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

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## Highlights

- A first comparative transcriptomic study of cattle lymph node responses to cattle tick infestations.
- First record of host responses to tick infestation for the South African Bonsmara cattle breed.
- Larval feeding stages suppresses the maturation of cattle host immunity.

## 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 constitute one of the major factors that decrease the livelihood of all farming communities, more so in resource-poor communities and developing countries. Understanding the immunological responses during tick infestation in cattle is instrumental in the development of novel and improved tick control strategies, such as vaccines. In this study, gene expression patterns were compared within the lymph nodes of three cattle breeds at different life stages of the cattle tick, *Rhipicephalus microplus* . For Bonsmara (5/8 *Bos taurus indicus* × 3/8 *B. t. taurus* ) cattle specifically, some 183 genes were found to be differentially expressed within the lymph nodes during larval and adult tick feeding, relative to uninfested cattle. Overall, the 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 found 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. This work provides a first transcriptome analysis of

bovine lymph node responses in tick-infested cattle. Findings show a dynamic immune response to tick infestation for the Bonsmara cattle breed, and that suppression of the maturation of the cattle hosts' immunity is especially evident during the larval feeding stages.

# 1 Introduction

In developing nations, livestock production is a fundamental source of revenue and nutrition, wherein cattle-farming constitutes one of the major agricultural industries[1]. The global demand for livestock products exceeding current production rates poses a threat to a sustainable food supply [2,3] In the livestock industry, vectors and vector-borne diseases have a major impact [1,4,5]and to alleviate the burden placed on the industry, control strategies (such as vaccines) are continuously being developed and improved [6]. Since few commercial vaccines are available for controlling ectoparasites, insight into host responses to ectoparasite infestation is vital for the rational formulation of protective vaccine antigens. Host immunity to ectoparasite infestation has been studied for several ectoparasitic species of economic importance in domestic ruminants, including: the sheep blow fly (*Lucilia cuprina*)[7]; the sheep scab mite (*Psoroptes ovis*)[8]; lice affecting cattle[9] and sheep[10]; as well as ticks affecting cattle[11] and sheep[12,13]. Of these, host responses to tick infestations have been studied in more detail.[11,14]

Variable resistance against tick infestation has been observed between cattle breeds from the more tick-susceptible *Bos taurus taurus* to the more tick-resistant *B. t. indicus* breeds, as well as between several crossbred cattle[11]. Several immunological factors such as presence of granulocytes and histamine have been well described in cutaneous responses, but the adaptive immune responses that confer resistance among different cattle breeds remain poorly understood and described in lymphoid tissues. Therefore, the identification of specific cellular immune markers and/or pathways that underlie this resistance will be invaluable for screening and breeding of more tick-resistant animals and improvement of tick vaccine formulations by impacting the choice of adjuvant.

Recent improvements in bovine functional genomics enable the linking of phenotypic traits with large-scale genotypic screens. The most used technologies to date include functional transcriptomics via either DNA microarrays[15] or RNA sequencing[16,17,18]. 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[19,20,21]. In the last decade, several studies have described the relative transcriptional profile of the skin[17,22,23,23,25,26,27,28,29] and blood[29,30] of more tick-resistant cattle compared to more tick-susceptible breeds.

The skin represents the first physical barrier to tick infestation, containing many immune cells within its dermal layers that mediate innate immunity[31]. Pattern recognition receptors (PRRs) recognizing pathogen-associated molecular patterns (PAMPs) have been described that are modulated by tick saliva that includes toll-like receptors (TLR) 4 [21], TLR-2 [33] and TLR-2/TLR-3 [34]. However, the downstream effects of saliva-induced cellular signalling via these PRRs, including subsequent proinflammatory cytokine release, and its linkage to the adaptive immune system remains unexplored. Bridging the innate and adaptive immune responses is mediated to a large extent by dendritic cells (DCs) that link the initial recognition of tick components with the development of an antigen-specific adaptive immune response. In subsequent exposures of cattle to ticks, adaptive immunity may be engaged by other antigen-presenting cells that is not limited to DC's alone [31]. 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-dependent or T-independent antigens [35,36]. Therefore, interrogating the transcriptional profile of lymph nodes as sites of immune cell differentiation and proliferation, in response to tick infestation, is vital. Since no research is available on the response of bovine secondary lymphoid organs to ectoparasites, this study could serve as a hallmark for the study of bovine immune responses to ectoparasites.

This pilot study aimed to compare the transcriptional regulation of regional draining lymph nodes in response to infestation by immature and adult *R. microplus* ticks in 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 cross) cattle. Bonsmara breed-specific findings in regards to identification of unique or shared transcriptional responses in the regional draining lymph nodes of this cattle breed is highlighted.

## 2 Materials and methods

### 2.1 Experimental animals and artificial tick challenge

Three of each breed for Holstein-Friesian, Bonsmara and Brahman calves (~9 months old and with minimal previous exposure to ticks) were housed in closed stables within the quarantine facilities of the University of Pretoria Biomedical Research Centre (ethical number: EC036-13) with governmental section 20 approval (DAFF reference number: 12/11/1/8/1). Bonsmara cattle were chosen as an important South African mixed breed that has been shown to display an intermediate level of tick resistance [37].

Calves were treated 14 days prior to the onset of the study 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 Amitraz 12.5% (100 ml/50 L water, Cooper) to ensure that cattle were free of parasites prior to the commencement of the study. Hatched larvae derived from a South African field strain of *R. microplus* (ClinVet Pty. Ltd., Bloemfontein, South Africa) were placed on cattle in two patches (~2,000 larvae; 0.1 g) attached bilaterally on each side of the animal around the draining region of the superficial cervical lymph node. Additional 6,000 larvae (0.3 g) 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. This study aimed to investigate the host immunological response to the tick life cycle and not to correlate results to the number of ticks attached to the cattle. Therefore, tick infestation was considered successful if a minimum of a 100 semi-engorged adult female *R. microplus* ticks were present in each patch at day 17 of the study.

### 2.2 Collection and processing of lymph node samples

Lymph node samples were taken at three time-points: prior to artificial tick infestation (i.e. ‘pre-infestation samples’), three days post tick infestation (larval life stage) and 17 days post tick infestation (adult tick life stage)( Supplementary Figure S1 ). The initial biopsy was collected after patches were attached bilaterally to the cattle. Lymph node biopsies were collected 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). For each time point, a wedge of 1 $cm^3$ from the superficial cervical lymph node, containing regions from the hilum to the capsule, was taken on alternating sides of the animal. To control for an inflammatory response for subsequent sampling, the "pre-infestation" lymph node biopsy was taken from both the left and right 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 [37]. 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 $H_2SO_4$), cut into smaller pieces, homogenised using liquid nitrogen and a QIAshredder (QIAGEN) column. Total RNA was isolated using TRI-reagent fractionation (Sigma-Aldrich) and the RNeasy mini kit (QIAGEN). Contaminating genomic DNA was removed by DNase I treatment. Final RNA concentrations, purity and integrity were assessed with the NanoDrop-1000 (Thermo Fisher Scientific, USA) and the Experion automated electrophoresis (Bio-Rad) systems.

### 2.3 Microarray assay of isolated cattle lymph nodes

Microarray assays were performed using the Agilent Bovine V2 4x44K slides (Agilent Technologies, USA). Total RNA with an $A_{260}$ :$A_{280}$ ratio of>1.7 and an RNA Quality Indicator of >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 three cattle breeds (Bonsmara, Brahman and Holstein-Friesian) was made. First-strand cDNA synthesis was performed by incubating 4 µg RNA with 250 pmol oligo($dT_{25}$) and 775 pmol random hexamer primer for 10 min at 70˚C, followed by cooling on ice for 10 min. Reverse transcription and aminoallyl-dUTP (5-(3-aminoallyl)-2′deoxyuridine-5′ triphosphate) incorporation was 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). 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, and the labelling efficiency and sample concentration determined using the NanoDrop® ND-1000 system. Cy3 and Cy5 labelled cDNA samples that had a labelling efficiency of>10 (i.e. a minimum ratio of 10:1000 of labelled to unlabelled nucleotides) 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. Hybridisation, washing and post-processing were performed at the ACGT Microarray Facility (University of Pretoria, South Africa) using the Axon GenePix 4000B scanner and Axon GenePix Pro 6.0 software (Molecular Devices) as described previously [38].

### 2.4 Microarray data analysis and functional annotation of transcripts

To identify significantly differentially expressed gene transcripts, 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 changes were determined between all transcripts within a cattle breed collected at different time-points (3 replicates) using empirical Bayesian statistics, which were subsequently expressed as *P* values (corrected for false discovery rates, FDR). Transcripts were regarded as differentially expressed if a>2-fold change (log2ratio > 1, log2ratio < -1) in

either direction with *P* value ≤ 0.05 were observed. Transcripts with M values > 0, a Cy5 intensity > 1,000 and a log2 fold expression of one or greater in a single state (i.e. "larvae" or "adult") relative to the reference pool, were considered differentially expressed. Pearson correlations were done in R to determine the correlation of the biological replicates within each group (Supplementary Table S1). Expression data has been submitted to the NCBI GEO database (accession nr.: GSE147918). 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 of normalised M (or intensity values). The methods for distance calculation and clustering were "Euclidean" and "ward.D", respectively. Differentially expressed transcripts were functionally annotated by first 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.uplib.idm.oclc.org/ ) databases to confirm gene entries and identify homologous transcripts with described functions [39]. Additional searches were also performed to provide gene ontology terms (GO) and protein families via SMART/Pfam databases using EggNOG (v.4.5.1) [40]. 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/ ). Transcript functional group classifications were based on the eukaryotic orthologous group terms for gene ontology (KOG) [41]. Differentially expressed genes (DEGs) were also analysed in the context of functional pathways using the KEGG database and ranking these pathways based on statistical overrepresentation with a fold enrichment (FE > 1) and *P* value (≤0.05) adjusted with the Benjamini–Hochberg correction in DAVID.

### 2.5 Validation of microarray results using qRT-PCR

Using the same RNA isolated from Bonsmara cattle lymph node tissues, 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 12 K Flex Real-Time PCR System (Applied Biosystems R, Life Technologies, Inc.) using the manufacturer's recommended cycling conditions. The resulting data were normalised against six endogenous controls, including: beta actin (ACTB) (NM_173979.3), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM_001034034.2), hypoxanthine phosphoribosyltransferase 1 (HPRT1) (NM_001034035.2), succinate dehydrogenase complex subunit A, flavoprotein (Fp) (SDHA) (NM_174178.2), TATA box binding protein (TBP) (NM_001075742.1) and mitochondrial ribosomal protein S9 (MRPS9) (NM_001035332.1). Data processing was done in ExpressionSuite v1.1. (ThermoFisher Scientific).

## 3 Results

### 3.1 Effect of *R. Microplus* attachment and feeding on the lymph node transcriptome of Bonsmara cattle

A total of 183 genes were found to be differentially expressed in Bonsmara cattle lymph nodes ( Fig. 1 A). Bonsmara at the larval stage represented the majority of DEGs (65 down-

regulated and 89 up-regulated). All shared DEGs in both tick life stages showed a similar expression profile for both post-infestation collection points ( Fig. 1 B). This suggests similar regulation of immunity in response to larval attachment and adult feeding and/or re-attachment. In addition to Bonsmara cattle, the transcriptional response of Brahman and Holstein-Friesian breeds were evaluated ( Supplementary Figure S2 ). However, weak correlation between individual replicate animals within Brahman and Holstein-Friesian groups, resulted in the identification of a low number of valid DEGs and was therefore omitted from further discussion. To verify the expression data, qRT-PCR was performed on four randomly selected transcripts that were specifically up-regulated during the immature feeding stages on Bonsmara cattle. These transcripts were: toll-like receptor 7 (NM_001033761.1); chemokine (C-C motif) ligand 14 (NM_001046585.1); defensin beta (4A and 5) (NM_174775.1) and CD40 (NM_001105611.2). Overall concordance in regard to the direction of change (i.e. up-, down- or unaffected) was observed between the array and qRT-PCR findings (Supplementary Table S2). However, in this pilot study, a small animal population (n = 3) was sampled and only four genes were used in array validation. This will have to be expanded upon in future studies to lend greater support to the current findings.

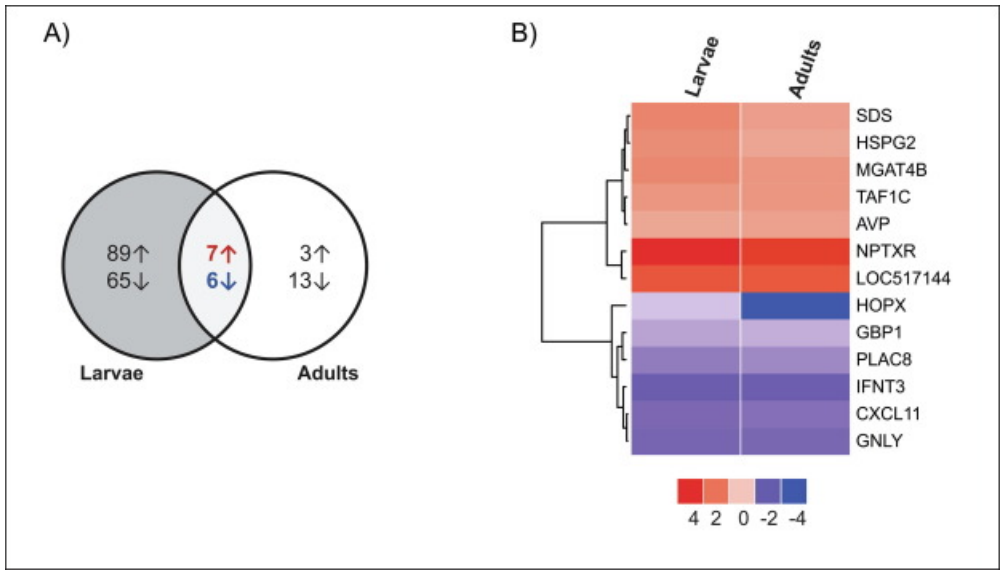


**Fig. 1.** The distribution of differentially expressed genes in the lymph nodes of Bonsmara cattle infested with *R. microplus* larvae and adult ticks. (A) A Venn diagram indicating the number of up/down regulated genes in the lymph nodes of Bonsmara cattle, specific for feeding of larvae, adult ticks or shared between both *R. microplus* life stages. (B) Heatmap of the 13 differentially expressed genes in lymph nodes of Bonsmara cattle shared between larvae or adult tick infested cattle. Colours represent the $\log_2$ transformed fold changes from red (up-regulated: +4) to blue (down-regulated: −4).

### 3.2 Functional annotation and hierarchical clustering of DEGs in lymph nodes of Bonsmara

Of the 183 unique transcripts identified as differentially expressed in Bonsmara lymph nodes ( $P \leq 0.05$) (Supplementary table S3), approximately 14% of transcripts (25 DEGs) could not be functionally annotated following manual curation of the data ( Fig. 2 A). The annotatable transcripts were separated using KOG identifiers into general functional classes and general functional processes, of which cellular and signalling processes represented the largest component of differentially expressed transcripts (~66%; Fig. 2 A). Overall, the largest subsets of DEGs were associated with the larval life stage, involved in signal transduction ( Fig. 2 B: T) and the formation of extracellular structures ( Fig. 2 B: W). Hierarchical clustering indicated that patterns during adult feeding and preinfested stages were similar, compared to the expression patterns during the larval feeding stage ( Fig. 3 A). 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 ( Fig. 3 A). 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 ( Fig. 3 D: Cluster 3). Similarly, some DEGs were down-regulated upon larvae infestation, and then returned to non-infested levels during adult feeding ( Fig. 3 B: Cluster 1). Cluster 2 ( Fig. 3 C) represents an intermediate cluster that presented a similar trend to Cluster 3 ( Fig. 3 D), but with less gene expression variability between the three sampling points. These results point towards an 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 [42], and current clustering results appear to support some of these findings. Pathway analysis showed over representation of four KEGG pathways (Supplementary Table S4), namely: (1) cytokine-cytokine receptor interactions (bta04060) (FE: 1.91; *P value* = 0.04), (2) the chemokine signalling pathway (bta04062) (FE: 2.07; *P value* = 0.02), (3) Leukocyte transendothelial migration (bta04670) (FE: 2.68; *P value* = 0.01) and (4) the Toll-like receptor signalling pathway (bta04620) (FE: 2.32; *P value* = 0.05). The comparable clustering patterns of these transcripts give an indication to their possible shared functions ( Fig. 3 B&D). Eight related chemokines (i.e CCL3 (LOC616364), CCL4, CCL5, CCL19, XCL1, CXCL9, CXCL10 and CXCL11) and two chemokine receptors (CCR6 and CX3CR1) were found as DEGs. All of the latter chemokines (except for CCL19) and chemokine receptor CX3CR1 group together in Cluster 1 ( Fig. 3 B&D), showing down-regulation during larval stages with only slight recovery during adult feeding. By contrast in expression Cluster 3 ( Fig. 3 D), CCL19 and CCR6 were shown as up-regulated upon tick attachment with subsequent down-regulation during adult feeding.

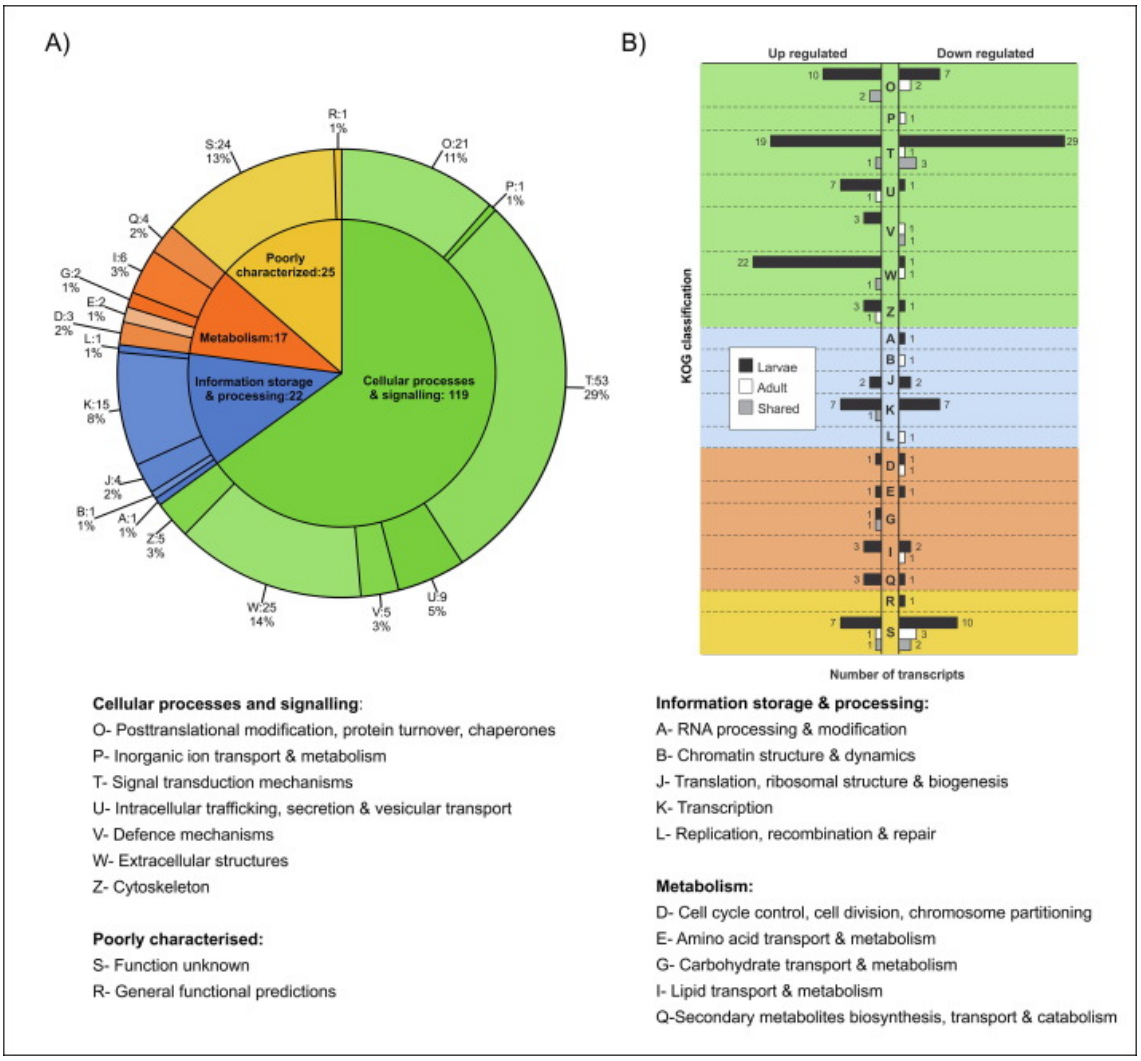


**Fig. 2**. Significantly (above threshold and *P* value ≤ 0.05) differentially expressed genes in the lymph node of Bonsmara cattle as classified according to their eukaryotic orthologous functional groups (KOGs). A) Classification of genes differentially expressed in Bonsmara lymph node tissues during 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. 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.

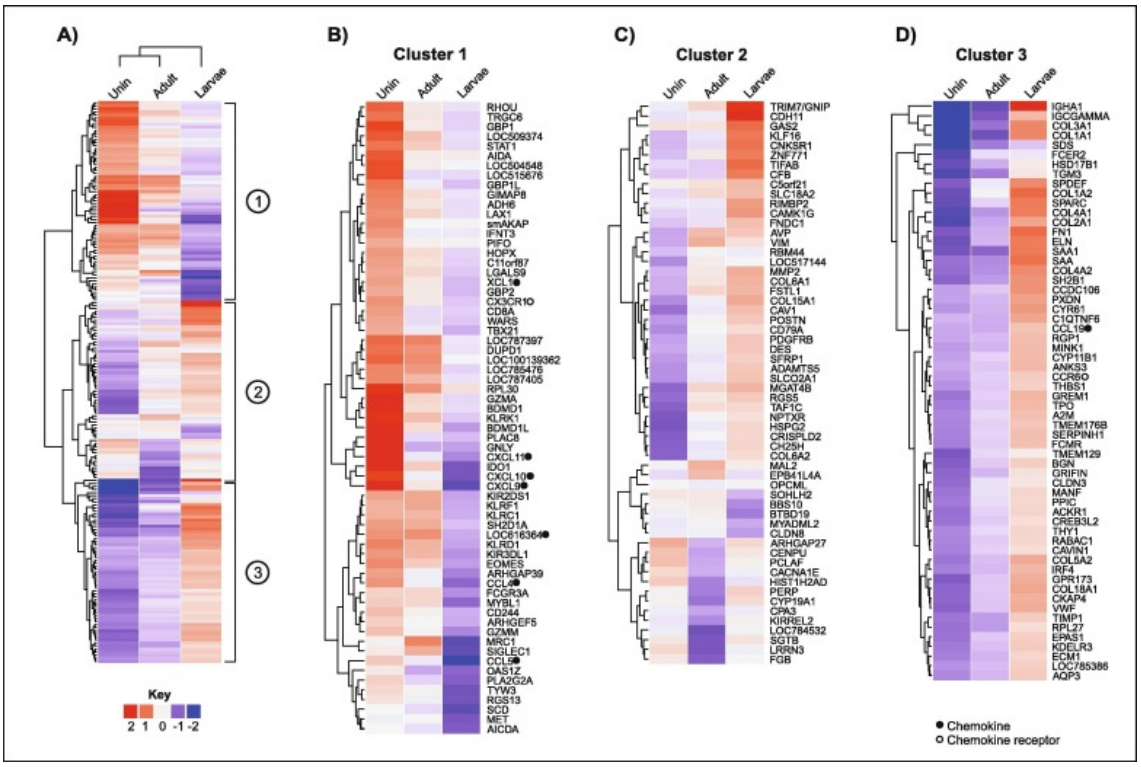


**Fig. 3**. Hierarchical clustering of expression pattern of 183 genes that are differentially expressed in the lymph nodes of Bonsmara cattle infested with larvae and adult *R. microplus* . The colour bar of the M−values indicates a higher expression in red (2) and a lower expression in blue (-2) of the identified DEGs at the relevant pre-infestation, larvae infested and adult-infested sampling points.

## 4 Discussion

Overall, the transcriptional data suggests that the majority of the immune response occurs upon larvae attachment and is maintained with more mature tick attachment and feeding. Since the lymph node is a dynamic organ where the spatial orientation of cells is related to their function [54], the following discussion aims to address the main steps in regulated lymphocyte trafficking in lymph nodes. These include: (4.1.) Leukocyte recruitment to the lymph node via chemokines and chemotaxis, (4.2.) Trans-endothelial and intranodal movement on the reticular network, (4.3.) Active regulation of cellular transcription and translation in the lymph node (including leukocyte associated cellular regulatory networks) and (4.4.) Chemokine receptors regulating the movement of cells out of the lymph node. The findings described in the following sections have been integrated into a proposed mechanism of immune-effects in Bonsmara cattle in response to *R. microplus* infestation ( Fig. 4 ). This hypothesis may be used in future as a scaffold for interrogating proposed pathways to finally obtain a more complete view of bovine immunity to *R. microplus* feeding.

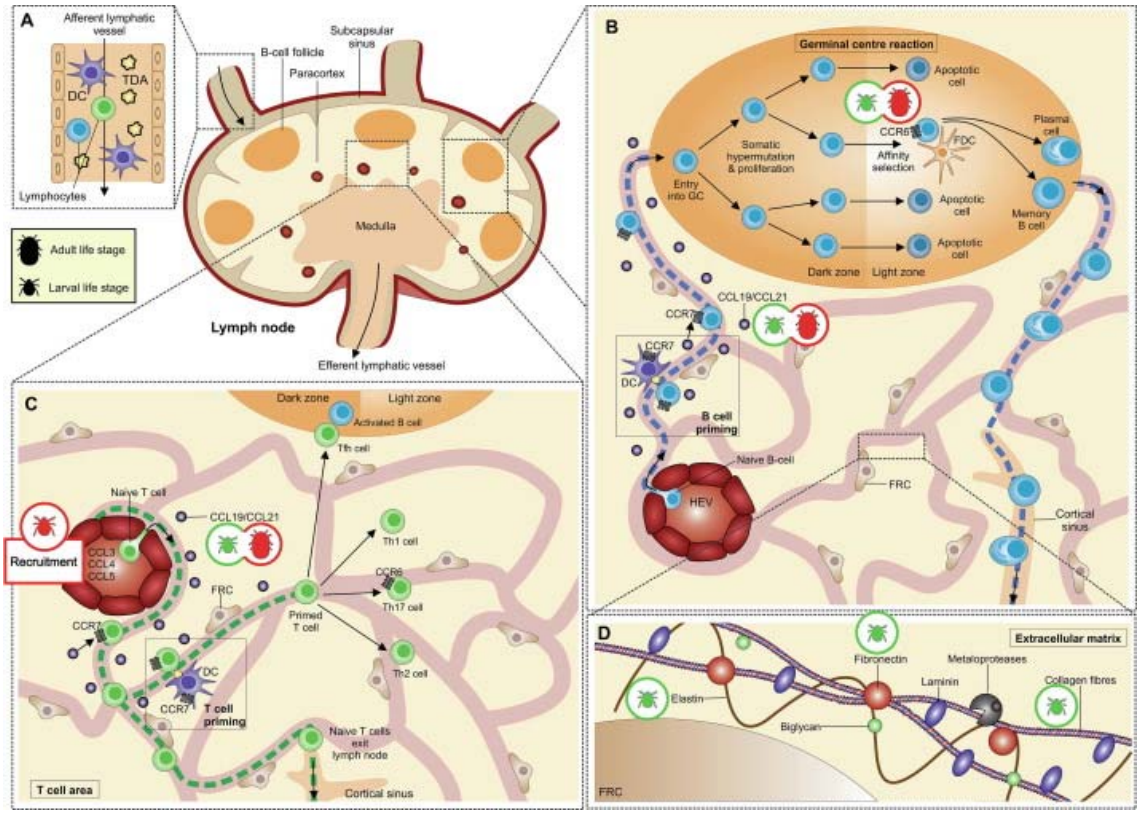


**Fig. 4**. The proposed effect of tick attachment and feeding on draining lymph nodes in Bonsmara cattle. Indicated is the typical processes of leukocyte recruitment to the lymph nodes via chemokines and chemotaxis (panels A, B and C); *trans* -endothelial and intranodal movement on the reticular network (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 Fig. 3 . Selected targets involved in lymphocyte recruitment and chemotaxis that showed transcriptional up- (green) and down-regulation (red) during larval and adult tick feeding are indicated. Abbreviations used in the figure: Tick-derived antigen (TDA); dendritic cell (DC); high endothelial venule (HEV); fibroblastic reticular cell (FRC).

### 4.1 Leukocyte recruitment via chemokines and chemotaxis

During lymphocyte trafficking naïve lymphocytes enter the lymph node 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 ( Fig. 4 B,C) [36,43,44,45]. Additionally, the afferent lymphatic vessels also deliver tissue-derived antigens and immune cells to the subcapsular sinus surrounding the lymph node [36,46]( Fig. 4 A). Several differentially expressed chemokines (i.e. CCL3, CCL4, CCL5, CCL19, XCL1, CXCL9, CXCL10 and CXCL11) were identified that are involved in the recruitment and entry of lymphocytes to the lymph node (Supplementary table S2). CCL5 was found to be differentially expressed in the skin of tick-naïve and tick-infested Holstein–Friesians [27], suggesting its role at the site of tick attachment in addition to its role in the lymph node. While CXCL10 expression was found to be significantly higher in the blood of tick infested Holstein-Friesian cattle (compared to Brahman) [27] and in larvae infested cross-bred cattle [30]. Currently, the human and murine chemokine system is well characterised [47] with limited data for bovine homologues [48]. As such, the following proposed functions will need to be validated in bovines. Three differentially expressed inflammatory chemokines CCL3 (MIP-1α, LD78a), CCL4 (MIP-1β, LAG-1) and CCL5 (RANTES) were identified ( Fig. 4 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 [49], wherein CCL4 is the most potent chemoattractant for CD4 + CD25 + regulatory T cells [50] in accordance with KEGG pathway analysis (bta04620) [51,52,53,54]. 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 [51,55,56,57]**.** In addition, CCL5 has also been identified to have a role in Paratuberculosis, mastitis and parasitic infections [51,58,59,60,61,61]. Overall, the down-regulation ( Fig. 3 , Supplementary table S2) 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 chemotactic pathways and up-regulation of anti-chemotactic proteins such as the olfactory-like receptors ( Fig. 4 ; panel C).

**4.2 Trans-endothelial and intranodal movement on the reticular network**

The chemokine CCL19 was found to be up-regulated during larval feeding ( Fig. 3 ). This chemokine ligand binds to its receptor, CCR7, which has several downstream effects in the lymph node ( Fig. 4 B&C). Upon recognition of pathogens/parasites 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 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 2.4B&C) [35]. Additionally, CCR7 is also expressed on naïve T [63] and B cells [36,45,64]. Its role in T cell migration within peripheral lymph nodes was also validated by gene knockout experiments in mice [49,65]. We propose that in Bonsmara cattle, up-regulation of CCL19 within lymph nodes sustain the follicular network and ensure T cell priming upon entry, but that the tick larvae may hinder recruitment and migration of immune cells to the lymphoid tissues. Migration of immune cells to the lymphoid tissues is enabled during adult feeding which suggests that resistance to host defences is already established during the larval stages.

Alpha-1,3-mannosyl-glycoprotein-4-beta-N-acetylglucosaminyltransferase B (MGAT4B) involved in N-linked glycoprotein biosynthesis was found to be up-regulated during both immature and adult life stages ( Fig. 3 , Supplementary table S2). This transferase could be important as recruited lymphocytes infiltrate the lymph node, they traverse 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 ( Fig. 4 B& C) [66,67]. It appears that during all stages, *trans* -endothelial migration of lymphocytes into lymph nodes is maintained ( Fig. 3 ). Following infiltration, T cells migrate to the paracortex while B cells localize to the cortical lymphoid follicles ( Fig. 4 B&C) [68]. 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) [69]. These follicular cells mediate the building of a reticular network by producing extracellular matrix components that interweave to form the reticular fibres criss-crossing the cortex ( Fig. 4 B-D) [70, 71,72,73,74]. Modification of this 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 found

as DEGs ( Fig. 3 ). Several secreted proteases, protease inhibitors ( Fig. 4 D) and their associated extracellular matrix receptor interaction pathways were identified (KEGG pathway ID: bta04512) in larvae infested Bonsmara tissues. Together these transcripts function in cell–cell and cell-extracellular matrix adhesion processes [75], subsequent T cell and monocyte migration [76], and signalling for T and B cell proliferation through interactions with fibronectin [75]. In this regard, fibronectin 1 (FN1) was identified as up-regulated upon larval attachment supporting this mechanism. In addition, the transcription factor SPDEF (up-regulated, larvae) appears to be co-regulated with several collagens (i.e. COL1A2, COL2A1, COL4A1 and A2), as well as fibronectin 1 (FN1) and elastin (ELN) ( Fig. 3 ). As it has been shown previously that SPDEF regulates expression of metalloproteinase 9 (a collagenase for type 4 collagen) [77], 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 bovine 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 when adult ticks are feeding. These changes suggest that modulation of lymph node physiology occurs during larval infestation, priming the lymph tissues for development of an immune response.

### 4.3 Regulation of transcription and translation

Evidence towards ticks affecting the maturation process of lymph node tissues via several transcriptional networks is also provide by this study. It includes, 12 DEGs involved in regulating gene expression; namely 5 transcription factors highly expressed during larval feeding (i.e. KLF16, SPDEF, ZNF771, CREB3L2 and EPAS1) and a TATA box binding protein-associated factor (TAF1C) that was up-regulated throughout infestation ( Fig. 3 ). TAF1C is involved in assembly of the preinitiation complex for RNA polymerase I-dependent transcription of ribosomal RNA genes [78], and indeed the two 60S ribosomal proteins (RPL27 and RPL10- LOC785386) were shown to be up-regulated during the larval feeding stage, forming a cluster with other similarly regulated transcripts involved in transcriptional initiation (EPAS1); protein trafficking (KDELR3) and extracellular matrix production (TIMP1 and ECM1). Both transcription factors KLF16 and ZNF771 appear to be co-regulated with a TRAF-interacting protein (TIFAB) and complement factor B (CFB) ( Fig. 3 ). Of great interest is the T bet transcription factor (TBX21) which remains largely suppressed throughout all stages of tick feeding. It 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, innate lymphoid cells, CD4 + and CD8 + T effector cells, B cells, γδ T cells and certain regulatory T cells [79].

The interferon regulatory factor (IRF4) was up-regulated during larval infestation and then diminished during the adult stages ( Fig. 3 D). The IRF family of transcription factors control differentiation of CD4 + follicular helper cells in secondary lymphoid germinal centres for the production of high-affinity neutralizing antibodies [80,81,82], as well as the development of cytotoxic T cell and CD8 + T lymphocyte responses. IRF4 can thus function in both humoral and cellular immunity.

Expansion and differentiation of CD8 + T cells are further regulated by T box transcription factors T bet (TBX21) and eomesodermin (EOMES), which was also detected ( Fig. 3 B) [79,81,83]. Both were down-regulated following immature tick infestation ( Fig. 3 B). T bet is also involved in differentiation/maturation of IFN-γ-producing T helper cell lineages [79], and acts as a repressor of the JAK/STAT1 pathway in macrophages [84]. This interplay is supported

by the differential regulation of TBX21, EOMES and STAT1, as well as IRF4 ( Fig. 3 B&D). An overall suppressive effect on T cell maturation via this mechanism/pathway seems thus evident during the larval feeding stage. Additional transcripts encoding receptors (i.e. CD224 (2B4), KLRF1 KLRC1, KLRD1 (CD94), KIR3DL1, FCGR3a or CD16a), receptor-signal activators on natural killer cells (KIR2DS1), and the secretion of their associated products granzymes A (GZMA), M (GZMM) and Y (GNLY) were shown to be co-repressed along with eomesodermin [85,86,87,88] ( Fig. 3 B). This DEG cluster supports the transcriptional link and down-regulation of differentiation of CD8 + T lymphocytes into NK cells or cytotoxic T cells during the immature stages . Such activity was also reported for salivary gland extracts of *Dermacentor reticulatus* [90], *Amblyomma variegatum, Haemaphysalis inermis* and *Ixodes ricinus* [91,92]. Combined, the latter suggests that in response to tick infestation there is a suppression of cytotoxic T cell-mediated immunity in the host.

## 4.4 Chemokine receptors regulating migration in lymph nodes

The chemokine receptor CRR6 was up-regulated following tick attachment, reverting to basal levels during adult feeding ( Fig. 3 D). It is known that human memory and effector CD4 + TH-17 cells, a subset of TH1 cells [93] and dendritic cells [94] express CCR6. This receptor with its ligand (CCL20) is responsible for the chemotaxis of dendritic cells, effector and/or memory T and B cells [95]. This ligand-receptor pair is also important in skin and mucosal surfaces under inflammatory conditions, where CCR6 is recruit by both proinflammatory IL17 producing Th and Treg cells to sites of inflammation. The up-regulation CCR6 support its importance in the movement of lymphocytes from the lymph node to the cutaneous site of tick attachment. Therefore, recruitment of cells to the tick feeding pool may not be directly influenced, but rather that further maturation of effector cells are influenced that would lead to a Th1/Th2 response. With regards to antibody production, immunoglobulin transcripts (IGHA1 and IGCGAMMA) followed a similar trend as CCR6 and CCL19 ( Fig. 3 D), that may translate into decrease immunoglobulin production by B cells during adult tick feeding. CX3CR1 (a chemokine receptor) was also down-regulated upon larval attachment. This receptor binds fractalkine (a chemokine) in humans, mediating T cell migration to sites of inflammation [54]. Ligand binding to CX3CR1 further mediates both the adhesive and migratory functions of T cells leading to migration of this cell type to its effector sites [97,99]. 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 [98]. 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 also contributes to the enhanced adhesive effects observed for effector T cells that expresses this receptor [97,98,99]. Therefore, the down-regulation of CX3CR1 suggests that the mechanism of T cell migration to the site of inflammation (tick attachment) is negatively affected during the larval feeding 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 [37]. Furthermore, previous studies showed a significant increase in CD3 + T lymphocytes in resistant compared to susceptible cattle [23,37]. Similar to current findings, these studies also showed minimal infiltration of CD3 + T lymphocytes during the immature feeding stages for susceptible breeds. For Bonsmara cattle at least, some recovery of CD3 + T lymphocytes is evident following immature tick feeding [37], which is also supported by the transcriptional data presented here. Overall, at a transcriptional level, there seems to be a possible suppression of lymphocyte maturation signals that is especially evident during the larval feeding stage.

## 5 Concluding remarks

This work provides the first comparative transcriptomic study of lymph node responses to *R. microplus* infestation. It is evident that bovine immune responses to tick infestation is a highly dynamic and complex process, stressing the need for caution when considering comparisons between tick life stages. For Bonsmara cattle several transcriptional immune related mechanisms could be identified that has provided some resolution regarding host responses during tick feeding. Transcriptional data suggests that lymph node maturation and *trans* -endothelial migration of lymphocytes appears to be maintained during tick attachment and feeding. In contrast, the maturation of host immunity is suppressed, especially 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 (i.e. tick attachment site). In contrast to studies performed on peripheral cutaneous responses, these results highlight the importance of understanding lymphocyte development in the secondary lymphoid organs, from the point of initiation to the mounting of an adaptive immune response. Cattle will most likely already be burdened with varying degrees of tick infestation in the field prior to application of a tick vaccine. As such, understanding how the hosts' immune system is compromised by these parasites will inform how next generation tick vaccines should be formulated (i.e. with adjuvants and other additives) to overcome these451 suppressive effects and increase the efficacy of vaccination. Additionally, vaccinating animals in the field when burdens are at their lowest (e.g. during winter, or following dipping), may be important to enable the host's immune system to achieve immunological memory prior to seasons when burdens are peaking. The data presented here shows the differential regulation of several key genes and further studies will be performed to improve on these findings.

### Author contributions

Luïse Robbertse performed all experimental work, as well as data analyses and drafting of the manuscript. Sabine Richards and Christian Stutzer assisted in experimental work, as well as data analyses and drafting of the manuscript. Nicholas Olivier assisted with microarray project design, microarray processing and data analyses. Andrew Leisewitz and Jan Crafford are the trained veterinary personnel responsible for tissue collection and additional support in the project. Christine Maritz-Olivier conceptualised the study, conducted data curation, funding acquisition, project administration, supervision and critical editing and reading of the article.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Funding

This work was funded by the Tshwane Animal Health Innovation Cluster as part of the Technology Innovation Agency (grant THAC12-00027) of South Africa.

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