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## ORIGINAL ARTICLE

Male Infertility

# Damaged male germ cells induce epididymitis in mice

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Epididymitis can be caused by infectious and noninfectious etiological factors. While microbial infections are responsible for infectious epididymitis, the etiological factors contributing to noninfectious epididymitis remain to be defined. The present study demonstrated that damaged male germ cells (DMGCs) induce epididymitis in mice. Intraperitoneal injection of the alkylating agent busulfan damaged murine male germ cells. Epididymitis was observed in mice 4 weeks after the injection of busulfan and was characterized by massive macrophage infiltration. Epididymitis was coincident with an accumulation of DMGCs in the epididymis. In contrast, busulfan injection into mice lacking male germ cells did not induce epididymitis. DMGCs induced innate immune responses in epididymal epithelial cells (EECs), thereby upregulating the pro-inflammatory cytokines such as tumor necrosis factor- $\alpha$  (*TNF- $\alpha$* ), interleukin-6 (*IL-6*), and interleukin-1 $\beta$  (*IL-1 $\beta$* ), as well as the chemokines such as monocyte chemoattractant protein-1 (*MCP-1*), monocyte chemoattractant protein-5 (*MCP-5*), and chemokine ligand-10 (*CXCL10*). These results suggest that male germ cell damage may induce noninfectious epididymitis through the induction of innate immune responses in EECs. These findings provide novel insights into the mechanisms underlying noninfectious epididymitis, which might aid in the diagnosis and treatment of the disease.

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## INTRODUCTION

Microbial infections and inflammatory conditions in the male genital tract are responsible for approximately 15.0% of male infertility cases in developed countries,<sup>1</sup> and this ratio can be much higher in undeveloped countries.<sup>2</sup> Epididymitis and orchitis are more likely to contribute to male infertility than inflammatory conditions in the accessory sexual glands, including the prostate and seminal vesicle glands.<sup>3</sup>

Epididymitis is more common than orchitis in outpatient visitors.<sup>4</sup> Orchitis often occurs with epididymitis and is termed epididymo-orchitis.<sup>5</sup> Epididymitis can take an acute form with symptoms lasting several weeks, or it can be chronic lasting more than 3 months. Acute epididymitis can be caused by the reflux of urine into the ejaculatory ducts or by retrograde bacterial infection ascending the urogenital tracts.<sup>6</sup> The mumps virus can induce epididymitis as epididymo-orchitis syndrome.<sup>7</sup> However, many epididymitis cases lack evidence of microbial infections in the reproductive tract. Various risk factors for noninfectious epididymitis have been identified including a previous systemic infection, adverse reaction to a medication, physical trauma of the scrotum, and prolonged sitting or bicycle riding.<sup>5,8</sup> The mechanisms by which these risk factors induce noninfectious epididymitis are unknown.

Most male germ cells are generated in puberty, when a considerable time has passed since the establishment of self-tolerance to autoantigens during the fetal and neonatal periods. Therefore,

male germ cells produce immunogenic antigens that may induce an autoimmune response. Male germ cells do not induce inflammation in the testis under physiological conditions because of the immunoprivileged status of the testis.<sup>9</sup> However, autoimmune orchitis can occur under certain pathological conditions, such as physical trauma, and exposure to chemical toxins or high temperature that may damage germ cells.<sup>10</sup> The accumulation of apoptotic germ cells related to defective clearance mechanisms in the testis favors autoimmune orchitis.<sup>11</sup> We previously demonstrated that damaged male germ cells (DMGCs) induce an innate immune response in Sertoli cells, thereby upregulating the expression of pro-inflammatory cytokines and chemokines that promote inflammation.<sup>12</sup> These previous studies suggested that DMGCs might induce endogenous inflammation in the testis.

Although the epididymis is also considered an immunoprivileged organ, immunosuppression in the epididymis is not as complete as in the testis.<sup>13</sup> Therefore, we hypothesized that DMGCs induce epididymitis when they are present in the epididymis. Busulfan is an alkyl sulfonate, a cell cycle nonspecific alkylating antineoplastic agent. Busulfan severely damages male germ cells. We used a busulfan-induced male germ cell damage model to provide substantial evidence that DMGCs induce epididymitis in mice. Therefore, DMGC might be an etiological factor in noninfectious epididymitis associated with various risk factors that may damage male germ cells.

## MATERIALS AND METHODS

### Animals

C57BL/6J mice were obtained from the Laboratory Animal Center of the Peking Union Medical College (Beijing, China). Tumor necrosis factor- $\alpha$  knockout (*TNF- $\alpha$ <sup>-/-</sup>*) mice (B6/129S6-*TNF<sup>tm1GK1</sup>/J*) on a C57BL/6J background were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Wild-type (WT) mice were obtained by backcrossing knockout mice to C57BL/6J mice. The mice were maintained in a specific pathogen-free facility with a 12 h/12 h light/dark cycle and were provided with food and water *ad libitum*. The mice were handled in compliance with the guidelines (ACUC-A01-2018-008) for the Care and Use of Laboratory Animals established by the Chinese Council on Animal Care. The experimental procedures were approved by the Institutional Animal Care and Use Committee of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China).

### Antibodies and major reagents

Rat monoclonal anti-F4/80 (ab6640), rabbit polyclonal anti-CD45 (ab10558), rabbit monoclonal anti-CD4 (ab183685), rabbit polyclonal anti-CD8 (ab203035), and rat monoclonal anti-B220 (ab64100) antibodies and pan-cytokeratin (ab7753) were purchased from Abcam (Cambridge, UK). Horseradish peroxidase (HRP)-conjugated secondary antibodies and 3,3'-diaminobenzidine (DAB) were purchased from Zhongshan Biotechnology Co. (Beijing, China). Collagenase type IV (C0130-100MG), hyaluronidase (H3506-100MG), and busulfan (S85543-229) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Busulfan injection

Busulfan was dissolved in dimethyl sulfoxide (DMSO) at 5 mg ml<sup>-1</sup> and diluted with 2× phosphate-buffered saline (PBS) at a ratio of 1:1. Ten-week-old male mice received an intraperitoneal injection of a single dose of busulfan (25 mg kg<sup>-1</sup>). Mice injected with the same volume of a mixture of DMSO and PBS served as the controls. Five mice per group were injected for one experiment, and the total number of mice in the study is listed in **Supplementary Table 1**.

### Histology and immunohistochemical staining

For histological analysis, the testes and epididymides were fixed in 4% (*w/v*) paraformaldehyde (Sigma-Aldrich) for 24 h. The tissues were embedded in paraffin and cut into 5- $\mu$ m-thick sections on a Leica CM1950 (Leica Biosystems, Nussloch, Germany). The sections were stained with hematoxylin and eosin (H and E) and mounted with neutral balsam (Zhongshan Biotechnology Co.) for observation by a light microscope (BX-51, Olympus, Osaka, Japan).

For immunohistochemical staining, the sections were soaked in citrate buffer (11 mmol l<sup>-1</sup>, pH 6.0; Zhongshan Biotechnology Co.) and heated in a microwave oven at 100°C for 10 min to retrieve the antigens. The sections were washed three times with 1× PBS and then incubated in 1× PBS containing 3% (*v/v*) H<sub>2</sub>O<sub>2</sub> (Zhongshan Biotechnology Co.) for 15 min to inhibit endogenous peroxidase activity. After being washed three times, the sections were blocked with 5% (*v/v*) normal goat sera (Zhongshan Biotechnology Co.) in PBS for 1 h at room temperature and then incubated with the primary antibodies overnight at 4°C. The sections were washed three times with PBS and incubated with the appropriate HRP-conjugated secondary antibodies at room temperature for 30 min. The HRP activity was visualized using the DAB method. The negative controls were incubated with preimmune rabbit sera (Zhongshan Biotechnology Co.) instead of primary antibodies. The sections were counterstained with hematoxylin and mounted with neutral balsam for observation.

### Cell isolation

Primary epididymal epithelial cells (EECs) were isolated from 4-week-old C57BL/6J mice based on previously described procedures.<sup>14</sup> In brief, mice were anesthetized with CO<sub>2</sub> and euthanized by cervical dislocation. The entire epididymis of each mouse was collected and incubated with 10 mg ml<sup>-1</sup> collagenase type IV (Sigma-Aldrich) in F12 Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc., Gaithersburg, MD, USA) at 37°C for 30 min to remove the interstitial cells. The epididymal tubules were collected after filtration through an 80- $\mu$ m copper mesh and then treated with 0.5 mg ml<sup>-1</sup> hyaluronidase (Sigma-Aldrich) in F12/DMEM at 37°C for 15 min to remove peritubular smooth muscle cells. The tubules were cut into small pieces (approximately 1 mm) and treated with 1.0 mg ml<sup>-1</sup> hyaluronidase at 37°C for 30 min with occasional gentle pipetting. The suspensions were filtered through an 80- $\mu$ m copper mesh. EECs were collected and cultured in a humidified atmosphere containing 5% (*v/v*) CO<sub>2</sub> at 37°C in F12/DMEM supplemented with 1.2 mg ml<sup>-1</sup> sodium bicarbonate (Sinopharm Chemical Reagent Co., Ltd., Beijing, China), 10% (*v/v*) fetal bovine serum (Life Technologies), 100 U ml<sup>-1</sup> penicillin, and 100  $\mu$ g ml<sup>-1</sup> streptomycin. The EEC purity was >95% from assessment by immunofluorescence staining for pan-cytokeratin, a marker of pan-epithelial cells.<sup>15</sup>

Male germ cells were isolated from 10-week-old mice on the basis of previously described procedures.<sup>16</sup> Briefly, the testes were decapsulated and incubated with 1 mg ml<sup>-1</sup> collagenase type IV at 37°C for 15 min with gentle oscillation. The suspensions were filtered through an 80- $\mu$ m copper mesh to remove the interstitial cells. The seminiferous tubules were cut into small pieces of approximately 1 mm and incubated with 0.5 mg ml<sup>-1</sup> hyaluronidase at 37°C for 10 min with pipetting. After filtration through an 80- $\mu$ m copper mesh, cell suspensions were collected and cultured in F12/DMEM at 37°C for 6 h. During that time, the testicular somatic cells attached to the culture dishes, and the germ cells were subsequently recovered by collecting the nonadherent cells. The purity of the germ cells was >95% from immunostaining for mouse vasa homolog (MVH), a marker of germ cells.<sup>17</sup> The germ cells were cultured in serum-free F12/DMEM to induce damage, and more than 80% of the germ cells underwent apoptosis or necrosis 24 h after culture. The apoptotic and necrotic cells, as well as cellular debris, were collected after centrifugation (Beijing Jingli Centrifuge Co., Ltd., Beijing, China) at 300 g for 5 min and were used as DMGCs.

### Immunofluorescence staining

For immunofluorescence staining, EECs were cultured on Lab-Tek chamber slides (Merck Millipore, Billica, MA, USA). The cells were fixed with methanol at -20°C for 30 min and then permeabilized with 0.5% (*v/v*) Triton X-100 in PBS for 10 min. After being blocked with 10% (*v/v*) normal goat sera in PBS at room temperature for 30 min, the cells were incubated with the primary antibodies at 37°C for 2 h. After being washed three times with PBS, the cells were incubated with appropriate tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC)-conjugated secondary Abs (Zhongshan Biotechnology Co.) for 30 min. The cells were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI; Zhongshan Biotechnology Co.) according to the manufacturer's instructions. The slides were mounted with Antifade Mounting Medium (Vector Laboratories, Inc., Burlingame, CA, USA) for observation by a fluorescence microscope BX-51 (Olympus, Tokyo, Japan).

### Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from EECs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's

instructions. RNA was treated with RNase-free DNase I (Invitrogen) to remove genomic DNA contaminants. RNA (1  $\mu\text{g}$ ) was reverse transcribed into cDNA in a 20- $\mu\text{l}$  reaction mixture containing 2.5  $\mu\text{mol l}^{-1}$  random primer, 2  $\mu\text{mol l}^{-1}$  dinucleotide triphosphates, and 200 U Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). PCR was performed with a Power SYBR Green PCR master mix kit (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM 7300 real-time cycler (Applied Biosystems). The relative mRNA levels were determined using the comparative  $2^{-\Delta\Delta\text{Ct}}$  method by normalizing to  $\beta\text{-actin}$  as described in Applied Biosystems User Bulletin No. 2 (P/N 4303859).<sup>18</sup> The primer sequences are listed in **Supplementary Table 2**.

#### Stimulation of EECs with DMGCs

EECs were cultured in 6-well plates at a density of  $5 \times 10^5$  cells per well. DMGCs ( $1 \times 10^6$  per well) were added to the EECs. At specific time points after stimulation, DMGCs were removed and EECs were collected for analysis of gene expression.

#### Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels were measured with ELISA kits according to the manufacturer's instruction. A mouse tumor necrosis factor (TNF- $\alpha$ ) ELISA kit (CME0004) was purchased from Beijing 4A Biotech (Beijing, China). ELISA kits for mouse monocyte chemoattractant protein-1 (MCP-1; BMS6005) and C-X-C motif chemokine 10 (CXCL10; BMS6018) were purchased from eBioscience (San Diego, CA, USA).

#### Statistical analyses

All data were presented as the mean  $\pm$  standard deviation (s.d.). Statistical significance between individual comparisons was determined using the Student's *t*-test. For multiple comparisons, one-way analysis of variance (ANOVA) with a Bonferroni (selected pairs) *post hoc* test

was used. The calculations were performed using SPSS version 13.0 (SPSS, Chicago, IL, USA), and  $P < 0.05$  was considered statistically significant.

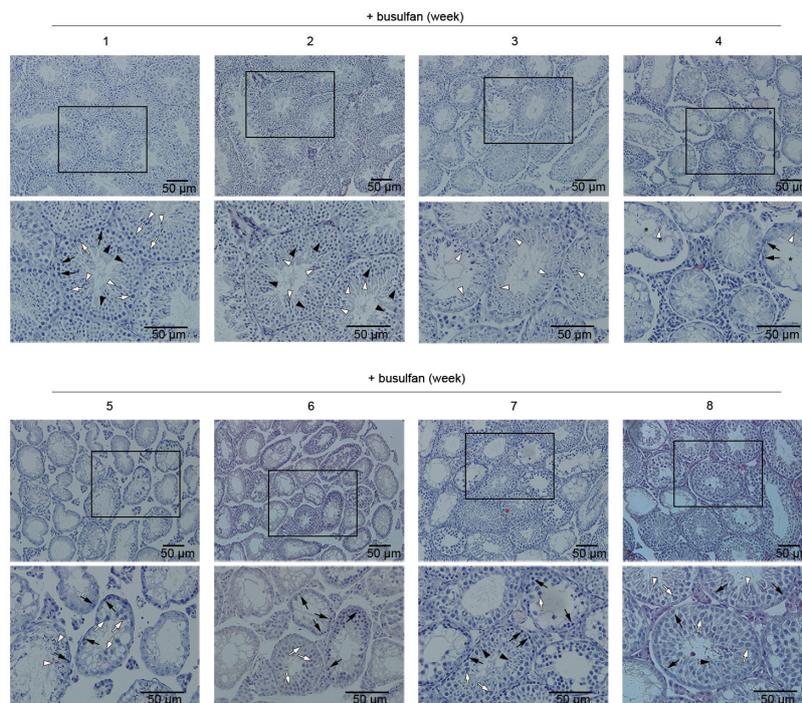
## RESULTS

### Busulfan-induced male germ cell damage

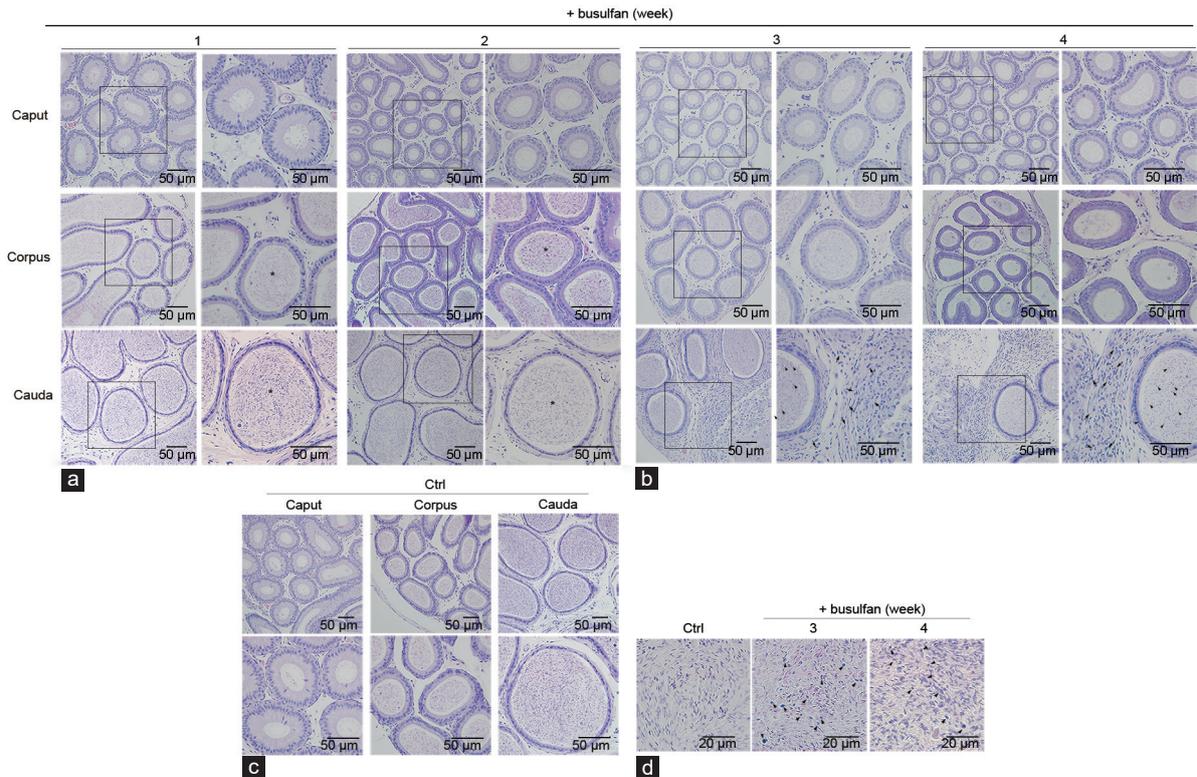
To determine the extent of busulfan-induced male germ cell damage, 25 mg  $\text{kg}^{-1}$  of busulfan was intraperitoneally injected into 10-week-old C57BL/6J mice. Germ cell damage was evaluated once each week for 8 weeks after busulfan injection (**Figure 1**). All stages of male germ cells, including spermatogonia (black arrows), spermatocytes (white arrows), round spermatids (black arrowheads), and elongating spermatids (white arrowheads), were observed 1 week after busulfan injection. Spermatogonia and spermatocytes had mostly disappeared at 2 weeks after busulfan injection, whereas round and elongating spermatids remained. Only elongating spermatids were observed in the seminiferous tubules at 3 weeks. The germ cells were almost completely absent except for a few elongating spermatids within a certain seminiferous tubule (asterisks) at 4 weeks. However, the germ cells were gradually recovered at 5–8 weeks after the busulfan injection (**Figure 1**, lower panels).

### Cell infiltration in the epididymis

The structure of the epididymis was histologically examined after H and E staining (**Figure 2**). At 1 week (**Figure 2a**, left panels) and 2 weeks (**Figure 2a**, right panels) after busulfan injection, the epididymides displayed a normal histological structure. The corpus and cauda epididymal tubules were filled with many elongated spermatozoa (asterisks). At 3 weeks (**Figure 2b**, left panels) after busulfan injection, massive cell infiltration (arrows) was observed in the stroma of 75.0% (15/20) of the cauda epididymal tubules. Cell infiltration was not evident in the caput or corpus epididymis 3 weeks



**Figure 1:** Busulfan-induced male germ cell damage. Busulfan was intraperitoneally injected into mice. The testes were collected at the indicated weeks after busulfan injection. Paraffin sections of the testes were stained with H and E. The marked squares (upper panels) are magnified for high resolution (lower panels). Black arrows, white arrows, black arrowheads, and white arrowheads indicate spermatogonia, spermatocytes, round spermatids, and elongating spermatids, respectively. Images are representative of at least five mice at each time point. H and E: hematoxylin and eosin stain.



**Figure 2:** Histology of the epididymis. The caput, corpus, and cauda epididymides were collected at the indicated weeks after busulfan injection; (a) 1 and 2 weeks; (b) 3 and 4 weeks. Paraffin sections of the epididymides were stained with H and E. The marked squares (left panels) are magnified for high resolution (right panels). Asterisks indicate the epididymal tubules containing spermatozoa. Arrows and arrowheads indicate cell infiltration and DMGCs, respectively. (c) Histology of the Ctrl epididymis. Mice were injected with DMSO for 4 weeks. Sections were stained with H and E. (d) High magnification of the epididymal tubules show DMGCs (arrowheads). Images represent the epididymides of at least 20 mice. H and E: hematoxylin and eosin stain; DMGCs: damaged male germ cells; DMSO: dimethyl sulfoxide; Ctrl: control.

after busulfan injection. Cell infiltrations were observed in 83.3% (25/30) of the cauda epididymis (**Figure 2b**, right panels) 4 weeks after busulfan injection. By contrast, cell infiltration was not present in the epididymides of the control mice 4 weeks after injection with DMSO alone (**Figure 2c**). Many degenerated spermatozoa (arrowheads) were observed within the lumen of the cauda epididymal tubules of mice 3 and 4 weeks after busulfan injection (**Figure 2d**). Degenerated spermatozoa were not observed in the epididymides of the control mice (**Figure 2d**, left panel). The percentage of epididymides with cell infiltrations was markedly decreased after 4 weeks post busulfan injection. Evident cell infiltration (data not shown) was observed in only 5.0% (1/20) of mice 7 and 8 weeks after busulfan injection. The incidence of epididymitis at different time points after busulfan injection is shown in **Supplementary Table 1**. Cell infiltration in the epididymis was not observed in the control mice injected with 50.0% DMSO alone (data not shown).

#### Immune cells in the epididymitis

Cell infiltration into the epididymis suggested epididymitis. Therefore, we characterized the immune cell types in the epididymitis. Immunohistochemical staining for immune cell markers showed that most infiltrating cells were macrophages (black arrows) positively stained for F4/80 (**Figure 3a**, middle and right panels). Few macrophages were detected in the epididymides of the control mice injected with DMSO (**Figure 3a**, left panel). Immunostaining for CD45 demonstrated that pan-lymphocytes (black arrowheads) were increased in the epididymitis (**Figure 3b**, middle and

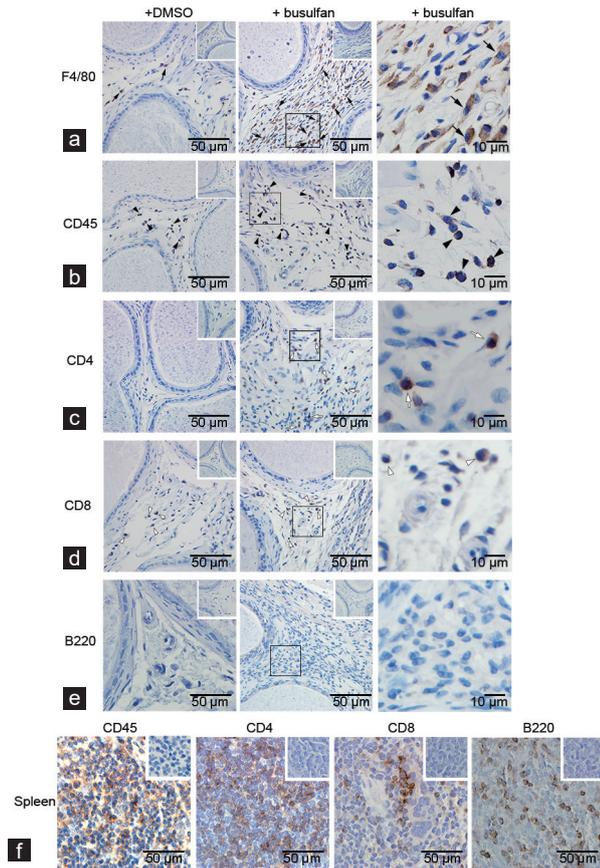
right panels) compared with the controls (left panel). CD4<sup>+</sup> lymphocytes (white arrows) were markedly increased in the epididymitis (**Figure 3c**). By contrast, CD8<sup>+</sup> lymphocytes (white arrowheads) were rare in both epididymitis and the controls (**Figure 3d**). B lymphocytes were not detected by staining for B220 (**Figure 3e**). The specificity of antibodies for detecting lymphocytes was confirmed by immunohistochemical staining of the mouse spleen (**Figure 3f**).

#### Busulfan injection and induction of epididymitis in mice lacking germ cells

To confirm that epididymitis was induced by DMGCs after busulfan injection, mice that lacked germ cells in the testis were injected with a second dose of busulfan, 4 weeks after the first busulfan injection. The testis and epididymis were analyzed 4 weeks after the second busulfan injection. Germ cells were completely lost in the testis of all 20 mice (**Figure 4a**). Only Sertoli cells (black arrows) were observed within the seminiferous tubules. Massive cell infiltration (black arrowheads) was found in 1 of the 20 mice (**Figure 4b**), but not in the other 19 mice (data not shown). Immunohistochemical staining showed many macrophages (white arrows) in the epididymitis (**Figure 4c**). However, CD45<sup>+</sup> lymphocytes (white arrowheads) were not increased (**Figure 4d**).

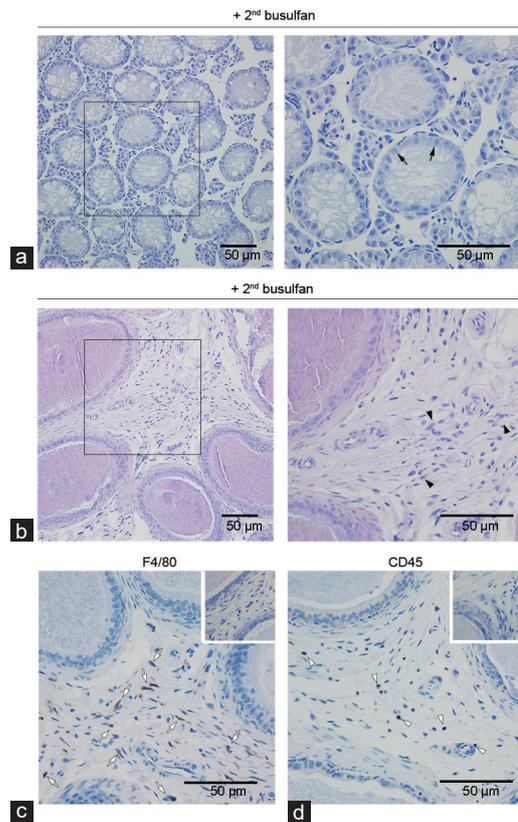
#### DMGCs and cytokine production in EECs

To explore the mechanisms underlying epididymitis, we examined the expression of various immune cytokines, including pro-inflammatory



**Figure 3:** Identification of immune cells. Mice were injected with busulfan for 4 weeks (middle panels) and with DMSO for the controls (left panels). Paraffin sections of the cauda epididymis were immunohistochemically stained for (a) macrophages, (b) pan-lymphocytes, (c) CD4, (d) CD8, and (e) B lymphocytes using specific primary antibodies against F4/80, CD45, CD4, CD8, and B220, respectively. Right panels show high magnification of the marked squares. (f) Assessment of antibody specificity. Paraffin sections of mouse spleens were immunohistochemically stained using the antibodies against the indicated markers. Insets in the upper right corners represent the negative controls. Images of the epididymis represent the results of at least three mice, and the spleen sections were from one mouse. DMSO: dimethyl sulfoxide.

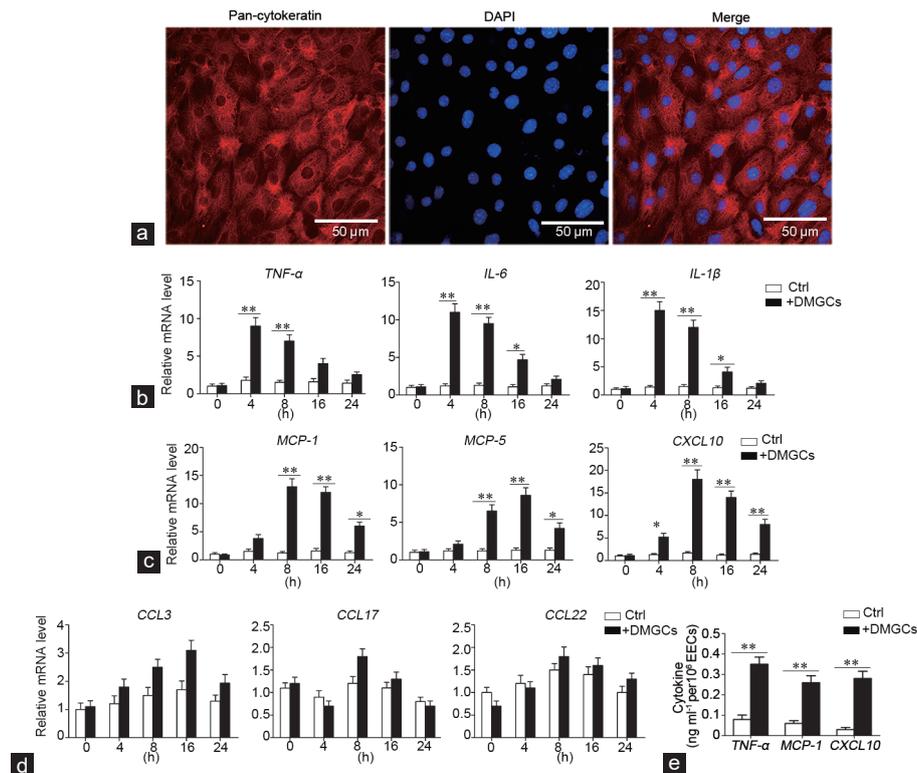
cytokines and chemokines in EECs after stimulation with DMGCs. The purity of EECs was >95.0% by immunofluorescence staining for pan-cytokeratin (Figure 5a). Real-time qRT-PCR results showed that the presence of DMGCs upregulated the expression of major pro-inflammatory cytokines, including *TNF- $\alpha$* , interleukin (*IL-6*), and *IL-1 $\beta$* , in a time-dependent manner (Figure 5b). The mRNA levels of the pro-inflammatory cytokines were significantly upregulated at 4 h ( $P < 0.01$ ), 8 h ( $P < 0.01$ ), and 16 h (*IL-6* and *IL-1 $\beta$* ,  $P < 0.05$ ) after the presence of DMGCs. Moreover, DMGCs also significantly upregulated the expression of several chemokines, including monocyte chemoattractant protein-1 (*MCP-1*), monocyte chemoattractant protein-5 (*MCP-5*), and chemokine ligand-10 (*CXCL10*), in EECs at 8 h ( $P < 0.01$ ), 16 h ( $P < 0.01$ ), and 24 h (*MCP-1*,  $P < 0.05$ ; *MCP-5* and *CXCL10*,  $P < 0.01$ ) (Figure 5c). In contrast, DMGCs did not significantly upregulate other chemokines, including chemokine (C-C motif) ligand 3 (*CCL3*), *CCL17*, or *CCL22* (Figure 5d). ELISA results confirmed that the presence of DMGCs significantly increased the secretion of *TNF- $\alpha$* , *MCP-1*, and *CXCL10* in the culture media of EECs at 24 h ( $P < 0.01$ ; Figure 5e).



**Figure 4:** Male germ cell damage and epididymitis after a second busulfan injection. Busulfan was intraperitoneally injected into mice for 4 weeks, and then the mice were given a second busulfan injection. The testes and epididymides were collected 4 weeks after the second busulfan injection. (a) Germ cell damage. The paraffin sections of the testis were stained with H and E. The marked square (left panel) is magnified for high resolution (right panel). Black arrows indicate Sertoli cells. (b) Cell infiltrations. Paraffin sections of the cauda epididymis were stained with H and E. Black arrowheads indicate cell infiltrations. (c) Macrophages and (d) pan-lymphocytes were identified by immunohistological staining using specific antibodies against F4/80 and CD45, respectively. The insets in the upper right corners represent the negative controls. The white arrow and arrowheads indicate macrophages and lymphocytes, respectively. Images are the results from one mouse. H and E: hematoxylin and eosin stain.

#### Role of *TNF- $\alpha$* in the epididymitis after busulfan injection

Given that *TNF- $\alpha$*  plays an important role in orchitis and epididymitis,<sup>19,20</sup> we assessed the role of *TNF- $\alpha$*  in the epididymitis after busulfan injection into *TNF- $\alpha$* <sup>-/-</sup> mice. Germ cells were comparably damaged in *TNF- $\alpha$* <sup>-/-</sup> (Figure 6a, right panels) and WT (left panels) mice 4 weeks after busulfan injection. In controls, mice injected with DMSO alone had normal germ cells. Only Sertoli cells (black arrows) were observed in the seminiferous tubules after busulfan injection (Figure 6a). Cell infiltration (black arrowheads) was observed in the epididymides of WT mice (Figure 6b, left panels). However, we did not find cell infiltration in *TNF- $\alpha$* <sup>-/-</sup> mice 4 weeks after busulfan injection (Figure 6b, right panels). DMGCs were abundant within the epididymal tubules (asterisks) of WT and *TNF- $\alpha$* <sup>-/-</sup> mice (Figure 6b). Immunohistochemical results confirmed many F4/80<sup>+</sup> macrophages (white arrows) in the caudal epididymides of WT mice (Figure 6c, left panel). There were considerably fewer macrophages in the cauda epididymides of *TNF- $\alpha$* <sup>-/-</sup> mice (Figure 6c, right panel) than in WT mice. More CD45<sup>+</sup> lymphocytes (white arrowheads) were



**Figure 5:** Expression of immune cytokines in EECs. (a) The purity of EECs. EECs were costained with DAPI and specific antibodies against pan-cytokeratin. (b) Pro-inflammatory factors. EECs were cultured in the presence of DMGCs or in the absence of DMGCs (Ctrl) for the indicated hours. The relative mRNA levels of *TNF- $\alpha$* , *IL-6*, and *IL-1 $\beta$*  were determined by real-time qRT-PCR. The relative mRNA levels of (c) *MCP-1*, *MCP-5* and *CXCL10*, (d) chemokine (C-C motif) ligand 3 (*CCL3*), *CCL17*, and *CCL22* were determined by real-time qRT-PCR. EECs were treated as described in b. (e) Cytokine secretion in culture medium. EECs were cultured in the presence or absence of DMGCs for 24 h. The protein levels of *TNF- $\alpha$* , *MCP-1*, and *CXCL10* were measured using ELISA. The data are the mean  $\pm$  s.d. of three independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$ . qRT-PCR: quantitative reverse transcription polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay; s.d.: standard deviation. EECs: epididymal epithelial cells; DAPI: 4',6-diamidino-2-phenylindole; DMGCs: damaged male germ cells; TNF- $\alpha$ : tumor necrosis factor- $\alpha$ ; IL: interleukin; MCP-1: monocyte chemoattractant protein-1; CXCL10: chemokine ligand-10.

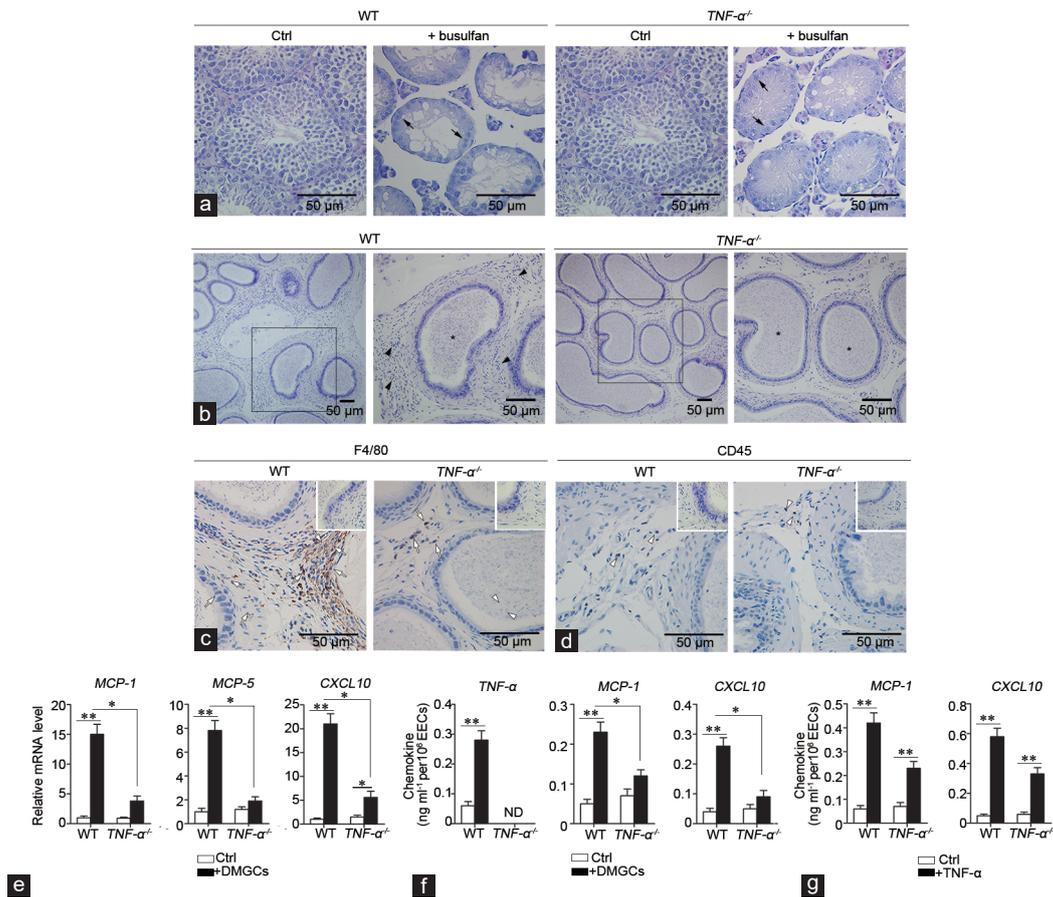
found in the cauda epididymides of WT mice (Figure 6d, left panel) than in *TNF- $\alpha$* <sup>-/-</sup> mice (right panel) 4 weeks after busulfan injection. The expression of chemokines in WT and *TNF- $\alpha$* <sup>-/-</sup> EECs after stimulation with DMGCs was also examined. The mRNA levels of *MCP-1* ( $P < 0.01$ ), *MCP-5* ( $P < 0.01$ ), and *CXCL10* ( $P < 0.01$ ) were markedly upregulated in WT EECs 8 h after the presence of DMGCs (Figure 6e). The upregulation was significantly lower in *TNF- $\alpha$* <sup>-/-</sup> EECs than in WT EECs. ELISA results confirmed that TNF- $\alpha$  was absent from the culture medium of *TNF- $\alpha$* <sup>-/-</sup> EECs (Figure 6f, left panel), whereas MCP-1 (middle panel) and CXCL10 (right panel) levels were insignificantly increased in *TNF- $\alpha$* <sup>-/-</sup> EECs 24 h after the presence of DMGCs. Recombinant TNF- $\alpha$  significantly induced the production of *MCP-1* ( $P < 0.01$ ) and *CXCL10* ( $P < 0.01$ ) in both WT and *TNF- $\alpha$* <sup>-/-</sup> EECs (Figure 6g).

## DISCUSSION

Epididymitis is the most frequently experienced case of intrascrotal inflammation, which can lead to male infertility.<sup>21,22</sup> While microbial infections are among the etiological factors responsible for inflammation in the male genital tract, a large number of epididymitis patients lack any evidence of microbial infections.<sup>1</sup> Knowledge regarding noninfectious epididymitis is rather limited. Understanding the mechanisms underlying noninfectious epididymitis might benefit the diagnosis and therapy of this disease.<sup>23</sup> The present study demonstrated that DMGCs induced epididymitis in mice.

Several risk factors for noninfectious epididymitis have been proposed including a previous systemic infection,<sup>24</sup> certain medications,<sup>25,26</sup> and vasectomy.<sup>27</sup> Moreover, strenuous labor and long-term sitting or bicycle riding are also associated with epididymitis. However, the causative agents of noninfectious epididymitis remain to be defined. A recent study demonstrated that intraperitoneal injection of lipopolysaccharide (LPS) resulted in epididymitis in mice.<sup>20</sup> Epididymitis after LPS injection was associated with an accumulation of DMGCs in the epididymis, suggesting that DMGCs trigger epididymitis caused by a postsystemic infection.<sup>20</sup> The present study confirmed that DMGCs induce epididymitis after an intraperitoneal injection of busulfan in mice through the activation of innate immune responses in EECs, which may be an etiological factor of medication-associated epididymitis. It would be interesting to clarify whether DMGCs are triggers of human noninfectious epididymitis associated with different risk factors because most risk factors can damage male germ cells.<sup>20,28–30</sup>

Most male germ cells develop a long time after the establishment of immune tolerance to self-antigens, thus allowing them to produce immunogenic antigens. Augmentation of the apoptosis of male germ cells resulted in inflammatory conditions in the testis.<sup>31,32</sup> Moreover, a defect in the phagocytic removal of apoptotic male germ cells by Sertoli cells favored autoimmune orchitis.<sup>11</sup> These early studies suggested that DMGCs might induce orchitis. Although DMGCs may be transmitted luminally to the epididymis from the testis, whether DMGCs induce epididymitis has not been investigated. The present study demonstrated



**Figure 6:** Role of *TNF-α* in epididymitis. (a) Germ cell damage in the testis. Busulfan was intraperitoneally injected into WT and *TNF-α*<sup>-/-</sup> mice for 4 weeks. Mice injected with DMSO alone served as theCtrls. Paraffin sections of the testes were stained with H and E. Black arrows indicate Sertoli cells. (b) Cell infiltrations. Paraffin sections of the cauda epididymides were stained with H and E at 4 weeks after busulfan injection. Asterisks and black arrowheads indicate epididymal tubules with DMGCs and cell infiltrations. (c) Macrophages. Paraffin sections were stained with specific antibodies against F4/80. (d) Lymphocytes. Paraffin sections of the epididymides were stained with primary antibodies against CD45. The insets in the upper right corners represent the negative controls. Images are representatives of at least five mice. (e) Expression of chemokines. EECs were cultured in the presence or absence (Ctrl) of DMGCs for 8 h. The relative mRNA levels of *MCP-1*, *MCP-5*, and *CXCL10* were determined by real-time qRT-PCR. (f) Cytokine secretion. EECs were treated as described in e for 24 h. The protein levels of *TNF-α*, *MCP-1*, and *CXCL10* in culture media were measured by ELISA. (g) Effect of *TNF-α* on *MCP-1* and *CXCL10* production. EECs were stimulated with 5 ng ml<sup>-1</sup> recombinant mouse *TNF-α* for 24 h. *MCP-1* and *CXCL10* levels in media were measured by ELISA. The data are expressed as the mean ± s.d. of three independent experiments. \**P* < 0.05 and \*\**P* < 0.01. qRT-PCR: quantitative reverse transcription polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay; s.d.: standard deviation; Ctrl: control; *TNF-α*: tumor necrosis factor-α; WT: wild-type; DMSO: dimethyl sulfoxide; DMGCs: damaged male germ cells; MCP: monocyte chemoattractant protein; CXCL10: chemokine ligand-10; EECs: epididymal epithelial cells.

that busulfan injection results in epididymitis concomitant with germ cell damage in the testis and an accumulation of DMGCs in the epididymis. Epididymitis was not induced after a second busulfan injection in mice lacking germ cells in the testis. These results provide substantial evidence that DMGCs induce epididymitis.

We did not observe orchitis when male germ cells were damaged in the testis after busulfan injection. The different inflammatory conditions in the testis and the epididymis may be explained by their immune microenvironments. The testis is a remarkable immunoprivileged organ that is tightly controlled by tissue structure, cellular interaction, and dense immunosuppressive networks.<sup>33</sup> Although the epididymis has immunoprivileged properties that protect spermatozoa from detrimental immune responses, the immune privilege of the epididymis is not as complete as that of the testis.<sup>13</sup> Many immune cells reside in the epididymis.<sup>34</sup> Notably, most dendritic cells are localized in the caput epididymis, which is thought to support self-tolerance toward sperm antigens.<sup>35</sup> Conversely, blood and lymphatic capillaries are

predominantly distributed in the cauda epididymal tubules.<sup>36</sup> The differential distributions of immune cells and circulating capillaries might lead to variable susceptibilities to antigen stimulation in different regions of the epididymis, resulting in the predominance of caudal epididymitis. The accumulation of DMGCs in the cauda epididymal tubules should also predominantly induce inflammation in the cauda segment. Accordingly, caudal epididymitis is frequently observed in outpatients, which corresponds to the observations in the present study.

To understand the mechanism by which DMGCs induce epididymitis, we examined the expression and function of cytokines that facilitate inflammation. Toll-like receptors (TLR) initiate innate immune responses in many cell types after challenge with TLR ligands, and this facilitates inflammatory conditions by inducing the expression of inflammatory cytokines.<sup>37</sup> Various TLRs are expressed in the epididymis and initiate an innate immune response in EECs.<sup>38,39</sup> High-mobility group box 1 (HMGB1) and heat shock proteins (HSPs) are well-defined endogenous TLR ligands.<sup>40–45</sup> HMGB1 and

certain HSPs are abundantly expressed in male germ cells and can be released under stress conditions.<sup>46,47</sup> Therefore, it is reasonable to speculate that DMGCs release endogenous TLR ligands that trigger innate immune responses in EECs. We previously demonstrated that DMGCs induce inflammatory cytokine expression in Sertoli cells through the activation of TLR2 and TLR4. However, we did not observe immune cell infiltration in the testis. The lower immune privilege of the epididymis may explain why epididymitis occurs more frequently than orchitis in humans.<sup>1</sup>

We demonstrated that DMGCs induce the expression of major pro-inflammatory cytokines in EECs, including *TNF- $\alpha$* , *IL-6*, and *IL-1 $\beta$* , suggesting that innate immune responses can be triggered by endogenous TLR ligands in EECs. Our recent study demonstrated that *TNF- $\alpha$*  is critical for LPS-induced epididymitis.<sup>20</sup> An early study showed that *TNF- $\alpha$*  has a role in the pathogenesis of experimental autoimmune orchitis.<sup>19</sup> Therefore, we assessed the role of *TNF- $\alpha$*  in the development of epididymitis after busulfan injection in the present study. Epididymitis was not observed in *TNF- $\alpha$* <sup>-/-</sup> mice after busulfan injection, suggesting that *TNF- $\alpha$*  also plays an important role in DMGC-induced epididymitis. This result confirms that DMGCs induce immune responses in wild-type mice, but this might be because *TNF- $\alpha$* <sup>-/-</sup> mice have a defective immune system.

The expression of major chemokines in EECs in the presence of DMGCs was examined to understand the mechanisms underlying leukocyte infiltration. We found that *MCP-1*, *MCP-5*, and *CXCL10* were significantly upregulated in EECs after challenge with DMGCs, whereas the expressions of *CCL3*, *CCL17*, and *CCL22* were unaltered. *MCP-1* and *MCP-5* promote macrophage migration. Accordingly, macrophages were dramatically increased in the epididymitis after busulfan injection. *CXCL10* is a pleiotropic cytokine involved in the pathogenesis of autoimmune diseases by recruiting leukocytes and inducing apoptosis.<sup>48</sup> We recently demonstrated that the production of *CXCL10* by Sertoli cells induced male germ cell apoptosis.<sup>49</sup> The role of *CXCL10* produced by EECs in the pathogenesis of epididymitis remains unclear. Notably, the upregulation of *MCP-1*, *MCP-5*, and *CXCL10* was significantly reduced in *TNF- $\alpha$* <sup>-/-</sup> EECs, suggesting that *TNF- $\alpha$*  is involved in the upregulation of chemokines. We confirmed that recombinant *TNF- $\alpha$*  significantly upregulated the production of *MCP-1* and *CXCL10* in EECs. These results correspond to previous observations in other cell types.<sup>49-51</sup> Therefore, *TNF- $\alpha$*  might play a central role in the initiation of epididymitis through the induction of chemokines in EECs.

Epididymitis was observed in 83.3% of mice 4 weeks after busulfan injection, and this incidence was markedly reduced thereafter. These results suggest that DMGCs might transiently induce epididymitis, which should be mostly recovered. These phenomena correspond to the clinical observations that only minor acute epididymitis advances to chronic disease in humans. Therefore, epididymitis in mice after busulfan injection should be a useful model to investigate the pathomechanism of human noninfectious epididymitis. However, we cannot explain why busulfan injection did not induce epididymitis in some mice.

## CONCLUSION

The present study showed that DMGCs induce epididymitis in mice. DMGC-induced *TNF- $\alpha$*  production in EECs plays a central role in the initiation of epididymitis. It would be interesting to clarify whether germ cell damage is a common etiological factor for human noninfectious epididymitis associated with various risk factors that may damage germ cells. The identification of the specific male germ

cell antigens that induce epididymitis might aid in the development of diagnostic and therapeutic strategies for noninfectious epididymitis.

## AUTHOR CONTRIBUTIONS

DSH and WHL conceived and designed the experiments and wrote the paper. FW, XQY, HW, MLG, RC, WJZ, RQH, AJL, and YMC performed all the experiments. All authors read and approved the final manuscript.

## COMPETING INTERESTS

All authors declared no competing interests.

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## REFERENCES

- Fijak M, Pilatz A, Hedger MP, Nicolas N, Bhushan S, *et al*. Infectious, inflammatory and 'autoimmune' male factor infertility: how do rodent models inform clinical practice? *Hum Reprod Update* 2018; 24: 416-41.
- Ekwere PD. Immunological infertility among Nigerian men: incidence of circulating antisperm auto-antibodies and some clinical observations: a preliminary report. *Br J Urol* 1995; 76: 366-70.
- Niederberger C. WHO manual for the standardized investigation, diagnosis and management of the infertile male. *Urology* 2000; 57: 208.
- Trojan TH, Lishnak TS, Heiman D. Epididymitis and orchitis: an overview. *Am Fam Physician* 2009; 79: 583-7.
- Kaver I, Matzkin H, Braf ZF. Epididymo-orchitis: a retrospective study of 121 patients. *J Fam Pract* 1990; 30: 548-52.
- McConaghy JR, Panchal B. Epididymitis: an overview. *Am Fam Physician* 2016; 94: 723-6.
- Emerson C, Dinsmore WW, Quah SP. Are we missing mumps epididymo-orchitis? *Int J STD AIDS* 2007; 18: 341-2.
- Chan PT, Schlegel PN. Inflammatory conditions of the male excurrent ductal system. Part I. *J Androl* 2002; 23: 453-60.
- Fijak M, Meinhardt A. The testis in immune privilege. *Immunol Rev* 2006; 213: 66-81.
- Schuppe HC, Meinhardt A, Allam JP, Bergmann M, Weidner W, *et al*. Chronic orchitis: a neglected cause of male infertility? *Andrologia* 2008; 40: 84-91.
- Pelletier RM, Yoon SR, Akpovi CD, Silvas E, Vitale ML. Defects in the regulatory clearance mechanisms favor the breakdown of self-tolerance during spontaneous autoimmune orchitis. *Am J Physiol Regul Integr Comp Physiol* 2009; 296: R743-62.
- Zhang X, Wang T, Deng T, Xiong W, Lui P, *et al*. Damaged spermatogenic cells induce inflammatory gene expression in mouse Sertoli cells through the activation of Toll-like receptors 2 and 4. *Mol Cell Endocrinol* 2013; 365: 162-73.
- Hedger MP. Immunophysiology and pathology of inflammation in the testis and epididymis. *J Androl* 2011; 32: 625-40.
- Buff S, Lambert V, Marchal T, Guerin P. Isolation, culture and characteristics of epididymal epithelial cells from adult cats. *Theriogenology* 2005; 64: 1603-18.
- Graves CL, Harden SW, LaPato M, Nelson M, Amador B, *et al*. A method for high purity intestinal epithelial cell culture from adult human and murine tissues for the investigation of innate immune function. *J Immunol Methods* 2014; 414: 20-31.
- Wang T, Zhang X, Chen Q, Deng T, Zhang Y, *et al*. Toll-like receptor 3-initiated antiviral responses in mouse male germ cells *in vitro*. *Biol Reprod* 2012; 86: 106.
- Encinas G, Zogbi C, Stumpp T. Detection of four germ cell markers in rats during testis morphogenesis: differences and similarities with mice. *Cells Tissues Organs* 2012; 195: 443-55.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001; 25: 402-8.
- Yule TD, Tung KS. Experimental autoimmune orchitis induced by testis and sperm antigen-specific T cell clones: an important pathogenic cytokine is tumor necrosis factor. *Endocrinology* 1993; 133: 1098-107.
- Wang F, Liu W, Jiang Q, Gong M, Chen R, *et al*. Lipopolysaccharide-induced testicular dysfunction and epididymitis in mice: a critical role of tumor necrosis factor alpha. *Biol Reprod* 2019; 100: 849-61.
- Haidl G, Allam JP, Schuppe HC. Chronic epididymitis: impact on semen parameters and therapeutic options. *Andrologia* 2008; 40: 92-6.



- 22 Luzzi GA, O'Brien TS. Acute epididymitis. *BJU Int* 2001; 87: 747–55.
- 23 Haidl G, Haidl F, Allam JP. Therapeutic options in male genital tract inflammation. *Andrologia* 2018; e13207.
- 24 Somekh E, Gorenstein A, Serour F. Acute epididymitis in boys: evidence of a post-infectious etiology. *J Urol* 2004; 171: 391–4.
- 25 Nikolaou M, Ikonomidis I, Lekakis I, Tsiodras S, Kremastinos D. Amiodarone-induced epididymitis: a case report and review of the literature. *Int J Cardiol* 2007; 121: e15–6.
- 26 Gasparich JP, Mason JT, Greene HL, Berger RE, Krieger JN. Amiodarone-associated epididymitis: drug-related epididymitis in the absence of infection. *J Urol* 1985; 133: 971–2.
- 27 West AF, Leung HY, Powell PH. Epididymectomy is an effective treatment for scrotal pain after vasectomy. *BJU Int* 2000; 85: 1097–9.
- 28 O'Neill DA, McVicar CM, McClure N, Maxwell P, Cooke I, *et al*. Reduced sperm yield from testicular biopsies of vasectomized men is due to increased apoptosis. *Fertil Steril* 2007; 87: 834–41.
- 29 Shiraiishi K, Naito K, Yoshida K. Vasectomy impairs spermatogenesis through germ cell apoptosis mediated by the p53-Bax pathway in rats. *J Urol* 2001; 166: 1565–71.
- 30 Yin Y, DeWolf WC, Morgentaler A. Experimental cryptorchidism induces testicular germ cell apoptosis by p53-dependent and -independent pathways in mice. *Biol Reprod* 1998; 58: 492–6.
- 31 Goldacre MJ, Wotton CJ, Seagroatt V, Yeates D. Immune-related disease before and after vasectomy: an epidemiological database study. *Hum Reprod* 2007; 22: 1273–8.
- 32 Nistal M, Riestra ML, Paniagua R. Focal orchitis in undescended testes: discussion of pathogenetic mechanisms of tubular atrophy. *Arch Pathol Lab Med* 2002; 126: 64–9.
- 33 Li N, Wang T, Han D. Structural, cellular and molecular aspects of immune privilege in the testis. *Front Immunol* 2012; 3: 152.
- 34 Flickinger CJ, Bush LA, Howards SS, Herr JC. Distribution of leukocytes in the epithelium and interstitium of four regions of the Lewis rat epididymis. *Anat Rec* 1997; 248: 380–90.
- 35 Da Silva N, Cortez-Retamozo V, Reinecker HC, Wildgruber M, Hill E, *et al*. A dense network of dendritic cells populates the murine epididymis. *Reproduction* 2011; 141: 653–63.
- 36 Hirai S, Naito M, Terayama H, Ning Q, Miura M, *et al*. Difference in abundance of blood and lymphatic capillaries in the murine epididymis. *Med Mol Morphol* 2010; 43: 37–42.
- 37 Leifer CA, Medvedev AE. Molecular mechanisms of regulation of Toll-like receptor signaling. *J Leukoc Biol* 2016; 100: 927–41.
- 38 Zhu W, Zhao S, Liu Z, Cheng L, Wang Q, *et al*. Pattern recognition receptor-initiated innate antiviral responses in mouse epididymal epithelial cells. *J Immunol* 2015; 194: 4825–35.
- 39 Cheng L, Chen Q, Zhu W, Wu H, Wang Q, *et al*. Toll-like receptors 4 and 5 cooperatively initiate the innate immune responses to uropathogenic *Escherichia coli* Infection in mouse epididymal epithelial cells. *Biol Reprod* 2016; 94: 58.
- 40 Chase MA, Wheeler DS, Lierl KM, Hughes VS, Wong HR, *et al*. Hsp72 induces inflammation and regulates cytokine production in airway epithelium through a TLR4- and NF-kappaB-dependent mechanism. *J Immunol* 2007; 179: 6318–24.
- 41 Roelofs MF, Boelens WC, Joosten LA, Abdollahi-Roodsaz S, Geurts J, *et al*. Identification of small heat shock protein B8 (HSP22) as a novel TLR4 ligand and potential involvement in the pathogenesis of rheumatoid arthritis. *J Immunol* 2006; 176: 7021–7.
- 42 Vabulac RM, Ahmad-Nejad P, da Costa C, Miethke T, Kirschning CJ, *et al*. Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. *J Biol Chem* 2001; 276: 31332–9.
- 43 Wheeler DS, Chase MA, Senft AP, Poynter SE, Wong HR, *et al*. Extracellular Hsp72, an endogenous DAMP, is released by virally infected airway epithelial cells and activates neutrophils via Toll-like receptor (TLR)-4. *Respir Res* 2009; 10: 31.
- 44 Curtin JF, Liu N, Candolfi M, Xiong W, Assi H, *et al*. HMGB1 mediates endogenous TLR2 activation and brain tumor regression. *PLoS Med* 2009; 6: e10.
- 45 Park JS, Gamboni-Robertson F, He Q, Svetkauskaite D, Kim JY, *et al*. High mobility group box 1 protein interacts with multiple Toll-like receptors. *Am J Physiol Cell Physiol* 2006; 290: C917–24.
- 46 Biggiogera M, Tanguay RM, Marin R, Wu Y, Martin TE, *et al*. Localization of heat shock proteins in mouse male germ cells: an immunoelectron microscopical study. *Exp Cell Res* 1996; 229: 77–85.
- 47 Zetterstrom CK, Strand ML, Soder O. The high mobility group box chromosomal protein 1 is expressed in the human and rat testis where it may function as an antibacterial factor. *Hum Reprod* 2006; 21: 2801–9.
- 48 Antonelli A, Ferrari SM, Giuggioli D, Ferrannini E, Ferri C, *et al*. Chemokine (C-X-C motif) ligand (CXCL)10 in autoimmune diseases. *Autoimmun Rev* 2014; 13: 272–80.
- 49 Jiang Q, Wang F, Shi L, Zhao X, Gong M, *et al*. C-X-C motif chemokine ligand 10 produced by mouse Sertoli cells in response to mumps virus infection induces male germ cell apoptosis. *Cell Death Dis* 2017; 8: e3146.
- 50 Mukaida N, Zachariae CC, Gusella GL, Matsushima K. Dexamethasone inhibits the induction of monocyte chemotactic-activating factor production by IL-1 or tumor necrosis factor. *J Immunol* 1991; 146: 1212–5.
- 51 Hu J, You S, Li W, Wang D, Nagpal ML, *et al*. Expression and regulation of interferon-gamma-inducible protein 10 gene in rat Leydig cells. *Endocrinology* 1998; 139: 3637–45.

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**Supplementary Table 1: Incidence of epididymitis**

<i>+Busulfan (weeks)</i>	<i>Total mice (n)</i>	<i>Incidence of epididymitis (n)</i>	<i>Percentage with epididymitis</i>
1	20	0	0
2	20	0	0
3	20	15	75.0
4	30	25	83.3
5	20	10	50.0
6	20	2	10.0
7	20	1	5.0
8	20	1	5.0

C57BL/6J male mice (10-old-week) were intraperitoneally injected with 25 mg kg<sup>-1</sup> body weight for the indicated durations. The incidence and percentage of males with epididymitis were determined from histological analysis and immunohistochemical staining

**Supplementary Table 2: Primers used for quantitative reverse transcription polymerase chain reaction**

<i>Primer pairs (5'→3')</i>		
<i>Target genes</i>	<i>Forward</i>	<i>Reverse</i>
<i>β-Actin</i>	GAAATCGTGCGTGACATCAAAG	TGTAGTTTCATGGATGCCACAG
<i>TNF-α</i>	CATCTTCTCAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
<i>IL-6</i>	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTTCATACA
<i>IL-1β</i>	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
<i>MCP-1</i>	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
<i>MCP-5</i>	CCTGTGGCCTTGGCCTCAA	GAGGTGCTGATGTACCAGTTGG
<i>CCL3</i>	TGTACCATGACACTCTGCAAC	CAACGATGAATTGGCGTGGAA
<i>CXCL10</i>	CCAAGTGCTGCCGTCATTTTC	TCCCTAAGGCCCTCATTCTCA
<i>CCL17</i>	TGCTCGAGCCACCAATGTAG	ACAGTCAGAAACACGATGGCA
<i>CCL22</i>	ACCTCTGATGCAGGTCCCTA	CTTGCGGCAGGATTTTGAGG

*TNF-α*: tumor necrosis factor-α; *IL-6*: interleukin-6; *IL-1β*: interleukin-1β; *MCP-1*: monocyte chemoattractant protein-1; *MCP-5*: monocyte chemoattractant protein-5; *CCL3*: chemokine (C-C motif) ligand 3; *CXCL10*: chemokine ligand-10