Comparative immune-profiling of tick vaccine formulations and the role of co-infestation with *Babesia microti* in *Ixodes ricinus*-infested BALB/c mice

by

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Submission declaration

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'Cause I'm on top of the world

I'm on top of the world

Waiting on this for a while now

Paying my dues to the dirt

I've been waiting to smile

Been holding it in for a while

Take it with me if I can

Been dreaming of this since a child

I'm on top of the world

~Imagen Dragons

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Summary

Parasites have evolved a wide variety of mechanisms to evade or manipulate their host's immune responses. Parasite interactions especially in regards to host cellular responses have been well studied *in vivo* using pathogen free laboratory rodents (e.g. mice). However, co-infestation (or infection) with multiple pathogens and/or parasites is common in animal hosts (e.g. cattle) under field conditions. Such co-infestations have the potential to modulate the host immune responses, enabling infection/infestation that makes the host more susceptible, as well as confounding the efficacy of treatments (i.e. vaccination).

A greater understanding of the immunological interplay elicited during co-infestation of a model host animal with parasites of economic importance could therefore influence the way that the next generation vaccines are designed. Consequently, a co-infection model with *Babesia microti* and *Ixodes ricinus* in BALB/c mice was interrogated in the presence and absence of immunisation with a mock antigen (i.e. ovalbumin) formulated in Freund's adjuvant. Results show that co-infestation of *I. ricinus* and *B. microti* skews the host immune response towards a Th1 immune response with a down-regulation in antigen specific IgE antibodies. Both B- and T lymphocyte data indicated that *I. ricinus* ticks have a negligible effect on lymph node, spleen and blood lymphocyte subpopulations, possibly due to the immunosuppressive effect of tick saliva. Secondly, mice infested with *B. microti* only or co-infested had a similar effect on the host immune response, with similar lymphocyte subpopulations being differentially regulated. Additionally, co-infestation resulted in significant up-regulation of IL-10 (Th2 cytokine) and TNF-α (Th1 cytokine) associated with potent anti-inflammatory properties, limiting host immune response to pathogens.

Similarly, the effect of formulation with different adjuvants to enhance host immune responses to vaccination were tested in mice using tick-specific antigens (Bm86 and TC-X) derived from the cattle tick, *Rhipicephalus microplus*. Mice immunised with a combination of Bm86 formulated with GLA-SE adjuvant had a significantly higher Bm86 specific IgG2a antibody titre and Th1 polarised immune response relative to mice vaccinated with Bm86 formulated with Alum. Furthermore, formulations containing Alum had significantly higher concentrations of IL-2 (in the spleen and lymph node) and TNF-α (in the spleen), indicating a more Th1 polarized immune response overall. The chosen adjuvants had negligible stimulatory effects on the *ex vivo* lymphocyte subpopulations when using control tissues. However, immunisation with both antigens resulted in significantly higher populations of memory B cells (CD80+) and T regulatory cells (CD3+CD4+CD25+). Both these lymphocyte subpopulations are essential in

host immune response and maturation following vaccination. Results from this study therefore indicated that vaccines formulated with both the Bm86 and TC-X antigens produces an optimal immune response compared to vaccines containing only Bm86. In regards to the adjuvants, Alum and GLA-SE, different effects were observed on the host immune maturation for the different antigen combinations.

The study was successful in describing the effect co-infestation of a tick and a tick-borne pathogen has on a model organism and how these simultaneous infestations may influence antigen-specific immune reactions. Furthermore, the study is the first to describe the immunological response in BALB/c mice when immunised with Bm86 antigen formulated with different adjuvants. The work completed in this study is essential to efforts being made to develop a *R. microplus* vaccine for cattle use, taking into account the effect co-infestation has on vaccine efficacy as well as conducting a preliminary vaccination study.

Table of Contents

Submission declaration i

Plagiarism declaration: ii

Acknowledgements iv

Summary v

Table of Figures x

List of Tables xii

List of Abbreviations xiii

Chapter 1: Literature review 1

1.1. Tick evolution and adaptation to hematophagy 1

1.2. *Ixodes ricinus*: a model tick for immune responses in vertebrate hosts 3

1.2.1. Ticks: Ancient master manipulators of host immunity 4

1.2.2. The effects of tick saliva on host immunity 5

1.3. Tick-borne pathogens and host immunity: a focus on *Babesia* 6

1.3.1. The co-evolution of pathogens and how they utilize tick vectors to infect vertebrate host 7

1.4. *Ixodes ricinus* and *Babesia microti*: a model system for the study of vector-pathogen-host interactions 8

1.5. Tick vaccines: Lack of antigens and data for adjuvant selection 11

1.5.1. *Rhipicephalus microplus*: a case study in increasing vaccine efficacy using a model organism 12

1.6. Adjuvants and their role in veterinary vaccines 14

1.7. Relevance of co-infestation in South African cattle herds 18

1.8. Biological questions forming the basis of this study: 19

1.9. Hypotheses: 22

1.10. Aims and specific objectives: 22

1.11. Outputs: 23

Chapter 2: Materials and Methods 25

2.1. Experimental animals, *B. microti* parasites and *I. ricinus* ticks 25

2.2. BALB/c mice co-infestation with *Babesia microti* and *Ixodes ricinus* and immunization 26

2.2.1. Experimental set-up and infestation protocols 26

2.2.2. Termination and blood collection 27

2.2.3. Spleen and lymph node collection, processing and leukocyte isolation 27

2.2.4. Serum antibody concentration determined using enzyme-linked immunosorbent assay 28

2.2.5. In vivo cytokine detection of lymph node tissue 30

2.2.6. Leukocyte staining and flow cytometry 31

2.3. *Rhipicephalus microplus* antigen(s) and adjuvant formulation optimization in BALB/c mice. 35

2.3.1. Bm86 and TC-X antigen production 35

2.3.2. Experimental set-up and vaccination regime 35

2.3.3. Tissue culturing and supernatant collection 36

2.3.4. Serum antibody titre determination using enzyme linked immunosorbent assay 37

2.3.5. *Ex-vivo* cytokine detection of cultured spleen and lymph nodes 38

2.3.6. Leukocyte staining and flow cytometry 38

2.4. Statistical and data analysis 38

Chapter 3: Results 39

3.1. Assessment of the immune response against ovalbumin in BALB/c mice during co-infestation with *Babesia microti* and *Ixodes ricinus* 39

3.1.1. Total IgG and IgG1, IgG2a and IgE responses to infestation and vaccinatio 39

3.1.2. *In vivo* cytokine analysis of lymph node tissues: Th1 responses 42

3.1.3. *In vivo* cytokine analysis of lymph node tissues: Th2 responses **43**

3.1.4. Lymphocyte populations produced during co-infection in the spleen, lymph node and blood of BALB/c mice **45**

3.2. Assessment of the immune response of BALB/c mice following immunization with the Bm86 and TC-X antigen combination formulated in two adjuvants **51**

3.2.1. Serum Bm86 and TC-X specific IgG1 and IgG2a antibody titres **51**

3.2.2. *Ex vivo* cytokine concentrations 24 hours after culturing **52**

3.2.3. Lymphocytes populations present in *ex vivo* cultures after 48 hours **58**

Chapter 4: Discussion: **62**

4.1. Assessment of the Immune response in BALB/c mice co-infected with *Babesia microti* and *Ixodes ricinus* and its implications for the development of tick vaccines. **62**

4.1.1. Day 16: Individual infection/ infestation of *B. microti* and *I. ricinus* versus co-infestation **62**

4.1.2. Day 38: Co-infestation and its effect on antigen-specific immune responses **68**

4.1.3. Effect of *Ixodes ricinus* nymph infestations in the presence and absence of immunisation with OVA and Freund's adjuvant **71**

4.2. The effect of adjuvant on the response towards two protective antigens from *Rhipicephalus microplus* in BALB/c mice: **74**

4.2.1. Vaccine formulation effect on cytokine production in spleen and lymph node tissues **76**

4.2.2. Vaccine formulation effect on lymphocyte subpopulations in the spleen and lymph nodes **77**

4.2.3. Vaccine formulation effect on antibody production for spleen and lymph nodes **78**

Chapter 5: Concluding discussion **79**

References: **83**

Table of Figures

Figure 1.1: Phylogenetic tree showing the evolutionary relationships among tick species based on the mitochondrial-genome **2**

Figure 1.2: Immunomodulary effects of *Ixodes* secreted protein families on vertebrate innate and adaptive immune responses **5**

Figure 1.3: Summary of study relevance **20**

Figure 2.1: Experimental set-up, infestation and immunisation protocol for study objective to evaluate co-infestation and its effect on immunisation **27**

Figure 2.2: Experimental set-up and immunization regime for study objective to evaluate adjuvants on immune response towards Bm86 and TC-X **34**

Figure 2.3: Spleen and lymph node culturing protocol **36**

Figure 3.1.1: Serum total IgG concentrations of mice euthanized on day 16 and 38. **38**

Figure 3.1.2: Anti-OVA IgG1 and IgG2a concentrations and calculated IgG2a/IgG1 ratio of mice euthanized on day 38 **39**

Figure 3.1.3: Anti-OVA IgE concentration of mice euthanized on day 38 **40**

Figure 3.1.4: *In vivo* lymph node supernatant Th1 associated cytokine concentrations on day 16 and 38 **41**

Figure 3.1.5: *In vivo* lymph node supernatant Th2 associated cytokine concentrations on day 16 and 38 **43**

Figure 3.1.6: Fold changes of the CD45+ CD19+IgM+ B-lymphocyte subpopulations in lymph nodes, spleen and blood of experimental groups relative to the negative control group **45**

Figure 3.1.7: T-lymphocyte subpopulation percentages relative to negative control groups in a heat map format. **48**

Figure 3.2.1: Antigen specific Bm86 and TC-X antibody response of BALB/c mice 30 days after primary immunization **50**

Figure 3.2.2: Culture supernatant Th1 associated cytokine concentrations after 24 hours **52**

Figure 3.2.3: Culture supernatant Th2 and Th17 associated cytokine concentrations after 24 hours. **55**

Figure 3.2.4: Lymphocyte subpopulations percentages in cultured spleen and lymph node tissue after 48 hours. **58**

Figure 4.1.1: Immune response development of mice terminated on day 16 **62**

Figure 4.1.2: Immune response development of treated mice terminated on day 38 **68**

Figure 4.2: Antigen(s) and adjuvant formulations effect on *in vivo* and *ex vivo* immune response. **73**

List of Tables

Table 1.1. Summary of *I. ricinus* infestation and *Babesia* infection induced immune responses in vertebrate hosts. **9-10**

Table 1.2. A summary of adjuvant classification and commonly used examples **16**

Table 2.1. Lymphocyte marker panels used for the quantification of T and B lymphocytes respectively. All antibodies were supplied by BD Pharmingen™ **32**

Table 5.1. Summary of immune reactions launched by co-infestation study **79**

List of Abbreviations

ANOVA	Analysis of variance
B	Blood
B.C	Before Christ
Bp	Base pair
Ca.	Circa
CD	Cluster of differentiation
ConA	Concanavalin A
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FC	Fold change
g	Centrifugal force
GLA-SE	Glucopyranosyl lipid A in sable emulsion
GPI	Glycosylphosphatidylinositol
HPR	Horseradish peroxidase
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
LN	Lymph node(s)
Min	Minutes
MYA	Million years ago
Nr	Number
OPD	o-Phenylenediamine dihydrochloride
OVA	Ovalbumin
PBS	Phosphate-buffered saline
PRR	Pathogen recognition receptors
RNAi	RNA interference
rpm	Resolutions per minute
RT	Room temperature
S	Spleen
SAT	Saliva-assisted transmission
SEM	Standard error of the mean
SGE	Salivary gland extract

SPF	Specific pathogen-free
Spp	Species
TBD	Tick-borne diseases
TBP	Tick-borne pathogens
TBEV	Tick-borne encephalitis virus
Th1	Type 1 T helper
Th2	Type 2 T helper
TLR	Toll-like receptors
TNF-α	Tumour Necrosis Factor alpha

Chapter 1: Literature review

1.1. Tick evolution and adaptation to hematophagy

Ticks (Order: *Ixodida*) have been well described as obligate hematophagous ectoparasites with some of the earliest references to ticks and tick-borne diseases dating as far back as ancient Egypt (ca. 1550 B.C.) and Greece (ca. 850 B.C.) (de la Fuente *et al.*, 2003). Following recent analysis of the 18S nuclear and 16S mitochondrial genes of the living fossil species, *Nuttalliella namaqua*, the origin of ticks is hypothesized to have occurred during the Late Permian period (260-270 MYA), with the majority of their evolution and dispersal occurring throughout the Tertiary period (de la Fuente et al., 2003; Mans *et al.*, 2011). Ancestral ticks have been proposed to have occurred as free-living scavengers or predators, feeding on lymphatic fluids of dead arthropods before finally adapting to a blood feeding lifestyle (Mans *et al.*, 2004; Walter *et al.*, 1998).

Currently, around 900 tick species have been classified (Barker *et al.*, 2004), that can be divided into three families: the *Ixodidae* (hard ticks); *Argasidae* (soft ticks) and the *Nuttalliellidae* that contains only one species namely *Nuttalliella Namaqua* (Hoogstraal, 1956) (Figure 1.1).

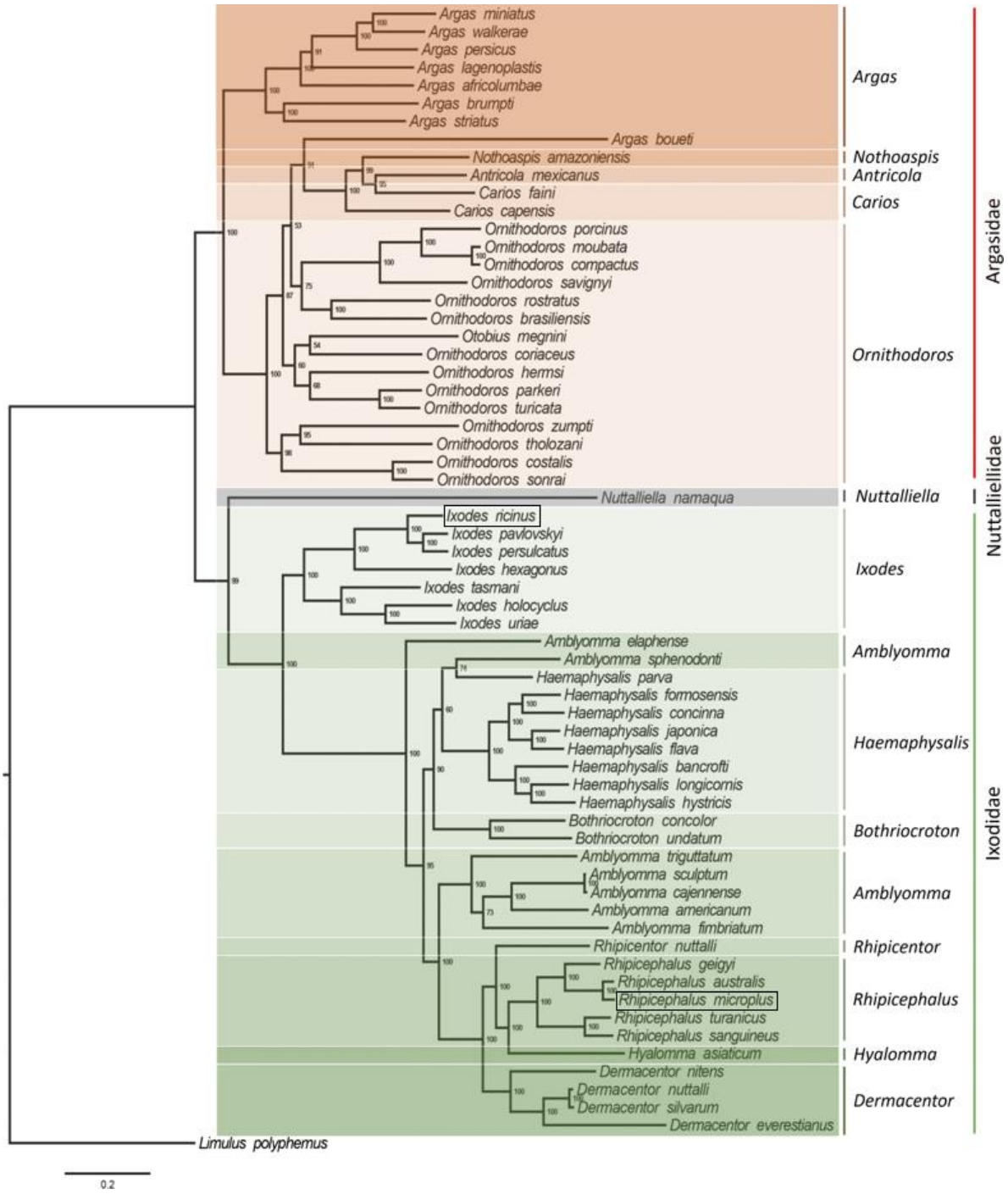

Figure 1.1: Phylogenetic tree showing the evolutionary relationships among tick species based on the mitochondrial-genome (Adapted from Wang *et al.* (2019)).

The tree was constructed using maximum likelihood analysis of the 13 protein coding genes and 2 rRNA nucleotide sequences (12,150 bp) of 63 tick species. The Atlantic horseshoe crab, *Limulus Polyphemus* (GeneBank nr: NC003057), is indicated as the outgroup of the phylogenetic tree. The scale-bar represents the number of expected nucleotide changes per site and the percentage of bootstrap support is given at each node. The grey, orange and green areas indicate the species that belong to the *Nuttalliellidae, Argasidae* and *Ixodidae* families, respectively. Black boxes indicate the two species that are relevant to the current study, *Ixodes ricinus* and *Rhipicephalus microplus*, respectively

The *Ixodidae* family comprises of 702 tick species distributed worldwide (Guglielmone *et al.*, 2010) with host specificities ranging from terrestrial (including wild and domesticated animals) to semi-aquatic vertebrates. Morphologically, *Ixodid* ticks possess a sclerotized scutum in all feeding stages, and usually feed for several days during which they ingesting a large amount of blood (Nava *et al.*, 2014).

Ticks from this family consists of 9 genera (Figure 1.1), with the *Ixodes* genus being the largest genera comprising of 247 species. The castor bean tick, *Ixodes ricinus*, the species relevant to this study will be discussed in section 1.2. The *Rhipicephalus* genus consists of 85 species. The majority of ticks from this genus have a three-host life-cycle while a select few have a two-host and one-host life cycle (Nava *et al.*, 2017). Ticks with a one-host parasitic life-cycle for part of the *Boophilus* sub-genera, and are important to the cattle industry of the tropical and sub-tropical areas of the world such as *Rhipicephalus microplus* (discussed in section 1.5.1).

Several ticks from the *Ixodes* genus have medical and veterinary importance and as such have been the focus of several studies. To date, *I. ricinus* laboratory reared ticks have been used to offer insight into the developmental cycle of these ticks (eggs, larvae, nymphs and adults), while *Ixodes* cell lines have been utilized to study tick-borne pathogens such as A*naplasma*, *Borrelia* and *Rickettsia* (Bell-Sakyi *et al.*, 2007). Other techniques such as RNA interference (RNAi) have been developed to determine gene function analysis and the screening of potential vaccine antigens (de la Fuente *et al.*, 2005). Lastly, a draft genome of *I. scapularis* cell lines has been assembled which will serve as an important reference for future tick research *(Miller et al., 2018).* Specifically, *I. ricinus,* is a candidate tick model, that has been used extensively in tick host research.

1.2. *Ixodes ricinus*: a model tick for immune responses in vertebrate hosts

Ixodes ricinus, is a European tick species of medical and veterinary importance. It is distributed in parts of Ireland, as well as from Northern Sweden throughout most of Europe as well as North Africa (Estrada-Peña *et al.*, 2013; Estrada-Peña, 2001). *Ixodes ricinus* has a complex 2 year life-cycle consisting of four life stages (egg, larva, nymph and adult), with each successive stage attaching to a new host to feed, then detaching to molt off-host (larvae and nymphs) or produce eggs (female adults) (Milne, 1943). A wide host preference has been documented for these ticks and adult females are known for parasitizing large mammals such as cattle, sheep

and deer, whereas larvae and nymphs prefer medium-sized mammals and birds as hosts (Hofmeester *et al.*, 2016; Medlock *et al.*, 2013).

Ixodes ricinus ticks have the ability to transmit more than twenty different pathogenic parasites, bacteria and viruses that infect vertebrate hosts (Sprong *et al*., 2018). The predominant pathogens that are transmitted are from the *Babesia* genus such as: *B. divergens*; *B. venatorum* (Becker *et al*., 2009; Bonnet *et al*., 2009; Bonnet *et al.*, 2007); *B. capreoli* (Nikol'skii *et al.,* 1972) and *B. microti* (Gray et al., 2002). Additionally spirochetes such *Borrelia burgdorferi sensu lato* and bacteria such as *Anaplasma phagocitophylum* (Estrada-Peña, 2004) are also transmitted. As co-infestation of these ticks with several pathogens has been well described, the likelihood of co-transmission of more than one tick-borne pathogen (TBP) to a vertebrate host poses a severe risk (Moutailler et al., 2016). It has been suggested that of the naturally occurring populations, at least half (~45%) of adult female *I. ricinus* are infected with one pathogen and of the already infected population, 22% of *I. ricinus* can be co-infected with up to five pathogenic species (Moutailler *et al.*, 2016). The geographical distribution of *I. ricinus* populations to peri-urban areas have also been on the rise in part due to climate change, urbanization and introduction of *I. ricinus* populations by rodents, domesticated animals and birds (Rizzoli *et al.*, 2014), all of which contribute to the increased incidence of transmitted TBPs (Lindgren *et al.*, 2000).

Despite= the latter, several studies have been dedicated to characterizing the immune response developed by vertebrate hosts when infested with *I. ricinus* (section 1.4). But there is limited information on the effect that tick infestation and simultaneous protozoan infection has on the development and maturation of the vertebrate host immune response.

1.2.1. Ticks: Ancient master manipulators of host immunity

Jawed vertebrate's immune system is estimated to have evolved some 450 MYA (Davidson *et al.*, 2003), consisting of a complex collection of cells, tissues and molecules tasked with preventing and eradicating established infections (Abbas *et al.*, 2014). When vertebrate skin is injured, a hemostatic defense mechanism is launched characterized by vasoconstriction, platelet plug formation, blood coagulation and fibrinolysis that collectively function to prevent blood loss (Hoffman *et al.*, 2013).

Ticks are able to obtain blood from vertebrate host(s) by suppressing and/or evading the host immune response by tick saliva injected into the feeding cavity (Kotál *et al.*, 2015). The anti-hemostatic and immune-modulation mechanism of ticks' saliva is mediated, in part, by an

array of salivary proteins that have evolved mainly through gene duplication events in an 'arms race' between ticks and their host (Chmelař *et al.*, 2016); examples of which are illustrated in Figure 1.2.

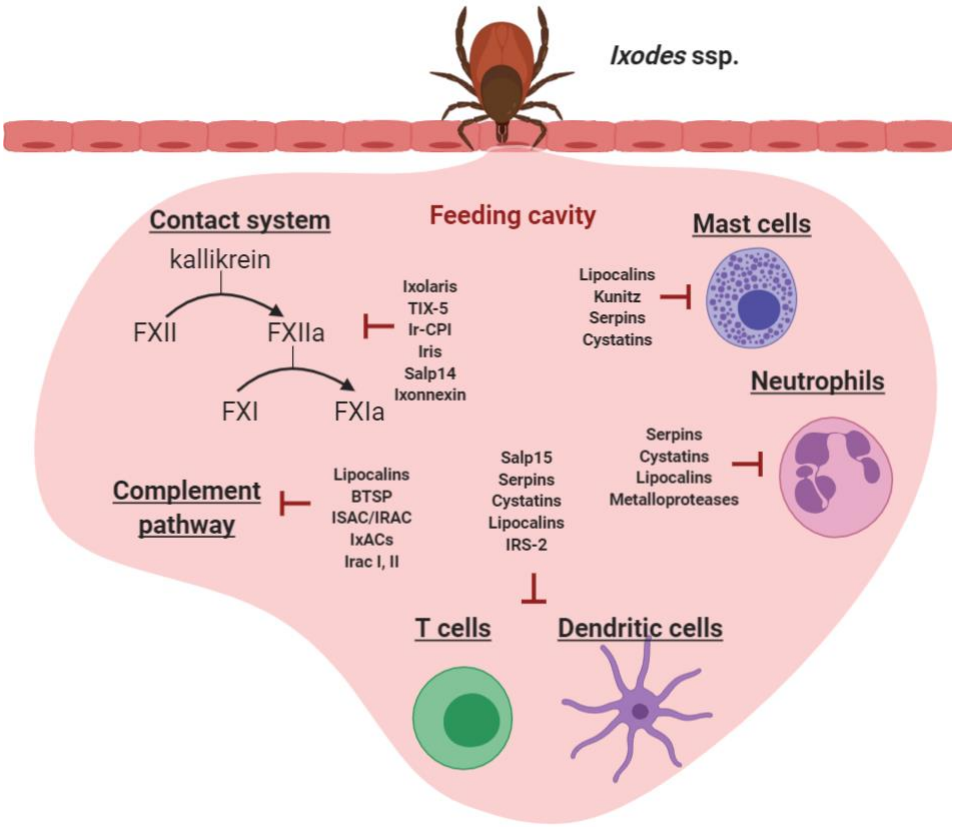

Figure 1.2: Immunomodulary effects of *Ixodes* secreted protein families on vertebrate innate and adaptive immune responses.
Abbreviations: Basic Tail-Secreted Proteins (BTSP), coagulation contact phase inhibitor from *I. ricinus (Ir-CPI), I. ricinus* serpin 2 (IRS-2), *I. ricinus* anticomplement *(Irac), I. scapularis* anticomplement *(ISAC), I. scapularis* salivary protein 14/15 (Salp14/15), *Ixodes* anticomplement proteins(IxACs), tick inhibitor of factor Xa toward factor V *(TIX-5).* Adapted from Štibrániová *et al.* (2019)and Chmelař et al. (2016).

1.2.2. The effects of tick saliva on host immunity

Tick saliva injected into the feeding site of the host contains a variety of pharmacological active molecules possessing anti-hemostatic, anti-inflammatory and immunosuppressing properties that can impair both the innate and adaptive immune responses of the host (Francischetti *et al.*, 2009; Ribeiro *et al.*, 1985; Wikel, 1999). This environment that is created in the tick feeding pool also contributes to the successful transmission of an array of tick-borne pathogens (Bowman *et al.*, 2008; Brake *et al.*, 2012). Additionally, tick saliva can also contain protein toxins (belonging to families also found in other venomous animals) that can induce paralysis and other toxicosis (Cabezas-Cruz *et al.*, 2014; Pienaar *et al.*, 2018).

Upon mechanical injury of the host skin by the tick mouthparts, the host innate defense mechanism, mainly hemostasis, which includes coagulation, vasoconstriction, and platelet aggregation (Figure 1.2) is activated. The initial innate immune response of the host is also affected, such as inhibition of complement activation and host inflammatory responses (Francischetti et al., 2009). The inhibition of the host alternative complement pathway is crucial for tick feeding, it has been found that this cascade can be inhibited by the saliva and/or salivary gland extracts (SGE) of several *Ixodes* species (Lawrie *et al.*, 2005; Ribeiro, 1987). Additional immune responses affected by tick saliva and/or tick SGE include: phagocytosis of pathogens, production of inflammatory cytokines by macrophages (Kýčková *et al.*, 2006; Ramachandra *et al.*, 1992), natural killer cell activity (Kubeš *et al.*, 2002) as well as T cell (Ferreira *et al.*, 1998; Urioste *et al.*, 1994) and B cell (Hannier *et al.*, 2003) proliferation.

Several studies have been completed that have evaluated murine immune responses to *Ixodid* saliva and/or SGE (Ferreira *et al.*, 1999; Kovář *et al.*, 2001, 2002; Leboulle *et al.*, 2002; Mejri *et al.*, 2007; Mejri *et al.*, 2001). It is proposed that adaptive immunity in resistant murine hosts to *I. ricinus* infestation is mediated by a polarized Th1 immune response involving IFN-γ production (Sauer *et al.*, 1995) and delayed hypersensitivity that results in IgG2a antibody production (Allen, 1973; Ganapamo *et al.*, 1995). In contrast, susceptible host adaptive immunity is characterized by a Th2 immune response involving IL-4 and IL-5 cytokines favoring the production of IgG1 and IgE antibodies (DeKruyff *et al.*, 1989; Molnár, 2007). A single study did describe a polarized Th2 response, increased IgG1 and IL-10 production that negatively effects the regulation of IFN-γ (Menten-Dedoyart *et al.*, 2008).

1.3. Tick-borne pathogens and host immunity: a focus on *Babesia*

Ticks are efficient vectors of disease with the ability to transmit a wide variety of TBPs that have significant medical and veterinary importance (de La Fuente et al., 2017a) including bacteria (e.g. *Borrelia, Anaplasma, Rickettsia* and *Francisella*) (de la Fuente *et al.*, 2008), viruses (e.g. *Asfarviridae*, *Reoviridae*, *Rhabdoviridae* and *Orthomyxoviridae*) (Labuda *et al.*, 2004) and protozoa (*Babesia* spp.) (Homer *et al.*, 2000). For the scope of this thesis the focus will be placed on *B. microti* infection and its effects on the vertebrate immune response.

Organisms belonging to the genus *Babesia* are intraerythrocytic apicomplexan parasites transmitted by ticks of the *Ixodid* family (Chauvin *et al.*, 2009) with more than 100 *Babesia* species identified to date (Levine, 1971; Telford III *et al.*, 1993). These parasites are closely related to the *Theileria* genus, and are one of the most widespread blood parasites, second to

trypanosomes, that have a major global economic, medical and veterinary impact (Homer et al., 2000). *Babesia* parasites are able to infect a wide variety of vertebrates, however, they require both a vertebrate host and non-vertebrate vector to maintain the transmission cycle (Duh *et al.*, 2001). The tick vector must feed on the vertebrate reservoir that is competent in maintaining the *Babesia* organism in an infectious state. In the vertebrate host, the parasite is able to directly invade the red blood cells for asexual production of daughter parasites (i.e. merozoites) through binary fusion. The merozoites are then released into circulation upon rupture of the host red blood cells (referred to as “free parasites”) to repeat additional cycles of red blood cell infection and replication (Homer et al., 2000; Rossouw *et al.*, 2015; Telford III et al., 1993). Vertebrate hosts infected with *Babesia* can experience malaria-like signs that are associated with hemolysis and occasionally in death if left untreated (Vannier *et al.*, 2009). The severity of *Babesia* infection is determined by several factors including age, immunocompetence, species involved and co-infestation with other pathogenic agents (Homer et al., 2000). Several *Babesia* species are able to infect humans, of which *B. microti*, a rodent-borne piroplasm, is the most prevalent and transmitted by ticks from the *Ixodes* genus (Telford III et al., 1993).

1.3.1. The co-evolution of pathogens and how they utilize tick vectors to infect the vertebrate host

Tick-borne pathogens co-evolved with tick vectors and their vertebrate hosts enabling manipulation of vector immune responses, facilitating transmission to the vertebrate host with subsequent multiplication (de La Fuente *et al.*, 2017a). In order for a TBPs to successfully infect a tick host and be transmitted via the tick saliva, the pathogen needs to survive the protease-rich environment within the tick midgut, cross the midgut lumen, overcome the tick innate immune response in the hemolymph, migrate to the salivary glands (Hajdusek *et al.*, 2013) and vector molting (de La Fuente *et al.,* 2017a). The latter is referred to as saliva-assisted transmission (SAT) and relates to the pathogen exploiting bioactive molecules in tick saliva to enable transmission, such as tick-derived anti-hemostatic, anti-inflammatory and immunomodulatory properties (Kazimírová *et al.*, 2013). Only a few tick salivary molecules associated with specific pathogen transmission have been identified to date, including limited studies on the SAT of *B. microti* by *I. ricinus (*both species focused on in this study).

To date, *I. ricinus* ticks feeding on animals experimentally inoculated with pathogen containing SGE, have been reported for tick-borne encephalitis virus (TBEV), resulting in enhanced transmission and infectivity (Labuda *et al.*, 1993).In *Borrelia burgdorferi*, SGE of *I. ricinus* resulted in spirochete proliferation (Macháčková *et al.*, 2006). Similarly, SGE of *I. ricinus*

resulted in proliferation of *Francisella tularensi* the causative agent of tularemia also known as rabbit fever (Kročová *et al.*, 2003).

1.4. *Ixodes ricinus* and *Babesia microti*: a model system for the study of vector-pathogen-host interactions

Th1 immune responses are associated with infections caused by protozoa, whereas Th2 immune responses are regarded as important in the immunity to extracellular parasites (Cox, 2001). To date, studies have identified mice than can develop immunity (partial resistance) against *I. ricinus* infection and *Babesia* infection, independently (Table 1.1. and Figure 1.3).

Bagg albino crossed (BALB/c) mice have been shown to be incapable of acquiring resistance to *I. ricinus* nymph infestation. Immune profiling using *in vivo* collected lymphocytes from pre-sensitized mice stimulated *in vitro* with *I. ricinus* SGE or Concanavalin A (Con A) resulted in lymphocyte proliferation, cytokine- and antibody production. Exposure to *I. ricinus* nymphs resulting in a Th2 polarized immune response (Table 1.1).

Table 1.1: Summary of *I. ricinus* infestation and *Babesia* infection induced immune responses in vertebrate hosts.

	Effect of *I. ricinus* nymph infestation on BALB/c immune response			***Babesia* ssp infection effect on vertebrate immune response**				
	Immune response (*in vivo/ vitro*)	***Agent used in ex vivo* stimulation**	**Reference.**	***Babesia* ssp.**	**Host**	**Immune response (*in vivo*)**	**Disease progression**	**Reference**
Lymphocyte maturation	Lymphocyte increase (LN)	SGE	Ganapamo et al. (1995); (Ganapamo *et al.*, 1997; Mejri *et al.*, 2002)	*B. microti*	BALB/c mice	Increased in $CD4^+$ T helper cells	Resolution [1]	Ruebush *et al.* (1986)
	T Lymphocyte decrease (LN)	SGE	Ganapamo *et al.* (1996b); (Ganapamo et al., 1997) (Mejri et al., 2002)					
	Th1 subpopulation decrease		Robbertse *et al.* (2018a)					
	Increase CD4+ T cells (S)	ConA	(Ganapamo et al., 1997)					
	CD4+ T cell decrease (LN)		Ganapamo et al. (1996b)					
Antibody production	Increase in IgE (B)		Christe *et al.* (1999)	*Babesia* ssp.	Bovine	Increase in IgG (non-specific)	Establishment [2]	Homer et al. (2000)
				B. bovis		Increase in IgG1 and IgG2	Resolution	Brown *et al.* (1999)

Cytokine production	Increase IL-10 (LN)	ConA	Ganapamo et al. (1995)	*B. microti*	BALB/c mice	Increased IFN-γ and TNF-α	Progression [3]	Igarashi *et al.* (1999)
	Increased IL-4 (LN)		Mejri et al. (2002)					
	Decrease IFN-γ	ConA/SGE	Ganapamo et al. (1995)	*B. bovis*	Bovine	Increased IL-12 and IL-10		Garcia *et al.* (2004); (Goff *et al.*, 2006)
	Increased IL-2, TNF-α and GM-CSF	ConA	(Christe et al., 1999; Mejri et al., 2002)					
		Con A or anti-CD3 antibodies	Ganapamo et al. (1996b)					

Abbreviations: Blood (B), Concovalin A (Con A), Lymph node (LN), Salivary gland extract (SGE)

***Babesia* infection state characteristics:**

1) Resolution: Falling parasitaemia due to *Babesia* degeneration inside the erythrocyte and clearance by the spleen, which is hyperreactive.

2) Establishment: *Babesia* organisms establish their intraerythrocytic infection

3) Progression: *Babesia* organisms succeed in invading the erythrocyte, and the resulting merozoites start proliferating and lyse the infected cell

Mammalian hosts are able to develop partial resistance to *Babesia* species through infection and recovery (Vannier *et al.,* 2009). Host *Babesia* infection progression can be divided into three broad stages namely the establishment, progression and resolution of the disease (Homer *et al.,* 2000). BALB/c mice that survive *B. microti*, have been shown to resolve infection after approximately 21 days (Skariah *et al.*, 2017). Development of host immunity to *Babesia* parasites results in a predominantly cellular immune responses characterized by induced CD4+ T lymphocyte proliferation and Th1 associated cytokines (IFN-γ and TNF-α) (Igarashi *et al.*, 1999; Zintl *et al.*, 2005) (Table 1.1. and Figure 1.3). *Babesia* infection of the vertebrate host results in an increased IgM and IgG antibody production which have a limited effect on *B. microti* disease resistance and resolution as antibody binding is limited to a short window in the time between the *Babesia* parasite gaining access to the blood stream and the time it invades the targeted cells (Abdalla *et al.*, 1978; Homer *et al.,* 2000).

Currently there is a lack of information regarding the effect that tick infestation and *Babesia* parasites co-infection may have on a vertebrate host immune response, as well as the implications of co-infestation on vaccine mediated immunity. As such, this forms the basis of the first biological question of this study, namely to evaluate the immunological implication of simultaneous tick-induced Th2 and *Babesia*-induced Th1 response has on a murine host (Figure 1.3).

1.5. Tick vaccines: Lack of antigens and data for adjuvant selection

Due to the growing medical and veterinary impact that TBPs have, more effective measures are needed to control ticks and the diseases they transmit. Various strategies for tick control have been tested including the use of acaricides on domesticated animals (Coles *et al.*, 2014), biological control through the use of entomopathogenic fungi (Fernandes *et al.*, 2012) and vaccination against ticks (Willadsen *et al.*, 1995) and TBPs (Dantas-Torres *et al.*, 2012). Acaricides are the most frequently used control method but have the drawbacks of chemical pollution of the food chain and environment (Playford *et al.*, 2005) and already established concern about the increasing frequency of acaricide resistance globally (Foil *et al.*, 2004; Rodriguez-Vivas *et al.*, 2011).

In contrast, tick vaccines are seen as an environmentally friendly, practical and economical solution for reducing tick loads (especially in acaricide resistant areas). A successful tick vaccine will not only reduce the tick load per host animal but will also indirectly reduce the transmission of TBPs (de la Fuente *et al.*, 2017b). To date, only one protective antigen, Bm86, has been commercialized (GAVAC™) and is used today in South America against the cattle

tick *Rhipicephalus microplus* (section 1.5.1). However, the efficacy of Bm86-based vaccines varies greatly (from 0-100%) depending on the geographical location and tick species (De Vos *et al.*, 2001b; García-García *et al.*, 1999). In addition to species diversity in the Bm86 sequence, other factors that can contribute to the lack of global protection with the B86 vaccine include genetic and immunological variance between bovine breeds as well as the nutritional and disease status of the animal. In an effort to improve upon the Bm86 vaccine, the research group of Prof. C Maritz-Olivier have identified two additional protective antigens that were validated in Holstein-Friesian cattle (section 1.5.1) (Maritz-Olivier, 2016). The vaccine reduced the tick load by 90% and none of the eggs produced by the engorged females were able to develop into larvae. All antigens tested to date were formulated in Montanide™, which resulted in severe inflammation at the injection site. Taking this vaccine further into costly field trials in Africa (where animals are plagued with co-infections such as *Babesia* spp.,) requires a better understanding of the immune responses during co-infection/infestation. Moreover, the resulted host response elicited by a specific adjuvant would provide invaluable information to guide future vaccine formulations. As such, the second biological question of this study is focused on evaluating a novel adjuvant and Alum in BALB/c mice.

1.5.1. Rhipicephalus microplus: a case study in increasing vaccine efficacy using a model organism

Rhipicephalus microplus, known as the Southern cattle tick, is considered the most economically important tick of cattle globally. (Jongejan *et al.*, 2004). *Rhipicephalus micoplus* is proposed to have originated in South-East Asia from where it has spread into the tropical and sub-tropical regions of South and Central America, Australia, Asia and Africa (Pipano *et al.*, 2003; Tanaka *et al.*, 1999). The short life cycle and fecundity of *R. microplus* enables the tick to outcompete other species, adapt more rapidly to different climatic zones (Adakal *et al.*, 2013; Nyangiwe *et al.*, 2013), and to rapidly develop acaricide resistance (Dela Fuente *et al.*, 2000; Rajput *et al.*, 2006; Rodriguez-Vivas et al., 2011). *Rhipicephalus micoplus* is also a vector for *Anaplasma, Borrelia* and *Babesia* spp. (Dela Fuente *et al.,* 2000; Jongejan *et al.*, 2004; Madder *et al.*, 2011), and it has been estimated that the animal losses and control of these tick-borne diseases costs South Africa approximately $21 million per annum (McLeod *et al.*, 1999). *Rhipicephalus microplus* infestation potentially also results in hide damage, anemia, weight loss and secondary infections (de la Fuente *et al.*, 2015).

Bm86: a promising vaccine antigen

The Bm86 antigen is a membrane bound glycosylphosphatidylinositol (GPI)-linked glycoprotein located on the gut lumen of the tick digestive tract (Gough *et al.*, 1993), suggested

to be involved in cell-cell or pathogen-gut cell interactions (Liao *et al.*, 2007). The Bm86 antigen is the basis for the only commercialized recombinant vaccine against *R. microplus*, currently sold as Gavac® (Herber-Biotec S.A., CIGB, Camagüey, Cuba) in Latin America. (De Vos et al., 2001b; Gough et al., 1993; Mulenga *et al.*, 2000; Pipano et al., 2003; Willadsen, 2001) and the discontinued vaccine TickGARD® and TickGARD® plus (Intervet Australia Pty. Ltd., Australia) in Australia (De la Fuente *et al.*, 2007). A Cuban vaccination study using the Gavac® vaccine have resulted in a ~60% reduction in acaricide use and lowered incidence of disease (transmission of *Babesia* and *Anaplasma* spp.). This has resulted in significant savings on production costs of around 6 million US$ (De la Fuente *et al.*, 2007). Similarly, a nation-wide vaccination study in Venezuela using1.9 million cattle resulted in a 83.7% reduction in chemical usage and 81.5% in other associated costs (Suarez *et al.*, 2016). Successive vaccination with the Bm86 vaccine was shown to result in an increase in antibody production and consequent protection against *R. microplus* (De La Fuente *et al.*, 1999; Redondo *et al.*, 1999), suggesting the production of memory B cell activation following vaccination. Furthermore, vaccination resulted in a reduction of engorged female ticks and their reproductive capacity, that ultimately resulted in a reduction of viable offspring in subsequent generations (Rodríguez *et al.*, 1994). The Bm86-based vaccine also confers limited protection against various other hard tick species, such as *R. appendiculatus* (Canales *et al.*, 2009), *Hyalomma anatolicum anatolicum, H. dromedarii, R. decoloratus* (De Vos *et al.*, 2001a) and *R. annulatus* (De La Fuente *et al*., 1999). Unfortunately, South African cattle vaccination trials using the Gavac® vaccine against *R. microplus* did not show the same promising results (Maritz-Olivier, 2016).

Multi-antigen immunizations: Bm86 formulated in combination with TC-X

Studies using Bm86 in combination with other tick antigens have shown promise against *R. microplus* infestation (Richards *et al.*, 2015; Schetters *et al.*, 2016). As such, in this study we tested a combinational formulation containing both Bm86 and TC-X (Patent, University of Pretoria pending) in a BALB/c model.

The TC-X antigen is a Kunitz domain-containing protein which in ticks are mostly serine protease inhibitors, such as inhibitors of blood coagulation (Blisnick *et al.*, 2017; Lwaleed *et al.*, 2006; Maritz-Olivier *et al.*, 2007). *In vivo* RNAi studies conducted by our group targeting TC-X showed a 77% mortality of attached ticks and a 10% reduction in replete female weights compared to controls. Further investigation of transovarial gene silencing indicated a 45% decrease in oviposition efficiency of *R. microplus* females and a 47% decrease in egg weights (Louw, 2013).

The results suggest that TC-X plays a significant role in ovary biology and egg development making it a novel vaccine target, since a decrease in egg weights and oviposition efficiency would result in less progeny in the next generation, thereby reducing the infestation load on cattle hosts. Pilot cattle vaccination trails using TC-X as a single antigen conferred no significant reduction in infestation and only resulted in a prolonged feeding time. However, when using TC-X in combination with Bm86 it resulted in a synergistic effect in reducing tick numbers, and an overall vaccine efficacy of 89% was achieved, relative to the 78% obtained for Bm86 alone without any viable offspring (Maritz-Olivier and Stutzer, unpublished data). However, the optimal vaccine formulation has not been addressed to date.

1.6. Adjuvants and their role in veterinary vaccines

Several methods have been proposed to enhance current commercial and next-generation vaccines efficacies (Stutzer *et al.*, 2018). This includes the use of exposed (antigens exposed to the host during feeding such as saliva proteins) vs. concealed antigens (not exposed to the host such as Bm86) (Nuttall *et al.*, 2006), multi-antigen containing formulations (Schetters *et al*., 2016) and the use of different adjuvant formulations (Valle *et al.*, 2001). Furthermore, antigens with poor immunogenicity can be altered by stabilizing the tertiary structure, changing the method of antigen production, formulation and delivery (Fox *et al.*, 2013). For the scope of the current study, antigens were formulated with two different adjuvants in an effort to increase antigen specific antibody titers and promote lymphocyte maturation. Additionally, cytokine profiles were quantified to determine immune polarization of individual formulations.

Adjuvants are key components of modern vaccines, capable of enhancing antigen specific immune responses. This is especially true for modern vaccines comprising of recombinantly produced proteins that lack the native heterogeneous components which may include particular forms of proteins, lipids and oligonucleotides that act as natural adjuvants of live attenuated or inactivated pathogen preparations (Cimica *et al.*, 2017; Reed *et al.*, 2013). Adjuvant selection plays a crucial part in vaccine formulation as an optimal formulation would result in a higher level of immunogenicity, boosting the potential and longevity of a specific immune response to an antigen while having low toxicity (Wack *et al.*, 2005). Additional advantages to optimal antigen/adjuvant selection included: dose sparing (Cox, 2011), enabling a more rapid immune response (Levie *et al.*, 2002; Tong *et al.*, 2005), broadening of antibody response via expansion of B cell diversity (Wiley *et al.*, 2011), increase magnitude and functionality of the antibodies produced, improved antigen stability, product safety,

biodegradability, and lower costs by improving effectiveness (Chauhan *et al.*, 2017; Jones *et al.*, 2013; Mohan *et al.*, 2013). The choice of adjuvant can also play a significant role in the level of involvement of Th1 and/or Th2 responses elicited during vaccination (Cribbs *et al.*, 2003).

To date, some 30 adjuvant molecules are in use that can be classified into three broad groups: delivery systems, immunomodulary molecules and a combination of the two former classes (combination system). Most adjuvants in advanced development and use are classified as combination systems (Jones *et al*., 2013; Pashine *et al.*, 2005).

Delivery systems are composed of non-immunostimulatory components usually phospholipid bilayers that associate or encapsulate antigens and/ immunomodulary molecules (Reed *et al*., 2013). These delivery systems function in presenting the vaccine antigen(s) to the immune system in an optimal manner, inducing controlled release and depot delivery systems (Reed *et al.*, 2013). Adjuvants functioning as immunomodulary molecules act directly on the host immune system to augment a response to vaccine antigen(s). These molecules are typically ligands for innate immune pathogen recognition receptors (PRRs), as well as bacterial endotoxins that stimulate an innate immune response by directly acting on the immune system thereby increasing responses to antigens (Reed *et al.*, 2009). The various adjuvant classes, as well as examples of each are summarised in Table 1.2.

Table 1.2: A summary of adjuvant classification and commonly used examples.

Type	Adjuvant/formulation	Reference
	Delivery systems	
Water-in-oil emulsion	Montanide™	Aucouturier *et al.* (2002)
	Freund's incomplete	Allison *et al.* (1974)
Oil-in-water emulsions	MF59	(Brito *et al.*, 2014); O'Hagan *et al.* (2012)
	AS03	Morel *et al.* (2011)
	Immunostimulatory molecules	
TLR ligands	Unmethylated CpG oligonucleotides	Klinman *et al.* (1999)
	MPLA, LPS (e.g. GLA-SE)	Mata-Haro *et al.* (2007) (Coler *et al.*, 2011); Skidmore *et al.* (1975)
Cytokines	IL-2, -12, -15, -18, -21 and GM-CSF	Tovey *et al.* (2010)
Saponins	QS-21	Zhu *et al.* (2016)
	Quil-A	Rajput *et al.* (2007)
	Combinational adjuvants	
Mineral salts	Aluminum salts (alum)	Bomford (1989)

Abbreviations: TLR (Toll-like receptors), GM-CSF (Granulocyte Macrophage Colony Stimulating Factor), QS-21 (*Quillaja Saponaria-21), MPLA (*Monophosphoryl lipid A), LPS (Lipopolysaccharides), GLA-SE (Glucopyranosyl lipid A in stable emulsion)

Montanide: The adjuvant of choice in the commercial Bm86 tick vaccine

Both commercialized Bm86-based *R. microplus* vaccines, GAVAC and TickGARD, are formulated with Montanide™ as an adjuvant. This adjuvant is composed of a mineral oil and a surfactant from the mannide monooleate family. Montanide™ works as a depot at the site of injection, enabling slow antigen release and stimulation of antibody producing plasma cells (Aucouturier *et al.*, 2001)**.** However the type of immunity induced (i.e. Th1 or Th2) remains unknown and may also be dependent on the antigen (Heegaard *et al.*, 2011). The use of Montanide™ as a cattle vaccine adjuvant is associated with several drawbacks including: mild to severe local inflammatory reactions, the formation of granulomas and ulcers at the injection site (Aguilar *et al.*, 2007; Reed et al., 2009; Wu *et al.*, 2008), as well as difficulty to achieve proper emulsion with the antigen in the vaccine formulation (Reed *et al*., 2009). In our research group, vaccination with Montanide did indeed result in severe inflammation and granuloma formation. These side effects are unwanted by farmers as it reduces the price of cattle meat and hides and in severe cases cause carcass trim losses. As such, an alternative adjuvant formulation for vaccines against *R. microplus* is required. For the purpose of this study two adjuvants, the classical aluminum salt (Alum) and, Glucopyranosyl lipid A in stable emulsion (GLA-SE) a new generation adjuvant, were evaluated. The ability of theses adjuvants to raise antibody titers, cytokine production and temporal changes in lymphocyte

subpopulations in response to vaccination with the Bm86 and TC-X combination vaccine was interrogated

Aluminium salts: the classical adjuvant

Aluminum salts (Alum) have been used for nearly 90 years in veterinary and human vaccines (Marrack *et al.*, 2009; White *et al.*, 1955). Alum consist of crystalline nanoparticles that aggregate to form a heterogeneous dispersion of particles of several microns (Kool *et al.*, 2012). The mechanism of action of Alum is not completely understood, with studies indicating that it is a combinational adjuvant, functioning as both a delivery system and immunostimulatory compound. It has been reported that formulations containing alum affects antigen uptake, induces uric acid formation which enhances danger signals, recruits various types of immune cells and elicits a Th2-mediated immune response (Kool *et al.*, 2008; Reed *et al*., 2013). More specifically, Alum used in murine immunization trials was shown to result in boosted humoral immunity by causing a polarized Th2 immune response (Brewer *et al.*, 1999), stimulating production of monocyte and granulocyte chemo-attractants and enhancing monocyte differentiation into dendritic cells (Kool *et al.*, 2008; Seubert *et al.*, 2008), causing the accumulation of eosinophils at the site of injection, as well as enhancing production of antigen-specific IgE and IgG1 (Eisenbarth *et al.*, 2008; Kuroda *et al.*, 2011; Reed *et al.*, 2009). Although Alum is the most widely utilized adjuvant, it has yet to be tested in a cattle tick vaccine formulation, especially with the Bm86 and/or combination of Bm86 and TC-X antigens.

Glucopyranosyl lipid A in stable emulsion

Glucopyranosyl lipid A in stable emulsion (GLA-SE) has shown promise as an adjuvant for veterinary and human vaccines (Falloon *et al.*, 2016; Heeke *et al.*, 2016; Pantel *et al.*, 2012; Santini-Oliveira *et al.*, 2016). In cattle vaccination studies, GLA-SE has been tested as an adjuvant against Bovine tuberculosis (TB) caused by the bacterial pathogen *Mycobacterium bovis.* Vaccination of cattle with sub-unit vaccines formulated with *Mycobacterium* proteins and GLA-SE adjuvants induced a strong humoral response (Jones et al., 2013). Regarding the use of the GLA-SE adjuvant in ectoparasites vaccines, GLA-SE is currently being utilized in an anti-helminth vaccine for humans clinical trials in combination with the *Schistosoma mansoni* antigen- Sm-p80 (Molehin *et al.*, 2016).

Glucopyranosyl lipid A (GLA) is a non-toxic immunostimulatory monophosphoryl lipid A (MPLA) derivative of lipopolysaccharides (LPS), which naturally are, inflammatory toxic components purified from the bacterial cell wall of *Salmonella minnesota* (Baldridge *et al.*, 1999). Monophosphoryl lipid A is a TLR 4 agonist, improving vaccine immunogenicity by

enhancing antigen presenting cell maturation (Baldridge *et al.*, 1999; Evans *et al.*, 2003; Okemoto *et al.*, 2006) with only a ~0.1 % inflammatory toxicity due to its parent LPS molecule (Evans et al., 2003; Qureshi *et al.*, 1982). Glucopyranosyl lipid A (GLA) formulation with a stable emulsion (SE), induces a balanced IgG1/IgG2 response in vaccinated hosts (Cauwelaert *et al.*, 2016; Dowling *et al.*, 2016), while a Th1-mediated immune response is observed in *ex vivo* antigen stimulated cultures (Coler *et al.*, 2015). The GLA-SE adjuvant is yet to be formulated in a tick vaccine.

1.7. Relevance of co-infestation in South African cattle herds

South Africa has approximately 14.1 million cattle (Newsletter, 2006), contributing significantly to livelihoods in the informal agricultural sector as a form of nutrition and income (Brown *et al.*, 1999). During the warmer wet seasons, cattle can be infested with several tick species at any given point, increasing the chance of simultaneous co-infection/infestation with several vector associated pathogens. South African cattle owned by resource-poor farmers are kept on communal rangelands characterized by poor management and sanitation, resulting in low productivity. Furthermore, these farmers spend limited capital on antibiotic and anti-protozoal agents, resulting in an increased incidence of diseases and parasitism within cattle herds (Kaewthamasorn *et al.*, 2006; Rajput *et al.,* 2006). In this regard, anaplasmosis and babesiosis are the most prominent constraints to the health and improved productivity of cattle in South Africa (Mtshali *et al.*, 2004; Thomson *et al.*, 1994).

Anaplasma marginale (*Rickettsiales: Anaplasmataceae*) is the causative agent of bovine anaplasmosis worldwide (Kocan *et al.*, 2004). The clinical symptoms of bovine anaplasmosis may include fever, weight loss, abortion, lethargy, icterus, and often death in animals older than two years (De Waal, 2000). Cattle persistently infected are able to develop resistance against the disease. Eastern Cape surveys have reported that 25.6% of communal cattle tested positive for *Anaplasma marginale* infection (Marufu, 2008)

Bovine babesiosis is responsible for large economic losses in the livestock industry worldwide (Gohil *et al.*, 2013), with infection usually resulting in mortality, abortions and a decrease in milk and meat production (Bock *et al.*, 2004). Common *Babesia* species affecting cattle include *B. bovis*, *B. bigemina* and *B. divergens* with the distribution of these pathogens depending on the spread of the Ixodid ticks responsible for their transmission (Gohil *et al*., 2013). *Babesia bovis* and *B. bigemina* are commonly transmitted by *R. microplus* and *R. annulatus* (*R. decoloratus* for *B. bigemina alone*) in tropical and sub-tropical regions of the world, including Africa, Asia, Australia and America. *Babesia divergens* is transmitted by *I. ricinus* which is

found in northern Europe (Bock *et al*., 2004; Chauvin *et al*., 2009; Zintl *et al.*, 2003). Currently bovine babesiosis is treated by the administration of chemotherapeutic drugs, resulting in short term protection from babesiosis (4-8 weeks depending on the *Babesia* species) (De Waal *et al.*, 2006; Mosqueda *et al.*, 2012). The administration of these compounds, however, have several drawbacks including drug residues in meat and milk products, as well as necessary repeated administration being impractical and costly *(Gohil et al.*, 2013). Additionally, the prolonged and misuse of these drugs can potentially result in the development of resistant strains (Rodriguez *et al.*, 1996). A study by Marufu (2008) reported that 44.6% and 45.9% of Eastern Cape communal cattle, tested positive for *B. bovis* and *B. bigemina*, respectively.

Due to the impact of *R. microplus* on cattle health and production (as an ectoparasite and vector of disease) the development of a vaccine is critical. As discussed above, South African cattle herds have a high prevalence of parasitic infection, including established *Anaplasma* and *Babesia* infections. The effect that such a multiplicity of infection/infestation may have on tick vaccine efficacy remains to be determined. However, a cost-effective model system within a controlled laboratory environment that can be used to gain insight into the interplay of cell-mediated and antibody-mediated immunity (Th1 vs. Th2) during vaccination and with different adjuvants and/or co-infection is lacking. Due to the cost and technical difficulty of working with large animals in vaccination trials (e.g. cattle), a murine model was evaluated in this study as an alternative small-animal model to study immune responses to vaccines formulated with different antigen(s) and adjuvants. In this study we made use of BALB/c mice which have a prototypical Th2 polarized immune system and are able to clear *Babesia* infection, compared to the commonly used C57BL/6 mouse model that has a more Th1 polarized immune system (Watanabe *et al.*, 2004). Thus, the BALB/c immune response may be more similar to that of a tick susceptible bovine host (Brake et al., 2012; Robbertse, 2018).

1.8. Biological questions forming the basis of this study:

The first aim of the proposed study is to analyze the BALB/c murine immune response when the host is infected with either *B. microti* and/or *I. ricinus* ticks in the presence and absence of ovalbumin (OVA) as antigen formulated in Freund's adjuvant. The OVA protein is a large glycol-protein and a constituent of the whites of chicken eggs that has been found to be mildly immunogenic (Caubet *et al.*, 2011). Freund's adjuvant is as a water-in-oil emulsion, prepared from non-metabolizable oils (paraffin oil and mannide monooleate) (Brancq *et al.*, 1995). Freund's adjuvant is designed to provide continuous release of antigens necessary for stimulating a strong, persistent immune response. Complete Freunds' adjuvant contains killed

Mycobacterium tuberculosis and attracts macrophages and other cells to the injection site which enhances the immune response (Mohan *et al*., 2013). Thus, the Complete Freund's adjuvant is used for the initial injections, while incomplete Freunds' adjuvant (lacking killed *M. tuberculosis*) is used in booster immunizations. BALB/c mice were chosen as model organisms for this study due to the low cost of purchasing, housing and feeding. BALB/c mice are also inbred, reducing background variability between biological repeats and because BALB/c mice are commonly used model organisms, markers associated with the immune response have previously been identified and studied.

The second aim entails the evaluation of two adjuvants on the Th1/Th2 response elicited against a combination tick vaccine (Bm86 and TC-X). In summary, with this study we aimed to obtain insight into the host-pathogen-parasite immune response (Figure 1.3).

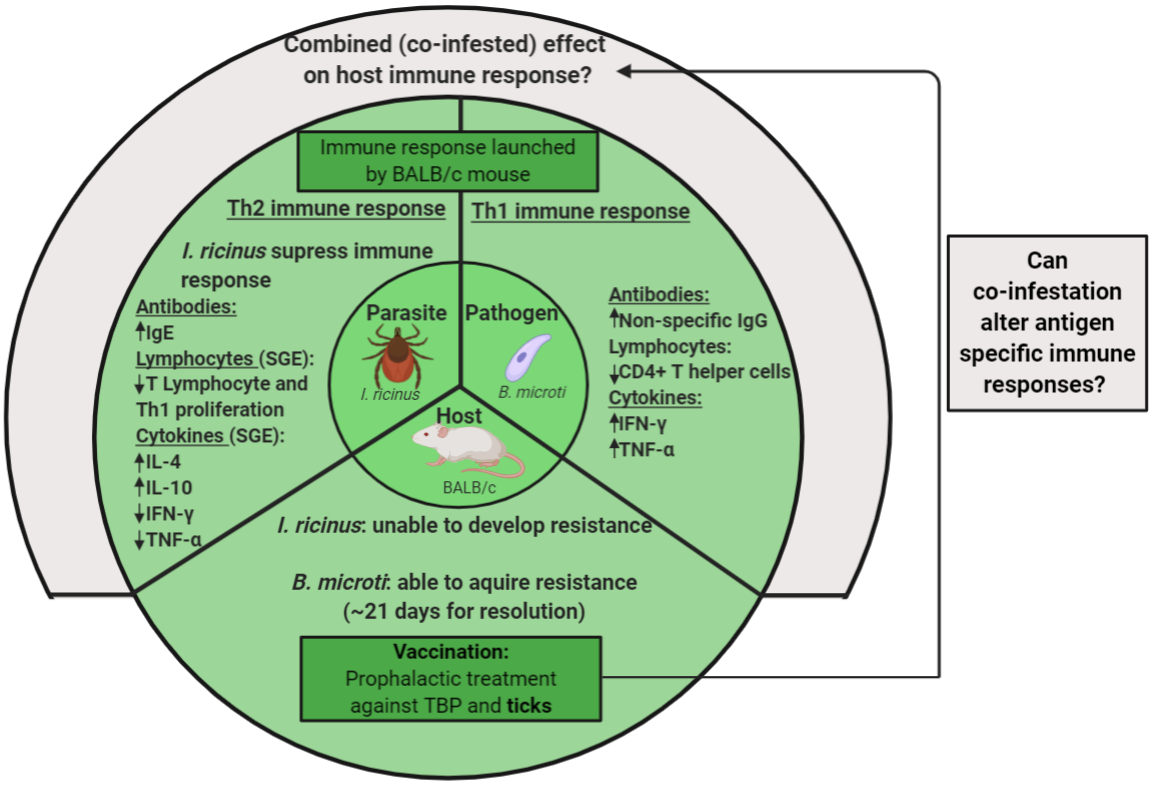

Figure 1.3: Summary of study relevance.

Ixodes ricinus (Th2) and *B. microti* (Th1) effect on BALB/c immune response is summarized in the top two triangles. Vaccination has been proposed as a prophylactic against tick infestation, which in turn will result in a decrease in TBP transmission e.g. *B. microti*. To date, only one protective tick antigen has been commercialized in a vaccine, namely Bm86 against the cattle tick *R. microplus*. Limited studies have been completed on the effect co-infection/infestation of a parasite and pathogen has on a vertebrate host, and in turn, how this may influence vaccine efficacy and immune response launched when vaccinated (Mabbott, 2018).

Abbreviations: Cluster of differentiation (CD), Interferon-gamma (IFN-γ), Immunoglobulin (Ig) Interleukin (IL), Salivary gland extract (SGE), Tick-borne pathogens (TBP), Tissue necrosis factor-alpha (TNF-α)

1.9. Hypotheses:

(a) Co-infection/infestation with *B. microti* and *I. ricinus* will result in different immune responses in the presence and absence of an antigen, compared to mice infested with ticks alone or infected with *B. microti* alone.

(b) GLA-SE and Alum vaccine formulations will modulate the Th1 and Th2 responses in BALB/c mice immunized with the combination of Bm86 and TC-X antigens compared to saline immunized controls.

1.10. Aims and specific objectives:

Aim 1:
To assess the immune response to ovalbumin in BALB/c mice during co-infection/infestation with *Babesia microti* and *Ixodes ricinus*.

Objectives:

- Establish a mono-infection of *B. microti*, a mono-infestation of *I. ricinus* and co-infected/infested BALB/c mice.
- Perform a time-dependent study with and without co-infection/infestation and/or vaccination with ovalbumin.
- Termination of mice at selected time points for collection of blood via jugular vein bleeding and harvesting of spleen and lymph node tissues.
- Monitor ovalbumin antigen-specific antibody production from collected blood using an enzyme linked immunosorbent assay.
- Perform Th1 and Th2 cytokine profiling on *in vivo* collected lymph node tissue supernatant.
- Assess the temporal changes in immunophenotypes, namely cell subpopulations in blood, spleen and lymph node tissues following termination at selected intervals of infection/infestation.
- Perform appropriate statistical analysis of data generated by enzyme linked immunosorbent assay, cytokine quantification and flow cytometry to compare host immunological responses between infected/infested and non-infected/infested host.

Aim 2:

Assessment of the immune response of BALB/c mice following immunization with Bm86 and TC-X antigen combination formulated in two different adjuvants.

Objectives:

- The primary and booster vaccination of BALB/c mice with Bm86 and TC-X antigen combination formulated in two different adjuvants.
- To terminate mice at the selected time points, collection of blood via jugular vein bleeding and harvesting of spleen and lymph node tissues.
- To culture single cell suspensions derived from spleens and lymph nodes in the presence of appropriate *ex vivo* stimulants/antigens in order to induce a targeted immune response to be quantified using cytokines and lymphocyte phenotype profiling.
- To monitor antigen-specific antibody production in serum collected at the selected time points using an enzyme linked immunosorbent assay.
- To determine if the *ex vivo* immune response in BALB/c mice is Th1 or Th2 biased by characterizing the cytokine profile in spleen and lymph node cell culture supernatants following 24 hours of culture.
- To assess the temporal changes in the immune cell phenotypes by means of flow cytometry from cultured spleen and lymph node tissue following 48 hours of culture.
- To perform appropriate statistical analysis of data generated by enzyme linked immunosorbent assay, cytokine quantification and flow cytometry to compare mice immunological responses between Bm86, TC-X vaccinated and saline vaccinated controls.

1.11. Outputs:

Research findings generated in this study were presented at the following seminars:

- Maritz-Olivier C, Robbertse L, Richards S, Leisewitz A, Crafford J, Greyling N, Barnard A, Clift S (Sept 2018) Analysis of lymphocyte subsets in the lymph nodes amongst three cattle breeds as potential mediators of immune-resistance to *Rhipicephalus microplus*. AITVM/STVM meeting, Buenos Aires, Argentina.
- Maritz-Olivier C, Robbertse L, Greyling N (2019) Comparative Immune-Profiling of Three Cattle Breeds Infested with *Rhipicephalus microplus* and *Ixodes ricinus*

infestation of BALB/c Mice During Co-infection with *Babesia microti*. African vaccine Network meeting, March 2019, ILRI, Nairobi.

- Greyling, N, Stutzer, C; Robbertse, L, Maritz-Olivier C (August 2019) Immune profiling of BALB/c mice co-infected with *Babesia microti* and *Ixodes ricinus* and its effect on immunization. Oral presentation, Biochemistry, Genetics and Microbiology seminar, University of Pretoria South Africa

Chapter 2: Materials and Methods

2.1. Experimental animals, *B. microti* parasites and *I. ricinus* ticks

All animal studies were conducted within the Institute of Parasitology (Biology Centre of the Czech Academy of Sciences, České Budějovice, the Czech Republic). All experimental animals were treated in accordance with the Animal Protection Law of the Czech Republic No. 246/1992 (ethics approval No. 161/2011), Specific pathogen-free (SPF) female BALB/c mice (6 to 8 weeks, ~20 g each) were bred from stock obtained from the Charles River Laboratories International Inc. (Germany). Mice were housed in plastic cages with sterilized wood-chip bedding, in a specific-pathogen free room under a constant temperature of 22°C and relative humidity of 65%. A 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, the Czech Academy of Sciences. Ticks were maintained in chambers with a humidity of about 95%, temperature 24°C and a day/night period set to 15/9 hours. *Babesia microti* (Franca) Reichenow (ATTC® PRA-99™, USA), was maintained by continuous passages in BALB/c female mice (Charles River Laboratories, Germany) and used for *in vivo* experiments.

2.2. BALB/c mice co-infestation with *Babesia microti* and *Ixodes ricinus* and immunization

2.2.1. Experimental set-up and infestation protocols

The study consisted of 40 BALB/c mice which were randomly divided into six experimental groups, a negative control group and five test groups (Group A-E). The negative controls and test Groups A-C consisted of 8 mice were further subdivided into 2 subgroups (n=4), that were euthanized on day 16 and 38, respectively. Mice were allowed to acclimatize 7 days prior to the start of the study. BALB/c mice from Groups A and B were injected intra-peritoneally on days 1 and 22 with 150 µl of *B. microti* infected blood (50% parasitemia, ~800 × 10^6 of infected red blood cells). The negative control group and Groups C-E were injected with 150 µl 1 x PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4) on the same days.

For tick infestations, the fur was clipped from the dorsal regions of each mouse and a capsule affixed between the shoulders. The capsules were made from modified 5.0 ml microcentrifuge tubes securely attached using a thin layer of adhesive (Pattex contact adhesive, Henkel corporation, USA) to yield a 2 cm^2 area for infestation. Groups B to D were infested with 20 *I. ricinus* nymphs on days 2 and 23 of the study. The ticks were allowed to attach to the host and feed to repletion. In contrast, an un-infested capsule was attached to mice from Group A, E and the negative control group on the same days in a similar manner.

Mice from Group A, B, D and E were immunized intraperitoneally with OVA (10 µg/mouse) (Sigma, St. Louis, MO) formulated with Freund's complete (day 16) or Freund's incomplete (day 38) adjuvant (Sigma, St. Louis, MO) in 1 x PBS (50:50, v/v) to a final volume of 200 µl per immunization. Mice from Group C and the negative control group were injected with 200 µl 1 x PBS on the same days. Experimental outline is summarized in Figure 2.1.

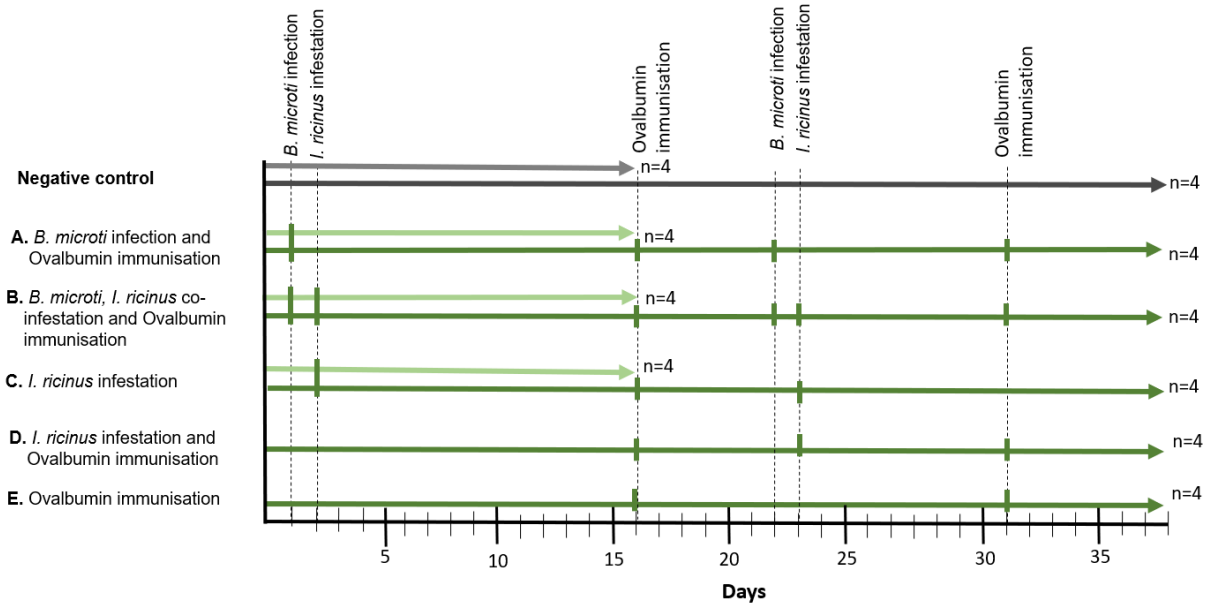

Figure 2.1: Experimental set-up, infestation and immunisation protocol for mice co-infested/infected and immunised
The study consisted of 40 BALB/c mice with the negative control (grey arrows) and group A-C having n=8 mice, and group D and E n=4 mice. Mice were allowed to acclimatize 7 days prior to the start of the study. Mice from the negative control group and groups A-C were euthanized and tissues were collected on day 16 (light green/gray arrows) and 38 (dark green/gray arrows). *Babesia microti* infections (group A and B) occurred on days 1 and 22, *Ixodes. ricinus* infestations (group B-D) on days 2 and 23. Ovalbumin immunizations (group A, B, D and E) were administered on day 16 and 31.

2.2.2. Termination and blood collection

Experimental animals were terminated on days 16 and 38 of the study. Mice where anaesthetized with a 150 µl intraperitoneal injection of ketamine (1.625% Narkamon) and xylazine (0.35% Rometar) (Bioveta, Ltd., Czech Republic), formulated in PBS. Blood was immediately collected via the jugular vein, and mice were euthanized via cervical dislocation. The collected blood was kept at 4°C for 30 min and then centrifuged at 2 000 x g for 20 min at 4°C for serum collection. The serum was stored at -20°C until further use.

2.2.3. Spleen and lymph node collection, processing and leukocyte isolation

Spleens and sub-iliac lymph nodes (identified as the regional draining lymph node) (Robbertse *et al.*, 2018b) were aseptically removed from all mice groups taking care to remove the surrounding fascia. Single cell suspensions were prepared from whole spleens in 1 ml and lymph nodes in 100 µl PBS by gentle extrusion through plastic sieves (Corning ® Cell Strainer, 70 µm). Samples were then centrifuged (500 x g, 10 min, 4˚C) and the resultant lymph node supernatant collected and used for cytokine detection (section 2.2.5).

For FACS analyses, erythrocytes were removed from the splenocyte and lymphocyte suspensions by incubation in an ammonium chloride-based lysing reagent (BD Pharm Lyse™, BD Biosciences, USA) at RT for 3 min as per manufacturer's instructions. Sample volumes were adjusted to 30 ml with PBS, centrifuged (500 x g, 10 min, 4˚C) and the cell pellet washed with FACS buffer (PBS, 0.05% NaN_3) and then centrifuged (500 x g, 10 min, 4˚C). The resultant single cell suspensions were suspended in 20 µl FACS buffer prior to being subjected to surface antigen detection using fluorescently labelled antibodies (section 2.2.6).

2.2.4. Serum antibody concentration determined using enzyme-linked immunosorbent assay

Total serum IgG (day 16 and 38) and anti-ovalbumin (anti-OVA) IgG1, IgG2 and IgE concentrations were determined using the total IgG, Anti-OVA IgG1 and IgE ELISA detection kits for mice supplied by the Cayman Chemical Company (USA), according to the manufacturer's guidelines. Buffers used in all of the ELISA analyses correspond to Immunoassay Buffer B concentrate (10 X concentrate), Wash Buffer concentrate (400 X concentrate), 3, 3', 5, 5'- tetramethyl-benzidine (TMB) substrate solution and HPR Stop solution. Immunoassay Buffer B concentrate (Assay buffer) was prepared by dilution in 90 ml PBS, to make up 1 X concentrate according to manufacturer's instructions. Similarly, Wash Buffer concentrate (Wash buffer) was prepared by diluting 5 ml Wash Buffer Concentrate in 2 L PBS and adding 1 ml of supplied Polysorbate 20 to make up 1 X concentrate according to manufacturer's instructions

Total IgG and anti-OVA IgG1 concentration

Supplied IgG (mouse) and Anti-OVA IgG1 (mouse) ELISA standards were prepared and serially diluted in Assay Buffer per manufactures instructions. Serum samples were used to make a 1:15 000 (IgG total) and 1: 4 000 (anti-OVA IgG1) dilution in Assay buffer. Samples and standards (100 µl/well) were added into appropriate wells of the supplied Goat anti-Mouse IgG and OVA pre-coated 96 well strip plates. The plates were covered with supplied 96 well cover sheets and incubated for 2 hours at RT on an orbital shaker (200 rpm). Following incubation, plates were washed three times with 300 µl/well of supplied Wash buffer. To each well 100 µl/well of supplied Mouse IgG Assay HRP-Conjugate and Goat Anti-Mouse IgG1 HRP Detection antibody (1 X concentrate working solution each) was added respectively. The plates were covered and incubated for 1 hour, then washed three times as described previously. Following the final wash steps a 100 µl/well of 3, 3', 5, 5'- tetramethyl-benzidine (TMB) substrate solution was added to each well and the plate incubated for 30 min at RT.

All final signal development reactions were stopped through the addition of 100 µl HRP Stop solution according to manufacturer's instructions. Absorbance at 450 nm was determined using the SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA). Plate negative control values were subtracted from the mean of duplicate values, and antibody concentrations were calculated using standard values. Briefly, concentrations were calculated by plotting a standard curve in Microsoft® Excel (ver. 8), anti-OVA IgG1, IgG2a and IgE concentrations were converted to mg/ml and IgG total to g/ml. The mean and standard error of the mean (SEM) for each antibody concentration was plotted and compared between relevant experimental groups terminated on the same days.

Anti-OVA IgE concentration

Supplied anti-OVA IgE (mouse) ELISA standard was prepared and serially diluted in Assay Buffer per manufactures instructions. Serum samples were used to make a 1:25 dilution in supplied Assay buffer. Samples and standards (100 µl/well) were added into appropriate wells of the supplied Goat anti-Mouse IgE pre-coated 96 well strip plates. The plates were covered with supplied 96 well cover sheets and incubated for 2 hours at RT on an orbital shaker (200 rpm) Following incubation, plates were washed three times as described previously. After washing, 100 µl/well of supplied OVA-biotin conjugated working solution reconstituted in Assay buffer was added and the plate incubated for 1 hour at RT on an orbital shaker (200 rpm). Following incubation, the plates were washed three times as previously described and 100 µl/well Streptavidin-HPR (1 X concentrate) working solution was added. The plates were covered and left to incubate for 30 min at RT on orbital shaker (200 rpm). After incubation the plates were washed as previously described. Following the final wash step, 100 µl/well of supplied 3, 3', 5, 5'- tetramethyl-benzidine (TMB) substrate solution was added and the plate incubated for 30 min at RT on orbital shaker (200 rpm). All final signal development reactions were stopped through the addition of 100 µl HRP Stop solution according to manufacturer's instructions and the Absorbance at 450 nm determined.

Anti-OVA IgG2a concentration

Mouse anti-OVA IgG2a concentration was determined using pre-coated OVA plates (Cayman Chemical Company, USA). The same dilution and reagents were used as outlined before for the anti-OVA IgG1 assay, and detection was achieved using Goat anti-Mouse IgG2a secondary antibody conjugated to HRP (Thermo Fisher Scientific, USA) at a 1:1000 dilution prepared in Assay buffer.

2.2.5. Ex vivo cytokine detection of lymph node tissue

Cytokine protein concentrations were determined from single cell suspensions (section 2.2.3) of isolated sub-illiac lymph nodes from BALB/c mice on the day of termination. A multiplex cytokine bead array (MILLIPLEX® MAP Mouse Th17 Magnetic Bead Panel, Merck, Germany) was used according to manufacturer's instructions. The panel used for the MILLIPLEX® mouse cytokine assay included markers for Th1- (IL-2, IFN-γ, TNF-α) and Th2- (IL-10, IL-4, IL-13, IL-25/IL-17E) specific cytokines. The immunoassays procedure was carried out according to manufacturer's instructions and is briefly stated below.

Supplied Mouse Th17 standards were reconstituted with 250 µl deionized water and serially diluted in Assay Buffer to obtain six working solutions. Provided Wash buffer (200 µl) was added each of the 96 wells after which the plate was sealed with provided plate sealer and set on orbital shaker (200 rpm) at RT for 10 min. Wash buffer was decanted and 25 µl of provided standards and controls were added to appropriate wells. Assay buffer (25 µl) was also added to each well after the addition of the samples and to empty wells serving as a background standard. After the addition of Assay buffer, 25 µl of provided appropriate Matrix solution was added to wells including the standards, samples and backgrounds wells. Following the addition of Matrix solution, 25 µl of undiluted (neat) samples were added to each well. Finally, 25 µl of provided Mixed beads were added to each well. After the addition of all the reagents, the plate was sealed and allowed to incubate overnight (16-18 hours) at 4°C on an orbital shaker (200 rpm). After incubation, the contents of the plate were gently removed and the wells washed twice with 200 µl Wash buffer. After washing 25 µl of provided Detection Antibodies were added to each well, plates were sealed and incubated for 1 hour at RT, shaking (200 rpm).

For detection of the cytokines, the 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 further analyzed in Graph Pad Prism (ver 6.01). To compensate for between-sample variation, values were normalized using the constant IL-5 cytokine concentration. The mean and standard error of each cytokine concentration was plotted and data points compared between day 16 (negative control and Group A-C) and day 38 (negative control and Group A-E).

2.2.6. Leukocyte staining and flow cytometry

Fluorescently labelled monoclonal antibody combinations to detect T and B-lymphocytes (BD Pharmingen™, Franklin Lakes, New Jersey) were diluted in 8 µl of FACS buffer as indicated in Table 2.1. Single cell suspensions (section 2.2.3) derived from the isolated blood, spleen and lymph node tissues (~2 x 10^6 cells/20 µl FACS buffer) were incubated with 2.1 µl prepared antibody dilution for 45 min at 4˚C. Undiluted antibodies are stored at 4°C.

Following incubation, the samples were washed twice with 200 µl FACS buffer via centrifugation (500 x g for 3 min). Samples were finally resuspended in 200 µl ice cold FACS buffer and transferred to 5 ml Falcon 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), 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 analyzed using the BD FACSDiva™ software (ver. 6.1.3). Final values were converted from percent of cell population to absolute cell numbers in Microsoft® Excel (ver. 8). The percent of cell population of the negative control and Group A-E was used to generate a heat map, indicating whether cell populations were increased or decreased compared to the control group.

Table 2.1: Lymphocyte marker panels used for the quantification of T and B lymphocytes respectively. All antibodies were supplied by BD Pharmingen™

Lymphocyte marker panel 1 (B-lymphocytes)				BD Pharmingen™ Antibody information		
Antibody	**Alternative Name**	**Conjugate**	**Isotype**	**Cat. nr**	**Conc. (mg/ml)**	**Vol used per reaction (µl)**
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	561047	0.2 mg/ml	0.2
CD19 Rat Anti-Mouse (clone 1D3)**	na	Alexa Fluor® 700	Rat LEW, also known as Lewis IgG2a, κ	557958	0.2 mg/ml	0.4
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	552867	0.2 mg/ml	0.2
CD21/CD35 Rat Anti-Mouse (clone 7G6)**	CR2/CR1	FITC	Rat SD, also known as Sprague-Dawley (outbred) IgG2b, κ	561769	0.5 mg/ml	0.4
CD27 Hamster Anti-Mouse (clone LG.3A10)*	na	APC	Armenian Hamster IgG1, κ	560691	0.2 mg/ml	0.2
CD80 Hamster Anti-Mouse (clone 16-10A1)*	Cd80; B7; B7-1; Cd28l; Ly-53; MIC17; TSA1	PE	Armenian Hamster IgG2, κ	561955	0.2 mg/ml	0.2

Lymphocyte marker panel 2 (T-lymphocytes)				BD Pharmingen™ Antibody information		
Anti-mouse antibody	**Alternative name**	**Conjugate**	**Isotype**	**Cat. nr**	**Conc. (mg/ml)**	**Vol used per reaction (µl)**
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	561047	0.2 mg/ml	0.2
CD3e Hamster anti-Mouse (clone 500A2)**	CD3ε chain	Alexa Fluor® 700	Syrian Hamster IgG2, κ	557984	0.2 mg/ml	0.4
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	553046/561835	0.5 mg/ml	0.2
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	561780	0.2 mg/ml	0.2
CD195 Rat Anti-Mouse (clone C34-3448)*	CCR5	PE	Rat IgG2c, κ	559923	0.2 mg/ml	0.2

CD184 Rat Anti-Mouse (clone 2B11/CXCR4)**	CXCR4, C-X-C chemokine receptor type 4; Fusin; LESTR; PB-CKR; Sdf1r	APC	Rat IgG2b, κ	558644	0.2 mg/ml	0.2

Markers and/or combinations of markers used for cell population analyses

Cell type / Cell population	Marker(s)	Reference
B lymphocytes		
Transitional	CD45+CD19+IgM^{low}	(Radtke *et al.*, 1999)
Follicular B cell	CD45+CD19+IgM^{high}	(Byth *et al.*, 1996)
Marginal and follicular	CD45+CD19+IgM+CD21+	(Won *et al.*, 2002)
Follicular and memory	CD45+CD19+IgM+CD27+	(Herrmann *et al.*, 2003)
Activated	CD45+CD19+IgM+CD27+CD80+	(Yang *et al.*, 2006)
Memory	CD45+CD19+IgM+CD21+CD27+CD80+	(Thorarinsdottir *et al.*, 2016)
T lymphocytes		
T cells	CD3+CD4+	(Petrie *et al.*, 1990)
T reg	CD3+CD4+CD25+	(Khattri *et al.*, 2003)
T cells	CD45+CD3+	(Byth et al., 1996)
Th1	CD45+CD3+CD195+	(Robbertse et al., 2018a)
Th1 + Th2	CD45+CD3+CD195+CD184+	(Nakae *et al.*, 2007)
Th2	CD45+CD3+ CD184+	(Dorfman *et al.*, 2003)

To visualize the change in B- and T lymphocyte subpopulations between different tissues (spleen and lymph node) and blood, experimental groups and time points (day 16 and 38) heat maps were generated from flow cytometry data using the online versatile matrix visualization and analysis software Morpheus (https://software.broadinstitute.org/morpheus). Fold changes for heat map visualizations were calculated using Equation 2.1.

Equation 2.1: $Heatmap\ fold\ change = Log2\ (\frac{mean\ experimtal\ \%\ population}{mean\ control\ \%\ population})$

Log_2 fold changes (Log_2FC) that were $-1 \leq Log_2FC \geq 1$ and *P* value <0.05 compared to the negative control % population is indicated with black squares. Lymphocyte subpopulations that had a higher population percentage compared to the negative control population were indicated in green ($0 \leq Log_2FC \geq 3$) and in red ($-3 \leq Log_2FC \geq 0$) if they had a lower population percentage compared to the negative control.

2.3. *Rhipicephalus microplus* antigen(s) and adjuvant formulation optimization in BALB/c mice.

2.3.1. Bm86 and TC-X antigen production

Recombinant Bm86 and TC-X-T (TC-X fused to a proprietary toxoid sequence) was produced in yeast and bacterial protein production systems, respectively by the Biomanufacturing technologies unit at the CSIR (Pretoria, South Africa). Antigens were received in PBS and diluted with 1xPBS to a concentration of 0.5 µg/µl before immunization.

2.3.2. Experimental set-up and vaccination regime

The study consisted of 30 BALB/c mice randomly divided into 3 groups (each group, n=10): control, Group A (immunized only with Bm86 antigen) or Group B (immunized with both Bm86 and TC-X antigens) and the control group. Groups A and B were sub-divided into two groups (n=5), and immunized with antigen(s) formulated with either Imject™ Alum (Thermo Fisher Scientific, USA) or GLA-SE (Afrigen, RSA) as an adjuvant (Figure 2.2).

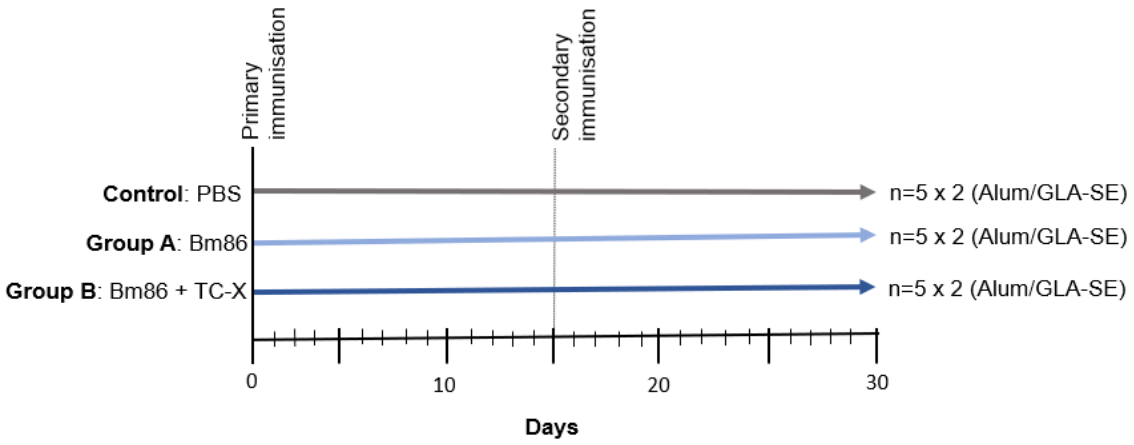

Figure 2.2: Experimental set-up and immunization regime for study objective to evaluate adjuvants on immune response towards Bm86 and TC-X.

BALB/c mice were immunized on day 0 and 15. On day 30 mice were euthanized, spleens, caudal, lumbar and inguinal lymph nodes were removed for culturing and subsequent cytokine analyses. Mice acclimatized for a period of 7 days before the start of experimental procedures.

The vaccine antigens were formulated with adjuvant according to manufacturer's guidelines. Control groups were immunized with 60 µl PBS combined with either Imject™ Alum (Thermo Fisher Scientific, USA) or GLA-SE (a kind gift from AgriGen Pty., South Africa), 60 µl each, in

a 1:1 ratio (50:50, v/v, total volume 120 µl). Mice from Group A were vaccinated with 30 µg Bm86 formulated with either Alum or GLA-SE in a 1:1 ratio. Similarly, Group B mice were vaccinated with an antigen combination of Bm86 and TC-X-T (30 µg each) that were formulated in either Alum or GLA-SE to a final volume of 120 µl. BALB/c mice were immunized intraperitoneally with 120 µl of vaccine formulation on day 0, with a subsequent booster injection given on day 15. Thirty days after the initial immunization, mice where anaesthetized with 150 µl of anesthetic as previously described (section 2.2.2.). Blood was then collected via the jugular vein, and mice were euthanized via cervical dislocation after which the spleen, caudal-, lumbar- and inguinal lymph nodes were aseptically removed for *ex vivo* culturing.

2.3.3. Tissue culturing and supernatant collection

Spleen, caudal-, lumbar- and inguinal lymph node tissues were collected in cold RMPI 1640 medium (Thermo Fisher Scientific, USA) containing 1% Penicillin-Streptomycin (10,000 U/ml) (Thermo Fisher Scientific, USA) and 0.1% 2-Mercaptoethanol (Thermo Fisher Scientific, USA). Single cell suspensions were prepared by gentle extrusion through plastic sieves (Corning ® Cell Strainer, 70 µm). The resulting suspensions were then washed with 15 ml RMPI 1640 media (containing antibiotics and mercaptoethanol), following centrifugation (500 x g for 10 min, 4ºC). Erythrocyte contamination in tissue was removed as described in section 2.2.3. Splenocytes and lymphocytes were resuspended in 1 ml and 200 µl of RMPI 1640 medium (containing antibiotics and mercaptoethanol), respectively. Cell counts were determined by Trypan blue exclusion immediately after recovery. Briefly, resuspended splenocytes and lymphocytes were diluted with RMPI 1640 media (containing antibiotics and mercaptoethanol) in a 1: 20 and 1: 5 ratios, respectively. Trypan blue solution, 0.4% (Thermo Fisher Scientific, USA) was added to diluted cells in a 1: 1 ratio. The solution was carefully mixed using a pipette and 20 µl of the solution was added onto a haemocytometer. The hemocytometer was placed onto a light microscope, only white cells were considered viable and thus counted. The number of cells per 1 ml was calculated using Equation 2.2

Equation 2.2: $\frac{nr\ of\ cells\ counted}{nr\ of\ blocks\ counted\ in} \times dilution\ factor\ \times 10^4 = nr\ of\ cells\ in\ 1\ ml\ sample$

The culturing protocol (Figure 2.3) was performed with 1 x10^6 to 1 x10^5 splenocytes and 1.5 x10^5 to 2.0 x10^4 lymphocytes into 96 wells TTP tissue culture plates (Merck, Germany). These cells were cultured in 200 µl RMPI 1640 containing antibiotics (1.1% Penicillin-Streptomycin), 10% heat inactivated fetal bovine serum (FBS) and 0.1% 2-Mercaptoethanol (Thermo Fisher Scientific, USA). Cells were cultured at 37ºC in 5% CO_2 in duplicate in the presence of 5 µg

(0.5 µg/µl) of the antigen(s) used for vaccination for 24 hours prior to supernatant collection. In the negative control reactions, cells were not stimulated with antigen(s). Following incubation, cells were centrifuged at 306 x g for 3 min after which 150 µl of supernatant was collected for cytokine profiling. The media was then replenished with an additional 150 µl culture RMPI. Cytokine concentrations (pg/ml) were transformed to represent the concentration. The cells were cultured for an additional 24-hour period, and re-stimulated with antigen(s) 24 hours before final cell collection (48 hours after the initial stimulation). The cells cultured for 48 hours were then processed for flow cytometry.

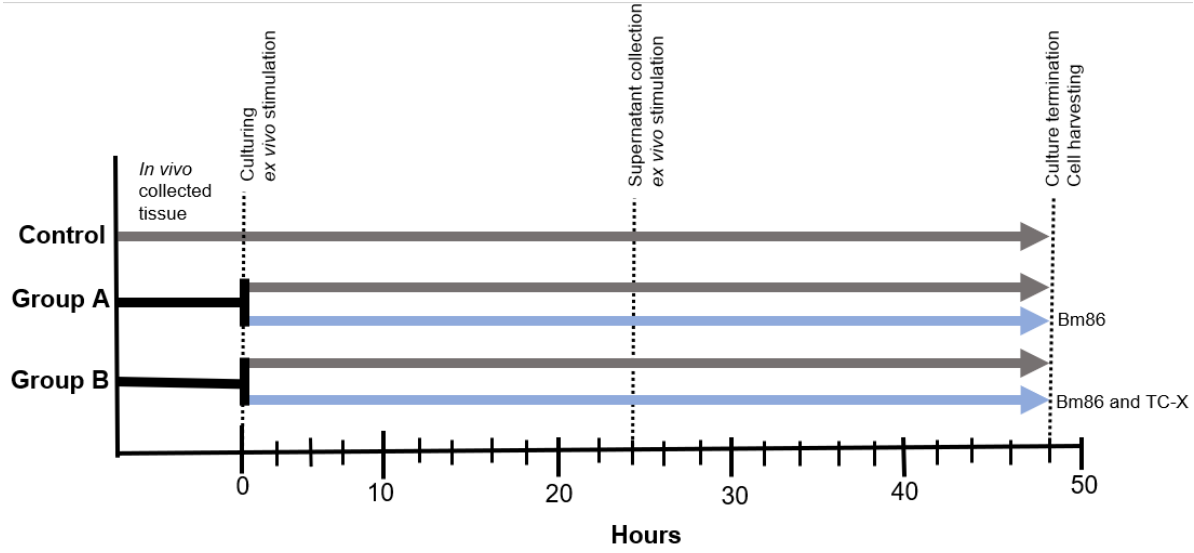

Figure 2.3: Spleen and lymph node culturing protocol.
Spleen and lymph node tissue collected from BALB/c mice on day 30 were cultured in the presence of antigen(s) (blue arrow) used for immunization or in the absence of antigen (gray arrows). After 24 hours of culturing supernatant was collected from cultures for *ex vivo* cytokine quantification. Cells were re-stimulated and after 48 hours in total cells were used to perform flow cytometry to detect lymphocyte subpopulations

2.3.4. Serum antibody titre determination using enzyme linked immunosorbent assay

Bm86 and TC-X specific IgG1 and IgG2a antibody titers were determined from the collected serum using direct enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Nunc MaxiSorp™ flat-bottom) were coated overnight at 4ºC with 50 µl/well of either Bm86 or TC-X-T (10 µg/ml) diluted in coating buffer (0.015 M Na_2CO_3, 0.035M $NaHCO_3$, pH 9.6). Following incubation, wells were blocked with 200 µl/well of blocking buffer (1.5% BSA in PBS, pH 7.4) for 45 min at 37ºC. Plates were then washed three times with PBS containing 0.05% Tween-20 (Sigma-Aldrich, Germany). Mouse primary serum was diluted 1: 1 000 in blocking buffer and 50 µl added per well. Plates were incubated for a further 45 min at 37ºC. Serum collected from mice prior to initial immunization was used as a negative and baseline control (i.e. naïve

serum). Plates were washed as described previously and incubated with 100 µl/well of secondary antibodies conjugated to HRP. Either Goat anti-Mouse IgG1 or Goat anti-Mouse IgG2a (Thermo Fisher Scientific, USA) was diluted 1:1000 with blocking buffer and added to the plates for incubation at 37ºC for 45 min. Plates were washed as previously described and 100 µl/well of substrate solution (0.1M Na_2HPO_4 and 0.05M citric acid, pH 5.0-5.5) containing 0.04 mg/well o-phenylenediamine dihydrochloride (OPD) and 0.04 µl/well 30% H_2O_2 was added. Following a 3 min incubation, the enzymatic reaction was stopped by the addition of 100 µl/well 2M H_2SO_4. Absorbance was measured at 490 nm using a microplate reader (Infinite® 200 PRO, Tecan). The antibody titer was estimated as the highest dilution factor, where the absorbance value was at least double that of the control group. The mean and SEM of each antibody titer was plotted and compared between relevant groups

2.3.5. Ex-vivo cytokine detection of cultured spleen and lymph nodes

Cytokine protein levels were measured in the supernatant of cultured spleen and lymph node tissue collected after the first 24 hours of antigen(s) stimulation. The supernatant cytokine levels were quantified using the Magnetic Luminex assay (mouse premixed multi-analyte kit, R&D systems, USA) according to manufacturer's instructions. The assay panel included markers for Th1- (IL-2, IFN-γ, TNF-α), Th2- (IL-10, IL-4, IL-12) and Th17- (IL-17A) specific cytokines. For detection of the cytokines, Luminex® detector parameters were adjusted similar to section 2.2.5. The mean and SEM of each cytokine concentration was plotted and data points compared between relevant groups.

2.3.6. Leukocyte staining and flow cytometry

After 48 hours incubation, cultured cells were treated similarly as described in section 2.2.6 with markers only included for CD80 (B lymphocyte), CD3, CD4, CD25 and CD 184 (T lymphocyte)

2.4. Statistical and data analysis

Data analyses was performed with GraphPad Prism® v.6.01 software (GraphPad Prism® software, Inc., San Diego, CA), using one-way analysis of variance (ANOVA), post hoc Tukey test for multiple comparisons, unless otherwise stated. The mean and SEM were calculated and graphed for each population subset. Statistically significant data was indicated on graphs as *P value $\leq$0.05, **P value $\leq$0.01, ***P value $\leq$0.001, ****P value $\leq$0.0001. Figures were generated using free online BioRender illustration software (https://biorender.com/).

Chapter 3: Results

3.1. Assessment of the immune response against ovalbumin in BALB/c mice during co-infection/infestation with *Babesia microti* and *Ixodes ricinus*

3.1.1. Total IgG and IgG1, IgG2a and IgE responses to infestation and vaccination

Total IgG concentration was determined for all experimental groups at both termination time points, namely day 16 and 38 (Figure 3.1.1).

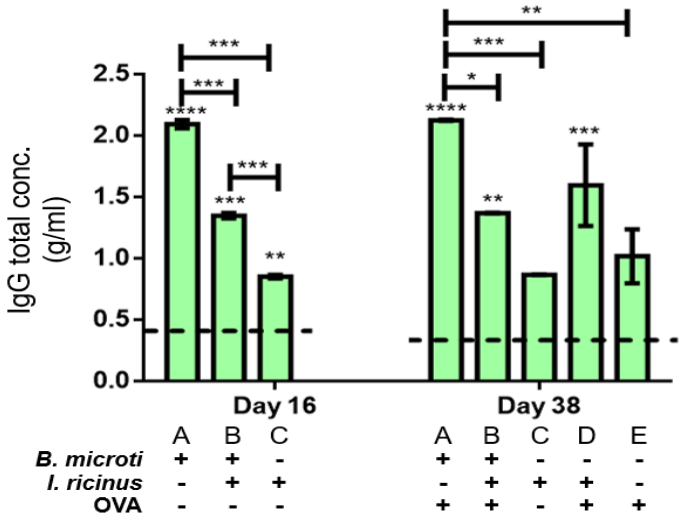

Figure 3.1.1:Serum total IgG concentrations of mice euthanized on day 16 and 38.
Serum total IgG concentrations (g/ml) of mice terminated on day 16 and 38 are shown. *Day 16* (**A**) mice infected with *B. microti*, (**B**) mice co-infected/infested with *B. microti* and *I. ricinus* and (**C**) mice infested with only *I. ricinus*. *Day 38* (**A**) mice infected with *B. microti,* immunized with OVA, (**B**) mice co-infected/infested with *B. microti* and *I. ricinus*, immunized with OVA, (**C**) mice infested with *I. ricinus*, (**D**) mice infested with *I. ricinus*, immunized with OVA and (**E**) mice immunized with OVA. Dashed lines indicate the mean IgG total concentration for the negative control group. Group numbers are indicated with letters A to E (see chapter 2). ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001.

Total IgG concentration: Day 16

It is evident that in mice terminated on Day 16, total serum IgG concentration was significantly higher for mice infected with *B. microti*, (1,35 ± 0,02 g/ml, *P*≤0.0001) followed by mice co-infected with *B. microti* and *I. ricinus* (0,86 ± 0,015 g/ml, *P*≤0.001) and lowest in mice only

infested with *I. ricinus* (0,86 ± 0,02 g/ml, $P \leq 0.01$), when compared to mice from the negative control group (0,45 ± 0,08 g/ml) (Figure 3.1, day 16).

Total IgG concentration: Day 38

For mice terminated on day 38, the serum IgG total concentration was significantly higher for mice infected with *B. microti* and OVA immunized (2,13 ± 0,005 g/ml, $P \leq 0.0001$), compared to mice co-infested with *B. microti* and *I. ricinus* and OVA immunized (1,37 ± 0,00 g/ml, $P \leq 0.01$) and mice infested with *I. ricinus* and OVA immunized (1,6 ± 0,33 g/ml, $P \leq 0.001$) compared to mice from the negative control group (0,4 ± 0,04 g/ml) (Figure 3.1.1, Day 38).

Serum OVA-specific IgG1, IgG2a and IgE concentrations

The anti-ovalbumin (OVA) specific IgG1, IgG2a and IgE antibody concentrations were only determined for Groups A, B, D and E on day 38 (Figure 3.2.1), as these were the only groups vaccinated with OVA on day 16 and 21 of the study.

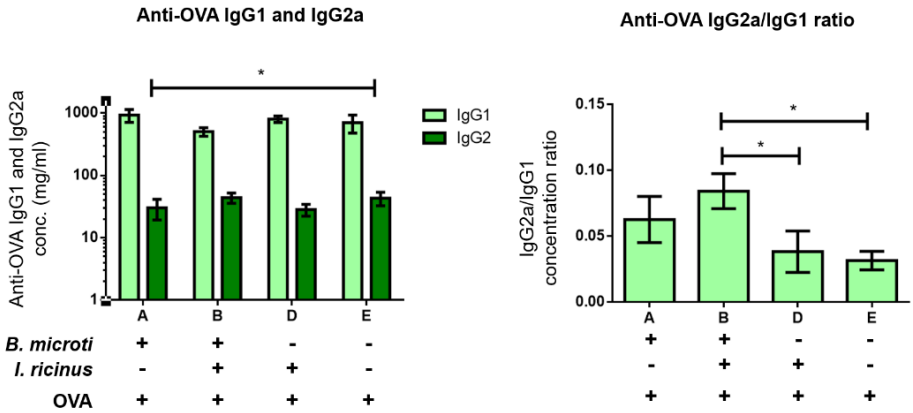

Figure 3.1.2: Anti-OVA IgG1 and IgG2a concentrations and calculated IgG2a/IgG1 ratio of mice euthanized on day 38.
Experimental groups*:* (**A**) mice infected with *B. microti*, immunized with OVA, (**B**) mice co-infected/infested with *B. microti* and *I. ricinus*, immunized with OVA, (**C**) mice infested with *I. ricinus*, (**D**) mice infested with *I. ricinus*, immunized with OVA and (**E**) mice immunized with OVA (as summarized in Figure 2.1). *$P \leq 0.05$, **$P \leq 0.01$

Anti-OVA specific IgG1 concentrations were consistently higher in all of the experimental groups and the anti-OVA specific IgG2a concentrations were consistently lower. Only one significant difference was observed in mice infected with *B. microti*, which had significantly higher OVA-specific IgG2a concentrations (49,86 ± 4,85 mg/ml) compared to mice only immunized with OVA (20,02 ± 5,11 mg/ml, $P \leq 0.05$)

The IgG2a to IgG1 ratio was calculated to determine whether a Th1 or Th2-biased immune response was elicited under the various conditions. The results indicate that mice co-infected with *B. microti* and *I. ricinus* had a significantly higher anti-OVA IgG2a/IgG1 ratio (0,08 ± 0,01) compared to mice only infected with *I. ricinus* and mice only immunized with OVA (0,04 ± 0,02 and 0,03 ± 0,01, respectively, $P \leq 0.05$). Therefore, a Th1-biased/polarized response is elicited in the presence of *B. microti* infection which is in line with a response towards a mostly intracellular occurring parasite, such as *B. microtti* (see chapter 4).

OVA-specific IgE serum concentrations (Figure 3.1.3) are significantly higher in mice infected with *I. ricinus* (2,37 ± 0,52 mg/ml) compared to both mice infected with only *B. microti* (0,61 ± 0,22 mg/ml, $P \leq 0.05$) and mice co-infected/infested with *B. microti* and *I. ricinus* (0,137 ± 0,09 mg/ml, $P \leq 0.01$). As such, the findings show that infection with *B. microti* does not illicit an antigen specific IgE-mediated response, while both *I. ricinus* infestation and OVA immunization produce an IgE response. The latter is in line with published findings showing increased IgE levels during tick infestation (see chapter 4).

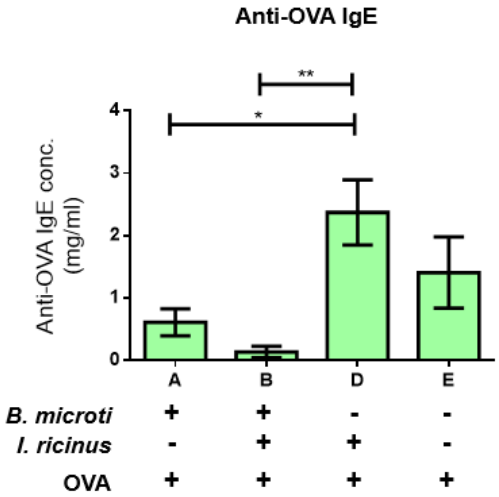

Figure 3.1.3: Anti-OVA IgE concentration of mice euthanized on day 38
Serum IgE anti-OVA concentrations (mg/ml) of mice immunized with OVA on day 16 and 21 and terminated on day 38. Experimental groups: (**A**) mice infected with *B. microti*, immunized with OVA, (**B**) mice co-infected with *B. microti* and *I. ricinus*, immunized with OVA, (**C**) mice infested with *I. ricinus*, (**D**) mice infested with *I. ricinus*, immunized with OVA and (**E**) mice immunized with OVA. Group numbers are indicated with letters A to E (as summarized in Figure 2.1). *$P \leq 0.05$, **$P \leq 0.01$

3.1.2. In vivo cytokine analysis of lymph node tissues: Th1 responses

The Th1 immune cytokine profile was further analyzed by evaluating the levels of TNG-α, IFN-γ and IL-2 (Figure 3.1.4).

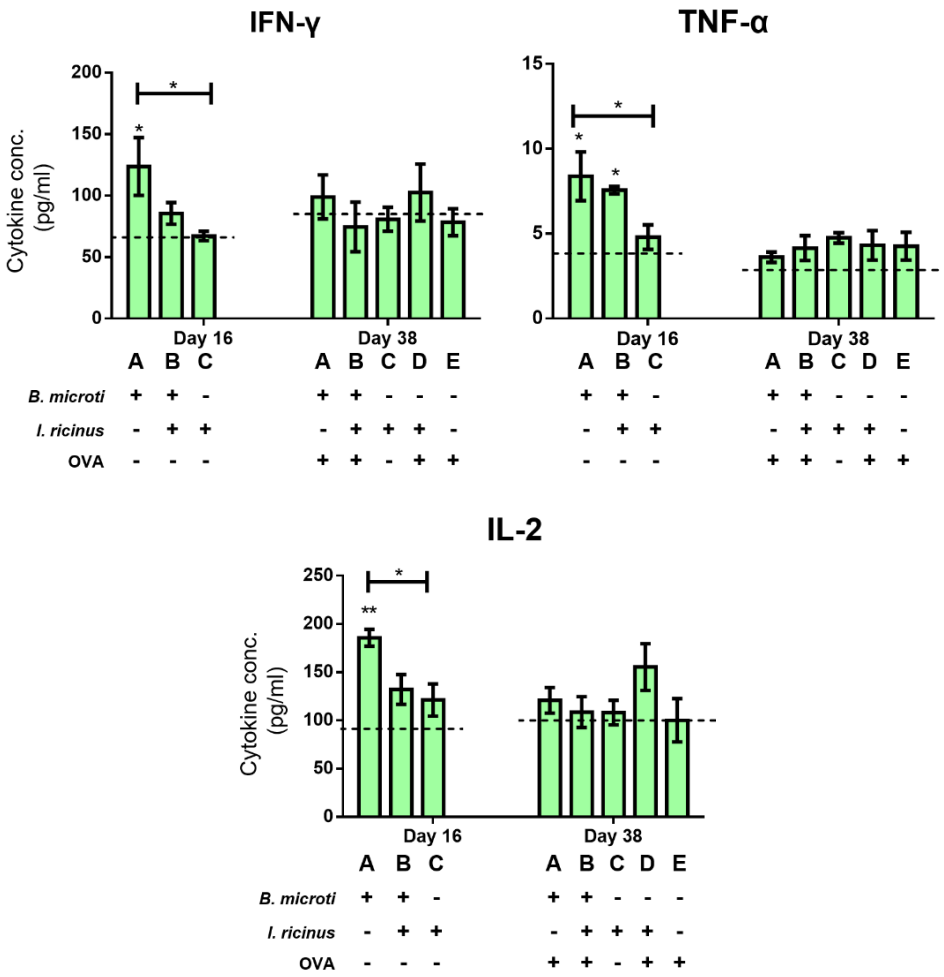

Figure 3.1.4: *In vivo* derived lymph node supernatant Th1 associated cytokine concentrations on day 16 and 38

Lymph node cytokine concentrations for TNF-α and IFN-γ and IL-2 is shown. Graphs are plotted for each termination time point (day 16 and 38). *Day 16* (**A**) mice infected with *B. microti*, (**B**) mice co-infected with *B. microti* and *I. ricinus* and (**C**) mice infested with *I. ricinus*. *Day 38* (**A**) mice infected with *B. microti*, immunized with OVA, (**B**) mice co-infected with *B. microti* and *I. ricinus*, immunized with OVA, (**C**) mice infested with *I. ricinus*, (**D**) mice infested with *I. ricinus*, immunized with OVA and (**E**) mice immunized with OVA. Dashed lines indicate the mean cytokine concentration for the negative control saline injected and un-infested/infected mice. *$P \leq 0.05$ and **$P \leq 0.01$.

Th1 responses at 16 days

On day 16, mice infected with *B. microti* has significantly higher TNF-α, IFN-γ and IL-2 (8,38 ± 1,44 pg/ml, 123,81 ± 23,52 pg/ml and 185,73 ± 8,85 pg/ml, $P \leq 0.05$) concentrations compared to mice from the negative control group (3,97 ± 0,23 pg/ml, 65,49 ± 6,08 p/ml and 94,41 ± 7,38 pg/ml) and mice only infested with *I. ricinus* alone (4,79 ± 0,72 pg/ml, 67,22 ±

3,86 pg/ml and 121,29 ± 16,83 pg/ml, $P \leq 0.05$). Mice co-infested with *B. microti* and *I. ricinus* also had a significantly higher TNF-α concentration (7,56 ± 0,22 pg/ml, $P \leq 0.05$) compared to the negative control group. All of the latter support the development of a Th1-biased response during *B. microti* infection (Fig.3.1.2.), which is somewhat suppressed during co-infestation with ticks. However, this suppression of the Th1-polarised response is not to the level observed in mice only challenged with ticks.

Th1 responses at 38 days

No significant differences in Th1 associated cytokine concentrations were observed on day 38 (Figure 3.1.4). The data indicates that the Th1-mediated responses predominate in lymph node tissues during *B. microti* infection with cytokines levels returning to baseline levels by day 38, possibly due to the resolution of the *B. microti* infection within 21 days (see chapter 1 section 1.4).

3.1.3. In vivo cytokine analysis of lymph node tissues: Th2 responses

The Th2 immune response was measured by IL-4, IL-10, IL-13 and IL-25 cytokine concentrations (pg/ml) (Figure 3.1.5). IL-13 and IL-25 cytokines did not show any significant difference in concentration between lymph node tissues of experimental groups and the negative control on both days 16 and 38 (Figure 3.1.5).

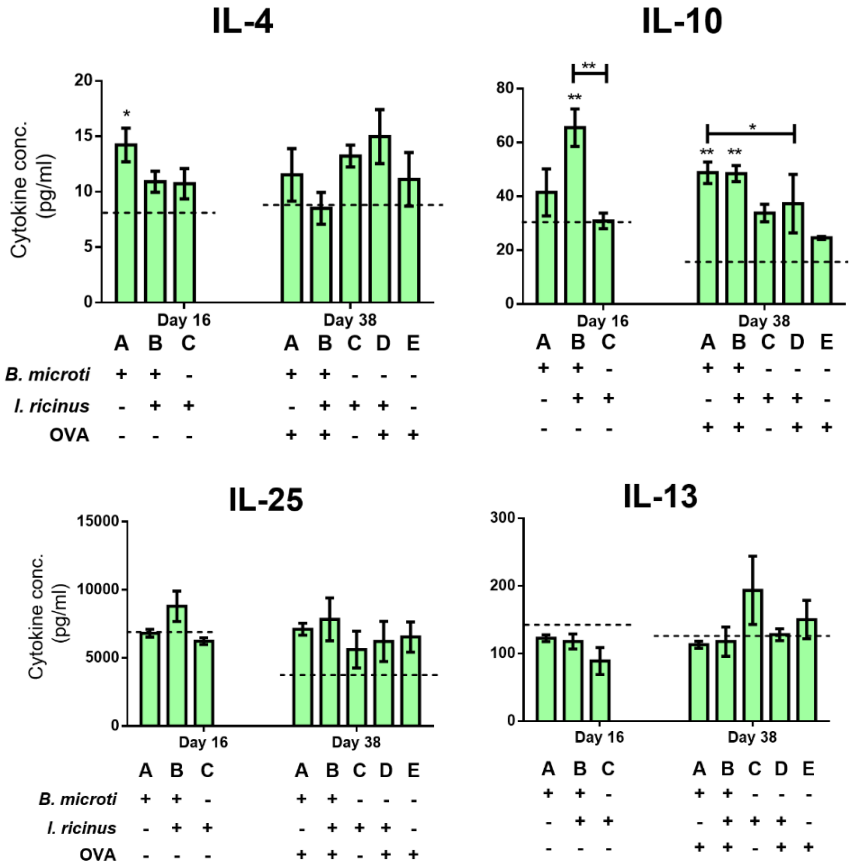

Figure 3.1.5: *In vivo* derived lymph node supernatant Th2 associated cytokine concentrations on day 16 and 38

Lymph node cytokine concentrations for IL-4, IL-10, IL-13 and IL-25. Graphs are plotted for each termination time point (day 16 and 38). *Day 16* (**A**) mice infected with *B. microti*, (**B**) mice co-infected with *B. microti* and *I. ricinus* and (**C**) mice infested with *I. ricinus*. *Day 38* (**A**) mice infected with *B. microti*, immunized with OVA, (**B**) mice co-infected with *B. microti* and *I. ricinus*, immunized with OVA, (**C**) mice infested with *I. ricinus*, (**D**) mice infested with *I. ricinus*, immunized with OVA and (**E**) mice immunized with OVA. Dashed lines indicate the mean cytokine concentration for the negative control saline injected and un-infested/infected mice. *$P \leq 0.05$ and **$P \leq 0.01$.

Th2 responses at 16 days

On day 16, mice infected with only *B. microti* had a significantly higher production of IL-4 (14,22 ± 1,52 pg/ml, $P \leq 0.05$) compared to the negative control (7,83 ± 0,72 pg/ml). No other significantly different IL-4 concentrations between experimental and negative control groups were seen on day 16 or day 38 of the study (Figure 3.1.4). Interleukin 10 concentrations on day 16 were significantly higher in mice co-infested with B. *microti* and *I. ricinus* (65,5 ± 7,0 pg/ml), as compared to both mice only infested with *I. ricinus* and mice from the negative control group (30,86 ± 2,9 pg/ml and 31,53 ± 2,61 pg/ml, $P \leq 0.01$).

Th2 responses at 38 days

Similarly, on day 38, IL-10 concentration was significantly higher for both mice infected with *B. microti* and OVA vaccinated, as well as mice co-infected with *B. microti* and *I. ricinus* and OVA vaccinated (48,8 ± 4.0 pg/ml and 48,5 ± 2,6 pg/ml, $P \leq 0.01$) compared to the negative control (16,4 ± 2,5). Mice infected with *B. microti* (OVA vaccinated) IL-10 concentration was significantly higher compared to mice infested with *I. ricinus* (or OVA vaccinated) (37,3 ± 9,4 pg/ml, $P \leq 0.05$) (Figure 3.1.5). Interleukin 10 production appears to be significantly influenced by infection with *B. microti*. Finally, no significant results were obtained for any of these cytokines from splenic supernatants (results not shown).

3.1.4. Lymphocyte phenotype populations during co-infection/infestation in the spleen, lymph node and blood of BALB/c mice

B-lymphocyte population

Five markers were analyzed for B-cells, namely CD19, IgM, CD21/CD35, CD27 and CD80 (See Table 2.1, Chapter 2) as well as the common leukocyte marker CD45. From the results 42.6% (results not shown) of the B lymphocyte populations were significantly differentially regulated at day 16 compared to day 38, with only 24.44% (results not shown). The significantly differentiated B cell populations between the various experimental conditions are shown in Figure 3.1.6.A as a heat map.

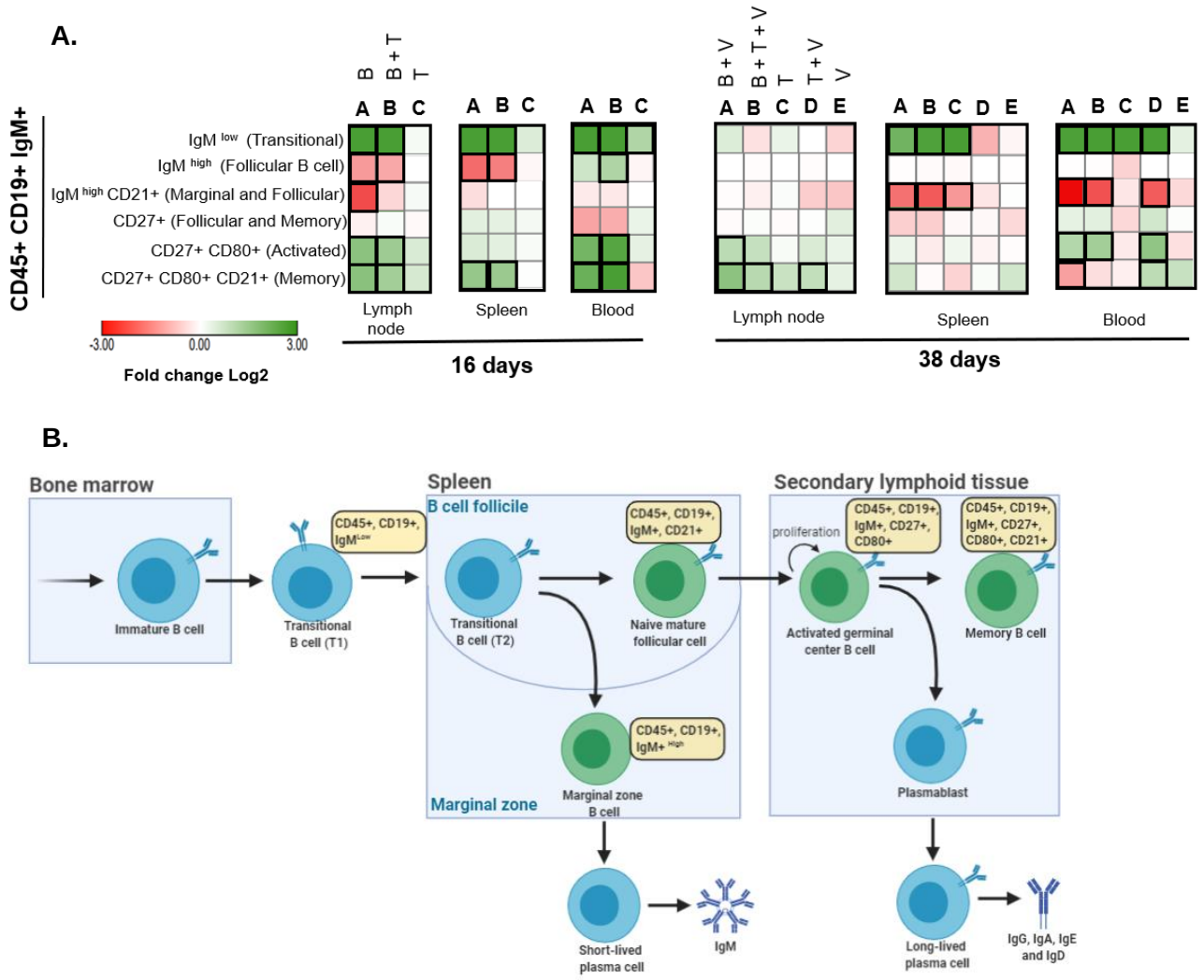

Figure 3.1.6: Fold changes of the CD45+CD19+IgM+ B-lymphocyte subpopulations in lymph nodes, spleen and blood of experimental groups relative to the negative control group.
In **figure A,** numerical values were used to generate the heatmap using Equation 2.1 (chapter 2) from flow cytometry cell population data. The lymphocyte sub-type associated with a marker combination is give in brackets. Data was generated for sub-illiac lymph node, spleen and blood on day 16 and 38 of the experiment. *Day 16* (**A**) mice infected with *B. microti*, (**B**) mice co-infected with *B. microti* and *I. ricinus* and (**C**) mice infested with *I. ricinus*. *Day 38* (**A**) mice infected with *B. microti*, immunized with OVA, (**B**) mice co-infected with *B. microti* and *I. ricinus*, immunized with OVA, (**C**) mice infested with *I. ricinus*, (**D**) mice infested with *I. ricinus*, immunized with OVA and (**E**) mice immunized with OVA. Black blocks around subpopulation indicate $-1 \leq Log_2FC \geq 1$ in subpopulation percentage compared to negative control saline injected and un-infested/infected mice with a *P value* ≤ 0.05. In **figure B** an illustration of B cell development and activation is shown with the corresponding cell markers per population.

B lymphocyte subpopulations on day 16

Most B lymphocyte subpopulations evaluated were significantly increased with a $Log_2FC \geq 1$ compared to the negative control (78.26%, results not shown) on day 16. Mice infected with *B. microti* and mice co-infected with *B. microti* and *I. ricinus* (Groups A and B), displayed similar regulation of lymphocyte subpopulations with the most up-regulated subpopulations in the

spleen, sub-iliac lymph nodes and blood (Figure 3.1.6.A, indicated in green). Both Groups A and B had a significant upregulation in the lymph node, spleen and blood of follicular B cells (CD45+CD19+IgM^{low}), activated B cells (CD45+CD19+IgM +CD27+CD80+) in lymph node and blood, as well as memory B cells (CD45+CD19+IgM+CD27+CD80+CD21+) in all three tissues evaluated. Under these two experimental conditions, two B cell subpopulations were found to be significantly decreased ($Log_2FC \leq -1$, indicated in red) compared to the negative control on day 16 (21.74%, results not shown). The decreased populations were the marginal B cells (CD45+CD19+IgM^{high}) in the lymph nodes and the marginal and follicular B cells in the spleen (CD45+CD19+IgM^{high}CD21+).

Mice infested with only *I. ricinus* (Group C) displayed no significant changes for B cell populations in the lymph node and the spleen. Only in blood was an increase in the follicular B cells (CD45+CD19+IgM^{low}) and a decrease in memory cells (CD45+CD19+IgM+CD27+ CD80+CD21+) detected. Considering the conventional pathway of B cell maturation (Figure 3.1.6.B) the data from day 16 suggests that *Babesia* infection as well as co-infection/infestation causes an influx of B cells into the lymph nodes, giving rise to activated and memory B cells that enter the blood. This influx is aligned with the efflux of precursor cells from the spleen. Based on the few differentially regulated B cells in mice only challenged with ticks (Group C), we propose that the observed affects are predominantly due to *Babesia* infection. This is also in line with literature that describes the strong immune-suppressive effects of ticks on their vertebrate hosts (see Chapter 4) and the reduced levels of total IgG observed (Figure 3.1.1). Again, similar to data shown in Figure 3.1.4 tick infestation was not sufficient to suppress all of the *Babesia*-induced immune effects.

B lymphocyte subpopulations on day 38

Of all the subpopulations differentially expressed on day 38, the majority of subpopulations (68,2%, results not shown) were found to be up-regulated compared to the negative control (results not shown). Most of the differentially regulated subpopulations occurred in the blood. Again, in mice that were infected with *Babesia* or co-infected there was a decrease in the marginal and follicular cells. This was also observed in mice that were vaccinated with OVA and then challenged with ticks. What is interesting is that in mice that only had ticks, this response was not detected. This once more points towards a unique response to ticks compared to the other groups. In Groups A-D an increase in the follicular B cells was observed. However, a decrease in the activated B cells was observed for only Groups A, B and D. Again, this response was not present in mice with ticks alone.

In the lymph node on day 38, mice infected with *B. microti* displayed an increase in memory B cells, with a slight increase also observed for co-infected/infested mice and tick with OVA vaccinated mice. Mice infected with *B. microti* also had an increase in activated B cells in the lymph node. In the spleen, there was a significant decrease in the marginal and follicular B cells in Groups A, B and C. In these group, all also showed an increase in follicular B cells.

T lymphocyte subpopulations on day 16

In this study, we evaluated five T cell markers namely CD3e, CD4, CD25, CD195 and CD184 (Table 2.1, Chapter 2) as well as the common leukocyte marker CD45. On day 16, some 53.7% (29/54) T lymphocyte subpopulations were found as significantly differentially expressed compared to day 38 which only had 20.00% (18/90). These ratios are similar to those observed for B lymphocytes. Most T lymphocyte subpopulations (68.97%) were significantly decreased ($Log_2FC \leq -1$) compared to the negative control on day 16 (Figure 3.1.7, indicated in red).

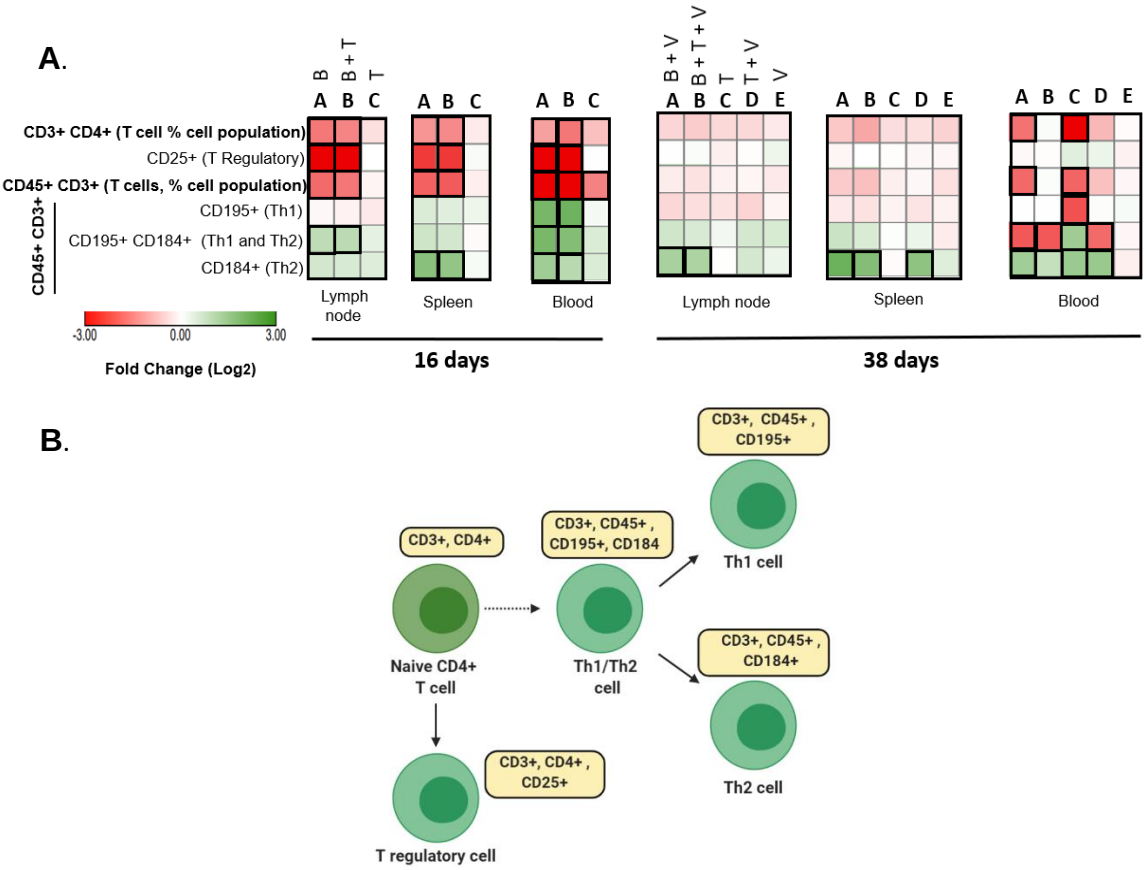

Figure 3.1.7: T-lymphocyte subpopulation percentages relative to negative control groups in a heat map format.
Numerical values used to generate the heatmap was calculated using Equation 3.1 from flow cytometry % cell population data., the lymphocyte sub-type associated with a marker combination is give in brackets. Data was generated for spleen, sub-illiac lymph nodes and blood on day 16 and 38 of the experiment. *Day 16* **A**) mice infected with *B. microti*, **B**) mice co-infected with *B. microti* and *I. ricinus* and **C**) mice infested with *I. ricinus*. *Day 38* **A**) mice infected with *B. microti*, immunized with OVA, **B**) mice co-infected with *B. microti* and *I. ricinus*, immunized with OVA, **C**) mice infested with *I. ricinus*, **D**) mice infested with *I. ricinus*, immunized with OVA and **E**) mice immunized with OVA. Black blocks around subpopulation indicate $-1 \leq Log_2FC \geq 1$ in subpopulation percentage compared to negative control saline injected and un-infested/infected mice with a *P value* < 0.05.

In *B. microti* infected and co-infected/infested mice all three tissues evaluated shared similar trends for T cell subpopulations. With regards to upregulated populations, the CD195+CD184+ (Th1 and Th2) T cells were highest in the blood followed by the lymph node. The CD184+ cells were highest in the spleen. In blood all of the latter two populations were high, as well as the CD195+ (Th1) cells. With regards to downregulation in these two experimental groups, T helper lymphocytes (CD3+CD4+ as well as CD3+CD45+) and T regulatory lymphocytes (CD3+CD4+CD25+) were both down-regulated.

As detected before, mice challenged with only ticks did not show any significant response in the lymph node and spleen. Only two populations (CD3+CD4+ and CD45+CD3+) were

downregulated in blood during tick infestation. The lack of any tick-induced effects on Treg cells (CD25+) is striking and provide further evidence that tick immune suppression hinder activation of Th1 and Th2 which then does necessitate activation of Treg cells.

T lymphocyte subpopulations on day 38

Similar to B lymphocyte subpopulations, the majority of significantly differentially expressed T cell subpopulations occurred in the blood (Figure 3.1.7). Firstly, in contrast to previous trends observed, CD3+CD4+ and CD45+CD3+ T cell populations were upregulated in both *Babesia* infected vaccinated mice and mice only challenged with ticks. This response was not observed for any of the other groups.

In mice challenged with ticks only, an additional upregulation of CD195+ Th1 cells were observed, which was not seen in any of the other experimental groups. This response needs to be further evaluated as it is not accompanied by the increase in Th1 activating cytokines, such as IL-2, TNF-α or IFN-γ on either day 16 or 38 (Figure 3.1.4). Tick-challenged mice also differed in regards to their CD195+CD184+ T cells (Th1 and Th2) which were upregulated, and not downregulated as in other Groups (A, B and D). In all groups, excluding for the mice only vaccinated with OVA, without any pathogen/parasite challenge, there was an increase in the CD184+ Th2 cells.

3.2. Assessment of the immune response of BALB/c mice following immunization with the Bm86 and TC-X antigen combination formulated in two adjuvants

3.2.1. Serum Bm86 and TC-X specific IgG1 and IgG2a antibody titres

IgG1 and IgG2a antibody titers specific for Bm86 and TC-X antigens were determined using ELISA (Figure 3.2.1).

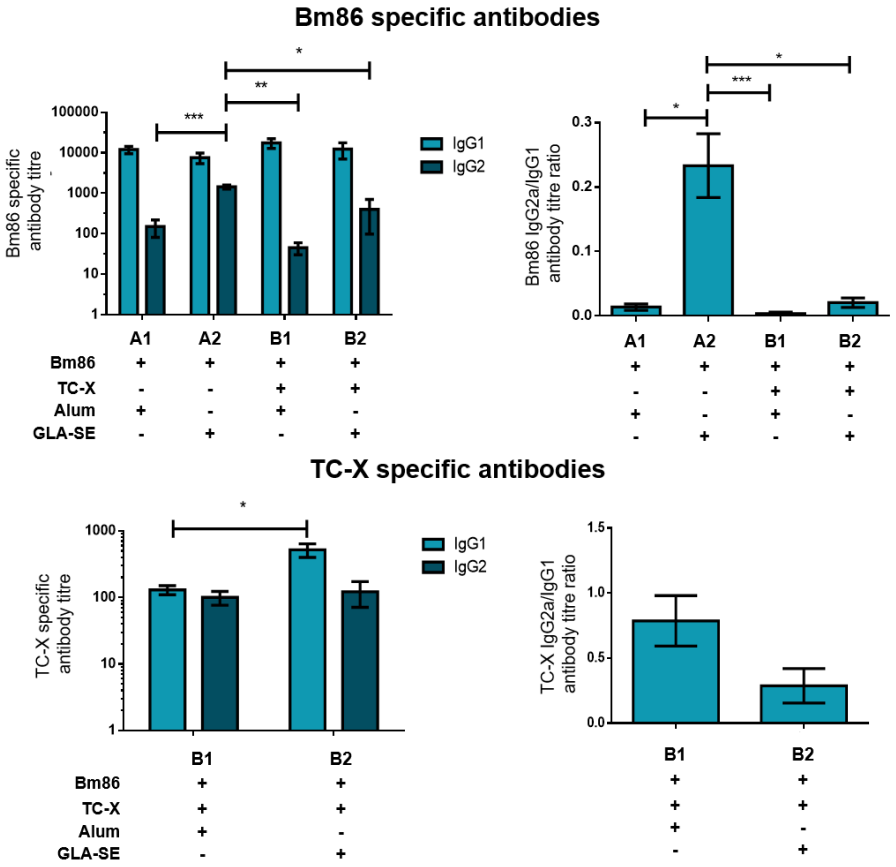

Figure 3.2.1: Antigen specific Bm86 and TC-X antibody response of BALB/c mice 30 days after primary immunization.
IgG1 and IgG2a antibody levels specific to Bm86 and TC-X as determined by ELISA are shown. The ratio of IgG2a/IgG1 antibody titers specific to Bm86 and TC-X is shown next to antigen-specific titer graph. Mice were immunized with formulations containing: A1) Bm86 and Alum, A2) Bm86 and GLA-SE, B1) Bm86, TC-X and Alum; and B2) Bm86, TC-X and GLA-SE *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$

Consistently high IgG1 antibody titers against Bm86 was observed between all experimental groups, with no significant differences observed between groups (Figure 3.2.1.). However for the anti-Bm86 IgG2 titers, mice immunized with Bm86 formulated with GLA-SE had the

highest serum IgG2a titer specific for Bm86 (1440,00 ± 143,11 titre), significantly higher than mice immunized with Bm86 + Alum (150 ± 61,64408 titre, $P \leq 0.001$), as well as mice immunized with Bm86 + TC-X + Alum (45,00 ± 13,04 titre, $P \leq 0.01$) or Bm86 + TC-X + GLA-SE (400,00 ± 269,44 titre, $P \leq 0.05$). Overall, GLA-SE induced elevated IgG2 titers compared to Alum in all experimental groups.

TC-X specific IgG1 antibody titers were significantly higher for mice immunized with Bm86 + TC-X + GLA-SE (520 ± 119,99 titer) compared to mice immunized with Bm86 + TC-X + Alum (130 ± 19,99 titer, $P \leq 0.05$) (Figure 3.2.1.). There were no significant differences observed for the TC-X specific IgG2a tires.

Further data analysis included calculating the ratio of Bm86 specific IgG2a to IgG1 (Figure 3.2.1) to evaluate the response as biased towards a Th1 or Th2 immune response. Mice immunized with Bm86 + GLA-SE had the highest IgG2a/IgG1 ratio (0,233 ± 0,049) pointing towards a slight Th1 biased response as reflected in the higher production of IgG2 under this condition. This was significantly higher compared to values obtained for mice immunized with Bm86 formulated with Alum (0,014 ± 0,005, $P \leq 0.05$), mice immunized with Bm86 and TC-X formulated with Alum (0,004 ± 0,002, $P \leq 0.001$) and mice immunized with Bm86 and TC-X formulated with GLA-SE (0,021 ± 0,01, $P \leq 0.05$) (Figure 3.2.1.). There were no significant differences in the TC-X specific IgG2a/IgG1 titer ratio (Figure 3.2.1.). No Bm86 or TC-X antigen specific IgG1 or IgG2a antibody tires were detected for mice vaccinated with PBS formulated with Alum or GLA-SE and therefore cross-reactivity of serum antibodies with the antigens can be excluded. This background signal was also deducted from all test samples prior to plotting of the data (see chapter 2).

3.2.2. Ex vivo cytokine concentrations 24 hours after culturing

Ex vivo cytokine concentrations were determined from lymph node or spleen cells cultured in the presence of antigen(s) stimulation. Overall, lymph node cultures had higher cytokine concentrations compared to spleen cultures.

Th1 cytokine concentrations of cultured spleen and lymph nodes

The presence of a Th1 immune response was evaluated by measuring the concentration of TNF-α, IFN-γ and IL-2 from spleen and lymph node supernatant after 24 hours of culturing in the presence of antigen stimulation (Figure 3.2.2).

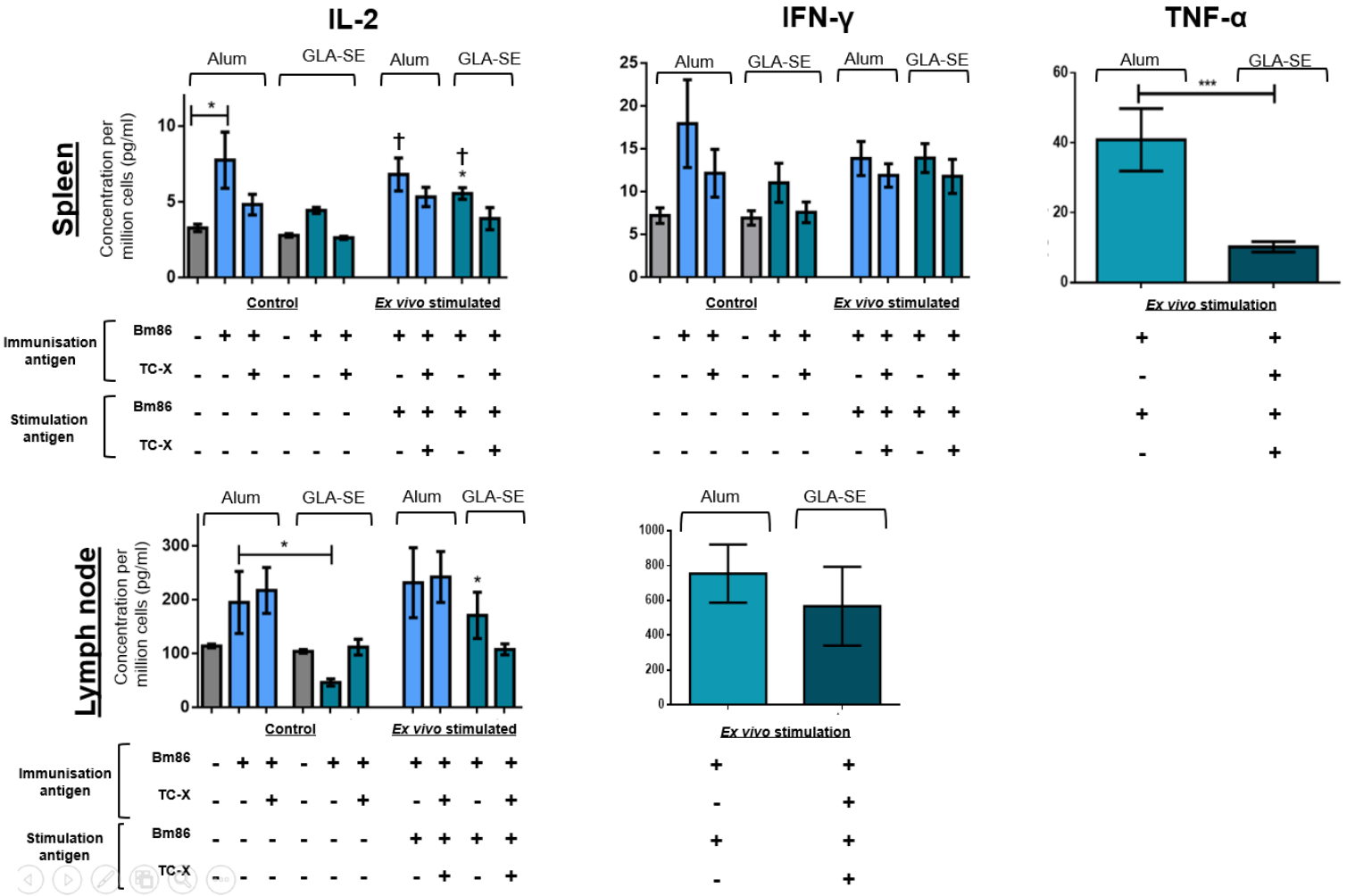

Figure 3.2.2: Culture supernatant Th1 associated cytokine concentrations after 24 hours.

Spleen cytokine concentrations for IL-2, IFN-γ and TNF-α and lymph node cytokine concentrations for IL-2 and IFN-γ. Grey bars indicate cytokine concentrations of control mice (immunized with only Alum/ GLA-SE), with no *ex vivo* stimulation, light blue - and dark blue bars indicate spleen and lymph node cultures of mice immunized with formulations containing either Alum or GLA-SE as adjuvants, respectively. First set of bars in graphs represent cultures that were not stimulated *ex vivo* with antigen(s), second set of bars are duplicate cultures that was stimulated *ex vivo* with antigen(s). Asterix (*) above bars indicate significance difference between the control and antigen(s) stimulated cultures (duplicates) from the same mouse. Cross (†) indicates significant difference between *ex vivo* antigen(s) stimulated cultures and control cultures of mice only immunized with adjuvants. Mice immunized with formulations containing: A1) Bm86 and Alum, A2) Bm86 and GLA-SE, B1) Bm86, TC-X and Alum and B2) Bm86, TC-X and GLA-SE *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

In the control spleen cells, the IL-2 concentration from mice immunized with Bm86 + Alum (7,78 ± 1,86 pg/ml) was significantly higher than that of the control group that received only Alum (3,30 ± 0,25 pg/ml, $P \leq 0.05$). This effect is regarded as significant, as the cytokine levels were calculated as per million cells. Upon comparing the control values for all mice vaccinated with Alum versus GLA-SE, a trend is seen where GLA-SE resulted in lower overall IL-2 levels (although not statistically significant). This trend is also seen in cells derived from the lymph nodes of these mice.

After *ex vivo* antigen stimulation, no significant differences in the IL-2 levels across all groups were detected. When comparing *ex vivo* stimulated versus non-stimulated groups, the IL-2 concentrations were significantly higher in two groups, namely in cultures of mice immunized with Bm86 formulated with either Alum (6,83 ± 1,09 pg/ml) or GLA-SE (5,62 ± 0,38 pg/ml).

In the cells derived from lymph nodes the trend of groups vaccinated with antigens formulated in Alum displaying a higher level of IL-2 compared to antigens formulated in GLA-SE was again evident. There was a significant difference between control cultures, with mice immunized with Bm86 + Alum (195,4 ± 57,7 pg/ml) having a significantly higher concentration of IL-2 than cultures of mice immunized with Bm86 + GLA-SE (46,865 ± 6,789 pg/ml, $P \leq 0.05$). In cells stimulated with antigen, the IL-2 concentrations were not significantly different between cultures (Figure 3.2.2 B). Upon comparing non-stimulated to stimulated cells, there was only one statistically relevant fining between the control and antigen stimulated cultures of mice immunized with Bm86 + GLA-SE. These cells showed a higher IL-2 concentration in *ex vivo* stimulated with Bm86 (171,46 ± 43,0 pg/ml) compared to cultures not stimulated (46,87 ± 6,8 pg/ml, $P \leq 0.05$). This is similar to the findings in the spleen, which also showed an increase in spleen IL-2 following stimulation with Bm86.

Cultured spleen IFN-y concentrations did not show a significant difference between control and antigen(s) stimulated cultures or experimental mice but did show a similar trend between cultures of mice vaccinated with the same antigen(s) but different adjuvants. In all cases the responses were: Bm86 + adjuvant > Bm86 + TC-X + adjuvant > only adjuvant. As such, it appeared that the inclusion of TC-X may cause a reduction in the Bm86 elicited IFN-γ responses. Cultured lymph node IFN-y concentrations (Figure 3.2.2) and cultured spleen TNF-α concentrations were only detected in cultures of mice immunized with Bm86 formulated with either Alum or GLA-SE (*ex vivo* Bm86 stimulation) with no significant difference in IFN-y between the two cultures. However, TNF-α concentration was significantly higher in spleen cultures of mice immunized with Bm86 formulated with Alum (40,820 ± 8,967 pg/ml) than GLA-SE (10,191 ± 1,491 pg/ml) ($P \leq 0.001$) as an adjuvant. After culturing and stimulating lymph

node cells, we were unable to detect any signal for TNF-α as the values obtained were in the range of the control values.

Th2 and Th17 cytokines concentrations of cultured spleen and lymph nodes

In this study an initial assessment of the Th2 and Th17 immune responses were analyzed by means of the concentrations of IL-4, IL-10 (Th2) and IL-17 (Th17) from spleen and lymph node supernatants after culturing and *ex vivo* stimulation (Figure 3.2.3).

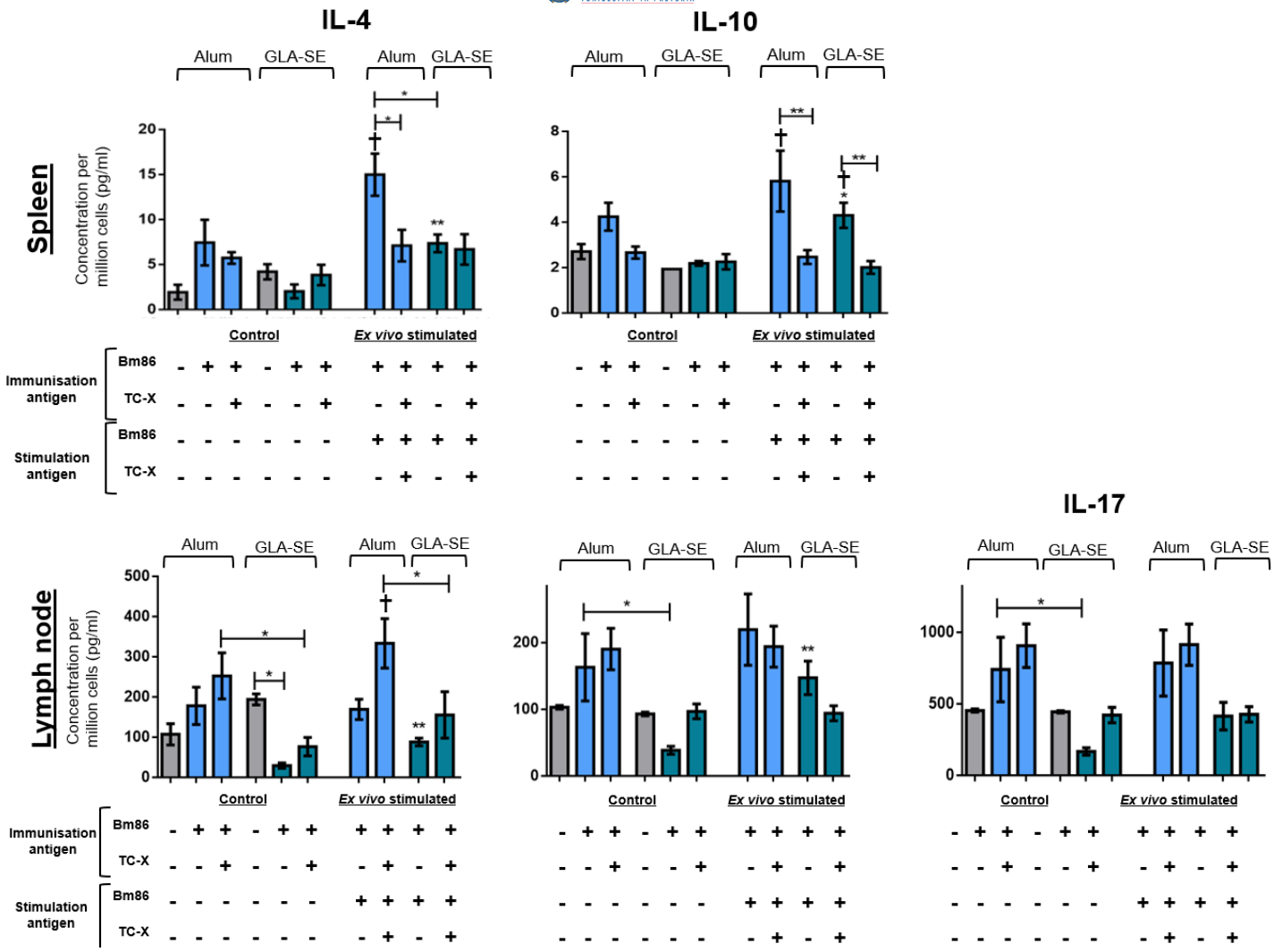

Figure 3.2.3 Culture supernatant Th2 and Th17 associated cytokine concentrations after 24 hours.
Grey bars indicate cytokine concentrations of control mice (immunized with only Alum/ GLA-SE) with no *ex vivo* stimulation, light blue and dark blue bars indicate spleen and lymph node cultures of mice immunized with formulations containing either Alum or GLA-SE as adjuvants, respectively. The first set of bars in each graph represent cultures that were not stimulated *ex vivo* with antigen(s), second set of bars are duplicate cultures that was stimulated *ex vivo* with antigen(s). Asterix (*) above bars indicate significance difference between the control and antigen(s) stimulated cultures (duplicates) from the same mouse. Cross (†) indicates significant difference between *ex vivo* antigen(s) stimulated cultures and control cultures of mice only immunized with adjuvants *$P \leq 0.05$, **$P < 0.01$, ***$P \leq 0.001$.

IL-4 concentrations in the control cultured spleen cells were not significantly different between all groups evaluated. In contrast, cultures stimulated with antigen(s) showed significant differences between the different experimental groups. Firstly, mice immunized with Bm86 + Alum + *ex vivo* Bm86 stimulation (14,05 ± 2,35 pg/ml) had a significantly higher IL-4 concentration compared to cultures from mice immunized with Bm86 + TC-X + Alum + *ex vivo* Bm86 (6,18 ± 1,75 pg/ml, $P \leq 0.05$) and TC-X stimulation (7,0 ± 0,99 pg/ml, $P \leq 0.05$) as well as mice immunized with Bm86 + GLA-SE + *ex vivo* Bm86 stimulation (1,96 ± 0,83 pg/ml, $P \leq 0.001$). Again, as described previously, the inclusion of TC-X with Bm86 into a formulation seem to reduce the Bm86 elicited response.

When analyzing the findings from the lymph nodes for IL-4 statistical variation occurred between control and antigen(s) stimulated cultures. In the control cultures, two values were significant. The first pertains to the mice immunized with Bm86 + TC-X + Alum (253,3 ± 57,18 pg/ml) that had a significantly higher IL-4 concentration compared to control cultures from mice immunized with Bm86 + TC-X + GLA-SE (77,27 ± 22,78 pg/ml, $P \leq 0.05$). Secondly, cultures of mice only immunized with GLA-SE (194,77 ± 13,70 pg/ml) also had a significantly higher IL-4 concentration compared to control cultures of mice immunized with Bm86 + GLA-SE (30,30 ± 6,62 pg/ml, $P \leq 0.05$).

In the *ex vivo* antigen(s) stimulated cultures, two values were noteworthy. Firstly, mice immunized with Bm86 +TC-X + Alum + *ex vivo* Bm86 and TC-X stimulation) had significantly higher IL-4 concentration (334,23 ± 61,52 pg/ml), compared to cultures from mice immunized with Bm86 + TC-X + GLA-SE + *ex vivo* Bm86 and TC-X stimulation (156,25 ±57,61 pg/ml, $P \leq 0.05$). This group also has a significant higher IL-4 concentration compared to control cultures from mice immunised with only Alum (1,96 ± 0,83 pg/ml, $P \leq 0.001$). Secondly, between control and antigen(s) stimulated cultures of mice immunised with Bm86 + GLA-SE + *ex vivo* Bm86 stimulation the stimulated cells showed a significantly higher IL-4 concentration (89,08 ± 8,63 pg/ml) compared to the control culture (30,30 ± 6,62 pg/ml, $P \leq 0.01$). This is similar to the IL-4 concentrations observed in the spleen.

Cultured spleen cells did not show any significant differences between control cultures with regards to IL-10 concentrations. Significant differences were however detected between the antigen(s) stimulated cultures. Cultures of mice immunized with Bm86 + Alum + *ex vivo* Bm86 stimulation (5,83 ± 1,34 pg/ml) was significantly higher compared to spleen control cultures from mice immunized with only Alum (2,72 ± 0,33 pg/ml, , $P \leq 0.01$) and cultures from mice immunized with Bm86 + TC-X + Alum + *ex vivo* Bm86 and TC-X stimulation (2,48 ± 0,30 pg/ml, $P \leq 0.01$). Similar results were also seen in antigen(s) stimulated cultures of mice immunized

with the GLA-SE as adjuvant. In this case, cultures of mice immunized with Bm86 + GLA-SE + *ex vivo* Bm86 stimulation had significantly higher IL-10 concentrations (4,32 ± 0,55 pg/ml) than mice immunized with Bm86 + TC-X formulated with GLA-SE + *ex vivo* Bm86 and TC-X stimulation (2,02 ± 0,28 pg/ml, $P \leq 0.01$), cultures of mice only immunized with GLA-SE (1,95 ± 0 pg/ml, $P \leq 0.05$) and duplicate cultures from the same mouse not stimulated with Bm86 (2,20 ± 0,10 pg/ml, $P \leq 0.05$). This was yet another example of where inclusion of TC-X appears to reduce the response elicited by Bm86 alone.

The trend that the response elicited by Alum is greater than that observed for GLA-SE is also evident in the lymph node IL-10 concentrations. Again, mice immunized with Bm86 + Alum (163,58 ± 50,45 pg/ml) showed a significantly higher concentration of IL-10 compared to cultures of mice immunized with Bm86 + GLA-SE (39,15 ± 6,04 pg/ml, $P \leq 0.05$). There were also significant differences between the control and antigen stimulated lymph node cultures from the same mouse, mice immunized with Bm86 + GLA-SE + *ex vivo* Bm86 stimulation (147,80 ± 25,05 pg/ml). All of the latter had a significantly higher IL-10 concentration compared to the culture that was not stimulated with Bm86 (39,15 ± 6,04 pg/ml, $P \leq 0.01$).

With regards to IL-17, levels of this cytokine in spleen cultures were below the detection limit and thus not included. Lymph node cultures had the highest IL-17 concentrations of all cytokines measured during the study, but only one significant difference was observed for control cultures where cultures from mice immunized with Bm86 + Alum (742,71 ± 226,0 pg/ml) displayed a significantly higher IL-17 concentration compared to mice immunized with Bm86 + GLA-SE (169,77 ± 25,60 pg/ml, $P \leq 0.05$). This again supported the finding that the response against Bm86 immunization is higher than that observed with Bm86 formulated with GLA-SE.

3.2.3. Lymphocytes populations present in ex vivo cultures after 48 hours

Lymphocyte subpopulations were analyzed by means of multi-color flow cytometry on spleen and lymph node cells cultured for 48 hours in the presence of antigen(s) (Figure 3.2.4). As with cytokine concentration determination, the control lymphocyte population percentages were compared to the stimulated cultures. Leucocyte population percentages were determined for CD80+ subpopulations (corresponding to mature B cells), CD3+CD4+ subpopulations (corresponding to T helper cells) and CD3+CD4+CD25+ subpopulations (corresponding to T regulatory cells).

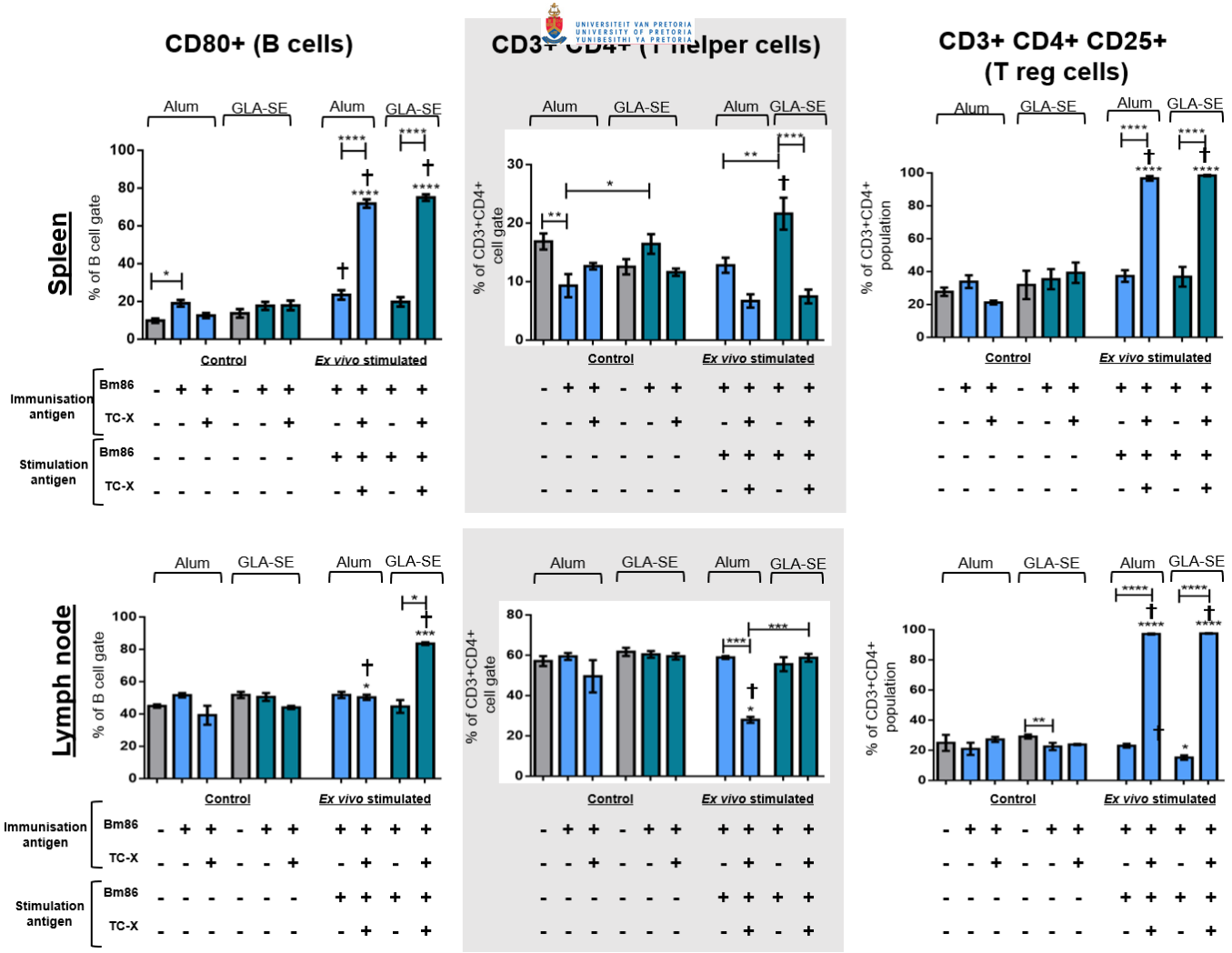

Figure 3.2.4: Lymphocyte subpopulations percentages in cultured spleen and lymph node tissue after 48 hours.

Grey bars indicate cytokine concentrations of control mice (immunized with only Alum / GLA-SE) with no *ex vivo* stimulation, light blue and dark blue bars indicate spleen and lymph node cultures of mice immunized with formulations containing either Alum or GLA-SE as adjuvant, respectively. The first set of bars in each graph represent cultures that were not stimulated *ex vivo* with antigen(s). The second set of bars are duplicate cultures that was stimulated *ex vivo* with antigen(s). Asterix (*) above bars indicate significance difference between the control and antigen(s) stimulated cultures (duplicates) from the same mouse. Cross (†) indicates significant difference between *ex vivo* antigen(s) stimulated cultures and control cultures of mice only immunized with adjuvants *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

Mature B lymphocytes population percentages

In the spleen the most significant finding was that mature B cell populations percentages were significantly higher for mice immunized with Bm86 + TC-X + *ex vivo* Bm86 and TC-X stimulation formulated with either Alum (72.0 ± 2,19) or GLA-SE (75,18 ± 1,73), compared to control and antigen(s) stimulated cultures. As such, B cell maturation did occur in mice vaccinated with the combination vaccine, independent of the adjuvant used.

Lymph node cultures had a higher CD80+ percentage than spleen cultures, except for cultures of mice immunized with Bm86 + TC-X + GLA-SE + *ex vivo* Bm86 and TC-X stimulation which was significantly increased (83,71 ± 0,82) compared to the controls and other *ex vivo* stimulated groups.

T helper lymphocyte population percentages

Un-stimulated spleen cell cultures showed variable CD3+CD4+ populations between mice immunized with Bm86 + Alum (9,36 ± 1,97) having lower CD3+CD4+ cell levels relative to control cultures from mice immunized with only Alum (16,91 ± 1,39, $P \leq 0.01$). A similar case is observed between mice immunized with Bm86 + alum and mice immunized with Bm86 + GLA-SE (16,50 ± 1,67, $P \leq 0.05$). Of the antigen(s) stimulated cultures, mice immunized with Bm86 + GLA-SE + *ex vivo* Bm86 stimulation (21,66 ± 2,73) had significantly higher CD3+CD4+ population percentages than all of the other stimulated groups. This points towards GLA-SE indicting a strong T helper response in the spleen. However, this is not reflected in the lymph node.

In the lymph node control cultures CD3+CD4+ population percentages stayed constant between experimental cultures after 48 hours, similar to the CD80+ subpopulations with the exception that population percentages were higher. Antigen(s) stimulated cultures showed variation with cultures from mice immunized with Bm86 + Alum + (59,05 ± 0,75) and cultures from mice immunized with Bm86 +TC-X + GLA-SE + *ex vivo* Bm86 and TC-X stimulation (58,92 ± 1,93), having significantly higher CD3+CD4+ population percentage than cultures from mice immunized with Bm86 +TC-X + Alum + *ex vivo* Bm86 and TC-X stimulation (28,10 ± 1,50 ,$P \leq$ 0.001). The latter culture also had significantly lower CD3+CD4+ population percentages than lymph node cultures from the same mouse cultured as a control (49,70 ± 7,60, $P \leq 0.05$) as well as mice immunized with only Alum (57,24 ± 2,23, $P \leq 0.0001$). As such it can be deduced that the combination immunization of Bm86 and TC-X with Alum resulted in a suppression of T helper cells in the lymph nodes. Future studies on the percentages of these

cells circulating in blood (via cannulation) would be valuable to track the maturation of T helper cells from spleen to lymph node to blood.

T regulatory lymphocyte population percentages

Spleen culture CD3+CD4+CD25+ population percentages showed no variation between control cultures. However, in the antigen(s) stimulated cultures two significant observations was made. Firstly, both mice immunized with Bm86 +TC-X + Alum + *ex vivo* Bm86 and TC-X stimulation (96,87 ± 1,45) or GLA-SE (98,59 ± 0,46) had significantly higher CD3+CD4+ population percentages than the rest of the evaluated groups. As such, it can be concluded that the combination vaccine, formulated in either Alum or GLA-SE, was successful in activation of T regulatory cells.

In the lymph nodes, the levels of CD3+CD4+CD25+ T regulatory populations were consistently lower between control cultures (similar to spleen control cultures), with the exception of cultures from mice immunized with only GLA-SE (29,33 ± 1,35) having a significantly higher population percentage than control cultures. Significant differences were observed in antigen(s) stimulated cultures (similar to spleen CD3+CD4+CD25+ and CD80+ population percentages), where both cultures of mice immunized with Bm86 + TC-X + Alum + *ex vivo* Bm86 and TC-X stimulation (97,35 ± 0,38) or GLA-SE (97,7 ± 0,27) had a significantly higher CD3+CD4+CD25+ subpopulation percentage than the other groups evaluated. As such, we propose that the combination vaccine, formulated in either Alum or GLA-SE, was successful in activation of T regulatory cells. Only one significant decrease in T regulatory cells were detected for mice immunized with Bm86 + GLA-SE + *ex vivo* Bm86 stimulation (15,36 ± 1,49) compared to mice immunized with the same formulation with lymph nodes cultured in control conditions (23,84 ± 0,39, $P \leq 0.05$) and mice only immunized with GLA-SE (29,33 ±1,35, $P \leq 0.01$) (indicated by cross).

Chapter 4: Discussion:

Although a considerable amount of research has been focussed on the immune response in murine models when infested with *I. ricinus* nymphs or infected with *B. microti* (section 1.3), the combined immunological effect of co-infection/infestation remains undescribed. It is therefore suggested that both the parasite and pathogen interfaces (i.e. their interactions with the host, as well as each other) needs to be taken into consideration when designing a tick vaccine, as vaccine efficacy may be compromised when immunising co-infected/infested hosts (Rego *et al.*, 2019). As such the first general aim of this study was to: (a) gather pilot data and set a baseline for future investigations into the immune response during co-infection/infestation with both *B. microti* and *I. ricinus* ticks in a murine model (section 4.1.1); and (b) determine if co-infestation alters antigen-specific immune responses in an immunised murine model (section 4.1.2).

In addition to the above-mentioned aims, this study also had a second aim which focussed on immunological profiling of BALB/c mice that were immunised with two protective antigens (against the cattle tick *R. microplus*) formulated in two different adjuvants. This part of the study was conducted to gain insight into: (a) the immunological consequences of vaccination with the Bm86 and/or TC-X antigen(s) in a murine model; and (b) determine the effect of the adjuvants on Th1 and Th2 responses in terms of modulation of lymphocyte subpopulations, as well as antibody and cytokine production (section 4.2).

4.1. Assessment of the Immune response in BALB/c mice co-infected with *Babesia microti* and *Ixodes ricinus* and its implications for the development of tick vaccines.

4.2. *Day 16: Infection/ infestation with B. microti or I. ricinus alone versus co-infection/infestation*

The *in vivo* immune responses elicited in the spleen, sub-iliac lymph nodes and serum of BALB/c mice that were terminated on day 16 are summarized in Figure 4.1.1.

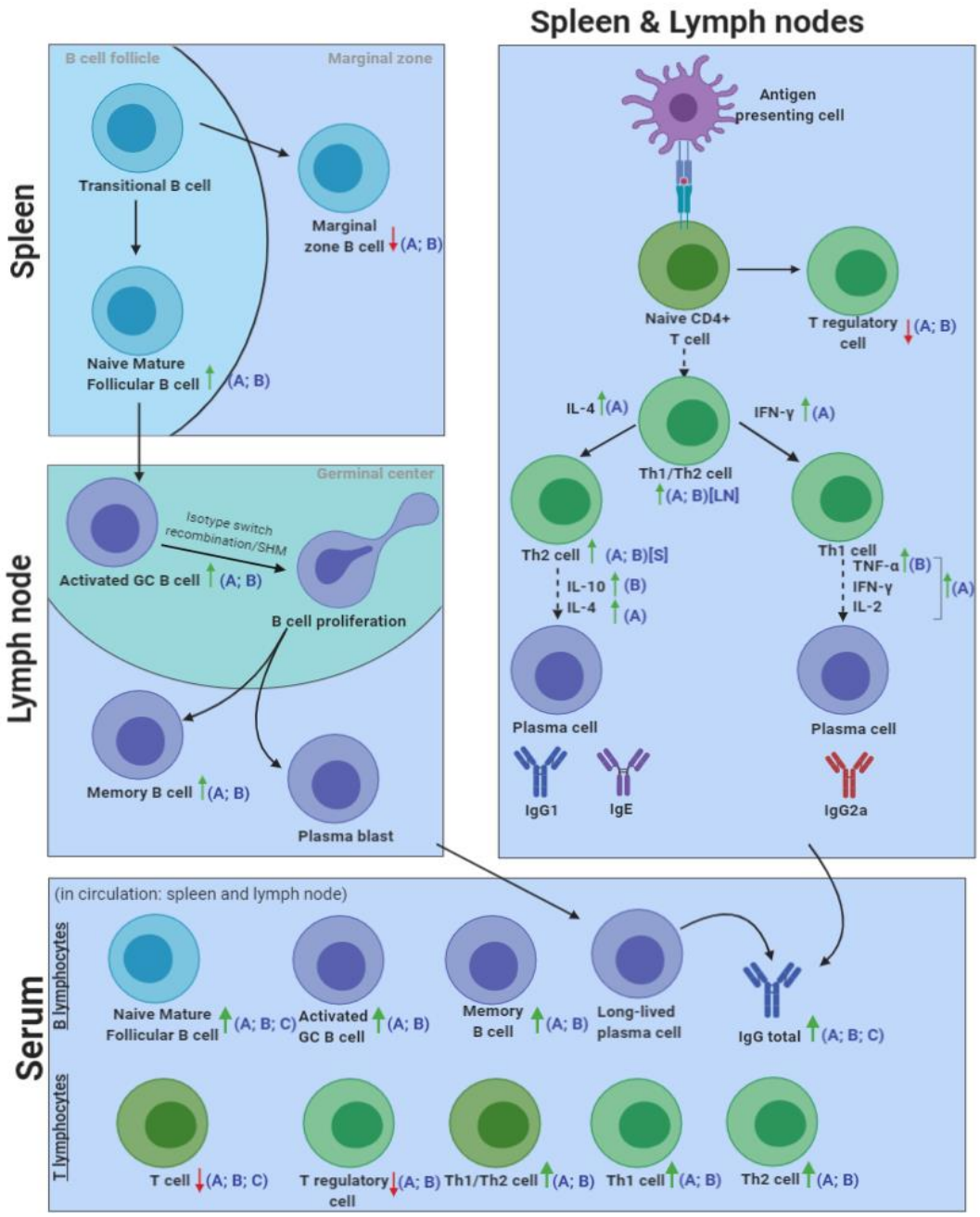

Figure 4.1.1: Evolution of the immune response in mice terminated on day 16

Immune response development of: **[A]** mice infected with *B. microti* on day 1; **[B]** mice co-infected/infested with *B. microti* (day 1) and *I. ricinus* (day 2); and **[C]** mice infested with *I. ricinus* on day 2. *In vivo* spleen, lymph node and serum samples were used to determine differential regulation of lymphocyte subpopulations associated with B- and T cell development and maturation. Lymph nodes were used to determine cytokine production and blood serum was used to determine total IgG concentration. Green arrows indicate significant up-regulation compared to control groups and red arrows indicate significant down-regulation compared to control groups. Abbreviations correspond to Spleen (S), Lymph nodes (LN), Somatic hypermutation (SHM) and Germinal centre (GC).

Similar immune responses were observed between mice infected with *B. microti* parasites alone and co-infected/infested with both *B. microti* and *I. ricinus* nymphs (Figure 4.1.1). In all of the results for day 16 isolates, near complete immune suppression of the responses by *I. ricinus* was evident. Therefore, an apparent polarisation of the co-infected/infested immune responses is observed that resembles that of a *B. microti* mono-infection. The immune suppressive effects of *I. ricinus* ticks, as well as some other tick species such as *R. microplus* on their hosts have been described to date (section 1.1.2). These immune suppressive effects of ticks are mediated by the various bioactive compounds that are secreted from the tick salivary glands and injected into the feeding pool during infestation. Examples of such bioactive proteins found in *I. ricinus* salivary glands include Kunitz inhibitors, serpins and cystatins that inhibit haemostatics while compounds such as Salp15, lipocalins and cystatins have *in vivo* immune suppressive effects (reviewed by Chmelař *et al.* (2017); Table 1.1 and Figure 1.2). In both *I. ricinus* (Kotál *et al.*, 2015) and in *R. microplus* (Robbertse, 2018), it has been shown that tick feeding supresses both B- and T cell maturation in the lymph nodes, similar to findings in our study. Future studies on the effect that vaccination has on hosts with high tick loads remain to be conducted to fully comprehend the effect of tick-mediated immune suppression on the development of a protective response.

Lymphocyte subpopulation regulation

Mice infected with *B. microti* or those co-infested/infected with *I. ricinus* and *B. microti* showed up-regulation of the follicular B cells (CD45+CD19+IgM^{low}) and the down-regulation of marginal zone B cells (CD45+CD19+IgM^{high} CD21+) in the spleen (Figures 3.1.6 and 4.1). In secondary lymphoid organs, marginal zone B cells differentiate into short lived plasma cells that secrete low affinity IgM antibodies (Martin *et al.*, 2001) that are associated with innate immune responses to blood-borne pathogens (Cerutti *et al.*, 2013). In contrast, follicular B cells are naïve precursors cells that start off within the germinal centres (De Silva *et al.*, 2015), and, when activated by antigens, differentiate in the secondary lymphoid tissue resulting in increased antibody affinity (Mesin *et al.*, 2016). The up-regulation of splenic follicular B cell populations appears to coincide with an up-regulation of activated germinal centre B cells (CD45+CD19+IgM+CD27+CD80+) in the lymph nodes (Figures 3.1.6 and 4.1). Germinal centre B cells, in turn differentiate into memory B cells (CD45+CD19+IgM+ CD27+CD80+CD21+) and plasma cells that maintain and increase serum antibody affinities (IgG, IgA and IgE) during both the primary immune response and during re-infection (Mesin *et al.*, 2016). The up-regulation of germinal centre associated lymphocyte subpopulations is possibly associated with an increased production of serum total IgG antibodies in co-infected/infested mice and mice only infected with *B. microti* (Figures 3.1.1 and 4.1 section

labelled as serum).

A previous study by Yi *et al.* (2018) has reported on lymphocyte development in the germinal centres of C57BL/6J mice spleen and lymph node tissues during *B. microti* infection. Their results indicated that the specific antibodies produced during infection were mostly directed against those antigens that were exposed during the extracellular life stages of *Babesia* infection. Thus, from the pattern of differentially regulated lymphocyte subpopulations in the secondary lymphoid organs of *B. microti* infected mice, as well as the co-infected/infested group, the results point towards the initiation and maturation of the adaptive immune response with the subsequent development of memory cells against *B. microti* infection. This fact is exemplified by the ability of BALB/c mice to acquire resistance to *B. microti* infection and resolve infections in some 20 days (Clawson *et al.*, 2002).

Considering the T lymphocyte populations of spleen and lymph node tissues in *B. microti* infected and co-infected/infested mice, similar developmental patterns were observed (Figures 3.1.7 and Figure 4.1 sections on spleen and lymph node). Both of the latter experimental groups had a down-regulation of T regulatory lymphocytes (CD3+CD4+CD25+) involved in regulating and modulating the host immune response thereby maintaining homeostasis and preventing inflammation (Smigiel *et al.*, 2014). Treg cells have the ability to inhibit Th1 and Th2 responses through the secretion of IL-10 (Biedermann *et al.*, 2004; Sundstedt *et al.*, 2003). Thus, the down-regulation of Treg lymphocytes observed in this study could possibly be supported by the up-regulation of the Th1/Th2 positive lymphocytes (CD3+CD45+CD195+CD184+) in the lymph nodes and the Th2 (CD3+CD45+CD184+) lymphocytes in the spleen and serum.

The differentially regulated lymphocyte subpopulations detected in spleen and lymph node tissues were also found to circulate in the blood (i.e serum)(Figure 4.1.1 section on serum). Once lymphocyte populations have matured and differentiated in the secondary lymphoid tissues, these populations are released into the blood from where they circulate to the various tissues and organs (Marti *et al.*, 2017). Future studies on following the trafficking of lymphocytes through the various secondary lymphoid organs, possibly via cannulation, would be beneficial in supporting this hypothesis.

Cytokine production

Mice infected with only *B. microti* had an up-regulation of cytokines associated with both Th1 (IFN-γ, TNF-α and IL-2) and Th2 (IL-4) immune responses. These results were similar to

previous infection studies performed by Djokic *et al.* (2018), where *B. microti* infection resulted in a mostly Th1 polarised immune response (section 1.3), typical of the response elicited towards intracellular parasites. The up-regulation of IL-4 during *B. microti* infections has also been observed in previous studies (Jeong *et al.*, 2012). These results also indicate an ongoing active *B. microti* infection where both Th1 and Th2 responses are elicited towards the *Babesia* antigens exposed during the extracellular and intracellular life stages of the pathogen, respectively. This has also been shown for *Plasmodium* spp., apicomplexan parasites, that also have extracellular and intracellular stages in their life cycles (Belachew, 2018; Schuster, 2002).

In contrast, during co-infection/infestation the up-regulation of both TNF-α (Th1) and IL-10 (Th2) cytokines were observed pointing towards the immune response not being polarised towards either a Th1 or Th2 mediated response. Tumour necrosis factor α is generally associated with a homeostatic role, limiting the extent of an inflammatory response (Marino *et al.*, 1997). However, in the field of tick mediated immunity, TNF-α transcripts have been shown to be highly up-regulated in peripheral blood mononuclear cells of tick resistant bovine hosts (Piper *et al.*, 2009) and in the skin tissue of mice following repeated infestation with *I. ricinus* (Mbow *et al.*, 1994). Tumour necrosis factor α has also been shown to be up-regulated in ConA and anti-CD3 antibody-stimulated lymph node cell cultures derived from BALB/c mice infested with *I. ricinus* nymph (Ganapamo *et al.*, 1996a). Also, in the initial stages of *B. microti* infection, TNF-α has been found to be up-regulated (Hemmer, 2000). Therefore, the elevated levels of TNF-α during co-infection/infestation is expected. What should be noted however, is that TNF-α levels remain low in BALB/c mice that are infested with only *I. ricinus*. Therefore, it is once again proposed that the main driver of TNF-α expression in the co-infected/infested experimental group is the *Babesia* parasite.

Interleukin 10 has both pro-inflammatory and anti-inflammatory properties to limit host immune responses to pathogens and prevent damage to the host, as well as maintaining normal tissue homeostasis (Iyer *et al.*, 2012). In the field of tick mediated immunity, IL-10 transcripts have been shown to be up-regulated in susceptible bovine hosts (Wang *et al.*, 2007). For *Babesia* infections, up-regulation of IL-10 has been linked to the resolution of *B. microti* infections (Hemmer *et al.*, 2000). Again, the up-regulation of IL-10 during co-infection/infestation is not surprising and it is mostly ascribed to the response elicited by *Babesia* as in *I. ricinus* infested mice, IL-10 concentrations remain low.

Total IgG production

Total IgG antibody concentrations were significantly up-regulated for all experimental groups compared to the negative controls (Figures 3.1.1 and 4.1 section on serum). Mice infected with *B. microti* had the highest concentration of total IgG antibodies detected, which is supported by serodiagnostic studies that have shown *B. microti* infections to result in such an increase in titre of *Babesia*-specific total IgG (Li *et al.*, 2012). This increase in antibody production, however, has a negligible impact on pathogen control and infection. It has been proposed that the antibodies produced during a *Babesia* infection, mainly neutralise free parasites and prevent entry into red blood cells (Li *et al*., 2012). Mice infected with *B. microti* had a 1. 6-fold significant increase ($P \leq 0.01$) of total IgG concentration compared to *B. microti* infected mice co-infested with *I. ricinus* ticks. This again may be attributed to the immune suppressive effects of tick saliva previously mentioned. Furthermore, basal levels of IFN-γ and IL-4 during co-infection/infestation could have contributed to the decrease in IgG total antibody concentration as these cytokines are associated with antibody class switching by B cells to IgG2a (Th1) and IgG1 (Th2) (Stevens *et al.*, 1988) (Figures 3.14., 3.1.5 and 4.1.1). The lowest levels of total IgG were observed for mice infested with *I. ricinus* only. This finding aligns with studies showing that in cattle infested with *R. microplus* ticks, there is a severe suppression of maturation of many lymphocyte lineages in the draining lymph nodes, migration into the germinal centres, lack of B-cell priming and by implication the downstream germinal centre reaction to yield activated B cells that can secrete IgG (Robbertse, 2018). In future, it would be of value to follow the clearance of *Babesia* infection over time in mice infested with ticks, as the ticks decrease the IgG levels (Figure 3.1.1) and the TNF-α levels (Figure 3.1.4) associated with clearance of the *Babesia* infection.

Ixodes ricinus nymph infestation effect on B- and T cell maturation

Mice infested with *I. ricinus* nymphs only had similar cytokine and antibody profiles compared to control mice (Figure 4.1.1 sections on spleen and lymph node and serum) supporting previous observations that ticks are able to severely suppress the immune responses in their vertebrate hosts. However, mice from this experimental group showed differential regulation of B- and T lymphocytes in blood, with an up-regulation of naïve mature follicular B cells, as well as the down-regulation of T cells. Again, just as observed in cattle infested with *R. microplus* (Robbertse, 2018), it is evident that in BALB/c mice there is no significant activation of follicular cells (CD45+CD19+IgM+CD21+), marginal zone B cells (CD45+CD19+IgM+), activated germinal B cells (CD45+CD19+IgM+CD27+CD80+) as well as memory B cells (CD45+CD19+IgM+CD25+CD80+CD21+), in response to tick infestation (Figure 3.6.1.). Results seem to point towards a lack of maturation of the transitional B cells (CD45+CD19+IgM^{low}) (Figure 3.1.6) to follicular B cells that will be discussed further in section

4.1.2. The latter has not been shown before and as such is a novel finding that may open the door of using a murine model to predict responses in large animals. These B cell maturation indicators, may also be used when planning expensive large animal studies, for the selection of immune markers/pathways to be evaluated.

In regards to the suppression of T cell proliferation, several studies have reported the ability of saliva from *Ixodid* ticks (such as *I. ricinus*) to supress the proliferation of this lymphocyte (Titus, 2000). The circulation of differentiated T lymphocytes (Figure 3.1.7), could indicate that the maturation of these populations has already taken place in the secondary lymphoid tissues (as described above). In summary, the suppression of both B- and T cell responses may explain why BALB/c mice are unable to acquire resistance to *I. ricinus* infestation (Christe *et al.*, 1998).

4.2.1. *Day 38: Co-infection/infestation and its effect on antigen-specific immune responses*

The *in vivo* immune responses elicited in BALB/c mice that were terminated on day 38 are summarised in Figure 4.1.2. Tissues used to monitor immune response development included the spleen, sub-iliac lymph nodes and serum.

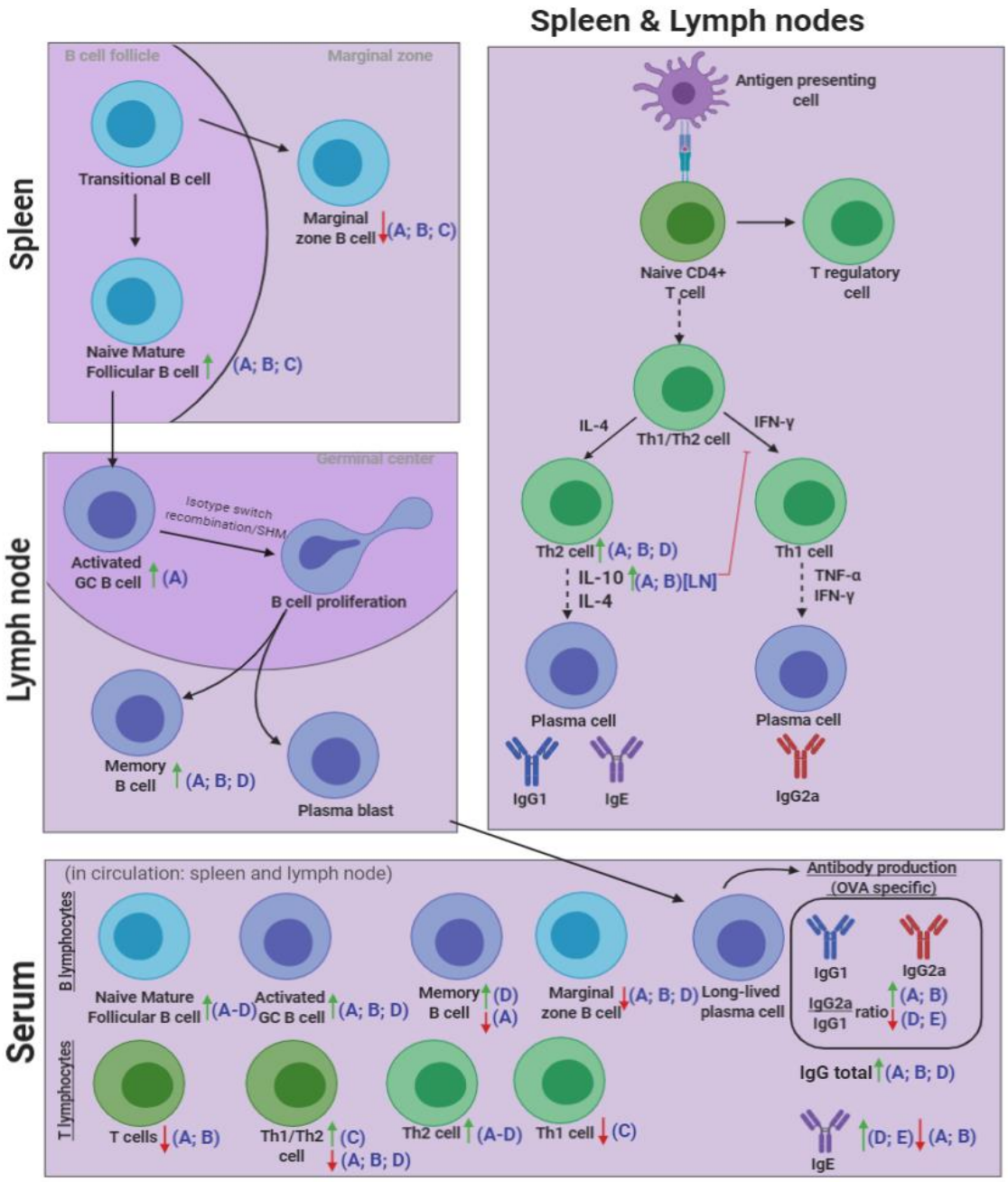

Figure 4.1.2: Immune response development of treated mice terminated on day 38
Immune response development of: **[A]**mice infected with *B. microti* and immunised with Ovalbumin; **[B]** mice co-infected with *B. microti* and *I. ricinus* and immunised; **[C]** mice infested with *I. ricinus*; **[D]** mice infested with *I. ricinus* and immunised with Ovalbumin; and **[E]** mice immunised with Ovalbumin. *In vivo* spleen, lymph nodes and serum were used to determine differential regulation of lymphocyte subpopulations associated with B- and T cell development and maturation. Lymph nodes were used to determine cytokine production and serum was used to determine the total IgG concentration. Green arrows indicate significant up-regulation compared to control groups while red arrows indicate significant down-regulation compared to control groups. Abbreviations corresponds to: Spleen (S), Lymph nodes (LN), Somatic hypermutation (SHM), Germinal centre (GC).

Babesia microti infection versus co-infection/infestation in the presence of immunization

Tissue and blood collected on day 38 from mice infested with *B. microti* alone (Group A) or with co-infection/infestation (Group B) and immunised with OVA, had similar immune responses compared to experimental groups from day 16 (see section 4.1.1 for discussion on day 16). However, on day 38 the majority of the lymphocyte populations were found to be differentially regulated in the blood, rather than in secondary lymphoid tissues (Figures 3.1.6 and 3.1.7 and 4.1.2). This may be indicative of an adaptive immune response maturation that has taken place in the secondary lymphoid tissues and that the lymphocyte subpopulations are now circulating in the blood enabling clearance of the *Babesia* infection.

Lymphocyte subpopulations that were differentially regulated in spleen and blood had similar patterns of regulation to that of *B. microti* mono-infected mice (Group A) analysed on day 16. These observed similarities could be attributed to the limiting effect that immunization with OVA in Freund's adjuvant has on lymphocyte subpopulations (further discussed in section 4.1.3). Group E mice that were immunised with OVA and Freund's adjuvant alone, in the absence of infection/infestation, had no differential regulation of the lymphocyte subpopulations in the secondary lymphoid organs and serum. Thus, the difference in lymphocyte subpopulation regulation and localization between day 16 and 38 is likely attributed to the effects of either *Babesia* or co-infection/infestation. However, as noted above the effects of *I. ricinus* alone were not significant and therefore we again propose that the observed changes are due to the *Babesia* infection. Differences between the two time points (day 16 and 38) include the re-establishment of the basal Treg cell population (CD3+CD4+CD25+) levels on day 38 (Figures 3.1.7 and 4.1.2). This could be attributed to the resolution of the *B. microti* infection and the feeding status of the *I. ricinus* nymphs (i.e. being replete and dropping), causing the host to experience limited immunomodulation from either infection or infestation and returning to a baseline condition.

In regards to cytokine production, IL-10 was up-regulated in both Groups A and B on day 38 (Figures 3.1.5 and 4.1.2). On day 16, IL-10 was only up-regulated in *B. microti* infected mice co-infested with *I. ricinus*. However, the up-regulation of IL-10 in mice infected with *B. microti* on day 38 is a novel finding not described in previous studies. The up-regulation of IL-10 could be linked to normalisation of the Treg cell levels (Chaudhry *et al.*, 2011), which have the ability to secrete IL-10 in an effort to regulate the immune response by supressing a Th1 polarised immune response (Couper *et al.*, 2008).

4.2.2. *Effect of Ixodes ricinus nymph infestations in the presence and absence of immunisation with OVA and Freund's adjuvant*

Lymphocyte populations following I. ricinus nymph infestations of vaccinated and non-vaccinated murine hosts

On day 38 mice infested with *I. ricinus* nymphs and euthanised on day 38 displayed no changes in cytokine and total IgG concentration for Group C and D in the presence or absence of immunization. Moreover, these groups displayed similar trends to mice form the negative control group, as well as mice immunized with OVA formulated in Freund's adjuvant only (i.e. Group E). No changes in cytokine and total IgG parameters were detected between day 16 and 38 for Group C. In regards to lymphocyte populations, there was differential regulation of various B- and T lymphocyte subpopulations in both the spleen and serum on day 38. Differential regulation of spleen localised lymphocytes for mice infested with ticks in the presence or absence of immunization, followed similar trends to mice from Group A and B that were immunized with OVA and finally euthanized on day 38 (Figure 3.1.6 and 3.1.7). Between day 16 and day 38, transitional B-cell populations (CD45CD19+IgM^{low}) increases significantly compared to negative control mice localized to the spleen and circulating in the blood (Figure 4.1.2 section on spleen). However, no further maturation of B-cell populations were observed on day 38 circulating in the blood or localized to the spleen or lymph node tissues for Group C. These results can again support the immune suppressive effects of *I. ricinus* ticks on B-cell maturation as discussed previously. Including immunization of infested hosts with OVA formulated with Freund's (i.e. Group D), limited activation of B-cells and memory B cells is achieved that is likely antigen/adjuvant specific (Figure 3.1.6). However, T cell maturation and signalling is coupled to B cell activation and maturation (Janeway Jr *et al.*, 2001).

The Th1 associated lymphocytes (CD3+CD45+CD195+) were only down-regulated in mice infested with *I. ricinus* nymphs (i.e. Group C), similar results were generated by Robbertse *et al*. (2018a) in mice euthanized 12 days post *I. ricinus* nymph infestation. The down-regulation of Th1 lymphocyte subpopulations and up-regulation of transitional Th1/Th2 (CD3+CD45+CD195+CD184+) and Th2 subpopulations (CD3+CD45+CD195+CD184+) (Figures 3.1.7 and 4.1.2), correlates with several studies where *I. ricinus* infestation results in a more Th2 polarised immune response (section 1.1.2). Similarly, the down-regulation of T lymphocyte cell populations was also observed in mice euthanized on day 16 (section 4.1.1). Following activation of circulating naïve T cells by antigen presenting cells, T cells migrate to

the secondary lymphoid organs (spleen and lymph tissue) (Alberts *et al.*, 2002). However, this is not the case during tick infestation only (Group C). There is no localization of T cells to the spleen or lymph tissue (Figures 3.1.7 and 4.1.2) as such, T cell dependent activation of B cells are not able to take place in the secondary lymphoid organs and only circulating transitional T cells (Th1/Th2) and Th2 cells are present after 38 days. This links to the observed stasis of B cells in a transitional state previously observed. Tick secreted modulatory molecules that are involved and at what states lymphocyte differentiation inhibition occurs, remains to be elucidated fully.

For Group D, as observed for B cell maturation, some circulation of Th2 cells in the blood occurs that could indicate an endpoint OVA-specific response after 38 days with two infestations and immunization. Some localization of Th2 cells to the spleen was also observed.

IgE responses following Ixodes ricinus nymph infestation in the presence/absence of immunizations in murine hosts

Mice immunised with OVA in Freund's adjuvant and then infested with *I. ricinus* nymphs (Group D) showed a unique immunological profile relative to mice that were only immunized or infested with *I. ricinus* (Figure 4.1.2). Mice from this experimental group had a significant up-regulation of total IgG and the highest production of OVA IgE-specific antibodies, indicating a Th2 polarised immune response. The elevated levels of IgE is in line with previous *I. ricinus* infestation studies in BALB/c mice that also developed an increase in IgE when infected with *I. ricinus* larvae and nymphs (Christe *et al*., 1998) or IgG1 antibodies when infected with nymphs only (Mejri *et al*., 2001). Additionally, mice from this experimental group had a low IgG2a/IgG1 antibody ratio compared to that of mice infected with *B. microti*, relating to aTh2 polarised immune response.

Mice immunized with OVA and Freund's adjuvant

Mice were immunised with OVA formulated with complete and incomplete Freund's adjuvant for the 1st and 2nd injections, respectively. Mice that were only immunised with OVA and Freund's (Group E) had similar cytokine and lymphocyte subpopulation profiles compared to mice from the negative control group (PBS alone) (Figures 3.1.4 - 3.1.7). In regards to antibody production, Group E had increased levels of OVA-specific IgE antibodies compared to mice with *B. microti* infection which displayed the lowest levels of IgE. Group E also had a low IgG2a/IgG1 ratio corresponding to a more Th2-mediated immune response (Sher *et al.*, 1992).

The data we obtained for the Th1 and Th2 results do not correlate with results obtained by

Chuang *et al.* (1997), where mice immunised with OVA formulated in complete Freund's adjuvant only, resulted in a Th1 polarised immune response after two immunisations. The latter study did not include a negative control group as included in the present study (i.e. immunised with PBS/saline), as results were only compared to other adjuvant formulations. Freund's complete adjuvant is known to cause a Th1 polarised immune response due to the presence of the attenuated *M. tuberculosis* (Coffman *et al.*, 2010), while ovalbumin immunisations induces a Th2 polarised immune response (Ellertsen *et al.*, 2010). As such, this finding needs to be validated further in future studies.

Mice that were infested with *I. ricinus* and vaccinated (Group D) had an up-regulation of Th2 lymphocyte in the spleen and blood (Figure 4.1.2, sections indicated by lymph node and spleen, respectively). Antigen-specific immune response of mice from Group D, are similar to that of mice from Group E (only immunised mice), both having similar OVA specific antibody profiles. These results may indicate that *I. ricinus* infestation in the presence of immunisation has negligible effect on antigen-specific immune response. This is a very important finding showing that a response can be elicited towards an antigen amid the severe immune suppressive effects of tick infestation. However, the vaccine formulation used (OVA formulated with Freund's) enlisted a poor immunological response in regards to B-and T lymphocyte differentiation and maturation. As such, the immunosuppressive effects of tick infestation on antigen-specific immune maturation was not determined. Additional *I. ricinus* infestation studies are required in the presence of an antigen/adjuvant formulation that is known to induce an antigen-specific lymphocyte differentiation and maturation. The significant up-regulation of total IgG from Group D, is similar to results from day 16 (Group C, mice only immunised). The possible reason for the significant upregulation of total IgG was discussed in section 4.1.1.

4.3. The effect of adjuvant on the response towards two protective antigens derived from *Rhipicephalus microplus* in BALB/c mice:

For this study, BALB/c mice were immunised with only Bm86 or in combination with TC-X formulated with either Alum or GLA-SE as adjuvants. The host immune response to each formulation was analysed by measuring cytokine concentrations and lymphocyte subpopulations following *ex vivo* antigen stimulation, as well as *in vivo* antigen-specific antibody production (Figure 4.3).

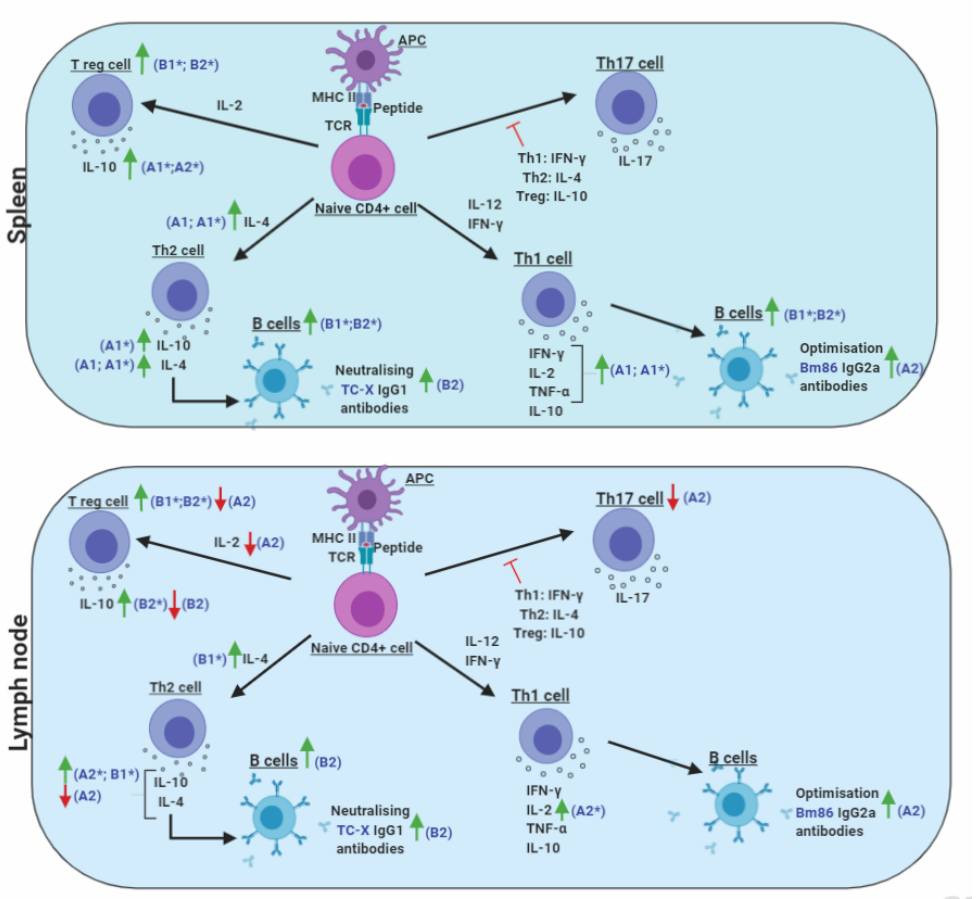

Figure 4.2.1: Antigen(s) and adjuvant formulations effect on *in vivo* and *ex vivo* immune response.
Immune response enlisted in terms of cytokines production, lymphocyte subpopulations and antibody production in spleen and lymph nodes of BALB/c mice. Mice immunised with: **[A1]** Bm86 + Alum, **[A2]** Bm86 + GLA-SE, **[B1]** Bm86 and TC-X + Alum, **[B2]** Bm86 and TC-X + GLA-SE. Up-regulation is indicated by a green arrow and down regulation by a red arrow. Inhibition is indicated by a red capped line. "*" indicates effect of *ex vivo* stimulation. Abbreviations correspond to: Antigen presenting cells (APC), Major histone compatibility molecules (MHC II) and T cell receptor (TCR).

Spleen and lymph node tissues from immunised mice were harvested 30 days after initial immunisation. The collected tissues were cultured *ex vivo* in the presence or absence of antigen(s) stimulation for 24 hours before cytokine production was determined. Cytokine

profiles in the spleen and lymph nodes showed similar differentially expressed cytokines and trends (Figure 3.2.2 and 3.2.3), with a few exceptions.

The effects of vaccine formulation on cytokine and lymphocytes in the spleen.

As there are significant differences in the spleen versus the lymph node, the discussion of the results will focus on each individual tissue. Therefore, in the spleen, it is evident that mice immunised with only the Bm86 antigen in either of the adjuvants resulted in increased IL-10 levels. For Bm86 formulated in Alum, significant changes are observed in the up-regulation of additional cytokines, namely IL-4, IL-10, IFN-γ, IL-2, TNF-α. As such, it can be deduced that Alum resulted in the activation of a more diverse immune response than initiated by GLA-SE. Alum impacted Treg, Th2 and Th1 responses (Figure 4.2.1). In contrast, the combination vaccine (i.e. containing both Bm86 and TC-X) formulated in either GLA-SE or Alum affected completely different pathways/cytokines. It affected Treg cells (CD3+CD4+CD25+) as well as CD80+ B cells (CD80+) (Figure 3.2.4). Therefore, the inclusion of TC-X into the formula significantly alter the response elicited in the spleen.

The effects of vaccine formulation on cytokine and lymphocytes in the lymph nodes

The effect of Bm86 formulated in Alum on the lymph nodes were not significant (Figure 4.2.1). However, Bm86 formulated in GLA-SE did alter the response in the lymph nodes significantly in six ways. Firstly, it decreased the levels of IL-2, IL-10, IL-4 as well as Treg and Th17 cells. Secondly, it up-regulated Bm86 specific IgG2a antibodies. However, during *ex vivo* stimulation of isolated Group A2 lymphocytes with Bm86 produced higher levels of IL-10, IL-4 and IL-2. Combined, these finding point towards GLA-SE causing a Th1 polarized response which is further discussed below.

When mice were immunised with the combination of the two antigens in either of the adjuvants, it resulted in up-regulation of Treg cells. Only in mice immunised with the combination of antigens in Alum, was an increase in IL-4 observed. Mice immunized with the combination formulated in GLA-SE (Group B2) resulted in up-regulation of CD80+ B cells and IgG1 against both Bm86 and TC-X (Figure 3.2.1). Combined, this indicates that the combination vaccine in GLA-SE resulted in a slight Th2 response. This a very interesting observation when compared with only Bm86 formulated in GLA-SE where a Th1 polarized response was elicited. As protection against ticks are believed to require a Th2 response (i.e. IgG1), the inclusion of TC-X and formulation in GLA-SE may be the most suitable to obtain IgG1 antibodies directed against both Bm86 and TC-X via this Th2 response.

Additional specific observations in regards to this aim of the study (section 4.2) is described in the following sections.

4.3.1. *Vaccine formulation effect on cytokine production in spleen and lymph node tissues*

Cytokine production in stimulated spleen cell cultures

In the spleen the majority of cytokines were differentially up-regulated in tissue cultures stimulated with Bm86 and mice immunised with Bm86 and Alum as an adjuvant. Vaccines containing TC-X as a combinational antigen and GLA-SE as an adjuvant had negligible effect on cytokine levels in the spleen.

Mice immunised with a formulation containing Bm86 and Alum, as well as cultures stimulated with Bm86 had an increase in IL-4 (Th2) as well as IFN-γ, IL-2 and TNF-α (Th1). These results may indicate that Bm86 and Alum formulations results in a balanced cytokine profile with the up- regulation of cytokines associated with both a Th1 and Th2 immune responses. Indeed, this formulation did not result in polarised antigen-specific antibody production (Figure 3.2.2 and 3.2.3). This type of response has also been reported in mice immunised with a DNA vaccine containing the Bm86 sequence. It resulted in similar cytokine levels in *in vivo* splenocytes, an up-regulation in IL-4 and IFN-γ and a mixed Th1/Th2 immune response (Ruiz *et al.*, 2007). Alum as an adjuvant has been shown to cause a Th2 biased immune response resulting in antibody production, failing to enlist a Th1/Th17 immune response (Garlapati, 2012; Pulendran *et al.*, 2011). The Bm86 *ex vivo* stimulation resulted in the up-regulation of IL-10 associated with a Th2 and Treg immune response. These results suggest that Bm86 as an antigen could cause a Th2 polarised immune response resulting in IgG1 antibody production. The lack of differential IL-17 cytokine expression in spleen tissue could be attributed to the up-regulation of Th1, Th2 and Treg cytokines, as it has been shown the up-regulation of these cytokines have a putative link to the suppression of Th17 immune response (Kaiko *et al.*, 2008).

Cytokine production in stimulated lymph node cell cultures

Cultured lymph node tissues showed some variation in cytokine expression compared to spleen tissue. However, similar to spleen cytokine expression, TC-X as a combination antigen

had no significant effect on cytokine levels (stimulated or un-stimulated) (Figure 3.2.2 and 3.2.3). Unlike in the spleen, GLA-SE played a role in differentially down-regulating cytokines in contrast to Alum that caused an up-regulation of selected cytokines (IL-4 and IL-10). Cytokines differentially down-regulated by Bm86 formulated with GLA-SE included: IL-2, IL-10 and IL-4. The down-regulation of IL-10 in mice immunised with GLA-SE as an adjuvant has been reported previously (Coler *et al.*, 2011). Since GLA-SE is a TLR-4 associated agonist (section 1.4.2), a Th1 biased immune response is expected and the down-regulation of both IL-10 and IL-4 appear to promote this. Similar to spleen cytokines, Bm86 *ex vivo* stimulation resulted in an increase of IL-4, IL-10 and IL-2 cytokines, suggesting that the Bm86 antigen results in a balanced immune response in the lymph nodes when used *ex vivo*.

4.3.2. *Vaccine formulation effect on lymphocyte subpopulations in the spleen and lymph nodes*

Secreted cytokines have the potential to alter lymphocyte subpopulations. A such, in an effort to identify the effector cells involved in the immune reactions enlisted by the different antigen(s) and adjuvant formulations, flow cytometry was used to identify general B cells (CD80+) (Good-Jacobson *et al.*, 2012), T helper cells (CD3+CD4+) and T regulatory cells (CD3+CD4+CD25+) (Rodríguez-Perea *et al.*, 2016) after 48 hours of culturing in the presence of antigen(s). *In vivo* immunisation and *ex vivo* stimulation with Bm86 had negligible effect on lymphocyte subpopulations in the spleen and lymph node (Figure 3.2.4). In contrast, *ex vivo* stimulation with both Bm86 and TC-X antigens resulted in a significant up-regulation of B cells and Treg cells in both cultured spleen and lymph nodes. These results suggest that immunisation with different adjuvants had negligible effects on *ex vivo* lymphocyte populations. However, the immunisation and *ex vivo* stimulation with the combination of Bm86 and TC-X resulted in enhanced B cell and Treg cell development and maturation. These results may suggest that vaccination with the combination antigen in GLA-SE will elicit good anti-Bm86 and anti-TC-X IgG1 antibodies, and that during follow up booster injections enhanced B cell and Treg cell responses can be anticipated. However, this needs to be validated. Another vital future study that should be conducted entails analyses of the affinity of the IgG antibodies raised to each antigen under the various formulation conditions. Techniques used to determine this could possibly include Monoclonal Antibody (MAb) titre analysis similar to a study by (Montgomery *et al.*, 2009).

The Bm86 antigen by itself formulated with Alum (Group A1) had a negligible effect on lymphocyte subpopulations stimulated with Bm86 *ex vivo.* However, the formulation containing Bm86 and GLA-SE down-regulated Treg cells in both the presence and absence of *ex vivo* Bm86 stimulation. This could be linked to the down-regulation of the regulatory cytokine IL-10 that was also reported by Coler *et al*. (2011) for murine lymph node tissues.

4.3.3. *Vaccine formulation effect on antibody production for spleen and lymph nodes*

Antibody titres (IgG1 and IgG2a) specific for Bm86 and TC-X antigens were determined using ELISA 30 days after the initial immunisation. The titre values in turn were used to calculate a IgG2a/IgG1 titre ratio indicative of a Th1 or Th2 polarised immune response relative to different experimental groups (Rostamian *et al.*, 2017).

All experimental groups had high IgG1 Bm86 specific antibody titres irrespective of antigen and adjuvant formulation. Mice immunised with Bm86 and GLA-SE had the highest production of IgG2a antibodies which resulted in a more polarised Th1 immune response relative to the other formulations (Figures 3.2.1 and 4.3). However, GLA-SE adjuvant had no significant impact on *ex vivo* stimulated spleen cell cultures in regards to cytokines and lymphocyte subpopulations produced. Moreover, this adjuvant caused a down-regulation of selected cytokines and lymphocyte subpopulations in *ex vivo* stimulated lymph node cell cultures. Antibody production results indicated that the GLA-SE adjuvanted Bm86, results in a polarised Th1 immune response *in vivo* compared to other experimental groups. Similar antibody ratios have been reported in mice and cattle immunised with Bm86 based vaccines (Valle *et al.*, 2001).

Mice immunised with Bm86 and TC-X in combination with the GLA-SE adjuvant had a significantly higher IgG1 TC-X-specific antibody titre compared to mice immunised with the same antigens that were formulated with Alum. The titre ratio indicates a Th2 polarised immune response relative to mice vaccinated with the same antigens formulated with Alum (indicated in Figure 4.2.1). Immunisation with an antigen fused to a toxoid peptide (as is the case with the TC-X antigen) have shown to result in Th2 polarised immune response (Baxter, 2007). Additionally, stimulating *ex vivo* lymph node cultures with Bm86 in formulation with TC-X resulted in the up-regulation of Th2 associated cytokines (IL-4 and IL-10). To date, TC-X has been utilised in a limited number of vaccination studies and as such the effect the antigen has on a host immune response has yet to be determined.

Chapter 5: Concluding discussion

Ticks and the pathogens they transmit are of growing medical and veterinary concern (de La Fuente *et al.,* 2017a). Limited research has been conducted on the immune effect of simultaneous pathogen-parasite infection on vertebrate immunity, despite the fact that co-infection is a reality in field conditions where vaccines need to be protective.

In this study, a murine model was use to evaluate the immunological implications of vector infestation (i.e. *I. ricinus*) and co-infection with an associated pathogen (i.e. *B. microti*). Additionally, the effect that co-infection/infestation may have on antigen-specific immune reactions in vaccinated hosts was also investigated. The results from the above-mentioned study indicated that co-infestation of a murine host alters the immune response, resembling immune reactions similar to that of *B. microti* infected hosts (i.e. a Th1 polarized immune response) rather than a host infested with *I. ricinus* nymphs. We hypothesized that this may be due to the immune suppression of *I. ricinus* saliva (Kotál *et al.*, 2015), that limits initiation and maturation of the host immune responses. Mice infected with *B. micorti* and co-infested with ticks had differential regulation of the same B- and T lymphocyte subpopulations. However, mice infected with *B. micorti* by itself had higher levels of total IgG antibody concentrations. Furthermore, co-infected hosts had a unique cytokine profile, with IL-10 and TNF-α cytokines being up-regulated, both cytokines associated with limiting inflammation and maintaining homeostasis. The study also indicated that co-infestation in the presence of a reference vaccine (OVA formulated with in/complete Freund's adjuvant), altered the antigen-specific immune response relative to that of an unchallenged immunised host. This finding indicates that the co-infestation of the vertebrate host could potentially alter vaccine efficacy, although additional studies are required to prove this.

To our knowledge, this is the first *in vivo* study describing the murine immune responses developed during infestation with a tick vector (i.e. *I. ricinus*) in the presence of a tick-borne pathogen (i.e. *B. microti*). Furthermore, experimental groups of mice infected/infested with only *B. micorti* or *I. ricinus* correlated with several findings from previous studies, indicating a successful study design and execution. The results from the present study indicated that co-infestation could potentially alter antigen-specific immune reactions elicited when vaccinating a host. As such this study is vital in an effort to increase field vaccine efficacy of other vertebrate hosts of agro-economic importance (e.g. cattle). Results from this study is summarised in Table 5.1.

Table 5.1: Summary of immune reactions launched by the co-infestation study

Markers			Day 16			Day 38				
			B. microti **[A]**	**Co-infestation [B]**	***I. ricinus*** **[C]**	***B. microti*** **and OVA [A]**	**Co-infestation [B]**	**I. *ricinus* [C]**	***I. ricinus*** **and OVA [D]**	**OVA [E]**
Antibodies (Serum)		Total IgG	↑	↑	↑	↑	↑		↑	
	OVA	IgE				↓	↓		↑	↑
		IgG2a/IgG1				↑	↑			↓
Cytokines (Lymph node)	**Th1**	IL-2	↑							
		TNF-a	↑	↑						
		IFN-y	↑							
	Th2	IL-4	↑							
		IL-10		↑		↑	↑			
Lymphocyte subpopulations (Lymph nodes, spleen and blood)	**B cells**	Marginal Zone B cells	↓ (LN)	↓(LN)		↓ (S, B)	↓ (S, B)	↓ (S, B)		
		Naïve mature follicular B cell	↑ (LN, S, B)	↑ (LN, S, B)	↑(B)	↑ (S, B)	↑ (S, B)	↑ (S, B)	↑ (B)	
		Activated GC B cells	↑ (LN, B)	↑ (LN, B)		↑ (LN, B)	↑ (B)		↑ (B)	
		Memory B cells	↑ (LN, S, B)	↑ (LN, S, B)		↑ (LN, B)	↑ (LN, B)		↑ (LN, B)	
	T cells	T cells	↓ (LN, S, B)	↓ (LN, S, B)	↓ (B)	↓ (B)		↓ (B)	↓ (B)	
		Treg	↓ (LN, S, B)	↓ (LN, S, B)						
		Th1/Th2	↑ (LN, B)	↑ (LN, B)		↓ (B)	↓ (B)	↑ (B)	↓ (B)	
		Th1	↑ (B)	↑ (B)				↓ (B)		
		Th2	↑ (S, B)	↑ (S, B)		↑ (LN, S, B)	↑ (LN, S, B)	↑ (B)	↑ (S, B)	

Abbreviations: LN (lymph node), S (spleen), B (blood/serum), OVA (ovalbumin)

The efficacy of a vaccine may depend on a particular set of factors in the immune response elicited by the host when immunised, which could be compromised/supressed by a parasite/pathogen's immune modulators (Mabbott, 2018). Given the previously mentioned results, the second part of the project aimed to evaluate *R. micoplus* antigen(s) and adjuvant formulations in the same murine model, in an effort to identify formulations with the ability to compensate for immunological parameters that are down-regulated during parasite/pathogen infections. To date, the immunological mechanism of the Bm86 antigen in cattle is yet to be fully understood. *Rhipicephalus micorplus* antigens, Bm86 and TC-X (as a novel antigen) were formulated with Alum and GLA-SE adjuvants. This study then monitored *in vivo* antigen-specific IgG1 and IgG2a antibody production, as an indication of immunological memory and immune polarisation (Ratajczak *et al.*, 2018). Additionally, *ex vivo* antigen stimulated cytokine production (Th1 and Th2 specific) and production of lymphocyte subpopulations associated with immune maturation were also taken into consideration, in an effort to elucidate secondary lymphoid tissue immunological responses elicited by the chosen antigen set.

Results indicated that immune responses launched by BALB/c mice vary depending on the antigen(s) and adjuvant formulations utilised for immunisation. The Bm86 antigen formulated with GLA-SE resulted in an increased production of Bm86 specific IgG2a antibodies compared to other formulations. Combinational antigen formulations resulted in increased lymphocyte subpopulations (B lymphocytes and Treg cells) required for immune maturation when recalled *ex vivo*. Formulations containing Bm86 formulated with Alum had balanced cytokine profiles with up-regulations in Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) cytokines.

Bovine babesiosis is responsible for large economic losses in the livestock industry worldwide (Gohil *et al.*, 2013), with infection usually resulting in mortality, abortions and a decrease in milk and meat production (Bock *et al.*, 2004).. Cattle infested with *B. bovis* elicits a Th1-mediated immune response, resulting in neutralising antibodies with mixed T and B cell responses (Ortiz *et al.*, 2019). Since cattle with prior exposure (i.e. infested and/or infected) could have a polarised immune response, it is proposed that cattle be treated with anti-protozoal agents before administration of vaccines to limit the polarization of the hosts immune responses due to *Babesia* infection (Mosqueda *et al.*, 2012).

Additional limitations, considerations and future perspectives:

The first part of the study, monitoring immune response development of BALB/c mice when infected with *B. microti* and co-infested *I. ricinus* was completed in the absence of a homologous tick antigen (i.e. *I. ricinus* protein homologs). Thus, the effect of co-infestation

and alternative antigen/adjuvant formulations has on vaccine efficacy was not determined, with the study focussing only on immune response and maturation in a murine model. In future, tick antigens will be utilised to (i) induce a greater immune response than OVA and Freund's adjuvant and (ii) to evaluate if co-infestation with *B. microti* influence vaccine efficacy.

The current study used the TC-X antigen conjugated to a tetanus toxoid in an effort to increase immunogenicity and to provide long-lasting immunity (Baxter, 2007). However, it should be considered that the experiment be repeated with the TC-X antigen in the absence of the toxoid conjugate. Results from this study will elucidate the immunological mechanism of the TC-X antigen and indicate if the TC-X antigen is worth including in future combinational vaccines.

In addition to the future studies mentioned in sections 4.1 and 4.2, future studies will aim to infest cattle hosts with the *R. microplus* in the presence of *B. bovis* or *B. bigemina* infection and then determine candidate vaccine efficacy by monitoring *R. microplus* survival parameters following feeding and includes: female numbers and weights, as well as egg laying capacity and occlusion (Stutzer, 2016). However, when such a study is to be undertaken, variation within and between cattle breeds need to be taken into consideration. The *R. microplus* resistant *Bos indicus* and susceptible *Bos taurus* cattle breeds have been shown to differ in regards to the percentage of immune cell subsets and transcripts produced during infestation (Piper *et al.*, 2009; Robbertse *et al.*, 2018b) potentially altering vaccine efficacy between different cattle. Continuation of the study in cattle hosts could verify host immune markers (or immunological biomarkers) compared to markers identified in the murine model host. These correlations can be utilised to compare specific immune responses to vaccine efficacy, further establishing mice as a model organism for optimising vaccine formulations before use in cattle.

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