Urothelial lesion formation is mediated by TNFR1 during neurogenic cystitis

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Urothelial lesion formation is mediated by TNFR1 during neurogenic cystitis. Am J Physiol Renal Physiol 291: F741–F749, 2006. First published April 18, 2006; doi:10.1152/ajprenal.00081.2006.—Interstitial cystitis (IC) is a chronic bladder inflammatory disease of unknown etiology that shares similarities with Crohn’s disease and psoriasis. IC, often regarded as a neurogenic cystitis, is associated with urothelial lesions that likely compromise the bladder permeability barrier and thereby contribute to patient morbidity. Here, we use a murine model of neurogenic cystitis to investigate the mechanism of urothelial lesion formation and find that urothelial apoptosis induces formation of lesions. Lesions formed in wild-type mice but not in mice deficient in TNF, TNF receptors, or mast cells. In urothelial cultures, only siRNAs targeting TNFR1, but not TNFR2, blocked TNF-induced apoptosis, indicating a primary role for TNFR1. Trans-epithelial resistance, a measure of bladder barrier function, decreased during neurogenic cystitis in wild-type and TNFR2−/− mice but was stabilized in TNF−/− mice. Anti-TNF antibodies both altered bladder mast cell localization and stabilized barrier function. Based on these findings, we conclude that mast cell activation and release of TNF drive urothelial apoptosis and lesion formation in a murine neurogenic cystitis model, and we hypothesize that anti-TNF therapy may stabilize bladder barrier function in IC patients.

It has been suggested that urothelial lesions contribute to decreased bladder barrier function (15). By allowing noxious urine components to cross the permeability barrier normally provided by intact urothelium, these lesions may result in the activation of sensory nerves and thus contribute to pain and voiding dysfunction. Consistent with this hypothesis, FIC cats show decreased bladder barrier function compared with normal cats that correlate with urothelial lesions (31).

Several mechanisms have been proposed for the bladder defects in IC, including a urinary antiproliferative factor (APF) and a neuroimmune interaction involving mast cells (14, 24). The APF hypothesis is supported by findings that urine of IC patients contains a glycopeptide that inhibits urothelial proliferation in vitro (25, 26). Alternatively, mast cells are thought to play a role in the pathogenesis of IC for at least some patients because a subset of IC patients have elevated counts of bladder mast cells that often appear partially activated and juxtaposed with sensory nerves (34, 42, 50). In this neuroimmune model, it is hypothesized that inflammatory mediators released by mast cells result in urothelial damage, and increased concentrations of methylhistamine and mast cell-specific tryptase have been detected in urine of IC patients (5, 13).

Mast cells have shown to mediate bladder inflammation in various rodent cystitis models. For example, instillation of substance P or lipopolysaccharide into the bladder via transurethral catheter resulted in cystitis that included leukocyte influx and vascular permeability (4, 41). In both models, cystitis was not observed in mast cell-deficient (W/Wv) mice, but sensitivity was restored upon reconstitution with wild-type (WT) bone marrow. At least some effects of mast cells in the bladder may be mediated by TNF, since mast cells were shown to directly induce urothelial inflammatory responses, and anti-TNF antibodies abrogated the urothelial responses in culture (3). Mast cells have been further implicated in bladder pathogenesis in a rat neurogenic cystitis model that employs the attenuated Barth’s strain of pseudorabies virus (PRV) to activate bladder afferents strictly via central mechanisms. Jasmin and colleagues (21, 22) observed that rats developed a cystitis associated with mast cell influx and urinary histamine metabolites. Thus mast cells play a role in several cystitis models, including a centrally mediated neurogenic cystitis that mimics aspects of IC.

We recently adapted the PRV-induced neurogenic cystitis model to the murine system to take advantage of more abundant genetic tools. We reported that PRV induced a neurogenic cystitis in mice that increased vascular permeability, induced leukocyte influx, and resulted in differential trafficking of mast cell pools within the bladder (10). Furthermore, these events

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did not occur in TNF receptor-deficient mice, suggesting that TNF is essential for mast cell trafficking in neurogenic cystitis. Because TNF is also a potent mediator of apoptosis, we examined the murine neurogenic cystitis model for evidence of apoptosis. Here, we demonstrate that PRV induced urothelial lesions and correlated with diminished bladder barrier function and these processes were dependent on intact TNF signaling mediated primarily by TNF receptor 1 (TNFR1). The data suggest that therapies targetting TNF signaling may stabilize the urothelial permeability barrier and thereby reduce symptoms in IC patients.

MATERIALS AND METHODS

Animals. Female, 4- to 6-wk-old, mice with targeted deletion of TNFR1 (TNFR1<sup>−/−</sup>; B6.129-Tnfrsf1a<sup>tm1(Majd)/J</sup>, TNFR2 (TNFR2<sup>−/−</sup>; B6.129S7-Tnfrsf1b<sup>tm1(Dam)/J</sup>, TNFR1 and TNFR2 (TNFR1<sup>/−/−</sup>; B6.129-Tnfrsf1a<sup>tm1(Dam)/J</sup>), and their wild-type C57BL/6J controls were purchased from Jackson Laboratory (Bar Harbor, ME). TNF-deficient mice (TNF<sup>−/−</sup>; C57BL/6J) were generously provided by Dr. M. Brown (Northwestern University, Chicago, IL). TNF<sup>−/−</sup> mice were backcrossed 10 generations to C57BL/6J mice. All experiments were performed using protocols approved by Northwestern University Animal Care and Use Committee. The mice were housed in containment facilities of the Center for Comparative Medicine and maintained on a regular 12:12-h light-dark cycle with food and water ad libidum.

Induction of neurogenic cystitis. PRV was prepared and titrated as previously reported (8). Neurogenic cystitis was induced by injection of 10 µl of Bartha strain of PRV containing 2.29 × 10<sup>8</sup> pfu into the abductor caudalis dorsalis muscle with a 26-gauge Hamilton syringe while maintaining the animals under anesthesia. Ultraviolet-irradiated PRV stocks were employed as negative control inocula in sham-treated animal groups. For anti-TNF antibody therapy experiments, mice were administered 250 µg anti-mouse/rat TNF monoclonal antibody (TN3–19.12; BD Pharmingen, San Jose, CA) or 250 µg of purified hamster IgG1 monoclonal antibody (G235–2356; BD Pharmingen) via intraperitoneal injection 1 day before PRV infection. Animals were monitored for full recovery from anesthesia and were euthanized at various times for tissue harvest.

Transferase-mediated dUTP nick-end labeling/uroplakin III staining. Urothelial apoptosis was assessed in tissue sections with transferase-mediated dUTP nick-end labeling (TUNEL) as previously described (30). Briefly, sections were deparaffinized, rehydrated, and incubated with 20 µg/ml nuclelease-free proteinase K at 37°C. After PBS rinse, blocking solution (3% BSA, 20% FBS) was added to the tissue section and incubated at room temperature. Sections were briefly rinsed with PBS, and TUNEL reaction mixture (Roche Diagnostics, Indianapolis, IN) was applied and incubated at 37°C. For quantifying urothelial lesions, a positive lesion was defined as three or more adjacent TUNEL-positive cells, and lesions were determined from TUNEL staining of two nonserial sections from each bladder. For detection of uroplakin III (UPS) immunoreactivity following TUNEL labeling, the sections were incubated with mouse anti-uroplakin III (1:100, clone AU1; Research Diagnostics, Concord, MA) at room temperature. Sections were then incubated with biotinylated goat anti-mouse (1:250; Jackson ImmunoResearch Laboratories, West Grove, PA) followed by incubation with streptavidin-conjugated Alexa Fluor 594 (1:1,000; Molecular Probes, Carlsbad, CA). Slides were stained with DAPI, and images were acquired using a Nikon E800 microscope equipped with a Spot Color RT camera (Diagnostic Instruments, Sterling Heights, MI).

Cell culture. The PD07i cell line was established by generating primary urothelial cultures from a pediatric bladder specimen, followed by immortalization with HPV16 E6E7, as previously described (27, 32). PD07i, PD08i, TEU-1 (27), and TEU-2 (3) cell lines were maintained in EpLife medium (Cascade Biologies, Portland, OR). All tissues were obtained under the sponsorship of the Internal Review Board of Northwestern University.

Immunoblot analysis of TNFR1, TNFR2, and cleaved caspase-8. Whole cell lysates from urothelial cells, which were lysed with RIPA buffer and protease inhibitors, were prepared and protein concentrations were measured by BCA (Pierce Biotechnology, Rockford, IL). SDS-PAGE was performed on 10% polyacrylamide gel (TNFR1, TNFR2) or 4–20% polyacrylamide gel (caspase-8). Proteins were transferred onto PVDF membrane and probed with mouse anti-human TNFR1 (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human TNFR2 (1:2,000; Santa Cruz Biotechnology), or anti-caspase-8, clone C15 (1:70) (45) followed by secondary antibody conjugated to horseradish peroxidase (1:30,000; Jackson Immunoresearch Laboratories) and detected with enhanced chemiluminescence reagent (Pierce Biotechnology). Equal loading of proteins was confirmed by immunoblot detection of GAPDH levels using mouse anti-GAPDH (1:5,000; Santa Cruz Biotechnology). Signal intensity was quantified by densitometry software.

Annexin-V staining. PD07i cells were cultured in chambered slides (Nunc, Rochester, NY). Cells were labeled with 10 and 1,000 ng/ml recombinant TNF (Biosource, Camarillo, CA) at 37°C for 24 h. Apoptosis was assessed using Annexin-V-FLUOS staining kit (Roche Diagnostics) according to manufacturer’s protocol. Images were acquired using a Nikon E800 microscope equipped with a Spot Color RT camera.

Caspase-3 activity assay. PD07i cells were stimulated with 0.1 µM staurosporine for 2 h (Calbiochem, La Jolla, CA) or 10 and 100 ng/ml TNF at 37°C for 24 h. Urothelial cells were lysed with RIPA buffer containing protease inhibitors, and protein concentrations were determined by BCA. Twenty micrograms whole cell extract were added to caspase assay buffer (100 mM HEPEs, 1% CHAPS, 1% sucrose, 2 mM DTT) containing 50 µM caspase-3 substrate, Ac-DEVD- AFC (AFC-138; MP Biomedicals, Irvine, CA). Caspase-3 activity was quantified by determining the rate of cleavage of the fluorescent substrate using a SpectraMax fluorimeter (400-nm excitation, 505-nm emission).

TNFR1 and TNFR2 gene silencing. Retroviruses encoding small-interference (si)RNAs were used to knockdown expression of TNFR1, TNFR2, and luciferase (control) in PD07i cells. The pSM2 retroviral vector (clone V2HS_95009) encoding short hairpin RNA (shRNA) to TNFR2 was obtained commercially (OpenBiosystems, Huntsville, AL). For targeted silencing of TNFR1, complimentary oligonucleotides specific for TNFR1 were synthesized (Oligo A: 5′-TGACGACAGGCCCCCTACCATGGGGAGCAAGCTTGGTTCTTCTAATGGTATTGCCCTTCTCATCATTTTT-3′; Oligo B: 5′-GATCAAAAATAGTAAGCAAGACGCCCTACATTAGAAGACCAAAGCCTGCTCCTCAATTGGGTACCG-3′), annealed, and ligated into the pSHAG vector, and recombined with pMSCV- puro vector using LR clonase (Invitrogen, Carlsbad, CA). The luciferase control plasmid contained the pSHAG vector encoding the shRNA firefly luciferase gene. Retroviral expression vectors were transfected into BOSC 23 cells using Lipofectamine 2000 (Invitrogen), supernatants were collected and used to infect PA 317 cells, and final supernatants containing amphotrophic virus were used to infect PD07i cells followed by selection in 1 µg/ml puromycin to create stable cell lines. Silencing of TNFR1, TNFR2, and luciferase gene expression was confirmed by Western blotting. The resulting stable cell lines that targeted the silencing of TNFR1, TNFR2, and luciferase expression were designated as PD07iTNFR1, PD07iTNFR2, and PD07iLuc, respectively.

IL-8 ELISA. PD07i cells were stimulated with two doses (1 and 2 µg/ml) of TNFR1 and TNFR2 agonist antibodies (R&D Systems, Minneapolis, MN) for 24 h at 37°C and PD07iLuc, PD07iTNFR1, and PD07iTNFR2 cell lines stimulated with 0.1, 1, 10, and 200 ng/ml TNF for 24 h at 37°C. Cell culture supernatants were collected and concentration of IL-8 was determined by the CXC13/IL-8 DuoSet ELISA kit (R&D Systems) relative to an IL-8 dilution series.
**Bladder TER measurements.** Bladders were harvested, rinsed with PBS, and carefully filleted open with the lumen facing upward. The tissue was carefully secured in an Ussing chamber (model CHM8; WPI, Sarasota, FL) and filled with 0.1 M KCl. Using electrodes cast in 2% agarose-0.1 M KCl solution, readings were performed using the EVMX epithelial voltmeter (WPI). Instrument baseline was subtracted from tissue readings. The TER was determined based on the average of the initial resistance and the resistance at 30 min. TER values were determined as the product of the average TER and the tissue area (0.126 cm²) and reported as Ω·cm².

**Acidified toluidine blue staining.** Tissue sections were deparafinized, rehydrated, and stained with acidified toluidine blue to visualize mast cells. Briefly, sections were immersed in 0.5% potassium permanganate. Following distilled water rinse, sections were immersed in 2% potassium metabisulfite, incubated in tap water, and washed with distilled water. Finally, sections were immersed in acidified toluidine blue solution (0.02% toluidine blue in 0.25% glacial acetic acid, pH 3.2). Sections were washed with distilled water and coverslipped. To determine mast cell numbers, the bladder cross-sections were divided visually into four layers: urothelium, lamina propria, proximal half of the detrusor muscle relative to the bladder lumen, and the distal half of the detrusor muscle relative to the bladder lumen; and mast cells were quantified from two nonadjacent sections (9). All values were reported as the mean of sections for each animal.

**Statistical analysis.** The results are expressed as means ± SD from three or more independent samples. When data from more than two groups were compared, the results were analyzed for statistical significance by one-way ANOVA followed by a posttest comparison using either Bonferroni’s or Tukey’s multiple comparison test. A value of P < 0.05 was considered statistically significant.

**RESULTS**

**Neurogenic cystitis induces urothelial lesions.** Employing a murine model of PRV-induced neurogenic cystitis (10), we examined the bladders of PRV-infected mice for evidence of apoptosis using TUNEL staining. At post-infection day (PID) 5, TUNEL labeling was not observed in bladders of sham-treated mice (Fig. 1A). However, in PRV-infected mice apoptotic cells were evident and were found in foci, reminiscent of punctate lesions in IC bladders (Fig. 1B). To determine whether the urothelial apoptosis induced by PRV resulted in the formation of urothelial lesions, bladder sections were also stained for UP3, a protein expressed at high levels in superficial urothelial cells and a marker of urothelial integrity (54, 55). Bladder sections from sham-treated mice displayed continuous immunostaining of UP3 consistent with intact urothelium (Fig. 1C), whereas bladders of PRV-infected mice exhibited disrupted UP3 staining that coincided with TUNEL staining (Fig. 1D). It is possible that the absence of UP3 staining observed in Fig. 1D resulted from decreased protein expression. However, we also observed apoptotic cells which were immunoreactive for UP3 and which were still attached to the urothelium (arrowheads) or in the process of exfoliation (Fig. 1E; arrow). The observation of TUNEL-positive superficial cells that display normal UP3 expression suggests that areas devoid of UP3 staining represented a loss of superficial cells and thus identifying a urothelial lesion. Taken together, these data suggest that urothelial apoptosis leads to the formation of lesions in PRV-induced neurogenic cystitis.

**TNF mediates urothelial lesions.** To test whether TNF plays a role in urothelial apoptosis, we infected a panel of mouse strains with PRV to determine the requirements for formation of urothelial lesions (Table 1). A majority of PRV-infected wild-type mice displayed urothelial lesions (11/21). In contrast, most of PRV-infected TNFR1/2−/− (0/5) and TNF−/− mice (1/8) were devoid of apoptotic foci, indicating a requirement for TNF in lesion formation. To determine a potential source for TNF that mediates urothelial lesions, W/W^v mice were also infected with PRV, and no lesions were observed (0/5). Thus these data suggest that mast cells and TNF signaling contribute to urothelial apoptosis during neurogenic cystitis.

**Urothelial cells undergo apoptosis in response to TNF treatment.** To determine the receptor(s) mediating urothelial responsiveness to TNF, we characterized a panel of urothelial cell lines for TNF receptor expression by immunoblotting (Fig. 2A). TNFR1 expression was detected in murine and human urothelium and in cell lines established from human bladder (PD07i, PD08i) and ureteral urothelium (TEU-1 and TEU-2). The finding of TNFR expression in TEU-2 cultures is consistent with our previous observation that TEU-2 inflammatory responses to mast cell supernatants were mediated by TNF (3). Although bladder and ureteral urothelial cells are morphologically and physiologically similar (12, 32) and express both TNFR1 and TNFR2, they are derived from distinct embryologic compartments. Therefore, we focused subsequent experiments on characterizing the urothelial line PD07i because bladder cell lines are likely more relevant for identifying mechanisms of bladder cell apoptosis, despite expressing lower levels of TNFR proteins (Fig. 2A).

We next examined whether TNF acted directly to elicit an urothelial apoptotic response by staining for annexin-V, a marker for early apoptosis (11, 16). PD07i cultures stimulated with TNF for 24 h exhibited annexin-V staining that was not detectable in untreated cultures (Fig. 2B). Caspase-8 is an initiator caspase immediately downstream of TNF receptor activation and which is cleaved to generate active caspase-8 (47). Accumulation of cleaved caspase-8 was detected as early as 4 h in PD07i cultures treated with TNF (Fig. 2C) and achieved a maximum of a 4.5-fold increase at 24 h (P < 0.05). Finally, we measured the activity of caspase-3, an executioner caspase (20). Caspase-3 activity was significantly induced in PD07i cultures treated with TNF for 24 h (4–4.5-fold, P < 0.05), approaching the level induced by staurosporine, a potent apoptotic inducer.

**TNF-induced urothelial apoptosis in culture is primarily mediated by TNFR1.** To characterize the relative contributions of TNFR1 and TNFR2 in urothelial responses, we stimulated PD07i cultures with agonist antibodies specific for TNFR1 and TNFR2 and measured IL-8 secretion by ELISA (Fig. 3A). TNF has been shown to induce IL-8 secretion via NF-kB activation, so we used IL-8 secretion as an indicator of urothelial responsiveness to TNF. IL-8 secretion was increased in a dose-dependent manner in cultures treated with TNFR1 agonist antibody (P < 0.001). Conversely, no dose-dependent increase in IL-8 release was seen in cells treated with TNFR2 agonist antibody (P > 0.05). These data suggest that TNFR1 is the major mediator of urothelial responses. To test this possibility, we utilized RNA interference (46, 56) to specifically knock-down TNFR expression. Cell lines were established that targeted TNFR1 expression, TNFR2 expression, and luciferase as a control (PD07isTNFR1, PD07isTNFR2, and PD07isLuc, respectively). PD07isLuc and PD07isTNFR2 cell lines were similarly responsive to TNF as determined by IL-8 ELISA.
However, PD07siTNFR1 cells were less responsive to TNF ($P < 0.05$ at 1 and 10 ng/ml). We next examined apoptosis of PD07siLuc, PD07siTNFR1, and PD07siTNFR2 cells in response to TNF stimulation (Fig. 3C). Annexin-V staining was detected in PD07siLuc cells stimulated with TNF for 24 h. In contrast, TNF-induced annexin-V staining was abrogated in PD07siTNFR1 cells. PD07siTNFR2 cells, however, remained sensitive to TNF-induced apoptosis. Therefore, these data suggest that TNFR1 is the major mediator of urothelial apoptosis.

TER decrease is TNF dependent. Because TNF-induced lesions may compromise the bladder permeability barrier, we assessed barrier function in bladders of PRV-infected mice by measuring transepithelial resistance (TER; Fig. 4). PRV infection induced a 61% decrease in bladder TER in mice compared with sham-treated controls ($P < 0.001$). In contrast, bladders of TNF$^{-/-}$ mice infected with PRV did not exhibit a significant decrease in TER relative to sham-infected WT mice ($P > 0.05$). These data suggest that PRV-induced neurogenic cystitis results in compromised bladder barrier function and that TNF

Table 1. Urothelial lesions require TNF and mast cells

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PRV, pseudorabies virus.
plays a role in the loss of the urothelial permeability barrier. These physiological data are also consistent with the microscopic data indicating a requirement for TNF signaling in the formation of urothelial lesions (Table 1).

**TNFR1 signaling mediates decreased bladder barrier function.** To further characterize the requirements for TNF-dependent loss of barrier function, we examined the effects of PRV on TER in wild-type, TNFR1−/−, TNFR2−/−, and TNFR1/2−/− mice (Fig. 5). PRV-infected R1/2−/− mice did not exhibit the same reduction in bladder TER observed in WT mice supporting the observations of stable TER in TNF−/− mice and demonstrating a requirement for signals mediated by TNF receptors (Fig. 4). TNFR2−/− bladders, however, exhibited the same decrease in TER seen in WT tissues, indicating full susceptibility to the effects of PRV and suggesting that TNFR2 does not play a major role in PRV-induced loss of bladder barrier function ($P > 0.05$). TNFR1−/− TER values were not significantly different from WT ($P > 0.05$). However, taken together with the in vitro findings (see Fig. 3), these data are consistent with a predominant role for TNFR1 in the loss of bladder barrier function during neurogenic cystitis, and TNFR2 mediates compensatory effects in the absence of TNFR1.

**Anti-TNF therapy abrogates mast cell trafficking and stabilizes barrier function.** Anti-TNF therapies have been successfully employed for the treatment of TNF-mediated human diseases as well as to mitigate the effects of TNF in animal models (33). We sought to determine the efficacy of anti-TNF therapy in ameliorating the effects of PRV-induced neurogenic cystitis. Previous studies revealed that mast cells trafficked from the detrusor toward the lamina propria during neurogenic cystitis (10), so we first examined the effect of anti-TNF antibodies on mast cell trafficking (Fig. 6A). Wild-type mice treated with isotype control antibody and PRV had a significant increase in lamina propria mast cells compared with sham-treated group ($P < 0.01$), consistent with previous observations. However, mast cells did not accumulate in the lamina propria of wild-type mice treated with anti-TNF antibody before PRV infection. Thus anti-TNF therapy blocks the trafficking of mast cells to the lamina propria during neurogenic cystitis. We next determined whether anti-TNF therapy stabilized urothelial barrier function during neurogenic cystitis (Fig. 6B). Bladders of mice that received injections of anti-TNF antibodies showed significantly enhanced TER relative to mice that received isotype control antibody ($P < 0.05$), whereas bladders of mice receiving isotype control antibodies showed a PRV-induced loss of TER similar to animals receiving no antibody. Thus anti-TNF therapy stabilizes the urothelial permeability barrier during neurogenic cystitis.

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**Fig. 2.** TNF induces urothelial apoptosis. A: Western blotting revealed protein expression of TNFR1 and TNFR2 in human urothelial cell lines established from pediatric bladder (PD07i, PD08i) and adult ureter (TEU-1, TEU-2) specimens. Both receptors were detected freshly isolated mouse (M) and human (H) urothelium. B: PD07i cells were incubated with medium alone (left) or medium containing 1,000 ng/ml TNF (right) for 24 h. Apoptosis was visualized by annexin-V staining and was only observed in TNF-treated cultures. Scale bar represents 20 μm. C: PD07i cells (4 × 10⁵) were incubated with 1 μg/ml TNF for 0, 4, 8, 24, 48, or 72 h. Total protein was prepared and analyzed for caspase-8 (C-8) cleavage by Western blotting. Densitometry of autoradiograms revealed that caspase-8 cleavage was greatest at 24 h (4.5-fold increase over control; *$P < 0.05$). All results were normalized to GAPDH expression. D: PD07i cells (7 × 10⁵) were incubated with 0.1 μM staurosporine (St) for 2 h or 10 or 100 ng/ml TNF for 24 h. Caspase-3 (C-3) activity was quantified by cleavage of the fluorogenic substrate Ac-DEVD-AFC, and results from 3 separate wells are reported as means ± SD. PD07i cells incubated with either 10 or 100 ng/ml TNF resulted in a 4-fold or greater increase in caspase-3 activity relative to controls (*$P < 0.05$). All results are representative of 3 independent experiments.
DISCUSSION

Parallels exist between IC and the chronic inflammatory conditions Crohn’s disease (CD) and psoriasis: these diseases are of unknown etiology, are remitting/relapsing, and result in epithelial lesions. TNF is central to the pathogenesis of psoriasis and CD (29, 35, 48, 52), and this observation has been exploited for highly successful therapies. For example, 82% of CD patients who received anti-TNF treatment (infliximab) had improvement in their signs and symptoms of disease, and 48% of these patients experienced complete remission (48). However, the potential role of TNF in the pathogenesis of IC has not been examined.

Fig. 3. TNF receptor 1 (TNFR1) mediates urothelial apoptosis. A: PD07i cells (4 x 10⁵) were incubated with either 1.5 ng/ml TNF, 1 or 2 μg/ml TNFR1 agonist antibody, or 1 or 2 μg/ml TNFR2 agonist antibody for 4 h, and IL-8 secretion was determined by ELISA. TNFR1, but not TNFR2, agonist antibody evoked dose-dependent IL-8 secretion from PD07i cells above baseline (*P < 0.001 and P > 0.05, respectively). B: PD07siLuc (■), PD07siTNFR1 (●), and PD07siTNFR2 (▲) stable cell lines were generated using target-specific small interfering RNAs to knockdown gene expression of luciferase (control), TNFR1, and TNFR2, respectively. Immunoblotting revealed that TNFR1 and TNFR2 protein expression was reduced in PD07siTNFR1 (siR1) and PD07siTNFR2 (siR2) cell lines, respectively (inset). siRNA cell lines were incubated with 0, 0.1, 1, 10, or 100 ng/ml TNF for 24 h, and IL-8 secretion was quantified by ELISA. PD07siTNFR1 cell line exhibited decreased IL-8 secretion in response to TNF (*P < 0.05 at 1 and 10 ng/ml). C: PD07siLuc, PD07siTNFR1, and PD07siTNFR2 cells were incubated with 0, 10, or 1,000 ng/ml TNF for 24 h and assessed for apoptosis by annexin-V staining. Annexin staining was not detected in PD07siTNFR1 cells treated with TNF. Scale bar represents 20 μm. A and B: results from 3 separate wells are reported as the means ± SD. Data shown are representative of 3 independent experiments.
The murine model of PRV-induced neurogenic cystitis, which shares similarities with IC including central nervous system involvement, mast cell trafficking/accumulation, and urothelial lesions, implicates TNF signaling in the disease process. Previously, PRV-induced neurogenic cystitis was shown to result in mast cell accumulation in the lamina propria due to mast cell trafficking from the proximal detrusor, but mast cell trafficking was not observed in TNFR1/2-deficient mice (10). In this study, we demonstrated that TNF mediated urothelial lesion formation and the loss of bladder permeability, principally through TNFR1. Administration of a monoclonal antibody to TNF to PRV-infected mice significantly abolished mast cell trafficking into the lamina propria and stabilized the bladder permeability barrier. Anti-TNF therapy has not yet been studied as a treatment for IC. Given the parallels between IC and other inflammatory diseases that are currently treated with anti-TNF therapy, and given the central role of TNF in our murine model of neurogenic cystitis, IC may be amenable to anti-TNF therapies.

The possibility of treating IC with anti-TNF therapies is also consistent with epidemiological and physiological similarities between IC and CD. Recent studies showed cross talk between the pathways that innervate and regulate reflexes in the bladder and gut, where noxious gut stimuli sensitize bladder afferents to mechanical stimuli in a rat model (38, 51). In humans, the incidence of CD is increased ~100-fold in the IC patient population relative to the general population (2), suggesting the possibility of common, underlying mechanisms. Indeed, clin-
TNI MEDIATES BLADDER LESIONS

The biological activities of TNF are mediated by two functionally distinct receptors, TNFRI (p55, CD120a) and TNFRII (p75, CD120b). The relative contributions of TNFRI and TNFRII to disease processes are not yet understood, although a picture is emerging for a dominant role for TNFRI. The data described here demonstrate a dominant role for receptor 1 in TNF-induced urothelial IL-8 secretion, an NF-κB-dependent process (3), and in urothelial apoptosis in vitro. The role of receptor 1 in neurogenic cystitis, however, is less clear. We observed that receptor 2 plays no role in the loss of bladder barrier function in the presence of receptor 1 but may serve a compensatory role in TNFR1−/− mice. Interestingly, TNFRI has also shown to be the major mediator of TNF signaling in CD and in the development of arthritic lesions (39, 57). The possibility of tissue-specific TNF receptor functional redundancy supports the use of therapeutics that target TNF signaling broadly, rather than the use of receptor-specific approaches.

Compromised bladder barrier function is thought to contribute to IC symptoms. We find that PRV results in both urothelial lesions and a loss of barrier function. Because anti-TNF antibodies stabilize barrier function in our neurogenic cystitis model, we speculate that anti-TNF therapy would relieve symptoms in IC patients by stabilizing the urothelial permeability barrier. This hypothesis is consistent with clinical findings using the “potassium sensitivity test” (PST), where concentrated potassium chloride solution is instilled into the bladder via a transurethral catheter. A PST evokes no response in normal volunteers, presumably due to an intact urothelial permeability barrier, but ∼70% of IC patients react strongly to a PST (36, 37). These data support the notion of defective barrier function in IC and are consistent with the correlation of microscopic urothelial lesions and increased permeability in FIC bladders (6, 7, 31).

Although TNF is required for urothelial lesion formation, it is possible that TNF acts via multiple mechanisms in this murine model of neurogenic cystitis. One mechanism is that TNF induces urothelial apoptosis directly, leading to the development of lesions. TNF also mediates mast cell trafficking toward the urothelium where mast cells are activated (10). This juxtaposition of activated mast cells and urothelium may result in a “proximity effect” whereby elevated local concentrations of TNF induce urothelial apoptosis and the formation of lesions. The mechanisms underlying bladder mast cell trafficking and which mediate this proximity effect are unknown. Mast cell homing to the gut and lung is mediated by the expression of mast cell chemokines in the target tissue and respective integrins on the mast cell surface (1, 18, 43). We showed that mast cells traffic toward the urothelium during neurogenic cystitis in a TNF-dependent process (10). We postulate that similar mechanisms underlie bladder mast cell trafficking during neurogenic cystitis. Preliminary results indicate that the urothelium expresses mast cell chemokine activity (Chen MC et al., unpublished observations). Future work will be necessary to elucidate the chemokine(s) that drives mast cell chemotaxis and determine whether inhibition of mast cell chemo-
kines abrogates neurogenic cystitis, thus providing a novel therapeutic modality.

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