Untersuchungen zur Bindung von Galektin-1 an testikuläre Zellen

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List of Abbreviations

A	Ampere
APS	Ammonium persulphate
Asn	L-Asparagine
bp	Base pairs
BSA	Bovine serum albumine
°C	Degree Celcius
CD	Cluster of differentiation
CRD	Carbohydrate-recognition domain
Da	Dalton, 1 g/mol, 1.66×10^{-27}kg
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DPBS	Dulbecco's phosphate buffered saline
DTT	1,4-Dithiothreitol
EAO	Experimental autoimmune orchitis
ECL	Enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
g	Gram or gravitational acceleration, 9.81 m/s², according to context
G	Gauge, non-metric unit of measurement for outer diameters of needles
Gal-1	Galectin-1
Gal	Galactose
GalNAc	N-Acetylgalactosamine
Glc	Glucose
GlcNAc	N-Acetylglucosamine
GnT5	N-Acetylglucosaminyltransferase 5

GSL	Glycosphingolipid
h	Hour
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulphonic acid
ICC	Immunocytochemistry
IFN	Interferon
IHC	Immunohistochemistry
IgG	Immunoglobulin G
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl β-D-thiogalactopyranoside
kbp	Kilobase pairs
kDa	Kilodalton
K-MES	2-(N-Morpholino)ethanesulphonic acid, potassium salt
l	Litre
λ	wave length of light
LB medium	Lysogeny broth medium (Bertani, 2004)
LacNAc	N-Acetyllactosamine
LPS	Lipopolysaccharide
M	mol/l or molar mass (in g/mol), according to context
mA	Milliampere
mAb	Monoclonal antibody
MCP-1	Monocyte chemoattractant protein-1
MEM	Minimum essential medium
mg	Milligram
MHC	Major histocompatibility complex
min	Minute
ml	Millilitre
mM	10^{-3} mol/l
MNC	Mononuclear cells
NeuNAc	Sialic acid
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Normal goat serum
NHS	N-Hydroxysuccinimide
µg	Microgram

µl	Microlitre
µM	10^{-6} mol/l
OD_{xy}	Optical density at xy nm wavelength
pAb	Polyclonal antibody
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
pH	$-\log_{10}[H^+]$
PHA	Phytohaemagglutinin
PMSF	Phenylmethylsulphonyl fluoride
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
s	Second
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	L-Serine
SOB medium	Super optimal broth medium
SOC medium	Super optimal broth catabolite repression medium
ST3Gal I	α2-3 Sialyltransferase I
ST6Gal I	α2-6 Sialyltransferase I
TBS	Tris-buffered saline
TAE buffer	Tris-acetate-EDTA buffer
TE buffer	Tris-EDTA buffer
TEMED	N,N,N´,N´-Tetramethylethylenediamine
TFB buffer	Transformation buffer
TGF	Transforming growth factor
Thr	L-Threonine
TNF	Tumour necrosis factor
T_{reg}	Regulatory T cell
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
% v/v	Volume-volume percentage
% w/v	Mass-volume percentage
wt	Wild-type

1. Introduction

1.1. Structure and Function of the Male Reproductive Tract

The male reproductive system is anatomically and functionally focused on two aspects of sexual reproduction: 1. on the creation, maturation and exocrine secretion of male gametes (spermatogenesis) and 2. on the endocrine secretion of male gonadal steroid hormones. The testis as a paired reproductive gland is the only site for spermatogenesis and the major source of male sex steroid hormones.

Despite its significance for understanding the genesis of somatic tumours in the testis, the embryological origin of somatic testicular cells is still largely unproven. Coelomic epithelial cells form the genital ridges and genital cords as somatic precursors of the sexually undifferentiated gonads, the male development of which is triggered by the sex determining region of the Y chromosome (Griswold and Behringer, 2009; Martineau et al., 1997). Sertoli cells seem to derive from the coelomic epithelium (Karl and Capel, 1998), while multiple origins of Leydig cells are discussed, e.g. neural crest cells (Griswold and Behringer, 2009). The peritubular cells are thought to originate from the mesonephros (Buehr et al., 1993). In contrast primordial germ cells migrate into the genital ridges from the ectoderm of the yolk sac to form the parenchymal cells (gametes) of the testis (Fujimoto et al., 1977). Maintaining its proximity to the inguinal region during fetogenesis, the testis is located into a peritoneal evagination – the scrotum – a process to be completed until birth (Hutson et al., 1997).

The mature testis consists of 250-350 lobules separated by connective tissue septa emanating from the tunica albuginea, which firmly encapsulates the organ. Each lobule contains two to four seminiferous tubules as sites of spermatogenesis. The tubules continue to the rete testis and eight to twelve efferent ductules leading to the epididymis, where spermatozoa are stored and mature. From there they are transferred into the vas deferens, which enters the prostatic urethra on the seminal collicle (Schill et al., 2005a).

The vast majority of seminal plasma is produced in accessory glands along the male reproductive tract. The seminal vesicle's secretion contributes to about 60% of the seminal plasma and is mainly composed of fructose, prostaglandins, transferrin and lactoferrin. In addition the prostate produces 15-30% of the seminal plasma and is primarily responsible for liquefaction of the semen and for sperm motility. Alongside these major accessory glands the bulbourethral and urethral glands (Cowper's and Littré's glands) are responsible for the preejaculate (Schill et al., 2005b).

1.1.1. The Tubular Compartment of the Testis

Seminiferous tubules are composed of a lamina propria and the seminiferous epithelium. The lamina propria of rodents is made of one layer of myofibroblastic peritubular cells surrounded by a basal lamina, while human testes contain three to four such layers (Schell et al., 2008). The seminiferous epithelium itself hosts the spermatogenic cells embedded into Sertoli cells, which form the nurturing framework of the tubules (Figure 1). At their basis Sertoli cells are linked to each other by gap and tight junctions (Dym and Fawcett, 1970).

During spermatogenesis the male sexual stem cell (spermatogonium) undergoes mitosis and forms a primary spermatocyte (alongside a renewed spermatogonium), which enters meiosis. Meiosis I leads to the formation of two secondary spermatocytes, which develop each into two spermatids during meiosis II. Maturation of spermatids (spermiogenesis) leads to release of spermatozoa into the lumen of the seminiferous tubules (spermiation) (Bergmann, 2005; de Kretser et al., 1998).

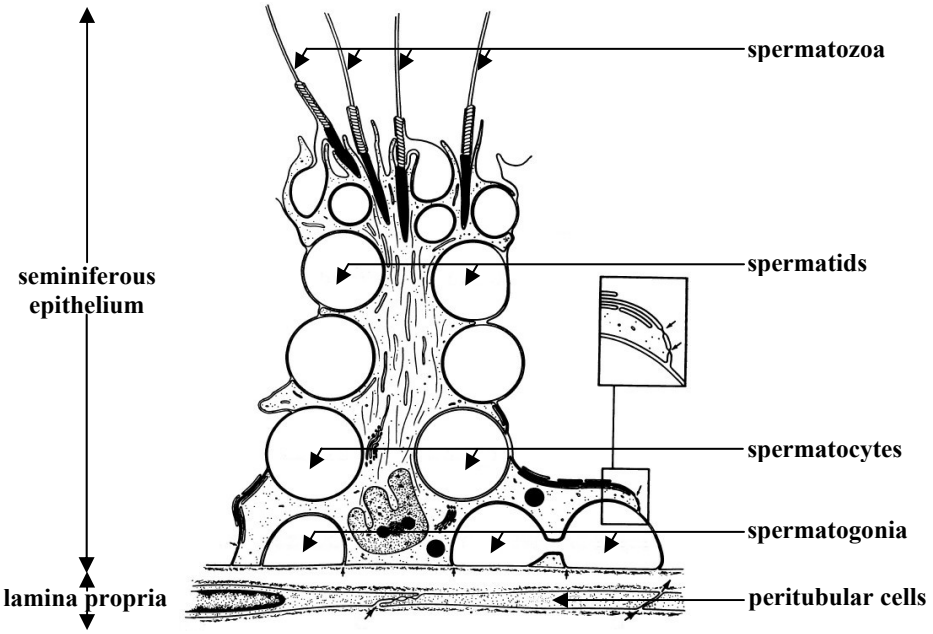


Figure 1: Architecture of the tubular compartment of the testis. A single Sertoli cell is shown with embedded developing germ cells and adjacent peritubular cells. Sertoli cells extend from the myofibroblastic peritubular cells to the apical lumen of the seminiferous epithelium and host spermatogenesis and spermiation. Linkage of Sertoli cells by gap and tight junctions (see enlarging box) creates the blood-testis-barrier and a basal and an adluminal compartment of the seminiferous epithelium. (Created based on Fig. 18 in (Dym and Fawcett, 1970).)

1.1.2. The Interstitial Compartment of the Testis

While 82% of the rat testis volume consist of seminiferous tubules, the interstitium comprises only 16% of the overall testicular volume. The remaining 2% are allocated to the tunica albuginea (Mori and Christensen, 1980). The interstitium features several cell types – predominantly Leydig cells – embedded into loose connective tissue and blood vessels:

Leydig cells, which produce the male gonadal steroid hormones, are the main interstitial cell type. They are located in clusters within the testis and are supplied by capillaries, which take up the secreted gonadal hormones. With one macrophage per four to five Leydig cells both cell types are located very close to each other and form digitations between their cell surfaces. Secreted mediators of **testicular macrophages** stimulate testosterone secretion in Leydig cells (Fijak and Meinhardt, 2006; Hedger, 1997; Hutson, 1992). Additionally macrophages are necessary for the development of Leydig cells and for their recovery after depletion and thus for the reproductive function of the testis (Fijak and Meinhardt, 2006). Vice versa Leydig cells determine the number of testicular macrophages (Meinhardt et al., 1998). Only in humans macrophages are also found in the lamina propria of seminiferous tubules (Frungieri et al., 2002a). **Testicular leukocytes** furthermore comprise lymphocytes, natural killer cells, mast cells, and dendritic cells (Hedger, 1997; Rival et al., 2006).

1.2. Immunological Privilege of the Testis

Similar to the central nervous system the testis is regarded as an immunologically privileged organ based on experiments showing long-term survival of xenogeneic tissue implanted into the testis (Bobzien et al., 1983; Head and Billingham, 1985b). Yet the testis is capable of mounting acute inflammatory responses to pathogenic agents like uropathogenic *Escherichia coli* with resulting epididymo-orchitis and possible infertility (Bhushan et al., 2009).

These immunological properties are needed to tolerate autoantigens, which arise after completion of immune competence when spermatogenic cells develop into mature spermatozoa in puberty for the first time (Fijak and Meinhardt, 2006). Nevertheless these antigens are immunogenic once they are found outside the testicular compartment: Injection of a testis homogenate into rats causes development of autoimmune orchitis severely impairing fertility (Tung et al., 1981; Tung et al., 1971).

Originally these distinct immunological characteristics were solely attributed to the blood-testis-barrier and its morphological correlate, specialized basal tight junctions between Sertoli cells (Dym and Fawcett, 1970). Considering the incomplete blood-testis-barrier of the rete testis and macrophages as well as T cells in the epididymis, from today's point of view hormonal and cellular factors together with cytokines have to contribute to tolerogenicity in the testicular compartment (Flickinger et al., 1997; Itoh et al., 1999) (Figure 2):

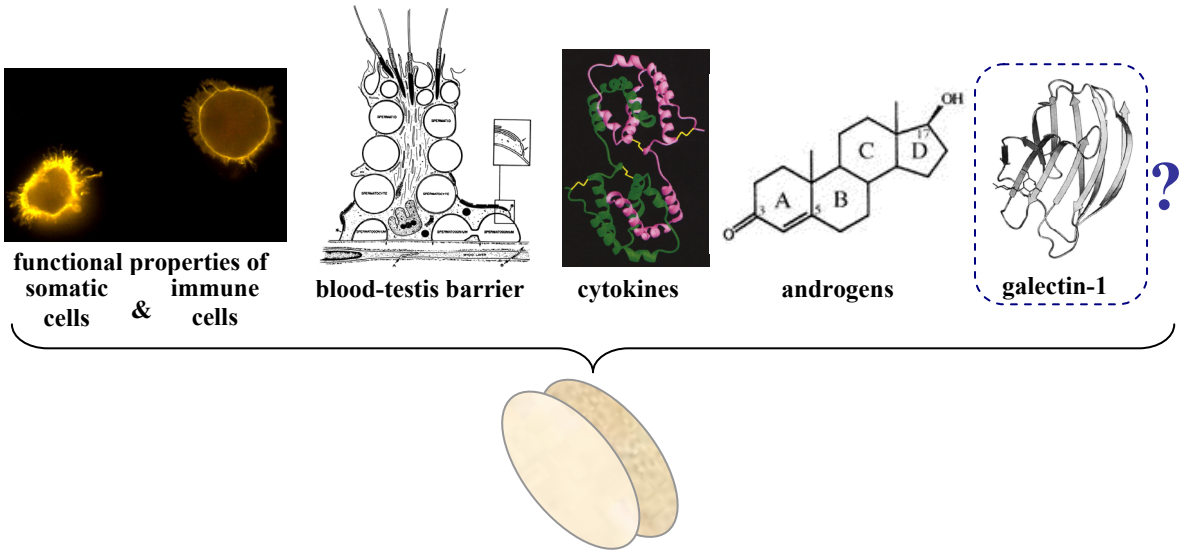


Figure 2: Factors involved in the immunological privilege of the testis. In addition to the blood-testis barrier somatic and immune cells together with cytokines and androgens are known to establish a tolerogenic microenvironment within the testis. Galectin-1 – an animal lectin with known anti-inflammatory properties *in vitro* and *in vivo* – is likely to be a new immunoregulatory factor in the testis. (From left to right: ED1/ED2-Cy3 staining of testicular macrophages (630x magnification). Scheme of blood-testis-barrier from (Dym and Fawcett, 1970). Ribbon diagram of IL-10 from (Zdanov et al., 1995). Molecule structure of testosterone from (Bennett et al., 1997). Ribbon diagram of galectin-1 from (Lobsanov et al., 1993).)

1.2.1. Testicular Immune Cells

Both somatic and immune cells of the testis have functional characteristics distinct from those found in other organs and tissues. These are therefore described more detailed below:

Testicular macrophages are regarded as essential for the immunologically privileged status of the testis. Testicular macrophages e.g. challenged by lipopolysaccharide (LPS) secrete only 2.8% of the interleukin 1 (IL-1) amount released by peritoneal macrophages under the same conditions (Hayes et al., 1996). They similarly bear a reduced capability of TNF-α secretion upon stimulation by LPS (Kern et al., 1995). Besides that a fraction of murine testicular macrophages found in density gradient centrifugation secrets the immunosuppressive and tolerogenic cytokine TGF-β (Bryniarski et al., 2004). Testicular macrophages are a heterogeneous cell population and can be divided into two classical subsets with distinct properties: Under normal conditions the majority (about 80%) is regarded as tissue resident macrophages due to their surface marker CD163 expression recognized by the anti ED2 antibody. They are an essential part of immunosuppression in the testis and likely to interact closely with Leydig cells (Chapter 1.1.2). On the other hand approximately 20% of testicular macrophages express CD68 (recognized by the anti ED1 antibody) and are described as having recently migrated to the testis (Hedger, 2002; Rival et al., 2008). Under inflammatory conditions the composition of testicular macrophage populations changes. During experi-

mental autoimmune orchitis (EAO) the number of $ED1^+$ macrophages increases. They are believed to take part in the autoimmune inflammation, which involves mononuclear cell infiltration, apoptosis of germ cells and impairment of seminiferous tubules (Rival et al., 2008). $ED1^+$ macrophages, but only a few $ED2^+$ macrophages, secrete monocyte chemoattractant protein-1 (MCP-1) and express inducible nitric oxide synthase (iNOS) (Gerdprasert et al., 2002a; Gerdprasert et al., 2002b). Half of the $ED1^+$ macrophages are $ED1^+ED2^+$ and believed to be in transition from the incoming to the resident phenotype (Hedger, 2002).

Dendritic cells (DCs) serve two tasks as antigen-presenting cells: 1. In peripheral tissues they patrol in search for foreign antigens, which they phagocytose. This initiates their maturation and migration into lymphoid tissues, where they clonally select and amplify $CD4^+$ and $CD8^+$ T cells. In addition, DCs activate quiescent T cells and (memory) B lymphocytes (Banchereau and Steinman, 1998). 2. DCs induce self-tolerance by causing anergy of self-reactive T cells and apoptosis of self-reactive thymocytes (peripheral and central tolerance) (Matzinger, 1994). Derived from bone marrow progenitor cells ($CD34^+$) and monocytes ($CD14^+$) human DCs are separated into interstitial DCs, Langerhans cells and plasmocytoid DCs (likely to equal the murine lymphoid DCs) (Banchereau et al., 2000). DCs are present both in normal and chronically inflamed testes of Wistar rats with numbers increasing during EAO (Rival et al., 2006). Testicular DCs in normal testes were found to be immature and tolerogenic, while DCs in testes undergoing EAO appear to be mature and autoimmunogenic contributing to autoimmune inflammation (Rival et al., 2007). These mature DCs were recently detected in testicular draining lymph nodes of EAO rats (Guazzone et al., 2010).

Mast cells are found around blood vessels near or in the tunica albuginea of rodent testes (Anton et al., 1998). In humans they are located in the testicular interstitium and tunica albuginea (Nistal et al., 1984). Mediators secreted by mast cells (e.g. histamine and TNFα) influence the permeability of blood vessels and mast cell activation by cell-cell interaction with activated T cells is suspected to contribute to autoimmune diseases such as multiple sclerosis (Bhattacharyya et al., 1998; Secor et al., 2000). Mast cell secretion of tryptase, a serine protease, is thought to cause fibrosis of the seminiferous epithelial wall in infertility and to participate in experimental autoimmune orchitis by increasing the expression of MCP-1, TGF-β_2 and cyclooxygenase-2 (COX-2) by peritubular cells via the proteinase-activated receptor-2 (Frungieri et al., 2002b; Iosub et al., 2006). Increased numbers of mast cells are described in the testis of infertile men (Meineke et al., 2000).

1.2.2. Testicular Somatic Cells

Somatic cells of the testis not only provide the basis of steroidogenesis and spermatogenesis but are also involved in immunosuppression and responses to pathogenic stimuli: **Sertoli cells** on the one hand prolong the survival of allo- and xenografts upon cotransplantation (Selawry and Cameron, 1993) and secrete the proapopototic and immunosuppressive Fas ligand (Filippini et al., 2001). On the other hand Sertoli cells secrete the proinflammatory cytokines IL-1α and IL-6 (Gerard et al., 1991; Riccioli et al., 1995) and protect spermatogenesis from bacterial infection by secretion of MCP-1 after stimulation of their toll-like receptors (Riccioli et al., 2006). As stated in chapter 1.2.1 **peritubular cells** secrete MCP-1, TGF-β_2 and cyclooxygenase-2 (COX-2) thus influencing leukocyte function within the testis (Maekawa et al., 1996; Müller et al., 2005).

Leydig cells, a functionally heterogeneous cell population, are responsible for testicular androgen biosynthesis. Interestingly, the intratesticular concentration of testosterone is much higher than serum levels (Jarow and Zirkin, 2005). Besides maintaining spermatogenesis, **androgens** influence testicular immunology by binding to Sertoli, Leydig and peritubular cells (Vornberger et al., 1994): Testosterone *in vitro* reduces the expression of proinflammatory cytokines (IL-1, IL-6 and TNF-α) and increases the expression of the anti-inflammatory cytokine IL-10 in macrophages and T cells (D'Agostino et al., 1999; Liva and Voskuhl, 2001). Accordingly allografts transplanted into the testis are rejected after suppression of androgens (Head and Billingham, 1985a).

Fibroblastoid **Co-cells**, which only exist in humans, compartmentalize the interstitial space and influence the local growth factor milieu by secreting extracellular matrix components, to which growth factors bind (Holstein and Davidoff, 1997; Schlatt et al., 1997).

Additionally reduction or even absence of HLA-A/B/C and HLA-DR (derived from the MHC I and II gene complex) on the surface of developing **germ cells** may protect spermatogenesis from antigen-specific immune responses (Pollanen and Niemi, 1987).

1.3. Biology and Chemical Nature of Galectins

Lectins, i.e. carbohydrate-binding proteins were discovered in the past two decades as factors of growing significance in the field of immune modulation. With galectin-1 a major member of this protein family is investigated in this study.

1.3.1. Origin and Architecture of Glycans

Diversity and biological wealth of glycans derive from chemical multitude of their monomers, i.e. monosaccharides, and structural flexibility of the glycosidic bonds by linking different anomers (α- or β-configuration of the anomeric carbon) and hydroxy groups (→ constitutional isomers) of monosaccharides to form e.g. α1-3 or β1-4 glycosidic bonds. Since each monosaccharide contains more than one hydroxyl group, branched glycans are common (Varki, 2009k). Glycans attached to target molecules are created during glycosylation – an enzymatic process centralized within the endoplasmic reticulum and Golgi apparatus. From there glycoproteins, glyco(phospho)lipids and proteoglycans are included into cellular surfaces as glycocalyx and released as the majority of secreted and extracellular matrix proteins. Only cytoplasmic and nuclear proteins are glycosylated in the cytoplasm (Farquhar and Palade, 1981; Varki and Varki, 2007). Glycosylation itself is carried out by glycosyltransferases, which synthesize glycans using nucleotide-linked and thus activated monosaccharides. Thereafter glycans are modified by methyl-, acetyl- and sulphotransferases (Varki, 2009b). N-linked glycans, O-linked glycans and glycolipids act as the major binding partners of galectin-1 and are therefore described below.

N-linked glycans (N-glycans) are characterized by their N-glycosidic attachment to L-asparagine in Asn-X-Ser/Thr sequences of polypeptides/proteins (X: any amino acid except L-proline). Their first carbohydrate is most commonly β-N-acetylglucosamine, forming a GlcNAcβ1-Asn linkage. Starting from there N-linked glycans share a common core: Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ1-Asn, which bears a Manα1-3 and a Manα1-6 arm (Kornfeld and Kornfeld, 1985). Thus N-glycans are diversified via the glycans attached to these arms leading to a high mannose, complex and hybrid type (Figure 3).

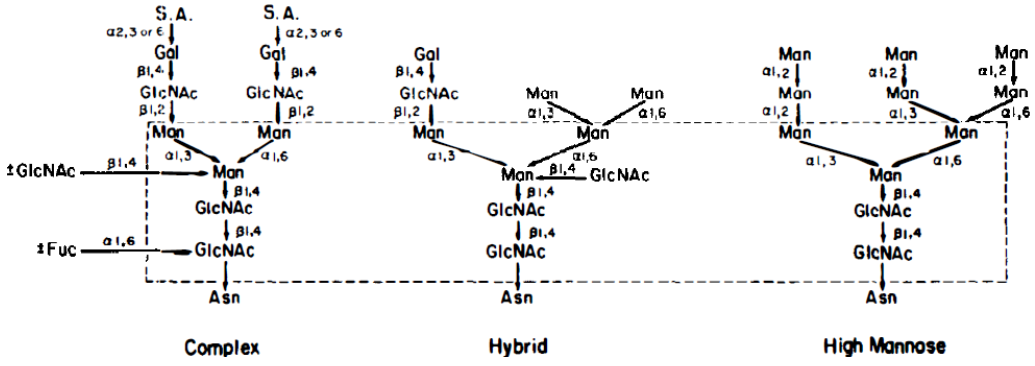


Figure 3: The N-glycan core (within dashed line) forms the common basis of N-linked glycans and bears a Manα1-3 arm and a Manα1-6 arm. Glycosylation of these arms, including further branching, leads to diversification and forms three major N-glycan types: In the high mannose type solely mannose residues are attached to the common core. In contrary the complex type bears various carbohydrates linked to the core by a β-N-acetylglucosamine residue. The hybrid type combines properties of both types: a mannose-only branch linked to the Manα1-6 arm and a complex branch attached to the Manα1-3 arm. (From (Kornfeld and Kornfeld, 1985).)

The synthesis of N-glycans starts with seven carbohydrates being attached to dolichol phosphate, a lipid carrier, on the cytoplasmic side of the endoplasmic reticulum (ER) wall. This interim glycan is flipped to the inner ER lumen and extended to fourteen carbohydrates ($Glc_3Man_9GlcNAc_2$-P-P-Dolichol), which are transferred as a whole to Asn-X-Ser/Thr sequences of nascent proteins. Prior to leaving the ER the glycan is cut down to $Man_8GlcNAc_2$ or $Man_9GlcNAc_2$ to facilitate glycoprotein folding. Further shortening in the cis-Golgi apparatus leads to $Man_5GlcNAc_2$, the precursor for hybrid and complex N-linked glycans. Sparing of this mannosidase activity creates high mannose type N-glycans (Varki, 2009g).

Accordingly linkage of one or two GlcNAc residues to each core Manα1-3 and Manα1-6 (e.g. by N-acetylglucosaminyltransferase 5) paves the way for bi-, tri- and tetra-antennary complex N-glycans or hybrid N-glycans, respectively (Figure 3) (Schachter, 2000). The trans-Golgi network introduces **binding partners of galectin-1** to complex and hybrid N-glycans:

Galactose is β-linked to terminal GlcNAc to form so-called type-2 N-acetyllactosamine (Galβ1-4GlcNAc, LacNAc, Figure 4). Repeated linking of galactose and GlcNAc to this first LacNAc establishes poly-N-acetyllactosamines (polyLacNAc), to which galectin-1 binds with high affinity (Varki, 2009g). The N-acetylglucosaminyltransferase 5 (GnT5) is thus essential for polyLacNAc creation (Rabinovich and Toscano, 2009). Lectin binding to these glycans is modulated by terminal caps made of an α-linked sugar, like sialic acid, or sulphate. In this respect the α2-6 sialyltransferase (ST6Gal I) is of exceptional importance (Amano et al., 2003; Rabinovich and Toscano, 2009).

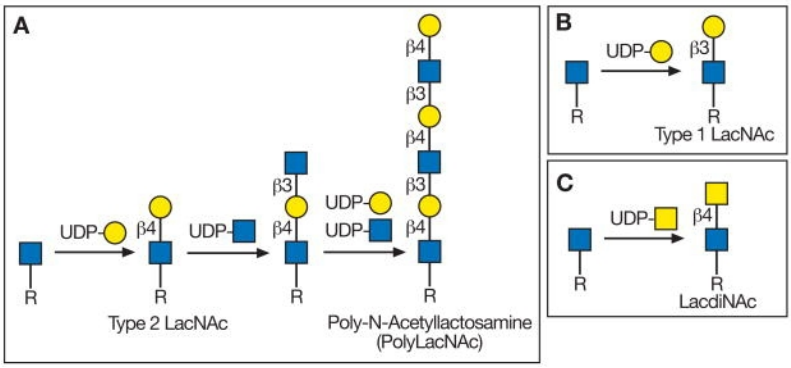


Figure 4: Formation of galectin-1 binding partners. Poly-N-acetyllactosamine branches of complex and hybrid N-glycans derive from repeated linkage of galactose and N-acetylglucosamine to the N-acetylglucosamine which is β1-2 linked to the N-glycan core. Galactose and N-acetylglucosamine are most commonly β1-4 linked (A), described as type-2 N-acetyllactosamine. Rarely (B) β1-3 linkage (→ type-1 N-acetyllactosamine) appears – and even β1-4 linking of N-acetylgalactosamine to N-acetylglucosamine (C, → LacdiNAc). (From (Varki, 2009g).) R: N-glycan core. ○: galactose. □: N-acetylgalactosamine. ■: N-acetylglucosamine.

O-linked glycans (O-glycans) are attached to Ser and Thr in proteins and polypeptides by an O-glycosidic bond. Primarily N-acetylgalactosamine (GalNAc) serves as the first carbo-

hydrate. Specific amino acid target sequences do not exist for O-glycosylation. Proline adjacent to Ser or Thr only increases the likeliness of glycosylation. O-glycans are synthesized directly on proteins. Members of this glycan family range from monosaccharides to large branched multimers and serve as ABO and Lewis blood group antigens, tethered O-glycans (e.g. MUC4, MUC16) and secreted mucins (Hattrup and Gendler, 2008; Varki, 2009h).

The simplest O-glycans GalNAcαSer/Thr (Tn antigen) and NeuNAcα2-6GalNAcαSer/Thr (sialyl-Tn antigen) are only found in tumours suggesting a loss of glycosyltransferases needed for the synthesis of more complex glycans. Larger O-linked glycans share eight core structures (Figure 5), but catalyzing enzymes are only known for cores 1 to 4 (Varki, 2009h).

Linkage of β-galactose to the Tn antigen by T synthase (C1GalT-1) leads to **core 1** (T antigen, Galβ1-3GalNAcαSer/Thr), which is often α2-3- and α2-6-sialylated. It is found on most cell types. **Core 2** O-glycans derive from core 1 by β1-6 linkage of GlcNAc to the initial GalNAc catalyzed by C2GnT (core 2 β1-6 N-acetylglucosaminyltransferase, GCNT1). C2GnT is influenced by cytokines and during lymphocyte activation. This new terminal GlcNAc serves as the starting point for LacNAc and poly-LacNAc residues (Varki, 2009h).

In contrary **core 3** O-glycans, which are found on proteins secreted by epithelia, start with GlcNAc β1-3 linked to the initial GalNAc. This bond is catalyzed by C3GnT (core 3 β1-3 N-acetylglucosaminetransferase). **Core 4** O-linked glycans derive from core 3 via addition of a second GlcNAc β1-6 linked to the initial GalNAc, which is carried out by a subtype of C2GnT. Distribution of core 4 O-glycans is similar to core 3 (Rabinovich and Toscano, 2009; Varki, 2009h).

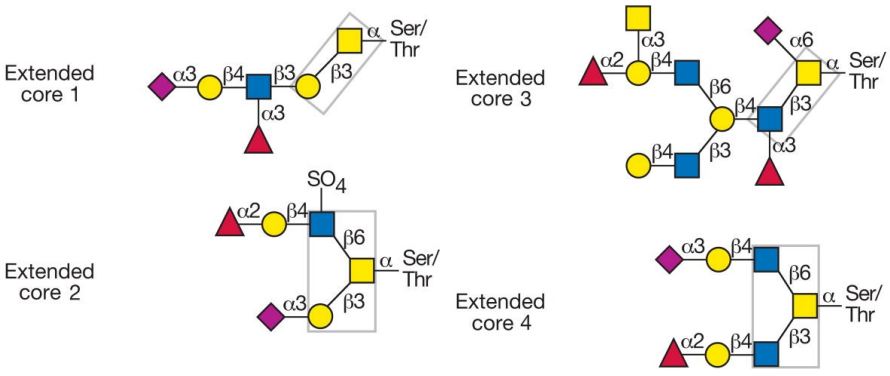


Figure 5: Despite structural diversity of O-linked glycans, distinct core structures (highlighted by boxes) can be distinguished and allocated to cell types and biological settings. While cores 1 and 2 are found in most cell types within mammalian organisms, the core structures 3 and 4 are restricted to secreted proteins of mucous membranes. Cores 1 and 3 are un-branched, while cores 2 and 4 are branched. (From (Varki, 2009d, fig. 9.2).) ○: galactose. □: N-acetylgalactosamine. ■: N-acetylglucosamine. △: fucose. ◇: sialic acid (=N-acetyl-neuraminic acid).

Larger glycans like poly-N-acetyllactosamine, LacdiNAc and repetitive Galβ1-3GlcNAc are linked to the cores to from mature O-linked glycans. They are branched by β1-6 linked GlcNAc and modified by terminal sulphate, fucose and sialic acid with impact on antigenicity and lectin binding (Figure 4) (Varki, 2009h).

Notably, terminal α2-3 linkage of sialic acid to core 1 O-glycans by α2-3 sialyltransferase I (ST3Gal I) inhibits core 2 O-glycan formation on CD43 and CD45. This blocks the linkage of polyLacNAc residues, to which galectin-1 binds with high affinity (Grabie et al., 2002; Rabinovich and Toscano, 2009). Since ST3Gal I has been found to be expressed in developing T_H2 cells but not developing T_H1 cells, it may attribute to differences in sensitivity towards galectin-1 induced apoptosis (Grabie et al., 2002). O-glycosylation furthermore changes during activation of lymphocytes thus influencing their homing (Fukuda, 2002).

When glycans are linked to lipids so-called **glycolipids** are formed. They are classified into glycosphingolipids (GSL), glycoglycerolipids and glycosylphosphatidylinositol anchors and located in cell membranes (Varki, 2009e). GSL are of interest for lectin-glycan interaction:

In GSL glycans are O-glycosidically linked to ceramide, which consists of a sphingoid base (e.g. sphingosine) and a covalently linked fatty acid. Since sphingoids, fatty acids and glycans vary, GSL are a divers class of molecules (Karlsson, 1970). According to the first carbohydrate a Glcβ1-type and a Galβ1-type are distinguished (Degroote et al., 2004). In Galβ1-type GSL, which are predominantly found in the central nervous system, the initial galactose can be linked to sialic acid, sulphate and galactose but their glycans are small. In contrary Glcβ1-type GSL, which are more common, may contain large, branched glycan structures and glycans common to O-glycans such as ABO blood group antigens (Degroote et al., 2004; Hakomori, 2003).

Glcβ1-type GSL are synthesized on the cytoplasmic, Galβ1-type GSL on the luminal side of the ER wall. Further glycosylation and sulphate linkage take place in the Golgi apparatus (Kolter et al., 2002). Terminal sugars are added by enzymes which also process glycoproteins leading to glycan structures shared by GSL and glycoproteins (Yamamoto, 2004).

Due to the hydrophobic properties of ceramide, GSL aggregate with cholesterol, other GSL, phosphosphingolipids and cell receptor molecules to form lipid rafts (Parton, 1994). This clustering increases the likeliness of lectin binding and influences growth factor signalling via cell surface receptors of the tyrosine kinase family (like the epidermal growth factor receptor) (Degroote et al., 2004). Due to its influence on cell-cell signalling this clustering has been described as the “glycosynapse” (Hakomori Si, 2002).

1.3.2. Lectin Biology

Glycan-binding proteins consist of lectins, glycosaminoglycan-binding proteins and glycan-specific antibodies. Lectins share conserved carbohydrate-recognition domains within their families and bind carbohydrates on cell surfaces (Chapter 1.3.3). Glycosaminoglycan-binding proteins in contrast lack conserved origins and recognize extracellular, sulphated poly-saccharides consisting of repeated disaccharide units (hexosamine + hexose/hexuronic acid) called glycosaminoglycans (Varki, 2009d, table 26.1; Varki and Angata, 2006).

Lectins were first described after discovering that plant extracts can agglutinate red blood cells and were accordingly termed agglutinins. The first lectin ever characterized was ricin, which was found in the seeds of ricinus communis by Hermann Stillmark in 1888 (Bies et al., 2004). Thereafter plant lectins were found in nearly all plant species, especially in the legume family. Meanwhile lectins are known throughout nature: in viruses (e.g. influenza haemag-glutinin), bacteria (e.g. adhesins of uropathogenic *E. coli*, toxin B of *V. cholerae*) and animals (e.g. selectins for leukocyte transmigration through vascular walls) (Varki, 2009c).

Six superfamilies of lectins can be classified according to sequence homologies in their carbohydrate-recognition domains (Figure 6). The first two superfamilies distinguished were C-type and S-type lectins (Drickamer and Taylor, 1993):

1. C-type lectins are defined by their need of Ca^{2+} to bind glycans and include the following: Selectins are found in the membrane of leukocytes (L-selectin), platelets (P-selectin) and activated endothelium (E- and P-selectin) and mediate leukocyte transmigration and lymphocyte homing. Collectins facilitate phagocytosis (mannose-binding protein) and suppress pulmonary immune cell function (surfactant protein A and D). As macrophage mannose receptor they are involved in endocytosis (Varki, 2009a). **2. S-type lectins** were originally defined by dependence on free thiols for glycan-binding – a property first described for galectin-1. Since not all S-type lectins need free thiols for their activity, they were termed galectins and defined by their β-galactoside binding specificity (Chapter 1.3.3) (Varki, 2009c). **3. P-type lectins** account for intracellular trafficking of newly synthesized lysosomal enzymes from the trans-Golgi network to the lysosomes. They target mannose-6-phosphate (M6P) residues on N-glycans and are therefore called cation-independent M6P receptor (CI-MPR) and cation-dependent MPR (CD-MPR). Failure in the creation of mannose-6-phosphate residues accounts for lysosomal storage diseases like mucolipidosis-II and -III (Varki, 2009i). **4. L-type lectins** are primarily plant lectins, which were found in the seeds of legume plants and characterized by their haemagglutinating capacities. Their capability to “read” erythrocyte

antigens coined the term “lectin” (from the Latin “legere”), but their physiological function is still largely unknown. Amongst non-plant L-type lectins calnexin and calreticulin serve as chaperones of glycoproteins in the endoplasmic reticulum (Varki, 2009f). **5. I-type lectins** bind to glycans via their immunoglobulin (Ig)-like domain. They include sialic acid-recognizing Ig-superfamily lectins (siglecs), which deserve sialic acid for binding. The siglec sialoadhesin is found on macrophages mediating internalization of pathogens and contact to lymphocytes. The siglec CD22 is expressed on B cells and negatively regulates signalling of the B cell receptor (Nitschke, 2005). **6. R-type lectins** have carbohydrate-recognition domains similar to ricin but are not only found in plants but also in bacteria and animals. As such they serve in animals as the mannose receptor family (containing a C-type lectin domain, too), which is used by Kupffer-Stern cells and macrophages to bind mannose residues on bacteria (Varki, 2009j).

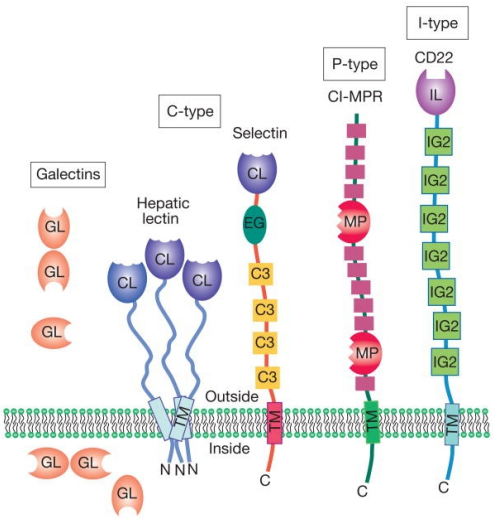


Figure 6: Lectin superfamilies are defined by their genetically conserved carbohydrate-recognition domains (CRD) common to all family members. They are specialized in binding specific carbohydrates at the outer end of glycan structures found in N-glycans, O-glycans and glycosphingolipids on cell surfaces. They are found as secreted proteins as well as membrane-bound and can bear more than one CRD per molecule (as depicted for P-type lectins). High binding avidity of lectins is generated by multiple binding of non-covalently linked lectin multimers, each monomer of which is binding with relatively low affinity. Typical CRD are the galectin (*GL*), C-type lectin (*CL*), cation-independent mannose-6-phosphate receptor/P-type lectin (*MP*) and I-type lectin (*IL*) CRD. (Image source (Varki, 2009d, fig. 26.1).) *C3*: complement regulatory repeat. *EG*: epidermal growth factor-like domain. *IG2*: immunoglobulin C2-set domain. *TM*: transmembrane region.

Accordingly the plant lectins, which we applied to study the glycans expressed on isolated testicular cells, bear the following properties:

PNA (affinity for the core 1 O-glycan) is known to cause mitosis of rat lymphocytes and human blood peripheral lymphocytes only after desialylation by neuraminidase (Novogrodsky et al., 1975). Studies of PNA binding to lymphocytes from lymph nodes lead to contradictive results (Cooper, 1984): While studies exist demonstrating binding to human B cells in lymph

node germinal centres and to peripheral blood T cells, other investigators obtained hardly any binding of PNA to germinal centre lymphocytes and T cells.

LEA requires three continuous Galβ1-4GlcNAc (=LacNAc) units for binding and thus prefers poly-N-acetyllactosamine units (Kawashima et al., 1990; Nachbar et al., 1980). Due to this specificity it has a binding pattern similar to galectin-1 (Stowell et al., 2008a).

MAA isolated from the tree Amur Maackia consists of two lectins: Maackia amurensis haem-agglutinin (MAH) and leukoagglutinin (MAL), which are both mitogenic for human peripheral lymphocytes. MAL shows high affinity for terminal sialic acid α2-3 linked to penultimate galactose in complex-type N-glycans (=sialyllactosamine, NeuNAcα2-3Galβ1-4GlcNAc, Figure 3) (Wang and Cummings, 1988). MAH preferentially binds NeuNAcα2-3Galβ1-3(NeuNAcα2-6)GalNAcα1-Ser/Thr in O-glycans (Imberty et al., 2000).

Elderberries contain four lectins: SNA-I/II/III and seed-SNA. **SNA-I**, which we used in this study, shows high specificity for terminal sialic acid α2-6 linked to penultimate galactose in N-glycans (NeuNAcα2-6Gal) as well as for terminal sialic acid α2-6 linked to GalNAc in O-glycans (NeuNAcα2-6GalNAc) (Shibuya et al., 1987a). In contrast SNA-II, SNA-III and seed-SNA bear higher affinity for galactose and GalNAc (Girbes et al., 1996; Kaku et al., 1990; Mach et al., 1991; Peumans et al., 1991). Thus SNA-I is applied to distinguish terminal α2-6 from α2-3 sialylation (Shibuya et al., 1987b), which is important since α2-6 sialylation of CD45 was shown to reduce galectin-1 induced apoptosis of T cells (Amano et al., 2003).

1.3.3. The Family of Galectins

Discovery of galectins originated from the idea that carbohydrates might be involved in cell adhesion (Leffler et al., 2004). Therefore, it was tested whether tissue extracts are able to agglutinate erythrocytes (Nowak et al., 1976). Since lactose and asialofetuin could already be bound to chromatography columns, these immobilized β-galactosides were used to fractionate tissue extracts. Lectins bound to the columns were eluted by lactose – a technique still applied today (Beyer et al., 1980). Similarly the first β-galactoside binding lectin was discovered in the electric organ of the electric eel (Teichberg et al., 1975).

Finally the criteria for membership in the galectin family and the name itself were defined by major researchers around Samuel H. Barondes in 1994 to standardize the nomenclature:

"Membership in the galectin family requires fulfillment of two criteria: affinity for β-galactosides and significant sequence similarity in the carbohydrate-binding site, the relevant amino acid residues of which have been determined by X-ray crystallography (Lobsanov et al., 1993). We further propose that the mammalian galectins be numbered sequentially […]." (Barondes et al., 1994)

The galectin family (at that time still called metal-independent β-galactoside-binding lectins) was divided into three subfamilies (Figure 7) according to the number of CRD per protein (Hirabayashi and Kasai, 1993):

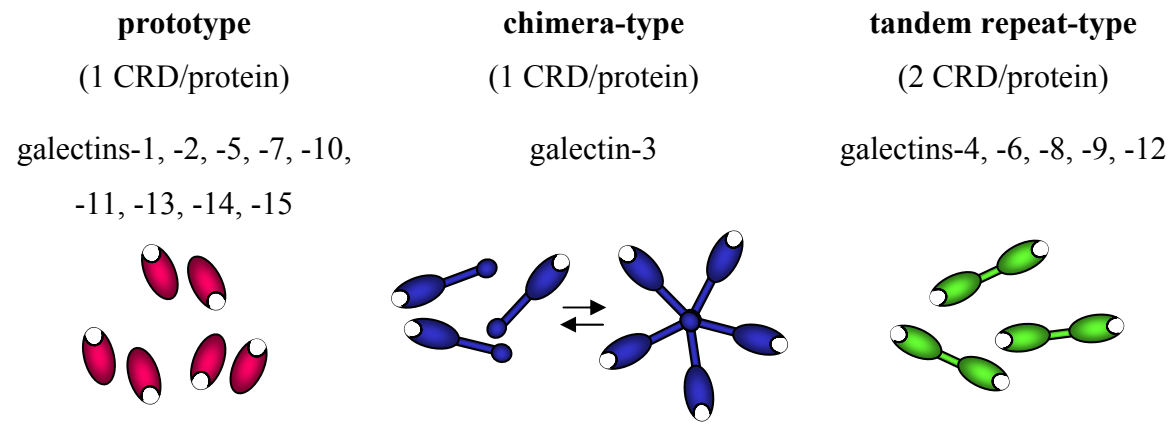


Figure 7: Subclassification of galectins according to the number of carbohydrate-recognition domains (CRD) per molecule as introduced by Jun Hirabayashi and Ken-ichi Kasai (Hirabayashi and Kasai, 1993). Galectins with one CRD ("prototype" subfamily) form monomers, dimers and oligomers depending on their concentration and carbohydrate binding partner. Although allocated to one group, dimers formed by prototype galectins are not homogenous. In galectin-3 – the only chimera-type galectin – the C-terminal CRD is neighboured by 18 N-terminal conserved amino acids and repeats of each 8-11 amino acids. This was regarded as a second domain and the galectin structure thus termed "chimeric". The two CRD in tandem repeat-type galectins can differ from each other. (Modified from (Leffler et al., 2004; Rabinovich and Toscano, 2009).)

In order to explain differences in binding strength of galectins to specific β-galactosides a model of the CRD consisting of four classical and one additional subsite was developed (Figure 8) (Knibbs et al., 1993; Leffler et al., 2004).

The CRD is imagined to recognize a tetrasaccharide structure with its subsites bearing different affinities to the individual carbohydrates, of which the tetrasaccharide is composed. The seven conserved amino acids of the carbohydrate-binding site form the subsites C and D: C specifically and strongly binds β-galactosides thus defining the galectin family. The adjacent subsite D (the second most firmly binding subsite) binds different carbohydrates and accounts for the differential characteristics of the galectin family members. Further variation between galectins derives from the binding characteristics of subsites A and B. Subsite E was introduced to account for the influences of the attached lipid or protein.

This model not only provides a way of explaining differences between galectins but also creates a framework for interpreting how binding of galectins to cell surface glycoproteins is regulated by modifying glycans, e.g. per terminal sialylation (Knibbs et al., 1993; Leffler et al., 2004; Seetharaman et al., 1998).

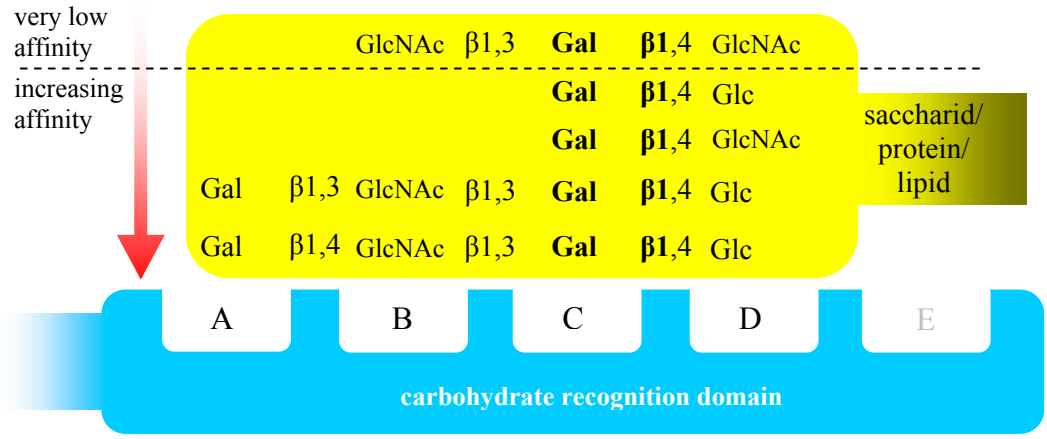


Figure 8: 4+1 subsite model of the carbohydrate-recognition domain (CRD) of galectins. This model was developed by comparing the capability of oligosaccharides to inhibit precipitation of laminin by carbohydrate-binding protein 35 (CBP35 = galectin-3). The depicted oligosaccharides showed 3- to 14-fold affinity for CBP35 in comparison to lactose (Galβ1-4Glc). As a necessity of galectin binding the terminal nonreducing galactosyl unit had to be unsubstituted and β1-3 or β1-4 linked to Glc or GlcNAc. Thus galectin affinity for cell surface glycoproteins can be influenced by modifying their terminal Gal residue e.g. by sialylation. Concluding from the most strongly bound oligosaccharides and from the high affinity of CBP35 to the polylactosamine-rich laminin, polylactosamines are the strongest target structures of galectins. Subsite B binds – according to the individual galectin – also Gal, GalNAc or NeuNAc. (Created based on Fig. 10 in (Knibbs et al., 1993) according to (Leffler et al., 2004).) *Gal* = D-galactose. *GalNAc* = N-acetyl-D-galactosamine. *Glc* = D-glucose. *GlcNAc* = N-acetyl-D-glucosamine. *NeuNAc* = N-acetylneuraminic acid = sialic acid.

Galectin-1 particularly binds complex-type N-glycans with high affinity, which is increased by the number of glycan branches (Hirabayashi et al., 2002; Lee et al., 1990). Furthermore galectin-1 binds terminal type 1 and type 2 LacNAc equivalently (Brewer, 2004). As means of modulation 3-O-sulphation of LacNAc increases binding of galectin-1 (Lee et al., 1990). α2-6 linkage of sialic acid by ST6Gal I but not α2-3 sialylation decreases galectin-1 binding to glycans (Stowell et al., 2008a).

A further common property of galectins is their cellular origin: They are synthesized on free cytosolic ribosomes, they are not equipped with a signal peptide and post-translationally their N-terminus is acetylated. Notably, galectins are secreted from the cell without the endoplasmic reticulum and Golgi apparatus, i.e. on a non-classical pathway, the details of which still remain elusive (Cooper and Barondes, 1990; Nickel, 2003).

The spectrum of biological functions attributed to the galectin family ranges from inflammation and immunity to cancer and structural development (Chapter 1.3.5).

1.3.4. Galectin-1 is Conserved Across Species

Since this study investigates target cells of galectin-1 in the testis using isolated testicular cells from Wistar Unilever rats and fluorescent labelled human galectin-1 C2S, the similarity of rat and human galectin-1 has to be clarified: Generally speaking galectins are conserved proteins found in all animals, plants and fungi. For galectin-1, respectively, the orthologue gene sequence of the carbohydrate-recognition domain shows 87% conservation between human and mouse genes (Leffler et al., 2004).

There is accordingly a high degree of congruence between the amino acid sequences of galectin-1 found in man, rat and mouse. This is demonstrated by aligning the corresponding sequences across species borders (Figure 9).

```
LEG1_HUMAN MACGLVASNLNLKPGECLRVRGEVAPDAKSFVLNLGKDSNNLCLHFNPRFNAHGDANTIV 60
LEG1_RAT   MACGLVASNLNLKPGECLKVRGELAPDAKSFVLNLGKDSNNLCLHFNPRFNAHGDANTIV 60
LEG1_MOUSE MACGLVASNLNLKPGECLKVRGEVASDAKSFVLNLGKDSNNLCLHFNPRFNAHGDANTIV 60
           ********************:****:*.**********************************

LEG1_HUMAN CNSKDGGAWGTEQREAVFPFQPGSVAEVCITFDQANLTVKLPDGYEFKFPNRLNLEAINY 120
LEG1_RAT   CNSKDDGTWGTEQRETAFPFQPGSITEVCITFDQADLTIKLPDGHEFKFPNRLNMEAINY 120
LEG1_MOUSE CNTKEDGTWGTEHREPAFPFQPGSITEVCITFDQADLTIKLPDGHEFKFPNRLNMEAINY 120
           **:*:.*:****:**..*******::**********:**:*****:*********:*****

LEG1_HUMAN MAADGDFKIKCVAFD 135
LEG1_RAT   MAADGDFKIKCVAFE 135
LEG1_MOUSE MAADGDFKIKCVAFE 135
           **************:
```

Figure 9: Alignment of galectin-1 amino acid sequences from man, rat and mouse. Comparison of the human and rat sequences shows 91% congruence and only 5 positions, where amino acids of differing physicochemical properties are found: (66): G→D. (68, 76, 86): A→T. (96): N→D. Sequences from man (accession no.: P09382, entry name: LEG1_human, revision: 133, 02/03/2010), rat (accession no.: P11762, entry name: LEG1_rat, revision: 83, 02/03/2010) and mouse (accession no.: P16045, entry name: LEG1_mouse, revision: 111, 02/03/2010) were obtained from the UniProt Protein knowledge base (UniProtKB/Swiss-Prot, UniProt Consortium, Cambridge (UK), Geneva (Switzerland), Washington (USA), www.uniprot.org/uniprot/). Multiple sequence alignment was carried out using CustalW2 version 2.0.12 (European Bioinformatics Institute, Cambridge, UK, www.ebi.ac.uk/Tools/clustalw2/index.html). Amino acid characteristics colour code: Red: small. Blue: acidic. Magenta: basic. Green: hydroxyl + amine. Grey: others.

The initially translated human galectin-1 protein is composed of 135 amino acids and then processed by removal of the initiator methionine, so that the final human galectin-1 is a 134 amino acid protein (Hirabayashi and Kasai, 1988). Wild-type human galectin-1 and the C2S mutant, which we used for assessing galectin-1 binding, are functionally equivalent. The C2S mutant bears higher stability in non-reducing conditions making it suitable for standardized experimental results (Hirabayashi and Kasai, 1991). Besides that, recent experiments have shown that substitution of all cysteine residues amplifies its stability even more with hardly any influence on its carbohydrate binding specificity (Nishi et al., 2008).

1.3.5. Modes of Galectin-1 Action in Biology

Interest in the biology of lectins and glycans derives from the fact that they are not static. Both are regulated in the course of innate and adaptive immunity: Functional changes of immune cells are linked to coordinated synthesis and modification of glycans (Marth and Grewal, 2008). Additionally expression levels of lectins vary during responses of the immune system (van Kooyk and Rabinovich, 2008).

The biological effect of galectin-1 is based on its bivalent binding properties as a non-covalent dimer: Galectin-1 cross-links glycans and thus approximates the proteins, to which they are N- or O-glycosidically linked. In consequence homogenous clusters of membrane proteins are created, which amplify their corresponding signals by neighbouring intracellular domains (Figure 10) (Fred Brewer, 2002). Such clustering was originally described while investigating the induction of apoptosis in human T cells by galectin-1 (Pace et al., 1999).

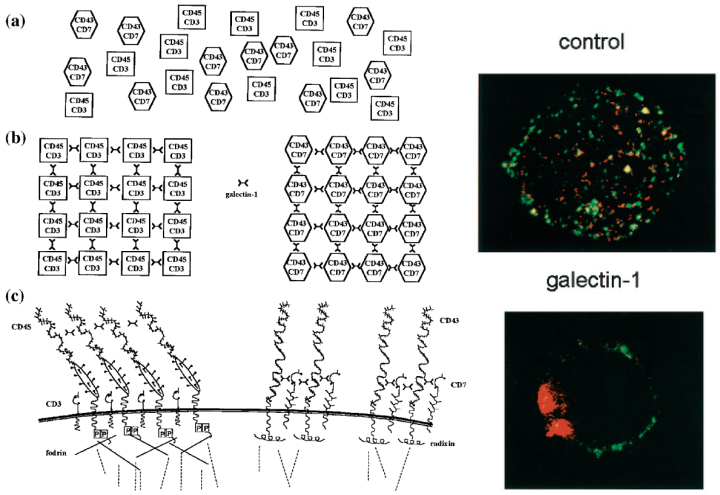


Figure 10: Key biological effects of galectin-1 base on the formation of glycan-galectin-1 lattices due to the bivalent binding capabilities of non-covalent galectin-1 dimers. *Left:* Binding of galectin-1 to human T cells separates CD43/CD7 and CD45/CD3 complexes *(a)* from each other to from homogenous clusters within the cell membrane *(b)*. Thus intracellular glycoprotein domains are closer to each other *(c)* amplifying their trans-membrane signal transduction. *Right:* Confocal microscopy images showing clustering of CD45 (red) and CD43 (green) upon addition of galectin-1. (Reproduced from (Pace et al., 1999).)

Galectin-1 is commonly regarded as a multipotent anti-inflammatory lectin: Binding of galectin-1 to CD7, CD43 and CD45 causes apoptosis of activated peripheral T cells (Hernandez et al., 2006; Perillo et al., 1995). In the same way galectin-1 causes apoptosis of immature double negative and double positive thymocytes (Stillman et al., 2006). Galectin-1 furthermore shows affinity for CD2 and CD3 without linked induction of apoptosis (Stillman et al., 2006). Several intracellular mechanisms have been considered as mediating galectin-1 induced apoptosis of activated T cells: Activation of transcription factor AP-1 and down-regulation of Bcl-2 (Rabinovich et al., 2000a), hyperpolarization of the mitochondrial mem-

brane and the ceramide pathway of apoptosis (Matarrese et al., 2005) as well as tyrosine phosphorylation in the $p56^{lck}$/ZAP-70/MAPK pathway (Ion et al., 2005). Notably, galectin-1 induced apoptosis has been described to be independent from activation of caspases and cytochrome c release from mitochondria (Hahn et al., 2004) and accordingly a later study showed that apoptosis of T cells caused by galectin-1 could not be inhibited by intracellular bcl-2, which regulates cytochrome c release from mitochondria (Bi et al., 2008).

Binding of galectin-1 to CD45 and consequent intracellular signalling have recently been shown to depend on the expressed CD45 isoform as well as on the presence of core 2 O-glycans and to be negatively regulated by α2-6 sialylation of N-glycans (Earl et al., 2010). In this context galectin-1 induced reduction of intracellular phosphatase activity of CD45 is regarded as essential for mediating apoptosis (Amano et al., 2003; Earl et al., 2010). Introducing CD45 isoforms as variables of susceptibility to apoptosis caused by galectin-1 may explain differences of B and T lymphocytes in galectin-1 signalling (Earl et al., 2010).

Galectin-1 not only causes apoptosis of T cells but also modulates their cytokines: It decreases the levels of cytokines like IL-2, which require costimulatory signals in addition to the T cell receptor (TCR), by phosphorylation of the TCR-ζ chain and an increase of inhibiting pp21ζ. In contrast cytokines which do not require costimulation are left unchanged – such as interferon γ (IFN-γ) (Chung et al., 2000).

Upon incubation with galectin-1 dendritic cells are pushed towards tolerogenicity, which leads to peripheral T cell tolerance: They secrete elevated levels of interleukin 10 (IL-10) and interleukin 27 (IL-27) (Ilarregui et al., 2009). IL-27 promotes the formation of IL-10 secreting type 1 regulatory T cells (T_{reg}) and increases the IL-10 secretion of T_H1, T_H2 and T_H17 cells (Saraiva and O'Garra, 2010; Steinman et al., 2003). Moreover, galectin-1 treatment *in vivo* ameliorates several chronic inflammations, such as experimental autoimmune encephalomyelitis (EAE) in mice, which serves as a model of multiple sclerosis (Ilarregui et al., 2009).

In addition, galectin-1 seems to be a key mediator of $CD4^+CD25^+$ regulatory T cells, which are important for the maintenance of immune tolerance: Galectin-1 is overexpressed in these cells, induced upon activation and $CD4^+CD25^+$ regulatory T cells of galectin-1 knockout mice bear a diminished regulatory potential (Garin et al., 2007). Besides that, expression of galectin-1 is especially found in activated B and T cells, inflammatory macrophages and decidual NK cells (Rabinovich and Toscano, 2009).

These links of galectin-1 with regulatory T cells and dendritic cells together with successful application in experimental autoimmune encephalomyelitis (EAE) point at a therapeutical potential of galectin-1 in autoimmune pathologies and possible significance in the testis.

Beside its role in immunology, galectin-1 has been demonstrated to be involved in numerous other biological processes (for a detailed review see (Camby et al., 2006)). These fields include cell growth and migration, embryonic tissue development and nerve growth as well as nerve reconstitution after injury. Notably, galectin-1 has also been described to be involved in tumour-immune escape by killing antitumour $CD4^+$ and $CD8^+$ T cells (Rubinstein et al., 2004). Additionally galectin-1 serves as a modulator of transendothelial migration of leukocytes: Galectin-1 expressed in endothelial cells of prostate cancer inhibits transmigration of T cells, most likely by clustering of CD43 (He and Baum, 2006).

1.3.6. Galectin-1 in the Context of T_H1-type Immunity and Male Infertility

Infertility is a common medical condition with one in ten couples seeking medical assistance because of fertility problems. Underlying pathologies are found in the female partner only (38%), the male partner only (20%) and in both partners (27%). In 15% of all infertility cases no distinct cause can be identified (de Kretser, 1997).

Infections of the genitourinary tract and immunological disorders are of growing significance for understanding male infertility. They extend the spectrum of urological, systemic, endocrine and genetic diseases accounting for infertility (e.g. varicocele, cystic fibrosis, diabetes, Y chromosome defects) (Bhushan et al., 2009; Irvine, 1998; Schuppe and Meinhardt, 2005).

Approximately 12% of male factor infertility cases are attributed to autoimmunity (Fijak and Meinhardt, 2006). They are characterized by autoantibodies against antigens on ejaculated spermatozoa and against testicular antigens (Bohring et al., 2001; Fijak et al., 2005; Naz, 2004). Consequently these autoantibodies either impair the fertilizing function of spermatozoa or initiate a chronic inflammation of the testis (Fijak and Meinhardt, 2006). The latter of these two pathogenic processes is studied in the model of experimental autoimmune orchitis (EAO) induced by immunization of rats with a testis homogenate and complete Freund's adjuvant (Adekunle et al., 1987; Doncel et al., 1989; Fijak et al., 2005; Tung et al., 1970).

Galectin-1 is a promising candidate for the amelioration of EAO and therapeutic intervention in infertility resulting from testicular autoimmune inflammation: It has successfully been applied in several experimental models of EAO-like chronic autoimmune inflammations *in*

vivo (Rabinovich et al., 2007) and has been shown to be expressed in Sertoli cells of rodent and human testis (Dettin et al., 2003; Wollina et al., 1999).

Models include – in addition to experimental autoimmune encephalomyelitis (EAE) (Chapter 1.3.5) – those for myasthenia gravis (Levi et al., 1983), collagen-induced arthritis (Rabinovich et al., 1999), concanavalin A-induced hepatitis (Santucci et al., 2000) and experimental autoimmune uveitis (Toscano et al., 2006). There are also hints that galectin-1 ameliorates acute inflammations, too (Rabinovich et al., 2000b). An imbalance of T_H1-type and T_H2-type immune responses with predominant T_H1-type cytokines are regarded as the underlying pathology of these disorders (Romagnani, 1994). Reduced severity and delayed onset of chronic inflammation were shown to be linked with the following mechanisms after administration of galectin-1 to mice and rats (Table 1).

Mechanism	Pathology/Model	Reference
T_H1 cells↓, apoptosis of T cells↑	Autoimmune diabetes	(Perone et al., 2006)
T_H1 response↓, T_H2 response↑	Autoimmune diabetes	(Perone et al., 2009)
T_H1 response↓, T_H2 response↑	Experimental autoimmune uveitis	(Toscano et al., 2006)
T_H1 response↓, T_H2 response↓↑	Graft-versus-host disease	(Baum et al., 2003)
Apoptosis of T_H1 and T_H17 cells↑	Experimental autoimmune encephalomyelitis	(Toscano et al., 2007)
Apoptosis of activated T cells↑, T_H2 response↑	Collagen-induced arthritis	(Rabinovich et al., 1999)
Apoptosis of activated T cells↑, T_H1 response↓	Con A-induced hepatitis, TNBS-induced colitis	(Santucci et al., 2000) (Santucci et al., 2003)
Macrophage recruitment↓	Nephrotoxic serum nephritis	(Tsuchiyama et al., 2000)

Table 1: Overview of immunoregulatory mechanisms of galectin-1 shown in experimental *in vivo* models of chronic autoimmune inflammations. (Modified from (Rabinovich and Toscano, 2009).) *Con A*: concanavalin A. *TNBS*: 2,4,6-trinitrobenzene sulfonic acid.

In general, naive $CD4^+$ T cells differentiate into T_H1 cells under the influence of dendritic cells when exposed to high levels of IL-12. On the contrary, low levels of IL-12 in the presence of dendritic cells lead the way to differentiation into T_H2 cells. T_H1 cells are regarded as a proinflammatory phenotype of T cells due to their secretion of IFN-γ as well as IL-2. They promote phagocytosis and are linked to delayed-type hypersensitivity. T_H2,

however, predominantly secrete IL-4 as well as IL-10 and support differentiation of B cells into plasma cells, thus causing a rather humoral immune response while being linked with allergies (Coffman, 2006; Steinman, 2007). T_H17 cells secrete high levels of IL-17 and are thought to be involved in autoimmune diseases (Mangan et al., 2006). Shifting immune responses by galectin-1 from T_H1 towards T_H2 answers has thus coined galectin-1 to be an anti-inflammatory mediator (Rabinovich et al., 2007).

Similar to these experimental models, in which a beneficial effect of galectin-1 has been demonstrated *in vivo*, immune regulation during EAO is characterized by a predominant T_H1-type response with increased secretion of the proinflammatory cytokines IL-2, TNFα and IFN-γ (Yule and Tung, 1993). Additionally loss of regulatory T cells promotes the T cell development towards a T_H1-dominated immune response (Itoh et al., 1992; Tung and Teuscher, 1995). In addition the number of testicular macrophages increases in the course of EAO. They release TNFα and thus cause apoptosis of germ cells (Rival et al., 2008; Theas et al., 2008). Testicular dendritic cells also increase in numbers and promote the autoimmune T_H1 cell function by presenting self antigens and secreting IL-12 (Rival et al., 2007).

1.4. Aim of the Study

Galectin-1, a lectin binding galactose β1-4 N-acetylglucosamine-containing oligosaccharides on the surface of immune and stromal cells, is of emerging importance in the understanding of T cell homeostasis and immunosuppression (Rabinovich and Toscano, 2009).

Its functional capability ranges from induction of T cell apoptosis, a shift of immune reactions from T_H1 to T_H2 and expansion of regulatory T cells (T_{reg}) to modulation of monocyte and macrophage functions (Camby et al., 2006). With its anti-inflammatory properties revealed in animal models of autoimmunity and chronic inflammation such as experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune uveitis (EAU), galectin-1 has become a factor of clinical significance (Rabinovich et al., 2007).

Although the testis is an immune privileged site and its immune and somatic cells are thought to collectively maintain an anti-inflammatory environment (Fijak and Meinhardt, 2006), paradoxically inflammatory based infertility is among the most important aetiologies of male infertility (Bhushan et al., 2009; Schuppe and Meinhardt, 2005).

Despite the finding that autoimmune inflammation of the testis is dominated by T_H1 cells (Yule and Tung, 1993), whose pathophysiological influence can be ameliorated by galectin-1, the role of galectin-1 in testicular immune regulation and inflammatory based infertility still remains unclear.

Using a flow cytometric approach, this study aims at identifying testicular cells targeted by galectin-1 and at investigating how the glycan structure on the cellular surface of these cells influences galectin-1 binding intensity.

2. Materials & Methods

2.1. Materials & Laboratory Animals

2.1.1. Laboratory Animals

	Supplier	Cat. Number
Male immature Wistar rats, 18 days old on day of delivery	Charles River Laboratories Germany GmbH, Sulzfeld, Germany	WIUSIFELACT (CRL:WI(WU))
Male Wistar Unilever rats, 250-274 g	Harlan Winkelmann GmbH, Borchen, Germany	HsdCpb:WU, 32418260

2.1.2. Chemicals

	Manufacturer	Cat. Number
Acetic acid (glacial)	Merck KGaA, Darmstadt, Germany	100063
Agarose (electrophoresis grade)	Invitrogen GmbH, Karlsruhe, Germany	15510-027
Albumine bovine fraction V, pH 7.0, standard grade, lyophilized	SERVA Electrophoresis GmbH, Heidelberg, Germany	11930
Alexa Fluor 647 carboxylic acid, succinimidyl ester, 1 mg	Invitrogen GmbH, Karlsruhe, Germany	A20006
Ammonium persulphate (APS)	Sigma-Aldrich Chemie GmbH, Munich, Germany	A3678
Ampuwa, water for injection	Fresenius Kabi AG, Bad Homburg, Germany	6605508
Bacto tryptone	BD Biosciences GmbH, Heidelberg, Germany	211705
Bacto-yeast extract	BD Biosciences GmbH, Heidelberg, Germany	210929
Certistain Erythrosin B	Merck KGaA, Darmstadt, Germany	115936

Coomassie Brilliant G-250	Bio-Rad Laboratories GmbH, Munich, Germany	161-0406
4-CORE Buffer Pack	Promega GmbH, Mannheim, Germany	R9921
(p-hydroxy) Coumaric acid	Sigma-Aldrich Chemie GmbH, Munich, Germany	C9008
Dimethyl sulphoxide (DMSO)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	A994.2
1,4-Dithiothreitol (DTT)	Roche Diagnostics GmbH, Mannheim, Germany	10 708 984 001
EDTA (Titriplex III)	Merck KGaA, Darmstadt, Germany	108418
Ethidium bromide, 10X stock, 10mg/ml	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	2218.2
Fetuin from fetal calf serum	Sigma-Aldrich Chemie GmbH, Munich, Germany	F2379
D(+)-Glucose-monohydrate, for microbiology	Merck KGaA, Darmstadt, Germany	108342
Glycerol	Merck KGaA, Darmstadt, Germany	356350
Goat serum (normal)	Dako Germany GmbH, Hamburg, Germany	X0907
Iodine (≥ 99.8%)	Sigma-Aldrich Chemie GmbH, Munich, Germany	207772
Isopropyl-β-D-thiogalacto-pyranoside (IPTG)	SERVA Electrophoresis GmbH, Heidelberg, Germany	26600.04
α-Lactose monohydrate, reagent grade	Sigma-Aldrich Chemie GmbH, Munich, Germany	L3625
Luminol	Sigma-Aldrich Chemie GmbH, Munich, Germany	A4685
Magnesium chloride	Merck KGaA, Darmstadt, Germany	814733

Magnesium sulphate	Sigma-Aldrich Chemie GmbH, Munich, Germany	M2643
Mayer's haemalaun solution	Merck KGaA, Darmstadt, Germany	109249
β-Mercaptoethanol for molecular biology, 14.3 M	AppliChem GmbH, Darmstadt, Germany	A1108
Methanol, ≥ 99.8%	Sigma-Aldrich Chemie GmbH, Munich, Germany	65543
2-Methylbutan	Merck KGaA, Darmstadt, Germany	106056
Non-fat dry milk, blotting-grade	Bio-Rad Laboratories GmbH, Munich, Germany	170-6404
Paraformaldehyde, extra pure DAC	Merck KGaA, Darmstadt, Germany	104005
Phytohaemagglutinin	PAA Laboratories GmbH, Pasching, Austria	J01-006
Ponceau S	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	5938.1
Potassium chloride	Merck KGaA, Darmstadt, Germany	104938
RBC lysis solution	QIAGEN GmbH, Hilden, Germany	158902
Rotiphorese Gel 30 acrylamide/ bisacrylamide mixture	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	3029.1
Roti-Quant, Bradford assay, 5X stock	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	K015.1
Sodium chloride	Sigma-Aldrich Chemie GmbH, Munich, Germany	S3014
Sodium dodecyl sulphate	Merck KGaA, Darmstadt, Germany	428023
Sodium hydrogen carbonate, GR for analysis, ACS, Reag. Ph Eur	Merck KGaA, Darmstadt, Germany	106329
D(+)-Sucrose, ≥ 99.5 %, p.a., for microbiol. and molecular biology.	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	4621.1

TEMED (N,N,N',N'-tetramethyl-ethylenediamine)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	2367.3
Tris Pufferan	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	4855.2
Triton X-100	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	3051.2
Tween 20	Carl Roth GmbH + Co. KG, Karlsruhe, Germany	9127
Xylene cyanol FF	Sigma-Aldrich Chemie GmbH, Munich, Germany	X-4126

2.1.3. Enzymes and Inhibitors

	Manufacturer	Cat. Number
Collagenase A	Roche Diagnostics GmbH, Mannheim, Germany	10103586001
DNase I, grade II	Roche Diagnostics GmbH, Mannheim, Germany	10104159001
Hyaluronidase	Roche Diagnostics GmbH, Mannheim, Germany	10106500001
Lysozyme, from chicken egg white	Sigma-Aldrich Chemie GmbH, Munich, Germany	L6876
NdeI, restriction enzyme	Promega GmbH, Mannheim, Germany	R6801
Trypsin	Roche Diagnostics GmbH, Mannheim, Germany	10109819001
Trypsin-EDTA, 0.05% w/v / 0.02% w/v in DPBS	PAA Laboratories GmbH, Pasching, Austria	L11-004
(Soybean) trypsin inhibitor	Roche Diagnostics GmbH, Mannheim, Germany	10109886001
XbaI, restriction enzyme	Promega GmbH, Mannheim, Germany	R6181
XhoI, restriction enzyme	Promega GmbH, Mannheim, Germany	R6161

2.1.4. Antibodies

Primary Antibodies	Dilution	Manufacturer	Cat. Number
Goat anti-human vimentin pAb	1:100 (ICC)	Sigma-Aldrich GmbH, Munich, Germany	V4630
Mouse anti-human α-smooth muscle actin mAb, $IgG_{2a,\kappa}$, clone 1A4	1:100 (ICC)	Dako Germany GmbH, Hamburg, Germany	M0851
Mouse anti-rat CD68 (ED1) mAb, IgG_1	1:50 (ICC)	AbD Serotec GmbH, Düsseldorf, Germany	MCA341R
Mouse anti-rat CD163 (ED2) mAb, IgG_1	1:50 (ICC)	AbD Serotec GmbH, Düsseldorf, Germany	MCA342R
Rabbit anti-human galectin-1 pAb	1:5,000 (WB)	Prof. G. Rabinovich, Lab. of Immunopath., Inst. of Biol. and Exp. Med., Nat. Council of Sci. and Tech. Research, Buenos Aires, Argentina	(Rabinovich et al., 1999)
Secondary Antibodies	**Dilution**	**Manufacturer**	**Cat. Number**
Donkey anti-goat IgG pAb, Cy3 conjugated	1:800 (ICC)	Chemicon International, Temecula, USA	AP180C
Donkey anti-mouse IgG pAb, Cy3 conjugated	1:1,000 (ICC)	Dianova GmbH, Hamburg, Germany	715-165-151
Goat anti-rabbit IgG pAb, horseradish peroxidase conjugated	1:10,000 (WB)	Cappel Antibodies, MP Biomedicals Germany GmbH, Berlin, Germany	0867437

2.1.5. Plant Lectins

Plant Lectins (Origin)	Conc.	Manufacturer	Cat. Number
LEA, FITC conjugated (Lycopersicon esculentum, tomato)	20 µg/ml	EY Laboratories, Inc., San Mateo, USA	F-7001-1
MAA, FITC conjugated (Maackia amurensis, Amur maackia)	20 µg/ml	EY Laboratories, Inc., San Mateo, USA	F-7801-2
PNA, FITC conjugated (Arachis hypogaea, peanut)	20 µg/ml	EY Laboratories, Inc., San Mateo, USA	F-2301-1
SNA-I, FITC conjugated (Sambucus nigra, elderberry)	10 µg/ml	EY Laboratories, Inc., San Mateo, USA	F- 6802-1

2.1.6. Bacterial Strains and Plasmid

	Manufacturer	Reference
E. coli BL21(DE3)pLysS	Laboratory stock.	
E. coli DH5α	Laboratory stock.	
pET-21a(+) vector containing the gene of human galectin-1 C2S	Dr. K. Kasai, Dr. J. Hirabayashi, Research Center for Medical Glycoscience, AIST, Tsukuba, Japan	(Hirabayashi and Kasai, 1991)

2.1.7. Columns for Plasmid and Protein Purification

	Manufacturer	Cat. Number
JETSTAR plasmid purification kit, mini column/maxi column	GENOMED GmbH, Löhne, Germany	200 050/ 220 020
NHS-activated Sepharose 4 fast flow	GE Healthcare Europe GmbH Life Sciences, Freiburg, Germany	17-0906-01
Poly-Prep column	Bio-Rad Laboratories GmbH, Munich, Germany	731-1550

2.1.8. Cell Culture Media and Antibiotics

	Manufacturer	Cat. Number
Ampicillin sodium salt	Sigma-Aldrich Chemie GmbH, Munich, Germany	A9518
Chloramphenicol	Sigma-Aldrich Chemie GmbH, Munich, Germany	C0378
DMEM/Ham's F-12, without L-glutamine	PAA Laboratories GmbH, Pasching, Austria	E15-012
Dulbecco's PBS, without Ca & Mg	PAA Laboratories GmbH, Pasching, Austria	H15-002
Fetal bovine serum "GOLD"	PAA Laboratories GmbH, Pasching, Austria	A15-151
L-Glutamine, 200 mM	PAA Laboratories GmbH, Pasching, Austria	M11-004
HEPES buffer solution, 1 M	GIBCO, Invitrogen GmbH, Karlsruhe, Germany	15630-056
MEM non-essential amino acid solution, 100×, without L-glutamine	Sigma-Aldrich Chemie GmbH, Munich, Germany	M7145
β-Mercaptoethanol, 50 mM	GIBCO, Invitrogen GmbH, Karlsruhe, Germany	31350-010
Penicillin/streptomycin, 100×, 10,000 units/ml, 10 mg/ml	PAA Laboratories GmbH, Pasching, Austria	P11-010
RPMI 1640 with L-glutamine, with phenol red	PAA Laboratories GmbH, Pasching, Austria	E15-840
Sodium pyruvate MEM, 100 mM	GIBCO, Invitrogen GmbH, Karlsruhe, Germany	11360-039

2.1.9. Specific Material for Electrophoresis and Western Blotting

	Manufacturer	Cat. Number
100 bp DNA size ladder	Promega GmbH, Mannheim, Germany	G2101
1 kbp DNA size ladder	Promega GmbH, Mannheim, Germany	G5711
Amersham Hyperfilm ECL, 18 x 24 cm	Amersham Biosciences Europe GmbH, Freiburg, Germany	28-9068-36
Hybond ECL membrane, 0.20 µm pore size	Amersham Bioscience Europe GmbH, Freiburg, Germany	RPN3032D
SERVA unstained SDS-PAGE protein marker 6.5 - 200 kDa	SERVA Electrophoresis GmbH, Heidelberg, Germany	39215.01
Whatman 3MM Chr blotting paper, 46 x 57 cm	Whatman GmbH, Dassel, Germany	3030-917

2.1.10. Consumables for Cell Culture and Cell Isolation

	Manufacturer	Cat. Number
BD Falcon cell strainer, 70 µm	BD Biosciences GmbH, Heidelberg, Germany	352350
BD Microlance needles, 18 G/25 G	BD Biosciences GmbH, Heidelberg, Germany	304622/ 300400
BD Plastipak syringes with BD Luer-Lok, 5 ml/10 ml/20 ml/50 ml	BD Biosciences GmbH, Heidelberg, Germany	300911/ 300912/ 300629/ 300865
Cell culture multiwell plates, 6/12/24 wells	Greiner Bio-One GmbH, Frickenhausen, Germany	657160/ 665180/ 662160
Cell culture flasks, 175 cm^2	Greiner Bio-One GmbH, Frickenhausen, Germany	660175

Petri dishes, 60 x 15 mm	Sarstedt AG & Co., Nümbrecht, Germany	83.1801
PP tubes with screw cap, conical, 15 ml/50 ml (=”Falcon tubes”)	Greiner Bio-One GmbH, Frickenhausen, Germany	188271/ 227261
Serological pipettes, 5 ml/10 ml/ 25 ml/50 ml	Greiner Bio-One GmbH, Frickenhausen, Germany	606180/ 607180/ 760180/ 768180
Trypan Blue Stain	Invitrogen GmbH, Karlsruhe, Germany	15250-061

2.1.11. Consumables for Flow Cytometry and Microscopy

	Manufacturer	Cat. Number
Cover slips, round, 12 mm	Gerhard Menzel GmbH & Co. KG, Braunschweig, Germany	CB00120RA1
Cover slips, 24 x 60mm	Gerhard Menzel GmbH & Co. KG, Braunschweig, Germany	BB024060A1
Dako delimiting pen	Dako Germany GmbH, Hamburg, Germany	S2002
Entellan new	Merck KGaA, Darmstadt, Germany	107961
PP tubes for FACScan flow cytometer, 5 ml, 12 x 75 mm	Sarstedt AG & Co., Nümbrecht, Germany	55.1578
SuperFrost plus slides	R. Langenbrinck, Labor- und Medizintechnik, Emmendingen, Germany	03-0060
Tissue-Tek O.C.T. compound	Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands	4583
Vectashield mounting medium with DAPI	Vector Laboratories, Inc., Burlingame, USA	H-1200

2.1.12. Equipment and Software

	Manufacturer	Cat. Number
(Analytical) balance Sartorius Research R200D	Sartorius AG, Göttingen, Germany	R200D
(Electronic) balance Scaltec SPB53	Scaltec Instruments GmbH, Göttingen, Germany	14805574
Bürker's cell counting chamber	LO - Laboroptik GmbH, Friedrichsdorf, Germany	1900000
(Mini) centrifuge MC6	SARSTEDT AG & Co., Nümbrecht, Germany	90.186.100
(Refrigerated) centrifuge Heraeus Labofuge 400R	Kendro Laboratory Products GmbH, Hanau, Germany	75008375
CO_2 incubator Binder CB 150	Binder GmbH, Tuttlingen, Germany	9040-0012
Confocal laser scanning microscope TCS SP2	Leica Microsystems GmbH, Wetzlar, Germany	TCS SP2
Cryostat CM 1900	Leica Microsystems GmbH, Wetzlar, Germany	CM 1900
Digital block heater HX-2	PEQLAB Biotechnologie GmbH, Erlangen, Germany	91-D1200
Electrophoresis power supply E143	Consort nv, Turnhout, Belgium	E431
Electrophoresis power supply E831	Consort nv, Turnhout, Belgium	E831
Flow cytometer BD FACSCalibur, 4 colours, incl. CellQuest Pro 4.0.2	Becton Dickinson GmbH, Heidelberg, Germany	342975
Fluorescence microscope Axioplan 2 imaging with AxioCam color, HBO 100 lamp and HAL 100 lamp	Carl Zeiss MicroImaging GmbH, Jena, Germany	000537, 412-312, 447219-1039-685
Gel Jet Imager, incl. Intas GDS software 3.28	Intas Science Imaging Instruments GmbH, Göttingen, Germany	n/a

Hellendahl cuvette	VWR International GmbH, Darmstadt, Germany	631-9130
Horizontal shaker	GFL Gesellschaft für Labortechnik GmbH, Burgwedel, Germany	3017
illustra NAP^{TM}-10 Columns	GE Healthcare Europe GmbH Life Sciences, Freiburg, Germany	17-0854-01
Inverted microscope Olympus CKX41SF equipped with Olympus Camedia C-7070	Olympus Optical Co., Ltd., and Olympus Imaging Corp., Tokyo, Japan	2G 1 2544, D33235
Laminar air flow cabinet, class II, BDK-S 1800	BDK Luft- und Reinraumtechnik GmbH, Sonnenbühl-Genkingen, Germany	S 1800
Magnetic stirrer RCT basic	IKA Werke GmbH & Co. KG, Staufen, Germany	3810000
Microsoft Excel 2003	Microsoft Corporation, Redmond, USA	n/a
Microwave oven	Samsung Electronics GmbH, Schwalbach, Germany	M1712N
Orbital shaker MS2 Minishaker	IKA Werke GmbH & Co. KG, Staufen, Germany	L002050
PerfectBlue Gelsystem Mini M chamber	PEQLAB Biotechnologie GmbH, Erlangen, Germany	40-0911
PerfectBlue Gelsystem Mini M comb, 1.5 mm/10 teeth	PEQLAB Biotechnologie GmbH, Erlangen, Germany	40-0911-10D
Pipettor Easypet 4421	Eppendorf AG, Hamburg, Germany	4421 000.013
Pipetus, pipette filler	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany	9907200
SDS gel electrophoresis chambers	Invitrogen GmbH, Karlsruhe, Germany	n/a

Semi-dry electro blotter PerfectBlue, 20 x 20 cm	PEQLAB Biotechnologie GmbH, Erlangen, Germany	52-2020
UV/visible light spectrophotometer Ultrospec 2100 Pro	Biochrom Ltd., Cambridge, United Kingdom	80-2112-21
WEASEL for flow cytometry data analysis 2.7	Walter and Eliza Hall Institute, Parkville, Australia	n/a
Zeiss AxioVision software 4.7.1	Carl Zeiss MicroImaging GmbH, Jena, Germany	410130-0500-000

2.2. Media and Buffers

2.2.1. Media and Buffers for Transformation of Bacteria

LB Medium (Sambrook and Russell, 2001b, p. A2.2)	
10 g	Bacto Tryptone
5 g	Yeast extract
10 g	NaCl
950 ml	Deionized H_2O
Adjust pH to 7.0 with 5 M NaOH, fill up to 1 l with deionized H_2O and autoclave.	

LB Agar (Sambrook and Russell, 2001b, p. A2.5)	
1.5% w/v	Agar
	LB medium
Autoclave and store at 4°C.	

SOB Medium (Sambrook and Russell, 2001b, p. A2.3)	
2% w/v	Bacto Tryptone
0.5% w/v	Yeast extract
10 mM	NaCl
2.5 mM	KCl
Fill up to 1 l with water and autoclave. Add 2 M Mg^{2+} (see below) prior to use for a Mg^{2+} concentration of 20 mM.	

SOC Medium (Sambrook and Russell, 2001b, p. A2.3)	
980 ml	SOB medium
20 ml	1 M Glucose
Sterile filter through a 0.20 µm filter.	

10X TE Buffer (pH 8.0) (Sambrook and Russell, 2001b, p. A1.7)	
100 mM	Tris-Cl (pH 8.0)
10 mM	EDTA (pH 8.0)
Fill up to a volume of 1 l with distilled water, autoclave and store at room temperature.	

TFB Buffer (Sambrook and Russell, 2001a, p. 1.107)	
10 mM	K-MES pH 6.3
100 mM	KCl
45 mM	$MnCl_2 \cdot 4\ H_2O$
10 mM	$CaCl_2 \cdot 2\ H_2O$
3 mM	$[Co(NH_3)_6]Cl_3$
Fill up to a volume of 1 l with distilled water, sterile filter and store at 4°C.	

2M Mg^{2+} Solution	
1 M	$MgCl_2$
1 M	$MgSO_4$
Fill up to a volume of 50 ml with distilled water, sterile filter and store at RT.	

2.2.2. Media and Buffers for Recombinant Expression of Proteins

2YT Medium (Sambrook and Russell, 2001b, p. A2.4)	
16 g	Bacto Tryptone
10 g	Yeast extract
5 g	NaCl
900 ml	Deionized H_2O
Adjust pH to 7.0 with 5 M NaOH, fill up to 1 l with deionized H_2O and autoclave.	

Cell Lysis Buffer	
50 mM	Tris-Cl (pH 8.0)
1 mM	EDTA (pH 8.0)
100 mM	NaCl
1 mM	DTT

STE Buffer (Sambrook and Russell, 2001b, p. A1.22)	
10 mM	Tris-Cl (pH 8.0)
0.1 M	NaCl
1 mM	EDTA (pH 8.0)
Autoclave and store at 4°C.	

2.2.3. Buffers for Purification of Recombinant Galectin-1

Extraction Buffer	
20 mM	Sodium phosphate buffer (pH 7.2)
0.15 M	NaCl
4 mM	β-Mercaptoethanol
2 mM	EDTA
1 mM	PMSF

Sodium Phosphate Buffer (pH 7.2) (Sambrook and Russell, 2001b, p. A1.5)	
68.4 ml	1 M Na_2HPO_4
31.6 ml	1 M NaH_2PO_4

2.2.4. Buffers for DNA Agarose Gel Electrophoresis

50X TAE Buffer (Sambrook and Russell, 2001b, p. A1.17)	
242 g	Tris base
57.1 ml	Glacial acetic acid
100 ml	0.5 M EDTA (pH 8.0)
Fill up to 1 l with bidistilled H_2O.	

DNA Sample Buffer	
3% v/v	Glycerol
0.25% w/v	Xylene cyanol FF
	Deionized H_2O

2.2.5. Buffers for SDS-PAGE

10X Tris-Glycine Buffer	
0.25 M	Tris base
1.92 M	Glycine
1% w/v	SDS
	Bidistilled water

Laemmli Sample Loading Buffer	
62.5 mM	Tris-Cl (pH 6.8)
2% w/v	SDS
5% v/v	Glycerol
0.3% w/v	Bromophenol blue
0.9% v/v	β-Mercaptoethanol

2.2.6. Buffers for Western Blotting

Concentrated Anode Buffer	
300 mM	Tris
20% v/v	Methanol

Anode Buffer	
30 mM	Tris
20% v/v	Methanol

Cathode Buffer	
25 mM	Tris
40 mM	6-Aminohexanoic acid
20% v/v	Methanol

Tris-Buffered Saline (TBS) (Sambrook and Russell, 2001b, p. A1.8)	
8 g	NaCl
0.2 g	KCl
3 g	Tris base
800 ml	Distilled H_2O
Adjust pH to 7.4 by HCl, and add distilled water to 1 litre.	

2.2.7. Media for Cell Isolation and Cell Culture

Complete DMEM Cell Culture Medium	
500 ml	DMEM/Ham's F-12 without L-glutamine
5 ml	100× Penicillin/streptomycin (stock concentration: 10,000 units/ml, 10 mg/ml)
6.5 ml	200 mM L-glutamine

Complete RPMI 1640 Cell Culture Medium	
430 ml	RPMI 1640 medium with L-glutamine
50 ml	Fetal calf serum "GOLD"
5 ml	100× Penicillin/streptomycin (stock concentration: 10,000 units/ml, 10 mg/ml)
5 ml	100 mM Sodium pyruvate MEM
5 ml	1 M HEPES buffer
5 ml	100× MEM non-essential amino acid solution without L-glutamine

PBS-A for Isolation of Sertoli and Peritubular Cells	
500 ml	Dulbecco's PBS without Ca and Mg
750 mg	D-Glucose
5 ml	100× Penicillin/streptomycin (stock concentration: 10,000 units/ml, 10 mg/ml)

RPMI-A (Sertoli cell-medium)	
500 ml	RPMI 1640 medium with L-glutamine
12.5 ml	1 M HEPES buffer
5 ml	100× Penicillin/streptomycin (stock concentration: 10,000 units/ml, 10 mg/ml)

RPMI-B (Peritubular cell-medium)	
450 ml	RPMI-A
50 ml	Fetal calf serum "GOLD"

Collagenase-Hyaluronidase-DNase I-Solution	
10 mg	Collagenase
10 mg	Hyaluronidase
200 µg	DNase I
10 ml	PBS-A

Hyaluronidase-DNase I-Solution	
10 mg	Hyaluronidase
200 µg	DNase I
10 ml	PBS-A

Trypsin-DNase I-Solution	
25 mg	Trypsin
200 µg	DNase I
10 ml	PBS-A

Trypsin Inhibitor-Solution A	
50 mg	Trypsin inhibitor
5 ml	PBS-A

Trypsin Inhibitor-Solution B	
25 mg	Trypsin inhibitor
10 ml	PBS-A

2.2.8. Buffer for Flow Cytometry

10X Phosphate-Buffered Saline (pH of 7.2) **Modified from (Sambrook and Russell, 2001b, p. A1.7)**	
82 g	NaCl
0.2 g	KCl
10 g	Na_2HPO_4
4 g	KH_2PO_4
800 ml	Distilled water.

Adjust pH to 7.2 with HCl, then fill up to 1 l.

2.3. Care and Use of Laboratory Animals

Healthy male Wistar Unilever rats weighing 250-274 g were obtained from Harlan Winkelmann GmbH and eighteen-day-old male Wistar rats from Charles River Laboratories Germany GmbH. Experimental procedures were approved by the local authority (Regierungspräsidium Gießen GI 20/23) and conform to the "Code of Practice for the Care and Use of Animals for Experimental Purposes".

2.4. Recombinant Expression of Human Galectin-1 C2S

A pET-21a(+) vector containing the full length coding sequence of human galectin-1 C2S was a generous gift of Dr. Ken-ichi Kasai and Dr. Jun Hirabayashi (Research Center for Medical Glycoscience, AIST, Tsukuba, Japan). Preparation of recombinant human galectin-1 C2S was carried out in three steps: Amplification of the plasmid in an *E. coli* DH5α strain, expression in an *E. coli* BL21(DE3)pLysS strain and purification of the recombinant protein.

2.4.1. Transformation of *E. coli* DH5α

Preparation of competent *E. coli* DH5α and transformation were performed according to a modified Hanahan's protocol (Hanahan, 1983).

E. coli DH5α were directly picked from a frozen glycerol stock and streaked onto an LB agar plate (Chapter 2.2.1) devoid of antibiotics by using an inoculating loop. The plate was incubated overnight at 37°C. A single colony was transferred into 5 ml of SOB medium (Chapter 2.2.1) and grown overnight in a shaking incubator (235 rpm) at 37°C.

50 ml of pre-warmed SOB medium were inoculated with 500 µl of overnight culture and cells were again incubated in a shaking incubator (235 rpm) for 2-3 h at 37°C. Bacterial cell growth was monitored by measuring OD_{600nm} in spectrophotometry every 30 min. When the cell density reached an OD_{600nm} of 0.45-0.50, the culture was incubated on ice for 10 min. The cell suspension was transferred into a 50 ml Falcon tube and kept on ice for further 10 min followed by centrifugation at 1,075 × g for 15 min at 4°C. The cell pellet was gently resuspended in 20 ml of TFB buffer (Chapter 2.2.1) and the cell suspension was kept on ice for further 10 min and pelleted by centrifugation at 1,075 × g for 15 min at 4°C. The resulting supernatant was carefully decanted and bacteria were resuspended in 3.9 ml of TFB buffer. The cells were then made competent for transformation by addition of 140 µl of DMSO and incubation on ice for 5 min. Then 140 µl of 1 M DTT were added and incubation was continued for 10 min before another 140 µl of DMSO were added for 5 min.

For transformation, 200 µl aliquots of competent bacteria were incubated on ice for 30 min with 50 ng of the human galectin-1 C2S pET-21a(+) vector in TE buffer (pH 8.0) (Chapter 2.2.1). Then the cells were transferred to a heat block pre-heated to 42°C for exactly 45 s (→ opening of cell membrane) and then cooled on ice again for 2 min (→ closing of cell membrane). After that 800 µl of warm (37°C) SOC medium (Chapter 2.2.1) were added to each tube and incubated for 1 h at 37°C in a shaking incubator (235 rpm). Transformation efficiency was checked by observation of colony formation after incubating 200 µl of the resulting cell suspension on pre-warmed LB agar plates with a final concentration of 50 µg/ml ampicillin overnight at 37°C. Until the cell suspension had entered the LB agar, the inoculated plates were stored at RT and then inverted for overnight incubation. The ampicillin resistance gene included in the pET-21a(+) vector enables selection of transformed bacteria. A single resulting colony was transferred into 5 ml of SOB medium containing ampicillin (50 µg/ml) for overnight culture in a shaking incubator (235 rpm) at 37°C. From there bacteria were stored at -80°C in a 30% v/v glycerol stock.

2.4.2. Plasmid Isolation

200 ml of LB medium containing ampicillin (50 µg/ml) were inoculated with 1 ml overnight culture of transformed *E. coli* DH5α and incubated overnight at 37°C in a shaking incubator (235 rpm). Plasmids were isolated using a JETSTAR plasmid purification kit with maxi column according to the manufacturer's instructions. Briefly, cell membranes were destabilized by resuspending the bacterial cell pellet in a 10 mM EDTA solution, which chelates bivalent cations. Cells were then lysed by the anionic detergent SDS (1 % w/v) and intracellular proteins, chromosomal and plasmid DNA were denatured by NaOH (200 mM). Finally, plasmid DNA was purified using its affinity for an anion exchange resin and utilizing the circumstance that plasmid DNA renatures faster than chromosomal DNA after neutralization with 3.1 M CH_3COOK/CH_3COOH (pH 5.5). Plasmids were eluted from the resin by an 1.25 M NaCl solution containing 100 mM Tris-Cl (pH 8.5), precipitated by isopropanol, washed by 70% v/v ethanol and resuspended in 100 µl of 10 mM Tris-Cl (pH 8.5). DNA concentration was assessed by measuring OD_{260nm} and plasmid solutions were stored at -20°C.

Purified plasmid DNA was analysed by a 1.5% w/v DNA agarose gel electrophoresis (Chapter 2.8). The plasmid was linearised by digestion with XbaI and also the insert was visualised by digestion with NdeI and XhoI. As a further control an empty pET-21a(+) vector as well as its linearised form were applied to 1.5% w/v DNA agarose gel electrophoresis. Restriction enzyme digestion was performed in 20 µl reaction volumes according to the

manufacturer's recommendations. 1 µg plasmid DNA was solved in an appropriate volume of sterile, deionized water, 2 µl of 10X restriction enzyme buffer (Promega 4-CORE buffer D: pH 7.9, 60 mM Tris-Cl, 60 mM $MgCl_2$, 1.5 M NaCl, 10 mM DTT) and 0.2 µl of 1% w/v acetylated bovine serum albumin (BSA). Digestion was carried out with 0.5 µl of XbaI or NdeI and XhoI (10 u/µl) for 2 hours at 37°C.

2.4.3. Transformation of *E. coli* BL21(DE3)pLysS

E. coli BL21(DE3)pLysS, an expression strain carrying a chloramphenicol resistance gene, was transformed according to the protocol described above (Chapter 2.4.1).

As minor modifications, colonies picked from a -80°C glycerol stock were streaked onto an LB agar plate containing a final concentration of 34 µg/ml chloramphenicol for overnight incubation at 37°C. Subsequently, a single colony was transferred into 5 ml of SOB medium supplemented with chloramphenicol (34 µg/ml). After overnight incubation at 37°C in a shaking incubator (235 rpm) the bacterial cell suspension was chemically transformed with 100 ng of the pET-21a(+) vector containing the full length coding sequence of human galectin-1 C2S.

2.4.4. Expression of Recombinant Human Galectin-1 C2S in *E. coli* BL21(DE3)pLysS

Expression of recombinant human galectin-1 C2S based on a modification of the methods described by Dr. Ken-ichi Kasai and Dr. Jun Hirabayashi (Hirabayashi et al., 1989; Hirabayashi and Kasai, 1991). For expression of the recombinant human galectin-1 C2S protein, *E. coli* BL21(DE3)pLysS competent cells were transformed with the pET-21a(+)-galectin-1 C2S construct. Transformed *E. coli* BL21(DE3)pLysS were streaked onto LB-agar plates containing ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml) for overnight incubation at 37°C.

One of the positive colonies was transferred into 5 ml of 2YT medium (Chapter 2.2.2) with ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml) and cultured at 37°C overnight in a shaking incubator (235 rpm). The next day 250 ml of 2YT medium supplemented with ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml) were inoculated with 1 ml of the overnight culture and incubated at 37°C in a shaker (235 rpm) until an OD_{600nm} of 0.5-0.7 was reached. The expression of human galectin-1 C2S was induced by addition of IPTG to a final concentration of 0.5 mM and the incubation was continued for 2 h in the shaking incubator (235 rpm) at 37°C. Cells were harvested by centrifugation at 3,000 × g for 15 min at 4°C,

washed in 250 ml of STE buffer (Chapter 2.2.2) and pelleted again. The resulting cell pellet was stored at -20°C. Success of induction was monitored by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Chapter 2.9) of 1 ml samples of the bacterial culture collected before and 2 h after induction with IPTG. Samples were pelleted by centrifugation, lysed in SDS-PAGE sample loading buffer (Chapter 2.2.5), boiled at 95°C for 5 min and then stored at -20°C before an analysis via SDS-PAGE.

2.4.5. Preparation of Asialofetuin-Sepharose for Purification of Recombinant Human Galectin-1 C2S

Recombinant human galectin-1 C2S was purified by affinity chromatography on an asialofetuin-Sepharose column following the method described by Adrian de Waard and Jun Hirabayashi (de Waard et al., 1976; Hirabayashi et al., 1989).

Asialofetuin was synthesized from fetuin and then covalently linked via its primary amino group to the N-hydroxysuccinimide (NHS)-activated Sepharose column. Sepharose itself comprises cross-linked agarose forming beads and serves as the matrix of the column.

Thus 100 mg fetuin from fetal calf serum were dissolved in 5 ml of 0.15 M NaCl to obtain a final concentration of 20 mg/ml. Asialofetuin was derived from fetuin by adjusting the pH to 2 by 1 M HCl and incubating the solution for 1 h at 80°C. Subsequently, the solution was neutralized to pH 7.0 by using 1 M NaOH. The solution was then dialyzed three times against 0.1 M $NaHCO_3$ (pH 6-9) each for 1-2 h.

Linking of asialofetuin to the Sepharose column was carried out just before use: 5 ml of the NHS-activated Sepharose were prepared by washing twice with 30 ml of cold 1 mM HCl in a 50 ml Falcon tube at 4°C. The washed NHS-Sepharose beads and the dialysate were then mixed and the pH was immediately adjusted to 8.3 to start the covalent linking process. The reaction mixture was incubated for 2 h at RT on a slowly rotating wheel.

After the incubation 5 ml of 0.1 M Tris-Cl (pH 8.5) were added and further incubated for 4 h at RT to block unreacted NHS groups. The Sepharose linked with asialofetuin was then filled into a Poly-Prep column and washed six times with 15 ml of 0.1 M Tris-Cl (pH 8.5). Finally, the column was washed with 15 ml of 20% v/v ethanol prior to storage at 4°C.

2.4.6. Purification of Recombinant Human Galectin-1 C2S

The pellet of *E. coli* BL21(DE3)pLysS containing recombinant human galectin-1 C2S, which was stored at -20°C, was gently thawed on ice, weighed and resuspended in 20 ml of extraction buffer (Chapter 2.2.3).

Then 80 µl of 10 mg/ml lysozyme were added per gram bacterial pellet and incubated for 15 min at RT on a horizontal shaker. The cells were then disrupted by sonication (10 s on ice, power setting 50%, 6 times with 15 s intervals). To determine the level of cell disruption, 30 µl of sonicated solution were collected and centrifuged for 5 min at maximum speed in a mini centrifuge and the protein concentration was measured by Bradford assay (Chapter 2.7).

The procedure was repeated until no additional increase in protein concentration was observed. The lysate was centrifuged at 14,000 × g for 10 min at 4°C and the obtained supernatant was applied to the asialofetuin-Sepharose column. Thereafter the column was washed with 25 ml of extraction buffer and human galectin-1 C2S was eluted with 10 ml extraction buffer containing 0.1 M lactose in 1 ml fractions. The protein concentration was measured by Bradford assay. Fractions containing recombinant human galectin-1 C2S were pooled and dialysed against PBS (pH 7.0-7.5) containing ß-mercaptoethanol (final concentration: 1 mM).

2.5. Fluorescent Labelling of Recombinant Human Galectin-1 C2S

Recombinant human galectin-1 C2S was conjugated with Alexa Fluor 647 – a 1.25 kDa sized fluorophore with excitation maximum at λ=650 nm and emission maximum at λ=668 nm. The carboxylic acid group of Alexa Fluor 647 is activated by linkage to N-hydroxysuccinimide and reacts with free primary amino groups of proteins once the pH is alkalized (Figure 11):

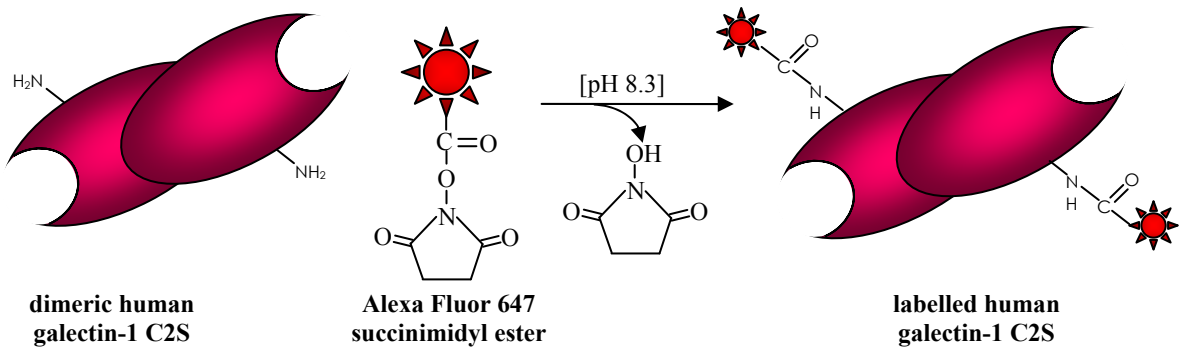


Figure 11: Scheme of fluorophore labelling of recombinantly expressed human galectin-1 C2S. Upon alkalizing of the reaction solution to pH ~ 8.3 the succinimidyl moiety of the Alexa Fluor 647 dye is destabilized and consequently reacts with the primary amino groups of human galectin-1 C2S, i.e. the ε-amino groups of the eight lysine residues of galectin-1 C2S (here only two amino groups are shown).

500 µl of a 2 mg/ml solution of recombinant human galectin-1 C2S were applied in the labelling reaction to assure a high number of fluorophores per galectin-1 C2S molecule, which contains eight lysine residues. Its N-terminus is not acetylated due to expression in bacteria – a known property with no influence on sugar binding (Hirabayashi et al., 1989; Leffler et al., 2004). During the conjugation reaction the dye-to-protein molar ratio was 11:1. The mass of dye to be applied was calculated as follows:

$$n(dye) = 11 \cdot n(protein)$$

$$\Leftrightarrow n(dye) = 11 \cdot \frac{m(protein)}{M(protein)} = 11 \cdot \frac{10^{-3}\,g}{29000\,\frac{g}{mol}} = 11 \cdot 34.5nmol = 0.379\mu mol$$

$$m = M \cdot n = 1250\,\frac{g}{mol} \cdot 0.379\mu mol = \underline{\underline{0.47mg}} \approx 0.5mg$$

In this context it has to be considered that human galectin-1 C2S is in a dynamic equilibrium between its monomeric and dimeric form. At a concentration of 2 mg/ml the majority of human galectin-1 C2S exists as non-covalently linked dimers based on its dissociation constant K_d of ~ 7 µM with a slow equilibration time of $t_{1/2}$ = 10 h (Cho and Cummings, 1995). Thus at the applied galectin-1 concentration of 2 mg/ml the molar concentration of dimers is 69 µM (M(dimer) = 29 kDa). Special attention was paid to avoid glutathione and any free primary amines – such as NH_4^+, Tris, glycine or ethanolamine – and triethylamine in the protein solution. Thus PBS (pH 7.0-7.5) containing β-mercaptoethanol (final concentration: 1 mM) was used as initial solvent and throughout all following steps for the recombinant human galectin-1 C2S. β-Mercaptoethanol was added as a reducing and thus stabilizing agent.

The molar extinction coefficient of human galectin-1 C2S for λ=280 nm was calculated according to Edelhoch's method by summing up the molar extinction coefficient of each aromatic amino acid and cysteine within the protein (Pace et al., 1995; Pingoud and Urbanke, 1997, p. 154): Based on the primary structure of galectin-1 C2S (Figure 12) with one tryptophan (ε = 5,500 $M^{-1}cm^{-1}$), two tyrosine (ε = 1,490 $M^{-1}cm^{-1}$) and five cysteine residues (regarded as cystine, ε = 125 $M^{-1}cm^{-1}$) the estimated molar extinction coefficient is ε_{280nm} = 9105 $M^{-1}cm^{-1}$.

```
        10         20         30         40         50         60
ACGLVASNLN LKPGECLRVR GEVAPDAKSF VLNLGKDSNN LCLHFNPRFN AHGDANTIVC

        70         80         90        100        110        120
NSKDGGAWGT EQREAVFPFQ PGSVAEVCIT FDQANLTVKL PDGYEFKFPN RLNLEAINYM

       130
AADGDFKIKC VAFD
```

Figure 12: Primary structure of wild-type human galectin-1 consisting of 134 amino acids. In human galectin-1 C2S the cysteine in position 2 (in red box) is replaced by a serine. (Source of primary sequence: Entrez Protein, NCBI; reference: NP_002296; analysed via ProtParam, Protein Identification and Analysis Tools on the ExPASy Server, http://www.expasy.ch/tools/protparam.html, Swiss Institute of Bioinformatics, Lausanne, Switzerland)

The concentration of the recombinant human galectin-1 C2S solution was adjusted to 2 mg/ml in a volume of 500 µl by adding distilled water. Then 50 µl of 1 M $NaHCO_3$ (= 1/10 of the galectin-1 C2S solution volume, pH 8.3) were added. Finally 0.5 mg of Alexa Fluor 647 succinimidyl ester were solved in 50 µl of distilled water (final concentration: 10 mg/ml) and immediately added to the pH adjusted protein solution. The dye was allowed to react with human galectin-1 C2S in the final volume of 600 µl for 1 h at RT protected from light on a shaker at 600 rpm.

2.6. Purification of the Protein-Dye-Conjugate

Purification of the galectin-1 C2S-dye-conjugate from free dye (M = 1250 Da ≈ 1×10^3 Da) was carried out by gel filtration chromatography on an illustra NAP-10 column. The exclusion limits of the column range from 1×10^3 to 5×10^3 Da due to its Sephadex G-25 DNA-grade resin.

The column was equilibrated by washing three times with 5 ml of Dulbecco's PBS containing ß-mercaptoethanol (final concentration: 1 mM; DPBS-βME). Then the 600 µl of labelling reaction mixture were adjusted to the minimal volume applicable to the column by adding 150 µl of DPBS-βME. This solution was pipetted onto the column followed by 250 µl of DPBS-βME. The resulting flow through fraction was collected. The final elution of Alexa Fluor 647 labelled galectin-1 C2S was carried out by applying 1200 µl of DPBS-βME to the column and collecting the resulting eluate. Additionally, four further eluting steps each with 1000 µl of DPBS-βME were carried out.

The obtained elution fractions were analysed by SDS-PAGE and Western blotting with subsequent detection by a rabbit anti-human galectin-1 polyclonal antibody (Chapters 2.9, 2.11 and 3.2). Prior to usage of the purified labelled human galectin-1 C2S in binding assays Tris (pH 8) was added to a final concentration of 1 mM to bind any free dye molecules.

2.7. Protein Concentration Measurement by Bradford Assay

Concentrations of protein solutions were determined according to the method described by Marion M. Bradford (Bradford, 1976). The assay reagent was prepared by diluting 1 volume of the 5X stock with 4 volumes of distilled water. The solution is brown coloured and bears a pH of 1.1. BSA at concentrations of 0, 250, 500, 1000, 1500, 2000 µg/ml were used as standards. Standards and samples were dissolved in PBS and 1ml assay reagent was mixed with 20 µl of sample or standard. After 5 min incubation OD_{595nm} was measured in a spectrophotometer to assess the protein concentration in reference to the standard solutions.

2.8. DNA Agarose Gel Electrophoresis

Agarose gels (1.5% w/v) were used to separate DNA fragments by length. The appropriate amount of agarose was dissolved in 1X TAE buffer (Chapter 2.2.4) and heated in a micro-wave oven (300-400 W) until agarose was dissolved. To stain DNA fragments ethidium bromide stock solution (1 mg/ml) was added to a final concentration of 0.1 µg/ml. The gel solution was cooled, cast in the gel chamber and an appropriate comb for preparation of sample wells was inserted. The gels were allowed to polymerize for 30-45 min before the comb was removed. Plasmid samples (100 ng DNA) and size markers were mixed with an appropriate volume of DNA sample buffer (Chapter 2.2.4) and pipetted into the wells of the agarose gel. The gels were run at 7.5 V/cm with a 100 bp and 1 kbp DNA size ladder as references and finally visualised on a 305 nm UV transilluminator and photographed using a gel documentation system.

2.9. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) applied here was first described by Ulrich Laemmli (Laemmli, 1970). The technique evolved from discontinuous electrophoresis (= disc electrophoresis) developed by Ornstein and Davis, which features a polyacrylamide matrix consisting of a stacking gel (pH 6.8, wide pores) and a resolving gel (pH 8.8, narrow pores) to avoid aggregation of proteins upon entering the gel and to increase the sharpness of protein bands (Davis, 1964; Ornstein, 1964).

The gel matrix was created by polymerising acrylamide (CH_2=CH-CO-NH_2) and bisacryl-amide (N,N'-methylenebisacrylamide, CH_2=CH-CO-NH-CH_2-NH-CO-CH=CH_2) catalysed by ammonium persulphate (APS, $(NH_4)_2S_2O_8$), which forms radicals facilitated by N,N,N',N'-tetramethylethylenediamine (TEMED). Concentrations of acrylamide (increases the length of resulting polyacrylamide chains) and bisacrylamide (increases the degree of cross-linking) determine pore size, gel density and elasticity.

The SDS-PAGE was run in vertical slab gel chambers assembled from glass plates (gel thick-ness: 1 mm). Using the Rotiphorese Gel 30 solution of 30% w/v acrylamide and 0.8% w/v bisacrylamide 7.5 ml of a resolving gel with an acrylamide concentration of 15% w/v were prepared, poured into the gel mould up to 1 cm below the upper end of the mould and covered with distilled water to avoid contact with oxygen, which would disturb polymerisation. After 30 min the water was removed and 2.5 ml of a stacking gel with an acrylamide concentration of 4% w/v were poured onto the resolving gel. An ethanol cleaned Teflon comb was

immediately inserted to create wells for sample application. After at least 30 min of polymerization the comb was removed. Gel concentrations were chosen in correspondence with the molecular weight of monomeric galectin-1 C2S of 14.5 kDa (Sambrook and Russell, 2001b, p. A8.42). Stacking and resolving gels were prepared with the following protocol:

4% w/v Stacking Gel	[µl]
Distilled water	3000
0.5 M Tris-Cl (pH 6.8)	1250
10% w/v SDS	50
30% w/v acrylamide mix	650
10% w/v APS	25
TEMED	5
Total	**5000**

15% w/v Resolving Gel	[µl]
Distilled water	2340
1.5 M Tris-Cl (pH 8.8)	2500
10% w/v SDS	100
30% w/v acrylamide mix	5000
10% w/v APS	50
TEMED	5
Total	**10000**

The gel was finally mounted in the electrophoresis chamber and 1X Tris-glycine buffer (Chapter 2.2.5) was poured into the upper and lower chamber of the electrophoresis unit. Each well of the gel was gently washed with 1X Tris-glycine electrophoresis buffer using a hypodermic needle. Before loading onto the gel the protein samples were denaturized by heating to 95°C for 5 min in Laemmli sample loading buffer (Chapter 2.2.5). The boiled samples were immediately stored on ice and spun down. Up to 50 µg of each protein sample were loaded onto the gel and a protein size marker was included. The gel was run at 8 V/cm until the bromophenol blue front left the stacking gel. The voltage was then increased to 15 V/cm and the gel run until the bromophenol blue front reached the end of the resolving gel.

2.10. Coomassie Brilliant Blue Staining

Proteins on SDS-PAGE gels were visualised by Coomassie Brilliant Blue staining. The gels were fixed for 1h in 7% v/v glacial acetic acid in 40% v/v methanol. This was followed by overnight incubation on a horizontal shaker in Coomassie Brilliant Blue G-250 staining solution diluted 4:1 with methanol. The next day the background signal was reduced by rinsing for 1 min with 10% v/v acetic acid in 25% v/v methanol. Further destaining – if necessary – was carried out by incubation in 25% v/v methanol. Finally the gel was digitalized by scanning.

2.11. Western Blotting

The proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane to identify them by an immunoblot. This technique – called Western blotting – was originally described by Towbin and Burnette (Burnette, 1981; Towbin et al., 1979).

Protein blotting was carried out in a semi-dry PerfectBlue electro blotter onto a Hybond ECL membrane. Four layers of Whatman 3MM paper saturated with concentrated anode buffer (Chapter 2.2.6) were placed onto the anode followed by four layers of Whatman 3MM paper equilibrated in anode buffer (Chapter 2.2.6), the Hybond ECL membrane (impregnated in anode buffer), the SDS-PAGE resolving gel (impregnated in cathode buffer (Chapter 2.2.6)), four layers of Whatman 3MM paper (equilibrated in cathode buffer) and the cathode itself.

Western blotting was carried out at 100 mA/membrane for 1.5 h and success of blotting was checked by reversibly staining the membrane in Ponceau S staining solution. To reduce background signal the membrane was washed with 0.01% v/v Tween 20 in TBS (TTBS, Chapter 2.2.6). After digital documentation by scanning and marking the spots of the protein size marker the membrane was completely destained by repeated washing with TTBS.

Unspecific binding sites for antibodies were blocked by incubating the nitrocellulose membrane for 1 h at RT with blocking solution (5% w/v non-fat dry milk in TTBS) on an orbital shaker. After that the membrane was washed two times with TTBS for 10 min.

The membrane was then incubated with the rabbit anti-human galectin-1 antibody diluted 1:5,000 in blocking solution for 1 h at room temperature. The primary antibody was removed by washing three times in TTBS for 10 min. The following incubation with a horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody was carried out at a dilution of 1:10,000 in TTBS containing 1% w/v non-fat dry milk for 1 h at RT. After washing the membrane again three times with TTBS for 10 min the antibody complexes were visualised by enhanced chemiluminescence (ECL).

0.3 µl of 30% v/v H_2O_2 were added to 1 ml of solution A (50 mg Luminol in 200 ml of 0.1 M Tris-Cl, pH 8.6) and mixed with 100 µl of solution B (11 mg of p-hydroxy coumaric acid in 10 ml of DMSO). This mixture was pipetted onto the membrane and incubated for 2 min at room temperature. Excessive volume was removed and the membrane was inserted between two plastic films before it was exposed to an X-ray film for 1 min, which was developed in a dark chamber.

2.12. Cell Isolation

2.12.1. Mononuclear Cells from Mesenteric Lymph Nodes

Mononuclear cells (MNC) from mesenteric lymph nodes were isolated from one male adult Wistar Unilever Rat by opening up the peritoneal cavity and careful blunt preparation of the mesenteries. Four mesenteric lymph nodes were collected and immediately stored in complete RPMI 1640 cell culture medium (Chapter 2.2.7) on ice. This medium was used throughout all following steps and during cell culture.

Lymph nodes were transferred into a Petri dish filled with cell culture medium under the flow bench. Each lymph node was infiltrated with 2-3 ml of cell culture medium using a 25 G needle and a 10 ml syringe. Afterwards the lymph nodes were cut into small pieces and aspirated into the syringe without an attached needle. This was followed by grinding the organs through a 70 µm nylon mesh. Finally the mesh was flushed with medium until the Falcon tube was filled up to a total volume of 45 ml. Pelleting of cells was carried out by centrifuging at 280 × g for 10 min at RT without break.

After discarding the supernatant, contaminating erythrocytes were lysed by incubation with 6 ml of QIAGEN RBC lysis solution for 2 min at 34°C in a water bath and for further 2 min at RT followed by filling up to a volume of 50 ml with complete RPMI 1640 cell culture medium and centrifugation at 280 × g for 10 min at RT. The supernatant was decanted and cells were resuspended in 10 ml of cell culture medium. After counting the cells in a Bürker's counting chamber they were seeded in a 24-well plate at 2×10^6 cells/well in a final volume of 1 ml per well in complete RPMI 1640 cell culture medium supplemented with β-mercapto-ethanol (final concentration: 50 µM). Cells were cultured for 48 h in the presence or absence of 6% v/v phytohaemagglutinin (PHA) (Figure 13) and analysed by flow cytometry in respect to binding of human galectin-1 C2S and plant lectins.

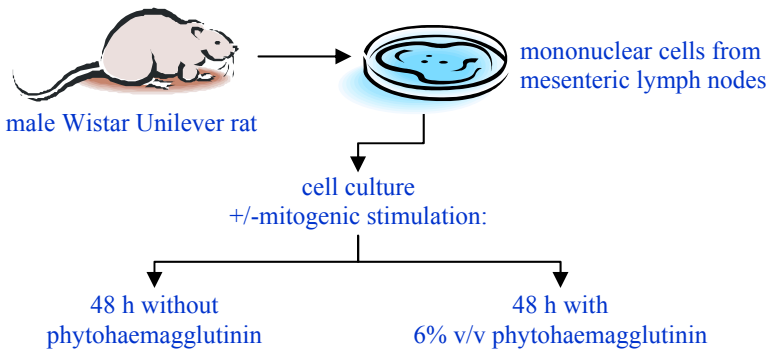


Figure 13: Isolation and mitogenic stimulation of mononuclear cells from mesenteric lymph nodes of healthy Wistar Unilever rats.

Mononuclear cells (MNC) from mesenteric lymph nodes were a suitable positive control for galectin-1 binding because this cell population consists of around 60-70% of T lymphocytes, which are known to be cells targeted by galectin-1 (Amano et al., 2003; Mertens and Krueger, 1976; Zwingmann-Cornelius, 2001).

2.12.2. Testicular Macrophages

For the isolation of testicular macrophages three male Wistar Unilever rats were sacrificed by an overdose of isoflurane. The obtained testes were decapsulated into Petri dishes filled with complete DMEM cell culture medium (Chapter 2.2.7). Special attention was paid to maintain the integrity of the seminiferous tubules and to remove large blood vessels between them. This was followed by gentle prizing apart of seminiferous tubules using curved forceps according to the method established by Hedger and Eddy (Hedger and Eddy, 1986).

The dissociated tubules were transferred into 50 ml Falcon tubes and complete DMEM cell culture medium was added up to a final volume of 50 ml. After settling for 5 min the supernatants were carefully transferred into new 50 ml Falcon tubes for centrifugation at 300 × g for 10 min at RT without brake. The resulting cell pellets from six testes were resuspended in 18 ml of culture medium and pooled. Then 2 ml were seeded out into each well of two 6-well plates. For fluorescence microscopy 0.5 ml were pipetted onto 12 mm cover slips in the wells of 24-well-plates followed by 500 µl of cell culture medium. The macrophages attached to the plastic surface during 30 min of incubation at 32°C. Then contaminating cells were removed by extensively washing the wells five times with warm (32°C) cell culture medium.

2.12.3. Peritubular Cells and Sertoli Cells

The isolation of peritubular and Sertoli cells followed a modified version of Hoeben's protocol (Hoeben et al., 1999). The applied media and enzyme solutions are described in Chapter 2.2.7 and were all passed through a 0.20 µm filter after preparation.

Ten male nineteen-day-old Wistar rats were sacrificed by cervical dislocation. Blood volume was reduced by immediate postmortem incision of the jugular veins and rinsing under flowing tap water. Twenty testes were removed via transabdominal route and collected in 20 ml of PBS-A. Then 20 ml of 1% w/v iodine in ethanol were added for disinfection. Afterwards, the testes were transferred into PBS-A and remaining iodine was removed by two further washing steps in PBS-A. The testes were carefully decapsulated into a sterile, dry Petri dish. Decapsulation was followed by enzymatic digestion.

The seminiferous tubules were incubated for 10-15 min in 10 ml of the trypsin-DNase I-solution in a shaking water bath (120 rpm) at 32 °C. The duration of digestion was adjusted to surveillance of progress by phase contrast microscopy. It was stopped by addition of 5 ml trypsin inhibitor-solution A. After settling of the seminiferous tubules for 5 min the supernatant was decanted and trypsin inhibitor-solution B added. After another 5 min of settling and removal of the supernatant the seminiferous tubules were washed nine times with 20 ml PBS-A. Each washing step was followed by eight minutes for sedimentation of the tubules.

2.12.4. Specific Steps in the Isolation of Peritubular Cells

After these washing steps the seminiferous tubules were incubated with the collagenase-hyaluronidase-DNase I-solution for 10-15 min at 32°C in a shaking water bath (120 rpm) until detaching of peritubular cells from the seminiferous tubules could be seen in phase contrast microscopy (Figure 14).

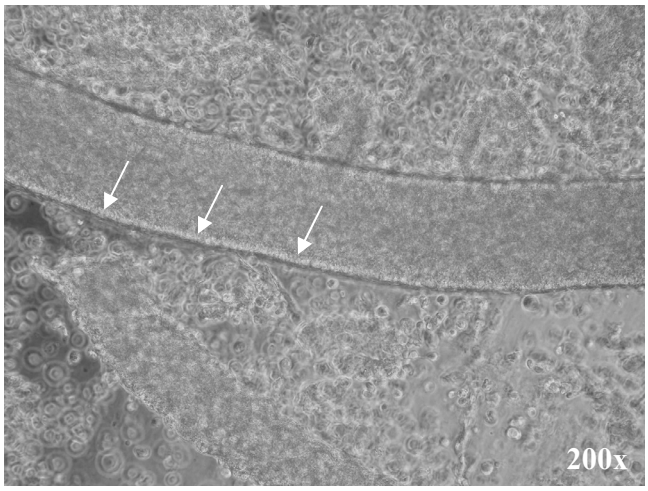


Figure 14: Surveillance of enzymatic digestion during isolation of peritubular cells. At this time point the lamina propria of seminiferous tubules (indicated by arrows) was still intact. Thus enzymatic digestion with collagenase, hyaluronidase and DNase I was carefully continued to detach peritubular cells without releasing Sertoli cells. (Phase contrast microscopy, 200x magnification.)

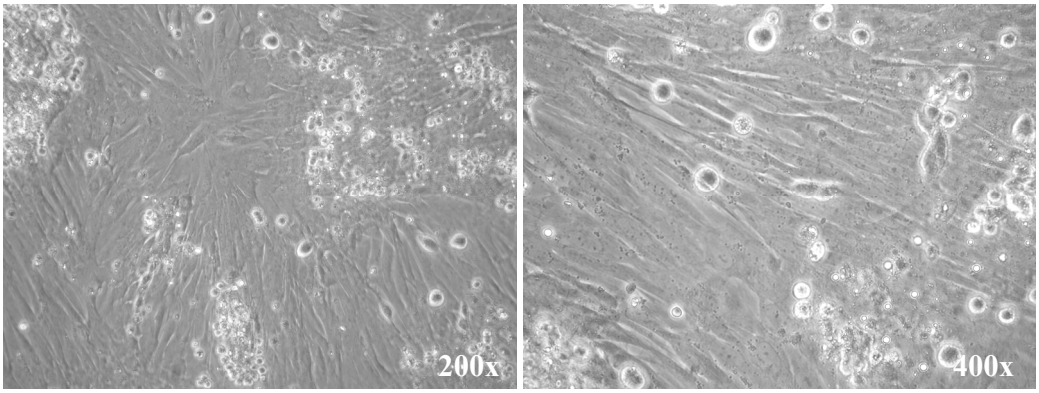
20 ml of PBS-A were added to the digested seminiferous tubules and allowed to settle for 10 min. The resulting supernatant containing the peritubular cells was harvested and the remaining seminiferous tubules were used for Sertoli cell isolation (Chapter 2.12.5). Further 20 ml PBS-A were added to the digested seminiferous tubules. After 10 min of sedimentation the supernatant was collected for peritubular cell isolation. Both supernatants were each adjusted to 40 ml with RPMI-B and centrifuged at 280 × g for 10 min at RT without break. The cell pellets each were resuspended in 15 ml of RPMI-B and pooled before filtering through a 70 µm nylon mesh. Then 30 ml of peritubular cell suspension were equally

distributed to six culture flasks, which were filled with RPMI-B to a final volume of 25 ml. The flasks were incubated at 37°C and 5% v/v CO_2.

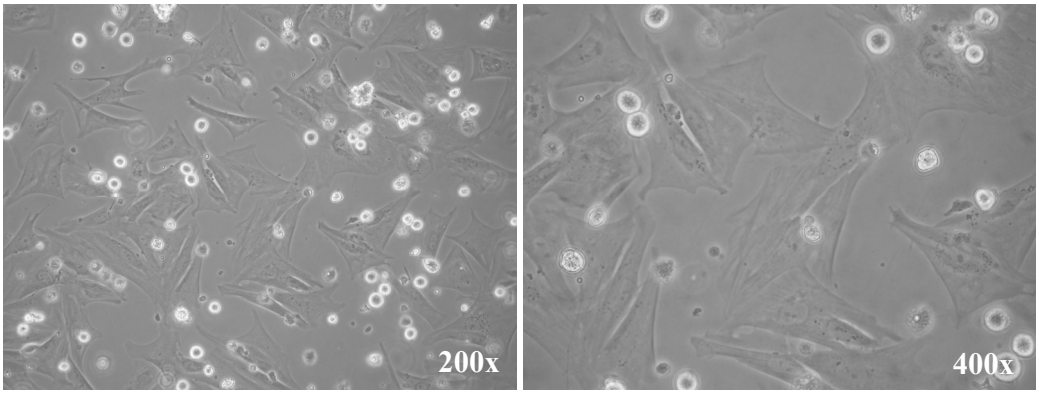
Splitting of peritubular cells was carried out on day 3 after cell isolation: After washing twice with 10 ml of PBS-A, 1 ml of pre-heated 0.05% w/v trypsin 0.02% w/v EDTA in DPBS solution was pipetted into each flask followed by exactly 3 min of incubation at 37°C. Detaching of cells was checked microscopically before the trypsin activity was stopped by adding 10 ml of RPMI-B into each flask. 6 ml of the resulting solution were transferred into a new culture flask to split the cells. After that 18 ml of RPMI-B were added into each flask. Culturing of cells at 37°C and 5% v/v CO_2 was continued after splitting.

5 days after isolation the cells were washed twice with PBS-A and trypsinised like before. After trypsinisation 7 ml of RPMI-B were immediately transferred into each flask and obtained cell suspensions were transferred into 50 ml Falcon tubes. The flasks were rinsed with further 7 ml of medium, which were collected in the same Falcon tubes. Three of these 14 ml cell suspensions were collected in one 50 ml Falcon tube and centrifuged for 10 min at 280 × g at RT without break. The pellet was resuspended in 20 ml of RPMI-B and cells were counted in a Bürker's counting chamber after trypan blue staining. 1×10^6 peritubular cells were seeded into each well of 6-well-plates (2 ml final volume per well). For fluorescence microscopy peritubular cells were seeded into 24-well-plates (1.25×10^5/well) containing 12 mm cover slips (1 ml final volume per well). Isolated peritubular cells were always used in experiments on day 7 after isolation. The following light microscopic images document the increase of purity in the course of culturing and washing steps of peritubular cells (Figure 15).

Peritubular cell culture on day 3 after isolation, before 1st splitting:



Peritubular cell culture on day 5 after isolation, before 2nd splitting:



Peritubular cell culture on day 7 after isolation:

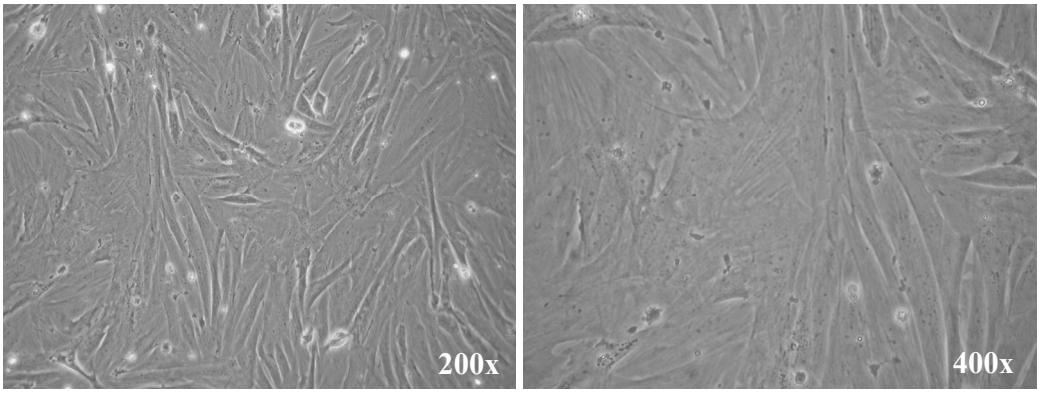


Figure 15: Light microscopy of cultured peritubular cells at three time points showing high homogeneity and spreading out of isolated peritubular cells. Due to mitotic activity of peritubular cells and washing steps the number of non-adhering cells, such as germ cells, decreased in the course of cultivation. (Phase contrast microscopy, 200x and 400x magnification.)

2.12.5. Specific Steps in the Isolation of Sertoli Cells

The seminiferous tubules, which remained after enzymatic detaching of peritubular cells, were washed five times with 20 ml PBS-A followed each time by 10 min for sedimentation. Subsequently they were digested by 10 ml hyaluronidase-DNase I-solution for 10-15 min at 32 °C in a shaking water bath (120 rpm) aiming for short tubular segments with a cobble stone-like surface in phase contrast microscopy (Figure 16). This was followed by six washing steps with 20 ml of PBS-A. Each time the tubular fragments were allowed to settle for 10 min before removal of supernatant. Finally, the cell pellet was resuspended in 20 ml of RPMI-A and dispersed by passing ten times through an 18 G needle. Cells were pelleted by centrifugation at 200 × g for 10 min at RT without break and resuspended in 15 ml of RPMI-A prior to passing through a 70 µm nylon mesh. 4×10^6 cells were seeded into each well of 6-well plates (2 ml final volume per well). For fluorescence microscopy Sertoli cells were distributed into 24-well plates (5×10^5/well) containing 12 mm cover slips (1 ml final volume per well). They were seeded out at a higher density than peritubular cells since they do not bear mitotic activity and cell-to-cell contacts are needed during cultivation.

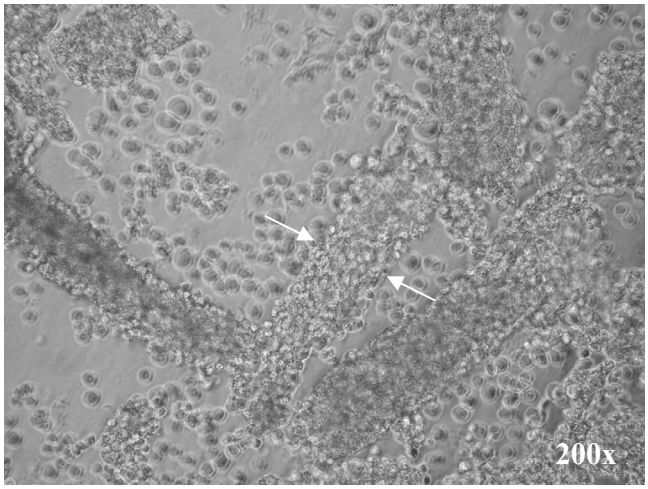
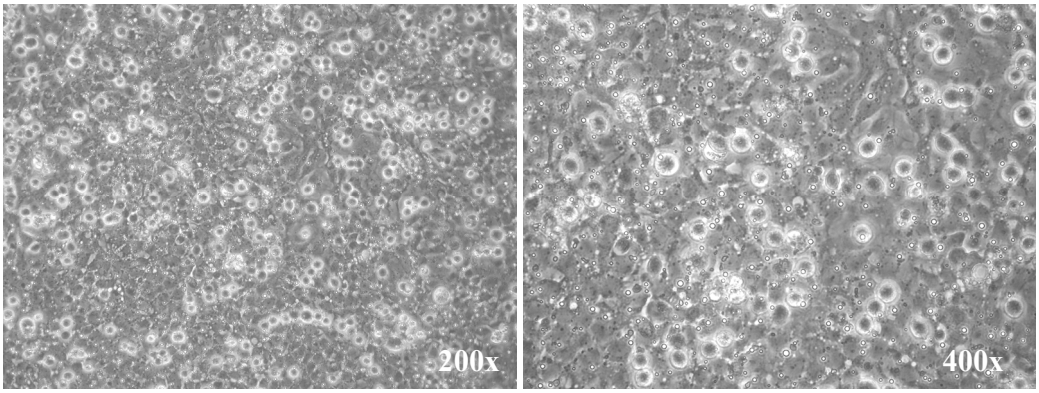


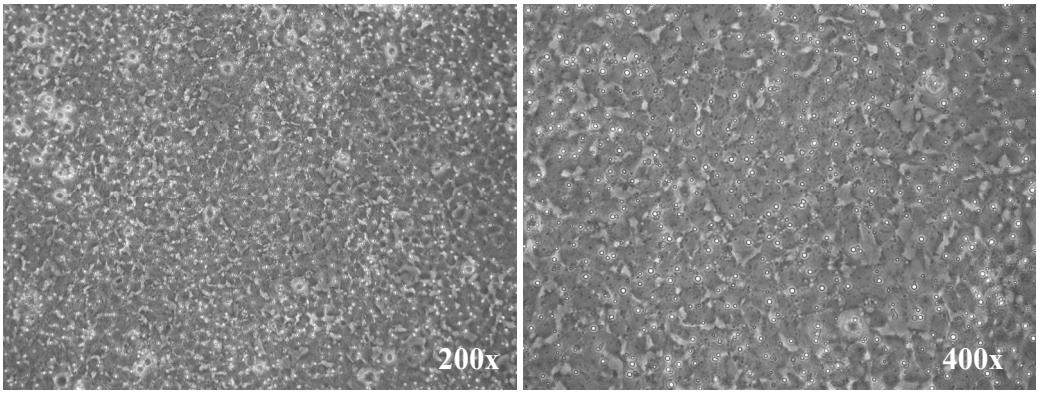
Figure 16: Microscopical check of Sertoli cell isolation. Progress of releasing Sertoli cells from the tubular epithelium (arrows) was indicated by lightened cell-to-cell contacts and shortening of tubular fragments. Duration of enzymatic treatment was adjusted accordingly. (Phase contrast microscopy, 200x magnification.)

On day 3 after isolation cells were washed four times with PBS-A followed by adding 2 ml of RPMI-A into each well of a 6-well-plate and 1 ml of RPMI-A into the wells of a 24-well-plate. Cellular morphology was checked microscopically before and after washing. On day 4/5/6 after isolation daily washing and microscopic surveillance were carried out. Special attention was paid to apply the isolated Sertoli cells in experiments constantly at the same time point, i.e. on the 6th day after their isolation. Light microscopy showed an increase of purity in the course of cultivation and washing steps of Sertoli cells (Figure 17).

Sertoli cell culture on day 3 after isolation:



Sertoli cell culture on day 4 after isolation:



Sertoli cell culture on day 5 after isolation:

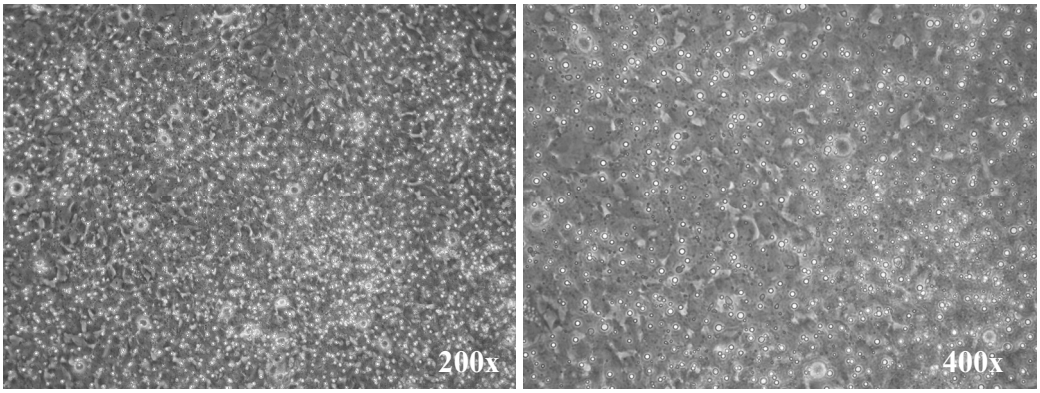


Figure 17: Light microscopy of cultured Sertoli cells at three time points showing high homogeneity of isolated cells, characteristic lipid droplets of Sertoli cells and a decrease of non-adhering cells throughout the washing steps carried out during culturing of the cells. (Phase contrast microscopy, 200x and 400x magnification.)

2.13. Immunofluorescence Staining of Isolated Testicular Cells

Purity of isolated cells was checked by fluorescence microscopy using cell type-specific primary antibodies detected by corresponding fluorescent labelled secondary antibodies.

Testicular macrophages seeded onto 12 mm cover slips in 24-well plates were washed with DPBS and fixed in 4% w/v paraformaldehyde (PFA) in DPBS for 20 min at RT. Unspecific binding sites of antibodies were blocked by incubating the cover slips for 1 h at RT with 5% v/v NGS 5% w/v BSA in DPBS after carefully washing with warm DPBS three times.

Blocking solution was removed by washing three times with warm DPBS. Then the cover slips were incubated at 4°C overnight with a mouse anti-rat CD68 (ED1) and/or a mouse anti-rat CD163 (ED2) monoclonal primary antibody diluted 1:50 in 3% w/v BSA in 0.05% v/v Tween 20 DPBS (T-DPBS). On the following day the slides were carefully washed three times in T-DPBS followed by incubation with a Cy3-conjugated donkey anti-mouse IgG secondary antibody dissolved 1:1,000 in 3% w/v BSA in T-DPBS for 1 h at RT. Unbound secondary antibodies were removed by three times washing with T-DPBS. The cover slips were finally mounted on microscopy slides using Vectashield mounting medium with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) and sealed by nail polish prior to visualisation with an Axioplan 2 epifluorescence microscope. Control slides were incubated only with the secondary antibody with omission of the primary antibody.

Staining of Sertoli and peritubular cells for fluorescence microscopy was carried out as follows: The cells seeded onto 12 mm cover slips were washed twice with 500 µl of 0.1% w/v BSA in PBS before fixation in 4% w/v PFA in DPBS for 30 min at 4°C. The slides were gently washed three times with 500 µl of DPBS and the cells were then permeabilized by incubation with 0.2% v/v Triton X-100 in DPBS for 15 min at RT. Unspecific binding sites were blocked by incubation for 1 h at RT with 5% v/v NGS 5% w/v BSA in DPBS. After further three times washing with DPBS the cells were incubated overnight at 4°C with a mouse anti-human α-smooth muscle actin monoclonal antibody (for peritubular cells) or a goat anti-human vimentin polyclonal antibody (for Sertoli cells) both diluted 1:100 in 3% w/v BSA in T-DPBS.

After washing of cover slips three times with T-DPBS the cells were incubated for 1 h at RT with a Cy3-labelled donkey anti-mouse IgG secondary antibody diluted 1:1,000 in 3% w/v BSA in T-DPBS (for peritubular cells) or with a Cy3-labelled donkey anti-goat IgG secondary antibody diluted 1:800 in 3% w/v BSA in T-DPBS (for Sertoli cells). Unbound secondary

antibodies were removed by three times washing with T-DPBS. The cover slips were finally mounted on microscopy slides using Vectashield mounting medium with DAPI and sealed by nail polish prior to visualisation with a confocal laser scanning microscope Leica TCS SP2.

2.14. Cryostat Sections of Rat Liver and Testis Tissue

Testes and liver were obtained from adult male Wistar Unilever rats. After excision, the organs were snap-frozen in liquid 2-methylbutan, which was cooled down nearly to its melting point of -160°C in liquid nitrogen (boiling point: -196°C). Afterwards the organs were either directly processed for cryosections or stored at -80°C beforehand.

Tissue cryosections were cut on a Leica CM 1900 cryostat at a thickness of 12 µm. The organs were allowed to equilibrate to block temperature and then thoroughly fixed on the object plate by Tissue-Tek O.C.T. compound. Cut sections were transferred onto SuperFrost plus slides by electrostatic attraction and air-dried for 15 min at RT before storage at -20°C.

2.15. Human Galectin-1 C2S Binding to Liver and Testis Cryosections

Cryostat sections of rat liver and testis were encircled by a hydrophobic delimiting pen and fixed in 4% w/v PFA in PBS for 30 min at 4°C. Afterwards, sections were washed three times in 0.1% w/v BSA in PBS for each 5 min in a Hellendahl cuvette placed on an orbital shaker.

Cryosections of liver and testis were incubated for 1 h at 4°C in darkness either with 1% w/v BSA in PBS alone or 10 µM labelled human galectin-1 C2S or 10 µM labelled human galectin-1 C2S pre-incubated for 30 min with 100 mM lactose or sucrose, respectively.

After the incubation the sections were washed four times with 0.1% w/v BSA in PBS for 5 min in darkness and finally mounted using Vectashield mounting medium with DAPI and large cover slips (24 x 60 mm). After sealing by nail polish the fluorescence signals were visualised with an Axioplan 2 epifluorescence microscope.

Maintenance of testicular morphology was checked by light microscopy of haemalaun-eosin stained cryostat sections applying the Axioplan 2 microscope in transmitted light microscopy mode. Therefore air-dried cryosections were hydrated by incubation in a decreasing series of ethanol solutions (100/90/80/70/50% v/v in distilled water) each for 10 min. After insertion in distilled water for 5 min sections were stained by Mayer's haemalaun. Tap water was used for consequent bluing and again 5 min of incubation in distilled water followed. Then sections were counterstained with 1% w/v Certistain Erythrosin B in distilled water for 1-2 min. After

further 5 min in distilled water sections were shortly inserted in 50% and 70% v/v ethanol in distilled water followed by 5 min each in 80% and 90% v/v ethanol in distilled water as well as 100% v/v ethanol for 10 min. Sections were mounted using Entellan after three incubation steps in xylene for each 5-10 min.

2.16. *In Vitro* Detection of Human Galectin-1 C2S Binding to Isolated Cells by Flow Cytometry

By flow cytometry binding of human galectin-1 C2S to isolated mononuclear and testicular cells was examined concentration-dependent and carbohydrate-specific. Isolated cells were incubated with different concentrations of galectin-1 C2S (0/0.1/1/10 µM) as well as with a complex of galectin-1 C2S with lactose or sucrose, respectively.

Mononuclear cells from mesenteric lymph nodes are non-adherent in cell culture and were directly used for flow cytometry. In contrast testicular macrophages, Sertoli and peritubular cells adhere to plastic cell culture wells and need to be detached carefully.

Testicular macrophages were detached by incubation with cold 0.02% w/v EDTA in DPBS for 15 min. This was followed by gentle pipetting up and down of the cell suspension to detach further cells.

Sertoli and peritubular cells were harvested by short trypsinisation: The adherent cells were washed twice with DPBS to avoid inhibition of trypsin activity by remaining cell culture medium. The incubation with pre-heated 0.05% w/v trypsin 0.02% w/v EDTA in DPBS was performed on a heating plate at 34°C for exactly 2 min. The level of cell detachment was checked under a phase contrast microscope and augmented by hitting the plates. Enzymatic activity was stopped by immediate addition of 3 ml of warm RPMI-B (Chapter 2.2.7) to avoid cell surface protein damage by trypsin. The resulting cell suspension was transferred into a 50 ml Falcon tube and – for Sertoli cells – repeatedly passed through a 1000 µl pipette to avoid formation of cell clumps. Afterwards the cells were pelleted by centrifugation at 440 × g for 10 min at RT without break. The cell pellet harvested from one 6-well plate was then resuspended in 10 ml of RPMI-A for Sertoli cells or RPMI-B for peritubular cells.

All isolated cell types were incubated with Alexa Fluor 647 labelled human galectin-1 C2S in the same way to assure comparability (Table 2).

Preincubation:	[Alexa Fluor 647 Galectin-1 C2S]			
	0 µM	**0.1 µM**	**1 µM**	**10 µM**
1% w/v BSA in PBS	+	+	+	+
+ 100 mM lactose		+	+	+
+ 100 mM sucrose		+	+	+

Table 2: Set-up of experimental conditions for flow cytometric measurement of human galectin-1 C2S binding to isolated testicular cells.

Accordingly fluorophore labelled human galectin-1 C2S was pre-incubated with 1% w/v BSA in PBS (Chapter 2.2.8) alone or with 100 mM lactose (or sucrose) in 1% w/v BSA in PBS for 30 min at 4°C.

A total of 5×10^5 cells were used for each experimental condition and therefore applied into flow cytometry tubes. The cells were washed with 1000 µl of 0.1% w/v BSA in PBS followed by centrifugation at 440 × g for 10 min at 4°C without break before the cells were incubated for 1 h at 4°C in darkness with 50 µl of the solutions of pre-incubated labelled galectin-1. Then the cells were washed three times by addition of 1000 µl of 0.1% w/v BSA in PBS and centrifuged at 440 × g for 10 min at 4°C without break. The cells were finally fixed in 500 µl of 2% w/v PFA in PBS.

Flow cytometric data acquisition was carried out by using a BD FACSCalibur flow cytometer equipped with an argon-ion laser (emission wave length: λ=488 nm) and a red diode laser (emission wave length: λ=635 nm).

Analysis of flow cytometry data was performed with WEASEL software for flow cytometry data analysis, version 2.7.

2.17. Verification of Plant Lectin Binding to Isolated Cells by Flow Cytometry

A set of fluorescent labelled plant lectins was used to investigate the cell surface glycosylation pattern of the isolated cell types assessed in respect to galectin-1 binding (Table 3).

Plant Lectins (Origin)	Conc.	Targeted Glycan Structure
LEA, FITC conjugated (Lycopersicon esculentum, tomato)	20 µg/ml	Oligomers of β1-4 linked GlcNAc (≤4 monomers); 3 repetitive LacNAc units like in type-2 poly-N-acetyllactosamine.
MAA, FITC conjugated (Maackia amurensis, Amur maackia)	20 µg/ml	Terminal sialic acid α2-3 linked to penultimate galactose in complex-type N-glycans.
PNA, FITC conjugated (Arachis hypogaea, peanut)	20 µg/ml	Core 1 O-glycan (T antigen, most common O-glycan, Galβ1-3GalNAcαSer/Thr).
SNA-I, FITC conjugated (Sambucus nigra, elderberry)	10 µg/ml	Terminal sialic acid α2-6 linked to penultimate galactose (like in N-glycans); terminal sialic acid α2-6 linked to GalNAc (like in O-glycans).

Table 3: Plant lectin array for flow cytometric assessment of glycosylation patterns of isolated cells.

Harvesting of cells for flow cytometry was performed as described for the human galectin-1 C2S binding assay (Chapter 2.16).

5×10^5 cells were washed with 1000 µl of 1% w/v BSA in PBS followed by centrifugation at 440 × g for 10 min at 4°C without break. Afterwards the cells were incubated for 1 h at RT in darkness with 50 µl of the corresponding plant lectin solution at the given final concentration (Table 3). Finally, the cells were washed three times with 1000 µl of 1% w/v BSA in PBS and centrifuged at 440 × g for 10 min at 4°C without break. Before flow cytometric analysis, the cells were fixed in 500 µl of 2% w/v PFA in PBS.

Flow cytometric data acquisition and analysis were carried out as described for the human galectin-1 C2S binding assay (Chapter 2.16).

3. Results

3.1. Recombinantly Expressed Human Galectin-1 C2S

In the first step a pET-21a(+) vector containing the full length coding sequence of human galectin-1 C2S was transferred into *E. coli* DH5α to amplify the plasmid DNA (Chapters 2.4.1 and 2.4.2). Afterwards, the linearised and digested plasmid DNA fragments were separated by 1.5% w/v DNA agarose gel electrophoresis (Figure 18).

Instead of recombinant wild-type human galectin-1 the C2S mutant was used, where cysteine at the 2nd position of the protein chain is replaced by serine. It bears increased resistance to oxidative inactivation while exhibiting unaltered carbohydrate specificity and a binding strength close to that of the wild-type protein (Chapter 4.1).

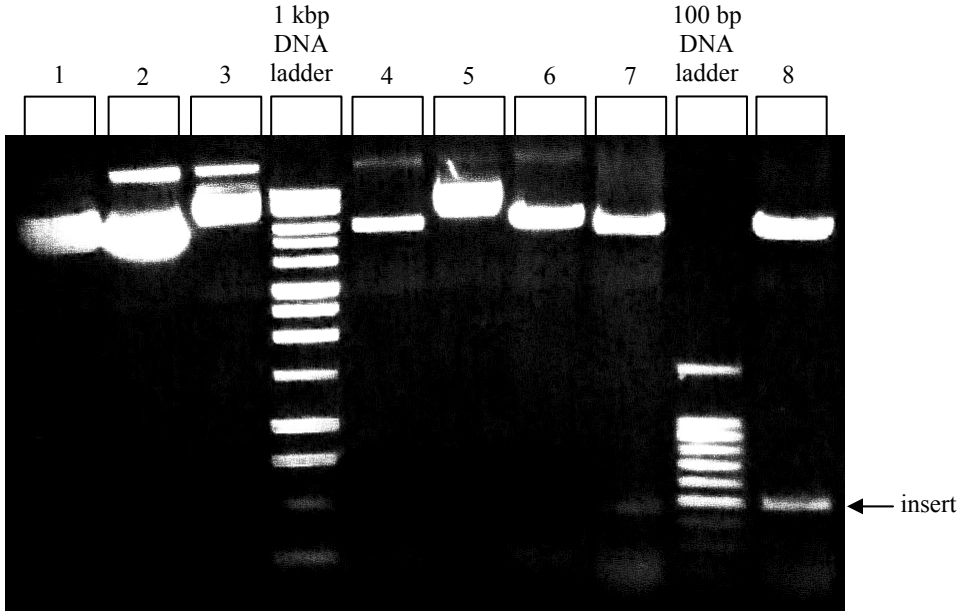


Figure 18: 1.5% w/v DNA agarose gel electrophoresis of pET-21a(+)-galectin-1 C2S construct. The pET-21a(+) vector carrying the human galectin-1 C2S gene was analysed alongside with its linearised form (digested by XbaI) and the form created by removal of the insert (by digestion with NdeI and XhoI). As a further control an empty pET-21a(+) vector as well as its linearised form were applied to the same DNA agarose gel electrophoresis.
lane 1: empty pET-21a(+) vector. *lane 2:* linearised empty pET-21a(+) vector (digested by XbaI). *lane 3:* pET-21a(+) vector with galectin-1 wt gene. *lane 4:* linearised pET-21a(+) vector with galectin-1 wt gene (digested by XbaI). *lane 5:* pET-21a(+) vector with galectin-1 C2S gene. *lane 6:* linearised pET-21a(+) vector with galectin-1 C2S gene (digested by XbaI). *lane 7:* pET-21a(+) vector with galectin-1 wt gene after removal of insert (digested by NdeI and XhoI). *lane 8:* pET-21a(+) vector with galectin-1 C2S gene after removal of insert (digested by NdeI and XhoI). Upper band in lanes 2, 3, 4, 5 and 6 due to genomic DNA.

The expression strain *E. coli* BL21(DE3)pLysS was transformed with the amplified plasmid. After that, protein expression was induced by IPTG (final concentration: 0.5 mM) and continued during 2 h of incubation at 37°C in a shaking incubator (Chapters 2.4.3 and 2.4.4). Galectin-1 C2S was found to be expressed in soluble form and was extracted from cells after disruption by lysozyme and sonication. The optimal time of expression after induction by IPTG was assessed by checking the amount of expressed recombinant protein at several time points (Figure 19).

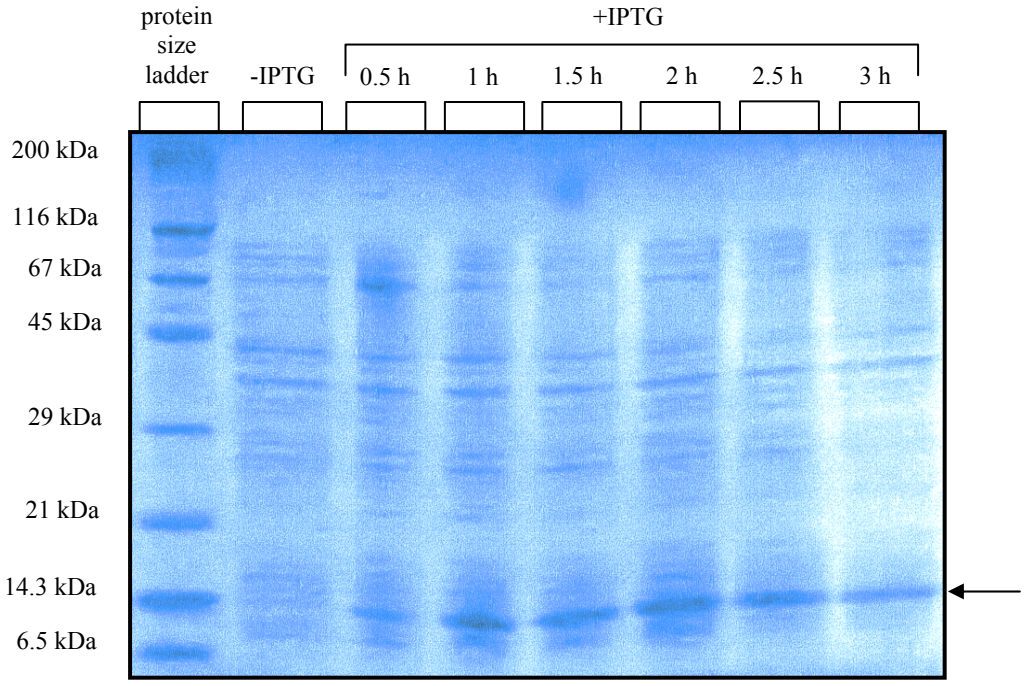


Figure 19: Determination of the optimal time of IPTG-induced expression of recombinant human galectin-1 C2S in *E. coli* BL21(DE3)pLysS. Expression was induced by IPTG (final concentration: 0.5 mM) at 37°C and culture samples were taken before induction with IPTG (*-IPTG*) and then every 30 min until 3h after induction (*+IPTG 0.5h/1h/1.5h/2h/2.5h/3h*). Samples were analysed by 15% w/v SDS-PAGE and visualised by Coomassie Brilliant Blue staining. The expression level reached a plateau 2h after induction so that large scale expression of galectin-1 C2S was conducted with 2 h of expression time.
Arrow: recombinant human galectin-1 C2S (M = 14.5 kDa).

Affinity chromatography on an asialofetuin-Sepharose column was used for purification of recombinant human galectin-1 C2S from the supernatant of the lysed cells after centrifugation. The protein was finally eluted from the column with a buffer containing 0.1 M lactose. The eluate was dialysed against PBS (pH 7.0-7.5) containing ß-mercaptoethanol (final concentration: 1 mM) (Figure 20).

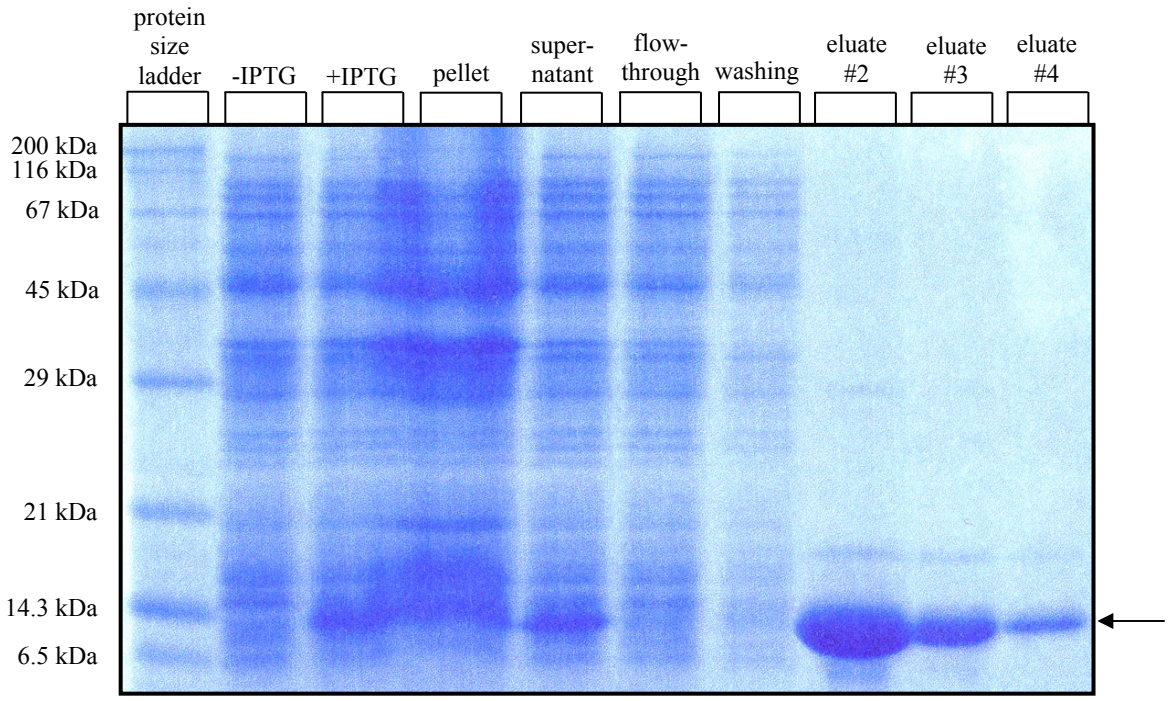


Figure 20: Purification of recombinant human galectin-1 C2S. After 2 h of expression *E. coli* BL21(DE3)pLysS were lysed by lysozyme and sonication. Cells were pelleted and the supernatant was applied to an asialofetuin-Sepharose column for purification of human galectin-1 C2S by affinity chromatography. The column was washed with 25 ml of buffer and the recombinant protein was eluted by 10 ml of buffer containing 0.1 M lactose in 1 ml fractions. Samples of each step were analysed by 15% w/v SDS-PAGE and visualised by Coomassie Brilliant Blue staining. *Arrow:* recombinant human galectin-1 C2S (M = 14.5 kDa).
Increase of galectin-1 C2S content in bacterial cell culture after 2 h of expression (*+IPTG*) compared to preinduction level (*-IPTG*). After lysing and pelleting bacteria, galectin-1 C2S was primarily found in the supernatant (*supernatant*) rather than in the pellet (*pellet*). The lectin showed high affinity to the asialofetuin-Sepharose column since it was not eluted by buffer alone (not found in *washing* fraction) but could be eluted in high purity by buffer containing 0.1 M lactose (*eluates #2-4*). (The *flow-through* fraction resulted from application of the cell lysate to the column.)

3.2. Alexa Fluor 647 Labelled Human Galectin-1 C2S

Gel filtration chromatography was used to purify the protein-dye conjugate from free dye after labelling recombinantly expressed human galectin-1 C2S with the fluorophore Alexa Fluor 647 (Chapters 2.5 and 2.6). The protein-dye conjugate was eluted from the column by 1200 µl of buffer containing β-mercaptoethanol (1 mM). The eluate contained recombinant human galectin-1 C2S at a w/v concentration of 0.83 mg/ml and a molar concentration of dimers of 28.6 µM. To proof that the majority of labelled galectin-1 C2S was eluted in these 1200 µl, four consecutive 1000 µl elution fractions were applied to the column. All fractions were analysed by SDS-PAGE (6 µg of protein per lane) (Figure 21).

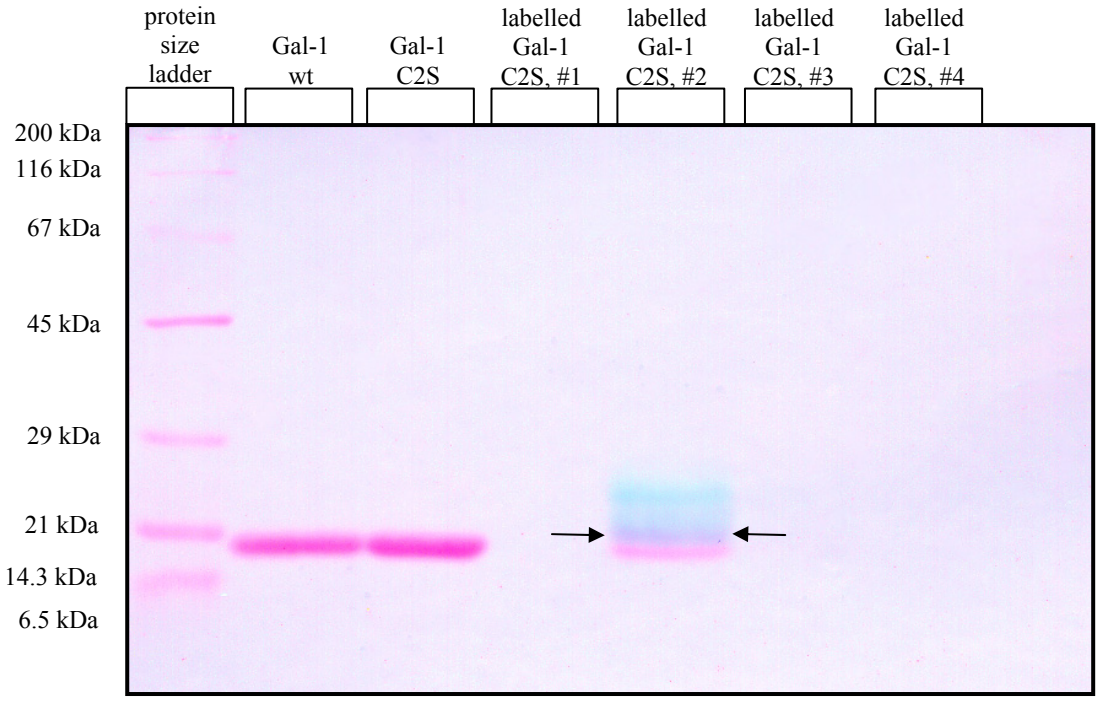


Figure 21: Ponceau S staining of recombinant human galetin-1 C2S (M = 14.5 kDa) after SDS-PAGE. Elution fractions 1-4 of Alexa Fluor 647 labelled human galectin-1 C2S and free Alexa Fluor 647 dye obtained by gel filtration chromatography: Elution fraction *#1* resulted from application of the labelling reaction mixture to the column. Labelled galectin-1 C2S (see arrows) mainly found in fraction *#2*. Increase in the size compared to unlabelled Gal-1 wt/C2S is due to conjugation of dye molecules (M(dye) = 1250 Da). *Gal-1 wt*: wild-type human galectin-1 as reference. *Gal-1 C2S*: unlabelled human galectin-1 C2S as reference. *labelled Gal-1 C2S, #3-4*: Elution fractions 3-4 of labelling products of human galectin-1 C2S and Alexa Fluor 647 dye.

Immunodetection of purified recombinant human wild-type and C2S mutant galectin-1 as well as purified Alexa Fluor 647 labelled Galectin-1 C2S was carried out.

Binding of the rabbit anti-human galectin-1 polyclonal antibody verified that the blotted reaction products were human galectin-1. In addition to monomers some non-lysed multimers of human galectin-1 C2S were detectable (M ≈ 45 kDa) (Figure 22).

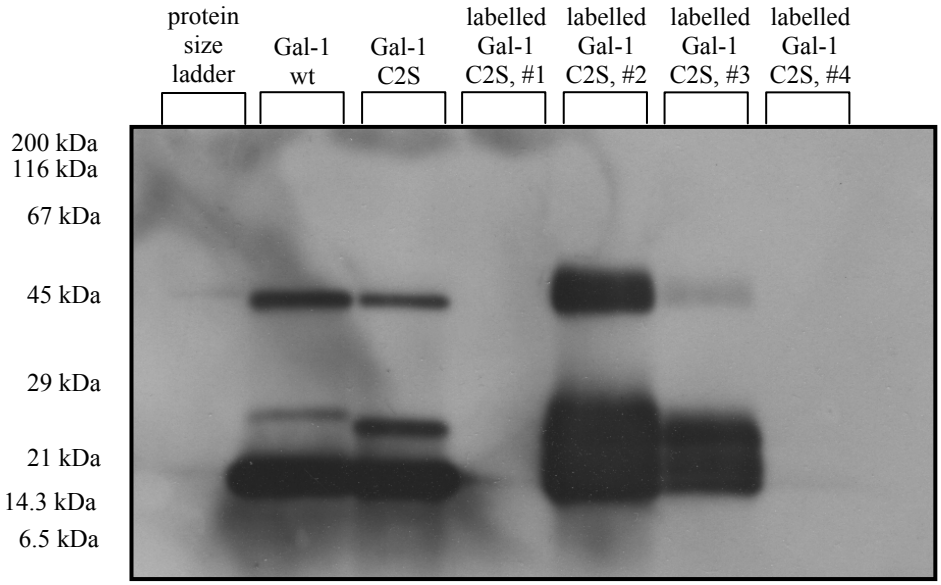


Figure 22: Immunodetection of purified recombinant human wild-type and C2S mutant galectin-1 as well as purified Alexa Fluor 647 labelled Galectin-1 C2S by a rabbit anti-human galectin-1 polyclonal antibody. As shown by this analysis the majority of eluted human galectin-1 C2S was present in fraction *#2*. Additionally some non-lysed multimers of human galectin-1 C2S were detectable (M ≈ 45 kDa). *Gal-1 wt*: wild-type human galectin-1 as reference. *Gal-1 C2S*: unlabelled human galectin-1 C2S as reference. *labelled Gal-1 C2S, #1-4*: Elution fractions 1-4 of labelling products of human galectin-1 C2S and Alexa Fluor 647 dye.

3.3. Purity of Isolated Testicular Cells

In order to verify the purity and identity of isolated testicular macrophages, Sertoli cells and peritubular cells phase contrast microscopy was carried out during all isolation steps and cell marker proteins were detected afterwards by immunofluorescence using specific primary antibodies recognizing epitopes present only on the cells of interest.

High autofluorescence and unspecific binding of secondary antibodies were excluded by examining the fluorescence signals after incubating cells only with the diluent used for the antibody solutions and after incubation with the secondary antibodies only.

Purity and quality of testicular macrophages were verified by incubation of isolated cells both with monoclonal antibodies directed against the lysosomal membrane glycoprotein CD68 (= ED1 antibody) and the cell surface glycoprotein CD163 (= ED2 antibody) (Figure 23).

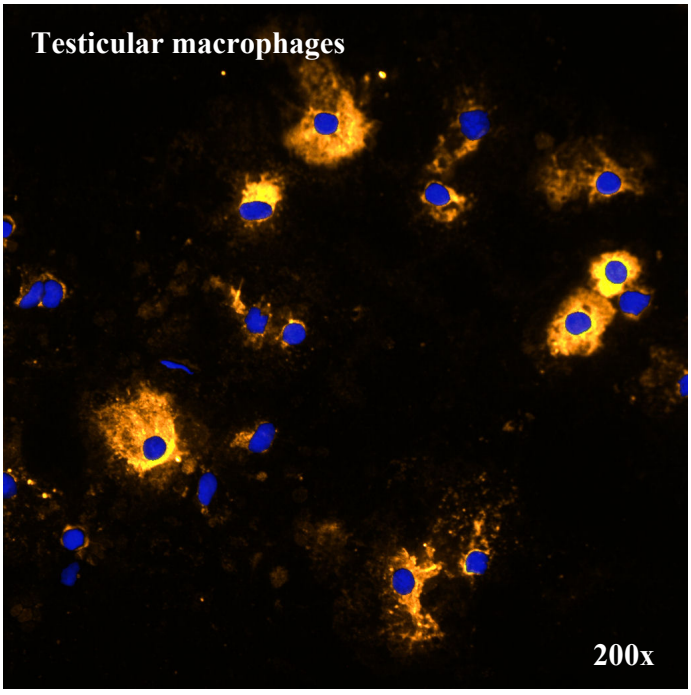


Figure 23: ED1/ED2 staining of testicular macrophages isolated from Wistar Unilever rats. Cells were incubated with mouse anti-rat CD68 (ED1) and mouse anti-rat CD163 (ED2) monoclonal antibodies followed by a donkey anti-mouse IgG polyclonal antibody labelled with Cy3. Nuclear staining was carried out with DAPI. No Cy3 staining was visible for testicular macrophages incubated only with the secondary antibody and DAPI. (Epifluorescence microscopy, Cy3-DAPI overlay, 200x magnification.)

In conclusion, testicular macrophages were isolated with a purity of about 90%. They were mainly contaminated by some developing germ cells and adhering spermatozoa, which are characterized by their unique morphology.

Purity of Sertoli cells was checked during isolation and culturing under a phase contrast microscope according to their cytoplasmatic lipid droplets and cobblestone morphology.

Additionally, the Sertoli cells were identified by vimentin staining. Immunofluorescence staining using a goat anti-human vimentin polyclonal antibody detected by a Cy3 labelled donkey anti-goat secondary antibody successfully demonstrated the isolated cells to be Sertoli cells. The purity of isolated Sertoli cells was estimated as >95% (Figure 24).

The morphology of peritubular cells was monitored during isolation and culturing using phase contrast microscopy based on their stretched, elongated cell shape. The cells were identified as peritubular cells by immunofluorescence staining with a mouse anti-human α-smooth muscle actin monoclonal antibody and a corresponding donkey anti-mouse secondary antibody conjugated with the fluorophore Cy3. The purity of isolated peritubular cells was estimated as >95% (Figure 24).

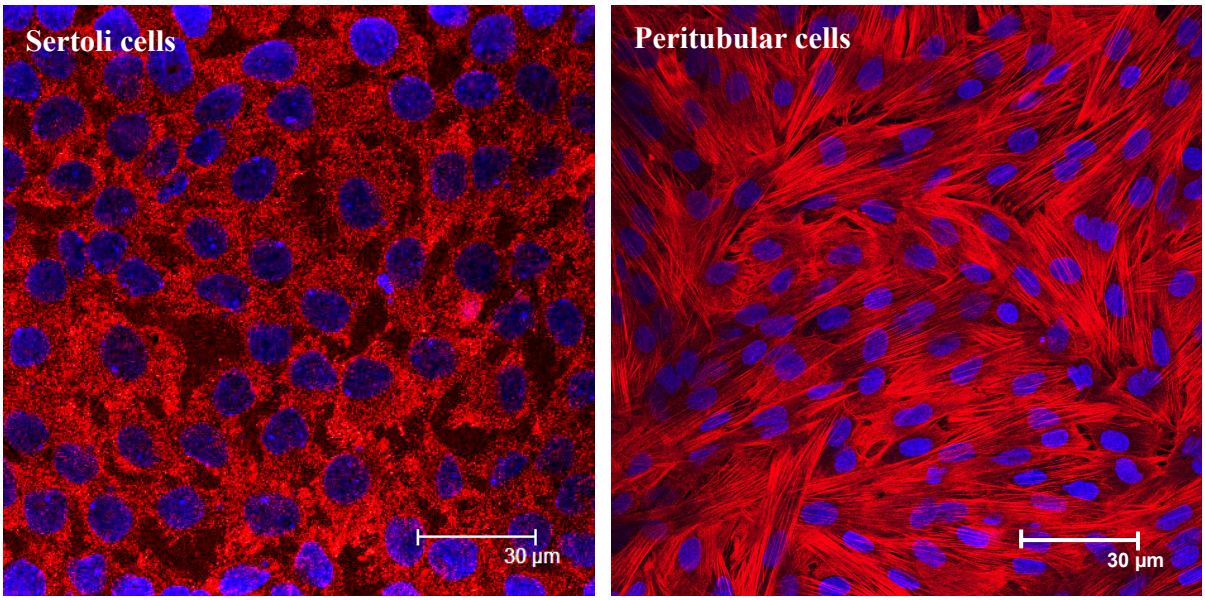


Figure 24: Immunofluorescence staining of Sertoli and peritubular cells isolated from Wistar Unilever rats. *Left:* Detection of vimentin using a goat anti-human vimentin polyclonal antibody followed by a corresponding secondary antibody labelled with Cy3. *Right:* Assessing purity of isolated peritubular cells by a mouse anti-human α-smooth muscle actin monoclonal antibody and a suitable Cy3 labelled secondary antibody. Nuclear staining was performed with DAPI in both cases. (Confocal microscopy, Cy3-DAPI overlay.)

3.4. Immunohistochemical Binding Pattern of Alexa Fluor 647 Labelled Human Galectin-1 C2S

Immunohistochemistry was used to obtain an overview of galectin-1 binding sites in rat testis. For this reason testicular cryosections were incubated with 10 µM Alexa Fluor 647 labelled galectin-1 C2S as a probe for galectin-1 binding sites. A fluorescence signal was mainly found on the surface of the tubular wall, vascular endothelium and vascular adventitia (Figure 25). To exclude false positive results, binding of labelled galectin-1 C2S to testicular cross sections was blocked by 100 mM lactose. Since 100 mM sucrose did not affect binding of galectin-1, sucrose did not bind to the labelled galectin-1 thus showing intact carbohydrate specificity of our fluorescent probe for lactose and polylactosamines (Figure 25).

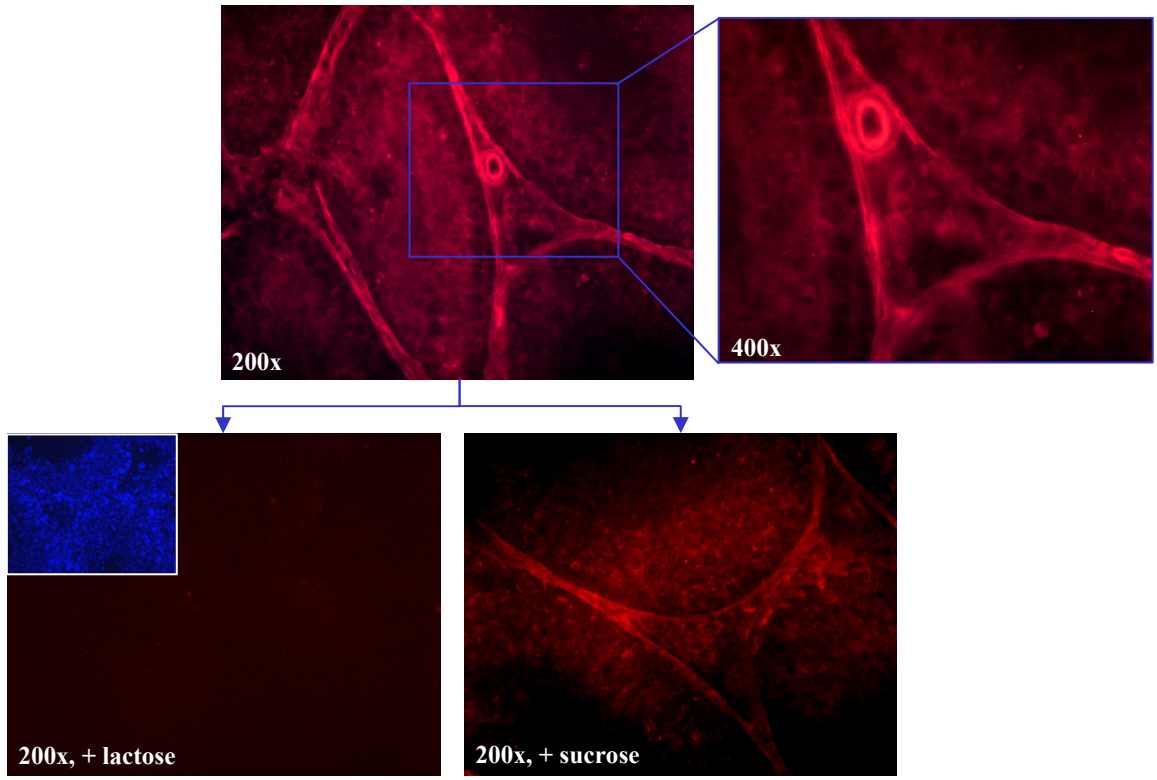


Figure 25: Frozen testicular sections obtained from Wistar Unilever rats were incubated with 10 µM Alexa Fluor 647 labelled human galectin-1 C2S +/- 100 mM lactose or 100 mM sucrose after fixation with 4% w/v PFA in PBS. Binding of galectin-1 to the testis was carbohydrate-specific since it was specifically inhibited by co-incubation with lactose, but not by sucrose. Testicular interstitial cells and seminiferous epithelium showed weak intensity while the tubular wall, vascular endothelium and adventitia bore strong fluorescence signals. (Nuclear staining with DAPI is shown for the section incubated with labelled galectin-1 C2S and lactose as proof of intact histology. Epifluorescence microscopy.)

Additionally cryosections of rat liver were incubated with Alexa Fluor 647 labelled galectin-1 C2S to compare the intensity of binding in another organ. The overall signal intensity was weak and galectin-1 did not to bind to hepatocytes. Only a weak binding to scattered cells occurred, the size and distribution of which resemble Kupffer cells (Figure 26).

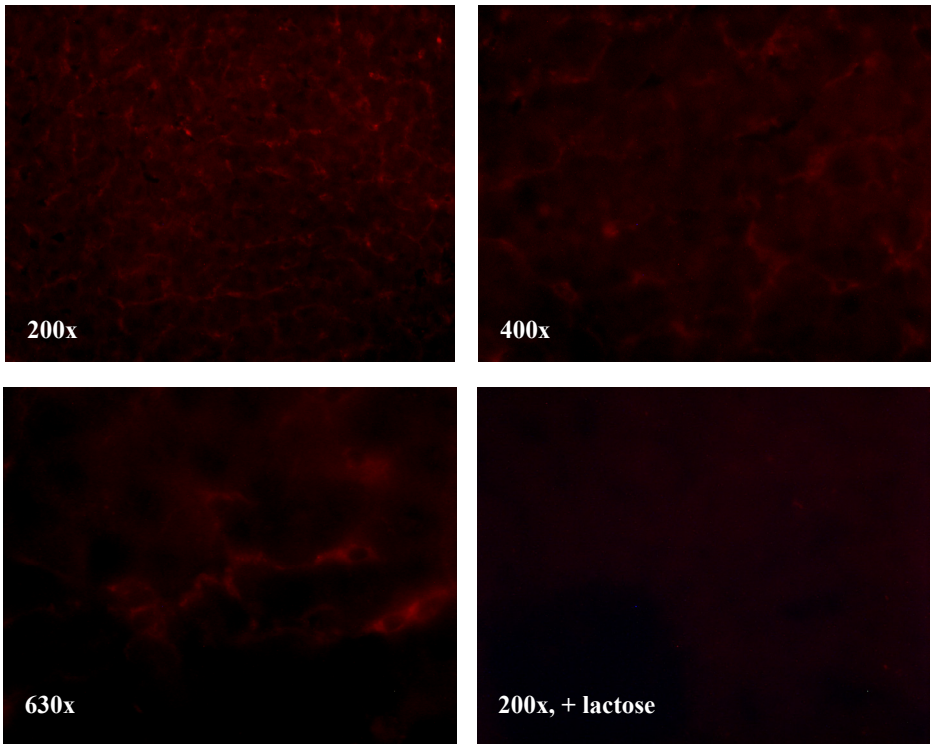


Figure 26: Cryostat sections of liver from Wistar Unilever rats were incubated with 10 µM Alexa Fluor 647 labelled human galectin-1 C2S, which did not bind to the surface of hepatocytes. A few scattered cells were weakly bound by galectin-1. Concluding from its known affinity, galectin-1 presumably bound to the common leukocyte marker CD45 expressed e.g. by Kupffer cells. The weak overall signal intensity was further reduced by co-incubation with 100 mM lactose. The same exposure time was applied as for the testis cross sections shown in Figure 25. (Epifluorescence microscopy.)

3.5. Affinity of Galectin-1 C2S to Isolated Cells

3.5.1. Stimulated vs. Unstimulated Mononuclear Cells

After conjugating recombinant human galectin-1 C2S with the fluorophore Alexa Fluor 647 the maintenance of its carbohydrate binding properties and specificity had to be proven.

The population of mononuclear cells (MNC) from mesenteric lymph nodes was chosen as positive control because it consists of around 60-70% T lymphocytes, which are known to bind galectin-1 (Chapter 1.3.5). Isolated MNC from mesenteric lymph nodes of healthy male Wistar Unilever rats were cultured for 48 h in the presence or absence of the mitogenic stimulus phytohaemagglutinin (PHA) (Chapter 2.12.1).

Incubation of these mononuclear cells with 1 µM conjugated galectin-1 C2S revealed strong binding of galectin-1 as compared to unstained MNC indicating functional integrity of the labelled lectin (Figure 27). The signal intensity was significantly higher than autofluorescence showing a good signal to background ratio.

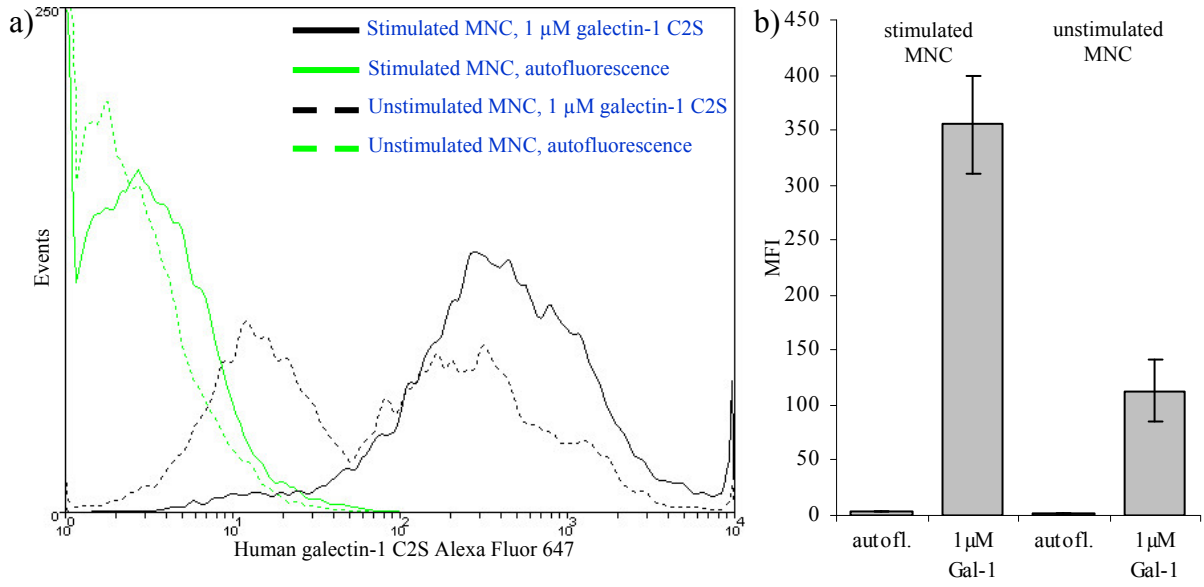


Figure 27: Mononuclear cells were obtained from the mesenteric lymph nodes of Wistar Unilever rats and cultured for 48 h in the presence or absence of the mitogen phytohaemagglutinin (PHA). Then cells were harvested, incubated with 1 µM labelled galectin-1 C2S and galectin-1 binding was analysed by flow cytometry. *a)* The graph shows that PHA stimulation increased the number of cells binding galectin-1 with high affinity documenting that our recombinant galectin-1 C2S re-folded properly after isolation. *b)* Median fluorescence intensities (*MFI*) with standard errors of the mean of autofluorescence (*autofl.*) and of cells incubated with 1 µM labelled galectin-1 C2S (*1 µM Gal-1*). Data shown are representative of two independent experiments.

Furthermore, concentration-dependent signal intensity of galectin-1 C2S binding was shown both for PHA-stimulated as well as non-stimulated mononuclear cells (Figure 28).

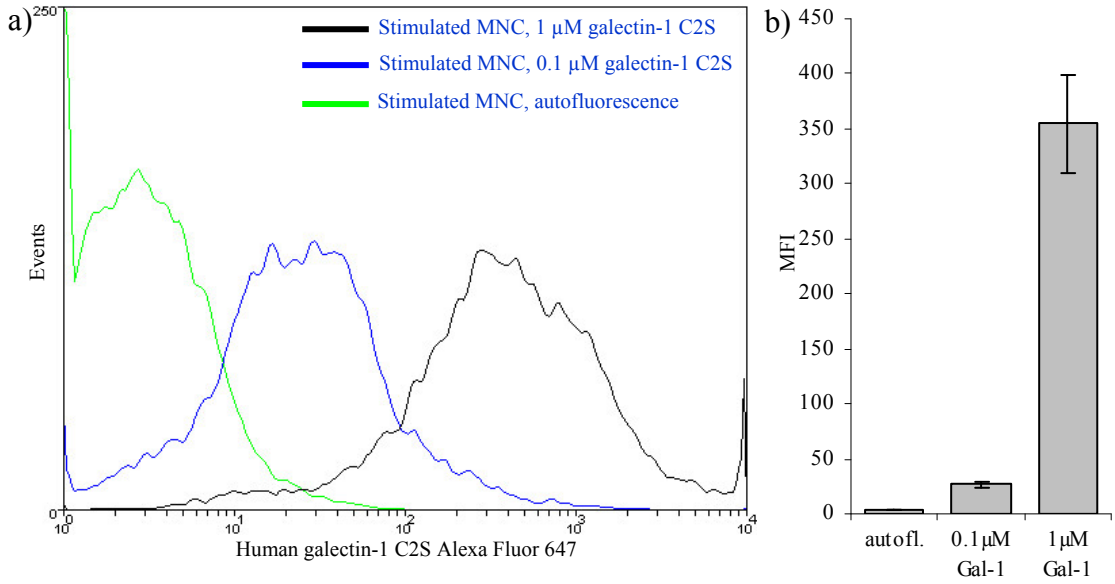


Figure 28: *a)* Dependency of signal strength on the concentration of labelled human galectin-1 C2S shown for PHA stimulated mononuclear cells from mesenteric lymph nodes. *b)* Median fluorescence intensities (*MFI*) with standard errors of the mean of autofluorescence (*autofl.*) and of cells incubated with 0.1 µM or 1 µM labelled galectin-1 C2S (*0.1 µM/1 µM Gal-1*). Data shown are representative of two independent experiments.

Finally, the specificity of labelled recombinant human galectin-1 C2S for polylactosamine residues of cell surface glycoproteins was shown by inhibition of binding to cells by 30 min

of pre-incubation of galectin-1 with 100 mM lactose (Figure 29). In agreement with the carbohydrate specificity of galectin-1 100 mM sucrose did not abolish binding to cells.

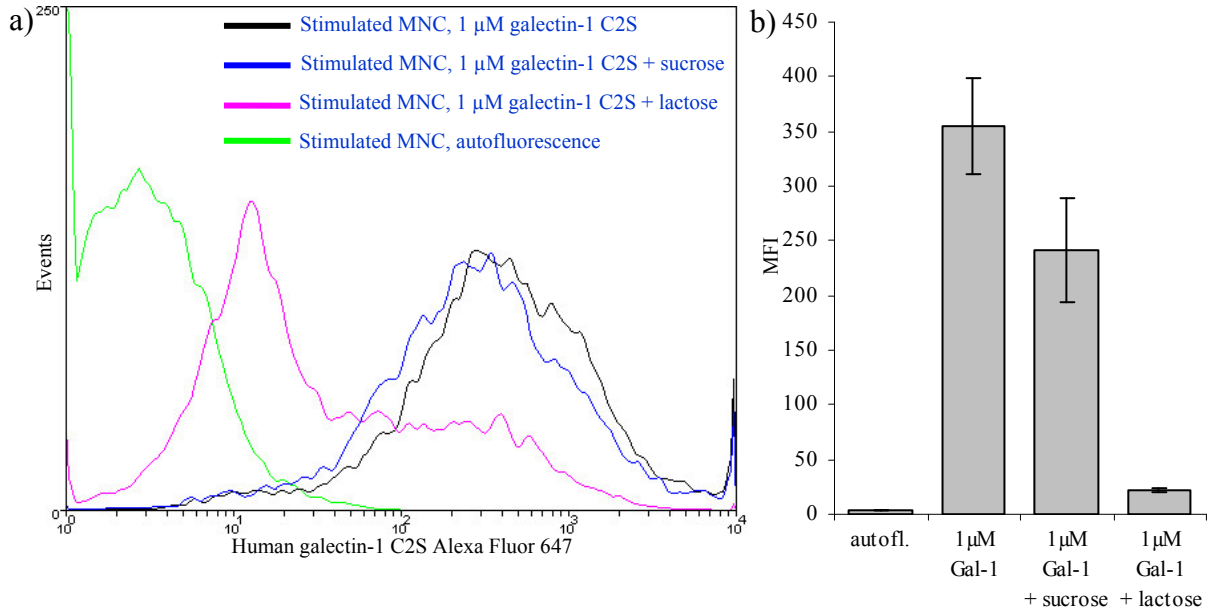


Figure 29: *a)* Pre-incubation of labelled galectin-1 C2S with 100 mM lactose, but not with sucrose abolished galectin-1 C2S binding to cells confirming the carbohydrate specificity of binding to the mononuclear cells. Data shown are derived from mononuclear cells stimulated with PHA and incubated with 1 µM galectin-1. *b)* Median fluorescence intensities (*MFI*) with standard errors of the mean of autofluorescence (*autofl.*) and of cells incubated with 1 µM labelled galectin-1 C2S alone (*1 µM Gal-1*) or galectin-1 C2S pre-incubated with sucrose (*+sucrose*) or lactose (*+lactose*). Data shown are representative of two independent experiments.

3.5.2. Tubular and Interstitial Testicular Cells

After confirming the carbohydrate specificity of Alexa Fluor 647 labelled recombinant human galectin-1 C2S by flow cytometry and immunohistochemistry, galectin-1 binding to isolated tubular and interstitial testicular cells was examined by the same method. Experimental conditions for each cell type were established and applied consistently to obtain comparable results.

Sertoli cells and peritubular cells showed high signal intensities and very strong binding of galectin-1. In both cases the intensity of the fluorescence signal was dose-dependent. To test the specificity of galectin-1 staining, pre-incubation of the fluorescent dye labelled lectin with 100 mM lactose or 100 mM sucrose was performed. Since pre-incubation with lactose, but not with sucrose, reduced the fluorescence intensity, galectin-1 binding to the cells was specific for the presence of lactosamine residues on the cell surfaces (Figure 30 and Figure 31).

In comparison to both aforementioned testicular cell types testicular macrophages bore weaker binding of galectin-1. As shown above, the specificity of concentration-dependent binding of galectin-1 was confirmed by reduction of fluorescence signal by using 100 mM lactose, but not 100 mM sucrose (Figure 32).

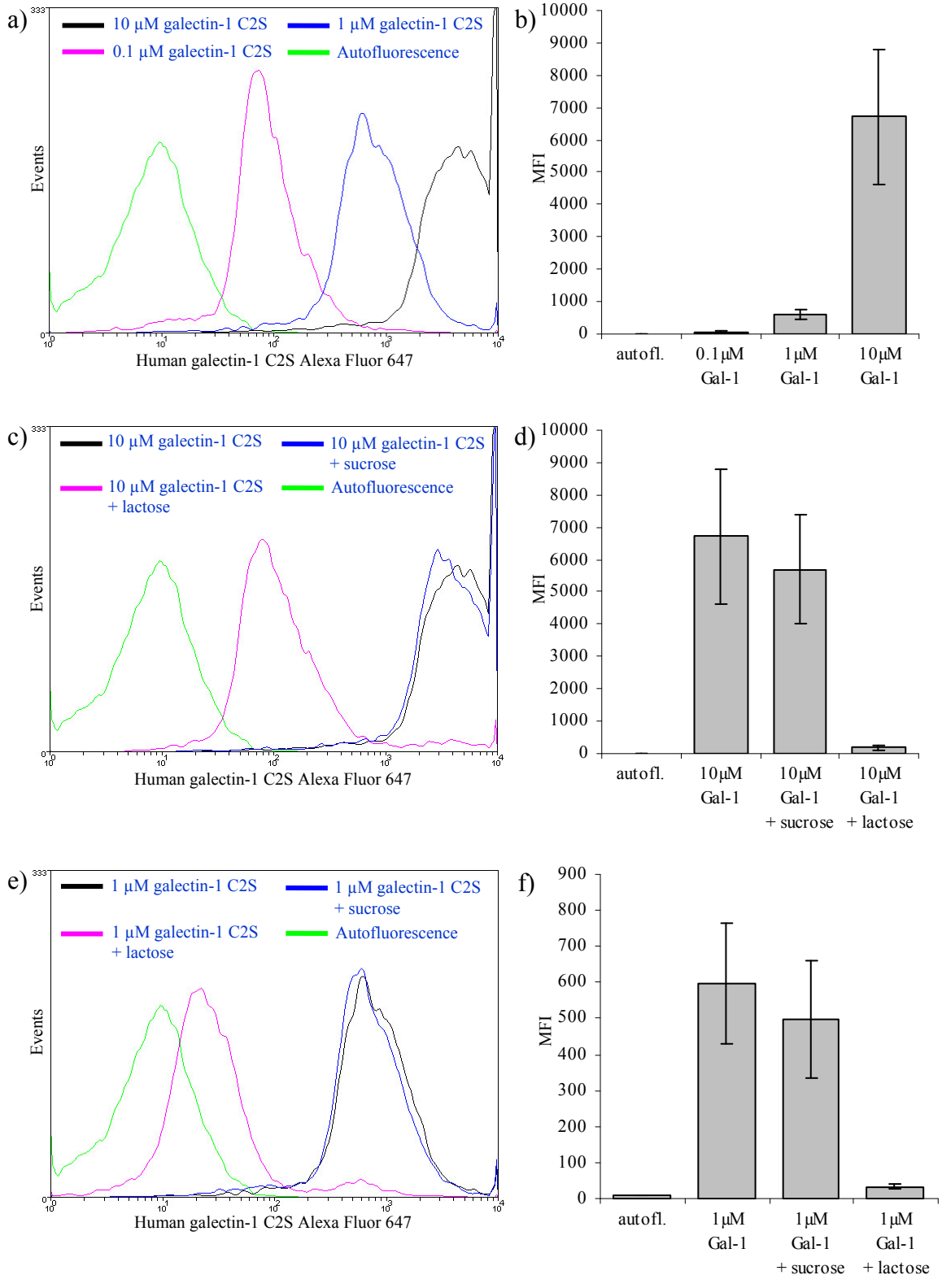


Figure 30: Galectin-1 C2S binding to isolated Sertoli cells. *a)* Dose response curve with three different concentrations (0.1 µM, 1 µM, 10 µM) of galectin-1 C2S-Alexa Fluor 647. *c), e)* Addition of lactose significantly reduced the intensity of galectin-1 binding. Pre-incubation with sucrose did not alter the signal strength. *b), d), f)* Median fluorescence intensities (*MFI*) with standard errors of the mean of autofluorescence (*autofl.*) and of cells incubated with 0.1 µM, 1 µM or 10 µM labelled galectin-1 C2S alone (*0.1 µM/1 µM/10 µM Gal-1*) or galectin-1 C2S pre-incubated with sucrose (*+sucrose*) or lactose (*+lactose*). Data shown are representative of two independent experiments.

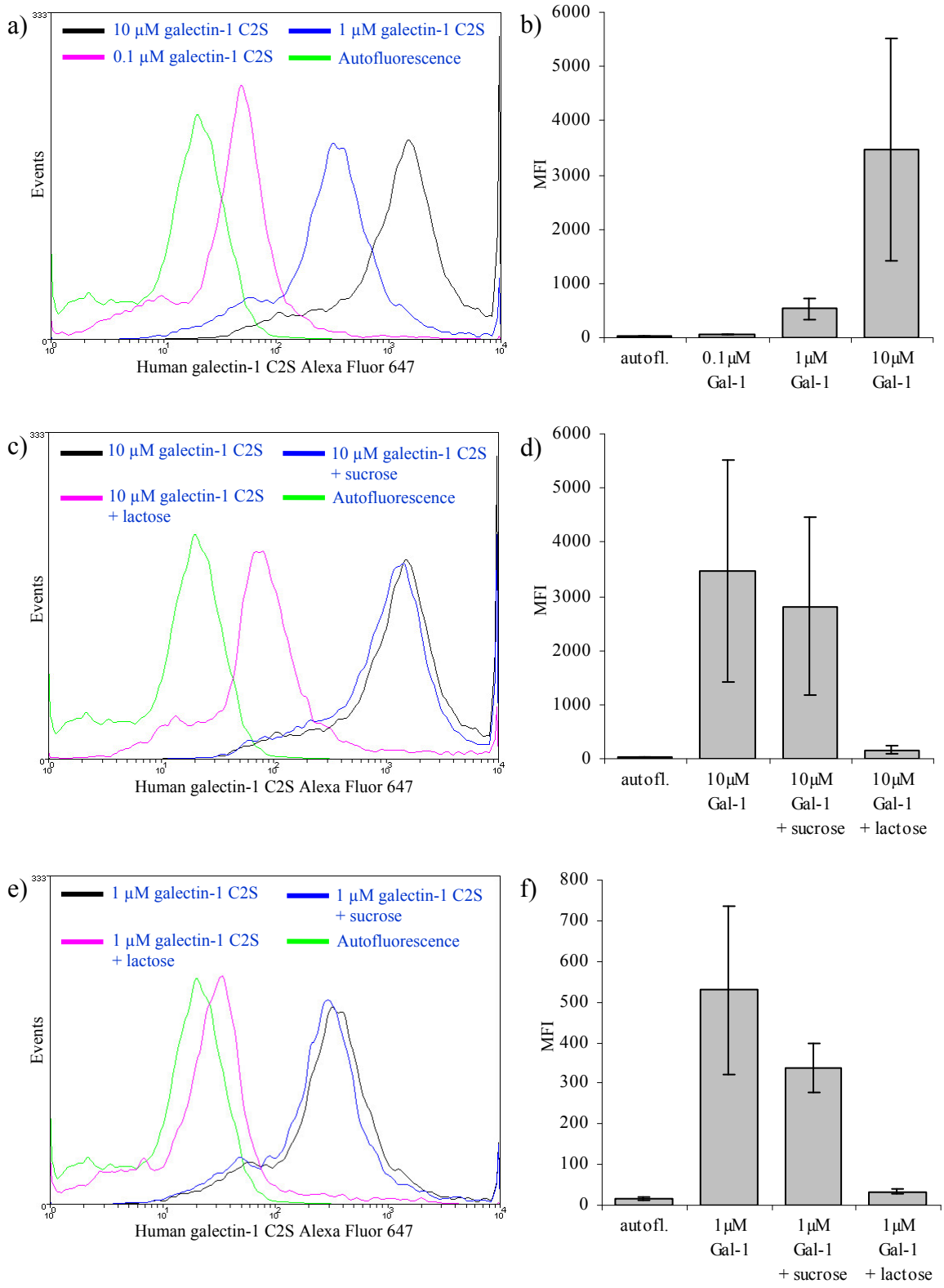


Figure 31: Binding of galectin-1 C2S to peritubular cells. *a)* Dose response curve with three different concentrations (0.1 µM, 1 µM, 10 µM) of galectin-1 C2S-Alexa Fluor 647. *c), e)* Carbohydrate specificity of galectin-1 binding to peritubular cells was confirmed by specific inhibition of lectin binding by pre-incubation with lactose, but not with sucrose. *b), d), f)* Median fluorescence intensities (*MFI*) with standard errors of the mean of autofluorescence (*autofl.*) and of cells incubated with 0.1 µM, 1 µM or 10 µM labelled galectin-1 C2S alone (*0.1 µM/1 µM/10 µM Gal-1*) or galectin-1 C2S pre-incubated with sucrose (+*sucrose*) or lactose (+*lactose*). Data shown are representative of two independent experiments.

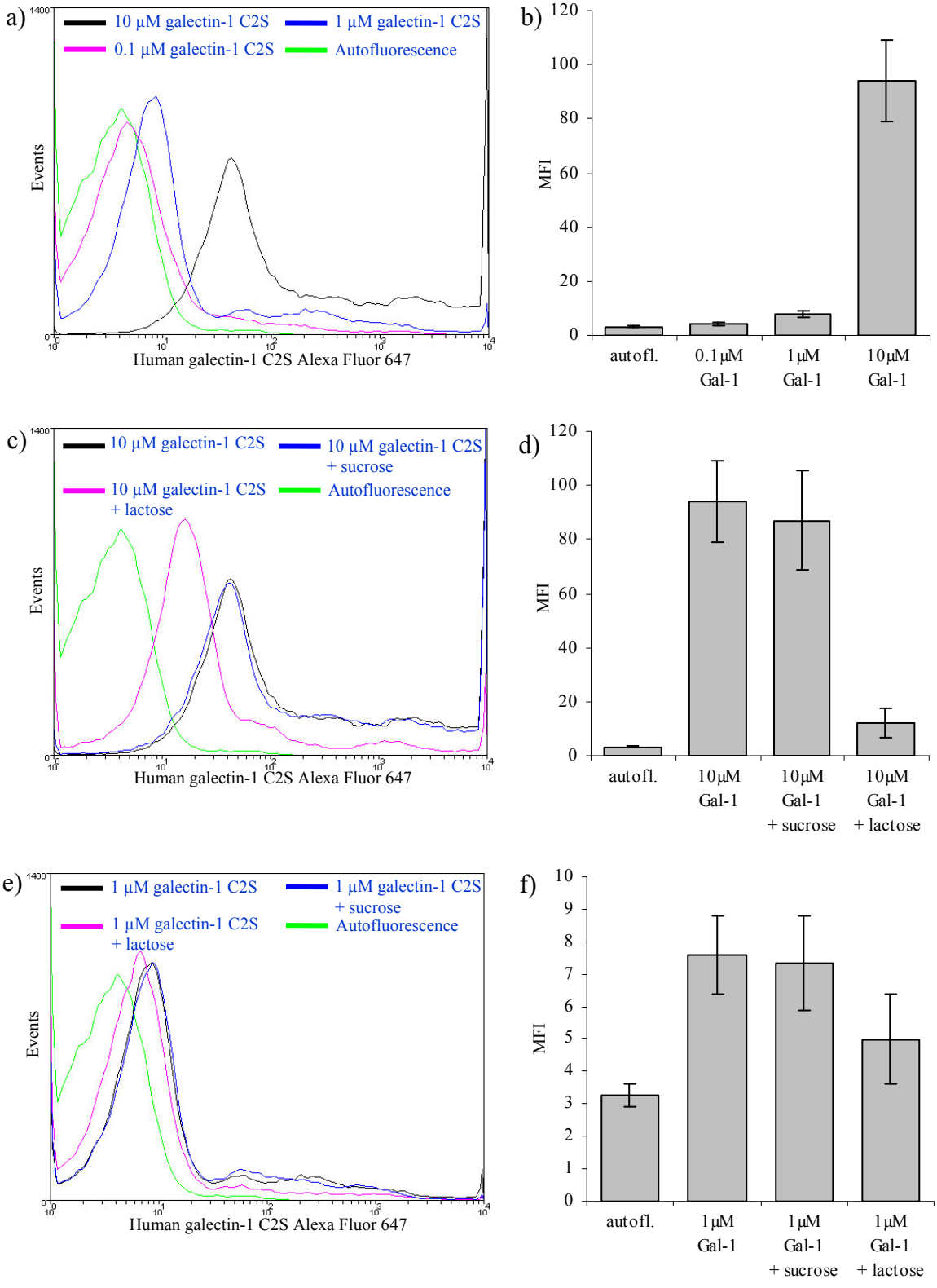


Figure 32: Galectin-1 C2S binding to testicular macrophages. *a)* Dose response curve with three different concentrations (0.1 µM, 1 µM, 10 µM) of galectin-1 C2S-Alexa Fluor 647. *c), e)* Pre-incubation of labelled galectin-1 C2S with lactose or sucrose confirmed specificity of galectin-1 binding to testicular macrophages. *b), d), f)* Median fluorescence intensities (*MFI*) with standard errors of the mean of autofluorescence (*autofl.*) and of cells incubated with 0.1 µM, 1 µM or 10 µM labelled galectin-1 C2S alone (*0.1 µM/1 µM/10 µM Gal-1*) or galectin-1 C2S pre-incubated with sucrose (*+sucrose*) or lactose (*+lactose*). Note the overall very low signal intensities in *f)* causing only minor differences between the signals yielded for the experimental conditions. Data shown are representative of two independent experiments.

3.6. Glycophenotype of Isolated Cells

After examining the intensity of binding of galectin-1 C2S to tubular and interstitial testicular cells the glycophenotype, i.e. structure of glycans on cell membrane proteins was analysed. The following panel of fluorescent labelled plant lectins was used to assess the glycosylation pattern of isolated cells in flow cytometry (Table 4).

Plant Lectin (Origin)	Targeted Glycan Structure
LEA (Lycopersicon esculentum, tomato)	Oligomers of β1-4 linked GlcNAc (≤4 monomers); 3 repetitive LacNAc (Galβ1-4GlcNAc) units like in type-2 poly-N-acetyllactosamine.
MAA (Maackia amurensis, Amur maackia)	Terminal sialic acid α2-3 linked to penultimate galactose in complex-type N-glycans.
PNA (Arachis hypogaea, peanut)	Core 1 O-glycan (T antigen, most common O-glycan, Galβ1-3GalNAcαSer/Thr).
SNA-I (Sambucus nigra, elderberry)	Terminal sialic acid α2-6 linked to penultimate galactose (like in N-glycans); terminal sialic acid α2-6 linked to GalNAc (like in O-glycans).

Table 4: Plant lectins used in flow cytometry for assessment of glycosylation patterns of isolated cells.

Experimental conditions were standardized and applied for every cell type in the same way.

3.6.1. Stimulated vs. Unstimulated Mononuclear Cells

Mononuclear cells from mesenteric lymph nodes of Wistar Unilever rats showed stronger binding of human galectin-1 C2S when they were stimulated by the mitogen phytohaemagglutinin (PHA) as compared to unstimulated cells (Chapter 3.5.1). In a first step PHA stimulated and unstimulated mononuclear cells from mesenteric lymph nodes were incubated with the plant lectins (Figure 33).

Stimulated mononuclear cells showed increased affinity for MAA, PNA and SNA-I. The weak binding of LEA was slightly stronger upon mitogenic stimulation.

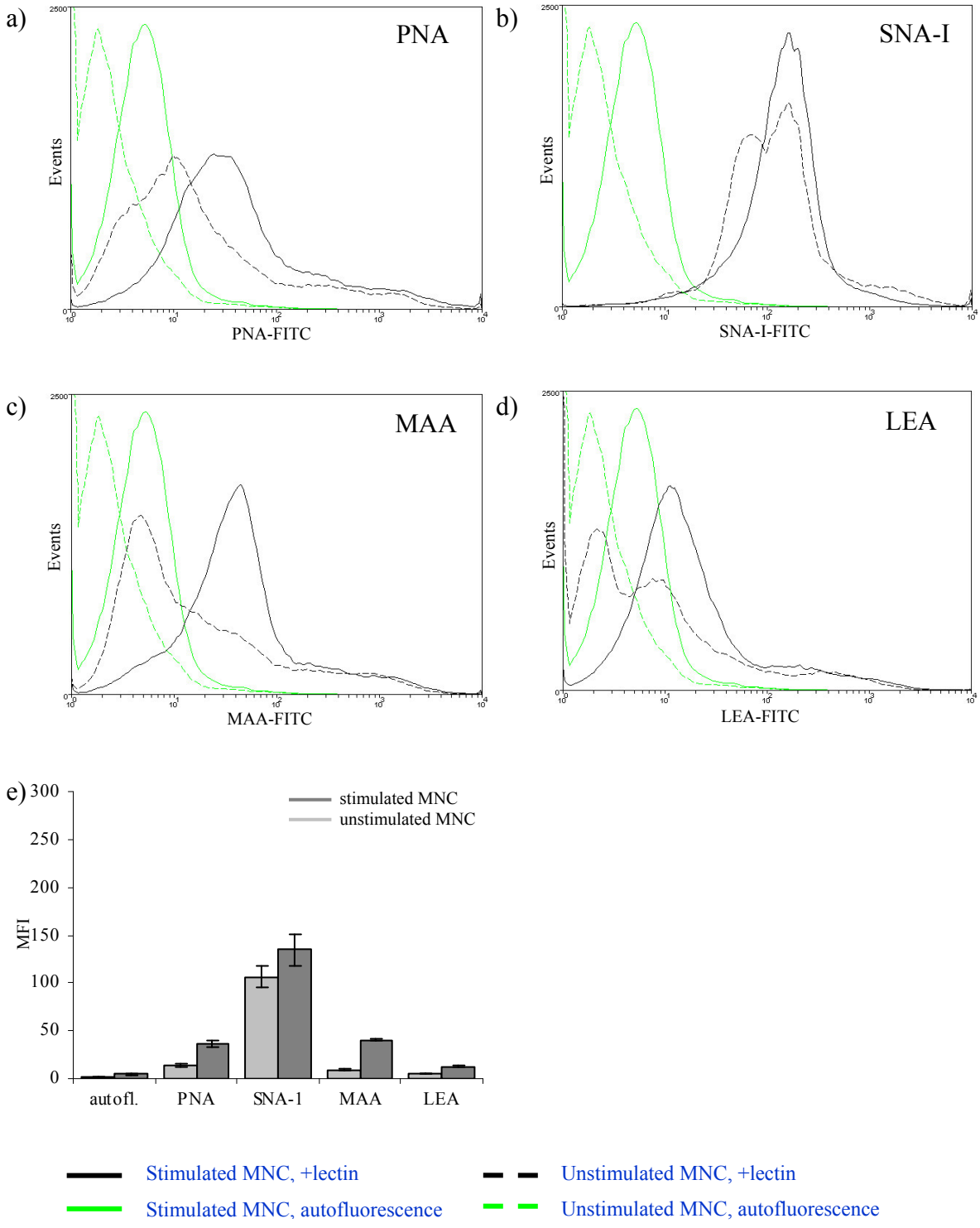


Figure 33: Binding of FITC-labelled plant lectins to mononuclear cells from mesenteric lymph nodes. Cells were cultured for 48 h in the presence or absence of the mitogen PHA. *a)* PNA: Arachis hypogaea lectin. *b)* SNA-I: Sambucus nigra lectin I. *c)* MAA: Maackia amurensis lectin. *d)* LEA: Lycopersicon esculentum lectin. *e)* Median fluorescence intensities (*MFI*) with standard errors of the mean of autofluorescence (*autofl.*) and of cells incubated with the aforementioned plant lectins. Data shown are representative of two independent experiments.

3.6.2. Tubular and Interstitial Testicular Cells

The strongest binding of human galectin-1 C2S was found for Sertoli and peritubular cells (Chapter 3.5.2), which were subsequently incubated with a panel of plant lectins to examine their specific cell surface glycan structures being of significance for the galectin-1 binding properties (Figure 34 and Figure 35).

The analysis of the glycophenotype demonstrated moderate binding of SNA-I and MAA by Sertoli cells in relation to their autofluorescence. Peritubular cells showed similar signal intensity for MAA, while binding of SNA-I was stronger. Binding of PNA and LEA was weak for both cell types in proportion to the respective autofluorescence level.

Testicular macrophages exhibited weaker binding of human galectin-1 C2S as compared to Sertoli and peritubular cells. Binding strength of the plant lectins PNA and SNA-I ranged from moderate to strong with a trimodal distribution, which is not depicted by the calculated median fluorescence intensities. Binding of MAA and LEA similarly ranged from weak to moderate (Figure 36).

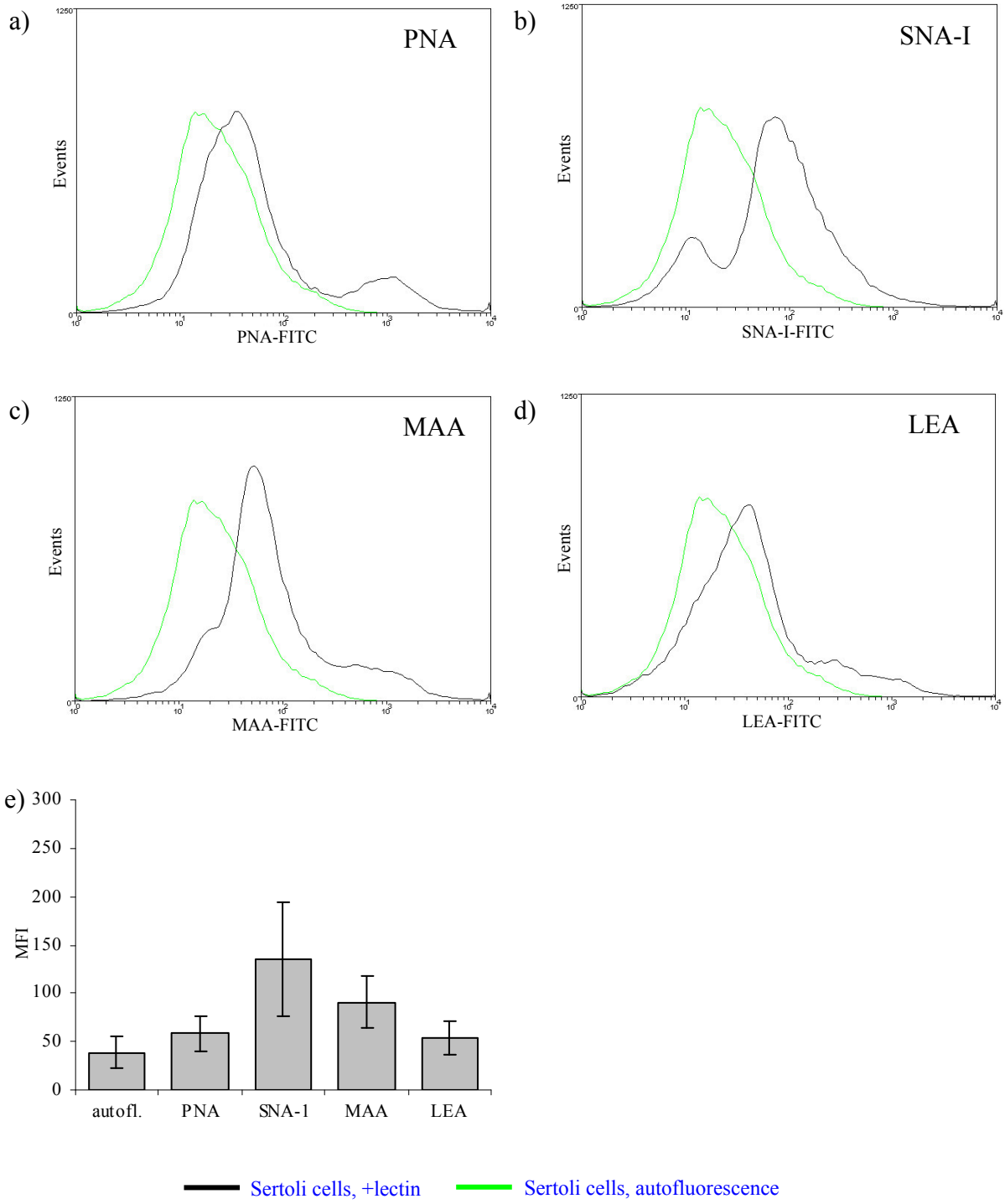


Figure 34: Binding of FITC-labelled plant lectins to Sertoli cells. *a)* PNA: Arachis hypogaea lectin. *b)* SNA-I: Sambucus nigra lectin I. *c)* MAA: Maackia amurensis lectin. *d)* LEA: Lycopersicon esculentum lectin. *e)* Median fluorescence intensities (*MFI*) with standard errors of the mean of autofluorescence (*autofl.*) and of cells incubated with the aforementioned plant lectins. Data shown are representative of two independent experiments.

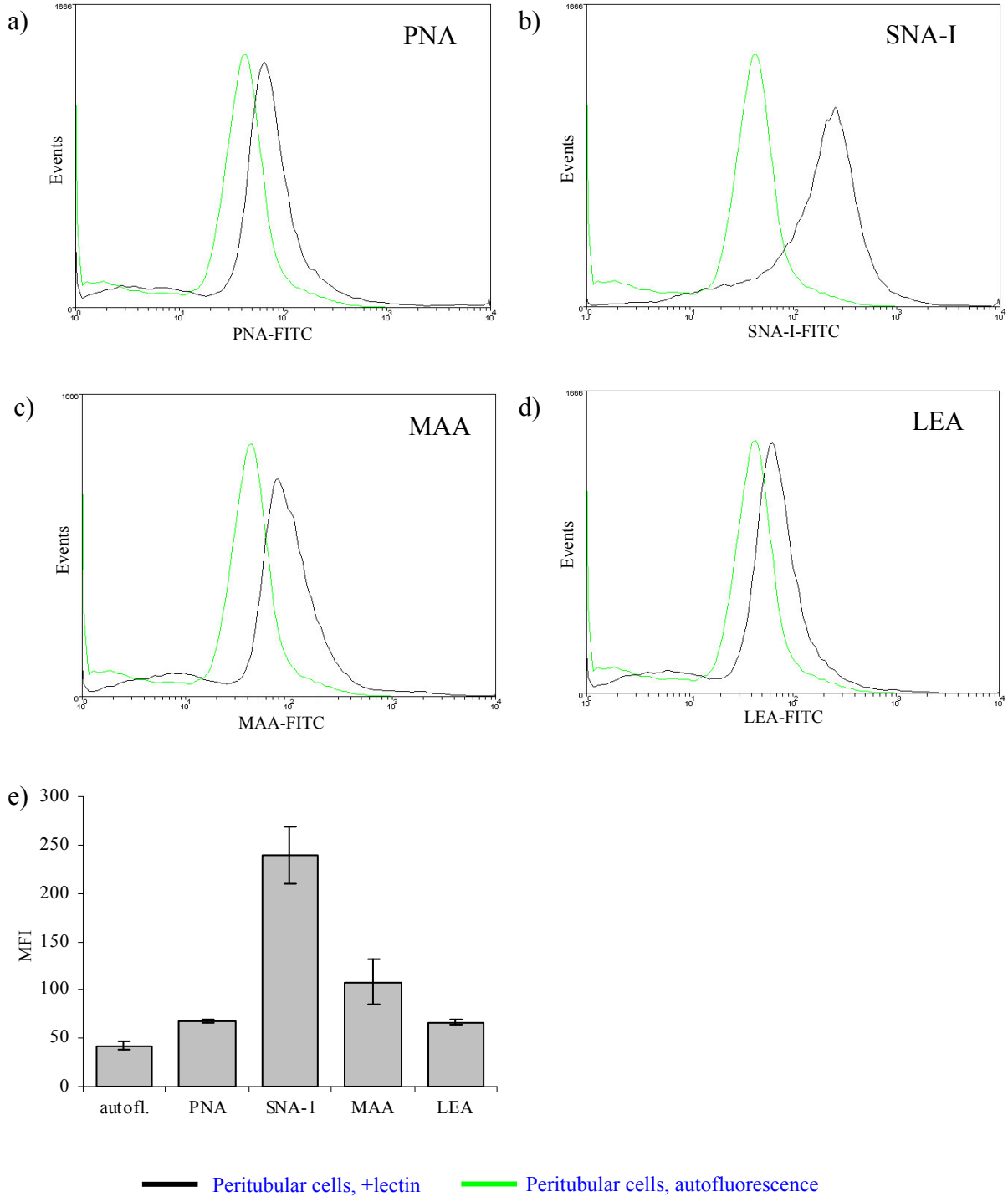


Figure 35: Binding of FITC-labelled plant lectins to peritubular cells. *a)* PNA: Arachis hypogaea lectin. *b)* SNA-I: Sambucus nigra lectin I. *c)* MAA: Maackia amurensis lectin. *d)* LEA: Lycopersicon esculentum lectin. *e)* Median fluorescence intensities (*MFI*) with standard errors of the mean of autofluorescence (*autofl.*) and of cells incubated with the aforementioned plant lectins. Data shown are representative of two independent experiments.

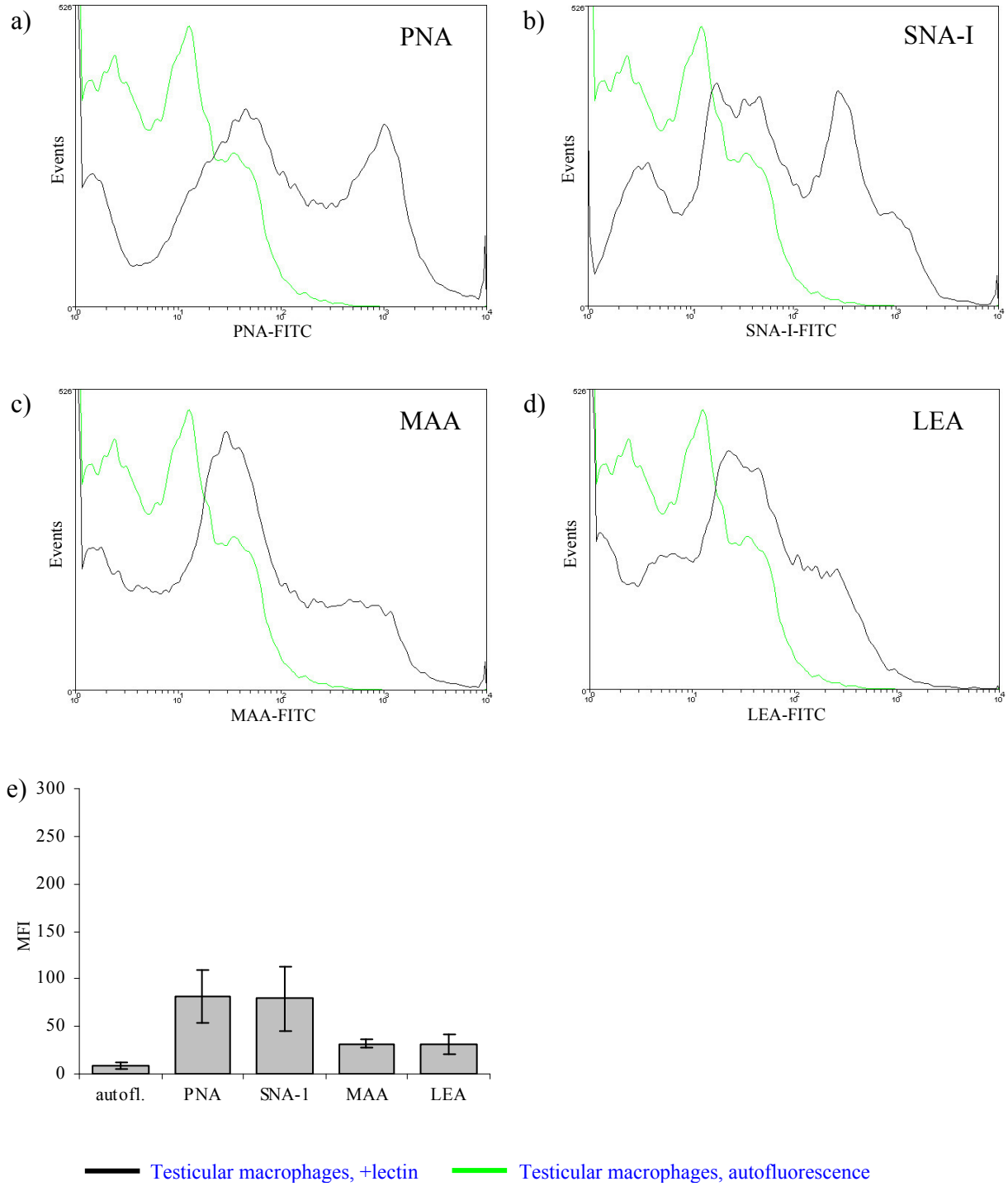


Figure 36: Binding of FITC-labelled plant lectins to testicular macrophages. *a)* PNA: Arachis hypogaea lectin. *b)* SNA-I: Sambucus nigra lectin I. *c)* MAA: Maackia amurensis lectin. *d)* LEA: Lycopersicon esculentum lectin. *e)* Median fluorescence intensities (*MFI*) with standard errors of the mean of autofluorescence (*autofl.*) and of cells incubated with the aforementioned plant lectins. Data shown are representative of two independent experiments.

4. Discussion

Interest in galectin-1, a 14.5 kDa sized evolutionary conserved animal lectin, arises from its capability to ameliorate acute (Rabinovich et al., 2000b) and chronic inflammatory processes (Liu and Rabinovich, 2010; Rabinovich et al., 2007) both *in vitro* and *in vivo*. Due to these tolerogenic properties galectin-1 has started to extend the classical spectrum of immunoregulatory factors consisting of cytokines and hormones. As a member of the galectin family it is characterized by its conserved carbohydrate-recognition domain and its affinity for β-galactosides on cell surface glycoproteins (Barondes et al., 1994). It shows preferential binding to poly-N-acetyllactosamine (poly-Galβ1-4GlcNAc) (Stowell et al., 2004).

Since the expression of galectin-1 was found also in the testis of rat (Dettin et al., 2003) and men (Wollina et al., 1999), a contribution of this lectin to the immunologically privileged status of the testis appears to be likely.

4.1. Recombinant Human Galectin-1 C2S as a Molecular Probe

In order to detect galectin-1 binding sites in the testis of Wistar Unilever rats and to assess its affinity for distinct testicular cells, we recombinantly expressed human galectin-1 C2S, labelled it with Alexa Fluor 647 and applied it in immunohistochemistry and flow cytometry. The functionally equivalent C2S mutant was chosen instead of wild-type galectin-1 to avoid false-negative study results caused by sensitivity of wild-type galectin-1 to oxidizing conditions. The C2S mutant bears increased resistance to oxidative inactivation, unaltered carbohydrate specificity and a binding strength close to that of the wild-type protein (Hirabayashi and Kasai, 1991). Specificity of this self-constructed molecular probe was proven by assessing galectin-1 binding to mononuclear cells from mesenteric lymph nodes using flow cytometry.

Applying PHA stimulated and unstimulated mononuclear cells as a positive control, the obtained signal intensity was confirmed to be dependent on the concentration of galectin-1 C2S, the specificity of which persisted despite conjugation to a fluorophore. Signal strength of galectin-1 binding was reduced nearly down to autofluorescence level by pre-incubation of galectin-1 C2S with its inhibitory sugar lactose. Pre-incubation with sucrose consistently did not affect lectin binding. Stimulated mononuclear cells bound galectin-1 more strongly than unstimulated cells (Moos et al., 2009).

Discrimination of functional states by our modified human galectin-1 corresponds with what is known about the biology of T cells in respect to glycans and galectin-1:

Activation of $CD4^+$ T cells and $CD8^+$ T cells leads to decreased expression of the glycosyltransferase ST6Gal I with consequent reduction of terminal α2-6 NeuNAc linkage (Comelli et al., 2006). This reduction of α2-6 linked sialic acid exposes galactose residues, which can serve as binding partners of galectin-1 (Amano et al., 2003). Similarly, activated $CD8^+$ T cells bear reduced expression of the sialyltransferase ST3Gal I. This enzyme α2-3 sialylates core 1 O-glycans, e.g. on CD43, and thus blocks further processing of glycans towards core 2 glycan structures, which serve as a starting point for the synthesis of galectin-1 binding motifs in O-glycans (Priatel et al., 2000; Rabinovich and Toscano, 2009). Although deletion of the ST3Gal I gene increased apoptosis of $CD8^+$ T cells in this study, Priatel et al. were unable to induce apoptosis of $CD8^+$ T cells by addition of galectin-1 (Priatel et al., 2000). Thus the involvement of galectin-1 in apoptosis of $CD8^+$ T lymphocytes remains to be elucidated. In addition to stimulation-induced changes in glycan motifs T cells themselves show increased expression of galectin-1 in the course of their activation (Blaser et al., 1998).

The circumstance that binding of galectin-1 in the flow cytometric assay could be strongly reduced, but not completely inhibited by pre-incubation with lactose indicates that some residual binding of the lectin might be due to interaction with neighbouring structures next to its specific target glycans (Damjanov, 1987) or that prolonged pre-incubation or higher concentrations of lactose should be applied.

4.2. Galectin-1 Target Cells in the Testis Identified by Immunohistochemistry

Our immunohistochemical findings demonstrate β-galactoside-specific binding sites of galectin-1 within the testis of healthy Wistar Unilever rats (Chapter 3.4):

The tubular wall, vascular endothelium and vascular adventitia exhibited strong signal intensities while the interstitium of the testis and the seminiferous epithelium showed weak fluorescence. Obtained signals were derived from carbohydrate-specific binding of galectin-1 since pre-incubation with lactose, but not with sucrose, reduced the signal strength to the signal level of autofluorescence in immunohistochemistry (Moos et al., 2009).

An early study conducted on paraffin sections of human testes demonstrated galectin-1 binding to Sertoli cells with moderate intensity and to germ cells with weak intensity. No binding was observed for the tubular wall (Wollina et al., 1999). The investigators used a mixture of biotinylated galectin-1 and biotinylated anti-galectin-1 polyclonal antibody to

increase the yielded signal. With the same polyclonal antibody galectin-1 itself was found in the cytoplasm of human Sertoli cells and in the human tubular wall (Wollina et al., 1999).

A later study showed galectin-1 expression in rat Sertoli cells to start 45 days after birth and to change according to the stages of spermatogenesis. It was found in the apical cytoplasm of Sertoli cells and on late spermatids during spermiation and thereafter in basal portions of the nurturing Sertoli cells during meiosis of spermatocytes. Interstitial testicular cells showed intense reactivity for the anti-galectin-1 polyclonal antibody (Dettin et al., 2003).

Looking at strong binding of galectin-1 to the compartment of peritubular cells – a finding not mentioned by Wollina et al. and Dettin et al. – it has to be considered that laminin is deposited in the layer of peritubular cells by secretion of Sertoli cells (Pollanen et al., 1985; Skinner et al., 1985) and that it is a strong binding partner of galectin-1 (Vicovac et al., 1998). Concluding from the presence of laminin within the tubular wall a strong galectin-1 signal in the tubular wall can be expected. Therefore, we measured galectin-1 binding to isolated peritubular cells by flow cytometry to distinguish between galectin-1 binding to extracellular matrix components and affinity for cellular surface glycans (Chapter 4.3).

In order to test the binding capacity of galectin-1 to other tissues, we also checked its binding to frozen sections of an unrelated organ – the liver. We could demonstrate a weak binding of fluorophore tagged human galectin-1 C2S only to single cells in rat liver and excluded unspecific binding to the surface of arbitrary cells. Due to their size and distribution they resemble Kupffer cells and accordingly galectin-1 most likely bound to the common leukocyte marker CD45 expressed by these cells (Klein et al., 2007).

Finally, the strong binding of galectin-1 to endothelial cells within the testis might be linked to its known affinity for neuropilin-1, which is expressed on endothelial cells and initiates their migration via the vascular endothelial growth factor receptor 2 pathway (VEGFR-2) (Hsieh et al., 2008). Since galectin-1 has been shown to be overexpressed in vascular endothelial cells of tumours, it may play a role in endothelial development within the testis (Hsieh et al., 2008). Galectin-1 has been demonstrated to interact with another vascular endothelial growth factor receptor, albeit with a different effect. It activates VEGFR-3 in choriocarcinoma cells as a possible pathway of inhibiting their proliferation (Fischer et al., 2009).

The emphasized galectin-1 binding to the blood vessel wall could be explained by its affinity to α1β1 integrin on vascular smooth muscle cells (Moiseeva et al., 2003) known to modulate spreading, migration and proliferation of vascular smooth muscle cells on laminin.

4.3. Differences in the *in Vitro* Binding Capacity of Galectin-1 C2S to Different Isolated Testicular Cell Types

For the first time galectin-1 binding has been assessed *in vitro* for isolated, single cells from the testis by flow cytometry using fluorophore labelled recombinant human galectin-1 C2S. This technique enables to compare binding affinities and define underlying glycocalyx structures for each cell type without interference with the extracellular matrix. This is of importance as galectin-1 bears high affinity e.g. for laminin (Vicovac et al., 1998).

Due to the binding of galectin-1 to a broad spectrum of immune cells via CD43 and CD45 our special interest was focused on its affinity to testicular macrophages. We also included non-immune testicular cells such as peritubular and Sertoli cells in our assessment as they are thought to play an important role in the testicular innate immune response and thus immune privilege (Fijak and Meinhardt, 2006).

In our study we have shown that all investigated testicular cell types were able to bind galectin-1, albeit with different intensity. The fluorescence signal of Alexa Fluor 647 conjugated galectin-1 C2S binding to peritubular and Sertoli cells was comparable to that of phytohaemagglutinin stimulated mononuclear cells, while in contrast galectin-1 binding to testicular macrophages was much weaker with an intensity lower than unstimulated mononuclear cells (Moos et al., 2009). The observed binding was dose-dependent and carbohydrate-specific.

Our demonstration of strong affinity of galectin-1 to isolated Sertoli cells by flow cytometry corresponds to Dettin's and Wollina's description of cells positive for galectin-1 binding in the rodent and human testis (Dettin et al., 2003; Wollina et al., 1999). The weak signal intensity of galectin-1 binding to the tubular epithelium in our immunohistochemical assay is most likely related to obscuring of galectin-1 targets in the process of tissue sectioning and fixation prior to incubation with Alexa Fluor 647 labelled human galectin-1 C2S.

Strong galectin-1 binding to the tubular wall of our formalin-fixed frozen testicular sections of rat was confirmed by flow cytometric analysis of *in vitro* cultured rat peritubular cells. However, these findings are not consistent with Wollina's et al. results obtained for cross sections of human testis embedded in paraffin after fixation in Bouin's solution (Wollina et al., 1999). These varying results may relate to the denoted technical differences (e.g. directly labelled recombinant galectin-1 vs. biotinylated galectin-1/biotinylated anti-galectin-1 anti-

body in Wollina's et al. assay), although our findings appear to be more likely than Wollina's et al. given the high affinity of galectin-1 to laminin found in the tubular wall (Chapter 4.2).

For peritubular cells and testicular macrophages both our flow cytometric and immunohistochemical findings correspond with each other.

In summary, we conclude from our studies that peritubular cells and Sertoli cells are the primary target cells for galectin-1, but surprisingly not as hypothesised testicular macrophages. It seems to be likely that galectin-1 contributes to the testicular immune privilege mainly by interaction with testicular somatic cells. This perspective centred on somatic cells is supported by the fact that galectin-1 is capable of inducing apoptosis of Leydig cells *in vitro* in a concentration-dependent and carbohydrate-specific manner (Martinez et al., 2004) with Sertoli cells being the primary source of galectin-1 in the testis of men and rats (Dettin et al., 2003; Wollina et al., 1999).

4.4. Glycophenotype of Cells Isolated from the Testis

To understand how glycan structures attribute to preferential binding of galectin-1, a new set of plant lectins has been applied in the analysis of isolated testicular cells and mononuclear cells from mesenteric lymph nodes in flow cytometry for the first time.

As compared to unstimulated mononuclear cells, phytohaemagglutinin (PHA) stimulated mononuclear cells showed higher α2-3 sialylation of N-glycans, slightly increased α2-6 sialylation and increased core 1 O-glycan expression. Oligomeric β1-4 linked GlcNAc and repetitive LacNAc – detected by the lectin LEA with weak intensity under both conditions – were expressed slightly stronger upon mitogenic stimulation. Thus an increase of repetitive LacNAc units rather than a decrease of α2-6 sialylation attributes to a higher affinity of galectin-1 to PHA stimulated mononuclear cells (Chapters 3.5.1 and 3.6.1).

The surface of Sertoli and peritubular cells, which showed the strongest binding of galectin-1 amongst the investigated testicular cells, exhibited weakly to moderately oligomeric β1-4 linked GlcNAc units and repetitive LacNAc residues according to the binding of LEA. Expression of the core 1 O-glycan (T antigen) was also weak. Concluding from moderate MAA and SNA-I binding, Sertoli cells bear α2-3 and α2-6 linked sialic acid residues, while peritubular cells showed a higher degree of α2-6 sialylation.

Testicular macrophages were found to exhibit a trimodal distribution of lectin affinity. This may be related to their functional division into tissue resident macrophages ($CD163^+$), monocytes or recently arrived macrophages ($CD68^+$) and those in transition between both

states ($CD68^+CD163^+$) (Hedger, 2002; Rival et al., 2008). Thus, testicular macrophages were in part found to highly express core 1 O-glycans and N-/O-glycans with α2-6 linked sialic acid. α2-3 sialylation and oligomeric β1-4 linked GlcNAc units as well as repetitive LacNAc were moderately present (Table 5).

	Gal-1	PNA	SNA-1	MAA	LEA
Sertoli cells	+++	+	++	++	+
Peritubular cells	+++	+	+++	++	+
Testicular Macrophages	+	++/+++	++/+++	+/++	+/++

Table 5: Overview of galectin-1 and plant lectin binding to isolated Sertoli cells, peritubular cells and testicular macrophages. Signal yields are described with the following symbols: +++/++/+: strong/moderate/weak binding. *Gal-1:* recombinant human galectin-1 C2S. *PNA:* Arachis hypogaea lectin. *SNA-I:* Sambucus nigra lectin I. *MAA:* Maackia amurensis lectin. *LEA:* Lycopersicon esculentum lectin.

To analyse the glycan structures more precisely, pure MAL should be applied in future experiments instead of MAA as the latter is composed of the two lectins MAL and MAH, which bear slightly varying specificities (Chapter 1.3.2) (Varki and Varki, 2007).

Lectin histochemistry studies on the testis of several species carried out in the past can partially help to assess our results for PNA, SNA-I and MAA, whilst the binding of LEA has not been investigated for testicular cells before.

In Wistar rat testis Arya and Vanha-Pertulla demonstrated strong affinity of PNA for early stages of the developing acrosome while binding to Sertoli cells, basement membrane and Leydig cells was weak. No affinity was found for peritubular cells, spermatogonia and spermatocytes (Arya and Vanha-Perttula, 1984). The study also applied the lectin WGA with affinity for oligomeric GlcNAc and sialic acid. A strong to moderate WGA signal was found for Sertoli cells and a weak signal for peritubular cells (Arya and Vanha-Perttula, 1984). A later study described PNA binding sites in rat germ and interstitial cells of Sprague-Dawley rats starting at 15-30 days after birth. No PNA signal was seen for Sertoli cells and basement membrane in the examined period, i.e. until 90 days after birth (Malmi et al., 1990).

In respect to testicular cross sections from Fischer 344 rats, affinity of MAA has been described for the cytoplasm of some spermatocytes/spermatids, elongated nuclei of spermatids, peritubular cells and testicular interstitium (Wine and Chapin, 1997).

In testes of men with normal spermatogenesis PNA presented cytoplasmic staining of all germ cells with emphasis on acrosomes of spermatids. Peritubular and interstitial cells were not recognized by PNA (Malmi et al., 1987), whereas WGA binding showed strong fluorescence

at interstitial cells, tubular wall and seminiferous epithelium surrounding spermatozoa. Since treatment with Limulus polyphemus agglutinin, which binds to sialic acid, decreased WGA binding, the tubular wall was regarded to contain oligomeric GlcNAc units and to be sialylated (Malmi et al., 1987).

A study on unfixed frozen sections of normal human testes showed staining of interstitial cells and tubular wall by PNA and WGA and binding of early acrosomes by PNA (Wollina et al., 1989). A successive study on human testis demonstrated MAA to weakly bind to all cells of the seminiferous epithelium, lamina propria and to Leydig cells (Arenas et al., 1998). Ultrastructurally MAA was shown to moderately bind to the rough endoplasmic reticulum of Sertoli cells. PNA showed weak affinity for spermatogonia, Sertoli and Leydig cells. Signal intensity of PNA was moderate for peritubular cells, spermatocytes and spermatids and could be increased by desialization. SNA did not bind to any testicular cell type while the signal for seminiferous epithelium and interstitium was strong with WGA (Arenas et al., 1998).

In conclusion, our results showing weak signal intensity of PNA binding to Sertoli cells correspond with the findings described in literature for Wistar rats as well as for men (Arenas et al., 1998; Arya and Vanha-Perttula, 1984). We found moderate affinity of both SNA-I and MAA for Sertoli cells, while Arenas et al. demonstrated also MAA binding to human Sertoli cells, however did not find any cells in the testis of men to be targeted by SNA (Arenas et al., 1998).

While we demonstrated weak PNA binding to peritubular cells, earlier studies performed in the testis of Wistar and Sprague-Dawley rats could not detect PNA binding (Arya and Vanha-Perttula, 1984; Malmi et al., 1990). Notably, there is discordance in the literature regarding PNA binding to human testis. Malmi et al. described peritubular cells as unstained by PNA (Malmi et al., 1987), while Wollina et al. as well as Arenas et al. documented PNA binding to these cells (Arenas et al., 1998; Wollina et al., 1989). We found strong affinity of SNA-I and moderate affinity of MAA to peritubular cells contradicting Arenas et al. finding of absence of SNA binding in human testis (Arenas et al., 1998). Our MAA binding results for peritubular cells are supported by findings for Fischer 344 rats (Wine and Chapin, 1997).

Our demonstration of in part strong PNA binding to testicular macrophages equals Malmi's et al. finding of PNA binding to interstitial cells in the testis of Sprague-Dawley rats (Malmi et al., 1990). Again results for men vary: Malmi et al. did not find an interstitial staining pattern for PNA (Malmi et al., 1987), while Wollina et al. did (Wollina et al., 1989). Our moderate binding of MAA to testicular macrophages is backed by findings for Fischer 344 rats (Wine

and Chapin, 1997). The partly strong affinity of SNA-I for testicular macrophages again contradicts Arenas' et al. finding in human testis (Arenas et al., 1998).

Differences between previous histochemical studies and our flow cytometric results may partially be due to accessing different cellular compartments by different techniques. By flow cytometry we looked at the cellular surface of unfixed and non-permeabilized cells while the histochemical studies examined both plasma membranes and cytoplasm of investigated cells.

4.5. Linking Glycan Structures with Galectin-1 C2S Affinity

In synopsis of our plant lectin and galectin-1 studies (Moos et al., 2009), we conclude that reduced binding of galectin-1 to testicular macrophages in comparison to Sertoli and peritubular cells could be put down to a high degree of terminal α2-6 sialylation in cell surface glycans of testicular macrophages (according to high levels of SNA-I reactivity).

Interestingly, a higher degree of terminal α2-6 sialylation has been shown to selectively protect T_H2 cells from galectin-1 induced apoptosis while T_H1 and T_H-17 cells remain sensitive to galectin-1 because of a lower level of α2-6 linked sialic acid (Toscano et al., 2007).

Since MAL-II, i.e. MAH, binding was demonstrated to be similar for all T cell subpopulations, α2-3 sialylation was regarded as not having an influence on galectin-1-mediated apoptosis. Furthermore, T_H2 cells showed highly reduced binding of PNA and of a core 2 O-glycan-specific anti-CD43 antibody indicating that both core 1 and core 2 O-glycans, i.e. the starting points of galectin-1 target motifs, were reduced – to protect cells from galectin-1 binding (Toscano et al., 2007). In addition galectin-1-deficient mice were more prone to T cell-mediated experimental autoimmune encephalomyelitis (EAE) and developed stronger T_H1 and T_H-17 immune responses (Toscano et al., 2007). Expression of ST6Gal I, which preferentially α2-6 sialylates terminal galactose in N-glycans, accordingly was shown to decrease galectin-1 induced apoptosis in a murine T cell line (Amano et al., 2003).

Yet there is another link between galectin-1 and the modulation of T cell responses: $CD4^+CD25^+$ regulatory T cells (T_{reg}) have been demonstrated to overexpress galectin-1, which might contribute to their immunosuppressive properties due to its pro-apoptotic effect (Garin et al., 2007).

4.6. Modulation of Sertoli and Peritubular Cells in Testicular Immunity by Galectin-1

Somatic cells are regarded as regulators of the testicular immune system and as contributors to the immune privilege of the testis (Fijak and Meinhardt, 2006). In view of our finding that galectin-1 preferentially binds to somatic cells of the testis (Moos et al., 2009), influence of this lectin on the immunology of somatic testicular cells has to be considered.

Sertoli cells bear far-reaching immunosuppressive properties. They prolong allo- and xeno-graft survival upon cotransplantation and secret the proapopototic Fas ligand (Bellgrau et al., 1995; Filippini et al., 2001; Selawry and Cameron, 1993). On the other hand they are capable of secreting the proinflammatory cytokines IL-1α and IL-6 and the chemokine MCP-1 to protect spermatogenesis from bacterial infection (Gerard et al., 1991; Riccioli et al., 1995; Riccioli et al., 2006). With regard to galectin-1, Sertoli cells are an autochthonal source of this lectin in the testis of men and rats (Dettin et al., 2003; Wollina et al., 1999). This has recently been confirmed in cryosections of human testis (Chui et al., 2010).

The presence of endogenous testicular galectin-1 suggests a role in the biology of the testis for this factor. In this respect galectin-1 was shown to induce apoptosis of rat Leydig cells *in vitro* (Martinez et al., 2004). Investigation of murine Leydig tumour cells revealed that high concentrations of galectin-1 cause apoptosis while low concentrations induce proliferation of Leydig tumour cells (Biron et al., 2006). Thus Sertoli cell-derived galectin-1 could be a regulator of Leydig cell viability. Leydig cells in turn downregulate proinflammatory cytokines by androgen secretion. Studies showed reduction of IL-1 and TNF-α secretion of human peripheral blood mononuclear cells and murine macrophages by testosterone *in vitro* (D'Agostino et al., 1999; Li et al., 1993) and *in vivo* inhibition of experimental autoimmune orchitis in rats (Fijak et al., 2011).

Distinct localization of galectin-1 in the compartments of the testis is essential for its biological activity. Human Sertoli cells were shown *in vitro* to bear polarized secretion of galectin-1 favouring high concentrations in the adluminal compartment of the seminiferous tubules (Chui et al., 2010), which corresponds to a linkage of galectin-1 expression in Sertoli cells to the seminiferous epithelial cycle (Dettin et al., 2003; Timmons et al., 2002).

As high concentrations of galectin-1 were also found in the layer of peritubular cells (Chui et al., 2010), galectin-1 is either secreted into the basal compartment of the seminiferous epithelium or synthesized in it. This is of importance as **peritubular cells** not only encapsulate the

seminiferous epithelium, but influence leukocytes in the testicular interstitium and promote experimental autoimmune orchitis by secretion of MCP-1, TGF-β_2 and cyclooxygenase-2 (COX-2) upon stimulation of proteinase-activated receptors-2 by mast cell tryptase (Iosub et al., 2006).

While little is known about the interaction of galectin-1 with stromal cells, growing evidence points at use of galectin-1 by stromal cells for immune modulation. For example mesenchymal stromal cells of human bone marrow use galectin-1 to suppress allogeneic T cells (Sioud, 2011). In the testis galectin-1 accordingly could mitigate and shift T cell activity towards T_H2 responses (Chapter 1.3.5). In a testis-specific way galectin-1 could furthermore increase the levels of the immunosuppressive Fas ligand, activin A, TGF-β_1 and indoleamine 2,3 dioxygenase secreted by Sertoli cells (Meinhardt and Hedger, 2011) – possibly by autocrine signalling – to inhibit adaptive immune responses. Galectin-1 could also influence TGF-β_1 expression in peritubular cells (Skinner and Moses, 1989).

Since adaptive immunity is suppressed in the immunologically privileged testis (Meinhardt and Hedger, 2011), toll-like receptors on Sertoli cells and testicular macrophages are crucial for recognition of infectious agents and thus for innate immunity in the testis (Bhushan et al., 2009).

As revealed by a recent study of LPS-challenged murine macrophage cell lines, galectin-1 is capable of inhibiting toll-like receptor (TLR)-induced activation of NFκB by binding to sialic acid α2-3 linked to β-galactosides in the glycans of TLR (Amith et al., 2010). In this way galectin-1 could modulate pathogen recognition of testicular macrophages, dendritic cells, Sertoli and peritubular cells as well as defensin secretion in the testis (Bhushan et al., 2008; Com et al., 2003). Modulation of TLR signalling by galectin-1 could change MCP-1 secretion and adhesion molecule expression of Sertoli cells (Bhushan et al., 2009; Riccioli et al., 2006) and recruitment of leukocytes in testicular inflammation (Gil et al., 2010).

In summary, by influencing Sertoli and peritubular cells galectin-1 could play a central role in exerting an immune regulatory effect on the testis as both cell types are considered essential for a unique testicular cytokine milieu with impact on innate and adaptive immunity thus supporting the immune privilege of the testis (Figure 37).

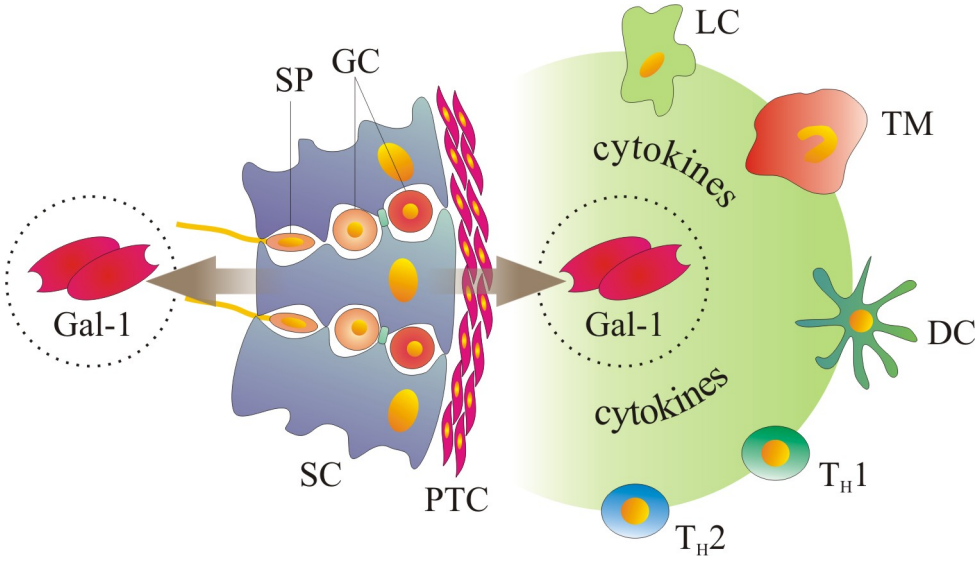


Figure 37: Galectin-1 tunes testicular immunity by interaction with somatic cells - a model. The lectin galectin-1 (*Gal-1*), which shifts T_H1- to T_H2-type immune responses (*T_H1*, *T_H2*), possibly modulates testicular immunity by targeting Sertoli (*SC*) and peritubular cells (*PTC*). Causing an enhanced secretion of immunosuppressive cytokines in Sertoli and peritubular cells, e.g. Fas ligand, activin A or TGF-β_1, could mitigate adaptive immunity involving T cells, dendritic cells (*DC*) and testicular macrophages (*TM*). Influencing Leydig cell (*LC*) viability could affect androgen-mediated immunosuppression and modulation of toll-like receptor signalling could change pathogen recognition of macrophages, dendritic cells, Sertoli and peritubular cells. Additionally Galectin-1 expression of Sertoli cells is also connected to spermatogenesis. *GC*: germ cells. *SP*: spermatids.

4.7. Future Steps in Testis Related Research on Galectin-1

With the demonstration of preferential binding of galectin-1 to non-immune cells in the testis and a lower affinity of galectin-1 to testicular macrophages, further studies are necessary to understand the contribution of this lectin to the immunology of the testis.

In a search for further cells targeted by galectin-1, testicular dendritic and Leydig cells are the next candidates for assessment in our flow cytometric assay of galectin-1 binding. Considering that endogenous expression of galectin-1 was found in T_{reg} cells (Garin et al., 2007) and that galectin-1 can cause apoptosis of Leydig cells *in vitro* (Martinez et al., 2004), dendritic and Leydig cells are both likely candidates of galectin-1 binding. Analysis of testicular glycan structures could be extended by lectin microarrays – a just recently introduced new technique examining adherence of live labelled cells to immobilized lectins resembling an enzyme-linked immunosorbent assay (ELISA) (Tateno et al., 2007).

Thus far, galectin-1 affinity studies have been restricted to normal testis. In a next step they have to be extended towards diseases of the testis, particularly inflammatory testicular infertility. In view of a role in maintaining the privileged immune status of the testis it would be challenging to look for changes in galectin-1 affinity and glycan structures on immunohistochemical and single cell level since failure of the testis to prevent chronic inflammation might be linked to altered galectin-1 binding and signalling.

In this regard testicular cells should be stimulated with proinflammatory stimuli like LPS and co-incubated with galectin-1 to examine a putative inhibition of proinflammatory mediators (such as MCP-1 and COX-2) or an increase of anti-inflammatory mediators (like TGF-β_1) (Iosub, 2005). A shift from T_H1-type (IL-2, IFNγ and TNFα) to T_H2-type cytokines (IL-4, IL-5 and IL-10) would be useful (Mosmann and Coffman, 1989; Romagnani, 1994). Moreover, mRNA levels of ST6Gal I could be assessed in those cells showing strongest and weakest impact of galectin-1 (Toscano et al., 2007). Additionally, an influence of galectin-1 on NO secretion and iNOS expression upon challenge by LPS could be examined for testicular cells to compare the reaction with rat peritoneal macrophages (Correa et al., 2003).

To evaluate the importance of large glycan structures in mediating an influence of galectin-1 on the immune status of the testis, cytokine levels following inflammatory stimuli may be examined when culturing testicular cells in the presence of inhibitors of complex-type N-glycan and complex O-glycan synthesis such as kifunensine or benzylGalNAc (Karmakar et al., 2008).

Besides causing apoptosis of activated T cells and influencing the balance between T_H1 and T_H2 responses, galectin-1 has been shown to cause intracellular Ca^{2+} mobilization and exposure of phosphatidylserine on the external side of the cell membrane in activated neutrophils and promyelocytic HL60 cells without consequent apoptosis. This was termed preaparesis (Stowell et al., 2007; Stowell et al., 2008b). Phosphatidylserine exposure following galectin-1 binding has been hypothesised to be involved in tagging cells for phagocytosis by macrophages and thus to be involved in leukocyte turnover (Stowell et al., 2007).

As a functional assay intracellular calcium concentration in living cells and exposure of phosphatidylserine after incubation with galectin-1 could be determined. Increase of intracellular calcium concentration is detectable by a calcium-dependent intracellular dye (Karmakar et al., 2008) – a technique already successfully applied in our laboratory (Iosub et al., 2006). Exposure of phosphatidylserine can be measured by fluorophore labelled annexin-V (Stowell et al., 2008a; Stowell et al., 2007).

In extension to investigating intracellular mechanisms of galectin-1 signalling, its influence on the development of autoimmune inflammation of the testis is a recommended topic.

Therefore, experimental autoimmune orchitis (EAO) can be induced in galectin-1 deficient and wild-type animals. Notably, galectin-1 knockout mice are available and have been shown to suffer from increased severity of experimental autoimmune encephalomyelitis (Toscano et al., 2007).

Considering the short half-life of wild-type human galectin-1 in non-reducing environments (Hirabayashi and Kasai, 1991) and the consequent need for continuous re-synthesis or re-application, the question arises how galectin-1 can be used as a therapeutic agent in humans. Two recent studies have addressed this question and shown two ways of successfully ameliorating chronic autoimmune diseases using recombinant human galectin-1 *in vivo*:

In a study using non-obese diabetic (NOD) mice as a model of type 1 diabetes mellitus, bone marrow-derived dendritic cells transfected to express transgenic galectin-1 were injected intravenously, a treatment that successfully managed to delay the onset of hyperglycaemia (after 60±10 days instead of 29±4 days) (Perone et al., 2006).

Repeated intraperitoneal injection of recombinant human galectin-1 proved to be even more effective. Starting this treatment in preclinical stages prevented the onset of hyperglycaemia. Furthermore, autoimmune inflammation and hyperglycaemia were reversed by intraperitoneal injection of galectin-1 after clinical manifestation of the disease. None of the animals became diabetic during the 37-week follow-up (Perone et al., 2009). In view of this sustained effect and the ineffectiveness of galectin-1 expressing dendritic cells to maintain normoglycaemia, the authors assumed that such dendritic cells transfected to express galectin-1 might bear limited migration and increased immunogenicity due to maturation caused by galectin-1 (Perone et al., 2009).

An alternative approach was applied earlier. Administration of recombinant galectin-1 in early or late stages of the T_H1-dominated experimental autoimmune uveitis (EAU) ameliorated the ocular pathology and shifted the immune reaction towards a T_H2-type response (Toscano et al., 2006). Notably, transfer of $CD4^+$ T cells including $CD4^+CD25^+Foxp3^+$ T_{reg} cells from galectin-1 treated animals suffering from EAU prevented pathogenesis in the host animals (Toscano et al., 2007).

In conclusion, the latter two approaches are capable of ameliorating T_H1-type autoimmune inflammations. They may provide novel treatments of autoimmune orchitis, which significantly attributes to what is clinically often diagnosed as idiopathic infertility of men, as studies of experimental autoimmune orchitis in rodents have shown that testicular autoimmune inflammation is mediated by T_H1 cells (Tung and Teuscher, 1995; Yule and Tung, 1993).

With the identification of testicular cells targeted by galectin-1 a step towards future therapeutic application of galectin-1 for the treatment of testicular autoimmune inflammation has been achieved (Moos et al., 2009).

5. Summary

Glycans linked to cell surface proteins encode a multitude of biological information decoded by specialized proteins termed lectins. Understanding this glycan based language and downstream signalling is of emerging significance due to research of the past two decades pointing at a large impact on regulating immune responses and maintenance of self-tolerance.

Galectin-1 is a 14.5 kDa lectin conserved across species, which binds galactose β1-4 N-acetylglucosamine containing oligosaccharides on the surface of immune and stromal cells. Its functional capability ranges from induction of T cell apoptosis, a shift of immune reactions from T_H1 to T_H2 and expansion of regulatory T cells (T_{regs}) to modulation of monocyte and macrophage functions. With its anti-inflammatory properties revealed in animal models of autoimmunity and chronic inflammation such as experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune uveitis (EAU), galectin-1 has emerged as a factor of clinical significance.

In view of the immune privileged status of the testis, we hypothesised that galectin-1 could play a role in maintaining the immunosuppressive phenotype of this organ. As a first step, human galectin-1 C2S was recombinantly expressed, purified, labelled with Alexa Fluor 647 and its binding was investigated by immunohistochemistry using testicular cryosections. In a second step, isolated testicular macrophages, Sertoli and peritubular cells as important cells of testicular innate immunity were used to test their binding capacity of galectin-1 by flow cytometry.

It was shown that all investigated testicular cell types are able to bind galectin-1, albeit with different intensity. The fluorescence signal of Alexa Fluor 647 conjugated galectin-1 C2S binding to peritubular and Sertoli cells is comparable to that of phytohaemagglutinin (PHA) stimulated mononuclear cells as positive control. In contrast galectin-1 binding to testicular macrophages is much weaker with an intensity lower than unstimulated mononuclear cells. The specificity of binding was verified by co-incubation with lactose or sucrose, respectively.

In order to examine the phenotype of cell surface glycans, which mediate the affinity of galectin-1 for the respective cells, we applied a new set of plant lectins (MAA, LEA, PNA, SNA-I) in the analysis of isolated testicular cells by flow cytometry.

It was found that reduced binding of galectin-1 to testicular macrophages in comparison to Sertoli and peritubular cells could result from a high degree of terminal α2-6 sialylation in cell

surface glycans of testicular macrophages – a regulatory mechanism known to modulate susceptibility of T_H1 and T_H2 cells to galectin-1 induced apoptosis.

In summation, it is concluded from these studies that peritubular cells and Sertoli cells are the primary target cells for galectin-1 amongst the investigated cell types in the testis, but surprisingly not testicular macrophages. It seems likely that galectin-1 contributes to the testicular immune privilege mainly by interaction with testicular somatic cells.

6. Zusammenfassung

Mit Zelloberflächenproteinen verbundene Glykane enthalten eine Vielzahl biologischer Informationen, die durch spezifische Bindepartner, die Lektine, gebunden und erkannt werden. Das Verständnis dieser auf Glykanen basierenden Sprache und ihrer nachfolgenden Signalwege hat in den letzten zwanzig Jahren zunehmende Bedeutung erlangt und schließt Prozesse wie die Regulation der Immunantworten und Aufrechterhaltung der Selbsttoleranz mit ein.

Bei Galektin-1 handelt es sich um ein 14,5 kDa großes, über Artengrenzen hinweg konserviertes Lektin, welches Galactose-β-(1→4)-N-Acetylglucosamin enthaltende Oligosaccharide auf der Oberfläche von Immun- und Stromazellen bindet. Seine Fähigkeiten reichen von der Induktion der Apoptose in T-Zellen, der Verschiebung von T_H1- zu T_H2-Immunantworten über die Vermehrung regulatorischer T-Zellen bis hin zur Modulation der Funktion von Monozyten und Makrophagen. Galektin-1 ist dabei von klinischer Bedeutung, da seine antientzündlichen Eigenschaften in Tiermodellen autoimmunologischer Erkrankungen und chronischer Entzündungen – wie z. B. in experimenteller Autoimmunenzephalomyelitis und experimenteller Autoimmunuveitis – nachgewiesen wurden.

Mit Blick auf die immunologisch privilegierte Stellung des Hodens vermuteten wir, dass Galektin-1 eine Rolle bei der Aufrechterhaltung des immunsuppressiven Phänotyps dieses Organs spielen könnte. In einem ersten Schritt wurde daher humanes Galektin-1 C2S rekombinant exprimiert, welches aufgereinigt und mit Alexa Fluor 647 markiert wurde. Anschließend untersuchten wir seine Bindung an Kryostatschnitte des Hodens in der Immunhistochemie. In einem zweiten Schritt wurden isolierte testikuläre Makrophagen, Sertoli- und Peritubulärzellen als wichtige Zellen der angeborenen Immunantwort des Hodens mittels Durchflusszytometrie auf Ihre Fähigkeit untersucht Galektin-1 zu binden.

Es konnte gezeigt werden, dass alle untersuchten testikulären Zelltypen Galektin-1 binden können, jedoch mit unterschiedlicher Intensität. Das Fluoreszenzsignal der Bindung von Alexa Fluor 647 konjugiertem Galektin-1 C2S an Peritubulär- und Sertoli-Zellen ist vergleichbar mit dem mononukleärer Zellen, die mittels Phytohämagglutinin stimuliert und als Positivkontrolle verwendet wurden. Im Gegensatz dazu zeigen testikuläre Makrophagen eine viel schwächere Galektin-1 Bindung, die geringer als die unstimulierter mononukleärer Zellen ausfällt. Die Spezifität der Bindung wurde durch die gleichzeitige Inkubation mit Lactose bzw. Saccharose nachgewiesen.

Um den Phänotyp der auf den Zelloberflächen befindlichen Glykane zu untersuchen, welche die Affinität von Galektin-1 für die jeweiligen Zellen vermitteln, verwendeten wir Pflanzenlektine (MAA, LEA, PNA, SNA-I) in der Analyse isolierter testikulärer Zellen mittels Durchflusszytometrie.

Aus den Ergebnissen der Pflanzenlektin- und Galektin-1-Bindung konnte gefolgert werden, dass die im Vergleich zu Sertoli- und Peritubulärzellen verminderte Bindung von Galektin-1 an testikuläre Makrophagen auf einem hohen Maß an endständiger, α2-6 konjugierter Neuraminsäure an den Glykanen der Zelloberfläche beruhen könnte. Von diesem regulatorischen Mechanismus ist bekannt, dass er die unterschiedliche Empfindlichkeit von T_H1- und T_H2-Zellen bei induzierter Apoptose durch Galektin-1 bedingt.

Zusammenfassend kann gesagt werden, dass peritubuläre und Sertoli-Zellen, aber überraschenderweise nicht testikuläre Makrophagen, die vorrangigen Zielzellen unter den untersuchten Zellen im Hoden für Galektin-1 darstellen. Damit kann Galektin-1 über eine Interaktion mit den somatischen testikulären Zellen zur immunologisch privilegierten Stellung des Hodens im Organismus beitragen.

7. References

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8. List of Own Publication

Conference Poster:

- Moos, S., Fijak, M., Klug, J., Bhushan, S., Hirabayashi, J., Rabinovich, G.A., Fröhlich, S., Schneider, E., Meinhardt, A. (2009). Analysis of Galectin-1 Binding to Isolated Tubular and Interstitial Testicular Cells in Rat. 5th International Workshop Molecular Andrology, Gießen, Germany, May 8-10.

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10. Ehrenwörtliche Erklärung

Ich erkläre:

Ich habe die vorgelegte Dissertation selbständig, 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.

Gießen, den 6. Juni 2011

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Sven Moos