Galectin-1 enhances TNFα-induced inflammatory responses in Sertoli cells through activation of MAPK signaling

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ABBREVIATIONS

ANOVA	Analysis of variance
AP-1	Activator protein 1
APS	Ammonium persulfate
BSA	Bovine serum albumin
BTB	Blood-testis barrier
bp	Base pair
BV	Blood vessels
cAMP	Cyclic adenosine monophosphate
°C	Degree Celsius
cDNA	Complementary DNA
CD	Cluster of Differentiation
CRD	Carbohydrate-recognition domain
COX-2	Cyclooxygenases-2
CSF1	Colony-stimulating factor 1
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
DAMPs	Danger associated molecular patterns
DC	Dendritic cells
DAPI	4', 6'-diamino-2-phenylindole
dH_2O	Deionized H_2O
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	deoxy-ribonucleoside triphosphate
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EAO	Experimental autoimmune orchitis
EB	Ethidium bromide
ECL	Enhanced chemiluminescence

EDTA	Ethylene diamine tetraacetic acid
EEA1	Early endosome antigen 1
FAS	The first apoptosis signal
FCS	Fetal calf serum
Foxp3	Forkhead box protein P3
FSC	Forward scatter
Gal-1	Galectin-1
Gcnt1	Core 2 glucosaminyl (N-acetyl) transferase 1
GDNF	Glial cell line-derived neurotrophic factor
H	Hour(s)
HMGB1	High mobility group box 1
Hprt	Hypoxanthine guanine phosphoribosyl transferase
H-Ras	Harvey rat sarcoma viral oncogene homolog
HRP	Horseradish peroxidase
IFN-γ	Interferon-γ
IDO	Indolamin-2, 3-dioxygenase
IgG	Immunoglobulin G
IL-1α	Interleukin 1α
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-10	Interleukin 10
iNOS	Inducible nitric oxide synthase
JAM-A	Junctional adhesion molecule-A
JNK	c-Jun N-terminal kinase
kDa	Kilo Dalton
LacNAc	N-acetyllactosamine
LC	Leydig cells
LH	Luteinizing hormone
L-PHA	phytohemagglutinin-L
LPS	Lipopolysaccharide

M	Molar
mA	Milliampere
MAA	Maackia amurensis lectin
MAPK	Mitogen activated protein kinase
MC	Mast cells
MCP-1	Monocyte chemoattractant protein-1
mg	Milligram
Mgat5	Mannosyl (α-1,3-)-glycoprotein ß-1,2-N acetylglucosaminyltransferases
MHC	Major Histocompatibility Complex
min	Minute
ml	Milliliter
mM	Milimolar
MW	Molecular weight
NaCl	Sodium chloride
NF-κB	Nuclear factor-Kb
NO	Nitric oxide
NP-40	Nonidet P-40
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PNA	Peanut agglutinin lectin
PMSF	Phenylmethylsulfonyl fluoride
P/S	Penicillin/Streptomycin
RA	Retinoic acid
RE	Relative expression
RIPA	Radioimmunoprecipitation assay

rMFI	Relative median fluorescence intensity
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodiumdodecylsulphate
Sec	Second
SEM	The standard error of the mean
SNA	Sambucus nigra lectin
SOX9	SRY-box 9
SRY	Sex-determining region of Y-chromosome gene
SSC	Side scatter
St6gal1	ST6 beta-galactoside α-2-6-sialyltransferase 1
StAR	Steroidogenic acute regulatory protein
TAE	Tris acetate EDTA
TBS	Tris buffered saline
TEMED	N, N, N`, N`-Tetramethylethylenediamine
TGFβ1	Transforming growth factor β1
TGFβ2	Transforming growth factor β2
Th1	T helper cells type 1
Th17	T helper cells type 17
TH	Testicular homogenate
TLR	Toll like receptor
TNFα	Tumor necrosis factor alpha
Treg	T regulatory cells
Tris	Tris (hydroxymethyl) amino methane
UV	Ultraviolet
µ	Micro
µg	Microgram

µl	Microliter
µM	Micromolar
V	Volt
VEGF	Vascular endothelial growth factor
v/v	Volume per volume
WHO	World Health Organization
w/v	Weight per volume

1. INTRODUCTION

1.1. Male reproductive system

In mammals the male reproductive system is composed of several different organs, namely the gonads (testis), epididymis, ductus deferens, prostate, and the penis (Meniru, 2001). Spermatozoa produced in the testes are transported to the epididymis through the rete testis and the efferent ducts, where they mature and acquire their capacity for motility to reach and fertilize the oocyte. The sperm maturation is also influenced during ejaculation by the secretions of the accessory sex glands (seminal vesicles, prostate and bulbourethral glands) (Knorr *et al.*, 1970).

1.2. Structure and function of the testis

The testes development from the sex cord is regulated by the sex-determining region of Y-chromosome gene (SRY) (Koopman, 1995). SRY and its downstream target SRY-box 9 (Sox9) induce the differentiation of somatic cell precursors to Sertoli cells, a critical step in testis morphogenesis (Morais da Silva *et al.*, 1996). The mammalian testis is constituted of two compartments: the seminiferous tubules and the interstitial space, which are separated by the basal lamina produced by the peritubular and Sertoli cells (Elftman, 1963; Fijak and Meinhardt, 2006; Li *et al.*, 2012; Zhao *et al.*, 2014). The two primary functions of the testis are the formation of sperms (spermatogenesis) taking place in the seminiferous epithelium and the production and secretion of the male steroid hormones (steroidogenesis) mainly synthesized by the Leydig cells in the interstitial space (Figure 1) (Zhao et al., 2014).

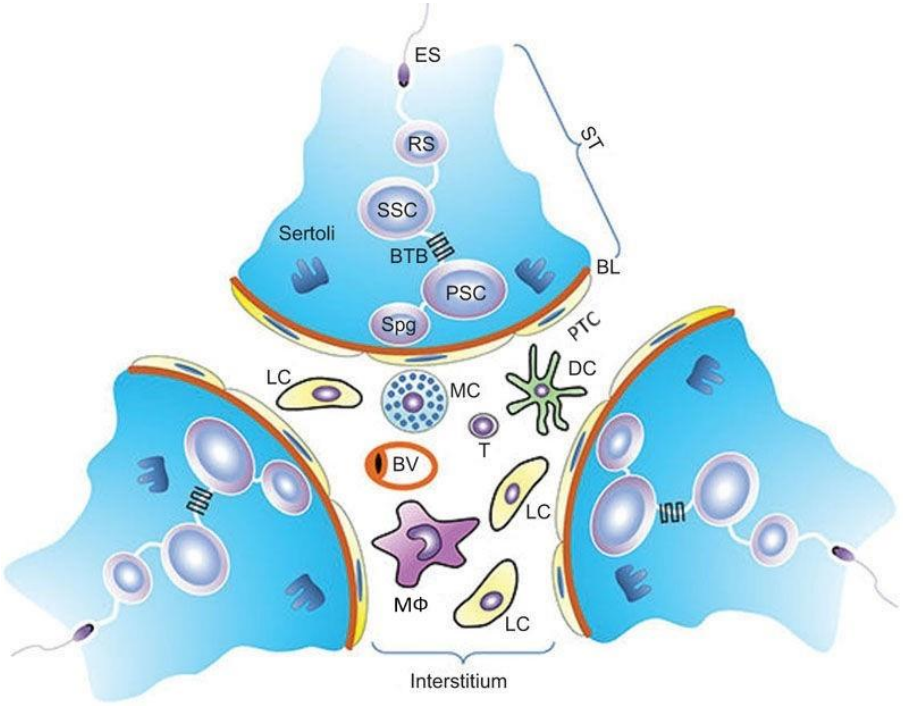

Figure 1. Scheme illustrating the structure of the testis. The mammalian testis is constituted of two compartments: the seminiferous tubules (ST) and the interstitial space (interstitium), which are separated by the basal lamina (BL) produced by the Sertoli and peritubular cells (PTC). In the seminiferous tubules, different stages of developing germ cells (spermatogonia (Spg), primary spermatocytes (PSC), secondary spermatocytes (SSC), round spermatids (RS), and elongated spermatids (ES)) are intimately associated with Sertoli cells. The tight junctions between neighboring Sertoli cells form the blood-testis barrier (BTB), which separates spermatogonia from most spermatocytes and all spermatids. Leydig cells (LC) are the major population of cells in the interstitial space. The interstitial space also contains various types of immune cells such as testicular macrophages (MΦ), dendritic cells (DC), few mast cells (MC), and blood vessels (BV). (Zhao *et al.*, 2014; with the permission of the publisher, license number: 4303600412665).

During spermatogenesis, spermatogonia undergo mitosis and form primary spermatocytes (Figure 1) (Zhao *et al.*, 2014). The primary spermatocyte enter meiosis and divide into two secondary spermatocytes (Meiosis I) (Bustos-Obregon *et al.*, 1975).

Then each secondary spermatocyte divides into two haploid spermatids (Meiosis II), which transform into spermatozoa (Bustos-Obregon *et al.*, 1975). The later process, called spermiogenesis, is characterized by the removal of spermatid cytoplasm, formation of the acrosome, condensation of DNA in the sperm head, and tail establishment (Bustos-Obregon *et al.*, 1975). Except germ cells, the seminiferous tubules also contain Sertoli cells surrounded by one (e.g. rats and mice) or more (e.g. humans) layers of peritubular cells (Maekawa *et al.*, 1996; Yang *et al.*, 2015). The Sertoli cells extend from the basal membrane to the lumen of the tubules and support the development of the different stages of germ cells (Figure 1) by providing essential nutrients and growth factors (Griswold, 1995; Fijak and Meinhardt, 2006). Sertoli cells also play a key role in the regulation of spermatogenesis and maintenance of testicular immune privilege primarily by secreting anti-inflammatory factors (e.g. galectin-1 (Gal-1), transforming growth factor β1 (TGFβ1) and indoleamine 2, 3-dioxygenase (IDO)) in addition to forming the BTB (Fijak and Meinhardt, 2006; Zhao *et al.*, 2014; França *et al.*, 2016; Gao *et al.*, 2016). Peritubular cells possess properties of smooth muscle like cells and surround the seminiferous tubules. They are responsible for tubular contractility and the transportation of immotile spermatozoa to the epididymis. Moreover, they are involved in the maintenance of the spermatogonial stem cell niche by secreting glial cell line-derived neurotrophic factor (GDNF) (Albrecht, 2009; Mayerhofer, 2013; Chen *et al.*, 2014).

In the interstitial space of the testis reside the Leydig cells and immune cells such as macrophages, dendritic cells, and mast cells (Figure 1) (Zhao *et al.*, 2014). In the Leydig cells steroidogenesis takes place, a process that is converting cholesterol to biologically active steroid hormones involving mitochondria and the smooth endoplasmic reticulum (Tremblay, 2015). In the testis, testosterone is the most important steroid hormone, which plays an important role in the formation of the male phenotype, sexual behavior and maintenance of spermatogenesis (Wilson, 1999). Steroidogenesis in Leydig cells is largely regulated by luteinizing hormone (LH) through affecting expression of steroidogenic acute regulatory protein (StAR) (Haider, 2007).

1.3. Immune privilege of the testis

The testis is an immune privileged organ as defined by the capacity to tolerate not only new testicular autoantigens but also allo- and xenografts experimentally transplanted into the testis environment. After the establishment of the immune system during puberty male germ cells enter meiosis and transform into specialized spermatozoa. During this process, a myriad of new autoantigens is produced, which has to be tolerated by the immune system (Fijak and Meinhardt, 2006). The immune privileged status of the testis has also been appreciated by studies showing that allo- and xenografts transplanted into the interstitial space of the rat testis could survive long term (Head *et al.*, 1983; Setchell, 1990). The tissues transplanted include skin, pancreatic islets, and parathyroid fragments (Ferguson and Scothorne, 1977; Whitmore and Gittes, 1977; Head *et al.*, 1983; Mital *et al.*, 2010). More interestingly, the transplantation of spermatogonia into the seminiferous tubules of infertile recipient mice could reestablish spermatogenesis and produce mature spermatozoa, even across species barriers in some cases (Brinster and Zimmermann, 1994; Dobrinski, 2008). These studies illustrate that the environment of the testis could protect allo- and xenografts from immune attack and define the testis as an immune privileged site. Therefore, several mechanisms that include the BTB, somatic and immune cells and hormones (testosterone) are involved in the maintenance of the testicular immune privilege (Figure 2) (Zhao *et al.*, 2014).

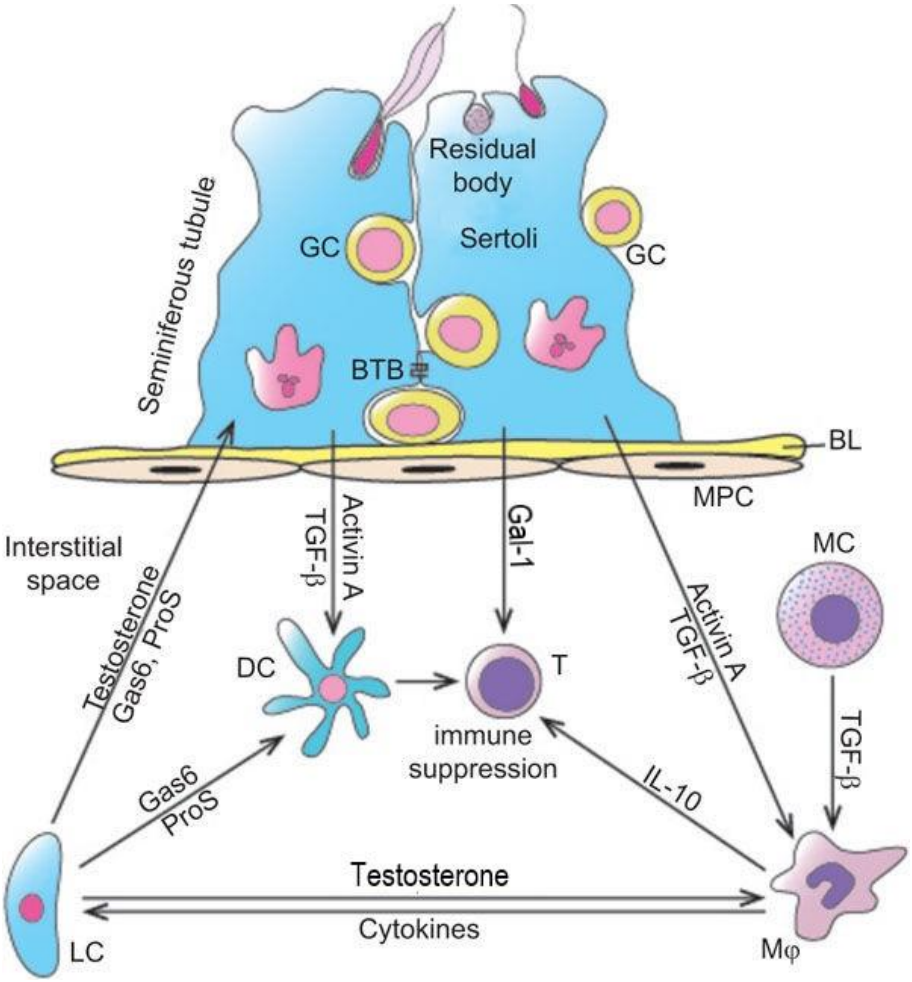

Figure 2. Schematic of the BTB, somatic and immune cells and molecules that support immune privilege in the testis. Sertoli and Leydig cells secrete multiple immunosuppressive factors (activin A, Gal-1, PDL-1, TGF-β, ProS and testosterone) which suppress of immune cell activation. Macrophages exhibit immunosuppressive properties by producing anti-inflammatory cytokines such as IL-10 and TGF-β. Gas6, growth arrest-specific gene 6; LC, Leydig cell; MC, mast cell; Mϕ, testicular macrophages; PDL-1, programmed death ligand-1. (Zhao *et al.*, 2014; with the permission of the publisher, license number: 4350711055905).

1.3.1. Blood – testis barrier

The BTB is the physical separation of newly developing germ cells from the immune system. It is established by tight junctions between Sertoli cells (Figure 2) (Zhao *et al.*,

2014). The BTB is made up of various integral membrane proteins including junctional adhesion molecules, claudin 1 and claudin 11. It divides the seminiferous epithelium into two different compartments: (a) the basal compartment containing the spermatogonia, pre-leptotene, and leptotene spermatocytes and (b) the abluminal compartment carrying zygotene, pachytene and secondary spermatocytes, haploid spermatids, and spermatozoa (Mital *et al.*, 2011). This barrier can limit the access of immune cells to autoantigens expressed in meiotic and haploid germ cells (Mital *et al.*, 2011). During infection, inflammation, and trauma, the integrity of the BTB is impaired, which is often association with germ cell loss (Dulin *et al.*, 2011; Meinhardt and Hedger, 2011). In the rete testis there is no BTB, so spermatozoa and immune cells could get into contact. This latter factor highlights the view that a physical barrier is not sufficient and for protective function further means by the testicular somatic and immune cells as well as hormones (testosterone) are needed to provide an immune privileged environment in the testis.

1.3.2. Role of somatic cells in the maintenance of testicular immune privilege

Most evidence for a role of Sertoli cells in immunosuppression is based on co-transplantation studies showing that Sertoli cells co-transplanted with pheochromocytes (neuroendocrine cells) or liver cells were creating an immune privileged environment (Mital *et al.*, 2010). In an autoimmune type 1 diabetes model, syngeneic pancreatic islets are rejected by autoimmune T cells and autoantibodies, while co-transplantation of Sertoli cells with allogeneic pancreatic islets can significantly prolong their survival (Suarez-Pinzon *et al.*, 2000). Gao *et al*. reported that Sertoli cells could secrete Gal-1, which is able to promote differentiation of tolerogenic dendritic cells and regulatory T cells (Treg cells) (Gao *et al.*, 2016). More recently, Tung *et al*. found that non-sequestered meiotic germ cell antigens are phagocytosed in the apical part of Sertoli cells and egress basally through intracellular transport which triggers a Treg cell-

dependent systemic tolerance (Tung *et al.*, 2017). Under stress conditions such as infection or inflammation, Sertoli cells can express IL-6, tumor necrosis factor alpha (TNFα), IL-1β, and Fas ligand, which are involved in the disruption of spermatogenesis and induction of germ cell apoptosis (O'Bryan and Hedger, 2008). Peritubular cells are also suggested to play a role in the maintenance of the testicular immune privilege (Schuppe and Meinhardt, 2005). Peritubular cells produce TGFβ, a cytokine which displays immunosuppressive and anti-inflammatory activities (Skinner and Moses, 1989). Under inflammatory conditions, they also express immune mediators, such as IL-6, cyclooxygenases-2 (COX-2), activin A and Toll-like receptors (de Winter *et al.*, 1994; Schell *et al.*, 2008; Mayerhofer, 2013; Mayer *et al.*, 2016). Nonetheless, the role of Sertoli and peritubular cells in the maintenance of immune privilege or inflammatory responses in the testis remains to be further elucidated.

1.3.3. Involvement of testicular immune cells in the formation of immune privilege

Testicular immune cells including testicular macrophages, dendritic cells, and T cells, all located in the interstitial space, are also involved in the maintenance of testicular immune privilege through several mechanisms (e.g. suppression of antigen-specific immunity and T cell activation, macrophage polarization).

1.3.3.1. Testicular macrophages and dendritic cells

Testicular macrophages are majorly involved in the regulation of spermatogenesis and steroidogenesis, the immune response to inflammation and infection, and the maintenance of testicular immune privilege. Testicular macrophages are residing in the interstitial space and comprising the largest population of immune cells (about 20–25% of the interstitial cells) in rodents (Niemi *et al.*, 1986; Itoh *et al.*, 1995). The majority

(~80%) of testicular macrophages possess an anti-inflammatory/immunoregulatory M2 phenotype, as defined by the expression of the scavenger receptor CD163 as well as the production of the anti-inflammatory cytokine IL-10 (Wang *et al.*, 2017).

Under normal or inflammatory conditions testicular macrophages express relatively low levels of pro-inflammatory cytokines (TNFα and IL-6) compared to macrophages from other organs, a means accompanied by the high expression of anti-inflammatory factors (IL-10) (Bhushan *et al.*, 2015). Mechanistically, expression of TLR pathway-specific genes such as CD14 and MyD88 associated with TNFα and IL-6 production is also relatively low in testicular macrophages (Bhushan *et al.*, 2015). However, testicular macrophages still preserve some capacity for innate immune responses by the activation of AP-1 and MAP kinase signaling pathways after LPS stimulation (Bhushan *et al.*, 2015). These contradictory features enable testicular macrophages to maintain the immune privilege of the testis under normal conditions, while protecting testicular cells from pathogens by the balanced pro-inflammatory activity. Moreover, testicular macrophages express several molecules involved in spermatogonial proliferation and differentiation, such as colony stimulating factor 1 (CSF1) and enzymes required for retinoic acid (RA) biosynthesis. In agreement, the transient depletion of macrophages in the adult mouse testis results in a reduction of spermatogonial numbers (DeFalco *et al.*, 2015). Under pathological conditions such as infection and/or sterile inflammation (orchitis), the number of M1 macrophages dramatically increases and disrupts the balance of M1/M2 macrophages. This is thought to a disturbance of the testicular immune privilege, impairment of the BTB and sloughing of germ cells, causing infertility (Rival *et al.*, 2008; Theas *et al.*, 2008; Fijak *et al.*, 2018).

Dendritic cells as professional antigen presenting cells are also present in the rodent testis under physiological conditions (Wang and Duan, 2016). Their maturation state is regarded as a key point in the induction of peripheral immune tolerance or contrary in the development of autoimmunity. Under normal conditions, testicular dendritic cells are immature and functionally tolerogenic characterized by the differential expression of

antigen presenting molecules (major histocompatibility complex class II (MHC class II)), co-stimulatory molecules (CD80, CD86), chemokine receptors (CCR2, CCR7) and cytokines (IL-10, IL-12, TNFα) (Rival *et al.*, 2007).

1.3.3.2. T cells

Besides testicular macrophages and dendritic cells, T cells are also involved in the maintenance of immune privilege in the testis. T cells such as suppressor CD8+ cells, NK T cells and Treg cells are rarely found in the rat, mouse and human testis (Mukasa *et al.*, 1995; Tompkins *et al.*, 1998; Schuppe *et al.*, 2008; Jacobo *et al.*, 2009; Duan *et al.*, 2011). Their function involves the modulation of immune responses by increasing cellular immune surveillance and inhibiting antigen specific T cell responses in the adult testis (Hedger, 1997; Li *et al.*, 2012). Specifically, the population of immunoregulatory T cells such as CD4+CD25+Foxp3+ T cells and CD8+Foxp3+ T cells are thought to suppress antigen specific T cell responses in the adult rodent testis (Fijak *et al.*, 2011b, 2015; Jacobo *et al.*, 2015; Tung *et al.*, 2017).

1.3.4. Testosterone has immunosuppressive functions in testicular immune privilege

An increasing body of evidence indicates immunosuppressive features for testosterone. Evidence derived from clinical and epidemiological studies revealed that women are more susceptible to autoimmune diseases compared to men, to which the immunosuppressive properties of androgens are thought to contribute (Cutolo *et al.*, 2004). Testosterone supplementation exerts an immunomodulatory and protective effect during development of experimental autoimmune diseases such as experimental autoimmune orchitis and adjuvant-induced arthritis (Harbuz *et al.*, 1995; Fijak *et al.*, 2011b). In this regard, testosterone can polarize blood monocytes towards M2 phenotype

macrophages mediated by elevated expression and autocrine action possibly of the anti-inflammatory cytokine IL-10 (Wang *et al.*, 2017). Moreover, testosterone can also promote the differentiation of rat splenic T cells towards Treg cells with immunosuppressive features (Fijak *et al.*, 2015). These data indicate that androgens play an important role in maintaining the balance between autoimmune responses and tolerance. A link between androgens and testicular immune privilege was further strengthened in experiments using conditional knockout of androgen receptors in Sertoli cells. Interestingly, specific deletion of the androgen receptor in Sertoli cells compromises testicular immune privilege, likely caused by disruption of the BTB (Meng *et al.*, 2011). In line with these data, previous studies suggested that androgens can regulate the permeability of the BTB by its effects on the kinetics of endocytosis and the recycling of integral membrane proteins such as occludin, JAM-A, and N-cadherin in Sertoli cells (Meng *et al.*, 2005; Yan *et al.*, 2008). Testicular fluid, which contains almost 10 times higher concentrations of testosterone than serum, displays the immunosuppressive properties, an effect partly abolished by using the anti-androgen flutamide (Wang *et al.*, 2017). In addition, administration of testosterone prior to stimulation with LPS significantly decreases the expression of TNFα in isolated Sertoli or peritubular cells (Fijak *et al.*, 2015). Taken together, there are clear indications that androgens have a critical function in maintaining testicular immune privilege.

1.4. Galectins

In all organisms, galectins are amongst the most broadly expressed types of lectins. A typical characteristic is a shared carbohydrate-recognition domain (CRD), which binds to β-galactose-containing glycoconjugates (Cummings and Liu, 2017). Galectins have a variety of biological functions, that include the regulation of immune cell activity, induction of effector T cell apoptosis, pathogen recognition, and promotion of tumor cell metastasis (Cummings and Liu, 2017).

Galectins are widely expressed in mammals and divergent galectins can be found in a single species (up to 15 in rodent) (Cummings and Liu, 2017). A list of galectins in vertebrate is shown in Figure 3a (Cummings and Liu, 2017). The shared galectin CRD has approximately 130 amino acids, although only eight residues, which have been shown to be related to glycan binding, are almost invariant (Barondes *et al.*, 1994). Part of the conserved sequence motif in galectins is shown in Figure 3b, along with a comparison between several human galectins.

According to the number and organization of their CRD, galectins have been divided into three major groups (Figure 3a), including (a) prototypical galectins (galectin-1, -2, -7, -10, -11, -13, -14, and -15) that contain one CRD. This could form monomers, dimers and oligomers in a concentration-dependent manner. (b) Chimera-type galectins (galectin-3), which have one C-terminal CRD connected with N-terminal conserved amino acids through which it could form oligomers, and (c) tandem-repeat galectins (galectin-4, -6, -8, -9, and -12, which consist of two CRDs connected by a peptide linker (Barondes *et al.*, 1994; Rabinovich and Toscano, 2009; Liu and Rabinovich, 2010). Some galectins display species specific characteristics, for example galectin-5 (prototype) and galectin-6 (tandem repeat) that are expressed in rodents, but not in humans. Galectin-11 (prototype) has only been found in sheep (Cummings and Liu, 2017).

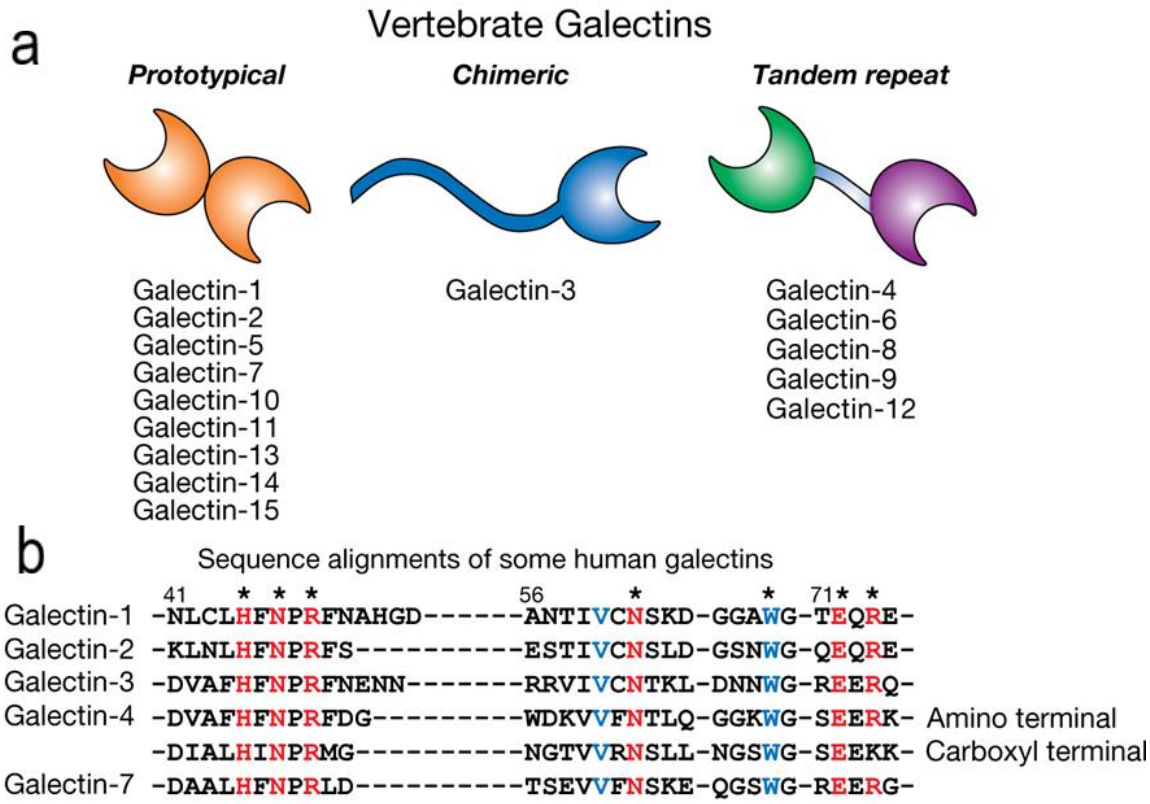

Figure 3. Different types of galectins, their organization and conserved amino acid sequences. (a) Fifteen different vertebrate galectins are classified into three groups: prototypical galectins, chimera-type galectins and tandem repeat galectins. (b) Examples of conserved amino acid sequences of some human galectins are shown. (Cummings and Liu, 2017; with the permission of the publisher, and copyright at the Consortium of Glycobiology Editors, La Jolla, California, published by Cold Spring Harbor Laboratory Press).

Galectins are synthesized on cytosolic ribosomes without a signal peptide, which results in the secretion of galectins through an endoplasmic reticulum- and Golgi- independent pathway (Nickel, 2005). However, the details of the secreting pathway of galectins are still elusive. Besides vertebrates, galectins are also widely found in fungi and invertebrates (Vasta, 2012).

1.4.1. Galectin-1 (Gal-1)

Gal-1 (a ~14 kDa protein) is the first described member of the galectin family. It is defined as an S-type lectin due to the abundance of cysteine residues (Cedeno-Laurent and Dimitroff, 2012; Cummings and Liu, 2017). Gal-1 is widely expressed in tissues of many vertebrates and invertebrates and requires reducing conditions to maintain its activity and stability (Cedeno-Laurent and Dimitroff, 2012; Cummings and Liu, 2017). Through binding to specific glycan structures, Gal-1 is involved in a variety of physiologic and pathologic processes including mRNA splicing, pathogen recognition, selective induction of Th1 and Th17 apoptosis, inhibition of T cell trafficking, expansion of tolerogenic dendritic cells and Treg cells, maintenance of the maternal-fetal tolerance, induction of pro-angiogenesis in anti-VEGF refractory tumors and suppression of autoimmune pathology (Toscano *et al.*, 2006, 2007; Norling *et al.*, 2008; Ilarregui *et al.*, 2009; Rabinovich and Croci, 2012; Croci *et al.*, 2014). Moreover, Gal-1 plays a role as a master regulator of clinically relevant inflammatory response gene expression in osteoarthritic chondrocytes by stimulating NFκB-mediated inflammation (Osório, 2016).

The structure of Gal-1 consists of two anti-parallel β-sheets with conserved amino acid residues in the CRD, which is also observed in other galectins (Figure 4a) (López-Lucendo *et al.*, 2004; Cedeno-Laurent and Dimitroff, 2012). Through van der Waals' force and hydrogen bond formation, the CRD of Gal-1 can recognize and bind lactosamine (LacNAc) (Figure 4b) (López-Lucendo *et al.*, 2004). Gal-1 affinity to LacNAc is largely mediated by defined key amino acids (His45, Asn47, Arg49, Val60, Asn62, Trp69, Glu72, and Arg74) (Cedeno-Laurent and Dimitroff, 2012). Gal-1 can bind to only the LacNAc sequence positioned on complex O-glycans and N-glycans linked glycoproteins or glycolipids, but not to soluble LacNAc disaccharides. The multivalent binding of Gal-1 results in high-affinity binding, because the affinity of each CRD to each glycan ligand is synergistic and additive (Thiemann and Baum, 2016).

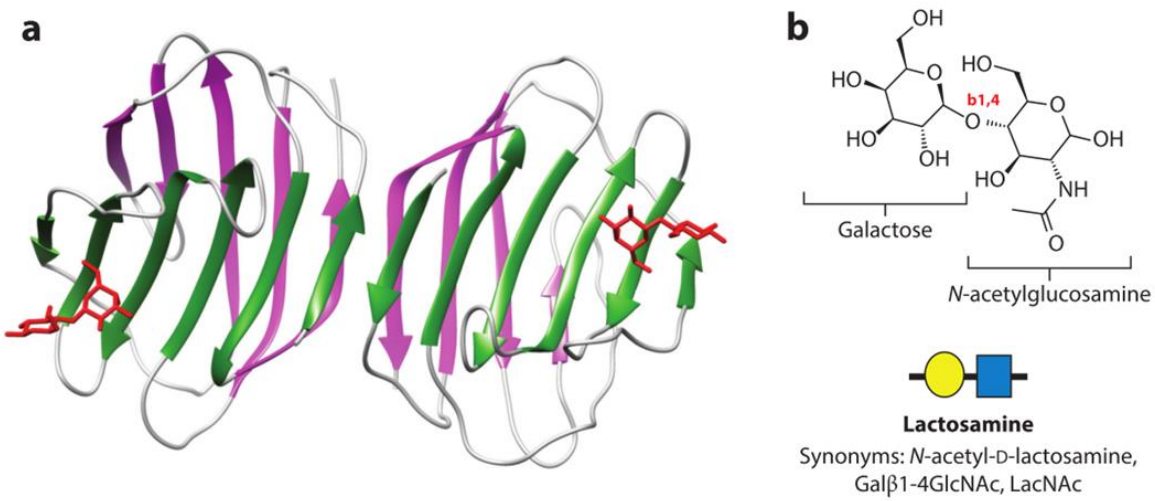

Figure 4. Ribbon diagram of the homodimeric structure and architecture of Gal-1 - ligand binding. (a) Two anti-parallel β-sheets (pink and green) fold into a binding pocket, the CRD, which binds to lactose (red). (b) Structure of lactosamine (LacNAc), consisting of galactose (yellow) and N-acetylglucosamine (blue). (Thiemann and Baum, 2016; with the permission of the publisher, license number: 4280080787751).

The function of Gal-1 is predominantly affected by its cellular location. Intracellular Gal-1 is mostly monomeric, and mediates cell growth, migration, and invasion through protein - protein interactions with cytoplasmic Harvey rat sarcoma viral oncogene homolog (H-Ras) (Paz *et al.*, 2001; Zhang *et al.*, 2014). Secreted Gal-1 spontaneously dimerizes and concentrates at the cell surface, which in turn binds to glycoconjugates located at the cellular surface and forms galectin - glycan complexes that influence intracellular signaling pathways and further regulates cell processes such as apoptosis, angiogenesis, migration and proliferation (Cedeno-Laurent and Dimitroff, 2012; Elola *et al.*, 2015).

The synthesis of complex glycans serving as Gal-1 binding molecules on cell membranes depends on the synchronized activities of glycan-modifying enzymes such as glycosyltransferases and glycosidases (Figure 5) (Cedeno-Laurent and Dimitroff, 2012). More specifically, expression of core 2 glucosaminyl (N-acetyl) transferase 1

(Gcnt1) for the synthesis of the backbone of Gal-1 ligands (core 2 O-glycans), promotes binding of Gal-1 to the cell surface (Cabrera *et al.*, 2006). Similarly, mannosyl (α-1,3-)-glycoprotein ß-1,2-N-acetylglucosaminyltransferases (Mgat) like Mgat5, which initiates the formation of the polylactosamine branch on N-glycans, also increase Gal-1 binding (Demetriou *et al.*, 2001). On the contrary, expression of ST6 beta-galactoside α-2-6-sialyltransferase 1 (St6gal1), which can add a terminal sialic acid to the galactose residue via an α-2-6-linkage on an N-glycan branch, abrogates Gal-1 binding to some terminal N-acetylglucosamines (Toscano *et al.*, 2007). In cultured T cells, overexpression of St6Gal1 prevents Gal-1 binding and thus cell death (Amano *et al.*, 2003).

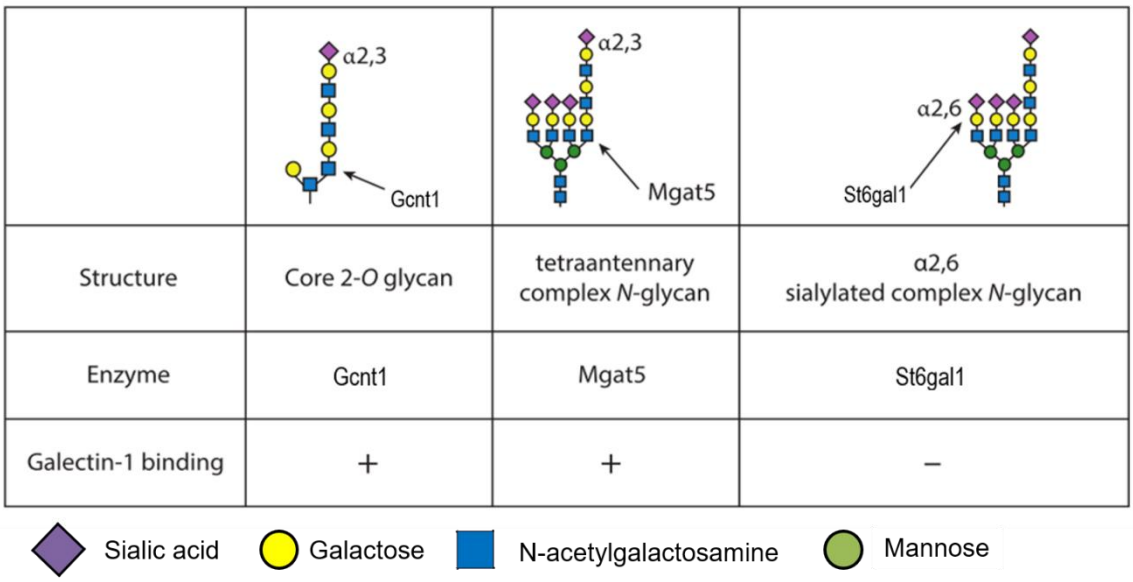

Figure 5. The function and influence of glycosyltransferases on Gal-1 binding to glycan ligands. There are three important post-translational mechanisms that influence the affinity of Gal-1 binding to glycan ligands: (a) activity of the core 2 glucosaminyl (N-acetyl) transferase 1 (Gcnt1) for the synthesis of core 2 O-glycans, which built the backbone of Gal-1 ligands, (b) branching of N-glycans by mannosyl (α-1,3-)-glycoprotein ß-1,2-N-acetylglucosaminyltransferases 5 (Mgat5, promotes Gal-1 binding), and (c) suppression of ST6 beta-galactoside α-2-6-sialyltransferase 1 (St6gal1) activity, that abrogates Gal-1 binding to some terminal N-acetylglucosamines by adding α-2-6-sialic acid. (Thiemann and Baum, 2016; with the permission of the publisher,

license number: 4280080787751).

1.4.2. Gal-1 in autoimmune diseases and the testis

By binding to specific cell surface glycan structures, Gal-1 acts as a homeostatic immune inhibiting factor, which is involved in the selective induction of Th1 and Th17 apoptosis (Toscano *et al.*, 2007), inhibition of T cell trafficking (Norling *et al.*, 2008), expansion of tolerogenic dendritic cells and regulatory T cells (Toscano *et al.*, 2006; Ilarregui *et al.*, 2009), decreased activation of M1 microglia (Starossom *et al.*, 2012), maintenance of maternal-fetal tolerance (Blois *et al.*, 2007), induction of pro-angiogenesis in anti-VEGF refractory tumors (Croci *et al.*, 2014) and suppression of autoimmune pathologies (Rabinovich and Croci, 2012).

Consequently, administration of Gal-1 reduces the severity and delays the onset in experimental *in vivo* models of chronic autoimmune diseases such as autoimmune diabetes, autoimmune retinal disease, experimental autoimmune encephalomyelitis, experimental autoimmune uveitis, collagen-induced arthritis, and experimental autoimmune orchitis (Rabinovich *et al.*, 1999; Toscano *et al.*, 2006; Perone *et al.*, 2009; Rabinovich and Toscano, 2009; Starossom *et al.*, 2012; Pérez *et al.*, 2015). In a glycosylation-dependent manner Gal-1 ameliorates inflammation in autoimmune disorders (Méndez-Huergo *et al.*, 2017).

At the same time, targeted deletion of Gal-1 significantly increases the severity of inflammation in mouse models of collagen-induced arthritis and experimental autoimmune encephalomyelitis (Starossom *et al.*, 2012; Iqbal *et al.*, 2013). On the contrary, mice lacking Gal-1 develop a reduced incidence and severity of symptoms in

experimental autoimmune orchitis (Pérez *et al.*, 2015), which indicates different functions for Gal-1 in the testis.

Interestingly, Gal-1 expression in the testis exhibits a stage-specific pattern during the spermatogenic cycle. Immunostaining of Gal-1 in Sertoli cells is found mainly at stages X–II and on apical stalks during spermiation (Dettin *et al.*, 2003). Gal-1 is located in cytoplasmic projections and nuclei of Sertoli cells as well as on heads and tails of late spermatids (Dettin *et al.*, 2003). Moreover, an *in vitro* study showed administration of recombinant Gal-1 can induce rat Leydig cell apoptosis and thus reduces testosterone production in a dose-dependent fashion (Martinez *et al.*, 2004). Interestingly, Gal-1 regulates the sperm fertilizing capacity both *in vitro* and *in vivo*. Mechanistically, Gal-1-deficient sperm display defects in hyperactivation and progesterone-induced acrosome reaction, features that could be rescued by exposure of the sperms to recombinant Gal-1 (Vasen *et al.*, 2015). Furthermore, Gal-1 is also expressed in human Sertoli cells (Wollina *et al.*, 1999; Chui *et al.*, 2011), but whether this affects the immunoregulatory functions of Sertoli cell has not been elucidated yet.

1.5. Male infertility

1.5.1. Epidemiology and etiology of male infertility

As defined by the World Health Organization (WHO): “Infertility is the inability of a sexually active, non-contracepting couple to achieve pregnancy in one year” (Rowe *et al.*, 2000). Approximately 50% of infertility cases are due to male factors (Rowe *et al.*, 2000). Male infertility could result from physical issues, infections, genetic reasons as well as environmental factors (Jungwirth *et al*, 2017). However, 30–45% of these deficiencies are still idiopathic. The etiology of male infertility can also be categorized in pre-testicular, testicular and post-testicular reasons (Henkel and Schill, 2003;

Gudeloglu and Parekattil, 2013; Kumar and Singh, 2015). Pre-testicular factors of male infertility are mainly related by endocrine disorders characterized by dysregulated sex steroid secretion (e.g. Kallmann syndrome, hypothalamic or pituitary disorders) (Gudeloglu and Parekattil, 2013). Testicular factors of infertility are categorized as congenital, acquired or idiopathic disorders that result in spermatogenic failure (Gudeloglu and Parekattil, 2013). Congenital testicular causes include testicular dysgenesis (cryptorchidism), genetic disorders (Klinefelter's syndrome: 47, XXY, or XXY syndrome, Y chromosome deletions), and spermatogenic arrest (maturation abnormalities). Acquired testicular factors consist of trauma, viral diseases (mumps), bacterial infections (*E. coli, Chlamydia, Enterobacter spp, Gonorrhea*), varicocele, or sterile autoimmune inflammation of the testes. Post-testicular factors consist of obstructions or ejaculatory disorders, which both affect the transport of spermatozoa (Henkel and Schill, 2003).

1.5.2. Male infertility associated with inflammation

Infection and inflammation of the male genital tract are considered as one of the most important identifiable etiologies of male infertility (Weidner *et al.*, 2013). Testicular inflammation (orchitis) is characterized by the presence of inflammatory infiltrates in the testicular interstitium and associated disruption of seminiferous tubules, that can lead to partial or total impairment of spermatogenesis (Schuppe and Meinhardt, 2005; Haidl *et al.*, 2008). Acute epididymitis, orchitis or combined epididdymo-orchitis caused by infection show clinical symptoms that can often be successfully treated with antibiotics and/or antiphlogistics. Post- or non-infectious chronic orchitis is hazardous because it is not associated with discomfort or pain (Fijak and Meinhardt, 2006; Schuppe *et al.*, 2008, 2017; Jungwirth *et al.*, 2012, 2018; Pilatz *et al.*, 2015; Fijak *et al.*, 2018). However, in a noninfectious environment inflammatory reactions in the testis can also be triggered by physical trauma or chemical noxae, which could induce germ cell apoptosis, increased production of pro-inflammatory cytokines as well as immune cell infiltration (Schuppe

et al., 2008). In the majority of patients, sub-acute or chronic inflammatory reactions in the testis remain asymptomatic until advanced stages of the disease. At the same time, no specific seminal or serum markers are available for diagnosis of chronic testicular inflammation. As the diagnosis of chronic orchitis is difficult, it is assumed that many cases are neglected in the clinic as an underlying reason of male infertility (Schuppe *et al.*, 2008; Pilatz *et al.*, 2015).

1.5.3. Experimental autoimmune orchitis

EAO is a rodent model to study organ-specific autoimmunity and chronic testicular inflammation that reproduces pathological changes also seen in some cases of human immunological infertility (Tung and Teuscher, 1995; Lustig *et al*., 2013; Aslani *et al.*, 2015; Fijak *et al.*, 2018). The EAO model in rodents is usually induced by immunization with homologous testicular protein extracts in incomplete or complete Freund's adjuvant, followed by administration of inactivated *Bordetella pertussis* bacteria or *Bordetella pertussis* toxin (Kohno *et al.*, 1983; Doncel *et al.*, 1989).

The initial phase of EAO involves the production of auto-antibodies against testicular antigens, increased migration and infiltration of leukocytes such as macrophages, CD4+ and CD8+ effector T cells and dendritic cells as well as the elevated production of pro-inflammatory cytokines like COX-2, IFNγ, TNFα and IL-6 or chemokines such as MCP-1 (Fijak *et al.*, 2005; Iosub *et al.*, 2006; Rival *et al.*, 2006, 2008; Nicolas *et al.*, 2017). The chronic phase of the disease consists of granuloma formation, progressive apoptosis of germ cells and shrinkage of seminiferous tubules, which can finally result in a complete absence of spermatogenesis and consequently decreased testicular weight (Guazzone *et al.*, 2003, Fijak *et al.*, 2011a). Comparable pathological changes were found in testicular biopsies from patients with chronic orchitis (Duan *et al.*, 2011; Aslani *et al.*, 2015; Nicolas *et al.*, 2017). Although the population of immunoregulatory T cells

such as CD4+CD25+Foxp3+ T cells (Treg) and CD8+Foxp3+ T cells is increased in inflamed testes, these cells were not able to suppress the inflammatory reaction and maintain organ homeostasis (Jacobo *et al.*, 2009, 2015; Fijak *et al.*, 2011b). Specifically, M1 macrophages expressing MHC class II, CD80 and CD86 costimulatory molecules as well as pro-inflammatory cytokines (IL-6 and TNFα) are much increased and thus develop as the main macrophage population in EAO testes (Rival *et al.*, 2008). This observation was accompanied by elevated expression of MCP-1 (Aslani *et al.*, 2015; Nicolas *et al.*, 2017). Besides cytokines and chemokines, molecules, like HMGB1, are also playing in testicular inflammation in human and rat. In this regard, blockade of upregulated HMGB1 by ethyl pyruvate after induction of EAO reduces disease progression and spermatogenic damage as documented by lower numbers of interstitial macrophages as well as decreased IL-6 mRNA expression levels (Aslani *et al.*, 2015).

1.6. Aim of the study

The testis is an immune privileged organ, where the somatic (mostly Sertoli and peritubular cells) and immune cells act collectively together to maintain an anti-inflammatory environment. Paradoxically, about 15% of all male infertility cases are related to testicular inflammation. Gal-1 plays a prominent role in the maintenance of immune homeostasis by binding to N-acetyllactosamine-containing oligosaccharides on the cellular surface. Its wide immune inhibitory capacity ranges from reduction of pathogenic Th1 and Th17 cell responses, promotion of Treg cell differentiation and polarization of M2 macrophages to the expansion of tolerogenic dendritic cells. This promoted us to investigate whether Gal-1 is involved in the maintenance of immune homeostasis in the testis.

The EAO model represents an *in vivo* system for studying organ-specific autoimmunity and chronic testicular inflammation that reproduces pathological changes also seen in some cases of human immunological infertility.

Therefore, the aim of this study was to investigate the expression of Gal-1 in rat EAO testes and the involvement of Gal-1 in testicular inflammation. Moreover, the glycan profiles in EAO testes and TNFα challenged Sertoli as well as peritubular cells were investigated by using lectin binding assay.

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
Ammonium persulphate (APS)	Sigma, Steinheim, Germany
BlueEasy prestained protein marker	Nippon Genetics Europe, Dueren, Germany
Bromophenol blue sodium salt	Sigma, Steinheim, Germany
Calcium chloride	Merck, Darmstadt, Germany
Coomassie Brilliant Blue G-250	Bio-Rad, Munich, Germany
DAPI mounting media	Vector, Burlingame, USA
Dimethyl sulfoxide	Merck, Darmstadt, Germany
Dithiothreitol (DTT)	Roth, Karlsruhe
DNA Ladder (100 bp)	Nippon Genetics Europe, Dueren, Germany
D-Glucose	Sigma, Steinheim, Germany
1,4-Dithiothreitol	Roche, Mannheim, Germany
Ethanol	Sigma, Steinheim, Germany
Ethidium bromide	Roth, Karlsruhe, Germany
Ethylene diaminetetraacetic acid disodium salt (EDTA)	Merck, Darmstadt, Germany
Glycerol	Merck, Darmstadt, Germany
Glycine	Sigma, Steinheim, Germany
Halt Phosphatase Inhibitor Cocktail	Thermo Fisher Scientific, Waltham, USA
Hydrochloric acid 37%	Sigma, Steinheim, Germany

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	Roth, Karlsruhe, Germany
Igepal CA-630 (NP-40)	Sigma, Steinheim, Germany
Isoflurane	Baxter, Unterschleißheim, Germany
α-Lactose monohydrate, reagent grade	Sigma, Steinheim, Germay
Mayer's hematoxylin solution	Merck, Darmstadt, Germany
Methanol	Sigma, Steinheim, Germany
β-Mercaptoethanol	AppliChem, Darmstadt, Germany
Non-fat dry milk	Roth, Karlsruhe, Germany
N, N, N`, N`-Tetramethylethylenediamine (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
ProLong Gold Antifade Mountant with DAPI	Thermo Fisher Scientific, Waltham, USA
Propidium iodide solution	Miltenyi Biotec, Bergisch Gladbach, Germany
Protease inhibitor cocktail	Sigma, Steinheim, Germany
Rotiphorese Gel 30	Roth, Karlsruhe, Germany
Sodium acetate	Roth, Karlsruhe, Germany
Sodium chloride	Sigma, Steinheim, Germany
Sodium dodecyl sulfate (SDS)	Merck, Darmstadt, Germany
Super Signal West Pico chemiluminescent substrate	Thermo Scientific, Waltham, USA
Tris (hydroxymethyl) aminomethane	Roth, Karlsruhe, Germany

Triton X-100	Sigma, Steinheim, Germany
Tween-20	Roth, Karlsruhe, Germany

2.2. PCR reagents

RNase-Free DNase Set	Qiagen, Hilden, Germany
Desoxy-ribonukleoside triphosphate (dNTP) 10mM	Promega, Mannheim, Germany
iTaq Universal SYBR Green Supermix	Biorad, München, Germany
Moloney Murine Leukemia Virus Reverse Transcriptase, (M-MLV RT)	Promega, Mannheim, Germany
Oligo dT 15 Primer	Promega, Mannheim, Germany
Recombinant RNasin® Ribonuclease Inhibitor	Promega, Mannheim, Germany
Taq polymerase	Promega, Mannheim, Germany

2.3. Cell culture reagents and equipment

Bovine serum albumin (endotoxin free)	Invitrogen, Karlsruhe, Germany
Cell culture CO_2 incubator	Binder, Tullingen, Germany
Cell strainer, 70µm	BD Falcon, NY, USA
Dulbecco´s PBS (1×) w/o Ca^{2+} & Mg^{2+}	Gibco, Darmstadt, Germany
Fetal bovine serum	Gibco, Darmstadt, Germany
JNK inhibitor	Sigma, Steinheim, Germay
Neubauer counting chamber	Boeco, Hamburg, Germany
Penicillin/Streptomycin (100×)	Gibco, Darmstadt, Germany
p38 inhibitor	Sigma, Steinheim, Germay
RPMI 1640 medium	Gibco, Darmstadt, Germany
Sterile plastic ware for cell culture	Sarstedt, Nümbrecht, Germany
Trypsin/EDTA	Gibco, Darmstadt, Germany

2.4. Miscellaneous

Enhanced chemiluminescence (ECL) reagents	Thermo Fisher Scientific, Waltham, USA
Entellan ®Neu	Merck, Darmstadt, Germany
Hybond ECL nitrocellulose membrane	Amersham, Freiburg, Germany
Nitrocellulose membrane (0.2 µm pore size)	GE Healthcare, Freiburg, Germany
RNeasy Mini Kit	Qiagen, Hilden, Germany
Pierce BCA Protein Assay	Thermo Fisher Scientific, Waltham, USA

2.5. Software

Bio-Rad CFX Manager 3.1	Bio-Rad, Munich, Germany
FlowJo V10	FlowJo LLC, Oregon, USA
FusionCapt Advance software	Witec Ag, Luzern, Switzerland
GraphPad Prism 5	GraphPad Software, San Diego, USA
Zeiss AxioVision software 4.8	Carl Zeiss, Göttingen, Germany

2.6. Equipment

Centrifuge (5424R)	Eppendorf, Hamburg, Germany
CFX96 Touch thermal cycler	Bio-Rad, Munich, Germany
Cryostat CM30509	Leica, Wetzlar, Germany
Desktop centrifuge Biofuge Fresco	Heraeus, Hanau, Germany

Electronic balance SPB50	Ohaus, Giessen, Germany
Fluorescent microscope Axioplan 2 Imaging	Carl Zeiss, Göttingen, Germany
Fusion FX7	Witec Ag, Luzern, Switzerland
Gel Jet Imager 2000 documentation system	Intas, Göttingen, Germany
Heat block DB-2A	Techne, Cambridge, UK
Horizontal mini electrophoresis system	PEQLAB, Erlangen, Germany
Labofuge 400R	Heraeus, Hanau, Germany
MACS Quant 10 flow cytometer	Miltenyi Biotec, Bergisch Gladbach, Germany
Microplate ELISA reader (Multiscan RC)	Labsystems, Vantaa, Finland
Microwave oven	Samsung, Schwalbach, Germany
Mini centrifuge Galaxy	VWR International, Germany
Mini-rocker shaker MR-1	PEQLAB, Erlangen, Germany
Mixer Mill MM 300	Retsch, Haan, Germany
NanoDrop ND 2000	Thermo Fisher Scientific, Waltham, USA
PCR thermocycler	Biozyme, Oldendor, Germany
Power supply units	PEQLAB, Erlangen, Germany
Pre-cast gel system	Invitrogen, Karlsruhe, Germany
SDS gel electrophoresis chambers	Consurs, Reiskirchen, Germany
Semi-dry electroblotter	Bio-Rad, Munich, Germany
Stainless steel beads, 5mm (#69989)	Qiagen, Hilden, Germany
SuperFrost Plus microscope slides	R.Langenbrinck, Emmendingen, Germany
Tips and tubes	Sarstedt, Nümbrecht, Germany
Thermo Shaker	PEQLAB, Erlangen, Germany
Vertical electrophoresis system	PEQLAB, Erlangen, Germany
UV visible spectrophotometer Ultrospec 2100 Pro	Biochrom, Cambridge, UK

3. METHODS

* Details of antibodies, primers, lectins, enzymes, buffers and solutions are listed in the appendix.

3.1. Animals

19 day old rats were used for Sertoli and peritubular cell isolation. Adult male Wistar rats (Charles River Laboratories, Sulzfeld, Germany) aged 60 - 70 days were used for EAO induction. All the 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 Sertoli and peritubular cell isolation, male rats were killed by CO_2 inhalation. During immunization procedure, rats were anaesthetized by intraperitoneal administration of 100 mg/kg b.w. ketamine and 10 mg/kg b.w. xylazine. All efforts were made to alleviate the suffering of animals.

3.2. Induction of EAO

To induce EAO, adult male Wistar rats (Charles River Laboratories, Sulzfeld, Germany) aged 60–70 days were immunized with testicular homogenate in complete Freund`s adjuvant as previously described (Aslani *et al.*, 2015). Briefly, animals were injected with testicular homogenate in complete Freund's adjuvant, followed by *i.v.* injection of inactivated *Bordetella pertussis* bacteria three times every 14 days (Figure 6). Control animals received 0.9% NaCl instead of testicular homogenate in complete Freund`s adjuvant. Normal untreated rats were also included. Animals were sacrificed 50 days after the first immunization, testes were removed and frozen in liquid nitrogen. All animal experiments were approved by the local animal ethics committee (Regierungspraesidium Giessen GI 20/23 – Nr. 33/2008).

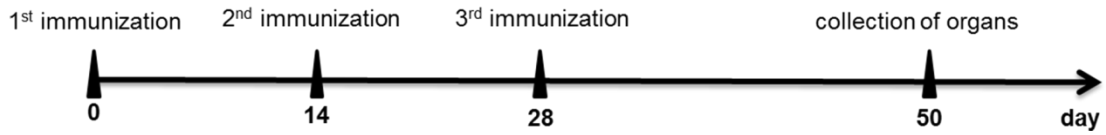

Figure 6. Schematic diagram illustrating induction of EAO in Wistar rats by active immunization with testicular homogenate (TH) in complete Freund's adjuvant followed by *i.v.* injection of inactivated *Bordetella pertussis* bacteria. Control adjuvant animals received saline instead of TH.

3.3. Hematoxylin-eosin staining

10 µm thick frozen sections were cut using a cryostat. Frozen samples were rehydrated through a series of decreasing ethanol concentrations starting from 100% to 40%, for 3 min each step and rinsed in deionized H_2O (dH_2O). Samples were stained with Mayer's hematoxylin solution for 5 min, left under flowing tap water for 10 min and rinsed with dH_2O for 5 min. Then samples were stained in 0.5% eosin solution containing acetic acid (2 drops in 100 ml solution) for 1-2 min and rinsed in dH_2O again. Subsequently, sections were dehydrated in increasing alcohol series starting from 40% to 100% for 3-5 min, and then rinsed in xylene for 2 × 5 min. Finally, sections were mounted using Entellan ®Neu (Merck, Darmstadt, Germany).

3.4. Immunofluorescence microscopy

Testicular cryosections (10 µm) were fixed in methanol (Sigma-Aldrich, Steinheim, Germany) at -20°C for 10 min and permeabilized in 0.1% Triton X-100. After blocking with 5% goat serum and 2.5% BSA (Carl Roth, Karlsruhe, Germany) in PBS-T (PBS containing 0.1% Tween 20), sections were incubated overnight with appropriate primary antibody in 2.5% BSA diluted in PBS-T at 4°C. Afterwards sections were washed in PBS-T and incubated with secondary antibody in PBS-T at room temperature (RT) for 1

h. Finally, sections were mounted with ProLong Gold Antifade Mounting media with DAPI (Thermo Fisher Scientific, Waltham, USA). Images were taken with an Axioplan 2 Zeiss microscope (Carl Zeiss, Göttingen, Germany).

3.5. Lectin binding assay

Testicular cryosections were fixed in 2% paraformaldehyde for 30 min. After blocking with 1% BSA in PBS, sections were washed with 0.1% BSA in PBS. Subsequently, sections were incubated with a specific lectin conjugated with FITC (EY Laboratories, San Mateo, USA) in 1% BSA-PBS for 30 min and rinsed in 0.1% BSA in PBS 3 × 5min. Finally, sections were mounted with ProLong Gold Antifade Mounting media with DAPI. Images were taken with an Axioplan 2 Zeiss microscope (Carl Zeiss, Jena, Germany).

3.6. Isolation of Sertoli and peritubular cells

Sertoli and peritubular cells were isolated from 19-day old Wistar rats. Ten male rats were killed by CO_2 inhalation. Blood was removed by immediate postmortem incision of the jugular veins under flowing tap water. The abdomen was disinfected with 70% ethanol and twenty testes were removed aseptically via transabdominal route and collected in 20 ml of PBS-A. Testes were rinsed by 20 ml of 1% (w/v) iodine alcohol for 30 sec to sterilize, and immediately washed two times with PBS-A. Then, the testes were decapsulated in a sterile Petri dish and dispersed by enzymatic digestion.

Seminiferous tubules were digested using 10 ml trypsin - DNase solution at 32°C in a shaking water bath (120 oscillations/min) for 4-6 min. When seminiferous tubules were separated, trypsin digestion was stopped by adding trypsin inhibitor solution A. After

settling down of the seminiferous tubules for 5 min the supernatant was discarded and 10 ml trypsin inhibitor solution B was added. Then seminiferous tubules were washed with PBS-A solution 7 times for 8 min each to remove unwanted interstitial cells. After washing steps, the seminiferous tubules were digested using collagenase - hyaluronidase - Dnase - solution in a shaking water bath (120 oscillations/min) at 32°C for 10-12 min. The detachment of peritubular cells from the seminiferous tubules was observed under the phase contrast microscope (Figure 7a - b). Then 20 ml PBS-A was added to the digested seminiferous tubule fragments. After settling down for 10 min, the supernatant with enriched peritubular cells was carefully collected. 20 ml peritubular cell medium was added and the resulting cell suspension was centrifuged at 280 g for 10 min without break. The peritubular cell pellet was dissolved in 20 ml peritubular cell medium and then seeded in 5 flasks containing 16 ml peritubular cell medium. Peritubular cells were passaged after 3 days` incubation. For treatment, peritubular cells were seeded into 6-well plates (5×10^5 cells/well).

For Sertoli cell isolation, the remaining seminiferous tubules were washed 4 times using PBS-A solution. Subsequently, the tubules were further digested by using 10 ml of hyaluronidase-DNase-solution at 32°C in a shaking water bath (120 oscillations/min) for 7-10 min, until short tubule fragments and released cells were visible (Figure 7c - d). The fragmented tubules were allowed to settle for 12 min and washed 4 times with PBS-A to remove contaminating germ and peritubular cells. The fragments were dispersed by passing 10 times through an 18G needle using a 20 ml syringe, followed by centrifugation at 300 × g for 10 min. After resuspending with 40 ml Sertoli cell medium, cells were passed through 70 µm nylon filter (BD Biosciences, Heidelberg, Germany) to get rid of undigested tubular fragments. Cells were counted using the Neubauer counting chamber (Boeco, Hamburg, Germany). Finally, 2×10^6 cells/well were seeded in 6-well plates and incubated at 32°C with 5% CO_2. After two-day culture hypotonic shock treatment by using 20 mM Tris-HCl (pH 7.5) for 1.5 min was performed to remove contaminating germ cells (Figure 7e). On day 4, a highly purified population of Sertoli cells was ready for further experiments (Figure 7f).

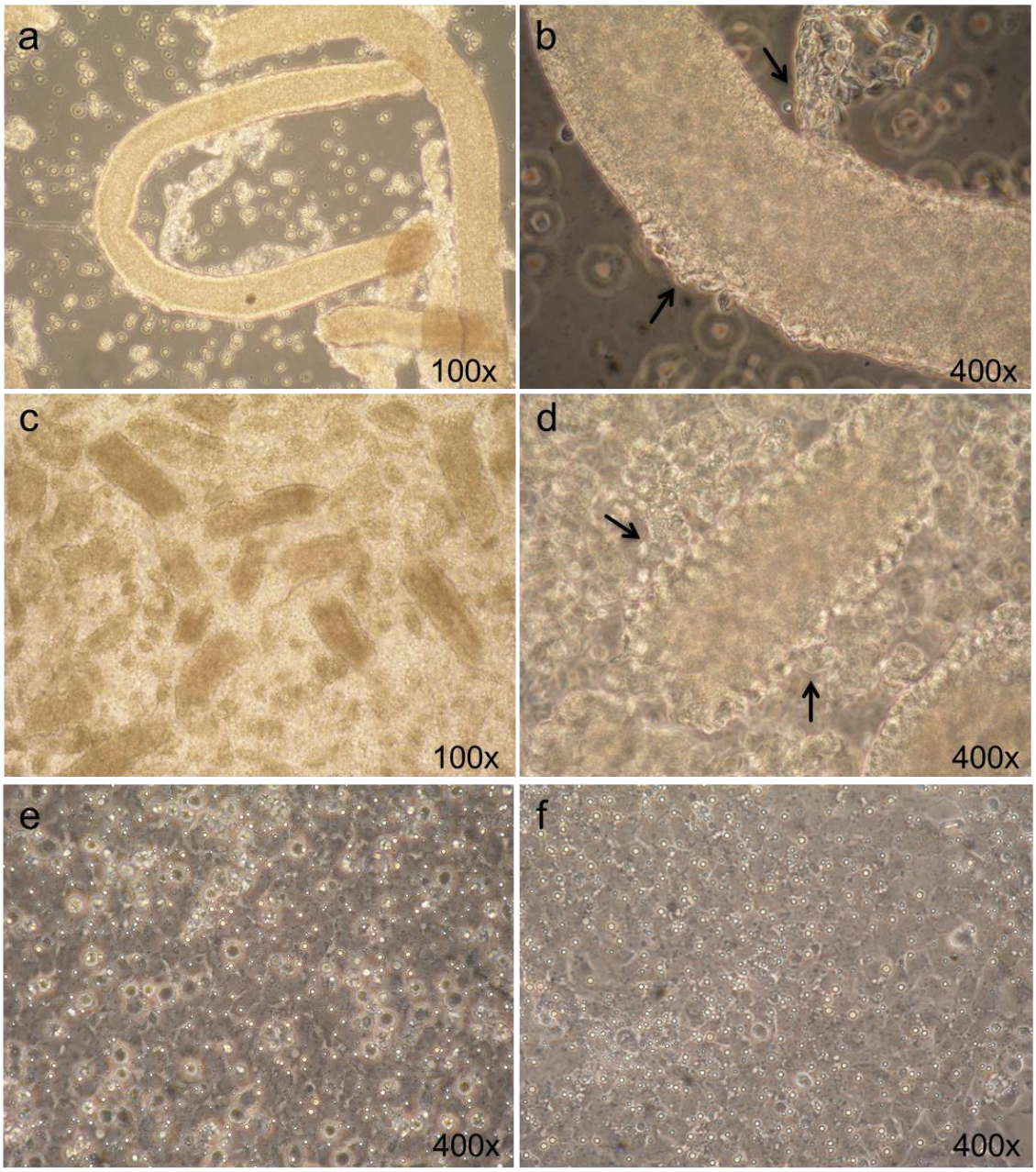

Figure 7. Isolation of peritubular and Sertoli cells from immature rat testes. The enzymatic digestion of testicular parenchyma with collagenase, hyaluronidase and DNase I led to detachment of peritubular cells (indicated by arrows) while the lamina propria of tubules was still intact as shown at 100x (a) and 400x (b) magnification. Subsequently, the seminiferous tubules became fragmented (c) and release of Sertoli cells was visible (arrows) (d). After two days, Sertoli cell culture still contained contaminating cells (e). A pure population of Sertoli cells was achieved after hypotonic shock (f).

By immunofluorescence using antibodies directed against α smooth muscle actin and vimentin, the purity of Sertoli (Figure 8a and 8b) and peritubular cell (Figure 8c and 8d) populations, respectively, were estimated > 95%.

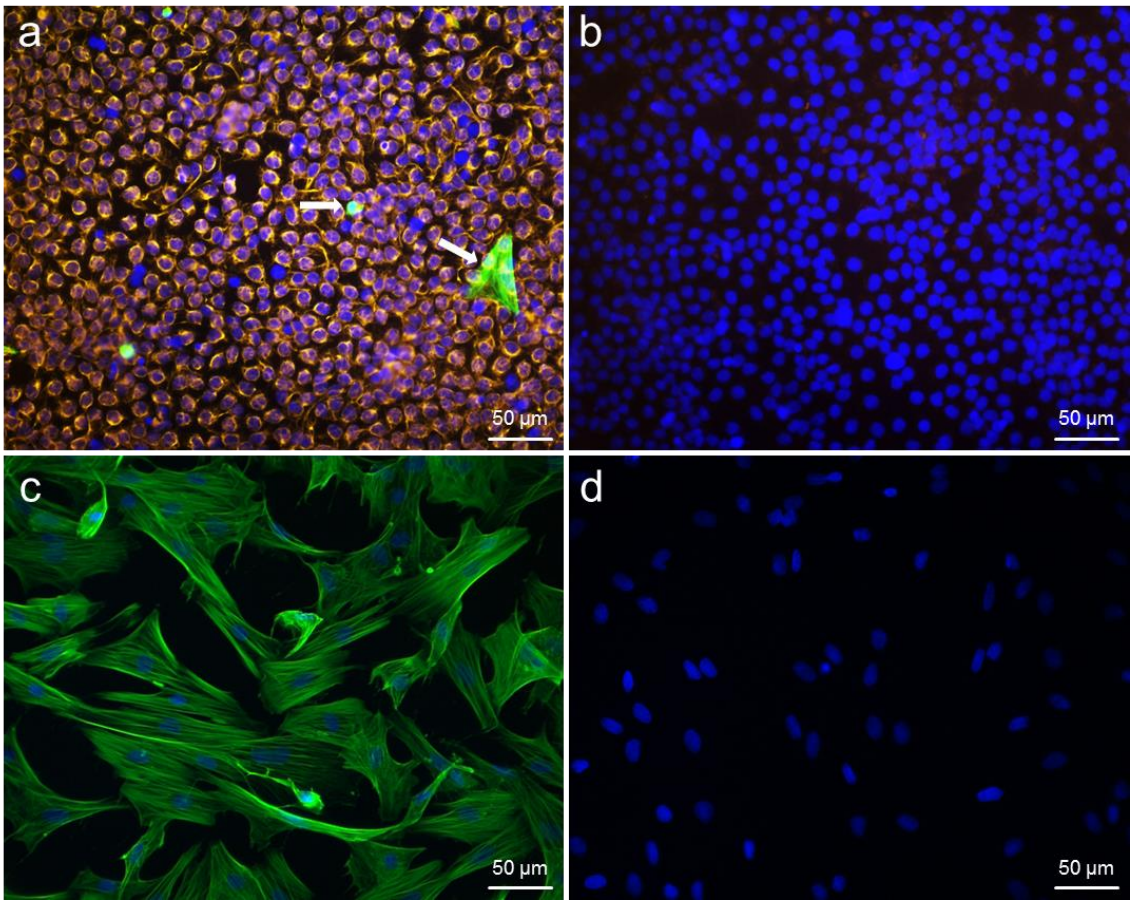

Figure 8. The purity of primary Sertoli and peritubular cells was analyzed by immunostaining. Double staining of vimentin (orange) and α smooth muscle actin (green) in Sertoli cell culture (a) after hypotonic shock displayed very high purity with only a few contaminating peritubular cells (white arrows) visible. Cultured peritubular cells (c) after passaging show no contaminating Sertoli cells. The negative control with omission of primary antibodies for Sertoli (vimentin; b) and peritubular cells (α smooth muscle actin; d) displayed no staining.

3.7. Western blotting

Western blotting is a technique applied to analyze the expression of proteins in tissue homogenates or cell extracts by using antibodies specifically targeting the desired protein. Frozen rat testes or Sertoli cells were collected into RIPA lysis buffer supplemented with proteinase inhibitor cocktail and Halt Phosphatase Inhibitor Single-Use Cocktail and homogenized using stainless steel beads and a tissue lyser (Qiagen, Hilden, Germany) at 30 oscillations/second for 5 min. The protein concentration was measured by using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA). Twenty µg protein of each sample were separated by SDS-polyacrylamide gel electrophoresis for about 2 h at 120 V. Separated proteins were electroblotted onto a 0.2 µm pore size nitrocellulose membrane (GE Healthcare, Freiburg, Germany) by using a semidry electroblotter (Bio-Rad, Munich, Germany) for 30 min at 100 mA. The efficiency of the transfer was checked by staining the membrane with Ponceau S solution for 10 s. Then membranes were washed in TBS containing 0.1% Tween (TBS-T). After incubation in blocking buffer (5% non-fat milk/TBS-T or 5% BSA/TBS-T (in case of phosphoprotein detection)) for 1 h at RT, the membranes were probed overnight with primary antibodies diluted in blocking buffer at 4°C. Subsequently, membranes were washed three times with TBS-T, and then incubated with HRP-conjugated anti-mouse or anti-rabbit secondary antibody in 5% non-fat milk diluted in TBS-T for 1 h at RT. Afterwards, the membranes were washed again three times in TBS-T. The blots were developed by using Hybond ECL (Amersham, Freiburg, Germany) and visualized by using the Fusion Imaging system (Witec Ag, Luzern, Switzerland).

In order to perform additional antibody detection on the same membrane, membranes were incubated with stripping buffer for 3 min at 60°C after washing once with TBS-T. The membranes were washed again three times in TBS-T and blocked in 5% non-fat milk for 1 h at RT. Subsequently, the procedures like antibody incubation and

visualization were performed as described above.

3.8. cDNA synthesis and real-time polymerase chain reaction (real-time PCR)

3.8.1. Extraction of RNA

Total RNA was isolated from fresh cells or frozen rat testes by using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, less than 5 mg of tissue or 5×10^5 cells were homogenized with 350 µl RLT lysis buffer (containing 1% β-mercaptoethanol) by using stainless steel beads and a tissue lyser (Qiagen, Hilden, Germany) at 30 oscillations/second for 3 min. Cell or tissue lysates were collected after centrifugation for 12 min at 12,000 x g and equal volume of 70% ethanol was added and mixed well. Up to 700 µl of the mixture was transferred to RNeasy mini spin column and centrifuged at 10,000 × g for 1 min. The flow-through was discarded and columns were washed with 350 ml RW1 buffer. Then genomic DNA digestion was performed on column by using DNase I (Qiagen, Hilden, Germany) as follows: 10 µl DNase I stock solution was added to 70 µl provided RDD buffer. Afterwards, 80 µl of the DNase solution was added to the column and incubated for 30 min at RT. Subsequently, 350 µl RW1 buffer was used to wash column membrane again. Then columns were washed twice with provided RPE buffer (containing 90% ethanol) to precipitate RNA followed by a centrifugation at 12,000 × g for 2 min to dry the membrane. The collection tube was replaced by a new tube and 30 µl RNase-free water was directly added to the column membrane. After incubation for 1 min, the RNA was eluted by centrifugation at 12,000 × g for 2 min. RNA concentration of each sample was measured using the NanoDrop ND2000 (Promega, Mannheim, Germany).

3.8.2. Test for genomic DNA contamination

3.8.2.1. Amplification of a housekeeping gene β-actin

To test a possible genomic DNA contamination, isolated RNA samples were used as template for amplification of a housekeeping gene (β-actin) transcription. A typical PCR mix is shown in Table 1.

Table 1. PCR reaction mix.

Reagents	Volume
RNA	1 µl
5 × Green Go Taq Flexi Buffer	5 µl
$MgCl_2$ 25 mM	2 µl
dNTP 10 mM	0.5 µl
Go Taq Polymerase Flexi	0.25 µl
H_2O	15.25 µl
Reverse primer 10 pM	0.5 µl
Forward primer 10 pM	0.5 µl
Total volume	25 µl

PCR was run on a PCR thermocycler (PeqLab, Erlangen, Germany) for 25 cycles as shown in Table 2.

Table 2. PCR conditions for β-actin amplification.

Number of cycles	Temperature (°C)	PCR step	Time
1	94	Denaturation	4 min
25	94	Denaturation	40 sec
	55	Annealing	40 sec
	72	Elongation	40 sec
1	72	Enzyme deactivation	10 min

3.8.2.2. Agarose gel electrophoresis

1.5 g of agarose was completely dissolved in 100 ml TAE buffer. Ethidium bromide solution (Roth, Karlsruhe, Germany; 2 drops/100 ml) was added to the agarose solution for detection of nucleic acids and gels were casted. A 100 bp DNA ladder (Promega, Mannheim, Germany) and samples from β-actin amplification were loaded. Then gel was run at 100 V (2-10 V/cm gel) in TAE buffer for 20 min. Finally, agarose gels were examined on a UV transilluminator and photographed using a gel jet imager 2000 documentation system (Intas, Göttingen, Germany). cDNA used as positive control displayed a β-actin transcript at the expected size of 232 bp. No amplification product was detected in negative control with H_2O instead of RNA and isolated RNA samples, indicating no presence of genomic DNA contamination in the extracted RNA samples (Figure 9). Only RNA samples without genomic DNA contamination were used for the following experiments.

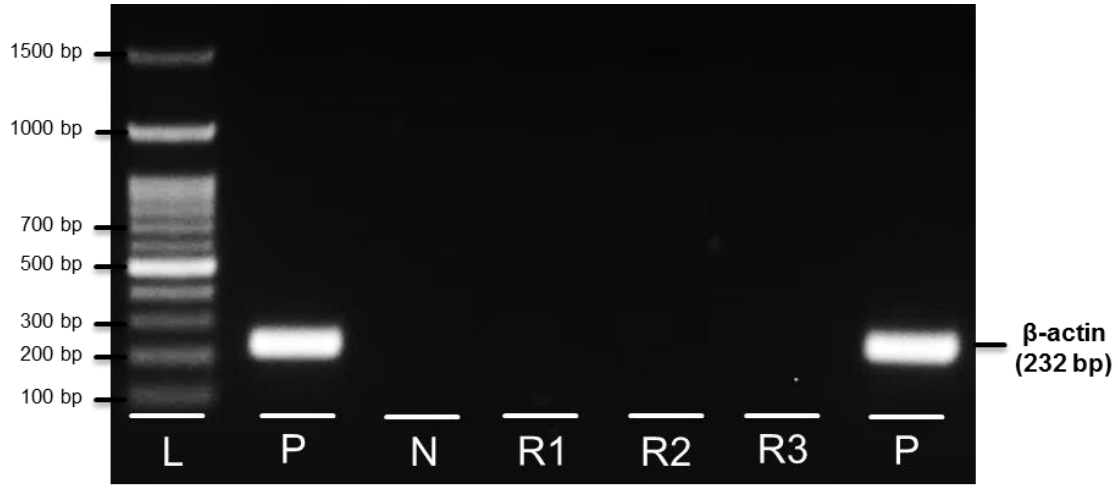

Figure 9. **Representative agarose gel with PCR products for testing genomic DNA contamination**. Positive control (P) containing testicular cDNA displayed a β-actin transcript at the expected size of 232 bp. No β-actin transcripts were detected in negative control (N) and RNA samples extracted from untreated testis (R1), adjuvant testis (R2) and EAO testis (R3). (L: 100 bp DNA ladder).

3.8.3. Reverse transcription PCR (RT-PCR)

The RNA samples without genomic DNA contamination were reverse transcribed by using M-MLV reverse transcriptase RNase (H-), point mutant (Promega, Mannheim, Germany). For each reverse transcription reaction 2 µg of RNA was used. RT-PCR mix (Table 3) was denatured for 10 min at 70°C.

Table 3. Composition of RT-PCR mix.

Reagents	Volume
RNA	Calculated volume
Oligo dT concentration (10 pM)	2 µl
H_2O	X µl
Total volume	22 µl

X = 20 µl – volume of RNA

In another reaction set up master mix (Table 4) was pre-warmed for 2 min at 42°C. Afterwards 18 µl of denatured RNA were mixed with 22 µl of RT-PCR master mix with addition of 1 µl of M-MLV reverse transcriptase. The samples were reverse transcribed at 42°C for 75 min, followed by inactivation of reverse transcriptase by incubating the samples at 70°C for 15 min. For further use cDNA samples were stored at -20°C.

Table 4. RT-PCR master mix.

Reagents	Volume
5 X RT-PCR buffer	8 µl
dNTP 10mM	2 µl
Recombinant RNasin® Ribonuclease Inhibitor (40 u/µl)	1 µl
H_2O	7 µl
Total volume	18 µl

3.8.4. Real-time PCR

Real-time PCR is a widely used technique to monitor and quantify the amplification of a targeted gene under real time conditions based on the polymerase chain reaction. The key feature of real-time PCR is that fluorescent dyes (e.g. SYBR green) intercalated in "real time" with the double stranded DNA (amplified products). Real-time PCR was performed by using CFX TouchTM Real-Time PCR detection system (Bio-Rad, Munich, Germany). The primers for rat galectin-1 (Lgals1), mannoside acetylglucosaminyltransferase 5 (Mgat5), core 2 glucosaminyl (N-acetyl) transferase 1 (Gcnt1), and ST6 beta-galactoside α-2-6-sialyltransferase 1 (St6gal1) were purchased as PrimePCR SYBR Green Assays from Bio-Rad (Munich, Germany). As housekeeping genes β-actin, hypoxanthine guanine phosphoribosyl transferase (Hprt) and 18s rRNA (Rn18s) were used. A typical 20 µl real- time PCR reaction mix (Table 5) was used as follows.

Table 5. Real-time PCR reaction mix.

Reagents	Volume per reaction
cDNA	1 µl
2 × iTaq™ Universal SYBR® Green Supermix	10 µl
Forward primer (10 pM)	0.5 µl
Reverse primer (10 pM)	0.5 µl
H_2O	8 µl
Total volume	20 µl

After Real-time PCR reaction, each targeted gene has a value of cycle threshold (Ct) defined as the number of cycles required for the fluorescent signal to cross the threshold. Relative gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Briefly, the $2^{-\Delta\Delta Ct}$ method is built on calculation of ΔCt by subtracting the Ct value of the housekeeping gene from the Ct value of the targeted gene in each sample. Then ΔΔCt is calculated by subtracting the mean of the ΔCt value of the

control samples from the ΔCt value of each sample. Finally, $2^{-\Delta\Delta Ct}$ is representative for the relative expression of the gene. Real-time PCR conditions are listed in Table 6.

Table 6. Real-time PCR program

Cycles	Temperature (°C)	PCR step	Time
1	95	Denaturation	30 sec
45	95	Denaturation	15 sec
	55	Annealing	30 sec
	72	Elongation	30 sec
Melt curve	50 - 95	Dissociation	5 sec

In addition during real-time PCR, analysis of the melt curve was also performed, which could assess the dissociation characteristics of double-stranded DNA during heating. When the temperature gets raised, double DNA strand starts to dissociate and then the inserted SYBR green fluorescent dye is released, leading to a decrease of the emitted fluorescent signal. The melt curve was obtained from a function of temperature plotted by the change in slope of the fluorescence curve. The melt curve analysis was used to monitor whether only one specific PCR product was produced during the target gene amplification. A single peak of the melt curve represents one single product. Figure 10 shows a representative melt curve for all the studied real-time PCR.

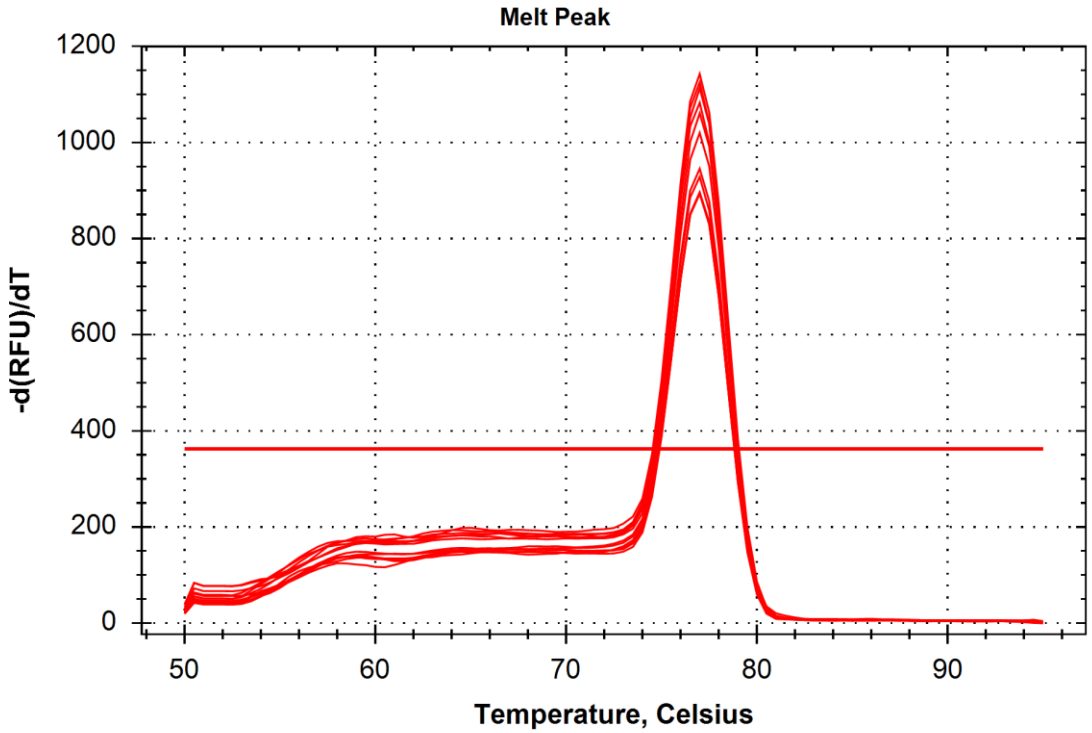

Figure 10. Representative melt curves from real-time PCR of Hprt amplification. The single peak represents one single product.

3.9. Measurement of lectin binding to Sertoli and peritubular cells by flow cytometry

A total of 2 x 10^5 Sertoli or peritubular cells were used for the lectin binding assay. After washing with 1% BSA in PBS, cells were incubated for 1 h at RT with 50 µl of the corresponding plant lectin solution. Afterwards the cells were washed 3 times with 1% BSA in PBS. In order to exclude dead cells propidium iodide (PI) was added before the measurement and samples were analyzed with a MACSQuant 10 flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). Data were collected from 20,000 events and analyzed with FlowJo soſtware version 10.0.8 (Ashland, Oregon, USA).

The gating strategy is presented below (Figure 11). After gating out cell debris, doublets

and non-viable cells (Figure 11a - c), the cells binding a specific lectin were selected in the FITC channel (Figure 11d). For further analysis, the median of fluorescence intensity from each lectin binding experiment were collected.

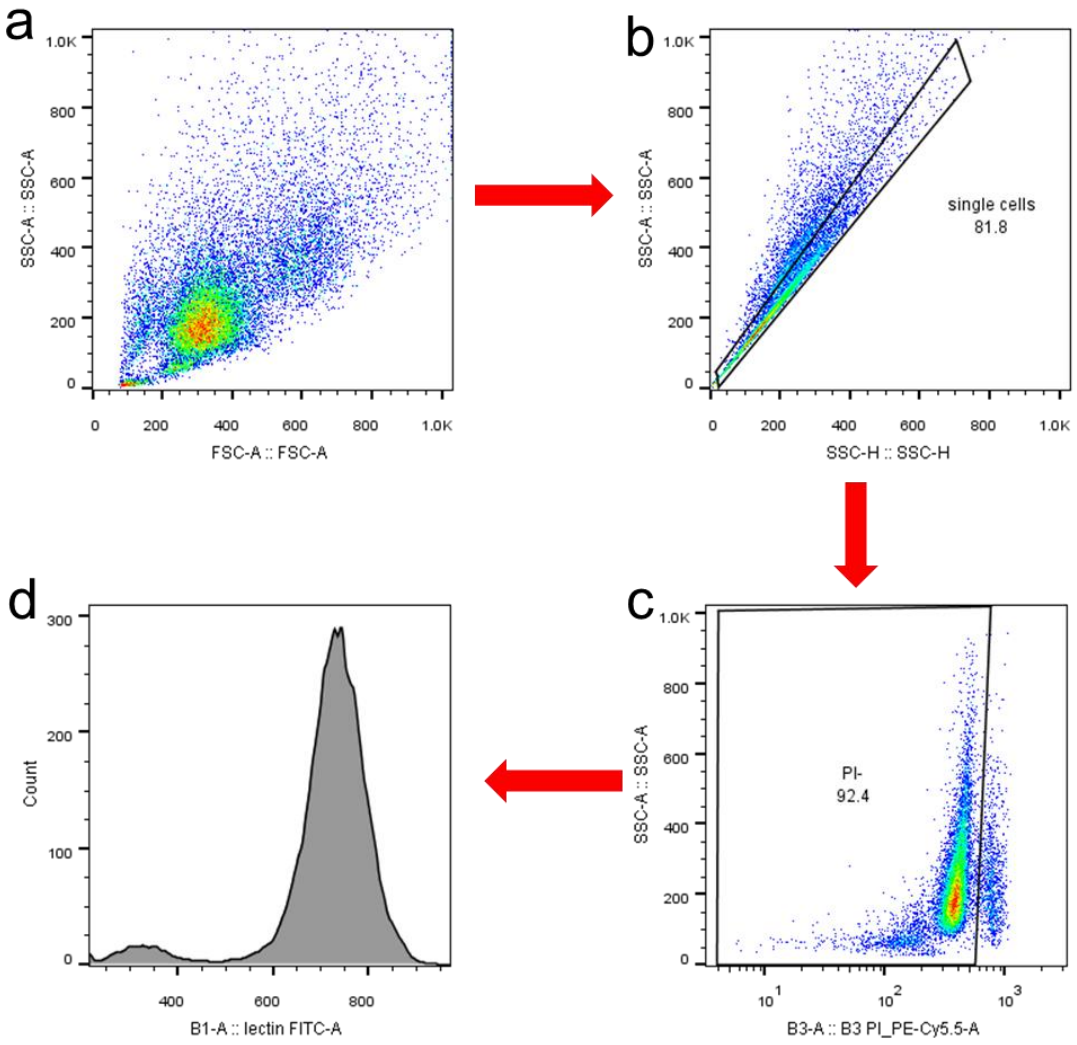

Figure 11. The gating strategy for flow cytometric analysis of lectin binding to Sertoli and peritubular cells. Detected cells were displayed by using forward scatter (FSC) and side scatter (SSC) (a). Doublets (b), cell debris and non-viable cells (c) were gated out. The FITC channel was selected to show indicated lectin binding (d).

3.10. Statistical analysis

Data are shown as mean ± SEM. Comparisons of lectin binding in untreated and TNFα treated cells were performed by a two-tailed t-test. One-way ANOVA followed by Tukey's multiple comparison post hoc tests were applied when more than two groups were compared. P-values < 0.05 were considered as statistically significant. All tests were performed using GraphPad Prism 5 software (GraphPad Software, San Diego, USA).

4. RESULTS

4.1. Histopathological changes in rat EAO

Fifty days after the first immunization 70% (7/10) of the animals manifested chronic testicular inflammation. EAO was evaluated by means of histopathological alterations and reduced testicular weight (Fijak *et al.*, 2005). Pathological changes in the testis included strong infiltration of the interstitium by leukocytes and cell loss in the germinal epithelium (Figure 12c) that was accompanied by a reduced testicular weight (Aslani *et al.*, 2015). Testes from untreated and adjuvant controls showed a completely normal morphology (Figure 12a and 12b).

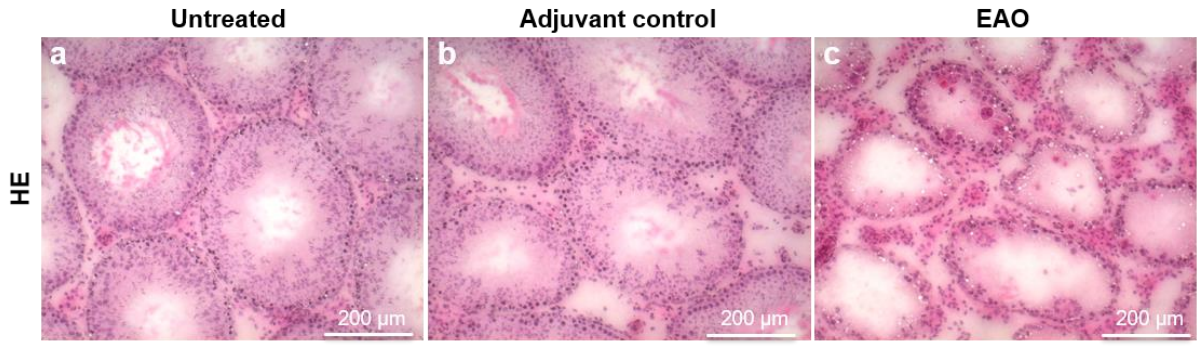

Figure 12. Testicular histopathological changes in EAO. Hematoxylin-eosin (HE) staining in cryostat sections from normal (a), adjuvant control (b) and EAO (c) rat testes.

4.2. Gal-1 is mainly expressed in Sertoli and germ cells

Immunofluorescence staining revealed that in normal testis Gal-1 was localized in seminiferous tubules, mainly in germ cells and in the cytoplasm of Sertoli cells as co-localization with vimentin showed (Figure 13a - d). In normal testis Gal-1 was detected in the basal and apical cytoplasm of Sertoli cells (Figure 13b and 13d). As shown in Figure 13e and 13f in inflamed testis only Sertoli cells expressed Gal-1 in otherwise 'empty' tubules depleted of germ cells.

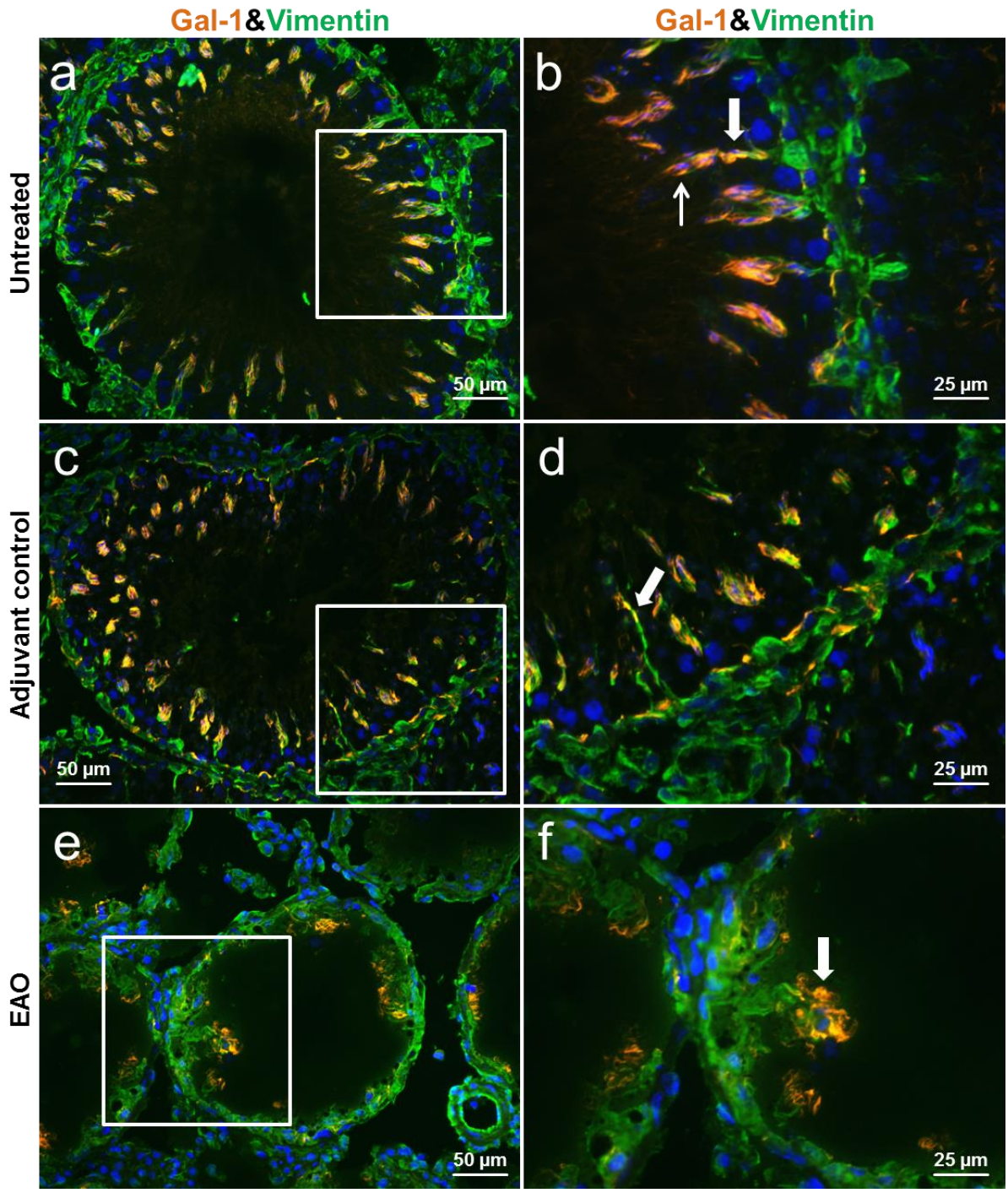

Figure 13. In normal rat testes Gal-1 is expressed mainly in Sertoli cells and germ cells. Localization of Gal-1 (Alexa 546, orange) in normal (a and b), adjuvant control (c and d) and EAO (e and f) testis. Vimentin (Alexa 488, green) was used as a marker of Sertoli cells. Right panels (b, d and f) show magnified areas in boxes (a, c and e), which display stained Gal-1 (Alexa 546, orange) in germ cells (thin arrow) and Sertoli cells (thick arrow) (b, d and f).

4.3. CD68+ macrophages in the vicinity of granulomas express Gal-1 in inflamed testes

To examine a possible localization of Gal-1 in testicular macrophages, two different macrophage makers (CD68 and CD163) were applied using double immunofluorescence. Co-staining revealed that in normal testes CD68+ macrophages were not positive for Gal-1 (Figure 14a and 14c). Interestingly, in inflamed testis Gal-1 was detected in a few CD68+ macrophages located in the vicinity of granulomas (Figure 14e and 14g). Of note, the expression of Gal-1 was not observed in CD163+ macrophages, both in normal and EAO testes (Figure 14b, 14d, 14f and 14h).

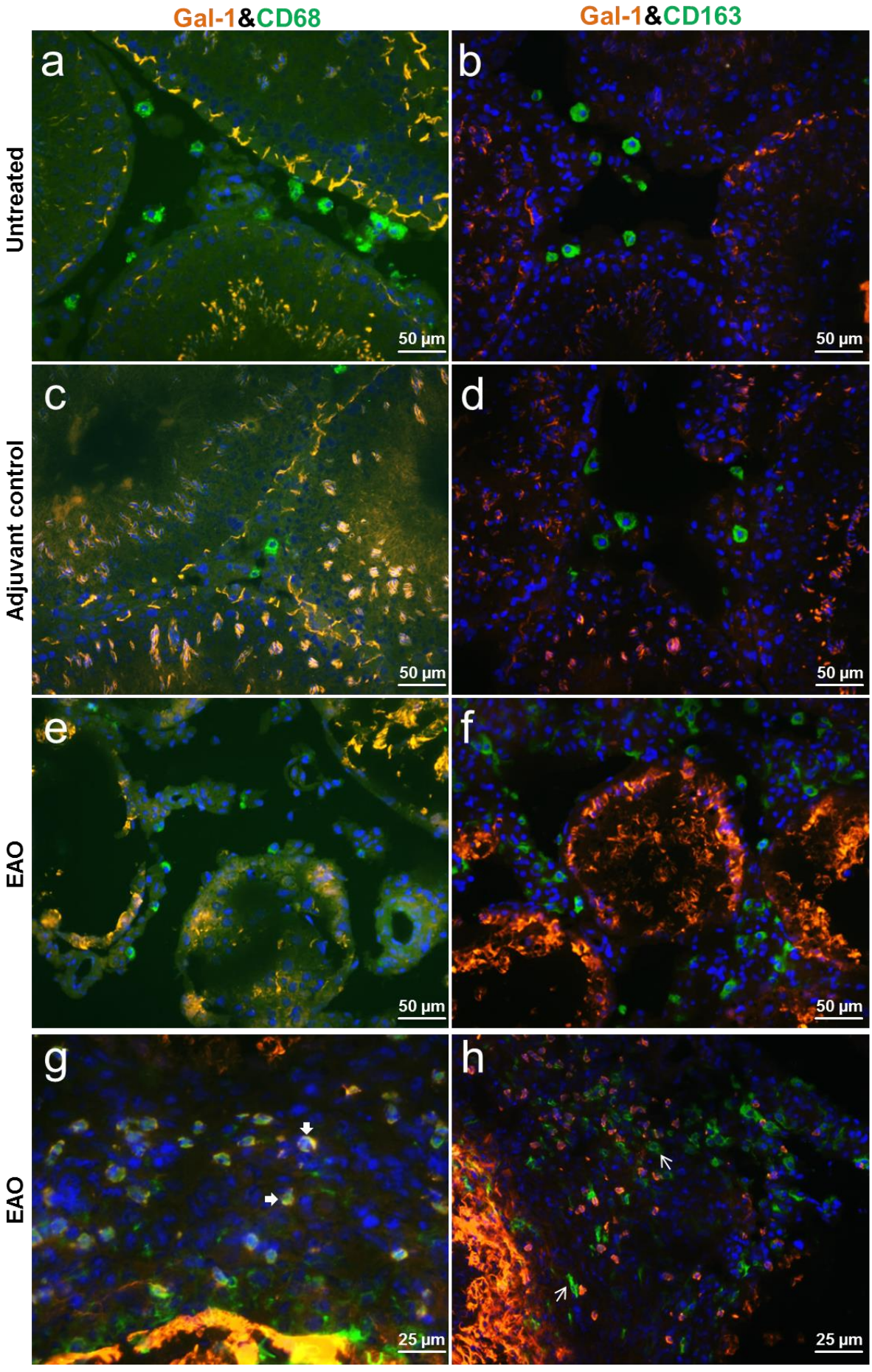
Gal-1&CD68
Gal-1&CD163
Untreated
Adjuvant control
EAO
EAO
a
b
c
d
e
f
g
h
50 µm
50 µm
50 µm
50 µm
50 µm
50 µm
25 µm
25 µm

Figure 14. CD68+ macrophages around granulomas express Gal-1 in inflamed testes. Localization of Gal-1 (Alexa 546, orange) in normal (a and b), adjuvant control (c and d) and EAO (e - h) testes. Staining of Gal-1 and CD68 (Alexa 488, green) or CD163 (Alexa 488, green) in the region of granulomas (g, h). Testicular macrophages were stained with CD68 and CD163 antibodies. Gal-1 was expressed in some CD68+ macrophages (g) found around granulomas (thick arrows), but not in CD163+ macrophages (h; thin arrows).

4.4. Expression of Gal-1 in EAO testis is decreased due to germ cell loss

In order to investigate testicular expression of Gal-1 in the EAO model, testes from untreated, adjuvant control and EAO rats were processed for Western blot and real-time PCR analyses. Gal-1 protein levels in EAO testes were downregulated as compared to normal and adjuvant control testes (Figure 15a - b). Similarly, relative expression of Gal-1 mRNA was also reduced in inflamed testes (Figure 15c). Because the ratio of testicular cell types in EAO testis is changed due to the loss of germ cells and infiltration of immune cells, the relative expression of Gal-1 mRNA was also normalized to the Sertoli cell specific transcript Sox9 (Figure 15d). These data indicate that the mRNA expression of endogenous Gal-1 in Sertoli cells was not changed in EAO testis as compared to control testis at the investigated time point.

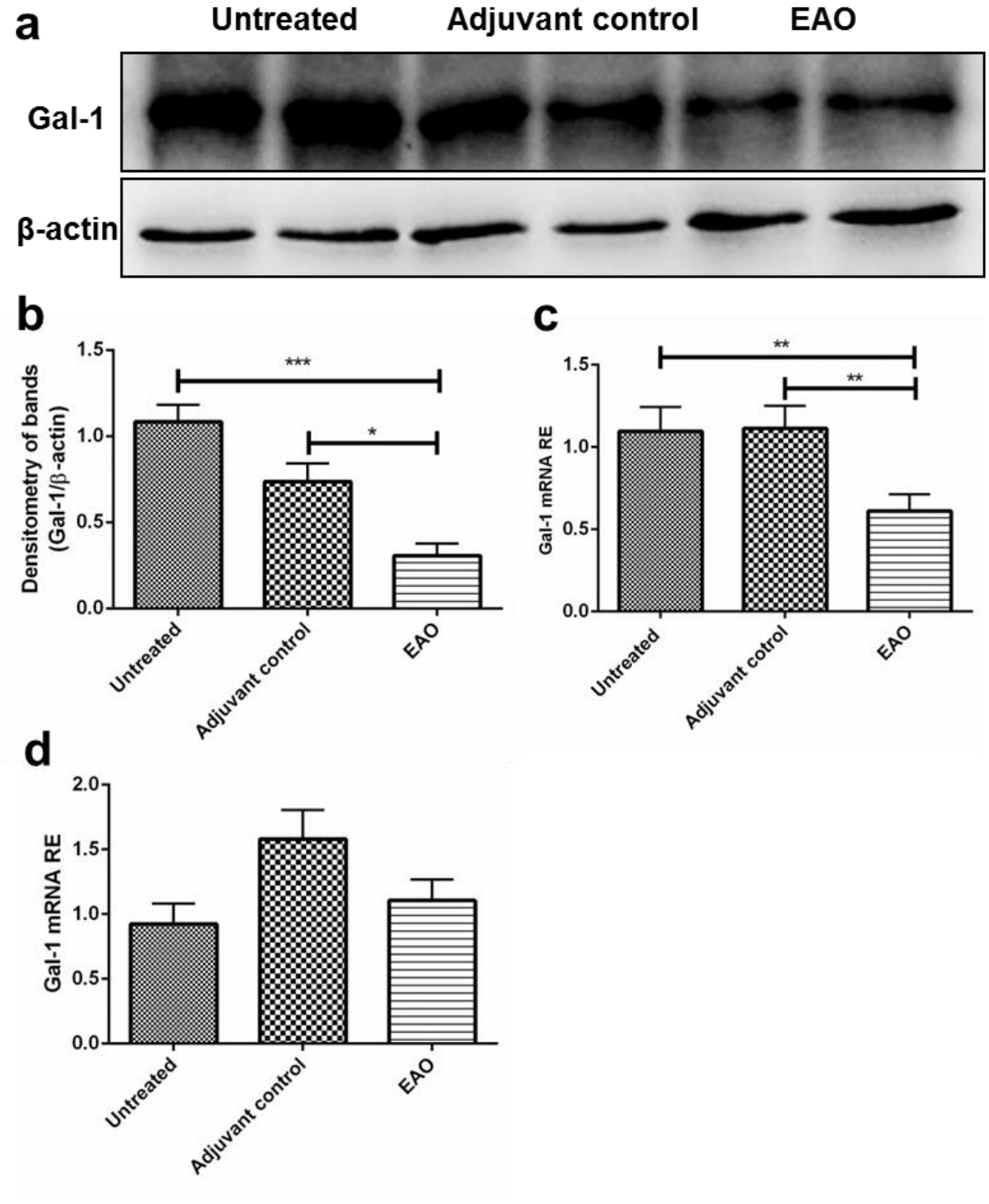

Figure 15. Changes in the expression of Gal-1 in EAO testes are due to germ cell loss. Western blot (a) and densitometric (b) analysis of Gal-1 expression in testes from untreated, adjuvant control and EAO animals. Gal-1 mRNA relative expression (RE) was normalized to three housekeeping genes (β-actin, Hprt and 18s rRNA) (c) or Sertoli cell marker Sox9 (d). (n = 5, * $P<0.05$, ** $P<0.01$, *** $P<0.001$).

4.5. Increase of St6gal1 mRNA expression and terminal α-2-6-sialylation in EAO testis

Gal-1 binds to N-acetyllactosamine (LacNAc) present on branches of N- and O-glycans on the cell surface (Cedeno-Laurent and Dimitroff, 2012). There are three important post-translational mechanisms to form Gal-1 binding sites including: (a) activity of core 2 glucosaminyl (N-acetyl) transferase 1 (Gcnt1) for the synthesis of core 2 O-glycans, which built the backbone of Gal-1 ligands, (b) suppression of ST6 beta-galactoside α-2-6-sialyltransferase 1 (St6gal1) activity, that abrogates Gal-1 binding to some terminal N-acetylglucosamines by adding α-2-6-sialic acid and (c) branching of N-glycans by mannosyl (α-1,3-)-glycoprotein ß-1,2-N-acetylglucosaminyltransferases (Mgat) like Mgat5 (Figure 16a) (Cedeno-Laurent and Dimitroff, 2012).

The results showed that the level of St6gal1 mRNA in EAO testes was upregulated (Figure 16b). In contrast, Mgat5 mRNA expression was downregulated as compared to control testes (Figure 16c).

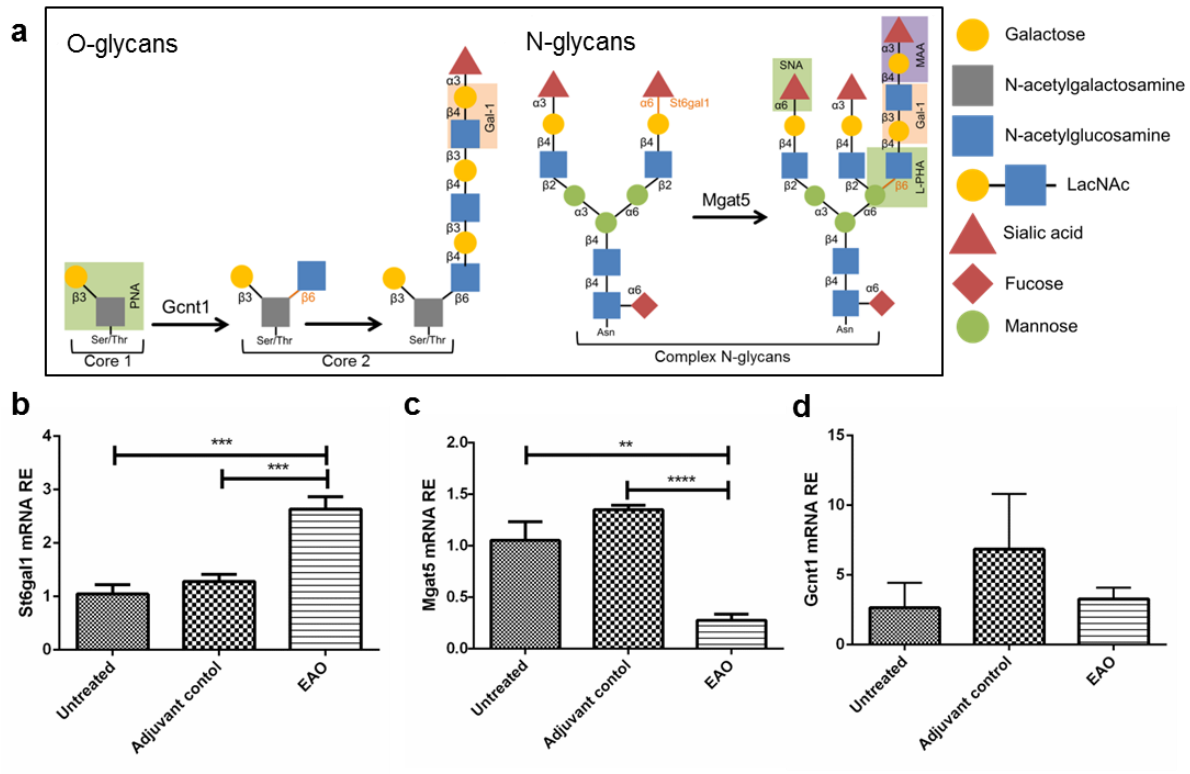

Figure 16. Expression analysis of transferases involved in glycan biosynthesis in EAO testes. (a) Schematic representation of N- and O-glycan biosynthesis. St6gal1 (b), Mgat5 (c) and Gcnt1 (d) relative mRNA expression was normalized to β-actin, Hprt and 18s rRNA. (n = 5, ** P<0.01, *** P<0.001).

At the same time, binding of SNA, that recognizes terminal α-2-6 sialic acid residues (red triangles in Figure 16a), was increased as compared to untreated and adjuvant control testis (Figure 17a - c). In contrast, binding of *Phaseolus vulgaris* agglutinin (L-PHA), that recognizes ß-5-ß-4-N-acetyl–glucosamine (blue squares in Figure 16a), was reduced in EAO testis (Figure 17d - f). These data indicate that α-2-6-sialylation of O- and N-glycans is elevated in inflamed testis. Notably, expression of Gcnt1 mRNA was unchanged (Figure 16d), whereas binding of peanut agglutinin (PNA), that recognizes asialo-galactose ß-1-3-N-acetylgalactosamine (core-1) in O-glycans, was decreased in EAO testis (Figure 17g - i). However, we did not observe any significant change in the binding of MAA, that is recognizing NeuNAcα (2-3) Galβ (1-4) GlcNAc/Glc, in EAO testis (Figure 17j - l).

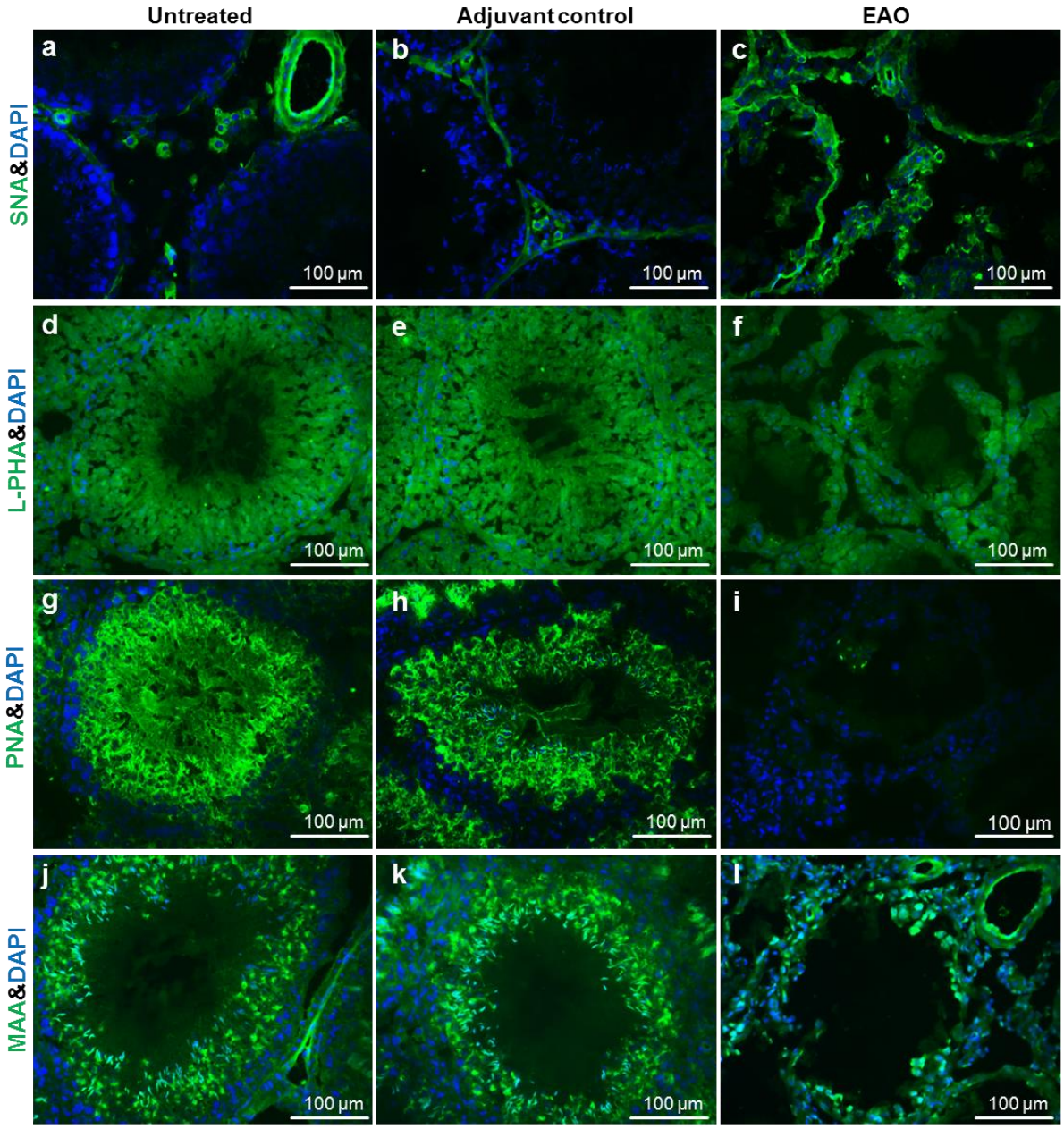

Figure 17. Expression analysis of lectin-FITC binding in EAO testes. Binding of FITC labeled SNA (a - c), L-PHA (d - f), PNA (g - i), and MAA (j - l) to testicular sections from untreated (a, d, g and j), adjuvant control (b, e, h and k) and EAO (c, f, i and l) animals.

4.6. The binding of SNA to Sertoli and peritubular cells is increased after TNFα stimulation, whereas binding of L-PHA is decreased

Since inflammatory conditions influence the glycophenotype of cells, we investigated the binding of different lectins that selectively recognize specific oligosaccharide structures to TNFα stimulated primary Sertoli and peritubular cells by flow cytometry (Figure 18a). The binding of SNA to Sertoli (Figure 18a, b) and peritubular cells (Figure 18a, c) challenged by TNFα was significantly increased compared to untreated cells. In contrast, the binding of L-PHA to TNFα stimulated Sertoli cells (Figure 18b) and peritubular cells (Figure 18c) was significantly reduced as compared to control cells. Increased binding of MAA was only found in TNFα stimulated Sertoli cells, whereas the binding of PNA to stimulated Sertoli and peritubular cells was unchanged (Figure 18b - c).

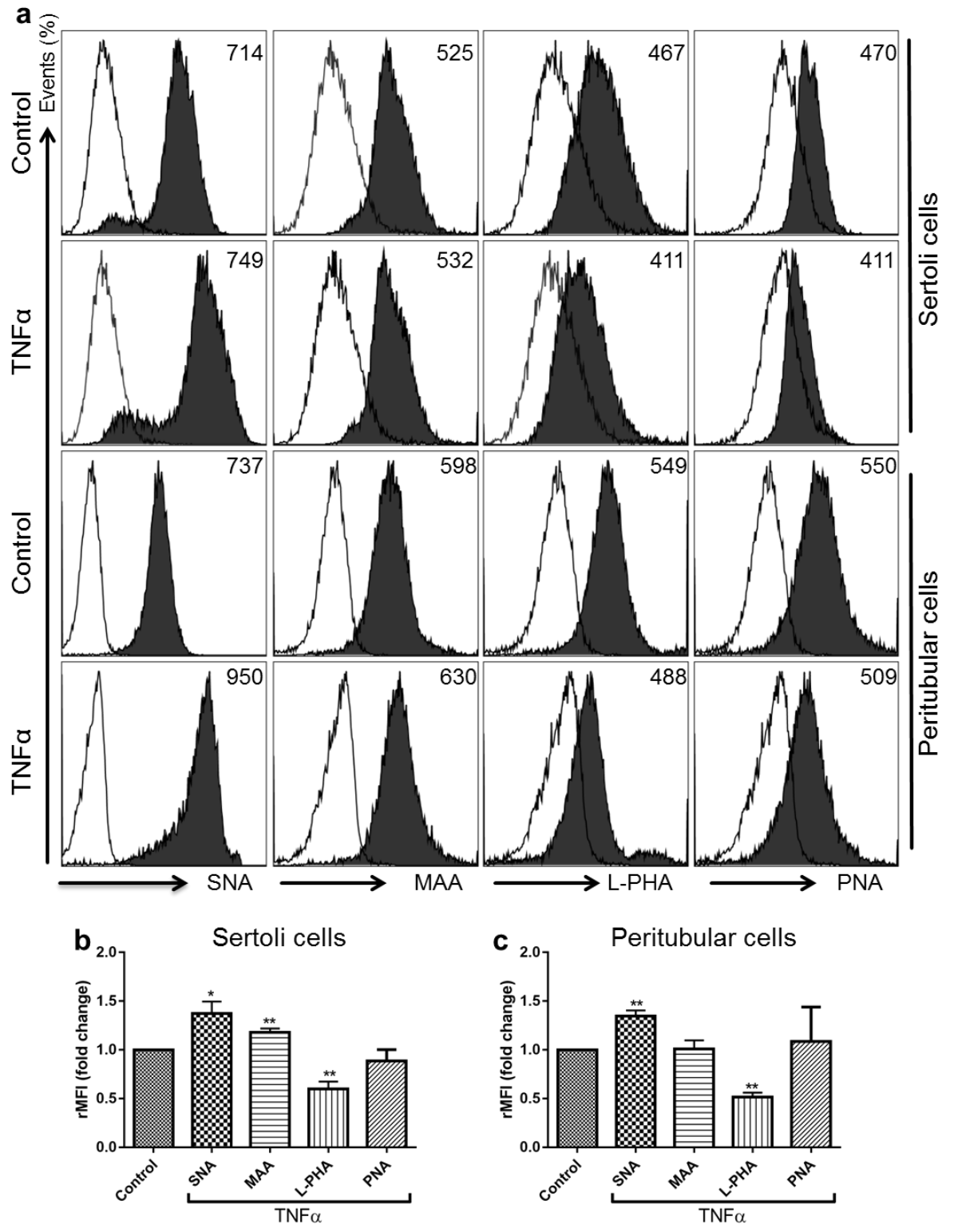

Figure 18. Influence of TNFα stimulation on the glycan profile of Sertoli and peritubular cells. (a) Flow cytometric analysis of cell-surface glycans in Sertoli and peritubular cells after stimulation with 25 ng/ml TNFα was detected by staining cells with FITC-labeled lectins (SNA, MAA, L-PHA, or PNA) (black filled histograms) or without

(open histograms). Numbers in the upper-right corner represent the median of fluorescence intensity. The binding of FITC-labelled lectins to Sertoli (b) or peritubular cells (c) was quantified as relative median fluorescence intensity (rMFI); (rMFI = (MFI with lectin – MFI without lectin)/MFI without lectin) (n = 3-5, * P<0.05, ** P<0.01, *** P<0.001).

4.7. Gal-1 is upregulated in Sertoli cells after TNFα stimulation

The inflammatory cytokine TNFα is highly upregulated in EAO testis (Theas *et al.*, 2008; Aslani *et al.*, 2015). To examine the influence of an inflammatory environment on Gal-1 expression, TNFα was used to stimulate primary Sertoli cells. After stimulation, a dose-dependent increase in Gal-1 expression was observed in Sertoli cells as compared to untreated cells (Figure 19).

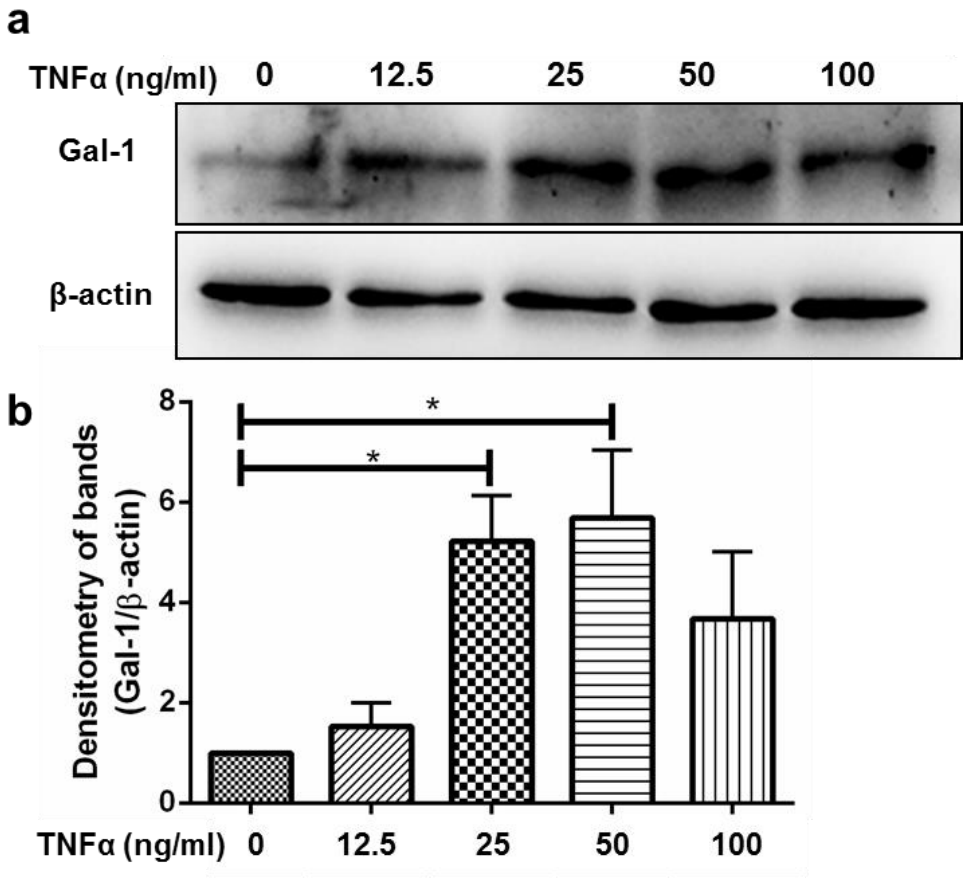

Figure 19. Analysis of Gal-1 expression in primary Sertoli cells. Western blot (a) and densitometric (b) analysis of Gal-1 expression in primary Sertoli cells after TNFα stimulation. (n = 5, * P<0.05).

4.8. Gal-1 and TNFα synergistically induce an inflammatory response in Sertoli cells

To determine whether binding of Gal-1 to Sertoli cells can modulate the inflammatory response, we analyzed expression of inflammatory cytokines in TNFα-stimulated (25 ng/ml) Sertoli cells and stimulated cells that were pretreated with Gal-1 (5 µg/ml). In Sertoli cells treated with TNFα only, mRNA expression levels of IL-1α (Figure 20a), MCP1 (Figure 20c) and IL-6 (Figure 20e) were increased. In contrast, mRNA expression of TGFβ2 was not affected after TNFα stimulation as compared to untreated cells, although it was increased when compared to stimulation with Gal-1 alone (Figure 20d). Pretreatment of Sertoli cells with recombinant Gal-1 prior to the addition of TNFα synergistically induced expression of IL-1α, TNFα, MCP1, and IL-6 mRNA (Figure 20a – c, e). These effects were abrogated by the addition of lactose to the Gal-1 solution 5 min prior to stimulation of Sertoli cells. Of note, Sertoli cells did not respond with an inflammatory response after treatment with Gal-1 alone (Figure 20).

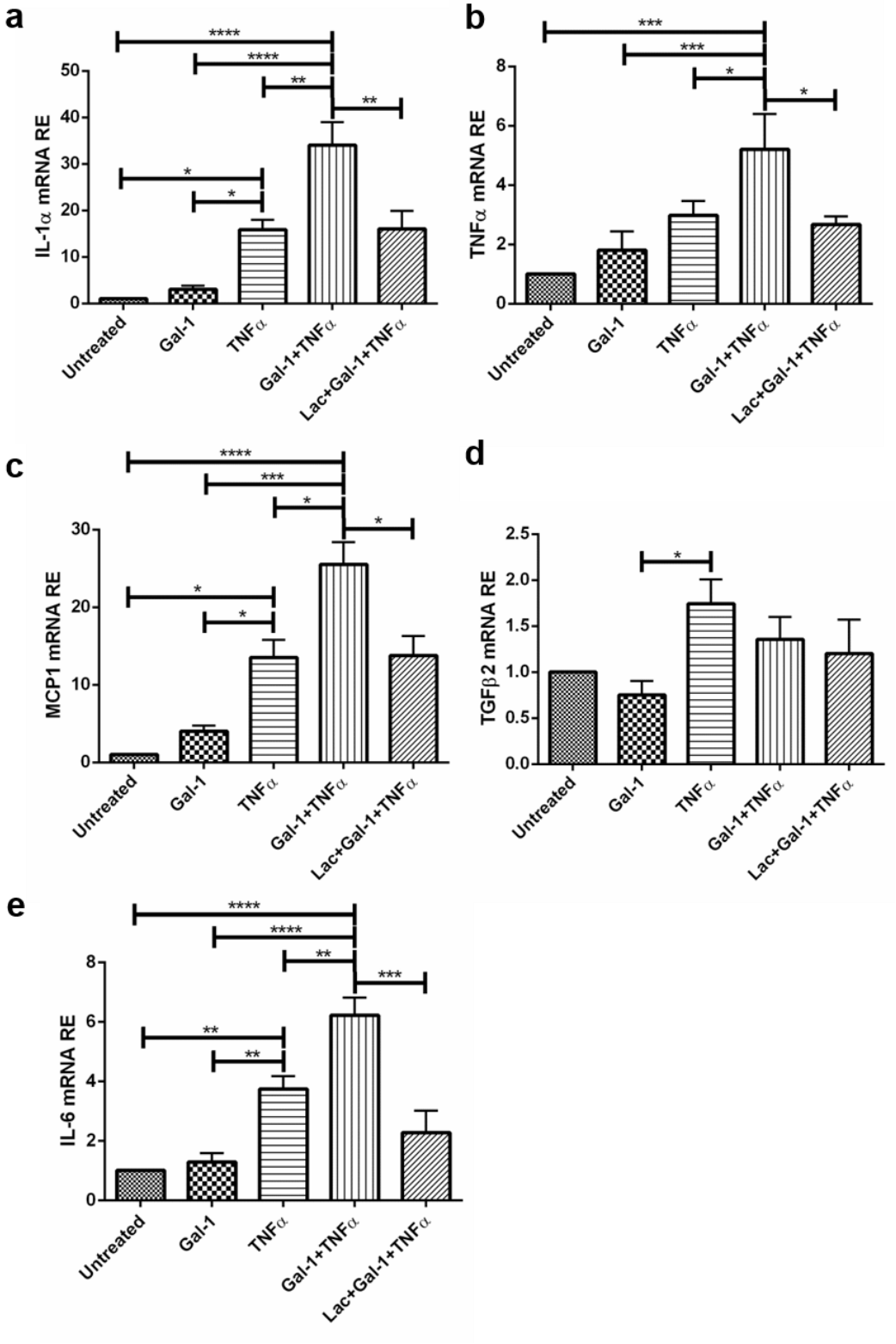

Figure 20. Gal-1 and TNFα act synergistically on the expression of pro-inflammatory mediators in Sertoli cells. Primary Sertoli cells were pretreated with Gal-1 (5 µg/ml) for 2h, and then stimulated with TNFα (25 ng/ml) for 6h. Lactose (Lac, 50 mM) was used as an inhibitor of Gal-1 binding. Relative mRNA expression of IL-1α (a), TNFα (b), MCP1 (c),

TGFβ2 (d), and IL-6 (e) was normalized to Hprt; (n = 3-5, * P<0.05, ** P<0.01, *** P<0.001).

4.9. Gal-1 and TNFα synergistically activate phosphorylation of MAPK p38 and JNK

To better understand the mechanisms underlying the synergistic effects of Gal-1 and TNFα on the expression of pro-inflammatory cytokines IL-1α and MCP1, we evaluated the activation kinetics of mitogen-activated protein kinases (MAPK) in Sertoli cells following TNFα and Gal-1 treatment. Sertoli cells stimulated with TNFα showed increased phosphorylation of p38 and JNK from 15-30 min after stimulation (Figure 21a, lanes 2 and 3). Interestingly, pretreatment of Sertoli cells with Gal-1 prior to TNFα stimulation synergistically enhanced phosphorylation of p38 and JNK 15-30 min after stimulation (Figure 21a, compare lanes 2 and 3 with lanes 7 and 8; Figure 21c and d).

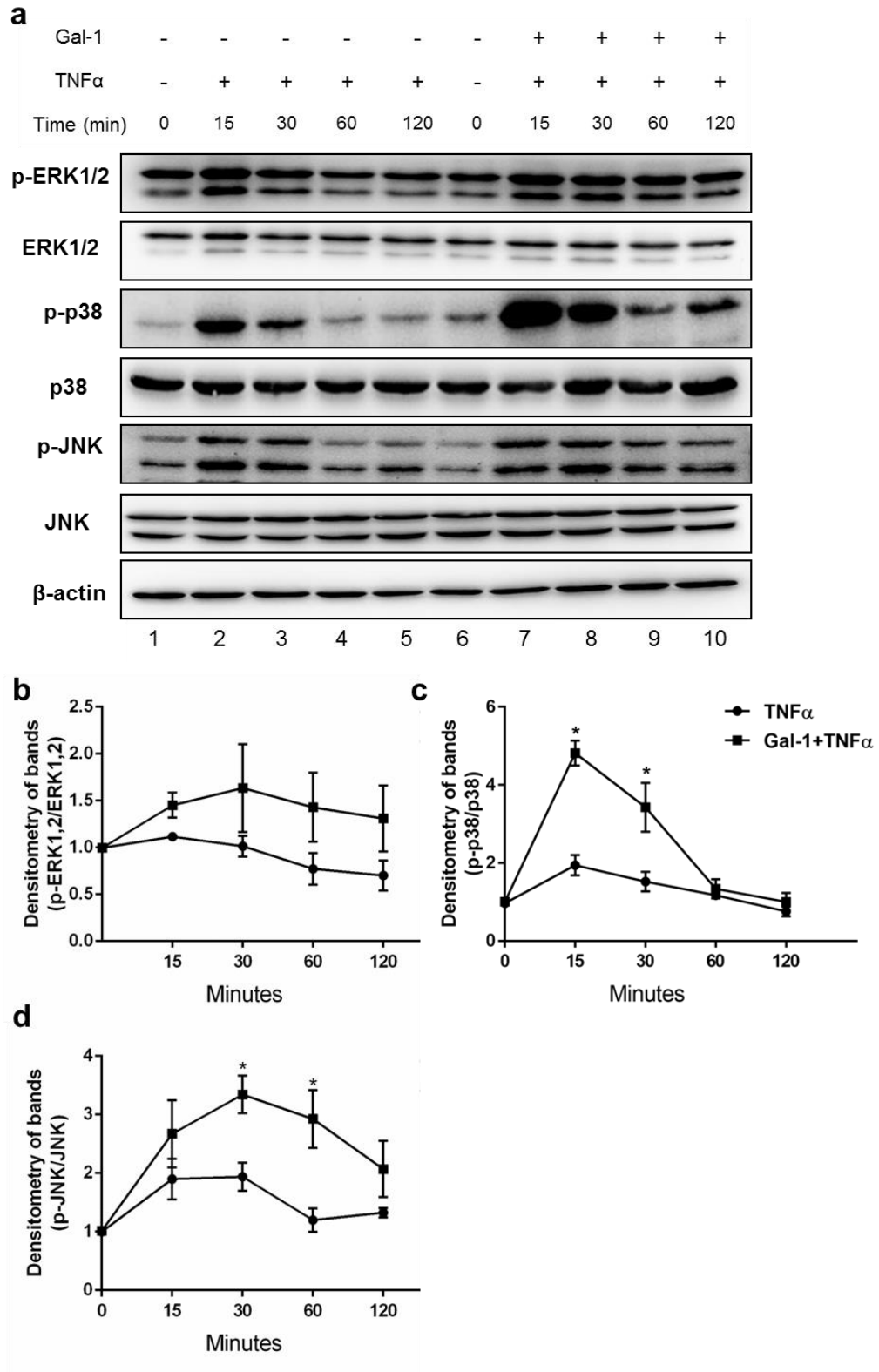

Figure 21. Effects of Gal-1 and TNFα on MAPK phosphorylation in Sertoli cells. (a) Isolated Sertoli cells were pretreated with Gal-1 (5 μg/ml; 2h) and then stimulated with TNFα (25 ng/ml) for 0-120 min. Subsequently, phosphorylation of MAP kinases ERK1/2, p38 and JNK was investigated by Western blotting. Densitometric analysis of ERK1/2 (b), p38 (c) and

JNK (d) phosphorylation in primary Sertoli cells after TNFα and Gal-1 stimulation (n = 3, * P<0.05).

In contrast, no activation of MAPK was detected when Sertoli cells were treated with Gal-1 alone (Figure 22a, compare lanes 1 and 2, and 6 and 7). Gal-1 induced phosphorylation of p38 and JNK in the presence of TNFα was specific, because the effect could be abrogated by adding 50 mM lactose (Figure 22a, lanes 5 and 10, Figure 22d, e, g).

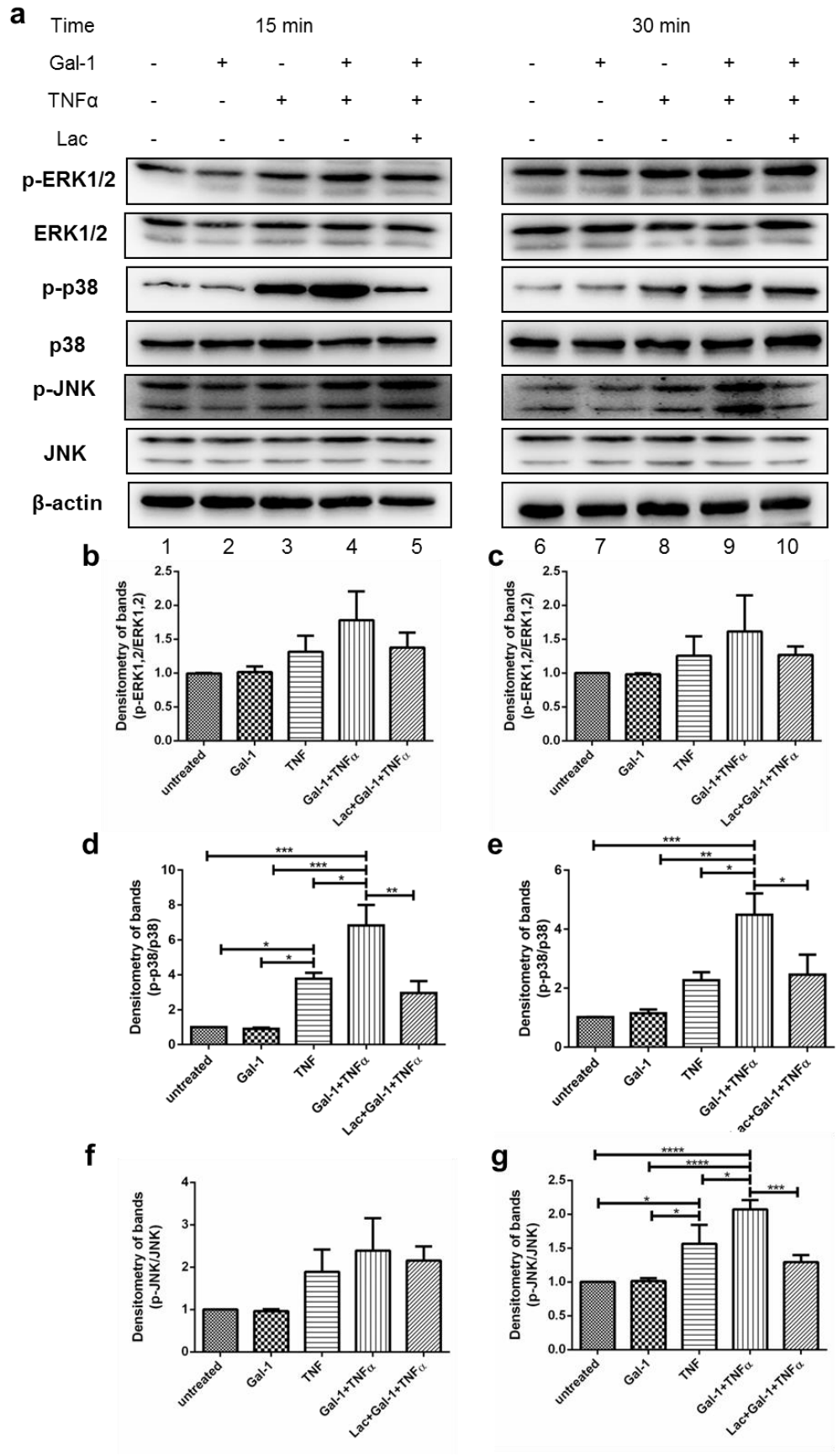

Figure 22. Lactose abrogates effects of Gal-1 on MAPK phosphorylation in TNFα treated Sertoli cells. (a) Gal-1 was pre-incubated with lactose (50 mM) for 5 min prior to

addition to Sertoli cells. After 2 h Sertoli cells were stimulated with TNFα (25 ng/ml) for the indicated times. Subsequently phosphorylation of MAP kinases ERK1/2, p38 and JNK was investigated by Western blotting. Densitometric analyses of Figure 22a are shown in Figure 22b – g. Stimulation with TNFα was for 15 min (b, d, f) or 30 min (c, e, g); (n = 3-4, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001).

4.10. Treatment of Sertoli cells with p38 and JNK inhibitors abrogates the Gal-1 and TNFα induced IL-1α, TNFα, IL-6 and MCP-1 mRNA expression

In order to determine whether the effect of Gal-1 and TNFα treatment on inflammatory cytokine response is induced specifically through the MAPK signaling pathway, we used a p38 inhibitor (SB 203580, 5 mM) and a JNK inhibitor (SP600125, 20 mM) during Gal-1 and TNFα stimulation. In the presence of both inhibitors the TNFα stimulated mRNA expression of IL-1α (Figure 23a), MCP-1 (Figure 23c) and IL-6 (Figure 23e) was completely abrogated. Furthermore, the synergistic effects of Gal-1 and TNFα on IL-1α (Figure 23a), TNFα (Figure 23b), MCP-1 (Figure 23c) and IL-6 (Figure 23e) mRNA expression levels were completely reversed.

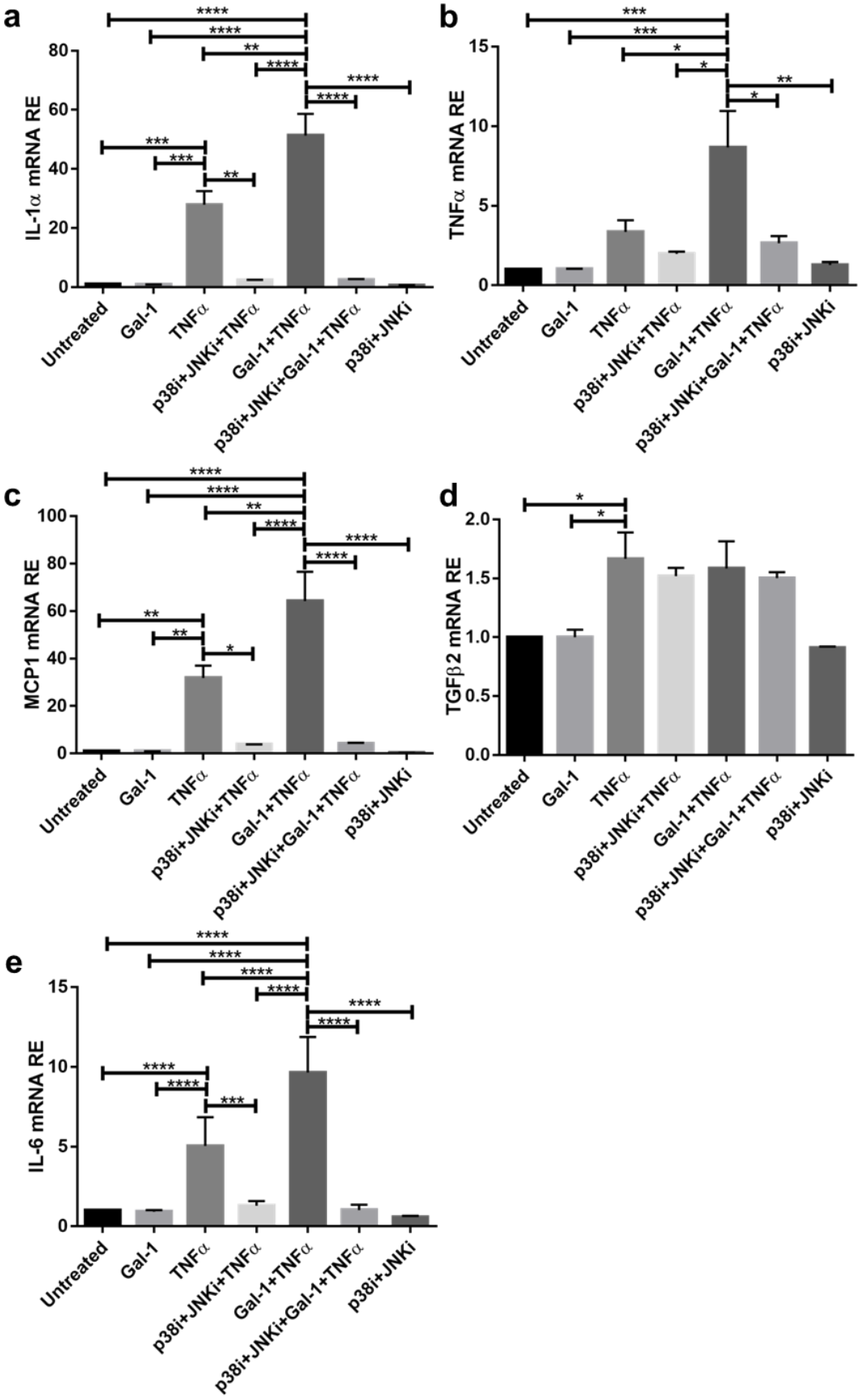
a
IL-1α mRNA RE
b
TNFα mRNA RE
c
MCP1 mRNA RE
d
TGFβ2 mRNA RE
e
IL-6 mRNA RE
Untreated
Gal-1
TNFα
p38i+JNKi+TNFα
Gal-1+TNFα
p38i+JNKi+Gal-1+TNFα
p38i+JNKi

Figure 23. Inhibitors of p38 and JNK MAP kinases reverse the synergistic effect of Gal-1 and TNFα on expression of inflammatory mediators in Sertoli cells. Primary Sertoli cells were pretreated with Gal-1 (5 µg/ml) for 1 h prior to addition of the p38 inhibitor SB 203580 (5 mM; p38i) and the JNK inhibitor SP600125 (20 mM; JNKi) for 1 h. Afterwards Sertoli cells were stimulated with TNFα (25 ng/ml) for 6 h. Relative mRNA expression of IL–1α (a), TNFα (b), MCP1 (c), TGFβ2 (d) and IL-6 (e) was normalized to Hprt; (n = 4-7, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001).

Treatment of Sertoli cells with either the p38 inhibitor alone (Figure 24) or the JNK inhibitor alone (Figure 25) considerably reversed synergistic expression of IL-1α, TNFα, MCP1 and IL-6 mRNA after induction with Gal-1 and TNFα.

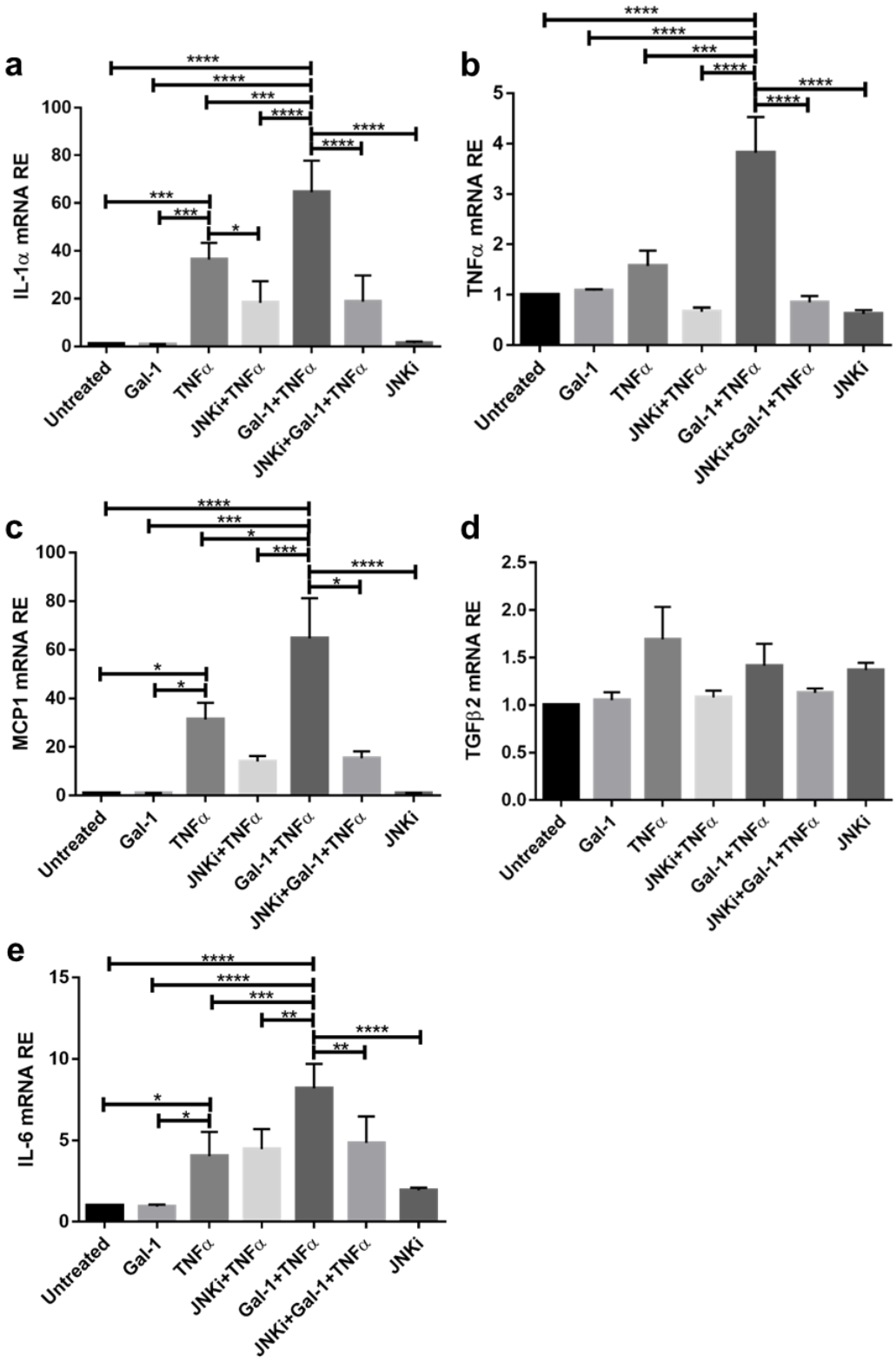

Figure 24. Inhibition of JNK MAP kinase partially reversed Gal-1 and TNFα induced expression of inflammatory mediators in Sertoli cells. Primary Sertoli cells were pretreated with Gal-1 (5 µg/ml) for 1 h prior to addition of JNKi for 1 h. Afterwards Sertoli cells were stimulated with TNFα (25 ng/ml) for 6 h. Relative mRNA expression of IL-1α (a),

TNFα (b), MCP1 (c), TGFβ2 (d) and IL-6 (e) was normalized to Hprt; (n = 4-7, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001).

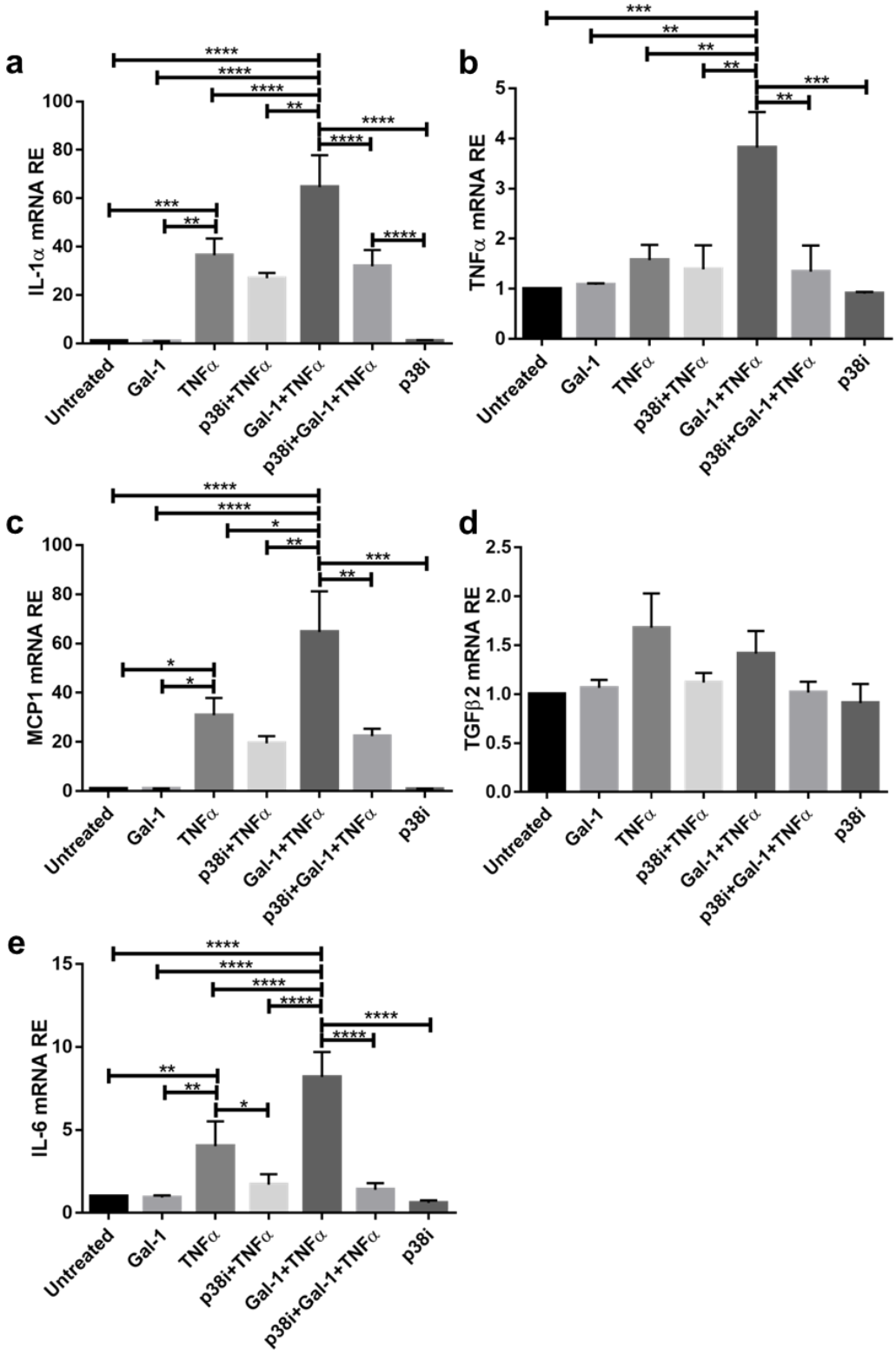

Figure 25. Inhibition of p38 MAP kinase partially reversed Gal-1 and TNFα induced expression of inflammatory mediators in Sertoli cells. Primary Sertoli cells were

pretreated with Gal-1 (5 µg/ml) for 1 h prior to addition of p38i for 1 h. Afterwards Sertoli cells were stimulated with TNFα (25 ng/ml) for 6 h. Relative mRNA expression of IL-1α (a), TNFα (b), MCP1 (c), TGFβ2 (d) and IL-6 (e) was normalized to Hprt; (n = 4-7, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$).

5. DISCUSSION

An increasing body of evidence indicates that Gal-1 has immunoregulatory functions in autoimmune disease models such as systemic lupus erythematosus, lysolecithin-induced demyelination, experimental autoimmune encephalomyelitis (EAE) and experimental arthritis (Iqbal *et al.*, 2013; Mari *et al.*, 2016; Rinaldi *et al.*, 2016; Hornung *et al.*, 2017). The immunoregulatory characteristics of Gal-1 range from induction of apoptosis in Th1 and Th17 cells (Toscano *et al.*, 2007), inhibition of T cell migration (Norling *et al.*, 2008), expansion of tolerogenic dendritic cells and regulatory T cells (Toscano *et al.*, 2006; Ilarregui *et al.*, 2009), to maintenance of the maternal-fetal tolerance (Blois *et al.*, 2007). Taking into account that Gal-1 is also abundantly expressed in immune privileged organs such as the placenta, eye, brain, ovary and testis (Rabinovich and Croci, 2012), we propose that Gal-1 may play a role in the maintenance of testicular immune privilege. We therefore investigated the expression of Gal-1 and relevant glycans in the inflamed testis by using a model of experimental autoimmune orchitis and asked whether Gal-1 could modulate the pro-inflammatory response of Sertoli cells challenged with the archetypal cytokine TNFα.

5.1. Testicular pathological changes in rat EAO

In order to mimic symptoms and pathological changes of human chronic testicular inflammation in an animal model, adult male Wistar rats were used to induce EAO. For this purpose, a rat EAO model was adopted using testicular homogenate in combination with complete Freund's adjuvant and inactivated Bordetella pertussis bacteria. This protocol has been used previously in rats and mice (Doncel *et al.*, 1989; Fijak *et al.*, 2011b; Aslani *et al.*, 2015; Nicolas *et al.*, 2017). Pathological changes in EAO testis include strong interstitial leukocytic infiltration of the interstitium and loss of the germinal epithelium, hallmarks also observed in previous studies (Doncel *et al.*, 1989; Fijak *et al.*, 2011b; Aslani *et al.*, 2015). Furthermore, the induction rate in this study was comparable to the induction rate mentioned in previous studies in rat (Doncel *et al.*, 1989; Fijak *et al.*, 2011b; Aslani *et al.*, 2015).

TNFα as a pro-inflammatory cytokine is highly upregulated in EAO testis. The factor is mainly produced by testicular macrophages and T lymphocytes, and is thought to play a major role in the progression of the disease (Yule and Tung, 1993; Suescun *et al.*, 2003; Theas *et al.*, 2008; Aslani *et al.*, 2015). In this regard, administration of TNFα directly to adult rat testes can disrupt the integrity of the BTB, impair Sertoli-germ cell adhesion, and induce germ cell apoptosis (Li *et al.*, 2006; Theas *et al.*, 2008). In contrast, antibody neutralization of TNFα could reduce the incidence and severity of autoimmune orchitis induced by adoptive transfer of T cells in mice (Yule and Tung, 1993). More recently, an *in vitro* study showed that the apoptotic effect of conditioned media obtained from testicular macrophages in EAO testes on germ cells is abrogated by the selective TNFα blocker Etanercept (Theas *et al.*, 2008). Thus, TNFα seems to play an important role in the pathogenesis of EAO and was therefore used in this study to mimic testicular inflammatory conditions during *in vitro* treatment of primary testicular cells.

5.2. Gal-1 is expressed in Sertoli cells, germ cells and macrophages around granuloma

In line with previous studies (Wollina *et al.*, 1999; Timmons *et al.*, 2002; Dettin *et al.*, 2003; Chui *et al.*, 2011), this study also shows that Gal-1 is mainly expressed in Sertoli cells and germ cells of the normal rat testis in a stage-specific pattern throughout the spermatogenic process. Dettin *et al.* described that Gal-1 is strongly expressed on apical stalks of Sertoli cells and on the head of spermatids during spermiation (stages VI - VIII) and restored at the basal part of Sertoli cells after spermiation (Dettin *et al.*, 2003). Martinez *et al.* found that *in vitro* treatment with recombinant Gal-1 induces Leydig cells apoptosis and thus reduces testosterone production in a dose-dependent manner (Martinez *et al.*, 2004). Gal-1 was also found in the epididymis where it is associated with sperm maturation (Vasen *et al.*, 2015). Exposure of sperm to Gal-1 resulted in an increased percentage of progesterone induced acrosome reaction and Gal-1 deficient sperms display defects in their capacity to develop hyperactivation a further indicator that Gal-1 is involved in sperm function and fertilization (Vasen *et al.*, 2015).

In the EAO testis, some CD68+ macrophages in the vicinity of granulomas were positive for Gal-1. Since 60-70% of macrophages in granuloma lesions are CD68 positive and local macrophage proliferation plays a key role in granuloma formation (Lan *et al.*, 1995), it is possible that Gal-1 induces macrophage proliferation and promotes granuloma formation. In support, Gal-1 can induce proliferation of vascular endothelial and smooth muscle cells and promote fibrosis during chronic pancreatitis/pancreatic cancer (Moiseeva *et al.*, 2000; Kanda *et al.*, 2015; Tang *et al.*, 2018). Several studies have also shown that Gal-1 could be involved in the resolution of inflammation by regulating inflammatory signaling as well as accumulation and phagocytosis of macrophages and microglial cells (Starossom *et al.*, 2012; Rostoker *et al.*, 2013; Gaudet *et al.*, 2015). Moreover, Gal-1 can modulate macrophages to a pro-regenerative phenotype characterized by increased arginase-1 and reduced IL-6 and iNOS production in mouse bone-marrow derived macrophages (Abebayehu *et al.*, 2017). Similar to the inflamed testis, Gal-1 expression was limited to macrophages in the vicinity of spinal cord lesions (Gaudet *et al.*, 2015). Moreover, Gal-1 was preferentially expressed by peritoneal $CD11b^{high}$ macrophages as compared to $CD11b^{low}$ macrophages. The $CD11b^{high}$ macrophages had a distinct phenotype characterized by a decreased expression of TNFα and IL-1β, and increased expression of TGFβ. Expression of Gal-1 declined once the cells were converted to the $CD11b^{low}$ phenotype as shown in a mouse peritonitis model (Rostoker *et al.*, 2013). It can be speculated that Gal-1 facilitates the resolution of macrophage-mediated inflammation during peritonitis and may have a similar function during orchitis.

5.3. Downregulation of Gal-1 in orchitis testis is due to germ cell loss

Gal-1 was lower expressed in the inflamed testis, whereas its mRNA expression levels did not change when real-time PCR results were normalized to the Sertoli cell specific transcript Sox9. This suggests that downregulation of Gal-1 in the EAO testis is the consequence of germ cell loss. These results are in line with data published in a mouse model of EAO (Pérez *et al.*, 2015). In contrast, in isolated Sertoli cells Gal-1 expression was upregulated after TNFα stimulation. Addition of exogenous Gal-1 potentiated the TNFα effect in Sertoli cells. Therefore, higher Gal-1 levels induced by inflammatory mediators in the inflamed testis

could play a pro-inflammatory role in contrast to an anti-inflammatory function as a means to sustain immune privilege in the normal testis. In the normal testis Gal-1 produced by Sertoli cells is able to promote differentiation of tolerogenic dendritic cells and Treg, further supporting a role of endogenous Gal-1 in the maintenance of testicular immune privilege (Gao *et al.*, 2016).

5.4. The change of glycocalyx signature in inflamed testis

Gal-1 binds to the disaccharide lactosamine, LacNAc, on complex O-glycans and N-glycans. Structural variations of glycans in mammalian cells can strongly affect the binding of galectins. As an example Gal-1 can bind to terminal α-2-3 sialic acid capping the lactosamine-containing sequence, but not to α-2-6 sialic acid (Stowell *et al.*, 2008). Gal-2 displayed significantly reduced binding to both α-2-3 sialic acid and α-2-6 sialic acid capping the lactosamine-containing sequence (Stowell *et al.*, 2008). In contrast, Gal-3 has a more flexible CRD showing that neither α-2-3 sialic acid nor α-2-6 sialic acid decorating the lactosamine-containing sequence affects Gal-3 binding (Hirabayashi *et al.*, 2002). Thus, the analysis of the glycocalyx signature on the cell surface by specific lectin binding can indicate potential binding capacity for different galectins.

The biosynthesis of glycan ligands for binding galectins is regulated by several glycosyltransferases (Méndez-Huergo *et al.*, 2017). Expression of Gcnt1 for the synthesis of the backbone on O-glycans increases Gal-1 binding to the cell surface (Cedeno-Laurent and Dimitroff, 2012). Similarly, activity of Mgat5 associated with the branching of N-glycans can also facilitate Gal-1 binding (Thiemann and Baum, 2016). In contrast, expression of St6gal1, which is adding a sialic acid to the lactosamine sequences, could abrogate Gal-1 binding (Thiemann and Baum, 2016). In Th1 and Th17 cells, overexpression of St6gal1 (adds α-2-6 sialic acid) could prevent Gal-1 binding and thus the death of cells (Toscano *et al.*, 2007). Conversely, masking glycan ends with α-2-6 sialic acid added by the enzyme St6gal1 also prevents galectin-9 binding to glycan ligands and induction of T cell death (Bi and Baum, 2009; Rabinovich and Croci, 2012).

As shown by Wang *et al*. the glycocalyx signature of cells is changed in autoimmune diseases like multiple sclerosis and its animal model EAE (Wang *et al.*, 2014). Deficiency of the N-glycan branching enzyme Mgat5 in mice promotes T cell activity, endocytosis of CTLA-4 and autoimmunity, including a spontaneous multiple sclerosis (MS)-like disease (Lee *et al.*, 2007). This study shows that in EAO presence of terminal sialic acid and the sialyltransferase St6gal1 was upregulated, while Mgat5 mRNA expression and L-PHA binding were decreased. Moreover, SNA binding to primary Sertoli and peritubular cells was increased after TNFα stimulation, whereas L-PHA was downregulated. Collectively, these findings suggest that under inflammatory conditions the glycan composition on the Sertoli and peritubular cell surface becomes less favorable for Gal-1 binding, a means to dampen excessive immune reactions elicited otherwise by concerted TNFα and Gal-1 action in sterile testicular inflammation. Similar to our findings, Benjamin *et al.* reported that α-2-6 sialic acid was significantly increased on the surface of adipocytes after induction of insulin resistance with TNFα (Parker *et al.*, 2016). Likewise, primary human umbilical vein endothelial cells showed considerable expression of L-PHA-reactive Mgat5-modified N-glycans, that decreased significantly following exposure to pro-inflammatory cytokines like IFNγ and IL-17, whereas α-2-6 sialic acid expression was increased following IFNγ/IL-17 stimulation (Croci *et al.*, 2014). Interestingly, upregulated expression of IFNγ and IL-17 were accompanied by increased numbers of Th1 and Th17 cells in EAO testes (Jacobo *et al.*, 2011). Thus it could be speculated that the inflammatory cytokines TNFα, IFNγ and IL-17 may be involved in the increased expression of α-2-6 sialic acid in EAO testis.

5.5. Gal-1 and TNFα synergistically increased expression of inflammatory cytokines through the MAPK p38 and JNK

Galectins as the soluble proteins function in the extracellular milieu by controlling signaling pathways through protein–glycan or protein–protein interactions (Cerliani *et al.*, 2017). These multivalent complexes (often named lattices) result in high-avidity binding that promote cross-linking, reorganization, and clustering of a preferential suit of glycoproteins and glycolipids, which could affect signal transduction, endocytosis of receptors, and signaling

thresholds of relevant receptors (Rabinovich *et al.*, 2007; Nabi *et al.*, 2015; Cerliani *et al.*, 2017; Godula, 2018). Likewise, Gal-1 can bind to cellular surface glycoproteins including CD3, CD7, CD43 and CD45 and cause an impressive redistribution of these receptors modulating signal transduction (Garner and Baum, 2008; Godula, 2018). Lectin-glycan interactions can control cell responses by adjusting thresholds of cellular activation.

Gal-1 can induce apoptosis of various cell types including glycosylated thymocytes, activated T cells, Leydig cells and breast tumor cells (Martinez *et al.*, 2004; Toscano *et al.*, 2007; Geiger *et al.*, 2016). Besides induction of cell death, Gal-1 binding can also prevent cell death, mediate epithelial-mesenchymal transition, induce M2 macrophage polarization, regulate adhesion and migration, modulate macrophage phagocytosis, promote fibrogenesis, and regulate responses to pathogens (Starossom *et al.*, 2012; Bacigalupo *et al.*, 2015; Gaudet *et al.*, 2015; Thiemann and Baum, 2016; Bunn *et al.*, 2017; Kathiriya *et al.*, 2017). Gal-1 could regulate cell signaling on immature dendritic cells and drive tolerogenic dendritic cells through binding to and clustering of CD45 and CD43 (Fulcher *et al.*, 2006, 2009). However, on mature, LPS-induced inflammatory dendritic cells, Gal-1 binding to CD43 and clustering can prevent cell migration (Thiemann *et al.*, 2015). In order to determine the function of Gal-1 on one cell type is very important to identify the glycoprotein(s) bound by Gal-1, detect the effect of Gal-1 binding on the receptor (such as clustering, internalization, retention on the cell surface, enhancing or inhibiting intracellular signal transduction), and determine the final outcome of signal transduction.

In this study, we could show that Gal-1 and TNFα synergistically increased the expression of inflammatory mediators in Sertoli cells, whereas Gal-1 alone had no effect. This synergistic effect is specific because it is abrogated in the presence of lactose and mediated through phosphorylation of MAPK p38 and JNK. Additionally, a previous study showed that binding of Gal-1 to N-glycan modified CD45 can prolong retention of CD45 on the surface of microglial cells by inhibiting early endosome antigen 1 (EAA1) induced endocytosis, and thus augment its phosphatase activity (Starossom *et al.*, 2012). Adams *et al*. showed that EEA1 as an endocytosis marker in Sertoli cells is involved in the internalization of intercellular junctions (Adams and Wayne Vogl, 2017). It could be speculated that the potential mechanisms behind the effect of Gal-1 on TNFα induced inflammatory cytokines in

Sertoli cells include (a) Gal-1 binding to cellular surface glycoproteins causes substantial receptor redistribution and then modulates signal transduction (Figure 26a), and/or (b) binding of Gal-1 prolongs retention of receptors and thus augments its activity (Figure 26b). For the further study, the glycoprotein(s) bound by Gal-1 on Sertoli cell surface need be also identified.

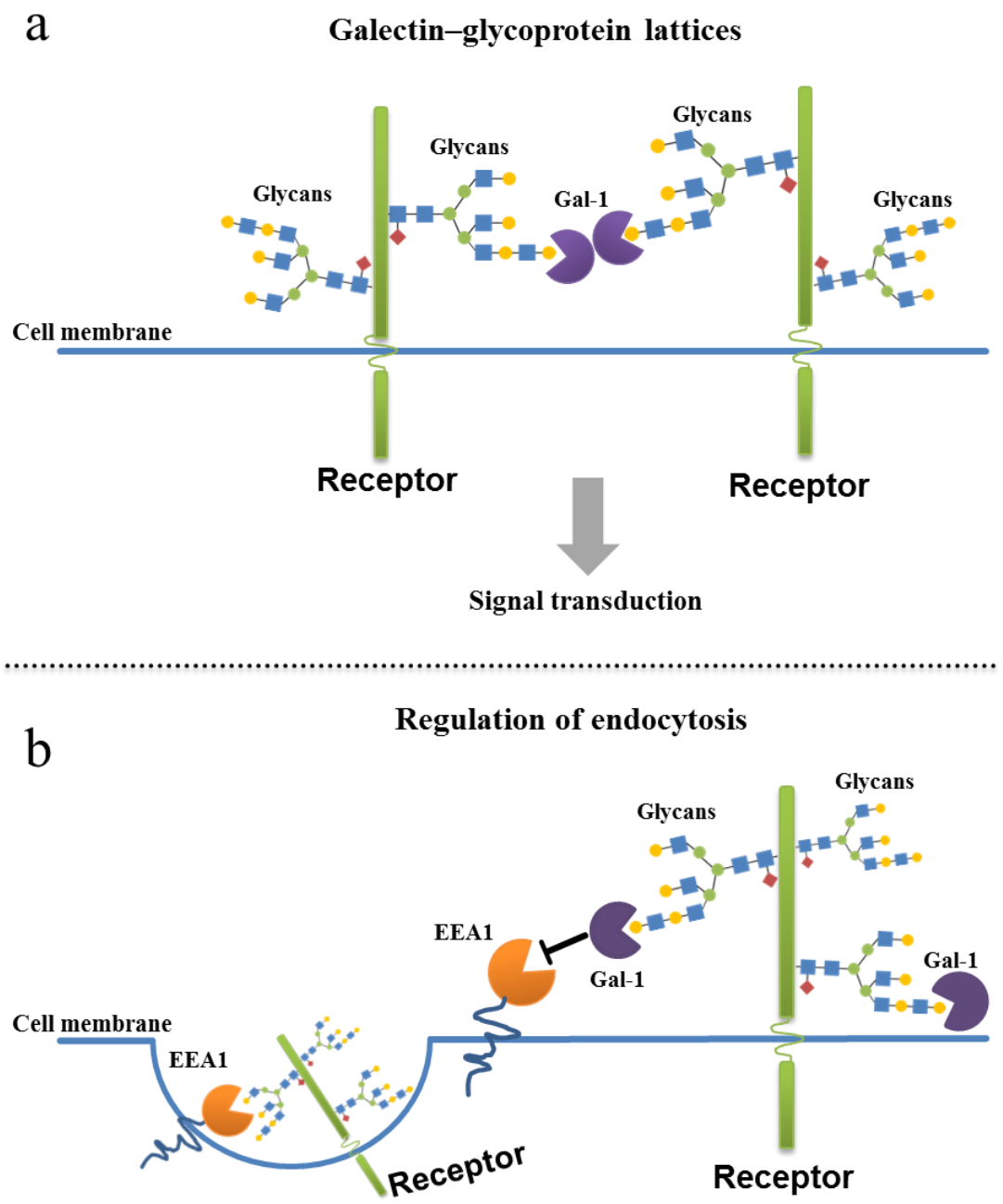

Figure 26. Galectin-glycoprotein lattices in the regulation of cell signaling and receptor turnover. Gal-1 could bind to cellular surface glycoproteins (receptors) on Sertoli cells and cause the redistribution of these receptors, and then modulates signal transduction (a). Binding of Gal-1 to cellular surface glycoproteins would prolong retention of receptors on the surface of Sertoli cells by inhibiting EAA1 induced endocytosis, and thus augment its activity (b).

Recent studies have shown that mice lacking Gal-1 developed a reduced incidence and severity of symptoms in experimental models of epileptic seizures (Bischoff *et al.*, 2012) and EAO (Pérez *et al.*, 2015). In EAO mice deficient for Gal-1 decreased numbers of apoptotic germ cells were found compared to testes from normal mice (Pérez *et al.*, 2015). Considering that Gal-1 was found to be strongly expressed on apical stalks of Sertoli cells during spermiation, involvement in the elimination of defective germ cells is a possible function. This hypothesis is supported by studies showing that Gal-1 can induce human T lymphocytes and Leydig tumor cell apoptosis by binding to Fas (Biron *et al.*, 2006; Brandt *et al.*, 2008). In this regard, upregulation of Fas expression was found in aberrant meiotic and postmeiotic germ cells (Francavilla *et al.*, 2002). Because an increased number of Fas positive apoptotic germ cells was found in EAO testes (Theas *et al.*, 2003), interaction of Gal-1 and Fas could possibly be also involved in germ cell apoptosis in orchitis.

In consideration of our findings that TNFα stimulates Gal-1 expression in Sertoli cells and the addition of exogenous Gal-1 enhances the pro-inflammatory effects of TNFα, we propose that under normal conditions Gal-1 induces apoptosis of defective germ cells. In contrast, under inflammatory conditions Gal-1 adopts a pro-inflammatory and pro-apoptotic function in the induction of immune responses and germ cell sloughing.

Taken together, the present data in light of the published literature indicate that under normal conditions Gal-1 (mainly produced by Sertoli cells) could be involved in the induction of germ cell apoptosis and the maintenance of immune privilege through inducing activated T cell apoptosis and promoting the differentiation of tolerogenic dendritic cells (Figure 27a). Under inflammatory conditions Gal-1 and TNFα synergistically act to increase expression of inflammatory cytokines (e.g. IL-6, MCP-1, TNFα), which may play a role in inflammatory reactions and associated testicular damage, characterized by the infiltration of activated T cells, M1 macrophage polarization, apoptosis of germ cells and increased terminal α-2-6 sialylation (Figure 27b). It needs to be considered in this regard that application of recombinant Gal-1 to treat autoimmune diseases may elicit an inflammatory response in Sertoli cells by the synergistic activity with TNFα.

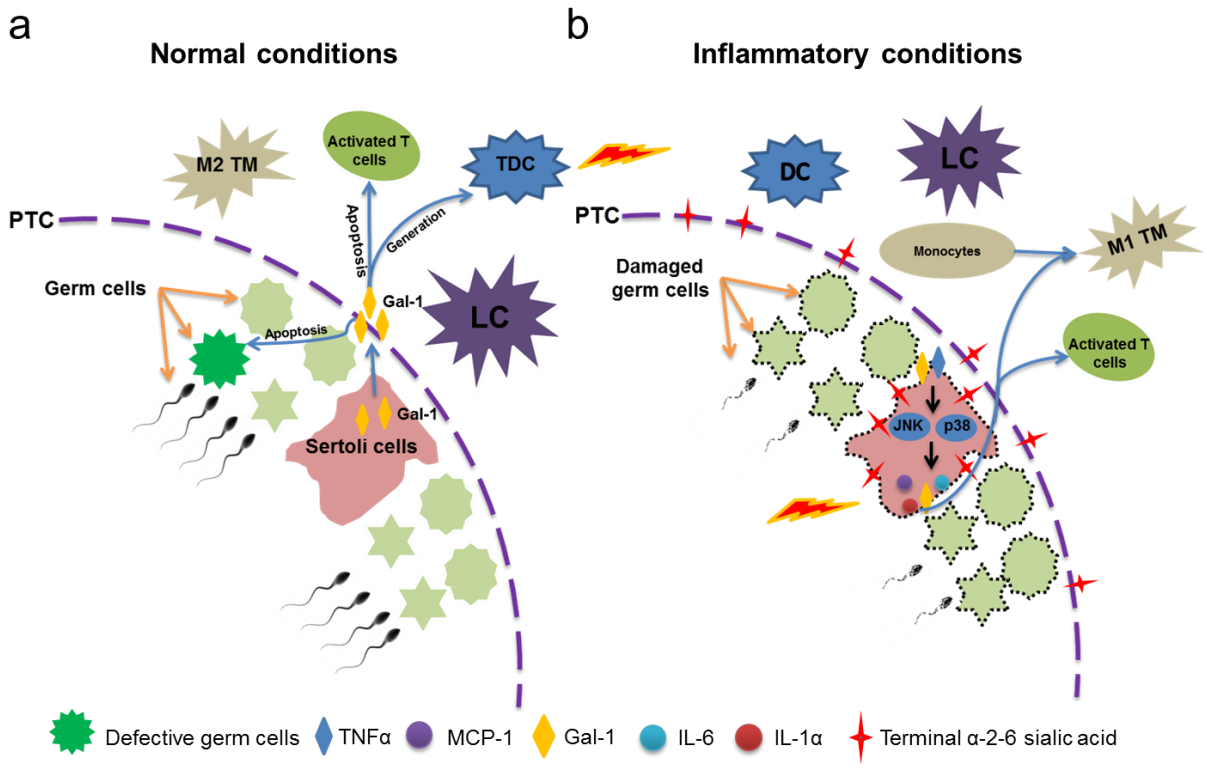

Figure 27. Schematic representation of possible roles of Gal-1 in testis under normal and inflammatory conditions. Under normal conditions Gal-1 could induce apoptosis of defective germ cells, activation of T cell apoptosis and generation of tolerogenic dendritic cells (TDC) in testis and thus maintain the immune privilege (a). In contrast, under inflammatory conditions Gal-1 and TNFα induce synergistically expression of inflammatory cytokines (e.g. IL-6, MCP-1, TNFα, and IL-1α) via stimulation of MAPK (p38, JNK) signaling (b). These cytokines are related to massive immune response (infiltration of activated T cells, M1 macrophage polarization), testicular damage, and changes in the glycocalyx signature, like increased terminal α-2-6 sialylation (b). (LC: Leydig cells, DC: dendritic cells, PTC: peritubular cells, TM: testicular macrophages).

Summary

Gal-1 is a multifunctional lectin involved in the modulation of immune responses. Using animal models of autoimmunity and chronic inflammation Gal-1 has been shown to have anti- as well as pro-inflammatory properties. Here, a model of rat experimental autoimmune orchitis (EAO) was used to investigate the role of Gal-1 in chronic testicular inflammation. EAO is characterized by leukocytic infiltration in the interstitium, spermatogenic damage and elevated production of inflammatory mediators like TNFα and MCP-1. In normal rat testis Gal-1 is predominantly expressed in Sertoli and germ cells. In extracts from inflamed testes a significant downregulation of Gal-1 mRNA and protein expression was observed. In contrast, relative expression of Gal-1 mRNA normalized to SOX9, a Sertoli cell marker, was not significantly changed. This suggests that downregulation of Gal-1 in the EAO testis is the consequence of germ cell loss.

In order to investigate an influence of Gal-1 on the immune properties of Sertoli cells, primary cells were isolated and challenged with the pro-inflammatory cytokine TNFα. Subsequently, Gal-1 expression was found to be upregulated. However, binding of the lectin SNA to Sertoli cells was increased as shown by flow cytometry. SNA is recognizing terminal α-2-6-sialic acid residues that block binding of Gal-1. In contrast, binding of L-PHA, that is recognizing β-1-6-branching of complex N-glycans, was downregulated in TNFα challenged Sertoli cells. These findings suggest that under inflammatory conditions the glycan composition on the Sertoli cell plasma membrane becomes less favorable for Gal-1 binding so that excessive immune reactions elicited by concerted action of TNFα and Gal-1 in sterile testicular inflammations may be dampened.

Gal-1 and TNFα synergistically increased expression of pro-inflammatory mediators such as MCP1, IL-1α, and IL-6 when applied to primary Sertoli cells. These effects were abrogated when Gal-1 binding was blocked by preincubation with the galactose containing disaccharide lactose. Combined stimulation of Sertoli cells with Gal-1 and TNFα lead to enhanced phosphorylation of MAP kinases p38 and JNK as compared to TNFα alone, whereas Gal-1 alone did not activate MAPK signaling. In addition, treatment of Sertoli cells with p38 and

JNK inhibitors abrogated Gal-1 and TNFα induced expression of IL-1 α, TNFα, IL-6 and MCP-1 mRNAs. These data show that Gal-1 enhances the pro-inflammatory activity of TNFα on Sertoli cells via stimulation of MAPK signaling.

Zusammenfassung

Gal-1 ist ein multifunktionelles Lektin, das an der Modulation von Immunantworten beteiligt ist. So konnten in Tiermodellen zu Autoimmunerkrankungen und chronischen Entzündungen sowohl anti- als auch pro-inflammatorische Wirkungen von Gal-1 gezeigt werden. In dieser Arbeit wurde an einem Modell der experimentellen Autoimmun-Orchitis (EAO) in der Ratte die Rolle von Gal-1 bei chronischer testikulärer Entzündung untersucht. Die EAO ist durch Leukozyteninfiltrate im Interstitium, Schädigung der Spermatogenese und die Produktion von Entzündungsmediatoren wie TNFα und MCP-1 gekennzeichnet. In normalen Rattenhoden wurde Gal-1-Expression hauptsächlich in Sertolizellen und Keimzellen gefunden. In Extrakten aus entzündeten Hoden war die Expression von Gal-1 auf mRNA- und Proteinebene dagegen signifikant erniedrigt. Wurde die Expression von Gal-1 mRNA jedoch mit der mRNA Expression von SOX9, einem spezifischen Marker für Sertolizellen, normalisiert, dann zeigte sich die Expression im EAO-Hoden unverändert. Die verringerte Expression von Gal-1 scheint also auf den Verlust der Keimzellen im entzündeten Hoden zurück zu führen zu sein.

Zur Untersuchung des Einflusses von Gal-1 auf die Immuneigenschaften von Sertolizellen wurden primäre Sertolizellen aus der Ratte verwendet, die zunächst mit dem proinflammatorischen Zytokin TNFα behandelt wurden. Dadurch wurde die Expression von Gal-1 sowie die Bindung des Lektins SNA erhöht, SNA erkennt terminale α-2-6-Sialylsäurereste, die die Bindung von Gal–1 inhibieren. Im Gegensatz dazu war die Bindung des Lektins L-PHA, das β-1-6-Verknüpfungen in komplexem N-Glykanen erkennt, an stimulierten Sertolizellen erhöht. Unter entzündlichen Bedingungen, wie sie in sterilen testikulären Entzündungen durch die gemeinsame Wirkung von TNFα und Gal-1 ausgelöst werden können, könnte also die veränderte Zusammensetzung der Glycane auf der Oberfläche von Sertolizellen die Bindung von Gal-1 herunter regulieren, um eine überschiessende Entzündung zu verhindern.

TNFα konnte die Expression pro-inflammatorischer Mediatoren wie MCP1, IL-1α und IL–6 in Sertolizellen induzieren, wobei die zusätzliche Inkubation mit Gal-1 die Wirkung von

TNFα synergistisch verstärken konnte. In Anwesenheit von Laktose, einem Galaktose enthaltenden Disaccharid, konnte dieser Effekt blockiert werden. Im Vergleich zu TNFα alleine verstärkte die kombinierte Stimulation von Sertolizellen mit Gal-1 und TNFα die Phosphorylierung der MAP-Kinasen p38 und JNK, wogegen Gal-1 alleine MAPK Signalwege nicht aktivieren konnte. Darüberhinaus verhinderte die Behandlung von Sertolizellen mit p38- und JNK-Inhibitoren, die durch Gal-1 und TNFα induzierte Expression von IL-1α, TNFα, IL-6 und MCP-1 mRNA. Diese Daten zeigen also, dass Gal-1 die pro-inflammatorische Aktivität von TNFα auf Sertolizellen über die Stimulation der MAPK Signaltransduktion erhöht.

6. APPENDIX

6.1. Enzymes for Sertoli and peritubular cell isolation

Table 7. List of enzymes used for Sertoli and peritubular cell isolation.

Enzyme	Company	Catalogue No.
Collagenase A	Roche Diagnostics, Mannheim, Germany	10103586001
DNase I	Roche Diagnostics, Mannheim, Germany	10104159001
Hyaluronidase	Sigma, Steinheim, Germany	H3506
Trypsin	Sigma, Steinheim, Germany	T5226
Trypsin inhibitor	Sigma, Steinheim, Germany	T6522

6.2. Antibodies

Table 8. List of antibodies and antibody dilutions used in this study.

Primary antibodies	**Manufacturer**	**Catalogue No.**	**Dilution**
Monoclonal rabbit anti galectin-1	GeneTex, USA	GTX62666	1:400*/1:200**
Polyclonal rabbit anti rat vimentin	Santa Cruz Biotechnology, USA	L4793.AB45	1:200*
Monoclonal mouse anti rat CD68 (ED1)	AbD Serotec, UK	MCA341R	1:200**
Monoclonal mouse anti rat CD163 (ED2)	AbD Serotec, UK	MCA342R	1:200**
FITC labelled monoclonal mouse anti α-smooth muscle actin	Sigma-Aldrich, Germany	F3777	1:1000*
Monoclonal mouse anti-β-actin	Sigma-Aldrich, Germany	A5441	1:10000*
Polyclonal rabbit anti rat phospho-p38 MAPK (Thr180/Tyr182)	Cell Signaling Technology, Germany	9211	1:1000*
Polyclonal rabbit anti rat p38 MAPK	Cell Signaling Technology, Germany	9212	1:1000*
Polyclonal rabbit anti rat phospho-p44/42 MAPK (ERK1/2)	Cell Signaling Technology, Germany	9101	1:1000*
Polyclonal rabbit anti rat p44/42 MAPK (ERK1/2)	Cell Signaling Technology, Germany	9102	1:1000*
Phospho-SAPK/JNK (Thr183/Tyr185)	Cell Signaling Technology, Germany	9251	1:1000*
SAPK/JNK	Cell Signaling Technology, Germany	9252	1:1000*
Secondary antibodies	**Manufacturer**	**Catalogue No.**	**Dilution**
Goat anti rabbit IgG-Alexa Fluor 546	Thermo Fisher Scientific, USA	A11071	1:1000**
Goat anti mouse IgG-Alexa Fluor 448	Thermo Fisher Scientific, USA	A10684	1:1000**
Goat anti rabbit IgG-HRP	ICN, USA	55676	1:10000*
Sheep anti mouse IgG-HRP	Sigma-Aldrich, Germany	A5906	1:10000*

IgG: Immunglobulin G, HRP: Horseradish peroxidase, *Western blotting, **Immuno-fluorescence.

6.3. Primers

Table 9. List of primers used in this study. F: forward primer, R: reverse primer.

Gene	Primer (5`→3`)	Catalogue No.	Gene ID	Amplicon size (bp)
Lgals 1 (Gal-1)	PrimePCR SYBR Green Assay	qRnoCED0001745 (Bio-Rad)	56646	63
Mgat5	PrimePCR SYBR Green Assay	qRnoCID0053085 (Bio-Rad)	65271	116
St6gal1	PrimePCR SYBR Green Assay	qRnoCED0004461 (Bio-Rad)	25197	63
Gcnt1	PrimePCR SYBR Green Assay	qRnoCED0008631 (Bio-Rad)	64043	93
Hprt	F: TCTGTCATGTCGACCCTCAG R: CCTTTTCCAAATCTTCAGCA	/	24465	109
Actb (β-actin)	F: ATGGTGGGTATGGGTCAGAA R: GGGTCATCTTTTCACGGTTG	/	81822	232
Rn18s (18s rRNA)	F: TACCACATCCAAGGAAGGCAGCA R: TGGAATTACCGCGGCTGCTGGCA	/	19791	180
Sox9	F: CTGAAGGGCTACGACTGGAC R: TACTGGTCTGCCAGCTTCCT	/	140586	140
Tnf (TNFα)	F: GCCTCTTCTCATTCCTGCTC R: CCCATTTGGGAACTTCTCCT	/	24835	101
Tgfb2 (TGFβ2)	F: CCGGAGGTGATTTCCATCTA R: GCGGACGATTCTGAAGTAGG	/	81809	201
Il6 (IL-6)	F: GCCCTTCAGGAACAGCTATG R: GTCTCCTCTCCGGACTTGTG	/	24498	119
Ccl2 (MCP-1)	QuantiTect Primer Assay	QT00183253 (Qiagen)	24770	117
Il1a (IL-1α)	QuantiTect Primer Assay	QT00183670 (Qiagen)	24493	101

6.4. List of lectins used in this study.

Table 10. List of conjugated lectins.

Lectin	Origin	Binding sites	Final concentration
MAA, FITC conjugated	*Maackia amurensis*	Terminal α-2-3-sialylation	20 µg/ml
PNA, FITC conjugated	*Arachis hypogaea*	Core 1 O-glycan	20 µg/ml
SNA, FITC conjugated	*Sambucus nigra*	Terminal α-2-6-sialylation	10 µg/ml
L-PHA, FITC conjugated	*Phaseolus vulgaris*	β-1-6 branching on complex N-glycans	20 µg/ml

6.5. Buffers for Sertoli and peritubular cell isolation

Table 11. PBS-A buffer

Reagents	Volume/weight
PBS Dulbecco's without Ca^{2+} and Mg^{2+}	500 ml
D-Glucose	750 mg
100 × Penicillin & Streptomycin	5 ml

Table 12. Trypsin-DNase-solution

Reagents	Volume/weight
PBS-A	10 ml
Trypsin	25 mg
DNase I	100 µg

Table 13. Trypsin inhibitor-solution A

Reagents	Volume/weight
PBS-A	5 ml
Trypsin inhibitor	50 mg

Table 14. Trypsin inhibitor-solution B

Reagents	Volume/weight
PBS-A	10 ml
Trypsin inhibiter	25 mg

Table 15. Collagenase-hyaluronidase-DNase-solution

Reagents	Volume/weight
PBS-A	10 ml
Collagenase A	10 mg
Hyaluronidase	10 mg
DNase I	100 µg

Table 16. Hyaluronidase-DNase-solution

Reagents	Volume/weight
PBS-A	10 ml
Hyaluronidase	10 mg
DNase I	100 µg

All enzyme solutions were sterilized by passing through a 0.20 µm filter (Sarstedt, Nuembrecht, Germany).

6.6. Culture medium for Sertoli and peritubular cell isolation

Table 17. Sertoli cell culture medium

Reagents	Volume
RPMI-1640 medium with L-glutamine	495 ml
100 x Penicillin & Streptomycin	5 ml

Table 18. Peritubular cell culture medium

Reagents	Volume
RPMI-1640 medium with L-glutamine	445 ml
Fetal bovine serum	50 ml
100 x Penicillin & Streptomycin	5 ml

6.7. Buffers and solutions for Western blotting

Table 19. RIPA buffer

Reagents	Concentration
Tris-HCl (pH 8.0)	50 mM
SDS	0.1%
Triton X-100	0.1%
EDTA	2 mM
Sodium deoxycholate	0.5%

1 × Proteinase inhibitor cocktail (Sigma-Aldrich, Steinheim, Germany) and 1 × Halt Phosphatase Inhibitor Single-Use Cocktail (Thermo Fisher Scientific, Waltham, USA) were added fresh just before cell lysis.

Table 20. Laemmli sample loading buffer

Reagents	Concentration
Tris-HCl (pH 6.8)	50 mM
SDS	2%
Glycerol	10%
β-mercaptoethanol	1%
EDTA	12.5 mM
Bromophenol blue	0.02%

Table 21. 10 × Tris buffered saline (pH 7.4)

Reagents	Weight
Tris base	24.2 g
NaCl	80 g

Dissolved in 1 L dH_2O.

Table 22. Separating gel

Reagents	10%	12.5%	15%
dH_2O	4.01 ml	3.17 ml	2.35 ml
1.5 M Tris-HCl pH 8.8	2.5 ml	2.5 ml	2.5 ml
10% (w/v) SDS	100 µl	100 µl	100 µl
Acrylamid	3.34 ml	4.17 ml	5 ml
10% (w/v) APS**	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl
Total	10 ml	10 ml	10 ml

* Separating gels with different percentages were used according to the molecular weight of target proteins (based on 37.5:1 acrylamide/bisacrylamide ratio). 10% gel: 120~40 kDa; 12.5% gel: 40~15 kDa; 15% gel: < 20 kDa.

** 10% APS was prepared fresh before each experiment.

Table 23. Stacking gel

Reagents	4%
dH_2O	3 ml
0.5 M Tris-HCl pH 6.8	1.25 ml
10% (w/v) SDS	50 µl
Acrylamide	0.65 ml
10% (w/v) APS**	25 µl
TEMED	5 µl
Total	5 ml

Table 24. Blocking buffer

Reagents	Volume/weight
1 × TBS	100 ml
Tween-20	0.1 ml
Non-fat dry milk	5 g

Table 25. 10 × Electrophoresis buffer (pH 8.3)

Reagents	Weight
Tris base	30.3 g
Glycine	144 g
SDS	10 g

Dissolved in 1 L dH_2O.

Table 26. Ponceau S staining solution

Reagents	Concentration
Ponceau S	2%
Trichloroacetic acid	30%
Sulfosalicylic acid	30%

Table 27. Stripping buffer

Reagents	Volume
1 M Tris-HCl (pH 6.8)	2 ml
10% SDS	700 µl
β-mercaptoethanol*	700 µl
Total	100 ml

* added freshly just before stripping of membrane.

Table 28. Semi-dry transfer buffer

Reagents	Concentration
Tris	48 mM
Glycine	39 mM
SDS	0.04%
Methanol	20% (v/v)

6.8. Buffer for PCR

Table 29. TAE Buffer

Reagents	Concentration
Tris-acetate	40 mM
EDTA, pH 8	1 mM

6.9. Buffer for immunofluorescence staining

Table 30. PBS (10x)

Reagents	Weight
KCl	2 g
NaCl	80 g
Na_2PO_4	14.4 g
KH_2PO_4	2.4 g

Dissolved in 1 L dH_2O, pH adjusted to 7.4 with NaOH.

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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.

10. LIST OF PUBLICATIONS

1. Publication originated from this thesis

Lei T, Moos S, Klug J, Aslani F, Bhushan S, Wahle E, Fröhlich S, Meinhardt A, and Fijak M. Galectin-1 enhances TNFα-induced inflammatory responses in Sertoli cells through activation of MAPK signalling. Sci Rep. 2018; 8(1):3741.

2. Other publications

Lei T, Freitag N, Bergmann M, Bhushan S, Almousa Y, Huang AC, Chen H, Liu F, Blois S, Andreas Meinhardt A, and Fijak M. Mice deficient for galectin 3 display disturbed maturation of Sertoli cells, delay of the first wave of spermatogenesis and increased apoptosis of germ cells. (Manuscript in preparation)

Lu Y, Rafiq A, Zhang Z, Aslani F, Fijak M, **Lei T**, Wang M, Kumar S, Klug J, Bergmann M, Chakraborty T, Meinhardt A, and Bhushan S. Uropathogenic Escherichia coli virulence factor hemolysin A causes programmed cell necrosis by altering mitochondrial dynamics. FASEB J. 2018:fj201700768R.

Lei T, Guo N, Liu JQ, Tan MH, and Li YF. Vitrification of in vitro matured oocytes: effects on meiotic spindle configuration and mitochondrial function. Int J Clin Exp Pathol. 2014; 7(3):1159-1165.

Lei T, Guo N, Tan MH, and Li YF. Effect of mouse oocyte vitrification on mitochondrial membrane potential and distribution. J Huazhong Univ Sci Technolog Med Sci. 2014; 34(1):99-102.

3. Conference abstracts

10th International Giessen Graduate Centre for the Life Sciences (GGL) Conference (27-28 September 2017) Giessen, Germany. Targeted disruption of the galectin 3 gene delays the first wave of spermatogenesis in mice. (Poster presentation)

9th International Giessen Graduate Centre for the Life Sciences (GGL) Conference (20-21 September 2016) Giessen, Germany. Galectin-1 enhances TNFα induced Monocyte Chemotactic Protein 1 expression in Sertoli cells through regulating the MAPK signaling pathway. (Poster presentation)

19th European Testis Workshop (11-15 June 2016) Saint Malo, France. Galectin-1

enhances TNFα induced Monocyte Chemotactic Protein 1 expression in Sertoli cells through regulating MAPK signaling pathway. (Oral presentation)

8th International Giessen Graduate Centre for the Life Sciences (GGL) Conference (30 September – 1 October 2015) Giessen, Germany. Expression of galectin-1, galectin-3 and sialylation pattern are changed in a rat model of the chronic testicular inflammation. (Poster presentation)

11. 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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Tao Lei