Surfactant Protein D regulates murine testicular immune milieu and sperm functions

Sushama Rokade[1], Uday Kishore[2], Taruna Madan [1,*]

[1]Department of Innate Immunity, National Institute for Research in Reproductive Health (NIRRH), Indian Council of Medical Research (ICMR), Parel, Mumbai 400012, India

[2]Biosciences, College of Health and Life Sciences, Brunel University London, Uxbridge UB8 3PH, UK

***Correspondence:**

Dr. Taruna Madan

Scientist 'E' and Head,

Department of Innate Immunity,

National Institute for Research in Reproductive Health (NIRRH)

Indian Council of Medical Research (ICMR), Parel, Mumbai 400012, India

Tel.: +91-022-24192049

Fax: +91-022-2413 94 12

E-mail: taruna_m@hotmail.com

Short Title: SP-D in testis and male fertility

ABSTRACT

Problem: Surfactant Protein D (SP-D), a pattern recognition protein that regulates inflammation and immune homeostasis, is expressed by testicular germ cells under the influence of testosterone. The present study investigates the role of SP-D in testicular immune-privilege and sperm functions.

Method of study: Testicular levels of cytokines and immunoregulatory molecules were evaluated in lipopolysaccharide (LPS) challenged SP-D gene knock-out mice ($SP\text{-}D^{-/-}$). Further, sperm functions were assessed by CASA and *in vitro* capacitation. The effect of a recombinant fragment of human SP-D (rhSP-D) on LPS induced testicular inflammation and sperm motility was assessed in wild type (WT) mice.

Result: Endogenous absence of SP-D led to significantly increased testicular levels of immunosuppressive molecules viz., Serpina3, IL-12p40, TGF-β1 and IL-10, and reduced levels of immune cell activation markers, CD86, IL-2 and ITGAX. These compensatory mechanisms resulted in markedly blunted levels of TNF-α, IL-12p40, MIP-1α, G-CSF and IL-6 in response to LPS challenge. Notably, exogenous supplementation of rhSP-D salvaged the WT mice from LPS-induced pro-inflammatory immune response and impairment of sperm motility by up-regulating the levels of TGF-β1 and IL-10.

Conclusion: The study highlights the involvement of SP-D in maintenance of testicular immune privilege and its indirect contribution to male fertility.

Key words: SP-D, testis, sperm motility, LPS, inflammation, capacitation, fertility

Introduction

It is estimated that nearly 15 % cases of male factor infertility are attributed to inflammatory or autoimmune conditions[1]. An exaggerated inflammatory response to male urogenital tract infections can have detrimental effect on testicular spermatogenesis and sperm motility leading to a loss of fertility[2]. The mammalian testis is an immune privileged site where adaptive immune responses are finely regulated in order to protect auto-immunogenic sperm antigens[3]. The testicular cells have been shown to express a number of pattern recognition receptors (PRRs) such as Toll-Like Receptors, RIG-I-Like receptors and NOD-like receptors, which are actively involved in the testicular innate immune response to infections [4, 5, 6]. The innate and adaptive immune responses in the testis should be finely balanced to protect the auto-antigenic spermatozoa and to provide protection against infections. Thus, understanding the regulation of innate and adaptive immunity within the male reproductive tract is important for designing strategies to improve infertility.

Surfactant Protein D (SP-D), a pattern recognition protein of the collectin family, is an integral component of the innate immune system[7]. We have recently reported the presence of SP-D in the murine testis and on the caudal sperm[8]. Several reports have previously demonstrated the presence of SP-D in male reproductive tract of human, rodent and stallion[9, 10, 11]. The protective role of SP-D in pulmonary infections and inflammation has been studied comprehensively[12]. Through its C-terminal lectin domain, SP-D binds to carbohydrate moieties on the surface of pathogens and mediates phagocytosis and clearance of pathogens through the interaction of its collagen region with phagocytic cells[7]. SP-D is also known to play an important role in the control of inflammation, clearance of apoptotic and necrotic cells, and modulation of the activity of immune cells [macrophages, dendritic cells (DCs) and T-lymphocytes] via its

direct interaction with cell surface receptors such as CD14, SIRP-α, and CD91-calreticulin[13, 14, 15]. It exhibits anti-inflammatory effects in the lung and inhibits human $CD3^+$/ $CD4^+$ Th cell proliferation and IL-2 production[15, 16].

The role of SP-D as a host defence molecule in the rodent prostate has been demonstrated[11]. Recently, we have shown the testicular germ cells as a source of SP-D and testosterone as a positive regulator of SP-D expression in the testis of mice[8]. Importantly, we have reported an up-regulation of SP-D in murine testis following lipopolysaccharide (LPS) challenge[8]. LPS, a structural component of the outer membrane of Gram-negative bacteria, is the primary pathological factor associated with chronic inflammatory diseases and male reproductive impairments[17]. SP-D is known to inhibit LPS mediated inflammation through direct binding to LPS receptor CD14[18]. The interaction of SP-D with the extra-cellular domains of TLR-2 and TLR-4 via its C-terminal domain has been reported[19]. SP-D has also been shown to inhibit the LPS- induced inflammatory response in alveolar macrophages and TLR4/MD-2-transfected HEK293 cells[20]. Therefore, examining the immunological role of SP-D in the testis and on sperm functions under inflammatory conditions is likely to shed light on its role in male fertility.

SP-D gene knock-out ($SP\text{-}D^{-/-}$) mice have been previously used to dissect the *in vivo* functions of SP-D in the lung, brain and female reproductive tract[21, 22, 23]. Despite being a systemic knock-down model, the genetic deficiency of SP-D was associated with tissue specific immuno-phenotypes at these sites. SP-D deficiency resulted in an exaggerated pulmonary inflammation with increased infiltration of inflammatory cells and pro-inflammatory cytokine levels following pathogen or allergen challenge[21]. We have recently shown that, the $SP\text{-}D^{-/-}$ female mice exhibit an extended estrous cyle with an altered serum profile of ovarian hormones,

significantly smaller litter size due to pre-implantation embryo loss and a blunted pro-inflammatory response to LPS administration in pregnant mice[23].

In view of the role of SP-D in the control of inflammation and its relevance in male fertility, we examined the functional importance of SP-D in testicular immune privilege and sperm functions. We show that SP-D$^{-/-}$ male mice have significantly reduced weight of the testis and epididymis. In addition, a reduced degree of protein tyrosine phosphorylation was observed in the capacitated sperm from SP-D$^{-/-}$ mice. Although mating studies did not reveal any significant defect in fecundity, we observed an altered immune milieu in the testis of SP-D$^{-/-}$ mice that resulted in a subdued testicular inflammatory response following LPS challenge. Importantly, our data reveals a protective role of exogenous rhSP-D on testicular inflammation and sperm motility in LPS challenged wild type (WT) mice. Taken together, our results highlight the importance and plausible contribution of SP-D in the testicular immune regulation and male fertility.

Materials and Methods

Animals

This study was reviewed and approved by the Institutional Animal Ethics Committee (IAEC No. 04/15). Mice with the targeted deletion of SP-D (SP-D$^{-/-}$) on a Swiss black background (outbred mice) and wild type mice (WT) of the similar genetic background were obtained from the laboratory of Dr. Jefferey Whitsett, Cincinnati Children Hospital Medical Centre, USA. The study was conducted in accordance with the Institutional Guidelines for the care and use of experimental animals. Animals were maintained under controlled conditions of temperature and humidity with 12 h light: 12 h dark cycle and were fed *ad libitum*.

Genotyping of SP-D$^{-/-}$ mice

The SP-D$^{-/-}$ mice have been generated using neomycin replacement strategy with the deletion of second exon and a part of intron 2 of SP-D gene by a homologous recombination[24]. These animals were genotyped as reported previously[23]. Briefly, DNA was extracted from tail clips using RED Extract-N-Amp Tissue PCR Kit (Sigma, St. Louis, MO, USA) as per the manufacturer's protocol. The genotyping was carried out by PCR analysis using primers specific for exon 2 of the SP-D gene and neomycin resistance (Neo) gene. The primers were designed using NCBI Primer Blast software (Table 1). The PCR products were run on a 2 % agarose gel (GeNei^{TM}, Bangalore, India) to confirm the presence or absence of the SP-D gene.

Histological analysis of testis

For histological evaluation, testes from 8 week old SP-D$^{-/-}$ and WT mice (n = 5 each) were fixed in 4 % (w/v) buffered formalin (Qualigens, Mumbai, India), embedded in paraffin,

and sectioned at a thickness of 5 μm. The testicular cross-sections were stained with hematoxylin and eosin (Qualigens), dehydrated and mounted using DPX mountant (Fisher Scientific, Mumbai, India). Images were captured using Leica AS LMD microsystem.

Hormone measurements

Serum and intra-testicular levels of testosterone and estradiol in 8 week old SP-$D^{-/-}$ and WT mice (n = 5 each) were measured using hormone specific ELISA kits according to the manufacturer's protocol (Diagnostic Biochem Canada Inc).

Fertility studies

The reproductive capacity of SP-$D^{-/-}$ male mice was evaluated by cohabitation with WT female mice. Briefly, sexually mature (2-4 month old) littermate males of SP-$D^{-/-}$ and WT genotype were caged individually with 2 mature WT females for 2 months (n = 5 each). The fertility was assessed by evaluating the percentage of females achieving pregnancy, number of offspring per litter, litter weight, and period between the beginning of mating and delivery of the pups.

Assessment of Sperm motility and *in vitro* capacitation

Caudae epididymis from 8- 10 week old SP-$D^{-/-}$ and WT mice (n = 5 each) were excised and rinsed with M199 media (HiMedia, Mumbai, India). Sperm were allowed to exude for 10 min at 37°C in 5 % CO_2 through three to five incisions in 500 μl of fresh M199 media. The sperm preparation was immediately subjected to motility analysis, using CASA (Computer Assisted Sperm Analyzer, Hamilton-Thorne Research, Beverly, Mass). For each sample, a 20 μl

of sperm suspension, in at least 5 different microscopic fields was scored at 37°C. The pipette tips, slides and coverslips, used for motility analysis, were maintained at 37°C throughout the analysis. The validated default Mouse 2 analysis settings, provided by Hamilton Thorne, were used in the present study. These default settings were: 60 frames per second; number of frames, 30; minimum contrast, 30; minimum cell size, 4 pixels; cell size, 13 pixels; cell intensity, 75; cells counted as progressive if average path velocity (VAP) > 50 μm/sec and straightness (STR) > 50%; cells counted as slow if VAP cutoff, 10 μm/sec and straight-line velocity (VSL) cutoff, 0 μm/sec; minimum static intensity gate, 0.10; maximum intensity gate, 1.52; minimum static size gate, 0.13 pixels; maximum static size gate, 2.43 pixels; minimum static elongation gate, 5 pixels and maximum static elongation gate, 100 pixels.

We also compared the sperm motility parameters between SP-$D^{-/-}$ and WT mice after an inflammatory episode by an i.p. injection of pyrogen free-saline with or without 5 mg/ kg body weight of LPS (from *E. coli*, 0111:B4, Sigma). Mice were sacrificed 24 h post-LPS challenge (n = 5 per group) by cervical dislocation and caudae epididymis were excised. The sperm suspension was prepared for motility analysis by CASA, as described above.

For *in vitro* capacitation, the caudae epididymis from SP-$D^{-/-}$ and WT mice were excised and rinsed with DMEM F-12 media (Sigma). The sperm were allowed to exude for 10 min at 37°C in 5 % CO_2 via three to five incisions in 500 μl of fresh DMEM F-12 media. The resulting sperm suspension was washed twice in DMEM F-12 media at 500 g for 5 min. For capacitation, the sperm pellet was suspended in DMEM F-12 media supplemented with 5 mg/ ml Bovine Serum Albumin (Sigma) and 15 mM $NaHCO_3$ (Sigma), adjusted to the final concentration of 1 x 10^7 sperm cells/ ml, and incubation was allowed to proceed for different time points viz., 0 min, 15 min, 60 min and 90 min at 37°C in 5 % CO_2. Following incubation, the sperm motility was

182 analysed by CASA and the degree of protein tyrosine phosphorylation was assessed by western
183 blotting.
184
185 **Sperm count**
186 Cauda epididymis from adult SP-D$^{-/-}$ and WT mice were placed in a small clean petri dish
187 containing 1 ml PBS (n = 5 per genotype). Up to three to five small incisions were made in the
188 cauda to release the sperm, and the suspension was then allowed to settle at 37°C for 30 min.
189 Sperm suspension was loaded on both sides of the Neubuaer's Chamber (Hemocytometer) and
190 the number of sperm was counted at 40 X magnification. Sperm count was performed in
191 triplicate for each sample. Replicate counts on two separate dilutions of each sample were
192 performed to account for plausible uneven distribution of spermatozoa despite thorough mixing.
193
194 **Collection of spermatozoa for western blotting**
195 Spermatozoa incubated under capacitating condition at different time points were
196 collected by centrifugation at 5000 g for 5 min at 4°C. The resulting sperm pellet was suspended
197 in lysis buffer containing 50 mM Tris HCl (Sigma), pH 7.6, 150 mM NaCl (Thermo Fisher), 1 %
198 (v/v) Triton X-100 (Sigma), 0.5 % (w/v) SDS (Sigma) and 1 % (v/v) protease inhibitor (GE
199 Healthcare, Little Chalfont, United Kingdom) and vortexed for 10 min. The lysate was
200 centrifuged at 10,000 g for 15 min at 4°C and the resulting supernatant was boiled with SDS-
201 PAGE sample buffer for 5 min. 40 µg of sperm protein was used to detect tyrosine
202 phosphorylation by western blotting.
203
204

Assessment of testicular immune milieu of SP-D$^{-/-}$ and WT mice

To evaluate the immune milieu, testis from 8 week old SP-D$^{-/-}$ and WT mice (n = 5 each), was collected for RNA and protein extraction to estimate the levels of immunoregulatory molecules [such as, CD274, indole-amine 2,3- dioxygenase (IDO), matrix metalloproteinase 2 (MMP-2), matrix metalloproteinase 9 (MMP-9), matrix metalloproteinase 12 (MMP-12), FasL, Surfactant Protein A (SP-A), Mannan Binding Lectin A (MBL-A) and serine proteinase inhibitor family A member 3 (serpina3)], and cytokines [such as, Activin-A, Activin-B, Inhibin-A, Inhibin-B, IL-1α, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-13, IL-17, keratinocyte chemoattractant (KC), monocyte chemoattractant protein -1 (MCP-1), macrophage migration inhibitory factor (MIF), macrophage inflammatory protein – 1β (MIP-1β), TGF-β1 and TNF-α] by real time RT-PCR, western blotting and multiplex cytokine assay (Bio-rad, USA). Flow cytometric analysis was performed to evaluate the number of immune cells (viz., CD4^{+} and CD8^{+} T- lymphocytes, F4/80^{+} macrophages and CD11c^{+} dendritic cells) in the interstitial spaces of the testis of SP-D$^{-/-}$ and WT mice (n = 10 each). The activation status of these immune cells was assessed by measuring the testicular levels of their activation markers (viz., CD40L, MHC class II, CD86 and IL-2) by real time RT-PCR and western blotting (n = 5 each). The primers for all immune molecules were designed using NCBI Primer Blast software and are listed in Table 1.

LPS induced inflammatory response in the testis of SP-D$^{-/-}$ and WT mice

To evaluate the degree of LPS mediated inflammatory response in the testis of 8- 10 week old SP-D$^{-/-}$ and WT mice, systemic inflammation was induced by an i.p. injection of 100 µl of pyrogen free-saline containing 0.1 mg/ kg body weight (low dose) or 5 mg/ kg body weight (high dose) of LPS (from *E. coli*, 0111:B4; Sigma). A separate group of mice (control) were

injected with equal volumes of saline (n = 5 per treatment group). Mice were sacrificed 6 and 24 h after LPS challenge by cervical dislocation and testis tissues were snap-frozen for analysing the expression of steroid hormones by hormone specific ELISA kits (DBC) and that of cytokines by multiplex cytokine assay (Biorad).

Administration of exogenous recombinant human SP-D (rhSP-D)

The expression and purification of a fragment of human SP-D (rhSP-D), containing 8 N-terminal Gly-Xaa-Yaa triplets followed by the α-helical coiled-coil neck region and a globular C-type lectin domain was carried out as described previously[25]. 8 week old WT mice were injected i.p. with 5 mg/ kg body weight of LPS in 100 µl of pyrogen free-saline followed by an i.p. administration of 2.5 mg/ kg body weight of rhSP-D or saline alone (n = 5 per treatment group). Animals were sacrificed 24 h post rhSP-D injection and testes tissues were processed for RNA extraction and histopathology, while the epididymis was processed for histopathology and sperm motility analysis.

Multiplex Cytokine analysis

Testis tissues collected from SP-$D^{-/-}$ and WT mice were rinsed in PBS and homogenized. Tissue lysates were prepared using Bio-Plex cell lysis kit (Bio-Rad) and protein concentration in the resulting supernatants was estimated using the Micro BCA protein assay kit (Thermo Fisher Scientific). Samples were pooled (5 mice/ genotype) and the levels of pro-inflammatory cytokines in the supernatants were analysed using the Bio-Plex Pro Mouse Cytokine 23-plex Assay (Bio-Rad) as per the manufacturer's protocol. The assay was performed twice to validate the results and values were plotted as mean (of 6 values) ± SD.

RNA isolation and real time RT – PCR analysis

Total RNA from the testis tissues of SP-D$^{-/-}$ and WT mice was extracted using TRIsoln reagent (GeNei^{TM}) and treated with DNase I (Thermo Scientific, Rockford, USA) at 37°C for 30 min to eliminate any potential genomic DNA contamination. The yield and quality of resulting RNA was evaluated by determining the A260: A280 ratio. 2-3 μg of RNA was reverse transcribed using Superscript^{TM} III first strand synthesis kit (Invitrogen, Carlsbad, CA, USA). 1 μl of cDNA was used for subsequent real time PCR reactions. For all experiments, murine 18s was used as an internal quality control. Primers for amplification of cDNAs were designed using NCBI Primer BLAST Software (Table 1). Real time PCR of diluted cDNA was carried out on Bio-Rad CFX 96^{TM} Thermal Cycler machine (Bio-Rad). The specificity of each PCR reaction was validated by a melt curve analysis followed by visualization of the amplification products on 2 % (w/v) agarose gel (GeNei^{TM}) for desired product size.

Western blotting

Testis tissues were homogenised in 400 μl of ice-cold cell lysis buffer containing protease inhibitor (GE Healthcare) and nuclease mix (GE Healthcare). After 1 h incubation on ice, the homogenates were centrifuged at 15000 g for 20 min at 4°C. The protein concentration in the resulting supernatant was estimated by Micro BCA^{TM} protein assay kit (Thermo Fisher Scientific). The aliquots of the supernatant were diluted in Laemmli sample buffer and boiled for 10 min. Equal amount of proteins (40 μg) from testis and sperm lysate were separated on 10 % SDS-PAGE polyacrylamide gel (Biorad). After electrophoresis, the separated proteins were transferred onto PVDF membranes (Pall Corporation, New York, USA), blocked for 45 min using 3 % (w/v) skimmed milk protein in TBS (Tris Buffered Saline) and probed overnight at

4°C with rabbit polyclonal anti-mouse primary antibodies against serpina3 antibody (1:800, 47 kDa, orb215432, Bioryt Ltd., Cambridge, UK), CD86 (1: 1000, 38 kDa, A1199, Abclonal, Baltimore Avenue, College Park, MD), TGF-β1 (1: 1000, 44 kDa A2124, Abclonal), IL-2 (1: 1000, 18 kDa, A8029, Abclonal), and pJAK-2 (Tyr 1007/Tyr 1008) (1: 500, 128 kDa, sc-16566, Santa Cruz Biotechnology, Inc., CA, USA). Next, the blots were washed in TBS containing 0.1 % (v/v) Tween 20 (GeNei ^{TM}) and probed for 1 h with goat polyclonal anti-rabbit IgG-horseradish peroxidase (HRP) (1:6000, AS014, Abclonal). ECL prime western blotting reagent (Thermo Scientific Pierce) was used for developing the blots on X-ray films. Equal loading and extract degradation were checked by Ponceau S (GeNei ^{TM}) staining and via anti- β-actin (1: 2500, 42 kDa, ab8227, Abcam, Cambridge, MA, USA) and anti- α- tubulin monoclonal antibodies (1: 10000, 50 kDa, T6074, Sigma).

Flow cytometry

The percentage of various immune cell populations in the testis of SP-D$^{-/-}$ and WT mice was evaluated by flow cytometry. Briefly, the testis tissues from 8 week old SP-D$^{-/-}$ (n = 10) and WT mice (n = 10) were dissected, washed three times in cold PBS and the tunica albuginea was removed. The seminiferous tubules were dispersed but not fragmented in 0.5 mg/ ml collagenase type I solution (Sigma) for 15 min at room temperature. The enzymatic digest was passed though 70 µm mesh (BD, USA) to separate interstitial cells and seminiferous tubules. The interstitial cell suspension containing the immune cells of the testis was washed twice with cold FACS buffer [PBS with 1 % BSA, (GeNei^{TM})]. Next, the cells were fixed in 4 % (w/v) formalin (Qualigens) and blocked with 3 % BSA (GeNei^{TM}) for 30 min. The cell suspension was incubated with the fluorochome tagged primary antibodies against immune cell specific markers,

such as rat monoclonal anti-mouse F4/80-phycoerythin (PE) (1:30 in 100 µl for 10^6 cells, ab105156, Abcam), Armenian Hamster monoclonal to CD11c-PE (1:50 in 100 µl for 10^6 cells, ab86901, Abcam) and mouse T lymphocyte subset antibody cocktail: PE-Cy™7 CD3e, PE CD4, APC CD8a (20 µl in 100 µl for 10^6 cells, 558431, BD Pharmingen) for 40 min at 4°C. After final wash in FACS buffer, the cells were analysed using BD FACS Aria™ SORP flow cytometer (BD Biosciences, San Jose, CA, USA). Matched isotype controls were used as negative controls for each experiment.

Statistical analysis

The data were analysed using GraphPad Prism Version 6 software (Graph Pad Inc., San Diego, CA, USA). Non-parametric Mann-Whitney test was used for a comparison between the two groups and Kruskal-Wallis test followed by post hoc test (Dunn's multiple comparison tests) for the LPS study. P-value < 0.05 was considered to be statistically significant.

Results

Normal morphology of testis and epididymis in SP-D$^{-/-}$ mice

In order to understand the importance of SP-D in male fertility, we first sought to compare the morphology of the testis and epididymis between SP-D$^{-/-}$ and WT mice. The weight of testis and epididymis in 8 week old SP-D$^{-/-}$ mice (64 ± 3.26 and 26.05 ± 1.79 mg, respectively) was significantly low as compared to the age matched WT mice (84 ± 5.99 and 34.58 ± 2.61 mg, respectively), (Fig 1A and B). Importantly, a decreased length of the epididymis with less convoluted cauda was also observed in SP-D$^{-/-}$ mice (Fig 1A). However, despite these phenotypical differences, the histological staining of testis and epididymis from SP-D$^{-/-}$ mice revealed a normal structure with no significant difference relative to the WT mice (Fig S2). Apparently, we did not find any significant difference in the body weights of SP-D$^{-/-}$ (26.65 ± 1.78 gm) and WT mice (27.90 ± 2.43 gm). Interestingly, measurement of the serum and intra-testicular levels of steroid hormones revealed significantly lower serum levels of testosterone (2500 ± 500 vs 4200 ± 840 pg/ ml) in SP-D$^{-/-}$ mice with no significant difference in the serum levels of estradiol (55.70 ± 5.50 vs 72.64 ± 7.20 pg/ ml respectively) than WT mice (Fig 1C). Moreover we did not find any substantial difference in the intra-testicular levels of these hormones between SP-D$^{-/-}$ and WT mice (100 ± 20 vs 80 ± 16 pg testosterone/ mg of testis and 0.608 ± 0.12 vs 0.59 ± 0.11 pg of estradiol/ mg of testis) (Fig 1D).

Motility of caudal sperm

The motility parameters of the sperm preparations from cauda epididymis of SP-D$^{-/-}$ and WT mice were evaluated by CASA. No significant difference in the total motility (79.7 ± 11.7 vs

89 ± 6.4 % respectively) (Fig 2A) and progressive motility (30.3 ± 4.7 vs 28 ± 2.5 % respectively) (Fig 2B) was observed in the sperm suspensions from SP-D$^{-/-}$ and WT mice. An assessment of the mean values of various motility parameters revealed decreased vigour parameters [curvilinear velocity (VCL), lateral amplitude (ALH) and beat frequency (BCF)] and increased progression parameters [progressive velocity (VSL), path velocity (VAP), straightness (STR) and linearity (LIN)] in the sperm preparation from SP-D$^{-/-}$ mice compared to WT mice, although only mean BCF and LIN values showed a statistically significant difference (Table 2).

We also determined the effect of LPS induced inflammation on the sperm count and motility following an i.p. administration of a high dose of LPS for 24 h. Although, the caudal sperm count in SP-D$^{-/-}$ mice (1.88 ± 0.2 x 10^6 cells/ cauda) was lower than the WT mice (3.94 ± 2.6 x 10^6 cells/ cauda) in control groups, the levels did not reach a statistical significance (Fig 2C). LPS treatment did not lead to any significant alteration in the sperm count in WT mice (3.1 ± 1.5 x 10^6 cells/ cauda) compared to its levels in control WT mice. However, it resulted in a ~ 4 fold decrease in the sperm count (4.8 ± 1.6 x 10^5 cells/ cauda) in the LPS treated SP-D$^{-/-}$ mice compared to the untreated control SP-D$^{-/-}$ mice (Fig 2C). While LPS challenge reduced the percentage of total and progressive sperm motility in SP-D$^{-/-}$ as well as WT mice, the difference in the values was not statistically significant (Fig 2A and B). Although LPS administration reduced the percentage of rapidly motile sperm and increased the percentage of static sperm in both SP-D$^{-/-}$ and WT mice, the differences in the values for velocity distribution between the two genotypes were not statistically significant (Fig 2D).

***In vitro* capacitation**

To further explore the functional differences in sperm derived from SP-D$^{-/-}$ and WT mice, sperm preparations were incubated under *in vitro* capacitating conditions at different time points. Immunoblotting analysis was performed to assess the protein tyrosine phosphorylation, which is a characteristic of capacitated sperm. Our results revealed a time dependent increase in the degree of protein tyrosine phosphorylation in JAK-2 pathway in sperm suspesion from both SP-D$^{-/-}$ and WT genotype (Fig 3A). Interestingly, the degree of protein tyrosine phosphorylation was considerably low in the sperm preparation from SP-D$^{-/-}$ mice compared to the WT (Fig 3B), suggesting a likely involvement of SP-D in the sperm capacitation process.

Fertility of SP-D$^{-/-}$ male mice

To determine the effect of SP-D deficiency on fecundity, we individually co-habited sexually mature SP-D$^{-/-}$ and WT males with WT female mice for 2 months. The mating ability of SP-D$^{-/-}$ male mice was comparable to that of WT mice, based on the pregnancy rate in female mice. Mating studies revealed no significant difference between the SP-D$^{-/-}$ and WT mice in the parameters indicative of fertility including the litter size and litter weight (Table 3).

Increased levels of immunoregulatory molecules in the testis

To evaluate the importance of SP-D in testicular immune privilege, we determined the levels of various immunoregulatory molecules in the testis of SP-D$^{-/-}$ mice compared to the WT mice under physiological conditions. The analysis of mRNA expression revealed significantly increased levels of immunosuppressive molecules, such as serpina3 (~ 2.6 fold), TGF-β1 (~ 1.9 fold) and IL-10 (~ 2 fold) in the testis of SP-D$^{-/-}$ mice than WT (Fig 4A and B). Interestingly, the testis of SP-D$^{-/-}$ mice also showed higher levels of pro-inflammatory cytokines, such as TNF-α (~

1.4 fold) and IL-1α (~ 1.3 fold), (Fig 4C). Significantly reduced levels of the immunosuppressive molecules, such as IDO (~ 1.6 fold), Activin-B (~ 2 fold), and Inhibin-B (~ 2.7 fold), and of pro-inflammatory cytokines, such as KC (~ 2 fold) and IL-6 (~ 2 fold) in SP-D$^{-/-}$ mice compared to the WT were observed (Fig 4A, B and C). There was no significant difference in the levels of MMP-2, MMP-9 and MMP-12. Although SP-A levels did not change, a ~ 2.3 fold higher level of MBL-A transcripts was observed in the testis of SP-D$^{-/-}$ mice (Fig 4D). Next, we assessed the protein levels of these molecules in the testis of mice from both the genotypes by multiplex cytokine assay and immunoblotting. Consistent with our transcript data, the testis of SP-D$^{-/-}$ mice exhibited significantly higher protein levels of Serpina3 (~ 2.2 fold), IL-12p40 (~ 7.5 fold), TGF-β1 (~ 2 fold), IL-10 (~ 1.6 fold) and TNF-α (~ 2 fold) than WT mice (Fig 4E, F, G and H). Thus, a significant alteration of testicular immune milieu in SP-D$^{-/-}$ mice was evident, which suggests a role for SP-D in the maintenance of immune homeostasis within the testis.

Reduced levels of immune cell activation markers

To further characterize the immune status of the testis of SP-D$^{-/-}$ mice, we enumerated the percentage of immune cells in testicular interstitial spaces. Flow cytometric analysis showed no significant difference (Fig 5A) in the percentage of testicular immune cells between SP-D$^{-/-}$ and WT mice; these included F4/80^{+} macrophages (5.7 ± 1.3 % vs 4.4 ± 1.8 %) (Fig S3 A), CD11c^{+} dendritic cells (5.0 ± 0.7 % vs 7.1 ± 2.2 %) (Fig S3 B), CD4^{+} T lymphocytes (0.2 ± 0.08 % vs 0.2 ± 0.1 %) (Fig S3 C), and CD8^{+} T lymphocytes (0.3 ± 0.1 % vs 0.3 ± 0.09 %) (Fig S3 D). Interestingly, comparison of the testicular levels of immune cell activation markers revealed significantly lower levels (~ 2 fold) of CD86 and IL-2 (Fig 5B, D and E) in the SP-D$^{-/-}$ mice, thus indicating a reduced activation status of immune cells in the testis of SP-D$^{-/-}$ mice. We also

evaluated the testicular mRNA expression of ITGAX and MRC1, markers for M1 and M2 macrophages, respectively and observed a significantly decreased (~ 3.85 fold and ~ 2.4 fold respectively) transcript levels of both the markers in the testis of SP-D$^{-/-}$ mice compared to WT mice (Fig 5C). Although the ratio of M2/M1 macrophages in both WT (~ 1.39) and SP-D$^{-/-}$ mice (~ 2.09) showed a predominance of M2 marker, the proportion of M2 marker was higher in the SP-D$^{-/-}$ than WT mice, suggesting an immuno-regulatory phenotype in the knockout mice testis.

Blunted inflammatory response to LPS in the testis

To analyse the relevance of SP-D in inflamed testis, we used LPS induced systemic inflammation model by intra-peritoneal administration of low and high dose of LPS. LPS administration to the WT mice resulted in an up-regulation of pro-inflammatory cytokines in the testis, while the SP-D$^{-/-}$ mice showed a blunted inflammatory response to LPS challenge. Though, LPS treatment up-regulated the testicular levels of MCP-1 and RANTES in SP-D$^{-/-}$ mice similar to the WT mice (Fig 6A and B), LPS challenged SP-D$^{-/-}$ mice showed blunted immune response for TNF-α, IL-10, IL-12p40, MIP-1α, G-CSF and IL-6 compared to their levels in WT mice (Fig 6).

We also analysed the levels of testosterone and estradiol in the testis of LPS treated mice. There was no significant difference in the basal testicular levels of both hormones in SP-D$^{-/-}$ and WT mice. Moreover, LPS treatment did not significantly alter the levels of testosterone at both low and high dose of LPS at 6 h and 24 h time points in murine testis from both the genotypes (Fig 7A). Interestingly, a significant increase in the intra-testicular level of estradiol was observed in both SP-D$^{-/-}$ and WT mice following LPS challenge (Fig 7B). This increase was

433 significantly higher in SP-D$^{-/-}$ mice in the high dose of LPS treated group at both 6 and 24 h and
434 in the low dose group at 6 h time point.
435
436 **Protective effect of exogenous rhSP-D on LPS induced testicular inflammation and sperm**
437 **motility**
438 To further confirm the role of SP-D in the regulation of LPS induced immune response in
439 the testis, we treated LPS challenged WT mice with exogenous rhSP-D and examined the
440 testicular histology, cytokine response, and sperm motility.
441 The histological analysis of testis from the LPS challenged mice showed vacuolization
442 and an increase in the intercellular gaps in seminiferous epithelium due to disruption of cell-to-
443 cell contact (Fig S4). Although the testis from rhSP-D treated and LPS challenged mice also
444 showed the presence of intercellular gaps and vacuoles in seminiferous epithelium, the number
445 of tubules showing such features were fewer, compared to untreated group (Fig S4).
446 As evident from the intra-testicular transcript levels of cytokines, the LPS treatment
447 resulted in a pro-inflammatory response involving a significant down-regulation of TGF- β1
448 levels (~ 6 fold) and a concomitant up-regulation of TNF- α (~ 15.22 fold) compared to the saline
449 control group (Fig S5). The rhSP-D treatment of the LPS challenged mice significantly
450 increased the levels of all thee cytokines: TGF-β1 (~ 2.79 fold), IL-10 (~ 27.66 fold) and TNF- α
451 (~ 73.26 fold) compared to the saline control group (Fig S5). We found a preponderance of an
452 anti-inflammatory milieu in the rhSP-D treated mice (viz., ~ 15.67 fold up-regulation of TGF-β1,
453 ~ 21.40 fold up-regulation of IL-10 and ~ 4.8 fold up-regulation of TNF- α), compared to the
454 untreated group (Fig 8A). It appears that SP-D could be an important molecule in the regulation

of inflammatory responses in the testis, thus impacting upon local immune homeostasis, and consequently, the male fertility.

Further analysis of the sperm motility of these mice by CASA revealed that LPS challenge led to a significant decrease in the percentage of total sperm motility (57 ± 8.5 %) and an increase in the percentage of static sperm (25 ± 10.6 %) compared to the saline control group (91.67 ± 2.5 %) and (2.6 ± 1.5 %) respectively (Fig 8B and C). Remarkably, treatment with rhSP-D was effective in restoring the total motility (90 ± 5.2 %) of sperm and in reducing the percentage of static sperm (1.67 ± 0.6 %) that was hampered by LPS challenge (Fig 8B and C) signifying the protective role of SP-D in inflammation associated male infertility.

Discussion

The present study demonstrates an integral role of SP-D in immune regulation of the testis and sperm functions. Our findings reveal that systemic absence of SP-D altered the immune milieu of the testis, leading to a reduced immune cell activation and subdued inflammatory response to LPS challenge. Importantly exogenous supplementation of rhSP-D negated the effect of LPS induced inflammation in the testis and on sperm motility in WT mice. Protein tyrosine phosphorylation was significantly reduced in capacitated sperm derived from SP-D$^{-/-}$ mice relative to WT mice, suggesting that SP-D may contribute to sperm capacitation process.

Increased infiltration of large, foamy macrophages, activated T lymphocytes and elevated proinflammatory cytokines was reported previously in the lungs of SP-D$^{-/-}$ mice[24, 26]. In contrast, in testis, an immune privileged organ unlike lungs, we observed significantly increased levels of immunoregulatory molecules in SP-D$^{-/-}$ mice indicating a dominance of immune-suppressive milieu in the testis of these mice. This inference was further supported by the reduced levels of immune cell activation markers, such as CD86 and IL-2 in the testis of SP-D$^{-/-}$ mice, suggesting a reduced activation status and function of testicular immune cells. In addition, we found a predominance of M2 macrophage marker (MRC1) associated with macrophages involved in immune suppression[27]. The M1 macrophages secrete inflammatory cytokines such as IFN-γ and TNF-α and produce nitric oxide (NO). The M2 macrophages are regulatory macrophages with high phagocytosis potential and produce IL-10. They clear apoptotic cells, can mitigate inflammatory response, and promote wound healing[27]. Collectively these data suggest the predominance of an overall immunosuppressive milieu in the testis of SP-D$^{-/-}$ mice.

A complex interplay between multiple immunological factors, is indispensible to protect auto-antigenic spermatozoa in the testis, which is a pre-requisite for normal fertility[3]. Therefore an increase in the other immune suppressive molecules and modulation of the immune cell functions could be a compensatory mechanism to restore the dysregulation caused by an absence of SP-D with an aim to preserve fertility.

Levels of SP-D are known to increase at the site of infection and inflammation[11]. Recently, we demonstrated an LPS induced increase in the levels of SP-D in murine testis[8]. SP-D inhibits LPS mediated effects via direct interaction with LPS as well as with LPS receptors TLR4/MD2, TLR2 and CD14[19, 20, 21]. In view of an overall increase in testicular immunosuppressive milieu, we hypothesized that the testis of SP-D$^{-/-}$ mice would exhibit alterations in inflammatory response to LPS challenge. As expected, we found a reduced severity of inflammatory reaction in the testis following LPS challenge, reaffirming immunosuppressive microenvironment within the testis of SP-D knock-out mice.

Previously, a decline in serum and intra-testicular levels of testosterone at 6 and 18 h after LPS administration has been reported in rats[28]. However, in our study, we did not find any significant difference in the intra-testicular levels of testosterone post LPS challenge. Notably, we found a significant increase in the levels of estradiol after LPS treatment. Earlier studies have reported estradiol mediated suppression of the LPS induced inflammatory responses at various sites including H9c2 cardiomyoblast cells, murine lung and bone marrow derived macrophages[29, 30, 31]. Thus an increase in the testicular levels of estradiol upon LPS challenge may be a specific feedback response to regulate inflammation in the absence of SP-D. It would be interesting to examine the testicular aromatase activity in this regard.

Another strategy to unravel the role of SP-D in testicular inflammation was exogenous supplementation of rhSP-D in the WT mice challenged with LPS. Consistent with the involvement of SP-D in controlling inflammation, we observed a protective effect of rhSP-D against LPS induced inflammatory response in the testis and sperm motility, thus highlighting the beneficial properties of SP-D in inflammation associated male infertility. Though endogenous absence of SP-D led to a pro-inflammatory response in the lungs[21], and murine uterus[23], a predominance of immune-suppressive milieu was observed in the testis, a unique phenomenon plausibly associated with the immune-privileged nature of this organ. Compensatory mechanisms could have led to the alteration in the levels of other immunoregulatory molecules to perform the role that was played by SP-D. Though observations from the two strategies seem to be contradictory, they converge to suggest that SP-D regulates the testicular immune milieu. On similar lines, Perez *et al.*, 2015, reported that exogenous administration of recombinant Gal-1 (galectin-1, a β-galactoside binding immunoregulatory protein) attenuates the severity of experimental autoimmune orchitis (EAO), whereas, Gal-1 deficient mice showed significant reduction in the incidence and severity of EAO[32].

Importantly, male mice with the targeted deletion of SP-D gene exhibited a decrease in certain components of masculine parameters. They had lower serum levels of testosterone and decreased weight of testes and epididymis. Though under normal physiological conditions the caudal sperm count of SP-D$^{-/-}$ mice was lower than WT mice, levels did not reach statistical significance. In spite of exhibiting a blunted response to LPS induced inflammation in testis, a significant reduction of caudal sperm count in LPS challenged SP-D$^{-/-}$ mice was surprising. In this regard, future studies involving DNA flow cytometry of testicular spermatogenic cells using propidium iodide staining will ascertain if the reduced sperm count is due to a defect at testicular

level. It will be relevant to evaluate the incidence of LPS induced germ cell apoptosis in SP-D$^{-/-}$ mice, as the testicular germ cells are a source of SP-D in the testis[8.] Importantly, SP-D$^{-/-}$ mice displayed a decreased length of the epididymis with less convoluted cauda. Changes in the convoluted nature of cauda can directly affect the quantity of spermatozoa retained in the epididymis, and could be a reason for the reduced caudal sperm count in SP-D$^{-/-}$ mice. Furthermore, there is a possibility that SP-D may have cauda specific roles.

We have previously reported the localization of SP-D on murine spermatozoa[8]. Therefore, to understand the putative role of SP-D in sperm functions, we first compared the sperm motility parameters of SP-D$^{-/-}$ mice with the WT. No difference in the percentage of total and progressive sperm motility was observed in sperm suspension from both the genotypes. In addition, we evaluated the capacitation induced protein tyrosine phosphorylation in sperm suspensions from SP-D$^{-/-}$ and WT mice. Many biochemical and physiological changes have been described for sperm incubated under capacitating conditions. These include an efflux of plasma membrane cholesterol, increased adenylate cyclase activity, increased cAMP levels, hyperpolarization of membrane potential and increased serine/threonine and tyrosine phosphorylation of some proteins[33, 34]. Thus, we evaluated the tyrosine phosphorylation in JAK2 pathway as a measure of capacitation for sperm suspensions incubated under *in vitro* capacitating conditions[35]. The observation that SP-D$^{-/-}$ mice exhibit considerably reduced tyrosine phosphorylation in capacitated sperm appears to implicate SP-D in the sperm capacitation process. Cytokines are known to influence sperm cell capacitation process in a positive or negative manner. Importantly, interferon – α (IFN- α), interferon- γ (IFN- γ) and TNF- α negatively affect sperm motility, while IL-6 is known to enhance sperm capacitation and acrosome reaction *in vitro*[36, 37]. With the ability to influence immune response and cytokine

production[15, 16], SP-D could be an important molecule involved in regulating sperm capacitation process.

Majority of the proteins that undergo phosphorylation at tyrosine residues during capacitation are localized on the sperm flagellum[38]. We have previously shown the localization of SP-D throughout the length of the flagellum of murine spermatozoa[8]. Our results of sperm motility analysis showed decreased vigour parameters (VCL, ALH and BCF) and increased progression parameters (VSL, VAP, STR and LIN) in SP-$D^{-/-}$ mice. Altogether, these findings highlight the involvement of SP-D in sperm capacitation, a process characterized by vigorous sperm motility. Thus, understanding the involvement of SP-D in sperm acrosome reaction as well as sperm- egg interaction via *in vitro* fertilization assay would be crucial in defining the specific role of SP-D in the fertilization process.

Despite relatively low caudal sperm count and decreased sperm vigour parameters in SP-$D^{-/-}$ mice, mating studies did not reveal any defect in the fecundity of SP-$D^{-/-}$ mice. This is not surprising since the sperm count should be reduced to 10% or less in order to influence fertility[39]. Previously knock-out male mice with targeted deletion of genes having important roles in the testis such as IL-12p40[40], ADP-Ribosylation Factor-Like 4 protein (Arl4)[41], follicle-stimulating hormone β (FSH β)[42] and activin II receptor A (ActRcIIA)[43], exhibited normal fertility even with the reduced sperm count or testicular weight. It is worth noting that the female reproductive tract is equipped with an efficient mechanism to select a few, but high quality fertilizing sperm that ultimately reach the ampulla, thus compensating for the reproductive deficiencies males may have. The defects in sperm functions could thus be masked by such proficient selection system. These sperm defects may possibly be evident when knock-out mice are mated under more challenging conditions such as by unilateral vasectomy to reduce the number of ejaculated

sperm, or by mating with super-ovulated females with high number of eggs in ampulla[44, 45]. Recently Cysteine-rich secretory protein 2 (Crisp2) and galectin-1 (Gal-1) gene knock-out mice were described to show defects in fertilization *in vivo*, only under such restricted mating conditions[44, 45].

A detailed understanding of the putative roles of SP-D in male fertility will require additional studies, ideally involving the use of testis-specific knock-down model, restricted mating trials, and *in vitro* fertilization assays. Nevertheless, the current study provides first evidence for the involvement of SP-D in regulating testicular immune privilege and male fertility.

Acknowledgements

We thank the Director, National Institute for Research in Reproductive Health, for providing necessary facilities in carrying out this research (NIRRH/RA/418/09-2016). Authors are extremely grateful to Dr. Jeffrey Whitsett, Cincinnati Children Hospital Medical Centre, USA, for kindly providing SP-D$^{-/-}$ mice in carrying out this study. We thank Jaymi Semona for her help with documentation for procuring these mice and Paul S. Kingma for providing primer details for genotyping. S. R. is thankful to the Council of Scientific and Industrial Research (CSIR), Government of India for providing Junior and Senior Research Fellowships. We also thank Dr. Geeta Vanage and Dr. Vikas Dighe for the histology and CASA facility, Dr. S. Mukherjee for the flow cytometry facility and Mr. S. D. Rawool for his help with animal handling.

References

1. Bachir BG, Jarvi K. Infectious, Inflammatory, and Immunologic Conditions Resulting in Male Infertility. *Urol Clin North Am* 2014;41:67–81.
2. Sarkar O, Bahainwala J, Chandrasekaran S, Kothari S, Mathur PP, Agarwal A. Impact of inflammation on male fertility. *Front Biosci (Elite Ed)* 2011;3:89-95.
3. Meinhardt A, Hedger MP. Immunological, paracrine and endocrine aspects of testicular immune privilege. *Mol Cell Endocrinol* 2011;335:60-68.
4. Winnall WR, Muir JA, Hedger MP. Differential responses of epithelial Sertoli cells of the rat testis to Toll-like receptor 2 and 4 ligands: implications for studies of testicular inflammation using bacterial lipopolysaccharides. *Innate Immun* 2011;17:123–136.
5. Zhu W, Chen Q, Yan K, Liu Z, Li N, Zhang X, Yu L, Chen Y, Han D. RIG-I-like receptors mediate innate antiviral response in mouse testis. *Mol Endocrinol* 2013;9:1455-1467.
6. Lech M, Avila-Ferrufino A, Skuginna V, Susanti HE, Anders HJ. Quantitative expression of RIG-like helicase, NOD-like receptor and inflammasome-related mRNAs in humans and mice. *Int Immunol* 2010;22:717–2810.
7. Nayak A, Dodagatta-Marri E, Tsolaki AG, Kishore U. An Insight into the Diverse Roles of Surfactant Proteins, SP-A and SP-D in Innate and Adaptive Immunity. *Front Immunol* 2012;3:131.
8. Rokade S, Madan T. Testicular expression of SP-A, SP-D and MBL-A is positively regulated by testosterone and modulated by lipopolysaccharide. *Immunobiology* 2016;221:975-85.

9. Beileke S, Claassen H, Wagner W, Matthies C, Ruf C, Hartmann A, Garreis F, Paulsen F, Schicht M, Brauer L. Expression and localization of lung surfactant proteins in human testis. *PLoS One* 2015;10:e0143058.
10. Kankavi O, Ata A, Celik-Ozenci C, Sati L, Ciftcioglu MA, Demir R. Presence and subcellular localizations of surfactant proteins A and D in human spermatozoa. *Fertil Steril* 2008;90:1904–1909.
11. Oberley RE, Goss KL, Quintar AA, Maldonado CA, Snyder JM. Regulation of surfactant protein D in the rodent prostate. *Reprod Biol Endocrinol* 2007;5:42.
12. Wright JR. Immunoregulatory functions of surfactant proteins. *Nat Rev Immunol* 2005; 5:58–68.
13. Kishore U, Greenhough T, Waters P, Shive AK, Ghai R, Kamran MF, Bernal A L, Reid KB, Madan T, Chakraborty T. Surfactant proteins SP-A and SP-D: structure, function and receptors. *Mol Immunol* 2006;43:1293–1315.
14. Gardai SJ, Xiao YQ, Dickinson M, Nick JA, Voelker DR, Greene KE, Henson PM. By binding SIRP alpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. *Cell* 2003;115:13-23.
15. Borron P, Mostaghel EA, Doyle C, Walsh ES, McHeyzer-Williams MG, JWright JR. Pulmonary Surfactant Proteins A and D Directly Suppress CD3 +/ CD4+ Cell Function: Evidence for Two Shared Mechanisms. *J Immunol* 2002;169:5844-5850.
16. Pastva AM, Wright JR, Williams KL. Immunomodulatory roles of Surfactant Proteins A and D: Implications in Lung disease. *Proc Am Thorac Soc* 2007;4:252-257.

17. Collodel G, Moretti E, Brecchia G, Kuželová L, Arruda J, Mourvaki E, Castellini C. Cytokines release and oxidative status in semen samples from rabbits treated with bacterial lipopolysaccharide. *Theriogenology* 2015;83:1233-1240.
18. Sano H, Chiba H, Iwaki D, Sohma H, Voelker DR, Kuroki Y. Surfactant proteins A and D bind CD14 by different mechanisms. *J Biol Chem* 2000;275:22442-51.
19. Ohya M, Nishitani C, Sano H, Yamada C, Mitsuzawa H, Shimizu T, Saito T, Smith K, Crouch E, Kuroki Y. Human pulmonary surfactant protein D binds the extracellular domains of Toll-like receptors 2 and 4 though the carbohydrate recognition domain by a mechanism different from its binding to phosphatidylinositol and lipopolysaccharide. *Biochemistry* 2006;18;45(28):8657-64.
20. Yamazoe M, Nishitani C, Takahashi M, Katoh T, Ariki S, Shimizu T, Mitsuzawa H, Sawada K, Voelker DR, Takahashi H, Kuroki Y. Pulmonary surfactant protein D inhibits lipopolysaccharide (LPS)-induced inflammatory cell responses by altering LPS binding to its receptors. *J Biol Chem* 2008;283:35878-88.
21. Madan T, Reid KB, Singh M, Sarma PU, Kishore U. Susceptibility of mice genetically deficient in the surfactant protein (SP)-A or SP-D gene to pulmonary hypersensitivity induced by antigens and allergens of Aspergillus fumigatus. *J Immunol* 2005; 174(11):6943-54.
22. Lambertsen KL, Østergaard K, Clausen BH, Hansen S, Stenvang J, Thorsen SB, Meldgaard M, Kristensen BW, Hansen PB, Sorensen GL, Finsen B. No effect of ablation of surfactant protein-D on acute cerebral infarction in mice. *J Neuroinflammation* 2014;11:123.

23. Kay S, Madan T. Fertility defects in Surfactant associated protein D knock-out female mice: altered ovarian hormone profile. *Mol Immunol* 2016;71:87-97.
24. Korfhagen T, Sheftelyevich V, Burhans M, Bruno M, Ross G, Wert S, Stahlman MT, Jobe AH, Ikegami M, Whitsett JA, Fisher JH . Surfactant Protein-D Regulates Surfactant Phospholipid Homeostasis in Vivo. *J Biol Chem* 1998;273:28438– 43.
25. Mahajan L, Madan T, Kamal N, Singh VK, Sim RB, Telang SD, Ramchand CN, Waters P, Kishore U, Sarma PU. Recombinant surfactant protein-D selectively increases apoptosis in eosinophils of allergic asthmatics and enhances uptake of apoptotic eosinophils by macrophages. *Int Immunol* 2008;20(8):993–1007.
26. Clark H, Palaniyar N, Hawgood S, Reid KB. A recombinant fragment of human surfactant protein D reduces alveolar macrophage apoptosis and pro-inflammatory cytokines in mice developing pulmonary emphysema. *Ann N Y Acad Sci* 2003;1010:113-116.
27. Murray PJ and Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nature Reviews Immunology* 2011;11:723-737.
28. O'bryan MK, Schlatt S, Phillips DJ, De Kretser DM, Hedger MP. Bacterial lipopolysaccharide-induced inflammation compromises testicular function at multiple levels *in vivo*. *Endocrinology* 2000;141(1):238-46.
29. Fan MJ, Huang-Liu R, Shen CY, Ju DT, Lin YM, Pai P, Huang PY, Ho TJ, Tsai FJ, Tsai CH, Huang CY. Reduction of TLR4 mRNA stability and protein expressions though inhibiting cytoplasmic translocation of HuR transcription factor by E_2 and/or ERα in LPS-treated H9c2 cardiomyoblast cells. *Chin J Physiol* 2014;57(1):8-18.

30. Qi D, He J, Wang D, Deng W, Zhao Y, Ye Y, Feng L.17β-estradiol suppresses lipopolysaccharide-induced acute lung injury though PI3K/Akt/SGK1 mediated up-regulation of epithelial sodium channel (ENaC) *in vivo* and *in vitro*. *Immunol Invest* 2014;43(7):693-704.
31. Liu L, Zhao Y, Xie K, Sun X, Jiang L, Gao Y, Wang Z. Estrogen inhibits LPS-induced IL-6 production in macrophages partially via the nongenomic pathway. *Chin J Physiol* 2014;57(1):8-18.
32. Perez CV, Gomez LG, Gualdoni GS, Lustig L, Rabinovich GA, Guazzone VA. Dual roles of endogenous and exogenous galectin-1 in the control of testicular immunopathology. *Sci Rep* 2015;5:12259.
33. Escoffier J, Krapf D, Navarrete F, Darszon A, Visconti PE. Flow cytometry analysis reveals a decrease in intracellular sodium during sperm capacitation. *J Cell Sci* 2012; 125:473–48.
34. Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P, Kopf GS. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development* 1995;121 :1139–1150.
35. Laflamme J, Akoum A, Leclerc P. Induction of human sperm capacitation and protein tyrosine phosphorylation by endometrial cells and interleukin-6. *Mol Hum Reprod* 2005;11(2):141–150.
36. Naz RK, Chaturvedi MM, Aggarwal BB. Role of cytokines and proto-oncogenes in sperm cell function: relevance to immunologic infertility. *Am J Reprod Immunol* 1994;32:26–37.

37. Naz RK, Kaplan P. Interleukin-6 enhances the fertilizing capacity of human sperm by increasing capacitation and acrosome reaction. *J Androl* 1994;15:228–233.
38. Sakkas D, Leppens-Luisier G, Lucas H, Chardonnens D, Campana A, Franken DR and Urner F. Localization of tyrosine phosphorylated proteins in human sperm and relation to capacitation and zona pellucida binding. *Biol Reprod* 2003;68:1463–1469.
39. Russel L, Ettlin R, Hikim A, Clegg ED. Histopathology of the testis. Quantitative evaluation of testis histopathology. In *Histological and histopathological evaluation of the testis*, U.S. Environmental Protection Agency, Washington, D.C., EPA/600/8-91/012.
40. Enright BP, Davila DR, Tornesi BM, Blaich G, Hoberman AM, Gallenberg LA. Developmental and reproductive toxicology studies in IL-12p40 knock-out mice. *Birth Defects Res B Dev Reprod Toxicol* 2011;92(2):102-10.
41. Schürmann A, Koling S, Jacobs S, Saftig P, Krau S,Wennemuth G,Kluge R, Joost HG. Reduced Sperm Count and Normal Fertility in Male Mice with Targeted Disruption of the ADP-Ribosylation Factor-Like 4 (Arl4) Gene. *Mol Cell Biol* 2002;22(8):2761–2768.
42. Kumar TR, Wang Y, Lu N, Matzuk MM. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat Genet* 1997;15:201–204.
43. Matzuk MM, Kumar TR, Bradley A.Different phenotypes for mice deficient in either activins or activin receptor type II. *Nature* 1995;374:356– 360.
44. Vasen G, Battistone MA, Croci DO, Brukman NG, Weigel Muñoz M, Stupirski JC, Rabinovich GA, Cuasnicú PS. The galectin-1-glycan axis controls sperm fertilizing capacity by regulating sperm motility and membrane hyperpolarization. *FASEB J* 2015;29(10):4189-200.

45. Brukman NG, Miyata H, Torres P, Lombardo D, Caramelo JJ, Ikawa M, Da Ros VG, Cuasnicú PS. Fertilization defects in sperm from Cysteine-rich secretory protein 2 (Crisp2) knock-out mice: implications for fertility disorders. *Mol Hum Reprod* 2016;22(4):240-51.

Figure Legends

Figure 1: Weight of the testis and epididymis and hormone levels

Size (A) and weight (B) of the testis and epididymis of SP-D$^{-/-}$ and WT mice. Both the size and weight of testis and epididymis are significantly smaller, with less convoluted cauda in SP-D$^{-/-}$ mice. Bar graphs representing (C) Serum and (D) Intra-testicular levels of testosterone and estradiol in WT and SP-D$^{-/-}$ mice assayed using specific ELISA kits. Significantly reduced serum levels of testosterone while no significant difference in the intra-testicular levels of both hormones was found in SP-D$^{-/-}$ mice. Data represents the mean of five independent determinations; error bar represents the S.D. *, $p < 0.05$.

Figure 2: Sperm motility parameters in the LPS treated SP-D$^{-/-}$ and WT mice. Assessment of motility by CASA revealed no significant difference in the (A) total and (B) progressive motility between the sperm suspensions from SP-D$^{-/-}$ and WT mice in both control and LPS treated groups. (C) Significantly reduced sperm count in SP-D$^{-/-}$ mice upon LPS challenge. (D) Comparison of velocity distribution between sperm suspension from SP-D$^{-/-}$ and WT mice in control and LPS treated groups revealed no significant differences. Data represents the mean of five independent experiments; error bar represents the S.D. *, $p < 0.05$.

Figure 3: Reduced degree of protein tyrosine phosphorylation in capacitated sperm from SP-D$^{-/-}$ mice. (A) Sperm suspensions from SP-D$^{-/-}$ and WT mice were incubated *in vitro* in capacitation media for 0, 15, 60 and 90 min respectively and protein tyrosine phosphorylation was assessed using anti-mouse pJAK-2 antibody by western blotting. (B) Densitometric analysis

showing significantly reduced degree of protein tyrosine phosphorylation in SP-D$^{-/-}$ mice relative to WT mice. α- tubulin was used as an internal loading control. The data are representative of 5 independent experiments; error bar represents the S.D. *, $p < 0.05$, **, $p < 0.001$.

Figure 4: Altered immune milieu in the testis of SP-D$^{-/-}$ mice.

(A- H) Levels of cytokines and immunosuppressive molecules were assayed in the testis of SP-D$^{-/-}$ and WT mice using real time RT-PCR, western blot and multiplex cytokine assay. Results revealed significantly different immune milieu in the testis of SP-D$^{-/-}$ mice with a pre-dominance of immunosuppressive molecules. 18s and β- actin were used as internal loading controls for transcript and immunoblot analysis, respectively. Data represents the mean of five independent experiments, error bar represents the S.D. *, $p < 0.05$, **$p < 0.001$.

Figure 5: Reduced activation status of immune cells in the testis of SP-D$^{-/-}$ mice.

(A) Bar graph summarizing the percentage of immune cells in the testis of SP-D$^{-/-}$ and WT mice. Data represents the mean of ten independent experiments, error bar represents the S.D. No significant difference in the number of intra-testicular immune cells was observed between the two genotypes. Real time RT- PCR (B), and western blot analysis (D) and (E) was carried out to evaluate the intra-testicular levels of immune cell activation markers in SP-D$^{-/-}$ and WT mice. The testis of SP-D$^{-/-}$ mice showed significantly reduced levels of CD86 and IL-2. (C) Transcript level analysis of M1 and M2 macrophage marker viz., ITGAX and MRC1 respectively revealed significantly reduced levels of both markers in the testis from SP-D$^{-/-}$ than WT mice. 18s and β-actin were used as internal loading controls for transcript and western blot analysis respectively.

Data represents the mean of five independent experiments; error bar represents the S.D. **, $p < 0.001$.

Figure 6: Blunted inflammatory response to LPS challenge in the testis of SP-D$^{-/-}$ mice. (A - H) Multiplex cytokine analysis of whole testicular protein lysates was carried out from SP-D$^{-/-}$ and WT mice injected with high (5 mg/ kg) or low dose (0.1 mg/ kg) of LPS, or saline for 6 h and 24 h. The testis of SP-D$^{-/-}$ mice showed a blunted response to LPS induced up-regulation of pro-inflammatory cytokines. (H6- high dose LPS treated for 6 h, H24- high dose LPS treated for 24 h, L6- low dose LPS treated for 6 h and L24- low dose LPS treated for 24 h). Bar graphs represent the mean of 6 values for each time point and dose of LPS; error bar represents S.D. *, $p < 0.05$, **, $p < 0.001$.

Figure 7: Significant increase in the levels of estradiol upon LPS challenge.

Levels of (A) Testosterone and (B) Estradiol were assessed in the testis of SP-D$^{-/-}$ and WT mice 6 h and 24 h following LPS challenge by hormone specific ELISA kits. No significant differences in the levels of testosterone (A) were observed in control and treatment group between the two genotypes. However, LPS treatment resulted in a significant increase in the levels of estradiol (B) in the testis of both WT and SP-D$^{-/-}$ mice. This increase was significantly higher in SP-D$^{-/-}$ mice than WT mice. Data represents the mean of five independent experiments; error bar represents the S.D. *, $p < 0.05$.

Figure 8: Protective effect of exogenous rhSP-D on LPS induced inflammation in testis and sperm motility.

High dose LPS challenged WT mice were treated with exogenous rhSP-D for 24 h and the levels of intra-testicular cytokines were assayed by real time RT-PCR, while the sperm motility was assessed by CASA. (A) Comparing the levels of cytokines in rhSP-D treated LPS challenged mice to that of LPS alone challenged mice showed a predominance of anti-inflammatory milieu in rhSP-D treated group. (B) Significant decrease in the percentage of total sperm motility and (C) increased percentage of static sperm in LPS challenged mice compared to the saline control group. Treatment with rhSP-D restored the total motility of sperm and reduced the percentage of static sperm that was hampered by LPS challenge to control levels. Data are representative of 5 independent experiments; error bar represents the S.D. *, $p < 0.05$.

Figure S1: Genotyping of SP-D$^{-/-}$ mice.

Representative agarose gel image showing PCR products amplified using SP-D (exon 2) and Neo gene specific primer pairs. Genomic DNA from SP-D$^{-/-}$ and WT mice was subjected to PCR and (A) SP-D gene specific amplicon (204 bp) was obtained only in WT mice while (B) Neo gene specific PCR amplification product (249 bp) was seen in the DNA sample from SP-D$^{-/-}$ mice. This confirmed the presence of normal and recombinant SP-D gene in WT and SP-D$^{-/-}$ mice respectively.

Figure S2: Morphology of the testis and epididymis.

Histology of the testis (a, b), cauda epididymis (c, d) and initial segment of epididymis (e, f) from 8 week old WT and SP-D$^{-/-}$ mice respectively showing normal architecture in SP-D$^{-/-}$ mice compared to the WT, (scale bar = 100 μm). Insets show higher magnification images (scale bar = 20 μm). Data are representative of five independent determinations.

Figure S3: Percentage of intra-testicular immune cells

Flow cytometric analysis was carried out to enumerate the percentage of (A) $F4/80^+$ macrophages, (B) $CD11c^+$ DC cells, (C) $CD4^+$ lymphocytes and (D) $CD8^+$ lymphocytes in the interstitial spaces of the testis from SP-$D^{-/-}$ and WT mice. Black line represents the fluorescence for stained population, while gray line represents the isotype control antibody fluorescence. Data are representative of mean ± S.D. of ten independent experiments.

Figure S4: Protective effect of exogenous rhSP-D supplementation on testicular morphology in LPS challenged WT mice.

The histological analysis of testis from (a) control, (b) LPS challenged and (c) LPS challenged group treated with exogenous rhSP-D. (a) Testicular section from saline control group showing normal architecture. (b) Morphology of the testis from LPS challenged group revealed vacuolization (yellow arrow-heads) and intercellular gaps (black arrow) in seminiferous epithelium due to disruption of cell-cell contact. (c) The testis from rhSP-D treated LPS challenged mice shows protective effect of rhSP-D on inflammation induced alterations in morphological features of the testis. (Scale bar = 100 μm). Insets represent higher magnification images (scale bar = 20 μm). Data are representative of five independent determinations.

Figure S5: Effect of exogenous rhSP-D treatment on transcript levels of cytokines in LPS induced inflammation in testis.

Analysis of transcript levels of cytokines viz., (A) TGF-β1, (B) IL-10 and (C) TNF-α revealed that the LPS treatment resulted in a pro-inflammatory response by significant down-regulation of

TGF- β1 levels with significant increase in the levels of TNF- α compared to the saline control group. Treatment with exogenous rhSP-D in LPS challenged mice significantly increased the levels of all three cytokines compared to the control and LPS challenged group with a predominance of anti-inflammatory cytokines. Data are representative of 5 independent experiments, error bar represents the S.D. *, $p < 0.05$.

925 **Table 1 List of murine primer pairs used**

Gene Target	Forward Primer (5' → 3')	Reverse Primer (5' → 3')	Amplicon Size (bp)
SP-D	GCAGGACATGCTGCCCTTTCT	ACCCTTCTCACCCCGTGGACC	204
Neo	TTCGGCTATGACTGGGCACAACAG	TACTTTCTCGGCAGGAGCAAGGTG	249
CD274	TGCTGCCCTTCAGATCACAG	TTCTGGATAACCCTCGGCCT	148
Serpina3	AACATGCCAGGCAAGCGGCA	AGCAGGGCAGATCCCAGCCAT	150
IDO	GCAGGCCAGAGCAGCATCTTCC	CGGTGGGCTGGAGGCATGTA	122
FasL	GGTTCTGGTGGCTCTGGTTGGA	GGGTGTACTGGGGTTGGCTATTTGC	146
TGF-β1	GCGTGCTAATGGTGGACCGCA	GCACGGGACAGCAATGGGGG	130
IL-10	GCACCCACTTCCCAGTCGGC	GGCTTGGCAACCCAAGTAACCCT	158
Activin-A	GGCTCAGGACATCACCCAGGTCC	CTCAAAGAGGAGGGCTGGGGCT	140
Activin-B	CCATCACAGAGGCCATCCAG	CAGTCGTTCCAGCCGATGAG	271
Inhibin-A	TTGCCCAGGAGGCTGAGGAAG	GGGTGGGTCAGCAGAGGGAAA	271
Inhibin-B	GGGTCCGCCTGTACTTCTTC	CCTGGATGGCCTCTGTGATG	243
KC	GGTGAGGACATGTGTGGGAG	CGAGACCAGGAGAAACAGGG	185
TNF-α	GGGCCACCACGCTCTTCTGTCT	GATCCATGCCGTTGGCCAGGAG	237
IL-1α	GCGCTCAAGGAGAAGACCAGCC	GTGCCAGGTGCACCCGACTTT	186
MIF	ATCGCCTGCACATCAGCCCG	TCTCCCGGCTGGAAGGTGGG	193
IL-6	AGACAAAGCCAGAGTCCTTCAGAGA	GCCACTCCTTCTGTGACTCCAGC	146
MMP-2	CAGGGCACCTCCTACAACAG	CCACTTCCGGTCATCATCGT	270
MMP-9	TTTGAGGGCCGCTCCTACTC	GATGTGGTCGCACACCAGAG	251
MMP-12	GCTGTCACAACAGTGGGAGA	GCAGAGAAGCCCAGGGAATA	257
SP-A	ACCTGGATGAGGAGCTTCAGACTC	TGCTTGCGATGGCCTCGTTCT	225

MBL-A	ACCAGGTCAAGGGCTCAGGGG	TGCCAGCTTCTCCTCAATGGCTC	182
CD40L	TTGCAGCACACGTTGTAAGC	TGAATGGGCGTTGACTCGAA	204
MHC II	AGACACCCAGGGCCTTTATG	AAAGCAAGTTGGGGGTCACT	154
CD86	ACGGAAGCACCCACGATGGA	CCTGTCAAAGCTCGTGCGGC	285
IL-2	TCCTTGTCAACAGCGCACCC	CATGCCGCAGAGGTCCAAGT	261
MRC1	TTTGCCTTTCCCAGTCTCCC	CCTCGCGTCCAATAGCTGAA	122
ITGAX	CTCACCCAGCCAGAGGATTT	CAGTGGCATCACTCAGTGTCT	263
18s	GGAGAGGGAGCCTGAGAAAC	CCTCCAATGGATCCTCGTTA	175

926 The primers for above listed genes were synthesized using NCBI Primer Blast software.

927

928

929

930

931

932

933

934

935

936

937

938

939

940

941

Table 2 Sperm motility parameters

Sperm Parameters		VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	BCF (Hz)	STR (%)	LIN (%)
Control	WT	278.9 ± 49.8	188.5 ± 24.3	523.2 ± 99.7	19.6 ± 2.2	20.1 ± 2.9	66 ± 3.5	37 ± 2.7
	SP-D$^{-/-}$	275.1 ± 106.8	199.2 ± 78.2	431.6 ± 199.5	13.8 ± 6.7	15.8 ± 2.5*	69.7 ± 0.6	47 ± 3.6*
LPS treated	WT	294.58 ± 17.41	205.92 ± 18.93	535.10 ± 46.83	20.72 ± 1.61	20.94 ± 4.46	67.60 ± 3.44	38.40 ± 1.95
	SP-D$^{-/-}$	265.13 ± 14.58*	178.17 ± 17.50*	425.93 ± 19.15*	16.2 ± 3.55*	17.2 ± 3.59	66.33 ± 7.23	43 ± 4.58

Various motility parameters of caudal sperm suspension from saline control and LPS treated WT and SP-D$^{-/-}$ mice were analyzed by CASA. VAP (Average path velocity), VSL (straight line velocity), VCL (curvilinear velocity), ALH (lateral amplitude), BCF (beat frequency), STR (straightness), LIN (linearity). Data are expressed as mean ± SD, n = 5 per group, *$p < 0.05$.

Table 3 Fertility of SP-D$^{-/-}$ male mice

Genotype	Pregnancy (%)	Litter Size (No. of pups)	Litter weight (gm)	Time to delivery (days)
WT	100.0	8.4 ± 0.5	13.0 ± 1.0	26.4 ± 3.9
SP-D$^{-/-}$	100.0	8.7 ± 1.1	13.1 ± 1.4	23.6 ± 2.3

Wild type (WT) or knockout (SP-D$^{-/-}$) males were caged with WT females for 2 months. The percentage of pregnant females, litter size, litter weight and the time to delivery from the day animals were kept for mating were recorded. Data are presented as mean ± SD, n = 5; n.s. (not significant).

Figure 1

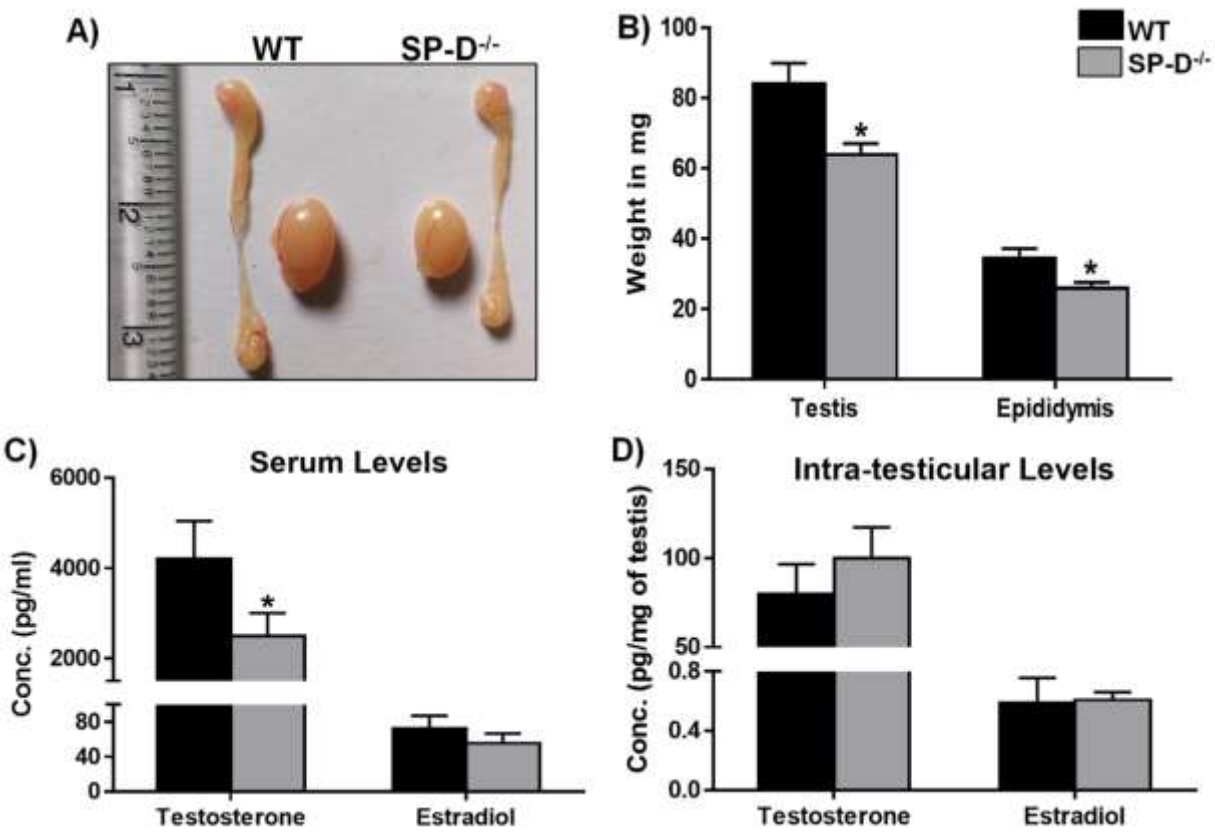
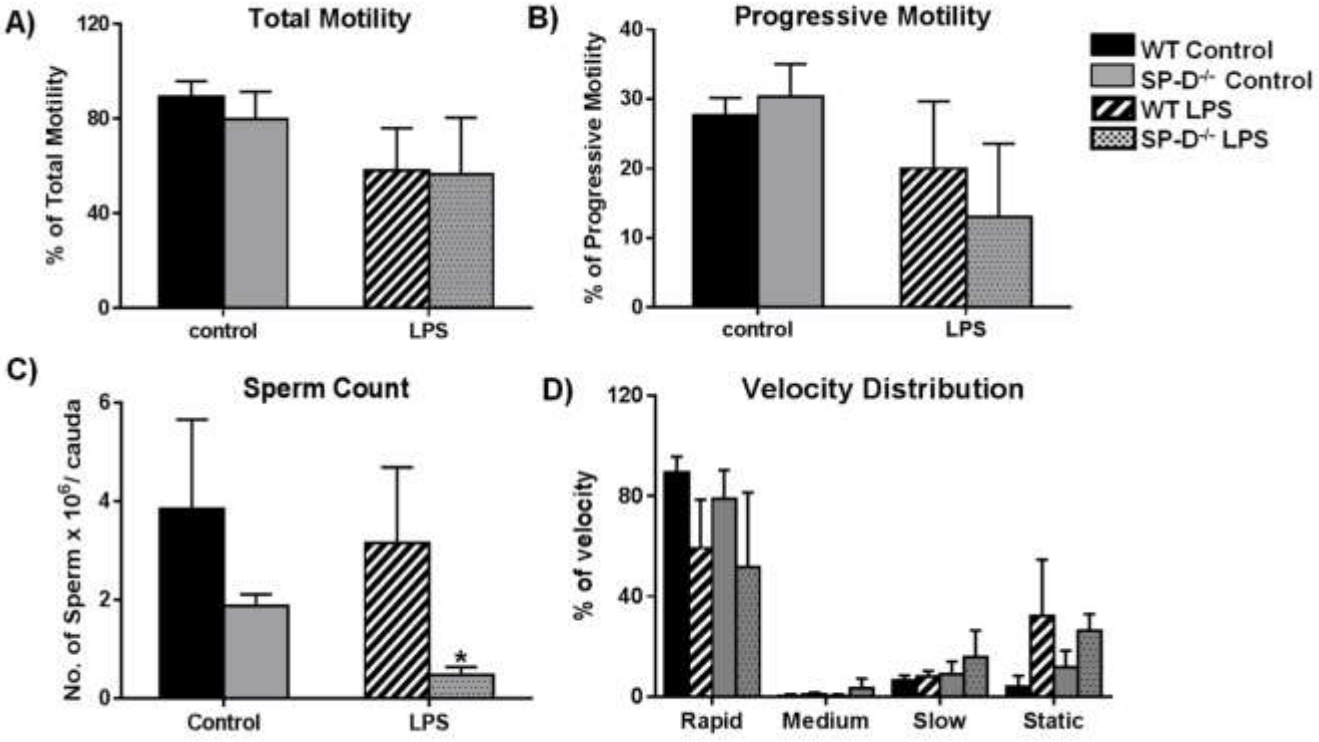

A)
WT
SP-D-/-
B)
WT
SP-D-/-
Weight in mg
100
80
60
40
20
0
*
*
Testis
Epididymis
C)
Serum Levels
Conc. (pg/ml)
6000
4000
2000
80
40
0
*
Testosterone
Estradiol
D)
Intra-testicular Levels
Conc. (pg/mg of testis)
150
100
50
0.8
0.4
0.0
Testosterone
Estradiol

Figure 2



985

986

987

988

989

990

991

992

993

994

995

996

997

Figure 3

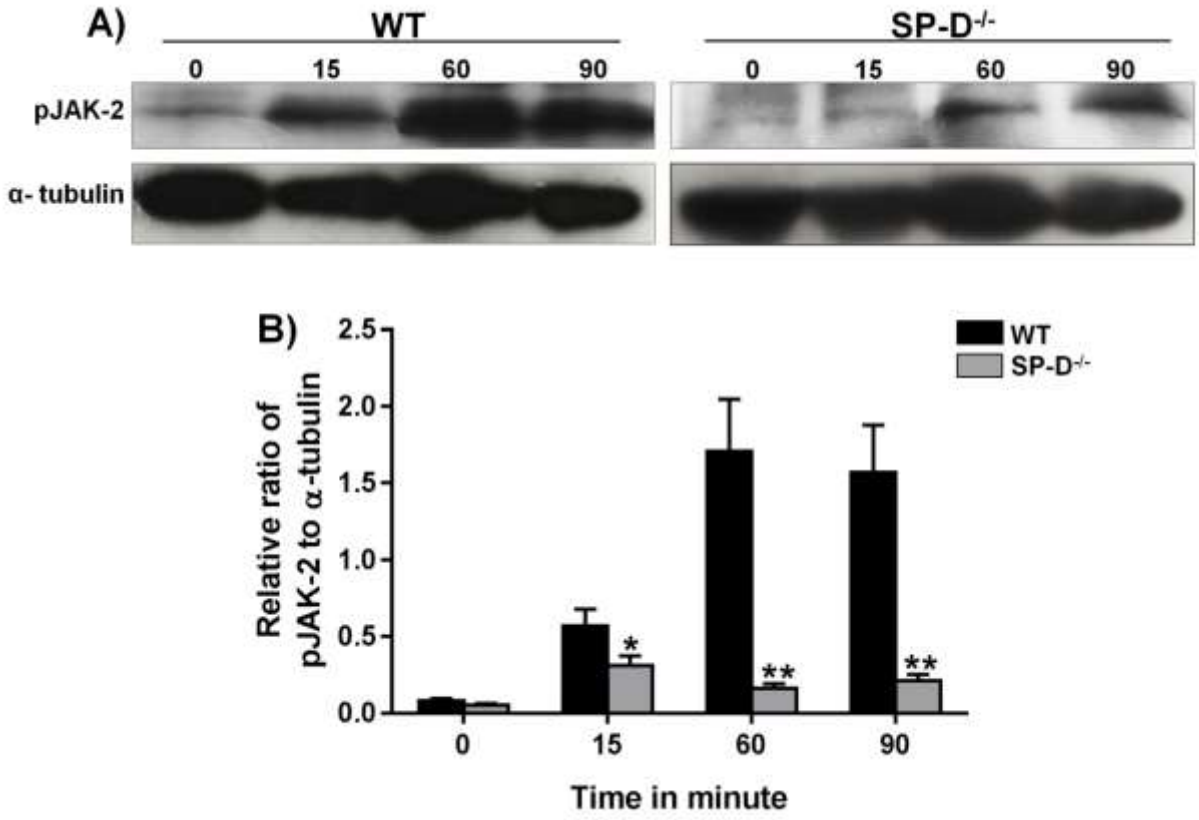
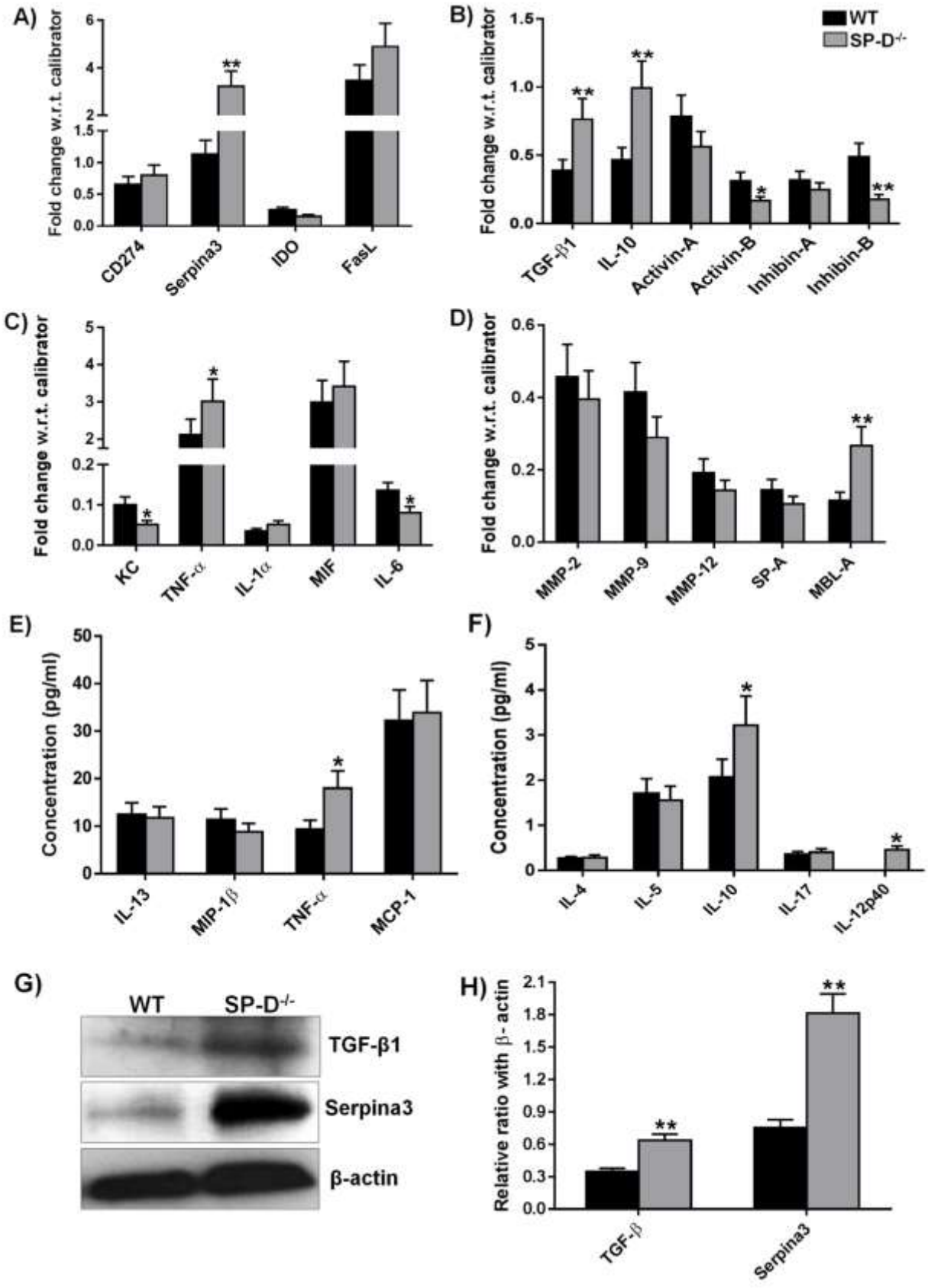

A)
WT
SP-D-/-
0
15
60
90
pJAK-2
α- tubulin
B)
Relative ratio of pJAK-2 to α-tubulin
2.5
2.0
1.5
1.0
0.5
0.0
*
**
**
WT
SP-D-/-
Time in minute

Figure 4



1011

Figure 5

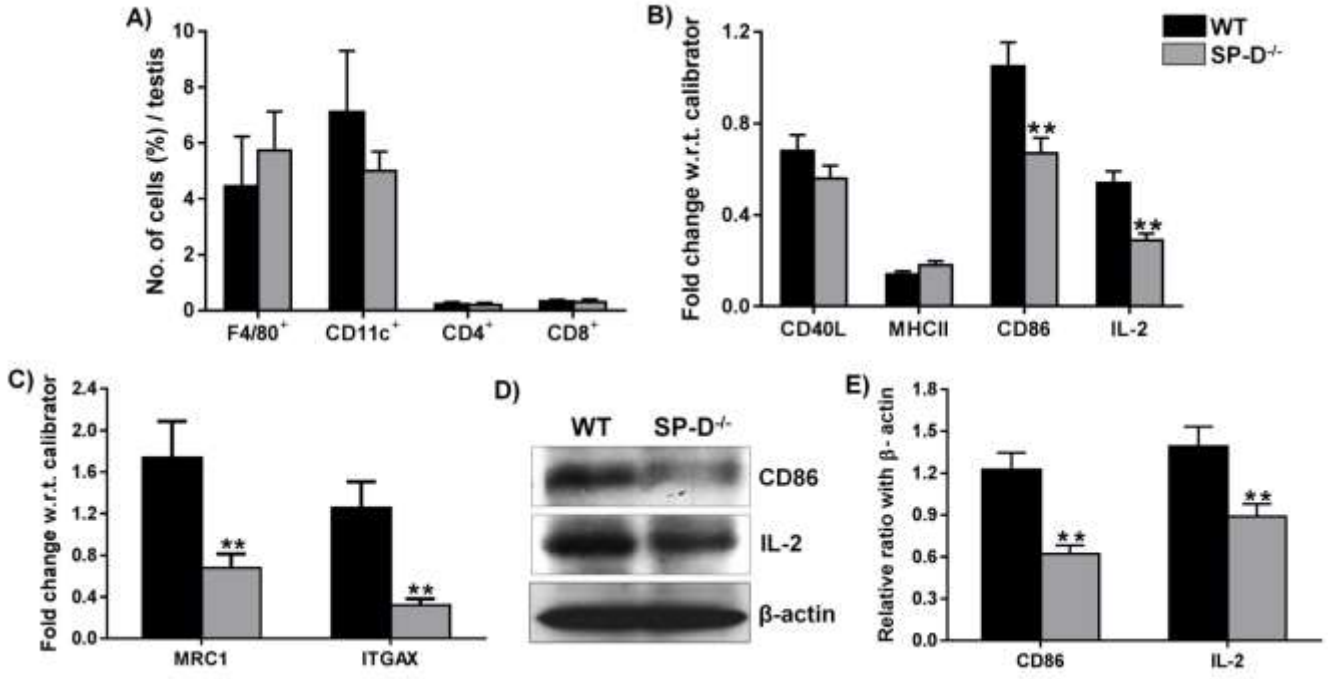
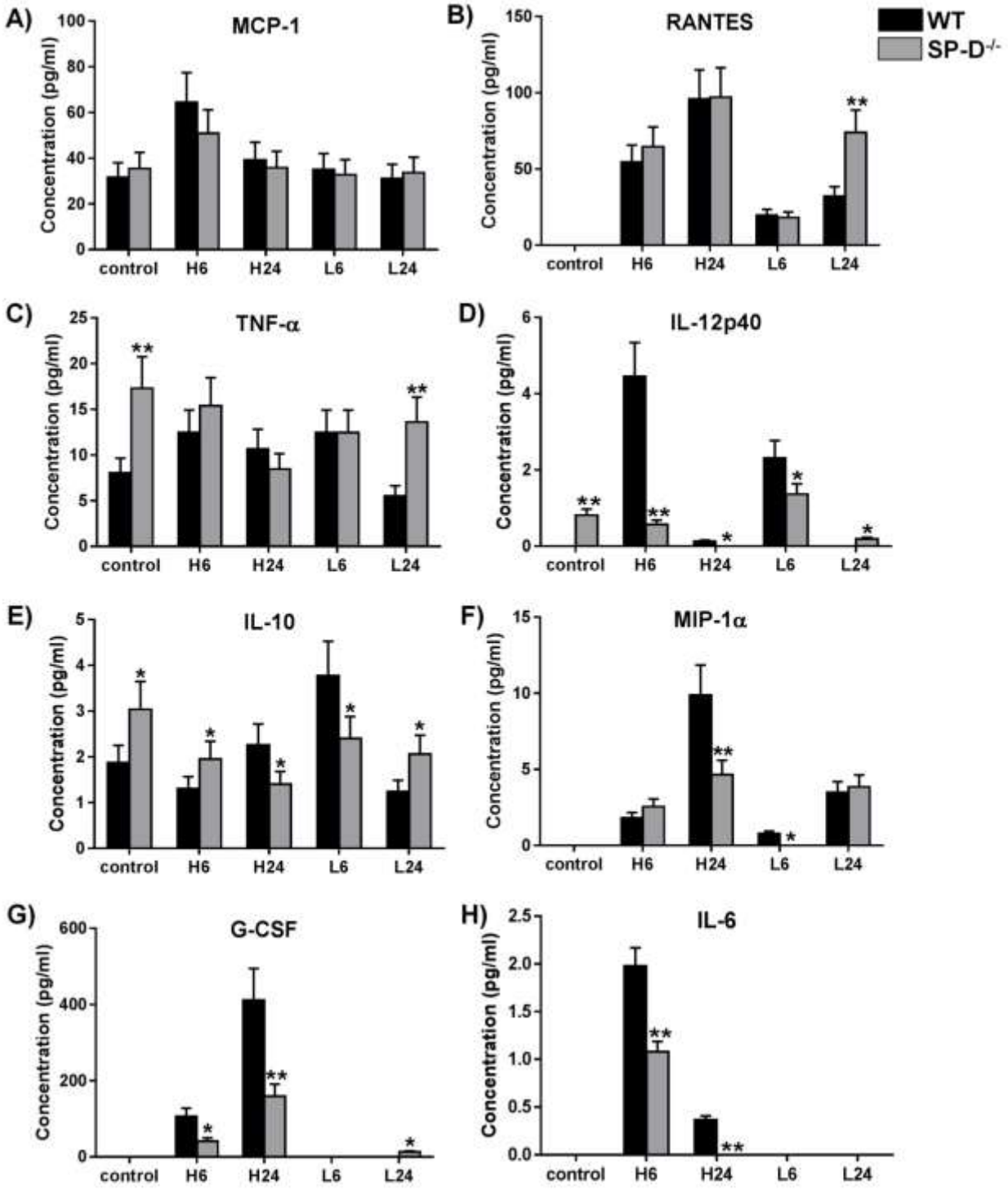

A)
No. of cells (%) / testis
F4/80+
CD11c+
CD4+
CD8+
B)
Fold change w.r.t. calibrator
WT
SP-D-/-
CD40L
MHCII
CD86
IL-2
C)
Fold change w.r.t. calibrator
MRC1
ITGAX
D)
WT
SP-D-/-
CD86
IL-2
β-actin
E)
Relative ratio with β- actin
CD86
IL-2

Figure 6



1028

1029

1030

1031

Figure 7

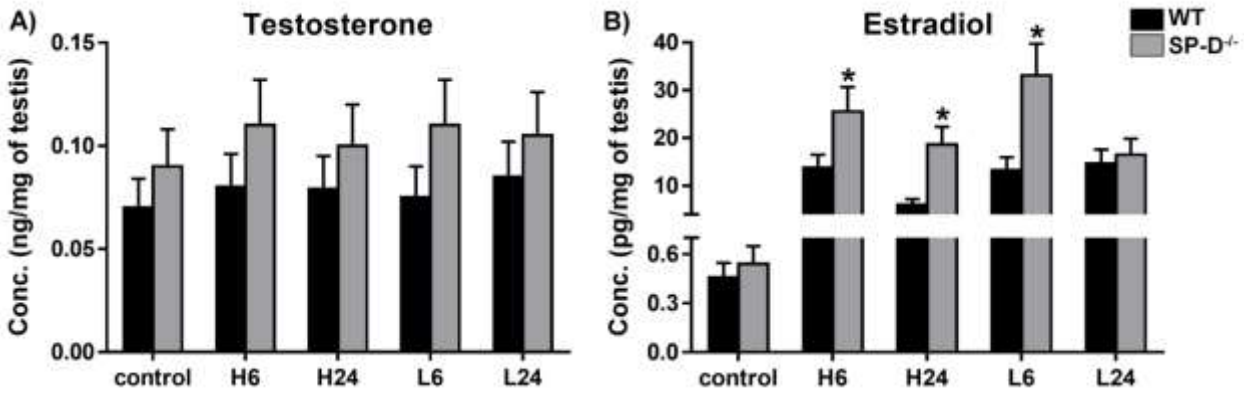


Figure 8

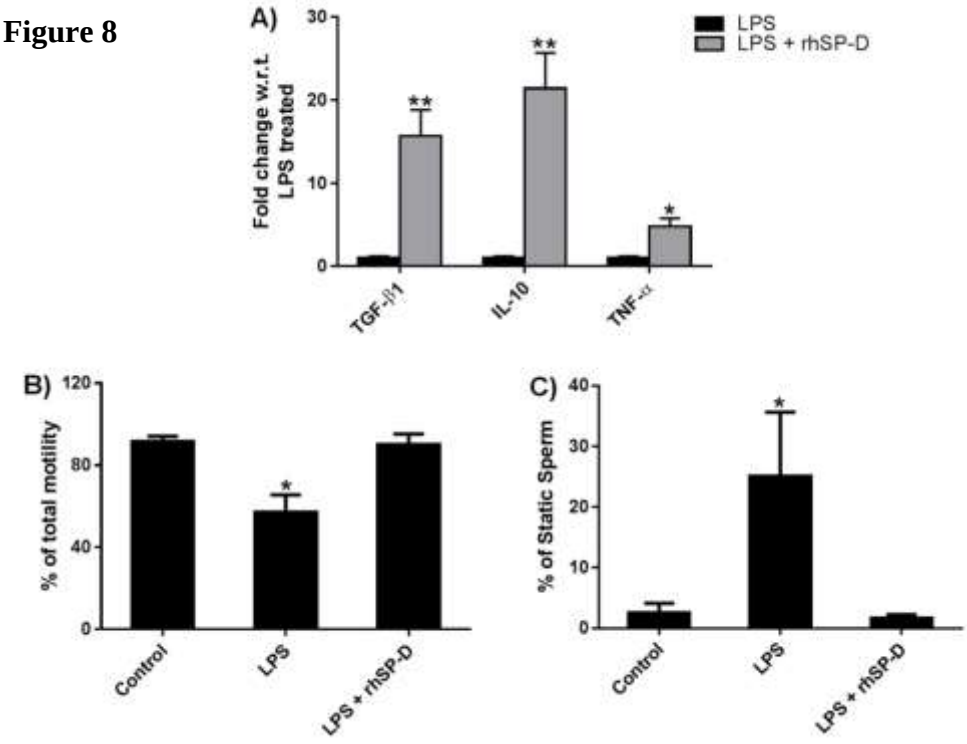


Figure S1

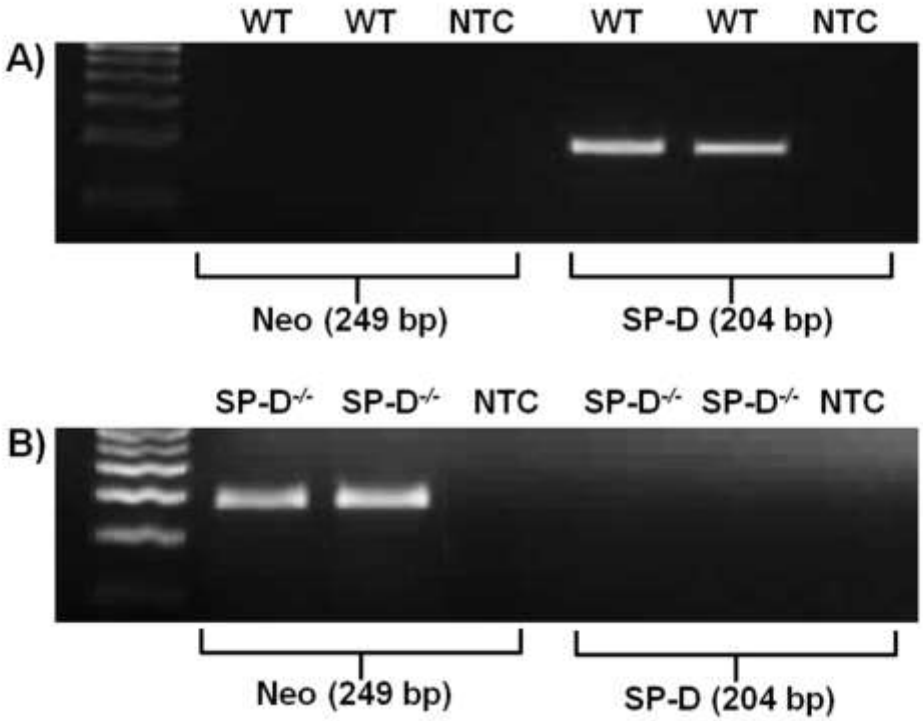

WT
WT
NTC
WT
WT
NTC
A)
Neo (249 bp)
SP-D (204 bp)
SP-D-/-
SP-D-/-
NTC
SP-D-/-
SP-D-/-
NTC
B)
Neo (249 bp)
SP-D (204 bp)

Figure S2

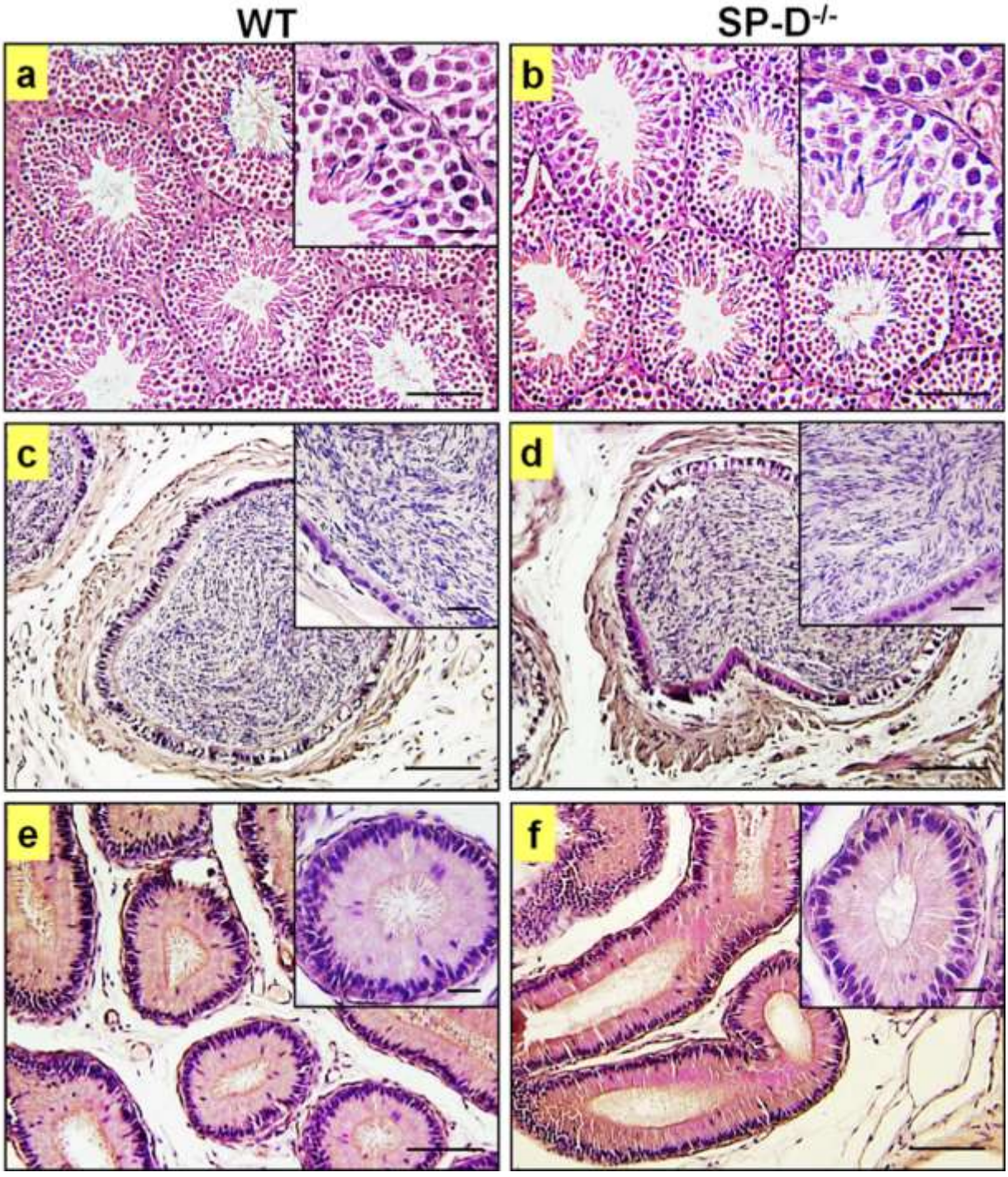


Figure S3

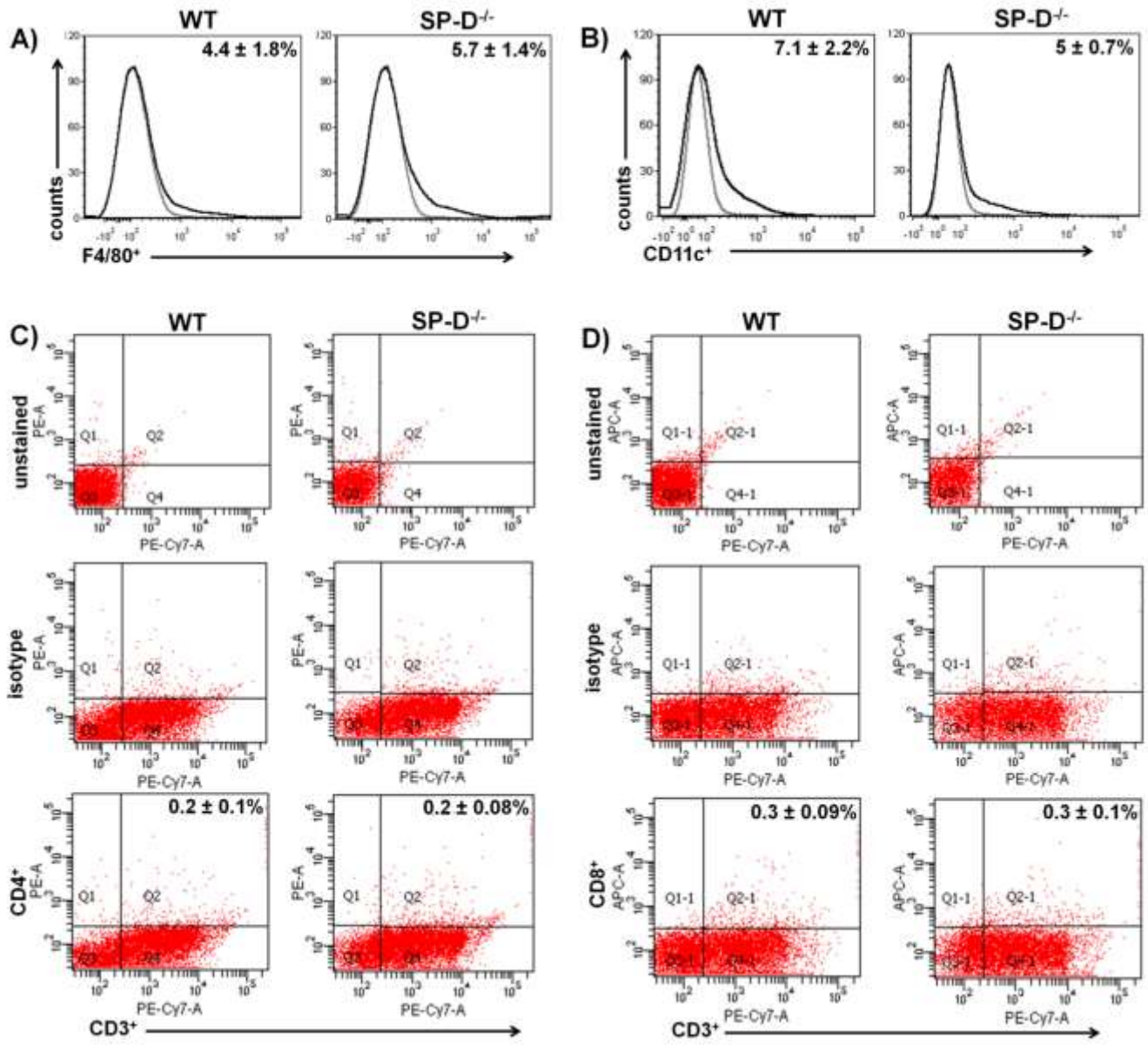
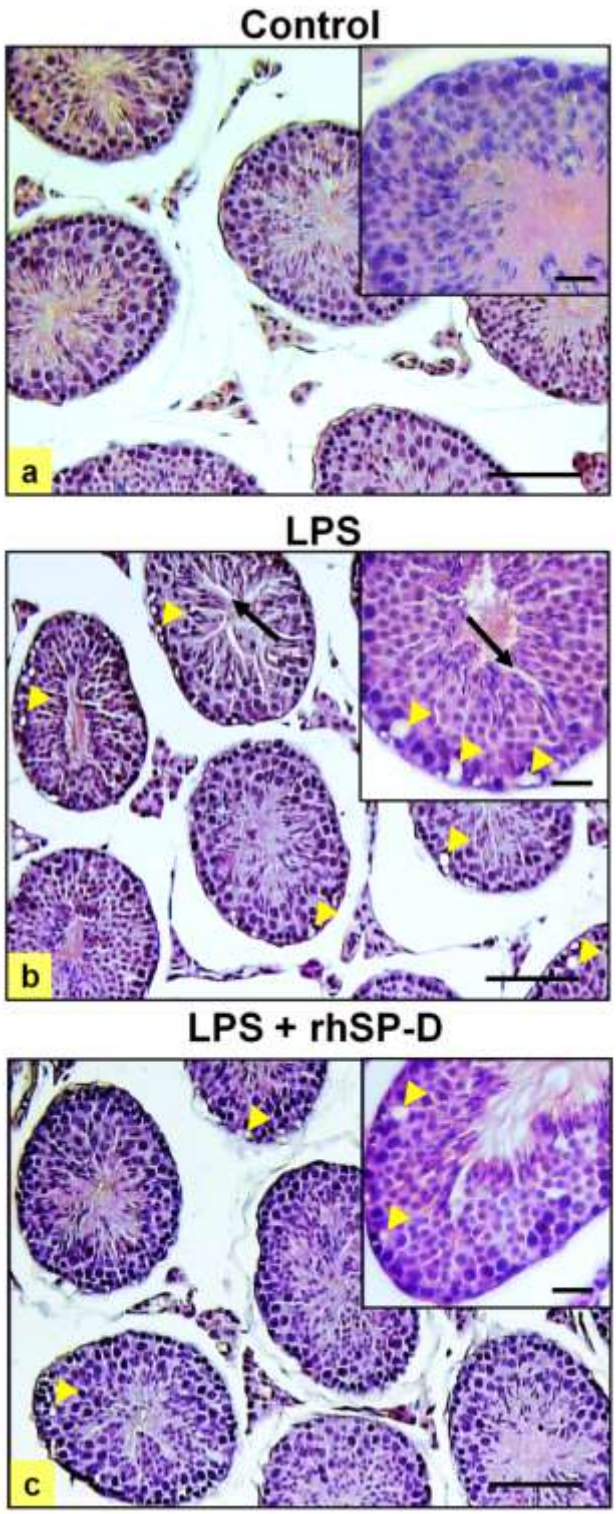

A)
WT
SP-D-/-
4.4 ± 1.8%
5.7 ± 1.4%
counts
F4/80+
B)
WT
SP-D-/-
7.1 ± 2.2%
5 ± 0.7%
counts
CD11c+
C)
WT
SP-D-/-
unstained
isotype
CD4+
0.2 ± 0.1%
0.2 ± 0.08%
PE-A
PE-Cy7-A
Q1
Q2
Q3
Q4
CD3+
D)
WT
SP-D-/-
unstained
isotype
CD8+
0.3 ± 0.09%
0.3 ± 0.1%
APC-A
PE-Cy7-A
Q1-1
Q2-1
Q3-1
Q4-1
CD3+

Figure S4

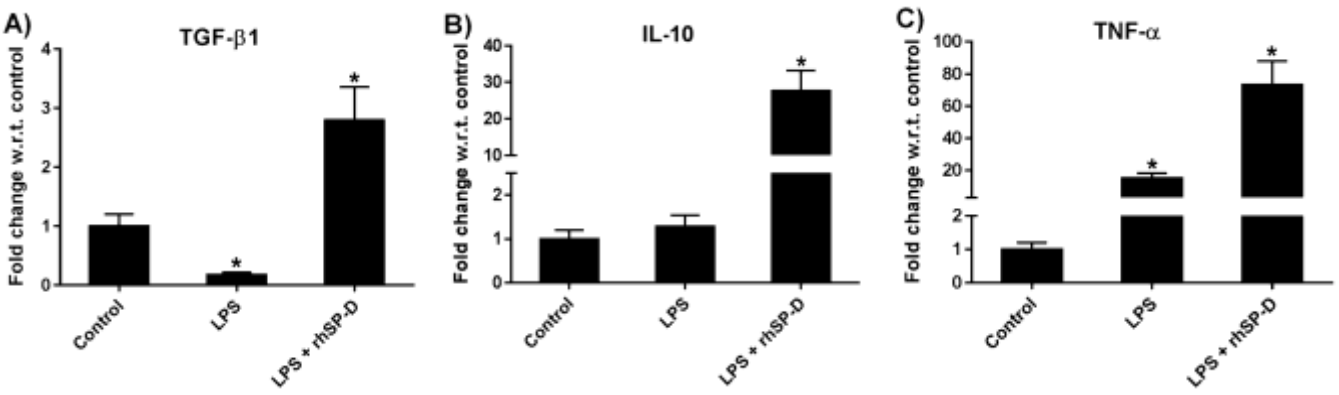


1067

1068

1069

Figure S5



1070