Comparative immune-profiling of three cattle breeds infested with *Rhipicephalus microplus* and *Ixodes ricinus*-infested BALB/c mice

by

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Submitted in partial fulfilment of the requirements for the degree

Philosophiae Doctor

In the Faculty of Natural & Agricultural Sciences

University of Pretoria

Pretoria

November 2018

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Acknowledgements

I wish to thank the following people, institutions and funding bodies without which my research would not have been possible:

- Prof Christine Maritz-Olivier, my project supervisor for accepting me into her research group, her time, input and guidance. Thank you for your support and motivation throughout my studies.

- Prof Andrew Leisewitz, my co-supervisor, for his time, helpful advice, input and always showing the positive in troublesome situations.

- RNDrs Petr Kopáček and Ondřej Hajdušek at the Institute of Parasitology, Biology Centre, Czech Republic for their generosity of opening their labs, enabling me to complete an important part of my studies. I would also like to thank RNDr Marie Jalovecká, Mgr Jan Kotál and Jan Erhart for their support and infectious enthusiasm.

- Dr Sabine Richards. Thank you for being a voice of reason, thank you for telling me when I was being irrational, and thank you for your unending support. Dr Christian Stutzer for your friendship, lengthy discussions and laughter.

- Drs Sarah Clift, Jan Crafford and Annette-Christi Barnard for their advice, support and insightful ideas during the research undertaken.

- I would like to acknowledge all other funding bodies for their financial support to help me in the completion of this degree: The University of Pretoria, The Meat Industry Trust of South Africa, The Technology and Human Resources for Industry Programme (THRIP), and The National Research Foundation.

- To my friends, words fail to describe how much I appreciate your love, support and encouragement over the course of my tertiary studies. Thank you for opening your hearts and home: Chanelle, Judd, Anton, Marius, Sabah, Thoko, Rosie, Anri, Ouma Marie and Oupa Hennie.

- I would additionally like to thank Anton for printing and submitting my thesis in my absence. May the 4th be with you!

- Thank you to my guardian angel, my aunt and my friend T. Annelie.

- Thank you to my parents, Sarel and Elna Robbertse, who never gave up on me and supported me wherever my path led me. You will never know how much you taught and still teach me; how grateful I am for everything you have done for me and how much I love you.

- Lastly, thank you to the studious crow for unlocking literature with its key rubeum.

Declaration

I, Luïse Robbertse declare that the thesis/dissertation, which I hereby submit for the degree *Philosophiae Doctor* specializing in Genetics at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this, or any other tertiary institution.

Luïse Robbertse

November 2018

Summary

Ticks and tick-borne diseases have a negative impact on the quality and quantity of livestock-derived products. As such, effective control strategies against these parasites are needed. Currently, acaricides are the most widely used control mechanism against ticks but due to the increase in resistance to these chemicals, new control strategies, like vaccination, need to be developed and improved. In cattle, increasing the understanding of the basic underlying variation in immunological responses to tick infestation may constitute the basis of improved tick control strategies in the future. While the identification of protective antigens is essential, the final formulation of vaccines is pivotal in the efficacy of a future vaccine. As such, increasing the understanding of the basic underlying variation in immunological responses to tick infestation may constitute the basis of improved tick control strategies in the future. Chapter 2 describes the differential regulation of T and B-lymphocyte subsets in the skin and lymph nodes amongst three cattle breeds as potential mediators of immune-resistance to *Rhipicephalus microplus* tick. This study has been the first to describe in detail the *in vivo* immune responses in lymph nodes of cattle following *Rhipicephalus microplus* infestation, attachment and continued feeding. To further compliment this study, Chapter 3 provides a temporal analysis of the bovine lymph node transcriptome during cattle tick (*Rhipicephalus microplus*) infestation. Here a detailed description on the specific transcriptional processes in the lymph nodes of Bonsmara cattle is given. These processes include: (1) Leukocyte recruitment to the lymph node via chemokines and chemotaxis, (2) Trans-endothelial and intranodal movement on the reticular network, (3) Active regulation of cellular transcription and translation in the lymph node (including leukocyte associated cellular regulatory networks) and (4) Chemokine receptors regulating the movement of cells out of the lymph node. In addition to studying the immune response in cattle, the viability of a mouse model was used for basic immune profiling during tick feeding and vaccination in Chapter 4. Here an *in vivo* evaluation of *Ixodes ricinus* induced effects on T and B-cell maturation in the spleen and lymph nodes of BALB/c mice is given.

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List of Abbreviations

AF488	Alexa Fluor 488
AF647	Alexa Fluor 647
APC	Allophycocyanin
BLAST	Basic Local Alignment Search Tool
C1QA	complement C1q subcomponent subunit
cAMP	cyclic AMP
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	cluster of differentiation
cDNA	complementary DNA
Con A	concanavalin A
CX3CR	CX3C chemokine receptor
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
Cy	Cyanine
DAVID	Database for Annotation, Visualization and Integrated Discovery
DC	dendritic cell
DEG	differentially expressed gene
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dpi	days post infestation
dpv	days post vaccination
dUTP	2´-Deoxyuridine, 5´-Triphosphate
EDTA	ethylenediaminetetraacetic acid
FCS	foetal calve serum
FDC	follicular dendritic cells
FITC	Fluorescein isothiocyanate
FRC	fibroblastic reticular cell
GC	germinal centre
GO	gene ontology
H&E	Haematoxylin and Eosin
HEVs	high endothelial venules
IHC	Immunohistochemistry
IL	interleukin
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOG	Eukaryotic Orthologous Groups
LIMMA	Linear Models for Microarray Data
MHC	major histocompatibility complex
NE	nymphal extract
PAMP	pathogen-associated molecular pattern

PBS	phosphate buffered saline
PRR	pattern recognition receptor
qRT-PCR	quantitative real-time PCR
Rcr	relative cytokine ratios
RNA	ribonucleic acid
RNase	ribonuclease
RPMI medium	Roswell Park Memorial Institute medium
SPF	specific pathogen-free
TBM	tingible body macrophage
TDA	tick-derived antigen
Th cell	T helper cell
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAF	TNF receptor associated factor
XCL	Chemokine (C motif) ligand
MGAT4B	alpha-1,3-mannosyl-glycoprotein-4-beta-N-acetylglucosaminyltransferase B

Chapter 1: Bovine immune factors underlying tick resistance: integration and future directions

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This chapter has been published as a global literature review in Frontiers in Cellular and Infection Microbiology (Research topic: Tick-host-pathogen interactions) (doi: 10.3389/fcimb.2017.00522). A copy of the published article is attached at the end of the thesis. For uniformity throughout the thesis, the references in the chapter are formatted to the Ticks and Tick-borne Diseases referencing format. Luïse Robbertse, Sabine A. Richards and Christine Maritz-Olivier conceptualized the manuscript focus, critically revised the draft manuscript, and approved the final manuscript. Luïse Robbertse and Sabine A. Richards accumulated data, designed figures and tables and wrote the first manuscript draft.

1.1. Abstract

The mechanisms underlying tick resistance within and between cattle breeds have been studied for decades. Several previous papers on bovine immune parameters contributing to tick resistance discussed findings across DNA, RNA, protein, cellular, and tissue levels. However, the differences between bovine host species, tick species and the experimental layouts were not always taken into account. This review aims to (a) give a comprehensive summary of studies investigating immune marker differences between cattle breeds with varying degrees of tick resistance, and (b) to integrate key findings and suggest hypotheses on likely immune-regulated pathways driving resistance. Experimental issues, which may have skewed conclusions, are highlighted. In future, improved experimental strategies will enable more focused studies to identify and integrate immune markers and/or pathways. Most conclusive thus far is the involvement of histamine, granulocytes and their associated pathways in the tick-resistance mechanism. Interestingly, different immune markers might be involved in the mechanisms within a single host breed in contrast to between breeds. Also, differences are evident at each tick life stage, limiting the level to which datasets can be compared. Future studies to further elucidate immune molecule dynamics across the entire tick life cycle and in-depth investigation of promising markers and pathways on both molecular and cellular level are in dire need to obtain a scientifically sound hypothesis on the drivers of tick resistance.

Keywords: cattle, tick, resistance, tick resistance, immune factors, parasite, host

1.2. Introduction

The economic importance of ticks and the need to control them was realized alongside the discovery of their potential as vectors of harmful parasites, particularly to livestock (Hunter and Hooker, 1907; Theiler, 1911). The variability in the degree to which cattle display resistance to ixodid ticks was first suggested by Johnston and Bancroft (1918). It is known that tick resistance in cattle varies from more tick-susceptible *Bos taurus taurus* (*B. t. taurus*) to more tick-resistant *B. t. indicus* breeds, between bovine crosses as well as within a single cattle breed (George et al., 1985; Rechav et al., 1991b; Mattioli and Cassama, 1995; Mwangi et al., 1998; Mattioli et al., 2000; Nascimento et al., 2011). However, the biological factors underlying bovine resistance to tick infestation are still poorly understood. Tick resistance is a multi-factorial trait suggested to involve host-related factors such as sex, age, lactation, grooming behaviour, skin composition and host surface area, coat length and environmental factors (Wharton et al., 1970; Seifert, 1971; Doube and Wharton, 1980; Binta and Cunningham, 1984; Ali and de Castro, 1993; Meltzer, 1996; Norval et al., 1996; Mattioli, 1998; Martinez et al., 2006; Kongsuwan et al., 2010). It is also well established that the tick-resistance phenotype is heritable, as is evident from breed-specific resistance patterns. Furthermore, it was proposed that tick attachment sites on resistant cattle rapidly become unsuitable for feeding due to host immune responses (Roberts, 1968b). The majority of studies indicate that resistance is acquired through exposure to ticks (Wagland, 1975, 1980; George et al., 1985; Momin et al., 1991) and that resistance is acquired sooner and to a higher degree in *B. t. indicus* than in *B. t. taurus* breeds (Riek, 1962; Wagland, 1978, 1980; Rechav et al., 1990). This phenotype only becomes apparent after subsequent (and not initial) tick exposure in *B. t. taurus*, *B. t. indicus* and mixed breed cattle (Roberts, 1968a; Wagland, 1975; Hewetson and Lewis, 1976).

To further elucidate potential mechanisms underlying differences in tick resistance, several studies have investigated host immune responses towards ticks on a cellular and molecular level. Gaining an understanding of the molecular mechanisms underlying tick-resistance mechanisms will be advantageous in the identification of specific genomic alterations or specific markers that could lead to the ability to screen cattle for their potential resistance status without prolonged tick infestation trials or counts. This would be helpful in breeding more tick-resistant cattle. In this regard, it was shown that although host resistance to tick infestation and product yield was not correlated in Holstein-Friesian cattle (Jonsson et al., 2000), carriers of both *B. t. taurus* and *B. t. indicus* genes may suffer from a trade-off between animal-derived product yield and tick resistance (Wang et al., 2007). A clear understanding of tick-resistance mechanisms would also be beneficial to vaccine trials, where a difference in the resistance status of individual animals could skew results and therefore make accurate data interpretation more difficult. Furthermore, more effective vaccine formulations could be devised as vaccine efficacy is hindered by the modulation of host immune responses through tick saliva (Kazimírová and Štibrániová, 2013). Knowledge regarding molecular mechanisms underlying tick resistance could allow for the optimal selection of appropriate adjuvant/vaccine formulation strategies to provide a cross-breed protective response.

This review therefore provides a summary of studies performed up to date in cattle blood and skin tissue, with critical evaluation of findings followed by hypotheses on key role players, possible immune-regulated pathways as well as improvements for consideration when planning future

experiments. Several recent studies were published pertaining to genetic associations with regards to the tick-resistance phenotype (Mota et al., 2016a, b, 2017; Junqueira et al., 2017; Sollero et al., 2017), however, these are outside the scope of this summary. This review should provide readers with the basic knowledge and a critical evaluation of findings to date to make informed decisions for future studies investigating the tick-host-interface with a focus on resistance. Due to differences in experimental layouts, which might skew data interpretation and comparisons such as tick life stage, tick species and type of bovine comparison (between or within breeds), are provided in Supplementary table 1.1.

1.3. Blood

Immune cells originate from hematopoietic stem cells in the bone marrow with naïve and mature forms of these cells circulating in the blood and lymphatic systems. Here, they encounter foreign molecules that lead to their proliferation, differentiation and maturation (Janeway et al., 2001). However, due to the constant circulation and changing dynamics of immune response components, experimental designs (especially time-points) must be chosen carefully. Various gene expression, translational and cytological studies have investigated blood to elucidate immune responses linked to tick resistance/susceptibility in cattle and these are described in the next section.

1.3.1. Gene expression studies in the blood of tick-infested cattle

Gene expression studies of peripheral blood mononuclear cells identified transcripts for IL-2, IL2Rα, TNFα, and CCR1 to be significantly up-regulated in resistant cattle relative to susceptible cattle, while a significantly higher expression of *CXCL10* was detected in susceptible Holstein-Friesian compared to resistant Brahman cattle (Piper et al., 2009). Pathway analysis indicated that genes that are more highly expressed in the resistant breed are associated with the hematopoietic cell lineage and cytokine-cytokine receptor interaction pathways. Another study found a significant up-regulation of *CD25*, *IL10*, *FoxP3*, and *CXCL10* in samples from cattle infested with larvae compared to samples obtained from uninfested animals. In susceptible animals, *CXCL8* was down-regulated in susceptible animals 24 and 48 h after infestation compared to samples from uninfested animals (Domingues et al., 2014). Although *CXCL10* was identified in both studies as differentially regulated, major differences in the study designs hinder any direct comparisons. Piper et al. (2009) obtained blood samples at the peak of tick infestation without reference to a specific time point after infestation and found significantly higher chemokine expression in susceptible compared to resistant cattle breeds. Domingues et al. (2014) on the other hand compared tick-infested versus tick-uninfested cattle of the same breed and identified an increase in *CXCL10* in resistant animals 48 h and an increase in susceptible animals 24 h after tick infestation. Therefore, the role of CXCL10 remains to be confirmed in future studies and its contribution to resistance pathways elucidated.

1.3.2. Translational studies in the blood of tick-infested cattle

1.3.2.1. Immunoglobulins

A link between tick resistance and immunoglobulins was proposed in 1987 by Rechav and colleagues who found a positive correlation between tick numbers and total serum gamma globulin levels in naturally infested *B. t. taurus* and *B. t. indicus* cattle. During the acquisition of tick resistance, a negative correlation between tick weight and total serum gamma globulin levels was, however, documented in *B. t. taurus* cattle infested with *Rhipicephalus decoloratus* (Rechav et al., 1991a). This discrepancy could be a result of differences in experimental design, as the animals studied by (Rechav et al., 1991a) were most likely in the process of acquiring tick resistance as opposed to the more established resistance of cattle studied by Rechav (1987). In this regard, resistance in the former study was supported by reduced tick weights only.

In general, the number of ticks feeding on cattle were found to positively correlate with salivary gland specific IgG levels in previously infested (Sahibi et al., 1998) and naïve (Cruz et al., 2008) *B. t. taurus* cattle. Cruz et al. (2008) furthermore reported that there was no change in the avidity of antibodies developed by either *Rhipicephalus microplus* resistant or susceptible animals against salivary soluble extracts (Cruz et al., 2008).

Differences in the IgG1 isotype was observed when comparing cattle breeds displaying varying tick-resistance phenotypes. After multiple infestations of tick-naïve cattle, IgG1 levels (against several tick extracts) were found to be significantly higher in susceptible compared to resistant cattle (Piper et al., 2017), with similar results obtained the studies of Garcia et al. (2017) and Piper et al. (2009). Although no differences were observed for tick-naïve animals at the beginning of the study by Piper et al. (2017), higher tick-saliva specific IgG1 levels were seen before and at the beginning of the first infestation in the tick-resistant cattle breed by Garcia et al. (2017). Yet, Kashino et al. (2005) reported a decrease of IgG1 upon heavy tick infestation in naturally infested susceptible animals, compared to resistant animals (Kashino et al., 2005). As such, the question arises whether studies done under controlled housing conditions and those done under field conditions with natural infestation, and possible co-infections/infestations, can be compared.

In contrast to IgG1 levels, no significant differences were identified for the IgG2 isotype between breeds (Piper et al., 2009, 2017), with similar results seen by Garcia et al. (2017) for resistant animals throughout the study. The latter study does however describe an increase of this isotype in the susceptible breed at the third infestation compared to the baseline. At the same time point, IgG2 levels were significantly higher in susceptible compared to resistant animals. Again, in contrast, Kashino et al. (2005) identified decreased levels of IgG2 in naturally infested susceptible compared to resistant animals during heavy infestation.

Only two studies have investigated IgE levels between resistant and susceptible cattle breeds. Garcia et al. (2017) determined levels of total IgE in the sera of cattle infested with *R. microplus*. No difference in these levels were noted between resistant and susceptible breeds. Tick-specific IgE antibody levels, however, were shown to be significantly lower in resistant compared to susceptible animals during heavy infestation as well as during subsequent light infestation (Kashino et al., 2005). This difference in IgE levels between cattle breeds seems to be a result of an increase in this immunoglobulin in susceptible animals instead of a decrease in resistant animals. The same trend was seen in some studies investigating IgG isotypes. IgE with

associated receptors and cellular responses are believed to have evolved to counter helminths and other parasites that cannot be phagocytosed (Fitzsimmons et al., 2014). In this paper, we propose a role for IgE-dependent responses as one of the drivers of resistance (see sections 1.5: Dynamics of granulocytes and histamine and their suggested involvement in the tick-resistance mechanism over the tick lifecycle and 1.6: Future directions: potential drivers involved in tick resistance), and as such, daily data on the IgE levels throughout the period of tick attachment and subsequent life stages will be of great importance.

Considering the consensus from the majority of studies, resistant animals seem to display a more constant tick-specific immunoglobulin isotype profile with fewer changes observed throughout infestation cycles. Susceptible animals on the other hand show an increase of tick-specific immunoglobulin levels over multiple infestations. Differences in host immune responses are furthermore evident by the observation that a great variation in tick salivary gland extract profiles are recognized between individual cattle sera (Cruz et al., 2008). Furthermore, sera from a resistant compared to a susceptible cattle breed reacted with more tick salivary proteins, which require further investigation (Garcia et al., 2017). On the other hand, differences in tick numbers and thus the amount of tick antigens in different hosts also requires more in-depth studies to determine immune responses independent of varying tick numbers.

1.3.2.2. Other

Additional host immune components have also been associated with tick resistance to date, including histamine, complement, acute-phase proteins and bovine lymphocyte antigens. Increased histamine and complement levels were found to be associated with lower tick numbers and resistant animals, respectively (Riek, 1962; Wambura et al., 1998; Zhao et al., 2013). Three proteins of the acute-phase response, which is generally initiated in response to tissue damage (Baumann and Gauldie, 1994), were linked to the tick-resistance mechanism by Carvalho et al. (2008). Briefly, susceptible Holstein-Friesian cattle showed a significant increase in haptoglobulin levels resulting from heavy tick infestation as well as constantly higher alpha-1 acid glycoprotein levels compared to resistant Nelore (*B. t. indicus*) animals. This might be a result of increased tick numbers and the associated increase in tissue damage on susceptible animals. Lastly, only during intense infestation did the more resistant cattle breed show higher levels of serum amyloid A when compared to the more susceptible cattle breed. In two separate studies, a total of 19 bovine lymphocyte antigens were tested in blood collected from a mixed breed cattle population with no overlapping findings. In total, two antigens were significantly associated with tick resistance (W8 and W16) and three with tick-susceptibility (W5, W6, CA31) (Stear et al., 1984, 1989). Additional data is therefore required to resolve knowledge gaps in the pathways associated with the above-mentioned compounds.

1.3.3. Cytological studies

1.3.3.1. Identification of immune cell subtypes in circulating blood of tick-infested cattle using associated markers

Identification and quantification of immune cell subtypes in circulating blood has been performed in *B. t. indicus*, *B. t. taurus* and mixed breed cattle with the use of associated markers. Piper et al. (2017) did not find any significant differences while Piper et al. (2009) identified significantly higher levels of CD4+, CD25+ activated and WC1+ γδ T-cell populations in more tick-resistant cattle. Significantly higher levels of CD14+ monocyte and MHC II presenting cells were obtained in more tick-susceptible cattle. As these cell subtypes are known to be associated with a variety of immune responses and pathways, linking them to a putative resistance mechanism will only be possible when analysing them in combination with additional markers (Figure 1.2).

1.3.3.2. Identification of immune cell subtypes in circulating blood of tick-infested cattle using morphological characteristics

The cellular composition of blood is regarded as an important identifier of the overall health of humans and animals and as such changes in the percentage of different white blood cell populations may be used as an indication of a systemic immune response. Three main studies have relied on the use of different white blood cell population counts (basophils, eosinophils, lymphocytes, total leukocytes, neutrophils, monocytes) in describing the immune response of cattle with varying levels of tick resistance with no differential regulation identified to date (Brown et al., 1984; Rechav, 1987; Rechav et al., 1990). Only blood eosinophil levels were significantly higher in the more susceptible *B. t. taurus* breed (which carried more ticks) in the study by Rechav et al. (1990). The investigation of cell subtypes in blood represents a daunting task as shown by the lack of identified differential regulation of markers obtained. In contrast, research to date has detected significant differences between hosts with varying tick-resistance status in skin tissue (refer to skin section below). This is the case since the dynamics of immune cells in blood only provide a snapshot of what is occurring at a specific time point. Experimental layouts must thus be considered carefully as studies incorporating and comparing several time-points and/or tissues might represent a more realistic view of immune drivers of resistance.

1.4. Skin tissue

The skin represents the first site of encounter to tick infestation and thus the first line of host immune defence. Upon penetration and successful attachment, ixodid ticks alternate salivation and blood intake every 5–20 min (Francischetti et al., 2009). Numerous salivary components mediate suppression of host responses such as blood coagulation, immunity, inflammation and the ability of the host to develop new blood vessels (Hovius et al., 2008; Kazimírová and Štibrániová, 2013; Kotál et al., 2015). The identification of immunological defence responses at the site of tick infestation have been extensively studied, as evident from the next section.

1.4.1. Gene expression studies in the skin of tick-infested cattle

Three transcriptional studies on cattle skin have showed the involvement of the complement cascade in the feeding of ticks in both susceptible and resistant cattle (Wang et al., 2007; Piper et al., 2010; Carvalho et al., 2014). The complement system is a part of the innate immune system and plays a vital part in the clearance of foreign cells via the activation of one of three biochemical pathways (Nesargikar et al., 2012). Up-regulation of gene expression for complement components in tick-resistant cattle (*C1QA*) (Wang et al., 2007) and tick-susceptible cattle (*complement component 3*) (Piper et al., 2010) have been shown, while the general pathway down-regulation of complement has also been described in susceptible animals (Carvalho et al., 2014). More in-depth studies are required regarding these components potentially involved in the tick-resistance mechanism. This is due to the various complement components investigated to date combined with differences amongst results reported. Interestingly, all immunoglobulin associated transcripts were identified to be more abundant in less resistant animals (Wang et al., 2007; Piper et al., 2010) which correlates with findings obtained from blood and could be linked to increased tick numbers on these animals. Furthermore, CD14 (on transcriptional level in skin and translational level in blood) was identified in both tissues to be associated with tick-susceptibility (Piper et al., 2008, 2009). CD14 is known to be a marker for monocytes and macrophages and can therefore be involved in several immune response mechanisms (Ziegler-Heitbrock and Ulevitch, 1993).

Other components that were found to be up-regulated in susceptible cattle include transcripts for IL13RA1, CD44, CD63, TNFα, IL-1β, IL-10, NFKBp50, CD1a, CCR-1, CCL2, CCL26, TLR9, MyD88, CD14, FTH1, BDA20, and Traf-6 (Wang et al., 2007; Piper et al., 2008; Nascimento et al., 2011). However, no transcript was reported in more than one of these studies and as such all require validation. Most recently, Franzin et al. (2017) reported on a microarray study of skin from uninfested cattle, larvae (2 days after larvae infestation) and nymph (9 days after larvae infestation) life stages fed on *B. t. taurus* and *B. t. indicus* breeds. Samples were compared within and between breeds. An observed allergic contact-like dermatitis was found to be delayed in susceptible animals (detected by the involvement of *IL-6*, *CXCL-8*, *CCL-2*, *HMGB1*, *ISG15*, and *PKR*) which in turn results in the production of chemokines and cytokines involved in the inflammatory response. In another study, down-regulation of inflammatory response gene expression was observed within 24 h after tick infestation in susceptible animals, while at the 48-h sampling point genes associated with antigen presentation and oxidative stress were found to be up-regulated in resistant cattle (Carvalho et al., 2014). One study identified *CXCL-8* expression as being down-regulated in resistant cattle between different genetic crossbred cattle groups from which skin and lymph node samples were obtained 9 days after larvae challenge (Regitano et al., 2008). However, it was unclear to which tissue this finding refers to. Differential gene expression was furthermore identified for genes encoding Blimp- 1 (Kongsuwan et al., 2010), cathepsin L2 precursor, MHC class antigen I (Nascimento et al., 2011), various adhesion molecules (Carvalho et al., 2010), TNF receptor-associated factor 6, TATA-binding protein, lumican and beta-2 microglobulin (Marima, 2017).

Due to the variation in experimental designs of different studies, caution should be taken when trying to compare results emanating from transcriptional studies, especially with regards to sampling time-points. Since gene expression rarely involves absolute quantification and is based

on the relative quantification of transcripts between two populations or between transcripts and reference genes under a specific set of conditions, the results generated may be study specific. RNA sequencing would be an alternative approach not yet utilised in this field of study for the obtainment of large-scale results based on absolute quantification which could allow the identification of novel transcripts. In addition to this, studies on a protein and cellular level, to validate potentially relevant findings from gene expression studies, should be undertaken.

1.4.2. Translational studies and metabolites in the skin of tick-infested cattle

Few studies investigated immune factors underlying the tick-resistance mechanism in skin of cattle on the protein or metabolite level. To date, only one paper has focused on proteins. However, no significant findings regarding proteins directly involved in immune response pathways were identified (Kongsuwan et al., 2010). On a metabolite level, higher histamine levels were linked to tick resistance by Schleger et al. (1981) and Willadsen et al. (1979). These findings also correlate with results obtained from studies done on blood (Riek, 1962; Zhao et al., 2013). Histamine is an immunomodulator produced by a variety of cell types including mast cells, basophils, dendritic cells, and T-cells and can regulate both innate as well as adaptive immune response cells (O'Mahony et al., 2011). The expression of the histidine decarboxylase, which results in the decarboxylation of L-histidine and subsequent production of histamine, is influenced by several immune factors including a variety of cytokines. This secondary metabolite regulates, amongst others, antigen-specific Th1 and Th2 cells in addition to antibody isotype responses (Jutel et al., 2006). Histamine seems to be an effector molecule in tick resistance. This is evident from studies showing that histamine injection at tick attachment sites lead to detachment of some tick larvae, indicating a direct involvement of histamine rather than a general inflammatory reaction being the cause of tick rejection (Kemp and Bourne, 1980).

Furthermore, higher tick numbers were observed in cattle treated with an antihistaminic drug (Tatchell and Bennett, 1969). Histamine is well documented to be involved in proinflammatory responses and the immediate-type hypersensitivity response, characterised by increased vascular permeability, smooth muscle contractions, activation of certain nerves, wheal-and-flare reactions and itch responses (O'Mahony et al., 2011). Acquired resistance was linked to the occurrence of a hypersensitivity reaction to tick salivary gland components (Riek, 1962). The type of hypersensitivity is, however, not confirmed since contradicting results have been obtained (Kemp et al., 1986; Smith et al., 1989; Latif et al., 1991; Bechara et al., 2000; Piper et al., 2010; Prudencio et al., 2011; Marufu et al., 2013).

1.4.3. Cytological studies

1.4.3.1. Identification of immune cell subtypes via surface markers at the site of tick attachment

On a cellular level, two potential cell subtypes have been identified across independent studies. Markers used for the identification of γδ T-lymphocytes were found to be present in higher levels in resistant compared to susceptible animals (Constantinoiu et al., 2010; Franzin et al., 2017). Gamma delta T-cells are suggested to function as regulatory T-cells in bovines (Hoek et al., 2009).

The expression of CD3+ T-lymphocytes was increased over time in *B. t. indicus* cattle (compared to *B. t. taurus*) in two studies (Constantinoiu et al., 2010; Franzin et al., 2017). This could be explained by different infestation protocols. Constantinoiu et al. (2010) made use of naïve cattle which were infested weekly with *R. microplus* larvae. Samples were taken at 1 day, one, 3 and 7 weeks post-primary infestation. CD3+ T-lymphocytes were found to be more abundant in resistant cattle at 1 day and at 3 weeks. In contrast, Franzin et al. (2017) used naïve cattle, which were challenged only once. Samples were taken at 2 and 9 days post infestation and significantly higher CD3+ T-lymphocyte levels were found in resistant compared to susceptible animals for the later sampling timepoint only. Based on the above studies and the importance of CD3+ T-lymphocytes in innate and adaptive immune responses, these cells are likely involved in the tick-resistance mechanism.

1.4.3.2. Neutrophils at the site of tick attachment

Although an increase in neutrophil levels at the site of tick attachment is well described and has been related to the number of previous tick exposures (Allen et al., 1977; Binta and Cunningham, 1984; Brown et al., 1984; Gill, 1986; Walker and Fletcher, 1986), no differences have been reported from studies investigating the association of neutrophils to tick resistance. This led to the hypothesis that this cell type is not linked to the tick-resistance mechanism. Latif et al. (1991) found a decrease in the infiltration of neutrophils in less resistant (*B. t. indicus* and *B. t. taurus*) animals when compared to resistant Zebu cattle infested with *Rhipicephalus appendiculatus* nymphs. In contrast, no differences for cattle infested with *Amblyomma variegatum* nymphs (comparing resistant and more susceptible Zebu animals) were found. Furthermore, no differences in the number of neutrophils at the site of adult *R. microplus* attachment could be observed between resistant and susceptible breeds by Carvalho et al. (2010). The same results were obtained by Marufu et al. (2014) for naturally infested cattle. It is evident that there is an increase in the number of neutrophils upon larvae and adult tick infestation, where equal numbers of this cell subtype were found to be present irrespective of the resistance classification of the host (Carvalho et al., 2010; Franzin et al., 2017). Although the reaction of neutrophils upon larval maturation to nymphs presents with conflicting results (Latif et al., 1991; Franzin et al., 2017), the lack of differential levels of this granulocyte seen in the larvae and adult life stage may support the hypothesis that this cell subtype remains unchanged across the tick life cycle.

1.4.3.3. Basophils at the site of tick attachment

The fluctuation in the number of basophils between tick-resistant and tick-susceptible cattle breeds infested with either one- or multi-host ticks have been studied. Interestingly, Latif et al. (1991) again identified a potential variation in host immune responses to different multi-host tick species. At *R. appendiculatus* nymph attachment sites, significantly fewer basophils were present in more susceptible compared to resistant cattle within and between breeds. In the same study, no significant differences in the number of basophils were identified between tick-susceptible and tick-resistant cattle within the *B. t. indicus* breed at the sites of *A. variegatum* nymph attachment. Yet, cattle of intermediate resistance showed the highest numbers of basophils. This can be

explained by the study layout as the cattle group of lower resistance had previous exposure to much lower *R. appendiculatus* numbers compared to *A. variegatum*. Therefore, animals could be presenting with a higher resistance level against the latter species and thus account for observed discrepancies.

In the one-host tick, *R. microplus*, basophil infiltration levels were also found to vary among cattle of varying resistance in response to tick infestation (Figure 1.1). Overall, basophil numbers at the site of adult tick attachment have been shown to be more abundant at tick attachment sites in resistant cattle than in their susceptible counterpart (Carvalho et al., 2010). Similarly, naturally infested cattle were found to have differing levels of basophils at the site of adult female *R. microplus* attachment, with tick counts negatively correlating with basophil counts (Marufu et al., 2014). The finding that basophil counts increase at the site of tick attachment in cattle was further corroborated by Franzin et al. (2017). This study showed that not only did both tick-resistant and tick-susceptible cattle recruit basophils at the site of *R. microplus* infestation, but also that upon maturation of *R. microplus* larvae to their nymph life stage, significantly more basophils were found to be present in resistant compared to susceptible hosts.

In summary, upon tick attachment basophil levels seem to increase in all cattle. The rate and level of increase is however dependent on the number of previous tick infestations and the level of tick resistance in the respective cattle breed. An increase in the number of infestations of a less susceptible breed to multi-host adult tick species not only showed an association with increased time taken to recruit basophils, but also increased number of basophils compared to previous tick infestations (Allen et al., 1977; Brown et al., 1984; Walker and Fletcher, 1986). Increased levels of this cell subtype in the nymph and adult life stages were found to be higher in resistant animals, with no difference identified between cattle breeds infested with tick larvae (Latif et al., 1991; Carvalho et al., 2010; Marufu et al., 2014; Franzin et al., 2017). These results suggest that it is not necessarily the difference in immune response pathway between cattle breeds that play a part in resistance but rather the level and reaction time of such immune responses.

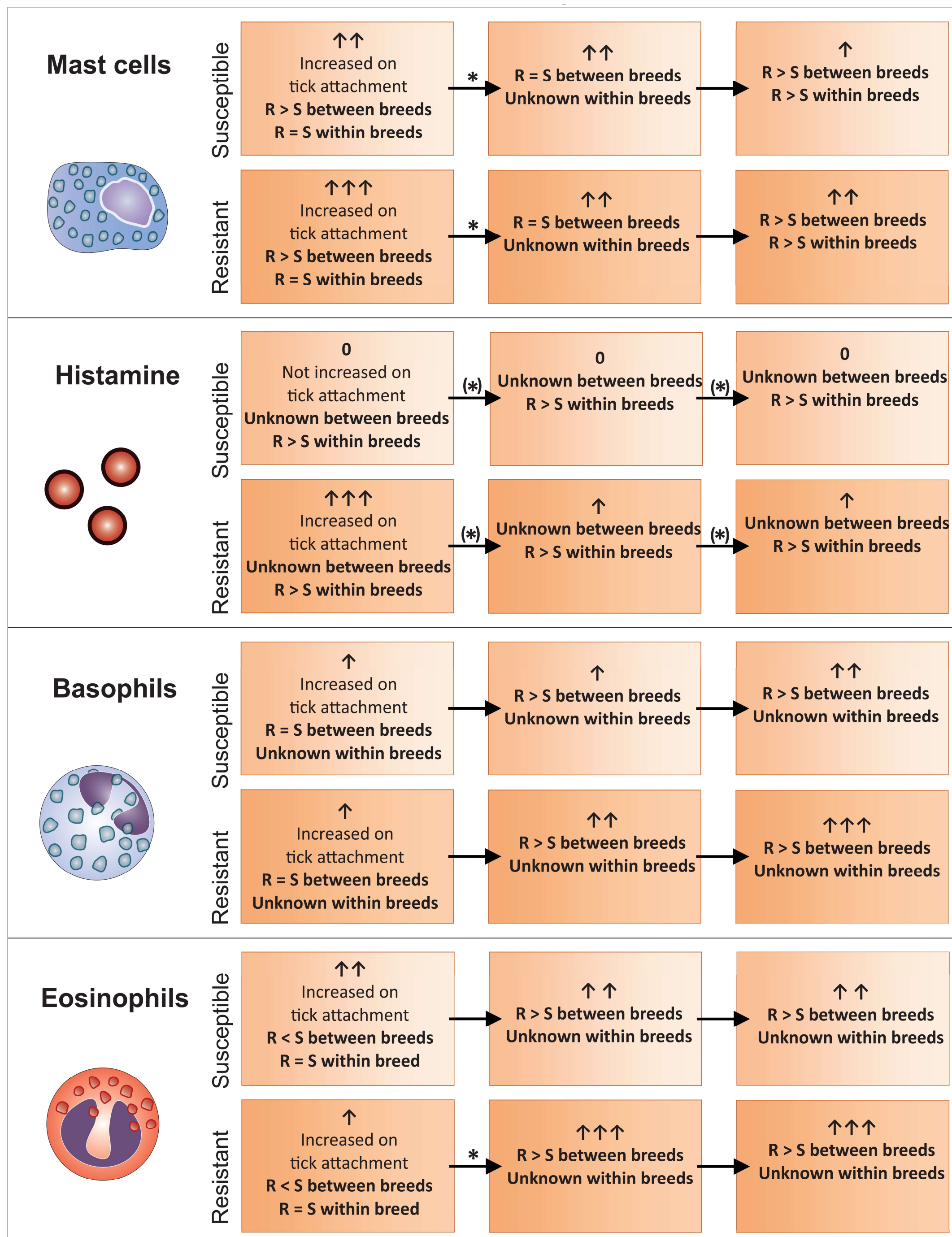

Figure 1.1: Mast cell, basophil, and eosinophil levels identified in the skin of cattle (sections on basophils (1.4.3.3), eosinophils (1.4.3.4) and mast cells (1.4.3.5) at the site of tick attachment) with different tick-resistance status and their correlation with blood (section 1.3.2: translational studies in the blood of tick-infested cattle) and skin (section 1.4.2: translational studies and metabolites in the skin of tick-infested cattle) histamine levels (based on literature investigating changes as result of *R. microplus* infestation). Granulocyte and histamine levels are indicated per tick life stage with arrows. An asterix indicates that a specific cell subtype dynamic between life stages was confirmed by literature as per relevant skin and blood section. Where dynamics were obtained from within breed comparisons the asterix was placed in brackets. Resistant and susceptible animals are abbreviated with “R” and “S”, respectively. Graphical representations correspond to the key of Figure 1.2.

1.4.3.4. Eosinophils at the site of tick attachment

1.4.3.4.1. Between breed comparisons of eosinophil levels

Altered patterns of eosinophil regulation were identified across different tick life stages and will hence be discussed accordingly. Three conclusions can be drawn regarding the attachment of larvae on cattle. Firstly, upon tick larvae attachment, there was an overall increase in the number of eosinophils at the site of tick attachment in all cattle breeds. Susceptible cattle did, however, display a higher influx of eosinophils compared to their tick-resistant counterparts (Figure 1.1; Moorhouse and Tatchell, 1969; Piper et al., 2010; Franzin et al., 2017). Secondly, the infestation history of the host does not seem to play an important role regarding the levels of eosinophils at the larval life stage. This is supported by the observation that a higher influx of eosinophils to the attachment site occurs in susceptible breeds in naïve cattle as well as in cattle that have been repeatedly infested (Piper et al., 2010; Franzin et al., 2017). Lastly, differences are observed amongst larval infestation using different tick species. In the case of Moorhouse and Tatchell (1969) it was found that hours after the attachment of *R. microplus* larvae to cattle (with previous tick exposure), susceptible cattle presented with a greater number of eosinophils. In contrast, no difference in the influx of eosinophils was observed between resistant and susceptible cattle breeds in response to infestation with the multi-host tick, *Haemaphysalis longicornis*.

With regards to nymph infestation, Franzin et al. (2017) showed that a reversal of the larval eosinophil response is observed, where upon maturation of tick larvae to nymphs, a greater number of eosinophils occur in resistant breeds (Figure 1.1). The same trend seen during the nymph life stage continues into the adult life stage (Figure 1.1). This has been confirmed in studies using Shorthorn-Zebu vs. Shorthorn (Riek, 1962) and Nelore vs. Holstein-Friesian (Carvalho et al., 2010) cattle infested with adult *R. microplus*. One study did, however, not confirm this observation. Marufu et al. (2014) identified that the more susceptible Bonsmara (*B. t. afrikanus*) cattle displayed higher eosinophil levels compared to that of the resistant Nguni (*B. t. indicus*) cattle breed. To date, it is unknown what the cause of this discrepancy could be.

1.4.3.4.2. Within breed comparisons of eosinophil levels

In contrast to studies between cattle breeds, no difference in eosinophil levels at the tick larval life stage was found between animals of the same breed (Figure 1.1). Schleger et al. (1976) showed that in *B. t. taurus* infested with *R. microplus* larvae, similar numbers of eosinophils were present between animals of varying resistance. However, eosinophils were more localized to the site of tick attachment in more resistant cattle (Schleger et al., 1976). This is in contrast to what is seen in blood, where eosinophil levels are significantly higher in susceptible cattle, even under conditions of natural infestation (Rechav et al., 1990).

Regarding nymph infestation, differences in the response were observed with regards to what tick species the cattle were infested with. In a study by Latif et al. (1991) Zebu cattle infested with *A. variegatum* have a greater influx of eosinophils as opposed to cattle infested with *R. appendiculatus*. However, upon conducting intra-breed comparisons, Zebu cattle that displayed resistance to *R. appendiculatus* had lower eosinophil levels while no difference in eosinophil

levels could be detected in *A. variegatum* susceptible or resistant animals. The latter observation could, however, be due to the presence of higher *A. variegatum* tick numbers compared to *R. appendiculatus* before the commencement of this study. As such, the tick species effect on eosinophil biology remains to be validated.

Studies focusing on the effects of multiple infestations (independent of the host resistance status), indicated that eosinophil and degranulation levels progressively increase with the number of infestations with Hyalomma anatolicum anatolicum (Gill, 1986) and R. appendiculatus (Walker and Fletcher, 1986) adults. In contrast, Allen et al. (1977) showed that all *B. t. taurus* cattle infested with adult *Ixodes holocyclus* had increased eosinophil levels, irrespective of whether the animals were previously infested or not. However, the latter study should be confirmed due to the low numbers of biological repeats per cattle group and low tick numbers used.

Discrepancies observed for within a single to between different cattle breeds infested with tick larvae could indicate that eosinophils play different roles in the resistance mechanism in genetically more resistant breeds compared to acquired resistance within breeds. Since studies looking at the changes in eosinophil levels within breeds have mainly focused on *B. t. taurus* cattle, more studies should investigate changes in *B. t. indicus* animals of various resistance.

1.4.3.5. Mast cells at the site of tick attachment

Differences in mast cell numbers have been related to the tick life stage and differences have furthermore been found when comparing results from within and between breed studies. Upon *R. microplus* larvae attachment, there is an increase in the number of mast cells at the site of tick attachment in all cattle (Figure 1.1). This increase is intensified in more tick-resistant cattle (*B. t. indicus*) when compared to more susceptible animals (*B. t. indicus*) (Franzin et al., 2017) which was not seen in a study investigating effects within a *B. t. taurus* breed (Schleger et al., 1976; Figure 1.1).

Yet, upon maturation to nymphs, the number of mast cells are similar for both resistant and susceptible animals while a significant decrease in the number of mast cells in more resistant hosts is seen in cattle infested with nymphs compared to larvae (Figure 1.1; Franzin et al., 2017). Similarly, no significant changes in the number of mast cells in the skin of resistant and more susceptible animals infested with *R. appendiculatus* or *A. variegatum* nymphs was found between and within cattle breeds except for a suggested decrease of cells in less resistant animals within the Zebu breed (Latif et al., 1991).

When ticks reached the adult life stage, a greater number of mast cells at the site of tick attachment in more resistant cattle was observed in all studies (Figure 1.1; Engracia Filho et al., 2006; Veríssimo et al., 2008; Marufu et al., 2014). In a study by Engracia Filho et al. (2006), Gyr x Holstein cattle were grouped into resistant and susceptible groups based on previous infestations. Upon adult attachment of *R. microplus* it was shown that the number of mast cells in the more resistant group was greater than in the more susceptible group (Engracia Filho et al., 2006). Furthermore, this was confirmed in a similar study using a wider range of cattle breeds and resistance groups including Nelore, Holstein-Friesian, Brown, Gyr and crossbred animals which showed that in the upper dermis of *R. microplus* adult-infested cattle skin there was a

negative correlation between the number of ticks on the animals and the number of mast cells present (Veríssimo et al., 2008). Both tick-susceptible and more tick-resistant cattle skin naturally infested with *R. microplus* adults also showed a negative relationship between tick counts and mast cell numbers (Marufu et al., 2014).

In addition, investigation of the skin of *B. t. taurus* cattle infested with adult *H. a. anatolicum* suggested that irrespective of previous exposure to ticks, there is a negative correlation between the number of mast cells in the skin of cattle and the number of ticks attached to these animals (Gill, 1986). An increased number of mast cells was found at the tick bite lesion of tertiary as compared to primary infested animals. Additionally, degranulation of mast cells was seen in tertiary infested animals as opposed to naïve cattle. Allen et al. (1977) showed that in the case of adult *I. holocyclus* attachment on European cattle breeds, the number of mast cells in the skin increased upon tick attachment irrespective of previous exposure. It was also shown that mast cell infiltration and mast cell degranulation increased in previously exposed cattle as opposed to naïve cattle (Allen et al., 1977). In contrast, to the above results, *B. t. taurus* cattle infested multiple times with adult *R. appendiculatus* showed a decrease of mast cells at the tick attachment site (Walker and Fletcher, 1986).

In summary, as for the dynamic of the eosinophil cell subtype, differences in results were seen within and between cattle breeds. Mast cells were found to be at similar levels in susceptible and resistant cattle within a cattle breed. While between breeds, resistant cattle showed higher mast cell levels for the larval life stage (Schleger et al., 1976; Franzin et al., 2017). Similar results were obtained for studies investigating tick nymph and adult life stages between breeds.

1.5. Dynamics of granulocytes and histamine and their suggested involvement in the tick-resistance mechanism over the tick lifecycle

Changes in histamine and cell infiltration patterns over the life cycle of *R. microplus* stress the importance of taking the dynamics of cellular changes in response to the maturing tick into account when planning a study (Figure 1.1). In the case of histamine, it is increased in the tick larval life stage (in resistant animals) pointing towards it acting as an effector molecule within cattle breeds. In addition, we hypothesise that histamine is increased in resistant animals throughout all tick life stages based on four observations. Firstly, a study comparing susceptible and intermediate-resistant cattle identified higher blood histamine levels throughout the tick life cycle for the latter group within a single cattle breed (Riek, 1962). Secondly, higher histamine levels were found at the site of larval attachment of more resistant cattle within the same breed (Willadsen et al., 1979; Schleger et al., 1981). Thirdly, resistant breeds have equal or higher basophil (Carvalho et al., 2010; Marufu et al., 2014; Franzin et al., 2017); and mast cell (Schleger et al., 1976; Engracia Filho et al., 2006; Marufu et al., 2014; Franzin et al., 2017) levels throughout all life stages compared to susceptible breeds. Lastly, as histamine can be released from mast cells as well as basophils via an IgE and/or eosinophil-dependent mechanism, the presence of these cells correlate with the increase in histamine observed (Ishizaka et al., 1972; Zheutlin et al., 1984; Janeway et al., 2001; Galli et al., 2005; Stone et al., 2010) (Figure 1.2).

1.5.1. Comparison of findings obtained within a single cattle breed

Although no differences were observed for mast cell and eosinophil levels at the larval life stage, histamine levels were found to be higher in resistant compared to susceptible animals (Figure 1.1) (Riek, 1962; Willadsen et al., 1979; Schleger et al., 1981). Potentially, histamine is thus released via basophils; however, no study up to date has extensively investigated this cell subtype within a single breed. It should be noted that Riek (1962) only investigated the dynamics of this compound during the larval life stage in a limited study using only one highly resistant animal. Higher histamine levels upon nymph and adult tick attachment in blood were also identified when comparing medium resistant to susceptible cattle (Figure 1.1; Riek, 1962). If histamine levels were linked to tick numbers as result of tissue damage, it would be expected that higher histamine levels are present in more susceptible animals which is not the case. This indicates that histamine could be involved in the tick-resistance mechanism within cattle breeds. Since not enough data regarding the number of granulocytes are available to date for the nymph and adult life stages, it cannot be hypothesised by which cell subtype histamine may be released. However, increased mast cell numbers in resistant compared to susceptible animals at the adult life stage could indicate a delay in mast cell dependent release of histamine as seen for interbreed comparisons at the larval life stage (as discussed in the next section: Comparison of findings obtained between different cattle breeds).

1.5.2. Comparison of findings obtained between different cattle breeds

Although mast cells increased upon larvae attachment for all cattle breeds evaluated, this cell subtype was found to be more abundant in resistant breeds (Figure 1.1) (Franzin et al., 2017). This, together with the equal levels of basophils in both resistant and susceptible cattle at the tick larval life stage (Figure 1.1; Franzin et al., 2017), indicates that histamine might be increased as a result of mast cell degranulation and contributes to the first line of tick defence. The higher eosinophil levels in susceptible compared to resistant cattle breeds at the larval life stage (Figure 1.1; Moorhouse and Tatchell, 1969; Piper et al., 2010; Franzin et al., 2017) furthermore suggests that the immune response in susceptible cattle increases histamine levels via an eosinophil-dependent mechanism. This mechanism might be less efficient and slower in susceptible cattle due to its involvement in late-phase reactions (Piliponsky et al., 2001). Mast cell levels in the nymph life stage were found to be present at similar levels in resistant and susceptible breeds (Latif et al., 1991; Franzin et al., 2017), while a relatively higher number of mast cells was seen in more tick-resistant cattle in the adult tick life stage (Figure 1.1). This could be a result of a decrease of this cell subtype in susceptible and not an increase in resistant animals. The apparent decline in the number of mast cells that was observed in the more resistant cattle breed from the larvae to the nymph life stage may thus be delayed and occurring in the more susceptible cattle at the adult life stage. Since the tick-resistance mechanism in *B. t. indicus* animals results in a generally faster response to tick infestation compared to *B. t. taurus* cattle (Riek, 1962; Wagland, 1978, 1980; Rechav et al., 1990), resistance within susceptible cattle breeds might be achieved through a delayed histamine release via basophils. This mechanism is suggested to occur at the nymph life stage in resistant cattle breeds (Figure 1.1). To specifically elucidate this resistance mechanism in-depth, especially granulocyte levels for animals within a breed, presenting with varying tick-resistance phenotypes, need to be determined throughout the tick life cycle.

Additionally, investigation of histamine within and between cattle breeds at all tick life stages are essential.

1.6. Future directions: potential drivers involved in tick resistance

This integrative discussion will give an evaluation of key role players investigated up to date to establish a global view of components potentially involved (directly or indirectly) in the tick-resistance mechanism. It must be kept in mind that some observed immune responses may be a by-product of the effector pathway/molecule or a response to tick infestation without any involvement in the actual resistance mechanism. For example, gene expression results and actual dynamics occurring on protein level often do not correlate due to post-transcriptional, posttranslational and degradation regulation (Vogel and Marcotte, 2012). Therefore, results from studies employing gene expression analysis were only included as part of this discussion if their findings have been validated by a second study. Furthermore, since gamma globulin levels, apart from IgE, were generally increased in susceptible cattle, this could be linked to elevated tick numbers. Even though antibody specificity could be a contributing factor, these molecules were thus not included as key role players in this section as evidence remains non-conclusive. Lastly, due to a constant dynamic of immune molecules, identified markers on translational and cellular levels were included if a significant difference (irrespective of the direction) was seen between animals with more and less resistance status. Figure 1.2 summarizes potential role players and their possible interactions driving resistance, based on findings up to date with the discussion providing an integrative explanation of identified immune marker interactions of the respective components supported by literature. Indicated with an asterix (*) in the text below and in Figure 1.2, are components that have not yet been identified to be linked to tick resistance/susceptibility in cattle.

During the process of tick attachment, the skin of the host is damaged/pierced, and tick saliva is exposed to sentinel cells, such as granulocytes. Several acute-phase proteins have been shown to be involved at the site of tick attachment (Carvalho et al., 2008). These proteins can be linked to granulocyte (Quaye, 2008; Stone et al., 2010; Eklund et al., 2012) and monocyte (Hochepied et al., 2003) recruitment and/or activation. Furthermore, tick secreted allergens can cross-link to IgE (Galli and Tsai, 2012) and binding of IgE to its high-affinity receptor (FcεRI) on dermal mast cells (and basophils) has been shown to lead to the release of inflammatory mediators (Stone et al., 2010) such as histamine (Galli et al., 2005).

Following activation, mast cells readily secrete IL-5*, IL-13*, and TNFα (Janeway et al., 2001; Stone et al., 2010). In addition, IgE binding leads to the enhancement of CCL2 (monocyte chemoattractant protein 1) transcription that promotes the migration of monocytes (Oliveira and Lukacs, 2001) and T-cells (Oliveira and Lukacs, 2003) to amplify the local inflammatory reaction. Lastly, due to the antigen-presenting nature of mast cells, following the uptake of the IgE-antigen complex, the allergen is presented on mast cells on MHC II, which in turn interacts directly with T-cell receptors (containing CD3) and induces antigen-specific clonal expansion of T-cell populations (Mekori and Metcalfe, 1999; Henz et al., 2001).

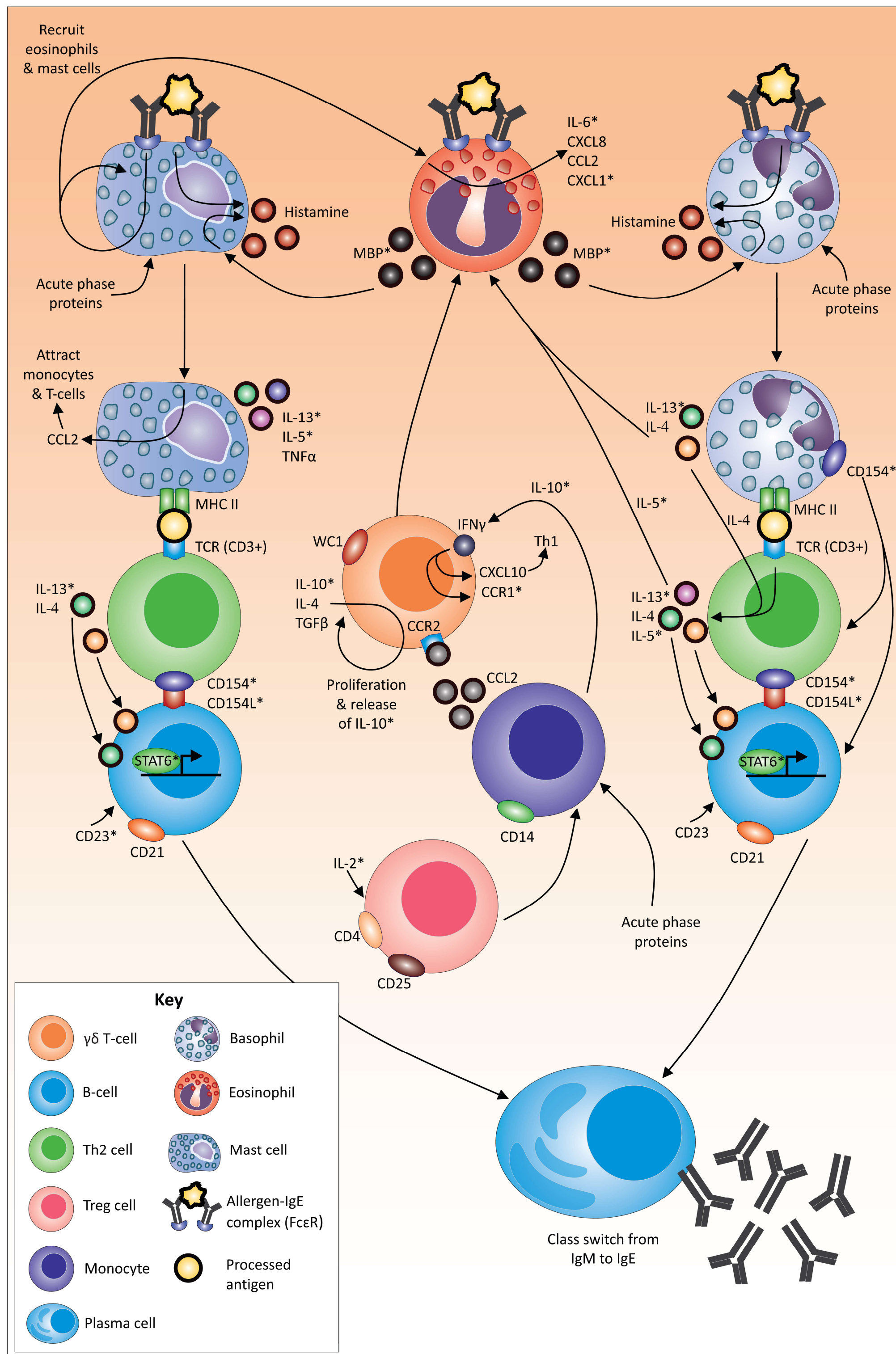

Figure 1.2: Proposed mechanism, role players and associated pathways in the tick-resistance mechanism. An asterix (*) indicates immune molecules that have not yet been identified to be linked to tick resistance/susceptibility in cattle (based on chosen exclusion criteria indicated in text). Molecules without an asterix have been linked to tick resistance/susceptibility in literature (as per relevant skin and blood section). Arrows indicate a direct or indirect link between molecules.

Basophils also function as antigen-presenting cells in response to certain allergens (Sokol et al., 2008, 2009). The binding of the IgE-allergen complex to FcεRI on basophils activates several pathways in the cell resulting in the release of histamine (Ishizaka et al., 1972) and the expression of IL-4 and IL-13* (Stone et al., 2010). These cytokines are important for the promotion of eosinophil trafficking (Stone et al., 2010) and are also secreted by Th2 cells in response to the presentation of allergen via MHC II and IL-4 production (Perrigoue et al., 2009; Yoshimoto et al., 2009) Activated Th2 cells also secrete cytokines (e.g., IL-5*) which increases eosinophil production (Janeway et al., 2001).

Antigen presentation to Th2 lymphocytes by mast cells and/or basophils, provide two essential signals for isotype switching. The first signal is IL-4 and/or IL-13* which bind to the respective receptors on B-cells and activate transcription at the IgE isotype-specific site via STAT6 (Stone et al., 2010). The second signal involves the binding of CD40L (CD154L*) to the relevant T-cell receptors, which in turn activates DNA switch recombination (Stone et al., 2010). Basophils express high levels of CD154* after activation and have been suggested to play a role in polyclonal amplification of IgE production and in the differentiation of Th2 cells (Stone et al., 2010). In addition, the binding of CD23* to CD21+ B-cells may participate in the control of IgE production (Aubry et al., 1992).

Histamine can also be released from mast cells and basophils via an IgE-independent mechanism (Siraganian and Hook, 1976; Piliponsky et al., 2001) utilising the major basic protein* released from eosinophils (Zheutlin et al., 1984; Janeway et al., 2001). The binding of the allergen-IgE complex to mast cells is suggested to drive the recruitment and activation of additional mast cells and eosinophils (Wong et al., 2009). Mast cells can also induce the release of IL- 6*, CXCL8, CCL2 and CXCL1* by eosinophils (Wong et al., 2009).

The development of eosinophilic allergic inflammation and the initiation of Th2-responses is regulated by a T-cell subtype (Zuany-Amorim et al., 1998). Regulatory T-cells are generally known for their ability to suppress putative deleterious activities of Th cells (Corthay, 2009), with IL-2* playing an important role in the survival and proliferation of CD4+CD25+ regulatory T-cells (Létourneau et al., 2009). The exact role of CD4+CD25+ regulatory T-cells in bovines is, however, unknown. It has been proposed that this cell population is neither anergic nor suppressive in cattle, and that their function(s) can to be linked to γδ T-cells (WC1.1+, WC1.2+) (Hoek et al., 2009). Together with the latter cell type, CD14+ monocytes have been linked to immune suppression in ruminants (Hoek et al., 2009). In addition, γδ T-cells bearing the lineage marker WC1 are associated with the production of the proinflammatory cytokine IFNg (Rogers et al., 2005), that furthers the action of CXCL10 (an INFg inducible protein, which recruits activated Th1 cells to the site of inflammation) (Dufour et al., 2002). Interferon gamma (Zella et al., 1998) as well as other cytokines such as IL-10* on monocytes (Loetscher et al., 1996; Sozzani et al., 1998) can mediate up-regulation of *CCR1** expression. The release of IL-10*, IL-4 and TGFβ can result in the proliferation of subsets of γδ T-cells (Guzman et al., 2014). The accumulation of γδ T-lymphocytes during allergic inflammation in turn is orchestrated by the CCR2/CCL2 pathway (Costa et al., 2009; de Oliveira Henriques and Penido, 2012).

Several immune markers associated with the above pathways, such as histamine, have been identified in section 1.3 and 1.4 (Figure 1.2). To date, histamine levels in the blood and skin were found to be increased in resistant cattle, while little variation was seen in susceptible animals

(Riek, 1962; Willadsen et al., 1979; Schleger et al., 1981). Degranulation of both mast cells (Riley, 1953; Mota et al., 1954) and basophils (Pruzansky and Patterson, 1970) is followed by the subsequent release of histamine in an immediate hypersensitivity reaction (Ishizaka et al., 1970, 1972). This reaction is well known to be linked to more frequent and intense grooming (O'Mahony et al., 2011), which was identified to play a role in tick-resistance (Riek, 1956; Snowball, 1956). Future studies are now required to elucidate these predicted pathways in-depth, focusing on likely markers not yet investigated (*) and molecular mechanisms/molecules that have resulted in contradicting findings up to date. Since opposing findings could be linked to varying study time-points, a dynamic investigation of all potential role players would be valuable and could lead to a better-defined picture of occurrences.

1.7. Critical evaluation and concluding remarks

To date, few immune markers have been investigated with sufficient depth to obtain a picture of events involved in the cattle tick-resistance mechanism. Especially large-scale transcriptome studies have identified various components with hardly any overlap of results between studies. One reason for this is the differences in experimental designs which can drastically influence the occurrence of immune events depicted at the chosen time point corresponding to a specific tick life stage(s). In addition, potential markers or pathways should not only be studied on a molecular level but also on a cellular level. The clearest pictures, as obtained for histamine, granulocytes and gamma globulin levels, were identified using markers on both translational and cellular level. In this regard, some immune markers seem to differ for the resistance mechanism within a host breed compared to between breeds as well as between tick life stages (see section 1.5: Dynamics of granulocytes and histamine and their suggested involvement in the tick-resistance mechanism over the tick lifecycle and Figure 1.1). It should be noted that even though the final effector molecules might be the same, different pathways are possibly involved in establishing this mechanism (see section 1.6: Future directions: potential drivers involved in tick resistance). Several experimental factors should thus be considered when comparing experiments as some of these parameters can potentially skew results and lead to contradicting findings if not addressed correctly (Table 1.1). This includes important aspects and potential solutions regarding (1) factors relating to the selection and treatment of host animals, (2) factors relating to infestation and sampling protocols, (3) selection of immune markers and (4) data analysis and interpretation.

Table 1.1: Problem identification and potential solutions for studies evaluating the interplay of cattle immune responses to tick infestation.

Problem statement/explanation	Possible solutions and future guidelines
Factors relating to the selection and treatment of host animals	
Extrapolation of findings between different host species (e.g., rodents and bovines).	A rodent model can be used to provide hypotheses, as numerous validated immune markers are available for murine models. However, a significant amount of results cannot be directly extrapolated from murine to bovine hosts and should therefore be confirmed in the appropriate host species to validate an immune response.
Not considering intra-breed differences (range of tick-resistance status between individual hosts within a breed).	Cattle should be sourced from registered breeders to limit genetic differences to a minimum. Additionally, the resistance status of each animal needs to be taken into account when analysing data and confirmed prior to the start of a study.
Lack of patient history of experimental animals.	Information on animal source, age and previous exposure(s) to ticks should be provided.
Reporting prophylactic treatments of host animals upon arrival and health status throughout study.	Upon arrival, all treatments should be reported with special emphasis on acaricide treatments and prophylactic treatments (antibiotics for infections, deworming strategies), as all of these influence immunity. General health parameters such as weight, temperature and hematocrits need to be reported as bovine studies are rarely conducted under biosafety level standards.
Factors relating to infestation and sampling protocols	
Comparison of immune responses between animals with different tick attachment efficiencies and thus tick numbers at the respective sampling time-points.	Comparing immune responses of susceptible and resistant hosts infested with the same number of ticks (especially at nymph and adult life stages) would allow for a better understanding of which mechanism is at play at which life stage.
Protocol for tick infestation and evaluation of specific tick life stage(s).	Biological question should take into account infestation protocol, as multiple infestations results in numerous life stages being present on a single animal that will bias detection of a life stage specific response.
Focus on between animal comparisons at specific time-points instead of immune dynamics.	Focus should be placed on the progression of immune responses within breeds and then determine between breed differences.
Choice of biological sample for analyses.	To date, skin and blood have been studied extensively. Insights from secondary lymphoid organs are in dire need to fully understand tick-mediated immune suppression as well as factors underlying tick resistance.
Selection of immune markers	
Translation of findings to all cellular levels.	Gene expression profiling studies should be validated using protein and/or cellular markers. Genotyping studies should take into account cellular immune markers to link genotypes and phenotypes.
Most immune markers are not confirmed as cross-reactive to specific cell subpopulations in bovines or are lacking.	Immune markers must be confirmed to be cross-reactive to a specific cell subpopulation.
Data analysis and interpretation	
Comparison between studies investigating different tick species (e.g. one-host vs. multi-host ticks).	Transcriptome analysis across ixodid tick species support differences in proteins being present in a specific species. Therefore, cross-species comparisons should be carefully considered.
Comparison between studies investigating different tick life stages.	A unique set of proteins/molecules can be secreted by each life stage of a tick species, with a unique subset of immune cells affected/targeted (e.g., see section on granulocytes).

To date, the only partially clear picture of immune events involved in the tick-resistance mechanism involves histamine and associated cell subtypes and molecules (Figures 1.1, 1.2). This pathway, with its effector molecules and modes of action, still requires additional in-depth investigation before focus is placed on the identification of other contributing mechanisms. The identified cell dynamics between tick life stages furthermore suggest that future studies should concentrate on the dynamics of immune responses at various time-points over the complete tick life cycle. This would reduce between study variations in addition to obtaining a temporal overview of events. In general, it is advisable that experiments are standardized as much as possible and more focus should lie on an in-depth investigation of markers/pathways across genetic, translational and cellular levels to successfully validate a response. Only then can one attempt to consolidate all information into a feasible blue print for the identification of major drivers underlying the bovine immune mechanism driving tick-resistance and subsequent postulation of viable and effective tick control strategies.

1.8. Aims and objectives of the study

Chapter 2 Aim: Comparison of the differential regulation of T and B-lymphocyte subsets in the skin and lymph nodes amongst three cattle breeds as potential mediators of immune-resistance to *Rhipicephalus microplus*

Objectives:

a) Compare the T and B-lymphocytes populations from skin of three cattle breeds (Brahman, Bonsmara and Holstein-Friesian) in response to immature and adult *R. microplus* tick infestation.

b) Compare the T and B-lymphocytes populations from lymph nodes of these cattle breeds in response to immature and adult *R. microplus* tick infestation.

Chapter 3 Aim: Temporal analysis of the bovine lymph node transcriptome during cattle tick (*Rhipicephalus microplus*) infestation

Objectives:

a) Identify the transcriptional response in regional draining lymph nodes from three cattle breeds (Brahman, Bonsmara and Holstein-Friesian) in response to immature and adult *R. microplus* tick infestation.

b) Compare the differentially expressed genes common among the three cattle breeds.

c) Identify key components and possible immune pathways for the Bonsmara cattle breed in response to tick infestation.

Chapter 4 Aim: *In vivo* evaluation of Ixodes induced effects altering T and B-cell maturation in the spleen and lymph nodes of BALB/c mice

Objectives:

a) Characterise the *in vivo* immune response of tick-naïve and immunised BALB/c mice infested with *Ixodes ricinus* nymphs.

b) Characterise the *in vivo* B-cell lineages of tick-naïve and immunised BALB/c mice infested with *I. ricinus* nymphs.

c) Characterise the *in vivo* T-cell lineages of tick-naïve and immunised BALB/c mice infested with *I. ricinus* nymphs.

1.9. Outputs from this study

Publications

Luïse Robbertse, Sabine A. Richards, Christine Maritz-Olivier (2017). Bovine Immune Factors Underlying Tick Resistance: Integration and Future Directions. Frontiers in Cellular and Infection Microbiology. doi: 10.3389/fcimb.2017.00522

Luïse Robbertse, Sabine A. Richards, Sarah J. Clift, Annette-Christi Barnard, Andrew Leisewitz, Jan E. Crafford, Christine Maritz-Olivier (2018). Comparison of the differential regulation of T and B-lymphocyte subsets in the skin and lymph nodes amongst three cattle breeds as potential mediators of immune-resistance to *Rhipicephalus microplus*. https://doi.org/10.1016/j.ttbdis.2018.03.034

Luïse Robbertse, Christian Stutzer, Sabine A Richards, Andrew L Leisewitz, Jan E Crafford, Christine Maritz-Olivier. Temporal analysis of the bovine lymph node transcriptome during cattle tick (*Rhipicephalus microplus*) infestation. To be submitted to PLOS Neglected Tropical Diseases.

Luïse Robbertse, Christian Stutzer, Marie Jalovecká, Christine Maritz-Olivier. *In vivo* evaluation of Ixodes induced mediated effects altering T and B-cell maturation in the spleen and lymph nodes of BALB/c mice. To be submitted to Parasite Immunology.

Conference outputs

Comparative immunoprofiling of three cattle breeds (Holstein-Friesian, Bonsmara and Brahman) against *Rhipicephalus microplus* infestation using cell subtype quantification methods and transcriptional profiling. Oral presentation at the first Joint International Conference of the Association of Institutions for Tropical Veterinary Medicine (AITVM) and the Society of Tropical Veterinary Medicine (STVM) at the Humboldt Universitaet zu Berlin (2016).

1.10. Additional outputs

Publications

Luïse Robbertse, Samantha Baron, Nicolaas A van der Merwe, Maxime Madder, Wilhelm H Stoltsz, Christine Maritz-Olivier (2016). Genetic diversity, acaricide resistance status and evolutionary potential of a *Rhipicephalus microplus* population from a disease-controlled cattle farming area in South Africa. Ticks and Tick-borne Diseases. doi: 10.1016/j.ttbdis.2016.02.018.

International conference presentations

Rhipicephalus microplus: Acaricide resistance screening and phylogenetic analysis in the Mnisi area. Oral presentation at the Joint 8th International Ticks and Tick-borne Pathogens (TTP-8) and 12th Biennial Society for Tropical Veterinary Medicine (STVM) Conference, Cape Town, South Africa (2014).

Genetic diversity, acaricide resistance status and evolutionary potential of a *Rhipicephalus microplus* population from a disease-controlled cattle farming area in South Africa. Oral presentation at the first Joint International Conference of the Association of Institutions for Tropical Veterinary Medicine (AITVM) and the Society of Tropical Veterinary Medicine (STVM) at the Humboldt Universitaet zu Berlin (2016)

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Chapter 2: Comparison of the differential regulation of T and B-lymphocyte subsets in the skin and lymph nodes amongst three cattle breeds as potential mediators of immune-resistance to *Rhipicephalus microplus*

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This chapter has been published in Ticks and Tick-borne Diseases (https://doi.org/10.1016/j.ttbdis.2018.03.034). A copy of the published article is attached at the end of the thesis. Luïse Robbertse performed sample preparation, immunohistochemical staining optimisation, histopathological and immunohistochemistry analysis of samples, all related quantification and analyses of results. The aforementioned work was done in collaboration with Sarah Clift at the Department of Paraclinical Sciences (Section Pathology), Faculty of Veterinary Sciences, University of Pretoria, South Africa. Additional flow cytometric analysis was performed by Sabine Richards and Annette-Christi Barnard. Experimental work for the flow cytometry work was conducted by Sabine Richards and Annette-Christi Barnard. Further analysis and interpretation of results was conducted by Luïse Robbertse and Sabine Richards. Andrew Leisewitz and Jan Crafford were the trained veterinary personnel responsible for tissue collection and additional support in the project. Christine Maritz-Olivier provided advice, direction, critical revision and supervision in planning of the project. All other technical assistance is acknowledged at the end of the chapter.

2.1. Abstract

Although varying natural resistance to ticks between highly resistant Brahman (*Bos taurus indicus)*, resistant Bonsmara (5/8 *B. t. indicus* x 3/8 *B. t. taurus*) and susceptible Holstein-Friesian (*B. t. taurus*) breeds is documented in skin and blood, little information is available describing draining lymph nodes. To elucidate the cellular dynamics during *Rhipicephalus microplus* induced immune responses, this study analysed immune factors from these cattle breeds using histology, immunohistochemistry and flow cytometry. Following the collection of skin and lymph node samples before artificial tick infestation, cattle were infested with *R. microplus* larvae. Subsequent sampling coincided with the tick larvae (day 3) and adult (day 17) developmental stages. A significant influx of CD20+ B-lymphocytes in the dermis of all cattle breeds was observed while CD3+ T-lymphocytes were significantly increased in more tick-resistant breeds. Eosinophil infiltration in germinal centres of lymph nodes was significant for all cattle breeds while tingible body macrophages were significantly increased for adult-infested Brahman animals. A negligible fluctuation in CD20+ and CD79α+ B-lymphocyte numbers was present in the lymph node of more resistant cattle breeds, while susceptible animals showed a decrease in B-lymphocytes after infestation, followed by an increase between larvae to adult-infested time-points. Increased variability of γδ T-lymphocyte populations in lymph nodes was statistically significantly associated with tick susceptible cattle breeds. In addition, a more stable T-helper lymphocyte population was identified in the lymph nodes for the Brahman cattle breed. Results suggest the association of tick susceptibility with differential B-lymphocyte regulation in lymph node tissues, increased variability of WC1+ γδ T-lymphocyte populations in the lymph node as well as a decrease in T-helper lymphocytes in the lymph node.

Keywords: *Rhipicephalus microplus*, tick resistance, lymph node, skin, cattle, immune response.

2.2. Introduction

Globally, livestock production accounts for 40% of the total value of the agricultural industry with the bovine meat commodity being the largest contributor to this industry projected to increase by 1.4% annually (Bruinsma, 2003). One of the most important tick species negatively affecting this industry is the cattle tick, *Rhipicephalus microplus* (Jongejan and Uilenberg, 2004). In contrast to a number of other *Rhipicephalus* species, which have a two or three-host lifecycle, *R. microplus* completes its lifecycle on a single host. As such *R. microplus* has a higher fecundity that has been linked to its ability to outcompete other species, adapt more rapidly to different climatic zones (Adakal et al., 2013; Nyangiwe et al., 2013; Tonnesen et al., 2006) and to rapidly develop acaricide resistance (de la Fuente et al., 2000; Rajput et al., 2006; Rodriguez-Vivas et al., 2011). In addition to the previous points, *R. microplus* is a vector for *Anaplasma*, *Borrelia* and *Babesia* spp. (de la Fuente et al., 2000; Jongejan and Uilenberg, 2004; Lynen et al., 2007; Madder et al., 2011). It is therefore a priority to develop and implement effective control strategies to address the increasing threat by this species on livestock and related sectors.

Currently, acaricides are the most widely used control strategy employed against all tick species as limited success has been reported pertaining to the successful implementation of biological- and immunological control strategies (Ghosh et al., 2007). Understanding the basic differences underlying variation in cattle resistance to ticks may constitute the basis of improved tick control strategies in the future. Natural resistance to tick infestation varies between as well as within different cattle breeds (reviewed in: (Robbertse et al., 2017)). For example, *Bos taurus indicus* breeds are generally highly tick-resistant, compared to *Bos taurus taurus* cattle breeds.

Host immunity to tick infestation already starts at the site of attachment when the tick firmly attaches itself to the host skin. Physical (e.g. skin composition) as well as mechanical (e.g. grooming behaviour) mechanisms have been linked to tick resistance (Jonsson et al., 2014). Several studies have also provided some insight into the immunological factors influencing resistance to tick infestation. Pertaining to cattle hosts, most results to date point towards the involvement of histamine, granulocytes and their associated pathways (Carvalho et al., 2010; Engracia Filho et al., 2006; Franzin et al., 2017; Marufu et al., 2014; Riek, 1962; Schleger et al., 1976; Schleger et al., 1981; Willadsen et al., 1979). However, the role of other immune factors present in the skin is not clear (such as the role of B-lymphocytes) and requires further elucidation (Hannier et al., 2004).

Skin as a first line of defence against tick infestation plays an important role in tick resistance and is therefore well studied (Constantinoiu et al., 2013, 2010, Piper et al., 2010, 2009, 2008; Wang et al., 2007). Although secondary lymphoid organs are important in the development of immune responses, very few studies have focused on their involvement. This is particularly true for regional lymph nodes draining the cutaneous tick attachment sites.

A limitation with a number of studies (except for Franzin et al. (2017)) is that they (a) only analyse a single time point in the tick lifecycle and do not take into account the underlying temporal dynamics of immune responses; (b) draw conclusions by comparing findings between different host and tick species as well as tick life stages; (c) directly compare absolute values to one another between different bovine breeds without normalisation for their respective immune statuses at the onset of the study; (d) lack confirmation with *in vivo* proteomics approaches.

Therefore, in this study, we aimed to compare immune cell populations from skin and draining lymph nodes to investigate the effects of *R. microplus* infestation per life stage in different cattle breeds. These results were used to gain insight into factors underlying the resistance mechanisms between breeds pertaining specifically to this one-host tick. This study was designed to include three lymphocyte phenotype quantification methodologies (histology, immunohistochemistry (IHC) and flow cytometry) to investigate changes in skin and/or lymph node tissues of Brahman (*B. t. indicus*), Holstein-Friesian (*B. t. taurus*) and Bonsmara (a *B. t. indicus* and *B. t. taurus* cross; 5/8 Afrikander, 3/16 Shorthorn and 3/16 Hereford) cattle.

2.3. Materials and methods

2.3.1. Experimental animals

All procedures were carried out as approved by the Animal Ethics Committee at the University of Pretoria (project number: EC036-13) and the Department of Agriculture, Forestry and Fisheries South Africa according to the section 20 regulations. Three Holstein-Friesian (*B. t. taurus*), three Brahman (*B. t. indicus*) and three Bonsmara (*B. t. indicus* and *B. t. taurus* cross) calves, all approximately 9 months of age, were housed at the University of Pretoria Biomedical Research Centre (UPBRC). Calves used in this study had minimal previous exposure to ticks. Upon arrival (14 days before onset of the study) all of the calves were treated with: Toltrazuril 5% (3 ml/10 kg, oral dose, Bayer AH); Albendazole 7.5% (1 ml/10 kg, oral dose, Zoetis); Oxyteracycline (20 mg/kg intramuscular divided over two sites, Intervet SA); Diminazene (3.5 mg/kg subcutaneous Intervet SA) and sprayed with Amitraz 12.5% (100 ml/50 L water, Cooper) to ensure that cattle were free of ticks prior to the commencement of the study. A combination of patch feeding and whole-body infestation was used. Two patches were attached bilaterally to the shoulder of each animal to coincide with the region that is drained by the superficial cervical lymph nodes. In each of the patches 2,000 *Rhipicephalus microplus* larvae (obtained from Clinvet, South Africa) (weighed on site; 0.1 g) were placed and an additional 6,000 larvae (weighed on site; 0.3 g) were placed on each animal for whole-body infestation. Since this study aims to look at the response of different cattle breeds to the tick lifecycle and not to correlate results to the number of ticks attached to cattle, tick infestation was considered successful if at least 100 *R. microplus* adult female ticks were present. Semiengorged female *R.microplus* ticks between 4.5 and 8 mm were counted at day 17 post infestation.

2.3.2. Tissue collection

Skin and lymph node samples were taken at three time-points, including before artificial tick infestation (labelled as uninfested samples throughout the paper), three days post tick infestation (larval life stage) and 17 days post tick infestation (adult tick life stage). Skin (including an attached tick) and lymph node biopsies were collected surgically under general anaesthesia using xylazine (0.05-0.2 mg/kg intramuscular, Bayer AH) as a premedication followed by ketamine (0.02-0.1 mg/kg intravenous, Bayer AH) and butorphanol (0.05-0.1 mg/kg intravenous, Intervet). Lidocaine 2% (10–20 ml divided between the various sites, Bayer AH) was used as a subcutaneous local anaesthetic line block. An 8 mm diameter skin biopsy punch was used for the collection of the skin biopsies that were collected from two different tick-infested areas on the flank of each animal. Skin biopsies were immediately immersed in 10% buffered formalin (pH 7.4). A superficial cervical lymph node wedge biopsy of

approximately 1 cm^3 was collected. This included regions from the hilum to the capsule. Biopsies were collected at each time point from alternate sides. Each biopsy wedge was trisected with a third being submitted for histology and IHC, a third used for flow cytometry and the remaining tissue was kept for future studies. Samples for flow cytometry were placed on ice and processed within 30 min of collection.

2.3.3. Histopathology of the skin and lymph nodes

Lymph node and skin samples were fixed for 24 hours before being embedded in paraffin and processed as per routine histological techniques for Haematoxylin and Eosin (H&E) staining (Fischer et al., 2008). Skin and lymph node samples were sectioned at 4 µm and stained with H&E to enable histological evaluation. Stained skin sections (collected from the flank of each animal) were analysed under a light microscope and areas of 1 mm^2 (including the dermis and epidermis) were selected for further analyses. The thickness of the skin was measured from the bottom of the dermis to the outermost aspect of the epidermis at three separate regions within the 1 mm^2 area using the Olympus Cellsens® digital imaging software. Separate measurements for the dermis and epidermis were recorded at three separate locations in the aforementioned 1 mm^2 area. Counting of selected different cell types in three germinal centres (GCs) of each lymph node section was done by subjective cell identification in the H&E stained sections under a light microscope (400x magnification). Cell types identified and counted included eosinophils and tingible body macrophages (TBMs).

2.3.4. Immunohistochemistry of skin and lymph nodes

For immunostaining, 3 µm-thick sections of formalin-fixed, paraffin-embedded lymph node and skin tissues were collected on positively charged glass slides and dried overnight at 50˚C. Routine dewaxing in xylol was followed by rehydration through graded ethanol and distilled water. Sections were treated with 3% hydrogen peroxide in methanol for 15 minutes to quench endogenous peroxidase activity and potential non-specific reactivity was blocked with 0.1 M of phosphate buffered saline (PBS), pH 7.6 containing 0.1% bovine serum albumin for 5 min. Deparaffinised sections were microwave-heated to 96˚C in a citrate buffer (pH 6) for 14 min prior to incubation with the primary antibodies. Primary antibodies used for immunostaining included an anti-CD3 polyclonal rabbit antibody (Dako) (a general T-cell receptor marker), anti-CD20 polyclonal rabbit antibody (ThermoFisher Scientific©) and anti-CD79α monoclonal mouse antibody (Bio-Rad AbD Serotec). The latter two markers were chosen for the identification of B-cells. CD20 is traditionally a marker present on both resting and activated B-cells and is lost during maturation to plasma cells (Tedder and Engel, 1994) while CD79α is present on B-cells throughout their life -cycle (Chu and Arber, 2001). The anti-CD3 and anti-CD20 primary antibodies were detected by means of a two-step polymer detection kit (BioGenex Super Sensitive IHC detection system) while the anti-CD79α antibody was detected using the Dako REAL™ EnVision™ Detection System After immunostaining, slides were counterstained with Lilly Mayer's haematoxylin.

Quantification of the lymphocyte subtypes in the skin was performed via manual lymphocyte counting under a light microscope. Cells were manually counted in a 1 mm^2 area adjacent to the tick bite lesion and included both the dermis and the epidermis of the skin. The quantification of cells in the lymph node was done by calculating the relative percentage of positively stained cells to the area of the region of interest as described in detail below. Virtual

slide images were generated using the VS120-S6-W slide loader system (Olympus) and the Olympus Cellsens® digital imaging software. Immunostained slides were scanned at 10 x magnification and a high resolution panoramic image was constructed. Regions of interest were defined and included the selection of two 2 mm^2 cortex and medulla sections, including follicular GCs in the cortical region. The full colour image was separated into its three colour phases with the blue phase corresponding to positive staining and thus used for quantification of lymphocyte types. With the full colour image, the colour threshold of the blue phase image was manually adjusted to ensure that only the areas of positive staining were counted. This value was then represented as a percentage of the positive staining in the selected area.

2.3.5. Flow cytometry

All procedures were carried out on crushed ice unless otherwise indicated. The tissue samples were each placed in 2 ml CTCM (RPMI containing 10% foetal calve serum (FCS), 200 U/ml Penicillin and 200 µg/ml Streptomycin) in a sterile environment. The tissue was cut into small pieces with a sterile scalpel and sheared and filtered using the Medimachine (BD Biosciences™, USA). A nucleated cell count of the lymph node cell suspension was performed using the ADVIA 2120 Hematology System (Siemens Healthcare GmBH, Germany). Viable cell numbers were confirmed using a haemocytometer and Trypan blue staining. All samples processed demonstrated a cell viability >95%. Two million cells per reaction were used incubated with 1% FCS in RPMI for 15 minutes. Two microliters of fluorochrome-labelled antibody were used in each reaction (final volume of 250 µl) and incubated for 30 minutes in the dark before cells were washed twice with BD CellWASH (BD Biosciences™, USA) by centrifugation at 450 x g followed by removal of supernatant and addition of 1 ml BD CellWASH. For antibodies requiring permeabilisation (for intracellular staining, indicated with an asterix in the following sections), cells were treated using Leucoperm™ (AbDSerotec®) as per manufacturer's instructions and 2 µl antibody were added. The following labelled antibodies were obtained from AbD Serotec® and were used against bovine lymphocyte markers: AF647-conjugated anti-CD4 and AF488-conjugated anti-CD3* for the detection of T-helper lymphocyte populations; FITC-conjugated anti-WC1 for the detection of gamma delta T-lymphocytes; AF647-conjugated anti-CD8 and AF488-conjugated anti-CD3* for the detection of cytotoxic T-lymphocytes. Samples were incubated at room temperature for 20 minutes, washed and fixed using BD Cell Fix™ (BD Biosciences™, USA). Fixed samples were used within 24 hours for flow cytometry analyses using the BDAccuri C6™ (BD Biosciences™, USA). Unstained cells treated in the same manner as stained cells for each calf and each time point were used as controls. Data acquisition was done using a threshold of 350,000 horizontal forward scatter (FSC-H) and a total of 30,000 events were acquired. Forward scatter light data was acquired using a linear amplifier, and side scatter light data was acquired with a logarithmic amplifier. Data analysis was performed using the commercially available software FlowJo v 10.0.8 (FlowJo, LLC). Gates for analyses were set around the lymphocyte population on a dot plot of forward angle versus side angle light scatter. Labelled lymphocyte populations were analysed using either dot plots or histograms for fluorescence, with gating parameters based on the unstained samples. Results are presented as the percentage of lymphocytes that emitted fluorescence above that of the negative population.

2.3.6. Statistical analyses and data interpretation

All data was firstly compared within a breed at different time-points. Due to the small sample size of the study population, the unequal variance t-test (Welch Two Sample t-test) was used for all histological and immunohistochemical evaluations (Ruxton, 2006). All analyses were conducted in the Bioconductor open source software platform utilising the R statistical programming language and statistical significance was assumed at $P < 0.05$ (Team, 2016). For flow cytometric analysis a single-factor one-way analysis of variance was performed using Microsoft Excel for each marker set on the three biological repeats of each cattle breed per time point. The standard error of the mean was calculated for each population subset. Data is represented in the text as mean of the ratios (uninfested:larvae-infested; uninfested:adult-infested and larvae-infested:adult-infested) per experimental animal. Only data found to be statistically significant within a breed were then used to identify statistically significant interbreed differences using the ratios calculated as described above. Ratios of the biological replicates were then used for statistical analyses.

2.4. Results

2.4.1. Histopathology of skin and lymph node biopsies

All samples were subjected to standard H&E staining for histomorphological analyses. Measurements of the overall skin thickness (dermis and epidermis) were comparable between the three cattle breeds (Figure 2.1a). Separate measurements of the dermis showed that dermal thickness did not differ statistically between the cattle breeds (Figure 2.1b). In contrast, the epidermis of Brahman (43.19 ± 2.75 µm, $P = 0.0173$) (mean ± SE) and Holstein-Friesian (36.36 ± 7.75 µm, $P = 0.02$) cattle were significantly thinner than that of Bonsmara cattle (74.01 ± 5.62 µm) (Figure 2.1c).

Histopathology of lymph nodes indicated changes in the cellular composition of the GCs of tick-infested cattle (Figure 2.2). A significant increase ($P < 0.05$) in GC eosinophils within each breed was observed between uninfested (i.e. prior to artificial infestation) and adult tick-infested samples with a highly significant increase ($P = 0.001$) seen in Holstein-Friesian cattle. The increase in the GC eosinophils translated to a 15, 15 and 10.48-fold increase for Brahman, Bonsmara and Holstein-Friesian cattle, respectively. Upon comparing eosinophil levels between breeds, using ratios to normalise the data, there were no statistically significant changes. Intra-breed analyses of GC TBMs showed an increase from samples obtained from uninfested to adult tick-infested cattle in all three breeds with the only statistically significant increase occurring in Brahman cattle ($P = 0.03$). This translated to a 2.7, 4.3 and 2.3-fold increase for Brahman, Bonsmara and Holstein-Friesian cattle, respectively.

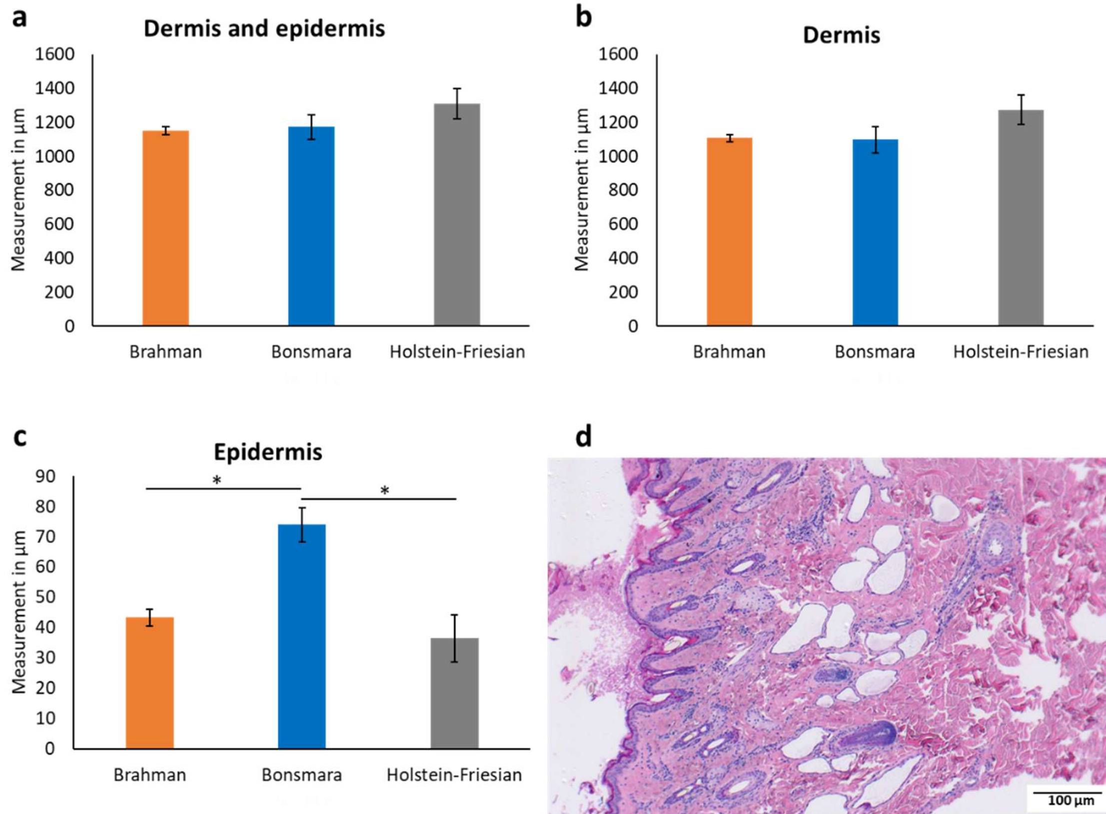

Figure 2.1: Thickness of the skin of Brahman, Bonsmara and Holstein-Friesian cattle. Represented is the thickness of the dermis together with the epidermis (a), dermis (b) and epidermis (c) of Brahman, Bonsmara and Holstein-Friesian cattle. The results are presented as the breed means with the standard error of the mean. Significant differences between breeds are indicated (*$P < 0.05$). A representative Figure of a bovine skin (Brahman, before tick infestation) section (bar = 100 µm) stained with Haematoxylin and Eosin is shown (d).

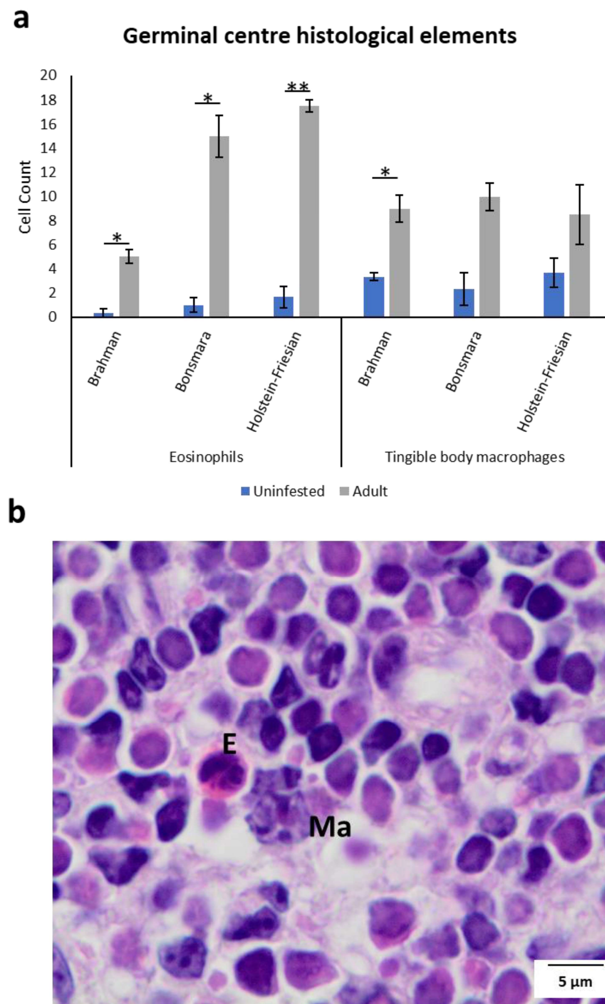

Figure 2.2: Eosinophils and tingible body macrophages in the germinal centres of cattle lymph nodes. The average of the cell counts of three germinal centres per biological replicate per breed (a). The results are presented as the breed means with the standard error of the mean. Significant differences between breeds are indicated (**P* < 0.05; ***P* < 0.001). A representative Figure (Brahman, adult tick-infested) of a germinal centre containing an eosinophil (E) and tingible body macrophage (Ma) (b) (bar = 5 µm) is shown.

2.4.2. Immunohistochemistry of skin and lymph node biopsies

The number of CD20+ B-lymphocytes and CD3+ T-lymphocytes was quantified for skin biopsy samples collected from uninfested, larvae infested and adult tick-infested cattle (Figure 2.3). Intra-breed analyses showed a statistically significant increase of CD20+ B-lymphocytes around the adult tick bite site when compared to uninfested cattle ($P < 0.05$) for all three breeds (Figure 2.3a). The increase in the same cells from uninfested to adult tick-infested samples translated to a 21, 13 and 5.6-fold increase for Brahman, Bonsmara and Holstein-Friesians, respectively. In addition, Brahman and Bonsmara cattle had a statistically significant (P = 0.034 and P = 0.022) increase in the number of CD20+ B-lymphocytes when the bite sites of larvae were compared to that of adult ticks with an 8.3 and 5-fold increase, respectively. Furthermore, using the ratios obtained from the intra-breed comparisons, allowed for the identification of a statistically significant difference between Brahman (21-fold increase) and

Bonsmara (13-fold increase) (*P* = 0.02) as well as Brahman (21-fold increase) and Holstein-Friesian (5.6-fold increase) (*P* = 0.04) (data not shown). A statistically significant, 5.75 and 1.57-fold, increase of CD3+ T-lymphocytes is seen in Brahman (*P* = 0.001) and Bonsmara cattle (*P* = 0.04), respectively, around the bite site of adult ticks when compared to uninfested skin (Figure 2.3c). A 3.3-fold statistically significant increase (*P* = 0.003) was also present in Brahman cattle upon tick maturation to adults.

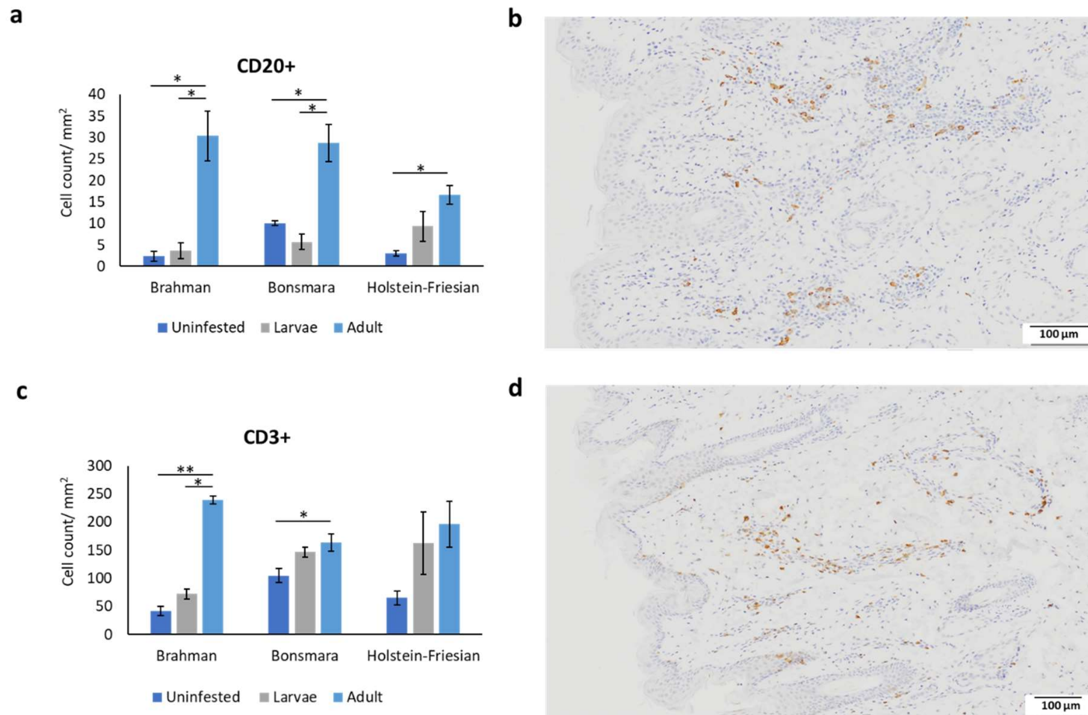

Figure 2.3: Cell counts of CD20+ B-cells (a) and CD3+ T-cells (c) in the region adjacent to the tick attachment site in the skin. The results are presented as the breed means with the standard error of the mean for the three cattle breeds over three time-points. Significant differences between breeds are indicated (**P* < 0.05; ***P* < 0.001). Representative images for the positive staining of CD20+ B-lymphocytes (Brahman, adult tick-infested) (b) and CD3+ T-lymphocytes (Holstein-Friesian, adult tick-infested) (d) are shown adjacent to their respective graphs (bar = 100 µm).

In lymph node tissues, the relative percentage of CD20+ and CD79α+ B-lymphocytes (Figure 2.4) and CD3+ T-lymphocytes (Figure 2.5) was quantified for uninfested, larvae-infested and adult tick-infested cattle. Histological analysis revealed CD20 and CD79α to be present on bovine plasma cells in the sampled lymph node tissue. CD20+ B-lymphocytes in the lymph node (medulla, cortex and GC alone) of Holstein-Friesian cattle showed a significant (*P* = 0.027) decrease (0.5-fold change) when uninfested cattle were compared to cattle infested with larvae (Figure 2.4a). In the same cattle breed, a significant (*P* = 0.011) increase in CD20+ B-lymphocytes (3.85-fold change) was seen from cattle infested with larvae to cattle infested with adult ticks (Figure 2.4a). A statistically significant (*P* = 0.022) increase (1.93-fold change) was also noted from uninfested cattle to cattle infested with adult ticks (Figure 2.4a). In addition, CD79α+ B-lymphocytes in the lymph node (medulla, cortex and GC alone) of Holstein-Friesian cattle showed a significant (*P* = 0.006) increase (3.22-fold change) when uninfested cattle were compared to cattle infested with adults (Figure 2.4b). A statistically

significant (P = 0.039) increase (1.76-fold change) was also noted in the same cattle breed when larvae-infested cattle were compared to cattle infested with adults (Figure 2.4b). The cortex of Bonsmara cattle had a significant (P = 0.049) increase (2.7-fold change) in the relative percentage of CD20+ B-lymphocytes from uninfested cattle to cattle infested with adults (Figure 2.4c). The GCs of Holstein-Friesian cattle showed a significant (P = 0.006) decrease (0.37-fold change) in CD20+ B-lymphocytes from uninfested cattle to cattle infested with larvae (Figure 2.4e).

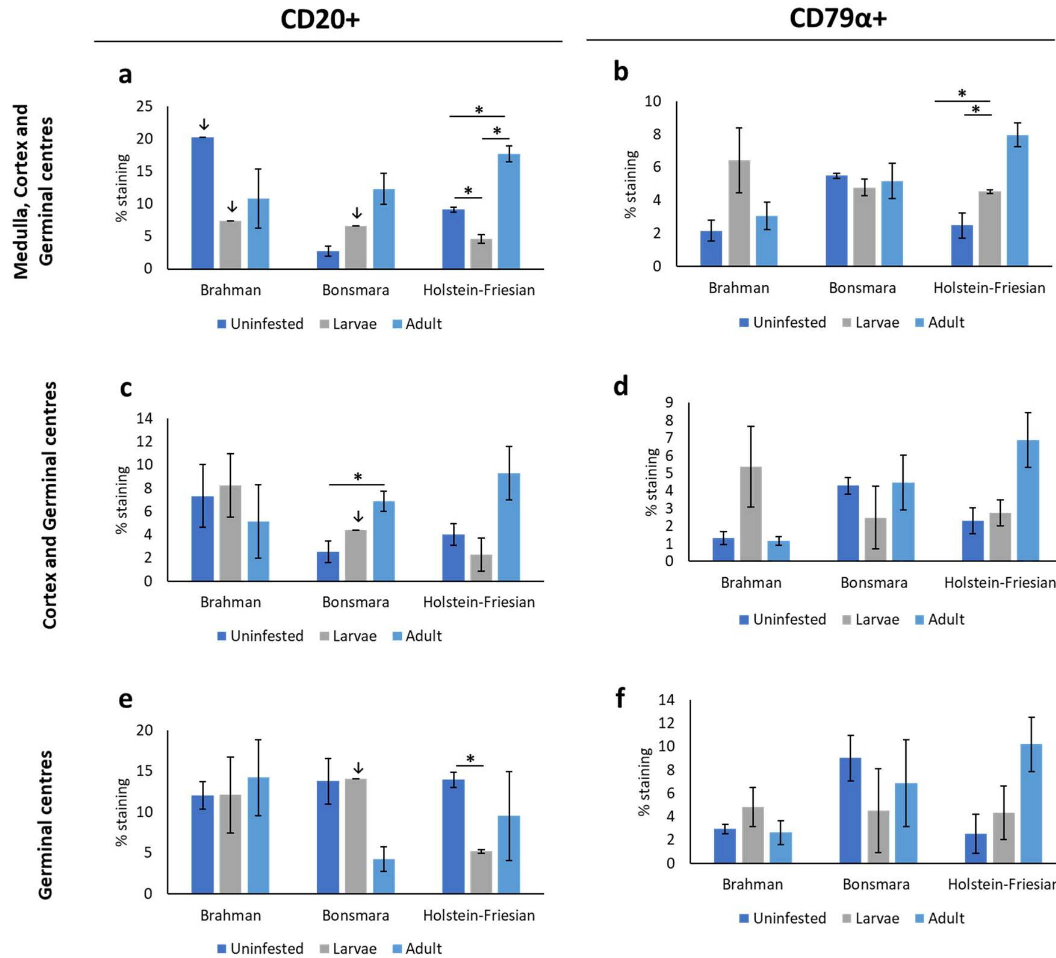

Figure 2.4: The relative percentage of positive staining for CD20+ and CD79α+ B-lymphocytes in the lymph nodes of cattle. The percentage of CD20+ staining regions in the lymph node (including the medulla, cortex and germinal centres) (a), the cortex (including the germinal centres) (c) and the germinal centres alone (e) are shown. The percentage of CD79α+ staining regions are indicated (b, d and f) corresponding to the above regions. The results are presented as the breed means with the standard error of the mean for the three cattle breeds over three time-points. Significant differences between breeds are indicated (*P < 0.05). Arrows (↓) indicate data missing at time-points resulting in an insufficient number of biological replicates for statistical analyses.

The cortex showed significant increases in the relative percentage of CD3+ T-lymphocytes in Bonsmara cattle. For this cell subpopulation, the statistically significant increase was seen for Bonsmara cattle from uninfested to adult-infested (P = 0.024) and from larvae- to adult-

infested (P = 0.02) animals indicated by the fold changes of 3.21 and 2.3, respectively (Figure 2.5a). Even though not statistically significant, Brahman cattle followed a similar trend. When only considering the GCs, the changes in the relative percentage of CD3+ T-lymphocytes are comparable to that of the entire cortex (Figure 2.5c). A significant (P = 0.045) fold increase of 5.08 was seen in Brahman cattle infested with larvae to infestation with adults. Significant increases in the relative percentage of CD3+ T-lymphocytes in Bonsmara cattle from uninfested cattle to cattle infested with adults (P = 0.028) with a fold change of 1.87 and from cattle infested with larvae to adult (P = 0.015) with a fold change of 3.06. No significant changes were observed in the lymph nodes of Holstein-Friesian cattle with regards to CD3+ T-lymphocytes.

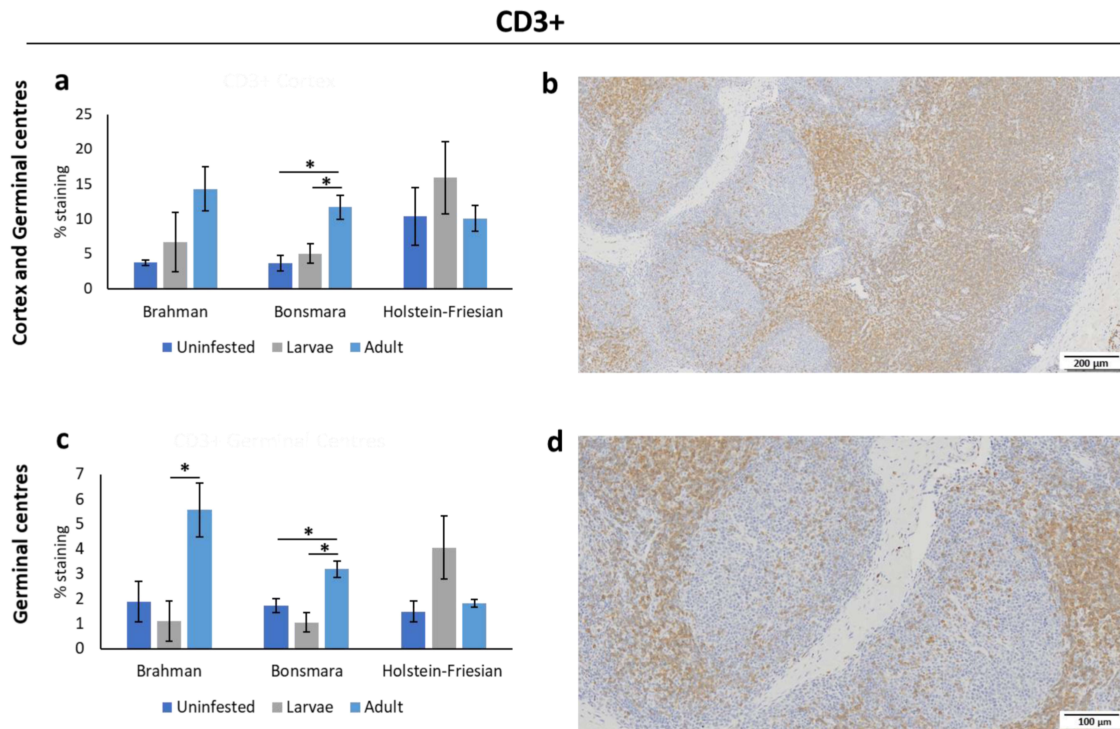

Figure 2.5: The relative percentage of positive staining for CD3+ T-lymphocytes in the lymph nodes of cattle. The percentage of CD3+ staining regions in the cortex (including the germinal centres) (a) and the germinal centres alone (c) are shown. The results are presented as the breed means with the standard error of the mean for the three cattle breeds over three time-points. Significant differences between breeds are indicated (*P < 0.05). Representative images (Holstein-Friesian, adult tick infestation) for the positive staining of CD3+ T-lymphocytes are adjacent to their respective graphs (b) (bar = 200 µm) and (d) (bar = 100 µm) is shown.

2.4.3. Flow cytometric evaluation of lymphocyte subtypes in cattle lymph node tissues upon tick infestation

To establish an overall picture of the immune response resulting from *R. microplus* infestation in different cattle breeds with varying degrees of tick resistance, lymph node and skin samples were also subjected to flow cytometric analyses. Cells counts for lymph node tissues were high enough to provide material for flow cytometry (ranging from $7x10^3$ to $25x10^3$ cells/µl), but, the number of cells that could be harvested from the skin biopsies was too low

to allow for this analysis (cell counts between 0.03x10^3 to 0.15x10^3 cells/µl for all three time-points).

In general, significant differences were only seen within the Bonsmara and Holstein-Friesian cattle groups and none within the Brahman cattle group. Investigation of the CD3+/CD4+ T-helper lymphocyte population showed that larvae-infested Bonsmara cattle demonstrated a significant 1.6-fold (P = 0.032) rise in the CD3+/CD4+ T-helper lymphocyte proportion with tick maturation (Figure 2.6a). A 1.2-fold significant reduction (P = 0.018) of this lymphocyte population upon tick infestation was seen in Holstein-Friesian cattle (uninfested to larvae-infested) (Figure 2.6a). The WC1+ (γδ T-lymphocyte) populations showed a highly significant (P = 0.0005) decrease of 3.35-fold in Holstein-Friesian cattle upon tick infestation and a significant (P = 0.008) decrease from uninfested to adult tick-infested animals as well as a significant increase (P = 0.041) upon tick maturation from larvae to the adult life stage (Figure 3.6b). The WC1+ population in Bonsmara cattle demonstrated a significant decrease (P = 0.016) upon tick infestation (Figure 2.6b). There were no changes in the CD3+/CD8+ (cytotoxic T-lymphocyte populations) in any breed at any time point (Figure 2.6c).

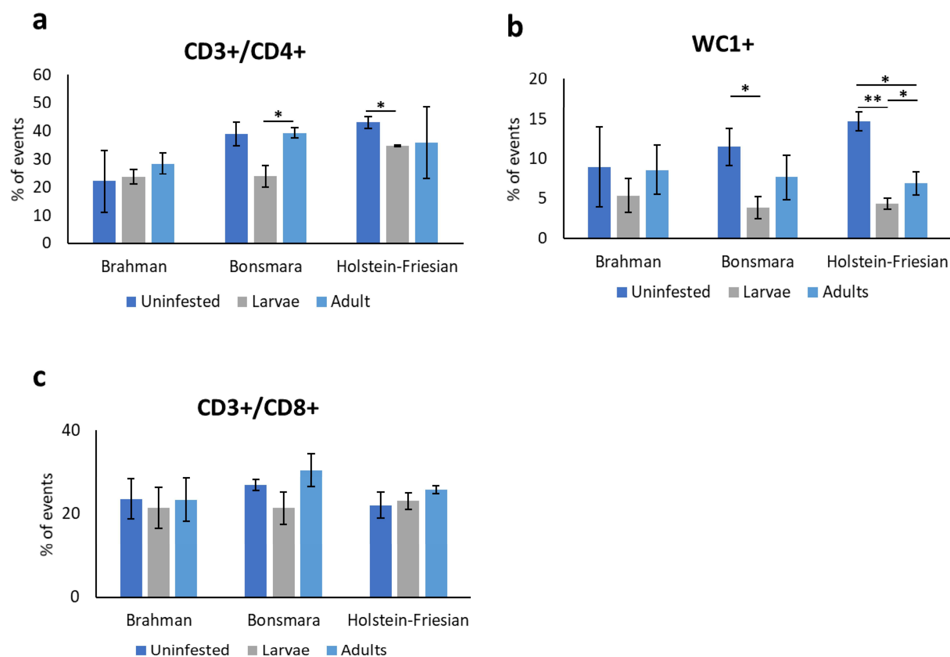

Figure 2.6: Percentage of the cellular subset comprising the T-helper cell (CD3+/CD4+) (a), γδ T-cell (WC1+) (b) and cytotoxic T-cell (CD3+/CD8+) (c) population of Brahman, Bonsmara and Holstein-Friesian cattle over three time-points of *R. microplus* infestation (uninfested, larvae and adult-infested cattle). Results are presented as the breed means of three time-points with standard errors of the mean. Significant differences between breeds are indicated (*P < 0.05; ** P < 0.001).

2.5. Discussion

The level of cattle infestation with *R. microplus* varies according to the host subspecies and it has been well documented that *B. t. taurus* breeds harbour significantly larger numbers of external parasites than *B. t. indicus* cattle which carry five to ten times less ticks than the former subspecies (Jonsson et al., 2014). The highly tick-resistant Brahman (*B. t. indicus*) cattle breed is generally used for meat production while the more susceptible Holstein-Friesian

(*B. t. taurus*) cattle breed is most often used in milk production (Johnston and Haydock, 1969; O'Kelly and Spiers, 1976; Rechav and Dauth, 1987; Utech et al., 1978). Another important cattle breed in South Africa is the Bonsmara which represents a mixed breed animal that shows intermediate tick resistance (Scholtz et al., 1991). In an attempt to obtain some insight into immunological events following tick infestation (including the time period of tick maturation on the host) we assessed the temporal changes in some measures of the immune response at different time-points in these three important South African cattle breeds. It should be noted that results from this study cannot necessarily be extrapolated to an entire breed since only three biological replicates were used to represent each breed. By using histopathological, immunohistochemical and flow cytometric analyses on cattle skin and lymph node tissues, we investigated the effect of *R. microplus* infestation on selected lymphocyte subpopulations. A summary of statistically significant results is presented in Figure 2.7.

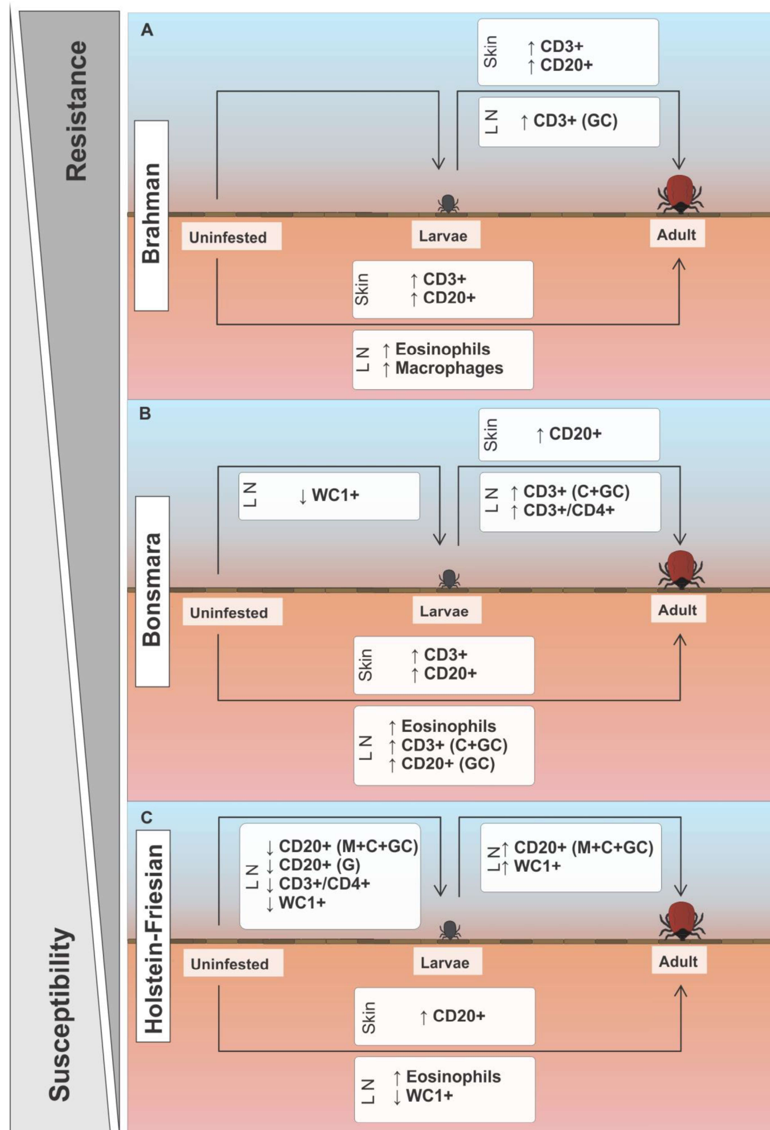

Figure 2.7: Summary of findings of cell subtype population dynamics due to *R. microplus* attachment and feeding in different cattle breeds and tissues. Represented is the summary of significant findings in this research paper for Brahman (a), Bonsmara (b) and Holstein-Friesian (c) cattle. Indicated are the results obtained from histopathology, flow cytometry and immunohistochemistry.

2.5.1. Bovine skin thickness is not related to tick resistance

Previous work suggests that phenotypic coat characteristics such as hair length, coat smoothness and coat colour have an influence on tick numbers and are related to tick resistance in cattle (Martinez et al., 2006). Other authors have reported that there are no relationships between morphological coat traits, such as skin thickness and tick resistance (Marufu et al., 2011; Spickett et al., 1989). Because of this discrepancy in the literature we further investigated the possible contribution of the dermal and epidermal thickness to tick resistance. Although the average skin thickness of the sampled Brahman, Bonsmara and Holstein-Friesian cattle in this study did not differ significantly, the Bonsmara cattle breed did have a significantly thicker epidermis than the other two cattle breeds. The lack of significance in epidermal thickness between Brahman and Holstein-Friesian cattle suggests that the epidermis may not be directly involved in the resistance of cattle to tick infestation.

2.5.2. CD20+ B-lymphocytes increase in the skin of tick-infested cattle

Until recently, the role of B-lymphocytes in the skin of tick-infested cattle was poorly described. Franzin and colleagues (Franzin et al., 2017), using IHC, observed a non-statistically significant infiltration of CD21+ B-lymphocytes around the feeding lesion of *R. microplus* in bovine hosts. Our results show a significant increase in CD20+ B-lymphocytes in the skin of all three studied cattle breeds. B-cells are known to be present in low numbers in normal skin and in higher numbers at sites of cutaneous inflammation (Egbuniwe et al., 2015). In both animal models and humans, they have been shown to play a role as proinflammatory and anti-inflammatory agents in inflammatory skin disorders (Egbuniwe et al., 2015). Tick attachment result in damage to the host skin and exposure of tick saliva to sentinel cells including granulocytes. Cross-linking of immunoglobulins (of B-lymphocyte origin) to tick allergens and binding to relevant receptors is followed by the release of inflammatory mediators including histamine from dermal mast cells and basophils (Galli et al., 2005; Galli and Tsai, 2012; Stone et al., 2010). Histamine release is involved in the immediate-type (type 1) hypersensitivity response resulting in pruritis, potentially leading to increased grooming behaviour and thus a reduction in tick numbers (O'Mahony et al., 2011). Acquired resistance to ticks has previously been linked to the occurrence of a hypersensitivity reaction to tick salivary gland components (Bechara et al., 2000; Kemp et al., 1986; Latif et al., 1991; Marufu et al., 2013; Piper et al., 2010; Prudencio et al., 2011; Riek, 1962; Smith et al., 1989).

2.5.3. CD3+ T-lymphocytes increase in the skin of tick-infested cattle

The results obtained from this study indicate that: (a) upon larval and adult tick attachment in Brahman cattle, a significant increase in the number of T-cells is seen and (b) Bonsmara cattle have a significant increase in T-lymphocytes at the area of larval tick attachment but not adult tick attachment and (c) Holstein-Friesian cattle do not have a significant difference in the number of T-lymphocytes at the area of tick attachment.

In murine studies, T-lymphocytes at the site of tick attachment are increased (Mbow et al., 1994; Schoeler and Wikel, 2001). Similar results have also been found in bovines. Two studies found CD3+ T-lymphocytes to be increased in *B. t. indicus* compared to *B. t. taurus* cattle (Constantinoiu et al., 2010; Franzin et al., 2017). While Constantinoiu et al. (2010) found a significant higher level in resistant cattle at one day and at three weeks post larvae infestation,

Franzin and colleagues (2017) found an increase nine days post larvae infestation. This suggests that a T-lymphocyte dependant mechanism may be correlated with tick resistance at the tick attachment site. The results of this study correlate well with these other studies. CD3 is a pan-T-lymphocyte marker, but since no further markers were used, conclusions cannot be drawn regarding the T-cell subtype population.

2.5.4. Adult tick attachment increases eosinophils and macrophages in the germinal centres

Eosinophils are capable of acting as antigen-presenting cells in a multitude of organisms (Padigel et al., 2006; Shi, 2004; Shi et al., 2000). After trapping the antigen, these "primed" eosinophils migrate to the T-cell rich regions of the lymph nodes. Here they aid in the development and maturation of T-helper cells (specifically Th2 cells) (Shi, 2004). Eosinophils are generally found in the GC linked to diseases (Faras et al., 2014). The significant eosinophil infiltration in the GCs of lymph node tissues in all three breeds as was found in this study is in line with previous studies that have described the up-regulation of the T-helper 2 pathway (Ferreira and Silva, 1999; Kovář et al., 2002; Mejri and Brossard, 2007; Schoeler et al., 2000, 1999; Schoeler and Wikel, 2001).

In the light zone of the GC, interaction of B-cells with the follicular dendritic cells assures the survival of the B-cells that had recently undergone clonal proliferation and somatic hypermutation (in the dark zone) (Dubois et al., 1999). In the dark zone of the GC, B-cell proliferation and isotype switching is stimulated by T-helper cells that are primed with antigen (Crotty, 2011). The presence of eosinophils in the dark zone may thus indicate that due to the antigen presenting capability of eosinophils they may function in addition to T-helper cells and follicular dendritic cells in the affinity maturation process. An additional reason may be their involvement via some as yet unknown mechanism.

Germinal centre reactions were suggested to be down-regulated with the involvement of TBMs (Smith et al., 1998). Apoptosis is an important process in B-cell clonal selection and TBMs specifically seem to be involved in the removal of apoptotic B-cells that failed to receive survival signals due to the low affinity of their B-cell receptors (Rahman et al., 2010). The interplay between TBM and CD20+ as well as CD79α+ cell populations identified in this study showed different cell population levels between the different cattle breeds. More research is, however, required to determine the exact differences and directions of underlying dynamics. Immune responses in Brahman cattle might simply be faster/more efficient thus leading to variable cell population levels at specific time-points.

2.5.5. Changes in CD20+ and CD79 B-lymphocytes in the lymph node of tick-infested cattle

Immunohistochemical analyses of bovine lymph nodes showed no significant change in the B-lymphocyte populations in the more resistant Brahman cattle. Cutaneous tick bite reactions in susceptible animals were suggested to be slower and more gradual compared to resistant breeds (Franzin et al., 2017). These breed differences, with regards to cell dynamics in the skin, could be hypothesised to be similar in the lymph node. In resistant animals a large portion of B-cell development and maturation process may have already occurred by the time tissue samples were collected. This could result in seemingly more stable cell populations in resistant

animals. Future studies could therefore focus on analysing Th1 and Th2 cytokines in the lymph nodes in combination with ratios of IgG1 to IgG2 throughout the lifecycle of *R. microplus*.

Previous studies have shown involvement of innate (O'Kelly and Spiers, 1976; Riek, 1962) and acquired immune responses to the acquisition of tick resistance mechanisms between breeds. Acquired resistance to tick infestation was found to be least apparent in *B. t. taurus* subspecies and significant resistance was found to appear only after the fourth round of infestation on tick-naïve Brahman animals compared to naïve Shorthorn (*B. t. taurus*) cattle (Riek, 1962; Wagland, 1975). The immunohistochemical findings in the current study show a significant increase for the Holstein-Friesian cattle group between the larval and adult tick life stage in the combined cortex, medulla and GC lymph node pertaining to the CD20+ B-lymphocyte population. This increase mainly seems due to a B-lymphocyte influx in the cortex. The same trend was apparent (significantly so) for Bonsmara cattle when comparing tick uninfested and adult-infested animals. Similar results were found for CD21+ lymphocytes in draining lymph nodes in the goat after tertiary tick infestation (Monteiro et al., 2011). B-lymphocyte proliferation as a result of tick infestation is furthermore supported by findings showing that lymph nodes draining tick attachment sites increased in weight upon tick infestation compared to controls (Boppana et al., 2005). An increased lymph node lymphocyte response has also been demonstrated to in response to lipopolysaccharide stimulation *in vitro* following tick infestation compared to controls (Gakuya and Mulei, 2005; Ganapamo et al., 1996, 1995). A decrease in CD20+ lymphocyte numbers could also be observed in Holstein-Friesian animals upon tick larvae infestation mainly due to reduced B-lymphocyte populations in the GCs. This could be a result of affinity maturation and subsequent B-lymphocyte apoptosis of low affinity B-lymphocytes followed by clonal expansion of the higher affinity specific lymphocytes and the subsequent increase of CD20+ lymphocytes over the time period between larval and adult tick infestation (MacLennan, 1994). Lymph nodes in *Ixodes ricinus* infested mice showed an increase in B-lymphocytes in vitro with only the occasional production of anti-tick IgG antibodies (Kashino et al., 2005).

2.5.6. Proportions of T-lymphocytes in bovine lymph nodes

In the present study, based on the IHC findings, there was a trend towards an increased number of CD3+ T-lymphocytes from uninfested and larvae-infested cattle to animals infested with adult ticks in the more resistant breeds. This finding is at least partially attributable to the increase in CD3+ lymphocytes in the GCs specifically, as per results shown. A closer look at the T-lymphocyte populations showed that the CD3+/CD4+ T-helper lymphocyte population identified via flow cytometric analyses increased significantly from larvae to adult-infested Bonsmara cattle and decreased significantly and almost significantly ($P = 0.059$) in Holstein-Friesian and Bonsmara cattle, respectively, upon larvae infestation. This could indicate that the more susceptible breeds in this study have declining CD3+/CD4+ T-helper lymphocyte populations while the most resistant breed showed a more stable T-helper lymphocyte response over time. It is known that tick infestation in general leads to a more pronounced T-helper 2 response (Ferreira and Silva, 1999). It has also been suggested that a T-helper 2 response is associated with tick susceptibility (Ferreira and Silva, 1999; Kovář et al., 2002; Mejri and Brossard, 2007; Schoeler et al., 1999; Schoeler and Wikel, 2001). The results from the present study contribute to the validity of this hypothesis with the observation of increased eosinophils and CD20+ B-lymphocytes and thus a potential T-helper 2 bias (Janeway, 2001; Spencer and Weller, 2010) during tick maturation in tick-susceptible animals. We therefore

hypothesise that the observed decrease in CD3+/CD4+ T-helper lymphocytes in more susceptible animals will result in a reduced T-helper 1 response or an associated T-helper 2 increase, with associated reduction in high-affinity IgG. This however will need to be confirmed using cytokine profiling in future studies. The increase seen for Bonsmara CD3+/CD4+ lymphocytes in the lymph node upon tick maturation might represent the development of a stronger T-helper 2 response in these animals with the associated development of high-affinity IgG. Presence of a stable T-helper lymphocyte population could indicate cross-regulation of the two T-helper cell populations. However, more in-depth investigation is required into T-helper lymphocyte subpopulations in lymph nodes from cattle breeds with variable tick resistance during the period of tick maturation.

CD3+/CD8+ cytotoxic T-lymphocytes showed no differential levels over time in any cattle breed, as expected, since these cells primarily play a role in defence against intracellular pathogens (Janeway, 2001). In the bovine, γδ T-lymphocytes have been found to act as a major T-lymphocyte regulatory subset with suggested involvement in immune suppression (Guzman-Villanueva et al., 2014). Increased variability correlating with tick susceptibility in the two more susceptible breeds can be seen for the WC1+ lymphocyte populations on flow cytometric results with no variation identified for the resistant Brahman cattle. The WC1+ γδ T-lymphocyte population in the Bonsmara cattle demonstrated a significant reduction upon larvae infestation. The same was true in the Holstein-Friesian animals indicating a decrease in WC1+ γδ T-lymphocytes upon larvae infestation followed by a rise of this lymphocyte population at the adult life stage. The observation that the resistant cattle breed did not show changes in the WC1+ γδ T-lymphocyte population size while the population size was negatively correlated with tick attachment in the susceptible breed, could indicate that a decreased level of this lymphocyte population is favourable for continued *R. microplus* infestation. As stated previously, different immune response dynamic between breeds could be at play.

Varying B-cell subpopulations have been identified in lymph nodes compared to skin and skin-draining lymph in a study investigating an ovine granuloma model of cutaneous inflammation (Geherin et al., 2011). In this study, more MHC II, CD1 and CD80/86 were expressed in skin B-cells compared to lymph node B-cells supporting their role in T-cell activation (Geherin et al., 2011, 2012). Furthermore, lymph nodes draining the skin are known to be the site where cutaneous antigens are exposed to naïve T-cells. Antigens encountered previously will be re-exposed to memory T-cells within the skin (Egbuniwe et al., 2015). Investigation into different T-cell subsets in tick-infested skin and draining lymph nodes is needed in order to better understand the mechanism of tick resistance.

2.6. Conclusion

This study found no correlation between skin layer thickness and tick resistance; however, a significant influx of B-lymphocytes into areas of tick attachment in all cattle investigated in this study indicated that B-lymphocytes are a crucial mediator of immune responses upon tick infestation. CD3+ T-lymphocyte populations in cattle skin were found to increase significantly only in the more tick-resistant breeds. Investigation of lymphocytes in the GCs of lymph nodes showed an increase in eosinophils in more susceptible breeds. Due to a link between eosinophils and T-helper 2 lymphocytes, the latter population needs to be studied. In addition, GC TBMs showed a significant increase between uninfested and adult tick-infested Brahman cattle suggesting that resistance is likely to be associated with a more pronounced affinity

maturation process. Furthermore, the B-lymphocyte numbers remained consistent in the lymph nodes in the more resistant cattle breeds over the chosen time-points. In contrast, susceptible animals showed a decrease of B-lymphocytes in lymph node tissue upon larvae infestation, followed by an increase from larvae to adult-infested cattle. The WC1+ T-lymphocyte population was not variable across tick life stages in the resistant Brahman breed with increased cell population dynamics associated with increased susceptibility and a possible suppression of WC1+ γδ T-lymphocyte production. In general, CD3+ T-lymphocytes increased in number towards adult infestation in more resistant breeds and a more stable CD3+/CD4+T-helper lymphocyte population was found in resistant animals. However, there was an opposing tendency, with observation of a decrease in CD3+ T-lymphocyte populations in the more susceptible breeds. Future studies are required to obtain a clearer picture especially in terms of T-helper 1 versus T-helper 2 profiles over tick infestation time-points in lymph nodes from various tick -naïve cattle breeds.

2.7. Acknowledgements:

The authors would like to thank Ms Rephima Phaswane in the Pathology Section, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, South Africa for her help in optimising the CD79α+ staining in cattle tissues.

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Chapter 3: Temporal analysis of the bovine lymph node transcriptome during cattle tick (*Rhipicephalus microplus*) infestation

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This chapter is in the process of being submitted to PLOS Neglected Tropical Diseases. For uniformity throughout the thesis, the references in the chapter are formatted to the Ticks and Tick-borne Diseases referencing format. Luïse Robbertse performed sample preparation, all related data analyses and writing of the initial draft of the manuscript. Sabine Richards and Christian Stutzer assisted in data analyses and drafting of the manuscript. Andrew Leisewitz and Jan Crafford were the trained veterinary personnel responsible for tissue collection and additional support in the project. Christine Maritz-Olivier provided advice, direction, critical revision and supervision in planning of the project. All other technical assistance is acknowledged at the end of the chapter.

3.1. Abstract

Livestock production is a fundamental source of revenue and nutrition, wherein cattle-farming constitutes one of the major agricultural industries. Vectors and vector-borne diseases have a major impact on the livelihood of all farming communities, but more so in resource-poor communities and developing countries. Understanding the mechanism(s) of tick resistance in cattle is instrumental in the development of novel and improved tick control strategies. In this study, gene expression patterns were compared within the lymph nodes of three cattle breeds at different tick life stages of the cattle tick, *Rhipicephalus microplus*. Results indicated 183 differentially expressed genes within Bonsmara (5/8 *Bos taurus indicus* x 3/8 *B. t. taurus*) lymph node tissues, while only 5 differentially expressed genes were identified in each Holstein-Friesian (*B. t. taurus*) and Brahman (*B. t. indicus*) cattle breeds during larval and adult tick feeding, compared to the baseline of uninfested cattle. Overall, all the latter data provides evidence for a transcriptional regulatory network that is activated during immature tick infestation but is down-regulated towards basal transcriptional levels when adult ticks are feeding. Specific processes in the lymph nodes of Bonsmara cattle were seen to be differentially regulated on a transcriptional level. These include: (1) Leukocyte recruitment to the lymph node via chemokines and chemotaxis, (2) Trans-endothelial and intranodal movement on the reticular network, (3) Active regulation of cellular transcription and translation in the lymph node (including leukocyte associated cellular regulatory networks) and (4) Chemokine receptors regulating the movement of cells out of the lymph node.

Keywords: *Rhipicephalus microplus*; immune response; lymph node; transcriptional response; infestation; host immune response

3.2. Introduction

In developing nations, livestock production is a fundamental source of revenue and nutrition, wherein cattle-farming constitutes one of the major agricultural industries, especially in the tropical and subtropical regions of the world (Randolph et al., 2002). Indeed, the global demand for livestock products such as milk and meat are rapidly rising, exceeding current production rates (Godfray et al., 2010; Prosperi et al., 2016). Also, in developing countries entering export markets, the quality of these products needs to be improved as to be competitive and allow for integration into the global market (Luo and Tung, 2007). The latter is mainly attributed to the success in optimising carcass value through growing exports of higher value cuts. In this regard, vectors and vector-borne diseases have a major impact (Delago, 1999; Jongejan and Uilenberg, 2004; Randolph et al., 2002). In attempting to alleviate the burden of vectors and their associated diseases, control strategies (such as vaccines) are continuously being developed and improved (Stutzer et al., 2018). Since few vaccines are available for controlling ectoparasites, insight into the host response to ectoparasite infestations may be beneficial in this regard.

The host immune response to ectoparasite infection has been studied for several ectoparasitic species of economic importance in domestic ruminants. The immune response to ectoparasites in ruminant hosts have been studied for the sheep blow fly (*Lucilia cuprina*) (Elhay et al., 1994); the sheep scab mite (*Psoroptes ovis*) (Van den Broek and Huntley, 2003); lice affecting cattle (Milnes et al., 2007) and sheep (Bany et al., 1995), as well as ticks affecting sheep (Ogden et al., 2002; Ramachandra and Wikel, 1992) and cattle as reviewed by Robbertse et al. (2017). Of these host parasite interactions, host responses to tick infestations have been studied in more detail (Robbertse et al., 2017; Tabor et al., 2017).

Variable resistance towards tick infestation has been observed between cattle breeds from the more tick-susceptible *Bos taurus taurus* (*B. t. taurus*) to the more tick-resistant *B. t. indicus* breeds, as well as between several crossbred cattle, with marked differences in tick numbers within breeds (Robbertse et al., 2017). Several cells and compounds, predominantly granulocytes and histamine (involved in innate immunity) have been well described to date as drivers of immunity during the different tick life stages as reviewed by Robbertse et al., 2017. However, the adaptive immune mechanisms responsible for the differences underlying tick resistance amongst different cattle breeds remain poorly understood. The identification of specific cellular immune markers and/or pathways underlying tick resistance will be invaluable in the screening and breeding of more tick-resistant animals (and by extension decreasing the spread of tick-borne diseases), the formulation of future tick vaccines (impacting the choice of adjuvant) and identification of correlates of protection during cattle vaccination trials.

Recent improvements in bovine functional genomics allow linking phenotypic traits with large-scale genotypic screens. The most used technologies to date include functional transcriptomics via either DNA microarrays (Pareek et al., 2011) or RNA sequencing (Wickramasinghe et al., 2012). DNA microarrays have been employed successfully to describe gene co-expression within cattle and in assessing bovine immunological responses, which when combined provided a broad overview of gene transcription under a given set of conditions (Beiki et al., 2016; Brym and Kaminski, 2017; Donaldson et al., 2005). In the last decade, several studies have described the relative transcriptional profile of the skin (Carvalho et al., 2014, 2010; Franzin et al., 2017; Kongsuwan et al., 2010; Nascimento et al., 2011; Piper et al., 2010, 2008; Regitano et al., 2008;

Wang et al., 2007) and blood (Domingues et al., 2014; Wang et al., 2007) of more tick-resistant compared to more tick-susceptible cattle.

The skin represents the first physical barrier to tick infestation, containing many immune cells within its dermal layers that mediate innate immunity (Mann et al., 2012). Recognition of pathogens and their products is mediated by a number of genetically conserved molecular pattern recognition receptors (PRRs) in response to pathogen-associated molecular patterns (PAMPs). For ticks, toll-like receptor (TLR) 4 (Zhao et al., 2013), TLR-2 (Oliveira et al., 2010) and TLR-2/TLR-3 (Bernard et al., 2016) have been proposed as being affected by tick saliva. The downstream effects of tick saliva induced cellular signalling via these PRRs with subsequent proinflammatory cytokine release and linkage to the adaptive immune system remains unexplored. Bridging of the innate and adaptive immune responses is mediated to a large extent by dendritic cells (DCs) that link recognition of pathogens or their products (such as tick saliva) with the development of an antigen-specific, adaptive immune response. In subsequent exposures, adaptive immunity may be engaged by a number of antigen-presenting cells as the process is not limited to DC's alone (Mann et al., 2012). Within this context, lymph nodes are one of the major loci where innate responses lead to acquired immunity and the subsequent development of B-lymphocyte immunity, driven by T-dependant or T-independent antigens (Milligan and Barrett, 2015; von Andrian and Mempel, 2003). As such, assessment of the transcriptional profile of lymph nodes, as sites of immune cell differentiation and proliferation, in response to tick infestation would be of interest. Since no research is available on the response of bovine secondary lymphoid organs to ectoparasites, this study will hopefully serve as a hallmark for the study of bovine immune responses to ectoparasites.

This paper aims to compare the transcriptional regulation of regional draining lymph nodes in response to immature and adult tick infestation of *R. microplus*. We describe transcriptional responses for lymph nodes of three cattle breeds, namely Holstein-Friesian (*B. t. taurus*), Brahman (*B. t. indicus*) and Bonsmara (a *B. t. indicus* and *B. t. taurus* cross; 5/8 Afrikander, 3/16 Shorthorn and 3/16 Hereford). Emphasis is placed on firstly, comparing transcriptional responses within a cattle breed, before and after infestation, and secondly, comparing differentially expressed genes common between the breeds. Findings of this study include the identification of breed-specific and shared transcriptional responses in the regional draining lymph nodes of cattle. Additionally, key role players and pathways in response to tick infestation are discussed for the Bonsmara cattle breed.

3.3. Materials and methods

3.3.1. Experimental animals and tick challenge

Holstein-Friesian, Bonsmara and Brahman calves (approximately 9 months old and with minimal previous exposure to ticks) were housed at the facilities of the University of Pretoria Biomedical Research Centre, South Africa. Institutional (ethical clearance number: EC036-13) and governmental (Section 20) clearances were obtained from the University of Pretoria Animal Ethics Committee (UP-AEC) and the South African Department of Agriculture, Forestry and Fisheries (DAFF). Three biological replicates of each breed were maintained and treated for internal (Toltrazuril 5% (3 ml/10 kg, oral dose, Bayer AH); Albendazole 7.5% (1 ml/10 kg, oral

dose, Zoetis); Oxyteracycline (20 mg/kg intramuscular divided over two sites, Intervet SA); Diminazene (3.5 mg/kg subcutaneous Intervet SA)) and external parasites (Amitraz 12.5% (100 ml/50 L water, Cooper)) to ensure that cattle were free of parasites prior to the commencement of the study.

Larvae derived from a South African field strain of *R. microplus* (ClinVet Pty. Ltd., South Africa) were placed on cattle in two sleeve-like patches (~2000 larvae per patch; 0.1 g; weighed on site) attached bilaterally on each side of the animal around the draining region of the superficial cervical lymph node. An additional 6,000 larvae (0.3g; weighed on site) were used for whole-body infestation, resulting in a total infestation of 10,000 larvae per animal. The larvae were allowed to advance through their life stages until mature females started to engorge. Tick infestation was considered successful if at least 100 *R. microplus* adult female ticks were present in each patch. Semiengorged female *R. microplus* ticks between 4.5 and 8 mm were counted at day 17 post infestation

3.3.2. Collection and processing of lymph node samples

Skin and lymph node samples were taken at three time-points, including before artificial tick infestation (labelled as uninfested samples throughout the paper), three days post tick infestation (larval life stage) and 17 days post tick infestation (adult tick life stage). Lymph node biopsies were collected under general anaesthetia (xylazine (0.05-0.2 mg/kg intramuscular, Bayer AH) as a premedication followed by ketamine (0.02-0.1 mg/kg intravenous, Bayer AH) and butorphanol (0.05-0.1 mg/kg intravenous, Intervet)). For each time point, approximately a 1 cm^3 wedge of the superficial cervical lymph node, containing regions from the hilum to the capsule, were taken on alternating sides of the animal. Each biopsy wedge was trisected with a third being submitted for transcriptome analysis while the remaining tissue was used in a complementary study (Robbertse et al., 2018). Samples for transcriptome analysis were placed on ice and transferred to an RNA stabilizing solution (0.5 M EDTA, 1 M ammonium citrate, 5.3 M ammonium sulphate, adjusted to pH 5.2 with H_2SO_4). A total of 27 samples were cut into smaller pieces using a scalpel and homogenized utilising liquid nitrogen and mechanical shearing with the aid of a mortar and pestle. Samples were further homogenized using a QIAshredder (QIAGEN, Germany) column. Total RNA was isolated using TRI-reagent fractionation (Sigma-Aldrich) and the RNeasy mini kit (QIAGEN, Germany) following the manufacturer's protocol. Contaminating genomic DNA was removed with a DNase I treatment (QIAGEN, Germany). Final RNA concentrations, purity and integrity were assessed with the NanoDrop-1000 (Thermo Fisher Scientific, USA) and the Experion automated electrophoresis system (Bio-Rad).

3.3.3. Microarray assay of isolated cattle lymph nodes

Microarray assays were performed using the Agilent Bovine V2 4x 44K slides (Agilent Technologies, USA). Total RNA with an A_{260}:A_{280} ratio of 1.7 and an RNA Quality Indicator of greater than 7 was selected for cDNA synthesis. A reference RNA pool consisting of equivalent quantities (4 µg) of RNA from the 27 lymph node samples collected from the three cattle breeds (including biological replicates and the three time-points) was made. First-strand cDNA synthesis

was performed by incubating 4 µg RNA with 250 pmol oligo(dT_{25}) and 775 pmol random primer 9 for 10 minutes at 70˚C, followed by cooling on ice for 10 minutes. Reverse transcription and aminoallyl-dUTP (5-(3-aminoallyl)-2'deoxyuridine-5' triphosphate) incorporation were performed simultaneously using 340 units SuperScript® III reverse transcriptase (Invitrogen™ life technologies, USA). Following standard hydrolysis of contaminating RNA, the cDNA samples were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany). cDNA samples were coupled to DMSO dissolved Cy3 (reference pool) and Cy5 (samples) fluorescent dyes (GE Healthcare Life Sciences) at pH 9. Unincorporated dye was removed using the same purification kit as used for cDNA purification. Labelling efficiency, the extent of dye incorporation and sample concentration was determined using the microarray settings on the NanoDrop® ND-1000 system. The measure represents a value of labelled nucleotides to unlabelled nucleotides, and the ratio should be at least 10:1000 (labelled to non-labelled nucleotides) to be considered acceptable for hybridisation. Cy3 and Cy5 labelled cDNA samples that had a labelling efficiency of more than 10 were selected for hybridisation. Equivalent picomoles (20 pmol) of Cy3-labelled cDNA from the common reference pool were hybridised with Cy5-labelled individual test cDNA. Overnight hybridisation at 65°C (rotation speed of 10), washing and post-processing were performed at the ACGT Microarray Facility (University of Pretoria). Prior to slide scanning with the Axon GenePix 4000B scanner (Molecular Devices) slides were washed and dried by centrifugation. Axon GenePix Pro 6.0 software (Molecular Devices) measured, recorded and analysed images based on the software's default settings as well as visual inspection of spots (adjusting the grid and flagging of low quality spots). Flagged features were ignored in subsequent analyses and given a zero-weight value.

3.3.4. Microarray data analysis, functional annotation and gene enrichment analysis

In order to identify genes for which the expression levels were significantly differentially expressed, the linear model for microarray data analysis (LIMMA) within the R statistical environment (http://cran.r-project.org/) was employed. Adaptive background correction (offset = 50) was followed by within-array normalisation (Robust Spline) and between-array normalisation (G quantile). Fold change was determined between all transcripts within a cattle breed collected at different time-points using the empirical Bayesian statistics, which were subsequently expressed as *P*-values (corrected for false discovery rate). Transcripts were regarded as differentially expressed if a greater than 2-fold change (log2ratio >1, log2ratio <-1) in either direction with *P*-value <0.05 were observed. Pearson correlations were done in R to determine the correlation of the biological replicates within each group. Uniquely differentially expressed genes in Bonsmara cattle were subjected to hierarchial clustering using the ComplexHeatmap-package in the R statistical environment (http://cran.r-project.org/). The heatmap function was used to make a data matrix if normalised M (or intensity values). The methods for distance calculation and clustering were "Euclidean" and "ward.D" respectively.

Differentially expressed transcripts were firslty functionally annotated using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov/). Outputs from DAVID were then futher analysed with BLAST searches (using default parameters) against the Uniprot (http://www.uniprot.org/blast/) and nonredundant NCBI (https://www.ncbi.nlm.nih.gov/) databases to confirm gene entries and identify homologous

transcripts with described functions (Consortium 2014). Additional searches were also performed to provide gene ontology terms (GO) and protein families via SMART/Pfam databases using EggNOG (v.4.5.1) (Huerta-Cepas, Szklarczyk et al. 2016). In the case of enzymes, outputs were manually inspected, and final annotations were based on consensus with the closest (>40%) reviewed database entries from Uniprot (http://www.uniprot.org/uniprot/) and BRENDA (http://www.brenda-enzymes.org/). Transcripts were finally classified into functional groups based on the eukaryotic orthologous group terms for gene ontology (KOG) (Tatusov et al., 2003). differentially expressed genes (DEGs) were also analysed in the context of functional pathways of the KEGG database and ranking these pathways based on statistical overrepresentation using DAVID.

3.3.5. Validation of microarray results using relative quantitative reverse transcriptase-PCR (qRT-PCR)

Using the same RNA isolated for microarray hybridisations, cDNA was synthesised in a 20 µl reaction using 2 µg of total RNA and the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). From this reaction, 100 ng of cDNA was combined with the TaqMan® Gene Expression Master Mix (ThermoFisher Scientific) in a 20 µl reaction. The samples were loaded on a custom designed TaqMan OpenArray® RT-PCR using the Accufill™ system. Quantitative-PCR was performed on the QuantStudioTM 12K Flex Real-Time PCR System (Applied Biosystems R, Life Technologies, Inc.) using the manufacturer’s recommended cycling conditions. The resulting data was normalised against six endogenous controls, including: beta actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1), succinate dehydrogenase complex subunit A, flavoprotein (Fp) (SDHA), TATA box binding protein (TBP) and mitochondrial ribosomal protein S9 (MRPS9). Data processing was done in ExpressionSuite v1.1. (ThermoFisher Scientific).

3.4. Results

3.4.1. Effect of *R. microplus* attachment and feeding on the lymph node transcriptional profile: Brahman, Bonsmara and Holstein-Friesians

In contrast to the 183 DEGs found in Bonsmara, few transcripts were identified as differentially expressed within the Holstein-Friesian (5 DEGs; Figure 3.1C) and Brahman (5 DEGs; Figure 3.1A) breeds during larval and adult tick feeding, compared to the baseline before artificial tick infestation. Bonsmara animals at the larval life stage (compared to uninfested animals) represented the majority of DEGs with 68 being down-regulated and 90 up-regulated. The variation within Brahman and Holstein-Friesian groups was validated by Pearson correlation analyses that indicated a weak correlation between individual replicate animals, resulting in the low number of valid DEGs identified for these two breeds in this study (Supplementary table 3.1). All transcripts identified as differentially expressed (in both tick life stages within a cattle breed) showed a similar expression profile for both time-points (Figure 3.1). This suggests similar regulation of immunity in response to larvae attachment and adult feeding and/or re-attachment. To verify the expression data, qRT-PCR was performed on five selected transcripts. These

transcripts were: toll-like receptor 7; chemokine (C-C motif) ligand 14; interleukin 8 receptor (beta); defensin beta (4A and 5) and CD40. Overall concordance was observed between the array and qRT-PCR findings (Supplementary table 3.2).

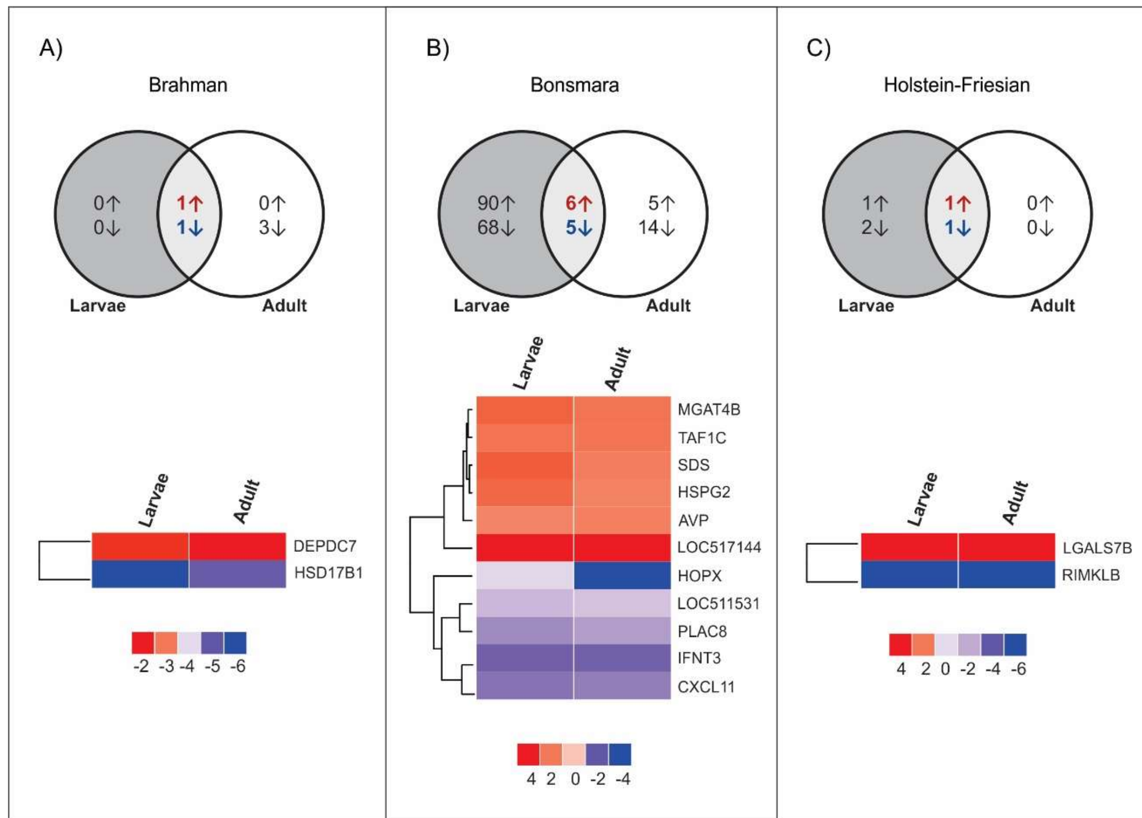

Figure 3.1: Differentially expressed genes shared between and specific to the larval and adult *R. microplus* life stages when compared to uninfested samples of the same cattle breed. Venn diagrams of DEGs and gene expression profiles of shared DEGs for (A) Brahman, (B) Bonsmara and (C) Holstein-Friesian cattle. Illustrated are Venn diagrams indicating the number of DEGs at the larvae and adult life stage compared to uninfested animals. Numbers of up-(red) and down-regulated (blue) genes are indicated. Corresponding gene expression profiles of genes differentially expressed in both life stages per cattle breed were highlighted using hierarchical clustering. Colours represent the log_2 transformed fold changes from red (up-regulated: +4) to blue (down-regulated: -6).

3.4.2. Functional annotation of differentially expressed lymph node transcripts in Bonsmara

Of the 183 unique transcripts identified as differentially expressed in Bonsmara lymph node tissues (above threshold and P-value ≤ 0.05) (Figures 3.2; Supplementary table 3.3), 13.7% could not be functionally annotated according to our outlined annotation criteria (Figure 3.2A). The remaining annotatable transcripts were separated using KOG identifiers into general functional classes and then into general functional processes, of which cellular and signalling processes represented the largest component of differentially expressed transcripts (~66%; Figure 3.2A). The former revealed four specific functional classes that were affected to a greater degree when

cattle were infested with *R. microplus*. These functional classes correspond to: transcription (K: ~8%); posttranslational modification, protein turnover and chaperones (O: ~11%); extracellular structures (W: ~14%); and signal transduction mechanisms (T: ~29%) (Figure 3.2A). Overall, the largest subsets of differentially expressed transcripts were associated with the larval life stage, especially transcripts involved in signal transduction mechanisms (Figure 3.2B: T). Moreover, the highest number of lymph node transcripts up-regulated during larval infestation was related to the extracellular structures functional class (Figure 3.2B: W).

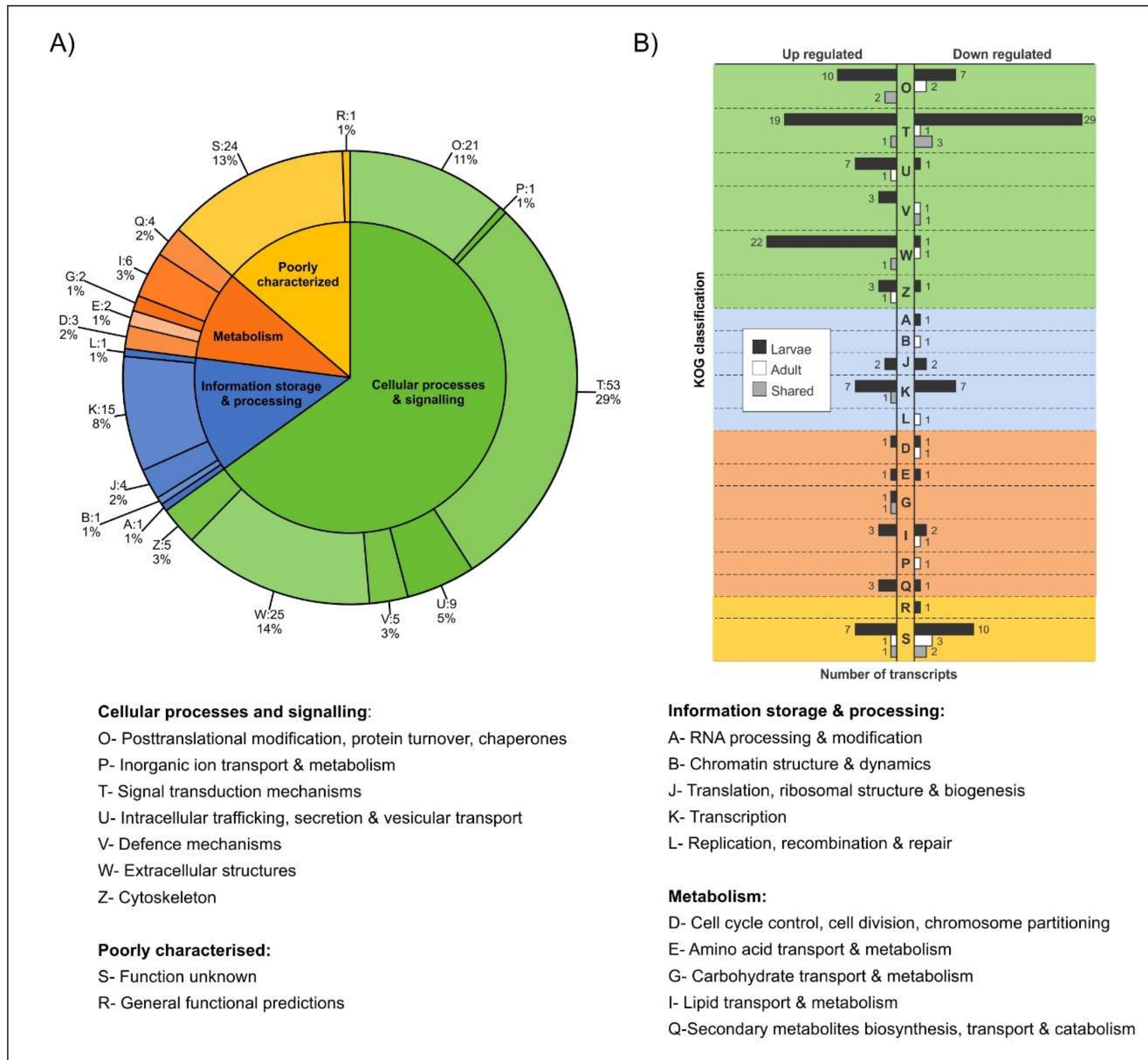

Figure 3.2: Differentially expressed genes in the lymph node of Bonsmara cattle as classified according to their eukaryotic orthologous functional groups (KOGs). A) Classification of genes differentially expressed in Bonsmara lymph node tissues during tick infestation (above threshold and *P*-value ≤ 0.05). The functional classifications are divided into three functional classes (metabolism, cellular processes and signalling, information storage and processing) and a “poorly characterised” class. B) Distribution, classification and level of differential expression of genes differentially expressed in Bonsmara lymph node tissues during tick infestation (above threshold and *P*-value ≤ 0.05). The number of transcripts either up-regulated or down-regulated as well as whether these occurred during the larvae, adult or both life stages are indicated.

3.4.3. Hierarchical clustering of differentially expressed transcripts: Bonsmara

Hierarchical clustering indicated that gene expression patterns within the lymph node tissues during adult tick feeding and uninfested cattle stages were more similar than when compared to the expression patterns during the larval feeding stage (Figure 3.3A). From Bonsmara cattle, the expression levels of the 183 DEGs at the three sampling points grouped into three main expression clusters determined from the pattern of the resulting branches (Figure 3.3A). Several of the DEGs were greatly up-regulated upon larvae infestation, but then expression reduced during the adult feeding stages to levels similar to that observed in non-infested conditions (Figure 3.3D: cluster 3). Similarly, some DEGs were down-regulated upon larvae infestation, and then returned to non-infested levels during adult feeding (Figure 3.3B: cluster 1). Cluster 2 (Figure 3.3C) represents an intermediate cluster that presented with a similar trend to cluster 3, but with less gene expression variability between the three sampling points in Bonsmara cattle. These results point towards a more intense response upon larval attachment with limited differential gene expression during adult feeding. Previous reports have suggested that the majority of tick rejection is seen during the larval life stage (Roberts 1968), and current clustering results appear to support some of these findings (Figure 3.3).

Pathway analysis with DAVID showed significant enrichment of three KEGG pathways namely, (1) cytokine-cytokine receptor interactions (bta04060), (2) the chemokine signalling pathway (bta04062) and (3) the Toll-like receptor pathway (bta04620). The comparable clustering patterns of these transcripts give an indication to their possible shared functions (Figure 2.3B and 2.3D). Eight related chemokines (CCL3 (LOC616364), CCL4, CCL5, CCL19, XCL1, CXCL9, CXCL10 and CXCL11) and two chemokine receptors (CCR6 and CX3CR1) were found as differentially expressed in Bonsmara lymph nodes. All of the latter chemokines (except for CCL19) and chemokine receptor CX3CR1 group together in cluster 1 (Figure 3.3B and 3.3D), showing down-regulation during larval feeding stages with only slight recovery during adult feeding. By contrast in expression cluster 3 (Figure 3.3D), CCL19 and CCR6 were shown as up-regulated upon tick attachment with subsequent down-regulation during adult feeding.

Figure 3.3: Hierarchical clustering of expression pattern of 183 genes that are differentially expressed in the lymph nodes of Bonsmara cattle infested with larvae and adult *R. microplus*. The colour bar of the M-values indicates a higher expression in red (2) and a lower expression in blue (-2) of the identified DEGs at the relevant uninfested, larvae infested and adult-infested sampling points.

3.5. Discussion

Findings indicate a dynamic immune response elicited in bovines during infestation of immature ticks (i.e. the larval life stage). A recent review on bovine immune responses to *R. microplus* infestation also indicated a dynamic regulation of cellular responses (e.g. granulocytes) between resistant and susceptible cattle breeds (Robbertse et al., 2017). An apparent delay in host immune responses was observed during the immature tick life stages (especially the larval life stage) in tick-susceptible breeds (Franzin et al., 2017; Robbertse et al., 2017) suggesting that animals of a susceptible breed develop a more gradual, slower cutaneous response to tick bites compared to resistant breeds. Several other studies have shown that resistance to ticks is acquired through tick exposure in susceptible, as well as resistant breeds (George et al., 1985; Hewetson and Lewis, 1976; Momin et al., 1991; Roberts, 1968; Wagland, 1980, 1975), with resistance being acquired sooner and to a higher degree in *B. t. indicus* breeds (Rechav et al., 1990; Riek, 1962; Wagland, 1980, 1978). As such, we propose that intra-breed comparisons performed at different tick life stages will provide more quality insights into the underlying dynamics of the development of an immune response in bovines.

3.5.1. Effect of tick attachment and feeding on the transcriptional profile of lymph nodes between Brahman, Bonsmara and Holstein-Friesians

To identify transcripts potentially involved in the host immune response to tick feeding, DEGs were subsequently compared between cattle breeds. Despite the few DEGs identified in the Holstein-Friesian and Brahman cattle used in this study, similarities in DEGs were noted between the cattle breeds.

Two similar shared transcripts were identified as differentially expressed in Bonsmara and Brahman cattle. Firstly, the transcript encoding hydroxysteroid 17-beta dehydrogenase 1 (HSD17B1) was found to be differentially expressed in Bonsmara (unique to the larval life stage) and Brahman (differentially expressed at the larvae and adult life stages) cattle breeds. Hydroxysteroid 17-beta dehydrogenases also catalyses the conversion of steroidal hormones between the active and inactive forms (Moeller and Adamski, 2009). In addition, the presence of hydroxysteroids 17-beta and 3-beta dehydrogenase have been reported in lymphoid organs during immunisation studies that were linked to inflammation (Mukhopadhyay et al., 2009). As no known link between HSD17B1 and bovine immunity is known, the significance of this result remains to be evaluated further.

Secondly, transcripts for olfactory-like G protein-coupled receptors LOC517144 (differentially expressed at the larvae and adult life stages) and OR9Q2 (unique to the adult life stage) were found to be differentially expressed in Bonsmara and Brahman, respectively. In 2016, Clark and colleagues described a possible role for these receptors on T-lymphocytes. They showed that ligand bound to the associated receptor increased cAMP levels and inhibited the rate of chemokine-driven chemotaxis of CD4+ T-cells from the footpad to the popliteal lymph node in mice (Clark et al., 2016). As T-cells within an affected tissue is a crucial component of adaptive immune inflammation, we propose that the up-regulation of this class of receptor throughout the life cycle of *R. microplus* could function to constantly hinder chemokine-driven chemotaxis of T-cells from the sites of inflammation to the lymph nodes. This hypothesis is supported by

observations that CD4+/CD3+ T-cell numbers are significantly decreased in the lymph nodes of Bonsmara (Robbertse et al., 2018).

The transcripts for two galectins (LGALS9 and LGALS7B) were identified in Brahman (unique to the larval life stage) and Holstein-Friesian (differentially expressed at the larvae and adult life stages) cattle, respectively. Galectins are a family of carbohydrate binding proteins that are expressed in both vascular and lymphatic endothelial cells, where they function in regulating leukocyte tissue entry during inflammation (Thiemann et al., 2015). In addition to playing a role in the regulation of DC migration, galectins also mediate DC activation via protein kinase C signalling (Thiemann and Baum, 2016). As DCs are also considered effective antigen-presenting cells in eukaryotes, they are a critical cell population that emigrates from inflamed tissue to regional lymph nodes. As such, the potential down-regulation of DC migration (via the galectins LGALS7B and LGALS9) and subsequent activation by the attachment and feeding of *R. microplus* larvae may offer a rapid way of supressing the activation of cellular immunity in the lymph nodes.

Another group of transcripts identified were claudins (CLDN2, CLDN3 and CLDN8). These transcripts were found to be differentially expressed in the larval life stage for Holstein-Friesian (CLDN2) and Bonsmara (CLDN3 and CLDN8) cattle. A claudin transcript (claudin-11) was also identified, where it was found to be differentially expressed in the skin of both tick-susceptible and resistant animals, compared to stressed (i.e. irritated) skin (Franzin et al., 2017). Claudins are tight-junction associated transmembrane proteins that control the contact between adjacent cells to create a barrier for paracellular movement of immune cells, water and other solutes across the endo- and epithelial cell layers (Turksen and Troy, 2004). The described claudin transcripts, however, have differential expression patterns compared to uninfested samples of the same breed (Supplementary table 2.3). This is in keeping with literature where it has been shown that differential expression of separate claudin genes regulate the “tightness” between adjacent cell contacts thereby affecting the permeability of the epi- and endothelial layers to facilitate immune cell infiltration (Turksen and Troy, 2004).

A small number of genes were found to be differentially expressed between the different sampling points for the Brahman and Holstein-Friesian cattle breed (Figure 3.1A and 3.1C). To overcome the latter, inbred animals (of known relatedness) and larger cohorts of animals will need to be tested in future to rule out the possibilities of (1) a lack of dynamic processes in the lymph nodes of these breeds, and (2) incorrect sampling point. Therefore, factors such as medical history and genetic variability may have contributed to the observed biological variation. All subsequent sections describe the genes found to be differentially expressed in Bonsmara cattle with a focus between life stages where applicable.

3.5.2. The effect of tick attachment and feeding on draining lymph nodes in Bonsmara cattle: An integrative perspective

Lymph nodes are encapsulated organs consisting of a matrix of fibroblastic reticular cells and mesenchymal tissues through which lymphatic fluids infiltrate to bring peripheral components of the immune response (such as professional antigen-presenting cells and free antigen) in contact with components of the adaptive immune response (consisting of B-cells and/or plasma cells, T-cells and macrophages) (Fletcher et al., 2015). Since the lymph node is a dynamic organ where

the spatial orientation of cells is related to their function, the following discussion aims to address the main steps in regulated lymphocyte trafficking in lymph nodes. These include: (1) Leukocyte recruitment to the lymph node via chemokines and chemotaxis, (2) Trans-endothelial and intranodal movement on the reticular network, (3) Active regulation of cellular transcription and translation in the lymph node (including leukocyte associated cellular regulatory networks) and (4) Chemokine receptors regulating the movement of cells out of the lymph node. The findings of the following sections have been integrated into a proposed mechanism of immune-effects in Bonsmara cattle in response to *R. microplus* (Figure 2.4, indicated in subheadings). This hypothesis may be used in future as a scaffold for testing of proposed pathways to finally obtain a more complete view of bovine immunity to *R. microplus* feeding.

3.5.2.1. Leukocyte recruitment to the lymph nodes via chemokines and chemotaxis (Figure 2.4; panel A, B and C)

During lymphocyte trafficking naïve lymphocytes enter the lymph node via the blood through the high endothelial venules (HEVs) and the afferent lymphatic vessels. Migration of naïve B and T-cells through the walls of the HEVs involves a multistep adhesion and migration cascade (Figure 3.4; panel B and C) (Butcher and Picker, 1996; Girard and Springer, 1995; Miyasaka and Tanaka, 2004; von Andrian and Mempel, 2003). Additionally, the afferent lymphatic vessels also deliver tissue-derived antigens and immune cells to the subcapsular sinus surrounding the lymph node (Forster et al., 1999; von Andrian and Mempel, 2003) (Figure 2.4; panel A).

Many differentially expressed chemokines (CCL3, CCL4, CCL5, CCL19, XCL1, CXCL9, CXCL10 and CXCL11) were identified in this study, that are important in the recruitment and entry of lymphocytes to the lymph node (Supplementary table 3.3). Chemokines function in coordinating the localization of immune cells to generate an immune response at very specific anatomic sites (Allen et al., 2007; Rot and von Andrian, 2004; Viola and Luster, 2008). These small chemotactic cytokines act by binding to specific target cell receptors that can bind multiple chemokine types (with high redundancy), whereby if one chemokine or receptor is not functioning, another can replace it (Malhotra et al., 2012). The human and murine chemokine system is well characterised (Zlotnik and Yoshie, 2012), however, data for bovine homologues is restricted to a handful of chemokines as reviewed by Widdison and Coffey (2011). As such, many of the following proposed functions are based on human and murine data and will need to be validated in bovines.

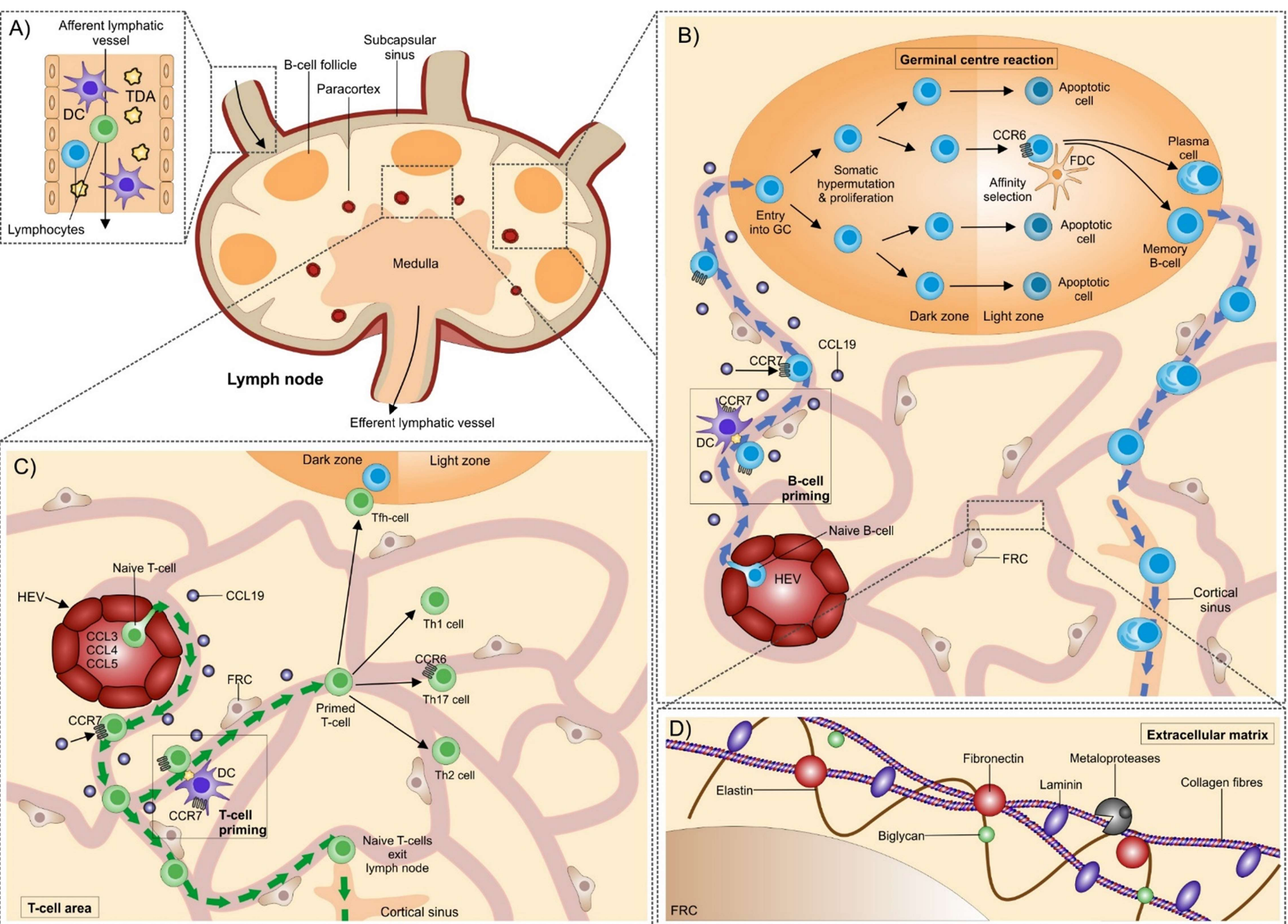

Figure 3.4: The effect of tick attachment and feeding on draining lymph nodes in Bonsmara cattle. Indicated is the process of leukocyte recruitment to the lymph nodes via chemokines and chemotaxis (panels A, B and C); Trans-endothelial and intranodal movement on the reticular network (panels B, C and D) and composition of the lymph node reticular network (panel D). The expression patterns of the transcripts indicated here are illustrated in Figure 2.3. Abbreviations used in the figure: Tick-derived antigen (TDA); dendritic cell (DC); high endothelial venule (HEV); fibroblastic reticular cell (FRC).

Three differentially expressed inflammatory chemokines CCL3 (MIP-1α, LD78a), CCL4 (MIP-1β, LAG-1) and CCL5 (RANTES) were identified (Figure 3.4, panel C), which are all CC chemokine ligand (CCL) subfamily members known to bind to the chemokine receptor CCR5. The CC chemokine ligand 3 and CCL4 are known to enhance T-cell recruitment to reactive lymph nodes (Tedla et al., 1998), wherein CCL4 is the most potent chemoattractant for CD4+ CD25+ regulatory T-cell populations (Bystry et al., 2001), in accordance with the KEGG pathway analysis (bta04620) (Baggiolini et al., 1997; Loetscher and Moser, 2000; Luster, 1998; Moser and Loetscher, 2001). A similar response has been described for bovine CCL4 and CCL5 during bacterial (*Mycobacterium bovis*) and viral (respiratory syncytial virus and bovine immune deficiency virus) infection (Werling et al., 2002; Widdison et al., 2008; Widdison and Coffey, 2011; Wright et al., 2002). In addition, CCL5 has also been identified to have a role in Paratuberculosis, mastitis and parasitic infections (Araujo et al., 2009; Buza et al., 2003; Griesbeck-Zilch et al., 2008; Pareek et al., 2005; Taubert et al., 2006; Widdison and Coffey, 2011). Overall, the down-regulation (Figure 3.3, cluster 1; Supplementary table 2.3) observed for these three chemokines upon larval infestation point towards an impaired recruitment of T-lymphocytes to the lymph nodes in Bonsmara cattle. This is in line with the observed suppression of KEGG chemotaxis pathways (as discussed previously) (Figure 3.4; panel C) and up-regulation of antichemotactic proteins such as the olfactory-like receptors.

3.5.2.2. Trans-endothelial and intranodal movement on the reticular network (Figure 2.4; panel B, C and D)

The chemokine CCL19 was found to be up-regulated in lymph node tissues during larval feeding (Figure 3.3, Cluster 3). This chemokine ligand binds to its receptor, CCR7, which has several downstream effects in the lymph node (Figure 2.4, panel B and C). In this study, CCR7 was found to be up-regulated using qRT-PCR during the adult feeding stage. Upon recognition of pathogen/parasite and/or their products, immature DCs begin a maturation process where the uptake and processing of pathogen-derived antigens is increased. This results in the activation of the CCR7 that is vital in the process of mediating homing of DCs to the T-cell zone of lymphoid tissues. In the current context, this involves processing, display and transport of tick-derived antigen(s) (TDAs) from the site of infestation to the site of immune response induction (Figure 2.4; panel B and C) (Milligan and Barrett, 2015). In addition, CCR7 is also expressed on naïve T-cells (Braun et al., 2011) and naïve B-cells (Forster et al., 2008; Miyasaka and Tanaka, 2004; von Andrian and Mempel, 2003) with its role in T-cell migration within peripheral lymph nodes validated by gene knockout experiments in murines (Forster et al., 1999; Gunn et al., 1999). We propose that Bonsmara cattle continue to up-regulate CCL19 in lymph nodes to sustain the follicular network and ensure T-cell priming upon entry, but that tick larvae may hinder migration of immune cells to lymphoid tissues. However, during adult feeding, *R. microplus* does allow for migration of immune cells to lymphoid tissues indicating that resistance is established during the larval stages (as supported by clustering of data, Figure 2.3).

In addition, alpha-1,3-mannosyl-glycoprotein-4-beta-N-acetylglucosaminyltransferase B (MGAT4B) was found in this study to be up-regulated during both immature and adult life stages (Figure 3.3, Supplementary table 3.3). This transferase functions in the biosynthesis of GlcNAcbeta1-4 branching within the N-linked glycan core structure. This is important as recruited

lymphocytes infiltrate the lymph node by traversing the endothelial layer of the HEVs using L-selectin receptors that recognize and bind to abundant 6-sulfo sialyl Lewis X (sialic acidα2-3Galβ1-4(Fucα1-3(sulpho-6))GlcNAcβ1-R) sugar moieties present in the N-and O-glycan cores of the sialomucins that decorate the HEV surface (Figure 3.6, panels B and C) (Kawashima et al., 2005; Uchimura et al., 2005). It appears that during tick attachment and feeding (at both the immature and adult life stages), trans-endothelial migration of lymphocytes into the lymph node are maintained.

Following infiltration, T-cells migrate to the paracortex while B-cells localize to the cortical lymphoid follicles (Figure 3.4, panels B and C) (Bajenoff et al., 2006). Infiltration and migration of these cell types within the lymph node tissues are mediated by chemokines such as the chemokine ligand CCL19 (up-regulated upon larvae attachment) that is produced by paracortical FRCs and by cortical follicular dendritic cells (FDCs) as reviewed by Girard et al. (2012). These stromal cells (including endothelial cells, FDCs and FRCs), provide a functional scaffold for migration mediated by attraction and adhesion of immune cells. Follicular dendritic cells are responsible for building the complicated reticular network by producing extracellular matrix components that interweave to form the reticular fibres criss-crossing the cortex (Figure 3.4; Panel B, C and D) (Gretz et al., 2000, 1997, 1996; Kaldjian et al., 2001; McKnight and Gordon, 1998). This network covers much of the lymph node and provides ultrastructural support, as well as spaces and pathways for immune cell movement.

Modification of the extracellular matrix was evident during tick infestation as several types of collagen (1-6, 15 and 18), as well as collagen-associated molecules such as fibronectin (FN1), elastin (ELN), biglycan (BGN), thrombospondins (THBS1 and AMTS5) were differentially expressed (Figure 3.3). Several secreted proteases and protease inhibitors were also identified as differentially expressed within Bonsmara lymph node tissues (Figure 3.4, Panel D; Supplementary table 3.3). These alterations in the lymph node environment through changes in the extracellular matrix structures facilitate leukocyte migration, differentiation and inflammatory functions at the early stages of infestation (Hemler, 1990). Many associated extracellular matrix receptor interaction pathways were identified (KEGG pathway ID: bta04512), containing several DEGs in lymph node tissues of larvae infested Bonsmara. These both function in cell-cell and cell-extracellular matrix adhesion processes (Holzmann and Wagner, 2012), which facilitate T-cell and monocyte migration (Hemler, 1990), as well as signal transduction to mediate T-cell and B-cell proliferation through interactions with fibronectin (Holzmann and Wagner, 2012). In this regard, fibronectin 1 (FN1) was identified as up-regulated upon larval attachment in this study (Supplementary table 3.3), supporting the latter effects on cellular adhesion changes in lymph node tissues. Of interest is the fact that the transcription factor SPDEF (up-regulated at larval tick attachment), appears to be co-regulated with several collagens (COL1A2, COL2A1, COL4A1 and A2), as well as fibronectin 1 (FN1) and elastin (ELN) (Figure 2.3, cluster 3). As it has been shown previously that SPDEF regulated expression of metalloproteinase 9 (a collagenase for type 4 collagen) (Johnson et al., 2010), it does lend some support for its co-regulation with collagen related genes. However, SPDEF has not been previously confirmed as transcription factor for these genes directly in bovines (or in other species) and requires further investigation.

Overall, all the data provides evidence for a transcriptional regulatory network that is activated during immature tick infestation that is down-regulated towards basal transcriptional levels

(relative to uninfested levels) when adult ticks are feeding. In regard to extracellular structures, almost all genes involved show the highest up-regulation upon larval attachment compared to adult tick-infested or uninfested cattle. These changes suggest that modulation of lymph node physiology is most obvious during larval infestation and does not persist into the adult phase of attachment and feeding. It thus appears that larval infestation does prime the lymph tissues for development of the immune response. To the best of the authors' knowledge no direct evidence has been provided that indicates that ticks are able to affect the maturation process of lymph node tissues. Several additional transcriptional networks were identified that are described in the following section.

3.5.2.3. Active regulation of cellular transcription and translation in the lymph node

Transcriptional initiation and control is a key process leading to cellular activation (metabolism and differentiation). Transcription factors and other proteins of the transcription initiation complex are responsible for a coordinated and regulated gene expression. Twelve genes related to these functions were found as differentially expressed in lymph tissues during tick feeding on Bonsmara cattle (Supplementary table 3.3). Of these, 5 transcription factors (KLF16, SPDEF, ZNF771, CREB3L2 and EPAS1) were highly expressed during feeding of immature stages, while a TATA box binding protein-associated factor (TAF1C) was up-regulated and maintained following tick infestation (Figure 3.3; Supplementary table 3.3). The latter is involved in assembly of the preinitiation complex for RNA polymerase I-dependent transcription of ribosomal RNA genes (Russell and Zomerdijk, 2005). Concomitantly, two 60S ribosomal proteins (RPL27 and RPL10 (LOC785386)) were shown to be up-regulated during the immature life stage, forming a cluster with other similarly regulated transcripts involved in: transcriptional initiation (EPAS1); intracellular protein trafficking (KDELR3); extracellular matrix (TIMP1 and ECM1) and membrane permeability for small solutes and water (AQP3) (Figure 3.3, Cluster 3; Supplementary table 3.3). The following sections will describe specific transcription factors and their associated cellular networks in leukocytes.

3.5.2.3.1. Leukocyte associated cellular regulatory networks

Both transcription factors KLF16 and ZNF771 are associated with cluster 2 and appear to be co-regulated with a TRAF-interacting protein (TIFAB) and complement factor B (CFB) (Figure 3.3). The TIFAB protein is a cytosolic protein that acts as a negative regulator of Toll-like receptor-TRAF6-mediated NF-kB signalling for immune cell maturation and has been shown to be highly expressed in immature dendritic cells and macrophages (from bone marrow), as well as naïve splenic B-cells (Matsumura et al., 2009; Varney et al., 2015). The CFB protein itself is produced mainly in the liver and extrahepatic macrophages/monocytes (includes fibroblasts and other epithelial and endothelial cells) functioning as a circulating component of the alternative pathway of complement activation in the blood that eventually leads to pathogen cytotoxicity, inflammation and immunological regulation (including B-cell proliferation, macrophage spreading and apoptosis) (Huang et al., 2002; Kerr, 2013; Merle et al., 2015). The rate of production of CFB is controlled by cytokines, such as interleukin-1 and interferons, as well as pathogen-derived lipopolysaccharides (Kerr, 2013). The regulation of CBF in macrophages is achieved by

proinflammatory cytokines TNF-α and IFN-γ through the intracellular action of STAT1 and IFN-regulatory factors (IRFs) (Huang et al., 2002). The signal transducer and activator of transcription (STAT1) was found to be down-regulated following infestation, though levels appear to have recovered in later feeding stages (Figure 3.3, Cluster 1; Supplementary table 3.3).

Of great interest is the T-bet transcription factor (TBX21) (Figure 3.3, Cluster 1), which remains largely suppressed throughout all stages of tick feeding. This protein is known to play a central role in both the adaptive and innate immune responses where it affects the survival, development and proper functioning of dendritic cells, natural killer (including natural killer T) cells, innate lymphoid cells, CD4+ and CD8+ T effector cells, B-cells, γδ T-cells and certain regulatory T-cells (Lazarevic et al., 2013). Lastly, an aminoacyl-tRNA synthetase (WARS) and a tRNA-yW synthesising methyltransferase (TYW3) were also down-regulated during initial infestation (Figure 3.3, Cluster 1), of which WARS has been indicated to play a regulatory function for activation of p53 via IFN-γ (Petrossian and Clarke, 2011; Yao et al., 2013). Since there is no published correlation between the significance of p53 activation and ectoparasite infestation, it is unclear what role it plays and requires further investigation.

3.5.2.3.2. T-lymphocytes

The interferon regulatory factor, IRF4, was also found to be up-regulated in lymph node tissues following tick infestation and then diminished during adult tick feeding stages (Figure 3.3, Cluster 3; Supplementary table 3.3). This protein is a member of the IRF family of transcription factors involved in differentiation of multiple lineages of CD4+ T-helper cells that is activated by certain conditions (such as cytokines in particular) to determine the fate of the T-helper cell (Huber and Lohoff, 2014; Xu et al., 2012). IRF4 is also involved in the development of cytotoxic T-cell responses, as well as differentiation of CD4+ follicular helper cells in secondary lymphoid germinal centres for production of high-affinity neutralizing antibodies (Crotty, 2011; Kaech and Cui, 2012; Kratchmarov et al., 2017). Moreover, due to the additional role of IRF4 in CD8+ T-lymphocyte responses this transcription factor has functions in both humoral and cellular immunity. Also, selective manipulation of IRF4 expression has been suggested as a strategy to boost immunity in people who are poor vaccine responders (e.g. infants and elderly) (Yao et al., 2013). It is unlikely that the tick directly affects IRF4 expression in its target cell, but rather the production of upstream cytokine production.

Expansion and differentiation of CD8+ T-cells are also regulated by T-box transcription factors T-bet (TBX21) and eomesodermin (EOMES) (Figure 3.3, Cluster 1) (Kaech and Cui, 2012; Lazarevic et al., 2013; Zhang and Bevan, 2011). Both these transription factors were down-regulated following immature tick infestation, with only eomesodermin transcription appearing to recover to a degree during the adult feeding stages (Figure 3.3, Cluster 1; Supplementary table 3.3). It has been shown that differential expression of T-bet is involved in differentiation/maturation of IFN-γ-producing T-helper cell sub lineages as reviewed by Lazarevic and colleugues (2013). However, IRF4 acts as a repressor of the JAK/STAT1 pathway in macrophages (Malyshev and Malyshev 2015). An interplay is evident from transcriptional profiles of TBX21, EOMES and STAT1 (Figure 3.3B), as well as IRF4 (Figure 3.3D). A suppressive effect of T-cell maturation via this mechanism/pathway is most apparent during the tick larval feeding stage.

3.5.2.3.3. Natural killer cells

Additional transcripts encoding receptors (including CD224 (2B4); KLRF1; KLRC1; KLRD1 (CD94); KIR3DL1; FCGR3a (CD16a)) and receptor-signal activators on natural killer cells (KIR2DS1), as well as the secretion of their associated products such as granzymes A (GZMA), M (GZMM) and Y (GNLY) that are involved in apoptotic cell death of infected target cells were co-repressed along with eomesodermin (Chowdhury and Lieberman, 2008; Dotiwala et al., 2016; Finton and Strong, 2012; van Stijn et al., 2008) (Figure 3.3, Cluster 1; Supplementary table 3.3). The latter indicates a possible transcriptional link and down-regulation of differentiation of CD8+ T-lymphocytes into natural killer T-cells or cytotoxic T-cells during the immature feeding stages of *R. microplus* on Bonsmara cattle (Connelley et al., 2014). Such activity was first reported from salivary gland extracts of *Dermacentor reticulatus* (Kubes et al., 1994), with similar suppressive activities recorded for salivary gland products of *Amblyomma variegatum, Haemaphysalis inermis* and *Ixodes ricinus* (Farber, 1997; Kotál et al., 2015). Natural killer cells are produced from innate lymphoid cells that can differentiate and mature in the lymph nodes (amongst other tissues) and can, apart from their cytotoxic functions, regulate T-cell interactions of endothelial, dendritic, and macrophage cell lineages (Kotál et al., 2015; Vivier et al., 2008). Combined, the latter suggests that in response to tick infestation there is a suppression of cytotoxic T-cell immunity in the host.

3.5.2.4. Chemokine receptors regulating the movement of cells out of the lymph node

The chemokine receptor CRR6 was found to be up-regulated upon tick attachment (reverting back to basal uninfested levels during adult feeding) in the lymph nodes of Bonsmara cattle (Figure 3.3, Cluster 3). It is known that human memory and effector CD4+ TH-17 cells and a subset of TH1 cells (Acosta-Rodriguez et al., 2007), as well as dendritic cells (White et al., 2013) express CCR6. This receptor together with its ligand (CCL20) is responsible for the chemotaxis of dendritic cells, effector and/or memory T-cells and B-cells (Homey et al., 2000). In addition, this ligand-receptor pair plays an important role at skin and mucosal surfaces under homeostatic and inflammatory conditions where CCR6 is essential for the recruitment of both proinflammatory IL17 producing helper T-cells and regulatory T-cells to sites of inflammation. The up-regulation of this chemokine receptor suggests its importance in the movement of immune cells from the lymph node to the site of infection/ tick attachment. Therefore, normal recruitment of cells to the feeding pool may not be directly influenced, but that the tick may influence the further maturation (signals/signal production) of effector cells that would lead to an antibody or cytotoxic-mediated response. With regards to antibody production it was seen that related immunoglobulin transcripts (IGHA1 and IGCGAMMA) followed a similar trend in expression as CCR6 and CCL19 (Figure 2.3D). This may indicate a decrease in immunoglobulin production by B-cell during the adult tick feeding stage.

The expression of CX3CR1, a chemokine receptor, was down-regulated upon larval tick attachment with slight up-regulation upon adult tick feeding. This receptor has been shown in humans to bind fractalkine (a membrane bound chemokine) which mediates T-cell migration to sites of inflammation (Moser and Loetscher, 2001). The binding of ligands to the CX3CR1 receptor mediates both the adhesive and migratory functions of T-cells leading to migration of this

cell type to its effector sites (Fujita et al., 2012; Imai et al., 1997). Efficient recruitment of T-cells into inflammatory lesions requires high-affinity adhesive interactions of effector T-cells with the inflamed endothelial cell lining of draining blood vessels (Schwab and Cyster, 2007). In addition to an increased number of integrin ligands such as Thrombospondin-1 (THBS1, which was up-regulated upon larval attachment), the ligand of CX3CR1 may contribute to the enhanced adhesive effect of effector T-cells that express the fractalkine receptor CX3CR1 which was down-regulated in the larval stage of tick feeding (Fong et al., 1998; Imai et al., 1997). The apparent down-regulation of the transcription of CX3CR1 suggests that the mechanism of T-cell migration to the site of inflammation (tick attachment) may be negatively affected by tick feeding at the larval life stage.

From a companion histological study, the recruitment of CD3+ T-lymphocytes to the site of tick attachment was only significantly increased during the adult tick feeding stage in Brahman and Bonsmara cattle (Robbertse et al., 2018). A study performed by Franzin and colleagues (2017), using tick-naïve cattle, also showed a significant increase in CD3+ T-lymphocytes in resistant compared to susceptible cattle. Moreover, the infiltration of CD3+ T-lymphocytes increased more drastically in tick-resistant Brahman cattle compared to the more susceptible Holstein-Friesian breed (Franzin et al., 2017; Robbertse et al., 2018). From both studies, infiltration of CD3+ T-lymphocytes are minimal during the immature feeding stages for susceptible breeds, which correlates with findings in this study. For Bonsmara cattle at least, some recovery of CD3+ T-lymphocytes is evident following immature tick feeding (Robberste et al., 2018), which is also supported by the transcriptional data presented in this study. Overall, at a transcriptional level, there seems to be a possible suppression lymphocyte maturation signals that is especially evident during the larval feeding stage.

3.6. Concluding remarks

This work provides the first comparative transcriptomic study of lymph node responses in tick-infested cattle, as well as the first transcriptome study performed for the South African Bonsmara breed. From these findings it is evident that bovine immune response to tick infestation is a highly dynamic and complex process, stressing the need for caution when considering correlations between life stages and time-points. The comparative transcriptomic analysis presented could only identify a limited number of DEGs in Brahman and Holstein-Friesian cattle. Therefore, additional deep sequencing will be performed to compliment and expand the current transcriptional data for the three breeds in this study. However, for Bonsmara cattle several transcriptional immune related mechanisms could be identified that has provided some resolution as to the host response during tick feeding. Transcriptional data suggests that lymph node tissue maturation and trans-endothelial migration of lymphocytes appears to be maintained during tick attachment and feeding. While the suppression of maturation of the cattle hosts immunity is especially evident during the larval feeding stages. The down-regulation of chemokines suggests altered recruitment of lymphocytes to the lymph node, while the down-regulation of components involved in maturation and up-regulation of proteins involved in cell repressor pathways suggests an immune-repressive action. Combined, this suggests that in response to tick infestation, there is a suppression of maturation of many cytotoxic lymphocyte lineages (on a transcriptional level), that may further impact lymphocyte migration to the site of inflammation (tick attachment). In

contrast to studies performed on peripheral (local skin) responses, results shown in this study highlight the importance of understanding lymphocyte development in secondary lymphoid organs, from the point of initiation to the mounting of an adaptive immune response. The data presented here shows the regulation of several genes and future studies should be included to improve on these findings. Firstly, the inclusion of proteomic and/or cellular responses for specific cell subtypes in the lymph node (in addition to transcriptomic studies) is needed to clearly define cell-specific responses. Secondly, to better understand the resultant immune response, concurrent studies of the tick together with the host may give better insight into this temporal interaction.

3.7. Acknowledgements

The authors would like to thank Mr Nicholas Olivier from the ACGT Microarray Facility, University of Pretoria, for his assistance and support with the microarray experiment.

3.8. References

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Chapter 4: *In vivo* evaluation of *Ixodes ricinus* induced effects on T and B-cell maturation in the spleen and lymph nodes of BALB/c mice

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This chapter serves as a draft manuscript to be submitted to Parasite Immunology. For uniformity throughout the thesis, the references in the chapter are formatted to the Ticks and Tick-borne Diseases referencing style. Luïse Robbertse, Christine Maritz-Olivier and Marie Jalovecká planned the initial experimental design. Luïse Robbertse designed figures and tables and wrote the first manuscript draft. Luïse Robbertse and Christian Stutzer performed the experimental procedures. Luïse Robbertse, Christine Maritz-Olivier and Christian Stutzer conceptualized the manuscript focus, critically revised the draft manuscript, and approved the final manuscript.

4.1. Abstract

Despite the inability of the BALB/c mice to acquire resistance against *Ixodes ricinus* nymphal attachment after multiple infestations, immune responses in naïve animals to tick infestations are inevitable. To investigate this, our study aimed to characterise the *in vivo* immune response of tick-naïve and nymph extract immunised BALB/c mice infested with *I. ricinus* nymphs. This was done by characterising B and T-lymphocyte populations in the draining lymph nodes and spleens of these BALB/c mice. Additionally, the T-helper 2/ T-helper 1 cytokine ratios from *in vivo* isolated lymphocytes from tick-infested mice were evaluated. Results indicate that immunising BALB/c mice with nymph extract does not provide protection to subsequent tick infestations. Additionally, a B-cell population (CD45+ CD19+ IgM+ CD27+ CD80+) was uniquely up-regulated during tick feeding and significantly down-regulated in immunised mice at 9 and 8 days post infestation, respectively. Regarding T-cells, no significant differences in the numbers of CD45+ CD3+ CD4+ T-helper cells were noted in tick-naïve or nymph extract immunised mice compared to their controls. Concurrently, a T-helper cell subset (CD45+ CD3+ CD4+ CD195+ CD184-) was significantly decreased in tick-naïve mice at 12 days post infestation. A slight significant decrease in T regulatory cells (CD45+ CD3+ CD4+ CD25+) was observed in only nymph extract immunised mice. The results presented in this study are the first to describe populations of T and B-lymphocytes in the lymph nodes of tick extract immunised and tick-naïve mice in response to infestation. Although additional studies with a finer sampling time scale and *in vitro* stimulation of lymphocytes are needed to further describe the observed lymphocyte populations, the results presented indicate that unique cell populations are affected by tick extract immunisation and tick feeding.

Keywords: Immune response; *Ixodes ricinus*; B-lymphocytes; T-lymphocytes, Flow cytometry

4.2. Introduction

Ticks have the ability to affect the immune system of their host (Mans et al. 2008; Wikel 2013; Wikel et al., 1994). They achieve this by inserting their mouth parts into the skin of their host and secreting a cocktail of salivary gland-derived molecules into the feeding lesion. It has been suggested that immunological interactions at the parasite-host interface are vital not only in tick feeding but also in the transmission of tick-borne pathogens (Wikel 2018). Ticks are known to transmit a wide range of pathogens resulting in the development of diseases that are detrimental to the health of the host (Jongejan and Uilenberg 2004). These pathogens include protozoa, bacteria and viruses transmitted, by an array of ixodid tick species (de la Fuente et al., 2008; Kazimírová and Štibrániová 2013; Shaw-Yhi 2015). Due to the impact of these parasites and their associated diseases on both animal and human health, studying the effects of tick attachment and feeding on host immunity in a model system within a controlled laboratory environment may provide insight into the mechanisms by which ticks evade host immune responses detrimental to their survival.

A number of laboratory models namely, guinea pigs, rabbits and mice have been used to study the acquisition of immunological resistance (represented by a decrease in tick numbers) to repeated tick infestations (Reviewed in Wikel 1996; 2013; 2018). With regards to studies in laboratory mice, host acquired resistance via tick saliva (by means of infestation) has only been documented for certain tick species, i.e. *Dermacentor variabilis* (Hollander and Allen 1985) and *Haemaphysalis longicornis* (Wada et al., 2010) in BALB/c and C57BL/6 mouse strains, respectively. In contrast, it has been found that mice do not acquire resistance against *Rhipicephalus sanguineus* in C3H/HeJ mice (Ferreira and Silva 1999); *Ixodes scapularis* in BALB/c and C3H/HeN mice (Schoeler et al., 1999; 2000); *Ixodes pacificus* in C3H/HeN mice (Schoeler et al., 2000) and *Ixodes ricinus* in BALB/c, C3H or C57BL/6 mice (Borsky et al., 1994; Christe et al., 1999). Despite the inability of the murine host to acquire a protective response, the host immune system responses (such as lymphocyte proliferation and cytokine production) are altered upon exposure to *I. ricinus* nymphs (Ganapamo et al., 1995; 1996a; 1996b; 1997; Christe et al., 1999; Mejri et al. 2002).

In this regard, proliferation of lymph node derived lymphocytes is increased by *in vitro* stimulation with salivary gland extracts from partially engorged *I. ricinus* females (Ganapamo et al., 1995; 1997; Mejri et al., 2002). The aforementioned lymph nodes were collected from BALB/c mice 9 days post infestation with *I. ricinus* nymphs. In contrast, a decrease in T-lymphocyte proliferation from both tick-naïve and BALB/c mice previously infested with *I. ricinus* larvae (Ganapamo et al., 1997) or nymphs (Ganapamo et al., 1996b; 1997; Mejri et al., 2002) is seen. Ganapamo and colleagues (1997), showed that 9 days post tick infestation with larvae or nymphs, BALB/c lymph nodes had a CD4+ T-cell specific immune response when stimulated with salivary gland soluble antigen from partially fed female adults. While lymph node derived CD4+ T-cells, collected 9 days after *I. ricinus* nymph infestation, have a decreased cell proliferation response to Concanavalin A (Ganapamo et al., 1996b). Meijri and colleagues (2002) additionally found that tick-naïve BALB/c mice spleen derived lymphocytes stimulated *in vitro* with *I. ricinus* adult female saliva had a decreased T-cell proliferation in response to Con A.

With regards to *in vitro* cytokine profiles, lymph node derived lymphocytes from BALB/c mice infested with *I. ricinus* nymphs 9 days prior to collection produced high levels of IL-10 (with no significant IL-5) (Ganapamo et al., 1996b) and high levels of IL-4 (with low levels of IFNγ) (Ganapamo et al., 1995) after *in vitro* stimulation with Con A. The higher expression of

IL-4 and lower expression of IFNγ (in response to Con A stimulation) was also confirmed by Christe et al. (1999) for BALB/c, DBA, C57BL/6, C3H, CBA, SJL and FVB strains of mice. In addition, *in vitro* stimulation of lymph node lymphocytes with salivary gland extracts from *I. ricinus* females only stimulates IL-4 (Ganapamo et al., 1995; Mejri et al., 2002) expression and not IFNγ (Ganapamo et al., 1995). In addition, lymph node derived lymphocytes from BALB/c mice infested with *I. ricinus* nymphs produced increased levels of IL-2, TNFα and GM-CSF when stimulated *in vitro* with Con A or anti-CD3 antibodies (Ganapamo et al., 1996a). Lymph node derived CD4+ T-cells (collected 9 days after *I. ricinus* nymph infestation) produced increased levels of IL-2 and GM-CSF, while repeated infestation led to an increase in IL-2 production (Ganapamo et al., 1996a).

As an extension to the immune response elicited by tick feeding, limited literature is available describing the effect of immunisation with whole tick extract on host immunity in the aim to elicit a protective immune response in murines. However, this has been described, to some extent, in guinea pigs. Immunity in guinea pigs has been raised against *Ixodes holocyclus* (Bagnall 1975); *Dermacentor andersoni* (Allen and Humphreys 1979); *Rhipicephalus sanguineus* (Garin and Grabarev 1972) and partial immunity to *D. variabilis* (Trager 1939).

The overall aim of this study was to characterise the *in vivo* immune response of tick-naïve and immunised BALB/c mice infested with *I. ricinus* nymphs. Here we immunised BALB/c mice with whole nymph extract prepared from *I. ricinus* ticks and subsequently infested these mice to see if any resistance to tick infestation was obtained. We characterised B-lymphocyte populations in the draining lymph nodes and spleens of these BALB/c mice. Additionally, we evaluated T regulatory and T-helper lymphocytes populations in these tissues. Finally, we compared T-helper 2 / T-helper 1 cytokine ratios from *in vivo* isolated (but not *in vitro* stimulated) lymphocytes from tick-infested mice.

4.3. Materials and methods

4.3.1. Experimental laboratory animals and ticks

Six- to eight-week-old specific pathogen-free (SPF) female BALB/c mice (Charles River Laboratories International, Inc.) were used for the experiments. The mice were housed in plastic cages with sterilized wood-chip bedding for three weeks prior to the start of the study in a specific pathogen free room under constant temperature of 22°C and relative humidity of 65%. Sterilized pellet diet and water was provided *ad libitum*. *Ixodes ricinus* nymphs were obtained from the breeding facility of the Institute of Parasitology, Biology Centre, CAS. Ticks were maintained in wet chambers with a humidity of about 95%, temperature 24°C and day/night period set to 15/9 h. All experimental animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 Sb., ethics approval No. 161/2011.

4.3.2. Mapping of regional draining lymph nodes for the site of tick infestation

The following method was used to map the lymph nodes draining the planned area of skin to be used to feed ticks. Three mice were anaesthetised with 150 μl of anaesthetic, consisting of 1.625% Narkamon and 0.35% Rometar (Spofa a.s., Czech Republic), formulated in 1 x PBS and injected interperitoneally. Dye injections were performed using 5% Evans Blue (Sigma, St. Louis, MO) in 25 μl PBS, delivered using a 0.5 ml syringe with a 30-gauge needle (Becton-

Dickinson). The dye was injected subcutaneously in two locations in the dorsal region in a 2cm^2 area located mid-way between the front and rear limbs. This region was selected as it matches the region of the tick-container placement. Mice were euthanised via cervical dislocation 30 minutes following injection of the dye and dissected to locate the lymphatic vessels and lymph nodes for evidence of the presence of the dye.

4.3.3. Experimental set-up and infestation protocols

Two complementary studies were performed, one with naïve tick-infested mice (Study A) and one with immunised tick-infested mice (Study B). The studies were conducted as outlined in Figure 4.1.

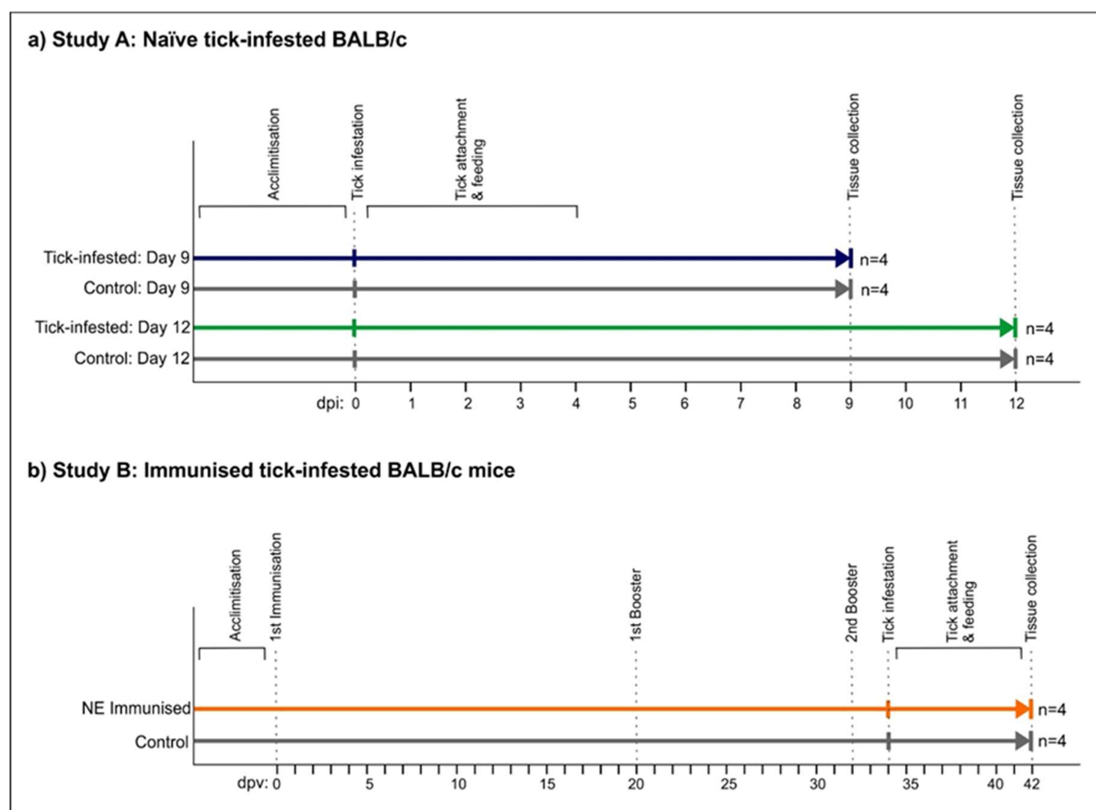


Figure 4.1: Experimental set-up and tick infestation protocol. a) Study A: Indicated are the experimental (tick-infested) and control groups for sampling points at 9 and 12 days post tick infestation (dpi). The mice in the tick-infested and control groups were sacrificed and tissues were collected at days 9 and 12 dpi. b) Study B: Indicated are the experimental (Nymphal extract (NE) immunised, tick-infested) and control (tick-infested) groups. Mice were initially immunised at day 0 and 2 booster immunisations were administered at 20 and 32 days post vaccination (dpv). Both control and experimental groups were infested at 34 dpv and samples were collected at 8 dpi (42 dpv). For both each experimental and control group in both study A and B consisted of four BALB/c mice. Mice acclimatized for a period of 21 days before the start of experimental procedures.

4.3.3.1. Study A: Naïve tick-infested mice

Two tick-infested groups (n = 4 per group) corresponding to 9 and 12 days post tick infestation (dpi) were run concurrently with their respective uninfested controls (n = 4 per group) as outlined in Figure 4.1a. On the day of tick infestation, the fur was clipped from the

dorsal regions of mice and capsules were placed on mice of the experimental and control group. The capsules were made from modified 5.0 ml microcentrifuge tubes securely attached to the dorsal region of each mouse to yield a 2 cm^2 area. The capsules were attached using a thin layer of adhesive (Pattex contact adhesive, Henkel corporation, USA). After assuring that the patches were attached securely and 20 *I. ricinus* nymphs were placed into the feeding chamber. Ticks were allowed to attach to the host and feed to repletion. The fully fed and dropped nymphs were collected, counted and weighed. Mice were euthanised on days 9 and 12 post tick infestation.

4.3.3.2. Study B: Nymph extract immunised tick-infested mice

For the immunisation study, a nymph extract was prepared consisting of engorged whole pathogen-free *I. ricinus* nymphs (n = 25) homogenized in 500 µl 1x PBS containing protease inhibitors (final concentration 0.37 mg/ml of cOmplete™ ULTRA protease inhibitor cocktail, Sigma-Aldrich, Germany). Ticks were homogenized using mechanical shearing and the resultant crude homogenate was resuspended with cold 1x PBS (also containing protease inhibitor) and washed twice via centrifugation (500 x g for 10 min at 4˚C). A final extract was prepared by additional homogenization by sonification of the cell pellet using an ultrasonic cell disruptor (Branson Ultrasonics, USA) for 10 pulses (1 sec pulse/rest, 3W output). The insoluble fraction (containing cellular debris) was removed by centrifugation at 10,000 xg (20 min, 4°C) and the supernatant was transferred and filled to a final volume of 1 ml with cold 1x PBS (containing protease inhibitor). The protein concentration of the nymphal extract (NE) immunogen was determined using a NanoDrop-1000 (Thermo Fisher Scientific, USA).

BALB/c mice were separated into control and test groups, 4 animals per group (n = 4) as described in Figure 4.1b. The test group was immunised with freshly prepared NE (30 µg) combined with Freund´s (complete and incomplete) adjuvant (50:50, v/v) in a final volume of 100 µl per immunisation. Control animals were injected with 50 µl PBS and 50 µl of adjuvant. Mice were injected intraperitoneally with the initial formulation containing Freund´s complete adjuvant (day 0), followed by two booster injections at 20 and 32 days post initial vaccination (dpv) with Freund´s incomplete adjuvant (Figure 4.1b). Capsules were placed on mice (as previously described) 35 dpv and 20 *I. ricinus* nymphs were placed into the feeding chamber. The nymphs were allowed to feed to repletion (8 dpi), collected and analysed (i.e. death on host, weight at fall-off and duration of obtaining the blood meal) (Prevot et al. 2007). Mice were finally euthanised (42 dpv) and whole blood collected via cardiac puncture. The collected blood was allowed to separate at 4°C and the blood cells were collected following centrifugation at 10,000 xg (20 min). Individual sera obtained from control and NE immunised animals were pooled and subjected to serum protein determinations. Total serum protein and IgG was determined using the QuikStart™ Bradford (Bio-Rad, USA) and Mouse total IgG (Cayman Chemical Company, USA) microtiter assays, respectively, according to manufacturer's guidelines. Final protein concentrations were expressed as g/dl as per Zaias et al. (2009).

4.3.4. Spleen and lymph node collection, processing and leukocyte isolation: naïve and immunised mice

Following the administration of anaesthesia, mice were euthanised via cervical dislocation. Spleens and subiliac lymph nodes (identified as the regional draining lymph node) were aseptically removed from all mice groups taking care to remove the surrounding fascia. Single

cell suspensions were prepared from whole spleens (in 1000 µl PBS, containing protease inhibitor) and lymph nodes (in 100 µl PBS, containing protease inhibitor) by gentle extrusion through plastic sieves (Corning ® Cell Strainer, 70 µm). Samples were centrifuged (500 xg for 10 min, 4˚C) and the resulting supernatant was collected and used for cytokine detection as described below. Erythrocytes in the splenocyte and lymphocyte suspensions were lysed with an ammonium chloride-based lysing reagent (BD Pharm Lyse™, BD Biosciences, USA) at room temperature for 3 min as per manufacturer's instructions. Sample volumes were adjusted to 30 ml with 1 x PBS then centrifuged (500 xg for 10 min, 4˚C). Samples were then additionally washed with FACS buffer (containing 1x PBS and 0.05% NaN_3) and then centrifuged (500 xg for 10 min, 4˚C). The resulting single cell suspensions were subjected to surface antigen detection using fluorescently labelled antibodies.

4.3.5. Leukocyte staining and flow cytometry: naïve and immunised mice

Single cell suspensions (consisting of ~2 x 10^6 cells) in 20 µl of FACS buffer (1x PBS, 0.05% NaN_3) derived from the isolated blood, spleen and lymph node tissues, were incubated for 45 min at 4˚C with two groups of labelled monoclonal antibody combinations for the detection of T and B-lymphocytes (Table 4.1). The dilution factor of these fluorescently labelled antibodies was optimised and are represented in Table 4.1. Following incubation for 45 min, stained samples were washed twice via centrifugation (500 xg for 3 min) with FACS buffer and then resuspended in 200 µl of ice cold FACS buffer before being transferred to the Falcon-5 ml polystyrene round bottom tubes (Corning Inc., USA) for flow cytometry. Fluorescence was measured using the BD FACS Canto™ II flow cytometer system (BD Biosciences, USA). This device is equipped with two lasers: Coherent® Sapphire™—Solid state (488 nm) and JDS Uniphase™ HeNe—Air cooled (633 nm). Thirty thousand events were collected for each sample and the resultant data analysed using the BD FACSDiva™ software (ver. 6.1.3) and converted from percent of cell population to absolute cell numbers in Microsoft® Excel (ver. 8).

Table 4.1: Experimental set-up and tick infestation protocol. a) Study A: Indicated are the experimental (tick-infested) and control groups for sampling points at 9 and 12 days post tick infestation (dpi).

Lymphocyte marker panel 1 (B-lymphocytes)			
Antibody	**Alternative name**	**Conjugate**	**Isotype**
CD45 Rat Anti-Mouse (clone 30-F11)*	Ptprc; LCA; Leukocyte common antigen; T200; Ly-5; Lyt-4	PerCP	Rat LOU, also known as Louvain, LOU/C, LOU/M IgG2b, κ
CD19 Rat Anti-Mouse (clone 1D3)**	na	Alexa Fluor® 700	Rat LEW, also known as Lewis IgG2a, κ
IgM Rat Anti-Mouse (clone R6-60.2)*	Ighm; Igh-M; Immunoglobulin M; Igh6; muH; immunoglobulin heavy constant mu	PE-Cy™7	Rat LOU, also known as Louvain, LOU/C, LOU/M IgG2a, κ
CD21/CD35 Rat Anti-Mouse (clone 7G6)**	CR2/CR1	FITC	Rat SD, also known as Sprague-Dawley (outbred) IgG2b, κ
CD27 Hamster Anti-Mouse (clone LG.3A10)*	na	APC	Armenian Hamster IgG1, κ
CD80 Hamster Anti-Mouse (clone 16-10A1)*	Cd80; B7; B7-1; Cd28l; Ly-53; MIC17; TSA1	PE	Armenian Hamster IgG2, κ
Lymphocyte marker panel 2 (T-lymphocytes)			
Anti-mouse antibody	**Alternative name**	**Conjugate**	**Isotype**
CD45 Rat Anti-Mouse (clone 30-F11)*	Ptprc; LCA; Leukocyte common antigen; T200; Ly-5; Lyt-4	PerCP	Rat LOU, also known as Louvain, LOU/C, LOU/M IgG2b, κ
CD3e Hamster anti-Mouse (clone 500A2)**	CD3ε chain	Alexa Fluor® 700	Syrian Hamster IgG2, κ
CD4 Rat Anti-Mouse (clone RM4-5)*	Cd4; CD4 antigen; L3T4; Ly-4; T-cell surface antigen T4/Leu-3	FITC	Rat DA, also known as DA/HA IgG2a, κ
CD25 Rat Anti-Mouse (clone PC61)*	Interleukin-2 receptor alpha chain; IL-2RA; IL-2Rα; Il2ra; IL-2R p55	PE-Cy™7	Rat OFA, also known as Outbred OFA IgG1, λ
CD195 Rat Anti-Mouse (clone C34-3448)*	CCR5	PE	Rat IgG2c, κ
CD184 Rat Anti-Mouse (clone 2B11/CXCR4)**	CXCR4, CXC chemokine receptor type 4; Fusin; LESTR; PB-CKR; Sdf1r	APC	Rat IgG2b, κ

*0.2 µl used per reaction
**0.4 µl used per reaction

4.3.6. *In vivo* cytokine detection

Cytokine protein levels were determined in the supernatant from single cell suspensions of spleen and lymph node tissues isolated from naïve tick-infested mice and corresponding controls. The supernatant cytokine levels were determined by a multiplex cytokine bead array (MILLIPLEX® MAP Mouse Th17 Magnetic Bead Panel, Merck, Germany) according to manufacturer's instructions. The panel used for the MILLIPLEX® mouse cytokine assay includes markers for Th1 (IL-2, IFNγ, TNFα) and Th2 (IL-10, IL-4, IL-13, IL-25/IL-17E) specific cytokines. For detection of the cytokines, Luminex® detector parameters were adjusted to include a five-parameter logistic (5PL) weighted algorithm for standard curve calculation (from in-array standards for T-helper 1 and T-helper 2 cytokines) with a minimum bead count of 50.

Cytokine values were reported in pg/ml and were further analysed in Microsoft® Excel (ver. 8). Since supernatant samples were collected from homogenized tissue solutions and not from cell culture or blood, these concentrations may vary significantly between samples. Therefore, cytokine ratios were determined to establish the relative contribution of different cytokine combinations to either a Th1 or a Th2 profile. To take into account the contribution of each cytokine associated with profiles for either Th1 (i.e. 3 cytokines) or Th2 (i.e. 4 cytokines), a weighted average value was calculated. This was done by using the proportion that each cytokine contributed to the Th1 (1:3) or Th2 (1:4) profile. The resulting Th2 and Th1 values were then expressed as a ratio (Th2:Th1) that was multiplied by a factor of 100.

4.3.7. Statistical analysis

An unequal variance t-test (Welch Two Sample t-test) was used for all samples (Ruxton 2006). All analyses were conducted in the Bioconductor open source software platform utilising the R statistical programming language (Team 2016). The standard error of the mean was calculated for each population subset. True statistical significance was assumed at 0.05 and lower, however, all *P*-values less than 0.1 were also considered to illustrate trends in the text and accompanying figures. For tick-related parameters a single-factor one-way analysis of variance was performed in Microsoft® Excel (ver. 8).

4.4. Results

4.4.1. Lymph node mapping to determine the regional draining lymph node

Upon visual inspection, lymphocyte mapping indicated that the regional draining lymph node draining the dorsal cutaneous area of the mouse to be the subiliac lymph node (nomenclature as described by (Van den Broeck et al., 2006)). Figure 4.2 provides a representative image of the site of dye injection and the resulting lymphatic drainage. The draining lymph node is located in the region of the hind limbs, cranial to the musculature of the thigh. Since this node was identified as the regional draining lymph node, it was used for subsequent flow cytometric analyses.

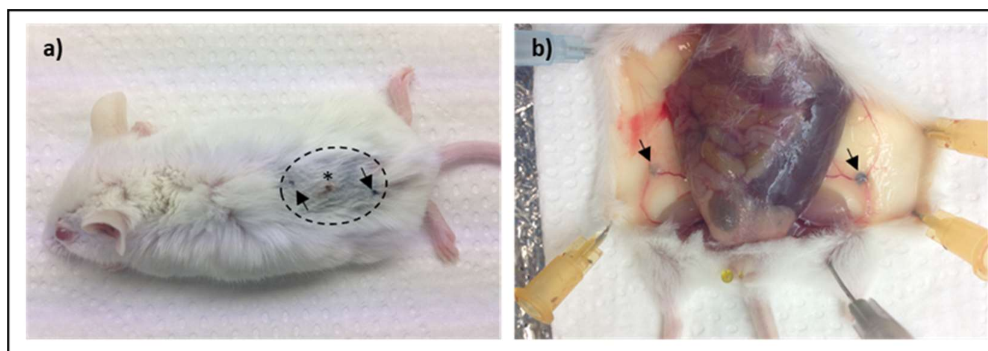


Figure 4.2: Lymphocyte mapping for the determination of regional draining lymph nodes from the site of tick infestation. a) The injection sights of the Evan's Blue dye solution (indicated with black arrows). The asterix (*) indicates an injury as a result of shaving. The dotted ovoid represents the placement site of the patch in which ticks were placed. b) The localization of the Evan's Blue dye solution that has migrated to the regional draining (subiliac) lymph nodes (indicated with black arrows).

4.4.2. B-lymphocytes in the draining subiliac lymph nodes and spleens from *I. ricinus* infested BALB/c mice

For BALB/c naïve mice infested with ticks (including their uninfested controls), a total of four CD45+ CD19+ IgM+ B-lymphocyte subpopulations were present in the subiliac lymph node tissues isolated at 9 and 12 dpi (Figure 4.3a-d). A CD27+ CD80- CD21- phenotypic subpopulation represented the majority of CD45+ CD19+ IgM+ B-lymphocytes identified for both tick-infested and uninfested (control) animals. This cell population constituted 80.79 ± 5.16 and 70.05 ± 2.61% of the CD45+ CD19+ B-lymphocyte population of the control and tick-infested groups at 9 dpi and 48.35 ± 4.11 and 45.98 ± 2.33% at 12 dpi (Figure 4.3a). Although no statistical significant differences were observed between the experimental and control groups, a statistically significant decrease was observed for both tick-infested (~24.07%, P = 0.002) and control (~32.44%, P = 0.041) groups from 9 dpi to 12 dpi.

The second largest B-lymphocyte subpopulation with a CD27- CD80- CD21+ phenotype produced an increasing trend between the sampling time-points for control and tick-infested groups, respectively (Figure 4.3b). This cell population was found to constitute 12.31 ± 3.83 and 20.41 ± 1.61% of the CD45+ CD19+ B-lymphocyte population of the control and tick-infested groups at 9 dpi and 18.99 ± 2.14 and 27.05 ± 4.40% at 12 dpi. A near statistically significant increase (~8.1%, P = 0.059) is noted between the control and experimental group at 9 dpi. In addition, a statistically significant increase (~6.68%, P = 0.025) was observed between 9 and 12 dpi for the control group. A similar trend was observed for a CD27- CD80+ phenotypic subpopulation, where a statistically significant increase (~0.829%, P = 0.025) was observed between 9 and 12 dpi for the control group only (Figure 4.3c). The least abundant B-lymphocyte subpopulation with a CD27+ CD80+ phenotype (Figure 4.4d), was significantly increased (~0.452%, P = 0.033) in the tick-infested group relative to control at 9 dpi and remained relatively unchanged at 12 dpi. The latter represents the only subpopulation of B-lymphocytes to be significantly increased during tick infestation in the murine subiliac lymph node.

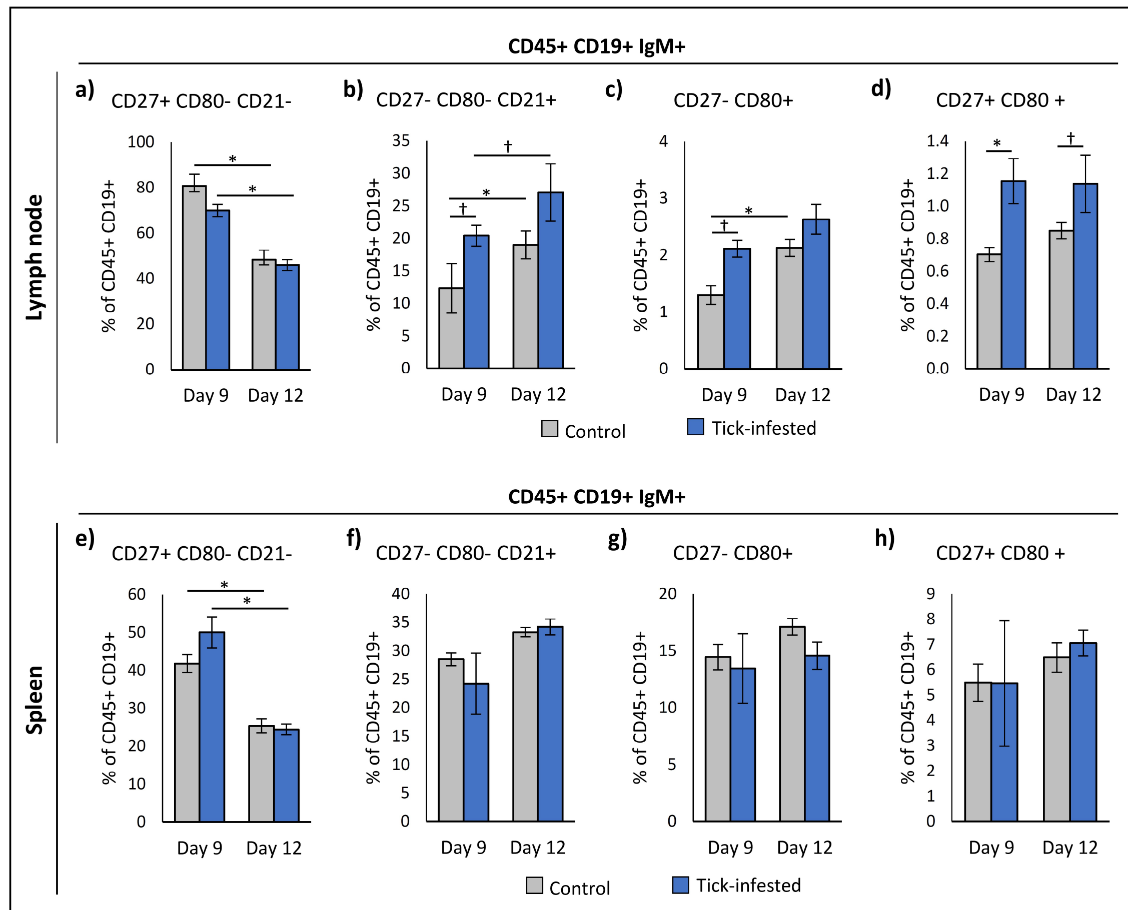


Figure 4.3: CD45+ CD19+ IgM+ B-lymphocyte percentages present in the subiliac lymph nodes (a-d) and spleens (e-h) of tick-infested BALB/c mice and their controls. Indicated are the percentages of B-lymphocyte subpopulations (relative to CD45+ CD19+ cells) present in BALB/c mice at 9 and 12 dpi together with their relative controls. The lymph node derived B-lymphocyte subpopulations presented are: a) CD45+ CD19+ IgM+ CD27+ CD80- CD21-, b) CD45+ CD19+ IgM+ CD27- CD80- CD21+, c) CD45+ CD19+ IgM+ CD27- CD80+ and d) CD45+ CD19+ IgM+ CD27+ CD80+. Corresponding spleen derived cell populations are also presented in (e-h). An asterix (*) indicates *P*-values < 0.05 while *P*-values larger than 0.05 but smaller than 0.1 are represented with a dagger (†).

In the spleen, little variability in the CD45+ CD19+ IgM+ B-lymphocyte numbers were observed between the infested and uninfested murine groups at all time-points tested (Figure 4.3e–h). Similar to the lymph node, the CD27+ CD80- and CD21- subpopulation represented the majority of the B-lymphocytes detected in the spleen for both tick-infested and control naïve animals (Figure 4.3e). This cell population constituted 50.06 ± 2.36 and 41.79 ± 4.04% (9 dpi) and 24.42 ± 1.85 and 25.35 ± 1.39% (12dpi) of the total B-lymphocyte population detected for control and tick-infested groups, respectively (Figure 4.3e). Although no statistical significant differences were observed between experimental and control groups at both sampling days, a statistically significant decrease was observed for both tick-infested (~16.44%, $P = 0.005$) and control (~25.64%, $P = 0.006$) groups between sampling points (Figure 4.3e). The same trend was observed for this subpopulation within the lymph node tissues (Figure 4.3a.). The remaining analysed splenic B-lymphocyte subpopulations were seen to be present at relatively constant levels with no clear increasing or decreasing trends detected between uninfested and infested groups, as well as between sampling time-points (Figure 4.3f-h).

4.4.3. B-lymphocytes in the draining subiliac lymph nodes and spleens from nymphal extract immunised and *I. ricinus* infested BALB/c mice

Regarding NE immunised and infested mice (Figure 4.5), the CD27+ CD80- CD21- phenotypic B-lymphocytes were the largest subpopulation in host subiliac lymph node tissues (8 dpi) for CD45+ CD19+ IgM+ B-lymphocytes with 57.17 ± 2.27 and 52.03 ± 2.07% detected for control and NE immunised mice (Figure 4.4a). A significant decrease (~5.14%, $P = 0.035$) was observed in NE immunised mice for this subpopulation relative to control animals. A similar trend was identified for the CD27+ CD80+ subpopulation (~0.55%, $P = 0.052$) that represented 2.00 ± 0.20 and 1.46 ± 0.05% of the total B-cell population detected in control and NE immunised mice, respectively (Figure 4.4d). No significant modulation was observed for the other B-lymphocytes populations tested within the murine lymph node tissues (Figure 4.4b & c).

The splenic B-lymphocyte populations for immunised and infested mice showed overall no significant variation between control and NE immunised groups (Figure 4.4e & f), with only a limited increase (~0.62%, $P = 0.048$) of the CD27- CD80- CD21+ subpopulation detected for the NE immunised mice relative to control (Figure 4.4f).

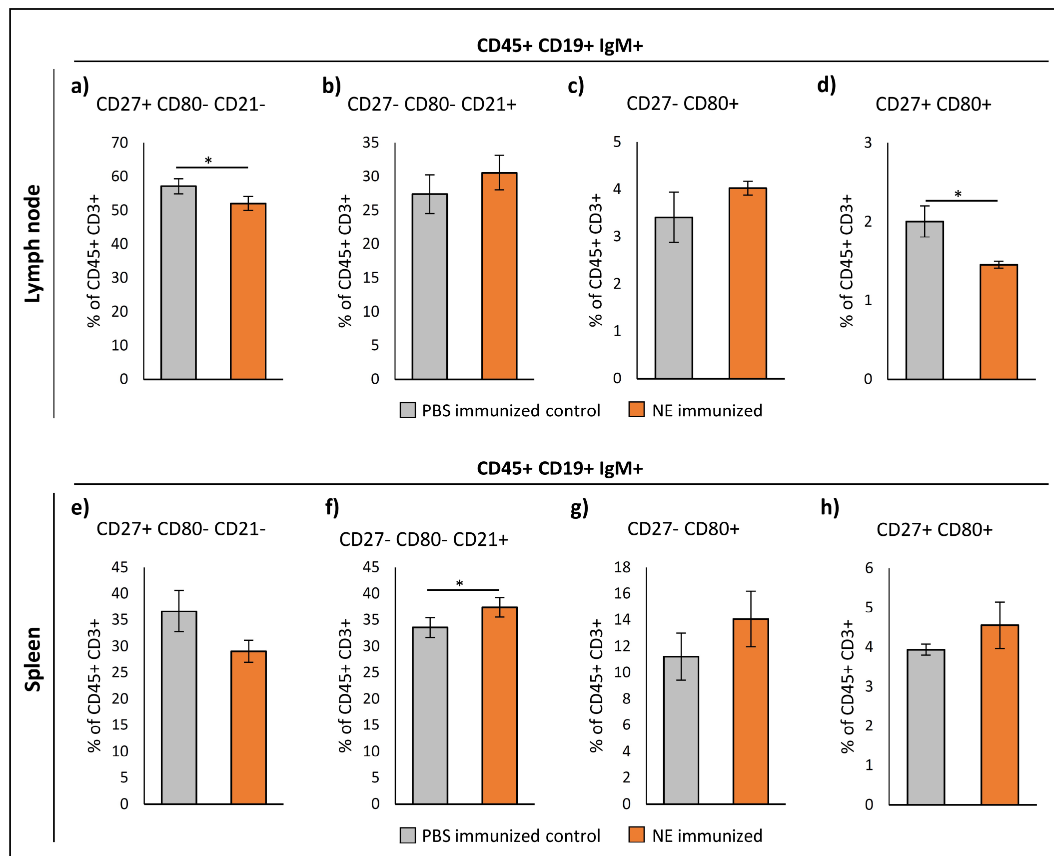


Figure 4.4: CD45+ CD19+ IgM+ B-lymphocyte percentages present in the subiliac lymph nodes (a-d) and spleens (e-h) of NE immunised and tick-infested BALB/c mice. Indicated are the percentages of B-lymphocyte subpopulations (relative to CD45+ CD19+ cells) present in BALB/c mice 42 dpv at 8 dpi together with their relative controls. The lymph node derived B-lymphocyte subpopulations presented are: a) CD45+ CD19+ IgM+ CD27+ CD80- CD21-, b) CD45+ CD19+ IgM+ CD27- CD80- CD21+, c) CD45+ CD19+ IgM+ CD27- CD80+ and d) CD45+ CD19+ IgM+ CD27+ CD80+. Corresponding spleen derived cell populations are also presented in (e-h). An asterix (*) indicates *P*-values < 0.05.

4.4.4. T regulatory cells in the spleen and draining lymph nodes from *I. ricinus* infested BALB/c mice

A CD45+ CD3+ CD4+ CD25+ population of T regulatory cells was identified in the subiliac lymph nodes and spleens of both control and infested BALB/c mice (Figure 4.5a & b). In the lymph nodes, this cell subpopulation constituted 31.94 ± 1.29 and 35.92 ± 1.93% of the total CD45+ CD3+T-lymphocyte population at 9 dpi, and 50.79 ± 2.03 and 43.42 ± 6.64% at 12 dpi, detected for control and infested groups, respectively (Figure 4.5a). A significant increase in cell population was detected for the control group from day 9 to day 12 (~18.85%, $P = 0.0006$) (Figure 4.5a). However, no significant changes could be determined for infested mice between sampling days, nor between treatment groups, and therefore no change in T regulatory lymphocytes in lymph node tissues can be inferred as a consequence of infestation. However, for the immunised and infested mice, a limited decrease in the NE immunised T regulatory lymphocyte subpopulation could be detected relative to control (~1.4%, $P = 0.050$) (Figure 4.6a).

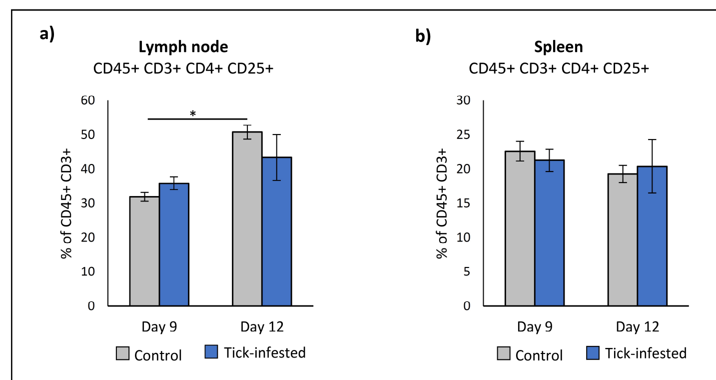


Figure 4.5: A population of T regulatory cells present in the subiliac lymph node and spleen of tick-infested BALB/c mice. Indicated are the percentages of CD45+CD3+CD4+CD25+ T-lymphocyte populations (relative to CD45+CD3+ cells) present in the subiliac lymph nodes (a) and spleens (b) of mice at 9 and 12 dpi together with their relative controls. The asterix (*) indicates *P*-values < 0.05.

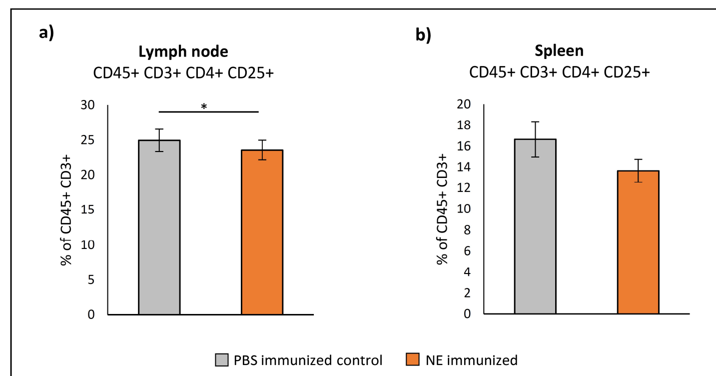


Figure 4.6: A population of T regulatory cells present in the subiliac lymph node and spleen of NE immunised and tick-infested BALB/c mice. Indicated are the percentages of CD45+CD3+CD4+CD25+ T-lymphocyte populations (relative to CD45+CD3+ cells) present in the subiliac lymph nodes (a) and spleens (b) of mice at 42 dpv at 8 dpi together with their relative controls. The asterix (*) indicates *P*-values = 0.05.

T regulatory cells population was also found to constitute 22.63 ± 1.45 and 21.29 ± 1.63% at 9 dpi, as well as 19.32 ± 1.24 and 20.41 ± 3.9% at day 12, of the CD45+ CD3+ splenic T-lymphocyte population for control and tick-infested mice, respectively (Figure 4.5b). However, no statistically significant differences could be detected between tick-infested and uninfested groups at either sampling times, indicating that these cells are present at a relatively constant level in the spleen. Similarly, no statistically significant change in this T-lymphocyte population could be detected in immunised and infested mice splenic tissues though a similar trend to lymph node cell populations is noted (Figure 4.6b).

4.4.5. T-helper lymphocytes in the spleen and draining subiliac lymph nodes from *I. ricinus* infested BALB/c mice

Activated T-helper cells (CD45+ CD3+ CD4+) were identified as a large subset of the CD45+ CD3+ T-lymphocyte population in the lymph nodes and spleen of both control and tick-infested groups (Figure 4.7a & e). In the lymph node, this cell population was found to constitute 59.8 ± 2.8 and 52.15 ± 4.23% (at 9 dpi), as well as 55.89 ± 2.06 and 49.19 ± 4.12% (at 12 dpi), of the total T-lymphocyte population tested for naïve control and infested groups, respectively (Figure 4.7.a). Three further subpopulations of activated T-helper (CD45+ CD3+ CD4+) cells could be detected in murine lymph node (Figure 4.7b-d) and splenic (Figure 4.7f-h) tissues that were separated by their expression (or lack of expression) of CD195 and CD184. Overall, no statistically significant changes could be detected between the sampling time-points for any of the subpopulations of T-helper lymphocytes analysed in lymph nodes (Figure 4.7b-d). However, a significant decrease in a CD195+ CD184- subpopulation (~7.587%, $P = 0.02$) could be detected between infested and uninfested mice at day 12 (Figure 4.7b). For the immunised and infested experimental groups, a similarly large T-helper (CD45+ CD3+ CD4+) population was detected from the overall nodal T-lymphocyte population in control (55.94 ± 2.50%) and NE immunised (52.07 ± 2.89%) mice (Figure 4.8a). However, similarly to the infested/uninfested experimental groups, no significant modulation in T-helper subpopulations could be detected (Figure 4.8a-d).

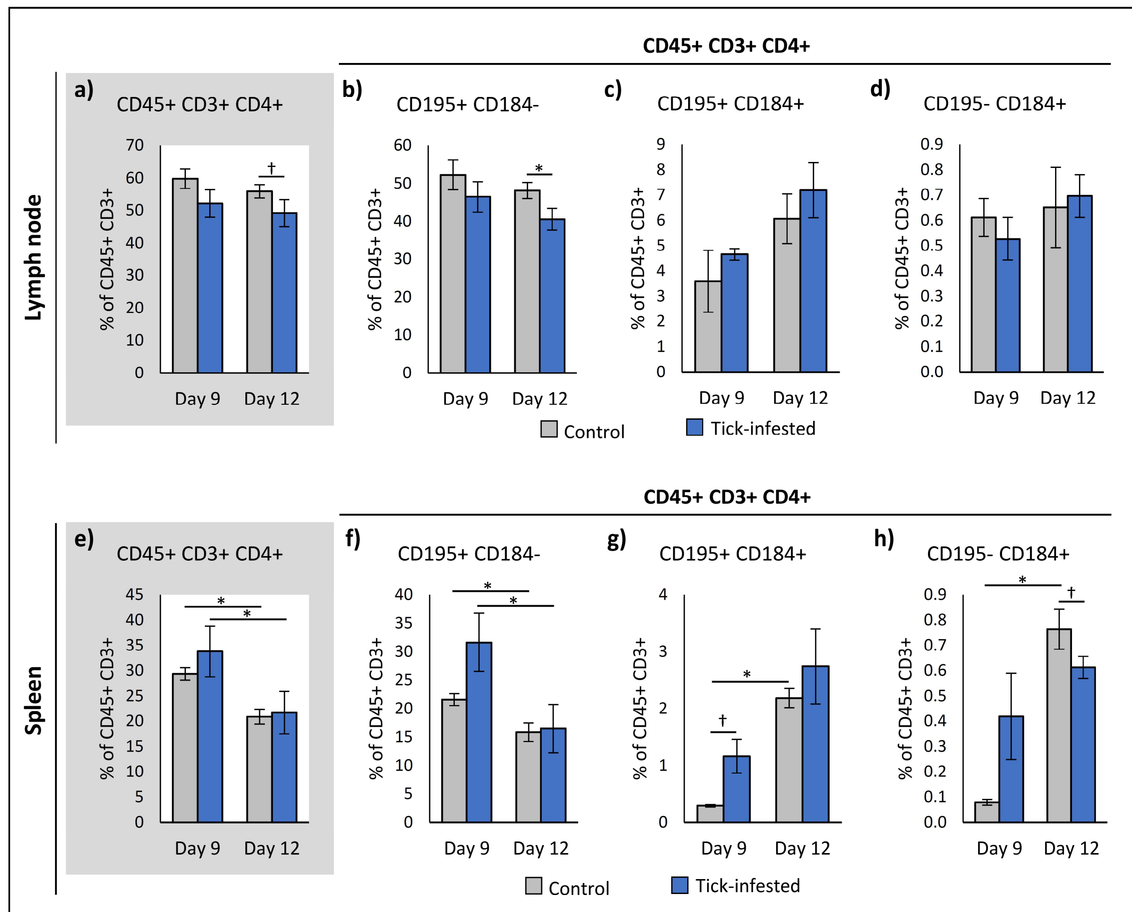


Figure 4.7: T-helper lymphocytes and related subpopulations in the lymph nodes and spleens of mice of tick-infested BALB/c mice. Represented are the percentages of CD45+ CD3+ CD4+ T-helper cell populations in the lymph nodes (a-d) and the spleen (e-h) of mice at 9 and 12 dpi. Panels a) and e) represent the parent CD45+ CD3+ CD4+ T-helper populations of the lymph nodes and spleens respectively. The adjacent three graphs for each parent population represent alternative phenotypes for the T-helper populations. The asterix (*) indicates *P*-values < 0.05 while *P*-values larger than 0.05 but smaller than 0.1 are represented with a dagger (†).

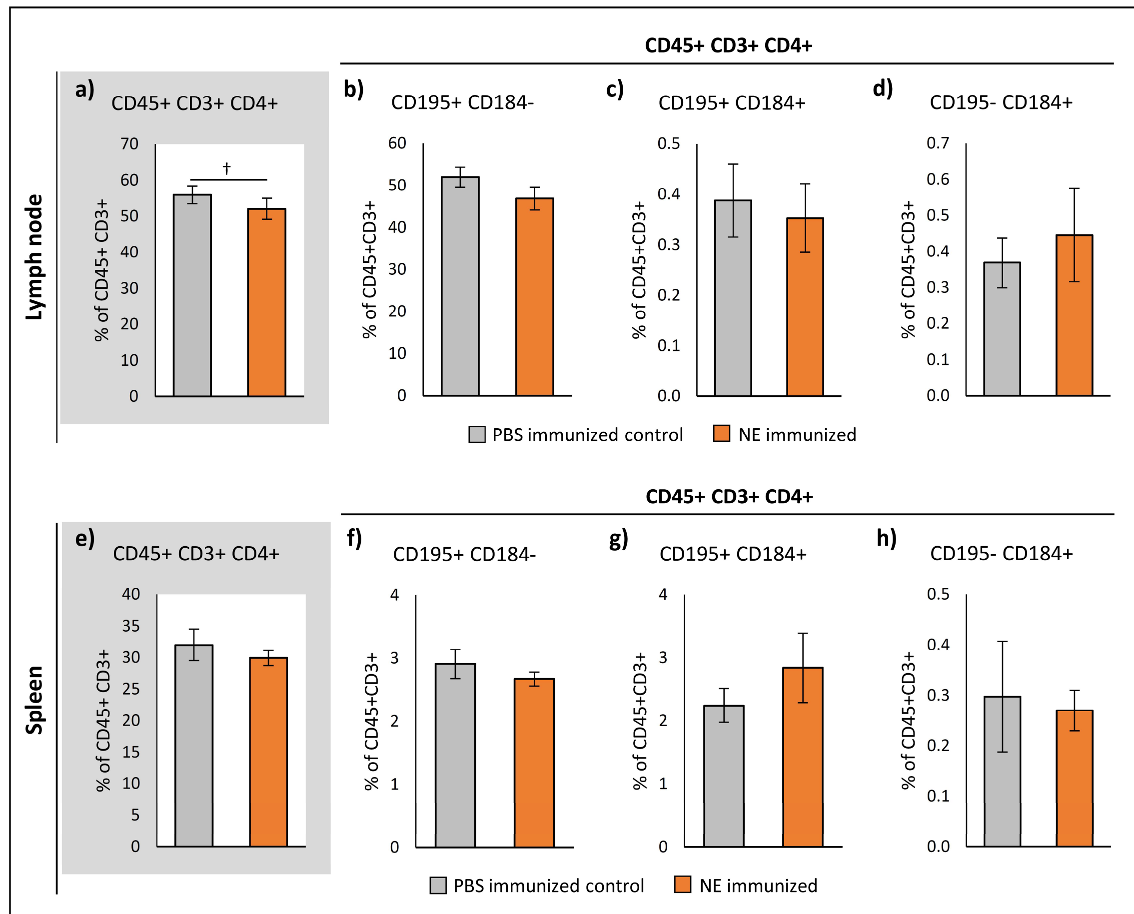


Figure 4.8: T-helper lymphocytes and related subpopulations in the lymph nodes and spleens of NE immunised and tick-infested BALB/c mice. Represented are the percentages of CD45+ CD3+ CD4+ T-helper cell populations in the lymph nodes (a-d) and the spleen (e-h) of mice 42 dpv and 8 dpi. Panels a) and e) represent the parent CD45+ CD3+ CD4+ T-helper populations of the lymph nodes and spleens respectively. The adjacent three graphs for each parent population represent alternative phenotypes for the T-helper populations. *P*-values larger than 0.05 but smaller than 0.1 are represented with a dagger (†).

In the spleen, the T-helper cell population was found to constitute 29.35 ± 1.24 and 33.8 ± 5.02% (9 dpi), as well as 20.88 ± 1.49 and 21.69 ± 4.21% (12 dpi), of the overall splenic T-lymphocyte population for control and experimental groups, respectively (Figure 4.7e). As observed for lymph node tissues, no statistically significant changes could be detected between control and tick-infested groups at the individual same sampling points for the T-helper population. However, significant decreases in the overall T-helper population was observed for both the naïve control (~8.468%, *P* = 0.005) and tick-infested (~12.109%, *P* = 0.032) groups from day 9 to day 12 (Figure 4.7e). A similar trend was detected in the CD195+ CD184- T-helper subpopulation between sampling time-points for naïve control (~5.767%, *P = 0.02*) and infested (~15.138%, *P* = 0.037) groups (Figure 4.7f). Additionally, significant increases in the CD195+ CD84+ (~1.894%, *P* = 0.001) and CD195- CD84+ (~0.685%, *P* = 0.004) splenic T-helper subpopulations were detected between sampling time-points for naïve uninfested mice (Figure 4.7g & h). Overall, no statistically significant differences were detected between control and tick-infested groups for all T-helper subpopulations. However, a near statistically significant increase in CD195+ CD184+ (9 dpi, ~1.581%, *P* = 0.075) and decrease in CD195- CD184+ (12 dpi, ~0.194%, *P* = 0.089) in T-helper subpopulations between naïve

control and infested murine groups were obtained, respectively (Figure 4.7g & h). Regarding the immunised and infested experimental groups, no significant modulation in splenic T-helper populations could be detected, similar to results obtained for the lymph node tissues (Figure 4.8e-h).

4.4.6. Cytokines in the draining subiliac lymph nodes from naïve *I. ricinus* infested BALB/c mice

Due to limitation in sample collection from a single cell suspension, a defined trend for individual cytokine profiles could not be determined. With regards to the overall profiles representing Th2/Th1, little variation was observed between the groups and time-points tested (Figure 4.9). Relative Th2/Th1 cytokine ratios (Rcr) for infested naïve mice at 9 dpi (Rcr = 90.36 ± 7.23) and 12 dpi (Rcr = 92.03 ± 7.15), together with their respective controls (Rcr = 76.64 ± 5.71 and 94.58 ± 5.29, respectively), were determined. Statistical analysis revealed no significant changes between experimental and control groups at 9 dpi and 12 dpi, as well as between sampling time-points for tick-infested mice. Only a limited significant increase in the Th2/Th1 ratio was obtained between naïve controls groups from day 9 and 12 (~17.94%, $P = 0.031$).

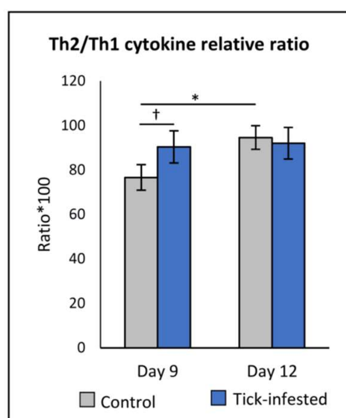
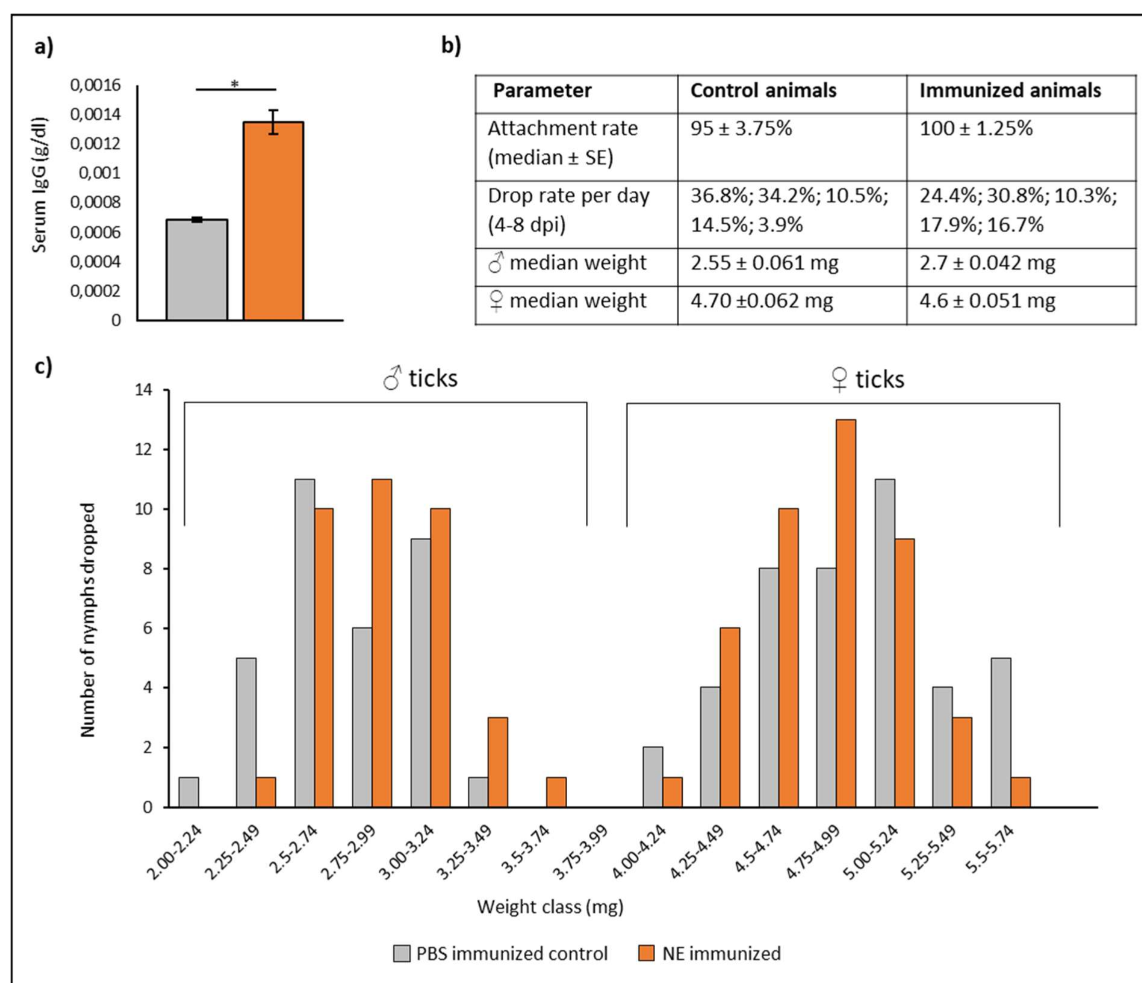


Figure 4.9: The relative ratios of lymph node derived T-helper 1 cytokines to T-helper 2 cytokines. Indicated are the ratios of T-helper 1 cytokines to T-helper 2 cytokines that originated from the lymph nodes of BALB/c mice 9 and 12 dpi. The asterix (*) indicates *P*-values < 0.05 while *P*-values larger than 0.05 but smaller than 0.1 are represented with a dagger (†).

4.4.7. Host serum IgG production and influence on tick infestation parameters in vaccinated mice

For the immunised infested mice, results indicated that immunisation with NE significantly increased total IgG (96.75%, $P = 0.017$) relative to PBS injected control mice (Figure 4.10a). Further assessment of nymph infestation parameters (tick survival, weight, feeding period) indicated no significant protective effects resulting from immunisation of mice with tick extracts relative to the mock injected control animals (Figure 4.10b & c). Two distinct distribution peaks for engorged nymph weights were observed for both control (2.55 ± 0.061 mg and 4.7 ± 0.062 mg) and NE immunised (2.7 ± 0.042 mg and 4.6 ± 0.051 mg) mice (Figure 4.10b & c). This corresponds to previously published data on *I. ricinus* nymphs that developed to male (2.61 ± 0.32 mg) and female (4.22 ± 0.05 mg) ticks (Dusbabek et al., 1995) (Figure 4.10b).



Parameter	Control animals	Immunized animals
Attachment rate (median ± SE)	95 ± 3.75%	100 ± 1.25%
Drop rate per day (4-8 dpi)	36.8%; 34.2%; 10.5%; 14.5%; 3.9%	24.4%; 30.8%; 10.3%; 17.9%; 16.7%
♂ median weight	2.55 ± 0.061 mg	2.7 ± 0.042 mg
♀ median weight	4.70 ±0.062 mg	4.6 ± 0.051 mg

Figure 4.10: Host serum IgG production and influence on tick infestation parameters NE immunised and tick-infested BALB/c mice. a) The total serum IgG concentration for PBS immunised and NE immunised mice at 42 dpv and 8 dpi. b) The tick attachment rate, tick drop rate (4-8 dpi) and median tick weights for nymphs collected in this study. c) Weight class and number of nymphs from control and NE immunised mice.

4.5. Discussion

In the present study we show that, although serum IgG increases in mice following NE immunisation, it does not offer any significant protection to subsequent tick infestations. Additionally, a B-cell population (CD45+ CD19+ IgM+ CD27+ CD80+) was uniquely up-regulated during tick feeding and significantly down-regulated in immunised mice at 9 dpi and 8 dpi, respectively. Regarding T-cells, no significant differences in the numbers of CD45+ CD3+ CD4+ T-helper cells were noted in tick-naïve and NE immunised mice compared to their controls. While a T-helper cell subset (CD45+ CD3+ CD4+ CD195+ CD184-) was significantly decreased in tick-infested mice (12 dpi). Lastly, a slight significant decrease in T regulatory cells (CD45+ CD3+ CD4+ CD25+) were observed in NE immunised mice.

4.5.1. Effects of tick infestation and immunisation on B-lymphocytes

As naïve B-lymphocytes encounter an antigen, they are activated and express antigen-specific IgM which is later accompanied by IgG and followed by the formation of germinal centres in secondary lymphoid organs (Takemori et al., 2014). In germinal centers, two weeks after immune stimulation, B-lymphocytes undergo affinity maturation and somatic hypermutations to increase the specificity of the immunoglobulins present on them (MacLennan 1994). To date, little information is available regarding the effect of tick feeding on murine B-cells. In a transcriptional study on the skin of BALB/c *I. scapularis* infested mice, a negative regulation of B-cell signalling clusters (6 hours after tick infestation) was found (Heinze et al., 2012). Regarding the effects on *I. ricinus* infested mice, Mbow and colleagues (1994) found no significant difference in CD45R+ B-cells in the skin of tick-infested BALB/c mice 72 hours post tick infestation. Since these studies looked at early immune responses at the site of tick attachment, changes in B-cells (which are part of the adaptive immune response) are not expected for naïve animals. In this study we therefore aimed to evaluate B-lymphocyte populations in the secondary lymphoid organs of tick-infested and/or NE immunised BALB/c mice.

In the current study, the lymph nodes and spleens of naïve tick-infested BALB/c mice, indicate no significant difference in the overall population of B-lymphocytes compared to the control mice (Figure 4.3a and e). Yet, a significant decrease was observed for this population in the lymph node of NE immunised mice (42 dpv and 8 dpi) (Figure 4.4a). This significant decrease in NE immunised mice suggests that the CD27+ CD80- CD21- subpopulation is uniquely affected (decreased) in response to immunisation with NE. While the absence of CD80 and CD21 on the surface of these cells points to the naïve nature of these cells (Alegre et al., 2001; Anderson et al., 2007), we propose that the CD45+ CD19+ IgM+ CD27+ CD80- CD21- B-lymphocyte population presented in Figure 4.4a represents an early stage of activated B-lymphocytes in the lymph nodes. The expression of murine CD27 has been found on distinct lineages of B-lymphocytes including, developing centroblasts within the germinal centre of the spleen and lymph node (Xiao et al., 2004) and on memory B-lymphocytes (Xiao et al., 2004). This may suggest the conversion of these naïve memory B-lymphocytes to a more mature state due to immunisation with NE.

Although an increasing trend is seen in the B-cell populations presented in Figures 4.3b & c and 4.4b & c, no modulation of these populations could be attributed to tick infestation and/or NE immunisation. This implies that CD27 negative B-cells are not affected by either tick infestation or immunisation with NE. The CD27+ CD80+ B-cell population shown in Figures 4.3d and 4.4d represents the only CD45+ CD19+ IgM+ population differentially regulated by tick infestation and/or NE immunisation. This population seems to be uniquely up-regulated during tick feeding (Figure 4.3d) and significantly down-regulated when mice are immunised (Figure 4.4d). These data suggest that this population is stimulated upon initial exposure to tick saliva (tick feeding, 9 dpi) but is supressed (8 dpi) in mice immunised with NE. However, the presence of CD80 on B-lymphocytes indicates these as having an antigen presenting capacity (Alegre et al., 2001; Bromley et al., 2001). In this regard, the co-occurrence of IgM and CD80 may be contradictory and should be further investigated. Yet, Dogan and colleagues (2009) have shown in a mouse model (after immunisation with sheep red blood cells) that memory B-lymphocytes are composed of an IgM+ in addition to an IgG+ subset. This indicates that in addition to the classical IgG+ long-lived B memory cell, an IgM+ subset is present. Regarding the expression of CD27 on these CD80+ B-lymphocytes (Figures 4.3d and 4.4d), Anderson and colleagues (2007) have suggested that CD27 does not have a function in the

antigen presenting capacity of these cells and may indicate recent B-cell activation and accelerated germinal centre formation (Xiao et al., 2004).

4.5.2. Effects of tick infestation and immunisation on T-lymphocytes and cytokines

T-helper cells are a crucial part of the adaptive immune system as they coordinate immune responses by the release of specific cytokines (Kara et al., 2014). In our study, no significant differences in the numbers of CD45+ CD3+ CD4+ T-helper cells were noted in tick-naïve (Figures 4.7a) and NE immunised (Figures 4.7b) mice compared to their controls. The lack of a significant increase or decrease in this cell population suggests that this cell population is not affected by a tick-induced immune response at given sampling points and should be evaluated using *in vitro* stimulation assays. T-helper lymphocytes can be further classified by their expression of CD195 (CCR5) and CD184 (CXCR4) (Kumar et al., 2006; Qin et al., 1998). Variable expression of these cell markers was not clearly detectable in the lymph nodes of tick-infested nor immunised mice. An exception to this is a statistical significant decrease seen in the tick-infested group at day 12 for T-helper lymphocytes expressing CD195 but lacking the expression of CD184 (Figure 4.7b). Qin and colleagues (1998), demonstrated that CD195 is a marker for T-lymphocytes associated with T-helper 1 inflammatory reactions. This marker is also accepted as a necessary receptor for the activation of T-helper 1 lymphocytes (Luther and Cyster 2001). A decrease in the prevalence of this cell marker may therefore be attributed to a decrease in the T-helper 1 cell population, yet this observation is not supported in the cytokine data reported in Figure 4.9. To date, murine studies have quantified the *in vitro* production of T-helper cytokines in *I. ricinus* exposed BALB/c mice (Ganapamo et al.,1995; Ganapamo et al.,1996a; Ganapamo et al.,1996b; Christe et al.,1999; Mejri et al., 2002). Therefore, to assess the direct *in vivo* cytokine response we aimed to quantify cytokine levels of lymph node derived lymphocytes from tick-naïve *I. ricinus* infested BALB/c mice. This approach was taken as relationships between cellular cytokines (following *in vitro* stimulation) and in vivo serum cytokines has been found in humans (Jason et al., 2001). As shown in Figure 4.9, the relative proportion of cytokines produced for the tick-infested mice remained constant between the two sampling days and no significant differences were noted between control and experimental animals. Since available literature supports the increased production of Th2 cytokines such as IL-4 (Ganapamo et al.,1995; Christe et al.,1999; Mejri et al., 2002) and IL-10 (Ganapamo et al., 1996b), a lack of significant results in this study points to a flaw in experimental design. Previous studies have stimulated lymphocytes *in vitro* with either salivary gland extracts from partially engorged *I. ricinus* females (Ganapamo et al.,1995; Ganapamo, et al.,1997; Mejri et al.,2002) or Con A (Ganapamo et al.,1996b; Mejri et al., 2002). The authors suggest a similar approach in future, such as stimulation with nymph extract.

Regulatory T-cells are generally known for their ability to suppress putative deleterious activities of T-helper cells (Corthay 2009). A population of T regulatory cells with the cell surface phenotype CD45+ CD3+ CD4+ CD25+ was identified in both the lymph nodes and spleens of the non-immunised and immunised BALB/c mice groups (Figure 4.5 & Figure 4.6). However, only a slight significant decrease in T regulatory cells in NE immunised mice was observed (Figure 4.6.a), pointing to a possible effect of NE immunisation in infested BALB/c mice. However, in naïve non-immunised groups, these cell populations remain relatively constant between 9 and 12 dpi between naïve uninfested and infested mice, and any changes that may have occurred were completed either before or after these sampling points and should be investigated in follow-up studies. Since literature pertaining to *I. ricinus* immune

responses in mice point to a T-helper cell regulation (Ganapamo et al.,1995; Ganapamo et al.,1996a, Ganapamo et al.,1996b; Christe et al.,1999; Mejri et al.,2002), the hypothesis that T regulatory cells may have an influence on the balance of T-helper 1 to 2 responses remains to be fully demonstrated. Overall, results do indicate some cessation of down-regulation to enable T-helper responses in vaccinated hosts, while an apparent "suppression" of responses are maintained in naïve hosts during infestation and tick feeding. This may be better defined if a protective immunogen is used.

4.6. Conclusion

To contribute to the understanding of the effect of tick feeding on the immune system of its host, this study aimed to characterise the *in vivo* immune response of tick-naïve and nymph extract immunised BALB/c mice infested with *I. ricinus* nymphs. Findings indicate that BALB/c mice do not acquire a protective response to *I. ricinus* ticks following immunisation with *I. ricinus* nymph extract. Only a single B-cell population (CD45+ CD19+ IgM+ CD27+ CD80+) was up-regulated during tick feeding and down-regulated in immunised mice. While no significant differences in the numbers of CD45+ CD3+ CD4+ T-helper cells were noted in tick-naïve or nymph extract immunised mice compared to their controls. Yet a T-helper cell subset (CD45+ CD3+ CD4+ CD195+ CD184-) was significantly decreased in tick-naïve mice at 12 days post infestation. Lastly, a slight significant decrease in T regulatory cells (CD45+ CD3+ CD4+ CD25+) was observed in only nymph extract immunised mice. The lack of a significant increase or decrease in a number of mentioned cell populations suggests that either these cell populations are not affected by a tick-induced immune response or that the assumed immune response had occurred prior to the sampling points. As such, future studies including immunisation with adult tick extracts and *in vitro* stimulation of lymphocytes for cytokine analysis is required to supplement this work.

4.6. References

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Chapter 5: Concluding discussion

5.1. Problem statement and rationale

The negative impact of ticks and tick-borne diseases on the quantity and quality of livestock products, advocates a need for effective control strategies against these parasites (Allen and Prosperi, 2016; Jongejan and Uilenberg, 2004). Due to the increasing resistance to the most widely used control strategy (acaricides), new control strategies need to be developed and improved (Abbas et al., 2014; Ghosh et al., 2007; Stutzer et al., 2018). In cattle, increasing the understanding of the basic underlying variation in immunological responses to tick infestation may constitute the basis of improved tick control strategies in the future. As highlighted in Chapter 1, the focus of the current literature is on characterisation of immune response in the blood and skin of tick-resistant and tick-susceptible cattle (Robbertse et al., 2017).

To contribute to the current understanding of the immune response elicited by cattle in response to tick infestation and continued feeding, this thesis primarily focuses on the immune response in Bonsmara cattle (Chapters 2 and 3). This cattle breed was chosen because Bonsmara cattle are an important breed in South Africa mainly used for beef production (Bosman et al., 2017; Scholtz et al., 1991). Developed to withstand the subtropical South African climate, this cattle breed is described as a composite cattle breed (a *B. t. indicus* and *B. t. taurus* cross; 5/8 Afrikaner, 3/16 Shorthorn and 3/16 Hereford) (Bonsma, 1980). Due to its mixed lineage, it has intermediate tick resistance (M C Marufu et al., 2011) and therefore provides an opportunity to study the effects of tick feeding on the immune response of its host. In addition to Bonsmara cattle, the immune response of Brahman and Holstein cattle was analysed as two polar opposites of tick resistance. Bahman (*B. t. indicus*) cattle are currently known as tick-resistant breed as they are known to harbour significantly less ticks than their tick-susceptible counterparts, Holstein-Friesians (*B. t. taurus*) (Jonsson et al., 2014). This study entails a novel systematic approach by combining both cell subtype quantification (histopathology, immunohistochemistry and flow cytometry) and transcriptional profiling (DNA microarrays) methodologies to elucidate the cellular responses that contribute to tick-induced immune responses.

In addition to studying the immune response in cattle, the viability of a mouse model was assessed for basic immune profiling during tick feeding and vaccination in Chapter 4. Due to the impact of these parasites and their associated diseases on both animal and human health, studying the effects of tick attachment and feeding on host immunity in a model system within a controlled laboratory environment may provide insight into the fundamental mechanisms by which ixodid ticks evade effective host immune responses.

5.2. Leukocytes in the skin of tick-infested cattle (Chapter 2)

The importance of CD3+ T-cells in the tick resistance mechanism has been suggested in *B. t. indicus* cattle (compared to *B. t. taurus*) (Constantinoiu et al., 2010; Franzin et al., 2017). To further evaluate this, we quantified CD3+ T-lymphocytes adjacent to the tick attachment site in Bonsmara, Holstein-Friesian and Brahman breeds using immunohistochemistry (IHC) (Figure 2.3c and d). The results correlated with published data and point to three main findings. Firstly, that upon larval and adult tick attachment in Brahman cattle, a significant increase in the number

of CD3+T-cells is seen. Secondly, that Bonsmara cattle have a significant increase in T-lymphocytes at the area of larvae attachment which is not seen during adult tick attachment. Lastly, that Holstein-Friesian cattle do not have a significant difference in the number of T-lymphocytes at the area of tick attachment. These results further confirm that a CD3+ T-lymphocyte dependent mechanism may be correlated with tick resistance at the tick attachment site.

In this study, B-lymphocytes were quantified in the skin of cattle due to their importance in the adaptive immune system and cutaneous inflammation (Egbuniwe et al., 2015). Franzin and colleagues previously demonstrated using IHC a statistically non-significant infiltration of CD21+ B-lymphocytes around the feeding lesion of *R. microplus* in bovine hosts (Figure 2.3a and b). Intriguingly, we found the presence of CD20 on immature and mature (including plasma cells) developmental stages of B-cells (confirmed by Dr. Sarah Clift, veterinary pathologist). The CD20 marker identifies a broader range of B-lymphocytes. A statistically significant influx of CD20+ B-lymphocytes was observed at the site of adult tick attachment in all three cattle breeds. Brahman and Bonsmara cattle also had a statistically significant increase in the number of CD20+ B-lymphocytes at the bite sites of larvae, compared to adult ticks.

5.3. Leukocyte dynamics in the lymph nodes of tick-infested cattle (Chapter 2)

Skin as a first line of defence against tick infestation plays an important role in the tick-host interaction and has therefore been well studied (Constantinoiu et al., 2013, 2010, Piper et al., 2010, 2009, 2008). Previous studies have shown involvement of innate (O'Kelly and Spiers, 1976; Riek, 1962) and acquired immune responses to the acquisition of tick resistance mechanisms between different cattle breeds. Although secondary lymphoid organs are important in the development of immune responses, and potentially immune responses against tick infestations, very few studies have focused on their involvement. This is particularly true for regional lymph nodes draining the cutaneous tick attachment sites. Therefore, in this thesis we aimed to study the immune response in the lymph nodes draining the cutaneous tick attachment site.

Immunohistochemistry data suggests that the percentage of CD20+ and CD79+ B-lymphocytes remained consistent in the lymph nodes in the more resistant cattle breeds over the chosen time-points (Figure 2.4) compared to susceptible breeds where we did not observe such an effect. This suggests that in resistant animals a large portion of B-cell development and maturation process may already have occurred by the time of tissue samples collection. In contrast to more tick-resistant breeds, Holstein-Friesian animals showed a decrease of B-lymphocytes in lymph node tissue upon larvae infestation; mainly due to reduced B-lymphocyte populations in germinal centres (Figure 2.4e). Furthermore, in Holstein-Friesian animals, the percentage of B-lymphocytes in the lymph nodes increased from larvae to adult-infested cattle. This increase mainly seems due to a B-lymphocyte influx in the cortex. This same increasing trend was seen for Bonsmara cattle when comparing tick uninfested and adult-infested animals.

The IHC analysis of T-lymphocytes in the more resistant breeds showed a trend towards an increased number of CD3+ T-lymphocytes from uninfested and larvae infested cattle to animals infested with adult ticks (Figure 2.5). To further elucidate the CD3+ populations in the lymph nodes, flow cytometric analysis of CD3+/CD4+ T-helper lymphocytes showed a significant

increase from larvae to adult-infested Bonsmara cattle and decreased in Holstein-Friesian cattle upon larvae infestation (Figure 2.6a). This could indicate that the more susceptible breeds showed declining CD3+/CD4+ T-helper lymphocyte populations while the most resistant breed showed a more stable T-helper lymphocyte response over time. In addition, CD3+/CD8+ cytotoxic T-lymphocytes showed no differential levels over time in any cattle breed, probably due to their role in defence against intracellular pathogens (Janeway et al., 2001) (Figure 2.6c). Flow cytometric analysis for WC1+ γδ T-lymphocytes populations revealed increased variability correlating with tick susceptibility in the two more susceptible breeds. This could be seen by the significant reduction of this cell population in the lymph nodes of Bonsmara and Holstein-Friesian cattle upon larval infestation. In addition, no variation for this cell population was noted for Brahman cattle. These results suggest that a decreased level of the WC1+ γδ lymphocyte population could be favourable for continued *R. microplus* infestation in cattle.

Additional analysis revealed the presence of tingible body macrophages and eosinophils in H&E stained lymph node samples. Investigation of lymphocytes in the germinal centres of lymph nodes showed an increase in eosinophils in more susceptible breeds (Figure 2.2a). The presence of eosinophils in lymph nodes is not well documented especially for parasite infested organisms. Due to the proposed antigen presenting capability of eosinophils (Padigel et al., 2006; Shi, 2004; Shi et al., 2000), their presence in the germinal centres of lymph nodes implies their role in the affinity maturation process of B-lymphocytes. To confirm this hypothesis, the role of eosinophils in cattle lymph nodes should form the basis of subsequent studies. Germinal centre tingible body macrophages also showed a significantly increased occurrence (Figure 2.2a) between uninfested and adult tick-infested Brahman cattle suggesting that more tick-resistant cattle breeds may be associated with a more pronounced affinity maturation process (Rahman et al., 2010). Although these results do not provide conclusive evidence about the function of subsets of T and B-cells in the lymph nodes of tick-infested cattle, they do provide intriguing suggestions as to the potential involvement of these cell types in the tick resistance mechanism. Thus, transcriptomics was used to further evaluate leukocyte activities in the lymph nodes of cattle (Chapter 3).

Additionally, it is necessary to concede that the exact resistance status of these animals should have been evaluated prior to the start of the experiment. Yet due to technical and the associated financial constraints this was not possible at the time of experimentation. Future studies

5.4. Lymph node transcriptional profiles of tick-infested cattle (Chapter 3)

Using conventional microarray assays and a reference pool design, differentially expressed genes (DEGs) were identified from the lymph nodes of tick-infested cattle. To minimise the identification of false positive transcripts, care was taken to use intra-breed comparisons performed at different tick life stages to provide quality insights into the underlying dynamics of the development of an immune response in bovines.

While only 5 DEGs were identified for each Holstein-Friesian and Brahman, 183 were identified in Bonsmara cattle during larval and adult tick feeding, compared to the baseline before artificial tick infestation (Figure 3.1). The small number of DEGs for Holstein-Friesian and Brahman cattle is attributed to the variation within these biological groups as confirmed by Pearson correlations. Despite the few DEGs in Holstein-Friesian and Brahman, similarities in DEGs were noted

between the cattle breeds. They include the transcript hydroxysteroid 17-beta dehydrogenase 1 (HSD17B1) and olfactory-like G protein-coupled receptors LOC517144 and OR9Q2 differentially expressed in Bonsmara and Brahman cattle, suggestive of inflammation at cutaneous regions (Moeller and Adamski, 2009; Mukhopadhyay et al., 2009) and T-cell chemotaxis (Clark et al., 2016), respectively. Secondly, transcripts for two galectins (LGALS9 and LGALS7B) were identified in Bahman and Holstein-Friesian cattle, linked to regulating leukocyte tissue entry during inflammation (Thiemann et al., 2015). Lastly, transcripts for claudins were found to be differentially expressed in larvae infested Holstein-Friesian (CLDN2) and Bonsmara (CLDN3 and CLDN8) cattle involved in regulating permeability of the epi- and endothelial layers to facilitate immune cell infiltration (Turksen and Troy, 2004). It was additionally noted that transcripts identified as differentially expressed (in both tick life stages within a cattle breed) showed a similar expression profile for both larvae and adult tick-infested cattle. This suggests similar regulation of immunity in response to larvae attachment and adult feeding and/or re-attachment.

5.5. The effect of tick attachment and feeding on the transcription profile of draining lymph nodes in Bonsmara cattle (Chapter 3, section 3.5.2.)

Since lymph nodes are dynamic organs where the spatial orientation of cells is related to their function the second half of Chapter 3 discussed the findings in relation to their functional role in lymphocyte trafficking (Figure 3.4). These include: (1) Leukocyte recruitment to the lymph node via chemokines and chemotaxis, (2) Trans-endothelial and intranodal movement on the reticular network, (3) Active regulation of cellular transcription and translation in the lymph node (including leukocyte associated cellular regulatory networks) and (4) Chemokine receptors regulating the movement of cells out of the lymph node.

5.5.1. Leukocyte recruitment to the lymph node via chemokines and chemotaxis

Numerous deferentially expressed chemokine transcripts relating to leukocyte recruitment to the lymph node were identified (CCL3, CCL4, CCL5, CCL19, XCL1, CXCL9, CXCL10 and CXCL11). The ligands CCL3 and CCL4 are known to enhance T-cell recruitment to reactive lymph nodes (Bystry et al., 2001; Tedla et al., 1998). The observed down-regulation for these two chemokines upon larval infestation points towards an impaired recruitment of T-lymphocytes to the lymph nodes in Bonsmara cattle.

5.5.2. Trans-endothelial and intranodal movement on the reticular network

The transcripts of the chemokine CCL19, which is essential for the migration of immune cells to and within lymphoid organs, were identified as differentially expressed (Braun et al., 2011; Forster et al., 2008, 1999; Gunn et al., 1999; Miyasaka and Tanaka, 2004; von Andrian and Mempel, 2003). The receptor to this ligand (CCR7) was found to be up-regulated during the adult ticks feeding stage. CCR7 is essential in the homing of dendritic cells, naïve T-cells and B-cells to lymphoid tissues (Braun et al., 2011; Forster et al., 2008; Milligan and Barrett, 2015). Yet, in the current study the role of CCR7 during tick infestations is unclear.

Additionally, a transcript necessary for trans-endothelial movement was identified (MGAT4B) (Kawashima et al., 2005; Uchimura et al., 2005) (Figure 3.3). Modification of the extracellular matrix was evident during tick infestation as several types of collagen (1-6, 15 and 18), as well as collagen-associated molecules such as fibronectin (FN1), elastin (ELN), biglycan (BGN), thrombospondins (THBS1 and AMTS5) were differentially expressed. In addition, several secreted proteases and protease inhibitors were also differentially expressed in Bonsmara lymph nodes tissue. Regarding transcripts related to extracellular structures, almost all studied genes involved show the highest up-regulation upon larval attachment compared to adult tick-infested or uninfested cattle, suggesting modulation of lymph node physiology and architecture (confirmed in Chapter 2) predominantly during larval infestation.

5.5.3. Active regulation of cellular transcription and translation in the lymph node (including leukocyte associated cellular regulatory networks)

Relating to leukocyte associated cellular regulatory networks, differentially expressed transcription factors KLF16 and ZNF771 appear to be co-regulated with a TRAF-interacting protein (TIFAB) and complement factor B (CFB) (Figure 3.3). The proteins of the latter two transcripts have been suggested to be involved in regulation of Toll-like receptor-TRAF6-mediated NF-kB signalling for immune cell maturation (Matsumura et al., 2009; Varney et al., 2015) and as a circulating component of the alternative pathway of complement activation in the blood (Huang et al., 2002; Kerr, 2013; Merle et al., 2015), respectively. Interestingly, the T-bet transcription factor (TBX21) transcript remained largely suppressed throughout all stages of tick feeding (Figure 3.3, Cluster 1). This protein is known to play a central role in both the adaptive and innate immune responses where it affects the survival, development and proper functioning of dendritic cells, natural killer cells (including natural killer T), innate lymphoid cells, CD4+ and CD8+ T effector cells, B-cells, γδ T-cells and certain regulatory T-cells (Lazarevic et al., 2013). Since suppression of this transcript in the hosts of other ectoparasites has not been described, its role during parasite infection and/or disease transmission remains elusive.

Regarding the regulation of T-lymphocytes, the interferon regulatory factor, IRF4, was found to be differentially expressed. As a member of the IRF family of transcription factors, this protein is essential in the differentiation of multiple T-cell lineages (Crotty, 2011; Huber and Lohoff, 2014; Kaech and Cui, 2012; Kratchmarov et al., 2017; Xu et al., 2012). Interestingly, selective manipulation of IRF4 expression has been suggested as a strategy to boost immunity in people who are poor vaccine responders (Yao et al., 2013). In addition to transcriptional regulation of T-lymphocytes, numerous transcripts were differentially expressed suggesting the down-regulation of CD8+ T-lymphocytes differentiation into natural killer T-cells or cytotoxic T-cells during feeding of immature stages of *R. microplu*s on Bonsmara cattle (Connelley et al., 2014). The down-regulation of genes related primarily to intracellular infections, and therefore to Th1 immune type of response, implies that feeding of *R. microplus* actively polarises the host immune response towards a Th2 response, as suggested previously (Brake and Perez de Leon, 2012).

5.5.4. Chemokine receptors regulating the movement of cells out of the lymph node.

A total of two transcripts coding proteins involved in regulating the movement of leukocytes out of the lymph node were identified in this study. Firstly, the chemokine receptor CRR6 which is expressed in TH-17 and TH1 cells (Acosta-Rodriguez et al., 2007) as well as dendritic cells (White et al., 2013) was up-regulated (Figure 3.3) upon tick attachment in the lymph nodes of Bonsmara cattle. The up-regulation of the chemokine receptor suggests its importance in the movement of immune cells from the lymph node to the site of infection/ tick attachment. Secondly, the expression of CX3CR1, a chemokine receptor which mediates T-cell migration to sites of inflammation (Moser and Loetscher, 2001), was down-regulated upon larval tick attachment with slight up-regulation upon adult tick feeding. The CX3CR1down-regulation can be connected to impaired T-cell migration to the site of tick attachment caused by tick larval stage feeding (Fujita et al., 2012).

To date, this is the first study describing the transcriptional responses and cell phenotype events in the lymph nodes of tick-infested cattle. This may be due in part to the technical difficulty of collecting lymph node samples. Although the transcriptional data presented in Chapter 3 is validated to a degree by cytological studies in Chapter 2, additional experiments are required to postulate a definitive conclusion about the roles of these transcripts (and their protein products) in the host parasite interaction.

5.6. B- and T-lymphocytes in the draining subiliac lymph nodes and spleens from *I. ricinus* infested BALB/c mice (Chapter 4)

Working with large animals under experimental conditions has limitations. These include the high cost and technical difficulty resulting in the inevitable decrease in biological replicates in a study. The use of a murine model would thus be useful in studying immunological responses towards in tick-exposed host animals and vaccine responses prior to costly cattle trials. Mice are routinely used in a laboratory setting as model organisms since: (1) they are easier to handle and care for than larger model organisms; (2) due to their inbred status their lack of genetic variability provides more consistent results; and (3) a vast number of markers for the identification of cell subtypes and cytokines is available. Although not suitable to study the differences between resistant and susceptible cattle breeds, for the development of tick control strategies it is important to evaluate other model organisms to aid in the improve insight into how ticks evade effective host immune responses.

Due to differential regulation of B-lymphocytes observed in *R. microplus* infested cattle in Chapter 2 we aimed to evaluate B-lymphocyte populations in the secondary lymphoid organs of tick-infested and/or NE immunised BALB/c mice. With the use of multicolour flow cytometry few B-lymphocyte populations were noted as differentially regulated. Firstly, a significant decrease in CD45+ CD19+ IgM+ CD27+ CD80- CD21- B-lymphocytes was in the lymph node of NE immunised mice and not in mice that were only infested with ticks (Figure 4.3a and 4.4a). The significant decrease in NE immunised mice suggests that this cell population is decreased in response to immunisation. The absence of CD80 and CD21 on the surface of these cells points to the naïve nature of these cells (Alegre et al., 2001; Anderson et al., 2007). Due to the pressence of murine CD27 on developing centroblasts within the germinal centre and on memory B-

lymphocytes (Xiao et al., 2004) we propose that the cell population in Figure 4.3a and 4.4a represents an early stage of activated B-cells in lymph nodes.

Secondly, the CD45+ CD19+ IgM+ CD27+ CD80+ population of B-cells was shown to be uniquely up-regulated during tick feeding (Figure 4.3d) and significantly down-regulated in immunised mice (Figure 4.4d). This suggests that this B-cell population is stimulated following the initial exposure of mice to tick saliva but is supressed in NE immunised mice. This cell type may be an IgM+ (Dogan et al., 2009) subset of antigen-presenting cells, as is evident by the presence of CD80+ (Alegre et al., 2001; Bromley et al., 2001).

Together with the regulation of B-lymphocytes observed in *R. microplus* infested cattle in Chapter 2, T-cells were also identified to be important in the tick-host interface. However, in this study, using *Ixodes ricinus*, no significant differences in the numbers of CD45+ CD3+ CD4+ T-helper cells were noted in tick-naïve (Figure 4.7a) and NE immunised (Figure 4.7b) mice. The data indicate that this cell population is not directly affected by tick feeding or NE immunisation at these sampling points. Yet, a statistically significant decrease in T-helper lymphocytes expressing CD195 (CCR5) was observed (Figure 4.7b). Since this marker is associated with T-helper 1 inflammatory reactions (Luther and Cyster, 2001), a decrease in this cell population was expected. This finding was not supported by cytokine data as collected cells were unfortunately not stimulated *in vitro* before cytokine detection. This experiment will need to be repeated in future with an *ex vivo* stimulant such as NE. Lastly, a population of T regulatory cells with the cell surface phenotype CD45+ CD3+ CD4+ CD25+ was significantly decreased in NE immunised mice (Figure 4.6a). These cells are primarily known to modulate deleterious activities of T-helper cells (Corthay, 2009) and may thus be important in the modulation of T-helper 1 and T-helper 2 cell responses observed in *I. ricinus* infested mice (Christe et al., 1999; Ganapamo et al., 1996a, 1996b, 1995; Mejri et al., 2002).

5.7. Conclusion

This study has been the first to describe in detail the *in vivo* immune responses in lymph nodes of cattle following *Rhipicephalus microplus* infestation, attachment and continued feeding. The identification of specific cellular immune markers and/or pathways underlying tick resistance will be invaluable in the screening and breeding of more tick-resistant animals (and by extension decreasing the spread of tick-borne diseases). Future studies involving the selective inhibition of immunological pathways will be needed to confirm hypotheses in this thesis. In addition, this study contributed to the development of a murine model to study the effects of tick feeding.

5.8. References

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