High mobility group box protein-1 is involved in modulation of cell specific immune responses during chronic testicular inflammation in rat

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ABBREVIATIONS

Å	Angstrom
APS	Ammonium persulfate
BSA	Bovine serum albumin
cDNA	Complementary DNA
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Desoxyribonucleosidtriphosphate
ECL	Enhanced chemiluminescence
EAO	Experimental autoimmune orchitis
EDTA	Ethylenediaminetetraacetic acid
EP	Ethyl pyruvate
FCS	Fetal calf serum
g	Gram
G	Gauge
h	Hour
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMGB1	High mobility group box protein 1
HRP	Horse radish peroxidase
IL	Interleukin
IPTG	Isopropyl-β-D-thiogalactopyranosid
kbp	Kilobase pair
kDa	Kilodalton
l	Litre
LB medium	Lysogeny broth medium
LC3	microtubule-associated protein1 light chain 3
LPS	Lipopolysaccharide
µg	Microgram
µl	Microlitre
min	Minute

ml	Millilitre
NaCl	Sodium chloride
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NGS	Normal goat serum
O/N	Over night
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PCR	Polymerase chain reaction
PTC	Peritubular cells
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
s	Second
SDS	Sodium-dodecyl-sulfate
SC	Sertoli cells
SOB	Super optimal broth medium
SOC	Super optimal broth catabolite repression medium
TAE	Tris acetate EDTA buffer
TE	Tris EDTA
TEMED	N,N,N´,N´-Tetramethylethylemediamine
TM	Testicular macrophages
TNF	Tumor necrosis factor
Tris	Tris(hydroxymethyl)-amino-methane
W	Watt

1 INTRODUCTION

1.1. Male reproductive system

The male reproductive system in mammals is made up of different parts namely the male gonad (testis), epididymis, accessory glands, the excretory ducts and the penis [1]. The proper function of all these parts will ensure normal fertility of a male individual. In mammals, the testis is the site for production of sperm and most of the circulating androgens with testosterone being most abundant in the lumen of the seminiferous tubules [2]. Spermatozoa are released and transported to the rete testis to enter the epididymis. There sperms maturate under the influence of epididymal epithelial cells and gain competence for fertilizing the ovum, a process completed by the secretions of the accessory glands (seminal vesicles, prostate gland and bulbo-urethral glands) during ejaculation [3].

1.2. Testis - structure and function

In the mammalian embryo the testis is formed from the sex cord following expression of the SRY gene. The mammalian testis is constituted of the seminiferous tubules and interstitial space. The seminiferous tubules are surrounded by myoid peritubular cells (PTC) and Sertoli cells (SC). Leydig cells (main producers of testosterone) and mononuclear cells reside in the interstitial space of the testis (Figure 1A). The contractile PTC form the seminiferous tubules and facilitate the movement of newly generated immotile testicular spermatozoa in the lumen of the seminiferous epithelium into the epididymis [4]. Depending on the species one or more layers of PTC are formed around the tubules. The role of PTC in spermatogenesis is largely unexplored. However, there is evidence showing a direct or indirect role of PTC in spermatogenesis. A recent study indicated that PTC express androgen receptor and mediate androgen actions on fetal SC proliferation [5]. SC extend from the basal lamina to the lumen, engulf the developing germ cells and provide them with necessary growth factors and nutrients for progression of normal spermatogenesis (Figure 1B) [6]. Functions of SC are regulated by androgens via the androgen receptor.

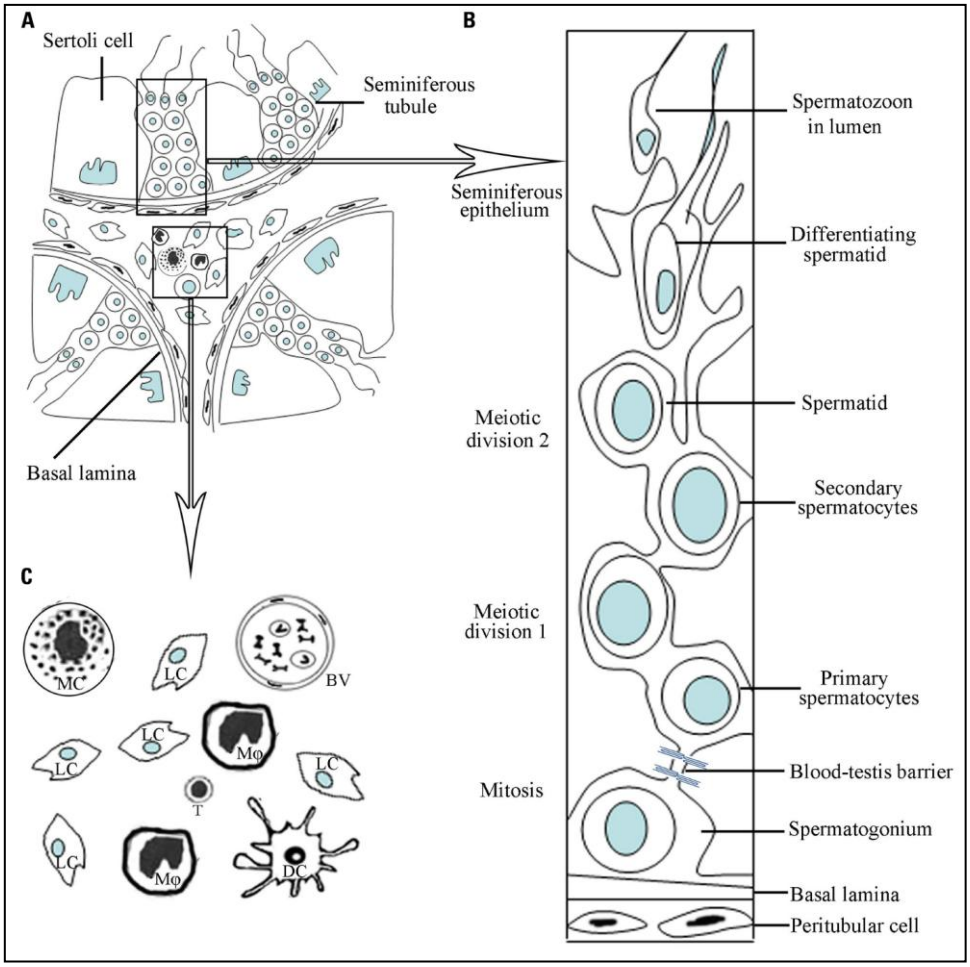


Figure 1. Structure and cellular compartments of the testis. The testis is composed of two compartments: 1) the seminiferous tubules and 2) the interstitial space (A). Sertoli cells act as a matrix to hold the germ cells. The tight junctions at the basal part of Sertoli cells form the blood-testis barrier which separates spermatogonia from spermatocytes (B). The largest population of cells in the interstitial space is the Leydig cells (LC). The interstitial space also contains immune cells such as testicular macrophages (MΦ), dendritic cells (DC), mast cells (MC) and blood vessels (BV) (Li et al., Frontiers in Immunology; 2012).

Synthesis of sex steroid hormones (steroidogenesis) takes place in Leydig cells in the interstitial space. Production of high levels of testosterone after puberty activates spermatogenesis in the testis. Leydig cells are the major cell type in the interstitium. Additionally, the testicular interstitium contains blood and lymphatic vessels as well as various types of immune cells including testicular macrophages (TM), dendritic cells, lymphocytes and mast cells (Figure 1C) [6,7].

1.3. Immune privilege of the testis

The testis is an immune privileged organ, providing tolerance to introduced antigens or grafts without eliciting an inflammatory immune response [8,9]. Exposure of antigens from newly produced germ cells to the immune system is inevitable. Therefore, the immune system has developed mechanisms to control the immune response against neo-self antigens. Immune privilege does not mean that immune responses are absent in a tissue, but that such responses are strictly controlled. Hence, immune privileged tissues are more susceptible to infections by exogenous antigens as the immune responses are actively suppressed via tightly regulated mechanisms in those tissues [8,10].

1.3.1. Sertoli cell junctions

One strategy to hamper provocation of immune responses against neo-self antigens is the physical separation of newly developing germ cells from the immune cells by SC' tight junctions called blood-testis barrier (BTB). However, this is not sufficient to shape the immune privilege of the testis as the SC barrier is not complete and it opens at points to let the developing spermatocytes move forward to the center of the seminiferous lumen [11]. The complete BTB serves three functions: anatomical, physiological, and immunological. The tight junctions (TJ) form the physical barrier that restricts passage of molecules and cells to the lumen. The physiological barrier is comprised of transporters that regulate movement of substances in or out of the lumen. The immunological barrier limits the access of immune system to autoantigenic germ cells [11]. It is now believed that physical barrier, differential action of testicular somatic and immune cells, cytokine profiles and production of anti-inflammatory molecules and hormones (testosterone) all together provide a tightly regulated immune response in the testis. There is no BTB in the rete testis, therefore spermatozoa in transit are not physically protected when they exit the testis and enter the epididymis [6,8,9].

To form the BTB, adjacent SC are tightly connected by junctions including tight junctions (TJ), basal ectoplasmic specializations, gap junctions, and desmosome-like junctions in the basal third of the seminiferous epithelium [11]. The tight junctions and other components of the BTB allow the cyclic passage of early primary spermatocytes while maintaining BTB integrity [12]. Permeability of tight junctions is governed by the membrane protein mainly claudins and occludins [13].

1.3.2. Role of Sertoli and peritubular cells

In addition to BTB formation, SC are recognized to have immunosuppressive characteristics [14]. Sertoli cells are known to provide an immunosuppressive environment which allows cotransplanted bovine adrenal chromaffin cells survive in adult rat [15]. Under stress conditions such as infection or inflammation SC release anti-inflammatory cytokines and molecules like activin which participate in the immune protection of the testis. Activin A is an immune regulatory molecule largely produced by SC which has a role in controlling inflammation and immunity in the testis in addition to its role in stimulating FSH production by pituitary gonadotrophs [16]. Additionally, rat Sertoli cells produce IL-1α which has an important role in BTB tight junctions dynamics during passage of spermatocytes [17]. Under inflammatory conditions SC express IL-6, TNF-α and its receptor TNFR2 and FAS ligand which altogether direct the germ cells to apoptotic programmed cell death [18]. PTC are also believed to participate in the maintenance of the testicular immune environment [19]. PTC express activin A, TNF-α receptors 1 and 2. Under inflammatory conditions they respond to TNF-α and express other inflammatory molecules, such as IL-6 and COX-2 [20]. The role of PTC in the testicular inflammatory responses remains to be further clarified.

1.3.3. Testicular macrophages

Testicular macrophages are specialized to provide protection for the newly produced germ cells and at the same time perform qualitatively normal inflammatory responses and protection against infections to maintain immune privilege of the testis. Under physiological conditions macrophages compose the majority of leukocytes (about 15-20%) which are present in the interstitial cells of rat testis [21]. Different subsets of macrophages can be distinguished in the rat testis which express either lysosomal glycoprotein CD68 (ED1+) or the scavenger receptor CD163 (ED2+) or both (ED1+/ ED2+). The major population (80%) of TM is the resident macrophages which are identified as ED1-/ED2+. ED2+ macrophages which display an immunosuppressive profile are responsible for the maintainance of immune privilege in the testis [19]. On the other hand, ED1+ macrophages, which presumably derive from circulating monocytes/macrophages infiltrating the testis, represent a minor (20%) proportion of the TM under normal conditions. ED1+ macrophages preserve the pro-inflammatory status of circulating monocytes and may have different functional roles within the testis [18,22]. Under normal non-inflammatory conditions, ED1+ macrophages express higher levels of IL-6 compared to ED2+ macrophages, [23], whereas ED2+ macrophages

produce significant amounts of the immunoregulatory cytokine IL-10 and to a lower level anti-inflammatory cytokine TGFβ1 confirming the distinct roles of the two types of macrophages in mediating inflammatory responses [24]. Production of immunosuppressive cytokines supports the fact that resident macrophages contribute to the testicular immune privileged status [24, 10].

Generally, immune responses elicited by TM are reduced as compared to peritoneal macrophages by blockage of NFkB activation, although TM maintain a general responsiveness by activation of MAP kinase and AP-1 signaling pathways following LPS stimulation [25]. These paradoxical features enable TM to protect testicular cells against microbes and at the same time maintain the immune privileged status of the testis. Cytokines produced by TM at low level under normal conditions such as TNF-α and IL-6 is shown to be crucial for the development and steroidogenesis of Leydig cells in adult rat [26]. Moreover, TM also influence SC functions and spermatogenesis [27].

Under pathological conditions such as testicular inflammation (orchitis) the number of ED1+ macrophages dramatically increases affecting the ratio of ED1+/ED2+ which could lead to progression of inflammation, impairment of testicular immune privilege and opening of the BTB and consequently triggering uncontrolled inflammatory reactions in the testis [28].

1.3.4. Cytokines of the testis

Cytokines are small proteins functioning in cell-cell communication which have an important role in a wide range of vital actions such as immune cell development, inflammation and immune responses [29]. Maintenance of normal testicular function and testicular immune privilege is strictly controlled by numerous paracrine and autocrine acting cytokines most of which belong to immune regulatory factors including those with immunosuppressive or anti-inflammatory activities such as TGF-β family members [30]. In addition, under physiological conditions some pro-inflammatory factors, including IL-1, IL-6, and TNF-α, are expressed in the testis and regulate testicular functions. As an example IL-1α facilitates BTB opening by affecting the SC actin cytoskeleton and expression of claudins and occludins [31]. Localized production of IL-1α by Sertoli and germ cells in vivo results in a change of BTB conformation, which triggers the movement of leptotene spermatocytes across the BTB towards the lumen [32]. IL-1β is also expressed in the testis under physiological conditions and is upregulated during inflammation and plays an important role in fueling

testicular inflammation [17]. Another cytokine with a high impact on testicular immune balance is IL-6. IL-6 has a role in T-cell activation and expansion [33]. IL-6 is expressed in testicular germ cells at different stages of differentiation, Leydig cells and peritubular cells in mouse testis. IL-6 production is upregulated in germ cells from autoimmune orchitis rats compared with control group [18]. Moreover, IL-6 expression in TM is significantly increased in chronic testicular inflammation. One of the most potent pro-inflammatory cytokines is TNF-α which is prominently synthesized by germ cells and interstitial cells, such as macrophages and mast cells [14]. Under non-inflammatory conditions, TNF-α protects the germ cells from apoptosis in normal testes. On the contrary, at onset of inflammation, TNF-α acts as an apoptotic factor that induces germ cell death. In rat autoimmune orchitis, production of TNF-α is upregulated in the testis and it is released mainly by TM trigger apoptosis of germ cells [28].

1.3.5. Testosterone as an immunosuppressive factor

It is known that female individuals are more susceptible to autoimmune reactions compared with males likely due to the immunosuppressive properties of androgens [34]. Testosterone has been successfully used to prevent onset of experimental autoimmune diseases such as experimental autoimmune orchitis and atherosclerosis [35,36,37]. Testosterone has a negative effect on TLR4 expression in macrophages [38]. Exhibiting immune modulatory capabilities, androgens play a role in maintaining the balance between autoimmunity and tolerance. Substantial evidence of the link between androgens and testicular immune privilege was found in investigations using mice conditional knockout of androgen receptors in SC. Interestingly, Sertoli cell-specific deletion of the androgen receptor in mice disrupts testicular immune privilege [39], possibly because androgens regulate SC tight junctions [40]. In agreement with these data, an earlier study indicated that androgens regulate the permeability of the BTB by regulating the expression of a SC tight junction protein, Claudin-3 [40,41]. The testicular endocrine environment also affects and modulates the action of immune cells in the testis [42]. Taken together, androgens play critical role in maintaining the integrity of testicular immune privilege by regulating local microenvironments [37,6].

1.4. Male infertility

Infertility is commonly defined as the failure to conceive a child after at least 12 months of unprotected intercourse. Male factor infertility roughly accounts for 40-50% of total cases

[43]. To achieve fertility a man requires normal spermatogenesis, successful epididymal maturation and storage of sperm, normal sperm transport via the efferent duct system and accessory gland function. Male infertility can result from physical problems, hormonal changes, genomic reasons as well as lifestyle or environmental factors. Male infertility can be categorized in pre-testicular, testicular and post-testicular factors [44,45]. Pre-testicular dysfunctions are mainly due to impaired gonadotropin levels. Gonadotropin-Releasing-Hormone (GnRH) induces LH and FSH release from pituitary gland which has a direct effect on testosterone production. Testicular disorders fall into different categories: 1) cryptorchidism: the absence of one or both testes from the scrotum; 2) genetic disorders such as Klinefelter's syndrome: 47, XXY, or XXY syndrome with damaged seminiferous epithelium and increased number of Leydig cells; 3) varicocele: abnormal enlargement of the veins in the scrotum draining the testes; 4) immunological disorders: infection and inflammation of the testes (orchitis) which can occur due to viral diseases (mumps), bacterial infections (E. coli, Enterobacter spp., Chlamydia and Gonorrhea) or sterile autoimmune inflammation of the testes [45,46].

1.4.1. Immunological male infertility

About 15% of all male infertility cases are due to inflammation of the male reproductive tract [47]. Autoimmune inflammation or bacterial and viral infections of the testes and/or epididymis (orchitis and epididymo-orchitis) can impair spermatogenesis and cause immunological male infertility [48,49]. Chronic inflammation of the testis (orchitis) is characterized with infiltration of immune cells in the testis which can disturb the seminiferous tubules and impair spermatogenesis [50].

Testicular inflammation (orchitis) is an acute, symptomatic disease due to local or systemic infection [51]. However, an inflammatory reaction in the testis can be triggered in a noninfectious environment, for example physical trauma or chemical noxae can induce germ cell apoptosis [49], enhanced pro-inflammatory cytokine production and inflammation [52]. Danger associated molecular patterns (DAMPs) are endogenous molecules with a physiological role in cell maintenance that can be secreted or release from the cell in response to shock or physical stress and signal danger by triggering *de novo* inflammation which in turn causes prolonged release of DAMPs and cytokines thus fueling the inflammation by recruiting more immune cells and finally initiate chronic autoimmune diseases such as

autoimmune orchitis. Cytokines [53], oxidized mitochondrial DNA [54], heat-shock proteins, uric acid [55,56] and high mobility group box protein-1 (HMGB1) [57] are examples of a diverse set of endogenous molecules with known danger-signaling capacities [58]. Release of such molecules can either be the result of an active process in living cells or be due to leakage from dying cells [59]. Autoimmune orchitis is characterized by type, location and extent of inflammatory infiltrates and according to its clinical course (acute versus chronic). Sub-acute or chronic inflammatory reactions in the testis remain asymptomatic until advanced phase of the disease in the majority of patients. As there are yet no specific seminal or serological markers available [51], diagnosis of chronic testicular inflammation remains difficult and is largely ignored as an underlying reason of male infertility [49].

1.4.1.1. Experimental autoimmune orchitis as a model of immunological infertility

Experimental autoimmune orchitis (EAO) is an animal model for immunological male infertility in human [60,61]. Different approaches have been used to induce EAO such as immunization of the animals using testicular antigens (germ cells or testis homogenate), adoptive T cell transfer from orchitis animals, or by neonatal thymectomy [61,62,63]. The rat model of EAO actively induced by testis homogenate in adjuvant is widely used [37,64]. At onset of disease auto-antibodies against germ cells are formed and testicular levels of cytokines and chemokines such as IL-6, TNF-α, or MCP-1are elevated followed by interstitial mixed cellular infiltrates [65]. Specifically, TNF-α plays a crucial role in mediating inflammation and administration of neutralizing TNF-α antibodies can block onset of disease [66]. As the disease progresses, infiltration of leukocytes into the interstitial space and germ cell sloughing occurs, culminating in apoptotic bodies and granuloma formation, necrosis, which can ultimately lead to complete absence of spermatogenesis and reduced testis weight [65,67].

1.5. High mobility group box protein-1

High mobility group box protein-1 (HMGB1), previously known as HMG-1 or amphoterin is a non-histone nucleosomal protein, expressed abundantly in almost all mammalian cells. HMG proteins were discovered as nuclear proteins more than 30 years ago and are named for their high mobility in electrophoretic polyacrylamide gels [68]. HMG proteins consist of three families namely HMGA, HMGB and HMGN, all of which have the capacity to bind and distort DNA but are not necessarily transcription factors. The HMGB family has four members -HMGB1, HMGB2, HMGB3 and HMGB4- with an 80% amino

acid sequence identity. HMGB1 expression is ubiquitous, HMGB2 is primarily expressed in the thymus and testes, and HMGB3 is localized to the bone marrow [59]. Unlike HMGB1, HMGB4 (which lacks the acidic tail) is a potent transcriptional repressor and is strongly expressed in the adult mouse testis and weakly in the brain, but not in many other tissues [69].

In the last decade a large amount of data has been presented showing how nuclear HMGB1 can be released under inflammatory conditions and act as a pro-inflammatory cytokine and chemoattractant. For the first time, Wang et al. showed that released HMGB1 acts as a late mediator of sepsis [57] and is a potent inducer of pro-inflammatory response when administered *in vitro* to cells or injected *in vivo* to animals [57]. Compared to other pro-inflammatory cytokines such as TNF-α or IL-6, HMGB1 is released at a later time point of infection/inflammation, which makes it a valuable therapeutic target [70].

1.5.1. HMGB1 structure

HMGB1 is highly conserved between different species throughout evolution. It is a 25-kDa protein composed of 215 amino acids (Figure 2). There are two positively charged DNA binding domains, A-box and B-box and a negatively charged C-terminal acidic tail composed of 30 glutamic and aspartic acids, which partly covers helical structured A and B boxes. HMGB1 has two nuclear localization signals (NLS) and two nuclear emigration signals (NES), which bind to nuclear exportin CRM1 [71]. All HMGB proteins bind to DNA via their HMG boxes, made up by amino acid sequences formed in α-helical structures with a nonspecific binding affinity for minor grooves of DNA [72]. The unique structure enables HMGB1 to specifically recognize and bind to chromatin, containing sharp bends or kinks, such as four way junctions, damaged DNA, or to induce bending in linear duplex DNA [73]. Architectural properties of HMGB1 explain the importance of HMGB1 in mediating fundamental cellular events such as transcription, recombination, replication and repair.

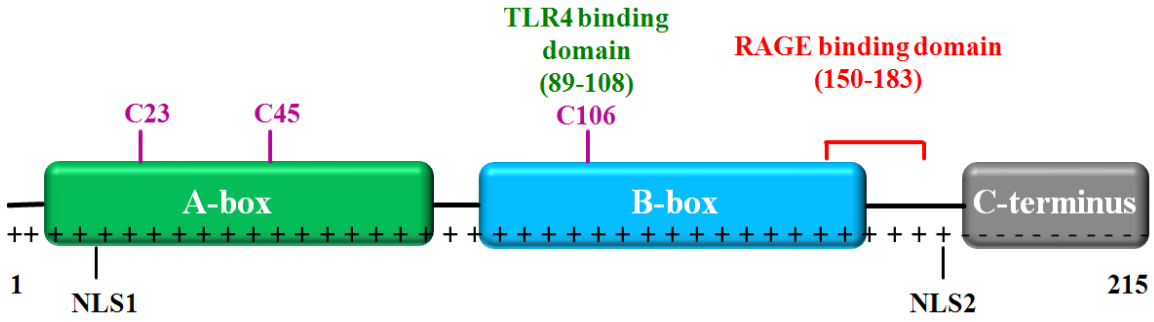


Figure 2. Structure of HMGB1 molecule. HMGB1 is composed of 215 amino acids with a molecular weight of 29 kDa, containing two DNA binding motifs (A-box and B-box) and the negatively charged acidic tail (C-terminus). Cystein residues C23, C45 and C106 have an important impact on HMGB1 cytokine activity. To bind TLR4, released HMGB1 should have an intact disulfide bond between C23 and C45 and the C106 in the TLR4 binding domain must be in reduced form. Amino acids 150 to 183 form the RAGE binding site. Shuttling of HMGB1 between nuclei and the cytoplasm is regulated by acetylation of two nuclear localization signals (NLS1 and NLS2).

Using truncated forms of the full-length HMGB1 it was demonstrated that the cysteine 106 in B box possesses the extracellular cytokine activity, given that oxidation or selective mutation of this residue abolishes the activity of HMGB1 signaling to activate cytokine release. This activity can be competitively inhibited by truncated A box protein. HMGB1 has a binding site for the Receptor of Advanced Glycation End products (RAGE) and a TLR4 binding site. The disulfide bridge between cysteins 23 and 45, and the reduced form of cystein 106 are crucial for TLR4 binding and cytokine activity of HMGB1 [74].

1.5.2. HMGB1 release

During inflammation or injury, nuclear HMGB1 can be secreted actively or released passively or it can be retained in the nuclei tightly bound to the DNA [75]. The behavior of HMGB1 under stress conditions is differentiated on the basis of molecular mechanisms such as the redox status of the HMGB1 molecule, post translational modifications which results in distinct release kinetics, and downstream signaling responses in different cells under different stress conditions. HMGB1 can be actively secreted through non-canonical mechanisms along with pro-inflammatory cytokines following inflammasome activation in stimulated and/or infected immune cells (Figure 3) [76,77]. Active secretion of HMGB1 occurs when immunologically competent cells such as monocytes, macrophages, natural killer cells, dendritic cells and endothelial cells are exposed to danger signals like other DAMP molecules, microbe associated molecular patterns (MAMPs), pathogen-associated molecular patterns (PAMPs), or some endogenous cytokines including TNF-α, IL-1 and IFN-γ. Like

other pro-inflammatory mediators that participate in feed-forward regulation, HMGB1 induces its own release *in vivo* and *in vitro* [74].

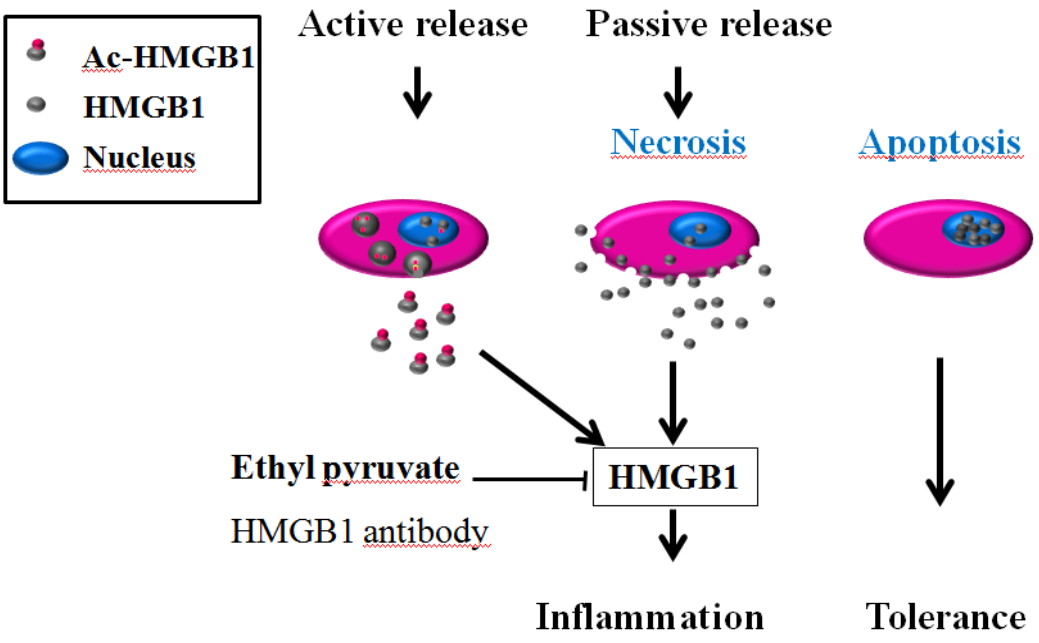


Figure 3. Extracellular HMGB1 release. Danger signals induce HMGB1 acetylation (Ac-HMGB1), which is then packed in secretory vesicles and actively released from immune cells. In necrotic cells, HMGB1 passively leaks out of the dying cells as a result of loss of integrity of the cell membrane. Released HMGB1 activates various inflammatory signaling pathways and further fuels the inflammation. Blocking HMGB1 release using antibodies or chemicals (such as ethyl pyruvate) inhibits inflammation. In contrast, in apoptotic cells HMGB1 binds irreversibly to the DNA to dampen further inflammatory signaling and elicits tolerance.

In contrast to passively released HMGB1, actively secreted HMGB1 is highly acetylated. For instance, monocytes hyperacetylate nuclear HMGB1 molecules in response to infammatory stimuli [71]. Passive release of HMGB1, initiated by impairment of cellular integrity, is a rapid event [52]. Interestingly, apoptotic cells do not release HMGB1 even after undergoing secondary necrosis, and therefore do not stimulate further pro-inflammatory reaction even when released in the cell environment. In apoptotic cells, HMGB1 is oxidized on cys106 utilizing caspase activity and mitochondrial reactive oxygen species (ROS) [78]. Oxidized cys106 loses its TLR4 binding capability, impairing the cytokine activity of the B box [79]. In apoptotic cells, HMGB1 binds irreversibly to the chromatin due to generalized hypoacetylation of histones. If chromatin deacetylation is prevented, the HMGB1 molecules can be released in the extracellular space and promote inflammation. Thus, cells undergoing apoptosis are programmed to elicit tolerogenic signals compared to cells that have been

damaged or killed by trauma, which send danger signals to the neighboring cells and potentiate persistent inflammatory reactions [52].

1.5.3. HMGB1 receptors

The multifunctional protein HMGB1 mediates a large variety of responses through binding and activation of different receptors. The elicited response relies on HMGB1 concentration in the microenvironment, the post-translational modifications of HMGB1, its redox status, receptor expression profile in the target tissue and complex formation with other endogenous or exogenous signaling molecules [80]. One important underlying reason making a nucleosomal protein with minimum immunological activity into a multipotent signaling molecule, is its ability to interact with several pattern recognition receptors that were previously identified to elicit danger signals from exogenous (toll-like receptors 2, 4 (TLR2, 4) and TLR9) and endogenous molecules such as the RAGE ligands (Figure 4). Additionally, via formation of complexes with other PAMPS or cytokines such as IL-1, HMGB1 can bind to specific receptors for these molecules and enhance the elicited responses [81].

1.5.3.1. Receptor for advanced glycation end products (RAGE)

The first HMGB1 receptor identified, was RAGE, a transmembrane, multi-ligand member of the immunoglobulin superfamily [82]. The primary structure of the cytoplasmic region of RAGE, which is critical for signal transduction, is conserved in various species including human, bovine, mouse, and rat [83]. RAGE can activate diverse intracellular signaling pathways, including the mitogen-actived protein kinase (MAPK) cascade leading to amplification or progression of various diseases including diabetes, inflammation, and tumor growth/metastasis [84]. Interestingly, *in vitro* stimulation of human fibrosarcoma HT-1080 cells with HMGB1, enhanced RAGE and extracellular signal-regulated kinase (ERK) interactions. Ligand induced interaction of ERK with RAGE leads to the enhancement of ERK kinase activity in the cell. It was shown that the membrane-proximal domain of the C-terminal cytoplasmic region of RAGE is required for the direct ERK/RAGE interaction [83].

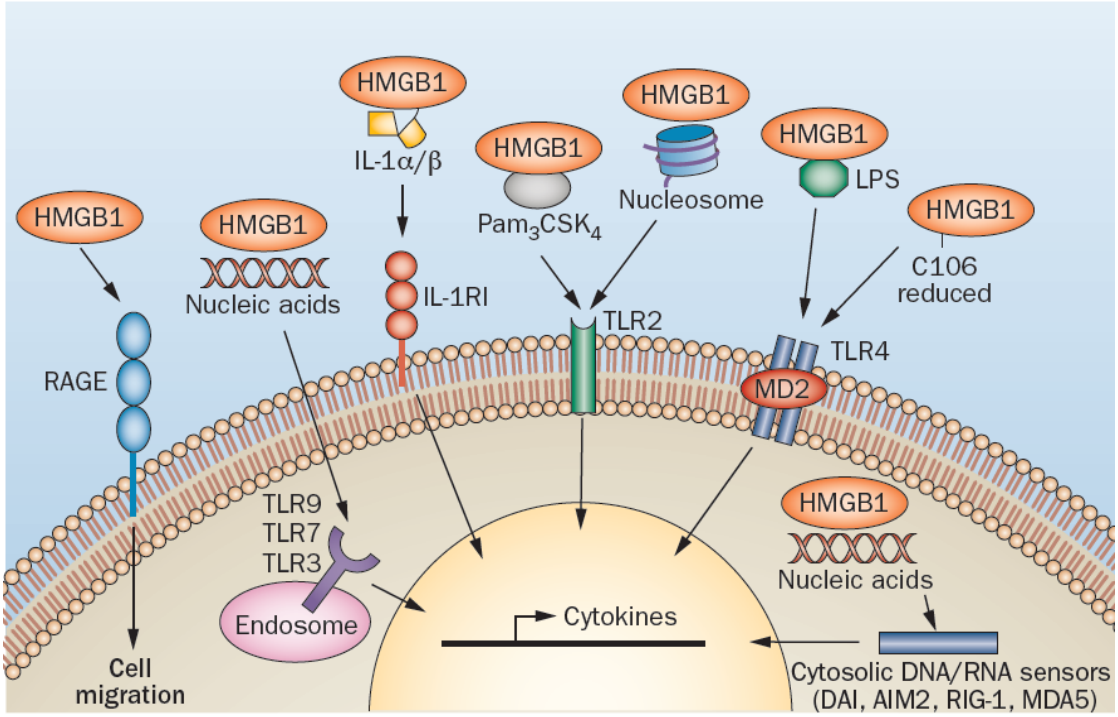


Figure 4. HMGB1 receptors and signaling. HMGB1 can activate a wide range of signaling pathways by binding to different receptors. HMGB1 either can directly bind to receptors such as RAGE and TLR4, or to other receptor in complex with their ligand molecule, e.g. IL-1 receptor. Post translational modification of the HMGB1 molecule, expression pattern of its receptors on the target cell and presence of molecules which form complexes with HMGB1 in the microenvironment is decisive of which pathway is activated in the cell (Harris *et al.* Nat Rev Rheumatol; 2012).

1.5.3.2. Toll-like receptor 4 (TLR4)

As a member of the TLR family, TLR4 is a pattern recognition receptor which can bind to LPS and activate the innate immune response. TLR4 plays a role in various inflammatory diseases such as diabetes type 2, periodontal disease, and Crohn's Disease (CD) [85]. Conventionally TLRs were known as a tool used by the innate immune system to distinguish self and non-self by recognizing pathogen associated molecular patterns (PAMPs). However, there are more than 20 endogenous molecules known to date that can bind to TLR4 and other pattern recognition receptors and therefore called danger associated molecular patterns (DAMPs) [86].

In regard to HMGB1 binding, TLR4 is the most studied receptor from the TLR family. Several TLRs are present in the rat testis, out of which TLR2 and 4 are highly expressed in TM and SC [85,86]. Interaction of a danger molecule with TLR induces production of inflammatory mediators, including cytokines, and increases phagocytosis [89]. It was shown that interaction with TLR4 is required for HMGB1 activation of cytokine release in

macrophages. In contrast to RAGE and TLR2 knockout macrophages, which produce TNF-α, when exposed to HMGB1, TLR4 knockout macrophages do not respond to HMGB1 stimulation [90]. As mentioned before, HMGB1 - TLR4 binding requires the reduced form of a crucial cysteine in position 106. As shown by Yang *et al.* synthetic 20-mer peptide containing cysteine 106 within the cytokine stimulating B box mediates TLR4-dependent activation of TNF-α release by macrophages [90].

1.5.4. HMGB1 in inflammation and autoimmunity

HMGB1 is the only nuclear protein which can obtain cytokine activity upon release from the nucleus to induce proinflammatory responses [59]. Alarmins such as HMGB1 are endogenous molecules, which are released form injured tissues to trigger homeostatic responses promoting tissue repair and activation of the immune system. Alarmins consist of heterogenous molecules with various functions within the cell that share some functional characteristics. HMGB1 is preferentially released during necrosis, however HMGB1 is oxidized and retained inside the cells dying via apoptosis. Released HMGB1 should promote tissue regeneration and directly or indirectly activate T-cell-dependent immune responses.

As an endogenous nuclear protein, translocation and release of HMGB1 also alerts the organism to sterile tissue damage [59]. Besides, HMGB1 secreted by maturing dendritic cells modulates the priming, activation, and Th1 polarization of T cells. In this manner, HMGB1 directs initiation/maintenance of autoimmune diseases by regulating the outcome of antigen presentation in case of tissue damage and infection. It seems that dendritic cells utilize the evolutionary conserved HMGB1 as a tool to control the activation of adaptive immunity by regulated secretion of HMGB1, therefore HMGB1 plays a key role at the intersection between innate and adaptive immunity [91].

Elevated levels of HMGB1 have been observed in patients with mechanical trauma, strokes, acute myocardial infarction, acute respiratory distress, and liver transplantation [80] as well as in chronic autoimmune disorders like rheumatoid arthritis [90,91].

HMGB1's immune regulatory actions are tightly modulated at different levels. In addition to regulated secretion/release from different types of dying cells, as already mentioned, HMGB1 loves company, which means that extracellular HMGB1 sustains significantly higher levels of inflammation, when it forms complexes with PAMPs or IL-1. These two different types of HMGB1 activities (alone or in company) allows this alarmin to signal tissue repair

when the danger is no longer existing (as in trauma not followed by infection) and to promote inflammation and innate immune cell activation (and thus, more collateral damage to the tissue), while the danger persists, and exogenous pathogens are still present [81,92].

1.6. Autophagy

Autophagy, apoptosis and necrosis are finely regulated and mechanistically related processes in the cell that are important to maintain cell homeostasis, cell survival as well as cell death [95]. Unlike apoptosis and necrosis, which are programmed cell death events leading to either tolerogenic clearance of the damaged cells or triggering inflammatory reactions to further signal danger, autophagy (a greek word meaning “self-eating”, brought up by Christian DeDuve about half a century ago [96]) can be either scheduled as a cell survival or a cell death program.

Every mammalian cell maintains basal levels of autophagy crucial to remove excess, old and unnecessary macromolecules or malfunctioning organelles [97]. Autophagy can be induced in a non-selective or selective manner [98]. Non-selective or bulk autophagy occurs during cell starvation to control cellular stress on nutrient deprivation. Cells degrade parts of the cytoplasm to provide energy and necessary building blocks for vital processes and cell survival [99]. In selective autophagy protein aggregates, pathogens and damaged organelles such as the mitochondria are labelled by specific markers detected by the autophagy machinery which then triggers the formation of endoplasmic reticulum or Golgi complex originating isolation membrane (phagophore) around the malfunctioning organelle. The phagophore elongates and wraps the target and is then closed to form an autophagosome. At a later stage, the autophagosome is translocated towards the lysosome and their membranes are fused (autophagolysosome). Damaged organelles are eventually removed by enzymatic degradation of the autophagolysosome cargo [99].

In the recent years, studies have shown that the autophagy machinery has a more sophisticated role than only bulk degradation of cellular material. Selective autophagy has been largely investigated in various diseases [98,99]. Recent data supporting selective autophagy shows that specific proteins are targeted to degradation earlier than other molecules and complexes under stress and starvation conditions. Therefore, the first targets are drawn towards autophagic degradation earlier by means of post translational

modifications such as acetylation and ubiquitination and the vital molecules are hidden from the autophagic machinery [102].

Mitophagy is a type of selective autophagic elimination of mitochondria, which is tightly regulated via Atg32 (in yeasts) and Parkin [98]. Mitophagy is a mechanism with which autophagy regulates inflammation. Damaged mitochondria release ROS resulting in caspase-1 activation and pro-inflammatory signaling. By degradation and removal of the damaged mitochondria ROS levels decrease [103]. Interestingly, induction of autophagy decreases apoptosis levels and inhibition of autophagy is compensated in the cells by upregulation of apoptosis [104]. However, it is also shown that autophagy can induce apoptosis or play in partnership with apoptosis and cause cell death [103,104]. Although autophagy can be an alternative cell death mechanism instead of apoptosis, it is still thought that at least in cells with intact apoptotic machinery, autophagy is primarily a pro-survival rather than a pro-death mechanism [107]. The complex signaling controlling the switch between autophagy, apoptosis and necrosis is not completely known, there is evidence that the metabolic energy sources such as intracellular calcium and glucose levels can influence this balance [100,93].

1.6.1. The molecular machinery of autophagy

Our current knowledge about autophagy genes (atg) is mainly based on studies using the yeast *S. cerevisiae* [108]. Most of the genes identified also exist in mammalian cells having similar functions. Upon starvation or stimulation of autophagy and downstream mTOR inhibitory complex, the ULK1 complex (normally localized in the cytosol) translocates into the ER lumen and activates autophagy-specific class III phosphatidylinositol-3-OH kinase (PI3K) complex, which together with autophagy initiation proteins such as ATG1 and Beclin-1 further activates other ATGs and lead to formation of an isolation membrane usually from a special part of the ER called omegasome. The phagophore contains phosphatidylethanolamine (PE)-conjugated LC3 (microtubule-associated protein 1 light chain 3) in the outer side as well as in the inner side [107,108]. Labeling of LC3 is widely used to visualize autophagosomes and autophagolysosomes. LC3-I is a cytosolic protein, which is converted to membrane-bound LC3-II by addition of PE to the C-terminal glycine residue upon induction of autophagy [111].

Beclin-1 is one of the key initiators of autophagy [97]. In inactivated mode it is bound to the pro-apoptotic protein BCL-2. Upon cell starvation or ERK1/2 pathway signaling

activation, Beclin-1 is activated after subsequent BCL2 dissociation and together with other ATGs initiates the formation of phagophore.

p62/sequestosome 1 (p62/SQSTM1) is an adaptor molecule, which binds with one end to ubiquitinated proteins (targeted for autophagic degradation) and with the other extreme to conjugated LC3 and therefore drives the cargo into the center of the forming autophagosome [112]. In later stages of autophagy P62 is degraded by the lysosomal enzymes and can be used as a marker to monitor autophagy flux [113].

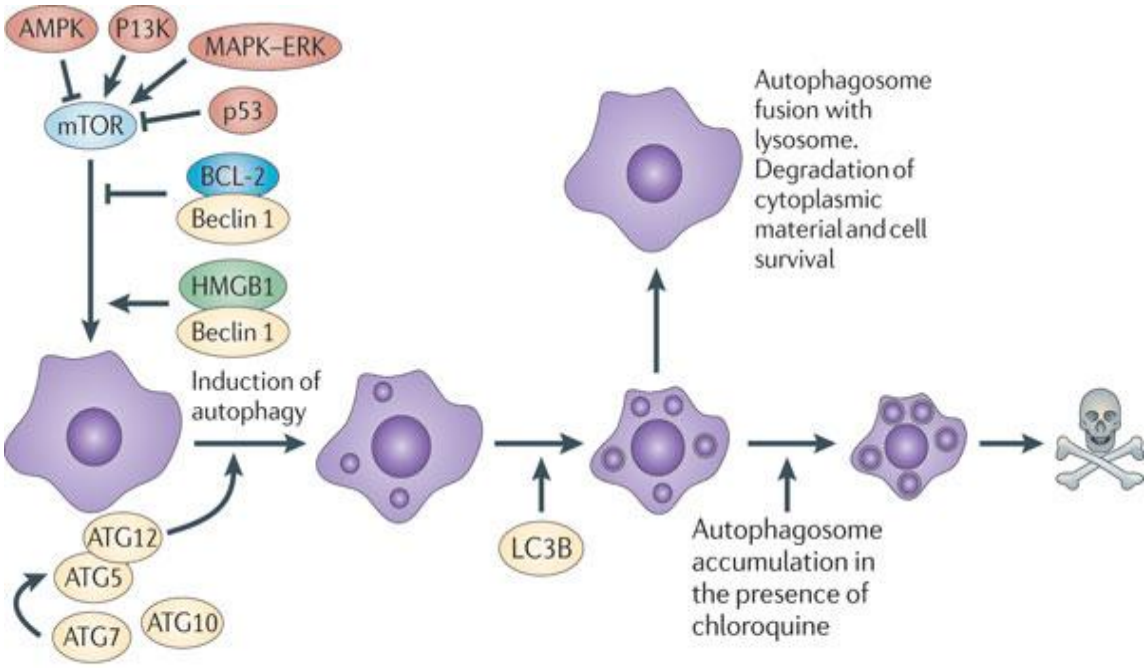


Figure 5. HMGB1 and autophagy signaling. Autophagy is a mechanism of self-eating in times of starvation to provide energy for cell survival. This process is also important for removal of damaged organelles as well as attacking pathogens. Autophagy is activated by various signals such as PI3K or MAPK-ERK. It is regulated by inhibitors such as the pro-apoptotic protein BCL-2 which binds to Beclin-1 and inhibits autophagy. HMGB1 can replace BCL-2 and bind to Beclin-1 and activate autophagy. Autophagy initiates by formation of an isolation membrane (phagophore containing LC3B) which engulf the cargo in the cytoplasm and eventually fuses with lysosomes to degrade the material. Chloroquine inhibits lysosome- autophagosome fusion and thus results in accumulation of autophagosomes (Figure taken from Espina V. *et al.* Nat Rev Cancer; 2011).

1.6.2. Autophagy-based unconventional protein secretion

Most secretory proteins posses signal peptides that direct them through the ER and Golgi complex-dependant secretion pathway [114]. However, accumulating data shows that several proteins lacking a signal peptide are also secreted through different mechanisms generally called unconventional protein secretion pathway. Unconventional secretion of proteins is broadly categorized as vesicular (eg. HMGB1, IL-1β) or non-vesicular (eg. fibroblast growth factor 2) pathways [113,114,115]. Unconventional protein secretion via autophagosome was introduced in recent years in yeasts where autophagosomes were programmed to avoid fusion with lysosomes and were directed to fuse with endosomes, move towards the plasma membrane and release their cargo to the extracellular milieu [118]. HMGB1 does not have a signal peptide to enter the ER and thus is packed in lysosomal vesicles when it is translocated from the nucleus [119]. Furthermore, activation of autophagy induces HMGB1 release in tumor cells [120]. Another advantage of the unconventional protein secretion is that in vesicular secretion format the cargo is covered and protected from modifications (such as

changes in the redox state), which might be important in protein function as for HMGB1 [121].

1.6.3. Autophagy in the testis

Autophagy plays a crucial role in the ovary and in the testis. Pro-survival side of autophagy controls germ cell survival in mouse ovary and its inhibition will lead to loss of germ cells [122]. Recent data shows that Sertoli cell specific overactivation of mammalian target of rapamycin (mTOR: an inhibitor of autophagy) in mouse testis, causes germ cell death and induces proliferation of Sertoli cells which in some cases leads to formation of Sertoli cell tumor [123]. Sertoli cells as highly differentiated and non-proliferating cells maintain a relatively high rate of basal autophagy. As autophagy is also a well-known tumor suppressive mechanism, disturbing the autophagy signaling pathway in Sertoli cells leads to germ cell loss and development of Sertoli cell tumors [122,121]. Administration of the carcinogene, Lindane, was shown to interrupt autophagy at the lysosomal degradation step [124]. Ethanol selectively induces mitophagy in Sertoli cells in contrast to many mammalian organs where it damages the mitochondria and forces the cell into apoptotic death [125]. The removal of germ cells in the testis is a tightly regulated process to keep the sperm production under control. Autophagy and apoptosis are known to regulate the induction of spermatocytes and germ cell death in rats and mice [124,104].

1.6.4. Role of HMGB1 in autophagy

High mobility group box 1 (HMGB1) has a dual active role in fine tuning autophagy in response to cell stress [127]. Extracellular HMGB1 activates ERK signaling pathway via RAGE binding and triggers autophagy initiation by mTOR dephosphorylation [126,127]. Endogenous HMGB1 is a novel Beclin-1 binding protein active in autophagy. As nuclear HMGB1 (acetylated form) translocates from the nucleus to the cytoplasm, it displaces BCL-2 from Beclin-1 (which requires C23/45) thus inducing autophagy [127]. Recent studies show that extracellular HMGB1 can be phagocytosed and enhance autophagy levels. Endocytosed extracellular HMGB1 have been co-localized with autophagolysosomes [130].

HMGB1 inhibitor ethyl pyruvate (EP) blocks autophagy activation by means of HMGB1 nuclear translocation. Under acute or chronic inflammation HMGB1 is released extracellularly, where it can act both in an autocrine and paracrine fashion either via RAGE

signaling or being uptaken by the neighbouring cells and induce autophagy or cell death [131]. However, whether released HMGB1 induces autophagy in a cell depends on several variables such as oxidative status of the cell´s microenvironment, cell type and HMGB1 receptor profile on the target cell [132].

1.7. Therapeutic value of targeting HMGB1

Targeting HMGB1 in acute or chronic inflammatory diseases has been proven to be a very promising tool to ameliorate inflammation. What makes this molecule very attractive is its late mode of release and action compared to other conventional cytokines (TNF-α and IL-1) [133]. Unlike TNF-α, blocking the action of HMGB1 is shown to be effective, even when applied 24 hours after onset of disease [59,132]. Additionally, as mentioned before manipulation of the redox status of the HMGB1 molecule can modulate its pro/anti-inflammatory activity and therefore could be used to elicit a specific response, which is beneficial at a certain stage of the disease. As an example, inhibition of HMGB1 induced autophagy in osteosarcoma decreases cell survival and drug tolerance and induces cell death, which is desired in this case [135].

In addition to HMGB1 neutralizing antibodies, other HMGB1 antagonists are HMGB1 A-box protein and ethyl pyruvate. A-box is comprised of 80 amino acids and has a 40% similarity to HMGB1 B-box. A-box binds to HMGB1 receptor competitively without inducing cytokine production and thus has an antagonistic role and has been successfully used to attenuate HMGB1 inflammatory effects [134]. Ethyl pyruvate is a lipophilic derivative of pyruvate and has anti-inflammatory effects. It is used as food additive and therefore is an interesting therapeutic tool. There are several studies showing that ethyl pyruvate inhibits HMGB1 induced cytokine production by blocking NF-κB and p38 MAPK signaling. This chemical has been successfully used to reverse established lethal sepsis in mice and reduce serum levels of HMGB1 in those animals [136].

1.8. Aim of the study

Sterile immunological impairment of the testis is frequently observed in testicular biopsies and can lead to focal or complete disruption of spermatogenesis. To date the underlying reasons of autoimmune reactions in the testis (orchitis) remain elusive and the asymptomatic nature of the incidence along with lack of diagnostic markers makes it difficult to diagnose and consequently treat the disease [49]. In the last decade it has been shown that the nuclear protein HMGB1 has cytokine activity and plays a key role in mediating inflammation in infectious as well as in sterile injuries [75]. Compared to conventional pro-inflammatory cytokines, HMGB1 is released and upregulated in the later phase of the disease and is successfully utilized as a valuable diagnostic marker and a therapeutic target in a wide range of infectious and sterile inflammatory diseases such as arthritis, colitis and different types of cancers [70,131,135,136].

In this study, a rat model of experimental autoimmune orchitis (EAO) will be used to investigate the role of HMGB1 in initiation and chronification of the disease. Moreover, putative target cells and the mode of action should be investigated to collect evidence if HMGB1 is a promising target for therapeutic intervention in inflammatory male factor infertility.

2. MATERIALS

2.1. Chemicals

Acetic acid	Merck, Darmstadt, Germany
Acrylamide 30% (w/v)	Roth, Karlsruhe, Germany
Agarose	Invitrogen, Karlsruhe, Germany
Ammonium solution 25%	Merck, Darmstadt, Germany
Benzyldimethylamine (BDMA)	Plano, Wetzlar, Germany
Bromophenol blue sodium salt	Sigma, Steinheim, Germany
Calcium chloride	Merck, Darmstadt, Germany
Coomassie Brilliant Blue G-250	Bio-Rad, Munich, Germany
Dimethyl sulfoxide	Merck, Darmstadt, Germany
DNA Ladder (100 bp)	Promega, Mannheim, Germany
DAKO fluorescent mounting medium	DAKO, CA, USA
DAPI mounting media	Vector, Burlingame, USA
di-potassium hydrogen phosphate	Merck, Darmstadt, Germany
di-sodium hydrogen phosphate	Merck, Darmstadt, Germany
1,4-Dithiothreitol	Roche, Mannheim, Germany
EPON (Agar 100)	Plano, Wetzlar, Germany
Ethanol	Sigma, Steinheim, Germany
Ethidium bromide	Roth, Karlsruhe, Germany
Ethylene diaminetetraacetic acid disodium salt (EDTA)	Merck, Darmstadt, Germany
Enhanced chemiluminescence (ECL) reagents	Amersham, Freiburg, Germany
Erythrosine B	Merck, Darmstadt, Germany
Ethyl pyruvate	Sigma, Steinheim, Germany
Fluorescein Calibration Dye	Bio-Rad, Munich, Germany
Formamide	Merck, Darmstadt, Germany
Formaldehyde solution 37%	Merck, Darmstadt, Germany
Glutaraldehyde EM grade 50%	EMS, München, Germany
Glycerol	Merck, Darmstadt, Germany
Glycine	Sigma, Steinheim, Germany
Hydrochloric acid 37%	Sigma, Steinheim, Germany
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	Roth, Karlsruhe, Germany
Igepal CA-630 (NP-40)	Sigma, Steinheim, Germany

Isofluran	Baxter, Germany
Isopropyl-β-D-thiogalactopyranosid (IPTG)	Sigma, Steinheim, Germany
Ketamine	Pharmacia GmbH, Germany
Lipopolysaccharide	Sigma, Steinheim, Germany
Mayer's hemalum solution	Merck, Darmstadt, Germany
Magnesium chloride	Merck, Darmstadt, Germany
Magnesium sulfate	Sigma, Steinheim, Germany
β-Mercaptoethanol	AppliChem, Darmstadt, Germany
Methanol	Sigma, Steinheim, Germany
Non-fat dry milk	Bio-Rad, Munich, Germany
N, N, N', N'-Tetramethylethylenediamin (TEMED)	Roth, Karlsruhe, Germany
Osmium tetroxide, 99%	Sigma, Steinheim, Germany
Paraformaldehyde	Merck, Darmstadt, Germany
Picric acid	Merck, Darmstadt, Germany
Phenylmethylsulfonyl fluoride (PMSF)	Sigma, Steinheim, Germany
Ponceau S	Roth, Karlsruhe, Germany
Potassium chloride	Merck, Darmstadt, Germany
1,2- Propylene oxide	Merck, Darmstadt, Germany
Protease inhibitor cocktail	Sigma, Steinheim, Germany
Protein size markers	Invitrogen, Karlsruhe, Germany
Rotiphorese Gel 30	Roth, Karlsruhe, Germany
Sodium acetate	Roth, Karlsruhe, Germany
Sodium azide	Merck, Darmstadt, Germany
Sodium chloride	Sigma, Steinheim, Germany
Sodium dodecyl sulfate (SDS)	Merck, Darmstadt, Germany
Supersignal west pico chemiluminescent substrate	Thermo Scientific, Waltham, USA
Tris (hydroxymethyl) aminomethane	Roth, Karlsruhe, Germany
Triton X-100	Sigma, Steinheim, Germany
Triton X-114	Sigma, Steinheim, Germany
Tween-20	Roth, Karlsruhe, Germany
Uranyl acetate dehydrate	Merck, Darmstadt, Germany
Urea	Merck, Darmstadt, Germany
Xylazine	Bayer, Leverkusen, Germany

2.2. Enzymes

Collagenase A (Cat. #10103586001)	Roche applied science, Germany
DNase I (Cat. #10104159001)	Roche applied science, Germany
Hyaluronidase (Cat. # H3506)	Sigma, Steinheim, Germany
Trypsin (from porcine pancreas) (Cat. # T5266)	Sigma, Steinheim, Germany
Trypsin inhibitor (Cat. # T6522)	Sigma, Steinheim, Germany

2.3. Cell culture reagents and equipments

Bovine serum albumin (endotoxin free)	Invitrogen, Karlsruhe, Germany
Ca-Mg-free HBSS (1x) medium	Invitrogen, Karlsruhe, Germany
Ca-Mg-free HBSS (10x) medium	Invitrogen, Karlsruhe, Germany
Cell strainer, 70µm	BD Falcon, NY, USA
DMEM/F12	Invitrogen, Karlsruhe, Germany
DMEM (high glucose) medium	PAA, Cölbe, Germany
Dulbecco´s PBS (1X) w/o Ca2+ & Mg2+	PAA, Cölbe, Germany
Fetal calf serum (FCS)	PAA, Cölbe, Germany
L-Glutamine 200 mM (100X)	PAA, Cölbe, Germany
Lipofectamine ^{TM} 2000	Invitrogen, Karlsruhe, Germany
MEM (EAGLE) medium	Invitrogen, Karlsruhe, Germany
Neubauer counting chamber	LaborOptik, Marienfeld, Germany
Penicillin/Streptomycin (100X)	PAA, Cölbe, Germany
RPMI 1640 medium	PAA, Cölbe, Germany
Trypsin/EDTA	PAA, Cölbe, Germany

2.4. Cell line

Sertoli cell line SCIT-C8: A gift from Dr. Konrad, Department of Obstetrics and Gynecology, Faculty of Medicine, Justus Liebig University, Giessen

2.5. Bacterial strains

Subcloning Efficiency™ DH5α™ competent cells	Invitrogen, Karlsruhe, Germany
BL21(DE3)pLysS competent cells	Stratagene, Heidelberg, Germany

2.6. Expression constructs

2.7. PCR reagents

DNase I	Qiagen, Hilden, Germany
Desoxyribonukleosidtriphosphate (dNTP)	Promega, Mannheim, Germany
Moloney Murine Leukemia Virus Reverse Transcriptase,	

(M-MLV RT)	Promega, Mannheim, Germany
Oligo dT	Promega, Mannheim, Germany
QuantiTect SYBR Green PCR Kit	Qiagen, Hilden, Germany
RNase A	Roth, Karlsruhe, Germany
RNase inhibitor	Promega, Mannheim, Germany
Taq polymerase	Promega, Mannheim, Germany

2.8. Kits

BCA Protein Assay Reagent	Thermo Scientific, IL, USA
Bio-Rad Protein Assay	Bio-Rad, Munich, Germany
Bradford kit	Roth, Karlsruhe, Germany
Duolink In Situ Orange Starter kit	Olink Bioscience, Sweden
Duolink In Situ Probemaker MINUS/ PLUS	Olink Bioscience, Sweden
Endotoxin LAL Assay kit	GenScript, Piscataway, USA
HMGB1 ELISA Kit	IBL, Hamburg, Germany
NucleoBond® Xtra Midi	Macherey Nagel, Düren, Germany
Ni-NTA Superflow	Qiagen, Hilden, Germany
Rat IL-6 ELISA development kit	Promokine, Heidelberg, Germany
Rat TNF-α ELISA development kit	Promokine, Heidelberg, Germany
Rat TNF-α ELISA Ready-SET-Go!®	eBioscience, Frankfurt, Germany
RNase-Free DNase Set	Qiagen, Hilden, Germany
RNeasy Micro kit	Qiagen, Hilden, Germany
RNeasy Mini kit	Qiagen, Hilden, Germany
SYBR Green PCR Kit	Qiagen, Hilden, Germany
QuantiTect primer assay	Qiagen, Hilden, Germany

2.9. List of Equipment

Cell culture CO_2 incubator	Binder, Tuttlingen, Germany
Desktop centrifuge Biofuge Fresco	Heraeus, Hanau, Germany
Electronic balance SPB50	Ohaus, Giessen, Germany
Fluorescent microscope Axioplan 2 Imaging	Carl Zeiss, Göttingen, Germany
Gel Jet Imager 2000 documentation system	Intas, Göttingen, Germany
Heat block DB-2A	Techne, Cambridge, UK
Horizontal mini electrophoresis system	PEQLAB, Erlangen, Germany

Hybond ECL nitrocellulose membrane	Amersham, Freiburg, Germany
Labofuge 400R	Heraeus, Hanau, Germany
LEO 906 E transmission electron microscope	Zeiss, Oberkochen, Germany
MIKROM HM560 Cryo-Star	Microm, Walldorf, Germany
Microplate reader	Berthold, Bad Wildbad, Germany
Microwave oven	Samsung, Schwalbach, Germany
Mini centrifuge Galaxy	VWR International
Mini-rocker shaker MR-1	PEQLAB, Erlangen, Germany
Mixer Mill MM 300	Retsch, Haan, Germany
MyiQTM2 Two-Color real-time PCR detection system	Bio-Rad, Munich, Germany
NanoDrop ND 2000	Promega, Mannheim, Germany
PCR thermocycler	Biozyme, Oldendor, Germany
Potter S homogenizer	B. Braun, Melsungen, Germany
Power supply units	Consurs, Reiskirchen, Germany
Pre-cast gel system	Invitrogen, Karlsruhe, Germany
SDS gel electrophoresis chambers	Consurs, Reiskirchen, Germany
Semi-dry electroblotter	PEQLAB, Erlangen, Germany
Ultrasonic homogenizer Bandelin Sonopuls	Bandelin, Berlin, Germany
UV visible spectrophotometer Ultrospec 2100 Pro	Biochrom, Cambridge, UK
Vertical electrophoresis system	PEQLAB, Erlangen, Germany
X-ray Hyperfilm	GE, Buckinghamshire, UK

3 METHODS

* All buffers and solutions are listed in appendix 1.

2.10. Animals and human biopsy sections

18 day old immature Wistar rats (Charles River Laboratories, Sulzfeld, Germany) were purchased for Sertoli cell (SC) and peritubular cell (PTC) isolation. The animals were housed in standard conditions (12 h light/dark cycle, 20~22°C), with access to water and food *ad libitum* for 24 hours and 19 day old rats were used for Sertoli cell isolation. All experiments involving animals were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the German law of animal welfare. For immunization procedure, Wistar Kyoto rats (180–220 g body weight (b.w.) were anaesthetized by intraperitoneal administration of 100 mg/kg b.w. ketamine and 10 mg/kg b.w. xylazine and all efforts were made to minimize suffering. For cell isolation experiments animals were deeply anesthetized and sacrificed by inhalation of an overdose of isoflurane (1ml/1 L volume of the executer).

2.11. Human biopsy samples

Paraffin sections from testicular biopsies from patients with leukocytic infiltrates or patients with normal spermatogenesis were provided by the Giessen Testicular Biopsy Repository. The study was approved by the local institutional review board (Ref. No. 100/07).

2.12. Induction of EAO

To induce EAO, adult inbred male Wistar Kyoto rats (Charles River Laboratories, Sulzfeld, Germany) aged 60-70 days were actively immunized with testicular homogenate [65]. Briefly, animals were immunized 3x every 14 days with testicular homogenate in complete Freund's adjuvant followed by *i.v. Bordetella pertussis* (DSMZ, Germany) injection. Control animals received NaCl instead of testicular homogenate. Animals were sacrificed 50 and 80 days after first immunization (EAO50 and EAO80 groups, respectively). All animal experiments were approved by local animal ethics committee (Regierungspraesidium Giessen GI 20/23 – Nr. 33/2008).

2.12.1. Hematoxylin and eosin staining

Hematoxylin and eosin (H&E) staining is a standard staining method used in histology, providing an overview of tissue structure enabling differentiation of structural changes during examination under normal, inflamed or degenerative changed conditions. During H&E staining, positively charged metal-hematein complexes bind in an acidic milieu to the negatively charged phosphate residues of the DNA in the nucleus. Eosin is a red dye used to stain cytoplasm, collagen and muscle fibers for examination under the microscope. The eosinophilic structures are generally composed of intracellular or extracellular proteins. Most of the cytoplasm is eosinophilic. Red blood cells are stained intensely red.

8-10 µm thick paraffin or frozen sections were cut using a microtome or a cryostat. Sections were deparaffinized by 3x5 min treatment with Xylene. Frozen samples or deparaffinized samples were rehydrated through a series of decreasing ethanol concentrations ranging from 100% to 50%, for 3-5 min and rinsed in dH_2O. Samples were stained with Mayer's hemalum solution for 5 min, rinsed in dH_2O and left under flowing tap water for 7-10 min and rinsed with dH_2O again. Subsequently, samples were stained in 0.5% Eosin (plus 2 drops of acetic acid glacial) for 1-2 min and rinsed in dH_2O. Sections were dehydrated in increasing alcohol series starting from 50% to 100% for 3-5 min, and finally treated with xylene for 2x5 min. Sections were mounted for viewing using a xylene based mounting medium.

2.12.2. Immunofluorescent microscopy

Cryosections, paraffin sections and isolated testicular cells were used for immunofluorescent labeling. Formation of methylene bridges during fixation in formalin fixed tissues cross-links proteins and therefore masks antigenic sites. Therefore for paraffin sections an antigen retrieval step was performed. A heat mediated antigen retrieval method was used to break the methylene bridges and expose the antigens for immunostaining. Sections were boiled in citrate buffer for 5 min at 600 W and then for 15 min at 350 W in the microwave oven. Sections were washed 3x5 min in PBS-0.1% Tween 20. The fixation step was performed for cryosections and isolated cells. Samples were fixed using 4% PFA for 10 min at room temperature (RT). For TLR4 and RAGE immunostaining samples were fixed with acetone for 10 min at -20°C. Samples were permeabilized using 0.5% Triton X-100 for 10 min. Non-specific binding of antibody was blocked using 5% BSA in PBS for 1h at RT and then incubated with primary antibody overnight (O/N) at 4°C. Later sections were washed

3x5 min in PBS-0.1% Tween 20 and further incubated with fluorescent conjugated secondary antibody for 1h at RT in a dark room. Sections were washed 3x5 min in PBS-0.1% Tween 20 and DAPI mounting medium was used to mount coverslips.

2.13. Isolation of testicular cells

2.13.1. Isolation of peritubular cells and Sertoli cells

Ten 19 -day old rats were killed by cervical dislocation. Blood was removed by opening the jugular vein under flowing water. Testes were collected and kept in PBS A and later disinfected in 20 ml 1% iodine-ethanol (30 sec). Testes were washed in PBS A (3x), then decapsulated and placed in PBS A. Seminiferous tubules were digested using trypsin-DNase solution for 6 min in a shaking water bath at 32°C (140 strokes/ min) (Figure 6A). All enzyme solutions were passed through a 0.2 µm filter. The seminiferous tubules were settled down for 5 min and the supernatant discarded. Trypsin digestion was stopped using trypsin inhibitor A and B. To remove contaminating cells such as interstitial cells and germ cells, tubules were washed 7x using PBS A. Tubules were further digested in collagenase-hyaloronidase-DNase solution in a shaking water bath at 32°C for 15 min. The tubules become shorter and PTC were released from the tubules (Figure 6B).

Digested tubules were left to settle for 10 min. Supernatant containing PTC was collected carefully and transferred into a new Falcon tube. PTC medium was added and the resulting suspension was centrifuged at 280 × g for 10 min without break. The cell pellet was dissolved in 20 ml of PTC medium and cultured at 37°C and 5% CO_2. PTC were passaged 3 days after isolation (Figure 6D). Two days prior to experiments, 5×10^5 cells/well were seeded into 6-well plates and cultured with 10% FCS in RPMI-1640 medium. Before experiments cells were starved O/N in RPMI 1640 medium with 1% FCS.

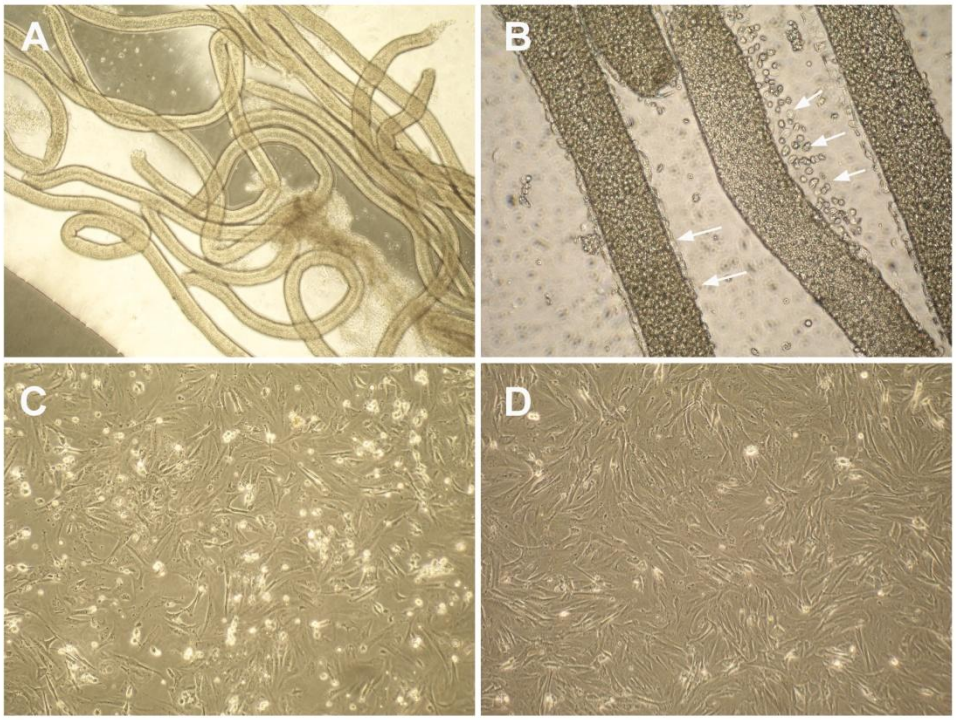


Figure 6. Isolation of peritubular cells from rat testis. Decapsulated testis from 19 days old rats were digested using trypsin-DNase for 5 min which causes seminiferous tubule release (A). Trypsin activity was inhibited using trypsin inhibitor. Peritubular cells were released in the supernatant following collagenase-hyaloronidase-DNase digestion for 15 min (arrows point to releasing peritubular cells) (B). Peritubular cell culture before splitting contains germ cell contamination (C). A pure population of peritubular cells is achieved after passaging the cells (D).

For isolation of SC, the remaining seminiferous tubules were washed 4x using PBS A. The tubular aggregates were further digested in hyaloronidase-DNase solution for 7-10 min in shaking water bath at 32°C, until shortened tubules and released cells were visible (Figure 7A). The tubules were washed 4x using PBS A. After the last washing step, 20 ml of Sertoli cell medium was added. The tubular aggregates were passed through an 18G needle (10x) to disperse the cell aggregates. The cell suspension was filtered through a 70µm cell strainer. Cells were centrifuged for 10 min at 200 × g. The supernatant was discarded carefully and the pellet was dissolved in SC medium. Cells were counted using the Neubauer counting chamber. 3×10^6 cell/ well were plated in 6-well plates and incubated at 32°C and 5% CO_2 (Figure 7B). To remove contaminating germ cells, hypotonic shock treatment was applied on the third day of isolation. Briefly, SC were rinsed two times with PBS and incubated with 20 mM Tris-HCl (pH 7.5) for 1.5 min. Cells were washed twice with PBS and cultured for two more days in RPMI-1640 medium (Figure 7D). Purity of PTC and SC was confirmed >95% using α-smooth muscle actin and vimentin staining, respectively (see appendix 3).

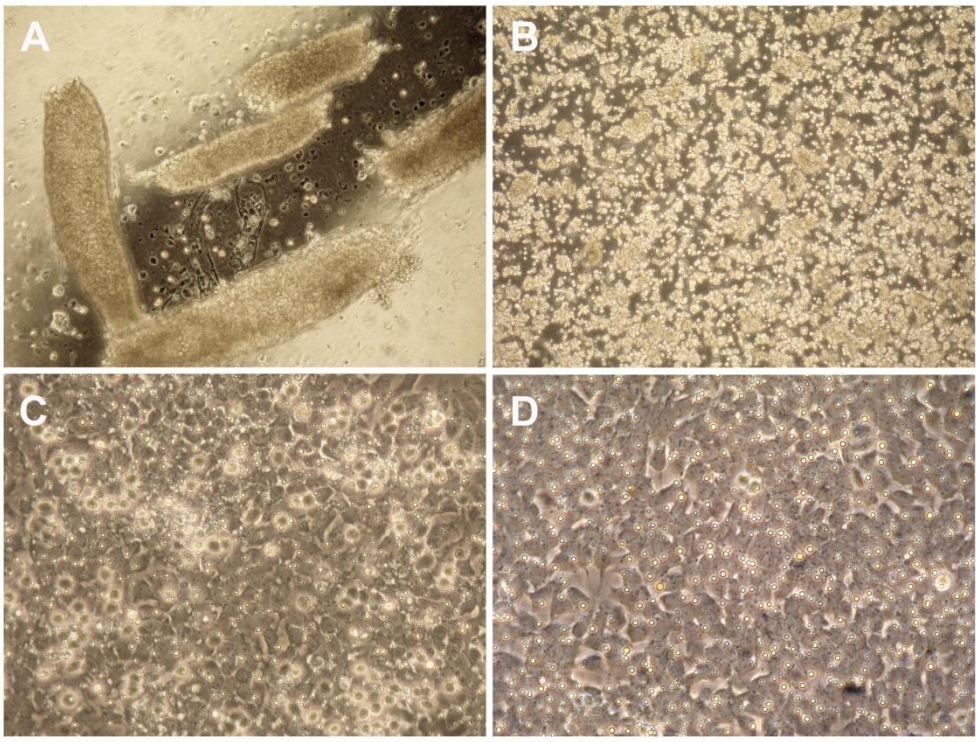


Figure 7. Isolation of Sertoli cells from rat testis. Seminiferous tubules from peritubular cell isolation were further digested for 8 min by hyaloronidase-DNase solution to form very small tubule fractions and free Sertoli cells (A). Sertoli cells were cultured immediately after seeding approximately 3×10^6 cell/ well in 6-well plates (B). Sertoli cell culture by day three contained contaminating cells (C). A pure population of Sertoli cells was achieved two days later after hypotonic shock (D).

2.13.2. Isolation of testicular macrophages

Testicular macrophages were isolated from adult outbred rat testis (10-12 weeks old). Testes were decapsulated and placed in TM medium. The supernatant was centrifuged at 300 × g for 10 min and subsequent pellet was resuspended in TM medium, seeded and maintained at 32°C and 5% CO_2. Contaminating cells were vigorously washed away while testicular macrophages adhered to the plate [25]. Purity of the cells was >80% as confirmed by ED1/ ED2 staining (see section 3.11).

2.14. Transfection of Sertoli cells

Introducing foreign nucleic acids into eukaryotic cells (transfection) can be done using various techniques. One of the commonly used transfection reagents is the Lipofectamin. It is a cationic-lipid transfection reagent which is mixed with the foreign DNA to form liposomes containing the DNA and later administered to the living cells. The liposome will fuse with the cell membrane and discharge the cargo inside the cell. Isolated SC were seeded on 24-well

plates containing 1.5 mm glass coverslips (Langenbrink, Teningen, Germany). Transfection was performed at least one day after hypotonic shock. For each well 400 ng DNA (GFP-LC3 or GFP control vector) was trasnfected using Lipofectamin 2000 according to manufacturer's protocol. Briefly, 2 µl Lipofectamin and 400 ng DNA were incubated separately in 50 µl medium without antibiotics for 5-10 min at RT. DNA was mixed with Lipofectamin and further incubated for 30-35 min. The DNA/Lipofectamin mixture was added drop wise to each well (24-well plate containing 500 µl medium per well). The medium was changed after 4-6 h with fresh medium containing antibiotics and cells were left O/N to express the GFP-tagged protein. After 24 h cells were stimulated using rHMGB1 or HBSS and later prepared for immunofluorescent imaging (see section 3.11).

2.15. Expression and purification of recombinant human HMGB1

2.15.1. Preparation of competent *E. coli* and transformation

Escherichia coli (*E. coli*) DH5α strain was streaked directly from a frozen glycerol stock onto an LB agar plate containing no antibiotics. The plate was incubated for 16 h at 37°C. A single colony was picked and grown in 5 ml SOB medium O/N on shaker (235 strokes/min) at 37°C. The following day, 50 ml of pre-warmed SOB medium were inoculated with 0.5 ml bacterial suspension from the O/N culture. Cells were grown for 2.5-3 h at 37°C. Culture growth was monitored by measuring optical density at 600 nm (OD_{600}) every 20 min. At OD_{600} of 0.45-0.50, cells were incubated on ice for 20 min, and harvested by centrifugation at 1075 × g for 15 min at 4°C. The supernatant was discarded and the cells were resuspended in 10 ml (1/3 of original volume) TFB buffer and incubated on ice for 10-15 min. Again, cells were centrifuged at 1075 × g for 12 min at 4°C. The resulting supernatant was discarded and cells were resuspended in 2.4 ml of TFB buffer plus 84 µl of DMSO and incubated on ice for 5 min. 84 µl DTT was added and incubated on ice for another 10 min. After that 84 µl DMSO was added and incubated on ice for 5 min. For transformation 100-300 ng of the pET-11d HMGB1 (rHMGB1) plasmid DNA were added to 200 µl of DH5α cells and kept on ice for another 30 min. Tubes were incubated on a preheated heat block at 42°C for 45 sec and immediately placed on ice for 2 min. Afterwards, 800 µl pre-warmed SOC medium (20 mM glucose in SOB medium) was added. Cells were incubated for a further 60 min at 37°C in a shaking incubator then centrifuged at 2000 × g for 1 min. Subsequently, 600 µl of supernatant was discarded, cells were resuspended in 200 µl of remaining medium, and streaked onto a 90mm LB agar plate containing ampicillin (50 mg/ml)/ chloramphenicol (25 mg/ml).

2.15.2. Plasmid DNA isolation

A single colony from the streaked LB agar plate was used for inoculating a 5ml LB medium containing ampicillin/ chloramphenicol. The bacterial culture was incubated O/N in a shaking incubator at 37°C. For the isolation of plasmid DNA, the NucleoBond® Xtra Midi was employed. DNA was isolated and purified according to the manufacturer's instructions. Isolated plasmid DNA was subjected to agarose gel electrophoresis.

2.15.3. DNA agarose gel electrophoresis

Agarose was dissolved in 1x TAE buffer. After cooling, ethidium bromide (0.5 µg/ml) was added to the agarose solution. Plasmid DNA sample was mixed 1:6 with 6x gel DNA loading buffer. Depending on fragment size, agarose gels were prepared at concentrations ranging from 0.8% to 2%. Gels were run at 100 V (2-10 V/cm gel). The gel was examined on a UV transilluminator and photographed using a gel documentation system.

2.15.4. Transformation of BL21(DE3)pLysS competent cells and induction of HGMB1 recombinant protein expression

E. coli BL21(DE3)pLysS strain was transformed with pET-11dHMGB1-T7 vector as described in section 3.4.1. A mini culture (5 ml) was prepared in 2YT expression media using a single colony of transformed BL21(DE3)pLysS cells from the agar plate for 8 h at 37°C. The second mini culture was prepared and incubated O/N at 37°C in a shaker (100 ml). When the optical density of bacterial suspension reached 0.5-1 (OD_{600nm}), the temperature was reduced to 30°C and 1ml from the culture was collected as negative control. The sample was centrifuged 2 min 2000 × g. Supernatant was discarded and the pellet was resuspended in 2x Laemmli buffer according to the $OD_{600nm.}$ Sample was boiled at 95°C for 5 min and kept at −20°C until use.

Protein expression was induced using 1mM Isopropyl-β-D-thiogalactopyranosid (IPTG) at 30°C for 4 h. Cells were harvested by centrifugation at 6000 × g at 4°C for 15 min. Resulting supernatant was discarded and the pellet was weighed. The pellet was washed with PBS and transferred to 50 ml Falcon tubes. Cells were kept frozen at -20°C until protein purification was performed.

2.15.5. Purification of recombinant HMGB1

His-tagged rhHMGB1 protein was affinity purified by Ni-NTA agarose (Qiagen, Germany) according to the manufacturer's instructions. rhHMGB1 was further purified using ion-exchange chromatography (RESOURCE™ S column; GE Healthcare) and dialyzed O/N at 4°C against dialysis buffer [139]. The identity of the purified rhHMGB1 was confirmed by Western blot using polyclonal anti-HMGB1 antibody. To confirm the identity of the product protein samples were separated on a 12.5% SDS-PAGE and stained by Commassie Blue. The respective band size for rhHMGB1 (~30 kDa) was cut out of the gel and used for mass spectrometry.

2.15.6. Endotoxin removal

Purified rHMGB1 was subjected to Triton X-114 extraction to remove endotoxin contamination [140]. Triton X-114 was added to protein sample in a final concentration of 1% (v/v). The solution was stirred at 4°C for 30 min and then heated at 37°C for 10 min and centrifuged at 10000 × g at 25°C for 10 min. Finally, the protein was aliquoted in Eppendorf Protein LoBind tubes and kept at -80°C until use. Endotoxin concentration was measured using chromogenic LAL assay according to manufacturer's protocol. Endotoxin level of the purified protein was lower than 2 EU/ml. Possible DNA or RNA contaminations in the sample were measured using a NanoDrop. No traces of DNA and RNA were detected.

2.16. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Discontinuous sodium-dodecyl-sulphate (SDS) polyacrylamide gel electrophoresis was performed to analyze recombinant protein expression and purity by Coomassie brilliant blue staining or to detect protein expression and modifications in tissue homogenate and cell lysates using western blot (appendix 2). For the separating gel proper gel percentage was chosen according to the size of the protein to be detected (from 10% to 20%). 5 ml of the gel solution was poured in between the two glass plates used for casting the gel. After polymerization 1.5 ml of the stacking gel was added and the comb was placed on the top. To load the samples the comb was removed and the gel was placed in a running chamber filled with electrophoresis buffer. 40 mg frozen testis tissue was homogenized in RIPA buffer containing proteinase inhibitor cocktail. For lysate preparation from cultured cells, cells were washed with 1x PBS and 100 µl/well of RIPA buffer was added (6-well plate format). Cells were scraped and transfered to 1.5 ml eppendorf tubes. Homogenized samples or cell lysates

were kept on ice for 30 min and centrifuged at 18000 × g for 15 min at 4°C. The supernatant was collected and mixed with 1 volume of 2x Laemmli loading buffer, boiled at 97°C for 5 min and kept on ice before loading onto appropriate SDS-PAGE gel. A protein marker suitable for the protein size to be detected was used. Gels were run at 120 V constant voltage for 2-3 h.

2.17. Coomassie Brilliant Blue staining

Among the available protein detection methods, staining with Coomassie Brilliant Blue (CBB) is most frequently used for detection of proteins in SDS-PAGE gels. The colloidal CBB staining procedure was used to visualize the proteins on the gel. Following electrophoresis, gels were washed in dH_2O for 2x10 min to remove the SDS buffer from the gel (otherwise high background and weak protein staining will be visible). Gels were immersed in CBB staining solution for 2 h at room temperature (RT). The staining solution was washed with dH_2O twice and gels were de-stained in destaining solution, until desired band intensity was obtained and finally washed with dH_2O again.

2.18. Western blotting

Western blotting is used to detect the expression of proteins present in tissue homogenates or cell extracts using antibodies specifically directed for detection against the desired protein. For detection of phosphorylated proteins 10-12% polyacrylamide gels were used and the proteins were blotted for 1 h 45 min. For detection of LC3 (~16-18 kDa) 17-20% gels were used and blotted for 1.5 h. Gels were washed with dH_2O and soaked in semi dry transfer buffer. Resolved proteins were transferred onto a nitrocellulose membrane using a semi-dry blotter. The efficiency of transfer was checked by Ponceau S staining. Membranes were washed with TBST buffer and blocked in blocking buffer (5% milk/TBST or 5% BSA/TBST (in case of phosphoprotein detection) for 1 h before incubating with the primary antibody diluted in blocking buffer O/N at 4°C. After incubation membranes were washed 3x with TBST for 5 min and incubated for 1 h at RT with horseradish peroxidase conjugated secondary antibody diluted in TBST. Membranes were then washed 3x10 min and incubated for 4 min in ECL solution (for β-actin and HMGB1 homemade ECL was used: 1 ml solution A plus 100 µl solution B). For Beclin-1 and phosphoprotein detection SuperSignal West Pico Substrate and for LC3 detection AceGlow™ Chemiluminescence Substrate was used. The membranes were exposed to X-ray film and developed.

2.19. ELISA

Enzyme-linked immunosorbent assay (ELISA) was employed to quantify concentrations of HMGB1, IL-6 and TNF-α protein in serum samples and testicular homogenate from EAO, adjuvant and control animal groups. Blood was collected directly from the heart and was allowed to clot O/N at 4°C. The clot was removed by centrifugation at 1500 × g for 15 min and the resulting supernatant, designated serum, was carefully removed using a Pasteur pipette. Serum samples were stored at -80°C until the ELISA was performed.

To measure concentration of HMGB1 a plate coated with anti-HMGB1 polyclonal antibody was used. Samples and standards were incubated on the plate overnight at 37°C. After a washing step HMGB1 antibody conjugated to peroxidase enzyme was added and incubated for additional 2 h. Wells were extensively washed and substrate (tetramethyl benzidine (TMB) plus buffer with 0.005 M hydrogen peroxidase) was added. Color development was stopped after 30 min using 0.35 M H_2SO_4 and the optical density were measured at 450 nm using the microplate reader. Protein concentrations were calculated according to the standard curve.

Concentrations of IL-6 and TNF-α were measured using plates coated with monoclonal antibody to rat IL-6 or TNF-α. Plates were washed twice prior adding the samples and standards and incubation for 2 h at RT. After washing avidin-HRP conjugate enzyme was added and kept for 30 min at RT. After extensive washing the substrate 2, 2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS) was added and kept for 20 min. Finally, the optical density was measured at 405 nm using the microplate reader.

2.20. Analysis of gene expression by quantitative real-time PCR (qRT-PCR)

qRT-PCR enables both detection and quantification of one or more specific sequences in a DNA sample. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes (housekeeping genes). To evaluate relative mRNA expression of IL-6 and TNF-α in treated testicular cells, RNA was isolated, reverse transcribed and qRT-PCR was used to check relative cytokine RNA levels compared to control samples.

2.20.1. RNA isolation

RNA was isolated from fresh cells or tissue or from material frozen at -80°C. For samples less than ≤5 × 10^5 numbers of cells or ≤5 mg tissue, mini columns were used otherwise the mini columns were applied. Lysis buffer RLT (containing guanidine salts) with 1% β-mercaptoethanol was used to lyse the cells or the tissue. All steps of RNA isolation and centrifugations were performed at RT (20-25°C).

To isolate RNA from rat testis tissue, 30-50 mg frozen tissue was lysed in 700 µl RLT buffer using a Potter-S homogenizer. The lysate was centrifuged for 20 min at ≥8000 × g at 4°C and supernatant was collected.

For RNA extraction from isolated testicular cells, 1×10^6 cells were seeded in 6-well plates. RNA was extracted from isolated TM using RNeasy micro kit. Alternatively, for RNA extraction from SC and PTC RNeasy mini kit was used. Cells were washed once with PBS and lysed in RLT buffer. DNA was sheared using a 21 G needle (5x) and a 1 ml RNAse-free syringe.

Lysate from cells or supernatant from homogenized tissue were mixed with equal volume of 70% ethanol and loaded on RNeasy columns. Columns were centrifuged at full speed (8000 × g) for 15 sec. Columns were washed once with RW1 wash buffer. RNase-Free DNase was used for on-column treatment and subsequent DNase removal. 10 µl DNase I stock solution was added to 70 µl buffer RDD. 80 µl of the DNase solution was administered to the column and incubated for 15-20 min at RT. Columns were washed once more and RNA was precipitated using 80% ethanol. Columns were dried by centrifugation with the tube cap open for 5 min at full speed. RNA was eluted in 30-50 µl RNase free dH_2O prewarmed at 65°C. RNA concentrations were quantified using the Nanodrop.

2.20.2. Reverse transcription (RT)

Approximately 80 ng of isolated RNA from TM, and 200 ng of RNA from SC and PTC were used for reverse transcription using M-MLV reverse transcriptase. The desired amount of RNA to be transcribed was mixed with 2 µl oligo dT in a total volume of 18 µl and denatured in 70°C. The RT master mix was prepared and pre-warmed at 42°C before adding the M-MLV enzyme. Denatured RNA was mixed with the RT master mix and reverse

transcribed (table 1). The quality of the synthesized cDNA for all samples was confirmed by performing β-actin PCR. cDNA samples were stored at -20°C until use.

2.20.3. qRT-PCR

To evaluate IL-6 and TNF-α gene expression at the mRNA level, QuantiTect SYBR green PCR Master Mix and the QuantiTect primer assay for IL-6 were employed. TNF-α and β-actin primers were designed and purchased from Eurofins MWG Operon (see appendix 3). PCR efficiency was higher than 99%. Relative gene expression was calculated using the ΔΔCt (*delta delta Ct*) method [141]. Real-time PCR was performed using I-cycler IQ5 detection system.

Table 1. Reverse transcription of isolated RNA. Recipe for RT master mix preparation and RT program.

Reverse Transcription (RT) master mix		
Volume	**Material**	
6µl	RT buffer 5x	
2µl	dNTP	
1µl	RNAsin	
2µl	dH_2O	
1µl	M-MLV	
Reverse transcription program		
Action	**Temperature**	**Time**
RNA denaturation	70°C	10 min
Denatured RNA was kept on ice before adding the RT master mix.		
Reverse transcription	42°C	75 min
Enzyme inactivation	72°C	15 min

Table 2. Real time polymerase chain reactions.

Real time PCR program		
Cycle	**Temperature**	**Time**
1	95°C	13 min 30 sec
45 cycle	94°C	15 sec
	55°C	30 sec
	72°C	15 sec
1	50°C	10 sec

2.21. Immunofluorescence staining

Immunofluorescent double staining was used to determine the localization of the target proteins. Paraffin sections from human testis were deparaffinized and submitted to antigen retrieval prior staining (see Appendix 1). For frozen tissue staining, 8 µm thick sections were cut from rat testis using a cryostat. Sections were air dried before fixation. For cell staining, cells were seeded on glass coverslips. After performing the stimulation, cells were washed once with 1x PBS-Tween (0.01%).

To stain receptors on cell surface sections were fixed with acetone for 10 min at -20°C and further blocked. To stain intracellular/ intranuclear proteins cells were fixed with 4% PFA for 10 min at RT followed by 10 min permeabilization with PBS-Triton X-100 (0.5%) at RT. Samples were blocked using 5% BSA-PBS for 1 h at RT and later incubated O/N at 4°C with specific primary antibody (see Appendix 3). Slides were washed 3x5 min at RT with 1x PBS/Tween (0.01%) and incubated with fluorescent-labeled secondary antibody for 1 h at RT. Finally sections were washed 3x5 min and were mounted for visualization with mounting medium containing DAPI.

To prepare section for laser scanning confocal microscopy, 1.5 mm glass coverslips were used in 24-well plates (Sarstedt, Nümbrecht, Germany). Cells were washed once with 1x PBS-Tween (0.01%) after last treatment time point fixed with 4% PFA for 10 min at RT and mounted using DAKO fluorescence mounting media. Edges of the coverslips were sealed with nail polish.

2.22. Proximity ligation assay (PLA)

PLA assay was used to analyze the interaction of two proteins in the cells or tissue sections, facilitating localization of the target proteins. It is a highly sensitive and specific method to study protein-protein interactions. Two primary antibodies labeled with MINUS and PLUS oligonucleotide probes recognize target antigens of interest (Figure 8).

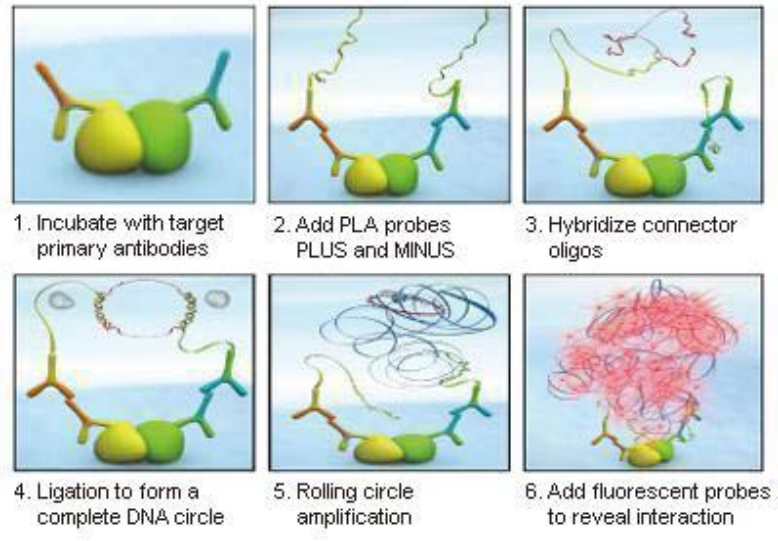


Figure 8. Principles of *in situ* proximity ligation assay. PLA assay enables detection of subcellular localization, modification and interaction of two proteins with high sensitivity and specifity within a cell or a tissue. Weak and transient interactions can be detected in situ and subpopulation of cells can be differentiated. Two different antibodies raised in different animals specifically bind to two target protein molecules. Secondary species specific antibodies conjugated with PLA probes are applied. In case of close proximity (<40 nm) PLA probes form a circular DNA using a ligase, and the signal is amplified by a rolling circle PCR reaction. The DNA product is labeled with complementary fluorescent probes and the signal can be visualizes using a microscope. (Figure was taken from http://www.olink.com/)

When the PLA probes are in close proximity (<40 nm), the DNA strands interacts through a subsequent addition of two other circle-forming DNA oligonucleotides. When the two added oligonucleotides have joined by enzymatic ligation, they are amplified via rolling circle amplification using a polymerase [142]. Following the amplification reaction, several-hundredfold replication of the DNA circle has occurred. The product is visualized by labeled complementary oligonucleotide probes. The resulting high concentration of fluorescence in each single-molecule amplification product is easily visible as a distinct bright dot using a fluorescence microscope.

In situ PLA was used to study the subcellular binding of HMGB1 to TLR4 in isolated testicular cells. Cells were fixed with acetone for 10 min at -20°C. Non-specific antibody binding was blocked using Duolink blocking solution for 30 min at 37°C. Samples were incubated O/N at 4°C with HMGB1 antibody labeled with PLA MINUS probe and TLR4 antibody labeled with PLA PLUS probe. For negative control a non- relevant antibody (beta-actin) was used together with TLR4 antibody. Ligation of the adjacent probes (<40 Å) was performed using ligase in ligation buffer for 30 min and finally rolling circle PCR reaction was done for 90 min. Amplification buffer contained fluorescently labeled complementary

probes which visualize every single binding of HMGB1 to TLR4. Images were captured using fluorescent microscope. Quantification of the data was done using Duolink Image Tool (Olink Bioscience, Uppsala, Sweden).

2.23. Transmission electron microscopy

Isolated SC were treated with 10 µg of rhHMGB1 or HBSS. Cells were washed 2x with 0.15 M HEPES buffer and fixed in TEM fixation buffer for 1-2 h at RT. Cells were additionally fixed in 1-4% osmium tetroxide in wash buffer for 1-2 h at RT and subsequently washed 2x3 min and stained in 0.5% uranyl acetate for 2 h at RT. Afterwards, samples were dehydrated in a series of alcohol dilutions (30% to 100%, each for 5-10 min), then treated with propylene oxide and 100% ethanol (1:1) for 10 min at RT. Cells were then treated with propylene oxide and epoxy resin (EPON plus 0.3% BDMA) (1:1) for 30 min at RT. Samples were embedded in EPON for 1.5 h and vacuumed in desiccator, then refilled with EPON and incubated at 60°C for 48 h. Blocks were trimmed and semi-thin sections (0.75 µm) were cut and stained by toluidine blue staining (1 g toluidine blue and 1 g sodium borate in 100 ml dH_2O). Ultrathin sections (70 nm) were cut using a diamond knife. Sections were placed on the grid and analyzed using the TEM LEO 906 microscope (Zeiss).

2.24. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM or LSCM) is a technique for obtaining high-resolution optical images with depth selectivity. The key feature of confocal microscopy is its ability to obtain in-focus images from selected depths [143] which provides the capablity of isolating and collecting a plane of focus from within a sample, thus eliminating the out of focus "haze" normally seen with a fluorescent sample. Isolated cells were seeded on 1.5 mm glass coverslips (thickness 0.17 mm) (Langenbrink, Teningen, Germany) as recommended for CLSM. After treatement cells were fixed using 4% PFA for 10 min and mounted. Drying the coverslip was avoided before mounting on a slide. The coverslip was sealed using a transparent nail polish. Slides were stored at 4°C.

2.25. 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, USA**)**

4 RESULTS

2.26. Testicular pathological changes in rat experimental autoimmune orchitis (EAO)

Fifty days after first immunization 70% (7/10) of the animals developed orchitis, while thirty days later all immunized animals (10/10) manifested chronic testicular inflammation. EAO was evaluated by means of pathological changes in the testicular morphology and reduced testicular weight [65]. A nearly 2-fold significant reduction in testicular weight was observed in orchitis animals 50 and 80 days after the first immunization (0.7643±0.05 g and 0.7167±0.03 g, respectively) compared to normal (1.58±0.02 g) and adjuvant control groups (1.563±0.03 g and 1.443±0.06 g). Pathological changes in the testis comprise leukocytic infiltration into interstitium, germ cell sloughing and apoptotic body formation in the seminiferous tubules, formation of granuloma and aspermatogenesis (Figure 9C, 9D).

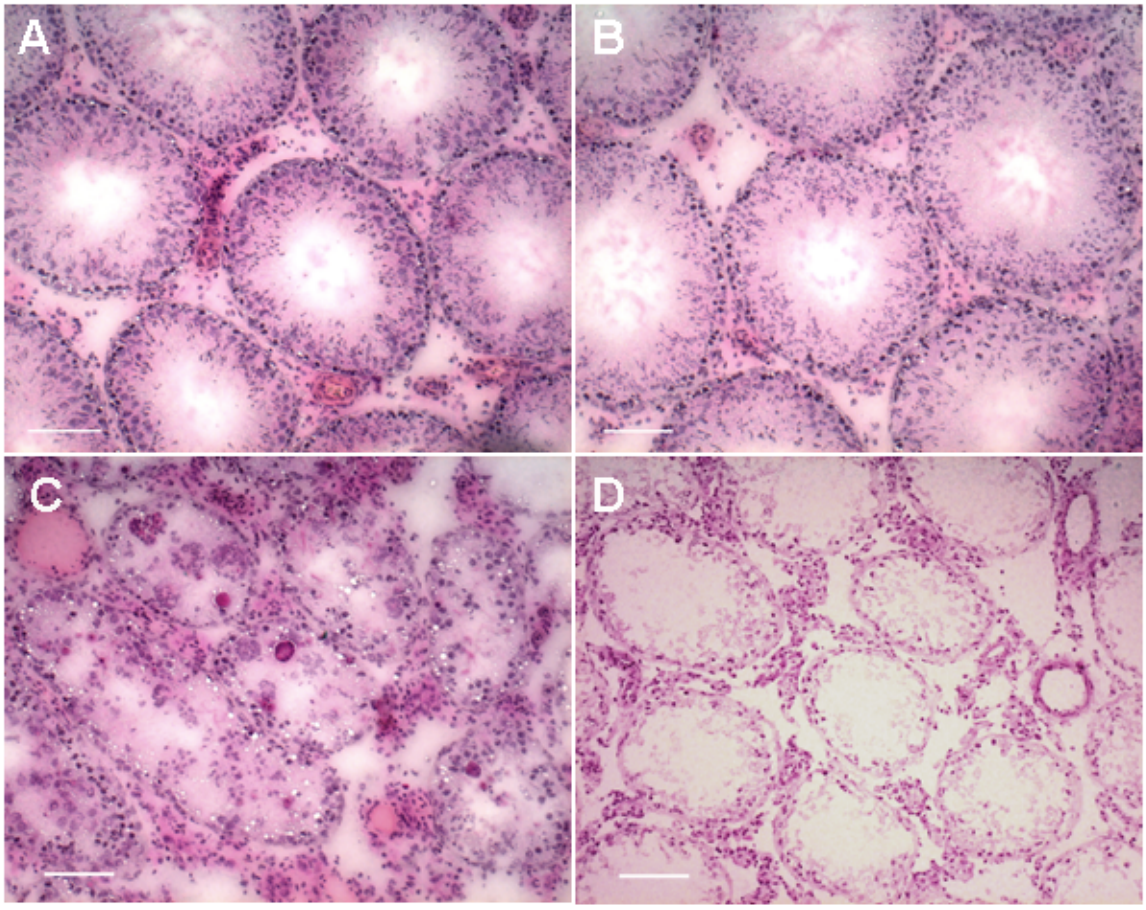


Figure 9. Progression of experimental autoimmune orchitis. Hematoxilin-eosin staining of testis cryosections from normal (A), adjuvant50 (B), EAO50 (C) and EAO80 (D) rats. Frozen testicular sections were rehydrated and stained with hematoxilin and eosin. Early EAO is characterized by massive leukocyte infiltration, apoptotic body formation and germ cell sloughing. At later stages of

the disease the majority of seminiferous tubules are devoid of germ cells and many of the tubues contain only some Sertoli cells.

2.27. HMGB1 expression in testicular cells

Immunofluorescent staining of isolated testicular somatic cells and control testis sections revealed that HMGB1 is mainly present in the nuclei of SC (Figure 10A), TM (Figure 10B), dendritic cells (Figure 10D), and Leydig cells. PTC seem to have considerable amounts of HMGB1 in the nucleus as well as in the cytoplasm (Figure 10C).

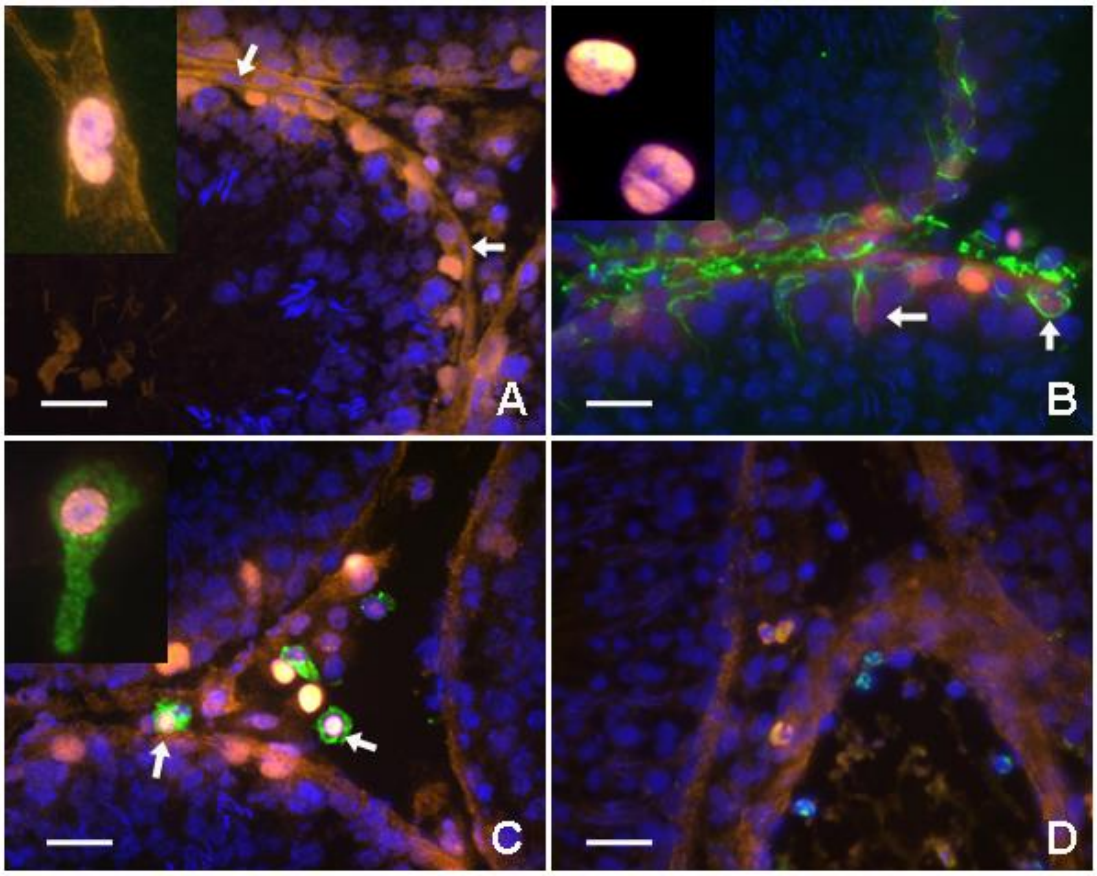


Figure 10. HMGB1 expression in rat testicular cells. Immunolabeling of HMGB1 (Cy3, orange) in isolated peritubular cells and in frozen sections from normal rat testis (A). Double staining of HMGB1 (Cy3, orange), Sertoli cell marker Vimentin (FITC, green) (B), monocyte/macrophage marker ED1/ED2 (FITC, green) (C), dendritic cell marker OX-62 (FITC, green) (D) and nuclei (DAPI, blue) in isolated testicular cell and sections from normal rat testis. Peritubular cells, Sertoli cells and macrophages (A, B and C respectively) are indicated by the white arrows. HMGB1 was mainly localized in the nuclei of Sertoli cells and macrophages (A, B) and in the nuclei as well as in the cytoplasm of peritubular cells (C). Dendritic cells in adjuvant rat testis express HMGB1 in the nuclei as well as in the cytoplasm (D). Scale bars represent 50 µm.

2.27.1. HMGB1 translocates from the nucleus into the cytoplasm in somatic cells in EAO and in human testis with leukocytic infiltrations

In human testis with normal spermatogenesis (Figure 11A) HMGB1 was predominantly expressed in the nuclei of testicular somatic cells, spermatogonia and also in some spermatocytes and elongating spermatids (Figure 11C). However, in human testis with inflammatory infiltrates (Figure 11B) HMGB1 was released from the nucleus into the cytoplasm and the interstitial space (Figure 11D). As shown in Figure 11D in inflamed testis PTC, SC and interstitial cells are devoid of nuclear HMGB1.

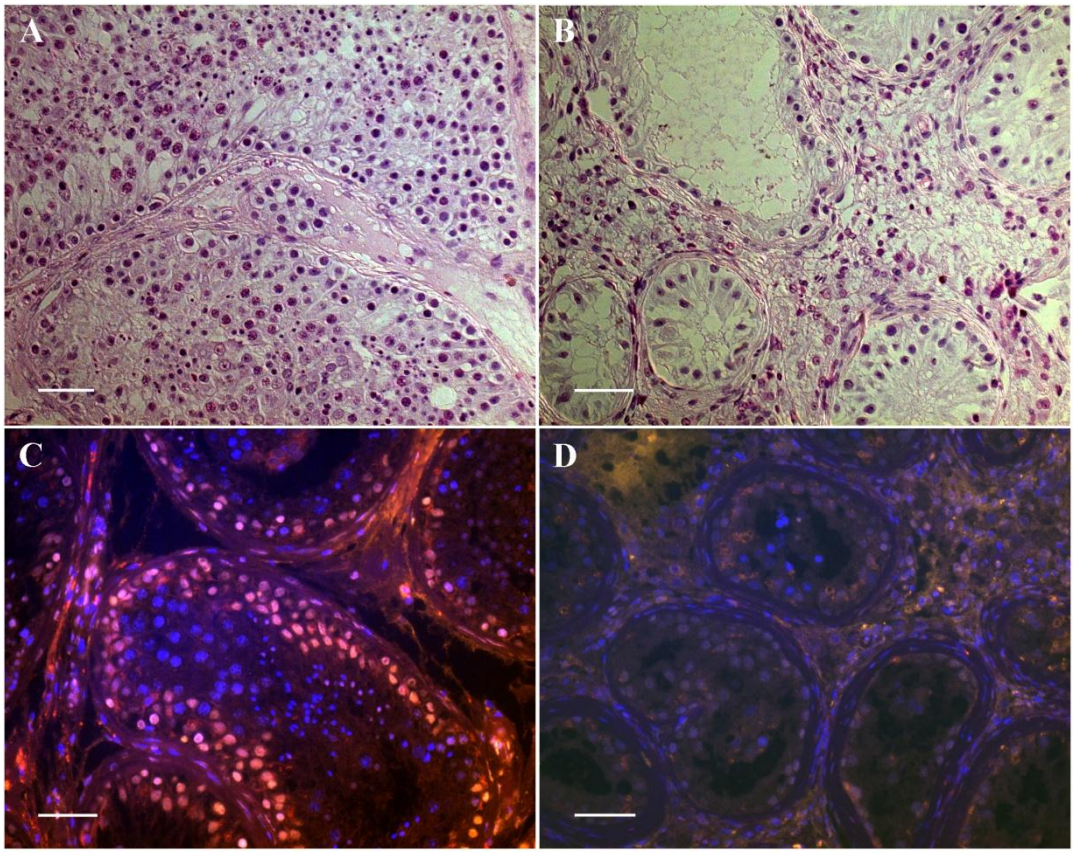


Figure 11. Translocation of HMGB1 in human testis with leukocytic infiltration. Histopathology of human testis shown using H&E staining. Seminiferous tubules with normal spermatogenesis containing cells from different stages of spermatogenesis (A). Figure B shows seminiferous tubules with impaired spermatogenesis. Massive infiltration of leukocytes is visible in the interstitial space, tubules show spermatogenesis arrest and are empty from germ cells. Immunofluorescent staining of HMGB1 (Cy3, orange) and nuclei (DAPI, blue) in paraffin sections of human testis (8 µm thick). HMGB1 was localized in the nuclei of Sertoli cells, spermatogonia and spermatocytes in testis with normal spermatogenesis (C). In contrast, nuclear HMGB1 was released from the remaining peritubular cells, Sertoli cells, interstitial cells, spermatogonia and spermatocytes in testis with inflammatory leukocytic infiltration and impaired spermatogenesis (D). Scale bar represents 30 µm.

Similarly in control and adjuvant rat testis, HMGB1 was localized in the nuclei of testicular somatic cells and immune cells (Figure 12A, B). In EAO50 HMGB1 was translocated from the nucleus into the cytoplasm of testicular somatic cells and in intracellular space (Figure 12C). A similar pattern was observed in EAO80, however some HMGB1 was detected in the nuclei (Figure 12D). Interestingly, HMGB1 was strictly retained in the nucleus in apoptotic bodies (germ cells undergoing apoptosis) in the lumen of seminiferous tubules in EAO50 testis, although it was released from adjacent cells (Figure 12F). To confirm the specificity of the antibody, the HMGB1 antibody was pre-absorbed for 2 h with recombinant HMGB1 (ratio 1:5) before incubation with the cryosection (Figure 12E).

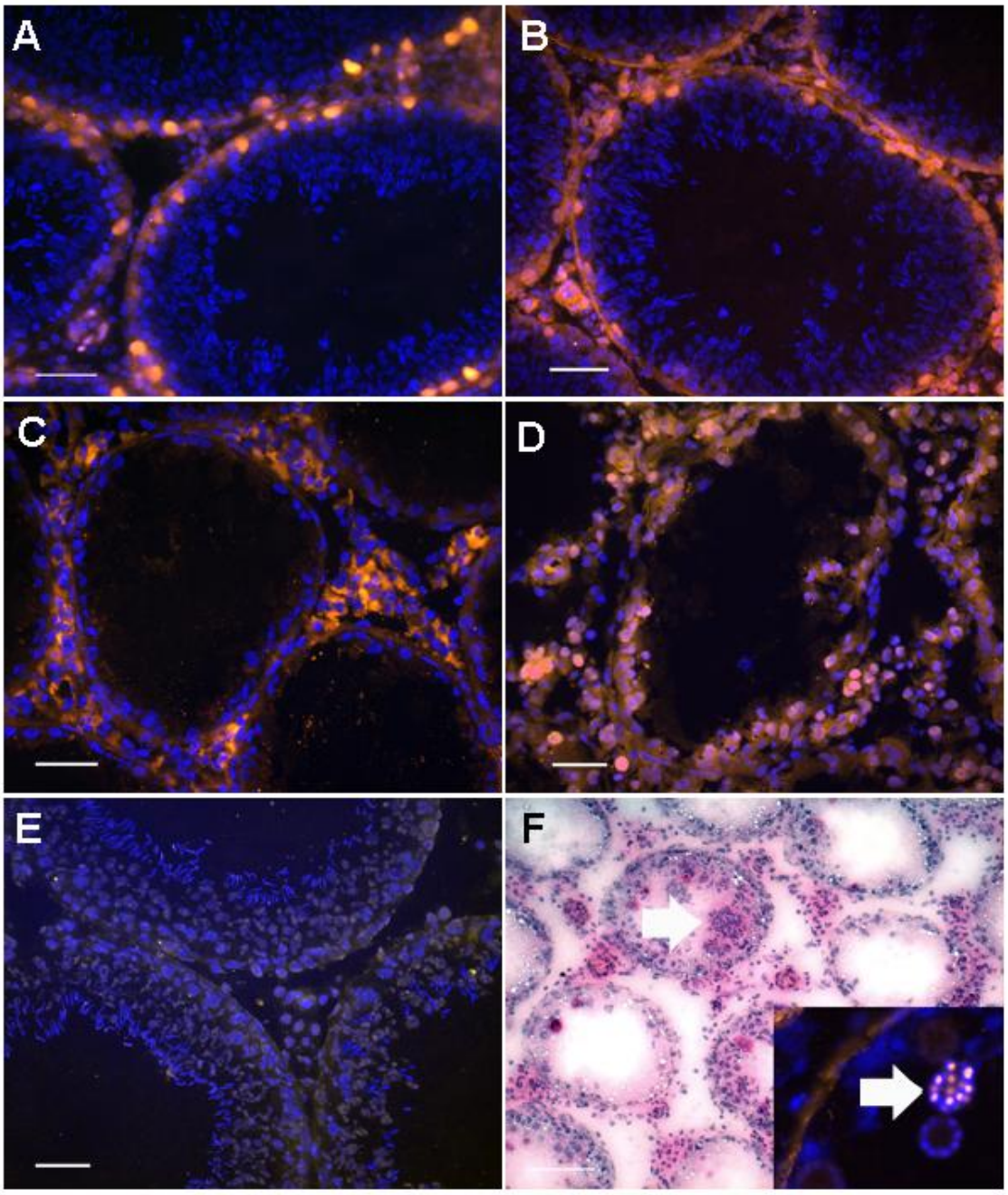


Figure 12. Localization of HMGB1 in rat testis with experimental autoimmune orchitis. Immunofluorescent detection of HMGB1 (Cy3, orange) and nuclei (DAPI, blue) in testicular cryosections from normal (A), adjuvant50 (B), EAO50 (C) and EAO80 (D) animals. In normal and adjuvant control testis, HMGB1 is localized in the nuclei of Sertoli cells, peritubular cells and some interstitial cells (A, B). In EAO50 nuclear HMGB1 is translocated out of the nucleus and is mainly present in the cytoplasm of Sertoli cells and peritubular cells or is released outside the cell (C). In the late phase of the disease (EAO80) extracellular and cytoplasmic HMGB1 is still present, however, nuclear HMGB1 is reappearing in some interstitial cells (D). Pre-absorption of HMGB1 antibody with rhHMGB1 for 2 h was used as a negative control (E). Apoptotic bodies (indicated by arrows) which are typically seen in the seminiferous tubules from EAO50 testis retain HMGB1 in the nucleus (F). Scale bars represent 50 μm in figures A-E and 30 μm in figure F.

2.27.2. Testicular HMGB1 levels are elevated in the chronic phase of EAO

Extracellular HMGB1 is a mediator of inflammation which has a late phase of action compared to early inflammatory cytokines such as TNF-α [144]. Levels of TNF-α and IL-6 in testis were significantly increased in EAO50, which confirms their action as pro-inflammatory cytokines. TNF-α levels remained elevated also in the late phase of the disease (EAO80; Figure 13A, 13B). In contrast, HMGB1 levels increased slightly in EAO50 testis. However, HMGB1 levels were significantly up-regulated at a later phase in testis of EAO80 rats indicating its significance in mediating inflammation in the chronic phase of the disease (Figure 13C).

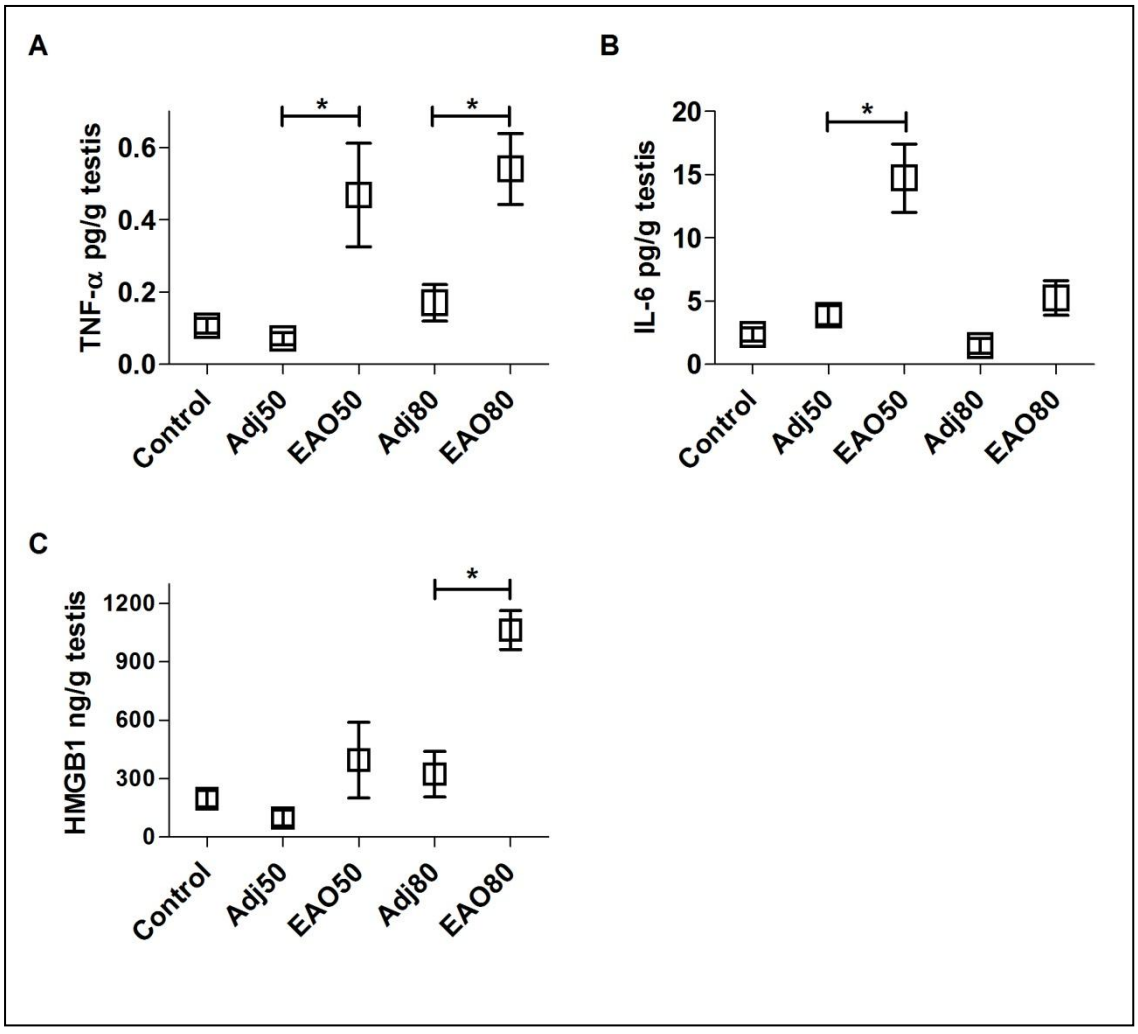


Figure 13. Levels of HMGB1, TNF-α and IL-6 in EAO rat testis. Protein levels of TNF-α (A), IL-6 (B) and HMGB1 (C) were measured in testicular homogenates from normal, adjuvant and EAO groups (50 and 80 days) using ELISA. Values from testicular homogenate ELISA were normalized according to total protein content of each sample and total testis weight. (n=3-7); *P<0.05.

2.27.3. Serum HMGB1, IL-6 and TNF-α levels remain predominantly unchanged in EAO

To prove that the cytokine increase is not due to systemic inflammation, serum levels of TNF-α, IL-6 and HMGB1 were analyzed. Serum levels of measured cytokines remained largely unchanged indicating that upregulation of inflammatory cytokines is testis specific, except IL-6 levels which were elevated in EAO80 group (Figure 14A, 14B, 14C).

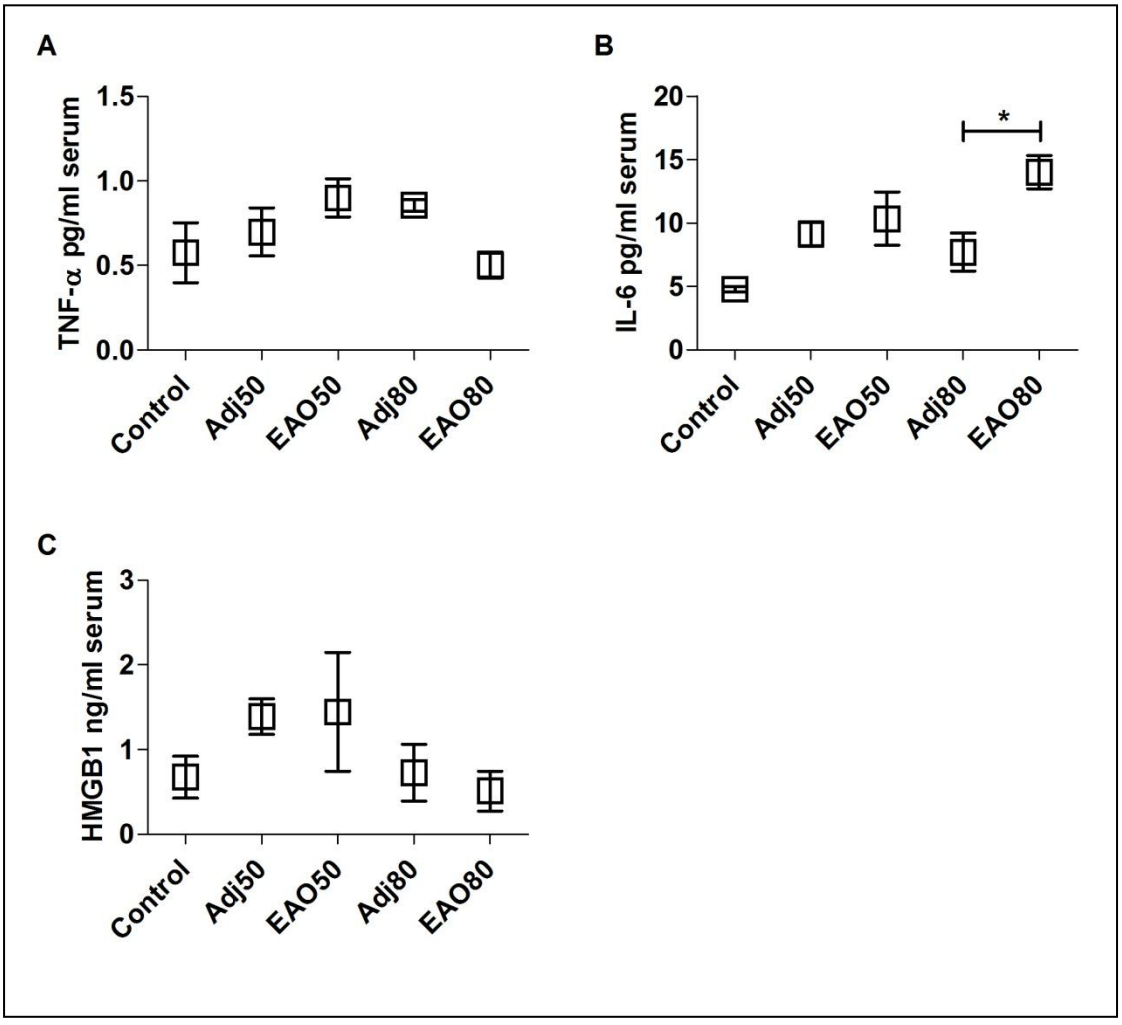


Figure 14. Levels of HMGB1, TNF-α and IL-6 in serum samples from EAO rats. Protein levels of TNF-α (A), IL-6 (B) and HMGB1 (C) were measured in serum samples from normal, adjuvant and EAO groups (50 and 80 days) using ELISA. (n=3-7); *P<0.05.

2.28. HMGB1 receptors

To date, several studies have shown that HMGB1 induces different cellular responses by binding to RAGE or TLR4 [126,143,144]. Using immunoblot and immunofluorescent analysis, we found that expression levels of RAGE and TLR4 proteins vary significantly between testicular somatic cells.

2.28.1. TLR4 expression in testicular cells

Immunofluorescent staining of TLR4 in cryosections of EAO rat testis showed that TLR4 is mainly expressed in testicular macrophages (Figure 14A). Using isolated SC and PTC we confirmed that TLR4 is also expressed in those cells, albeit in lower levels (Figure 14B, 14C).

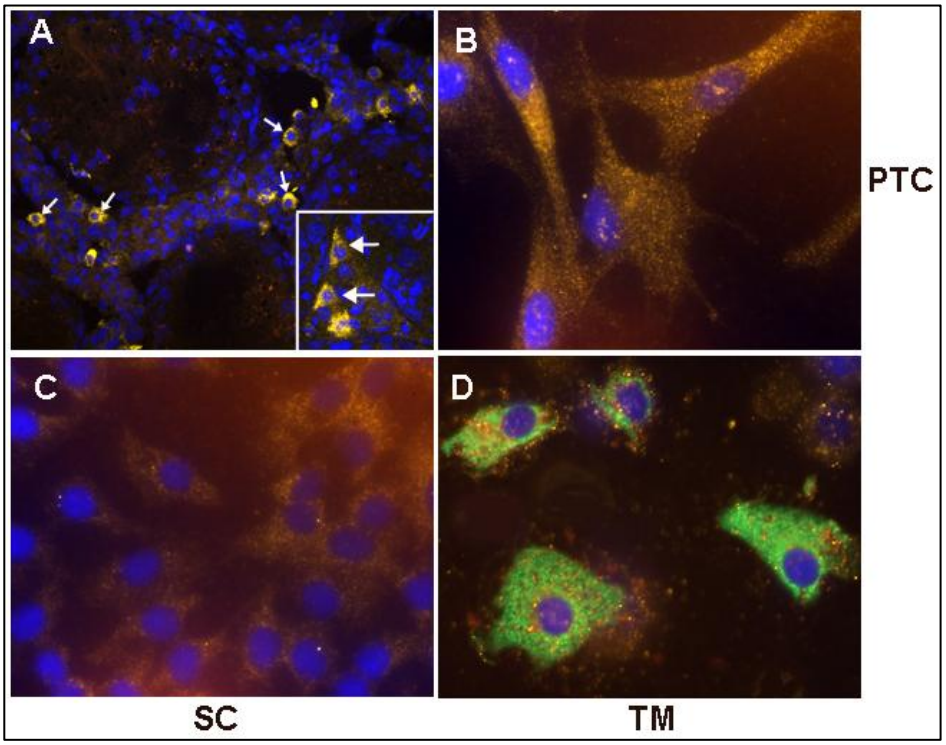


Figure 14. TLR4 expression pattern in testicular cells. Immunofluorescent staining of TLR4 (Cy3) in EAO50 rat testis (A), isolated peritubular cells (B), Sertoli cells (C) and testicular macrophages double labelled by ED1/ED2 staining (FITC) (D). TLR4 is dominantly expressed in testicular macrophages in rat. However, Peritubular cells and to a lower extent Sertoli cells also express TLR4 (B and C respectively). Images were taken by a fluorescent microscope. (PTC: peritubular cells, SC: Sertoli cells, TM: testicular macrophages)

2.28.2. HMGB1 - TLR4 binding

HMGB1-TLR4 binding was evaluated in testicular macrophages, PTC and SC using Proximity Ligation Assay (PLA) assay. The level of HMGB1-TLR4 binding was significantly higher in TM (Figure 15C) than in SC and PTC (Figure 15B, 15A). Duolink image tool was used to quantify the dots per cell (Figure 15D).

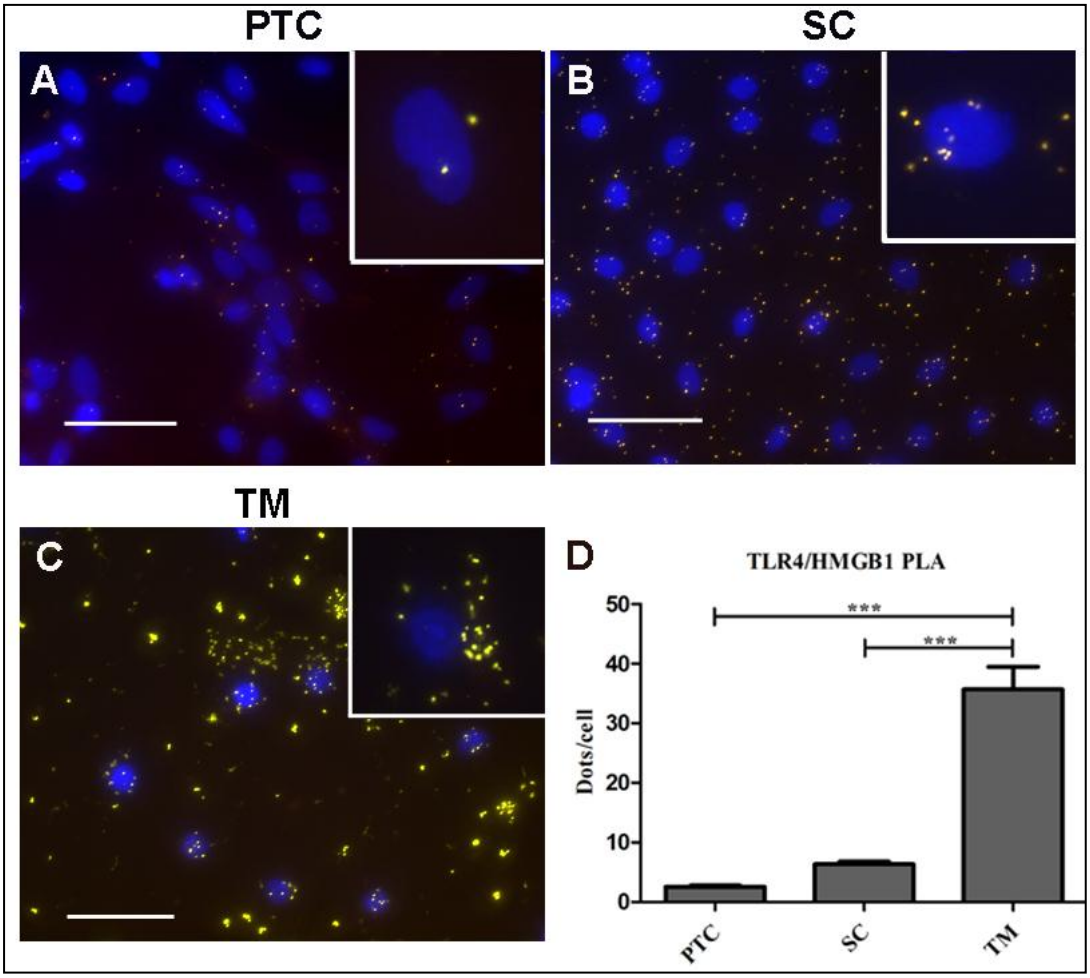


Figure 15. HMGB1–TLR4 interaction in testicular cells. Proximity ligation assay was performed in isolated peritubular cells, Sertoli cells and testicular macrophages. Isolated peritubular cells, Sertoli cells and testicular macrophages were seeded on 8-well culture slides (BD Biosceinces). Cells were treated with 1µg/µl rHMGB1 for 2 h. HMGB1-TLR4 interaction was detected in all three cell types, but it was significantly higher in testicular macrophages. Scale bars represent 50 µm. ***P<0.001; PTC=peritubular cells, SC=Sertoli cells, TM=testicular macrophages.

2.28.3. RAGE expression in testicular cells

RAGE expression was stronger in the basal compartment of seminiferous tubules of normal rat testicular cryosection which is corresponding to SC and PTC. However, weak staining was seen in the intratesticular space (Figure 16A). RAGE expression was confirmed by immunofluorescent staining in isolated PTC, SC and TM (Figure 16B, 16C, 16D). Similarly, Western blot analysis showed that RAGE is expressed at higher levels in SC and PTC as compared to TM (Figure 16E).

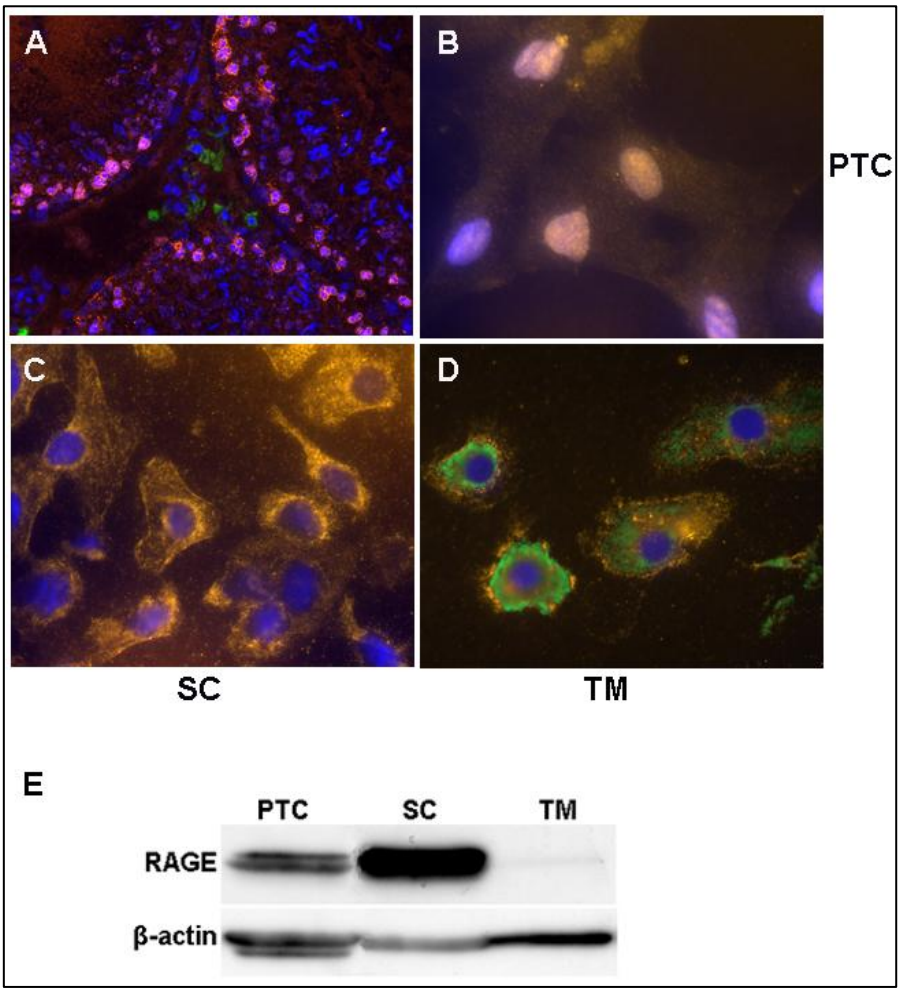


Figure 16. RAGE receptor expression pattern in testicular somatic cells. Immunofluorescent labelling of RAGE receptor (Cy3) in cryosection from normal rat testis (A), isolated peritubular cells (B), Sertoli cells (C) and testicular macrophages double marked by ED1/ED2 staining (FITC) (D). Sertoli cells express higher levels of the RAGE receptor. RAGE is also present at lower levels in peritubular cells and testicular macrophages. Immunoblot analysis was perfomed using RAGE primary antibody and β-actin as loading control (E). (PTC: peritubular cells, SC: Sertoli cells, TM: testicular macrophages)

2.28.4. HMGB1 – RAGE binding

PLA assay was used to quantify the levels of HMGB1-RAGE binding in different testicular somatic cells. Interestingly, PTC showed the highest level of HMGB1-RAGE binding (Figure 17A). HMGB1-RAGE binding was significantly lower in SC as compared to PTC (Figure 17B). In contrast, TM showed the lowest HMGB1-RAGE binding levels among all three cell types (Figure 17A, 17D).

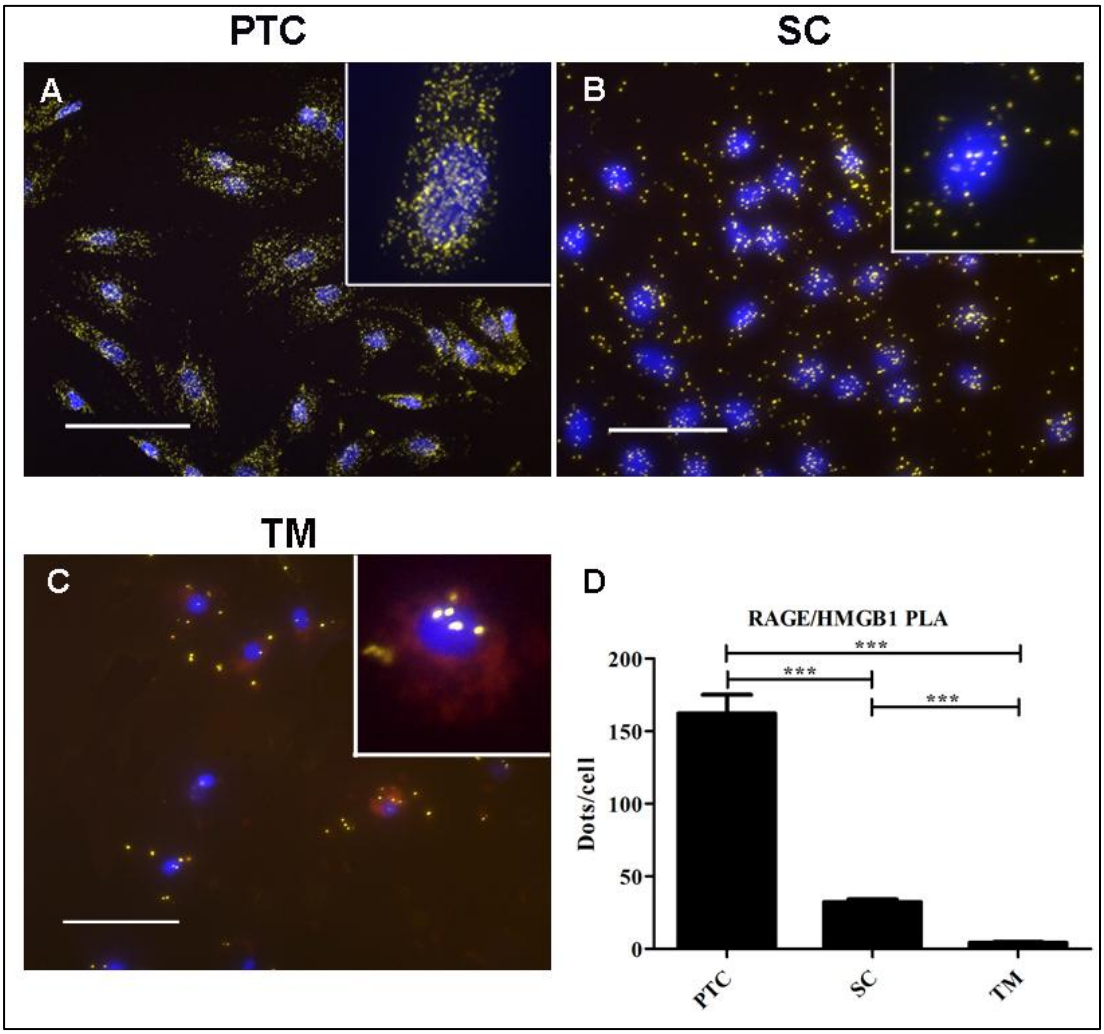


Figure 17. **HMGB1 – RAGE interaction in testicular cells.** Proximity ligation assay was used to quantify HMGB1 binding to RAGE in different testicular somatic cells. Isolated peritubular cells, Sertoli cells and testicular macrophages were seeded on 8-well culture slides (BD Biosceinces). Cells were treated with 1µg/µl rHMGB1 for 2 h. High levels of HMGB1-RAGE binding was observed in peritubular cells (A). The binding level was lower in Sertoli cells (B) and testicular macrophages showed very low HMGB1-RAGE binding (C). Pictures were taken by a fluorescent microscope. Scale bars represent 50 µm. ***P<0.001; PTC=peritubular cells, SC=Sertoli cells, TM=testicular macrophages.

2.29. Recombinant expression and purification of HMGB1

His-tagged recombinant human HMGB1 (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). Over-expression of rhHMGB1 was induced in BL21(DE3)pLysS cells. A new band representing rhHMGB1 expression appeared in the cell lysate supernatant 4h after IPTG induction (Figure 18 lane 2, 3). His-tagged rhHMGB1 was enriched using Ni-NTA agarose (Figure 18 lane 8), followed by further HPLC purification (Figure 18, lanes 10-15) resulting in 3 different protein bands in different elution fractions. The 3 protein bands were identified as HMGB1 by Western blotting and mass spectrometry analysis. The largest protein band corresponding to the molecular size of full length HMGB1 (Figure 18 lane 10, 11, 12) was used for *in vitro* experiments. These fractions were pooled and then dialyzed to get highly purified protein (Figure 18 lane 17). To remove endotoxin contamination, protein solution was treated with Triton X-114 before aliquoting in protein LoBind tubes and stored at -80°C.

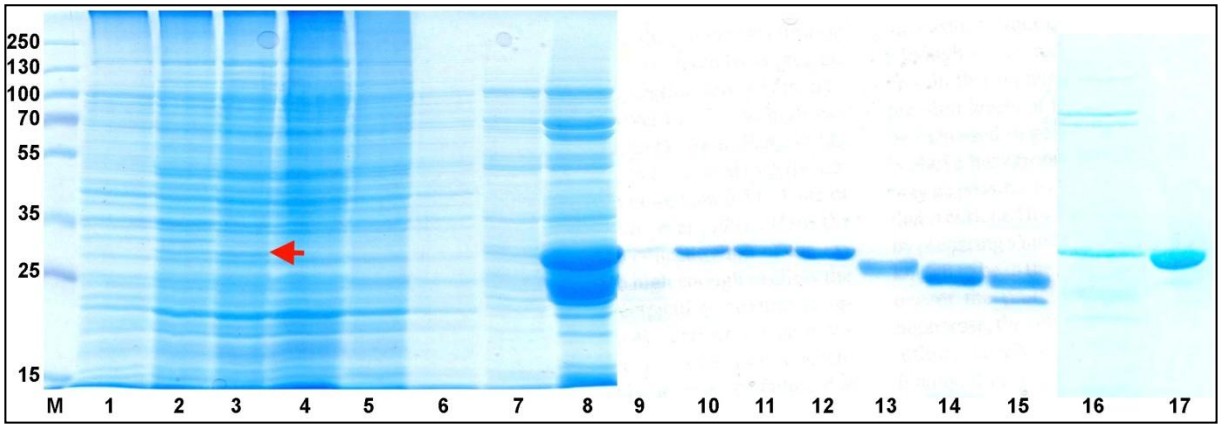


Figure 18. Expression and purification of human recombinant HMGB1 (rhHMGB1). Expression of His-tagged rhHMGB1 was induced by IPTG treatment for 4 h in BL21(DE3)pLysS cells (Lane 1: IPTG negative, Lane 2: IPTG 4 h; the appearing rHMGB1 band is marked by the red arrow). Cells were lysed using lysozyme and sonication. Resulting supernatant (Lane 3) was used for His-tagged protein purification using Ni-NTA agarose beads (Lane 4: flow-through fraction, Lanes 5, 6: wash fractions, Lanes 7, 8: first and second eluates). Second eluate from purified His-tagged rhHMGB1 was further purified using affinity chromatography. The protein was eluted using PMSF- and DTT-free Tris-KCl buffer. The eluates show two different peaks corresponding to two differet protein bands (Lanes 10, 11, 12 and Lanes 13, 14, 15) which were confirmed to be human HMGB1 using mass spectrometry. The full length rhHMGB1 (Lanes 10, 11, 12) was used for further *in vitro* experiments. The purified rhHMGB1 was dialyzed against Tris-HCl buffer pH 8.0 and 10% glycerol overnight at 4°C (Lane 16: rhHMGB1 before dialysis, Lane 17: rhHMGB1 after dialysis). All purified products were loaded on a 12.5% SDS gel and stained overnight by Coommassie Brilliant Blue staining. (M: protein molecular marker).

2.30. HMGB1 activates different signaling pathways in testicular somatic cells

HMGB1 is known to activate several signaling pathways via addressing different receptors (Figure 4) [146]. Moreover, HMGB1 has been shown to bind to cell specific receptors in different testicular somatic cells. To analyze the role of HMGB1 in activating different signaling pathways, isolated testicular macrophages, PTC and SC were stimulated with 5 µg/ml rhHMGB1.

2.30.1. NF-κB and Akt signaling pathways

Stimulation with rhHMGB1 differentially activates NF-κB and Akt signaling pathways in testicular somatic cells. TM stimulated with rhHMGB1 activated the NF-κB P65 pathway which induces pro-inflammatory cytokine production. However, in rhHMGB1 treated PTC the Akt signaling pathway was activated which can trigger a wide range of different responses including CREB phosphorylation and expression of survival genes. In contrast, treatment of SC with rhHMGB1 did not induce a significant change in NF-κB and Akt phosphorylation (Figure 19).

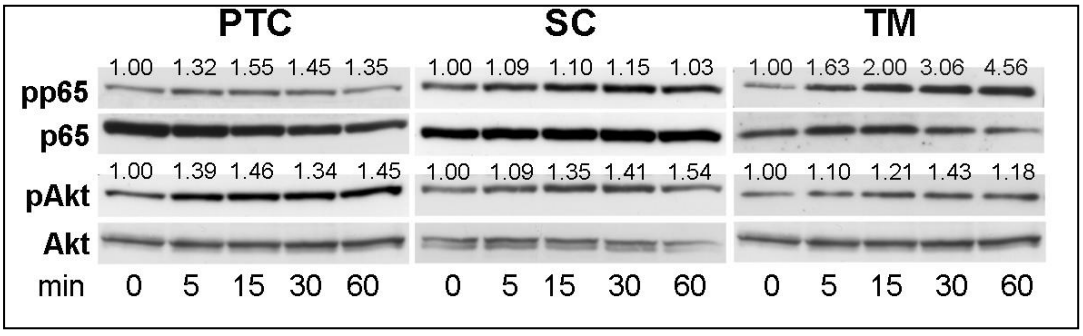


Figure 19. NF-κB and Akt signaling in peritubular cells, Sertoli cells and testicular macrophages. Western blotting was used to detect phosphorylation of p65 and Akt compared to total protein level. Isolated peritubular cells, Sertoli cells and testicular macrophages were stimulated using 10 µg/µl rhHMGB1 for indicated time points. Cells were lysed in RIPA buffer and loaded on a 10% SDS-PAGE gel. WB membranes were incubated with phosphorylated and total protein antibodies for p65 and Akt overnight. Secondary anti-rabbit IgG antibody conjugated with HRP was used and the signal was visualized using SuperSignalWest Pico Substrate. Images were obtained using the chemiluminescence imaging system (PEQLAB)). Numbers above the blots are representative of the band intensity compared to total protein levels. PTC=peritubular cells, SC=Sertoli cells, TM=testicular macrophages.

2.30.2. MAPK signaling pathways

HMGB1 induces pro-inflammatory responses by activating NF-κB and p38 signaling pathways [147]. In TM, rhHMGB1 induced phosphorylation of p38 mitogen-activated protein

kinase (MAPK), but no discernible effect on ERK1/2 was detected (Figure 20). Conversely, rhHMGB1 stimulation in SC and PTC led to low phosphorylation levels of p38 MAPK. Interestingly, ERK 1/2 was activated in rhHMGB1 treated SC and PTC (Figure 20).

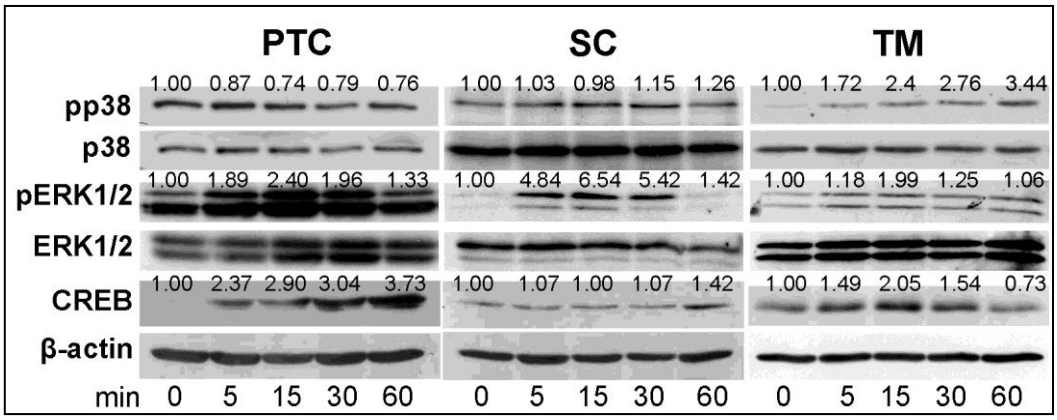


Figure 20. p38, ERK1/2 and CREB signaling in peritubular cells, Sertoli cells and testicular macrophages. rhHMGB1 induced phosphorylation of p38, ERK1/2 and CREB proteins was monitored using Western blot. Isolated peritubular cells, Sertoli cells and testicular macrophages stimulated with 10 µg/µl rhHMGB1 for indicated time points. WB membranes were incubated with respective phosphorylated and total protein antibodies overnight at 4°C. Images were taken using the chemiluminescence imaging system (PEQLAB). Numbers above the blots are representative of the band intensity compared to total protein levels. PTC=peritubular cells, SC=Sertoli cells, TM=testicular macrophages.

2.31. HMGB1 induces IL-6 and TNF-α expression in testicular macrophages and peritubular cells but not in Sertoli cells

To study the effects of alternative signaling pathway activation by HMGB1 in testicular cells in regulating cytokine production during testicular inflammation, isolated testicular macrophages, peritubular and SC were stimulated either with rhHMGB1 or LPS as a positive control or pretreated with ethyl pyruvate. IL-6 and TNF-α mRNA expression were analyzed by quantitative real-time RT-PCR. rhHMGB1 treated PTC demonstrated a significant upregulation in TNF-α mRNA levels, whilst IL-6 mRNA levels remained unchanged (Figure 21A, 21B). In SC treated with rhHMGB1 cytokine levels remained unaffected (Figure 21C, 21D). In TM, IL-6 and TNF-α mRNA levels increased significantly after rhHMGB1 treatment. Ethyl pyruvate pretreatment inhibited inflammatory effects of rhHMGB1 in TM (Figure 21E, 21F).

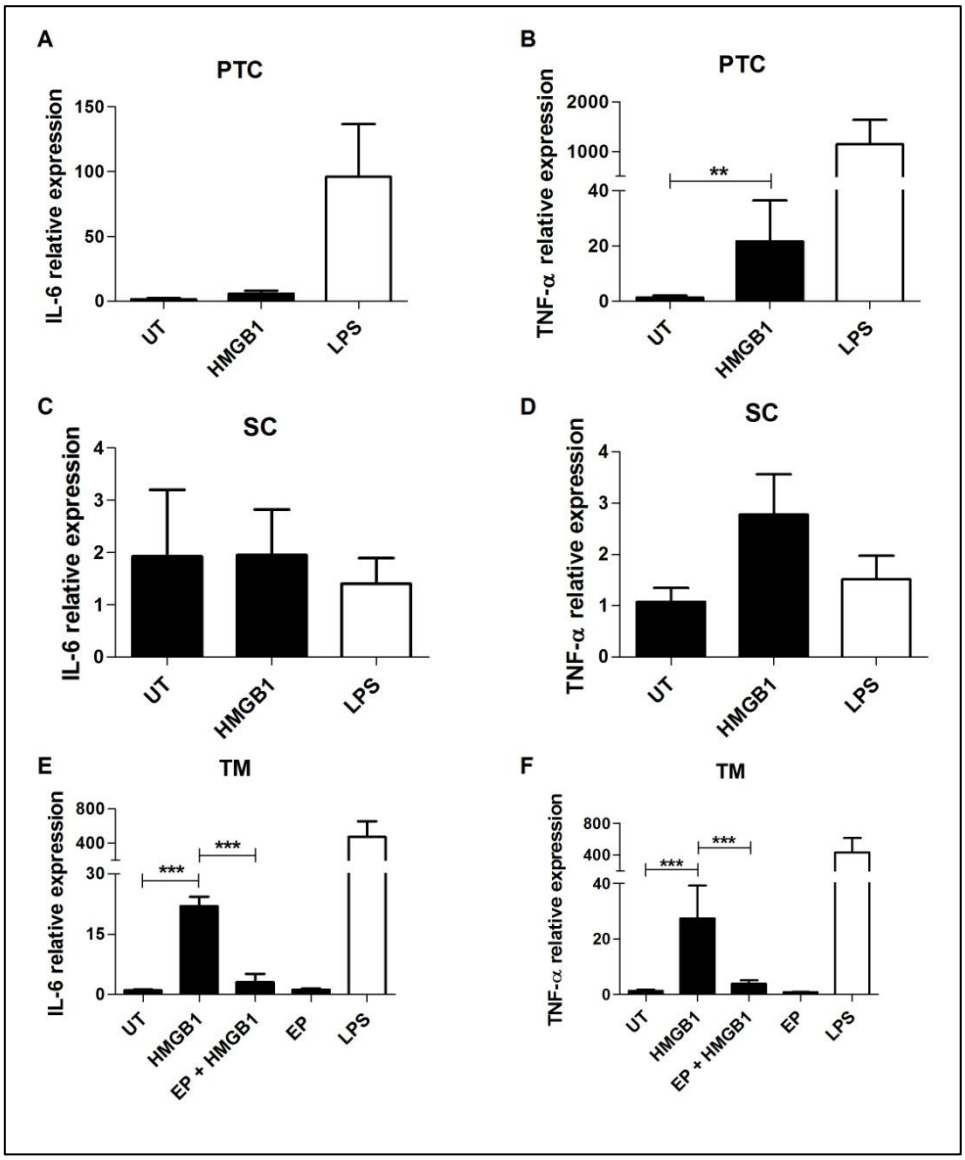


Figure 21. Quantitative real-time RT-PCR analysis of TNF-α and IL-6 mRNA expression. Quantitative real-time PCR was performed to analyze the relative expression of IL-6 and TNF-α mRNA levels in testicular cells treated with rhHMGB1. Isolated peritubular cells (A, B), Sertoli cells (C, D) and testicular macrophages (E, F) were treated with 5 µg rhHMGB1 and/or 10 mM ethyl pyruvate or 10 µg LPS for 6 h or left untreated. Cells were lysed in RLT buffer and total RNA was isolated. RNA was reverse transcribed into cDNA. Equal amounts of cDNA were used for real-time PCR. Relative gene expression was normalized to β2-microglobulin; (n=4); **P<0.01; ***P<0.001. PTC=peritubular cells, SC=Sertoli cells, TM=testicular macrophages, EP=ethyl pyruvate.

2.32. Recombinant HMGB1 enhances formation of autophagosome/ autophagolysosomes in Sertoli cells

HMGB1 is known as a critical pro-autophagic protein [127]. To investigate an alternate role of HMGB1 in SC, formation of autophagosomes in HMGB1 treated SC was monitored usinf transmission electrone microscopy (TEM). Isolated SC were seeded on glass coverslips or in 3.5 cm diameter cell culture Petri dishes. One day after hypotonic shock SC were treated

with rhHMGB1 for 1 h to 6 h or with HBSS for 1.5 h to induce autophagy. Cells were fixed in 1.5% paraformaldehyde and 1.5% glutaraldehyde and further stained for TEM analysis. Number of autophagosomes/ autophagolysosomes was increased in rhHMGB1 treated SC and HBSS starved cells compared to untreated cells (Figure 22).

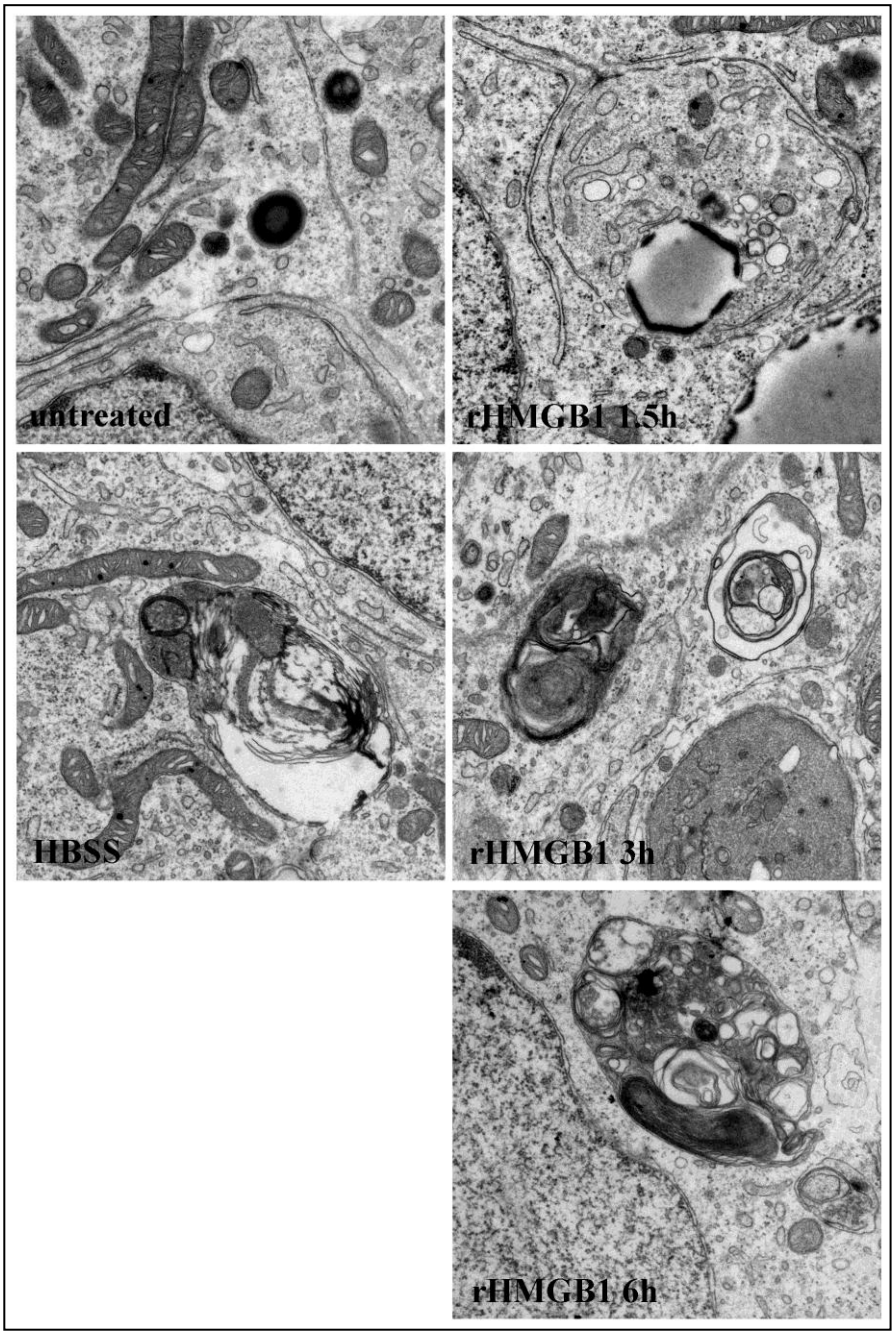
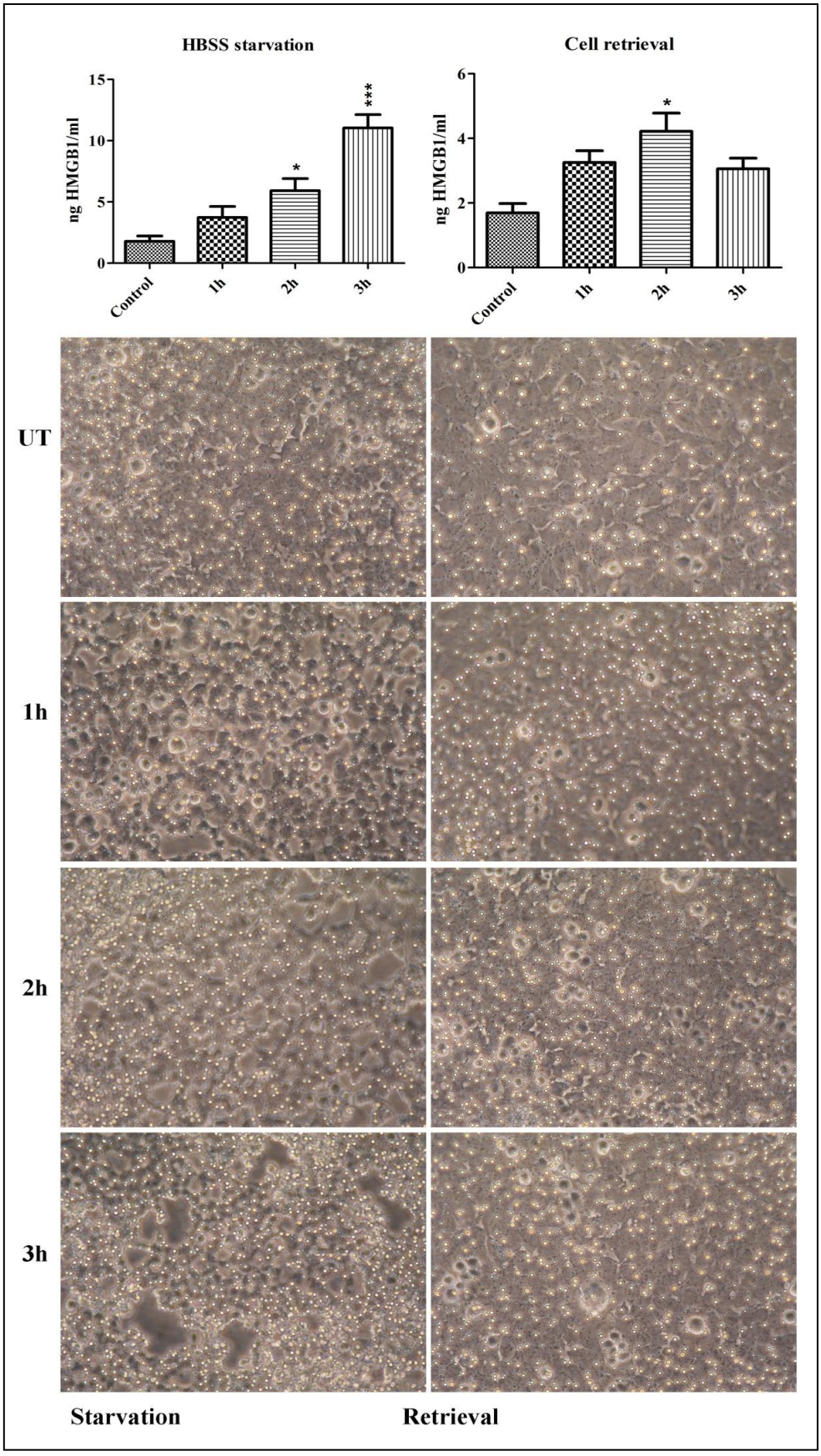


Figure 22. Recombinant HMGB1 triggers the formation of autophagosomes in rat Sertoli cells. Isolated rat Sertoli cells were stimulated with 10 µg/µl rhHMGB1 for indicated time points. For controls cells were left untreated or starved using Hank's Balanced Salt Solution (HBSS) to induce autophagy. Cells were fixed and stained for TEM analysis. (n=3)

2.32.1. Serum starvation induces HMGB1 release from Sertoli cells

Necrotic cells release HMGB1 passively to signal danger and apoptotic cells keep HMGB1 in the nucleus to avoid further inflammatory signaling [75]. To check the fate of HMGB1 in starving cells undergoing autophagy, isolated SC were starved using HBSS medium and appearance of HMGB1 in the supernatant was measured by HMGB1 ELISA. The content of HMGB1 in the supernatant from starving SC was increased by time (Figure 22). Significant increase of HMGB1 levels in the SC undergoing autophagy started already 2 h after starvation. To confirm the integrity of the starving cells and that HMGB1 release is not due to cell death and necrosis, starving cells were recovered by adding RPMI-1640 medium for 3 h after starvation and HMGB1 content in the supernatant was measured. Starving cells started obtaining round shape after 2 h HBSS treatment. Normal morphology of the cells was retrieved after adding RPMI-1640 medium. HMGB1 release was significantly reduced after cell retrieval, however HMGB1 levels remained significantly higher in 2 h HBSS treated SC compared to untreated control.

Figure 22. Serum starvation induces HMGB1 secretion and release in rat Sertoli cell. Isolated Sertoli cells were seeded on 24-well cell culture plates. Two days after hypotonic shock cells were starved using HBSS medium for 1, 2 and 3 h and the resulting supernatant was collected. Starved cells were left in RPMI-1640 medium for further 3 h to revive and the supernatant was collected. HMGB1 levels in the cell supernatant were measured using HMGB1 ELISA and compared to the untreated control. The experiment was performed three times using cells from separate preparations. *P<0.05; ***P<0.001. (figure on the next page)


HBSS starvation
ng HMGB1/ml
15
10
5
0
*
***
Control
1h
2h
3h
Cell retrieval
ng HMGB1/ml
6
4
2
0
*
Control
1h
2h
3h
UT
1h
2h
3h
Starvation
Retrieval

4 DISCUSSION

In the present study, we showed that nuclear HMGB1 was redistributed into the cytoplasm and interstitial space in testis sections from EAO animals. In EAO50 testis sections, the nuclei from the remaining testicular cells (mainly testicular somatic cells as the germ cells underwent apoptosis), namely Sertoli cells, peritubular cells and also infiltrating cells such as testicular macrophages, were devoid of HMGB1. Later on, in EAO80 nuclear HMGB1 was detected indicating new HMGB1 synthesis at the chronic phase of the disease. In contrast to EAO testis, HMGB1 was located mainly in the nuclei of testicular somatic cells but not in germ cells in normal rat testis. HMGB1 was localized exclusively in the nuclei of Sertoli cells and testicular macrophages and in the nuclei as well as in the cytoplasm of peritubular cells. The identity of Sertoli cells and testicular macrophages was confirmed by vimentin and ED1/ED2 double staining, respectively. In parallel, isolated peritubular cells, Sertoli cells and testicular macrophages were also used for HMGB1 staining. These findings are in line with the previous study which showed that HMGB1 was mainly expressed in rat Sertoli cells [148]. In contrary to the previous study HMGB1 was not detected in spermatogonia. This may be due to different antibodies or fixation protocols used. However, the localization to Sertoli cells is unarguably the same in both studies.

Nuclear translocation of HMGB1 under inflammatory conditions has been reported in several inflammatory disease models such as polymiositis, colitis, vasculitis, systemic lupus erythematosus, myositis, Sjögren's syndrome and experimental arthritis and is considered to have a role in directing inflammatory reactions and pathogenesis of these diseases [75,136] [148,149]. Furthermore, in patients suffering from rheumatoid arthritis (RA) significant elevation of extracellular HMGB1 in blood, synovial tissue and fluid was reported [150,151] [153]. Similar results were shown in rodent models of RA [154]. Under inflammatory conditions, the nucleosomal structural protein, HMGB1, is translocated from the nucleus and is either actively secreted by immune cells and autophagic cells or passively leaks out from necrotic cells with compromised integrity of the cell membrane into the extracellular space where it acts as a cytokine to signal a wide range of pro- or anti-inflammatory responses such as inflammation, cell proliferation and migration, tumorigenesis and autophagy through a repertoire of receptors like TLR2, TLR4 and RAGE [142,118,154,155]. It is important to note that 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 [59,71]. HMGB1 can be acetylated on its two nuclear localization signals (NLS). Under normal conditions there is a balance between acetylation and deacetylation of HMGB1 therefore HMGB1 shuttles between the nucleus and the cytoplasm. Upon LPS stimulation in monocytes HMGB1 is hyperacetylated and exits the nucleus either actively via nuclear export channels or diffuses out passively. Hyperacetylated HMGB1 cannot re-enter the nucleus and thus accumulates in the cytoplasm and is later packed in secretory vesicles which are released to the extracellular milieu upon stimulation [71].

Additionally, HMGB1 was translocated from the nuclei into the cytoplasm in dendritic cells in orchitis. In our previous study, it was demonstrated that number and maturation status of dendritic cells were significantly increased in rat EAO. Isolated DCs in orchitis showed significantly higher mRNA expression of CCR7 as well as IL-12 and the ability to stimulate proliferation of T cells, which points to their immunogenic phenotype and migratory-ready state [156,157]. Dumitriu *et al.* have already shown that extracellular HMGB1 is essential for the migration of maturating dendritic cells [159]. Moreover, HMGB1 is able to induce secretion of multiple pro-inflammatory cytokines like TNF-α, IL-1α, IL-6, IL-8 and IL-12 in dendritic cells [160]. Although not directly investigated in this study, it is supposed that HMGB1 has a stimulatory effect on the process of DC maturation in EAO testis and induces synthesis of pro-inflammatory cytokines in an autocrine/paracrine manner by DCs as well as adjacent cells.

Interestingly, apoptotic bodies visible mainly in the lumen of seminiferous tubules from EAO50 testis retained nuclear HMGB1, although HMGB1 was released from all other neighboring cells. Several studies focusing on HMGB1 release during different types of cell death have demonstrated that HMGB1 is irreversibly bound to the chromatin in cells undergoing apoptosis which is a skill apoptotic cells have obtained to avoid further inflammatory signaling to the adjacent cells and hence elicit tolerogenic signals hampering prolonged inflammation [75].

Despite testicular immune privilege the testis is prone to infection and inflammation. Inflammatory reactions in the testis may overcome its immune suppressive characteristics and result in disruption of testicular immune privilege followed by onset of autoimmune reactions in the testis and antibody production against spermatogenic cells [10,160]. Sub-acute or

chronic testicular inflammation (orchitis) appears asymptomatic until late stages of the disease and yet there are no serological or seminal markers enabling diagnosis of the disease at earlier stages, therefore the underlying causes of testicular autoimmune inflammation remain largely unknown [49]. Interestingly, in human testis with normal spermatogenesis, HMGB1 was mainly present in the nuclei of cells localized in the basal part of the seminiferous tubules such as Sertoli cells, spermatogonia and early spermatocytes. In peritubular cells HMGB1 was present only in the cytoplasm. In line with results from EAO testis, in testis sections from infertile men showing inflammatory leukocytic infiltrations and impaired spermatogenesis, nuclear HMGB1 was completely translocated out of the nucleus. To further analyze the mediating role of HMGB1 in the testis the rat model of experimental autoimmune orchitis (EAO) was used. This model serves as a model to study chronic testicular inflammation in the testis, reflecting pathological changes also seen in human testis with immunological infertility [49,61,65]. Characteristics of an EAO testis are highly similar to human testis with inflammatory infiltrations. Animals immunized with testis homogenate in complete Freund's adjuvant followed by Bordetella pertussis (BP) injection developed organ-specific autoimmunity in the testis. Animals were sacrificed 50 (early EAO) or 80 days (late EAO) after the first immunization. H&E staining of frozen testis sections showed massive leukocytic infiltration in the peritubular space of EAO testis. Seminiferous tubules were damaged and germ cells formed apoptotic bodies inside the lumen which led to impaired spermatogenesis and eventually complete absence of spermatogenesis at later stages in EAO80. EAO is widely used to study the mechanism of immune privilege and autoimmune inflammation in the testis. Interestingly, depletion of regulatory T cells in vasectomized mice induced autoantibody production against orchitogenic antigens and developed a CD4+ T cell mediated autoimmune orchitis [162]. Administration of viable syngenic spermatogenic cells without adjuvants also caused induction of EAO [163]. Recently, it was also shown that placement of a syngeneic donor's testes, epididymides and vasa deferentia into the abdominal cavity of the recipient animal is sufficient to induce autoimmune orchitis [164]. Taken together, EAO is accepted as a helpful model to study testicular chronic inflammation and autoimmunity.

In contrast to early pro-inflammatory cytokines TNF-α and IL-6, HMGB1 was significantly up-regulated in the chronic phase of the disease (EAO80). Testis-specific upregulation of HMGB1 together with the reappearance of nuclear HMGB1 in EAO80 testis

implies a role for HMGB1 as a late mediator of testicular inflammation. It seems that increasing concentrations of pro-inflammatory mediators at early stages of the disease stimulates release of nuclear HMGB1 ultimately reaching high levels during the later chronic phase of EAO. Upregulation of HMGB1 protein in EAO testis at a later phase is in agreement with the previous work from Wang *et al.* which introduced HMGB1 as a late mediator of sepsis [144]. This study showed that compared to TNF-α and IL-1 levels, which are elevated within hours from the onset of disease, HMGB1 is increased days later and parallels the onset of lethality [165].

In immune-privileged testis TNF-α together with other cytokines involved in the inflammatory cascade like IL-1α, IL-1β or IL-6 play a dual physiological function in modulating steroidogenesis and spermatogenesis. During development of testicular inflammation, a disturbed balance of these cytokines and elevated secretion levels are involved in testicular damage, apoptosis of germ cells and leukocytic infiltration of the interstitium [17,37]. For the first time, it is shown that an upregulation of TNF-α as well as IL-6 in EAO testis at the onset of EAO (EAO50) is followed by translocation of HMGB1 from the nuclei into the cytoplasm of testicular cells (EAO50) with a subsequent release to the extracellular space (EAO80). Late kinetics of HMGB1 upregulation and release as compared to classical pro-inflammatory cytokines has significant implications in understanding and manipulating inflammatory reactions in innate immune responses [133]. It is important to note that, HMGB1 upregulation time point differed significantly compared to classical pro-inflammatory cytokines. In contrast to classical pro-inflammatory cytokines such as TNF-α and IL-6 which were elevated in testis from EAO50 rats, HMGB1 protein levels were significantly upregulated in the late chronic phase of the disease (EAO80). However, HMGB1 translocation from the nuclei into the cytoplasm in testicular cells was visible already 50 days after first immunization (EAO50). It seems that increasing concentration of pro-inflammatory mediators at early stages of the disease stimulates the release of HMGB1 ultimately reaching high levels during late chronic phase of EAO.

Actively secreted or passively released HMGB1 can signal directly through RAGE or TLR4 [75]. In addition to pro-inflammatory signals, RAGE also elicits tolerogenic signals upon activation with HMGB1. In contrast, HMGB1 activated TLR4 elicits pro-inflammatory responses [80]. It was found that RAGE and TLR4 are differentially expressed in testicular somatic cells. Immunofluorescence staining and Western blot results using rat testis or isolated testicular cells showed that RAGE is abundantly expressed in peritubular cells and

Sertoli cells. Very low levels of RAGE were detected in testicular macrophages. Opposite, TLR4 was predominantly expressed in testicular macrophages. However, Sertoli cells and peritubular cells expressed lower amounts of TLR4 protein. In agreement with differential expression levels of HMGB1 receptor in testicular cells, HMGB1-receptor binding performed by PLA assay showed a cell-specific pattern. Higher levels of HMGB1-TLR4 binding were observed in testicular macrophages, whilst strong HMGB1-RAGE binding was detected in peritubular and to a lower extent in Sertoli cells.

RAGE is a multi-ligand receptor which regulates different cellular processes such as metabolism, inflammation and cell growth reactions upon binding to different DAMP molecules such as HMGB1 and S100 proteins [166]. Recently, it has been shown that RAGE promotes autophagy and inhibits apoptosis which leads to tumor cell survival. RAGE activation caused decreased mTOR phosphorylation and increased Beclin-1/ PI3K interaction [167]. Additionally knock down of RAGE, but not TLR4 reduced autophagy levels [132]. Given that RAGE is a phosphatidylserine receptor and plays a role in the clearance of apoptotic cell bodies [168], it might be that RAGE dependant upregulation of autophagy in Sertoli cells plays a major role in clearance of apoptotic germ cells during EAO progression. TLR4, a primary receptor for extracellular HMGB1, is responsible for macrophage migration and activation, cytokine production and tissue injury [168,169]. It is known that TLR4 induced cytokine production occurs via IκB degredation and NFκB translocation [171]. Interestingly, HMGB1-receptor binding to different testicular cell types is fine tuned via cell-specific receptor expression profile of adjacent cells. This might be an indication of different roles of these cells in orchestrating inflammatory reactions when exposed to extracellular HMGB1. Regulation of TLR4 and RAGE receptors have been implicated in pathogenesis of several diseases and is a regulatory mechanism to define HMGB1 signaling. For example, upregulation of RAGE in cancer tissue caused rapid pancreatic tumor growth and diminished apoptosis [172]. In contrast, an increased level of TLR4 mediated pro-inflammatory responses were observed due to mechanisms that can activate B cell TLR4 expression in patients with Crohn's disease and inflammatory bowel disease [143,144]. However, to date there is no other study demonstrating similar type of delicate navigation of HMGB1 actions through differential expression pattern of its receptors in cells within the same organ like in the testis.

Additionally, to augment an inflammatory response HMGB1 can form complexes with immune mediators that include IL-1, endotoxin or DNA [172,173,174]. Forming a complex

with other cytokines or danger associated molecular patterns (DAMPs) HMGB1 can bind to a wide range of receptors such as IL1-RI, TLR2, TLR3, TLR7 and TLR9 thus inducing a broad repertoire of immunological responses that include cytokine release, induction of chemotaxis and angiogenesis as well as cell differentiation and proliferation [81,75]. In contribution to autoimmune pathogenesis, when forming an immune complex containing DNA, HMGB1 will induce cytokine production via binding to RAGE [176]. On the contrary, DAMPS such as HMGB1 when forming complexes with TLR ligands inhibited the inflammatory action of TLR9 and TLR2 via a RAGE dependant pathway [176,177].

For *in vitro* experiments recombinant His-tagged human HMGB1 was expressed and purified. All buffers used for purification of the recombinant protein were devoid of DTT or any other reducing agent as DTT has been shown to reduce the crucial cysteine important for TLR4 binding and pro-inflammatory activity of HMGB1 [90]. At the molecular level, activation of different signaling pathways were observed by applying rhHMGB1 to isolated testicular cells. In testicular macrophages stimulated with rhHMGB1, MAPK p38 and p65 NFκB was phosphorylated, whilst in Sertoli and peritubular cells the ERK1/2 MAPK signaling pathway was activated. As shown by Park *et al.* stimulation of neutrophils with HMGB1 led to p38 MAPK activation and to a lesser extent PI3K, Akt and ERK1/2 phosphorylation. Inhibition of the molecules involved in this pathway decreased HMGB1-induced cytokine production through reduced NFκB activation [147]. ERK1/2 MAPK activation in peritubular cells and Sertoli cells correlates with high levels of HMGB1-RAGE binding in these cells, as previously shown that RAGE interacts directly to ERK [83]. In testicular macrophages, HMGB1 induced NFκB phosphorylation subsequently stimulated pro-inflammatory cytokine release and upregulation of HMGB1 receptors' expression [179]. In this view, increased secretion of HMGB1 and elevation of its receptors can sustain and amplify the inflammatory response which leads to progression and/or chronification of EAO.

In line with the results from HMGB1 signaling pathway activation, *in vitro* stimulation of testicular macrophages using rhHMGB1 caused a significant increase in TNF-α and IL-6 mRNA expression levels. Release of cytokines was abolished by ethyl pyruvate pre-treatment, an inhibitor of HMGB1 action. Peritubular cells treated with rhHMGB1 produced elevated amounts of TNF-α, but not IL-6. Interestingly, in Sertoli cells no change in inflammatory cytokine levels was observed after rhHMGB1 administration. This phenomenon could be explained by the fact that for cytokine induction by HMGB1, TLR4 signaling is a crucial requirement [180]. As shown previously that TLR2 and RAGE knockout macrophages

exposed to HMGB1 produced TNF, whilst in TLR4 knockouts TNF production was inhibited [90].

In contrast to normal conditions, macrophages in EAO testis actively release HMGB1, which in an autocrine/paracrine manner interacts with TLR4 to trigger production of IL-6 and TNF-α. This inflammatory cascade (loop) seems to play a peculiar role during autoimmune reactions in the testis. The onset of EAO parallels infiltration of testicular interstitial space with lymphocytes, mainly lymphocytes, macrophages, T cells and dendritic cells [21,65,157,180]. Testicular macrophages play an important role in preserving testicular immune privilege. Ablation of testicular macrophages resulted in decreased incidence and severity of testicular damage in EAO [181]. Under normal conditions rat testicular resident macrophages expressing scavenger receptor CD163 (ED2+ cells) display an alternatively activated phenotype, constitutively produce IL-10 and have reduced co-stimulatory activity on T cell proliferation. In contrast, during inflammation newly arrived migrating monocytes/macrophages (CD68+/ ED1+ cells) increase expression of immune mediators (e.g. MCP-1, TNF-α, IL-6, iNOS) and shift the macrophage subpopulation balance to overcome immune privilege [9,24,181]. When exposed to a non-inflammatory testicular milieu ED1+ macrophages alter their phenotype to a reduced capacity of pro-inflammatory cytokine production, increased IL-10 production and impaired ability to induce T cell activation, a phenotype compatible with their role in maintaining testicular immune privilege [24].

Accumulating data shows that the redox state of the three critical cysteine residues in the HMGB1 molecule determines its release during different types of stress (Figure 23). HMGB1 only exhibits a pro-inflammatory effect when C106 is in reduced form and C23 and C45 form a disulfide bridge [183]. The cytokine activity of HMGB1 was totally abolished when C106 was substituted with an alanin or if this residue was oxidized [184]. Caspase dependent ROS production during apoptosis results in oxidation of extracellular HMGB1. Oxidized HMGB1 (in C106 as C23-C45 disulfide band is already oxidized) is devoid of inflammatory activity [78]. The mammalian immune system discriminates between different types of cell death. Basically necrosis, apoptosis and autophagy are closely related and finely regulated processes which determine the fate of each cell depending on the cell type and cell microenvironment [185]. Necrotic cell death often leads to inflammation and adaptive immunity, whereas apoptotic cell death is known to elicit immune tolerance. Immune tolerance can be established via apoptotic antigen presentation by MHC class I to $CD8^+$ T cells [186]. However, the MHC

class III-encoded receptor RAGE can trigger the switch from apoptosis to autophagy [172]. A recently reported mechanism of apoptosis/autophagy switch regulation shows that under stress conditions p53 forms a complex with HMGB1 in the nucleus and in the cytoplasm in colorectal cancer. Lower levels of p53 induced accumulation of cytoplasmic HMGB1 and Beclin-1 binding and favored autophagy. In contrast loss of HMGB1 increased p53 levels and apoptosis and reduced autophagy [187]. It seems that the microenvironment is decisive in manipulating the redox state of extracellular HMGB1 guiding the HMGB1 molecule to preferentially bind the suitable receptor and convey the necessary responses. With the knowledge of the importance of the HMGB1 redox status in modulating its pro-inflammatory actions, tailored therapies can be develop to target only the pro-inflammatory state of the molecule.

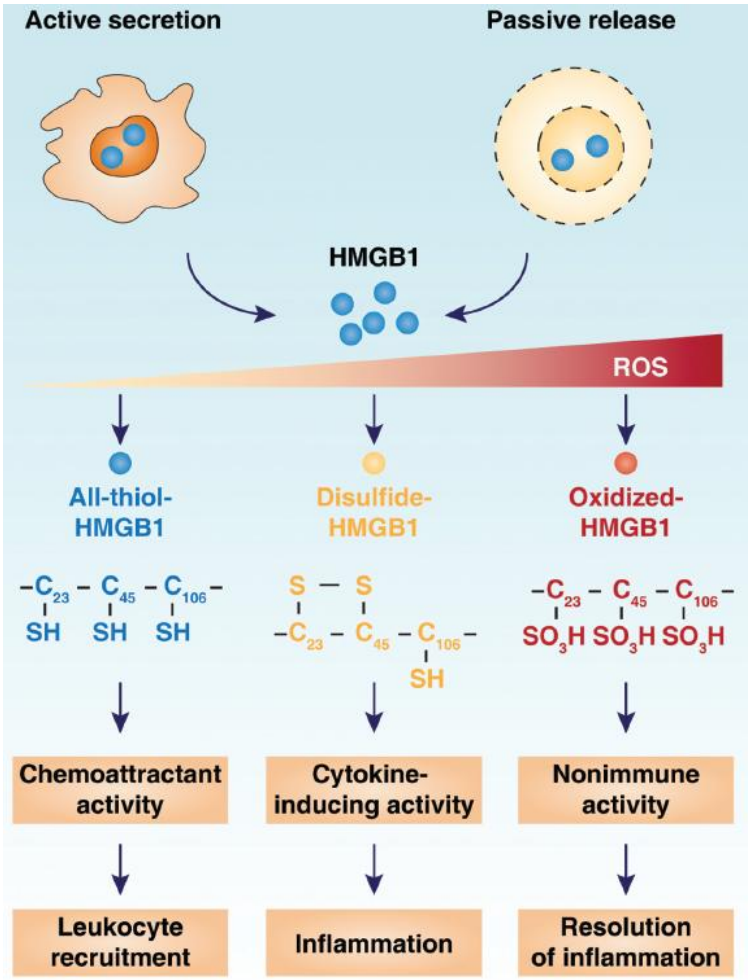


Figure 23. Redox status defines HMGB1 extracellular activity. In stress conditions, HMGB1 is actively secreted by immune cells or passively released from dying necrotic cells, due to loss of cell integrity. Although different in post translational modifications, the redox state of those types of extracellular HMGB1 can be modified according to the microenvironment. In reduced form all-thiol-HMGB1 acts as a chemoattractant to recruit immune cells. For pro-inflammatory action, formation of the C23/C45 disulfide bridge is crucial. However, in the case of apoptotic cell death and a milieu with elevated ROS levels the HMGB1 molecule is oxidized, loses its pro-inflammatory characteristics and preferentially conveys tolerogenic signals which lead to resolved inflammation or enhanced tumorogenesis (Tang et al. Mol Med; 2012).

In this study, *in vitro* HMGB1 stimulation did not induce pro-inflammatory cytokines production in Sertoli cells. Additionally, IL-6 levels remained unchanged also in stimulated peritubular cells. Furthermore, HMGB1-RAGE signaling was detected at significantly high levels in peritubular and Sertoli cells. Unlike germ cells, Sertoli cells survive the

inflammatory environment and are present also in chronic phase of the disease when the inflammation is resolved [163]. As it has been shown that RAGE activation induces autophagy and limits apoptosis [128], HMGB1-RAGE induced activation of autophagy may be an explanation on how these cells manage to survive longer than other testicular cells. Autophagy (macroautophagy), the lysosomal digestion of cell organelles or cytoplasm which are sequestered by a double membrane (phagophore) that later forms the autophagosome [188]. Taking part in cell death and cell survival processes, autophagy has been implicated to play a fundamental regulatory role in many biological and pathological processes such as development, aging, autoimmunity and cancer. The level of autophagy activity determines whether the cell dies via apoptosis, necrosis or autophagy and if the dead cell clearance is a silent or inflammatory process [103]. For example reduced autophagy levels in dying cells may lead to necrosis hence triggering further inflammation. As autophagy plays a role in acquired immunity as well as defense against intracellular pathogens the term "immunolophagy" has been proposed recently [189]. Interestingly, new data shows that autophagy can be regulated by HMGB1 (reduced form containing intramolecular C23/C45 disulfide bridge). On the one hand, cytoplasmic HMGB1 replaces BCl-2 and binds to Beclin1 to induce autophagy. On the other hand, extracellular HMGB1 activates MAPK signaling via RAGE binding and leads to phosphorylation of BCl-2 and induction of autophagy [125,130].

Starvation induced autophagy activation resulted in HMGB1 release from Sertoli cells in a time dependent manner. In addition to its function in cell survival, autophagy has been recently shown to have a role in biogenesis and secretion of cytokines such as IL-1β and macrophage migration inhibitory factor (MIF) [114,189]. Autophagy is triggered as a programmed cell survival pathway in case of nutrient deprivation. After 3 h starvation Sertoli cells underwent morphological changes. However, cells restored normal shape again after adding medium containing nutrients. Autophagic cells are known to release HMGB1 selectively without losing membrane integrity. The HMGB1 molecule released from autophagic cells has distinct properties and elicits further pro-autophagy signaling. In contrast, HMGB1 released from apoptotic cell is oxidized and will induce further apoptotic cell death avoiding inflammation [120].

Analyzing the ultra-structure of Sertoli cells challenged with HMGB1 demonstrated elevated formation of double membrane vesicles representing autophagosomes within these cells. Interestingly, morphological changes and engulfment of mitochondria by

autophagosomes (mitophagy) were observed. These observations suggest that HMGB1 may induce or enhance mitophagy in Sertoli cells. It is known that HMGB1 is necessary for gene expression of heat shock protein B1 which has a role in actin cytoskeleton regulation and cellular transport eventually required for mitophagy [102,127]. In addition to increased number of autophagosomes in HMGB1 treated Sertoli cells, the tight junctions were impaired at some regions between two adjacent cells. However, the possible effects of extracellular HMGB1 on Sertoli cell tight junctions are not known and remain to be elucidated.

In EAO testis, various cells commence a complex series of different stress responses thus finding the underlying mechanisms and the starting point of inflammation is challenging. It is shown that CD4+ but not CD8+ T cells are crucial for the onset of EAO [61]. More specifically TNF producing CD4+ cells play a key role in induction of disease [63]. However, Jacobo *et al.* showed that both CD4+ and CD8+ positive T cells are involved in initiation and progression of EAO [63]. Furthermore CD4+ T cells and Th17 subsets rather modulate the onset of EAO however CD8+ T cells contribute to chronification of the disease [191]. At the same time, large numbers of monocytes/macrophages infiltrate the testis actively producing pro-inflammatory cytokines. Upregulation of cytokines at early stages of EAO caused damage to the blood testis barrier by down regulation of tight junction proteins such as occludin which eventually leads to breaking the immune privilege of the testis [192]. Exposure of unprotected germ cells to high levels of cytokines and DAMP molecules produced by activated testicular cells or by dying cells together with upregulation of apoptotic signaling molecules such as Fas-Fas ligand in the intratesticular fluid from EAO animals causes excessive apoptosis which at later stages impairs spermatogenesis [193]. ROS released by apoptotic cells maintains an oxidative microenvironment around germ cells. This results in HMGB1 oxidation which induces further apoptosis [132]. However, in Sertoli cells a couple of mechanisms e.g. inhibition of apoptosis by 17β-estradiol and 5α-dihydrotestosterone as well as receptor expression profile lead to tolerogenic signaling activation and may redirect these cell into autophagic pathway. Autophagic Sertoli cells secrete HMGB1. Therefore, the autophagy inducing form of HMGB1 which is abundantly present in the microenvironment around Sertoli cells will continue triggering autophagy in these cells. It is supposed that selective induction of autophagy in Sertoli cells may explain the mechanism of how these cells face a different fate and survive for a significantly longer time in the inflamed testis of EAO animals.

In the present study it was demonstrated that administration of ethyl pyruvate successfully inhibited HMGB1 induced IL-6 and TNF-α production in testicular macrophages. Ethyl pyruvate is a derivative of the endogenous metabolite, pyruvic acid. Ethyl pyruvate treatment is established to have anti-inflammatory effects. Administration at clinically relevant doses is proved to be safe for human [194]. Interestingly, ethyl pyruvate has been shown to attenuate HMGB1 release by inducing necrosis-to-apoptosis switch in lung adenocarcinoma cells and to inhibit NFκB signaling by directly targeting p65 in LPS stimulated murine macrophage-like RAW 264.7 cells [136,194]. Ethyl pyruvate treatment of septic mice with systemic inflammation successfully resulted in increased survival rate of the animals [136]. Considering the fact that pre-treatment with ethyl pyruvate inhibited HMGB1 induced inflammatory cytokine production in testicular macrophages, this molecule seems to be a promising tool for therapy of EAO animals. Considering that non-infectious orchitis is asymptomatic at early stages, using a late phase target widens the therapeutic window for treatment strategies to rescue remaining foci of seminiferous tubules with intact spermatogenesis. In addition to ethyl pyruvate, monoclonal anti-HMGB1 antibodies, recombinant A box domain of HMGB1 with anti-inflammatory effects, or soluble RAGE have been also used to neutralize the action of HMGB1 in animal models of RA showing beneficial effects to reduce inflammatory reactions and to attenuate disease development even when used after onset of disease [149,195,196]. Administration of A box and monoclonal anti-HMGB1 antibodies also ameliorated the inflammatory responses in a mouse model of ConcavalinA induced hepatitis [198]. All these promising data indicate that blockade of HMGB1 might be a new potent target of therapy in autoimmune and chronic inflammatory diseases.

Taking together, the present study shows that early upregulation of pro-inflammatory cytokines (TNF-α, IL-6) released from different testicular cells can trigger HMGB1 nuclear translocation and release (Figure 24). In a regulatory loop to sustain and prolong the immune response, released HMGB1 binds to TLR4 on testicular macrophages to induce the synthesis and release of TNF-α and IL-6 by activating p38 and p65 signaling pathways. Administration of ethyl pyruvate attenuates HMGB1 pro-inflammatory action in testicular macrophages. In contrast, in Sertoli cells HMGB1/RAGE binding induces a rather tolerogenic immune response via activating autophagy in these cells. In addition to the crucial role that the redox state of the microenvironment plays on the action of extracellular HMGB1, the cell specific

expression profile found in this study adds a further level of local regulation on HMGB1 function. Consequently, the response of neighboring, but different cell types will vary upon stimulation with HMGB1.

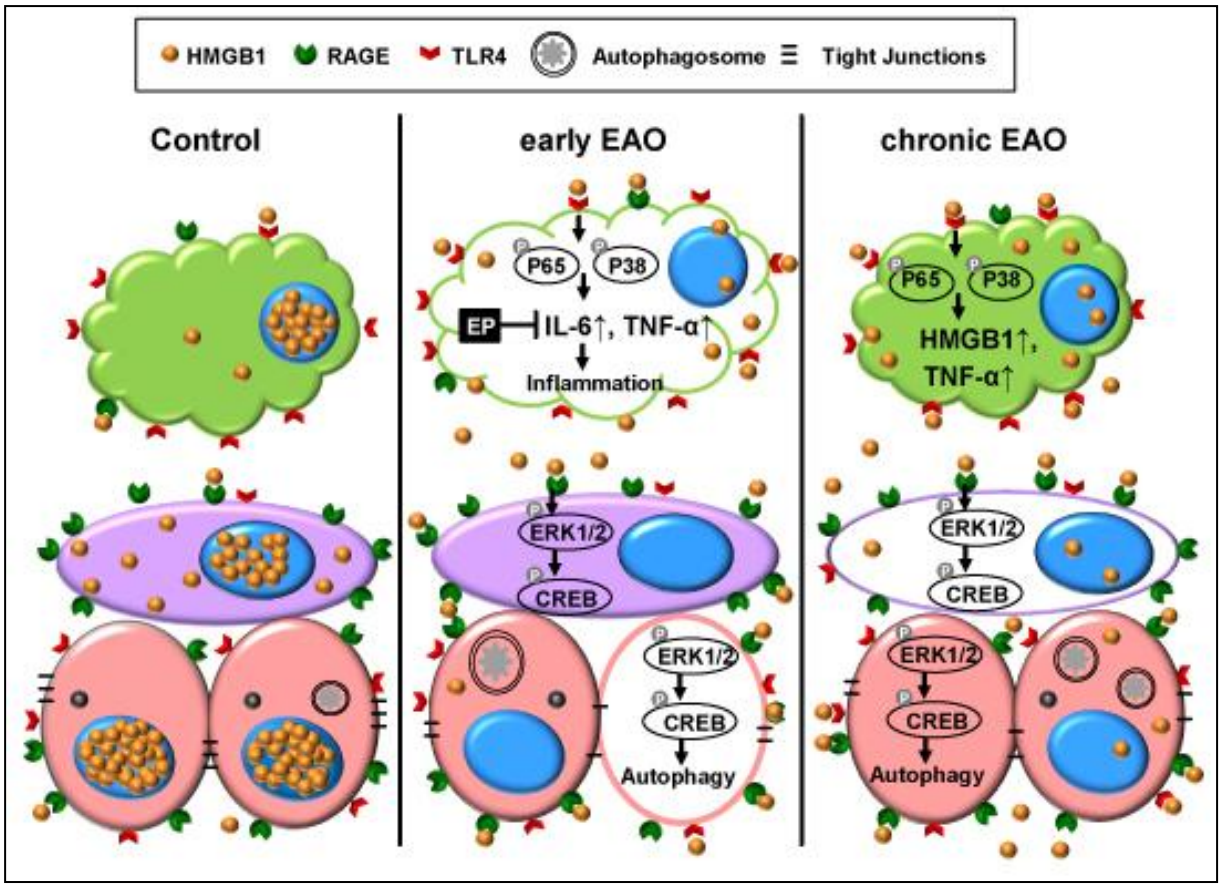


Figure 24. Hypothetical model how HMGB1 mediates immune responses in EAO testis in a cell-specific manner. In normal rat testis, HMGB1 is localized in the nucleus of testicular macrophages (green), peritubular cells (violet) and Sertoli cells (pink). In early stages of EAO development increased numbers of newly arrived macrophages in the testis coincides with upregulation of IL-6 and TNF-α in EAO50 and HMGB1 nuclear translocation in the cytoplasm and extracellular space. However, HMGB1 levels in the testis are increased at a later time point in the chronic phase of the disease. Extracellular HMGB1 binds differentially to RAGE (green round shapes) receptor in peritubular cells and Sertoli cells and to TLR4 (red pointed shaped) in testicular macrophages and induces ERK1/2, CREB or p38, p65 activation, respectively. Testicular macrophages fuel the ongoing inflammation. However, peritubular cells and Sertoli cells choose to elicit a more tolerogenic signal. Specifically Sertoli cells, although damaged under stress accompanied by opening of the tight junctions in some parts, survive the inflammatory challenge of EAO testis. The reason may be that HMGB1 induces activation of pro-survival pathways such as elevated autophagy levels in Sertoli cells.

3. APPENDIX

Appendix 1

Buffers and solutions for cell culture

PBS A
1.5mg/ml D-glucose
1% penicillin/streptomycin (100x)

Sertoli cell isolation medium
1% penicillin/streptomycin in RPMI 1640

Peritubular cell isolation medium
1% penicillin/streptomycin
10% Fetal Calf Serum (FCS) in RPMI 1640

Testicular macrophage isolation medium
1% penicillin/streptomycin in DMEM:F12 medium

Trypsin-DNase solution
2.5mg/ml trypsin
30µg/ml DNase I

Trypsin inhibitor A
10mg/ml Trypsin Inhibitor
Trypsin inhibitor B
2.5mg/ml Trypsin Inhibitor

Collagenase- Hyaluronidase- DNase solution
1mg/ml Collagenase
1mg/ml Hyaluronidase
30µg/ml DNase

Hyaluronidase- DNase solution
1mg/ml Hyaloronidase
30µg/ml DNase

Buffers and solutions for bacterial experiments

LB medium (pH 7.4)
1% (w/v) bactotryptophan
0.5% (w/v) yeast extract
1M NaCl

LB-Agar

1.5% bactoagar in LB medium

2YT medium (pH 7.0)

2% (w/v) bactotryptophan
1% (w/v) yeast extract
100mM NaCl

SOB medium

2% (w/v) bactotryptophan
0.5% (w/v) yeast extract
10mM NaCl
2.5mM KCl

TFB buffer (pH 6.2)

10mM 2-*(*N-morpholino) ethanesulfonic acid (MES)
45mM $MnCl_2$
10mM $CaCl_2$
100mM KCl

Buffers and solutions for biochemical experiments

PBS (10x) (1L, pH 7.4) (Phosphate Buffered Saline)

80g	NaCl
2.0g	KC
14.4g	Na_2PO_4
2.4g	KH_2PO_4

TBS (0.1% Tween) (10x) (1L, pH 7.6) (Tris Buffered Saline)

24.23g	Tris-HCl
80.06g	NaCl
10ml	Tween 20

HEPES buffer (pH 7.5)

700ml	dH_2O
238.30g	HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

Dialysis buffer

25mM	Tris-HCL (pH 8.0)
150mM	KCl
10%	Glycerol

RIPA buffer (Radio Immuno Precipitation Assay buffer)

150mM	NaCl
1.0%	NP-40
0.5%	sodium deoxycholate
0.1%	SDS (sodium dodecyl sulphate)
50mM	Tris-HCl pH 8.0
1%	Protease inhibitor cocktail

Laemmli 2x buffer (pH 6.8)

4%	SDS
10%	2-Mercaptoethanol
20%	glycerol
0.004%	bromophenol blue
0.125M	Tris-HCl

Running buffer (pH 8.3)

25mM	Tris base
190mM	glycine
0.1%	SDS

Transfer buffer (semi-dry)

48mM	Tris
39mM	glycine
20%	methanol
0.04%	SDS

Blocking buffer

5% milk or BSA (Bovine Serum Albumin) in TBST

Home made ECL solution

Solution A

25mg/ml Luminol in TRIS-HCl(pH 8.6)(0.1M)

solution B

1.1mg/ml para-Hydroxycoumarinacid in DMSO

0.3µl/ml H_2O_2 (35%)

Paraformaldehyde (4%)

4% Paraformaldehyde in PBS (1x), heat up to 60°C, add 1N NaOH dropwise until the solution is clear.

TEM fixation buffer

1.5% glutaraldehyde

1.5% paraformaldehyde in 0.15M HEPES buffer

Sodium Citrate buffer (pH 6.0)

10mM Sodium citrate (Tri-sodium citrate (dihydrate))

0.05% Tween 20

Tris-EDTA buffer (pH 9.0)

10mM Tris base

1mM EDTA

0.05% Tween 20

Coomassie Brilliant Blue staining solution

0.02% CBB G250

5% aluminum sulfate-(14-18)-hydrate

10% ethanol

2% ortho-phosphoric acid

Coomassie Brilliant Blue destaining solution

10% ethanol

2% ortho-phosphoric acid

Ponceau S staining solution

2% Ponceau S

30% trichloroacetic acid

30% sulfosalicylic acid

1x TAE buffer

40mM Tris-acetate

1mM EDTA (pH8.0)

6x Gel Loading buffer

0.25%(w/v)Bromophenol blue
30% Glycerol

Reverse transcription and PCR buffers

Real-time PCR master mix

1x quantitect SYBR green PCR kit
10pM forward primer
10pM reverse primer
(1x quantitect primer assay)
20nM fluorscein

Appendix 2

Polyacrylamide SDS gel

Stacking gel	**4%**	**Separating gel**	**10%**	**12.5%**	**17%**
DDW	3 ml	DDW	4.01ml	3.17 ml	1.672 ml
0.5 M Tris-HCl pH 6.8	1.25 ml	1.5 M Tris-HCl pH 8.8	2.5 ml	2.5 ml	2.5 ml
10% (w/v) SDS	50 µl	10% (w/v) SDS	100 µl	100 µl	100 µl
Acrylamide	650 µl	Acrylamide	3.34 ml	4.17 ml	5.678 ml
10% (w/v) APS	25 µl	10% (w/v) APS	50 µl	50 µl	50 µl
TEMED	5 µl	TEMED	5 µl	5 µl	5 µl
Total volume	5 ml	Total volume	10 ml	10 ml	10 ml

Appendix 3

Antibodies

Primary antibodies	**Manufacturer**	**Catalogue No.**	**Dilution** (**IF;*WB)
Monoclonal rabbit anti rat Phospho-Akt (Ser473) (D9E) XP®	Cell signaling, Germany	4060	1:1000**
Polyclonal rabbit anti rat Akt1/2/3 (H-136)	Santacruz, USA	8312	1:1000*
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*
Polyclonal rabbit anti rat Toll-like Receptor 4 Ab (Rodent Specific)	Cell signaling, Germany	2219	1:1000* 1:50**
Polyclonal rabbit anti rat LC3B (D11) XP®	Cell signaling, Germany	3868	1:500*
Polyclonal rabbit anti rat Beclin 1	Cell signaling, Germany	3738	1:1000* 1:150**
Polyclonal rabbit anti rat SQSTM1 (p62) (D-3)	Santacruz, USA	28359	1:500*
Polyclonal rabbit anti rat RAGE	Thermo Scientific, USA	PA1-075	1:1000* 1:100**
Polyclonal rabbit anti rat Vimentin (c-20)	Santacruz, USA	L4793.AB45	1:100**
Monoclonal mouse anti rat Actin smooth muscle [1A4] antibody (FITC)	GeneTex,, USA	GTX72531	1:100**
Polyclonal rabbit anti rat HMGB1	Abcam, UK	18256	1:1000* 1:200**
Monoclonal mouse anti rat CD68 (ED1)	AbD Serotec, Germany	MCA341R	1:100**
Monoclonal mouse anti rat CD163 (ED2)	AbD Serotec, Germany	MCA342R	1:100**

Secondary antibodies	**Manufacturer**	**Catalogue No.**	**Dilution (****IF; *WB)
Donkey anti mouse IgG-Cy3	Dianova, Hamburg, Germany	715-165-151	1:1000**
Donkey anti rabbit IgG-Cy3	Chemicon, Hampshire, UK	AP182C	1:1000**
Donkey anti goat IgG-HRP	Santa Cruz, California, USA	SC-2020	1:1000*
Goat anti rabbit IgG-HRP	ICN, Ohio, USA	55676	1:10000*
Sheep Anti mouse-HRP	Sigma-Aldrich, Steinheim, Germany	A5906	1:10000*

Primer sequences

Gene	**Primer Sequences (5´→ 3´)**	**PubMed Accession No.**	**Product length (bp)**
β-actin	F: ATGGTGGGTATGGGTCAGAA R: GGGTCATCTTTTCACGGTTG	NM_0031144.2	232
beta-2 microglobulin (β2M)	F: CCGTGATCTTTCTGGTGCTT R: AAGTTGGGCTTCCCATTCTC	NM_012512	113
TNF-α	F: GCCTCTTCTCATTCCTGCTC R: CCCATTTGGGAACTTCTCCT	NM_012675.3	101
IL-6	Quantitect Primer Assay Rn_Il6_1_SG (Qiagen)	NM_012589	128

4. SUMMARY

Despite its immune privileged status, the testis is prone to inflammatory infertility. However, sterile inflammation of the testis (autoimmune orchitis) is neglected as a cause of male factor infertility due to lack of non-invasive diagnostic tools. Accumulating data shows that high mobility group box protein 1 (HMGB1), a non-histone chromosomal protein, plays an important role in onset and chronification of autoimmune diseases once released from the nuclei. In this study, we analyzed how HMGB1 can regulate inflammatory reactions *in vivo* using a rat model of experimental autoimmune orchitis (EAO) and *in vitro* in primary testicular cells. HMGB1 was translocated from the nuclei in EAO testis and in the testis of infertile men with leukocytic infiltrates. Interestingly, HMGB1 levels in EAO testis were elevated at the late chronic phase of disease as compared to early proinflammatory cytokines such as IL-6 and TNF-α. We found that testicular somatic cells show a cell-specific expression profile of HMGB1 receptors TLR4 and Receptor for Advanced Glycation End products (RAGE). The highly sensitive and specific proximity ligation assay was used to analyze HMGB1 receptor binding in testicular cells. HMGB1-TLR4 binding was dominant in testicular macrophages. However, Sertoli and peritubular cells showed higher levels of HMGB1-RAGE interaction. In support, HMGB1 triggered RAGE-dependent ERK1/2 MAPK and CREB activation in Sertoli and peritubular cells, whilst in testicular macrophages HMGB1 induced TLR4-signaling as evidenced by p38 MAPK and p65 NF-κB phosphorylation which stimulated an increase in mRNA levels of TNF-α and IL-6. Recent studies showed that RAGE induced ERK activation leads to enhanced autophagy levels. In line with these data, in our study extracellular HMGB1 triggered formation of autophagosomes in Sertoli cells. Increased autophagy levels in isolated Sertoli cells may explain how these cells survive the inflammatory environment in EAO testis. Considering HMGB1's late phase of action, inhibition of HMGB1 may be a putative target for therapeutic intervention in treatment of chronic testicular inflammation.

5. ZUSAMMENFASSUNG

Der Hoden ist ein immun-privilegiertes Organ, dessen Immunstatus Toleranz gegenüber den Neoantigenen der Keimzellen gewährt. Paradoxerweise sind sterile Entzündungen relativ häufig in testikulären Biopsien von Männern zur Abklärung einer Infertilität zu finden. Mangelnde Kenntnis der Pathomechanismen, der asymptomatische Verlauf und ein Fehlen geeigneter Markern erlauben jedoch bisher keine kausale Therapie.

Zahlreiche Arbeiten zeigen, dass das chromosomale Nicht-Histon-Protein High Mobility Group Box Protein 1 (HMGB1) eine wichtige Rolle bei der Entstehung und Chronifizierung von Autoimmunerkrankungen und sterilen Entzündungen spielt, sobald es aus dem Zellkern freigesetzt/transloziert wird. In dieser Studie wurde untersucht, wie das HMGB1 Protein entzündliche Reaktionen in einem *in vivo* Rattenmodell der experimentellen Autoimmun-Orchitis (EAO) und *in vitro* in isolierten testikulären Zellen regulieren kann. Im Hoden von EAO Tieren und unfruchtbaren Männern mit leukozytären Infiltraten wurde eine HMGB1 Translokation aus den Zellkernen beobachtet. Interessanterweise war der HMGB1 Level in EAO Hoden, im Gegensatz zu den proinflammatorischen Zytokinen wie IL-6 und TNF-α, erst in der späten chronischen Phase der Erkrankung erhöht. Zudem konnte gezeigt werden, dass die HMGB1-Rezeptoren TLR4 und RAGE (Receptor of Advanced Glycation End Products) in verschiedenen Typen testikulärer somatischer Zellen unterschiedlich exprimiert werden. Mit Hilfe des Proximity Ligationsassays wurde nachgewiesen, dass die HMGB1-TLR4 Bindung in testikulären Makrophagen signifikant höher ist als in Sertoli und peritubulären Zellen, die im Gegensatz dazu eine höhere HMGB1-RAGE Interaktion zeigten. Entsprechend löst HMGB1 eine RAGE-abhängige ERK1/2 MAPK- und CREB-Aktivierung in Sertoli und peritubulären Zellen aus, während es in testikulären Makrophagen den TLR4-Signalweg induziert, der durch eine Phosphorylierung von p38 MAPK und p65 NF-κB und folgender Induktion der mRNA Expression von TNF-α und IL-6 charakterisiert ist.

Neueste Daten zeigen, dass eine RAGE induzierte ERK Aktivierung Autophagie induzieren kann. In Übereinstimmung mit früheren Forschungsergebnissen konnte in dieser Arbeit gezeigt werden, dass extrazelluläres HMGB1 zur verstärkten Bildung von Autophagosomen in Sertoli-Zellen führt. Erhöhte Autophagie in Sertoli-Zellen könnte erklären, wie diese Zellen den Entzündungsprozess in EAO Hoden überleben. In Anbetracht, dass HMGB1 in der späten Phase der Erkrankung agiert, könnte die Hemmung von HMGB1 ein mögliches Ziel für die therapeutische Intervention bei der Behandlung von chronischer Hodenentzündung darstellen.

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Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

9. OWN PUBLICATIONS

a. Publication originally from this thesis

- **Aslani F**, Schuppe H-C, Bhushan S, Wahle E, Meinhardt A, Fijak M. High mobility group box protein-1 is involved in modulation of cell specific immune responses during chronic testicular inflammation in rat. (Manuscript in preparation)

b. Other publications

- **Aslani F**, Modaresi MH, Soltanghoraee H, Akhondi MA, Shabani A, Lakpour N, Sadeghi MR. Seminal molecular markers as a non-invasive diagnostic tool for the evaluation of spermatogenesis in non-obstructive azoospermia. *Syst Biol Reprod Med;*57(4):190-6, Aug 2011.
- **Aslani F**, Akhondi MA, Modaresi MH, Shabani A, Arabi M, Sadeghi MR. Evaluating the Presence of Testis-specific Transcripts in Mature Human Spermatozoa. *Medical Journal of Reproduction & Infertility;* Vol.8, Issue3, 2007 [Article in Persian].
- Ardekani MA, **Aslani F**, Lakpour N. Application of Genomics and Proteomics Technologies to Early Diagnosis of Reproductive Organ Cancers. *Medical Journal of Reproduction & Infertility;* Vol.8, Issue3, 2007 [Article in Persian].

c. Conference Abstracts

- 7th European Congress of Andrology (ECA); Berlin, Germany. (28 Nov.- 1 Dec. 2012) [Oral Presentation]
- The 10th Joint Annual Meeting of the International Cytokine Society (ICS) and the International Society for Interferon and Cytokine Research (ISICR); Geneva, Switzerland.(11-14 Sep. 2012) [Poster Presentation]
- Joint International Congress of the American Society for Reproductive Immunology (ASRI) and the European Society for Reproductive Immunology (ESRI); Hamburg, Germany. (31 May- 2 Jun. 2012) [Poster Presentation]
- 4th meeting of International Network of Young Researchers in Male Fertility; Edinburgh, UK. (29 Sep.- 1 Oct. 2011) [Poster Presentation]
- 4th GGL annual meeting; Giessen, Germany. (21-22 Sep. 2011) [Oral Presentation]
- 9th Iranian Congress of Biochemistry and Molecular Biology; Shiraz, Iran. (Nov. 2007) [Poster Presentation]
- 14th Middle East Fertility Society annual meeting; Antalya, Turkey. (31 Oct.- 3 Nov. 2007) [Oral Presentation]
- 7th International Congress on Obstetrics and Gynecology; Teheran, Iran. (9-13 Nov. 2007) [Oral Presentation]

10. EHRENWÖRTLICHE ERKLÄRUNG

Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Giessen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Giessen, den

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