

Upregulation of Macrophage Migration Inhibitory Factor (MIF) and CD74, Receptor for MIF, in Rat Bladder During Persistent Cyclophosphamide-Induced Inflammation

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The objective of this study was to determine if macrophage migration inhibitory factor (MIF) is upregulated in the bladder during persistent cystitis. MIF is a pro-inflammatory cytokine found pre-formed in the urothelium. Previous findings showed that acute bladder inflammation increased MIF release into the bladder lumen while upregulating MIF and CD74 (MIF receptor) in the bladder. Because the effects of persistent cystitis on MIF and CD74 are not known, MIF and CD74 changes in the bladder were examined after short-term (1-day) or persistent (8-day) cyclophosphamide (CYP)-induced bladder inflammation. Anesthetized male Sprague-Dawley rats received either a single CYP treatment (150 mg/kg, ip; saline, control) and examined 1 day after treatment (short-term), or repeated CYP doses (20–75 mg/kg, ip; saline, control; every third day for 8 days) and examined after 8 days of treatment (persistent). MIF protein levels in urine and bladder were determined. In addition, *Mif*, *CD74*, and *cox-2* expression in the bladder was determined. Histology verified cystitis and MIF and CD74 immunoreactivity in the bladder. Repeated CYP doses were decreased to avoid toxicity. Short-term or repeated low CYP doses (40 mg/kg; 8 days) increased urinary MIF and decreased bladder MIF amounts while upregulating bladder *Mif* and *CD74* mRNA expression. Persistent CYP-induced bladder inflammation (even at 40 mg/kg; 8-day treatment) also upregulated other inflammatory cytokines (CCL5, IL-11, iNOS) in the bladder. Short-term and persistent (low dose) CYP cystitis are associated with markedly increased MIF release into the urine and upregulation of *Mif* and *CD74* in bladder. This supports the hypothesis that MIF and CD74 play a

significant role in both acute and persistent stages of bladder inflammation. *Exp Biol Med* 233:620–626, 2008

Key words: bladder; inflammation; cytokines; cox-2

Introduction

Macrophage migration inhibitory factor (MIF), one of the first cytokines to be identified (1, 2), is recognized to play a pivotal role in inflammation (3). MIF was initially described as released by activated lymphocytes to stop the random migration of macrophages (1, 2), but it is also synthesized by a variety of cells outside the immune system, including the pituitary gland (4). MIF is now recognized as a pluripotent mediator with pro-inflammatory cytokine activities and hormonal properties that mediate cell growth and proliferation (5). MIF is unique since it stands upstream of other inflammatory mediators (e.g., TNF- α , IL-8), it is also constitutively expressed and thus important in regulating the inflammatory cascade, and finally, it directly regulates the immunosuppressive action of glucocorticoids (for recent reviews see (6, 7)).

The urothelium synthesizes, stores and releases MIF (8, 9). In addition, acute (4 hours or less) experimental cystitis resulted in: 1) release of MIF into the intraluminal fluid, 2) upregulation of bladder *Mif* (10, 11), and 3) increased CD74 (MIF receptor) and CD44 (signalling molecule for MIF-CD74 receptor complex) (12, 13) in the urothelium along with upregulation in the bladder (14). Blockade of released MIF with intravesical antibodies to MIF significantly decreased experimental inflammation (15). Thus experimental observations support the hypothesis that MIF plays a pro-inflammatory role in cystitis by binding to its receptor (CD74) in the urothelium.

However, since previous experimental cystitis models involved acute inflammation (1–4 hours) (10, 11, 14, 16), it was important to ascertain whether cystitis-associated changes in MIF were only present during the early part of

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inflammation or were also observed in persistent, established cystitis. Therefore, the effects of longer term inflammation on bladder MIF and CD74 levels using cyclophosphamide (CYP) as a model were investigated. CYP-induced cystitis (produced by the urotoxic effects of the cyclophosphamide metabolite, acrolein) in rodents is a well-characterized model of bladder inflammation lasting for several days (17–24).

Consequently, changes in urinary and bladder MIF levels were examined as well as *Mif*, *CD74*, and *cox-2* (as a confirmatory marker of inflammatory changes already reported by others (20) to be upregulated by CYP) mRNA in the bladder following either 1-day or 8-day CYP treatment. In addition, since the standard repeated CYP protocol had to be modified (using lower doses of CYP), inflammatory cytokine arrays were used to examine and document inflammatory changes in the bladder produced by 8-day CYP treatment. Present results show that persistent (8-day) CYP-induced cystitis (even at a reduced CYP dose) increased MIF release into the lumen and upregulated bladder *Mif* and *CD74*.

Materials and Methods

All experiments were approved by the Institutional Animal Care and Use committee at Bay Pines VA Medical Center. Male Sprague-Dawley rats (350–390 g; Harlan; Indianapolis, IN) were used in all experiments. The rats had free access to food (Teklad 2215, Harlan) and water throughout the experiments.

CYP-Induced Cystitis. One-Day CYP Treatment.

Rats were anesthetized with halothane and CYP (N = 5; 150 mg/kg; intraperitoneal [ip]) injected to induce acute inflammation (17, 18, 20, 21, 23, 24). Rats received buprenorphine (0.03 mg/kg; sc q. 12 hours; 2 doses total) at the time of the injection to prevent and alleviate distress. Control rats received saline (ip) and buprenorphine. Rats were placed in metabolic cages to collect urine (with general protease inhibitor cocktail added; Sigma, St. Louis, MO). At the end of the survival period, rats were reanesthetized with halothane, bladder was removed and rats were euthanized. Half of the bladder was placed in 4% paraformaldehyde for histology while the remainder was snap frozen at -80°C .

Eight-Day CYP Treatment. It was originally intended to use the commonly published protocol for persistent CYP-induced cystitis in female Wistar rats, i.e., 75 mg/kg every third day for ten days (18, 20, 21, 24). However, this protocol produced severe weight loss and animal death (See Results below), and therefore lower concentrations of CYP were tested and the exposure time was reduced to only 8 days. Under halothane anesthesia, rats received injections of saline (n = 5; ip), or CYP at concentrations of 75 (N = 6), 60 (N = 3), 40 (N = 3), and 20 (N = 3) mg/kg (ip) every third day. Rats received buprenorphine (0.03 mg/kg; sc; single dose) after every CYP (or saline) injection. Rats were placed in metabolic

cages to collect urine and reanesthetized (after 8 days of treatment) to remove the bladder as described above.

MIF Protein, *Mif*, *CD74*, and *Cox-2* mRNA Determination. Western blotting (denaturing, non-reducing conditions) and/or ELISA (Chemicon, Temecula, CA) determined MIF levels as described (25). Briefly, 15 μl of urine or 6 μg of bladder protein per rat were loaded into NuPAGE Bis-Tris gels (4–12%, Invitrogen; Carlsbad, CA). Separated proteins were transferred to a polyvinylidene fluoride membrane and MIF protein bands detected using biotinylated polyclonal antibody (1:1000, BAF289, R&D Systems, Minneapolis, MN), streptavidin-horseradish peroxidase conjugates and chemiluminescent substrate (Pierce, Rockford, IL). Band intensities were quantified using Kodak Image Station (Kodak, Rochester, NY) and normalized to creatinine (urine) or mg organ weight (bladder). ELISA data are expressed as ng MIF/mg creatinine (urine) or ng MIF/mg of protein (bladder).

Bladder total RNA was isolated using TriZol reagent (Invitrogen), with 1 μg of total RNA reverse transcribed and the resulting cDNA (2 μl) used for relative quantitative polymerase chain reaction (PCR) using conditions as described by the manufacturer (4:6 primer:competimer ratio, Ambion, Austin, TX). *Mif* and *cox-2* were amplified along with the 18S rRNA internal standard using high stringency conditions: 30 cycles of 94°C 1 min, 63°C 1 min, 72°C 1 min as described previously (10). PCR generated band intensities were calculated by dividing total gene of interest band intensity by 18S rRNA band intensity (internal standard), and fold change in expression was determined by dividing each treatment relative band intensity by mean control relative band intensity. Data represent the mean $\pm$ SEM of two separate PCR reactions per experimental animal. Reaction without reverse transcriptase served as a negative control.

Inflammatory Cytokine Targeted Array. Total RNA (1 μg) from saline (n = 3) and 40 mg/kg CYP-treated (n = 3) animals were separately pooled and biotinylated using TrueLabeling-AMP kit with the manufacturer's protocol (GA-030, SuperArray Bioscience; Fredrick, MD). The resulting biotinylated cRNA was used to probe inflammatory cytokine targeted arrays (ORN-011; SuperArray Bioscience). Briefly, the cRNA probes were denatured and hybridized to the array membranes at 60°C overnight with constant agitation. The array membrane was washed at 60°C for 15 minutes under low stringency conditions (2X SSC, 1% SDS) followed by a 15-minute high stringency wash (0.1 X SSC, 0.5% SDS). Hybridized biotinylated cRNA probes were detected using alkaline phosphatase streptavidin and a chemiluminescent substrate. The resulting image was analyzed using the GEarray analyzer program (SuperArray). Spot intensity was normalized by background subtraction and normalized to ribosomal protein RPL13A. Arrays were repeated three times; data represents mean fold variation of pooled samples. CYP-induced differences (fold change ≥ 2.0) in bladder gene

expression when compared with saline treated controls are reported.

Histology. Bladder paraffin sections (4 μ m) were stained with hematoxylin and eosin and in order to examine and document bladder inflammation with reduced CYP doses and exposure, the sections were evaluated by one of us (XW) while blind to the experimental treatment. The sections were scored for histopathologic changes as follows: (0) no edema or inflammation observed; (1) slight edema and inflammation (few polymorphonuclear leukocytes or lymphocyte infiltration); (2) moderate edema and inflammation (interstitial polymorphonuclear leukocytes, lymphocyte infiltration; vascular congestion); (3) severe edema and inflammation (numerous polymorphonuclear lymphocytes, lymphocyte infiltration; vascular congestion, fibroblastic proliferation; ulceration; partial absence of epithelium). Bladder sections were also immunostained for MIF and CD74. Briefly, sections were deparaffinized, subjected to antigen retrieval (citrate buffer, pH 6), and then exposed to rabbit anti-MIF (Torrey Pines; Houston, TX; 1:500) or rabbit anti-CD74 (Santa Cruz, CA; 1:500) overnight (4°C) followed by standard avidin-biotin peroxidase reaction (Vector; Burlingame, CA). Digital images were captured using a Nikon microscope and a Pixera (Los Gatos, CA) camera.

Data Analysis. Data were analyzed using Student's *t*-tests (1-day experiments) or analysis of variance (ANOVA; 8-day experiments), followed by post-hoc tests (Bonferroni adjusted *t*-tests; GraphPad Prism 4, San Diego, CA) if the initial ANOVA was significant ($P < 0.05$).

Results

CYP Effects on Animal Weight and Survival.

Hemorrhagic cystitis was evident in every 1-day CYP-treated (150 mg/kg) rat, and also every 8-day (75 mg/kg) and two out of three 8-day (60 mg/kg) CYP-treated rats. However, 20 and 40 mg/kg CYP did not cause hemorrhagic cystitis. None of the control animals showed hemorrhagic cystitis. In addition, repeated doses of 75 mg/kg CYP produced urinary incontinence, markedly decreased body weight ($64 \pm 0.83\%$ of original weight), and three out of six rats did not survive to day 8. A significant decrease in body weight was also observed after 60 mg/kg CYP ($84 \pm 2.8\%$ of original weight). No changes in body weight or continence were noted for 20 and 40 mg/kg CYP groups or for saline-treated rats. Because of profound (and unexpected) weight loss and incontinence at higher doses (affecting the viability of the animals and experimental results), only the 20 and 40 mg/kg CYP groups were included in the analysis of repeated CYP treatment.

CYP Increased Urinary MIF and Upregulated Bladder MIF. Using acute models (4 hours or less) of bladder inflammation, previous studies reported increased MIF release into the bladder lumen (11, 25). In the present study, either 1-day or 8-day CYP (20 or 40 mg/kg) produced significantly increased urinary MIF levels (Fig.

1) with greater effects seen in the 1-day group (22-fold increase at 1 day vs 5-fold increase at 8 days for CYP 40 mg/kg; Fig. 1B, C).

CYP (1-day or 8-day; 40 mg/kg) also caused a significant increase in bladder weight, suggesting edema (Fig. 2A). In addition, 1-day or 8-day (40 mg/kg) CYP significantly decreased bladder MIF levels (Fig. 2B) while upregulating *Mif* mRNA, as determined by RT-PCR (Fig. 2C). Changes in *cox-2* expression in the bladder after CYP treatment have been well-described in female Wistar rats (20). In the present study, bladder *cox-2* mRNA was also upregulated by 1-day CYP, while 20 and 40 mg/kg CYP for 8 days markedly upregulated (>10 -fold) *cox-2* in the bladder (Fig. 2D).

Histology showed that while the bladders from saline treated rats showed no signs of inflammation (Fig. 3A), 1-day CYP produced marked edema in the bladder (including the muscular layer), marked epithelial denudation and deposition of fluid and plasma components on the bladder lumen (Fig. 3B). MIF immunostaining was located in basal and intermediate layers of the urothelium in saline treated rats (Fig. 3F) as reported previously (8, 10, 11, 26), whereas only occasional basal immunostaining (due to epithelial denudation) was observed in CYP-treated rats (Fig. 3G). After 8-day treatment with saline, inflammation was not observed in the bladder (Fig. 3C; mean edema score = 0.2 ± 0.2), whereas after 8 days CYP (either 20 or 40 mg/kg), bladders showed submucosal edema and inflammation (Fig. 3D, E) with significantly greater inflammatory scores than control bladders (1.7 ± 0.3 , $P < 0.05$; 2.3 ± 0.7 , $P < 0.01$; respectively). Urothelial MIF immunostaining appeared normal in saline treated (8-day) rats (Fig. 3H) but was decreased in 20 and 40 mg/kg treated groups (Fig. 3I, J), corresponding to decreased MIF levels in bladder homogenates shown by ELISA (Fig. 3C).

Persistent (Low Dose; 40 mg/kg) CYP Cystitis Increased CD74 in Urothelium and Upregulated Bladder CD74. It was previously reported that one hour following substance P treatment, CD74 (receptor for MIF; Ref. 13) was upregulated in the bladder (14). The effects of longer term (8 days) inflammation on CD74 were examined in the bladder. In this study, the urothelium of 8-day treated rats showed a small increase in CD74 immunostaining (Fig. 4A–C) while upregulation of *CD74* mRNA was documented in bladder homogenates (Fig. 4D). Therefore, upregulation of CD74 during acute cystitis has been confirmed and extended to sustained (8 days) cystitis.

Persistent (Low Dose; 40 mg/kg) CYP Cystitis Increased Other Inflammatory Markers in the Bladder. Since the standard CYP protocol was modified by reducing the dose administered and the duration of treatment, changes in other inflammatory markers were examined to document bladder inflammation caused by the modified CYP protocol. Using a targeted array analysis (targeted rat inflammatory array containing sequences for 96 different rat inflammatory genes), 40 mg/kg CYP induced

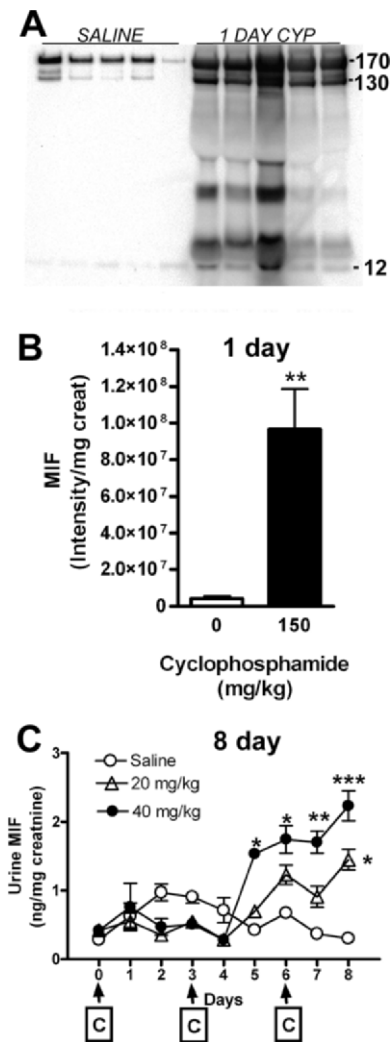


Figure 1. CYP increased urinary MIF levels. (A) Western-blotting analysis of urine from saline (N = 5) and 1-day (N = 5) CYP treated rats. Each lane is urine from a single animal. Urine from saline treated rats showed prominent high molecular weight MIF bands (170 and 130 kDa) corresponding to MIF- α 1-inhibitor-3 as documented by us recently (25, 26), in addition to a faint 12 kDa band corresponding to monomeric MIF. After 1-day CYP treatment, there is marked increase in the intensities of all the bands, particularly the 170 and 130 kDa MIF-bands. (B) Densitometric analysis of MIF Western blot shown in (A), where the intensity of MIF bands at 170, 130, and 12 kDa have been added together to describe changes in total amount of MIF in the urine. Intensity was normalized to creatinine in the urine. (C) MIF ELISA showed increased urinary MIF levels in 8-day CYP treated rats, with the 20 mg/kg group showing a significant increase only at day 8 of treatment, whereas the 40 mg/kg showed significant increases urinary MIF at day 5 and this was sustained through to day 8 of treatment. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

statistically significant upregulation in only the following genes: CCL5 (8.1-fold), IL-11 (9.6-fold), MIF (5.9-fold), and inducible nitric oxide synthase (iNOS) (3.1-fold).

Discussion

The deleterious effects of repeated CYP treatment on male Sprague-Dawley rats following the same protocol used for female Wistar rats (20, 21) were unexpected. Buprenor-

phine was administered on the day of CYP injection to relieve discomfort and, to our knowledge, represents a departure from published protocols. It is possible that buprenorphine may have impaired micturition and thus exacerbated acrolein's effects in the bladder. Although unable to rule out this possibility, it is considered unlikely and it does not account for profound weight loss and increased mortality. More likely, gender and strain differences in sensitivity to repeated CYP are involved. Female Sprague-Dawley rats showed less hemorrhagic cystitis than males treated with CYP, suggesting that gender confers a protective effect on the bladder (22). In addition, CYP produced more severe histological changes in the bladders of ovariectomized rats compared with intact rats, and this effect was lessened by estrogen treatment (27). Also, Rodo et al (22) reported significant weight loss in male and female Sprague-Dawley rats (8.1% vs 4.7%) after a single dose (200 mg/kg) of CYP. Finally, differences in CYP's ability to produce bladder inflammation were reported for different strains of mice (28), probably related to differential metabolism of cyclophosphamide into acrolein (29). Our results indicate that, at least for Sprague-Dawley male rats, the standard CYP doses using commonly in the literature (150 mg/kg for short-term; 75 mg/kg repeated every three days for persistent inflammation) are deleterious to the animals and may represent a model of severe hemorrhagic cystitis, but may not be a relevant model for persistent cystitis.

In the present study, reducing the CYP dose administered to male Sprague-Dawley rats produced persistent (8 days) bladder inflammation (increased bladder weight; histological signs of inflammation; upregulation of inflammatory markers) without overtly affecting the animals' health (as evidenced by lack of weight loss, gross hematuria, or incontinence in the 20 and 40 mg/kg CYP groups).

CYP treatment increased urinary levels of MIF and decreased bladder levels of MIF, in agreement with our earlier findings using substance P (11, 15). Whereas 1-day CYP treatment (with a large CYP dose) caused a marked increase in urinary MIF, repeated treatment with 20 or 40 mg/kg CYP produced increased urinary MIF levels at day 8 and 5 (respectively) of treatment, suggesting that the bladder inflammation produced by significantly reducing the dose of CYP elicited a slower inflammatory reaction. Bladder *Mif* was also upregulated after 1-day or 8-day CYP (40 mg/kg), corroborating earlier findings with acute models of cystitis (LPS or substance P) (10, 11, 15). Bladder *cox-2* was also upregulated after either 1-day or 8-day CYP (even at our lowest dose of 20 mg/kg), in agreement with previous reports on the effects of CYP (using 75 mg/kg in female Wistar rats) on the bladder (20). CD74 (receptor for MIF) urothelial immunostaining showed a slight increase after 8-day CYP, along with upregulation of CD74 mRNA consistent with previous results (14).

The present MIF findings differ from earlier findings using a different model of chemical cystitis (intravesical

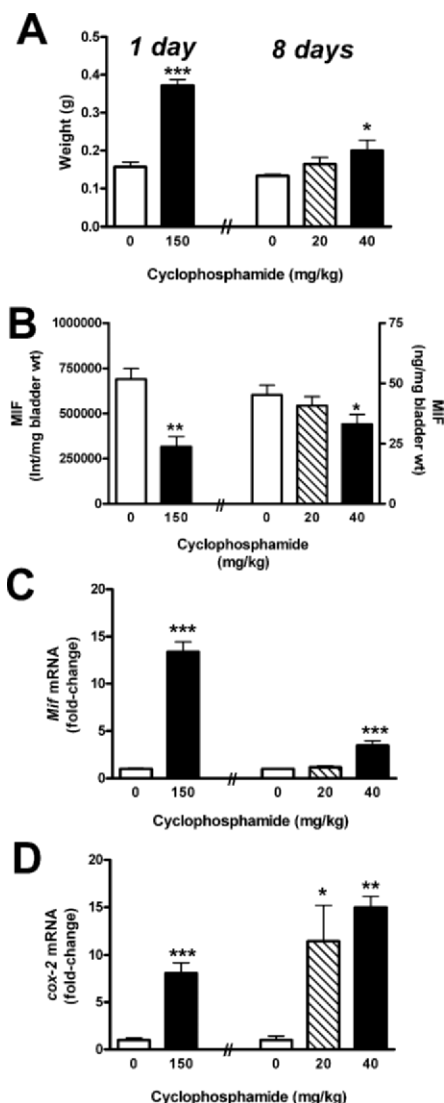


Figure 2. CYP induced changes in the bladder. (A) CYP treatment, either 1 day or 8 day (40 mg/kg) increased bladder weight (indication of edema). (B) Bladder MIF protein levels were decreased by 1 day or 8 day (40 mg/kg) CYP treatment (left y-axis presents MIF Western blot intensity/mg bladder while right y-axis shows ELISA results as ng MIF/mg bladder), consistent with release of MIF from the bladder. (C) Concomitantly, bladder MIF mRNA was upregulated by CYP treatment (1 or 8 day). (D) Cox-2 mRNA was also upregulated by all CYP treatments in the bladder. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

hydrochloric acid) (30). In that study, two hours after acid infusion, bladder MIF levels were decreased and no *Mif* upregulation was observed, contrary to effects seen in the present study using a different agent and much longer observation intervals (CYP, 24 hr and 8 days). Differences in methodology (e.g., extent of injury caused by acid infusion may be greater than by reduced repeated CYP treatment) and in observational time points may account for these differences and suggest that examination of changes at very acute times (hours), short-term (1 day), and more sustained (8 days) of inflammation is an important factor in interpreting cytokine changes (31).

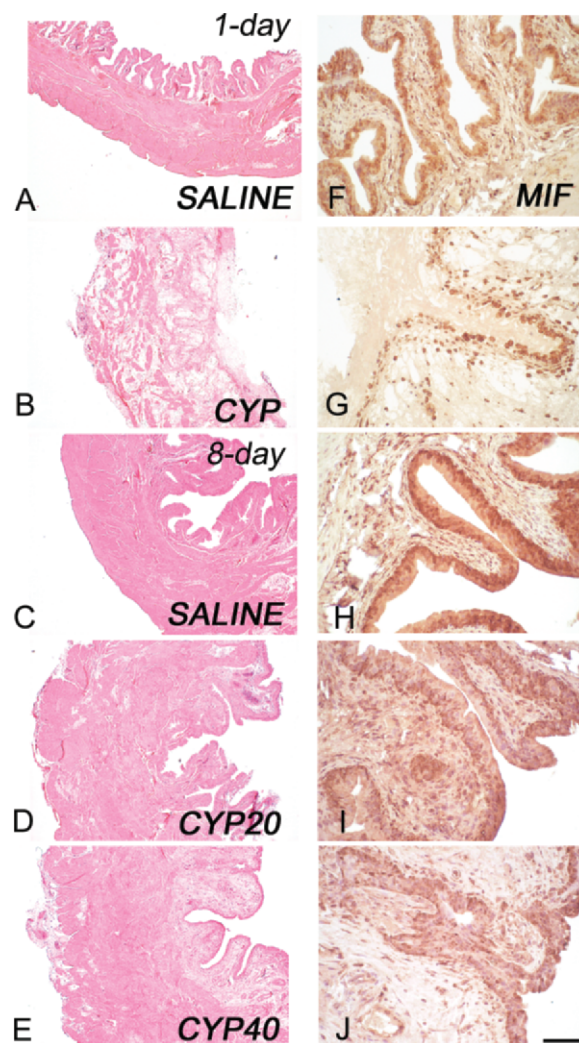


Figure 3. Morphological bladder changes caused by CYP treatment. Hematoxylin and eosin (HE) paraffin sections from representative bladders after 1-day saline (A) or CYP treatment (B). While saline treated bladders appeared normal with no inflammation (A), 1-day CYP treatment produced marked epithelial denudation (B) and widespread edema (including the muscle layer), and fibrinogen deposits along the luminal surface. MIF immunohistochemistry showed MIF located in the basal and intermediate cells of the urothelium in saline treated rats (F), whereas (because of marked denudation) only occasional basal cells were immunostained with MIF in 1 day CYP treated rats (G). After 8-day saline treatment HE sections showed normal morphology (C), whereas submucosal edema and inflammatory changes were evident after 8-day treatment with 20 or 40 mg/kg CYP (D, E). MIF immunohistochemistry showed normal appearance (basal and intermediate urothelial staining; H) in saline treated rats (D), but decreased intensity of immunostaining in the urothelium was observed after 20 (I) and 40 (J) mg/kg of CYP for 8 days. Calibration bar = 500 μ m in A–D; 100 μ m in F–J.

Upregulation of inflammatory cytokines in the bladder in response to CYP treatment has already been examined in female Wistar rats (21). Although the emphasis of the present study is on MIF and MIF's receptor (CD74) as upstream regulators of the inflammatory cascade, the contribution of other cytokines also needs to be examined. In the present study, inflammatory cytokine gene array data

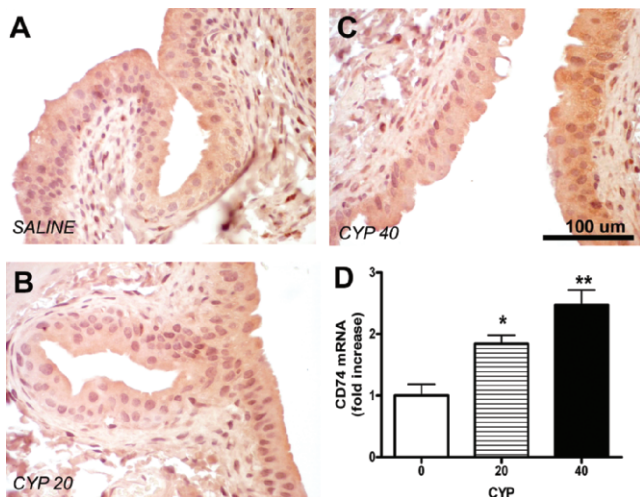


Figure 4. CYP induced increased CD74 immunostaining in the urothelium. (A) Urothelium of saline treated rats showed very slight CD74 immunoreactivity, whereas increased immunostaining was observed after 20 and 40 mg/kg CYP (B, C). Controls sections, where primary antibody had been omitted, showed no immunoreactivity (not shown). (D) RT-PCR of bladder homogenates showed upregulation of CD74 by 8-day CYP treatment.

confirmed that persistent inflammation produced by 8-day CYP treatment (40 mg/kg) upregulated expression of other inflammatory mediators, such as CCL5 (cysteine-cysteine chemokine ligand 5) (formerly known as RANTES [Regulated on Activation, Normal T Expressed and Secreted]), interleukin 11 (IL-11), iNOS, and MIF. To our knowledge, CCL5 and IL-11 upregulation in the bladder after CYP have not been reported previously and thus represent a novel finding. However, detrusor smooth muscle cells were shown to release RANTES in response to inflammatory cytokines (32), and RANTES was also recently reported to be involved in neurogenic cystitis (33). Thus, RANTES may be a central mediator of inflammatory processes in the bladder. CYP (150 mg/kg) was shown to upregulate iNOS in the bladder of Sprague-Dawley rats (34) after 48 hours. In this study, we confirm that persistent CYP induced cystitis (even at much lower doses; 40 mg/kg for 8 days) upregulates iNOS in the bladder.

Acute experimental cystitis (1–4 hr) has been previously documented to elicit MIF release into the intraluminal fluid (11, 25, 26). The present experiments show increased MIF release in persistent (8-day) cystitis. Similarly, increased urinary MIF levels in patients with bacterial cystitis has been recently reported (35). Therefore, taken together, these findings suggest that increased urinary MIF is associated with bladder inflammation. The working hypothesis is that intraluminal MIF may be exerting pro-inflammatory effects on the bladder following binding to urothelial CD74 (receptor for MIF; Ref. 13) that is upregulated either by acute cystitis (14) or shown in the present study to increase in the urothelium following repeated CYP treatment. Blocking released MIF with intraluminal antibodies prevents

binding to urothelial CD74 thus blocking MIF's pro-inflammatory cascade in the bladder and prevented or reduced acute inflammatory changes in the bladder (15), again suggesting that released MIF exerted pro-inflammatory effects in the bladder directly.

In conclusion, our findings showed that persistent CYP-induced cystitis caused MIF release (increased urinary MIF levels along with decreased bladder MIF levels) and upregulated *Mif* and *CD74* in the bladder, along with other cytokines. These findings support the hypothesis that MIF may exert pro-inflammatory effects on the bladder by acting on its receptor (CD74) that is upregulated during inflammation (short-term and persistent). Whether blockade of intraluminal MIF or intravesical CD74 will affect either the development of persistent cystitis or treat established cystitis remains under investigation.

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