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RESEARCH ARTICLE

Impaired cytokine expression, neutrophil infiltration and bacterial clearance in response to urinary tract infection in diabetic mice

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One sentence summary: This study shows that the cytokine-driven neutrophil response to bacterial infection of the bladder is deficient in a mouse model of type 1 diabetes mellitus compared with healthy mice.
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ABSTRACT

Diabetic patients have increased susceptibility to infections, and urinary tract infections (UTI) are the most common type in women with diabetes mellitus. Knowledge of bacterial clearance effectiveness following UTI in diabetics is sparse. In this study, the effects of diabetes on bacterial clearance efficiency and components of the innate immune system in response to UTI in a murine model were investigated. Streptozotocin-induced diabetic and control female C57BL/6J mice were infected with uropathogenic *Escherichia coli*, and bacterial load, expression of chemokines, and neutrophil infiltration in the bladder over time were investigated. Expression levels of histone deacetylases were also measured to address a potential mechanism underlying the phenotype. Bacterial clearance during UTI was significantly prolonged in diabetic mice relative to controls. Neutrophil infiltration in bladder tissue and urine, and both mRNA and protein expression of chemokines MIP-2, KC, MCP-1 and IL-6 in bladder tissue were diminished at early time points after infection in diabetic mice relative to controls. In addition, mRNA levels of histone deacetylases 1–5 were increased in diabetic mice. This is the first study to show an association of impaired bacterial clearance in diabetic mice with suppression of UTI-induced chemokine expression and neutrophil infiltration in the bladder.

Key words: type 1 fimbria; myeloperoxidase; quantitative real-time RT-PCR; ELISA

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INTRODUCTION

Urinary tract infections (UTI) by uropathogenic bacteria are the most common urological disorders, and lower UTI involving bladder infections (or cystitis) specifically affect millions of individuals each year, carrying an annual cost of more than 3.5 billion dollars in the US (Griebling 2005a,b). Surveys have suggested that women are about four times more susceptible to UTI than men (Griebling 2007). Individuals with diabetes mellitus (DM) have a higher prevalence of several types of infection, including asymptomatic bacteriuria, lower UTI and acute pyelonephritis compared with people without DM (Scholes *et al.*, 2005; Geerlings 2008). The documented increasing prevalence of DM in the US and throughout the world portends an increasing prevalence of lower UTI (Centers for Disease Control and Prevention 2012). The mechanistic basis of this association is still unclear; corollaries of DM that may be contributing factors include glucosuria, which can enhance growth of bacteria (Rosen, Hung and Kline 2008), alterations in the composition of the luminal urothelial surface that may enhance bacterial adherence and impaired innate immunity (Geerlings 2008).

Uropathogenic *E. coli* (UPEC) is the predominant uropathogen, responsible for approximately 80% of community-acquired UTIs (Ronald 2002). UPEC UTI is a complex process, and its fate in certain mouse strains is determined within the first 24 hours through dynamic host–pathogen interactions with various checkpoints and population bottlenecks (Hannan *et al.*, 2012). UPEC adhere to the urothelium by means of adhesin-tipped fimbriae (pili), among which the type 1 fimbria is the predominant adhesion appendage on almost all cystitis-causing UPEC strains (Hultgren *et al.*, 1985; Langermann *et al.*, 1997). The FimH adhesin on type 1 fimbriae has been shown to mediate invasion and colonization of urothelial cells *in vivo* in murine models (Connell *et al.*, 1996; Martinez *et al.*, 2000), and clinical studies have provided strong circumstantial evidence for a critical role for type 1 fimbria in human UTI (Hannan *et al.*, 2012). Host resistance to UTI is dependent on a strong innate immune response, initiated primarily by toll-like receptor 4 (TLR4) (Haraoka *et al.*, 1999; Schilling *et al.*, 2003), which can bind both lipopolysaccharide (LPS) and FimH on UPEC (Hoshino *et al.*, 1999; Mossman *et al.*, 2008). TLR4 signaling results in expulsion of UPEC via exocytosis (Song *et al.*, 2007a) and activation of NF-κ B-dependent and -independent signaling pathways that lead to the production of cytokines and recruitment of neutrophils to the bladder (Song *et al.*, 2007b). Although LPS-independent activation of TLR4 by FimH has been reported (Fischer *et al.*, 2006; Mossman *et al.*, 2008), both LPS and FimH may be required to yield the strongest cytokine and neutrophil response (Hedlund *et al.*, 2001; Schilling *et al.*, 2001).

Neutrophils and macrophages are the major phagocytic cells of the innate immune response against bacterial infections, and neutrophils are the major early responders recruited to the bladder during UTI (Haraoka *et al.*, 1999). TLR4-defective C3H/HeJ mice (Poltorak *et al.*, 1998) are deficient in both the neutrophil response to UPEC and in bacterial clearance compared with wild-type C3H/HeN mice, suggesting a role for neutrophils in clearance of UTI (Shahin *et al.*, 1987). Further suggestive evidence for the importance of neutrophils was shown by impaired UPEC clearance from the urinary tract of C3H/HeN mice after pretreatment with an antibody that depletes peripheral neutrophils, as well as subsets of other leukocytes (Haraoka *et al.*, 1999; Daley *et al.*, 2008).

Rosen *et al.* showed that UPEC infection of female C3H/HeN mice with streptozotocin (STZ)-induced type 1 DM yielded bladder titers 10 000 times higher than in non-diabetic C3H/HeN mice, whereas the difference in titers between UPEC-infected diabetic and non-diabetic C3H/HeJ mice was 100-fold, suggesting deficiencies in both TLR4-dependent and -independent responses to UPEC in diabetic mice (Rosen, Hung and Kline 2008). Numerous studies have shown functional defects in neutrophils from diabetic patients and animals (Alba-Loureiro *et al.*, 2007), including impairments in chemotaxis (Pereira, Sannomiya and Leme 1987; Mowat and Baum 1971), phagocytosis (Bagdade, Nielson and Bulger 1972; Panneerselvam and Govindasamy 2003), and bactericidal activity (Tan *et al.*, 1975; Yokoo, Kumamoto and Hirose 1994). However, while the initial recruitment of macrophages to the bladder after UPEC instillation has been reported to be higher in STZ-diabetic mice than in controls (Yokoo, Kumamoto and Hirose 1994), the neutrophil and related cytokine/chemokine responses to UTI in animal models of DM have not been studied.

Several cytokines with roles in neutrophil trafficking are expressed in response to UPEC in the bladder and in epithelial cell lines, and their expression can be induced by TLR4. These include interleukin (IL)-8 (CXCL8), two functional homologues of IL-8 that are also expressed in mice, namely macrophage inflammatory protein 2 (MIP-2, CXCL2) and keratinocyte chemoattractant (KC, CXCL1), as well as monocyte chemoattractant protein 1 (MCP-1, CCL2) and IL-6 (Boyd *et al.*, 2006; Song *et al.*, 2007b; Ao *et al.*, 2009). In humans, IL-8 is the major attractant for neutrophils, and in mice, MIP-2 enabled neutrophils to traverse epithelial cells in UPEC-infected kidneys (Hang *et al.*, 1999). Furthermore, both MIP-2 and KC have been shown to contribute to neutrophil infiltration of the kidney in a mouse model of renal inflammation (Roche *et al.*, 2007). MCP-1, while primarily a monocyte chemoattractant, has been shown to promote neutrophil infiltration into injury sites in murine models of corneal and lung infection (Xue *et al.*, 2007; Balamayooran *et al.*, 2011). Incubation of type 1-fimbriated UPEC with human kidney carcinoma cells caused secretion and mRNA expression of MCP-1, KC and IL-8, but not LPS-induced CXC chemokine (LIX, CXCL5), a third functional homologue of IL-8 (Godaly *et al.*, 2007). IL-6 is an important mediator of inflammation and has been shown to inhibit neutrophil apoptosis (Biffl *et al.*, 1996; Gabay 2006). It is the most prominent cytokine detected in urine in response to UTI in humans and animals (de Man *et al.*, 1989; Hedges *et al.*, 1991) and is secreted by urothelial cells in response to FimH and LPS in a TLR4-dependent manner (Hedlund *et al.*, 2001; Schilling *et al.*, 2001).

Diabetic complications such as retinopathy and associated changes in gene expression persist even after good glycemic control is achieved, suggesting involvement of epigenetic modifications such as alterations in acetylation of histones (Intine and Sarras 2012). Consistent with that hypothesis, STZ-diabetic rats were found to have increased levels of three histone deacetylases (HDACs) and a reduced level of acetylated histone 3 in the retina, which persisted after restoration of good glycemic control (Zhong and Kowluru 2010).

In the current study, we aimed to evaluate the efficiency of neutrophil infiltration into the bladder during acute UTI in diabetic mice, with respect to the expression profiles of regulatory chemokines and HDACs, the latter as a potentially targetable mechanism underlying altered expression of the chemokines.

MATERIALS AND METHODS

Propagation and characterization of type 1-fimbriated *E. coli*

Growth of type 1-fimbriated UPEC and characterization of the fimbriae were conducted as described with minor modifications (Martinez *et al.*, 2000). UPEC strain 53498 (American Type Culture Collection, Manassas, VA, USA) was freshly streaked from a frozen glycerol stock onto a Luria agar plate and grown at 37°C overnight. To stimulate expression of type 1 fimbriae, a single colony was inoculated into Luria-Bertani (LB) broth and incubated at 37°C overnight without shaking (static conditions), and then the bacterial suspension was subcultured at 1:1000 into 2 ml of fresh LB broth and incubated again at 37°C overnight without shaking. Bacteria were washed and concentrated to 1.6 $\times$ 10^9 cells ml^{-1} in 1 $\times$ PBS, and type 1 fimbria expression was confirmed by mannose-sensitive agglutination of a 2% solution of guinea pig erythrocytes. Bacteria were maintained on ice for a maximum of 2 h before installation into the bladder.

Type 1 DM induction in mice

C57BL/6J female mice (Jackson Laboratory, Bar Harbor, ME, USA) 8 weeks old received two intraperitoneal injections of high-dose STZ (150 mg kg^{-1} mouse) on consecutive days to induce DM, or 0.1 M sodium citrate vehicle, pH 4.5, as described (Rosen, Hung and Kline 2008). Blood glucose levels of mice were monitored weekly until UPEC inoculation 4 weeks after STZ or vehicle injection, and only STZ-injected mice that maintained glucose levels >300 mg dl^{-1} were used in experiments. Mice received food and water ad libitum. All protocols were pre-approved by the IACUC of Case Western Reserve University in compliance with the Public Health Service policy on humane care and use of laboratory animals.

Experimental UTI in mice and bacterial clearance assay

We followed the transurethral inoculation and clearance assay protocols of Mulvey, Schilling and Hultgren (2001) with some modifications. Twelve week-old female diabetic or control C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) were anesthetized with isoflurane. Prior to inoculation, the bladder was emptied by gentle abdominal massage, while collecting the urine for detection of pre-existing bacteriuria. Fifty microliters of UPEC suspension (1.6 $\times$ 10^9 CFU ml^{-1}) in 1 $\times$ PBS were injected into the lumen via transurethral catheterization, using a 24 Ga. $\times$ 0.75 in. shielded I.V. catheter (BD Angiocath™ Autoguard™, BD Medical Systems, Sandy, UT, USA). After 30 s, the catheter was removed. At the indicated times after UPEC inoculation, urine was collected aseptically by massaging the mouse abdomen over a sterile Eppendorf tube, and then mice were euthanized by cervical dislocation under anesthesia. Bladders were removed aseptically, weighed and homogenized in 1 ml of 0.025% Triton X-100 in 1 $\times$ PBS. Serial dilutions of urine samples or bladder homogenates were spread on LB agar plates and incubated at 37°C overnight. Bacteria numbers were counted and calculated as per ml of urine, or per whole bladder. Examination of urine prior to inoculation confirmed that none of the mice had pre-existing bacteriuria.

Neutrophil counts in urine

At different times after UPEC inoculation of mice, beginning 4 weeks after STZ or vehicle injection, urine was collected aseptically by massaging the mouse abdomen over a sterile Eppendorf tube. Urine was mixed 10:1 with Turk's stain (0.05 mg ml^{-1} crystal violet, 3% glacial acetic acid in distilled water), and neutrophils were counted with a Bürker chamber under a microscope. Examination of urine prior to inoculation of UPEC confirmed that none of the mice had pre-existing leukocyturia.

Neutrophil MPO assay

Urine was collected aseptically and bladders were harvested for ELISA of myeloperoxidase (MPO) at the times following UPEC inoculation indicated in Fig. 3 (beginning 4 weeks after injection of STZ or vehicle). Bladders were homogenized for 3 min on ice using PowerGen 125 homogenizer (Fisher Scientific, Waltham, MA, USA), and urine and bladder tissue neutrophils were quantified using a MPO ELISA kit from Hycult Biotech (Plymouth Meeting, PA) as described by Haraoka *et al.* (1999) and the manufacturer's protocol.

Quantitative real-time RT-PCR (qRT-PCR)

Bladders were harvested for RNA isolation and qRT-PCR 4 weeks after injection of STZ or vehicle (before UPEC inoculation), and in the case of the chemokines, at the times following UPEC inoculation indicated in Fig. 3. Total RNA was isolated using Trizol (Life Technologies, Grand Island, NY, USA), and first-strand complementary DNA was synthesized from 1 μg of total RNA using a high-capacity cDNA synthesis kit (Life Technologies). cDNA was amplified in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), using SYBR Green PCR master mix with primer pairs for chemokines, HDACs and β-actin (Life Technologies). The primer sequences (Table 1) were designed using the online Universal Probe Library Assay Design Center (Roche, Mannheim, Germany). Chemokine and HDAC gene expression levels were normalized to the corresponding β-actin gene expression level and calculated relative to the mean level in healthy control mice before UPEC inoculation (set to 1.0) by the comparative C_T method (Livak and Schmittgen 2001). The mean levels of β-actin mRNA did not differ significantly between the control and diabetic mice before or after inoculation.

Chemokine ELISA

Bladders were harvested for ELISA of chemokines at the times following UPEC inoculation indicated in Fig. 3 (beginning 4 weeks after injection of STZ or vehicle). Bladders were homogenized for 3 min on ice using PowerGen 125 homogenizer (Fisher Scientific), and levels of MIP-2, KC, MCP-1 and IL-6 (RayBiotech, Norcross, GA, USA) were quantified by ELISA as indicated in the manufacturer's protocols. Absorbances at 405 and 450 nm were measured using a FLUOstar OPTIMA Fluorescence plate reader (BMG LABTECH GmbH, Ortenberg, Hesse, Germany).

Statistical analysis

Data are expressed as means $\pm$ 95% confidence intervals (95% CI) or SEM. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). Urine (serial) measures were analyzed by calculating the area under the curve (AUC) for each mouse and comparing the AUC values in diabetic vs control groups by Student's *t* test. Comparisons of measures in bladder tissues were performed with the Student's *t* test for each time point, using the Holm–Sidak method to correct for

Table 1. Primer sequences used for qRT-PCR.

Gene	Forward primer	Reverse primer
MIP-2α	5′-AAAATCATCCAAAAGATACTGAACAA	5′-CTTTGGTTCTTCCGTTGAGG
KC	5′-AGACTCCAGCCACACTCCAA	5′-TGACAGCGCAGCTCATTG
MCP-1	5′-CATCCACGTGTTGGCTCA	5′-GATCATCTTGCTGGTGAATGAGT
IL-6	5′-GCTACCAAACTGGATATAATCAGGA	5′-CCAGGTAGCTATGGTACTCCAGAA
β-actin	5′-GGTCATCACTATTGGCAACG	5′-ACGGATGTCAACGTCACACT
HDAC1	5′-TGGTCTCTACCGAAAAATGGAG	5′-TCATCACTGTGGTACTTGGTCA
HDAC2	5′-CTCCACGGGTGGTTCAGT	5′-CCCAATTGACAGCCATATCA
HDAC3	5′-TTCAACGTGGGTGATGACTG	5′-TTAGCTGTGTTGCTCCTTGC
HDAC4	5′-AATCCTGCCCGTGTGAAC	5′-GTAGGGGCCACTTGCAGA
HDAC5	5′-GCATGAACTCTCCCAACGAG	5′-TTCACCTCCACTGCCACAG
HDAC6	5′-GGCTGAGATTCGGAATGG	5′-CCCATCCATAAGATTGTGCTG
HDAC7	5′-CGCCAGTTGGAAACAATGAT	5′-GCTGAGAGCCTGGTGTGTCT
HDAC8	5′-GCAGCTGGCAACTCTGATT	5′-GTCAAGTATGTCCAGCAACGAG
HDAC9	5′-CAAGAAGCGAGTGTTTGAGGT	5′-GTTTGGTGAACTGGGACCTG
HDAC10	5′-ACCTTGCAGATGATGGGAGA	5′-GCTCAGAAACCCTCCAGTTG
HDAC11	5′-TGCAGACATCACACTGGCTAT	5′-GGTGGGCATCGAGATCAA

Table 2. General characteristics of DM and age-matched control mice.

Group	Blood glucose (mg dl^{-1})	Body weight (g)	Bladder weight (mg)	Bladder weight/body weight (mg g^{-1})
Control	106.4 ± 3.5	22.4 ± 0.75	21.5 ± 0.45	0.97 ± 0.02
DM	524.2 ± 9.7*	21.6 ± 0.63	23.3 ± 1.36	1.10 ± 0.09

Values are means ± SEM, $n = 10$ mice per group.
*significantly different from controls by unpaired, two-tailed Student's t test, $P < 0.0001$.

multiple comparisons. Differences were considered significant for $P < 0.05$.

RESULTS

Characteristics of diabetic and control mice

At the time of euthanasia, the mean blood glucose level in diabetic mice was more than five times higher than in controls, while there were no statistically significant differences in body weights, bladder weights or bladder weight/body weight ratios between the two groups (Table 2).

Reduced bacterial clearance in diabetic mice

Lower UTI is an acute disease with a typical clearance pattern within 2 weeks in healthy C57BL/6 mice, but the process is determined within first 5 days post-infection, with a gradual decrease in bacterial load after a peak at about 24 h post-infection (Ingersoll *et al.*, 2008; Rosen, Hung and Kline 2008). Here we show that, during the course of UTI from 3 h to 5 days post-infection, the overall bacterial count in urine was significantly higher in diabetic mice than in the control group (Fig. 1a, $P < 0.0001$). Control mice cleared the majority of UPEC from the bladder by day 5, whereas diabetic mice had significantly higher numbers of bacteria in the bladder at every time point (Fig. 1b). The data indicate less efficient bacterial clearance ability in these diabetic mice, resulting in prolonged infection.

Reduced neutrophil infiltration in response to UTI in diabetic mice

To evaluate the effect of DM on neutrophil infiltration into the bladder in response to UTI, we evaluated the neutrophil numbers in urine and bladder tissues of diabetic and control mice at the times after UPEC inoculation indicated in Fig. 2. In control mice, UPEC administration resulted in a strong increase in the number of neutrophils in urine, peaking at 6 h, followed by a sharp partial decrease by 12 h, and then a gradual reduction through 72 h. On the other hand, diabetic mice demonstrated a markedly attenuated influx of neutrophils in urine, peaking at 12 h and then gradually decreasing through 72 h (Fig. 2a, $P < 0.0001$ by Student's t test comparison of AUC values over the 3–12 h period in diabetic vs control mice). Neutrophil infiltration after UPEC administration was also assessed by measuring levels of the neutrophil marker MPO by ELISA. The time courses of MPO protein levels in urine and bladder paralleled the course of neutrophil counts in urine, with a robust peak at 6 h post-UPEC installation in controls that was markedly attenuated in diabetic mice (Fig. 2b, urine, $P < 0.0001$ by Student's t test comparison of AUC values over the 3–12 h period in diabetic vs control mice; Fig. 2c, bladder, $P < 0.0001$ comparing DM with control at 3 and 6 h).

Attenuated chemokine expression in response to UTI in diabetic mice

Expression levels of MIP-2, KC, MCP-1 and IL-6 were measured at both the transcription and protein levels over time in response to UPEC installation (Fig. 3) Elevated chemokine mRNA levels were evident by 3 h post-infection, and peaked at 3 h (MCP-1), 6 h (MIP-2 and IL-6) or 12 h (KC) in both control and diabetic mice. However, the levels were significantly lower in the diabetic mice at multiple early time points (Fig. 3, left column). Chemokine protein levels followed the same pattern, except the MCP-1 protein level peaked later than its mRNA, at 6 h (Fig. 3, right column). The results show that early release of

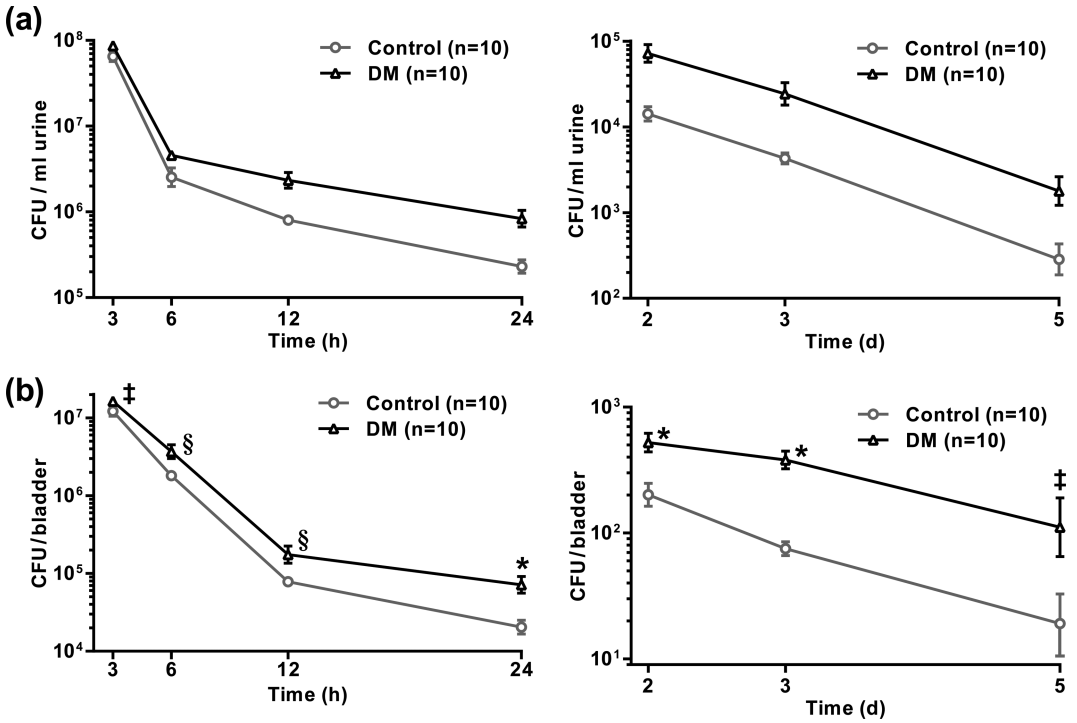

Figure 1. *In vivo* kinetics of UPEC infection and clearance in the bladder. Shown are UPEC colony forming units in urine and bladder tissue at the indicated times after inoculation of bacteria. Examination of urine prior to inoculation confirmed that none of the mice had pre-existing bacteriuria. Bacterial clearance assays were performed as described in the section 'Materials and Methods'. (**a**) Bacterial counts per ml of urine were significantly higher in diabetic compared with control mice, determined by calculating area under the curve over the entire time course for each mouse and comparing the area values in diabetic vs control mice by a Student's *t* test ($P < 0.0001$). (**b**) Bacterial counts per whole bladder were significantly higher in diabetic compared with control mice at each time point, determined by multiple Student's *t* tests with corrections for multiple comparisons by the Holm–Sidak method. $^{‡}P < 0.02$, $^{§}P < 0.001$, and $^{*}P < 0.0001$. Data in (a) and (b) are expressed as means ± 95% CI.

neutrophil-attractant chemokines upon infection with type 1-fimbriated UPEC is severely attenuated in female diabetic mice.

Altered HDAC gene expression in DM

Using qRT-PCR, we observed significantly increased expression of mRNAs for several proteins in the TLR4 signaling pathway to NF-κB activation, including MyD88, IRAK1 and IRAK4, in uninfected bladders of diabetic mice compared with controls, suggestive of sustained NF-κB activation and expression of proinflammatory cytokines (data not shown). Those results led us to investigate potential involvement of chromatin remodeling enzymes such as HDACs in the impaired cytokine response to UPEC infection in diabetic mice. We determined the effect of DM on gene expression of HDACs 1–11 in the bladder in uninfected mice. The mRNA levels of HDACs 1–5 were significantly higher in diabetic mice bladders compared with controls, suggesting a potential inhibitory effect on the expression of chemokines during DM (Fig. 4). On the other hand, the expression levels of HDACs 9 and 11 were significantly lower in the bladders of diabetic mice compared with non-diabetic control mice.

DISCUSSION

This study demonstrated that clearance of UPEC strain 53498 is impaired in diabetic female C57BL/6 mice, characterized by diminished infiltration of neutrophils into the bladder and markedly attenuated induction of chemokine/cytokine expression in the bladder compared with non-diabetic controls. In contrast, the bladder chemokine response to experimental UTI in healthy mice was rapid and robust, and was accompanied by substantial accumulation of active neutrophils in the bladder and urine.

In the experimental UTI model, type 1 fimbriae promote colonization of the urothelium and initiate an early innate host response (Connell *et al.*, 1996; Martinez *et al.*, 2000; Hedlund *et al.*, 2001; Schilling *et al.*, 2001). An essential role for neutrophils in the innate immune response was suggested in a report demonstrating that pretreatment of female C3H/HeN mice with the neutrophil depleting antibody RB6–8C5 drastically impaired clearance of UPEC from the bladder and kidneys (Haraoka *et al.*, 1999). However, subsequent work revealed that RB6–8C5 also depletes peripheral dendritic cells and a subset of macrophages in mice (Tvinnereim, Hamilton and Harty 2004; Daley *et al.*, 2008). Although dendritic cells have been reported to be dispensable for UPEC clearance (Engel *et al.*, 2006), the relative roles of neutrophils and macrophages remain to be clarified.

Others have shown that UTI can trigger local mucosal expression of certain chemokines from the two main categories, CXC (MIP-2 and KC) and CC (MCP-1) (Hang *et al.*, 1999; Mittal *et al.*, 2004; Godaly *et al.*, 2007; Duell *et al.*, 2012). Treatment of mice with MIP-2 and KC neutralizing antibodies before *E. coli* Shiga toxin- and LPS-induced renal injury blocked neutrophil infiltration into the kidney in an additive manner (Roche *et al.*, 2007). More recently, neutrophil influx into and bacterial clearance from the lungs of mice after intratracheal instillation of *E. coli* were markedly reduced by genetic disruption of MCP-1 (Balamayooran *et al.*, 2011). Those results indicate important roles for MIP-2, KC and MCP-1 in neutrophil recruitment, and suggest that the impaired chemokine responses to UTI that we

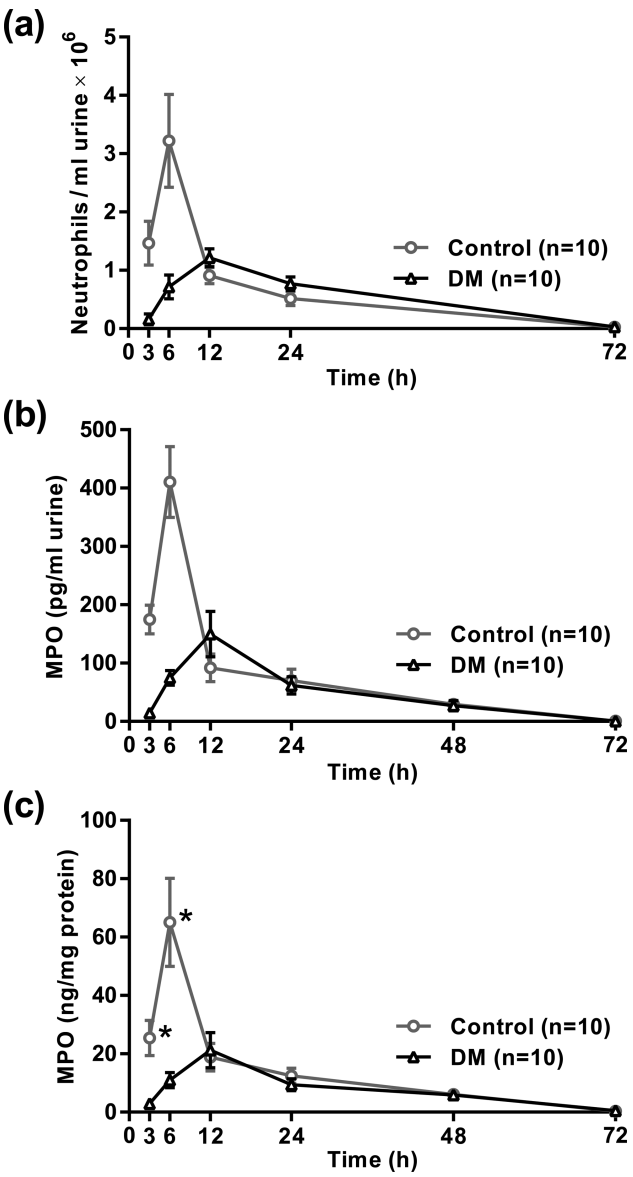

Figure 2. Influx of neutrophils into the urine, with confirmatory MPO kinetics in the urine and bladder. Urine was collected aseptically and bladders were harvested from diabetic and control mice at the indicated times after UPEC inoculation. (a) Urine was stained with Turk's solution and neutrophils were counted using a hemacytometer. The initial neutrophil response was markedly attenuated in diabetic mice compared with controls ($P < 0.0001$ by Student's t test comparison of AUC values over the 3–12 h period in diabetic vs control mice). (b) Results of ELISA quantification of the neutrophil marker MPO in urine, shown as ng MPO per ml of urine. $P < 0.0001$ by Student's t test comparison of AUC values over the 3–12 h period after UPEC challenge in diabetic vs control mice comparing DM with control at the indicated times. (c) ELISA analysis of MPO concentrations in bladder homogenates, shown as concentrations of MPO per mg total protein. *$P < 0.0001$ by multiple Student's t tests corrected for multiple comparisons by the Holm–Sidak method. Data in (a)–(c) are expressed as means ± 95% CI.

observed in diabetic mice are a likely cause of the diminished neutrophil infiltration into the bladder and impaired bacterial clearance.

In our time course, the levels of MIP-2, MCP-1 and IL-6 proteins in the bladder and neutrophils in the bladder and urine peaked at 6 h after UPEC instillation. A similar time course of MIP-2 induction accompanied by neutrophil infiltration was found in a mouse model of *Pseudomonas aeruginosa*-induced UTI (Mittal *et al.*, 2004). Also, MIP-2 and KC levels in the lungs of mice peaked at 6 h after intratracheal installation of *E. coli*, although MCP-1 and neutrophil levels continued to increase through 24 h in that model (Balamayooran *et al.*, 2011). In our model, KC mRNA and protein levels peaked at 12 h, and the mean MCP-1 mRNA level peaked at 3 h after UPEC instillation, suggesting different influences on the regulation of transcription of those chemokines relative to MIP-2 and IL-6. Our expression results are roughly consistent with Duell *et al.* (2012), who found increases of 67-, 22-, 28- and 133-fold in the mRNA levels of MIP-2, KC, MCP-1 and IL-6, respectively, 2 h after infection of mice with type 1-fimbriated UPEC strain CFT073.

Others have reported that expression of MIP-2, KC, MCP-1 and IL-6 can be induced by TLR4, likely through activation of the transcription factor NF-κB (Boyd *et al.*, 2006; Song *et al.*, 2007b; Ao *et al.*, 2009). Our finding that the responses of all four of those cytokines to UPEC are impaired in diabetic mice, without altering the different individual time courses of expression, suggests that the impairment is due to a defect common to the regulation of the four cytokines, possibly at the level of TLR4 signaling to NF-κB, or epigenetic regulation of transcriptional activation by NF-κB through chromatin remodeling.

Although NF-κB is persistently activated in many tissues in DM, suggesting a sustained proinflammatory state (Patel and Santani 2009), its function is regulated at multiple levels. Our observation of increased expression of TLR4 signaling pathway genes in uninfected diabetic bladders (data not shown), which is consistent with sustained signaling to NF-κB, suggested possible involvement of epigenetic modifications such as protein deacetylation in the impaired cytokine response to UTI in diabetic mice. HDACs are important chromatin remodeling enzymes that can inhibit the transcription of genes by deacetylating lysine residues on histones, resulting in increased chromatin condensation. HDACs can also deacetylate and thereby regulate the activity of a variety of non-histone proteins, including transcription factors (Villagra, Sotomayor and Seto 2010). HDACs have been implicated in both pro- and antiinflammatory actions (Halili *et al.*, 2009). HDACs 1, 2 and 3 have been shown to inhibit NF-κB activation by deacetylating or binding to NF-κB subunits, resulting in binding to IκB and export from the nucleus; on the other hand, HDACs have also been implicated in maintaining Iκ B kinase activity, leading to dissociation of IκB from and activation of NF-κB (Grabiec, Tak and Reedquist 2011). HDAC1 has also been shown to participate in the suppression of proinflammatory cytokine expression, including IL-6 and IL-8, in human breast cancer cells via chromatin modifications (Janzer *et al.*, 2012). Thus, one or more of the three class I HDACs we found to be upregulated in diabetic bladders (HDACs 1, 2 and 3) could have contributed to the attenuation of the chemokine/cytokine response to UPEC at one or both of two levels: by promoting NF-κB binding to IκB, or by increasing chromatin condensation in the regions of the chemokine/cytokine gene promoters.

Our observation of increased expression of class I HDACs 1, 2 and 3 in the bladder in STZ-diabetic mice is consistent with a report that STZ-diabetic rats had increased expression of class I HDACs 1, 2 and 8 in the retina (Zhong and Kowluru 2010). We also observed upregulation of class IIa HDACs 4 and 5, consistent with a recent study showing increased expression of HDACs 2, 4 and 5 in both STZ-induced and db/db diabetic rat kidneys (Wang *et al.*, 2014). On the other hand, class IIa HDAC 9 and class IV HDAC 11 were downregulated in diabetic bladders. Deletion studies have indicated that individual HDACs of both class I (widely expressed in mammalian tissues) and

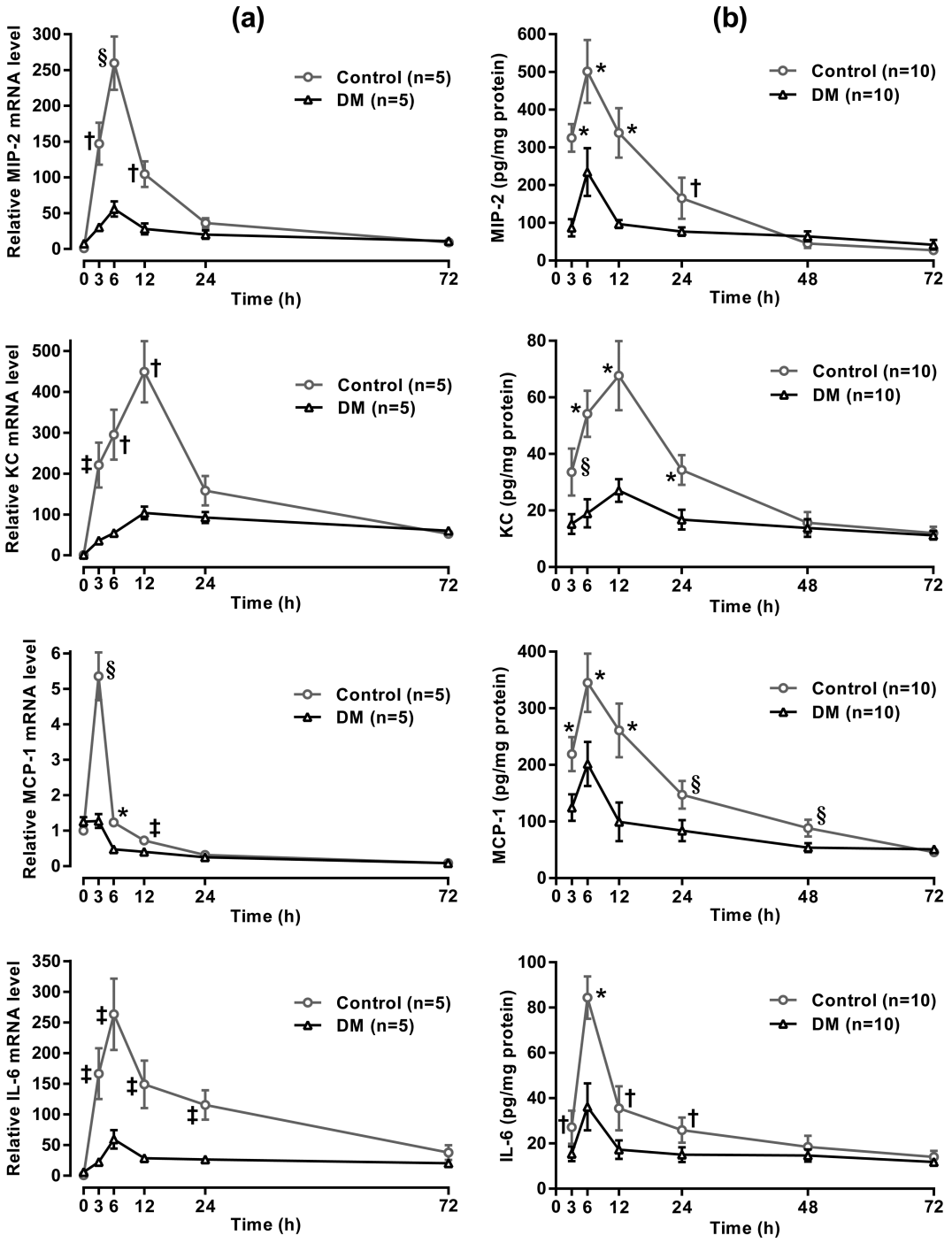

Figure 3. Proinflammatory cytokine response to UPEC in the bladder. (**a**) qRT-PCR. Bladders were harvested from 4-week diabetic mice and age-matched controls at the indicated time points following UPEC inoculation ($n = 5$ per group). Total RNA was isolated, cDNA was synthesized, and MIP-2, KC, MCP-1 and IL-6 mRNA levels were quantified by qRT- PCR as described in the section 'Materials and Methods'. Using the comparative C_T method, chemokine threshold cycles (C_T) were normalized to the corresponding β-actin C_T values, and expression levels were calculated relative to the average normalized values in uninoculated (time 0), non-diabetic control mice (set at 1.0). Each bladder sample was assayed in triplicate. qRT- PCR data are expressed as means ± SEM. (**b**) ELISA. Bladders were harvested from 4-week diabetic mice and age-matched controls at the indicated time points following UPEC inoculation ($n = 10$ per group). Bladders were homogenized, and chemokine levels were quantified by ELISA. MIP-2, KC, MCP-1 and IL-6 MPO levels were calculated from the corresponding standard curves as pg/mg protein. Each bladder sample was assayed in quadruplicate. ELISA data are expressed as means ± 95% CI. Statistical analyses of qRT-PCR results and ELISA results in DM compared with control mice at each time point were performed by multiple Student's t tests with corrections for multiple comparisons by the Holm–Sidak method ($^{\ddagger}P < 0.02$, $^{\dagger}P < 0.005$, $^{\S}P < 0.001$ and $^{*}P < 0.0001$).

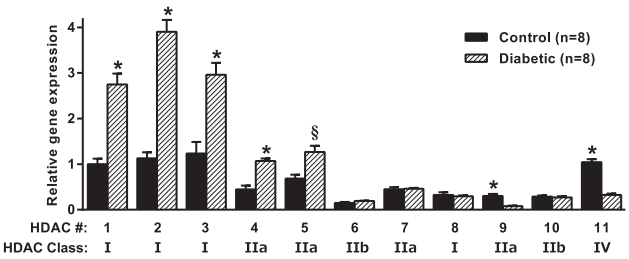

Figure 4. Relative HDAC mRNA levels in diabetic and control mice. Bladders were harvested from 4-week diabetic mice ($n = 8$) and age-matched controls ($n = 8$). Total RNA isolation, cDNA synthesis and qRT-PCR were performed as described in the section 'Materials and Methods'. The results, normalized to β-actin expression, are expressed relative to the level of HDAC 1 in control mice, set at 1.0. $^{*}P < 0.0003$, $^{\S}P = 0.002$ for DM vs control by multiple Student's t tests with corrections for multiple comparisons by the Holm–Sidak method.

class II (limited expression patterns) can affect the expression of limited sets of genes (Witt *et al.*, 2009). The novel class IV HDAC, HDAC11, has been shown to inhibit the expression of plasminogen activator inhibitor type-1, a promoter of macrophage migration, in a murine model of kidney ischemia and reperfusion injury (Kim *et al.*, 2013). Such a role is consistent with our observation of lower HDAC11 expression in STZ-diabetic mice, a DM model previously reported to exhibit increased recruitment of macrophages to the bladder after UPEC instillation (Yokoo, Kumamoto and Hirose 1994). Further studies are required to clarify the roles of the specific HDACs up- or downregulated in the bladder in diabetic mice in the gene expression machinery of the immune response to UTI.

In conclusion, we showed that bacterial clearance is impaired in diabetic mice, likely due in part to attenuated expression of cytokines in response to UPEC challenge, resulting in lower recruitment of neutrophils to the bladder. Further characterization of the bacterial clearance defect in DM will include studies of upstream control of the expression of the related genes, as suggested by altered expression of HDACs.

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