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ANTIMICROBIAL AND XENOBIOTIC DEFENCE IN THE MAMMALIAN TESTIS

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Stockholm 2012

Published by Karolinska Institutet. Printed by Universitetsservice US-AB

Cover photo: Sertoli cells, germ cells and the blood– testis barrier by Anna-Karin Sundström

ISBN 978-91-7457-762-4

ABSTRACT

Different mechanisms exist in the mammalian testis to protect the germ cells from autoimmune attack, invading microbes, and xenobiotic induced toxicity, which would otherwise be deleterious to the preservation of fertility. The general aim of this thesis was to study these testicular defence mechanisms, including the structural building blocks of the blood– testis barrier (BTB), antimicrobial peptides, proinflammatory cytokines and ATP-binding cassette (ABC) transporter proteins.

Coxsackie and Adenovirus Receptor (CAR) is a cell adhesion molecule present in tight junctions of epithelial cells. We found that CAR was expressed in male germ cells in the human, the rat and the mouse testis, with a localization corresponding to the acrosomal structure of the elongated spermatids as well as in mature spermatozoa. CAR co-localized and co-immunoprecipitated with JAM-C in the mouse testis, suggesting that CAR may function in germ cell differentiation and polarization, similar to JAM-C.

Interleukin-18 (IL-18) is a proinflammtory cytokine that contributes to host defence in epithelial barrier tissues. We found that IL-18, the IL-18 receptor and the interleukin-1β converting enzyme (ICE) was expressed in the rat testis throughout postnatal development. IL-18 protein and messenger ribonucleic acid (mRNA) were mainly localized in meiotic and post-meiotic germ cells. Only Pro-IL-18 was detected in interstitial fluid and in testicular cell extracts, suggesting that IL-18 normally is in its non-active form in the healthy testis, but may become activated during testicular infection. Recombinant IL-18 stimulated spermatogonial proliferation and thus may act as a growth factor during patho-physiological conditions in the testis.

The nuclear protein High mobility group box chromosomal protein-1 (HMGB1) does also have extracellular immune-functions, including cytokine stimulation and direct antimicrobial activity. We found that HMGB1 was expressed in the nucleus and in the cytoplasm of Sertoli cells in the human and rat testis. HMGB1 protein was also present in interstitial fluid collected from non-treated rat testis, indicating extracellular release. HMGB1 purified from human and rat testis demonstrated strong and direct antibacterial activity and thus may act as an antimicrobial peptide in the seminiferous epithelium, contributing to the protection of the developing germ cells against invading pathogens.

The ABC transporter family is responsible for active transport of substrates across membranes. They are located in barrier tissues such as the BTB and the blood– brain barrier (BBB) and protect against uptake of xenobiotics. We found that testes from immature (6-day-old) rats accumulated significantly higher levels of the ABC transporter substrate and anticancer drug doxorubicin, compared to the testes from 16- and 24-day old rats. This correlated with a significantly lower level of testicular expression of ABC transporters compared to 16- and 24-day-old rats. Thus, lack of a mature ABC transporter efflux system may render the immature testis more susceptible to xenobiotic-induced toxicity.

The studies described here support the view that the mammalian testis has evolved several defence mechanisms to preserve fertility.

LIST OF PUBLICATIONS

I. Mirza M, Hreinsson J, Strand ML, Hovatta O, Söder O, Philipson L, Pettersson RF, Sollerbrant K. Coxsackievirus and Adenovirus receptor (CAR) is expressed in male germ cells and forms a complex with the differentiation factor JAM-C in mouse testis. Experimental Cell Research.2006, 312: 817-30.

II. Strand ML, Wahlgren A, Svechnikov K, Zetterström C, Setchell BP, Söder O. Interleukin 18 is expressed in rat testis and may promote germ cell growth. Molecular and Cellular Endocrinology. 2005, 240: 64-73.

III. Zetterström C, Strand ML, Söder O. The high mobility group box chromosomal protein 1 (HMGB1) is expressed in the human and rat testis where it may function as an antibacterial factor. Hum Reprod. 2006 Nov;21(11):2801-9.

IV. Strand ML, Jahnukainen K, Sultana T, Hou M, Eksborg S, Ritzmo C, Melaine M, Jégou B and Söder O. The age dependent testicular expression pattern of ABC transporters renders the immature testis more susceptible to adverse actions of cytotoxic drugs Manuscript.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ATP	Adenosine-tri-phosphate
BBB	Blood– brain barrier
Bcrp	Breast cancer resistance protein
BTB	Blood– testis barrier
CAR	Coxsackievirus and adenovirus receptor
cDNA	Complementary deoxyribonucleic acid
CTX	Cortical thymocyte xenopus
DAB	3,3-Diaminobenzidine
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ES	Ectoplasmic specializations
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
GSH	Glutathione
GST	Glutathione *S*-transferase
hCG	Human chorionic gonadotropin
HIV	Human immunodeficiency virus
HMGB1	High mobility group box chromosomal protein-1
HPLC	High performance liquid chromatography
ICE	Interleukin-1β converting enzyme
IF	Immunofluorescence
IFNγ	Interferon gamma
IHC	Immunohistochemistry
IκB	inhibitor of kappa B
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-1RI	Interleukin-1 receptor type I
IL-1RII	Interleukin-1 receptor type II
IL-1ra	Interleukin-1 receptor antagonist
IL-1RAcP	Interleukin-1 receptor accessory protein
IL-18	Interleukin-18
IL-18BP	Interleukin-18 binding protein
IL-18R	Interleukin-18 receptor
IL-18RAcP	Interleukin-18 receptor accessory protein
IRAK	Interleukin-1 receptor associated kinase
JAM	Junctional adhesion molecule
LH	Luteinizing hormone
LNX	Ligand of numb protein X
LNX2	Ligand of numb protein X2
LPS	Lipopolysacharide
MDR	Multidrug resistance
Mdr1	Multidrug resistance gene 1
MIF	Macrophage migration inhibitory factor

MIS	Müllerian inhibiting substance
mRNA	Messenger ribonucleic acid
Mrp1	Multidrug resistance protein 1
MyD88	Myeloid differentiation primary response gene (88)
NALP-1	Nacht -LRR-PYD containing protein-1
NFκB	Nuclear factor kappa B
NLR	Nod like receptor
PAMP	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
PNA	Peanut agglutinin
RIA	Radioimmuno assay
RT-PCR	Reverse transcriptase PCR
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBC	Tubulobulbar complex
TGFβ	Transforming growth factor beta
TIR	Toll/interleukin-1 receptor homology domain
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
Traf 6	TNF receptor associated factor 6
ZO-1	Zonula Occludens 1

1 INTRODUCTION

1.1 TESTICULAR DEFENCE MECHANISMS

Protection of developing germ cells against noxious influences has high priority in all species to preserve fertility. Post meiotic germ cells appear long after the maturation of the immune system and the establishment of self-tolerance. Thus, they express antigens that are regarded as non-self by the immune system. To prevent an autoimmune attack, the germ cells therefore need to be protected from the immune system. At the same time, the testis faces the challenge to prevent invasion of pathogenic microbes, which also would be deleterious to the developing germ cells. Moreover, the testis is (already during fetal development) exposed to a constantly growing mixture of environmental pollutants, drugs and endocrine disruptors and therefore dependent on barrier strategies that protects against xenobiotic induced toxicity. This thesis describes different mechanisms that exist in the mammalian testis to protect the germ cells from autoimmune attack, invading microbes and xenobiotic induced toxicity. In brief, these strategies encompass epithelial cell barriers, secretion of immune-regulatory and immune-stimulatory cytokines and antimicrobial peptides as well as the testicular expression of ABC transporter proteins. Other protective mechanisms also exist, however a thorough investigation of these was beyond the scope of this thesis.

1.2 CLINICAL IMPLICATIONS

1.2.1 Autoimmune infertility

Local and systemic infection and inflammation leading to disruption of the controlled immune balance in the testis may impair both steroid synthesis and sperm production. This can lead to temporary or permanent infertility. Such autoimmune infertility accounts for 5-10 % of male infertility cases in developed countries, but can be even higher in countries were investigations of reproductive health is absent (Meinhardt and Hedger, 2011). Orchitis (testicular inflammation) is mainly seen as a complication of systemic infection of paramyxovirus (mumps orchitis) or human immunodeficiency virus (HIV). Nevertheless, asymptomatic inflammatory reactions in the testis may also be an important contributor to or an underlying cause of male infertility (Schuppe et al., 2008).

1.2.2 Infertility- a late side effect of childhood cancer therapy

Almost every day, one child in Sweden is diagnosed with cancer (approximately 300 cases per year, according to the Swedish Children´s Cancer Foundation). Worldwide, the incidence is 110-130 cases per one million people per year (Bleyer, 1990). Previously, most children died from their cancer, but today around 75% of all children diagnosed with cancer will become long-term survivors (Bleyer, 1990). The positive development has led to the increased awareness of late side effects of childhood cancer therapy and the pediatric oncologists today face the challenge to keep the high survival rates, while minimizing the risks for late sequele. One of the most common late side effects is infertility.

The developing testis is sensitive to both chemotherapy and irradiation. However, the risk of developing infertility due to childhood cancer therapy depends largely on the type of treatment and the doses that have been given (Jahnukainen and Soder, 2003). Chemotherapy treatment usually involves a combination of drugs that may have synergistic activity. Thus, it can be difficult to elucidate the specific contributions of each individual agent to the overall toxicity.

Furthermore, it is difficult to estimate or predict testicular side effects from pre-pubertal chemotherapy treatment due to a lack of proper methods to detect early signs of cytotoxic damage to the immature testis. It is not until puberty arrives and shows absence of testicular growth and sperm production and elevated levels of gonadotropins that the toxicity is noticed (Wallace et al., 1991, Muller et al., 1988). Moreover, the lack of studies investigating the maturational development of xenobiotic defence mechanisms, such as the testicular expression of ABC transporter proteins in pre-pubertal boys may contribute to the above problems.

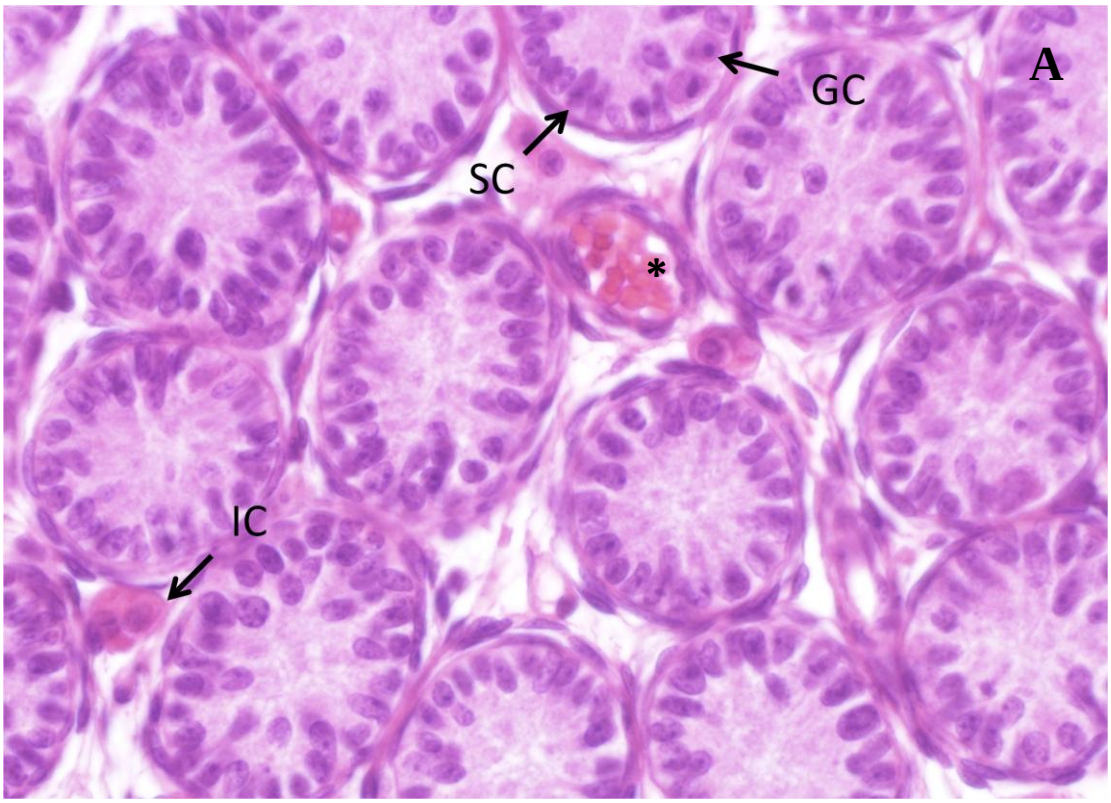
In contrast to the rodent testis, there are no studies in humans demonstrating that treatment with cytotoxic drugs during the prepubertal period would result in more severe gonadal damage than treatment after puberty. In fact, the gonadal damage caused by doxorubicin as well as several other drugs appears to be less pronounced in humans than in rodents.

1.3 THE POSTNATAL TESTIS

A proper development of the testis is thought to be required for the determination of testicular size and for creating the germ line stem cell pool. Damage to the developing testis may thus have serious consequences for the function of the adult testis. Normal development of the postnatal testis involves both endocrine communication between the testis and the hypothalamic-pituitary axis as well as paracrine communication between the testicular cells. In the neonatal testis, the germ cells (gonocytes) are located in the center of the seminiferous cords, while the Sertoli cells and peritubular myoid cells are located at the basement membrane (Figure 1A). The interstitial tissue of the newborn testis contains fetal Leydig cells, lymphocytes, macrophages, blood and lymph vessels and nerves.

During the neonatal period in the rat (postnatal day 0-6), the gonocytes, which have been in mitotic arrest, resume their mitotic activity. They also begin to migrate from the center of the seminiferous cords to the basement membrane, forming the stem cell pool (McGuinness and Orth, 1992). Here they differentiate further into type A spermatogonia. The proliferation of type A spermatogonia indicates the beginning of spermatogenesis. Thus in rats the neonatal period overlaps with the onset of puberty, but in humans there is a long delay between birth and the start of puberty.

Sertoli cells divide rapidly after birth but cease their proliferation around postnatal day 15-18 in the rat and around 11-14 years in humans, when they also start to develop tight junctions, the structural components of the BTB (Vitale et al., 1973, Steinberger and Steinberger, 1971). Follicle stimulating hormone (FSH) is an important stimulator of



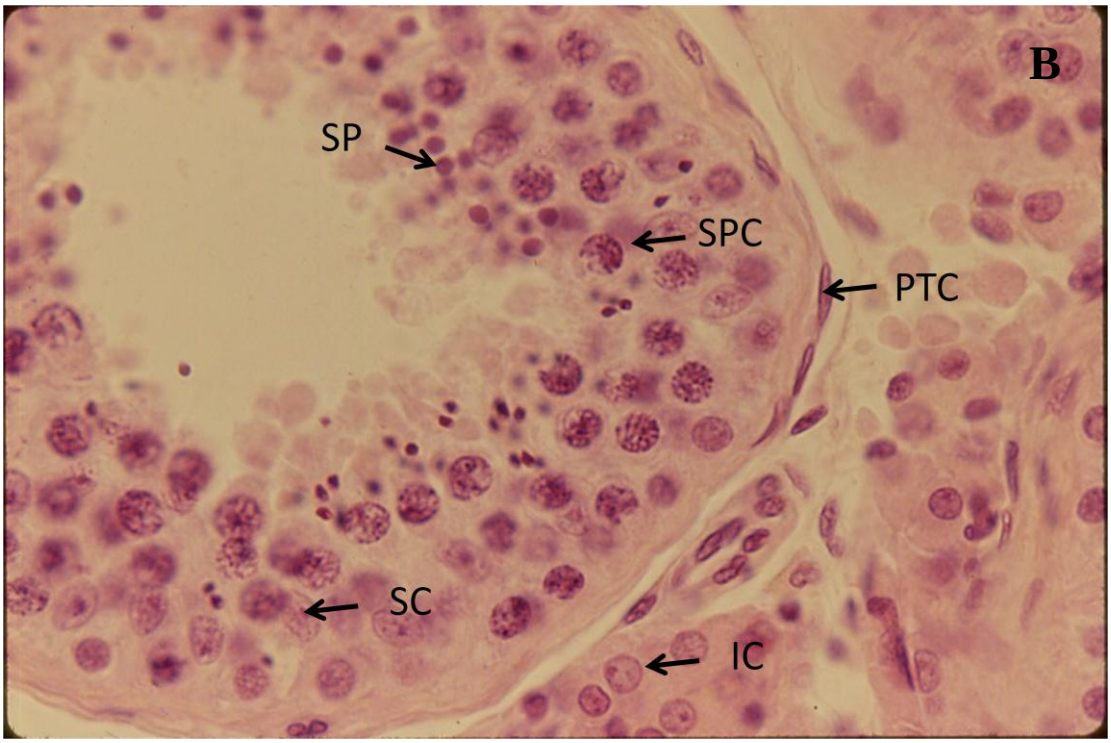


Figure1. Cross section of an immature, 6-day-old rat testis (A) and an adult human testis (B). SC= Sertoli cell, GC= gonocyte, IC= interstitial cell, PTC= peritubular myoid cell, SPC= spermatocyte, SP= spermatid, * = blood vessel. By David King, with permission.

Sertoli cell proliferation, but stimulation by locally produced cytokines and growth factors may also be involved in the process (Griswold et al., 1977, Petersen et al., 2002, Petersen et al., 2000, Petersen et al., 2004). No further proliferation occurs after this active period and the Sertoli cell number is considered to be constant. The number of the Sertoli cells is important because they determine the amount of developing germ cells that can be supported, and therefore also testicular size. As puberty progresses, the Sertoli cells elongate and stretch from the basal to the lumen of the seminiferous tubules, while supporting the developing germ cells located in crypts between adjacent Sertoli cells.

Peritubular myoid cells surround the seminiferous tubules and function as structural support, as well as an aid in the transport of released spermatozoa. In the rat, they are contractile from postnatal day 15 and it is these contractions that facilitate the movement of tubular fluid and the transport of spermatozoa in the seminiferous tubules (Kormano and Hovatta, 1972). The peritubular myoid cells also participate in paracrine regulation of the seminiferous epithelium and cooperate with the Sertoli cells (Skinner et al., 1985, Tung et al., 1984).

Prenatally, the fetal Leydig cells secrete testosterone and other androgens needed for development of internal and external genitalia. After birth, the fetal Leydig cells are still present in both the human and rat testis. In the rat, progenitors of the adult type Leydig cells start to differentiate and replace the fetal Leydig cells during the second week of postnatal life (Lording and De Kretser, 1972). The progenitors differentiate into immature Leydig cells by day 28 in the rat. These cells express high levels of testosterone metabolizing enzymes, resulting in relatively low testosterone production (Ge and Hardy, 1998). By day 56 in the rat, the immature Leydig cells have differentiated to adult Leydig cell, which have increased in number and size, as well in their capacity to produce testosterone (Bortolussi et al., 1990).

Leydig cells and testicular macrophages live in close relation to each other. In addition to their immune functions, the testicular macrophages are also involved in paracrine regulation of normal testicular functions and have been shown to influence the secretion of testosterone by the Leydig cells (Yee and Hutson, 1985). Moreover, testicular macrophages decrease in size when Leydig cell function is impaired, suggesting a dual dependence on each other (Bergh, 1985). Testicular macrophages are initially found in the fetal testis around gestational day 19 in the mouse. They then increase in concentration in the interstitial compartment of the testis between postnatal days 13-20 and increase in size between postnatal days 20-47 (Hutson, 1990).

1.4 THE ADULT TESTIS

The adult testis has two major functions, i.e. the production of testosterone and the production of germ cells. The testosterone synthesis (steroidogenesis) takes place in the Leydig cells located in the interstitial compartment of the testis. The differentiation of the germ cells (spermatogenesis) takes place in the seminiferous tubules (Figure 1B).

The germ cells are located in "pockets" between adjacent Sertoli cells and are transported from the basal to the adluminal compartment as they differentiate. The Sertoli cells provide nutrients and physical support to the differentiating germ cells and also regulate the spermatogenic process by secreting factors that are important for germ cell maturation. Spermatogenesis can be divided in four phases including mitosis (self renewal of spermatogonia), meiosis (formation of spermatids), spermiogenesis (differentiation of spermatids to spermatozoa) and spermiation (release of the spermatozoa). Mitosis and the decision to enter meiosis are largely controlled by paracrine factors secreted from the somatic cells of the testis, while successful completion of meiosis is androgen-dependent.

The duration of spermatogenesis in rats and in humans is 52 and 74 days, respectively (Clermont and Harvey, 1965, Heller and Clermont, 1964, Amann, 2008). The germ cells together with the Sertoli cells are arranged in special cellular associations, known as stages. These cellular associations develop cyclically in time. There are 14 stages in the rat (Leblond and Clermont, 1952), while in humans there are only six (Clermont, 1966).

1.5 ENDOCRINE REGULATION OF THE TESTIS

The testis is regulated by pituitary hormones, FSH and luteinizing hormone (LH). FSH acts on G-protein coupled receptors located on the Sertoli cells, to stimulate Sertoli cell proliferation during the perinatal period (Griswold, 1998, Griswold et al., 1977). In the adult testis, FSH has tropic functions including, among others, the regulation of secretion of transferrin, androgen binding protein, Müllerian inhibiting substance (MIS) and inhibin B. Inhibin B in turn regulates FSH via negative feedback (Jegou, 1992, Griswold, 1998).

LH acts on G-protein coupled receptors located on the Leydig cells to stimulate their testosterone production. Testosterone then binds to the nuclear androgen receptor on Sertoli-, Leydig- and peritubular myoid cells exerting paracrine and autocrine effects. Germ cells lack androgen receptor expression and therefore testosterone regulates spermatogenesis via the Sertoli cells. Testosterone also has endocrine effects and regulates the production of LH through negative feedback (Figure 2).

1.6 THE BLOOD-TESTIS BARRIER

Spermatogenesis is a vulnerable process and protection of germ cells against noxious influences is thus needed, as mentioned in chapter 1.1. Part of this protection is mediated by the BTB that physically separates the meiotic germ cells from the blood circulation. The BTB is created by the Sertoli cells (Dym and Fawcett 1970, Setchell 1980) and it divides the seminiferous epithelium into the basal and adluminal compartments, respectively. However, the peritubular and endothelial cells also contribute to the function of the barrier (Ploen and Setchell, 1992, Holash et al., 1993).

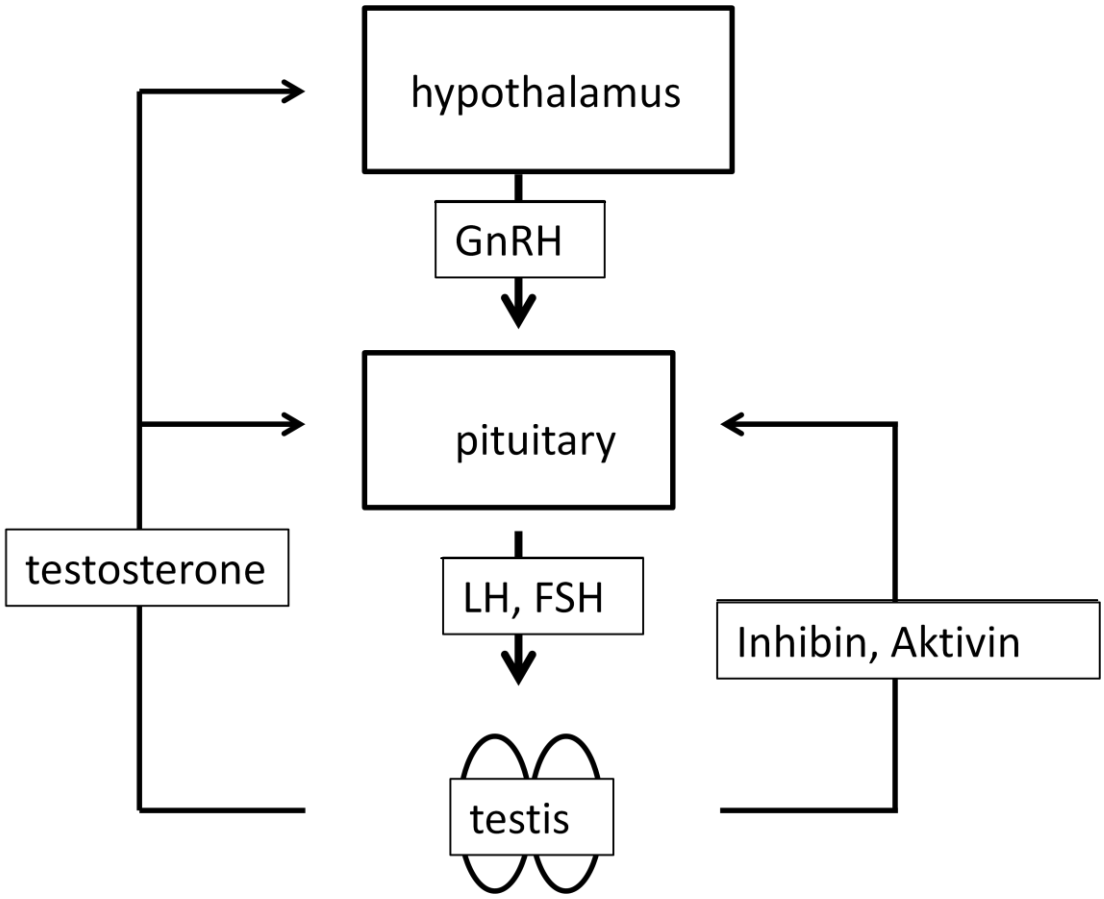


Figure 2. Endocrine regulation of the testis.

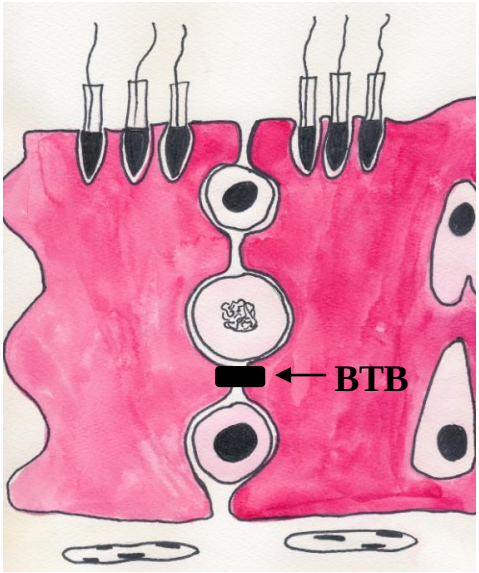


Figure 3. The BTB consists mainly of tight junctions between adjacent Sertoli cells. The meiotic and post meiotic germ cells are located above the BTB and are protected from the blood circulation. Illustration by Anna-Karin Sundström.

The BTB segregates the post meiotic germ cells from the immune system, creates polarity and confers a unique environment for germ cell differentiation (Figure 3). It starts to form at postnatal day 15 to 18 in the rat and it is one of the tightest barriers in the body (Cheng et al., 2010). Everything that enters the adluminal compartment has to pass via the Sertoli cell. On the other hand, the BTB must also be able to dismantle during spermatogenesis to allow the passage of preleptotene and leptotene spermatocytes from the basal compartment to the adluminal compartment, where the meiosis can be completed. This junctional disassembly and reassembly occurs at stage VIII in the rat and is tightly regulated by cytokines and testosterone (Xia et al., 2005, Cheng et al., 2010, Li et al., 2009).

1.6.1 Testicular junctions

Cell adhesion is mediated by sub-cellular structures that can be divided into three major groups: anchoring-, gap- and tight junctions. The BTB consists mainly of tight junctions and the testis-specific type of anchoring junction known as the basal ectoplasmic specialization (ES), located between adjacent Sertoli cells (Wong and Cheng, 2005, Mruk and Cheng, 2004, Li et al., 2009). Besides tight junctions and basal ES, desmosome-like anchoring junctions and gap junctions are also integral components of the BTB. In contrast to other barriers, the tight junctions at the BTB are located on the basal and not the apical side of the epithelium. Different types of junctions between the Sertoli cells and germ cells also exist, depending on the maturational status of the germ cells. Desmosome-like junctions are formed between primary spermatocytes or round spermatids and Sertoli cells. In contrast, when the step 8 spermatids start to elongate, the anchoring junctions are replaced by apical ES (Mruk and Cheng, 2004, Li et al., 2009). Basal and apical tubulobulbar complexes (TBC) are other junctional types specific to the testis (Mruk and Cheng, 2004, Li et al., 2009).

1.6.2 Junctional proteins

At the molecular level, the BTB is composed of integral transmembrane proteins, peripheral adaptor proteins, associated signaling molecules and cytoskeletal proteins (Wong and Cheng, 2005). The integral membrane proteins known to be present in the tight junctions in the testis include junctional adhesion molecules (JAMs), claudins and occludins (Cheng and Mruk, 2002, Xia et al., 2005). These junctional transmembrane proteins are linked to actin, via adaptor proteins zonula occludens (ZO) 1–3 (Cheng and Mruk, 2002). Modulation of these structural proteins, such as their degradation, internalization, dissociations-associations and recycling are the events that are involved in junctional restructuring and movement of the preleptotene spermatocytes across the BTB, as well as the release of step 19 spermatids into the lumen, which all takes place in spermatogenic stage VIII in the rat. Knockout studies of integral transmembrane proteins including nectin-2, nectin-3 and JAM-C have resulted in mice with impaired spermatogenesis, illustrating important functions of these proteins in germ cell differentiation (Mueller et al., 2003, Inagaki et al., 2006, Gliki et al., 2004).

1.6.3 Coxsackie and adenovirus receptor

CAR belongs to the same transmembrane immunoglobulin super family as the JAMs and necdins (Bergelson et al., 1997). It consists of a pair of immunoglobulin-like

domains in its extracellular region, a single transmembrane domain and a cytoplasmic tail (Bergelson et al., 1997). The cytoplasmic tail is highly conserved, suggesting an important role in the function of CAR. The C-terminal part of the cytoplasmic tail contains a (type 1) PDZ recognition motif, which is involved in the binding of CAR to several PDZ domain containing proteins (Cohen et al., 2001, Coyne et al., 2004, Excoffon et al., 2004). Due two alternative splicing, there are two isoforms of CAR, harboring distinct PDZ binding motifs (Philipson and Pettersson, 2004).

CAR is a structural component of tight- and adherence junctions and is an adhesion molecule that is engaged in homotypic interactions with CAR molecules on neighboring cells (Figure 4) (Cohen et al., 2001, Honda et al., 2000). It also mediates heterophilic interactions with JAM-L, which is important for the migration of neutrophils across epithelial tight junctions (Zen et al., 2005).

CAR is expressed in different tissues during embryonic development including CNS, liver, lung, heart, testis and skeletal muscle, among others (Nalbantoglu et al., 1999, Mirza et al., 2005, Tomko et al., 2000). In many tissues, the high level of CAR expression during embryonic development indicates that CAR may be involved in tissue formation. Indeed, CAR was shown to be required for heart development since CAR knockout mice die *in utero*, due to heart failure (Asher et al., 2005, Chen et al., 2006, Dorner et al., 2005).

In humans, CAR mRNA is highly expressed in the adult heart, brain and pancreas. Significant CAR expression was also found in the testis and in the prostate (Tomko et al., 1997, Bergelson et al., 1998). Another study reported CAR expression in germ cells and Sertoli cells in the rat testis, with a localization corresponding to the Sertoli-Sertoli and Sertoli-germ cell interface (Wang et al., 2007). Recently, we found that CAR was expressed in the acrosomal region of the mouse, rat and human spermatozoa. This will be discussed in detail in section 4.1.

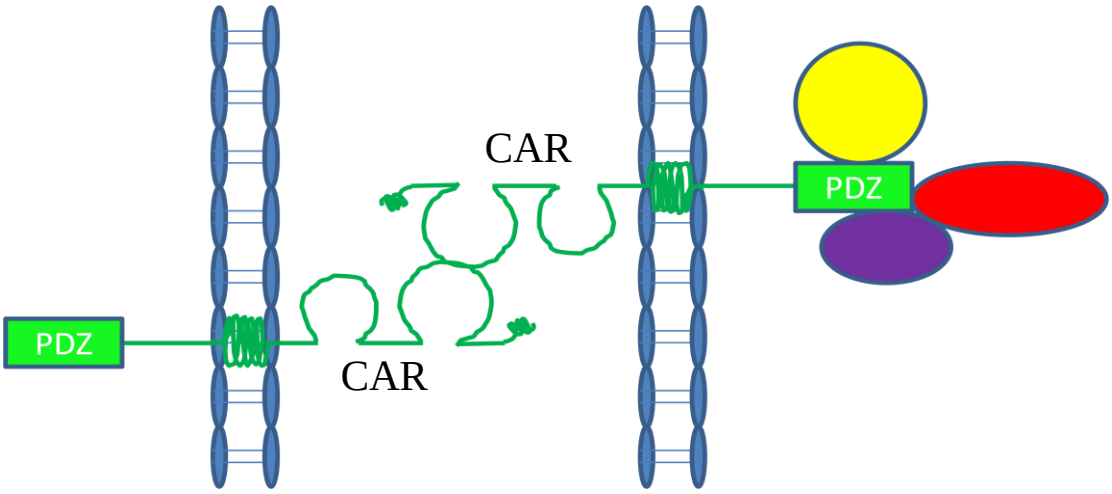


Figure 4. CAR is a structural component of tight junctions and adherence junctions and is engaged in homotypic interactions with CAR molecules on neighboring cells.

1.7 PARACRINE REGULATION OF THE TESTICULAR IMMUNE SYSTEM

The BTB itself does not count for all the protection from autoimmune attack on the germ cells. Instead, the whole testis can be considered as an immune-privileged site. This was initially shown by prolonged survival of allo- and xenografts that had been transplanted into the interstitium of the rat testis and survival of ectopically transplanted Sertoli cells, co-transplanted with allogenic pancreatic tissue (Fijak and Meinhardt, 2006). The immune privilege in the testis depends on multiple mechanisms including normal strategies for tolerance control, reduced immune activation, local immunosuppression and antigen specific immune-regulation (Meinhardt and Hedger, 2011). An important contributor to the immune privilege is the testicular secretion of hormones, peptides and cytokines, including androgens, the immunosuppressive transforming growth factor beta (TGFβ) family and cytokines with complex immunoregulatory/immunostimulatory functions such as members of the interleukin-1 (IL-1), macrophage migration inhibitory factor (MIF) and others (Schuppe and Meinhardt, 2005, Hedger and Meinhardt, 2003, Fijak and Meinhardt, 2006, Meinhardt and Hedger, 2011).

However, it is important to understand that testicular immune privilege does not mean that the testis is incapable of mounting an efficient immune response when needed. Instead, infections of the testis appear to be less common than infections in the epididymis (Krieger, 1984). Since the adaptive immune response in the testis is suppressed, there is a need for greater reliance on the fast, evolutionally old innate immune response. This involves rapid mobilization of phagocytic cells, natural killer cells as well as the secretion of proinflammatory cytokines, antimicrobial peptides and interferons (Grandjean et al., 1997, Agerberth et al., 1995, Dejucq et al., 1997, Meinhardt and Hedger, 2011). The innate immune defence mechanisms that are discussed in more detail in this thesis involve mainly the testicular secretion of proinflammatory cytokines of the IL-1 family, as well as the testicular production of the antimicrobial peptide HMGB-1.

1.7.1 Cytokines

Cytokines are small signaling molecules, originally described as the hormones in the immune system. Later it became evident that many different cell types can secrete cytokines and they are involved in many different processes, such as regulation of the immune response, growth, differentiation and tissue remodeling. Cytokines act in an autocrine, paracrine or endocrine fashion in nano- to femtomolar concentrations. They can roughly be divided into proinflammtory and anti-inflammatory cytokines. Proinflammatory cytokines stimulate the immune response and promote inflammation. IL-1 and tumor necrosis factor alpha (TNFα) are classical proinflammatory cytokines. They produce fever, inflammation, tissue destruction and in some cases even shock and death, when administered to humans (Dinarello, 2000). Other important proinflammatory cytokines are IL-6, Interferon gamma (IFNγ) and IL-2. The anti-inflammatory cytokines are immunoregulatory molecules that control the proinflammatory cytokine response. Interleukin -1 receptor antagonist (IL-1ra), IL-4, IL-6, IL-10 among others, belong to this group (Opal and DePalo, 2000). A homeostatic balance of pro- and anti- inflammatory cytokines is important for the maintenance of health.

1.7.2 Interleukin-18 and the interleukin-1 family of cytokines

IL-1 is a potent proinflammatory cytokine and has several functions that are important for the innate immune defence (Dinarello, 2009, Dinarello, 2006). The IL-1 family consists of the agonists IL-1α and IL-1β and the antagonist IL-1ra. There are two IL-1 receptors described, IL-1RI and IL-1RII, where the IL-RI is the signaling receptor and IL-RII act as a scavenger receptor (O'Neill, 2008, Colotta et al., 1993). IL-18 also belongs to the IL-1 family of cytokines and even though it has structural similarities to IL-1β, it signals through its own receptor, IL-18R (Nakamura et al., 1989, Parnet et al., 1996, Born et al., 1998). There is also a naturally occurring antagonist to IL-18, the IL-18 binding protein (IL-18BP) (Novick et al., 1999). The IL-1 family is constantly growing and several new members have been described (Dinarello, 2009).

The IL-1RI and the IL-18R are members of the IL-1RI/Toll-like receptor (TLR) family (O'Neill, 2008). Upon ligand binding the receptor-ligand complex recruits a coreceptor, the IL-1 receptor accessory protein (IL-1RAcP) and the IL-18 receptor accessory protein (IL-18RAcP), respectively (Kim et al., 2001, Born et al., 1998, Greenfeder et al., 1995). The formation of the heterodimeric receptor is important for signal transduction, involving the Toll/interleukin-1 receptor (TIR) homology domains, recruitment of myeloid differentiation primary response gene 88 (MyD88) and IL-1 receptor associated kinases (IRAKs) ultimately leading to activation of nuclear factor kappa B (NFκB) (Dunne and O'Neill, 2003, Burns et al., 1998) (Figure 5).

IL-1α, IL-1β as well as IL-18 are produced as precursor proteins. The proforms of IL-1β and IL-18 need to be processed to a mature protein before they exhibit biological activity, whereas IL- α has activity both as a mature and a precursor protein (Mosley et al., 1987). Pro-IL-1α is processed intra-cellularly by calpain, while both IL-1β and IL-18 are cleaved by ICE (also known as caspase-1) (Black et al., 1988, Ghayur et al., 1997, Kobayashi et al., 1990). IL-1 and IL-18 lack a signal peptide and their secretion does not follow the normal Golgi route. Instead, IL-1β and IL18 maturation and secretion requires assembly and activation of a multi-protein complex called inflammasome, that directs activation of ICE for cleavage of the precursor forms of IL-1β and IL18 (Martinon et al., 2002, Martinon et al., 2009) (Figure 6). Both IL-1β and IL-18 can also be cleaved by proteinase 3 (Coeshott et al., 1999, Sugawara et al., 2001).

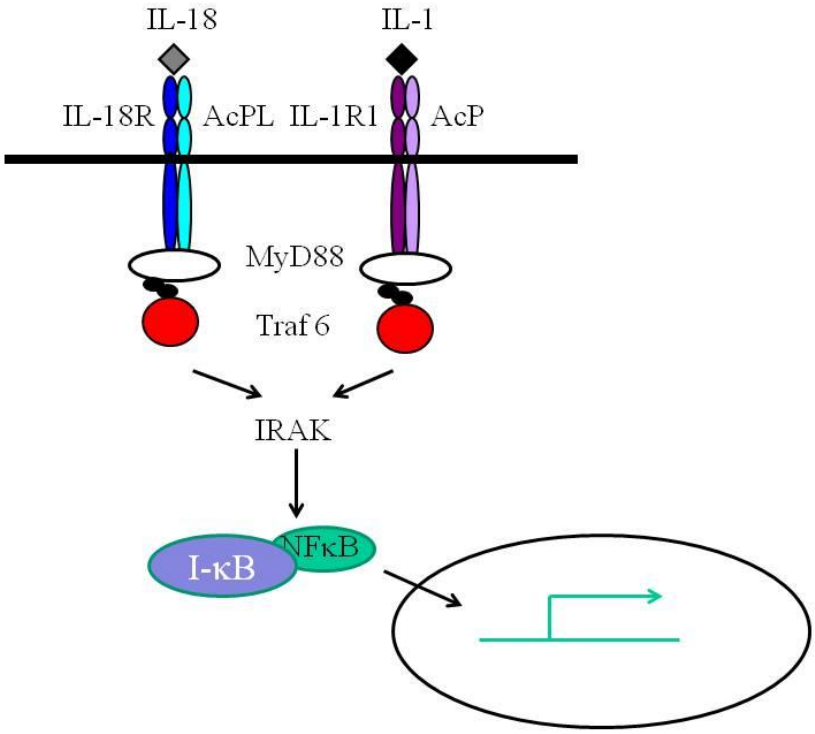


Figure 5. IL-18 and IL-1 signaling pathways involving binding of each ligand to their own receptor, interaction of the receptor with the adaptor protein MyD88, followed by Traf 6 and IRAK activation and finally nuclear translocation of NFκB.

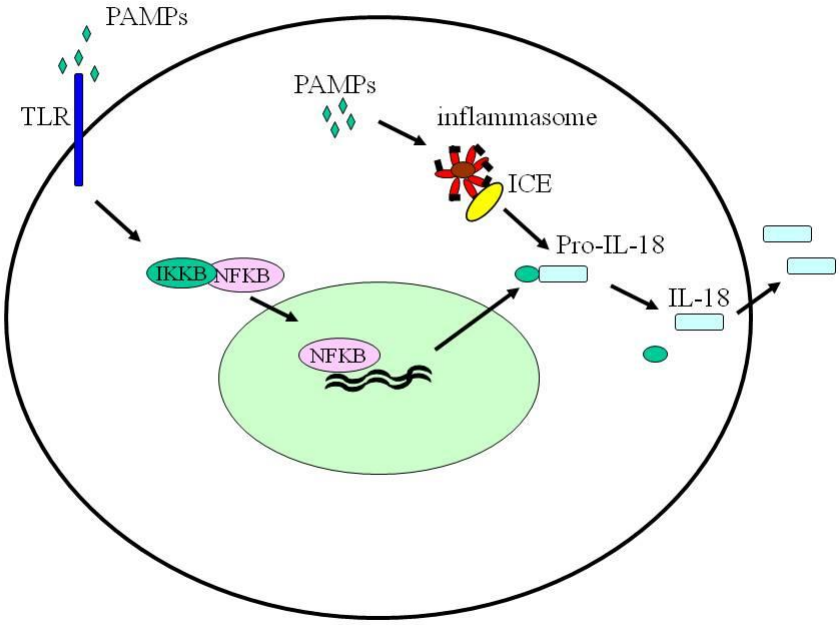


Figure 6. Extracellular and intracellular PAMPs bind to membrane- and intracellular receptors, respectively, leading to assembly of the inflammasome complex and activation of ICE. Pro-IL-18 is cleaved by ICE, followed by extracellular release.

1.7.3 **The IL-1 family in the mammalian testis**

IL-1 has important functions with regard to the immune defence, but IL-1 also plays paracrine physiological roles in many tissues. In the testis, IL-1α is produced and secreted constitutively by adult rat Sertoli cells (Syed et al., 1988, Gerard et al., 1991), but others have also reported IL-1α expression in germ cells (Haugen et al., 1994). The production of IL-1α in the Sertoli cells is dependent on interactions with germ cells (Jonsson et al., 1999). The expression initiates during puberty and is stage dependent (Soder et al., 1991, Syed et al., 1988, Wahab-Wahlgren et al., 2000). IL-1α is present throughout the epithelial cycle, except in stage VII, which lack mitotic and meiotic DNA synthesis (Jonsson et al., 1999, Wahab-Wahlgren et al., 2000). Several forms of IL-1α are expressed in the testis, including the 31kD proform, the 17kD mature form and a 24kD form that is produced from an alternatively spliced transcript (Sultana et al., 2000).

IL-1β is, in contrast to IL-1α, not produced under constitutive conditions in the rat testis, but the immunoreactive protein was induced in testicular macrophages, after systemic lipopolysacharide (LPS) treatment (Jonsson et al., 2001). In contrast, LPS treatment had no effect on the total level of IL-1 bioactivity in the adult testis, suggesting that the agonist activity of IL-1 might be regulated by IL-1ra that also was found to be induced in the testis by LPS treatment.

There are some studies reporting IL-1ra expression in mouse Sertoli cells grown *in vitro* and immunoreactive protein has been found in Leydig cells in the human testis biopsies (Zeyse et al., 2000, Abu Elheija et al., 2011). However, Jonsson et, al did not detect constitutive expression of IL-1ra in the rat testis (Jonsson et al., 2001).

The mRNA species encoding IL-1RI and IL-1RII have been found in isolated Sertoli-, peritubular- and Leydig cells and testicular macrophages in the rat, mouse and human testis (Gomez et al., 1997). IL-1 receptors mRNAs were also found in isolated rodent germ cells, but not in the germ cells of the human testis. Localization of IL-1RI mRNA by *in situ* hybridization also showed a wide distribution of the receptor in the mouse, but not in the rat testis (Gomez et al., 1997). In addition, high affinity binding sites for the IL-1R has also been found to be heterogeneously distributed in the mouse testis, with highest density present in the luminal border of the epididymis and interstitial areas of the testis (Takao et al., 1990).

The constitutive production of testicular IL-1α suggests that this cytokine may be involved in the physiological processes of the rat testis. Indeed, it has been shown that IL-1α stimulates germ-, peritubular- and immature Sertoli cell proliferation (Parvinen et al., 1991, Pollanen et al., 1989, Petersen et al., 2002, Svechnikov et al., 2004). IL-1α is also found in interstitial fluid (Gustafsson et al., 1988, Hedger et al., 1998), suggesting a paracrine role of IL-1α in the interstitial compartment. Supporting this, IL-1α has been shown to inhibit human chorionic gonadotropin (hCG) stimulated testosterone production, but to stimulate basal Leydig cell steroidogenesis (Svechnikov et al., 2001).

Recently, it was found that IL-1α affected the permeability of the BTB during spermatogenesis, suggesting a role for IL-1α in junctional dynamics (Sarkar et al., 2008). Intratubular injection of IL-1α was shown to perturb Sertoli-germ cell adhesion and to compromise the integrity of the BTB. Both *in vivo* and *in vitro* studies showed that IL-1α treatment led to F-actin reorganization (Sarkar et al., 2008, Lie et al., 2011). These results indicate that IL-1 may stimulate BTB disassembly by inducing actin remodeling, thereby facilitate the movement of preleptotene spermatocytes across the BTB.

1.7.4 Antimicrobial peptides

Antimicrobial peptides have been found in plants, insects and mammalian cells and represent an ancient defence system. Over 1200 antimicrobial peptides have now been identified or predicted from various organisms (Nakatsuji and Gallo, 2012). Many of the antimicrobial peptides share common features with positively charged and hydrophobic/amphipathic structure. This amphiphatic structure is believed to facilitate interactions with negatively charged phospholipids and hydrophobic fatty acids located in the microbial membranes, resulting in pore formation. The expression of antimicrobial peptides can be stimulated by TLR signaling and release of proinflammatory cytokines (Lai and Gallo, 2009). Antimicrobial peptides also contribute to host defence by influencing cytokine release, chemotaxis and antigen presentation (Lai and Gallo, 2009).

The antimicrobial peptides can be divided in three different groups, including peptides with α-helical structure, cysteine-containing peptides with β-sheeted structure, stabilized with disulfide bonds and peptides with extended loop structure (Lai and Gallo, 2009). The two most studied mammalian gene families of antimicrobial peptides are the cathelicidins and defensins. Cathelicidins have been described in both invertebrates and mammals, but only one human cathelicidins gene has been found. This gene encodes an 18 kD precursor-protein, named hCAP18 (Lai and Gallo, 2009). hCAP18 is stored in granules in neutrophils. During neutrophil activation, hCAP18 is cleaved and released as an antimicrobial peptide of 37 amino acids, beginning with two leucins and therefore named LL-37. In addition to neutrophils, LL-37 has also been described in the testis, epididymis, skin, and respiratory epithelium (Bals et al., 1998, Agerberth et al., 1995, Malm et al., 2000, Murakami et al., 2004).

Several mammalian α-and β-defensins have been identified. The defensins were originally found in the granules of neutrophils and macrophages, but later also shown to be produced by keratinocytes and in epithelial cells of the respiratory, digestive and urinary tract (Lai and Gallo, 2009, Lehrer et al., 1993).

1.7.5 Antimicrobial peptides in the testis

Several defensins has previously been detected in the rat, mouse and human reproductive tract (Grandjean et al., 1997). In the rat testis, most of the defensins are expressed in testicular macrophages and in the Sertoli- and peritubular myoid cells. Spermatogonia expressed only α-defensins, meiotic cells only β-defensins and post meiotic cells expressed both. We have recently found testicular expression of the

nuclear factor HMGB1 and showed that it possesses antibacterial activity, as described in section 4.3 in this thesis.

1.7.6 High mobility group box protein-1

Recently, it was found that the nuclear DNA binding protein HMGB1, isolated from the human adenoid, possessed potent antimicrobial activity (Zetterstrom et al., 2002). The antimicrobial activity of HMGB1 was rapid, even compared to well known antimicrobial peptides and it was suggested that HMGB1 was an antibacterial peptide, involved in the barrier defence in the adenoid.

HMGB1 is expressed in most cells and was originally described as a nuclear protein that bound to bent DNA and thought to be involved in gene regulation and recombination (Bianchi et al., 1989, Bianchi, 1988, Maher and Nathans, 1996, Murphy et al., 1999). Since then, several extracellular functions of HMGB1 have been found, including the stimulation of neurite outgrowth (Huttunen et al., 1999), promoting tumor invasion and metastasis (Taguchi et al., 2000), being a late mediator of endotoxin lethality (Wang et al., 1999a), stimulating cytokine release (Andersson et al., 2000) and acting as a proinflammatory cytokine itself.

HMGB1 have been shown to interact with several extracellular receptors including the receptor for advanced glycation end products (RAGE), Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4), thereby elucidating immune stimulatory processes (Park et al., 2004, Huttunen et al., 1999, Kokkola et al., 2005, Yang et al., 2010). The role of HMGB1 as a danger signal and a proinflammatory cytokine has on the other hand been questioned (Tsan, 2011). Recent data indicates that highly purified HMGB1 lack cytokine activity and that the immune stimulatory capacity of HMGB1 requires complex formation of HMGB1 and LPS or HMGB1 and other pathogen-associated molecular patterns (PAMPs) (Pisetsky, 2011).

1.8 XENOBIOTIC DEFENCE MECHANISMS IN THE TESTIS

Xenobiotics are chemicals that are found within an organism, but which are not normally produced there. Drugs are xenobiotics in humans because our bodies do not produce the drugs by itself and they are not part of our normal diet. Natural compounds can also be regarded as xenobiotics if they are taken up by another organism that does not produce the compound in question. An example of this is the uptake of natural human hormones by fish. Pollutants are also regarded as xenobiotics, since they are chemical compounds that are considered foreign or artificial to the whole biological system.

Our bodies have evolved defence systems consisting of transporter proteins located in epithelial cell barriers to limit the uptake of xenobiotics to the tissues. Even though tight junctions in epithelial barriers are efficient in preventing the entry of large hydrophilic substances, they are less efficient in preventing the entry of small hydrophobic molecules, including xenobiotic compounds such as clinically important drugs, environmental pollutants, dietary toxins etc., but also endogenous (non-xenobiotic) compounds such as hormones, peptides and lipids.

The role of ABC transporter proteins in pharmacokinetics and in the prevention of xenobiotic uptake in barrier tissue has become increasingly clear. Originally, the ABC-transporter proteins, encoded by so called multidrug resistance (MDR) genes, gained interest due to their over expression in tumor tissue and their diverse substrate specificity, leading to the development of the phenomena MDR (Ambudkar et al., 1999). Several MDR genes have also been found in many tissues important for drug extrusion and elimination as well as in barrier tissues such as the BTB and the BBB (Leslie et al., 2005). Even though the ABC transporter proteins play an important role in the protection of tissues against xenobiotic-induced toxicity, their expression may also hinder the delivery of clinically important cytotoxic drugs to sanctuary sites such as the testis and the brain, which may be a disadvantage in the treatment of leukemia, brain tumors and other cancers.

1.8.1 ABC transporter protein family

The ABC transporter family is a large and widely expressed protein family, responsible for active transport of a wide range of substrates across membranes. The substrates include both endogenously produced compounds including phospholipids, ions, peptides, steroids, polysaccharides among others (Klein et al., 1999) as well as xenobiotic compounds such as drugs, pesticides, flavonoids (plant derived antioxidants) and food toxins (Table 1).

Classical ABC proteins are composed of two or three membrane spanning domains, consisting of several transmembrane α-helices and two hydrophilic, intracellular nucleotide binding domains (Leslie et al., 2005) (Figure 7). Binding of ATP (adenosine-tri-phosphate) at the nucleotide binding domains, followed by hydrolysis of ATP is required for their transport of substrates across membranes (Leslie et al., 2005). The ABC transporter protein family can be divided into seven subfamilies (A-G) in the human (Dean and Allikmets, 2001, Klein et al., 1999).

The most well known ABC transporter proteins that confer drug resistance include P-glycoprotein (P-gp) encoded by multidrug resistance gene 1, (gene symbol *ABCB1*, former *MDR1*), the multidrug resistance protein 1 (MRP1, gene symbol *ABCC1* and the breast cancer resistance protein (BCRP, gene symbol *ABCG2*) (Leslie et al., 2005). These ABC transporters have some overlapping substrate specificity (Table 1). All three are for instance involved in transport of the clinically important anticancer drug doxorubicin, which was used as a xenobiotic model in paper IV in this thesis (Leslie et al., 2005, Schinkel and Jonker, 2003).

However, even though P-gp, Mrp1 and Bcrp show substrate overlap, the mechanism for transport may be different. P-gp has been shown to be a primary active transporter. In primary active transport, substrates are transported in one direction (i.e. out of the cell) using hydrolysis of ATP. In contrast, Mrp1 efflux is also ATP-driven, but occurs through a co-transport mechanism with reduced glutathione (GSH) (Leslie et al., 2005, Schinkel and Jonker, 2003).

Table 1. Xenobiotic and endogenous substrates of P-gp, Mrp1 and Bcrp.

ABCB1 (MDR1/P-gp)	**ABCC1 (MRP1)**	**ABCG2 (BCRP)**
Anticancer drugs Anthracyclines (doxorubicin, daunorubicin, epirubicin) Vinca Alkaloids (vinblastine, vincristine) Epipodophyllotoxins (etoposide, teniposide) Actinomycin D Taxanes (paclitaxel, docetaxel)	***Anticancer drugs*** Anthracyclines (doxorubicin, daunorubicin, epirubicin) Vinca Alkaloids (vinblastine, vincristine) Epipodophyllotoxins (etoposide, teniposide) Camptothecins (topotecan, irinotecan Anthracenedion (mitoxantrone) Heavy metal oxyanions (arsenite, trivalent antimony) Flutamid (antiandrogen)	***Anticancer drugs*** Anthracyclines (doxorubicin, daunorubicin Anthracenedion (mitoxantrone) Campothecins
HIV protease inhibitors Saquinavir Ritonavir Nelfinavir Lopinavir		
Analgesic Morphine Asimadoline		
Corticoids Dexamethasone Hydrocortisone Corticosterone		
Cardiac glycosides Digoxin		
Diagnostic Dyes Hoechst Rhodamine		
Antibiotics Erythromycin Gramicidin		
Dietary/environmental PhIP	***Dietary/environmental*** Aflatoxin B_1 Nitrose-amine conjugates (NNAL-O-gluc)	***Dietary/environmental*** Flavonoids (genistein, flavopiridol, pheophorbide) PhIP
Pesticides ivermectin chlopyrifos	***Pesticides*** metholachlor-GS metoxychlor fenitrothion	***Pesticides***

ABCB1(MDR1/P-gp	**ABCC1 (MRP1)**	***ABCG2 (BCRP)***
Endogenous (non-xenobiotic) Phosphatidylcholine	***Endogenous (non-xenobiotic)*** LTC_4 estrone 3-sulfate $E_2$17βG sulfated bile acids folates GSH and GSH disulfide Oxidized glutathione (GSSG)	***Endogenous (non-xenobiotic)*** folic acid estrone 3-sulfate 17β-estradiol-3-sulfate

Modified from (Schinkel and Jonker, 2003, Kruh and Belinsky, 2003, Leslie et al., 2005).

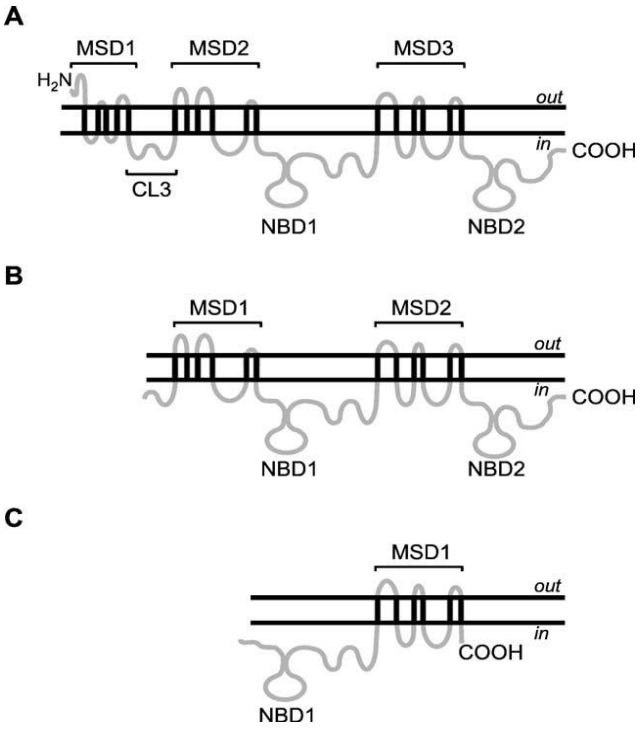


Figure 7. Predicted secondary structures of P-gp (A), Mrp1 (B) and Bcrp (C). MSD= membrane spanning domain, NBD= nucleotide binding domain, CL= cytoplasmic loop. With permission, E.M. Leslie et al. / Toxicology and Applied Pharmacology 204 (2005) 216–237.

1.8.2 P-glycoprotein

P-gp was discovered by Juliano and Ling and is the most studied member of the ABC transporter protein family (Juliano and Ling, 1976). It is encoded by both *Mdr1*a (*Abcb1a*) and *Mdr1b* (*Abcb1b*) in rodents (Devault and Gros, 1990, Silverman et al., 1991, Schinkel, 1997). Together the two isoforms have the same distribution as human P-gp and suggested to have similar physiological functions (Schinkel, 1997).

It has been suggested that P-gp mediates substrate efflux by acting as either a flippase or a hydrophobic vacuum cleaner (Schinkel and Jonker, 2003, Higgins and Gottesman, 1992). In the former model, the substrate first has to insert to the inner leaflet of the plasma membrane lipid bio-layer, before being "flipped" to the outer leaflet. In the latter model the substrate is directly bound by P-gp in the plasma membrane and extruded to the extracellular environment.

P-gp is expressed in a variety of tissues with excretory/secretory functions, including the gastrointestinal tract, liver, kidney, pancreas and adrenals, but also in tissue barriers such as the placenta, BBB and the testis. (Thiebaut et al., 1987, Cordon-Cardo et al., 1990, Leslie et al., 2005). P-gp is usually located on the luminal sides of tissue barriers where it provides extrusion of substrates to the blood. This expression pattern indicates that P-gp may play an important function in reducing the exposure of tissues to xenobiotic agents. Indeed, *Mdr1a* and *Mdr1a/b* knockout mice support this hypothesis by demonstrating reduced uptake of P-gp substrates in the brain (Schinkel et al., 1997, Schinkel et al., 1995, Schinkel et al., 1994).

1.8.3 P-gp expression and function in the testis

In the testis, P-gp is expressed at the luminal side of the endothelial cells of the capillaries. Supporting a protective role in the testicular barrier defence against xenobiotics, *Mdr1a* knockout mice accumulate more of the P-gp substrates ivermectin and vinblastine within the testis, compared to wild type mice (Schinkel et al., 1994). *Mdr1a/b* knockout mice also demonstrate enhanced penetration of digoxin into the testis. (Schinkel et al., 1997). The protective role of P-gp in the testis have also been demonstrated by pharmacological inhibition of P-gp, leading to increased testicular uptake of the HIV inhibitor nelfinavir (Choo et al., 2000).

The testicular expression of P-gp is not limited to the endothelial cells, but also located to Leydig cells, macrophages, peritubular myoid cells, Sertoli cells and late spermatids (Melaine et al., 2002) (Figure 8). P-gp is not expressed in mitotic and meiotic germ cells, which could at least partly explain the susceptibility of these cell types to anticancer therapy. Recently it was shown that P-gp is an integrated component of the BTB, where it co-localizes with several tight junctional proteins and suggested to participate in the regulation of BTB dynamics (Su et al., 2009).

1.8.4 Testicular expression of Mrp1 and Bcrp

Mrp1 expression is found in several organs including the brain, kidney, placenta and testis (Bart et al., 2004). In the testis Mrp1 is localized to the basal side at the Sertoli cells and Leydig cells (Wijnholds et al., 1998). *Mrp1* knockout mice are healthy and fertile, but etoposide treatment resulted in disrupted spermatogenesis and transient

infertility (Wijnholds et al., 1998). This was not seen in etoposide-treated wild type mice. Mrp1 have also been shown to protect the seminiferous tubules against damage mediated by the pesticide methoxychlor (Tribull et al., 2003). Mrp1 transports sulfated estrogens and the co-localization of Mrp1 and estrogen sulfotransferase in Leydig cells may suggest that Mrp1 is involved in maintaining the low estrogen levels in the testis (Leslie et al., 2005)

The testicular expression of Bcrp is located in myoid cells and the luminal side of endothelial cells in humans (Bart et al., 2004). Bcrp limits the testicular entry of the phyto-estrogen genistein (Enokizono et al., 2007). Phyto-estrogens are plant compounds that can bind to estrogen receptors on the Leydig- and Sertoli cells, inducing estrogenic or anti-estrogenic effects. It has also been reported that Bcrp is regulated by estrogen, testosterone and progesterone (Hartz et al., 2010, Merino et al., 2005, Tanaka et al., 2005). Bcrp itself also functions as a transporter of sulfonate-, GSH- or glucuronide-conjugated steroid hormones (Mao and Unadkat, 2005). Thus, Bcrp, similar to Mrp1, may be involved in physiological steroid transport in the testis in addition to its role in xenobiotic protection.

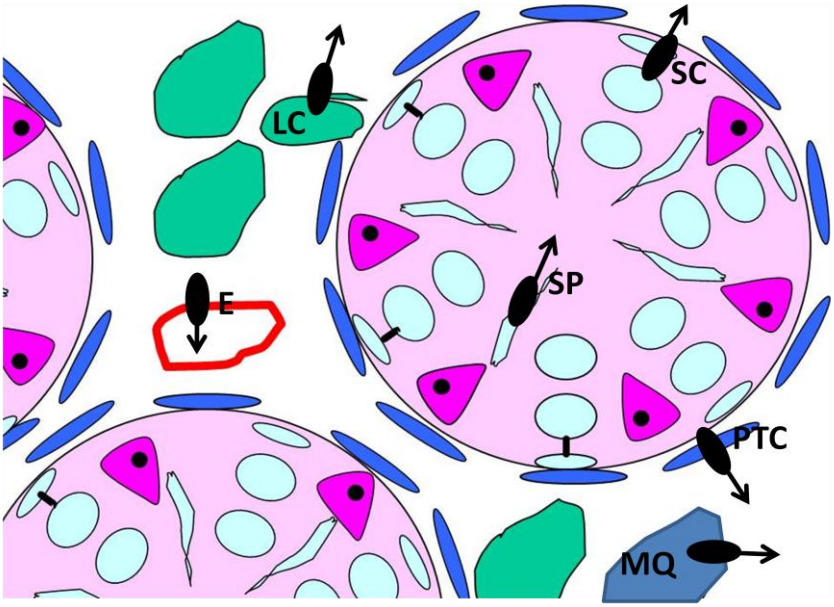


Figure 8. Expression of P-gp in different cell layers of the testis. Endothelial cell expression confer efflux of xenobiotics to the blood circulation, while Sertoli cell and peritubular myoid cell expression leads to xenobiotic efflux from the seminiferous tubules, aiding in germ cell protection. P-gp expression in Leydig cells may indicate involvement of P-gp in steroid transport. Elongated spermatids express P-gp and may protect these cells from xenobiotic toxicity during epididymal maturation or in the female genital tract. E= endothelial cell, MQ= testicular resident macrophage, LC= Leydig cell, SC= Sertoli cell, PTC= peritubular myoid cell, SP= elongated spermatid.

2 AIMS OF THE STUDY

The general aim of this work was to study defence mechanisms that contribute to the antimicrobial, immunological and xenobiotic protection of developing germ cells in the mammalian testis.

The specific aims were:

1. To study the expression, localization and function of CAR in the mouse, rat and human testis as well as to investigates its role in mature spermatozoa.

2. Investigate the expression and paracrine function of IL-18 in the developing postnatal rat testis.

3. Study the expression, localization and antimicrobial activity of HMGB-1 in the human and rat testis.

4. To study the ontogeny of the testicular ABC transporter dependent protection against xenobiotic-induced toxicity of the rat testis.

3 MATERIALS AND METHODS

3.1 ANIMAL EXPERIMENTS

3.1.1 Testis donors (paper I-IV)

Male Sprague Dawley rats in different postnatal ages (6, 10, 16, 20, 24, 30, 40 and 60-day-old) were used as testis donors for the polymerase chain reactions (PCRs), western blot, *in situ* hybridizations, immunohistochemistry (IHC) and indirect immunofluorescence (IF) analyses. Testis biopsies from three men (30-40 years old) undergoing fertility examination, but with qualitatively and quantitatively normal spermatogenesis, were used for the reverse transcriptase PCR (RT-PCR) analyses in paper III. The testis that was used for *in situ* hybridization (paper III) was obtained from a 70-year-old man with prostate cancer and the material used for IHC was obtained from the healthy testis from a 33-year-old man with seminoma.

3.1.2 LPS treatment of rats (paper II)

LPS is a component of the cell wall of gram negative bacteria and induces a strong inflammatory response when injected to animals. To study the activation of IL-18 under inflammatory conditions, 60-day-old male Sprague Dawley rat were treated with a single intraperitoneal injection (i.p.) of 50 and 100 µg/kg LPS for 6 hours, before the testes, liver and spleen were removed for subsequent analysis.

3.1.3 Doxorubicin treatment of rats (paper IV)

6-, 16- and 24 day-old Sprague Dawley rats were injected i.p. with 3 mg/kg of the anticancer drug doxorubicin. The animals were sacrificed after 5, 20 and 60 minutes, followed by removal of the testes for subsequent doxorubicin measurements. Rats in the same ages also received the same treatment for 4 hours and 24 hours, before testes were removed and frozen at -80 °C.

3.2 ISOLATION AND *IN VITRO* CULTURE OF TESTICULAR CELLS

3.2.1 Staging of rat seminiferous tubules (paper I and II)

In the rat testis, an epithelial cycle can be divided in 14 stages, where each stage refers to a unique association of different germ cell types with the Sertoli cells (Clermont, 1972). The different stages can be identified by the use of a transillumination microscope, combined with a normal light microscope and the squash preparation technique (Parvinen and Vanha-Perttula, 1972, Soderstrom and Parvinen, 1976). In brief, the testes are decapsulated and the seminiferous tubules washed in phosphate buffered saline, under a transillumination microscope. The different stages are identified on the basis of their different light absorption, which depends on the level of chromatin condensation in the haploid cells. This technique was employed to isolate two millimeter segments from spermatogenic stages I, V, VIIa and VIII-IX, followed by *in vitro* culture for 24, 48 and 72 hours and subsequent proliferation analyses.

Half millimeter segments of seminiferous tubules were also squashed carefully against a cover slip, snap frozen and fixed for subsequent immunohistochemical analyses to be able to identify germ cells in the different developmental phases.

3.2.2 Isolation of mouse and human spermatozoa (paper I)

Several mouse epididymides were pooled, cut into small pieces in PBS and spermatozoa were isolated by filtration. The cell suspension was centrifuged to pellet the cells. Human spermatozoa were donated from patients at the fertility unit in the Karolinska University Hospital, Sweden. The spermatozoa were separated by the swim up technique. This method utilizes the motility of spermatozoa to separate them from seminal fluid. In brief, the semen is overlaid by pre-equilibrated culture medium in a culture tube, incubated for 40-60 min where after the sperm is collected from the top milliliter, washed and pelleted by centrifugation.

3.2.3 *In vitro* induction of the acrosomal reaction (paper I)

During fertilization the sperm must penetrate the hard shell of the egg. In order to do so the sperm goes through an acrosomal reaction. During this event, the membrane of the acrosome (the cap structure of the sperm) fuses to the zona pellucida, releasing its acrosomal contents. The acrosome reaction can also be stimulated *in vitro* by the calcium ionophore A23187. We employed this to induce the acrosome reaction in purified human spermatozoa, followed by centrifugation and washing of the cell pellet, that was subjected to IF omitting the fixation and permeabilization step.

3.2.4 Isolation and *in vitro* culture of Leydig cells (paper II)

Leydig cells were isolated from 40-day-old rats, using a two step purification method that yields 90 % purity and viability (Svechnikov et al., 2001). These cell cultures were incubated in the presence or absence of different concentrations of recombinant rat IL-18, with or without hCG. The testosterone concentration in the culture supernatant was then measured by radioimmunoassay (RIA). Leydig cells from 20-day-old rats were also isolated and cultured *in vitro* for subsequent proliferation analyses. In contrast to cell-lines, primary cultures of Leydig cells exhibit native steroidogenic activities and thus give reliable results regarding the intracellular functions of mammalian Leydig cells.

3.3 METHODS TO STUDY APOPTOSIS

3.3.1 Cell death ELISA (paper II)

The ELISA kit for detection of cell death was used for the detection of apoptosis in cell lysates from stage I segments of seminiferous tubules after incubation with or without IL-18. The cell death ELISA allows for rapid quantification of histone-complexed DNA fragments which are released from apoptotic or necrotic cells.

3.3.2 *In situ* 3’ end (TUNEL) labeling (paper II)

Terminal deoxynucleotidyl transferase (TdT) is a DNA polymerase that adds nucleotides to the 3’ end of gene segments without the use of a template strand. It is

therefore used for the biotin-labeling of blunt ends of fragmented double stranded DNA found in apoptotic cells. This method was used to quantify apoptosis of spermatogonia in stage I segments of seminiferous tubules after incubation with or without recombinant IL-18.

3.3.3 Cleaved caspase 3 (paper II)

Another marker of apoptosis is the presence of the cleaved or activated form of the apoptosis executor protein caspase-3, which can be detected by specific antibodies and used to visualize apoptotic cells in fixed tissue preparations. This method was used in combination with *in situ* 3' end labeling and cell death ELISA to determine possible anti-apoptotic effects of IL-18 on differentiating spermatogonia.

3.4 METHODS TO STUDY GENE EXPRESSION

3.4.1 Reverse transcriptase PCR (paper I, II & III)

RT-PCR was employed for the detection of CAR, IL-18 and HMGB1 mRNA expression in tissue extracts from mouse, rat and human testes. In brief, the RNA is extracted and reversed transcribed into cDNA (complementary deoxyribonucleic acid) using the enzyme reverse transcriptase, followed by amplification of the cDNA by PCR. This requires the use of gene specific primers that binds to the specific cDNA, allowing the Taq polymerase chain reaction to occur. The PCR products are then visualized by agarose gel chromatography followed by ethidium bromide staining. In contrast to real-time PCR, conventional RT-PCR measures the endpoint of the PCR reaction and can only be used in a semi-quantitative way due to saturation effects etc.

3.4.2 Real-time PCR (paper IV)

Real-time PCR was used to quantify the developmental expression of ABC transporters in the postnatal rat testis. This method is based on RT- PCR combined with the use of fluorophores, which allows for the direct measurement of the DNA amplification instead of the end point. During each cycle, the dye SYBR green incorporates to the cDNA and cause an increase in fluorescent intensity that can be measured "in real time" and thus used for accurate quantification of gene expression. The limitation of using CYBR green as a flourophore is that it binds to any double stranded DNA, including primer dimers or nonspecific PCR products and this may interfere with the results.

3.4.3 S^{35} *In situ* hybridization (paper II & III)

S^{35} *In situ* hybridization was employed to localize the expression of IL-18 and HMGB1 transcripts in testicular tissue. This method utilizes S^{35}-labeled oligonucleotide DNA probes that are complementary to the target mRNA of interest. After hybridization of the radioactive probe to the tissue, the tissue sections are washed, dipped into film emulsion, exposed and developed. The positive cells are identified using dark and bright field microscopy. In contrast to PCR, *in situ* hybridization allows for the cellular localization of gene expression in tissues.

3.5 METHODS TO STUDY PROTEIN EXPRESSION AND INTERACTIONS

3.5.1 Western blot analysis (paper I, II & III)

The presence of CAR, IL-18 and HMGB1 protein in testicular tissue extracts was studied by western blot. Western blot was also employed for detection of multiple forms of the proteins, such as activated IL-18 that are processed by cleavage.
The total cell protein concentration was measured by Bradford and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer and incubation of the membrane by specific antibodies against the protein of interest.

3.5.2 Immunohistochemistry & Immunofluorescence (paper I, II, III & IV)

We performed IHC or IF to study the localization of CAR, IL-18, HMGB1 and P-gp in the mammalian testis. In both methods antibodies are utilized to identify and localize proteins in tissue sections. The major difference between the two methods is that in IHC the primary protein-antibody complex is bound to a biotin-conjugated secondary antibody and thereafter amplified by a biotin-streptavidin step, followed by a color reaction, most commonly 3,3-Diaminobenzidine (DAB). In IF the primary protein-antibody complex is detected by a secondary antibody conjugated with a fluorescent dye. IF was also used to study the co-localization of P-gp with the junctional proteins ZO-1 and occludin, employing two secondary antibodies with different fluorophores. Addition of an antigen-retrieval step to the IHC/IF protocol was done in some cases to prevent false negative results due to fixation-induced masking of epitopes in the tissues.

3.5.3 GST pull-down assay (paper I)

Glutathione S- transferase (GST) -fusion proteins can be constructed to investigate direct protein-to-protein interactions in a GST-pull down assay. Following binding of potential binding partners to the GST-fusion protein, the GST-complex is bound to GSH-coated beads. The interacting proteins may then be identified using specific antibodies. In order to isolate CAR from mouse testis, two GST constructs of the p70 and p80 ligand of numb (LNX) protein isoforms were used as baits. The GST-LNX fusion proteins were incubated with testicular homogenates and bound proteins were separated on SDS-PAGE, transferred to filters and analyzed by western blot, using antibodies against CAR.

3.5.4 Co-immunoprecipitation (paper I)

Co-immunoprecipitation of was performed to investigate if CAR and JAM-C could form a protein complex *in vivo*. Proteins in mouse testicular homogenates were precipitated employing an anti JAM-C antibody and separated by SDS-PAGE, followed by transfer and Western blot. Coprecipitated CAR protein was visualized by the specific CAR antibody RP1284.

3.6 H^3-THYMIDINE INCORPORATION (PAPER II)

Spermatogonial, as well as Leydig cell DNA synthesis was measured by H^3-thymidine incorporation. Two mm segments of seminiferous tubules in defined stages and isolated Leydig cell were cultured *in vitro* for 24 and 48 hours. Four hours before harvest, the cells were pulsed with 0.5 µCi H^3-thymidine, which is incorporated into the DNA of

dividing cells. Incorporated radioactivity (counts per minutes, cpm) was measured in a β-scintillation spectrometer. The limitation of this method is that measuring thymidine incorporation does not exclusively give a value of DNA replication, but also measures DNA repair.

3.7 RADIOIMMUNO ASSAY (PAPER II)

RIA was used for the determination of the testosterone production by Leydig cells after exposure to IL-18. This method measures hormone concentrations in cell culture media and in blood. It is based on the competitive antibody binding of radio-labeled hormone and the hormone present in the sample.

3.8 REVERSED PHASE HPLC (PAPER III &IV)

Reversed phase high performance liquid chromatography (HPLC) is a method used to separate and purify proteins as well as other molecules in tissue or blood samples. The stationary phase contains lipophilic groups. When the sample is passed through the system the lipophilic proteins/molecules will remain in the column, while the hydrophilic proteins will be eluted. Reversed phase HPLC was employed to purify and isolate peptides with antibacterial activity from testicular extracts (paper III) and for the determination of the doxorubicin concentrations in testicular tissue extracts after *in vivo* treatment (paper IV).

3.9 ZONE INHIBITION ASSAY (PAPER III)

The inhibition zone assay is an antibacterial assay and was employed for determination of the antibacterial activities in the HMGB1 positive and negative fractions obtained by the reversed phase HPLC. Small wells are punched out in an agar plate and seeded with a bacterial strain. The sample to be tested is added to the well and incubated for appropriate time. The diameters of the inhibition zones are then measured as reflections of the ability of the test substance to kill the bacteria.

4 RESULTS AND DISCUSSION

4.1 CAR IS EXPRESSED IN MALE GERM CELLS

Several junctional proteins of the CTX (cortical thymocyte xenopus) family are expressed in the mammalian testis and play important roles in spermatogenesis. Targeted deletion of JAM-C revealed an important role of this CTX protein in creating polarity in round spermatids (Gliki et al., 2004). Other adhesion molecules such as nectin-2 and 3 have also been shown to be important for spermatogenesis (Mueller et al., 2003, Inagaki et al., 2006).We therefore wanted to investigate the testicular expression and localization of the CTX member CAR. Endpoint RT-PCR of total RNA isolated from mouse testis, as well as western blot and GST-pull down experiments from mouse testis homogenates showed expression of CAR at the mRNA and protein level. IF localized CAR protein to the lumen of the seminiferous tubules in cells corresponding to elongating spermatids. The CAR staining was sickle shaped and in close association with the condensed nuclei, indicating acrosomal localization. Weak CAR staining was also observed in more immature germ cells. Indirect immunofluorescent staining of CAR in staged segments of rat seminiferous tubules demonstrated CAR expression in round spermatids and in late stages of elongated spermatids, most pronounced in stage VIII.

A previous study, using the Rmcb antibody on sections from mouse testis, showed a different CAR expression pattern than what we found (Peters et al., 2001). In this study, CAR was found in all germ cell types, as well as in Leydig- and Sertoli cells. However, in our hands we failed to detect mouse CAR with the Rmcb antibody. Our results are mainly in agreement with the later study by Wang et al (Wang et al., 2007), showing strong CAR expression on the convex side of the elongate spermatid head in stage VIII of the rat testis. Wang et al, also demonstrated CAR expression in round spermatids and, in contrast to us, in the BTB of the Sertoli cells. The discrepancies may be due to the use of different antibodies and immune-protocols, or by the fact that we performed testicular CAR-localization studies on mouse tissue. Another study by our laboratory confirmed the absence of CAR in the tight junctions of the Sertoli cells (Mirza et al., 2007).

At spermiation, the stage VIII spermatozoa are released and transported to the epididymis for further maturation. The presence of CAR in late spermatids in stage VIII prompted us to look for CAR in released spermatozoa. We found that CAR co-localized with the acrosomal marker peanut agglutinin (PNA) in spermatozoa isolated from mouse epididymis. Using isoform specific antibodies we found that CAR-1 was expressed all over the acrosome, while CAR-2 was restricted to the inner acrosomal membrane, suggesting that the two different CAR isoforms may have different functions.

Many spermatozoa isolated from epididymis are not fully mature and fertilization competent. Therefore, we wanted to investigate CAR expression in mature spermatozoa, isolated from human ejaculates. CAR was found to be co-expressed with PNA in the acrosome of mature human spermatozoa.

During fertilization, the sperm binds to the zona pellucida, followed by an acrosomal reaction involving fusion of the outer acrosomal membrane and the plasma membrane, release of acrosomal enzymes and exposure of the inner acrosomal membrane. The acrosomal reaction is needed for the sperm to be able to penetrate the zona pellucida. The proteins of the inner acrosomal membrane then adheres to the plasma membrane of the oocyte (Kaji and Kudo, 2004). To see whether CAR could be exposed to the surface of acromsome-reacted sperms, an acrosome reaction was induced *in vitro*, followed by IF staining of the immunoglobulin domains of CAR. We found that CAR was exposed to the surface of acrosome-reacted but not acrosome-intact sperms, indicating that CAR may be involved in the events following the acrosome reaction such as passage through the zona pellucida or interaction with the egg plasma membrane.

CAR has been shown to interact with the two cytoplasmic proteins LNX and LNX2. These are PDZ-domain containing scaffolding proteins involved in protein– protein interactions (Rice et al., 2001). We found acrosomal localization of LNX2, while LNX was located in a structure called acroplaxome, located below the acrosome. The role of these two proteins in spermatozoa and their interaction with CAR needs further investigation.

Our finding that CAR co-localized and co-immunoprecipitated with JAM-C in the mouse testis, suggests that the two proteins interact *in vivo*. JAM-C is expressed in the junctional plaques that anchor the spermatids to the Sertoli cells, which is necessary for germ cell differentiation (Gliki et al., 2004). Similar to CAR, we also found that JAM-C is localized to the acrosome in isolated spermatozoa. Round spermatids from JAM-C deficient mice lack acrosomal structures and lack all morphological signs of polarity. No further differentiation, such as condensation of nuclei and formation of flagella, occurs in these mice, leading to infertility. It might be that CAR also is involved in germ cell differentiation and polarization. Since CAR deficient mice die *in utero*, conditional knock outs need to be done to investigate the role of CAR in spermatogenesis.

The acrosomal membrane and the plasma membrane are in close connection in elongated spermatids and therefore it is difficult to rule out the exact spermatid localization of CAR by IF. Co-localization of CAR and the cytoplasmic specialization marker Espin, as well as localization of CAR to spermatogonia, lacking acrosomes, suggest that CAR localizes to the plasma membrane (Wang et al., 2007). We found that CAR co-localizes with the acrosomal marker PNA in human and mouse spermatozoa, suggesting that CAR is a multifunctional protein localized to the germ cell plasma membrane at spermatid-Sertoli cell junctions, mediating spermatid-Sertoli cell adhesion or migration of spermatocytes across the BTB (Mirza et al., 2007, Wang et al., 2007), but also expressed in the acrosome in mature spermatozoa, playing a role in the fertilization of the oocyte.

CAR expression in endothelial cells is down-regulated by the inflammatory cytokines IFNγ and TNFα (Vincent et al., 2004). A down-regulation of CAR protein expression in Sertoli cells was also seen after TNFα treatment *in vitro* (Wang et al., 2007). Cytokine-mediated down-regulation of CAR may have implications for germ cell–

Sertoli cell adhesion and germ cell differentiation during patho-physiological conditions in the testis, such as testicular inflammation.

4.2 EXPRESSION OF INTERLEUKIN-18 IS IN THE RAT TESTIS

The expression of cytokines and antibacterial peptides in the testis may reflect local host defence mechanisms with the functions to protect developing germ cells from harmful effects of infections. The IL-1 system in the testis has previously been studied (for review see Svechnikov et, al. (Svechnikov et al., 2004)). Since IL-18 is a relatively new member of the IL-1 family, we wanted to investigate the expression and paracrine function of this proinflammatory cytokine in the rat testis.

We found that IL-18 mRNA is expressed in a constitutive manner during postnatal testicular development in the rat, as well as in the adult rat testis. Constitutive mRNA expression of IL-18R and ICE was also found in the developing and adult rat testis. The expression of IL-18 and IL8R showed no signs of stage dependency by RT-analyses of pooled segments of staged seminiferous tubules. Although a slight decrease in the mRNA species encoding ICE was observed in stage XIV-VI. Western blot analysis of rat testicular extracts from different postnatal ages revealed constitutive production of the immature (24 kD) form, but not the processed (18 kD) form of IL-18. Treatment of 60-day-old rats with 50 or 100 µg/kg LPS, a treatment that is known to induce IL-1β expression in the rat testis, failed to induce processing of pro-IL-18 (Jonsson et al., 2001). *In situ* hybridization and IF analysis showed that the IL-18 mRNA expression was localized to spermatocytes and round spermatids, while the most prominent site for the IL-18 protein expression was the round and elongating spermatids.

Our data on testicular IL-18 expression differ from a later study by Elhija et, al. (Abu Elhija et al., 2008), in which IL-18, IL-18R and ICE was found to be expressed at higher levels in sexually immature mice, compared to adult mice. In this study, constitutive production of the cleaved form of IL-18 was found, both in immature and adult testes. In contrast to our results, IL-18 was localized to the spermatogonia and the interstitial cells. The underlying reason for these discrepancies is not clear, but could be due to different species, antibodies and protocols used.

Processing of proIL-18 requires cleavage by active ICE (Ghayur et al., 1997). We found abundant testicular mRNA expression of this enzyme. However, we did not investigate the presence of active ICE protein in the rat testis. Active ICE was indeed found in the mouse testis (Abu Elheija et al., 2011). Activation of ICE requires the assembly of a multiprotein complex called inflammasome (Martinon et al., 2002). At least three types of inflammasomes have been identified, consisting of ICE and different members of the NOD-like receptor family (NLR) (Martinon and Tschopp, 2005). This protein family acts as sensors for bacterial components or danger signals, triggering an innate immune response, similar to TLRs. The NLR-family member Nacht- LRR-PYD containing protein 1 (NALP-1) has been found in spermatogonia and round spermatids in the human testis (Kummer et al., 2007), suggesting that the testis may have the capacity to process ICE, leading to IL-18 activation, in response to invading pathogens.

IL-18 has been shown to stimulate growth of chondrocytes and osteoblasts (Cornish et al., 2003). We also found that recombinant rat IL-18 stimulates spermatogonial DNA synthesis in stage I segments of seminiferous tubules cultured *in vitro*, suggesting a paracrine, growth promoting role for IL-18 in the testis. Similar to IL-18, IL-1α has been shown to stimulate spermatogonial DNA synthesis (Parvinen et al., 1991). In contrast to IL-18, IL-1α shows a stage dependent expression pattern that mimics the DNA synthesis pattern (Soder et al., 1991). No effect of IL-18 on the level of apoptosis in cultured stage I segments of seminiferous tubules was observed, indicating that the enhanced DNA synthesis in response to IL-18, did not reflect a decrease in apoptotic activity.

Western blot analysis revealed the presence of proIL-18 in interstitial fluid, which may reflect secretion of IL-18 into this compartment. On the other hand, since we detected IL-18 protein production in spermatids which are located behind the BTB, a secretion of IL-18 to interstitium is difficult to explain, but could be a consequence of the incompleteness of the BTB at the rete testis. In addition, IL-18 may also be secreted by interstitial cells. Indeed, we found expression of IL-18, as well as IL-18R and IL-18AcPL mRNA species in isolated Leydig cells cultured *in vitro*, which may indicate a role for IL-18 in paracrine or autocrine Leydig cell regulation. However, positive RT-PCR results obtained from *in vitro* cultured cells should always be interpreted with caution, since cell isolation and *in vitro* culture may induce expression of cytokines and receptors. We could not detect any significant effect of IL-18 on testosterone synthesis in immature Leydig cells, either in the presence or absence of hCG, albeit we saw a weak but non-significant stimulation of basal testosterone production. Neither did IL-18 stimulate Leydig cell proliferation as measured by thymidine incorporation. In contrast to IL-18, IL-1α and IL-1β have been shown to regulate Leydig cell steroidogenesis, as well as proliferation of immature Leydig cells *in vitro* (Svechnikov et al., 2001, Svechnikov et al., 2004, Khan et al., 1992).

Taken together, our findings show a constitutive production of proIL-18, as well as expression of ICE and IL-R in the immature and adult rat testis and the localization of IL-18 production to the meiotic and post-meiotic germ cells. In addition, we found that IL-18 could stimulate germ cell proliferation *in vitro.* Considering the innate immune-functions of IL-18 and the presence of only the pro-form of IL-18 in the testis, we suggest that IL-18 activity in the testis is tightly controlled to prevent harmful effects of inflammatory reactions. At the same time, upon invasion of pathogens, the testis is likely to mount an efficient innate immune response resulting in activation of ICE and IL-18. Therefore, we suggest that IL-18 may act as a mitogenic factor for germ cells during patho-physiological conditions. Further studies regarding the danger- or pathogen signals that trigger ICE activation in the testis and the following IL-18 response to this will be necessary in order to understand the testicular role and regulation of IL-18.

IL-18 is not a requirement for fertility under physiological conditions, since IL-18 knockout mice produce offspring at the predicted mendelian ratio (Takeda et al., 1998). Nevertheless, challenging these IL-18 deficient mice with systemic or uro-genital infection may give clues to the patho-physiological role of IL-18 in testis.

4.3 HMGB1 1 IS EXPRESSED IN THE TESTIS WHERE IT MAY FUNCTION AS AN ANTIBACTERIAL FACTOR

Previous studies in our laboratory have shown that the DNA-binding, nuclear protein HMGB1, isolated from the adenoid, possesses potent antibacterial activity comparable to that of classical antimicrobial peptides (Zetterstrom et al., 2002). It was therefore suggested that HMGB1 is playing a role in the innate immune defence against invading microbes in the adenoid. Since the testis is an important barrier tissue designed to protect the germ cells from harmful influences, we wanted to investigate the expression and antibacterial functions of HMGB1 in the rat and human testis.

RT-PCR analyses revealed expression of HMGB1 mRNA in adult human and rat testis, as well as in immature rat testis. The mRNA expression was localized to the Sertoli cells and possible also germ cells in the adult human and rat testis as shown by *in situ* hybridization. In the testes from 10-day-old rats, HMGB1-transcripts were observed in the central portion of the seminiferous tubules, corresponding to the localizations of immature Sertoli cells at this age. IF staining of rat and human tissues showed a similar localization of HMGB1 protein in Sertoli cells as well as in immature germ cells. Moreover, we also found expression of HMGB1 in interstitial cells, peritubular myoid cells and endothelial cells. The cellular localization was mainly nuclear for all cell types except for the Sertoli cells were HMGB1 also was found in the cytoplasm. In some of the interstitial cells, HMGB1 co-localized with the testicular macrophage marker ED2.

Our results are in line with a previous study, showing the presence of HMGB1 both in the nuclear and cytosolic fractions of homogenized testis tissue (Mosevitsky et al., 1989). Since the first discovery of HMGB1 as a nuclear protein, several extracellular functions of HMGB1 have been found, including the stimulation of inflammatory responses and tissue repair (Wang et al., 2004, Wang et al., 1999a, Andersson et al., 2000). The sub-cellular localization of HMGB1 in monocytes is dependent on the acetylation status of the nuclear localization signal of the HMGB1 protein (Bonaldi et al., 2003). Upon inflammatory stimulation, HMGB1 becomes hyper-acetylated and re-locates to the cytoplasm, where it, followed a second stimulus, is released extra-cellularly (Gardella et al., 2002). Other studies have shown that also phosphorylation of HMGB1 is important for its cytoplasmic localization (Oh et al., 2009, Youn and Shin, 2006). Whether similar mechanisms determines sub-cellular localization of HMGB1 in Sertoli cells remains to be investigated. There are indeed many similarities between Sertoli cells and macrophages, including the ability to phagocytose and produce defensins (Grandjean et al., 1997).

We found HMGB1 protein to be present in interstitial fluid collected from non-treated rat testes, indicating extracellular release. HMGB1 can be released to the extracellular milieu passively by necrotic cells or actively by immune cells, where it is believed to act as a danger signal, warning for tissue injury or invading pathogens (Andersson et al., 2002, Yang et al., 2007). Interestingly, inflammasomes, the large multi-protein complexes involved in ICE activation and subsequent release of IL-1β and IL-18 were recently shown to mediate extracellular release of also HMGB1 (Vande Walle et al., 2011). In addition to immune cells, it has been shown that viable, non-immune cells

can secrete HMGB1 in response to cytokine or LPS stimulation (Xu et al., 2010, Liu et al., 2006, Wang et al., 1999b). Whether the presence of HMGB1 in testicular interstitial fluid is due to passive leakage or active secretion needs further investigation.

We demonstrated that HMGB1 purified from the human and rat testis possessed strong antimicrobial activity in antibacterial assays, suggesting that HMGB1, similar to LL-37 and the defensins, is part of a surveillance system designed to protect the testis against bacterial infections. The presence of innate defence mechanisms based on testicular production of antimicrobial peptides may explain the relative rareness of testicular infection, compared to the rest of the urogenital tract. Because HMGB1 has been proposed to posses extracellular immune-stimulatory functions we speculate that the production of HMGB1 in Sertoli cells serves dual tasks, that is to directly kill invading microbes and to act as a co-stimulator of the immune-response. Considering the potent immune-stimulatory properties of HMGB1 and the immune-privilege of the testis it is very likely that extracellular release of HMGB1 is under tight control in the testis. The signals triggering cytoplasmic localization and possible secretion of HMGB1 are unknown, but may involve pattern recognition receptors such as extracellular TLRs or intracellular NLRs, as well as intracellular signaling molecules including kinases. Future studies involving LPS treatment or cytokine treatment of *in vitro* cultured testicular cells may answer these questions.

4.4 THE AGE DEPENDENT TESTICULAR EXPRESSION PATTERN OF ABC TRANSPORTERS RENDERS THE IMMATURE TESTIS MORE SUSCEPTIBLE TO ADVERSE ACTIONS OF XENOBIOTICS

The clinically important anticancer drug Doxorubicin is a substrate for the ATP-dependent cassette transporter proteins P-gp, Mrp1 and Bcrp. These efflux pumps are expressed in important tissue barriers to protect against toxicity from exogenous harmful agents (Ambudkar et al., 1999, Schinkel and Jonker, 2003). We used doxorubicin as a xenobiotic model to study the development of the ABC transporter dependent efflux system in the rat testis. Doxorubicin have been shown to be more toxic towards the reproductive system of immature rats, compared to older animals and to enhance apoptosis of germ cells in 6-day-old, but not in 16- and 24 day-old rats (Bechter et al., 1987, Hou et al., 2005). We hypothesized that this higher level of testicular toxicity seen in 6-day-old rats was due to the absence of ABC transporter expression, leading to higher uptake of doxorubicin in immature rat testes.

Indeed, we found that the uptake of doxorubicin in rat testes was age-dependent. One hour after intra-peritoneal treatment with a single dose of 3 mg/kg doxorubicin, 6-day-old rats and 16-day-old rats had accumulated 8-fold and 6-fold higher levels of the drug, compared to the testes of 24-day-old rats. The higher level of doxorubicin in the immature testis was not due to a higher vascular blood volume, since point counting revealed that the vascular space in the 6-day-old testis did not differ from that of 16- and 24-day-old rat testis.

We therefore wanted to investigate the developmental expression of ABC-transporter proteins in the rat testis, to see whether a lower level of ABC transporter expression

could explain the higher level of doxorubicin uptake in immature testes. Real-time PCR analyses revealed significantly lower levels of P-gp/Mdr1a, Mrp1 and Bcrp expression in 6-day-old rats compared to 16- and 24 day-old rats. Similar to the testis, ABC transporter expression at the BBB has also been shown to limit the entrance of xenobiotic compounds to the brain (Schinkel et al., 1994, Schinkel et al., 1997). A maturational difference in P-gp/Mdr1a gene expression, analogous to that we detected in the rat testis, has also been observed in the mouse and rat brain (Tsai et al., 2002, Matsuoka et al., 1999). In addition it was shown that reduced P-gp gene expression in the neonatal brain, compare to the adult, resulted in greater P-gp substrate accumulation in the brain of the neonatal mouse (Goralski et al., 2006). This maturational difference in P-gp substrate accumulation was not observed in *mdr1a* knockout mice.

Intra-peritoneal treatment with a single dose of doxorubicin did not seem to have a stimulatory effect on ABC transporter expression in the testis. However, we detected a small, but significant decrease in Mrp1 mRNA expression in 24-day-old rat testes, 4 hours after treatment and a similar decrease in Bcrp expression in the testes of 6-day-old rats, 24 hours after treatment. This is in contrast to other studies, where treatment with different MDR gene substrates has shown to stimulate MDR gene-expression (Chin et al., 1990, Chaudhary and Roninson, 1993, Abolhoda et al., 1999). These studies have been conducted on *in vitro* cultured cells or on tumor tissue, which may explain the different results, compared to our *in vivo* approach.

In the adult rat, human, mouse and guinea pig testis, P-gp is localized to endothelial cells of the testicular capillaries, as well as all other somatic cell types (Melaine et al., 2002). P-gp expression was lacking in spermatogonia, spermatocytes and early spermatids, but was present in late spermatids. Recently it was also shown that P-gp is localized to the BTB in the rat (Su et al., 2009). Since we were interested in the ABC transporter function in the developing testis we investigated the localization of P-gp in the testis from 6-, 16- and 24 day-old rats by IF. We could hardly detect P-gp expression in the 6-day-old testis, but in 16-day-old rats, there was prominent expression in Sertoli cells, endothelial cells as well as some interstitial cells. In the 24-day-old rat testis, the tubular expression of P-gp showed a clear ring-like pattern, corresponding well to the location of the BTB. Our results support our hypothesis, that the ABC transporter efflux-pump system is not fully developed in 6-day-old rats, leading to a higher uptake of drug and thereby increased testicular toxicity at this young age, compared to older animals.

P-gp has been shown to co-localize with several junctional proteins at the BTB in the adult rat testis (Su et al., 2009). We found co-localization of P-gp with the BTB proteins occludin and ZO-1, already at postnatal day 16 in the rat testis, which is the approximate time for when the BTB is formed. In 24-day-old rats, there was clear co-localization of P-gp, occludin and ZO-1 at the BTB. Recently it was shown that the BTB localization of P-gp and its interaction with occludin, claudin-11 and JAM-A increased after adjudin treatment, suggesting that P-gp may decrease luminal entry of adjudin by increasing the tightness of the BTB (Su et al., 2009). In contrast to these results, we found no effect of doxorubicin treatment on the localization or intensity of the P-gp immunostaining at the BTB. Nor did we see an increase in P-gp co-localization with occludin and ZO-1, suggesting that P-gp does not prevent doxorubicin

uptake by increasing the tightness of the BTB, seen after adjudin treatment. Rather, it is likely that P-gp prevents luminal entry of doxorubicin by its efflux function.

There are no studies in humans, showing that treatment with doxorubicin at the pre-pubertal age would result in a higher degree of gonadal damage compared to treatment after puberty. In fact, the gonadal damage caused by doxorubicin and other drugs seems to be less pronounced in humans, compared to rodents. One possible explanation could be species differences in the ontogeny of ABC transporter protein expression. Nevertheless, the ontogeny of ABC transporter expression in the human brain follows a similar pattern as in rodents, with lower ABC transporter gene expression in the immature brain, compared to adults (Daood et al., 2008). A study of P-gp function in the brain of nonhuman primates also showed a developmental difference, with higher uptake of P-gp substrate in the infant, compared to adult brain (Takashima et al., 2011). Since gonadal toxicity still is a major adverse side-effect of chemotherapeutic treatment of childhood cancer, characterization of the developmental expression of ABC transporter proteins in the human testis is of clinical importance to identify potential age groups which may have a higher risk for such toxicity.

It is also important to understand that the combined use of drug-reversed agents (inhibitors of ABC transporter proteins) and chemotherapeutic drugs may increase the risk for side effects, including enhanced neurotoxicity (Greenberg et al., 2005, Hanko et al., 2003). It seems likely that such ABC transporter modulating drugs also may enhance the testicular toxicity associated with chemotherapy, especially in individuals that are at higher risk, due to immature ABC transporter function.

In conclusion, we have found that testes from pre-pubertal rats (6-day-old) accumulate higher levels of the anticancer-drug doxorubicin compared to 16- and 24-day-old rats and that this increased drug-uptake correlates with a very low level of testicular ABC transporter proteins. During postnatal development of the rat testis, there is a gradual increase in ABC transporter protein expression, correlating with a reduced testicular uptake of doxorubicin as the rat testis matures. We suggest that lack of a mature ABC transporter dependent efflux system renders the immature testis more susceptible to xenobiotic-induced toxicity. Further studies employing ABC transporter protein inhibitors need to be done to confirm our results.

4.5 SUMMARY AND CONCLUSIONS

The work of this thesis deals with antimicrobial and xenobiotic protection of the mammalian testis, focusing on the role of CAR, IL-18, HMGB1 and P-gp.

- CAR is expressed in round spermatids, in late stages of elongated spermatids and in released spermatozoa, where it localizes to the acrosomal region.

- CAR was exposed to the surface of acrosome-reacted but not acrosome-intact sperms, indicating a role for CAR in oocyte fertilization.

- CAR co-localized and co-immunoprecipitated with JAM-C in the mouse testis, suggesting that CAR may function in germ cell differentiation and polarization, similar to JAM-C.

- IL-18, IL-18R and ICE were expressed in a constitutive manner during postnatal testicular development in the rat, as well as in the adult rat testis.

- IL-18 protein and mRNA were mainly localized to meiotic and post-meiotic germ cells. Only Pro-IL-18 was detected in interstitial fluid and in testicular cell extracts, suggesting that IL-18 normally is in its non-active form in the healthy testis.

- IL-18 stimulated spermatogonial proliferation and thus may act as a mitogen during pathophysiological conditions in the testis.

- HMGB1 was produced in the Sertoli cells of the human and rat testis and showed both nuclear and cytoplasmic localization.

- HMGB1 purified from human and rat testis demonstrated potent antibacterial activity and may act as an antimicrobial peptide, as well as an immune-stimulatory molecule in the testis.

- Immature rats (6-day-old) accumulated more ABC transporter protein substrates in their testes compared to older rats (16- and 24-day-old), after *in vivo* treatment.

- The higher concentration of doxorubicin in immature rat testes correlated with a lower level of P-gp, Mrp1 and Bcrp1 expression.

- The age-dependent expression pattern of ABC transporters may render the immature testis more susceptible to side effects of cytotoxic drugs as well as other xenobiotics.

5 ACKNOWLEDGEMENTS

Olle Söder
Tack för ditt fantastiska stöd under min långa tid som doktorand i din grupp. Jag vill speciellt tacka dig för att du alltid har uppmuntrat mig att arbeta självständigt, pröva mina idéer och inte vara för blygsam, utan stolt över mitt arbete. Tack även för alla julluncher med efterföljande glögg hemma hos dig och Irene.

Kirsi Jahnukainen
Jag vill tacka dig för ditt utmärkta jobb som min bihandledare. Framförallt vill jag tacka dig för att du noggrant läst mina manuskript och alltid lyckats hjälpa mig att skala bort allt onödigt så att budskapet kommer fram klart och tydligt. Jag vill även tacka för hjälp med djurförsök och på hela labbets vägnar för att du kommer hit då och då och livar upp tillvaron.

Staffan Eksborg
Tack så mycket för ditt fina stöd under tiden som min bihandledare och speciellt för att du hjälpt mig så tålmodigt med figurerna och statistiken. Tack också för alla intressanta samtal om forskning, men också om livet utanför Karolinska, om hästresor och om fotografering.

Yvonne Löfgren
Tusen tack för allt pipetterande och alla PCR:er som du hjälpt mig med. Tack också för den hjälp som jag fått med allt som ingår i arbetet på labbet. Tack framförallt för alla goda skratt som vi har haft i labbet och för trevligt sällskap på våra testis workshop resor.

Aida Wahlgren
Tack för allting från trevliga middagar hos dig och Hasse till alla vetenskapliga och icke vetenskapliga samtal som vi haft, tack för sällskap på resor och konferenser, tack för all hjälp i labbet med transilluminationsteknik, stadiedissektion och squashpreparationer och tack för allt roligt som vi gjort tillsammans.

Konstantin Svechnikov
Thank you for always being so helpful with everything in the lab concerning testosterone measurements, Leydig cell isolation and western blots. Also thank you for all the things I have learned about Russia.

Nina Renlund
Tack för allt roligt som vi har gjort tillsammans både med labbet och utanför. Tack för allt stöd och för alla trevliga luncher som vi haft. Tack också för att du fortsätter försöka hålla kontakt trots utomlandsvistelse, flytt och småbarnsliv.

Cecilia Zetterström
Tack för ett trevligt och givande samarbete med lab-projekt och publikationer. Tusen tack också för att du trots tidsbrist alltid är så hjälpsam och som nu senast grävde igenom dina lådor efter ett urgammalt etiskt tillstånd som jag behövde.

Christin Skwirut
Tack för allt uppmuntrande och positivt som du alltid lyckas säga om mig (och alla andra också). Självklart så är jag också tacksam över hjälp med allt smått och gott som ingår i ett laboratorium.

Martin Ritzén
Tack för att du varit med och grundat barnendokrinlab som är en mycket trevlig arbetslpats att få jobba på.

Cecilia Petersen
Tack för din hjälpsamhet och din välvilja. Tack också för alla trevliga samtal både på labbet och på testis workshop resor. Du har alltid kloka saker att säga och jag har lärt mig mycket av dig.

Mi Hou
Thank you for all the help with animal and laboratory experimental work, for your help with solving scientific problems and for being so friendly and easy to work with.

Taranum Sultana
Thank you for helping me with animal experiments, manuscripts and for scientific input regarding my last project. Also thank you for your generosity regarding antibodies.

Jan-Bernd Stukenborg
Thanks for that you, together with Luise, have contributed to make the testis group even a nicer and better research group.

Luise Landreh
Tack också du för att du bidragit med så mycket vetenskapligt, laboratoriemässigt och social till vår lilla testis grupp och gjort barnendokrinlab lite roligare och lite trevligare. Tusen tack för att du hjälpt till att språkgranska min avhandling.

Emelie Benyi
Tack för all trevliga lunchdiskussioner om småbarns-och forskarlivet som vi har haft och för roliga fester o utflykter tillsammans med barnen. Det uppskattas med lite roliga avbrott i vardagsträsket!

Mirja Nurmio
Tack för allt roligt som vi haft, bland annat när vi firade Vaphu i Turku, när vi startade INYRMF och på konferenser och möten. Tack också för att vi fick möjligheten att komma på en finsk disputaion!

Ulrika Berg
Dig vill jag också tacka för alla intressanta samtal om forskningen, dansen och livet och också stort tack för trevligt sällskap både i doktorandrummet och på danstillställningar.

Britt Massironi
Tack för alla värdefulla tips och troubleshooting vad det gäller PCR- och immunohistokemi experiment och annat som rör forskningen och labbet (inte minst datorproblem).

Carina Ritzmo
Tack så mycket för att du hjälpt mig med alla HPLC mätningar och för all hjälp med manuscript.

Paola Fernandez-Vojvodich
Thank you for your company in the PhD room as well as the coffie room and for the nice chats that we have had. Thank you also for our joyful midsummer party last summer!

Michael Landreh
Thank you so much for correcting my thesis manuscript.

Christian Sköld
Tusen tack för granskningen av min avhandling, samt för att du ställt upp som barnvakt och lekledare.

Mari Strand
Tack min lilla syster för hjälp med granskning av avhandlingen och inte minst barnpassning och annan markservice.

Tack alla mina kära medarbetare både på och utanför barnendokrinlab, *Susanne Hallberg, Lena Sahlin ,Momina, Mirza, Kerstin Sollerbrant, Berit Fröjsa, Gunnie Westerholm, Martti Parvinen, Lars Hagenäs, Lars Sävendahl, Mikael Holst, Dionisios Chrysis, Ola Nilsson, Vichit Supornsilchai, Blesson Selvanesan, Maryana Hulchiy, Lin Ma, Mikael Holst, Peter Bang, Mikael von Euler, Maria Ahlsen, Sara Phil, Irina Svechnikova, Brian Setchell, Giedre Grigelioniene, Eugenia Colón, Shazad Akram, Ahmed Reda, Juliia Savchuk, Rós Kjartansdóttir, Emma Eriksson, Andrei Chagin, Farasat Zalman, Katja Sundström,Therese Cedervall, Elham, Karimian, Bettina Cederqvist, Hong Su, Maryam Iravani, Sanne van Raaij ,Juanjuan Zhang.*

Tack min kära familj, mina (världens bästa) föräldrar *Mona* och *Benny* som ställer upp och är barnvakt titt som tätt, min syster *Malin* med familj som också alltid ställer upp och mina svärföräldrar *Ingvor* och *Stig* som hjälper oss med våra små pojkar.

Tack mina fina, trogna vänner som följt med genom åren av pluggande, forskande och dans, *Marie Björklund, Marie Carlén, Anna-Karin Sundström, Malin Sköld* och *Veronika Nilsson*. Äntligen ska ni få gå på (min) disputationsfest! Extratack till *Anna-Karin Sundström* för dina illustrationer av Sertoli celler, könsceller och blod–testisbarriären.

Framförallt vill jag tacka min stora kärlek och klippa *Christer*, som alltid stöttat mig och som stått ut med mig och ”mina testiklar” under alla dessa år som doktorand. Att denna avhandling till sist blev klar är till stor del din förtjänst som skött markservicen där hemma.

Sist, men inte minst vill jag tacka mina små juveler och ”hjälpforskare” *Maximilian*, som tålmodigt försöker få mig att upptäcka det fascinerande och intressanta med centrifuger och tvättmaskiner, *David* för att du roar mig med diverse spännande experiment och kreativa uppfinningar hemma i köket och så lilla *Dante*, som snällt har spenderat timmar i sjalen när jag suttit och mikroskoperat.

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