

**Towards improving Subolesin and MSP-based vaccines targeting
Rhipicephalus microplus and *Anaplasma phagocytophilum***

by

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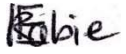
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Submission declaration

I, Elsje Christine Rabie declare that the dissertation, which I hereby submit for the degree Master of Science (MSc) Genetics at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: 

Date: 2024/08/23

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Executive Summary

Ticks are ectoparasites that both directly and indirectly impact livestock. Moreover, ticks transmit pathogens that cause tick-borne diseases that severely affect livestock industries. Consequently, the control of ticks and their tick-borne diseases is imperative. However, the current chemical tick control paradigm is marred by the increasing development of acaricide resistance. As such, tick control should be supplemented with complementary strategies, such as vaccines. Several tick vaccines have been evaluated in pre-clinical and clinical trials, however, even the most efficacious candidates were hindered by antigenic diversity and inconsistent protection, within and between tick species and geographical regions. Therefore, a universal tick vaccine requires an antigen that is highly conserved within and between tick species.

A potential candidate for this role is the tick antigen Subolesin. Although several studies have evaluated the efficacy of this antigen in vaccine trials, there is still limited knowledge regarding the gene structure of this protein, as well as its gene expression and diversity in the prominent tick species, *Rhipicephalus microplus*. Given the importance of antigen diversity in tick vaccine efficacy, understanding the diversity of Subolesin in *R. microplus* ticks would inform the design of future vaccines. Consequently, the expression and diversity of Subolesin (and its many variants) in *R. microplus* ticks from different tissues (ovary, gut and gland), life stages (larvae, nymph and adult) and several South African locations (Mpumalanga and a pathogen-free laboratory-strain) were elucidated for the first time on a RNA (ribonucleic acid) sequencing level. Results indicate that Subolesin has similar expression levels across all life stages and tissues. In addition, this study elucidates for the first time the gene structure of Subolesin in *R. microplus*. Furthermore, it was found that Subolesin has four potential mRNA (messenger ribonucleic acid) variants which could translate into three protein variants. Thus far, this finding is exclusive to *R. microplus*, as Subolesin in other tick species have been reported as a single mRNA and protein. Lastly, Subolesin was found to be highly conserved across all samples, which emphasises the promise of this antigen as a vaccine candidate targeting South African *R. microplus* ticks.

Another factor emphasising the need for tick control is the devastating effects of tick-borne pathogens, such as *Anaplasma phagocytophilum*. This pathogen is the causative agent of granulocytic anaplasmosis which affect several hosts, including humans and most livestock animals, like cattle and sheep. However, there are virtually no prophylaxes for preventing this disease, and current vaccines are not protective and face the same problems as tick vaccines

with regards to antigenic diversity. Therefore, the aim of controlling the tick vector has become even more important. Given Subolesin's promise as a potential tick vaccine candidate, this study sought to improve upon previous studies by adsorbing Subolesin onto Poly D-L-lactide-co-glycolide acid (PLGA) microparticles. PLGA microparticles overcome many challenges associated with traditional subunit vaccines, such as poor immunogenicity, antigen instability and poor thermostability. Moreover, it has been shown that multiple vaccinations increase the risk of injection site reactions, as well as increases the burden on animals, farmers and veterinarians. These effects are exacerbated by the administration of separate vaccines for ticks and each tick-borne pathogen. Therefore, the development of combination vaccines targeting both the tick and tick-pathogen would be advantageous.

Consequently, this study evaluated the immune profile elicited by vaccination with antigen coated PLGA microparticles simultaneously targeting both the tick vector (Subolesin) and the tick-borne pathogen *A. phagocytophilum* (MSP4) in a murine model. Sera results indicated that Subolesin antigens (soluble and adsorbed onto particles) were not immunogenic after ELISA (enzyme linked immunosorbent assay) analysis and were consequently excluded from further analysis. Nevertheless, ELISA results demonstrated that all MSP4-receiving groups elicited a Th2 biased response in sera. Furthermore, this study offers the first insights into the T and B cell profile elicited by immunization with MSP4 in any animal model. Therefore, this study offers valuable insights into the design and application of future PLGA microparticle based vaccines for the control of ticks and *A. phagocytophilum*.

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List of Abbreviations

A	alanine
aa	amino acids
ADCC	antibody dependent cell-mediated cytotoxicity
Adj	adjuvant
Ag	antigen
Ak2	Akirin 2
<i>A. americanum</i>	<i>Amblyomma americanum</i>
<i>A. variegatum</i>	<i>Amblyomma variegatum</i>
<i>A. marginale</i>	<i>Anaplasma marginale</i>
<i>A. phagocytophilum</i>	<i>Anaplasma phagocytophilum</i>
ANOVA	analysis of variance
AO/PI	Acridine Orange/Propidium Iodide
APC	antigen presenting cell
BCA	Bicinchoninic acid
BLAST	Basic Local Alignment Search Tool
Bm86	Boophilus microplus protein 86
<i>B. indicus</i>	<i>Bos indicus</i>
<i>B. taurus</i>	<i>Bos taurus</i>
bp	base pairs
C	cytosine
CAGE	cap analysis of gene expression
cDNA	complementary DNA
CDS	coding sequence
CLA3	Clare A
combi	combination
Con A	concanavalin A
CO ₂	carbon dioxide
CPM	counts per million
CV	ClinVet
DC	dendritic cell
DEPC	diethyl pyrocarbonate
<i>D. silvarum</i>	<i>Dermacentor silvarum</i>
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease

EF-1 α	Elongation Factor-1 alpha
ELISA	enzyme linked immunosorbent assay
EOs	essential oils
EOCs	essential oil compounds
E-value	expect value
FAO	Food and Agriculture Organization
FasL	Fas ligand
FBS	foetal bovine serum
FC	flow cytometry
FMO	fluorescence minus one
FPLC	Fast Protein Liquid Chromatography
F+R	forward and reverse sequencing reads
Gbp	giga-base-pair
<i>H. flava</i>	<i>Haemaphysalis flava</i>
<i>H. anatolicum</i>	<i>Hyalomma anatolicum</i>
HSP	Heat shock protein
IEDB	Immune Epitope Database and Analysis Resource
IFN- γ	Interferon gamma
IgE	immunoglobulin E
IgG	immunoglobulin G
IgG1	immunoglobulin G1
IgG2a	immunoglobulin G2a
IgM	immunoglobulin M
IGV	Integrative Genomics Viewer
IKK	I-KB kinase
IL	interleukin
IMD	Immune deficiency
iNOS	Inducible nitric oxide synthase
INR	transcription initiation sequence
IUPAC	International Union of Pure and Applied Chemistry
<i>I. pacificus</i>	<i>Ixodes pacificus</i>
<i>I. persulcatus</i>	<i>Ixodes persulcatus</i>
<i>I. ricinus</i>	<i>Ixodes ricinus</i>
<i>I. scapularis</i>	<i>Ixodes scapularis</i>
groEL	chaperone protein encoding gene
K	lysine

KZN	KwaZulu-Natal
LB	Luria-Bertani
LC	Liquid chromatography
LRS	Long-Read Sequencing
MBC	memory B cell
MCP-1	Monocyte-chemoattractant protein-1
MHC	major histocompatibility complex
MPs	microparticles
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MSP4	major surface protein 4
MyD88	Myeloid differentiation primary response 88 protein
NCBI	National Centre for Biotechnology Information
N.D.	not determined
NF-κB	Nuclear Factor Kappa B
N-Rel	N-terminal domain of Relish
Ni-NTA	nickel-nitrilotriacetic acid
NLS	nuclear localisation signal
n.s.	not specified
N.S.	Not significant
nt	nucleotide
ONT	Oxford Nanopore Technologies
OPD	o-phenylenediamine dihydrochloride
ORF	open reading frame
<i>O. erraticus</i>	<i>Ornithodoros erraticus</i>
<i>O. moubata</i>	<i>Ornithodoros moubata</i>
OVA	ovalbumin
OVARU	Onderstepoort Veterinary Animal Research Unit
PacBio	Pacific Biosciences
PAE	predicted aligned error
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pLDDT	predicted local distance difference test
PLGA	poly(lactic-co-glycolic acid)
Poly-A	polyadenylation
pre-mRNA	primary transcript

PVA	polyvinyl alcohol
P1/P2/P3	Subolesin protein variants
RACE	rapid amplification of cDNA ends
RATE	rapid amplification of transcript ends
<i>R. appendiculatus</i>	<i>Rhipicephalus appendiculatus</i>
<i>R. annulatus</i>	<i>Rhipicephalus annulatus</i>
<i>R. decoloratus</i>	<i>Rhipicephalus decoloratus</i>
<i>R. haemaphysaloides</i>	<i>Rhipicephalus haemaphysaloides</i>
<i>R. microplus</i>	<i>Rhipicephalus microplus</i>
<i>R. sanguineus</i>	<i>Rhipicephalus sanguineus</i>
rpm	Revolutions per minute
RNA	ribonucleic acid
RNAi	RNA interference
RNA-pol II	RNA polymerase II
RNA-seq	RNA sequencing
RPMI medium	Roswell Park Memorial Institute medium
RSA	Republic of South Africa
qRT-PCR	quantitative real-time PCR
sc	subcutaneous
SCID	severe combined immunodeficiency
SD	Standard deviation
SEM	Scanning Electron Microscopy
SLO	secondary lymphoid organ
SPF	specific pathogen-free
SNV	single nucleotide variant
SUB	Subolesin
T	threonine
TAB2	TAK1-binding protein
TAK1	TGF- β activated kinase
TBD	tick-borne disease
TBS	tris buffered saline
Th cell	T helper cell
TLR2	Toll-like receptor 2
TMM	trimmed mean of M values
TNF	tumour necrosis factor
TSS	transcription start site

v/v	volume per volume
WR	working reagent
WV	Wolverdend
w/v	weight per volume
UBQ	ubiquitin
USA	United States of America
USD	United States Dollars
UTA	Utah B
UTR	untranslated region
X1, X2, X3, X4	Subolesin mRNA species
$\gamma\delta$ T-cells	Gamma delta T cells

Chapter 1

Review of literature

1.1. The cattle tick: *Rhipicephalus microplus* and the impact of ticks

1.1.1. *Rhipicephalus microplus*: An overview of taxonomy, lifecycle and distribution

The hematophagous ectoparasite *Rhipicephalus microplus* belongs to the kingdom Animalia, phylum Arthropoda, order Acari, class Arachnida, family Ixodidae (hard-bodied), and sub-family *Metastrata* (Guglielmone et al., 2010; Horak et al., 2002; Walker, 2003). Furthermore, *Rhipicephalus microplus* (formerly known as *Boophilus microplus*), is also colloquially known as the Asian or Asiatic blue tick (Nyangiwe et al., 2017), the pantropical blue tick (Barker and Murrell, 2004; Madder et al., 2011) and the southern cattle tick (Yawa et al., 2019). *Rhipicephalus microplus* has a karyotype of 22 diploid chromosomes in females (XX) and 21 diploid chromosomes in males (XO) (J H Oliver 1977; Dorn et al. 2017).

These one-host ticks have a relatively short life cycle that can be completed in approximately three to four weeks (Jongejan and Uilenberg, 2004; Walker, 2003). The cattle tick's rapid life cycle occurs faster and yields more eggs than the closely related endemic species *Rhipicephalus decoloratus* (*R. decoloratus*), which gives it the potential to outcompete these ticks (Tonnesen et al., 2004; Walker, 2003). Furthermore, up to three or four tick generations can occur per annum, which contributes to the rapid spread of *R. microplus* (George et al., 2002; Marques et al., 2020).

The first report of *R. microplus* in South Africa was in 1908 in the Eastern Cape (Howard, 1908), and has since then been reported in all nine provinces (Horak, 2018). Globally, *Rhipicephalus microplus* is mostly found within the tropical and sub-tropical regions of the world (Madder et al., 2011). From Figure 1.1, it is evident that the global distribution and density of the primary host of these ticks, cattle, coincides with the incidence of *R. microplus* and their ecologically favoured niches. Although *R. microplus* is not the only tick affecting cattle, it is evident from Figure 1.1 that eight of the top ten beef producing countries have regions of high suitability for the cattle tick. Since *R. microplus* ticks transmit high morbidity and mortality diseases, these ticks pose a significant threat to the cattle industry and other livestock and associated industries (Table 1.1).

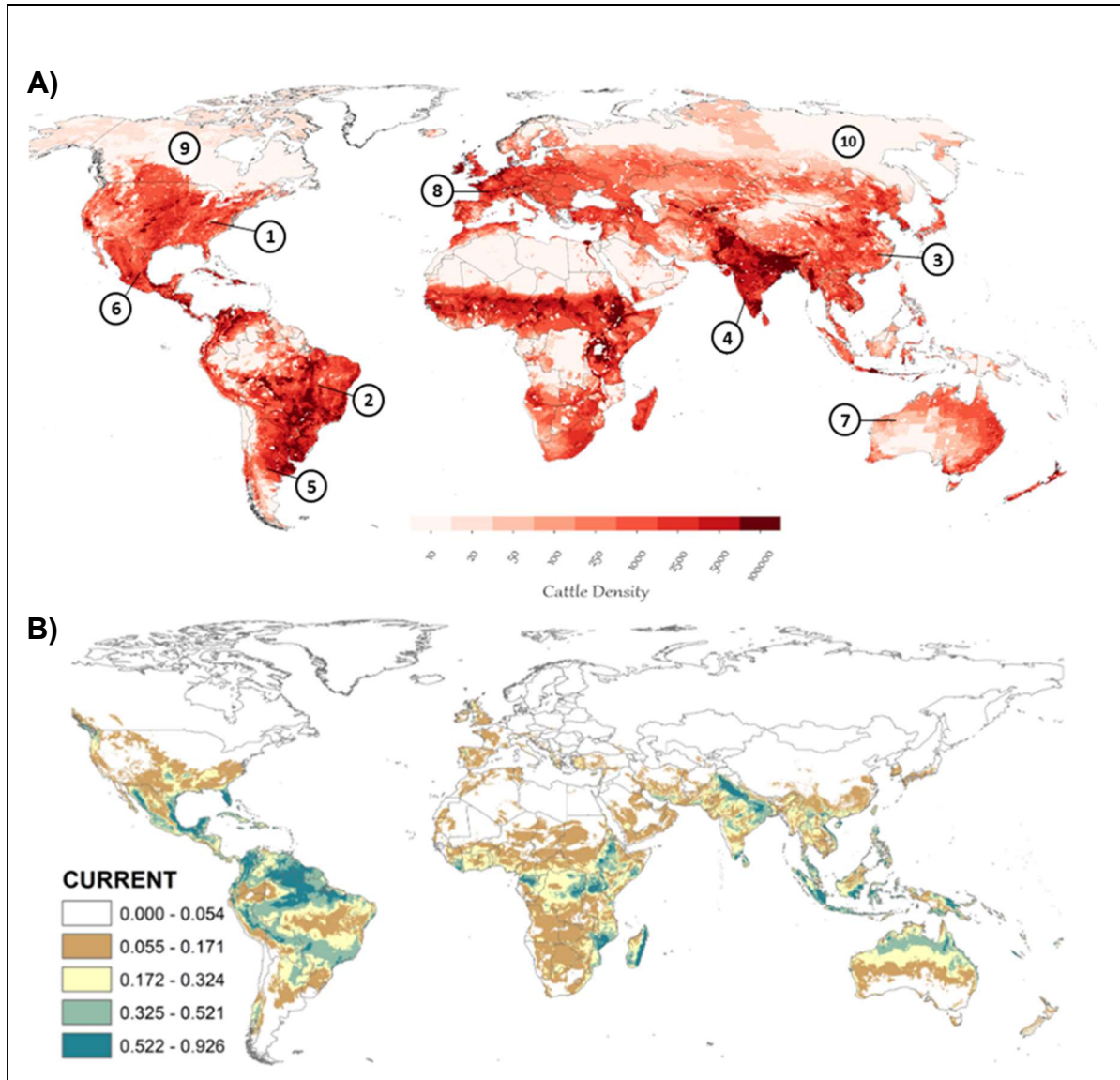


Figure 1.1. Global distribution of cattle and *Rhipicephalus microplus* ticks. (A) Map depicting the global density of cattle based on census data from 2010. Map was taken from Symington (2023) based on the study by Gilbert (et al., 2018) . The numbers indicate the top ten beef producing countries globally during 2022 in order, namely: (1) USA, (2) Brazil, (3) China, (4) India, (5) Argentina, (6) Mexico, (7) Australia, (8) France, (9) Canada and (10) Russia. Data obtained from Dias Sousa (2024). **(B)** Global map depicting the ecological niches where *R. microplus* have been found from 1981-2021. The colour key indicates areas of suitability for *R. microplus*, where greater values indicate greater suitability (0.000-0.926). Taken from Perez-Martinez et al. (2023).

1.1.2. Impact of tick infestations

Tick infestations are responsible for the loss of millions of United States Dollars (USD) annually in industries associated with livestock, such as leather, milk and meat production (Table 1.1). The already significant burden created by direct losses alone (Table 1.1.A) is exacerbated by indirect losses, which includes secondary infections forming due to tick infestations, the transmission of tick-borne diseases (TBDs), as well as the cost of treatment (Table 1.1.B).

Table 1.1. Impact of tick infestation and tick-borne diseases (TBDs) on various industries associated with livestock.

Impact	Industry	Country of origin for statistics	Specific loss(es) to animal production parameters	Cost (USD) of losses incurred and/or savings due to effective control	Reference(s)
A] Direct:					
Tick bite	Leather	Pakistan	-Overall damaged skin and hides represented 33.88% of the total hide count.	N.D. ¹	(Chaudhry et al., 2011)
	Leather	India	-Tick damage is reported for 3.08% of hides sampled. Depreciation of cost of leather by tick bite marks and the cost of treatment.	USD 69.49 million (cattle) and USD 22.61 million (buffaloes)	(Singh et al., 2022)
Tick burden & blood feeding	Milk and meat	India	Milk loss due to <i>R. microplus</i> was 13.91 L, 56.89 L and 85.34 L in low, moderate and high tick burdens, respectively.	USD 502.48 million per annum	(Singh et al., 2022)
	Livestock and meat	Brazil	Infestation with <i>R. microplus</i> lead to 6.89% of reduction in cattle live weights.	A loss of USD 4713.0 in a study with 120 animals	(Calvano et al., 2019)
	Livestock and meat	-	Live weight loss of 0.6g potential growth per engorged female tick.	N.D.	(Walker, 2003)
	Livestock and meat	-	Average loss of 1.37 ± 0.25 g bodyweight (<i>B. taurus</i>) and 1.18 ± 0.21 g (<i>B. taurus</i> $\times$ <i>B. indicus</i>) bodyweight per engorged <i>R. microplus</i> female tick ²	N.D.	(Jonsson, 2006)

¹ N.D. - not determined

² Average estimated from 19 studies from 1960-1998 that estimated the damage coefficient, *d*, as defined by Sutherst et al. (1983).

Impact	Industry	Country of origin for statistics	Specific loss(es) to animal production parameters	Cost (USD) of losses incurred and/or savings due to effective control	Reference(s)
B] Indirect					
TBDs	Livestock	USA	A rate of 26.8% anaplasmosis-induced abortion.	USD 424.17 per clinical case	(Alderink and Dietrich, 1983)
	Livestock	RSA	Cost of Bovine Anaplasmosis (<i>Anaplasma</i> spp. not specified).	USD 9.6 million per annum ³	(Hove, 2018)
	Livestock	USA ⁴	Average cost impact <i>Anaplasma marginale</i> in cattle industry.	USD 793.0 per animal ⁵	(Railey and Marsh, 2021)
	Livestock	USA	Since eradication of Babesia in USA, significant savings have been made.	USD 3 billion per annum	(Schnittger et al., 2012)
	Livestock productivity	China	1% increase in zoonotic diseases leads to 0.022% and 0.036% loss in total factor productivity of beef and dairy livestock, respectively.	N.D.	(Gong et al., 2021)
Cost of treatment	Livestock	USA	Anaplasmosis treatment: diagnostic tests, preventative vaccines, preventative feed additives, post-infection antibiotics for cattle.	USD 67.7 per animal ⁶	(Railey and Marsh, 2021)

³ Cost in reference was estimated using estimated South African cattle population size from 2015 (13.7 million cattle) and the pre-weaning mortalities of calves from commercial and emerging sectors from 2010 (3% annually), however the reason for mortality was not specified (Scholtz and Bester, 2010).

⁴ USA – United States of America

⁵ Reference calculated value using 2016 inflation rates and an upper-bound estimate model assuming that infected animals do not recover weight over a two-year period (Alderink and Dietrich, 1983).

⁶ Original article listed the cost for preventative vaccination and feeding, diagnostic testing and post-infection antibiotics. The value in this table represents the sum of these costs.

Impact	Industry	Country of origin for statistics	Specific loss(es) to animal production parameters	Cost (USD) of losses incurred and/or savings due to effective control	Reference(s)
Cost of treatment	Livestock	RSA	Increase of years of productive life in bulls (26.3%-29.7%) and oxen (15.98%-19.02%) due to introduction of tick control.	N.D.	(Salih et al., 2023)
C] Cumulative costs (per country and globally):					
National	-	India	Ticks and TBDs	USD 787.6 million per annum	(Singh et al., 2022)
National	-	Brazil	<i>R. microplus</i> infestations on cattle	USD 3.2 billion per annum	(Grisi et al., 2014)
National	-	Mexico	<i>R. microplus</i> infestations on cattle	USD 573.6 million per annum ⁷	(Rodríguez -Vivas et al., 2017)
National	-	Tanzania	Ticks and TBDs	USD 364.0 million	(Kivaria, 2006)
National	-	Puerto Rico	Ticks and TBDs	USD 6.7 million	(Senbill et al., 2018)
National	-	Australia	Losses due to ticks, TBD and treatment and prevention strategies	USD 175.0 million per annum	(Playford, 2005)
Global	-	Globally	Losses due to ticks and TBDs	USD 22-30 billion per annum ⁸	(Lew-Tabor and Rodriguez Valle, 2016)

⁷ Another report found that the annual loss due to the misuse of chemical control in Mexico is USD 942.3 million based on a national cattle population of 30 million in Mexico, and an exchange rate of 13 pesos/dollar (García et al, 2016).

⁸ Value in table obtained by using Food and Agriculture Organization (FAO) estimated global cattle population size at the time (1.47 billion) and inflation rates from 1996 to 2015 at 52.3% and calculated based costs estimated from Australia and using the method from de Castro (1997).

Furthermore, secondary infections at bite sites raise the cost of rearing livestock and increases the risk for the development of anti-microbial resistance (Tiseo et al., 2020). Ticks transmit the greatest variety of pathogenic organisms, which include viruses, fungi, protozoa and bacteria (Sonenshine 2014). Infection with TBDs escalates the costs of maintaining livestock, where the average cost impact of bovine anaplasmosis in the United States of America (USA) is estimated at 793.0 USD per animal (Table 1.1). Furthermore, some tick-borne pathogens are zoonotic (i.e., *Anaplasma phagocytophilum*) and threaten both human and animal health (Gong et al., 2021).

1.2. The zoonotic pathogen: *Anaplasma phagocytophilum*

In 1932, the causative agent of an infectious disease transmitted by ticks to sheep was described in Scotland (Foggie, 1951). Since then, this Gram-negative bacterium has been found in various countries across the northern hemisphere and has more recently been reported across the southern hemisphere, establishing its status as a global threat (Figure 1.2).

The spread of this pathogen has been partially attributed to the expanding geographic range of its vectors, *Ixodes scapularis*, *Ixodes ricinus*, *Ixodes pacificus* and *Ixodes persulcatus*, as shown in Figure 1.2 (Woldehiwet, 2010). Other potential vectors include *Dermacentor*, *Rhipicephalus*, *Hyalomma* and *Haemaphysalis* spp. (Dunkel, 2018), which are established globally in regions currently unaffected by the established *Ixodes* vectors (Figure 1.2) (Bonnet et al., 2022; Martin et al., 2022; Raghavan et al., 2019). Therefore, although vector competence has not yet been proven, these ticks pose a potentially significant threat (Dugat et al., 2015; Stuen et al., 2013b).

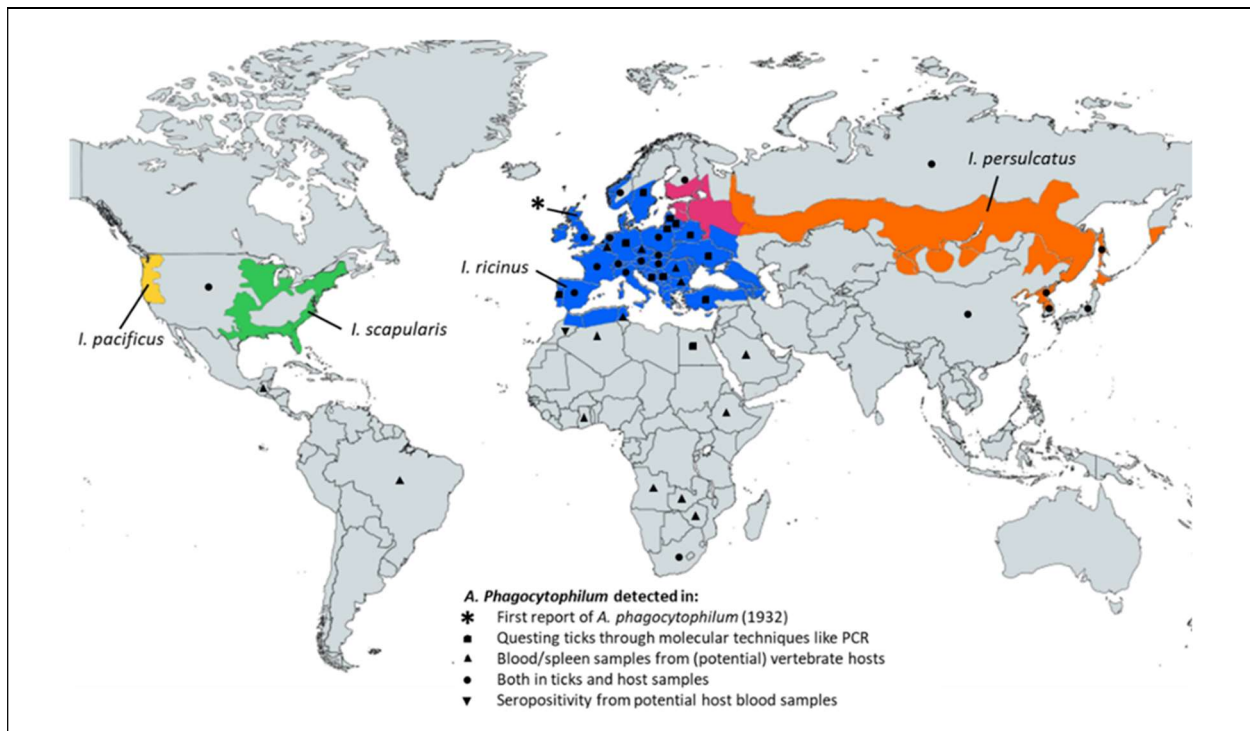


Figure 1.2. Map depicting the global distribution of *Anaplasma phagocytophilum* and its four *Ixodes* tick vectors. The geographic distribution of *Ixodes* ticks in the USA is split with *Ixodes pacificus* (yellow) on the west coast and *Ixodes scapularis* (green) on the east coast. *Ixodes ricinus* (blue) is primarily found in Europe, however there is some overlap around the Baltic Sea area and north-western Russia (pink) with *Ixodes persulcatus* (orange), which predominantly found in Asia. Countries where *A. phagocytophilum* has been detected is indicated with symbols, also denoting the method used for detection. The distribution of *Ixodes* ticks was taken from Diuk-Wasser et al. (2016). The first report of *A. phagocytophilum* was made in Scotland in 1932 (indicated by asterisk), and has since spread globally (Foggie, 1951). The distribution of *A. phagocytophilum* was partly based on the review by Stuenkel et al. (2013a) regarding the presence of *A. phagocytophilum* in the Americas, Europe, Asia and Africa and supplemented with recent articles (Barradas et al., 2021; Dahmani et al., 2015; Efstratiou et al., 2021; Elhamiani Khatat et al., 2016; Ghafar and Amer, 2012; Hodžić et al., 2017; Iqbal et al., 2019; Kallio et al., 2014; Kelly et al., 2014; Kolo, 2023; Kolo et al., 2020; M'ghirbi et al., 2012; Nakayima et al., 2014; Narankhajid et al., 2018; Nelder et al., 2019; Sormunen et al., 2016; Teshale et al., 2018; Wyk et al., 2022). Image created with MapChart.net.

The life cycle of *A. phagocytophilum* is dependent on reservoir hosts which can be persistently infected and continuously infect previously uninfected vectors (Figure 1.3) (Jaarsma et al., 2019). These hosts typically do not develop clinical symptoms and include *Odocoileus virginianus* (white-tailed deer), *Peromyscus leucopus* (white-footed mouse) in the US, and other small mammals such as short-tailed shrews, chipmunks, skunks and opossums to name a few (Keesing et al., 2012). Non-reservoir hosts include most livestock and domestic animals (Table 1.2) which can develop mild to severe symptoms that can have significant impacts on animal health and productivity.

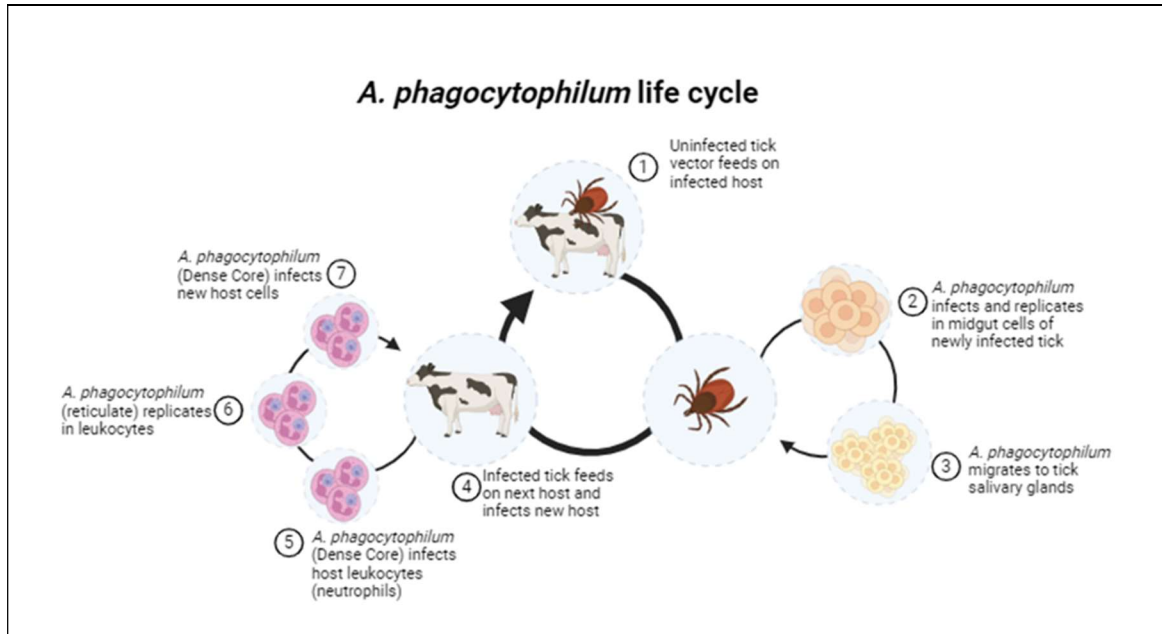


Figure 1.3. Depiction of the life cycle of *Anaplasma phagocytophilum*. (1) A previously uninfected tick feeds on an infected host, (2) *A. phagocytophilum* reaches the tick midgut cells through the bloodmeal taken from the infected host, where it replicates, whereafter (3) it migrates to the salivary glands (Hajdušek et al., 2013; Liu et al., 2011). (4) The newly infected tick feeds on a new host (previously uninfected) and transmits *A. phagocytophilum*, where (5) the dense core morphotype infects the host's leukocytes (mostly neutrophils) before entering (6) a replicative phase (reticulate morphotype) within membrane-derived vacuoles in the cytoplasm of host leukocytes (Troese and Carlyon, 2009). (7) Once sufficient replication has taken place, *A. phagocytophilum* condenses and organises into the dense core morphotype again and infects new host cells (Troese and Carlyon, 2009). Therefore, transmission is exclusively transstadial (Jaarsma et al., 2019). Image was created in BioRender.com.

Table 1.2. Summary of the known hosts of *Anaplasma phagocytophilum* and the host-specific symptoms and the respective immune response/immunopathology elicited after infection.

Host	Symptoms	Immune response/Immunopathology	Reference
Known hosts			
A] Sheep	High fever, malaise, neutropenia and thrombocytopenia, impaired spermatogenesis and abortion in pregnant animals (aborted foetus often shows signs of leukomalacia or hypoxia) and vulnerability to secondary infections (i.e., septicaemia, arthritis and pneumonia).	-Suppressed immune response due to reduced antibody production (especially in persistently infected sheep) -Reductions in B-cells, $\gamma\delta$ T-cells ⁹ , CD4+ T-cells and CD25 expression on lymphocytes after infection.	(Chianini et al., 2004; Garcia-Perez et al., 2003; Stuen, 2007; Stuen et al., 2001; Whist et al., 2003)
B] Cattle	Anorexia, reduced milk production, coughing, respiratory distress, fever, lethargy, dullness, abortion in pregnant animals/stillbirths. <u>Opportunistic secondary infections:</u> - <i>Staphylococcus aureus</i> mediated tick pyaemia, pasteurellosis, septicaemic listeriosis.	-Limited research beyond damage to humoral and cellular immune responses.	(Atif, 2015; Spickler, 2013)
C] Goats	Clinical illness is rare, however there have been reports of febrile syndrome, fever, apathy, poor appetite, reduced milk production, hyperaemic stains on udder skin and fatality.	- <i>A. Phagocytophilum</i> specific antibodies have been detected, but protective capacity has not been shown.	(Paternolli, 2016)
D] Horses	<u>Mild:</u> -Apathy, weakening and increased body temperature, stiffness/aversion to movement, lameness. <u>Acute/Severe:</u> -Fever, bleeding from mucosa, weight loss, enlarged spleen and lymph nodes, physical collapse, multiple organ failure, mortality.	-High antibody titres raised (44% of patients), but protective role for antibodies in pathogen clearance was not shown. -Upregulation of pro-inflammatory cytokines IL ¹⁰ -1 β , TNF ¹¹ - α and IL-8 across 20-day infection period.	(Artursson et al., 1999; Dzięciel et al., 2013; Kim et al., 2002)

⁹ $\gamma\delta$ – Gamma delta

¹⁰ IL - interleukin

¹¹ TNF – tumour necrosis factor

Host	Symptoms	Immune response/Immunopathology	Reference
E] Dogs	<u>Mild:</u> -Haematologic abnormalities (thrombocytopenia, leukopenia, and anaemia, lymphocytopenia).	-Not characterised beyond symptoms of anaemic inflammation.	(Granick et al., 2009; Lillieh et al., 1998)
	<u>Severe:</u> -Diarrhoea, vomiting, meningitis, endocarditis.		
F] Cats¹²	Anorexia, arthralgia, lethargy, lameness, hyperesthesia, myalgia, conjunctivitis, incoordination, neutrophilia, lymphopenia, thrombocytopenia and anaemia.	Not characterised.	(Schäfer and Kohn, 2020; Stuen et al., 2013a)
G] Humans	<u>Mild:</u> -Fever, chills, headaches, myalgia, malaise, haematologic abnormalities (leukopenia, anaemia, thrombocytopenia).	-High antibody titres raised in 40% of patients -Induces proinflammatory response via release of cytokines (IL-10, IL12, and IFN- γ ¹³) which contributes to tissue injury.	(Carlyon and Fikrig, 2003; Dugat et al., 2015; Dumler and Bakken, 1998; Guzman, 2023; Ismail et al., 2010; Zhang et al., 2013)
	<u>Severe:</u> -Facial oedema, mental confusion, multiple organ dysfunction syndrome, haemorrhagic and gastrointestinal complications, Rhabdomyolysis and a 5% fatality rate.	Tissue injury prevents neutrophils from exerting anti-microbial response.	

¹² Literature review analyzed 34 reports of cats diagnosed with *A. phagocytophilum* infection according to ABCD guidelines.

¹³ IFN- γ – Interferon gamma

Furthermore, this zoonotic pathogen can also have severe health consequences for humans (Table 1.2). The manifestation and severity of the symptoms of *A. phagocytophilum* infection is host specific and it has been shown that both can be attributed to the host immunopathology resulting from infection (Severo et al., 2012). It is evident that this pathogen has immunosuppressive effects and triggers an inflammatory immune response that contributes to the resulting immunopathology in all hosts. Given the spread of this pathogen, its vectors (Figure 1.2) and its zoonotic action (Table 1.2), understanding how this pathogen causes infection and crucially how to clear it, is imperative.

Several reviews have detailed extensively how *A. phagocytophilum* manipulates and evades the host immune system (Brown, 2012; Scorpio et al., 2018; Woldehiwet, 2008). Briefly, *A. phagocytophilum* evades the immune system through (1) suppression of the host adaptive immune system (Eskeland et al., 2023; Woldehiwet, 2008), (2) antigenic variation, which leads to a lack of antibody and T cell recognition (Brown, 2012; Granquist et al., 2010), (3) modulation of host lymphocyte functions (Whist et al., 2003), (4) incorporation in host cell endosomal compartments (Lin and Rikihisa, 2003; Mott et al., 1999), and (5) mechanisms of survival within neutrophils, such as the inhibition of phagosome-lysosome fusion (Gokce et al., 1999; Rikihisa, 2011), disruption of neutrophil functions (Londoño et al., 2023; Rikihisa, 2011; Woldehiwet, 2008) and delaying neutrophil apoptosis (DeLeo, 2004).

Despite knowing the mechanisms that collectively enable *A. phagocytophilum* to cause infections in the host, the knowledge of what is required for pathogen clearance has predominantly been characterised in mice (Table 1.3). Typically, the clearance of intracellular bacterial infections requires both CD4 T cell dependent immunity as well as other antimicrobial effectors (Londoño et al., 2023; von Loewenich et al., 2004). For mice in particular it has been shown that clearance of *A. phagocytophilum* infection is primarily dependent on a CD4 T cell immune response (Birkner et al., 2008). However, the mechanism through which CD4 T cells accomplish pathogen clearance is still not understood. Although antibodies do seem to offer some protection against infection, it is unlikely that a humoral response alone is sufficient for control of this pathogen (Sun et al., 1997). Lastly, although B cells are not required for pathogen clearance, they could play a role in preventing the death of T-cell deficient mice following infection (Birkner et al., 2008). Therefore, the development and design of control measures like vaccines should take these immune factors that are essential for pathogen clearance into account.

Table 1.3. Summary of murine host immune responses after infection with *Anaplasma phagocytophilum*. The ability of the mice to clear infection with *A. phagocytophilum* in the absence of certain immune components/molecules are indicated.

Murine model	Immune component knocked out	Immune component essential for pathogen clearance	Reference
SCID ¹⁴	B and T cells	Yes	(von Loewenich et al., 2004)
iNOS ¹⁵ deficient	iNOS	No	
phox deficient	phox		
TNF ¹⁶ deficient	TNF		
TLR2 ¹⁷ deficient	TLR2		
MyD88 ¹⁸ deficient	MyD88		
Fas/FasL ¹⁹ deficient	Fas/FasL		
Perforin deficient	Perforin		
IL ²⁰ -12 deficient	IL-12		
IFN- γ ²¹ deficient	IFN- γ		
MCP-1 ²² deficient	MCP-1		
CD4 T cell deficient	CD4 T cells	Yes	

1.3. Parasite and pathogen control: developing the next generation of drug and prophylactic treatments.

1.3.1. Tick control: the status quo and new directions

Currently, chemical acaricides remain the main form of tick control, since it is widely applicable and relatively affordable (Table 1.4). However, ticks have very short generational times, which in conjunction with the misuse/overuse of acaricides promotes the rapid development of resistance against most acaricide classes soon after its introduction, as reviewed by (Obaid et al., 2022). Consequently, several alternative control strategies have been investigated. However, most of these strategies still have limited field efficacy and cannot replace acaricides (Table 1.4). Therefore, chemical control at this stage must be supplemented with alternative tick control strategies. Moreover, it has been demonstrated that the simultaneous use of acaricides along with tick vaccines is more effective than either alone (Arocho Rosario et al., 2022). Consequently, tick vaccines are the most promising complementary control measure identified thus far (Table 1.4).

¹⁴ SCID – severe combined immunodeficiency

¹⁵ iNOS – Inducible nitric oxide synthase

¹⁶ TNF – Tumour necrosis factor

¹⁷ TLR2 – Toll-like receptor 2

¹⁸ MyD88 – Myeloid differentiation primary response 88 protein

¹⁹ FasL – Fas ligand

²⁰ IL – Interleukin

²¹ IFN- γ – Interferon gamma

²² MCP-1 – Monocyte chemoattractant protein-1

Table 1.4. Summary of the benefits and drawbacks of different tick control strategies.

Tick Control Strategy	Pro(s)	Con(s)	Reference(s)
A] Chemical acaricides	-Affordable -Universal cross-protection	-Not environmentally friendly -Reduced efficacy due to increasing resistance in ticks -Susceptible to weather conditions, i.e., dilution of acaricide dip due to rainfall	(Beltrán Zapa et al., 2020; Chevillon et al., 2007; Obaid et al., 2022; Santos et al., 2018)
B] Vaccines (i.e., live, subunit, particle, peptide etc.)	-Environmentally friendly -Promising <i>in vitro</i> and <i>in vivo</i> efficacy - Anamnestic responses that offer long-term protection	-No universal cross-protection (antigenic diversity) -Cold-chain requirements -Requires multiple doses	(Abbas et al., 2023; Kasaija et al., 2023; Parham, 2015)
C] Biological control (i.e., entomopathogenic fungi and bacteria, ectoparasite eating birds, parasitoid wasps and flies, and nematodes etc.)	-Has shown efficacy in reducing tick populations in controlled environmental studies	-Challenges with application in natural settings -Does not affect all life stages of ticks -Ethical and environmental concerns with using GMO's -Production of inundative quantities would be prohibitively expensive as a control measure	(Nepveu-Traversy et al., 2024; Plantan et al., 2013; Ramos et al., 2023)
D] Environmental control (i.e., tick traps/tick tubes and habitat management)	-User friendly	-Limited to ticks affecting small animals i.e., rodents/mice	(Diuk-Wasser et al., 2021; Jordan et al., 2022; Yans et al., 2022)
E] Essential oils (EOs) and isolated compounds (EOCs)	-Promising <i>in vitro</i> results	-Lack of field trials corroborating <i>in vitro</i> efficacy studies -Reduced cost-effectiveness due to required high concentrations -Potential side effects (behavioural, health and welfare) -Limited market availability	(Gonzaga et al., 2023)

Tick Control Strategy	Pro(s)	Con(s)	Reference(s)
F] Genetic control (i.e., acquired tick resistance, breeding of livestock for resistance)	-Resistance can be inherited -Reduced labour required in long term	-Difficult to phenotype resistance -Costly to identify individual variation in resistance -Acquired tick resistance not fully understood	(Cardoso et al., 2021)
G] Paratransgenic/ RNAi control	-Results show ticks are good candidates due to their slow feeding and digestion.	-Delivery of recombinant bacteria to midgut of tick would be challenging, especially in field conditions -Not tested yet in ticks in trials for efficacy	(Koosha et al., 2019)
H] Inert dusts/desiccants (i.e., Kaolin, Silica gel/dust, perlite etc.)	-Low risk application (no toxicity to vertebrates) -Long-lasting -High efficacy against immature ticks	-Most effective before feeding starts -Slow action compared to acaricides -Less effective after being wetted	(Showler and Saelao, 2022)
I] Growth inhibitors (i.e., Fluzuron, Novaluron, Pyriproxyfen etc.)	-High efficacy against ticks	-Variable results suggest more testing is required (Novaluron, Pyriproxyfen) -Possible contamination of milk with fluzuron, which is problematic given its genotoxicity in humans	(Ribeiro et al., 2019; Showler and Saelao, 2022; Suárez et al., 2021)
J] Anti-tick microbiota vaccines	-Manipulates tick microbiome against tick-transmitted pathogens -No negative side effects on host	-Severely limited efficacy data (only tested in mice)	(Mateos-Hernández et al., 2020; Mateos-Hernández et al., 2021; Wu-Chuang et al., 2023)

Numerous tick vaccine candidate antigens have been investigated in the search for the universal tick vaccine that offers cross-protection against multiple tick species (Table 1.5). However, many of these vaccines are plagued by low efficacies, limited cross-protection and requires multiple doses.

Table 1.5. List of tick vaccine antigen candidates being studied in the clinical and pre-clinical stages evaluated in cattle. Table was modified from Abbas et al. (2023) and Nepveu-Traversy et al. (2024) and updated. The tick species each antigen was derived from is included in parenthesis in the 'antigen candidate' column and the tick species it was tested against in trials is listed in the 'species of tick' column.

Antigen Candidate	Characterization	Vaccination Regime	Dose per injection	Adjuvant	Tick species	Breed	Efficacy in bovine trials	Reference(s)
Egg-associated antigens								
A] BYC (<i>R. microplus</i>)	Boophilus yolk pro-cathepsin	Four vaccinations with two-week intervals	100 µg	Quil-A®	<i>R. microplus</i>	Hereford (6-month-old)	Total efficacy: 14-36%	(da Silva Vaz et al., 1998)
B] VTDCE (<i>R. microplus</i>)	Vitellin-degrading cysteine endopeptidase	Five vaccinations with 10-day intervals (test group received 5 boosters after challenge)	1 ml VTDCE (100 µg)	Marcol 52 (Exxon Mobil Corporation, USA) and Montanide 888 (Seppic, France)	<i>R. microplus</i>	Hereford (14-month-old)	Total efficacy: 21%	(Seixas et al., 2008)
C] BmTI (<i>R. microplus</i>)	<i>R. microplus</i> trypsin inhibitor	Three vaccinations with three-week intervals	100 µg	Freund's complete adjuvant	<i>R. microplus</i>	Nelore calves (6-month-old)	Total efficacy: 72.80%	(Andreotti et al., 2002)
D] Synthetic BmTI N-terminal fragment (<i>R. microplus</i>)	<i>R. microplus</i> trypsin inhibitor	Three vaccinations with three-week intervals	100 µg	1.5 mg Saponin in deionized distilled water	<i>R. microplus</i>	Nellore x Simmental (8-month-old)	Total efficacy: 18.4%	(Andreotti, 2007)
E] rRmLTI (<i>R. microplus</i>)	Recombinant <i>R. microplus</i> larvae trypsin inhibitor	Three vaccinations at days 0, 15 and 30	100 µg	Montanide ISA 61 VG (Seppic, Paris)	<i>R. microplus</i>	Holstein Calves (1-year-old)	Total efficacy: 32%	(Andreotti et al., 2012)

Antigen Candidate	Characterization	Vaccination Regime	Dose per injection	Adjuvant	Tick species	Breed	Efficacy in bovine trials	Reference(s)
Gut associated antigens								
F] Bm95 (<i>R. microplus</i>)	<i>R. microplus</i> 95, peptidase	Three vaccinations at weeks 0, 4 and 7	100 µg	Montanide 888 (Seppic, France)	<i>R. microplus</i>	Holstein calves (1-year-old)	Total efficacy: 85-89%	(García-García et al., 2000)
	Recombinant <i>R. microplus</i> 95 protein	Four vaccinations with 4-week intervals	200 µg	Algel (Indian Immunological Ltd., India)	<i>R. microplus</i>	Crossbred calves (<6-month-old)	Total efficacy: 81.27%	(Kumar et al., 2009)
G] SBm4912, SBm7462, SBm19733 (<i>R. microplus</i>)	Synthetic Bm86 derived peptides	Three vaccinations with on days 1, 30 and 60	2 mg	1.5 mg saponin in 4 ml distilled deionized water	<i>R. microplus</i>	Jersey-breed (8–10-month-old)	Total efficacy: SBm4912: 72.4% SBm7462: 81.05% SBm19733: 35.87%	(Patarroyo et al., 2002)
H] Bm7462® (<i>R. microplus</i>)	Synthetic Bm86 epitope construct peptide	Three vaccinations with on days 1, 30 and 60	2 mg	1.5 mg saponin in 4 ml distilled deionized water	<i>R. microplus</i>	Holstein Friesian calves (6-10-month-old)	Total efficacy: 72.4%	(Patarroyo et al., 2020)
I] Ba86 (<i>Rhipicephalus annulatus</i>)	<i>R. annulatus</i> 86 protein (Bm86 homolog)	Three vaccinations at weeks 1, 3 and 7	100 µg	Montanide ISA 50 V (Seppic, France)	<i>R. microplus</i>	Crossbred calves	Total efficacy: 71.5% ²³	(Canales et al., 2009)

²³ Cross-protection observed above 60%

Antigen Candidate	Characterization	Vaccination Regime	Dose per injection	Adjuvant	Tick species	Breed	Efficacy in bovine trials	Reference(s)
J] Haa86 (<i>H.a. anatolicum</i> ²⁴)	<i>H. a. anatolicum</i> 86 protein (Bm86 homolog)	Three vaccinations with on days 0, 30 and 60	100 µg	10% Montanide 888 (Seppic, France) in mineral oil	<i>H. a. anatolicum</i> and <i>R. microplus</i>	<i>Bos taurus</i> x <i>Bos indicus</i> (10-12-month-old)	Total efficacy: <i>R. microplus</i> : 36.5% <i>H. a. anatolicum</i> larvae: 68.7% <i>H. a. anatolicum</i> adults: 45.8%	(Kumar et al., 2012)
K] TROSPA (<i>R. microplus</i>)	Tick receptor for outer surface protein A	Three vaccinations on days 0, 28 and 49	100 µg	Montanide ISA 50 V (Seppic, France)	<i>R. microplus</i>	Calves	No effect	(Merino et al., 2013)
L] RmAQP1 (<i>R. microplus</i>)	Aquaporin 1	Three vaccinations with two-week intervals	100 µg	Montanide ISA 61 VG (Seppic, France)	<i>R. microplus</i>	Holstein calves (1-year-old)	Total efficacy: 68-75%	(Guerrero et al., 2014)
M] RmAQP2 peptides (<i>R. microplus</i>)	Synthetic Aquaporin 2 peptides	Four vaccinations on days 0, 21, 42, and 63	0.05 mg of each peptide	Peptides conjugated to keyhole limpet hemocyanin (KLH); 0.75 mg Quil-A (saponin) in 0.5 ml	<i>R. microplus</i>	Holstein calves (≥ 2-month-old)	Total efficacy: 25%	(Scoles et al., 2022)

²⁴ *H. a. anatolicum* – *Hyalomma anatolicum anatolicum*

Antigen Candidate	Characterization	Vaccination Regime	Dose per injection	Adjuvant	Tick species	Breed	Efficacy in bovine trials	Reference(s)
N] rGST-HI (<i>H. longicornis</i> ²⁵)	Glutathione S-transferase	Six vaccinations with 15-day intervals	1 st /2 nd /3 rd /4 th dose: 200 µg; 5 th /6 th dose: 400 µg	Montanide 888 (Seppic, France) and Marcol 52 (Exxon Mobil Corporation, USA)	<i>R. microplus</i>	Cattle	Total efficacy: 57%	(Parizi et al., 2011)
O] ATAQ peptide (<i>R. microplus</i>)	Bm86 homolog peptide	Three vaccinations at days 0, 15 and 30	200 µg	Peptide conjugated to KLH; Montanide ISA 61 VG (Seppic, France)	<i>R. microplus</i>	Holstein-Friesian (12-18-month-old)	Total efficacy: 35%	(Aguirre Ade et al., 2016)
P] 5'-nucleotidase (<i>R. microplus</i>)	Membrane-bound ectoenzyme	Three vaccinations at 1-month intervals	100 µg	Quil-A and ISA50	<i>R. microplus</i>	Hereford x Angus (9-month-old)	No effect	(Hope et al., 2010)
Salivary gland associated antigens								
Q] Bm91 (<i>R. microplus</i>)	<i>R. microplus</i> 91	Four vaccinations at weeks 0, 3, 6 and 26	200 µg	Montanide ISA 50 V (Seppic, France)	<i>R. microplus</i>	Lamphun (3-year-old)	Reduction in reproductive efficiency index (6%) and egg viability (8%) compared to control	(Lambertz et al., 2012)

²⁵ *H. longicornis* - *Haemaphysalis longicornis*

Antigen Candidate	Characterization	Vaccination Regime	Dose per injection	Adjuvant	Tick species	Breed	Efficacy in bovine trials	Reference(s)
R] SILK (<i>R. microplus</i>)	Flagelliform silk protein	Three vaccinations on days 0, 28 and 49	100 µg	Montanide ISA 50 V (Seppic, France)	<i>R. microplus</i>	Calves	Total efficacy: 62%	(Merino et al., 2013)
S] pP0 (<i>R. microplus</i> and <i>R. sanguineus</i>)	Ribosomal protein P0 peptide	Four vaccinations on days 0, 21, 36 and 60	500 µg	Peptide conjugated to KHL; VG Montanide 888 (prepared to 10% in mineral oil)	<i>R. microplus</i>	Holstein (1-2-year-old)	Total efficacy: 96% ²⁶	(Rodríguez-Mallon et al., 2015)
T] UBQ (<i>R. microplus</i>)	Ubiquitin	Three vaccinations at weeks 1, 4 and 6	100 µg	Montanide ISA 50 V (Seppic, France)	<i>R. microplus</i>	European crossbred calves (1-year-old)	Total efficacy: 55%	(Almazán et al., 2010)
Other antigens								
U] rHa-CRT (<i>H. anatolicum</i>)	Calreticulin	Three vaccinations at days 0, 28 and 49	100 µg	Montanide ISA 50 V (Seppic, France)	<i>H. anatolicum</i> and <i>R. microplus</i>	<i>Bos indicus</i> x <i>Bos taurus</i>	Total efficacy against <i>H. anatolicum</i> : 41.3% Total efficacy against <i>R. microplus</i> : 37.6%	(Kumar et al., 2017)

²⁶ Cross-protection observed above 60%

Antigen Candidate	Characterization	Vaccination Regime	Dose per injection	Adjuvant	Tick species	Breed	Efficacy in bovine trials	Reference(s)
VJ rHa-CathL (<i>H. anatolicum</i>)	Cathepsin L-like cysteine proteinase	Three vaccinations at days 0, 28 and 49	100 µg	Montanide ISA 50 V (Seppic, France)	<i>H. anatolicum</i> and <i>R. microplus</i>	<i>Bos indicus</i> x <i>Bos taurus</i>	Total efficacy against <i>H. anatolicum</i> : 30.2% Total efficacy against <i>R. microplus</i> : 22.2%	(Kumar et al., 2017)
WJ RmFER2 (<i>R. microplus</i>)	Ferritin 2	Three vaccinations at weeks 1, 4 and 6	50 µg	Montanide ISA 50 V (Seppic, France)	<i>R. microplus</i> and <i>R. annulatus</i>	European crossbred calves	Total efficacy against <i>R. annulatus</i> : 72% ²⁷ Total efficacy against <i>R. microplus</i> : 64%	(Hajdusek et al., 2010)
XJ IrFER2 (<i>I. ricinus</i>)	Ferritin 2	Two vaccinations with 6-week intervals	100 µg	1.5 mg saponin with Montanide ISA 50 V (Seppic, France)	<i>I. ricinus</i>	Holstein-Friesian (6-month-old)	No effect	(Knorr et al., 2018)

²⁷ Cross-protection observed above 60%

Antigen Candidate	Characterization	Vaccination Regime	Dose per injection	Adjuvant	Tick species	Breed	Efficacy in bovine trials	Reference(s)
Y] rHaFER2 (<i>H. anatolicum</i>)	Ferritin 2	Three vaccinations on days 0, 28 and 49	450 µg	Montanide ISA 50 V (Seppic, France)	<i>H. anatolicum</i>	<i>Bos indicus</i> x <i>B. taurus</i>	Total efficacy: 51.7% against larvae 51.2% against adults	(Manjunathach ar et al., 2019)
Z] rHaTPM (<i>H. anatolicum</i>)	Tropomyosin	Three vaccinations on days 0, 28 and 49	300 µg	Montanide ISA 50 V (Seppic, France)	<i>H. anatolicum</i>	<i>Bos indicus</i> x <i>B. taurus</i>	Total efficacy: 63.7% against larvae 66.4% against adults	(Manjunathach ar et al., 2019)
Multi-antigen combinations								
AA] Rm39 + Rm76 + Rm180 + Rm239 (<i>R. microplus</i>)	Recombinant <i>R. microplus</i> proteins 39, 76, 180, 239	Three vaccinations on days 0, 21 and 42	Rm76: 25 µg Rm39, Rm180, Rm239: 100 µg	Aluminium hydroxide 1:8 (protein:adjuvant) ratio	<i>R. microplus</i>	Holstein calves (3–7-month-old)	Total efficacy: 73.2%	(Maruyama et al., 2017)
AB] Bm95-MSP1a (<i>R. microplus</i>)	<i>R. microplus</i> 95-Major surface protein 1a	Three vaccinations at weeks 0, 4, and 8	120 µg	Montanide ISA 50 V (Seppic, France)	<i>R. microplus</i>	Beefmaster x Charolais (6-month-old)	Total efficacy: 64%	(Almazán et al., 2012)
AC] RmLTI-BmCG-LTB (<i>R. microplus</i>)	<i>R. microplus</i> larvae trypsin inhibitors, <i>B. microplus</i> Campo grande heat-labile enterotoxin B chimera protein	Three vaccinations with 15-day intervals	200 µg	Montanide ISA 61 VG (Seppic, France)	<i>R. microplus</i>	Angus heifers (3–6-month-old)	Total efficacy: 55.6%	(Csordas et al., 2018)

Antigen Candidate	Characterization	Vaccination Regime	Dose per injection	Adjuvant	Tick species	Breed	Efficacy in bovine trials	Reference(s)
AD] UBQ-MSP1a (<i>R. microplus</i>)	Ubiquitin-major surface protein 1a	Three vaccinations at weeks 0, 4, and 8	120 µg	Montanide ISA 50 V (Seppic, France)	<i>R. microplus</i>	Beefmaster x Charolais (6-month-old)	Total efficacy: not determined	(Almazán et al., 2012)
AE] Q38 (<i>R. microplus</i>)	<i>R. microplus</i> SUB-AKR chimera	Three vaccinations on days 0, 28 and 49	100 µg	Montanide ISA 50 V (Seppic, France)	<i>R. microplus</i>	Calves	Total efficacy: 75%	(Merino et al., 2013)
AF] EF1a-MSP1a (<i>R. microplus</i>)	Elongation factor 1 alpha-Major surface protein 1a	Three vaccinations at weeks 0, 4, and 8	120 µg	Montanide ISA 50 V (Seppic, France)	<i>R. microplus</i>	Beefmaster x Charolais (6-month-old)	Total efficacy: 38%	(Almazán et al., 2012)
AG] BrRm-MP4 (<i>R. microplus</i>)	<i>R. microplus</i> salivary gland metalloprotease 4	Four vaccinations with 15-day intervals	1 st and 2 nd doses: 100 µg 3 rd and 4 th doses: 200 µg	Montanide 888 (Seppic, France) and Marcol 52 (Exxon Mobil Corporation, USA)	<i>R. microplus</i>	Calves (18-month-old)	Total efficacy: 60%	(Ali et al., 2015)

Briefly, vaccines should ideally have the following qualities: (1) The components of a vaccine formulation should physically access the lymph node or spleen to elicit an immune response through interaction with T and B cells that circulate in those organs (Moyer et al., 2016), (2) Vaccines should induce long-term protective immunity (i.e., epitope identification improves immunogenicity and (3) vaccines should be accessible (i.e., cost, number of doses, route of administration etc.) (Amanna and Slifka, 2020; Parvizpour et al., 2020; Pollard and Bijker, 2021). Therefore, tick vaccines should be developed with these requirements in mind, as well as to overcome the previously identified shortcomings (Table 1.5).

1.3.2. Subolesin: A promising tick vaccine candidate

1.3.2.1. The evolution and conservation of Subolesin (tick akirin)

Subolesin is the functional homolog of the vertebrate specific Akirin 2 and is therefore also known as “tick akirin” (Macqueen and Johnston, 2009). Invertebrate Akirin typically has four exons and three introns, which coincides with the findings for Subolesin (Artigas-Jerónimo et al., 2018; Macqueen and Johnston, 2009). However, characterization of the Subolesin gene in *I. scapularis* uncovered five exons and four introns (Naranjo et al., 2013). This gene organization pattern has been found in *Dermacentor silvarum* ticks as well, but not in *Rhipicephalus sanguineus* ticks (de la Fuente et al., 2006c; Hu et al., 2014). Thus far, the Subolesin gene structure has not been elucidated in *R. microplus* (Ghosh et al., 2019; Octavio Merino-Charrez et al., 2019).

Furthermore, a characteristic N-terminal nuclear localization signal (NLS) motif is conserved in Type-2 Akirins (Artigas-Jerónimo et al., 2018; Goto et al., 2008; Macqueen and Johnston, 2009). Both NLSs were largely conserved in Subolesin, albeit with a few differences in a few binding sites (Artigas-Jerónimo et al., 2018) (Figure 1.4).



Figure 1.4. Amino acid sequence alignment of *Ixodes scapularis* Subolesin (Sub) and *Rattus norvegicus* Akirin 2 (Ak2) with accession numbers indicated. The red boxes indicate the NLS1, and the orange residues indicate extensions of the NLS domains. DNA binding sites (green and enclosed in a cyan box), binding sites 1-5 (bold underlined in Ak2 and cyan for Sub) are shown. Taken from Artigas-Jerónimo et al. (2018).

Moreover, DNA binding sites were also largely conserved between Akirin and Subolesin, albeit with a few residues that differed (Figure 1.4). Both Subolesin and its vertebrate homolog Akirin 2 has shown localisation to both the nucleus and cytoplasm (Antunes et al., 2014; Krossa et al., 2015; Pavithiran et al., 2018). Currently, there are no resolved Subolesin or akirin protein structures, however, Subolesin is predicted to have helical regions connected by highly disordered regions (Lagunes et al., 2016). Nevertheless, it is evident from Figure 1.5 that the Subolesin protein is highly conserved across most tick species. The high levels of conservation of Subolesin across tick species suggest that this protein has a crucial function in ticks.

		Rhipicephalus										Ixodes				Dermacentor				Amblyomma						Hyalomma			Haemaphysalis			Omitochondros spp.							
		GG4622.1_subolesin_Reversi	MM6231.1_subolesin_Rappendiculatus	AB28974.1_subolesin_Rmicroplus	GG4621.1_subolesin_Rangitaneus	AKM12409.1_subolesin_Rhaemaphysaloides	GG4620.1_subolesin_partial_Rannulatus	GG4619.1_subolesin_partial_Rectoratus	AA58806.1_subolesin_Rpudchellus	AAV6703.1_subolesin_Iscapularis	AY5547.1_subolesin_Ipersulatus	GG4693.1_subolesin_Ikonus	AF9698.1_subolesin_Iscapularis	AY5259.1_subolesin_Dolium	AY5673.1_subolesin_Dolium	PH1054.1_subolesin_Dmagnatus	AY6734.2_subolesin_Dvariabilis	GG4623.1_subolesin_partial_Dreticulatus	GG4601.1_subolesin_partial_Avariegatum	ABY845.1_subolesin_partial_Ahebraeum	GG4694.1_subolesin_Acajenense	GG4695.1_subolesin_Americanum	GG4601.1_subolesin_partial_Amaculatum	AT9326.1_subolesin_Aureolatum	AC3262.1_subolesin_Atriste	AU0371.1_subolesin_Ascutulum	MM7011.1_subolesin_Hanaticum	GG4625.1_subolesin_partial_Hufipes	MM6233.1_subolesin_partial_Hmagnatum	MM7677.1_subolesin_Hexavatum	ACA804.1_subolesin_partial_Hongicornis	GG4626.1_subolesin_Helipica	ACA097.1_subolesin_Hqinghaiensis	AU2155.1_subolesin_Hflava	MM6236.1_subolesin_Hpunctata	GG4628.1_subolesin_Omoubata	GG4627.1_subolesin_Oswignyi	MM6605.1_subolesin_partial_Oerraticus	
Rhipicephalus	AG4622.1_subolesin_Reversi	100	98.76	99.38	98.14	97.52	90.06	90.68	99.38	77.72	77.72	77.72	74.35	80.57	93.33	93.33	92.12	82.42	78.53	78.53	76.84	77.97	77.97	83.71	85.88	86.29	90.8	83.44	83.44	92.64	59.22	84.57	82.12	82.12	80.45	78.61	77.59	67.04	
	ABA6231.1_subolesin_Rappendiculatus		100	98.14	98.14	97.52	88.82	89.44	99.38	77.17	77.17	77.17	73.82	80.57	93.33	93.33	91.52	81.82	77.97	77.97	76.27	77.4	77.4	83.71	85.31	85.71	90.8	83.44	83.44	92.64	59.22	84	82.12	82.12	80.45	78.61	77.59	67.04	
	AB28974.1_subolesin_Rmicroplus			100	97.52	96.89	90.68	91.3	98.76	77.17	77.17	73.82	80	92.73	92.73	92.73	91.52	81.82	77.97	77.97	76.27	77.4	77.4	83.15	85.31	85.71	90.18	82.82	82.82	92.02	58.66	84	81.56	81.56	79.89	78.03	77.01	66.48	
	AG4621.1_subolesin_Rangitaneus				100	98.76	88.2	88.82	98.76	77.72	77.72	73.82	80.57	93.94	93.94	93.94	92.12	82.42	76.84	76.84	75.14	76.27	76.27	82.58	84.18	84.57	90.8	83.44	83.44	92.64	59.22	85.14	81.56	81.56	79.89	78.61	77.59	67.04	
	AKM12409.1_subolesin_Rhaemaphysaloides					100	87.58	88.2	98.14	77.72	77.72	73.82	80.57	92.73	92.73	92.73	90.91	81.21	76.27	76.27	74.58	75.71	75.71	82.02	83.62	84	90.8	83.44	84.05	92.64	58.66	84.57	82.68	82.68	81.01	78.61	77.59	67.6	
	AG4620.1_subolesin_partial_Rannulatus						100	99.32	89.44	69.02	69.02	69.02	65.97	71.43	83.64	83.64	83.64	81.03	88.74	84.05	84.05	82.21	81.82	83.44	74.72	76.84	77.14	81.6	90.6	90.6	83.44	63.03	75.43	73.18	73.18	71.51	70.52	69.54	72.12
	AG4619.1_subolesin_partial_Rectoratus							100	90.06	69.57	69.57	69.57	66.49	72	84.24	84.24	84.24	81.64	89.4	84.66	84.66	82.82	82.42	84.05	75.28	77.4	77.71	81.6	90.6	90.6	83.44	63.64	76	73.74	73.74	72.07	70.52	69.54	72.12
	AA58806.1_subolesin_Rpudchellus								100	77.72	77.72	77.72	74.35	80.57	93.94	93.94	93.94	92.12	81.82	77.97	77.97	76.27	77.4	77.4	83.71	85.31	85.71	90.18	84.05	84.05	93.29	59.22	84.57	82.12	82.12	80.45	78.61	77.59	67.04
	AAV6703.1_subolesin_Iscapularis									100	99.46	98.91	85.86	88.04	80.43	80.43	80.43	80.43	72.28	73.37	73.91	72.83	73.91	74.46	80.98	82.07	80.98	77.72	71.2	71.74	79.35	60.33	82.61	83.15	83.15	80.43	76.63	77.72	68.48
	AY5547.1_subolesin_Ipersulatus										100	99.46	98.91	85.86	88.04	80.43	80.43	80.43	72.28	73.37	73.91	72.83	73.91	74.46	80.98	82.07	80.98	77.72	71.2	71.74	79.35	60.33	82.61	83.15	83.15	80.43	76.63	77.72	68.48
AG4693.1_subolesin_Ikonus											100	99.46	98.91	85.86	88.04	80.43	80.43	72.28	73.37	73.91	72.83	73.91	74.46	80.98	82.07	80.98	77.72	71.2	71.74	79.35	60.33	82.61	83.15	83.15	80.43	76.63	77.72	68.48	
Dermacentor	AF9698.1_subolesin_partial_Iariandinae												100	84.29	76.96	76.96	76.96	67.54	71.2	71.73	70.16	71.2	71.73	78.01	79.06	74.35	68.06	68.95	75.82	59.16	79.58	81.15	81.15	79.58	72.25	72.77	65.45		
	AG4694.1_subolesin_Ihexagonus													100	83.43	83.43	83.43	83.43	74.29	74.44	75	73.33	74.44	75	82.22	82.78	81.67	80.57	73.71	74.29	82.29	61.33	83.98	84.53	84.53	82.87	79.43	80	68.89
	AF5259.1_subolesin_Dolium														100	100	100	98.18	86.67	82.49	81.92	80.79	81.36	81.36	88.2	89.27	90.29	94.55	87.27	87.27	96.36	61.11	86.93	85	85	83.33	82.66	81.61	70.39
	AL5673.1_subolesin_Dolium															100	100	98.18	86.67	82.49	81.92	80.79	81.36	81.36	88.2	89.27	90.29	94.55	87.27	87.27	96.36	61.11	86.93	85	85	83.33	82.66	81.61	70.39
	APH1054.1_subolesin_Dmagnatus																100	98.18	86.67	82.49	81.92	80.79	81.36	81.36	88.2	89.27	90.29	94.55	87.27	87.27	96.36	61.11	86.93	85	85	83.33	82.66	81.61	70.39
	AAV67034.2_subolesin_Dvariabilis																	100	86.06	81.92	81.36	80.23	80.79	80.79	87.08	88.14	89.14	93.94	87.27	87.27	95.76	60.56	85.8	83.89	83.89	82.22	82.66	81.61	70.95
	AG4623.1_subolesin_partial_Dreticulatus																		100	87.73	87.12	87.12	86.06	87.73	79.21	80.79	81.71	81.21	90.07	90.07	83.03	65.66	77.27	76.67	76.67	75	75.14	74.14	76.36
	AG4600.1_subolesin_partial_Avariegatum																			100	99.39	98.16	96.36	98.16	88.76	90.4	88.7	77.97	85.89	85.89	79.66	69.88	80.34	80.56	80	79.44	73.6	72.63	77.58
	ABY845.1_subolesin_partial_Ahebraeum																				100	97.55	95.76	95.55	88.2	89.83	88.14	77.4	85.28	85.28	79.1	70.48	80.9	81.11	80.56	80	73.03	72.07	78.18
	AG4699.1_subolesin_partial_Acajenense																					100	96.97	97.55	99.33	89.83	89.27	76.27	84.05	84.05	77.97	68.67	79.21	80	79.44	78.89	73.6	72.63	76.36
AG4695.1_subolesin_Americanum																						100	95.76	89.33	89.83	89.27	77.4	83.64	83.64	79.1	68.45	79.78	80.56	81.11	79.44	74.16	73.18	74.85	
Hyalomma	AG4601.1_subolesin_partial_Amaculatum																							100	88.2	92.09	88.7	76.84	84.66	84.66	78.53	69.28	80.34	80.56	80	78.89	73.6	72.63	76.97
	JAT9326.1_subolesin_Aureolatum																								100	96.07	95.51	83.71	76.97	76.97	58.39	63.89	86.59	89.44	88.89	88.33	82.02	81.01	70.95
	JAC3262.1_subolesin_Atriste																									100	96.61	84.75	77.97	77.97	86.84	63.89	88.2	88.33	87.78	86.67	80.9	79.89	70.95
	JA03174.1_subolesin_Ascutulum																										100	85.71	78.86	78.86	87.43	62.22	87.5	87.22	86.67	85.56	81.46	80.46	69.27
	AL8701.1_subolesin_Hanaticum																											100	88.96	88.96	98.16	57.78	82.95	81.67	81.67	80	80.35	79.31	68.16
	AG4625.1_subolesin_partial_Hufipes																												100	98.66	90.8	63.25	63.25	75	75	73.33	73.99	72.99	75.15
	AB6233.1_subolesin_partial_Hmagnatum																													100	90.8	63.25	76.14	75.56	75.56	73.89	74.57	73.56	75.54
	JAP6767.1_subolesin_Hexavatum																														100	58.89	84.66	83.33	83.33	81.67	82.08	81.03	69.83
	ACA8004.1_subolesin_partial_Hongicornis																															100	69.83	67.04	67.04	66.48	57.22	57.78	62.05
	AG4626.1_subolesin_Helipica																																100	91.26	91.06	88.83	79.33	79.44	71.11
Haemaphysalis	ACA0971.1_subolesin_Hqinghaiensis																																100	99.44	96.09	78.89	78.89	73.33	
	AIL0212.1_subolesin_Hflava																																	100	95.53	78.89	78.89	73.33	
	AB6236.1_subolesin_Hpunctata														</																								

1.3.2.2. The function of Subolesin

Subolesin was first identified in *I. scapularis* derived IDE8 derived tick cells as 4D8 and classified as an endopeptidase (Almazán et al., 2003). It was also identified as a promising protective antigen as it was associated with the inhibition of tick infestation in mice (Almazán et al., 2003). Since then, through several RNA interference (RNAi) studies it was demonstrated that Subolesin plays an essential role in tick reproduction, development and feeding (de la Fuente et al., 2006a; Kocan et al., 2007; Nandy et al., 2023; Nijhof et al., 2007). Furthermore, Subolesin is also thought to play a role in the arthropod IMD (immune deficiency) pathway as depicted in Figure 1.6. Although the exact function of Subolesin is still unknown, it is evident that Subolesin has many roles that are crucial in tick biology. Despite its intracellular localization, Subolesin progressed to immunization trials.

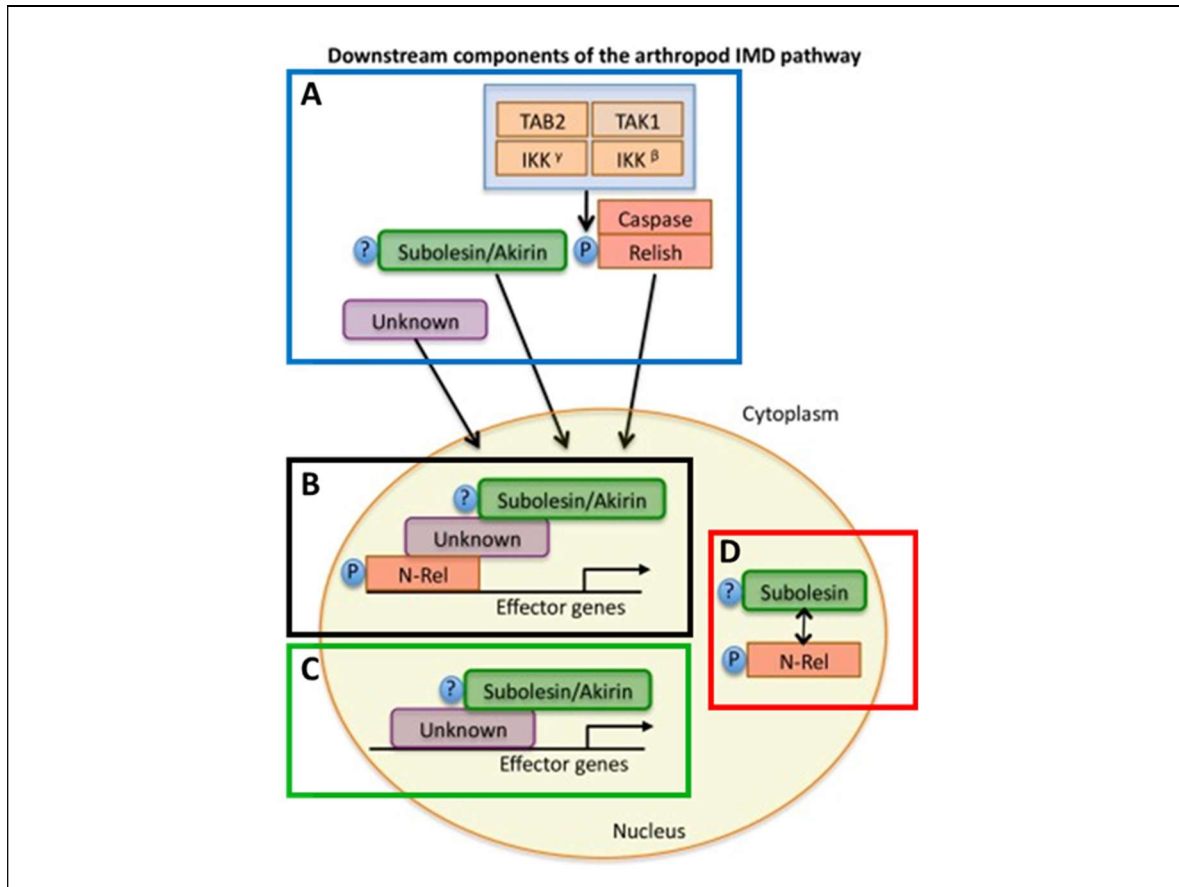


Figure 1.6. A model with simplified annotations for the role of Subolesin (tick akirin) in the downstream components of the arthropod IMD pathway. (A) The TGF-β activated kinase (TAK1), Tak1-binding protein (TAB2) and the I-κB kinase (IKK) complex are recruited after the arthropod IMD pathway is activated, resulting in the phosphorylation of Relish, a Nuclear Factor Kappa B (NF-κB) transcription factor. Thereafter the N-terminal domain of Relish (N-Rel) is cleaved by Caspase-8 homolog DREDD or a similar Caspase before translocation to the nucleus where it induces production of antimicrobial peptides. Subolesin/Akirin may be post-translationally modified and translocated to the nucleus. (B) In the nucleus, Subolesin/Akirin may interact with N-Rel unknown proteins and indirectly drive the production of anti-microbial peptides and other effector genes, or (C) Subolesin/Akirin could potentially act as a transcription factor and directly influence downstream effector genes. (D) N-Rel and Subolesin may be reciprocally regulated in ticks (Naranjo et al., 2013). Modified from Artigas-Jerónimo et al. (2018).

1.3.2.3. Subolesin as vaccine antigen: Outcome of vaccination trials

Vaccination of cattle with Subolesin followed by challenge with ticks has resulted in reduced tick infestations, weight, feeding to repletion, moulting from larvae to nymphs and oviposition in adult female ticks (Table 1.6). Although Subolesin did not offer better protection than Bm86 (gold standard) (Table 1.6.A), the combination of Subolesin and Bm86 in a different adjuvant increased efficacy to 97% (Table 1.6.E). Furthermore, vaccination with Subolesin derived from *R. microplus* demonstrated cross-protection against several tick species, including *R. decoloratus*, *R. appendiculatus* and *Hyalomma anatolicum* (Table 1.6.D and Table 1.6.L-M). However, the variability of efficacy between different breeds and tick species highlights the importance of antigen choice (Table 1.6.J). Nevertheless, high efficacies have been achieved using Subolesin in vaccines. However, given the intracellular location of Subolesin and its proposed function in the nucleus, the mechanism of action through which Subolesin elicits protection as a vaccine antigen remains unknown (Chapter 4).

Subolesin has also been shown to affect pathogen transmission of *Anaplasma marginale* (*A. marginale*), *A. phagocytophilum*, and *Borrelia burgdorferi* by ticks (Bensaci et al., 2012; de la Fuente et al., 2006b; de la Fuente et al., 2006d). Moreover, Subolesin has been used in a combination vaccine (SUB-MSP1a) targeting both *R. microplus* and *A. marginale* simultaneously, and demonstrated enhanced immunogenicity compared to the other immunization- and control groups (Contreras et al., 2015). However this was not tested in cattle and the efficacy was determined by measuring the weight of ticks (n=15) through capillary feeding on blood from immunized pigs (Contreras et al., 2015). In a similar trial using the same Subolesin-MSP1a chimeric antigen, significant reductions in tick infestation and pathogen infection levels were observed in both cattle and sheep (Torina et al., 2014). Therefore, Subolesin has potential as an antigen for developing pathogen transmission blocking vaccines.

Table 1.6. Overview of Subolesin-based vaccines evaluated in bovine vaccination trials listed in chronological order. The major antigen(s) (Ag) are indicated, as well as which tick species the respective Subolesin (SUB) antigen was derived from, as well as which tick species it was tested against in infestation trials.

Major antigen(s) (spp. sequence used for Ag production)	Tick spp. targeted in infestation trial	Breed	Adjuvant	Dose	Vaccination Regime	Efficacy in Bovine Trials	Reference(s)
A] SUB ²⁸ (<i>R. microplus</i>), UBQ ²⁹ , Bm86 ³⁰	<i>R. microplus</i>	European crossbred calves (1 year old)	Montanide ISA 50 V2 (Seppic, France)	100 µg	Three vaccinations at weeks 1, 4 and 6	Total efficacy: SUB: 51% UBQ: 55% Bm86: 60%	(Almazán et al., 2010)
B] SUB (<i>A. americanum</i> ³¹), Threonyl-tRNA synthetase (2C9), 60S ribosomal protein L13a (2D10), 60S ribosomal protein L13e (2B7), Interfase cytoplasmic foci protein 45 (2G7)	<i>A. americanum</i> nymphs and adult females	Crossbred calves	Montanide ISA 50 V2 (Seppic, France)	100 µg	Three vaccinations at weeks 0, 4, and 6	Total efficacy: SUB: 70% 2C9: 49% 2D10: 43% 2B7: 33% 2G7: 73%	(de la Fuente et al., 2010)
C] SUB (<i>R. microplus</i>)	<i>R. microplus</i> (control and SUB dsRNA injected larvae)	European crossbred calves (7 months)	Montanide ISA 50 V2 (Seppic, France)	100 µg	Three vaccinations on days 0, 28 and 49	Total efficacy: Vaccine alone + control ticks: 44% Vaccine + 100 % RNAi knockdown ticks: 75% Vaccine + 50% RNAi knockdown ticks: 22%	(Merino et al., 2011)

²⁸ SUB - Subolesin

²⁹ UBQ - ubiquitin

³⁰ Bm86 – *Boophilus microplus* antigen 86

³¹ *A. Americanum* – *Amblyomma americanum*

Major antigen(s) (spp. sequence used for Ag production)	Tick spp. targeted in infestation trial	Breed	Adjuvant	Dose	Vaccination Regime	Efficacy in Bovine Trials	Reference(s)
D] SUB-MSP1a ³² (<i>R. microplus</i>), EF-1 α ³³ -MSP1a, UBQ-MSP1a, BM95a ³⁴ -MSP1a	<i>R. microplus</i> and <i>R. annulatus</i>	Beefmaster x Charolais calves (6-month-old)	Montanide ISA 50 V2 (Seppic, France)	120 μ g	Three vaccinations at days 0, 30 and 60	Total efficacy against <i>R. microplus</i> : SUB-MSP1a: 81% EF1 α -MSP1a: 38% UBQ-MSP1a: N.D. BM95-MSP1a: 64% Total efficacy against <i>R. annulatus</i> : SUB-MSP1a: 67% EF1 α -MSP1a: N.D. UBQ-MSP1a: 22% BM95-MSP1a: 74%	(Almazán et al., 2012)
E] Bm86 + SUB (<i>R. microplus</i>)	<i>R. microplus</i> and <i>R. annulatus</i>	Hereford/Holstein (<i>Bos taurus taurus</i>) mixed breed	W/O: Saponin Quil-A® (water) in Montanide ISA 50 V2 (Seppic, France) (oil)	100 μ g	Three vaccinations at weeks 0, 3 and 6	REF: 97% against both tick spp.	(Schetters, 2014)
F] SUB (<i>R. microplus</i>)	<i>R. microplus</i>	Cross-bred male calves (6-8 months) (<i>Bos taurus</i> x <i>Bos indicus</i>)	10% Montanide 888 VG-mineral oil	100 μ g	Three vaccinations on days 0, 30, and 60	Total efficacy: First challenge: 44.1% Second challenge: 37.2%	(Shakya et al., 2014)

³² SUB-MSP1a - subolesin-major surface protein 1a

³³ EF-1 α - elongation factor-1 alpha

³⁴ Bm95 - *Boophilus microplus* antigen 95

Major antigen(s) (spp. sequence used for Ag production)	Tick spp. targeted in infestation trial	Breed	Adjuvant	Dose	Vaccination Regime	Efficacy in Bovine Trials	Reference(s)
G] SUB-MSP1a (<i>R. microplus</i>)	<i>Hyalomma</i> spp., <i>Rhipicephalus</i> spp. and <i>Haemaphysalis</i> spp.	Cattle	Montanide ISA 50 V2 (Seppic, France)	100 µg	Two vaccinations on days 0 and 30	32-39% reduction in tick weight of <i>Hyalomma</i> and <i>Haemaphysalis</i> spp. (Tick infestations were not reduced, but <i>R. microplus</i> was not found on cattle among infesting ticks)	(Torina et al., 2014)
H] SUB peptide (<i>R. microplus</i>)	<i>R. microplus</i>	Calves	Montanide ISA 50 V2 (Seppic, France)	100 µg	Two vaccinations at weeks 1 and 5	Reduction in tick number: 70% Reduction in egg hatching: 30% Reduction in tick weight and egg mass: N.S. ³⁵	(Lagunes et al., 2016)
I] SUB (<i>R. microplus</i>)	<i>R. microplus</i>	European crossbred calves	IV (heat inactivated <i>Mycobacterium</i> <i>bovis</i>)	200 µg	Two vaccinations at days 0 and 22	Total efficacy 65%	(Contreras et al., 2019)

³⁵ N.S. – not significant

Major antigen(s) (spp. sequence used for Ag production)	Tick spp. targeted in infestation trial	Breed	Adjuvant	Dose	Vaccination Regime	Efficacy in Bovine Trials	Reference(s)
J] SUBrd (<i>R. decoloratus</i>) + SUBra (<i>R. appendiculatus</i>) + SUBav (<i>A. variegatum</i>) combined (SUB combi) and individually	<i>R. appendiculatus</i>	<i>Bos indicus</i> and Crossbred (<i>Bos indicus</i> x <i>Bos taurus</i>)	Montanide ISA 50 V2 (Seppic, France)	100 µg	Three vaccinations at days 0, 30, and 60	Total efficacy in <i>Bos indicus</i> : SUBra: 47% SUBrd: 66% SUBav: 86% SUB combi: 92% Total efficacy in Crossbred: SUBra: 90% SUBrd: 89% SUBav: 83% SUB combi: 74%	(Kasaija et al., 2020)
	<i>A. variegatum</i>	<i>Bos indicus</i> and Crossbred (<i>Bos indicus</i> x <i>Bos taurus</i>)	Montanide ISA 50 V2 (Seppic, France)	100 µg	Three vaccinations at days 0, 30, and 60	Total efficacy in <i>Bos indicus</i> : SUBra: 50% SUBrd: 58% SUBav: 47% SUB combi: 51% Total efficacy in Crossbred: SUBra: 89% SUBrd: 94% SUBav: 76% SUB combi: 69%	
	<i>R. decoloratus</i>	Crossbred (<i>Bos indicus</i> x <i>Bos taurus</i>)	Montanide ISA 50 V2 (Seppic, France)	100 µg	Three vaccinations at days 0, 30, and 60	Total efficacy in Crossbred: SUBra: 51% SUBrd: 69% SUBav: 72% SUB combi: 71%	

Major antigen(s) (spp. sequence used for Ag production)	Tick spp. targeted in infestation trial	Breed	Adjuvant	Dose	Vaccination Regime	Efficacy in Bovine Trials	Reference(s)
K] Bm86 + pSUB ³⁶ (<i>R. microplus</i>)	<i>R. microplus</i>	<i>Bos taurus</i> x <i>Bos indicus</i> and 5/8 Holstein	Montanide ISA 50 V2 (Seppic, France)	100 µg	Three vaccinations at weeks 0, 4, and 9	Total efficacy: Peptide SUB alone: 67% Bm86 alone: 56% Peptide SUB + Bm86%: 49%	(Mendoza-Martínez et al., 2021)
L] SUB (<i>R. microplus</i>)	<i>R. decoloratus</i> <i>R. appendiculatus</i>	<i>Bos indicus</i> x <i>Bos taurus</i>	IV (heat inactivated <i>Mycobacterium bovis</i>)	200 µg	Two vaccinations at days 0 and 21	Total efficacy against <i>R. appendiculatus</i> : - SUB: 91% - SUB + IV: 94% Total efficacy against <i>R. decoloratus</i> : - SUB: 71% - SUB + IV: 96%	(Kasaija et al., 2022)
M] Bm86 + SUB (<i>H. anatolicum</i>) + tropomyosin (TPM)	<i>R. microplus</i>	<i>Bos taurus</i> x <i>Bos indicus</i>	Montanide ISA 50 V2 (Seppic, France)	100 µg	Three vaccinations at weeks 0, 4, and 8	Total efficacy: <i>H. anatolicum</i> larvae 1 st challenge: 86.62% <i>H. anatolicum</i> larvae 2 nd challenge: 88.24% <i>H. anatolicum</i> adults: 86.2% <i>R. microplus</i> larvae 1 st challenge: 88.9% <i>R. microplus</i> larvae 2 nd challenge: 84.6%	(Parthasarathi et al., 2023)

³⁶ pSUB - Subolesin peptide

1.3.3. Zoonotic anaplasmosis: Treatment and prophylaxes

The treatment of granulocytic anaplasmosis in humans primarily relies on tetracycline antibiotics, and most commonly doxycycline (Bakken and Dumler, 2015; Spickler, 2013). However, tetracycline treatment is not suitable in certain instances (e.g., pregnant women or individuals with a drug allergy), consequently patients are treated with Rifampin instead (Bakken and Dumler, 2015; Spickler, 2013). Similarly, animals afflicted with granulocytic anaplasmosis are also treated with tetracyclines, where oxytetracycline is used in ruminants as well as horses, whereas doxycycline is used in canines and felines (Atif, 2015; Stuen and Bergström, 2001).

Prophylaxes against *A. phagocytophilum* infection in humans include wearing protective clothing, using tick repellents and manually checking for and removing ticks (Spickler, 2013). Prophylaxes for livestock include the use of chemical acaricides in endemic areas, which mainly targets the tick vector (Stuen et al., 2013a) (Table 1.4). Rodent-targeted approaches have also been investigated (Table 1.4), however, the robustness of this method is still unknown (Eisen, 2023). Therefore, other control measures like vaccines are essential. Currently there are no commercial vaccines available for preventing transmission and infection of humans or animals with this pathogen (Contreras et al., 2017a; Stuen et al., 2013a). However, several antigens have been identified as potential targets for subunit vaccine development that have been tested in animal trials (Table 1.7). However, the protective effects for most vaccines tested have only been evaluated *in vitro*, and *in vivo* trials have demonstrated low levels of efficacy (Table 1.7). This highlights the gap in effective disease control and is mostly attributed to: (1) antigenic diversity of candidate antigens between different *A. phagocytophilum* strains (Stuen et al., 2013a) (2) immunogenic antigens and (3) *in vivo* testing of candidate antigens using suitable animal models.

Table 1.7. Overview of vaccines targeting *Anaplasma phagocytophilum* in chronological order.

Major antigen(s)	Animal model	Adjuvant	Dose	Vaccination Regime	Efficacy	Reference(s)
A] Formaldehyde inactivated <i>A. phagocytophilum</i>	Norwegian White Sheep	Montanide ISA 61VG (Seppic, France)	1 ml of 1×10^8 inactivated <i>A. phagocytophilum</i>	Two vaccinations at week 0 and 4	No protection	(Stuen et al., 2015)
B] MSP4 ³⁷ and HSP70 ³⁸	Latxa breed sheep	Montanide ISA 50 V2 (Seppic, France)	100 µg	Three vaccinations at days 0, 30 and 55	-MSP4-HSP70 immunized group: Decreased levels of <i>A. phagocytophilum</i> infected neutrophils (0% 10 days post infection)	(Contreras et al., 2017b)
C] VirB9-1 ³⁹ , Vir9-2, VirB10 plasmid DNA and recombinant proteins	C3H/HeN mice	15 µg of Quil-A® adjuvant (Sergeant Adjuvants, USA)	- 100 µg DNA plasmid - 100 µg recombinant protein	Heterologous prime boost: - Two plasmid DNA injections at weeks 0 and 2 - Two recombinant protein vaccinations at weeks 4 and 6	-Significant reduction of bacterial load in rVirB10 immunized group: Mean ± SEM peak bacterial load of 530 ± 159.9 , $P = 0.032$	(Crosby et al., 2018)
D] rAsp14 ⁴⁰ and rOmpA	Norwegian White Sheep	Montanide ISA 61VG (Seppic France)	1 mg of rAsp14 or rOmpA	Two vaccinations at days 0 and 21	No protection	(Eskeland et al., 2019)

³⁷ MSP4 – major surface protein 4

³⁸ HSP - Heat shock protein

³⁹ VirB9-1, Vir9-2, VirB10 – Components of the *A. phagocytophilum* Type 4 Secretion System (T4SS)

⁴⁰ rAsp14, rOmpA- *A. phagocytophilum* bacterial surface membrane proteins

Major antigen(s)	Animal model	Adjuvant	Dose	Vaccination Regime	Efficacy	Reference(s)
E] KLH ⁴¹ + and KLH conjugated peptides (OmpA59-74 ⁴² + Asp14113-124+ AipA9-21)	C57BL/6 mice	Alum-based Imject adjuvant (Thermo Fisher Scientific, USA)	- 100 µg KLH - 33.3 µg of each KLH-conjugated peptide	Three vaccinations on days 0, 21, and 42	-Ab's raised against Asp14113-124 and AipA9-21 peptides prevented <i>A. phagocytophilum</i> infection of myeloid cells	(Naimi et al., 2020)
F] Himar1 ⁴³ transformed live attenuated <i>A. phagocytophilum</i>	Norwegian White Sheep	-	1 ml	One inoculation (Day 0)	No clinical protection	(Eskeland et al., 2021)
G] MSP4 chimeric antigen	Sheep, Rabbits	Montanide ISA 50 V2 (Seppic, France)	100 µg 50µg	Three vaccinations on days 0, 20 and 55; weeks 0, 2 and 4	Antibody inhibition assays: -Peptide immunized sheep serum 7 arbitrary units of <i>A. phagocytophilum</i> levels in HL60 cells compared to 25 in control -Peptide immunized rabbit serum showed 11 arbitrary units of <i>A. phagocytophilum</i> levels in HL60 cells compared to 20 and 19 in controls	(de la Fuente et al., 2022)

⁴¹ KLH - keyhole limpet hemocyanin

⁴² OmpA59-74, Asp14113-124, AipA9-21 – KLH conjugated peptides mimicking OmpA, Asp14, and AipA adhesin domains

⁴³ Himar1 – DNA transposon element

1.3.4. The promising *Anaplasma phagocytophilum* vaccine candidate: MSP4

MSP4 is one of the six major surface proteins (MSPs) found in the outer membrane of the *Anaplasma spp.* bacterium (Contreras et al., 2017b). This protein is encoded by a single gene, with approximately 849 bp of coding region, and is highly conserved across most *Anaplasma* species (de la Fuente et al., 2005). Although the biological function of MSP4 is still unknown, it has been proposed that this protein plays a vital role in the bacterial infection of tick (vector) and vertebrate (host) cells (de la Fuente et al., 2001; de la Fuente et al., 2016; Dugat et al., 2015; Villar et al., 2015).

More specifically, MSP4 has been shown to play a role in facilitating the adhesion between *Anaplasma* bacterial cells and tick cells during infection (bacteria-tick cell interaction) (Villar et al., 2015). It has been demonstrated that *A. phagocytophilum* potentially uses a mechanism of increasing infection by elevating the activation of stress response proteins, which facilitates increased levels of bacteria-tick cell interactions and the protection of bacteria against stress (Villar et al., 2015). Heat shock proteins 60 and 70 (HSP60/GroEL and HSP70) may interact with MSP4 and bind to tick cells to increase tick-pathogen interactions, as they are localized on the bacterial membrane (Villar et al., 2015). Further testing concluded that antibodies against HSP70 and MSP4 inhibited the pathogenic infection of tick cells, which supports the previous finding that both these proteins are involved in bacteria-tick cell interactions (Table 1.7.B) (Contreras et al., 2017b; Villar et al., 2015). Similarly, immunization with a MSP4 chimeric antigen comprised of MSP4 epitopes reduced levels of *A. phagocytophilum* infection in human and tick cells as demonstrated through antibody inhibition assays (de la Fuente et al., 2022) (Table 1.7.G).

However, insights into MSP4's ability to offer effective *in vivo* protection against *A. phagocytophilum* infection are still lacking. Vaccination with MSP4 and HSP70 did not protect lambs from developing symptoms but did significantly lower the *A. phagocytophilum* DNA and infection levels in neutrophils at an accelerated rate, compared to both the MSP4 and control groups (Table 1.7.B). In addition, the MSP4 antigen has demonstrated serological cross-reactivity between *A. marginale* and *A. phagocytophilum* in bovine, ovine and equine sera in immunization trials against *A. marginale* (Dreher et al., 2005; Watthanadirek et al., 2019). Furthermore, the MSP4 antigen is commonly used in vaccines against the pathogens causing Malaria (*Plasmodium spp.*) and has demonstrated protection against lethal challenge in mice (Goschnick et al., 2004). Furthermore, MSP4 is a highly conserved protein (Figure 1.7).

	Host	Human			Dog			Bovine			Sheep			Horse		Deer						Chipmunk	Rat	Bison	Donkey		
	Host MSP4 Sample (accession, strain, country)	AAS99869.1_HZ_USA	AKZ20807.1_NY18_Spain	AHJ80824.1_Sic_HCase2012_Italy	KJV67070.1_ApNP_Austria	KJV64546.1_ApMUC09_Netherlands	AKZ20806.1_DogL610_Spain	AAX40464.1_Bovine-2654/1_Spain	AAS99873.1_Elsa_Switzerland	CEH11152.1_BOV-10_179_France	AEI91037.1_150S_Central Europe	ARO49593.1_AD50_Turkey	AKZ20808.1_Sheep_Spain	AAW32619.1_ovine 5_USA	AAS99871.1_MRK_USA	AAW32618.1_horse31_Germany	AAX40465.1_Roe-deer-1539/25_Spain	AAW32614.1_roe_Germany	AVH68965.1_d438_Hungary	QTZ21550.1_LU-A20_Poland	QTZ21549.1_P-C03_Poland	AAS99872.1_AP-V1_USA	KJZ99346.1_CR1007_USA	ABS32272.1_ZI-China_China	AAW32616.1_bison 12_Poland	AAW24032.1_Italy136_Italy	
Human	AAS99869.1_HZ_USA	100	99.65	98.58	100	100	99.65	100	99.29	100	99.29	97.16	98.94	98.94	99.65	100	98.58	91.84	98.58	99.29	98.58	99.29	100	91.13	99.65	99.65	
	AKZ220807.1_NY18_Spain		100	98.23	99.65	99.65	99.29	99.65	98.94	99.65	98.94	96.81	98.58	98.58	99.29	99.65	98.23	91.49	98.23	98.94	98.23	98.94	99.65	90.78	99.29	99.29	
	AHJ80824.1_Sic_HCase2012_Italy			100	98.58	98.58	98.23	98.58	99.29	98.58	99.29	96.45	97.52	97.52	98.23	98.58	97.87	90.78	97.16	98.58	97.16	97.87	98.58	90.07	98.94	98.23	
Dog	KJV67070.1_ApNP_Austria				100	100	99.65	100	99.29	100	99.29	97.16	98.94	98.94	99.65	100	98.58	91.84	98.58	99.29	98.58	99.29	100	91.13	99.65	99.65	
	KJV64546.1_ApMUC09_Netherlands					100	99.65	100	99.29	100	99.29	97.16	98.94	98.94	99.65	100	98.58	91.84	98.58	99.29	98.58	99.29	100	91.13	99.65	99.65	
	AKZ220806.1_DogL610_Spain						100	99.65	98.94	99.65	98.94	96.81	98.58	98.58	99.29	99.65	98.23	91.49	98.23	98.94	98.23	98.94	99.65	90.78	99.29	100	
Bovine	AAX40464.1_Bovine-2654/1_Spain							100	99.29	100	99.29	97.16	98.94	98.94	99.65	100	98.58	91.84	98.58	99.29	98.58	99.29	100	91.13	99.65	99.65	
	AAS99873.1_Elsa_Switzerland								100	99.29	99.29	97.16	98.23	98.23	98.94	99.29	98.58	91.49	97.87	99.29	97.87	98.58	99.29	90.78	99.65	98.94	
	CEH11152.1_BOV-10_179_France									100	99.29	97.16	98.94	98.94	99.65	100	98.58	91.84	98.58	99.29	98.58	99.29	100	91.13	99.65	99.65	
Sheep	AEI91037.1_150S_Central Europe										100	97.16	98.23	98.23	98.94	99.29	98.58	91.49	97.87	99.29	97.87	98.58	99.29	90.78	99.65	98.94	
	ARO49593.1_AD50_Turkey											100	97.87	97.87	96.81	97.16	98.23	91.84	97.52	97.16	97.52	98.58	97.16	91.13	96.81	96.81	
	AKZ220808.1_Sheep_Spain												100	100	98.58	98.94	99.65	92.55	99.65	98.94	99.65	97.16	98.94	91.84	98.58	98.58	
Horse	AAW32619.1_ovine 5_USA													100	98.58	98.94	99.65	92.55	99.65	98.94	99.65	98.94	98.94	91.84	98.58	98.58	
	AAS99871.1_MRK_USA														100	99.65	98.23	91.49	98.23	98.94	98.23	98.94	99.65	90.78	99.29	100	
	AAW32618.1_horse31_Germany															100	98.58	91.84	98.58	99.29	98.58	99.29	100	91.13	99.65	99.65	
Deer	AAX40465.1_Roe-deer-1539/25_Spain																100	92.55	99.29	98.58	99.29	98.58	98.58	91.84	98.23	98.23	
	AAW32614.1_roe_Germany																	100	92.2	91.84	92.2	92.2	91.84	91.84	91.49	91.49	
	AVH68965.1_d438_Hungary																		100	98.58	98.58	98.58	98.58	91.49	98.23	98.23	
	QTZ21550.1_LU-A20_Poland																			100	98.58	99.29	99.29	91.13	99.65	98.94	
	QTZ21549.1_P-C03_Poland																				100	98.58	98.58	91.49	98.23	98.23	
Chipmunk	AAS99872.1_AP-V1_USA																					100	99.29	91.49	98.94	98.94	
Rat	KJZ99346.1_CR1007_USA																							100	91.13	99.65	99.65
Bison	ABS32272.1_ZI-China_China																								100	90.78	90.78
Donkey	AAW32616.1_bison 12_Poland																									100	99.29
	AAW24032.1_Italy136_Italy																										100

Figure 1.7. Percentage of identity among MSP4 amino acid sequences from different *Anaplasma phagocytophilum* strains sampled from different hosts and geographical locations. Complete MSP4 sequences (282 aa) were obtained from The National Center for Biotechnology Information (NCBI). Representative sequences were chosen per host, strain and country. Sequences were aligned using MAFFT (Multiple Alignment using Fast Fourier Transform) v.7 server (Katoh et al., 2017) with default settings, except for the gap opening penalty which was increased to 5.0. The percent identity was calculated using Ident and Sim (Stothard, 2000).

It is evident that the amino acid sequence of MSP4 is highly conserved (>90%) across *A. phagocytophilum* strains from different hosts and geographical regions (Figure 1.7). Therefore, although the *in vivo* protective capacity of MSP4 requires further testing, the highly conserved nature of MSP4 and promising *in vitro* results make it a promising vaccine candidate.

1.4. Micro-vaccines: a customisable multi-purpose platform

Traditional subunit vaccines contain components or purified pieces of the targeted pathogen which have been selected due to their immunogenicity, i.e., recombinant proteins (Kozak and Hu, 2023). Although the methods for designing subunit vaccines are well established, there is still room for improvement (Table 1.8.A). Microparticle based vaccines overcome some of these problems, such as the low immunogenicity and unstable nature of traditional subunit vaccines (Table 1.8.B). Therefore, microparticle based vaccines are an attractive alternative to traditional subunit vaccines.

Table 1.8. Advantages and disadvantages of traditional subunit vaccines and microparticle-based vaccines.

Vaccine type	Pro(s)	Con(s)	Reference(s)
A] Subunit	- Scalable in production - Readily modifiable -High safety profile	-Low immunogenicity without adjuvant -Identification of suitable antigens, development and manufacture are typically time-consuming -Unstable formulations and cold-chain requirements -Expression systems are often not feasible (lack of post-translational modifications and folding requirements)	(Celis-Giraldo et al., 2021; Kozak and Hu, 2023)
B] Microparticle	-Self-adjuvanting -Stable at room temperature -Sustained release of antigens -Protects fragile proteins from degradation -Highly customisable	-Problems with scaling up production -Formulation reproducibility issues	(Hua et al., 2021; Kohane, 2007; Leclercq et al., 2011; Lim et al., 2022; Maina et al., 2020)

Micro-vaccines are based on microparticles, which are typically between 1 and 1000 µm in size (da Silva et al., 2023; Gheibi Hayat and Darroudi, 2019; Zhao et al., 2014), and can be

synthesised from several materials including (but not limited to), lipids, polymers and metals (Celis-Giraldo et al., 2021; Pudlarz and Szemraj, 2018). These particles have three main roles in vaccinology, namely act as (1) adjuvants; (2) carriers for antigens or other immunostimulatory substances; and (3) as antigen presentation platforms (Celis-Giraldo et al., 2021; Kheirollahpour et al., 2020; Nguyen and Tolia, 2021). Antigens can be loaded onto microparticles through encapsulation, physical adsorption and surface conjugation (Garg and Dewangan, 2020). Although encapsulation protects the antigens from proteolytic degradation, the entrapment procedure could also cause physical or chemical damage to the antigens and increase protein instability (Garg and Dewangan, 2020). Consequently, loading antigens using physical adsorption is considered a more stable alternative (Allahyari and Mohit, 2016; Jain et al., 2011).

1.4.1. Poly D-L-lactide-co-glycolide acid (PLGA) microparticles

Commonly used microparticles are synthetic polymer-based PLGA (Poly D-L-lactide-co-glycolide acid) microparticles, which are composed of lactic- and glycolic acid monomers. PLGA-based nanoparticles have been approved for vaccine and drug delivery by the FDA especially due to an excellent safety profile in humans (Jain et al., 2011). Additional benefits associated with PLGA nanoparticles include: (1) Antigens loaded onto PLGA nanoparticles are protected from proteolytic degradation that increases their half-life in plasma (Mahboubian et al., 2010); (2) PLGA nanoparticles have no adverse effect on dendritic cell (DC) functions like migration, maturation, cytokine secretion and costimulatory properties (Waeckerle-Men and Groettrup, 2005); (3) non-specific DC targeting can be achieved by modifying the several customisable factors e.g. size, hydrophobicity, charge etc. (Allahyari and Mohit, 2016; Kheirollahpour et al., 2020); (4) PLGA nanoparticles can be customized to facilitate the sustained release of antigens (Kheirollahpour et al., 2020); and (5) PLGA microparticles are biodegradable and can be metabolized back into its lactic and glycolic acid monomers (Makadia and Siegel, 2011).

1.4.2. PLGA microparticle antigen release

The release of antigens by PLGA particles are influenced by several design and environmental factors. Briefly, PLGA particles typically follow a tri-phasic antigen release profile that spans several days, which includes (I) an initial burst stage, (II) a slow release phase followed by (III) a faster release, which is also called a second burst release (Figure 1.8) (Fredenberg et al., 2011). Prolonging the time that immunomodulatory molecules are exposed to immune cells is crucial for eliciting a robust immune response, therefore, the sustained antigen release profile

(among other characteristics) make PLGA microparticles an ideal vaccine delivery platform (Ou et al., 2022).

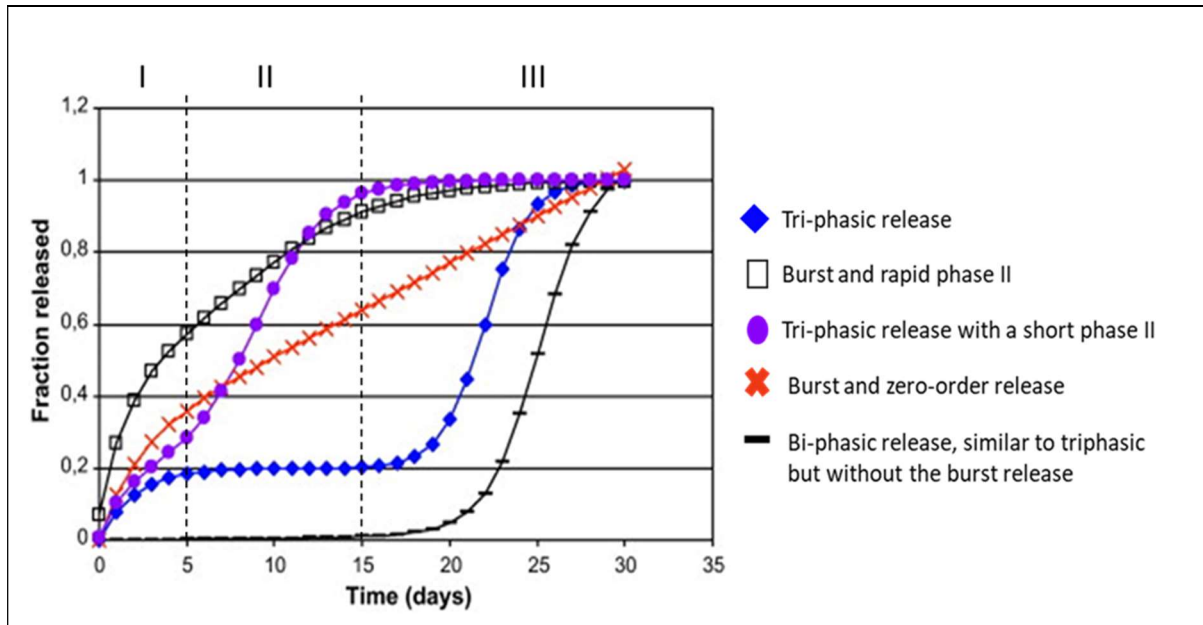


Figure 1.8. The profile of antigenic release from PLGA particles. Release profiles consisting of different phases. The burst observed in phase I is attributed to proteins that were weakly bound/adsorbed to the particle surface, which are easily accessible for hydration, or through the formation of cracks and the disintegration of particles (Fredenberg et al., 2011; Huang and Brazel, 2001; Wang et al., 2002). The slow release observed in phase II is facilitated by the slow diffusion of antigens through the dense polymer or through existing pores (Fredenberg et al., 2011). Polymer degradation and hydration continues simultaneously. Lastly, the faster release observed in phase III is usually attributed to the erosion of the particle (Fredenberg et al., 2011). Modified from Fredenberg et al. (2011).

1.5. Research Gaps to be addressed.

Gap 1:

The Subolesin gene structure has thus far only been elucidated in *I. scapularis* ticks (Naranjo et al., 2013). Given the variation in percentage identity of the Subolesin amino acid sequence between Ixodes and Rhipicephalus ticks (Figure 1.5), it is important to determine the gene structure in *R. microplus* ticks for mapping of mRNA sequences and mapping localization predictions.

Gap 2:

Antigenic diversity can be an obstacle hindering the efficacy of tick vaccines against different geographical strains and across tick species (Abbas et al., 2023; Bishop et al., 2023). Tick Subolesin has been evaluated in immunization trials and shown to afford cross-protection

against multiple tick species (Table 1.6). The variability of this vaccine candidate in South African field populations of *R. microplus* ticks has yet to be evaluated. This includes evaluating the variability in the predicted Subolesin epitope regions and the expression of Subolesin on a transcriptional level.

Gap 3:

Additional challenges faced by traditional livestock vaccines include costs and vaccine loss due to cold-chain requirements, as well as unstable vaccine formulations with commonly used water-in-oil emulsion adjuvants (Leclercq et al., 2011; Maina et al., 2020; Plewes et al., 2023). PLGA microparticles can be used as vaccine platforms to increasing the stability of the vaccine antigens, thereby creating vaccines that are stable at room temperature (Maina et al., 2020). Furthermore, PLGA microparticle-based vaccines which facilitate the slow sustained release (Figure 1.8) of antigens could reduce the number of doses required to achieve the same levels of immunogenicity as subunit Subolesin vaccines, which typically receive three doses (Table 1.6) (Ou et al., 2022). Subolesin has not yet been tested using PLGA microparticles as a platform (Table 1.6), therefore this study will address this gap for the first time.

Gap 4:

The burden of livestock vaccinations on farmers is exacerbated by the administration of separate vaccines targeting ticks and their transmitted pathogens (Martinod, 1996). Surveys have shown that farmers would prefer to use a highly effective multivalent vaccine compared to their monovalent counterparts (Masemola et al., 2021). Therefore, combination vaccines targeting both the tick vector and pathogen(s) it transmits is crucial to simplify treatment regimes. In this study the Subolesin and MSP4 antigens will be combined with PLGA microparticles to target both the tick vector and the intracellular pathogen *A. phagocytophilum*.

Gap 5:

The evaluation of immunological responses to vaccination with vaccine candidate antigens targeting *A. phagocytophilum* has largely been limited to measuring antibody responses (i.e., enzyme linked immunosorbent assays) and *in vitro* antibody-inhibition assays (Table 1.7). Furthermore, it has been demonstrated that the clearance of *A. phagocytophilum* infection is not dependent on antibodies (Table 1.3). Additionally, partial protection against *A. phagocytophilum* infection was only observed in the immunization group that elicited an

increased IFN- γ CD4 T cells (Table 1.7.C), as previously established (Table 1.3). Therefore, only evaluating the antibody response is not a good indicator of potential protection and clearance. In this study, the T and B cell populations elicited after immunization and stimulation will be identified in both the spleen and lymph nodes.

In conclusion, this study aims to address these gaps as listed in Table 1.9.

Table 1.9. Summary of the gaps identified in the literature review and how these gaps will be addressed in their respective chapter with aims and objectives.

Chapter(s)	Gap(s)	Aim(s)	Objective(s)
Chapter 2	1+2	Evaluate gene structure and nucleotide diversity of Subolesin in South African field samples of <i>R. microplus</i> ticks.	1. Determine the gene structure of Subolesin in <i>R. microplus</i> ticks 2. Determine how the Subolesin gene is transcribed in <i>R. microplus</i> based on transcriptional regulatory sequences. 3. Determine the diversity among South African <i>R. microplus</i> Subolesin sequences and global Subolesin sequences.
Chapter 3	3+4+5	Evaluate a combination microparticle-based vaccine targeting both the tick vector (Subolesin) and its transmitted pathogen, <i>A. phagocytophilum</i> (MSP4) in a murine model	1. Profile the in sera antigen-specific response observed during the vaccination trial 2. Profile the antigen-specific cell populations in lymph node and spleen tissues.

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Chapter 2

Characterising the expression and variation of Subolesin in South African *Rhipicephalus microplus* field populations.

2.1.1. Introduction

Chemical acaricides have always formed the cornerstone of tick control. However, the exclusive long-term use of acaricides is not feasible, since ticks quickly develop resistance against chemical acaricides after they have been introduced (Chevillon et al., 2007). Moreover, chemical acaricides negatively impact the environment and are therefore unsustainable as the exclusive control measure for ticks (Santos et al., 2018). Although several alternatives have been investigated (Table 1.4), one of the most promising and widely investigated candidates remains the use of livestock vaccines targeting ticks.

The “gold-standard” of tick vaccines is still the Bm86 antigen, however, evaluation of this antigen has shown varying efficacies across geographical areas and between cattle breeds (Bishop et al., 2023). Therefore, the development of tick vaccines is ongoing and many antigens that have been evaluated in bovine trials (Table 1.5). One of these antigens is the tick protein Subolesin. Several RNA interference studies demonstrated that Subolesin is crucial for tick reproduction and survival (de la Fuente et al., 2006a; Kocan et al., 2007; Nandy et al., 2023; Nijhof et al., 2007). Shortly after this discovery, Subolesin was evaluated in vaccine trials, with some success (Table 1.6). Typically, tick vaccine antigens elicit protection against ticks by inducing a humoral immune response in the host, whereafter antigen specific antibodies can recognise and interfere with tick proteins exposed to the host immune system, which are secreted proteins in the feeding pool or bloodmeal, or extracellular tick proteins exposed to the host immune system on tick cell membranes (Nuttall et al., 2006). However, since Subolesin is an intracellular protein (Antunes et al., 2014; Trentelman et al., 2019) proposed to function in the nucleus (Figure 1.6), the manner through which protection against tick infestations is achieved is still unknown (Chapter 4).

One possible explanation could be that a different variant of Subolesin that is exposed to the host immune system could be made. A time-efficient and high throughput way of investigating this is by using RNA sequencing data (Gao et al., 2017). This approach has several other advantages: (1) analysing transcriptome data generated by RNA-sequencing does not require a reference genome (Wang et al., 2009). This is especially beneficial in ticks, since the most complete *R. microplus* genome assembly is still only partially annotated and has highly

repetitive content (Jia et al., 2020); and (2) RNA-sequencing data generates more complex transcriptome data that can provide insights into transcript splicing isoforms as well as their expression levels (Ozsolak and Milos, 2011).

To date, the Subolesin gene structure has only been elucidated in *I. scapularis* (Naranjo et al., 2013), with preliminary studies predicting the exon/intron organization in other tick species (de la Fuente et al., 2006b; Hu et al., 2014). Moreover, there is little known about the Subolesin RNA species in *R. microplus* with regards to diversity and expression levels. Elucidating the gene structure and transcriptional regulatory sequences of Subolesin in *R. microplus* could offer important insights into whether there could be more than one copy of the Subolesin gene or alternative splicing resulting in alternative Subolesin variants (e.g. mRNA/protein isoforms). Therefore, this study will use transcriptome data to determine on both a gene and transcript level whether an alternative Subolesin variant could potentially be accessible to the immune system e.g. secreted or exposed on a cell membrane, which could explain the observed efficacy in bovine vaccine trials.

Furthermore, a significant challenge to vaccine efficacy is antigenic diversity within and between tick populations (Parizi et al., 2023). Although the amino acid sequence of Subolesin is highly conserved (Figure 1.5), there is still some diversity between genera. Antigenic diversity can significantly influence the recognition of epitopes, with a single amino acid change having the potential to abrogate recognition (Rojas, 2022). Furthermore, amino acid changes can alter the sequence and structure (conformation) of proteins, thereby potentially impacting both continuous and discontinuous epitopes (Prabantu et al., 2020). Therefore, evaluating the antigenic diversity of candidate antigens, i.e., Subolesin, is an important step in predicting tick vaccine efficacy. Therefore, this study will use RNA sequencing to evaluate the diversity of Subolesin within South African *R. microplus* field samples (Mpumalanga) and a laboratory-reared strain, as well as compared to global sequences.

2.1.2. Biological questions:

1. What is the Subolesin gene structure in *R. microplus*, and how does it compare with other tick species?
2. Based on the transcriptional regulatory sequences, how is the Subolesin gene transcribed in *R. microplus*?
3. What is the diversity among the South African and global Subolesin sequences?

2.2. Materials and Methods

2.2.1. Tick collection and tick dissection

Engorged or partially engorged adult female *R. microplus* ticks were collected from cattle at five communal dip stations in the Mnisi area, a closed community in Mpumalanga where cattle movement is restricted (Figure 2.1).

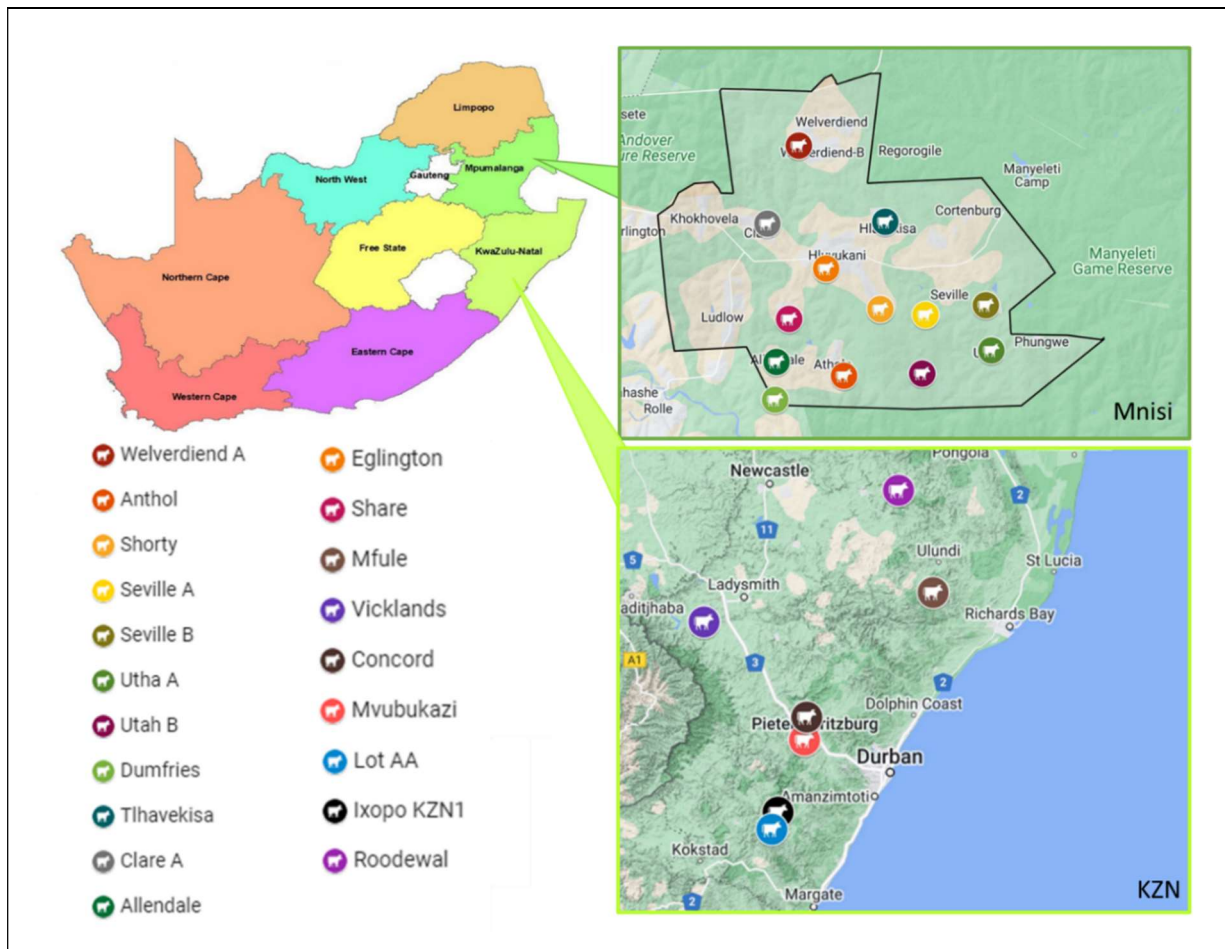


Figure 2.1. A map of South Africa, indicating the locations of the ticks collected in the Mnisi area (outlined in black) in Mpumalanga and KwaZulu-Natal. The sampling locations are indicated by the coloured markers and the names of the corresponding dip station/farm is indicated on the left.

Tick collection was performed according to the terms and provisions of the National Environmental Management: Biodiversity Act (Act of 2004, permit number: 2787NT (12/11/1/1). Dissections were performed with the help of Dr C. Stutzer and Miss. M. Archary. Briefly, ticks were washed in SABAX Pour Saline (0.9%) solution before dissection. A transverse incision was made (between the mouth parts and the first pair of legs) to remove the head, where after all internal organs were gently removed from the exoskeleton through

this opening. The organs were rinsed in SABAX Pour Saline (0.9%) solution, and the individual organs (salivary gland, ovary, and gut) were collected and immediately placed in TRI Reagent® (Sigma-Aldrich, USA) and stored at -80°C in the biobank at the Hans Hoheisen Wildlife Research Station (Mpumalanga, University of Pretoria) until further processing. Ticks were randomly selected for each dip station and a minimum of ten organs were pooled per dip station.

2.2.2. RNA isolation and RNA-Sequencing

Total RNA was extracted from samples using a modified TRI Reagent® (Sigma-Aldrich, USA) protocol followed by purification using the RNeasy® Mini Kit (Qiagen, Germany) by Miss M. Archary and Miss L.J. Bishop. Briefly, tick tissues were homogenized in a ratio of 200 µl homogenate and 800 µl of TRI Reagent® using the Ultra Turrax homogeniser (IKA-Werke, Germany). Contamination between samples were prevented by washing the blades with a 1% volume per volume (v/v) sodium hydroxide solution in Diethylpyrocarbonate (DEPC; Sigma-Aldrich, USA) water, followed by a 70% (v/v) ethanol solution (Protea Laboratory Solutions (Pty) Ltd, South Africa) in ddH₂O. A volume of 500 µl homogenate was aliquoted and centrifuged at 10,000 x g for 30 seconds to separate the cellular debris. After aspiration, 200 µl of chloroform (Merck, Germany) was added to the supernatant, before the mixture was mixed via inversion and incubated for 15 minutes at room temperature. The samples were centrifuged for 15 minutes at 12,000 x g (4°C) and the aqueous phase containing RNA was added to 1 ml µl of ice-cold isopropanol (Sigma-Aldrich, USA), followed by inversion and incubation at -80°C overnight. Once defrosted, samples were centrifuged for 30 minutes at 12,000 x g at 4°C before the supernatant was removed. The RNA pellet was washed with ice-cold 70% ethanol, vortexed, and centrifuged for 30 minutes at 12,000 x g and the supernatant was discarded. The residual ethanol was allowed to evaporate in the laminar flow-hood by air-drying the RNA pellet at room temperature. The pellet was resuspended in 50 µl DEPC-treated water (pre-warmed to 55°C) via a 15-minute incubation followed by 10 seconds of vortexing. This step was repeated once more.

For RNA purification, the RNeasy® Mini Kit (Qiagen, Germany) was used. Briefly, 350 µl of Buffer RLT was added to the sample as per manufacturer's recommendations, followed by the addition of cold 250 µl absolute ethanol and vortexing. The resulting mixture was transferred to a RNeasy spin column for centrifugation at 8,000 x g for 1 minute, before the eluent was discarded. On-column DNase I digestion was performed with the addition of 350 µl RW1 buffer before centrifugation at 8,000 x g and the eluent was discarded. RDD and 10 µl of DNase I

enzyme (27 Kunitz units) were combined before being added to the column for a 15-minute incubation. Thereafter, 350 µl of RW1 buffer was added and incubated for a further 5 minutes before centrifugation at 8,000 x g for 1 minute before discarding the eluent. The membrane was washed with 500 µl RPE wash buffer and centrifuged at 8,000 x g for 15 seconds. The wash step was repeated with an additional 500 µl RPE wash buffer that was incubated for 2 minutes before centrifugation at 14,000 x g for 2 minutes. The RNA was eluted through the addition of 25 µl of DEPC-treated water at 30°C, followed by a 10-minute incubation and centrifugation at 8,000 x g for 1 minute. The elution step (excluding the incubation) was repeated to increase the yield. The purity and concentration of the RNA was determined using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA). Eukaryotic RNA Analysis was performed using the High Sensitivity RNA Screen Tape (Agilent Technologies, USA) to assess the integrity of the extracted RNA.

A minimum of 2 µg of total RNA was precipitated using 0.1 sodium acetate (3M, pH 5.2) and 2.5 absolute ethanol (v/v) in order to preserve the quality for shipping to Macrogen, Inc. (South Korea) where RNA sequencing was conducted. Library preparation was performed by Macrogen, Inc. (South Korea) using the TruSeq® Stranded mRNA Kit (Illumina, USA), which was then sequenced using the Illumina NovaSeq 6000 sequencing instrument. A minimum of 80 million read-pairs per sample at 150 bp paired-end sequencing was specified for sequencing.

2.2.3. RNA-Sequencing data quality control and trimming

The RNA-seq data was subjected to quality control using FastQC v0.11.7 (Andrews, 2010), and quality filtering, trimming and the removal of all Illumina primer/adaptor sequences and the first 11 nucleotides from each read using Trimmomatic v0.36 (Bolger et al., 2014). Once the RNA-seq data was imported into CLC Genomics Workbench (Qiagen, v.23.0.4), the reads were trimmed once again using the default parameters.

2.2.4. *De novo* transcriptome assembly

A *de novo* assembly with all available RNA-sequencing data for *R. microplus* sequenced by the Tick and Tick-borne Diseases group (University of Pretoria) was prepared. The RNA-sequencing data included the Mnisi RNA-sequencing data and historical sequencing data from other locations in Mnisi, KwaZulu-Natal, and tissue-specific data from a laboratory-reared *R. microplus* tick samples from (ClinVet Pty Ltd., South Africa). The ClinVet ticks were cycled on pathogen-free cattle previously untreated with any acaricides, and ticks were confirmed

pathogen free. The *de novo* assembly was created using the *de novo* assembly tool (https://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=De_Novo_Assembly.html) in CLC Genomics Workbench (Qiagen, v.23.0.4) with the parameters listed in Table 2.1.

Table 2.1. Parameters for representative South African *de novo* transcriptome assembly (CLC Genomics Workbench (Qiagen v.23.0.4)).

Parameter	Input
Mapping mode	Create simple contig sequences (fast)
Automatic bubble size	Yes
Minimum contig length	50
Automatic word size	Yes
Perform scaffolding	Yes
Auto-detect paired distances	Yes

2.2.5. Consensus sequence of Subolesin RNA species

To obtain a South African *R. microplus* reference sequence of each Subolesin mature mRNA species to which RNA sequencing data could be mapped to for variant detection, a consensus sequence was created. This was achieved by conducting blastn (Altschul et al., 1990) using the NCBI (The National Center for Biotechnology Information) listed sequence of the appropriate mRNA species as the query sequence and using the RSA representative *de novo* transcriptome assembly (section 2.2.4) as the database. The BLAST parameters were adjusted until the entire query sequence was represented in the BLAST results and the corresponding reads were extracted and used to make a consensus sequence for each mRNA species.

2.2.6. Annotation of Subolesin gene, mRNA species and protein variants

The location of the Subolesin gene was determined manually through BLAST with the *I. ricinus* Subolesin sequence (accession number AGI44593.1) against the *R. microplus* genome assembly (accession number GCF_013339725.1) (Jia et al., 2020), which corresponded with the annotated gene location (LOC119175104). The gene features were manually annotated using consensus sequences obtained from literature and IUPAC (International Union of Pure and Applied Chemistry) (Table S1). Furthermore, several online prediction tools were used at default settings to predict gene and mRNA features, including TSSW (Solovyev et al., 2010), Nsite (Shahmuradov and Solovyev, 2015) and POLYAH (Salamov and Solovyev, 1997).

Protein domains and features were predicted using several online prediction tools, namely, InterPro v.95.0. (Paysan-Lafosse et al., 2023), SignalP-6.0 (v.6.0.) (Teufel et al., 2022), TMHMM-2.0 (Krogh et al., 2001) and DeepTMHMM (v.1.0.24.) (Jeppe et al., 2022). Furthermore, the secondary structure of each protein variant was predicted using the secondary structure prediction tool in CLC Genomics Workbench (Qiagen, v.23.0.4) (https://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=Secondary_structure_prediction.html), which utilizes a hidden Markov model to predict secondary structures based on protein sequences from the protein data bank (<http://www.rcsb.org/pdb/>). Protein variant 3D structures were created using ColabFold v.1.5.3. (Mirdita et al., 2022) with the default parameters, which utilizes AlphaFold2 (Jumper et al., 2021), HH-suite3 (Steinegger et al., 2019) and MMseqs2 (Steinegger and Söding, 2017). Two confidence scores were used to assess the predicted structures in this study. Firstly, the predicted local distance difference test (pLDDT) scores were used as a per-residue measure of local confidence indicating how well the predicted structure would compare to an experimental structure (Jumper et al., 2021). Scores are based on the local distance difference test Ca (lDDT-Ca), which assesses the correctness of the local distances (Mariani et al., 2013). Secondly, the predicted aligned error (PAE) score indicates the confidence of the relative position of two residues within the predicted structure and is defined as the expected positional error at residue X if the predicted and actual structures were aligned on residue Y, measured in Ångströms (Å) (Jumper et al., 2021). The interpretation of the output of these confidence scores are explained in detail in Figure 2.6.

2.2.7. Comparison of the expression of Subolesin in South African tick populations

The differences in expression between the different mature mRNA species of Subolesin was determined by creating an expression browser (https://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=Create_Expression_Browser.html) in CLC Genomics Workbench (Qiagen, v.23.0.4). Each sample was subjected to TMM (Trimmed Mean of M values) normalization, which normalizes the RNA-sequencing reads based on the sequencing depth of each sample, to enable comparison between all samples. Thereafter, the TMM-adjusted log CPM (counts per million) counts were calculated for each sample with default settings. The log CPM threshold was chosen from literature (Law et al., 2018), and set so that the cutoff would exclude mRNA species with less than 10 read counts and calculated as follows:

$$\log CPM \text{ cutoff} = 10 \div \text{median library size (million)}.$$

Finally Sashimi plots (Katz et al., 2015) were created using the “sashimi plot” function on Integrative Genomics Viewer (IGV, v.2.16.2) (Robinson et al., 2011) with default parameters. The plots were created using TPM (transcript per million)-normalised RNA-sequencing reads that mapped only to the subolesin gene region. Mapping of the RNA-seq reads was done using CLC Genomics Workbench (Qiagen, v.23.0.4), exported and visualised in IGV.

2.2.8. Variant detection workflow

The variant detection workflow consisted of extracting RNA sequencing reads from each sample that mapped exclusively to the Subolesin gene region, before the extracted reads were mapped to the appropriate mRNA species' consensus sequence (section 2.2.6). Variants were detected using the fixed ploidy variant detection tool (https://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/index.php?manual=Fixed_Ploidy_Variant_Detection.html), and an amino acids variant track was created to detect amino acid changes. The required variant probability was set at 90% with a ploidy of 2. The variant detection tool predicts variants utilising a Bayesian model to estimate the posterior probabilities of site types, whereafter the variant with the highest likelihood (estimated using Expectation Maximization procedure accounting for sequencing error and prior site type probabilities) and posterior probability is called. All the above was completed using CLC Genomics Workbench (Qiagen, v.23.0.4).

2.2.9. Amino acid sequence alignment of Subolesin protein sequences

Global Subolesin amino acid sequences (*R. microplus* and other tick species) were obtained from NCBI (Sayers et al., 2022). Sequences were aligned using the MAFFT (Multiple Alignment using Fast Fourier Transform) v7 server (Kato et al., 2017) with default settings, except for the gap penalty which was increased to 5.0. The alignment was visualised in CLC Genomics Workbench (Qiagen, v.23.0.4). Epitopes in the South African *R. microplus* amino acid sequence (P1) were based on previously identified epitope regions (Lagunes et al., 2016; Zeb et al., 2024). Epitope sequences are listed in Table S4.

2.3. Results

2.3.1. South African *de novo* transcript assembly

The draft genome assembly used in this study (accession number GCF_013339725.1) was created using sequencing data from *R. microplus* ticks from China (Jia et al., 2020). Therefore, to exclude variance due to geographical location it was necessary to create a South African representative reference against which variants could be called. The RNA-sequencing reads used to assemble the *de novo* transcriptome are listed in Table 2.2 and the *de novo* assembly statistics are included in the supplementary data (Table S2).

Table 2.2. RNA-sequencing reads before and after quality trimming.

RNA-Sequencing reads used in <i>de novo</i> transcriptome				
	Sample	Raw reads number (F+R) ⁴⁴	Trimmed reads number (F+R)	Trimmed data rate (%) ⁴⁵
ClinVet	Larval	92,869,634	92,849,325	99.98
	Nymph	99,912,014	99,885,647	99.97
	Ovary	78,923,042	78,911,203	99.98
	Gut	79,454,546	79,441,508	99.98
	Salivary gland	79,454,784	79,443,437	99.99
Mnisi	Utah A	44,039,250	43,237,414	98.18
	Athol	76,791,490	76,678,077	99.85
	Shorty	71,520,624	71,039,860	99.33
	Thlavekisa	78,827,098	64,784,376	82.19
	Seville A	55,273,058	55,054,684	99.60
	Seville B	65,183,846	64,784,376	99.39
	Dumfries	94,933,516	94,422,258	99.46
	Share	27,700,176	22,150,982	79.97
	Eglington	104,322,796	102,179,445	97.95
	Allendale	34,171,008	27,453,308	80.34
	Clare A Ovary	111,515,978	108,014,437	96.86
	Clare A Gut	111,704,028	111,671,140	99.97
	Clare A Salivary gland	117,933,746	117,902,575	99.97
	Utah B Ovary	116,124,206	112,546,057	96.92
	Utah B Gut	114,849,306	114,801,940	99.96
	Utha B Salivary gland	115,877,372	115,840,963	99.97
	Wolverdiend A Ovary	111,296,746	107,624,953	96.70
	Wolverdiend A Gut	103,977,528	103,937,684	99.96
	Wolverdiend A Salivary gland	11,0913,592	110,874,248	99.96
KZN	Ixopo	70,200,225	69,685,704	99.27

⁴⁴ F+R – forward and reverse sequencing reads

⁴⁵ *Number of trimmed reads/number of raw reads

RNA-Sequencing reads used in <i>de novo</i> transcriptome			
Lot AA	68,549,754	67,907,286	99.06
Mfule	70,408,538	69,361,063	98.51
Roodewal	70,503,670	68,646,615	97.37
Roodewal	69,659,722	67,640,799	97.10
Mvubukazi	68,770,300	67,367,947	97.96
Vicklands	70,838,018	69,587,267	98.23
Concord	70,376,074	68,872,405	97.86
Total	2,656,875,685	2,604,598,983	98.03

2.3.2. Annotation of the *Rhipicephalus microplus* Subolesin gene and RNA species

The *R. microplus* Subolesin gene (NC_051171.1) occurs on chromosome 7 and is approximately 60,903 nucleotides in length, with five predicted exons and four introns (Figure 2.2).

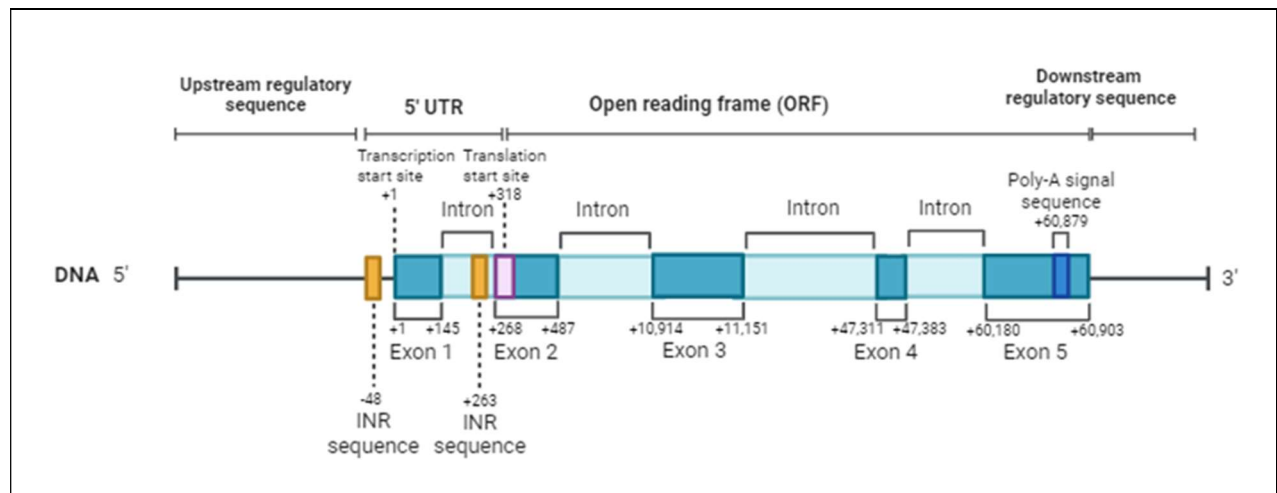


Figure 2.2. Schematic representation of the proposed *Rhipicephalus microplus* Subolesin gene structure. Different sections of the *R. microplus* Subolesin gene are indicated, including the 5' untranslated region (5'UTR) and the open reading frame (ORF). The Subolesin gene has five exons and four introns. Transcription initiation sequences (INR) are indicated in orange, and the translation initiation site (Kozak sequence) is indicated in pink. The polyadenylation (Poly-A) signal sequence is indicated in dark blue with its nucleotide position. Image was created with BioRender.com.

Contrary to the gene sequence determined for *I. scapularis* Subolesin (Figure 2.3.A), no TATA boxes were found and only two putative arthropod initiation factor sequences ([TAG]-C-A-[GT]-T) sequences were identified in the predicted 5' UTR of the proposed *R. microplus* Subolesin gene sequence (Figure 2.3.B).

A

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CCAGATTTAATTCTACGAGGGGCTGCGGCAAAAAGTCACGGCTGCATCGCGTCGGGAGCCACAG
TGTATTAATAAAAAGTGTCTTTCCTTTATCAGCTTTATGTGGAGTTTATAGGTTTATCTATCTAAA
ACAAAAAATTTAGACGTCATAGGGTTTTTTTTTTTTTTTTTTTCTTTCTCCTATTTGAAACCG
AAAATGCTACTGACAGAATCCGACACCCAGATAGAATGGATGGCTTATCTTTTCATTTCCAGCAC
AAGTGAAGAGTTGGACGAGGAGAAAACACGCAAAATGCGTCCAATTAAACGTAATATAAATTAA
AGAATACACGGATTATTTTATTACGAAAAAATCACCGGAAGAACTTGGGAAGTAAACGAAGCCGTTCG
CACGTTCCAAACAACCAACCAATCGTAGCAGACAATAAAGCAAAATCGGCTGCGGTGGCCATTTTGGAG
TTTCGAGGAGGTTTGTGCTGTAAAGTGTGTGCGTCTCGC...GGTTTTTCGTCTCTATTTTCAGCTTGGAAAAT
CCACCAGGAGCCTGCAACCGCAAAAAAGTTTCATCATCCGGAGCTAAGCCTATCGCAGCATGGCTTGCCGCA
ACATTAAAGCG...GCCACCGAAATTAACCTTCAGTAAGGCTGCGCTTTAGACT...TTCCCGTCTCCCCCTGA
CAGAGGAGATAGCGGCCAACATT...TCTGTCTGCCAAGCTGGCAGGTGAGTGAACCCACCAGGAA...CTCTT
TCTTTTCTCTTACAGAGCAGTACGACACATTGTGCAAGTTTACGTACGACCAAAATTCAGAAGCGGTTTGA
GGGTGCCACTCCAAGCTGTAAAGTGCCTTTTTTTTTT...TGCAATTTTCTTTTCTAGATTGTGCTATAAC
ATGATGGG...AAGGCACTCTCTCAGTACCT
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B

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TACACCGGCAGTCTCGACAAAGTATTGTCATAAATGTAATAGCTACAACCTCAACATGAAATTAACTTATGCTGAAGTCGTGA
AAAAAATACACAGTGTGTTGAACGCAGTTTTCAAAACTTGGCCAATCACAGTGTTAAAACTAGAAGCGCCGCCGTTGTCC
GGTGGCCATATTGAGGGACAGAGCAGCTCGAACCGGCAAGTAGTGATTGTTGTGT...TCCTATCTTCTCGATTTCAGGTG
AAGTGACGAGCAGGCAGGAGCGTCCCGAGTTAGGCTTACGTCTGAGTGATGCCTTGTGCGACATTAAAGCGGACACATG
ACTGGGACCCCTTGACAGTCCGAGTGGCAGATCGCCGAAGCGACGCGGATGTATGCCTCTGTCCCCGCTCTACAAGG
GCGCACCAGCATCGATCCCTCGCCCTTCGGAGACGTGCCACAAAGTTGACTTCAGGTAGTAATCCCTCTCGCATG...GCCGC
CTTGTCTGCTGCAGAGGAGATAGCAGCAACATCCGTGAGGAGATGCGACGGCTACAACGGCGCAAGCAGCTCTGTTTC
CAGGGCGCTGACCCTGAATCCAGCATAACAGTGGTCTCTCGTACCAGTGCATCGAGACCAGCCCTGTTACGTTCCGC
CAGGTTGGGCTCATTGCGAACGAATGATGAAGGAGCGAGAGAGCAAGATACGGGAGGAGTATGACCATGTGCTATCTAC
CAAACTCGCAGGTGAGGCATTTACATTTTCA...TTTGACTTTCTTTTGCAAAAGTACGACACATTTGTAAATTACCTA
CGACCAATTGAGAAGCGGTTGAAGGTGCCACGCCAGCTGTAAGTGGTCAACCGTTTTT...CTGTTACGATTCCGTTTCA
GATTGTGCTGAACAGTCGAAGAAGGACTCCTCTCTGCAGCAGGCAACATGATCCAGCTGAGCATGCCGGCCCTGATTGG
CAGTGACTTGACACAAACTGCTGGTGCAATCATTCTCAACATCTACAAGCCCTACTGTGTTGGCAATGTGTGTGCTGTTG
CCACCTGTGATGACGTGCAAGACTGCTTTGTATGCCAAGGAGTGAAGTTTAGTACGTTTCTATGGTGGTGCATTACGA
TTGCTTACTTCTTTCTGAATGAACGCTTATGTTTTTGACCGTTGGATACTGCAGCTGCCAAGGGGTGGCTTCACAATTAACT
TAGTGACAGATGTATAATATAAATGCACTGCAGGGTGTGCATGCCAAGCTTAATGCATTACTTTTCTACCACTGTGCTTTT
TCTTGCCCTTGCTTTCGCAATGTTTGACTGGCTCATGTACATAGGTTAGCATGAATTAGCCACATTACTCTCGGTCTAAAA
TGCCGACTGCCAGTGCTTATTGGTAGTGTAGGAGAACCGCTGAAATGTGGTTTCTAGGATTGAAATGCCAGGCTTT
CACATGCATTGGCTCTCGAGTTGGGATGAGGTCAACATTAGATCATCGCTGAAGTATTGCAAGTTTGCTTTTTTATGCTTT
CATTTGCTTTTCAGATTGTGTAACTGGATGGTATTAGATAAACTCACTTGAGATACACGCTTTTGCTGTGTGCTGTAGCA
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Figure 2.3. (A) Nucleotide sequence of the *Ixodes scapularis* Subolesin gene, containing promoter (grey), exon (green) and intron (white) regions first and translation start and stop codons (yellow). Shown additionally is the putative NF-kB binding site (bold underlined), major (red) and minor (light purple) transcription start site, translation start and stop codons (yellow). Only the exon/intron junction sequences are shown. In the mRNA, Poly-A starts after the last nucleotide in exon 5. Taken from Naranjo et al. (2013). **(B)** Nucleotide sequence of the *Rhipicephalus microplus* Subolesin gene, containing 5' UTR (dark yellow), exon (light blue) and intron (white) regions, putative transcription start sites (dark purple), arthropod initiation factors (bold and underlined), Kozak sequence (dark green) and polyadenylation signal sequence (dark blue).

Arthropod initiation factor sequences (INRs) are typically found within arthropod promoter sequences close to transcription initiation sites (Cherbas and Cherbas, 1993). However, this kind of INR is typically found within -10/+10 of the transcription start site (TSS), whereas the INR before the TSS for exon 1 is found at -48, now referred to as the X1 mRNA. Another arthropod INR was found just before exon 2 at position +263, which corresponds to the

proposed transcription start site for the X2 mRNA species. Of the four putative pre-mRNA (primary transcript) species (i.e., X1-X4) (Figure 2.4), only the first pre-mRNA species has five exons (X1), while all the others maintain only four exons.

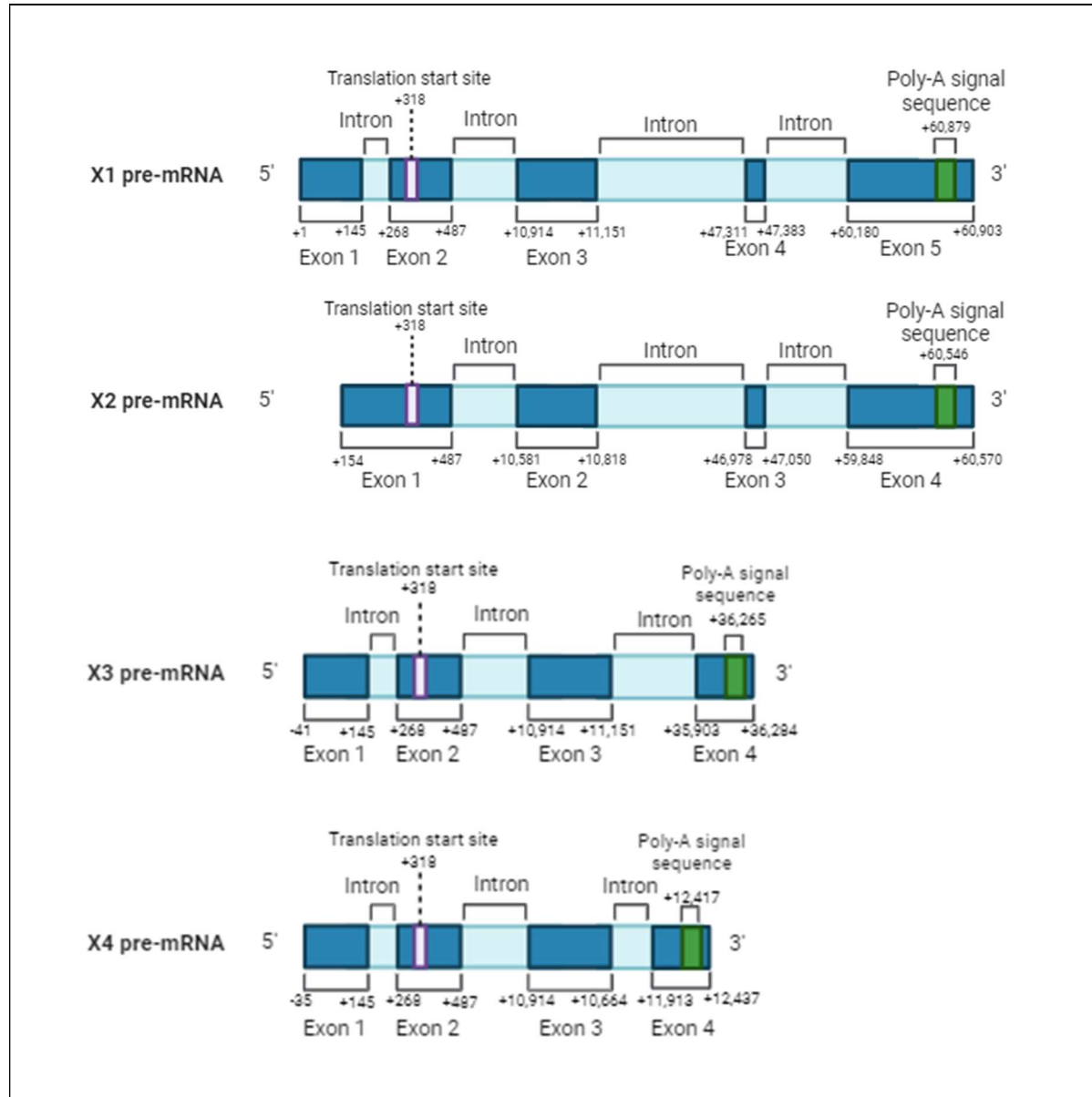


Figure 2.4. Schematic representation of four putative *Rhipicephalus microplus* Subolesin pre-mRNA species. The pre-mRNA species appear in numerical order (X1-X4) and decrease in length from X1-X4. The translation initiation site (Kozak sequence) is indicated in pink, and each mRNA species' Poly-A signal is indicated in green. Image was created with BioRender.com.

The first three exons of the putative X1, X3, and X4 pre-mRNA species are identical, with a different fourth exon (Figure 2.4). Furthermore, the first exon of the X2 mRNA species is a combination of a part of the first and second exon of the X1 mRNA species. The rest of the X2 mRNA variant's exons are identical to that of the X1 mRNA species (Figure 2.4). A Kozak

sequence was found at the same nucleotide position in all pre-mRNA species (Figure 2.4). Lastly, a polyadenylation signal (Poly-A) was found in the last exon of each of these mRNA species. Of the four putative pre-mRNAs, three unique putative protein variants (i.e., P1-P3) could be translated (Figure 2.5).

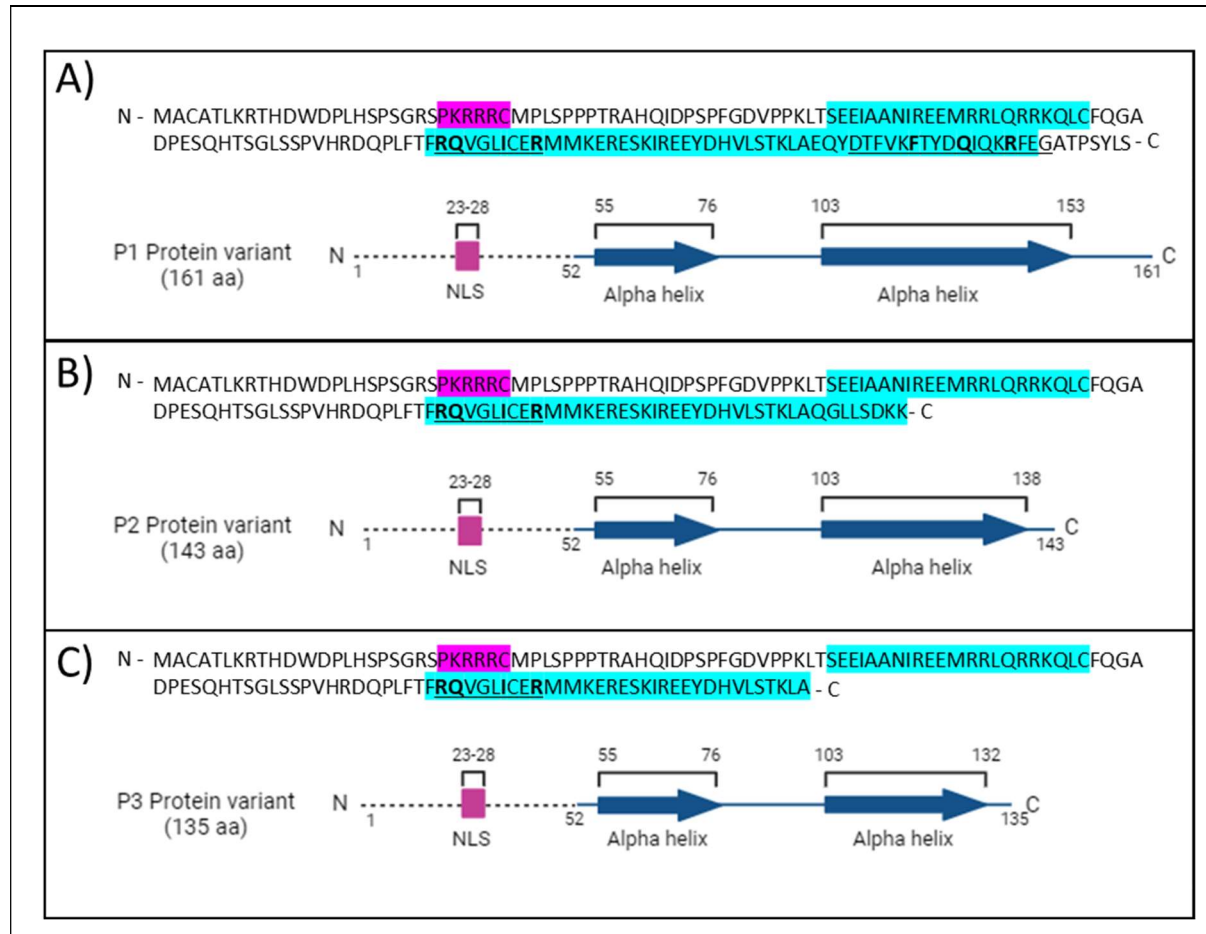


Figure 2.5. Figure depicting the putative *Rhipicephalus microplus* Subolesin protein variants (P1-P3) amino acid sequences and secondary structure as predicted by different platforms. The amino acid sequences and schematic representations of the (A) first (P1), (B) second (P2) and (C) third (P3) *R. microplus* Subolesin protein variants are shown. Nuclear localisation signal (NLS) sequences are shown in pink, with predicted alpha-helices indicated in blue. Amino acid residues that form previously predicted DNA binding sites are underlined (Artigas-Jerónimo et al., 2018). In the schematic diagrams, disordered regions are indicated by dashed lines (1-52) and two alpha-helices (blue arrows) are indicated. Domains were predicted by InterPro (v.9.5.0.) and CLC Genomics Workbench (v.23.0.4) and include a nuclear localisation signal (NLS) sequence and alpha-helices at the amino acid positions indicated. Schematic created in BioRender.com.

Both X1 and X2 putative pre-mRNAs encode the same putative protein variant (P1), of 161 amino acids in length (Figure 2.5.A). The X3 pre-mRNA encodes a second putative variant (P2) of 143 amino acids in length (Figure 2.5.B), whereas the X4 pre-mRNA encodes a third putative variant (P3) of 135 amino acids in length (Figure 2.5.C). According to both TMHMM-2.0 (Krogh et al., 2001) and DeepTMHMM (Jeppe et al., 2022) there are no predicted

transmembrane regions for any of the Subolesin variants, and all the proteins were predicted to be intracellular. The latter being supported by the lack of signal peptides in any protein using SignalP-6.0 (v.6.0.) (Teufel et al., 2022).

However, all protein variants shared a nuclear localisation signal (PKRRRC, amino acid position 23-28), as predicted by InterPro (v.9.5.0) (Paysan-Lafosse et al., 2023) (Figure 2.5). In regards to protein structure, a predicted disordered region (position 1-52), followed by a coil region (position 52-72), was identified for all variants using InterPro (v.9.5.0) (Figure 2.5) (Paysan-Lafosse et al., 2023). Two additional alpha-helices were predicted for each protein variant using the CLC Genomics Workbench platform. The first alpha-helix was predicted to be between amino acids 55-76 and was shared by all the protein variants (Figure 2.5). In contrast, the second alpha-helix was predicted between amino acids 103-153 for P1 (Figure 2.5.A), while shorter helices were predicted for P2 and P3, at positions 103-138 (Figure 2.5.B) and 103-132 (Figure 2.5.C), respectively. Similarly, two alpha-helices are present in the putative P1 3D predicted structure (Figure 2.6.A) with relatively high confidence scores (pLDDT and PAE).

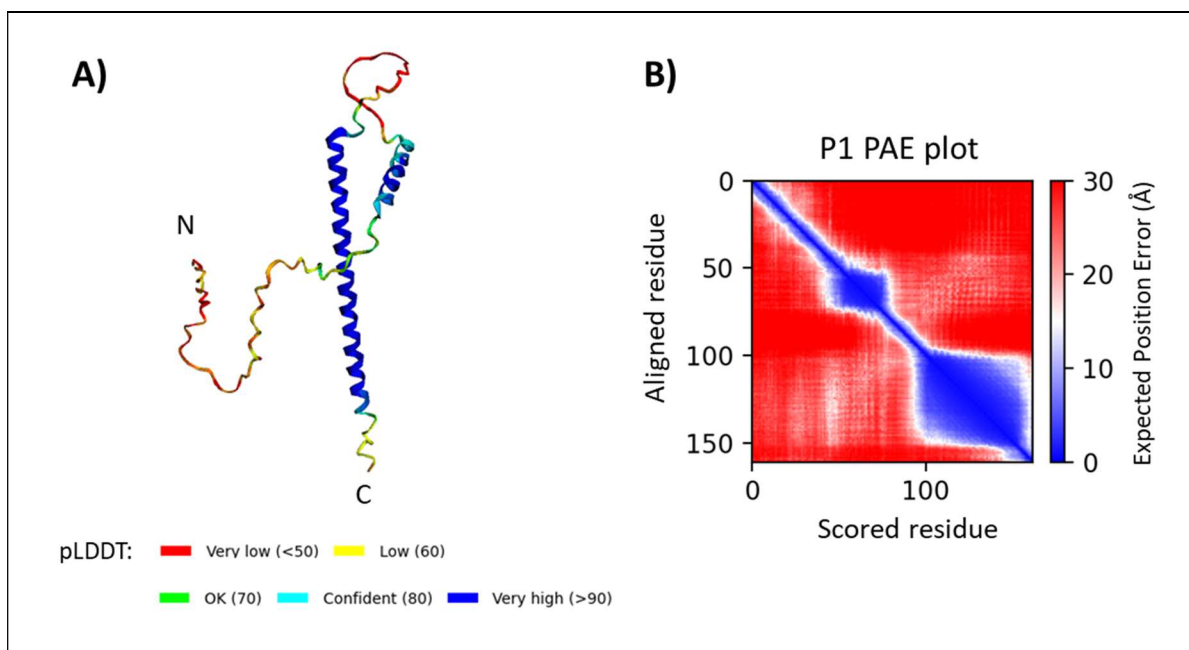


Figure 2.6. Predicted 3D structure of the putative P1 Subolesin protein variant and pLDDT and PAE confidence scores. (A) Predicted 3D structure of the P1 Subolesin protein variant created using ColabFold v.1.5.3. The N and C terminals are indicated on the structure. The structure is coloured according to the pLDDT (predicted local distance difference test) score, which increases in confidence from 0-100. High confidence means that the predicted structure could have a favourable comparison to experimental models. **(B)** The PAE plot of indicates the PAE (predicted aligned error) score for each residue of the predicted 3D structure of the P1 Subolesin variant. The aligned residues are indicated on the left y-axis and on the x-axis, and the expected position error (PAE score) is indicated on the right y-axis. A high PAE score represents a high expected position error, thus low confidence in the predicted relative position of the residues.

However, beyond the alpha-helix domains, the rest of the structure has very low confidence scores (both pIDDT and PAE) (Figure 2.6). Therefore, no conclusions can be made regarding the possible conformation of the protein or the interactions between the potential domains. Similar results were observed for the two other putative *R. microplus* Subolesin protein variants (Figure S1), therefore, only the putative P1 Subolesin protein variant was included as an example.

2.3.3. Expression levels of Subolesin in South African *Rhipicephalus microplus* populations

The expression levels of each putative transcript from different samples were compared by using logCPM (TMM-adjusted) as the expression value. Introns were removed from pre-mRNA sequences (X1-4) to construct mature mRNAs encoding the 3 protein variants (Figure 2.4 and Figure 2.5), and these were used in further analyses. The logCPM cutoff value was determined as 0.1 (Table S3), meaning that mRNA species values above 0.1 were considered expressed.

It is evident from Figure 2.7 that the highest expression levels for tick subolesin in *R. microplus* (ClinVet strain) was of the putative X1 mature mRNA species that was consistently detected across adult female and immature life stages (nymph and larvae). Furthermore, the X1 mature mRNA species had higher expression values than the X2 mature mRNA species for most other field samples (Figure 2.7), except for UTA (Utah B) Gut, WV (Wolverdam) and UTA Ovary. However, transcription of the X3 and X4 mature mRNA species are much lower than the X1 and X2 species across all samples (tissues and life stages), with the X3 species having the lowest expression and negligible expression in the gut (SAMPCLA3_Gut) and salivary glands (SAMPUTA_SG) of two samples from Mnisi (Figure 2.7).

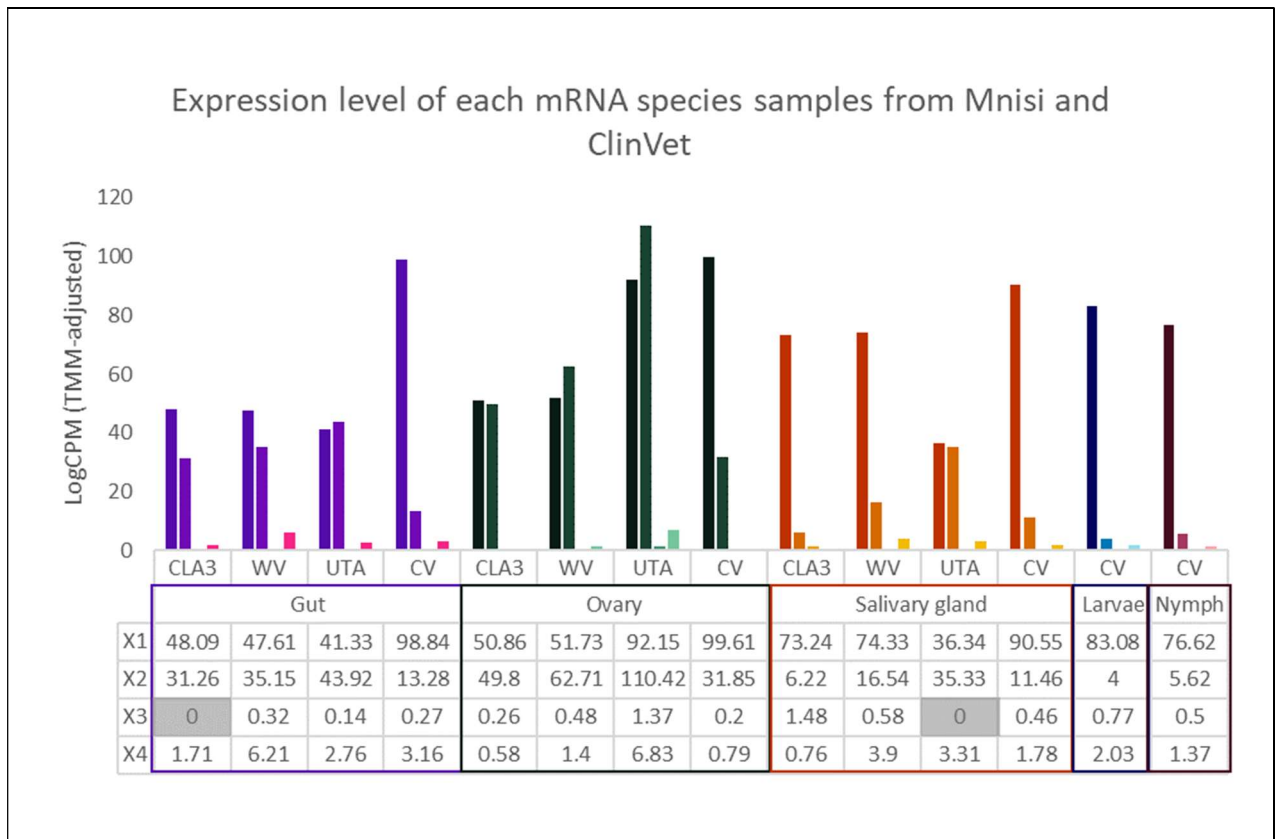


Figure 2.7. Graph depicting the level of expression of the Subolesin mRNA species (X1-X4) in different tissues/life stages in tick samples collected from Mnisi and ClinVet. The expression is split by tissue (gut, ovary, salivary gland) and life stage (larvae, nymph) and then location. The Mnisi (Mpumalanga) locations are indicated according to the names of the dip stations where the sample was collected from, namely Clare A (CLA3), Welverdiend (WV) and Utah B (UTA), and the ClinVet samples are indicated as CV. In each coloured division, the mRNA species are arranged by location from dark to light from X1-X4. The expression value of each mRNA species is indicated on the y-axis as the TMM-adjusted logCPM (counts per million), and species with logCPM values above 0.1 were considered expressed (See Table S3). Species below this threshold are highlighted in grey.

For further support of each pre-mRNA species, a Sashimi plot was constructed (Figure 2.8), which indicates the number of RNA sequencing reads supporting each intron/exon junction. Table 2.3 lists the support for each intron/exon junction, and it is evident that between the expression levels of the X1 and X2 pre-mRNA, X2 dominates (Intron B, E and F, Table 2.3. and Figure 2.8). This is evidenced by the lower support for the first exon/intron junction of the X1 pre-mRNA species (Intron A, Figure 2.8 and Table 2.3), which is more than two times less than the support for its other intron/exon junctions (Intron B, E and F, Table 2.3 and Figure 2.8). Furthermore, the support for the intron that links to the unique fourth exon of the X3 pre-mRNA species is very low (Intron D, Figure 2.8 and Table 2.3), with some samples having no supporting RNA sequencing reads for this intron/exon junction. The same trend was observed in the case of the intron/exon junction supporting the unique fourth exon of the putative X4 pre-mRNA species (Intron C, Table 2.3 and Figure 2.8).

Table 2.3. Support for each intron/exon junction as determined by Sashimi Plot. Each value represents the number of junction RNA-sequencing reads (TPM-normalised) that connect exons to each other.

RNA sequencing reads supporting each intron as indicated in Figure 2.8						
Sample ID	Intron A	Intron B	Intron C	Intron D	Intron E	Intron F
Rm_Gland	435	902	13	4	638	734
Rm_Gut	323	877	35	1	619	697
Rm_Larvae	454	1014	15	10	625	701
Rm_Nymph	408	887	11	7	596	684
Rm_Ovary	538	1432	14	3	1195	1352
SAMPCLA3_Gut	140	659	10	0	900	586
SAMPCLA3_Ovary	303	1566	26	6	2296	1086
SAMPCLA3_SG	125	500	2	0	765	453
SAMPUTA_Gut	62	599	30	0	851	388
SAMPUTA_Ovary	284	2156	108	0	2889	1380
SAMPUTA_SG	81	541	74	0	1208	536
SAMPWV1_Gut	147	653	107	7	698	386
SAMPWV1_Ovary	257	1862	32	21	2044	1254
SAMPWV1_SG	184	733	62	22	978	680

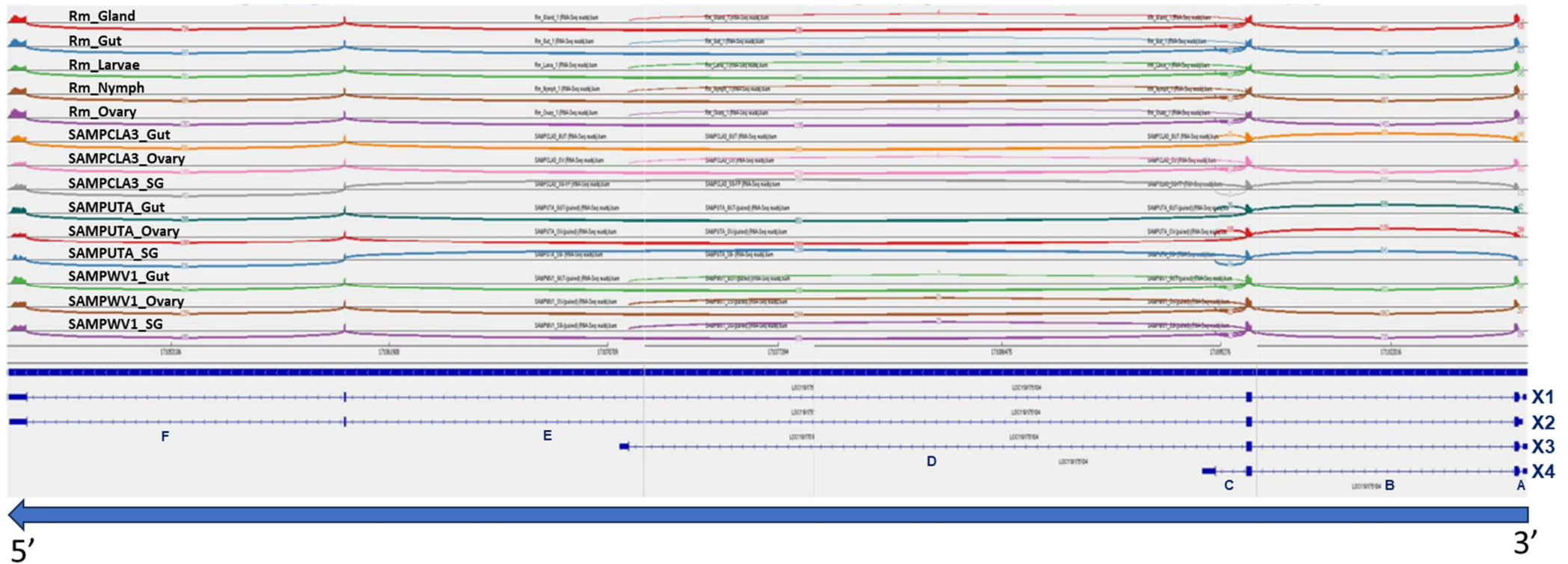


Figure 2.8. Sashimi Plot indicating the read coverage for each exon junction of the *Subolesin* gene. The name of each sample is indicated in black text above the curving lines. The mRNA species (X1-X4) are indicated in dark blue at the bottom of the image and the arrow at the bottom indicates the direction of transcription/translation, with dark blue bars representing exons and striped lines indicating introns. Each capital letter (A-F) refers to the intron indicated above it. The number of RNA sequencing reads (TPM normalised) supporting each intron is indicated by the curved line connecting exons to each other and corresponds to the support values in Table 2.3. The Sashimi plot was generated using Integrative Genomics Viewer (IGV, v.2.16.2).

2.3.4. Variant detection

The choice of the reference sequence used for variant detection is crucial. If the South African RNA sequencing data is mapped against the Chinese genome assembly, then the variants detected would represent geographical diversity between China and South Africa. Therefore, to determine the diversity amongst South African sequences, an RSA reference sequence is needed. As such, following the creation of the RSA *de novo* assembly (Section 2.3.1), a consensus nucleotide sequence for each mature mRNA species was created to use in the variant detection pipeline. This was accomplished by using BLAST to locate RNA sequencing reads that span the full-length of the open reading frame of each mature mRNA including the CDS (Table 2.4), before the reads were extracted and combined to create consensus sequences.

Table 2.4. Final BLAST parameters used to obtain the consensus sequence of each mRNA species, X1-X4 by using blastn against the RSA representative *de novo* transcriptome assembly. Gap cost and match/mismatch parameters were kept as default. The BLOSUM62 matrix was used, lower complexity regions were masked, and redundant results were not filtered out.

Parameter	X1	X2	X3	X4
Expectation value	1.0E-27	1.0E-55	1.0E-26	1.0E-26
Word size	7	40	4	4

After consensus sequences were created, the variant detection analysis was completed for each mRNA species. No variants were detected within the coding sequence (CDS) region of the X1 mRNA species, across all tissues and life stages. Variants were detected outside of the CDS however, these variants did not affect the resulting protein (Figure 2.9). Furthermore, all variants detected were single nucleotide variants (SNVs) and were common across all samples, regardless of life stage, tissue, and sampling location (Figure 2.9).

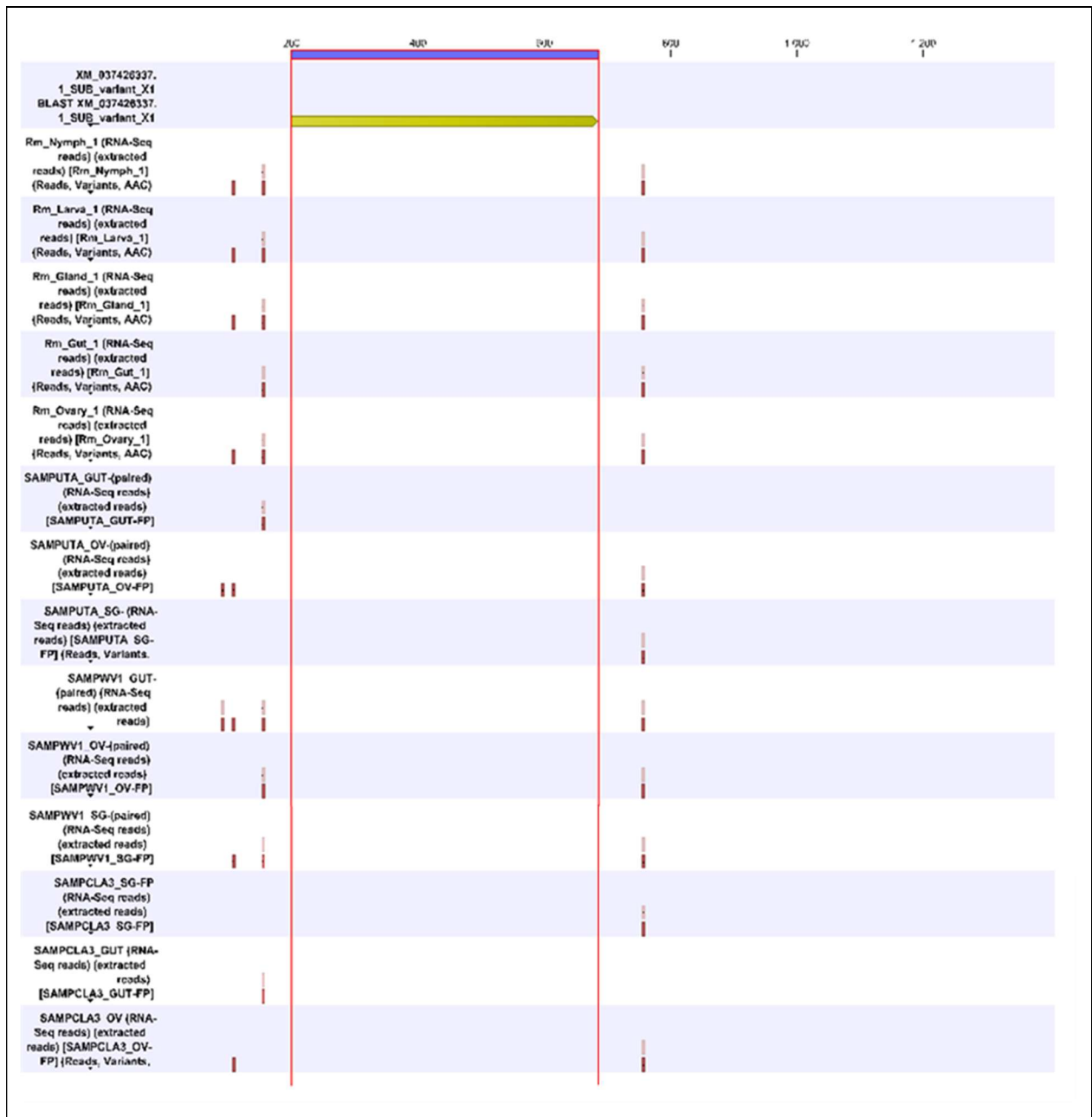


Figure 2.9. Variants detected in the Subolesin X1 mRNA species in RNA sequencing data from tick samples collected from Mnisi and ClinVet. The name of each sample is indicated on the left side, and the section enclosed by vertical red lines indicates the coding sequence (CDS). Nucleotide changes are indicated by the small red squares. A single dark red square indicates that only a single variant was called at a certain position, whereas more than one square indicates that more than one variant was called at that position. Tabulated statistics on variants detected are listed in supplementary.

The same trend was observed in the case of the X2 (Figure 2.10) and the X3 mRNAs (Figure 2.11), where there were no variants detected within the CDS. Furthermore, all variants detected were outside the CDS region and were either SNVs or deletions for both the X2 (Figure 2.10) and X3 mRNA species (Figure 2.11). There were many more variants observed

for the X2 mRNA species (Figure 2.10) than the X1 and X3 mRNA species. As before these variants were not unique to any sampling location, tissue, or life stage.

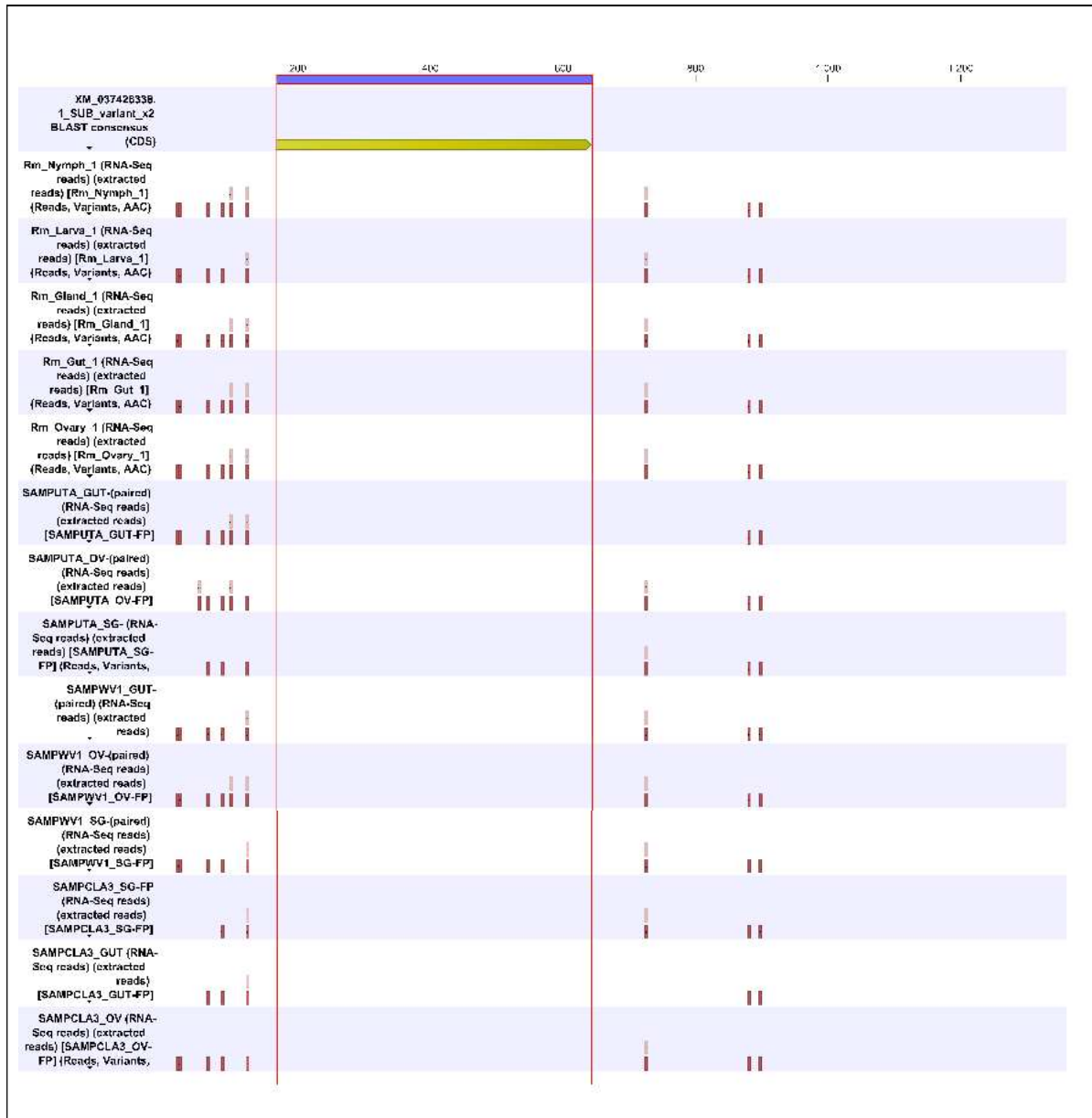


Figure 2.10. Variants detected in the Subolesin X2 mRNA species in RNA sequencing data from tick samples collected from Mnisi and ClinVet. The name of each sample is indicated on the left side, and the section enclosed by vertical red lines indicates the coding sequence (CDS). Nucleotide changes are indicated by the small red squares. A single dark red square indicates that only a single variant was called at a certain position, whereas more than one square indicates that more than one variant was called at that position. Tabulated statistics on variants detected are listed in supplementary.



Figure 2.11. Variants detected in the Subolesin X3 mRNA species in RNA sequencing data from tick samples collected from Mnisi and ClinVet. The name of each sample is indicated on the left side, and the section enclosed by vertical red lines indicates the coding sequence (CDS). Nucleotide changes are indicated by the small red squares. A single dark red square indicates that only a single variant was called at a certain position, whereas more than one square indicates that more than one variant was called at that position. Tabulated statistics on variants detected are listed in supplementary.

Despite the X4 mRNA species having the shortest length, it had the most variants of all the mRNA species (Figure 2.12). Furthermore, a variant was detected in the CDS of the X4 mRNA species in the ovary tissue sample from the Clare A dip station in Mnisi (SAMPCLA3_OV) (Figure 2.12).

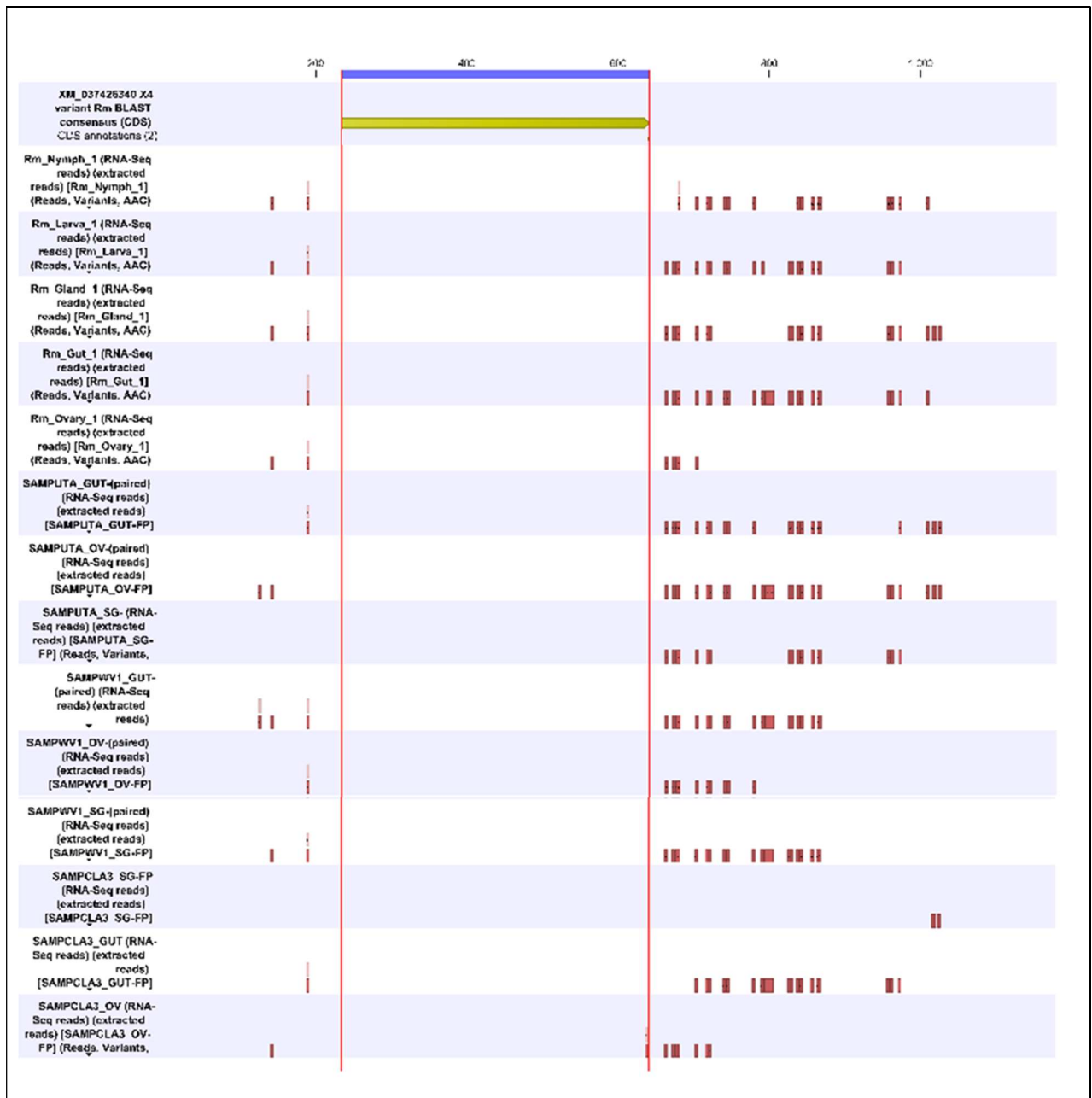


Figure 2.12. Variants detected in the Subolesin X4 mRNA species in RNA sequencing data from tick samples collected from Mnisi and ClinVet. The name of each sample is indicated on the left side, and the section enclosed by vertical red lines indicates the coding sequence (CDS). Nucleotide changes are indicated by the small red squares. A single dark red square indicates that only a single variant was called at a certain position, whereas more than one square indicates that more than one variant was called at that position. Tabulated statistics on variants detected are listed in supplementary.

Figure 2.13 depicts the single nucleotide variant found in the CDS of the X4 mRNA species. The variant resulted in a non-synonymous change from thymine to cytosine at nucleotide position 639 (639 T>C), changing a stop codon to an arginine at amino acid position 136 that resulted in the addition of four more amino acids (i.e., ATTK) before the next stop codon. Consequently, change leads to a new protein variant that is 140 amino acids long (Figure 2.13). From the current data, the influence on the predicted tertiary structure of this addition

on the new Clare variant is unknown (Figure S2). Furthermore, this variant is only present at a very low frequency of only 3.15789E+11 of countable reads covering this nucleotide position.

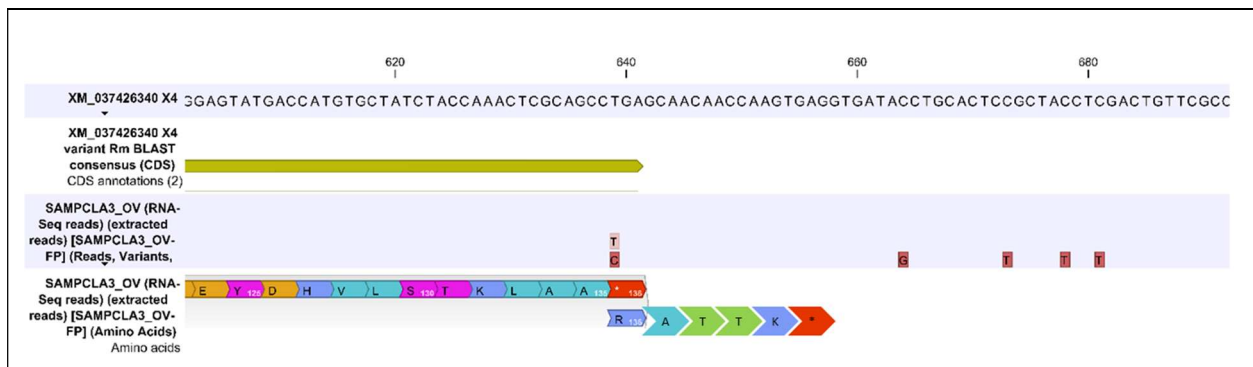


Figure 2.13. Single nucleotide variant detected in the X4 mRNA species CDS of the Subolesin sequence identified from Clare dip station and its translational consequences. The variant includes a change from a thymine to a cytosine (639 T>C). This variant is non-synonymous and changes a stop codon to an arginine, resulting in the addition of four more amino acids, namely alanine (A), threonine (T), threonine (T) and lysine (K) before the next stop codon.

2.3.5. Subolesin sequence analysis across global *Rhipicephalus microplus* data

An alignment of South African and global *R. microplus* Subolesin amino acid sequences are shown in Figure 2.14. Previously, linear B cell epitopes and conformational discontinuous epitopes were predicted from the Subolesin *R. microplus* amino acid sequence from Mexico (ABA62329.1) (Lagunes et al., 2016). These epitopes were found to be conserved across all South African Subolesin amino acid sequences (P1-P3) (Figure 2.14). The linear B cell and conformational discontinuous epitopes are highly conserved across global *R. microplus* populations, with the exception of some variants that were observed in several sequences from India (position 42) and Mexico (position 80) (Figure 2.14). The other variants observed had low frequencies and did not appear in more than one sample. Therefore, these variants could be sequencing errors and this should be confirmed in future.

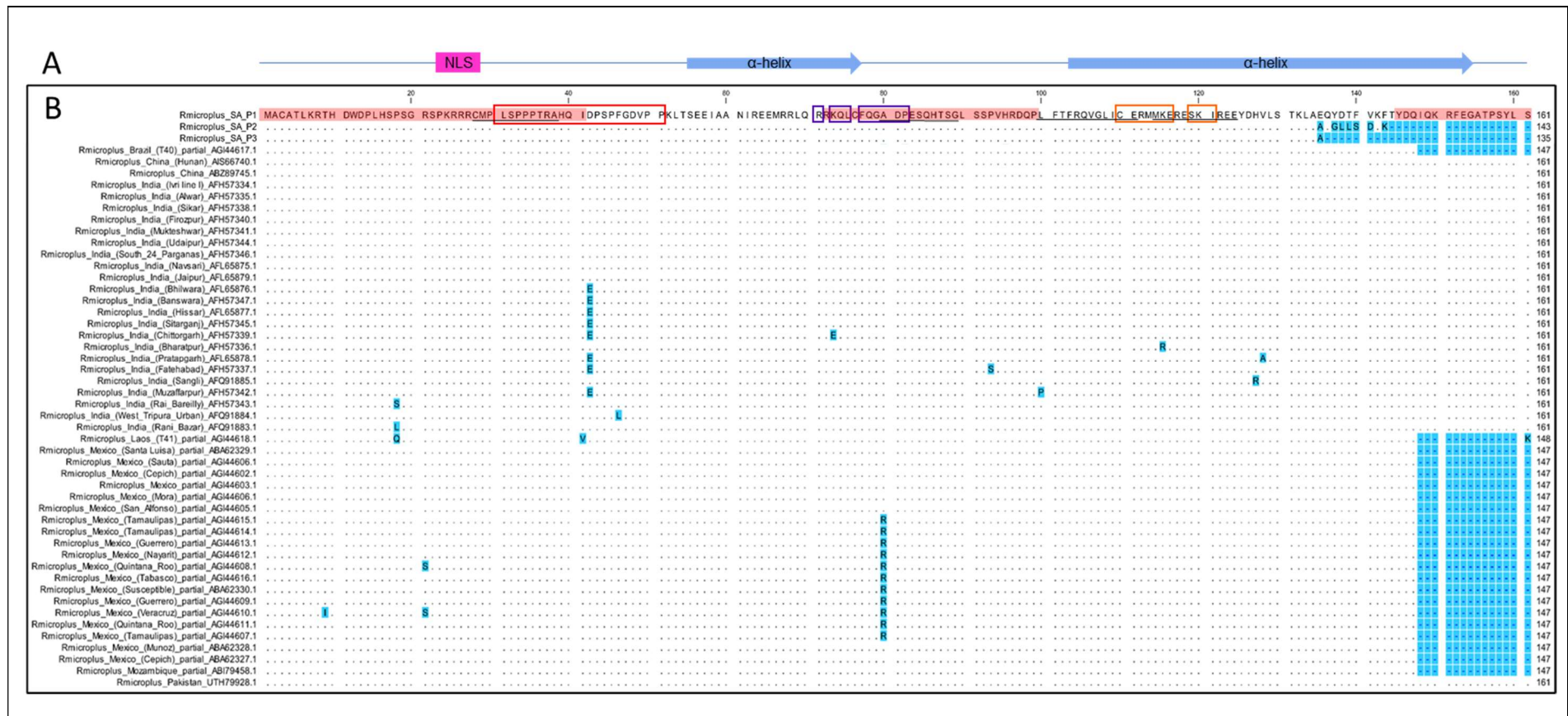


Figure 2.14. Amino acid sequence alignment of Subolesin protein from *Rhipicephalus microplus* ticks. (A) The secondary structure of *R. microplus* Subolesin P1 is indicated at the top, with alpha-helices (blue arrows) and NLS sequence (pink). **(B)** Subolesin sequences from global *R. microplus* strains were obtained from NCBI and were aligned to the representative South African Subolesin sequence from this study (P1). Mismatched/missing amino acids are indicated in blue. Linear B cell epitope regions predicted by Lagunes *et al.* (2016) are underlined, and conformational discontinuous epitopes are enclosed in red, purple and orange borders. B cell epitope regions predicted by Zeb *et al.* (2024) are highlighted in light red. Sequences were aligned in MAFFT (v.7.0.) using default settings, except for the gap penalty which was increased to 5.0.

In a recent study, different linear B cell epitope regions for *R. microplus* Subolesin was predicted (Zeb et al., 2024). As before, the differences in the predicted epitope regions are present in the *R. microplus* populations from India and Mexico (Figure 2.14). However, the last predicted epitope region covers the C-terminus (145-161 amino acids (aa)) of the Subolesin amino acid sequence. Since the amino acid length of the *R. microplus* Subolesin variants decrease from P1 to P3, the P2 and P3 variants do not have this terminal epitope (Figure 2.14).

Regardless, it is evident from Figure 2.14 that there was some overlap between these predicted epitope regions. Interestingly, two of these regions coincide with regions of low structural organisation, as predicted previously (Figure 2.5 and Figure 2.6). Moreover, the most variation between *R. microplus* strains were observed in the regions without an organised secondary structure, whereas the regions corresponding to the alpha-helices were highly conserved (Figure 2.14).

2.3.6. Subolesin sequence analysis across genera

In contrast, Subolesin is less conserved across tick species from the genera *Rhipicephalus*, *Amblyomma*, *Dermacentor*, *Ixodes*, *Hyalomma*, *Haemaphysalis* and *Ornithodoros* (Figure 2.15). It is evident from Figure 2.15 that the region (154-170 aa) show considerable overlap between epitopes recognised by IgG antibodies raised in two cattle breeds immunized with a cocktail of Subolesin antigens and Subolesin derived from *R. appendiculatus* (Figure 2.15). In *R. appendiculatus* and *R. decoloratus* this region was recognised by antibodies from all immunisation groups, and in *A. variegatum* this region was recognised by antibodies from three immunisation groups (Figure 2.15). Furthermore, this region overlapped with the linear B cell epitope (160-169 aa) predicted in *I. scapularis* (Prudencio et al., 2010), but not with epitopes predicted in any other species. However, this region formed part of the Q38 subolesin/akirin chimera vaccine tested in mice (Moreno-Cid et al., 2013), which exhibited extensive overlap with the Subolesin peptide sequence derived from *R. microplus*, as seen at 48-55 aa, 83-86 aa, and 112-154 aa (Figure 2.15).

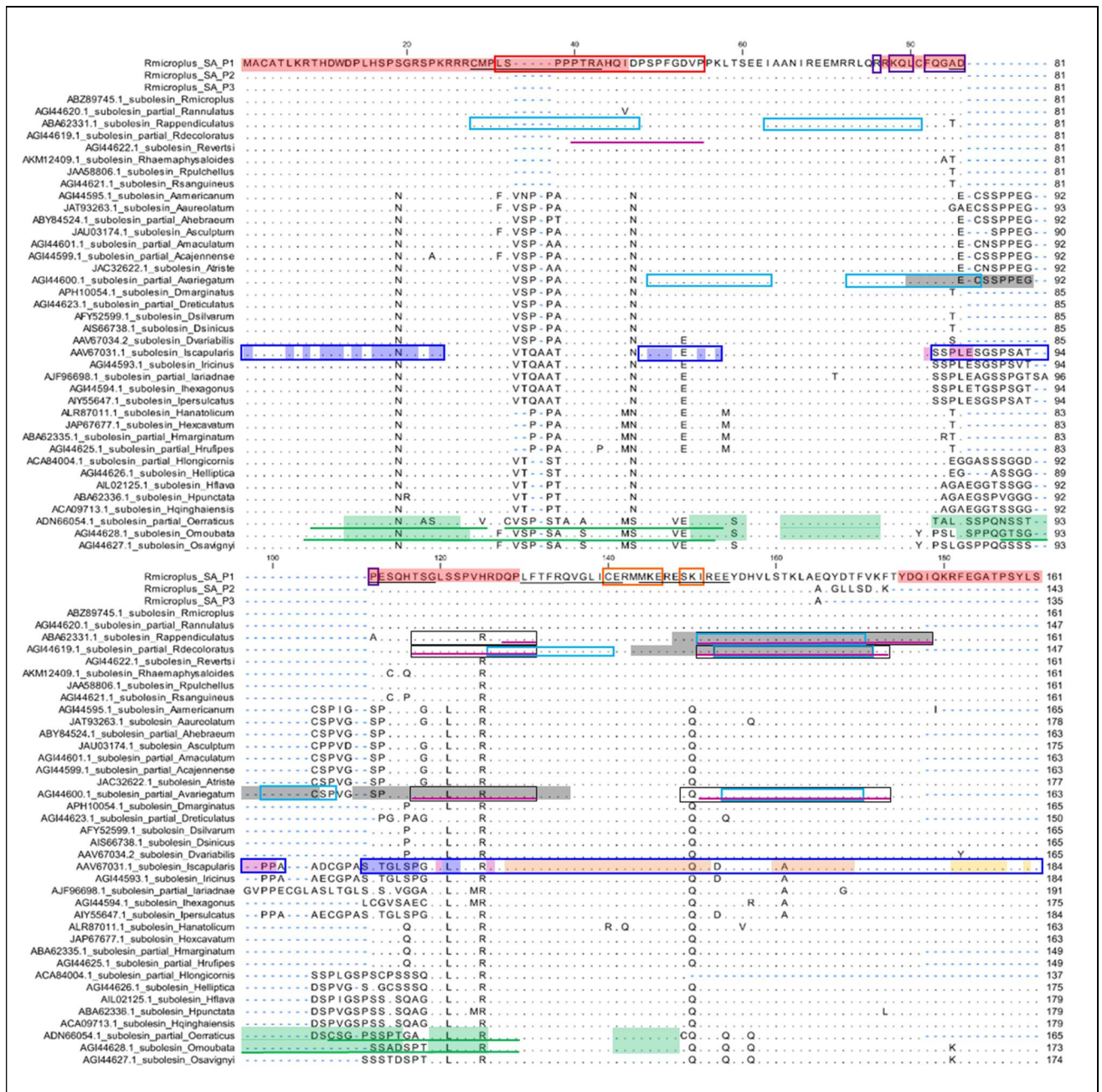


Figure 2.15. Amino acid sequence alignment of the Subolesin protein from different tick species. Subolesin sequences from tick species from the genera *Rhipicephalus*, *Amblyomma*, *Dermacentor*, *Ixodes*, *Hyalomma*, *Haemaphysalis* and *Ornithodoros* were aligned to the representative South African Subolesin sequence from this study (P1). Residues matching the P1 sequence are indicated by black dots, missing residues are indicated as blue dashes and mismatched residues are indicated as the corresponding single letter amino acid code. Linear B cell epitope regions predicted by BCEpred, Antigenic, ABCpred, and IEDB (Immune Epitope Database and Analysis Resource) (at least three) are underlined in black, and correspond to the epitope regions contained in the Subolesin peptide vaccine evaluated in calves (Lagunes et al., 2016), and conformational discontinuous epitopes are enclosed in a red, purple and orange borders. B cell epitope regions predicted by IEDB are highlighted in light red (Zeb et al., 2024). Linear B cell epitope regions were predicted using BcePred (highlighted in dark green) and ABCPred (underlined in dark green) for *Ornithodoros* spp (Manzano-Román et al., 2015). Conformational discontinuous epitopes predicted through epitopes peptides identified from IgG raised in sera in rabbits and sheep immunized with Subolesin, including conformational discontinuous (highlighted in dark blue, pink and yellow) and linear B cell (highlighted in orange) epitopes (Prudencio et al., 2010). Epitope regions recognised by IgG from crossbred (highlighted in grey) and *Bos indicus* (light blue border) cattle immunized with SUB cocktail, and crossbred cattle (black border) and *Bos indicus* (underlined in pink) immunized with SUBra (*R. appendiculatus* Subolesin antigen) (Contreras et al., 2022). The amino acids that constitute the Q38 chimera is enclosed in a dark blue border (Contreras et al., 2022). The sequence of the epitopes used in this figure are listed in Table S4. Sequences were aligned in MAFFT (v.7.0.) using default settings, except for the gap penalty which was increased to 5.0.

2.4. Discussion

Despite evidence showing Subolesin as essential in numerous tick biological processes, much about the Subolesin gene remains unknown (Artigas-Jerónimo et al., 2018). To date, the Subolesin gene structure has only been fully elucidated in *I. scapularis*, whereas in *D. silvarum* and *R. sanguineus* the gene organization pattern has been revealed through annotation software (de la Fuente et al., 2006b; Hu et al., 2014; Naranjo et al., 2013). Furthermore, previous studies have investigated the similarity of the Subolesin gene between tick species and concluded that *R. microplus* Subolesin has a 77% degree of identity among the genera *Hyalomma*, *Amblyomma*, and *Ixodes* (Lagunes Quintanilla, 2019).

Recently, the full-length open reading frame (ORF) of *R. microplus* was confirmed through the generation of cDNA (complementary DNA) and subsequent PCR (polymerase chain reaction) amplification (Zeb et al., 2024). However, the gene structure was not elucidated. Therefore, this study elucidated for the first time the gene structure of Subolesin in *R. microplus*. In this regard, there are several key differences between the Subolesin genes in *I. scapularis* and *R. microplus*. Firstly, the *R. microplus* Subolesin gene is approximately 60,903 nt in length, which is more than double the length of the *I. scapularis* Subolesin gene (24,504 nt) (Figure 2.3)(Naranjo et al., 2013). Additionally, NF- κ B- binding sites were found in the *I. scapularis* Subolesin gene, which were lacking in the *R. microplus* Subolesin gene (Figure 2.3)(Naranjo et al., 2013). Furthermore, the TATA box is responsible for initiating transcription in the *I. scapularis* Subolesin gene (Naranjo et al., 2013), whereas only arthropod initiation factors that can initiate transcription in both the presence and absence of a TATA box (Cherbas and Cherbas, 1993) were identified in the *R. microplus* gene (Figure 2.3).

Similarities between *R. microplus* and *I. scapularis* Subolesin include the gene organization pattern, where both have five exons and four introns (Naranjo et al., 2013). Furthermore, as found in *R. microplus*, two putative transcription start sites (TSSs) were found in the *I. scapularis* Subolesin gene, and the TSSs were confirmed as major and minor TSSs through the rapid amplification of cDNA ends (5'-RACE) (Naranjo et al., 2013). However, both TSSs were found in the first exon, whereas in *R. microplus*, the difference between the TSSs means the inclusion or exclusion of the first exon. Furthermore, arthropod initiation factors, such as identified in exon 2 of the *R. microplus* gene, are usually found within a ± 10 nt region of a TSS (Cherbas and Cherbas, 1993). In contrast, the arthropod initiation factor found in the first exon of the *R. microplus* gene is approximately 48 nt upstream from the putative TSS. Moreover, the arthropod INR found upstream of the first putative TSS is one of the less

dominant cognates, whereas the arthropod INR upstream of the second TSS is the most dominant cognate (Figure 2.3) (Cherbas and Cherbas, 1993). Combined with the low levels of support observed for the exon/intron junctions of exon 1, these results suggest that the second TSS is the major TSS. However, in future both putative TSSs in *R. microplus* could be confirmed using complementary techniques such as cap analysis of gene expression (CAGE) or robust analysis of 5'-transcript ends (5'-RATE) (Gowda et al., 2006; Shiraki et al., 2003).

In addition to these two transcripts resulting from alternative TSSs (X1 and X2), there are two more transcripts (X3 and X4) which most likely arise through alternative splicing, since they are identical apart from their last two exons which are unique due to differences in sequence and length (Figure 2.4). For translation to occur, pre-mRNA is matured via the spliceosomal complex in the nuclear compartment to remove intronic sequences (Latchman, 2015). The pre-mRNA requires further processing with the addition of both a 5' cap and 3' poly-A tail, before the now mature mRNA can be exported from the nucleus to the cytoplasm (Kelly and Corbett, 2009; Latchman, 2015). The Subolesin mRNA species are most likely transcribed by RNA polymerase II (RNA pol II), since most protein coding genes are transcribed by this enzyme complex, as well as due to the RNA pol II associated arthropod initiation factors found near the TSSs (Figure 2.2 and Figure 2.3) (Cherbas and Cherbas, 1993; Latchman, 2015). Since elongation requires the phosphorylation of the C-terminal repeat (S2) on RNA pol II through pTEF-b, which is a cap dependent kinase, it can be assumed that the mRNA species have 5' caps (Latchman, 2015). Furthermore, each Subolesin mRNA species' terminal exon is punctuated by a Poly-A signal, therefore, suggesting that each mRNA species can become mature mRNA that has both a 5' cap and a 3' poly-A tail. Consequently, each mRNA species has the potential to be translated. Currently, *R. microplus* is the only tick species that appears to have more than one putative mRNA species that can be transcribed from the Subolesin gene predicted to yield three protein isoforms after translation (Figure 2.4).

However, in future the presence of these mRNA species/isoforms should be confirmed using Long-Read Sequencing (LRS) technologies such as those from Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT) (Branton et al., 2008; Rhoads and Au, 2015). Since Illumina sequencing uses short read technology, studying alternative splicing and mRNA isoforms is limited by the recovery of splice junction sites from short reads (Su et al., 2024). Therefore, LRS technologies would enable full length mRNA species to be sequenced and overcome this limitation.

It has been shown that alternative splicing isoforms/species could have tissue specific functional roles. However, studies investigating the functional and tissue-specific differences between the potential protein variants, discovered that most of the alternative splicing isoforms are not detectable as proteins, even when low peptide coverage is taken into account (Ezkurdia et al., 2015). It has been suggested that this could be due to a low abundance of these protein isoforms/variants or that most alternative splicing isoforms/species are the result of erroneous splicing (Pozo et al., 2021). Another explanation posits that there is usually one main functional isoform that is translated, whereas the other mRNA species created may have an unknown function (Ezkurdia et al., 2015; Pozo et al., 2021). This might be the case for *R. microplus* Subolesin, based on the comparatively lower levels of expression of the other pre- and mature mRNA species (Figure 2.7, Figure 2.8 and Table 2.3).

The third mRNA species (X3) and fourth mRNA species (X4) both share the same first three exons as the X1 mRNA species, but each have a unique fourth exon and translates to shorter proteins of 143 and 135 amino acids, respectively (Figure 2.5). Although the X1 and X2 mRNA species differ, both encode the same protein (P1, 161 amino acids) (Figure 2.5). Despite this, the secondary structure of the three protein variants is very similar (Figure 2.5), which is reflected in the predicted tertiary structures (Figure 2.6 and Figure S1). However, this contradicts what was predicted in other ticks.

In *Ornithodoros* spp. (*O. moubata* and *O. erraticus*) three alpha-helices were predicted in the secondary structure of Subolesin (Manzano-Román et al., 2015), of which the latter two corresponded to the amino acids forming the two alpha-helices predicted in this study (Figure 2.5). Furthermore, the tertiary structure for *I. scapularis* was modelled on the Sleeping Beauty transposase due to its potential participation in NF-κB signalling pathway, which Subolesin is a potential effector in (Artigas-Jerónimo et al., 2018) (Figure 1.6). Based on the modelling, *I. scapularis* Subolesin has 4 alpha-helices (Artigas-Jerónimo et al., 2018), compared to the two predicted in *R. microplus* in this study (Figure 2.6). Furthermore, a DNA binding site (clamp loop) was predicted in the last two alpha-helices in *I. scapularis* Subolesin and was modelled on the DNA-bound transposase, Mos1, from *Drosophila mauritiana* (Artigas-Jerónimo et al., 2018; Voigt et al., 2016). The residues that form the predicted DNA binding site were fully conserved, however it was located in a single alpha-helix (103-153 aa) (Figure 2.5) of *R. microplus* in contrast to being found in two alpha-helices as predicted in *I. scapularis* (Artigas-Jerónimo et al., 2018). However, this was only the case for the P1 *R. microplus* protein variant, as the DNA binding site residues in the second alpha-helix were altered and missing in the shorter protein variants (P2 and P3) (Figure 2.5). This suggests that the P2 and P3 protein

variants likely do not have DNA binding capabilities. Furthermore, since the DNA binding residues are found within a single alpha-helix, the formation of a clamp loop structure as predicted for *I. scapularis* seems unlikely (Artigas-Jerónimo et al., 2018). However, since there are no crystal structures for Subolesin, and the tertiary structure and DNA binding sites were modelled on the Sleeping Beauty transposase and Mos 1 transposase respectively and had very low residue conservation (Sleeping Beauty transposase 7% and Mos 1 transposase 5%) (Artigas-Jerónimo et al., 2018), these results must be confirmed.

Aside from the predicted alpha-helices, the rest of the *R. microplus* Subolesin protein structure consists of highly disordered regions (Figure 2.5), which is consistent with findings in other tick species (Artigas-Jerónimo et al., 2018; Ghosh et al., 2019; Manzano-Román et al., 2015). Intrinsically disordered regions in proteins are considered flexible functional regions, that can undergo ‘induced folding’ or conformational changes when interacting with binding partners and consequently can have multiple binding partners (Trivedi and Nagarajaram, 2022). Since Subolesin is thought to interact with transcription factors e.g. GII protein has elongation factor 1-alpha (EF-1α) II and III domains (de la Fuente et al., 2008), it is possible that Subolesin needs to have a flexible structure to interact with multiple binding partners (Goto et al., 2008; Naranjo et al., 2013). Therefore, the presence of these disordered regions suggests that the Subolesin protein could be a very dynamic protein (Trivedi and Nagarajaram, 2022). Moreover, the same nuclear localisation signal (PKRRRC) was identified in all protein variants, supporting trafficking to the nucleus (Latchman, 2015), as shown in *R. microplus* (Antunes et al., 2014) and *I. scapularis* (Artigas-Jerónimo et al., 2018). It has been suggested that Subolesin potentially affects transcriptional regulation which takes place in the nucleus (Goto et al., 2008). No evidence was found in this study to suggest an extracellular role for any of the putative Subolesin protein variants (e.g. secretory function), therefore this study strengthens the support for Subolesin’s functional role in the nucleus. However, since post-translational modifications such as phosphorylation has also been predicted in *I. scapularis* Subolesin, the possibility of post-translational modifications affecting the structure and function of the protein variants should not be excluded (de la Fuente et al., 2008; Ramazi and Zahiri, 2021).

The expression levels of Subolesin differed between samples on the mRNA species level, where the mature forms of X3 and X4 mRNAs had low levels of expression, in contrast to that of X1 and X2 mature transcripts. Although both the X1 and X2 transcripts had the highest expression levels, the X2 pre-mRNA species appears to be the most highly expressed according to the Sashimi plot (Figure 2.8). This supports the possibility that the X2 transcript

is the primary Subolesin variant (Ezkurdia et al., 2015). However, in future the results from the Sashimi plot can be strengthened by determining isoform expression estimates by using the MISO algorithm (Katz et al., 2015). Alternatively, further confirmation of expression levels of all the putative mRNA species can include molecular techniques, e.g. quantitative real-time PCR (Camacho Londoño and Philipp, 2016), as well as proteomics techniques, e.g. Edman or LC-MS-MS to confirm whether these transcripts are really translated into functional proteins. In addition, tissue isolation from ticks followed by differential centrifugation and a western blot using anti-Subolesin sera may be performed to determine the tissue-specific subcellular expression of Subolesin. Finally, Long-Read Sequencing (LRS) technologies can also be used along with software capable of detecting and quantifying transcript isoform abundance (Su et al., 2024), which would negate the need for quantifying the RNA sequencing reads spanning the intron/exon junctions using Sashimi plots and MISO .

Subolesin was determined to be expressed across all life stages of *R. microplus* ticks, which is consistent with findings in other tick species (Lu et al., 2015; Sultana et al., 2015). However, there were no notable differences between adults and other developmental stages identified in this study. This expression pattern contradicts what was found in “soft” ticks (*Ornithodoros turicata*), where Subolesin had significantly higher expression levels in adult life stages, compared to larvae and nymphs (Sultana et al., 2015). However, there does not seem to be a conserved expression pattern for the life stages in “hard” ticks either, as it was shown that Subolesin was highly expressed in *R. haemaphysaloides* eggs compared to other life stages (Lu et al., 2015). Whereas for *Haemaphysalis flava*, Subolesin had significantly higher expression levels in the engorged adult females (Liu et al., 2016). Moreover, the expression of Subolesin in this study was relatively conserved across tissues similar to findings in *R. haemaphysaloides*, but contrary to findings in *H. flava* (Liu et al., 2016; Lu et al., 2015). However, it should be noted that the variation between these studies could also be due to differences in the times of/since feeding (fed or unfed) of ticks before collection, as well as the age or sex of the ticks collected (Liu et al., 2016; Lu et al., 2015; Sultana et al., 2015). Nevertheless, the expression of Subolesin seems to vary on a species level.

In early bacterial studies, it was shown that essential genes are more conserved than non-essential genes (Jordan et al., 2002; Luo et al., 2015; Rocha and Danchin, 2004). Similarly, essential genes have since been shown to be highly conserved in eukaryotes (Krylov et al., 2003). These high levels of conservation are attributed to slower evolution rates (sequence divergence) due to selective pressure constraints, as well as a lower propensity for gene loss due to the essentiality of their function (Krylov et al., 2003). Since Subolesin is a highly

conserved protein, the results from variant detection were unsurprising. There were no variants detected within the CDS of the X1 and X2 transcripts, coding for the full-length Subolesin protein (possibly the main functional species). This coincides with the 'knockout rate' hypothesis, which posits that the greater the effect a gene knockout has on overall fitness, the slower the evolutionary rate (Wilson et al., 1977). Several studies have shown that knockout of Subolesin results in tick mortality and significantly lower fertility rates, therefore, it would be possible that the Subolesin gene evolves slowly (de la Fuente et al., 2005; de la Fuente et al., 2006a; de la Fuente et al., 2006c; de la Fuente et al., 2008). Furthermore, since Subolesin (tick akirin) is conserved across the metazoa, it most likely has a low propensity of gene loss (Artigas-Jerónimo et al., 2018; Krylov et al., 2003). In eukaryotes, genes with this characteristic are associated with a lower rate of sequence evolution, high expression and are more likely to have several interacting partners (Krylov et al., 2003). Therefore, considering the findings from aforementioned studies and the findings from this study, further support has been provided for Subolesin as an essential gene for tick survival.

In regard to tick vaccine development, a significant challenge impacting vaccine efficacy is antigenic variation, especially within epitope regions (Parizi et al., 2023). Epitopes can further be divided into linear (continuous) and conformational (discontinuous) epitopes (Zhang et al., 2011). Linear epitopes consist of a sequence of continuous amino acids that are sufficient for binding, whereas conformational epitopes are formed by key amino acid residues that are united through protein conformation (Zhang et al., 2011). From Figure 2.14 it is evident that the predicted epitope regions within the alpha-helix regions are the most conserved, within *R. microplus* strains (Figure 2.14). In contrast, the amino acid regions with no predicted structural organisation have the most variation (Figure 2.14). Typically, most B cell epitopes do not have an organised secondary structure like alpha-helices or beta sheets (Kringelum et al., 2013). Furthermore, epitopes are generally more surface exposed or protrude from the antigen surface, which coincides with the observation that protein-protein interaction sites are typically flexible regions (Kringelum et al., 2013). However, since there are no crystal structures available for Subolesin, the exact conformation is still undetermined (Figure 2.6). Therefore, the influence of protein conformation on the accessibility of these epitopes cannot yet be confirmed. Consequently, the predicted conformational discontinuous epitopes can only be considered preliminary. Furthermore, although the epitope regions are mostly conserved, there are some amino acid residues that differ (Figure 2.14). However, future work should determine whether these differences are key residues essential for epitope recognition/binding and could potentially contribute to immune evasion.

As expected (Figure 1.5), Subolesin was highly conserved within genera, with the most variation occurring between species from different genera (Figure 2.15). As observed previously (Figure 2.14), the highest amino acid residue conservation between tick species was observed in regions corresponding to the predicted *R. microplus* alpha-helices (Figure 2.15). However, there is still extensive antigenic variation between Subolesin from different tick species within the predicted disordered regions (Figure 2.15). Epitope regions recognised by IgG from crossbred and *Bos indicus* cattle immunized with a SUB cocktail (Subolesin derived from *R. decoloratus*, *A. variegatum*, and *R. appendiculatus*) or with SUBra (Subolesin derived from *R. appendiculatus*) were identified (Contreras et al., 2022). Although there were several overlapping regions, there was only one region that was identified in both cattle breeds and immunization groups in *R. appendiculatus* (151-180 aa) and in *R. decoloratus* (153-171 aa) (Figure 2.15). This region was also partially recognised by SUB cocktail immunized *Bos indicus* IgG and both cattle breeds immunized with SUBra in *A. variegatum* (Figure 2.15)(Contreras et al., 2022). However, this region only partially overlaps with one predicted linear B cell epitope in *I. scapularis* (Prudencio et al., 2010), but not any predicted epitopes from any other species (Figure 2.15). The *R. microplus* derived Subolesin peptide (Lagunes et al., 2016) had considerable overlap with the amino acid regions (48-55 aa, 83-86 aa, 112-154 aa) that constitutes the Q38 subolesin/akirin chimera (Figure 2.15) (Moreno-Cid et al., 2013).

However, the *in silico* predicted epitopes only showed partial overlap with the epitopes recognized in sera from vaccinated animals (Contreras et al., 2022; Prudencio et al., 2010) (Figure 2.15). These results indicate that current *in silico* epitope predictions are limited. One reason could be due to the omission of bovine specific major histocompatibility complexes I and II (MHCI and MHCII) epitopes, since bovines are non-model organisms and have high diversity in their MHC genes (Vasoya et al., 2021). However, a database has recently been made available where bovine MHCI and MHCII repertoires can be recognized in tandem (Vasoya et al., 2021), and a prediction tool that improves the evaluation and selection of bovine helper T cell epitopes has recently been made available (Fisch et al., 2021). Therefore, although bovine epitope prediction is not yet a foolproof method, the current epitope prediction paradigm could be improved by incorporating bovine specific databases with immunoinformatic tools, as comprehensively described in a novel pipeline by Bishop, L.J. (PhD).

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Chapter 3

Evaluation of host immune responses following immunization with parasite antigens adsorbed onto PLGA microparticles in a BALB/c murine model

3.1.1. Introduction

For the design of effective vaccines, an in-depth knowledge about the biological system where the vaccine is acting is essential. The current immunological tick control paradigm is focussed on antibodies which disrupt tick feeding and digestion of the blood meal. Briefly, ingested antibodies elicited upon vaccination recognise target tick proteins. This is effective due to the binding of antibodies which can follow two pathways: (1) steric hindrance (blocking) of the target protein function, e.g. antibody recognition of secreted salivary proteins (Narasimhan et al., 2004), and (2) the recruitment of effector proteins and/or cells, which leads to antibody dependent cell-mediated cytotoxicity (ADCC), e.g. antibodies binding to Bm86 exposed extracellularly on tick gut cells leads to the recruitment of cell lysis proteins (i.e., complement) which leads to cell death and interferes with gut cell endocytic abilities (Kemp et al., 1989; Willadsen et al., 1989).

However, the life cycle of *A. phagocytophilum* is more complex, since the parasite can reside intracellularly in infected host cells, circulate in the host blood or be taken up in the host blood meal and escape to replicate in the midgut/salivary gland tick cells (Figure 1.3). To target circulating *A. phagocytophilum*, MSP4 has been suggested as a good vaccine candidate, since MSP4 has been shown to play a role in tick-pathogen and host-pathogen cell adhesion interactions (Contreras et al., 2017; Villar et al., 2015). When targeting intracellular parasites, the control of infected cells requires the presentation of parasite proteins to the host immune system. In vertebrate hosts, this can occur via either the expression of parasite proteins on the host cell membrane or via the MHC I pathway (Janeway CA Jr, 2001). However, *A. phagocytophilum* infects neutrophils, eosinophils, and monocytes, and disrupts their functions and suppresses the immune system (Woldehiwet, 2008). Which in turn suppresses the presentation of parasite proteins via the MHC I pathway. Fluorescent microscopy has shown that MSP4 is present on the *A. phagocytophilum* bacterial cell membranes (Alberdi et al., 2015; Villar et al., 2015), however no studies have conclusively shown that the MSP4 protein is expressed on surface of the infected tick or host cells. Therefore, targeting intracellular *A. phagocytophilum* may require a different approach (Figure 3.1).

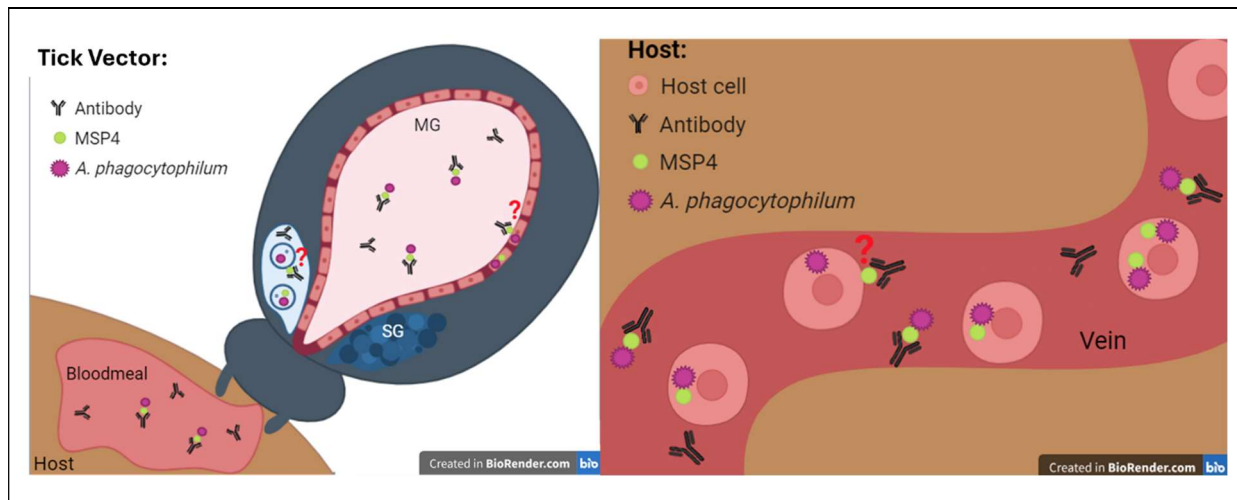


Figure 3.1. Accessibility of MSP4-specific antibodies to the *Anaplasma phagocytophilum* MSP4 protein in the tick vector and the host. In the tick vector, MSP4-specific antibodies can access circulating *A. phagocytophilum* in the bloodmeal and midgut lumen. However, the antibodies cannot access the MSP4 protein when *A. phagocytophilum* is inside tick cells since MSP4 has not yet been shown to be expressed on the tick cell surfaces (red question marks). In the host, MSP4-specific antibodies can access *A. phagocytophilum* circulating in the blood and/lymph, however, there has been no evidence that the MSP4 protein is expressed on the surface of infected host cells (red question mark).

One of the shortcomings of vaccines is their dependence on maintaining a cold chain during storage and distribution, as interruptions may result in potency loss (Chen and Kristensen, 2009; Kumru et al., 2014). Therefore, the development of thermotolerant formulations remains an intense field of study. Furthermore, several adjuvants cause unfavourable local inflammatory responses at the injection sites, which reduces farmers' willingness to use vaccines (Marit et al., 2022; van Doorn et al., 2016). Therefore, alternatives offering more stability are essential, such as PLGA microparticles. These particles are thermostable at room temperature and have been shown to increase the stability of antigens bound to them (Maina et al., 2020). There have been many studies using particles to target tick-borne diseases in cattle, as reviewed by (Maina et al., 2020). However, there have been few targeting ticks (Sales-Junior et al., 2005) and none using a combination approach to target both vector and pathogen.

The efficacy of particles as drug carriers or vaccine platforms is greatly influenced by design factors that determine the uptake of particles by the immune system, particle distribution, and immunogenicity (Horvath and Basler, 2023). This includes, but is not limited to particle size, surface charge and route of administration, which all greatly influences the avenue of uptake by the immune system (Horvath and Basler, 2023). Furthermore, the route of administration influences particle trafficking, since local or systemic administration recruits different immune cells as effectors (Horvath and Basler, 2023). Subcutaneous injection typically deposits

particles or antigens in the hypodermis, where the particles consequently trigger uptake, processing, and maturation via Langerhans dendritic cells (epidermis) and cutaneous dendritic cells (dermis) (Fathallah et al., 2013; Hwang, 2012). Larger microparticles or high concentrations of smaller particles can create a “depot” effect which elicits an acute inflammatory response and can recruit macrophages and neutrophils and prolong T cell stimulation (Koerner et al., 2021; Kohane, 2007). Microparticles internalised by dendritic cells (DCs) are transported to secondary lymphoid tissues via lymphatic vessels.

An immune response is triggered in lymphoid tissues (i.e., lymph node or spleen) when the particles are released into the endosome, which undergoes acidification once it fuses with a lysosome and could speed up the particle degradation and antigen release (Horvath and Basler, 2023). This is followed by antigen presentation of the degraded antigen cargo as peptides via major histocompatibility complex II (MHCII) molecules to CD4⁺ T cells (Figure 3.2). However, it has been shown that particles do manage to escape into the cytoplasm and undergo cross-presentation via MHCI molecules to CD8⁺ T cells (Figure 3.2) (Schliehe et al., 2011; Shen et al., 2006).

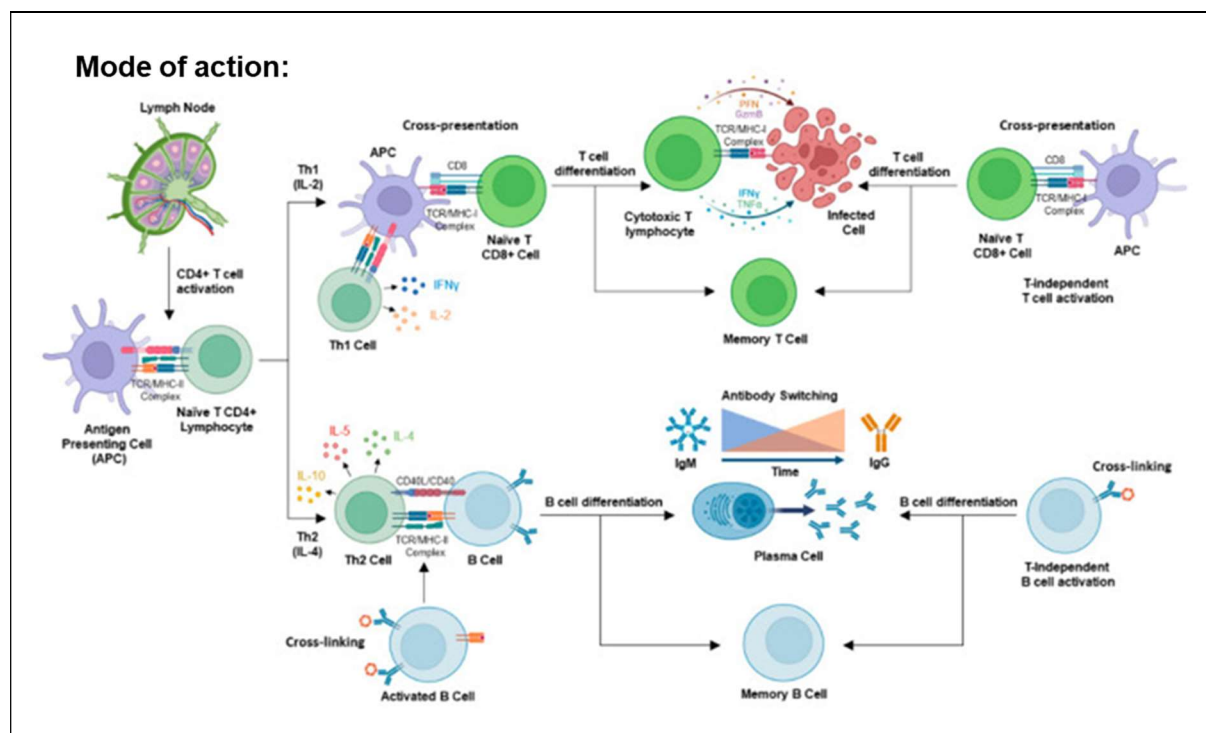


Figure 3.2. Activation of both humoral and cellular immune responses by microparticles. Following antigen presentation by antigen presenting cells (APCs), CD4⁺ T cells can differentiate into T helper 1 (Th1) cells, which promote the generation of cytotoxic and memory T cells by cross-presentation, or T helper 2 (Th2) cells, which enhance the differentiation of B cells into plasma or memory B cells. Microparticles can also promote B- and T-cell activation independent of Th cells. Modified from Jiménez-Cabello et al. (2022).

Long-lasting protection against ticks induced by tick vaccines depends on strong humoral responses (Ndawula, 2021). Ideally vaccination will lead to the formation of a strong memory B cell (MBC) response in the host, which can rapidly proliferate and produce large quantities of antibodies (Parham, 2015). These cell populations can circulate within the host for a long time after immunization (Parham, 2015). Ticks take up these antibodies through a blood meal, after which their fitness is negatively impacted (Willadsen, 2006). Therefore, the promotion of a Th2 driven B cell response via microparticles is an important added benefit for tick vaccines (Figure 3.2). In addition, in mice it has been shown that although a humoral response does play a role, a CD4+ T cell response is essential for *A. phagocytophilum* clearance (Table 1.3). Furthermore, the clearance of intracellular bacteria typically relies on a strong cellular response, including memory T cell and CD8+ T cells, which are promoted by Th1 cell activity (Alberts B, 2002; van Schaik et al., 2024).

Therefore, considering the biology of ticks and that of *A. phagocytophilum*, one will need both humoral and cellular immune responses for efficient control. Microparticles can elicit both cellular and humoral immune responses and this dual vaccine will benefit from this ability. In conclusion, this study investigates for the first time the immune profile elicited by immunization with Subolesin and MSP4 antigens adsorbed onto the surface of PLGA microparticles in a murine model by evaluating both the humoral and cellular response.

3.1.2. Biological questions

1. How does the addition of PLGA microparticles change the immune response elicited compared to the response elicited to the soluble antigen?
2. How does the addition of Montanide (adjuvant) influence the elicited immune response?

3.2. Materials and methods

3.2.1. Experimental animals

All animal studies were conducted at the Onderstepoort Veterinary Animal Research Unit (OVARU, South Africa). Experimental animals were treated in accordance with the Animal Protection Law of the Republic of South Africa and were approved in terms of Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) (approval No: 12/11/11/1 2386KL) and the Animal Ethics Committee of the University of Pretoria (approval No. NAS081/2022). Specific Pathogen Free (SPF) female BALB/c mice (6-8 weeks, ~20g each) were obtained from breeding stock maintained at the Animal Unit of the South African Vaccine Producers, a subsidiary of the National Health Laboratory Service (Johannesburg, South Africa). Mice were housed under a constant temperature of $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and relative humidity of $40\text{-}60\% \pm 20\%$. Individual treatment groups were housed separately (maximum 5 individuals per cage) in cages lined with sterilised sawdust bedding. Animals received sterilised water and pellets *ad libitum*.

3.2.2. Antigen production and purification

Recombinant antigens (Subolesin: AGI44593.1 and MSP4: AFD54597.1) were provided by a collaborator Prof. J. de la Fuente (Spain) and produced as follows: For the production of the heterologous proteins, One Shot™ BL21 Star™ (DE3) chemically competent *Escherichia coli* (Invitrogen, USA) were transformed with the target gene cloned into pET101/D-TOPO® expression vector (Ref. K101-01, Invitrogen, USA). One vial of *E. coli* cells was thawed on ice per transformation, before 10 ng plasmid DNA was added into each vial and mixed by stirring gently with a pipette tip. The cells were incubated on ice for 30 minutes and given a heat-shock for 30 seconds at 42°C without shaking, then the tubes were immediately transferred to ice. A volume of 250 μl room temperature Luria-Bertani (LB) medium was added to the cells and incubated at 37°C for 30 minutes with shaking (200 rpm). Then the entire transformation reaction was inoculated into 10 ml of LB containing 50 $\mu\text{g}/\text{ml}$ ampicillin (Sigma-Aldrich, St Louis, MO, USA) and 0.5% glucose (Laboratorios CONDA S.A., Madrid, Spain) and kept growing overnight at 37°C with shaking. Finally, 9 ml of the overnight culture was propagated into 1000 ml flasks containing 300 ml LB broth, 50 $\mu\text{g}/\text{ml}$ ampicillin and 0.5% glucose for 2 h at 37°C and 200 rpm, and then after 4-6 h Isopropyl β -d-1-thiogalactopyranoside (IPTG; 0.5 mM w/v; Sigma-Aldrich, USA) was added for induction of heterologous protein production. The cells were harvested by centrifugation at $3,900 \times g$ for 15 minutes at 4°C and stored at -80°C for purification procedure.

For protein purification, 1 g of the cells harvested from the expression procedure were resuspended in 5 ml of lysis buffer (50 mM potassium phosphate, pH 7.8, 400 mM NaCl, 100 mM KCl, 7 M Urea, 10 mM Imidazole) containing protease inhibitor (Ref. 04693132001, Roche, Spain) and disrupted using a cell sonicator (Model MS73; Bandelin Sonopuls, Germany). After disruption, the insoluble protein fraction containing the antigens as inclusion bodies were collected by centrifugation at 15,000 x g for 15 minutes at 4°C and stored at -20°C until purification procedure. Recombinant proteins were purified by Ni-NTA affinity chromatography using 1 ml HisTrap™ Fast Flow columns (Merck, Germany) mounted on the AKTA-FPLC system (GE Healthcare, USA) in the presence of 7 M urea. The eluted fraction containing the purified proteins was dialyzed against 1000 volumes of PBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) for 12 h at +4°C. The protein concentration was determined using a bicinchoninic acid assay (Ref. 23225, Pierce™ BCA Protein Assay Kit, Thermo Scientific, USA) (Section 3.2.3.). Finally, the recombinant proteins were concentrated using an Amicon® Ultra Centrifugal Filter (Merck, Germany) with a 3 kDa molecular weight cutoff and lyophilized.

3.2.3. Microparticle production and vaccine formulation

The PLGA microparticles were produced in our collaborator's laboratory, Dr. S. Murdan (UCL School of Pharmacy, UK) using a double emulsion solvent evaporation method as described by (McConnell et al., 2008). Briefly, 5 ml of 5% weight per volume (w/v) PLGA in dichloromethane and 1 ml of 5% (w/v) polyvinyl alcohol (PVA) were homogenised for 3 minutes at 24,500 rpm in deionised water. The resulting primary water-in-oil emulsion was added dropwise to 40 ml of a 5% (w/v) PVA solution while homogenising for 5 minutes at 10,000 rpm to form a double water-in-oil emulsion. The emulsion was stirred overnight to allow solvent evaporation and microparticle hardening. The microparticles were then harvested through centrifugation at 15,000 rpm for 10 minutes. The resulting pellet was collected, resuspended in water, centrifuged once more. The process was repeated to remove any remaining PVA before lyophilising the microparticles and storing it at 2-8°C.

The tick and bacterial antigens were adsorbed onto different particles (MSP4 and Subolesin separately). Antigens were adsorbed onto the particle surface at a 1:5 ratio as follows: 1000 µl of stock antigen solution (200 µg/ml) was added to a vial of 1000 µg of PLGA MPs and was mixed by pipetting multiple times. The resulting suspension was left on a shaker overnight at 4°C and then freeze-dried. The adsorption of antigens (%) on PLGA MPs were determined using a micro-BCA assay (Ref. 23225, Pierce™ BCA Protein Assay Kit, Thermo Scientific, USA). Briefly, the freeze-dried sample was resuspended in 1000 µl PBS, then centrifuged at

3,000 rpm for 5 minutes. The supernatant was collected for assay. Working Reagent (WR) was prepared by mixing 25 parts of Micro BCA Reagent A and 24 parts Reagent B with one part of Reagent C (25:24:1, Reagent A: B: C). 150 µl of each sample (supernatant) was pipetted in 5 replicates into a microplate well, plus 5 replicates of the blank (PBS pH 7.4) and incubated at 37°C for 2 hours. After the 2 hours plates were cooled to room temperature and absorbance was measured at 562 nm. The average absorbance of the blank standard replicates was subtracted from the reading of all other individual sample replicates. The standard curves for ovalbumin (OVA) prepared previously was used to determine amount of protein in each unknown sample. Best-fit curve fitting, and its equation was used in calculations. The morphology of the microparticles were assessed, briefly, samples were mounted onto aluminium stubs and allowed to dry overnight before being sputter coated with gold in a high-vacuum evaporator for three minutes at 30 mA (Emitech K550, Ashford, England) and photographed using a scanning electron microscope (SEM, Philips XL30, Eindhoven, Holland). The average size (nm) and the surface properties (Zeta potential; mV) of the microparticles (blank and antigen adsorbed) were determined.

For vaccine formulation, microparticles and recombinant antigens were resuspended in 1 × phosphate buffered saline (PBS). Each dose contained 20 µg of the appropriate antigen and 100 µg of PLGA microparticles as appropriate for the immunization group. The adjuvant Montanide™ ISA 50 V2 (Seppic, France) was combined at a 1:1 volume ratio with the PBS microparticle formulation or soluble recombinant antigen for vaccination.

3.2.4. Immunisation trial setup

The BALB/c mice were randomly divided into eight experimental groups (n=5) and allowed to acclimatise for seven days prior to the start of the study. Mice received subcutaneous immunizations under the loose skin between the shoulders and neck area on day 0 and day 14 before finally being euthanized on day 21. The eight groups received the following formulations: (A) MSP4 coated microparticles without adjuvant, (B) Subolesin coated microparticles without adjuvant, (C) Subolesin coated microparticles with adjuvant, (D) soluble Subolesin antigen with adjuvant, (E) Subolesin and MSP4 coated microparticles with adjuvant, (F) MSP4 coated microparticles with adjuvant, (G) soluble MSP4 antigen with adjuvant, and (H) blank uncoated microparticles with adjuvant (Figure 3.3).

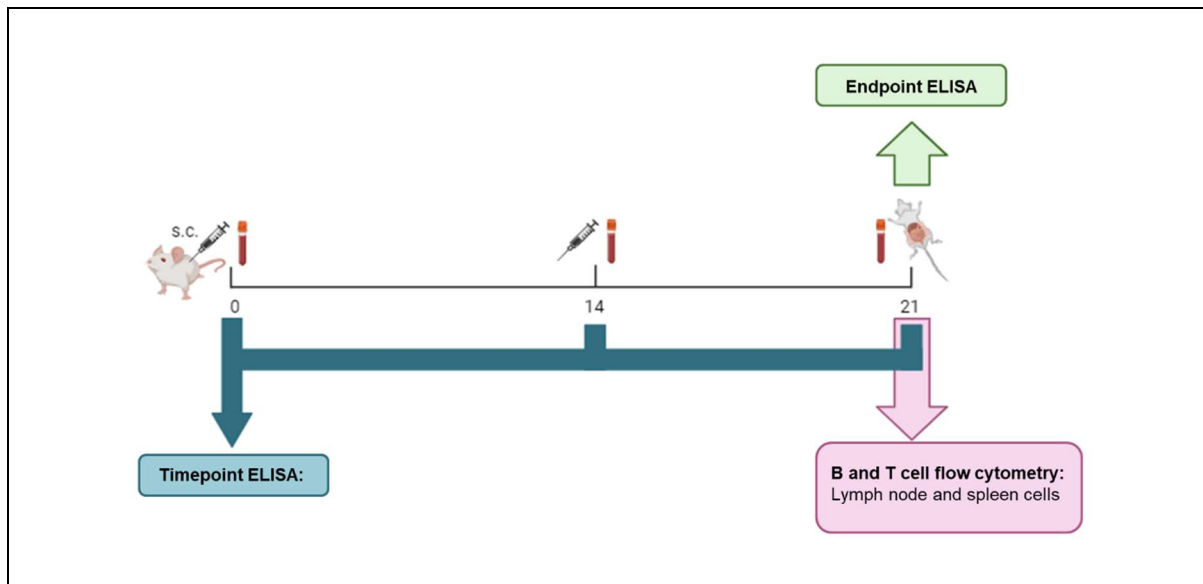


Figure 3.3. Diagram depicting the immunisation trial setup. Mice were immunized on days 0 and 14, before euthanization on day 21. Blood was collected on days 0, 14 and 21. After euthanization on day 21, spleen and lymph node tissues were collected from mice. Endpoint enzyme linked immunosorbent assays (ELISAs) were performed with serum collected on day 21 and timepoint ELISAs with serum from all collection timepoints. Flow cytometry was performed on spleen and lymph node tissues collected on day 21.

3.2.5. Collection and processing of blood and tissues from immunized animals

Blood ($\pm 200 \mu\text{l}$) was collected from mice on day 0 before immunisation and on day 14 via the submandibular vein (1.5 ml Eppendorf tubes). On day 21, mice were euthanised using isoflurane inhalation and a terminal bleed was conducted via cardiac puncture. Blood was centrifuged for 20 minutes at $400 \times g$ for serum collection, which was stored at -20°C (0.6 ml Eppendorf tubes) until later use. Brachial-, axillary- and inguinal lymph nodes, as well as spleen tissues, were excised from euthanized animals with care taken to remove the surrounding fascia. Single cell suspensions of the lymph and spleen were prepared by gentle extrusion of the tissue and $1 \times \text{PBS}$ through a sieve (Corning® Cell Strainer, $70\mu\text{m}$) and washing with cold $1 \times \text{PBS}$. The cell suspension was finally passed through a sterile syringe-filcon (BD Biosciences, USA). Spleen cell suspensions were centrifuged at $400 \times g$ (ROTOFIX 32A, Hettich Lab, Germany) for 10 minutes before resuspension in 2 ml of $1 \times \text{BD Pharm Lyse}^{\text{TM}}$ Lysing Buffer (BD Biosciences, USA) to remove contaminating erythrocytes. Consequently, cells were resuspended in the lysis buffer and incubated for 2 minutes, followed by centrifugation for 5 minutes and washed with 2 ml $1 \times \text{PBS}$ before centrifugation and resuspension in 5 ml of non-reconstituted RPMI 1460 Media (Thermo Fisher Scientific, USA) (1% Penicillin-Streptomycin (1000 U/ml) and 0.1% 2-Mercaptoethanol) before cell counting. Lymphocyte suspensions were treated similarly, excluding the erythrocyte lysis step.

3.2.6. Cell culturing, stimulation, and supernatant collection

Resuspended cells were counted using the LUNA-FL™ Dual Fluorescence Cell Counter (Logos Biosystems, South Korea). Briefly, each cell suspension was diluted and stained with Acridine Orange/Propidium Iodide (Logos Biosystems, South Korea) cell viability dye (18 µl cell suspension: 2 µl AO/PI dye). The volume of cells that need to be added to each well was calculated using the following equation:

$$\left(\frac{\text{no. of cells}}{\text{no. of blocks counted}} \right) \times \text{Dilution factor} \times 10^4 = \text{no. of cells in 1 ml of sample}$$

The dilution factor refers to the cell suspension:viability dye dilution ratio and 10^4 refers volume of the squares on the haemocytometer (0.1 mm^3). Lymph and spleen cells were resuspended in warm (37°C) reconstituted RPMI 1460 Media (Thermo Fisher Scientific, USA) containing 10% Foetal Bovine Serum, 1% Penicillin-Streptomycin (10000 U/ml) and 0.1% 2-Mercaptoethanol and seeded at a density of $\pm 5 \times 10^5$ (lymph node) and $\pm 1 \times 10^6$ (spleen) cells per well (final volume of 200 µl per well) in flat bottom 96 well plates (Corning, USA), respectively. Cells were incubated in duplicate at 37°C in 5% CO_2 and stimulated with 3 µg of the appropriate antigen per well. In the negative control reactions, cells were not stimulated with any antigens only PBS was added. Following 24 hours, cells were collected via centrifugation at $300 \times g$ for 5 minutes and the resultant supernatants were collected and stored at -80°C until later use. Cell pellets were resuspended in fresh reconstituted media before repeating the stimulation and supernatant collection at 48 hours. Following 48 hours, cells pellets were resuspended in 200 µl cold fixing buffer (2% Paraformaldehyde in $1 \times \text{PBS}$) per well and incubated for 20 minutes at 4°C . Cells were again pelleted before resuspending in 150 µl of cold freezing medium (RPMI 1460 media, 40% FBS, 10% DMSO) and storing at -80°C until later use.

3.2.7. Enzyme linked immunosorbent assays.

Subolesin and MSP4 specific total IgG, IgG1, IgG2a, and IgE were determined from serum collected on day 0, 14, and 21. Endpoint titration assays were completed to determine the optimal serum dilution by using serum from day 21. Serum collected from mice prior to initial immunisation via submandibular vein (day 0) was used as a negative and baseline control (i.e., naïve serum). Thereafter, timepoint ELISAs were completed at the calculated endpoint titration using serum from each timepoint.

Briefly, Nunc Maxisorp clear flat 96-well plates (Thermo Fisher Scientific, USA) were coated overnight at 4°C with 50 µl/well of the appropriate antigen (0.002 µg/µl) diluted in blocking buffer Tris Buffered Saline (TBS-T) solution containing (1% (v/v) Tween 20® (Sigma-Aldrich, USA) and 0.5% (w/v) casein pH 7.5-7.6 (Sigma-Aldrich, USA). Supernatants were discarded and all wells were blocked with 300 µl of blocking buffer followed by incubation for 2h at room temperature. Plates were subsequently washed three times with 300 µl 1×TBS and incubated with primary serum for 1h at room temperature. Wells were washed again and then incubated at room temperature for 45 min with 100 µl of the appropriate rabbit anti-mouse HRP-conjugated secondary antibodies (Nordic MUBio, Netherlands and Rockland; Immunochemicals, Inc., USA) (1:6000 in blocking buffer). Plates were again washed and 200 µl/well of substrate solution (0.102M Na₂HPO₄ and 0.05M citric acid, pH 5.0) that contained o-phenylenediamine dihydrochloride (OPD) (0.4 mg/ml) and 30% H₂O₂ (0.4 µl/ml) was added. After 30 minutes, the enzymatic reaction was stopped by the addition of 3M H₂SO₄ and the absorbance was measured at 492 nm using the SpectraMax® Paradigm® Multi-Mode Detection Platform (Molecular Devices, USA).

All samples were analysed as an average of the two technical repeats per biological sample, subtracting the background signal (no antigen) to determine antigen specific responses. The optimal dilution from the endpoint titration series was determined by calculating cut-off values using the statistical prediction method as described previously (Frey et al., 1998) at a significance level of 95%. Outliers were identified using the GraphPad Prism (v.9.5.0) ROUT method using a false discovery rate of 1%. A mixed-effects two-way ANOVA was performed at a 95% confidence level followed by Tukey's multiple comparison's test ($\alpha=0.05$). Data was represented as standard deviation of the mean.

3.2.8. Leukocyte staining for flow cytometry.

Fluorescently labelled monoclonal antibody combinations were used to detect T and B lymphocytes (BD Pharmingen™, USA), and includes markers for CD80, CD40, IgM, and CD19 (B lymphocyte) and CD3, CD4, CD195, and CD184 (T lymphocyte) respectively (Table 3.2 and Table 3.3).

For flow cytometry, frozen cells were thawed for 10 minutes at 30°C and then centrifuged at 700 × g for 5 minutes. The freeze media was carefully removed and the cells washed with 150 µl 1 × PBS per well, followed by centrifugation and removal of the supernatant. Each well received 5 µl of Mouse BD FC Block™ (Purified Rat Anti-Mouse CD16/CD32, BD Pharmingen™, USA) and was allowed to incubate in the dark for 5 minutes at room

temperature. Master mixes for T and B cell panels were prepared separately, which consisted of antibody (volume used per reaction listed in Table 3.2 and Table 3.3) and Stain Buffer (2% FBS, 0.09% Sodium Azide, Dulbecco's Phosphate-Buffered Saline pH 7.4) (BD Pharmingen™, USA) made up to a total volume of 50 µl per reaction. Appropriate antibody mixes were added to each well and was left to incubate at 4°C for 30 minutes in the dark. The cells were centrifuged again, the supernatant removed, before and cells resuspended in 150 µl of Stain Buffer. The cells were finally stored at 4°C before analyses using the BD Accuri™ C6 flow cytometer system (BD Biosciences, USA).

3.2.9. Flow cytometry and data analysis

A dilution series was performed to obtain the optimal antibody concentrations for the T and B cell panels (Table 3.2 and Table 3.3). Unstained and fluorescence minus one (FMO) controls were included to determine the true cell subpopulations. One replicate per biological sample was used. Flow cytometry results were analysed in FlowJo v10.8.1. Unstimulated population frequencies were subtracted from stimulated population frequencies to obtain the value of antigen specific responses. Cell populations (percentages) were converted to absolute values before a mixed-effects one-way ANOVA was performed at a 95% confidence level followed by Tukey's multiple comparison's test ($\alpha=0.05$) ($p \leq 0.05$) using GraphPad Prism (v.9.5.0). Cells were gated using a time gate before populations as described in Table 3.1. An example of the gating strategies is depicted in Figure S3.

Table 3.1. Markers and/or combinations of markers used for cell population analysis.

T cells		
Cell type/ cell population	Marker(s)	Reference
Th1	CD3+ CD4+ CD195+	(Robbertse et al., 2018)
Th2	CD3+ CD4+ CD184+	
B cells		
Cell type/ cell populations	Marker(s)	Reference
Inactive transitional B cells	CD45+ CD19+ IgM+	(Brennecke et al., 2018)
Activated B cells	CD45+ CD19+ IgM+ CD80+	(Anderson et al., 2007)

Table 3.2. Lymphocyte and splenocyte marker panels used for classification of T cell subpopulations.

Lymphocyte marker Panel 1 (T cells)				BD Pharmingen™ Antibody information		
Antibody	Alternative name	Conjugate	Isotype	Cat nr.	Conc. (mg/ml)	Volume used per reaction (µl)
PerCP-Cy™5.5 Rat Anti-Mouse CD3 Molecular Complex (Clone 17A2)	n.a.	PerCP-Cy™5.5	Rat SD, also known as Sprague-Dawley (outbred) IgG2b, κ	560527	0.2 mg/ml	1.25
BD Pharmingen™ FITC Rat Anti-Mouse CD4 (Clone GK1.5)	Cd4; CD4 antigen; L3T4; Ly-4; T-cell surface antigen T4/Leu-3	FITC	Rat LEW, also known as Lewis IgG2b, κ	553729	0.5 mg/ml	1.25
BD Pharmingen™ PE Rat Anti-Mouse CD195 (Clone C34-3448)	Ccr5; chemokine (C-C) receptor 5; C-C CKR-5; CC-CKR-5; CCR-5; AM4-7	PE-Cy5	Rat IgG2c, κ	559923	0.2 mg/ml	1.25
BD Pharmingen™ Purified Rat Anti-Mouse CD184 (Clone 2B11/CXCR4)	CXCR4, C-X-C chemokine receptor type 4; Fusin; LESTR; PB-CKR; Sdf1r	APC	Rat IgG2b, κ	551852	0.5 mg/ml	1.25
BD Pharmingen™ Purified Rat Anti-Mouse CD16/CD32 (Clone 2.4G2)	FcγRIII/FcγRII; Fcgr3/Fcgr2	n.a.	Rat SD, also known as Sprague-Dawley (outbred) IgG2b, κ	553142	0.5 mg/ml	5

Table 3.3. Lymphocyte and splenocyte marker panels used for classification of B cell populations.

Lymphocyte marker Panel 2 (B cells)				BD Pharmingen™ Antibody information		
Antibody	Alternative name	Conjugate	Isotype	Cat nr.	Conc. (mg/ml)	Volume used per reaction (µl)
BD Pharmingen™ APC Hamster anti-Mouse CD80 (Clone 16-10A1 (RUO))	Cd80; B7; B7-1; Cd28l; Ly-53; MIC17; TSA1	APC	Armenian Hamster IgG2, κ	560016	0.2 mg/ml	1.25
BD Pharmingen™ PE Rat Anti-Mouse IgM (Clone R6-60.2 (RUO))	Ighm; Igh-M; Immunoglobulin M; Igh6; muH; immunoglobulin heavy constant mu	PE	Rat LOU, also known as Louvain, LOU/C, LOU/M IgG2a, κ	553409	0.2 mg/ml	1.25
BD Pharmingen™ FITC Rat Anti-Mouse CD19 (Clone 1D3 (RUO))	n.a.	FITC	Rat LEW, also known as Lewis IgG2a, κ	553785	0.5 mg/ml	2.5
PerCP-Cy™5.5 Rat Anti-Mouse CD45 (Clone 30-F11 (RUO))	Ptprc; LCA; Leukocyte common antigen; T200; Ly-5; Lyt-4	PerCP-Cy™5.5	Rat LOU, also known as Louvain, LOU/C, LOU/M IgG2b, κ	550994	0.2 mg/ml	1.25
BD Pharmingen™ Purified Rat Anti-Mouse CD16/CD32 (Clone 2.4G2)	FcγRIII/FcγRII; Fcgr3/Fcgr2	n.a.	Rat SD, also known as Sprague-Dawley (outbred) IgG2b, κ	553142	0.5 mg/ml	5

3.3. Results

3.3.1. Antigen production and PLGA microparticle characterisation

Recombinant proteins were produced by Professor J. de la Fuente and used to create the antigen adsorbed PLGA microparticles by Dr S. Murdan. After the PLGA particles were produced, the average size and average Zeta potential of the microparticles (blank and antigen adsorbed) was determined (Table 3.4).

Table 3.4. Results of PLGA microparticle characterisation* (provided by Dr. S. Murdan)

Particles	Average size (nm) $\pm$ SD ⁴⁶	Average Zeta potential (mV) $\pm$ SD	% Adsorption
Blank PLGA	1292 $\pm$ 323	-0.61 $\pm$ 1.763	-
SUB PLGA	5197.4 $\pm$ 839	-2.356 $\pm$ 0.346	100
MSP4 PLGA	1362.6 $\pm$ 210	-5.92 $\pm$ 1.198	94

*All measurements and assays were conducted using PBS buffer (pH 7.2) as solvent/dispersant.

It is evident from Table 3.4 that there was a large difference in average size between the SUB PLGA microparticles and the other microparticle groups. Furthermore, the average Zeta potential of each particle type was negative, and the adsorption percentage of SUB loaded microparticles was higher (100%) than that of MSP4 loaded microparticles (94%).

3.3.2. Serum response

Endpoint (day 21) ELISAs were conducted with serum to blocking buffer dilutions ranging from 1:50 to 1:3200 and 1:50 to 1:6400 (Figure 3.4 and Figure 3.5, respectively). Upon comparison between Group H (background non-specific response) and the immunized groups (Group B-E), all Subolesin-specific responses were lower. As such, it is evident that the anti-SUB IgG response raised by all of the groups was negligible across all the dilutions tested (Figure 3.4). Even at a 1:10 dilution, there was no significant signal detected (not shown). Due to the lack of antigen-specific (i.e., anti-Subolesin) response, the scope of the study was narrowed and further analyses focused only on the groups receiving MSP4 formulations.

⁴⁶ SD – standard deviation

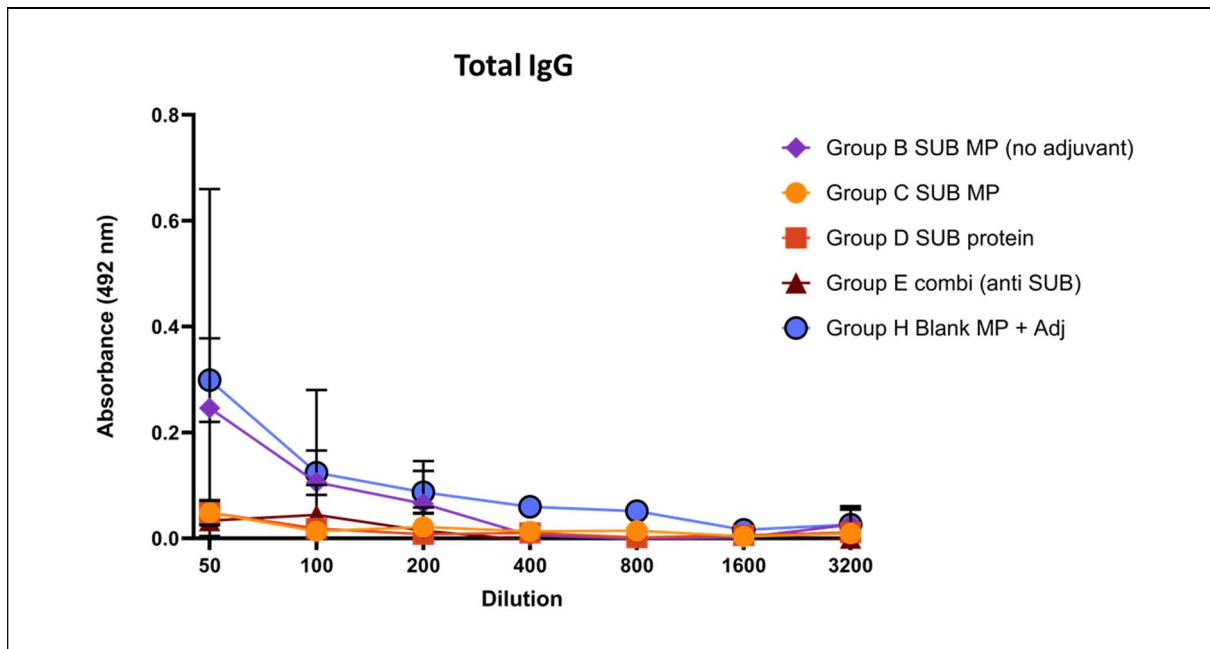


Figure 3.4. Endpoint titration series of Subolesin-specific antibody responses in female BALB/c mice immunized with soluble Subolesin antigen or adsorbed to PLGA microparticles in the presence or absence of Montanide™ adjuvant. Serum endpoint titration series (1:50-1:3200) of total Subolesin-specific IgG responses in female BALB/c mice immunized with SUB coated microparticles with (Group B) and without adjuvant (Group C), SUB antigen with adjuvant (Group D), SUB and MSP4 coated microparticles with adjuvant (Group E) and blank microparticles not coated with any antigen and adjuvant (Group H).

Considering serum antibody responses, a clear anti-MSP4 total IgG and IgG1 response could be seen across all dilutions tested (Figure 3.5.A and Figure 3.5.B). In contrast, a much lower anti-MSP4 IgG2a response was detected across all the dilutions tested, which suggests that no IgG2a signal above baseline was generated (Figure 3.5.C). Similarly, low levels of IgE were generated for all groups, which indicates that there were no significant allergenic responses associated with the vaccine formulations (Figure 3.5.C). For the groups receiving MSP4, a statistical prediction method was used to calculate the cutoff values from the endpoint titration series (Figure 3.5.A and Figure 3.5.B) at a 95% confidence level, which was determined to be 1:6400 (Frey et al., 1998). The cutoff calculation was not calculated for IgG2a and IgE, since the MSP4-specific antibody response was negligible for both (Figure 3.5.C).

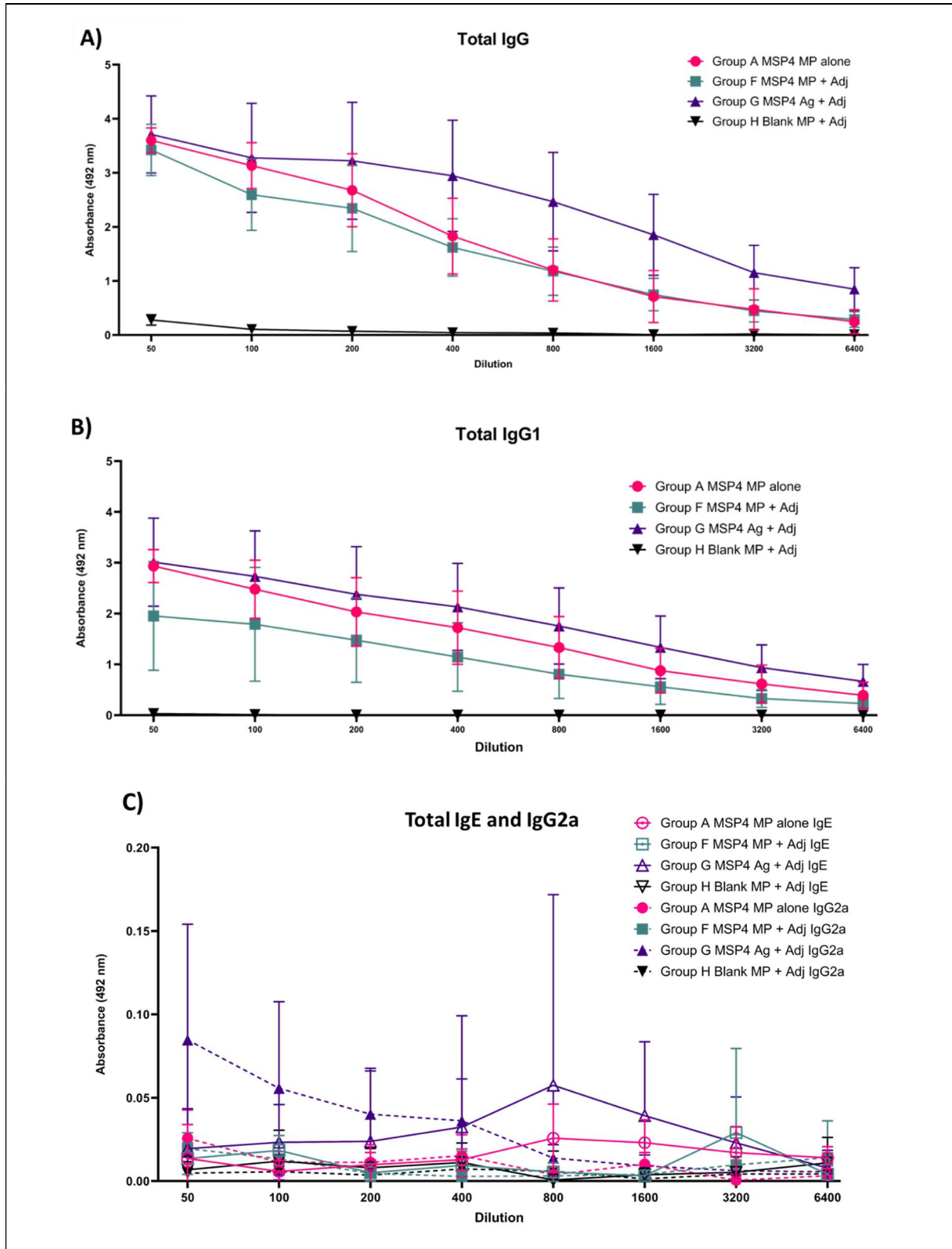


Figure 3.5. Endpoint titration series of MSP4-specific antibody responses in female BALB/c mice immunized with MSP4 antigen free or adsorbed to PLGA microparticles in the presence or absence of Montanide™ adjuvant. Serum endpoint titration series (1:50-1:6400) of MSP4-specific total IgG (A), IgG1 (B) and IgG2a (dotted line) and IgE (solid line) (C) responses in female BALB/c mice immunized with MSP4 coated microparticles without (Group A) and with adjuvant (Group F), MSP4 antigen with adjuvant (Group G) and blank microparticles not coated with any antigen and adjuvant (Group H).

Timepoint ELISAs were conducted at a 1:6400 dilution with serum collected on day 0, day 14, and day 21 (Figure 3.6).

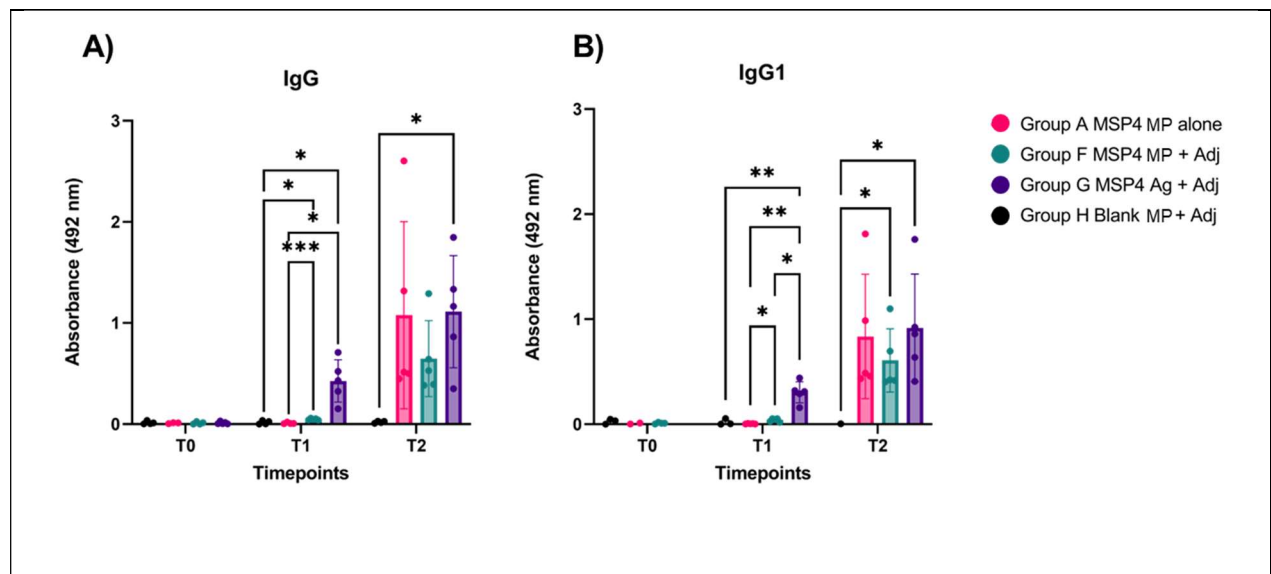


Figure 3.6. MSP4-specific antibody responses in female BALB/c mice immunized with MSP4 antigen free or adsorbed to PLGA microparticles in the presence or absence of Montanide™ adjuvant over three timepoints. The MSP4-specific antibody response was determined for immunization groups A, F, G and H from serum collected at pre- (day 0) and post-immunization (day 14 and 21) timepoints at a 1:6400 titre. **(A)** Total IgG response across three timepoints for all groups, **(B)** IgG1 response across three timepoints for all groups. A Mixed-effects two-way ANOVA was performed at a 95% confidence level followed by Tukey's multiple comparison's test ($\alpha=0.05$) (** $p \leq 0.01$, * $p \leq 0.05$).

As expected, the MSP4-specific IgG and IgG1 response increased over time, with the highest absorbances present at day 21 (Figure 3.6). Furthermore, it is evident from Figure 3.6.A that Group G (MSP4 antigen and adjuvant) had a significantly higher anti-MSP4 total IgG response than Group A (MSP4 microparticle only) at day 14. However, there were no significant differences between the test groups (Group A, F and G) at day 21 (Figure 3.6.A), where only Group G total IgG responses were significantly greater ($p \leq 0.05$) than the negative control Group H. A similar trend is observed for the anti-MSP4 IgG1 response at day 14 (Figure 3.6.B), where Group G (MSP4 antigen and adjuvant) had a significantly higher response than all the other groups (Group A, F and H). However, there were no significant differences between the test groups (Group A, F and G) at day 21 (Figure 3.6.B). Furthermore, it should be noted that both the IgG and IgG1 responses for Group F was significantly higher than Group A, at day 14 which indicates that the addition of an adjuvant resulted in an earlier antibody response compared to the microparticles alone. Furthermore, since both groups receiving microparticles (Group A and Group F) has a significantly lower IgG and IgG1 response at day 14 than the free MSP4 antigen group (Group G), it can be concluded that microparticles delayed the antibody response. The IgG (Figure 3.6.A) and IgG1 (Figure 3.6.B) responses for the group

receiving MSP4-coated microparticles in the absence of adjuvant (Group A) and the group receiving soluble MSP4 with adjuvant (Group G) had large standard deviations on day 21. This indicates that there was some variation between the individuals within these groups, however, none of the individuals were removed as outliers.

As the IgG1 and IgG2a isotypes can also be used as indicators for the underlying Th response. (Comoy et al., 1997), it is evident from the higher MSP4-specific IgG1 response, compared to the MSP4-specific IgG2a response (Figure 3.5.C), that a Th2-biased response was elicited by all test groups.

3.3.3. Flow Cytometry

Flow cytometry was performed to identify the T and B cell subpopulations from lymph node cells and splenocytes after *in vitro* stimulation with the MSP4 antigen. The T cell populations of interest are Th1 and Th2 cells, which are distinguished by the presence of either the CD195 or CD184 marker, respectively (Figure 3.7). From Figure 3.7 it is evident that the percentage of MSP4-specific Th1 and Th2 cell responses detected in lymph node tissues were very low (<0.15% population frequency). Group A (MSP4 microparticles without adjuvant) had a significantly higher ($p \leq 0.0001$) CD195+ T lymph node population frequency than Group F (MSP4 microparticles with adjuvant) (Figure 3.7.A). However, a high non-specific background response was observed to be elicited by the blank microparticle and adjuvant based on the response in the negative control Group H (Figure 3.7.A). This implies that the CD195+ responses observed for Group G and Group F were negligible. In contrast, none of the CD184+ lymph node population frequencies from the test groups (Group A, F, and G) were significantly different from each other, only from the negative control group, where Group F and Group G had significantly higher ($p \leq 0.01$) population frequencies than Group H (Figure 3.7.B). These results suggest that the MSP4 microparticle vaccine elicited a Th1 cellular response even in the absence of the adjuvant.

Similar trends were observed in the splenocytes. Although Group F appeared to have a higher CD195+ T cell population frequency than the other groups, this difference was not significant (Figure 3.7.C). Furthermore, there were no significant differences observed between the CD184+ population frequencies of any groups (Figure 3.7.D). Furthermore, the results suggest that there was also a Th1 biased cellular response in the splenocytes, however these results were not significant (Figure 3.7.C and Figure 3.7.D). Lastly, the large standard deviation observed for some groups (Group H, Figure 3.7.A; Group G, Figure 3.7.C) suggests that there was some variation between the individuals in within these groups.

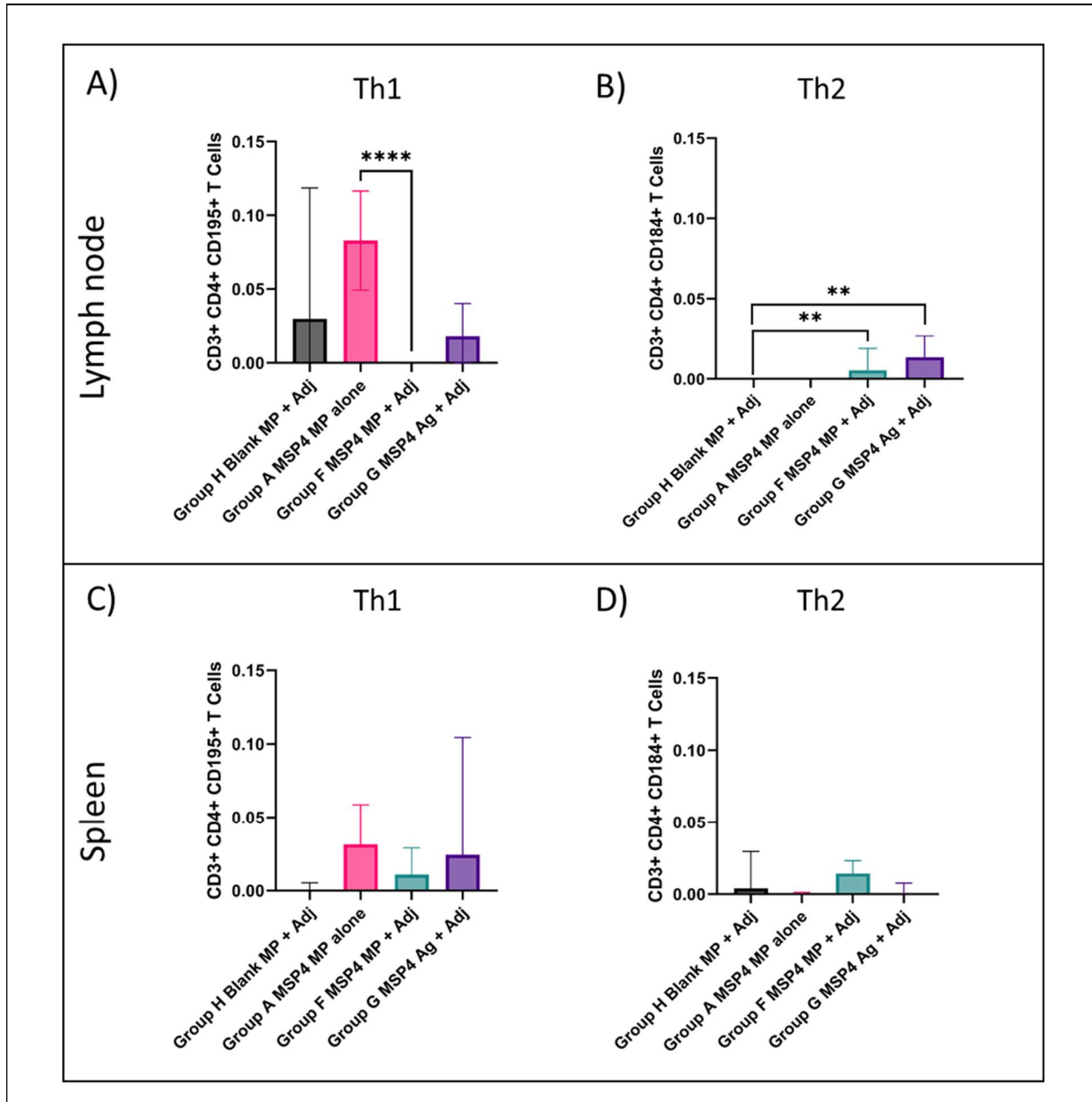


Figure 3.7. T cell analysis of lymph node and spleen cells isolated from immunized female BALB/c mice. The population frequency (%) of CD184+ and CD195+ T cells are indicated for (A-B) lymphocytes and (C-D) splenocytes. Graphs were plotted as the mean of each marker per group with the standard deviation. A Mixed-effects one-way ANOVA was performed at a 95% confidence level followed by Tukey's multiple comparison's test ($\alpha=0.05$) (**** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$).

The B cell populations that are identifiable with the markers used in this study (Table 3.3) are limited to transitional B cells (CD45+ CD19+ IgM+) and activated B cells (CD45+ CD19+ IgM+ CD80+). Similar to the T cell responses, is evident from Figure 3.8, that the percentage of MSP4-specific B cell populations observed were low (<0.20%).

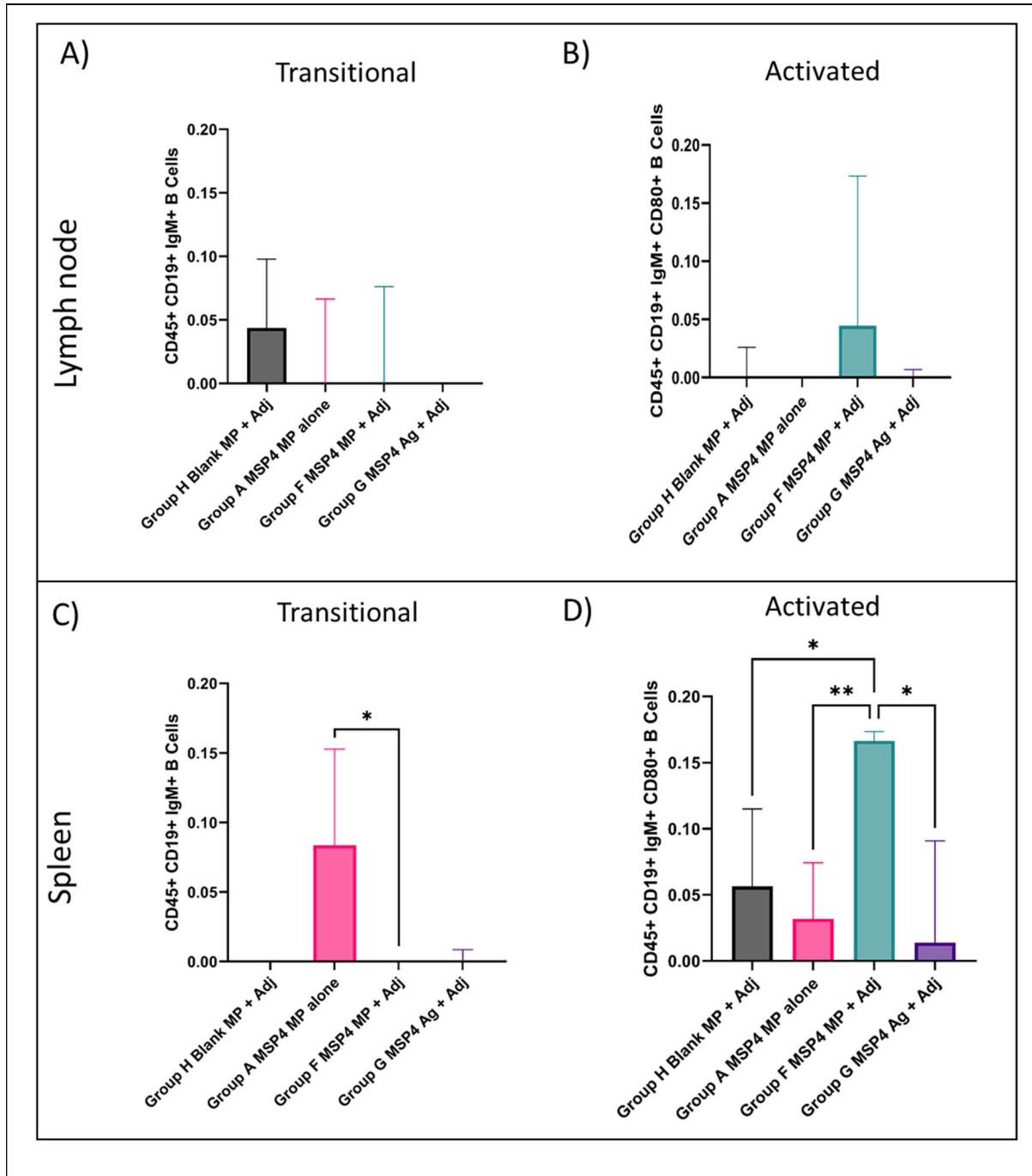


Figure 3.8. B cell analysis of lymph node and spleen cells isolated from immunized female BALB/c mice. The population frequency (%) of IgM+ and IgM+ CD80+ B cells are indicated for (A-B) lymphocytes and (C-D) splenocytes. Graphs were plotted as the mean of each marker per group with standard deviation. A Mixed-effects one-way ANOVA was performed at a 95% confidence level followed by Tukey's multiple comparison's test ($\alpha=0.05$) (**** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$).

It is evident from Figure 3.8.A, that there were no significant differences between the transitional B cell lymph node population frequencies of the different immunization groups, nor the activated B cell lymph node population frequencies (Figure 3.8.B). Furthermore, given the high non-specific background response observed for the negative control Group H, the

response for the other groups (Group A, F, and G) were negligible (Figure 3.8.A). These results suggest that there was no significant activated B cell response present in the lymphocytes at the time of tissue isolation.

In the splenocytes, the transitional B cell population frequency of Group A (MSP4 microparticles no adjuvant) was significantly higher ($p \leq 0.05$) than Group F (MSP4 microparticle with adjuvant) (Figure 3.8.C). However, there were no significant differences between the population frequencies of the other groups (Figure 3.8.C). In contrast to the activated B cell population frequencies of the lymph node, there were significant differences in the splenocytes (Figure 3.8.D). Group F (MSP4 microparticles with adjuvant) had a significantly higher population frequency than the negative control group, Group H ($p \leq 0.05$), and both the other test groups, Group A ($p \leq 0.01$) and Group G ($p \leq 0.05$) (Figure 3.8.D). However, given the high non-specific background response observed for the negative control Group H, the responses observed for Group A and Group G were negligible (Figure 3.8.D). Lastly, the large standard deviations observed suggests that there was some variation between the individuals in within these groups (Group F, Figure 3.8.B; Group A, Figure 3.8.C).

3.4. Discussion

It has been established that the control of ticks and *A. phagocytophilum* require strong humoral and cellular immune responses respectively. This study aimed to determine whether the use of PLGA microparticles would change the immune response elicited after immunization of female BALB/c mice with Subolesin and MSP4 antigens.

The humoral response elicited by immunization with Subolesin was assessed using an indirect ELISA. However, the results indicated that immunization of mice with either Subolesin or Subolesin-coated microparticles did not elicit a significant humoral response in sera, either in the absence or presence of an adjuvant relative to the negative control group (Figure 3.4). Previous studies with Subolesin typically detected significant Subolesin specific antibody titres at serum dilutions of 1:1000, although some determined the optimal dilution to be 1:100 (Table 1.6). Nevertheless, in this study a negligible response was observed across all dilutions, even as low as 1:50 (Figure 3.4). Therefore, it was concluded that the absence of an anti-Subolesin response in the host was due to the quality of the Subolesin antigen. Consequently, the immunization groups receiving Subolesin were excluded from further analysis.

Immunization with soluble MSP4 antigen (Group G) elicited significantly higher MSP4-specific responses (IgG and IgG1) in sera (relative to the negative control group) at both day 14 and 21 (Figure 3.6). In contrast, the same was not observed for groups immunized with MSP4 coated microparticles (Figure 3.6). However, given the expected antigenic release profile of PLGA microparticles (Figure 1.8), which facilitates a slow sustained release of antigens, it is unsurprising that these groups demonstrated a delayed antibody response compared to the free MSP4 antigen group (Group G) (Figure 3.6). In addition, by combining Montanide™ adjuvant with the MSP4 coated microparticles (Group F) facilitated an earlier response at day 14 for both IgG and IgG1. These results were expected, since Montanide™ has been shown to stimulate humoral immune responses in cattle and mice (Dummer et al., 2014; Jones et al., 1990; Raman et al., 2004). Other studies in mice have found persistent humoral protection up until 10 weeks after the first immunization with PLGA particles (Demento et al., 2012; Sales-Junior et al., 2005; Wang et al., 2014). Therefore, it is possible that the timeline for this immunization trial was too short to see the immune response to the antigen coated microparticles develop completely. Therefore, future studies should include more serum sampling points and a longer time after the final vaccination before the final sampling timepoint.

Moreover, a Th2 humoral response was observed in sera for all the MSP4 immunized groups, based on the dominant IgG1 response (Figure 3.6.B). These results were expected since it has been shown that particles in the microparticle size range (2-8 μm) promote Th2 biased responses, and the MSP4-coated microparticles used in this study are approximately 5197.4 ± 839 nm in size (Joshi et al., 2013; Kanchan and Panda, 2007). However, since a Th1 (cellular) response is more important for the clearance of intracellular bacterial infections, such as *A. phagocytophilum*, future work should focus on using particles in the nano size range, since these particles have been associated with Th1-biased responses based on size alone (Kanchan and Panda, 2007; Londoño et al., 2023; von Loewenich et al., 2004). Previous studies that evaluated IgG responses showed that high IgG antibody levels only offer partial protection against *A. phagocytophilum* infection in lambs (Contreras et al., 2017). As such, a humoral response is not sufficient to accurately predict *in vivo* protection, and the cellular immune response should be investigated too.

Flow cytometry was conducted on cells isolated from secondary lymphoid organs (lymph nodes and spleen). The development of T and B cells in secondary lymphoid organs are summarized in Figure 3.9, with the T and B cells identified in this study indicated with their respective surface markers.

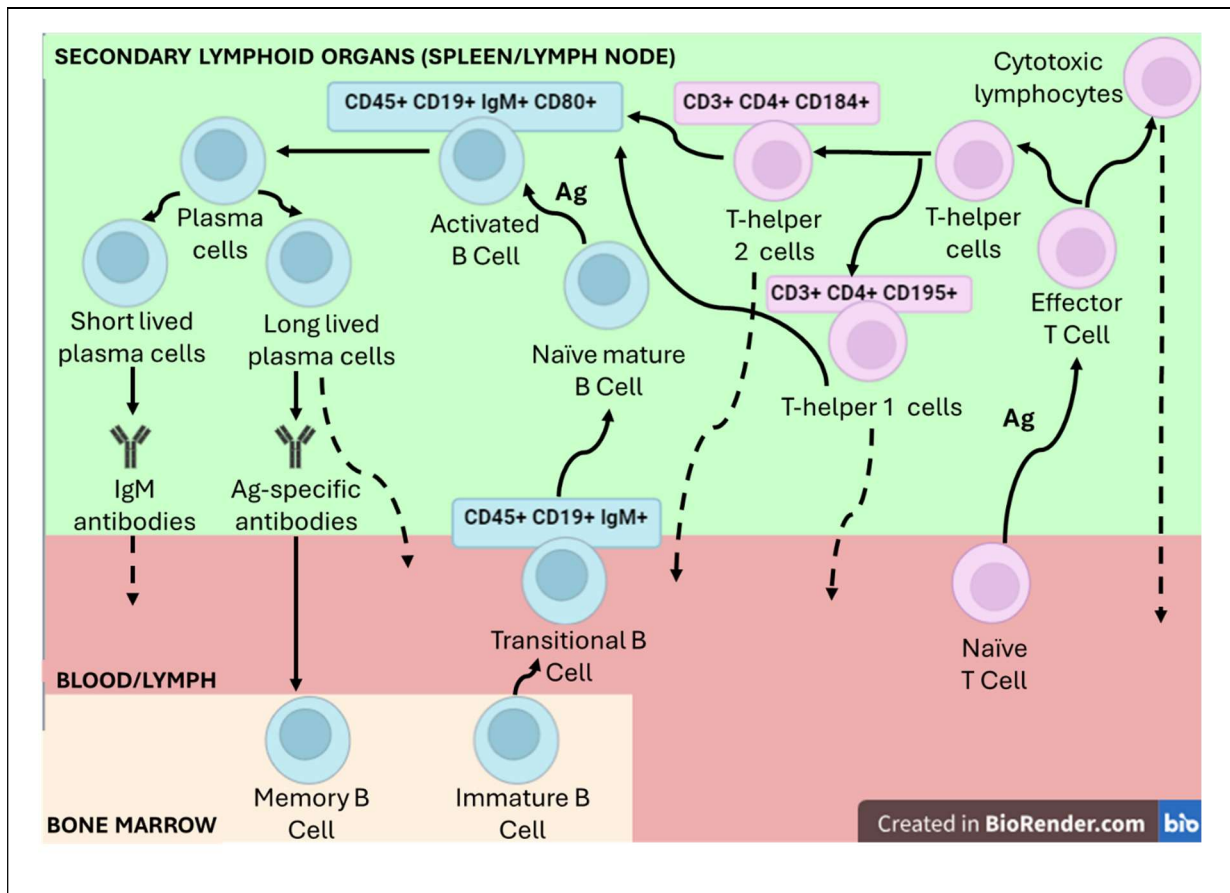


Figure 3.9. A schematic summarising the development of T and B cells in secondary lymphoid organs (SLOs), such as the spleen and lymph node. Dashed arrows indicate movement of cells into circulation (blood/lymph fluid). Naïve T cells enter the SLOs through either the blood or lymph fluid. Naïve T cells encounter antigens (Ag) in the SLO via presentation by APCs (antigen presenting cells), upon which it differentiates into effector T cells, namely cytotoxic lymphocytes or T helper (Th) cells. In this study, both Th1 (CD3+ CD4+ CD195+) and Th2 (CD3+ CD4+ CD184+) cells can affect B cells by influencing the type of class-switched antibodies produced: Th1 drives IgG2a production and Th2 drives IgG1 production. Both Th cell types can also leave the SLO and migrate to the sites of the original antigen entry (immunization/tissue injury). The development of B cells originates in the bone marrow until it reaches the immature B cell stage. Naïve immature B cells (transitional/inactivated) (CD45+ CD19+ IgM+) travel through the blood/lymph fluid to the SLO, whereafter it becomes a mature naïve B cell once it passed through a primary follicle (not pictured). After encountering its specific antigen in the SLO, the B cell becomes activated (CD45+ CD19+ IgM+ CD80+), after which it can differentiate into two types of plasma cells: short-lived plasma cells (secretes IgM antibodies) or long-lived plasma cells (secretes high affinity isotype-switched antibodies) which matures in either the bone marrow or sites in SLO (medullary cords in lymph node and red pulp in spleen). Both plasma cell types may leave the SLO to enter circulation. After the primary response has subsided, some B cells become Memory B cells that can remain in the SLO, recirculate through the body or return to the bone marrow. Image was created in Biorender.com based on information from Parham (2015) and van Schaik et al. (2024) and Table 3.1.

Activated B cells can typically be identified using the CD80 marker, whereas its absence is typically characteristic of naïve B cells (Anderson et al., 2007) (Figure 3.9). More specifically, the CD80 marker has been used to exclusively identify the memory B cell (MBC) phenotype

in mice and is highly upregulated in both human and murine MBCs (Good-Jacobson et al., 2012; Tomayko et al., 2010; Zuccarino-Catania et al., 2014). Through flow cytometry it was shown that groups that received PLGA microparticles elicited a delayed B cell response compared to the group that received the soluble MSP4 antigen with adjuvant. Given the significantly higher levels of IgG1 observed in sera for the soluble MSP4 group (Group G) at day 14 compared to both microparticle groups (Group A and Group F) (Figure 3.6), and the significantly lower population of activated B cells compared to Group F (MSP4 MPs with adjuvant) (Figure 3.8.D), it is evident that the IgG1 antibody producing B cells for the microparticle group without adjuvant (Group A) had already peaked. These results are supported by the low levels of Th2 cells observed for the group that received soluble antigen (Group G) (Figure 3.7.D), given that Th2 cells drive the production of IgG1 antibodies, it is most likely that the Th2 response had already matured and subsided by the time these cells were isolated.

From Figure 3.8.D it is evident that the microparticle group that received adjuvant (Group F) had a significantly higher activated spleen B cell population, while most of the spleen B cells identified in the microparticle group that did not receive adjuvant were still inactivated transitional B cells (Figure 3.8.C). Therefore, the presence of an adjuvant appeared to accelerate the process of B cell maturation, as it appears as though the activated B cell populations for Group F (MSP4 MPs with adjuvant) were peaking at the time of sample collection, whereas Group A (MSP4 MPs without adjuvant) have not yet peaked. These results were unsurprising, since Montanide™ is known to boost humoral immune responses by entrapping antigens in water droplets which promotes antigen uptake and presentation by APCs (Burakova et al., 2017).

It is evident that a delayed Th1 response was elicited by the PLGA microparticles, since Group A (MSP4 MPs only) had a significantly higher ($p \leq 0.0001$) lymph node CD195+ population frequency than Group F (MSP4 MPs and adjuvant), and had a higher population frequency than Group G (MSP4 antigen and adjuvant) (Figure 3.7.A). Furthermore, it is evident from Figure 3.7.C, that the presence of an adjuvant seemed to reduce this delay slightly, as Th1 cells elicited in Group F (MSP4 MPs and adjuvant) were only detected in the spleen. Furthermore, given the low IgG2a levels observed in sera for Group G (MSP4 antigen and adjuvant) as well, it is possible that there was an overall delayed Th1 response, given the small amount of Th1 cells observed in the spleen (Figure 3.7.C). Therefore, if samples were collected at a later timepoint, then the Th1 response and activation of IgG2a producing B cells might have been observable. Furthermore, Th1 cells are known for secreting cytokines that

activate macrophages and cytotoxic T cells, that work together to target intracellular pathogens (Alberts B, 2002). Given the importance of strong cellular immune responses in clearing infections with intracellular bacterial infections like *A. phagocytophilum* (Birkner et al., 2008; Joshi et al., 2013), the development of a Th1 response could be an important predictor of *in vivo* protection. Therefore, future studies should investigate the potential correlation between a cellular immune response and protection against challenge with *A. phagocytophilum*.

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Chapter 4: Concluding Discussion

The possibility of designing a vaccine against ticks that will offer protection across the various species has always been enticing. One antigen that has been proposed for a universal tick vaccine is Subolesin. This protein has been shown to date to be conserved across a number of tick species (Figure 1.5) (Artigas-Jerónimo et al., 2018) and to have a severe impact on tick fecundity when knocked out via RNA interference in ticks (de la Fuente et al., 2006a; Kocan et al., 2007; Nandy et al., 2023; Nijhof et al., 2007). To date, all literature indicates that Subolesin is strictly an intracellular protein (Antunes et al., 2014; Trentelman et al., 2019) that functions in the nucleus (Figure 1.6). However, there is a paradox between using Subolesin as an antigen and its nuclear localization in tick cells, which renders this antigen inaccessible to the host immune response. Typically, the induction of antibody dependent cell mediated cytotoxicity (ADCC) requires antibodies to bind to the target protein on the surface of a cell and recruit additional immune factor to clear the infection/target (Zahavi et al., 2018). Alternatively, in vertebrates it has been demonstrated that antibodies can launch an intracellular immune response if they enter a cell while bound to the invading pathogen (McEwan et al., 2013). However, this has not been shown in ticks.

Although antibodies have been shown to escape the tick midgut and enter the tick haemolymph (Ackerman et al., 1981), the mechanism through which this trans-tissue migration occurs remains to be fully elucidated. Tick digestive cells in the gut tissue is known to be phagocytic cells with elevated rates of endocytosis (Sonenshine, 2014). As such, uptake of antibodies along with the bloodmeal, is expected. However, following phagocytosis, intracellular digestion takes place where the vesicles fuse with lysosome which contains enzymes to break down complex molecules including sugars and proteins into usable energy needed by the cell to survive (Anderson et al., 2008; Lara et al., 2005; Sonenshine, 2014). In a single study using fluorescent microscopy of *I. scapularis* ISE6 tick cells, the authors showed that anti-actin antibodies do occur in the cytoplasm after an overnight incubation in the absence of cell membrane permeation (de la Fuente et al., 2011). Based on the results, it was proposed that antibodies can cross the plasma membrane of tick cells and bind to intracellular proteins. This statement is very controversial, as there is limited evidence suggesting that some antibodies can cross the plasma membrane of cells. Normal antibody uptake occurs via Fc receptor-mediated uptake (only occur in cells that have Fc receptors that can bind the Fc

region of antibodies, facilitating internalization), which is common in immune cells, but not in most other cell types (Parham, 2015).

Nevertheless, several vaccines using “concealed” antigens – typically hidden from the host immune system – as opposed to antigens that are exposed to the host immune system, such as salivary proteins secreted during feeding, have been evaluated as tick vaccines (Table 1.5) with only Bm86 reaching commercialization (Jonsson et al., 2000; Rodríguez et al., 1995; Willadsen et al., 1995). Dual antigens have also been evaluated, which have both secreted and concealed antigenic epitopes (e.g. truncated constructs of the 64P cement protein) whose efficacy was partially attributed to cross-reactive epitopes and not the presence of 64P in the midgut, haemolymph and salivary glands (Havlíková et al., 2009; Trimnell et al., 2005; Trimnell et al., 2002). Therefore, answering the question whether (a) there are splice variants of Subolesin that yield an exposed variant that mediate protection upon vaccination, or (b) if the response raised against Subolesin during vaccination led to a cross-reaction with a protective antigen that has never been identified, remains crucial.

In the first section of this study (Chapter 2), we showed that there is a single Subolesin gene in the *R. microplus* genome and that four possible mRNA species can be transcribed. All three predicted *R. microplus* Subolesin protein variants had nuclear localization signals, which is consistent with previous findings (Naranjo et al., 2013). Furthermore, signal peptide sequences were absent from all putative protein variants. Therefore, this paradox cannot be explained on a genome or transcriptome level. Future studies should investigate the potential cross-reactivity of anti-Subolesin antibodies with other tick proteins. This can be done by performing a Western blot using polyclonal antisera raised after vaccination with recombinant full-length Subolesin. Furthermore, future studies should confirm the expression of these putative mRNA species and protein variants using Long-Read Sequencing technologies.

Another ambitious goal is the development of a single vaccine that can target the tick vector along with a tick-borne pathogen in a single formulation. Given previous studies showing the effect of Subolesin on pathogen transmission (de la Fuente et al., 2006b) and reduced pathogen infection and tick infestation levels following immunization with a Subolesin-MSP1a combination vaccine (Contreras et al., 2015; Torina et al., 2014), the combination of using Subolesin (tick antigen) with MSP4 (*A. phagocytophilum* antigen) has been proposed.

However, the complexity of the tick vector and the tick-borne pathogen lifecycles combined with the specific immune response that needs to be elicited to offer a protective response makes this endeavor challenging. For example, *A. phagocytophilum* has both a free circulating life stage as well as an intracellular life stage (Figure 1.3), which will predominantly require an antibody response or T cell response, respectively. This is similar to the complexity observed with *Plasmodium falciparum* infection (causative agent of malaria) and the complex vaccines required to address the free and intracellular life stage of the parasite, as reviewed by Bonam et al., (2021).

MSP4 is one of the six major surface proteins on the surface of *A. phagocytophilum* (Contreras et al., 2017). However, there have been no studies conclusively demonstrating that the MSP4 protein can be displayed on the cell surface of infected host cells (Alberdi et al., 2015; Villar et al., 2015). In contrast, it has been shown that *A. phagocytophilum* resides and replicates in vacuoles within the host cell and does not express any of its proteins on the host cell surface (Rikihisa, 2010). Therefore, immunization with MSP4 will need to yield high affinity, high titers of protective antibodies to target the circulating life stage (Figure 3.1). Although several immune effectors have been identified as essential for *A. phagocytophilum* clearance, the mechanism of protection has not yet been elucidated. It has been shown that CD4+ T-cells are the most important immune effector needed for *A. phagocytophilum* clearance (Table 1.3). This correlates to other studies which indicated that antibodies offer some protection against *A. phagocytophilum*, but that it is unlikely that it can exclusively control this pathogen (Sun et al., 1997).

Antigens delivered using microparticles have been shown to improve humoral responses due to their slow antigenic release profile (Figure 1.8). In mice, it was shown that the IgG response raised by ovalbumin delivered via PLGA microparticles was significantly higher than that raised by soluble ovalbumin after two weeks, and continued to increase until it peaked at ten weeks post immunization (O'Hagan et al., 1993). Therefore, in this study, we set out to evaluate the effect of PLGA microparticles on the immune system when coated with MSP4, Subolesin or both antigens versus vaccination with soluble antigen (Chapter 3). Data showed that no significant anti-Subolesin IgG response was raised at any dilutions in the sera of immunized female BALB/c mice, which led to the exclusion of Subolesin from further analyses. Nevertheless, regarding MSP4-coated microparticles, significant T and B cell responses were identified in spleen and lymph node cells. Although MSP4-coated microparticles did not elicit

significantly higher antibody titers than soluble MSP4 at the timepoints evaluated in this study, high antibody titers were still obtained. Nevertheless, despite the small sample size, this pilot study demonstrated the importance of vaccine formulation and regime and its effects on the development of an immune response. As this study did not include a challenge to determine the *in vivo* protective efficacy, future studies should include *in vitro* evaluation of *A. phagocytophilum* uptake into tick cells in the presence of the antisera raised from this study.

In conclusion, this study confirmed the intracellular localization of Subolesin and stressed the need for future research to explain the observed protective response in bovines vaccinated with Subolesin. In regard to the use of MSP4-coated microparticles, this study showed that high antigen specific antibody titers could be raised and emphasized the need for future research to investigate the *in vivo* protective efficacy of MSP4.

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Supplementary:

Table S1. Table with consensus sequences used to annotate gene and mRNA features of Subolesin.

Name	Consensus sequence*	Description	Reference
TATA Box	TATAWAWN	Binds TATA-binding protein (TBP)	(Juo et al., 1996)
Arthropod initiation factor (INR)	DCAKT	25% of arthropod RNA polymerase II-transcribed promoters contain one or more copies of the sequence TCAGT beginning within the interval (-10, +10)	(Cherbas and Cherbas, 1993)
DPE	RGWYVT	Binds Transcription factor IID (TFIID)	(Bhagavan and Ha, 2015)
GC Box	GGGCGG	Binds SP1 transactivator Sp1	
CAAT Box	GGCCAATCT	Binds CAAT enhancer binding protein (C/EBP)	
TCA	TCAKTY	Inr consensus in Drosophila	(Kadonaga, 2012)
TCT	YYCTTTY	Binds TBP-related factor 2 (TRF2)	
BREu	SSRCGCC	Binds Transcription factor IIB (TFIIB)	
BREd	RTDKKKK	Binds Transcription factor IIB (TFIIB)	
Focused INR Drosophila	TCAGTY	(A+1) for focused promoters in Drosophila based on	(Proudfoot, 2011)
Polyadenylation (Poly-A) signal	AAUAAA		
NFkB	GGGRNYYYCC	Consensus binding sequence of NFkB	(Chen et al., 1998)

*The International Union of Pure and Applied Chemistry (IUPAC) standard single character code for DNA bases was used to represent the consensus sequences.

Table S2. Table summarising the statistics of the *de novo* transcriptome assembly representing South Africa.

Summary statistics of <i>de novo</i> assembled transcriptome of <i>R. microplus</i> in South Africa.				
Representative assembly	Number of contigs	Longest contig (bp)	Shortest contig (bp)	Mean contig size (bp)
<i>R. microplus</i> South Africa	1 738 120 480	48 888	50	123

Table S3. Summary of the RNA sequencing library size (RNA-sequencing reads) for each sample. The median library size and logCPM cutoff value is also listed.

	Sample Name	RNA-sequencing library size (reads)
ClinVet	Rm_Gland	79,454,546
	Rm_Gut	79,454,784
	Rm_Larva	92,869,634
	Rm_Nymph	99,912,014
	Rm_Ovary	78,923,042
Mnisi	SAMPCLA3_GUT	111,704,028
	SAMPCLA3_Ovary	108,044,142
	SAMPCLA3_SG	117,933,746
	SAMPUTA_GUT	114,849,306
	SAMPUTA_Ovary	112,586,438
	SAMPUTA_SG	115,877,372
	SAMPWV1_GUT	103,977,528
	SAMPWV1_Ovary	107,658,188
	SAMPWV1_SG	110,913,592
Median library size		107,851,165
logCPM cutoff value*		0.1

*logCPM Cutoff = $10 \div \text{median library size (million)} = 10 \div 107.851165 \times 10^6 = 0.09272037071 \approx 0.1$

Table S4. Table listing the sequences of the predicted epitopes of Subolesin from different tick species based on literature. The epitope type is given, along with the species it was identified in, as well as the sequence of the epitope and its position in the alignment depicted in Figure 2.14 and 2.15.

Epitope type	Species	Sequence and position	Reference
Linear B cell	<i>R. microplus</i>	28-CMPLSPPPTRA-46	(Lagunes et al., 2016)
		85-QGADPESQHTSG-119	
		130-LFTFRQVGLICE-141	
		143-MKERESKIREE-154	
		1-MACATLKRTHDWDPLHSPSGRSPKRRRCMPLSPPPTRAH QI-46	(Zeb et al., 2024)
	<i>R. appendiculatus</i>	77-RKQLCFQGADPESQHTSGLSSPVHRDQP-112	
		175-YDQIQKRFEGATPSYLS-191	
		28-CMPLSPPPTRAHQID-47	
		67-IREEMRRLQRRKQLC-81	
		117-TSGLSSPVRRDQPLF-131	
	<i>R. decoloratus</i>	128-QPLF-131	(Contreras et al., 2022)
		148-ESKIREEYDHVLSTKLAEQYDTFVKFTYDQI-178	
		151-IREEYDHVLSTKLAEQYDTF-170	
		40-PTRAHQIDPSPFGDVP-55	
		117-TSGLSSPVHRDQPLF-131	
<i>A. variegatum</i>	<i>A. variegatum</i>	126-RDQPLFTFRQVGLIC-140	
		143-MMKERESKIREEYDHVLSTKLAEQYDTFV-171	
		151-IREEYDHVLSTKLAEQYDTFVKF-173	
		153-EEYDHVLSTKLAEQYDTFV-171	
		49-SPFGDVPPKLTSEEI-63	
		73-RLQRRKQLCFQGAEC-89	

Table S4. Table listing the sequences of the predicted epitopes of Subolesin from different tick species based on literature. The epitope type is given, along with the species it was identified in, as well as the sequence of the epitope and its position in the alignment depicted in Figure 2.14 and 2.15.

Epitope type	Species	Sequence and position	Reference
Linear B cell	<i>A. variegatum</i>	80-LCFQGAECSSPEGC-105	(Contreras et al., 2022)
		91-SSPEGCSP-107	
		117-TSGLLSPVRRDQPLF-131	
		149-SQIREEDHVLSTKLAEQYDTF-170	
		151-IREEDHVLSTKLAEQYDTF-170	
		154-EYDHVLSTKLAEQYDTF-170	
	<i>O. erraticus</i>	9-THDWDPLHSPNGRASKRRRCV-29	(Manzano-Román et al., 2015)
		13-DPLHSPNGRASKRR-26	
		32-CVSPSTAPARAHQMSPSPFVEVPPK-57	
		54-VPPKLSS-60	
		65-ANIREEMRRLQR-76	
		83-TALDSSPQNSSTDSCSGPSSPT-115	
		107-CSGPSSPTGASGLLSPVRRDQP-129	
		119-GLSPVR-125	
	<i>O. moubata</i>	141-ERMMKERE-148	(Prudencio et al., 2010)
		8-RTHDWDPLHSPNGRASKRRRCVPLCVSPSTAPARAHQMS PPFVEVP-56	
		13-DPLHSPNGRASKRRR-27	
		54-VPPKLSS-60	
		65-ANIREEMRRLQR-76	
		86-DSPPQGTSGSSAD-115	
		92-GTSGSSADSPTGLLSPVRRDQP-129	
		141-ERMMKERE-148	
	<i>I. scapularis</i>	128-QPLFTFRQVGLICERMMKERESKIR-152	(Prudencio et al., 2010)
		160-SAKLAEQYDT-169	
Conformational discontinuous*	<i>R. microplus</i>	31- <u>LSPPPTRAHQIDPSPFGDVPPK</u> -57	(Lagunes et al., 2016)
		76- <u>RRKQLCFQGADP</u> -112	
		140- <u>CERMMKERESKI</u> -151	
	<i>I. scapularis</i>	1- <u>MACATLKRTHDWDPLHSPNGRSPK...SPFGEVPPK...SPTGLSPGGLS</u> -122	(Prudencio et al., 2010)
		82- <u>FSSPLESGSPSATPPADCGGPASPTGLSPGGLLSPVRR</u> -126	
		181- <u>RFEGATPSYL</u> -191	

*Predicted epitope regions of the possible conformational discontinuous epitopes are underlined and indicated in bold.

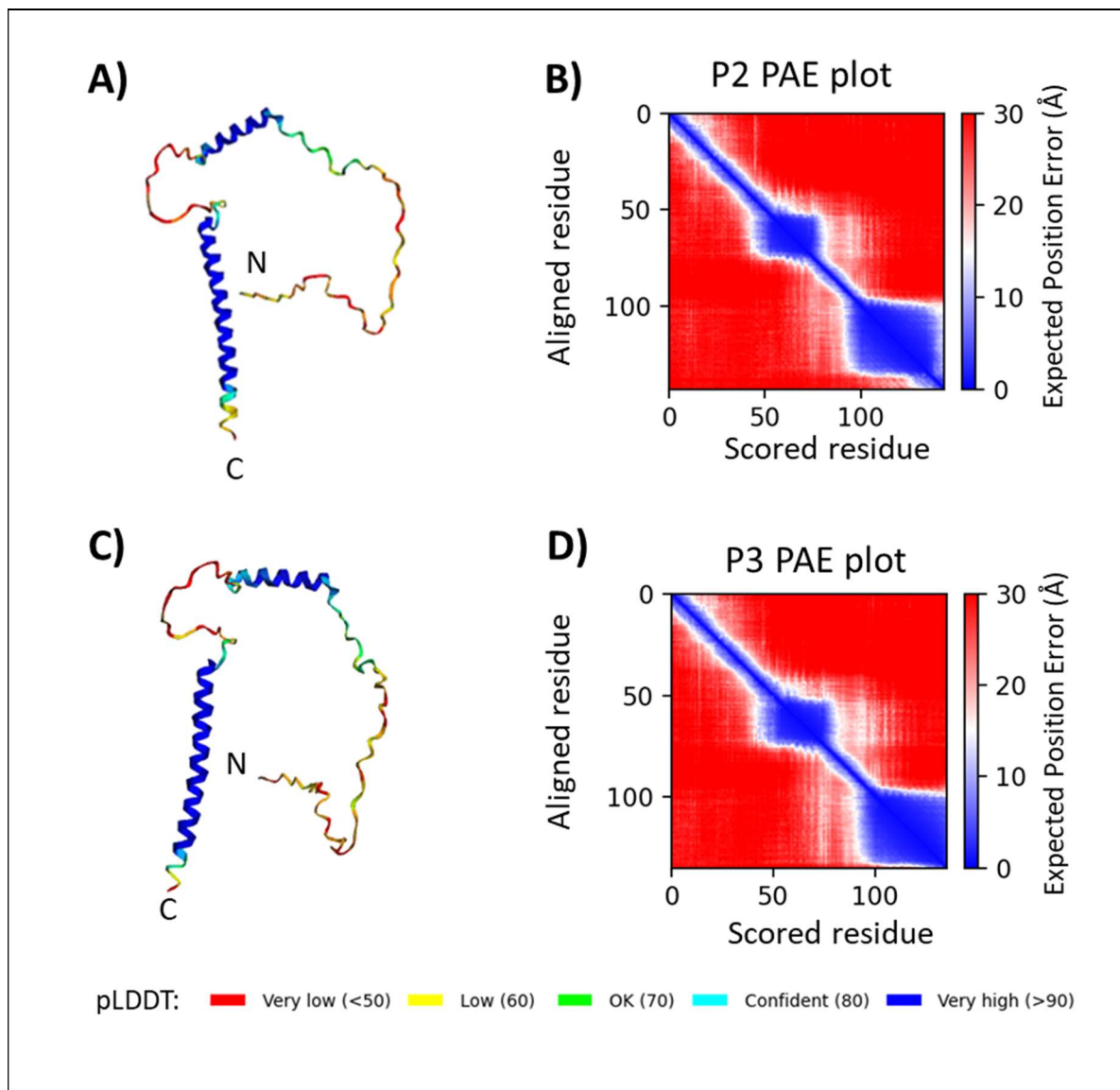


Figure S1. Predicted 3D structure of the putative P2 and P3 Subolesin protein variant and pLDDT and PAE confidence scores. (A) Predicted 3D structure of the P2 Subolesin protein variant created using ColabFold v.1.5.3. The N and C terminals are indicated on the structure. The structure is coloured according to the pLDDT (predicted local distance difference test) score, which increases in confidence from 0-100. High confidence means that the predicted structure could have a favourable comparison to experimental models. (B) The PAE plot of indicates the PAE (predicted aligned error) score for each residue of the predicted 3D structure of the P2 Subolesin variant. The aligned residues are indicated on the left y-axis and on the x-axis, and the expected position error (PAE score) is indicated on the right y-axis. A high PAE score represents a high expected position error, thus low confidence in the predicted relative position of the residues. Similarly, the (C) predicted 3D structure and (D) PAE plot of the P3 Subolesin protein variants are indicated.

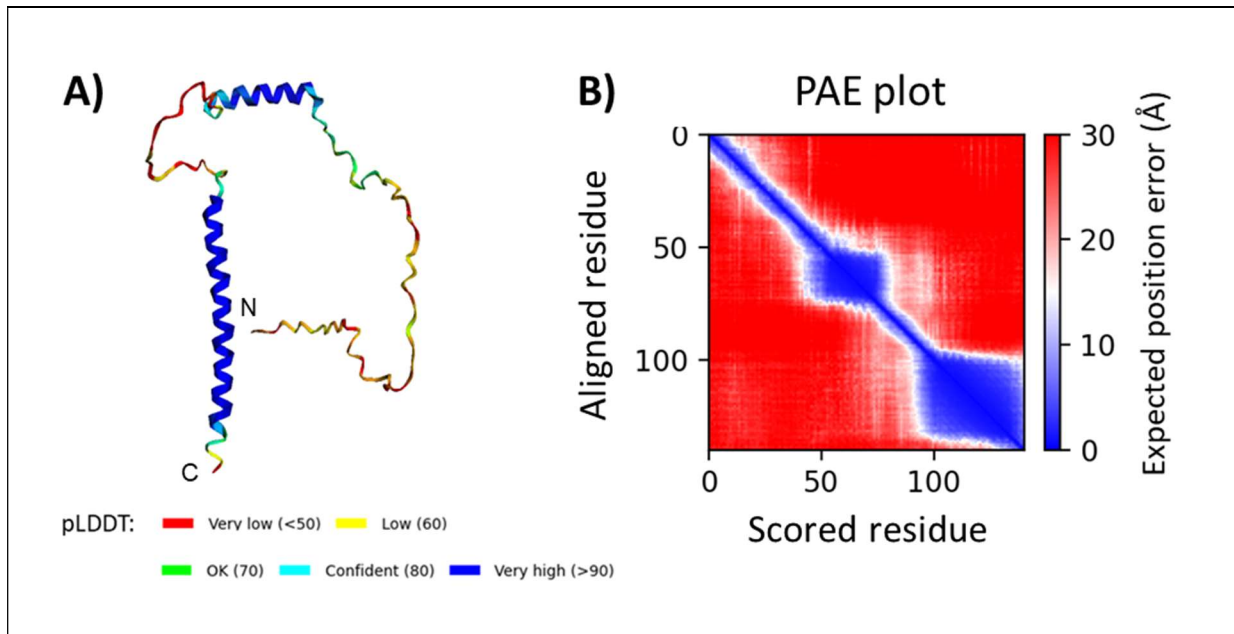


Figure S2. Predicted 3D structure of the putative Clare P3 Subolesin protein variant and pLDDT and PAE confidence scores. (A) Predicted 3D structure of the Clare P3 Subolesin protein variant created using ColabFold v.1.5.3. The N and C terminals are indicated on the structure. The structure is coloured according to the pLDDT (predicted local distance difference test) score, which increases in confidence from 0-100. High confidence means that the predicted structure could have a favourable comparison to experimental models. (B) The PAE plot indicates the PAE (predicted aligned error) score for each residue of the predicted 3D structure of the Clare P3 Subolesin variant. The aligned residues are indicated on the left y-axis and on the x-axis, and the expected position error (PAE score) is indicated on the right y-axis. A high PAE score represents a high expected position error, thus low confidence in the predicted relative position of the residues.

Supplementary available upon request:

1. Table with summary statistics of BLAST results used to create the consensus sequence for each mRNA species (X1-X4) as described previously (**2.2.5. Consensus sequence of Subolesin RNA species**).
2. Table with summary statistics of variants detected for each putative mRNA species (X1-X4) as described previously (**2.2.8. Variant detection workflow**).

Table S5. Summary of the amounts of antigens, microparticles, adjuvant per injection

Group	Antigen	Dose (µg)	PLGA particles (µg)	Adjuvant (µl)	Finale volume of formulation per dose
1	MSP4+SUB	20+20	100+100	100	200
2	MSP4	20	100	n.a.	100
3	MSP4	20	100	50	100
4	MSP4	20	n.a.	50	100
5	n.a.	n.a.	100	100	200
6	SUB	20	100	50	100
7	SUB	20	100	50	100
8	SUB	20	n.a.	100	100

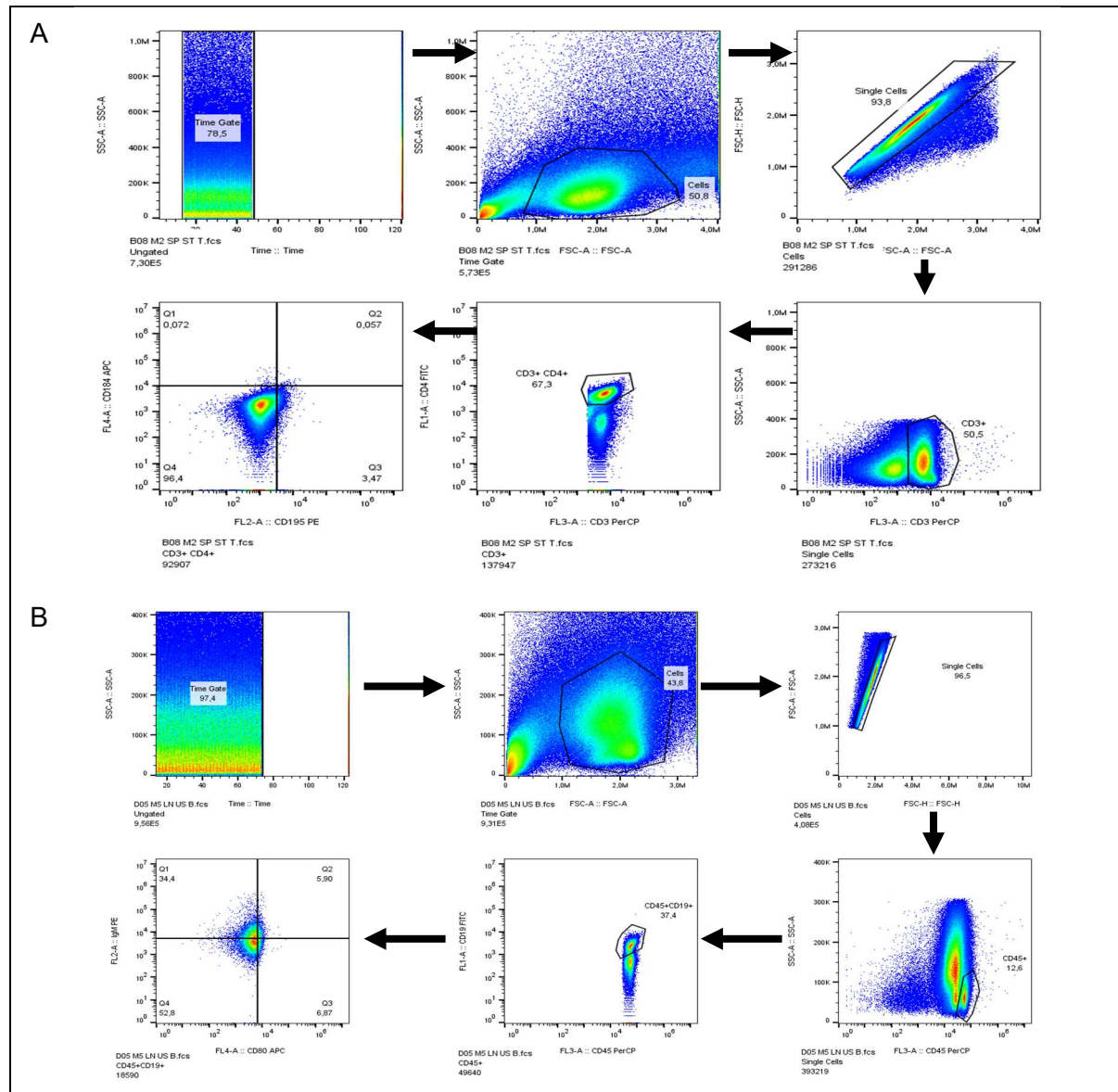


Figure S3. Example of gating strategy used to identify subpopulations for (A) B cells and (B) T cells.

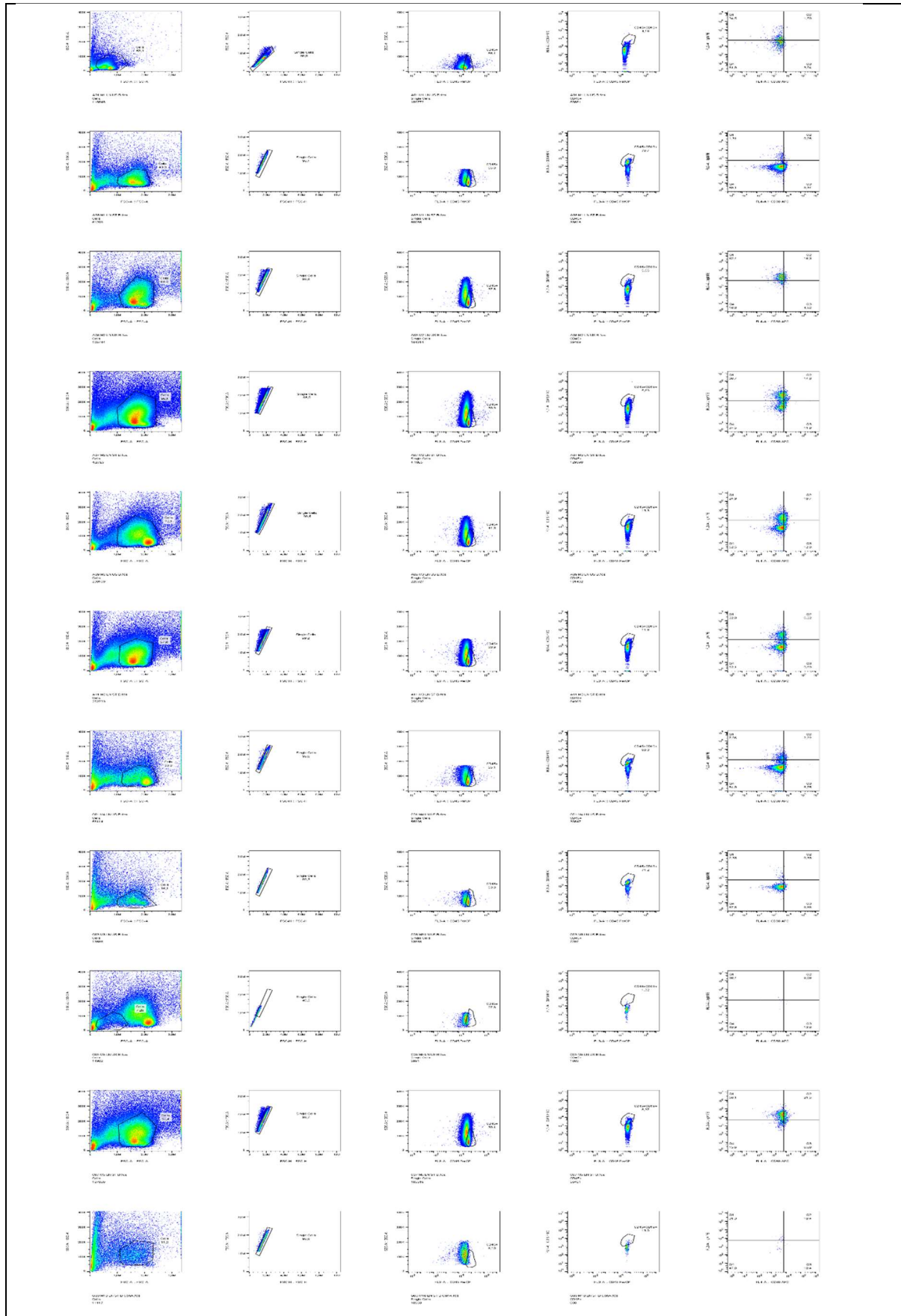


Figure S4. Pseudocolour plots depicting Group A B-Cell populations with each gate derived from murine lymph node cells.

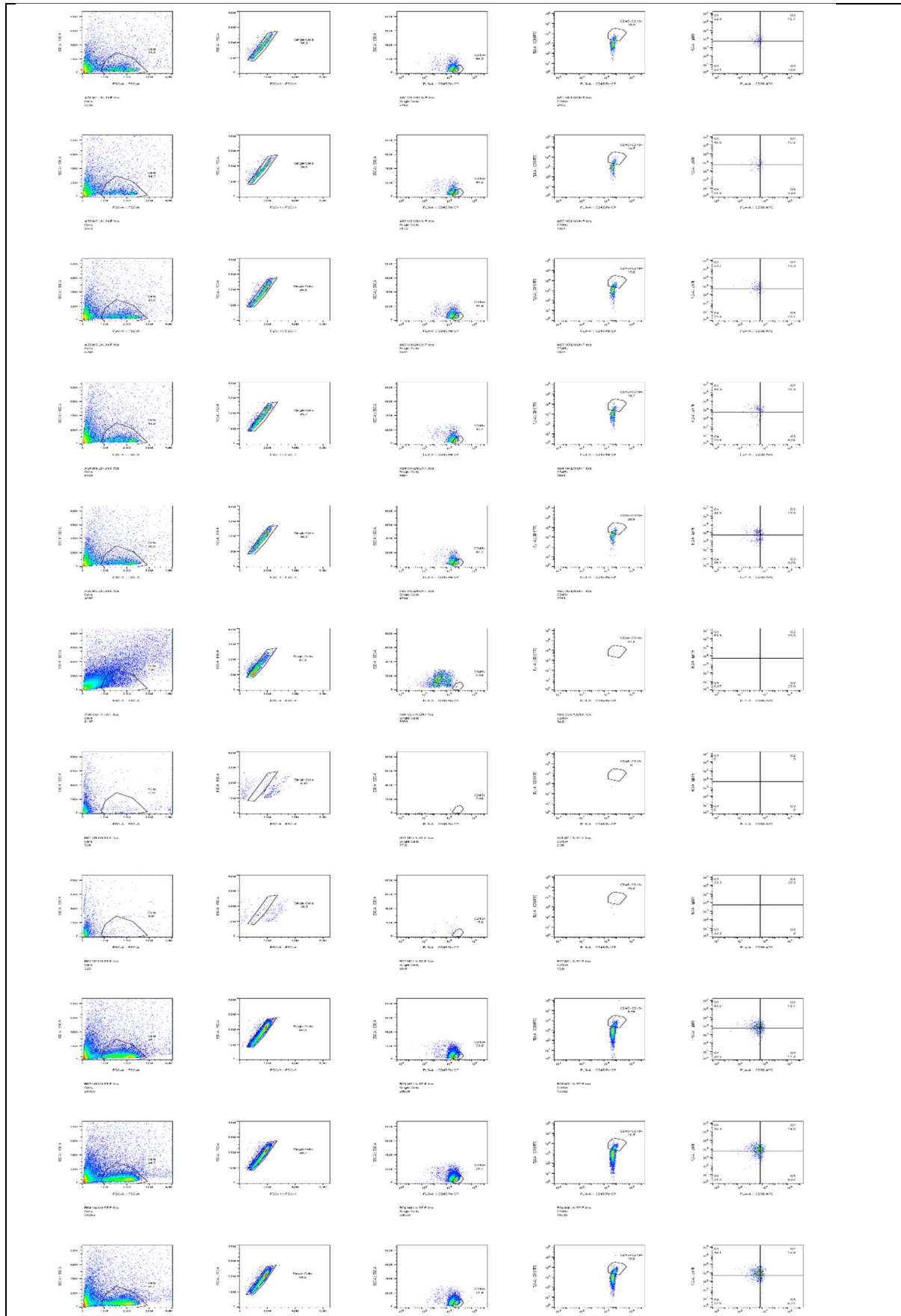


Figure S5. Pseudocolour plots depicting Group F B-Cell populations with each gate derived from murine lymph node cells.

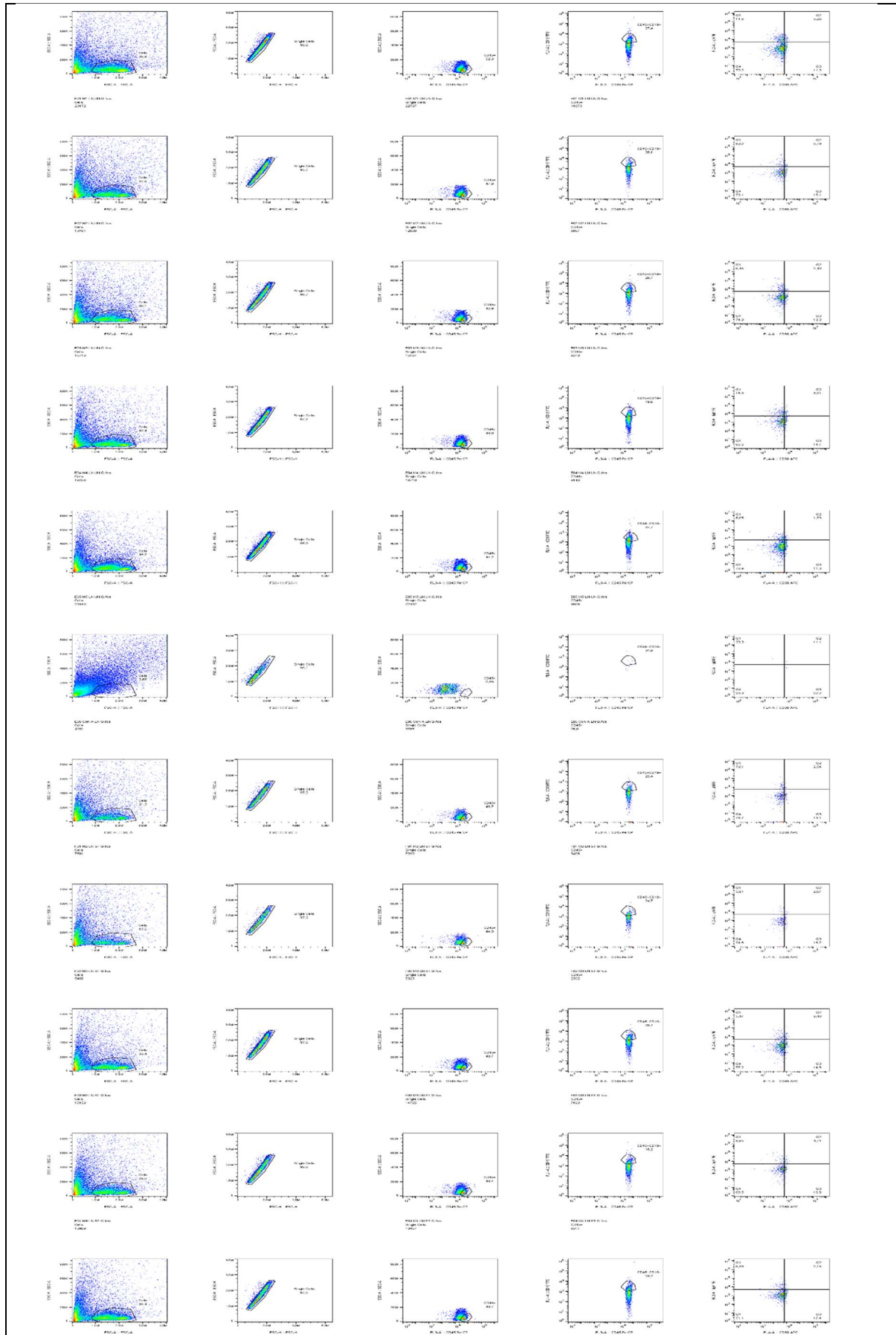


Figure S6. Pseudocolour plots depicting Group G B-Cell populations with each gate derived from murine lymph node cells.

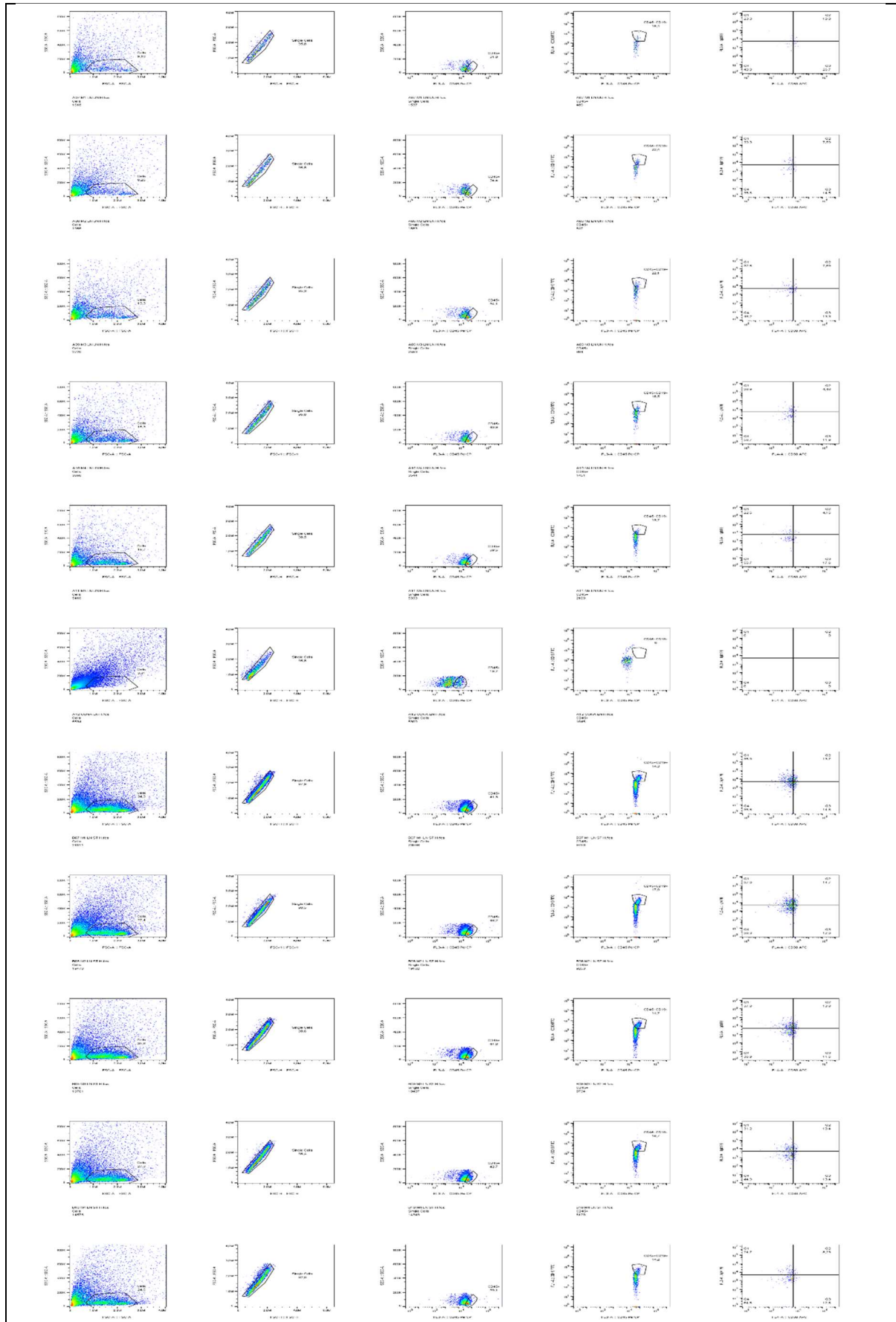


Figure S7. Pseudocolour plots depicting Group H B-Cell populations with each gate derived from murine lymph node cells.

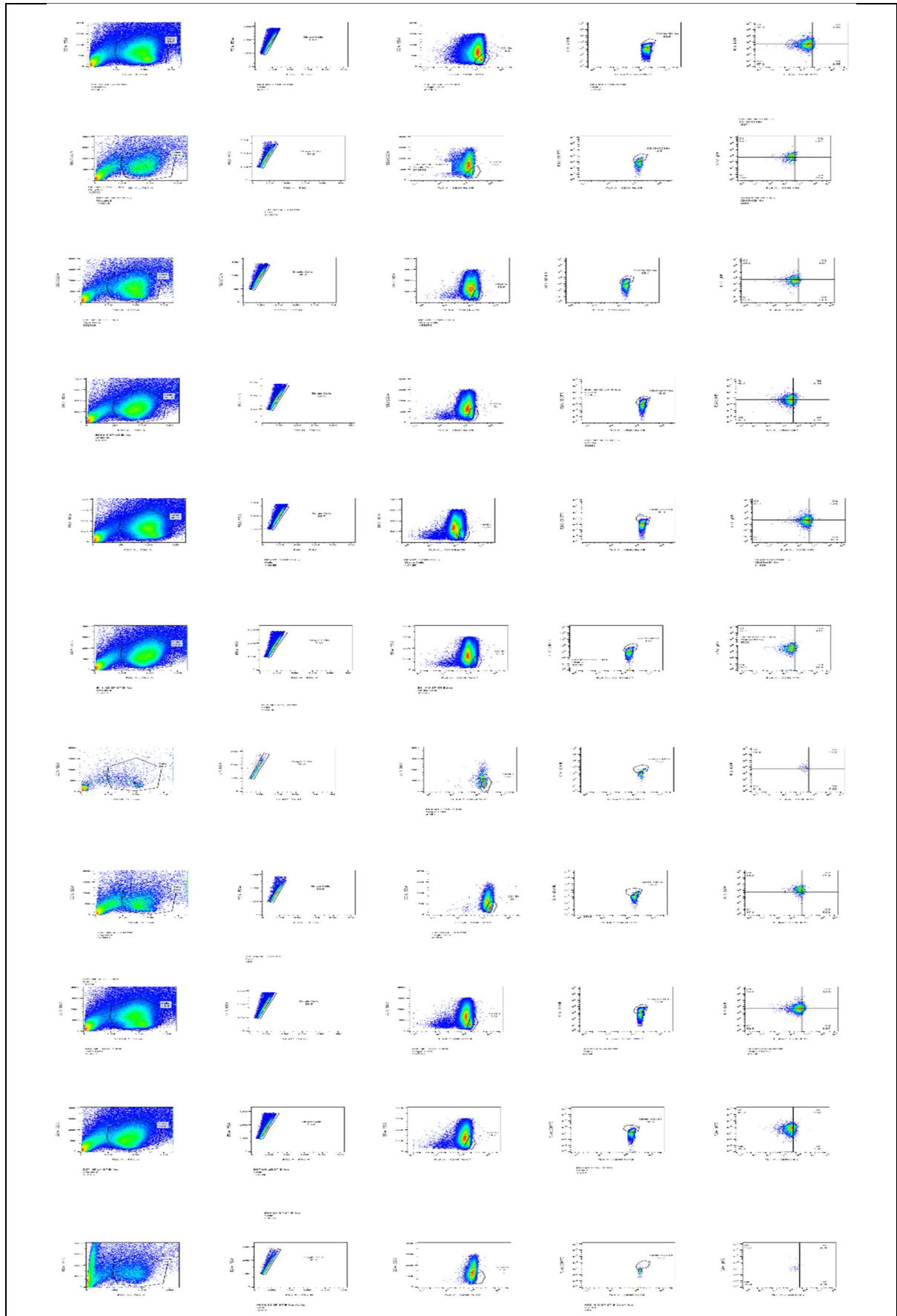


Figure S8. Pseudocolour plots depicting Group A B-Cell populations with each gate derived from murine spleen cells.

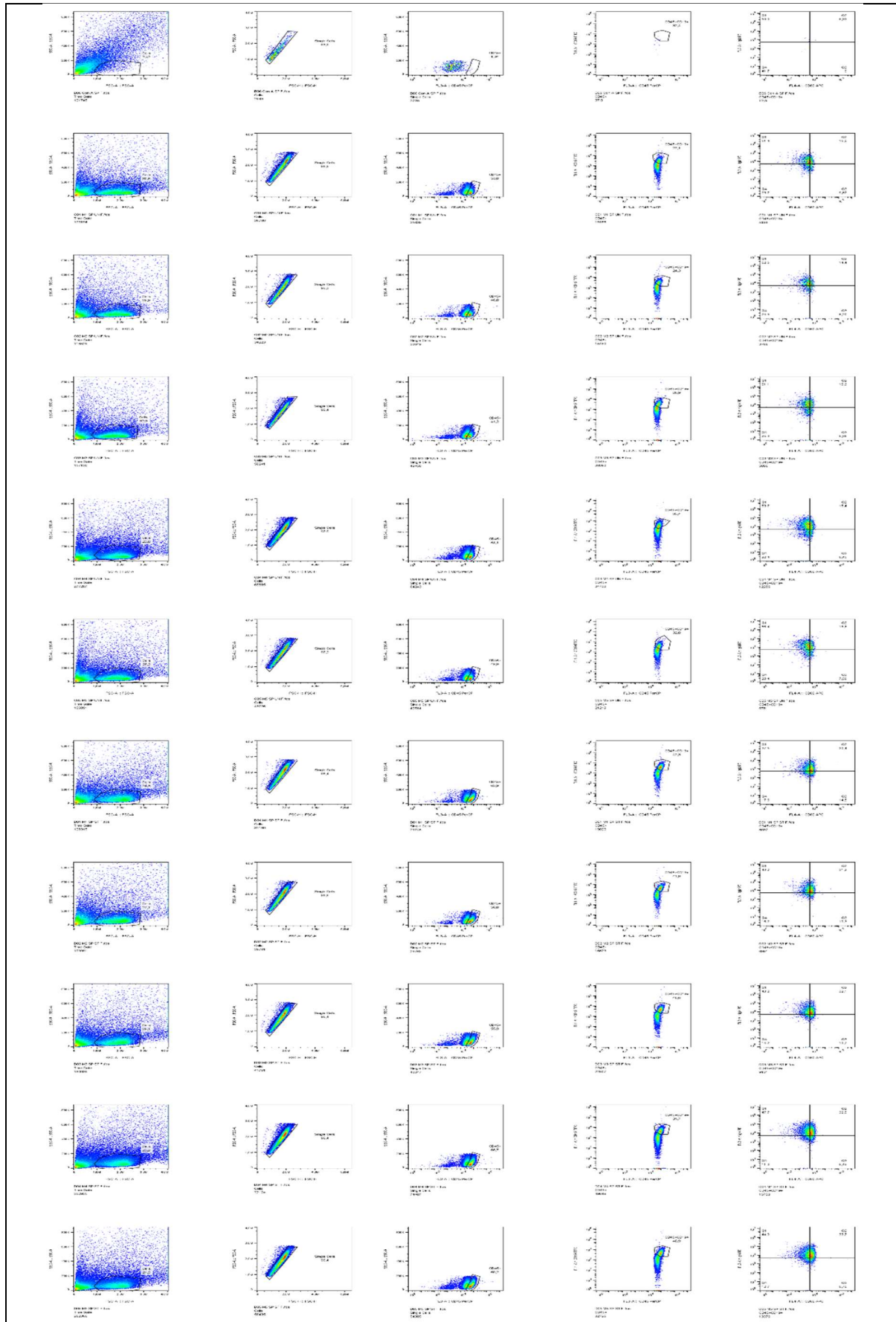


Figure S9. Pseudocolour plots depicting Group F B-Cell populations with each gate derived from murine spleen cells.

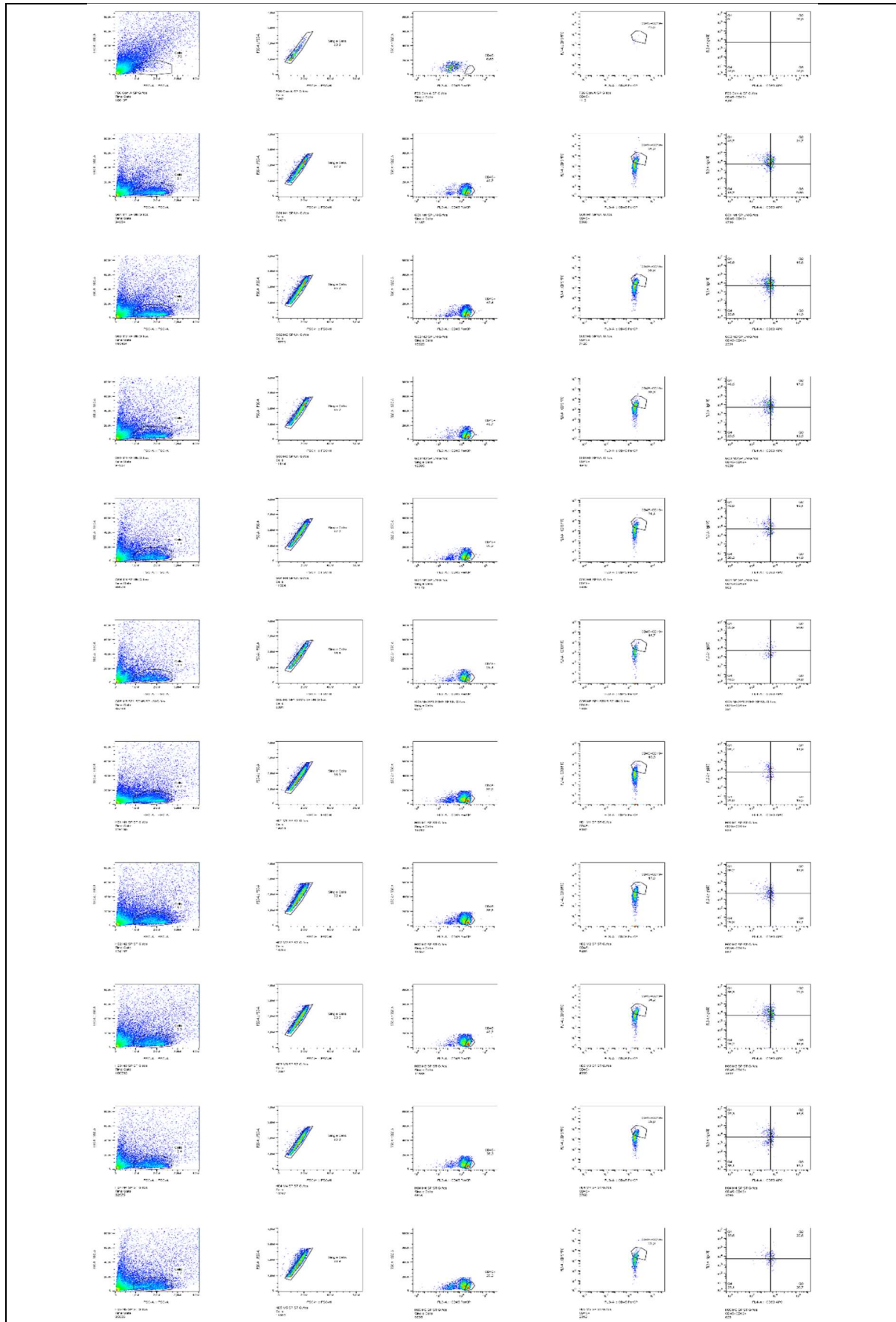


Figure S10. Pseudocolour plots depicting Group G B-Cell populations with each gate derived from murine spleen cells.

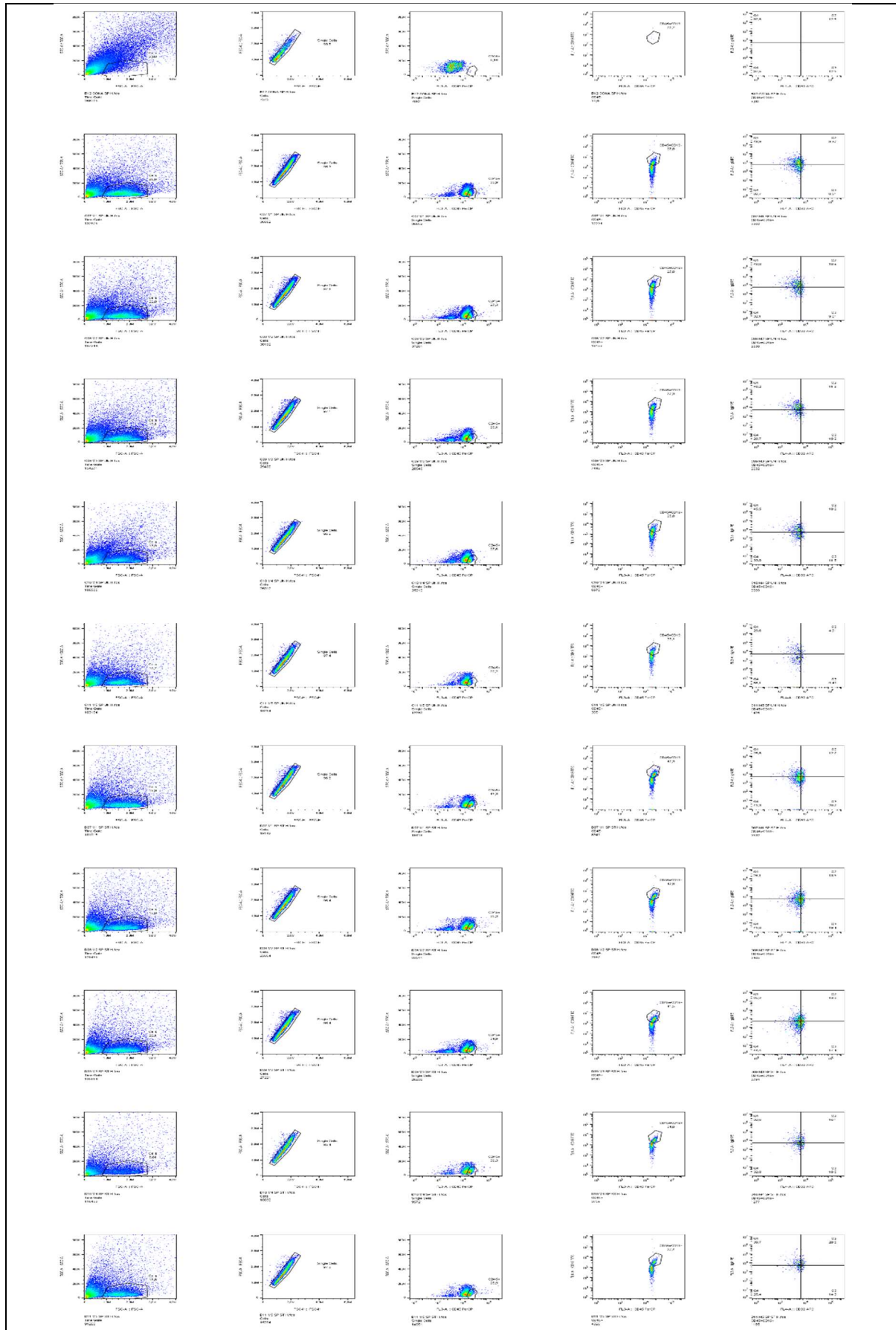


Figure S11. Pseudocolour plots depicting Group H B-Cell populations with each gate derived from murine spleen cells.

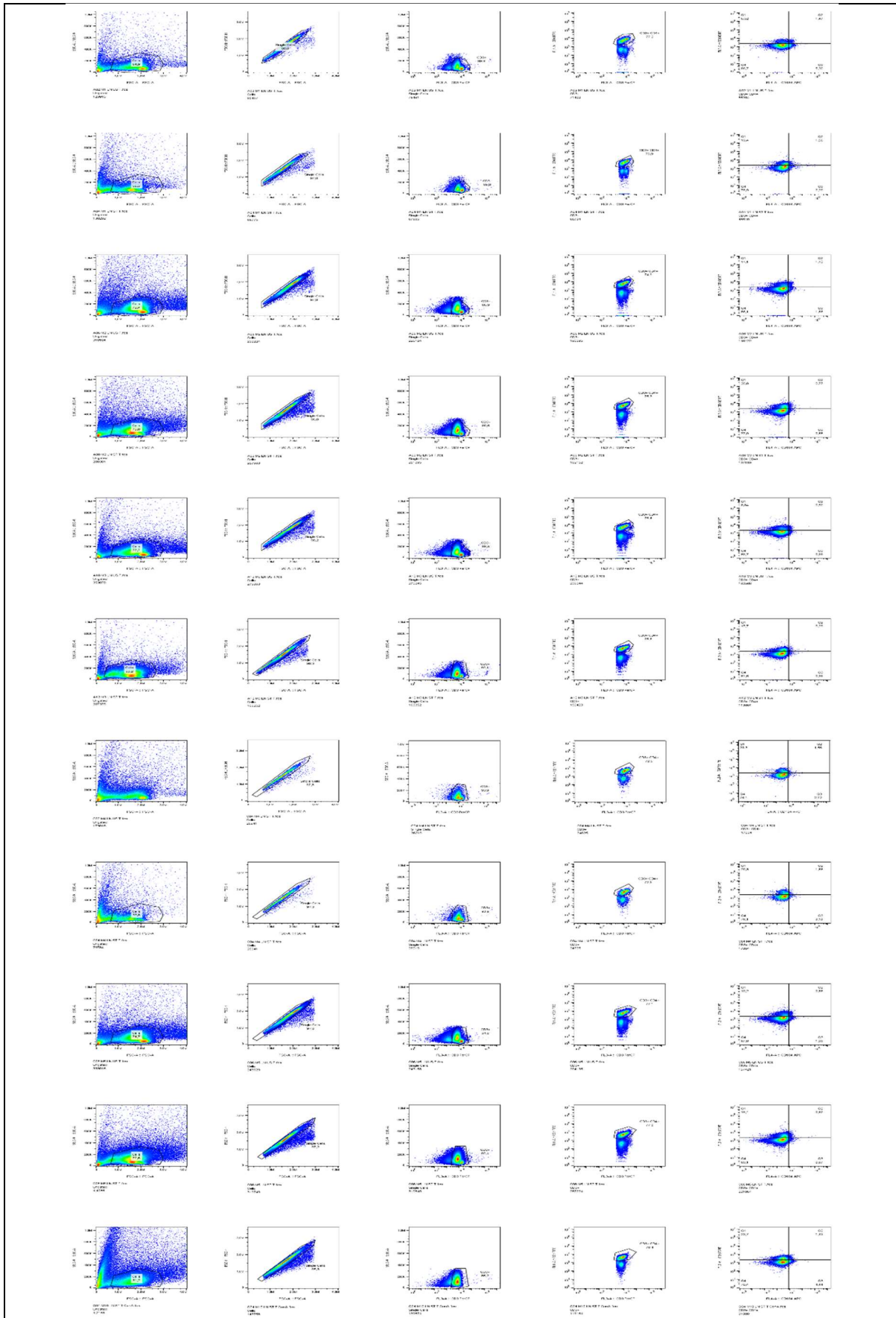


Figure S12. Pseudocolour plots depicting Group A T-Cell populations with each gate derived from murine lymph node cells.

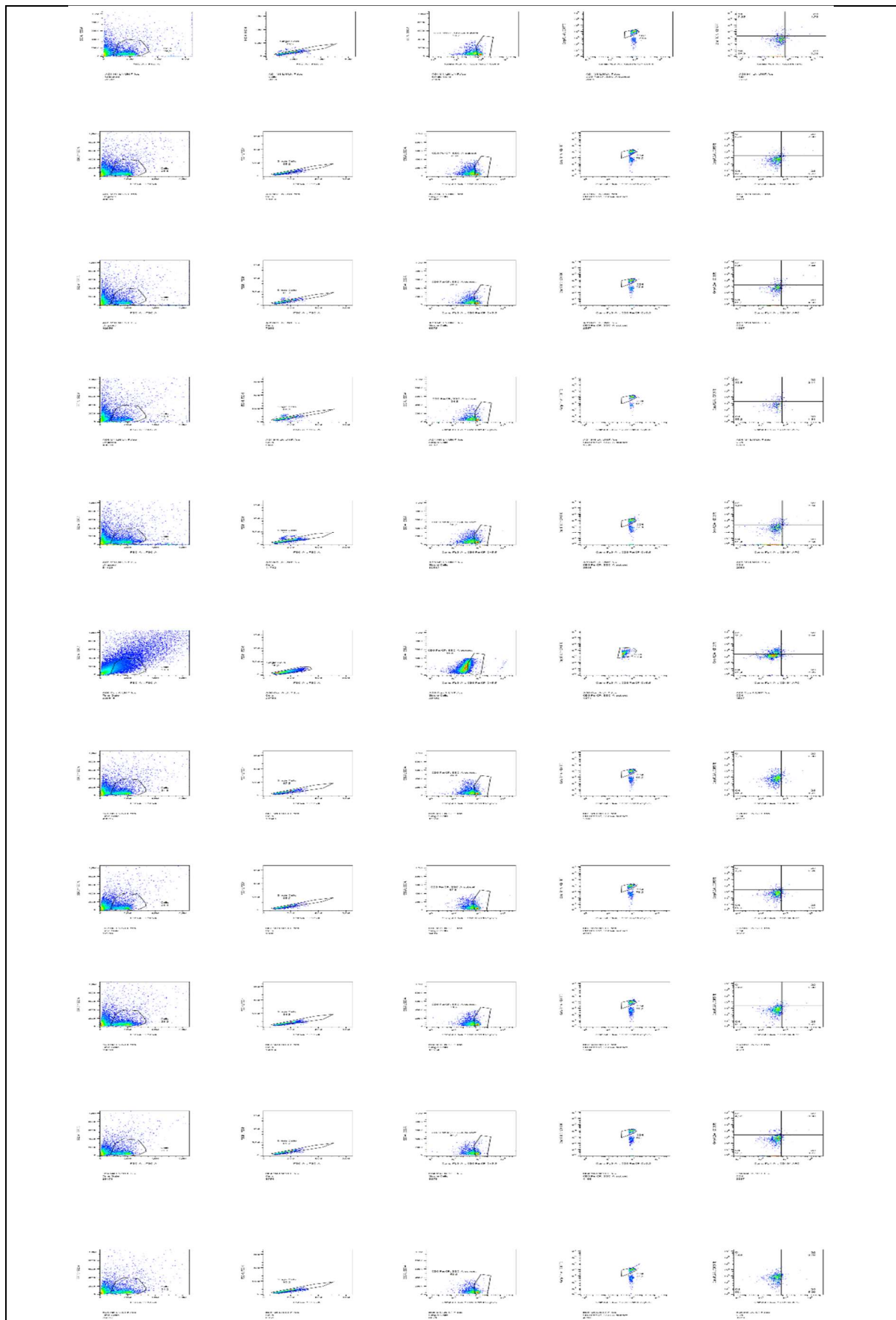


Figure S13. Pseudocolour plots depicting Group F T-Cell populations with each gate derived from murine lymph node cells.



Figure S14. Pseudocolour plots depicting Group G T-Cell populations with each gate derived from murine lymph node cells.

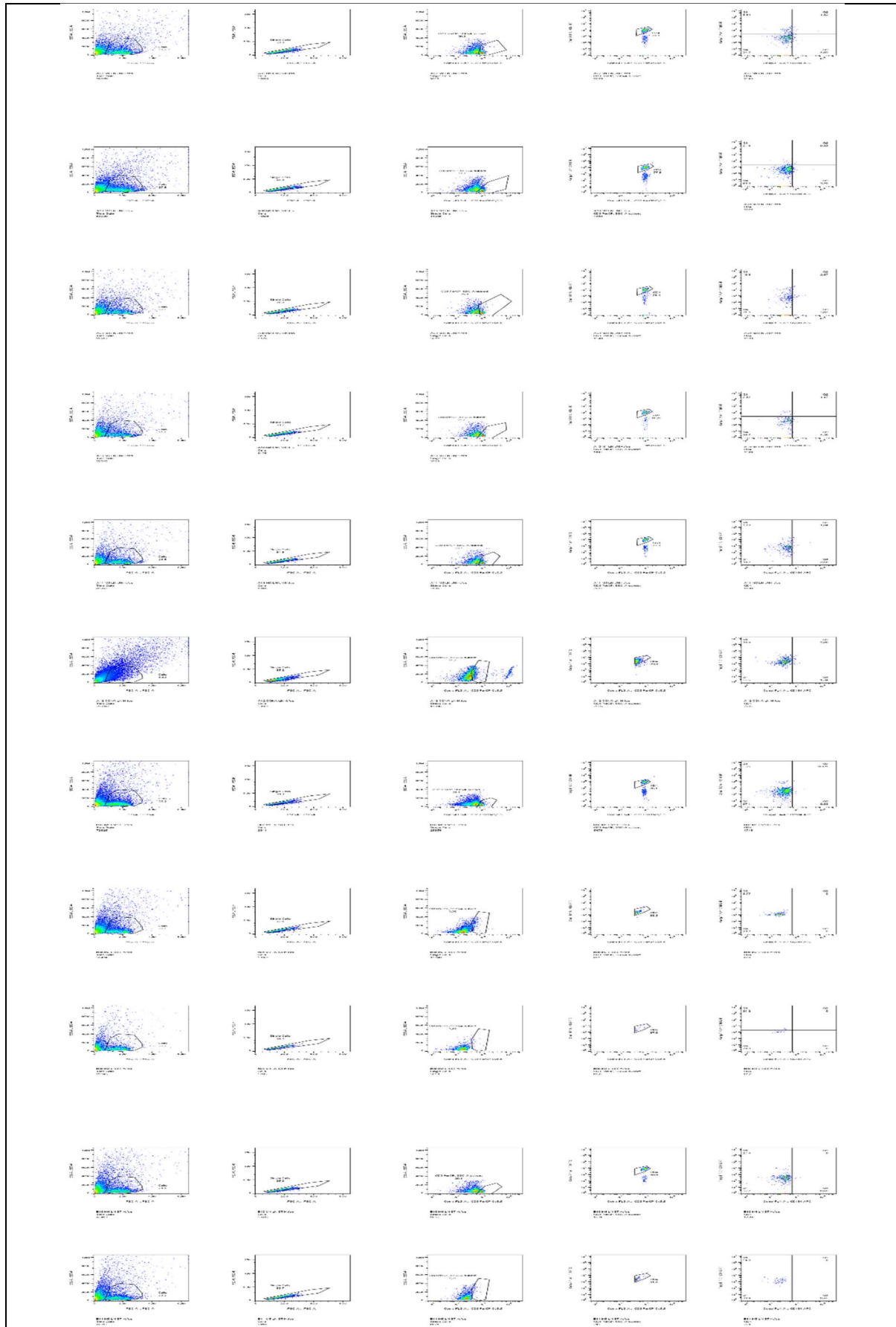


Figure S15. Pseudocolour plots depicting Group H T-Cell populations with each gate derived from murine lymph node cells.

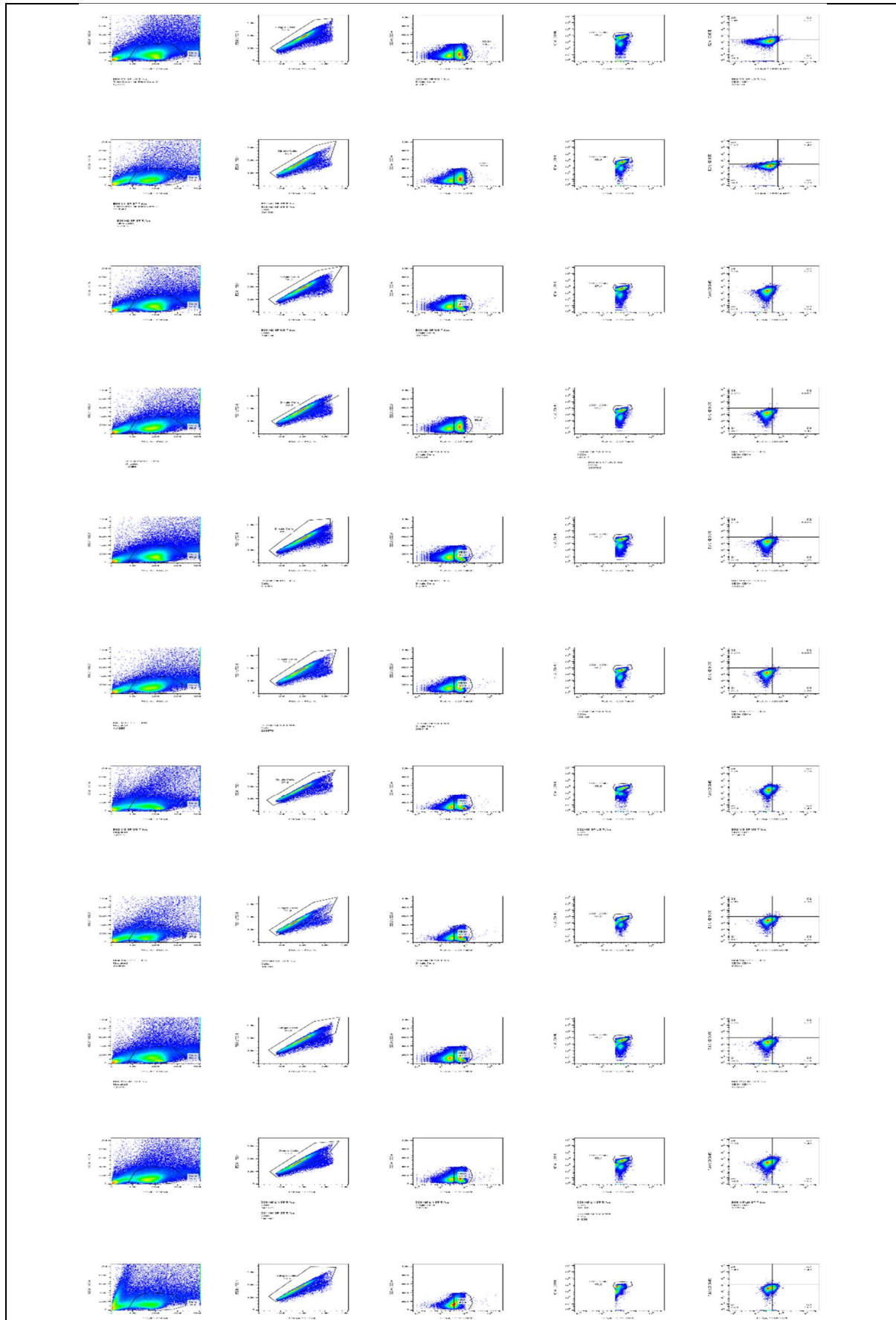


Figure S16. Pseudocolour plots depicting Group A T-Cell populations with each gate derived from murine spleen cells.

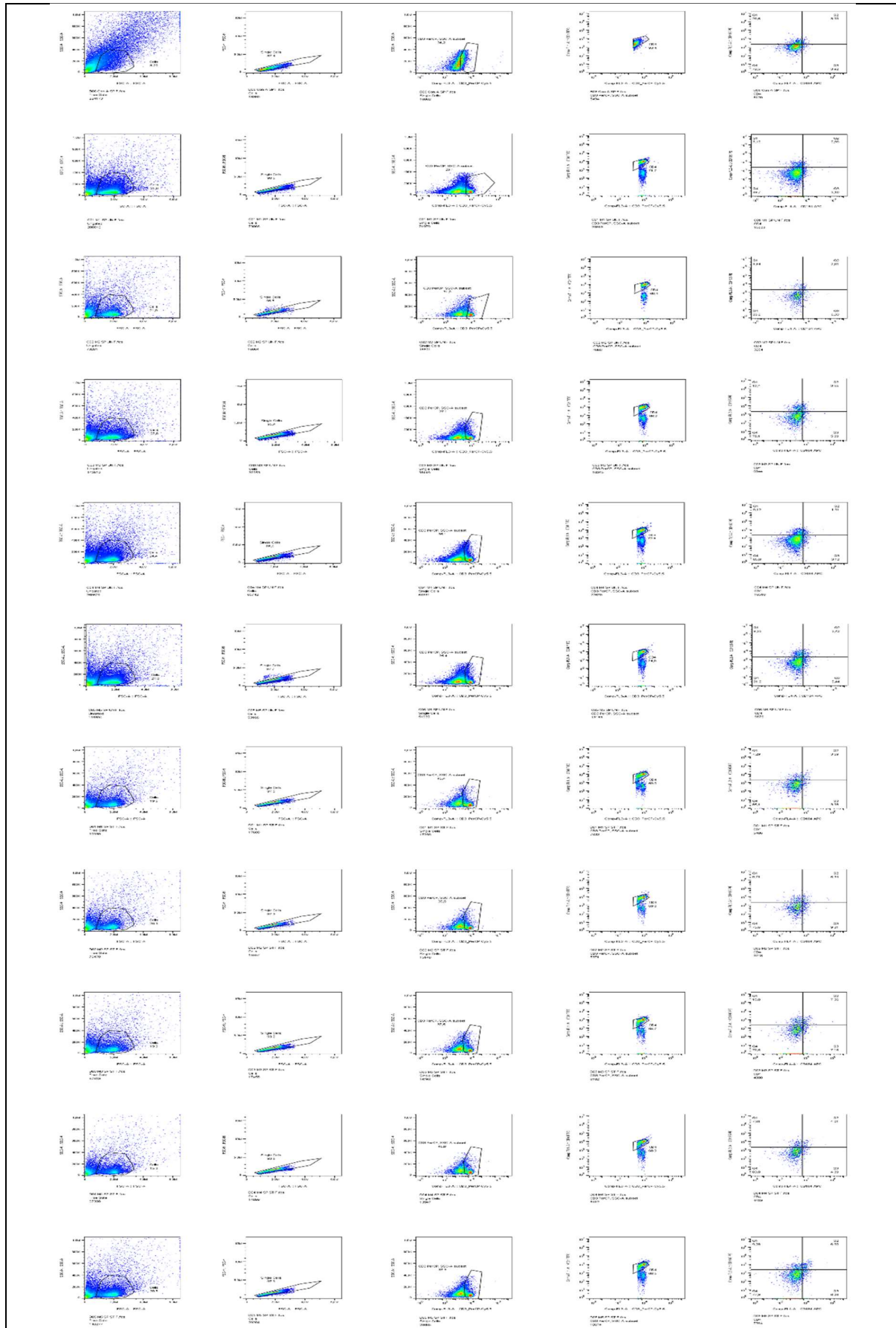


Figure S17. Pseudocolour plots depicting Group F T-Cell populations with each gate derived from murine spleen cells.

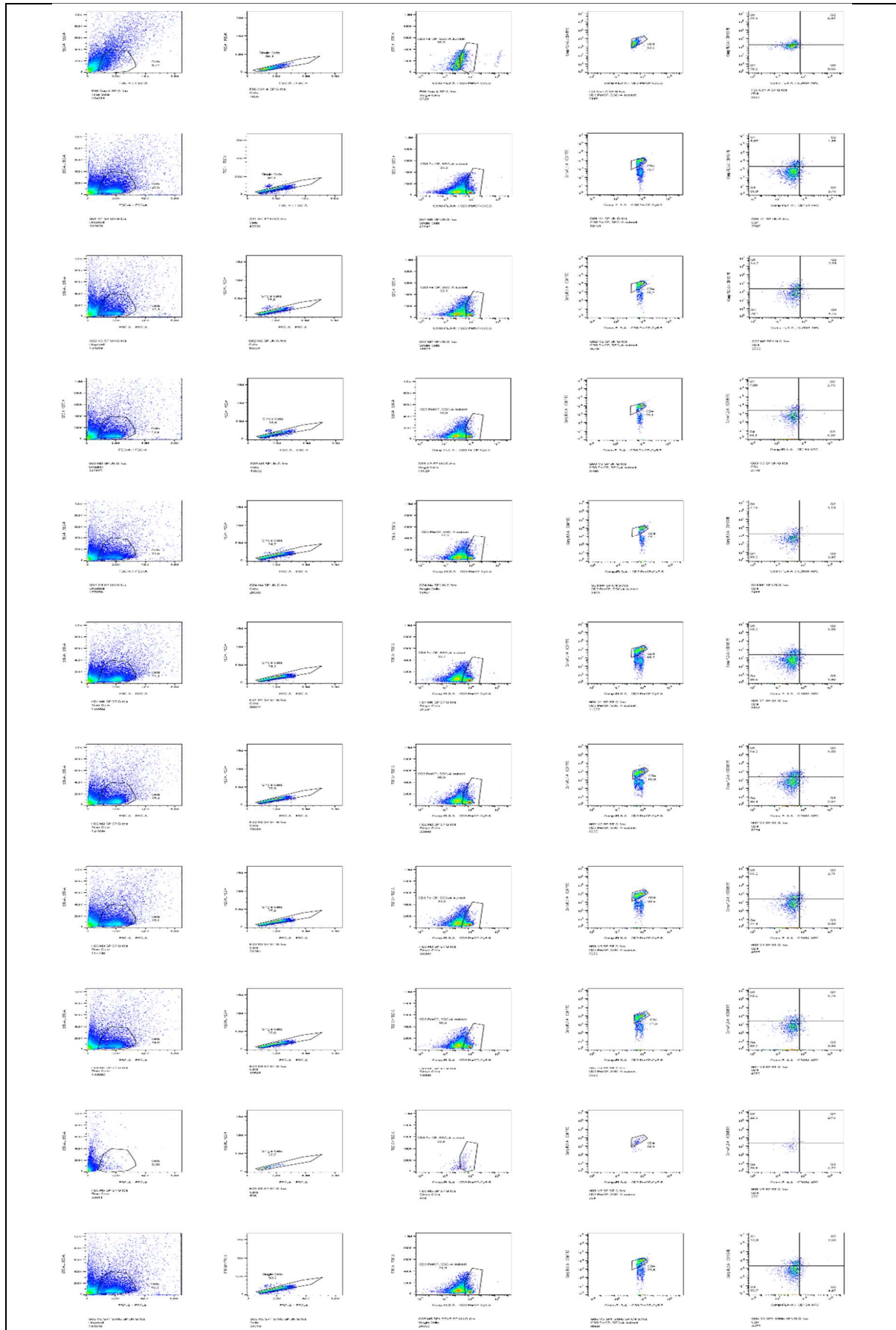


Figure S18. Pseudocolour plots depicting Group G T-Cell populations with each gate derived from murine spleen cells.

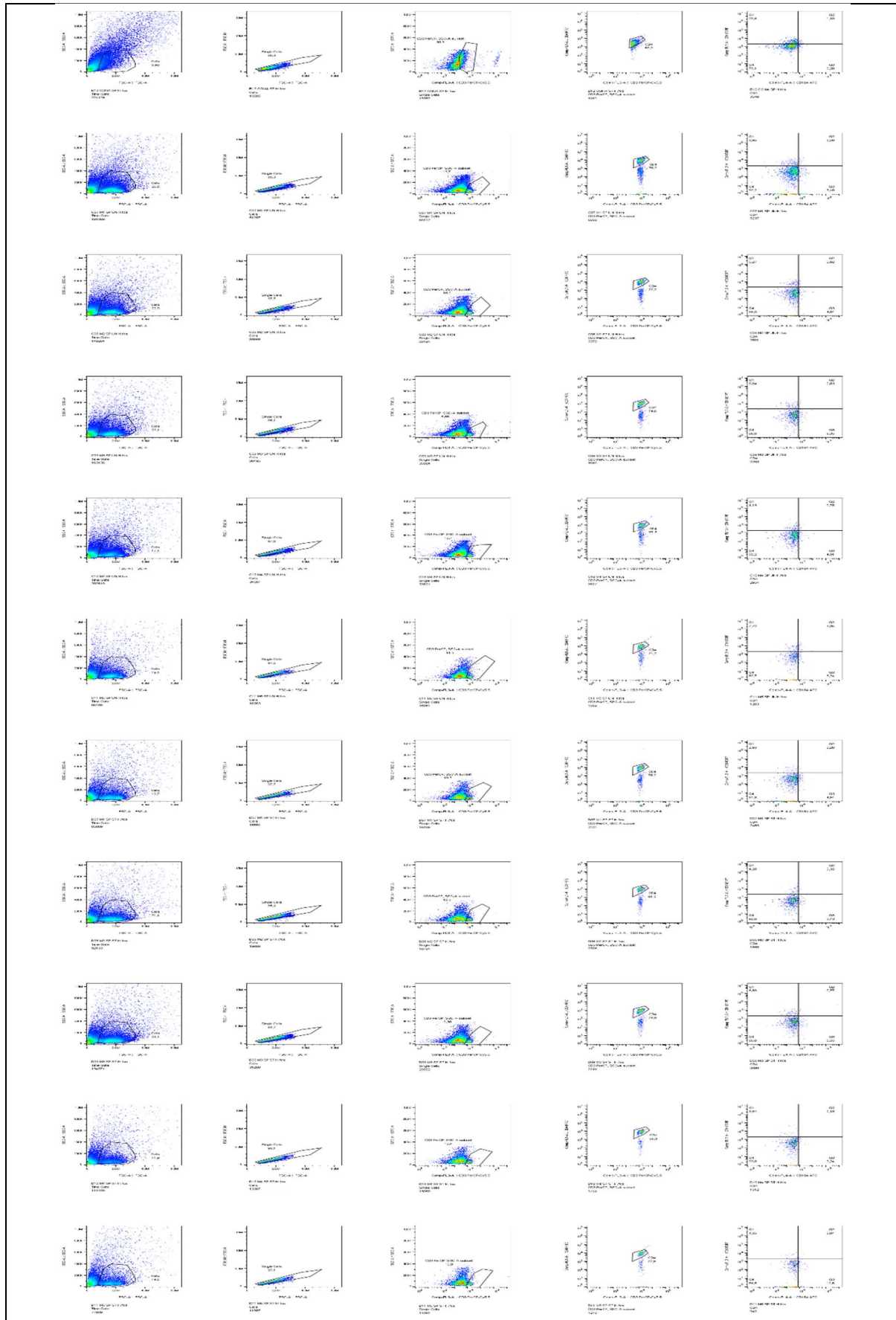


Figure S19. Pseudocolour plots depicting Group H T-Cell populations with each gate derived from murine spleen cells.