MONASH University

Characterising the equine antibody response to snake venom

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Master of Philosophy

A thesis submitted for the degree of *Master of Philosophy* at
Monash University in 2018

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Abstract

The treatment for snake bite envenomation involves the administration of snake antivenom. Snake antivenoms are composed of antibodies, proteins that are capable of binding and neutralising snake venom toxins. While antivenom to snake bite has been the standard method of treatment for envenomation and available for over 100 years, snake bite envenomation is still a major cause of morbidity and mortality worldwide. The antivenom antibodies, typically derived from horses that have been immunised multiple times with snake venom, can display varying amounts of cross-protection. While this usually relates to the venom species used in the immunisation, there is a degree of inter-species protection presumably due to antibody cross-reactivity. Despite the utility and importance of snake antivenom, there is limited understanding of the nature, kinetics and binding properties of the neutralising antibodies generated in the immunised horses. There is therefore a need to improve the understanding and characterisation of the horse response to snake venom and we have elected to do this in the long term by isolating venom-specific B cells through the course of the immunisation protocol and characterising the genes encoding their B cell receptors.

To study the antibody response to snake venom, immunological and molecular approaches were undertaken and developed, with varying degrees of success. This process began with the isolation and characterisation by flow cytometry of B cells from a mixed population of peripheral blood mononuclear cells (PBMCs) in an effort to define antibodies that would function with horse lymphocytes. I then attempted to isolate, directly from blood, antigen-specific B cells. Progress has been made in this project in being able to isolate equine B cells from PBMC, despite the paucity in flow cytometry antibodies for equine surface markers. However, antigen-specific B cells could not be isolated as of yet. Following isolation of B cells, the next step of the project involved attempts to create a cell culture system to support the proliferation and differentiation of B cells into Antibody Secreting Cells (ASCs). The cell culture experiments have so far yielded limited success in converting B cells into ASCs. The final aspect of this thesis involved the amplification and cloning of equine antibody heavy chain and light chain genes, with the aim of using the isolated antibody genes from single B cells to reconstruct the original anti-venom immunoglobulin. Progress was achieved in this phase of the project, when the heavy chain and light chain genes of equine B cells were isolated and subsequently cloned.

Although this project achieved only some of its aims, it forms a stepping stone to study the antibody response to snake venom. With greater understanding, a better designed antivenom and refined process to developing antivenom can be developed, progressing from the initial antivenom discovery made over a century ago. This project aims to provide a means to better understand the antibody response to snake venom, and develop a process for investigating broader questions in the field of snake antivenom.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature: 

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Acknowledgements

I would like to begin by thanking my primary supervisor Professor David Tarlinton for giving me the opportunity to do this interesting and unique project. David has provided me with insight and guidance throughout my postgraduate education, for which I am incredibly grateful. Even while being the head of a large department and a laboratory of at least ten people, David always had spare time for me to discuss my project, experiments, and to provide feedback on a regular basis.

I thank my two co-supervisors, Professor Andrew Lew and Associate Professor Steven Rockman, for being an excellent source of knowledge on many aspects of my project and for their willingness to attend my group meeting presentations. They, along with David, have established a triumvirate supervisory role in enabling me to do the best that I can, as well as imparting guidance and advice whenever I requested. I am grateful to each of my three supervisors for demonstrating an ideal example of collaboration between academia and industry, and how to carry out a scientific endeavour. Further gratitude to my supervisors for critique of my written work and presentations, and for their edits during the writing of my thesis.

I give thanks to members of my committee, Dr Dan Andrews, Associate Professor Menno Van Zelm and Associate Professor Frank Alderuccio, for contributed valuable critique and ideas during project meetings and seminars. I also give thanks to Frank Alderuccio for enabling me the opportunity to be a teaching associate at Monash University, providing me with experience and confidence in teaching valuable skills to undergraduate students.

The members of the Tarlinton lab, or Tarlie's Angels, I thank immensely for their assistance throughout my project, providing feedback of my experiments and presentations, as well as their immeasurable kindness and collegiality. This includes long time members, such as Catherine Pitt, Kristy O'Donnell, Dimitra Zotos, Marcus Robinson, Erica Brodie, Simona Infantino, Isaak Quast and Michael Low. Newer or short time members I also thank include Lorena Goldring, Cassandra Zakrisson, Rohia Farighi and Zoe Deng, who are welcome additions to the group. I also give thanks to Amanda Light, a long-time member of the group, whose lifetime worth of experience and willingness to provide help is greatly valued. I also give thanks to Marcus for offering to read and critique my thesis drafts.

Although not an official supervisor, I would also like to thank Kirsten Vandenberg at Seqirus, who has been a wonderful source of advice and technical knowledge whenever I carried out experiments at Seqirus. She has also made me learn that there are great people in industry that have quirks and personalities like any from academia. I would also like to extend my thanks to Seqirus for their stipend support during my project, as well as Seqirus animal staff for performing horse immunisations and providing me with the cellular material to perform my experiments.

Many people of the department of Immunology and Pathology helped me to have an enjoyable time during my project, especially during social event, board game nights and daily lunch time quizzes. This includes but not limited to Angela Nguyen, Samuel De Jong, Timothy Gottschalk, Anisha Ansari, Mahtab Parvaresh, Craig McKenzie, Pei Mun Aui, Cassandra Castelino, Lakshanie Wickramasinghe, Jennifer Boer, Jodie Abramovitch, Eva Pesce, Jade Jaffar, and Katharine Goodall. Many thanks to Darren Partridge and Rachael Borg for their lab management assistance, and to Kate York for helping me with administrative tasks and general inquiries. I would also like to thank the Baker student society, for allowing Immunology students such as myself to partake in the annual student retreat. The retreats were wonderful experiences, filled with wonderful talks, fun activities, social parties, and lifelong memories that I will fondly remember. The Monash support staff were of great support and assistance during my project. I thank members current and prior of the AMREPFlow cytometry facility, Geza Paukovics, Eva Orlowski-Oliver, Magdaline Costa and Phil Donaldson for teaching me proper flow cytometry use, and for their assistance during the sorts. I thank Monash IT support for quickly resolving my IT related issues. I also thank Sharmila Ramesh with administrative issues related to my degree. Although a large part of my project was carried out at Monash, my project began at the Walter and Eliza Hall Institute (WEHI), and I would like to thank many people at WEHI who helped in varying ways and/or were great social connections at WEHI. They include but not limited to, Charlotte Slade, Robert Hennessey, Tobias Kratina, Kim Pham, Susanne Heinzel, Andrey Kan, Patrick Leung, Julia Marchingo, Merle Dayton and Shalin Naik. I give special thanks to Jamie Brady, who provided enormous help and guidance with many aspects of the molecular side of my project. I also give thanks to Sue Hardy and Keely Bumsted-O'Brien for assistance with my initial enrolment to WEHI.

There are many friends who have been of immense support and source of sanity during my project including Rick Tankard, Thomas Scerri, Billy Tankard, Angela Nguyen, Mahtab Parvaresh, Samuel De Jong, Christopher & Stephanie Haggarty-Weir, Anisha Ansari, Cassandra Castelino, Luke Tellis, Aaron Eastlake, Mathew Preston, James Schwarz, Becky Loh, Catriona Nguyen-Robertson and Larissa Ratten. Many others contributed to my knowledge and enjoyment during my project that I have not listed here, but their assistances are appreciated nevertheless. I would also like to thank Rick and Tom for help in proof reading my thesis and advice on statistics.

I would like to thank my brother Pece, and Rebecca Tomasiello for their never-ending emotional support during my project, especially during the tough times, and for Pece for also helping to edit my thesis. Also my father and mother for their support throughout the years and their belief that my happiness and wellbeing is all that matters for them. I also thank my aunty, uncle and cousins for their support.

Finally, I would like to give special thanks to my long time academic mentor, Steven Turner. Steven has been a constant source of support even before I was an honours student. Steven's guidance, encouragement and willingness to chat is greatly treasured. Steve is also the big reason I began this project and this journey, all those year ago.

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Abbreviations

2-ME – Beta-Mercaptoethanol

^{o}C – Degrees centigrade

Ab – Antibody

ABTS – 2’2-Azinobis (3-ethylbenzthiazoline Sulfonic Acid)

AEC – 3-amino-9-ethyl Carbazole

APC – Allophycocyanin

ASC – Antibody Secreting Cell

BCR – B cell receptor

BSA – Bovine Serum Albumin

BD – Becton Dickinson

CSR – Class Switch Recombination

CD – Cluster of Differentiation

cDNA – complementary deoxyribonucleic acid

CMV – CytomegaloVirus

CTV – Cell Trace Violet

DMF – N,N-dimethyl formamide

DNA – Deoxyribonucleic Acid

DTT – Dithiothreitol

ELISA – Enzyme Linked Immunosorbent Assay

ELISPOT – Enzyme Linked ImmunoSPOT

FACS – Fluorescence Activated Cell Sorting

FCS – Foetal Calf Serum

FITC – Fluorescein isothiocyanate

GC – Germinal Centre

GFP – green fluorescent protein

H_20_2 – Hydrogen Peroxide

HRP – Horse Radish Peroxidase

Ig – Immunoglobulin

IgG – Immunoglobulin G

IgM – Immunoglobulin M

KLH – Keyhole Limpet Haemocyanin

Mab – monoclonal antibody

Min – Minute

mRNA – messenger Ribonucleic acid

PBS – Phosphate Buffered Saline

PC – Plasma Cell

PCR – Polymerase Chain Reaction

PE – Phycoerythrin

Poly A – Poly Adenosine

qPCR – quantitative Polymerase Chain Reaction

RNA – Ribonucleic acid

RPM – Revolutions per minute

SD – Standard Deviation

SHM – Somatic hypermutation

Th – T Helper Cell

PBMC – Peripheral Blood Mononuclear Cells

1. Introduction

Snake antivenom is currently the most effective treatment for snakebite envenomation worldwide. It is a therapeutic preparation, designed as an effective treatment for envenomation. Antivenom is composed of antibodies, which are proteins capable of neutralising the activity of snake venoms in the body and their toxic activity. There are however issues with the production of antivenom in its current state, which includes issues of cost, reproducibility, and effectiveness to target multiple species of snakes (1, 2). Furthermore, the immunology involved in generating neutralising snake venom antibodies, such as the degree of affinity maturation, recognition of epitopes and cross-reactive potential, is not well understood (3, 4). In order to understand how to optimise snake antivenom production, one needs to understand snake venom toxins, the underlying immunology of how the antibodies are generated and the structure of these antibodies.

1.1.1 Snake venom toxins

Snake venom glands contain a toxic mixture of proteins (including enzymes), peptides and ions. The venom is injected via hollow fangs into the tissue of the bitten organisms and it is then carried in the bloodstream (3-6). The toxic effects can be due to the venom interacting with blood (pro-coagulant or anti-coagulant), tissues or cellular components. The toxin interactions can cause many symptoms, including neuromuscular paralysis, haemorrhaging, necrosis, renal failure, hypotension and blood clotting (3, 4). The toxins in venom that cause these harmful effects exist in diverse forms, such as neurotoxins, proteases, phospholipase enzymes, C-type lectins, and others (3, 4). Additionally, the number of toxin groups/families present in venom varies from species to species, with some like Tiger Snake (*Notechis scutatus)* having 42 different toxins (7), while the beaked sea snake (*Enhydrina schistosus)* has a much simpler arsenal of 18 proteins (8). In the case of Australian terrestrial and sea snakes, some of the common toxins groups are neurotoxins and phospholipase enzymes (7-15).

Despite the diversity of snake species and therefore, the diversity of venoms, recent proteomic analysis of the venom compartments from disparate Australian snakes has revealed high degrees of evolutionary conservation of the classes of proteins from apparently unrelated snake groups (Table 1.1), as reported in a study by Dr. Bryan Fry (6). Furthermore, among the main clinically relevant snakes to which Seqirus Ltd (previously CSL and Commonwealth Serum Laboratories) develops antivenom (Table 1.2), there is a high degree of similarity between the different symptoms that occur with the particular snake species and the types of toxins present although the full proteomic (or venomic) profile of these snakes has not been fully

characterised (7-9, 11-13, 16-27). Nevertheless, this highlights a major point about snake venoms; that with Australian snakes, there is potentially a high degree of similarity between the toxins.

Species/Toxin	**CRiSP**	**fXaTx**	**fVaTx**	**Kunitz**	**Lectin**	**Natriuretic**	**PLA_2**	**SVMP**	**3FTx**	**Waprin**
Pilbara Death Adder *(Acanthophis wellsi)*	X			X	X	X	X	X	X	X
Northern Shovel-nosed Snake *(Brachyurophis roperi)*	X			X	X	X	X		X	
Golden-crowned snake *(Cacophis squamulosus)*	X			X	X	X	X		X	X
Mud Adder *(Denisonia devisi)*	X			X	X	X	X	X	X	X
Bardick *(Echiopsis curta)*	X			X	X	X		X	X	X
Orange-naped Snake *(Furina ornate)*	X			X	X				X	
Black-bellied Swamp Snake *(Hemiaspis signata)*	X	X		X	X	X	X	X	X	
Broad-headed Snake *(Hoplocephalus bungaroides)*	X	X		X	X	X	X		X	
Ringed Brown Snake *(Pseudonaja modesta)*	X		X	X	X	X	X		X	X
Rosen's Snake *(Suta fasciata*)	X	X		X	X	X	X	X	X	X
Bandy-Bandy *(Vermicella annulata)*	X			X	X			X	X	X

Table 1.1: Diversity of snake venom proteins found in distinct snake species. Adapted from reference (6). "X" denotes presence of toxin group.
fXaTx = factor Xa; fVaTx = factor Va; PLA2 = phospholipase A2; SVMP = snake venom metalloprotease; 3FTx = three finger toxin; CriSP = Cysteine-rich secretory protein. Waprins, Kunitz, and Natriuretics are peptides. Lectins are carbohydrate binding proteins.

	Snake venom toxins					
Australian Snakes	Presynaptic Neurotoxin	Post-Synaptic Neurotoxin	Pro-coagulant	Anti-coagulant	Phospholipase A2	Myotoxin
Brown Snake (*Pseudonaja textilis*)	X		X			
King Brown (*Pseudechis australis*)	X			X	X	?
Death Adder (*Acanthophis antarcticus*)		x				
Taipan (*Oxyuranus scutellatus*)	x	x	x		X	x
Tiger Snake (*Notechis scutatus*)	x	x	x			x
Beaked Sea Snake *(Enhydrina Shistosus)*		x			x	
Spine Bellied Sea snake *(Hydrophis curtus)*		X			x	

Table 1.2: Australian snakes and the toxins known to be present based on clinical symptoms and/or proteomic analysis. "X" denotes known presence of the particular toxin, "?" denotes uncertain presence of toxin type.

1.1.2 Immunity to snake venom toxins

When venom is injected into a horse, there is an initial innate immune response followed by the stimulation of the adaptive immune system (3, 4). Venom antigens are carried as native protein or acquired by dendritic cells (DC), the major antigen presenting cells (APC), to secondary lymphoid organs where venom is presented to antigen specific CD4 T cells as processed peptides bound on MHC Class II on DCs. This priming stimulates both migration of the CD4 T cells to the T cell–B cell border, and establishes the cytokine profile of the subsequent response. Antigen specific B cells that bind venom antigen through their B cell receptor (BCR), also move to the borders of the T cell areas of secondary lymphoid organs where their chances of engaging their cognate, activated antigen-specific T cells is significantly improved. Such an interaction will stimulate the CD40 receptor on the B cells in the presence of secreted cytokines. This promotes the extra-follicular proliferation of the responding B cell clones, which in turn leads to three outcomes: early B cell memory; extra-follicular foci of antibody secreting cells (ASC) and the Germinal Centre (GC) reaction, within which proliferation, somatic hypermutation (SHM) and affinity maturation of the activated B cells occurs and clones are induced to differentiate towards the memory B cell or plasma cell fates (3, 4, 28-33).

After undergoing rounds of activation and affinity selection within the GC, the B cells express high affinity BCR. ASCs derived from these B cells secrete the high affinity antibodies into the circulation and this begins to neutralise and clear the venom proteins. This occurs by binding to enzyme active sites, by sequestering them away from potential toxin target sites, and by promoting toxin clearance from the body (34).

However not every component of venom is targeted by the polyclonal antibodies elicited by immunisation. Some toxins such as the short neurotoxins in Beaked Sea Snake are poorly immunogenic. This means that the capacity of antivenom to neutralise such toxins is poor (8). Moreover, some venom components have little clinical effect e.g. brown snake neurotoxin has little potency in humans so neurotoxicity is not observed in brown snake bites (35).

1.1.3 Antibodies and cross reactivity to snake venoms

Although the composition of snake venoms vary between species (6), it has been observed that antivenom developed against one species of snake can react to the venom of a different species, a phenomenon referred to as cross-reactivity (4, 9, 16, 34, 36-39). This phenomenon was observed by Calmette in his original antivenom research (40). Cross reactivity arises because of the antigenic similarities in venom toxins that an antibody can recognise. For example, Isbister *et al*. demonstrated that rabbit antibodies

against brown snake venom were able to cross-neutralise the pro-coagulant effect of taipan venom equally as well as brown snake venom (18). Additionally, Kornhauser *et al.* demonstrated cross-neutralisation by Tiger Snake antivenom of the neurotoxic effect of a geographically distant Egyptian cobra (37). A study by Wagstaff *et al.,* utilised DNA immunisation to develop cross-neutralising antibodies for the snake venom metalloproteinases of two African vipers (41). While these and other studies provide evidence for venom immunisation producing cross reactive antibodies to multiple species of snakes, the specificity of this cross-neutralisation is difficult to define using polyclonal antibodies. Additionally, the antibodies that are useful/necessary to neutralise the toxicity of the venom is not known when using polyclonal antibodies. Furthermore, many of the antivenoms are polyvalent/polyclonal, and as such, it is difficult to deduce cross reactivity. Deciphering these cross reactivities with monoclonal antibodies may lead to a better understanding of the antivenom response, and to a better designed antivenom.

1.2 Immunology and snake venom

Despite long periods of development and use, there remain aspects of antivenom that are poorly understood. For example, it is not understood which toxins are the key targets for neutralisation, which toxins are immunogenic and how much antibody is needed to neutralise a given snake venom. These sorts of questions cannot be easily answered with current antivenom, since they are composed of antibodies from many B cells of varying specificities and affinities. However, these questions could be addressed by probing the individual B cell immune response to the snake venom toxins.

1.2.1 Adaptive immune system

The mammalian immune system is composed of adaptive and innate arms (42), the former to be discussed here in greater detail. Within the adaptive arm of the immune system are two major immune cell types, B lymphocytes (B cells), and T lymphocytes (T cells). These two cell types are distinguished phenotypically and functionally from other immune cells by the presence of unique antigen receptors that are capable of recognising a unique antigen and after initial encounter, by their ability to mount an improved response upon rechallenge by the same or a highly related antigen (42, 43). This improved response upon antigen re-exposure is referred to as the memory response, a key hallmark of adaptive immunity (28, 43).

T cells, which mature in the thymus, are broadly demarcated as either CD8 T cells or CD4 T cells, both of which take part in the cellular or cell-mediated adaptive immune response (44, 45). CD8 T cells are cytotoxic cells that are primed to eliminate virus infected host cells or tumours (45). In contrast, CD4 T cells comprise T regulatory (Treg) cells that act to maintain tolerance in the body (46, 47), and T helper cells (Th

cells), which act to help other immune cells in their activation, proliferation or secretion of molecules, thereby tailoring immune responses to meet specific immune challenges (44).

B cells develop in the bone marrow, while T cells develop in the thymus from hematopoietic progenitor cells that originated in the bone marrow (48, 49). B cells are distinguished from T cells by, among other things, their capacity to produce and secrete antibodies. Within secondary lymphoid organs, naive B cells are primed by binding their specific antigen through their BCR, after which they migrate within the lymphoid organ, such as the spleen, to the border of the B and T cell areas, to maximise the possibility of an encounter with CD4 T cells that recognise a peptide derived from the same protein antigen. The CD4 T cells are primed by exposure to DC within secondary lymphoid organs such as spleen or lymph node. After initial contact with the T cells and a brief period of extra follicular proliferation and differentiation that gives rise to GC independent memory and extra-follicular foci of short-lived ASCs, some of the expanded B cell clones accumulate within the follicle to form a GC, a site of extensive B-cell proliferation, mutation, death and differentiation (Figure 1.1) (43, 50). Within the GC, B cells undergo processes that modify their immunoglobulin genes, including SHM (50, 51). In SHM, the immunoglobulin (Ig) V genes are subject to high rates of mutation, which modifies the binding affinity of the resulting BCR. This diversification of BCR affinity permits competition between the many B cells within the GC, to receive stimulation from the antigens localised on the surface of follicular dendritic cells (FDCs), located in the GC (43). The GC is composed of two zones, called the Dark zone and the Light zone (50, 52). Within the Dark zone are rapidly proliferating B cells experiencing SHM, whereas the light zone contains B cells that are being selected for either re-entry into the dark zone, or exit out of the GC as a memory B cell, Plasma cell, or to commit to apoptosis (50, 52). B cells that have antibodies of low affinity are likely to progress towards a memory B cell pathway (28, 43, 50), while B cells with higher affinity antibodies are more likely to enter the ASC pathway (50, 52). The B cells of higher affinity preferentially survive the GC reaction, and through this mechanism drive affinity maturation (53). Antigen-activated B cells may additionally undergo class-switch recombination (CSR), which causes the B cell to produce different classes of antibodies, specifically switching from IgM, to IgE, IgA or IgG, which have distinct effector functions and thus distinct roles in the immune system (54). Most class switching is completed in the extra-follicular phase of the response, prior to GC formation (50). Throughout the GC reaction, progeny of the initial naïve B cell differentiate into one of two B cell fates, the antibody secreting Plasma Cell, or Memory B cells (50, 52) such that by the end of the reaction, both compartments are populated by significant numbers of cells. Current models suggest memory is produced first (55), potentially explaining its mixed affinity, while plasma cells are produced later in the response, again potentially explaining their high affinity (56). The former produces and secretes antibody, while the latter acts as the memory aspect of adaptive immunity. Plasma cells sequester into bone marrow niches, where they continuously produce antibodies for long periods of time (33). The memory B cells circulate within the body, and are capable of reactivation upon re-encountering the same

antigenic challenge (28, 50). Memory B cells that are re-challenged can differentiate into ASC, which can clear the antigen faster than naïve B cells, by virtue of their rapid production and secretion of antibody (28). The antibodies derived from the re-activated memory B cell are also usually of higher affinity than their naïve B cell counterparts (28, 50).

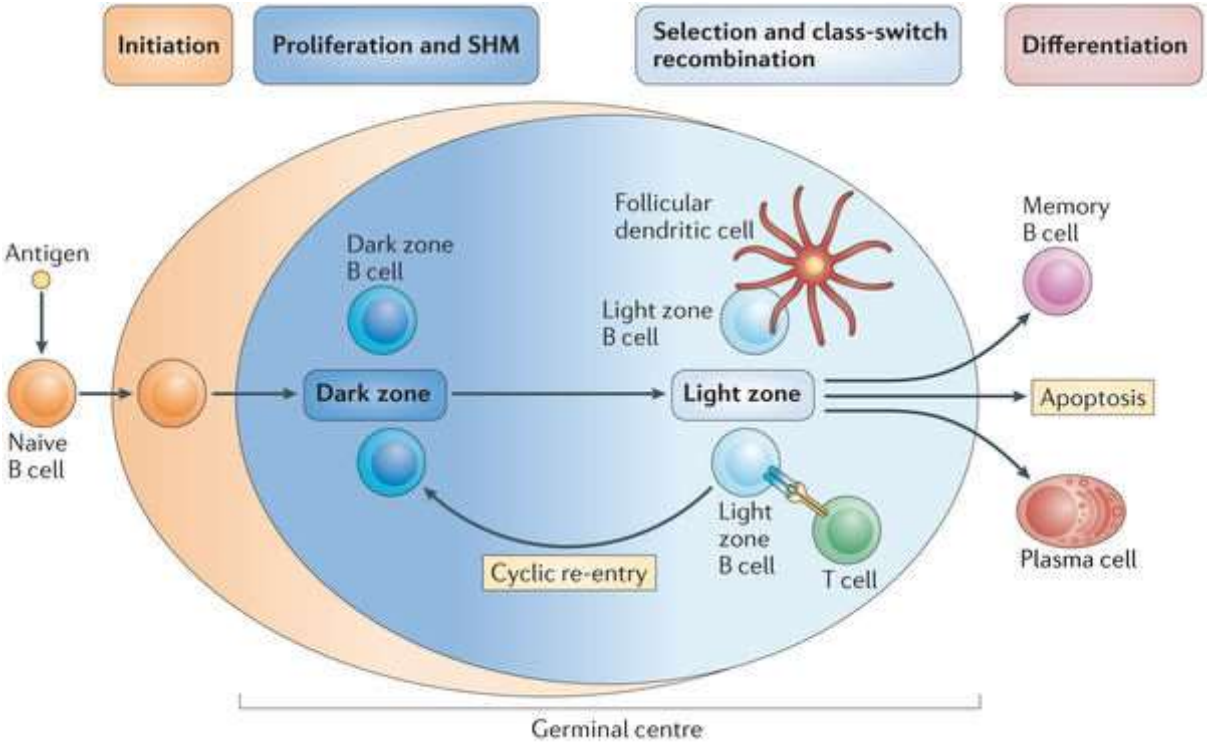

Figure 1.1: Germinal centre reaction driving differentiation of a naïve B cell towards a memory B cell or antibody secreting plasma cell fate. Following initial contact with antigen, the naïve B cell enters a GC and begins a cyclic process of proliferation within the dark zone, and selection within the light zone. From the light zone, the activated B cell can exit the GC as a differentiated memory B cell or an antibody secreting plasma cell. Obtained from (50)

1.2.2 Antibody structure

An understanding of antibody formation through genetic recombination events assists in comprehending the development of antivenoms and the complexity of the response via the generation of antibody affinity and isotypes.

As mentioned above, B cells have a unique, membrane-bound antigen specific receptor, the BCR (Figure 1.2)(57). An alternatively spliced form of the B cell receptor is secreted by plasma cells as immunoglobulin. Immunoglobulin is composed of two proteins called the Heavy chain and the Light chain (54, 58). Each

chain has a variable region and a constant region. The variable regions binds antigen and the constant region of the heavy chain determines effector function e.g. to activate complement, mediate antibody dependent cytotoxicity, enter tissues, cross membranes, bind Fc receptors (54). There are two isotypes of light chain, lambda and kappa, which are expressed in different ratios in different species e.g. for horses, ~90% of B cells express lambda (58-63).

Each immunoglobulin heavy chain constant region is encoded by a unique set of exons, distributed along the length of the chromosome, starting with those encoding IgM and IgD as being V region proximal and then typically IgGs and finishing with IgE and IgA. In the constant region of IgG, there are three domains, termed CH1, CH2 and CH3, which form the basis of the constant region (54, 57, 64). Horses have up to ten immunoglobulin isotypes (61). Within the horse, the IgG antibody class is further divided, based on sequence homology into seven subclasses, IgG1-7, which are found in different distributions in circulation, and vary in their specific roles (61, 65). As a general example, IgG1, IgG3, IgG4 and IgG7, were found to have a strong capacity for complement activation (58). In the case of snake venom, a study by Fernandes *et al.* found that IgG3 and IgG5 had the greatest capacity for neutralising the lethal activity of their tested South American snake venoms, in assays involving envenomated mice and administration of purified IgG3 and IgG5 (66). In the light chain, one exon forms the constant region, and there are seven lambda constant region subclasses.

To form a unique binding region for Heavy chain, VDJ segment recombination occurs early in B cell development. In this process the variable region assembles from the genetic recombination of individual elements selected from a large number of germline gene segments comprising Variable (V), Diversity (D) and Joining (J) gene segments. The light chain, however, forms from V and J gene segments only. With the heavy chain, a random D segment joins with a random J segment, followed by a random V segment joining the DJ element. In the case of the light chain, a random V and J segment are joined together. V(D)J recombination is mediated by the action of two enzymes, called recombination-activating genes (RAGs) 1 and 2, during B cell development in the bone marrow (54, 64).

While there are 52 V, 40 D and 8 J segments for heavy chain and 144 V and 7 J segments for lambda in the horse, the functional V genes are sorted into families or subgroups of V genes. The families are organised on the basis of having at least 75% nucleotide identity (58-60, 67) . Using this criterion, there are seven heavy chain V gene families (58-60). With the lambda light chain, there are at least nine functional families expressed in an adult horse, with each of the 7 J segments paired with one of the 7 lambda constant regions, forming an IGLJ-IGLC cluster (58, 60, 67). There are some V genes segments positioned after the IGLJ-IGLC cluster, which are found in an opposite transcriptional orientation to the V genes upstream of the IGLJ-IGLC cluster (58, 60).

In the process to form complete V gene segments, further sequence diversity is achieved at the V(D)J junctions via exonuclease digestion during recombination. Within the variable portion of the antibody, there are three regions of high diversity termed the complementarity-determining regions (CDR1, CDR2, CDR3) (54, 57, 68). CDR1 and CDR2 are encoded in the V regions themselves while CDR3 spans the region of V(D)J and VJ recombination. These three CDRs form the basis of unique antigen binding of the antibody. In between the CDRs are the framework regions of the antibody (FR1, FR2, FR3, FR4), that are more conserved and provide the overall structure of the VH and VL domains. In addition to rearrangement and base additions generated during development, non-templated nucleotide bases can become included by the action of Activation-induced cytidine deaminase (AID), an important enzyme for SHM and CSR during the GC reaction (54, 64), giving further diversity to the antibody response during the GC reaction (69). These changes are typically selected to occur in the CDR, consistent with these regions binding antigen.

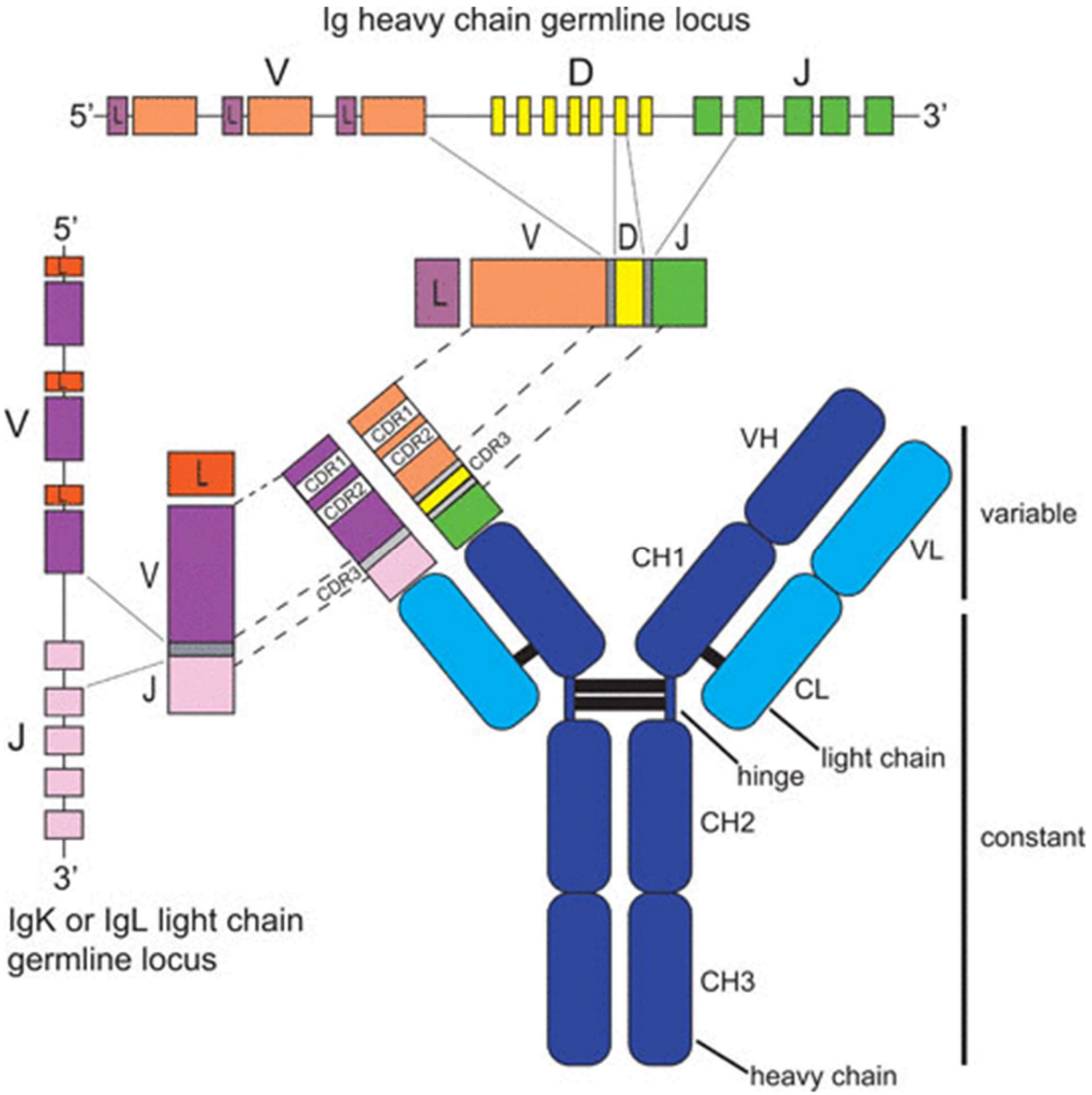

Figure 1.2: Structure of a prototypical human IgG antibody. The heavy chain V, D, and J segments are located above as in the germline, with the V and J segments of the light chain germline located on the left. Following V(D)J recombination, the variable region of the antibody is formed as variable heavy (VH) and variable light (VL) chains. The highly variable complementarity determining regions (CDR) are located within the variable regions. The heavy and light variable regions are joined to their respective constant regions. Black lines denote disulfide bridges joining the protein regions. Obtained from (57)

1.3. Snake antivenom production and epidemiology of snakebite

The development of an effective treatment to snakebite envenomation began over a century ago, commonly attributed to research undertaken by Alberte Calmette (40, 70) with other researchers such as Cesaire Phisalix and Gabriel Bertrand making their own independent discoveries (70). The antivenom that the French researchers developed was a breakthrough in snakebite treatment, created in a time of great immunological and microbiological research, most notably the diphtheria antitoxin work of Emil von Behring and Kitasato Shibasaburo (70). Although unknown to Calmette at the time, it was the presence of high affinity IgG antibodies, which gave antivenom its neutralising effect on snake venom (40). From this initial work, an effective treatment for snakebite emerged.

1.3.1 Antivenom production

The process for generating snake antivenom largely remains the same as for the original Parisian workers (71), and current manufacturing practices are based on this antivenom work (3, 34, 71-73). This process begins when large animals, typically horses, are injected with initially small doses of snake venom to trigger an immune response. The animals are then subject to increasing doses of snake venom over time, to achieve high titre of antivenom, in accordance to manufacturer's immunisation programme (2, 71).

The blood is collected from the immunised animals and then fractionated to separate antibodies from the blood plasma, such as by Ammonium sulfate precipitation or Caprylic acid precipitation (71). Specifically, the IgG fraction of antibody is collected, because this subtype is found to be the most efficient at neutralising snake venom toxins (66). Additionally, IgG is found at a greater concentration than other subtypes within the horse (61), and the IgG response to snake venom is detectable years after the initial snake venom immunisation (3), consistent with plasma cell longevity and germinal centre origin.

The collected antibodies are subjected to venom-neutralising tests to determine potency in terms of the efficacy of the collected antibodies in neutralising the snake venom toxins. Sequential bleeding and testing of serum continues until sufficient potency has been achieved, then the antivenom is formulated to a

specific form, as either whole IgG or antibody fragments (Fab or $F(ab')_2$) (34) and then it is ready for distribution and available when required for patient administration (34, 71, 73). Antivenom developed for a single species of snake is referred to as monovalent, whereas antivenom developed for multiple species is called polyvalent. At Seqirus Ltd, where antivenom in Australia is made, all horses used for antivenom production are injected with snake venom from two or five species of snakes. Monovalent antivenom from Seqirus Ltd is defined as the highest titre of antibody toward a particular snake species, as many of these monovalent preparations from Seqirus contain reactivity to other snake toxins due to previous/multiple envenomations of the same animal.

Domestic horses (*Equus caballus)* are the most commonly used animal for snake antivenom production for several reasons (71, 73). They are domesticated and docile (especially draft breeds), yield high volumes of blood, and are adept at a wide range of environments. Moreover, horse antibodies have been used in humans for over a century and found to be efficacious and safe for human and veterinary use (12, 18). Nevertheless, there is some risk of allergic reaction to horse immunoglobulins in xenogeneic hosts (71, 73, 74).

Although the current method of antivenom production has the advantage of being a relatively straightforward process, there are some disadvantages to the current method, from a scientific perspective (73). Specifically, immunising horses with snake venom generates a heterogeneous antibody profile that results in large batch to batch variation of the resulting antivenom (3, 34, 73, 75), which occurs even when using venom from the same species of snake (3). There are even cases where the antivenom titre has been inadequate (75). The high variation of antivenom batches, the expense and time needed to make them (34, 71) and frequent shortage of supply, especially in developing countries (2, 76-78), foreshadow a need for a more rationally designed antivenom.

1.3.2 Epidemiology of snakebite envenomation

While the creation of snake antivenom over a 100 years ago certainly saved countless lives, both people and animals (72), there are still deaths associated with snake bite. A 2008 report estimated between 400,000 to 2,000,000 snake bites and 20,000 to 90,000 attributed deaths per year, globally (79). In Australia, the number of deaths from snake bites is <10/year from 1400 bites (80). However, other consequences, such as economic or physical disability, have not been detailed (79), perhaps underestimating the problems to people and to the health care system that result from snake bites. Since the majority of snake bites and deaths occur in tropical and subtropical regions of the world (2, 71, 79), many experts argue snake bites are a neglected tropical disease that deserves more attention than it currently receives (1, 71, 77, 79, 81).

1.3.3 Monoclonal antibody development

Antibodies that target a defined antigenic epitope and are derived from single, antigen specific B cells are called monoclonal antibodies (82). A panel of such unique and defined antibodies specific for venom would enable a greater understanding of the phenomenon of cross reactivity and the B-cell response. With monoclonal antibodies, one could determine which toxic proteins are the most relevant to target, which toxins are immunogenic, and enable greater characterisation of the venom arsenal. Furthermore, monoclonal antibodies would allow insight into the cross reactivity phenomenon and to the potential of cross neutralisation across snake species, which polyclonal antibodies could never elucidate (3, 4, 34). Monoclonal antibodies may also lead the development of antivenom that is consistently produced and reduces batch to batch variability (71). Research by Frauches *et al.,* (83) highlights the utility in using monoclonal antibodies to treat snake envenomation. Frauches *et al.,* developed three monoclonal antibodies to some venom components of the *Bothrops atrox* snake, a common cause of snake bite injury in Brazil (83). The three monoclonal antibodies were evaluated for their capacity to neutralise the activity of 3 major components of *B atrax* venom. Frauches *et al.,* observed that the three monoclonal antibodies neutralised the lethal activity of their respective targets, but also observed that mice treated with the monoclonal antibodies survived envenomation. This study indicated that it is possible for a discrete number of monoclonal antibodies to provide protection against the envenomation of a particular snake. While the study did not investigate whether the antibodies were cross reactive with other species of snake, the researchers acknowledge this, with the need for further examination of cross examination of the antibodies to other species.

Monoclonal antibodies can be developed by a number of methods, each having advantages and disadvantages (84). The method that will be the focus of this thesis is single cell cloning expression, a technique that uses reverse transcriptase polymerase chain reaction (RT-PCR) to isolate the unique immunoglobulin heavy and light chain genes from antigen specific B cells (84). These extracted heavy and light chains are then cloned and expressed from DNA vectors that produce recombinant antibodies, originating from the single, antigen specific B cells. This methodology has been widely used to study cross reactive antibodies in research involving HIV by Nussenzweig (85-89), and influenza by Lanzavecchia (90-93) and Wilson (94-99) among many others. These studies demonstrated a capacity to identify and isolate broadly neutralising antibodies to specific antigens using antigen specific B cells. These monoclonal antibodies were also informative in determining the molecular basis of the cross neutralisation and could inform the design of better antibody based therapeutics in their respective diseases.

1.4 Research Goal

1.4.1 Hypothesis and aims.

I hypothesise that there are B cell epitopes present on the venoms from multiple species of snakes, to which monoclonal antibodies can be raised that will cross-react and cross-protect. I further propose that such antibodies already exist within the population of antibodies generated in horses by venom immunization and can be recovered as clonal entities by standard molecular and immunological techniques.

To investigate this hypothesis, three aims will be pursued. The first aim is to isolate a venom specific memory B cell from the blood of horses immunised with snake venom. The second aim is to develop a system of activating and inducing proliferation of such specific memory B cells. The third and final aim is to develop a system for extracting the unique antibody genes from individual B cells, to produce and then test these antibodies for their snake venom cross-reactivity and neutralisation capability.

2. Methods and Materials

2.1 Media

Media were purchased from Thermo Fisher Scientific unless otherwise specified.

CHO cell culture for recombinant antibody expression: **DMEM/F-12** (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) was combined with 10% v/v foetal calf serum (FCS), and 100 µM of penicillin/streptomycin.

Phosphate Buffered Saline (PBS): was composed of Potassium Phosphate monobasic (KH_2PO_4) at 1 mM, Sodium Chloride (NaCl) at 155 mM, and Sodium Phosphate dibasic (Na_2HPO_4-$7H_2O$) at 3 mM.

PBS/FCS: PBS was supplemented with 2% v/v FCS and used for flow cytometry and handling/washing cells prior to cell culture.

B cell culture media: RPMI 1640 (Roswell Park Memorial Institute 1640) glutaMAX™ was combined with 10% v/v FCS, 100 µM β-mercaptoethanol (2-ME) and 100 U/mL and 100 µg/mL of penicillin/streptomycin.

TAE buffer diluted with Milli Q H_2O from a 50X stock to a 1x working stock and stored at room temperature.

2.2 Solutions and supplements:

2'2-Azinobis (3-ethylbenzthiazoline Sulfonic Acid) Diammonium salt (ABTS) was purchased from Sigma-Aldrich and prepared and stored at 4°C protected from light as a 50x stock solution by dissolving 10g in 365mL of distilled water to make a 27 mg/mL solution. ABTS was used in ELISAs at 0.54 mg/mL, diluted in 0.01M citric acid with freshly added 0.1% v/v H_20_2.

3-amino-9-ethyl carbazole (AEC) was purchased from Sigma-Aldrich and prepared as a stock solution of 250 mg/mL in 0.05 M sodium acetate buffer, pH 5. The stock AEC was stored at 4°C and protected from light. AEC was used in ELISPOTs, filtered through a 0.45 µm membrane prior to use. After filtration, H_2O_2 was added to be 0.1% v/v.

B-mercaptoethanol (2-ME) was purchased from Sigma-Aldrich and diluted to 100 mM in HEPES Eagle's Medium (HEM). HEM was purchased from the Walter and Eliza Hall Institute (Parkville, Melbourne, Australia). For cell culture experiments, 2-ME was diluted in RPMI 1640, to a final concentration of 100 µM.

Block buffer was composed of PBS with 0.6% w/v skim milk powder, 1% v/v FCS and 0.05% v/v tween-20 (Sigma Aldrich). The solution was used for both ELISA and ELISPOT experiments. Block buffer was stored at 4°C and used within one month of preparation.

Citric Acid Buffer was assembled to a 1 M concentration by dissolving 52.5 g citric acid and 75 g tri-sodium citrate.$2H_20$ in 500 mL distilled water. The pH was adjusted to 4.4 and filter sterilised (0.2 µm). The 10x stock solution was diluted in water to 0.1 M and used to prepare ABTS preparation.

Foetal Calf Serum (FCS) was supplied by Gibco. The FCS was heat-inactivated in a 56°C water bath for 35 minutes, aliquoted and stored at -20°C, with aliquots tested for sterility by 37°C culture for one week. Batches in which FCS remained transparent, not cloudy or opaque after 4 and 7 days were considered suitable for culture work.

Freezing media was composed of 10% v/v dimethyl sulphoxide (DMSO), with 90% v/v FCS and used for long term liquid nitrogen storage of horse peripheral blood mononuclear cells (PBMC).

Lysis buffer used to extract RNA from FACS sorted single horse PBMC was composed of 1 µl RNase inhibitor 20-40 U/µL (Promega), 2 µL PBS, 0.4 µL 100 mM dithiothreitol (DTT), 0.1 µL 3 µg/µL Random Hexamers (Invitrogen), 1 µL 10% v/v NP40 (Sigma Aldrich) and 6.5 µL Diethyl pyrocarbonate (DEPC)-treated water.

Propidium Iodide (PI) was assembled as a 100x stock at 100 µg/mL in PBS, shielded from light and stored at 4°C. PI was used at a final concentration of 1 µg/mL to exclude dead cells in FACS analysis and sorting.

Sytox Blue was purchased from ThermoFisher and stored in freezer (-5 to -30°C), protected from light, either dessicated or resuspended at 1 mM in DMSO. Sytox blue was used at a final concentration of 0.5 mM to exclude dead cells in FACS analysis and sorting.

Antibodies

Table 2.1 Antibodies used for Flow cytometry, ELISA and ELISPOT.

Specificity	Clone name	Conjugate	Source	Application
Human CD21*	Bly-4	APC	BD Pharmingen	FACS
Equine CD4	CVS4	PE	Bio-Rad	FACS
Equine CD8	CVS8	PE	Bio-Rad	FACS
Equine IgG	Polyclonal	Unlabelled	Seracare (prior KPL)	ELISA, ELISPOT
Equine IgG	Polyclonal	HRP	Seracare (prior KPL)	ELISA, ELISPOT
Rat IgG	Polyclonal	Unlabelled	Southern Biotech	ELISA**
Rat IgG	Polyclonal	HRP	Southern Biotech	ELISA**

*Cross reactive to horse CD21 (100)

** For chimeric recombinant horse antibody detection

Table 2.2 Additional antibodies tested for cross reactivity to horse surface markers in flow cytometry

Specificity	**Clone name**	**Conjugate**	**Source**
Mouse CD19	ID3	Ef450	eBioscience
Mouse CD21	7G6	APC	BD pharminigen
Mouse CD23	B3B4	A680	WEHI monoclonal facility
Mouse CD38	NIMR5	A680	WEHI monoclonal facility
Mouse IgD	1126C	A680	WEHI monoclonal facility
Mouse IgM	331.12	A680	WEHI monoclonal facility
Human CD19	SJ25C1	PeCy7	BD
Human CD20	2H7	Bv605	BD
Human CD21	Bu32	APC	Biolegend
Human CD21	HB5	PE	Miltenyi
Human CD38	HI12	APC-Cy7	Biolegend
Human CD38	HB7	Bv605	BD
Human CD40	5C2	PerCpCy5.5	Biolegend
Human CD275	2D3	APC	Australian Biosearch
Human IgD	IA6-2	Percp	Australian Biosearch

2.3 Methods

2.3.1 Preparation of Horse PBMC via Sepmate™/Lymphoprep™

PBMC were isolated from heparinised blood collected from horses previously immunized as part of CSL antivenom production. Approximately 120 mL of blood was pooled from seven horses, seven days after their last immunisation with venom from multiple snakes. A sample of 4 mL of plasma was collected for antibody analysis following a centrifugation of 10 mL of whole blood. To separate the PBMC from red blood cells and dense granulocytes, Sepmate™ (STEM CELL Technologies) tubes were used. These tubes have a central barrier, where red blood cells and granulocytes pellet through to the bottom of the tube during centrifugation while PBMCs remain above the insert. Sepmate™ prevents the layers from mixing as the centrifuge slows down and keeps the sample separated from the density gradient medium. First, 15 mL of Lymphoprep™ (STEM CELL Technologies) density gradient solution was aliquoted per Sepmate™ tube through the central hole in the insert following the manufacturer's instructions. The pooled blood was mixed with equal volume of PBS/FCS. Following mixing of blood with PBS/FCS, with the Sepmate tube was kept upright and 25 mL of blood was quickly pipetted down the side of the tube. These steps were done at room temperature (RT). Next, samples were centrifuged at 1200 x g for 10 min at RT with the brake set on. After centrifugation, PBMC were harvested by decanting the solution above the insert into a new 50 mL tube in one smooth motion to limit red blood cell and granulocyte escape.

Finally, recovered cells were washed with PBS/FCS twice, via centrifugation at RT for 5 minutes at 2000 rpm, then resuspended in 25 mL PBS/FCS and counted via haemocytometer. Following counting, PBMC were resuspended at ~5-10x10^6 /mL in freezing media for long term storage in liquid nitrogen in cryovials in 1 mL aliquots. PBMC were thawed by warming the cryovial in a waterbath set at 37°C, followed by quickly aliquoting the PBMC into a 10 mL tube containing B cell culture media, that was prewarmed in a waterbath set at 37°C.

2.3.2 Enzyme-Linked ImmunoSpot (ELISPOT)

The ELISPOT assay for detecting IgG antibody secreting B cells was performed using goat anti-horse IgG capture and anti-horse IgG-Horseradish peroxidase (HRP) antibodies. The plates used for ELISPOT were Millipore MultiScreen - HA sterile plates, containing a 0.45 µM, surfactant-free, mixed cellulose ester membrane. Plate coating occurred using 100 µL of sterile PBS with goat anti-horse IgG at a concentration of 2 µg/mL, with an incubation of either four hours at RT, or overnight at 4°C. After incubation, the plate was sterile washed 3 times with PBS prior to addition of cells. ELISPOTs were used to detect the presence of ASC from freshly thawed PBMC, which were flow cytometrically sorted based on CD4/CD8 and CD21 expression. Cells were incubated at 37°C overnight for no more than 20 hours in fresh B cell media. After incubation, the ELISPOT plate was washed as follows: washed 3 times in 3 separate containers of PBS with 0.05% Tween 20, PBS, and distilled H_20, respectively. Secondary HRP-conjugated goat anti-horse IgG antibody was added in 100 µl block solution at 0.2 mg/mL. The plate was incubated for four hours at RT, and then washed as before. For colour development, AEC substrate was added (100 µL/well), and the plate was allowed to develop for 15-30 minutes. After colour development occurred, substrate was flicked off, and plate extensively washed with water, with the plastic backing removed for the washing step. Brown/red spots were counted using a dissecting microscope or automated ELISPOT reader.

2.3.3 Flow cytometry analysis and sorting of equine PBMC

PBMC were stained for flow cytometry using the following method unless otherwise indicated. After thawing PBMC, viable cells were counted via haemocytometer. A minimum of 100,000 cells were stained in V-bottom 96 well plates in a final volume of 50 µL of PBS/FCS, containing one or more fluorochrome antibodies (or none in the case of unstained controls), as well as the addition of live/dead marker, either PI or Sytox blue. Cells were stained for a minimum of 30 minutes at 4°C or RT for sorting of cells (100). After staining for 30 minutes, cells were washed with cold PBS/FCS and then resuspended in PBS/FCS and transferred to a Corning polystyrene FACS tube for analysis. Flow analysis was performed on Becton Dickinson (BD) Canto (II). Sorting was performed on either the BD INFLUX or ARIA (BD biosciences) with

assistance from the AMREP Flow Core Flow Cytometry facility staff. FACS data analysis was performed using Flowjo (TreeStar version 10) and statistical analysis performed using Graphpad Prism.

2.3.4 Enzyme linked immunosorbent assay (ELISA)

ELISAs for detecting horse IgG were performed using goat anti-horse IgG capture and anti-horse IgG HRP antibodies. Beginning with polystyrene round bottom, non-treated, sterile 96 well plates (Corning), coating of the wells was performed with 2 µg/mL of polyclonal goat anti-horse IgG antibody and incubated at 4 hours at RT (50 µL/well). After 4 hours, the plate was washed three times in three separate containers with solutions of, 1) PBS plus 0.1% tween 20; 2) PBS; and 3) dH_20. Plates were dried and samples to be tested added within one hour. Samples were added diluted 1:1 in a solution of blocking buffer. Blank controls were included to have only primary (and subsequent) secondary antibody coating. Antibody positive control, using total horse plasma (isolated during PBMC isolation procedure), was used starting at 1:1000 (assumed to be 20 mg/mL stock), and diluted across the plate with mid log dilutions. Plates were incubated at room temperature overnight. After incubation, plates were washed as before in the 3 solution containers outlined above. The secondary antibody of 0.2 µg/mL polyclonal goat anti-horse IgG HRP diluted in blocking buffer was added to each well for 4 hours of incubation at RT (50 µL/well). Following incubation of 4 hours and subsequent washing, plates were developed with the substrate ABTS in the dark for 30-45 minutes. Plates were read at 415 nm with reference wavelength of 492 nm on a Microplate reader. Data were stored as raw Excel files and analysed using Excel and/or Prism.

2.3.5 Quantifying IgG and IgM heavy chain mRNAs with qPCR.

A qPCR method for IgG and IgM mRNA quantification was developed, using primers for IgG and IgM heavy chain, and published equine actin primers (101, 102), as shown in Table 2.3. The qPCR was performed using a QuantiNova SYBR Green PCR Kit (Qiagen). The qPCR master mix was a 10 µL reaction comprising forward and reverse primers of 0.5 µL at 10 µM each, SYBR master mix at 5 µL, ROX buffer at 0.1 µL, H_20 at 2.9 µL, and cDNA at 1 µL. To ensure reproducibility of triplicates, SYBR mastermix, ROX buffer and cDNA were mixed and aliquoted into 384 well plates, followed by addition of primers. The plate was sealed with adhesive film, then inserted into a Quantstudio qPCR machine. Amounts of IgG and IgM heavy chain mRNA were normalised to equine actin mRNA.

Table 2.3 QPCR primers for Immunoglobulin IgG and IgM heavy chain

QPCR	Primer	Primer Sequence	Target
IgG	QPCR IgG forward	5' AAGGAGTTCAAGTGTAAGGTCAACAAC	IgG constant region CH2
	QPCR IgG reverse	3' GTGTGGGGCCAGGACGTA	IgG constant region CH3
IgM	QPCR IgM forward	5' CTGTGGGCCCTCTCTTGATG	IgM constant region CH1
	QPCR IgM reverse	3' AGACGGTCACATTTGGGGAC	IgM constant region CH2
Actin	Equine actin forward	5' CCAGCACGATGAAGATCAAG	Equine actin Exon 4
	Equine Actin reverse	3' GTGGACAATGAGGCCAGAAT	Equine Actin exon 5

2.3.6 Single cell sorts, RNA extraction and first strand cDNA synthesis

Single cells that were $CD21^+$ $CD4/CD8^-$ were sorted on a FACS Aria machine within the AMREPFlow core flow cytometry facility. Single cells were sorted directly into a 96 well plate, containing lysis buffer, centrifuged briefly, and stored at -80°C until processed further. For first strand cDNA synthesis, cells were brought to 65°C for 2 minutes, then to 10°C for 5 minutes using a PCR thermocycler (Biorad). cDNA was synthesised by the addition of a reverse transcriptase mix (14 µL) to each well, which contained the following components: 5 µL of 5x reverse transcriptase buffer, 1 µL of dNTPs 25 mM, 2 µL DTT 100 mM, 5.25 µL DEPC water, 0.25 µL RNase inhibitor rRNasin® 40 U/µL (Promega) and 0.5 µL reverse transcriptase Superscript II 200 U/µL (Invitrogen). A thermal program was run at 22°C for 10 minutes, 42°C for 50 minutes, 70°C for 15 minutes and 12°C holding temperature. The resulting cDNA (25 µL/well) was stored at -20°C.

Table 2.4 Nested PCR primers for equine immunoglobulin heavy chain (IgG). Restriction enzyme sites in **BOLD** and Kozak consensus underlined.

PCR	Primer	Primer Sequence	Target
1st Round	Heavy chain Leader	**5' TCTAGA**GCCACCATGARTCACCTGTGGTTCTTCC	Heavy chain leader
	IgG constant 1st round	3' TCCACCTTGRWGYTGCTGGC	IgG exon 1
2nd round	Heavy chain Leader	**5' TCTAGA**GCCACCATGARTCACCTGTGGTTCTTCC	Heavy chain leader
	IgG constant 2nd round	3' AAGTADYYRGAGACCAGGCAG	IgG exon nested

R=AG Y=CT M=AC K=GT S=GC W=AT
H=ACT B=GCT V=AGC D=AGT N=AGCT

Table 2.5 Nested PCR primers for equine immunoglobulin light chain (lambda). Restriction enzyme sites in **BOLD** and Kozak consensus underlined.

PCR	Primer	Primer Sequence	Target
1st Round	5' Light chain UTR	5' GCCACAGAAGGCAGGACTCGG	5' UTR
	3' light chain UTR	3' **ACGCGT**GGAGAAGGAGGTGTCGGAGT	3' Light chain UTR
2nd round	5' Light chain Leader	**5' GCTAGC**GCCACCATGGCCTGGWSHSBKCTYYYBY	5' light chain leader
	3' Light chain UTR	3' **ACGCGT**GGAGAAGGAGGTGTCGGAGT	3' Light chain UTR

R=AG Y=CT M=AC K=GT S=GC W=AT
H=ACT B=GCT V=AGC D=AGT N=AGCT

2.3.7 Single cell PCR amplification of equine heavy chain and light chain immunoglobulin

The PCR amplification of the antibody heavy and light chains from single cells was carried out in a two round, nested format in order to amplify product from low cDNA input. The first round PCR was performed using 2.5 µL of 25µL cDNA stock for separate heavy and light chain PCRs. The first round heavy chain PCR reaction mix contained 1 µL of each forward and reverse primer of 10 µM stock (table 2.4 for heavy chain), 10 µL of Redtaq master mix (Bioline), with addition of 1.25 mM of $MgCl_2$, followed by inclusion of nuclease free H_2O (Bioline) to a total reaction volume of 20 µL. The first round light chain PCR reaction mix contained 1 µL of each forward and reverse primer of 10 µM stock (table 2.5 for light chain), 10 µL of Redtaq master mix (Bioline) followed by inclusion of nuclease free H_2O to a total reaction volume of 20 µL. For the heavy chain first round reaction, the first cycle of the program was specified as 5 minutes at 95°C. Cycles 2-35 were comprised of 30 seconds at 95°C, 59°C and 72°C. The final cycle was 5 minutes at 72°C, with a holding cycle at 12°C. For the light chain first round reaction, the first cycle of the program was specified as 5 minutes at 95°C. Cycles 2-35 were comprised of 30 seconds at 95°C, 62°C and 72°C. The final cycle was 5 minutes at 72°C, with a holding cycle at 12°C. Second round was performed using 1 µL of first round PCR reaction as template. The PCR master mix for the second round for heavy chain and light chain PCRs was identical except for the use of the appropriate respective second round nested primers (table 2.4 and table 2.5). The PCR master mix for the second round for light chain was identical except for the use of second round nested light chain primers.

For the heavy chain second round reaction, the first cycle of the program was specified as 5 minutes at 95°C. Cycles 2-35 were comprised of 30 seconds at 95°C, 55°C and 72°C. The final cycle was 5 minutes at 72°C, with a holding cycle at 12°C. For the light chain second round reaction, the first cycle of the program was specified as 5 minutes at 95°C. Cycles 2-35 were comprised of 30 seconds at 95°C, 66°C and 72°C. The final cycle was 5 minutes at 72°C, with a holding cycle at 12°C. PCR products were analysed on 2% agarose gels made with TAE buffer (ThermoFisher Scientific). The gels were run at 120 V for 50-60 minutes and bands were visualised on a Chemidoc illuminator (Bioline).

2.3.8 Cloning of equine heavy chain and light chain immunoglobulin variable region genes

Heavy chain and light chain variable region gene cloning was performed using the TOPO cloning kit from Thermo Fisher Scientific. Following a heavy chain or light chain PCR, the products were run on a 2% agarose gel. Bands of correct size were isolated via gel extraction, using a QIAGEN gel extraction kit. A TOPO cloning reaction was 6 µL reaction mixture that comprised 1 µL of TOPO vector, 1µL of salt solution (from the kit),

fresh PCR product at 1-4 µL, and the remainder with water (from the kit). The TOPO cloning reaction was done at RT for at least 10 minutes after which it was aliquoted to chemically competent *E coli* for a 30 minutes incubation on ice. Following transformation, the bacteria were heat shocked for 30 seconds at 42^{o}C (heat block or water bath), then returned to ice. 200 µL of SOC medium (from TOPO cloning kit) was added to bacteria and the bacteria were incubated on a shaker for 30-45 minutes at 37^{o}C. Fresh ampicillin agar plates were prepared on the day, using imMedia™ Growth Medium that contained premixed agar and ampicillin (ThermoFisher). Following incubation on the shaker, the transformed bacteria were plated on the fresh-made ampicillin agar plates for overnight growth at 37^{o}C. After overnight growth, colonies were analysed via PCR to determine successful ligation of PCR product into TOPO vector, using M13 primers. Successful ligation was determined by the presence of a band of 750 base pairs (bp) for the heavy chain or 900bp for the light chain. Successful ligation colonies were selected for overnight growth in imMedia™ Growth Medium containing premixed ampicillin (ThermoFisher) for subsequent plasmid miniprep. Miniprep was performed following the manufacturer's guidelines of QIAGEN miniprep kit. Sequencing of plasmids were performed at Micromon Genomics facility at Monash, Clayton using M13 primers that flank the heavy chain and light chain inserts.

2.3.9 Recombinant antibody production using mammalian cell line

Recombinant antibody production was carried out following published methodology (103). After cloning into TOPO vector for sequencing, plasmids containing verified, expressible heavy or light chains were digested with restriction enzymes to release heavy and light chain fragments with appropriate "sticky" ends for cloning (Figure 2.1). TOPO plasmids containing the IgG heavy chain were digested with Xba1 and Xcm1, while TOPO plasmids containing light chain were digested with Nhe1 and Mlu1. All restriction enzymes were purchased from New England Biolabs (NEB). Expression vectors, kindly donated by Andrew Lew (WEHI), were digested with restriction enzymes to enable compatible end joining with the exception of the heavy chain expression vector digested with Nhe1 instead of Xba1, leaving a compatible end. Ligation reactions of expression vector and insert (heavy chain or light chain) were performed with T4 ligase (Promega) enzyme at 4^{o}C overnight or 3 hours at 24^{o}C. Once ligation was completed, competent *E. coli* were transformed with the ligated plasmids. The colonies that grew were picked for overnight growth in imMedia (ThermoFisher) at 37^{o}C. Plasmids were extracted using Qiagen miniprep extraction kit. For the horse heavy chain cloning, the ligated plasmid was digested with Xho1 and Xcm1 to reveal one band (horse insert) of approximately 1000bp plus vector. For light chain containing plasmids, enzyme digestion occurred with Nhe1 and Mlu1 restriction enzymes. Schematic for ligation is shown in Figure 2.1.

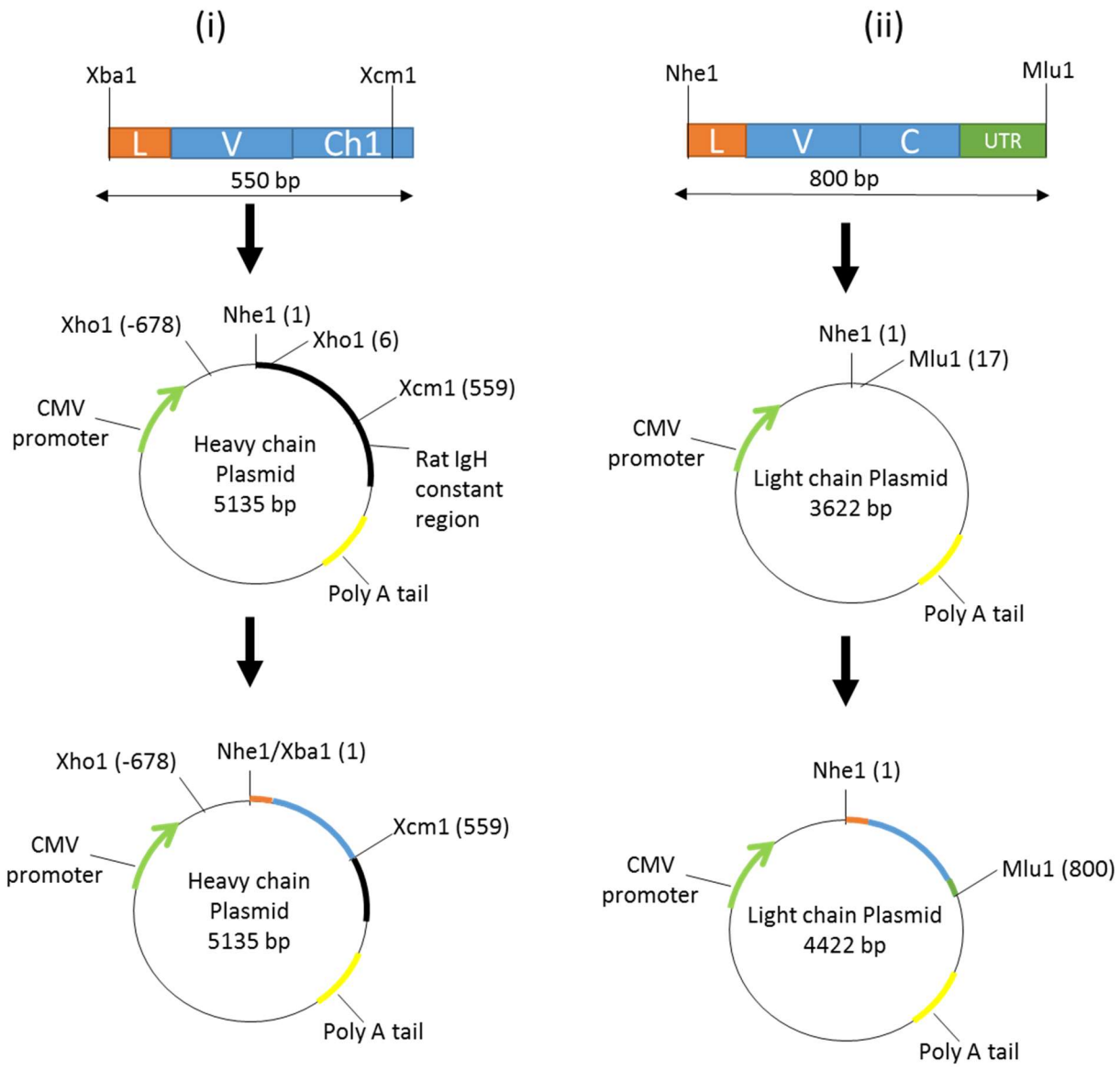

Figure 2.1: Schematic of ligation strategy for expressible plasmids. Heavy chain plasmid is enzyme digested with Nhe1 and Xcm1 to form compatible ends. This allows in-frame ligation of heavy chain insert (i). Light chain plasmid is enzyme digested with Nhe1 and Mlu1 to form compatible ends. This allows in-frame ligation of light chain insert (ii). Inserts were comprised of Leader (L), Variable region (V) and constant region (C), with the Light chain insert containing an Untranslated Region (UTR). Restriction enzyme cut sites are shown at ends of the inserts. Restriction enzyme cut sites on plasmids are shown with their relative position (in base pairs), to the Nhe1 site. Poly A tail and CytomegaloVirus (CMV) promoter is shown. Heavy chain plasmid contains rat IgH constant region.

Recombinant antibody production was performed using Chinese hamster ovary (CHO) cells, originally grown in DMEM/F12. The day before transfection, between 20,000 and 100,000 CHO cells were plated into a 6 well plate (Corning). The plates were set up in duplicate to have no DNA (negative control), GFP only

(positive control for transfection), or a GFP plasmid + Ig ligated plasmids (both heavy and light chain plasmids). Transfection was performed using Fugene 6 transfection reagent (Promega), following the manufacturer's instructions. The CHO cells were transfected overnight, and checked for successful transfection by the presence of GFP on a widefield fluorescent microscope. After 3-4 days of incubation, plates were centrifuged and supernatants collected for analysis using an ELISA, as outlined at 2.3.4, but modified to use a goat anti-rat IgG antibody as capture (1 µg/mL) and enzyme linked detection (0.1 µg/mL) reagents.

2.3.10 Equine B cell culture

Despite established protocols for B cell cultures in mouse and human, no such system is readily apparent in the literature for horse B cells. As such, I attempted to develop a B cell culture system for horses using the mitogenic factor Pokeweed Mitogen (PWM). PBMC were thawed, counted and then cultured in 96 well flat bottom plates (Corning) in B cell media, with or without 5 µg/mL PWM for up to 7 days. Cells and supernatants were analysed via Flow cytometry and ELISA, respectively at various time points. In stated experiments, equine IL-2, IL-4, IL-5, IL-6, IL-10 and/or CD40L (Kingfisher Biotech) were also added to the culture medium.

2.3.11 FITC conjugated Tiger Snake venom as a flow cytometry reagent

A novel method of isolating venom-specific B cells was performed using fluorophore-conjugated snake venom (FITC-venom) as a flow cytometry reagent. FITC-Venom was developed at CSL by Kirsten Vandenberg (Seqirus) and myself, using Tiger Snake venom (Seqirus) and the Invitrogen FluoReporter FITC Protein Labelling Kit, following the manufacturer's instructions. Approximately 200 µL (0.2 mg) of whole Tiger Snake venom stock, was used in the labelling procedure. After labelling, FITC-Venom was resuspended to 800 µg/mL for use in flow cytometry. Since the Tiger Snake venom contained proteins of size ranging between 40-90 kDa by Western blot (Seqirus data), a range of conjugation ratios of FITC dyes was used to create three conjugates corresponding to 4, 8 or 12 FITC dye molecules per protein molecule. In the pilot experiment, conjugate 4 was used as a starting point. PBMC from naïve (Serum Australis) or snake-venom immunised horses (Seqirus) were prepared for flow cytometry analysis. PBMC were thawed and stained with antibodies specific for CD4/CD8, CD21, together with a viability dye (Sytox blue), washed and stained for 30 minutes on ice with the FITC-Venom at 80 µg/mL. Analysis was carried out with assistance from AMREPFlow cytometry facility.

2.3.12 Cell trace violet (CTV) Labelling of PBMC

To assess proliferation of equine B cells in the B cell culture system, PBMC were labelled with CTV and cultured as above. CTV labelling was performed using ThermoFisher Scientific CellTrace™ Violet Cell Proliferation Kit for flow cytometry. Prior to labelling, cells were washed in a solution containing PBS + 0.1% Bovine serum albumin (BSA) and resuspend at a concentration of $2x10^7$/mL. CTV was made into a stock of 5 mM in DMSO. For cell labelling, CTV was diluted to 500 µM in PBS + 0.1% Bovine Serum Albumin (BSA). The 500 µM CTV was added to the cells to a final concentration of 5 µM and vortexed. Cells were placed in a 37^{o}C water bath for 20 minutes, with the tube gently flicked every 5 minutes. Labelling was stopped by adding cold PBS/FCS until the 10 mL tube was full. Cells were washed twice with PBS/FCS and stored on ice until seeding for culture.

2.3.13 Statistical analysis

For the majority of experiments, Mann-Whitney non-parametric U tests were performed to determine significance. No significance was left blank or labelled 'ns', and significance was indicated by $p < 0.05$ *. All statistical analyses were performed using GraphPad Prism software (version 7.01, La Jolla, CA, USA), unless otherwise indicated.

3. Characterising and Isolating B cells From Horse Blood

3.1: Introduction

The first aim was to isolate a venom specific memory B cell from the blood of horses immunised with snake venom. To study the antibodies of an antivenom response, it is critical to be able to separate the potentially rare B cells responsible for developing these antibodies from the other, irrelevant B cells. Proceeding from a mixed population of PBMC to a single, antigen specific B cell is challenging enough in the human or mouse system where there are verified reagents available to commence such an analysis (84, 98, 99, 104). It is even more formidable in the horse system, where there is a paucity of reagents that have been found to work for horse B cells in terms of flow cytometry (105-108). Furthermore, the B cell system of the horse is not as well characterised as that of the mouse or human, which is reflected in the relatively low number of published flow cytometry studies on horses (108). This chapter will focus on addressing these issues and developing a system for isolating B cells from the horse and forming a foundation for subsequent chapters of this thesis.

3.2: Results

3.2.1 Flow cytometry profile of equine PBMC with anti-CD4/CD8 and anti-CD21

There are some flow cytometry antibodies suitable for horses, although the range is not as extensive as that for mice or humans. Nevertheless, a recent study highlighted the use of an anti-human CD21 (clone Bly-4) antibody to stain equine B cells (100). On that basis, this antibody clone was acquired and tested to determine if equivalent staining could be reproduced. Furthermore, antibodies specific to horse T cell markers are available and these were also tested to determine if they would be useful in delineating putative B cells and putative T cells in a mixed PBMC population. Additionally, the T cell marker antibodies could act to enrich for B cells by negative selection.

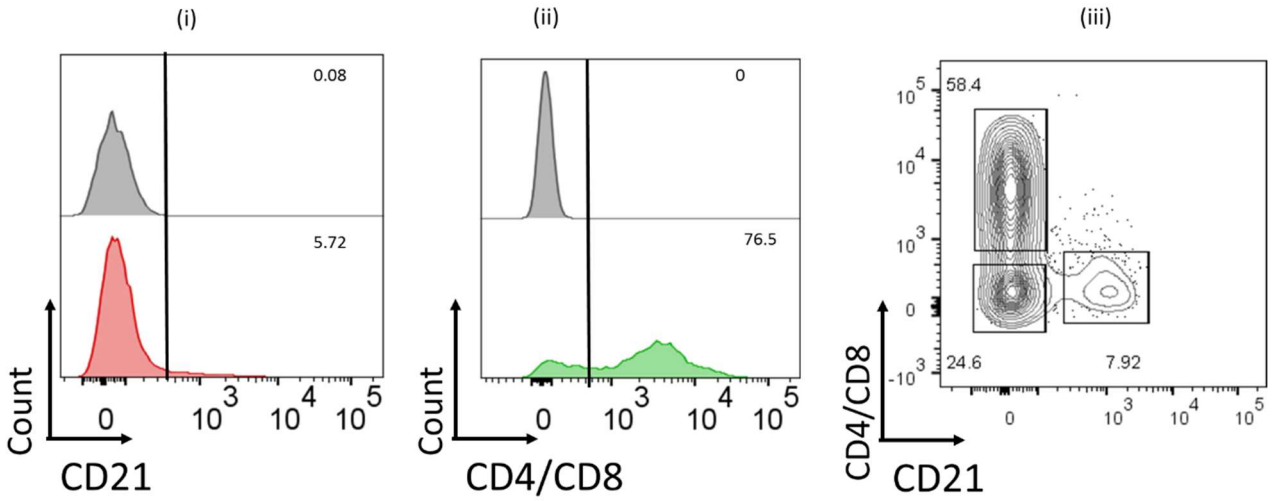

Figure 3.1: Staining equine PBMC with anti-CD21 and anti-CD4/CD8. Histograms and plots of PBMC thawed from cryovials, stained for CD21 alone (i), CD4/CD8 alone (ii), and in combination (iii). Histograms and plots are representative and used as basis for subsequent experiments. Grey histograms in (1) and (2) are unstained controls. Numbers represent frequency of population, pre-gated for forward & side scatter, doublet exclusion, and viability.

As demonstrated in Figure 3.1(i), anti-CD21 (Bly-4) stained a small but discreet population of cells, while in Figure 3.1(ii), a larger population of cells stained for the CD4/CD8 T cell marker antibodies (CVS4 and CVS8 in combination). When combined in the same sample, as shown in Figure 3.1(iii), a delineation is observed among PBMC that reveals putative B cells ($CD21^+$ $CD4/CD8^-$), putative T cells ($CD21^-$ $CD4/CD8^+$), and an additional population that is negative for both CD21 and CD4/CD8, which will be referred to as "double negatives" (-/-), with their relevance a focus in section 3.2.3. The frequencies of these three subpopulations are shown in table 3.1, where the highest proportion of the live PBMC are putative T Cells at ~70%, the double negatives are ~20%, and the smallest fraction being putative B cells at ~4-5%. The high frequency of T cells has been reported in the literature (106, 107, 109), with the relatively low frequency of B cells also reported of ~10% or less (105-107). The staining pattern with the Bly-4 clone of CD21 and CD4/CD8 formed the basis of subsequent experiments in isolating putative B cells.

	Average frequency ± SD
T cell	66.2 ± 6.8
-/-	20.8 ± 3.4
B cell	4.3 ± 1.9

Table 3.1: Average frequencies of PBMC subpopulations. PBMC were isolated from pooled blood of seven horses, one week after boosting. Frequencies of T cells, double negatives (-/-) and B cells are as a proportion of live PBMC. Average and standard deviation from three independent samples of PBMC-containing cryovials, from the PBMC of seven horses.

3.2.2 Flow cytometry profile of other antibodies tested for B cell markers

While it was encouraging that the CD4/CD8 and CD21 stained discreet populations of PBMC, other antibodies were tested to determine if a more extensive flow cytometry panel could be developed, identifying not just all B cells, but revealing subsets such as memory B cells. Antibodies specific for mouse or human antigens (Table 2.2) were compared to PBMC stained with the anti-CD21 (Bly-4) from Figure 3.1(i, iii). As demonstrated in Figure 3.2, these other antibodies failed to stain putative B cells in PBMC, even other clones targeting CD21, indicating the epitopes targeted by these clones are sufficiently different between mouse and horse or human and horse, so as to lack cross reactivity. Overall, the reactivity of these other antibodies was inadequate for further inclusion in this project, suggesting additional reagents should necessarily be evaluated.

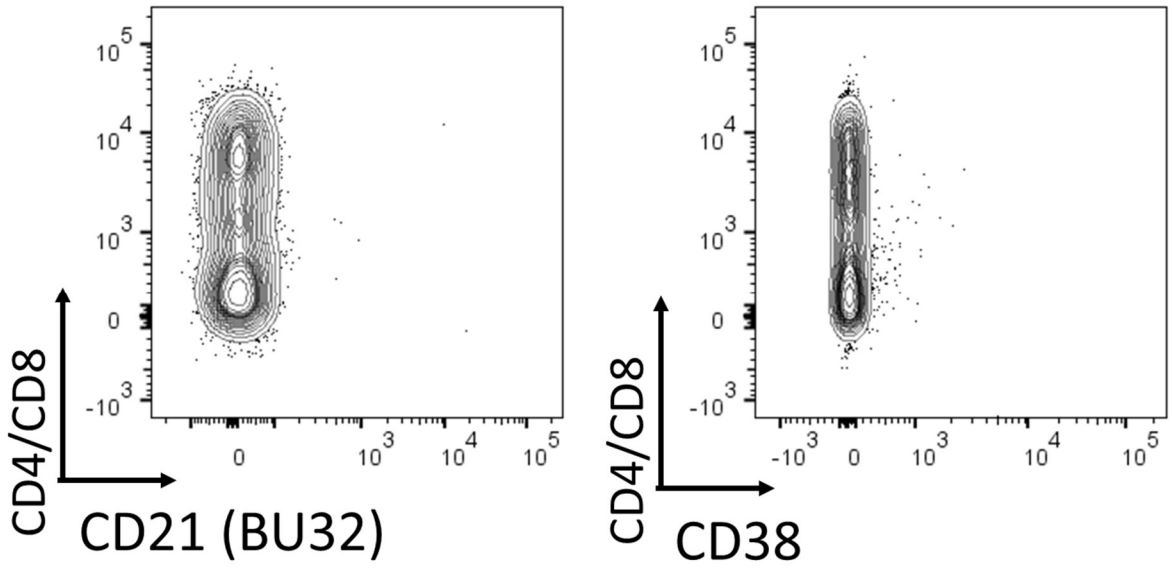

Figure 3.2: Flow cytometry plots of select additional antibodies tested for reactivity to horse PBMC. Failure of these antibodies and others (Anti-mouse CD19, CD21, CD23, CD38, IgD, IgM, anti-human CD19, CD20, CD40, CD275 and IgD) to adequately stain B cells lead to the discontinuation of their use for further analyses. Other antibodies tested are listed in Chapter 2, Table 2.2.

3.2.3 Pilot experiment in probing for snake venom specific B cells with venom-FITC conjugate on PBMC from naïve and immunised horses

One method of isolating antigen specific B cells is to use the antigen itself as a flow cytometry material, such as in the cases of bee venom (110), HIV (87, 111), the hapten 4(hydroxy-3-nitrophenyl) acetyl (NP) (112-114). In the case of isolating snake venom-specific B cells, this method would entail the use of snake venom conjugated to a fluorochrome like FITC as a flow cytometry reagent. This approach was attempted in a pilot experiment.

Whole Tiger Snake venom was conjugated to FITC, using the FluoReporter FITC Protein Labelling Kit, following the manufacturer's instructions. As a test for the successful conjugation of the FITC dye to snake venom proteins, my collaborator at Seqirus, Kirsten Vandenberg, performed a SDS PAGE of each of the venom-FITC conjugates. A Coomassie was performed on the SDS PAGE gel. This was followed by probing for FITC fluorescence on the bands, via a Chemidoc "Oriole" setting. As shown in Figure 3.3, FITC fluorescence was observed for each of the conjugates, with matching bands on a Coomassie stained SDS PAGE gel.

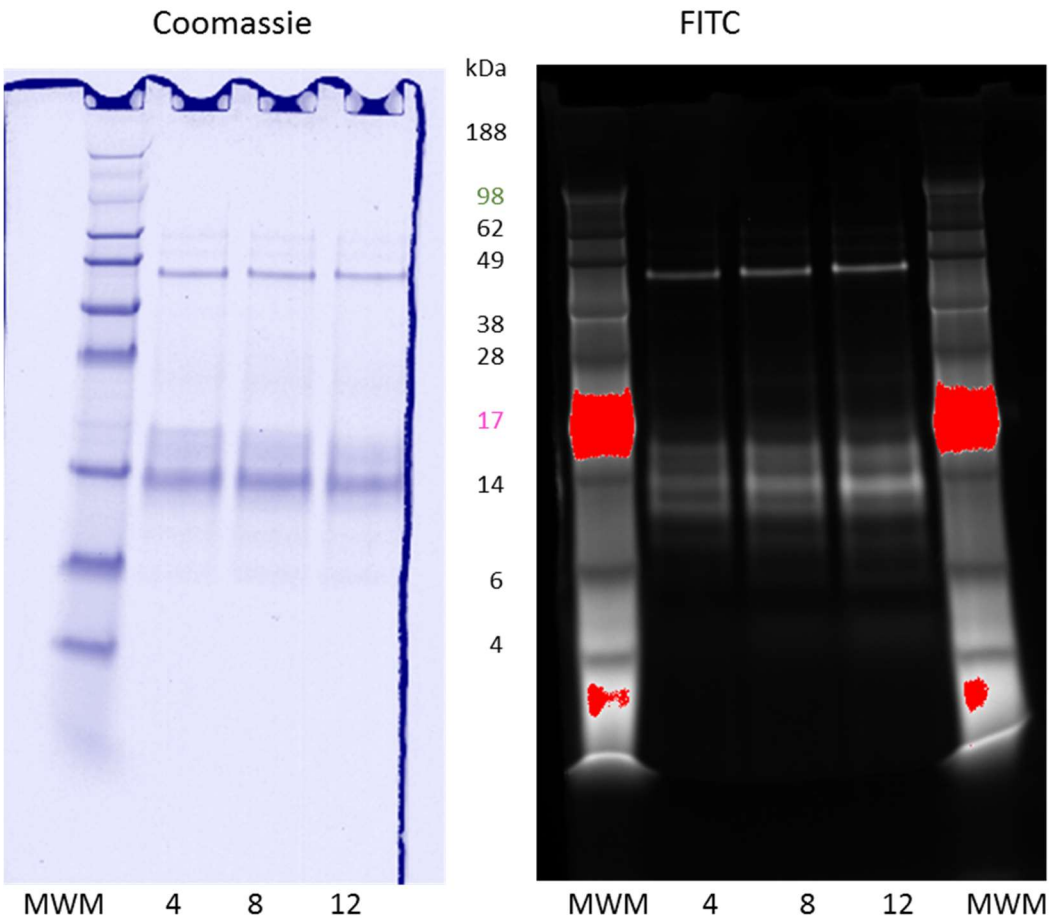

Figure 3.3: Examination of Tiger Snake venom – FITC conjugate. SDS PAGE gel of FITC conjugated Tiger Snake venom samples, loaded with approximately 5 µg/well. The Coomassie stained samples are on the left, with molecular weight marker (MWM). The right hand side gel was viewed with a ChemiDoc, using the "Oriole" setting to fluoresce the FITC bands. Lanes 4, 8 and 12 correspond to the µl of FITC used per conjugation reaction. Sample was prepared 1 in 2 in 4X LDS (NuPage) and incubated at 37^{o}C for 10 min. Samples were loaded on a gel which was 12% BisTris NuPAGE (non reducing), and run for 35 min at 200 Volts with MES running buffer. Gels were developed and analysed by Kirsten Vandenberg (Seqirus).

Following confirmation of successful venom-FITC conjugation, the venom-FITC was used in a flow cytometry experiment, to determine its utility as a flow cytometry reagent. PBMC from naïve and immunised horses were stained with the CD21 and CD4/CD8 specific antibodies, followed by a stain with venom-FITC at 80 µg/ml. The PBMC from naïve horses acted as a negative control, with the expectation that there would be few venom-FITC staining cells. As shown in Figure 3.4, among the PBMC subpopulations, the venom-FITC stained the CD21^{+} cells, but also stained high proportions of both the CD4/CD8^{+} cells and the double negative cells, indicating there was a lot of non-specific staining with the

venom-FITC at this concentration. This non-specific staining was present in PBMC from both naïve and immunised horses. Therefore, we decided not to continue on this path.

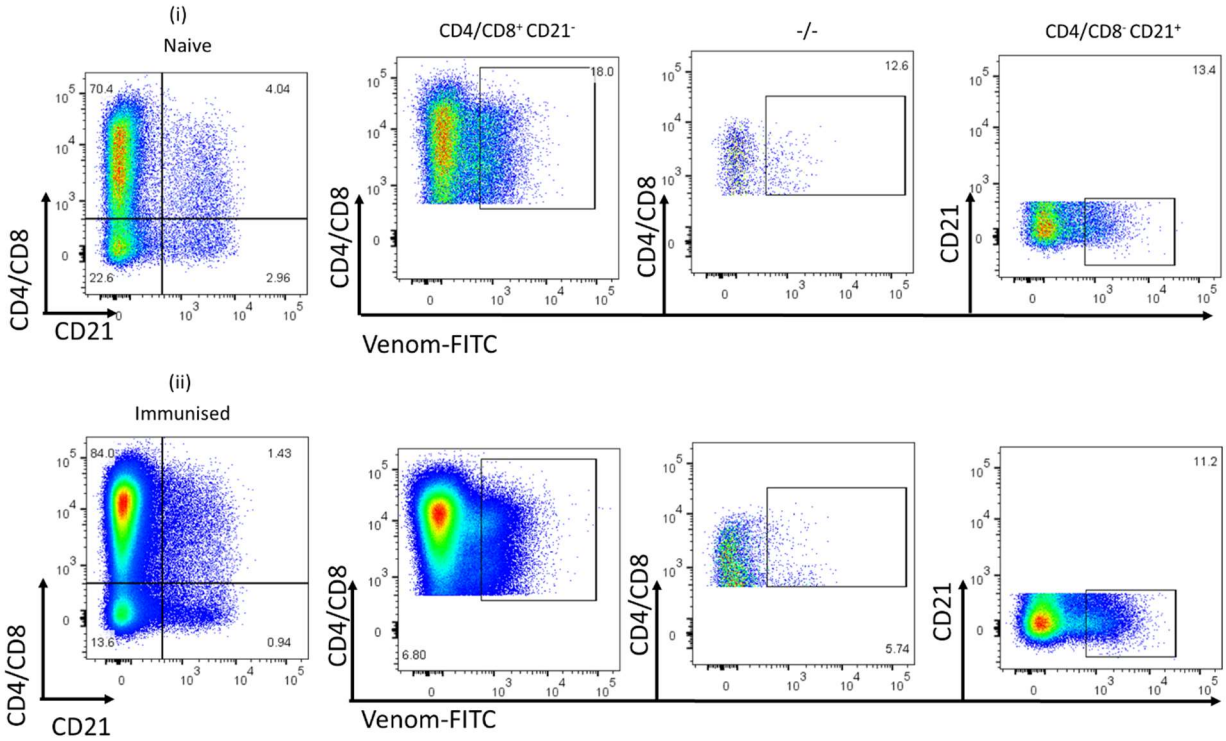

Figure 3.4: Staining equine PBMC with venom-FITC. Plot of PBMC stained with anti-CD4/CD8 and anti-CD21 from naïve (i) and snake venom immunised horses (ii). Histogram of the amount of staining with venom-FITC on T cells (CD4/CD8$^+$ CD21$^-$), double negatives (-/-) and B cells (CD4/CD8$^-$ CD21$^+$), with bisector line drawn from PBMC not stained with venom-FITC. Numbers represent frequency of cells staining with venom-FITC.

3.2.4 Quantifying the degree of enrichment of B cells in using anti CD21 and anti-CD4/CD8 as sorting antibodies

To determine if B cells were being selectively enriched when CD21 and CD4/CD8 were used as flow cytometry sorting antibodies, quantitative PCR (qPCR) was used to measure B cell content, using the delta-delta Ct method to measure relative gene expression (115). This was determined by comparing the relative amount of IgG and IgM transcripts among putative B cells (CD21$^+$ CD4/CD8$^-$), putative T cells (CD21$^-$ CD4/CD8$^+$), "double negatives" (CD21$^-$ CD4/CD8$^-$) and the total unsorted PBMC population as a relative comparison. The amount of IgG and IgM transcript was normalised to endogenous horse actin.

Starting with the unsorted PBMC, as shown in Figure 3.5(i), cells were sorted as either CD4/CD8$^+$ CD21$^-$, CD4/CD8$^-$ CD21$^-$, or CD4/CD8$^-$ CD21$^+$. The unsorted PBMC and three sorted PBMC subgroups were subjected to RNA extraction, and the mRNA was converted into cDNA. The cDNA was used to perform qPCR for equine IgG and IgM. The amounts of IgG and IgM heavy chain mRNA were quantified and normalised to equine actin mRNA. It was expected that the enrichment for IgG and/or IgM would be greatest among those sorted as CD21$^+$, if they were indeed B cells.

As shown in the fold change in amount of IgG (ii) or IgM (iii) mRNA relative to the amount in total unsorted PBMC, there was a consistent diminution of IgG and IgM transcript among the CD4/CD8$^+$ fraction relative to unsorted PBMC. As such, this is consistent with the notion that the CD4/CD8 antibodies are not recognising B cells. In the CD21$^+$ fraction however, while there was a degree of enrichment for IgG and IgM, the degree was lower than anticipated, since sorting a population that represents ~3% of the total to homogeneity should give greater than 4-5 fold enrichment for either IgG or IgM if all the IgG and IgM producing cells are within the sorted population. Furthermore, and surprisingly, the double negative group showed IgG and IgM mRNA, which was unexpected as the B cells should have been segregated into the CD21$^+$ group.

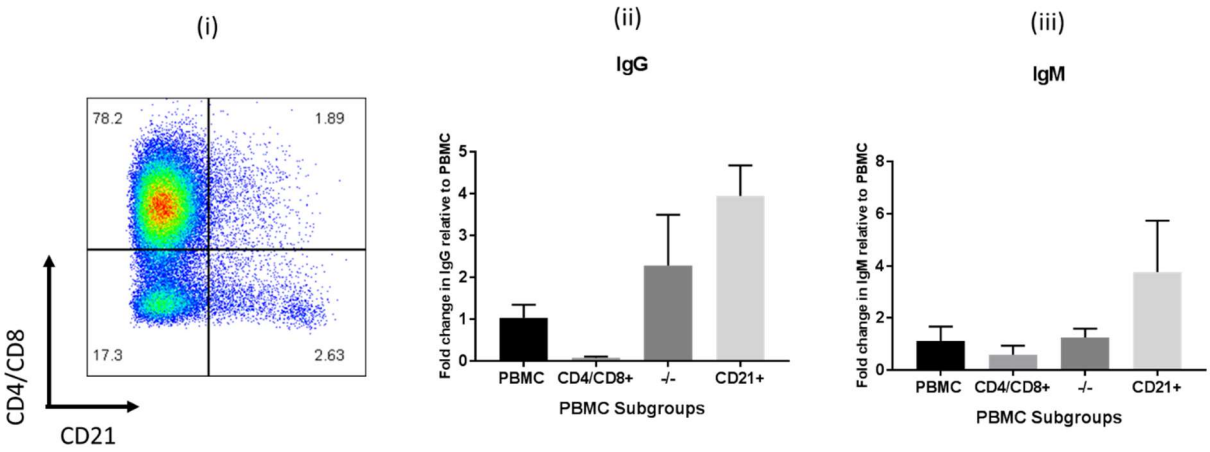

Figure 3.5: IgG and IgM transcript enrichment in populations sorted on CD4/CD8 vs CD21 from PBMC. FACS profile of PBMC sorted on the basis of CD4/CD8$^+$ CD21$^-$, CD4/CD8$^-$ CD21$^-$, or CD4/CD8$^-$ CD21$^+$, representing T cells, double negatives (-/-) or B cells, respectively (i). qPCR of IgG (ii) and IgM (iii) expression from PBMC sorted on basis of CD4/CD8 vs CD21. Graphs represent fold change in gene expression relative to unsorted PBMC group, normalised to endogenous actin. Each graph is representative of 3 independent experiments, indicating Mean ± SD, from PBMC of seven horses.

The qPCR data showed a reduction of IgG and IgM mRNA when CD4/CD8$^+$ cells were sorted from PBMC, but while the qPCR data trended for enrichment with CD21 expressing cells, the difference was not statistically significant between CD21$^+$ putative B cells and either total PBMC or the double negatives. The experiment

was underpowered to find any difference with a Mann-Whitney test. This prompted the idea that there may be some contaminating B cells among the double negative cells, suggesting further investigation.

3.2.5 Identifying antibody secreting cells within total and sorted PBMC via an ELISPOT

The observation that double negative PBMC were able to generate an IgG and IgM signal in the qPCR (Figure 3.5), prompted the concept that there were B cells in the double negative population contributing to IgG and IgM expression. One type of B cell that could explain this observation is an ASC, which normally have low or no surface expression of CD21, as demonstrated in humans (116). As such, an ELISPOT was established to test for the presence of these ASCs. Furthermore, the ELISPOT was used to determine the ASC frequency within the different PBMC subsets, as depicted in Figure 3.6(i). Total unsorted PBMC was used as a comparison for total ASC.

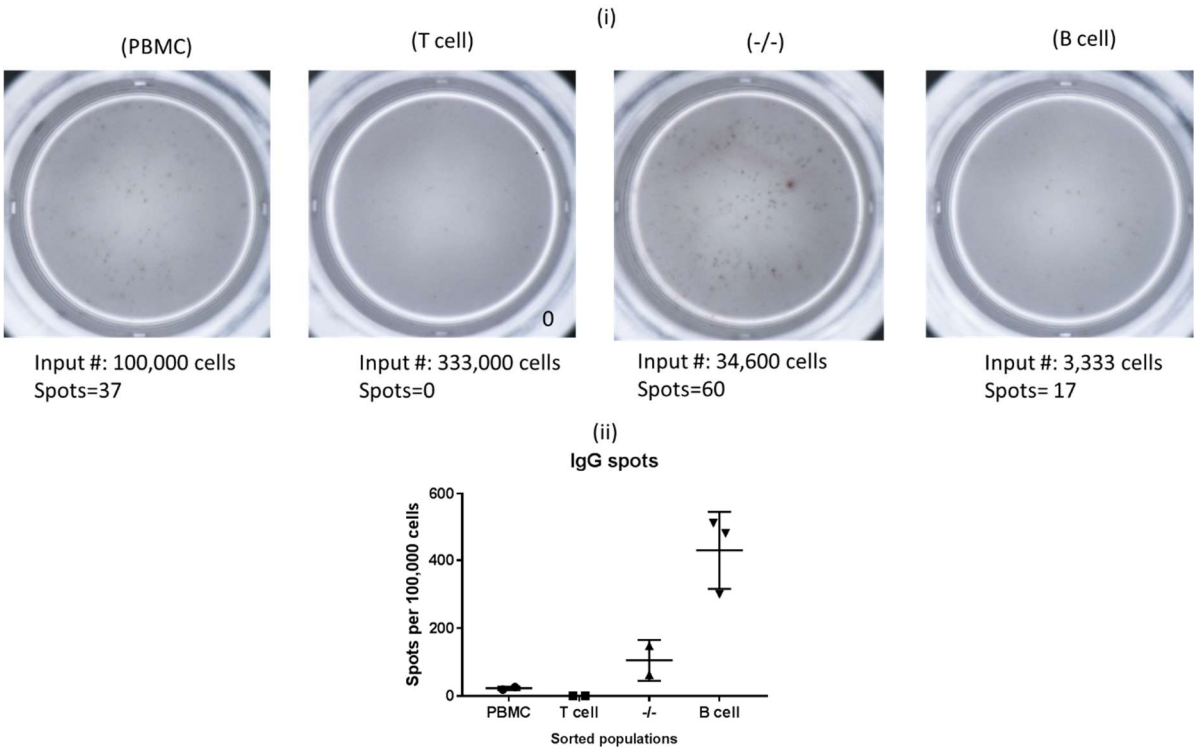

Figure 3.6: ASC detection in total and fractionated PBMC. Representative IgG ELISPOT wells from total PBMC, T cells, double negative cells and CD21+ putative B cells are shown, from one of two independent experiments (i). Input number of cells are stated below images, with number of IgG spot counts. Spots are normalised to per 100,000 input cells, representing the average of triplicate ELISPOT wells from two independent experiments, mean ± SD (ii), with caveat that for one ELISPOT experiment, the B cell group was below the detection limit, and shown as the average of triplicate wells. Graph is representative of 2 independent experiments, from PBMC of seven horses, mean ± SD.

	Percent of IgG spots (mean ± SD)	Cells seeded per well; experiment 1, experiment 2
PBMC	0.02 ± 0.005	10000, 10000
T Cell	0	258000, 333333
Double Negative	0.11 ± 0.06	34666, 42666
$CD21^+$ B cell	0.43	3333, 1666

Table 3.2: Percentage of IgG spots in total and fractionated PBMC with detection limit of IgG spots. The percentage of IgG spots as % of ASC/100,000 cells. Number of cells seeded per well is shown for both experiments. No spots were observed in the T cell group for both experiments. Spots were only observed in the B cell fraction in one experiment. Average ± standard deviation from 2 independent experiments, from PBMC of seven horses.

As shown in Figure 3.6, it was apparent that ASCs were present among total PBMC and, as shown in table 3.2, the IgG ASC frequency was 0.02%, calculated from 100,000 input cells, and this reflected a detection limit of there being at least one IgG spot per 10,000 input cells. Among the three sorted populations, the T cells were found to be free of any ASC, shown as a 0% frequency. Among the double negatives, more spots were observed than in the PBMC. As shown in Table 3.2, the frequency of IgG ASC increased approximately 5-fold over the total PBMC, with a detection limit reflecting more IgG spots per 1700 cells. Among the B cells, there were few spots, possibly due to the low seeding number of $CD21^+$ cells from the sort (1666-3333 cells/well), but the frequency was in one sample high (400/100,000). I found no spots in the other sorted B-cell sample, in which 1666 cells were plated, with an expected occurrence of 0.43%, there was the expectation that there would be approximately 716 spots. Nevertheless, as shown in Table 3.2, the frequency of IgG ASC in the B cell fraction tended to be greater than total PBMC.

3.3 Discussion

3.3.1 Identifying equine B cells

One of the main challenges in this project is the lack of verified reagents for isolating antigen specific B cells. Despite this difficulty, some progress has been made in identifying and isolating equine B cells from a mixed population of PBMC. With the currently available flow cytometry antibodies, CD21 appears to resolve at least some B cells from a mixed population of PBMC. This was supported by the qPCR data, where there was a trend towards enrichment of IgG and IgM mRNA amount, on PBMC sorted with the anti-human CD21 antibody. Furthermore, in the ELISPOT data, in the experiment in which ASC were detected in the $CD21^+$ fraction, IgG secreting ASCs were enriched when the PBMCs were sorted with the anti-CD21 antibody than in either the double negative fraction or the unfractionated PBMC.

While CD21 expression identifies B cells among the PBMC, within the CD21+ compartment itself exist both naïve and memory B cells, with lower expression of CD21 indicative of Memory B cells in humans (117), but it is not known if this is also observed in horses. However, the current panel of flow cytometry antibodies is not able to distinguish between memory B cells and naïve B cells, with the former population expected to contain B cells that would have developed during the venom-immunisation procedure. That is, we would expect some of the horse memory B cells to be specific for snake venom and indeed, some of these to express antibodies of high affinity to snake venom antigens. These B cells would be of great interest to isolate and from which to develop recombinant antibodies. As such, to be able to identify and isolate memory B cells, it might be warranted to identify and then generate fluorochrome conjugated antibodies to memory B cell surface markers. For example, one could make an anti-equine CD27 antibody, as a putative memory marker, and conjugate it to a fluorochrome. Such an antibody could be generated using a strategy employed by the Wagner group at Cornell University (118-120), which has developed many anti-equine antibodies to leukocyte surface markers. Using their anti-equine CD14 antibody as an example, the development strategy was as follows. Recombinant CD14/IgG1 fusion protein was generated, by cloning the extracellular region of CD14 into an expression vector. Then BALB/c mice were immunised with purified equine recombinant CD14 protein. Hybridomas were generated and selected, using particular protocols (121). The hybridoma supernatants were screened for monoclonal antibodies specific to CD14/IgG1 fusion protein (119) and then tested for their specificity to the CD14 portion of the CD14/IgG1 fusion protein (119, 122). The antibodies were then tested for their reactivity to CD14 on horse PBMC via flow cytometry (118). Following the testing of newly generated monoclonal antibodies to B cell surface markers via flow cytometry, additional validation of the antibodies can be achieved by qPCR as done in Figure 3.4.

3.3.2 Validating cell type identity by the use of qPCR

The qPCR experiments were used to validate the flow cytometry staining. That is, I am confident that the CD4/CD8 antibodies are not staining B cells because there was a consistent diminution of IgG and IgM transcript among the $CD4/CD8^+$ sorted cells, relative to total PBMC. These two antibodies can therefore

perform the role of a "dump channel". That is, during a flow cytometry sort, these two antibodies can be used to remove the large proportion of the PBMC that comprises T cells. The CD21 antibody typically revealed a small, discreet positive staining population, which generated an IgG and/or IgM signal by qPCR that was greater than in unsorted PBMC although the difference did not reach statistical significance and which was potentially enriched in ASC by ELISPOT, indicating the presence of B cells. However, as mentioned above, the presence of ASCs within the double negative fraction of PBMC might explain the lack of statistical significance in IgG enrichment between double negatives and total PBMC, as well as between B cells and total PBMC. Had all of the IgG expressing cells been sorted into one population, rather than two, the maximum amount of IgG expression might have been observed. To prove these ASCs are contributing to the lack of statistically significant enrichment in IgG expression, an experiment would involve extracting these cells from the double negative subpopulation, followed by a qPCR analysis on IgG enrichment on $CD21^{+}$ versus a double negative sample depleted of ASCs (123). However, the practical difficulty in carrying out this experiment is the lack of antibodies that can stain for ASCs markers in the equine system, such as CD138/syndecan-1 (124). Additionally, it may be warranted to determine how different the compartments are, following stimulation on the ELISPOT, as the ELISPOT experiment were performed with thawed PBMC only. Nevertheless, the observation of a B cell signal (via IgG) and verification by ELISPOT, from cells sorted with the CD21 marker, but not the $CD4/8^{+}$ population, provides confidence that the cell type being analysed and sorted as $CD21^{+}$ by flow cytometry includes B cells. The two assays also provide validation that the CD21 antibody is targeting the desired cell group. The use of qPCR as a method to validate the identity of cells and by extension flow cytometry antibodies, has been performed in other studies (125, 126). For example, Camilleri *et al.* used qPCR to validate their flow cytometry antibody panel and enable better discrimination of different mesenchymal stromal cell subtypes (125). Furthermore, in a study by Prieto *et al.,* they used the mRNA expression amounts of CD5 to confirm that they were enriching for $CD5^{hi}$ or $CD5^{lo}$ expressing B cells in their flow cytometry sorts (126). Additionally, a study by Tomlinson *et al.,* used qPCR to validate the identity of equine cells sorted with their chosen antibodies (127).

3.3.3 Snake venom antigen as a flow cytometry reagent

As a way to isolate antigen specific B cells, some research groups used the antigen itself as a probe for these cells via flow cytometry. Using fluorophore-tagged antigen as a flow cytometry reagent has been used in multiple studies for other antigenic systems, such as in Bee venom(110), HIV (87, 88, 111), phycoerythrin (128), and with NP coupled to the protein Keyhole Limpet Haemocyanin (NP-KLH) (112-114, 129-131), among many others. Isolating antigen specific cells by using the antigen has multiple utilities, such as being able to develop monoclonal antibodies specific to the antigen, for example as performed by Schneid *et al.* for HIV (88). More broadly, isolating antigen specific cells with the antigen, can enable a greater characterisation of the B cells responding to antigenic immunisation, such as the changes in

frequency of the antigen-specific cells over time, rates of SHM and changes in affinity. For example, a study by Zotos *et al.* (112), using the NP-KLH immunisation system in wild type and IL-21 deficient mice, was able to track the changes in NP-specific B cells in terms of frequency and SHM rates, and how these parameters were altered between wildtype and IL-21 deficient mice. Using snake venom antigen as a flow cytometry reagent, to the best of my knowledge, has not been reported before. The initial concern of this pilot experiment was a possibility that there would be a lack of any detectable staining of venom-FITC on the PBMC, which might have rendered the use of this experimental approach null. However, as observed, the larger problem seems to be that there was notable non-specific staining of venom-FITC among all PBMC including T cells. This high background staining reveals the need to use a lower concentration of antigen, at least lower than the 80 µg/mL concentration tested in my study, as well as other ratios of FITC conjugation to test. Therefore, an experimental approach would be to further titrate the venom-FITC, to a point where background staining to non-B cells reduces to autofluorescence levels. Presumably only the B cells specific to a snake venom antigen should stain positive for the venom-FITC. We would expect these B cells to be a rare population of the PBMC, on the basis of antigen specific B cells in other antigenic systems. Examples include the 0.3% of ASC specific to influenza (132), 1-2% memory B cells recognising pneumococcal polysaccharide (133), 1.2% of IgG memory B cells for pandemic influenza (134), 2% of IgG^+ B cells specific for HIV (87), or <1% of IgG1+ cells specific for NP (113).

There are other aspects of this venom-FITC process that may make isolating venom-specific B cells challenging. One is that, unlike the other antigen systems mentions above, venom from any snake species is a cocktail of many proteins (3, 4, 135) at varying concentrations and with varying levels of immunogenicity (3). For example, the neurotoxins of Beaked sea snakes are a major cause of the lethality in sea snake bites, but the neurotoxins are found to be poorly immunogenic (9, 16, 36). Therefore, the issue with finding venom specific B cells may be in finding the B cells that are making antibodies that target the relevant venom components. Another aspect is the safety risk of handling the toxins.

3.3.4 ASCs isolated from cryopreserved PBMC in antigen boosted horses

The ELISPOT data have revealed that freshly thawed PBMC from CSL horses, boosted a week prior to bleeding, contain ASCs. Specifically, while total PBMC yields a low frequency of IgG antibody secreting cell at 0.02%, the frequency of IgG spots increases in sorted B cells by 20 fold in the experiment in which ASCs were detected, compared to total PBMC, with a 0.43% of IgG producing cells in $CD21^+$ B cells. This value is low when compared to other studies investigating the frequency of IgG ASC. For example, in studies of influenza in humans (132, 136, 137), Halliley *et al.*, found approximately 0.8% of PBMC were IgG ASC (132). The Halliley *et al.* study found the peak of the ASC response to occur between days 5-8, following immunisation (132). Additionally, their study measured the frequency of total influenza specific ASC to be

at 0.3%. In a study by Pinna *et al.* on boosting influenza vaccine in humans (138), they observed that circulating influenza-specific memory B cells were at their highest frequency of 1% of all IgG+ B cells, at about 14 days after vaccination, with circulating plasmablasts reaching a peak between 6-8 days. The caveat with the ELISPOT data is that I cannot make a statement as to the frequency of antigen specific ASC that were present in my ELISPOT data, as I was measuring total IgG. Additionally, the ASCs detected in my ELISPOT experiments were isolated from cryopreserved PBMC, which may have impacted ASC viability. However, Trück *et al.* found no difference in B cell ELISPOT assays, using fresh or cryopreserved human PBMC, following 6 days of culture with a mixture of killed *Staphylococcus aureus* Cowan strain (protein A on its surface binds IgG including horse IgG), CpG oligodeoxynucleotide 2006 and Pokeweed Mitogen (PWM) (139). However, whether the lack of difference would also occur when culturing horse PBMC under the same conditions as the Trück *et al.* study is unknown. To determine the frequency of venom-specific ASC would involve performing an ELISPOT with antigens purified from snake venom coated on the ELISPOT wells, a procedure that has been performed for influenza antigens (140), but to the best of my knowledge, an ELISPOT with venom antigens has not been performed. Reliably identifying ASC from seven day boosted horse PBMC, and then working back to be able to isolate them by FACS could help in making monoclonal antibodies to snake venoms because the yield of antibody mRNA is greater in an ASC. It should therefore be easier to amplify the heavy and light chain genes than those of resting, quiescent memory B cells (123).

An alternative process of isolating and developing monoclonal antibodies from these ASC without the need for sorting is a PCR-based cDNA library method (141, 142), a technique of generating monoclonal antibodies from libraries of heavy chains and light chains cDNA, similar to antibody phage display (84, 143, 144). These cDNA libraries are first generated by extracting the mRNA from a mixture of cells containing the ASCs of interest, converted the mixture of mRNA into a cDNA pool. From the cDNA pool, heavy chain and light chain templates are PCR amplified, purified and sequenced to screen for repeating IgG heavy chain and light chain sequences. That is, a greater proportion of the cDNA would originate from the ASCs because of their higher antibody mRNA content. Therefore, the plan would be to screen for IgG heavy chain and light chain sequences that are amplified repetitively.

To carry out the PCR-based cDNA library method of isolating and generating monoclonal antibodies, some procedures need to be established. The first would be to determine the frequency of venom specific B cells in a PBMC mixture, which would involve carrying out an ELISPOT on total venom. The use of venom on an ELISPOT plate has yet to be reported in the literature, although the process could be performed in the same way as that of other antigens, such as influenza (140). The second procedure would be to assemble a cDNA library of the PBMC that originated from 7 days post boost. The third step would be to PCR amplify the heavy chain and light chain mRNA from the pool of cDNA. The fourth procedure would be to carry out cloning, sequencing and recombinant antibody expression of the PCR amplified products, following the methodology utilised in chapter 5; essentially the methodology of RT-PCR and expression vector cloning of

antibody genes (89). This method would be a faster method of generating monoclonal antibodies, as it would circumvent the issue in the current project that are preventing the development of monoclonal antibodies from single venom specific B cells viz. the lack of flow cytometry antibodies for identifying the antigen specific B cells from all B cells.

3.3.5 Concluding remarks

Overall, a method of identifying and isolating equine B cells has been developed using the currently limited selection of flow cytometry antibodies, supported with qPCR data. The equine PBMC contain IgG ASCs at a frequency comparable to other systems, and these ASC might be useful to develop antigen-specific antibodies. The use of snake venom antigen as a flow cytometry reagent is in need of further refinement, if it is to be used in a similar manner to other antigen-specific flow cytometry detection systems.

4 *In vitro* culture system for equine B cell growth and antibody production

4.1: Introduction

The second aim was to develop a system of activating and inducing proliferation of antigen specific memory B cells. Chapter 3 established a method of identifying and isolating equine B cells from a mixed population of PBMC using flow cytometry, a useful approach to studying B cells (145, 146). While B cells could be enriched with anti-CD21, one issue of the flow cytometry staining panel was that I could not identify venom-specific B cells. The B cell compartment includes naïve B cells, many of which would not be specific to venom antigens. Likewise, all B cells would also include memory B cells, which may be enriched for B cells specific to the immunising antigen (i.e., venom), and potentially have improved affinity to the antigens of interest (i.e., venom antigens). These venom-specific B cells, would be the optimal B cells to identify venom-specific antibody genes from which to make anti-venom antibodies (84, 89).

Another approach to characterising the memory B cell response is a cell culture method of expanding and differentiating memory B cells into ASCs. This approach has been utilised in many studies (91, 138, 147-151). Using the work of Pinna *et al.*(138) as an example, they were able to determine the frequency of memory B cells that produce antibodies that neutralise influenza, as well as the kinetics of the influenza response following boosting. They were able to determine these parameters, even when culturing from a bulk PBMC culture (138). Therefore, while I am currently unable to obtain a single venom specific B cell by flow cytometry, I could take the Pinna *et al.* approach of culturing bulk B cells, culture with cytokines, and develop an antibody library from an enriched memory B cell pool. Additionally, as memory B cells are more proficient at proliferation and differentiation than naïve B cells (29, 152, 153), this culture system could provide a natural system of enrichment from a starting total B cell compartment.

As there is currently no established cell culture system for equine B cell activation and differentiation, I attempted to develop an equine B cell culture system, to determine the conditions required to expand B cell numbers and/or differentiate memory B cells into ASCs, using approaches from the research of Pinna *et al.,* as a starting point. The ASC would be in many respects the ideal cell to recover as we can test the specificity by ELISpot or ELISA and then extract antibody genes more easily, because about 70% of the mRNA produced in an ASC is related to antibodies, in comparison to 2% for a Memory B cell (123).

4.2: Results:

4.2.1 Time course of equine PBMC over seven days of culture

As there is currently no established cell culture system for the growth and activation of horse B cells into ASC, I utilised an approach from the Pinna *et al.* study (138) in an attempt to create a simple culture system that would enable B cell growth and antibody secretion. Pinna *et al.* tested various combinations of TLR agonists, cytokines and CD40L-transfected cells on their PBMC cultures and sorted B cells, from which they found IL-2 and R848 (a TLR agonist) effective at expanding and differentiating memory B cells.

The first approach was to culture unseparated equine PBMC and determine if the B cells contained therein underwent growth and initiated antibody production. PBMC were cultured in the presence of Pokeweed Mitogen (PWM), a compound used in other cell culture systems to stimulate B cells to proliferate and differentiate (29, 138, 154-156), and that the Pinna *et al.* group used at 5 µg/mL. PBMC were cultured for seven days in the presence PWM at different concentrations, to determine if there was an increase in antibody production. After performing a titration of PWM concentration between 2.5, 5, 10 and 20 µg/mL, I found 5 µg/mL trended toward greater antibody output (Figure 4.1).

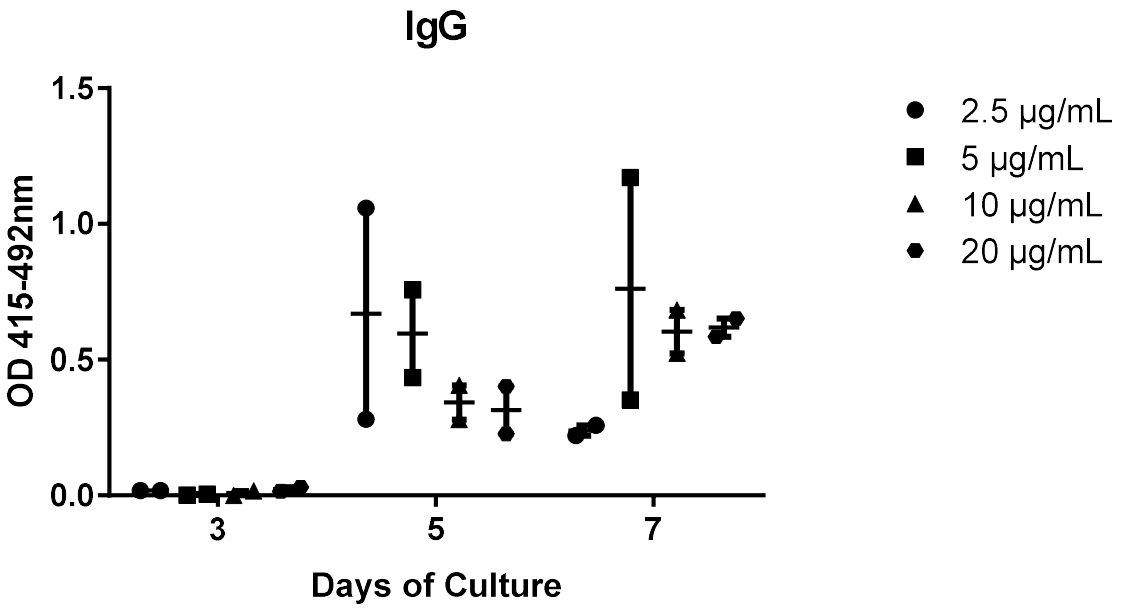

Figure 4.1: Testing concentrations of Pokeweed Mitogen in PBMC to probe for antibody production. PBMC were cultured for up to 7 days, in the presence of different concentrations of Pokeweed Mitogen (PWM) as indicated, with supernatant harvested on days 3, 5 and 7 for analysis via ELISA to determine antibody secretion. Graphs show IgG antibody as measured by ELISA and presented as optical density at one dilution. Graph is from one experiment with duplicate wells, plotting mean ± range.

The flow cytometry antibodies, anti-CD4/CD8 and anti-CD21, enabled analysis of the cultured cells to determine if B and/or T cells were specifically increasing in frequency over time of the culture, as shown in Figure 4.2 (i). As shown in Figure 4.2 (ii), there appeared to be a difference in the frequency of B cells from

the start of culture (day 0) to the end point (day 7), but only without PWM. Although the frequencies of B cells may have changed over time, the total number of cells may not have changed. This experiment was underpowered to find any difference with a Mann-Whitney test.

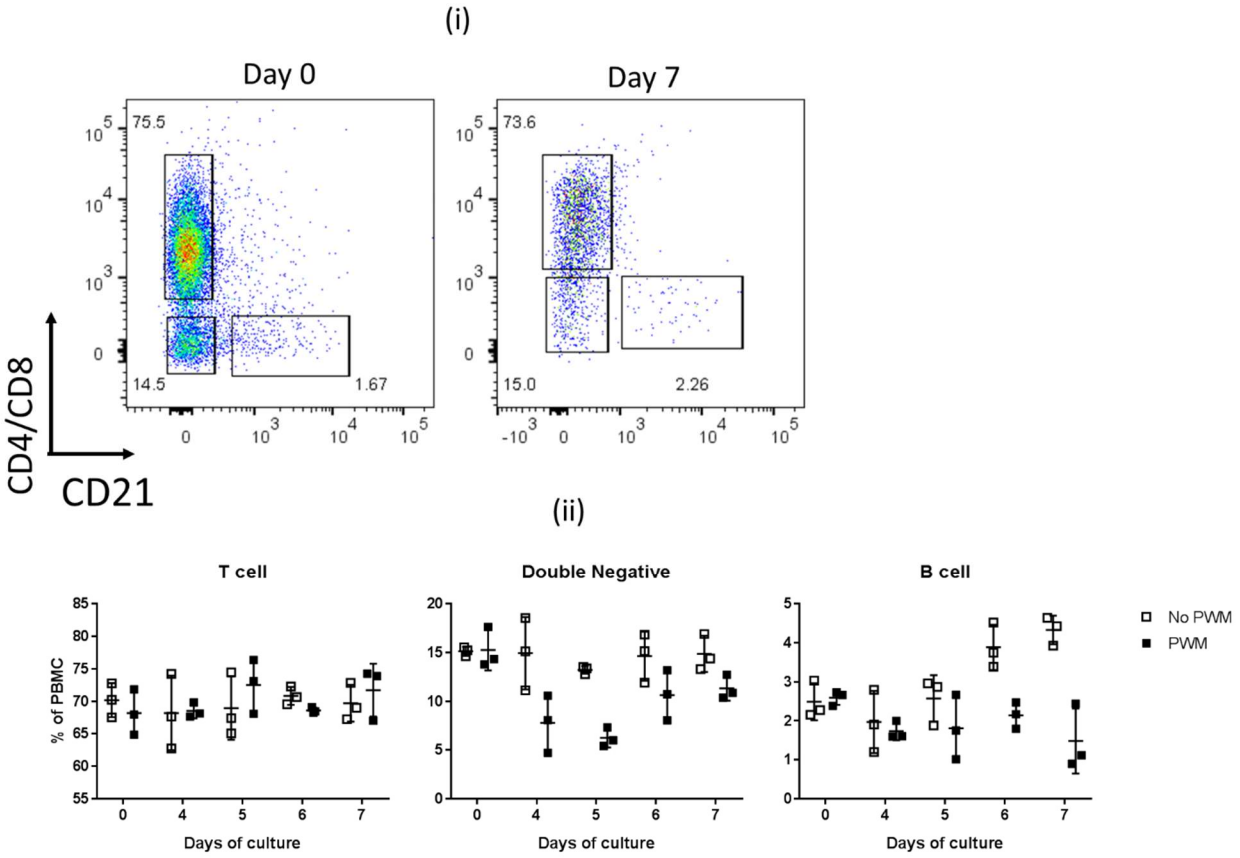

Figure 4.2. Frequency of T cells, double negatives and B cells in PBMC cultures over time. PBMC were cultured for 7 days in the presence or absence of Pokeweed Mitogen (PWM), with cells harvested on days 0, and 4-7 for flow cytometry analysis to determine the frequency of cell subsets. (i) Representative flow cytometry plots of expression of CD4/CD8 versus CD21 in PBMC cultures from day 0 and day 7 time points, with PWM. (ii) Frequency of T cells (CD4/CD8+), double negatives (CD4/CD8-CD21-) and B cells (CD21+) within PBMC culture wells over the time course of the experiment. Graphs are representative of 3 independent experiments, from PBMC of seven horses, mean ± SD.

As shown in Figure 4.3, there was no increase in total cell number over the 7 days of culture (i), and no increase in B cell (ii) or T cell (iii) numbers during the time course. However, there was a trend for an increase in the output of culture supernatant IgG antibody (Figure 4.2 iv), starting at day 6 of culture. However, the experiment was underpowered to find any difference with a Mann-Whitney test. Double

negative cells were not considered in further analysis, since the specific cell types that were present or proliferating cannot be determined with the current flow cytometry antibodies.

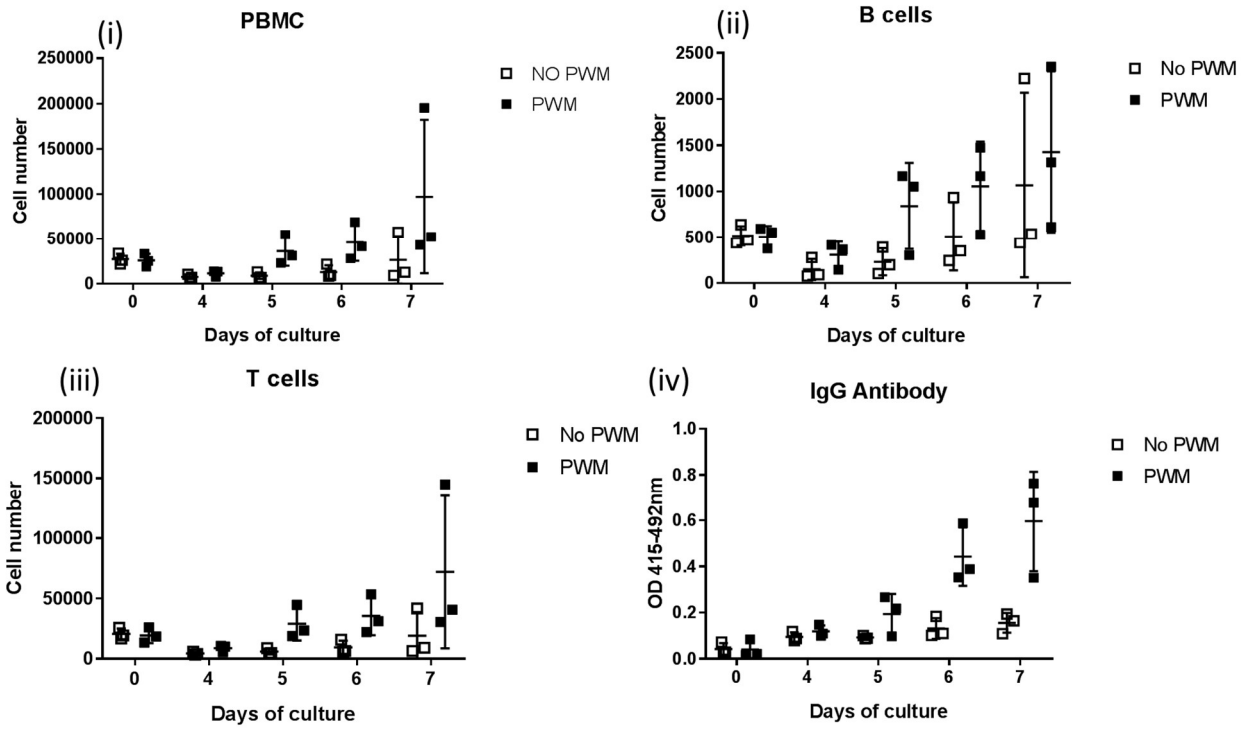

Figure 4.3: Numbers of cells and antibody production in PBMC cultures over time. PBMC were cultured for 7 days, in the presence or absence of Pokeweed Mitogen (PWM) as indicated, with cells and supernatant harvested on days 0 and days 4-7 for flow cytometry analysis and ELISA, to determine cell number and antibody secretion, respectively. Graphs show numbers of (i) total PBMC, (ii) B cells, (iii) T cells, and (iv) IgG antibody as measured by ELISA and presented as optical density at one dilution. All graphs are representative of 3 independent experiments, from PBMC of seven horses, mean ± SD.

The lack of PWM-induced cell number increase led me to investigate proliferation in the cell culture system further by labelling PBMC with Cell Trace Violet (CTV). This dye works by tracking the division of cells, whereby daughter cells receive half the dye of their immediate progenitor (157). PBMC were labelled with CTV and then cultured for seven days in the presence or absence of PWM. As shown in Figure 4.4, there was B cell proliferation with PWM. There was also proliferation with the T cells, and this may be a contributor for the proliferation observed with CTV labelled PBMC. When quantified, as presented in Figure 4.5, there was trend for a loss of cell number with T cells and B cells. Additionally, there was lack of difference between PBMC cultured with or without PWM, in terms of cell number at day seven. So while there was proliferation of B cells and T cells on the basis of CTV dilution, there was also a loss of these cell types in overall number. The experiment was underpowered to find any difference with a Mann-Whitney test.

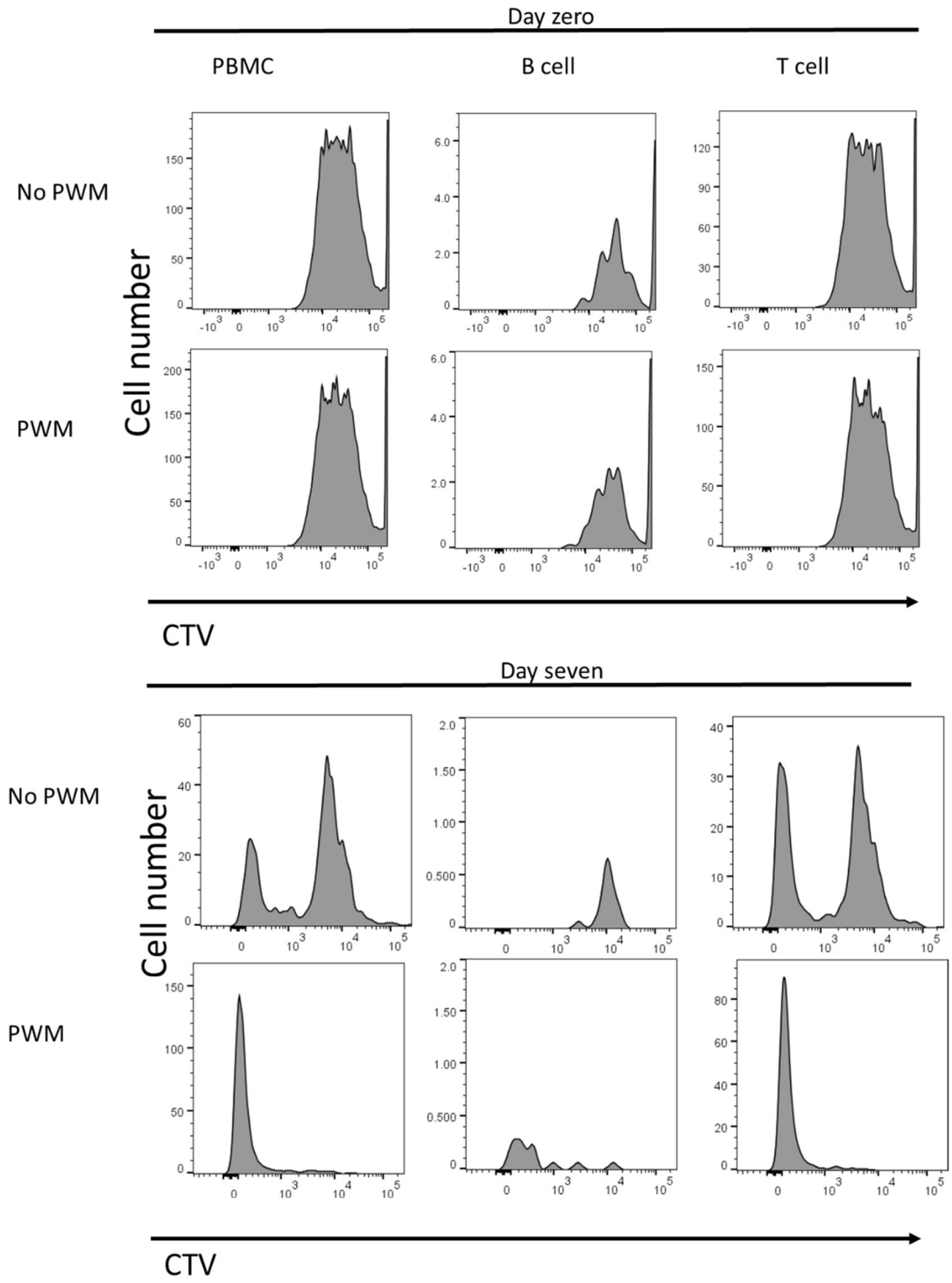

Figure 4.4: Examining cell proliferation in PBMC cultures using Cell Trace Violet (CTV). Flow Cytometry plots of CTV labelled PBMC at the outset (upper panels) of culture and after seven days (lower panels) in the presence or absence of PWM, as indicated, and after subdivision by FACS into indicated cell subsets. Representative plots of replicates within 1 experiment, from PBMC of seven horses.

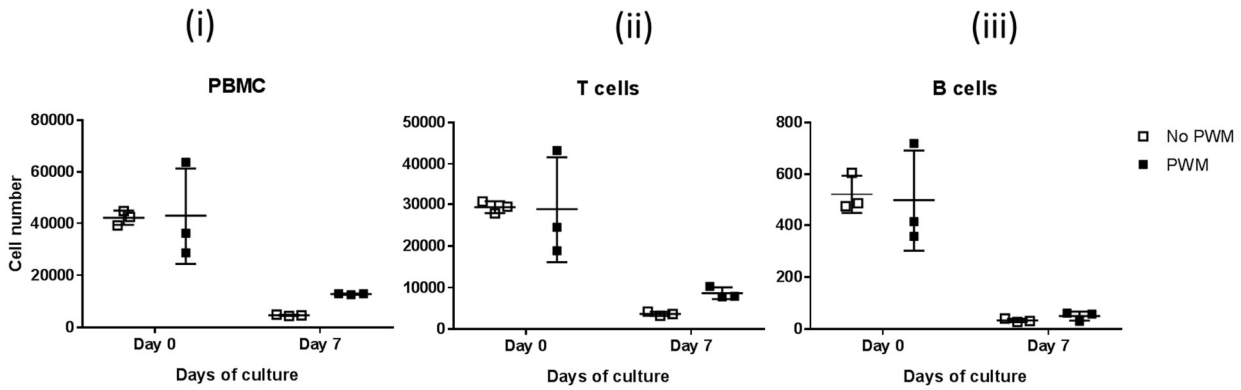

Figure 4.5: Numbers of cells in CTV labelled PBMC cultures over time. Numbers of indicated cell subsets in CTV labelled PBMC cultures after seven days in the presence or absence of PWM. Graphs show numbers of (i) total PBMC, (ii) T cells and (iii) B cells. Graphs depict mean ± SD of 3 technical replicates.

4.2.2 Testing if exogenous equine cytokines improve the B cell response to PWM

Culturing PBMC for seven days in the presence of PWM failed to produce a statistically significant increase in the number of B cells (or other cell types). Although there was IgG antibody detected after 5 days of culture, to improve the culture system in a manner to facilitate B cell proliferation and antibody production, a range of equine cytokines was tested in various combinations based on cytokines used by Pinna *et al.* and other prior literature (29, 138, 154, 155, 158-160). A total of thirteen groups were assembled, where total PBMC were cultured with PWM for seven days, together with no, one or more equine cytokines. The PWM-only group served as a baseline for comparing antibody and proliferation output. Surprisingly, as displayed in Figure 4.6, there was no apparent difference in antibody output from any of the combination of cytokines compared to control cultures, and the positive control sample (PWM only), did not reach a similar OD value as in Figure 4.3 (iv). Additionally, and as shown in Figure 4.7, there were no differences in any of the combinations of cytokines in terms of the proliferation of total PBMC (i), B cells (ii) or T cells (iii), when compared to the PWM only group of PBMC. The exception was the IL-4 group, where there was a trend of an increase in the number of B cells, between PBMC cultured with PWM and IL-4 against the PWM only PBMC group. This potentially indicated that B cells were either proliferating more between the start and end of the culture period with the inclusion of IL-4, or were surviving better in the presence of IL-4 when stimulated with PWM. However, these experiments were underpowered to find any difference with a Mann-Whitney test.

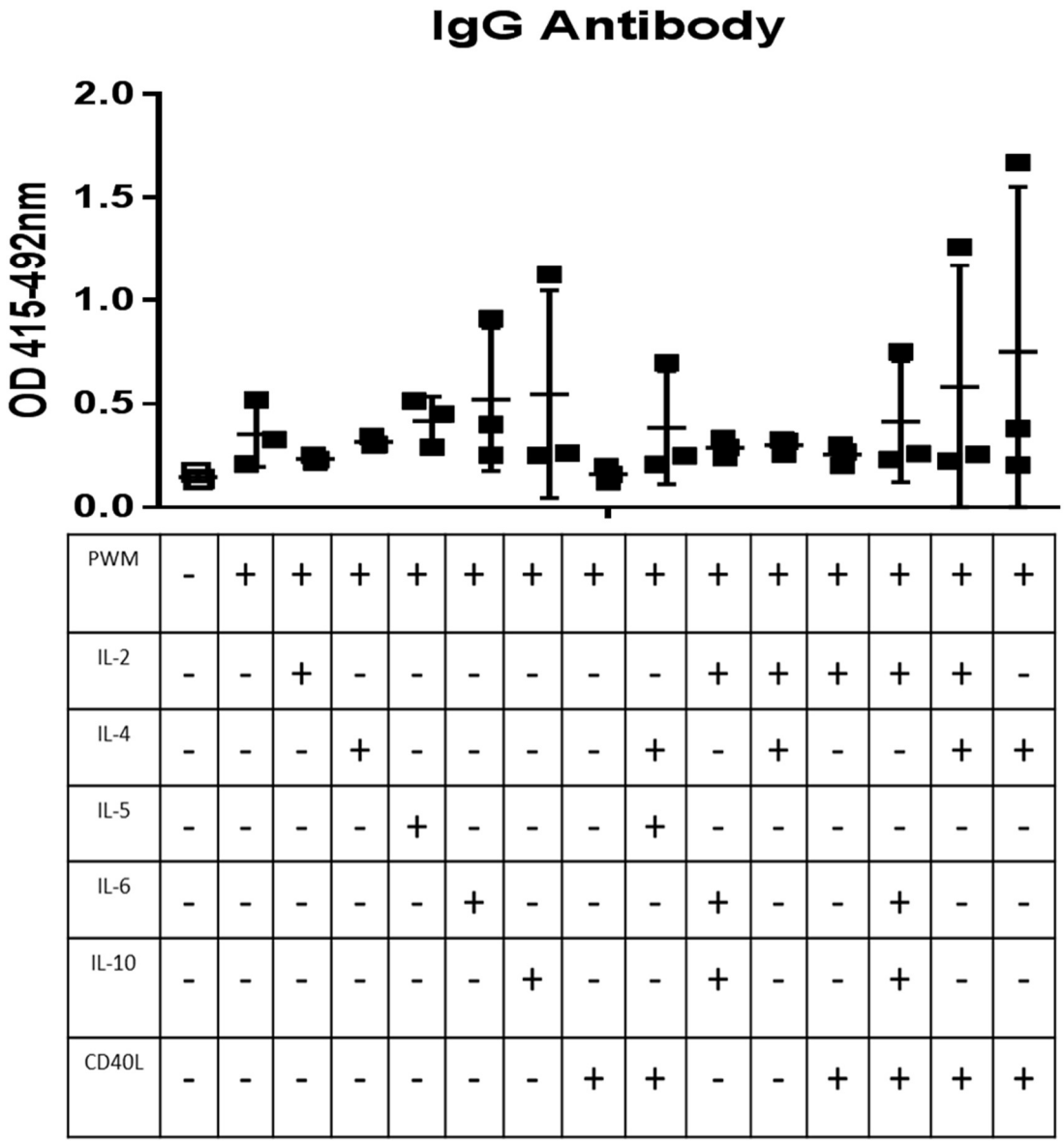

Figure 4.6: Impact of added cytokines on antibody output of cultured PBMC. PBMC were cultured with or without PWM, and with or without the indicated cytokine(s) for seven days and IgG antibody output measured via ELISA. Equine Cytokines added were IL-2, IL-4, IL-5, IL-6, IL-10 and CD40L. Graph represents output of one experiment with 3 replicates, from PBMC of seven horses, mean ± SD.

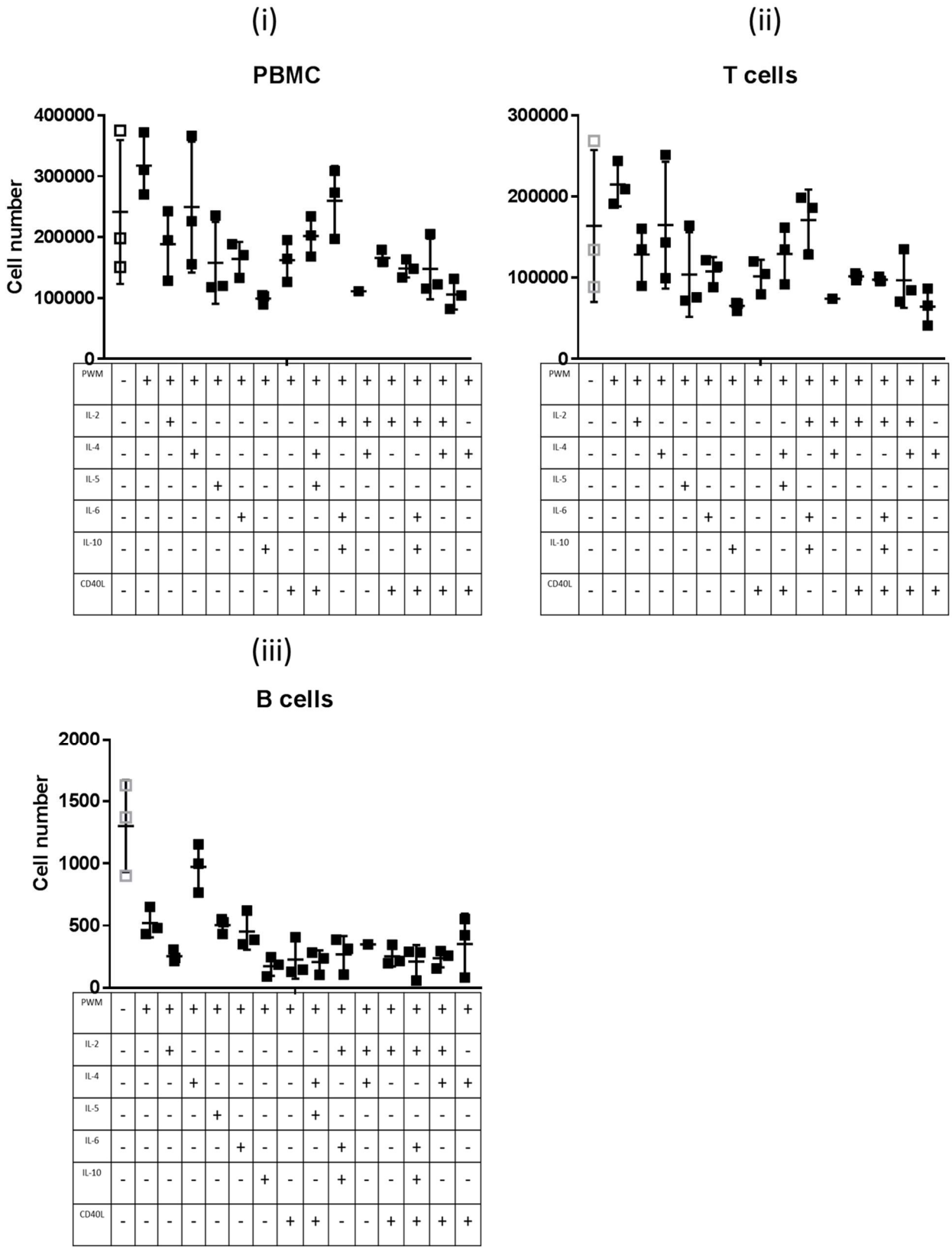

Figure 4.7: Impact of added cytokines on cell numbers in cultured PBMC. PBMC were cultured with or without PWM, and with or without additional cytokine(s) for 7 days, harvested, counted and analysed by flow cytometry to identify cell subsets. Equine cytokines were IL-2, IL-4, IL-5, IL-6, IL-10 and CD40L. Graph are mean ± SD of 3 replicates from one experiment, from PBMC of seven horses.

4.3: Discussion

4.3.1: Detection of IgG antibody from *in vitro* PBMC cultures with a lack of significant B cell growth

Memory B cells can be found in PBMC (113) and have an important role in providing long term protection from dangerous antigens (28, 151). When rechallenged by the same antigen, memory B cells activate, proliferate and differentiate into the ASC, the type of B cell that rapidly produces high affinity antibodies to the antigenic challenge. In this chapter, I aimed to develop a simple *in vitro* cell culture system to enable the activation of memory B cells into a state of proliferation and antibody secretion, as has been achieved for human B cells by Pinna *et al.*, (138), and Muir *et al.*, (151). However, while I was able to induce B cell proliferation and some IgG antibody production in response to PWM, I was unable to increase PBMC or B cell numbers over the culture period, suggesting cell death is a major limitation of the current culture.

When PBMC were cultured with PWM, there was a trend for IgG antibody production. As there was no apparent IgG antibody detected within the supernatant of PBMC cultured with no PWM, this suggests that the ASC were likely generated during culture, rather than transferred from the thawed PBMC instilled into the wells on day 0. However, it remains possible that the PWM maintained the survival of a small number of pre-existing, seeded ASC: in the absence of stimulation, both B cells and ASC die of neglect (161). One method of testing whether the antibodies observed in the culture supernatant were due to surviving ASC (rather than differentiated memory B cells) would involve performing an ELISPOT on PBMC, cultured with or without PWM, and comparing the number of antibody spots at the start of culture versus various days during culture. The survival hypothesis would be that the PWM cultured group would have a steady number of antibody spots over time, compared to the non-PWM cultured group. Alternatively, the increase in antibody production might be due to an increased production per ASC. This can be tested by an ELISPOT assay, by culturing PBMC overtime, with and without PWM, and measuring the size of antibody spots, with the hypothesis that the antibody spots are of greater size in a PWM cultured PBMC group, compared to PBMC cultured without PWM.

Regarding proliferation and cell number, it was apparent that there was a large decline in cell number over the culture course, despite observable CTV dilution of PWM-stimulated PBMC. The rapid rate of death might be caused by stimulation-induced apoptosis as observed in the work of Parry *et al.*, who observed apoptosis in B cells when there was hypercross-linking of surface IgM or IgD on mature B cells (162). They observed the B cell death rate was attenuated when IL-4 and/or anti-CD40 was included in cell culture. In my experiments, PWM acted to stimulate the PBMC, but in a way that resulted in high degree of cell loss. In my cultures, IL-4 was also able to increase B cell numbers in PWM-stimulated cultures, and one possibility is that this is due to similar survival effects of the equine IL-4 on stimulated B cells (159, 163).

Although there was no statistically significant increase in cell number, there was the trend suggesting that after a lot of death in the first four days of culture, there was a subsequent increase in cell number over the remaining 3 days of culture. As indicated by the CTV experiment, there was proliferation of both T and B cells. However as discussed above, the high amount of cell death probably accounts for the lack of cell number increase. One way to counter this high rate of death, might be to culture cells freshly isolated from PBMC, as opposed to my liquid nitrogen frozen stocks of PBMC. However, Truck *et al.*, found little difference between fresh and frozen PBMC cultures in terms of cellular activity or output (139), but whether this lack of difference between fresh or frozen PBMC cell culture output would occur with the equine system is unknown. Another way to counter the high death rate might be to modify the cryovial thawing process to a method used by Fecher *et al.*, who found that the gradual removal of DMSO in physiological temperatures reduced the amount of B cell death (164).

4.3.2: Use of equine cytokines to improve B cell culture system

Another possible explanation for the detection of IgG antibodies in the cell culture, was that helper T cells within the cultures were also stimulated by the PWM, providing help to the B cells in the form of cytokines. A study in Bovine PBMC by Norian *et al.*,(156) found that PWM was a very potent activator of T cells, in terms of the production of cytokines, such as IL-2, IL-4, IL-5, IL-6 and IL-10. These cytokines have various roles in B cell activation, survival and differentiation (154, 155, 158-160). IL-4 is a potent cytokine involved in B cell survival by limiting apoptosis (159), and in my cultures, the inclusion of IL-4 helped to increase B cell number. IL-5 and IL-6 have roles in antibody production, with IL-5 also having a role in B cell proliferation (160). IL-2 has a role in pushing the B cell toward the plasma cell phenotype (158). IL-10 acts synergistically with IL-2 to promote immunoglobulin production (154, 165). Although it was not discussed in the Norian *et al.,* study, CD40L is also another important B cell activating factor, present on activated T cells, and functions as a trimeric protein (29, 31, 153, 166-168). Furthermore, IL-21 is a cytokine that would be ideal to use in a B cell culture system, because of its important role in promoting an ASC phenotype (112, 149, 152, 153, 155). As recombinant equine IL-21 was not available from commercial companies, it may be warranted to produce our own recombinant equine IL-21 for future cell culture experiments.

Given the modest antibody output and lack of growth with B cells, cytokines were tested in the culture experiments to help the B cells within the PBMC to be activated, proliferate and produce antibody. The equine cytokines mentioned above were tested to determine if antibody output and growth could be improved by an amount greater than that of PBMC cultured with PWM alone. Using an approach based on Pinna *et al.,* study (138) and Muir *et al.* (151), various equine cytokines were tested with the PBMC cultures. However, no significant differences were observed among cultures that had cytokine(s) added in terms of antibody output or proliferation. The sole exception was the IL-4 & PWM group, which trended towards a

significant increase in B cell number, compared to the PWM alone group. As discussed earlier, IL-4 has the effect of limiting apoptosis in B cells (159). However, this did not translate into increased IgG antibody output. This observation could be attributed to naïve B cells or non-IgG expressing B cells surviving for a longer period of time and no effect on existing ASC. One possible explanation for the lack of significant differences with the other cytokines, is that the cultures may already contain high concentrations of endogenously produced cytokines with PWM stimulation of the T cells and thus be saturated. Alternatively, cytokines or T cells may have effects that are largely masked by the cell death occurring in this system. As mentioned previously with the Norian *et al.* study (156), PWM stimulated the release of cytokines from among the T cells within their PBMC cultures and this may occur with our equine PBMC cultures. Finally, while I used methods from the Pinna *et al.* study (138) as the initial step in developing an equine B cell culture, there were differences between my approach and their study. While I did test a range of cytokines on bulk PBMC with PWM for seven days of culture, I did not use an autologous irradiated feeder layer, or a CD40L expressing cell line, to support my PBMC cultures. These feeder cells may have been necessary components for supporting B cell growth and differentiation in my PBMC cultures. Furthermore, Pinna *et al.,* tested a range of mitogenic stimuli, such as R848, LPS and CpG, in addition to PWM, to determine which factor provided the most effective support for B cell growth and differentiation into ASCs.

4.3.3 Concluding Statements

This chapter explored a novel culture system for equine B cells. It provides an inroad, but is currently inadequate to be used to systemically identify horse IgG heavy and light chains from single cells. An improved cell culture system, producing a statistically significant degree of antibody output and memory B cell differentiation, similar to that in studies such as Pinna *et al.*, might be achieved by using sorted B cells, cultured with the various combinations of cytokines, an approach used in other studies (29, 153, 155, 158, 159). However, Pinna *et al.* found high yields of antibody with total/unsorted PBMC, hence my initial approach of culturing total PBMC, with the inclusion of various cytokines. However, my method was a simplified approach, compared to Pinna *et al.,* and may have benefited from following their procedures more thoroughly. Furthermore, as for the human B cell studies of both Pinna *et al*., (138) and Muir *et al.,(151),* other mitogen factors could also be tested to see if they can preferentially activate equine B cells; Toll-like receptor agonist factors such as R848, a CD40L expressing cell line instead of soluble CD40L, or irradiated feeder cells. Ultimately, given the limited progress in the B cell culture aspect of the project, the remaining focus of this thesis was on developing the B cell PCR approach for isolating and producing venom-specific antibodies.

5 Recombinant antibody production from equine B cells

5.1: Introduction

The third and final aim was to develop a system for extracting the unique antibody genes from individual B cells, to produce and then test these antibodies for their snake venom cross-reactivity and neutralisation capability. To better understand the antivenom response in horses, one needs to understand the kinetics of the response and knowledge of the diversity of the antibodies (138). Gaining this understanding can be facilitated by an efficient process of isolating venom-specific B cells and extracting their unique antibody genes. To generate monoclonal antibodies, as done by other researchers for influenza (98, 99) or HIV (87, 89, 169), the ideal cell would be antigen-specific memory B cells. From the previous chapters, progress has been made in isolating B cells from a mixed population of PBMC. The B cell to isolate would be a memory B cell, converted into an ASC. This is because a memory B cell would be more likely specific to the antigen of interest, as the memory B cell compartment contains cells that were generated during immunisation (138, 170, 171), as compared to a naïve B cell. Furthermore, converting a memory B cell into an ASC would result in a more efficient PCR, since the majority of the ASC mRNA is antibody related (123). However, no antigen specific memory B cells could be isolated with current reagents. The next approach would be to differentiate memory B cells into ASCs (93, 138). However, there was limited success in differentiating and expanding memory B cells into ASCs.

Despite these issues, it might be possible to develop anti-venom antibodies from single B cells, which has been the approach of a number of other studies (84, 87, 89, 98, 99, 169). This can be achieved by probing for repeated sequences in vaccinated horses, the assumption being that repetitively found sequences would correspond to the antigen of interest. In a human study by Truck *et al.*,(172) following vaccination for tetanus, influenza B and meningococci, they were able to identify antigen specific sequences by comparing the CDR3, a region of high variability (68), before and after vaccination. Therefore, I have tried to develop a single cell PCR approach to extracting antibody genes, an approach used by many groups to develop monoclonal antibodies (84, 87, 89, 98, 99, 169).

5.2 Results

5.2.1 Amplification of equine heavy chain and light chain gene segments from bulk PBMC

Before proceeding to the single cell PCR, confirmation that the antibody genes of any B cell could be amplified and cloned into our expression vectors was required, with additional verification that the amplified PCR product was equine IgG heavy chain (59, 65) and lambda light chain (63, 67). Lambda light

chain was selected for amplification since approximately 92% of all equine B cells express the lambda light chain (63, 67). The strategy for cloning heavy chain and light chain is shown in Figure 5.1.

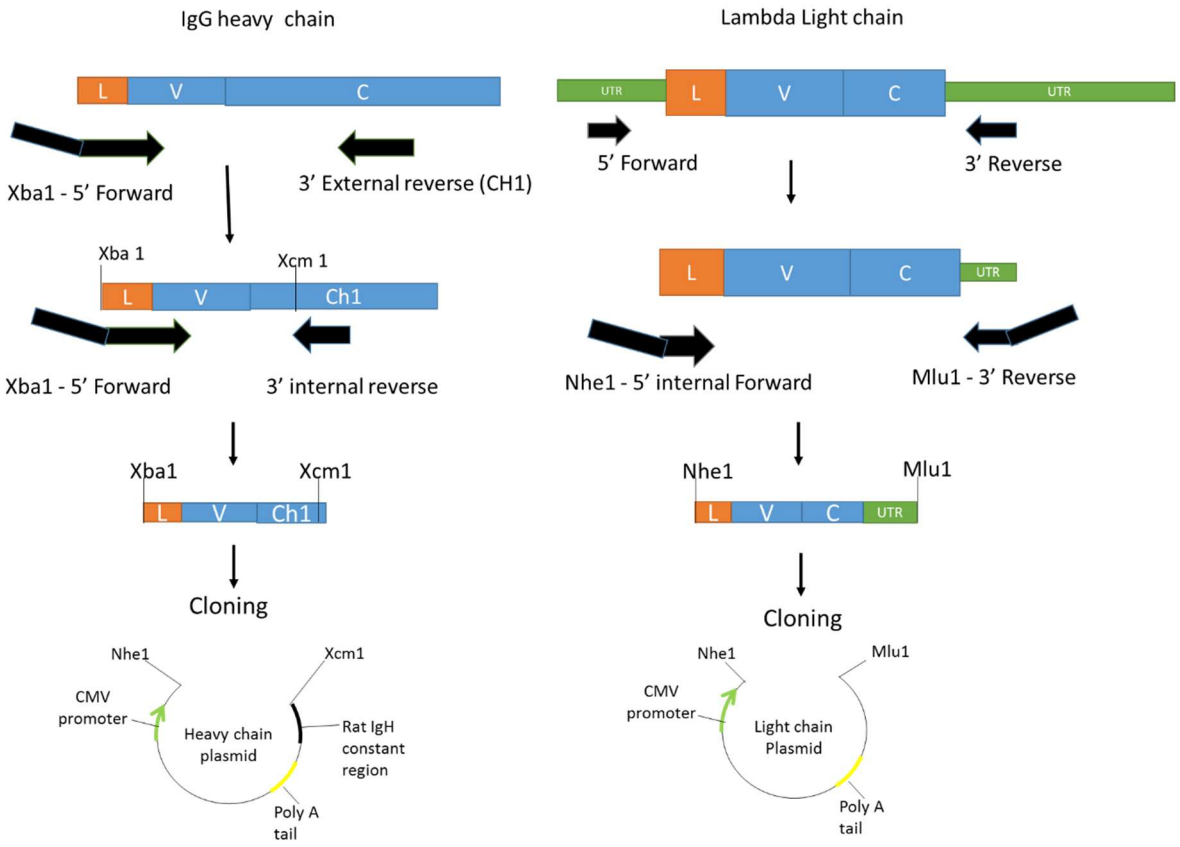

Figure 5.1: Strategy of cloning equine heavy chain and light chain variable region genes. IgG heavy and lambda light chain genes were amplified by semi-nested PCR from cDNA, before ligation into expression vectors. 1st round PCRs were performed with forward primer mixes specific for the leader region and reverse primers specific for the IgH constant region and light chain 3' UTR, respectively. 2nd round PCRs for heavy chain were performed with internal reverse primer, with light chain PCR performed using a forward primer mix specific for leader regions. Each expression vector contains a CytomegaloVirus (CMV) promotor and Poly Adenosine (A) site. Heavy chain plasmid contains rat IgH constant region.

To amplify equine heavy chains, a set of nested primers was developed, using strategies developed for human (173) or mouse (174) variable region gene primers (Figure 5.1). The forward primers of the heavy chain were designed to cover the V-region leader sequence, based on NCBI BLAST results of equine heavy chain variable leader sequences. From the approximately 400 known equine heavy chain variable sequences, it was found that two leader sequences were used extensively (about 95%), and the heavy chain forward primer was designed to cover these common leader sequences. Two unique reverse primers were

designed (forming a nested pair), with each reverse primer designed to cover all seven IgG constant regions (65). The degenerate internal reverse primer included a region of the IgG exon 1 that contains a common Xcm1 restriction cut site, a cut site that was also present in the expression vector.

To amplify the equine light chain, a set of nested primers was developed using a similar strategy to the heavy chain. Two nested, forward primers were developed, with the outer forward primer located upstream of the leader sequences in the untranslated region and the inner forward primer designed to cover the light chain leader sequences. Based on NCBI BLAST results of equine lambda light chain variable leader sequences, approximately 350 equine light chain variable sequences are known and these sequences formed the basis of a degenerate internal forward primer. A reverse primer was designed to bind a region downstream of the light chain stop codon and cover the seven lambda light chain constant regions (63).

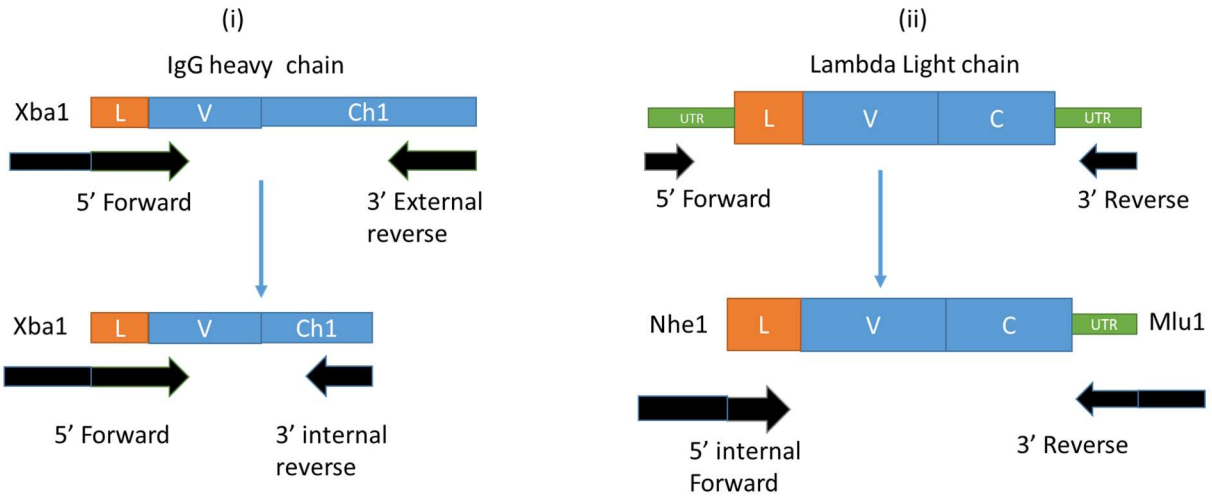

Figure 5.2: Strategy for PCR amplification of equine heavy chain and light chain variable genes. Schematic for amplifying heavy chain of horse IgG with a semi-nested PCR (i). In the first round of PCR, the 5' forward primer containing the restriction enzyme cut site is positioned at the start of the leader sequence, and the reverse primer is positioned at the end of the CH1 exon of the Cγ constant region. For the second round a reverse primer is positioned internal to the external reverse primer and the same forward primer is used. Schematic for amplifying the lambda light chain of horse immunoglobulin with a semi-nested PCR (ii). Forward and reverse primers for the first round PCR are positioned within the 5' and 3' untranslated regions (UTR), respectively. In the second round, the 5' forward primer is internal to the first round with the reverse primer unchanged in binding site. Both of the light chain second round primes have restriction endonuclease sites added to facilitate cloning into the expression vector. L denotes the leader sequence, V denotes the variable region, and C denotes the constant region.

Using nested primers that I designed to cover all known equine heavy chain and light chain leader sequences, and shown as a schematic in Figure 5.2, heavy chain and light chain PCR products were amplified in a semi-nested PCR comprising two rounds, with PBMC cDNA as a starting material. The PCR products were extracted with Gel extraction kits, following the manufacturer's instructions. The PCR products were cloned into a TOPO vector following the manufacturer's instructions. Chemically competent bacteria were transformed with the TOPO ligated heavy chain or light chain PCR plasmid, and colonies screened by PCR for successful ligation of heavy chain or light chain PCR products. Successful ligation of heavy chain or light chain was shown by the presence of bands of approximately 800 or 900bp, respectively. The bacterial colonies containing plasmids with ligated heavy chain or light chain were selected for overnight growth and the plasmids then purified using Qiagen Miniprep spin columns, following the manufacturer's instructions. Each heavy chain or light chain positive TOPO plasmid was sent for Sanger sequencing at Micromon Facility (Monash University, Clayton), with M13 forward and reverse primers, and the sequences were verified as genuine horse heavy and light chain by determining similarity to published sequences using NCBI BLAST. Heavy chains were verified by comparing the PCR product sequences to a randomly selected equine heavy chain sequence (GenBank ID: ADK09420) and they were found to have at least 75% homology, which is used as a criteria to determine subgroup membership (60). Light chains were verified by comparing the PCR product sequences to a randomly selected equine light chain sequence (GenBank ID: AIY24764), and they were found to have at least 75% homology, which is used as a criteria to determine immunoglobulin subgroup (60).

```
ADK09420          MSHLWFFLFQVAAPTCVLSQVQLKESGPGLVKPSQTLSLTCTVSGFSLSGYDVGWVRQAP      60
PBMCheavy3        MSHLWFFLFLVAAPTCVLSQVQLRESGPGLVKPSQTLSLTCTVSGLSLT-NGIGWVRQAP      59
PBMCheavy6        MKHLRFFLFLVAAPTCVLSQVQMKVSGPGLLKPSQTLSLVCDVSGFPLTDYDVGWVRQAP      60
PBMCheavy8        MSHLWFFLFLMAAPTCVLSQVQLKESGPSRVKPSQTLSLTCAVSGLSLDSNAAGWVRQAP      60
PBMCheavy9        MRPLWFFLFLVAAPTCVLSQVQLKESGPGLVKPSQTLSLTCTVSGFSLSSYGVGWVRQAP      60
PBMCheavy11       MNHLWFFLFLVAAPTCVLSQVILKESGPGLVKPSQTLSLACTVSGTTLSNVAVGWVRQGP      60
                  *  * **** :********** :: ***. :********.* ***  *     *****.*

ADK09420          GKGLEYVGEITRSG---SANYNPALKSRASITKDTSKSQVYLTLDSLTGEDTAVYYCARH      117
PBMCheavy3        GKRLQWIGDAGVGGRVNSVNINPVLKSRANITWVEAKRQVYLTLNRLTGEDTAVYYCASF      119
PBMCheavy6        GRGLEFVAGIWANG--RDANMPPNLISRARISRDVEKSQVYLSLSDLTDVDTAVYYCARG      118
PBMCheavy8        GKGPEYVAGIASNG---GPNYNPALKSRASITRDTSKSQVYLTLNSLTSEDTAVYYCAGG      117
PBMCheavy9        GKGLEYVGTIVIKN--GNAMYNPALKSRASITKDTSKSQVYLTLNSLTGEDTAVYYCVRS      118
PBMCheavy11       GKGLEWVG-LDGRG--GYGQYSPALKSRADITRDVSKSQIYLTMTGLTREDAAVYYCVGD      117
                  *:  :::.     .        * * *** *:    * *:**::  **  *:*****.

ADK09420          YMD-----------SFLFEYWGQGTLVTVSSESTMTPDLFPLVSCGPSLDESLVAVGCLA      166
PBMCheavy3        RSVNSVGMV------TYFEYWGQGILVSVSEASTTAPKVFPLASHSAATSGSTVALGCLV      173
PBMCheavy6        SE------HGW----YSFDYWGQGTLVTVSSASTTPPKVFQLASHSAGTSDSTVALGCLV      168
PBMCheavy8        GAVYK---EGW----LEIMYWGQGIMVTVSSASTTAPKVFPLAPSCGTTSDSTVALGCLV      170
PBMCheavy9        GFDSNWGSRAWFSVHYSMDSWGQGTLVTVSSASTTPPKVFQLASHSAGTSDSTVALGCLV      178
PBMCheavy11       DNFDKG-------FAEGIKYWGQGIPVTVSTASTTAPKVFALAPGCGTTSDSTVALGCLV      170
                             :  ****  *:**  **  *.:* *.      . * **:***.

ADK09420          RDFLPK------------------------------------------------------      172
PBMCheavy3        SSYFPEPVTVSWNSGALTSGVHTFPSVLQSSGLYSLSSMVTVPASSLKSQTYICNVAHPA      233
PBMCheavy6        SSYIPEPVTVSWNSGTLTSGVHTFPSVRQSSGLYSLSSMVTVPASSLKSQTYICNVAHPA      228
PBMCheavy8        SSYFPEPVTVSWNSGTLTSGVRTFPSVLQSSGLYSLSSMVTVPASSLESKTYICNVAHPA      230
PBMCheavy9        SSYFPEPVTVSWNSGALTSGVHTFPSVRQSSGLYSLSSMVTVPASSLKSQTYICNVAHPA      238
PBMCheavy11       SGYFPEPVKVSWNSGSLTSGVHTFPSVLQSSGFYSLSSMVTVPASTWTSETYICNVVHAA      230
                   .::*:

ADK09420          ----- 172
PBMCheavy3        SNSKV 238
PBMCheavy6        SSTKV 233
PBMCheavy8        SNIKV 235
PBMCheavy9        SNIKV 243
PBMCheavy11       SSTKV 235
```

Figure 5.3: Equine heavy chain variable region sequences recovered by semi-nested PCR amplification of cDNA from an unseparated population of PBMC. Alignment of IgG heavy chain V-region sequences from the PBMC that generated both heavy and light chains. PCR products were cloned into TOPO vector and sequenced using primers flanking plasmid insertion site. PCR products were verified as equine heavy chain by comparison to published sequence (ADK09420) using 75% homology as a cut-off (60). Entire sequenced V region and partial constant region is shown. CDR regions are in bold underlined. Constant region is downstream of CDR3, which is highlighted in bold only. Residues are highlighted at the bottom of each line as conserved (*), highly similar (:), similar (.) and gaps (-) inserted to maximise alignment. Highly similar residues have similar biochemical properties, similar have some shared biochemical properties (175, 176).

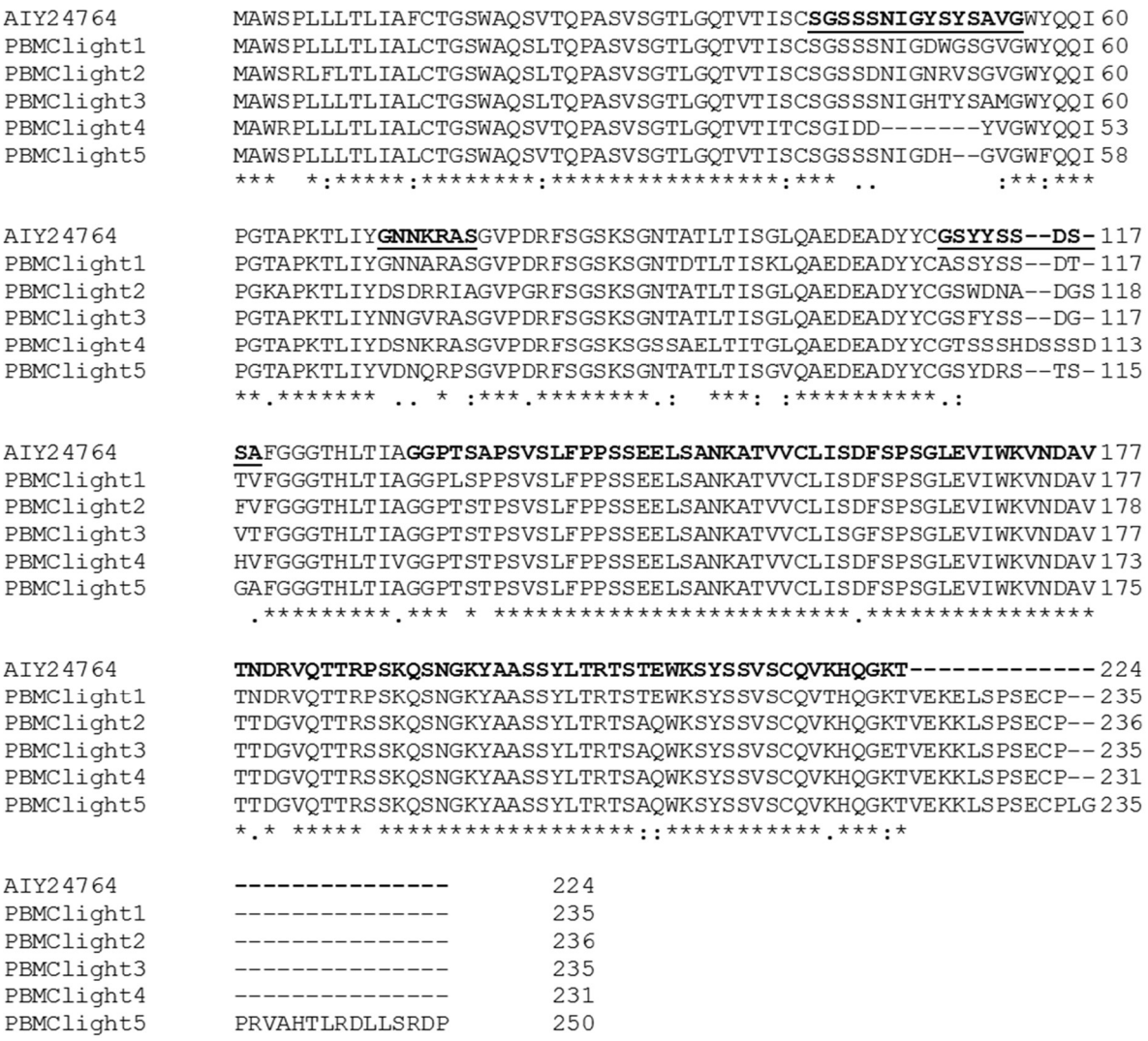

Figure 5.4: Equine light chain variable region sequences recovered by semi-nested PCR amplification of cDNA from an unseparated population of PBMC. Alignment of lambda light chain sequences from the PBMC used to generate both heavy and light chains. PCR products were cloned into TOPO vector and sequenced using primers flanking plasmid insertion site. PCR products were verified as equine light chain in comparison to published sequence (AIY24764) using 75% homology as a cut-off (60). Entire lambda light chain V region and constant region is shown. CDR regions are highlighted in bold and underlined. Constant region is downstream of CDR3, which is highlighted in bold only. Residues are highlighted at the bottom of each line as conserved (*), highly similar (:), similar (.) and gaps (-) inserted to maximise alignment. Highly similar residues have similar biochemical properties, similar have some shared biochemical properties (175, 176).

As shown in Figures 5.3 and 5.4, five different heavy and 5 different light chain sequences, respectively, were amplified from a mixed population of PBMC. Leader sequence is shown by the starting methionine

residue. The three CDRs and constant region are also shown. Each of the sequences had different CDRs, with CDR3 having a high degree of variability. Additionally, the heavy chain sequences were of the IgG subtype of antibody (65).

5.2.2 Cloning of recombinant chimeric equine antibodies

Having the capacity to amplify equine heavy chains (IgG) and equine (lambda) light chains, the next step was to clone the heavy chain and light chain into expression vectors that enable production and secretion of recombinant antibodies. The strategy for cloning heavy chain and light chain PCR products is shown in Figure 5.1.

Using previously sequenced heavy chain and light chain containing TOPO plasmids from 5.2.1, cloning was achieved by ligating the heavy chain insert into the heavy chain vector. The heavy chain insert was comprised of the heavy chain variable region and exon 1 of the IgG constant region. Exon 1 of the constant region was chosen because all equine IgG constant regions were recognized to contain a common Xcm1 enzyme site. This Xcm1 enzyme site was also present in exon 1 of the rat IgG heavy chain vector. The ligation created a full length chimeric horse-rat heavy chain. The horse light chain was ligated into an empty expression vector, which contained no light chain insert. A schematic for determining successful ligation is shown in Figure 5.5.

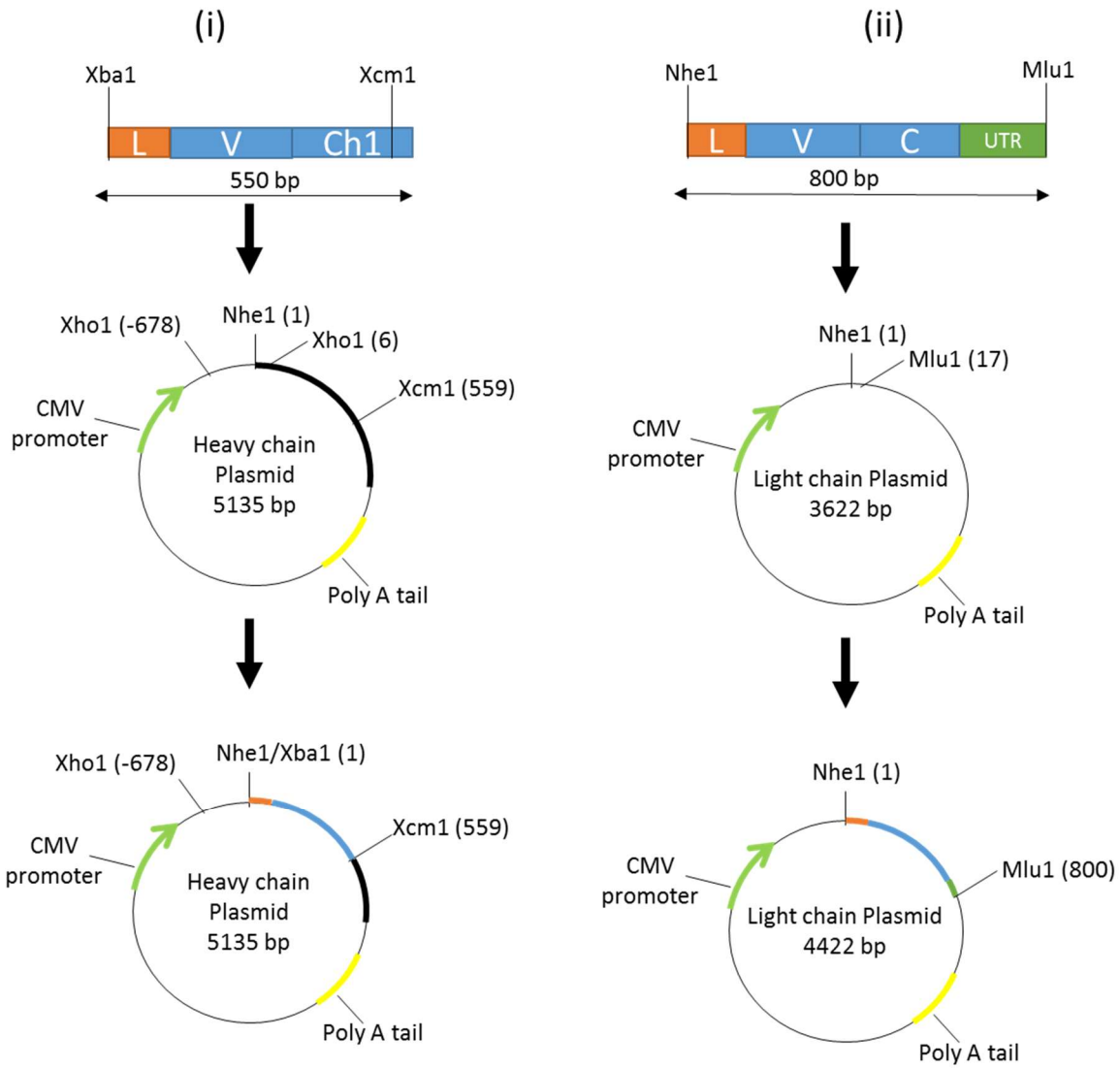

Figure 5.5: Schematic of successful ligation strategy. With the heavy chain IgG (i), the expectation from the successful ligation of the insert is a single 1237 bp product following digestion with Xho1 and Xcm1, due to the removal of the vector Xho1 site adjacent to the Nhe1 site during cloning. With the light chain (ii) the expectation from the successful ligation of the insert is a single 800 bp product following digestion with Nhe1 and Mlu1 digestion. Inserts were comprised of Leader (L), Variable region (V) and constant region (C), with the Light chain insert containing an Untranslated Region (UTR). Restriction enzyme cut sites are shown at ends of the inserts. Restriction enzyme cut sites on plasmids are shown with their relative position (in base pairs) to the Nhe1 site. Poly A tail and CMV promoter are shown.

As demonstrated in Figure 5.6, successfully ligated heavy chain Vh region into the heavy chain expression vector was demonstrated by the removal of an internal Xho1 cut site that is present in the parent plasmid. Further, determination of the light chain being successfully ligated was made by the presence of an appropriately sized insert.

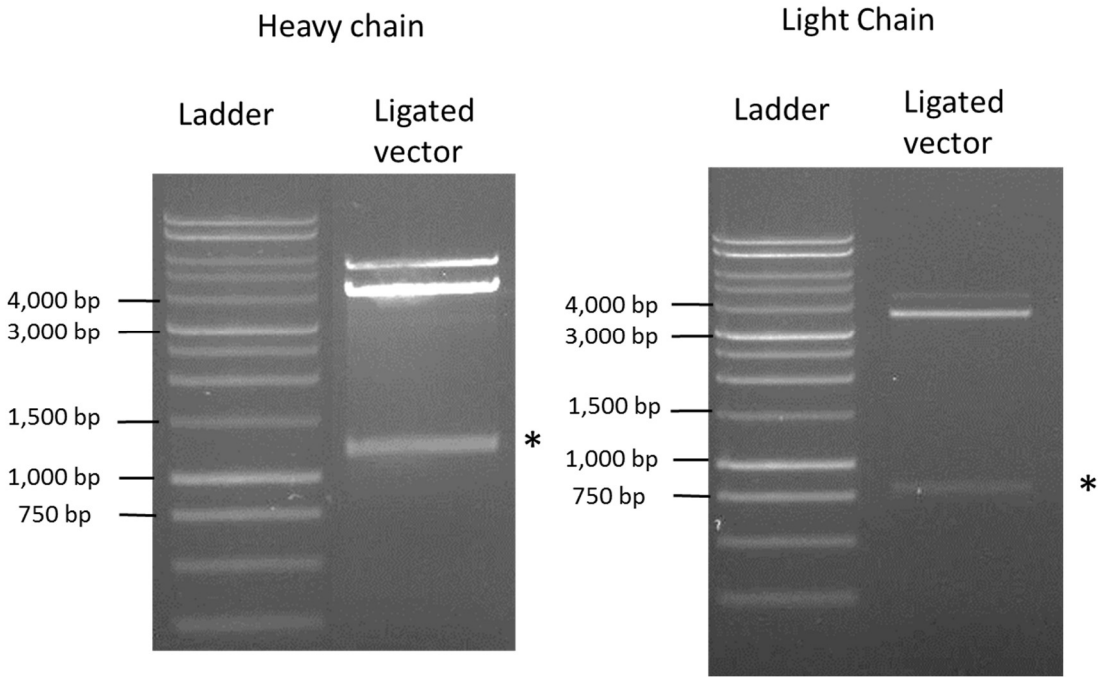

Figure 5.6: Confirmation of successful ligation of heavy chain and light chain into expression vectors. Restriction digestion of ligated heavy chain and light chain plasmids. For the heavy chain gel, plasmid containing ligated equine heavy chain variable region is shown, following digestion with Xho1 and Xcm1. For the light chain gel, ligated chain plasmid is shown, following digestion with Nhe1 and Mlu1. * denotes the presence of the expected heavy or light chain inserts. Molecular ladder and size of bands in base pairs (bp) is shown at left.

5.2.3 Production and detection of recombinant chimeric equine antibodies

With the successful ligation of equine heavy chain V region and lambda light chain, into their respective expression vectors, the next step was to demonstrate production of functional antibody from these ligated plasmid constructs, i.e. recombinant horse antibodies. To test this, I performed a transient transfection of the Chinese Hamster Ovary (CHO) cell line, which is commonly used to produce recombinant antibodies (177, 178), with 20,000 to 100,000 seeded CHO cells per well in a 6 well plate. Fugene 6 transfection reagent (Promega) was used to mediate the transfection, following the manufacturer's instructions, with around 1 µg of DNA for each plasmid.

The parent plasmids containing the original rat heavy chain and its light chain partner were also transfected to act as a positive control for antibody production. I also included an "empty" transfection no DNA as a negative control in parallel. The CHO cells were cultured for 4 days after transfection, and antibody

production into the supernatant was assessed via an ELISA for rat IgG. As shown in Figure 5.7, some antibody was produced with the ligated horse Ig-expressing plasmids, although it appeared less than the positive control plasmids encoding a fully rat IgG. No test of statistical significance was performed, due to the small sample size.

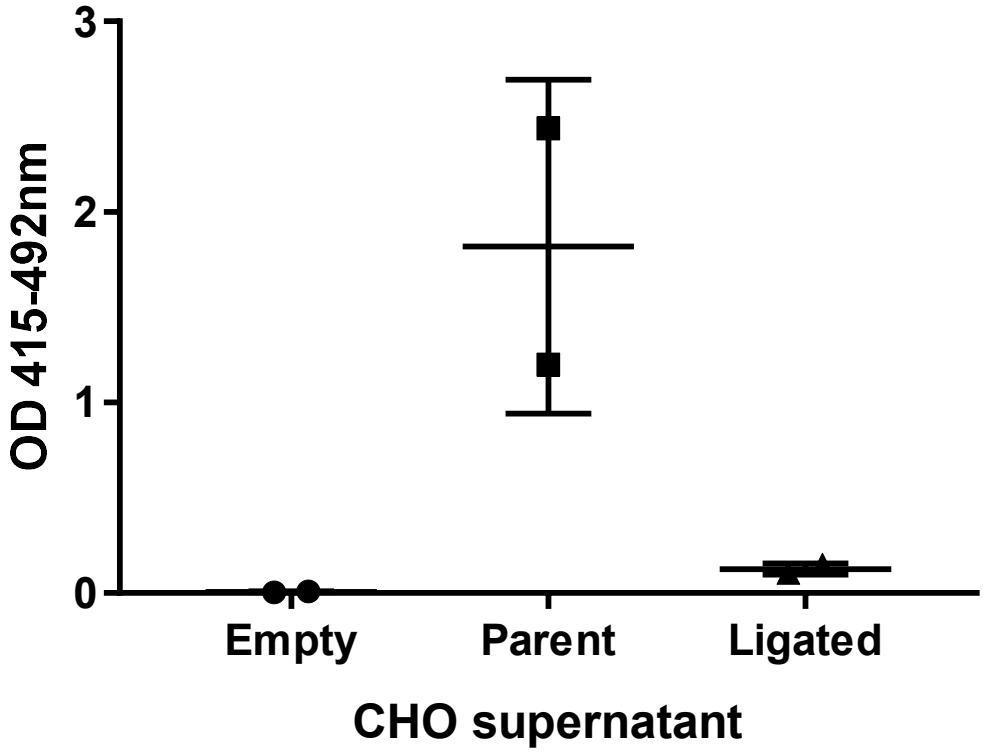

Figure 5.7: Production of recombinant horse immunoglobulin following transfection of CHO cells. Supernatant from CHO cells transfected with either Empty (no DNA), parental rat IgG expressing plasmids or recombinant horse plasmids, was probed for antibody production using an ELISA specific for rat IgG constant region. Graph is from one experiment with duplicate wells, plotting mean ± range.

5.2.4 V gene PCR and sequencing of single equine B cells

The next step towards creating a system for the isolation of monoclonal antibodies from horses was to determine if the cloning approach was sensitive enough to isolate and amplify the rearranged heavy chain V region and the full length rearranged lambda light chain from a single B cell.

A single B cell sort was carried out, using the anti-CD4/CD8 and anti-CD21 antibodies as described in chapter 3. A set of 36 $CD4/CD8^-$ $CD21^+$ individual B cells was sorted into the wells of 96 well plates containing lysis buffer. The whole RNA, prepared as per chapter 2, was then converted to cDNA using

Superscript II with random hexamer primers and PCR performed as above in 5.2.1 starting with 2.5 µL of cDNA, which was one tenth of the total. The light chain PCR was performed first, because 92% of all equine B cells contain lambda light chain (63, 67). Of the 36 single sorted cells, 10 were found to generate a light chain product (27.7% recovery rate). The heavy chain semi-nested PCR was performed on these 10 single cells using reverse primers specific for IgG. Overall, 5 single cells were found to have generated an IgG heavy chain product, resulting in a 50% frequency of IgG expression among the IgL PCR-positive B cells. Sequences of heavy chain VDJ and light chain VJ rearrangements from the 5 single, double PCR positive cells are shown in Figures 5.8 and 5.9, respectively.

Performing sequence analysis, using NCBI BLAST to compare the heavy chain V-region sequences from the single sorted cells to the NCBI databases, revealed that the 5 single cells had between 79-95% nucleotide sequence identity to published sequences, with V-Heavy22 having the greatest (95%) sequence identity with other heavy chain sequences, such as ADK09420. Similarly, analysis of the Light chain V-region sequences revealed that the 5 single cells had between 84-95% sequence identity to published sequences, with V-Light6 and V-Light23 having the greatest (95%) sequence identity. The CDRs were found to be the regions of highest diversity with CDR3 having the greatest variation.

```
ADK09420      MSHLWFFLFQVAAPTCVLSQ-----VQLKESGPGLVKPSQTLSLTCTVSGFSLSGYDVGW  55
Heavy6        MNHLWFFLFLVAAPTCVLSQ-----VQLKESGPGLVKPGQTLSLGCTVSGPTLRDYGVGW  55
Heavy15       MSHLWFFLFLVAATCCVVSQ-----AALKESGPGLMKPSQTLSLTCTSSDFVLRDHCIIW  55
Heavy22       MSHLWFFLFLVAAPTCVLSQ-----VQLKESGSGLVKPSQILSLTCTVSELSLSSNTVGW  55
Heavy23       MNHLWFFLFLVAAPTCVLSQ-----VQLKESGPGLVKFSQTLSLTCTVSGLSWNRDAVGW  55
Heavy24       MNHLWFFLFLVAAPTCVLSQWQLPQAQLKESGPGLVKPSQTLSLTCTVEGMSFGTFGVTW  60
              *.******* ***  **:**     . ***** **:* .* *** ** .       : *

ADK09420      VRQAPGKGLEYVGEITRSGSANYNPALKSRASITKDTSKSQVYLTLDSLTGEDTAVYYCA  115
Heavy6        VRQAPGKGLEFIGAIGSVGR-VVNPALKSRASVGKDTAQSQVYLTLDSLTTEDTAVYYCV  114
Heavy15       VRQRPGQGLEYVGDIEGSGSTNYHPALKSRASITKDNSLGRIYLTLNDLTGEDTAVYYCA  115
Heavy22       VRQAPGKGLEYVGLIASSGLTNYNPALKSRVSITKDASKSQVYLTLNSLTSEDTAVYYCA  115
Heavy23       VRQAPGKGLEWVGDVEGDGTINYAPALKSRASITTDTSKMQLFLTLNSLTSVDTAVYYCA  115
Heavy24       IRQVPGKGLEFVAALGSSDSVSYNPDLKSRATISIDGSKRKVILTLDSLTSDDTAVYYCA  120
              :** **:***::. :   .     * ****.::  * :  :: ***:.**  *******.

ADK09420      RHYMD---------SFLFEYWGQGTLVTVSSESTMTPDLFPLV-SCGPSLDESLVAVGCL  165
Heavy6        GGPDGFGS-TYFAYYRGIVYWGQGFLVTVSSASTTAPKVFALAPGCGPHLHTPP------  167
Heavy15       RLPTRYGYGSVNVFNYGVDDWGQGILVTVSSASTTAPKVFPLASHSAPHLLQRPP-----  170
Heavy22       GWEN----------LYDFAYWGQGALVAVSSASTTAPKVFALAPGCGPHLIHGP------  159
Heavy23       GARYT-GA-PSTSLIGGIDYWGQGTLVTVSSASTTAPKVFALAPGCGPHLPHYP------  167
Heavy24       GGIYGNDG-FYHFYTHALLWWGQGTLVIVSSASTTAPKVFALAPGCGPHLTTRP------  173
                           .  **** ** *** ** :*.:* *.  ..* *

ADK09420      ARDFLPK  172
Heavy6        -------  167
Heavy15       -------  170
Heavy22       -------  159
Heavy23       -------  167
Heavy24       -------  173
```

Figure 5.8: Equine heavy chain V-region sequences from FACS sorted, single B cells. Alignment of IgG heavy chain VDJ sequences from the five cells that generated both heavy and light chain PCR products. VDJ sequences, amplified by semi-nested PCR from cDNA of single sorted B cells, were sequenced with the final reverse primer. PCR products were verified as equine heavy chain by comparison to published sequence (ADK09420) using 75% homology as a cut-off (60). Entire sequenced V region and partial constant region is shown. CDR regions are in bold underlined. Constant region is downstream of CDR3 and is highlighted in bold only. Residues are highlighted at the bottom of each line as conserved (*), highly similar (:), similar (.) and gaps (-) inserted to maximise alignment. Highly similar residues have similar biochemical properties, similar have some shared biochemical properties (175, 176).

```
AIY24764      MAWSPLLLTLIAFCTGSWAQSVTQPASVSGTLGQTVTISCSGSSSNIGYSYSAVGWYQQI  60
Light6        MAWCLLFLTLIALCTGSWAQSLTQPGSVSGTLGQTVTISCSGSSSNIGHSTSAVGWYQQI  60
Light15       MAWCLLFLTLIALCTGSWAQSLTQPTSVSGTPGQTVTISCSGTSSNIGWEWSTVAWYQQV  60
Light22       TAWCRLLLTLIALCTGSWAQSVTQPASVSGTLGQTVTISCSGSSSNIGKYYA--VWFQQI  58
Light23       TAWCLLFLTLIALCTGSWAQSLTQPASVSGTLGQTVTISCSGSTSNIGNAYGLVGWYQQV  60
Light24       TAWCLLFLTLIALCTGSWAQSLTQPASVSGTLGQTVTISCSGTDSNIGHRVGAVGWYQQV  60
               **. *:*****:********:*** ***** **********: ****   .   *:**:

AIY24764      PGTAPKTLIYGNNKRASGVPDRFSGSKSGNTATLTISGLQAEDEADYYCGSYYSS-DSSA  119
Light6        PGTAPKTLIYENNKRASGVPDRFSGSKSDNTATLTISGVQAEDEADYYCSAGDSTDSSTV  120
Light15       PGTAPRTIIWRDHARTAGVPDRFSGSKHGNTATLTISGVQEEDEAVYYCATGDNTLRMFL  120
Light22       PGTAPKTLIHDSDKRASGVPERFSGSASGNTATLTISGVQAEDEADYYCTAYDEESNSHV  118
Light23       PGTALKTLIIRTNTRASGVPDRFSGSKSGNTATLTISGVQAEDEADYYCASGDSALG-LV  119
Light24       PGTAPKTVIWNTEERASGVPDRFSGSKSGNTATLTISGIQAEDEAIYYCAAGDSGLRTDV  120
               **** :*:*   . *::***:*****  .*********:* **** *** :  .

AIY24764      FGGGTHLTIAGGPTSAPSVSLFPPSSEELSANKATVVCLISDFSPSGLEVIWKVNDAVTN  179
Light6        FGGGTHLTIAGGPTSTPSVSLFPPSSEELSANKATVVCLISDFSPSGLEVIWKVNDAVTT  180
Light15       FGGGTHLTVAGGPTSTPSVSLFPPSSEELSANKATVVCLISDFSPSGLEVIWKVNDAVTT  180
Light22       FGGGTYLSIAGGPTSTPSVSLFPPSSEELSANKATVVCLISDFSPSGLEVIWKVNDAVTT  178
Light23       FGGVTHLTIAGGPTSTPSVSLFPPSSEELSANKATVVCLISDFSPSGLEVIWKVNDAVTT  179
Light24       FGGGTHLTVAGGPTSTPSVSLFPPSSEELSANKATVVCLISDFSPSGLEVIWKVNDAVTT  180
               *** *:*::******:*****************************************.

AIY24764      DRVQTTRPSKQSNGKYAASSYLTRTSTEWKSYSSVSCQVKHQGKT-----------      224
Light6        DGVQTTRSSKQSNGKYAASSYLTRTSAQWKSYSSVSCQVKHQGKTVEKKLSPSECP      236
Light15       DGVQTTRSSKQSNGKYAASSYLTRTSAQWKSYSSVSCQVKHQGKTVEKKLSPSECP      236
Light22       DGVQTTRSSKQSNGKYAASSYLTRTSAQWKSYSSVSCQVKHQGKTVEKKLSPSECP      234
Light23       DGVQTTRSSKQSNGKYAASSYLTRTSAQWKSYSSVSCQVKHQGKTVEKKLSPSECP      235
Light24       DGVQTTRSSKQSNGKYAASSYLTRTSAQWKSYSSVSCQVKHQGKTVEKKLSPSECP      236
               * ***** ******************::*****************
```

Figure 5.9: Equine light chain V-region sequences from FACS sorted, single B cells. Alignment of light chain VJ sequences from the five cells that generated both heavy and light chain PCR products. VJ sequences, amplified by semi-nested PCR from cDNA of single sorted B cells, were sequenced with the final forward primer. PCR products were verified as equine light chain in comparison to published sequence (AIY24764) using 75% homology as a cut-off (60). Entire lambda light chain V region and constant region is shown. CDR regions are highlighted in bold and underlined. Constant region is downstream of CDR3 and is highlighted in bold only. Residues are highlighted at the bottom of each line as conserved (*), highly similar (:), similar (.) and gaps (-) inserted to maximise alignment. Highly similar residues have similar biochemical properties, similar have some shared biochemical properties (175, 176).

5.3 Discussion

5.3.1 Demonstration of single cell PCR of immunoglobulin from equine B cells

Due to the lack of reagents and the inconsistency of culture methods, a single cell cloning approach and PCR amplification of Ig genes was undertaken. This approach has been used extensively to study the antibody response to influenza (84, 95, 98, 99) and HIV (87-89). More broadly, single cell PCR approaches have been used to develop monoclonal antibodies in species other than humans, such as rabbits (179) and

mice (180). Although the literature on equine B cells is sparse compared to that of mouse and human (108), there are publications detailing the heavy chain and light chain variable region genes of equine B cells (58, 59, 63, 65, 67). However, to the best of my knowledge, there has not been a study that demonstrated the isolation of antibody genes from single equine B cells. Therefore, the research presented within this thesis may be the first demonstration of antibody gene amplification from equine B cells at a single cell level. This can represent a new tool for immunologists to better study equine B cells, which are less studied than other equine immune cells, such as T cells (58, 108). When the initial indications achieved from the flow cytometry and *in vitro* cell culture approaches, investigated in chapters 3 and 4, respectively, are progressed to the point at which venom-specific B cells can be isolated, this PCR approach is a system by which their BCR can be unambiguously determined. In a recent study by Tallmadge *et al.*, the researchers were able to bulk isolate antigen specific B cells from equine PBMC (100), using fluorochrome conjugated antigens of either KLH or influenza, and then amplify the antibody genes. However, they did not carry out single cell sorting and V gene amplification, which would enable a reconstruction and characterisation of the complete BCR from antigen specific B cells, and specifically map the heavy and light chain pairs used by the individual cells.

5.3.2 Sequences of IgG heavy chain and light chain variable genes from single B cells

The antibody heavy chain and light chain sequences were found to share between 79-95% sequence identity to published sequences. Most of the differences occurred within the highly variable CDRs and in particular CDR3, which is the part of the antibody that has the most sequence variability (68). However, to determine if the sequences I recovered are from B cells that have undergone high amounts of somatic hypermutation and affinity maturation, the ideal approach would be to compare our sequences to those of the corresponding germline sequences from the originating horses (58, 181). These germline sequences in principle could be obtained from non-B cells sorted from the same PBMC samples, such as the T cells present in horse PBMC. This can give insight into how variable the B cell sequences are from a multi-immunised horse, compared to a naïve horse.

The recovery of IgG in the 10 single cells at a 50% frequency is an intriguing finding, since from my ELISPOT data from chapter 3, IgG secreting cells were at a frequency of less than 1% from sorted B cells. From the published literature on mouse or human B cells, when measured for antigen specificity in ELISPOTs, the frequency of IgG ASCs is less than 2% (112, 132, 136, 137), so although these results come from a single experiment, this is a high frequency of IgG expressing B cells, more than that of Human or Mouse studies (112, 113, 132, 136, 137, 182). However, there are at least four possibilities to account for this high frequency. One is that the high frequency was due to the use of the specific antigen, i.e., snake venom. The PBMC were isolated from horses that were boosted with snake venom a week prior. This boosting would

have presumably resulted in an increase in circulating IgG expressing B cells, which Hallilley *et al.* found 5-8 days after antigenic boosting in humans for example (132), and also in other examples from humans (136) and mice (112). A high frequency of circulating IgG B cells would not be unfounded following snake venom immunisation, since it has been reported in the literature that in the case of snake venom immunisation, IgG antibody was found to be at higher frequency and longer duration than IgM in serum (3, 4, 183-185). A second possibility might involve the immunisation process. Specifically, this process at Seqirus involved eight immunisations, starting from a very low dose (0.1 mg), through progressively higher doses, at different anatomical locations of the horses. This multi-dose and multi-site immunisation may be responsible for the high frequency of circulating IgG antibody. Other groups (186, 187) found high yields of antibodies via this method. A third possibility is that the high recovery arose as a statistical anomaly of small sample size. That is, if 100 or 1000 single B cells were isolated and probed for their heavy chain subtype, the mean frequency of IgG may be closer to the real mean. This statistical anomaly can be resolved by repeating the experiment. A fourth possibility may be that 50% of circulating horse B cells express IgG as a normal frequency, but this would suggest horses are substantially shifted from other mammals, since in prior studies of human B cells, IgG is usually less than 2% of blood B cells (132, 136, 137).

5.3.3 Chimeric recombinant horse antibody production

The recombinant antibody transfections yielded a low amount of antibody in culture supernatant, although it trended to be greater than the empty group. However, this low yield could be the result of a lack of secretion of the antibody produced in the cells, an issue that can sometimes occur with antibody production in CHO cells (177). To determine if this was the case, an intracellular flow cytometry assay could be performed on the transfected CHO, to detect the presence of non-secreted antibody. The low yield of recombinant antibody in my experiment could be because of a processing problem in the CHO cells. Flow cytometry was used in a study by Xu *et al.*(188) to detect the presence of their recombinant human/mouse chimeric CD19 antibody within cells, but not in the supernatant (188). If this turns out to be the case, that antibody is retained inside the CHO cells, the approach of Le Fourn *et al.* (177), may be warranted as a way to increase yield in the suprenatant. In this case, they modified their CHO cells' secretory and processing pathways by over-expressing human signal receptor protein SRP14. They found this approach lead to a high yield of recombinant trastuzumab and infliximab antibodies, by "rescuing" the processing and secretion of CHO cells overexpressing exogenous IgG protein (177). Another possibility for the low yield may be due to having chosen at random the heavy chain and light chains for our recombinant antibody, resulting in a suboptimal pairing of the complete antibody (84). However, studies by Brezinschek *et al.(189)* and Novobrantseva *et al.*(190), in humans and mice respectively, found that random heavy chains and light chains could usually pair and make productive antibodies. Therefore, a random horse heavy chain and light

chain is also likely to pair, so this random association may not be the cause of the low yield. A third possibility is that the chimeric nature of the antibody (equine heavy variable region with rat heavy chain constant region) leads to inadequate formation of antibody (191). To circumvent this, it may be necessary to clone in the remainder of the equine heavy chain constant region, to create a fully equine recombinant antibody or to use a rat lambda light chain.

5.3.4 Concluding statements

Isolating a single B cell and amplifying equine antibody genes is not a simple procedure compared to the more established human immune system (84, 87, 88, 99). To be able to perform the method in the relatively unknown equine B cell system is further complicated by the lack of verified reagents and less characterised equine antibody gene sequence database. Despite these challenges, single B cell PCR amplification of unique heavy chain and light chain antibody genes was achieved, demonstrating that the method could be applied to the characterisation of equine anti-venom responses and the isolation of monoclonal antibodies to Australian snake venom.

6. Discussion

6.1 Overview

The antibody response to snake venom is an understudied phenomenon (3, 4), despite the treatment being developed over a century ago (40). The process to study the antibody response to snake venom begins with the isolation of B cells that make the specific antibodies. Progress has been made in this project in being able to isolate equine B cells from PBMC, despite the paucity in flow cytometry antibodies for equine surface markers. Following the isolation of B cells, the next step of the project involved attempts to create a cell culture system of activating B cells into ASCs. The cell culture experiments have so far yielded limited success in converting B cells into ASCs. The final part of this work involved the amplification and cloning of antibody heavy chain and light chain genes. Progress was achieved in this phase of the project, when the heavy chain and light chain genes of single B cells were able to be isolated, and subsequently cloned. This unprecedented success occurred in spite of the considerable challenges that arose from a lack of verified reagents with which to study equine antibody responses, as emphasised in the preceding chapters. Nevertheless, this project acts as a stepping stone towards a better understanding of the antivenom response to snake venom, and a method of investigating broader questions concerning the field of snake antivenom.

6.1.1 Monoclonal antibodies as a way to neutralise relevant snake venom toxin components

Current antivenom to snake bite is composed of a polyclonal mixture of antibodies (71). These antivenom antibodies are usually produced in horses, following immunisation with one or more snake venoms (71). The aim of the envenomation is to generate a sufficient amount of antibody to neutralise snake venom toxins (2, 71, 192). However, a major issue with the current process of generating snake antivenom is that many of the generated antibodies target non-toxic components within snake venom (192, 193). Therefore, many of the polyclonal antibodies administered to snake bite patients are not of therapeutic benefit (192, 193). Further, each immunisation produces a different polyclonal mixture of antibodies that require testing and therefore the quality control of producing anti-snake venom medicines is a considerable burden (71). Characterised mixtures of monoclonal antibodies could alleviate this.

To counter the problem of irrelevant antibodies in snake antivenom, one method involves the development of monoclonal antibodies specific to toxic components in snake venom. Research by Frauches *et al.,* (83) highlights the utility in using monoclonal antibodies. In their research, Frauches *et al.,* developed three monoclonal antibodies to some venom components of the *Bothrops atrox* snake, which is a common cause

of snake bite injury in Brazil (83). The three monoclonal antibodies were evaluated for their capacity to neutralise the activity of 3 major components of *B atrax* venom. Frauches *et al.,* observed that the three monoclonal antibodies neutralised the lethal activity of their respective targets, but also observed that mice treated with the monoclonal antibodies survived envenomation. This study indicated that it is possible for a discrete number of monoclonal antibodies to provide protection against the envenomation of a particular snake.

While the study highlights the utility of using monoclonal antibodies as antivenom, one potential issue is venom toxin epitope variability within a species. As stated by Frauches *et al.,* a limitation of their three monoclonal antibodies is that there is intraspecific variability in the venom profile of *B atrax,* across the different regions in which the snake is located (194). As such, it is possible that the three monoclonal antibodies may not be effective when used against the venom of *B atrax* found in other regions. Therefore, for the development of neutralising monoclonal antibodies, it is necessary to know what the key toxins are, and ensure that the antibodies can target slight variations in the toxins.

6.1.2 Targeting cross reactive epitopes of venom toxins from multiple snake species

Snake antivenom is developed for the medically significant snake species in a given geographic region, usually as “monovalent” antivenom (2, 71, 193). Monovalent refers to the product derived from immunisation with venom from a single species of snake. One issue with the monovalent approach to antivenom design is that, antivenom developed for one species is typically more efficacious against that particular snake species. However, if in a geographical location there are multiple venomous snakes, there is an issue with delivering an appropriate medicine that can cover all possible species (71). To overcome this issue, antivenom manufacturers develop polyvalent antivenom for groups of medically significant snakes in a given geographic region (2, 71). However, the polyvalent approach necessitates a higher dosage of antivenom to be administered, leading to the higher possibility of allergic reactions to antivenom (195). Therefore there needs to be a better designed antivenom that can target multiple species of snakes, while being efficacious at an acceptable dosage.

Snakes are a diverse group of animals, and their venom arsenal is varied by multiple characteristics. These include the presence/absence of different toxin families, different concentrations of particular toxins, among other characteristics (6, 193). Despite the diverse number of toxin groups in any particular species, research by Jackson *et al.* in Australian snakes, revealed there is overlap in the type of toxins found in phylogenetically distant snake species (6). This means that there are possibly cross-reactive epitopes present in the distinct snake species that an antibody could recognise, which has been demonstrated in a number of studies (9, 16, 36, 38, 194, 196, 197).

In studies involving the common beaked sea snake *Enhydrina schistosus*, Tan *et al. (9, 36)* observed that venom lethality was neutralised using antivenom designed for Thai cobras. That is, there was cross reactivity and cross neutralisation of the antivenom to snake venom it was not originally designed for (9, 36). This indicated that there were epitopes that were similar enough to be recognised by antibodies in the heterologous antivenom. In a study of Australian snakes, Isbister *et al. (38)* demonstrated that rabbit antibodies against Brown snake venom were able to cross-neutralise the pro-coagulant effect of Taipan venom equally as well as Brown snake venom. This indicated that the pro-coagulant toxin components in Brown snake and Taipan were similar enough to induce cross reactive to antibodies developed for either snake. These studies indicate that cross neutralising antibodies are possible to be developed and isolated from immunised animals. However, these studies still involved a polyclonal mixture of antibodies.

6.1.3 Use of monoclonal antibodies to assess key toxin components

Snakes produce many distinct toxins as part of their venom arsenal, targeting various components to induce lethality (193, 198, 199). One of the major issues with current antivenom is that many antibodies are not therapeutic (192, 193). This is partly explained by the presence of non-lethal components of snake venom against which antibody responses are raised (192, 193). Determining which components of venom are the key toxins, and those which are non-toxic is an important, but challenging endeavour.

One approach to assess the key components of snake venom that are not neutralized in current antivenoms is termed antivenomics or immunodepletion assays (198, 199). In these assays, venom is incubated with antivenom antibodies, followed by a secondary antibody against the antivenom antibodies. The venom-antibody complexes are immunoprecipitated, and separated from venom that failed to be bound by antibodies. With the immunodepleted fraction, the fraction composed of venom that was not bound by antivenom antibodies, the identity of the remaining venom proteins can be determined by chromatography assays (194, 199, 200). Furthermore, the immunodepleted fraction can be tested for lethality, as a way to determine if the toxic components have been bound by the antivenom. There are studies that have demonstrated the capacity of monoclonal antibodies to neutralise specific toxins .e.g. for *B atrax* (83), *Naja kaouthia* (197), or even for cross neutralising toxins from multiple species, such as *Bothrops. jararacussu* and *Crotalus durissus terrificus* (196).To the best of my knowledge, the use of immunodepletion or antivenomics with snake venom has not been performed with monoclonal antibodies. The advantage with monoclonal antibodies is that they target only one protein in a complex venom mixture. By removing individual toxins from a mixture, the immunodepleted fraction could be assessed for lethality. If the immunodepleted fraction fails to induce lethality, this result would imply that the one toxin removed by the monoclonal antibody is sufficient to provide protection.

6.1.4 Developing antivenom in species other than horses

Current antivenom is largely developed from horses, because of their high yield of antibodies (71) and that horse antibodies are found to be effective and safe for both humans and veterinary use (73, 195). Although a useful animal to develop antivenom from, there are studies investigating the possible use of alternative animals to develop snake antivenom, including camelids (201-203) and chickens (204, 205).

Camelid (Camel, Llama and Alpaca) antibodies lack a light chain and are smaller than equine antibodies (202, 203). As such, camelid antibodies are found to be less immunogenic and less likely to trigger an allergic response (193, 206). In terms of generating anti-snake venom antibodies, camelids were found to have a similar immunological response to horses (207). A case report involving Tiger Snake envenomation on a dog, Padula *et al.* demonstrated the utility of camelid (alpaca) antivenom (201). The alpaca antivenom was able to successful treat the Tiger Snake bite, and that there was no complications from the camelid antivenom. Furthermore, camelid antibodies were found to be able to target epitopes that IgG from other mammals were not able to (208, 209). This is an interesting finding, because an issue with equine derived antivenom is that toxins with low immunogenicity, such as neurotoxins, are found to be poorly neutralised (16, 36). This is an important consideration in snake antivenom design, to target toxins that are important, but poorly immunogenic, such as neurotoxins in sea snakes (9, 16, 36). While camelid antibodies seem promising, they have yet to be tested in humans for safety and efficacy (193).

Chicken antibodies are considered an alternative to equine antibodies, primarily on the basis of manufacturing and cost saving parameters compared to developing antivenom in horses (205, 210-212). From an immunological perspective, chicken antibodies lack the capacity to activate mammalian complement system and do not interact with mammalian Fc receptors, avoiding the allergic issues that can occur with equine antibodies. While a promising alternative from a manufacturing/cost-saving perspective, there needs to be comparison between chicken antibodies and horse antibodies on their respective ability to neutralise snake venom. In a study of Taipan venom by Navarro *et al., (204)* equine and chicken derived antibodies were tested for their capacity to neutralise individual Taipan toxins, as well as neutralisation of lethality. Navarro *et al*., (204) observed that the chicken derived antibodies were less effective in neutralisation than the equine derived antibodies in their assays. However, their results could not be extended directly to other snake species, which they acknowledge (204).

While camelid and chicken antibodies show promise from theoretical, manufacturing, or pre-clinical studies, these alternative antibody sources need to address the fundamental question in snake antivenom research: are they more effective than current horse derived antivenom? The answer can be achieved with further investigation. Moreover, the regulatory hurdles with producing antivenom from an untried source should not be underestimated, nor the difficulty of conducting clinical trials in this setting (71).

6.2 Concluding remarks

Snake antivenom, derived predominantly in horses, remains a standard treatment for snake bite envenomation, even after a hundred years of development. While efficacious, the molecular and immunological development of snake antivenom is still largely unknown and understudied. Although this project achieved some modest aims, it acts as a stepping stone to study the antibody response to snake venom. With greater understanding, a better designed antivenom and refined process to developing antivenom can be developed, progressing from the initial antivenom discovery made over a century ago.

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