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# Animal Model

## Modulation of Inflammation by Slit Protein *In Vivo* in Experimental Crescentic Glomerulonephritis

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**A basic conservation of cell migration guidance mechanisms in the nervous and immune systems was proposed when Slit, known for its role in axon guidance, was found to inhibit chemokine-induced leukocyte chemotaxis *in vitro*. These studies examined the role of Slit2 in modulating inflammation *in vivo*. In a rat model of glomerulonephritis, endogenous glomerular Slit2 expression fell after disease induction, and its inhibition during the early disease period accelerated inflammation. *Ex vivo* glomerular leukocytes showed decreased chemokine and chemoattractant-induced chemotaxis in response to Slit2, suggesting an anti-inflammatory role for glomerular Slit2. In contrast to the effect of inhibition, glomerulonephritis was ameliorated by systemic Slit2 administration. Slit2 treatment improved disease histologically and also improved renal function when given early in the disease course. Leukocytes harvested from rats receiving Slit2 showed decreased monocyte chemoattractant protein-1 (MCP)-1-mediated migration, consistent with a peripheral Slit2 effect. In keeping with this functional alteration, Slit2-mediated inhibition of RAW264.7 cell chemotaxis was associated with decreased levels of active cdc42 and Rac1, implicating GTPases in leukocyte Slit2 signaling. These findings suggest a role for endogenous Slit2 in the inhibition of chemoattractant-mediated signals, demonstrate a potentially important anti-inflammatory effect for Slit2 *in vivo*, and provide further evidence for conserved mechanisms guiding the process of migration in distinct cell types. *(Am J Pathol 2004, 165:341–352)***

During inflammation, leukocytes have to migrate from the blood circulation into the interstitium of the involved tissue. Inflammatory cells respond to an orchestra of signals and eventually arrive at the appropriate site. While a large number of studies have established the importance of positive regulators in controlling leukocyte chemotaxis, there has been only limited evidence for the existence of endogenous inhibitory regulators of this process. Among the positive regulators, the best known are the secreted proteins of the chemokine family. Chemokines regulate leukocyte trafficking and inflammation through seven transmembrane receptors coupled to G proteins (GPCRs).[1,2] Although there are exceptions, chemokines generally act by establishing a concentration gradient in the interstitium by binding to heparan sulfate proteoglycans (HSPG). Inflammatory cells move toward the origin of the chemokines through a complex array of rearrangements of their cytoskeleton. Guidance cues provided by the cells and interstitium surrounding the inflammatory cells drive them further along the path toward the source of the chemokines and the eventual site of inflammation.

Based on the repellent function of Slit in axon guidance and neuronal migration,[3–7] an important role for similar molecules in the negative regulation of leukocyte migration was hypothesized. The Slit family of secreted proteins were subsequently shown to have no direct chemo-

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tactic activity of their own on leukocytes, but to act as negative regulators of chemokine-induced leukocyte chemotaxis *in vitro*.[8] Although the Drosophila *Slit* gene was discovered over a decade ago,[9–11] its vertebrate homologues including three mammalian *Slit* genes (*Slit*1, 2, and 3) were only recently identified. They are highly homologous to each other and encode ligands for the Roundabout (Robo) receptors.[3–5,12–15] It is now clear that *Slit* and *Robo* genes are expressed in a range of tissues in addition to the brain.[8] Slit proteins are secreted proteins containing four leucine-rich domains, nine EGF-like repeats, and a cysteine-rich carboxyl domain. They interact with glypican-1,[16–18] and as is the case with many of the chemokines, also appear to act by establishing concentration gradients through HSPG binding.

Although a potential role for Slit proteins in the negative regulation of leukocyte migration was shown through *in vitro* studies,[8] the role of these molecules in regulating leukocyte chemotaxis *in vivo* has not been reported. Furthermore, although the inhibitory effect of Slit *in vitro* suggests that it may be helpful in treating inflammatory diseases, the practical benefit of manipulating Slit activity *in vivo* has not been previously examined.

A model of crescentic glomerulonephritis (GN) in Wistar-Kyoto (WKY) rats, induced by the administration of anti-glomerular basement membrane (GBM) antibodies[19,20] was used to investigate the *in vivo* role and therapeutic effect of Slit protein. Multiple chemokines are known to be up-regulated in crescentic GN in both animal models and patients. Inhibition of chemokine signaling can attenuate crescentic GN,[21–24] making this disease a useful model to study the role of negative regulators of leukocyte chemotaxis.

The findings reported here demonstrate a role for endogenous Slit2 in the inhibition of leukocyte chemotaxis *in vivo*, and suggest a potentially important anti-inflammatory function for Slit2. The effect on leukocytes appears to be mediated through signaling pathways responsible for cell migration and motility, consistent with the effect of Slit proteins in the nervous system.

## Materials and Methods

All work with animals was performed in accordance with National Institute of Health guidelines and with protocols approved by the Baylor College of Medicine Animal Ethics committee.

### Crescentic Glomerulonephritis: Induction, Treatment, and Analysis

Male WKY rats, (180 to 200 g; Harlan Laboratories, Madison, WI), received one injection of anti-GBM serum (25 $\mu$l/100g) on day 0, as described previously.[20] Slit and Robo mRNA expression was monitored using RNase protection assay at various time points in unmodified disease for 30 days after induction. As anti-GBM antibody binding to the GBM occurs within the first hour after injection,[25,26] to eliminate concerns regarding an effect on this process, Slit2 antibody and early recombinant human Slit2 (rhSlit2) treatment (described below) commenced after this time (6 hours later). Proteinuria was assessed by the sulfosalicylic method.[27] Serum creatinine levels were measured using a kit (Sigma, St. Louis, MO, USA). Kidney tissues were fixed in formalin or methanol-Carnoy solution and paraffin-embedded. Five-$\mu$m paraffin sections were stained with periodic acid-Schiff (PAS) reagent to assess glomerular crescent formation. Infiltrating macrophages were identified by staining for ED-1 as previously described.[19] Crescents and ED-1-positive (ED-1$^+$) cells were scored by a researcher blinded to the experimental set up.

### Slit2 Antiserum Administration

Several rabbit polyclonal antisera against human Slit2 protein (production described below) were tested in *in vitro* chemotaxis assays. One antiserum was shown to completely block the ability of Slit2 to inhibit chemotaxis of rat SVZa neuronal cells in our standard neuronal migration assays[28,29] at an antiserum concentration of 1 in 500 (data not shown). Neutralizing anti-Slit2 antiserum was administered daily by intravenous (tail vein) injection (0.5 ml/rat/day for 7 days) commencing 6 hours after disease induction. Control rats received pre-immune serum. A total of 36 rats were examined with sacrifice occurring on days 3 ($n = 6$), 5 ($n = 6$), and 7 ($n = 6$). The experiments were performed in three separate sets (12 rats at a time, $n = 2$ per time point) and results pooled at the end. For the proteinuria and creatinine measurements, six rats per time point were also analyzed.

#### *Early Recombinant Human Slit2 (rhSlit2) Treatment*

Rats received daily intravenous injections of rhSlit2 (production described below). Each rat received seven injections in total with each dose delivering approximately 500 ng of rhSlit2 (in 0.5 ml). The first injection was given 6 hours after disease induction. Control rats received injections of vehicle buffer (Tris-HCl). A total of 36 rats were examined in a manner similar to that for the antibody treatment described above (sacrifice day 3, 5, and 7 with $n = 6$ per group).

#### *Delayed rhSlit2 Treatment*

Rats received daily intravenous injections as above for 5 days, commencing on day 7 after the induction of GN. Control rats received injections of vehicle buffer (Tris-HCl). All rats were sacrificed on day 12 ($n = 5$ per group).

### RNase Protection Assay

RNase protection assays were performed as described[30] using a kit (Torrey Pines Biologicals, Houston, TX) with corresponding probes labeled with [$^{32}$P]UTP. Total RNA from organs and cells was isolated using Trizol (Gibco, BRL, Grand Island, NY). Previously described riboprobes

specific for rat Slit1, Slit2, and Slit3, and the housekeeping gene L32 were used.[8,19]

Glomeruli were obtained by standard sieving techniques[27,31] and Slit2 mRNA expression was assessed. Although three separate assays were performed, each used individual rat samples with only $n = 1$ or 2 available for each time point, thus no statistical analysis was made. Densitometry was performed on all three assays and similar results were obtained (Alphaease Software 5.0, Alpha Innotech Corp, CA). Slit2 mRNA expression was expressed as a percentage of that in normal glomeruli.

## *Reverse Transcriptase PCR*

Rat peripheral blood mononuclear cells were assessed for Robo1 mRNA expression. Five $\mu$g of total RNA was reverse transcribed (20 $\mu$l reaction, 250 ng random primers, 10 mmol/L each dNTP) at 42°C for 50 minutes in the presence of Superscript II (Invitrogen, NY). RNase H (2u) was then added (37°C for 20 minutes) and 5 $\mu$l of the first-strand reaction was used in the PCR (100 $\mu$l volume, 10 $\mu$mol/L primers, 20mMdNTP, 150 mmol/L MgCl2, 5u *Taq* polymerase). Rat Robo1 primers used were: 5′GAGTCCTGTGTCTACAGACAG and 3′GCCCACATTTGCTTGCTCTCTC. The mixture was amplified for 40 cycles, denatured at 93°C, annealed at 62°C, and elongated at 72°C. Expected size of the PCR product was 547 bp. In control PCR reactions, reverse-transcribed samples following RNase and DNase pretreatment were used.

## *Production of Rabbit Polyclonal Anti-Human Slit2 Antisera*

Our general methodology for polyclonal antibody production has been previously described.[30] The cDNA sequence encoding the human Slit2 protein from amino acids 1–300 (GenBank Accession No. XM 039647) was generated by PCR. Human Slit2 shows a remarkable degree of homology with Slit proteins from other species. For example, the homologies for human Slit2 with rat Slit2, xenopus Slit, and drosophila Slit are 95%, 87% and 40%, respectively.[5] The alignment of human Slit2 with rat Slit2 for the peptide sequence used to generate the antisera reveals very close identity (Figure 1A). The *His*-tagged human Slit2 cDNA sequence was subcloned to pET20 (Novagen, Madison, WI), expressed in *E. coli*, and refolded to generate soluble peptide. The peptide was affinity purified by a Ni-nitrilotriacetic acid affinity column and run on a SDS-PAGE gel for verification (size ~25 kd) before immunizing rabbits. Recombinant peptide (100 $\mu$g) in complete Freund's adjuvant was administered to rabbits subcutaneously. Fifty-$\mu$g injections in incomplete Freund's adjuvant were repeated every 2 weeks. Serum was collected at various time points after the sixth week, and assessed for specificity against human and rat Slit2 in Western blot assays. The antiserum (at a dilution of 1:5000) identified Slit2 protein produced by 293T cells transfected with the full-length cDNA sequence for both human and rat Slit2[5] (Figure 1B). Briefly, $2 \times 10^6$ cells in

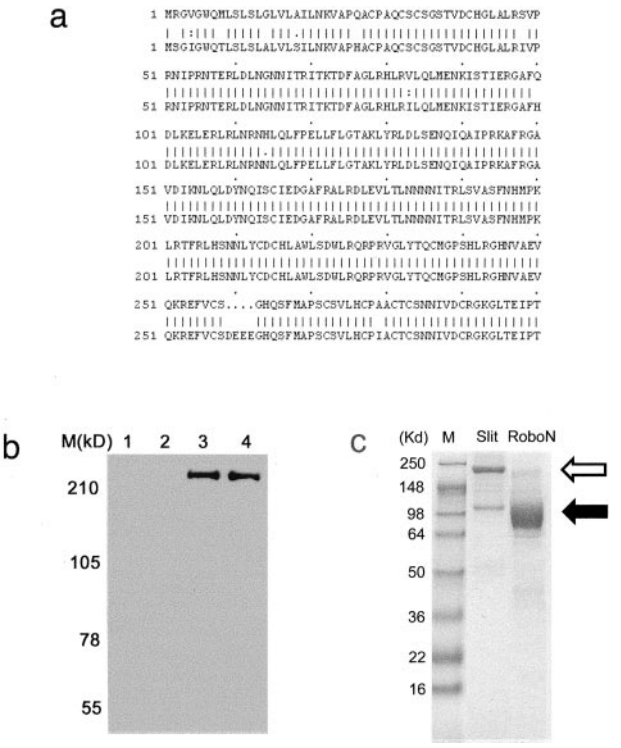


**Figure 1. a:** Alignment of human Slit2 peptide with rat Slit2. **b:** Western blot showing specificity of the Slit2 antibody for both human and rat Slit2. **c:** Coomassie Blue stain of purified rhSlit2 and RoboN using immobilized immunoaffinity chromatography. **a:** Alignment of the human peptide sequence (**top**) with rat Slit2 (**bottom**) showing 95% homology. This human peptide sequence was used to generate the antiserum used in rats. **b:** Anti-human Slit2 antiserum was used in Western blotting experiments (at 1 in 5000) and was able to detect both human (**lane 3**) and rat (**lane 4**) Slit2 from transfected 293T cells. A single band of approx 230 kd was seen. Wild-type 293T cells (**lane 1**) and 293T cells transfected with vector alone (**lane 2**) did not show evidence of Slit2 expression. **c: Lane 1**, protein size markers (M); **lane 2**, 1 $\mu$g of rhSlit2; **lane 3**, 1 $\mu$g of RoboN. A prominent band representing rhSlit2 (slit) was seen at 220 kd (**second lane**, **open arrow**). A smaller, less prominent protein species was also seen at ~100 kd. As seen in Figure 1B, this was not detected by the slit antibody; RoboN was seen as a single smear of molecular weight between 85 and 95 kd, possibly due to the heavy glycosylation (**third lane**, **black arrow**).

400 $\mu$l, were electroporated (at room temperature, 300V for 25ms) with 20 $\mu$g of plasmid (pcDNA3.1) containing either the human or rat Slit2 sequence. Controls were also performed with the vector alone. Electroporated cells were recovered in 20% fetal bovine serum (FBS) Dulbeccos modified Eagle's media (DMEM) medium at 37°C for 24 hours. By Western blotting, bands of the appropriate size (~220 to 240 kd) were seen in the 293T cells transfected with Slit2 but not in the control cells (vector alone, Figure 1B).

## *Production of rhSlit2 and RoboN*

Both rhSlit2 and RoboN were produced from stably transfected 293T cells. The methods required have been extensively described previously.[5,6] The full-length human Slit2 cDNA sequence and the extracellular domain of Robo1 were tagged at the carboxy terminus with c-*myc* and HA, respectively. Cells were cultured in DMEM supplemented with 5% fetal bovine serum and media was collected 3 days after cells became confluent. In brief,

slit- and RoboN-conditioned media were harvested from stable 293T cells (grown in DMEM with 5% fetal calf serum (FCS)) transfected with c-*myc*-tagged Slit2 or HA-tagged RoboN constructs. The pH of the conditioned media was adjusted to 7.5 before being passed three times through agarose-linked columns containing either monoclonal 9E10 (for c-*myc* tag) or 12CA5 (for HA tag) antibodies (Berkley Antibody Co. BAbCo, Richmond, CA). Columns were washed with phosphate-buffered saline (PBS), and eluted with 0.1 mol/L glycine (pH 2.9). The pH was immediately adjusted back to 7.5 with Tris buffer by adding appropriate amounts of 1 mol/L Tris (pH 7.5). Since rhSlit2 was used *in vivo*, a large preparation was produced, and about 11 $\mu$g of purified rSlit2 protein was typically obtained from 100 ml of Slit-2 stable transfectant culture. RoboN was diluted to 1 nmol/L and used in chemotaxis assays. The Slit2 protein was used in chemotaxis assays as described or at full strength (1 $\mu$g/ml) in the *in vivo* experiments. Endotoxin contamination of the reagents was excluded using the Limulus Amebocyte assay (BioWhittaker Inc., Walkersville, MD), indicating a concentration of $<$0.015 endotoxin U/ml. The purity of both rhSlit2 and RoboN is shown in Figure 1C.

## *Glomerular Leukocyte Chemotaxis Assays*

For each set of assays, glomerular leukocytes from three rats with GN were isolated and used. Experiments were repeated on two further occasions ($n = 3$ each time) and the findings were shown to be reproducible. Harvested cells from each rat were run in duplicate in the chemotaxis assays and the average of these two numbers was used as the cell migration for that particular rat.

In brief, 6 days after disease induction, kidneys were decapsulated and sieved. Suspensions were checked for glomerular purity ($>$95%), pooled, and incubated with digestive enzymes.[19,31,32] Both resident glomerular cells and inflammatory cells were obtained in the final "soup". The inflammatory cells were characterized by examination of stained cytospins, and consisted primarily of macrophage/monocytes ($\sim$75%) with some lymphocytes (25%). Cells were resuspended ($4 \times 10^6$ cells/ml) in DMEM with 3% normal rat serum and kept on ice before use. Chemotaxis was measured by transfilter assays in 48-well chemotaxis chambers (Neuroprobe, Cabin John, MD).[8,19] Different chemoattractants (chemokines or the bacterial chemoattractant product *N*-formyl peptide f-Met-Leu-Phe; fMLP, all at 10 nmol/L) were placed in lower wells of chambers (30 $\mu$l/well) and separated from cell suspension (50 $\mu$l) in upper wells by 5-$\mu$m pore-size PVP-free polycarbonate filters.

The effect of Slit2 was assessed in two different ways: first, by adding it to lower wells only (12.5 to 200 pM) and second, by pre-incubating the cells for 30 minutes in Slit2 (on ice) and then adding them directly (no washing) to the upper chambers. In these experiments the upper well Slit2 concentrations were the same as the lower well Slit2 concentrations. Cells not pre-incubated with Slit2 were similarly placed on ice for 30 minutes before the assay. In the assays with Slit2 pre-incubation and addition to both upper and lower chambers, the effect of adding RoboN was also assessed. Here, RoboN was added (final concentration, 1 nmol/L) at the same time as the Slit2.

Chambers were incubated at 37°C in 5% $CO_2$ for 90 minutes. The filter upper surface was washed three times with PBS and scraped to remove cells that had settled. Cells trapped in filter pores or adherent to the undersurface were fixed in methanol and stained. Transfilter migration was expressed as the total number of cells seen migrating in five 0.1 $mm^2$ fields. Results were expressed as mean $\pm$ SD. All counts were obtained by a researcher blinded to the experimental set-up.

## *Chemotaxis Assays on Peripheral Blood Mononuclear Cells from Rats Receiving rhSlit2*

Normal WKY rats received a single intravenous injection of rhSlit2 (approx 500 ng in 0.5 ml) through the tail vein. Control rats received a vehicle buffer (Tris-HCl). Thirty minutes later, rats were anesthetized, bled, and sacrificed. Blood counts and smears were examined for total and differential white cell counts. Peripheral blood mononuclear cells (PBMC) were isolated using Lympholyte rat (Accurate Chemical and Scientific Corp, Westbury, NY). Heparinized blood was diluted 1:2 with normal saline and layered over an equal volume of the Lympholyte. The mixture was centrifuged at room temperature ($1500 \times g$ for 20 minutes) allowing a mononuclear cell layer to form at the interface between the two solutions. The PBMC layer was removed, checked for purity ($\sim$90%) and incubated with red cell lysis buffer for 10 minutes on ice. The PBMC suspensions were rechecked for viability and purity, and washed three times with DMEM containing 3% normal WKY rat serum. Suspensions were then resuspended in DMEM (3% normal WKY rat serum) to a concentration of $1 \times 10^6$ cells/ml and immediately used in the chemotaxis assays (apparatus and membrane as described above). Media (DMEM + 3% WKY rat serum) with or without MCP-1 (10 nmol/L) was placed in lower wells of chambers (30 $\mu$l/well) and separated from cell suspension (50 $\mu$l) in upper wells by 5-$\mu$m pore-size filters. Chambers were incubated, disassembled, and filters were processed as described above. In addition to an analysis of the cells trapped in filter pores as described (transfilter migration), the total number of cells reaching the lower chamber were also counted (transwell migration) to further verify the result. This added verification step (assessing transwell counts) was not performed in the glomerular leukocyte assays because of the large number of wells to assess. Results were expressed as mean $\pm$ SD ($n = 4$ per group). All counts were obtained by a researcher blinded to the experimental set-up.

## *Assays for Active Rac1 and cdc42*

To obtain enough protein to perform these assays ($5 \times 10^6$ cells/sample, with the experiment repeated three times), murine macrophage-like cells (RAW264.7) were used rather than pooled PBMCs from rats. Despite being a cell line, these cells are well recognized as having

many characteristics of macrophages and have been used extensively by other investigators to study macrophage function.[33,34]

After verifying that rhSlit2 significantly inhibits chemokine-induced chemotaxis in this cell line (see supplementary Figure 1) we further examined the signaling pathways involved in the Slit2-mediated effect. The RAW 264.7 cells were maintained in complete medium (DMEM, 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin), serum-deprived for 48 hours (DMEM, 1% FBS) and rhSlit2 (100 pM) was then added to the media for 20 minutes. Cell lysates were incubated with pak-1 PBD agarose (Upstate Biotech, Lake Placid, NY), a glutathione-S-transferase (GST) fusion protein corresponding to the p21-binding domain of human pak-1, in accordance with the manufacturer's instructions. Rac1 and cdc42 activation were assessed by Western blotting of pak-1 PBD affinity-precipitated samples with monoclonal antibodies against Rac1 (BD Pharmingen, San Diego, CA) and cdc42 (Santa Cruz Biotech, Santa Cruz, CA). The protocol was performed according to the manufacturer's instructions on the use of the pak-1 PBD (Upstate Biotech, Lake Placid, NY). An aliquot of each cell lysate that had not been affinity precipitated was assessed in a similar way for levels of total Rac1, cdc42, and actin.

### *Flow Cytometry*

Single-cell suspensions were prepared from spleens or whole blood of rats ($n = 3$) 24 hours after rhSlit2 injection (500 ng in Tris-HCl); control rats ($n = 3$) were injected with Tris-HCl. Red blood cells were depleted by incubation in 0.83% $NH_4Cl$; cells were then washed with PBS (pH 7.4) containing 2% fetal calf serum and 0.08% sodium azide at 4°C. PE-conjugated anti-CD45-positive (OX-1, BD PharMingen) cells, were assessed for CCR2 expression using FITC-labeled rabbit anti-CCR2 IgG (Torrey Pines Biolabs, Houston, TX). Pre-immune rabbit IgG labeled with FITC was used as the isotype control. Stained cells were analyzed by flow cytometry on a FACS caliber with CellQuest software (version 3.01; BD Biosciences, San Diego, CA).

### *Statistics*

The unpaired *t*-test was used to assess differences between treatment groups in the *in vivo* studies. Chemotaxis assay results were assessed for statistical significance using analysis of variance and the Bonferroni multiple-comparison test. *P* values less than 0.05 were considered statistically significant.

## *Results*

### *Slit2 mRNA Is Down-Regulated in Experimental Crescentic Glomerulonephritis*

To determine whether alterations in Slit expression are associated with the process of inflammation, glomerular

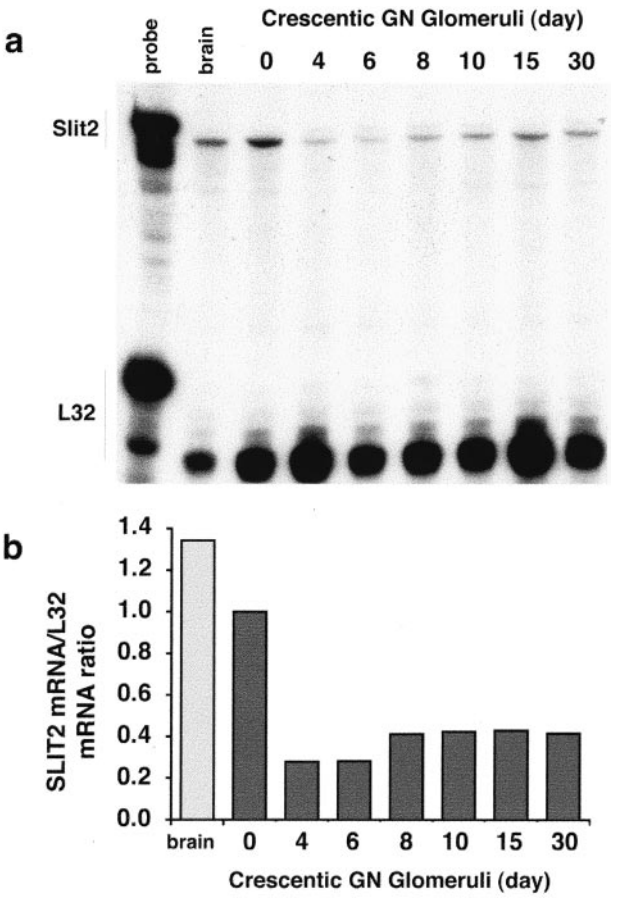


**Figure 2.** Glomerular Slit2 mRNA expression in crescentic glomerulonephritis. RNase protection assay (**a**) showing expression of glomerular Slit2 mRNA relative to L32 mRNA (days 0 to 30). Probes (**lane 1**) contain polylinker regions and are longer than protected bands. Brain Slit2 mRNA was used for comparison (**lane 2**). Each sample in **lanes 3–11**, represents glomerular RNA from one rat. Densitometric analysis of blots (**b**) showing glomerular Slit2 mRNA expression falling to approximately 25% of normal on days 4 and 6 after disease induction and remaining markedly suppressed until day 30 (days 8 to 30, approximately 40% of normal rats).

mRNA expression of Slit1, Slit2, and Slit3 in WKY crescentic GN was examined. The expression of the three known Slit genes in adult tissues (including kidney) and cell lines has previously been described.[8] Slit1 appears to be specific to the nervous system, whereas Slit2 and Slit3 are also expressed in other tissues. *In situ* hybridization using adult human kidney tissue sections has previously revealed the expression of Slit2 in mesangial and epithelial cells of glomeruli, and in tubular epithelial cells and endothelial cells of both arterioles and venules.[8] Consistent with these previous findings, glomerular Slit1 and Slit3 mRNA expression in WKY crescentic GN was undetectable at all time points examined. By contrast, a significant level of Slit2 mRNA expression was detected in normal glomeruli before the injection of anti-GBM antibodies on day 0 (Figure 2a). Following disease induction, glomerular Slit2 mRNA was markedly decreased, falling to approximately 25% of the normal level at days 4 and 6 and remaining significantly suppressed at days 15 and 30 (Figure 2, a and b). Peak macrophage infiltration occurred on days 5 to 6, as demonstrated by staining with an anti-ED-1 antibody.

**Table 1.** Effects of Slit2 Inhibition in Rats with Crescentic GN

| | Anti-Slit2 antibody treated | Pre-immune sera treated |
|---|---|---|
| Glomerular crescents (%) | | |
| Day 3 | 12.7 ± 2.1** | 8.8 ± 1.6 |
| Day 5 | 25.2 ± 2.6* | 21.5 ± 2.9 |
| ED-1$^+$ cells/glomerulus | | |
| Day 3 | 6.8 ± 1.3** | 4.6 ± 0.4 |
| Day 5 | 13.8 ± 0.6** | 11.8 ± 0.8 |
| Proteinuria (mg/day) | | |
| Day 3 | 9.2 ± 2.1** | 4.6 ± 1.5 |
| Day 5 | 26.1 ± 2.9 | 21.5 ± 5.4 |
| Creatinine (mg/dl) | | |
| Day 4 | 0.67 ± 0.09 | 0.61 ± 0.09 |
| Day 6 | 1.65 ± 0.12 | 1.60 ± 0.09 |

Glomerular crescents (%), infiltration of ED-1$^+$ cells and proteinuria were measured in rats with crescentic GN following treatment with either anti-Slit2 neutralizing antibody or control pre-immune sera. $N = 6$ for each of the time points shown.

**, $P < 0.01$ and *, $P < 0.05$, relative to control groups.

## *Slit2 Inhibition Accelerates Inflammation* in Vivo

To investigate the *in vivo* role of endogenous glomerular Slit2 during the early phase of inflammation, rabbit polyclonal anti-Slit2 antiserum was injected into glomerulonephritic rats daily for 7 days, commencing 6 hours after disease induction. This antiserum has been shown to block the inhibitory activity of rhSlit2 *in vitro* (see Materials and Methods). Rats receiving anti-Slit2 antiserum had accelerated disease associated with increased proteinuria, crescent formation, and macrophage infiltration during the early disease period compared to control rats (Table 1). There were significantly more glomerular crescents and ED-1$^+$ cells in the anti-Slit2 antiserum-treated rats at day 3 (Table 1; **, $P < 0.01$ for both) and day 5 (**, $P < 0.01$ and *, $P < 0.05$, respectively). Proteinuria was significantly higher in the anti-Slit2 group at day 3 (**, $P < 0.01$) but was no longer significantly different by day 5. Serum creatinine levels were not significantly different between the two groups at days 4 or 6.

## *Slit2 Inhibits Chemokine-Induced Chemotaxis of* ex Vivo *Inflammatory Glomerular Leukocytes*

To determine whether the loss of endogenous glomerular Slit2 could promote leukocyte infiltration into glomeruli during crescentic GN, infiltrating glomerular leukocytes were harvested after GN induction and the effect of rhSlit2 on chemotaxis was examined using transfilter cell migration assays.[8,19] *Ex vivo* inflammatory glomerular leukocytes were examined for their chemotactic response to the chemokines fractalkine and RANTES, and to the bacterial chemoattractant *N*-formyl peptide f-Met-Leu-Phe (fMLP). Pre-incubation of the inflammatory leukocytes with rhSlit2 before their addition to the chemotaxis chambers significantly decreased chemotaxis induced by various doses of fractalkine, RANTES, and fMLP (Figure 3a to d). The inhibitory effect of rhSlit2 was blocked when the soluble extracellular domain of Robo (RoboN) was added to the upper and lower chambers, suggesting that the inhibitory activity of rhSlit2 on leukocyte chemotaxis was mediated through Robo receptors expressed on the inflammatory cells. Interestingly, rhSlit2 inhibition of chemotaxis induced by fractalkine and fMLP (Figure 3, a and c) did not require pre-incubation with rhSlit2, and addition of rhSlit2 in the lower chambers was sufficient for the inhibition. In contrast, rhSlit2 inhibition of RANTES-induced chemotaxis required the pre-incubation (Figure 3b). The mechanism underlying this difference remains unclear at the present time, although one explanation may be that the different chemoattractants used had different potencies.

The inhibitory effect of different doses of rhSlit2 (0 to 200 pM) on fractalkine-induced chemotaxis (10 nmol/L) was also tested. The rhSlit2-mediated inhibition was shown to be dose-dependent (Figure 3d). Chemotaxis

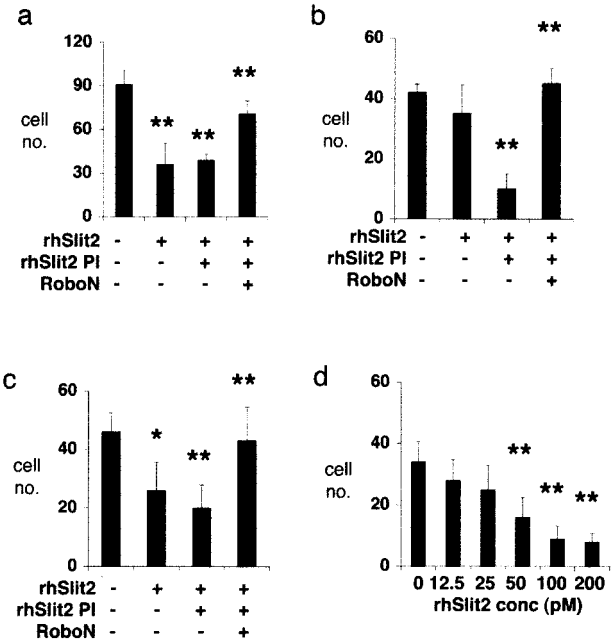


**Figure 3.** Slit2 inhibits chemotaxis of crescentic glomerulonephritic inflammatory leukocytes, ex vivo. Graphs (**a–c**) show the ability of rhSlit2 to inhibit fractalkine- (**a**), RANTES- (**b**), or fMLP-induced (**c**) chemotaxis of *ex vivo* inflammatory glomerular leukocytes. Cells were added to upper chambers; chemoattractants to lower chambers (10 nmol/L). RhSlit2 (100 pM) was added to lower chambers only (rhSlit2; **second bar**; **a–c**) or to both upper and lower chambers at the same concentration (rhSlit2 PI; **third bar**; **a–c**). Where Slit2 was added to upper chambers, cells were also pre-incubated (rhSlit2 PI) for 30 minutes with rhSlit2 (100 pM). Finally, the effect of adding the extracellular domain of the Slit receptor, RoboN (1 nmol/L), at the same time as rhSlit2 in the pre-incubation experiments, was assessed (RoboN pre-incubation of cells then addition to both upper and lower wells, **fourth bar**; **a–c**). RhSlit2 inhibited cell migration in response to all three agents (**a–c:** **, $P < 0.01$; *, $P < 0.05$; with *versus* without rhSlit2). With RANTES, rhSlit2 pre-incubation (**b**; rhSlit2 PI; **third bar**) of cells was required for the inhibitory effect to be observed. RoboN reversed the inhibitory effect of rhSlit2 on chemotaxis for all agents (**a—c**; **, $P < 0.01$). RhSlit2 inhibition of fractalkine-induced chemotaxis was dependent on the rhSlit2 dose (**d**). Concentrations ≥50 pM in lower chambers, significantly reduced fractalkine-induced chemotaxis (**d**; **, $P < 0.01$). Maximal inhibition was seen at ≥100 pM. Control experiments were performed without chemoattractant in lower chambers (with and without rhSlit2/RoboN as above). These all showed low level migration which was unaffected by the Slit2 (mean migration 0 to 10 cells per 5 high power field (hpf). Each graph shown represents one set of experiments ($n = 3$). All results were verified on two further occasions. The total number of migrating cells in 5 hpf are indicated on the *y* axis (mean ± SD).

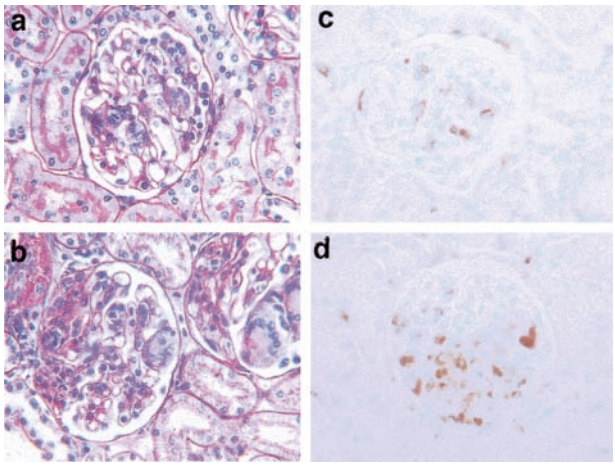


**Figure 4.** Early treatment with rhSlit2 protein reduces glomerular crescent formation and macrophage infiltration in crescentic glomerulonephritis (also see Table 2). Rats with crescentic GN received daily injections of rhSlit2 commencing 6 hours after disease induction (total of 7 injections). Control animals received vehicle (Tris-HCl). Histological appearances at day 7 in rhSlit2- (**a** and **c**) and vehicle-treated (**b** and **d**) rats are shown using PAS (**a** and **b**) and ED-1 (**c** and **d**) to assess crescents and macrophages, respectively. Rats treated with rhSlit2 showed significantly fewer glomerular crescents (**a**) compared to controls (**b**). Similarly, there were less glomerular ED-1-positive (ED-1$^+$) cells in the rhSlit2-treated rats (**c**) compared to controls (**d**).

induced by 10 nmol/L fractalkine was inhibited by rhSlit2 at a concentration of 50 pM or greater. In the presence of 50 pM, 100 pM, and 200 pM of rhSlit2, the proportion of migrated cells fell to 47%, 26%, and 23%, respectively, compared to fractalkine alone (Figure 3d; **, $P < 0.01$ for all).

## *Systemic rhSlit2 Administration Ameliorates Inflammation* in Vivo

To test the potential therapeutic effect of Slit2 on the inflammatory process, rhSlit2 was injected intravenously into WKY crescentic GN rats. Two groups of experiments were performed. In the first, rats received rhSlit2 protein daily for 7 days (days 0 to 6), commencing 6 hours after disease induction ("early rhSlit2 treatment"). In the second, rats received rhSlit2 daily for five days (days 7 to 11), commencing on the day proteinuria was first detected ("delayed rhSlit2 treatment").

In the early treatment study, rhSlit2 protein significantly ameliorated GN during the initial phase of the disease as was evident both functionally and histologically (Figure 4 and Table 2). Rats treated with rhSlit2 showed significantly fewer glomerular crescents, ED-1$^+$ cells, and reduced levels of proteinuria at day 5 and day 7 when compared to controls (Table 2; **, $P < 0.01$ for all). Serum creatinine levels on day 6 were also significantly lower in the rhSlit2-treated rats compared to controls rats treated with the Tris-HCl vehicle buffer only (**, $P < 0.01$). Representative glomeruli demonstrating these differences are shown (Figure 4).

In the delayed treatment study, rhSlit2 protein significantly improved histological parameters of GN but not the functional parameters (Figures 5 and 6). Histology

**Table 2.** Effects of Early Treatment with rhSlit2 Protein in Rats with Crescentic GN

| | rhSlit2 Treatment | Control |
|---|---|---|
| Glomerular crescents (%) | | |
| Day 5 | 14.7 ± 1.9** | 20.5 ± 2.9 |
| Day 7 | 36.8 ± 4.1** | 48.5 ± 3.7 |
| ED-1$^+$ cells/glomerulus | | |
| Day 5 | 7.2 ± 0.4** | 11.8 ± 0.8 |
| Day 7 | 14.4 ± 2.0** | 22.1 ± 2.7 |
| Proteinuria (mg/day) | | |
| Day 5 | 14.9 ± 2.4** | 22.8 ± 4.1 |
| Day 7 | 39.4 ± 3.9** | 54.3 ± 4.8 |
| Creatinine (mg/dl) | | |
| Day 4 | 0.57 ± 0.09 | 0.62 ± 0.08 |
| Day 6 | 1.06 ± 0.2** | 1.52 ± 0.1 |

Functional and histological alterations were examined in rats treated with rhSlit2 protein following the induction of crescentic GN. $N = 6$ for each of the time points shown.
**, $P < 0.01$ relative to control rats.

was assessed at the end of the treatment period on day 12, while function was assessed at various points during the course of the treatment. The proportion of glomeruli with crescents was greater in the control rats compared to the rhSlit2-treated rats (Figure 5a; 53.8 ± 4.6% *versus* 42.2 ± 5.2%; $P < 0.01$) as was the number of ED-1$^+$ cells per glomerulus (Figure 6a; 29.9 ± 4.6 *versus* 17.8 ± 2.9 cells; $P < 0.01$). When crescents were further analyzed by counting those associated with a greater than 50% collapse of the glomerular tuft (as seen in the lower glomerulus, Figure 5d), control rats were found to have approximately twice as many as the rhSlit2-treated rats (Figure 5b; control 19.8 ± 3.1% *versus* rhSlit2 9.9 ± 5.3; $P < 0.01$). Representative glomeruli showing these differences are shown (Figures 5 and 6). Despite the morphological differences in the delayed treatment studies, proteinuria and creatinine were not significantly different between the two groups. However, proteinuria levels appeared to show a trend with the control rats displaying slightly higher levels at all time points assessed (day 8, control 53.5 ± 15 *versus* rhSlit2 40.7 ± 19.8 mg/day; day 9, control 64.6 ± 17.5 *versus* rhSlit2 50.2 ± 21.8 mg/day; day 11, control 65.6 ± 17.7 *versus* rhSlit2 56.6 ± 18.3 mg/day). Creatinine levels at sacrifice were control 1.41 ± 0.14 *versus* rhSlit2 1.33 ± 0.13.

## *Systemic Administration of rhSlit2 Inhibits MCP-1-Mediated Chemotaxis of* ex Vivo *Circulating Peripheral Blood Mononuclear Cells*

To determine the mechanism by which peripherally injected rhSlit2 could ameliorate crescentic GN, normal WKY rats received a single injection of either rhSlit2 or vehicle (Tris-HCl), and peripheral blood mononuclear cells (PBMC) were harvested 30 minutes later to assess their response to MCP-1 in *ex vivo* chemotaxis assays. Total and differential white cell counts were not affected by the injection. PBMCs from rhSlit2-injected animals showed a complete inhibition of MCP-1-mediated chemotaxis in both the transfilter and transwell assays (sec-

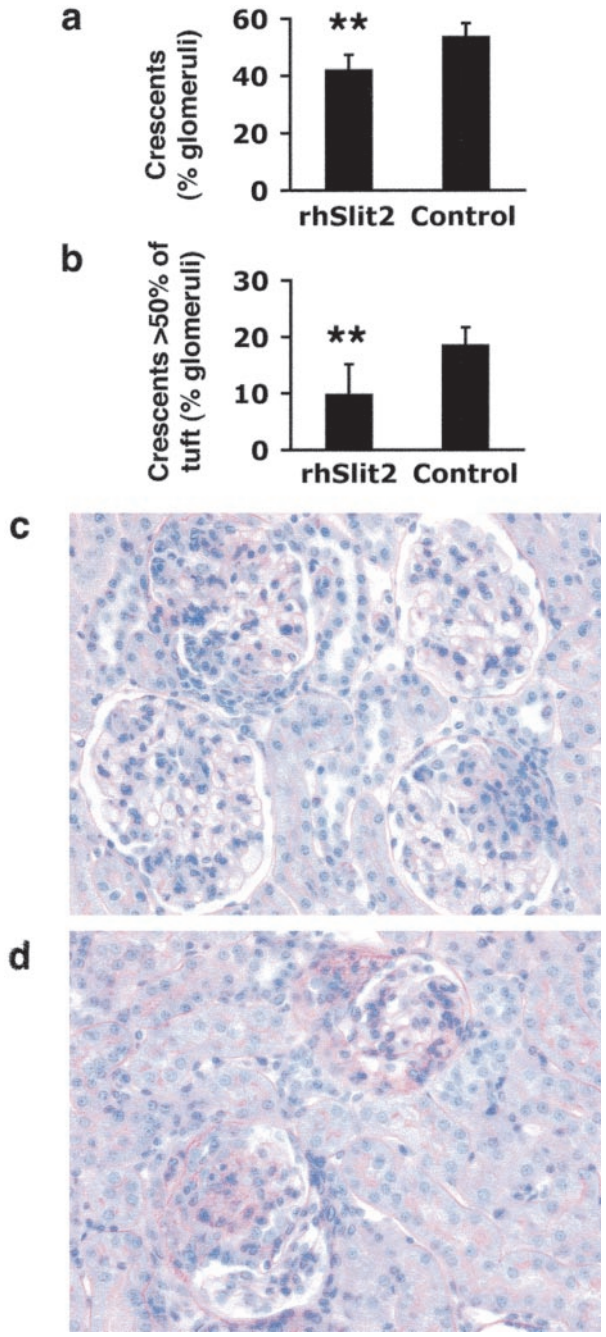


**Figure 5.** Delayed treatment with rhSlit2 protein reduces glomerular crescent formation in crescentic glomerulonephritis. Treatment of glomerulonephritic rats with rhSlit2 protein commenced 7 days after GN induction when the disease had been established (five rats per group). **a:** Quantification of crescentic glomeruli. Both groups showed the formation of glomerular crescents (**a–d**), although the percentage of crescentic glomeruli was significantly lower in rhSlit2-treated rats compared with controls (**a**; **, $P < 0.01$). Glomeruli demonstrating crescents in rhSlit2-treated animals (**c**) and control animals (**d**), are shown. Control rats, in addition, had an increased proportion of crescents that were large (defined as leading to >50% collapse of the tuft) compared to rhSlit2-treated rats (**b**; 19.8 ± 3.1 *versus* 9.9 ± 5.3; $P < 0.01$). A glomerulus with a large crescent is shown (**d**; lowest glomerulus) in one of the control rats.

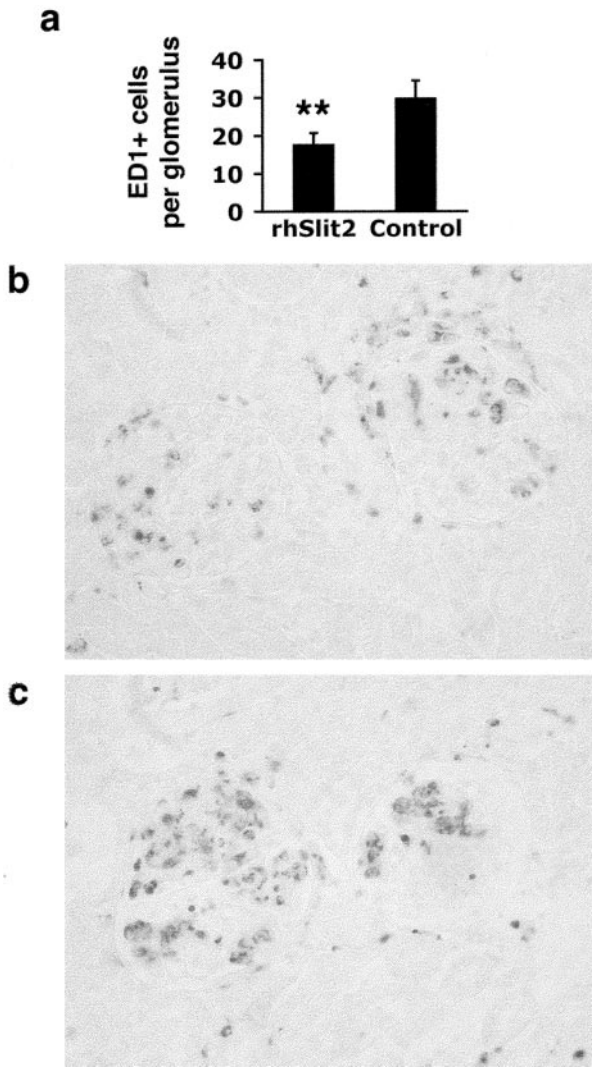


**Figure 6.** Delayed treatment with rhSlit2 protein reduces macrophage infiltration in crescentic glomerulonephritis. Rats with crescentic GN received daily rhSlit2 injections once proteinuria had developed (from day 7), for 5 days. Control rats received vehicle (Tris-HCl). Glomerular macrophage infiltration, as indicated by ED-1$^+$ staining (**a-c**) was significantly lower in rats treated with rhSlit2 compared to control rats (**a**; **, $P < 0.01$). Representative glomeruli showing macrophage infiltration in rhSlit2-treated animals (**b**) and control animals (**c**) are shown.

ond bar, Figure 7, a and b) with the number of migrated cells being similar to baseline levels (40 to 50% of maximum). PBMCs from vehicle- (Tris-HCl) injected animals showed a normal chemotactic response to MCP-1 (fourth bar, Figure 7, a and b) with an approximate doubling of migrated cells compared to media without MCP-1. MCP-1-mediated migration in vehicle-treated animals was significantly higher than that seen in all other groups (**$P <$ 0.01, Figure 7, a and b). Representative membrane appearances for MCP-1-mediated migration in rhSlit2- (Figure 7c) and vehicle- (Figure 7d) treated animals are shown. Expression of CCR2 (the receptor for MCP-1) on peripheral blood and spleen derived leukocytes was not affected by injection of Slit2 as assessed by flow cytometry (see supplementary Figure 2).

## *Rat Peripheral Blood Mononuclear Cells Express Robo1 mRNA*

As Robo1 is known to be one of the receptors for Slit2, reverse transcriptase PCR was used to assess rat PBMC

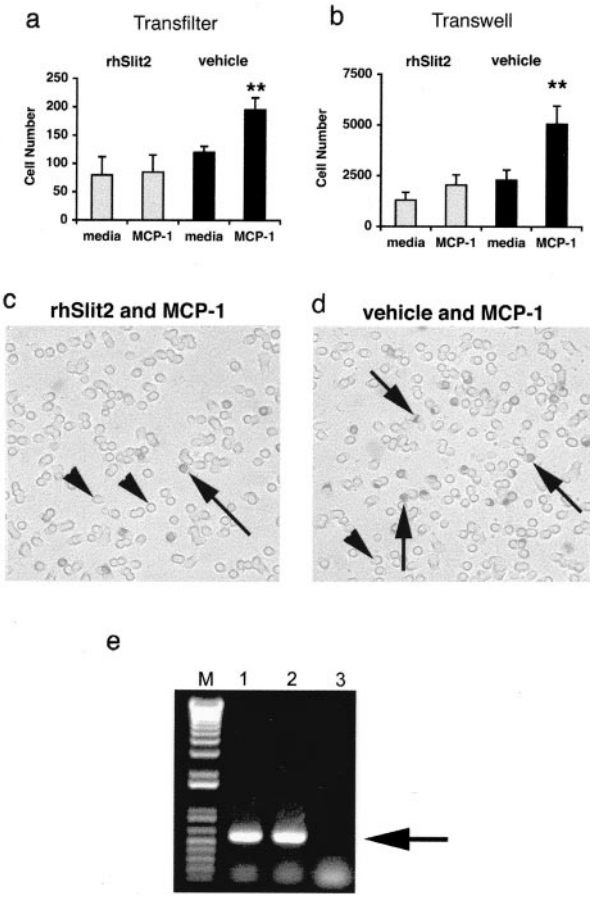


**Figure 7.** MCP-1-mediated migration by circulating PBMC is inhibited in normal WKY rats following a single IV rhSlit2 injection. MCP-1-mediated chemotaxis of peripheral blood mononuclear cells (PBMC) was assessed *ex vivo,* 30 minutes after a single injection of rhSlit2 or vehicle (Tris-HCl). Cells were placed in the upper wells of chemotaxis chambers and assessed for their ability to migrate in response to media or MCP-1 (10 nmol/L) in the lower chamber. The number of cells trapped in the pores (transfilter, **a**) and reaching the lower chamber (transwell, **b**) were assessed. Slit2-injected animals showed a complete inhibition of MCP-1-mediated chemotaxis (**second bar**, **a** and **b**) with the number of migrated cells being similar to or lower than baseline levels (40 to 50% of maximum). PBMCs from animals receiving vehicle showed a normal chemotactic response to the MCP-1 (**fourth bar**, **a** and **b**) with an approximate doubling of migrated cells compared to media without MCP-1. MCP-1-mediated migration in vehicle-treated animals was significantly higher than that seen in all other groups (**$P < 0.01$). Representative membrane appearances for MCP-1-mediated migration in rhSlit2- (**c**) and vehicle- (**d**) treated animals are shown. Empty 5-$\mu$m pores (**arrowheads**) and cells trapped in membrane pores (**arrows**) are indicated. There were significantly more migrating cells in vehicle-treated animals compared to all other groups ($n = 4$/group). Rat PBMC were found to express Robo1 mRNA by RT-PCR (**e**). **Lanes**: **M**, ladder; **1**, PBMC with no pretreatment of RNA; **2**, PBMC with DNase pretreatment of RNA; **3**, PBMC with RNase A pretreatment of RNA. The PCR product was of the predicted size (547 bp; **arrow**).

Robo1 mRNA expression. Using the rat Robo1 specific primers, a PCR product of the predicted size was amplified. This was not obtained when RNA samples were pretreated with RNase A (Figure 7e).

## *Slit2 Decreases GTP-Bound Forms of Rac1 and cdc42 in RAW264.7 Murine Macrophage-Like Cells*

In neuronal migration, Slit-Robo signaling appears to modulate the activity of GTPases such as cdc42.[28] To understand the mechanisms underlying the effect of Slit2 on leukocyte chemotaxis, levels of active cdc42 and Rac1 were examined in inflammatory cells following incubation with rhSlit2. To obtain a high enough quantity of protein from the assays, the murine macrophage-like cell line RAW264.7 was used in the affinity precipitation experiments. Inhibition of chemokine-induced chemotaxis by rhSlit2 was confirmed in these cells *in vitro* (unpublished data), consistent with the findings seen in other leukocyte cell lines.[8] GTP-bound active Rac1 and cdc42 proteins were pulled down from RAW264.7 cells following treatment with rhSlit2 protein, using a glutathione-S-transferase (GST) fusion protein corresponding to the p21-binding domain of human pak-1 (pak1-PBD). Pak-1 is the downstream effector of both cdc42 and Rac1 and hence it binds only to the GTP-bound forms of these molecules. Incubation of cells with rhSlit2 for 20 minutes led to a significant decrease in the levels of active Rac1 and cdc42 (Figure 8, a and b). As would be expected, levels of total Rac1 and cdc42 protein (both the GDP- and GTP-bound forms) and levels of actin, remained unchanged (Figure 8c).

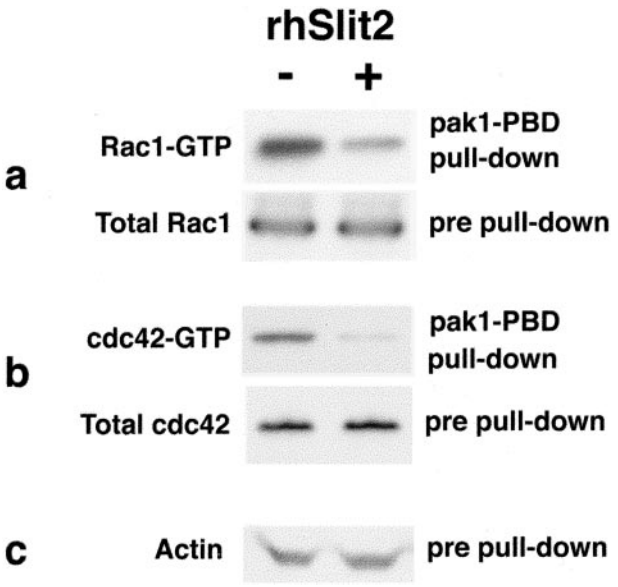


**Figure 8.** Levels of active Rac1 and cdc42 in RAW264.7 murine macrophage-like cells are reduced by rhSlit2 incubation. Levels of active, GTP-bound Rac1, and cdc42 were decreased on treatment of the RAW264.7 cells with rhSlit2 protein (**a** and **b**, pak1-PBD pull-down). As expected, lysates that had not been affinity precipitated with pak1-PBD, were shown to have similar amounts of total Rac1 and cdc42 proteins (**a** and **b**, pre-pull-down). The levels of total actin also remained unchanged (**c**).

## *Discussion*

The important role of chemokines in the process of leukocyte migration during the inflammatory response has been well established.[1,2,23] To date, apart from some reports of virally induced inhibitors of chemokines,[35,36] there have been no endogenous molecules identified which can directly oppose the effects of these pro-inflammatory factors. The data presented here supports a role for the Slit family of proteins as endogenous inhibitors of

the chemokines, acting on signaling pathways that control cell migration. This is analogous to the effect that has been shown for Slit proteins in the nervous system[3–7] and suggests that these molecules may have a conserved role in the regulation of cell migration in other diverse cell types.

In the nervous system, Slit proteins bind to their receptor, Robo, and act as repellents regulating the migration of neurons and axons. The demonstration of Slit and Robo isoforms in a variety of organs and cell types[8,37,38] is consistent with a wide role for these molecules and led to the hypothesis that Slit proteins may be important in regulating leukocyte migration. In support of this, Robo isoforms have been found on a variety of leukocyte cell lines and primary inflammatory cells.[8,39] Furthermore, Slit2 inhibition of chemokine-induced chemotaxis has been demonstrated in human embryonic kidney cells, after co-expression of chemokine receptors with Robo1.[8] In support of a possible action for Slit2 via Robo1 in inflammatory cells, we found Robo1 mRNA expression in rat PBMC. While specific ligand-receptor interaction and downstream signaling was not examined in these cells, Robo1 is known to be one of the cognate receptors for Slit2 in neurons.

To determine whether altered Slit2 expression is associated with the inflammatory process, glomerular Slit2 mRNA levels were examined after the induction of crescentic GN. A marked down-regulation of Slit2 mRNA was found early in the disease period and this persisted for up to 30 days after disease induction. A similar down-regulation of Slit isoforms was found in an inflammatory lung model (unpublished data) and in endothelial cells incubated with the inflammatory mediators TNF-$\alpha$ and IL-1$\beta$.[39] Anti-GBM disease in the WKY rat is well characterized as an acute, severe and progressive, cell-mediated, and chemokine-dependent model of inflammation.[19,20,25,26,40] Peak leukocyte infiltration occurs between days 5 and 7, and inflammatory cells can be found in the glomeruli for several weeks after induction.[25] Infiltrating leukocytes are generally mononuclear, with neutrophil infiltration and complement fixation not being major features.[40–42] Thus, the down-regulation of Slit2 correlated strongly with leukocyte infiltration, being maximally down-regulated on days 4 and 6 to approximately 25% of normal. The administration of a neutralizing Slit2 antibody daily for the first 7 disease days markedly accelerated the inflammatory process, suggesting that inhibition of endogenous glomerular Slit2 during this time could further promote leukocyte infiltration. The demonstration that rhSlit2 could inhibit RANTES, fractalkine, and fMLP-induced chemotaxis of *ex vivo* glomerular leukocytes harvested on day 6 of the model supports this hypothesis, as does the demonstration of rhSlit2 dose-dependency. The ability of the Robo extracellular fragment (RoboN) to block the inhibitory effect of rhSlit2 on leukocyte chemotaxis was consistent with a Robo-mediated effect.

To assess the potential therapeutic effect of Slit2 administration during inflammation, rats with crescentic GN were treated at both early and late phases of the disease. Treatment that commenced 6 hours after disease induction was associated with the most benefit, resulting in improved renal function during the early disease period. This correlated with histological improvement with fewer glomerular crescents and less infiltrating macrophages being evident in the rhSlit2-treated animals. These observations suggest that rhSlit2, given early, was able to inhibit leukocyte recruitment during the initiation period and thus attenuate the disease process. In contrast, treatment which commenced on day 7 did not improve renal function although histology was somewhat improved. As documented, the rats had already developed proteinuria before rhSlit2 injections were commenced, consistent with significant glomerular leukocyte infiltration already being present. These results suggest that late treatment with rhSlit2 was unable to resolve inflammation that was already present but may have been able to inhibit ongoing leukocyte recruitment after day 7 of the disease.

It is important to note that modulation of chemotactic factors rarely results in complete inhibition of inflammation.[43–45] This differs from the almost complete inhibition often observed when whole subpopulations of inflammatory cells are depleted.[46–48] The impact of rhSlit2 treatment was consistent with that seen in other studies of this model where individual or multiple chemoattractants are inhibited.[19,20,41] Since rhSlit2 appears to act by opposing the chemoattractant properties of chemokines, the disease amelioration observed was entirely consistent with this mechanism.

To determine the potential mechanism by which peripherally injected rhSlit2 could ameliorate inflammation, *ex vivo* PBMCs from normal WKY rats were assessed in chemotaxis assays after a single rhSlit2 injection. A complete loss of MCP-1-mediated chemotaxis was observed in PBMCs from the rhSlit2-injected rats, consistent with a peripheral effect of the administered rhSlit2. Thus the mechanism in the disease animals would appear to involve a rhSlit2-mediated prevention of migration by circulating leukocytes, rather than a local kidney effect.

Based on the studies in neuronal cells[3–7] the "chemorepulsive" effect of Slit requires a gradient. The results from the *ex vivo* PBMC chemotaxis assays suggest that this mechanism was not important in the animals treated with rhSlit2. As hypothesized by Wong et al,[28] the repulsive action depends on the Slit concentration gradient establishing cell polarity. Thus movement away from the Slit would occur through a differential effect on actin polymerization signaling pathways at each pole of the cell.[28] This is analogous to the manner in which chemokines work on leukocytes[49] although their effect is one of "chemoattraction". It is hypothesized that the peripheral injection of rhSlit2 effectively "disarmed" the circulating leukocytes, making them refractory to the effects of chemokines through some mechanism which is yet to be fully determined. The ability of rhSlit2 to inhibit the chemotactic effect of multiple chemokines/chemoattractants including MCP-1, fractalkine, RANTES and fMLP (presented here), and SDF-1 (reported previously[8]), supports the hypothesis that Slit2 acts on a pathway which is common to these chemokines. For this reason and based on the recent reports in neurons,[28] the effect of rhSlit2 on

GTPases was examined. These studies showed that levels of active, GTP-bound Rac1 and cdc42 were decreased in murine macrophage-like RAW264.7 cells after incubation with rhSlit2. Interestingly, these cells showed a pattern of GTPase changes that were distinct from those observed in SVZa neuronal migration[28] where GTP-Rac1 was not found to be reduced.

In summary, the results presented here demonstrate a potentially important role for endogenous Slit2 in modulating the inflammatory response. Rapid down-regulation of Slit2 in affected tissues may promote leukocyte migration from the circulation into these areas. Treatment of inflammatory cells with Slit2 is associated with a loss of function as indicated by their reduced ability to respond to multiple chemoattractants. Finally, the mechanism of action of Slit2 appears to involve an effect on the signaling pathways through which chemoattractants also act, making the GTPases very likely candidates for this effect. Further in depth studies are necessary to understand the similarities and differences of Slit-Robo signaling in neuronal migration and in leukocyte chemotaxis.

## Acknowledgments

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