UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
CENTRO DE BIOTECNOLOGIA
PROGRAMA DE PÓS GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

BENVINDO CAPELA JOÃO

RESPOSTA IMUNE LOCAL DE BOVINO INFESTADO COM *Rhipicephalus microplus*

Porto Alegre, 2021

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Resposta imune local de bovino infestado com *Rhipicephalus microplus*

Monografia submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS como requisito parcial para obtenção do título de Mestre em Ciências

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Porto Alegre, março de 2021

Instituições e Fontes Financiadoras

Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), Brasil.

Fontes financiadoras:

Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brasil.

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasil.

Instituto Nacional de Ciência e Tecnologia - Entomologia Molecular (INCT-EM), Brasil.

Dedicatória

Dedico esta monografia a todas as mães. “Minha Mãe (todas as mães negras cujos filhos partiram) tu me ensinaste a esperar como esperaste nas horas difíceis. Mas a vida matou em mim essa mística esperança. Eu já não espero sou aquele por quem se espera ...” (Agostinho Neto *in Sagrada Esperança*).

Agradecimentos

Agradeço ao meu orientador, professor Dr. Itabajara da Silva Vaz Junior, pela orientação incansável, que nos momentos mais difíceis do andamento da formação mostrou-se sempre otimista e acreditou neste trabalho. Agradeço também por todas as oportunidades que colocou ao meu alcance para que eu pudesse crescer na área da iniciação científica. Agradeço por todos os investimentos que fez em mim, quer a nível intelectual, material quanto moral e emocional.

Agradeço ao meu co-orientador, Dr. Luis Fernando Parizi, pelos préstimos no trabalho prático desta monografia e pelo humor positivo nas horas de trabalho infindáveis.

Agradeço a Bianca Cecco e Cintia De Lourenço pelo processamento e análise especializada quando do desenvolvimento da parte de histologia relativa a este trabalho.

Agradeço o Professor Dr. David Driemeier que colocou à minha disposição as condições do seu laboratório. Serei infinitamente grato pela aposta neste trabalho.

Agradeço a professora Dra. Luciane Sonne que revisou os primeiros manuscritos deste trabalho.

Agradeço a todos os colegas do Laboratório de Imunologia Aplicada a Sanidade Animal-Cbiot e do Laboratório do Setor da Patologia-Favet, ambos da UFRGS, por todos os momentos que partilhamos na vivência acadêmica.

Agradeço e saúdo todos os funcionários do Cbiot e do PPGBCM, em especial a Senhora Silvia Centeio pela simpatia e trabalho incansável para servir os alunos.

Lista de abreviaturas

CD	Grupamento de diferenciação
PRR	Receptor de reconhecimento de padrões
IL	Interleucina
γδT	Linfócito gama delta
DC	Célula dendrítica
mRNA	Ácido ribonucléico mensageiro
DNA	Ácido desoxiribonucleico
RNA	Ácido ribonucléico
ATR	Resistência adquirida
IgE	Imunoglobulina E
IgG	Imunoglobulina G
IgM	Imunoglobulina M
Th	Linfócito T auxiliar
APC	Célula apresentadora de antígeno
MHC	Complexo de histocompatibilidade
TLR	Receptor tipo *toll*
FcεRI	Receptor de IgE de alta afinidade
TNF	Fator de necrose tumoral

Resumo

Introdução: Carrapatos são ectoparasitas hematófagos que transmitem agentes patogênicos aos animais e aos humanos, fato que destaca sua importância na saúde animal e na saúde pública. As infestações pelos carrapatos e as doenças causadas pelos agentes veiculados por estes causam grandes perdas na economia agrária. O método de controle mais eficaz e amplamente difundido é o uso de acaricidas, porém o uso destes provoca a seleção de populações resistentes aos acaricidas. Dentre os vários métodos alternativos para combater os carrapatos, o método imunológico é o mais promissor, por isso urge a necessidade de se entender os mecanismos celulares e moleculares envolvidos na resistência imunológica dos bovinos contra o carrapato para se desenvolver vacinas mais eficazes no seu controle. **Objetivo**: Caracterizar *in vivo* a resposta imune local de bovino infestado com *R. microplus* e aferir quais são as subpopulações de células do sistema imune da pele dos bovinos da raça Hereford que estão envolvidas na resposta imune contra o carrapato *R. microplus*. **Métodos**: Para avaliação do infiltrado celular no local da picada do carrapato, foram coletadas biópsias da pele do bovino com carrapato fixado e em um estádio adulto de 17 e 21 dias parasitando bovinos da raça Hereford. As amostras foram fixadas em paraformaldeido 10% e embebidos em álcool e parafina e posteriormente corados com hematoxilina e eosina. Para marcação de linfócitos T e B na pele do bovino foram usados os anticorpos anti-$CD3^+$ e anti-$CD79^+$. **Resultados e discussão**: Bovino infestado com carrapato adulto *R. microplus* mostrou no local da picada do carrapato e nas áreas adjacentes um perfil de células inflamatórias compostas majoritariamente por um número acentuado de eosinófilos, moderada quantidade de linfócitos e raros mastócitos. Quanto as amostras submetidas a imuno-histoquímica, anticorpos monoclonais marcaram fortemente linfócitos T $CD3^+$, enquanto que a marcação de linfócitos B $CD79^+$ foi fraca na pele de bovino com carrapato adulto de 17 e 21 dias. **Conclusão**: A resposta imune local do bovino contra o carrapato é manifestada

pela resposta imune inata que é composta por granulócitos e células mononucleares. É verificado, portanto, um aumento de infiltrado celular composto majoritariamente por eosinófilos, seguidos por aumento de linfócitos e plasmócitos. Contudo, as técnicas utilizadas no presente trabalho não foram adequadas para determinação de outros tipos celulares, como basófilos, subpopulações de células dendríticas e linfócitos se estão ou não envolvidos na resposta imune na pele de bovino contra o carrapato *Rhipicephalus microplus* e, portanto o presente trabalho deve ser expandido considerando-se outras metodologias. Também deve ser considerado o tempo inicial de aquisição de resistência pelos bovinos e considerar diferentes estádios de desenvolvimento do carrapato, além de se estudar a interação do carrapato com vários componentes da estrutura da pele.

Palavras-chave: *Rhipicephalus microplus,* resposta imune, pele, bovino, saliva, linfócito.

Abstract

Introduction: Ticks are hematophagous ectoparasites which transmit pathogenic agents to animals and humans. Ticks are important parasites in the animal health and public health as well. Tick infestation and tick-borne diseases causes economic losses in the livestock industry. Amongst the methods used to tick control, the chemical method is the most widely used, but it selects populations resistant to acaricides. Alternative methods have been developed to tick control and the immunological is the more promising because it is environmentally friendly and effective. There is a necessity to understand the cellular and molecular basis underlining the interaction between tick and their hosts to better develop methods for tick control, such as vaccines. **Objective**: To characterize in vivo the local immune response of bovine infested with *R. microplus* and to investigate which subsets of cells of the immune system in the skin of the cattle are evolving in the immune response against tick. **Methods**: to evaluate the cellular infiltrate at the tick attachment site, skin biopsies were collected from the cattle with tick attached and considering different stages of tick development and attached for 17 and 21 days of the parasitic cycle. Samples were fix in paraformaldehyde 10%, imbibed in alcohol and paraffin and then stained with hematoxylin and eosin. To mark lymphocyte T and B in the skin biopsies, were used anti-CD3+ and anti-CD79+. **Results and discussion**: cattle infested with tick *R. microplus* showed at the tick attachment site and at the areas around a profile of inflammatory cells composed mostly by accentuated number of eosinophils, mild percentage number of lymphocytes and rare mast cells. Samples treated for immunohistochemistry, monoclonal antibodies CD3+ marked strongly lymphocyte T CD3+, while lymphocyte B CD79+ the marker was weak in all the skin biopsies with adult tick attached. **Conclusions**: local immune response of the cattle used in the present study against tick is manifested by the innate immune response composed by granulocytes

and mononuclear cells. There was verified the increment of cellular infiltrate composed mostly by eosinophils followed by the increment of lymphocyte and plasmocytes.

However, the methodology used in the present study should be improved to determine whether basophils, dendritic cells subsets and gamma delta T lymphocytes are involved or not in the cattle immune response against tick using another approach. The present study is an inclusive case of study and it should be expanded considering initial tick life stages and more skin structure components and its interaction with tick.

Keywords: *Rhipicephalus microplus*, immune response, skin, cattle, saliva, lymphocyte.

Sumário

Introdução

Carrapatos são ectoparasitas hematófagos que transmitem patógenos os quais causam doenças de importância na saúde animal e na saúde pública em geral (Dantas-Torres, 2012). Na produção animal destacam-se alguns carrapatos de maior importância, entre as mais de 720 espécies de carrapatos duros já descritas no mundo (Guglielmone *et al.*, 2014) .

Rhipicephalus microplus é um dos carrapatos de importância para a pecuária nas regiões dos trópicos e subtrópicos (Vancová *et al.*, 2020), por transmitir os agentes patogênicos do complexo da tristeza parasitária causada pelas espécies de *Babesia spp.* e *Anaplasma spp.*, as quais são também limitantes para o aumento da produção pecuária se considerarmos as perdas de ganho de peso vivo por animal nas unidades pecuárias (Guglielmone *et al.*, 2014). Em regiões como o Brasil e alguns países do continente africano onde este ectoparasita ocorre, as doenças transmitidas pelos protozoários que infectam o carrapato *R. microplus* estão em determinada época do ano em estabilidade enzoótica, deste maneira que a maioria dos animais ficam imunes aos agentes da tristeza parasitária (Santos *et al.*, 2009) . Porém, no campo, as infestações por carrapatos são de múltiplas espécies e, neste caso, existem vários outros fatores contribuindo para prejudicar o bom desempenho dos animais na produção, concomitantemente com os efeitos espoliativos diretos e indiretos causados pelos carrapatos (Reck *et al.*, 2014). Existe evidências da expansão competitiva e estabelecimento desta espécie de carrapato em regiões onde outrora não eram encontradas (Carvalho, 2016). Portanto, o controle estratégico do carrapato *R. microplus* é de suma importância quando aliado com outros métodos de controle.

Quanto aos efeitos na cadeia produtiva de bovinos, considera-se que os prejuízos econômicos causados pelo carrapato *R. microplus* podem potencialmente alcançar cerca de USD 3,24 bilhões de dólares por ano no Brasil (Grisi *et al.*, 2014), e de US$ 20 a US$ 30 bilhões de dólares de perdas anuais, quando considerado seu efeito econômico na cadeia produtiva de bovinos no mundo (Constantinoiu *et al.*, 2018). Porém, os efeitos econômicos na cadeia produtiva de animais de produção em algumas regiões do continente africano, por exemplo, ainda carecem de avaliação mais detalhada (Carvalho, 2016). O controle desta espécie de carrapato nos trópicos e nos subtrópicos é fundamental para controlar os agentes patogênicos do complexo da tristeza parasitária que têm sido também uma limitante na criação de animais e para o controle extensivo de outras espécies de carrapatos que afetam os animais nestas regiões.

É importante ressaltar que existem outros agentes infeciosos causadores de doenças transmitidas ao redor do mundo por diferentes espécies de carrapatos. Por exemplo a Febre Maculosa Brasileira, responsável por alta taxa de letalidade em humanos infectados pela bactéria *Rickettsia rickettsii*, cujo agente vetorial são os carrapatos das espécies *Amblyomma sculptum* e *Amblyomma cajannense* (De Oliveira *et al.*, 2016). Esta enfermidade está distribuída geograficamente em países como os Estados Unidos, Brasil, México e Colômbia, tornando-a uma das doenças de maior importância na saúde pública, juntamente com aquelas cujo agente causal é veiculado por carrapatos. Como exemplo citam-se a doença de Lyme (Biggs *et al.*, 2016), *Crimean-congo hemorragic fever*, causado pelo Naiovirus cujo vetor são os carrapatos das espécies *Amblyomma spp.*, *Hyalomma spp.* (Eisen *et al.*, 2008), *African tick bite fever* com seus vetores carrapatos das espécies *Amblyomma hebraeum* e *A. variegatum* (Dantas-Torres, 2012), *Tick borne encephalitis* cujos agentes vetores são carrapatos das espécies *Ixodes persulcatus, I. ricinus, H. concinna, H. punctata* (Asebe, 2016), *Powassan encephalitis* causados por

agentes infeciosos transmitidos por *Dermacentor andersoni, Haemaphysalis longicornis, I. cookei, I scapularis* , 2012), *Rocky mountain spotted fever* cujos agentes causais são transmitidos pelos carrapatos *Amblyomma americanum, A. aureolatum, A. cajenense, Dermacentor anderson, D. variabilis, R. sanguineus* (Carvalho, 2016).

Estratégias têm sido desenvolvidas para controlar os carrapatos e as doenças causadas pelos agentes patogênicos transmitidos por estes, como protozoários, bactérias, vírus e fungos (Frazzon *et al.*, 2000; García-García *et al.*, 1999; Gazim *et al.*, 2010; Wikel, 1996; Willadsen *et al.*, 1989). Existem vários métodos de controle aplicados para controlar os carrapatos, dentre os quais o mais comumente utilizado é o método que faz uso de acaricidas químicos. Todavia, o uso de acaricidas tem sido questionado devido ao potencial de selecionar espécies de carrapatos resistentes aos acaricidas, e essa resistência tem sido reportada para a maioria dos acaricidas convencionais utilizados em diferentes países para diferentes espécies de carrapatos (Abbas *et al.*, 2014; De La Fuente, 2018; Rodrigues *et al.*, 2019) e também devido a poluição ambiental e contaminação de produtos de origem animal que os acaricidas causam (Batista; Franco; Roehe, 2018; Tirloni *et al.*, 2014b).

Assim sendo, pelos motivos relativos a inadequação dos métodos aplicados para o controle e pelos prejuízos causados pelos carrapatos de forma direta e indireta aos animais e aos humanos, métodos alternativos têm sido sugeridos, incluindo o método biológico, químico e imunológico (Ali *et al.*, 2015; Parizi *et al.*, 2020; Rodrigues *et al.*, 2019).

O método imunológico potencialmente é mais sustentável (Kim *et al.*, 2015). Este método é baseado no estudo, identificação e testagem de moléculas presentes no carrapato como antígenos vacinais para o controle dos mesmos (Mulenga *et al.*, 2001). Por

exemplo, demostrou-se o sucesso da vacina Bm86 (GAVAC ™ e Tick-Gard™) quando usada para controlar o carrapato *R. microplus* em Cuba e na Austrália, respectivamente (Blecha *et al.*, 2018). Em contrapartida, o mesmo antígeno induziu uma proteção não satisfatória quando usada para controlar a mesma espécie de carrapato na América do Sul, incluindo Brasil (Andreotti *et al.*, 2012; Blecha *et al.*, 2018). Embora vários antígenos estejam sendo investigados (Ali *et al.*, 2015; Fragoso *et al.*, 1998; Parizi *et al.*, 2012, 2020; Prevot *et al.*, 2007), pesquisadores ainda estão buscando melhores antígenos que possam induzir uma resposta imunológica protetora nos hospedeiros contra as espécies de carrapatos.

A observação de que bovinos expostos repetidamente aos carrapatos desenvolvem resistência imunológica contra estes parasitas (Boppana *et al.*, 2005; Constantinoiu *et al.*, 2018; Wikel, 2013), tem sido explorada nas estratégias de desenvolvimento de vacinas que podem induzir proteção contra infestação múltipla de carrapato, uma realidade que é observada nas condições naturais de infestação dos animais no campo (Ndawula *et al.*, 2019). Além do controle da infestação pelos carrapatos nos bovinos, a busca por antígenos vacinais visa também interferir na transmissão de patógenos para os seus hospedeiros (Chmelař *et al.*, 2017a; Heinze *et al*., 2012, Hermance *et al*., 2014).

Os bovinos desenvolvem uma resposta inflamatória peculiar provocada pelo parasitismo do carrapato (Boppana *et al.*, 2005; Buczek *et al.*, 2020; Constantinoiu *et al.*, 2018; Piper *et al.*, 2009; Willadsen *et al.*, 1989). Todavia, os carrapatos desenvolveram mecanismos de evasão da reposta imune do hospedeiro (Martins, *et al*., 2019). Porém, esta é uma relação onde é balanceado a evasão do carrapato e a resposta do hospedeiro para combater o parasitismo (Mans, 2019). A evasão se dá pela saliva do carrapato que

contém um conjunto de proteínas que são produzidas diferencialmente durante aquisição de sangue em seus hospedeiros (Kim *et al.*, 2016; Tirloni *et al.*, 2016).

A saliva secretada pelos carrapatos, tal como outros artrópodes hematófagos, contém moléculas ativas com diversas funções e ações anticoagulante, anti-inflamatória e imunodepressora que facilitam a modulação do sistema homeostático do hospedeiro, na cascata da coagulação, inflamação e sistema imune (Nuttall, 2018). Assim os carrapatos podem se alimentar de sangue nos hospedeiros e manter seu ciclo biológico e também transmitir patógenos (Heinze *et al.*, 2012a; Ribeiro, 1989; Tirloni *et al.*, 2014b, 2016). Estas moléculas bioativas modulam também a resposta imune inata e adaptativa dos hospedeiros especificamente, conforme afirmado por Kotal *et al*., (2015) e reafirmado e testado *in vitro* (Tirloni *et al.*, 2014b, 2016) e *in vivo* (Coutinho *et al*., 2019).

O mecanismo através do qual os carrapatos modulam a resposta imune dos seus hospedeiros e como os bovinos adquirem resistência imunológica é alvo de pesquisas, e vários autores desenvolveram métodos para compreensão deste fenômeno (Allen, 1977; Robbertse *et al*., 2018; Narasimhan *et al*., 2020; Ohta *et al*., 2017), com esforço de elucidar os mecanismos biológicos envolvidos na relação carrapato-hospedeiro por exemplo (De La Fuente *et al*., 2017; Parizi *et al*., 2012). A compreensão dos mecanismos moleculares envolvidos nessa interação e a investigação como os hospedeiros montam a resposta imune contra o carrapato permitirá melhorar o desenvolvimento de vacinas efetivas para controlar os carrapatos (De La Fuente *et al*., 2017).

Em resposta a picada e inoculação da saliva do carrapato, o hospedeiro manifesta uma reação inflamatória cutânea induzida e caracterizada por células inflamatórias do sistema imune inato e adaptativo, tais como neutrófilos, macrófagos, mastócitos e linfócitos, os quais se hipotetiza prejudicarem a fixação, alimentação e a muda da larva

para outros instares subsequentes (Constantinoiu *et al.*, 2010; Heinze *et al.*, 2012a; Piper *et al.*, 2017a). A saliva do carrapato modula a ação dos linfócitos B e T, os quais são fundamentais na resposta imune adaptativa do hospedeiro contra o carrapato (Kotál *et al.*, 2015; Kumar *et al.*, 2016; Kotál et al., 2015). Consequentemente, a reposta imune do hospedeiro é comprometida e suprimida (Sajiki *et al*., 2021), o que reforça o entendimento de que a resposta imune adaptativa celular e humoral periférica não seja suficiente para o controle da infestação por carrapatos (Singh; Girschick, 2003).

A imunidade na pele, local onde o carrapato se fixa, tem um papel importante na resposta imune montada pelo hospedeiro contra o carrapato, havendo a necessidade de se investigar as características da reação inflamatória desenvolvida pelos hospedeiros e a resposta imune local celular contra o carrapato (De La fuente *et al*., 2019). Este estudo tem o objetivo de contribuir para o entendimento da relação carrapato-hospedeiro com o intuito de se descobrir vacinas capazes de induzir proteção e/ou interferir na transmissão de patógenos.

2 Objetivos

Objetivo geral:

Caracterizar *in vivo* a resposta imune local de bovino infestado com *R. microplus*.

Objetivos específicos:

Descrever as células envolvidas na resposta inflamatória na pele infestada com carrapatos.

Identificar alterações na população de células do sistema imune presentes na pele durante diferentes momentos da infestação com o carrapato *R. microplus*.

3 Resultados

Esta seção da monografia foi dividida em dois capítulos.

O Capítulo 1 é constituída de uma revisão focada na literatura da resposta imune cutânea contra carrapato.

O Capítulo 2 é composto de resultados experimentais da análise de células na pele bovina em regiões da picada de carrapato.

Ambos são apresentados na forma de manuscritos em redação.

3.1. Capítulo 1

Host skin during tick infestation: unearthing the immunity mechanisms to lead an anti-tick vaccine

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Planejamento da revisão da literatura: B.C.J., I.S.V.J., e L.F.P.
Escrita do artigo: B.C.J., L.F.P., e I.S.V.J.,
Revisão do artigo: B.C.J, L.F.P., L.T., J.Z e I.S.V.J.

Abstract

Host skin is the first line of defense against most microorganisms as bacteria, and fungi, as well as ectoparasites, including ticks. The skin immune system takes part of this defense firstly by physical and chemical barriers, as well the hemostatic system, followed by use of a wide range of antimicrobial molecules and specialized immune cells, responsible for inflammatory processes, antigen uptake and presentation, allergic responses and untimely destroying the pathogens. Specifically, against tick parasitism, skin immunity has a paramount role to disrupt tick attachment and blood feeding by development of both innate and adaptive responses. In recent years, an increasing number of researches in tick physiology showed a more detailed description of the role of immune cells and their mediators during tick parasitism. Accordingly, a summarization of this information can shed light on orchestration of diverse and complex host immune response mechanisms to reject tick parasitism, and the potential applications to the development new methods for tick control. In this review, we describe and discuss the major cellular responses, functional diversity, and effector mechanisms of host skin immunity against ticks.

Key words: Tick, skin, immunity, vaccine, parasite.

1. Introduction

1.1 Tick distribution

Ticks are obligate blood-feeding ectoparasites of a wide number of domestic and wild animals and humans (Showler *et al*., 2020). Ticks transmit pathogenic agents during host infestation such as protozoa, virus, bacteria, and helminths, causing tick-borne diseases (Dantas-torres *et al*., 2012). They are distributed all over the world, becoming a serious threat to animal husbandry and to public health, fact that make them one of the major constraints to the strategic control of infectious diseases worldwide (De la Fuente *et al*., 2008). These parasites are responsible to transmitting the higher number of distinct pathogen agents to animals and are secondly to mosquitoes as vector of agents that cause diseases in humans (De la Fuente *et al*., 2008). Considerable are the efforts to control or eradicate ticks and minimize its direct and indirect effect along the last decades (Willadsen *et al*., 1989; Gazim *et al*., 2010). Despite this effort, outbreaks of tick populations in previously tick free areas are becoming common for instance for *Rhipicephalus microplus* (Neves *et al*., 2018). In addition, it is important to observe that invasive tick species are spreading to new regions, as *Haemaphysalis longicornis* in the United States (Tufts *et al*., 2019) and *R. microplus* in many East African countries (Muhanguzi *et al*., 2020). Furthermore, these parasites are also found infesting new host species, including wild animals, broadening tick host accidental parasitism (Lohmeyer *et al*., 2018). Cases of Lyme disease are increasing in the north hemisphere due to the dispersion of tick by the migratory birds, constituting a serious threat to the public health and the control of the tick *Ixodes scapularis* in America, Europe and Asia (Ozdenerol, 2015), for instance. Therefore, methods to control ticks and tick-borne diseases needs

strategic improvement to contemplate the range of tick species, and their increasing host diversity.

1.2. Host immune resistance mechanisms against ticks

Ticks started to evolve mechanisms for hematophagous feeding approximately 100 million years ago (Klompen *et al*., 1996). Hard ticks feed on the host for several days to weeks, ingesting blood and increasing hundred times their body weight at the attachment site (Sauer *et al*., 1986). In other hand, soft ticks feed faster (minutes to an hour) increasing two to ten times their initial bodyweight (Mans *et al*., 2011, 2019). It is not surprising that ticks developed different adaptations to modulate host immune system to allow specific blood-feeding behaviors (Sonenshine, 1999). Accordingly, ticks also evolved to inject into the host molecules that inhibit host hemostatic system such as coagulation and platelet aggregation (Nuttal *et al*., 2019; Chmelar *et al*., 2017), by distinct mechanism of action (Ribeiro *et al*., 1987). In another hand, host hemostatic system emerged before the tick anti-hemostatic mechanisms (Ribeiro, 1992, Mans, 2019), which makes more interesting and complex to understand the parasite-host relationship.

Specifically, the understanding of skin cellular immune response against ticks for most of host species remains insufficient (Ohta *et al*., 2017; Karasuyama *et al*., 2020). Other immune mechanisms such as innate components, skin physical barriers and inducible mechanisms amplified by engagement of pattern recognition receptor (PRRs) and antigen-specific receptors at the skin are also important to be elucidated, since it is rarely studied in tick-host interaction, but more expanded for interaction of the microorganisms with their hosts, as reviewed elsewhere by Paludan *et al.,* (2020). In this sense, despite the progress to use tick compounds as antigens for vaccine development, the lack of high protection levels and efficacy improvement of such proteins could be

furthering by the comprehension of the cellular immune response in tick attachment site (Garcia *et al*., 2020; De la Fuente *et al*., 2019). The immunological control method based on the use of vaccines focused for many decades on the empirical selection and characterization of tick proteins (Ali *et al*., 2015; Fragoso *et al*., 1998; Parizi *et al*., 2012, 2020; Prevot *et al*., 2007; Maruyama *et al*., 2017), resulting in limited host protection levels. Although potential antigen showing promising results were analyzed along the time, the goal to develop an effective universal anti-tick vaccine remains to be uncovered.

Basic studies of interaction between ticks and their hosts are important to understanding tick blood-feeding behavior, salivary gland functions, blood digestion, and pathogen transmission (Wikel, 1996, Chmelar *et al*., 2017; Kurokawa *et al*., 2020). Accordingly, understanding specific immunological mechanisms in host tissues against ticks is important to direct anti-tick control strategies (Maruyama *et al*., 2017, Robbertse *et al*., 2020). In this way, typical macroscopic and microscopic characteristics at the skin associated with tick bite is well stablished for some tick-host infestation models (Allen *et al*., 1977; Boppana *et al*., 2005; Buczek *et al*., 2020; Piper *et al*., 2010; Frazin *et al*., 2017; Kurokawa *et al*., 2020; Anderson *et al*., 2017; Heinze *et al*., 2012; Glatz *et al*., 2016, Hermance *et al*., 2016; Heinze *et al*., 2012, Robbertse *et al*., 2020, Heinze *et al*., 2014, Carvalho *et al*., 2010).

1.3. Distinct degree of resistance against ticks among hosts

Different methodologies have been applied to determine the host immunological response against tick infestations (Tabor *et al*., 2017; Burrow *et al*., 2019). Since Trager (1939), demonstrated that rabbits repeatedly infested with ticks were able to impair the parasite reproductive parameters, many other authors followed this outstanding discovery and continued to demonstrate that not only the humoral and systemic immune response

was important, but local immune response also plays a pivotal role impairing tick attachment, feeding and development (Wikel *et al*., 1982; Allen, 1994; Karasuyama *et al*., 2018). Moreover, not all hosts mount a strong immune response against ticks. For example, dogs do not mount an effective immune response against *Rhipicephalus sanguineus* ticks. Similarly, the white footed mice *Peromyscus leucopus,* a natural host for *Ixodes scapularis* in the north hemisphere, do not develop an effective immune response against *Ixodes scapularis* (Anderson *et al*., 2017). Interestingly, guinea pig rejects most of the tick species used in the experimental infestation (Allen, 1973). Mice develop immunity against *H. longicornis* (Tabakawa *et al*., 2018; Ohta *et al*., 2017). In the other hand, hosts as guinea pigs, cows and sheep mount an efficient immune response capable of impairing the tick (Bechara *et al*., 1996; Szabó *et al*., 1995; Boppana *et al*., 2005; Wikel, 2013, Anderson *et al*., 2017). Taking altogether, these studies demonstrated that the immune response differs from natural hosts to the accidental hosts for specific species of ticks and the status of host immunity. In general, tick acquired immune response is normally observed in non-natural hosts, but not in the natural ones. This review will focus on local immune response developed by tick infestation on host skin, describing the main immune cells and the factors involved in such response.

2. Host skin immune system cells against ticks

Skin is the major body physical barrier and the first line of defense against external injuries, including tick mouthpart injury, and it is also the interface between the tick and the host (Heinze *et al*., 2012). Moreover, the skin contains resident immune cells that engage in the host defense against tick parasitism such as eosinophils, mast cells, dendritic cells, macrophages and keratinocytes (Francischetti *et al*., 2009). In this sense, the success of modulation of immune system in skin is essential to tick adaptation. Tick mouthparts

penetrate skin epithelial layers and secreted saliva countermeasure the host immune system locally, modulating essential mechanisms to control the parasitism by different proteins that are secreted into the host (Chmelar *et al*., 2017). Higher level of chemoattractant for neutrophil and macrophages mRNA were found at the human skin infested with tick *Ixodes ricinus* tick, suggesting a strong stimulation of the innate immune system in the skin earlier to tick attachment (Glatz *et al*., 2016).

Molecules injected into host skin for distinct parasite species can attract different combinations of host immune cells populations to the parasite attachment site. This phenomenon is observed for ectoparasites as *Aedes aegypti* mosquitoes infesting rabbits, which showed at skin bite area a differential profile of host immune cells affected by saliva and the status of the host immunity (Henrique *et al*., 2019). Hamster injected with sand flies saliva showed considerably an increase of neutrophils influx at the sand fly attachment site, evidence that saliva from sand fly is also essential for the migration and modulation of the host local cellular response at the skin of the hamster model (Vasconcelos *et al*., 2014). Pigs parasitized with crusted scabies upregulated genes at the skin related to dendritic cell activation process, maturation and ability to present antigens (Bhat *et al*., 2020). Skin immune response against helminth parasites is also well defined with the *Nippostrongylus brasiliensis*. *Nippostrongylus brasiliensis* model of infection, at the first stage the L3 penetrating the mouse skin there is a local leucocyte infiltration composed by the neutrophils and eosinophils, and the host skin injury defines the type 2 immune response against the parasite in which participate local cells producing type 2 interleukins (Allen *et al*., 2014). In IL-5 transgenic mouse which are resistant to first infection to *N. brasiliensis* there were found at the skin a load of larval stages in subcutaneous infection after 48 hours, meaning that some larvae did not molt to another stage and migrate to the lung. This result was correlated to the infiltrated predominant

composition at the skin where they play role to trapping the parasite (Daly *et al*., 1999). In contrast, filarial parasite developed at the skin of IL-5 deficient mouse (IL-5−/−) despite strong skin eosinophilia and did not impeding the larvae to molt (Babayan *et al*., 2010). The immune response in the host skin to ectoparasites that causes myiasis in human and animals are varied depending of the parasite species and the physiologic/immunologic status of the host. They are manifested with non-specific inflammatory reaction, cellular and humoral immune response at the skin layer logged the parasite (Otranto, 2001). Observations shows that not only the sentinels and migratory cell in the skin have an important role in parasite host interaction, skin outer layer keratinocytes also have a very important role in this interaction, reviewed in Briant *et al*., (2014). The latter cell type should be investigated its role at the parasite host interface, including ticks. These examples demonstrated that different local factors responsible for skin immune response as the first line against ectoparasites are common among hosts showed in several parasite host interaction.

Skin immune system is especially important against parasites as ticks that stay many hours attached into the host. It is not a surprise that many different immune system arms are summoned to deal with tick parasitism. For example, it was reported that *Ixodes scapularis* feeds uninterruptedly in white-footed mouse despite strong inflammatory response at the tick bite site, broadening the complex skin immune response to tick infestation in their host (Anderson *et al*., 2017). Basophils accumulated nearby tick *H. longicornis* mouthparts in mouse skin, releasing histamine; however, resident mast cells are not involved in the local immune resistance against ticks (Tabakawa *et al*., 2018). Another factor underling skin immune response against ticks was demonstrated by Ohta *et al*., (2017), describing skin CD4+ memory T cells expressing IL-3 at the skin which are essentially important to basophils migration and accumulation at the *H. longicornis*

attachment site in mouse, as well as memory and effector role in the tick host skin. It was observed that during *Ixodes scapularis* infected with POWV (Powassan virus) biopsies from mouse collected at the 3 hours post infection showed a differential increase of number and type of cell in the skin composed mainly by neutrophils, even though the macrophages were the first infected cells (Hermance *et al*., 2016). However, in the latter study there were not differences in the histopathological features in the later times of 6, 12 and 24 hours. Skin resident memory CD4+ T cell has been also explored in mouse model infested with many microorganisms vectored by ectoparasites (Glennie *et al*., 2015). Neutrophil was increasing at the skin after 12 hours *Ixodes scapularis* infestation on mouse and was associated with up regulation of gene expression related to antimicrobial responses, reactive oxygen species, wound healing and chemokines and cytokines consistent with the influx of this type of cell (Heinze *et al*., 2012).

Cattle primarily infested with *Hyalomma anatolicum anatolicum* showed at the tick bite site increased number of neutrophils, followed by mononuclear cells, in contrast with the prominent differential and higher increase of number of basophils and even neutrophils and mononuclear in the tertiary infestation in 24, 72 and 144 hours post infestation (Gill, 1986). The latter findings were correlated to the manifested immunological resistance at the tick bite site by the impaired tick biological parameters.

Regarding ticks and cattle interaction, Constantinoiu *et al*. (2010) investigated the local immune response of bovines infested with *R. microplus* larvae and suggested that CD3+, CD25+ and γδT cell have a role in skin immune response against tick larvae. However, the role of the latter cells was not determined at the skin during tick infestation. Furthermore, other host cells were found to increase in number at tick attachment site such as eosinophil, neutrophils and macrophages in the beginning of the infestation period

in bovines infested with *R. microplus* (Engracia *et al*., 2017). Interestingly, even inside of tick body was found host immune cells as neutrophils during cattle infestation with *R. microplus* larvae (Constantinoiu *et al*., 2010). Indigenous East African Zebu cattle infested with *R. appendiculatus* and *A. variegatum* ticks presented predominance of inflammatory cell infiltrating the skin composed by higher number of eosinophils and neutrophils when compared to other cells types at the tick attachment site. In contrary, when compared Zebu to the lower resistant cattle (*B. t. indicus and B. t. taurus*), the number of neutrophils decreased in all the cattle infested with the *R. appendiculatus* nymphs (Latif *et al*., 1991). No differences in the number of neutrophils were found at the adult tick *R. microplus* in the resistant and susceptible breeds in the work of Carvalho *et al*., (2010). The latter corroborate to Latif *et al*., (1999) which could not find any difference of neutrophils in the skin of Zebu infested with *A. variegatum* when compared to less resistant animals of Zebu and Holstein Friesian breed. This type of cell is augmented at the tick attachment site independently of the tick larvae or adult life stage, or the tick susceptibility of host (Carvalho *et al*., 2010; Franzin *et al*., 2017). The effect of tick species in the biology of the infiltrated cell to the tick bite site remains to be elucidated.

In the following section, the characterization of the main immune cell population in tick-host interaction are described in more details.

2.1 Dendritic cells

Dendritic cells (DCs) are the major antigen presenting cells of skin immune system (Xu *et al*., 2019; Yazdi *et al*., 2015). During blood feeding, parasite injected molecules are potentially source of many antigens to DC uptake, inducing the adaptive immune response (Henrique *et al*., 2019). DCs are crucial to define the outcomes in the

battlefield between parasite and their hosts, leading the host susceptibility or resistance depending on the modulation of these cells that the parasite engages (Anderson and Oliveira, 2020). Mouse DCs at *Aedes aegypti* mosquito attachment site was diminished after 24 hours of challenge, showing the possible role of DCs in the parasitism of mouse by mosquitos (Henrique *et al*., 2019). Pigs infested with *Sarcoptes scabiei* showed down regulation of genes related to DC subpopulation CD1B maturation and antigen presentation (Bhat *et al*., 2020). Otherwise, DC CD207 antigen presentation genes were upregulated (Bhat *et al*., 2020).

Several studies have demonstrated the effect of saliva and salivary molecules from many tick species, effecting the functions of DCs. It is known that some of molecules secreted in tick saliva also impairs DCs maturation and antigen presenting capability (Skallová, *et al*., 2008; Sokol *et al*., 2009), including pattern recognition receptors (PRR) blockade (Kotal *et al*., 2015). Interestingly, expression of C-type lectin genes in mouse skin infested with *Dermacentor andersoni* were up regulated (Heinze *et al*., 2012). C-type lectin receptor may be related to phagocytosis process and antigen processing mechanism and tick saliva molecules may engage this receptor in the DC (Heinze *et al*., 2012). However, the function of this type of receptor is not defined in the tick host interaction. Langerhans cells, a subset of skin DCs, at the host skin participate to interact with tick saliva antigen as shown in guinea pig infested with *Dermacentor andersonii* larvae (Allen *et al*., 1979). This work showed salivary gland antigens associated with suprabasal layer DC in the epidermis, that trapped these antigens during secondary, but not primary infestation (Allen *et al*., 1979). Tick serpin from *Rhipicephalus haemaphysaloides* inhibits DC co-stimulators B7 (CD80 and CD86) which in turn may influence the inflammatory cytokines and therefore impaired the tick feeding process (Xu

et al., 2019), which reinforce the need to study the DC subsets been induced by tick antigens and tick secreted small molecules (Martins *et al*., 2019). Guinea pigs infested with *Dermacentor andersoni* had increased Langerhans cell at the tick attachment site during infestations, reinforcing that DC subpopulations have a critical role at the skin tick attachment site (Nithiuthai and Allen, 1983). The inhibition of DC functions by tick saliva also reveals that DC has an importance in the skin at the tick-host interface (Esteves *et al*., 2019; Anderson *et al*., 2007). For example, mRNA levels of the dendritic cell marker CD11c and other mRNA related to innate immune response were significantly increased in skin of humans infested with *Ixodes ricinus* less than 24 hours after infestation compared to control normal skin (Glatz *et al*., 2016). Despite all the studies about tick-host cellular DC subpopulations, it is still unknown the contribution of each subpopulation of DC in the skin regarding tick-host interaction (Anderson *et al*., 2020).

2.2 Basophils

Basophil is a granulocyte related with inflammatory process during skin damage by parasites. It was demonstrated in human skin, where infestations by *Sarcoptes scabiei*, and *Cimex lectularius* resulted in basophils infiltration in skin lesions (Ito *et al*., 2011). Evidences suggesting that basophil participate in protective immune response against ectoparasites has been reviewed by Wada *et al*., (2011). Basophils were also augmented in its numbers in skin of animals infested with adult ticks. Moreover, these cells were increased in resistant cattle skin compared to the susceptible phenotypes (Carvalho *et al*., 2010). Similarly, the number of basophils in the skin of Nguni cattle was higher compared to skin of Bosmara cattle infested with *R. microplus* (Marufu *et al*., 2014).

In mouse, basophil is recruited to the tick attachment site through the interleukin 3 produced by peripheral and resident memory CD4+ T cells in skin (Ohta *et al*., 2017).

Ablation of the basophil in guinea pig have demonstrated that these cells have an essential role in acquired host immunity against ticks (Allen, 1973). Histamine released by basophil are essential mediators of the acquired tick resistance and play a fundamental role in tick rejection because it acts on skin epithelium, causing cell hyperplasia, itching and grooming (Tabakawa *et al*., 2018). Although basophils are very few in the circulating blood, these cells are concentrated in skin of crossbred Holstein cattle infested with *R. microplus* and found incremented its amount at tick bite site (Engracia *et al*., 2017). Since basophils are essential for the development of acquired tick resistance in the host, several studies had elucidating their role (Wada *et al*., 2011, 2010). Since seminal studies of Trager in 1939, basophils infiltration of skin with other granulocytes cells was related to acquired tick resistance (ATR). This was supported by the work of Brown *et al*., (1982), which infested guinea pigs with *A. americanum* and promoted the depletion of basophil and therefore the depletion of the ATR as well. In the latter, was demonstrated the role of basophils infiltrated in the skin of cattle and even though the quantity varies depending on the tick species, in cattle the amount of basophils increased in the primary infestation with *A. americanum,* and *Ixodes holocyclus* adults tick infestation to cattle previously infested, basophils was the primary infiltrate at the tick bite site (Brown *et al*., 1984; Allen *et al*., 1977). The role of basophils in these hosts need to be clarified dependent on the species of the tick that infest them.

Mouse infested with *H. longicornis* recruited basophils to the tick bite site at the second infestation (Wada *et al*., 2010). Another report in 1990 by Masuda *et al*., demonstrated that basophils were infiltrated at the tick feeding site during a *H. longicornis* reinfestation in mouse. Basophils are difficult to detect due to the characteristic few granules, but they were observed infiltrating and increased in cell-sufficient mouse and mast cell deficient mouse in the third infestation with the *D. variabilis.* The studies

developed by Ohta *et al*., (2017), confirmed the infiltration of basophil at the second infestation by specific basophil migration by the action of IL-3 produced by CD4 memory T cell. Depletion of basophils in mouse impaired the ATR completely (Wada *et al*., 2010) and did not affect the amount of other cell that infiltrate the feeding site in mouse infested with *H. longicornis*. In human skin infested with *Ixodes persulcatus,* was found a positive correlation and activation of basophil and eosinophil in various skin inflammatory diseases (Ito *et al*., 2011). Furthermore, histamine released by basophil and not by other granulocyte cells is essential for ATR and consequently for tick bite impairment in mouse (Tabakawa *et al*., 2018). Skin injected with histamine had more epidermal hyperplasia showing that histamine released by basophils is important for the host immunological local resistance capable of impairing ticks (Tabakawa *et al*., 2018).

Karasuyama *et al*., (2018), proposed a mechanism of basophil defense against tick infestation in a murine model. In such a model, during the first tick infestation, DC take up tick saliva antigens and move to draining lymph node where they present the antigens to the CD4+ T cells, activating the liberation of IL-4 by T cells. IL-4 derived from T cell stimulates B cells to produce IgE to tick antigens, which in peripheral blood bind to basophils. In turn, some of the CD4+ T cell migrate from the lymph node to the skin. At the second infestation, CD4+ T cell are reactivated by tick antigens and promote the liberation of IL-3 which recruit basophils to tick bite site and release histamine that acts on keratinocytes and therefore resulting in the epithelial hyperplasia that causes tick impairment.

2.3 Mast cells

Mast cells are another immune cell involved in host skin response against tick parasitism (Robbertse *et al*., 2017; Tabakawa *et al*., 2018). Tick secrete lipocalin which

in turn bind to histamine released by mast cell, that actions have been widely studied in the setting of parasite host relationship such as binding tick lipocalins (Kim *et al*., 2017). However, the degree of mast cells participation in ATR is related with tick and host species involved in the infestation (denHollander and Allen, 1985; Steeves and Allen, 1991). For example, FcεRI signaling related to mast cell is up regulated at the skin bite site of guinea pig infested with *Ixodes scapularis* nymphs (Kurokawa *et al*., 2020), suggesting their participation in mediated hypersensitivity processes (Karasuyama *et al*., 2017; Ohta *et al*., 2017). This same pathway is not activated in mouse submitted to the same infestation protocol, suggesting specific host immunological responses regarding FcεRI signaling pathways. These differences in such pathways may be influenced by natural or non-natural host status and tick species. Interestingly, mast cell deficient mouse failed to show ATR, but when mast cell was adopt transferred, ATR was recovered in the same mice (Matsuda *et al*., 1990), suggesting that the mast cell is essential for the ATR in some mouse associated tick species. Mast cells number increase at the tick bite site in normal mouse when infested with *D. variabilis* and not in mouse mast cell-deficient W/Wv mouse genetic controls (Steeves, 1991). In studies developed by Steeves and Allen, 1991, was demonstrated in histological skin analyses that mast cells had relevant role in the mice ATR against *D. variabilis* infested in mast cell-deficient W/Wv mouse. In contrary, mast cell contributed in mice infested with *H. longicornis*. Mast cells degranulation was incremented at the skin in the first 12 hours after infestation with *Ixodes scapularis* in mouse (Heinze *et al*., 2012).

Although these studies, mast cell specific mechanisms in skin tick attachment site remains unknown for the development of host protection.

2.4 Eosinophil

Eosinophils are produced in large number in allergic inflammation and helminth infections; however, eosinophilia reactions are also found in the ectoparasite infections, including tick infestations (Bath *et al*., 2017; Henrique *et al*., 2019). Eosinophils are recruited to host skin during inflammation where enzymes that are harmful to ectoparasites are release from their granules accordingly to (Janeway, 2000). Elucidation of the role of this type of cell in the host skin immune response against ectoparasites will provide a better understanding of the host parasite relation as suggested by (Engracia *et al*., 2017).

Higher number of eosinophils was observed during infection by *Sarcoptes scabiei* in humans (Bath *et al*., 2017). Histopathological studies of human scabietic infection showed increase cases of dermal eosinophil infiltrates (Elwood *et al*., 2015). Moreover, patients infected with crusted scabiei presents elevated eosinophils number in the skin in the Walton *et al*, (2008) study. Eosinophils play a role in the host anti helminthic defense as well (Janeway, 2000) which is not the scope of this review, but worth it cited. Sheep and cattle also infested with *Psoroptes ovis* showed in their histological lesions inflammatory infiltrate dominated with eosinophils (Van den Broek *et al*., 2000) and in red fox as well (Little *et al*., 1998).

The role of eosinophil has been widely studied in the cattle comparing the possible differences of the eosinophil influx in the skin attachment site infested with tick of different life stage and the effect in the influx of this type of cell (Engracia *et al*., 2017; Franzin *et al*., 2017). In practical way, it is observed that susceptible breed and naïve cattle and those that have been previously infested with tick present a greater influx of eosinophil (Piper *et al*., 2010). Differences were demonstrated in the eosinophils influx

in the skin infested with the larvae of different tick species and life stage. Eosinophil was much higher in the skin of the susceptible animal than to the resistant, even previously infested and decrease upon infestation with adult ticks of *R. microplus* (Carvalho *et al*., 2010). One phenomenon encounter in the field is that animals are infested with multiple species of tick and in the experiment with *Hyalomma a. anatolicum* and *R. appendiculatus* adult tick induced a progressively increase number of eosinophils and degranulation with the number of infestations as it was observed in seminal works (Gill, 1986; Walker; Fletcher, 1986).

The eosinophil amount was high in the dermal tick attachment site in the crossbred Holstein cattle infested with *R. microplus* (Engracia *et al*., 2017) and in the guinea pig infested with *I. scapularis* (Kurokawa *et al*., 2020). This type of cell composed the major number (47.8%) of cell that infiltrate the profound dermis of the cattle skin infested with the tick *R. microplus* (Engracia *et al*., 2017). During infestation of cattle with larvae Franzin *et al* (2017) observed that when larvae molt to nymph there were an increase of the influx of eosinophil and this pattern continues into the adult life stage that confirms works developed by Carvalho *et al*., (2010) with cattle infested with adult tick *R. microplus.* Eosinophils were different in number in two resistant specie of cattle (Bosmara and Nguni). The numbers of eosinophils in the skin infested with *R. microplus* was higher in the Nguni heifers than in the Bosmara heifers (Marufu *et al*., 2014). Eosinophils were augmented in number at the tick *A. variegatum* and *R. appendiculatus* attachment site in indigenous East African Zebu cattle when compared to susceptible Holstein Friesian cattle (Latif *et al*., 1999). The influx of eosinophil also was observed and increased at the tick *Hyalomma a. anatolicum* bite site in cattle after 24, 72 and 144 in the tertiary infestation after the animals had manifested the resistant phenotype (Gill, H. S. 1986). Mouse infested with *Ixodes scapularis* showed an increase of eosinophil after

3 hours and subsequently this amount augmented at the skin attachment site (Heinze *et al*., 2012). The role of eosinophil in the host immune response against the tick requires further investigation of the molecular mechanisms involve in such response and throughout all tick parasitic life cycle in its hosts.

2.5 γδT cells

Experiments conducted to investigate γδT cell role in immune response at the site of tick attachment found γδT cell augmented in its number, raising speculations about the participation of these cells in immunity against ticks (Constantinoiu *et al*., 2010; Piper *et al*., 2017). For example, the involvement of these cells was demonstrated in experiments showing that γδ T cells are enriched at the tick bite site in cattle (Carvalho *et al*., 2010). Constantinoiu *et al*., (2010) found that γδT cell were in higher levels in resistant compared to susceptible animals. This pattern was also observed in the work of Franzin *et al*., (2017). Moreover, Hoek *et al*., (2009) suggested that γδ T cell play a role of regulatory cell in bovines. However, the role of γδ T of cell in host immune defense against tick needs be further investigated.

3. Tick-host interface

3.1 Tick interaction with the host skin immune system

Tick evolved to interact with their hosts (Mans *et al*., 2011) and both tick and host determine the features of attachment site interface (Chmelar *et al*., 2017). On the skin, not only tick mouthparts injury induces host inflammation processes, but molecules injected by ticks also develop hyperemia, edema and secondary infections processes. Tick secretes along feeding time specifically proteins which modulate host skin defense mechanisms, such as inflammation, coagulation, wound repairing and immune response

(Francischetti *et al*., 2009; Tirloni *et al*., 2014). And earlier after tick attachment they secretes salivary gland proteins to form the cement cone that serves as adhesive for tick fixation in the host skin (Fawcett *et al*., 1986; Bullard *et al*., 2016). The most well-known and abundant protein family from tick cement are glycine-rich proteins (Suppan *et al*., 2017), that mimic host molecules for cement hardening, binding between cement and host tissue, and to avoid host rejection (Bishop *et al*., 2002; Trimnell *et al*., 2002). Furthermore, glycine-rich proteins were suggested be involved in antimicrobial mechanisms and immune evasion (Francischetti *et al.*, 2009; Havlikova *et al*., 2009). Nevertheless, is still unknown the functions of the majority of families of cement proteins. Another function of cement cone suggested was to build one physical barrier to avoid host immune molecules and the tick mouthparts contact (Binnington & Kemp (1980); Mulenga *et al*. 1999; Bishop *et al*. 2002). Due to these immune system related functions, many of these proteins have been exploited as anti-tick antigens with promising results (Shapiro *et al*., 1989; Bishop *et al*., 2002; Mulenga *et al.*, 1999; Trimnell *et al*., 2002, 2005; Zhou *et al*., 2006; Harnnoi *et al*., 2006).

Tick saliva proteins also induce changes in gene expression profile at tick skin attachment site, interfering in the pathways involved in skin defense, and similarly, host local response defines the genes encoding secreted tick proteins (Franzin *et al*., 2017; Garcia *et al*., 2020). Differentially expressed genes related to inflammation were up regulated in the skin of susceptible cattle during larvae and nymph infestation (Franzin *et al*., 2017). By consequence of tick parasitism, inflammation reaction changes dermal architecture and migration of inflammatory cell such as granulocytes, monocytes to the tick attachment site (Anderson *et al*., 2017). Phagocytosis-mediated by neutrophils and macrophages are the front line of defense for the clearance of skin of foreigner substances and necrotic debris (Constantionoiu *et al*., 2010; Anderson *et al*., 2017). The contribution

of these cells at molecular level is not well elucidated needing a more accurate analyses at the host skin interface to determine the factors underlining the host defense mechanism against parasites as cited and reaffirmed by several studies in this matter. Recent reviews mentioned the cellular count at the host skin and suggest many gaps that should be directing studies in the future concerning the skin immune response (Robbertse, 2017; Tabor *et al*., 2017; Karasuyama *et al*., 2017).

For example, Zhang *et al*., (2020) found at the tick bite site in rabbit infested with *Heamaphisalis longicornis* larvae, nymph and adults, that a subunit of the NADPH oxidase was upregulated and reveling that this system was active at each time interval of the tick stage development, and also that the host antagonize the action of tick foreign active substances from the tick saliva. In this study, they deduce also that the presence of ROS (reactive oxygen species) produced in the phagocytes shows that the phagocytosis activity is been activated and functioning to eliminate exogenous substances invading the host and tissue debris present at tick bite site (Zhang *et al*., 2020).

Ticks have evolved mechanisms to impair the antigen presenting process by APCs (antigen presenting cells) during the period of host skin attachment (Francischetti *et al*., 2009). In this sense, calreticulin and calnexin participate directly and indirectly in the process of phagocytosis (Gao *et al*., 2002) and play a very important role in the antigen presentation process. These proteins were upregulated in skin of rabbit infested with tick *Heamaphisalis longicornis* (Zhang *et al*., 2020). Moreover, the increase of the expression of the lectin in the skin of infested rabbits suggest that it can be applied as a marker of the inhibition of the skin immune response when rabbits are bitten by ticks. Future works should include all the tick parasitic cycling to full understanding of host skin anti-tick immune system mechanisms as well.

3.2 Cellular migration and inflammation

Inflammation is part of the frontline of the host defense against tick feeding (Tirloni *et al*., 2016), process that trigger immune cells migration to the site of bite (Ohta *el al*., 2017). Differential expression of adhesion molecules by skin cells determines the kind immune cells present at tick bite site. The kind of adhesion molecule expressed are affected by the genetic composition of the host and the level of tick infestation (Carvalho *et al*., 2010). In this sense, upregulation was observed in the skin of pig infested with mites *Sarcoptes scabiei* genes related to adhesion molecules as ICAM3 and molecules related recruitment and trafficking of immune cell were downregulated (Bhat *et al*., 2020). Selectins and integrins are adhesions molecules that ticks modulate its expressions in the endothelial cells surface and in at the leucocyte surface to impair leucocyte migration (Kotal *et al*., 2015, Carvalho *et al*., 2010)**.**

Adhesion molecules are upregulated at the tick host interface in mouse infested with *H. longicornis* (Zhang *et al*., 2020). Also, cattle with different phenotypes infested with adults of *R. microplus* showed in skin a differential expression of mRNA of ICAM-1, VCAM-1, LFA-1, P-selectin and E-selectin (Carvalho *et al*., 2010). Except for the LFA-1, which was upregulated in the susceptible animals *Bos t. indicus* during low or high infestation when compared with resistant animals (Carvalho *et al*., 2010).

Wound repairing, inflammation, coagulation and platelet aggregation depends on the leucocyte migration and the expression of adhesion molecules. *In vitro* studies demonstrate that tick saliva molecules bind and inhibit many proteases involved in homeostatic mechanisms of the host such as chymotrypsin, cathepsin G, factor Xa, trypsin, chymase, plasmin (Tirloni *et al*., 2016) and molecules that bind to adhesion molecules (Carvalho *et al*., 2010). Ticks secrete inhibitors proteins of proteases that

participate in many hemostatic systems of the host and migration of leucocytes (Mulenga *et al*., 2000 and Kotal *et al*., 2015).

4. Animal model to study skin immune response against tick

It is been demonstrated that repeated tick infestation of non-natural host elicited immune responses and impairing tick feeding and pathogen transmission (Trager, 1930; Allen, 1989; Wikel, 1982; Narasimhan *et al*., 2007; Narasimhan *et al*., 2020), making alternative models necessary to improve analysis of tick physiology and development of the control methods. Rabbits, guinea pigs, mice, hamsters stand as animal models used to investigate the immunity of the host against different species of tick (Zhang *et al*., 2020, Ohta *et al*., 2017, Allen, 1989, Robbertse, 2020). However, expanding animal models are necessary to increase the precision of findings on immune response against specific tick-host interactions, as different physiological mechanisms exist among ticks in natural and non- natural host interactions. Current tick management controls face a challenge that tick is a very promiscuous animal and the hematophagous behavior turn it to adapt to other hosts when the preferable host is not present in the environment (Showler *et al*., 2020).

It has been demonstrated that guinea pig, rabbit and bovine acquire an effective immune response against ticks (Allen, 1979; Kurokawa *et al*., 2020). Cattle acquired immunological resistance during the first infestation direct primarily against the larvae (Constantinoiu *et al*., 2010). This acquired immunity is manifested by the diminution of number and weight of engorged female or still the death of engorged tick in primary or subsequent infestations (Trager, 1939; Kurokawa *et al*., 2020). *Bos t. taurus* cattle and *Bos t. indicus* differ in their local immune response to tick infestation. Franzin *et al*., 2017 demonstrate that upon infestation of the Holstein *Bos t. taurus* and Nelore *Bos t. indicus* infested with larvae, nymph, showed different profile of cell at the skin. Bonsmara and

Nguni cattle induces infested with adults *R. microplus* also presented at the skin, basophils, mast cell and mononuclear cells higher in Nguni compared to Bonsmara skin cellular immune profile (Marufu *et al*., 2014). In contrary Carvalho et al., sampled the skin host infested and the findings that adhesion molecules were differentially and significantly different between Nelore and Holsltein. Constantinoiu *et al*., (2010) demonstrated that Brahman showed a delayed hypersensitivity compared to Holstein which showed more neutrophils at the tick attachment site. These studies demonstrate that cattle breeds respond differently to tick infestation concerned local immune response.

The manifestation of the acquired immunity depends on the tick and host specie, because ticks secreted different molecules to the host and the host respond based in it immune heritable characteristics (Robbertse, 2020; Kim *et al*., 2016; Kurokawa *et al*., 2020). Tick saliva secretion is based on the host genetic composition and specific stage of development secrete different salivary molecules (Tirloni *et al*., 2014; Kim *et al*., 2016 and Garcia *et al*., 2020) that depends as well the outcome of the manifestation of reactions and cellular profile at the tick host interface. White footed mice do not develop immunity against the *Ixodes scapularis*, even though, it is the natural host and despite the strong inflammatory reaction elicited at the tick bite site (Anderson *et al*., 2017). In contrary, guinea pig mount immune response right at the first hours after tick attachment (Anderson *et al*., 2017), which observed dermal inflammation at the 6 hours post tick infestation that was capable of reject the tick *I. scapularis.* Guinea pig also reject tick *R. sanguineus* (Bechara *et al*., 1995). Rabbit developed a strong tick rejection (Trager, 1930) with impairment of the tick physiology represents by the number of ticks that engorge and complete the parasitic cycle. The actual challenge is the translate the data encounters in these models to the natural and accidental host in order to control tick infestation. The advantage of these model is the facility to manage and are cost effective in the laboratory

setting, but there still the need to corroborate the results using the natural host of each tick species.

Conclusion

The local immune response to tick and other ectoparasite infestations is leading by the microenvironment created by the parasite protein secreted components, which in turn attract and engage the local immune cells. It is also governed by the host immune status. In the majority parasite host interaction at the skin is driven by the polarization from the Th1 response into the Th2 response as consequence of the mechanical rupture of the skin layers by the parasite and the cells producing Th2 response cytokines which many of them act as effectors molecules of the protective immune response (Table 1). Furthering the comprehension of the whole mechanism of Th2 immune response provoked by parasites will improve the recent control methods of these parasites, including tick control.

Table 1. Late contribution on the RI at the tick attachment site, cell and analysis

Tick species	Host	Cell characterization in this study	Techniques applied	References
R. microplus	Cattle	Increase of CD20+ B cell, CD3+ T cell	Histology, IHC	Robbertse *et al*., 2018
R. microplus	Cattle	Increase and identified WC1+ γδ T, B cell and CD4+ T helper	Histology, IHC, Transcriptome	Robbertse *et al*., 2017
R. microplus	Cattle	Increased number of eosinophils, mononuclear, mast cell at the tick attachment site	Biopsy, RNA extraction, Histology, Affymetrix microarray, qRT-PCR	Piper *et al*., 2010
R. microplus	Cattle	Increased number of eosinophils, macrophages, mast cell	Histology	Engracia Filho *et al*., 2017
R. microplus	Cattle	Identification of CD45+ cells, CD45RO cells, CD3+ T cell, CD8+ T cytotoxic, CD79α B cells, γδ T cells, MHCII macrophages, DC, B cells, activated T cells, CD4+ T, CD25+, CD21 B cells at the skin	Biopsy, Immunofluorescence	Constantinoiu *et al*., 2013
R. microplus larvae	Cattle	Identified increased number of CD3+, CD25+, γδ T, neutrophils, but unknown function of γδ T	Histology, IHC	Constantinoiu *et al*., 2010
R. microplus larvae	Cattle	Increased number of CD3+, CD4+, CD8+, CD25+, γδ T cells, CD45 and CD45RO cells at the tick attachment	Biopsy, Histology, Light microscopy, Immunofluorescence	Constantinoiu *et al*., 2018
R. microplus larvae and nymphs	Cattle	Increased of basophils at the second infestation, neutrophils, eosinophils and mast cells, mononuclear, CD3+ T, WC1+T and CD21+ B identified at the tick bite site	Biopsy, Histology, IHC, RNA extraction, Metaskin analysis, Affymetrix GeneChip, Tick behavior assay, Tick tissue dissection, Transcriptome	Franzin *et al*., 2017

Table 1. Continued

Tick species	Host	Cell characterization in this study	Techniques applied	References
R. microplus larvae	Cattle	Degree of mast cell disruption, eosinophil concentration and degranulation, and epidermal vesiculation	Biopsy, Histology	Schleger *et al*., 1976
R. microplus natural infestation	Cattle	Increased eosinophil	Biopsy, Histology	Rechav et al., 1990
R. microplus	Cattle	Increased number influx of neutrophil, basophil, eosinophil	RT-PCR, RNA extraction, Histology, Adhesion assay	Carvalho *et al*., 2010
R. microplus	Cattle	Increased of mast cell	Biopsy, Histology	Veríssimo *et al*., 2008
R. microplus adults	Cattle	Increased number of mast cell, eosinophil, basophil tick bite site	Biopsy, Histology	Marufu *et al*., 2014
Natural infestation - various - count *R. microplus*	Cattle	Augmented number of innate cells	PCR	Zhao, 2013
Ixodes scapularis nymph	Balb C/mice	Increased of neutrophil, macrophages, Th1 and Th2, T cell regulatory	Biopsy, Histology, PCR, Affymetrix mouse genome 430A 2.0 arrays and Histopathology	Heinze *et al*., 2012
Ixodes scapularis	White footed mice and guinea pig	Increased in higher number of neutrophils, macrophages, lymphocytes and plasma cells	Biopsy, Histology, IHC	Anderson *et al*., 2017
Ixodes scapularis	Balb C/mice	Identified activated fibroblast at the submuscular, neutrophils, macrophages	Biopsy, Histopathology and Immunofluorescence	Hermance *et al*., 2016
Ixodes scapularis nymps	Guinea pig and mice	Increased number of basophil and mast cell, eosinophil at the secund infestation	Biopsy, RNA-sequencing, Histology, ELISA	Kurokawa *et al*., 2020

Table 1. Continued

Tick species	Host	Cell characterization in this study	Techniques applied	References
Ixodes ricinus	Human	Accumulation of neutrophil, eosinophil, macrophages, dendritic cells and lymphocytes Th1 and Th2.	Biopsy, PCR, Histology, IHC	Glatz *et al*., 2017
Amblyomma cajennense and A. dubitatum	Capybara	Hyperplasia, cellular edema, necrosis of keratinocytes, polymorphonuclear leukocytes with eosinophilic granules: eosinophils or heterophils. Predominance of heterophils to eosinophils. increased of mononuclear, mast cell and basophil	Biopsy, Histology, Ultraestrutural analysis	Heijdeb *et al*., 2005
Haemaphisalis longicornis	Mice (Wild and Knockout for T, B cells, FcREI, IL3)	Attraction and increased number of basophils to the bite site through IL3 produced by CD4+ TRM	Biopsy, Flow cytometry, PCR, Intravital fluorescence microscopy	Ohta *et al*., 2017
H. longicornis	Mice	Abled identification of basophils	Histology, IHC (mba mMCP 8)	Wada *et al*., 2010
H. longicornis	Mice (WT and Knockout)	Basophil histamine release is essential for T cell attraction and ATR	Flow cytometry, cell proliferation assay, cell culture, Confocal fluorescence microscopy	Tabakawa *et al*., 2018
Rhipicephalus appendiculatus adults	Rabbit and cattle	Neutrophil and macrophages similar in both hosts, increased number of eosinophil and basophils. lymphoblasts and plasmacytes present in both hosts	Biopsy, Histochemistry, light microscopy and electron microscopy	Walker and Fletcher, 1986
R. appendiculatus e A. variegatum nymps	Cattle	Increased number of eosinophils in *R. appendiculatus* and neutrophils in the *A. variegatum*	Biopsy, Histology	Latif *et al*., 1991

Table 1. Continued

Tick species	Host	Cell characterization in this study	Techniques applied	References
Hyalomma anatolicum anatolicum	Cattle	Increased number of eosinophils, neutrophils, degranulation of mast cell, basophils	Biopsy, Histology	Gill, 1986
Hyalomma a. anatolicum	Sheep	Identification and increased number of neutrophils, macrophage, dendritic cell, lymphocytes CD8+ and γδ T cells	Biopsy, Immunohistochemistry	Boppana *et al*., 2005
Ixodes holocyclus	Cattle	Increased number of eosinophils, mast cell, neutrophil, basophil	Histology	Allen, 1977
Ixodes ricinus	Rabbits	Unknown	Surgical, Histochemical, Histology	Brossard, 1982
Rhipicephalus sanguineus	Dog, guinea pig	Increased eosinophil, neutrophil, eosinophil, basophil, mast cell, mononuclear	Biopsy, Histology, Electron microscopy	Szabó *et al*., 1999
Dermacentor andersoni nymph	Balb C/mice	Increased number of Inflammatory cells at the tick attachment site	Biopsy, Histology, PCR, Affymetrix mouse genome 430A 2.0 arrays histopathology	Heinze *et al*., 2014
Dermacentor andersoni larvae	Guinea pig	Increased number of basophils at the second infestation	Histology	Allen, 1973
Dermacentor andersoni	Guinea pig	Langerhans cell	Histochemical	Nithiuthai and Allen 1983
Dermacentor andersoni	Guinea pig	Decreased number of LC at primary infestation and increased at the secondary infestation	Biopsy, Histology	Nithiuthai and Allen, 1984
Dermacentor variabilis	3 Mice strains	Increased number of mast cell	Biopsy, Histology	denHollander, 1985

Table 1. Continued

Tick species	Host	Cell characterization in this study	Techniques applied	References
Haemaphysalis longicornis, Boophilus microplus, Boophilus decoloratus, R. appendiculatus	Cattle	Increased of eosinophils, monocytes and neutrophils	Biopsy, Histology	Moorhouse and Tatchel, 1969

Acknowledgements

This study was financed in part by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES): Finance Code 001, # Procad 88881.068421/2014-01; PGCI 23038.005296/2014-37; 88881.153222/2017-01; Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq): Grant # 465678/2014-9; 405763/2018-2, 302360/2018-2 and 441092/2016-0 and CNPq-INCT (Brazil), by The Agricultural Science and Technology Innovation Program (ASTIP) of China (China) and by Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health (USA).

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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3.2. Capítulo 2

Skin local immune response during *Rhipicephalus microplus* adult parasitism

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Abstract

Tick *Rhipicephalus microplus* is a hematophagous ectoparasite spread worldwide. This parasite is responsible to cause tick-borne diseases to animals by transmission of *Babesia* spp. and *Anaplasma* spp. and consequently causing economic losses. Ticks in their parasitic interaction with the host circumvent the immune response in order to maintain the hematophagous feeding behavior and to transmit tick-borne diseases. Tick saliva proteins modulate the homeostasis, response of the host as blood coagulation, inflammation, hemostasis and immune response. The present study aimed to evaluate the cellular inflammatory response of the Hereford cattle infested experimentally with adults *R. microplus* tick. To evaluate the cellular infiltrate at the tick attachment site, bovine skin biopsies were collected with different stages of tick development in 17th and 21st days of the parasitic phase. Samples were fixed, processed for histology, and stained with hematoxylin and eosin. In addition, immunohistochemistry using antibodies anti-CD3 and anti-CD79α was used to identify the presence of lymphocytes T and B in the skin. The bovine infested with *R. microplus* showed around the tick attachment site a profile of inflammatory cells composed mostly by accentuated number of eosinophils, mild percentage number of lymphocytes and rare plasmocytes. In samples treated for immunohistochemistry, monoclonal antibodies identified lymphocytes T CD3+, and B CD79α+ in all skin biopsies with an adult tick attached.

Keywords: *Rhipicephalus microplus*, histology, immunohistochemistry, skin immune response, cattle, tick saliva, lymphocyte.

1. Introduction

Ticks are hematophagous ectoparasites that belong to the phylum arthropods and are distributed worldwide. Tick importance arises because of their capacity to transmit several tick-borne disease agents to animals and humans (Dantas-Torres, 2012). For instance, Lyme disease has been reported in human in North America, Europe and Asia where *Ixodes* tick species are involved in transmission as competent vectors of the bacteria *Borrelia burgdorferi* to human host (Biggs *et al.*, 2016). *Rhipicephalus microplus* a tick which is distributed in tropical and subtropical regions of the world, transmits *Babesia* spp. and *Anaplasma* spp. that cause tick fever in cattle (Carvalho, 2016). The incidence of tick-borne diseases is increasing (Buczek *et al.*, 2020). For example, cases of diseases have been reported affecting humans such as Brazilian rocky mountain spotted fever caused by *Rickettsia rickettsia* which is transmitted by *Amblyomma sculputum* and *Amblyomma cajenense* in brazil (De Oliveira *et al.*, 2016). Crimean-Congo hemorrhagic fever caused by Naiovirus from *Amblyomma* spp., *Hyalomma* spp., and *Rhipicephalus* spp.. These diseases are of concern for public health in other countries also in Africa, Asia and Europe (Brites-Neto, 2015; Palomar *et al.*, 2016). Moreover, tick causes damage in animal skin, losses in animal gaining weight, in milk production and consequently causes economic losses in animal husbandry and industry directly (Grisi *et al.*, 2014).

Chemical, biological and immunological strategies to control tick infestations the pathogen transmission are been developed (Frazzon *et al.*, 2000; García-García *et al.*, 1999; Gazim *et al.*, 2010; Willadsen *et al.*, 1989). However, control of ticks and tick-borne diseases is increasingly difficult because of the constraint in developing effective alternative methods more environmentally friendly and cost-effective (Batista *et al.*, 2018; Parizi *et al.*, 2012; Tirloni *et al.*, 2014). Efforts have been in the direction of developing a vaccine that impairs tick infestation and their ability to transmit infectious

agents (Chmelař *et al.*, 2016; Kotál *et al.*, 2019; Tabor *et al.*, 2017). Thus, characterizing the interactions between the tick and the host immune system is crucial to understanding the tick biology, pathogen transmission, host defense response and to improve and develop new methods of tick control and eradicating tick-borne diseases (De La Fuente; Estrada-Peña, 2019).

Tick saliva proteins impair the hemostatic response of the host, such as coagulation, inflammation, and immune response (Chmelař *et al.*, 2017b; Nuttall, 2019; Ribeiro, 1989; Tirloni *et al.*, 2014a) and thus diminish inflammatory response to control initial infestation in the naïve susceptible cattle breeds (Carvalho *et al.*, 2010; Piper *et al.*, 2009). Therefore, with the lack of the essential other elements involved in the immune response against tick and the pathogens transmitted (Piper *et al.*, 2010) the host immune response is compromised and suppressed initially (Singh; Girschick, 2003). These numerous pharmacologically active molecules that modulate the host hemostatic response for successful blood feeding and transmission of infectious agents to the host are secreted in a redundant manner (Ribeiro, 1989; Henrique *et al.*, 2019). This modulation occurs in the level of innate immune response when feeding on naïve hosts and both innate and adaptive immune responses when feeding on tick-experienced animals (Engracia *et al*., 2017; Robbertese *et al*., 2018; Heinze *et al.*, 2012a, 2014).This behavior supports the evolution of tick-host relationship (Mans, 2019).

Tick immune response starts at the skin, the interface between the parasite and the host (Piper *et al.*, 2009). The skin contains cellular resident immune cells such as mast cells, dendritic cells, macrophages, and keratinocytes associated with its physical natural barrier function(Janeway; Medzhitov, 2002). These components of the local immune system is suggested to impair tick attachment, feeding and their potential to transmit bacteria, virus and protozoa to animals and humans (Francischetti et al., 2009; Heinze *et*

al., 2012). Evidence shows that in cattle, 90% of larvae applied in experimental infestation do not attach and their parasitic life cycle is interrupted (Tabor *et al.*, 2017).

Several attempts to investigate tick-host interaction at the molecular, cellular and tissue levels have found different patterns in the skin (Engracia *et al.*, 2017; Marufu, 2010). Cattle acquire immunity against tick following infestations (Roberts, 1968; Willadsen, 1980). And tick immunological resistance is manifested firstly against the larvae tick instar (Ribeiro, 1989; Roberts, 1968; Wikel, 1999). This acquired resistance to tick is expressed as a reduced engorgement weight, longer duration of feeding, reduced numbers of ova, blocked molting, and death of the tick (Wikel, 1999; Willadsen, 1980). Previous studies investigated dendritic cells, T and B lymphocytes, immunoglobulins, complement, granulocytes, monocytes/macrophages, cytokines, and biologically active molecules involved in the interaction between tick and host (Brossard, 2004; Nuttall; Labuda, 2003; Wikel, 2013, 1996; Willadsen, 1980).

The cutaneous immune response of the resistant cattle breed differs from the susceptible cattle (Constantinoiu *et al*., 2018, 2010; Piper *et al*., 2017). Cattle Holstein Friesian (*Bos taurus taurus*) and cattle Brahman (*Bos taurus indicus*) when examined their skin response upon *R. microplus* tick infestation showed a differential immune profile (Piper *et al.*, 2009, 2010). Differences from the host skin response have been demonstrated in a range of hosts as well (Boppana *et al.*, 2005; Buczek *et al.*, 2020; Franzin *et al.*, 2017; Heinze *et al.*, 2014; Ribeiro, 1989).

Specific reactions occur at the tick host interface depending on the tick species and the host (Otto *et al.*, 2018; Rechav; Goldberg; Fielden, 1997). For instance, in BALB/c and in C3H/HeN mice infested with *Ixodes scapularis* do not acquired resistance during the course of repeated infestation. Guinea-pig is resistant to *Dermacentor andersoni* larvae (Allen, 1973), and *Bos taurus* breed cattle previously exposed to *Ixodes*

holocyclus developed accumulations of basophils in the tick bite site, suggesting a cutaneous basophil hypersensitivity response against tick on the tick sensitized animals (Allen, 1977).

Observations made in the resistant and susceptible cattle infested with *R. microplus* showed intense and differential type of cellular infiltration in the skin (Engracia Filho *et al.*, 2017; Marufu, 2014; Riek, 1962; Stone, 2010). Studies have examined the humoral immune response and found that the titer of IgG1 and IgG2 antibody differ between less susceptible *Bos t. indicus* breed compared to the susceptible *Bos t. taurus* when infested with *R. microplus* (Piper *et al*., 2017) In addition, gene expression in the skin examination of the cattle Holstein Friesian infested with *R. microplus* larvae revealed expression associate with inflammation response and innate cell and *Bos t indicus*, instead, showed a T cell-mediated immune response. Many other cell subsets in the skin have been investigated applying different approaches to elucidate the local immune response in the tick host interface (Engracia Filho *et al.*, 2017; Marufu, 2014).

Several studies had explored the tick-host relationship by analyzing and using vast techniques at the level of DNA, RNA, protein, cellular and tissues (Piper *et al*., 2009, 2010; Franzin *et al*., 2017; Constantinoiu *et al*., 2010). However, findings in the RNA sequencing need to be validated by studies on the proteins and cellular levels at the tick host interface and considering tick stages of development between and within cattle breed, hence developing animal models to study tick host interaction (Robbertse, 2017).

In this paper, we aim to evaluate the cellular inflammatory response of the Hereford cattle infested experimentally with adults *R. microplus* tick.

2. Materials and methods

2.1. Ethics approval

The study was approved by Animal Experimentation Ethics Committee of Universidade Federal do Rio Grande do Sul (UFRGS). Project number 36228.

2.2. Experimental animals and ticks

The experiment was conducted using 12 months of pathogen-free Hereford cattle that were obtained from a tick-free area in Santa Vitoria do Palmar, Brazil, transported and kept at Faculdade de Veterinária, UFRGS. The animal was housed and maintained separately in tick-proof pens with a slatted floor. The ticks used in the study (Porto Alegre strain) are *R. microplus* pathogen-free, maintained in the laboratory of tick colony as the protocol previously described by RECK *et al.*, 2009.

2.3. Tick-animal infestation

Ten days after egg hatching, the larvae were applied on the dorsum of the animals. The infestation was conducted monthly. In the first infestation, approximately 10,000 larvae were applied and in the subsequent experiment infestations the number of larvae was increased to 40,000 ticks applied to each animal. Overall, 6 per annum infestations were performed of which the highest number of larvae applied was 40,000 ticks.

2.4. Collection of skin biopsies

First, the cattle were anesthetized with 1 mL of 2% lignocaine (Biocare, Pacheco, California, USA). Precisely, for the infested cattle, the anesthesia was subcutaneously inoculated adjacent to the site of the attached-feeding tick. After 15 minutes, skin biopsies were randomly taken from the neck, shoulders, withers and back from the cattle using an 8 mm biopsy punch. The biopsies were taken at an interval during six experimental infestations with 3 weeks interval between each infestation period. Eleven biopsies were

taken from the animals body at the different times of adult tick development after the infestation (17 days and 21 days partially engorged tick female and 21 days fully engorged tick female) while they were still attached in the skin. Overall, eleven biopsies from the parasited skin cattle and two biopsies from the unparasited skin were collected and immediately immersed in 10% buffered formaldehyde until subsequent analyses. Unparasited cattle skin biopsies were used as control.

2.5. Histological sample treatment

The sample fragments were fixed in 10% formaldehyde and processed routinely for histology, embedded in paraffin wax, cut into 3 μm sections, and stained with hematoxylin and eosin (HE). The samples were then examined with a routine light microscope searching for any alteration such as connective tissue proliferation, fibrinoid vascular degeneration, vacuolar epithelial degeneration, eosinophilic degranulation. Inflammatory cells were also identified (eosinophils, mast cells, macrophages, lymphocytes and plasma cells) and quantified as absent (-), mild (+), moderate (++), and severe (+++) accordingly with the presence percentage of cell observed by an experienced pathologist.

2.6. Immunohistochemistry

Another slide of the processed skin was submitted to immunohistochemistry, using reagents specific for $CD3^+$ T lymphocyte (1:250; Dako, St. Clara, California, USA) and CD79α (1:10; Biocare, Pacheco, California, USA). Antigen retrieval for $CD3^+$ was performed in XIV Protease (Sigma) for 15 minutes at room temperature. And CD79α antigen retrieval was performed using citrate buffer, pH 6.0, in a pressure cooker at 96 ºC for 20 minutes. Amplification was performed by using the Mach4-HRP Universal kit (Biocare, Pacheco, California, USA) and labeling was possible using 3-amino-9-etil-carbazole (AEC; Biocare, Pacheco, California, USA). Sections were counterstained with

Mayer's hematoxylin. Positive and negative controls were used for both $CD3^+$ and CD79α, respectively. $CD3^+$ and CD79α slides were evaluated and classified according to the intensity of labeling cells as weak labeling (25% of labeled cells), moderate labeling (25% to 75% of the labeled cells) and marked labeling (more than 75% of the labeled cells).

3. Results

3.1. Histological

Histological examination of eleven sections of bovine skin parasited with adult *R. microplus* showed inflammatory infiltrate in the skin, of which 45,45% (5/11) of the samples classified as moderate infiltrate, 27,28% (3/11) as accentuated, and in the remaining 27,27% (3/11) as mild, compared to the control. Infiltrate extended from the superficial to the profound dermis and surrounding the mouthpart of the tick inserted hypostome that causes rupture of the epidermis and the blood vessels in the profound dermis in most of the samples collected. Infiltrate consisted of degranulated eosinophils, lymphocytes, plasma cells and a few mast cells. in the control skin presented normal epidermis and dermis, normal blood vessels not dilated, beyond that there was no hyperplasia, hyperkeratosis (Figure 1 E and F)

Samples collected at the 17 days post infestation showed moderate inflammation and focal, epidermal hyperplasia, blood vessels augmented and accentuated hyperkeratosis in the external layer of the epidermis (Figure 1C and D). Histological sections collected with the adult tick of 17 days of growth, showed an increment of lymphocytes in 67% (2/3) of the samples, plasma cell in 100% (3/3) and eosinophils in 33% (1/3), compared with the control skin biopsies (Table 2).

Skin parasited with the tick of 21 days post infestation had accentuated infiltrate extended from epidermis, superficial and profound dermis until the muscular layer. The

blood vessels augmented their size, focal hyperplasia next to the tick attachment site, hyperkeratosis orthokeratosis accentuated and with infiltrate composed with eosinophils (Figure 1A and B) compared to control (Figure 1 E and F).

At the time when tick was 21 days feeding on the host and been partially engorged female (Table 3) and fully engorged female (Table 4), skin biopsies did not show any alteration in the type and the number of inflammatory cells. The exception was observed in samples with a fully engorged female of 21 days feeding on the host (67% (2/3) which presented an infiltrate composed predominantly by eosinophils classified as severe compared with the unparasited skin (Table 4) and (Figure 1A and B). Eosinophils and lymphocytes were located in a high percentage surrounding the tick mouthpart insertion and blood vessels. Less percentage of these cell types were found in other parts of the skin adjacent to the tick attachment site.

Fibrinoid vascular degeneration was present in 19% of samples (2/11) and absent in 81% (9/11) of samples collected. Vacuolar epithelial degeneration was observed in 28% (3/11) and not observed in 72% (8/11). The eosinophilic degranulation process was highly marked in 9% (1/11) of the samples, moderate in 36% (4/11), and mild in 18% (2/11). The remaining samples did not show eosinophilic degranulation. Myxoid tissue was present in more than half of the samples 45% (5/11) classified as moderate and in the other half of the samples 45% (5/11) was classified as mild.

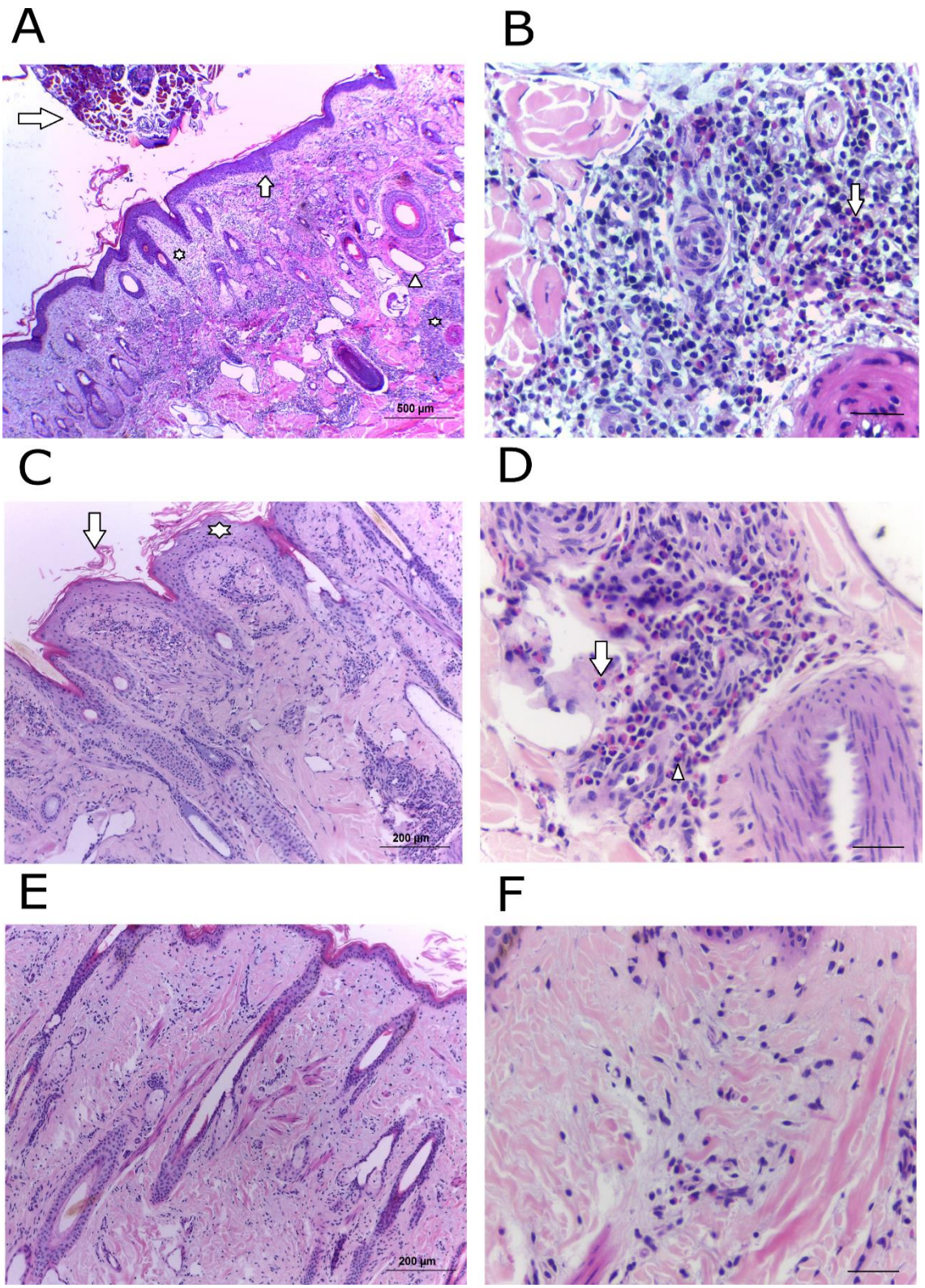

Figure 1 Inflammatory reactions at the tick attachment site in the host skin.

(A) and (B)- Skin parasited with a tick of 21 days post infestation. Tick head (big arrow), infiltrate (star), blood vessels (short arrow), eosinophil (arrow). (C) and (D) skin parasited with 17 days post infestation. hyperkeratosis (arrow), hyperplasia (star), eosinophil (arrow). (E) and (F)- Control. Bar – 50-500µm.

3.2. Immunohistochemical

Lymphocytes $CD3^+$ were present in all biopsies at the time of the tick stages of development considered in this study. There were more cells labeled with anti-CD3 than cells labeled with anti-CD79α in the skin biopsies compare to skin control (Figure 2). A moderate number of cells labeled with anti-CD3 were positive in 33% (1/3) of the samples collected with an adult tick of 17 days of development. Moreover, 33% (1/3) samples were labeled with anti-CD3 moderately, while another 33% (1/3) of samples were marked with anti-CD3 in skin biopsies collected with the tick of 21 days of development, but fully engorged female. For cells labeled with anti-CD79α, there was a moderate immunolabeling of 100% (3/3) of the samples collected with a tick of 21 days fully engorged compared with other instars of tick growth and with the control. All these values were compared with the positive and negative controls (Table 5) and (Figure 2).

Table 2 Leucocyte infiltrate in the cattle skin at the time of 17 days *R. microplus* feeding.

Leucocytes	% (n=3)	Parasited skin	Unparasited skin
Neutrophils	100% (3/3)	-[d]	-
Lymphocytes	67% (2/3)	++[b]	+
	33% (1/3)	+[c]	+
Plasmocytes	100% (3/3)	++[b]	+
Mast cell	100% (3/3)	+[c]	+
Eosinophils	33% (1/3)	++[b]	+
	67% (2/3)	+[c]	+
Macrophages	100% (3/3)	-[d]	-

[a] accentuated. [b] moderate, [c] mild, [d] absent.

Table 3 Leucocyte infiltrate in the cattle skin at time of *R. microplus* partially engorged female of 21 days.

Leucocytes	% (n=3)	Parasited skin	Unparasited skin
Neutrophils	100% (3/3)	-[d]	-
Lymphocytes	67% (2/3)	++[b]	+
	33% (1/3)	+[c]	+
Plasmocytes	100% (3/3)	++[b]	+
Mast cell	100% (3/3)	+[c]	+
Eosinophils	33% (1/3)	++[b]	+
	67% (2/3)	+[c]	+
Macrophages	100% (3/3)	-[d]	-

[a] accentuated. [b] moderate, [c] mild, [d] absent.

Table 4 Leucocyte infiltrate in the cattle skin at time of *R. microplus* fully engorged female of 21 days.

Leucocytes	% (n=3)	Parasited skin	Unparasited skin
Neutrophils	100% (3/3)	-[d]	-
Lymphocytes	67% (2/3)	++[b]	+
	33% (1/3)	+[c]	+
Plasmocytes	100% (3/3)	++[b]	+
Mast cell	100% (3/3)	+[c]	+
Eosinophils	33% (1/3)	++[b]	+
	67% (2/3)	+[c]	+
Macrophages	100% (3/3)	-[d]	-

[a] accentuated. [b] moderate, [c] mild, [d] absent.

Table 5 Cell labeled by immunohistochemistry in the cattle skin parasited with adult *R. microplus*.

Instars	CD3	CD79α	Parasited skin	Unparasited skin
17th dpi	67% (2/3)	67% (2/3)	+[c]	+
	33% (1/3)		++[b]	+
		33% (1/3)	-[d]	+
21st dpi	67% (2/3)	67% (2/3)	+	+
		33% (1/3)	-[d]	+
21st dpi	34% (1/3)	100% (3/3)	+[c]	+
	33% (1/3)		++[b]	+
	33% (1/3)		+++[a]	+

PEF., partially engorged female; FEF., fully engorged female; [a] more than 75% of the labeled cells;
[b] 25% to 75% of the labeled cells; [c] 25% of the labeled cells; [d] Not labelled.

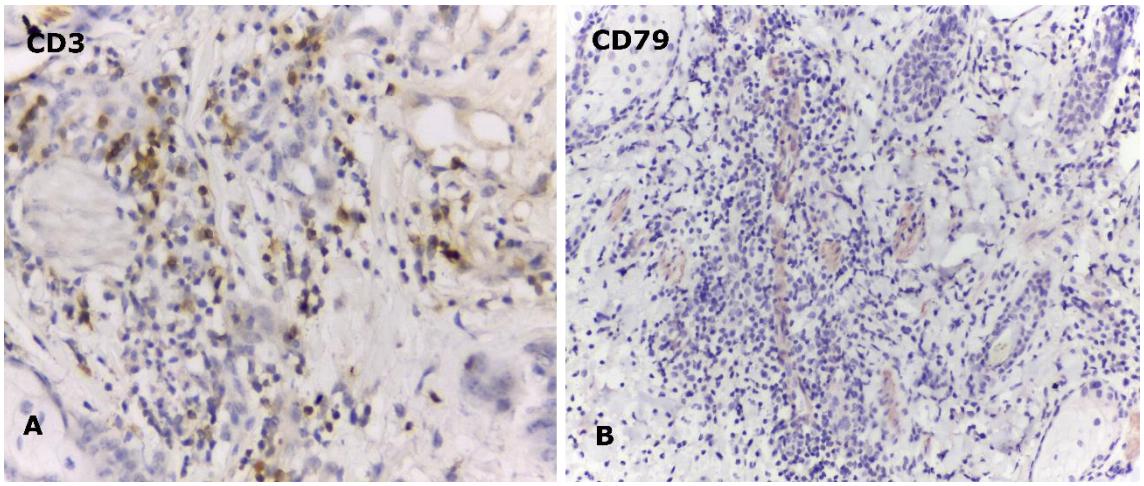

Figure 2. Immunohistochemistry. A - moderate number of lymphocytes showing labeling for $CD3^+$. IHQ anti-$CD3^+$. B - mild number of lymphocytes showing labeling for CD79α. IHQ anti-CD79α. 10x

4. Discussion

Ticks are parasite by arthropod that evolved to feed blood from their host to satisfying their physiological necessities (Carvalho, 2016). Tick attempts for several times to find and to attach to the suitable host (Engracia Filho *et al.*, 2017; Furlong, 2002). Tick hypostome insertion and the array of saliva molecules injected during feeding on the skin of the host causes local inflammation with characteristics that could help, for instance, in the diagnosis of tick bite and tick-borne diseases skin symptoms thus to establish differential diagnosis of diseases caused by hematophagous ectoparasites and choose the correct pharmacological treatment (Buczek *et al.*, 2020; Haddad *et al.*, 2018). In the present study the tick bite site presented rupture of the epidermis and dermis by the tick hypostome. The tick attachment bite site developed also superficial diffuse eosinophilic dermatitis with orthokeratotic hyperkeratosis in most of the samples, which is in accordance with the skin immediate hypersensitivity caused by most tick bites, reported by (Haddad *et al.*, 2018).

In several tick-borne diseases as Lyme disease, *Ixodes scapularis* bite and *Borrelia burgdorferi* transmission causes erythema migrans which are formed with deep perivascular and interstitial infiltrate of lymphocytes and in some cases plasma cell and eosinophil are present as well (Heinze *et al*., 2012b). It was also observed in the present study that findings showed the same type of cells in the tick bite site. These characteristics are of a late reaction when humans and animals are bitten by a tick (Haddad *et al.*, 2018). Mechanical stimuli caused by the tick and additional tick saliva proteins injected to the host contribute to the cellular outcome observed in the tick host interface (Tirloni *et al.*, 2016). Buczek *et al.* (2020), investigating the differences between bites of *Ixodes ricinus* and fly *Lipoptena cervi* in humans found that there were differences in those two cases due to the time these two ectoparasites fed on the host. For instance, tick feed for several

days or months on the host and vary its salivary gland components to countermeasure the host inflammatory reactions (Ribeiro, 1989). On the contrary *Lipoptena cervi* feed for several hours and the array of its saliva components changes as well in a short space of time and its components may differ from the tick salivary molecules (Buczek *et al.*, 2020). Correct pictures, laboratory tests associated with the patient history is necessary for differential diagnosis in tick-borne diseases affecting humans and animal. In the field conditions animals are infested with multiple types of ectoparasite that can hinder the diagnosis of certain parasites involved in the symptoms of the cutaneous reaction (Buczek *et al.*, 2020).

The disruption of the skin tissue and the injury of cells present in the epidermis and dermis plays an initial role in elicited the inflammatory reactions and consequently attracting cells to the tick bite site and to the subjacent areas of the skin (Mihara, 2017). In this study inflammatory infiltrate was located in the epidermis and dermis where resident cells are located and migratory inflammatory cells in response to external stimulus, in this case by tick bite and salivary gland proteins injected to the host-parasite interface. This finding is similar to the study developed by (Boppana *et al.*, 2005) in which sheep infested with *Hyalomma a. anatolicum* had an increase of inflammatory cells that migrate to the epidermis and dermis of the sheep. Tick saliva contains molecules of small molecular weight such as cystatins and serpins for example (Larissa, Kotál, Bensaoud and Kotsyfakis, 2019), that are ligands of antigen-presenting cell receptors that activate and induces migration of these cells to the lymph nodes to prime and activate $CD4^+$ T helper cell when this APC (antigen-presenting cells) carrying and present these antigens binding to the MHCII (Boppana *et al.*, 2005). The prime and activation of $CD4^+$ T helper cell activated downstream pathways in the T cell and then proliferate and undergo clonal expansion specific for the antigen or antigens been presented in the tick host interface and

the inflammatory cell migrate to the site of injury or where the antigen are been presenting (Constantinoiu *et al.*, 2010).

Few neutrophils degenerating were observed in skin biopsies collected with the stages of tick development. The results are not similar to with the study of Boppana *et al.*, (2005) where neutrophil and mononuclear cells were much more abundant in the skin of sheep infested with the *Hyalomma a. anatolicum*. Constantinoiu *et al*., (2018), during Santa Gertrudis cattle infested with *R. microplus* larvae the skin showed neutrophils at the early primary infestation compared to the later stages in subsequent infestations. Neutrophils are part of the early inflammatory reactions (Heinze *et al.*, 2012a).

The intensity of the infiltrate and the abundance of the type of cells in the skin of infested host depends on the species of the tick and the host (Ribeiro, 1989; Wikel, 1999). Riek, (1962) observed differences in the type and intensity of cellular infiltration in the skin of resistant and susceptible cattle infested with *R. microplus*. The findings of the present study demonstrated variable intensity and differences in the type of cells present in the skin. We have found more eosinophils surrounding the tick mouthpart inserted in the skin and in the adjacent areas of the tick attachment site. This type of cell was also found surrounding blood vessels. These findings corroborate with previous studies developed by (Carvalho *et al.*, 2010; Marufu, 2014).

The animal used in the present study had already been infested previously and the profile of cells present in the skin corroborate with a phenotype of the resistant animal to tick *R. microplus*. Piper *et al*., (2009) reported that resistant *Bos t taurus* cattle presented gene expression profile matching with cell mediated immune response compare with *Bos t. taurus* susceptible cattle that presented a profile of gene expression in the skin involved in the inflammatory response. The limitation of our study is that the comparison between

tick stages might not be accurate due to the design experiment that did not consider the cattle immunological acquiring process.

Proteomic and transcriptomic studies had revealed that the salivary gland protein profile of the tick changes when feeding in different hosts (Coutinho *et al.*, 2020; Kim *et al.*, 2016; Lewis *et al.*, 2015; Tirloni *et al.*, 2014b). This study was considered only adults tick stages feeding on the cattle. Contrary Santa Gertrudis cattle infested with *R. microplus* larvae induced a different type of immune cell in the skin and the profile of cell encountered had a tendency for more $CD3^+$, $CD4^+$, $CD8^+$, $CD25^+$ and γδ T cells, with significant differences in resistant cattle in γδ T cells and $CD4^+$. Findings of our study showed no differences in the intensity of inflammatory cell in the skin biopsies performed with tick of 17 days, 21 days (partially engorged female) and of 21 days (fully engorged female). The profile of cells maintained and showed a tendency of infiltrate of a resistant animal comprised mostly by eosinophil, followed by lymphocytes and a few mast cells, when infested with *R. microplus*. Previous studies demonstrated that lymphocytes, mast cells, eosinophils, basophils play a crucial role in acquired protective immunity to tick infestation (Karasuyama *et al.*, 2018). Constantinoiu *et al*., (2018) reported that not all cell infiltrates in the tick-host interface participate in the immune process against tick. Its duet that tick injects in a very small amount saliva molecules with a small molecular weight that acts as an antigens and many of them are TLR ligands that modulate the activation of cells (Martins *et al*., 2019).

Karasuyama *et al.* (2018) proposed a mechanism by which the basophils, mast cell and naïve lymphocytes and skin-resident memory $CD4^+$ T cells contribute to the development of tick immunological resistance. The study proposed that in the first infestation tick attach and inject antigens that are capture and process by the resident dendritic cells which migrate to the lymph node and activate naïve $CD4^+$ T cells (Chmelař

et al., 2017b; Nuttall, 2019). This activated T cell proliferate and differentiate into resident skin T cells that migrates to the skin and into interleukin 4 producing effector T cell. The IL-4 T cell activates and induce B cell to produce a tick specific antigen Immunoglobulin E that in turn circulate and bind to the FcεRI in mast cells or in basophils and causes these cells to degranulate when they encounter the tick specific antigen-binding the IgM and the FcεRI in the APCs (Stone, 2010). At the second tick infestation causes epithelial hyperplasia by the actions of the histamine and enzymes released by these cells. It is a basophil-mediated immunological resistance mechanism to tick (Karasuyama *et al.*, 2018). Basophils were not characterized in the skin biopsies. Eosinophil finding in the study showed degranulation in accordance to the hypothesis that supports the idea that immunological cellular response of the cattle was influenced by mast cell and basophil degranulation stimulated by major basic protein secreted by eosinophil, thus, in general drives Th2 mediated immune response of the host.

Eosinophil is associated with the acquired tick immunological resistance mediated by T helper 2 cells, it is been demonstrated that mice-infested by larval *Haemaphysalis longicornis* tick had an increase in the number of eosinophils in the skin when compared with the peripheral blood eosinophil and with the steady skin (Ushio *et al.*, 1993).

Eosinophil, mast cell and basophils are of importance in the cattle tick immunological response (Stone, 2010). Following activation by the tick antigen proteins, mast cells readily secrete IL5, IL13, and TNFα (Janeway; Medzhitov, 2002). In addition, IgE binding leads to the increment of CCL2 (monocyte chemoattractant protein 1) transcription that promotes the migration of monocytes (Oliveira; LukacS, 2001) and T-cells (Oliveira; Lukacs, 2003), to amplify the local inflammatory reaction. Due to mast cell antigen-presenting function, an antigen is presented to the MHCII, which in turn interacts directly with T-cell receptors (containing $CD3^{+}$) and induces antigen-specific

clonal expansion of T-cell populations (Mekori; Metcalfe, 1999). We found using immunohistochemistry that skin biopsies infested with *R. microplus* tick showed significantly increased infiltration of $CD3^+$ T and CD79α B lymphocytes in both epidermis and dermis of the skin with tick of 21 days feeding on the host compare to the control skin which is in agreement with Boppana *et al*., (2005) who found an increased number of these cell population next to the APC cells in the epidermis and dermis of the sheep infested with *Hyalomma a. anatolicum*.

Concerning cells labeled with immunohistochemistry, Constantinoiu *et al*., (2010) encountered $CD3^+$ populations of T cell in the skin of *Bos t indicus* tendering to be higher than *Bos t taurus* infest with adults *R. microplus*. The same pattern was observed in the present study and its due to the animal be already resistant because it was previously infested. Piper *et al.* (2017) analyzed the peripheral immune response of the resistant tick cattle and the less resistant tick cattle and found out that the resistant one had lymphocyte-mediated immune response compared to the less resistant one.

5. Conclusions

The local immune response of the Hereford cattle against *R. microplus* is manifested by innate response composed of by granulocytes such as eosinophil infiltrate in higher number, few mast cells. Cells of the adaptive immune response marked strongly by $CD3^+$ T and CD79 B lymphocytes induced by adult ticks during feeding play a significant role in the resistant animal which was not determined in the present study. Moreover, the profile of cells presented at the tick attachment site is influenced by the tick proteins injecting during feeding of the *R. microplus* in manner saliva dependent and it induces a Th2 mediated host immunity. This experiment might be expanded by increasing the biopsies samples between breed, within breed during different stages of the parasitic life cycle of *R. microplus* for furthering the study. Also, further studies should be investigating the basophil, γδ T cell and subsets of dendritic cells and keratinocyte roles in the host immune response against tick *R. microplus*. The acquired immunological resistance of cattle and other laboratory animals is well established, but studies developed in the natural conditions of infestation should be persuaded in the future as well. The techniques used in this study were not sufficient to investigate the subpopulations of cells and other factors involved in the bovino skin immune response during *Rhipicephalus microplus* adult parasitism.

Acknowledgments

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES): # Procad 88881.068421/2014-01; PGCI 23038.005296/2014-37; 88881.153222/2017-01; Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); CNPq-INCT.

Benvindo Capela João is a student under the CAPES master number of process 88882.346625/2019-1

Conflict of interest

The authors certify that they have no affiliations with, or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

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4 Discussão

A descoberta da importância dos carrapatos na saúde animal e saúde pública foi impulsionado juntamente com a observação de que estes eram vetores de patógenos aos animais e humanos (Rochlin e Toledo, 2020). As doenças transmitidas pelos agentes patogênicos veiculados pelos carrapatos são de preocupação atual pelo aumento de notificações destas em vários locais do mundo devido a expansão dos carrapatos para áreas do hemisfério norte onde não ocorriam as espécies de carrapatos que transmitem doenças importantes e cuja prevalência aumenta nestes lugares (Tufts *et al*., 2019; Muhanguzi *et al*., 2020).

Os carrapatos infestam seus hospedeiros animais de maneira espécie-específica, porém podem se fixar em humanos ou em outros hospedeiros, que não sejam específicos, classificados como sendo hospedeiros simpráticos, nos quais se alimentam e também transmitem patógenos (Rochlin e Toledo, 2020; Heinze *et al*., 2010; Furlong, 2002, Buczek *et al*., 2020). Assim sendo, urge a necessidade do estudo da relação carrapato-hospedeiro para se aprofundar o conhecimento dos fatores relacionados a imunidade desenvolvida por esses hospedeiros durante infestações e com isso desenvolverem-se modelos de infestações para entendimento dos correlatos de imunidade protetiva, da patogênese das doenças causadas pelos agentes patogênicos veiculados pelos carrapatos, da resposta imune montada por esses e para desenvolvimento dos métodos de controle através da mimetização e/ou manipulação do sistema imune dos hospedeiros (Robbertse *et al*., 2017).

A pele representa a primeira linha de defesa imunológica do hospedeiro contra os carrapatos. Os carrapatos Ixodídeos dilaceram com suas quelíceras a pele do hospedeiro e se fixam por intermédio do hipostômio e do cone cemento. Carrapatos alternam a salivação e o repasto sanguíneo a cada 5-20 minutos quando se alimentam (Franscischetti

et al., 2009). Várias componentes da saliva medeiam a supressão da resposta homeostática do hospedeiro, tais como coagulação sanguínea, imunidade, inflamação e a capacidade do hospedeiro em desenvolver o reparo de vasos sanguíneos (Kotal *et al*., 2015; Chmelar *et al*., 2017). O repertório de células inflamatórias presente neste estudo pode revelar uma mudança de proteínas injetadas pelo carrapato no hospedeiro que altera durante o período de alimentação. Alternância na quantidade e qualidade da saliva foi confirmado por dados proteómicos da espécie *R. microplus* e *Amblyomma americanum* (Tirloni *et al*., 2016; Kim *et al*., 2016). Esta alternância da composição da saliva se supõe que polariza a resposta imune dos hospedeiros, frente a infestação, para uma resposta Th2.

Ainda no presente estudo considerou-se estudar a resposta imune local de bovino infestado com carrapato adulto de *R. microplus*. O comportamento hematófago dos carrapatos permite que se fixem e se alimentem de sangue de seus hospedeiros (Tirloni *et al*., 2016, Maressa *et al*., 2019). Insetos também evoluíram no sentido de se alimentarem em seus hospedeiros vertebrados (Carvalho, 2016). Durante o processo de sondagem e de alimentação os insetos hematófagos induzem uma resposta inflamatória celular que é saliva-dependente, além do trauma causado pela inserção do aparelho bucal (Ribeiro *et al*., 1989, Henrique *et al*., 2019, Franzin *et al*., 2017). A fixação do carrapato na pele do bovino resultou em uma ruptura da epiderme e derme pelo aparelho bucal do carrapato. No local da picada do carrapato desenvolveu-se uma dermatite difusa eosinofílica com ortoqueratótica hiperqueratose em todas as biópsias de pele no estádio adulto do carrapato estudado. Estes dados corroboram com uma reação de hipersensibilidade imediata na pele observada na maioria dos hospedeiros no local de picada de carrapatos, por exemplo o caso que foi reportado pelo Haddad *et al*., (2018). Reações cutâneas a picada de artrópode *Lipoptena cervi* em veados foram relatadas, sendo suas características

histopatológicas similares com as lesões encontradas no presente estudo onde era evidente a formação de dermatite ortoqueratótica hiperqueratoses com infiltrado composto por eosinófilo, linfócito e com mastócitos (Buczek *et al*., 2020; Lazar *et al*., 2017). Este padrão histopatológico se repete em infestações de animais com uma vasta gama de artrópodes (Lazar *et al*., 2017, Hestvik *et al*., 2007, Maressa *et al*., 2019, Miteva *et al*., 2008).

Todavia as características histopatológicas são contrastantes na picada de *Ixodes scapularis* e pela infecção por *Borrelia burgdorferi* que causa erythema migrans na pele de indivíduos com um forte infiltrado perivascular e intersticial composto por linfócitos e em alguns casos está presente plasmócitos e eosinófilos (Heinze *et al*., 2012b). Neste estudo os resultados encontrados foram similares quanto a composição celular do infiltrado em todas as biópsias de pele com carrapato adulto fixo no hospedeiro. Porém as células mais abundantes encontradas em pele infestada com adultos *R. microplu*s foram eosinófilos. Estes dados corroboram com Engracia *et al*., (2017) onde bovinos durante a infestação com *R. microplus* apresentaram aumento do número de eosinófilos, macrófagos e mastócitos na pele.

Bucszek *et al*., (2020), reportou que as lesões causadas a pele de humanos picados pelo *Ixodes ricinus* eram caracterizadas por uma lesão eritematosa-infiltrativa não demarcada. Uma reação cutânea com predominância de eritema depois da picada de *I. ricinus* é o sintoma mais comum em pacientes na Europa, por exemplo. Quanto as células em pele de humanos infestados com *Ixodes ricinus* um aumento da acumulação de quantidade de neutrófilos, eosinófilos, macrófagos, células dendríticas, linfócitos Th1 e Th2 foram observadas em Glatz *et al*., (2017).

Em todas amostras de pele apresentaram também carrapato fixo e com formação da cavidade de alimentação do carrapato no qual foram encontrados neutrófilos e

macrófagos degenerados como células inflamatórias com maior predominância seguidos por eosinófilos em zonas subjacentes ao local da fixação do carrapato. De forma similar, *Lipoptena cervi* forma este bolsão de alimentação (Lazar *et al*., 2008). Porém, mosquitos se alimentam diretamente dos vasos sanguíneos não formando qualquer tipo de bolsão de alimentação (Henrique *et al*., 2019). Entretanto existem outros fatores que determinam o grau de severidade registrados pelas imagens e o tamanho das lesões em humanos e animais, como a intensidade da invasão, o local da picada de artrópodes no corpo do hospedeiro e característica individuais dos hospedeiros tais como o estado do sistema imune dos indivíduos (Matieva *et al*., 2008).

Os resultados mencionados acima podem ser fundamentais para se diagnosticar e estabelecer diagnóstico diferencial de picada e de enfermidades veiculadas pelos artrópodes hematófagos, para além de se escolher um tratamento farmacológico correto. A correta identificação do carrapato, tamanho do seu aparelho bucal e extensão da penetração na pele, forma do cone do cemento e seus estádios são essenciais para o diagnóstico, tratamento e profilaxia das doenças transmitidas pelos carrapatos (Rochlin e Toledo, 2020). Por exemplo, a profilaxia da doença de Lyme requerer não somente o uso de antibiótico, mas também uma correta identificação da duração da alimentação do carrapato.

O infiltrado inflamatório e o tipo celular encontrado neste estudo com predominância de eosinófilos já tem sido reportado em estudos anteriores (Franzin *et al*., 2017; Carvalho *et al*., 2010), sendo um padrão que se repete em bovinos resistentes a infestação por carrapatos (Piper *et al*., 2010; Engracia *et al*., 2017). Por exemplo, bovinos infestados com carrapatos adultos de *R. microplus* apresentaram aumento no número de eosinófilos, mastócitos e basófilos no local da picada na pele (Marufu *et al*., 2014).

A função de eosinófilos como mediadores da degranulação de basófilos já foi elucidada por (Wen e Rothenberg, 2016), porém sua função na relação carrapato com seus hospedeiros é desconhecida. No presente trabalho de pesquisa não foi identificado basófilos pela limitação da técnica utilizada. Carvalho *et al*., 2010 encontrou em biópsias de pele de animais susceptíveis infestados com *R. microplus* aumentado o número de basófilos quando comparado aos animais resistentes. Neste mesmo estudo não houve alterações na intensidade do infiltrado na pele dos dois tipos geneticamente diferentes, mas houve diferenças nos tipos celulares que constituíam este mesmo infiltrado. Aumentos no número de eosinófilos e basófilos foram encontrados no estudo desenvolvido por Franzin *et al*., (2017) em animais resistentes infestados com larvas e ninfas de *R. microplus*, contrariamente aos animais susceptíveis.

Metaloproteases de *R. microplus* têm a capacidade de inibir moléculas de adesão leucocitária, como moléculas de adesão intercelular 1 (ICAM-1), molécula de adesão vascular 1 (VCAM-1), P-selectina, E- selectina (Tirloni *et al*., 2016) e, estes fatores, como demostrado *in vitro* por (Carvalho *et al*., 2010), podem influenciar as características da inflamação celular observada em pele infestado de bovinos.

As diferenças encontradas nas lesões na pele causadas pela infestação pelos carrapatos adultos de *R. microplus* estão provavelmente relacionadas a composição e a quantidade de saliva secretada e também relacionada as diferenças fisiológicas do carrapato durante a alimentação, para além do estado imune dos hospedeiros (Tirloni *et al*., 2015, 2016; Kim *et al*., 2016).

Tirloni *et al* (2016) pesquisou a composição da saliva de fêmeas adultas de *R. microplus* e verificou que inibidores de serino protease eram secretadas na saliva de todas as fases de carrapatos analisadas, sendo que uma percentagem considerável de proteínas secretadas na saliva eram inibidores de serino protease (10% em fêmeas adultas

parcialmente ingurgitadas e 13% em fêmeas adultas totalmente ingurgitadas). Porém, o efeito de algumas serpinas secretadas e que entram em contato com hospedeiro ainda precisa ser estudado o seu efeito *in vivo*. Supõe-se que inibidores de serino proteases de *R. microplus* estejam envolvidos na relação carrapato-hospedeiro (Tirloni *et al*., 2015, 2016; Kim *et al*., 2016, Chmelar *et al*., 2017). A intensidade do infiltrado, as lesões causadas e o tipo celular encontrado devem estar relacionados com a interação destas moléculas nas cascatas dos processos homeostáticos do hospedeiro para facilitar a alimentação do carrapato. Os tipos celulares encontrados de forma acentuada como eosinófilos, seguido de uma presença moderada de linfócitos e mastócitos é um padrão encontrado em bovinos infestados com carrapatos e estão relacionados com a resposta imunológica do hospedeiro no local da picada em bovinos resistentes segundo (Robbertse *et al*., 2017; Constantinoiu *et al*., 2010; Franzin et al., 2017; Engracia et al., 2017).

Os mastócitos depois de ativados pelos antígenos, rapidamente secretam IL5, IL13 e TNFα (Janeway e Medzhitov, 2002). A ligação do receptor IgE e antígeno provoca o aumento da secreção e transcrição de CCL2 que promove a migração de monócitos, linfócitos T como encontrado em (Oliveira e Lukacs, 2003) para amplificar a reação inflamatória local. Também encontramos no presente trabalho, usando a técnica de imuno-histoquímica, que biopsias de pele com carrapato adulto de 21 dias apresentou um aumento de infiltrado de linfócito T $CD3^+$ e B $CD79^+$ na epiderme e derme quando comparados com a pele intacta. Estes dados estão de acordo com Boppana *et al*., (2005) o qual observou um aumento de células T junto as células apresentadoras de antígenos em epiderme e derme de carneiro infestado com *Hyalomma a. anatolicum*. Porém, Constantinoiu *et al*., (2018) encontrou diferentes tipos celulares, constituídos por $CD4^+$ e linfócito γδ nos animais resistentes comparados aos susceptíveis, no local da picada do carrapato quando bovinos foram infestados com larvas de *R. microplus*, fase na qual a

resistência imunológica contra o carrapato é manifestada. Estas diferenças podem ter sido registradas devido as técnicas utilizadas em cada um dos experimentos. Animais resistentes, fenótipo manifestado pelo animal utilizado no presente trabalho depois de várias infestações, expõem o carrapato a uma resposta inflamatória prematura que corresponde com a fase em que a expressão de genes que codificam proteínas que suprimem a imunidade do hospedeiro, inflamação e coagulação são baixas Franzin *et al*., (2017). O que não corresponde com carrapatos que se alimentam em hospedeiros susceptíveis e que os transcritos que codificam para as proteínas com ação imunomoduladoras são mais abundantes como demostrado em Franzin *et al*., (2017). Carrapatos alternam seu estado fisiológico quando alimentados em diferentes hospedeiros (Kim *et al*., 2016).

Ao passo que Carvalho *et al*. 2010, em experimento encontrou em animais resistentes aumentados o número de eosinófilos, basófilos e altos níveis de expressão de E-selectinas, uma proteína de adesão de linfócitos T de memória residente, linfócitos T γδ e basófilos. Animais resistentes deverão ser mais refratários as proteínas do carrapato com efeito antiadesivas da saliva. No presente trabalho níveis consideráveis de linfócitos T $CD3^+$ foram marcados e que podem ser majoritariamente linfócitos T γδ na pele do bovino e cuja a função na resposta imunológica contra o carrapato ainda carece de mais estudo. Linfócitos $CD3^+$ e γδ em animais susceptíveis provavelmente são a fonte para citocinas importantes que regulam os mecanismos efetores contra os carrapatos no endotélio e a memória imunológica.

O tratamento histológico das amostras, especificamente os produtos usados na fixação como paraformolaldeido 4% e etanol e posterior embebição em parafina das coletas de pele do bovino, mostraram-se efetivos e não tiveram efeito na recuperação antigênica das proteínas alvos dos anticorpos utilizados no presente trabalho ($CD3^+$ e

$CD79^{+}$), tal como revelado pelos resultados da imuno-histoquímica apresentados nos resultados. De modo que permite que os dados do presente trabalho sejam expandidos em trabalhos futuros sobre a relação carrapato hospedeiro ou em trabalhos semelhantes, utilizando os mesmos anticorpos.

Os anticorpos utilizados para marcar células que expressam $CD3^{+}$ e CD79α na pele do bovino, reagiram fortemente com as células localizadas junto ao local da picada do carrapato e próximo das áreas adjacentes ao hipostômio do carrapato. Isso indica que estes anticorpos possuem excelente reatividade com os antígenos das células presentes na pele do bovino e que podem ser usados para aferir a participação das células que expressam estes antígenos na resposta imune local do bovino contra a infestação com *R. microplus*. Constantinoiu *et al*., (2013), analisando os padrões de coloração de subpopulações de leucócitos na pele de raças taurinas e indicinas, utilizou quatro métodos de fixação dos quais a fixação com etanol mostrou menos efeito severo no reconhecimento do epítopo pelos anticorpos conservando deste modo a conformação dos epítopos dos antígenos frente ao tratamento com os fixadores. O método usado neste estudo também preservou a estrutura dos tecidos analisados. Portanto, os anticorpos anti-$CD3^{+}$ e anti-CD79α podem ser usados para analisar a resposta imune local no local da picada do carrapato em bovinos. Embora que exista a necessidade de se estabelecer as diferenças de reatividade destes anticorpos em relação a composição genética em diferentes raças de bovino para melhor se avaliar a imunidade local tendo em conta a sensibilidade e a especificidade dos anticorpos usados para tal efeito (Constantinoiu *et al*., 2010, 2013).

No presente trabalho de pesquisa o anticorpo específico para o antígeno $CD3^{+}$ reagiu com células presentes em todas biópsias de pele. O complexo $CD3^{+}$ foi expresso em linfócitos presente na pele do bovino. Porém houve mais células marcadas fortemente

com anti-$CD3^+$ do que células marcadas com anti-$CD79^+$ considerando os diferentes estágios adultos de desenvolvimento do carrapato fixo na pele do bovino. Em contrapartida Piper *et al*., (2009, 2017) identificou significativamente altos níveis de $CD4^+$, $CD25^+$ e $WC1^+$ linfócitos T γδ de populações de células no sangue periférico de animais resistentes. Enquanto que em animais suscetíveis foram obtidos significativamente altos níveis de monócitos $CD14^+$ e células apresentadoras de antígeno expressando MHCII. Franzin *et al*., (2017), encontrou na pele de animais resistentes o número de células $CD3^+$ aumentado. Neste estudo os animais utilizados já tinham sido previamente infestados com *R. microplus* e, portanto, para o estudo e aprofundamento e associação desses fatores com mecanismo de resistência imunológica deverá ser combinado o uso de mais marcadores assim como o aprimoramento dos experimentos considerando as fases iniciais do estádio dos carrapatos e animais não expostos ao carrapato.

5 Conclusão

A resposta imune local do bovino contra o carrapato é manifestada pela resposta imune inata no local da picada do carrapato que é composto pelas barreiras físicas na pele, célula imune residentes e pelas células que migram para o local da picada. Modelos bovinos e outros hospedeiros para estudo da resposta imune na pele contra carrapatos demostram majoritariamente um perfil celular polarizada de resposta Th1 para Th2 na maioria dos casos revisados no presente estudo.

É verificada, portanto um aumento de infiltrado celular composto majoritariamente por eosinófilos, seguidos por aumento de mastócitos. Porém as técnicas utilizadas no presente trabalho não foram suficientes para se determinar a percentagem de basófilos nas biópsias de pele analisadas. Podendo desta forma ser o alvo de trabalhos futuros para se determinar as funções destas células na relação carrapato-bovino. Células da resposta imune adaptativa foram marcadas fortemente por anti-CD3 comparativamente aquelas marcadas com anti-CD79α. Estas células possuem um papel importante na resistência imunológica do bovino contra o carrapato. A presença dessas células no local da picada do carrapato em proporção aumentada sugere que essas foram induzidas pelos carrapatos adultos com os quais foi coletada as amostras de pele. Além disso, o perfil de células presentes no local de fixação do carrapato é atraído de modo saliva-dependente e de acordo ao estado imune do bovino estudado, que neste caso em particular, já era um animal resistente.

O presente estudo e experimento deverá ser expandido, aumentando-se e tendo em conta amostras de pele entre espécies bovinas e dentro da mesma raça durante diferentes estádios de desenvolvimento do carrapato no seu ciclo parasitário. Também trabalhos futuros deverão investigar a função de basófilos, linfócitos γδ, subpopulações de células dendríticas e queratinócitos e fibroblastos na resposta imune contra o carrapato.

Acrescentando-se a este facto urge a necessidade de desenvolvimento de um modelo experimental de *R. microplus* para estudo dos correlatos da resposta imune do seu hospedeiro.

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Anexo A

Curriculum Vitae

BENVINDO, C.J.

1. Dados Pessoais

Nome: Benvindo Capela João

Local de nascimento: Huambo - Angola

Data de nascimento: 29/04/1987

Endereço eletrônico: benvindocapelajoao@gmail.com

2. Formação Acadêmica

2010 Graduação em Medicina Veterinária
Universidade José Eduardo dos Santos, Huambo, Angola
Título: Avaliação da qualidade da água em algumas indústrias alimentares na cidade do Huambo
Orientador: Joaquim Morais
Co - orientador: Débora Carneiro

2018 - presente Mestrado em Biologia Celular e Molecular
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
Título: Resposta imune local de bovinos infestados com carrapato *Rhipicephalus microplus*
Orientador: Itabajara da Silva Vaz Junior
Co - orientador: Luis Fernando Parizi
Bolsista: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

3. Formação complementar

2013 – 2015 Curso de Comunicação e Cultura V, HMT 247. (Carga horária: 2 anos).
University of the Nations, Worcester, África do Sul.

2015 Treinamento transcultural, DSP 212, com práticas desenvolvidas na Malásia, Singapura e Porto Elisabeth (Carga horária: 3 meses).
University of the Nations, Worcester, África do Sul.

2010 Estágio - extracurricular no laboratório de Imunologia Aplicada a Sanidade Animal, Centro de biotecnologia (Carga horária: 2 meses).
Universidade do Rio Grande do Sul-UFRGS, Brasil.

2009 Curso de Contabilidade Geral. (Carga horária: 3 meses).
Centro de Formação Profissional CONSULT, Huambo, Angola.

2**019** Estágio prático no laboratório de Histologia do Departamento de Biologia do Instituto de Biociências da UNESP. (Carga horária: 160 horas).
Universidade Estadual de São Paulo

2020 Participou do 1° Simpósio sobre Inovação e Tecnologia em Parasitologia da UFSC. (Carga horária: 20 horas).
Universidade Federal de Santa Catarina.

2020 Curso de curta duração - Biologia, importância e controle de carrapatos. (Carga horária: 32 horas).
Colégio Brasileiro de Parasitologia Veterinária, Brasil.

2019 Curso de curta duração - Análise de imagens utilizando o imageJ - Nível básico. (Carga horária: 5 horas).
Universidade Federal do Rio Grande do Sul, Departamento de Ciências Morfológicas.

2020 Curso de curta duração - Treinamento sobre funcionamento do Portal de Periódicos da CAPES. (Carga horária: 2 horas).
Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

2020 Participou do I Simpósio Internacional On-line do Programa de Pós-Graduação em Ciência Animal. (Carga horária: 20 horas).
Universidade Estadual do Maranhão.

2020 Curso de curta duração - Internacional Online de Asesoría Científica, Técnica y Gerencial del Conocimiento. PROYECTO INTERNACIONAL DE ASESORAMIENTO ONLINE, CON CONTINUACIÓN Y CONCLUSIÓN PRESENCIAL. CÓDIGO: MA06-CIOACTGC-290489. (Carga horária: 48 horas).

4. Atuação profissional

2016 - atual
Empresa ELFAR Ltda distribuidora, Namibe, Angola
Vínculo: Sócio gerente
Enquadramento funcional: Gerente administrativo e financeiro

2015 – atual
Ordem dos Médicos Veterinários de Angola
Vínculo: Membro
Enquadramento funcional: Membro

2020 – atual
Sociedade Americana de Entomologia
Vínculo: Membro
Enquadramento funcional: Membro do terceiro mundo

2012 - 2015

Empresa Ecovisão Angola

Vínculo: Técnico superior do ambiente

Enquadramento funcional: Técnico de laboratório

2012

ONG MENTOR initiative

Vínculo: Estagiário

Enquadramento funcional: Administrativo

Regime: Parcial

2009 - 2011

Empresa de Soluções e Gestão de Saúde, AMOSMID Ltda

Vínculo: Estagiário

Enquadramento funcional: Assistente Administrativo

Regime: Parcial

2007 - 2009

Universidade José Eduardo dos Santos, Huambo, Angola

Associação dos estudantes da Faculdade de Medicina Veterinária

Enquadramento: Vice - presidente

5. Artigos completos publicados em periódicos

Sajiki, Y., Konnai, S., Ikenaka, Y., Gulay, K. C. M., Kobayashi, A., Parizi, L. F., ... & Ohashi, K. (2021). Tick saliva-induced programmed death-1 and PD-ligand 1 and its related host immunosuppression. **Scientific Reports**, 11(1), 1-11.

6. Trabalhos publicados em eventos

FURQUIM, K. C. S. ; CAMARGO-MATHIAS, M.I.; BENVINDO, C.J. Morphohistochemical investigation of the feeding, digestive and reproductive systems of female dog ticks in rapid feeding stage. 2019. (Apresentação de poster em congresso e publicação na **Revista Histology and Histopathology: from cell biology to tissue engineering**)

Benvindo C. J.; Luis F. P.; Bianca S. C.; Cintia D. L.; David D.**;** Itabajara V. J.. Skin local immune response during *Rhipicephalus microplus* adult parasitism. In: Encontro Anual do Programa de Pós graduação em Biologia Celular e Molecular. 2020.

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João B.C.; Morais J. e Carneiro D. 2013. Avaliação da qualidade da água em algumas industrias alimentares no município do Huambo. Monografia. Faculdade de Medicina Veterinária. Universidade José Eduardo dos Santos. Trabalho de Fim de Curso.

João B.C. 2008. Caracterização da espécie forrageira *Cenchrus ciliares*. I jornadas de pastos e forragens da faculdade de Ciências Agrárias, Universidade Agostinho Neto.

João B.C., Alberto P.N.. 2007. Avaliação do comportamento alimentar de caprinos na fazenda experimental da Chianga. Faculdade de Ciências Agrárias, Universidade Agostinho Neto. I Jornadas Científicas Estudantis.

7. Organização de evento

Joao, B.C. e Alberto P.N. Primeiras Jornadas Estudantis da Faculdade de Ciências Agrárias. Universidade Agostinho Neto. 2007