UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

Instituto de Biociências de Botucatu - Unesp

Universidade Estadual Paulista "Júlio de Mesquita Filho"
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Programa de Pós-Graduação em Farmacologia e Biotecnologia

Alexandre Dorth de Andrade

Regulação da expressão de genes da família Wfdc (*Whey-acidic protein four disufide core*) por estímulo inflamatório no epidídimo de camundongos: potenciais funções imunológicas e reprodutivas

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Dissertação de Mestrado apresentada ao Programa de Pós-graduação em Farmacologia e Biotecnologia do Instituto de Biociências de Botucatu
Candidato: Alexandre Dorth de Andrade

Orientador: Prof. Dr. Erick José Ramo da Silva

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RESUMO

O epidídimo é responsável por transformar os espermatozoides imaturos em células funcionalmente competentes, capazes de adquirir motilidade progressiva e de reconhecer e fertilizar o oócito. Além disso, o epidídimo é primordial para proteger e armazenar o gameta masculino antes da ejaculação. Alterações da fisiologia epididimária podem afetar a sua função, causando infertilidade masculina. A inflamação do epidídimo, conhecida como epididimite, é uma das doenças mais prevalentes do trato urogenital masculino, sendo um fator relevante de infertilidade masculina. Sua principal etiologia envolve a invasão de bactérias, como *Escherichia coli*, via ascensão retrógrada pela uretra. O epidídimo expressa constitutivamente diversos componentes do sistema imunológico inato, incluindo os receptores do tipo Toll (TLRs), como o TLR4, que é ativado pelo lipopolissacarídeo (LPS) de bactérias Gram-negativas, além de proteínas com atividade antimicrobiana, como os inibidores da protease do tipo WFDC (*Whey-acidic protein four disufide core*), indicando que este órgão está em constante estado de alerta para combater infecções bacterianas. Além da ação antimicrobiana e inibidora de protease, as proteínas WFDC apresentam ação anti-inflamatória, imunomoduladora e sobre a função espermática, sendo proteínas multifuncionais no trato reprodutor masculino. Neste estudo, testamos a hipótese de que a expressão de genes *Wfdc* no epidídimo é modificada por estímulos inflamatórios, como parte da resposta tecidual à agressão e de mecanismos celulares de defesa. Para tal, caracterizamos dois modelos de indução de epididimite em camundongos induzida pelo LPS ultrapuro de *E. coli* via interstício do segmento inicial e ducto deferente, provocando assim, eventos inflamatórios nas regiões proximal e distal do epidídimo. Processamos o segmento inicial, para o primeiro modelo de epididimite, e a cauda do epidídimo, para o segundo modelo de epididimite, para investigação dos efeitos do LPS sobre a expressão de citocinas, por ensaios de RT-qPCR e multiplex, e para avaliação histopatológica. Nossos resultados mostram que o epidídimo responde rapidamente ao estimulo inflamatório, mas com padrão e intensidade diferentes para cada região do órgão, sendo o segmento inicial mais sensível ao LPS do que a cauda do epidídimo quanto ao aumento da expressão de citocinas pró-inflamatórias (IL1B, IL6, TNF e IFNG). Para a investigação do perfil de expressão dos genes *Wfdc* processamos o segmento inicial e a cauda do epidídimo para os ensaios de RT-PCR e qPCR. Nossos resultados mostram que a expressão dos genes *Wfdc* é alterada frente ao desafio infamatório nas diferentes regiões do epidídimo, e que sua expressão apresenta perfis diferentes para cada região estimulada. Observamos regulação negativa dos níveis de *Wfdc6a_v2, Wfdc7, Wfdc8, Wfdc11, Wfdc15b_v1-2* e *Wfdc16* no segmento inicial 6 h após a injeção intersticial de LPS, e de *Wfdc6a_v1*, *Wfdc6a_v2*, *Wfdc6b*, *Wfdc11*, *Wfdc15b_v1_v2* e *Wfdc16* na cauda do epidídimo 24 ou 72 h após a injeção intraductal de LPS. Por outro lado, observamos regulação

positiva da expressão dos transcritos *Wfdc2, Wfdc3, Wfdc4, Wfdc5, Wfdc6b, Wfdc10, Wfdc12, Wfdc15a* e *Wfdc15b_v1-2* no segmento inicial 24 e/ou 72 h após a injeção intersticial de LPS, enquanto apenas os níveis de *Wfdc2* e *Wfdc5* foram aumentados na cauda do epidídimo após a injeção intraductal de LPS no mesmo período. O tratamento dos animais como inibidor do fator de transcrição NFKB PDTC (100 mg/kg, i.p.) 1 h antes da indução da epididimite pelo LPS revelou que o aumento da expressão de alguns transcritos *Wfdc*, incluindo *Wfdc2*, *Wfdc6b* e *Wfdc10* no segmento inicial e *Wfdc5*, na cauda do epidídimo foram dependentes da ativação deste fator de transcrição. Em conjunto, nossos resultados fornecem novas informações sobre como o epidídimo expressa os genes *Wfdc* frente ao desafio inflamatório e destacam os possíveis papeis da família *Wfdc* no combate da inflamação e manutenção da homeostase do epidídimo. Nossos resultados sugerem que proteínas WFDC podem ser exploradas como alvos terapêuticos adjuvantes para o tratamento da epididimite.

ABSTRACT

The epididymis is responsible for transforming immature sperm into functionally competent cells capable of acquiring progressive motility and recognizing and fertilizing the oocyte. In addition, the epididymis is paramount for protecting and storing the male gamete before ejaculation. Changes in epididymal physiology may affect sperm maturation, transport or storage, causing male infertility. Epididymal inflammation, known as epididymitis, is one of the most prevalent diseases of the male urogenital tract, being a relevant factor of male infertility. Its main etiology involves the invasion of bacteria, such as *E. coli*, through retrograde ascension through the urethra. The epididymis constitutively expresses several elements of the innate immune system, including Toll-like receptors (TLRs), like the TLR4, which is activated by the lipopolysaccharide (LPS) of Gram-negative bacteria, as well as proteins with antimicrobial activity, like the WFDC (Whey-acidic difulfide core)-type protease inhibitors, indicating that this organ is constantly alert to fight bacterial infections. In addition to antimicrobial and protease inhibitory activities, WFDC proteins show anti-inflammatory, immunomodulatory and reproductive functions, being multifunctional proteins in the male reproductive tract. In this study, we tested the hypothesis that *Wfdc* gene expression in the epididymis is modified by inflammatory stimuli as part of tissue defense mechanisms to microbial aggression. For that, we characterized two mouse models of epididymitis induction induced by ultrapure LPS from *E. coli* via interstitial of the initial segment and vas deferens, causing inflammatory events in the proximal and distal regions of the epididymis. We processed the initial segment, for the first epididymitis model, and cauda epididymis, for the second epididymitis model, to investigate the effects of LPS on cytokine expression by RT-qPCR and multiplex assays and for histopathological evaluation. Our results show that the epididymis rapidly responds to inflammatory stimuli, but with different patterns and intensity for each organ region, and the initial segment is more sensitive to LPS than the cauda epididymis to increased expression of pro-inflammatory cytokines (IL1B, IL6, TNF e IFNG). In order to investigate the expression profile of the *Wfdc* genes, we processed the initial segment and the cauda epididymis for the RT-PCR and qPCR assays. Our results show that the expression of *Wfdc* genes is modified by the inflammatory challenge in the epididymis in a region-specific fashion. We observed a negative regulation of the levels of *Wfdc6a_v2, Wfdc7, Wfdc8, Wfdc11, Wfdc15b_v1-2* and *Wfdc16* transcripts in the initial segment 6 h after interstitial LPS injection, and *Wfdc6a_v1*, *Wfdc6a_v2*, *Wfdc6b*, *Wfdc11*, *Wfdc15b_v1_v2* and *Wfdc16* in the cauda epididymis 24 or 72 h after intravasal LPS injection. On the other hand, we observed a positive regulation of *Wfdc2, Wfdc3, Wfdc4, Wfdc5, Wfdc6b, Wfdc10, Wfdc12, Wfdc15a* and *Wfdc15b_v1-2* transcripts in the initial segment 24 and/or 72 h after interstitial LPS injection, while only *Wfdc2* and *Wfdc5* transcripts were increased in the cauda epididymis after intravasal LPS injection at the same time-point. Treatment with PDTC (100 mg/kg, i.p.), an inhibitor of

NFKB transcription factor, 1 h before inducing epididymitis revealed that up-regulation of some Wfdc transcripts, including *Wfdc2, Wfdc6b* and *Wfdc10* in the initial segment and Wfdc5 in the cauda epididymis were dependent on the activation of this transcription factor. Altogether, our results provide new information on how *Wfdc* gene expression is modulated in the epididymis during acute inflammation and highlight the possible roles of the *Wfdc* family in combating inflammation and restoring epididymis homeostasis. Our results suggest that WFDC proteins may be exploited as adjuvant therapeutic targets for the treatment of epididymitis.

LISTA DE SIGLAS E ABREVIATURAS

DHT: diidrotestosterona

AR: receptor de androgênios

PRRs: pattern recognition receptors, do inglês

WFDC: whey-acidic protein four disulfide core, do inglês

TLRs: toll-like receptors, do inglês

PAMPs: pathogen-associated molecular patterns, do inglês

LPS: lipopolissacarídeo

LTA: ácido lipoteicoico

KDO: ácido 3-deoxi-D-mano-oct-2-ulosônico

LBP: proteína de ligação do LPS

CD14: cluster of differentiation 14, do inglês

MD2: myeloid differentiation protein 2, do inglês

MyD88: Myeloid differentiation protein 88, do inglês

NFKB: nuclear factor kappa B, do inglês

PDTC: ammonium pyrrolidinedithiocarbamate, do inglês

SUMÁRIO

Capítulo 1

INTRODUÇÃO

1. Epidídimo

A espermatogênese é um processo contínuo de divisão e diferenciação celular que leva à produção diária de milhões de espermatozoides. Em mamíferos, o espermatozoide testicular recém-liberado na luz do túbulo seminífero, no entanto, é ainda funcionalmente imaturo, imóvel e incapaz de reconhecer e fertilizar o oócito. Essas características essenciais para a reprodução são adquiridas no processo de maturação espermática, que ocorre durante passagem dos espermatozoides pelo epidídimo, órgão acessório do sistema genital masculino formado por um ducto longo e altamente enovelado, que liga os dúctulos eferentes ao ducto deferente (Hinton & Robaire, 2015). Além de promover a maturação do gameta masculino, o epidídimo também é essencial para o seu transporte, concentração, armazenamento e proteção previamente à ejaculação (Turner, 2008; Cornwall, 2009).

Anatomicamente, o epidídimo está localizado no escroto, aplicado à margem posterior do testículo e conecta os dúctulos eferentes ao ducto deferente. Na idade adulta, o ducto epididimário, quando desenovelado, atinge aproximadamente 6 m no homem, 3 m no rato e 1 m no camundongo (Hinton & Robaire, 2015). O epidídimo apresenta quatro compartimentos principais: 1) luz tubular, que contém os espermatozoides banhados pelo fluído epididimário; 2) epitélio do tipo colunar pseudoestratificado, composto por diferentes tipos celulares cuja abundância varia ao longo do órgão; 3) camada de músculo liso, que envolve o epitélio e atua no transporte dos espermatozoides ao longo do órgão e na sua emissão, durante a ejaculação; e 4) interstício, que contém fibras nervosas, vasos sanguíneos e linfáticos, além de fibroblastos e células imunológicas (Figura 1) (Robaire & Viger, 1995; Hinton & Robaire, 2015).

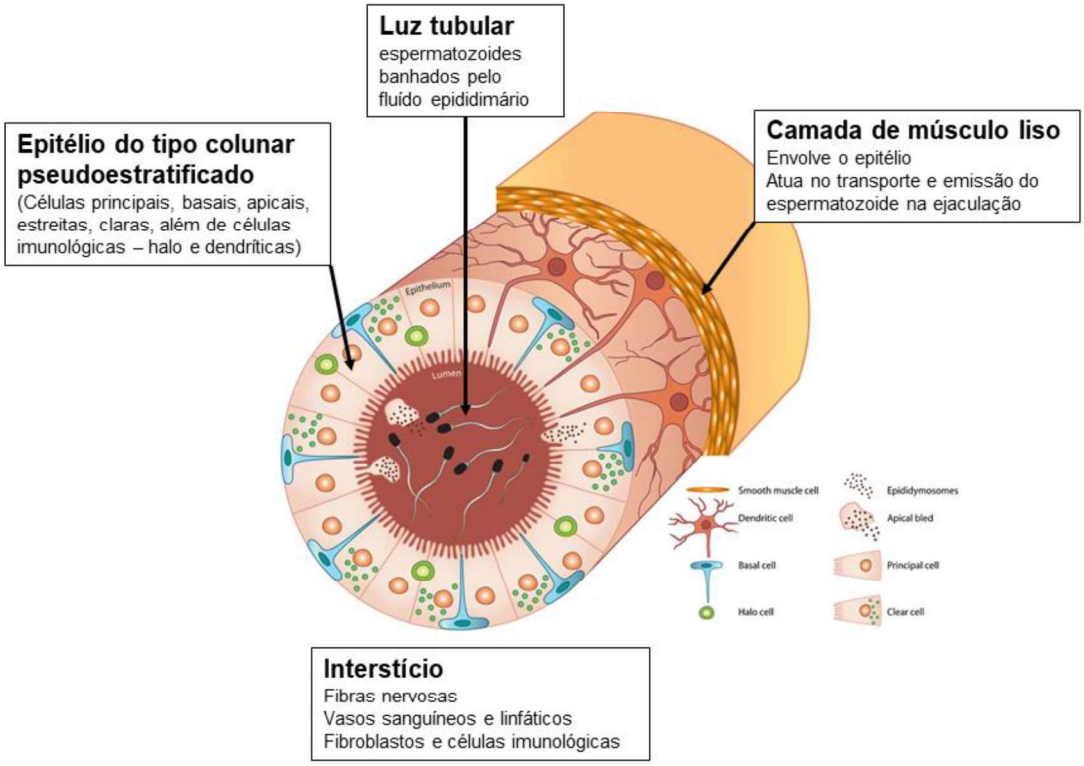

Figura 1: Representação esquemática de um corte transversal do ducto do epidídimo. Os tipos celulares do epitélio epididimário, bem como os compartimentos epitelial, luminal e muscular liso estão ilustrados. Adaptado de Belleannée et al. (2012).

O epidídimo pode ser dividido em diferentes regiões a partir de critérios anatômicos e morfológicos. Em roedores (ratos e camundongos), o epidídimo é dividido, da região proximal para distal, em segmento inicial, cabeça, corpo e cauda. Além disso, cada região do epidídimo pode ser subdividida em segmentos separados por septos intersticiais de tecido conjuntivo, apontando a presença de 10 e 19 segmentos no epidídimo de camundongos e ratos, respectivamente (Figura 2) (Jelinsky et al., 2007; Turner, 2008). A análise da expressão gênica por *microarray* revelou diferenças na expressão de uma série de genes entre os diferentes segmentos epididimários, fazendo com que a expressão de muitos genes apresente padrão diferencial de acordo com o segmento, ou ainda, seja restrita a alguns deles (Johnston et al., 2005; Jelinsky et al., 2007; Sipilä & Björkgren, 2016). Esse controle preciso e segmentado da expressão gênica no epidídimo influencia a atividade secretória e absortiva do epitélio, gerando microambientes luminais distintos entre seus diferentes segmentos, os quais

gradativamente interagem com os espermatozoides, promovendo sua maturação e culminando no seu armazenamento em estado quiescente nas porções distais desse órgão (Belleannée et al., 2012; Sipilä & Björkgren, 2016).

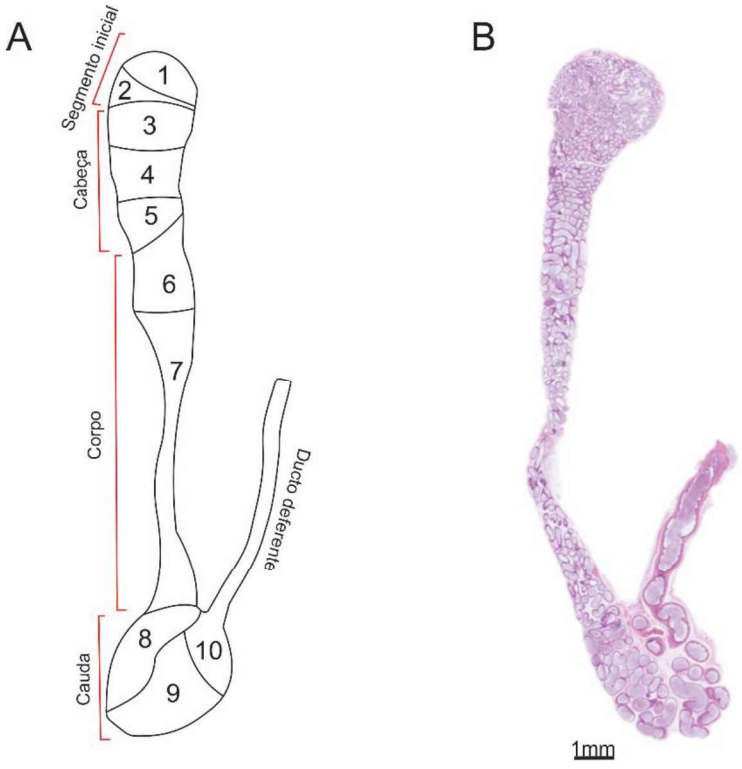

Figura 2: Anatomia do epidídimo de camundongo. (A) Representação esquemática do epidídimo de camundongo, indicando as principais regiões do epidídimo (segmento inicial, cabeça, corpo e cauda), e os 10 segmentos epididimários separados por septos de tecido conjuntivo. (B) Corte longitudinal do epidídimo corado com hematoxilina/eosina. Adaptado de Jelinsky et al. (2007) e Sullivan & Mieusset (2016).

O epitélio epididimário é formado por diversos tipos celulares, a saber: células principais e células basais, presentes em todas regiões epididimárias, células apicais e células estreitas, restritas ao segmento inicial e à zona intermediária (região de transição entre o segmento inicial e a cabeça do epidídimo), células claras, presentes na cabeça, corpo e cauda do epidídimo, além de células de origem imunológica, como as células halo e células dendríticas, cuja distribuição varia ao longo do órgão (Hinton & Robaire, 2015). A presença

de *junções estreitas* na porção luminal de células principais adjacentes forma a barreira hemato-epididimária, que é fundamental para a manutenção do microambiente luminal e proteção do espermatozoide em maturação (Hinton & Palladino, 1995; Mital et al., 2011; Avellar & Hinton, 2019). Essa distribuição diferencial das células epiteliais entre as regiões do epidídimo juntamente com o perfil segmentado de expressão gênica conferem grande complexidade estrutural e funcional a este órgão (Cornwall, 2009; Da Silva et al., 2011; Hinton & Robaire, 2015).

Vale destacar que os mecanismos moleculares, bioquímicos e celulares pelos quais o epidídimo exerce suas funções ainda permanecem pouco conhecidos. A importância de se investigar os processos epididimários responsáveis pela geração de espermatozoides competentes é enfatizada pelo fato de que quase metade dos casos de infertilidade masculina ainda não apresentam causas conhecidas, sendo que muitos podem estar associados a alterações durante a passagem dos espermatozoides pelo epidídimo (Turner, 2008; Cornwall, 2009).

Os gametas chegam ao epidídimo impulsionados pelo fluído testicular, pelo movimento ciliar dos dúctulos eferentes, que gera uma turbulência fluídica, e pela atividade contrátil da musculatura lisa, servindo como força motriz para impulsionar os espermatozoides para frente (Yuan et al., 2019). Durante o processo de maturação, o transporte de espermatozoides pelo epidídimo depende, sobretudo, das contrações da musculatura lisa ao redor do ducto epididimário (Avellar et al., 2019). Na maior parte das regiões do epidídimo essas contrações são rítmicas, com exceção da cauda do epidídimo, onde são esporádicas na ausência de estímulos específicos, como aqueles oriundos do sistema nervoso autônomo (Elfgen et al., 2018). Vale ressaltar, ainda, que tanto a espessura da camada muscular lisa que circunda as células epiteliais do epidídimo quanto a densidade da inervação autonômica aumentam do epidídimo proximal para o distal (Ricker, 1998).

As funções do epidídimo são reguladas por uma complexa rede de fatores hormonais, neuronais, testiculares e imunológicos. Os androgênios, sobretudo a testosterona e seu metabólito ativo diidrotestosterona (DHT), membros da família dos hormônios esteroides, são os principais promotores da diferenciação, morfologia e função do epidídimo (Patrão et al., 2009; Hamzeh & Robaire, 2010). Eles promovem seus efeitos a partir da interação com o receptor de androgênios (AR), membro da superfamília de receptores acoplados a fatores de transcrição, expresso pelas células epiteliais, intersticiais e da musculatura lisa ao longo do epidídimo (Patrão et al., 2009; O'Hara et al., 2011; Sipilä et al., 2011). Sabe-se que alterações na sinalização androgênios/AR tanto por mudanças nos níveis desses hormônios quanto na expressão/sinalização do AR podem afetar a estrutura, desenvolvimento e funções do epidídimo, resultando em infertilidade ou subfertilidade (Avellar & Hinton, 2019).

O impacto da interação entre o epidídimo e o sistema imunológico na fertilidade masculina tem atraído diversas pesquisas nas últimas décadas. O estabelecimento da tolerância imunológica acontece antes da geração do gameta maduro que, portanto, é considerado corpo estranho pelo sistema imunológico do indivíduo. Dessa forma, os espermatozoides são células autoantigênicas, capazes de induzir resposta autoimune e inflamatória se expostos ao ambiente extraluminal testicular e epididimário (Hedger & Hales, 2006). Entretanto, na luz dos túbulos seminíferos e do ducto epididimário os gametas masculinos são bem tolerados tanto durante a espermatogênese quanto na maturação epididimária, o que indica a existência de mecanismos de tolerância imunológica no testículo e epidídimo, protegendo o espermatozoide de ataques imunológicos (Hedger, 2011). O ambiente imunológico no testículo é relativamente bem estudado; do contrário, pouco se conhece sobre os mecanismos pelos quais o epidídimo protege o espermatozoide de um ataque autoimunitário (para revisão ver Hedger, 2011; Schagdarsurengin et al., 2016).

Paradoxalmente ao ambiente de tolerância imunológica do órgão, o epidídimo possui um vasto arsenal imunológico, composto por células imunitárias encontradas no epitélio, interstício, e algumas vezes na luz tubular, e pela expressão abundante e constitutiva de diversos elementos do sistema imunológico inato, como receptores de reconhecimento de

padrões (PRRs), citocinas, quimiocinas e proteínas antimicrobianas, incluindo membros da família da β-defensinas e dos inibidores de protease do tipo WFDC (*Whey-acidic protein four disulfide core*) (Clauss et al., 2002; O'rand et al., 2011; Ribeiro et al., 2016). Acredita-se que esse sistema esteja em constante estado de alerta para proteger os espermatozoides e o próprio tecido contra estímulos infecciosos e inflamatórios (Fijak et al., 2018).

É reconhecido que qualquer desequilíbrio entre eventos pró- e anti-inflamatórios no epidídimo pode comprometer a sua função e, consequentemente, a fertilidade. Embora uma proporção significativa dos casos de infertilidade masculina seja, de fato, atribuída a causas inflamatórias ou imunológicas (Hedger, 2011), os mecanismos celulares e moleculares básicos envolvidos na resposta do epidídimo em condições de inflamação ainda são pouco conhecidos (Avellar et al., 2019). Nesse ponto, vale destacar que doenças inflamatórias do epidídimo são altamente prevalentes em homens de todas as idades e importante fator etiológico de infertilidade masculina (Nicholson et al., 2010; Pilatz et al., 2015).

2. Epididimite

A inflamação é uma resposta biológica do tecido vascularizado contra estímulos nocivos de naturezas diversas, a qual envolve a produção e recrutamento de inúmeros mediadores humorais e celulares, que atuam de forma orquestrada visando combater o estímulo e restaurar a homeostasia do sistema. Nesse contexto, a inflamação do epidídimo, conhecida como epididimite, é um fator relevante para a perda de bem-estar e produtividade em homens de todas as idades (Tracy et al., 2008; Pilatz et al., 2015). Estudos epidemiológicos indicaram que a epididimite apresenta incidência anual de 250-650 casos/100000 homens no Reino Unido, sendo a condição inflamatória mais frequente do trato reprodutor masculino (Nickel et al., 2005; Nicholson et al., 2010). Trata-se do quinto diagnóstico urológico mais comum em homens com idades entre 18 e 50 anos, atingindo aproximadamente 600.000 casos por ano nos EUA (Tracy et al., 2008).

Fatores de risco para epididimite na maioria dos homens incluem: história de infecções do trato urinário ou infecções sexualmente transmissíveis, anormalidades anatômicas

(obstrução da saída da bexiga), além de cirurgias de próstata ou do trato urinário ou outros traumas escrotais (Taylor, 2015; McConaghy & Panchal, 2016). Dentre esses, infecções por bactérias, como *Chlamydia trachomatis, Escherichia coli*, *Enterococcus faecalis* e *Staphylococcus aureus*, compõem a etiologia mais comum de epididimite (Pilatz et al., 2015). Acredita-se que a principal rota de invasão bacteriana no epidídimo seja pela ascensão retrógrada via uretra/ducto deferente (Michel et al., 2015).

As lesões teciduais induzidas pela epididimite bacteriana, tipicamente observadas na cauda do epidídimo de homens e em modelos experimentais em roedores e lagomorfos, incluem infiltrado inflamatório intersticial, edema, destruição do epitélio, fibrose intersticial e obstrução do ducto epididimário (Hackett et al., 1988; Tanaka et al., 1995; Turner et al., 2011; Stammler et al., 2015; Michel et al., 2016; Silva et al., 2018). Além disso, observa-se mudança no perfil de expressão gênica ao longo do epidídimo, incluindo aumento ou diminuição da expressão de genes relacionados à resposta inflamatória (citocinas, quimiocinas, cicloxigenases, NO sintases, etc.), variando de acordo com o tipo de estímulo inflamatório (Turner et al., 2011; Lang et al., 2014; Michel et al., 2016; Silva et al., 2018).

As alterações morfológicas e funcionais no epidídimo induzidas pela epididimite podem causar quadros de infertilidade ou subfertilidade temporária ou permanente (Michel et al., 2015). De fato, cerca de 40% dos pacientes diagnosticados com epididimite bacteriana apresentam alterações persistentes de parâmetros espermáticos, como oligospermia ou azospermia, mesmo meses após o tratamento adequado da doença com antibióticos e anti-inflamatórios e supressão dos sintomas (Eickhoff et al., 1999; Rusz et al., 2012). Além disso, 20% dos pacientes evoluem para quadros de epididimite recorrente ou crônica, associada a dor escrotal crônica, podendo exigir a remoção cirúrgica do epidídimo (Nickel et al., 2002). Dessa forma, o melhor conhecimento da fisiopatologia da epididimite contribuirá para o desenvolvimento de novas estratégias terapêuticas visando a reversão dos seus efeitos nocivos e a preservação da fertilidade e bem-estar do homem.

3. Epidídimo e a imunidade inata

As interações entre o sistema imunológico inato e o trato reprodutor masculino são importantes para a fertilidade. De fato, diversos microrganismos patogênicos, incluindo bactérias, vírus e fungos, podem invadir e colonizar os tecidos do trato reprodutor masculino, provocando quadros inflamatórios que podem promover infertilidade temporária ou definitiva (Girling & Hedger, 2007).

Como comentado anteriormente, uma característica importante do epidídimo é o elevado número de componentes da imunidade inata, normalmente apresentando expressão constitutiva, dependente de androgênios e que varia conforme a região do órgão e tipo celular. Dentre estes componentes encontram-se proteínas com atividade antimicrobiana e imunomoduladora (tais como cistatinas, catelicidinas, lipocalinas, lactotransferinas, beta-defensinas e inibidores de protease do tipo WFDC), mediadores inflamatórios (citocinas, por exemplo), receptores de reconhecimento de padrões e componentes associados a respostas ao estresse oxidativo (Hall et al., 2002; Ribeiro et al., 2012, 2016). Estes elementos da imunidade inata epididimária atuam como a primeira barreira na defesa contra uma ampla gama de desafios (infecciosos e não-infecciosos) e estão criticamente envolvidos na regulação de respostas imunológicas inatas e adaptativas. Dentre eles, destacamos os receptores do tipo Toll (TLRs), envolvidos no reconhecimento de patógenos e disparo inicial de mecanismos de defesa contra infecções, e os inibidores de protease do tipo WFDC, cujos aspectos moleculares e funcionais no epidídimo vêm sendo estudados pelo nosso grupo.

3.1 *Receptores do tipo Toll (Toll-like receptors*;TLRs)

Estudos realizados nos últimos 15 anos demonstraram que o epidídimo expressa de forma abundante e constitutiva uma série de elementos do sistema imunológico inato associado à detecção de patógenos, incluindo os *Toll-like receptors* (TLRs) (Browne et al., 2018; Chapman et al., 2007; Palladino et al., 2007; Palladino et al., 2008; Rodrigues et al., 2008; Wu et al., 2016; Zhao et al., 2008; Zhu et al., 2015). Os TLRs compõem uma família de 13 receptores transmembranares (TLR1-TLR13) presentes em células imunológicas e não-

imunológicas, responsáveis pelo reconhecimento de *pathogen-associated molecular patterns* (padrões moleculares associados a patógenos; PAMPs), que são essenciais para a sobrevivência do microrganismo e, portanto, altamente conservados (Akira et al., 2006; Kawai & Akira, 2005; Kumar et al., 2009). É reconhecido que a ativação dos TLRs por diferentes PAMPs faz parte dos eventos iniciais da resposta imunológica contra infecções. A expressão diferencial de TLRs (TLR1 ao TLR11) foi observada em células imunológicas (macrófagos e células dendríticas) e não-imunológicas (células epiteliais, intersticiais e da musculatura lisa) em diferentes tecidos do trato reprodutor masculino de roedores, incluindo testículo, epidídimo e próstata (Quintar et al., 2006; Palladino et al., 2007; Bhushan et al., 2008; Rodrigues et al., 2008).

O TLR4 foi o primeiro membro descoberto dessa família de receptores, apresentando papel essencial na detecção e disparo de resposta imunológica contra infecção por bactérias Gram-negativas, via interação com lipopolissacarídeo (LPS), localizado na parede celular dessas bactérias (Poltorak et al., 1998). O LPS é um PAMP bem caracterizado, com estrutura composta por três regiões (Figura 3A): 1) Lipídio A (endotoxina), que ancora o LPS na membrana externa da bactéria; 2) um núcleo polissacarídeo que se liga ao lipídio A pelo ácido 3-deoxi-D-mano-oct-2-ulosônico (*3-deoxy-D-manno-oct-2-ulosonic acid*; KDO); 3) Antígeno-O, que consiste em unidades de repetição de oligossacarídeos (Maeshima & Fernandez, 2013). O lipídio A contém duas glicosaminas fosforiladas ligadas a 6 cadeias lipídicas, que consiste no componente responsável pela atividade antigênica da endotoxina, ou seja, é o padrão molecular conservado responsável pela indução da resposta ao LPS (Raetz & Whitfield, 2002; Park et al., 2009).

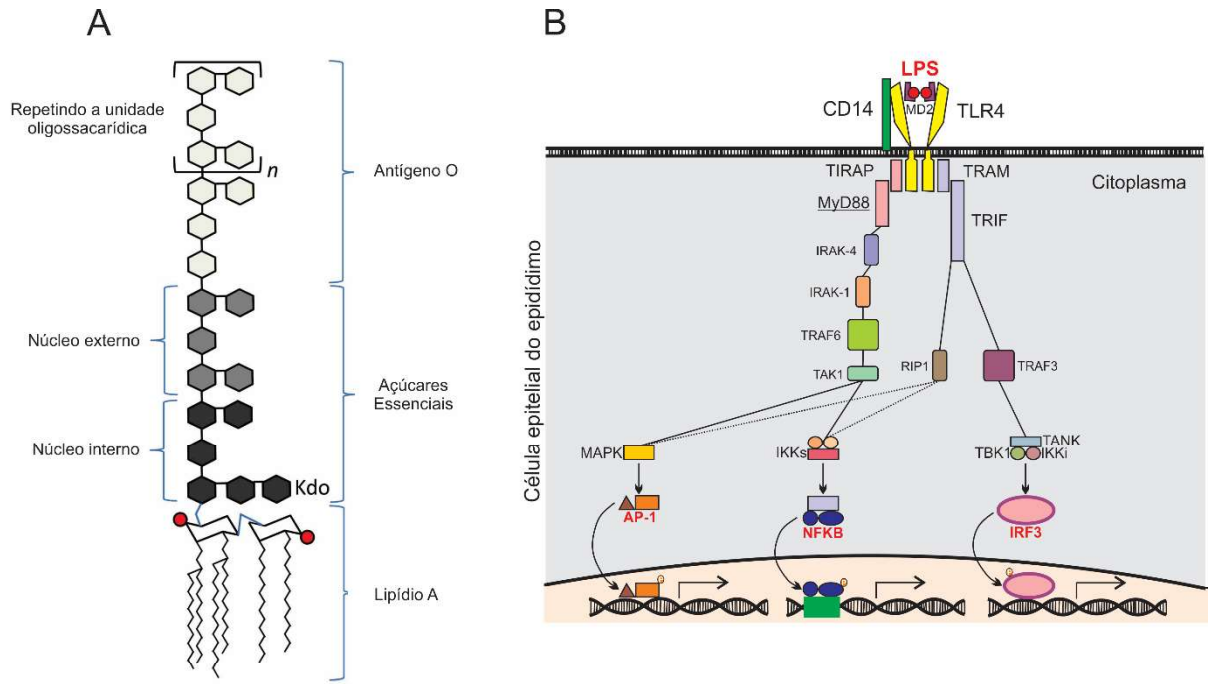

Figura 3. Esquema ilustrativo da via de sinalização mediada por TLR4 ativada por LPS. (A) Estrutura básica do lipopolissacarídeo (LPS). (B) Visão geral da sinalização celular associada ao receptor TLR4. Adaptado de Maeshima & Fernandez (2013), e Lu et al. (2008).

O reconhecimento do LPS pelo TLR4 requer o envolvimento de proteínas acessórias (Figura 3B). O LPS interage com a proteína de ligação do LPS (LBP) solúvel, que é uma proteína de transporte e que facilita a associação do LPS ao CD14. A proteína CD14 facilita a transferência do LPS ao complexo TLR4/MD2, modulando o reconhecimento do LPS. A proteína MD2 é um co-receptor especializado na interação com a porção lipídica do LPS e que interage com TLR4, provavelmente porque o LPS com suas 6 cadeias lipídicas é grande demais para interagir diretamente com o TLR4 (Lu et al., 2008; Park et al., 2009; Kawai & Akira, 2010). A ativação do TLR4 leva à dimerização dos domínios extracelulares do receptor, disparando vias de sinalização intracelular associadas, de acordo com o tipo de proteínas adaptadoras recrutadas: 1) dependente da proteína de diferenciação mieloide 88 (MyD88), sinalização celular canônica, que culmina na ativação do fatores de transcrição como o NFKB e AP-1, com consequente aumento da transcrição de genes envolvidos na resposta inflamatória, tais como citocinas e quimiocinas; e 2) e a via independente de MyD88, que

resulta na ativação do fator de transcrição regulado por interferon 3 (IRF3), capaz de modular a expressão de genes tipicamente envolvidos com respostas antivirais, como interferon-α (IFNα) e –β (IFNβ) (Figura 3B) (Lu et al., 2008).

3.2 Proteínas da família Whey-acidic protein four disufide core (WFDC)

A família WFDC é formada por proteínas que apresentam pelo menos um domínio de 40-50 resíduos de amino ácidos contendo oito cisteínas altamente conservadas que se organizam em quatro pontes dissulfeto intramoleculares [C1-(Xn)-C2-(Xn)-C3-(X5)-C4-(X5,X6)-C5-C6-(X2,X3)-C7-(X3,X4)-C8] (Bingle & Vyakarnam, 2008). A maioria dos genes que codifica proteínas WFDC é formada por um primeiro exon, que codifica o peptídeo sinal, seguido de um ou mais exons centrais, cada um codificando um domínio WFDC, e um último exon, que codifica a porção final da proteína (Clauss et al., 2002). Em humanos, 14 genes *WFDC* foram descritos em um cluster no cromossomo 20q12-q13 (Clauss et al., 2002, 2005). O *locus WFDC* no cromossomo 20 pode ser dividido em centromérico, contendo os genes *WFDC5*, *WFDC12*, *PI3* (*WFDC14*) e *SLPI* (*WFDC4*), e telomérico, onde se encontram os genes *WFDC2*, *WFDC6*, *EPPIN* (*WFDC7*), *WFDC8*, *WFDC9*, *WFDC10A*, *WFDC11*, *WFDC10B*, *WFDC13* e *WFDC3* (Figura 4) (Clauss et al., 2011; Ferreira et al., 2013; Small et al., 2016). Em ratos e camundongos foram identificados 18 e 16 genes *Wfdc*, localizados em *clusters* gênicos ortólogos aos do humano nos cromossomos 3 e 2, respectivamente (Clauss et al., 2005). Em camundongos, os genes *Wfdc5*, *Wfdc12*, *Wfdc15a*, *Wfdc15b* e *Wfdc4* são encontrados no sublocus centromérico, enquanto os genes para *Wfdc3*, *Wfdc2*, *Wfdc6a*, *Wfdc6b*, *Wfdc7*, *Wfdc8*, *Wfdc9*, *Wfdc10, Wfdc11* e *Wfdc13* no sublocus telomérico (Figura 4) (Clauss et al., 2005). Vale destacar que alguns dessa família possuem dois ou mais domínios WFDC em sua estrutura, como a WFDC13, enquanto outros possuem estrutura mista, apresentando domínio inibidore de protease do tipo Kunitz além do WFDC, como a WFDC8, WFDC6 e EPPIN, resultando em uma variedade biológica extraordinária dentre os membros dessa família. A estrutura genômica dos genes *Wfdc* murinos é apresentada na Figura 5.

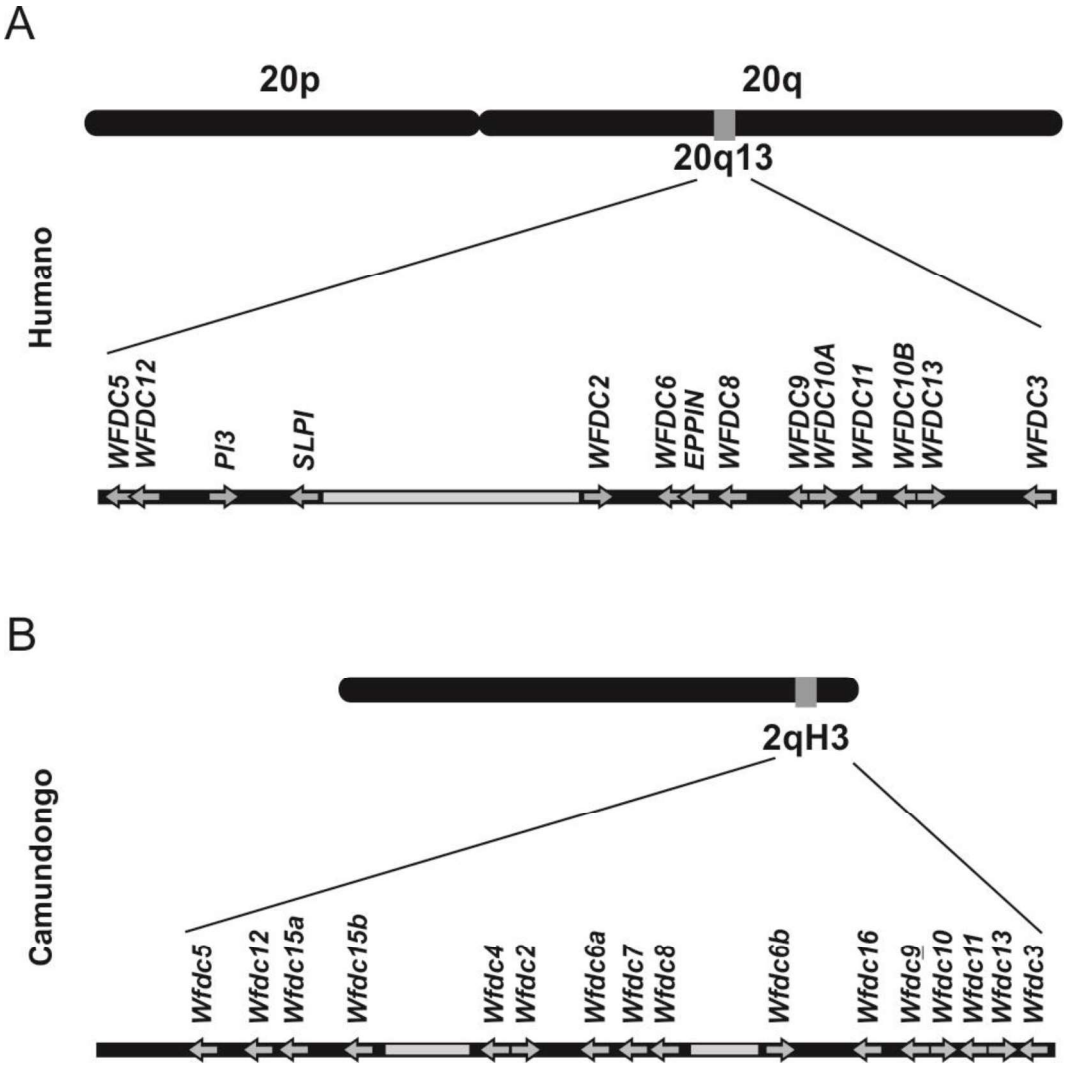

Figura 4. Diagrama esquemático do locus *WFDC* humano (A) e *Wfdc* murino (B) e as localizações relativas desses genes. A direção das setas representam o sentido de leitura dos genes *Wfdc* (Clauss et al., 2005; Small et al., 2016).

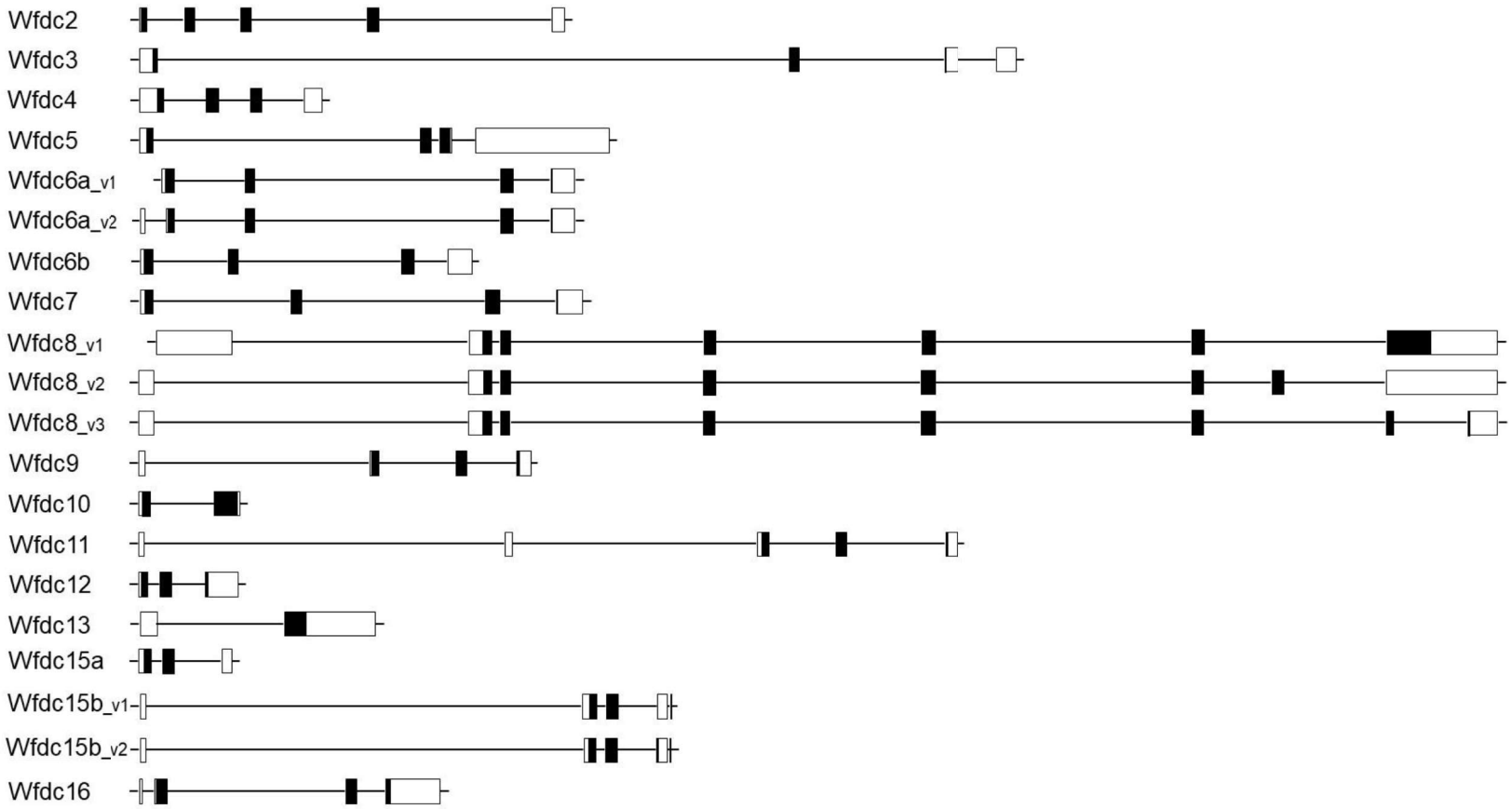

Figura 5. Estrutura dos genes *Wfdc* murinos localizados no cluster gênico do cromossomo 2 (ver figura 1B). As caixas e linhas representam as regiões de exons e de íntrons, respectivamente. As caixas pretas representam a região codificadora de cada transcrito. As estruturas foram desenhadas com base nas informações contidas em *National Center for Biotechnology Information* (NCBI; https://www.ncbi.nlm.nih.gov/), segundo os seguintes números de entrada (gene ID): *Wfdc2* (67701), *Wfdc3* (71856), *Wfdc4* (20568), *Wfdc5* (209232), *Wfdc6a* (209351), *Wfdc6b* (433502), *Wfdc7* (75526), *Wfdc8* (277343), *Wfdc9* (629754), *Wfdc10* (629756), *Wfdc11* (629761), *Wfdc12* (192200), *Wfdc13* (408190), *Wfdc15a* (68221), *Wfdc15b* (192201) e *Wfdc16* (277345). Os genes *Wfdc6*, *Wfdc8* e *Wfdc15b* apresentam 2, 3 e 2 variantes de *splicing* alternativo de RNAm, respectivamente, conforme apresentado.

A análise de genes *Wfdc* mostra que o *locus* gênico *Wfdc* é conservado em roedores murinos em relação ao humano, com algumas exceções onde a duplicação de genes e/ou o silenciamento gênico afetaram o número de genes ativos (Clauss et al., 2005). Muitos dos genes evoluíram rapidamente desde a separação das linhagens de primatas e murinos, como indicado por uma conservação de sequência relativamente baixa, na gama de 50-70% (Lundwall & Clauss, 2011). Por outro lado, alguns genes *WFDC* mantiveram alto grau de conservação em suas sequências, tal como *WFDC3*, cujo produto proteico apresenta cerca de 80% de identidade entre humanos e camundongos (Clauss et al., 2005). Tem sido sugerido que a rápida evolução desses genes pode ter sido impulsionada por funções putativas na reprodução, como indicado pela expressão abundante de vários genes *WFDC* no trato reprodutivo masculino (Lundwall & Clauss, 2011). Vale ressaltar, entretanto, que alguns representantes dessa família de genes também podem apresentar expressão diferencial em tecidos não-reprodutivos como rim, pulmão, glândula salivar, esôfago, amígdala, língua, entre outros (Hagiwara et al., 2003; Small et al., 2016). Uma segunda força que tem sido sugerida para impulsionar a evolução dos genes *WFDC* é o seu papel em eventos de defesa contra microrganismos (Clauss et al., 2005). Esta hipótese se baseia nas propriedades antimicrobianas (antibacteriana, antiviral e antifúngica) demonstradas para diversas proteínas WFDC, que então podem refletir a adaptação às diferenças na microbiota em diferentes animais (Lundwall & Clauss, 2011).

Nesse contexto, o papel das proteínas WFDC na inflamação, como imunomoduladores (Nukiwa et al., 2008) e na imunidade inata, como antimicrobianos (Bingle & Vyakarnam, 2008; Rajesh et al., 2011; Bingle, 2011) vem recebendo atenção. Potente atividade bactericida *in vitro* foi demonstrada para diferentes proteínas WFDC, incluindo EPPIN (WFDC7), SLIP (WFDC4) e WFDC8, cujo mecanismo de ação envolve a formação de poros na parede celular bacteriana resultando no extravasamento do conteúdo citoplasmático e morte da bactéria (Sallenave, 2002; Yenugu et al., 2004; McCrudden et al., 2008; Rajesh et al., 2011). A HE4 (WFDC2), SLPI, EPPIN e PI3 (WFDC14) também apresentaram atividade inibitória frente a proteases endógenas secretadas por células inflamatórias, como a elastase e a catepsina G,

e exógenas, secretadas por vírus, bactérias e fungos (Hellström et al., 2003; Verrier et al., 2012; Hua et al., 2014; Majchrzak-Gorecka et al., 2016). O mecanismo de sua atividade inibitória de protease envolve a ligação não-covalente à fenda catalítica da protease, inibindo assim a ligação ao substrato competitivamente (Hiemstra et al., 1996; Krowarsch et al., 2003; McCrudden et al., 2008; Small et al., 2016).

Apesar das proteínas WFDC serem classicamente vistas como uma família de proteínas com funções de inibidores de protease e agentes antimicrobianos, estudos mais recentes demonstraram que elas também podem atuar no controle da resposta inflamatória (Small et al., 2016). Por exemplo, foi demonstrado, por modelos *in vitro* e *in vivo*, que a EPPIN atua como imunomodulador durante a inflamação pulmonar aguda induzida pelo LPS, via inibição da sinalização mediada pelo NFKB (Scott et al., 2017). Essas observações indicam o envolvimento de proteínas WFDC no controle da resposta imunológica inata contra infecções. Considerando seu alto grau de expressão no trato reprodutor, podemos inferir que as proteínas WFDC também podem apresentar papeis importantes na resposta imunológica de órgãos reprodutivos contra agressões patogênicas.

De fato, expressão preferencial e abundante de genes WFDC foi detectada no trato reprodutor masculino humano e de roedores, em especial no epidídimo (Richardson et al., 2001; Sivashanmugam et al., 2003; Jalkanen et al., 2006; Rajesh et al., 2011; Silva et al., 2012). Destaca-se, nesse contexto, que algumas proteínas WFDC, como a EPPIN, interagem com espermatozoides (Richardson et al., 2001; Sivashanmugam et al., 2003; Silva et al., 2012), dando suporte à hipótese de que essa família de proteínas pode ter papeis relacionados à maturação espermática. Em harmonia com essa hipótese, demonstramos anteriormente que a distribuição celular da EPPIN na superfície de espermatozoides coletados de diferentes regiões do epidídimo variou conforme a região do epidídimo, indicando que a EPPIN secretada pelas células do epitélio epididimário interage de forma dinâmica com o espermatozoide de acordo com o seu estágio de maturação (Silva et al., 2012). De fato, devido ao seu papel crítico na modulação da motilidade do espermatozoide ejaculado, a EPPIN é considerada um alvo para o desenvolvimento de fármacos

contraceptivos masculinos (O'Rand et al., 2016). Apesar desses estudos, o perfil de expressão dos genes *Wfdc* no trato reprodutor masculino de camundongos ainda carece de maior aprofundamento. Além disso, o papel desses genes em eventos de defesa do epidídimo contra infecções *in vivo* ainda é pouco explorado.

Em conjunto, esses estudos sustentam a hipótese de que as proteínas WFDC são elementos multifuncionais no trato reprodutor masculino, atuando tanto como proteínas antimicrobianas e reguladoras da resposta imunológica inata quanto em eventos relacionados à promoção da fertilidade. Os mecanismos pelos quais essas proteínas exercem suas funções no epidídimo, bem como a regulação de sua expressão em condições normais ou patológicas, no entanto, ainda não são conhecidos. Considerando que a infecção e inflamação do epidídimo estão entre as causas mais comuns de infertilidade masculina (Hedger, 2011) e vislumbrando ampliar nosso conhecimento sobre as funções imunológicas e reprodutivas atribuídas aos genes *Wfdc*, é nossa intenção testar a hipótese de que a epididimite bacteriana modifica a expressão de membros dessa família nas diferentes regiões do epidídimo como parte da resposta inflamatória ao estímulo infeccioso, o que pode ter consequências para a função epididimária e para a maturação do espermatozoide. Para tal, no presente trabalho investigamos o perfil de expressão de genes *Wfdc* no epidídimo de camundongos submetidos a dois modelos experimentais de epididimite induzida por LPS ultrapuro de *E. coli* 055:B55.

OBJETIVOS

1. Objetivo geral

Estudar os efeitos da inflamação aguda sobre a expressão dos genes *Wfdc* no epidídimo de camundongos em modelos experimentais de epididimite induzida pelo LPS ultrapuro de *E. coli* 055:B55, bem como seu impacto na fisiologia epididimária.

2. Objetivos específicos

- Caracterizar o perfil de expressão dos transcritos *Wfdc2*, *Wfdc3*, *Wfdc6a*, *Wfdc6b*, *Wfdc7*, *Wfdc8*, *Wfdc*9, *Wfdc10*, *Wfdc11*, *Wfdc12*, *Wfdc15a*, *Wfdc15b* e *Wfdc16* no trato reprodutor masculino de camundongos adultos.
- Avaliar se a epididimite induzida pelo LPS ultrapuro de *E. coli* 055:B55 via interstício do segmento inicial ou luz do ducto deferente altera a expressão dos transcritos dos genes *Wfdc2*, *Wfdc3*, *Wfdc6a*, *Wfdc6b*, *Wfdc7*, *Wfdc8*, *Wfdc9*, *Wfdc10*, *Wfdc11*, *Wfdc12*, *Wfdc15a*, *Wfdc15b* e *Wfdc16* no epidídimo de camundongos.
- Investigar se as alterações da expressão dos genes *Wfdc* no epidídimo de camundongos submetidos aos modelos de epididimite induzida pelo de LPS ultrapuro de *E. coli* 055:B55 via interstício do segmento inicial ou luz do ducto deferente dependem da ativação do fator de transcrição NFKB.
- Avaliar o impacto da epididimite induzida pelo LPS ultrapuro de *E. coli* 055:B55 via interstício do segmento inicial ou luz do ducto deferente sobre a motilidade de espermatozoides de camundongos.

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Region-specific regulation of *Wfdc* gene expression in the mouse epididymis to intravasal and interstitial lipopolysaccharide-induced epididymitis

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ABSTRACT

Whey-acidic difulfide core (WFDC)-type protease inhibitors are a large family of multifunctional proteins with roles in innate immunity and fertility. Despite their abundant expression in the epididymis, the roles of WFDCs during epididymitis, a relevant factor of male infertility, remain largely unexplored. We hypothesize that *Wfdc* gene expression is modified during bacterial epididymitis as part of the tissue defense mechanism against infections. For that, we submitted mice to two experimental models of lipopolysaccharide (LPS)-induced epididymitis: 1) interstitial injection of LPS into initial segment (IS), and 2) luminal injection of LPS into the vas deferens. LPS induced acute inflammation in the initial segment and cauda epididymis, as demonstrate by up-regulation of inflammatory mediators and the presence of immune cell infiltrates and edema. In the IS, interstitial LPS injection induced a time-dependent regulation of several *Wfdc* transcripts, since down-regulated *Wfdc6a*, *Wfdc7*, *Wfdc8*, *Wfdc11, Wfdc15b* and *Wfdc16* transcript levels 6 h post-treatment, while up-regulated *Wfdc2*, *Wfdc3*, *Wfdc4*, *Wfdc5*, *Wfdc6*, *Wfdc10*, *Wfdc12*, *Wfdc15a* and *Wfdc16* at 24 h and 72 h. In the cauda epididymis, intravasal LPS injection down-regulated *Wfdc6a_v1, Wfdc6a_v2, Wfdc6b, Wfdc11 Wfdc15b_v1-2* and *Wfdc16* transcript levels 24 h and 72 h post-treatment, while up-regulation *Wfdc2* and *Wfdc5* at 24 h. Changes in the expression of *Wfdc2, Wfdc6b, Wfdc10, Wfdc12* and *Wfdc15b_v1-2* transcripts in the initial segment and cauda epididymis was prevented by pretreatment with PDTC, a NFKB inhibitor, suggesting an involvement of this pathway in regulating Wfdc gene expression. These data suggest a possible antimicrobial and immunomodulatory role of Wfdc genes in the inflamed epididymis, suggesting their potential exploration as therapeutic targets for adjuvant therapies of epididymitis.

INTRODUCTION

The epididymis is responsible for transforming functionally immature testicular spermatozoa into fully competent cells, capable of acquire progressive motility and fertilize the oocyte [1,2]. It is known that changes in epididymal physiology may induce male infertility,

which highlights the relevance of this organ to male reproductive potential and further studies on epididymal biology [3].

Interactions between the innate immune system and the male reproductive tract are relevant for fertility [4]. An important feature of the epididymis is its vast arsenal of immune cells and other innate immunity components, which are constitutively expressed in a region-dependent manner [5,6]. For instance, the epididymis abundantly expresses a wide number of antimicrobial/immunomodulatory proteins, such as cystatins, catelicidins, lipocalins, lactotransferins, beta-defensins and WFDC (Whey-acidic difulfide core)-type protease inhibitors; as well as inflammatory mediators, including cytokines and chemokines; under normal physiological conditions [5,7-9]. It is though that this defense system is in a constant state of surveillance, thus protecting the epididymis and its important load – spermatozoa – against pathological threats, which may affect the tissue and jeopardize fertility [10].

The inflammation of the epididymis, known as epididymitis, is a relevant factor of infertility, as well as loss of well-being and productivity in men of all ages [11,12]. Epidemiological studies have indicated that epididymitis has an annual incidence rate of 250-650 cases/100000 men in the UK, being one of the most common inflammatory conditions in the male reproductive tract [13,14]. Bacterial infections, such as *Escherichia coli*, make up the most common etiological factor of epididymitis [12].

Tissue lesions induced by bacterial epididymitis, typically seen in the cauda epididymis of men include interstitial inflammatory infiltrate, edema, epithelial and smooth muscle cell destruction, interstitial fibrosis, and epididymal duct obstruction [10,15,16]. These morphological changes are reproduced in experimental models of epididymitis in rodents and lagomorphs induced by the injection of bacteria or their purified products into the lumen of the vas deferens or epididymal mesenchyme [15-21]. Furthermore, it has been shown that acute inflammation induces changes in gene expression profile throughout the epididymis, including a differential effect on the expression of genes related to the inflammatory response and innate immunity, such as cytokines and chemokines, according to the type of inflammatory stimulus [16,19,20,22].

It is known that inflammation has an impact on expression of antimicrobial genes in the epididymis, which could affect its functions [21,23]. Experimental models of acute epididymitis in rodents have shown that the expression of several members of the β-defensin family, a large family of androgen-dependent antimicrobial peptides, is downregulated in the epididymis upon a bacterial-derived inflammatory stimulus, an effect that contributed to sperm motility impairment [21]. Furthermore, the overexpression of the β-defensin SPAG11A (also known as BIN1B and SPAG11E) in the epididymis resulted in resistance against *E. coli* infection and its harmful effects on sperm function [24]. These results indicate that antimicrobial genes play crucial roles in epididymal functions under normal and pathological conditions. Little is known, however, about the effects of inflammation on the expression of *WFDC* genes, another family of antimicrobial genes abundantly expressed in the epididymis, and its consequences for male fertility.

The *WFDC* family is composed of proteins having at least one domain of 40-50 amino acid residues containing eight highly conserved cysteines which are organized into four intramolecular disulfide bridges. In humans, 14 *WFDC* genes were described in a cluster on chromosome 20q12-q13, while in mice 16 *Wfdc* genes were identified, located in an orthologous cluster on chromosome 2 [26,27]. Evolution of these WFDC genes indicate their relevance for male fertility, as many of these genes are expressed in the epididymis and other male reproductive tissues [28,29]. Indeed, expression of different WFDC genes, such as *WFDC2*, *WFDC4*, *WFDC5*, *WFDC6*, *WFDC7 (EPPIN)*, *WFDC8*, *WFDC9*, *WFDC10*, *WFDC11*, *WFDC13* and *WFDC14*, was detected in the human and rodent male reproductive tract, in which the epididymis rose as an the most abundant source of their protein products [20,26,30-33]. Potent in vitro bactericidal activity has been demonstrated for different WFDC proteins, including *WFDC4*, *WFDC7*, *WFDC8*, *WFDC12*, whose mechanism of action involves pore formation in the bacterial cell wall resulting in extravasation of cytoplasmic content and bacterial death [33-37]. Although antimicrobial and protease inhibitor activities have been classically attributed to several WFDC proteins, studies further suggest that they also play crucial roles as immunomodulatory and anti-inflammatory molecules [38]. Furthermore, WFCD

proteins, such as EPPIN, bind to testicular and epididymal spermatozoa and play a role on sperm function [30,31,39,40].

Therefore, the multifunctional properties of WFDC proteins in immune and reproductive events, associated with their selective expression in the epididymis, warrant further investigation on their expression and potential roles during epididymitis, as well as on their contribution to disease outcomes. In the present study, we tested the hypothesis that LPS-induced epididymitis modifies the expression of *Wfdc* family members in different regions of the mouse epididymis as part of the tissue response against this stimulus. For that, we first characterized two experimental models of LPS-induced epididymitis in mice, which allowed us to study the effect of inflammatory stimuli via lumen and interstitial compartment of the epididymis. We also investigate the role of the transcription factor NFKB in LPS-induced changes in *Wfdc* gene expression.

MATERIAL AND METHODS

Animals

Adult male mice C57BL/6 (90-day-old) were purchased from Anilab Laboratory (Paulinia/SP). We maintained the animals at Department of Pharmacology, IBB/UNESP under controlled light (12 h light/dark cycle) and temperature (22–24ºC), with food and water ad libitum. We conduct all procedures according to the guidelines for animal care and use of laboratory animal, approved by the Research Committee from UNESP (process #1029).

Experimental models of epididymitis

Mice were anesthetized with ketamine/xylazine (60/20 mg/kg, i.p.) and then submitted to either of the following experimental models of LPS-induced epididymitis: 1) inflammation of the initial segment via interstitial LPS injection: we made a double incision in the abdomen to expose the epididymal portion of the initial segment, the injection was carefully made bilaterally in interstitium of the initial segment (segment 1; [41]) keeping the epididymal duct intact: 2) inflammation of the cauda epididymis via intravasal LPS injection: we made a single scrotal

incision to expose the epididymal portion of the vas deferens, the injection was made bilaterally in a retrograde direction as near as possible to the cauda epididymis. In both epididymitis models 10 µl of sterile saline solution containing different doses of ultrapure LPS from *E. coli* 055:B55 (12.5, 25 and 50 µg, Invivogen, cat. Tlrl-pb5lps) were injected using a needle 30-G and a 25-µl Hamilton syringe. This LPS preparation contains insignificant amounts of contaminating lipoproteins that could stimulate TLR2. LPS doses were equivalent to 10000 (12.5 µg), 20000 (25 µg) and 40000 (50 µg) endotoxin units (EU), according to chromogenic LAL endotoxin assay (see below). Sham-operated mice underwent a similar procedure, but were treated with sterile saline only (saline-control). Mice were euthanized at different post-treatment time points (2, 6, 24 and 72 h) for mRNA, protein and histological studies. In an additional group, mice were pretreated with the NFKB inhibitor PDTC (ammonium pyrrolidinedithiocarbamate; 100 mg/kg body weight, i.p.) for 1 h before LPS injection treatment. In this case, the mice were killed 24 h after treatment with LPS. Another group of mice that were not submitted to any surgical procedure where obtained for mRNA studies. From these animals we collected the following organs: testis, epididymis (initial segment, caput, corpus, cauda), vas deferens, prostate, seminal vesicle, liver, lung and brain.

Histopathological evaluation

Whole epididymis from saline-control or LPS-treated mice were immersed in Bouin's fixative (75 ml saturated picric acid, 5 ml glacial acetic acid, 25 ml 37% formaldehyde) and processed for paraffin embedding. Two non-consecutive longitudinal tissue sections (5 µm) were stained with hematoxylin/eosin, and observed for general histopathological examination under light microscope. Samples were evaluated in a blinded-fashion method.

Reverse transcriptase (RT) reaction, conventional polymerase chain reaction (PCR) and real-time quantitative PCR (qPCR).

We extracted total RNA from tissues from saline-, LPS- and non-treated mice using TRIzol® reagent (Life Technologies, cat. 15596026) following manufacturer's

recommendations. We analyzed total RNA samples in a spectrophotometer Biodrop µLite (Biodrop) to determine the RNA concentration. Integrity and purity of total RNA samples were evaluated by agarose gel electrophoresis and A260/A280 ratio, respectively. Samples that did not present well-defined 18S and 28S bands and A260/A280 ratio <1.8 were discarded.

For conventional PCR we performed first-strand cDNA synthesis from total RNA samples (2.5 µg) using Thermoscript™ RT-PCR System (Life Technologies, cat. 11146-016) kit following the manufacturer's recommendations. Reactions in the absence of reverse transcriptase were performed as negative control. PCR assays were performed by adding cDNA (250 ng) to PCR buffer containing MgCl2 (1.5 mM), dNTPs (0.2 mM each), sense and antisense oligonucleotides for the target genes (4 µM each, table 1) and Taq DNA polymerase (1 U, cat. 10342-020) in final volume of 20 µl. The PCR conditions were: 95ºC/1min, followed by 95ºC/1min – 60ºC/1min – 72ºC/1min for 30 cycles, and a final extension of 72ºC/3min. At the end, we mixed the DNA products with loading buffer and run on agarose gel (1.5%) prepared in TBE buffer. Results were documented using a GBOX imaging system (Syngene).

For real-time qPCR we performed first-strand cDNA synthesis from total RNA samples (2.5 µg) using kit Maxima First Strand cDNA Synthesis Kit with dsDNase (Thermo Fisher, cat. K1671) following the manufacturer's recommendations. We performed qPCR experiments in duplicate or triplicate, with absence of cDNA as a negative control, using a QuantStudio 3™ (Applied Biosystems) thermal cycler. We used Luna SYBR Green Master Mix Universal kit (New England Biolabs, cat. M300L) for the relative expression assays. The cDNAs (2.5-10 ng/reaction depending on the target, table 1) were amplified under the following conditions: initial denaturation at 95°C/1 min, followed by 40 cycles of denaturation at 95°C (15 s) and annealing at 60°C (1 min). Dissociation curve analysis was performed at the end of each reaction for quality control.

Table 1. Oligonucleotides primers used in the RT-PCR and qPCR assays.

Transcript	NCBI Access no.	Sense	Sequence (5'-3')	Amplification efficiency (%)	Amplicon (bp)
Wfdc2	NM_026323.2	+	GTTGCTACTGTTCACCCCCA	104,8	189
		-	CCTTCGCTCGGTCCATTAGG		
Wfdc3	NM_027961.1	+	AGCAGCCAATCACTGTTCTC	93,7	246

		-	CCTCTCAATGCGTGCTCTCC		
Wfdc4	NM_011414.3	+	GCGGCCTTTTACCTTTCACG	97,7	248
		-	CTTCCTCCACACTGGTTTGC		
Wfdc5	NM_145369.3	+	TCTGAGCTTATTGGGTGCCA	97,9	268
		-	CCGACTTTACAAAGACACGAG		
Wfdc6a_v1	NM_001177848.1	+	CTGACAAACTCCTCCTGGTT	106,7	169
		-	CTTCACTCTGACTCTTGGGC		
Wfdc6a_v2	NM_001033240.4	+	GACCCTGGGAAAAGCTACTG	98,3	188
		-	CTGTCGGTGCCTTGTACAC		
Wfdc6b	NM_001012725.2	+	CCTCCTGGTTTAATGCCTC	98,1	140
		-	CATGTTCTTATAAAGACCCCC		
Wfdc7	NM_029325.2	+	ACTTGCTGTTTCCCAGGAGAT	103,5	204
		-	ACCAACGACGAAAGTAAGCC		
Wfdc8_v1-2-3	NM_001276432.1 NM_001080550.2 NM_001276431.1	+	CGGACTTCAACTGTAAGGCG	109	236
		-	CATGCAAGCGTTTCTACAG		
Wfdc9	NM_001160414.1	+	CTCGGAAGCCTCAAGGATGAC	99,9	271
		-	CTTAGCTGGAGCGAGTGTCC		
Wfdc10	NM_001039501.2	+	AAGGCAGAGATATGAAGCACC	98,2	188
		-	TGACAGGCATCTCCCATACG		
Wfdc11	NM_001161806.1	+	GGAAGAACAAGTATTATCCTG	97,8	134
		-	GTAGGTCCAGCAGCATGTG		
Wfdc12	NM_138684.2	+	AGTCAAAGGCGAGGAGAAAAG	110,1	159
		-	CTGTTGTCTTTCACCGGCAG		
Wfdc15a	NM_183271.2	+	CTAGGAAAGGAGTTACGCCG	100	160
		-	GTTGTCCAGGGTTCCACAC		
Wfdc15b_v1-2	NM_138685.2 NM_001045554.1	+	GCTGTAACATGGCTCGACCT	103,4	167
		-	GCGCATCTGGCAGTAGAAGA		
Wfdc16	NM_001012723.2	+	TCGGTGAGCACAGTTGTACC	103,7	110
		-	ACCCCTACCCACACCTGATT		
Il6	NM_031168.2	+	CTTAATTACACATGTTCTCTG	99,7	92
		-	CAAGTGCATCATCGTTGTTC		
Il1b	NM_008361.4	+	TGCCACCTTTTGACAGTGATG	99,8	136
		-	ATGTGCTGCTGCGAGATTTG		
Tnf	NM_001278601.1	+	CCTATGTCTCAGCCTCTTCTCA	81,9	100
		-	CTTCTCATCCCTTTGGGGAC		
Ppia	NM_008907	+	GTCTCCTTCGAGCTGTTTGC	97	150
		-	GCGTGTAAAGTCACCACCCT		
Hprt1	NM_013556.2	+	TCCATTCCTATGACTGTAGA	106,5	90
		-	ATCATCTCCACCAATAACTT		

Cytokine multiplex bead assay

Total protein extracts from initial segment and cauda epididymis from saline-control or LPS-treated mice were prepared according to Silva et al [20]. Briefly, frozen initial segment (~25 mg) and cauda epididymis (~30 mg) were homogenized using a Polytron PT 1300 D in lysis buffer (9.1 mM NaH2PO4, 1.7 mM Na2HPO4, 150 mM NaCl, 0.5% Tween-20, containing 80 µM leupeptin, 1.5 µM aprotinin, 29 µM pepstatin A, 1 mM phenylmethylsulfonil fluoride, and

0.5 mM pefabloc). After 30-min incubation on ice, samples were centrifuged twice (10,000 × g, 4°C, 10 min). Supernatants were aliquoted and stored at −80°C until use. Total protein concentration was determined with BioRad protein assay reagent (BioRad Laboratories, cat. 5000006), using bovine serum albumin as standard.

Tissue concentrations of INFγ, IL1B, IL2, IL6, IL10 and TNF were simultaneously determined by using a magnetic bead assay (Millipore, cat. MCYTOMAG-70K). All procedures were performed according to manufacturer's instructions, at room temperature and protected from light. Samples were analyzed in a Luminex MAGPIX system (Millipore). Cytokines and chemokines concentrations in each epididymal sample were calculated using Analyst software (Millipore) with a three-, four- or five-parameter logistic curve-fitting method. Data were normalized to the amount of total protein in each epididymal sample and expressed as pg of target protein/mg of tissue total protein.

Endotoxin concentration assay

Endotoxin concentrations in the initial segment and cauda epididymis were measured using the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript, cat. L00350), according to Fabienne et al. (2015). Briefly, frozen initial segment and cauda epididymis were homogenized using a Polytron PT 1300 D, in sterile saline solution (pyrogen-free). After two freeze-thaw cycles, the samples were incubated on ice for 30-min and were centrifuged (10,000 × g, 4 °C, 10 min). Supernatants were aliquoted and stored at −80 °C until use. Total protein concentration was determined as previously described.

Endotoxin concentrations (EU/ml) in the samples were determined from a standard curve using pure endotoxin standards. The detection range of this kit was 0.05 EU/ml to 0.5 EU/ml. To inactivate endotoxin neutralizing agents that may inhibit endotoxin activity in the LAL assay, we heated the diluted sample to 70°C for 10 min. The sample was then subjected to an ultrasonic bath (37°C, 10 min) to improve recovery of measurable LPS activity. One hundred microliters of the diluted sample were incubated with 100 µl of LAL reagent in pyrogen free vials for 30 min at 37°C. After incubation we added 100 µl of reconstituted chromogenic

substrate to each vial and incubated again at 37°C for 6 min. Finally, we added 500 µl of each of the three color-stabilizer buffers. Endotoxin concentration was determined by reading the absorbance at 545 nm using the standard curve as reference.

Statistical Analysis

We expressed the results as mean ± standard error of the mean (SEM). We applied the Kolmogorov-Smirnov test to evaluate the normality of the data, and F test or Brown-Forsythe test to evaluate the homoscedasticity of the data. Statistical differences between two groups were analyzed using Student's t test, while the differences between more than two groups, by One-way analysis of variance (One-way ANOVA), followed by the Bonferroni test. We considered significant the differences associated with the probability $p < 0.05$. The statistical treatment of the data was implemented by the program GraphPad Prism 5.01 (GraphPad Software).

RESULTS

Acute inflammatory responses of the mouse initial segment and cauda epididymis to interstitial and intravasal LPS stimuli.

We explored the inflammatory responses of mouse initial segment and cauda epididymis using two models of epididymitis induced by LPS injection via: 1) interstitial space of the initial segment; and 2) lumen of the vas deferens towards cauda epididymis. Using Blue Evans dye as a tracer injected into the interstitial space of the initial segment, we showed that its distribution was restricted to this region by connective tissue septae up to 90 min after injection (Supplementary Figure S1). Consistently, the Blue Evans dye injected into the lumen of the vas deferens ascended into the epididymal duct of the cauda epididymis (Supplementary Figure S1). These results showed that small molecules are distributed into the epididymis in a segment-specific fashion according to the site of injection.

We evaluated the acute inflammatory responses of the initial segment and cauda epididymis 6 h after interstitial and intravasal injection of different doses of ultrapure LPS from

E. coli (12.5, 25 or 50 µg), using the transcript levels of inflammatory cytokines *Il1b*, *Il6* and *Tnf* as inflammatory markers. Upon interstitial LPS injection, we observed that all LPS doses tested up-regulated *Il6* (19-, 22- and 23-fold change for 12.5, 25, and 50 µg, respectively) and *Tnf* (20-, 22- and 24-fold change for 12.5, 25, and 50 µg, respectively) mRNA levels in the initial segment in comparison to saline-control, whereas only the highest dose up-regulated *Il1b* mRNA levels (3-fold change) (Figure 1A). Intravasal LPS injection also up-regulated *Il6* mRNA levels in the cauda epididymis at all doses tested (11-, 10- and 16-fold change for 12.5, 25, and 50 µg, respectively), whereas only the highest dose up-regulated *Tnf* mRNA levels (3-fold change) (Figure 1B). On the other hand, only 12.5 µg intravasal LPS injection up-regulated *Il1b* mRNA levels (3-fold change) in comparison to saline-control (Figure 1B).

Using 50 µg LPS in a time-course study (2, 6, 24 and 72 h), we observed that interstitial LPS injection up-regulated *Il1b*, *Il6* and *Tnf* mRNA levels in the initial segment at all time-points analyzed (Figure 2A). Interstitial LPS challenge induced a peak in *Il6* and *Tnf* mRNA levels at 6 h, while relative *Il1b* levels peaked later at 72 h post-treatment only (Figure 2A). Interestingly, intravasal LPS injection induced time-dependent differential changes on the relative expression of these inflammatory markers in the cauda epididymis (Figure 2B). Intravasal LPS injection up-regulated *Il1b* mRNA levels 2, 24 and 72 h post-treatment in comparison to their respective saline-controls, whereas *Tnf* mRNA levels were up-regulated at 2, 6 and 24 h (Figure 2B). We observed an up-regulation of *Il6* mRNA levels at 6 and 24 h in a bell-shape pattern reaching a peak at 6 h (Figure 2B).

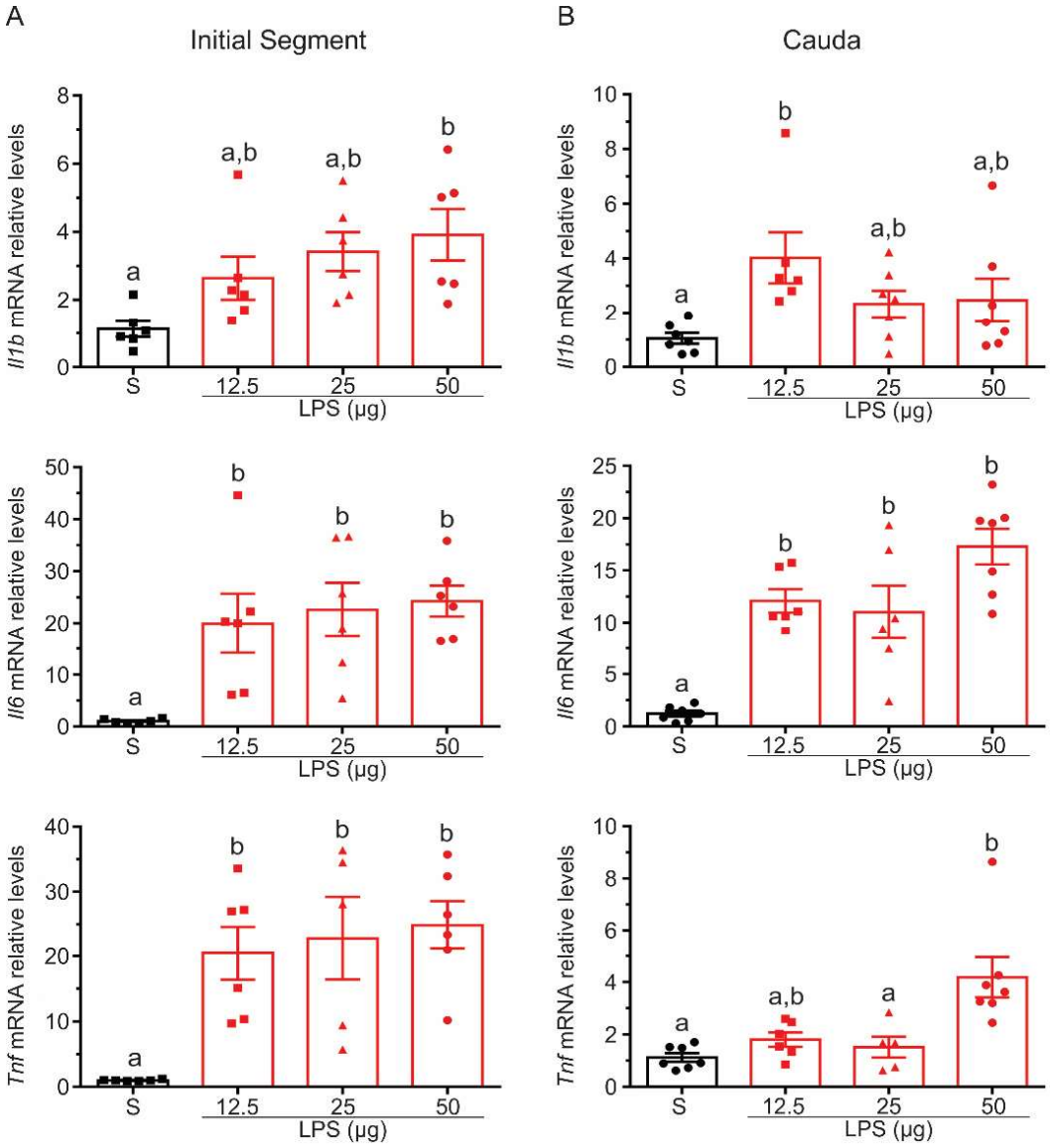

Figure 1. Effects of interstitial (A) and intravasal (B) LPS-induced epididymitis on the mRNA levels of the pro-inflammatory gene markers interleukin-1beta (*Il1b*), Interleukin-6 (*Il6*) and tumoral necrosis factor-alpha (*Tnf*). Relative quantification of *Il1b*, *Il6* and *Tnf* transcripts by RT-qPCR in the initial segment (A) and cauda epididymis (B) from saline-control (S), LPS-treated mice. Tissues were collected 6 h after treatment. Samples were normalized to their internal control (*Hprt1*) and expressed as relative values of their respective saline-control groups. Results are expressed as mean ± SEM of experiments performed in duplicate with samples from 6-7 mice per group, as indicated. Different letters indicate differences between groups ($p < 0.05$, ANOVA followed by Bonferroni test).

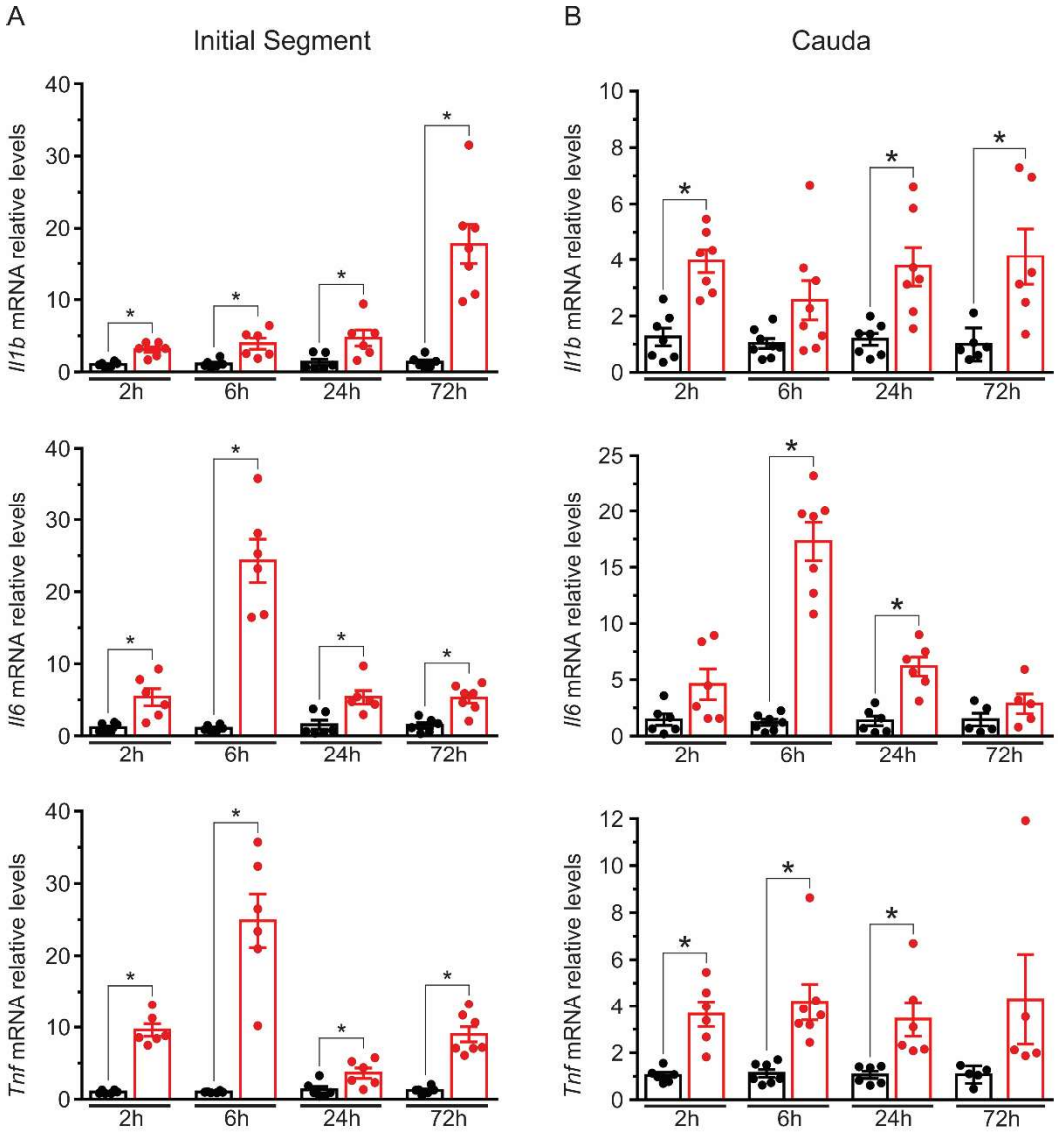

Figure 2. Time-course effects of interstitial (A) and intravasal (B) LPS-induced epididymitis on the mRNA levels of the pro-inflammatory gene markers interleukin-1beta (*Il1b*), Interleukin-6 (*Il6*) and tumoral necrosis factor-alpha (*Tnf*). Relative quantification of Il1b, Il6 and Tnf transcripts by RT-qPCR in the initial segment (A) and cauda epididymis (B) from saline-control (S), LPS-treated mice. Tissues were collected 2, 6, 24 and 72 h after treatment. Samples were normalized to their internal control (*Hprt1*) and expressed as relative values of their respective saline-control groups. Results are expressed as mean ± SEM of experiments performed in duplicate with samples from 6-8 mice per group, as indicated. Asterisks indicate differences between groups ($p < 0.05$, Student t test).

Consistently with mRNA data, we observed that interstitial LPS injection rapidly increased tissue concentrations of pro-inflammatory cytokines INFG (8-fold change), IL1B (6-fold change), IL6 (8-fold change), TNFα (23-fold change) in the initial segment in comparison to saline-control groups 6 h after challenge (Table 2). At the same time-point, intravasal LPS injection also up-regulated, but to a lesser extent tissue concentration of IL1B (2-fold change),

IL6 (3-fold change) and TNF (6-fold change), while did not change IFNG levels (Table 2). Additionally, both interstitial and intravasal LPS treatment increased tissue concentrations of the anti-inflammatory cytokine IL10 in the initial segment (9.8-fold change) and cauda epididymis (3.6-fold change), respectively (Table 2). Neither interstitial nor intravasal LPS changed IL2 production (Table 2).

Table 2. Effects of interstitial and intravasal LPS-induced epididymitis on the cytokine expression profile in the mouse initial segment and cauda epididymis.

Concentration (pg/mg of protein)	**Initial Segment**		**Cauda**	
	Saline-control (n=5)	LPS 50 µg (n=6)	Saline-control (n=5)	LPS 50 µg (n=6)
INFG	1.87 ± 0.18	15.41 ± 2.51*	4.97 ± 0.59	6.98 ± 0.85
IL1B	2.91 ± 0.23	17.09 ± 2.6*	4.97 ± 0.47	8.12 ± 0.44*
IL2	0.28 ± 0.02	0.38 ± 0.05	0.96 ± 0.1	0.97 ± 0.06
IL6	414.8 ± 76.37	3159 ± 62.97*	1656 ± 206,9	4101 ± 160.6*
IL10	1.54 ± 0.19	15.12 ± 2*	3.69 ± 0.39	13.29 ± 1.52*
TNF	0.34 ± 0.07	7.93 ± 0.4*	0.37 ± 0.02	2.38 ± 0.07*

Data represent mean ± SEM of experiments performed with samples from the indicated number of mice. Each sample was normalized to its respective total protein concentration and expressed as pg/mg of protein.

Asterisks indicate statistically different from the respective saline-control group (Student t test, $p<0.05$).

Macroscopic and histopathological analysis of the mouse epididymis upon interstitial and intravasal LPS-induced epididymitis

Next, we evaluated morphological changes in the epididymis from mice submitted to interstitial and intravasal LPS-acute epididymitis. We did not detect any obvious macroscopic changes in the epididymis from mice submitted to either epididymitis models in comparison to saline-control 6, 24 and 72 h after challenge, except by a slightly hyperemia in the initial segment 24 h upon interstitial LPS injection (Supplementary Figures S2, S3). We further observed an increase on the weight of the epididymis 6 h after interstitial LPS injection, followed by a decrease on the weight of the testis and the epididymis at 72 h (Supplementary

table S1). Conversely, intravasal LPS injection did not affect testis and epididymis weight, except by a slightly decrease on the weight of cauda epididymis 24 h after challenge (Supplementary table S1).

Our morphological analysis revealed that interstitial or intravasal sterile saline injection did not affect the histological architecture of epididymides from saline-control animals at 6, 24 and 72 h post-treatment (Figures 3 and 4). On the other hand, interstitial or intravasal LPS challenge induced signs of acute inflammation, which were mainly restricted to the segment directly exposed to the challenge and less intense in the neighbor segments (Figures 3 and 4). Upon interstitial LPS injection, the initial segment showed interstitial edema and intense interstitial, intra-epithelial and intraluminal infiltrates 24 and 72 h post-treatment (Figure 3). In intravasal LPS-treated animals, morphological changes in the cauda epididymis appeared as early as 6 h and included interstitial edema and immune cell infiltrate, which persisted until 72 h after treatment (Figure 4). At 24 and 72 h post-injection, we also observed the presence of inflammatory cells in the lumen of the cauda epididymis (Figure 4).

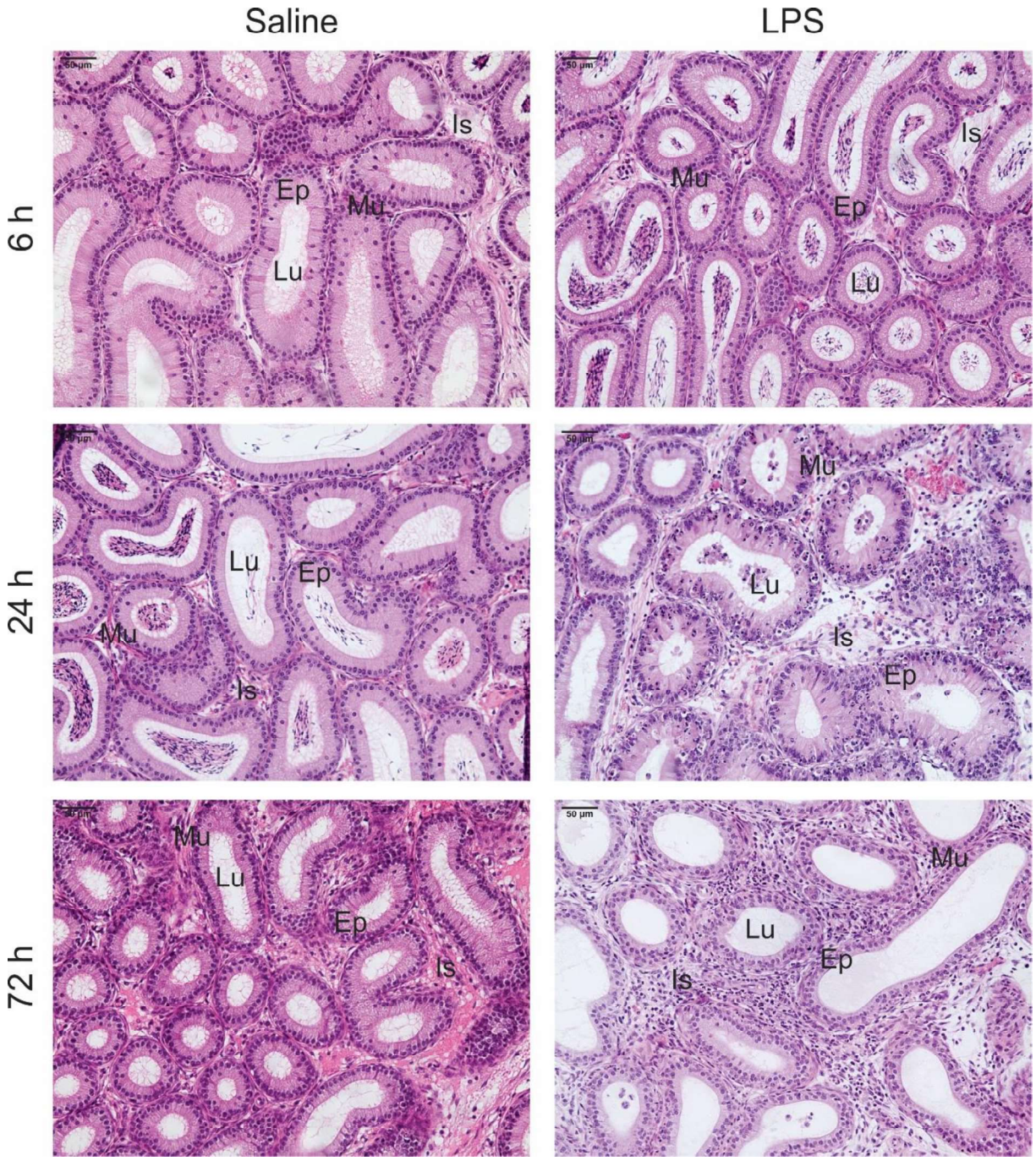

Figure 3. Effects of interstitial LPS-induced epididymitis on the morphology of the initial segment. Representative photomicrographs of epididymides collected from saline-control and LPS-treated mice 6, 24 and 72 h after treatment. Epididymal cross sections were stained with hematoxylin/eosin. Results are representative of experiments performed with tissues from three mice per group. Ep: epithelium, Is: interstitial space; Lu: lumen; Mu: smooth muscle. Scale bar = 50 µm.

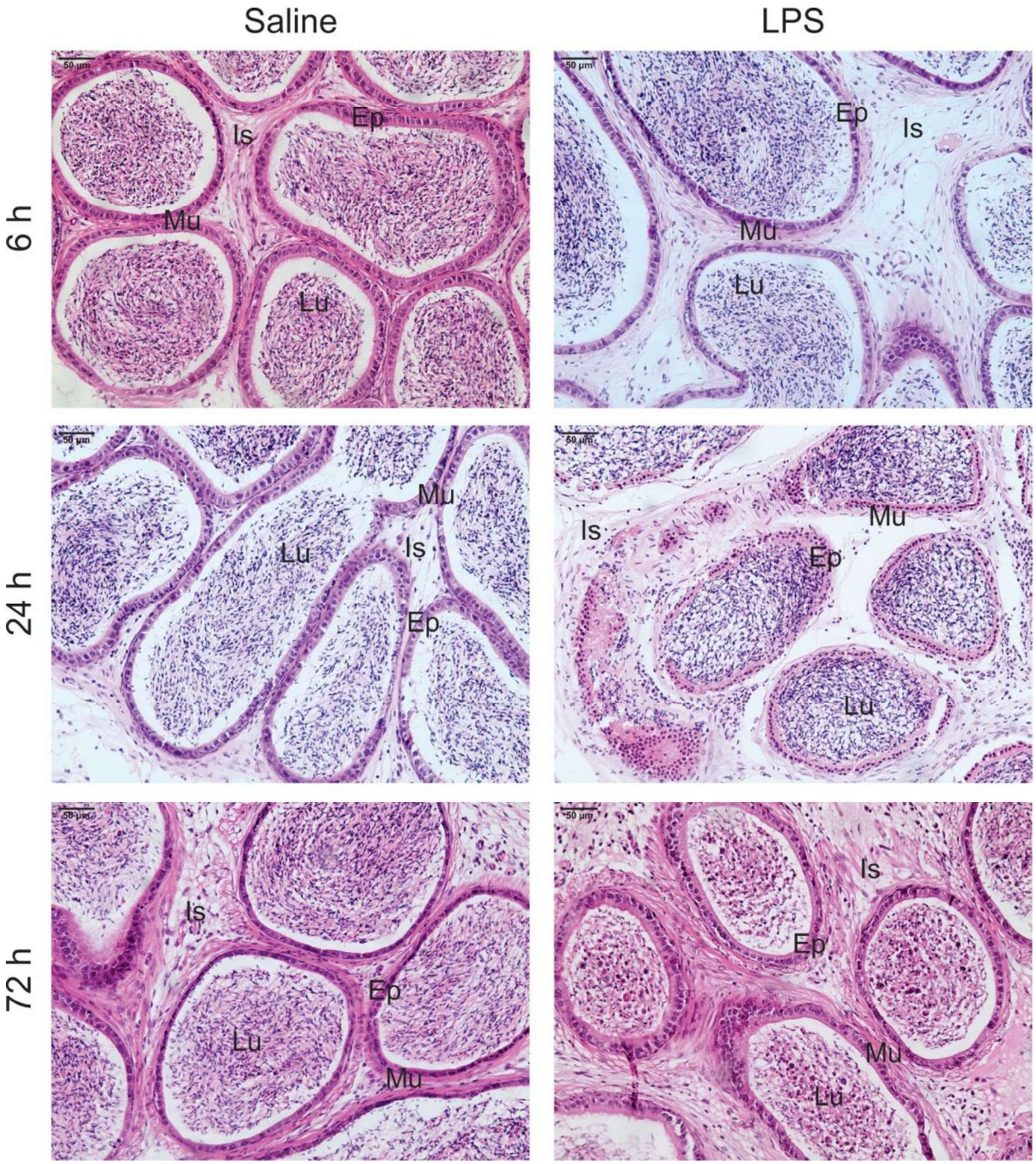

Figure 4. Effects of intravasal LPS-induced epididymitis on the morphology of the cauda epididymis. Representative photomicrographs of epididymides collected from saline-control and LPS-treated mice 6 h, 24 h and 72 h after treatment. Epididymal cross sections were stained with hematoxylin/eosin. Results are representative of experiments performed with tissues from three mice per group. Ep: epithelium, Is: interstitial space; Lu: lumen; Mu: smooth muscle. Scale bar = 50 µm.

Endotoxin clearance kinetics from the initial segment and cauda epididymis upon interstitial and intravasal LPS injection

We evaluated the clearance of LPS from the epididymis after interstitial and intravasal challenges. We observed that LPS concentrations were similar in the initial segment (65 ± 2

EU/mg of tissue) and cauda epididymis 2 h after treatment (58 ± 12 EU/mg of tissue) (Figure 5). LPS concentrations were reduced 5- and 9-fold in the initial segment and cauda epididymis, respectively, at 6 h in comparison to the earlier time-point (Figure 5). LPS clearance was not completed at 24 h, as we still detected low endotoxin levels, but above the method detection limit, in the initial segment (5.3 ± 0.4 EU/mg of tissue) and cauda epididymis (3.9 ± 0.1 EU/mg of tissue) at this time-point (Figure 5).

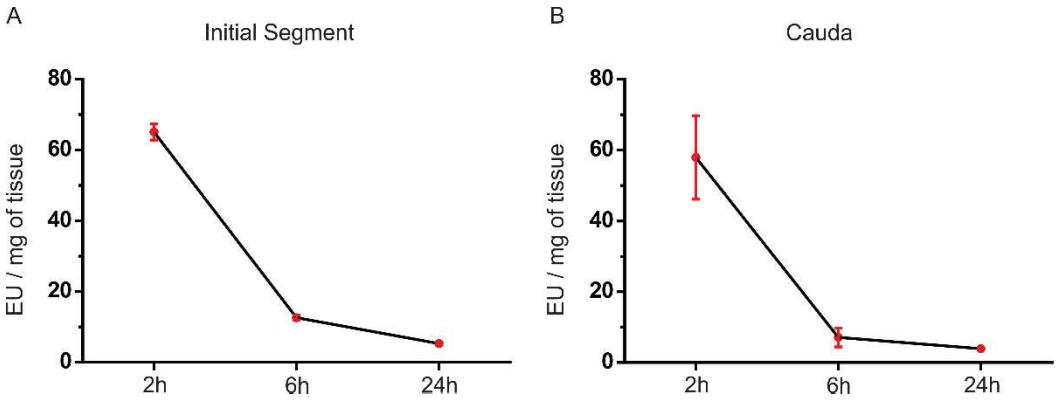

Figure 5. LPS clearance from the initial segment (A) and cauda epididymis (B) in LPS-induced experimental models of epididymitis. Tissues were harvested 2, 6 and 24 h after interstitial (A) and intravasal (B) LPS injection. Values normalized by tissue weight (mg). Results are expressed as mean ± SEM from tissues collected from 3 animals.

Basal expression profile of WFDC transcript in the mouse male reproductive tract

We performed RT-PCR analysis to characterize the constitutive expression of *Wfdc* genes in the male mouse reproductive tract under normal physiological conditions. We observed that *Wfdc* genes are differentially expressed in male reproductive tissues, most of them being abundantly expressed in the testis and epididymis (Figure 6). All *Wfdc* transcripts were detected in at least one epididymal region (Figure 6). More specifically, we detected *Wfdc2*, *Wfdc4, Wfdc6a_v1, Wfdc6a_v2, Wfdc6b, Wfdc7, Wfdc8, Wfdc9, Wfdc10, Wfdc11, Wfdc15b_v1-2* and *Wfdc16* transcripts in all epididymal regions (Figure 6). Regarding the *Wfdc* transcripts presenting a region-specific expression pattern in the epididymis, we observed that *Wfdc3* transcript was restricted to proximal epididymis (initial segment and caput), whereas *Wfdc12 and Wfdc15a* transcripts were found in distal regions (corpus and cauda). Furthermore, we found *Wfdc5* transcript in the cauda epididymis only (Figure 6).

Additionally, we found *Wfdc2*, *Wfdc3*, *Wfdc4*, *Wfdc6a_v1*, *Wfdc6a_v2*, *Wfdc6b*, *Wfdc7*, *Wfdc8*, *Wfdc10*, *Wfdc12*, *Wfdc15a* and *Wfdc16* transcripts in the testis (Figure 6). The seminal vesicle showed positive expression for *Wfdc2, Wfdc4, Wfdc6a_v1, Wfdc6a_v2, Wfdc6b, Wfdc7, Wfdc8, Wfdc9, Wfdc10, Wfdc12, Wfdc 15a* and *Wfdc15b_v1-2* transcripts (Figure 6). The prostate showed the lowest profile of *Wfdc* gene expression, being positive for *Wfdc2*, *Wfdc3*, *Wfdc4*, *Wfdc6a_v1*, *Wfdc8* and *Wfdc12* transcripts (Figure 6). Regarding the expression profile of *Wfdc* genes outside the male reproductive tract, we observed that *Wfdc2*, *Wfdc4*, *Wfdc6a_v1* and *Wfdc12* transcripts in the liver, lung and brain, whereas *Wfdc15b_v1-2* was expressed only in the liver. The expression of *Wfdc6a_v2*, *Wfdc6b*, *Wfdc8*, *Wfdc10* and *Wfdc16* was also detected in the lung (Figure 6).

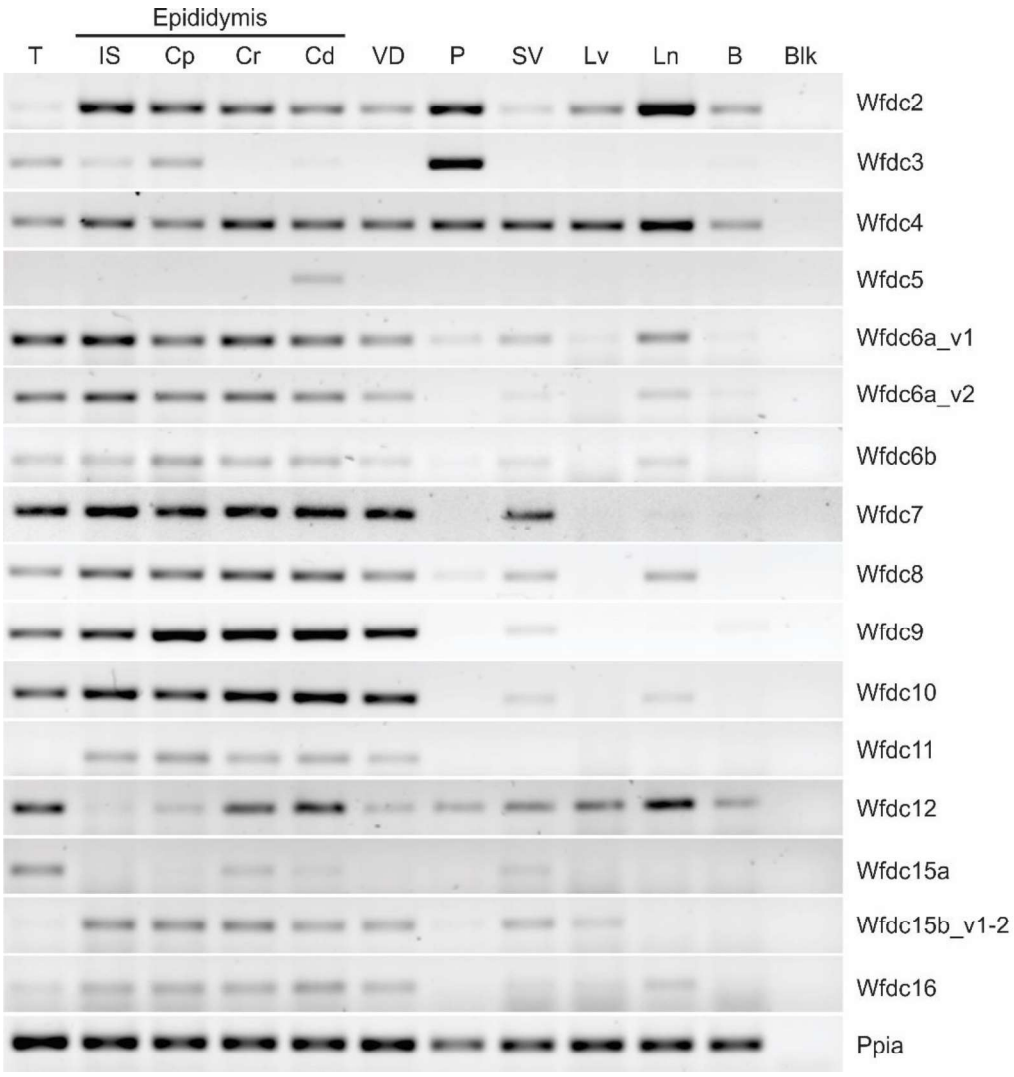

Figure 6. Expression profile of *Wfdc* genes in adult mouse reproductive and non-reproductive tissues. Representative inverted images of agarose gels showing the expression of *Wfdc2*, *Wfdc3*, *Wfdc4*, *Wfdc5*, *Wfdc6av_1*, *Wfdc6a_v2, Wfdc6b*, *Wfdc7*, *Wfdc8*, *Wfdc9*, *Wfdc10*,

Wfdc11, *Wfdc12*, *Wfdc15a*, *Wfdc15b*, and *Wfdc16* transcripts by RT-PCR. *Ppia* transcript was used as endogenous control. Testis (Te), epididymis (initial segment - IS, caput - Cp, corpus - Cr, and cauda - Cd), vas deferens (VD), seminal vesicle (VS), prostate (P), brain (B), lung (Ln), and Liver (Lv). Negative control (BLK) was performed in the absence of cDNA. Results in (A) and (C) are representative of experiments performed with samples from one mouse.

Effects of interstitial and intravasal LPS-induced epididymis on the expression of *Wfdc* genes

We performed qPCR experiments to investigate the impact of interstitial and intravasal LPS-induced acute epididymitis on the expression of *Wfdc* genes in the epididymis. Both interstitial and intravasal LPS injections did not affect the expression of *Wfdc* transcripts in the initial segment and cauda epididymis 2 h after treatment (Supplementary Figures S4, S5, S6 and S7). Interstitial LPS treatment did not change the relative transcript levels of *Wfdc6a_v1* and *Wfdc9* in the initial segment (Supplementary Figure S5). On the other hand, interstitial LPS injection down-regulated *Wfdc6a_v2* (0.36-fold change), *Wfdc7* (0.27-fold change), *Wfdc8* (0.43-fold change), *Wfdc11* (0.29-fold change), *Wfdc15b* (0.34-fold change), and *Wfdc16* (0.65-fold change) relative transcript levels in the initial segment in comparison to saline-control group 6 h post-treatment (Figure 7). The expression of these transcripts returned to saline-control levels 24 and 72 h post-treatment (Figure 7). At 24 h post-treatment, however, we observed that interstitial LPS injection up-regulated *Wfdc2* (1.3-fold change), *Wfdc3* (2.5-fold change), *Wfdc4* (1.5-fold change), *Wfdc5* (11.9-fold change), *Wfdc6b* (2.7-fold change), *Wfdc10* (1.1-fold change), *Wfdc12* (17.0-fold change), *Wfdc15a* (4.8–fold change) and *Wfdc15b_v1-2* (2.8-fold change) (Figure 8). Among these transcripts, relative levels of *Wfdc2* (2.6–fold change), *Wfdc3* (4.3-fold change), *Wfdc4* (7.2-fold change), *Wfdc5* (3.1-fold change), *Wfdc12* (1.1-fold change) and *Wfdc15a* (1.9-fold change) remained increased 72 h post-injection (Figure 7).

In the cauda epididymis, changes in the relative levels of *Wfdc* transcripts were detected later at 24 and 72 h and to a lesser extent than in the initial segment (Figure 8). Intravasal LPS treatment did not change the relative transcript levels of *Wfdc3*, *Wfdc4*, *Wfdc7*, *Wfdc8*, *Wfdc9*, *Wfdc10*, *Wfdc12* and *Wfdc15a* in the cauda epididymis at any time-point

analyzed (Supplementary Figures S4, S7). Intravasal LPS injection up-regulated *Wfdc2* (2.0-fold change), *Wfdc5* (2.0-fold change) transcripts, while down-regulated *Wfdc6b* (0.8-fold change) *Wfdc6a* (variants 1 and 2; 0.4- and 0.5-fold change, respectively), *Wfdc11* (0.7-fold change), *Wfdc15b* (0.7-fold change), and *Wfdc16* (0.8-fold change) transcripts 24 h or 72 h post-treatment in comparison to saline-control group (Figure 8).

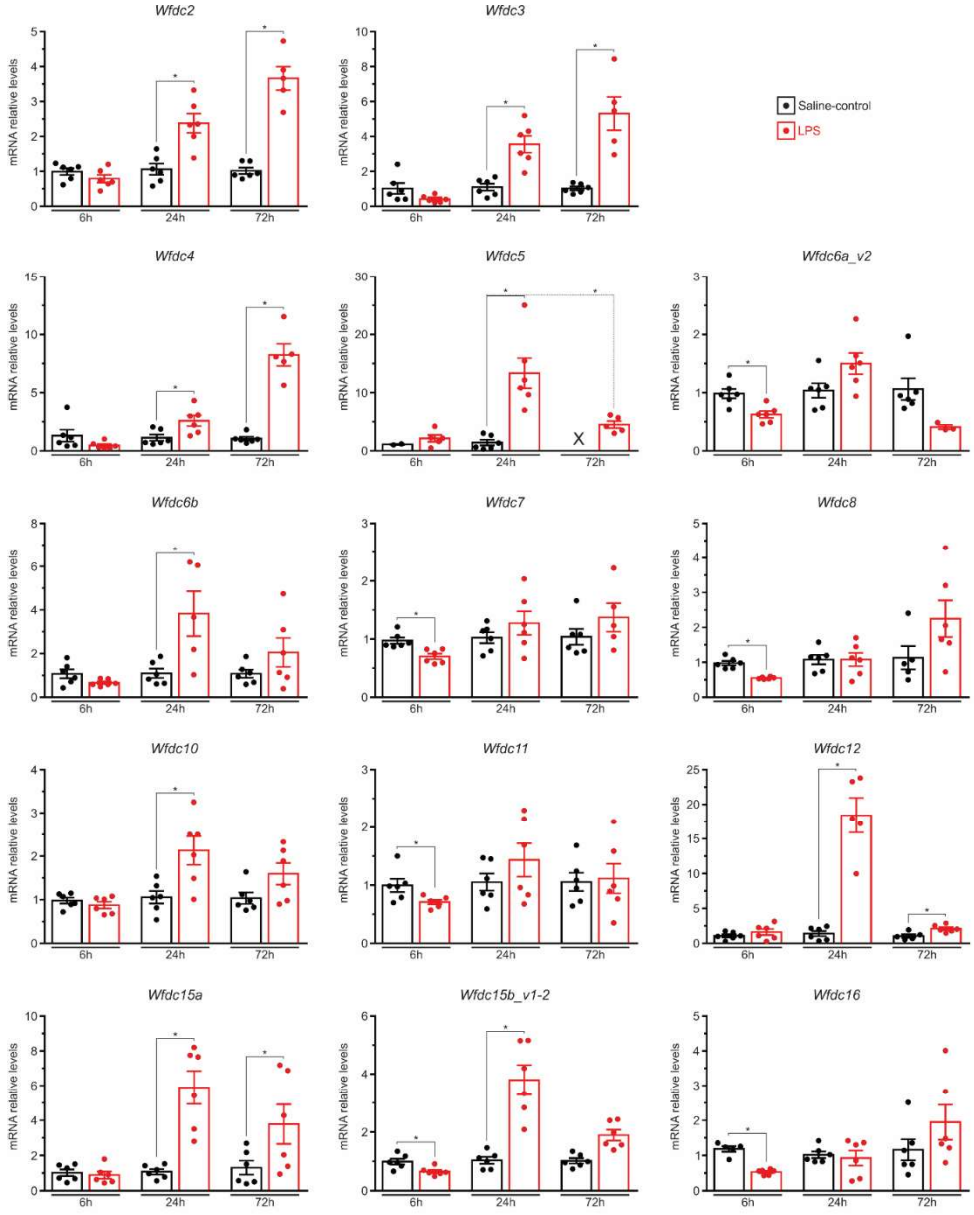

Figure 7. Effects of interstitial LPS-induced epididymitis on the relative levels of *Wfdc* transcripts in the initial segment. Relative quantitation of *Wfdc2, Wfdc3, Wfdc4, Wfdc5, Wfdc6a_v2, Wfdc6b, Wfdc7, Wfdc8, Wfdc10, Wfdc11, Wfdc12, Wfdc15a, Wfdc15b_v1-2* and Wfdc16 transcripts by RT-qPCR in the initial segment from saline-control and LPS-treated mice 6, 24 and 72 h after treatment. Data were normalized to their internal control (*Hprt1*) and expressed as relative values of their respective saline-control groups. X means not detected. Results are expressed as mean ± SEM of experiments performed in duplicate with samples

from 5-6 mice per group, as indicated. Asterisks indicate differences between groups (p <0.05, Student t test).

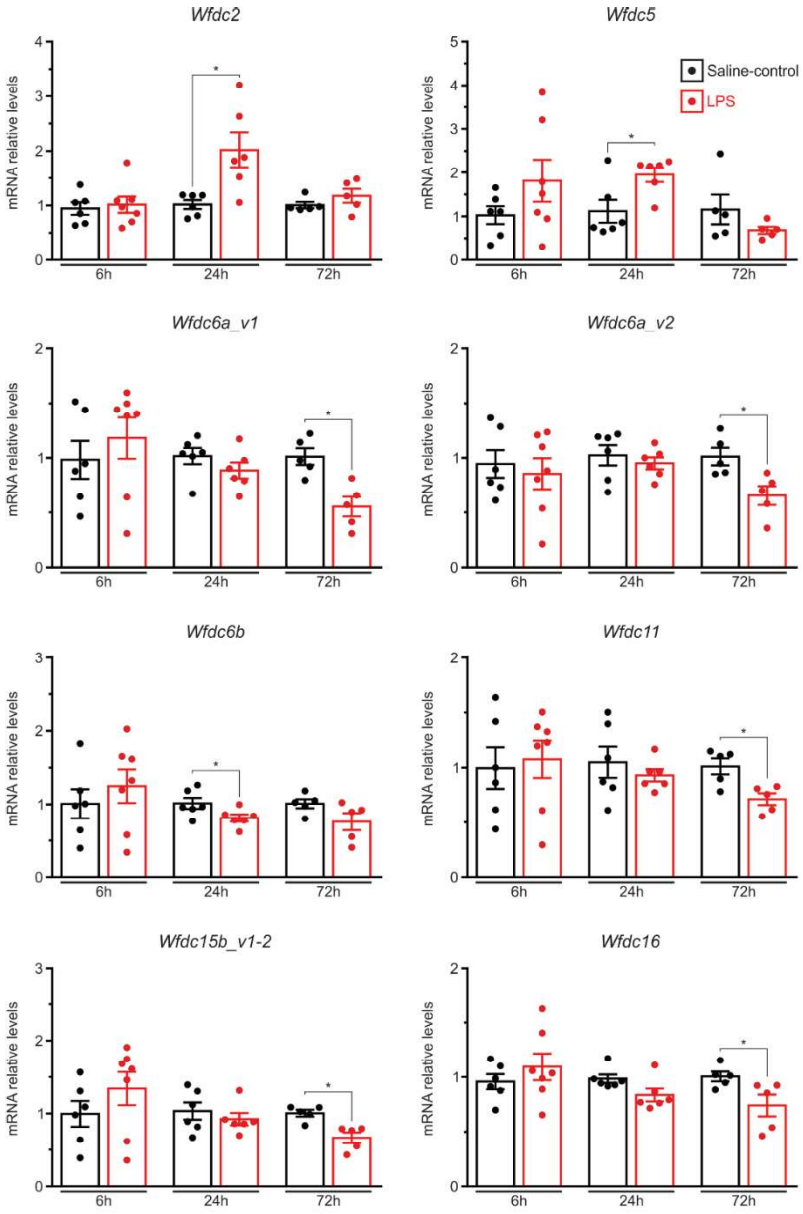

Figure 8. Effects of intravasal LPS-induced epididymitis on the relative mRNA levels of *Wfdc* transcripts in the cauda epididymis. Relative quantitation of *Wfdc2, Wfdc5, Wfdc6a_v1*, *Wfdc6a_v2, Wfdc6b*, *Wfdc11, Wfdc15b_v1-2* and *Wfdc16* transcripts by RT-qPCR in the cauda epididymis from saline-control and LPS-treated mice 6, 24 and 72 h after treatment. Data were normalized to their internal control (*Hprt1*) and expressed as relative values of their respective saline-control groups. Results are expressed as mean ± SEM of experiments performed in duplicate with samples from 5-6 mice per group, as indicated. Asterisks indicate differences between groups (p <0.05, Student t test).

Impact of inhibition on LPS-induced modulation of *Wfdc* genes in the inflamed epididymis

To investigate the involvement of NFKB activation on the LPS-induced regulation of *Wfdc* transcripts in the epididymis, we treated mice with the NFKB inhibitor PDTC (100 mg/kg, i.p.) 1 h prior inducing epididymitis via interstitial or intravasal LPS injection. Pre-treatment with PDTC prevented the up-regulation of *Il1b* and *Il6* transcript levels in the cauda epididymis 24 h after intravasal LPS challenge (Supplementary Figure S8). At this time-point, however, pre-treatment with PDCT was unable to prevent the up-regulation of these transcripts in the initial segment upon interstitial LPS injection (Supplementary Figure S8).

Pre-treatment with PDTC prevented the interstitial LPS-induced up-regulation of *Wfdc2*, *Wfdc6b*, and *Wfdc10* transcript levels in the initial segment (Figure 9). Changes in the relative levels of *Wfdc12* and *Wfdc15b* transcripts were only partially prevented by PDCT, while the relative levels of *Wfdc3*, *Wfdc4*, *Wfdc5*, and *Wfdc15a* transcripts remained elevated (Figure 9).In the cauda epididymis, pre-treatment with PDCT prior intravasal LPS injection prevented LPS-induced up-regulation of *Wfdc2* transcript levels, while partially prevented the up-regulation of *Wfdc5* transcript (Figure 10).

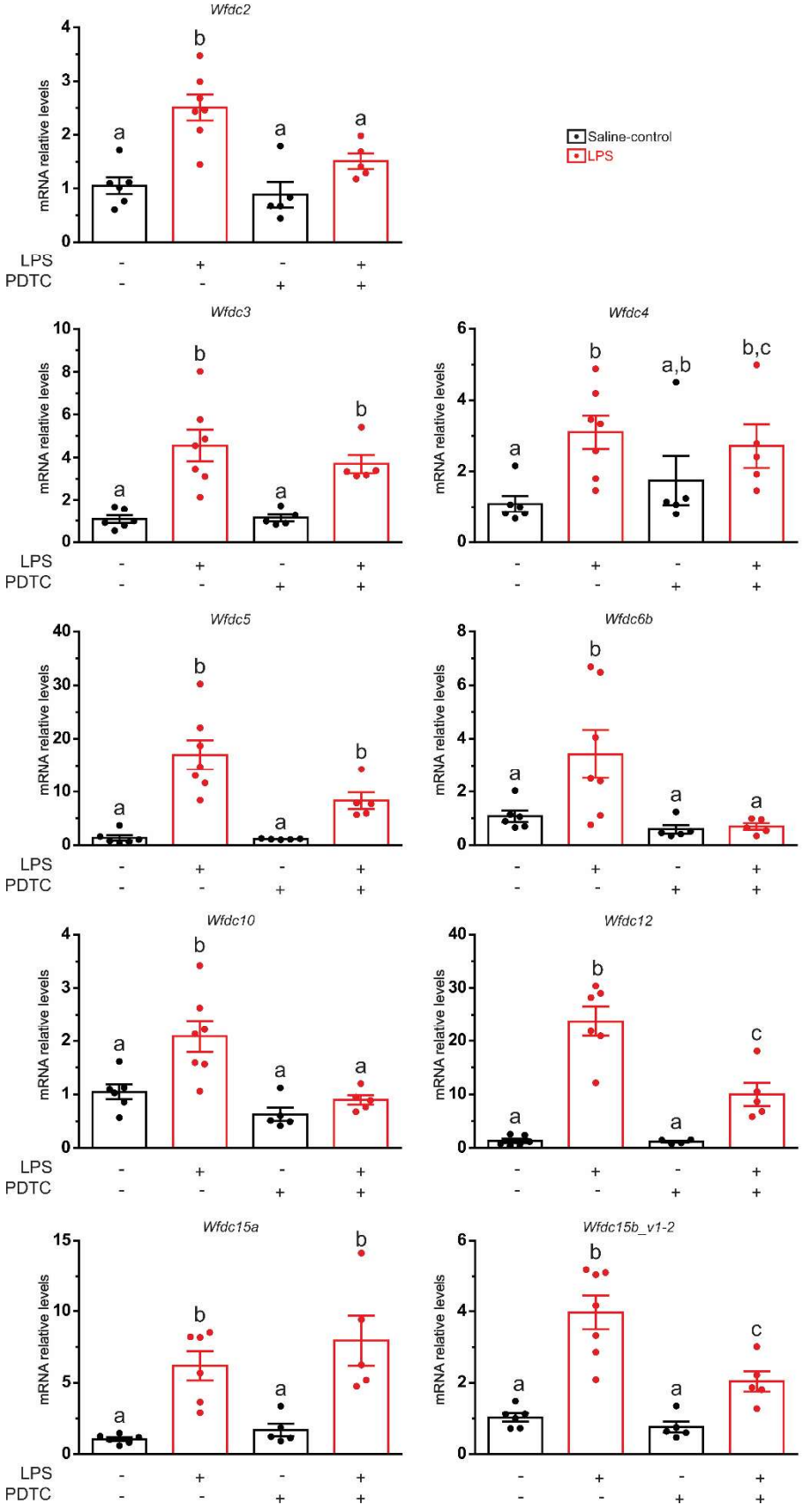

Figure 9. Effects of the NFKB inhibitor PDTC on the interstitial LPS-induced changes in the expression of *Wfdc* genes in the initial segment. Relative quantitation of *Wfdc2, Wfdc3, Wfdc4, Wfdc5, Wfdc10, Wfdc12, Wfdc15a,* and *Wfdc15b_v1-2* transcripts by RT-qPCR in the initial segment from mice pre-treated or not with PDTC (100 mg/kg, i.p.) 1 h prior interstitial saline or

LPS injection. Mice were killed 24 h after inducing epididymitis. Data were normalized to their internal control (*Hprt1*) and expressed as relative values of their respective saline-control groups. Results are expressed as mean ± SEM of experiments performed in duplicate with samples from 5-6 mice per group, as indicated. Different letters indicate differences between groups ($p < 0.05$, ANOVA followed by Bonferroni test).

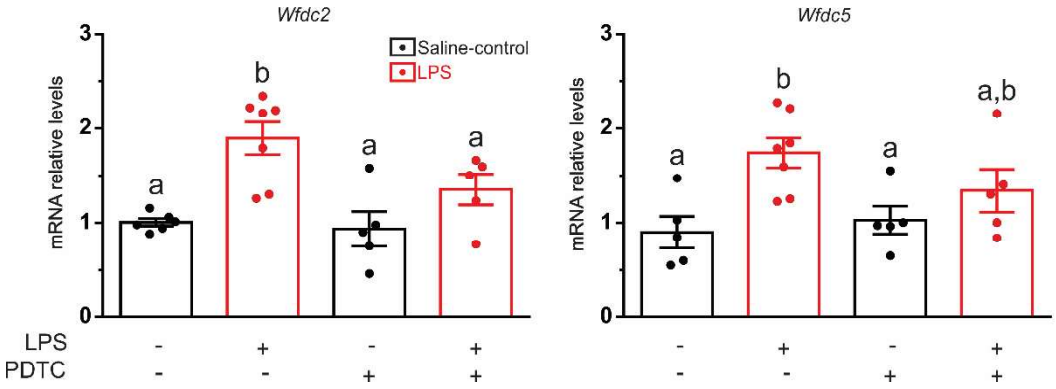

Figure 10. Effects of the NFKB inhibitor PDTC on the intravasal LPS-induced changes in the expression of *Wfdc* genes in the cauda epididymis. Relative quantitation of *Wfdc2* and *Wfdc5* transcripts by RT-qPCR in the cauda epididymis from mice pre-treated or not with PDTC (100 mg/kg, i.p.) 1 h prior intravasal saline or LPS injection. Mice were killed 24 h after inducing epididymitis. Data were normalized to their internal control (*Hprt1*) and expressed as relative values of their respective saline-control groups. Results are expressed as mean ± SEM of experiments performed in duplicate with samples from 5-7 mice per group, as indicated. Different letters indicate differences between groups ($p < 0.05$, ANOVA followed by Bonferroni test).

DISCUSSION

Inflammatory conditions of the epididymis pose serious risks to its functions and, consequently, to the reproductive health of men [11,12]. We characterized herein two mouse models of LPS-induced epididymitis to study the impact of inflammatory stimuli on the expression of members *Wfdc* of gene family in different epididymal regions. We showed that ultrapure LPS from *E. coli*, the most common etiological factor of epididymitis [12], induced an intense inflammatory response in the initial segment and cauda epididymis, which led to a differential regulation of *Wfdc* transcripts in a time- and region-dependent fashion. The regulation of *Wfdc* gene expression in the inflamed initial segment and cauda epididymis was partially dependent on NFKB activation by LPS/TLR4 signaling pathway. Our results indicated that WFDC proteins could play differential roles on innate immune responses of the epididymis

to bacterial infections, shedding new light into the contributions of antimicrobial genes to the pathophysiology of epididymitis.

Animal models of epididymitis are valuable tools for the study of its pathophysiology *in vivo*, since they produce similar features changes in the epididymis than to those observed in the clinic [10,43]. These experimental models are usually induced by the injection of pathogens or their derived-molecules directly into the epididymis via vas deferens toward the cauda (luminal route) or into the interstitial compartment of an epididymal region [10,43,44]. We found that the interstitial and intravasal LPS challenges rapidly induces an intense inflammatory reaction in the initial segment and cauda epididymis, respectively, characterized by immune cell infiltrates and interstitial edema, as well as up-regulation of inflammatory mediators at mRNA and protein levels. Our results confirmed that ability of the epididymis to generate a rapid inflammatory response to LPS, independently of its administration route, which is consistent with previous studies in the literature [20,21,23,45].

Interestingly, the inflammation of the initial segment and cauda epididymis resulted in different cytokine response patterns via up-regulation of different sets of inflammatory mediators. Indeed, interstitial LPS-induced epididymitis increased tissue concentrations of pro-inflammatory cytokines IFNG, IL1B, IL6, and TNF 6 h after challenge in the initial segment, which were consistent with changes in the mRNA profile of *Il1b*, *Il6* and *Tnf* transcripts. Conversely, intravasal LPS-induced epididymitis, induced a milder effect on the production of IL1B, IL6 and TNF, while did not change IFNG tissue concentrations. In mice, the initial segment/caput epididymis presented a larger population of resident immune cells, such as macrophages, dendritic cells and T lymphocytes, than the cauda [46], which could contribute to the more intense response of the proximal epididymis to LPS challenge. In fact, the initial segment is considered the “final barrier” protecting the testis and gamete cells to ascending pathogens, making this region a key site to the immune response in the epididymis [46]. Our study highlights the relevance of the epididymal regionalization to tissue immune responses to pathogens, warranting further investigations on how immune cells are recruited to its segments during inflammation, and its impact on fertility.

An important feature of the epididymis is the high number of innate immunity components, usually presenting androgen-dependent expression, as well as region- and cell-specific profiles [5,7,9]. Abundant expression of *WFDC* genes was detected in the human and rodent epididymis [30-33,39]. Our results confirmed these observations, as we detected basal expression of all *Wfdc* transcripts in at least one segment of the mouse epididymis under normal physiological conditions. The presence of several proteases, such as serine proteases, in the epididymis is likely to require different protease inhibitors to control their activity during sperm maturation or stress-related events, highlighting the putative roles of *WFDC* genes in epididymal function and protection [31,32]. Interestingly, we observed a variable expression profile of *Wfdc* transcripts along the epididymis. While some *Wfdc* genes, such as *Wfdc2, Wfdc6a, Wfdc7, Wfdc8, Wfdc9, Wfdc10*, *Wfdc11* and *Wfdc16* were detected in all epididymal regions, others were preferentially or exclusively detected in the proximal (*Wfdc3*) and distal (*Wfdc15a* and *Wfdc5*) regions. Despite the need to further investigate their expression at protein level in the epididymis, it is likely that this region-specific profile is related to functions in sperm protection, as well as maturation and storage, which occur in the proximal and distal regions of the epididymis, respectively [2,47].

There is overwhelming evidence indicating that epididymal antimicrobial proteins, such as β-defensins, are multifunctional elements, exhibiting additional activities related to sperm function and modulation of immune response [7,9]. This is likely to be the case for *Wfdc* genes, as it was previously shown that their protein products, such as WFDC2, WFDC4, WFDC7 and WFDC8, are microbicide agents [33,36,37,48,49], but also display immunomodulatory functions during inflammatory events [25,34,37,49,50]. Additionally, the sperm-binding ability of WFDC proteins, such as WFDC7 [30,31,39], indicates that they could further protect spermatozoa from both pathogenic and immune threats during their journey towards the egg, while playing roles on sperm function. Our results highlight the complex biological functions of WFDC proteins in different aspects of epididymal physiology.

Inflammation results in a striking disturbance of tissue homeostasis that aims to eliminate a hazardous stimulus. Under this condition, there are changes in the expression of

genes related to cell-defense mechanisms that protect cells from further damage [51,52]. In bacterial-induced epididymitis, understanding how LPS and other bacterial PAMPs affect the expression of *WFDC* genes helps us to pinpoint their contributions to tissue defense mechanisms against infections of the male reproductive tract. Our study suggests that some members of the WFDC family may be associated with epididymal protection during bacterial infection playing roles as tools to kill bacteria that reach the organ via luminal or interstitial compartments, while preventing epididymal cell damage due to acute inflammation. Consistently with this hypothesis, it has been demonstrated that WFDC7 (EPPIN), which display potent bactericidal activity, was up-regulated in the lung exposed to LPS and in turn inhibit neutrophil recruitment [50]. WFDC4 and WFDC12 expression also increased in inflammatory lung diseases, indicating their involvement in inflammatory disorders [53,54]. Interestingly, our results showed a transient down-regulation of some *Wfdc* transcripts, such as *Wfdc*7, *Wfdc8* and *Wfdc11*, in the initial segment 6 h after interstitial LPS stimulus, which could contribute to the initial inflammatory response. Indeed, the initial segment was more sensitivity to LPS in comparison to the cauda epididymis, where no *Wfdc* mRNA levels were affected at this time-point. The increased expression of other *Wfdc* transcripts, such as *Wfdc2*, *Wfdc4* and *Wfdc12*, at later time-points, on the other hand, may indicate their involvement in the homeostasis recovery of both initial segment and cauda epididymis. Indeed, inflammation-induced NFKB activation was inhibited by recombinant WFDC4, WFDC7 and WFDC12 in experimental models of lung inflammation [38,55,56]. Interestingly, *Wfdc5*, which has no defined function yet [40] and showed a cauda epididymis-specific expression under normal physiological conditions, was strongly up-regulated in the initial segment after LPS treatment suggesting that it could play important roles in immune events in this region during acute inflammation. Although many WFDC proteins do not have defined functions yet, such as WFDC5, WFDC9, WFDC10, and WFDC11 [40], our results suggest that they play roles both as antimicrobial and anti-inflammatory molecules in the inflamed epididymis.

Considering that activation of TLR4 by LPS lead to NFKB activation in the epididymis [45], this transcription factor emerges as a modulator of *Wfdc* gene expression in this organ

following an inflammatory stimulus. To address this hypothesis, we treated mice with PDTC, a NFKB inhibitor, previous interstitial and intravasal LPS injections. Our data pointed that LPS-induced up-regulation of *Wfdc2*, *Wfdc6b, Wfdc10* was NFKB-dependent. On the other hand, NFKB inhibition did not change the effects of LPS stimulation on the expression of *Wfdc3*, *Wfdc4* and *Wfdc15a*, indicating that other transcription factors are involved in their up-regulation by LPS. Furthermore, the NFKB-dependence of *Wfdc5* was region-specific, as its up-regulation was not affected by PDCT in the initial segment, but partially blocked in the cauda region. Additional experiments will be required to pinpoint the precise molecular mechanisms responsible for the differential expression profile of *Wfdc* genes in the initial segment and cauda epididymis during acute inflammation.

In conclusion, we show that the epididymis rapidly responds a bacterial-derived product via luminal or interstitial routes, but with different intensity and progression according to stimulated regions. The differential expression of the *Wfdc* genes indicates a role of this family in combating bacterial infection while keeping inflammation under control and helping restoring epididymis homeostasis. Considering potential physiopathological functions of WFDC proteins in epididymitis associated with their ability to be developed as drug targets, these proteins could emerge as putative targets to adjuvant therapies for epididymitis.

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SUPPLEMENTARY MATERIAL

Region-specific regulation of Wfdc gene expression in the mouse epididymis to intravasal and interstitial lipopolysaccharide-induced epididymitis

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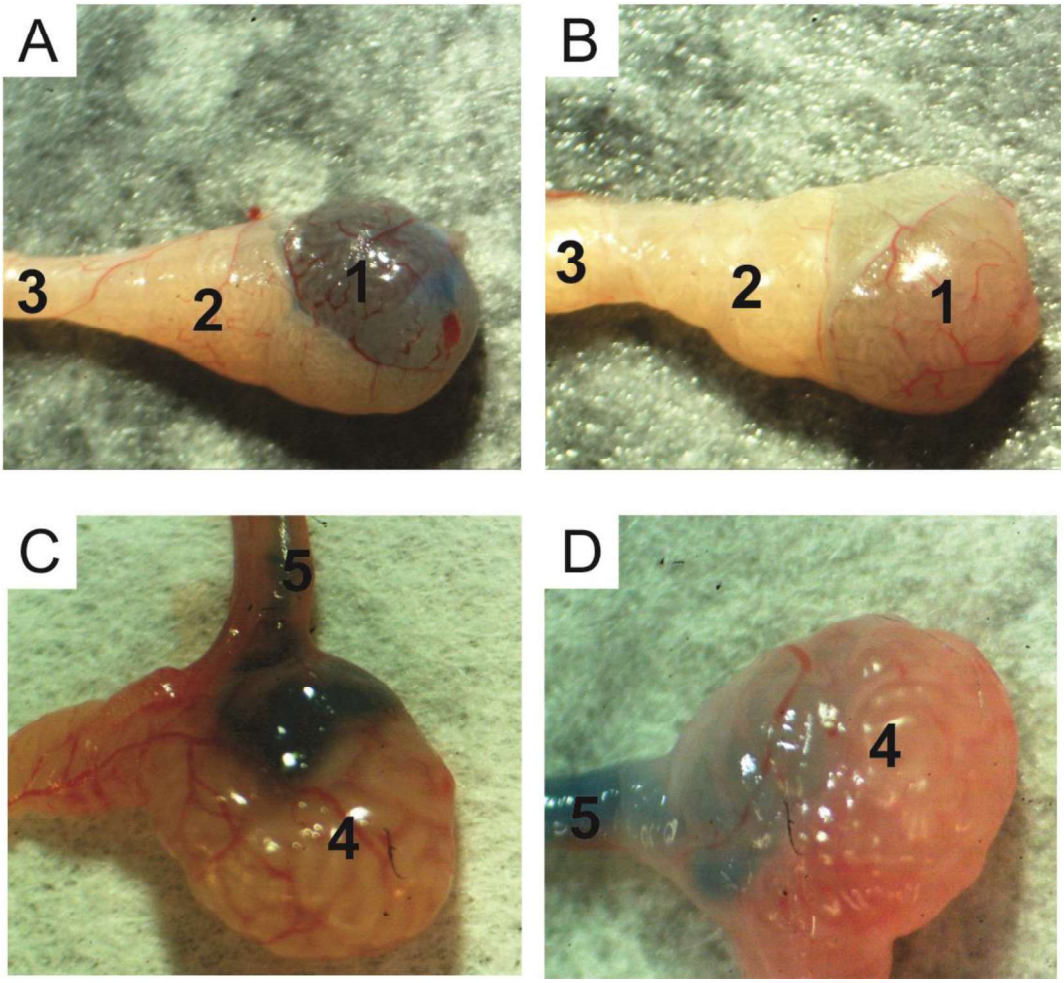
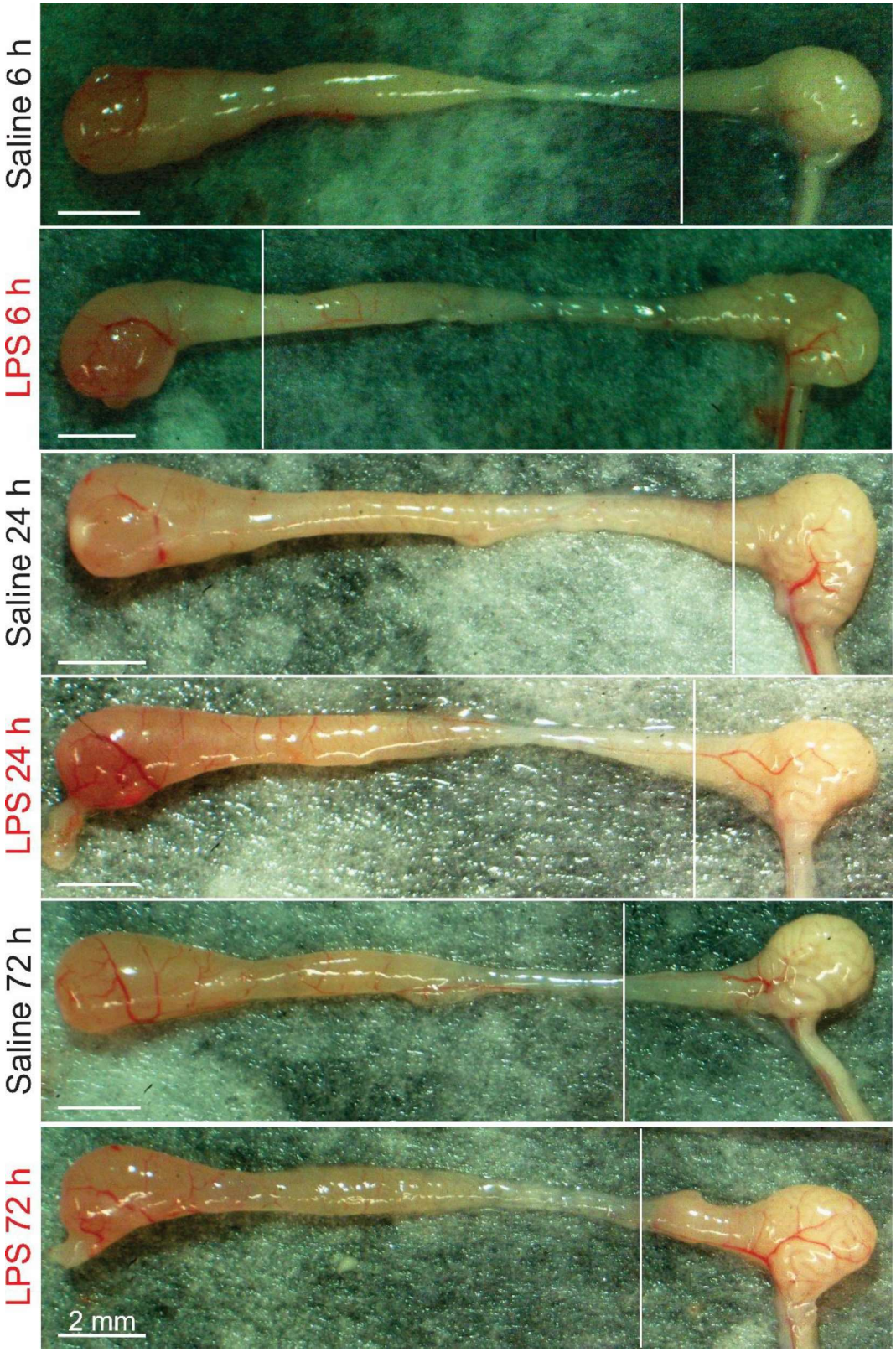
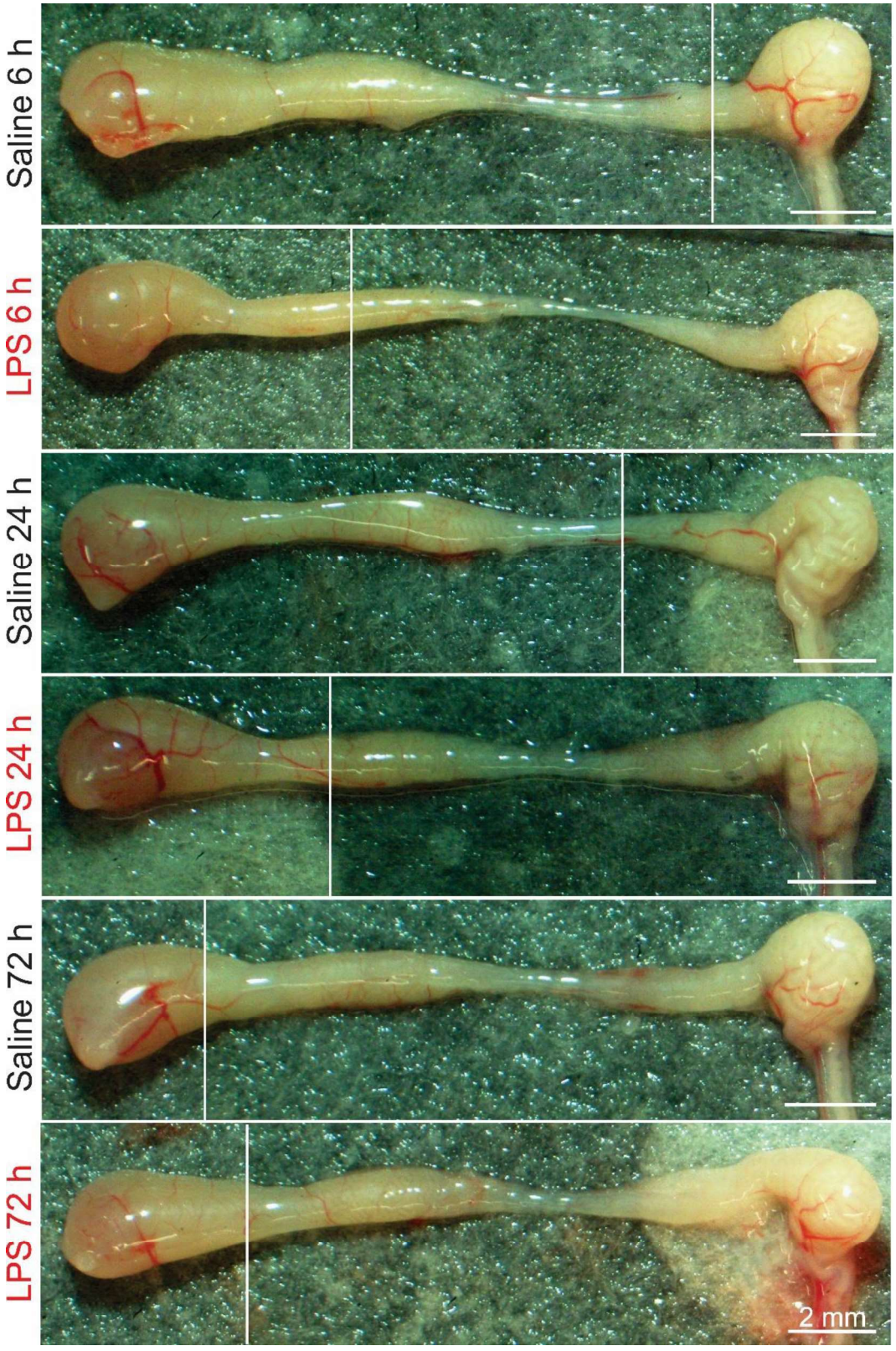
supplementary figures and tables



Supplementary Figure S1. Interstitial (A, B) and intravasal (C, D) injection for epididymitis induction in mice. Distribution of Blue Evans dye in the mouse initial segment 60 (A) and 90 (B) min after its interstitial injection. Distribution of Blue Evans dye in the mouse cauda epididymis 5 (C) and 15 (D) min after its retrograde injection into the lumen of the vas deferens. 1: Initial segment, 2: caput epididymis, 3: corpus epididymis, 4: cauda epididymis, 5: vas deferens.



Supplementary Figure S2. Gross anatomy of epididymides from mice submitted to the interstitial LPS-induced epididymitis. Typical appearance of epididymis from saline- and LPS-treated mice 6, 24 and 72 h after treatment.

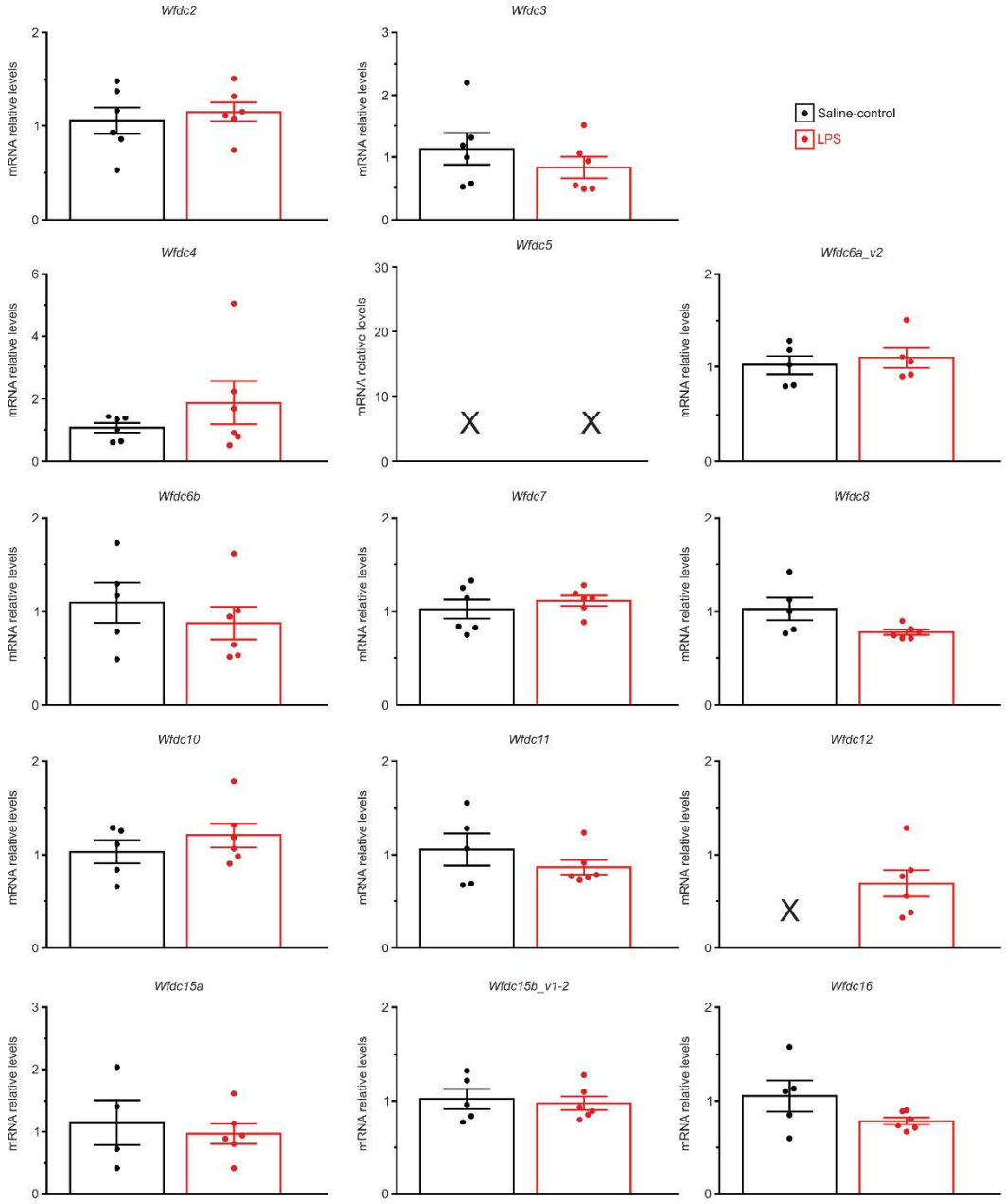


Supplementary Figure S3. Gross anatomy of epididymides from mice submitted to the intravasal LPS-induced epididymitis. Typical appearance of epididymis from saline- and LPS-treated mice 6, 24 and 72 h after treatment.

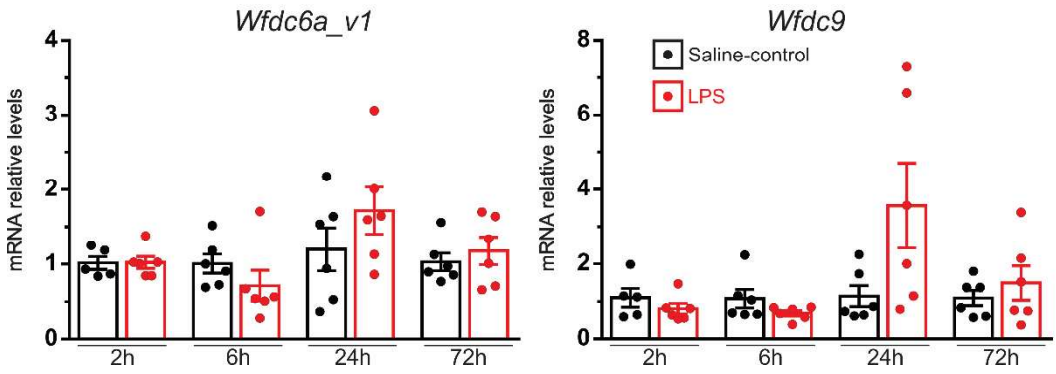
Supplementary table S1. Effects of interstitial and intravasal LPS-induced epididymitis on relative mass of testis and epididymis (initial segment, caput, corpus and cauda).

	Experimental group	Time (h)	Relative organ weight (mg/g body weight)					
			Testis	Epididymis	Initial Segment	Caput	Corpus	Cauda
Interstitial injection	Saline-control	2	8,03 ± 0,16	3,55 ± 0,12	0,74 ± 0,01	1,02 ± 0,07	0,81 ± 0,04	0,98 ± 0,03
	LPS		7,85 ± 0,20	3,40 ± 0,08	0,71 ± 0,01	0,94 ± 0,04	0,79 ± 0,03	0,96 ± 0,04
	Saline-control	6	7,77 ± 0,25	3,50 ± 0,12	0,69 ± 0,03	1,14 ± 0,07	0,76 ± 0,02	0,89 ± 0,04
	LPS		8,14 ± 0,29	3,90 ± 0,07*	0,84 ± 0,04*	1,19 ± 0,02	0,86 ± 0,03*	1,01 ± 0,03*
	Saline-control	24	8,51 ± 0,40	3,54 ± 0,09	0,76 ± 0,03	1,09 ± 0,02	0,84 ± 0,03	0,85 ± 0,04
	LPS		8,22 ± 0,41	3,54 ± 0,09	0,78 ± 0,02	1,04 ± 0,02	0,82 ± 0,03	0,89 ± 0,05
	Saline-control	72	9,63 ± 0,69	3,93 ± 0,19	0,90 ± 0,06	1,15 ± 0,04	0,87 ± 0,07	0,99 ± 0,05
	LPS		7,90 ± 0,30*	3,29 ± 0,18*	0,69 ± 0,04*	0,89 ± 0,03*	0,79 ± 0,06	0,87 ± 0,06
Intravasal injection	Saline-control	2	7,73 ± 0,13	3,60 ± 0,08	0,83 ± 0,03	1,06 ± 0,03	0,79 ± 0,02	0,91 ± 0,02
	LPS		7,68 ± 0,14	3,50 ± 0,08	0,76 ± 0,02	1,05 ± 0,04	0,78 ± 0,02	0,90 ± 0,04
	Saline-control	6	7,78 ± 0,34	4,65 ± 0,83	1,14 ± 0,39	1,51 ± 0,29	0,86 ± 0,14	1,14 ± 0,13
	LPS		8,37 ± 0,24	3,97 ± 0,19	0,79 ± 0,05	1,17 ± 0,11	0,90 ± 0,04	1,10 ± 0,06
	Saline-control	24	8,29 ± 0,25	3,89 ± 0,13	0,83 ± 0,05	1,14 ± 0,04	0,91 ± 0,04	1,01 ± 0,03
	LPS		8,36 ± 0,21	3,71 ± 0,07	0,90 ± 0,09	1,05 ± 0,06	0,87 ± 0,05	0,89 ± 0,03*
	Saline-control	72	7,82 ± 0,34	3,69 ± 0,13	0,82 ± 0,03	1,07 ± 0,04	0,82 ± 0,03	0,99 ± 0,04
	LPS		7,74 ± 0,37	3,69 ± 0,17	0,79 ± 0,03	1,10 ± 0,06	0,82 ± 0,03	0,97 ± 0,07

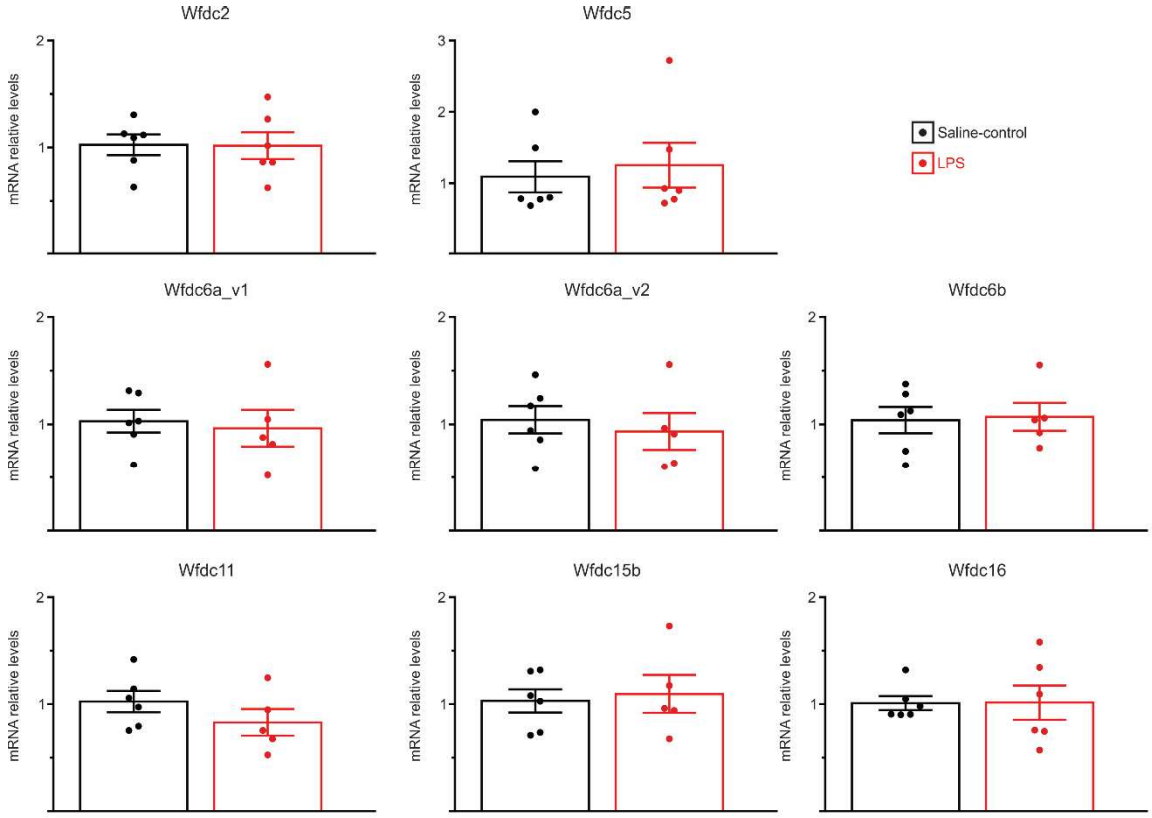
Results represent mean ± standard error of the mean (n = 6-8 animals per group). Organ mass was normalized by each animal's body mass and expressed as mg tissue/g body mass. The organs were collected 2, 6, 24 and 72 h after epididymitis induction. Asterisks indicate differences between groups ($p < 0.05$, Student t test).



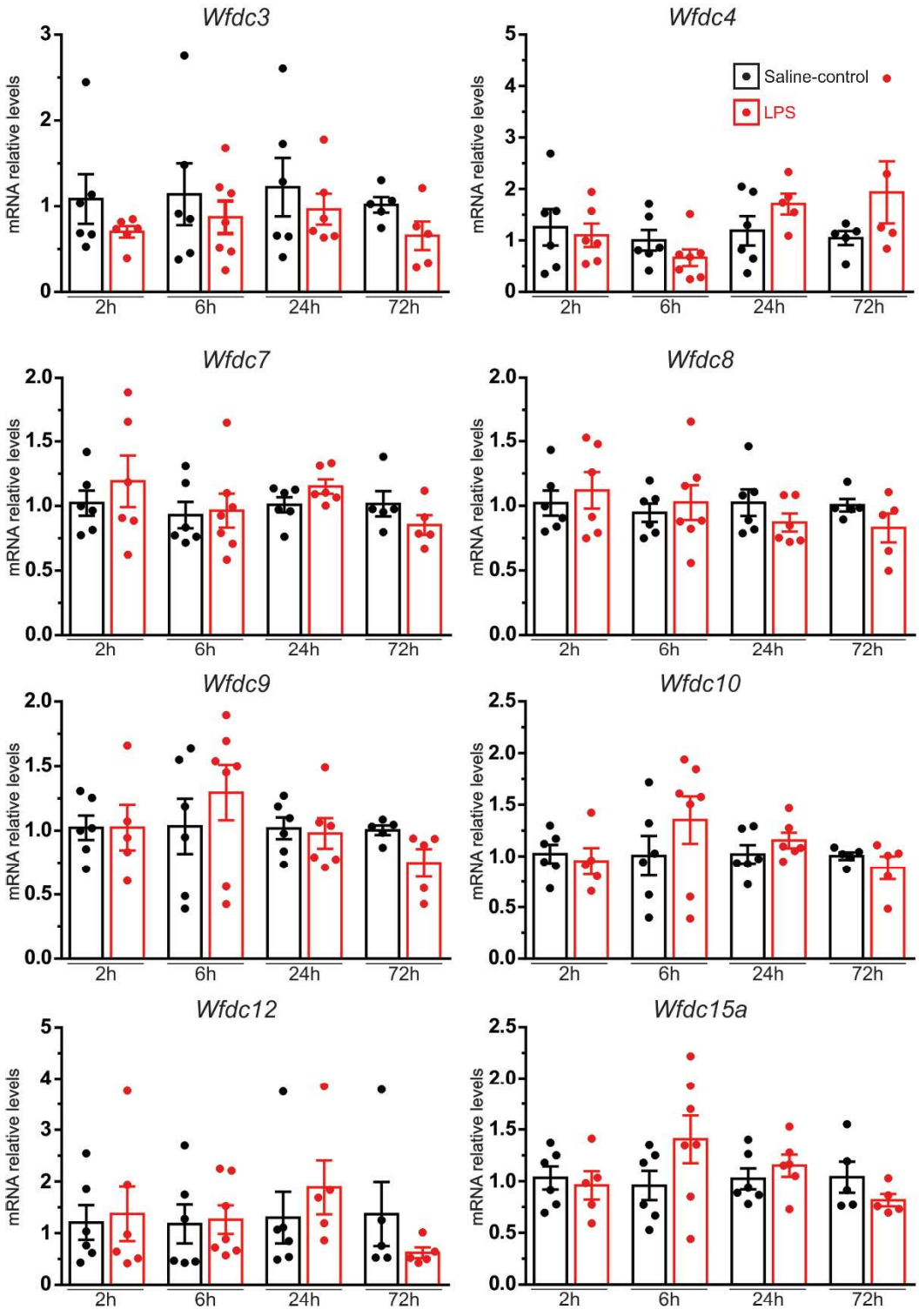
Supplementary Figure S4. Effects of interstitial LPS-induced epididymitis on the relative levels of *Wfdc* transcripts in the initial segment. Relative quantitation of *Wfdc2, Wfdc3, Wfdc4, Wfdc5, Wfdc6a_v2, Wfdc6b, Wfdc7, Wfdc8, Wfdc10, Wfdc11, Wfdc12, Wfdc15a, Wfdc15b_v1-2* and Wfdc16 transcripts by RT-qPCR in the initial segment from saline-control and LPS-treated mice 2 h after treatment. Data were normalized to their internal control (*Hprt1*) and expressed as relative values of their respective saline-control groups. X means not detected. Results are expressed as mean ± SEM of experiments performed in duplicate with samples from 5-6 mice per group, as indicated.



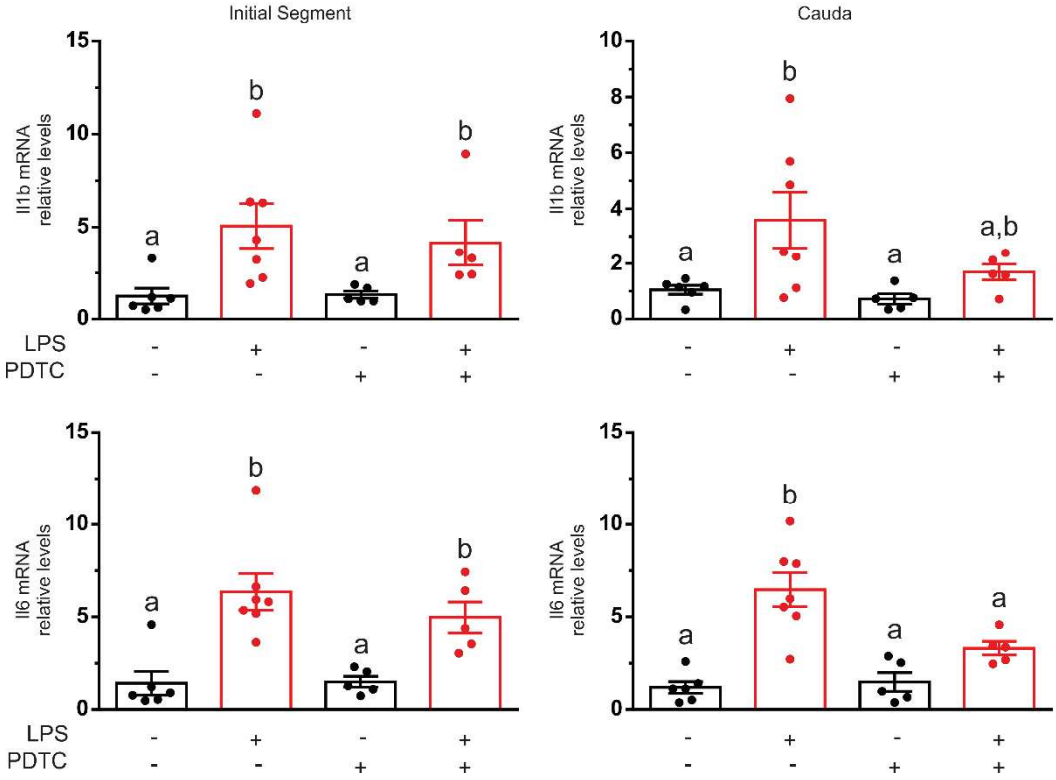
Supplementary Figure S5 Effects of interstitial LPS-induced epididymitis on the relative levels of *Wfdc6a_v1* and *Wfdc9* transcripts in the initial segment. Relative quantitation of *Wfdc6a_v1* and *Wfdc9* transcripts by RT-qPCR in the initial segment from saline-control and LPS-treated mice 2, 6, 24 and 72 h after treatment. Data were normalized to their internal control (*Hprt1*) and expressed as relative values of their respective saline-control groups. Results are expressed as mean ± SEM of experiments performed in duplicate with samples from 5-6 mice per group, as indicated.



Supplementary Figure S6. Effects of intravasal LPS-induced epididymitis on the relative levels of *Wfdc* transcripts in the cauda epididymis. Relative quantitation of *Wfdc2, Wfdc5, Wfdc6a_v1, Wfdc6a_v2, Wfdc6b, Wfdc11, Wfdc15b,* and *Wfdc16* transcripts by RT-qPCR in the cauda epididymis from saline-control and LPS-treated mice 2 h after treatment. Data were normalized to their internal control (*Hprt1*) and expressed as relative values of their respective saline-control groups. Results are expressed as mean ± SEM of experiments performed in duplicate with samples from 5-6 mice per group, as indicated.



Supplementary Figure S7. Effects of intravasal LPS-induced epididymitis on the relative levels of *Wfdc* transcripts in the cauda epididymis. Relative quantitation of *Wfdc3, Wfdc4, Wfdc7, Wfdc8, Wfdc9, Wfdc10, Wfdc12,* and *Wfdc15a* transcripts by RT-qPCR in the cauda epididymis from saline-control and LPS-treated mice 2, 6, 24 and 72 h after treatment. Data were normalized to their internal control (*Hprt1*) and expressed as relative values of their respective saline-control groups. Results are expressed as mean ± SEM of experiments performed in duplicate with samples from 5-7 mice per group, as indicated.



Supplementary Figure S8. Effects of the NFKB inhibitor PDTC on the interstitial (left panel) and intravasal (right panel) LPS-induced changes in the expression of pro-inflammatory gene markers interleukin-1beta (Il1b) and Interleukin-6 (Il6) in the initial segment and cauda epididymis. Relative quantitation of *Il1b* and *Il6* transcripts by RT-qPCR in the initial segment and cauda epididymis from mice pre-treated or not with PDTC (100 mg/kg, i.p.) 1 h prior saline or LPS injection. Mice were killed 24 h after inducing epididymitis. Data were normalized to their internal control (*Hprt1*) and expressed as relative values of their respective saline-control groups. Results are expressed as mean ± SEM of experiments performed in duplicate with samples from 5-7 mice per group, as indicated. Different letters indicate differences between groups ($p < 0.05$, ANOVA followed by Bonferroni test).

Capítulo 3

Efeito da epididimite intersticial e intraductal induzida por LPS sobre a motilidade espermática: consequências para a função do espermatozoide.

É conhecido que durante o processo de maturação os espermatozoides passam por mudanças no seu perfil proteico, devido, em parte, à atividade secretora do epitélio epididimário (Hinton & Robaire, 2015). Dessa forma, novas proteínas são adicionadas à superfície do espermatozoide durante a sua passagem pelo epidídimo, as quais podem atuar em processos associados à função espermática (motilidade ou fertilização do oócito, por exemplo) ou de proteção contra ameaças patogênicas (Hinton & Robaire, 2015). Vale ressaltar que uma das funções primordiais do epidídimo é transformar o espermatozoide imóvel em uma célula móvel, capaz de se movimentar ativamente pelo trato reprodutor feminino para cumprir sua função primordial: entregar o material genético do macho ao oócito.

A motilidade espermática é definida como a propagação de ondas transversais ao longo do flagelo no sentido proximal-distal em relação à cabeça do espermatozoide, gerando uma propulsão que o move em diferentes direções (Turner, 2005). De acordo com o tipo de movimento do espermatozoide, os parâmetros de cinemática podem auxiliar na identificação de trajetórias progressivas (mais lineares), associadas à motilidade ativada, e/ou vigorosas (menos lineares), associadas à motilidade hiperativada (Mortimer, 2000).

A partir dos dados levantados no capítulo 2, buscamos saber se as diferenças na reação inflamatória das diferentes regiões do epidídimo ao LPS poderiam resultar em diferenças na motilidade espermática. Como o epidídimo possui o papel crucial na reprodução, com a maturação, a proteção e o armazenamento dos espermatozoides antes da ejaculação (Silva et al., 2018), abordamos esse tópico avaliando a motilidade dos espermatozoides após induzir a epididimite com LPS pelas vias intersticial e intravasal.

Materiais e métodos

A análise da motilidade dos espermatozoides da cauda do epidídimo foi realizada pela plataforma CASA (*computer-assisted sperm analysis*), utilizando o sistema CEROS II equipado com um aquecedor de estágio miniTherm (Hamilton-Thorne, Bervely, MA, EUA).

Camundongos (n= 5/por grupo) foram submetidos aos modelos de epididimite induzidas pela injeção intersticial e intraductal de LPS ultrapuro, conforme descrito no capítulo 2. Os camundongos foram eutanasiados 1 ou 7 dias após a indução da epididimite.

Para o isolamento dos espermatozoides, a cauda do epidídimo foi dissecada, lavada com salina estéril e colocado em 1,7 ml de meio HTF (Irvine Scientific, cat. 90125) suplementado com 0,75% de BSA a 37°C sob 5% de CO_2 e ar. Três pequenos cortes foram cuidadosamente feitos na cauda do epidídimo permitindo a liberação dos espermatozoides no meio. Após 10 min de incubação, o tecido foi removido e alíquotas da suspensão de espermatozoides foram diluídas 1:5 a 1:25 em meio fresco.

Uma alíquota de 16 µl da suspensão de espermatozoides diluída foi colocada em lâminas de 80 µm 2XCELL (Hamilton Thorne) para avaliação da motilidade espermática. Os traçados dos espermatozoides foram capturados a 37°C com uma aquisição de taxa de quadros de 60 Hz por 1,5 s (90 quadros capturados) e objetiva de contraste de fase negativa de 4X usando a configuração padrão do instrumento. Registramos pelo menos 200 espermatozoides e 10 campos para cada amostra analisada e cada tempo analisado (0 e 60 minutos). Os vídeos gerados foram analisados identificando e deletando trajetos de espermatozoides com colisões ou falso-negativos. Registramos as trajetórias e os parâmetros cinemáticos individualmente e removemos os trajetos espermáticos com menos de 40 pontos de aquisição. Os seguintes parâmetros cinemáticos foram registrados: VAP= velocidade média da trajetória (µm/s); VSL= velocidade linear (µm/s); VCL= velocidade curvilínea (µm/s); STR= retilinearidade (%) e LIN= linearidade (%). Os espermatozoides foram classificados como móveis, progressivos (VAP≥50 µm/s e STR≥50%), lentos (VAP≤30 µm/s e VSL≤20 µm/s) e estáticos. Os espermatozoides foram classificados como hiperativados quando VCL≥180 µm/s, LIN<23,6% e ALH≥9,5& (ALH= amplitude lateral de deslocamento da cabeça).

Análise estatística

Os resultados foram expressos como média ± erro padrão da média (EPM) ou mediana. As diferenças estatísticas foram analisadas pelo teste t de Student, para os dados com distribuição paramétrica, e Mann-Whitney, para os dados com distribuição não-paramétrica. Foram consideradas significativas as diferenças associadas à probabilidade $p < 0,05$. O tratamento estatístico dos dados foi operacionalizado pelo programa GraphPad Prism 6 (GraphPad Software).

Resultados e Discussão

Nessa etapa utilizamos espermatozoides isolados da cauda do epidídimo para analisar o impacto dos dois diferentes modelos de epididimite induzida por LPS (intersticial e intraductal) sobre os parâmetros de motilidade espermática. Para o modelo de epididimite intersticial não observamos alterações nos parâmetros de motilidade (móvel, progressivo, lento e estático) nos camundongos analisados 1 e 7 dias após a indução da epididimite (Figura 6 A, C). Da mesma forma, não observamos alterações nos parâmetros de motilidade nos camundongos analisados 1 dia após a indução da epididimite intraductal (Figura 6 B). Neste modelo de epididimite, entretanto, observamos redução do percentual de células móveis e progressivas e aumento de células estáticas 7 dias após a injeção intraductal de LPS (Figura 6D). De maneira interessante, Silva et al. (2018), explorando modelo experimental de epididimite induzida pela injeção LPS e LTA (ácido lipoteicoico, agonista TLR2/TLR6) na luz do ducto deferente de ratos, não observaram modificações na motilidade espermática 7 dias após a indução da epididimite. Diferenças no modelo animal (camundongo x rato) e no tipo de endotoxinas utilizados nos dois estudos podem explicar esses resultados contraditórios. De fato, é conhecido que os espermatozoides de mamíferos expressam diferentes TLRs, incluindo o TLR2 e TLR4 (Palladino et al., 2008; Fujita et al., 2011). A incubação de espermatozoides com agonistas tanto do TLR4 quanto do TLR2 promoveu redução da

motilidade espermática, além de apoptose, prejudicando significativamente o seu potencial de fertilização (Fujita et al., 2011).

Quanto aos parâmetros de cinemática observamos que tanto a injeção intersticial quanto intraductal de LPS promoveram redução VAP e VSL, parâmetros que descrevem a progressividade (Verstegen et al., 2002), dos espermatozoides 1 e 7 dias após a indução de epididimite (Tabela 3). Paralelamente, observamos redução do VCL, parâmetro que descreve movimento vigoroso (Verstegen et al., 2002), dos espermatozoides de camundongos 7 dias após a indução da epididimite por ambos os modelos experimentais. promoveu também observamos redução do STR, parâmetro que descreve o movimento progressivo (Verstegen et al., 2002), nos espermatozoides de camundongos analisados 1 dia após a indução de epididimite intraductal (Tabela 3).

Por fim, investigamos o efeito da epididimite intersticial e intravasal sobre a motilidade hiperativada. A hiperativação é um processo que o espermatozoide apresenta durante o trajeto percorrido no oviducto da fêmea, como parte do processo de capacitação (Suarez, 2008). Esse movimento é descrito como vigoroso, não-progressivo, não-linear, sendo fundamental para a penetração do espermatozoide na zona pelúcida (Suarez, 2008; Goodson et al., 2011). É sabido que a incubação dos espermatozoides em condições capacitantes (contendo $NaHCO_3$ e BSA), por 60 a 120 min, aumenta o percentual de células hiperativadas (Goodson et al., 2011). Não observamos diferenças significativas na hiperativação dos espermatozoides dos camundongos submetidos aos dois modelos de epididimite com relação aos seus respectivos controles salina, incubados por 60 min em meio de cultura capacitante (Figura 7).

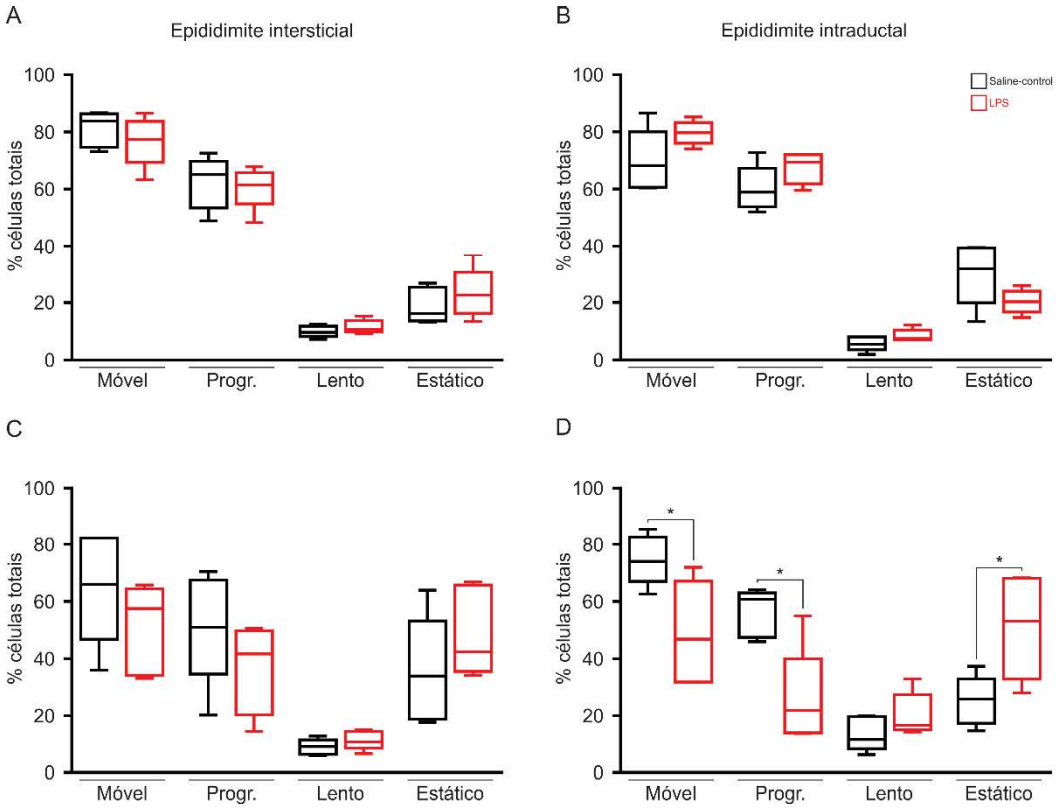


Figura 6. Efeitos da epididimite induzida pela injeção intersticial (A e C) e intravasal (B e D) de LPS sobre a motilidade espermática. Motilidade dos espermatozoides da cauda do epidídimo de camundongos tratados com salina-controle e LPS, sacrificados 1 (A e B) e 7 (C e D) dias após o tratamento e analisado pelo CASA. Os gráficos de caixa (mediana, intervalo interquartil e *whiskers* min.-máx.) representam a porcentagem de espermatozoides móveis, progressivos, lentos e estáticos. Os experimentos foram realizados com amostras de cinco animais por grupo ($p<0,05$, teste de Mann-Whitney).

Tabela 3. Efeitos da epididimite intersticial e intravasal induzida por LPS nos parâmetros de cinemática (VAP: Velocidade da trajetória média; VSL: velocidade linear progressiva; VCL: Velocidade curvilinea; STR: Retilinearidade; e LIN: Linearidade), de espermatozoides da cauda do epidídimo.

			Parâmetros de cinemática				
	Grupo	Tempo (dias)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	STR (%)	LIN (%)
Epididimite intersticial	Salina	1	121,3 ± 1,8	96,2 ± 2,7	202,9 ± 3,9	77,3 ± 1,2	49,2 ± 1,4
	LPS		113,2 ± 0,9*	87,0 ± 1,9*	193,3 ± 4,9	73,1 ±1,5	43,8 ± 1,8
	Salina	7	111,3 ± 6,6	88,9 ± 6,9	177,6 ± 10,4	76,2 ± 2,7	49,4 ± 2,1
	LPS		89,6 ± 5,9*	72,2 ± 6,8	143,3 ± 7,3*	77,8 ± 3,3	52,0 ± 4,1
Epididimite intraductal	Salina	1	124,2 ± 3,28	101,9 ± 2,9	209,1 ± 9,7	79,8 ± 0,4	49,2 ± 1,0
	LPS		115,7 ± 1,7*	92,3 ± 0,9*	195,7 ± 5,1	77,2 ±0,2*	47,2 ± 0,8
	Salina	7	109,9 ± 4,1	86,6 ± 4,4	179,2 ± 3,5	73,8 ± 2,0	48,2 ± 2,1
	LPS		78,7 ± 6,1*	58,1 ± 6,8*	126,7 ± 9,1*	63,0 ± 3,0	40,2 ± 2,4

Os dados para os parâmetros de STR e LIN foram transformados em raiz quadrada do arco-seno antes da análise.

Os asteriscos indicam estatisticamente diferentes do respectivo grupo controle salina.

Resultados representam a média ± EPM de experimentos independentes realizados com amostras de cinco camundongos por grupo ($p<0,05$, teste t de Student).

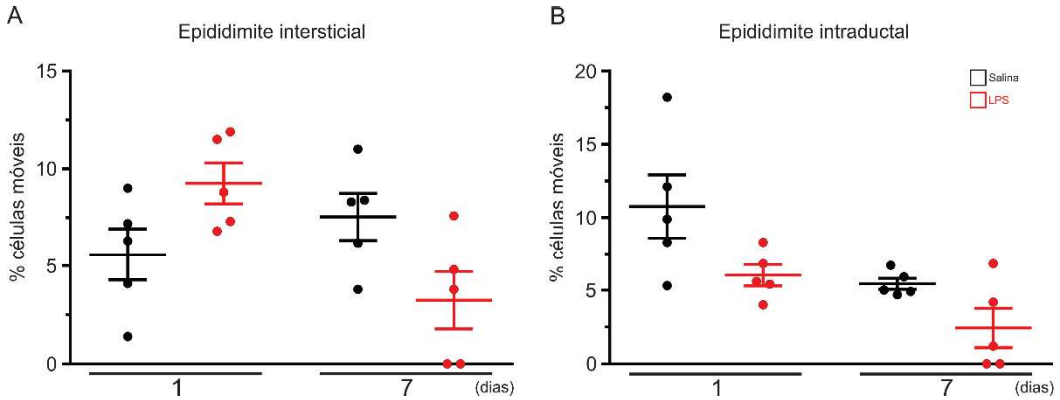


Figura 7. Efeitos da epididimite induzida pela injeção intersticial (A) e intravasal (B) de LPS sobre a hiperativação espermática. Motilidade dos espermatozoides da cauda do epidídimo de camundongos tratados com salina-controle e LPS, sacrificados 1 e 7 dias após o tratamento e analisado pelo CASA. A hiperativação foi determinada 60 min após a incubação dos espermatozoides em meio capacitante. Os gráficos de caixa (mediana, intervalo interquartil e whiskers min.-máx.) representam a porcentagem de espermatozoides móveis, progressivos, lentos e estáticos. Os experimentos foram realizados com amostras de cinco animais por grupo ($p<0,05$, teste de Mann-Whitney).

Capítulo 4

CONCLUSÃO

No presente trabalho, demonstramos que o desafio inflamatório intersticial e intravasal com LPS induziu reação inflamatória aguda no segmento inicial e na cauda do epidídimo, caracterizada pela regulação diferencial da expressão de mediadores inflamatórios entre as regiões do epidídimo. Descobrimos uma resposta diferencial da expressão dos genes *Wfdc* nas diferentes regiões epididimárias frente ao desafio inflamatório, sendo está parcialmente dependente da ativação do NFKB. Essas alterações induzidas pelo LPS foram acompanhadas por um efeito negativo sobre a motilidade e parâmetros cinemáticos dos espermatozoides, conforme a via de injeção do LPS. Esses resultados sustentam nossa hipótese inicial e sugerem que os membros da família *Wfdc* podem exercer funções no epidídimo buscando combater o estímulo nocivo e restaurar a homeostasia do órgão.

Nossos dados também incitam novos estudos que busquem o melhor entendimento do papeis das proteínas WFDC na fisiopatologia da epididimite bactéria, bem como a correlação entre seus níveis de expressão e a manutenção dos parâmetros de motilidade e de cinemática espermática.

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Anexo

Título do Projeto: Regulação da expressão de genes da família *Wfdc* (Whey-acidic protein four disufide core) por estímulo inflamatório no epidídimo de camundongos: potenciais funções imunológicas e reprodutivas;

Orientador: Prof. Dr. Erick José Ramo da Silva

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Campus de Botucatu

Instituto de Biociências de Botucatu - Unesp

Certificado

Certificamos que o projeto intitulado "Regulação da expressão de genes da família Wfdc (Whey-acidic protein four disulfite core) por estímulo infeccioso no epidídimo de camundongos: potenciais funções imunológicas e reprodutivas", Protocolo nº 1029-CEUA, sob a responsabilidade de Erick José Ramo da Silva, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 9 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela *COMISSÃO DE ÉTICA NO USO DE ANIMAIS* (CEUA), nesta data.

Finalidade:	( ) Ensino	(x) Pesquisa Científica
Vigência do Projeto:	Início: 7/8/2017	Término: 7/8/2019
Espécie/linhagem:	*Camundongo Mus musculus C57BL/6J*	
Nº de animais:	*100*	
Peso:	*25-50g*	Idade: 90 dias
Sexo:	*Macho*	
Origem	*Biotério do Instituto Nacional de Farmacologia e Biologia Molecular da Universidade Federal de São Paulo – UNIFESP*	

Botucatu, 14 de agosto de 2017.

Prof. Dr. Bruno Cesar Schimming
Presidente da CEUA

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Comissão de Ética no Uso de Animais IBB-Unesp