Cyclophosphamide-Induced Cystitis Increases Bladder CXCR4 Expression and CXCR4-Macrophage Migration Inhibitory Factor Association

Pedro L. Vera1,2*, Kenneth A. Iczkowski3, Xihai Wang1,2, Katherine L. Meyer-Siegler1,4

1 Bay Pines VA Healthcare System, Research & Development (151), Bay Pines, Florida, United States of America, 2 Department of Surgery, College of Medicine, University of South Florida, Tampa, Florida, United States of America, 3 Prostate Cancer Research Laboratories, University of Colorado-Denver, Aurora, Colorado, United States of America, 4 Department of Molecular Medicine, College of Medicine, University of South Florida, Tampa, Florida, United States of America

Abstract

Background: Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine involved in cystitis and a non-cognate ligand of the chemokine receptor CXCR4 in vitro. We studied whether CXCR4-MIF associations occur in rat bladder and the effect of experimental cystitis.

Methods and Findings: Twenty male rats received saline or cyclophosphamide (40 mg/kg; i.p.; every 3rd day) to induce persistent cystitis. After eight days, urine was collected and bladders excised under anesthesia. Bladder CXCR4 and CXCR4-MIF co-localization were examined with immunohistochemistry. ELISA determined MIF and stromal derived factor-1 (SDF-1; cognate ligand for CXCR4) levels. Bladder CXCR4 expression (real-time RT-PCR) and protein levels (Western blotting) were examined. Co-immunoprecipitations studied MIF-CXCR4 associations. Urothelial basal and intermediate (but not superficial) cells in saline-treated rats contained CXCR4, co-localized with MIF. Cyclophosphamide treatment caused: 1) significant redistribution of CXCR4 immunostaining to all urothelial layers (especially apical surface of superficial cells) and increased bladder CXCR4 expression; 2) increased urine MIF with decreased bladder MIF; 3) increased bladder SDF-1; 4) increased CXCR4-MIF associations.

Conclusions: These data demonstrate CXCR4-MIF associations occur in vivo in rat bladder and increase in experimental cystitis. Thus, CXCR4 represents an alternative pathway for MIF-mediated signal transduction during bladder inflammation. In the bladder, MIF may compete with SDF-1 (cognate ligand) to activate signal transduction mediated by CXCR4.

Citation: Vera PL, Iczkowski KA, Wang X, Meyer-Siegler KL (2008) Cyclophosphamide-Induced Cystitis Increases Bladder CXCR4 Expression and CXCR4-Macrophage Migration Inhibitory Factor Association. PLoS ONE 3(12): e3898. doi:10.1371/journal.pone.0003898

Editor: Patricia Bozza, Instituto Oswaldo Cruz and FIOCRUZ, Brazil

Received August 11, 2008; Accepted November 15, 2008; Published December 10, 2008

This is an open-access article distributed under the terms of the Creative Commons Public Domain declaration which stipulates that, once placed in the public domain, this work may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose.

Funding: This material is based upon work supported (or supported in part) by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development (PLV; KLMS). This work was also supported by the National Institute of Diabetes and Digestive and Kidney Diseases DK075059 (PLV; KLMS; XW), and the Bay Pines Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: pvera@health.usf.edu

Introduction

Macrophage migration inhibitory factor (MIF) is an ubiquitous pleiotropic cytokine involved in cell proliferation and inflammation [1,2]. MIF plays an important and unique role in inflammation since MIF stands upstream of other pro-inflammatory mediators and it can counter-regulate the anti-inflammatory effects of glucocorticoids [2]. MIF is implicated in animal models of inflammatory diseases, including arthritis, glomerulonephritis, acute lung injury and sepsis [for recent review [3]].

Our recent experimental evidence indicates that MIF participates in bladder inflammation since: (1) MIF is constitutively expressed in the urothelium [4,5]; (2) bladder MIF expression is upregulated in different models of experimental cystitis in animals [6,7]; (3) MIF is released from the bladder during experimental cystitis [6,8,9] and urinary tract infections in humans [10] and finally, (4) neutralizing MIF with intravesical antibodies decreased experimental bladder inflammation [7]. Thus, based on our experimental observations, our hypothesis of a pro-inflammatory role for MIF during bladder inflammation agrees well with MIF’s pro-inflammatory role in several disease models (e.g. arthritis, Crohn’s disease) where treatment with neutralizing MIF antibodies results in decreased inflammation [11,12].

The mechanism for MIF’s action is not completely defined and remains an active area of investigation. MIF may exert autocrine effects through binding to intracellular JAB1 [13] and also paracrine effects by binding to cell-surface receptors [14]. Until recently, complex formation between MIF and cell-surface CD74 was the only described mechanism for MIF-receptor interaction [15]. CD74 is part of the major histocompatibility complex-II (MHC-II) complex; however, a small amount of CD74 can be found on the cell-surface not associated with MHC-II [16]. MIF binds to cell-surface CD74 [13] and the MIF-CD74 complex then activates signal transduction by binding to another cell-surface receptor, CD44 [14]. We showed that MIF, CD44 and CD74 are all upregulated in the urothelium after experimental inflammation in humans 7].
rats [6,17]. Therefore, all of the components are in place during bladder inflammation for MIF-activated signal transduction to occur.

Recently, however, a novel functional association between MIF and chemokine receptors CXCR2 and CXCR4 was described in T cells in vitro [18]. Chemokines are small proteins that direct leukocyte traffic to sites of inflammation or injury [19]. CXCR4 is a G-protein coupled receptor for stromal cell-derived factor-1 (SDF-1/CXCL12). Although chemokines typically display a high degree of receptor promiscuity, CXCR4 was (until recently) thought to bind only to SDF-1 [19]. MIF, however, competed with the recognized ligand for CXCR4 (SDF-1/CXCL12) for binding to CXCR4 [18].

CXCR4 is expressed by normal urothelium and may be associated with bladder cancer [20,21]. Therefore, we hypothesized that CXCR4-MIF complex formation may also occur in the bladder (as described occurring in vitro [18]). Such associations, if present, would indicate another possible receptor target for MIF during cystitis, aside from the already described MIF-CDF4 association [15].

The object of the present study was to determine if there was an association between MIF and CXCR4 receptors in the bladder. Therefore, we examined: 1) location of cytokine receptor CXCR4 in the rat bladder; 2) baseline bladder levels of SDF-1 (cognate ligand for CXCR4) and changes in response to a chemically-induced (cyclophosphamide; CYP) model of bladder inflammation; 3) CXCR4 expression changes after CYP-induced cystitis; and 4) association between CXCR4 and MIF in the bladder before and after CYP-induced cystitis.

Our results show that both CXCR4 and SDF-1 are constitutively expressed in normal rat bladder and upregulated during CYP-induced cystitis. Using dual immunohistochemistry we show that MIF and CXCR4 are colocalized within the same cells in the urothelium and co-immunoprecipitation studies demonstrate MIF-CXCR4 associations in the bladder. These MIF-CXCR4 associations are increased during CYP-induced cystitis.

Results

Cyclophosphamide-induced bladder inflammation

Repeated measures ANOVA showed differences between saline- and CYP-treated rats in body weight, with significant decreases observed in CYP-treated rats as early as day 3 and continuing throughout the experiment (day 8; Table 1) but remaining below a 10% weight-loss threshold established as a protocol endpoint.

In agreement with our previous findings in male Sprague-Dawley rats [6], multiple CYP injections (40 mg/kg every third day for 8 days, a lower dose than reported effective for female Wistar rats; 75 mg/kg [22]) induced bladder inflammation. Hemorrhagic cystitis, however, was not observed in any of the saline-treated rats.

Table 1. Effect of cyclophosphamide on body weight (g)

| Treatment | Day 0 | Day 3 | Day 6 | Day 8 |
|-----------|-------|-------|-------|-------|
| Saline (N = 10) | 321 ± 2.6 | 322 ± 2.8 | 326 ± 3.5 | 326 ± 3.6 |
| CYP (N = 10) | 320 ± 1.6 | 303 ± 2.5*** | 300 ± 3.0*** | 295 ± 3.0*** |

Mean ± S.E.M. Comparisons were made between Saline and CYP groups at each time point using post-hoc Bonferroni t-tests.*** = p < 0.001
doi:10.1371/journal.pone.0003898.t001

Cyclophosphamide increased bladder SDF-1 levels

We measured bladder levels of SDF-1, the cognate ligand for CXCR4, using ELISA. There was a significant difference between saline (4.4 ± 1.0) and CYP-treated bladders (7.6 ± 0.7 ng SDF-1/mg protein; p < 0.05) in the levels of SDF-1. Spleen and skin were assayed as positive controls and showed the amount of SDF-1 in spleen is comparable to that found in the bladder (7.9 ± 1.0 ng SDF-1/mg protein). Skin, on the other hand, had greater amounts of SDF-1 (16.04 ng SDF-1/mg protein) corresponding to approximately four and two times the amount of SDF-1 in saline and CYP-treated bladders, respectively.

CXC4R-MIF in Cystitis
Figure 1. Effect of cyclophosphamide (CYP) treatment on bladder histology, bladder and urine MIF levels. Bladder paraffin sections stained with H&E showed normal morphology, as represented in A,B. CYP treatment however, caused significant edema and chronic inflammation (C,D), and some bladders had acute inflammatory cells. Asterisks in C,D mark areas of edema and high numbers of cellular infiltrates. Urothelial hyperplasia was also observed. In addition, urinary MIF levels were increased by CYP treatment (E; * = p<0.05), while bladder MIF levels were increased by CYP treatment (F; ** = p<0.01). Calibration bar: 1A,1C = 500 μm; 1B,1D = 100 μm.

doi:10.1371/journal.pone.0003898.g001
SDF-1 immunofluorescence was readily seen SDF-1 in skin keratinocytes and in endothelial cells in skin blood vessels (Fig 4A) as has been reported before [23]. However, SDF-1 immunostaining was not detected in bladder (Fig. 4C) or spleen (not shown).

Cyclophosphamide upregulated bladder CXCR4 expression: MIF-CXCR4 associations in the bladder

Real-time PCR showed that CXCR4 mRNA was significantly upregulated in the bladder following CYP treatment (Figure 5A; ~9-fold increase) when compared to saline. Western-blotting, on the other hand, showed equivalent levels of CXCR4 protein in the bladders of saline treated versus CYP-treated rats (Figure 5B).

Co-immunoprecipitation studies with CXCR4 antisera to “pull-down” CXCR4 protein complexes in bladder homogenates were followed by MIF Western-blotting in order to detect CXCR4-MIF complexes in bladder homogenates. Figure 5C shows a representative experiment where MIF western-blotting of fractions from bladders of saline and CYP-treated rats were collected from the CXCR4 antibody column. “Flow through” refers to fractions that did not adhere to the CXCR4 antibody column (and thus do not contain CXCR4-complexes), whereas “Anti-CXCR4” refers to fractions eluted from the CXCR4 antibody column (thus containing CXCR4 complexes). Note that most of the bladder MIF did not stick to the CXCR4 antibody column (“Flow-through”) and is not associated with CXCR4. However, a small amount was found co-immunoprecipitated with CXCR4 in the saline-treated rats and these CXCR4-MIF complexes increased after CYP treatment. Densitometric analysis showed an increase of 3.5 fold in CXCR4-MIF complexes in CYP treated rats compared to saline controls.

Discussion

The results from the present study demonstrate that CXCR4, a chemokine cell-surface receptor, is constitutively expressed in normal rat urothelium localized to basal and intermediate cells. CYP treatment (aside from producing bladder inflammation and urothelial hyperplasia, well-described effects of cyclophosphamide in the bladder [24,25]) also resulted in up-regulation of bladder CXCR4 mRNA and redistribution of CXCR4 to the entire urothelial area (including apical area of superficial cells previously devoid of CXCR4; Fig. 2D). Our findings of apical CXCR4 staining in superficial urothelial cells are in agreement with observations in colonic epithelial cells [26].

CYP treatment although producing CXCR4 mRNA upregulation (a novel finding) did not result in increased CXCR4 protein levels, and scoring of CXCR4 immunostaining actually showed a decrease in staining intensity following CYP treatment. A similar discrepancy between CXCR4 mRNA expression and protein levels has been reported in the rat neurons and shown to reflect activation, increased internalization and degradation of CXCR4 receptors [27]. Such activation, internalization and degradation of CXCR4 receptors may also account for the patchy CXCR4 immunostaining in the urothelium (especially with immunostaining in the apical surface of superficial cells) and may represent focal areas of CYP-induced CXCR4 response.

CXCR4 mRNA expression in normal human urothelium, bladder cancer and also bladder cancer cell lines [J82 and T24] was previously reported [20]. Addition of SDF-1 (presumably activating CXCR4 receptors) increased Matrigel invasion and cell
growth but was not effective in increasing intracellular calcium in these particular urothelial cancer cells [20]. However, other investigators using a different bladder cell line (RT-4) did report an increase in intracellular calcium upon stimulation with SDF-1 [21]. Taken together these results suggest that CXCR4 receptors are functional in the urothelium. There is also evidence of CXCR4 mRNA expression in other areas of the human urogenital system (e.g. urethra, cervix) [28].

We examined protein levels of SDF-1 in the bladders of both saline-treated and CYP-treated rats using ELISA. We report constitutive levels of SDF-1 in saline-treated bladders which increase after CYP treatment. Although SDF-1 immunofluorescence was readily detectable in skin keratinocytes, we were unsuccessful in detecting SDF-1 by immunohistochemistry in the bladder (or the spleen). Since the levels of SDF-1 in the skin (measured by ELISA) are approximately twice the levels found in bladder or spleen, we consider it likely that the levels in these organs were below the detection level for immunohistochemistry.

Recently, down-regulation of SDF-1 mRNA expression in the bladder (and other pelvic viscera) was reported after vaginal distension [29]. To our knowledge, our findings represent the first demonstration of SDF-1 protein levels in the bladder. Thus our findings indicate that bladder injury produced by CYP-treatment results in mRNA upregulation of the chemokine receptor CXCR4 and increased protein levels of its cognate ligand, SDF-1. In addition to several cytokines reported upregulated in the bladder after CYP treatment [30], changes in another chemokine (CX3CL1) and its receptor (CX3CR1) were described as a result of cyclophosphamide treatment [22]. Therefore, chemokines (in addition to cytokines) likely represent important mediators of bladder injury and possible targets for ameliorating bladder inflammation.

Until recently, CXCR4 was considered to bind exclusively to SDF-1 [19]. However, recent in vitro evidence showed that CXCR4 is also capable of binding MIF [18]. In this study we confirm these in vitro finding since we demonstrate 1) co-localization of CXCR4 and MIF in the urothelium, both in saline treated rats and after CYP treatment; 2) CXCR4-MIF associations are present in saline-treated bladder and increase after CYP treatment. Therefore, although, not directly tested in this study, our results suggest that MIF in the bladder may participate in bladder inflammation either through binding to CXCR4 in the urothelium (formerly thought to only bind SDF-1 but recently shown to also bind MIF [18]) or to CD74 (recognized binding protein for MIF which is upregulated in bladder inflammation [6]) to activate signal transduction pathways that result in the production of other inflammatory cytokines. Given that bladder MIF concentrations are approximately 30-fold greater than bladder SDF-1 concentrations, it is possible that MIF may be the primary ligand at the CXCR4 receptor in the urothelium. Moreover, CYP-treatment induced immunostaining of CXCR4 in superficial urothelial cells previously devoid of CXCR4 (or MIF) immunostaining. This raises the possibility that these cells will be activated by MIF present in the urine and in fact, CYP (present study) and other inflammatory stimuli [8–10] increase luminal MIF release. We cannot rule out a contribution of renal or ureteral MIF release to increased urine MIF levels observed in this study after CYP-treatment, Yet our current findings of increased urine

**Figure 3. Co-localization of CXCR4 and MIF in urothelium.** Representative sections from rats treated with saline (A–C) or CYP (D–I) are shown. The figure shows MIF immunostaining (green immunofluorescence), CXCR4 immunostaining (red immunofluorescence) and an overlay panel combining both immunostaining and a DAPI nuclear stain. MIF immunostaining is seen in basal and intermediate cells and in fibroblasts in the lamina propria of saline treated rats (A), while superficial cells do not stain for MIF. Arrows show luminal edge of urothelium. CXCR4 is restricted to basal and intermediate cells of urothelium (B) and lamina propria is not stained. Overlay of these panels (C) demonstrate co-localization of MIF and CXCR4 as orange coloring of cells. CYP treatment resulted in superficial cell staining for MIF (D,G) and CXCR4 (E,H) and overlay panels (F,I) demonstrate co-localization as orange color in urothelial cells. Arrows point to superficial cells showing MIF-CXCR4 co-localization. Calibration bar = 50 µm.

doi:10.1371/journal.pone.0003898.g003
MIF with concomitant decrease in bladder MIF protein levels are consistent with earlier findings where, in animals with bladders isolated from the kidneys (thus removing potential renal and ureteral contributions), we observed similar results [7,9]). Thus, based on our experimental evidence we consider likely that MIF is released into the lumen from pre-formed stores in the bladder during inflammation. Therefore, luminal MIF may contribute to bladder inflammation through binding to at least these two urothelial cell-surface receptors and suggests that blocking MIF or cell-surface receptors associated with MIF may prevent or ameliorate bladder inflammation.

The exact role of CXCR4 and MIF-CXCR4 associations in the bladder was not addressed in the present study and remains to be investigated. However, recent evidence from other models suggests, at least, two interesting and important possibilities for the involvement of CXCR4 in bladder inflammation and repair from injury. First, CXCR4 may be mediating urothelial cell proliferation and repair. CXCR4 and SDF-1 are also expressed in human intestinal epithelial cells where recent evidence indicates it participates in epithelial repair following injury and maintaining mucosal barrier integrity [31,32]. In this model then, chemokines and chemokine receptors (and particularly CXCR4/SDF-1) may represent an autocrine/paracrine loop that helps maintain mucosal barrier integrity and repair as well as regulating mucosal pathogenesis (including chronic inflammation as seen inflamma-
tory bowel disease and progression to colon cancer) [33]. The results from the present study indicate that MIF, by associating with CXCR4 receptors in the bladder (presumably urothelial in origin), may be competing with SDF-1 at CXCR4 receptors to participate in epithelial repair following injury. Second, CXCR4 activation may mediate pain hypersensitivity in the bladder. Recent evidence has clearly shown expression of CXCR4 (and other chemokine receptors) on dorsal root ganglion (DRG) neurons [34]. In addition, activation of these receptors produced excitatory effects on DRG neurons and stimulated release of Substance P [34]. Also, CXCR4 (and other chemokine receptors and chemokines) expression was reported in DRG neurons following a rodent model of persistent neuropathy [35,36]. These receptors were functional since intracellular calcium was increased following administration of SDF-1 in vitro [35]. Therefore, based on their findings, these authors suggest that chemokines and chemokine receptors may be important targets in the treatment of chronic pain. In CYP-induced cystitis, then, activation of CXCR4 receptors, either by its recognized ligand SDF-1 (upregulated as a result of CYP) or due to interaction with MIF (also upregulated as a result of CYP and present in greater quantities than SDF-1) may in fact be contributing to pain hypersensitivity. A similar suggestion has already been made for another chemokine, CX3CL1, and its receptor during CYP-induced cystitis [22]. Our results contribute to such a hypothesis and expand it to a different chemokine/receptor system.

In summary, the chemokine receptor CXCR4 is constitutively expressed in rat urothelium (in basal and intermediate cells) while CYP-induced bladder inflammation resulted in upregulation of CXCR4 and immunostaining of superficial cells (previously devoid of CXCR4). Its cognate chemokine ligand, SDF-1 is also upregulated by CYP-treatment. CXCR4 and MIF are co-localized in cells of the urothelium and CYP induced co-localization of CXCR4 and MIF to superficial cells of the urothelium. Immunoprecipitation demonstrated an association between CXCR4 and MIF in the bladder. Therefore, our results suggest that CXCR4, as a receptor for MIF in urothelial cells, may contribute to MIF-mediated bladder inflammation. Examining the differences between MIF activation of CXCR4 receptors versus MIF activation of CD74 receptors then might provide insights into the actual mechanism for MIF-mediated effects in the bladder and possibly other sites.

Methods

All experiments were conducted after obtaining full institutional animal care and use committee approval and conformed to NIH Guide for animal experimentation. Bladder inflammation was produced using a recently published protocol whereby a reduced dose of cyclophosphamide (CYP) is administered to male Sprague-Dawley rats in order to avoid profound weight loss and mortality [6].

Cyclophosphamide-induced cystitis

Male Sprague-Dawley rats (N = 20; 250–300 g; Harlan, IN, USA) were anesthetized with halothane and received either saline (vehicle control group; 0.1 ml/100 g body weight; i.p.; N = 10) or cyclophosphamide (CYP; Sigma, St Louis, MO, USA; in saline; 40 mg/kg; 0.1 ml/100 g body weight; i.p.; N = 10) every third day (vehicle control group; 0.1 ml/100 g body weight; i.p.; N = 10) for 14 days. Eight days after the first injection, ten rats (5 = saline; 5 = CYP) were anesthetized with sodium pentobarbital (60 mg/kg; i.p.; Ovation Pharmaceuticals, Deerfield, IL, USA) and perfused with saline followed by 4% paraformaldehyde and the bladders collected for histology. Alternatively, rats were re-anesthetized with halothane (N = 10; 5 = saline; 5 = CYP-treated), bladders were exposed through an abdominal incision and urine collected (using a syringe with a 32 gauge needle). Bladders were excised and quickly frozen (−80°C) for protein or mRNA extraction and the rats were euthanized.

Histology and Immunohistochemistry

Formaldehyde-fixed bladders were cut coronally through the mid-detrusor region and embedded in paraffin. Paraffin sections (4 μm) were stained with hematoxylin and eosin or processed for CXCR4 immunohistochemistry as follows: Slides were deparaffinized and subjected to antigen retrieval using citrate buffer (pH 6.0; 95°C for 30 min). Endogenous peroxidase was blocked by incubating the slides in 3% H2O2 for 3 min. The section were exposed to CXCR4 antisera (1:2000; rabbit-polyclonal; Sigma; #C3116) overnight at 4°C and then processed using a standard ABC reaction according to the manufacturer’s protocol (HIC Select; Chemicon, Temecula, CA, USA). Sections were lightly counterstained with hematoxylin, coverslipped and examined using a Leica inverted microscope. Immunostaining intensity was rated by a pathologist (KAI) blind to experimental conditions from 0 (no staining) to 4 (strong immunostaining). In addition, digital images were analyzed for CXCR4 immunostaining intensity using Image J (NIH; Bethesda, MD) and proprietary custom written plug-ins (University of Colorado-Denver; Prostate Diagnostic Laboratories, Denver, Aurora).

Frozen (coronal) bladder sections (12 μm) of mid-detrusor were exposed simultaneously to both MIF (1:200; goat-polyclonal; Novus Biological, Littleton, CO, USA; #NB100-1789) and CXCR4 antisera (1:200; rabbit-polyclonal; Sigma; #C3116). The ability of this MIF antibody to recognize MIF was verified by preliminary western-bLOTS using rat recombinant MIF (gift from Torrey Pines) and rat tissue homogenates (data not shown). Primary antisera were visualized using appropriate secondary antisera conjugated to fluorescein isothiocyanate (FITC; Jackson Immunochemicals; West Grove, PA, USA; #705-095-147) or tetramethylrhodamine isothiocyanate (TRITC; Jackson Immunochemicals; #711-025-152). Sections were coverslipped with fade-retardant medium (Prolong Gold with DAPI; Invitrogen; Carlsbad, CA, USA) and examined using a Leica (Leica microsystems, Wetzlar, Germany) inverted microscope, equipped with a Leica digital camera. Overlay pictures showing MIF (green; FITC), CXCR4 (red; TRITC) and DAPI nuclear counterstaining were obtained with Leica software. Control sections included omission of either or both primary antisera, or omission of either or both secondary antisera.

For SDF-1 immunohistochemistry, frozen bladder sections were exposed to SDF-1 antisera (rabbit polyclonal; Torrey Pines Biolabs, Houston, TX, USA; TP201;1:200; overnight 4°C) and visualized with TRITC. Spleen and skin sections were used as positive controls.

MIF and SDF-1 ELISA

Urine and bladders from saline and CYP-treated rats were assayed for levels of MIF and SDF-1 (bladders only) using enzyme-linked immunosorbent assay (ELISA). MIF ELISA was also tested for its ability to detect serum MIF. Briefly, high binding ELISA plates (Microlite 2, ThermoScientific, Waltham, MA, USA) were coated with 100 μl of primary antibody (1 μg/ml; anti-MIF; Abcam; Cambridge, MA, USA; #ab7207 or anti-SDF-1; Torrey Pines Biolabs; TP201) at room temperature overnight. The ability of this MIF antibody to recognize MIF was verified by
preliminary western-bLOTS using rat recombinant MIF (gift from Torrey Pines) and rat tissue homogenates (data not shown). Plates were blocked with 200 μl reagent diluent (1 % BSA in PBS pH 7.4) for 1 h at room temperature. Recombinant rat MIF (gift from Torrey Pines) or recombinant SDF-1 (Preprotech Inc, Rocky Hill, NJ, USA) was used to generate a standard curve from 1.6 to 100 ng/ml in reagent diluent. Samples were diluted to the appropriate concentration in reagent diluent and applied in duplicate wells. Plates were covered with adhesive tape and incubated 2 h at room temperature. Individual wells were then washed three times with wash buffer (PBS containing 0.05 % Tween-20, pH 7.4) using an automated plate washer (Thermo-Scientific). Detection antibody (biotinylated-goat anti-MIF, BAF289, R&D Systems, Minneapolis, MN, USA) or biotinylated goat anti-SDF-1, R&D Systems) was added at a final concentration of 200 ng/ml, the plates recovered with adhesive and incubated 2 h at room temperature. The wells were washed as described above, 100 μl streptavidin-horseradish peroxidase (1:200 dilution in reagent diluent, DY998, R&D Systems) added to each well and the covered plate was incubated for 20 minutes at room temperature. The wells were washed as described above, 100 μl peroxidase substrate (R&D; DY999) was added and each well read using a plate reader (Biotek, Winooski, VT, USA). A standard curve was created using linear regression and sample concentrations calculated by interpolation (PRISM 4.02, GraphPad Software, La Jolla, CA, USA). Assays had an inter- and intraplate coefficient of variation of 15.3 % and 18 %, respectively for MIF and 7.2 % (both for inter- and intraplate) for SDF-1. Bladder MIF and SDF-1 levels are expressed normalized to protein (BCA, Thermo Scientific) present in the samples. Urine creatinine was determined using a commercially available assay (Exocell, Philadelphia, PA, USA) and performed according to the manufacturer’s protocol. Urine MIF levels are normalized to urine creatinine levels in the samples.

Bladder CXCR4 mRNA expression, Western blotting and CXCR4-MIF co-immunoprecipitation

Total RNA was isolated from bladder tissues using Trizol (Invitrogen) and CXCR4 (SuperArray primer; PPR06440A; SABIosciences, Frederick, MD, USA) and 18S rRNA (control; SuperArray: PPR57734E) gene expression was determined by Real-time RT-PCR (Opticon, Bio-Rad, Hercula, CA, USA) using SYBR Green incorporation and the ΔΔCT method of analysis. Differences in bladder CXCR4 mRNA expression between saline-treated and cyclophosphamide-treated rats were determined using a Student’s t-test. p<0.05 was considered significant.

Western-blotting of bladder homogenates was performed under non-reducing conditions following the manufacturer’s protocols (NuPAGE Bis-Tris gels, Invitrogen) as described previously [9]. Briefly, 20 μl of bladder homogenates were loaded onto NuPAGE Bis-Tris gels (4–12 %; Invitrogen). After electrophoresis, separated proteins were transferred to a polyvinylidene fluoride membrane. CXCR4 protein bands were detected using a polyclonal antibody to CXCR4 (Sigma; C3116) and chemiluminescent substrate (Sigma 1:1000). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Band intensities were quantified using Kodak Image Station (Kodak, Rochester, NY, USA) and expressed as a ratio of the saline group.

In order to detect MIF binding to CXCR4 in the bladder, we used CXCR4 co-immunoprecipitation followed by MIF western blotting. CXCR4 was precipitated from frozen bladder tissue homogenates (200 μg of total protein) using CXCR4 antibody (5 μg; Sigma; #C3116). CXCR4 containing protein complexes were isolated using Protein G agarose beads and then separated by denaturing, reducing SDS electrophoresis. MIF containing bands were identified by Western blotting using biotinylated anti-MIF antibody (1:1000 dilution, R&D Systems).

Statistical Analysis

Generally, data are presented as Mean±S.E.M and group differences were determined with t-tests. Visual scoring of CXCR4 immunostaining is presented as Median±Interquartile range and differences between the two groups were assessed using a Wilcoxon rank sum test, whereas digitized CXCR4 immunostaining are presented as Mean±S.E.M of arbitrary intensity scores and differences are analyzed using t-tests. Body weight loss as a function of cyclophosphamide treatment was tested using two-way (Treatment x Time) repeated-measures analysis of variance (ANOVA). Bonferroni post-hoc t-tests examined body weight differences between saline and cyclophosphamide groups at specific time points. Analyses were conducted using statistical software (R; http://www.r-project.org/).

Acknowledgments

Gary A. Smith Jr., provided excellent technical assistance.

Author Contributions

Conceived and designed the experiments: PLV KLMS. Performed the experiments: PLV XW KLMS. Analyzed the data: PLV KAI XW KLMS. Contributed reagents/materials/analysis tools: PLV KLMS. Wrote the paper: PLV KAI XW KLMS.

References

1. Bucala R, Donnelly SC (2007) Macrophage migration inhibitory factor: a probable link between inflammation and cancer. Immunity 26: 201–205.
2. Flaster H, Bernhagen J, Calandra T, Bucala R (2007) The macrophage migration inhibitory factor-glucoctocytin dyad: regulation of inflammation and immunity. Mol Endocrinol 21: 1287–1298.
3. Javed A, Zhao Y, Zhao Y (2008) Macrophage-migration inhibitory factor: role in inflammatory diseases and graft rejection. Inflamm Res 57: 45–50.
4. Meyer-Siegler KL, Ordoñez RC, Vera PL (2004) Macrophage migration inhibitory factor is upregulated in an endotoxin-induced model of bladder inflammation in rats. J Interferon Cytokine Res 24: 55–63.
5. Vera PL, Meyer-Siegler KL (2003) Anatomical location of macrophage migration inhibitory factor in urogenital tissues, peripheral ganglia and hemorrhoidal spinal cord of the rat. BMC Neuroscience 4: 17.
6. Vera PL, Wang X, Meyer-Siegler KL (2008) Upregulation of Macrophage Migration Inhibitory Factor (MIF) and CD74, receptor for MIF, in rat bladder during persistent cyclophosphamide-induced inflammation. Exp Biol Med (Maywood) 233: 620–628.
7. Meyer-Siegler KL, Vera PL (2004) Intraluminal antibodies to macrophage migration inhibitory factor decrease substance P induced inflammatory changes in the rat bladder and prostate. J Urol 172: 1504–1509.
8. Vera PL, Iczkowski KA, Leng L, Bucala R, Meyer-Siegler KL (2005) Macrophage migration inhibitory factor is released as a complex with alpha1-inhibitor-3 in the intraluminal fluid during bladder inflammation in the rat. J Urol 174: 338–343.
9. Meyer-Siegler KL, Vera PL (2004) Substance P induced release of macrophage migration inhibitory factor from rat bladder epithelium. J Urol 171: 1698–1703.
10. Meyer-Siegler KL, Iczkowski KA, Vera PL (2006) Macrophage migration inhibitory factor is increased in the urine of patients with urinary tract infection: macrophage migration inhibitory factor-protein complexes in human urine. J Urol 175: 1523–1528.
11. Ohkawara T, Nishihira J, Takeda H, Hige S, Kato M, et al. (2002) Amelioration of dextran sulfate sodium-induced colitis by anti-macrophage migration inhibitory factor antibody in mice. Gastroenterology 123: 256–270.
12. Mikolajowska A, Metz CN, Bucala R, Holmdahl R (1997) Macrophage migration inhibitory factor is involved in the pathogenesis of collagen type II-induced arthritis in mice. J Immunol 158: 5514–5517.
13. Luo H, Thiele M, Franz J, Dahl E, Speckens S, et al. (2007) Macrophage migration inhibitory factor (MIF) promotes cell survival by activation of the Akt pathway and role for CSN5/JAB1 in the control of autocrine MIF activity. Oncogene 26: 5046–5055.
14. Leng L, Bucala R (2006) Insight into the biology of macrophage migration inhibitory factor (MIF) revealed by the cloning of its cell surface receptor. Cell Research 16: 162–168.

15. Leng L, Metz CN, Fang Y, Xu J, Donnelly S, et al. (2003) MIF signal transduction initiated by binding to CD74. J Exp Med 197: 1467–1476.

16. Starles D, Gore Y, Binsky I, Haran M, Harpaz N, et al. (2006) Cell-surface CD74 initiates a signaling cascade leading to cell proliferation and survival. Blood 107: 4807–4816.

17. Meyer-Siegler KL, Vera PL (2005) Substance P induced changes in CD74 and CD44 in the rat bladder. J Urol 173: 615–620.

18. Bernhagen J, Krohn R, Lüe H, Gregory JL, Zernecke A, et al. (2007) MIF signal transduction initiated by binding to CD74. J Exp Med 197: 1467–1476.

19. Starles D, Gore Y, Binsky I, Haran M, Harpaz N, et al. (2006) Cell-surface CD74 initiates a signaling cascade leading to cell proliferation and survival. Blood 107: 4807–4816.

20. Bernhagen J, Krohn R, Lüe H, Gregory JL, Zernecke A, et al. (2006) Cell-surface CD74 initiates a signaling cascade leading to cell proliferation and survival. Blood 107: 4807–4816.

21. Murdoch C, Monk PN, Finn A (1999) Functional expression of chemokine receptor CXCR4 on human epithelial cells. Immunology 98: 36–41.

22. Yuridullah R, Corrow KA, Malley SE, Vizzard MA (2006) Expression of fractalkine and fractalkine receptor in urinary bladder after cyclophosphamide (CYP)-induced cystitis. Auton Neurosci 126–127: 380–389.

23. Fedoruk ER, Jones D, Critchley HO, Phillips RP, Bleden TM, et al. (2001) Expression of stromal-derived factor-1 is decreased by IL-1 and TNF and in dermal wound healing. J Immunol 166: 5519–5524.

24. Moss LG (1967) A light and electron microscopic study of the effects of a single dose of cyclophosphamide on various organs in the rat. II. The urinary bladder. Lab Invest 14: 46–63.

25. Philips FS, Sternberg SS, Cronin AP, Virdal PM (1961) Cyclophosphamide and urinary bladder toxicity. Cancer Res 21: 1577–1589.

26. Dwinnell MB, Ogawa H, Barrett KE, Kagnoff MF (2004) SDF-1/CXCL12 regulates cAMP production and ion transport in intestinal epithelial cells via CXCR4. Am J Physiol Gastrointest Liver Physiol 286: G844–50.

27. Kolodziej A, Schulz S, Guyon A, Wu D, Pfeiffer M, et al. (2000) Tonic activation of CXCR chemokine receptor 4 in immature granule cells supports neurogenesis in the adult dentate gyrus. J Neurosci 20: 4807–4809.

28. McClure CP, Tighe PJ, Robins RA, Bansal D, Bowman CA, et al. (2005) HIV coreceptor and chemokine ligand gene expression in the male urethra and female cervix. AIDS 19: 1257–1263.

29. Woo LL, Hijaz A, Kwan M, Penn MS, Damaser MS, et al. (2007) Over expression of stem cell homing cytokines in urogenital organs following vaginal distention. J Urol 177: 1568–1572.

30. Malley SE, Vizzard MA (2002) Changes in urinary bladder cytokine mRNA and protein after cyclophosphamide-induced cystitis. Physiol Genomics 9: 5–13.

31. Moyer RA, Wendt MK, Johanesen PA, Turner JR, Dwinnell MB (2007) Rho activation regulates CXCL12 chemokine stimulated actin rearrangement and restitution in model intestinal epithelia. Lab Invest 87: 807–817.

32. Smith JM, Johanesen PA, Wendt MK, Binion DG, Dwinnell MB (2005) CXCL12 activation of CXCR4 regulates mucosal host defense through stimulation of epithelial cell migration and promotion of intestinal barrier integrity. Am J Physiol Gastrointest Liver Physiol 288: G316–26.

33. Zimmerman NP, Vongsa RA, Wendt MK, Dwinnell MB (2008) Chemokines and chemokine receptors in mucosal homeostasis at the intestinal epithelial barrier in inflammatory bowel disease. Inflamm Bowel Dis 14: 1000–1011.

34. Oh SB, Tran PB, Gillard SE, Hurley RW, Hammond DL, et al. (2001) Chemokines and glycoprotein120 produce pain hypersensitivity by directly exciting primary nociceptive neurons. J Neurosci 21: 5027–5035.

35. Bangs SK, Men D, Miller RJ, Henry KJ, Lieneswala J, et al. (2007) Delayed functional expression of neuronal chemokine receptors following focal nerve demyelination in the rat: a mechanism for the development of chronic sensitization of peripheral nociceptors. Mol Pain 3: 38.

36. Bangs SK, Men D, Miller RJ, Chen DM, Ripach MS, et al. (2007) CXCR4 chemokine receptor signaling mediates pain hypersensitivity in association with antiretroviral toxic neuropathy. Brain Behav Immun 21: 581–591.