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Laboratório de Imunoparasitologia "Dr. Mario Endsfeldz Camargo"

Estimulação de vias inatas em células uterinas e ensaios de imunoprofilaxia em modelo murino visando o controle da neosporose bovina

Eliézer Lucas Pires Ramos

Uberlândia – MG

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Tese apresentada ao Colegiado do Programa de Pós-Graduação em Imunologia e Parasitologia Aplicadas como parte de obtenção do título de Doutor.

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Paz e igualdade!

Com todo meu amor e carinho

à minha gata Rop, seja onde ela

estiver, à minha Mãe Patrícia

e aos meus avós Francisco e Tereza!

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RESUMO

Neospora caninum tem causado grande interesse na pesquisa devido a sua associação com repetidos abortamentos no rebanho de bovinos, gerando bilhões de dólares de prejuízo na pecuária anualmente. Classificado como um protozoário intracelular obrigatório, a transmissão vertical é comum em hospedeiros bovinos e as consequências geradas dependerá do perfil de resposta imune do hospedeiro. Além disso, *N. caninum* apresenta diversas moléculas secretórias importante no processo de invasão. Dentre elas, a NcROP4 é uma proteína de roptria que possui caráter imunogênico importante para o processo de invasão e evasão na célula hospedeira. Aqui nós objetivamos em dois manuscritos compreender os mecanismos inatos envolvidos na resposta contra *N. caninum* em células uterinas de bovinos, além de avaliar a capacidade imunomodulatória de NcROP4 recombinante (rNcROP4) no desenvolvimento de protocolos vacinais e a partir disso, testar protocolos imunoterapêuticos de doenças inflamatórias agudas. Os resultados expostos no capítulo II da tese demonstraram que *N. caninum* é capaz de induzir produção de IFNγ, IL-10, IL-6 e IL-8 em células epiteliais e estromais de endométrio bovino. Além disso, a produção de IFNγ foi dependente das moléculas TLR3 e MyD88, pois quando essas moléculas foram silenciadas houve a diminuição da produção desse mediador. Esse silenciamento gerou também um aumento na produção de IL-10, IL-6 e IL-8 após infecção. Em contrapartida, a inibição da MAPK p38 e ERK induziram uma redução de IFNγ, e aumento de IL-10, IL-6 e IL-8, exibindo efeito antagônico à TLR3 e MyD88. Adicionalmente, nós demonstramos que a produção de IFNγ é dependente de STAT1 enquanto que a produção de IL-10 é dependente de STAT3 nas células uterinas bovinas estudadas. Houve ainda um aumento na produção de IL-10 quando STAT1 foi silenciada e o mesmo se observou com IFNγ quando STAT3 foi silenciada, evidenciando um balanço entre essas duas citocinas durante a infecção pelo patógeno em células de endométrio bovinos. No capítulo III nós identificamos inicialmente que NcROP4 ligante ao mAb20D2. Essa região de ligação foi quimicamente sintetizada e denominada (PepNcROP4). A rNcROP4 foi produzida em *Escherichia coli*. Tanto o peptídeo quanto a proteína foram colocados em cultura de macrófagos e no peritônio murino e induziram uma alta na produção de IL-10 e pouca produção de IFNγ em comparação com o extrato do parasito (NLA). Nós usamos rNcROP4, PepNcROP4 e NLA em protocolos de imunização e verificamos que todos os grupos induziram produção de anticorpos específicos. Após o desafio com o parasito, rNcROP4 induziu

aumento no peso corpóreo enquanto os demais antígenos utilizados. Adicionalmente, a imunização com esta proteína resultou na redução da carga parasitária e considerável aumento na sobrevida em comparação aos demais grupos. Por fim, o efeito regulatório de rNcROP4 foi testado no tratamento de ileíte induzida por *Toxoplasma gondii* em modelo murino. Os resultados evidenciaram que o tratamento com esta proteína reduziu o processo inflamatório no íleo, evidenciando que rNcROP4 apresenta efeito regulatório em outras patologias inflamatórias em camundongos. A partir do exposto acima podemos concluir que receptores e vias de sinalização inatas participam da resposta à *N. caninum* em células endometriais bovinas e a proteína rNcROP4 exibe potencial regulatório capaz de aumentar a capacidade protetiva na neosporose e reduzir a ileíte induzida por *T. gondii* em camundongos.

Palavras-chave:
Neospora caninum, células endometriais, bovinos, rNcROP4, vacina, imunoterapia.

ABSTRACT

Neospora caninum has caused great interest in the research due to its association with repeated abortion in the cattle herd, entailing billions of dollars of loss in livestock annually. Classified as a mandatory intracellular protozoan, vertical transmission is common in bovine hosts and the consequences generated will depend on the host immune response profile. In addition, *N. caninum* presents several secretory molecules important in the invasion process. Among them, NcROP4 is a rhoptry protein that has immunogenic character important for the process of invasion and evasion in the host cell. Here we aimed at two manuscripts to understand the innate mechanisms involved in the response against *N. caninum* in bovine uterine cells and to evaluate the immunomodulatory capacity of recombinant NcROP4 (rNcROP4) in the development of vaccine protocols and from there to test the immunotherapeutic protocols of an acute inflammatory pathology. The results set forth in Chapter II of the thesis demonstrated that *N. caninum* is able to induce IFNγ, IL-10, IL-6 and IL-8 production in bovine endometrial cells. In addition, the IFNγ production was TLR3 and MyD88-dependent, because when these molecules were silenced there was a decrease in the production of this mediator. This silencing also generated an increase in the IL-10, IL-6 and IL-8 production after infection. In contrast, inhibition of MAPK p38 and ERK induced a reduction of IFNγ, and an increase in IL-10, IL-6 and IL-8 production, exhibiting an opposite effect to TLR3 and MyD88. In addition, we have demonstrated that IFNγ production is STAT1-dependent whereas IL-10 production is STAT3-dependent on bovine uterine cells studied. There was also an increase in IL-10 production when STAT1 was silenced and the same was observed with IFNγ when STAT3 was silenced, showing a balance between these two cytokines during pathogen infection in bovine endometrial cells. In chapter III we initially identified that NcROP4 binding to mAb20D2. This binding region was chemically synthesized and named (PepNcROP4). rNcROP4 was produced in *Escherichia coli*. Both the peptide and the protein were placed in mice macrophage and culture peritoneum and induced a high IL-10 production and low IFNγ production compared to the parasite extract (NLA). We also used rNcROP4, PepNcROP4 and NLA in immunization protocols and found that all groups induced specific antibody production. After challenge with the parasite, rNcROP4 induced increase in body weight while the other antigens used induced reduction of body weight. In addition, immunization with this protein resulted in a reduction of parasite burden and a considerable increase in survival compared to the other groups. Finally, the

regulatory effect of rNcROP4 was tested in the treatment of *Toxoplasma gondii*-induced ileitis in murine model. The results showed that the treatment with this protein reduced the inflammatory process in the ileum, evidencing that rNcROP4 presents regulatory effect in other inflammatory diseases in mice. From the foregoing, we can conclude that innate signaling receptors and pathways participate in the response to *N. caninum* in bovine endometrial cells and the rNcROP4 protein shows regulatory potential able to increase the protective capacity in neosporosis and reducing *T. gondii*-induced ileitis in murine model.

Key words:
Neospora caninum, endometrial cells, bovine, rNcROP4, vaccine, immunotherapy.

LISTA DE ABREVIATURAS E SIGLAS

BL21DE	Cepa de *Eschirichia coli*
BSA	Soroalbumina bovina
cDNA	DNA complementar
DAPI	4',6-diamidino-2-phenylindole
DO	Densidade ótica
ELISA	Ensaio imunoenzimático (*Enzyme-Linked Immunosorbent Assay)*
GRA	Proteínas de grânulos densos
IA	Índice Avidez
IE	Índice ELISA
IEDB	Immune Epitope Database
IFN-γ	Intérferon gama
IgG	Imunoglobulina G
IgM	Imunoglobulina M
IL-12	Interleucina 12
IL-15	Interleucina 15
IPTG	Isopropil β-D-1-thiogalactopiranosideo
LB	Meio de cultura Lúria Bertani
M13	Bacteriófago filamentoso
mAb	Anticorpo monoclonal
MIC	Proteínas de Micronemas
MOI	Quantidade de parasito por célula
MyD88	Fator de diferenciação mieloide 88 (*Myeloid differentiation factor 88*)
Nc-1	Isolado de *Neospora caninum*

NcROP4	Proteína de roptria 4 de *N. caninum*
NK	Células Natural Killer
NLA	Antígeno solúvel de *N. caninum*
NO	Óxido nítrico
OD	Densidade ótica (*Optical density*)
PBS	Solução salina tamponada com fosfato
PBS-T	Solução salina tamponada com fosfato com 0,05% de Tween
PBS-TM	Solução salina tamponada com fosfato com 0,05% de Tween e leite desnatado
PBT	Solução salina tamponada com fosfato com 0,1% de Triton-X100
PCR	Reação em cadeia da polimerase
RIFI	Reação de imunofluorescência indireta
rNcROP4	Proteína de roptria 4 de *N. caninum* recombinante
ROP	Proteínas de roptrias
SAG	Antígeno de superfície
SDS-PAGE	dodecil-sulfato de sódio de poliacrilamida
TBS	Solução salina tamponada com Tris
TBS-T	Solução salina tamponada com Tris com 0,05% de Tween
TBS-TM	Solução salina tamponada com Tris com 0,05% de Tween e leite desnatado
TLR	Receptores do tipo *Toll*
VP	Vacúolo parasitóforo

Sumário

SUMÁRIO

Capítulo 1

CAPÍTULO I

1.1 REFERÊNCIAL TEÓRICO

1.1.1 *Neospora caninum* – características gerais

Neospora caninum é um protozoário intracelular obrigatório, pertencente ao filo Aplicomplexa que apresenta estreita relação filogenética com o protozoário *Toxoplasma gondii*. O parasito apresenta ciclo de vida heteroxênico no qual os canídeos são hospedeiros definitivos e vários animais, incluindo aves e mamíferos, podem atuar como hospedeiros intermediários. Tornou-se alvo de estudos nas últimas décadas por ser o maior causador de abortamento em bovinos com consequentes perdas econômicas na pecuária, além de provocar doenças neuromusculares em cães (DUBEY, 2003; REICHEL e ELLIS, 2009; REICHEL et al., 2013; REICHEL et al., 2014; BARROS et al., 2018).

A primeira identificação da infecção por *N. caninum* ocorreu em 1984 na Noruega em cães com distúrbios neurológicos semelhantes aos causados por *T. gondii*, todavia os animais não apresentavam sororeatividade a este parasito (BJERKÃS et al., 1984). Poucos anos depois, através de análise histológicas em cérebros de cães que apresentavam cistos teciduais semelhantes ao de *T. gondii,* o parasito foi identificado como uma espécie distinta. Essa distinção aconteceu após observarem algumas características diferenciais tais como paralisia dos membros posteriores, características ultraestruturais de parasitos livres e dos cistos teciduais, além dos testes sorológicos e imunoistoquímicas negativos para *T. gondii*. Desse modo, foi estabelecida uma nova espécie de protozoário denominado *N. caninum*, posteriormente identificado em bovinos e isolado em cultura celular (DUBEY et al., 1988a; DUBEY et al., 1988b; THILSTED e DUBEY, 1989). Características bioquímicas, morfológicas e vitais fortemente semelhante a *T. gondii* encobriu sua identificação por décadas (HEMPHILL et al., 2006). Entretanto, desde sua distinção os estudos demonstraram que além de características patológicas, tais espécies de protozoários também se diferem nas interações com o hospedeiro e ciclo de vida (HEMPHILL et al., 2006; INNES e MATTSSON, 2007).

1.1.2 Ciclo biológico

N. caninum apresentam canídeos como hospedeiro definitivo, além de várias espécies de mamíferos e aves como hospedeiros intermediários (THILSTED e DUBEY, 1989; BARR et al., 1991; GONDIM et al., 2004; COSTA et al., 2008). O ciclo evolutivo deste parasito envolve basicamente três formas evolutivas: esporozoítas presentes em oocistos, taquizoítas, e bradizoítas no interior de cistos teciduais (Figura 1). A transmissão da neosporose pode ser tanto horizontal quanto vertical, sendo que a primeira ocorre em hospedeiros definitivos e intermediários (DUBEY et al., 1999; GOODSWEN, KENNEDY e ELLIS, 2013; DONAHOE et al., 2015). A infecção nos canídeos ocorre após a ingestão de cistos teciduais contendo bradizoítas, no estômago se dá a digestão química e enzimática, liberando as formas infectantes para a luz do intestino (DUBEY et al., 1999; McALLISTER, 2016) onde há merogonia, um tipo de reprodução assexuada e gamogonia tipo de reprodução sexuada culminando na formação de oocistos não esporulados. Essas estruturas com formato ovalado são eliminados no ambiente junto às fezes do animal e em condições propícias de umidade e temperatura esporulam-se, formando uma estrutura contendo dois esporocistos com quatro esporozoítas (McALLISTER et al., 1998; RINALDI et al., 2005; KUL et al., 2015; McALLISTER et al., 2016). Oocistos esporulados, quando ingeridos por bovinos ou outros hospedeiros intermediários, atingem o lúmen entérico, liberando esporozoítas capazes de invadir o epitélio intestinal transformando-se em taquizoítas que se multiplicam no interior de células nucleada por endodiogenia. Taquizoítas são disseminados pelo organismo do animal, através da circulação linfática, sanguínea e também via ascendente por tecidos nervosos periféricos, atingindo diretamente o sistema nervoso central (SAWADA et al., 1997; SHIBAHARA et al., 1999; McALLISTER, 2016).

A formação de bradizoítas se da pela pressão do hospedeiro sobre o parasito, principalmente mediada por ação do sistema imunológico. Esta forma evolutiva é capaz de formar uma parede espessa ao redor de vários parasitas, tal estrutura é denominada cisto tecidual (DUBEY et al., 1988a; DUBEY et al., 1988b). A intensa multiplicação no início da infecção caracteriza a fase aguda da neosporose (COLLANTES-FERNANDÉZ et al., 2006; AGUADO-MARTINEZ et al., 2008), já a formação de cistos teciduais corresponde a fase crônica da infecção que persiste por toda a vida do hospedeiro (SHIBAHARA et al., 1999; COLLANTES-FERNANDÉZ et al., 2006; AGUADO-MARTINEZ et al., 2008; McALLISTER, 2016).

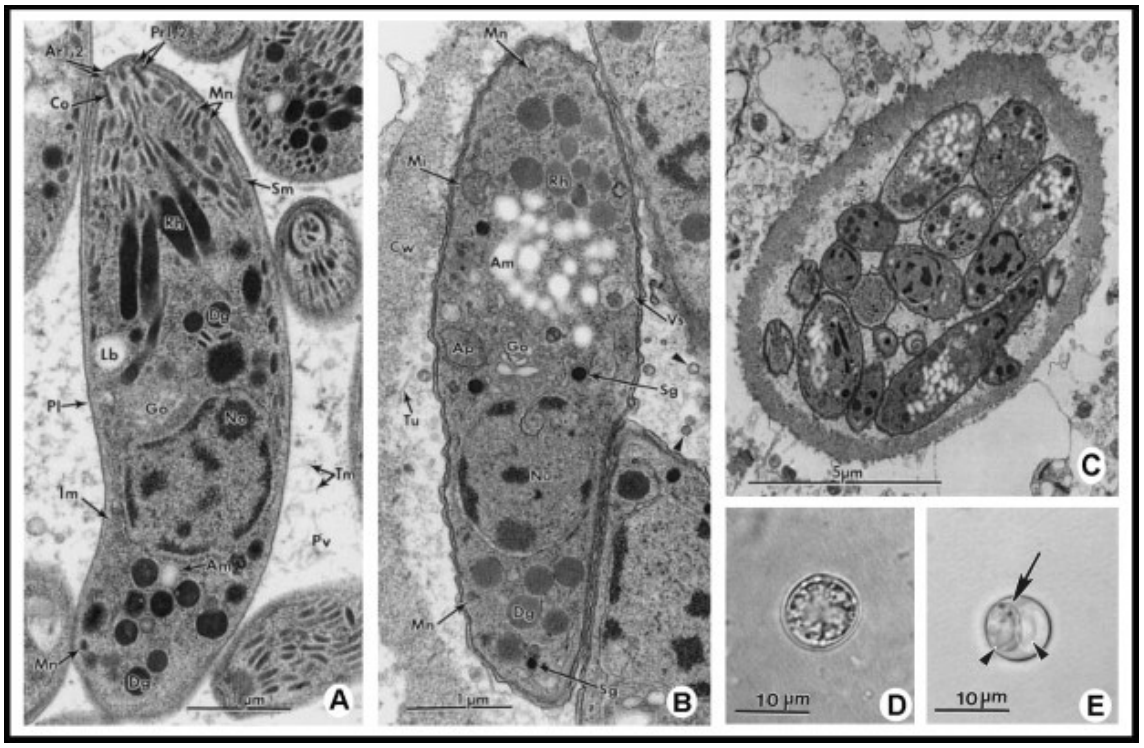


Figura 1- Ultra-estrutura de (A) taquizoíto, (B) bradizoíto, (C) cisto, (D) oocisto não-esporulado e (E) oocistos esporulados com dois esporocistos (seta) e dois esporozóitos (cabeça da seta) do parasito *N. caninum*. Fonte: Goodswen (2013).

A transmissão vertical é uma alternativa do parasito se propagar e ocorre devido à capacidade deste parasito ultrapassar a barreira transplacentária. Essa forma de transmissão já foi descrita em diversas espécies de animais e apresenta considerável importância em bovinos, pois durante a infecção do feto pode ocorrer abortamento (DUBEY, 2003; TREES e WILLIAMS, 2005; REICHEL e ELLIS, 2009; HORCAJO et al., 2016; McALLISTER et al., 2016; ALMERÍA et al., 2017). A transmissão vertical pode ocorrer de duas formas distintas: a primeira se dá pela infecção de fêmeas durante a gestação e a segunda ocorre pela reativação da infecção decorrente de variação hormonal e mudança no perfil de resposta imunológica, de pró-inflamatória (Th1) para anti-inflamatória (Th2), sendo o primeiro importante para manter a latência do parasito (INNES et al., 2001; QUINN et al., 2002 McALLISTER et al., 2016; ALMERÍA et al., 2017) (Figura 2).

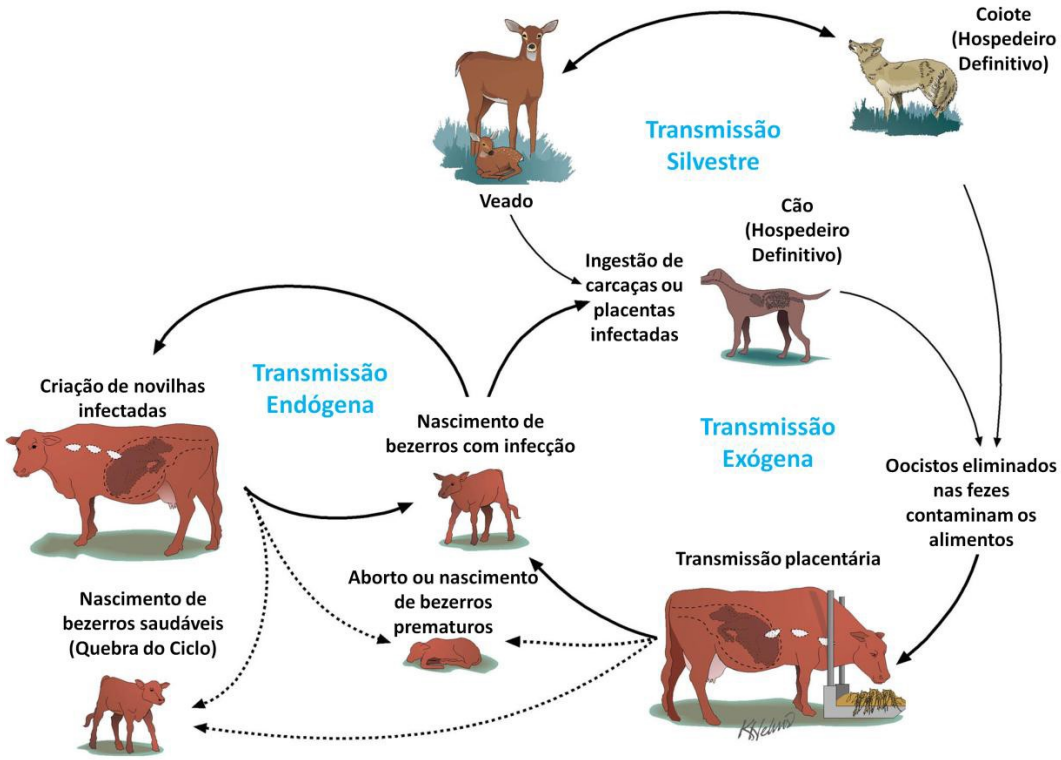


Figura 2 - Ciclo vital de *N. caninum* (adaptado de Milton M. McAllister, 2007).

1.1.3 Hospedeiros

De acordo com os relatos iniciais cães domésticos são os hospedeiros definitivos de *N. caninum* devido à reprodução sexuada que ocorre em seu intestino (McALLISTER et al., 1998; LINDSAY et al., 1999). Contudo, estudos posteriores consideraram coiotes (*Canis latrans*), dingos (*Canis lupus dingo*) e lobos cinzas (*Canis lúpus*) como hospedeiros definitivos adicionais de *N. caninum*, apontando a possibilidade de outros canídeos exercerem o papel de hospedeiro definitivo para o parasito (GONDIM et al., 2004; KING et al., 2010; DUBEY et al., 2011).

A infecção por *N. caninum* é frequentemente relatada em cães e bovinos, contudo, há relatos sorológicos da presença do parasito em outros organismos como carneiros, cabras, cavalos, búfalos, raposas, coiotes, lobos, veados, alpacas, camelos, galinhas e até psitacídeos (DUBEY, 2003; COSTA et al., 2008; MINEO et al., 2011; GOODSWEN, KENNEDY e ELLIS, 2013; GONDIN, MINEO e SCHARES, 2017).

Experimentalmente, relatos na literatura demonstraram que tal microrganismo é capaz de infectar com êxito espécies de mamíferos como bovinos, cães, cabras, carneiros, camundongos, gerbis, gatos, macacos, pombos e porcos (DUBEY et al., 2002; GONDIM et al., 2010). Embora não haja estudos que comprovem a infecção por *N. caninum* em humanos, alguns autores relatam evidências sorológicas da exposição do homem ao parasito em diferentes populações (NAM et al., 1998; TRANAS et al., 1999; LOBATO et al., 2006; ROBERT-GANGNEUX e KLEIN, 2009).

1.1.4 Patogênese e Patologia

Os sinais gerados pela infecção são bem descritos principalmente em cães e bovinos. Os canídeos apresentam grave comprometimento neuromuscular, podendo haver infecções congênitas com neonatos apresentando patogênese avançada dentro do primeiro ano de vida. Pode ainda haver o acometimento de outros órgãos como pulmões, coração, intestino e pele o que irá depender da virulência do isolado e carga parasitária, (ORDEIX et al., 2002; DUBEY, 2003; BASSO et al., 2005; DUBEY e SCHARES, 2011). A encefalomielite causada pelo protozoário torna-se cães adultos mais susceptíveis e se caracteriza por diversos sinais neurológicos dependentes do local parasitado no Sistema Nervoso Central (SNC) (BUXTON, McALLISTER e DUBEY, 2002; DONAHOE et al., 2015). Embora principalmente reconhecida como uma doença neuromuscular em cães, a neosporose pode induzir outras disfunções menos comuns que incluem dificuldade na deglutição, paralisia da mandíbula, flacidez muscular, atrofia muscular, paralisia de nervos faciais, miocardite, pancreatite, pneumonia e até falência cardíaca dependendo das células parasitadas. Dessa forma, a doença exibe características clínica localizada ou generalizada, envolvendo todos os órgãos, inclusive podendo causar a neosporose cutânea (PERLÉ et al., 2001; ORDEIX et al., 2002; MANN et al., 2016). O intenso parasitismo é marcante nestes casos, com um grande número de taquizoítas nas lesões cutâneas, sugerindo uma falta de controle imune do hospedeiro sobre a multiplicação dos parasitos (DUBEY, 2003).

A maioria das infecções por *N. caninum* em bovinos exibe caráter subclínico, contudo o abortamento é o principal problema podendo ser o único sinal clínico observado, apresentando caráter esporádico, endêmico ou epidêmico (DUBEY, 2003; DONAHOE et al., 2015; McALLISTER, 2016). Tal fator nos rebanhos geram perdas

econômicas consideráveis que ultrapassam bilhões de dólares anualmente (WILLIAMS et al., 2000; INNES et al., 2005; REICHEL et al., 2013; REICHEL et al., 2014). O abortamento pode ocorrer entre o quarto mês até o final da gestação, sendo mais comum entre o quinto e sétimo mês. A morte do feto depende de danos gerados à placenta de modo que torna-se inviável prosseguir com a gestação (DONAHOE et al., 2015; McALLISTER, 2016). O tecido fetal lesado decorrente da multiplicação do parasito gera insuficiência de oxigênio/nutrição e os danos induzidos na placenta pelo parasito podem pôr em risco diretamente a sobrevivência fetal ou induzir a liberação de prostaglandinas que por sua vez induzem o abortamento (DUBEY et al., 2002; CANTÓN et al., 2014).

Ocorrem também danos à placenta materna por taquizoítas devido à interrupção do fornecimento de nutrientes (DUBEY et al., 1992). Dependendo da fase da gestação em que ocorre a infecção intra-uterina, além de abortamento, pode ocorrer lesões degenerativas ou inflamatórias no feto, natimorto ou fetos mumificados, nascimento de bezerros fracos com sintomas no sistema nervoso central ou nascimento de bezerros clinicamente saudáveis, mas cronicamente infectados (ANDRIANARIVO et al., 2001; INNES et al., 2002). Tais consequências dependem da resposta imune contra esse parasito durante a transmissão vertical (KHAN et al., 1997; BASZLER et al., 1999; QUINN et al., 2002; ROSBOTTOM et al., 2008; ALMERIA et al., 2011).

O parasito exibe virulência variável que está relacionada ao crescimento *in vitro* e a diferentes isolados *in vivo*, como o de cães e bovinos sintomáticos, bezerros natimortos ou fetos abortados os quais apresentam virulência acentuada (ATKINSON et al., 1999; SCHOCK et al., 2000; QUINN et al., 2002; PÉREZ-ZABALLOS et al., 2005; COLLANTES-FERNÁNDEZ et al., 2006). Já isolados obtidos de bezerros saudáveis, mas infectados congenitamente, foram associados à baixa virulência (SHIBAHARA et al., 1999; MILLER et al., 2002; ROJO-MONTEJO et al., 2009).

1.1.5 Invasão celular no filo Apicomplexa

Parasitos do filo Apicomplexa invadem a célula hospedeira utilizando mecanismo conservado e extremamente bem-sucedido. O processo de invasão é totalmente dirigido pela forma taquizoíta do parasito, num processo conhecido por entrada ativa, diferindo da entrada induzida, provocadas por bactérias ou outros protozoários. O processo exige que o parasito utilize seu próprio sistema de citoesqueleto para induzir a invasão, ou seja,

a célula hospedeira não apresenta função ativa neste processo, como ocorre a indução da fagocitose por alguns protozoários como *Leishmania* sp (DOBROWOLSKI e SIBLEY, 1996; DOBROWOLSKI et al., 1997; SOLDATI et al., 2001; MEISSNER et al., 2002; JEWETT e SIBLEY, 2003). Há a penetração na célula sem alteração da actina do hospedeiro, em um processo independente de cálcio. De forma contrária, o parasito utiliza-se de um mecanismo de secreção regulado por cálcio intracelular (CARRUTHERS e SIBLEY, 1999). Organelas do complexo apical do parasito como micronemas e roptrias e distribuídas no citoplasma como grânulos densos estocam diversas moléculas fundamentais para o processo de invasão. O parasito ainda apresenta um complexo actina/miosina, denominado "movimento de deslizamento" que permite a sua junção a célula hospedeira. Além disso, diversos receptores de superfície associado a uma série de proteínas liberadas por essas organelas estão relacionadas aos processos de reconhecimento, movimentação e replicação (CESBRON-DELAUW et al., 2008; DAHER e SOLDATI-FAVRE, 2009; LOURIDO et al., 2010; COWPER, MATTHEWS e TOMLEY, 2012; FRÉNAL et al., 2017).

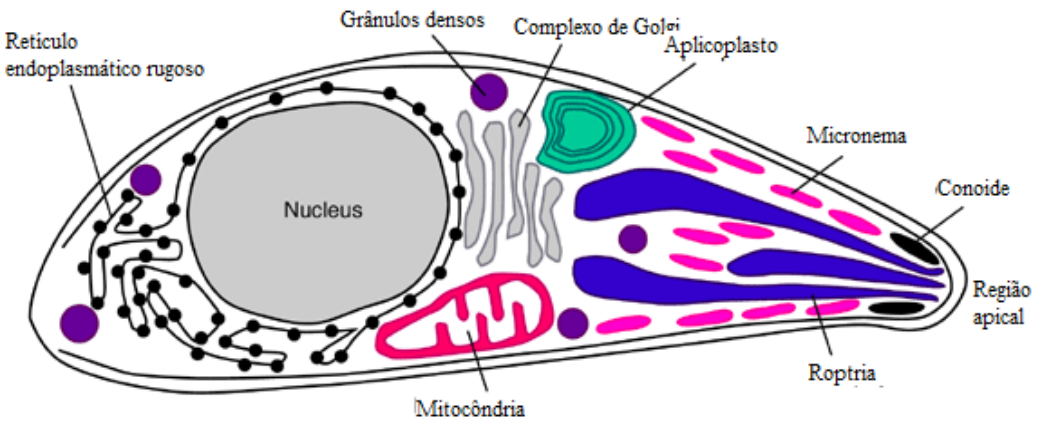
Micronemas e roptrias estão localizados na porção anterior do parasito (complexo apical) enquanto os grânulos densos estão mais amplamente distribuídos no citoplasma (JOINER e ROOS, 2002; RAVIDRAN e BOOTHROYD, 2008; COWPER, MATTHEWS e TOMLEY, 2012; FRÉNAL et al., 2017). Ambas são organelas fundamentais na invasão, processo que leva aproximadamente 15 a 20 segundos (RAVINDRAN e BOOTHROYD, 2008), enquanto os grânulos densos são fundamentais para o estabelecimento do parasito no vacúolo formado, bem como sua replicação (JOINER e ROOS, 2002) além de modular as vias de sinalização intracelular da célula hospedeira e manter sua permanência até o momento de evasão (ROSOWSKI et al., 2011; HUNTER e SIBLEY, 2012; HAKIMI, OLIAS e SIBLEY, 2017).

Resumidamente, o processo de invasão celular pode ocorre em três etapas: (1) adesão da forma taquizoíta à célula hospedeira, processo que não envolve a orientação do parasito e que requer antígenos de superfície glicosilados como SAG1 e SAG2. Após tal adesão, o parasito posiciona a extremidade anterior (região apical) para extrusão do conoide, seguida pela invaginação da membrana celular, formando o vacúolo parasitóforo (VP), momento em que proteínas oriundas dos micronemas (MIC1, 2, 3 e 4) são secretadas e funcionam como adesinas. Estudos recentes indicaram que a ligação de proteínas do tipo MIC a receptores de célula hospedeira, desencadeiam a secreção dos

componentes proteicos de roptrias (SINGH et al., 2010). A aderência conferida permite o movimento da junção ao redor do taquizoíta, que juntamente ao citoesqueleto do parasito, impulsiona-o para o interior do VP, culminando na invasão. O VP é revestido pela membrana celular do hospedeiro o que não permite a fusão aos lisossomos. (2) Na etapa seguinte, proteínas de roptrias são secretadas no interior do VP e ligam-se à membrana deste para formar associação com organelas do hospedeiro, de modo que a mitocôndria e o retículo endoplasmático são posicionados adjacentes ao VP. (3) A etapa final consiste na secreção de proteínas de grânulos densos que modificam a membrana do VP, o que contribui na remodelação e maturação do VP, formando uma rede de membrana intravacuolar, metobolicamente ativa para a multiplicação parasitária. Os taquizoítas no interior do VP proliferam-se por endodiogenia, produzindo novos parasitos em poucas horas, os quais subsequentemente lisam a célula hospedeira e ficam livres para invadir novas células (HELMPHILL et al., 2006; FRÉNAL et al., 2017) (Figura 3).

No caso de *T. gondii*, o parasito exibe um conjunto de 8 a 12 roptrias que apresentam forma de clava, consistindo de um corpo bulboso e um pescoço estreito e eletro denso. Já se conhece mais de 44 proteínas de roptrias, sendo parte transmembrana e parte constituindo o ambiente interno (PEIXOTO et al., 2010; ALI-HAKIMI, OLIAS e SIBLEY, 2017). Algumas proteínas de roptrias são homólogas a quinases, fosfatases e proteases, revelando sua importância para as funções do parasito (FLEIGE e SOLDATI-FRAVE, 2008). Algumas dessas proteínas de *T. gondii* como ROP5, ROP16 e ROP18 funcionam como proteínas quinases ou pseudoquinases que modulam ou auxiliam na modulação da resposta imune do hospedeiro, funcionando com um mecanismo de evasão do parasito (TAYLOR et al., 2006; SAEIJ et al., 2006; SAEIJ et al., 2007; KHAN et al., 2009; PEIXOTO et al., 2010; REESE et al., 2011; BUTCHER et al., 2011; BEHNKE et al., 2011; HUNTER e SIBLEY, 2012).

A



B

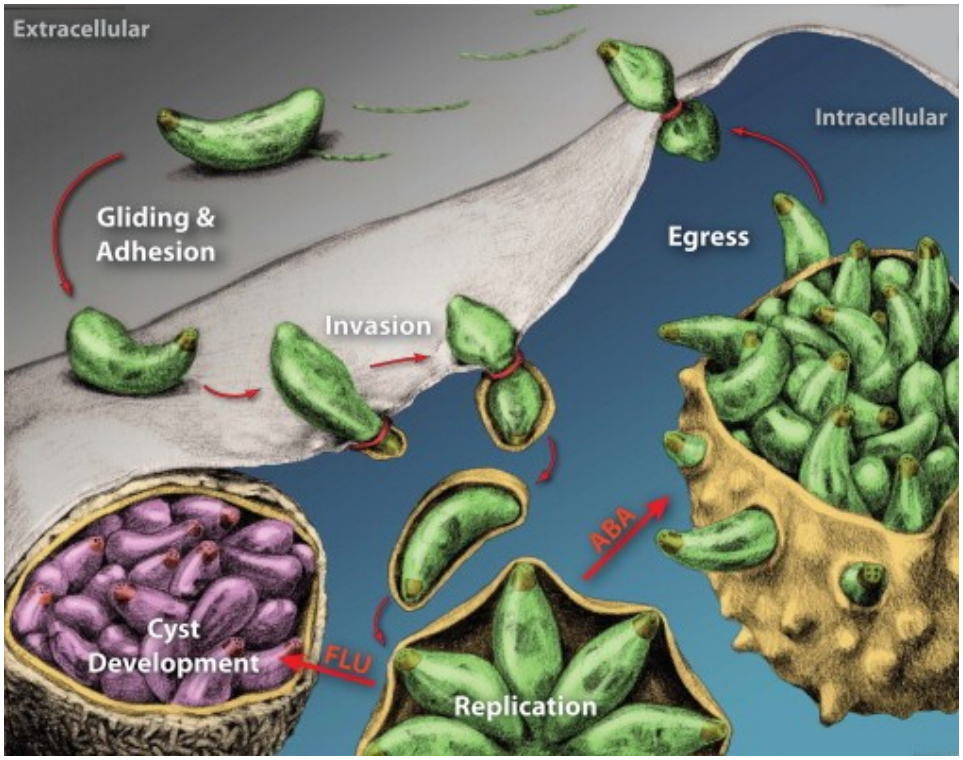


Figura 3 – Esquema da estrutura de taquizoíta (A) e representação do processo de invasão por taquizoítas de *T. gondii*. (A) O parasita é formado por duas membranas, a membrana plasmática e a membrana interna. Na região apical do parasito encontram-se estruturas conoide e as organelas apicais micronemas e roptrias. Grânulos densos são distribuídos ao longo do parasito. Outras organelas também são presentes como apicomplasto, mitocôndria, complexo de Golgi e retículo endoplasmático (Adaptado de Joiner e Roos, 2002). (B) A porção esquerda superior da figura representa a região extracelular da célula hospedeira com membrana plasmática em azul. Em 1, ocorre o contato iniciação entre o parasito (verde) e a célula hospedeira via antígenos de superfície. Em 2 ocorre a reorientação do parasito e as junções (vermelho) entre a região apical e a membrana celular com participação de proteína micronêmica e de roptrias. Em 3 há a representação do processo de invasão ativa e início da formação do vacúolo parasitóforo. Em 4 o vacúolo parasitóforo está formado e inicia-se a secreção de proteínas de grânulos densos (Adapatado de Billker et al., 2009).

A descarga de proteínas de roptrias ocorre seguindo a descarga das proteínas micronêmicas, o que coincide e contribui para formação do vacúolo parasitóforo (CARRUTHERS; SIBLEY, 1999). Após esta descarga, tais proteínas permanecem associadas ao vacúolo parasitóforo ou são exportadas para célula hospedeira onde participam dos processos de remodelamento celular (ROY et al., 2006; DUBREMETZ, 2007; BESTEIRO et al., 2011; HAKIMI, OLIAS e SIBLEY, 2017).

Das proteínas de roptrias de *T. gondii* identificadas e estudadas, sabe-se que ROP1 é um componente ativo na penetração do parasito na célula (NORRBY e LYCKE, 1967; OSSORIO et al., 1992; CHARRON e SIBLEY, 2004), no entanto, tal proteína não é essencial para o processo de invasão (KIM et al., 1993). Já ROP2 é liberada no interior do vacúolo parasitóforo e induz a associação de tal estrutura à mitocôndria da célula hospedeira, além de ser importante na biogênese da roptria (SINAI e JOINER, 2001; NAKAAR, et al., 2003; CLOUGH e FRICKEL, 2017). ROP4 é um membro da família ROP2 e é secretada durante a invasão, associando-se à membrana do vacúolo parasitóforo, onde torna-se fosforilada e exerce atividade quinase, o que permite a ativação de fatores da célula hospedeira (CAREY et al., 2004).

Em relação a ROP4 de *N. caninum* (NcROP4) ainda pouco se sabe a respeito das funções desta proteína. A proteína apresenta 594 aminoácidos, foi identificada como componente secretor do parasito em 2011 e também é denominada membro 1 da família das ROP2 (ALAEDDINE et al., 2013). Um anticorpo monoclonal identificado como 20D2 foi desenvolvido e certificou-se que ele se liga à NcROP4 e que a proteína apresenta peso molecular de 65 kDa (SOHN et al., 2011). Além disso, apesar de algumas proteínas de roptrias exibirem propriedade quinase, NcROP4 aparentemente não apresenta sítio catalítico. Entretanto, tal proteína exibe capacidade imunogênica e sua importância durante a invasão já foi demonstrada. Constatou-se que anticorpos gerados contra a proteína são capazes de neutralizá-la e prejudica a capacidade de invasão celular pelo parasito "*in vitro*" (ALAEDDINE et al., 2013). Contudo, não foi relatado até o momento qual a participação da proteína durante o processo de invasão e a localização dentro da célula hospedeira, além do seu participação durante a resposta imune contra *N. caninum* ou sua capacidade vacinal.

1.1.6 Epidemiologia e diagnóstico da neosporose

A neosporose tem emergido como uma patologia grave em bovinos e cães em todo o mundo (DUBEY, 2003). Já foi relatado que a infecção em bovinos por *N. caninum* está presente em diversos continentes, dentre eles: Europa, Oceania, Ásia, América do Norte e Sul (DUBEY, SCHARES e OTEGA-MORA, 2007; SPILOVSKÁ et al., 2009; YU et al., 2009; PANADERO et al., 2010, REICHEL et al., 2013).

No Brasil, a soroprevalência da neosporose bovina pode variar de 7 a 97%. Estas diferenças estão predominantemente relacionadas aos diversos fatores de risco como: tipo de bovino (corte ou leite), número de gestações, presença e número de cães na fazenda, idade do gado, número de oocistos ingeridos, etapa da gestação, resposta imunológica e o tipo de teste sorológico utilizado para determinar a exposição ou infecção, uma vez que não há um padrão nos métodos empregados na rotina laboratorial (GOODSWEN, KENNEDY e ELLIS, 2013; CALERO-BERNAL, DUBEY E GENNARI, 2017).

O diagnóstico da infecção por *N. caninum* pode ser realizado tanto pela detecção direta do parasito, bem como de modo indireto, pela detecção de anticorpos circulantes específicos a antígenos parasitários, sendo o segundo mais comumente empregado. A detecção do protozoário em amostras biológicas ocorre pela Reação em Cadeia da Polimerase (PCR), testes imunohistoquímicos e através de bioensaios em cultura de células e animais experimentais (DUBEY et al., 2007). Mais recentemente, a detecção e identificação de novos isolados estão sendo feitas pela técnica de microssatélite (BROM et al., 2014; SALEHI et al., 2015; QIAN et al., 2016; MEDINA-ESPARZA et al., 2016). Dentre os ensaios sorológicos comumente utilizados, destaca-se a Reação de Imunofluorescências Indireta (RIFI) e ensaios imunoenzimáticos (ELISA), sendo o primeiro considerado o padrão ouro para o sorodiagnóstico da infecção (DUBEY et al., 2007). Entretanto, uma das maiores deficiências das técnicas sorológicas é que estas geralmente indicam somente a exposição prévia do hospedeiro ao parasito, não permitindo informações sobre o estágio de infecção. Tal contratempo é parcialmente solucionado pela aplicação de técnicas baseadas em isotipos de anticorpos. Um marcador utilizado para determinação da fase aguda de infecção é a soropositividade de IgM, enquanto o que determina a fase crônica é presença de anticorpos IgG antígeno-específicos (BJORKMAN et al., 1999). Outra forma de se determinar o estágio da infecção é utilizando técnicas que mensuram a avidez de IgG frente aos antígenos de interesse, sendo que tal tipo de técnica utiliza-se dos fenômenos relacionados a força de

ligação do anticorpo a seus alvos (HEDMAN et al., 1989), a qual torna-se progressivamente maior no decorrer da infecção. Um índice avidez alto está geralmente relacionado às fases crônicas de infecção e a baixa avidez geralmente correlaciona-se a infecções ativas ou recentes (SENSINI et al., 1996 e COZON et al., 1998; SCHARES et al., 2002, AGUADO-MARTINEZ et al., 2005 AND BJORKMAN et al., 2006; DUBEY et al., 2013). Contudo, apesar de tais evidências, não é possível inferir com exatidão a fase de infecção que o hospedeiro se encontra, pois não há como garantir que tais fenômenos sejam estáveis em cada indivíduo testado (ASHBURN et al., 1998; BERTOZZI et al., 1999; LEFEVRE-PETTAZZONI et al., 2006; IQBAL e KHALID, 2007; HUANG et al., 2007).

Uma ferramenta alternativa hoje aplicada com frequência para se avaliar a soropositividade a diferentes infecções é a utilização de proteínas recombinantes. Trabalhos empregando proteínas recombinantes como NcSAG1, NcSRS2, NcGRA6, NcGRA7 e NcSAG4 de *N. caninum* no sorodiagnóstico apresentaram resultados satisfatórios como marcadores de fase aguda, crônica e até mesmo recrudescência da neosporose (LOUIE et al., 1997; NISHIKAWA et al., 2001; HOWE et al., 2002; CHAHAN et al., 2003; JENKINS et al., 2005; AGUADO-MARTÍNEZ et al., 2008; HIASA et al., 2012; TAKASHIMA et al., 2013).

Obter um diagnóstico eficaz capaz até de diferenciar a fase de infecção por *N. caninum* se faz importante pois as perdas médias estimadas devido ao abortamento induzidos por *N. caninum* é de 1 bilhão de dólares anualmente e possivelmente chegue a 2-4 bilhões de dólares por ano em apenas 10 países que representa aproximadamente dois terços dos prejuízos nas indústrias de laticínios e mais de um terço nas de carne (REICHEL et al., 2013). Nos Estados Unidos acredita-se que infecção pelo parasito gera prejuízos a indústria de laticínios de cerca de 546 milhões de dólares e na indústria de carne 111 milhões de dólares por ano (McALLISTER, 2016). No Brasil, cerca de 51,3 milhões de dólares e 101,0 milhões dólares por ano na indústria de laticínios e carne, respectivamente (REICHEL et al., 2013).

1.1.7 Resposta Imune contra *N. caninum*

O entendimento da resposta imune contra *N. caninum* é de fundamental importância para que estratégias eficientes de controle e tratamento sejam empregadas,

visto que não há vacinas ou tratamentos comercialmente disponíveis para a neosporose bovina (WALLACH et al., 2008). A primeira barreira física é representada por enterócitos e espessas junções intercelulares da mucosa intestinal que podem deter a invasão do parasito transmitido pela via oral. Quando às células epiteliais intestinais (enterócitos) são infectadas secretam moléculas citotóxicas como o Óxido Nítrico (NO), citocinas (IL-15) que ativam as células *natural killer* (NK), induzindo a produção de IFN-γ e de quimiocinas, recrutando leucócitos polimorfonucleares, macrófagos e células dendríticas. A partir deste recrutamento, ocorre a ativação de células da imunidade inata como macrófagos e células dendríticas via reconhecimento pelo TLR2 (*Toll like receptor 2*) dependente da molécula adaptadora MyD88 levando à secreção de IL-12 que ativa células NK e induz a diferenciação de células TCD4+ em uma subpopulação Th1 produtora de IFN-γ (KHAN et al., 1997; NISHIKAWA et al, 2001; MINEO et al., 2009; MINEO et al., 2010; DION, et al., 2011; ABE et al., 2015).

Contudo, para se entender melhor o comportamento da resposta imune adaptativa é de fundamental importância compreender a imunidade inata contra o patógeno. Os estudos da imunidade inata induzida pelo parasito se iniciou na última década e abrange principalmente o modelo experimental murino. Tais trabalhos demonstraram que *N. caninum* é capaz de ativar diferentes componentes da imunidade que contribuem para a eliminação do parasito ou resistência à infecção. Os eventos relacionados a este tipo de imunidade são desencadeados por reconhecimento de padrões moleculares associados a patógenos (PAMPs), pelos receptores de reconhecimento de padrão (PRRs) tais como receptores do tipo *Toll* (TLRs), *Nod* (NLRs) e lectinas do tipo C (CLRs) que reconhecem várias classes de protozoários, incluindo membros do filo Aplicomplexa, tais como *Eimeria* sp., *Plasmodium* sp., e *Toxoplasma gondii* (ADACHI et al., 2001; KIM et al., 2008; KOBLANSKY et al., 2013; GAZZINELLI et al., 2014; MAC-DANIEL e MENARD, 2015).

Já foi evidenciado que em camundongos geneticamente deficientes para a molécula adaptadora MyD88 (uma proteína crucial para o receptor de IL-1 e a maioria dos TLRs) e para TLR2 ocorre ausência total da produção de IL-12 e consequente síntese de IFN-γ em reposta a infecção por este parasito, resultando no aumento substancial da mortalidade. Além disso, a resposta adequada de células T e a produção de anticorpos estão diretamente associadas à redução de carga parasitária durante as fases aguda e crônica da infecção (MINEO et al., 2009; MINEO et al., 2010). Adicionalmente, um

estudo sugeriu que a resposta do hospedeiro contra *N. caninum* é dependente de TLR3 e da proteína adaptadora TRIF uma vez que o RNA do parasito induz produção de interferon do tipo I dependente de TLR3 (BEITING et al., 2014). O receptor ligante de açúcar Dectina-1 é capaz de favorecer o protozoário, funcionando como um mecanismo de escape o que aumenta a susceptibilidade à infecção, por meio de uma baixa produção de espécies reativas de oxigênio que é letal ao parasito (SILVA, et al., 2017). Além disso não somente receptores de membrana participam da imunidade inata contra *N. caninum*, mas também o receptor citoplasmático Nod2 que é altamente expresso além de ser recrutado para o vacúolo parasitóforo durante a infecção, particularmente participando do processo inflamatório, aumentando assim a letalidade ao hospedeiro (FERREIRA et al., 2016).

Já foi constatado que várias moléculas intracelulares também participam da susceptibilidade ou resistência à infecção por *N. caninum.* O parasito é capaz de modular a resposta imune do hospedeiro através da ativação da fosforilação da MAPK p38 uma vez que a sua inibição resultou na regulação positiva da produção de IL-12p40, além de levar em uma redução da carga parasitária e aumento da sobrevivência em camundongos durante a infecção. Adicionalmente, p38 é induzido por um mecanismo que depende das vias de sinalização GPCR, PI3K e AKT. Portanto, *N. caninum* utiliza da fosforilação de p38 em seu favor, a fim de regular negativamente as respostas imunitárias inatas do hospedeiro (MOTA et al., 2016).

Apesar dos estudos de imunidade contra *N. caninum* em bovinos serem bem reduzidos, já foi demonstrado que as células *natural killer* (NK) representam a primeira linha de defesa durante a infecção, pois são capazes de lisar fibroblastos infectados pelo parasito e produzir IFN-γ, facilitando uma resposta imune celular do subtipo Th1 (WILLIAMS et al., 2000; KLEVAR et al., 2007; MALEY et al., 2006; ROSBOTTOM et al., 2007). Além disso, observou-se que a produção desta citocina durante a gestação previne o abortamento em bovinos infectados (LOPEZ-GATIUS et al., 2007; ALMERIA et al., 2009). Entretanto, no útero há uma alta produção de IL-4, IL-10 e TGF-β que inibem o caráter pró-inflamatório e podem facilitar a proliferação e transmissão vertical (INNES et al., 2000; ENTRICAN, 2002; INNES, 2007). Com relação à imunidade inata, um recente estudo certificou que a imunização com antígenos inativados de *N. caninum* induz a expressão de TLR3, 7, 8 e 9 na interface materno-fetal de novilhas grávidas infectadas (MARIN et al., 2017). Há ainda outros estudos utilizando bovinos como

modelo de infecção que, todavia, se concentraram no entendimento da imunidade celular e/ou humoral (ANDRIANARIVO et al., 2005; ROSBOTTOM et al., 2011; SANTOLARIA et al., 2011).

N. caninum é capaz ainda de infectar células endometriais bovinas tais como células epiteliais e estromais (OROZCO et al., 2013). Tais células em cultura primária tem sido alvo de estudos de diversas patogêneses a fim de compreender a resposta imune inata durante infecções bacterianas e virais. Estudos estes bem estabelecidos e que apresentam resultados muitas vezes condizentes com o perfil geral de resposta do animal (CRONIN et al., 2012; HEALY et al., 2014; TURNER et al., 2014 CARNEIRO et al., 2017). Assim sendo o estudo da imunidade inata durante a infecção por *N. caninum* nessas células podem resultar na melhor compreensão dos mecanismos utilizados pelo parasito, devido a praticidade, padronização dos protocolos e disponibilidade de reagentes no mercado.

Assim sendo torna-se importante compreender melhor a resposta imune contra a infecção por *N. caninum*, para assim selecionar alvos capazes de levar a uma imunidade protetora e insumos profiláticos especificamente em modelo bovino visto que a extensa maioria dos trabalhos utilizam modelo murino para compreender os processos imunológicos. Os estudos até hoje desenvolvidos para gerar vacinas contra neosporose bovina se detém em investigar principalmente fatores imunes adaptativos. Sabe-se que LPS e outros constituintes bacterianos são grandemente influenciados por vias imunes inatas que levam a polarização da resposta imune celular durante a infecção (BASTO et al. 2012; BASTO e LEITÃO, 2014). Este fato pode ser explorado e PAMPs do parasito investigados por realmente gerar formulações de candidatos a vacinas de subunidade (BASTOS et al., 2015 e HEMPHILL et al., 2016).

1.1.8 Imunoprofilaxia da neosporose

Não há vacinas disponíveis no mercado para o controle de *N. caninum* e considerando a magnitude do impacto na pecuária desta infecção sugere-se que uma vacina seria a alternativa melhor empregada para controlar a doença. Em adição, vacinas não deixam resíduos de longa duração em carne ou leite ao contrário de tratamentos com fármacos. A melhor estratégia de controle é desenvolver uma vacina capaz de prevenir o abortamento e a transmissão transplacentária. Apesar de ter havido uma vacina comercial,

denominada NeoGuardTM, a mesma foi retirada do mercado devido sua baixa eficácia em ensaios de campo (GOODSWEN et al., 2015; MARUGAN-HERNANDEZ, 2017).

Atualmente, os estudos de imunoprofilaxia da neosporose se concentra em gerar proteção através de proteínas que ativa tanto a imunidade inata quanto a imunidade adaptativa. Através disso, diversos modelos vêm sendo utilizado levando em consideração as fases da doença, por meio de proteína de taquizoítas e bradizoítas, parasitas vivos ou organismos atenuados (BARTLEY et al., 2008), antígenos particulados (INNES e VERMEULEN, 2006), proteínas de membrana purificadas (HALDORSON et al., 2005), lisado de parasita, peptídeos imunodominantes ou ainda proteínas recombinantes (BENMOHAMED et al., 2002; CHO et al., 2005; EL-MALKY et al., 2014; RODRÍGUEZ-GASCÓN et al., 2014).

Vacinas baseadas em peptídeos e proteínas recombinantes apresentam vantagens potenciais em termos de pureza e especificidade na resposta imune quando comparadas às vacinas baseadas em lisado do parasita. Estudos utilizando antígenos de superfície recombinantes do parasito como NcSAG1 e NcSRS2 também revelaram proteção de moderada a elevada (CANNAS et al., 2003; CHO et al., 2005; HALDORSON et al., 2005; PINITKIATISAKUL et al., 2005; PINITKIATISAKUL et al., 2007).

Como citado anteriormente, outras moléculas importantes para estabelecimento da infecção pelos apicomplexa são as proteínas de micronemas, roptrias e grânulos densos que podem ser alvo no desenvolvimento de protocolos vacinais. Estudos revelaram que rGRA4 e rROP2 de *T. gondii* recombinantes aumentam a proteção de camundongos (MARTIN et al., 2004). Ainda na infecção por *T. gondii*, empregando rGRA2 e rGRA6 como imunógenos verificaram redução no número de cistos cerebrais após desafio em camundongos imunizados somente com rGRA2, constatando que a combinação de ambos antígenos não aumentava a proteção (GOLKAR et al., 2007). Em contrapartida, outro estudo utilizando junção de antígenos como SAG1, GRA1 e MAG1 certificou redução no número de cistos cerebrais até em 89% (GATKOWSKA et al., 2008). Bem como Jongert e colaboradores (2008) que observaram que a combinação das proteínas GRA7, MIC2, MIC3 e SAG1 acarretou na diminuição de 79% no número de cistos cerebrais.

Em relação a *N. caninum*, vacinas utilizando diferentes proteínas secretórias revelaram proteção diversificada. rNcMIC1 exibe proteção moderada (ALAEDDINE et al., 2005), bem como rNcGRA7 (JENKINS et al., 2004), rNcROP2 (DEBACHE et al., 2008), rNcROP2 e rNcMIC1 combinadas (DEBACHE et al., 2009) e rNcROP2,

rNcMAG1 e rNcPDI combinadas (DEBACHE et al., 2010). Em contrapartida a imunização com rNcMIC3 (CANNAS et al., 2003a) revelou elevada proteção em modelo murino. Mais recentemente, um estudo utilizando NcROP2 combinada com NcROP40 demonstrou indução na produção de IFNγ e do anticorpo IgG porém ainda houve transmissão vertical em camundongos (PASTOR-FERNÁNDEZ et al., 2015).

Esses achados demonstram que apesar dos avanços no desenvolvimento de protocolos imunoprofiláticos para a neosporose, ainda não se chegou a uma vacina efetivamente protetora. Essa situação deve-se a dificuldade em utilizar modelos animais adequados, além das escassas informações sobre a biologia do parasito com os diferentes hospedeiros. Com isso, avaliar a participação de proteínas do parasita durante a infecção e sua participação na resposta imune pode oferecer informação sobre a relação patógeno-hospedeiro e consequentemente, gerar estudos mais elaborados no desenvolvimento de vacinas para a neosporose.

1.2 Justificativa

A importância da infecção por *N. caninum* para a medicina veterinária se dá devido às perdas econômicas induzidas na pecuária. Tais perdas são decorrente da eficácia na transmissão vertical que leva ao abortamento, aumento na frequência de natimortos, absorção fetal e nascimento de animais persistentemente infectados. Essas características atingem diretamente a cadeia produtiva de leite e carne. De modo que a neosporose tornou-se extremamente prejudicial no mundo e no Brasil onde o setor agropecuário é responsável por um terço do Produto Interno Bruto (PIB) direto.

A resposta imune contra *N. caninum* ainda não foi muito bem elucidada, principalmente em bovinos. A compreensão da imunidade contra o parasito é de fundamental importância para que estratégias eficientes na prevenção e tratamento sejam empregadas, visto que não há vacinas ou tratamentos comercialmente disponíveis para a doença. O entendimento claro da resposta imune adaptativa necessita de uma exploração melhor sobre a resposta imune inata contra o patógeno. A maior parte dos estudos relacionados à imunidade inata contra *N. caninum* utiliza modelos experimentais que algumas vezes pode não corresponder exatamente aos hospedeiros do parasito. Assim sendo, o uso de alternativas experimentais a fim de entender os mecanismos imunes inatos em células bovinas é necessário, considerando às consequências que essa doença pode gerar nestes animais.

Outro ponto a ser considerado é a necessidade de gerar insumos imunoprofiláticos para a doença. Como não há vacinas disponíveis no mercado e considerando a magnitude do impacto na pecuária desta infecção, novos alvos devem ser investigados com relação à resposta imune contra as moléculas e estrutura do patógeno. A partir disso, há uma maior compreensão da biologia do patógeno e da imunidade induzida, e consequentemente tais alvos podem ser empregados em modelos vacinais ou até em protocolos imunoterapêuticos de outras patologias.

1.3 Objetivo geral

Avaliar a resposta imune inata de células bovinas durante a infecção por *N. caninum* e avaliar a capacidade de rNcROP4 na imunoprofilaxia da neosporose experimental e na imunoterapia de ileíte infecciosa.

A presente tese foi dividida em dois manuscritos presentes nos capítulos II e III

Objetivo geral do capítulo II:

Avaliar a participação *in vitro* de receptores e vias de sinalização intracelulares inatas em células epiteliais e estromais de endométrio bovino durante a infecção por *N. caninum*

Objetivos específicos do capítulo II:

- Determinar a produção de IFNγ, IL-10, IL-6 e IL-8 após a infecção com diferentes MOIs de *N. caninum* em células epiteliais e estromais de endométrio bovino.
- Avaliar a participação do receptor TLR3 e da molécula adaptadora MyD88 na produção de IFNγ, IL-10, IL-6 e IL-8 após a infecção por *N. caninum* em células epiteliais e estromais de endométrio bovino.
- Investigar a participação das MAPK p38, ERK e JNK na produção de IFNγ, IL-10, IL-6 e IL-8 após a infecção por *N. caninum* em células epiteliais e estromais de endométrio bovino.
- Avaliar o papel de STATs (STAT1 e STAT3) na produção de IFNγ, IL-10, IL-6 e IL-8 após a infecção por *N. caninum* em células epiteliais e estromais de endométrio bovino.

Objetivo geral do capítulo III:

Investigar a potencialidade imunoprofilática de rNcROP4 de *N. caninum* durante neosporose experimental murina e imunoterapêutica durante a ileíte induzida por *T. gondii*

Objetivos específicos do capítulo III:

- Predizer, por análise de bioinformática, os possíveis epítopos de células B em NcROP4.
- Investigar a produção de IFNγ e IL-10 em macrófagos e esplenócitos murinos estimulados por rNcROP4.
- Determinar a cinética de produção de anticorpos IgG anti-NcROP4 em camundongos após imunização pela proteína.
- Caracterizar o perfil inflamatório e quantificar a carga parasitária cerebral de camundongos imunizados por *N. caninum*.
- Determinar a taxa de sobrevivência em camundongos imunizados por *N. caninum*.
- Avaliar a produção de IFNγ e IL-10 no íleo de animais tratado com rNcROP4 e infectados por *T. gondii.*

Capítulo 2

Capítulo II

IFNγ/IL-10 balance is dependent of Pattern Recognition Receptor (PRRs) and innate signaling pathways during *Neospora caninum* infection in bovine endometrium cells

2.1 Introduction

Neospora caninum is an obligate intracellular protozoan parasite belonging to the Apicomplexa phylum causing neosporosis. It was initially described for inducing neuromuscular disease in dogs (DUBEY et al., 1988). This parasite presents a heteroxenic life cycle with canids as definitive host and a wide rate as intermediate hosts, including bovine (DUBEY and SCHARES, 2011). Neosporosis causes considerable concern in cattle breeding because when infected, cattle increase the probability of miscarriage due to the high efficacy of vertical transmission. These conditions can lead to considerable economic losses that are around billions of dollars (INNES et al., 2005; REICHEL et al., 2013; REICHEL et al., 2014). Depending on the stage of gestation in which intrauterine infection occurs, bovine neosporosis can be associated with abortion due to degenerative or inflammatory lesions in the fetus, stillborn or mummified fetuses, birth of weak calves with symptoms in the central nervous system or birth of calves clinically healthy, but chronically infected (ANDRIANARIVO et al., 2001; INNES et al., 2002). These consequences will depend of immune response against this parasite during the vertical transmission (KHAN et al., 1997; BASZLER et al., 1999; QUINN et al., 2002; ROSBOTTOM et al., 2008; ALMERIA et al., 2017). It is well established that the IFNγ is an important proinflammatory cytokine for the parasite replication control through the induction of effector molecules such as nitric oxide (NO) and reactive oxygen species (ROS) and acts as an Th-1-subtype lymphocytes inducer (BAZLER et al., 1999). The IFNγ production is also critical for infection control in cattle (INNES et al., 1995; DARWICH et al., 2016). However, although IFNγ is crucial for the host, during gestation the response generated by this cytokine can cause fetal rejection or abortion due to the

induced inflammatory response (KHAN et al., 1997; BASZLER et al., 1999; QUINN et al., 2002; ROSBOTTOM et al., 2008; ALMERIA et al., 2011).

Therefore, the understanding of the immune response against *N. caninum* is of fundamental importance for effective control and treatment strategies, since there are no commercially available vaccines or treatments for bovine neosporosis (WALLACH et al., 2008). Futhermore, it is important to understand the innate immunity against the pathogen to understand better the behavior of the adaptive immune response in bovine cells. Studies of innate immunity induced by the parasite have started in the last decade and the most of information come from murine experimental model. They have shown that the adapter molecule MyD88 participates of Pattern Recognition Receptor (PRR) TLR2 pathway being fundamental to IL-12 and IFNγ production (MINEO et al., 2009; MINEO et al., 2010). Additionally, this parasite is TLR3 and TRIF adapter protein dependent since parasite RNA induces TLR3-dependent type I interferon production (BEITING et al., 2014). There are few studies of innate immune response against *N. caninum* in cattle, mainly to understand the signaling pathway activate during the infection. It occurs due to the difficulty of using these animals in the research and the limited amount of inputs to evaluate specific immune signaling pathways.

Neospora caninum is able to infect uterine cells such as bovine endometrial epithelial and stromal cells that may facilitate vertical transmission (OROZCO et al., 2013). Thus, understanding the involvement of these cells in the immune response against *N. caninum* as well as describing intracellular mechanisms is important to better understand the innate immune response against the parasite in cattle.

Here we demonstrate that both PRRs and intracellular recognition molecules participate in the induction response against this pathogen, in addition we indicated some crucial signaling pathways in the immunity against *N. caninum* in the uterine microenvironment. Thus, the results presented here are relevant for the study of new prophylactic and therapeutic targets during bovine neosporosis.

2.2 Materials and methods

2.2.1 Endometrial bovine cells isolation and culture

Healthy uteri were collected from cattle processed as part of the normal work of an abattoir, as described previously (CRONIN et al., 2012; TURNER et al., 2014). Briefly, the endometrium was dissected from the uterus and enzymatically digested, and the endometrial epithelial and stromal cell populations were isolated by size difference using cell scraper and by their differential adhesion to cell culture flasks.

Endometrial cells were maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), 50 IU/mL penicillin, 50 µg/mL streptomycin, and 2.5 µg/mL amphotericin B (Sigma, St Louis, MO, USA). Cells were plated at a density of 1×10^5 cells/mL in 6-, 12-, 24-well plates (Helena Bioscience, Gateshead, UK) or in 25, 75, or 150 cm2 flasks (Nunc, Rochester, NY, USA).

2.2.2 Parasites

Neospora caninum tachyzoites (Nc-1 isolate) were kindly provided by Dr. Elisabeth Innes from Moredun Research Institute (UK), maintained by continuous passages in a diploid-immortalized cell line derived from cervical cancer (HeLa; CCL-2, ATCC, Manassas, VA, USA). Briefly, HeLa cells were cultured in RPMI-1640 medium (Thermo Scientific Inc., Waltham MA, USA) supplemented with 25mM HEPES, 2 mM l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated calf fetal serum [fetal calf serum (FCS); Thermo Scientific Inc., Waltham MA, USA] in an incubator under controlled temperature and atmosphere (37°C, 5% CO_2, 95% relative humidity; Thermo Scientific Inc., Waltham MA, USA). After HeLa infection with *N. caninum*, these cells have been cultured in RPMI-1640 medium without FCS. Extracellular parasites were washed twice (720 × g, 10 min, 4°C) with phosphate-buffered saline (PBS, 0.01M, pH 7.2), and the resulting pellet was resuspended in RPMI. Finally, the parasites were suspended in RPMI-1640 medium, and numbers of viable tachyzoites were determined by Trypan blue exclusion (Sigma-Aldrich, St. Louis, MO, USA) in a hemocytometer chamber (MOTA et al., 2014).

2.2.3 Endometrial cell culture infection

Ninety per cent confluent endometrial stromal and epithelial cells were infected with MOI 0,1, 0,5 or 1 *N. caninum* tachyzoites (Nc-1 strain) during 12 or 24 hours in 24-

wells plate. Each experiment used at least three animals, and treatments were replicated at least twice. The supernatant of the cells was collected for ELISA analysis to quantified IFNγ, IL-10, IL-6 and IL-8 production.

2.2.4 Enzyme linked immunosorbent assay

Concentrations of bovine IFNγ and IL-6 were measured in supernatants by ELISA according to the manufacturer's instructions (Thermo fisher scientific, Cramlington, UK). Bovine IL-8 and IL-10 was measured by ELISA as described previously (CRONIN et al., 2015).

2.2.5 Short interfering RNA

Endometrial epithelial and stromal cells were transfected with Lipofectamine RNAiMAX Reagent (Invitrogen, Waltham, MA, USA) and siRNA (Cell Signaling Technology, Denver, MA, USA) targeting MyD88, TLR3, STAT1 and STAT3. Briefly, RNAiMAX–RNAi duplex complexes were formed by adding 50 pmol of siRNA to 500 µL of Opti-MEM I medium (11058-021, Invitrogen, Waltham, MA, USA) in each well of a six-well plate, with 50 pmol of non-targeting siRNA (Cell Signaling Technology, Denver, MA, USA) as a control. After, 7.5 µl RNAiMAX was added to each well containing the diluted RNAi molecules and incubated for 20 min at room temperature. Exponentially growing cells (7×10^5 epithelial cells, 5×10^5 stromal cells) were then seeded in 2.5 mL per well of RPMI 1640 growth media, supplemented with 10% FBS. All transfections were replicated in triplicate, and *N. caninum* infection (MOI 0,5) were carried out in RPMI 1640 growth media, without FBS within 12 h and 24 h. The supernatant of the cells was collected for ELISA analysis to quantified IFNγ, IL-10, IL-6 and IL-8 production.

2.2.6 Inhibitors

To further evaluate the role of intracellular signaling pathways in the response to *N. caninum*, inhibitors targeting MAPK were tested using previously validated concentrations for bovine cells (CRONIN et al., 2012). Epithelial or stromal cells cultured

in 24-well plates were treated with an inhibitor of ERK1/2 activation (10 μM in solution, UO126, Sigma-Aldrich, St. Louis, MO, USA), an inhibitor of p38 (10 μM in solution, SB203580; Merck Chemicals, Burlington, MO, USA) and an inhibitor of JNK (10 μM in solution, JNK Inhibitor III; Merck Chemicals, Burlington, MO, USA) for 30 minutes before the infection with *N. caninum* in endometrial cell. The controls for each experiment were an equivalent volume of medium and vehicle, and an equivalent volume of medium and each inhibitor. After 12 and 24 hours after infection, cell supernatants were collected and stored at −20°C for analysis of IFNγ, IL-10, IL-6 and IL-8 by ELISA. The experiments were repeated on 3 independent occasions, with treatments applied to duplicate replicate wells.

2.2.7 Statistical analysis

The data were analyzed using GraphPad Prism 6.0 software package (GraphPad Software Inc., San Diego, USA). Differences were analyzed using One-way ANOVA with Tukey's multiple comparison post-test when necessary. Data were expressed as mean ± standard deviation (SD). Values of $P < 0.05$ were considered statistically significant.

2.3 Results

2.3.1 N. caninum *induces IFNγ, IL-10, IL-6 and IL-8 production in epithelial and stromal cells of bovine endometrium*

Epithelial and stromal cells of bovine endometrium were infected for 12 and 24 h with different *N. caninum* MOIs (0.1, 0.5 and 1) to check the IFNγ, IL-10, IL-6, IL-8 cytokines production (Figure 1). IFNγ production in epithelial cells occurred only 24 h post infection in the lowest MOI (0.1) and MOI 0.5. Whereas although there was production of this cytokine 12 hours after infection, this production was not statistically significant in MOI 0.5. Additionally, there was no IFNγ production when MOI 1 was used in this cell type (Figure 1A). Similarly, stromal cells have produced IFNγ only 24 h post infection when MOI 0.1. Although there was a production of this cytokine 12 h post infection without significant difference. However the MOI 0.5 induced IFNγ production

only 12 h post infection (Figure 1B) and there was no production of this cytokine using MOI 1. Highest IL-10 production was detected in epithelial and stromal cells 12 h post infection independent of MOI used (Figure 1C and 1D). We detected IL-6 production 12 and 24 h after infection in epithelial cells when they were infected with MOI 0.5 and 1 (Figure 1E). Similarly, IL-6 production in stromal cells occurred in the same condition, however in this type of cell, there was a low production 24 h after infection with the lowest MOI 0.1 (Figure 1F). IL-8 production in epithelial cells was crescent during the infection (12 and 24 h) in all MOIs studied. MOI 1 induced the highest IL-8 production independent of cell type. Epithelial cells had higher IL-8 production than stromal cells in the different MOI (Figure 1G and 1H). From these results the next experiments were performed with MOI 0.5.

2.3.2 Upregulation of IFNγ and downmodulation of IL-10, IL-6 and IL-8 are TLR3 and MyD88 dependent in bovine endometrium cells during N. caninum *infection*

To characterize the participation of TLR3 and adapter molecule MyD88 in the innate immune response of the endometrium to *N. caninum*, epithelial and stromal cells of bovine endometrium were transfected with siRNA targeting these genes and infected by *N. caninum* for 12 and 24 h. Both epithelial and stromal cells transfected with siRNA targeting *TLR3* and *MyD88* showed a reduction in IFNγ production comparing to scramble transfected cells (Figure 2A and 2B). Whereas the IL-10 production occurred only 12 h after infection in both cells types and increased when transfected with siRNA targeting *TLR3* and *MyD88* (Figure 2C and 2D). Similarly, the IL-6 production in epithelial cells was higher when transfected with siRNA targeting *TLR3* 12 and 24 h after infection. In other hands siRNA targeting *MyD88* showed no difference in the IL-6 production 12 h after infection and induced the increase of this cytokine 24 h after infection (Figure 2E). In stromal cells the IL-6 production was higher than the control (scramble) when transfected such with siRNA targeting *TLR3* as targeting *MyD88* 12 and 24 h after infection (Figure 2F). The effect of siRNA targeting *TLR3* was induced an increase of IL-8 production in epithelial cells 12 and 24 h after infection, this effect was also observed in siRNA targeting *MyD88* only 24 h after infection (Figure 2G). In stromal cells, siRNA targeting both *TLR3* and *MyD88* induced higher IL-8 production than scramble only 24 h after infection (Figure 2H). Therefore, *N. caninum* can activate TLR3

and MyD88 and these molecules participates in the IFNγ production and in the IL-10, IL-6 and IL-8 downmodulation.

2.3.3 Downmodulation of IFNγ and upregulation of IL-10, IL-6 and IL-8 are p38 and ERK dependent in bovine endometrium cells during N. caninum *infection*

To evaluate the MAPK participation during *N. caninum* infection in epithelial and stromal cells of bovine endometrium, these cells were treated with inhibitors to the MAP kinase p38 (SB203580), ERK (UO126) and JNK (JNK Inhibitor III), infected with *N. caninum* and then IFNγ, IL-10, IL-6 and IL-8 production were determined (Figure 3). Both epithelial and stromal cells showed a higher IFNγ production than the control (DMSO) when treated with p38 inhibitor (Figure 3A and 3B). However, the treatment with ERK inhibitor also induced a considerable higher IFNγ production in stromal cells (Figure 3B). In contrast IL-10 production was reduced when both cell types were treated with the p38 inhibitor, no differences for ERK or JNK inhibitors were observed (Figure 3C and 3D). In addition, the p38 and ERK inhibition were able to dramatically decrease or even abolish IL-6 production in both cell types (Figure 3E and 3F). There was also a decrease in IL-8 in both epithelial and stromal cells treated by p38 and ERK inhibitors (Figure 3G and 3H). These results showed that p38 downmodulates IFNγ and upmodulates IL-10, IL-6 and IL-8 during *N. caninum* infection in epithelial and stromal. ERK is also able to downmodulate the IFNγ production in stromal cell but not in epithelial cells and is able to upmodulate IL-6 and IL-8 but not IL-10.

2.3.4 The modulation of IFNγ, IL-10, IL-6 and IL-8 production are STAT1 and STAT3-depending in stromal endometrium cells of bovine during N. caninum *infection*

To evaluate if IFNγ, IL-10, IL-6 and IL-8 production was STAT1 and STAT3 pathway depending in the endometrium cells during *N. caninum* infection, stromal cells of bovine endometrium were transfected with siRNA targeting *Stat1* and *Stat3* and infected by *N. caninum* by 12 and 24 h (Figure 4). Our results demonstrated that the transfection using siRNA targeting *Stat1* cells produced less IFNγ than the control cells (scramble) both 12 and 24 h after infection and as expected the production of this cytokine was higher in siRNA targeting *Stat3* cells than in control cells (Figure 4A). In contrast,

the transfection with siRNA targeting *Stat1* led to an increase in IL-10 production while siRNA targeting *Stat3* caused a reduction of this cytokine at the time points evaluated when compared with the scramble cells (Figure 4B). Both transfection with siRNA targeting *Stat1* and *Stat3* reduced in IL-6 and IL-8 production when compare with the control 12 and 24 h after infection (Figure 4C and 4D). Therefore, the results demonstrated that Stat1 and Stat3 act on the balance of IFNγ and IL-10 production, besides inducing IL-6 and IL-8 production in an independent way during *N. caninum* infection in stromal cells of bovine uterus.

Figure Legends

Figure 1. Cytokines production in endometrial bovine cells after 12 and 24 h of *N. caninum* infection. IFNγ (A), IL-10 (C), IL-6 (E) and IL-8 (G) production of epithelial cells of bovine endometrium. IFNγ (B), IL-10 (D), IL-6 (F) and IL-8 (H) production of stromal cells of bovine endometrium. Detection limit IFN = 10-100 pg/mL. IL-10 = 10 - 100 pg/mL. IL-6 = 10 - 1000 pg/mL. IL-8 = 10 – 2000 pg/mL. *Statistically significant in relation to the control uninfected (ANOVA and Tukey's multiple comparison post-test; $P < 0.05$).

Figure 2. Cytokines production in endometrial bovine cells after 12 and 24 h of *N. caninum* infection using siRNA target TLR3 (siTLR3) and MyD88 (siMyD88). IFNγ (A), IL-10 (C), IL-6 (E) and IL-8 (G) production of epithelial cells of bovine endometrium. IFNγ (B), IL-10 (D), IL-6 (F) and IL-8 (H) production of stromal cells of bovine endometrium. *Statistically significant in relation to the control uninfected (ANOVA and Tukey's multiple comparison post-test; $P < 0.05$).

Figure 3. Cytokines production in endometrial bovine cells after 12 and 24 h of *N. caninum* infection pretreated with p38 (SB203580), ERK (UO126) and JNK (JNK Inhibitor III) inhibitors. IFNγ (A), IL-10 (C), IL-6 (E) and IL-8 (G) production of epithelial cells of bovine endometrium. IFNγ (B), IL-10 (D), IL-6 (F) and IL-8 (H) production of stromal cells of bovine endometrium. *Statistically significant in relation to the control uninfected and pretreated with vehicle DMSO (ANOVA and Tukey's multiple comparison post-test; $P < 0.05$).

Figure 4. Cytokines production in stromal bovine cells after 12 and 24 h of *N. caninum* infection using siRNA target Stat1 (siSTAT1) and Stat3 (siSTAT3). IFNγ (A), IL-10 (B), IL-6 (C) and IL-8 (D) production of stromal cells of bovine endometrium. *Statistically significant in relation to the control uninfected (ANOVA and Tukey's multiple comparison post-test; $P < 0.05$).

Figure 1

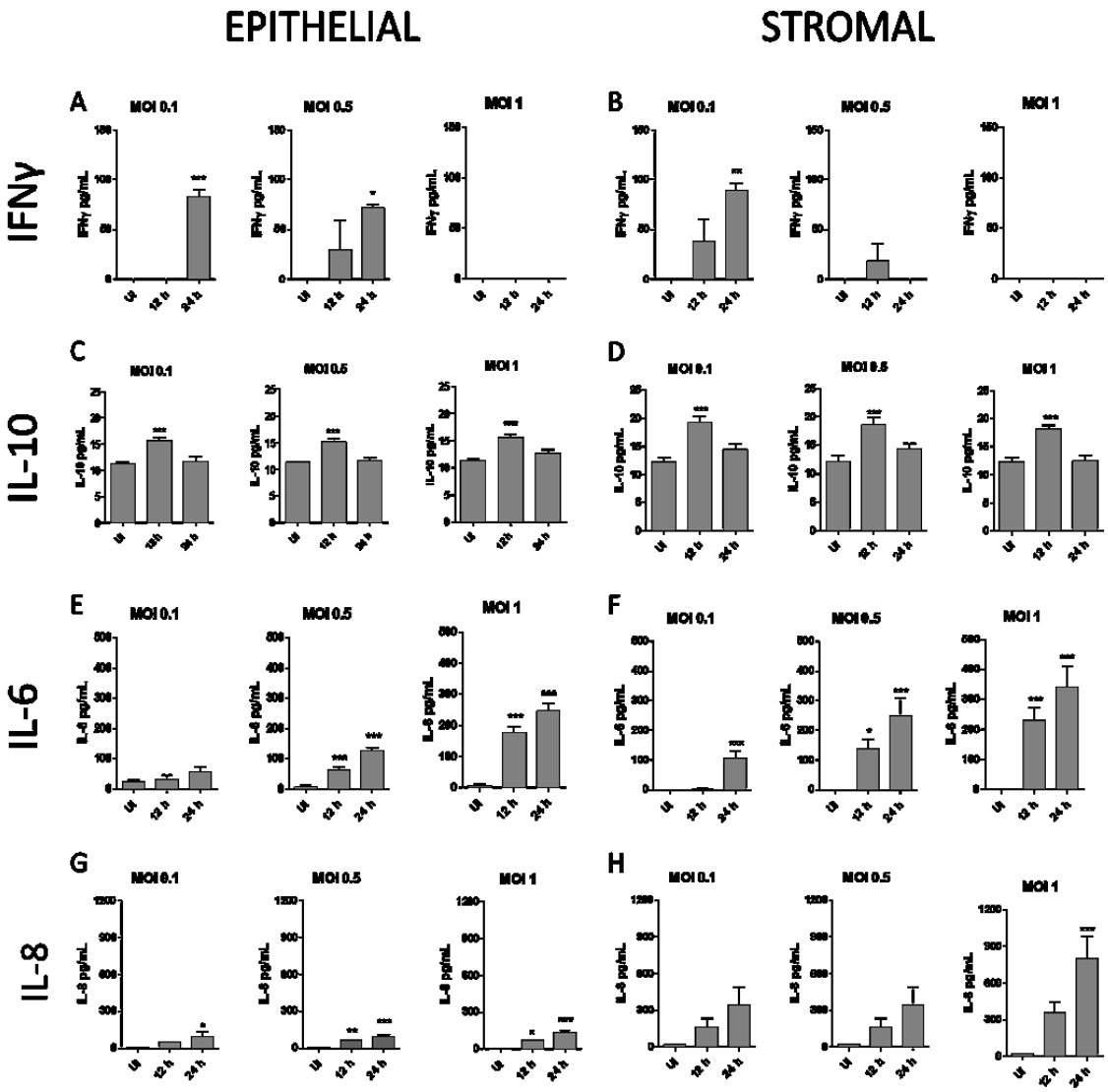

EPITHELIAL
STROMAL
IFNγ
IL-10
IL-6
IL-8
A
B
C
D
E
F
G
H
MOI 0.1
MOI 0.5
MOI 1
IFNγ pg/mL
IL-10 pg/mL
IL-6 pg/mL
IL-8 pg/mL
UI
12 h
24 h

Figure 2

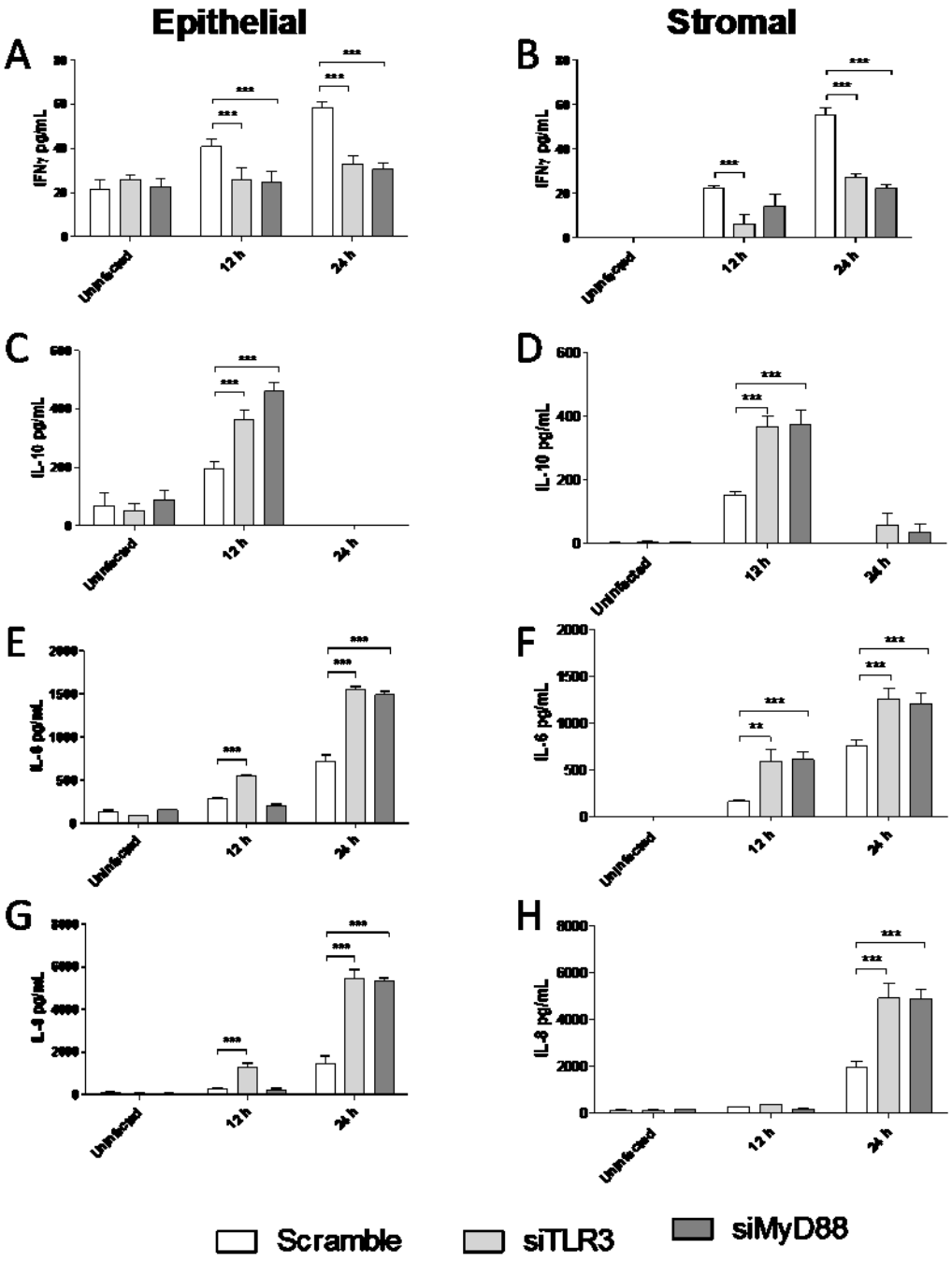

Epithelial
Stromal
A
B
C
D
E
F
G
H
IFNγ pg/mL
IL-10 pg/mL
IL-6 pg/mL
IL-8 pg/mL
Uninfected
12 h
24 h
Scramble
siTLR3
siMyD88

Figure 3

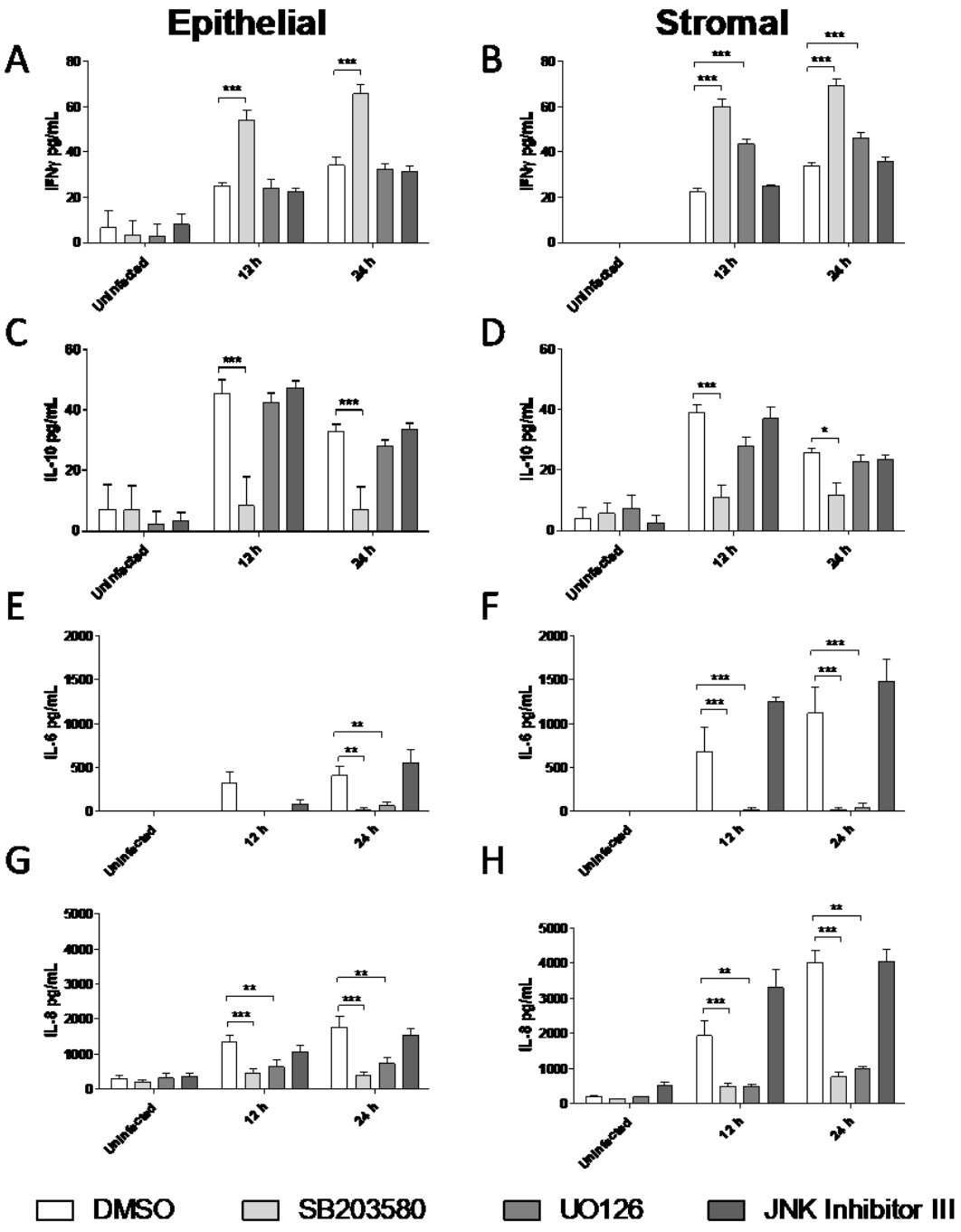
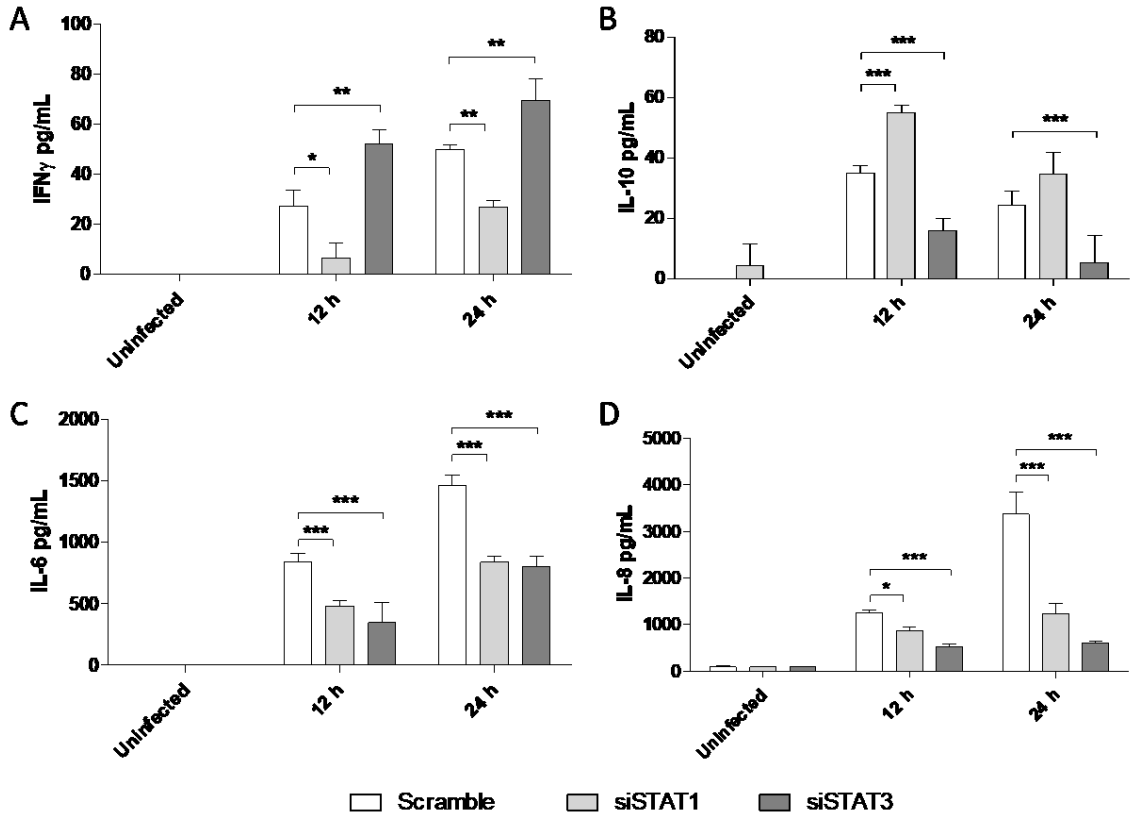

Epithelial
Stromal
A
B
C
D
E
F
G
H
IFNγ pg/mL
IL-10 pg/mL
IL-6 pg/mL
IL-8 pg/mL
Uninfected
12 h
24 h
DMSO
SB203580
UO126
JNK Inhibitor III

Figure 4


A
IFNγ pg/mL
100
80
60
40
20
0
Uninfected
12 h
24 h
B
IL-10 pg/mL
80
60
40
20
0
C
IL-6 pg/mL
2000
1500
1000
500
0
D
IL-8 pg/mL
5000
4000
3000
2000
1000
0
Scramble
siSTAT1
siSTAT3

2.4 Discussion

Our results demonstrated that both epithelial and stromal cells induce the crucial cytokines production for the control or resistance to *N. caninum* infection such as IFNγ, IL-10, IL-6 and IL-8. The IFNγ and IL-6 production increased during the times evaluated (12 and 24 h after infection) and the increase of IL-10 production occurred only 12 h after infection, demonstrating that these cells alone are able to induce a protective immunity against *N. caninum*. IFNγ is an important proinflammatory cytokine for the control of the replication of the parasite and other intracellular microorganisms through the induction of effector molecules such as nitric oxide (NO) and reactive oxygen species (ROS) and acts as the main inducer of subtype lymphocytes Th1 (MOSSMAN et al., 1989; SEDER et al., 1994.; GAZZINELLI et al., 1994; BAZLER et al., 1999). IL-6 is a multifunctional cytokine that participates in the infection and damage response, with role in both the inflammatory response and the induction of tissue homeostasis (CRONIN et al., 2012). Studies in bovine macrophages derived from PBMCs infected with *N. caninum* have indicated that the IL-6 production is increased which hampers the parasite invasion process in these cells through the Th17 lymphocytes activation, showing the role in the parasitic control of this cytokine (FLYNN and MARSHALL, 2011). Another cytokine that has shown a growing production just in the first 12 h of *N. caninum* infection in the cells studied here was IL-8. IL-8, also called CXCL8, is a pro-inflammatory cytokine responsible for the neutrophils recruitment to the site of infection, which is important for generating an effective pro-inflammatory response (McLOUGHLIN et al., 2004). Studies using bovine endothelial cells and macrophages derived from human monocytes have demonstrated that *N. caninum* induces a high IL-8 production within the first few hours of infection and this fact can aid in the infection control (TAUBERT et al., 2006; BOUCHER et al., 2017).

Here we find that IL-10 production was significantly higher 12 h after *N. caninum* infection in both stromal cells and epithelial cells. The regulatory character of IL-10 may favor the infection capacity of this parasite, but can reduce the inflammatory process at the site of infection and consequently the abortion (JESUS et al., 2013). The IL-10 production is important for tolerance in the fetal-maternal interface, however this cytokine is antagonized with IFNγ, which inhibits the major mechanism of parasitic control. This process is able to reduce the inflammatory process in that microenvironment

which facilitates the vertical transmission (TANGRI and RAGUPATHY, 1993; ENTRICAN, 2002).

We have demonstrated that gene silencing technology and the use of pharmacological inhibitors are viable alternatives to better understand the immune response against the parasite in epithelial and stromal endometrial bovine cells. The use of these technologies in the response against viruses and bacteria has generated important findings in the understanding of the innate immunity against these pathogens (SHELDON et al., 2009; SHELDON et al., 2010; TURNER et al., 2014; CRONIN et al., 2012; HEALY, et al., 2015; CARNEIRO et al., 2017).

The study of innate immunity against *N. caninum* has shown the importance of PRRs for the control or resistance to the infection through the induction or inhibition of several chemical mediators such as cytokines (MINEO et al., 2009; MINEO et al., 2010, MOTA et al., 2016; SILVA et al., 2017). Here we showed that both TLR3 and MyD88 molecules with siRNA silencing led to reduce the IFNγ production and increased the IL-10, IL-6 and IL-8 production during infection in the endometrial cells studied. A study in cattle found that immunization with inactivated *N. caninum* antigens induces TLR3, 7, 8 and 9 expressions in the placenta of infected pregnant heifers (MARIN et al., 2017). In addition, previous studies developed by our group corroborated these results showing that MyD88 and TLR2 genetically deficient mice there is a complete absence of IL-12 production and consequent IFNγ induction beside the reduction of IL-10 production in response to the parasite infection, resulting in a substantial increase in mortality (MINEO et al., 2009; MINEO et al., 2010). Another study has suggested that host response against *N. caninum* is TLR3 and TRIF-dependent since parasite RNA induces production of TLR3-dependent type I interferon (BEITING et al., 2014). Although studies show that MyD88 induces the IL-6, IL-8 and other cytokines production and our results show the opposite, increased IL-6 production in cells with silenced TLR3 and MyD88 can be related to the higher IL-10 production induced by the parasite, since when the gene for Stat3 was silenced there was a lower IL-6 production (CRONIN et al., 2012; KAWASAKI and KAWAI, 2014). This cytokine as well as IL-10 can induce the STAT3 phosphorylation, with consequent increase in the production of both (HEINRICH et al., 2003; WILSON, 2014; BABON et al., 2014; COKIC et al., 2015). Although the rise of IL-8 occurred in cells TLR3 and MyD88 knockdown, there was a decrement in such cytokines in cells treated with p38, ERK inhibitors and Stat3 knockdown in response to

the parasite. The production of this cytokine can be induced through phosphorylation of NF-κB via MyD88 or TRIF, p38 and ERK and in bovine endometrial cells it has already been found that the phosphorylation of STAT3 also induces the IL-8 production when stimulated by LPS (ANTONIAK and MACKMAN, 2014; LONG et al., 2015; CRONIN et al., 2016). Our results demonstrated that the block or silencing of other pathways (p38, ERK and STAT3) reduced the production of this cytokine corroborating with the literature data using LPS or other PAMPs (CRONIN et al., 2012; LONG et al., 2015; CRONIN et al., 2016). Thus, we believe that the parasite induces a greater p38, ERK and STAT3 response in IL-8 induction in TLR3 or MyD88 knockdown cells.

We found that p38 inhibition induced the increase of IFNγ and the decrease of IL-10 and IL-6 during *N. caninum* infection. Similarly, but only in stromal cells, ERK inhibition increased IFNγ production, reduced IL-10 production, and in both cell types IL-6 production also decreased. These results are comparable to the TLR3 and MyD88 silencing and can be related to the low IL-10 levels and consequent low STAT3 activity. Our group has also inferred that the parasite can modulate the host immune response through the activation of p38 MAPK phosphorylation since its inhibition resulted in the IL-12p40 positive regulation, the main IFNγ inducer, which can control infection, but the results demonstrated that there is also an increase in IL-6. Therefore, it is noted that *N. caninum* uses p38 phosphorylation in its favor, in order to down-regulate innate immune responses of the host (MOTA et al., 2016). In contrast, a study inhibiting ERK in mouse peritoneal macrophages infected with *N. caninum* showed a reduction in IL-12 production using the UO126 inhibitor (JIN et al., 2017). JNK inhibition did not influence the production of any cytokines evaluated here.

Finally, we sought to understand whether the changes in cytokine production studied here were influenced by STAT1 or STAT3. Silencing the STAT1 gene reduced the IFNγ, IL-6 and IL-8 production and enhanced IL-10 production in response to *N. caninum* infection. IFNγ is the main inducer of STAT1 phosphorylation which consequently leads to the SOCS1 and SOCS3 synthesis and STAT3 pathway blocking, thereby lowing the IL-6 and IL-10 production (GAO et al., 2012). Thus, the increase of IL-10 can be related to the decrease of IFNγ. The IL-6 reduction can be related to the ability of this cytokine to induce the phosphorylation of STAT1 leading to what increases its production (SEHGAL et al., 2002). On the other hands, Stat3 silencing led to the enhance of IFNγ and reduction of IL-10, IL-6 and IL-8 in response to the parasite. STAT3

is phosphorylated either via IL-6R or via IL-10R (LÜTTICKEN et al., 1994). It has already been shown that the IL-10-STAT3 pathway is able to block the NF-κB activation by reducing the inflammatory response (HUTCHINS et al., 2013) which may explain the high IFNγ production by silenced cells. In addition, a study using bovine endometrial cells demonstrated that Stat3 silencing leads to reduction of IL-6 and IL-8 after stimulation with LPS (CRONIN et al., 2016).

This study represents the first characterization of bovine endometrial cells response to *N. caninum*. The results demonstrated that both PPRs and intracellular recognition molecules participate in the induction of a pathogenic response, besides indicating crucial signaling pathways in the immunity against *N. caninum* in the uterine microenvironment. Thus, the results presented here are relevant for the study of new prophylactic and therapeutic targets during bovine neosporosis.

Capítulo 3

CAPÍTULO III

Regulatory effect of recombinant NcROP4 (rNcROP4) from *Neospora caninum* protects during neosporosis experimental murine and reduce ileitis in treatment of acute *Toxoplasma gondii* infection

3.1 Introduction

Neospora caninum is an intracellular obligated protozoan parasite of Aplicomplexa phylum that causes similar pathologies in different animals, a disease called neosporosis. This pathology generates loss of billions of dollars per year in the livestock economy in the United States due to abortion process in cattle (INNES et al., 2007; REICHEL et al., 2013). An essential process of the *N. caninum* life cycle is the cell invasion that generates parasite/host cell interaction. It is a complex mechanism that seems to be similar to other species of Apicomplexa mainly *Toxoplasma gondii*, since both are obligate intracellular organisms. Micronemes, rhoptry and dense granules are parasite organelles that secret proteins with different functions. These and some surface parasite proteins are related to recognition, movement and replication processes. Actually it has been showed that proteins from secreted organelles are important in immune response modulation (DUNN et al., 2008; ELLIS et al., 1994; JENSEN et al., 2015; JOINER and ROOS, 2002; ROSOWSKI et al., 2011).

Studies with *T. gondii* show that proteins of these organelles are able to modulate of host immune response (JENSEN et al., 2015). These characteristics are keys factors in parasite virulence. Some proteins have been tested, showing vaccine potential during the neosporosis. In relation to ROP4 from *N. caninum* (NcROP4) it has known that this protein displays 594 aminoacids and it was identified as a parasite secretory component (SOHN et al., 2011). Moreover, recent studies certified that NcROP4 displays high expression during invasion and evasion process on the host cell and occur phosphorylation during egress cell (ALAEDDINE et al., 2013).

Studies of neosporosis prophylaxis are focused in protection using proteins that activate as innate as adaptive immune response. Vaccine based in peptides and recombinant proteins produces vantages in relation of purity and specificity in response

when compared with vaccine based in parasite lysate (CANNAS et al., 2003; CHO et al., 2005). Research using recombinant surfaces antigens as NcSAG1 and NcSRS2 demonstrated high or moderate protection against *N. caninum* (HALDORSON et al., 2005; PINITKIATISAKUL et al., 2005). Others studies certified that vaccine using different secretory proteins carried to diversify protection. Experiments with rNcMIC1 (ALAEDDINE et al., 2005) displayed moderate protection against the parasite, such as rNcGRA7 (JENKINS et al., 2004), rNcROP2 (DEBACHE et al., 2008), rNcROP2 with rNcMIC1 (DEBACHE et al., 2009). Other combination as rNcROP2, rNcMAG1 and rNcPDI also revealed moderate protection against *N. caninum* (DEBACHE et al., 2010). In other hands, immunization with rNcMIC3 showed high protection in murine model (CANNAS et al., 2003). These findings demonstrate that despite the advances in vaccine protocols development for neosporosis, it does not have an effective vaccine yet. This occurs due the difficult to use appropriated animal models in experiments and the lack of knowledge related to the immune response of the immunogens used. Besides this, there is still few information on the parasite biology in the different hosts (MARUGAN-HERNANDEZ, 2017).

In other hands, closely related to *N. caninum*, *Toxoplasma gondii* infects around 50% of the human population around the world. In the acute phase, this parasite can cause severe ileitis. It is well described that mice infected with *T. gondii* succumb the infection within 13 days of oral infection with 100 cysts of parasite from Type II strain due developing inflammatory process (LIESENFELD et al., 1996). Mortality is related with a high Th1-like immune response with intense necrosis of the villi and mucosal cells in the ileum particularity, with Th1 cells, IFN-γ, TNF and nitric oxide (NO) mediating the development of intestinal lesions (LIESENFELD et al., 1999; LIESENFELD et al., 1996). New approaches in acute toxoplasmosis treatment are still being necessary.

The knowledge of the immune response of different proteins from parasites could lead to the generation of new approaches for vaccines or immunotherapy. Here we describe the regulatory capacity of the recombinant protein NcROP4 (rNcROP4) and from that we developed vaccine protocols for neosporosis and immunotherapy for ileitis caused by acute toxoplasmosis in murine model.

Thereby, to evaluate the participation of parasite proteins during infection and their capacity to modulate or activate the immune response could offer information about the pathogen-host relationship. Finally, we could generate studies more elaborate in

development of vaccines to neosporosis control and treatment of infections inflammatory disease. For this purpose, we aimed to evaluate the immune activation capacity of rNcROP4 and a derived peptide from NcROP4 (pepNcROP4) and to apply these targets in immunization protocols against *N. caninum* infection and in the *T. gondii*-induced ileitis treatment in mice model.

3.2 Material and methods

3.2.1 Animals and parasites

All experiments were carried out with 6–10 week-old female C57BL/6 mice (Centro de Bioterismo e Experimentação Animal - CBEA) from Federal University of Uberlândia (UFU), Brazil. All procedures were conducted according to institutional guidelines for animal ethics and the study received approval of the Ethics Committee for Animal Experimentation of the Institution (CEUA-UFU), under the protocol #029/12.

N. caninum tachyzoites of the Nc-liv isolate were maintained in HeLa cells (ATCC No. CCL-2) with RPMI 1640 medium (Gibco, Paisley, UK), supplemented with HEPES (25 mM), penicilin (100 U/mL), streptomicin (100 µg/mL), L-glutamin (2 mM), sodium bicarbonate (3 mM) (Sigma Chemical Co., St Louis, USA) under an atmosphere of CO_2 (5%) at 37°C until reached confluence. By scraping off the cell monolayer after 2–3 days of infection (SILVA et al., 2007). Tachyzoites were purified by forcible extrusion through a 26-gauge needle to lyse any remaining intact host cells, which were also removed by centrifugation at low speed. The supernatant containing parasite suspension was collected and then washed twice (720 × g, 10 min, 4°C) in PBS and the resulting pellet was resuspended in PBS for lysate antigen (NLA) preparation.

T. gondii tachyzoites (RH strain) were maintained by intraperitoneal serial passages in outbred Balb/c mice at regular 48 hours intervals (MINEO et al., 1980). Mouse peritoneal exudates were harvested when the majority of tachyzoites were extracellular, and then washed twice (720 × g, 10 min, 4 °C) in phosphate-buffered saline (PBS, pH 7.2). The resulting pellet was resuspended in PBS for soluble antigen (STAg) preparation.

It also was used the ME49 strain of *T. gondii* to experimental infection in mice. Therefore, such parasites were maintained in Balb/c mice by intraperitoneal infection

with 10 cysts of the parasite at regular intervals of 30 days. Brains were collected from animals were macerated in sterile PBS to obtain a homogeneous solution. It was conducted count cysts in 20 μL of the homogeneous solution in an optical microscope and inoculated at animals.

3.2.2 Obtaining soluble antigens of N. caninum *(NLA) and* T. gondii *(STAg)*

Soluble antigens of *N. caninum* and *T. gondii* were prepared as previously described (SILVA et al., 2007). Parasite suspensions were treated with protease inhibitors (Complete, Roche, Switzerland) and lysed by ten freeze–thaw (liquid nitrogen and water bath at 37 °C) cycles and further by ultrasound (six 60 Hz cycles for 1 min each) on ice. After centrifugation (10,000 × g, 30 min, 4 °C), supernatants were collected and the protein concentration was determined (BRADFORD, 1976). Soluble antigen aliquots were stored at −20 °C until use.

3.2.3 Hybridoma culture and obtaining the monoclonal antibody 20D2

Hybridoma secreting the monoclonal antibody 20D2 was produced by the Department of Microbiology, Immunology and Molecular Genetics, University of California at Los Angeles (USA) and gently provided for this study. These cells were cultured in RPMI supplemented with 20% fetal bovine serum, 2 mM glutamine, 50 mM 2-β-mercaptoethanol, 40 mg/mL gentamicin. After the cell monolayer formation, hybridomas were detached and transferred to the flasks containing the same culture medium without the addition of fetal bovine serum. Subsequent to formation of a new cell monolayer the culture supernatant was collected containing the monoclonal antibody 20D2 that was stored at -20°C until use in immunoassays.

3.2.4 Phage display and bioinformatics analysis

To evaluate the binding site of the mAb 20D2 in NcROP4 protein, the phage diplay technology was employed. Mimetics peptide (mimetopes) were obtained from two commercial libraries (Ph.D.-12 Phage Display Peptide Library Kit - New England Biolabs Ph.D.-C7c and Phage Display Peptide Library Kit - New England Biolabs, New

England, USA) which express sequences with 12 and 7 amino acids respectively, randomly arranged and connected to filamentous phage M13. Microtiter plates (NUNC maxisorpRT- Nalge Nunc International Co., Rochester, USA) were coated with 20D2 mAb (15 µg/well) in 0.06 M carbonate buffer, pH 9.6 overnight at 4 °C. Plates were washed with 0.1% Tween 20 in 50 mM Tris-buffered saline (TBS) and blocked with 5 mg/mL of BSA in TBS. Plates were then incubated with 1×10^{10} phages of each library diluted in TBS. The unbound phage particles were removed by washing with 0.1% Tween 20 in TBS. The specifically bound phage particles were then eluted with elution buffer (0.2 M glycine-HCl, pH 2.2, containing 1 mg/mL BSA) for 10 minutes at room temperature and immediately neutralized with neutralization buffer (1 M Tris-HCl, pH 9 2). The recovered phages were amplified in *E. coli* ER2738 and partially purified by adding PEG 8000 (Sigma-Aldrich, St. Louis, MA, USA). After the titration, the eluted phage particles were submitted to three additional rounds of binding and amplification.

Phage DNAs were extracted with iodide buffer/ethanol and submitted to sequencing according to the procedures of the automatic sequencer MegaBace (GE Healthcare Life Sciences, Piscataway, USA). For the sequencing reaction, the study used the M13 primers (5'-HOCCCTCATAGTTAGCGTAACG-3'-Invitrogen), f88-4/15-mer (5'-HOAGAAGTCCGAAGACGATGA - 3'- Invitrogem) and fUSE5/6-mer (5 '-HOGGAGTATGTCTTTTAAGT-3' - Invitrogem) that amplify regions of the corresponding phage DNA inserts encoding the amino acid random peptides fused protein PIII. The analysis of DNA sequences derived was processed in self software (Sequence Analyser Base Caller, Cimarron 3.12, Phred 15).

Epitopes predictions to B cells of NcROP4 were done in data bank IEDB (http://tools.immuneepitope.org/main/html/bcell_tools.html). Tertiary structure of the protein NcROP4 was obtained by using Modeller 8V2 and SWISSMODEL program (http://swissmodel.expasy.org/) as ROP2 of *T. gondii* structure as model.

3.2.5 Recombinant NcROP4 production and PepNcROP4 synthesis

To evaluate the potential of recombinant NcROP4 protein for diagnosis of neosporosis in mice were expressed residues 86-509 of the protein in *E. coli*. To obtain the cDNA for protein expression, total RNA was isolated from extracellular *N. caninum* parasites by the Trizol method (Invitrogen) as the manufacturer's instructions and reverse

transcription was performed for NcROP4 using the primer TAGCCTCGTGTCCTCCGTTTC. This RT reaction was then used as a template for PCR using the same 3′ primer and the 5′ primer CACCCAAGAAGAGGTCGAGCAAGTGC for NcROP4. The product was directionally cloned into the pET161 vector which encodes a C-terminal hexahistidine tag for detection and purification (BRADLEY et al., 2005). The constructs were sequenced at the ends to verify the coding region, transformed into BL21DE3 strain *E. coli*, and induced for 5 hours with 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG; Sigma Chemical Co., St Louis, USA). Cells were harvested by centrifugation, the pellet was resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 200 mM sucrose, 500 mM NaCl, one tablet proteases inhibitors and 1mg/mL lysozyme), and disrupted with freeze-and-thaw (six cycles) and sonication (ten 20-s cycles at 90% of power). Insoluble debris was removed by centrifugation at 10,000 × g for 20 min at 4°C and the supernatant was loaded into a NTA-Ni column (Qiagen, Hilden, Germany) previously equilibrated in 50 mM phosphate buffer, pH 8.0. The unbound proteins were removed by washing buffer (50 mM NaH2PO4 and 300 mM NaCl, pH 8.0). The rNcROP4 protein was eluted from the column with the same equilibrium buffer supplemented with 300 mM imidazole. rNcROP4 fractions were pooled and dialyzed against PBS. Purified protein was loaded into a Polimyxyn B column (Thermo Scientific, Waltham, Massachusetts, USA) to remove LPS. Finally, the protein concentration was determined by the method of Bradford (BRADFORD, 1976) and aliquots were stored at −20 °C.

Peptide sequences from NcROP4 were designed and chemically synthesized by Peptide 2.0 Inc. (Chantilly, USA). To increase immunogenicity, peptides were coupled to bovine serum albumin (BSA) and denominated PepNcROP4. PepNcROP4 were lyophilized and later solubilized in Dimethylsulfoxide (DMSO-Sigma, St. Louis, USA).

3.2.6 Immunofluorescence and Immunoeletronic microscopy

To observe if the monoclonal antibody 20D2 was connected to NcROP4, indirect immunofluorescence assay was performed. *N. caninum* intact tachyzoites were prepared as previously described by (CAMARGO, 1964). Briefly, parasite suspensions were treated with formaldehyde (4%) in PBS for 30 minutes at room temperature under slow stirring and centrifuged at 720 × g for 10 minutes at 4°C. The pellet was resuspended in

sterile distilled water and added demarcated areas of microscope slides for immunofluorescence. Indirect immunofluorescence assay proceeded with described previously (BRADLEY et al., 2005) and the analysis was performed on confocal microscope Zeiss LM 510 Meta.

For electronic immunomicroscopy, tachyzoites were centrifuged at 720 × g per 10 minutes at 4° C and fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in PBS. The parasites were washed with PBS, fixed in 1% osmium tetroxide solution, contained 2% agar, dehydrated in ethanol (increasing concentrations of 50 to 100%) and included in LR White resin (Sigma-Aldrich, St Louis, USA). The resin was sectioned (~60 nm) by ultramicrotomy. The cuts were placed in 200-mesh nickel screens and processed for immuno-labeling with colloidal gold. The screens were blocked in TBS 5% skim milk containing 0.05% Tween 20 (TBS-TM) and blocked again with 2.5% goat serum in TBS. Hybridoma supernatant containing mAb 20D2 was used as primary antibody which was incubated for two hours at 37° C. After wash cycles, the anti-mouse IgG secondary antibody conjugated to colloidal gold (Sigma-Aldrich, St. Louis, USA) was inserted into the screens and incubated for an additional hour at 37º C, washed again and contrasted with uranyl acetate and lead citrate. Ultrastructure and label were analyzed and documented in a transmission electron microscope (EM-109, Zeiss, Germany) coupled to an image capture system (MegaviewG2 / Soft Imaging Solutions, Olympus, Japan).

3.2.7 Peritoneal inoculum and peritoneal macrophages culture

To determine the capacity of immune response activation of rNcROP4 and PepNcROP4 C57Bl/6 mice were inoculated with 25 µg of these antigens and NLA intraperitoneal route. After 24 hours or seven days after inoculation we did peritoneal lavage. Fluid peritoneal was centrifugated (400 × g, 10 min, 4°C) and supernatant was used in ELISA to determine IL-12, IL-10 and IFNγ concentration.

Peritoneal lavage was made to obtain peritoneal macrophages. These cells was placed in 96-well plates (1×10^6 cells/mL) prior to stimulation. The macrophages were stimulated with 25 µg/mL of rNcROP4, PepNcROP4 and NLA. We also used 1 µg/mL of LPS and RPMI as control. After 24 hour of stimulus the supernatant was collected and stored at -70°C until cytokines production analysis.

3.2.8 Immunization with N. caninum *antigens*

Four groups of 7 mice were immunized subcutaneously with the following formulations: 25 μg of NLA, rNcROP4 and PepNcROP4 in 200 μL of sterile PBS added to 100 μL Freund's adjuvant (Sigma, St. Louis, USA). Immunizations were carried out at regular intervals of 15 days in three successive inoculations. First immunization was performed with Freund's complete adjuvant and the two remaining with incomplete Freund's adjuvant. Blood samples were collected at 0, 15, 30, 45 days after immunization and the sera analyzed for the presence of specific antibodies. Two weeks after the last immunization (45 d.a.i.), seven animals per group were challenged orally with 1×10^7 or 2×10^7 tachyzoites (lethal dose) of *N. caninum* (Nc-live strain). All surviving animals were euthanized at 30 days after challenge (75 d.a.i.), the brain tissues were collected, sliced longitudinally and stored at −70°C for quantification of parasite burden by real-time PCR or maintained in formaldehyde for histological analysis by HE.

3.2.9 T. gondii-*ileitis induction and treatment with antigens*

Four groups with 6 animals were intraperitoneally injected with 25 μg of rNcROP4, PepNcROP4 or STAg per mouse or with PBS and orally infected 48 hr later with 50 *T. gondii* cysts (ME49 strain). The mice were analyzed on day 7 post-infection. Blood samples were collected for serological assays. Tissue samples of small intestine was collected, fixed in 10% buffered formalin, and processed routinely for paraffin embedding and sectioning. For each organ, tissue sections of 5 μm thickness were mounted on slides and the sections stained with hematoxylin and eosin. The histological analysis was done using a 40 × objective.

3.2.10 Cytokines measure

Cytokine concentrations were measured by ELISA using assay kits for IL-12, IL-10 and IFN-γ (OpTEIA, BD Bioscience, San Diego, CA, USA) according to the manufacturer instructions. Supernatant of macrophages culture was used to IL-12 and IL-10 concentration. Peritoneal fluid was used to IL-12, IL-10 and IFNγ concentration. Ileum tissue samples (100 mg) obtained from mice were homogenized in 0.5 mL of PBS

containing protease inhibitors. Each sample was centrifuged for 10 min at 3,000 × g, and the supernatant used for ELISA. The cytokine concentrations in the samples were calculated based on standard curve generated with murine recombinant cytokines.

3.2.11 Specific antibody detection against N. caninum *by indirect ELISA*

To evaluate the seroconversion of immunized animals specific IgG anti-*N. caninum* was detected by indirect ELISA using NLA. For this purpose, microtiter plates were coated with NLA (10 μg/mL) and then blocked with PBS containing 0.05% Tween 20 (PBS-T) and skim milk (PBS-TM) at 5%. Next, plates were incubated with mice sera (1:25) in PBS-TM 1%. Subsequently, plates were incubated with peroxidase-labeled goat anti-IgG of mice (1:1,000) diluted in PBS-TM 1%. The reaction was developed by adding the enzyme substrate (0.03% H2O2 and 0.01 M 2,2′-azino-bis-3-ethyl-benzthiazoline sulfonic acid) (ABTS; Sigma) and the optical density (OD) was read at 405 nm in spectrophotometer (SpectraMax M2e, Molecular Devices).

The cut off was calculated as the mean OD negative control sera plus three standard deviations. Antibody titers were expressed as ELISA index (EI) as described previously (SILVA et al., 2002) following the formula: EI = OD sample/OD cut off. Values of EI ≥ 1.2 were considered to be positive in order to exclude borderline reactivity values close to EI = 1.0.

3.2.12 Detection of parasite burden by quantitative real-time PCR

Chronic parasitism was determined in brain tissue from mice after 30 days of infection with *N. caninum* by real-time quantitative polymerase chain reaction as described elsewhere (RIBEIRO et al., 2009). Primer pairs designed for the Nc-5 region (Np6/Np21) of *N. caninum* (sense: 3′-GCTGAACACCGTATGTCGTAAA-5′; antisense: 3′-AGAGGAATGCCACATAGAAGC-5′) were used in assays based on SYBR green detection system (Promega Co., Madison, USA). DNA extraction was performed from 20 mg of murine brain tissues using a commercial kit (Wizard SV Genomic DNA kit, Promega, Madison, USA) according to the manufacturer instructions. DNA concentrations were determined by UV spectrophotometry (260 nm; Nanodrop Lite,

Thermo Scientific, Massachusetts, USA) and adjusted to 200 ng/µL with sterile DNAse free water. Assays to determine *N. caninum* parasite loads were performed through real-time PCR (StepOnePlus, Thermo Scientific, Massachusetts, USA) and calculated by interpolation from a standard curve with DNA equivalents extracted from tachyzoites included in each run. Brain tissue from naïve mice was analyzed in parallel as negative control.

3.2.13 Statistics

Statistical analysis was carried out using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, USA). Differences between groups were analyzed using ANOVA or Kruskal-Wallis test, when appropriate, followed by Bonferroni or Dunn multiple comparison post-tests to examine all possible pairwise comparisons. The Kaplan-Meier method was applied to estimate the survival percentage of mice at each time point after challenge and survival curves were compared using the Log-rank test. Values of $P < 0.05$ were considered statistically significant.

3.3 Results

3.3.1 *mAb 20D2 recognize NcROP4 that is expressed in apical complex of* N. caninum

We performed immunofluorescence (Figure 1A and 1B) and immunelectronic microscopy (Figure 1C and 1D) assays to determinate region binding of monoclonal antibody 20D2 in parasite. Next step we applied phage displays technology to certify the binding region in NcROP4. Our results demonstrated that the mAb 20D2 recognized the apical complex of *N. caninum*, specifically in rhoptry (NcROP4). Phage display analysis showed that the monoclonal antibody 20D2 bind in 360-400 amino acid of NcROP4 (Figure 1E). Bioinformatics structure analysis demonstrated that this region is a B-cell epitope potential (Figure 1F). This region was chemically synthetized and the complex peptide-BSA was denominated PepNcROP4 and used in future experiments.

3.3.2 rNcROP4 induces a modulation of immune response in peritoneal macrophages and peritoneal cavity with IL-10 up-regulation

rNcROP4 was used in immune response analysis to evaluate the response profile induced by this protein and its peptide derivate (PepNcROP4). Our results demonstrated that after 24h with different stimulus (NLA, rNcROP4 and PepNcROP4) in peritoneal murine macrophages these cells showed higher IL-10 production of and lower IL-12 production when stimulated with rNcROP4 and PepNcROP4 than when stimulated with NLA. As previously described LPS induced a high IL-12 and IL-10 production (Figure 2A). This profile also was observed within 24h after peritoneal inoculation with different antigens, however it was not detected IFNγ in this time (Figure 2B). With 7 days after inoculum it was not seen IL-12 and IL-10 production, only NLA induced IFNγ production (Figure 2C). Therefore, we can observe that NcROP4 and PepNcROP4 modulate immune response with a regulatory profile.

3.3.3 Immunization with rNcROP4 induces protection against N. caninum *infection in mice*

C57Bl/6 mice were separated in four groups (PSB, NLA, rNcROP4 and PepNcROP4) as described. These animals were intradermal immunized three times fortnightly with different antigens (NLA, rNcROP4 and PepNcROP4). The results showed that 15 days after first immunization there was specific antibody production against *N. caninum* that remained during the immunizations in all groups (Figure 3A). We also observed that body weight of immunized animals increased until challenge (Figure 3B).

Fifteen days after last immunization the animals were challenged with *N. caninum*. It was seen that all groups decreased the body weight in the first day after challenged. We also observed that the PBS group showed a trend to loss body weight 20 days post challenged while NLA and PepNcROP4 groups maintained and rNcROP4 group increased body weight until 30 days post challenged (Figure 3C).

Thirty days after challenged the brain was collected to analyze the inflammation process and parasitism burden. Brain inflammation analysis demonstrated that PBS and NLA groups had more inflammatory process than PepNcROP4. While rNcROP4 group

did not show any inflammatory foci (Figure 4A). Brain parasite burden analysis displayed that NLA and rNcROP4 immunized animals showed lower parasites DNA when compared with PBS group while PepNcROP4 group did not have any statistic difference with PBS group (Figure 4B). Finally, we evaluated the survival of animals challenged with lethal dose of *N. caninum*. We certified that rNcROP4 immunized animals presented 80% of survival while NLA and PepNcROP4 immunized animals presented just 40% of survival. However, we observed that all groups had greater survival than control group (Figure 4C). Such results demonstrated that vaccine potential of NcROP4 during *N. caninum* infection.

3.3.4 Treatment with recombinant rNcROP4 is able to reduce the T. gondii*-induced ileitis*

Considering the immunomodulatory effect of rNcROP4 with IL-10 high production and IL-12 low induction, this protein was used in treatment protocols to toxoplasmosis in murine model. For this purpose, C57Bl/6 mice were treated with rNcROP4, STAg (soluble *T. gondii* antigens) and PBS. STAg was used due benefices results using this extract (BENEVIDES et al., 2013) in *T. gondii*-induced ileitis treatment. We observed that rNcROP4-treated animals showed a high IL-10 production and low IFNγ production in ileum (Figure 5a), histological analysis demonstrated that the treatment with rNcROP4 and STAg showed reduction in inflammatory process when compared with control (PBS) (Figure 5b). Therefore, we certified that rNcROP4 is a promising target in the immunotherapy of acute toxoplasmosis.

Figures legends

Figure 1 – Analysis of bind of mAb 20D2 in NcROP4 protein and bioinformatics design with B-cell epitopes. Indirect immunofluorescence (A and B) and immunoelectron microscopy (C and D) showing that the monoclonal antibody recognizes to NcROP4 protein (green). In silico analysis of the three-dimensional structure of the NcROP4 and alignment of the protein sequence with the peptides selected by phage display (E). Prediction of B-cell epitopes of NcROP4 according to "Bepipred Linear Epitope Prediction". Y-axis starts with the prediction threshold 350. X-axis represents the native amino acid sequence (F).

Figure 2 – IL-12 and IL-10 (A) production by peritoneal macrophages stimulated with different doses of NLA, rNcROP4, PepNcROP4 and LPS. IL-12, IL-10 and IFN-γ production after 24 hours (B) and seven days (C) by mice inoculated intraperitoneally with PBS, NLA, rNcROP4 and PepNcROP4. * $p < 0.05$.

Figure 3 – Production IgG antibodies anti-*N. caninum* from C57Bl/6 mice inoculated with PBS or immunized with NLA, rNcROP4 and PepNcROP4 (A). Body weight variation of C57Bl/6 mice after immunization with NLA, rNcROP4 PepNcROP4 (B) and after challenge with *N. caninum* (C).

Figure 4 – Inflammatory infiltrates (A) and parasitic brain burden (B) of C57Bl/6 mice inoculated with PBS or immunized with NLA, rNcROP4 and PepNcROP and subsequently challenged with *N. caninum* Nc-liv stain. Survival curve of C57Bl/6 mice immunized with NLA, rNcROP4 and PepNcROP4 and challenged with a lethal dose of *N. caninum* in comparison with PBS group (C). * $p < 0.05$.

Figure 5 – IFNγ and IL-10 concentration (A) and histological inflammatory profile (B) in ileum from C57Bl/6 mice treated with rNcROP4 and STAg, uninfected or inoculated with PBS seven day after infection with 50 cysts of *T. gondii* ME49 strain. * $p < 0.05$.

Figure 1

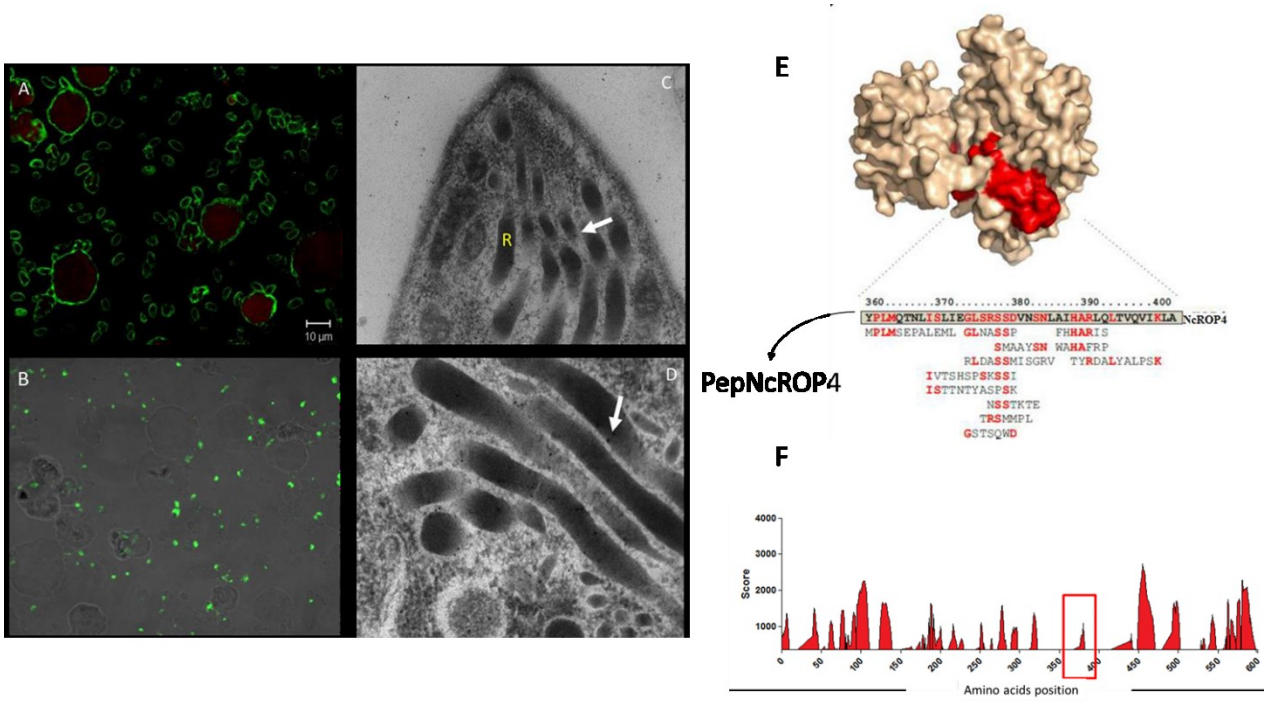

A
B
C
D
R
10 µm
E
PepNcROP4
360.....370.....380.....390.....400
YPLMQTNLISLIEGLSRSSDVNSNLAIHARLQLTVQVIKLA NcROP4
MPLMSEPALEML GLNASSP FHHARIS
SMAAYSN WAHAFRP
RLDASSMISGRV TYRDALYALPSK
IVTSHSPSKSSI
ISTTNTYASPSK
NSSTKTE
TRSMMPL
GSTSQWD
F
Score
4000
3000
2000
1000
0
50
100
150
200
250
300
350
400
450
500
550
600
Amino acids position

Figure 2

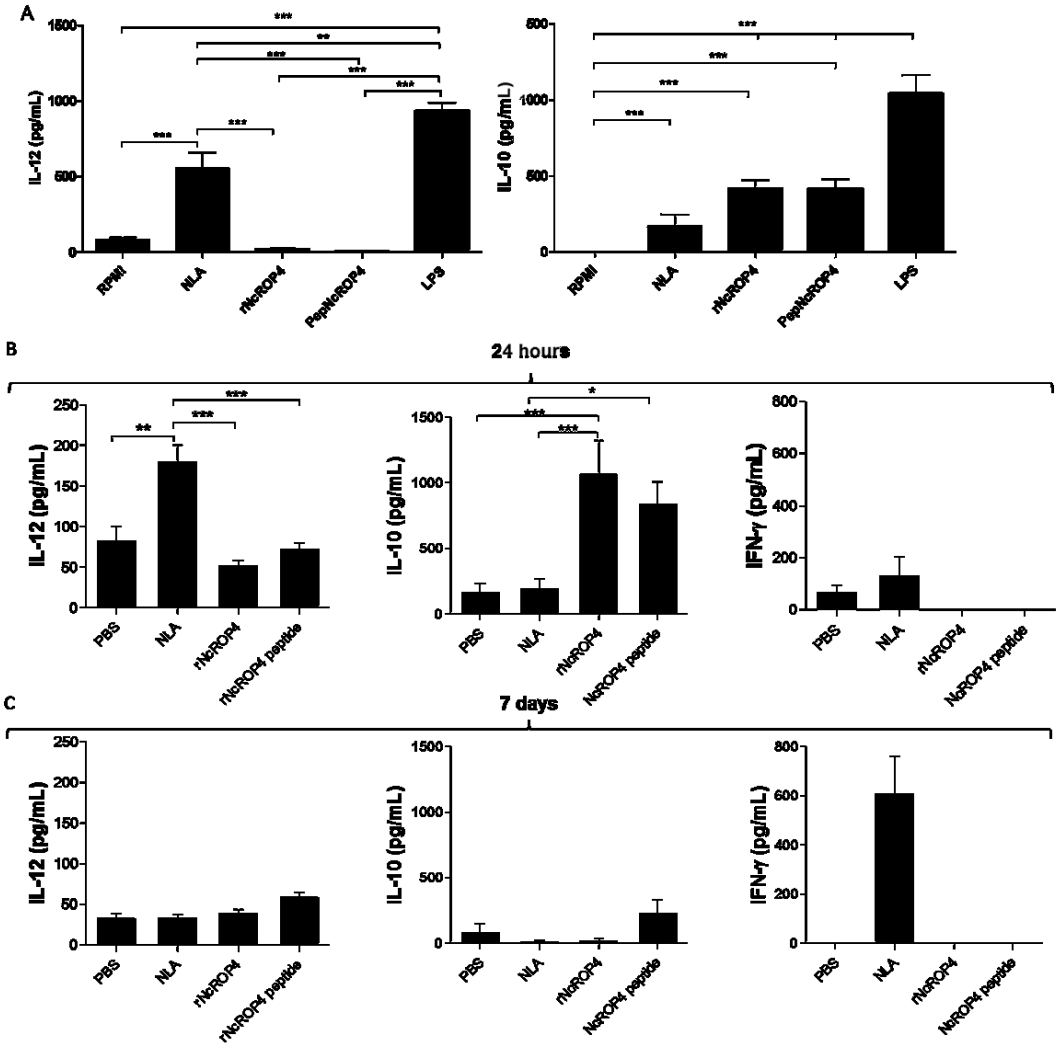

A
IL-12 (pg/mL)
IL-10 (pg/mL)
RPMI
NLA
rNcROP4
PepNcROP4
LPS
B
24 hours
IL-12 (pg/mL)
IL-10 (pg/mL)
IFN-γ (pg/mL)
PBS
NLA
rNcROP4
rNcROP4 peptide
C
7 days
IL-12 (pg/mL)
IL-10 (pg/mL)
IFN-γ (pg/mL)
PBS
NLA
rNcROP4
NcROP4 peptide

Figure 3

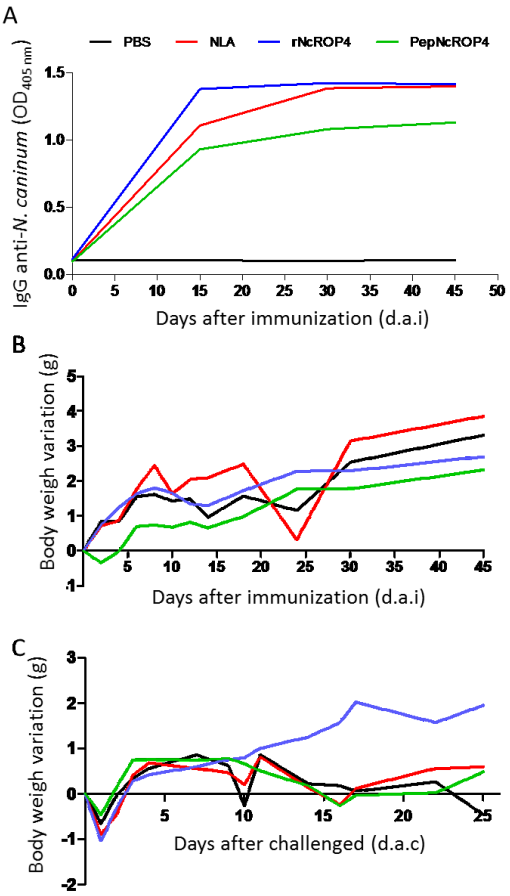

A
PBS
NLA
rNcROP4
PepNcROP4
IgG anti-N. caninum (OD405 nm)
Days after immunization (d.a.i)
B
Body weigh variation (g)
Days after immunization (d.a.i)
C
Body weigh variation (g)
Days after challenged (d.a.c)

Figure 4

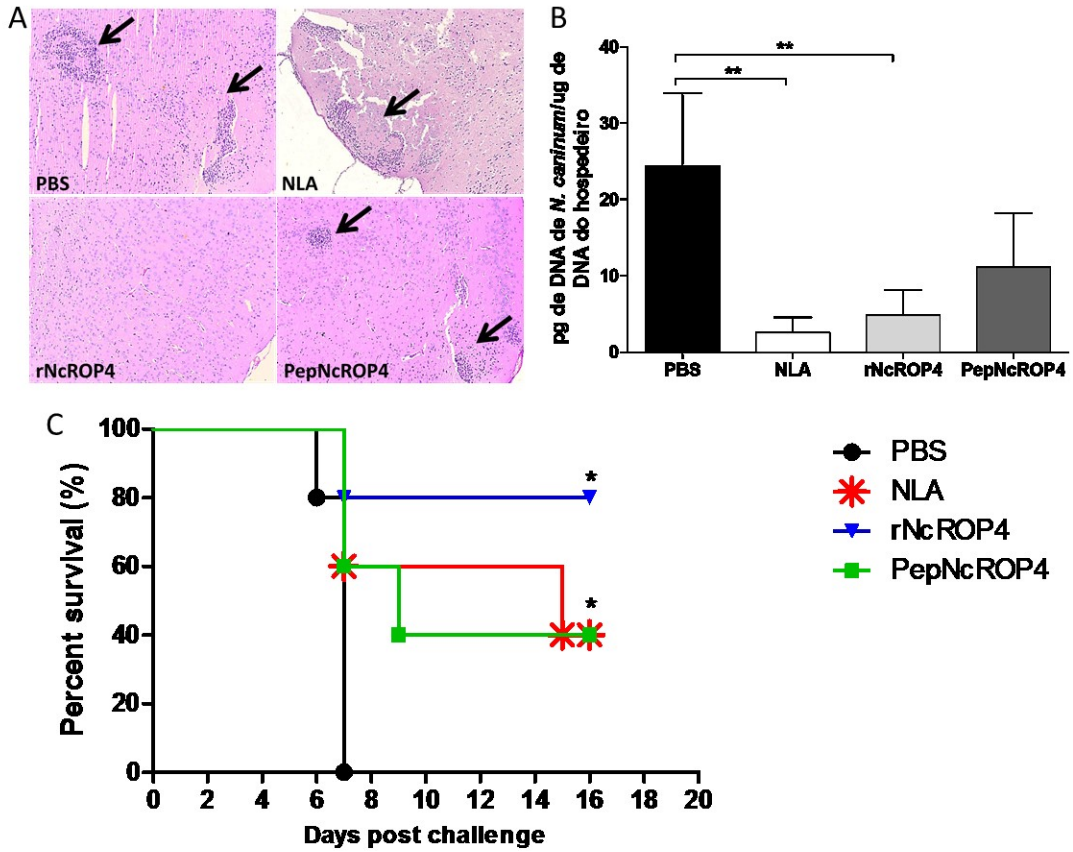
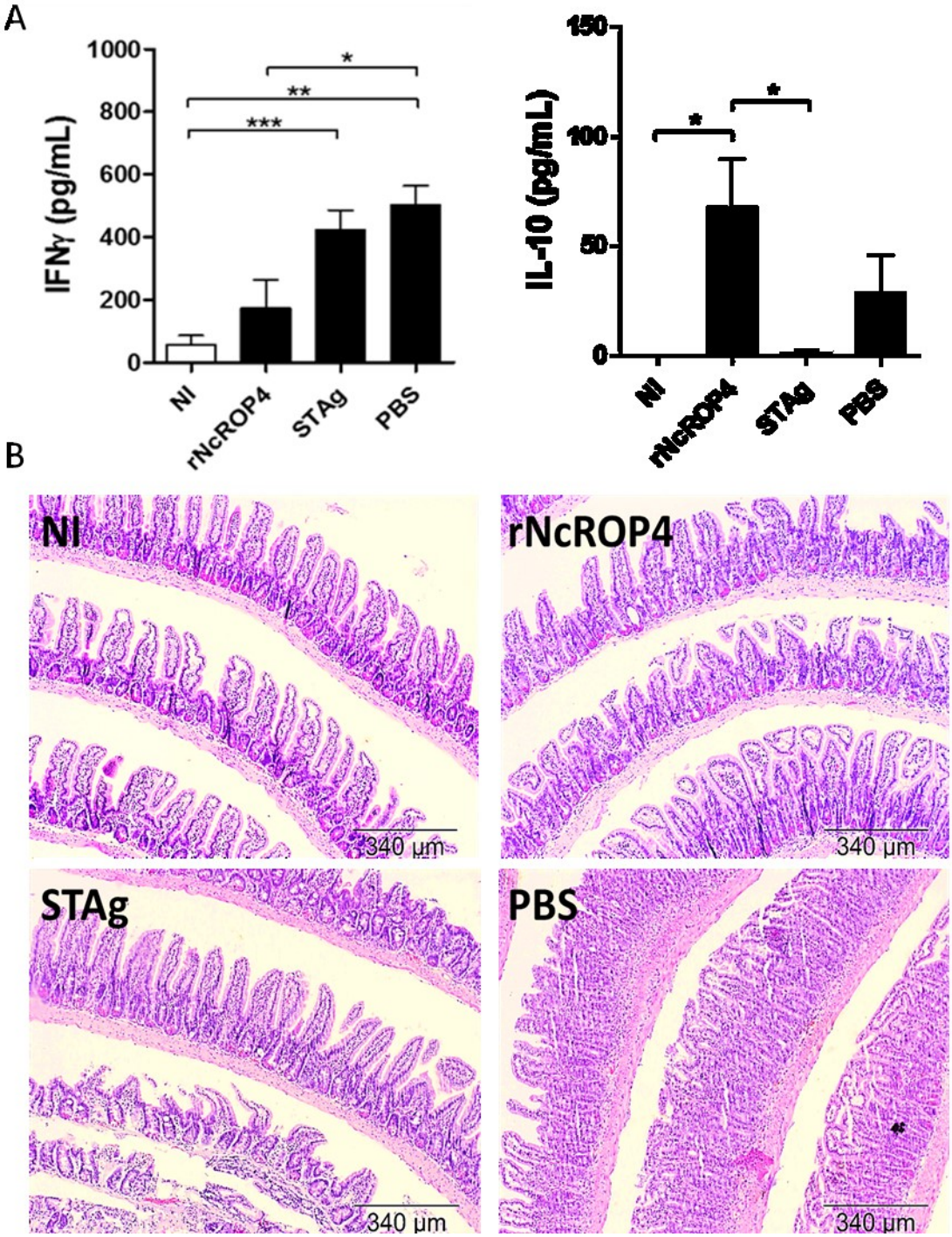

A
PBS
NLA
rNcROP4
PepNcROP4
B
pg de DNA de N. caninum/ug de DNA do hospedeiro
**
**
40
30
20
10
0
PBS
NLA
rNcROP4
PepNcROP4
C
Percent survival (%)
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20
Days post challenge
*
*
PBS
NLA
rNcROP4
PepNcROP4

Figure 5


A
IFNγ (pg/mL)
1000
800
600
400
200
0
*
**
***
NI
rNcROP4
STAg
PBS
IL-10 (pg/mL)
150
100
50
0
*
*
NI
rNcROP4
STAg
PBS
B
NI
rNcROP4
STAg
PBS
340 µm
340 µm
340 µm
340 µm

3.4 Discussion

To determine whether the immunodominant region binding to 20D2 mAb is immunogenic and protective, phage display technology was employed and the mAb binding region was chemically synthetized. There are several studies using mAb to obtain immunodominant regions from proteins to be used in vaccine protocols for protozoan parasites (BASTOS et al., 2016; CAO et al., 2015; HAN et al., 2017; MANSILLA et al., 2016).

Our results demonstrated that NcROP4 and PepNcROP4 are associated in a high induction of IL-10 and low induction of IL-12, consequently in regulatory profile. Our results agree with a study of Butcher et al. (2001) on what tachyzoites of *T. gondii* inhibit inflammatory cytokines production. This fact result in blockade of NF-kappaB nuclear translocation. Complementary results demonstrated that *T. gondii* activates STAT3 independently of IL-10 leaving to IL-12 and TNF down-regulation in macrophages (BUTCHER et al., 2005). These authors (BUTCHER et al., 2011) also discovered that using *T. gondii* with depleted ROP16 protein kinase occurs high IL-12p40 production and this production was related with STAT3 and STAT6 inactivation. They concluded that the parasite through ROP16 activates both STATs modulating the immune response of host cells.Additionally, studies observed that ROP5 and ROP18 proteins from *T. gondii* acts together inactivating Immunity Related Guanosine Triphosphatases (IRG's) and blocking the IFNγ secretion, primary cytokine to pathogen infection control, generating a regulatory profile during infection (NIEDELMAN et al., 2012) .

Here we demonstrated the immunomodulate effect of NcROP4 and its capacity to generate almost complete protection against *N. caninum* infection in mice. Other studies using proteins from rhoptry also obtained satisfactory results. Pastor-Fernandez et al. (2015) used NcROP40 and NcROP2 proteins in immunization protocols and certified fetal survival in mice after challenged with *N. caninum* in a murine model. Debache et al. (2009) demonstrated that the recombinant NcROP2, NcMIC1 and NcMIC3 combined immunization reduce the brain infection and the vertical transmission in experimentally *N. caninum*-infected mice. It was also showed that rNcROP2 decreased the brain parasite burden and increased the survival in mice (DEBACHE et al., 2008). Khosroshahi et al. (2012) demonstrated that association of ROP2 with SAG1 from *T. gondii* can generate moderate protection during *T. gondii* infection in mice. Using these proteins associated

with IL-12, Zhang et al. (2007) demonstrated high protection during *T. gondii* infection. Yuan et al. (2011) also observed that ROP16 protein from *T. gondii* can protect during infection in mice.

Although Th1 response has been reported to be extremely important for parasite elimination. We have found that the regulatory profile induced by rNcROP4 associated with Freund's adjuvant was important for protection against *N. caninum* infection. Immunization with rNcROP4 decreased the brain parasite burden 30 days after infection and increased the survival using lethal dose compared with non-immunized mice. Our group had previously described this fact in another study where it was observed that mice deficient in the Nod2 receptor had lower concentrations of inflammatory cytokines and higher concentrations of regulatory cytokines compared to wild type (WT) animals. In addition, it was observed that the deficient Nod2 animals presented higher survival than the WT animals, however the parasitism was higher in the acute phase (DAVOLI-FERREIRA et al., 2016). Other study using *N. caninum* profilin demonstrated moderate protection with high regulatory T-cell response (MANSILLA et al., 2016b). The discussion is the tolerance process that is very important to homeostasis, thus our results suggest that high IL-10 production during *N. caninum* infection leads to decrease in the inflammatory profile that may be benefic to host tolerance before a parasitic inoculum.

Additionally, we observed high production of specific antibodies against *N. caninum* in mice immunized with all antigens. The property of antibody neutralization is well described, so we believe that this neutralization also showed a protective effect generated by rNcROP4. Peptide (PepNcROP4) used in this work did not generate protection against the parasite. Although predicted as a B-cell epitope and binding to the monoclonal antibody (20D2), this prediction showed low antigenicity and this fact may be related to the low protection generated by the peptide.

It is being establish that *T. gondii* induces severe ileum inflammation by IFNγ high production and other mediators (LIESENFELD et al., 1996). As well as in the results presented in this study, a work developed by Benevides et al. (2013) found that C57Bl/6 mice treated with STAg and subsequently infected with *T. gondii* exhibited lower tissue damage in the ileum compared to untreated animals. In addition, it was found that the IL-10 and IFNγ production was lower in these animals compared to the control group (PBS). As noted in our study, ileum from rNcROP4-treated animals showed little or no tissue injury and high levels of IL-10. It is well described that during absence of IL-10, *T.*

gondii-infected mice succumb infection due to high production of IL-12, IFNγ and TNF. In addition, this cytokine prevents small intestinal necrosis in mice (GAZZINELLI et al., 1996; SUZUKI et al., 2000). Others studies demonstrated that parasites depleted of ROP16 protein showed a higher induction of intestinal lesions than wild type parasites, evidencing that STAT3 and STAT6 phosphorylation by this parasite control the inflammatory effects generated during infection (ONG et al., 2010; JENSEN et al., 2011). NcROP4 exhibits structure highly like many rhoptry proteins from *T. gondii* as ROP2, ROP4, ROP5, ROP8, ROP16 and ROP18, thus we hypothesize that these proteins may act in a manner analogous to other *T. gondii* proteins. Finally, we may certify that rNcROP4 proteins could be an alternative in immunotherapy protocols of inflammatory infection diseases due regulatory profile induced by this protein. For this purpose is necessary more studies using new experimental models and analyzing all protein effects. Moreover, studies focusing in discovery a region of the protein that have capacity to modulate the immune response is important for development of new discoveries using simpler molecules.

In conclusion, the compilation of our results demonstrated that the regulatory profile induced by rNcROP4 may be a future alternative in treatment of acute inflammation caused by *T. gondii* infection. We also observed the participation of this protein in protection of *N. caninum* infection in mice (Figure 6).

Figure 6

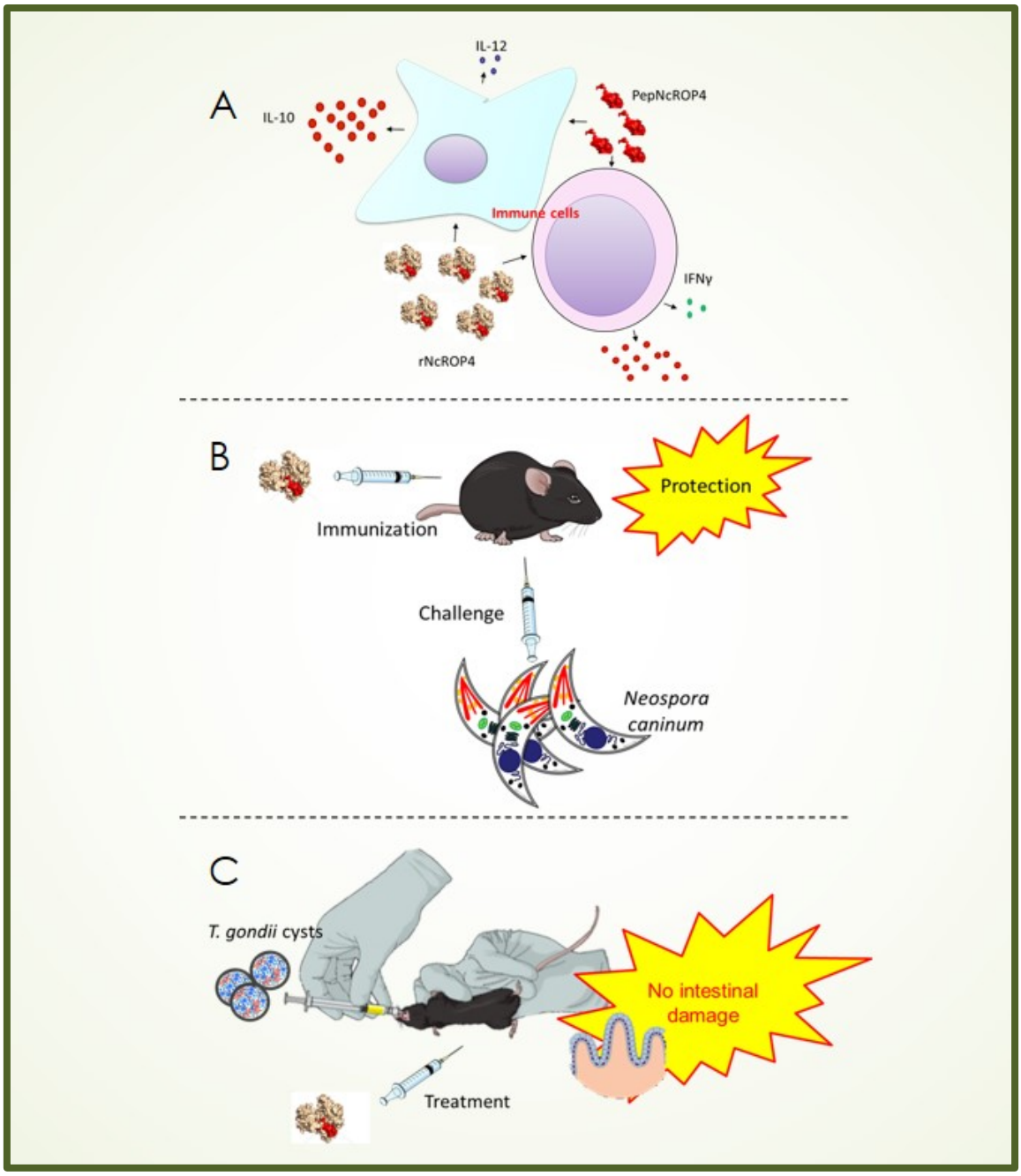


Figure 6 – Graphical abstract. rNcROP4 and PepNcROP4 are able to induce high IL-10 production in macrophages (A). rNcROP4 can protect against *N. caninum* in C57Bl/6 mice (B). The treatment with rNcROP4 can reduce the *T. gondii*-induced ileitis in C57Bl/6 mice (C).

Conclusão

Conclusão geral

Receptores e vias de sinalização inatas participam da resposta à *N. caninum* em células endometriais bovinas e a proteínas rNcROP4 exibe potencial regulatório capaz de aumentar a capacidade protetiva na neosporose e reduzir a ileíte induzida por *T. gondii.*

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