Characterization of the micro-environment of the testis that shapes the phenotype and function of testicular macrophages

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CONTENTS

ABBREVIATIONS

ACAID	Anterior chamber-associated immune deviation
ADX	Adrenalectomy
AM	Alveolar macrophage
ANOVA	Analysis of variance
APC	Antigen presenting cells
APS	Ammonium persulfate
AqH	Aqueous humor
AR	Androgen receptor
ASA	Anti-sperm antibodies
BMM	Bone marrow derived macrophages
BMP-6	Bone morphogenetic protein 6
BMP-7	Bone morphogenetic protein 7
BRB	Blood-retina barrier
BSA	Bovine serum albumin
BTB	blood-testis barrier
°C	Degree Celsius
CC10	Clara cell protein 10
CCL-4	Chemokine (C-C motif) ligand 4
CCL7	Chemokine (C-C motif) ligand 7
CCL21	Chemokine (C-C motif) ligand 21
cDNA	Complementary DNA
CD4	Cluster of Differentiation 4
CD25	Cluster of Differentiation 25
CD32	Cluster of Differentiation 32
CD45	Cluster of Differentiation 45
CD68	Cluster of Differentiation 68
CD80	Cluster of Differentiation 68
CD86	Cluster of Differentiation 86
CD163	Cluster of Differentiation 163
CNS	Central nervous system
COX	Cyclooxygenases
CREB	cAMP response element binding protein
CSF-1	Colony-stimulating factor 1
2D	two-dimensional
DC	Dendritic cells
DAPI	4’, 6’-diamino-2-phenylindole
DHT	Dihydrotestosterone
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid

DNase	Deoxyribonuclease
dNTPs	2'-deoxynucleoside-5'-triphosphate
DTT	Dithiothreitol
EAO	Experimental autoimmune orchitis
EB	Ethidium bromide
ECL	chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
EDS	Ethane dimethane sulphonate
ELISA	Enzyme-linked Immunosorbent Assay
FCS	Fetal calf serum
Foxp3	Forkhead box P3
GC-MS/MS	Gas Chromatography mass spectrometry
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR	Glucocorticoid receptor
GREs	GCs response elements
HRP	Horseradish peroxidase
HSC	hematopoietic stem cell
HSPC	hematopoietic stem and progenitor cells
IF	interstitial fluid
IFN-γ	Interferon-γ
IDO	Indolamin-2, 3-dioxygenase
IL-1α	Interleukin 1α
IL-1β	Interleukin 1β
IL-2	Interleukin 2
Il-6	Interleukin 6
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-15	Interleukin 15
IL-18	Interleukin 18
IRAK-M	IL-1R-associated kinase-M
JNK	c-Jun N-terminal kinase
kD	Kilo Dalton
LPS	Lipopolysaccharide
LC	Leydig cells
LC-MS/MS	liquid chromatography–MS/MS
LH	luteinizing hormone
M	Molar
MALDI-TOF-MS	Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony stimulating factor
MEM	Minimum essential medium

mg	Milligram
MHC	Major Histocompatibility Complex
MIF	Macrophage migration inhibitory factor
min	Minute
MIP	macrophage inflammatory protein
ml	Milliliter
mM	Milimolar
MOI	Multiplicity of infection
MS	Mass spectrometry
MW	Molecular weight
MYD88	Myeloid differentiation primary-response protein 88
MZ	Marginal zone
NaCl	Sodium chloride
NF-κB	Nuclear factor-Kb
NO	Nitric oxide
NOS	Nitric oxide synthase
NP-40	Nonidet P-40
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death receptor 1
PD-L1	Programmed death ligand-1
PG	Prostaglandin
PI3K	Phosphatidylinositol 3-kinase
PM	Peritoneal macrophage
PMSF	Phenylmethylsulfonyl fluoride
PPAR-γ	peroxisome proliferator-activated receptor-γ
PRR	Pattern recognition receptor
P/S	Penicillin/Streptomycin
PTC	Peritubular cells
RA	Retinoic acid
RE	relative expression
RIG-I	Retinoic acid-inducible gene I
RIP	Receptor interacting protein
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS-2	Reactive oxygen species -2
RT-PCR	Real time polymerase chain reaction
qRT-PCR	Quantitative real-time RT PCR
rpm	Revolutions per minute
RT	Room temperature

SC	Sertoli cells
SDS	Sodiumdodecylsulphate
sec	Second
SOCS	suppressor of cytokine signaling
SRC-1	steroid receptor coactivator-1
STAT3	Signal transducer and activator of transcription 3
TAE	Tris acetate EDTA
TAM	Axl and Mer
TBS	Tris buffered saline
TGF-β	Transforming growth factor β
TJ	Tight junctions
TLR	Toll like receptor
TM	Testicular macrophage
TNF-α	Tumor necrosis factor alpha
TRAF6	TNF receptor-associated factor 6
Tregs	T regulatory cells
Tris	Tris (hydroxymethyl) amino methane
UV	Ultraviolet
µ	Micro
µg	Microgram
µl	Microliter
µM	Micromolar
V	Volt
v/v	Volume per volume
w/v	Weight per volume

1. INTRODUCTION

1.1 The testis is an immune privileged organ

1.1.1 The definition of testicular immune privilege

Immune privilege of an organ is defined as the capability of tolerating foreign graft for prolonged periods of time without eliciting an inflammatory immune response (Forrester et al., 2008). Besides the testis, immune privileged organ include the central nervous system (CNS), the anterior chamber of the eye, the pregnant uterus as well as tumor-draining lymph nodes (Asano et al., 2015; Fijak and Meinhardt, 2006; Mellor and Munn, 2008). Over the past decades, these immune privileged organs are believed to be segregated from the lymphatic system, inhibiting access of innate and adaptive immune cells at these sites (Benhar et al., 2012). As a plethora of neo-autoantigens are expressed during spermatogenesis, the immune privilege of the testis is critical to protect the developing germ cells from auto-immune attack (Arck et al., 2014; Fijak and Meinhardt, 2006; Zhao et al., 2014). First observations were made in 1767 by John Hunter, who transplanted a testis from cock into the belly of hen, with the normal structure of the testis being preserved (Zhao et al., 2014). Two centenary later, Head and colleagues transplanted skin and parathyroid allografts to the interstitial space of the testis, both of which survived for a longer period in comparison to orthotropic grafts (Head et al., 1983). Hence, testicular immune privilege is considered to be an evolutionary adaptation to safeguard reproductive capability (Fijak and Meinhardt, 2006). In pathological conditions, the testicular immune privilege will be compromised. As a consequence, the production of anti-sperm antibodies (ASA) can be induced, which could lead to male infertility by affecting the sperm's fertilizing capacity (Wenes et al., 2016). A number of recent studies were devoted to investigate the underlying mechanisms of testicular immune privilege, which suggested that multiple mechanisms including testicular physical barriers, local immunosuppression and systemic immune tolerance are involved (Bhushan and Meinhardt, 2016; Zhao et al., 2014).

1.1.2 The contribution of structural elements to testicular immune privilege

The blood-testis barrier (BTB) is formed by tight junctions (TJ), basal ectoplasmic specializations, gap and desmosome-like junctions between the Sertoli cells (SC), which

divide the seminiferous tubule into a basal and apical (adluminal) compartment (Arck et al., 2014; Jiang et al., 2014). This physical barrier can sequestrate the auto-antigens of germ cells from the immune system. Moreover, SC have inherent immunosuppressive properties by secreting immunosuppressive factors, including anti-inflammatory proteins such as transforming growth factor-β (TGF-β), activin, complement, and proteinase inhibitors as well as indoleamin-2,3-dioxygenase (IDO) (Fallarino et al., 2009; Lee et al., 2007; O'Bryan et al., 2005; Sipione et al., 2006; Suarez-Pinzon et al., 2000). The immunosuppressive properties of SC are corroborated by SC co-transplantation studies, where pancreatic islets survived longer if applied with SC. Germ cells undergo apoptosis during spermatogenesis and these apoptotic cells are able to induce inflammatory responses, if not eliminated timely (Li et al., 2012; Zhao et al., 2014). In order to avoid autoimmune responses, SC can also engulf residual bodies from spermatids beside apoptotic germ cells, as a means for maintaining the testicular immune privilege and thus normal spermatogenesis (Asano et al., 2015).

Initially it was assumed that the tight junctions between SC are essential for protecting seminiferous tubules from a potent immune response. However, in early spermatogenesis, preleptotene spermatocytes and spermatogonia are located outside of the BTB without eliciting any autoimmune response (Setchell, 1990; Yule et al., 1988; Zhao et al., 2014). This implies that there are mechanisms other than the BTB to contribute the testicular immune privilege (Fijak and Meinhardt, 2006; Shechter et al., 2013). Data accumulated over the last decades implied that localized active immunosuppression rather than simple sequestration of auto-antigens by the BTB is central in the establishment and maintenance of testicular immune privilege (Fijak and Meinhardt, 2006). Mechanism implicated in the control of immune privilege seems to include high levels of intratesticular androgens and particularly the immunosuppressive properties of local cells such as SC in the seminiferous epithelium, regulatory T cells, macrophages and possibly Leydig cells (LC) (Bhushan and Meinhardt, 2016; Fijak and Meinhardt, 2006; Goldberg, 2015; Li et al., 2012; Meinhardt and Hedger, 2011; Zhao et al., 2014).

1.1.3 The role of the testicular interstitial cells in the maintenance of testicular immune privilege

In the testis of most mammals, the interstitial space is composed of a variety of different cells. The largest population of interstitial cells, steroidogenic LC, are critical for the production of androgens, which play a vital role in spermatogenesis (Fijak and Meinhardt, 2006; Zhao et al.,

2014). It has been demonstrated that androgens have anti-inflammatory functions and are able to inhibit the autoimmune response of the testis (Fijak and Meinhardt, 2006). In this regard, testosterone promotes the generation and polarization of regulatory T cell in the testis *in vivo* and inhibits the transcription of TNF-α in SC and peritubular cells（PTC）under inflammatory conditions *in vitro* (Fijak et al., 2015). Moreover, LC are able to affect macrophage function by producing a number of nonsteroidal immunoregulatory factors such as macrophage migration inhibitory factor (MIF), β-endorphin and prodynorphin (Marvizon et al., 2009; Meinhardt et al., 2000).

Testicular macrophages (TM) represent the principal immune cell population residing in the interstitial space of the testis (Bhushan and Meinhardt, 2016). In contrast to macrophages in other tissues, TM exhibit a reduced capacity to synthesize pro-inflammatory cytokines, most notablably TNF-α and IL-1β, concomitant with an enhanced ability to produce anti-inflammatory cytokines such as interleukin 10 (IL-10) (Bhushan et al., 2015). Under physiological conditions more than 80% of TM display an immunosuppressive M2 phenotype characterized by the expression of CD163, a marker of M2 macrophages (Bhushan and Meinhardt, 2016; Meinhardt and Hedger, 2011; Winnall and Hedger, 2013). The immunosuppressive M2 TM phenotype has been proposed to be relevant for the maintenance of testicular immune privilege (Bhushan et al., 2015).

Dendritic cells (DC) as antigen presenting cells (APC) are also found to be located in the interstitial space of the testis. They play an important role in the initiation and regulation of adaptive immune response (Guazzone et al., 2011). DC can induce the activation and differentiation of lymphocytes in response to allo-antigens, thereby minimizing autoimmune responses (Banchereau and Steinman, 1998; Rival et al., 2006). In mouse experimental autoimmune orchitis (EAO), the number of DC was dramatically increased in the testis, mostly in granulomas, but also in testicular draining lymph nodes (Rival et al., 2006), suggesting that DC may play a role in testicular autoimmune responses. Despite the assumed importance of DC in maintaining testicular immune privilege, this relatively small subpopulation of testicular interstitial cells has not been extensively investigated and their function in regulation of testicular immune homeostasis warrants further study.

Mast cells prominently participated in allergic as well as immune responses to parasites (Hussein et al., 2005). However, there are few studies showing that mast cells also play an important role in adaptive and innate immune responses. Mast cells can induce tissue fibrosis

and sclerosis by promoting fibroblast proliferation and collagen synthesis (Abe et al., 1998). Studies have demonstrated that increased mast cell numbers are associated with abnormal spermatogenesis and male infertility, related to mast cell induced tissue fibrosis and sclerosis in the testis (Fijak and Meinhardt, 2006; Jezek et al., 1999; Meineke et al., 2000). In the rat EAO model, the number of testicular mast cells is significantly increased, which suggests a role in disease development (Iosub et al., 2006). In contrast to the above mentioned, mast cells have also been demonstrated to be relevant in $CD4^+CD25^+Foxp3^+$ regulatory T-cell-dependent peripheral tolerance (Lu et al., 2006). In view of their restricted localization and relatively small numbers in the testis, their role in testicular immune privilege remains elusive.

A variety of lymphocyte subpopulations including $CD4^+$ T, $CD8^+$ T and $CD4^+CD25^+$ regulatory T cells (Tregs) have been identified in the testis (Hedger and Meinhardt, 2000). Most of the lymphocytes are $CD8^+$ T cells, with a small subpopulation of $CD4^+$ T cells. In contrast, no B cell have been identified in the testis under normal conditions (Lustig et al., 1993). In the EAO model, the number of both $CD4^+$ and $CD8^+$ lymphocytes were significantly increased in the early stage of disease, whilst the number of $CD4^+$ T cells decreased. Numbers of $CD8^+$ T cells did not change during the development of EAO, suggesting that rather the $CD4^+$ T cells play a role in initiation of chronic EAO (el-Demiry et al., 1987; Lustig et al., 1993). Furthermore, adoptive transfer of T cells from EAO animals can cause orchitis in healthy recipients, pointing to a role of T cells in the pathogenesis of orchitis. In addition, $CD4^+CD25^+$ Tregs have also been found in the testicular interstitium, with these cells believed to play an important role in immune privilege due to their immunosuppressive function by inhibition immune responses (Dai et al., 2005; Wheeler et al., 2011; Zhao et al., 2014).

1.1.4. Maintenance of testicular immune privilege by local immunosuppressive factors and systemic immune tolerance

Increasing evidence indicates that the local immunosuppressive milieu can also contribute to the immune privileged status of respective organs (Fijak and Meinhardt, 2006; Li et al., 2012). For instance, when antigenic material entered into the anterior chamber of the eye, it can initiate a systemic immune response and result in immune deviation (Zhou and Caspi, 2010). The anterior chamber-associated immune deviation (ACAID) is able to suppress the T cell-mediated immune response and induce the production of non-complement-fixing

antibodies, which avoid the occurrence of inflammation (Benhar et al., 2012).

Androgens are synthesized in the testis by LC upon stimulation with luteinizing hormone (LH). They play an important role in spermatogenesis, development and maintenance of male secondary sex characteristics, but also in the suppression of immune responses (Bhushan et al., 2015; Cutolo, 2009). Several studies have demonstrated that androgen treatment can ameliorate autoimmune diseases such as rheumatoid arthritis by suppressing aggressive autoimmune responses (Cutolo, 2009). On the molecular level, both experimental and clinical studies have illustrated the role of androgens on the regulation of pro-inflammatory cytokine expression. The pro-inflammatory cytokines including TNF-α, IL-1 and IL-6 were down regulated by testosterone stimulation, whereas the anti-inflammatory cytokine IL-10 was up-regulated (Bhushan et al., 2015). *In vivo*, in the EAO model testosterone substitution can inhibit the systemic immune responses to auto-antigens by preventing the accumulation of macrophages and $CD4^+$ T cells, whilst simultaneously increasing the number of immunosuppressive $CD4^+CD25^+Foxp3^+$ Tregs (Fijak et al., 2011). Collectively, androgens are proposed to maintain testicular immune privilege by inducing systemic tolerance to auto-antigens and suppressing the local immune response.

Besides of steroid hormones, testicular cells produce various immunosuppressive factors, which are also of particular importance in regulating the immune privileged status of the testis. IL-10, mainly secreted by TM, can significantly suppress the inflammation in EAO (Li et al., 2012). Moreover, TGF-β, a highly immunosuppressive molecule can be produced by SC and LC (Ko et al., 2016). Another anti-inflammatory cytokine, activin A, which belongs to TGF-β family, is expressed in most of the testicular somatic cells. Activin A can suppress the production of pro-inflammatory cytokines thus maintaining the immune privilege of the testis (Phillips et al., 2009).

In addition to immunosuppressive factors, there are various negative regulatory systems which also contribute to the testicular immune privilege. For example, the FasL/Fas system involving SC and germ cells can inhibit the immune responses by inducing T cell apoptosis (Glass and Natoli, 2016). The tyrosine kinase receptor Tyro3 family, which including Tyro3, Axl and Mer (TAM) and their ligands growth-arrest-specific gene 6 (Gas6) and Protein S (ProS), were found to be expressed in mouse testis, which can negatively regulate immune response (Rothlin et al., 2007). Moreover, Gas6/ProS-TAM can also promote phagocytosis of apoptotic germ cells to avoid autoimmunity (Wang et al., 2005). Hence, the Gas6/ProS-TAM

system is important to maintain testicular homeostasis by suppressing local inflammatory responses and promoting the clearance of auto-antigens in the testis (Wang et al., 2005; Zhao et al., 2014). Furthermore, programmed death ligand-1 (PD-L1) and programmed death receptor (PD-1) are constitutively expressed in the testis, which can suppress T cell activation (Keir et al., 2008).

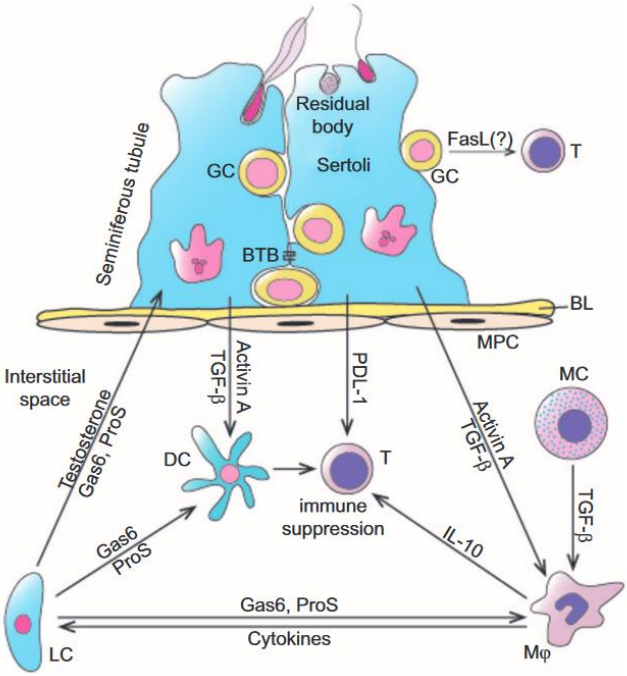


Figure 1. Schematic overview of immunosuppressive molecules that support immune privilege in the testis （Zhao et al., Cellular & Molecular Immunology, 2014, 11, 428-437. With the permission of the publisher, license number: 4142951190701).

1.2 Introduction to macrophage biology

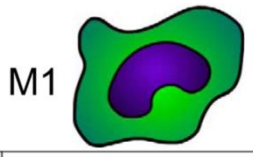
Macrophages belong to the APC and are present in almost all tissues. They play an essential role in numerous vital functions of the body including tissue development, homeostasis, tissue repair and immunity (Bhushan et al., 2015; Okabe and Medzhitov, 2016). In the late nineteenth century, Eli Metchnikoff first described the role of macrophages in immunity (Okabe and Medzhitov, 2016; Tauber, 2003). Macrophages are the first line of defense against the invading pathogens including bacteria, viruses and parasites. Besides activating innate and adaptive immunity, macrophages play an essential role in maintaining tissue homeostasis, inflammation resolution and tissue repair (Ferrer et al., 2017; Olingy et al., 2017). Conventionally, macrophages are classified into a classical inflammatory-activated (M1) state and an alternatively activated immunosuppressive (M2) state. M1 macrophages facilitate the

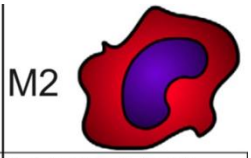
clearance of invading pathogens by secreting pro-inflammatory mediators, whereas M2 macrophages are essential for tissue homeostasis, immune surveillance, and inflammation resolution (Gordon et al., 2014; Italiani and Boraschi, 2014; Wynn et al., 2013). Among myeloid cells, macrophages show remarkable plasticity features which enable them to change their phenotype according to the type of stimuli received from the surrounding microenvironment (Mosser and Edwards, 2008; Okabe and Medzhitov, 2016). Macrophage precursor cells can be polarized to different macrophage phenotypes upon specific stimulation (Serbulea et al., 2017; Vergadi et al., 2017). For example, granulocyte macrophage colony stimulating factor (GM-CSF), the Th1 cytokine interferon-γ (IFNγ), and Toll-like receptor (TLR) ligands such as lipopolysaccharide (LPS) or tumor necrosis factor (TNF-α) can induce macrophage precursor cells to differentiate into M1 macrophages. M1 macrophages secrete high amounts of pro-inflammatory cytokines, including IL-1, IL-6, IL-12, IL-23, TNF-α, and reactive oxygen species -2 (ROS-2), which can promote inflammatory responses to ensure efficient microbial killing (Gombozhapova et al., 2017). In contrast, M2 macrophages are induced by multiple mediators such as macrophage colony stimulating factor (M-CSF), IL-10, transforming growth factor β (TGF-β), prostaglandins (PG), glucocorticoids, IL-4 and IL-13 (Maresz et al., 2008; Martinez et al., 2009). M2 macrophages are characterized by the expression of the scavenger receptor cluster of differentiation 163 (CD163), the secretion of large amounts of the anti-inflammatory cytokine IL-10 and low amounts of pro-inflammatory cytokines such as TNF-α (Bhushan et al., 2015; Italiani and Boraschi, 2014). In addition, mature macrophages also can switch phenotype upon adequate stimulation.

During embryo development and throughout adult life, macrophages are remarkably phenotypically diverse. Until last century, macrophages were considered to differentiate from circulating peripheral blood mononuclear cells (PBMCs), with subsequent migration into tissues to replace the aged resident tissue macrophages in both steady and inflammatory conditions (Prinz and Priller, 2014). However, in the advent of new technologies such as fate mapping, lineage tracing, parabiotic mice and transgenic mouse models, the origin of resident tissue macrophages and the ontogeny of macrophages has been re-written (Prinz and Priller, 2014). Accumulating data have demonstrated that most of the resident tissue macrophages in fetal or adult tissue have at least three different sources, including yolk sac, fetal liver and bone marrow derived monocytes (Ginhoux and Guilliams, 2016). During embryonic development, macrophages emerged from the yolk sac and migrated to different fetal organs. In adult life, however, they are sustained via longevity and local self-renewal (Okabe and

Medzhitov, 2016). The resident macrophages of the brain (microglia) exclusively originate from yolk sac macrophages, whereas skin macrophages (Langerhans cells) are derived from both yolk sac and fetal liver monocytes (Prinz and Priller, 2014). In turn, the fetal liver monocytes contribute to the macrophage population of the lung (alveolar macrophages) and the liver (Kupffer cells) (Guilliams et al., 2013). In contrast, macrophages of the gut, dermis, and heart - although derived from primitive yolk sac macrophages at the neonatal stage-are being constantly replaced by bone marrow derived circulatory monocytes in adult mice (Ginhoux and Guilliams, 2016; Okabe and Medzhitov, 2016).

Resident macrophages elicit distinct tissue-specific functions based on their phenotypes and the local microenvironment. For example, microglia has special roles in the development and function of the central nervous system (CNS) including maintenance of brain immune privilege (Prinz and Priller, 2014; Walker and Lue, 2015). Similarly, intestinal macrophages maintain the intestinal homeostasis by displaying inflammation anergy upon encountering with inflammatory stimuli (Cipriani et al., 2016; Kuhl et al., 2015). Lung alveolar macrophages (AM) play a relevant role in recycling of surfactant molecules and surveillance of inhaled pathogens. In contrast to peritoneal macrophages (PM), AM show higher efficient phagocytic activity and a stronger inflammatory response by secreting high levels of TNF-α upon stimulation with pro-inflammatory stimuli. In addition, the macrophage specific markers CD14, CD163 are highly expressed in AM, but not PM, suggesting an influence of the local microenvironment in macrophage function (Cai et al., 2014; Pilling et al., 2009; Tedesco et al., 2015). Kupffer cells in liver are critical for sustaining the normal function of the organ by a series of processes including toxin removal, iron recycling, erythrocyte clearance, lipid metabolism, clearance of microbes and cell debris from blood (Krenkel and Tacke, 2017; Nairz et al., 2017; Theurl et al., 2016). In summary, macrophages are unique immune cells in regards to plasticity, heterogeneity and origin. Tissue resident macrophages are derived from embryonic sources, followed by migration to target tissues during embryo development, where they differentiate to tissue specific macrophages with great plasticity in terms of theirs phenotypes and function based on influence of their local environment. Eliciting tissue specific programs is complex and mediated by local signals and finally implemented by interaction of metabolic pathway, signaling cascades, transcription factors and epigenetic changes.





Polarizing stimulus	IFN-γ, LPS, IFN-γ+LPS	IL-4, IL-13, Ic, IL-10, GC, GC+TGFβ
Phenotype	Pro-inflammatory	Anti-inflammatory
In vitro morphology	Round/oval	Elongated, fibroblast-like
Products/Markers	TNFα, IL-1β, IL-6, IL-12, IL-23, CXCL10, pSTAT1, MMP9	IL-10, TGFβ, CCL17, CCL22, CD163, CD206, pSTAT3/6
Phagocytic activity	High	Low
Antigen presentation	High	Low
Arginine metabolism	iNOS: Arginine -> NO	Arg1: Arginine -> Ornithine
Antibacterial capacity	High	Low
Effect on tumors	Tumoricidal	Protumorigenic

Figure 2. Macrophage polarization and distinguishing features of M1 and M2 macrophages (Raymond A et. al, American Journal of Physiology, 2016. With the permission of the publisher).

1.3 Testicular macrophages

TM represent the largest immune cell population of the testis (Bhushan and Meinhardt, 2016; Hussein et al., 2005). It is well documented that TM play a vital role in testis development, steroidogenesis, and spermatogenesis (DeFalco et al., 2015). In mice and rats, the two species most commonly studied, TM represent around 20% of total testicular interstitial cells, excluding blood vessels. They are localized exclusively outside of the seminiferous epithelium under normal conditions (Bhushan and Meinhardt, 2016). In comparison to macrophages in other organs, TM express remarkably low levels of pro-inflammatory genes, with concomitant high expression of anti-inflammatory factors, a balance that helps to sustain the testicular immunosuppressive environment (Li et al., 2012). In addition, TM display the M2 phenotype under normal conditions, which is critical for maintaining the immunosuppressive testicular milieu. *In vitro* studies indicated that TM display immunosuppressive features with a decreased capacity to produce pro-inflammatory cytokines TNF-α and IL-1β (Bhushan and Meinhardt, 2016). Mechanistically, our laboratory has shown that this is medicated by suppressing the NF-κB signaling pathway via impaired ubiquitination of IκBα, leaving this inhibitor bound to NF-κB in spite of upstream activation of the pathway. Moreover, TLR pathway genes were reduced in expression (Bhushan and Meinhardt, 2016).

1.3.1 The phenotypic heterogeneity of testicular macrophages

Macrophages can originate from different sources and show high heterogeneity and plasticity. There is accumulating evidence indicating that TM are heterogeneous in their function and phenotype (Bhushan and Meinhardt, 2016). In the last decade, more specific markers have been applied to investigate the function and phenotype of macrophages. In this regard, CD34 and CD68 are originally defined as specific markers for monocytes and macrophages, respectively, whilst CD163, arginase-1 and IL-10 are markers for tissue resident macrophages (Zhao et al., 2014). CD68 or CD163 are now widely used to categorize macrophage in the M1 and M2 phenotype. These two receptors can divide TM into three populations, $CD68^{+}CD163^{-}$ were considered as "newly arrived" cells with monocyte properties. $CD68^{-}CD163^{+}$ are mature tissue-resident macrophages representing the major population of the testis. In consequence, $CD68^{+}CD163^{+}$ are "intermediate" TM, meaning "newly-arrived" monocytes that are about to differentiate to mature tissue resident macrophages. It is believed that approximately 80% of resident TM display the M2 macrophage phenotype characterized by expressing the scavenger receptor CD163, whereas approximately 20% represent the M1 phenotype identified by the scavenger receptor CD68 (Winnall and Hedger, 2013).

More mechanistically, when comparing TM to bone marrow derived macrophages (BMM), BMM were found to express higher amounts of pro-inflammatory cytokines such as TNF-a and IL-1β after challenge with a combination of LPS and interferon-γ (IFN-γ) (Winnall et al., 2011). In contrast, TM instead produced higher levels of the anti-inflammatory cytokines IL-10, macrophage inflammatory protein (MIP)-2α/CXCL2 and suppressor of cytokine signaling (SOCS)-1 (Winnall et al., 2011). Those data indicated that TM respond with an alternative activated inflammatory response to inflammatory stimuli. The M2 phenotype of TM was further corroborated by other *in vitro* studies, in which the treatment of TM with inflammatory stimuli induced the secretion of a large amount of IL-10 and a low amount of TNF-α in comparison to PM (Bhushan and Meinhardt, 2016). Moreover, an anti-inflammatory STAT3 signaling pathway was activated in TM and low levels of NO were secreted (Bhushan et al., 2015). By separating rat TM into two population either negative or positive for CD163 expression by FACS sorting, $CD163^{+}$ TM produced significantly higher amounts of IL-10 compared to $CD163^{-}$ TM (Winnall and Hedger, 2013). Taken together, the majority of the TM population displays an alternative activated immunosuppressive M2 phenotype under normal conditions.

The phenotypic heterogeneity of TM has also been confirmed in other species. In mouse testis, F4/80 is established as a specific macrophage marker, but cells lack some other well defined macrophage markers such as CD80, CD86, MOMA1 and MOMA2. The lectin-binding protein Ym was identified as an alternatively activated cell marker to distinguish the TM population (Webb et al., 2001). The Ym^+ cells represent a large and macrophage-like population in the testis, may be categorized as M2 macrophages, although further experiments need to be re-examined (Maresz et al., 2008).

1.3.2 Innate immune functions of testicular macrophages

In spite of the immune privileged status of the testis, the organ can initiate innate immune responses against invading pathogens. Studies form Jegou and colleagues showed the presence of several defense factors such as interferons (IFNs) and antiviral proteins, in murine and human testis . Increasing evidence indicates that pattern recognition receptors (PRRs) are generally expressed in testicular cells, as a means to initiate testicular innate immune responses. Once the immune response is activated, it can override testicular immune privilege (Iwasaki and Medzhitov, 2010).

As the best characterized PRRs, toll-like receptors (TLRs) were found to be expressed in most testicular cells (Hedger, 2011). TLRs play a critical role in early host defense against invading pathogens. For example, in mouse SC it has been demonstrated that functional TLRs, including TLR2, TLR3, TLR4, TLR5 and TLR6 can be addressed by their respective ligands with subsequent secretion of immunoregulatory cytokines such as IL-1, IL-6, TNF-a, type 1 IFNs and monocyte chemoattractant protein-1 (MCP-1) (Wu et al., 2008). In addition, TLRs were also identified to be expressed in different stages of germ cell development. Wang et al. have found that TLR3 is present in spermatogonia and spermatocytes, and TLR11 is expressed in spermatids (Wang et al., 2012). Besides SC and germ cells, TLR3 and TLR4 are also found to be expressed in murine LC. Addressing them with their cognate ligands polyinosinic-polycytidylic acid (Poly (I:C)) and LPS, production of inflammatory factors such as TNF-α and IL-6 was subsequently induced (Zhao et al., 2014). Notably, it is shown that Gas6/ProS-TAM negatively regulates the TLR-initiated innate immune response in LC and SC as a means to avoid damage of the testis in inflammation (Lemke and Rothlin, 2008). Interestingly, the expression of TLRs in TM is significantly reduced compared to other macrophages, a means proposed to be associated with its immunosuppressive properties.

As the first line of defense against microorganisms, macrophages play an essential role in the host's innate immune system. Generally, macrophages elicit an immune response by recognizing pathogen associated molecular patterns (PAMPs) of microorganism (Kumar et al., 2011). Macrophages express a diverse range of pattern-recognition receptors such as TLRs, helicase retinoic acid-inducible gene I (RIG-I)–like receptors (RLRs), C-type lectin receptors and biosensor NOD–like receptors. These receptors play a vital role in activation of innate immune responses (Iwasaki and Medzhitov, 2010). PAMPs such as bacterial and viral nucleic acids, peptidoglycans, bacteria lipopeptids, double-stranded RNA and LPS can bind to TLRs of host cells to activate the signaling pathway. Except for TLR3, all TLRs share a common adaptor protein, the myeloid differentiation primary-response protein 88 (MYD88), which can recruit tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase (IRAK). Further downstream the Jun N-terminal kinase (MAPK8), p38 mitogen-activated protein kinase (MAPK14), nuclear translocation of the transcription factors, activated protein-1 (AP-1) and nuclear factor kappa B (NF-κB) are reactivated (Bhushan and Meinhardt, 2016). The activation of TLRs lead to the production of pro-inflammatory cytokines such as TNF, IL-1α, IL-1β, IL-6, IL-8, IL-12, caspase, inducible prostaglandin-endoperoxide synthase and nitric oxide synthase (NOS2). Besides TLRs, some NLRs can also activate NF-κB and MAP kinases upon bacteria stimulation (Hedger, 2011).

Compared to other tissue macrophages, TM exhibit unique resistance to pro-inflammatory challenge. This can be explained by the following reasons. Firstly, TM express high levels of negative regulators of TLR signaling pathway genes such as *Sarm, Rp105* and *Ikbα*. They concomitantly express low levels of TLRs related inflammatory genes including *Trif, Trak4, Irak4, Cd14, Md2, Mal, Irf3, Tak1,Traf6, Rip1, Btk1, Socs1 and Socs3* (Zhao et al., 2014). Secondly, IκBα polyubiquitination, an essential step to liberate NFκB, is inhibited in TM when challenged with LPS. Blocking the NFκB signaling pathway dampens the production of pro-inflammatory cytokines substantially (Bhushan and Meinhardt, 2016). Although TM fail to activate the NF-κB signaling pathway, pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, iNOS, monocyte chemoattractant protein-1 (MCP-1/CCL-2) and MIF are produced at moderate levels (Bhushan et al., 2011). Recently, our group showed that this was possible through activation of an alternative pathway CREB and AP-1 involving the upstream MAP kinase signaling pathway Erk1/2 and p38 (Bhushan and Meinhardt, 2016; Zhao et al., 2014).

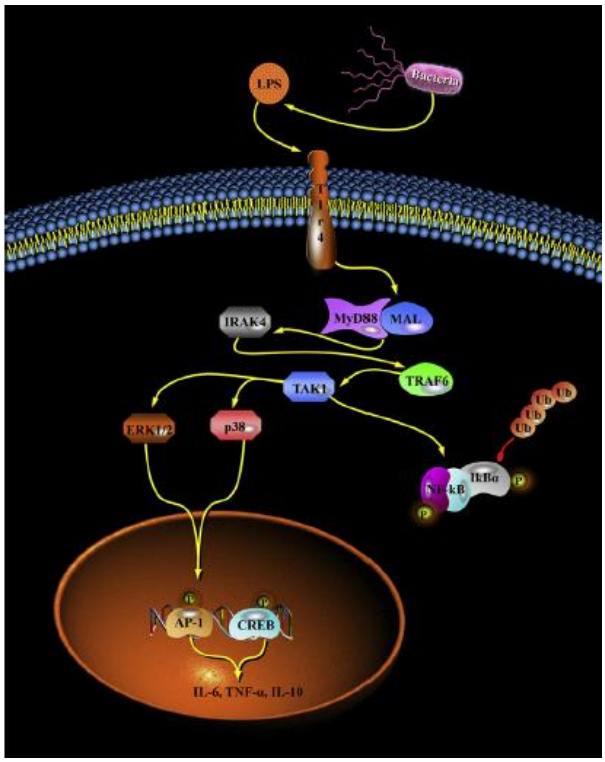


Figure 3. Model of TLR signaling pathway in TM (Bhushan et.al, JRI 2016. With the permission of the publisher, license number: 4143080637999).

1.4 Testicular interstitial fluid

It is generally accepted that a normal testicular micro-environment including proper intercellular communication is important for spermatogenesis and steroidogenesis (Stanton et al., 2016). As an example, PTC are essential for maintaining SC function and thus sperm production, whilst likewise SC are important for modulating LC steroidogenesis and PTC function. Moreover, TM also play a role in regulating LC function (Ariyaratne et al., 2009; Ehrchen et al., 2007; Mills and O'Neill, 2016; Varga et al., 2008). Communication between these cells is mostly mediated through soluble factors secreted into the testicular fluid. Testicular interstitial fluid (IF), as the major part of the testicular fluid, contains numerous proteins produced by different testicular cells including SC, PTC, LC, germ cells and TM (Stanton et al., 2016).

Accumulating evidence suggests that generally local micro-environmental factors contribute to the specialization of the tissue specific macrophage phenotype and function (Amit et al., 2016; Gosselin et al., 2014). In the brain, locally produced TGF-β influences the phenotype of

microglia through Smad and IRF7 pathway (Cohen et al., 2014). Similarly, retinoic acid (RA) contributes to functional polarization of peritoneal macrophages through the GATA6 transcription factor (Okabe and Medzhitov, 2014). CSF-2 controls the differentiation and polarization of alveolar macrophages after birth. TM may acquire an M2 macrophage phenotype due to an irreversible differentiation program controlled by master regulators and uniquely expressed transcription factors or as a consequence of the unique testicular microenvironment, in particular testicular IF, to which TM are persistently exposed. Numerous studies have indicated that the testicular IF has immunosuppressive properties which can influence the activity of the immune cells (Hedger et al., 1998; O'Bryan et al., 2005; Sainio-Pollanen et al., 1991). Amongst them, adult rat testicular IF was shown to inhibit stimulated T cell activation and/or proliferation (Hedger et al., 1998). Similar to IF, aqueous humor (AqH) also maintains the immune privileged status of the eye. AqH inhibits T cell proliferation (Wilbanks and Streilein, 1992), suppresses IFN-γ production and promotes TGF-β production by $CD4^+$ T cells (Taylor et al., 1997). Furthermore, AqH inhibits the capacity of monocyte derived DC to induce naive $CD4^+$ T cell proliferation, cytokine secretion and is associated with reduced expression of MHC as well as co-stimulatory molecules on DC (Denniston et al., 2011). These immunosuppressive functions of AqH are mainly mediated by growth factors, neuropeptides, soluble factors, and cytokines. The immunosuppressive properties of IF are known for long, but the responsible molecules have only very partially been characterized. Moreover, whether and how chronic exposure to IF can polarize macrophages to the M2 phenotype is completed unknown.

Androgens

Androgens are known to play a critical role in the development of the testis, reproductive activity, and male fertility beyond other functions (Cutolo, 2009). Moreover, androgens also play an important role in sustaining the testicular immune privilege. In this regard, it has been shown that androgen treatment *in vitro* suppresses the inflammatory responses by inhibiting the secretion of pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α, whilst promoting the production of anti-inflammatory mediators like IL-10 and TGF-β (Bhushan et al., 2015). Testosterone, the predominant androgen for testicular function, is synthesized by the interstitial LC (O'Hara et al., 2015). Testosterone affects the immune response under physiological conditions by inducing the generation and functional differentiation of Tregs. It also has direct effects on SC and PTC in testicular inflammation (Bhushan et al., 2015).

Besides testosterone, two other androgens namely dihydrotestosterone (DHT) and androstenedione are likewise important for the development of male reproductive organs. DHT (or more precise 5α-dihydrotestosterone, 5α-DHT), is responsible for the differentiation of the scrotum, penis and prostate during development. In the adult, DHT induces prostate growth and sebaceous gland activity (Connolly et al., 2013).

Glucocorticoids

Besides androgens, glucocorticoids (GC) comprise another class of steroid hormones. They are mostly synthesized and secreted by the adrenal glands (Ehrchen et al., 2007). GC are known for dampening inflammation, and are widely used to treat diseases based on an overactive immune response. GC elicit their immunosuppressive functions by binding to the glucocorticoid receptor (GR), which is very widely expressed in most vertebrate cells (Oppong and Cato, 2015). A study from Hazra et al. has documented that GC are considered to maintain normal testicular functions including SC development, LC maturation and steroidogenesis (Hazra et al., 2014). Beyond, corticosterone has other varied functions such as the regulation of glycolysis and spermatogenesis (Maeda et al., 2015). Intriguingly, adrenalectomy (ADX) caused a reduction of sperm numbers and damage of seminiferous tubules in rats (Silva et al., 2014). Interestingly, corticosterone can be synthesized and secreted by the testis itself (Maeda et al., 2015). These observations indicate that corticosterone in IF can originate from both the adrenal glands and the testis. Nevertheless, it remained elusive which testicular cell type are producing corticosterone and how it mediates its function mechanistically.

Prostaglandins

Prostaglandins (PG) such as prostaglandin E_2 (PGE_2), PGD_2, PGI_2, $PGF_{2\alpha}$ and PDJ_2 are important for regulating numerous processes in the body including neurotransmitter release, platelet aggregation and modulation of immune function (Harris et al., 2002). As the most widely characterized lipid mediator, the role of PGE_2 has been extensively studied in terms of its critical function in regulating immune responses during both acute and chronic infections (Agard et al., 2013). PGE_2 can control the activation, maturation, migration, and cytokine secretion of several immune cell types, especially those related to innate immunity such as macrophages, DC and neutrophils. Synthesis of PGE_2 is controlled by cyclooxygenases (COX) upon the stimulation of pro-inflammatory stimuli such as IL-1β, TNF-α and LPS

(Ricciotti and FitzGerald, 2011; Tassorelli et al., 2007). During the early stage of inflammation, macrophages can be locally attracted by PGE_2. Subsequently, PGE_2 inhibits the activation of macrophages via its receptor EP_2. As an example, during the infection of *Klebsiella pneumoniae* in rat, the phagocytic function of alveolar macrophages (AM) were inhibited by PGE_2, relayed by EP_2 signaling (Aronoff et al., 2004). Upon PGE_2 stimulation, the function of bacterial clearance in macrophages was blunted, which was regulated by the immunosuppressive IL-1R-associated kinase-M (IRAK-M) (Aronoff et al., 2004). In addition, macrophage phagocytic activity also can be inhibited by PGE_2 through decreasing the production of nitric oxide radicals (Agard et al., 2013). Furthermore, strong immunosuppressive properties of PGE_2 were visible by inhibiting the production of IL-15 through a cAMP-dependent modulation of the NF-κB pathway (Sainio-Pollanen et al., 1991)(Sainio-Pollanen et al., 1991)(Sainio-Pollanen et al., 1991)(Sainio-Pollanen et al., 1991). Most importantly, the immunosuppressive function of PGE_2 has been demonstrated by inhibiting the production of the archetypical pro-inflammatory cytokine TNF-α and concomitant upregulation of the production of IL-10 in macrophages (Kalinski, 2012). Similarly to PGE_2, PGI_2 also has anti-inflammatory functions as evidenced by suppressing the production of LPS-induced macrophage inflammatory protein 1α (MIP-1α) via the upstream IP receptor and cAMP pathway (Ricciotti and FitzGerald, 2011). However, an influence of PGI_2 on the macrophage phenotype is not investigated yet. PGI_2 assists the production of IL-10 and inhibits the expression of TNF-α, IL-1β, IL-12 in APCs including macrophages and DC (Winnall et al., 2007). Hence, PG are suspected as possible relevant factors in the testis helping to sustain the testicular immunosuppressive microenvironment.

Besides the androgens, GC and PG, IF contains high amounts of immunomodulatory molecules such as inhibin and activin A (Phillips et al., 2009). Activin A synthesized and secreted by SC, is well-documented to play an important role in regulating cell proliferation, differentiation, apoptosis, metabolism, homeostasis, immune responses and wound repair. Activin A, together with follistatin and inhibin B, is functionally related to regulate spermatogenesis and testicular steroidogenesis (Phillips et al., 2009). As an immunoregulatory protein, activin A may also be a candidate for maintaining esticular immune privilege (Meinhardt and Hedger, 2011). The balance of these immunoregulatory factors in IF may be critical for sustaining testicular homeostasis. Therefore, to clarify how these factors function alone or in combination on testicular cells is relevant for our understanding of testicular function.

1.5 Aim of study

The testis has evolved a strategy known as immune privilege to protect the neoantigens on developing germ cells from auto-immune attack. Testicular immune privilege is mechanistically established and maintained by a number of factors, that include the blood–testis barrier (BTB), high levels of intratesticular androgens, and somatic cells with immunosuppressive characteristics (Fijak and Meinhardt, 2006; Shechter et al., 2013). Notably, recent advances now imply that TM also contribute to the maintenance of the testicular immune privilege by skewing innate immune responses (Bhushan and Meinhardt, 2016; Bhushan et al., 2015; Winnall and Hedger, 2013). TM represent the largest pool of immune cells in the testis (Bhushan and Meinhardt, 2016). In contrast to other tissue macrophages, TM are refractory to inflammatory stimuli such as LPS and poly (I:C). TM display an immunosuppressive M2 macrophage phenotype evidenced by secreting low amounts of TNF-α and IL-6 with concomitant high production of the anti-inflammatory cytokine IL-10 (Bhushan et al., 2015). TM are chronically exposed to immunosuppressive IF. It appears tempting to hypothesize that the IF could influence the phenotype and function of TM. Although the immunosuppressive properties of IF are generally known, the molecules involved have only been begun to be characterized. Moreover, whether and how chronic exposure to IF can polarize macrophages to the M2 phenotype is unknown.

Therefore, the objective of this study was to investigate the influence of the testicular IF on the establishment of the macrophage phenotype and the associated molecular mechanism causing the immune-suppressive properties of TM.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Cell culture CO_2 incubator	Binder, Tuttlingen, Germany
Desktop centrifuge Biofuge Fresco	Heraeus, Hanau, Germany
Electronic balance SPB50	Ohaus, Giessen, Germany
Fluorescent microscope Axioplan 2 Imaging	Carl Zeiss, Göttingen, Germany
FUSION-FX7 Advance	PEQLAB, Erlangen, Germany
Flow cytometer	Miltenyi Biotec, Bergisch Gladbach, Germany
FlowJo software version 10	Tree Star, Ashland, USA
Gel Jet Imager 2000 documentation system	Intas, Göttingen, Germany
Heat block DB-2A	Techne, Cambridge, UK
Horizontal mini electrophoresis system	PEQLAB, Erlangen, Germany
Hybond ECL nitrocellulose membrane	Amersham, Freiburg, Germany
iCycler iQ® System	Bio-Rad, Münich, Germany
Microwave oven	Samsung, Schwalbach, Germany
Mini centrifuge Galaxy	VWR International, Darmstadt, Germany
Mini-rocker shaker MR-1	PEQLAB, Erlangen, Germany
Mixer Mill MM 300	Retsch, Haan, Germany
NanoDrop ND 2000	Thermo Fisher Scientific, Waltham, USA
PCR thermocycler	Biozyme, Oldendor, Germany
Potter S homogenizer	B. Braun, Melsungen, Germany
Power supply units	PEQLAB, Erlangen, Germany
SDS gel electrophoresis chambers	Consurs, Reiskirchen, Germany
Semi-dry-electroblotter	PEQLAB, Erlangen, Germany
TCS SP2 confocal laser scanning microscope	Leica, Wetzlar, Germany
Typhoon 9100	GE Healthcare, Freiburg, Germany
Thermo Shaker	PEQLAB, Erlangen, Germany

Tristar LB941	Berthold, Bad Wildbad, Germany
Ultrasonic homogenizer Bandelin Sonopuls	Bandelin, Berlin, Germany
UV visible spectrophotometer Ultrospec 2100	Biochrom, Cambridge, UK
Vertical electrophoresis system	PEQLAB, Erlangen, Germany

2.1.2 Chemicals

37% Formaldehyde solution	Sigma-Aldrich, Steinheim, Germany
Acrylamide 30% (w/v)	Roth, Karlsruhe, Germany
Agarose	Invitrogen, Karlsruhe, Germany
Bromophenol blue sodium salt	Sigma-Aldrich, Steinheim, Germany
Bradford	Bio-Rad, Munich, Germany
Coomassie brilliant blue G-250	Bio-Rad, Munich, Germany
Calcium chloride	Merck, Darmstadt, Germany
Dimethylsulfoxide	Merck, Darmstadt, Germany
Dithiothreitol (DTT)	Roth, Karlsruhe, Germany
DNA ladder (100 bp)	Promega, Mannheim, Germany
Ethanol	Sigma-Aldrich, Steinheim, Germany
Ethidium bromide	Roth, Karlsruhe, Germany
Ethylene diaminetetraacetic acid disodium salt	Merck, Darmstadt, Germany
Enhanced chemiluminescence (ECL) reagents	Amersham, Freiburg, Germany
Formamide	Merck, Darmstadt, Germany
Glycerol	Merck, Darmstadt, Germany
Glycine	Sigma-Aldrich, Steinheim, Germany
Glycogen	Invitrogen, Karlsruhe, Germany
Halt Phosphatase Inhibitor Cocktail	Thermo Fisher Scientific, Waltham, USA
Igepal CA-630 (NP-40)	Sigma-Aldrich, Steinheim, Germany
Lipopolysaccharide (LPS)	Sigma, Steinheim, Germany
Magnesium chloride	Merck, DarmstadtSigma, Germany
Magnesium sulfate	Sigma-Aldrich, Steinheim, Germany
β-Mercaptoethanol	AppliChem, Darmstadt, Germany
Methanol	Sigma-Aldrich, Steinheim, Germany
Non-fat dry milk	Roth, Karlsruhe, Germany

N, N, N', N'-Tetramethylethylenediamin (TEMED)	Roth, Karlsruhe, Germany
Paraformaldehyde	Merck, Darmstadt, Germany
Picric acid	Merck, Darmstadt, Germany
Phenylmethylsulfonyl fluoride (PMSF)	Sigma, Steinheim, Germany
Ponceau S	Roth, Karlsruhe, Germany
Potassium chloride	Merck, Darmstadt, Germany
Proteinase inhibitor cocktail	Sigma-Aldrich, Steinheim, Germany
Roti®-Phenol	Roth, Karlsruhe, Germany
Sodium acetate	Roth, Karlsruhe, Germany
Sodium chloride	Sigma-Aldrich, Steinheim, Germany
Sodium dodecyl sulfate (SDS)	Merck, Darmstadt, Germany
Sodium deoxycholate	Roth, Karlsruhe, Germany
Tris (hydroxymethyl) aminomethane	Roth, Karlsruhe, Germany
Triton X-100	Sigma-Aldrich, Steinheim, Germany
Tween-20	Roth, Karlsruhe, Germany
Uranyl acetate dehydrate	Merck, Darmstadt, Germany
Urea	Merck, Darmstadt, Germany
Xylazine	Bayer, Leverkusen, Germany

2.1.3 PCR reagents

DNase I	Invitrogen, Carlsbad, Germany
Desoxyribonukleosidtriphosphate (dNTP)	Promega, Mannheim, Germany
Ethylendiamin-tetraacetat (EDTA)	Invitrogen, Carlsbad, Germany
Moloney Murine Leukemia Virus Reverse Transcriptase, (M-MLV RT)	Promega, Mannheim, Germany
Oligo dT	Promega, Mannheim, Germany
RNase A	Invitrogen, Karlsruhe, Germany
SuperScript® II Reverse Transcriptase	Invitrogen, Carlsbad, Germany
SYBR green	Bio-Rad, Münich, Germany
Taq polymerase	Promega, Mannheim, Germany

2.1.4 Cell culture reagents

Bovine serum albumin (endotoxin free)	Invitrogen, Karlsruhe, Germany

Ca-Mg-free HBSS (1x) medium	Invitrogen, Karlsruhe, Germany
Ca-Mg-free HBSS (10x) medium	Invitrogen, Karlsruhe, Germany
DMEM-F12	PAA Lab, Cölbe, Germany
Fetal bovine serum (FBS)	Gibco, Darmstadt, Germany
Fetal calf serum (FCS)	PAA Lab, Cölbe, Germany
Goat serum	Dako, Glostrup, Denmark
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	PeproTech, Hamburg, Germany
Penicillin/Streptomycin (100×)	Gibco, Darmstadt, Germany
Phosphate buffer saline (PBS)	Gibco, Darmstadt, Germany
RPMI 1640 medium	Gibco, Darmstadt, Germany
Trypsin/EDTA	Gibco, Darmstadt, Germany
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Gibco, Darmstadt, Germany
Sodium pyruvate	Gibco, Darmstadt, Germany
MEM Non-essential Amino acid solution	Gibco, Darmstadt, Germany
Minimum essential medium (MEM)	Sigma-Aldrich, Steinheim, Germany
2-Mercaptoethanol	Gibco, Darmstadt, Germany
Trypsin/EDTA	PAA, Cölbe, Germany

2.1.5 Kits

Anti-rat TNF-α ELISA kit	eBioscience, San Diego, Germany
Anti-rat IL-10 ELISA kit	BD Bioscience, Heidelberg, Germany
Bio-Rad Protein Assay	Bio-Rad, Munich, Germany
Proteominer Enrichment Kit	Bio-Rad, Münich, Germany
CodeLink Expression Assay Kit	GE Healthcare, Buckinghamshire, UK
PureLink® RNA Mini Kit	Invitrogen, Carlsbad, Germany
Rat corticosterone ELISA kit	Enzo Life Sciences, Lörrach, Germany
RNase-Free DNase Set	Qiagen, Hilden, Germany
RNeasy Micro kit	Qiagen, Hilden, Germany
Zymosan A bioparticles	Thermo Fisher Scientific, Waltham, USA

2.1.6 Miscellaneous

Cell culture plate (6/12/24 well)	Greiner Bio-One, Frickenhausen, Germany
Collagenase A	Roche, Münich, Germany
Fixation/Permeabilisation working solution	eBioscience, San Diego, Germany
Filter (0.45 µM)	BD Bioscience, Heidelberg, Germany
Ficoll-Paque PLUS gradient	GE Healthcare, Uppsala, Sweden
Falcon tube (15/50ml)	Greiner Bio-One, Frickenhausen, Germany
Giemsa stain	Sigma-Aldrich, Steinheim, Germany
Goat serum	Dako, CA, USA
Hybond ECL nitrocellulose membrane	GE Healthcare, Cambridge, UK
Macrophage detachment buffer	PromoCell GmbH, Heidelberg, Germany
May Grünwald	Sigma-Aldrich, Steinheim, Germany
Neubauer counting chamber	LaborOptik, Marienfeld, Germany
Permeabilisation washing buffer	eBioscience, San Diego, Germany
Protein size markers	Thermo Fisher Scientific, Waltham, Germany
Sterile plastic ware for cell culture	Sarstedt, Nümbrecht, Germany
Syringe	BD Bioscience, Heidelberg, Germany
TO-PRO-3 dye	Invitrogen, Carlsbad, USA
Trypan Blue	Gibco, Darmstadt, Germany
RBC lysis buffer	Qiagen, Hilden, Germany
MACS column	Miltenyi Biotec, Bergisch Gladbach, Germany
4', 6-diamidino-2-phenylindole (DAPI)	Vector Laboratories, Burlingame, USA
8-well cell culture plates	BD Biosciences, Heidelberg, Germany
70-µm cell strainer	BD Bioscience, Heidelberg, Germany

2.1.7 Antibodies

Primary Antibody	**Manufacturer**	**Catalogue No.**	**Dilution (application)**

Rabbit anti IkBα	Santa Cruz, Dallas, USA	SC371	1:1000 (WB)
Rabbit anti p-STAT3	Cell Signaling Technology, Danvers, USA	9145	1:500 (WB)
Rabbit anti p-CREB	Cell Signaling Technology, Danvers, USA	9198	1:1000 (WB)
Mouse anti β-actin	Sigma-Aldrich, Steinheim, Germany	A5441	1:2500 (WB)
Mouse anti CD68	AbD serotec Raleigh, North Carolina, USA	MCA-341A488	1:100 (IF)
Mouse anti CD163	AbD serotec Raleigh, North Carolina, USA	MCA-342A647	1:100 (IF)

Table 1： Information for primary antibodies used in Western blotting (WB) and Immunofluorescence (IF) analyses.

Secondary antibody	**Manufacturer**	**Catalogue No.**	**Dilution**
Goat anti rabbit IgG-HRP	Cell Signaling Technology, Danvers, USA	7074	1:10,000 (WB)
Horse anti mouse IgG-HRP	Cell Signaling Technology, Danvers, USA	7676	1:10,000 (WB)
Goat anti mouse IgG-Alexa Fluor 488	Thermo Fisher Scientific, California, USA	A10684	1:1000 (IF)

Table 2: List of secondary antibodies for Western blot (WB) and Immunofluorescence (IF) analyses.

IgG: Immunoglobulin G

HRP: Horseradish peroxidase

Antigen Flow Cytometry	**Fluorchrome Antibodies**	**Manufacturer**	**clone**	**Cat Number**
CD45	PE-Cy7	Biolegend San Diego, California, USA	OX-1	202214
CD68	AlexaFluor 488	AbD serotec Raleigh, North Carolina, USA	ED1	MCA-341A488
CD163	AlexaFluor 647	AbD serotec Raleigh, North Carolina, USA	ED2	MCA-342A647
CD80	PE	Biolegend	3H5	200205

		San Diego, California, USA		
CD86	PE	BDbioscience Franklin Lakes, New Jersey, USA	24F	551396
MHCII	PE	BDbioscience Franklin Lakes, New Jersey, USA	OX-6	554929
CD4	PE	Biolegend San Diego, California, USA	OX-35	203307
CD25	FITC	Biolegend San Diego, California, USA	OX-39	202103
FoxP3	eFluor 660	eBioscience Santa Clara, California, USA	FJK-16s	50-5773-80

Table 3: Antibodies for flow cytometric (FACS) staining.

2.1.8 Primers

All primer pairs were designed online at *http://www.ncbi.nlm.nih.gov/tools/primer-blast/* and purchased from MWG-Biotech (Ebersburg, Germany). The sequences of primer pairs are shown in Table 4. Primers were diluted in RNA-free water.

Genes	Primer sequence (5'->3')
Inos-fw	CCTCTTCCAAGGTGTTTGCCT
Inos-rv	CCTCTTCCAAGGTGTTTGCCT
Cd68-fw	CGCATCTTGTACCTGACCCA
Cd68-rv	TGAGAGAGCCAAGTGGGGAT
Cd163-fw	TCCGGTTGAAGTTTTGTGACC
Cd163-rv	GTGGTCCCGATGACCGTATT
Il-6-fw	GCCCTTCAGGAACAGCTATG
Il-6-rv	GTCTCCTCTCCGGACTTGTG
Il-10-fw	CATCCGGGGTGACAATAACT
Il-10-rw	TGTCCAGCTGGTCCTTCTTT
Ccl2-fw	CAGGTCTCTGTCACGCTTCT
Ccl2-rw	AGTATTCATGGAAGGGAATAG
Ifn-α-fw	AGCAGATCCAGAAGGCTCAA
Ifn-α-rw	TCCGTCCTGTAGCTGAGGTT
Ifn-β-fw	GGTGGACCCTCCACATTGCGT
Ifn-β-rw	ACGGGTGCATCACCTCCATAGGG
Tnf-α-fw	GCCTCTTCTCATTCCTGCTC
Tnf-α-rw	CCCATTTGGGAACTTCTCCT

β2-microglobulin-fw	CCGTGATCTTTCTGGTGCTT
β2-microglobulin-rv	AAGTTGGGCTTCCCATTCTC

Table 4: Information on sequences of forward (fw) and reverse (rv) primers used in quantitative real-time PCR.

2.2 Methods

2.2.1 Animals

All adult male Wistar rats were bought from Charles River Laboratories (Sulzfeld, Germany) and kept under standard conditions (22°C, 12 h light/dark cycle) with pelleted food and water ad-libitum. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the German law of animal welfare. The experiments were performed according to the guidelines of the local authority (Regierungspraesidium, Giessen, Germany) and conformed to the Code of Practice for the Care and Use of Animals for Experimental Purposes (permission no. M_545). The rats were scarified by CO_2 inhalation, and all efforts were made to minimize suffering.

Male Sprague Dawley（SD） rats (8 weeks of age) for adrenalectomy （ADX）were bred in the Center of Experimental Animals, Zhejiang Chinese Medical University, (Hangzhou, China), where a specific pathogen free (SPF)-level laboratory has been authorized by the Zhejiang provincial government. All rats were housed under conditions of controlled humidity (50–60%) and maintained under controlled light (12 h light/dark cycle) with free access to water and rodent chow. All animal experiments were performed in consistency with the license from the Zhejiang Province Science and Technology Office (Hangzhou, China) and with approval from the animal ethics committee of Zhejiang Chinese Medical University (permit number ZSLL-2016-22).

2.2.2 Testicular interstitial fluid and serum collection

Testicular interstitial fluid (IF) was collected from adult Wistar rats (10-12 weeks old). Testes were washed several times with cold phosphate-buffered saline (PBS, Gibco, Darmstadt, Germany) to remove residual blood. To collect IF, a small incision was made in the distal end of the testicular capsule, and then hanged in a 15 ml falcon tube (Greiner Bio-One GmbH,

Kremsmünster, Austria) for 16-20 h at 4°C. The obtained IF was centrifuged at 500 ×g for 5 min, and subsequently passed through 0.45 µM membrane filters (BD Bioscience, San Jose, USA) to remove the cellular contamination. To obtain rat serum, blood was collected from the inferior cava vein and allowed to clot overnight at 4 °C. The clot was removed by centrifugation at 1000 ×g for 5 min, and the supernatant was carefully collect and filtered through 0.45 µM membrane filters (BD Bioscience). Serum and IF were stored at -80°C for further analyses or use.

2.2.3 Isolation of testicular macrophages

Isolation of TM from adult Wistar rats was performed as previously described (Bhushan et al., 2011; Bhushan et al., 2008a, b). Briefly, Rat testes were decapsulated and placed into 10 ml of ice-cold endotoxin-free DMEM-F12 culture medium (Gibco). Interstitial cells were released from seminiferous tubules by mechanically separating tubules by curved forceps. The separated seminiferous tubules weretransferred into a new 50 ml falcon tube (Greiner, Frickenhausen, Germany) filled with DMEM-F12 culture medium (Gibco). The seminiferous tubule fragments were allowed to settle down for 5 min. The supernatants were collected and centrifuged at 1000 rpm for 7 min. The resultant interstitial cell pellet was resuspended in DMEM-F12 culture medium. The cells (1×10^6) were seeded into wells of 24-well plates (Greiner Bio-One) and incubated at 32°C for 30 min. Contaminating cells were removed by extensive washing with PBS for 3-5 times. TM were cultivated in DMEM-F12 medium with 10% FCS (PAA Laboratories, Cölbe, Germany) and 1% penicillin/streptomycin (P/S, PAA Laboratories) at 32°C. Purity of TM were (≈ 85-90%) determined by using a CD68 (AbD serotec, Raleigh, North Carolina, USA) and CD163 antibodies (AbD serotec, Raleigh, North Carolina, USA) in a combined immunofluorescent staining.

2.2.4 Isolation of blood-derived monocytes

Rat blood-derived monocytes were isolated according to previously described methods (Marcos et al., 2000) with some modifications. Primarily, the rats were euthanized in a CO_2 chamber and the peripheral blood was collected immediately. Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation at 2000 rpm for 20 min with Ficoll-Paque PLUS gradient (GE Healthcare, Uppsala, Sweden) solution after dilution of the blood with PBS at a ratio of 1:2. Next, cells were collected from the white layer of the interface and further purified by washing with PBS for 2 times with gradually decreasing centrifugation (1500 rpm

at the first washing and then 1200 rpm) to remove lymphocyte. Enriched PBMCs were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FCS, 1% P/S, 10 mM HEPES (Gibco), 1 mM sodium pyruvate (Gibco), 1% MEM non-essential amino acid solution (Sigma, Life science, St. Louis, USA) and 50 μM 2-Mercaptoethanol (Gibco) at 37°C. The viability of cells was determined by the Trypan Blue (Gibco) exclusion method and 1×10^6 cells were seeded into each well of 24 well plate. After 6 h, floating cells were removed carefully by washing with PBS, and attached cells were cultured in medium containing 50 ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF, PeproTech, humburg, Germany) alone or together with IF (10%) or rat serum (10%) for 6 days. On the 7th day, cells were washed with fresh RPMI 1640 medium 2 times and then treated with 100ng/ml lipopolysaccharide (LPS, Invivogen, San Diego, USA) for 3 h.

2.2.5 Isolation of splenic T cells

T cells were isolated from adult healthy rats by centrifugation of splenic solenocyte suspension with Ficoll-Paque PLUS gradient (GE Healthcare). Rat spleen was washed 2 times in cold PBS. Single cell suspension of spleen was obtained by injecting RPMI 1640 medium, dissecting, and passing it through a 70 μM filter (BD Bioscience, Heidelberg). Red blood cells were removed by using RBC lysis buffer (Qiagen, Hilden, Germany). T cells were isolated through positive selection by using magnetic beads coated with monoclonal mouse anti-rat pan $CD4^+$ T cell Ab and a MACS column (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated T cells were suspended in RPMI-1640 medium containing 10% FCS, 1% minimal essential medium (Sigma-Aldrich, Steinheim, Germany), 1 mM sodium pyruvate, 10 mM HEPES, 100 U/m penicillin (PAA Laboratories), 100 mg/mL streptomycin (PAA Laboratories), and 50 μM 2-mercaptoethanol (Gibco). Purity of the T cells was determined by flow cytometric analysis using anti-CD4 antibodies (BD Bioscience). To determine immunosuppressive properties of TM and IF, isolated TM or IF were co-cultured with T cells.

2.2.6 Isolation of peritoneal macrophages

For peritoneal macrophage (PM) isolation, adult rats were euthanized and the abdominal skin was carefully removed avoiding a cut in the peritoneal wall. Cold PBS (50 ml) was injected slowly in the intraperitoneal cavity of the rats with a 50 ml syringe. The rat abdominal wall was massaged gently, and after 5 min, PBS was retrieved from the peritoneal cavity using 50 ml syringe (BD Bioscience, Heidelberg, Germany). The retrieved peritoneal lavage was transferred

into a sterile 50 ml falcon tube. The cell pellet was collected by centrifugation at 1000×g for 7 min. The pelleted cells were washed once again with PBS and resuspended into 10 ml of cell culture medium RPMI 1640. A total of 5×10^5 cells were seeded into the wells of 24-well plates for 30 min. Non-adherent cells were removed by washing extensively with PBS after seeding. PM was incubated at 37°C 5% CO2 in culture medium containing 10% FCS and 1% P/S for further experiments. Purity of PM was (≈ 90-95%) determined by using CD68 and CD163 antibodies in combined immunofluorescence.

2.2.7 Isolation of Sertoli cells, peritubular cells and Leydig cells

Sertoli cells (SC), peritubular cells (PTC) and Leydig cells (LC) were isolated as previously described (Bhushan et al., 2016; Fijak et al., 2015). Isolated SC were cultured in RPMI 1640 medium without FSC (to avoid the contamination with PTC). PTC were incubated in RPMI 1640 medium supplemented with 10% FCS. Purified LC were cultured in DMEM-F12 supplemented with 0.1% BSA, penicillin (100 U/mL) and streptomycin (100 ng/mL). Purity of SC (>95%) was examined by immunofluorescence using antibody directed against vimentin (Sigma-Aldrich, Steinheim, Germany). Purity of the PTC cell preparation was >95% as verified by smooth muscle actin (DakoCytomation, Hamburg, Germany). Purity of LC was >80% as estimated by combined cytochrome P450 and CD68/CD163 immunofluorescence staining.

2.2.8 cDNA synthesis and quantitative real-time PCR

2.2.8.1 Reagents for agarose gel electrophoresis

50 × TAE electrophoresis buffer (1L)

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA	14.6 g
H_2O	943 ml

adjust the PH to 8.0 with HCl.

1× TE buffer (1L)

1 M Tris-HCl (pH 7.5)	10 ml
0.5 M EDTA (pH 8.0)	2 ml
H_2O	988 ml

2.2.8.2 RNA isolation

RNA from each sample (GM-CSF±IF treated monocytes or PM cultured in conditional supernatant) was extracted by using the PureLink® RNA Mini Kit (Invitrogen, Carlsbad, USA). Following treatments, cells were washed 2 times with cold PBS and collected in 350 µl RLT buffer (containing 1% β-mercaptoethanol) by using cell scrapers. To avoid cell clumps, the cell lysate was passed through a 21G needle (BD Biosciences) on a 1 ml syringe (BD Biosciences) 9-12 times and then mixed with 1 volume (350 µl) of 70% ethanol. The total mixture (700 µl) was transferred to spin cartridge for centrifugation at 12,000 × g for 15 sec. To wash the column membrane, wash buffer 1 (500 µl) and wash buffer 2 (500 µl) were added to the spin column sequentially by centrifugation at 12,000 × g for 15 sec, respectively. The flow through was discarded. Next, the column was centrifuged at 12,000 × g for 1 min with the lid open allowing to dry the membrane. The RNA was dissolved by adding 14 µl RNase-free water to the column membrane directly. After 1 min incubation, the RNA was eluted to a new 1.5 ml collection tube by centrifugation at 13,000 × g for 2 min. The RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham).

2.2.8.3 DNA digestion

Prior to RT-PCR amplification, DNase I (Invitrogen, Carlsbad, USA) was used to eliminate the putative contamination of genomic DNA from each RNA sample. Approximately 2 µg of isolated RNA from cultured monocytes or PM was treated with 2 µl DNase I at room temperature for 15 min in the reaction mixture given below. To inactivate DNase I, 2 µl of 25 mM EDTA (pH 8.0, Invitrogen) solution was added to each sample and subsequently heated at 65°C for 10 min. Standard PCR was used to amplify DNase I digested RNA samples to detect the expression of a housekeeping gene (β-macroglobulin). The absence of PCR products were proved by agarose gel electrophoresis and ethidium bromide (EB) staining to confirm there were no DNA contaminations in each samples.

DNase digestion reaction mix:

Component	**Volume**
2 µg RNA	x µl
DNase I (1 U/µl)	2.0 µl
10 × DNase I buffer	2.0 µl
RNase free water	16-x µl

2.2.8.4 Reverse transcription

The purified RNA sample was reverse transcribed by using SuperScript® II Reverse Transcriptase kit (Invitrogen, Carlsbad, USA). For each sample, 2 µg of RNA was mixed with 2 µl of Oligo (dT) 15 Primer and 2 µl dNTP (10 mM), followed by denaturation at 65°C for 5 min and subsequently snap chilled on ice. The RT mix was prepared by adding 8 µl 5 × first strand buffer, 4 µl DTT (0.1M), and 4 µl RNase-free water to mix with the denatured RNA samples. Following, the samples were pre-warmed 2 min at 42°C and 1 µl of reverse transcriptase (SuperScript® II Reverse Transcriptase, 200 U/µl) was added to each sample. The reaction mix was incubated at 42°C for 50 min, and then heated at 70°C for 15 min to inactivate the reverse transcriptase. Standard PCR were performed to confirm the quality of the isolated cDNA form all samples by detection of β-macroglobulin expression. The cDNA samples were stored at -20°C till use.

2.2.8.5 Quantitative real-time RT PCR (qRT-PCR)

To measure Cd68, Inos, Il-6, Tnf-a, Il-10, Cd163, Ccl2, Ifn-αand Ifn-β expression, primer pairs were designed by "Primer BLAST" and synthesized by Eurofins MWG Operon (Table 4). The appropriate annealing temperatures were determined by gradient PCR. A typical 20 µl qRT-PCR reaction mix was used as follows.

Component	**Volume per reaction**
cDNA	1 µl
2 × iQ SYBR green supermix	10 µl
Forward and reverse primer mix (10 pM/µl)	1 µl
DNase/RNase free water	8 µl
Total volume	20 µl

Real-time PCR was performed in duplicate to measure the expression of target genes by using the iCycler iQ® System (Bio-Rad, Munich, Germany) according to the manufacturer's procedure.

The quantitative real-time RT-PCR program was as follows:

Action		Light Cycler
Initial Melting		95°C, 10 min
45 cycles	Melting	95°C 20 sec
	Annealing	59°C 30 sec
	Extension	72°C 30 sec
Melting point		50°C 10 sec
Cool to		4°C

Melt curve examination was used to confirm the specificity of the PCR product. Real-time RT PCR results were analyzed with the delta threshold cycle method using β-microglobulin as an internal standard to normalize the amount of mRNA. Data were analyzed by Bio-Rad CFX Manager 3.1 software and presented as relative expression (RE).

2.2.9 Immunoblotting

2.2.9.1 Buffers and solutions

RIPA buffer (10ml)

1 mM Tris-HCl (pH 7.4)	100µl
5 M NaCl	300 µl
NP-40	25 µl
Triton X-100	100ul
0.5 M EDTA	40 µl
100 mM PMSF *	100ul
100 × Proteinase inhibitor cocktail*	100 µl
100 × Halt Phosphatase Inhibitor Single-Use Cocktail*	100ul

* Added fresh just before cell lysis

10 × phosphate buffered saline (PBS)

KCl	4 g
KH_2PO_4	4 g
NaCl	160 g
$Na_2HPO_4 * H_2O$	23 g
H_2O	1L

Adjust pH to 7.4 with HCl.

10 × Tris buffered saline (TBS)

Tris base	24.2 g
NaCl	80 g
H_2O	1L
Adjust pH to 7.4 with HCl.	

Washing buffer TBS/T (1L)

1 × TBS	100ml
Tween-20	1ml
H_2O	900ml

Blocking buffer

1 × TBS	100 ml
Tween-20	100 µl
Non-fat dry milk	5 g

10 × Electrophoresis buffer (pH 8.3)

Tris base	30.3 g
Glycine	144 g
SDS	10 g
H_2O	1000ml
Adjust pH to 8.3 with HCl.	

Stripping buffer (100 ml)

1 M Tris-HCl (pH 6.8)	6.25 ml
10% SDS	2 ml
β-Mercaptoethanol*	700 µl
H_2O	91 ml
* added freshly just before stripping of membrane	

Cathode buffer

Tris base	30,03 g
6-amino-hexanoic acid	3,25 g
Methanol	100 ml
H_2O	400 ml

10 × Anode buffer

Tris base	36,3g
Methanol	100ml
H_2O	400 ml

1 × Anode buffer

Tris base	3,63g
Methanol	100ml
H_2O	400 ml

Separating gel:

	7.5%*	10%*	12.5%*	15%*
Water	4.85 ml	4.01 ml	3.17 ml	2.35 ml
1.5 M Tris-HCl pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml
10% (w/v) SDS	100 µl	100 µl	100 µl	100µl
Acrylamid	2.5 ml	3.34 ml	4.17 ml	5 ml
10% (w/v) APS**	50 µl	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl	5 µl
Total	10 ml	10 ml	10 ml	10 ml

Stacking gel:

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

* Separating gels with different percentages were used according to the molecular weight of target proteins (based on 37.5:1 acrylamide/bisacrylamide ratio). 7.5% gel: 250~120 kDa; 10% gel: 120~40 kDa; 12.5% gel: 40~15 kDa; 15% gel: < 20 kDa.

** Ammoniumpersulfate (APS) was prepared fresh before each experiment.

2.2.9.2 Western blotting

Following treatment, cells were washed twice with cold PBS and lysed in freshly made RIPA lysis buffer. Cell extracts were obtained by rigorously vortexing each sample at 5 min interval for 30 min. Cell debris was removed by centrifugation at 16000 rpm at 4°C for 20 min. The protein concentrations in cell lysates were determined by the Bradford Method (Bio-Rad,

Munich, Germany). Twenty µg protein of each sample were separated on SDS-polyacrylamide gels and separated proteins were electrophoretically transferred onto a 0.2 µm Hybond ECL nitrocellulose membrane (GE Healthcare, Cambridge, UK) by using a PerfectBlue^{TM} semidry electroblotter. Ponceau S staining was performed for each experiment to confirm the efficiency of protein transfer. Membranes were washed with TBS-T buffer and blocked in blocking buffer (5% non-fat milk in TBS-T buffer) for 1 h before incubating with the primary antibody (Table 1) diluted in 5% BSA/TBST overnight at 4°C. After washing three times with TBS-T, membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse antibody (Table 2) for 1 h at room temperature. Subsequently, the membranes were rinsed again for three times. The blots were developed by enhanced chemiluminescence (ECL) reagents (Thermo Fisher Scientific) and visualized by using the Fusion Imaging system (PEQLAB, Erlangen, Germany).

2.2.10 Immunofluorescence staining

Treated and untreated monocytes were grown on glass coverslips in 8-well cell culture plates (BD Biosciences). Cells were washed two times with ice cold PBS, fixed with 4% precooled paraformaldehyde (Merck, Darmstadt, Germany) for 15 min and subsequently permeabilized with 0.3% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) for 10 min. Non-specific binding of antibodies was blocked with 10% normal goat serum (Dako, Glostrup, Denmark) for 1 h, and then incubated with CD68 or CD163 (Table 1) antibodies overnight at 4°C. Samples were rinsed thoroughly and then incubated with goat anti mouse Alexa Fluor 488 secondary antibody (Thermo Fisher Sceintific, Carfornia, USA) for 1 h. Nuclei were counterstained with DAPI (Vector Laboratories, Burlingame, USA) or Cy5-conjugated TO-PRO-3 dye (Invitrogen, Carlsbad, USA). Images were taken either by the Fluorescence Microscope Axioplan 2 imaging system (Carl Zeiss, Göttingen, Germany) or a TCSSP2 confocal laser-scanning microscope (Leica, Wetzlar, Germany).

2.2.11 ELISA

The levels of TNF-α (eBioscience, San Diego, CA, USA) and IL-10 (BD Bioscience, San Jose, CA, USA) in cell culture-conditioned media, rat serum and rat IF were quantified by using specific sandwich ELISA. The concentration of TGF-β1 in serum and IF were measured by using the TGF-β1 (R&D Systems, Minneapolis, USA) ELISA kit. Corticosterone was measured using an ELISA kit from Enzo Life Sciences (Lörrach, Germany). All of the ELISA procedures were following the manufacturer's protocols.

2.2.12. May Grünwald–Giemsa staining

20 ×Giemsa Stain (pH 6.9)

Giemsa stain	0.4 g
Methanol	70ml
H_2O	30ml
Adjust pH to 6.9 with HCl.	

Dilute to 1× before use. To observe the morphological appearance of rat TM, May Grünwald–Giemsa staining was performed. Cultured TM on glass slides were fixed with cold methanol at -20°C for 10 min. The slides were air dried and stained with May Grünwald (Sigma-Aldrich, Steinheim, Germany) stain for 5 min at room temperature and subsequently washed two times with PBS. Next, slides were stained with 1×Giemsa stain for 15-20 min at room temperature. Slides were rinsed briefly in deionized water and air dried at room temperature. Images were visualized using microscope imaging system (Leica, Wetzlar, Germany).

2.2.13 Assessment of phagocytosis

The phagocytic activity of TM was measured by the Zymosan A bioparticles kit (Thermo Fisher Scientific, Waltham, USA). TMs were isolated from adult rats and cultured in 6-well plates for 3 h. To opsonize BioParticles yeast, equal volumes (50 ul) of reconstituted opsonizing reagent and BioParticles yeast suspension (20 mg/ml) were mixed and incubated at 37°C for 1 hour. The yeast particles were washed 2 times with 1 ml PBS, followed by centrifugation at 1000×g to form loosely packed pellets allowing to remove the excess antibody and azide. TMs were detached from the 6-well plated and 1×10^5 cells /well were seeded in 2 wells of 24-well plate. 1×10^6 particles were cultured with TM at 4°C (control) or 37°C for 1 h. Unbound particles were washed away by cold PBS 2 times and TM were harvested for immunofluorescence and flow cytometric analyses.

2.2.14 Flow cytometric analysis

To determine the TM population in whole testis, rats were euthanized by CO_2 asphyxiation and cervical dislocation. To obtain single-cell suspensions, the testes were decapsulated and enzymatically digested in a shaking water bath for 30 min at 37°C in RMPI 1640 medium containing collagenase A (Roche, Münich, Germany) (1 mg/ml), DNAse I (Invitrogen) and 10% FCS. The digested cells were further dispersed mechanically with a 1-ml syringe and filtered

through a 70-µm cell strainer. The cells were pelleted by centrifugation at 350×g for 7 min, and RBCs were lysed in RBS lysis buffer (Qiagen, Hilden, Germany). The cells were stained with antibodies after one time wash with PBS. To label the attached monocytes derived macrophages or splenic T cells, macrophage detachment buffer (PromoCell GmbH, Heidelberg, Germany) was used to free the cells from the plate. Then the cells were washed one time in culture medium and prepared for further staining. For flow cytometric analyses, 5×10^5 cells were incubated in washing buffer (with 0.5% BSA, 2mM EDTA in 1×PBS) before blocking with Fc block anti-CD16/32 (D34-485, 5µg/ml, BD Bioscience) at 4°C for 10 min. Then CD45 (OX-1, 2 µg/ml, Biolegend), CD163 (ED2, 2.5µg/ml, AbD serotec), CD80 (3H5, 2 µg/ml, Biolegend), CD86 (24F, 2µg/ml, BD bioscience) and MHCII (OX-6, 2µg/ml, BD bioscience) were used as cell surface markers for macrophage staining. CD4 (OX-35, 2 µg/ml, Biolegend), CD25 (OX-39, 5µg/ml, Biolegend) were used as T cells markers. Cells were incubated with antibodies for 30 min at 4°C followed by one wash with 1×washing buffer. For intracellular staining of CD68 (2.5µg/ml ED1, AbD serotec) and Foxp3 (FJK-16s, 2 µg/ml, eBioscience), cells were incubated with the antibodies after fixation and permeabilization using freshly prepared 1×Fixation/Permeabilisation working solution (eBiosceience) at 4°C for 30 min. Cells were washed one more time in 1× Permeabilisation washing buffer (eBioscience) and then tested by MACSQuant analyzer 10 flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). Date were collected for 30,000 events using a FACS Canto flow cytometer, analyzed with FlowJo software version 10 (Tree Star, Ashland, USA).

2.2.15 Mass spectrometry

2.2.15.1 Sample preparation

Serum and testicular fluid samples (three replicates each) were clarified by centrifugation (20,000g for 10 min at 10°C). One milliliter of the supernatant was applied to the Proteominer Enrichment Kit (large capacity kit, BioRAD) according to the manufacturer's instructions. Eluents were diluted with three-fold with H_2O and subjected to acetone precipitation.

2.2.15.2 Two-dimensional gel electrophoresis

For 2-D gel electrophoresis proteins were solubilized in 6 M urea, 2 M thiourea, 4% CHAPS, 1% DTT and 2% Pharmalyte 3-10. IPG-strips (pH 3-10 nl) were rehydrated at 20°C with the protein extract. On each strip, 125 or 250 µg proteins were applied and isoelectric focusing was

performed with 32.05 kVh. After focusing, the IPG-strips were equilibrated for 10 min in 2 ml equilibration stock solution (ESS; 6 M urea, 0.1 mM EDTA, 0.01 % bromphenol blue, 50 mM Tris-HCl pH 6.8, 30 % glycerol) for 15 min in 2 ml ESS I (10 ml ESS containing 200 mg SDS, 100 mg DTT) followed by 15 min in ESS II (10 ml ESS containing 200 mg SDS, 480 mg iodacetamide). Protein separation in the second dimension was performed by electrophoresis on 12.5% SDS polyacrylamide gels according to Laemmli (Laemmli, 1970). Electrophoresis was carried out in a Hoefer 600 system with the following program: 15 min at 15 mA/gel and 5 h at 110 mA at 25°C. Gels were stained with Flamingo (BioRAD) and scanned with a Typhoon 9100 (GE Healthcare). Densitometric analysis of the gels were done with PDQuest (BioRAD). Protein spots showing statistical significant differences in abundance between serum and testicular fluid samples were selected for further analysis.

2.2.15.3 Tryptic in-gel digestion of proteins

Selected spots were digested after reduction and carbamidomethylation with trypsin using an automated liquid handling system (MicroStarlet, HamiltionRobotics, Martinsried, Germany). Tryptic peptides were eluted from the gel plugs with 1% trifluoric acid.

2.2.15.4 Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

MALDI-TOF-MS was performed on an Ultraflex TOF/TOF mass spectrometer equipped with a nitrogen laser and a LIFT-MS/MS facility. The instrument was operated in the positive-ion reflectron mode using 2.5-dihydroxybenzoic acid and methylendiphosphonic acid as matrix. Sum spectra consisting of 200–400 single spectra were acquired. For data processing and instrument control the Compass 1.1 software package consisting of FlexControl 2.4, FlexAnalysis 3.0 and BioTools 3.0 was used.

2.2.15.5 Database search

Proteins were identified by MASCOT peptide mass fingerprint search (http://www.matrixscience.com) using the rat IPI database v3_73 (39711 sequences; 21109946 residues). For the search a mass tolerance of 75 ppm was allowed and carbamidomethylation of cysteine as global modification and oxidation of methionine as variable modification were used. A false positive rate of 5% was allowed.

Proteomic experiments were performed by PD Dr. Günter Lochnit from institute of biochemistry, Justus Liebig University of Giessen.

2.2.16 Prostaglandin determination

Prostaglandin (PG) were extracted and determined by liquid chromatography–MS/MS (LC-MS/MS). For liquid-liquid-extraction, 200-µl samples were incubated with extraction buffer containing 600 µl of ethyl acetate, 100 µl of 150 mM EDTA, and 20 µl of internal standard solution (25 ng/ml each of of [$2H^4$]-PGE_2, [$2H^4$]PGD_2 and [$2H^4$]TXB_2, and 10 ng/ml each of [$2H^4$]$PGF_{2\alpha}$ and [$2H^4$]-6-keto-$PGF_{1\alpha}$ in methanol). The samples were homogenized using a Mixer Mill MM400 (Retsch, Haan, Germany) and centrifuged at 20,000 x *g* for 150 s. The extraction was repeated, and extraction solvents were removed at 45°C under a gentle stream of nitrogen and stored at −80°C until measurement. The residues were reconstituted with 50 µl of acetonitrile:water:formic acid (20:80:0.0025, v/v, pH 4.0) and injected into the LC-MS/MS. PGs were separated with a Synergi 150x2.0 mm Hydro-RP column (Phenomenex, Aschaffenburg, Germany) and determined with a triple quadrupole MS (5500 Q-TRAP Sciex, Darmstadt, Germany (Brenneis et al., 2011).

Prostaglandin measurement was finished by Prof. Dr. Stefan Alexander Wudy from Steroid Research and Mass Spectrometry Unit, Pediatric Endocrinology and Diabetology, Center of Child and Adolescent Medicine, Justus Liebig University of Giessen.

2.2.17 GC-MS/MS steroid hormone measurement

These measurements were collected using 0.5 ml of rat serum or up to 10 µl of IF spiked with a cocktail of internal standards containing [16,16,17-$^2H_3$]testosterone (d3-T), [7,7-$^2H_2$]4-androstenedione (d2-4A), [16,16,17-$^2H_3$]5α-androstane-3α,17β-diol (d3-AD), [11,11,12,12-$^2H_4$]17-hydroxyprogesterone (d4-17OHP), [1α,2α-$^2H_2$]11-deoxycortisol (d2-S) and [2,2,4,6,6,17α,21,21-$^2H_8$]corticosterone (d8-B). After equilibration, extraction with ethyl acetate and purification by gel chromatography (Sephadex LH-20), the samples were derivatized using heptafluorobutyric anhydride (Sanchez-Guijo et al., 2013) Via GC-MS/MS analysis (Thermo Scientific Trace 1310 Gas Chromatograph with a TriPlus RSH Autosampler coupled to a TSQ8000 Triple Quadrupole mass spectrometer), the following transitions were observed for the analyses and their corresponding internal standards: m/z 665/665 for T, 668/668 for d3-T, m/z 482/482 for 4A, 484/484 for d2-4A, m/z 455/241 for AD, 458/244 for

d3-AD m/z 465/109 for 17OHP, m/z 469/113 for d4-17OHP, m/z 465/109 for S and m/z 467/109 for d2-S and 705/355 for B, 712/359 for d8-B.

GC-MS/MS steroid hormone measurement was finished by Prof. Dr. Rolf Micheal Nüsing from Institute of Clinical Pharmacology, Goethe University.

2.2.18 Microarray analysis

2.2.18.1 cRNA synthesis and Hybridization

Extracted RNA was subjected to cRNA synthesis, cRNA fragmentation and finally hybridization on CodeLink Rat Whole Genome using the CodeLink Expression Assay Kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) according to manufacturer's instructions. Bioarrays were stained with Cy5™-streptavadin (GE Healthcare) and scanned using the GenePix® 4000 B scanner and the GenePix Pro 4.0 Software (Axon Instruments, Arlington, USA). A total of 2 × 2 = 4 array images were subjected to data analysis. Spot signals of CodeLink bioarrays were quantified using CodeLink System Software 5.0.0.31312 which generated local background corrected raw as well as median centered intra-slide normalized data.

2.2.18.2 Quality control of microarray data

The genes represented by probe sets were annotated using the biocLite package (BioConductor) with the library “rwgcod.db” for CodeLink Rat Whole Genome arrays. The intra-slide normalized data were processed by an automated workflow that includes omitting of controls genes, removal of genes with poor QC or negative sign, removal of probe sets with too high proportion (>= 50%) of missing values per group or without any group having at least 50% of values flagged as “G = good” and 50% values above threshold, and removal of outliers (expression values deviating more than 4-times from the median). A total of 23879 probe sets remained after quality control with missing values imputed by probabilistic principal component analysis (PPCA) using the R-package pcaMethods. Imputed dataset was quantile normalized using the R-package limma, and logarithmized to the base of 2. Rank Products test (Breitling et al., 2004) has been applied and significant differential gene expression has been postulated for probe sets with a false discovery rate (FDR) < 0.05.

Microarray analysis was performed by Prof. Dr. Hamid Hossain from Institute of Medical

Microbiology, Justus Liebig University of Giessen.

2.2.19 Adrenalectomy

Nine weeks male SD rats were provided by the Animal House of the Zhejiang Chinese Medical University and maintained in a well ventilated animal room (22°C, 12 h light/dark cycle) in polypropylene cages with stainless steel grills. Twelve animals were randomly divided into two groups of 6 animals each: the sham operated and the adrenalectomized (ADX). The sham operation and bilateral adrenalectomy were performed under ether anaesthesia through a dorsal approach. The anesthetized rats were placed in ventral recumbency position; subsequently an area on the mid-dorsum (thoracolumbar junction) was shaved and prepared for aseptic surgery. A long midline incision (1-2 cm) was made just caudal to the peak of the animal's dorsal hump. Hemostats or blunt-tipped scissors were inserted subcutaneously through the incision to bluntly dissect the connective tissue a short. The skin incision was then pulled laterally to one side to expose the muscle just caudal to the last rib and a small incision was made in the muscle to enter the peritoneal cavity; through the incision, the adrenal glands can be found, which are small, pink organs located near the upper pole of each kidney. A second pair of forceps was used to move the incision over the gland, which is usually surrounded by adipose tissue just in front of the kidney. Once the gland was found, it was exteriorized by grasping periadrenal fat. Before removing the adrenal gland, the fascial connections between the kidney and adrenal gland should be dissected. Clamp the vessels at the base of the adrenal gland with both forceps. The tissue stump was then returned to the abdomen. On the right side, similar procedure was made to exteriorize the adrenal gland. The peritoneum and subcutaneous tissues were sutured by simple interrupted sutures with cat gut thread and the skin by ethicon silk thread. Rats had free access to standard food and 0.9 % NaCl solution ad libitum. Twelve animals were sacrificed on day 14. Blood samples were collected through cardiac puncture and testicular extracellular interstitial fluid (IF) was obtained by drip-collection. Serum and IF were stored at -80°C until corticosterone measurement. Removal of the adrenal glands was confirmed after the dissection of the rat by the detection of corticosterone in the blood, as shown in Fig 24.

The ADX animal operation was performed by Lizong Zhang from Zhejiang Chinese Medical University.

3. RESULTS

3.1 TM display immunosuppressive phenotypic characteristics

To characterize the macrophage population of the adult rat testis, a gating strategy was employed to exclude cell debris, doublets and dead cells. The TM population was readily identified in enzymatically digested live testicular $CD45^+$ ($efluor^-$) cells by the expression of the surface receptor markers CD68 and CD163. The TM population of the testis was heterogeneous and comprised two distinct populations: $CD68^+$-$CD163^-$ and $CD68^+$-$CD163^+$ (Figure 4 A). The $CD68^+$-$CD163^-$ population represented only a small proportion (≈ 25%), whereas the $CD68^+$-$CD163^+$ represented the majority (≈75%) of the TM population (Figure 4 C). TM were further characterized by the expression of co-stimulatory molecules, namely CD80, CD86 and MHC class II. In the $CD68^+$-$CD163^-$ macrophage population, approximately 75% were MHCII positive, whereas in the $CD68^+$-$CD163^+$ macrophage population only ca. 40% were positive for MHC class II (Figure 4 A). Notably, the expression of both CD80 and CD86 was almost neglectable in both populations (Figure 4 B).

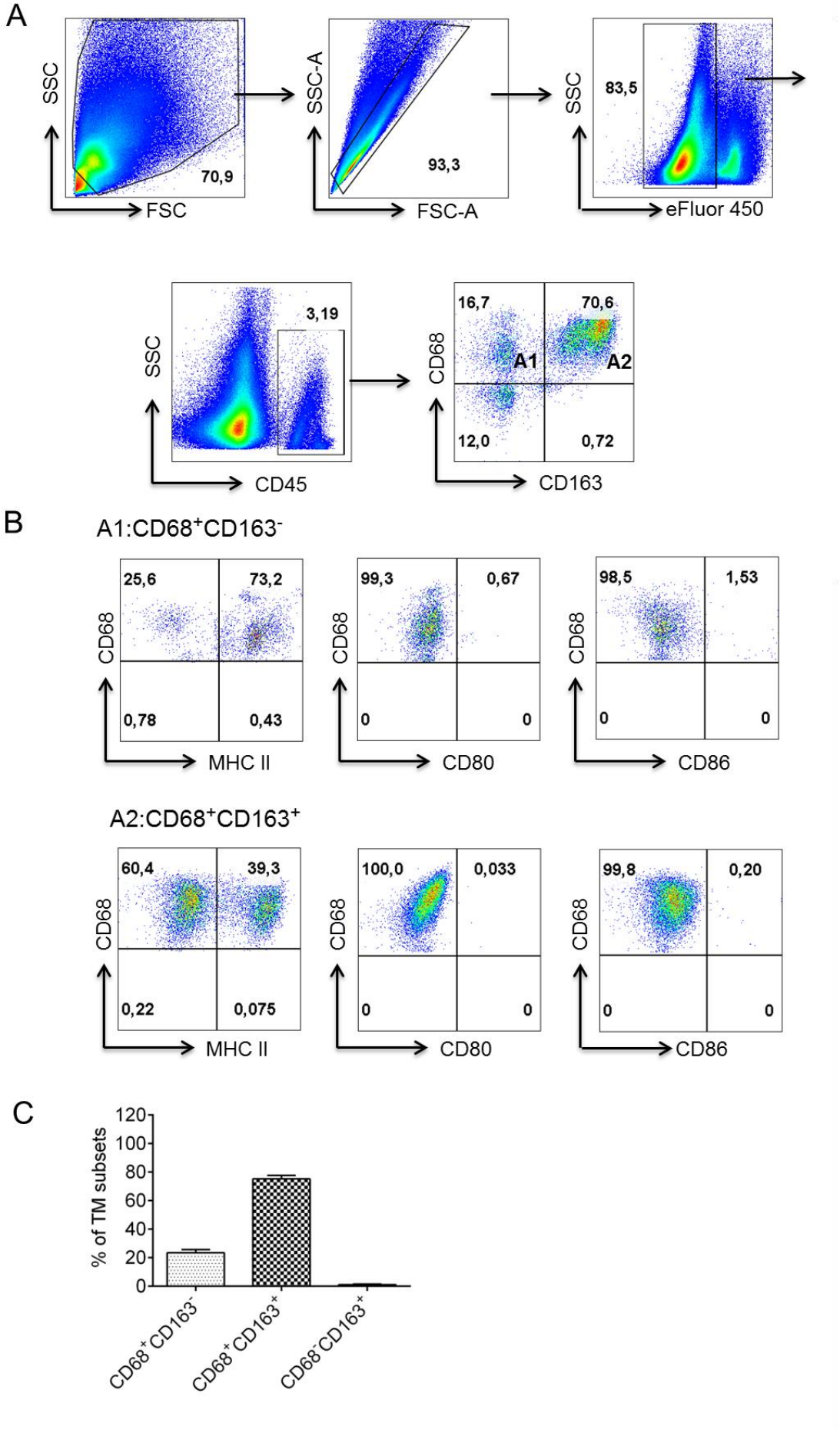

A
SSC
FSC
70,9
SSC-A
FSC-A
93,3
SSC
eFluor 450
83,5
SSC
CD45
3,19
CD68
CD163
16,7
70,6
A1
A2
12,0
0,72
B
A1:CD68+CD163-
CD68
MHC II
25,6
73,2
0,78
0,43
CD80
99,3
0,67
0
0
CD86
98,5
1,53
0
0
A2:CD68+CD163+
60,4
39,3
0,22
0,075
100,0
0,033
99,8
0,20
C
% of TM subsets
120
100
80
60
40
20
0
CD68+CD163-
CD68+CD163+
CD68-CD163+

Figure 4. Original gating strategy for identification of TM subsets. (A) Testicular single cell suspension was obtained by collagenase digestion. Cell aggregates and doublets were excluded on the basis of SSC-A versus FSC-A plot, whilst dead cells were excluded by eFluor 450 staining. The total leukocyte population was identified in live cells by the expression of CD45, and gated for identification of macrophage subsets by the expression of CD68 and CD163 surface markers. (B) The population of $CD68^+$ and $CD163^+$ were further stratified by analyzing the expression of costimulatory molecules CD80 and CD86 and MHC class II. (C) TM subset identified by CD68 and CD163 expression were gated from the total leukocyte population ($CD45^+$ cells). Contour plots (A, B) are representative of three independent experiments from 6 rats and the histogram (C) shows the summary of all the experiments (n=3).

3.2 TM display high phagocytic activity

After the initial characterization of the phenotypic heterogeneity of TM, it was next evaluated whether TM were functionally comparable to other macrophages by examining their phagocytic activity. Indeed, TM displayed high phagocytic activity as evidenced by engulfing fluorescence-labeled Zymosan A *S. cerevisiae* bioparticles (Figure 5A-C). In support, the high phagocytic activity of TM was further corroborated by the appearance of large vacuolar spaces in the TM (Figure 5 D).

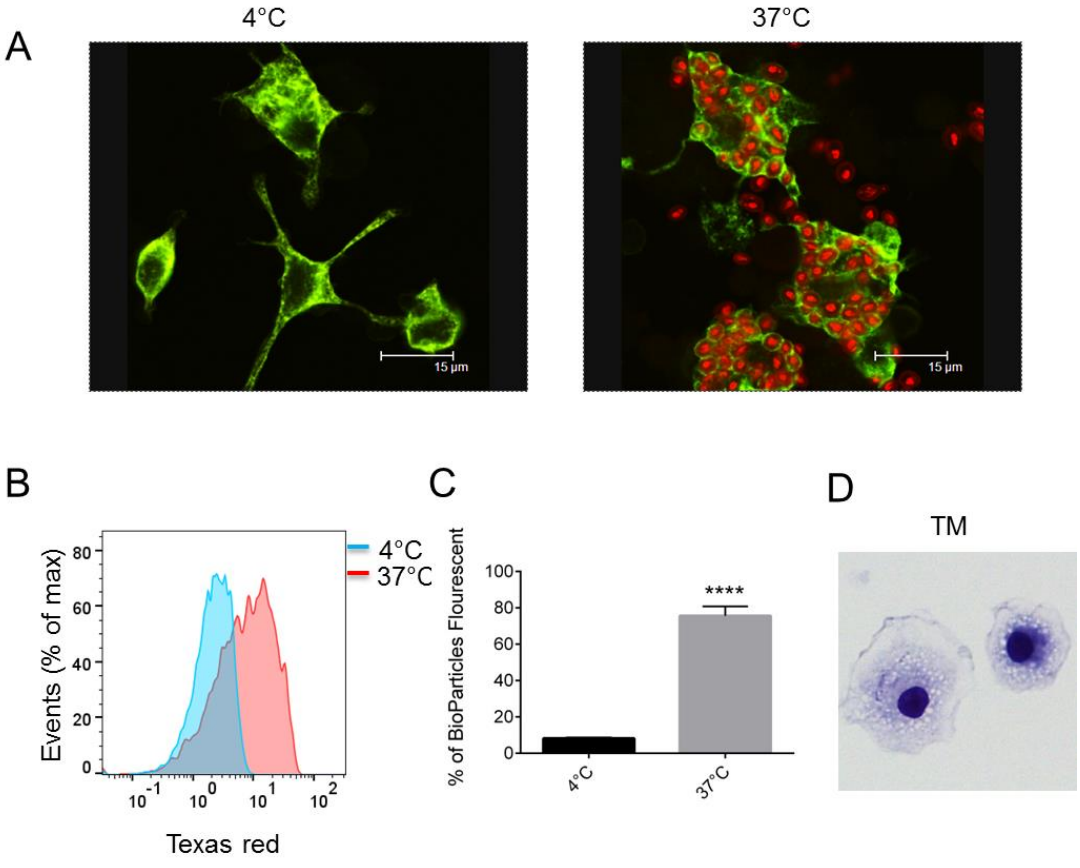


Figure 5. TM show phagocytic activity. (A) The phagocytic activity of TM from adult rat testis was assessed by uptake of fluorescence-labeled Zymosan A *S. Cerevisiae* bioparticles into acidified vesicles for 30 minutes at 37°C and 4°C. Immunofluorescence staining was performed to investigate the expression of CD68 (green), engulfed bioparticles appear as red dots in TM. (B) Flow cytometric analysis was used to confirm the phagocytic activity in $CD68^+$ TM. The blue area indicates the negative staining without the expression of Texas red, whilst the red area points out the TM with the engulfed fluorescence-labeled Zymosan A *S. cerevisiae* bioparticles. (C) Percentage of bioparticles taken up by TM at 4°C and 37°C. Histogram represents the summary of all three independent experiments. The unpaired Student's *t* test was employed for statistical analysis. ****$p < 0.0001$. (D) The appearance of large vacuolar spaces in TM was analyzed by May Grünwald–Giemsa staining.

3.3 TM induce the differentiation of splenic T cells to T regulatory cells

TM are known to exhibit phenotypic immunosuppressive properties characterized by the expression of CD163 and large amounts of the anti-inflammatory cytokine IL-10 (Bhushan et al., 2015; Winnall et al., 2011). To demonstrate functional immunosuppressive properties of TM, we established an *in vitro* co-culture system by cultivating TM and splenic T cells for 4 days with subsequent analysis of the expansion of immunosuppressive T regulatory cells (Tregs) by flow cytometry analysis. Indeed, TM induced the expansion of immunosuppressive Tregs by increasing the expression of $CD25^+$ and $Foxp3^+$ cells (Figure 6 A and B).

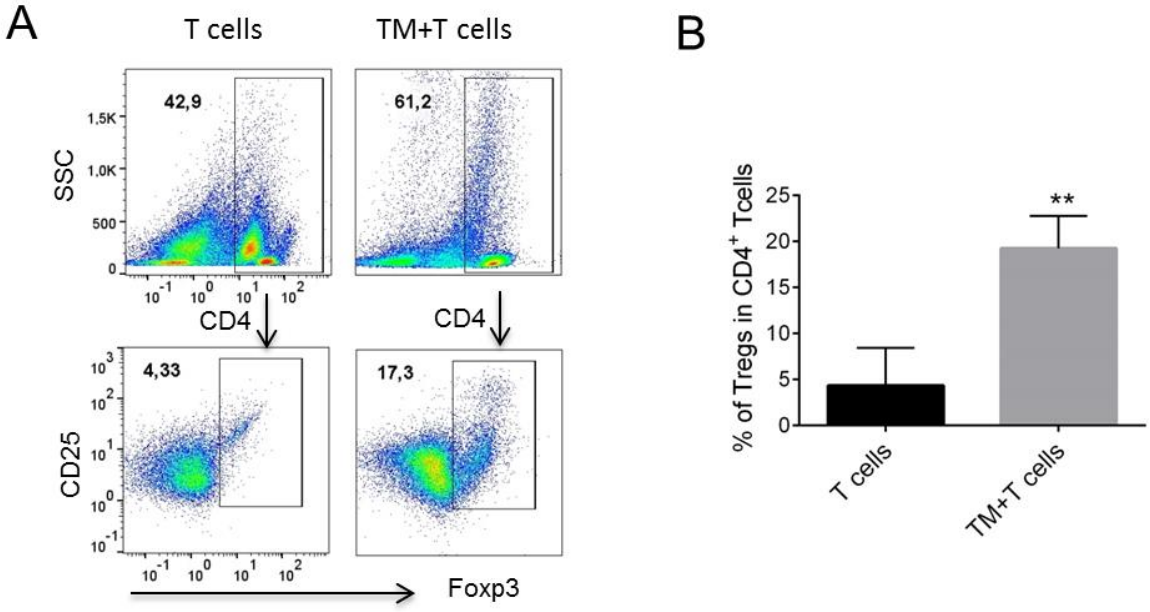


Figure 6. TM polarize splenic T cells towards Tregs. (A) TM and splenic T lymphocytes were co-cultured for 96 h, before determining the population of regulatory T cells ($CD25^+$ $Foxp3^+$) in the $CD4^+$ population. Contour plots are representative of three independent experiments, and (B) the histogram represents the summary of all the experiments. The unpaired Student's *t* test was employed for statistical analysis. ** $p < 0.01$

3.4 IF promote the polarization of splenic T cells to T regulatory cells

To investigate possible immunosuppressive proprieties of IF, which we speculated may influence the TM phenotype, an effect of IF on splenic T cells was tested. Splenic T cells were co-cultured with 10% IF for 4 days, and the differentiation of T cells to Treg was identified by flow cytometric analysis using antibodies against CD25 and Foxp3. IF treatment significantly increased the number of $CD25^+$ $Foxp3^+$ Tregs within the $CD4^+$ population (Figure 7 A and B).

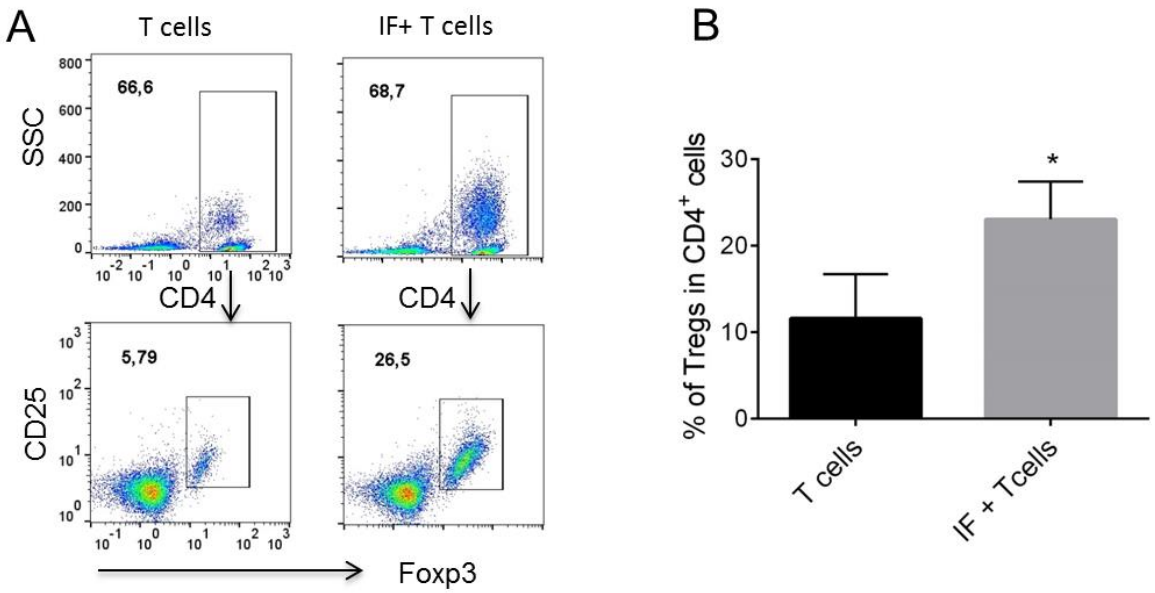


Figure 7. IF treatment induces a differentiation of Treg. Isolated splenic T cells were purified using magnetic beads coated with monoclonal mouse anti-rat pan $CD4^+$ antibody and a MACS column. 5×10^5 T cells were treated with IF for 4 days at 37°C. (A) Flow cytometric analysis was performed to quantify the expression of Treg markers CD25 and Foxp3 in untreated T cells (control, bottom left) or treated with IF (bottom right) from total $CD4^+$ splenic T cells. Contour plots are representative of three independent experiments, and (B) the histogram represents the summary of all the experiments. The unpaired Student's *t* test was employed for statistical analysis. *$p < 0.05$

3.5 IF polarizes blood monocytes derived M1 macrophages towards the M2 macrophage phenotype

As it was shown that IF displays immunosuppressive properties, it was next investigated whether IF could influence the macrophage phenotype. For this purpose, blood monocytes were cultured in the presence of granulocyte macrophage colony stimulating factor (GM-CSF) with/without the addition of IF or serum. Treatment with GM-CSF alone promoted the polarization of blood monocytes to the M1 macrophage phenotype, whereas further addition of IF caused a shift in the GM-CSF-induced M1 macrophage to the M2 macrophage phenotype, as determined by the increased number of $CD163^+$ cells (Figure 8 A, B, C). In contrast, serum treatment did not change the macrophage phenotype.

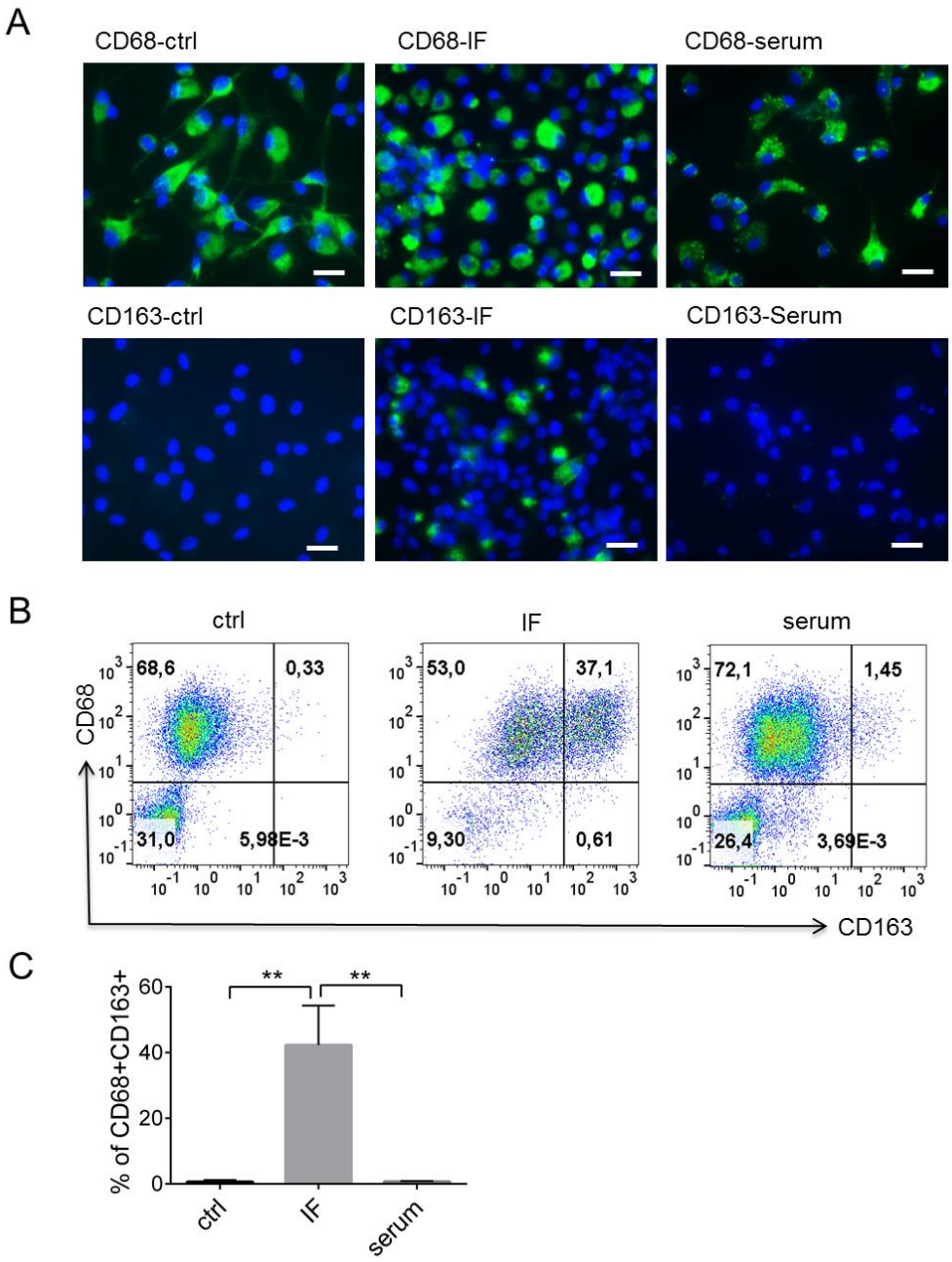


Figure 8. IF treatment differentiates M1 macrophages to the M2 macrophage phenotype. (A-B) Blood monocytes were stimulated with GM-CSF alone or GM-CSF with IF/serum for 7 days. (A) For immunofluorescence staining, cells grown on chamber glass were fixed with cold methanol and blocked by 10% goat serum, M1 and M2 macrophage were identified by mouse anti rat CD68 and CD163, respectively. Primary antibodies were visualized with Cy3-labeled secondary antibody and nuclei were counterstained with DAPI. Photos were taken using an Axioplan 2 fluorescence microscope (× 40 objective). Scale bars = 20 µm. (B) Flow cytometric analysis were used to analyze the differentiation of blood monocyte to M1 ($CD68^+$ $CD163^-$) or M2 macrophage ($CD68^+$ $CD163^+$). Contour plots are representative of three independent experiments, and (C) Histogram represents the summary of all three experiments. The one-way ANOVA was employed for statistical analysis. **$p < 0.01$

3.6 M2 macrophage specific genes are highly expressed in IF induced macrophage

The shift of the macrophage phenotype was verified by qRT-PCR results. Following treatment with IF, the expression levels of M1 macrophage marker genes, such as *Inos*, *Il-6* and *Tnf*-α were significantly reduced, whereas the expression levels of the M2 macrophage markers *Il-10* and *Cd163* were significantly increased (Figure 9).

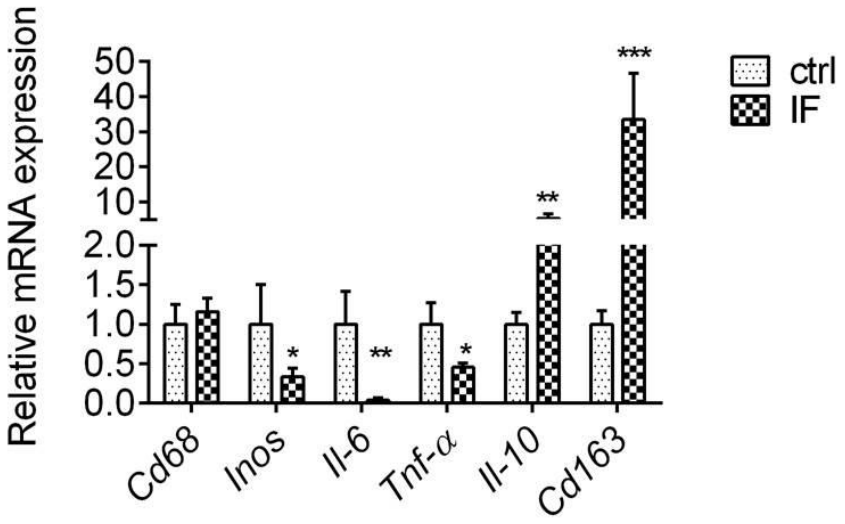


Figure 9. IF increases the expression of M2 macrophage associated genes. The mRNA levels of M1 and M2 macrophage related genes were quantified by qRT-PCR in GM-CSF or GM-CSF+IF treated macrophages. Results were normalized to expression level of β-macroglobulin. All data are shown as mean ± SD of at least three independent experiments. Student's *t* test was employed for statistical analysis. *p < 0.05, **p < 0.01, ***p < 0.001

3.7 IF treatment induces the production of IL-10

The change in the macrophage phenotype after IF treatment was further confirmed by analyzing the secretion of both the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokine TNF-α. ELISA results demonstrate that IF-induced M2 macrophages secrete high amounts of IL-10 with concomitantly low levels of TNF-α upon challenge with LPS (Figure 10 A and B). Notably, the addition of serum instead of IF did not change the phenotype of GM-CSF-induced M1 macrophages (Figure 10 A and B). The levels of IL-10 and TNF-α were similar in serum treatment and control group.

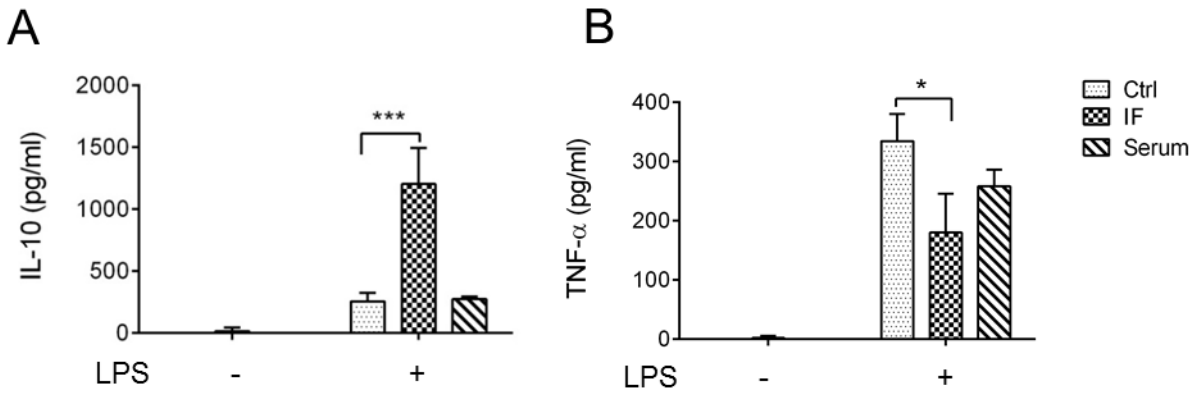


Figure 10. Secretion of the anti-inflammatory cytokine IL-10 was induced by IF. (A, B) Macrophages polarized by treatment with GM-CSF alone or GM-CSF with IF/serum were challenged with LPS (100 ng/ml) for 3 h. Conditioned media from cells were analyzed for IL-10 and TNF-α secretion by specific sandwich ELISA. Data are presented as the mean ± SD of 3-4 independent experiments. The one-way ANOVA was employed for statistical analysis. *** $p < 0.001$, * $p < 0.05$

3.8 IF suppresses the activation of the NF-κB signaling pathway

To analyze the activation of inflammatory signaling pathways in GM-CSF- and IF-polarized macrophages cells were stimulated with LPS. In GM-CSF-polarized macrophages, IκBα (an inhibitor of the pro-inflammatory transcription factor NF-κB) was readily degraded upon LPS treatment, whereas in IF-polarized macrophages, the degradation of IκBα was attenuated and the recovery rate was faster. The results indicate that IF treatment could suppress the activation of the NF-κB signaling pathway (Figure 11). Importantly, the activation of the anti-inflammatory signaling pathways CREB and STAT3 was much more pronounced in IF-polarized macrophages than in control macrophages.

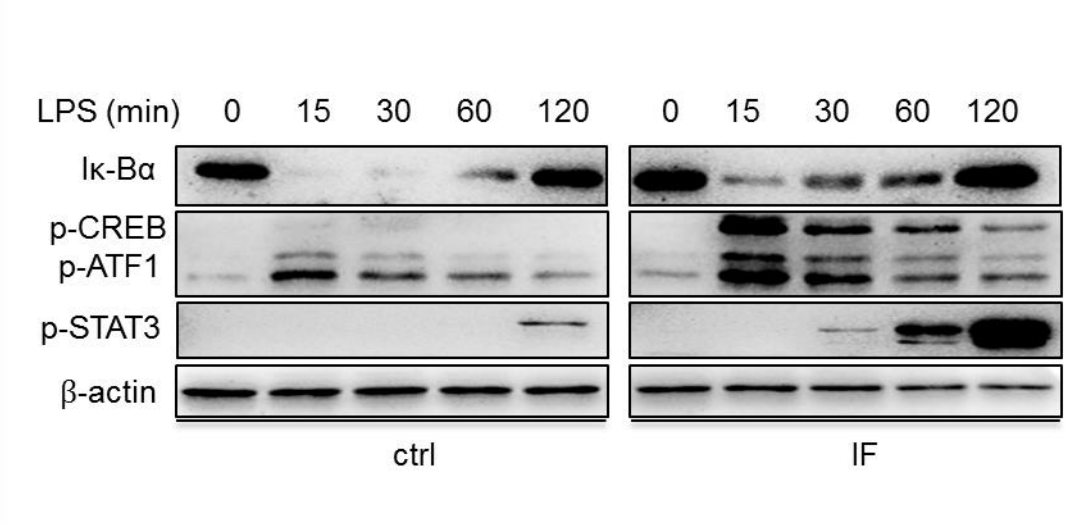


Figure 11. The NF-κB signaling pathway was attenuated in IF-polarized macrophages. Blood derived monocytes were treated with GM-CSF with or without IF for 7 days, and then stimulated with LPS (100 ng/ml) for the indicated time points. Cell lysates were subjected to Western blot analysis using antibodies directed against IκBα, phosphorylated CREB and phosphorylated STAT3. β -actin was used as a loading control. Representative blots from three different experiments are shown here.

3.9 IF-polarized macrophages polarize splenic T cells towards T regulatory cells

Previous studies and data form our lab demonstrate that TM display immunosuppressive properties (Schmidt et al., 2016). To determine whether IF polarized macrophages can functionally mimic TM, firstly, monocytes were polarized into M2 macrophages with GM-SCF and IF for 7 days, and then secondly co-cultured with splenic T cells for 4 days. Flow cytometric data clearly demonstrated that IF derived macrophages could induce the expansion of Treg similar to TM (Figure 12). These combined results clearly indicate that testicular IF has the capacity to influence the phenotype and function of TM.

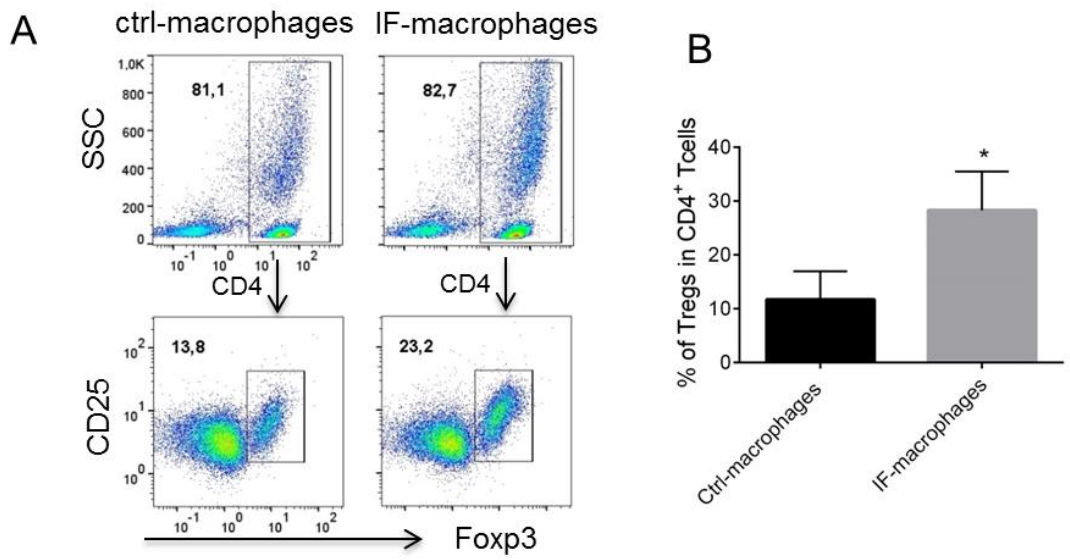


Figure 12. IF polarized-macrophages promote the differentiation of splenic T cells to Treg. Blood monocytes were stimulated with GM-CSF alone or GM-CSF with IF for 7 days. 5×10^5 splenic T cells were added to monocytes derived macrophages and co-cultured for 4 days. The expansion of Tregs ($CD25^+$ $Foxp3^+$) were analyzed by flow cytometry. Contour plots are representative of three independent experiments, and (B) the histogram represents the summary of all the experiments. The unpaired Student's *t* test was employed for statistical analysis. *$p < 0.05$

3.10 No indication for differences in immunosuppressive proteins in serum and IF

TM in their testicular environment are found exclusively in the interstitial space and are exposed to IF. IF in contrast to serum displays immunosuppressive properties as shown above. To characterize the nature of the molecules in the IF that are responsible for the observed effect on the polarization of blood monocytes to the M2 phenotype, IF and serum were analyzed by a proteomic approach consisting of a combination of two-dimensional (2D) gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Although a total of 68 proteins were found at different levels in IF and serum, no such differences were observed in the levels of established immunosuppressive proteins (Figure 13 and Table 5).

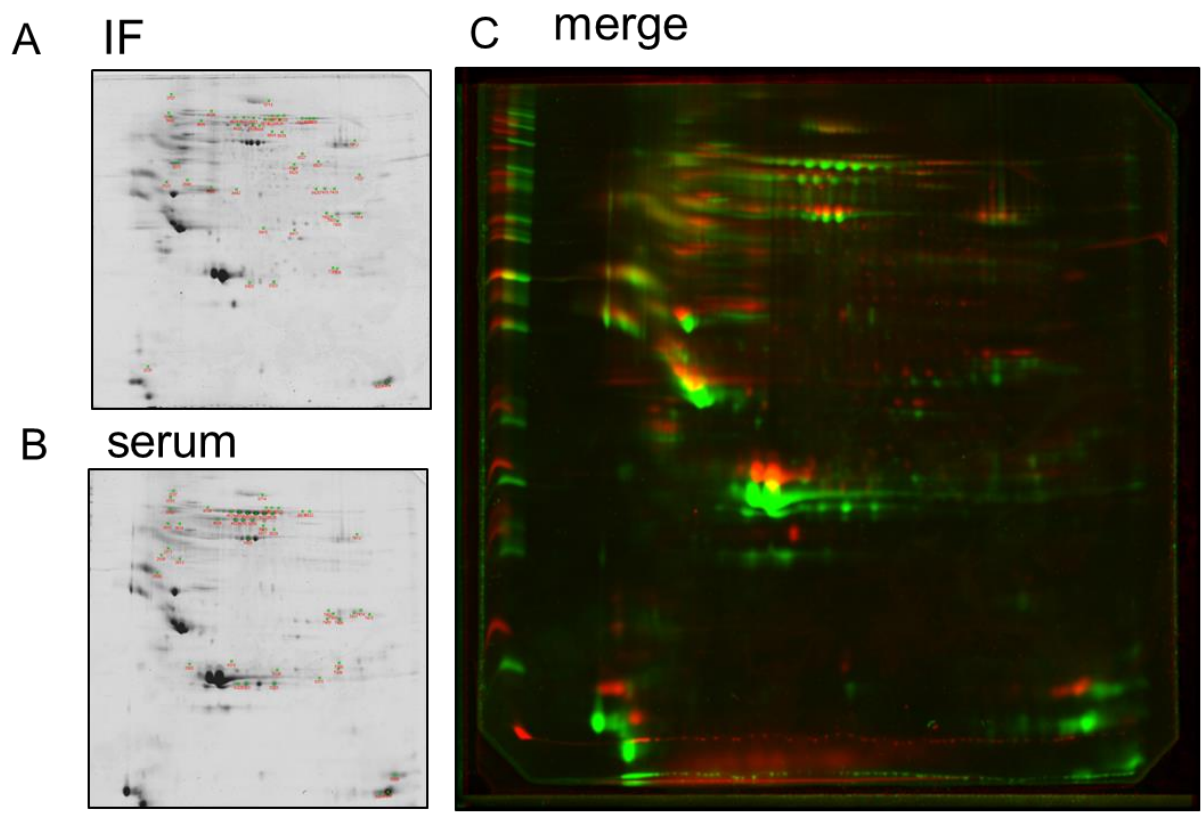


Figure 13. No differential in immunosuppressive proteins was found in the IF and serum. (A, B) Equal amounts of proteins from testicular IF and serum were resolved using two dimensional gel electrophoresis. (C) 2D-gel image overlay of protein from IF (red) and serum (green) is shown.

Table 5

Location	Accession	Name
2505	IPI00555299	Tax_Id=10116 Gene_Symbol=Pon1 paraoxonase 1
2508	IPI00211075	Tax_Id=10116 Gene_Symbol=Serpina3n Serine protease inhibitor A3N
2513	IPI00372372	Tax_Id=10116 Gene_Symbol=Serpinc1 Serine (Or cysteine) peptidase inhibitor, clade C (Antithrombin), member 1
2522	IPI00230941	Tax_Id=10116 Gene_Symbol=Vim Vimentin
2615	IPI00568259	Tax_Id=10116 Gene_Symbol=Itih4 Putative uncharacterized protein Itih4
3512	IPI00195673	Tax_Id=10116 Gene_Symbol=Tubb6 Tubulin, beta 6
3512	IPI00197579	Tax_Id=10116 Gene_Symbol=Tubb5 Isoform 1 of Tubulin beta-5 chain
3512	IPI00362160	Tax_Id=10116 Gene_Symbol=Tubb3 Tubulin beta-3 chain
3512	IPI00475639	Tax_Id=10116 Gene_Symbol=Tubb2a Tubulin beta-2A chain
3512	IPI00400573	Tax_Id=10116 Gene_Symbol=Tubb2c Tubulin beta-2C chain

3614	IPI00568259	Tax_Id=10116 Gene_Symbol=Itih4 Putative uncharacterized protein Itih4
3640	IPI00370411	Tax_Id=10116 Gene_Symbol=Fbln1 Putative uncharacterized protein Fbln1
3702	IPI00370411	Tax_Id=10116 Gene_Symbol=Fbln1 Putative uncharacterized protein Fbln1
3707	IPI00422076	Tax_Id=10116 Gene_Symbol=Thbs1 Thrombospondin 1
4323	IPI00476458	Tax_Id=10116 Gene_Symbol=Gpx3 Glutathione peroxidase 3
4422	IPI00189819	Tax_Id=10116 Gene_Symbol=Actb Actin, cytoplasmic 1
4422	IPI00480639	Tax_Id=10116 Gene_Symbol=C3 Complement C3 (Fragment)
4614	IPI00209973	Tax_Id=10116 Gene_Symbol=C4bpa Complement component 4 binding protein, alpha
4624	IPI00189981	Tax_Id=10116 Gene_Symbol=F2 Prothrombin (Fragment)
4624	IPI00372372	Tax_Id=10116 Gene_Symbol=Serpinc1 Serine (Or cysteine) peptidase inhibitor, clade C (Antithrombin), member 1
4627	IPI00209973	Tax_Id=10116 Gene_Symbol=C4bpa Complement component 4 binding protein, alpha
4632	IPI00372372	Tax_Id=10116 Gene_Symbol=Serpinc1 Serine (Or cysteine) peptidase inhibitor, clade C (Antithrombin), member 1
4635	IPI00209973	Tax_Id=10116 Gene_Symbol=C4bpa Complement component 4 binding protein, alpha
4704	IPI00188541	Tax_Id=10116 Gene_Symbol=Itih4 inter-alpha-inhibitor H4 heavy chain
5303	IPI00476458	Tax_Id=10116 Gene_Symbol=Gpx3 Glutathione peroxidase 3
5323	IPI00476458	Tax_Id=10116 Gene_Symbol=Gpx3 Glutathione peroxidase 3
5415	IPI00421781	Tax_Id=10116 Gene_Symbol=Krt72 keratin, type II cytoskeletal 72
5503	IPI00191737	Tax_Id=10116 Gene_Symbol=Alb Serum albumin
5603	IPI00372372	Tax_Id=10116 Gene_Symbol=Serpinc1 Serine (Or cysteine) peptidase inhibitor, clade C (Antithrombin), member 1
5603	IPI00189981	Tax_Id=10116 Gene_Symbol=F2 Prothrombin (Fragment)
5603	IPI00360140	Tax_Id=10116 Gene_Symbol=Tubgcp2 Tubulin, gamma complex associated protein 2
5612	IPI00372372	Tax_Id=10116 Gene_Symbol=Serpinc1 Serine (Or cysteine) peptidase inhibitor, clade C (Antithrombin), member 1
5617	IPI00656380	Tax_Id=10116 Gene_Symbol=Igh-6 Igh-6 protein
5618	IPI00656380	Tax_Id=10116 Gene_Symbol=Igh-6 Igh-6 protein
5620	IPI00189981	Tax_Id=10116 Gene_Symbol=F2 Prothrombin (Fragment)
5620	IPI00372372	Tax_Id=10116 Gene_Symbol=Serpinc1 Serine (Or cysteine) peptidase inhibitor, clade C (Antithrombin), member 1
5626	IPI00779823	Tax_Id=10116 Gene_Symbol=Kif20b Putative uncharacterized protein Kif20b
5626	IPI00372372	Tax_Id=10116 Gene_Symbol=Serpinc1 Serine (Or cysteine) peptidase inhibitor, clade C (Antithrombin), member 1
5629	IPI00656380	Tax_Id=10116 Gene_Symbol=Igh-6 Igh-6 protein

5714	IPI00208659	Tax_Id=10116 Gene_Symbol=Cfh Complement inhibitory factor H
5714	IPI00778265	Tax_Id=10116 Gene_Symbol=Cfh Platelet complement factor H
5717	IPI00331776	Tax_Id=10116 Gene_Symbol=C6 Complement component C6
5721	IPI00331776	Tax_Id=10116 Gene_Symbol=C6 Complement component C6
5723	IPI00331776	Tax_Id=10116 Gene_Symbol=C6 Complement component C6
6315	IPI00197703	Tax_Id=10116 Gene_Symbol=Apoa1 Apolipoprotein A-I
6417	IPI00361597	Tax_Id=10116 Gene_Symbol=Rnf213 Putative uncharacterized protein ENSRNOP00000049231
6425	IPI00211127	Tax_Id=10116 Gene_Symbol=Ass1 Argininosuccinate synthase
6425	IPI00959884	Tax_Id=10116 Gene_Symbol=Ccdc110 Ccdc110 protein
6523	IPI00948614	Tax_Id=10116 Gene_Symbol=Fgb Putative uncharacterized protein Fgb
6527	IPI00360340	Tax_Id=10116 Gene_Symbol=Ehd1 EH domain-containing protein 1
6537	IPI00948614	Tax_Id=10116 Gene_Symbol=Fgb Putative uncharacterized protein Fgb
6616	IPI00206780	Tax_Id=10116 Gene_Symbol=Plg Plasminogen
6622	IPI00206780	Tax_Id=10116 Gene_Symbol=Plg Plasminogen
6638	IPI00206780	Tax_Id=10116 Gene_Symbol=Plg Plasminogen
7401	IPI00203319	Tax_Id=10116 Gene_Symbol=Fcn1 Ficolin-1
7402	IPI00554039	Tax_Id=10116 Gene_Symbol=RGD1565368 glyceraldehyde-3-phosphate dehydrogenase-like
7402	IPI00555252	Tax_Id=10116 Gene_Symbol=LOC685186;Gapdh Glyceraldehyde-3-phosphate dehydrogenase
7405	IPI00203319	Tax_Id=10116 Gene_Symbol=Fcn1 Ficolin-1
7411	IPI00554039	Tax_Id=10116 Gene_Symbol=RGD1565368 glyceraldehyde-3-phosphate dehydrogenase-like
7411	IPI00567177	Tax_Id=10116 Gene_Symbol=- Putative uncharacterized protein Gapdh-ps2
7414	IPI00554039	Tax_Id=10116 Gene_Symbol=RGD1565368 glyceraldehyde-3-phosphate dehydrogenase-like
7414	IPI00555252	Tax_Id=10116 Gene_Symbol=LOC685186;Gapdh Glyceraldehyde-3-phosphate dehydrogenase
7415	IPI00195861	Tax_Id=10116 Gene_Symbol=Defb36 DEFB36
7416	IPI00211127	Tax_Id=10116 Gene_Symbol=Ass1 Argininosuccinate synthase
7612	IPI00422037	Tax_Id=10116 Gene_Symbol=C4-2 complement component 4, gene 2
7612	IPI00213036	Tax_Id=10116 Gene_Symbol=C4b Complement C4
8209	IPI00206634	Tax_Id=10116 Gene_Symbol=Pf4 Platelet factor 4
2505	IPI00555299	Tax_Id=10116 Gene_Symbol=Pon1 paraoxonase 1

Table 5. List of differentially expressed of proteins in IF and Serum identified by a combination of two-dimensional (2D) gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

3.11. Key immunomodulators are not differentially found in IF and serum

Although a total of 68 proteins were found at different levels comparing IF and serum, no such differences were observed in the level of established immunosuppressive proteins (Supplementary table 1). IL-10 and TGF-β were not identified in this proteomics approach and also the subsequent analysis by ELISA did not detect IL-10, TNF-α or the inactive form of TGF-β1 in either IF or serum (Figure 14). However, following acid activation, the level of TGF-β1 was significantly higher in the serum than in the IF (Figure 14). Taken together, these results did not suggest a role for known immunosuppressive proteins in IF in subverting the TM phenotype.

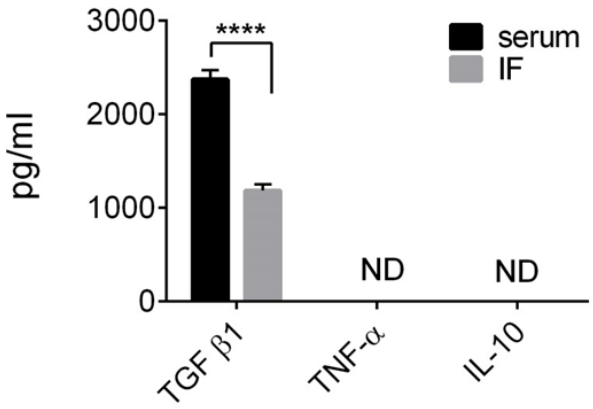


Figure 14. The cytokines TGF-β1, IL-10 and TNF-α were analyzed in serum and IF. Levels of TGF-β1 (IF and serum samples were activated with acid), IL-10 and TNF-α were measured in IF and serum by using specific sandwich ELISA. The unpaired Student's *t* test was employed for statistical analysis. ****$p < 0.0001$

3.12 High levels of prostaglandins (PG) in testicular IF

PG are established modulators of the macrophage phenotype (Kalinski, 2012). Liquid chromatography–MS/MS (LC-MS) analyses revealed that IF contains substantial amounts of PGE_2 and PGI_2 (each ca. 3,000 × higher than serum), PGD_2 (ca. 35 × higher than serum) and $PGF_{2\alpha}$ (ca. 12 × higher than serum). As the concentration of $PGF_{2\alpha}$ (ca. 3ng/ml) in IF

was very low in comparison to those of PGE_2, PGD_2 and PGI_2, $PGF_{2\alpha}$ was exluded from further experiments. Serum concentrations were consistently close to the detection limit in all cases. In contrast, the levels of thromboxane B2 (TXB_2) were significantly lower (ca. 50%) in IF as compared to serum (Figure 15).

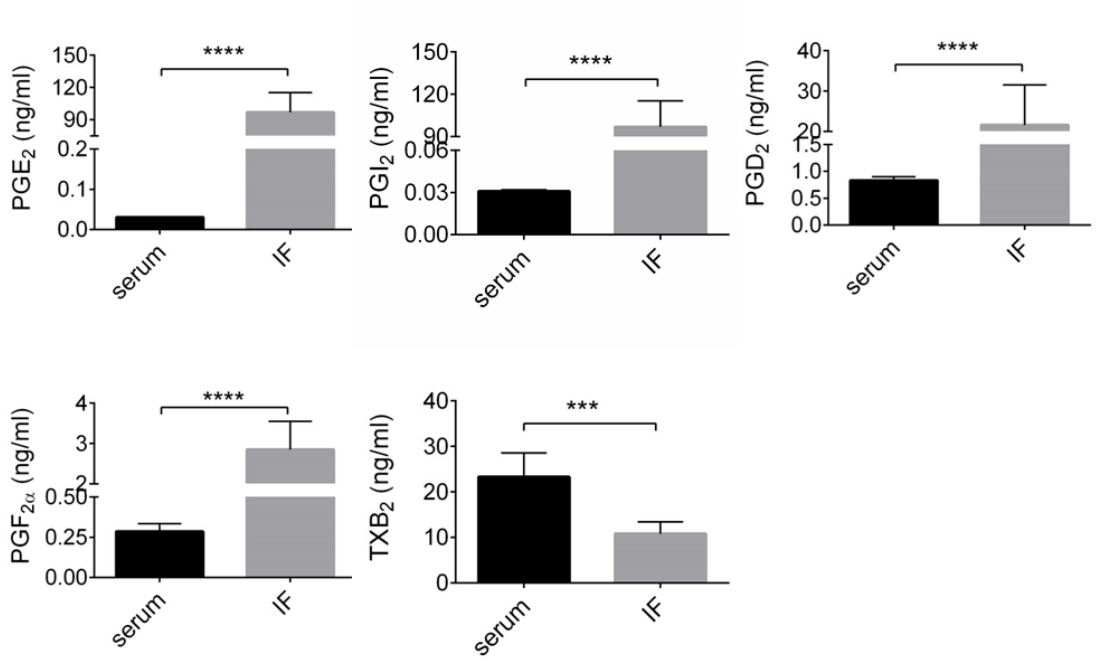


Figure 15. The levels of PG in serum and IF were measured by LC-MS. The levels of PG (PGE_2, PGI_2, PGD_2, PGF2α, TXB_2) were analyzed in rat testicular IF and serum by liquid chromatography–mass spectrometry (LC-MS). Data were obtained from seven rats and are presented as mean ± SD. The unpaired Student's *t* test was employed for statistical analysis. ***$p < 0.001$, ****$p < 0.0001$

3.13 PGE_2 and PGI_2 shift macrophages towards the M2 macrophage phenotype

Based on the high levels of PG detected in IF, the influence of PG on the M2 macrophage polarization was examined next. The treatment of GM-CSF-stimulated monocytes with PGE_2 and PGI_2 shifted the macrophage phenotype from M1 to M2 as shown by an increased number of $CD163^+$ cells within the $CD68^+$ cell population (PGE_2 from 0.21 to 7.53%; PGI_2 from 0.21% to 3.49%; Figure 16 A-C).

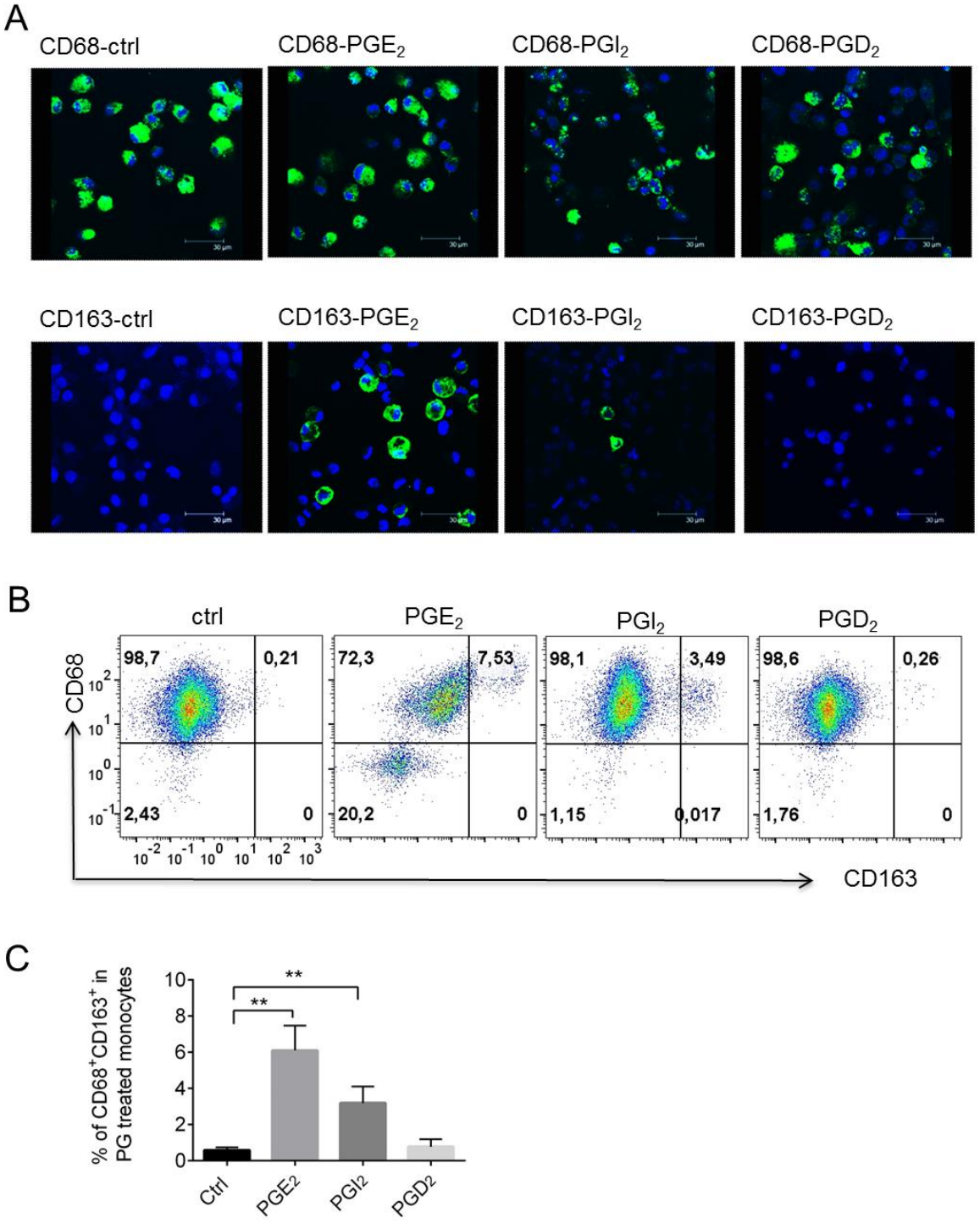


Figure 16. PGE_2 and PGI_2 induce the differentiation of M2 macrophages. Blood monocytes were stimulated with GM-CSF alone or GM-CSF with PG (PGE_2, PGD_2 and PGI_2) for 7 days. Immunofluorescence (A) and flow cytometric analysis (B) were used to determine the phenotype of macrophages. Contour plots are representative of three independent experiments, and (C) the histogram represents the summary of all the experiments. The unpaired one-way ANOVA was employed for statistical analysis. **$p < 0.01$.

3.14 PGE_2 or PGI_2-polarized macrophages secrete high amounts of IL-10

As PGE_2 and PGI_2 are able to polarize blood monocytes into the M2 macrophage phenotype, the cytokine levels were determined in PG (PGE_2, PGI_2 and PGD_2) pre-treated monocytes that were subsequently challenged with LPS. Both PGE_2 and PGI_2 can significantly enhance the secretion of IL-10 and have significantly decreased TNF-α levels in LPS stimulated macrophages (Figure 17 A and B). In contrast, PGD_2 had no effect on macrophage polarization and cytokine expression.

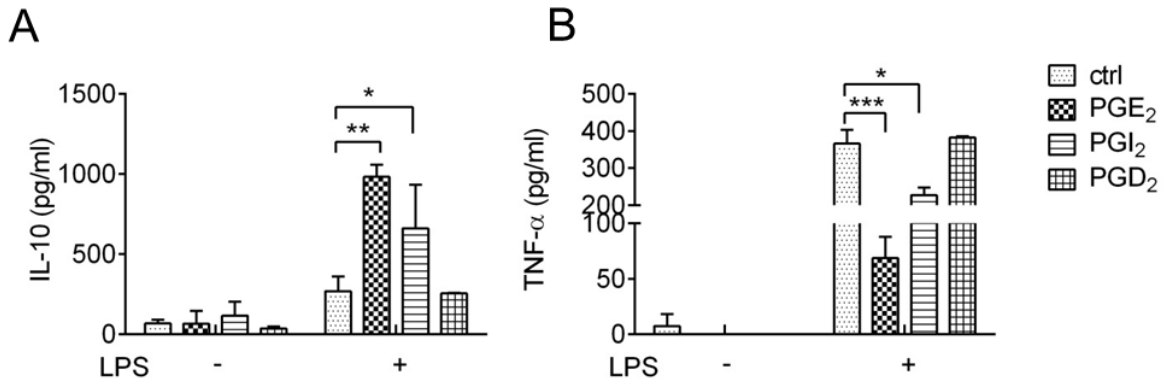


Figure 17. PGE_2 and PGI_2 induce the secretion of the anti-inflammatory cytokine IL-10. (A, B) Blood derived monocytes were incubated with GM-CSF or +/- PG (PGE_2, PGD_2, PGI_2) for 7 days followed by treatment with LPS (100 ng/ml) for 3 h. The secretion of IL-10 and TNF-α was analyzed by ELISA in conditioned media. Data are presented as the mean ± SD of 4 independent experiments. The one-way ANOVA was employed for statistical analysis. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

3.15 PGE_2 suppresses the activation of the NF-κB signaling pathway

As PGE_2 and PGI_2 treatment could induce the polarization of M2 macrophages and the production of IL-10 the activation of the inflammatory signaling pathway in GM-CSF ± PGE_2-polarized macrophages following LPS stimulation was analyzed as the next step,. Compared with GM-CSF-polarized macrophages, PGE_2 suppressed the activation of the NF-κB signaling pathway by deferring IκBα degradation. The results suggest that PGE_2 treatment could also suppress the activation of the NF-κB signaling pathway (Figure 18). Similarly, the

activation of the anti-inflammatory CREB and STAT3 signaling pathways was much more pronounced in PGE_2-polarized macrophages.

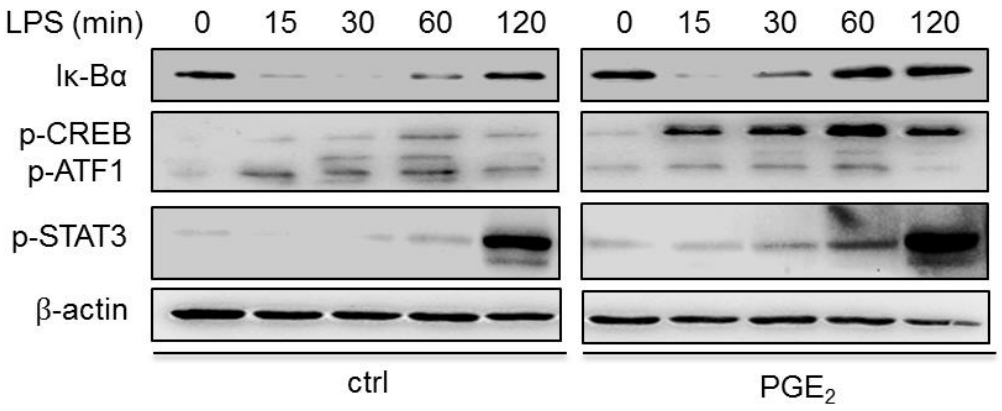


Figure 18. The NF-κB signaling pathway was inhibited in PGE_2-polarized macrophages. Blood derived monocytes were incubated with GM-CSF ± PGE_2 for 7 days followed by treatment with LPS (100 ng/ml) for the indicated time points. Cell lysates were analyzed by Western blot using antibodies against IκBα, p-CREB, p-STAT3. β-actin served as a loading control. Each experiment has been performed at least three times and a representative experiment is shown.

3.16 IF contains high amounts of steroid hormones

As a first step to determine a possible role for steroid hormones in influencing the TM phenotype, the concentrations of steroid hormones were analyzed in IF by gas chromatography tandem mass spectrometry (GC-MS/MS). In agreement with previous studies, significant levels of testosterone, 4-androstenedione and 5α-androstanediol (5α-androstane-3α, 17β-diol) were found in the IF (Figure 19). Importantly and previously unnoticed, IF contains extremely high levels of corticosterone. Although established to be synthesized mainly by the adrenal gland, the corticosterone levels in IF were approximately 7 times higher than in serum (Figure 19). Moreover, the level of 5α-androstanediol, dihydrotestosterone and 17OH-progesterone was also much higher in IF compared to serum. The influence of these steroid hormones on the macrophage phenotype is largely unknown and thus was further investigated.

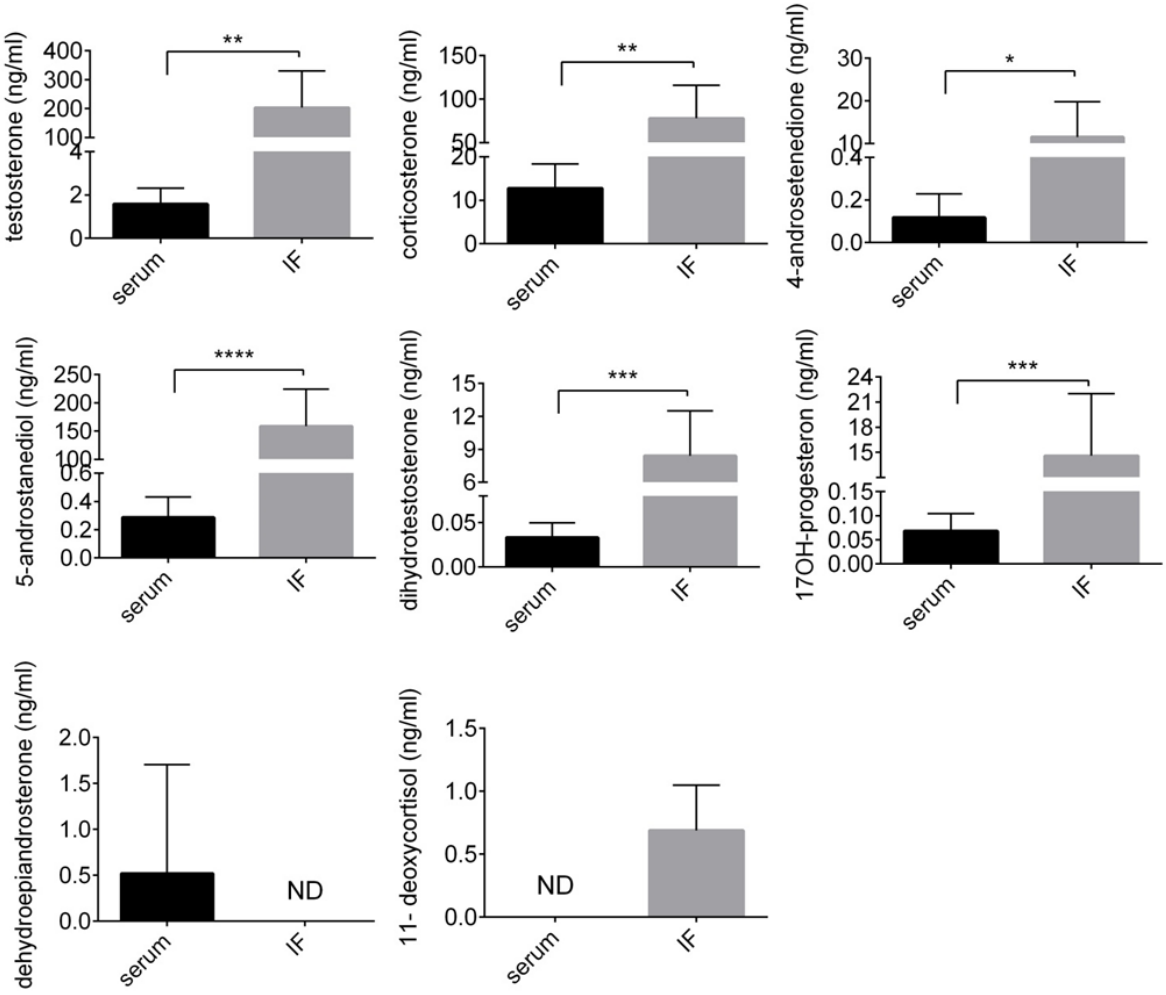


Figure 19. High levels of steroid hormones were found in IF. The concentrations of steroid hormones (testosterone, corticosterone, 4-androstenedione, 5-androstanediol, dihydrotestosterone, 17OH-progesterone, dehydroepiandrosterone and 11-deoxycortisol) in rat testicular IF and serum were measured by gas chromatography tandem mass spectrometry (GC-MS/MS). Data obtained from 5 rats are presented as mean ± SD. The unpaired Student's *t* test was employed for statistical analysis. ***$p < 0.001$, ****$p < 0.0001$

3.17 Testosterone and corticosterone polarize macrophages towards the M2 macrophage phenotype

As the next step, it was examined whether testosterone, corticosterone, 4-androstendion and 5-androstanediol could influence the macrophage phenotype. Flow cytometric analyses demonstrated that testosterone and corticosterone polarized GM-CSF-induced blood monocytes from M1 to the M2 macrophage phenotype as seen by a significantly increased

numbers of $CD163^+$ macrophages within the $CD68^+$ population. In this regard, corticosterone (from 0.75% to 45.8%) was much more potent in polarizing the macrophage phenotype than testosterone (to 8.05%) (Figure 20 A and B). Notably, 4-androstenedione (Figure 20 A and B) and 5-androstanediol (data not shown) did not influence the macrophage phenotype.

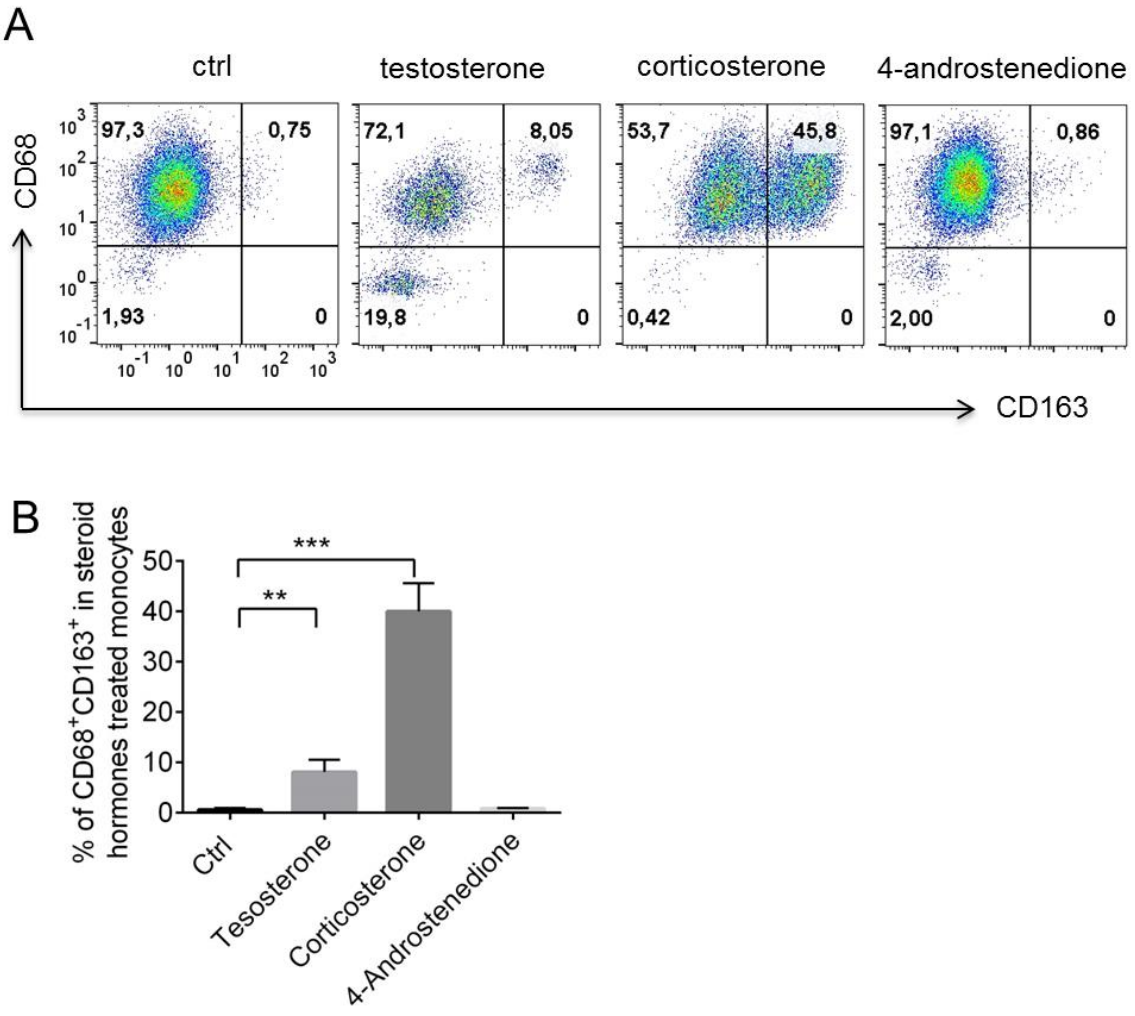


Figure 20. Testosterone or corticosterone can induce the differentiation of M2 macrophages. (A) The percentage of $CD163^+$ in $CD68^+$ macrophage population was analyzed by flow cytometry after steroid hormone (testosterone, corticosterone and 4-androstenedion) treatment. Contour plots are representative of three independent experiments, and (B) the histogram represents the summary of all the experiments. Data were obtained from seven rats and presented as mean ± SD. One-way ANOVA test was employed for statistical analysis. $^{**}p < 0.01$, $^{***} p < 0.001$

3.18 Testosterone or corticosterone induce the production of IL-10

In accordance with their potential to increase the number of $CD163^+$ macrophages,

testosterone and corticosterone polarized macrophages were characterized by the secretion of higher amount of IL-10 and lower level of TNF-α after LPS stimulation compared to control. Notably, 4-androstenedione did not influence the cytokine levels of both IL-10 and TNF-α (Figure 21).

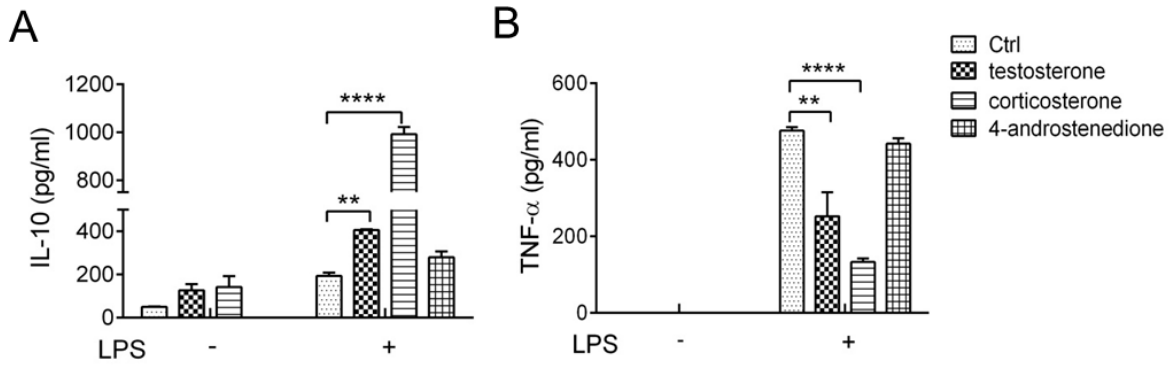


Figure 21. Testosterone or corticosterone induced the secretion of the anti-inflammatory cytokine IL-10. (A, B) Blood derived monocytes were incubated with GM-CSF +/- steroid hormones (testosterone, corticosterone and 4-androstenedione) for 7 days followed by treatment with LPS (100 ng/ml) for 3 h. The secretion of IL-10 and TNF-α were measured by ELISA in conditioned media. Data are presented as the mean ± SD of 3-4 independent experiments. One-way ANOVA test was employed for statistical analysis. **p<0.01; ****p<0.0001

3.19 Testosterone but not corticosterone suppresses the activation of the NF-κB signaling pathway

An impaired activation of the NF-κB signaling pathway was visible by only a partial degradation of IκBα in testosterone treated and LPS stimulated macrophages. In contrast, treatment with corticosterone did not attenuate the activation of the NF-κB signaling pathway. Although testosterone and corticosterone differentially regulated the NF-κB signaling pathway, both of them were found to activate CREB signaling. Of note, activation of STAT3 was similar to control in corticosterone stimulated cells, whereas testosterone activated STAT3 signaling (Figure 22).

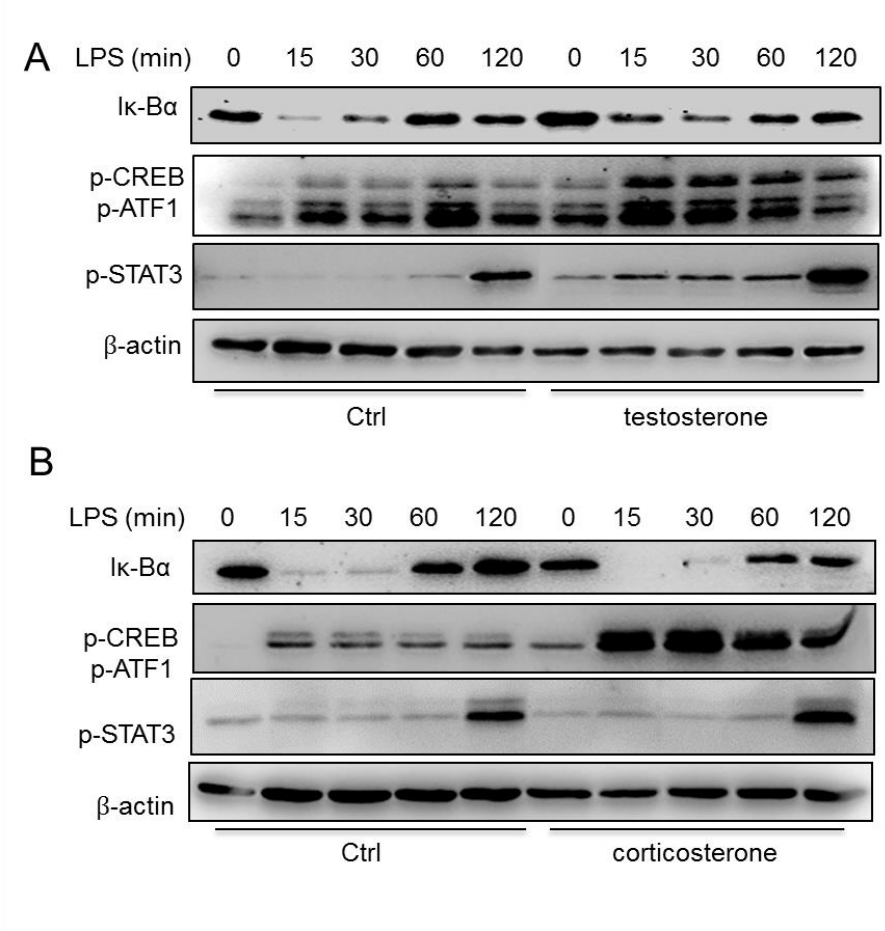


Figure 22. The NF-kB signaling pathway was attenuated in testosterone polarized macrophage. (A, B) Blood derived monocytes were incubated with GM-CSF ± steroid hormones (testosterone and corticosterone) for 7 days followed by treatment with LPS (100 ng/ml) for the indicated time periods. Cell lysates were analyzed by Western blot using antibodies against IκBα, p-CREB, p-STAT3. β-actin served as a loading control. Each experiment has been performed at least three times and a representative experiment is shown.

3.20 Corticosterone is responsible for maintaining the immunosuppressive phenotype of TM

Amongst the PG and steroid hormones tested, testosterone and especially corticosterone demonstrated the highest immunosuppressive potency and thus represent promising candidate molecules responsible for the establishment and maintenance of the immunosuppressive effect of IF on TM. As testosterone and corticosterone mediate their functions through the androgen receptor (AR) and glucocorticoid receptor (GR), respectively, specific AR and GR antagonists (flutamide and RU486, respectively) were used for subsequent studies. Of note, RU486 could significantly suppress the IF-induced M2 macrophage polarization, whereas the effect of flutamide did not reach statistical significance (Figure 23).

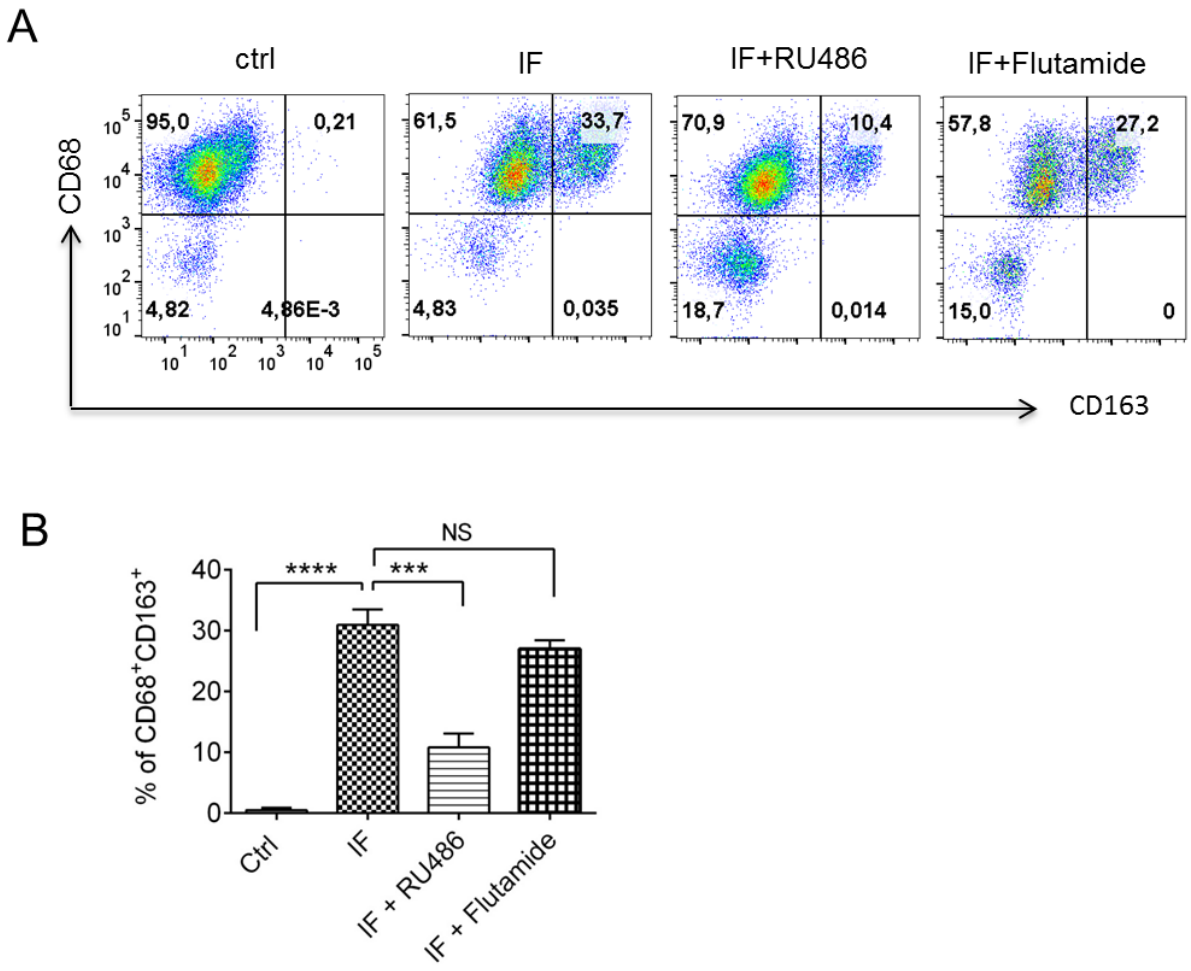


Figure 23. RU486 suppresses the differentiation of IF-polarized M2 macrophage. (A) Blood monocytes were stimulated with GM-CSF alone or GM-CSF with IF/serum for 7 days. The differentiation of M2 macrophage by IF treatment was blocked by the glucocorticoid receptor inhibitor (RU486), but not by an androgen receptor (flutamide) antagonist. Flow cytometric analysis was used to analyze the M1 ($CD68^{+}$-$CD163^{-}$) and M2 macrophage ($CD68^{+}$-$CD163^{+}$) population. Contour plots are representative of three independent experiments, and (B) the histogram represents the summary of all the experiments. Data were obtained from six rats and presented as mean ± SD. The one-way ANOVA test was employed for statistical analysis. p-values, ***p<0.001, ****p<0.0001.

3.21 Adrenalectomy decreases the corticosterone level in both IF and serum, but leaves IF levels higher

The above results suggested that corticosterone is the principal molecule in IF that influences the TM phenotype. Hence, a possible intratesticular origin of corticosterone supplementing production from the adrenal gland was investigated. To evaluate this possibility, bilateral

adrenalectomy (ADX) was performed in rats with subsequent analysis of the corticosterone levels by ELISA. In ADX animals the concentration of corticosterone was dramatically reduced in both serum and IF, suggesting that adrenal corticosterone production is the main provider of intratesticular corticosterone (Figure 24).

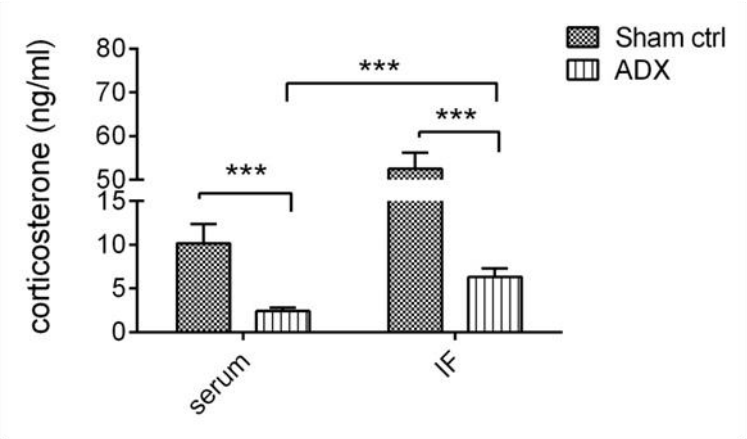


Figure 24. The corticosterone level in both IF and serum were reduced in ADX rats. Levels of corticosterone were measured by ELISA in serum and IF collected from sham control (n = 6) and ADX rats (n =5). Data are presented as the mean ± SD. Student's t-test was employed for statistical analysis. ***p <0.001.

3.22 ADX does not change the phenotype of TM

Of note, although a lower level the corticosterone concentrations in the IF of ADX rats was seen, it remained significantly higher than those in serum, which indicates local production by the testis. Analyzing the TM phenotype in ADX rats, flow cytometry data proved no difference in the TM phenotype in sham control and ADX rats (Figure 25 B, C). In accordance, the M2 macrophage population in ADX rats did not show any change comparing to control. The levels of IL-10 and TNF-α production were also not different following LPS challenge (Figure 25 D).

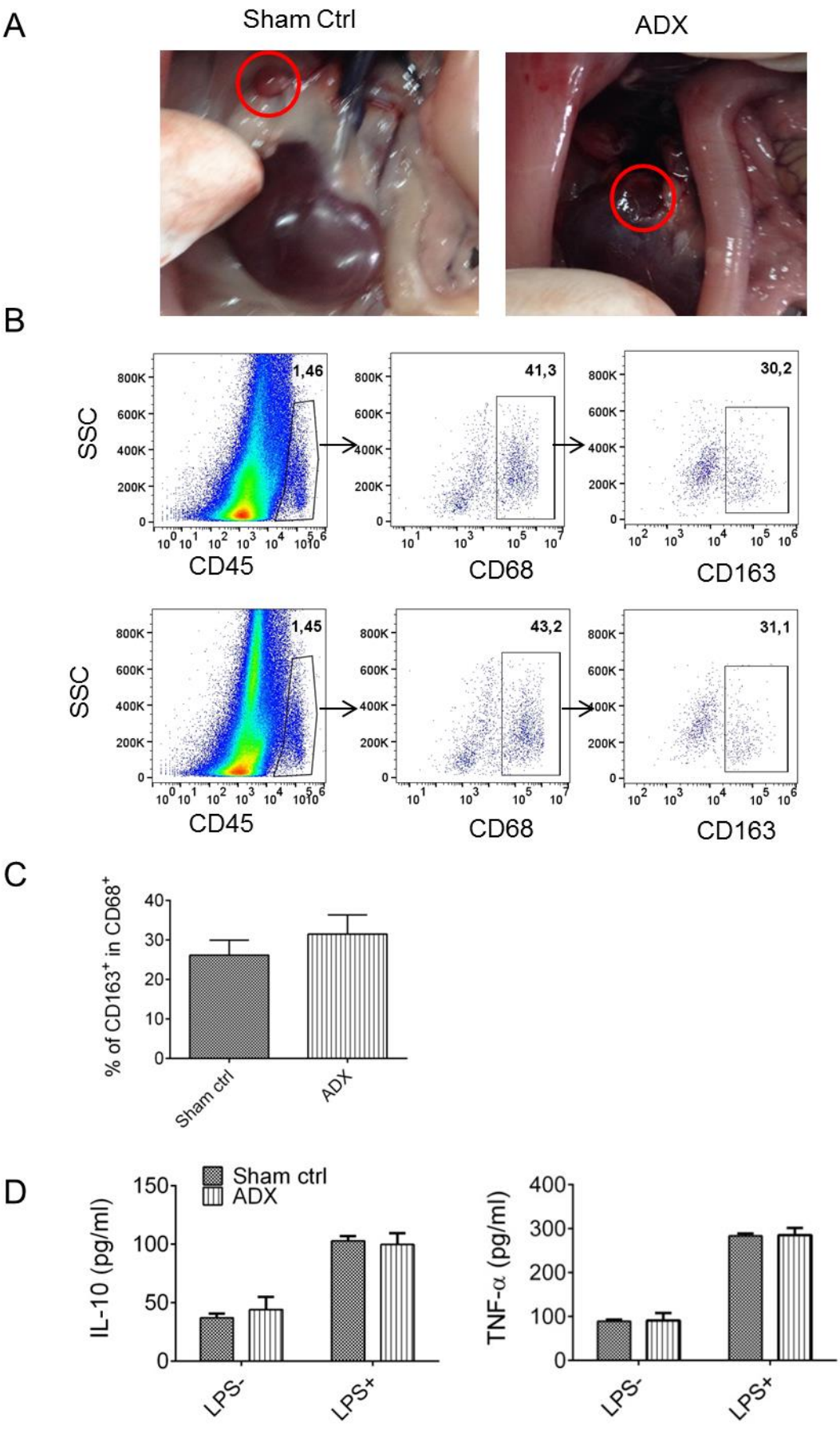

A
Sham Ctrl
ADX
B
SSC
CD45
CD68
CD163
1,46
41,3
30,2
1,45
43,2
31,1
C
% of CD163+ in CD68+
Sham ctrl
ADX
D
Sham ctrl
ADX
IL-10 (pg/ml)
TNF-α (pg/ml)
LPS-
LPS+

Figure 25. ADX did not change the phenotype of TM. (A) Sham ctrl and ADX rats showed the presence and absence of adrenal glands (B) Gating strategy was used to identify total CD68 and CD163 cells in $CD45^+$ immune cells. Cell aggregates and dead cells were excluded on the basis of SSC and PI staining. Contour plots are representative of five independent experiments, and (C) histogram represents the summary of all the experiments. (D) TM were isolated from sham control and ADX rat testes, and treated with LPS for 24 h. The production of IL-10 and TNF-α in cell supernatants was analyzed by ELISA. Data were collected from sham control (n = 6) and ADX rats (n =5) and presented as the mean ± SD. The Student's *t* test was performed for statistical analysis. p>0.05

3.23. TM are the main producers of corticosterone in the testis

To examining a possible intratesticular production of corticosterone as an explanation for the higher levels in IF both in sham and ADX animals compared to serum, basal secretion of corticosterone was assessed in isolated testicular cell types, namely Sertoli cells (SC), peritubular cells (PTC), Leydig cells (LC) and TM. Corticosterone production in supernatants of isolated SC, PTC and LC was barely detectable, whilst surprisingly TM were found to secrete substantial amounts of corticosterone (Figure 26). Thus, locally produced corticosterone could be a factor to maintain the immunosuppressive phenotype and function of TM, also in ADX animals.

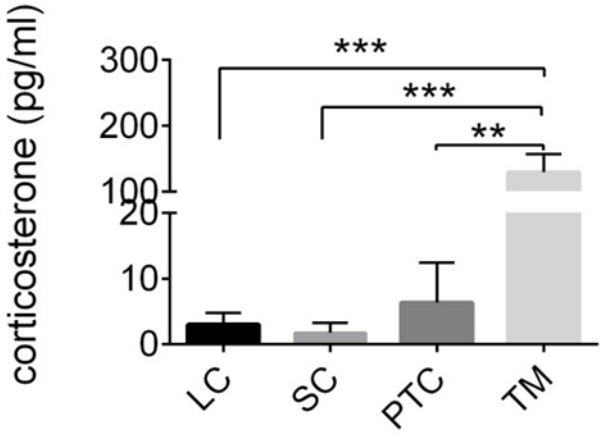


Figure 26. TM constitutively secrete corticosterone. Corticosterone levels were measured in conditioned medium of isolated testicular somatic cells, namely Leydig cells (LC), Sertoli cells (SC), peritubular cells (PTC) and testicular macrophages (TM). All data are shown as mean ± SD of at least three independent experiments. The one-way ANOVA test was employed for statistical analysis. **p < 0.01, ***p < 0.001

3.24 Corticosterone inhibits inflammatory responses through glucocorticoid receptor action.

TM are refractory to inflammatory stimuli relative to other tissue macrophages such as PM. To demonstrate that secreted corticosterone could suppress the secretion of proinflammatory cytokines in TM, the basal secretion of corticosterone by TM was compared to that of PM. In contrast to TM, PM secrete significantly lower levels of corticosterone (Figure 27 A). PM cultured in TM conditioned medium for 24 h, prior to LPS challenge showed significantly reduced levels of TNF-α, which was abrogated by addition of the GR inhibitor RU486 to TM conditioned medium (Figure 27 B). Thus, results obtained so far strongly indicate that the attenuated inflammatory response of TM was attributable to the constitutive endogenous secretion of corticosterone by TM.

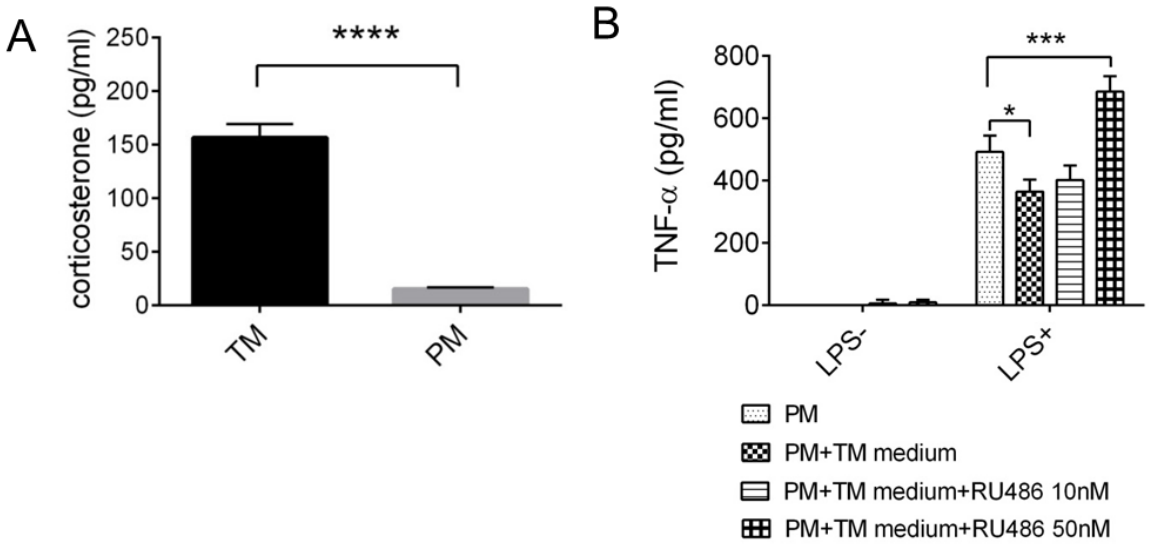


Figure 27. TM secreted corticosterone attenuates secretion of pro-inflammatory cytokine in PM. (A) Corticosterone levels were measured by ELISA in conditioned medium of TM and PM. All data are shown as mean ± SD of at least three independent experiments. Student's *t* test was employed for statistical analysis. ****$p < 0.0001$. (B) PM were cultured in TM conditioned medium with or without the presence of the glucocorticoid receptor antagonist RU486 (50 nM/ml) for 24 h followed by treatment with LPS (1 µg/ml) for 6h. Cell culture supernatants were analyzed for secretion of TNF-α by ELISA. All data are shown as mean ± SD of at least three independent experiments. The one-way ANOVA test was employed for statistical analysis. *$p < 0.05$, ***$p < 0.001$

3.25. TM display diminished expression of inflammatory genes

To determine possible principal differences in the constitutive gene expression profile of TM and PM, whole genome transcriptome analysis was performed to investigate the differential expression of pro-inflammatory genes in TM and PM. The basal expression levels of several important pro-inflammatory genes, such as *Il-1α, Il-1β, Il-2, Il-6, Il-2α, Il-15, Il-18, Ccl4, Ccl7, Ccl21* and *Tnf-α* were higher in PM than in TM (Figure 28). However，the anti-inflammatory genes including *Il-12, Il13, Bmp-6, Bmp-7* were found highly expressed in TM. This microarray data further confirmed the immunosuppressive properties of TM.

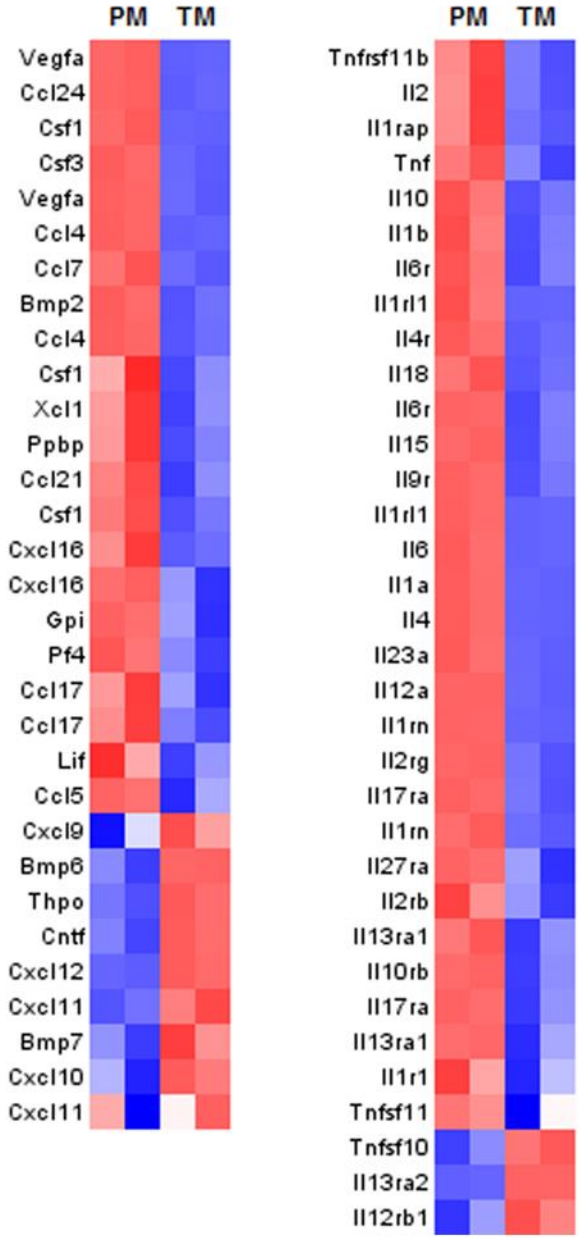


Figure 28. Differential expression of inflammatory genes in TM and PM. Gene expression profile for chemokines (left) and interleukins and corresponding receptors (right) were determined by CodeLink Rat Whole Genome arrays. The gene expression values are centered and scaled (mean of all data points subtracted from each individual data point, then divided by the standard deviation of all points) with red showing expression above mean and blue expression below mean.

3.26. Corticosterone treatment suppresses the expression of inflammatory genes in PM

To determine whether the high basal expression of inflammatory genes in PM can be attributed to the low endogenous secretion of corticosterone in these cells, subsequent qRT-PCR analyses of corticosterone treated PM was performed. Results revealed a significant suppression of the expression of pro-inflammatory genes, such as *Il-6, Inos, Ccl2* and *Ifn-β*. Notably, corticosterone treatment of PM induced the expression of the anti-inflammatory cytokine *Il-10* and increased the expression of the M2 macrophage gene *Cd163* (Figure 29). Hence, these results indicate that endogenous corticosterone secreted by TM suppresses the basal expression of pro-inflammatory cytokines and chemokines and thus this steroid represents a major factor responsible for the subdued immune response characteristic for these cells.

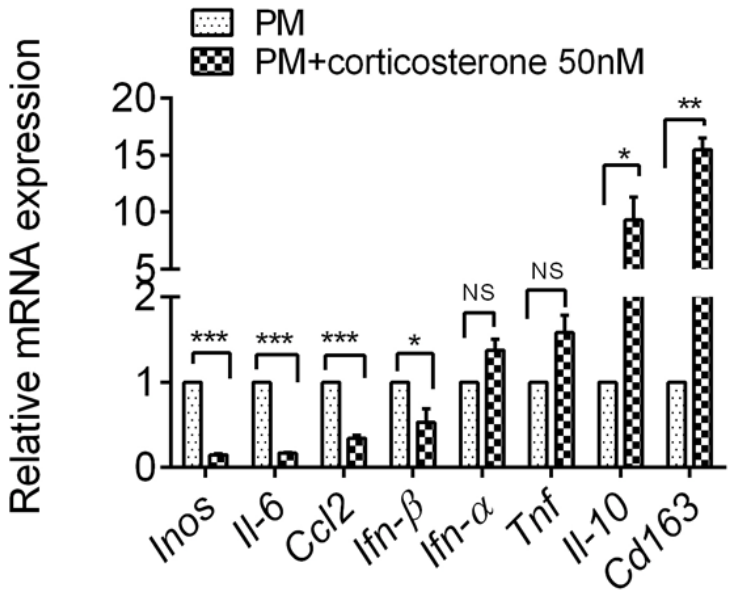


Figure 29. PM express higher level of anti-inflammatory genes after corticosterone treatment. PM were treated with corticosterone and RU486 as indicated in the figure for 24 h. Expression levels of inflammatory genes were analyzed by qRT-PCR. The results were normalized to expression levels of β-microglobulin. Student's *t* test was employed for statistical analysis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4. DISCUSSION

The principal aim of testicular immune privilege is to protect the neo-autoantigens of meiotic and post-meiotic germ cell from autoimmune attack (Li et al., 2012). A number of recent studies have suggested that not solely the sequestration of autoantigens by the BTB, but also the local micro-environment are critical for maintaining testicular immune privilege (Arck et al., 2014; Hedger and Meinhardt, 2000). TM, the largest immune cell population of the testis, play an important role in preserving the immuno-compromised environment of the testis by displaying an immunosuppressive M2 macrophage phenotype (Bhushan et al., 2015). TM reside in the interstitial space of the testis and are constantly exposed to immunosuppressive IF. It is therefore likely that the local immunosuppressive environment of the IF may thus influence the phenotype and function of TM. The immunosuppressive properties of testicular IF have been documented in many studies, but very little is known about their influence on the phenotype and function of TM (Wang et al., 2017). In this study, we demonstrate that testicular IF skews classical M1 macrophages towards the alternative M2 macrophage phenotype. Using GC–MS/MS analysis, we identified corticosterone as one of the most potent molecules present in IF that could shape the phenotype and function of TM. Notably, TM constitutively secrete corticosterone at basal levels that in turn can substitute systemic levels originating from the adrenal gland by acting in a paracrine/autocrine manner to maintain the TM in the immunosuppressive M2 macrophage phenotype.

4.1 TM show an immunosuppressive phenotype

TM are the most abundant immune cells present in the testis (Winnall and Hedger, 2013; Winnall et al., 2011). However, their phenotypic and functional heterogeneity are still poorly characterized (Hutson, 1990). In previous studies, the phenotypic characterization of rat TM was mainly achieved through immunohistochemical analysis, which revealed that TM express either CD68 or CD163 as surface marker. In addition, it was believed that $CD68^+$ macrophages (approximately 20%) are newly arrived macrophages originating from blood monocytes, whereas $CD163^+$ macrophages are resident macrophages (Li et al., 2012; Winnall and Hedger, 2013). In this study, we readily identified a unique heterogeneous macrophage population in the testis, which is composed of a mixture of $CD68^+$-$CD163^-$ and $CD68^+$-$CD163^+$ macrophages. In contrast to other studies, we showed that all macrophages are positive for $CD68^+$ and that

approximately 80% of macrophages are positive for both CD68 and CD163. Macrophages as antigen-presenting cells express high levels of co-stimulatory molecules to activate T cells, thereby inducing adaptive immunity (Iwasaki and Medzhitov, 2015). Surprisingly, approximately half of the TM population did not express MHC class II, and the expression of CD80 and CD86 was completely absent (Figure 4B). The lack of co-stimulatory molecules in TM could result in impaired presentation of antigens to T cells and thus hinder the activation of adaptive immunity, a means to maintain the immune privileged status of the testis. Similar to TM, intestinal and lung macrophages also do not express CD80 and CD86 to maintain the homeostasis of their respective organ (Chelen et al., 1995; Rogler et al., 1998). Although TM show an attenuated inflammatory response and lack CD80 and CD86 costimulatory molecules, they isplay normal phagocytic activity by engulfing fluorescence-labeled Zymosan A *S. cerevisiae* bioparticles. These results obtained here clearly suggest that adaptive immune responses are effectively suppressed in TM, but 'basic non-adaptive' functions are maintained .

4.2 TM show immunosuppressive functions

It is widely known that Tregs play a pivotal role in maintaining the homeostasis of the immune system by preserving immunologic self-tolerance and by negatively regulating immune responses (Rudensky, 2011; Smigiel et al., 2014). In addition, Tregs have the capacity to attenuate autoimmune responses and limit excessive inflammatory responses during viral and bacterial infections (Navegantes et al., 2017; Schmidt et al., 2016). Naturally arising Tregs inhibit the activation of T cells, whilst in turn block the activation of adaptive immune responses (Nguyen et al., 2006; Sojka et al., 2008). Recently, several studies reported that M2 macrophages inhibit $CD4^+$ T cell proliferation, but expand the Treg population. The supernatants of IL-10/TGF-β polarized M2 macrophages significantly induced the generation of Tregs with high expression of Foxp3 and Treg-specific molecules such as cytotoxic T-lymphocyte-associated protein 4 （CTLA-4） and IKAROS Family Zinc Finger 4（IKZF-4） (Schmidt et al., 2016). Moreover, compared to control T cells, the M2 induced Tregs displayed functional immunosuppressive activity by producing low levels of the pro-inflammatory cytokines IL-17 and IFN-γ (Schmidt et al., 2016). In our study, immunosuppressive properties of TM were indicated by increased expression of CD163, high production of the anti-inflammatory cytokine IL-10 and concomitant low production of the pro-inflammatory cytokine TNF-α. The immunosuppressive properties of TM were further demonstrated in our study by co-culturing TM with splenic T cells, in which TM induced the differentiation of T

cells to immunosuppressive Tregs (Figure 6A-B).

4.3 Characterization of immunosuppressive molecules in IF

Tissue-resident macrophages are highly heterogeneous and persistently exposed to tissue-specific signals that determine their development, phenotype and function. For example, in the human brain, TGF-β is essential for the development of microglia determined by the expression of microglial molecular signature characteristics (Butovsky et al., 2014; Nayak et al., 2014). In support, the number of microglia is significantly decreased in the CNS of TGF-β1 deficient mice (Butovsky et al., 2014). Another study has also reported that the phenotype of CNS resident macrophages and newly recruited infiltrating blood monocyte-derived macrophages are influenced by the local microenvironment (Lavin et al., 2014; Perry and Teeling, 2013). Similarly, heme induces blood monocyte differentiate into splenic red pulp macrophages, whereas CSF-2 controls the phenotypic and functional polarization of AM in the lung (Butovsky et al., 2014; Cohen et al., 2014; Guilliams et al., 2013; Haldar et al., 2014; Okabe and Medzhitov, 2014).

Over the years, a plethora of studies have been devoted to investigate how these environmental factors control the polarization of macrophages. Accumulating evidence highlights that environmental factors mediate the phenotypic and functional macrophage differentiation by specific transcriptional factors (Lavin et al., 2014; Wynn et al., 2013). For example, chronic exposure to TGF-β1 can decrease the capacity of myeloid cell differentiation into the M2 anti-inflammatory phenotype, and the TGF-β1 signaling pathway controls the switch of differentiation from the M1-to-M2 macrophage phenotype by down regulating the expression of the transcription factor interferon regulatory factor 7 (IRF7) (Jiang et al., 2016; Zhao et al., 2017). Similarly, retinoic acid (RA) mediates the tissue specific localization and functional polarization of PM through the GATA6 transcription factor (Arnold et al., 2015; O'Hara et al., 2015). In addition, the Spi-C transcription factor plays an important role in regulating the development of red pulp macrophage. Spi-C $^{-/-}$ mice display a cell-autonomous defect which fails to engulf senile red blood cells efficiently, thereby incapacitating the degradation of hemoglobin and thus impairing iron homeostasis (Davies et al., 2013; Kohyama et al., 2009). Moreover, interferon regulatory factor 5 (IRF5) as well as signal transducer and activator of transcription 1 (STAT1) can regulate monocyte-derived M1 macrophage polarization, whereas the monocyte-derived M2 macrophage phenotype is controlled by STAT6, IRF4 and peroxisome proliferator-activated receptor γ (PPARγ) transcription factors (Lawrence and

Natoli, 2011; Tugal et al., 2013; Wang et al., 2014). The nuclear receptor LXRα is critical for the differentiation of macrophages in the splenic marginal zone (MZ). This is corroborated by using LXR-deficient mice, which show substantial reduction in the generation of metallophilic macrophages and MZ (Bronte and Pittet, 2013; N et al., 2013). Taken together, these findings indicate that a number of key transcription factors are responsible for macrophage polarization and each organ macrophage is regulated and maintained by a specific transcription factor.

TM play a significant role in the activation of innate immune responses and aid normal testicular functions such as steroidogenesis and spermatogenesis (Bhushan and Meinhardt, 2016; Fijak and Meinhardt, 2006). Although several studies investigated the importance of TM in normal function of the testis and regulated on immunosuppressive the phenotype of TM, very few studies investigated the role of the local microenvironment in shaping the phenotype and function of TM. In this study, we have demonstrated that the testicular IF has immunosuppressive characteristics which can induce the polarization of both macrophages and T cells towards an immunosuppressive phenotype.

In this study, a proteomic approach combining 2D gel electrophoresis and MALDI-TOF-MS was used to identify relevant molecules in IF (Figure 13). A total of 68 proteins (Table 5) were identified at different levels in testicular IF compared to serum, albeit none was established to have immunosuppressive functions. This is in spite of previous studies who have indicated that the testicular microenvironment contains abundant immunosuppressive proteins such as TGF-β and IL-10 (Yang et al., 2010; Yotsukura et al., 1997). However, the results of the current study revealed that the testicular IF is devoid of such high levels of immunosuppressive proteins confirming earlier results which demonstrated that TGF-β was not required for the inhibitory activity of IF (Hedger et al., 1998). However, the IF is known to contain non-proteinaceous factors such as the T cell inhibitor lyso-glycerophosphocholines and PG (Foulds et al., 2008).

4.3.1 Prostaglandins in IF

PG are potent modulators of pro-inflammatory or anti-inflammatory responses and modulate immune cell phenotypes and functions (Boniface et al., 2009; Kalinski, 2012; Mitson-Salazar et al., 2016). As the most widely characterized lipid mediator, the role of PGE_2 has been extensively studied regarding its critical role in regulating immune responses during both acute and chronic inflammation (Chen et al., 2015; Ricciotti and FitzGerald, 2011). In this study, LC-MS analyses had revealed that IF contains substantial amounts of PGE_2, PGI_2, PGD_2 and $PGF_{2\alpha}$

(Figure 12). In the testis, PG are mainly produced by SC, LC, PTC, and TM (Carpenter, 1974; Frungieri et al., 2015; Winnall et al., 2007). The synthesis of PG is controlled by the rate-limiting enzyme cyclooxygenase (COX), which in SC and LC is regulated by PG levels in the testis (Ricciotti and FitzGerald, 2011; Tassorelli et al., 2007). Moreover, the expression of COX_2 is significantly increased in the testis under inflammatory conditions (Frungieri et al., 2015). The increased expression was mainly contributed by the TM and mast cells (Frungieri et al., 2002; Winnall et al., 2007). As the most commonly investigated type of PG, PGE_2 is more and more considered a vital factor in the immune repression of chronic diseases. It is generally accepted that PGE_2 is critical for increased T helper 2 (Th2) responses with concomitant decrease in Th1 responses (Kalinski, 2012; Ricciotti and FitzGerald, 2011). In addition, PGE_2 treatment increased the expression of IL-4 and IL-10 in the human Jurkat T cell line, whereas expression of IL-2 and IFN-γ was significantly reduced(Agard et al., 2013; Aronoff et al., 2004; Barnes, 2011; Fijak et al., 2015; Fijak et al., 2011; Harris et al., 2002; Jiang et al., 2016). Similarly, PGE_2 upregulates the production of IL-10 in macrophages and DC, with concomitant decrease in the secretion of TNF-α, IL-1β, IL-12 (Harris et al., 2002). More recently, a study has provided evidence that PG are implicated in polarizing the macrophage phenotypes by inhibiting the production of TNF-α and upregulating IL-10 via the cAMP-CREB signaling pathway (Harris et al., 2002; MacKenzie et al., 2013). Hence, PG are recognized as important immunomodulatory molecules also present in the testis, which putatively can contribute to the testicular immunosuppressive function by altering the phenotype of TM.

Based on these earlier observations, the high levels of PG in the IF suggested that the TM phenotype and function may be influenced by locally produced PG. Indeed, our data demonstrate that PGE_2 and PGI_2 polarize blood derived M1 macrophages towards the M2 macrophage phenotype as evidenced by increased CD163 expression and the secretion of large amounts of IL-10 parallel to low TNF-α production (Figure 16, 17). In support, PGE_2 suppresses NF-κB activation and triggers STAT3 signaling (Figure 18). Although the level of PGD_2 was likewise high in IF, PGD_2 mediated polarization of M1 macrophages to the M2 phenotype was not observed. Thus, PGE_2 and PGI_2 present in the IF may be possible candidates in sustaining the immunosuppressive M2 phenotype of TM.

4.3.2 Steroid hormones in IF

The testis is the principal production site for androgens, whereas corticosterone, another steroid, is synthesized and secreted by the adrenal gland (Dewan et al., 2000; Hines et al., 2015;

Jarow et al., 2005). In this study, we have analyzed the concentration of steroid hormones in IF and serum using GC-MS/MS. Whilst androgen levels were as high as expected, surprisingly, the level of corticosterone in IF was found much higher than in serum and even approximately 10-fold higher than in the aqueous humor (AqH) of the eye, another immune privileged organ. Similar to IF, AqH maintains the immune privilege status of the eye (Zhou and Caspi, 2010).

Accumulating evidence points to a role of testosterone in playing an important immunosuppressive function (Trigunaite et al., 2015). For example, testosterone can inhibit the production of TNF-α and IL-1β in human macrophages by the stimulation of production of oxidized low density lipoproteins (Frantz et al., 2005). Moreover, testosterone treatment increases the production of the anti-inflammatory cytokine IL-10 and down-regulates synthesis of the pro-inflammatory cytokine IL-6 in LPS stimulated human PBMC (Stenvinkel et al., 2005) . In the RAW 246.7 mouse macrophage-like cell line and also in primary macrophages, testosterone stimulation decreased the expression of *Tlr4*, thus limiting the expression of inflammatory cytokines upon challenge of cells (Rettew et al., 2008). Similarly, long exposure of this cell line with testosterone attenuated the activation of p38 MAP kinase signaling pathway and suppressed the production of nitric oxide (NO) (Fijak et al., 2015). In the testis, testosterone inhibits development of experimentally induced autoimmune orchitis likely by differentiation of naive T cells into immunosuppressive Treg (Fijak et al., 2015). Although the immunosuppressive character of testosterone is mostly accepted, it remained elusive if this steroid can influence the macrophage phenotype. This study provides first data that testosterone can polarize GM-CSF induced monocytes derived M1 macrophages to the M2 macrophage phenotype (Figure 20). In addition, testosterone treatment suppresses NF-κB signaling and downstream production of pro-inflammatory cytokines (Figure 21, 22). Taken together, similar to PGE_2 and PGI_2, testosterone is another important molecule in IF which can mediate immune responses and seems to play a function in maintaining the M2 phenotype of TM.

Since the discovery of GC in the 1940s (Cain and Cidlowski, 2017; Talaber et al., 2013), they have long been recognized as the most important agents for modulating immune responses by altering the phenotype and function of many leucocytes including macrophages, DC, T cells, mast cells and also non-immune cells such as neutrophils, eosinophils, endothelial and epithelial cells (Oppong and Cato, 2015). Accumulating evidence has pointed out that GC could be involved in macrophage differentiation. However, possible underlying molecular mechanisms remained elusive. Combined microarray and PCR analysis data revealed that GC

promote the differentiation of human monocytes into the anti-inflammatory M2 macrophage phenotype by up-regulating the expression of anti-inflammatory molecules including *Cd1d, Il1R2, Cd163,* and *Il-10*. The anti-inflammatory function of GC was further confirmed by the up-regulation of the anti-inflammatory cytokine IL-10 as well as CD163, a scavenger receptor and M2 alternative activated macrophage marker (Ehrchen et al., 2007). Moreover, GC support the survival of anti-inflammatory type monocytes during inflammatory responses by blocking apoptotic cell death pathways induced by oxidative stress (Varga et al., 2008). GC (dexamethasone) treatment mediates the up-regulation of CD163 and Gr-1, but decreases the expression of CX3CR1, a key mediator of inflammatory type monocytes (Tedesco et al., 2015). In addition, dexamethasone stimulation also elevates the production of IL-10 in monocytes. Our study has demonstrated that corticosterone in IF is approximately seven times higher in comparison to serum (Figure 19). The high concentration of corticosterone in IF could be necessary for maintaining the testicular immunosuppressive microenvironment.

GC mediate their function through the GR (Liberman et al., 2007; Petta et al., 2016). In this, GC bind to the GR and bind as GC-GR complex to GC-responsive DNA elements, which for example causes inhibition of inflammatory gene expression (Xavier et al., 2016). Moreover, the GR interacts with pro-inflammatory transcription factors such as AP-1 and NF-κB as co-factors which in turn provide a further level in the regulation of the expression of pro-inflammatory cytokines (Baschant and Tuckermann, 2010). Studies from our group have shown that the GR is expressed in all testicular cells and is also found in other immune cells tested such as PM. This is in agreement with previous findings showing that the GR is ubiquitously expressed in almost all organs of mammals in two isoforms, GRα and GRβ. GRα is important for binding to the GC-responsive elements (GREs) to regulate immune responses. The function of GRβ is not yet clear, but it may negatively regulate GRα mediated gene expression during inflammatory responses (Smoak and Cidlowski, 2004). In contrast, GRα induces repression of gene transcription by binding to negative GREs (nGREs) (Hapgood et al., 2016; Smoak and Cidlowski, 2004). For instance, the transcription of GC related pro-inflammatory genes is affected by nGREs, which regulate the GRs and some other transcription factors including AP-1 and NF-κB via protein-protein interaction. This leads to an inhibition of the synthesis of inflammation cytokines such as IL-6, TNF-α, IL-1β, IL-2, and IL8 (Barnes, 2006; Baschant and Tuckermann, 2010). These regulatory mechanisms are consistent with our findings that corticosterone significantly suppressed LPS-induced TNF-α production. In PBMC an activation of NF-κB signaling did not seem to be impaired by corticosterone (Figure 19).

Besides corticosterone, testosterone and PGE_2 were also able to skew macrophages towards the M2 phenotype. Besides the magnitude of the effect also their mode of action is distinct from another. Testosterone and PGE_2 inhibited the activation of NF-κB by stabilizing IκBα, whereas corticosterone can suppress the activation of the NF-κB signaling pathway by directly interacting with the NF-κB transcription factor without prior influence on IκBα degradation (Barnes, 2006). IL-10 exerts its anti-inflammatory property through activation of STAT3 signaling (Wang et al., 2017). In our study, corticosterone, testosterone and PGE_2 all significantly induced the secretion of IL-10. In contrast to testosterone and PG, the activation of STAT3 was not observed in corticosterone polarized M2 macrophages. To elucidate the molecular basis of these differences remains a challenge for the future.

In addition to the molecules described above, there are other steroid hormones present in IF. In our study, it has been shown that the levels of 4-androstenedione and 5α-androstanediol are also much higher in IF compared to serum (Figure 19), although an influence on macrophage phenotype polarization has not been observed (Figure 20). In our study, treatment of GM-SCF induced M1 macrophages with 4-androstenedione did not increase the production of the anti-inflammatory cytokine IL-10 after stimulation with LPS (Figure 21). This may be due to the fact that 5α-androstanediol mediates its function largely through the estradiol receptor, which is not shown to be expressed in TM. Collectively, corticosterone was identified in IF as the most potent molecule with the potential to establish and maintain the immunosuppressive M2 phenotype of TM. Additionally, PG (PGE_2 and PGI_2) and testosterone may also exert some minor influence on the phenotype and function of TM.

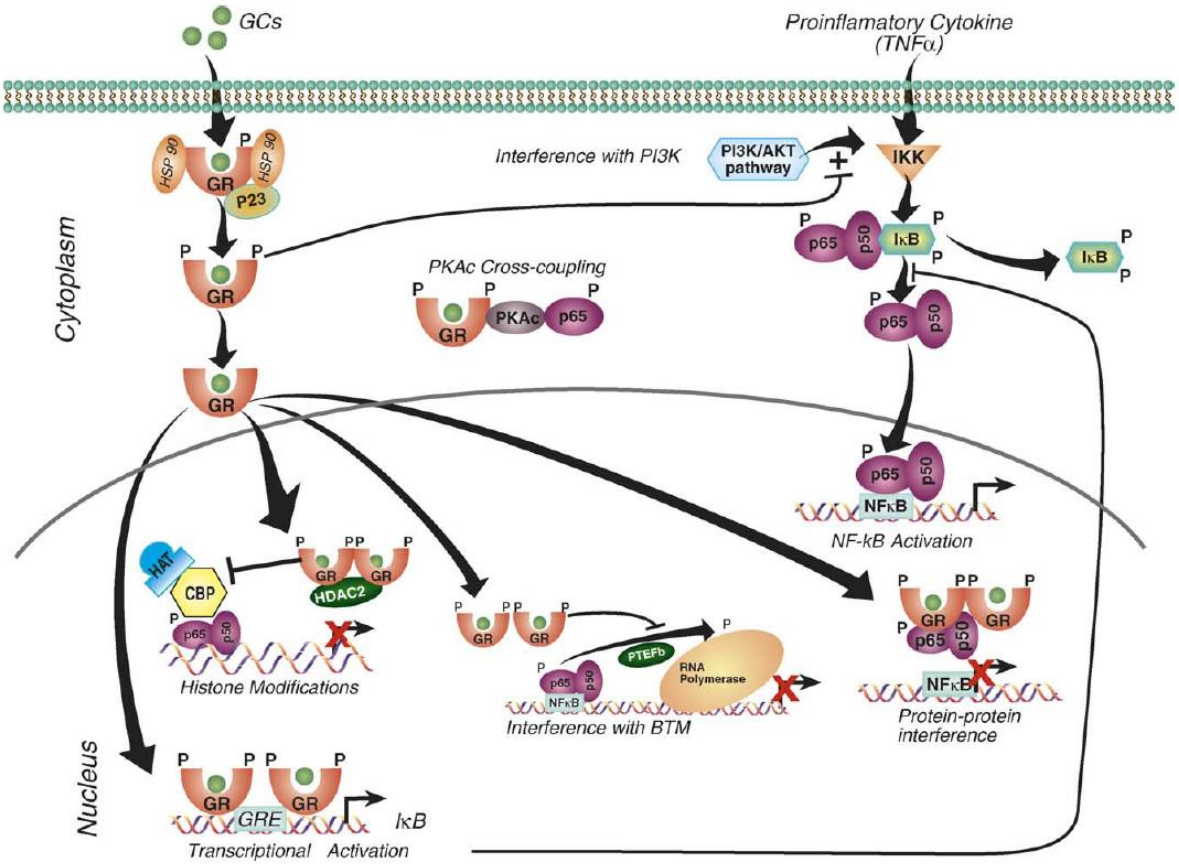


Figure 28. GRα-mediated repression of NF-kB signaling (Mechanisms of Ageing and Development 125, 2004, 697–706. With the permission of the publisher, license number: 4143080772257).

4.4 Corticosterone is the principal factor which maintains the TM phenotype

Outside of the adrenal local biosynthesis of GC has been documented in a number of organs namely intestine, thymus, skin and brain. However, the majority of GC in all organs originates from *de novo* synthesis in the adrenal cortex and is controlled by the hypothalamus-pituitary-adrenal-axis (Cain and Cidlowski, 2017; Talaber et al., 2013). In order to examine the hypothesis whether the high level of corticosterone in the IF is derived from adrenal gland production only, we have performed an ADX experiment to evaluate possible other sources of testicular corticosterone in view of their influence on TM. The results obtained from this study clearly demonstrate that corticosterone in the testis was primarily supplied by the adrenal glands. In the ADX rats, the corticosterone level was significantly decreased in both serum and testicular IF (Figure 24). Although the level of corticosterone was substantially lower in ADX rat, the TM phenotype did not change *in vivo* compared to sham control as evidenced by

$CD68^+CD163^+$ marker expression and production of characteristic cytokines IL-10 and TNF-α (Figure 25). Interestingly, the corticosterone concentration was 10 times lower in ADX testicular IF compared to the sham control at 2 weeks after surgery, though it remained approximately 3 folds higher than in ADX rat serum (Figure 24). These finding indicate the possibility that corticosterone may be produced locally. In support, an *in vitro* study from Naoyuki Maeda demonstrated that corticosterone may be synthesized and secreted by the testis (Maeda et al., 2015). Similarly, it was demonstrated that corticosterone levels in the brain were much higher than those in serum of adrenalectomized (ADX) mice, suggesting that corticosterone can also be produced locally in the mouse brain (Croft et al., 2008). In combination, this prompted the question which cell types in the testis could be the production site. We speculated that TM and LC are the most likely candidates to secrete corticosterone, since both of them express 11-dehydrocorticosterone 1 enzyme, which converts 11-dehydrocorticosterone into corticosterone (Usa et al., 2007). In this study, using isolated TM, PTC, SC and LC, it was illustrated for the first time that TM are the principal cells in the testis synthesizing corticosterone, which in turn could act in an autocrine manner back on TM as a means to maintain the immunosuppressive phenotype and function of these macrophages in an autocrine fashion.

Substantial evidence shows that locally synthesized GC can have relevant functions in normal tissue homeostasis, cell development and immune cell activation (Oppong and Cato, 2015). As an example, it was illustrated that corticosterone released form the murine intestinal mucosa is critical for T cell activation and the regulation of local immune cell responses (Cima et al., 2004). TM express low levels of pro-inflammatory cytokines in comparison to PM, suggesting that the secreted corticosterone may play a role in suppressing the expression of pro-inflammatory cytokines. When PM were stimulated with TM supernatant, the secretion of TNF-α was significantly reduced upon stimulation with LPS, an effect abolished using a GC inhibitor. Similarly, exposure of PM to corticosterone can induce down-regulation of pro-inflammatory genes.

Taken together, evidence obtained from this study supports the hypothesis that the testicular microenvironment establishes and sustains the immunosuppressive M2 macrophage phenotype of TM. Our data provide strong support that PGE_2, PGI_2, testosterone and corticosterone are important immunoregulatory molecules present in the IF, with corticosterone playing a particularly dominant role in determining the phenotype of TM. All of these factors, except

corticosterone, are able to inhibit the NF-κB signaling pathway to suppress the production of pro-inflammatory cytokines. Moreover, this study demonstrated for the first time that corticosterone in the testicular IF does not only originate from the adrenal glands, but levels can be supplemented TM, though in moderate amounts. Of note, our results indicate that even these moderate amounts of corticosterone secreted by TM may be sufficient to maintain the characteristic immunosuppressive M2 macrophage phenotype of TM.

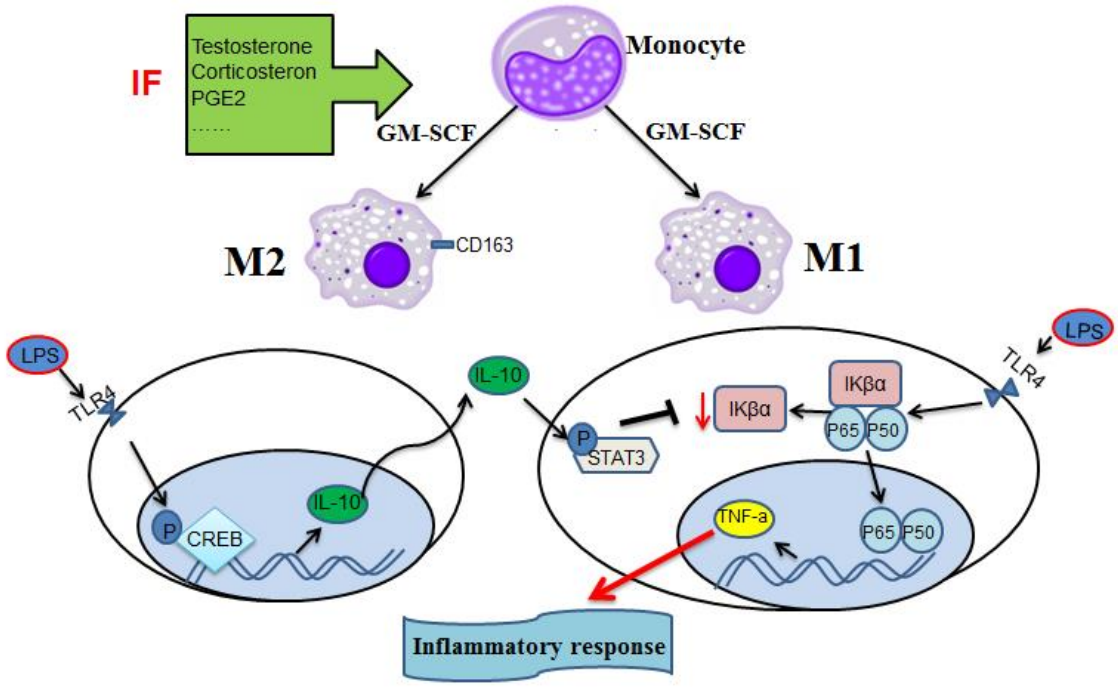


Figure 29. Schematic diagram summarizing the main findings of this thesis. The chart of testicular IF maintains the TM phenotype and immunosuppressive function. PGE_2, PGI_2, testosterone and corticosterone were identified as important immunoregulatory molecules present in the IF. Amongst the corticosterone being the most powerful mediator in shaping the phenotype and function of TM.

5. SUMMARY

Tissue-specific macrophages are important for the activation of innate immune responses and general organ homeostasis. Testicular macrophages (TM) reside in the testicular interstitial space and comprise the largest leukocyte population in the testis and are assumed to play a role in maintaining testicular immune privilege. Numerous studies have indicated that the interstitial fluid (IF) surrounding the TM has immunosuppressive properties, which may influence the TM phenotype. However, the identity of the immunosuppressive molecules present in the IF is poorly characterized. In this thesis it is shown that in the rat, IF shifts the M1 phenotype of granulocyte macrophage-colony stimulating factor induced bone marrow derived macrophages towards the M2 phenotype. M2 macrophages polarized by IF mimic the properties of TM such as increased expression of CD163, high secretion of IL-10 and low secretion of TNF-α. In addition, IF-polarized macrophages display immunoregulatory functions by inducing the expansion of immunosuppressive regulatory T cells. This thesis provides evidence that PGE_2, PGI_2, testosterone and corticosterone are important immunoregulatory molecules in the IF, playing a relevant role in determining the phenotype of TM. Except corticosterone, all of these factors are able to inhibit the NF-κB signaling pathway to suppress the production of pro-inflammatory cytokines and thus maintain an immunosuppressive microenvironment of the testis. Corticosterone was found to be the principal immunosuppressive molecule in the IF. Its receptor, the glucocorticoid receptor, was found to be present in TM immunohistochemically. In addition, TM locally produce small amounts of corticosterone, which suppress the expression of inflammatory genes and render TM refractory to inflammatory stimuli. Taken together, these results suggest that testicular corticosterone shapes the immunosuppressive function and phenotype of TM. This steroid hormone may therefore play also an important role in the establishment and maintenance of the immune privilege of the testis.

6. ZUSAMMENFASSUNG

Gewebsspezifische Makrophagen haben eine wichtige Funktion bei der Aktivierung angeborener Immunantworten und der Organhomeostase. Testikuläre Makrophagen (TM) befinden sich im Interstitium des Hodens und stellen die größte Leukozytenpopulation in der männlichen Gonade dar. Es wird angenommen, dass sie eine wichtige Funktion in der Aufrechterhaltung des Immunprivilegs des Hodens ausüben. Studien haben gezeigt, dass die interstitielle Flüssigkeit (IF), wleche die TM umgibt, immunsuppressive Eigenschaften aufweist, die den Phänotyp der TM beeinflussen könnten. Allerdings konnten immunsuppressive Moleküle in der IF bislang kaum charakterisiert werden.

In der vorliegenden Arbeit wird für die Ratte als Modell gezeigt, dass die IF den durch Granulozyten- Makrophagen-Kolonie-stimulierenden Faktor (GM-CSF) induzierten M1 Phänotyp von Makrophagen, die aus dem Knochenmark isoliert wurden, in Richtung des M2 Phänotyps verschieben kann. IF-polarisierte M2-Makrophagen zeigen damit charakteristische Eigenschaften von TM, wie z. Bsp. erhöhte Expression von CD163, hohe Level von sezerniertem IL-10 bei geringer TNF-α Sekretion. Darüber hinaus zeigen IF-polarisierte Makrophagen immunoregulatorische Funktionen, indem sie die Expansion von immunsuppressiven regulatorischen T-Zellen induzieren. In dieser Studie werden erstmals auch Ergebnisse vorgestellt, die zeigen, dass PGE2, PGI2, Testosteron und Corticosteron wichtige immunregulatorische Moleküle in der IF darstellen und eine wesentliche Rolle bei der Bestimmung des TM-Phänotyps spielen. Mit Ausnahme von Corticosteron sind die genannten Faktoren in der Lage, den NF-κB-Signalweg zu hemmen, und damit die Produktion von entzündungshemmenden Zytokinen zu unterdrücken. Bei Corticosteron war der NFκB Signalweg bei der Immunsuppression nicht blockiert. Corticosteron konnte als wichtigster immunsuppressiver Faktor in der IF identifiziert werden. Dessen Rezeptor, der Glucocorticoidrezeptor, konnte in TM mittels Immunhistochemie gefunden werden. TM produzieren lokal moderate Mengen an Corticosteron, die die Expression inflammatorischer Gene unterdrücken und TM unempfindlich gegenüber entzündlichen Stimuli machen können.

Zusammengenommen zeigen diese Ergebnisse, dass testikuläres Corticosteron maßgeblich für die immunsuppressive Funktion und den spezifischen Phänotyp der TM verantwortlich ist. Damit könnte das Steroidhormon auch eine wichtige Rolle bei der Etablierung und Aufrechterhaltung des Immunprivilegs im Hoden spielen.

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10. LIST OF PUBLICATION

1. Publication originally from this thesis

- **Ming Wang**, Monika Fijak, Hamid Hossain, Melanie Markmann, Rolf M. Nüsing, Günter Lochnit, Michaela F. Hartmann, Stefan A. Wudy, Lizong Zhang, Huanpeng Gu, Lutz Konrad, Trinad Chakraborty, Andreas Meinhardt and Sudhanshu Bhushan. Characterization of the micro-environment of the testis that shapes the phenotype and function of testicular macrophages. Journal of Immunology. 2017 Jun 1; 198(11): 4327-4340.
- Zhengguo Zhang, **Ming Wang**, Florian Eisel, Svetlin Tchatalbachev, Trinad Chakraborty, Andreas Meinhardt and Sudhanshu Bhushan. Uropathogenic Escherichia coli Epigenetically Manipulate Host Cell Death Pathways. Journal of Infection Disease. 2016 Apr 1; 213(7): 1198-207.
- Pradeep Kumar Kudipudi, **Ming Wang**, Nour Nicolas, Vera Michel, Lutz Konrad, Monika Fijak, Andreas Meinhardt, Sudhanshu Bhushan. Signaling via the IL-10/STAT3 pathway establishes the immunosuppressed phenotype of testicular macrophages. In final stage of preparation.

2. Other publications

- Yijun Qi, **Ming Wang** (Co-first author), Ruimin Liu, Hua Wei, Weixia Chao, Tian Zhang, Qiang Lou, Xiumin Li, Jin Ma, Han Zhu, Zhenhua Yang, Haiqing Liu and Yuanfang Ma. Downregulation of 14-3-3σ correlates with multistage carcinogenesis and poor prognosis of esophageal squamous cell carcinoma. PLoS One. 2014 Apr 17; 9(4): e95386.
- **Ming Wang**, Zhihong Huang, Peng Lei, Mingli Wang， Weixia Chao, Jin Ma, Wangyu Bu, Juan Zhang, Baohua Niu, Yuanfang Ma, Yijun Qi. Proteomic profiling of N-linked glycoproteins involved in hepatocellular carcinoma by two-dimensional electrophoresis and mass spectrometry. Chinese Journal of Biochemistry and Molecular Biology, 2013, 5: 482-489.

- **Ming Wang**, Yijun Qi, Ruimin Liu, Jin Ma. Clinical application of tumor associated antigens and its autoantibodies in early diagnosis of cancer. Chinese Pharmaceutical Biotechnology, 2013, V8(5): 376-380.

11 EHRENWÖRTLICHE ERKLÄRUNG

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

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

Giessen, den

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Ming Wang