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Damaged spermatogenic cells induce inflammatory gene expression in mouse Sertoli cells through the activation of Toll-like receptors 2 and 4

Xiaoyan Zhang, Tao Wang, Tingting Deng, Weipeng Xiong, Peng Lui, Nan Li, Yongmei Chen, Daishu Han *

Department of Cell Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, School of Basic Medicine, Peking Union Medical College, Beijing, China

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ABSTRACT

Testicular inflammation, including noninfectious inflammatory responses in the testis, may impair male fertility. Mechanisms underlying the initiation of noninfectious testicular inflammation are poorly understood. In the current study, we demonstrate that damaged spermatogenic cell products (DSCPs) induce expression of various inflammatory mediators, including TNF-α, IL-1β, IL-6, and macrophage chemotactic protein 1 (MCP-1), in Sertoli cells. Notably, the DSCP-induced inflammatory gene expression was significantly reduced by knockout Toll-like receptor (TLR)2 or TLR4, and abolished by double knockout TLR2 and TLR4 ($TLR2^{-/-}TLR4^{-/-}$). MCP-1 secreted by Sertoli cells after stimulation with DSCPs promotes macrophage migration. We also provide evidence that busulfan-induced spermatogenic cell damages *in vivo* upregulate TNF-α and MCP-1 expression in Sertoli cells, and facilitate macrophage infiltration into the testis in wild-type mice. These phenomena were not observed in $TLR2^{-/-}TLR4^{-/-}$ mice. Data indicate that DSCPs induce inflammatory gene expression in Sertoli cells via the activation of TLR2 and TLR4, which may initiate noninfectious inflammatory responses in the testis. The results provide novel insights into the mechanisms underlying damaged spermatogenic cell-induced testicular inflammation.

1. Introduction

Although the testis is an immune privilege site, the inflammatory condition of the testis is one of etiological factors of male infertility (Schuppe et al., 2008). Testicular inflammation can be caused by local or systemic infectious and noninfectious origins. In contrast to testicular inflammation due to infectious origin, the mechanisms underlying the initiation of noninfectious inflammation in the testis are poorly understood. Involvement of Toll-like receptors (TLRs) in testicular innate immune responses to alloantigens has been recently revealed (Hedger, 2011). In the present study, we investigate roles of TLRs in noninfectious testicular inflammation in response to spermatogenic cell damage that may occur in some pathological conditions.

Toll-like receptors (TLRs) are a family of triggers of the innate and adaptive immune responses against invading pathogens (Akira, 2009). To date, 13 distinct TLR members have been identified in mammals (Takeda and Akira, 2005). TLRs recognize highly conserved, pathogen-coded molecular structures termed pathogen-associated molecular patterns (PAMPs) and trigger immune responses. TLRs can be also recognized and activated by endogenous ligands released from damaged tissues and necrotic cells, which are termed damage-associated molecular patterns (DAMPs) (Seong and Matzinger, 2004). Numerous TLR endogenous ligands have been identified (Yu et al., 2010). Most of them activate TLR2 and TLR4 (Asea et al., 2002; Vabulas et al., 2002). The mRNA released from necrotic cells may activate TLR3 (Kariko et al., 2004). TLR9 can be activated by chromosomal DNA from the damaged cells (Viglianti et al., 2003). Activation of TLRs by DAMPs induces inflammatory gene expression, which may facilitate injury repair and lead to further pathological conditions such as autoimmune diseases (Piccinini and Midwood, 2010; Yu et al., 2010).

TLR activation triggers two signaling pathways: myeloid differentiation protein 88 (MyD88)-dependent and Toll/interleukin-1 receptor domain-containing adaptor inducing interferon-β (TRIF)-dependent pathways (Yamamoto and Takeda, 2010). The (MyD88)-dependent pathway leads to the activation of nuclear factor κB (NF-κB) and MAPKs, thus inducing expression of various inflammatory cytokines and chemokines, including TNF-α, IL-1β, IL-6, and macrophage chemotactic protein 1 (MCP-1). The TRIF-dependent pathway leads to predominantly the activation of interferon regulatory factor 3 (IRF3), which induces production of type 1 interferons (IFN-α and IFN-β). With the exception of TLR3 and TLR4, all other TLRs trigger immune response exclusively through the MyD88-dependent pathway. TLR3 signals exclusively through the TRIF-dependent pathway, whereas TLR4 initiates both the two pathways (Wang et al., 2009). The TLR-induced cytokines participate in the host defense against pathogens through the

* Corresponding author. Address: Department of Cell Biology, PUMC & CAMS, 5 Dong Dan San Tiao, Beijing 100005, China. Tel.: +86 10 65296457; fax: +86 10 65296466.
E-mail address: dshan@ibms.pumc.edu.cn (D. Han).

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regulation of immune responses or the direct killing of invading pathogens.

A growing body of evidence shows that TLRs play broad roles in testicular function and inflammation (Hedger, 2011). Various TLRs are expressed in the testicular cells in different species (Bhushan et al., 2008; Fujita et al., 2011; Palladino et al., 2008). Mouse Sertoli cells express TLR2 to TLR5, which can be activated by their respective PAMPs (Riccioli et al., 2006; Starace et al., 2008; Sun et al., 2010; Winnall et al., 2011; Wu et al., 2008). Expression, as well as activation, of TLR3 and TLR4 in mouse Leydig cells, and TLR3 in male germ cells were recently demonstrated (Shang et al., 2011; Wang et al., 2012). These studies have suggested that TLRs in the testicular cells play a role for the testis to mount appropriate local responses to invading microbial antigens. By contrast, TLR signaling and its role in response to endogenous agonists in the testis has not been extensively studied. Here, we demonstrate that damaged spermatogenic cell products (DSCPs) induce inflammatory mediator expression in Sertoli cells through the activation of TLR2 and TLR4, which may trigger noninfectious testicular inflammation in response to damaged spermatogenic cells.

2. Materials and methods

2.1. Animals

C57BL/6 strain mice were obtained from the Laboratorial Animal Center of Peking Medical College (Beijing, China). TLR knockout ($TLR2^{-/-}$, $TLR3^{-/-}$, $TLR4^{-/-}$, and $TLR5^{-/-}$) mice were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). TLR2 and TLR4 double knockout ($TLR2^{-/-}TLR4^{-/-}$) and wild-type (WT) mice were produced by cross-mating of $TLR2^{-/-}$ and $TLR4^{-/-}$ mice. The animals were maintained in a pathogen-free, temperature- and humidity-controlled room on a 12 h light/dark cycle, and had free access to food and water. All the measures taken for the mice were in accordance with guidelines for the Care and Use of Laboratory Animals approved by the Chinese Council on Animal Care.

2.2. Busulfan treatment

Male mice of 8–10 weeks of age were treated with a single intra-peritoneal injection of 25 mg/kg busulfan (Sigma). Busulfan was first dissolved in DMSO before equal volume of distilled water added.

2.3. Antibodies

Rabbit anti-NF-kBp65 (sc-372), rat anti-p38 (sc-1749), rat anti-JNK (sc-7345), goat anti-ERK (sc-94) and goat anti-phospho-ERK (sc-7976R) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-phospho-p38 (No. 4631), rabbit anti-phospho-p65 (No. 5970), rabbit anti-phospho-JNK (No. 9251), rat anti-phospho-p38 (No. 4631) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-TNF-α (Ab34674), rat anti-F4/80 (Ab664) antibodies were purchased from Abcam (Cambridge, UK). Goat anti-MCP-1 (AF-479-NA) antibody was purchased from R&D systems (Minneapolis, MN, USA). Monoclonal antibody to LPS core was purchased from HyCutt Biotechnology (Uden, The Netherlands).

2.4. Isolation of Sertoli cells

Three-week-old mice were used for isolation of Sertoli cells based on a procedure described previously with modifications (Wang et al., 2006). Briefly, decapsulated testes were incubated with 0.5 mg/ml collagenase (Sigma, St. Louis, MO, USA) at room temperature for 15 min with gentle oscillation, and then filtered through 80 μm copper meshes to remove interstitial cells. The interstitial cells can be collected at this step. The seminiferous tubules were resuspended in the 0.5 mg/ml collagenase at room temperature for 20 min to remove myoid cells. After filtration through 80 μm copper meshes, the tubules were cut into small pieces (~1 mm) and incubated with 0.5 mg/ml hyaluronidase (Sigma) for 15 min with oscillation. After washing thrice with PBS, the dispersed cells were cultured in F12/DMEM medium (Life Technologies, Grand Island, NY, USA) supplemented with sodium bicarbonate (1.2 mg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal calf serum (FCS, Life Technologies) in a humidified atmosphere with 5% CO_2 at 32 °C. The spermatogenic cells that did not adhere on the culture dishes at 24 h post-culture were removed by washing with PBS, and those that adhered to Sertoli cells were removed by treatment with a hypotonic solution (20 mM Tris, pH 7.4) for 1 min. Twenty-four hours later, the Sertoli cells were detached and reseeded in serum-free F12/DMEM for additional 24 h before treatments. The purity of Sertoli cells was more than 96% based on immunostaining for Wilms' tumor nuclear protein 1, a marker of Sertoli cells (Sharpe et al., 2003). Contaminants were mainly peritubular myoid cells.

2.5. Isolation of spermatogenic cells and preparation of DSCPs

Spermatogenic cells were isolated from 7-week-old C57BL/6 mice. Briefly, after removal of interstitial and peritubular myoid cells according to the procedures for Sertoli cell isolation mentioned above, serminiferous tubules were cut into small pieces, and germ cells were dispersed by gentle pipetting. After filtration through 80 μm copper meshes, cell suspensions were collected and cultured in F12/DMEM medium containing 10% FCS for 4 h at 32 °C in a humidified atmosphere with 5% CO_2 to further eliminate the contaminating Sertoli cells and peritubular myoid cells. The spermatogenic cells were identified by examining cell nuclear morphology after staining with 4′,6-diamidio-2-phenylindole (DAPI, Zhongshan Biotechnology Co., Beijing, China) based on manufacture's introduction. Somatic cell contaminants were excluded by analyzing mRNA of testicular somatic cell marker genes: Wt-1 for Sertoli cells, 3β-hydroxysteriod dehydrogenase (3β-Hsd) for Leydig cells (Teerds et al., 2007), fibronectin (Fn) for peritubular myoid cells (Tung et al., 1984). The spermatogenic cells were resuspended in serum-free F12/DMEM medium containing penicillin (100 U/ml) and streptomycin (100 μg/ml) to a density of 1×10^8 cells/ml, and incubated in a humidified atmosphere with 5% CO_2 at 32 °C for 48 h. During this time, most spermatogenic cells were damaged through apoptosis and necrosis. Apoptotic and necrotic rates of the spermatogenic cells were assessed by flow cytometry. The conditioned media of the damaged spermatogenic cells were collected after centrifugation at 1500*g* for 5 min at room temperature, and used as DSCPs. All the solutions for the isolation and culture of spermatogenic cells were filtered through a Charged Durapore Hydrophilic Cartridge Filter (Merck Millipore, Darmstadt, Germany) to remove the possible endotoxin contamination. This filter contains positively charged resins that can aid in the removal of the negatively charged soluble bacterial endotoxins (LPS) from the solutions.

2.6. Flow cytometry

Spermatogenic cells were washed with cold PBS, and stained with fluorescein isothiocyanate (FITC)-conjugated annexin V (FITC-AnxV, Zhongshan Biotechnology Co.) and propidium iodide (PI, Sigma) following manufacturer's instructions. The cells were analyzed using BD FACSSanto flow cytometer (BD Biosciences, San Jose, CA, USA).

2.7. Isolation and chemotactic migration of peritoneal macrophages

Peritoneal macrophages were isolated from 8-week-old mice based on a previous description (Chong et al., 2005). The peritoneal cavities were lavaged with 5 ml cold 1 × PBS to collect peritoneal cells. The cells were cultured in RPMI-1640 (Life Technologies) supplemented with 10% FCS on culture dishes in humidified atmosphere containing 5% CO_2 at 37 °C. Non-adherent cells after 24 h post-culture were removed by washing thrice with PBS, and more than 95% attached cells were macrophages based on immunostaining for F4/80, a marker of macrophages (Hume et al., 1984). Contaminants were mainly fibroblasts based on cell morphology.

The macrophages were assayed for their ability to migrate through a polycarbonate membrane with 8 μm pore diameter in Corning Transwell Supports (3422, Corning Incorporated, NY, USA) according to manufacturer's instruction. Briefly, the cells were seeded on the upper chambers of the wells at a density of 1×10^5/well in serum-free RPMI-1640 medium, conditioned medium (CM) of Sertoli cells after stimulation with DSCPs (CM + DSCPs), CM of Sertoli cells without DSCP stimulation, or DSCPs alone were placed in the lower chambers. In control, serum-free F12/DMEM media were placed in the lower chambers. The Transwell Supports were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h. The membranes were fixed with 90% ethanol and stained with Coomassie blue. The cells on the lower surface of membranes were counted under a microscope (IX-51, Olympus. Tokyo, Japan).

2.8. Real-time quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using Trizol™ reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was treated with RNase-free DNase 1 (Invitrogen) to remove potential contamination by genomic DNA. The absence of genomic DNA was confirmed by PCR to amplify β-actin using the RNA without reverse transcription. The primers for amplifying genomic DNA of β-actin were: upper stream 5′- GAG GGA AAT CGT GCG TGA C -3′, and down stream 5′- CTG GAA GGT GGA CAG TGA G -3′. The RNA (1 μg) was reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WT, USA). PCR was performed with Power SYBR® Green PCR master mix kit (Applied Biosystems, Fostercity, CA, USA) using an ABI PRISM 7300 real-time cycler (Applied Biosystems). The transcript levels of target genes were normalized to β-actin using the comparative threshold cycle method, as described in Applied Biosystems User Bulletin No. 2 (P/N 4303859). The sequences of primer pairs for PCRs are listed in Table 1.

Table 1
Primers used for real-time PCRs.

Target genes	Primer pairs (5′–3′)	
	Forward	Reverse
c-Kit	ACTTCGCCTGACAAGAGTTG	CGTACGTCAGGATTTCTGGTT
CD68	AGAGGGGCTGGTAGGTTGAT	CCAGCTGTTCACCTTGACCT
CD163	GGTGGACACAGAATGGTTCTTC	CCAGGAGCGTTAGTGACAGC
FN	ATGTGGACCCCTCCTGATAGT	GCCCAGTGATTTCAGCAAAGG
3β-HSD	TATTCTCGGTTGTACGGGCAA	GTGCTACCTGTCAGTGTGACC
IFN-α	GACCTCCACCAGCAGCTCAA	ACCCCCACCTGCTGCAT
IFN-β	GACGTGGGAGATGTCCTCAAC	GGTACCTTTGCACCCTCCAGTA
IL-1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
IL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
MCP-1	TTAACGCCCCACTCACCTGCTG	GCTTCTTTGGGACACCTGCTGC
Prm-2	GCCTCCTACATTTCCTGCAC	AGAAGGCGGAGGAGACACTC
SCP-2	AGGCCTCCACTAGGGTTGAT	TCACTTACGAAGCACTGGGG
TNF-α	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
WT-1	TCTTCCGAGGCATTCAGGAT	TGCTGACCGGACAAGAGTTG
β-actin	GAAATCGTGCGTGACATCAAAG	TGTAGTTTCATGGATGCCACAG

FN, figronectin; 3β-HSD, 3-hydroxysteroid dehydrogenase; MCP-1, macrophage chemotactic protein 1; Prm-2, protamin 2; SCP-2, synaptonemal complex protein 2; WT-1, Wilms' tumor nuclear factor 1.

2.9. ELISA

Sertoli cells were cultured in six well plates at the density of 5×10^5 cells/well containing 1 ml serum-free F12/DMEM and treated with DSCPs for 16 h. The concentration of cytokines in the medium was measured using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. LPS level in DSCPs was measured using ELISA kit (MyBioSource, San Diego, CA).

2.10. Western blot analysis

Sertoli cells and seminiferous tubules were lysed using lysis buffer (Applygen Technologies Inc., Beijing, China). Protein concentration of the cell lysates was determined using bicinchonic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of protein (20 μg) were separated on 10% SDS–PAGE gel, and subsequently electrotransferred onto polyvinyl difluoride membranes (Millipore, Bedford, MA, USA). After blocking with 5% nonfat milk in Tris-buffered saline (TBS, pH 7.4) for 1 h, the membranes were incubated with the primary antibodies overnight at 4 °C. After washing twice with TBS containing 0.1% Tween-20, the membranes were incubated with the appropriated peroxidase-conjugated secondary antibodies (Zhongshan Biotechnology Co.) at room temperature for 1 h. Peroxidase color visualization was achieved using an enhanced chemiluminescence detection kit (Zhongshan Biotechnology Co.).

2.11. Immunohistochemistry

The testes were fixed in 4% paraformadehyde for 24 h. Frozen sections were prepared for detecting cytokines and macrophages. After cryoprotection in 30% sucrose, frozen sections were cut to a thickness of 8 μm using Leica CM1950 (Leica Biosystems, Germany). Paraffin sections were prepared to detect apoptotic cells. After embedding in paraffin, the testes were cut in thickness of 5 μm. The sections were incubated with 1 × PBS containing 3% H_2O_2 for 15 min to block endogenous peroxidase activity. After blocking with 5% normal rabbit serum in PBS for 1 h at room temperature, the sections were incubated overnight at 4 °C with primary antibodies. After rinsing with PBS, the sections were incubated with biotinylated appropriated secondary antibodies (Zhongshan Biotechnology Co.) at room temperature for 30 min. The streptavidin-peroxidase activity was visualized with diaminobenzidine method. Negative controls were incubated with the pre-immune sera instead of the primary antibodies. TUNEL staining was performed to detect apoptotic cells using TUNEL assay kit (Zhongshan) based on the manufacture's instructions. The sections were counterstained with hematoxylin and mounted with mounting solution.

2.12. Statistical analysis

Data were presented as mean ± SEM. The data were analyzed by SPSS Version 11.0 statistic software package (Chicago, IL, USA). Student's tests were used to determine significance between groups of cells types, and one-way ANOVA tests with Bonferroni's (selected pairs) post hoc test were used to calculate significance for multiple comparisons. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Identification and damage of spermatogenic cells

Spermatogenic cells were identified based on the analysis of cell nuclear morphology after DAPI staining. As shown in Fig. 1A, the spermatogenic cells contained spermatogonia, spermatocytes, round and elongated spermatids. The purity of spermatogenic cells was more than 95%. Contamination of major somatic cells (Sertoli, Leydig and peritubular myoid cells) was excluded by detecting mRNAs of respective marker genes using qRT-PCR. The results showed that WT-1, 3β-HSD for Leydig cells and fibronectin for myoid cells were negative in germ cell preparation (Fig. 1B). In contrast, mRNA levels of three marker genes for germ cells (c-Kit for spermatogonia, synaptonemal complex protein 2 for spermatocytes, and protamin 2 for spermatids) were relatively high in germ cell preparation compared to the testis. The spermatogenic cells were damaged during culture in vitro. Apoptosis and necrosis of spermatogenic cells were assessed using flow cytometry after double staining with FITC-AnxV and PI. The cells were gating to exclude cell debris (Fig. 1C). Only limited numbers of apoptotic (~11%) and necrotic (~4%) cells were detected in the newly isolated spermatogenic cell population (Fig. 1D). After 48 h culture in vitro, about 85% of spermatogenic cells entered into apoptosis and necrosis (Fig. 1E). The conditioned media of the damaged spermatogenic cells at 48 h after culture were collected and used as DSCPs.

To exclude bacterial contaminants, an aliquotted DSCPs were cultured in an agar plate and Luria–Bertani medium without antibiotics at 37 °C for 48 h. Bacteria were not detected under these conditions. Moreover, ELISA results showed that LPS level was 0.23 ± 0.04 ng/ml in DSCP preparations ($n = 3$).

3.2. DSCPs induce inflammatory gene expression in Sertoli cells

Sertoli cells were stimulated with DSCPs and the expression of major inflammatory genes was determined using qRT-PCR. We found that 10^7 DSCPs/ml (conditioned media from 10^7 damaged spermatogenic cells) induce significant upregulation of various inflammatory genes in Sertoli cells. Peak mRNA levels of TNF-α and IL-1β were observed at 4 h after treatment with DSCPs (Fig. 2A), and those of IL-6 and MCP-1 appeared at 8 h (Fig. 2A and B). By contrast, IFN-α and IFN-β mRNA levels were not significantly upregulated by DSCPs (Fig. 2B).

Based on an ELISA, the concentrations of TNF-α, IL-1β, IL-6 and MCP-1 in media of Sertoli cells were significantly increased at 16 h after DSCP treatment (Fig. 2C). Markedly, TNF-α and MCP-1 were increased by more than 10-fold, although certain levels of TNF-α and MCP-1 were also detected in the control media of Sertoli cells without DSCP stimulation. However, IFN-α and IFN-β proteins were not detectable in the media of Sertoli cells with and without DSCP stimulation (Fig. 2C). Since only faint IL-1β and IL-6 were detected in media, we focused the following studies on the expression of TNF-α and MCP-1. DSCPs induced secretion of TNF-α and MCP-1 in a dose-dependent manner (Fig. 2D). The plateau concentrations of TNF-α and MCP-1 were observed in media of Sertoli cells 16 h after treatment with 10^7 and 10^8 DSCPs/ml. Both TNF-α and MCP-1 were not detectable in the DSCPs. The results indicate that DSCPs induce significantly TNF-α and MCP-1 production in Sertoli cells.

To compare the effect of CM of germ cells cultured for different durations in vitro, we measured cytokine levels in the media of Sertoli cells after stimulation with CM of germ cells cultured for different time. As shown in Fig. 2E, while CM of germ cells 12 h after culture induced significantly TNF-α and MCP-1 production,

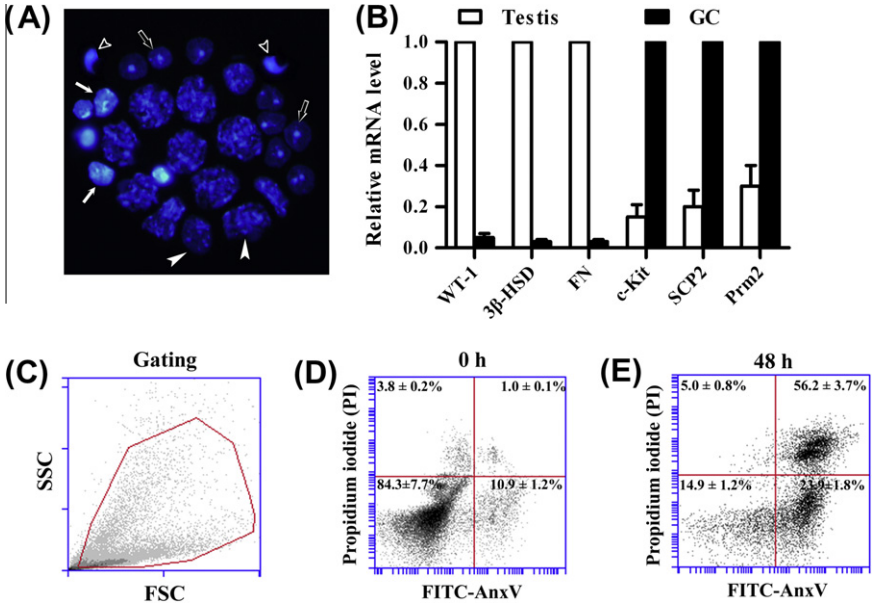

Fig. 1. Spermatogenic cell damage during culture in vitro. (A) Identification of spermatogenic cells. Based on nuclear morphology after staining with DAPI, spermatogenic cells contain spermatogonia (white arrows), spermatocytes (white arrowheads), round spermatids (black arrows), and elongated spermatids (black arrowheads). (B) Expression of marker genes in the testis and germ cell preparation. Total RNA was extracted from germ cells and the whole testes of 7-week-old mice and analyzed for the following testicular cell marker genes using qRT-PCR: Wilm's tumor nuclear factor 1 (WT-1) for Sertoli cells, 3β-hydroxysteroid dehydrogenase (3β-HSD) for Leydig cells, fibronectin (FN) for peritubular myoid cells, c-Kit for spermatogonia, synaptonemal complex protein 2 (SCP2) for spermatocytes, and protamin 2 (Prm2) for spermatids. (C-E) Representative flow cytometry density plots for apoptosis and necrosis of spermatogenic cells. Spermatogenic cells were stained with FITC-AnxV and PI. The spermatogenic cells were gated to exclude debris (C). Apoptotic and necrotic rates of newly-isolated spermatogenic cells (D), and of spermatogenic cells 48 h after culture in vitro were analyzed by flow cytometry (E). Living cells were defined as $AnxV^-PI^-$. $AnxV^+PI^-$ and $AnxV^-PI^+$ cells represent apoptotic and necrotic cells, respectively. $AnxV^+PI^+$ cells represent secondary necrotic cells from apoptotic cells. Data are the mean value of triplicate assays.

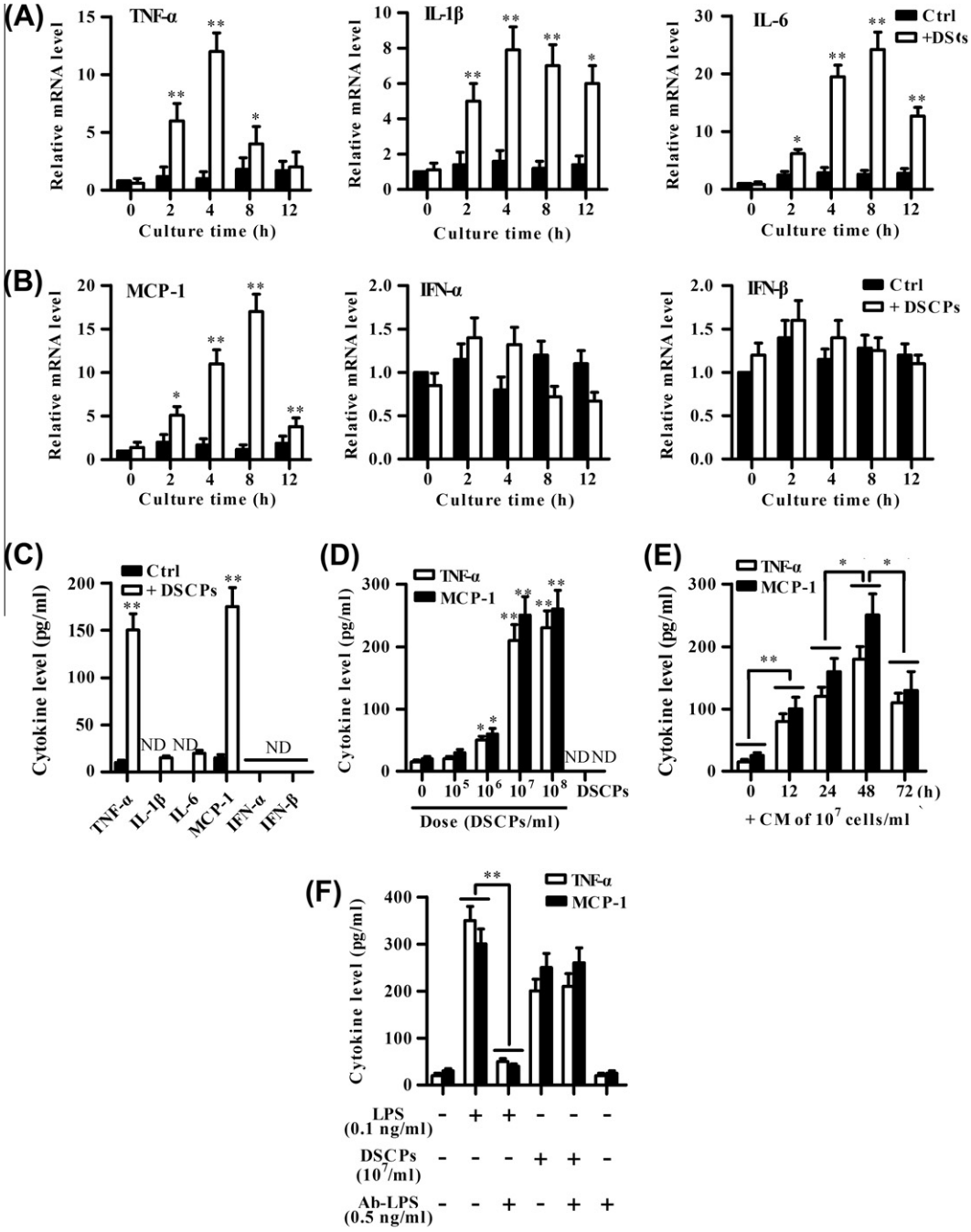

Fig. 2. Expression of inflammatory cytokines in Sertoli cells. (A and B) Upregulation of gene expression by damaged spermatogenic cell products (DSCPs). Sertoli cells were cultured in serum-free F12/DMEM media (Ctrl), and the media containing 10^7 DSCPs (from 10^7 cells)/ml (+DSCPs) for the indicated duration. The mRNA levels of the inflammatory genes were determined by qRT-PCR. (C) Secretion of inflammatory cytokines. Sertoli cells were treated with 10^7 DSCPs/ml for 16 h. The cytokine levels in the media were measured by ELISA. ND, no detectable. (D) Dose-dependent effect of the DSCPs on TNF-α and MCP-1 secretion. Sertoli cells were stimulated with different doses of DSCPs for 16 h. TNF-α and MCP-1 in the media were measured by ELISA. ND, no detectable. (E) Effects of the conditioned media (CM) from germ cells cultured for different durations on TNF-α and MCP-1 secretion. Sertoli cells were stimulated for 16 h with CM from 10^7 germ cells at the indicated times after culture. TNF-α and MCP-1 in the media were measured by ELISA. (F) Effects of neutralizing antibodies against LPS (Ab-LPS) on TNF-α and MCP-1 secretion. Sertoli cells were treated with the indicated conditions for 16 h, and TNF-α and MCP-1 levels in the media were measured by ELISA. Data are the mean ± SEM of three experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

the maximum levels of the two cytokines were detected in media of Sertoli cells treated with CM of germ cells 48 h after culture. Further, we examined the effect of antibodies neutralizing LPS on the DSCP-induced production of TNF-α and MCP-1 by Sertoli cells. A monoclonal antibody to LPS core (Ab–LPS) efficiently inhibited LPS-induced secretion of TNF-α and MCP-1 (Fig. 2F). By contrast, the Ab-LPS did not affect DSCP-induced production of the cytokines. These results confirmed that the SDCP-induced inflammatory cytokine expression in Sertoli cells could not be attributable to LPS contamination.

3.3. TLR2 and TLR4 synergically mediate DSCP-induced TNF-α and MCP-1 upregulation

Given that necrotic cells may release endogenous TLR ligands to induce inflammatory cytokine production in various kinds of cells

(Yu et al., 2010), we speculated that DSCPs may activate inflammatory signaling in Sertoli cells through the TLR activation. Therefore, Sertoli cells from TLR knockout mice were analyzed to test this hypothesis. The absence of TLRs in the respective knockout mice were confirmed by genotyping and analyzing proteins (data not shown). As shown in Fig. 3A, DSCPs upregulated significantly TNF-α and MCP-1 mRNAs in WT and single TLR knockout (including TLR2$^{-/-}$, TLR3$^{-/-}$, TLR4$^{-/-}$ and TLR5$^{-/-}$) Sertoli cells. Notably, the DSCP-induced TNF-α and MCP-1 upregulation in TLR2$^{-/-}$ or TLR4$^{-/-}$ Sertoli cells were significantly low compared with WT cells. By contrast, expression of the two genes was comparable among WT, TLR3$^{-/-}$ and TLR5$^{-/-}$ cells. Further, the DSCP-induced expression of TNF-α and MCP-1 was almost completely abolished in double knockout TLR2$^{-/-}$TLR4$^{-/-}$ cells. TNF-α and MCP-1 secretion by TLR2$^{-/-}$ or TLR4$^{-/-}$ Sertoli cells after DSCP treatment, consistent with mRNAs, was significant low compared to WT cells, and completely abolished in TLR2$^{-/-}$TLR4$^{-/-}$ cells (Fig. 3B). These data imply that DSCPs upregulate TNF-α and MCP-1 expression through the activation of TLR2 and TLR4 in Sertoli cells.

3.4. DSCPs induce activation of NF-κB and MAPKs in Sertoli cells

TLR-mediated inflammatory gene expression depends on the activation of NF-κB and MAPKs. Therefore, we analyzed the activation of NF-κB and MAPKs in Sertoli cells after DSCP stimulation. DSCPs induce efficiently NF-κBp65 phosphorylation in Sertoli cells after treatment with 10^7 DSCPs/ml, and peak levels of phosphorylated p65 were observed at 1 and 2 h after the treatment (Fig. 4A left). At 1 h post-stimulation, a plateau of p65 phosphorylation was observed after treatment with 10^7 and 10^8 DSCPs/ml (Fig. 4A right). Moreover, MAPKs p38 and JNK were dramatically phosphorated by the DSCPs in a time-dependent manner (Fig. 4B). The peak levels of phosphorated p38 and JNK were observed at 1 or 2 h post-stimulation. By contrast, phosphorated ERK was evident in Sertoli cells at basal condition, and not significantly increased by DSCPs. Notably, DSCPs did not induce evident activation of NF-κB, p38 and JNK in TLR2$^{-/-}$TLR4$^{-/-}$ Sertoli cells (Fig. 4C).

3.5. Involvement of NF-κB and MAPK activation in TNF-α and MCP-1 expression

The signaling pathways that are involved in the DSCP-induced TNF-α and MCP-1 upregulation were examined. The DSCP-induced phosphorylation of NF-κB and MAPKs was efficiently blocked by the pre-incubation of cells with the respective inhibitors (BAY11-7082 for NF-κB, SB202190 for p38, U0126 for ERK, and SP600125 for JNK) (Fig. 5A). BAY11-7082 significantly inhibited the DSCP-induced TNF-α and MCP-1 mRNAs (Fig. 5B). MAPK inhibitors individually did not significantly inhibit TNF-α expression (Fig. 5B upper panel). By contrast, MCP-1 mRNA was significantly reduced by BAY11-7082 or SB202190 alone (Fig. 5B lower panel). Inhibitors of NF-κB and MAPKs together completely abolished the DSCP-induced TNF-α and MCP-1 upregulation. In control, the inhibitors did not affect TNF-α and MCP-1 expression at basal condition. These results imply that DSCPs upregulate TNF-α through NF-κB activation, and induce MCP-1 via the activation of both p38 and NF-κB.

3.6. Busulfan-induced spermatogenic cell damage upregulates TNF-α and MCP-1 expression in Sertoli cells

In vivo studies were performed using busulfan-treated model to examine effect of damaged spermatogenic cells on inflammatory gene expression in Sertoli cells. Busulfan induced spermatogenic cell apoptosis in a time-dependent manner (Fig. 6A). Massive apoptotic spermatogenic cells appeared in WT and TLR2$^{-/-}$TLR4$^{-/-}$

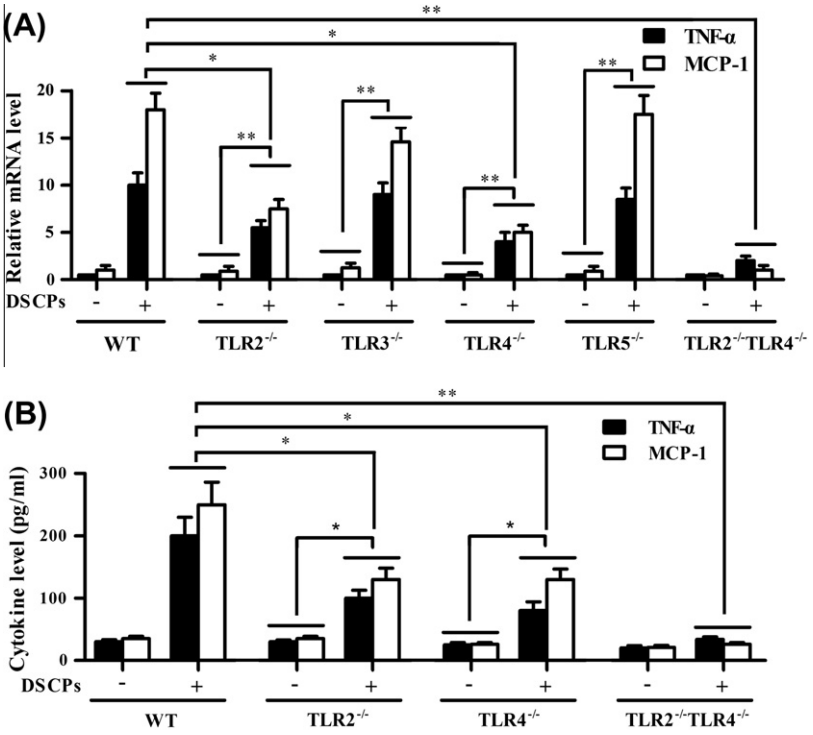

Fig. 3. Involvement of TLRs in the DSCP-induced gene expression. (A) Expression of TNF-α and MCP-1. Sertoli cells from WT and TLR knockout mice were stimulated with 10^7 DSCPs/ml (4 h for TNF-α, and 8 h for MCP-1 expression). Relative mRNAs were analyzed by qRT-PCR. (B) Secretion of TNF-α and MCP-1. Sertoli cells were treated with 10^7 DSCPs for 16 h. The concentrations of TNF-α and MCP-1 in media were measured using ELISA. Data represent the mean values ± SEM of three experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

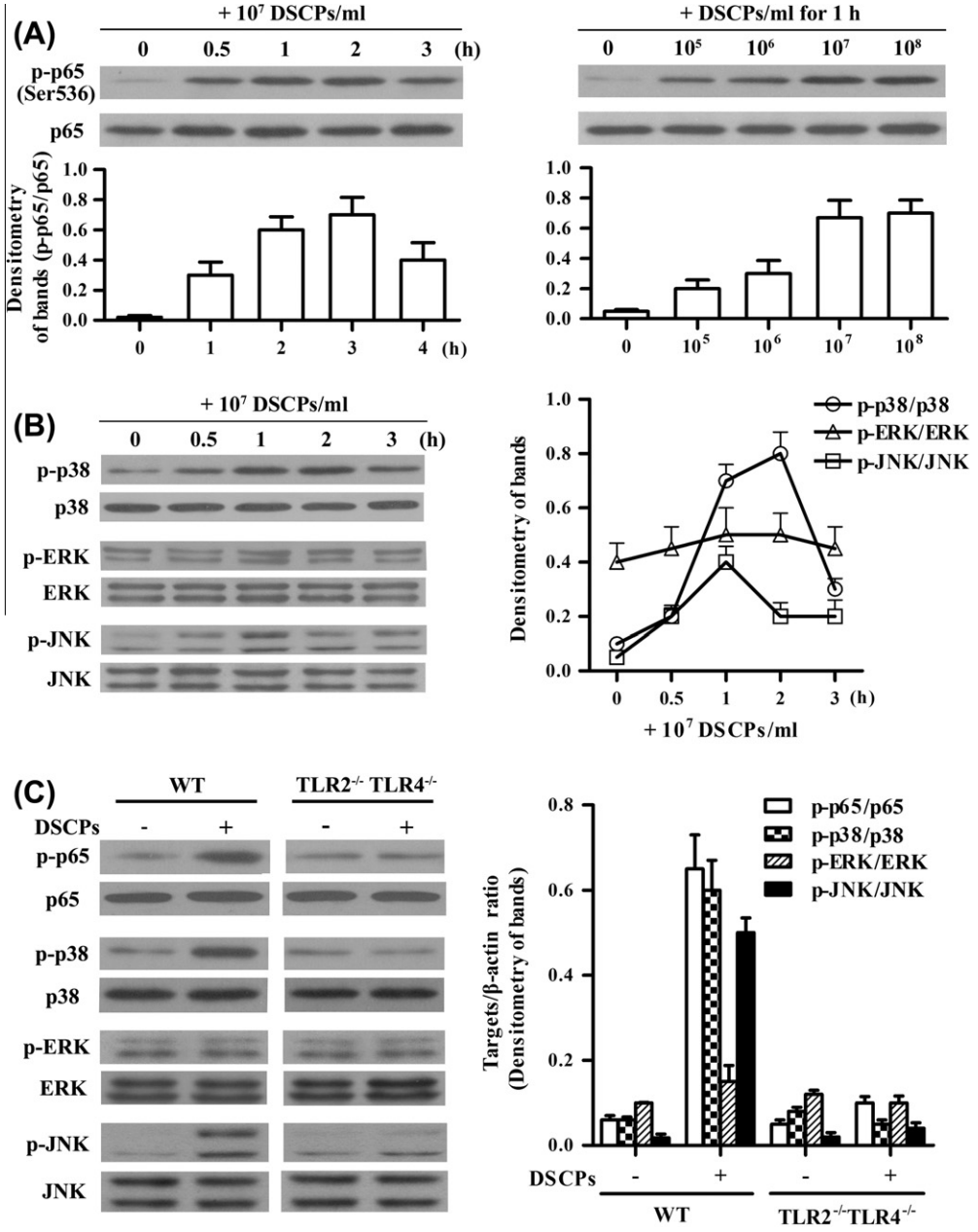

Fig. 4. Activation of nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPKs). (A) DSCP-induced phosphorylation of NF-κBp65. Sertoli cells were treated with 10^7 DSCPs/ml for the indicated duration (left panel), and with different doses of DSCPs for 2 h (right panel). Cell lyastes were analyzed by Western blotting using antibodies against phosphor-p65 (p-p65). (B) DSCP-induced phosphorylation of MAPKs. At the indicated duration after treatment with 10^7 DSCPs/ml, the cell lysates were subjected to Western blotting to probe p38, ERK and JNK using specific antibodies. (C) Comparison of DSCP-induced activation of NF-κB and MAPKs in WT and $TLR2^{-/-}TLR4^{-/-}$ Sertoli cells. Sertoli cells were treated with 10^7 DSCPs/ml for 2 h. Phosphorylation of NF-κB and MAPKs were determined using Western boltting. Images are the representatives of at least three experiments. Data are the mean value of three experiments.

mice 10 days after busulfan injection. Coincidently, both TNF-α and MCP-1 proteins were evidently elevated within seminiferous tubules of WT mice, and predominantly located in Sertoli cells (Fig. 6B left panel). By contrast, TNF-α and MCP-1 were not evidently upregulated in Sertoli cells of $TLR2^{-/-}TLR4^{-/-}$ mice (Fig. 6B right panel). In controls, only weak signals of TNF-α and MCP-1 were detected in Sertoli cells of mice without busulfan treatment (Fig. 6B upper panel). TNF-α and MCP-1 protein levels in the isolated seminiferous tubules after removing interstitial cells were quantitatively analyzed using Western blotting (Fig. 6C). Both TNF-α and MCP-1 were significantly increased in the tubules of WT mice after busulfan treatment, which was not observed in those of $TLR2^{-/-}TLR4^{-/-}$ mice.

3.7. MCP-1 secreted by Sertoli cells promote macrophage migration

Gavin that MCP-1 promotes macrophage recruitment, we examined the effect of MCP-1 secreted by Sertoli cells on macrophage migration. In vitro assays showed that CM of Sertoli cells after treatment with DSCPs (CM + DSCPs) promote transmigration of the macrophages across the filters (Fig. 7A). The increased macrophage migration was inhibited significantly by the pre-incubation of CM + DSCPs with MCP-1 neutralizing antibodies. By contrast, CM of Sertoli cells without DSCP stimulation or DSCPs alone did not promote macrophage transmigration.

We also examined migratory macrophages in the testis of mice after busulfan injection. Based on immunohistochemistry targeting

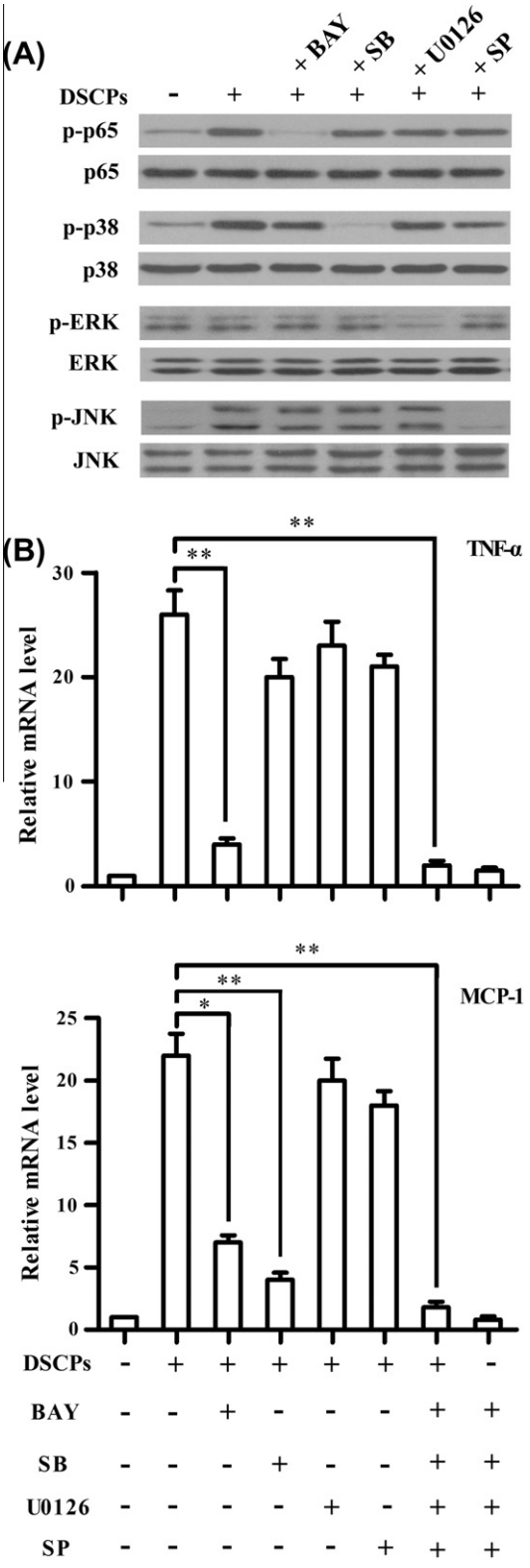

Fig. 5. Effect of NF-κB and MAPK inhibitors on TNF-α and MCP-1 expression. (A) Suppression of NF-κB and MAPK activation by inhibitors. Sertoli cells were treated for 2 h with 10^7 DSCPs/ml alone or with the DSCPs containing the respective inhibitors [10 μm BAY11-7082 (BAY) for NF-κB, 5 μm SB202190 (SB) for p38, 5 μm U0126 for ERK, and 5 μm SP600125 (SP) for JNK]. The cell lysates were subjected to Western blotting to probe the indicated proteins using specific antibodies. (B) Expression of TNF-α and MCP-1. Sertoli cells were treated in the indicated conditions 4 h for TNF-α (upper panel) and 8 h for MCP-1 (lower panel) expression analysis. The relative mRNA levels were determined by qRT-PCR. The images of Western bloting are the representatives of at least three separate experiments. Data are the mean ± SEM of the three experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

F4/80, dramatically increased numbers of macrophages were observed in the testicular interstitial spaces of WT mice 15 days after busulfan treatment (Fig. 7B upper panel). We did not found macrophage infiltration in the seminiferous epithelium. Notably, busulfan treatment did not increase macrophage number in the testes of TLR2$^{-/-}$TLR4$^{-/-}$ mice (Fig. 7B lower panel).

To confirm the increased macrophages were migratory rather than resident cells, the testicular interstitial cells were collected and mRNAs of F4/80 (a marker of total macrophages), CD68 (a marker of newly-arrived macrophages) and CD163 (a marker of resident macrophages) were analyzed using qRT-PCR. As shown in Fig. 7C, mRNAs of F4/80 and CD68 were increased by about 5-fold and 15-fold, respectively, in the testicular interstitial cells of WT mice after busulfan treatment. However, CD163 mRNA was reduced by ~3-fold, which may reflect an increased total interstitial cell number due to the infiltration of migratory macrophages. By contrast, no significant changes in the levels of the macrophage markers were observed in TLR2$^{-/-}$TLR4$^{-/-}$ mice with or without busulfan treatment.

4. Discussion

Damaged tissues, apoptotic and necrotic cells may release endogenous TLR agonists to trigger noninfectious inflammatory responses, thus leading to chronic inflammation and autoimmune diseases (Lin et al., 2011). Extensive apoptosis of spermatogenic cells occur under some traumatic and pathological conditions, such as vasectomy and cryptorchidia (O'Neill et al., 2007; Shiraishi et al., 2001; Yin et al., 1998), which correlate with an elevated risk of orchitis/epididymitis and orchitis (Goldacre et al., 2007; Nistal et al., 2002). A recent study showed that defect in the phagocytic removal of apoptotic spermatogenic cells by Sertoli cells favors autoimmune orchitis (Pelletier et al., 2009). These previous observations have suggested that the damaged spermatogenic cells may induce the noninfectious orchitis. However, the direct evidence and mechanism underlying damaged spermatogenic cell-induced orchitis remain unknown. We hypothesize that massive numbers of apoptotic spermatogenic cells will be further damaged via secondary necrosis if they are not properly removed, which may release endogenous TLR ligands to induce inflammation. In the present study, we provide evidence that DSCPs induce inflammatory mediator production in Sertoli cells through the activation of TLR2 and TLR4, thus initiating testicular inflammation.

Spermatogenic cells die significantly during culture in vitro (Mizuno et al., 1996). We confirmed a great proportion (~85%) of spermatogenic cells entered apoptosis and necrosis at 48 h after culture in vitro. Conditioned media of these damaged cells were used as DSCPs. Although TNF-α mRNA is predominantly detected in mouse spermatogenic cells *in vivo* (De et al., 1993), we did not detect TNF-α in DSCPs using an ELISA. This discrepancy may be explained by the factor that most spermatogenic cells died during culture in vitro, and that different approaches were used in two studies. However, DSCPs induced significantly inflammatory gene expression in Sertoli cells. In particular, TNF-α and MCP-1, two critical inflammatory mediators, were dramatically upregulated at protein levels in Sertoli cells after DSCP stimulation. Notably, the DSCPs induced TNF-α and MCP-1 production by Sertoli cells at a significant high level compared to CM of germ cells 12, 24 and 72 h after culture. The results suggest that DSCPs from cells 48 h after culture contain maximum inducers of inflammatory genes. Although more necrotic cells and cell debris were observed at 72 h after culture (data not shown), the CM from these cells induced less TNF-α and MCP-1 production compared to the DSCPs. This observation could be explained by a possibility that the inducers were degraded during longer time culture. Therefore, we fo-

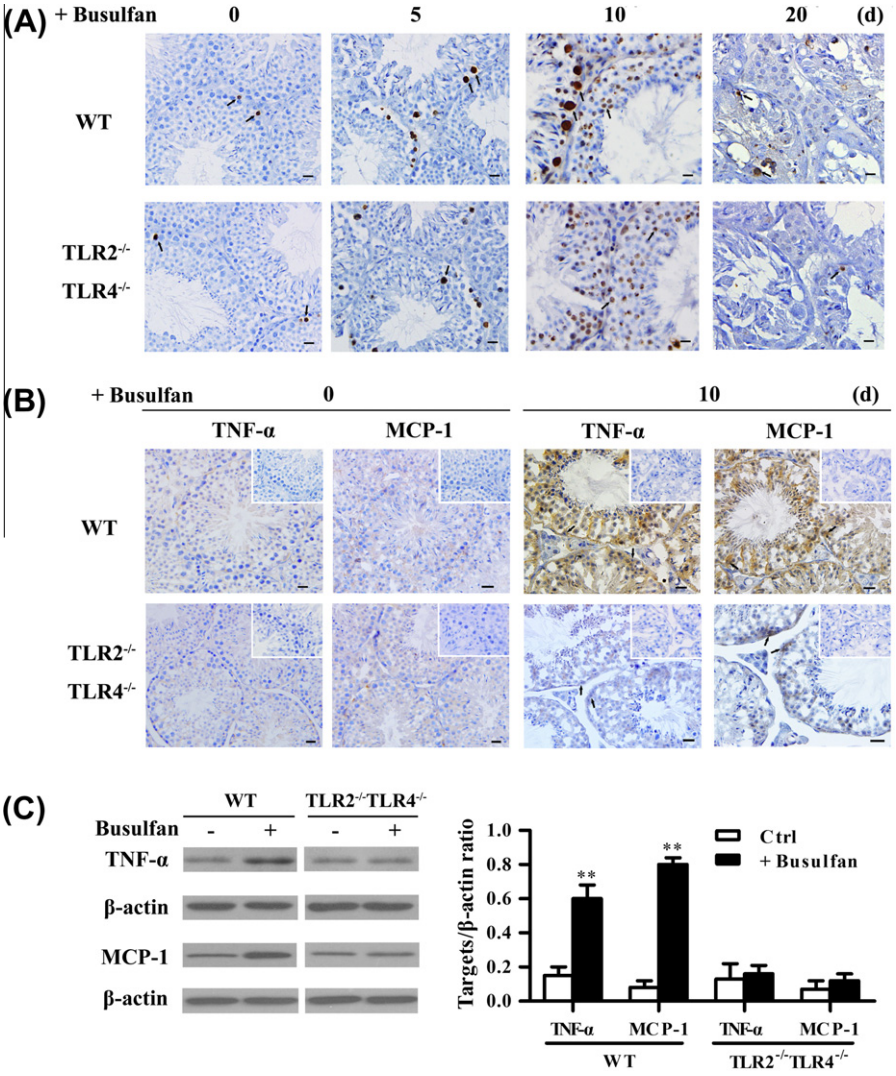

Fig. 6. TNF-α and MCP-1 expression in the testis of mice treated with busulfan. (A) Spermatogenic cells apoptosis induced by busulfan. At 10 days after busulfan injection intra-peritoneally, TUNEL staining was performed on the paraffin sections of the testis to determine apoptotic cells (arrows). (B) Immunohistochemistry was performed on the cryo-sections of the testis to determine TNF-α and MCP-1 proteins, which predominantly located in Sertoli cells (arrows). The insets in upper right corners are negative controls. (C) TNF-α and MCP-1 levels within the seminiferous tubules. The seminiferous tubules were isolated from the testes by removing interstitial cells. TNF-α and MCP-1 levels were analyzed by Western blotting. Images are the representatives of at least three experiments. Data are the mean values ± SEM of three experiments. Bar = 20 μm. $^{**}p < 0.01$.

cused on the DSCP-induced expression of TNF-α and MCP-1, and the involvement of signaling pathways. To exclude the possibility of LPS contamination in DSCPs, we measured LPS level using ELISA. We detected less than 0.3 ng/ml LPS in DSCP preparations, which is much lower than an effective dose (100 ng/ml) of LPS to induce inflammatory cytokine expression in Sertoli cells based on our previous studies (Wu et al., 2008; Sun et al., 2010). Moreover, we confirmed that neutralizing antibodies against LPS do not affect DSCP-induced production of TNF-α and MCP-1, whereas the antibodies efficiently inhibited LPS-induced expression of the cytokines in Sertoli cells. Taken together, the inflammatory trigger in DSCPs could be derived from damaged germ cells, rather than LPS contamination.

Given that damaged cells can release endogenous TLR agonists (Seong and Matzinger, 2004), and mouse Sertoli cells express several TLRs that mediate inflammatory cytokine expression (Riccioli et al., 2006; Wu et al., 2008), we speculate that DSCPs may upregulate inflammatory gene expression in Sertoli cells through TLR activation. Therefore, the involvement of TLRs in the DSCP-induced TNF-α and MCP-1 upregulation was examined. Using TLR knockout mice, we demonstrated that TLR2 and TLR4 are critical to mediate the DSCP-induced inflammatory gene expression in Sertoli cells. Overall the observations correspond to the previous findings that most endogenous TLR ligands activate TLR2 and TLR4 (Yu et al., 2010). Specifically, various heat shock proteins (HSPs) activate TLR2 and TLR4 in different cell types (Chase et al., 2007; Roelofs et al., 2006; Vabulas et al., 2001, 2002; Wheeler et al., 2009). High-mobility group box 1 (HMGB1) can activate several TLRs, including TLR2 and TLR4 (Curtin et al., 2009; Park et al., 2006, 2004). HSPs and HMGB1 are abundantly expressed in spermatogenic cells and can be released under stress conditions (Biggiogera et al., 1996; Zetterstrom et al., 2006). It should be worthwhile to define specific endogenous TLR ligands released from damaged spermatogenic cells.

DSCPs induced evident activation of NF-κB, MAPKs p38 and JNK in Sertoli cells. The observations are largely in agreement with previous reports that TLR ligands activate NF-κB (Wu et al., 2008), as well as p38 and JNK, in mouse Sertoli cells (Riccioli et al., 2006). We also demonstrated that DSCP-induced TNF-α upregulation is predominantly attributable to NF-κB activation. Whereas, DSCPs

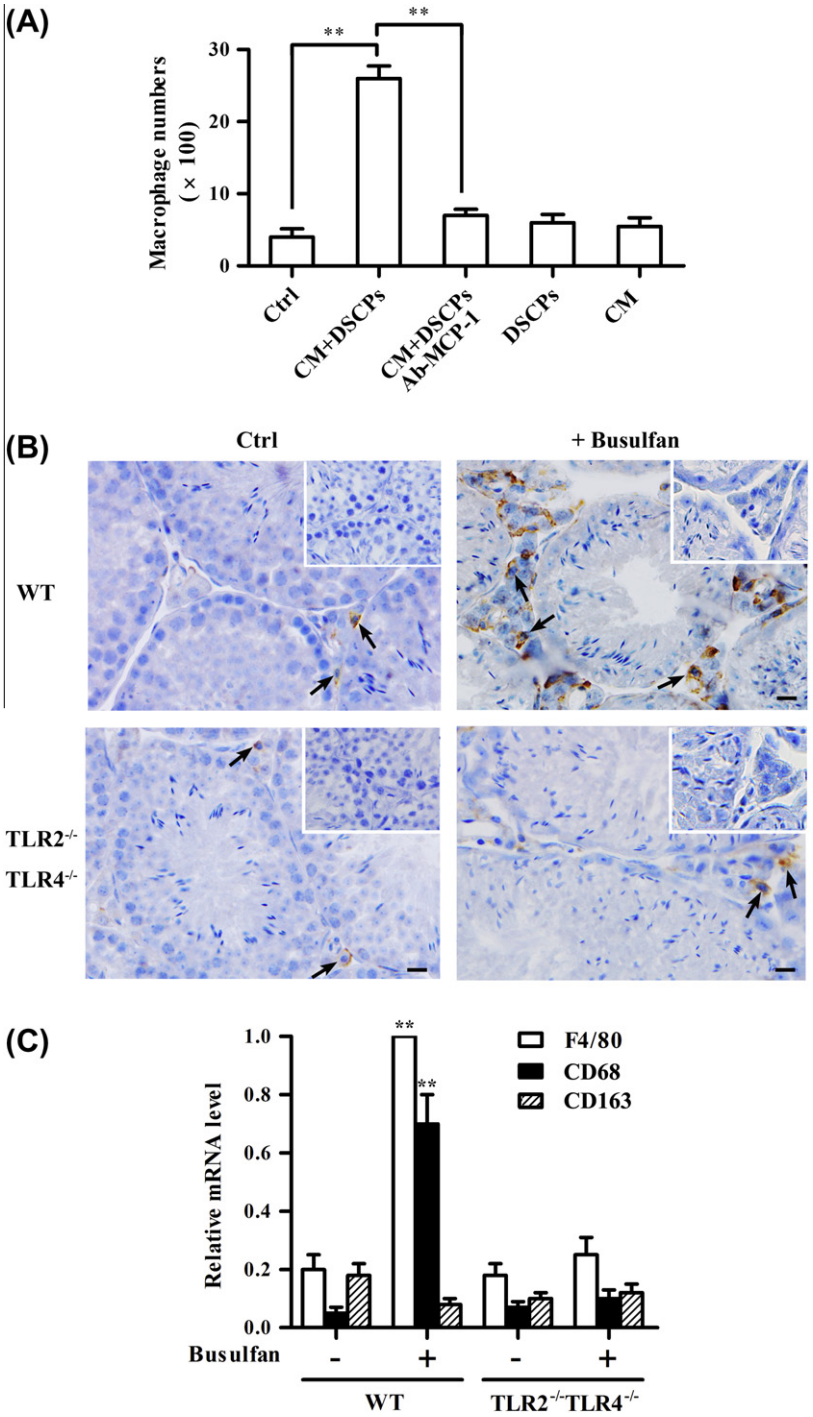

Fig. 7. Macrophage migration. (A) Chemotactic migration of macrophages in vitro. Mouse peritoneal macrophages were seeded in the upper chambers of Transwells with 8 μm pore filters, and the indicated media were placed in the lower chambers: Ctrl, serum-free F12/DMEM media; CM + DSCPs, conditioned media of Sertoli cells after stimulation with DSCPs; Ab-MCP-1, antibodies against MCP-1; CM, conditioned media of Sertoli cells without DSCP treatment. Migration activity of macrophages was assessed by counting cell numbers on the lower surface of the filter. (B) Macrophage infiltration in the testis. At 15 days after busulfan injection, immunohistochemistry was performed on the cryo-sections using antibodies against F4/80 to determine macrophages (arrows). The insets in upper right corners are negative controls. (C) Expression of macrophage marker genes in the interstitial cells. The interstitial cells were cultured in vitro for 4 h. The cells that adhered on the dishes were collected, and mRNAs of F4/80, CD68 and CD163 were determined using qRT-PCR. Data represent the mean values ± SEM of three independent experiments. $^{**}p < 0.01$.

upregulate MCP-1 through NF-κB and p38 activation. MAPK activation should also be involved in the DSCP-induced TNF-α upregulation because blockade of NF-κB and MAPKs together completely abolished TNF-α upregulation. The data provide further insights into the mechanisms underlying DSCP-induced inflammatory mediator production in Sertoli cells. Although TLR4 signaling also upregulates type 1 interferons through TRIF-dependent pathway in some cell types such as macrophages, we did not detect significant upregulation of IFN-α and IFN-β in Sertoli cells after DSCP stimulation. This may be explained by the fact that Sertoli cells display different cytokine responses to the agonists of TLR2 and TLR4 compared to macrophages (Winnall et al., 2011).

Inflammatory cytokines, including TNF-α, IL-1 and IL-6, play an important regulatory role in spermatogenesis and testicular steroi-

dogenesis under physiological conditions (Hedger and Meinhardt, 2003). However, these factors can be upregulated in the testis during inflammatory conditions, and impair normal spermatogenesis (Rival et al., 2006; Theas et al., 2008). Therefore, the regulation of these inflammatory cytokines is important in pathophysiology of the testis. Several early studies established that germ cells and residual bodies can induce the cytokine production in rat Sertoli cells (Jonsson et al., 1999; Stephan et al., 1997; Syed et al., 1993, 1995). Most these previous studies focused on the cytokines IL-1α and IL-6, which are believed to be critical in regulating germ cell proliferation and differentiation (Jegou, 1993). In the present study, we focused on TLR-mediated proinflammatory mediators TNF-α and MCP-1, because both them play important roles in the pathogenesis of autoimmune orchitis (Jacobo et al., 2011). MCP-1 is a potent chemokine that promotes migration and infiltration of monocytes and macrophages in experimental autoimmune orchitis (Guazzone et al., 2003). We provide evidence that DSCP-induced MCP-1 production by Sertoli cells promotes macrophage migration. Monocytes and macrophages that migrate into the testis may act as the spermatic antigen-presenting cells to induce autoimmunity against germ cells (Rival et al., 2008). The migratory macrophages might also produce a large amount of TNF-α in experimental autoimmune orchitis, thus inducing germ cell apoptosis (Theas et al., 2008). Moreover, high level of TNF-α may disrupt tight junction of blood–testis barrier, thus leading to release of spermatic antigens (Xia et al., 2005). Therefore, augmentation of MCP-1 and TNF-α could facilitate testicular inflammation and autoimmune orchitis.

The damaged spermatogenic cell-induced inflammatory responses were observed in the testes of busulfan-treated WT mice. By contrast, these phenotypes were not evident in TLR2$^{-/-}$TLR4$^{-/-}$ mice. While the observations *in vivo* were not direct evidence that damaged germ cells themselves induce inflammation in the testis, several indirect clues support that busulfan-induced damaged germ cells involved in the upregulation of TNF-α and MCP-1 in Sertoli cells: (1) The elevated TNF-α and MCP-1 in Sertoli cells are correlated to the extensive apoptosis of germ cells at WT mice 10 days after busulfan injection, whereas evident macrophage infiltration was firstly observed in the testis later than 10 days (15 days) after busulfan treatment. Therefore, infiltrated macrophages could not contribute to the induction of TNF-α and MCP-1 in Sertoli cells. (2) If the immune cells, such as resident macrophages, in the testis were activated by busulfan, they should produce elevated cytokines. Although some cytokines may stimulate Sertoli cells to produce TNF-α and MCP-1, they should not be TLR-dependent. (3) It has been known that most of known endogenous ligands are proteins such as heat shock proteins and high-mobility group box 1. If busulfan treatment promoted release of endogenous TLR ligands into the peripheral circulation from other sources, these high molecules should limitedly reach to Sertoli cells due to the structure of basement membrane and blood–testis barrier within seminiferous tubules. Finally, (4) in vitro data provided evidence that DSCPs are able to activate TLR2 and TLR4 in Sertoli cells. Taken together, we believe that damaged germ cells in busulfan-treatment models release endogenous TLR ligands to activate TLR2 and TLR4 in Sertoli cells, thus initiating endogenous inflammation in the testis. This mechanism may explain, at least in part, the correlation of extensive apoptotic germ cells and chronic orchitis.

The testis is a remarkable immune privilege organ. However, inflammatory conditions in the testis are important etiological factors of male infertility. The testicular inflammation caused by noninfectious origin should be particularly considered. We demonstrate that DSCPs induce inflammatory mediator production in Sertoli cells through the activation of TLR2 and TLR4, which may trigger testicular inflammation in response to damaged spermatogenic cells. Moreover, the involvement of TLR signaling pathways in the DSCP-induced inflammatory mediator production was dissected. The results provide novel insights into the mechanisms underlying damaged spermatogenic cell-induced testicular inflammation.

Acknowledgments

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