in 50 μl of sterile PBS with or without 1 μg of Link_TSG6. Animals were sacrificed after 4 h, and skin biopsies (frozen until required) were homogenized in 0.5 ml 0.5% hexadecyltrimethylammonium bromide (Sigma) in PBS. Myeloperoxidase activity was measured in the supernatants, clarified by centrifugation (13,000 × g for 5 min), using the H$_2$O$_2$ oxidation of 3,3′,5,5′-tetramethylbenzidine (SKBbio) as described before (36).

**Statistical Analysis of In Vivo Data**—Statistical differences were calculated on original data using analysis of variance followed by the Bonferroni test for intergroup comparisons or by unpaired Student’s *t*-test.
test (two-tailed) when only two groups were compared. A threshold value of \( p < 0.05 \) was taken as significant.

**Isothermal Titration Calorimetry (ITC)**—The interactions between the mutant Link_TSG6 proteins and an octasaccharide of HA (HAs; purified as in Mahoney et al. (37)) were determined on a MicroCal VP-ITC instrument at 25.0 °C in 5 mM Na-MES, pH 6.0, as described before (26); the interaction between Link_TSG6 and HA is maximal at pH 6.0 (38). A 158 \( \mu \)M solution of HA was added in 5-\( \mu \)l injections (54 in total) to protein (ranging from 15.0 to 19.5 \( \mu \)M) in the 1.4-ml calorimeter cell; the concentration of the HA was determined on the basis of an ITC titration with wild-type Link_TSG6 as discussed previously (30). For the Y12F mutant, additional experiments were conducted using 790 \( \mu \)M HAs and 59.7 \( \mu \)M protein. Data were fitted to a one-site model by nonlinear least squares regression with the Origin software package after subtracting the heats resulting from the addition of HA into buffer alone (see Ref. 30). Two separate experiments were done for each mutant, and the mean value of the binding constant was compared with that of wild-type Link_TSG6, determined previously under identical conditions (see Ref. 26).

**Formation of TSG-6-Id Complex**—Full-length recombinant TSG-6Q (at 80 \( \mu \)g/ml final concentration; 2.7 \( \mu \)M based on a molecular weight of 30 kDa) or Link_TSG6 (at either 29.2 \( \mu \)g/ml final concentration (2.7 \( \mu \)M) or 146 \( \mu \)g/ml final concentration (13.4 \( \mu \)M)) were incubated with Iol (320 \( \mu \)g/ml final concentration; 1.8 \( \mu \)M based on a molecular weight of 180 kDa) in 20 mM HEPES-\( \cdot \)HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl\(_2\) (total volume 25 \( \mu \)l) for 2 h at 37 °C; these conditions give maximum conversion of free TSG-6 into complex using purified Iol.\(^2\) TSG-6Q, Link_TSG6, or Iol were incubated alone under identical conditions as controls. Protein from 7.5 \( \mu \)l of each sample was analyzed on 10% (w/v) Tris-Tricine SDS-PAGE (39) after reduction and alkylation with dithiothreitol and iodoacetamide, respectively.

**Analysis of Plasmin Inhibition**—The effects of WT and mutant Link_TSG6 on the potentiation of plasmin inhibition by Iol were determined in an assay that employs the chromogenic substrate tosyl-Gly-Pro-Lys-4-nitranilide acid (Chromozyme PL) essentially as described for recombinant full-length TSG-6 in Wisniewski et al. (8). Iol (8.7 \( \mu \)g/ml; 48 nm) was preincubated in the presence of Link_TSG6 proteins (740 nM) in either pH 7.4 buffer (10 mM HEPES-\( \cdot \)HCl, pH 7.4, 150 mM NaCl, 0.02% (v/v) Tween 20) or pH 6.0 buffer (10 mM sodium acetate, pH 6.0, 150 mM NaCl, 0.02% (v/v) Tween 20) for 20 min at 37 °C in a total volume of 100 \( \mu \)l. An equal volume of plasmin/Chromozyme PL (Roche Diagnostics) in either pH 7.4 or pH 6.0 buffer was added (to give final concentrations of 3.4 \( \mu \)M plasmin, 197 \( \mu \)M Chromozyme PL, 370 nM Link_TSG6, and 24 nM Iol) and incubated for 20 min at room temperature; this is a similar molar ratio of Link_TSG6:Iol as reported previously for the full-length protein (8), and this gives maximum inhibition of plasmin with WT Link_TSG6.\(^3\) Plasmin activity was determined by measuring the absorbance at 405 nm and subtracting values from assays performed under identical conditions but that contained plasmin or Link_TSG6 (to correct for background color). Statistical differences were calculated using analysis of variance (Bonferroni test), and a threshold value of \( p < 0.05 \) was taken as significant.

**RESULTS**

**Anti-inflammatory Effect of Link_TSG6 in a Mouse Air Pouch Model**—Subcutaneous injection of air on the back of a mouse results in the formation of a stable, air-filled pouch (35). The tissue lining the air pouch has a similar morphology to the synovial membrane and has, thus, been used as a model for the articular synovium (40). The recombinant Link module from human TSG-6 (Link_TSG6; 10.9 kDa) caused a dose-dependent reduction of PMN migration into the air pouches (stimulated with zymosan A) when it was administered in the range of 9–183 pmol (0.1–2.0 \( \mu \)g) per mouse intravenously. As can be seen from Fig. 1A the most effective doses were 92 pmol (1 \( \mu \)g) and 183 pmol (2 \( \mu \)g), which caused 34 ± 7 and 41 ± 7% inhibition of cell influx, respectively; subcutaneous injection of 92 pmol of Link_TSG6 at a site remote from the air pouch resulted in 35 ± 10% inhibition, whereas a denatured preparation of the protein was inactive (1 \( \mu \)g boiled for 10 min and

![Fig. 1](http://www.jbc.org/)
for which single site mutants were available (26). In addition, from Fig. 1 it is apparent that the Arg (R) and Gln (Q) allotypes of TSG-6 do not exert significantly different effects on neutrophil migration in this model system.

Administration of Link_TSG6 (at the active dose of 1 μg intravenously per mouse) significantly reduced the levels of the chemokine KC (34 ± 4%), TNF-α (44 ± 7%), and PGE$_2$ (60 ± 7%) in the air pouch supernatants from T.O. mice (Fig. 2). Thus, Link_TSG6 decreases both PMN influx and the production of inflammatory mediators in the zymosan-inflamed air pouch model.

The anti-inflammatory effect of the TSG-6 Link module was tested in air pouches activated with IL-1β. Fig. 3 shows that injection of Link_TSG6 (1 μg intravenously) caused a significant reduction in PMN migration in this model (i.e. 43 ± 5% of values from animals treated only with PBS vehicle intravenously); a higher degree of inhibition (65%) can be calculated if values from animals treated only with PBS vehicle intravenously per mouse) significantly reduced the levels of the HA-binding activity (26). H4K was tested because His-4 lies close to Lys-11 and Tyr-12 (in the three-dimensional structure (29)), and thus, its mutation could affect the HA binding process. From Fig. 5 it can be seen that H4K, E6K, K13E, and Y78F caused a significant inhibition of neutrophil migration in vivo (*, p < 0.05). The Y78F mutant showed somewhat reduced activity compared with the wild-type protein (66% of WT, respectively), reducing PMN accumulation by 70 ± 7, 58 ± 7, and 67 ± 5%, respectively, but none of these differences were statistically significant. However, the mutants Y12F (20 ± 4% inhibition; p > 0.05) and F70V (13 ± 9% inhibition; p > 0.05) did have significantly reduced inhibitory activity compared with wild-type Link module (43 and 27% of WT activity, respectively; Table I).

Effect of Mutation on the Anti-inflammatory Activity of Link_TSG6—Six Link_TSG6 mutants (i.e. H4K, E6K, Y12F, K13E, F70V, and Y78F) were analyzed in the zymosan air pouch model to investigate the involvement of His-4, Glu-6, Tyr-12, Lys-13, Phe-70, and Tyr-78 in the inhibition of neutrophil migration; NMR spectroscopy indicated that these mutations do not affect the structural integrity of the protein (Ref. 26 and data not shown). E6K and K13E were chosen because the effects of equivalent mutations in full-length human TSG-6 (i.e. TSG-6E6K and TSG-6K13E, respectively) on neutrophil migration in vivo and on potentiation of LLO anti-plasmin activity had been reported previously (8). Tyr-12, Phe-70, and Tyr-78 form part of the HA-binding site (along with Lys-11 and Tyr-59), and mutations of these residues all result in greatly reduced HA-binding activity (26). H4K was tested because His-4

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**Fig. 2.** Link_TSG6 reduces production of inflammatory mediators in zymosan-inflamed air pouch model. Cell-free lavage fluids collected 4 h after zymosan injection from control (PBS vehicle only)- and Link_TSG6-treated mice (1 μg intravenously into the tail vein) were analyzed for KC (KC; n = 8), TNF-α (n = 14), and PGE$_2$ (n = 8) levels. Data (ng/cavity) are reported as the mean ± S.E. *p < 0.05 versus respective PBS group.

**Fig. 3.** Link_TSG6 inhibits PMN accumulation in IL-1β-inflamed air pouch model. Mice were treated either intravenously (i.v.) in the tail vein or locally in the air pouch (a.p.) with 100 μl of PBS alone or PBS containing 1 μg Link_TSG6 15 min before local injection of IL-1β (10 ng in 0.5% (w/v) CMC in PBS); the control group (Control) received local IL-1β only. The number of PMN in the air pouch was measured 4 h after the inflammatory challenge. Administration of 0.5% (w/v) CMC in PBS alone caused mild inflammation with an influx of 2.0 ± 0.1 × 10^6 PMN per mouse (n = 4). Data are the mean ± S.E. of n = 8 mice per group. *p < 0.05 versus the respective PBS group.
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**Fig. 4.** Link_TSG6 reduces PMN accumulation in a skin model of inflammation. Mice were given intradermal injections of IL-1β or fMLP alone or in combination with 1 µg Link_TSG6. Control skin sites were injected with sterile PBS alone. Neutrophil accumulation was determined 4 h later by myeloperoxidase activity. Data are the mean ± S.E. of n = 6 mice per group; *, p < 0.05 versus control group.

**Fig. 5.** Effect of mutagenesis on Link_TSG6 activity in zymosan-inflamed air pouch model. Mice were treated intravenously (tail vein) with 100 µl of PBS alone (n = 17) or PBS containing 1 µg of WT (n = 10) or mutant Link_TSG6 (H4K, n = 12; E6K, n = 10; Y12F, n = 8; K13E, n = 10; F70F, n = 8; Y78F, n = 10) 15 min before local injection of zymosan A. The number of PMNs in the air pouch was measured 4 h after the inflammatory challenge, and these data, represented as % of PBS control, are shown as the mean value ± S.E. WT and the H4K, E6K, K13E, and Y78F mutants showed significant (*, p < 0.05) inhibition of neutrophil migration compared with control.

**TABLE I**

The functional activities of Link_TSG6 mutants

| Mutant | Neutrophil inhibition | Hyaluronan binding | Potentiation of Iol |
|--------|-----------------------|--------------------|---------------------|
|        | % of WT               | % of WT            | pH 6.0             | pH 7.4             |
| H4K    | 152                   | 77                 | 77                  | 76                 |
| E6K    | 124                   | 399                | 100                 | 84                 |
| Y12F   | 43                    | 1                  | 18                  | 12                 |
| K13E   | 143                   | 33                 | 104                 | 100                |
| F70V   | 27                    | 10                 | 94                  | 99                 |
| Y78F   | 66                    | 6                  | 70                  | 63                 |

the corresponding binding constants (Kₜ), stoichiometries, and the errors of the fit given in Table II. From this table it can be seen that H4K had a HA binding affinity that is slightly lower than that measured previously for the wild-type protein (77% of WT), whereas Y12F, K13E, F70V, and Y78F all showed larger reductions in functional activity with 1, 33, 10, and 6%, respectively, of WT binding. E6K, however, has an ~4-fold increase in HA binding affinity (399% of WT). It should be noted that although these experiments were conducted at pH 6.0, we believe that the relative affinities of the wild-type and mutant Link modules measured here provide an accurate reflection of the relative activities of these proteins at pH 7.4.

In the case of Lys-13, we have shown before that this amino acid is not involved in HA binding because mutation to alanine does not affect the functional activity of Link_TSG6 (26). The fact that the K13E mutant has reduced ability to interact with HA indicates that this mutation affects the ligand-binding site in some way. Lys-13 is in close proximity to Lys-11, a residue that is important in binding HA, possibly by making a salt bridge to a carbonyl group on the sugar (26). Therefore, replacing Lys-13 with a negatively charged amino acid may perturb such interactions.

ITC data reported here for Y78F show that mutation of Tyr-78 to phenylalanine causes an ~15-fold reduction in HA binding affinity; this is consistent with our previous investigation of Y78F using a microtiter plate assay (26). ITC analysis of another Tyr-78 mutant (Y78V) indicated that mutation of this residue to valine gave rise to an identical loss of function (i.e. 6% of WT HA binding activity (26)). Interestingly, mutation of Tyr-12 to phenylalanine (see Table II) or valine (26) has similar effects on the HA binding activity of Link_TSG6 (i.e. 1 and 3% of WT, respectively). The value obtained here for F70V (10% of WT) is similar to that determined for this mutant using a microtiter plate assay (7% of WT (26)) but is somewhat lower than our previous ITC measurement (22% of WT (26)).

The finding that E6K has an increase in binding affinity for HA (Table I and II) was unexpected. The E6A mutant studied previously (26) had wild-type functions, indicating that this residue is not directly involved in HA binding. At present it is not known why this lysine mutant should have enhanced HA binding properties, but this may become clearer once we have completed the three-dimensional structure of Link_TSG6 in complex with HA₄.

Comparison of the effects of mutagenesis on the inhibition of neutrophil migration in vivo and on HA binding (Fig. 5 and Table I) indicate that these properties are not linked; e.g. Y78F causes significant reduction in PMN influx into the air pouch (30 ± 8% inhibition; 66% of WT activity) but has markedly reduced HA binding function (6% of WT), and K13E is a potent inhibitor of neutrophil migration (143% of WT) but has impaired ability to interact with HA (33% of WT). The conclusion that the HA binding function of TSG-6 does not mediate its inhibition of neutrophil migration is consistent with previous studies in vivo (42).

**Ability of Link_TSG6 to Form a Stable Complex with Iol—**Wisniewski et al. (8) hypothesize that inhibition of neutrophil migration by TSG-6 occurs via its potentiation of Iol antiplasmin activity, which leads to a down-regulation of the protease network. In this regard, it has been suggested that formation of a stable (probably covalent) complex between these proteins is necessary for TSG-6 to enhance the inhibitory activity of Iol. Given our findings that the Link module of TSG-6 is a potent inhibitor of neutrophil migration, we investigated whether Link_TSG6 could form a stable complex with Iol. As can be seen from Fig. 7, incubation of full-length TSG-6 and Iol (for 2 h at 37 °C) resulted in the formation of three novel bands (labeled 1–3) not present in TSG-6 or Iol alone. On Western blots these three bands were all immunoactive with an anti-Iol antibody, but only band 2 (apparent molecular mass ~116 kDa) was also detected by an anti-TSG-6 antisera (data not shown). N-terminal sequencing and mass spectrometry have confirmed that species 2 corresponds to a covalent complex of
TSG-6 with I, whereas bands 1 and 3 derive from I. However, when Link_TSG6 was incubated with I under identical conditions (at the same molar ratio as used for full-length TSG-6 or at a 5-fold higher concentration) no novel species were generated. This was confirmed by Western blotting with an anti-Link_TSG6 antiserum (data not shown). These results clearly indicate that Link_TSG6 and I are unable to form a complex that is stable on SDS-PAGE. Thus, the anti-inflammatory activity of Link_TSG6 in vivo is not reliant on covalent complex formation with I.

**Ability of Link_TSG6 to Potentiate the Anti-plasmin Activity of I**—Although the isolated Link module is unable to form a covalent complex with I, it was thought possible that Link_TSG6 could potentiate its anti-plasmin activity. Therefore, the effect of Link_TSG6 on the inhibition of plasmin by I was tested in a chromogenic assay (Fig. 8). The assay was carried out at pH 7.4, as reported previously for full-length TSG-6 (8), and also at pH 6.0, as some Link_TSG6 functions are maximal at this pH (e.g., HA and aggrecan binding (38)). Fig. 8 shows that WT Link_TSG6 can enhance I anti-plasmin activity at both pH 7.4 and pH 6.0 (40 and 39% inhibition, respectively).

![Fig. 6. Analysis of the interactions of Link_TSG6 mutants with HA by ITC.](image-url)

**Table II**

| Mutant | Stoichiometry (HA:protein) | $K_b$ | Mean $K_b$ | % of wild type |
|--------|---------------------------|-------|------------|----------------|
| H4K    |                           |       | $10^6$ u$^{-1}$ | $10^5$ u$^{-1}$ |
| E6K    |                           |       | 1.02 ± 0.01 | 2.88 ± 0.75 | 92.9 | 399 | 1 |
| Y12F   |                           |       | 1.02 ± 0.01 | 2.88 ± 0.75 | 92.9 | 399 | 1 |
| K13E   |                           |       | 1.02 ± 0.01 | 2.88 ± 0.75 | 92.9 | 399 | 1 |
| F70V   |                           |       | 1.02 ± 0.01 | 2.88 ± 0.75 | 92.9 | 399 | 1 |
| Y78F   |                           |       | 1.02 ± 0.01 | 2.88 ± 0.75 | 92.9 | 399 | 1 |

*Mean of the two experiments shown.*

*Wild-type value (48.32 ± 4.64 × 10^5 u$^{-1}$) is taken from Blundell et al. (44).*

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The TSG-6 Link Module Inhibits Neutrophil Migration in Vivo

**Results**

A novel finding of this study is that the isolated Link module from human TSG-6 (Link_TSG6) is a potent inhibitor of PMN migration in vivo. Link_TSG6 showed a similar effect to that of the full-length protein in the zymosan-stimulated air pouch model in two different mouse strains (see Fig. 1); for example, in T.O. mice 183 pmol of Link_TSG6 caused 41 ± 7% inhibition, whereas 200 pmol of TSG-6 proteins gave between 41 ± 8 and 46 ± 9% reduction in neutrophil influx. These results indicate that all of the inhibitory activity of TSG-6 is likely to reside within the Link module domain.

Dose-dependent reduction in neutrophil influx was seen in the zymosan air pouch model when Link_TSG6 was given intravenously (Fig. 1A); denatured protein was inactive, indicating that its anti-inflammatory effect is reliant on the Link module being correctly folded. As little as 1 μg (92 pmol) per mouse had a significant effect when administered intravenously into the tail vein or subcutaneously at a site remote from the air pouch (34 ± 7 and 35 ± 10%, respectively). This suggests that for Link_TSG6 to exert its effect, it does not need to be local to the site of inflammation but, rather, acts via the circulation. Consistent with this notion, Link_TSG6 (at the active dose of 1 μg/mouse) also caused similar neutrophil inhibition in IL-1β-stimulated air pouches when it was injected either intravenously or directly into the air pouch (43 ± 5 and 43 ± 9%, respectively; Fig. 3). In addition, this dose of Link_TSG6 could attenuate the effects of IL-1β and fMLP in a skin model of PMN migration (Fig. 4). These results demonstrate that the inhibitory activity of Link_TSG6 on neutrophil migration is not limited to a single site or inflammatory stimulus.

Here we tested two allotypes of TSG-6 that differ by a single amino acid (i.e., TSG-6Q, a newly discovered variant with a Gln at residue 144 in the preprotein (27), and TSG-6R, which has the protease network as proposed previously (8). To investigate this possibility the Link_TSG6 mutants were tested in the plasmin assay, and these results were compared with their effects in the air pouch model. As can be seen from Fig. 8, all of the mutant proteins (except Y12F) caused a significant potentiation of the anti-plasmin activity of IoI (*, p < 0.05), with values ranging from 29% plasmin inhibition with Y78F at pH 7.4 (63% of WT; Table I) to 46% inhibition with K13E at pH 7.4 (100% of WT). Y12F was the only mutant that did not potentiate IoI to a significant extent (6 and 4% inhibition at pH 6.0 and pH 7.4, respectively). The mutants H4K (at pH 7.4), E6K (at pH 7.4), Y12F (at pH 6.0 and pH 7.4), and Y78F (at pH 6.0 and pH 7.4) had significantly (p < 0.05) reduced functional activity compared with the wild-type protein at the equivalent pH. It should be noted that none of the mutant proteins exhibited a significantly increased potentiation of IoI compared with WT.

From Table I it is apparent that there is a poor correlation between the effects of the Link_TSG6 mutants on potentiation of IoI and on neutrophil migration in vivo. For example, F70V does not significantly inhibit PMN influx (13 ± 9% inhibition; 27% of WT) but has essentially wild-type potency (94 and 99% of WT at pH 6.0 and pH 7.4, respectively). Therefore, it seems unlikely that the inhibition of neutrophil migration by TSG-6 in vivo (seen here and in Wisniewski et al. (8)) can be explained by its potentiation of the anti-plasmin activity of IoI. Interestingly, it has been reported that the movement of neutrophils across pulmonary endothelial cell layers is not blocked by matrix metalloproteinase or serine protease inhibitors, indicating that extracellular matrix digestion by these enzymes is not a prerequisite for successful PMN transendothelial migration (43).

**Discussion**

A novel finding of this study is that the isolated Link module from human TSG-6 (Link_TSG6) is a potent inhibitor of PMN migration in vivo. Link_TSG6 showed a similar effect to that of the full-length protein in the zymosan-stimulated air pouch model in two different mouse strains (see Fig. 1); for example, in T.O. mice 183 pmol of Link_TSG6 caused 41 ± 7% inhibition, whereas 200 pmol of TSG-6 proteins gave between 41 ± 8 and 46 ± 9% reduction in neutrophil influx. These results indicate that all of the inhibitory activity of TSG-6 is likely to reside within the Link module domain.

Control experiments with IoI alone (data not shown) demonstrate that this protein has little inhibitory activity in the absence of Link_TSG6 (5 and 8% inhibition at pH 7.4 and pH 6.0, respectively), as was noted previously (8). Assays using Link_TSG6 in the absence of IoI resulted in 2 and 5% inhibition of plasmin at pH 7.4 and pH 6.0, respectively (data not shown).

These data clearly show that the isolated Link module of human TSG-6 can potentiate the anti-plasmin activity of IoI. Therefore, the inhibition of neutrophil migration by Link_TSG6 in vivo could be explained by its induction of IoI anti-plasmin activity and the consequent down-regulation of
an Arg at this position, as described in the original published sequence (7). We found that TSG-6Q and TSG-6R (expressed in Drosophila S2 cells (27)) had similar activities in vivo at doses of 100 pmol (T.O. mice, 38 ± 7 and 48 ± 6% inhibition, respectively; BALB/c mice, 75 ± 4 and 67 ± 3%, respectively) and 200 pmol (T.O. mice, 41 ± 8% and 46 ± 9%, respectively) per mouse (intravenously). These data indicate that there is no significant difference in the activities of these allotypes with respect to neutrophil migration. To date we have been unable to find any functional difference between TSG-6R and TSG-6Q (see Ref. 27).

Our experiments with full-length recombinant TSG-6 confirm the earlier finding from Wisniewski et al. (8) that TSG-6 is a potent inhibitor of PMN infiltration in vivo; this was observed using human TSG-6R, expressed using a baculovirus system, in an essentially identical model (i.e. carrageenan- or IL-1β-inflamed murine air pouches (8)). However, in the Wisniewski study maximum inhibition (~38% reduction in PMN influx) was seen with TSG-6R at a dose of 20 μg in carrageenan-stimulated air pouches, whereas we observed a 67 ± 3% inhibition with 3 μg (100 pmol) TSG-6R (intravenously) in the same mouse strain (i.e. BALB/c). Thus, the full-length TSG-6R protein used here appears much more potent than that described previously (approximately 10 times more active). There are several possible explanations for this. First, it could result from a genuine difference in the specific activities of the recombinant protein used in the two studies. However, this seems somewhat unlikely given that the full-length proteins were both produced in insect cell-based secretion systems. A second possibility is that the difference results from the site of TSG-6 administration (directly into the air pouch in (8) and intravenously here). This also seems unlikely because we found no significant difference in the inhibition of IL-1β-elicted neutrophil infiltration when Link_TSG6 was injected directly into the air pouch or given systemically (Fig. 3). It should be noted that in the IL-1β model, treatment with ~660 pmol of TSG-6R (injected into the air pouch) inhibited PMN migration by 32% (8), whereas we observed 43 ± 9% inhibition with 92 pmol of Link_TSG6 (into air pouch). Therefore, a large difference in inhibitory effect is seen in the two studies using an identical route of administration and the same inflammatory stimulator, albeit in a different mouse strain. A third possible explanation relates to the formulation of the recombinant proteins. The full-length TSG-6R used in the Wisniewski study was purified by ion exchange and gel filtration chromatography providing protein that was ≥95% pure in 20 mM MES, pH 6.5, 500 mM NaCl (10). This high salt preparation was diluted with saline before being injected into the air pouch. In our study, the full-length TSG-6 allotypes were purified by a combination of ion exchange chromatography and reverse-phase HPLC, allowing the production of lyophilized protein that is salt-free (27). It seems possible that the different salt concentrations of the protein preparations used in vivo could account for the difference in potency.

Six single site mutants of Link_TSG6 (i.e. H4K, E6K, Y12F, K13E, F70V, and Y78F) demonstrated to have wild-type folds were tested for their ability to inhibit neutrophil migration in vivo (Fig. 5), bind HA (Fig. 6), and potentiate the anti-plasmin activity of Iα1 (Fig. 8); these data are summarized in Table I. The fact that there is no obvious correlation between any of these activities indicates that the effect of Link_TSG6 on neutrophil migration is not dependent on HA binding nor is it mediated via potentiation of Iα1. As discussed below, this latter conclusion is quite different from that made by Wisniewski et al. (8).

Previously, it has been suggested that inhibition of neutrophil migration by TSG-6 occurs via its potentiation of the anti-plasmin activity of Iα1. This is a reasonable hypothesis given the importance of plasmin in local extracellular matrix degradation (both directly and by its up-regulation of matrix metalloproteinase activity), which is associated with cellular infiltration. These two properties were linked because a 120-kDa TSG-6α1 complex was detected in murine air pouch exudates after treatment with TSG-6R (albeit at low levels compared with free TSG-6) and because of the finding that two mutants of TSG-6R (i.e. TSG-6E41K and TSG-6K48E, which were expressed in insect cells) had corresponding effects on PMN infiltration and potentiation of Iα1 (these activities were significantly reduced or abolished, respectively). Here we have found that the equivalent Link_TSG6 mutants (E6K and K13E, respectively) have very different properties (see Figs. 5 and 8, and Table I). Neither mutation caused any impairment in the inhibition of neutrophil migration in vivo (124 and 143% of WT Link_TSG6, respectively). In addition, K13E (at pH 6.0 and pH 7.4) and E6K (at pH 6.0) had wild-type activities in the plasmin assay. The reason for these differences is not clear. It should be noted, however, that here we characterized our Link_TSG6 mutants by NMR spectroscopy, whereas no such structural analysis was carried out in the study of Wisniewski et al. (8). It is possible therefore that these Glu → Lys and Lys → Glu mutations, when expressed in the context of full-length TSG-6, adversely affect the folding of the protein.

If indeed the inhibitory effect of TSG-6 on neutrophil migration is not connected to its ability to down-regulate the protease network (as our data suggest), then what is the mechanism of its action? One possibility is that TSG-6 could affect some aspect of PMN adhesion/extravasation (e.g., attachment and rolling, tight adhesion, or transendothelial migration). Work is currently in progress to investigate the effect of TSG-6 on these processes using intravital microscopy on the mouse mesenteric microcirculation.

Given the finding that Link_TSG6 can inhibit PMN infiltration in more than one model of inflammation (see “Results”), it seems likely that native TSG-6 may be involved in the regulation of neutrophil migration in vivo. In this regard, TSG-6 expression is rapidly induced in many different cell types in response to inflammatory mediators, and the protein has been detected in inflammatory conditions (e.g., rheumatoid arthritis and septic shock) as well as being up-regulated in inflammation-like processes such as ovulation (see the Introduction). Here we have shown for the first time that TSG-6 treatment in vivo results in a reduction in the levels of inflammatory mediators (i.e. TNF-α, KC, and PGE2; see Fig. 2) in addition to inhibiting PMN migration. This finding is consistent with the hypothesis proposed by Wisniewski et al. (8) that endogenous TSG-6 can be part of a negative feedback loop in the inflammatory response.

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The Link Module from Human TSG-6 Inhibits Neutrophil Migration in a Hyaluronan- and Inter- α-inhibitor-independent Manner

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