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Targeting high mobility group box protein 1 ameliorates testicular inflammation in experimental autoimmune orchitis

Ferial Aslani[1], Hans-Christian Schuppe[2], Vanesa A. Guazzone[3], Sudhanshu Bhushan[1], Eva Wahle[1], Günter Lochnit[4], Livia Lustig[3], Andreas Meinhardt[1], and Monika Fijak[1,*]

[1]Department of Anatomy and Cell Biology, Justus-Liebig-University of Giessen, 35392 Giessen, Germany [2]Department of Urology, Pediatric Urology and Andrology, Justus-Liebig-University of Giessen, 35392 Giessen, Germany [3]Instituto de Investigaciones Biomédicas UBA-CONICET, Facultad de Medicina, Universidad de Buenos Aires, C1121 ABG, Buenos Aires, Argentina [4]Institute of Biochemistry, Faculty of Medicine, Justus-Liebig University of Giessen, 35392 Giessen, Germany

*Correspondence address. Department of Anatomy and Cell Biology, Justus-Liebig-University of Giessen, Aulweg 123, D-35392 Giessen, Germany. Tel: +49-641-9947032; Fax: +49-641-9947049; E-mail: monika.fijak@anatomie.med.uni-giessen.de

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STUDY QUESTION: Does high mobility group box protein 1 (HMGB1) regulate inflammatory reactions in a rat model of experimental autoimmune orchitis (EAO)?

SUMMARY ANSWER: HMGB1 appears to be involved in regulating inflammatory reactions in testes, as HMGB1 is translocated from testicular cells during the course of EAO and blocking its action by ethyl pyruvate (EP) reduces disease progression and spermatogenic damage.

WHAT IS KNOWN ALREADY: Despite its immune privileged status, the human testis is prone to inflammatory lesions associated with male factor infertility. Accumulating evidence shows that HMGB1 plays an important role in onset and progression of autoimmune diseases.

STUDY DESIGN, SIZE, DURATION: This is a cross sectional and longitudinal study involving Wistar male rats immunized with testicular homogenates to induce EAO 50 (EAO50; $n = 10$) and 80 (EAO80; $n = 10$) days after first immunization. Control adjuvant animals received saline instead of testicular homogenate ($n = 16$). Untreated animals ($n = 10$) were also studied. An interventional study was performed to block the action of HMGB1 starting 20 days after first immunization in EAO animals and respective controls ($n = 17$). Rats were treated i.p. with EP and the effect of EP treatment on testicular pathogenesis was evaluated 30 days later. Moreover, human testicular biopsies from infertile men with focal lymphocytic infiltrates ($n = 7$) and sections with intact spermatogenesis ($n = 6$) were probed with antibodies against HMGB1.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Testicular RNA and protein extracts from EAO animals, EAO animals treated with EP and relevant controls were used for analysis of cytokine expression by real-time RT–PCR and enzyme-linked immunosorbent assay. HMGB1 was co-localized on rat testicular cross sections with antibodies against testicular macrophages (TM), peritubular cells (PTC) and Sertoli cells (SC). Interaction of HMGB1 and its receptors (RAGE, TLR4) as well signaling pathways after HMGB1 stimulation were studied in isolated TM, PTC and SC by proximity ligation assay and western blot, respectively. Furthermore, HMGB1 immunofluorescence on human testicular biopsies was performed.

MAIN RESULTS AND THE ROLE OF CHANCE: HMGB1 was translocated from the nuclei in EAO testes and testes of infertile men with impaired spermatogenesis and lymphocytic infiltrates. Elevated HMGB1 levels were observed during late phase of EAO. In testicular somatic cells HMGB1 receptors Toll-like receptor 4 (TLR4) and *receptor* for advanced glycation end products (RAGE) were differentially expressed: HMGB1-TLR4 binding was predominant in TM, while HMGB1-RAGE interaction was prevalent in SC and PTC. In support, HMGB1 triggered extracellular signal regulated kinase (ERK)1/2 and cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) activation in SC and PTC, while TM responded to HMGB1 stimulation with p38 mitogen-activated protein kinase (MAPK) and p65 nuclear factor Kappa B (NF-κB) phosphorylation followed by increased tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) mRNA levels. *In vivo* treatment of EAO animals with EP 20 days after induction of disease revealed beneficial effects, as documented by

reduced disease progression and spermatogenic damage, lower macrophage numbers, as well as decreased concentrations of HMGB1 and IL-6 in the testis compared with EAO controls.

LIMITATIONS, REASONS FOR CAUTION: The ability of HMGB1 to bind to a wide range of receptors makes it difficult to prevent its action by blockade of a specific receptor; therefore we applied EP, a drug preventing HMGB1 release from cells. Due to its mode of action EP decreases also the secretion of some other pro-inflammatory cytokines.

Using isolated primary cells imposes limitations for cell transfection studies. As a compromise between purity and yield primary cells need to be isolated from animals of different age, which has to be considered when comparing their responses.

WIDER IMPLICATIONS OF THE FINDINGS: HMGB1 could be a promising target in attenuating testicular damage caused by inflammatory reactions.

STUDY FUNDING/COMPETING INTEREST(S): This study was supported by a research grant of the University Medical Centre Giessen and Marburg to M.F. and H.-C.S. A.M., H.-C.S. and M.F. are supported by the LOEWE focus group 'MIBIE' (Male infertility during infection & inflammation)—an excellence initiative of the government of the German state of Hessen. The authors have nothing to disclose.

Key words: testicular inflammation / experimental autoimmune orchitis / high mobility group box protein-1 / receptor for advanced glycation end products / ethyl pyruvate

Introduction

High mobility group box protein 1 (HMGB1) is a nucleosomal protein which is secreted during inflammation from activated immune cells or is passively released from necrotic cells (Wang *et al.*, 1999). Accumulating evidence points to the crucial role of HMGB1 in mediating late events in *de novo* inflammation and autoimmunity (Andersson and Harris, 2010; Yang and Tracey, 2010). Extracellular HMGB1 acts as a cytokine and induces a wide range of responses such as inflammation, autophagy, cell survival and tissue repair depending on its redox status, concentration, post-translational modification, specific receptor binding and complex formation with other inflammatory modulators (Andersson and Tracey, 2010; Tang *et al.*, 2010; Davis *et al.*, 2012; Hreggvidsdottir *et al.*, 2012; Yang *et al.*, 2012). HMGB1 binds to different receptors, among which the receptor for advanced glycation end products (RAGE) and toll-like receptor 4 (TLR4) are the best known (Harris *et al.*, 2012). Differential expression of these receptors at different stages of the disease is a regulatory mechanism to fine tune HMGB1 signaling. For example, up-regulation of RAGE in cancer tissue causes rapid tumor growth and diminishes apoptosis (Kang *et al.*, 2011), whereas increased levels of TLR4 mediate pro-inflammatory responses (Brint *et al.*, 2010; Ganley-Leal *et al.*, 2010).

Blocking HMGB1 release by using the anti-inflammatory drug ethyl pyruvate (EP) or neutralizing its action by anti-HMGB1 antibodies is shown to be effective in ameliorating chronic inflammatory and autoimmune diseases in animal models of colitis, hepatitis, arthritis or experimental autoimmune encephalomyelitis (Kokkola *et al.*, 2003; Dave *et al.*, 2009; Robinson *et al.*, 2013; Shen *et al.*, 2014). Since HMGB1 release occurs at later stages of the disease compared with conventional pro-inflammatory cytokines, it provides a broader time window for application of therapeutics to block HMGB1's action and therefore ceasing or delaying further inflammation (Yang and Tracey, 2010).

The testis is an immune privileged organ with several mechanisms to protect neo-antigens on meiotic and post-meiotic germ cells from host immune responses (Meinhardt and Hedger, 2011). Testicular somatic cells play an important role in the establishment and maintenance of testicular tolerance to germ cells and HMGB1 mRNA and protein are found in these cells in normal human and rat testis (Zetterstrom *et al.*, 2006). Corruption of immune privilege in the testis causes testis specific autoimmunity along with anti-germ cell antibody production and local inflammation of the male gonad (Fijak *et al.*, 2005; Fijak and Meinhardt, 2006). Severe infection and inflammation of the testis, which becomes clinically apparent as symptomatic orchitis or a combined epididymo-orchitis, can impair spermatogenesis and ultimately cause infertility. Post- or non-infectious chronic, low-grade orchitis is also of concern as it remains in most cases asymptomatic (Schuppe *et al.*, 2008). The prevalence of these testicular inflammatory lesions may well exceed the overall rate of 15% reported for infection and inflammation as underlying cause of male infertility (Schuppe *et al.*, 2008). However, to date no non-invasive tool is available to diagnose autoimmune reactions in the testis, a reason why this etiology is thought to be underestimated in men with idiopathic infertility (Rusz *et al.*, 2012; Fijak *et al.*, 2014).

Experimental autoimmune orchitis (EAO) is a rodent model of chronic testicular inflammation, which reproduces pathological changes seen in human immunological infertility. The initial phase of EAO involves infiltration and increased migration of monocytes/macrophages, T lymphocytes and dendritic cells into the testis followed by increased production of pro-inflammatory cytokines, among which tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) play a prominent role in promoting inflammation. The chronic phase of EAO is manifested by progressive loss of germ cells, seminiferous tubule atrophy and reduced testicular weight (Doncel *et al.*, 1989; Tung, 1995; Guazzone *et al.*, 2009; Fijak *et al.*, 2011a,b).

As the underlying mechanisms for onset and progression of autoimmune processes in the testis remain unknown, we hypothesized that HMGB1 may play a role in promoting testicular inflammation by prolonging pro-inflammatory cytokine release from testicular cells. Considering the fact that chronic, asymptomatic inflammation of the human testis shows characteristics of autoimmune orchitis and no established treatment regimens are available, the deciphering of mechanisms involved in the disease development is a matter of particular importance to identify molecules involved in the continuation of testicular inflammation, which could serve in the future as therapeutic targets.

Materials and Methods

Induction of EAO

To induce EAO 20 adult male Wistar rats (Charles River Laboratories, Sulzfeld, Germany) aged 60–70 days were actively immunized as previously

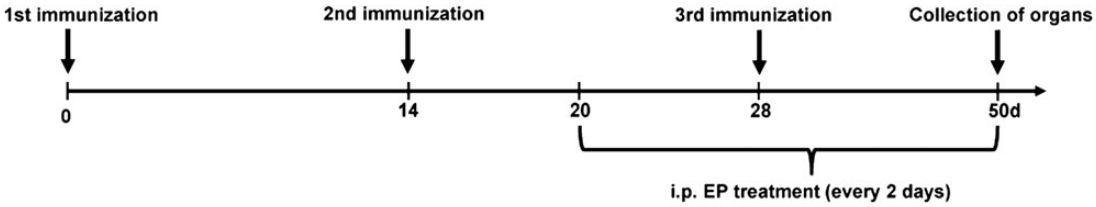

Figure 1 Schematic diagram illustrating the time course of interventional ethyl pyruvate (EP) treatment *in vivo* during experimental autoimmune orchitis (EAO) induction.

described (Fijak *et al.*, 2005). Briefly, animals were injected three times every 14 days with testicular homogenate in complete Freund's adjuvant followed by *i.v. Bordetella pertussis* (DSMZ, Germany) administration. Control adjuvant animals ($n = 16$) received NaCl instead of testicular homogenate. Ten untreated normal rats were also studied. Animals were sacrificed 50 and 80 days after first immunization (EAO50 and EAO80, respectively). In this progressive disease model, tissue remodeling is milder at 50 days than in fully developed EAO at 80 days (Doncel *et al.*, 1989). Animals were kept at 22°C with a 14 h light, 10 h dark schedule and fed with standard food pellets and water *ad libitum*.

EP treatment

To block HMGB1-induced inflammation, EAO (EAO+EP; $n = 9$) and adjuvant control (Adj+EP; $n = 8$) animals received i.p. injections of EP (40 mg/kg body weight; Sigma-Aldrich, Seelze, Germany) diluted in Ringer's solution (130 mM NaCl, 4 mM KCl, 2.7 mM $CaCl_2$; pH 7.00) every other day starting on Day 20 after first immunization (Fig. 1). EAO ($n = 7$) and adjuvant (Adj; $n = 8$) control animals received additional Ringer's solution as vehicle. Animals were sacrificed 50 days after first immunization.

Ethical approval

All animal experiments were approved by the local animal ethics committee (Regierungspraesidium Giessen GI 20/23 – Nr. 33/2008). Interventional study was performed at the School of Medicine, University of Buenos Aires in Argentina and was approved by the local ethics committee (2957/10, CICUAL – School of Medicine, University of Buenos Aires).

Human testis specimens

Paraffin sections from human testicular biopsies were provided by the Giessen Testicular Biopsy Repository. The specimens had been obtained from infertile men with non-obstructive azoospermia and a histological diagnosis of focal inflammatory lesions associated with disturbed spermatogenesis (Schuppe and Bergmann, 2013); biopsies from patients with obstructive azoospermia, i.e. intact spermatogenesis without any signs of inflammation served as control (Supplementary Fig. S1). From all men undergoing testicular biopsy written informed consent was obtained. The study was approved by the local institutional review board (Ref. No. 100/07).

Immunofluorescence staining

Deparaffinized and rehydrated Bouin fixed paraffin sections (5 μm) from human testis specimens were submitted to antigen retrieval by boiling for 20 min in 10 mM sodium citrate buffer containing 0.05% Tween 20, pH 6.0. Rat testicular cryosections (8 μm) were fixed in 4% paraformaldehyde (Merck, Darmstadt, Germany) and permeabilized in 0.1% Triton X-100 (Sigma-Aldrich, Steinheim, Germany). After blocking with 5% BSA (Carl Roth, Karlsruhe, Germany), sections were incubated overnight with the specific primary antibody (Table I). Sections were washed with PBS, incubated with appropriate fluorescent-labeled secondary antibody for 1 h at room temperature and coverslipped using mounting medium with DAPI (Vector Laboratories, Burlingame, USA). Pre-absorption of HMGB1 antibody with recombinant human HMGB1 (rhHMGB1) for 2 h was used as a negative control. Images were acquired using an Axioplan 2 microscope and AxioVision 4.8.2.0 software (Carl Zeiss, Jena, Germany).

Measurement of HMGB1, IL-6 and TNF-α by ELISA

Concentrations of HMGB1, IL-6 and TNF-α were determined in sera and testicular protein extracts using enzyme-linked immunosorbent assay (ELISA) according to the user's manual (HMGB1 ELISA kit, IBL International, Hamburg, Germany; IL-6 ELISA, BD Bioscience, Heidelberg, Germany; TNF-α ELISA kit, eBioscience, San Diego, USA).

Expression and purification of recombinant HMGB1

His-tagged rhHMGB1 construct in pET11d vector was a kind gift from Dr Patrick Swanson (Department of Medical Microbiology and Immunology, Creighton University Medical Center, NE, USA). Overexpression of rhHMGB1 was induced in BL21 (DE3) pLysS cells (Bergeron *et al.*, 2006). The resulting protein was affinity purified by Ni-NTA agarose (Qiagen, Germany) according to the user's manual. Further purification was performed by ion-exchange chromatography (ÄKTA FPLC, GE Healthcare, Uppsala, Sweden). Purified rhHMGB1 was dialyzed overnight at 4°C against 25 mM Tris–HCl (pH 8.0), 150 mM KCl, 10% glycerol (Bergeron *et al.*, 2006) and later subjected to Triton X-114 (Sigma-Aldrich) extraction to remove endotoxin contamination (Aida and Pabst, 1990). Endotoxin concentration was measured using a chromogenic LAL assay following the manufacturer's protocol (GenScript, Piscataway, NJ, USA). Endotoxin level was lower than 2 EU/ml for the purified recombinant protein. No traces of DNA and RNA were detected in the sample. All buffers and reagents were prepared without dithiothreitol (DTT).

Isolation and *in vitro* treatment of testicular cells

Testicular macrophages (TM) were isolated from adult Wistar rat testis (Charles River Laboratories) as previously described (Bhushan *et al.*, 2011). Briefly, testes were decapsulated and seminiferous tubules were separated mechanically. The tubule fragments were allowed to settle, the supernatant was centrifuged and the cell pellet was resuspended in Dulbecco's modified Eagle's medium F12 medium, plated and kept at 32°C. Contaminating cells were removed by vigorous washing, while TM adhered to the plate. Purity of the cells was >80% as verified by CD68/CD163 double staining

Table I Detailed list of antibodies and antibody dilutions used in this study.

Primary antibodies	Manufacturer	Catalogue No.	Dilution
Polyclonal rabbit anti rat phospho-p38 MAPK (Thr180/Tyr182)	Cell Signaling, Germany	9211	1:1000*
Polyclonal rabbit anti rat p38 MAPK	Cell Signaling, Germany	9212	1:1000*
Polyclonal rabbit anti rat phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204)	Cell Signaling, Germany	9101	1:1000*
Polyclonal rabbit anti rat p44/42 MAPK (ERK1/2)	Cell Signaling, Germany	9102	1:1000*
Monoclonal rabbit anti rat phospho-NF-κB p65 (Ser536) (93H1)	Cell Signaling, Germany	3033	1:1000*
Monoclonal mouse anti rat NF-κB p65 (L8F6)	Cell Signaling, Germany	6956	1:1000*
Monoclonal rabbit anti rat phospho-CREB (Ser 133) (87G3)	Cell Signaling, Germany	9198	1:1000*
Polyclonal rabbit anti rat TLR4 (Rodent Specific)	Cell Signaling, Germany	2219	1:50**
Polyclonal rabbit anti rat RAGE	Thermo Scientific, USA	PA1-075	1:100**
Polyclonal rabbit anti rat vimentin (c-20)	Santa Cruz Biotechnology, CA, USA	L4793.AB45	1:100**
Monoclonal mouse anti rat smooth muscle actin [1A4] (FITC)	GeneTex, USA	GTX72531	1:100**
Polyclonal rabbit anti rat HMGB1	Abcam, UK	18256	1:200**
Monoclonal mouse anti rat CD68 (ED1)	AbD Serotec, UK	MCA341R	1:100**
Monoclonal mouse anti rat CD163 (ED2)	AbD Serotec, UK	MCA342R	1:100**
Monoclonal mouse anti-β-actin	Sigma-Aldrich	A5441	1:10000*
Secondary antibodies	**Manufacturer**	**Catalogue No.**	**Dilution**
Donkey anti rabbit IgG-Cy3	Chemicon, Hampshire, UK	AP182C	1:1000**
Donkey anti mouse IgG-FITC	Dianova, Hamburg, Germany	715-095-151	1:1000**
Goat anti rabbit IgG-HRP	ICN, OH, USA	55676	1:10000*
Sheep anti mouse-HRP	Sigma-Aldrich, Steinheim, Germany	A5906	1:10000*

CD, cluster of differentiation; CREB, cyclic adenosine monophosphate (cAMP) response element-binding protein; Cy3, cyanine dye 3; ERK, extracellular signal regulated kinase; FITC, fluorescein isothiocyanate; HMGB1, high mobility group box protein 1; HRP, horseradish peroxidase; IgG, Immunoglobulin G; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor Kappa B; RAGE, receptor for advanced glycation end products; Ser: Serin; Thr, Threonin; Tyr, Tyrosin; TLR4, Toll-like receptor 4.
**Immunofluorescence.
*Western blotting.

Table II List of PCR primers used in this study.

Gene	Primer (5′→ 3′)	Catalogue no.	Entrez gene ID	Amplicon size (bp)
β-Actin	F: ATGGTGGGTATGGGTCAGAA R: GGGTCATCTTTTCACGGTTG	–	81822	232
beta-2 microglobulin (β2M)	F: CCGTGATCTTTCTGGTGCTT R: AAGTTGGGCTTCCCATTCTC	–	24223	113
TNF-α	F: GCCTCTTCTCATTCCTGCTC R: CCCATTTGGGAACTTCTCCT	–	24835	101
IL-6	QuantiTect Primer Assay Qiagen	QT00182896	24498	128

TNF-α, tumor necrosis factor α; IL-6, interleukin 6.

(Table I). Peritubular cells (PTC) and Sertoli cells (SC) were isolated from 19-day-old Wistar rats (Charles River Laboratories) using enzymatic digestion as described previously (Hoeben *et al.*, 1999; Bhushan *et al.*, 2008). Purity of the isolated PTC and SC was confirmed to be >95% using α-smooth muscle actin (PTC marker) and vimentin (SC marker) immunolabeling, respectively (Table I). HMGB1 concentrations were determined in intratesticular fluid of EAO animals (data not shown) and corresponding levels of 5 μg/ml of rhHMGB1 were used for subsequent *in vitro* experiments. To verify bioactivity of recombinant HMGB1, equal concentrations of rhHMGB1 were heat inactivated at 90°C for 20 min and used as a control. For RNA expression analysis, cells were treated with rhHMGB1 or *E. coli* 0111:B4 lipopolysaccharide (LPS) (Winnall *et al.*, 2011) as a positive control (Sigma-Aldrich) for indicated time points or pretreated for 1 h with 10 mM EP (Sigma-Aldrich).

In situ proximity ligation assay

To analyze the binding of HMGB1 to TLR4/RAGE in isolated testicular cells *in situ* proximity ligation assay (PLA) was used. PLA assay allows detection of specific protein-protein interaction using antibodies which are conjugated to complimentary oligonucleotides. Once the two antibodies are positioned closer than 40 Å the complimentary oligonucleotides will hybridize and are then amplified using rolling circle PCR. The product is visualized by

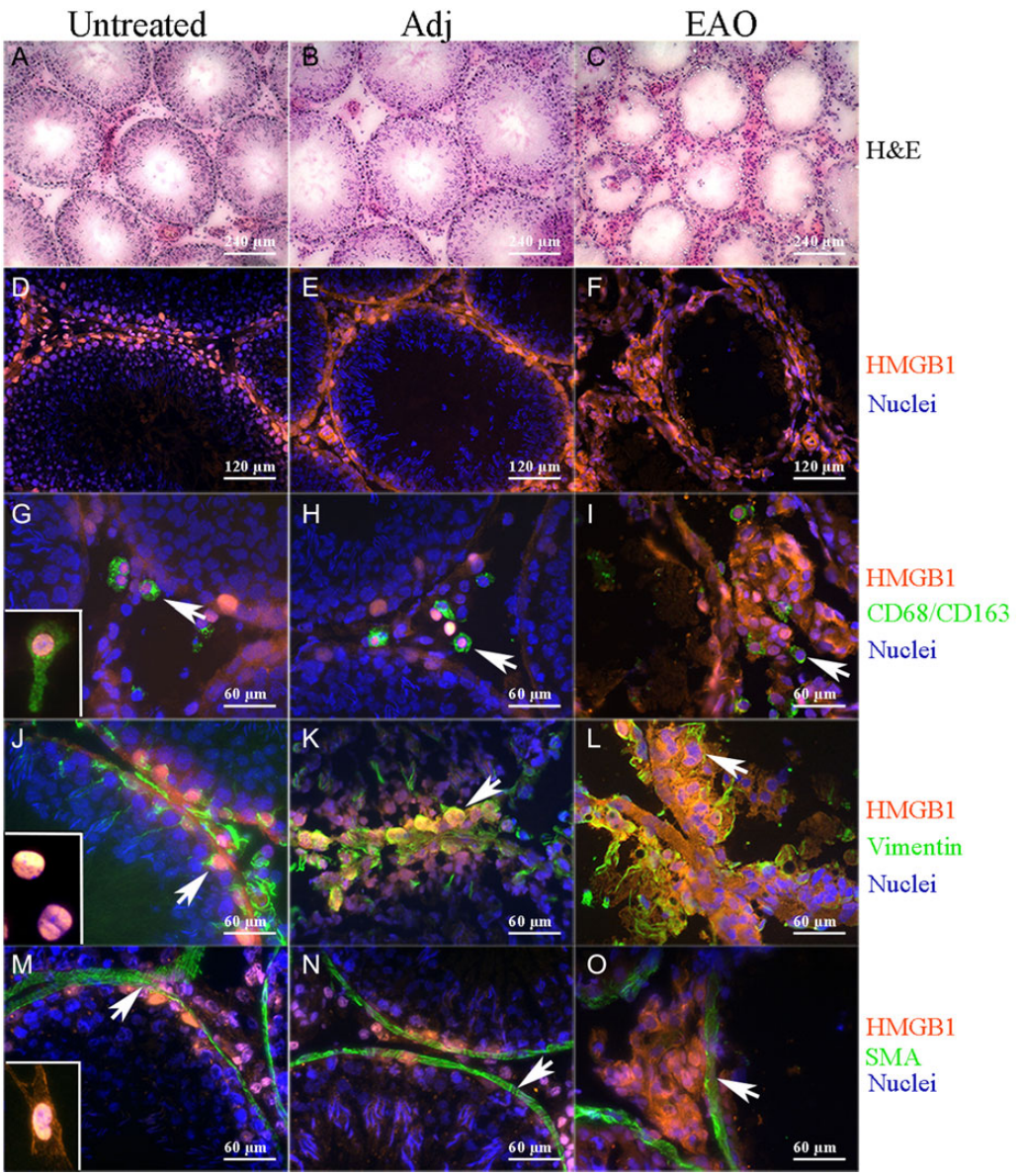

Figure 2 Nuclear high mobility group box protein 1 (HMGB1) is translocated in rat EAO testis. Representative hematoxilin-eosin staining (H&E) of testis cryosections from untreated (**A**), adjuvant control (Adj, **B**) and EAO50 (**C**) rat. EAO is characterized by interstitial leukocyte infiltration and impaired spermatogenesis. Double staining of HMGB1 (Cy3, orange) and monocyte/macrophage marker CD68/CD163 (FITC, green) (**G**–**I**), Sertoli cell (SC) marker vimentin (FITC, green) (**J**–**L**) and peritubular cell (PTC) marker smooth muscle actin (SMA, green) (**M**–**O**) in testicular cryosections from untreated (**D**, G, J, M), adjuvant control (**E**, H, K, N) and EAO (**F**, I, L, O) animals. Nuclei are counterstained using DAPI staining (blue). Insets show HMGB1 staining (Cy3, orange) in isolated testicular macrophages (TM) (G), SC (J) and PTC (M). Under non-inflammatory conditions HMGB1 was mainly localized in the nuclei of SC (D, E, J, K), TM (D, E, G, H) and PTC (D, E, M, N). Cytoplasmic translocation of HMGB1 was observed in some TM (F, I), SC (F, L) and at lower level in PTC (F, O) in EAO testis. Notable is a very strong intensity of HMGB1 staining in the intercellular space in inflamed EAO testis. TM (G–I), SC (J–L) and PTC (M–O) are indicated by the white arrows, respectively.

fluorescently labeled oligonucleotide tags and is detectable using a fluorescent microscope (Soderberg *et al.*, 2008).

Cells were fixed with acetone and incubated overnight at 4°C with the HMGB1 antibody labeled with PLA MINUS probe and PLA PLUS probe conjugated either to TLR4 or to RAGE antibody. PLA reactions were performed according to the manufacturer's manual (Olink Bioscience, Uppsala, Sweden). Images were captured using the Axioplan 2 microscope (Carl Zeiss). Quantification of the data was performed using Duolink Image Tool software (Olink Bioscience).

Western blotting

Cells were lysed in Laemmli buffer containing protease inhibitor cocktail (Sigma-Aldrich) and boiled. Proteins were resolved by 12% SDS

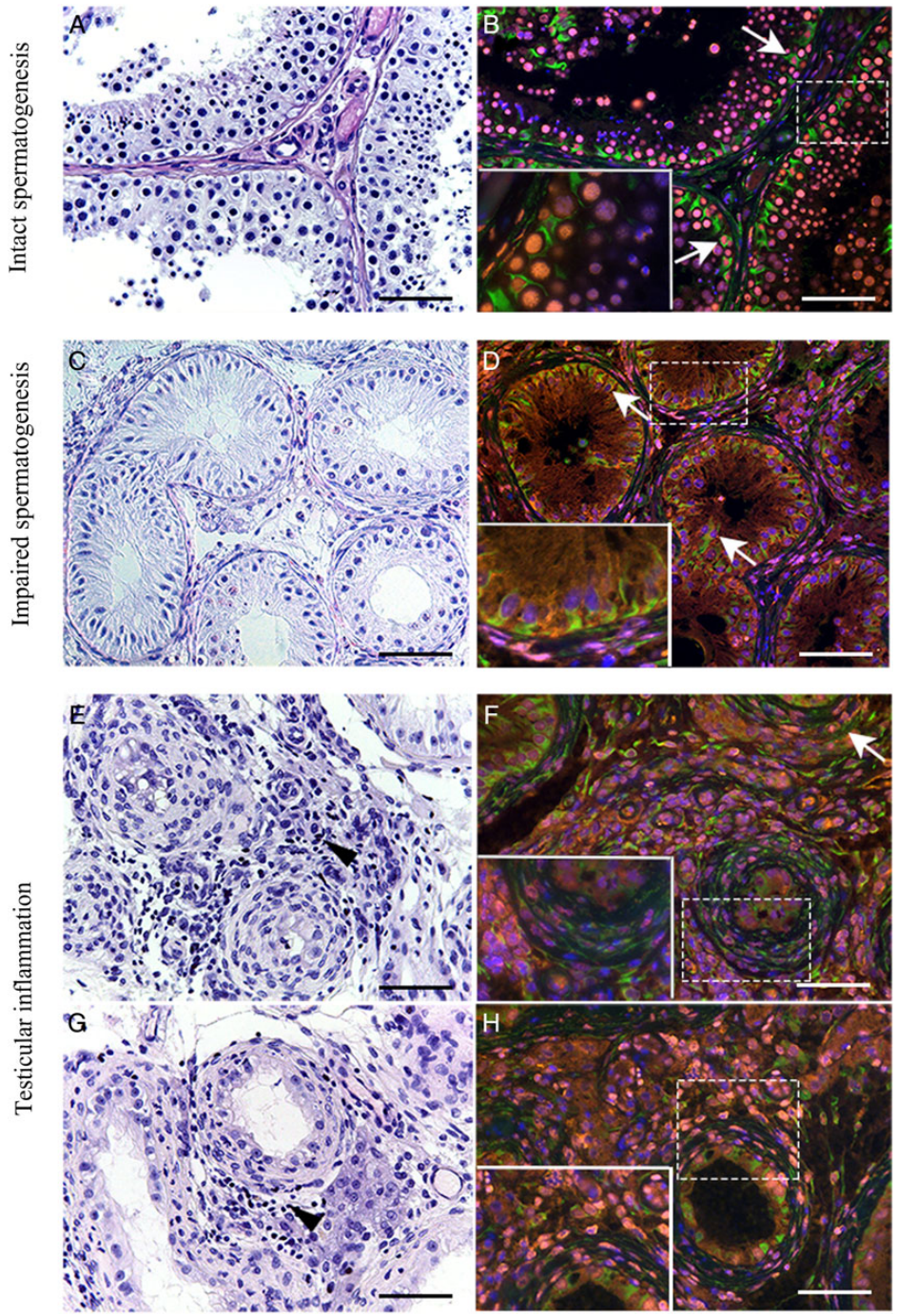

Figure 3 Cytoplasmic translocation of HMGB1 in human testis with focal inflammation and impaired spermatogenesis. H&E staining (**A**, **C**, **E**, **G**) and co-localization of HMGB1 (Cy3, orange) and vimentin (FITC, green) (**B**, **D**, **F**, **H**) on paraffin sections from human testicular biopsy showing seminiferous tubules with intact spermatogenesis and no signs of inflammation in the interstitial compartment (A, B), impaired spermatogenesis comprising seminiferous tubules with spermatogenetic arrest or Sertoli-cell-only pattern (C, D) and focal inflammatory lesions with peritubular lymphocytic infiltrates involving the lamina propria of affected seminiferous tubules as well as adjacent blood vessels (E–H). Nuclei are counterstained using DAPI staining (blue). In testis with intact spermatogenesis (B) HMGB1 is visible in the nuclei of SC, germ cells, PTC and some interstitial cells. In contrast, in the testis with impaired spermatogenesis (D) or inflamed testis (F, H) HMGB1 is translocated in the majority of cells out of the nucleus. Moreover, in the testis with inflammatory lesions (F, H) stronger vimentin staining was visible in interstitial cells. Insets show magnified areas in dotted boxes. Scale bars represent 50 μm.

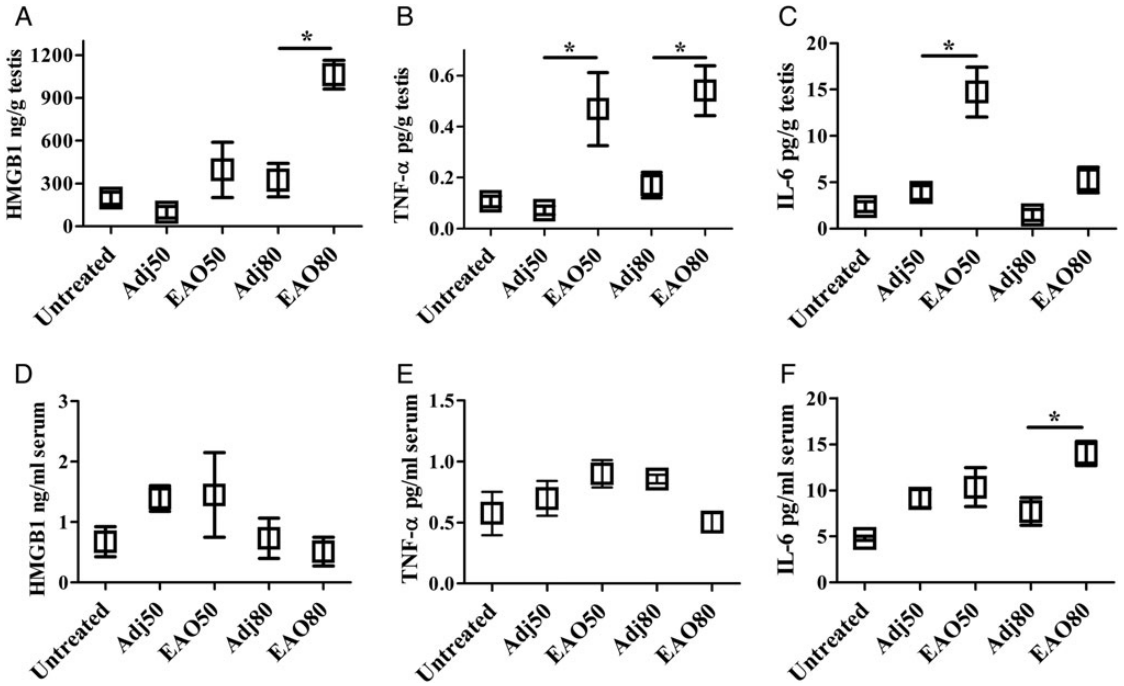

Figure 4 Relative levels of HMGB1, TNF-α and IL-6 are differentially expressed in rat EAO testis. Protein levels of HMGB1 (**A**, **D**), TNF-α (**B**, **E**) and IL-6 (**C**, **F**) were measured in testicular homogenates and serum from normal, adjuvant and EAO groups (50 and 80 days) using specific enzyme-linked immunosorbent assay; ($n = 3–7$); *$P < 0.05$. Data are presented as box-whisker plots ± SEM.

polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (GE Healthcare, Freiburg, Germany), blocked either with 5% non-fat dry milk or 5% BSA (Roth, Karlsruhe, Germany) and incubated overnight with indicated primary antibodies followed by incubation with HRP-conjugated secondary antibody for 1 h (Table I). Supersignal West Pico Chemiluminescent substrate (Thermo Scientific, Waltham, MA, USA) was used to visualize the signal using X-ray film (GE Healthcare, Buckinghamshire, UK).

RNA extraction and quantitative real-time RT–PCR

Total RNA was isolated using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Real-time RT–PCR was performed using iCycler iQ5 thermal cycler (Bio-Rad, Munich, Germany). To evaluate IL-6 and TNF-α mRNA expression, QuantiTect SYBR green PCR Master Mix and QuantiTect primer assay (Qiagen) were used (Table II). Relative gene expression was calculated using the ΔΔCt method.

Statistical analysis

Data are shown as mean ± SEM from at least three independent experiments. Comparisons of untreated and rhHMGB1 treated cells were performed by two-tailed *t*-test. One-way ANOVA followed by Tukey's multiple comparison *post hoc* tests were used when more than two groups were compared. *P*-values <0.05 were considered as significant. All tests were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

Results

Pathological changes in EAO

Successful induction of EAO was monitored by means of characteristic histopathological changes (edema formation, leukocytic infiltration, presence of apoptotic bodies in the seminiferous epithelium, germ cell loss; Fig. 2C) and reduced testicular weight (Fijak *et al.*, 2005). A significant reduction of nearly 2-fold in testicular weight was observed in orchitis animals 50 and 80 days after the first immunization (0.76 ± 0.05 g and 0.72 ± 0.03 g, respectively) compared with the normal (1.58 ± 0.02 g) and adjuvant control groups (1.56 ± 0.03 g and 1.44 ± 0.06 g). Fifty days after the first immunization 70% (7/10) of the animals developed orchitis, while 30 days later all immunized animals (10/10) revealed severe chronic testicular inflammation.

HMGB1 translocates from the nucleus into the cytoplasm in somatic cells in EAO testis

Immunofluorescence staining revealed that in untreated and adjuvant control groups HMGB1 is present mainly in the nuclei of somatic and germ cells (Fig. 2D and E). Using cell-specific markers HMGB1 was detected in the nuclei of TM, SC and PTC (Fig. 2). Moreover, in isolated PTC HMGB1 was detected in nuclei and cytoplasm (Fig. 2M inset). Similarly, in human testis with normal spermatogenesis HMGB1 was localized in the nuclei of SC, PTC, some interstitial cells and germ cells (Fig. 3). In the EAO50 (Fig. 2F) and EAO80 (data not shown) groups HMGB1 was translocated from the nuclei into the cytoplasm in the majority of TM and

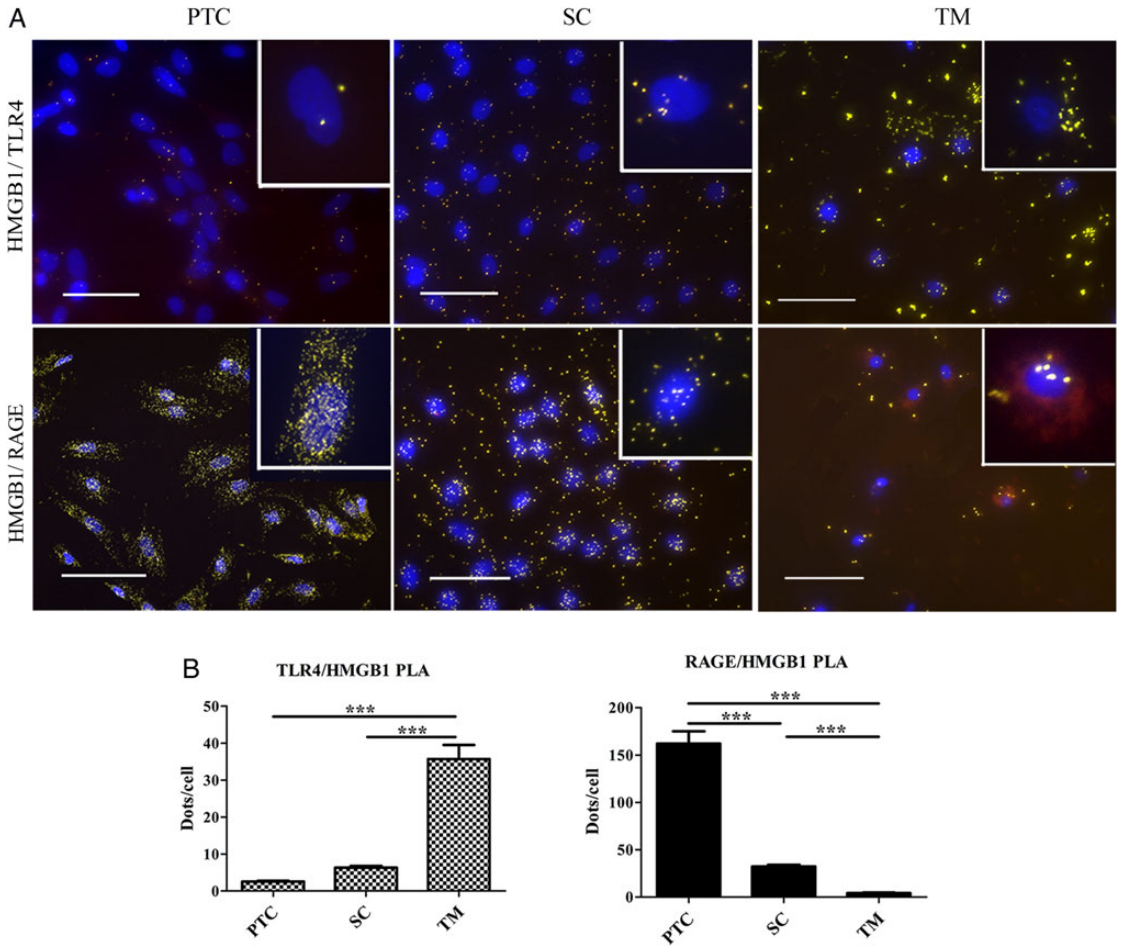

Figure 5 Cell-specific interaction of HMGB1 with Toll-like receptor 4 (TLR4) and receptor for advanced glycation end products (RAGE). (**A**) HMGB1-TLR4/RAGE binding in isolated rat testicular somatic cells. PTC, SC and TM were treated with 5 μg recombinant human (rh)HMGB1 for 2 h and proximity ligation assay (PLA) was performed. Yellow dots indicate HMGB1-TLR4/RAGE binding. Scale bar represents 50 μm. (**B**) Quantification of HMGB1-TLR4/RAGE binding in isolated testicular cells using the Duolink Image Tool; ***$P < 0.001$.

SC and likely also to the testicular interstitial space (Fig 2I and L). In some PTC, translocation of nuclear HMGB1 to the cytoplasm was evident (Fig. 2O). Likewise, in human testis biopsies with focal inflammatory infiltrates HMGB1 was found to be translocated from the nuclei when compared with specimens with seminiferous tubules revealing intact spermatogenesis in the absence of any signs of inflammation (Fig. 3). Notably, cytoplasmic translocation of HMGB1 in SC was not restricted to foci with peritubular lymphocytic infiltrates and severe damage of affected seminiferous tubules (partial to complete loss of the germinal epithelium, thickening of the lamina propria to complete tubular fibrosis), but was also detectable in areas revealing impaired spermatogenesis without infiltration of non-resident immune cells.

Testicular HMGB1 is a late mediator of inflammation in EAO

Levels of TNF-α and IL-6 in the testis were significantly up-regulated in EAO50 and only TNF-α remained elevated during the later phase of the disease (EAO80; Fig. 4B and C). In contrast, levels of HMGB1 started to increase in the testis of EAO50 animals reaching significantly elevated levels in the EAO80 group, suggesting a role during aggravation of disease (Figs 4A and 8E).

Serum levels of indicated cytokines did not increase significantly (except for IL-6 in EAO80 animals) excluding the possibility that elevated testicular levels result from a systemic inflammation (Fig. 4D–F).

RAGE and TLR4 are differentially expressed in testicular cells

To date, several studies have shown that HMGB1 induces different cellular responses depending on binding to various receptors. We focused on HMGB1 binding to RAGE and TLR4 as promiscuous receptors known to be expressed by testicular somatic cells (Mallidis *et al.*, 2007; Bhushan *et al.*, 2008; Brint *et al.*, 2010; Ganley-Leal *et al.*, 2010; Kang *et al.*, 2011). Using immunofluorescence analysis, expression levels of RAGE and

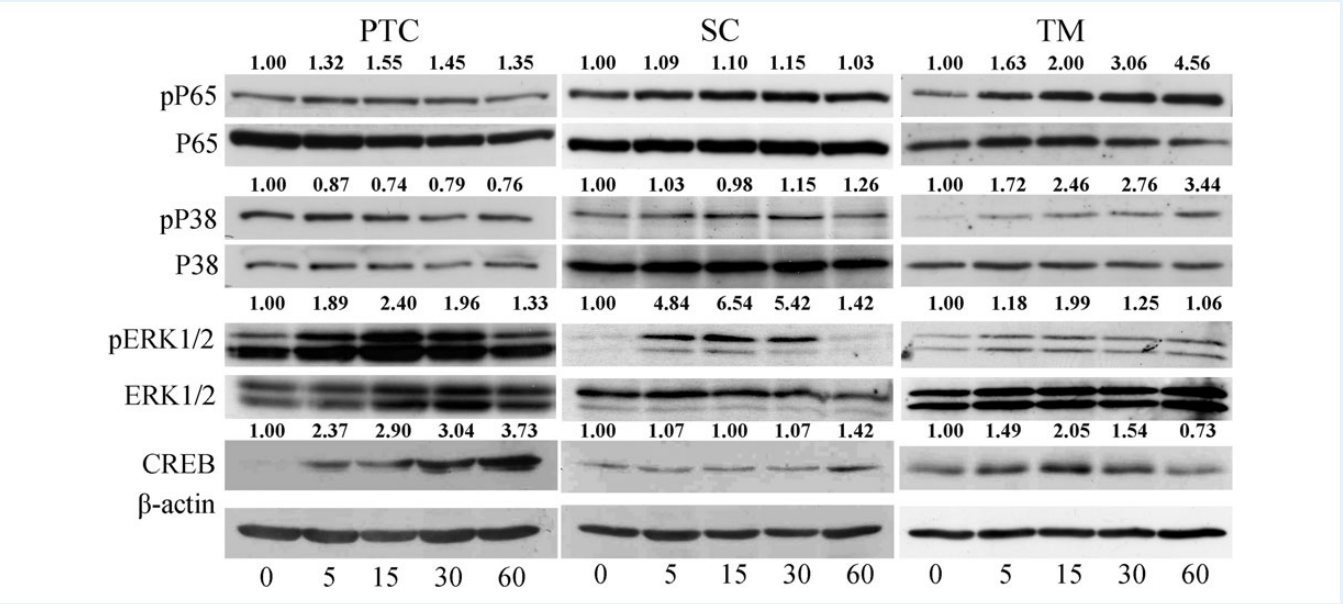

Figure 6 rhHMGB1 modulates activation of specific signaling pathways in rat testicular somatic cells. Isolated PTC, SC and TM were treated with 5 μg rhHMGB1 for 5–60 min. Phosphorylation of the nuclear factor Kappa B (NF-κB) subunit p65, mitogen-activated protein (MAP) kinases p38, extracellular signal regulated kinase (ERK) 1/2 and cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) were monitored by western blot analysis. β-Actin was used as a loading control. Numbers on top of each blot represent quantification of the relative abundance of each band in comparison to the respective total protein. Blots are representative of at least three independent experiments.

TLR4 proteins were found to vary substantially between isolated testicular cells (Fig. 5A and B).

HMGB1-TLR4 as well as HMGB1-RAGE binding was evaluated in TM, PTC and SC using Proximity Ligation Assay (PLA). Highest levels of HMGB1-TLR4 binding were detected in TM followed by SC with only little interaction visible in PTC. In contrast, significant HMGB1-RAGE interaction was observed in PTC compared with SC and TM. Of note, in TM only a few HMGB1-RAGE binding spots were visible (Fig. 5A and B).

HMGB1 has a cell-specific mode of action in inflamed testis

Based on differential receptor expression levels, activation of different signaling pathways by HMGB1 was examined in isolated TM, PTC and SC. rhHMGB1 induced phosphorylation of p38 mitogen-activated protein kinase (MAPK) in TM, but no substantial effect on ERK1/2 phosphorylation was detected (Fig. 6). Conversely, rhHMGB1 stimulation in SC and PTC caused strong activation of ERK 1/2 and CREB, while p38 MAPK showed only a minor increase in SC (Fig. 6). Moreover, strong phosphorylation of NF-κB subunit p65 was detected in TM and to a lesser extent in PTC (Fig. 6).

HMGB1 induces IL-6 and TNF-α mRNA expression in TMs and PTC, but not in SC

To study the effects of alternative signaling pathway activation by HMGB1, target gene expression levels of pro-inflammatory cytokines IL-6 and TNF-α were quantified using real-time RT–PCR analysis. In TM, IL-6 and TNF-α mRNA levels were significantly elevated after rhHMGB1 treatment, an effect that was effectively inhibited by pretreatment with EP (Fig. 7C and F). Additional control experiments in TM with heat inactivated rhHMGB1 showed no effect on IL-6 or TNF-α mRNA expression (Fig. 7C and F). Similarly, rhHMGB1 treated PTC demonstrated a significant up-regulation of TNF-α mRNA, but not IL-6 levels (Fig. 7A and D). In rhHMGB1 treated SC, IL-6 and TNF-α mRNA levels remained unchanged (Fig. 7B and E).

Interventional therapy with EP during EAO ameliorates testicular inflammation

To monitor whether inhibition of HMGB1 release after induction of EAO can attenuate progression of testicular inflammation, EP was administered intraperitoneally every second day starting from Day 20 after the first immunization (EAO+EP group) when the first signs of disease become apparent (Fijak *et al.*, 2005). Outcome of EP treatment on EAO development was evaluated 50 days after first immunization. In adjuvant control group treated with EP (Adj+EP group) no alterations in testis weight or morphology were detected (Fig. 8A–C). In the EAO animals treated with EP (EAO+EP group) a significant increase in testis weight was observed, which was accompanied by a significantly decreased number of aspermatogenic (devoid of germ cells) seminiferous tubules as compared with the EAO group (Fig. 8A–C). Morphological analysis of testicular architecture revealed that only 44% (4 of 9) of animals developed severe EAO compared with 100% (7 of 7) of control EAO animals.

To test the putative inhibitory properties of *in vivo* EP administration on the translocation/release of HMGB1 from testicular cells, localization of HMGB1 in testes from EAO+EP animals was analyzed. In intact tubules of EAO rats treated with EP, HMGB1 was visible in the nuclei in the majority of PTC, SC, spermatogonia and interstitial cells similar to control

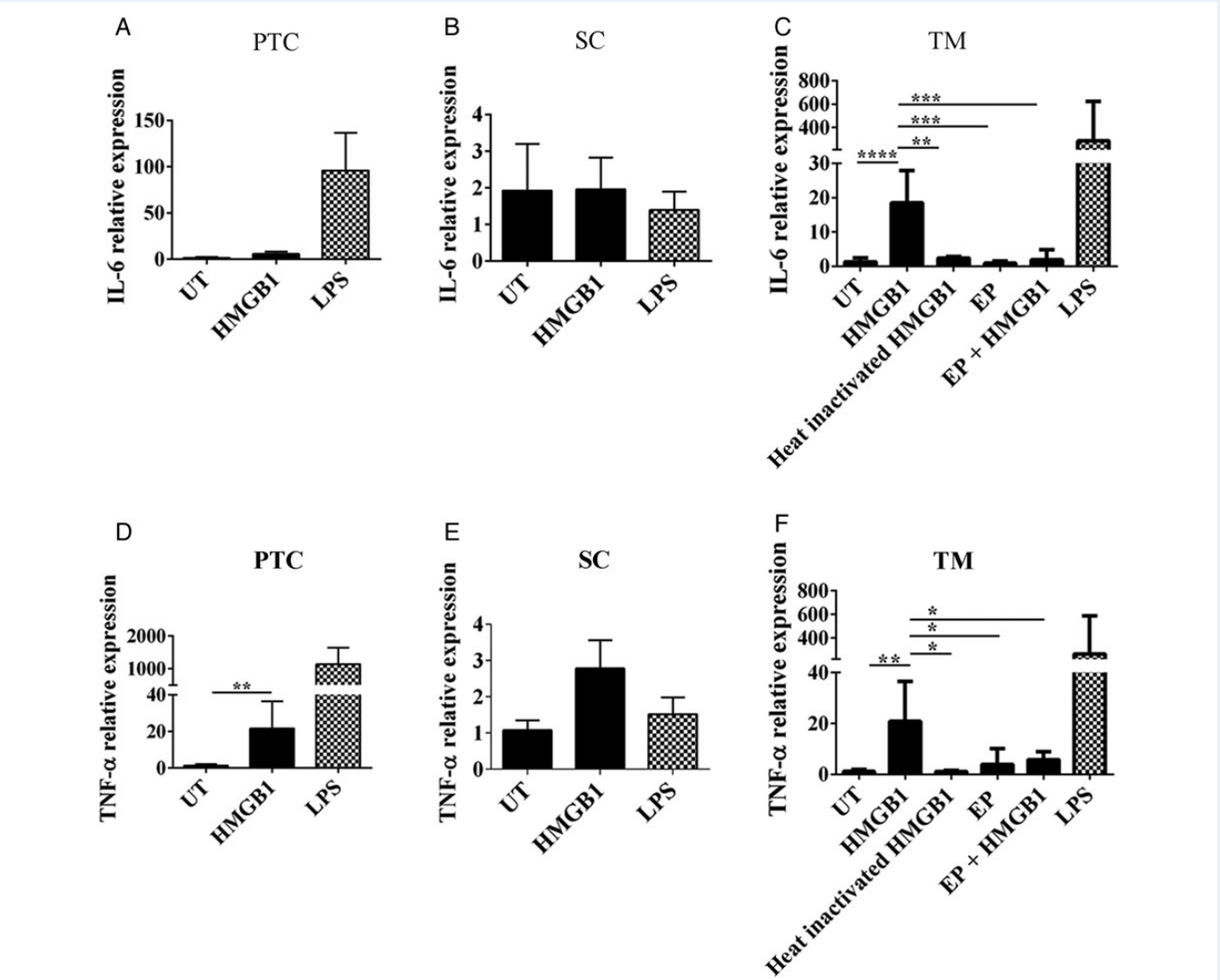

Figure 7 rhHMGB1 induces enhanced interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) expression in rat TM. mRNA expression of IL-6 and TNF-α was analyzed using quantitative real-time RT–PCR in isolated PTC (**A**, **D**), SC (**B**, **E**) and TM (**C**, **F**). Cells were treated with 5 μg rhHMGB1 or 10 μg LPS for 6 h or left untreated. TM were also treated with heat inactivated rhHMGB1 or pre-treated with 10 mM EP for 1 h before stimulation with rhHMGB1. Relative gene expression was normalized to β2-microglobulin; ($n = 4–7$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

groups (Fig. 8A). In contrast, in tubules showing germ cell loss HMGB1 was translocated out of the nucleus in most SC and PTC (Fig. 8A).

Accumulation of macrophages in the testicular interstitial space during EAO development plays an important role during immune response and aggravation of the disease (Rival *et al.*, 2008). Following EP treatment a significant decrease in the number of TM in the EAO group when compared with EAO control animals was evident (Fig. 8A and D). Interestingly, TM in EAO+EP group retained HMGB1 inside the nucleus in contrast to the EAO control group, where HMGB1 showed cytoplasmic translocation (Fig. 8A).

Furthermore, the analysis of relative testicular HMGB1 and IL-6 concentrations revealed beneficial effects of EP treatment on the levels of these pro-inflammatory cytokines. To better evaluate the effect of EP treatment on the production of HMGB1 and IL-6, EAO animals treated with EP (EAO+EP) were subdivided into two subgroups: (i) EAO+EP S—animals developing severe disease symptoms and (ii) EAO+EP M—rats showing only mild or no signs of testicular inflammation. The relative levels of HMGB1 and IL-6 were significantly lower in the EAO+EP M group compared with EAO control animals. In contrast, in the EAO+EP S animals relative testicular concentrations of HMGB1 and IL-6 were similar to the EAO group without EP treatment (Fig. 8E and F).

Discussion

Our results demonstrate that HMGB1 is involved in the development of chronic testicular inflammation in a rat model of EAO. Evidence is provided at multiple levels *in vitro* and *in vivo*. HMGB1, under normal conditions found in nuclei of cells in the testicular interstitium (macrophages) and seminiferous epithelium (SC and PTC, spermatogonia), was translocated into the cytoplasm and presumably extracellular space during

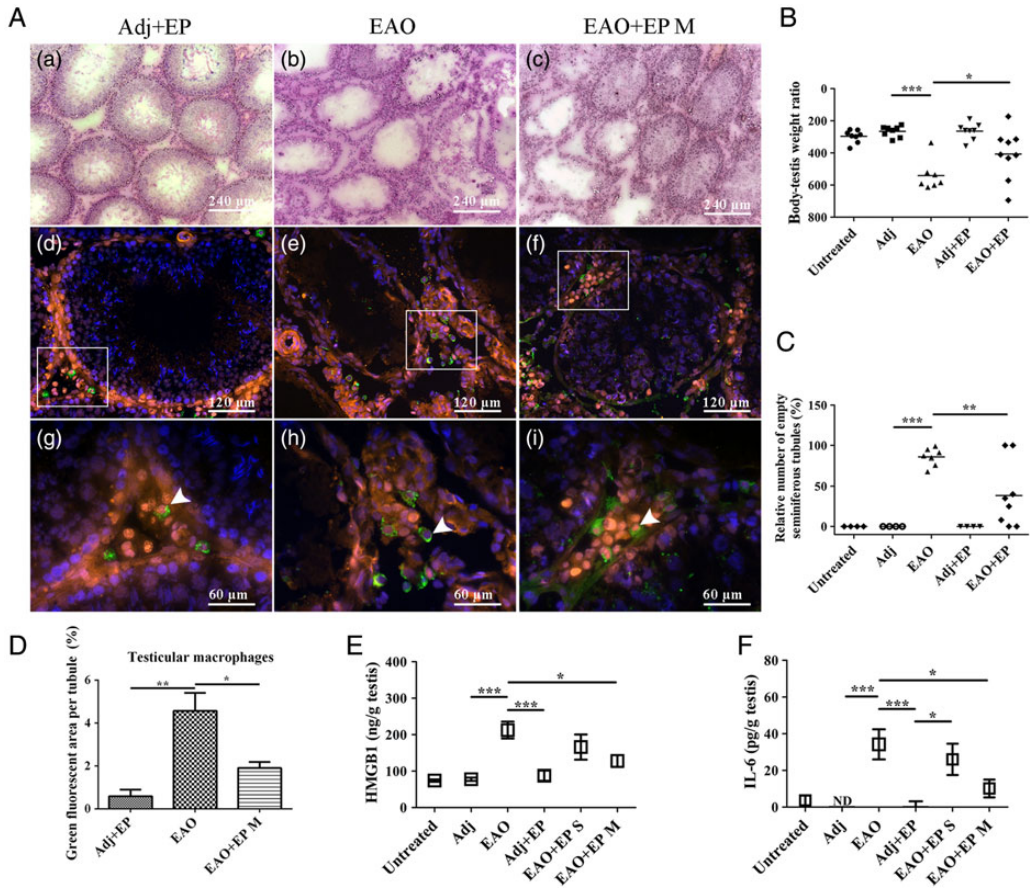

Figure 8 *In vivo* treatment with EP (40 mg/kg body weight, i.p.) attenuates the development and progression of rat EAO. (**A**) H&E staining of testes cryosections from Adj+EP (a), EAO (b) and EAO+EP M rats (c). Immunofluorescence detection of HMGB1 (Cy3, orange) and TM (CD68/CD163, FITC, green) in testicular cryosections from Adj+EP (d, g), EAO (e, h) and EAO+EP M (f, i) groups. Nuclei were counterstained using DAPI staining (blue). (**B**) Body to testis weight ratios in different treatment groups. (**C**) Relative number of aspermatogenic seminiferous tubules within the testis. *In vivo* treatment with EP protects spermatogenesis. (**D**) Area occupied by TM in relation to the number of seminiferous tubule cross-sections was quantified using Image J software. A significant decrease of TM accumulation was observed in the EAO+EP M group compared with EAO control alone. Relative concentration of HMGB1 (**E**) and IL-6 (**F**) was measured in testicular homogenates from untreated, Adj, EAO control animals and EP treated adjuvant (Adj+EP) as well as EAO+EP rats. EAO+EP group was subdivided into animals with severe disease symptoms (EAO+EP S) and rats showing only mild or no symptoms of EAO (EAO+EP M); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; $n = 4–9$ animals per group.

development of EAO. Interestingly a similar pattern of nuclear translocation of HMGB1 was evident in human testicular biopsies with focal or multifocal inflammatory lesions and concomitant damage of seminiferous tubules as seen in the rat model. Translocation of HMGB1 from the nucleus to the cytoplasm followed by passive or active release to the extracellular environment is a decisive step in its pathological function. Released HMGB1 acts as damage-associated molecular pattern (DAMP) and alarmin to augment rather than to elicit inflammatory responses (Harris *et al.*, 2012). Testicular concentrations of HMGB1 rose significantly (3-fold) in the late phase of EAO80 indicating a sustaining role in the aggravation of disease. In line with this finding, reports have shown the involvement of HMGB1 in the pathogenesis of several autoimmune diseases ranging from rheumatoid arthritis, vasculitis, systemic lupus erythematosus to myositis or Sjögren's syndrome (reviewed by Harris *et al.*) (Harris *et al.*, 2012). A pathogenic role of HMGB1 is strengthened by targeting this molecule for therapeutic intervention, which can be achieved by using ethyl pyruvate, neutralizing antibodies to HMGB1, quercetin, green tea extracts, curcumin, recombinant A box domain of HMGB1 or soluble RAGE, all resulting in beneficial effects with regard to the development of different autoimmune diseases in animals (Ulloa *et al.*, 2002; Kokkola *et al.*, 2003; Li *et al.*, 2007; Zetterstrom *et al.*, 2008; Dave *et al.*, 2009; Tang *et al.*, 2009; Kalariya *et al.*, 2011; Schierbeck *et al.*, 2011; Tu *et al.*, 2012; Robinson *et al.*, 2013).

For our *in vivo* approach, we selected EP—the stable lipophilic ester derivative of pyruvic acid—that has proved to be effective in a number of autoimmune diseases/models including autoimmune hepatitis (Shen *et al.*, 2014). Although pharmacologic effects of EP include protection of the cells from reactive oxygen species-mediated damage, down-regulation of NF-κB expression and decrease in the secretion of pro-inflammatory cytokines among others, the exact mechanism how EP causes a decrease in (bioactive) HMGB1 levels remains elusive (Yang *et al.*, 2002; Fink, 2007; Dave *et al.*, 2009; Hu *et al.*, 2012). In our model, treatment with EP

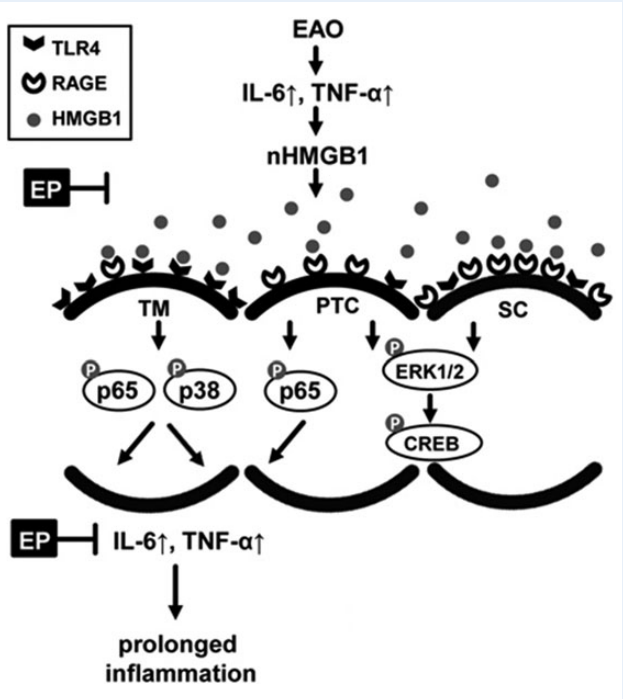

Figure 9 Hypothetical model of cell-specific action of HMGB1 on testicular somatic cells. In the early stage of development of EAO, levels of IL-6 and TNF-α are rapidly elevated followed by translocation of nuclear (*n*)HMGB1 into the extracellular space. Depending on the receptor expression profile on each cell type, HMGB1 induces activation of different signaling pathways. In adult TM high levels of HMGB1-TLR4 interaction trigger p38 MAPK and p65 NF-κB activation and subsequently elevated production of IL-6 and TNF-α which can be blocked by EP. In contrast, in immature SC with higher levels of HMGB1-RAGE binding, ERK1/2 is activated and cytokine up-regulation does not occur. On the other hand, in PTC from immature testis, both ERK1/2 and p65 are activated leading to TNF-α increase. Furthermore, *in vivo* blocking of HMGB1 release using EP treatment ameliorates disease symptoms by reduction of IL-6 and HMGB1 levels in the testis.

commenced with the first signs of autoimmune reaction detectable by the formation of auto-antibodies against testicular antigens as early as Day 20 after the first immunization (Fijak *et al.*, 2005). Of note, *in vivo* blockade of HMGB1 action by EP during the course of the disease led to significant amelioration of testicular inflammation as evidenced by protection of spermatogenesis, lack of macrophage invasion and lower IL-6 and HMGB1 levels.

Next, we were interested in elucidating the mechanism of HMGB1 contribution to autoimmune based testicular inflammation. In our *in vitro* experiments, addition of purified rHMGB1 to rat TM significantly stimulated the release of TNF-α and IL-6. In confirmation of the *in vivo* experiments, release of cytokines was abolished by addition of EP. A similar effect was observed in cultured PTC for TNF-α. Interestingly, SC did not respond to rHMGB1 administration by significant up-regulation of TNF-α or IL-6. This phenomenon could be explained by the fact that for cytokine induction by HMGB1, TLR4 signaling is an absolute requirement (Yang *et al.*, 2012). As shown in our study higher levels of HMGB1-TLR4 interaction were detected in TM, while increased expression of RAGE and concomitant HMGB1-RAGE binding was observed in SC and PTC. As the role of the RAGE pathway in the induction of cytokine release is less certain (Harris *et al.*, 2012), it may serve as an explanation for the lack of TNF-α and IL-6 production by HMGB1-stimulated Sertoli cells, where the RAGE pathway seems to be dominant. These data suggest that the action of HMGB1 in the testis might may be controlled by differential expression of TLR4 and RAGE receptors in populations of testicular cells that are known to be implicated in immune responses, a fact that could also be related to the different age of animals used for cell preparations (Arck *et al.*, 2014; Fijak *et al.*, 2011a,b).

Actively secreted or passively released HMGB1 can signal through RAGE and toll-like receptors such as TLR2 or TLR4 thus inducing a broad repertoire of immunological responses that include cytokine release, induction of chemotaxis and angiogenesis as well as cell differentiation and proliferation. HMGB1 receptor interaction and consequently downstream signaling can be influenced by various post-translational modifications of HMGB1 such as acetylation, phosphorylation, methylation and redox changes of cysteine residues (Harris *et al.*, 2012). Thus it is noteworthy that we rather used the oxidized form of rHMGB1—lacking dithiothreitol as a reducing agent, which otherwise could disrupt the disulfide bond at Cys23-Cys45 essential for TLR4 stimulation (Yang *et al.*, 2012). In the normal rat testis TLR4 is expressed predominantly by TM and at lower levels also by SC, PTC and dendritic cells (Bhushan *et al.*, 2008). In support of a role for TLR4 in mediating TNF-α production, Yang *et al.* (2010) showed that HMGB1-stimulated TLR4-deficient macrophages did not release TNF-α. Furthermore, inhibition of TLR4 binding with neutralizing anti-HMGB1 antibodies or by mutating cysteine 106 prevents HMGB1-induced cytokine release (Yang *et al.*, 2010).

At the signal transduction level, stimulation of TM with rHMGB1 activated MAPK p38 and p65 NF-κB, while in SC and PTC the ERK1/2 MAPK signaling pathway was dominant. These findings suggest a cell-specific and receptor-dependent mechanism of HMGB1 action in controlling immune responses in the testis. As shown by Park *et al.* (2003), stimulation of neutrophils with HMGB1 activated p38 MAPK and to a lesser extent PI3K, Akt and ERK1/2. Inhibition of the molecules involved in this pathway down-regulated HMGB1-induced cytokine production through a decrease in NFκB activation (Park *et al.*, 2003). In TM, HMGB1 induced NFκB phosphorylation, which can subsequently stimulate the release of pro-inflammatory cytokines and expression of HMGB1 receptors (van Beijnum *et al.*, 2008). Therefore, increased secretion of HMGB1 as well as up-regulated receptor expression can sustain and amplify the inflammatory response and lead to progression of EAO. HMGB1 translocation from the nucleus into the cytoplasm and increasing concentrations of pro-inflammatory cytokines (TNF-α, IL-6) in testicular cells were visible already 50 days after first immunization (EAO50). These findings support our hypothesis that increasing concentrations of pro-inflammatory mediators at early stages of the disease combined with high levels of HMGB1 later (EAO80) lead to progression and worsening of testicular inflammation.

Onset and progression of EAO are accompanied by interstitial infiltration with leukocytes, mainly macrophages, T cells and dendritic cells (Fijak *et al.*, 2005; Rival *et al.*, 2006, 2008). On the other hand TM play an important role in the preservation of immune privilege, at least in the normal testis (Meinhardt and Hedger, 2011). Interestingly, HMGB1 is also involved in attraction of immune cells by forming complexes with CXCL12 (Schiraldi *et al.*, 2012). Our results indicate that in contrast to normal conditions, cytoplasmic translocation of HMGB1 occurs in macrophages in EAO testis, which via an autocrine mechanism interacts with TLR4 to trigger production of IL-6 and TNF-α. This inflammatory cascade (loop) could play a relevant role

during the autoimmune reaction and aggravation of disease in the testis, as supported by the successful blockade of IL-6 and TNF-α secretion in TM *in vitro* and prevention of disease development *in vivo* by EP application. Moreover, EP administration during development of EAO inhibits translocation of HMGB1 in TM. Although SC show translocation of HMGB1 in EAO testis, they lack production of pro-inflammatory cytokines after HMGB1 stimulation in contrast to TM (hypothetical model in Fig. 9). Furthermore, in EAO testis we observed strong migration and maturation of dendritic cells as well as production of auto-antibodies against testicular antigens (Fijak *et al.*, 2005; Rival *et al.*, 2007). It is possible that HMGB1 may be involved in the establishment of autoimmunity in the EAO testis, acting as an endogenous adjuvant and trigger for maturation of antigen presenting cells (Rovere-Querini *et al.*, 2004).

Taken together, our results suggest that early pro-inflammatory cytokines (TNF-α, IL-6) secreted from different testicular cells at the onset of EAO can trigger cytoplasmic translocation of HMGB1. In a regulatory loop to sustain and prolong the immune response, subsequent binding of HMGB1 to TLR4 on TM induced the synthesis and release of TNF-α and IL-6 thus fueling the inflammation and finally leading to worsening of EAO. *In vivo* blocking of HMGB1's inflammatory action by EP attenuated or even fully inhibited testicular inflammation thus protecting against germ cell loss. Translocation of HMGB1 in inflammatory lesions of testicular biopsies indicates a similar role for this mediator in inflammation-based male factor sub-/infertility.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

F.A. contributed to study design, performance of experiments, interpretation and discussion of data, drafting of the article. H.-C.S. was involved in conception of the study, clinical evaluation, patient screening, collection of samples, interpretation and discussion of data and drafting of the manuscript. V.A.G. and L.L. performed interventional animal studies and contributed to data analysis and drafting of the manuscript. S.B. and G.L. performed experiments and were involved in interpretation of results. E.W. contributed to performance of experiments for the study. A.M. contributed to conception and study design, interpretation and discussion of data, drafting of the article. M.F. contributed to conception and study design, performance of experiments, interpretation and discussion of data as well as drafting of the article. The final version of article was revised and approved by all authors.

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Conflict of interest

None declared.

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