DEVELOPMENT AND EVALUATION OF OVINE POLYCLONAL ANTIBODY THERAPIES FOR THE TREATMENT OF SEVERE INFLUENZA INFECTION AND BACTERIAL SEPSIS

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LIST OF ABBREVIATIONS

µg	microgram
Ab(s)	Antibody(ies)
ADCC	Antibody-mediated cell cytotoxicity
AID	Activation-induced cytidine deaminase
ANOVA	Analysis of variance
APACHE II	Acute physiology and chronic health assessment II
APTT	Activated partial thromboplastin time
BALF	Broncho-alveolar lavage fluid
BCR	B-cell receptor
BMDC	Bone marrow derived dendritic cells
CARS	Compensatory anti-inflammatory response syndrome
CCS	Composite cytokine score
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CFU	Colony-forming units
CLP	Caecal-ligation and puncture
cRPMI	Complete Roswell Park Memorial Institute medium
CTL	Cytotoxic T lymphocyte
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C motif receptor 4
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DIC	Disseminated intravascular clotting
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FA	Freund's adjuvant
Fab	Antigen binding antibody fragment
FACS	Fluorescence-activated cell sorting
Fc	Constant fragment
FcR	Fc receptor

G-	Gram negative
G+	Gram positive
gDNA	Genomic deoxyribonucleic acid
GM-CSF	Granulocyte macrophage colony stimulating factor
HA	Haemagglutinin
HAI	Haemagglutination-inhibition
HMGB1	High mobility group box 1
ICAM-1	Intercellular cell adhesion molecule-1
ICU	Intensive care unit
IFN	Interferon
IL	Interleukin
IL-1RA	Interleukin-1 receptor agonist
IN	Intranasal
IP	Intraperitoneal
iRPMI	Incomplete Roswell Park Memorial Institute medium
IV	Intravenous
LPS	Lipopolysaccharide
mAb(s)	Monoclonal antibody(ies)
MAC	Membrane attack complex
MAP	Mean arterial pressure
MARS	Mixed antagonist response syndrome
MCP	Monocyte chemotactic protein
mg	Milligram
MHC	Major histocompatibility complex
min	minute(s)
mm	Millimetre
mRNA	Messenger ribonucleic acid
NA	Neuraminidiase
NF-κB	Nuclear-factor kappa-B
ng	nanogram
NorA	Noradrenaline (norepinephrine)
O/N	Overnight
OPD	Ortho-phenylenediamine
OSICS	Ovine sepsis with intensive care supports
pAb(s)	Polyclonal antibody(ies)
PAF	Platelet activation factor

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
pDC	Plasmacytoid dendritic cells
pg	Picogram
PR8	Influenza A/Puerto Rico/8/1934
PRR	Pathogen recognition receptor
PT	Prothrombin time
RAGE	Receptor for advanced glycation endproducts
RAH	Royal Adelaide Hospital
RAW	Murine macrophage-like RAW 264.7 cells
RBC	Red blood cell
RIG-1	Retinoic-acid inducible gene 1
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SC	Subcutaneous
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sf	Spodoptera frugiperda
SIRS	Systemic inflammatory response syndrome
SOC	Super optimal broth with catabolite repression
SOFA	Sequential organ failure assessment
SP-1	Specificity protein 1
SPF	Specific pathogen free
TCID	Tissue culture infective dose
TCR	T-cell receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TSB	Trypticase soy broth
VCAM-1	Vascular cell adhesion molecule 1
WBC	White blood cell
WHO	World Health Organisation

SUMMARY

Severe infections and their complications are growing health burdens in Australian society, with an increasing number of elderly, chronically ill and immunosuppressed people at high risk of contracting infections requiring costly critical care supports. While preventative measures for severe infections are undoubtedly important, there remains a need for additional therapies for such diseases where patients are dependent on supportive care. Passive immunotherapies utilising polyclonal antibodies (pAbs) are suitable platforms for the development of critical care therapies; they have a good safety profile and the broad reactive capacity of pAbs enable simultaneous targeting of multiple pathways. The application of ovine pAbs to the clinical scenarios of severe influenza infection and bacterial sepsis were explored herein.

Passive immunotherapy may have particular benefits for the treatment of severe influenza infection in at-risk populations; however the impact of passive immunotherapy on the formation of adaptive responses to the virus is unknown. Ovine pAbs were generated against H1N1 influenza haemagglutinin (HA) using different immunisation regimens and it was found that proprietary adjuvant CoVaccine HT^{TM} induced tenfold higher anti-HA pAb titres than Freund's adjuvant. Purified pAbs exhibited potent therapeutic capacity in a murine model of lethal influenza infection and treated mice generated normal antibody and cytotoxic T lymphocyte responses to the virus. These findings support the development of ovine pAbs for treatment of severe influenza infection.

Bacterial sepsis has heterogeneous causes and often a polymicrobial source of infection; thus the development of a broadly-acting pAb therapy for sepsis required a different approach. Consequently, pAbs were generated against high mobility group box 1 (HMGB1) protein, a late mediator of sepsis pathogenesis which was significantly elevated in non-surviving septic shock patients in a clinical study. Ovine anti-HMGB1 pAbs improved survival in murine endotoxaemia and caecal ligation and puncture models of sepsis, and altered early cytokine profiles in surviving mice to those associated with survival in the clinical cohort. Anti-HMGB1 pAb treated murine sepsis survivors were also more resistant to secondary bacterial infection and exhibited altered innate immune cell phenotypes and cytokine responses. In order to recapitulate the physiological manifestations of sepsis in a large animal, a pilot study was conducted using an ovine sepsis model incorporating intensive care support. While no significant impact of anti-HMGB1 pAb administration was observed, these studies provide a framework for future evaluation of anti-HMGB1 and other immunomodulatory therapies.

Together, these findings demonstrate that anti-HMGB1 pAbs reduce sepsis mortality without potentiating immunosuppression associated with survival and support the development of anti-HMGB1 therapy for sepsis treatment.

Collectively, these studies support two potential applications for ovine pAb therapies in infectious diseases: as potent antiviral agents and as immunomodulatory agents. These findings will assist in the development of passive approaches to the prevention and treatment of severe influenza infection in at-risk populations, and HMGB1-blockade therapies for the treatment of bacterial sepsis and septic shock. In addition to these disease scenarios, these findings may have relevance for the development of antiviral passive immunotherapeutics for emerging diseases, or for HMGB1 blockade therapy in other inflammatory conditions.

DECLARATION

I declare that:

this thesis presents work carried out by myself and does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; to the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, is clearly acknowledged.

Natalie Emilia Stevens

CONTRIBUTIONS

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CHAPTER 1.

GENERAL INTRODUCTION

1.1 Passive antibody immunotherapy

1.1.1 Overview

Antibodies (Abs) have been used for the prevention or treatment of infectious diseases for over a century. Disease-specific protection from convalescent serum was first noted in the 1890's and triggered widespread use of 'serum therapy' as the first application of passive immunotherapy [1]. Administration of disease-specific sera or serum fractions was the prevalent antimicrobial strategy until the discovery of sulphonamide antibiotics in the 1930's [2]. Thereafter, antibiotic use largely replaced passive immunotherapy strategies due to logistical advantages in their production and use. Similarly, the discovery and refinement of active immunisation for prevention of infectious diseases reduced the reliance on passive methods for infectious disease treatment [3]. Consequently, passive immunotherapy strategies were relegated to diseases where no other therapies or effective vaccines were available, such as viral infection and the neutralisation of toxins.

Fuelled by the discoveries of passive immunotherapy and immunisation, basic understanding of Ab structure and function grew remarkably in the mid 20th century. By the late 1970's, advances in physics and chemistry had led to the use of new techniques for the study and treatment of diseases. The subsequent development of monoclonal Ab (mAb) technology triggered a renaissance in the application of Ab therapies for a variety of conditions. The first mAb therapy approved for use was muromonab, a murine anti-CD3 Ab that was used to reduce organ rejection in transplant recipients [4] and since this time mAb therapies have been employed in the treatment and prevention of a wide variety of diseases. The ability to generate Abs with defined specificity and function has been particularly useful for the application to cancer [5] and inflammatory diseases [6], where Ab targets are often host proteins involved in cell signalling. However, polyclonal Ab (pAb) therapies generated by a natural immune response still maintain a niche role in the treatment of infectious diseases such as botulinum infection [7], cytomegalovirus [8] and vaccinia virus [9]. Intravenous immunoglobulin (IVIG) prepared from pooled sera of immunocompetent individuals is commonly used as replacement therapy for immunodeficient individuals to prevent infectious diseases, or to treat refractory infections [10]. Rapidly evolving pathogens with dynamic antigen expression are often not effectively controlled by an Ab of a single specificity, as identification of a cross-neutralising epitope is elusive particularly for emerging diseases, and pAb therapies can be applied in these scenarios. The discovery of serum therapy by Emil Behring, for which he was awarded the Nobel Prize in 1901, laid the foundations for further

work to describe the kinetics of the Ab response, allergy and rheumatic diseases, and antibody-mediated complement activation. The understanding of Ab generation and structure enabled the development of mAb technology and facilitated the advances in the application of pAb therapies.

1.1.2 The humoral immune system involves the selection and secretion of specific antibody clones of different isotypes

The production of a diverse pool of Abs is one of several immunological mechanisms which evolved to distinguish self from non-self for defence against foreign invaders and the maintenance of homeostasis [11]. The immune system can be divided into two arms: innate immunity, which can be rapidly inducted for non-specific defence against a range of pathogens; and adaptive immunity, which achieves specific defence with the formation of enduring immunological memory. The innate immune system is evolutionarily ancient - as evidenced by the existence of innate immunological receptors in a wide array of organisms including vertebrates, invertebrates and plants - and constitutes the dominant immune defence in most living organisms [12]. Innate immunity is conferred through the presence of physical barriers and signalling through pattern recognition receptors (PRR), which bind pathogen-specific carbohydrates, lipids and nucleic acids to instigate signalling cascades in innate immune cell types. Innate cells such as neutrophils, macrophages and natural killer cells are able to attack pathogens through phagocytosis, degranulation and release of lytic enzymes or by triggering apoptosis of foreign or infected cells. In higher order vertebrates, the innate immune system also serves another crucial purpose: the recruitment and facilitation of adaptive immunity. The adaptive immune response is broadly divided into two lymphocyte-driven functions: humoral mechanisms, which are mediated through B-cell bound and circulating Abs; and cell-mediated mechanisms which are mediated through direct action of T cells. The functionality of these lymphocytes rely on the broad antigen specificity of dedicated immunological receptors, with both T-cell receptor (TCR) and B cell receptor (BCR) repertoires generated by somatic mutation of encoding genes in the developing lymphocyte populations. Upon binding their cognate antigens through their receptors, lymphocytes undergo a series of cellular signalling events to induce proliferation and differentiation and form a pool of highly specific effectors. As the drivers of cell-mediated immunity, T cells engage with antigens in the context of major histocompatibility complex (MHC) on the host cell surface via their TCR. Antigens must be presented by MHC to interact with TCR, thus intracellular proteins or phagocytosed and presented extracellular particles are common targets of cell mediated immune responses. Extracellular antigens are

commonly targeted by B cells which differentiate into plasma cells which secrete their BCR in the form of a soluble immunoglobulin (Ig).

Naïve B cells reside in lymphoid follicles where they are exposed to a constant source of antigen through circulating lymphatic fluid. Upon activation through BCR binding exogenous antigen, B cells may differentiate into short lived Ab-secreting plasmablasts, however the majority migrate to the interfollicular region to undergo affinity maturation in the germinal centre reaction (GCR) [13]. Within the GCR, naïve B cells are exposed to either circulating antigen or antigen bound to follicular DC's and endocytose the antigen to present it to follicular T helper cells. The interaction with T helper cells is crucial to the activation of the B cell; T cells provide co-stimulation via CD40-ligand binding to expressed CD40 on B cells which proliferate in response. Proliferating B cells alter affinity for their antigen via mutations induced in the Ig variable (V) gene region which corresponds to the antigen-binding site [14]. These mutations occur at a rate approximately 1 million times higher than that found in normally dividing somatic cells, and is thus referred to as somatic hypermutation. Resultant cells compete for antigen displayed on follicular DC, with B cells expressing the highest affinity Ab able to acquire the most antigen to present to follicular T helper cells and receive signals essential to their survival. Reduced affinity clones are not selected for survival and subsequently undergo apoptosis; thus the polyclonal Ab response is shaped from a diverse pool of B cells [13]. Throughout the process, a proportion of activated B cells differentiate into memory B cells, which endure to provide immunological memory. These cells are responsible for the accelerated secondary Ab response following re-encounter with antigen (Figure 1.1), as they are able to rapidly proliferate to produce large populations of Ab-secreting plasma cells.

Mammalian Abs are classed into several isotypes based on structure. As 'class-switching' to a new isotype involves deletion of gene segments within the Ab heavy chain locus, individual B cells are generally committed to secretion of Ab of only one isotype. The initial encounter of membrane-bound BCR with antigen triggers production of plasmablasts that secrete Ab of the IgM isotype into circulating blood and lymph fluids; this constitutes the primary Ab response (Figure 1.1). These early IgM Abs generally have low affinity for antigen, however as IgM commonly circulates as a pentamer or hexamer it efficiently opsonises circulating antigen for phagocytosis. Additionally, antigen-bound IgM activates circulating complement proteins, which can either directly kill pathogens or stimulate further neutrophil recruitment or mast cell activation. Mature naïve B cells can co-express IgD alongside IgM, however the IgD isotype tends not to be secreted and serves as a cellular receptor for signal transduction.

Upon re-encounter with antigen the secondary Ab response, characterised by a second wave of B cell proliferation and class-switching of plasma cells to the IgG isotype, may be instigated. High-affinity monomeric IgG constitutes 75% of circulating Ig [15] and can perform multiple effector functions through binding Fc receptors. Additionally, IgG Abs are able to partake in a natural form of passive immunotherapy in pregnant mothers whereby they undergo placental transfer to provide fetal protection. After birth IgG is also supplied to the fetus through breastmilk, and are passed from the gut to the circulation to provide immunoprophylaxis in the first months of life. Additionally, IgG is the most common isotype to be exploited in monoclonal or polyclonal passive immunotherapeutics due to their established safety profile, and indeed all currently approved mAb therapies are of the IgG isotype [16].

The most abundant Ab isotype within breastmilk and other secreted fluids is IgA [17-20], and thus this isotype is a major component of immunity at mucosal surfaces. They are secreted by plasma cells within the mucosal tissues and are transported to the surface by Fc receptors on secretory epithelial cells where they exist primarily as dimers, though circulating IgA is generally monomeric. Passive immunotherapy based on an IgA subtype has been explored for the prevention of respiratory diseases such as tuberculosis [21] and influenza [22] and some cancers [23]. The least abundant Abs in the circulation are the IgE isotype, however these Abs are often bound to Fc receptors on circulating basophils and tissue-associated mast cells where they act as surrogate cellular antigen receptors. Signalling mediated through IgE plays an important role against the defence of parasitic pathogens [24, 25], but also mediates allergic type hypersensitivity [26]. Though no therapies consisting of IgE Ab are currently approved, the potent IgE-mediated response in allergy and their exceptional affinity for Fc receptors has prompted interest in IgE as the basis for mAb therapies for the treatment of malignancy [27, 28]. Depletion of IgE via anti-IgE Abs is currently employed for the treatment of allergic asthma and chronic idiotypic urticaria [29-31] and has also exhibited efficacy for other hypersensitivity conditions [32-34].

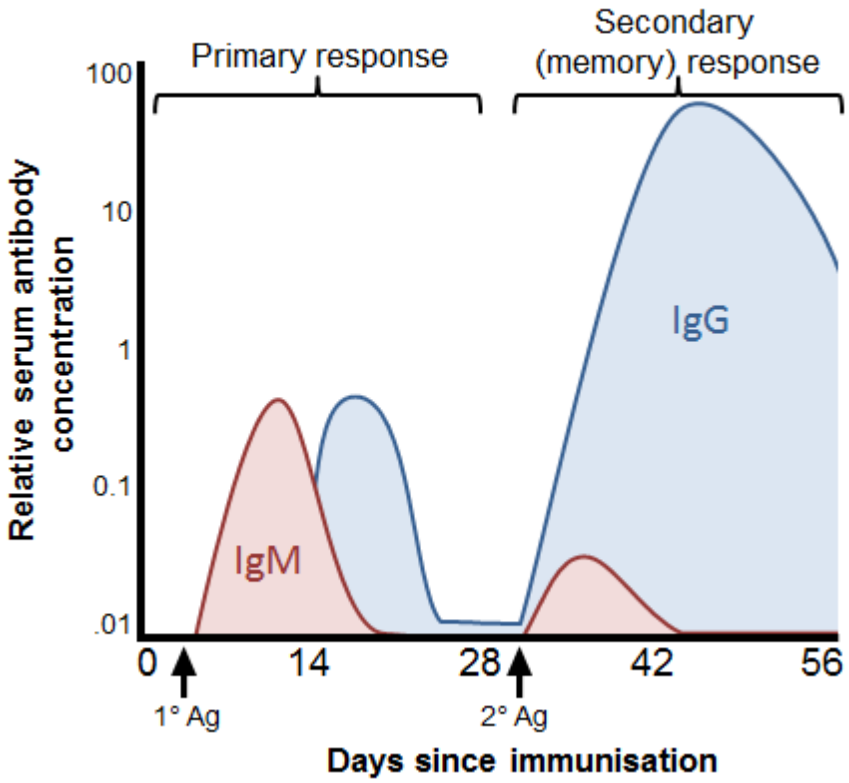


Figure 1.1 Primary versus secondary antibody responses

In the primary response to antigen, Abs of IgM and later of IgG isotype are secreted from plasmablasts and mature plasma cells. Upon re-exposure to the same antigen, memory B cells rapidly differentiate into plasma cells that produce a greater amount of Ab which is predominantly of the IgG isotype.

1.1.3 Structural antibody diversity is generated by genetic recombination and mutation

The general structure of the Ab molecule was determined as a consequence of several seminal studies in the late 1950's to early 1960's [35]. Experiments within this period by Rodney Porter focused on enzymatic cleavage of rabbit IgG with papain to yield three fragments of approximately 50,000 Da [36]. Two of these fragments appeared near-identical and retained antigen binding capacity, whereas the third fragment did not bind antigen but could be precipitated by anti-rabbit IgG Abs. Further work revealed that IgG Abs of different specificities had antigen binding fragments with heterogeneous structures but the third fragment was highly homogenous between Abs, and thus these fragments were later designated the antigen-binding fragment (Fab) and constant fragment (Fc) [37]. Parallel work by Gerald Edelman demonstrated that chemical reduction of the Ab structure yielded four smaller subunits comprised of two large heavy chains and two smaller light chains which were likely linked through disulphide bonds [38], and proposed and confirmed the Y-shaped

structure of the molecule (depicted in Figure 1.2). Edelman went on to solve the amino acid structure of a monoclonal human IgG sourced from a multiple myeloma patient [39]. Serum Abs sourced from myeloma patients were crucial to the study of antibody structure as they were monoclonal and could be crystallised, whereas Abs generated from immunisation were polyclonal which made their study difficult. Further studies demonstrated that heavy and light chains each contain a constant and variable region and that whole Abs are comprised of repeated Ig motifs which designate variable (V) and constant (C) domains [40]. Structural analyses of monoclonal Abs clones from myeloma patients revealed that different clones often had similarities within the C regions but diverse V regions and thus Ab specificity was found to be dependent on the amino acid sequence and subsequent structure of the V region. Studies of the polyclonal response revealed huge diversity in the Ab clones produced in healthy individuals, with over 10^{11} V-region clones potentially present in one individual.

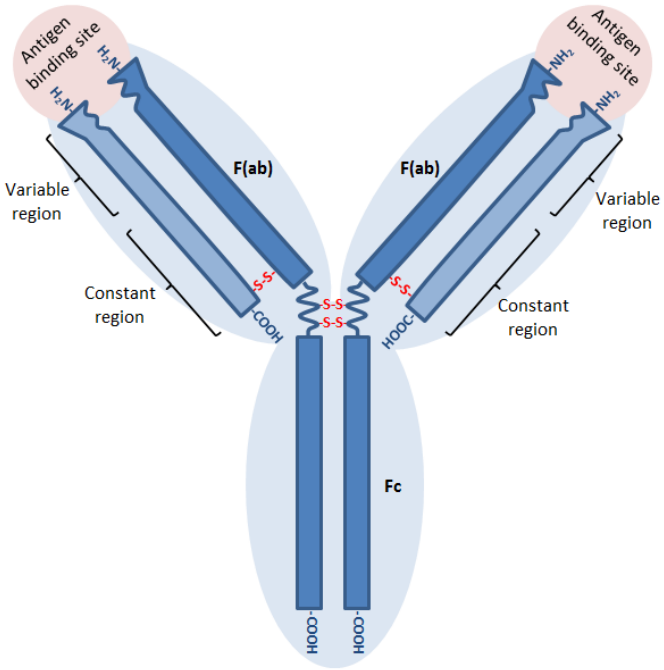


Figure 1.2 Structure of an antibody molecule.

Monomeric Ab are composed of four polypeptide chains: two heavy chains (dark blue) and two light chains (light blue). Antigen binding sites are each formed by the variable N-terminal regions of a light chain and a heavy chain. The C-terminal regions of the heavy chains form the tail (Fc region). The two Fab regions and Fc region are linked by a flexible hinge region that improves the ability of the Ab to bind antigen. (Adapted from Alberts et. al, 2002)

These early structural analyses confirmed that Ab specificity is indeed conferred by structure, however a problem became apparent: how can a higher number of Ab clones be generated than there are genes contained within the human genome? The first direct evidence of gene recombination in B cells came in the 1970's through genetic analysis of murine B-cell tumour tissue and murine embryos [41]. Molecular biologist Susumu Tonegawa digested and elecrophoretically separated the purified DNA and used radio-labelled mRNA encoding the expressed light chain of the B cell tumour to probe the DNA fragments. He found that mRNA probes corresponding to V and C regions bound to the same DNA fragment from B cell tumour DNA but separate fragments from embryo DNA, indicating that within B cells two different regions of the genome must be brought together to form the DNA sequence from which Ab light chains are translated. It is now known that Ig gene segments combine to form unique heavy and light chains during B cell development within the bone marrow [42]. Light chain V-domain gene sequences are formed by splicing a selected V region gene to a joining (J) region gene which combines imprecisely to amplify diversity. This V domain sequence is then combined with a C domain sequence to form the complete light chain sequence on chromosome 2 [40]. Heavy chain sequences are similarly generated through recombination of loci present on chromosome 14, where a V region sequence and a J region sequence are joined by a short sequence designated the diversity (D) region to produce the heavy chain V domain sequence. The VDJ construct is then combined with C domain sequence that designates the Ab isotype. The major mechanisms that contribute to structural diversity of the antigen binding site are summarised in Figure 1.3.

While combinatorial joining, junctional diversification and combination of H and L chain sequences occur in developing B cells within the bone marrow, somatic hypermutation occurs in activated proliferating B cells within lymphoid follicles. The process of somatic hypermutation is controlled by activation-induced cytidine deaminase (AID) in dividing cells [43]. The enzyme deaminates cytosine residues on single-strand copies of the V region gene sequence, and specific repair mechanisms are initiated which induce nucleotide base substitutions at these sites. Activity of AID in somatic hypermutation is focused on 'hotspot' nucleotide sequence motifs that follow the designation DGYW [44]. The rate of mutation is higher in antigen-binding sites of the V region gene, for reasons that are incompletely understood [45]. Resultant mutants are then competitively selected in the germinal centre reaction, with the highest affinity clones receiving essential stimulation from follicular T helper cells.

The AID enzyme is also involved in Ab class-switching between isotypes, where it selectively deaminates cytidine residues in repetitive 'switch' sequences which flank C region genes on the Ig heavy chain locus. The deamination results in the conversion of cytosine residues to uracil, and subsequent mismatch repair enzymes convert these sites to double-strand breaks, effectively deleting one or several C domain sequences. Deletional end joining via repair enzyme activity juxtaposes the VDJ segment to a new downstream C domain. In this way an Ab with identical specificity but a new isotype can be produced. As class switching involves the deletion of gene segments, it can only occur in one direction, in the order IgM, IgD, IgG3, IgG1, IgA, IgG2, IgG4, IgE, IgA2 [46].

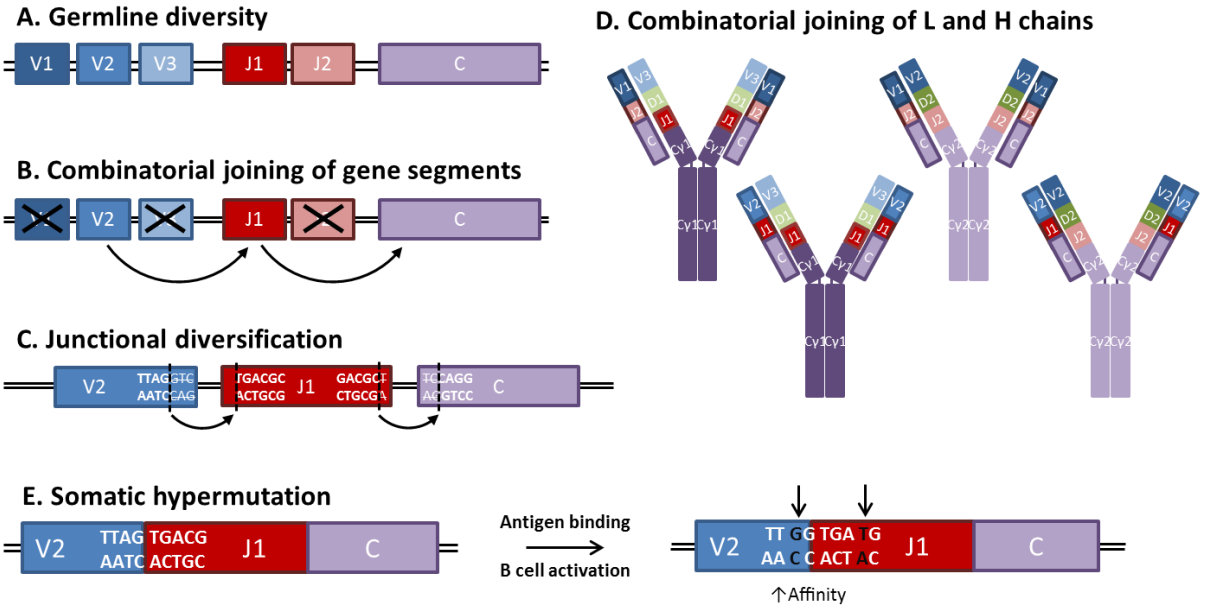


Figure 1.3 Generation of diversity in the antibody variable region

Mechanisms that contribute to diversity in antibody specificity alter the composition of the variable region. (A) In human, germline diversity exists in the inherited combination of immunoglobulin loci alleles in both heavy chain (V, D and J segments) and light chain (V and J segments; depicted). (B) Site-specific recombination facilitates joining of random combinations of gene segments to generate multiple antibody specificities. (C) Gene segments recombine imprecisely via specialised enzyme complexes that cleave the segments whilst including random base deletions and insertions to form a unique gene sequence. (D) Independently-formed heavy (H) and light (L) chain gene sequences are combined to result in a unique antigen binding site on the expressed antibody. (E) Following interaction with antigen, proliferating B cells undergo somatic hypermutation which directs a high frequency of random base mutations in the gene sequence corresponding to the variable antibody domain. Cells expressing higher affinity forms of their antibody are then selected for further proliferation. Together, these mechanisms enable the development of millions of antibody clones from a comparatively small number of genes.

1.1.4 Antibodies perform effector functions to protect against a diverse range of pathogens

As soluble proteins, Abs are effective sentinels that circulate the body to bind foreign particles and direct cells to mount additional responses. The ability for Abs to specifically bind to foreign sequences is exploited through a number of additional functions (summarised in Figure 1.4) that can be broadly classified as neutralisation, activation of cellular processes or the activation of the complement cascade. The simplest Ab function is the neutralisation of pathogens or toxins by direct binding to block functional sites [47]. Neutralisation of toxins generally occurs by preventing attachment and uptake by host cells, which is the primary mechanism of action for anti-venom therapies [48]. Direct neutralisation of pathogens can occur to prevent attachment of bacteria or virions to host cells, either by binding attachment sites or by agglutinating pathogens and thereby preventing their encounters with host cells. This is a common mechanism of IgA Abs located on mucosal membranes to defend against respiratory or gastrointestinal pathogens [49]. Intracellular pathogens can also be neutralised after attachment to host cells via Abs binding to fusion or internalisation proteins. Influenza glycoprotein haemagglutinin (HA) is frequently targeted by specific Abs which prevent viral entry into cells by blocking HA attachment to cellular receptors or by sterically hindering the low-pH induced conformational changes in HA necessary for fusion of the viral membrane [50]. Finally, Ab neutralisation of pathogens can occur after entry into host cells as in the case of some antiviral IgA that can inhibit viral replication [51, 52].

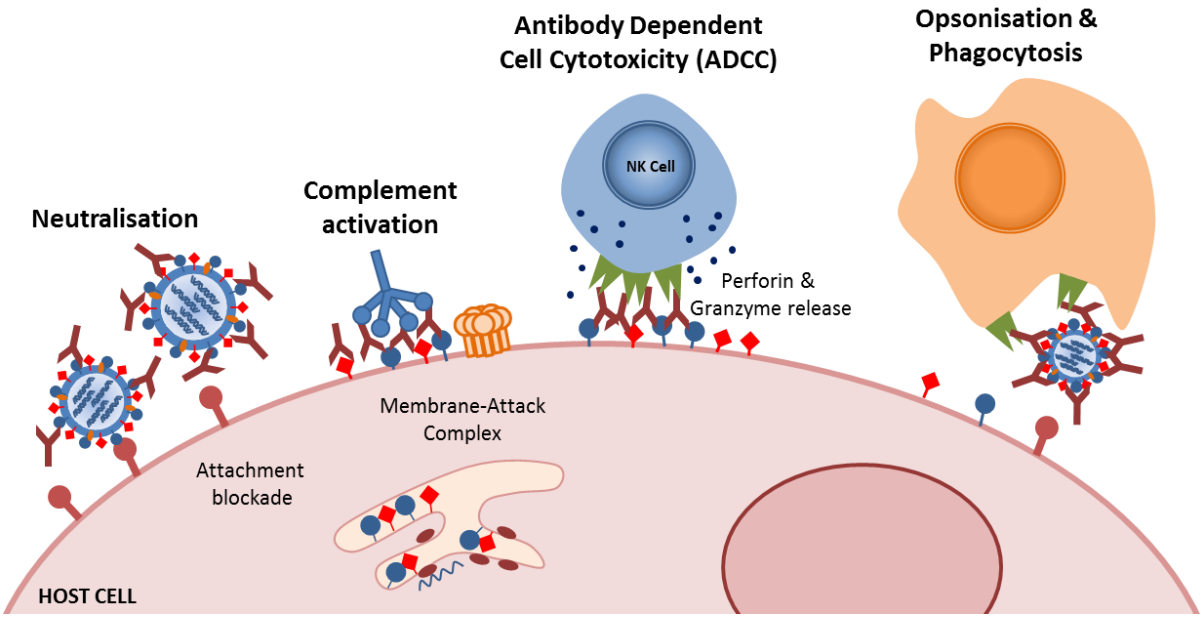


Figure 1.4 Effector functions mediated by antibodies

Antibodies produced following infection function through several chief mechanisms, depicted here in the context of viral infection. Direct pathogen neutralisation occurs to prevent pathogen attachment to host cells. Binding of antibodies to expressed viral proteins (or proteins on bacterial/fungal surfaces) triggers the activation of the complement cascade. Complement activation promotes inflammation, attracts innate cell types to infected tissues and culminates in the formation of a membrane-attack complex structure which disrupts the cell membrane to cause cell lysis and death. Bound antibody can signal through Fc receptors on macrophages, granulocytes or natural killer cells (depicted) to instigate direct cytotoxicity via the release of perforins and granzymes. Opsonisation of pathogens or infected cells with antibody stimulates phagocytosis via antibody interaction with Fc receptors on phagocytic cells. Together, these mechanisms work to prevent pathogen interaction with host cells and destroy infected or foreign cells.

The complement system is able to opsonize pathogens and assist in the development of inflammation and adaptive responses for the defense against invading pathogens. Formation of an Ab complex activates the classical complement pathway through the binding of C1q protein to motifs on antigen-bound IgG and IgM molecules. The increased affinity of C1q for IgM compared to IgG suggests that complement activation is more useful in the early phase of the humoral immune response. The complement cascade culminates in the formation of a pore-like membrane attach complex (MAC) on the surface of pathogenic bacteria, fungi or infected allowing diffusion of substances in and out of the cell and ultimately cell lysis [53]. Secondary effects of complement can also augment innate inflammatory process. The activation of the complement cascade generates anaphylatoxins C3a and C5a that trigger mast-cell degranulation and increased local vascular permeability. Neutrophils also invade the local site, attracted by chemotactic C5a where they release potent lytic enzymes in an attempt to phagocytose immune complexes.

The Fc portion of an Ab molecule can mediate a number of effector functions through specific Fc receptors (FcRs) [54]. Phagocytic antigen-presenting cells (APCs) sense immune complex formation via FcRs to phagocytose and present foreign material to T cells, thereby enhancing the adaptive response [55]. Signaling through FcR can instigate direct killing of the pathogen or infected cell, termed antibody dependent cell-mediated cytotoxicity (ADCC), by release of proteases and perforin from effector cells. Effector cells that mediate ADCC are predominantly natural killer (NK) cells [56], although granulocytes including neutrophils [57], eosinophils [58], and monocytes/macrophages [59, 60] can also perform ADCC functions. Effective ADCC through NK cells has been implicated as a major immunological mechanism for control of human immunodeficiency virus (HIV) infection [61, 62]. Additionally, ADCC function has also been attributed to effector-memory CD8 T-cell populations [63] which may play a role in viral defense, including chronic Epstein-barr and Hepatitis C [64] virus infections.

1.1.5 Passive immunotherapy for infectious diseases

Passive immunotherapy represents one of the oldest and most successful approaches to infectious disease treatment; however its use diminished with the advent of antimicrobial and chemotherapeutic agents. Ironically, it can be argued that subsequent misuse of antibiotics has given rise to the situation whereby passive immunotherapy may again become an essential approach to treat or prevent infections. The development of sophisticated molecular

techniques has ushered in a renaissance for passive antibody therapies which are being applied to a vast array of conditions including malignancy [65], autoimmune disease [66, 67], and neurodegenerative disorders [68] in addition to infectious disease. Current therapeutic Abs are used predominantly for anti-cancer applications and are the fastest growing group of new candidates in clinical trials, with a market value over US $20 billion [69, 70]. Therapeutics based on Abs have several advantages over small-molecule drugs, which have contributed to their extraordinary success. Abs can be generated to have exquisite specificity to almost any antigen with minimal or predictable off-target effects, unlike small molecule drugs which act upon signalling molecules likely present in multiple host cell types [71]. The extended *in vivo* half-life of Abs compared to many small-molecule enzyme inhibitors simplifies dosing requirements for administration. However, for many applications Ab-based therapies exhibit challenges in comparison to small molecule drugs. As large proteins of 150kDa, Abs do not readily pass the cell membrane to bind intracellular epitopes and [72] may exhibit reduced tissue penetration in comparison to small molecules [73], however Ab penetration may be improved with the application of Ab engineering techniques [73-75]. As with other biologicals, Ab stability and function may be affected by temperature, rendering manufacture, storage and standardisation of Ab therapeutics more complex than small molecules [76-78], however techniques which stabilise protein drugs through chemical modifications are being developed for clinical use [77]. Highly specialised assays are required for monitoring Ab therapy in comparison to small molecules as measurement of circulating therapeutic human Abs may be complicated by potential interference from endogenous Abs [79-81], and this represents an area of ongoing development. While the vast majority of Ab-based therapies are mAbs, pAb therapies may confer advantages over mAb based methods, particularly for infectious diseases which can escape immunity from mAb therapies through antigenic drift [82].

Technology for the development of Ab therapies is constantly evolving. The first technique to generate mAbs utilised fusion of myeloma cell lines with Ab-producing B cells to create an immortalised ‘hybridoma’ cell line that secreted the Ab of interest [83]. This advancement was followed a decade later by techniques to ‘humanise’ the Ab expressed by the generated hybridoma using chimerisation to combine the V domains of murine Abs with human C domains. These Abs were structurally 70% human however were still immunogenic to humans, thus more specific grafting of the antigen-binding site onto a human framework was subsequently used to generate the first mAbs approved for human use [84]. Since this discovery, computer-aided design of mAbs has enabled the development of mAbs with optimal binding affinity while reducing impact of the grafted sequence on mAb function.

Expression of mAb sequences using phage display also allowed the expression of fully human mAbs and the development of bispecific mAbs. Bispecific mAbs have the benefit of targeting two different epitopes using the one molecule and thus they may be used to target cancerous cells with higher avidity, neutralise multiple components of a pathogen or bring two targets in close proximity to promote interaction [85].

Generation of pAbs relies on the immunisation of an Ab-producing organism with a target sequence to produce high-titre antiserum. Immunisations can be performed using modified antigens, a sequence of antigens or novel adjuvants to generate the desired Ab response [86-88]. As generated pAbs are products of the natural immune response, they exhibit increased variability and heterogeneity compared to mAbs. The polyclonal response generates thousands of specificities capable of binding to many different epitopes, which may be of particular value for the targeting of rapidly-changing pathogens. Additionally, Abs of multiple specificities may combine on the surface of pathogens to yield synergistic effects on pathogen neutralisation and opsonisation [89]. Indeed, such synergistic activities are also being evaluated in mAb combination therapies and the development of bispecific Abs [85]. After collection and validation of sera, purification steps are applied to separate pAbs from other serum proteins which may be nonspecific processes that isolate total Abs or total IgG such as anion exchange chromatography or precipitation, or specific processes that isolate specific Abs using affinity chromatography. The most common pAb therapeutics are prepared from human donors and processed via nonspecific purification techniques [87].

Currently, passive Ab therapies for infectious disease represent a small portion of antimicrobial therapies approved for use. Passive Ab therapies approved or currently under investigation for the treatment of infectious disease are summarised in Table 1.1. Human-derived pAb products are licenced for use against anthrax [90], infant botulism [91], cytomegalovirus [92], hepatitis B virus [93], vaccinia virus [94] and varicella zoster virus [95]. A major advantage of Ab therapies derived from human Ab is the Fc portion, which mediates additional immune functions such as ADCC, opsonisation and increased phagocytosis of antigen [40, 96]. However, purification of human-derived Ab involves costly screening and treatment processes to minimise lot-to-lot variation and remove the possibility of disease transmission. Humanising Ab, typically murine, involves grafting complementarity-determining regions from mouse Ab onto a human Ab framework and permits the generation of large quantities of specific mAb with consistent function. The first and most successful anti-infectious humanised mAb is Palivizumab, which targets the F-protein of respiratory syncytial virus (RSV) [97-99]. Palivizumab is indicated for prophylaxis

against RSV in at-risk infants and has been associated with a 55% reduction in RSV-related hospitalisation [100]. However, humanised mAb therapies have high production costs which can make them prohibitively expensive [101] and suggest that timely, large-scale production for use in an emergency outbreak setting is unlikely to be practical.

Animal-derived Abs are more cost effective to produce, and exhibit reduced lot-to-lot variation than human-derived Ab therapies. Currently, animal-derived therapeutic Abs are predominantly used for anti-venom production and as anti-infectives for botulism [102], though development of animal-origin pAbs for rare and emerging diseases is ongoing [103]. In particular, the recent Ebola epidemic has revived interest in anti-infective pAb therapies [104, 105], where pAb therapies being perused over mAb approaches due to the potential for the development of mAb escape mutants [106, 107].

Table 1.1 Infectious disease antibody therapeutics currently approved or under clinical development

Name	Target	Antibody type	Indication	Route of administration	Current stage of development	Company
Monoclonal therapies						
Synagis: Palivizumab	RSV Protein F	Humanised IgG1 mAb	RSV treatment; Prevention of RSV in at-risk infants	Intramuscular injection	Approved in 1998	MedImmune
ABthrax: Raxibacumab	*B. anthracis* protective antigen	Human IgG1 mAb	Treatment of anthrax infection	IV infusion	Approved in 2012	GSK
Anthim: obiltoxaximab	*B anthracis* protective antigen	Humanised IgG1 mAb	Treatment of anthrax infection	IV infusion	Approved 2016	Elusys Therapeutics
Bezlotoxumab	*C. difficile* toxin B	Human IgG1 mAb	Treatment for *C. difficile* infection	IV infusion	Completed phase III	Merck (MSD)
PRO-140	Human CCR5 (HIV receptor)	Humanised IgG4 mAb	HIV treatment	SC injection	Phase III	Cytodyn, Inc.
Ibalizumab	Human CD4	Humanized IgG4 mAb	HIV treatment	IV infusion	Phase III	TaiMed Biologics
ShigaMab: Combination cαStx1/cαStx2	Shiga toxins 1 and 2 respectively	Both humanised IgG1 Mabs	Treatment of shiga-toxin-producing bacterial infection	IV infusion	Completed Phase II	Thallion Pharmaceuticals
Aurexis: Tefibazumab	*S. aureus* adhesion protein clumping factor A	Humanised IgG1 mAb	Severe *S. aureus* infection	IV infusion	Completed phase II	BMS, Co.
VRC01	HIV CD4 binding site	Human IgG1 mAb	HIV prevention and treatment	IV or SC injection	Phase II	National Institute of Allergy & Infectious Diseases
3BNC117	HIV CD4 binding site	Human IgG1 mAb	HIV treatment	IV infusion	Phase II	Rockefeller University
MHAA4549A	Influenza HA stalk	Human IgG1 mAb	Influenza treatment	IV infusion	Phase II	Genentech, Inc.
MEDI4893	*S. aureus* α-toxin	Human IgG1 mAb	*S. aureus* Pneumonia	IV infusion	Phase II	MedImmune LLC
Pascolizumab	Human IL-4	Humanised IgG1 mAb	Pulmonary Tuberculosis	IV infusion	Phase II	GSK
MEDI3902	*P. aeruginosa* PsI and PcrV	Bispecific IgG1	Prevention of nosocomial *Pseudomonas* infection	IV infusion	Phase II	MedImmune LLC

CR6261	Influenza HA stalk	Human IgG1 mAb	Experimental Influenza infection	IV infusion	Phase II	National Institute of Allergy & Infectious Diseases
GC1102	HBV surface antigen	Human IgG	Treatment of HBV infection	IV infusion	Phase II	Green Cross Corporation
Avastin: Bevacizumab	Human vascular endothelial growth factor	Humanised IgG1	Treatment of respiratory papillomatosis (HPV)	Intralesional injection	Phase II	National Institute of Respiratory Diseases, Mexico
MEDI8852	Influenza HA stalk	Humanised IgG1	Treatment of uncomplicated Influenza A infection	IV infusion	Phase II	MedImmune LLC
Salvecin: AR-301	*S. aureus* alpha-hemolysin	Human IgG1 mAb	Treatment of *S. aureus* pneumonia	IV infusion	Phase I & II	Aridis Pharmaceuticals, Inc
CHIKIVIG-01: Anti-CHIKV Hyperimmune IV Immune Globulin	Chikungunya virus	Human serum polyclonal IgG (Cold alcohol fractionated)	Prophylaxis in neonates at –risk or exposed to Chikungunya virus	IV infusion	Phase I & II	Pointe-à-Pitre University Hospital Centre
514G3	*Staphylococcus* species	Human IgG mAb ("True human")	*S. aureus* bacteraemia	IV infusion	Phase I & II	XBiotech, Inc.
MB66: Combined Anti-HIV (VRC01-N) and Anti-HSV (HSV8-N)	HIV CD4 binding site and HSV gD protein	Both human IgG1 Mabs	Prevention of HIV and HSV infection	Vaginal film	Phase I	LeafBio, Inc.
VRC01LS (Modified VRC01)	HIV CD4 binding site	Modified human IgG1 Mab (modified Fc domain for extended half life)	HIV prevention and treatment	IV or SC injection	Phase I	National Institute of Allergy & Infectious Diseases
Polyclonal therapies						
Anthracil: Anthrax Immune Globulin	*B. anthracis* protective antigen	Human serum polyclonal (anion exchange chromatography purified)	Treatment of inhalational anthrax	IV infusion	Approved 2015	Cangene Corporation
BabiBIG: Botulism Immune Globulin	Botulinum toxoid	Human serum polyclonal IgG (Cold alcohol fractionated)	Infant botulism	IV infusion	Approved 2003	California Department of Health Services
Nabi-HB: Hepatitis B Immune Globulin	HBV surface antigen	Human polyclonal (Cold alcohol fractionated)	Post-exposure prophylaxis	Intramuscular injection	Approved 2013	Nabi Biopharmaceuticals
HepaGam B: Hepatitis B Immune Globulin	HBV surface antigen	Human serum polyclonal IgG (anion exchange chromatography purified)	Prevention of HBV after liver transplantation	IV or intramuscular injection	Approved 2006	Cangene Corp
Vaccinia Immune Globulin Intravenous	Vaccinia virus	Human serum polyclonal IgG (anion exchange chromatography purified)	Treatment of Vaccina infection resulting from vaccination	IV infusion	Approved 2005	Cangene Corp
VARIZIG: Varicella Zoster Immune Globulin	Varicella Zoster Virus	Human serum polyclonal IgG (anion exchange chromatography purified)	Postexposure prophylaxis for Varicella	Intramuscular injection	Approved 2012	Cangene Corp
Cytogam: CMV Hyperimmune globulin	CMV	Human polyclonal (Cold alcohol fractionated)	Prophylaxis for CMV in transplant recipients	IV infusion	Phase III	CSL Behring
Anti-Influenza Intravenous Hyperimmune Immune Globulin	Influenza virus	Human polyclonal (Cold alcohol fractionated)	Influenza treatment	IV infusion	Phase III	National Institute of Allergy & Infectious Diseases

Abbreviations – CMV: cytomegalovirus; HBV: Hepatitis B virus; HIV: Human immunodeficiency virus; HPV: Human papilloma virus; HSV: Herpes simplex virus; RSV: Respiratory syncytial virus; IV: Intravenous; SC: Subcutaneous

1.1.6 Development and production of polyclonal antibody therapeutics

Polyclonal Abs have advantages over their mAb counterparts due to their ability to bind to a number of different epitopes [108]. Neutralisation capacity of an Ab of a single specificity may be synergistically-enhanced when combined with other Abs to bind to additional receptors, inhibit conformational change or induce viral aggregation [109]. Furthermore, pAb therapy may be particularly useful for treating viruses with a high rate of antigenic variability, as this treatment reduces selective pressure on the viral strain to generate resistance-conferring mutations. Human convalescent serum is often difficult to obtain in large quantities in the case of emerging or rapidly changing pathogens, thus animal-derived therapeutics are being considered for these purposes.

Concerns with adverse reactions to animal-derived Ab preparations have severely limited their use as anti-infective agents. Soon after the first application of 'serum therapy' for the treatment of diphtheria and tetanus in the 1890's, the treatment was associated with the delayed onset of widespread urticaria, fever, swelling and intense pain [110]. Further investigations into the syndrome by paediatricians Clemens von Pirquet and Bela Schick in 1906 revealed an increased frequency of respiratory abnormalities, proteinuria, joint symptoms and lymphadenopathy after administration of large quantities of foreign Ab-laden serum. These symptoms were later found to be the result of a Type III hypersensitivity reaction mediated through persistence of immune complexes. Serum sickness reactions can vary with the type and dose of the administered antigen which alter the size of antibody complexes and the location of their deposition [111]. Sites where plasma filtration occurs, such as renal glomeruli and synovial membranes within joints, are common sites of immune complex deposition and thus these sites are frequently sites of serum sickness pathology. The development of hypersensitivity symptoms can be greatly reduced with the application of modern purification techniques [103], and modification of the Ab.

As serum sickness is mediated through the Fc portion of foreign Ab, the use of Ab fragments can prevent hypersensitivity reactions [112]. Digestion of whole Abs with papain enzyme cleaves heavy chains above the hinge region to result in two identical Fab fragments, whereas pepsin enzymatic digestion fragments the Fc portion below the hinge region to produce a divalent molecule termed $F(ab)_2$ [113]. These Ab fragments retain antigen binding and neutralization capacity, which is particularly important for the neutralization of pathogen-associated toxins or the prevention of attachment to host cells [114]. Fragmented Ab products have exhibited the most success as anti-toxins in critical situations, where they have good

safety profiles [48]. A meta-analysis of reactions observed after administration of an ovine polyclonal Fab antivenom (FabAV), revealed a low incidence of immediate hypersensitivity, with symptoms reported in 0.08% of recipients [115] however delayed 'serum sickness' hypersensitivity reactions were more common, occurring in 0.13% of patients. In contrast, analysis of equine $F(ab')_2$ based scorpion antivenom were associated with adverse reactions, with 0.2% and 0.5% of patients exhibiting immediate or delayed hypersensitivity reactions respectively [116]. Fragmented ovine pAbs have also been applied to drug toxicity, such as DigiFab™ (BTG International Inc.), an ovine polyclonal Fab therapy indicated for digoxin overdose or suspected digoxin toxicity. Ovine polyclonal Fab and $F(ab)_2$ antibody fragments have a number of advantages over humanised mAbs; they can bind to multiple sites, thus increasing neutralising potential, and they are cost-effective to produce in large quantities. In the clinic, ovine pAb fragments have been associated with a low incidence of adverse events [117]. This technology poses a real opportunity with unrealised potential, being highly amenable to the timely development, assessment and production of new bio-therapeutics to treat and prevent infectious diseases in an economically-feasible manner.

1.2 Passive immunotherapy for influenza infection

1.2.1 Influenza infection

Influenza refers to an infection caused by any of the *Influenza* viruses of the family *Orthomoxyviridiae.* It is an acute respiratory illness that is manifested in seasonal epidemics, and occasional pandemics. Annually, influenza is attributed to 250,000- 500,000 deaths worldwide [118, 119]. Influenza infection is commonly contracted by inhalation of virus-containing respiratory secretions which enable inhaled virions to attach to respiratory epithelium [120]. Inflammation and cytopathy in the upper respiratory tract result in the onset of classic respiratory symptoms of sore throat, rhinitis and cough accompanied by malaise and high fever [121].

Influenza viruses are classified into three major subtypes; types A and B are common causes of human influenza infection whereas influenza C does not commonly cause infections in humans. The three influenza virus types share a similar structure of round budded virions approximately 90-100nm wide encapsulated by a lipid bi-layer envelope [121, 122].
Influenza A subtypes constitute the majority of circulating strains [123, 124] and are further classified based on the content of outer glycoprotein residues HA and Neuraminidiase (NA). At least eleven forms of NA and eighteen HA subtypes that are capable of potentially being incorporated into circulating human viruses through genetic recombination have been documented [125], giving the capacity for almost two-hundred antigenically distinct subtypes to be generated. Antigenic shift is triggered by a recombination between two separate viral strains that merge within a single host to create a novel replicative strain, and is responsible for the emergence of radically different strains of influenza that have caused pandemic infection in the past [119]. Current circulating influenza strains are primarily of the H1N1 and H3N2 subtypes, in addition to influenza B strains.

Gradual changes in circulating strains are promoted by antigenic drift of outer glycoprotein genes due to the rapid, low-fidelity replication of the influenza virus [126, 127]. In tropical regions, influenza circulates year-round, providing a reservoir for the genesis of antigenically diverse strains through interaction of strains from the southern and northern hemispheres [126]. Thus, every season new influenza strains emerge that are antigenically-distinct and can escape the immunity conferred from previous strains [123].

While influenza infection is generally self-limiting in healthy individuals, it poses a greater risk of mortality in individuals with comorbidities, supressed immunity and the elderly [128].

These populations often exhibit suboptimal responses to vaccination [129, 130] and are at higher risk of 'severe' influenza infection [131] which is defined as influenza infection accompanied by complications that require hospitalisation, and is attributed to over 3.4% of all critical hospitalisations during influenza season [132].

1.2.2 Immune response to influenza infection

The human immune system is capable of defending against invading viral pathogens, including influenza, in a number of ways. Initially, innate immunity prohibits viral entry by nonspecific anatomical and inflammatory barriers [133]. Membrane fusion and entry of the influenza virus into a target cell is mediated through the initial binding of HA to sialic acid residues on host cell surfaces [125, 134, 135]. Within the host cell cytoplasm, viral RNA is replicated and translated which allows assembly of new virions. The host cell membrane, studded with expressed viral proteins, forms the viral envelope. The other major glycoprotein NA facilitates budding of the virions from the host cell via cleavage of sialic acid residues. Viral pathogens are commonly identified through the presence of intracellular viral nucleic acids by PRRs, including toll-like receptors (TLRs) on endosomes [136, 137], stimulating the release of cytokines which aid the generation of an adaptive immune response (Figure 1.5). Influenza virus can be recognised by RNA receptors including TLR3, TLR7 and retinoic-acid inducible gene-I (RIG-I) in various cell types which stimulates production of type I interferons and inflammatory cytokines [137-140]. Phagocytosis of influenza-infected cells by macrophages and plasmacytoid dendritic cells (pDC) enable signalling through PRR to drive innate responses and also constitutes a major mechanism for influenza viral clearance.

If innate barriers and phagocytosis fail to control influenza infection, adaptive responses are required for eradication of the pathogen. Viral infections can stimulate both humoral and cell-mediated arms of adaptive immunity [136, 137, 141]. As intracellular pathogens, most viral diseases stimulate a predominantly cell-mediated adaptive response, however Ab play an important role in the defence of influenza [142]. Viral neutralisation by Ab binding to HA is considered a major component of influenza-specific immunity, as anti-HA Abs prevent attachment to host cells and opsonise extracellular virions for phagocytosis. Additionally, Ab-bound influenza virions may be directly targeted by complement-mediated lysis of enveloped viruses [143]. Anti-HA Abs effectively bind expressed HA on influenza infected cells to stimulate effector functions which aid in the clearance of the virus. Phagocytosis of infected cells is mediated through IgG-binding FcR [96], and Ab bound cells may also undergo

complement-mediated lysis [144] or ADCC [145]. Anti-NA Abs are also formed after influenza infection which prevent viral release from infected cells and similarly mark cells to be targeted by effector functions.

Cell-mediated adaptive responses also contribute to the clearance and control of influenza infection, and are particularly important for immunity against antigenically distinct influenza strains [146]. Epitopes targeted by cell-mediated responses are often present on internal proteins of the influenza virus which are more conserved than surface glycoproteins. Highly cross-reactive responses induced by seasonal influenza exposure, with the ability to offer heterosubtypic protection, have been documented in humans [147]. These epitopes are currently being investigated for vaccine development, however identifying epitopes that can provide population coverage across different human leukocyte antigen (HLA) types is a considerable challenge [148]. Additionally, concerns that increased or ill-timed cell mediated immune responses might contribute to detrimental immunopathology have implications for development of a vaccine to stimulate cell-mediated responses [149].

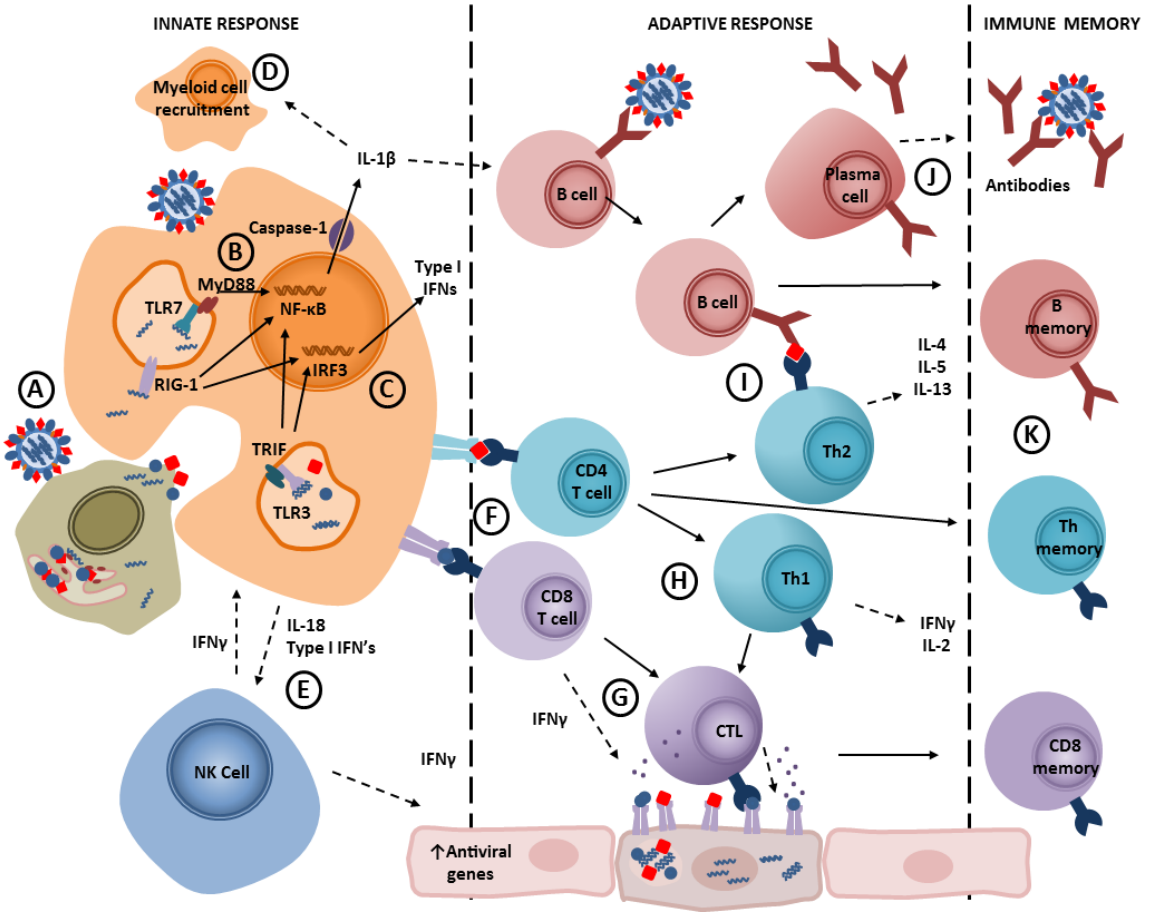


Figure 1.5 Innate and adaptive processes that instigate influenza-specific immune memory

Influenza infection is associated with a potent innate immune response and for formation of both humoral and cell mediated responses. (A) Resident phagocytes engulf infected cells or influenza virions. (B) Viral RNA is sensed via pattern recognition receptors in phagocytic cells: RIG-1 in the cytoplasm which promotes NF-κB and IRF-3-controlled gene transcription; TLR7 in endosomes which triggers MyD88 signalling and NF-κB gene transcription and (C) TLR3 in phagosomes which signals through TRIF to promote both NF-κB and IRF-3-controlled gene transcription. (D) Expression of IL-1β and other proinflammatory cytokines promotes the recruitment of myeloid cells into the lung to phagocytose infected cells. (E) Expression of IL-18 and type I interferons activates NK cells, which directly kill infected cells and secrete IFNγ to promote the expression of antiviral genes in lung epithelial cells. (F) Interaction of APC with naive T cells results in the generation of antigen specific Th1 or Th2 cells, and prompts the generation of CD8 effector cells. (G) Cytotoxic lymphocytes directly kill infected cells by the release of perforin and granzymes, which promotes viral clearance. (H) Specific Th1 cells stimulate cytokine-mediated viral replication blockage and generation of CD8 effector cells whereas (I) Specific Th2 provide help to B cells for clonal expansion. (J) Expansion of B cell clones leads to generation of antibody secreting plasma cells. Antibodies persist in the circulation to provide seroprotection against re-infection and (K) formation of CD8, Th and B memory cells enables recall responses if the virus is re-encountered.

1.2.3 Strategies for influenza prevention and treatment

The burden of influenza infection is predominantly controlled by a world-wide, co-ordinated vaccine effort [150]. However, seasonal vaccines may provide sub-optimal protection particularly in populations at risk of complications following influenza infection. Treatment options for those that succumb to influenza infection are predominantly limited to antiviral therapies.

Vaccination

Vaccination is the most widely used strategy to combat the morbidity and economic burden of influenza infection. In Australia, split-virion and subunit vaccines which contain antigens from three (trivalent) or four (quadrivalent) circulating strains without an adjuvant are recommended [151]. An exception Retrospective studies have found seasonal influenza vaccines to vary in effectiveness between seasons from 40% to 80% of all laboratory confirmed strains [128, 152, 153]. Data collected by the WHO Global Influenza Surveillance Network is used twice annually to make recommendations for the formulation of the influenza vaccine for the upcoming season [119, 150]. Unfortunately, time spent developing and manufacturing substantial quantities of the vaccine means the formulation often protects against a fewer circulating strains in a given season than existed in the previous season, as viral epitopes change over time to elude the vaccine [153]. Novel approaches to the development of influenza vaccines are currently being explored in an effort to improve efficacy of seasonal vaccination. The use of different adjuvants in subunit vaccines has been well explored [154], with several oil-in-water adjuvants currently in use in addition to traditional alum adjuvants, including MF59 and AS03 [155, 156]. Experimental adjuvants based on saponins, bacterial components and immunomodulatory proteins are also in clinical development for influenza vaccination [157]. Vaccines based on nucleic acids have been evaluated in early trials and are promising candidates for the development of cross-protective immunity due to their ability to induce effective CD8 responses [158]. However, concerns with unwanted genetic changes in cells producing influenza antigens have limited the use of DNA vaccines until further study can be performed [159]. Viral vector technology has also demonstrated clinical efficacy [160], however the development of vector-specific immunity and tolerance induction remains an inherent risk to this approach [161].Whether these novel approaches can provide effective immunity for at-risk immunocompromised populations remains to be investigated.

Vaccination against influenza has long been encouraged as a preventative measure for the entire population and is particularly recommended in susceptible populations including the elderly, who account for the majority of influenza-related deaths [119, 153], people with chronic conditions and healthcare workers. Although seasonal influenza vaccination has been shown to be beneficial for these populations, outcomes in the elderly, the immunosuppressed and those with chronic illness may not be optimal as the immune response may be inhibited or inadequately stimulated [128]. In the elderly this is likely to be a consequence of immunosenescence, the natural decline of immune function with age which manifests in a number of characteristic changes [162, 163]. Thymic involution in elderly persons produces a significantly decreased naïve T-cell population, impairing both cell-mediated and humoral immune responses. Accumulating B cell defects result in poor Ab responses with low-affinity Ab and decreased success of class-switching [163]. Additionally, proliferation of effector CTLs skews the adaptive immune response towards cell-mediated mechanisms and decreases vaccination efficacy in aged persons [162]. Vaccination is not recommended for infants under 6 months [164] as it is not highly efficacious due to the underdeveloped nature of the infant immune system. However, studies suggest influenza infection is often under-diagnosed in this group [165] and poses a significant health threat. Infants largely rely on passive protection from maternal IgG obtained through placental transfer and, later, breastmilk for prevention of influenza infection [166]. Consequently, influenza vaccination is encouraged in pregnant woman [167].

Antiviral medications

Current treatment for severe influenza generally incorporates supportive care and antiviral medications such as NA-inhibitors oseltamivir (Tamiflu^{TM}) or zanamivir (Relenza^{TM}) [168]. Though NA inhibitors are the most common antiviral drug for influenza, RNA polymerase inhibitors and M2 ion channel blockers are used for influenza treatment. The mechanisms of these antiviral agents are summarised in Figure 1.6. The efficacy of antiviral medications in reducing severity of infection is highly dependent on their timely administration. In a recent retrospective analysis, administration of antiviral drugs was associated with a modest 11% reduction in the risk of influenza complications [169]. Viral resistance to NA inhibitor medications is most often acquired by infective viruses during hospitalisation and treatment [170] which is a significant risk for immunocompromised patients hospitalised for extended durations.[171] During the H1N1 pandemic of 2009, NA inhibitors were crucial treatment options used in patients with an established H1N1 infection. Resistant strains detected in immunocompromised patients [172] were found to retain pathogenicity despite the resistance-conferring mutation [173]. Recently peramivir, an IV NA-inhibitor has been approved for

influenza treatment [174] and has exhibited similar efficacy to other NA-inhibitors particularly in at-risk populations [175]. However, concerns over an increased incidence of adverse effects to peramivir [176] may restrict its use to only oseltamivir-resistant strains.

Novel RNA polymerase inhibitors have been evaluated for the treatment of influenza infection, in addition to other viral diseases. While broad antiviral efficacy of these drugs has been demonstrated in several clinical trials, their use has also been associated with the development of haemolytic anaemia [177, 178]. Most recently, favipiravir has been trialled in influenza infection [179] however it is as yet unclear whether this approach could lead to the development of resistant strains in the clinic [180]. Adamantanes, or M2 channel blockers, have been successfully used in the past for the treatment of severe influenza infection [181]. However, Adamantane is not currently recommended for use due to the high frequency of circulating resistant strains. The widespread use of all antiviral drugs for influenza predisposes hosts to infection by resistant strains [112]; thus polyclonal passive immunotherapy may be a viable alternative to drug therapies for influenza [108].

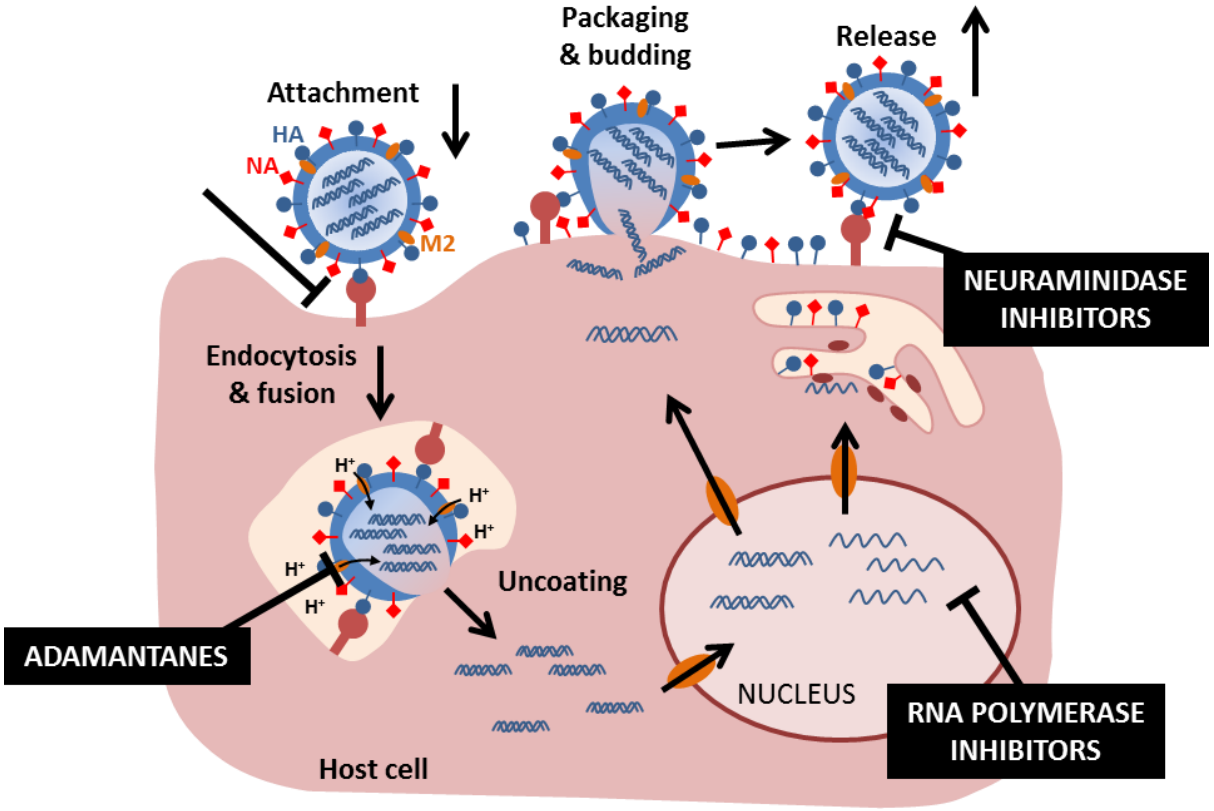


Figure 1.6 Mechanisms of antiviral drugs in the inhibition of influenza virus infection

Influenza virions attach to host cells and gain entry through endocytosis. The low pH environment of the endosome activates the M2 ion channel, which triggers acidification of the viral core and fusion of the viral and endosomal membrane. Adamantanes inhibit M2 activity and thus prevent viral uncoating. Once liberated, viral nucleic acids enter the nucleus and become translated to form a viral RNA polymerase capable of replicating viral RNA for the formation of new virions. Viral RNA polymerase inhibitors prevent the activity of the polymerase and thus inhibit the formation of new virions. Viral proteins assemble on the host cell membrane and form the viral envelope in which replicated viral RNA is packaged. Fusion of the viral envelope forms a new virion which is released from the cell via neuraminidase activity which cleaves sialic acid host receptors from viral haemagglutinin. Neuraminidase inhibitors function to prevent this cleavage and thus inhibit the release of new virions from the infected cell surface.

1.2.4 Passive immunotherapy for influenza infection

Passive immunotherapy using neutralising Ab may be the ideal rapid treatment strategy for influenza infection that functions independently of the individual's immunocompetency [182] as highlighted by previous animal [22, 183, 184] and human [185, 186] studies. Administration of pre-formed Ab can eradicate viral pathogens through different mechanisms, particularly Ab of human origin, however current Ab therapies are selected predominantly on their ability to neutralise virions *in vivo* [187]. Virus neutralisation occurs when the administered Ab binds to exposed sialic-acid binding sites on HA residues, which prevents cell binding and entry [99, 188]. This mechanism is supported by *in vitro* studies that correlate the effective dose of Ab fragments delivered with the number of targeted surface proteins expressed on virions [99, 125], a process mediated by the Fab portion of an Ab that can occur in the absence of the Fc portion. Additionally, Ab that react with the stalk region of the HA glycoprotein have been detected following infection and recovery which are able to neutralise multiple influenza subtypes [50, 189]. Even in cases where Ab are unable to neutralise all present virions, they may also assist in pathogen defence by temporarily reducing viral load and allowing time for active immunity to develop [187]. The process of blunting viral propagation could be valuable in individuals with suppressed immunity, such as those with chronic illness or the elderly, who fail to seroconvert effectively [108]. Hyperimmune IVIG has been used to treat severe influenza infection, with varied success. Recently, a multicentre clinical trial demonstrated that IVIG prepared from donors exposed to pandemic H1N1 influenza significantly reduced viral load in infected individuals compared to IVIG prepared from donations received before the 2009 pandemic.[190] However, IVIG therapies from convalescent sera are expensive to purify, and can exhibit considerable lot-to-lot variation [191].

Broadly-neutralising mAbs are being investigated as potential immunoprophylactics for the prevention of influenza, and the treatment of established infection. While a recent trial of an anti-M2 mAb in an experimental human H3N2 influenza model was not able to achieve the primary objective of the prevention of infection [192], mAb use was associated with reduced symptom development and a reduction in viral load. Some experimental cross-reactive anti-influenza mAbs are thought to function through the instigation of antibody-mediated cell cytotoxicity rather than direct viral neutralisation [193] and as such, the efficacy of these mAbs in immunocompromised individuals may be reduced. Whether anti-influenza mAbs are efficacious in treating severe infection in at-risk populations remains to be investigated, however difficulty in identifying a broadly neutralising epitope [194, 195] and concerns over

the development of resistant strains [196, 197] have delayed their clinical translation.

Polyclonal Abs (pAbs) can overcome these deficiencies by their intrinsic ability to target multiple epitopes, which can increase cross-reactivity and reduce the development of resistant strains.[87, 114] Approaches using pAbs sourced from animal sera are particularly advantageous as large quantities of pAb can be collected and batched for reduced variability [87]. Furthermore, pAbs of animal origin have simplified screening procedures compared to human-derived Abs. The formation of broadly-neutralising pAbs to influenza can be prompted through sequential immunisation with multiple HA subtypes [198], or the application of novel adjuvants to enhance humoral responses [199, 200]. Ovine pAbs are particularly efficient and cost-effective to produce, and exhibit reduced immunogenicity compared to those sourced from horses or other large mammals [201]. Ovine pAb fragments have been used with success in the clinic, and are currently available as critical care therapies for life-threatening digoxin toxicity (DigiFab™, DIGIBIND™) [202] and crotalid snake envenomation (CroFab™) [203]. This technology may have unrealised potential in the treatment of severe influenza infection and may provide efficacious adjunctive or stand-alone therapy for difficult to treat severe influenza infections.

1.3 Passive immunotherapy for sepsis treatment

Sepsis is defined as an excessive inflammatory response following infection and is the leading cause of death from infection [204]. A 2004 study of adult sepsis patients in Australian and New Zealand hospitals estimated the incidence of sepsis to be 0.08% of the population, with an in-hospital mortality rate of 37.5% [205]. While these findings were reportedly within sepsis estimates from the USA and UK, it is important to note that incidence and mortality rates from sepsis are higher in underdeveloped countries. Overall sepsis incidence and mortality is increasing [206] as more patients are admitted to hospitals with cancer, immunosuppressive conditions such as HIV and other chronic diseases which are associated with increased susceptibility to sepsis [207, 208]. As the pathology of sepsis is largely attributed to damage conferred from an exuberant host response, rather than direct action of pathogenic factors, there is an ostensibly unmet need for therapies that specifically target the host response.

1.3.1 Pathophysiology of sepsis and septic shock

Sepsis is characterised by a systemic inflammatory cascade termed systemic inflammatory response syndrome (SIRS) that is triggered by a pathogenic insult, thus the catastrophic pathology of sepsis exists as a consequence not of pathogenic factors but of the host's own immune response [209]. In some cases, sepsis may progress to 'septic shock', characterised by hypoperfusion associated with peripheral vasodilation, disseminated intravascular coagulation (DIC) and multiple organ failure.

The uncontrolled release of proinflammatory cytokines and systemic inflammation in sepsis occurs as an innate response to a pathogen or damage associated stimulus, and triggers a complex network of signalling cascades that damage host tissues in an effort to control infection (Figure 1.7). The overenthusiastic release of cytokines, termed the 'cytokine storm' stimulates vascular events, fever and the systemic release of acute-phase proteins. Direct action of bacterial factors in the circulation may activate the complement cascade, further fuelling the inflammatory response. Coagulation systems are activated and fibrinolysis is simultaneously supressed [210-212]. Nervous system function is also affected as immune cells release active neurotransmitters resulting in an imbalance between adrenergic and cholinergic pathways [213]. This imbalance can interfere with myocardial contractility, resulting in decreased perfusion of peripheral tissues [214, 215], potentiating liver, renal or

pulmonary failure, acute myocardial infarction and death. Release of platelet activation factor (PAF) coupled with significant endothelial damage trigger a coagulation cascade which can potentiate to DIC which represents a significant cause of organ failure in itself [210, 212]. The proinflammatory imbalance characterising sepsis can give rise to a subsequent immunosuppressive condition due to apoptosis of immune cells, 'immune paralysis' of activated neutrophils and suppression of adaptive immunity [216]. This phenomenon is called compensatory anti-inflammatory response syndrome (CARS) and has often attributed to the failure of anti-inflammatory medications in clinical trials. Consequently, secondary infection, or re-infection, is also a major cause of sepsis-related mortality [217].

Survivors of sepsis and septic shock often have a reduced quality of life and are at increased risk of ongoing long-term morbidities including motor, cognitive, neurosensory and visual impairments [218-220]. A recent comparison of sepsis survivors with other intensive care unit (ICU) critical illness survivors revealed that sepsis survivors had higher one year mortality rates and an increased incidence of depression and anxiety [221] compared to non-septic survivors. Sepsis is a significant cause of death in neonatal ICU wards, with a mortality rate approaching 50% in at-risk preterm infants. Neonatal sepsis survivors are often at increased risk of neurodevelopmental disorders such as cerebral palsy when survivors also exhibit comorbidities such as severe bronchopulmonary dysplasia [220, 222]. As cognitive symptoms during sepsis including delirium [223] and encephalopathy [224] are linked to the cytokine storm, it is postulated that inflammatory modulating therapies may have a beneficial role in not only improving sepsis survival, but improving longterm outcomes after sepsis.

International clinical consensus sepsis definitions proposed in the early 1990's highlighted the identification of SIRS as central to the diagnosis of sepsis. However, this definition was considered too broad for many intensivists as patients may present with SIRS in the absence of infection. In February 2016 an international task force presented their consensus definitions and recommendations for the clinical diagnosis of sepsis based on systematic review of large international datasets, surveys and cohort studies [225]. These new definitions, termed Sepsis-3, highlighted the downstream pathobiology of the host immune response and resultant organ dysfunction [226]. Diagnosis of sepsis under these revised criteria require a sequential organ failure assessment (SOFA) score of two or more, based on the identification of respiratory, haemostatic, cardiovascular, metabolic, renal or cognitive abnormalities [225]. Retrospective validation of these new criteria have indicated that SOFA scores may be superior to SIRS criteria for predicting patient mortality, however true clinical utility of Sepsis-3 will only be evident after prospective investigation.

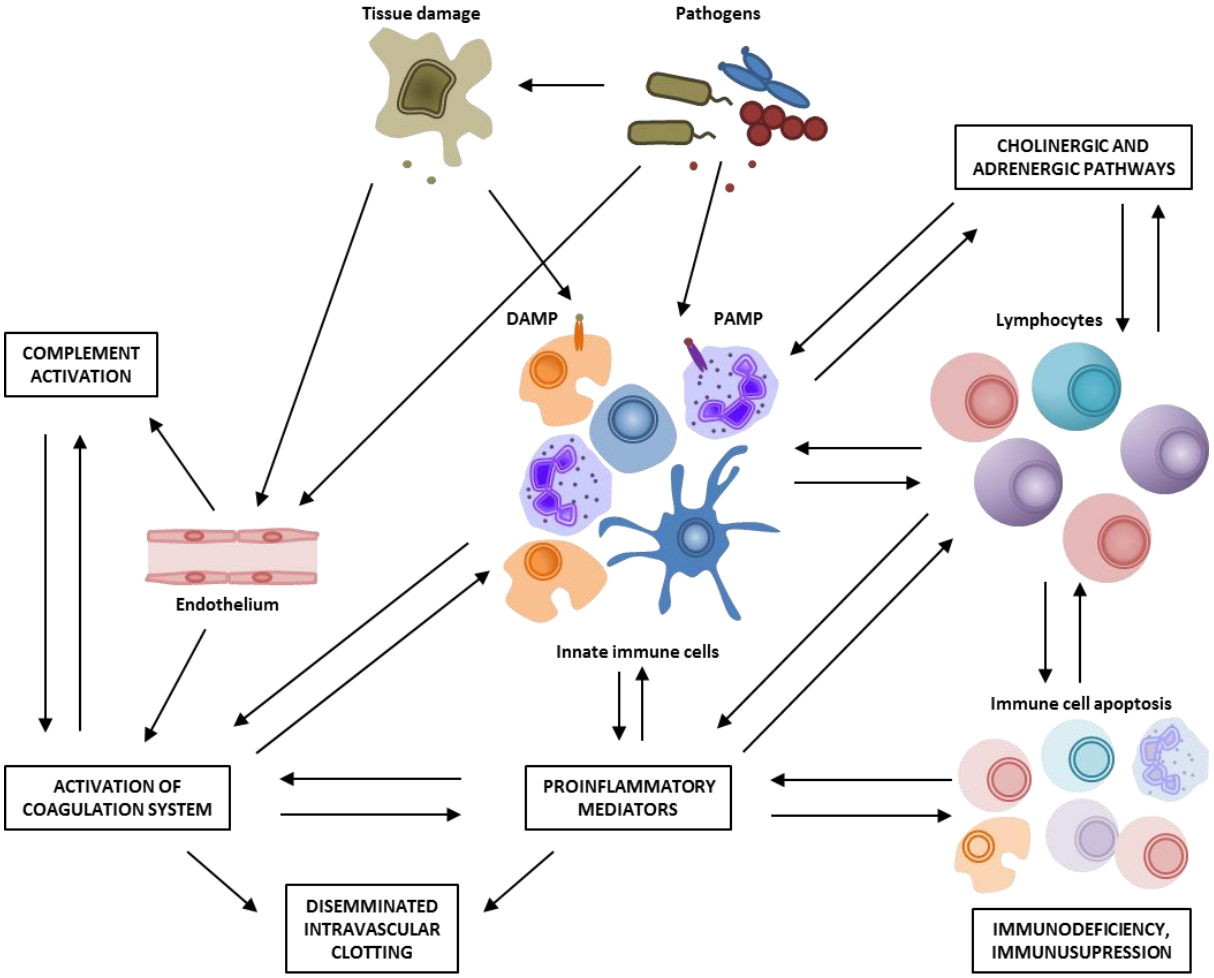


Figure 1.7 The inflammatory network in sepsis

Pathogen-associated molecular patterns or host damage-associated molecular patterns from damaged cells (DAMPs) stimulate PRRs on immune cells resulting in exuberant systemic cytokine production termed the 'cytokine storm'. Activation of adrenergic pathways and suppression of cholinergic pathways of the autonomic nervous system and endothelial expression of pro-coagulant, chemotactic and proinflammatory factors further amplify proinflammatory signalling. Imbalances in complement and coagulation cascades are intensified in a positive-feedback loop and contribute to disseminated intravascular clotting, multiple organ failure and mortality. Finally, the sustained proinflammatory environment affects immune effector cells, eventually causing cellular apoptosis and 'immunoparalysis'. Alterations in leukocyte apoptosis in the later stages of sepsis further account for increased susceptibility to secondary infection. (Adapted from Rittirsch et al, 2008 [214])

1.3.2 Current sepsis treatments

Fifty percent of deaths from sepsis occur within the first two days, and as it can take days to obtain blood culture and antibiotic sensitivity results, initial treatment options are primarily limited to empirical broad spectrum antimicrobials and supportive care. More recently, the aggressive use of the correct antibiotic as early as possible has been presented as the most effective way to reduce sepsis mortality [227]. The appropriate management of fluids and electrolyte balance also become a critical supportive measure and circulatory support is often required in the form of sympathomimetic amines such as dopamine to raise the heart rate and systolic blood pressure. Most recent therapies that have been incorporated into sepsis treatment regimens include the use of activated protein C, insulin therapy for glycaemic control and corticosteroids [228]. However, some doubt still remains as to whether these approaches offer any genuine benefits [229].

Sepsis and septic shock decrease levels of circulating protein C, a coagulation factor with important regulatory roles in coagulation, inflammation and vascular permeability. Since the first major study on the use of activated protein C in sepsis was published in 2001 [230], activated protein C has been pursued as a treatment for sepsis especially in patients presenting with DIC or at high risk of death. Multiple clinical trials have been conducted assessing the clinical utility of activated protein C since this time, and ultimately no significant clinical benefit has been deduced. Several studies associated activated protein C with adverse outcomes, including increased bleeding risk [231, 232] and higher mortality [233]. However, the conflicting and disappointing results from activated protein C trials have shed light onto the conduct and interpretation of clinical trials for sepsis and popularised the view of sepsis heterogeneity as the key to developing effective therapies.

Corticosteroids have previously been administered for chronic inflammatory conditions to reduce pathology, however they are often associated with risks including adrenal suppression, hypertension and osteoporosis [234]. Early trials indicated that high dose corticosteroid therapy could provide a survival benefit in clinical sepsis [235]; however more comprehensive investigations revealed no benefit to this approach and that high dose steroids may potentiate immunosuppression and death following secondary infection [236, 237]. Consequently, corticosteroid therapy in sepsis is limited to low-dose steroids as replacement therapy in cases of adrenal insufficiency [238, 239] and is associated with better responses to adrenergic agonists used to reverse shock, reduced reliance on intensive care and the restoration of organ function [240]. Additionally, a recent meta-analysis suggests that the use

of steroids provides the most benefit in the sickest patients [241]. A longer course of corticosteroid treatment has been attributed to long-term genomic effects, including sequestration of proinflammatory nuclear transcription factors NF-κB and AP-1 [240]. However, significant concerns exist in the medical community as to whether steroid therapy provides significant benefit for all patients or just specific sub-groups [240]. Patients with meningococcal infections are not likely to benefit from steroid treatment and evidence suggests that steroids may have adverse effects in cerebral malaria, RSV and HBV infection [239, 242]. As contraction of nosocomial infection is a potential consequence of steroid therapy in sepsis the use of corticosteroids is often restricted to sub-populations of critically ill patients with poor response to vasopressors [243].

1.3.3 Immune modulating therapies for sepsis

As outcomes of sepsis are intrinsically linked to the magnitude and duration of immune signalling in both SIRS and CARS phases, agents that aim to prevent or modulate this signalling are currently being explored in human studies. However, immune suppression may exacerbate disease pathogenesis in many patients as the initial inflammatory response is generally followed by an immunosuppressed CARS phase [244], which is poorly replicated in preclinical models. This is important as many patients survive the initial infection and associated systemic inflammation only to succumb to secondary nosocomial infections, most commonly pneumonia [217]. Consequently, there remains divergence in the scientific community as to whether treatments should seek to suppress the immune system or to activate it in response to sepsis, or to do both at different times [245, 246].

Agents that function to prevent PRR signalling by neutralisation of pathogen components or blockade of their receptors have been trialled in the prevention [247] and treatment [248] of sepsis, with limited success. Many infections caused by gram negative organisms release lipopolysaccharides (LPS) which can potentiate inflammation through TLR4 signalling. Consequently, a plethora of approaches to block TLR4 or LPS have been explored in preclinical models. A small molecule TLR4 antagonist, E5564, has been trialled with considerable success in both animal models and human experimental endotoxaemia, but did not confer a survival advantage in clinical trials despite a trend towards reduced mortality rates observed in the most severely ill patients [248]. Polymyxin B is a polypeptide antibiotic that has the ability to neutralise LPS, however it also potentiates nephrotoxicity and neurotoxicity [249], making it unsuitable for administration to septic patients. Technological

advancements in haemoperfusion technology have led to the development of blood filtration systems that pass blood through polymyxin B adsorbed filters, thereby removing circulating LPS [250, 251]. Early trials indicated the treatment was associated with improved haemodynamic function in gram-negative septic shock patients and large-scale trials of similar devices are ongoing. However, clinical utilisation of such methods may be hindered by views that absorptive haemoperfusion devices are inconvenient and time-consuming to set up [252] and only reliably effective in a subset of patients with high circulating LPS levels. Infection with staphylococcal species is particularly harmful in preterm infants and is a significant cause of late-onset neonatal sepsis [253]. As preterm infants are at increased risk of contracting infections that potentiate late-onset sepsis due to their prolonged hospital stay, there is increased interest in prophylaxis against pathogens in these infants. High dose prophylactic Pagibaximab, a mAb against staphylococcal lipoteichoic acid, completely prevented staphylococcal sepsis when administered to a small cohort of preterm infants, though therapeutic efficacy has not been established [247].

Despite exhibited efficacy in some patient sets, approaches to block pathogen signalling are unlikely to serve as ‘magic bullet’ therapies for the majority of patients, as they rely heavily on the identification of infective organisms for greatest efficacy. It has been postulated that, even when instigated as early as possible, blockade of PRR signalling is a poor strategy for sepsis treatment as the majority of harmful PRR signalling occurs in the first hours after infection and therapy may have been initiated too late [254].

As dysregulated cytokine expression has been associated with sepsis mortality, including interleukin 6 (IL-6) [255], tumour necrosis factor alpha (TNFα), IL-1 [256] and IL-8 [257], approaches to block the function of these mediators have been explored for sepsis treatment.
One of the first cytokines to be targeting in sepsis is TNFα, with approaches utilising fragmented pAbs [258], whole mAbs [259, 260], fragmented mAbs [261, 262] and soluble TNF receptors [263] trailed over the last 25 years. A recent meta-analysis of all anti-TNFα approaches revealed only a modest benefit, with a 2% improvement to mortality rates in treated individuals compared to placebo [264]. Similarly, blockade of IL-1 signalling via administration of IL-1 receptor agonist (IL-1RA) has exhibited limited success in clinical trials [265]. The poor success of cytokine directed therapies have been linked to the timing of administration as release of these cytokines are considered ‘early-phase’ sepsis signalling events and in many cases the treatment may have been initiated too late. This approach may also have little clinical utility in patients where CARS has been initiated [266]. Alternatively, administration of anti-inflammatory cytokines such as IL-10 has shown promise in reducing

morbidity from SIRS in preclinical models [267, 268] however IL-10 has been associated with sepsis-induced immune suppression [269], where administration to patients could potentiate CARS. Approaches targeting 'late-phase' inflammatory mediators or master regulators of the inflammatory response may be more promising in the clinic. One such mediator is high mobility group box 1 (HMGB1), which acts as an indicator of tissue damage to drive inflammation for tissue homeostasis.

1.3.4 Immune functions of HMGB1

Nuclear protein HMGB1 was originally identified as a DNA-binding protein with roles in DNA expression and regulation. In 1999, the first research attributing cytokine-like properties to HMGB1 was published and since HMGB1 has emerged as a therapeutic target for inflammatory conditions [270]. It has been specifically implicated in sepsis pathogenesis, where it functions as the archetypal DAMP [271, 272]. The inflammatory sequalea following HMGB1 release results from a complex series of interactions with immune mediators, receptor for advanced glycation end products (RAGE), TLR2, TLR4 and TLR9. Nuclear High Mobility Group (HMG) proteins are a group of chromatin-associated proteins that were found to have high-mobility in polyacrylamide gel electrophoresis separation after dissolution in 2% trichloroacetic acid [273]. These proteins are predominantly found in the cell nucleus bound to DNA, where they can exert a number of effects on cell cycle and metabolism through alterations in transcription and chromatin folding. The protein designated HMGB1 is one of four identified HMGB proteins and has two sequence dependant DNA-binding HMG-boxes and an acidic carboxyl-terminal tail.

The HMGB1 gene is located on human chromosome 13 and encodes a 214-amino acid protein. It is expressed in humans from development in the zygote stage and highly expressed in adult cells of all mammals and most eukaryotic organisms [274]. Studies on genetically modified $HMGB1^{-/-}$ mice have shown HMGB1 to be essential for development; knockouts fail to mature to adulthood and exhibit multiple organ failure and hypoglycaemia [275]. *In vitro* analysis of $HMGB1^{-/-}$ cells have suggested a role for HMGB1 in genome stability as the cells exhibit altered expression of genes involved in cell signalling, cell cycle and apoptotic pathways [276, 277]. It has been postulated that HMGB1 may mediate these effects by influencing DNA repair and telomerase function [278].

In addition to nuclear roles, HMGB1 has a growing number of roles within the cytoplasm or

extracellularly where it exerts its DAMP functions. The basic contributions of HMGB1 to sepsis pathogenesis are described in Figure 1.8. Interaction with RAGE, a multiligand transmembrane receptor involved in inflammation and immunity, promotes chemotaxis of immune cells and production of cytokines regulated by nuclear-factor kappaB (NF-κB) [279]. Stimulation of the HMGB1-RAGE axis has also been shown to upregulate intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 on endothelial cells in addition to RAGE itself [280]. Direct binding of HMGB1 to endothelium stimulates the release of fibrinolytic regulators tissue plasminogen activator and plasminogen activator inhibitor-1 [280], which deregulates haemostasis and triggers DIC. While HMGB1-RAGE binding has largely been thought to instigate the majority of the immunostimulatory activity attributed to HMGB1, the use of RAGE agonists has not been able to completely abrogate HMGB1-related immunostimulation [281]. The HMGB1-TLR4 signalling axis has been demonstrated to be crucial for HMGB1-mediated activation of dendritic cells [282] and T cells [283] and HMGB1-mediated disease pathology in myositis [284], cancer [285] and autoimmunity [286]

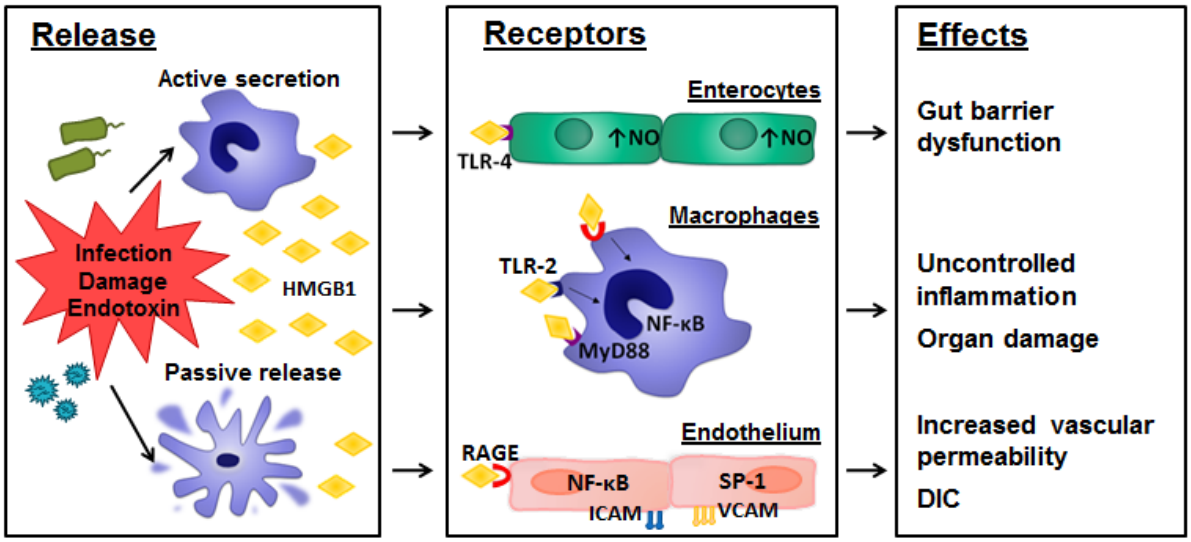


Figure 1.8 Contributions of HMGB1 to sepsis pathogenesis

HMGB1 is released passively or actively during sepsis following damage or pathogen sensing. HMGB1 binds to TLR-4 on enterocytes to stimulate nitric oxide production and gut barrier dysfunction. Sensing of HMGB1 via TLR-2, TLR-4 and RAGE may trigger activation of phagocytic cells, resulting in NF-κB transcription and production of inflammatory cytokines and chemokines. HMGB1 also binds to RAGE on endothelial cells and evokes nuclear translocation of transcription factors NF- B and SP-1. In response, endothelial cells express RAGE and adhesins, and secrete cytokines and regulators of fibrinolysis which potentiate vascular permeability and disseminated intravascular clotting.

Interaction of HMGB1 with both TLR2 and TLR4 have been shown to induce production of proinflammatory cytokines through NF-κB signalling pathways [287, 288] and HMGB1 stimulation of neutrophils through TLR4 promotes the production of reactive oxygen species. Binding of HMGB1 to C-X-C motif chemokine 12 (CXCL12) forms a chemoattractive heterocomplex that instigates inflammation via C-X-C receptor 4 (CXCR4) signalling. This mechanism has been implicated in the development of anaemia in sepsis [289, 290], in addition to wound healing [291] and tumour growth [292]. A number of roles of HMGB1-DNA complexes generated from necrotic release of nuclear components have also been described. HMGB1 signalling through TLR9 triggers cytokine secretion [293] and also promotes some immunosuppressive functions [294] which may implicate HMGB1 in the pathogenesis of CARS as well as SIRS. In particular, the suppressive action of HMGB1-DNA on peripheral dendritic cells may have clinical relevance in increasing susceptibility of septic patients to secondary nosocomial infections.

The alarmin functions of HMGB1 can also be modified by post-translational modifications, including redox modifications. Active release of HMGB1 in response to cell activation requires the acetylation of specific lysines which enable translocation from the nucleus into cytoplasmic lysosomes [295]. Fusion of the lysosomal membrane to the cell surface allows release of HMGB1 into the extracellular space where it can interact with receptor on neighbouring cells or bind to other inflammatory mediators. Hyperacetylated HMGB1 is associated with increased proinflammatory function [296], and is the predominant form released following pyroptosis [297, 298]. Passively released HMGB1 from necrotic tissues is not acetylated however it is often associated with inflammatory ligands including DNA and histone proteins and exerts an adjuvant function [299, 300]. Release of HMGB1 following apoptosis is poorly understood as early evidence indicated that apoptotic cells did not secrete HMGB1 which aligns well with the concept of apoptosis as immunologically ‘silent’ [301]. However, it has since been elucidated that HMGB1 is contained within microvesicles [302] released following pyroptosis and apoptosis [303, 304]. Once released from the cell, HMGB1 can be reversibly reduced and oxidised at three cysteine residues (positioned at C23, C45 and C106) which confers a significant structural and functional change (Figure 1.9).

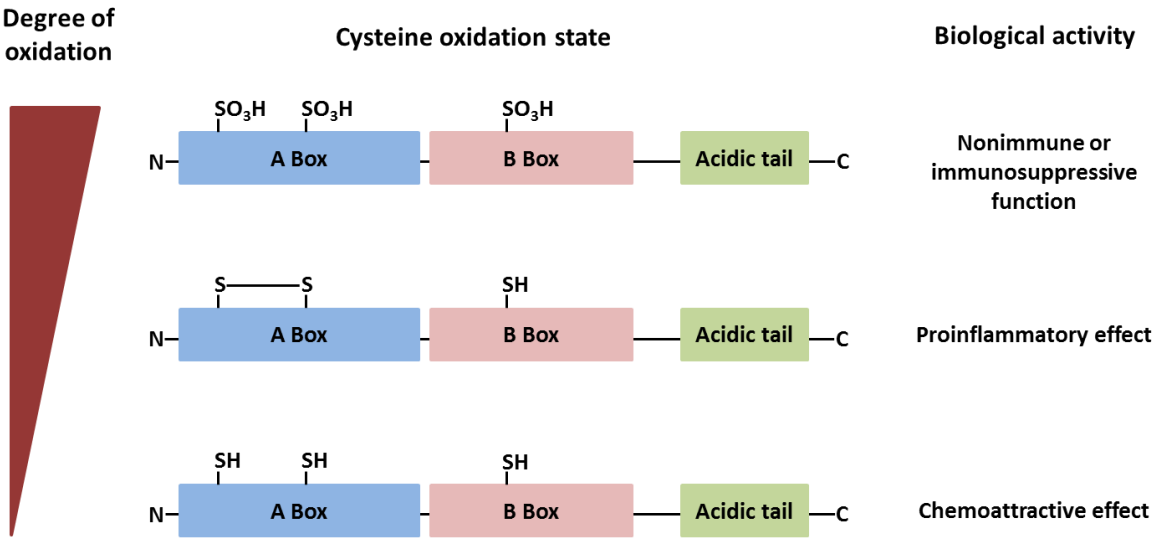


Figure 1.9 The redox state of HMGB1 impacts its biological function

Full-length HMGB1 contains three cysteine residues at C23, C45 and C106 that undergo redox modifications and confer functional variability. Terminally reduced all-thiol HMGB1 forms of a heterodimer with CXCL12 to signal through CXCR4 and promote chemotaxis. Partially oxidised HMGB1, characterised by a C23-C45 disulfide bond and a thiol-form C106, induces cytokine-like proinflammatory effects via interaction with RAGE and TLR4. Terminally oxidised sulphonyl-form HMGB1 lacks inflammatory activity, however it has been postulated that immunoregulatory functions of HMGB1 are attributed to this redox state.

In proinflammatory HMGB1, the first two cysteines are oxidised and form a disulphide bond whereas C106 is in a reduced (thiol) form. Thiol-C106 has been demonstrated as essential for HMGB1 binding to TLR4 [305]. The presence of a disulphide bond between C23 and C45 is thought to provide stability, and reduction or substitution of these residues markedly reduces HMGB1 cytokine activity [306]. The fully reduced form of HMGB1 also has roles in inflammatory disease as it performs chemotactic roles *in vivo*. Originally thought to occur from the 'cytokine' activity of HMGB1, it was elucidated in 2012 that cytokine and chemotactic functions of HMGB1 are mutually exclusive [307] and are performed by partially-oxidised and reduced forms of the protein respectively. Terminally oxidised HMGB1 does not exert cytokine or chemotactic activity, however it has been postulated that it may have a role in the resolution of immune responses and tissue regeneration [305, 307, 308]. *In vitro* studies have demonstrated that HMGB1 is able to exert a number of immunosuppressive effects, predominantly through RAGE signalling, which include the migration, enhancement and survival of regulatory T cells and restriction of conventional T cell proliferation [309]. It has been demonstrated *in vitro* that regulatory T cells express higher levels of RAGE than conventional T cells, and exposure to HMGB1 promotes IL-10 release. Additionally, HMGB1 appears to supress the production of IFNγ from conventional T

cells *in vitro*. However, *in vivo* studies have correlated increased HMGB1 expression with reduced regulatory T cell numbers and increased numbers of the Th17 subset [310, 311]. It is unknown whether the observed differential effects of HMGB1 on T cell subsets is due to the presence of different redox forms in different scenarios. As HMGB1 can be reversibly reduced and oxidised *in vivo* by natural antioxidants and reactive oxygen species, this may provide a mechanism in inflammatory conditions whereby HMGB1 can act as a biological 'switch': first promoting chemotaxis and then inflammation, and then becoming non-functional or anti-inflammatory as reactive oxygen species accumulate from excessive inflammation.

1.3.5 HMGB1 as a therapeutic target in sepsis

The release of HMGB1 can be classified into three mechanisms: active secretion from immune cells such as monocytes and dendritic cell subsets [295, 312]; passive release from cells undergoing autophagic stress [313] and from necrotic cell death [314, 315]. Necrotic release of HMGB1 is nearly instantaneous [301], whereas secretion from stressed but viable cells occurs later and is generally triggered by inflammation. As such, the kinetics of release of HMGB1 are distinct from those of other early proinflammatory mediators such as IL1β and TNFα. Significantly higher plasma HMGB1 has been observed in sepsis non-survivors at 3, 7 and 14 days post-admission to the ICU compared to survivors. The levels of HMGB1 also correlated with measures of sepsis severity; however it was found that HMGB1 was not an effective prognostic marker in determining outcome of septic shock.

Early *in vivo* experiments demonstrated that serum levels of HMGB1 increase in LPS-induced murine endotoxemia and lethality is completely abrogated following treatment with neutralising pAb [316]. Likewise, murine polymicrobial sepsis and septic shock is ameliorated following administration of anti-HMGB1 Abs or competitive antagonists [317]. Furthermore, mice are protected from sepsis mortality when the sphingosine kinase 1 pathway is blocked, which also inhibits HMGB1 release [318]. Administration of ethyl pyruvate to mice following CLP inhibited release of HMGB1 and TNFα and correlated with an increased survival rate from 30% to 88% [319]. Inhibitory A-box HMGB1 preparations have also been evaluated in preclinical studies and function via out-competing full-length HMGB1 for RAGE binding, without activating the receptor [308]. This approach has been used successfully to reduce inflammation in acute lung injury [320], autoimmune disease [321] and sepsis [317]. Studies of HMGB1 kinetics in septic mice revealed that HMGB1 release

parallels the onset of sepsis lethality, with peak levels occurring several days after the procedure. This is in contrast to other proinflammatory mediators that are released hours after CLP and decline [322].

Human studies have shown HMGB1 to be elevated in the serum of patients with severe sepsis and septic shock [323], and recently, survival from septic shock was found to be associated with the emergence of HMGB1 auto-Abs [324]. Preclinical modelling has shown that any approach used to neutralise or inhibit HMGB1 activity has a very wide therapeutic window [272], thus these findings present a strong argument for passive immune targeting of HMGB1 in bacterial sepsis and septic shock. As HMGB1 can complex with multiple binding partners, an approach to neutralise HMGB1 function ideally would need to target multiple binding sites that are differentially exposed on different forms of the protein. Combinations of traditional mAb therapies could be directed to target HMGB1, however these may require complex pharmaceutical formulation, thus pAb approaches are also being explored for anti-HMGB1 therapeutics. While the efficacy of HMGB1 neutralisation has been demonstrated in several animal models of inflammation, the contribution of HMGB1 to haemodynamic changes, organ damage and susceptibility to secondary infection require investigation in increasingly complex models of sepsis.

1.3.6 Approaches to modelling sepsis in the laboratory

Sepsis is a highly complex and often fatal syndrome which varies widely in its clinical presentation [211]. The pathogenesis of sepsis is influenced by multiple factors: patient characteristics such as age, comorbidities, and immune function; the nature of infection including infective site and type of pathogen; and the standard of care available including the time to intervention and supportive medications. Development of an animal model that appropriately recapitulates the complexity of sepsis is crucial to the development of effective therapeutics, however the majority of therapeutics that show efficacy in preclinical models do not exhibit similar clinical benefits [325]. This discrepancy highlights the need for multiple animal sepsis models and the refinement of existing models to better correlate with clinical outcomes. The use of relevant animal models is also imperative to gain insights into sepsis physiology, investigate long-term effects in survivors and improve treatment paradigms.

Selection of an animal species for preclinical sepsis models is often dictated by the need for a balance between ease of execution and complexity of the model. Small animal models favour quick, cost effective and easily executed studies whereas larger animal models are often more

appropriate to measure multiple clinical parameters.

Rodent models, including mice and rats, are among the simplest, most cost effective and accessible sepsis models for preclinical research. The availability of inbred strains with defined genetic characteristics and the wide array of reagents for immunologic studies in rodents enable in-depth evaluation of immunological parameters in sepsis that would not be easily achievable in large animal species [326]. Rodent models are commonly utilised as initial screening systems to identify and test new therapeutics, however these models have fallen under criticism following genome-wide expression analyses of human and rodent inflammatory responses, which revealed no more similarities between human and rodent gene expression following sepsis than could be found by random chance [327]. However, a second analysis restricted to genes differentially expressed in disease states revealed consistencies between human and murine gene expression in inflammatory diseases [328], and thus rodent models still provide useful data in the study of sepsis from a molecular perspective. Large animal models offer considerable benefits for the study of sepsis, as they are more likely to recapitulate pathophysiologic changes seen in humans such as alterations in cardiovascular function. However, as large animal models are less frequently performed, genomic comparison data is not yet available for the most common large animal sepsis models. In addition to the selection of an appropriate species, the method used to induce sepsis profoundly impacts the performance of the model. Preclinical sepsis models may be classified by the administration of an initiating agent: administration of a toxin; administration of a viable pathogen (such as bacteria); or alteration of the host barrier to allow dissemination of endogenous factors [329].

Simple toxaemia models involve the administration of a pathogen-derived toxin in lieu of a live organism in order to engage immune receptors to trigger inflammation and a shock-like state. In addition to being simple to perform, toxin administration in inbred rodents is highly reproducible. Toxin models are often used for acute sepsis studies where the initial innate-driven inflammatory response is of particular interest. Lipopolysaccharide (LPS) is the most common toxin used for these models and rapidly induces inflammation and clinical symptoms such as lethargy, piloerection, hypothermia and shivering. Cytokine production after LPS administration is also rapid but transient and the pattern of sustained cytokine release observed in clinical sepsis is only achievable with a continuous infusion of toxin. Furthermore, mice are highly resistant to LPS and require approximately 250 times the dose required in humans to achieve a similar cytokine response [330]. Consequently, while LPS administration is a convenient model for the study of transient inflammation it has limited

translational utility for the study of sepsis.

Live challenge models retain the ease of toxaemia models but introduce the complexity of a live, replicating organism, frequently bacteria, to the development of sepsis. Live challenge models can be tailored to achieve an appropriate mortality rate by selection of the infective organism and the route, dose and frequency of administration. For example, IV administration of *E. coli* induces a robust cytokine response however peritoneal administration promotes cytokine responses which are more mild [331]. The rate of administration can greatly impact the course of sepsis as a bolus dose of bacteria will induce similar responses to endotoxin administration whereas a continuous infusion of bacteria can more appropriately mimic a sustained clinical infection. A more complex, but arguably more relevant, bacterial administration model involves surgical implantation of a fibrin clot or agarose beads which are loaded with viable bacteria [332]. The implant can slowly disseminate bacteria to mimic an established focus of infection however the use of a single organism raises concerns that this does not appropriately mimic natural infection. In a clinical infection, the ratio of infective aerobic to anaerobic species is altered as the infection is controlled however polymicrobial bacterial implant models are not able to recapitulate this [333].

The gastrointestinal tract is often utilised as a source of endogenous bacteria in experimental sepsis models. A number of surgical sepsis models that aim to inoculate the peritoneum with gut bacteria have been developed, which range from injection of donor caecal contents into the peritoneum [334], to more complex surgical procedures. One of the most well-characterised endogenous sepsis models is caecal ligation and puncture (CLP), where a portion of the caecum is ligated from the gastrointestinal tract and punctured to induce peritonitis. The induction of sepsis in rodents through CLP has several consistencies with human sepsis, including a polymicrobial infection, development of acute organ injury [335, 336] and hyperdynamic circulation [337]. However, the CLP model bears the same criticisms as most rodent sepsis models, as they are generally not amenable to the same standard of supportive medications that are used clinically. While some supportive therapies have been used in the CLP model, including antibiotic administration [338] and fluid replacement therapy [337], the application of further supportive therapies are likely to be expensive and may not necessarily increase the similarity to human sepsis [325].

Sepsis models that can incorporate clinically relevant supportive medications are often performed in large animal species. Sepsis has previously been modelled in pigs [339], sheep [340], dogs [341] and cats [342]. Sheep models are particularly advantageous due to the

haemodynamic similarity between sheep and humans [343] and the availability and ease of handling of sheep. Septic sheep have previously been used to study clinically relevant parameters including the effects of inotropes [344], organ blood flow [343] and respiratory function [345, 346]. Recently, the ovine sepsis model has also been applied to study thyroid hormone function [347] and kidney pathology [348] in sepsis. These previous studies improve the understanding of ovine sepsis to enable the comparison to human sepsis using clinically-relevant parameters.

1.4 Aims and rationale

There exists an unmet need for infectious disease therapies that are efficacious in at-risk individuals and can be rapidly administered in time-sensitive critical care scenarios when limited diagnostic or prognostic data are available. Cost-effective passive immunotherapies based on ovine pAbs may fulfil these requirements and represent a highly customisable platform for the development of bio-therapeutics for a range of clinical needs. The works described within this thesis utilised current methods of therapeutic ovine pAb production for the production of novel therapeutics and assessed their function in animal models. The aim of this thesis was to explore the application of ovine pAbs in two clinical scenarios: as direct anti-infective agents in severe influenza infection and as immunomodulatory agents in the treatment of sepsis and septic shock.

Part A explores the application of ovine pAbs generated against HA to the treatment of severe influenza infection. The approach towards the generation of anti-HA ovine pAbs was empirically optimised using different adjuvants, immunisation routes and antigen doses to efficiently elicit high ovine pAb titres. The generated pAbs were applied to a murine model of influenza infection to explore not only therapeutic efficacy but also the consequences of this approach including immunogenicity of the therapy and the subsequent generation of adaptive influenza-specific responses. The aims of this work were to provide proof-of-principle for the development of an ovine pAb targeting influenza and to investigate the impact on subsequent anti-influenza responses.

Part B of this thesis details an approach towards the development of HMGB1-neutralising ovine pAbs for the treatment of sepsis and septic shock. A clinical study was performed to profile HMGB1 release in the context of the inflammatory milieu in the early phase of septic shock and ovine anti-HMGB1 pAbs were subsequently assessed in three murine sepsis

models to elucidate the effects of this approach on septic inflammation and survival. Finally, anti-HMGB1 pAbs were applied to an ovine sepsis incorporating intensive care supports to assess the effects of anti-HMGB1 pAbs on physiological sepsis endpoints. The aims of this work were to determine the feasibility and efficacy of anti-HMGB1 ovine pAbs in the treatment of sepsis using a clinically-directed approach to preclinical sepsis models.

CHAPTER 2.

MATERIALS AND METHODS

2.1 Cell culture media, buffers and solutions

2.1.1. Diluent solutions

Saline

Saline solution was prepared by the Media Production Unit (MPU), Infectious Diseases Laboratories, SA Pathology, Adelaide, Australia as a 0.85% (w/w) solution of NaCl in Milli Q water before filter sterilisation.

Phosphate buffered saline (PBS) 20x concentrate

A 20x concentrate of phosphate buffered saline (PBS) was prepared by the MPU (SA Pathology) and consisted of 2.7 M NaCl, 54 mM KCl, 30 mM KH_2PO_4 and 160 mM of anhydrous Na_2HPO_4 in Milli Q water, and was diluted 1:20 in reverse osmosis (RO) water for a working concentration of 1x PBS.

Endotoxin-free PBS (EF-PBS)

Endotoxin-free PBS (EF-PBS) was prepared MPU (SA Pathology) as 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH KH_2PO_4 and 8 mM of anhydrous Na_2HPO_4 dissolved in Baxter water (Baxter Laboratories), adjusted to pH 7.4, and filter sterilised. The endotoxin level was confirmed to be below 0.15 EU/mL via limulus amebocyte lysate assay (Charles River Laboratories).

2.1.2. Cell culture media

Mammalian cell culture

Roswell Park Memorial Institute (RPMI) medium (Sigma Aldrich) was supplemented with 100 U/mL penicillin, 100 µg/mL gentamycin, 2 mM L-glutamine (replaced every six weeks) and 10 mM (4-(2-hydroxyethyl)-1-peprazineethanesulfonic acid (HEPES) to produce incomplete RPMI (iRPMI). Complete RPMI (cRPMI) was prepared for use by the addition of 10% heat inactivated (56°C, 30 min) foetal calf serum (FCS; JRH Biosciences) to iRPMI.

Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich) was supplemented with penicillin, gentamycin, L-glutamine, HEPES and FCS as above, to produce complete DMEM.

Insect cell culture

Sf900™ III serum-free medium (SFM; Thermo Fisher Scientific) was supplemented with 100 U/mL penicillin, 100 µg/mL gentamycin, 2 mM L-glutamine and 10 mM HEPES for sf21 cell culture.

2.1.3. Bacterial culture media

Luria Bertrani Broth and Agar

Luria Bertrani (LB) broth was prepared by addition of Bacto™ typtone (1% w/v; BD Biosciences), Bacto™ yeast extract (0.5% w/v) and NaCl (1% w/v) to RO water; the pH was adjusted to 7.5 before autoclaving (25 min, 121 °C). For LB agar preparation, 1.5% (w/v) Bacto™ agar was added to the broth before autoclaving. Molten LB agar (20 mL) was poured into sterile 100 mm polystyrene petri dishes and allowed to harden. Antibiotic selection plates were prepared by addition of ampicillin (100 µg/mL), kanamycin sulphate (50 µg/mL), gentamicin (7 µg/mL) or tetracycline (10 µg/mL) to molten agar directly before plates were poured. For blue-white colony selection, poured plates were spread with IPTG (100 µL of 25 mg/mL stock; Sigma Aldrich) and 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal; 40 µL of 20 mg/mL stock; Sigma Aldrich) and allowed to dry (37 °C, 30 min) before use.

Super optimal broth with catabolite repression medium

Super optimal broth with catabolite repression (SOC) medium was prepared by addition of Bacto™ typtone (2% w/v), Bacto Yeast Extract (0.5% w/v), NaCl (10mM), KCl (2.5 mM), $MgCl_2$ (10mM) and $MgSO_4$ (10mM) to RO water; the pH was adjusted to 7.5 before auoclaving. Once cooled, filter sterilised 20% glucose solution was added (20 mL per L of broth).

Tryptic Soy Broth and Agar

Tryptic soy broth (TSB) was reconstituted in RO water from dehydrated media (30 g/L; Merck Millipore) and autoclaved (25 min, 121° C). For TSB agar preparation, 1.5% (w/v) Bacto™ agar (BD Biosciences) was added to the broth before autoclaving. Molten TSB agar (20 mL) was poured into sterile 100 mm polystyrene petri dishes and allowed to harden.

2.1.4. His-tagged protein purification buffers

Insect-cell lysis buffer was prepared from a buffered saline base consisting of 50 mM NaH_2PO_4 and 300 mM NaCl (pH 8) in Milli-Q water and was supplemented before use with 10 mM Imidazole, 1% Igepal and 1x cOmplete EDTA-free protease inhibitor (Roche Applied

Science). Wash buffer was prepared from buffered saline base supplemented with 20mM Imidazole. Elution buffer was prepared as a solution of 50 mM NaH_2PO_4, 300 mM NaCl and 250 mM Imadazole in Milli-Q water and adjusted to pH 8.

2.1.5. Red blood cell (RBC) lysis buffer

Red blood cell lysis buffer consisted of 150 mM NH_4Cl, 1mM $KHCO_3$ and 0.1 mM Na_2EDTA in Milli Q water, pH adjusted to 7.3.

2.1.6. Fluorescence-activated cell sorting (FACS) buffer

Fluorescence-activated cell sorting (FACS) buffer was prepared by dissolving 0.5% (w/v) bovine serum albumin (BSA; Sigma Aldrich) in 1x PBS without agitation. To minimise contamination, 0.04% (w/v) $NaN_3$was added and the final solution stored at 4° C.

2.1.7. SDS-PAGE loading dye (2x)

A 2x concentrate of SDS-PAGE-loading dye was prepared by adding 17.5% (v/v) glycerol, 4% (w/v) SDS, 10% (v/v) 2-ME and 0.001% (w/v) bromophenol blue to 0.125 M Tris base solution (pH 6.8).

2.1.8. SDS-PAGE running buffer (10x)

SDS-PAGE running buffer was prepared as a 10x concentrate with a final concentration of 250mM Tris base, 1.9 M glycine and 35 mM SDS in Milli Q water. Concentrate was diluted 1:10 in RO water prior to use.

2.1.9. Coomassie stain solutions

Coomassie stain was prepared by dissolving 0.1% Brilliant Blue (w/v), 10% acetic acid (v/v) and 40% methanol in RO water. Coomassie destaining solution consisted of 10% acetic acid (v/v) and 40% methanol in RO water.

2.1.10. Western blot buffers

Transfer buffer

Western blot transfer buffer was prepared by dissolving 7.5g Tris base and 36g glycine in RO water, with the volume made up to 2 L. Solution was magnetically stirred to dissolve solids before the addition of 500 mL methanol. Transfer buffer was stored at 4° C prior to use.

TBS and TBS-T

Tris buffered saline (TBS) was prepared as a 10x stock by dissolving 200 mM Tris base and 1.4M NaCl in MilliQ water. Stocks of TBS were diluted 1 in 10 in RO water prior to use. TBS-T was prepared by the addition of 0.1% (v/v) Tween-20 to prepared TBS and was used as diluent in blocking and antibody solutions.

2.1.11. Saturated ammonium sulphate (SAS)

Saturated ammonium sulphate (SAS) solution was prepared for the precipitation of polyclonal antibodies (pAbs) from whole serum. To yield 1 L of solution, 570g of ammonium sulphate solid (Sigma Aldrich) was added to 750 mL of Milli-Q water and solution magnetically stirred over a hot plate until 75°C was reached and all salt crystals had dissolved. The solution was stored at RT and if small ammonium sulphate crystals were observed in the bottom of the bottle after ~24 hours, then the clear solution above was considered 100% SAS.

2.1.12. Antibody purification buffers

Protein G columns (GE Healthcare) were used to purify ovine pAbs from whole ovine serum or dissolved serum precipitate. Filtered 1 x PBS was used for antibody (Ab) binding and column washing, and bound pAb were eluted by the addition of 0.15 M glycine (pH 2.5) prepared in Milli-Q water. Before use all buffers were passed through a 0.22 µm filter attached to a vacuum manifold and allowed to degas under vacuum pressure for 10 min.

2.2 Animals

2.2.1. Mice

Normal Balb/c females (6-8 weeks old) were obtained from Laboratory Animal Services (The University of Adelaide) and normal C57Bl/6 (B6) mice (8-12 weeks old) were bred in-house

at the Reid Animal Facility (RAF), University of South Australia. Mice were housed in individually ventilated cages and provided with standard chow and water *ad libitum*. All mice were housed at the RAF under specific-pathogen-free (SPF) and PC2 conditions, unless administered infectious organisms such as influenza virus or *P. aeruginosa* bacteria where mice were transferred to a dedicated infectious containment area. All animal studies were conducted with approval from the University of South Australia Animal Ethics Committee (AEC) and followed institutional and national ethical guidelines according to the principles outlined in the 'Australian code for the care and use of animals for scientific purposes (2013)'.

2.2.2. Sheep

Border Leicester x Merino ewes were housed in groups at South Australian Health and Medical Research Institute (SAHMRI) Preclinical, Imaging and Research Laboratories (Gilles Plains, South Australia) where they were given free access to food and water. Ewes between 9 months – 3 years old were used for pAb production, and ewes between 2.5-4 years old were used in the septic sheep model. All sheep studies were approved by SAHMRI AEC and conducted following institutional and national guidelines as above.

2.3 Clinical study

2.3.1. Human ethics

A prospective cohort study on septic shock patients admitted to the Royal Adelaide Hospital (RAH) Intensive Care Unit (ICU) between Dec 2012 - Dec 2013 was approved by the RAH Research Ethics Committee, the University of South Australia and The University of Adelaide Human Research Ethics Committees. Informed consent was sought from patients or next-of-kin as appropriate, and all samples obtained from patients were deidentified prior to storage and analysis. All procedures relating to the enrolment, sampling and analysis of patients conformed to the provisions of the National Statement on Ethical Conduct in Human Research, Australian National Health and Medical Research Council (NHMRC).

2.3.2. Inclusion criteria and sampling

Patients ≥ 18 years old admitted to the ICU with septic shock were identified by clinical staff, with septic shock defined as a diagnosis consistent with sepsis (including pneumonia, urinary

tract infection and peritonitis) in addition to hypotension despite adequate fluid resuscitation, adequate intravascular volume status and the need for vasopressors. Vasopressors included any infusion of noradrenalin or adrenalin. Patients on steroid therapy were excluded from the study, with the remaining patient cohort characteristics summarised in Table 4.1 (Chapter 5). Arterial blood was collected from patients into sodium citrate tubes on the first morning after admission (1-12 hours post admission) and every morning and afternoon thereafter for as long as haemodynamic intervention was required or a maximum of 7 days. Blood samples were immediately centrifuged (10 minutes, 1,000 x g) and the plasma aliquoted and frozen (-80˚C).

2.4 Recombinant protein production

2.4.1. Preparation of electrocompetent E. coli

The process of making electrocompetent *E. coli* involves washing away salts from culture medium to yield cells in a non-ionic buffer that can sustain DNA transformation through electroporation. A 2.5 mL aliquot of a fresh O/N culture of XL-1 *E. coli* inoculated from a single colony was used to inoculate fresh LB broth (500 mL). The broth was incubated (37 °C with shaking at 220 rpm) for approximately 4 hours to achieve a bacterial culture in log-phase growth, as indicated by an OD_{600} of 0.5-0.7. The culture was subsequently transferred to two chilled 250 mL centrifuge pots and allowed to cool on ice. Cultures were centrifuged (2°C, 5000 xg) for 20 minutes, with the resulting pellets initially gently resuspended in 5 mL ice-cold sterile Milli-Q water and then adjusted to a final 500 mL volume. Bacteria were centrifuged and washed once more before being carefully resuspended in sterile 10% glycerol in Milli-Q water (40 mL) and transferred to a 50 mL tube. The cells were again pelleted by centrifugation before addition of a quantity of cold 10% glycerol equivalent to the volume of the pellet (approximately 1.5 mL). Aliquots (40 µL) of the cells were stored at -80 °C before use as electrocompetent bacteria.

2.4.2. Sequence isolation and PCR

For production of recombinant influenza haemagglutinin (HA), the HA sequence of A/Puerto Rico/8/1934 (PR8) H1N1 (NCBI accession: AF389118) was obtained from gDNA prepared from PR8-transfected AB1 malignant melanoma cells (AB1-HA). The sequence was amplified via PCR with HotStarTaq Plus polymerase (Qiagen) according to the manufacturer's instructions and used specific primers modified to incorporate the appropriate restriction sites and a C-terminal 6x-His tag (Table 2.1). The resultant PCR product was

verified by 1% agarose gel electrophoresis visualised with 1x GelRed (Biotium) and corresponded to the predicated size of 1745bp (Figure 2.1a).

For production of recombinant human high mobility group box 1 (HMGB1), the full-length human HMGB1 sequence was cloned from mRNA obtained from donor white blood cells. Briefly, freshly drawn blood (8 mL) was placed into heparinised collection tubes and centrifuged (2,500 xg) for 10 min. A pasteur pipette was used to isolate the subsequent buffy coat layer residing on the surface of the RBC pellet which was placed into a fresh 10mL tube containing 1 x PBS. Cells were pelleted by centrifugation (10 min, 500 xg) and the entire pellet homogenised in TRIzol reagent (Thermo Fisher Scientific). Total RNA was isolated according to the manufacturer's instructions and cDNA was generated using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) according to manufacturer's instructions using oligo(dT) primers. The generated cDNA was subjected to PCR to amplify the HMGB1 sequence using specific primers encoding a C-terminal 6x-His tag and appropriate restriction sites (Table 2.1). Presence of a PCR product corresponding to the predicted size of 678bp was verified by 1% agarose gel electrophoresis (Figure 2.1b).

Table 2.1 PCR primers and cycling parameters used

Target sequence	Primer sequence	PCR program	
		Step	Parameters
Influenza HA (1745 bp)	Forward Primer: 5' TCG GTC GAC (Sal I) ACC ATG GAT GAA GGC AAA CCT ACT GGT C 3' Reverse Primer: 5' AA T*GC GGC CGC* (Hind III) TCA **ATG ATG ATG ATG ATG ATG** GCT AGC GAT GCA TAT TCT GCA CTG CAA 3'	Step 1 Step 2 Step 3 Step 4 Step 5 Step 6 Step 7	95˚C for 5 min 94˚C for 1 min 59˚C for 2 min 72˚C for 1 min Go to Step 2, 35 x 72˚C for 10 min 4˚C forever
Human HMGB1 (678 bp)	Forward Primer: 5' TCG GAA TTC (Eco RI) AGA ATG GGC AAA GGA GAT CCT AAG 3' Reverse Primer: 5' AAT GGC ATG CAA CCT (Sph I) AGG TTA **ATG ATG ATG ATG ATG ATG** TTC ATC ATC ATC ATC TTC 3'	Step 1 Step 2 Step 3 Step 4 Step 5 Step 6 Step 7	95˚C for 5 min 94˚C for 1 min 60˚C for 2 min 72˚C for 1 min Go to Step 2, 35 x 72˚C for 10 min 4˚C forever

Primers and conditions used for PCR amplification of target sequences. Specific primers are included in 5' to 3' notation with His-tag sequence represented by bold text and restriction sites indicated.

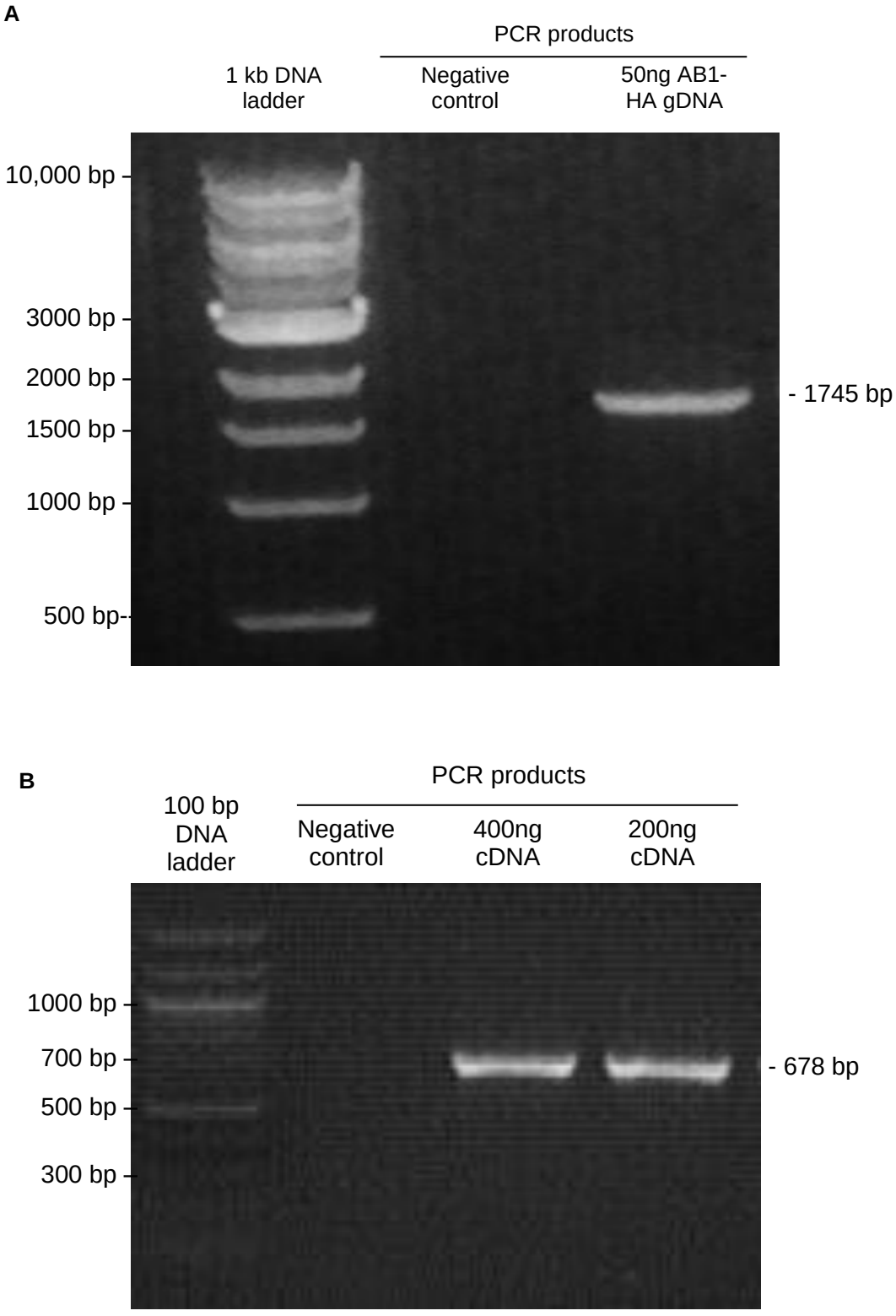


Figure 2.1 PCR product amplification of HA and HMGB1 gene sequences.
PCR amplifications of (A) influenza HA or (B) human HMGB1 target sequences were analysed via 1% agarose gel electrophoresis and visualised with GelRed under UV transillumination.

2.4.3. Generation of HA and HMGB1 expressing bacmid

Amplified HA or HMGB1 PCR products were initially cloned into the pGEM-T Easy vector system (Promega,) using the included T4 DNA ligase to ligate the products containing 3' adenine overhangs to the complementary 5' thymine overhangs present on the linearized vector. After incubation (4°C, O/N), the ligation reactions were added to electrocompetent XL-1 *E. coli*, transferred to a 2mm cuvette and electroporated at 2.5kV using a MicroPulser (Bio-rad). Transformants were resuspended in 1 mL SOC medium and incubated for 1 hour (37°C) before being plated onto ampicillin selective plates for blue-white colony selection. Positive (white) clones potentially containing the inserted PCR product were expanded in LB broth (37°C shaking, O/N) after which plasmid DNA was purified from the cultures using the QiaSPIN Miniprep kit (Qiagen). Insertion of the correct sequence was confirmed by using the BigDye V3 Sequencing kit (Thermo Fisher) according to the manufacturer's instructions. Briefly, purified plasmids were subjected to PCR cycle sequencing with vector-specific T7 and SP6 primers and the resulting DNA precipitated with 75% isopropanol. Dried DNA pellets were submitted to the SAPathology Molecular Pathology Department where sequences were analysed using an Applied Biosystems 3730xl DNA analyser.

Confirmed plasmid DNA containing correct HA and HMGB1 sequences were subsequently used to produce recombinant baculoviruses using the Bac-to-Bac expression system (Invitrogen). Briefly, inserts were digested from pGEM-T Easy vector using the restriction enzymes indicated in Table 2.1 and ligated into pFASTBac 1 vector which had been linearized with the same enzymes. Ligation mixtures were transformed into XL-1 *E. Coli* by electroporation as described above, with putative transformants grown on LB agar plates supplemented with ampicillin as a selective agent. Selected colonies were screened via PCR with sequence specific primers (as indicated in Table 2.1), and plasmids were purified from positive colonies using QiaSPIN Miniprep kits. Purified pFastBac 1 plasmids were used to transform chemically competent DH10-Bac *E. coli* using heat-shock transformation to generate a recombinant bacmid, according to the manufacturer's instructions. Transformation mixtures were cultured in SOC medium for four hours before streaking onto LB agar for blue-white selection with added kanamycin, gentamicin and tetracycline as selective agents. After growth for 48 hours, white colonies (indicating successful transposition of the pFastBac plasmid expression cassette into the baculovirus shuttle vector) were selected and regrown to confirm a white phenotype before inoculation of a broth culture containing selective antibiotics and grown at 37°C with shaking for 24 hours. Bacmid DNA was purified from the culture (25 mL) using a Qiagen Midiprep Kit.

2.4.4. Protein production

Semi adherent insect cells (Sf21) were maintained in sf900 SFM III media supplemented as described in 300 mL or 500 mL roller bottle flasks in a rolling incubator (27 °C between 0.5-2 x 10^6 cells/mL). Cells were split upon reaching a concentration of 2 x 10^6 cells/mL by addition of fresh media to the suspension culture to achieve a 1:2 or 1:4 split every 1-2 days.
Recombinant baculovirus was generated via the Bac-to-Bac expression system (Invitrogen) according to the manufacturer's instructions. Briefly, recombinant bacmid DNA (2 μg) was diluted in 200 μL antibiotic-free sf900 SFM containing 3% CellFECTIN II reagent and incubated for 25 min before the addition of 800 μL fresh antibiotic-free medium. Cultured sf21 cells (>95% viability) were adhered in a 6 well plate (9 x 10^5 cells/well) for 1 hour before washing with antibiotic-free medium. Transfection mixtures (1 mL) were added to adhered cells and incubated at 27°C for 4 hours; negative control wells were also included which contained CellFECTIN II reagent with no DNA. Transfection mixtures were aspirated and 1mL of supplemented sf900 III SFM (with antibiotics) was added. The plates were incubated at 27°C for 5 days and supernatants were harvested to yield passage 1 (P1) virus. Cells were also harvested via vigorous pipetting and the resultant pellet resuspended in 1 x SDS-PAGE loading dye for SDS-PAGE and Western blot protein expression analysis. Once protein expression had been confirmed, viral stock was amplified twice by incubation with roller bottle cultures of sf21 cells in suspension (1 x 10^6 cells/mL) for 48 hours to generate high-titre P3 stock. The quantity of viral stock needed for infection to achieve optimum protein expression was empirically determined for each viral stock by small-scale infections in 12 well plates (1 x 10^6 cells/well). Cells were incubated with 0, 25, 50, or 100 μL of viral stock added to media (1 mL) and wells were harvested at 48, 72, and 96 hours for analysis of protein in cell lysates by SDS-PAGE and Western blot. The conditions yielding the highest protein quantity were selected for scale-up. Large-scale protein production was performed by infection of 300-500 mL roller bottle cultures of sf21 cells (2 x 10^6 cells/mL) with 25-40 mL P3 baculovirus stock. Infected cultures were maintained for 4 days and supplemented with 100 μg/mL L-glutamine 24 hours prior to harvest.

2.4.5. His-tagged protein purification

His-tagged proteins were purified from baculovirus-infected cells using Ni-NTA agarose beads (Qiagen) according to the manufacturer's instructions. Beads were washed before use in 2 x 10 mL His-tag wash buffer to remove storage buffer, and beads were resuspended to a

50% slurry. Thawed cell pellets from large-scale infections of sf21 cultures were lysed by resuspension in ice-cold insect cell lysis buffer, at a ratio of 10 mL lysis buffer per 100 mL culture volume for 10 min before centrifugation (15,000 xg for 10 min) to remove membrane-bound material and nuclear debris. Harvested supernatant was incubated with 1.5-2 mL washed 50% Ni-NTA bead slurry for 4 – 18 hours (4°C) on a rotator to bind protein. Beads were pelleted and washed with 3 x 20 mL changes of wash buffer. Bound protein was eluted from beads by addition of 1 mL elution buffer followed by brief vortex mixing and centrifugation to collect beads. Supernatant containing eluted protein was collected in 1 mL fractions and elution repeated to achieve a total of 5-6 fractions. Used beads were stored in elution buffer at 4°C and reused after washing with 0.5 M NaOH according to manufacturer's instructions. Eluted protein fractions were analysed by UV spectroscopy (280nm; NanoDrop, Thermo Fisher Scientific), with the Beer-Lambert equation used to determine approximate protein concentration and stored at 4°C. Protein-containing fractions were pooled, added to dialysis tubing and dialysed against 3 changes of 1x PBS (1 L).

2.4.6. SDS-PAGE and Western Blot

Protein samples (5-10 µg) were diluted 1:2 in 2x SDS loading dye, boiled (95°C, 5 min) and resolved (200 V, 30 min) on a 10% polyacrylamide gel (10% acrylamide, 0.375 M Tris pH8, 0.1% SDS, 0.1% ammonium persulphate, 0.1% TEMED) with a 4% stacking gel layer (4% acrylamide, 0.126 M Tris pH6.8, 0.1% SDS, 0.1% ammonium persulphate, 0.1% TEMED) until the dye front reached the bottom of the gel. Gels were stained with Coomassie Blue stain on an orbital shaker O/N and excess stain removed with destaining solution (3 x 50 mL) until total protein could be visualised.

For detection of HA protein, electrophoresed gels were equilibrated in transfer buffer and protein transferred onto Amersham Hybond ECL membrane via wet electrophoretic transfer (GE Healthcare; 350 mA, 1 hour). Membranes were blocked with 5% skim milk in TBS-T (1 hour) before O/N incubation with purified rat anti-HA monoclonal antibody sourced from H36-4-5.2 hybridoma (gifted from Dr Raman Kumar; 500 ng/mL, 4 °C, O/N). After 3 x 50 mL TBS-T washes on an orbital shaker (60 rpm), bound Ab was detected by incubation with horseradish peroxidase (HRP)-conjugated anti-rat IgG Ab (1:5000; Rockland, Cat # 612-103-120). Membranes were washed as described, covered with ECL substrate (1 min; Abcam) and imaged on a ChemiDoc MP imaging system (BioRad).

For detection of HMGB1 protein, proteins were transferred (350 mA, 40 min) onto Amersham Hybond 0.45 µm PVDF membrane (GE Healthcare) that had been pre-wetted with

methanol. After transfer, the membrane was blocked by incubation in 5% BSA in TBS-T (1 hour). Protein was detected with monoclonal anti-HMGB1 rabbit Ab (1:1000; Abcam, cat # ab6721) and HRP-conjugated polyclonal goat anti-rabbit IgG Ab (1:5000; Abcam, cat # ab18256) diluted in BSA blocking buffer. Blots were imaged using ECL substrate, as described.

2.5 Antibody generation and purification

2.5.1. Ovine polyclonal antibody generation

To produce ovine pAbs against HA, purified recombinant HA antigen was diluted in PBS and emulsified with an equal volume of complete Freund's adjuvant (FA; for prime immunisation) or incomplete FA (for boost immunisation) (Gibco). Groups of five 9-month old or 3-year old Border Leicester x Merino ewes were immunised with 200 or 20 μg HA in 4 mL emulsion, either subcutaneously (SC) at 4 axillary sites or as a bolus intraperitoneal (IP) injection (prime only). Alternatively, antigen in PBS was gently mixed with an equal quantity of CoVaccine HT^{TM} suspension (Protherics Medicines Ltd) and sheep were immunised with 2 mL mixture SC at 4 axillary sites. Sheep were administered a prime immunisation and a boost dose every 14 days for a total of five boosts. Blood was sampled (8 mL) prior to each immunisation in addition to a test bleed (500 mL) performed at 8 weeks; serum was isolated and stored at -20° C.

To produce ovine pAbs against HMGB1, adult sheep were immunised with 200 μg recombinant HMGB1 in 1x PBS emulsified at a 1:1 ration with complete FA distributed over four axillary sites as SC injections. Sheep were boosted every two weeks with 200 μg recombinant protein emulsified in incomplete FA, for a total of four doses. Serum samples collected from jugular vein bleeds prior to each dose were stored for specific ELISA analysis. When high titres were confirmed, sheep were terminally bled and aliquoted serum stored at -20 °C in preparation for Ab purification.

2.5.2. Antibody purification

Crude pAb purification was performed by initial ammonium sulphate precipitation of total immunoglobulin from prepared serum. Briefly, thawed terminal bleed serum from HA or HMGB1 immunised sheep was filtered through packed glass wool and centrifuged (20 min, 8,000 xg) to remove particulates. Saturated ammonium sulphate solution was then added to

45% (v/v), mixed (30 min) and centrifuged (20 min, 8,000 x g). The resultant pelleted protein precipitate was subsequently dissolved in milli-Q water and dialysed into PBS before application to a gravity flow column packed with 2 mL Protein G agarose media (GE Healthcare). Excess protein was washed through the column with 1 x PBS (50 mL) and pAbs eluted with 10 mL glycine elution buffer (pH 2.5). Elutions (5 mL) were neutralised with 250 µL 1.5 M Tris Base (pH 8) immediately after collection.

For large scale pAb purification, dissolved serum proteins were applied to a 5 mL Protein G HiTrap HP agarose column (GE Healthcare) on a GE AKTA Pure FPLC system (2 mL/min flow rate; pressure limit 0.45 mPa). The column was subsequently washed with 8 column volumes (4 mL/min) and bound IgG eluted with 2.5 column volumes of glycine elution buffer (2 mL/min) collected in 10 mL fractions in prepared tubes with 500 µL 1.5 M Tris Base (pH 8) added.

Approximate concentration of pAb in eluates was determined by spectrophotometric analysis via absorbance at 280 nm as above. Eluted fractions containing significant concentrations of protein were pooled and dialysed into PBS before concentration determination via BCA assay (Thermo Fisher Scientific) according to manufacturer's instructions. Concentrated IgG was subsequently analysed for purity via SDS-PAGE. Control serum was sourced from non-immunised sheep and total IgG purified as described.

2.5.3. Functional assessment of anti-HA via haemagglutination inhibition (HAI) assay

All HAI assays were performed as previously described [184]. Briefly, purified pAb (500 µg/mL; 30 µL) was pipetted into duplicate wells of a round-bottom 96-well plate and serially diluted two-fold in PBS before the addition of either PR8 influenza virus (5 haemagglutination (HA) units in 30 µL) to all wells. After 30 minutes incubation at room temperature, 0.5% (v/v) chicken red blood cells (cRBC) in PBS (30 µL) was added to each well and gently mixed. Plates were visualised over a light box after 45 minutes. The endpoint HAI titre was recorded as the highest dilution of test sample that was able to completely inhibit the agglutination of cRBC by virus in duplicate wells.

2.5.4. Functional assessment of anti-HMGB1 polyclonal antibodies via in vitro macrophage stimulation

Total IgG purified from the serum of HMGB1-immunised sheep was assayed for neutralisation capacity against commercial active HMGB1 (Abcam, Cat #ab167718). Murine

macrophage RAW 264.7 cells were grown in complete DMEM to confluence, detached from flasks using TrypLEselect (Thermo Fisher Scientific) and added to 96 well plates (3.3 x 10^5 cells/well) before incubation for 24 hours (37°C). Sterile HMGB1 (1 μg/mL) was mixed with anti-HMGB1 pAbs (0.2 μg/mL) and incubated for 30 minutes prior to addition to cells. Supernatants were then aspirated from adherent cells and prepared mixtures added (200 μL). Cells were cultured for 18 hours and cell-free supernatants harvested for analysis of TNFα via ELISA. It was determined that generated anti-HMGB1 pAbs significantly reduced TNFα production from RAW cells compared to control pAb (Figure 2.2).

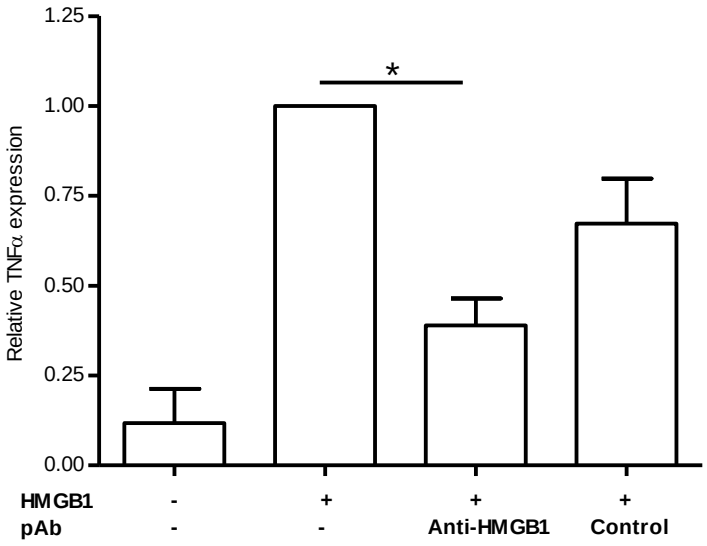


Figure 2.2 Ovine anti-HMGB1 pAbs inhibit the immunostimulatory capacity of HMGB1 *in vitro*

Cultured RAW 264.7 cells were incubated with recombinant human HMGB1 (1 μg/mL) in the presence of anti-HMGB1 or control ovine pAbs (0.2 μg/mL) for eighteen hours and supernatants were collected for analysis of TNFα content via ELISA. Measured TNFα concentrations were normalised to HMGB1-only wells; data are representative of two independent experiments. Data were compared via two-tailed T-tests, with significance denoted as thus: * = $P<0.05$.

2.6 Mammalian cell culture

2.6.1. Generation and stimulation of bone marrow-derived dendritic cells

Conditioned media from B16-GM-CSF cells was utilised as a source of GM-CSF for the generation of bone marrow-derived dendritic cells (BMDC). For preparation of conditioned medium, B16-GM-CSF cells were thawed and cultured in a T75 flask to 80% confluence in cRPMI (25 mL; 3-4 days). Cells were washed in EF-PBS (10 mL) and detached from the flask by addition of TrypLEselect (Thermo Fisher Scientific). Detached cells were washed in cRPMI and split into two T75 flasks each containing 40 mL cRMPI. Cells were cultured for 72 hours and conditioned medium was collected, centrifuged (1000 x g) and frozen.

Murine bone marrow was flushed from the tibia and femur bones of B6 mice and homogenised via passage through 19G, 21G and 23G needles before lysis of RBC with RBC lysis buffer (500 µL, 5 min). Isolated bone marrow cells were centrifuged (400 xg, 5 min), counted and adjusted to 5 x 10^5 cells/mL in cRPMI medium supplemented with 10% B16-GM-CSF conditioned medium. Cell suspensions were added to 6 well plates (5 mL/well) and cultured for 7 days. Media volume was replenished by 50% on Day 3 and Day 5 with cRPMI/10% B16-GM-CSF medium. On day 7, supernatants were removed and semi-adherent BMDC's were harvested from the plate via gentle pipetting. Harvested BMDC's were washed, counted and adjusted to 2 x 10^5 cells/mL in cRPMI. Cell suspensions (250 µL) were plated in duplicate wells of 48-well-plates, with or without added LPS (100 ng/mL) and cultured at 37 °C for 18 hours. Supernatants were harvested, centrifuged (500 xg, 5 min) to remove cellular debris and stored at -20 °C for analysis.

2.6.2. Isolation and stimulation of peritoneal macrophages

Mice were humanely killed via CO_2 inhalation and pinned ventral-side up on a surgical mat. The abdomen was sterilised with 70% ethanol and the outer skin was incised, pulled from the abdominal wall and pinned to the surgical mat. In mice that had had caecal ligation and puncture (CLP) surgery, the surgical site was clamped using a haemostat clamp to prevent leakage. A syringe with a 23G needle attached was used to inject 3 mL ice-cold EF-PBS with 1% FCS into the abdomen. The abdomen was massaged gently and fluid was aspirated into the syringe before being dispensed into a 10mL tube stored on ice. The lavage was repeated twice more with 2 mL EF-PBS, making a total of ~7 mL lavage fluid collected from each mouse.

Peritoneal cells were pelleted by centrifugation (300 xg, 5 min) and resuspended in 250 µL red blood cell lysis buffer before incubation (5 min, room temperature). Cells were washed twice with warm cRPMI, counted, adjusted to 5 x 10^5 cells/mL and 400 µL of cell suspension added into duplicate wells of a 48-well plate. Plates were incubated for 90 min at 37°C 5 % CO_2 to allow macrophages to adhere. Supernatants and non-adherent cells were subsequently removed, and adherent cells washed with EF-PBS before incubation with or without LPS (1µg/mL) in 250 µL cRPMI for 18 hours. Supernatants were subsequently harvested and stored at -20°C before analysis.

2.7 Immunoassays

2.7.1. Detection of antibodies via ELISA

ELISA analysis was used for the detection of specific Abs against immunogens, therapeutic Abs or influenza virus, with ELISA reagents summarised in Table 2.2. Enzyme ImmunoAssay (EIA) high-binding plates (Corning, Sigma Aldrich) were coated with 100 µL of the relevant coating protein diluted in 100mM $NaHCO_3$ buffer (pH9) and incubated at 4°C O/N. Plates were washed, then blocked with 2% (w/v) BSA in 1x PBS (1 hour, 37°C). The relevant samples were diluted 1:2 for Ab quantification, or serially diluted for endpoint analysis in 1% (w/v) BSA in 1x PBS with 0.05% Tween (PBS-T). After 3 washes with PBS-T (200 µL), diluted samples or serially diluted ovine pAb standards (32,000 – 2 ng/mL in PBS-T) were added to duplicate wells (100 µL) before incubation for 2 hours at 37°C. Plates were subsequently washed and bound serum Abs detected by addition of the relevant detection Ab (100 µL) followed by incubation for 1 hour (37°C). Plates were washed and prepared OPD substrate (Sigma) was added to each well and the colour allowed to develop in the dark until colour was visible in blank wells (37 °C). The reaction was subsequently stopped with 3 M HCl (50 µL), and the absorbance read at 490 nm on an EL808 model Ultra Microplate Reader (Bio-Tek instruments Inc.). For endpoint ELISA methods, endpoint absorbance values were defined as the mean absorbance plus two standard deviations of negative control samples diluted 1:100.

Table 2.2 Coating and detection reagents used for measuring antibodies by ELISA.

Detected Analyte	Sample	Coating protein	Coating Concentration	Detection Ab	Concentration
Murine anti-PR8 IgG	Murine sera	PR8 virus	500 $TCID_{50}$/mL in 100mM $NaHCO_3$	HRP-conjugated rabbit anti-murine IgG (eBioscience)	1:10,000 in 1% BSA PBS-T
Ovine IgG in circulation	Murine sera	Highly cross-adsorbed donkey anti-sheep IgG (Sigma)	5 µg/mL in 100mM $NaHCO_3$	HRP-conjugated donkey anti-sheep IgG (Sigma))	1:15,000 in 1% BSA PBS-T
Murine anti-ovine IgG	Murine sera	Ovine pAb	10 µg/mL in 100mM $NaHCO_3$	HRP-conjugated rabbit anti-murine IgG (eBioscience)	1:10,000 in 1% BSA PBS-T
Ovine anti-PR8 IgG	Ovine pAb	PR8 virus	500 $TCID_{50}$/mL in 100mM $NaHCO_3$	HRP-conjugated donkey anti-sheep IgG (Sigma)	1:15,000 in 1% BSA PBS-T
Ovine anti-A/PC IgG	Ovine pAb	A/PC virus	500 $TCID_{50}$/mL in 100mM $NaHCO_3$	HRP-conjugated donkey anti-sheep IgG (Sigma)	1:15,000 in 1% BSA PBS-T
Ovine anti-HMGB1 IgG	Ovine sera	Recombinant HMGB1	5 µg/mL in 100mM $NaHCO_3$	HRP-conjugated donkey anti-sheep IgG (Sigma)	1:15,000 in 1% BSA PBS-T

2.7.2. Detection of cytokines via ELISA

Enzyme ImmunoAssay (EIA) high-binding plates (Corning) were coated with the appropriate capture Ab (Table 2.3) diluted to the indicated concentrations in 0.1M Na_2HPO_4 (pH 9.6). Plates were incubated at 4 °C (O/N), washed (3x 1x PBS) and blocked (2% BSA/1xPBS) for 1 hour (37°C). Following washing (3x PBS-T), protein standards for TNF-α, IL-10, IL-6 or IL-12 and samples (diluted in 1% BSA/PBS-T) were added to duplicate wells and incubated for 2 hours, 37°C. Plates were then washed (3x PBS-T) and cytokines detected by incubation of plates with corresponding detection Ab (Table 2.3) diluted to the indicated concentration in 1% BSA/PBS-T. Plates were incubated for a further 1 hour (37°C) and following further washing, biotinylated Abs were detected by incubation of plates with streptavidin-conjugated HRP (Rockland) for 45 min (37°C). Plates were washed and prepared OPD substrate (Sigma) was added to each well and the colour allowed to develop in the dark until colour was visible in blank wells (37 °C). The reaction was subsequently stopped with 3 M HCl (50 µL), and the absorbance read at 490 nm on an EL808 model Ultra Microplate Reader (Bio-Tek instruments Inc).

Table 2.3 Reagents for cytokine detection in cell culture supernatant or mouse serum by ELISA

Detected Cytokine	Coating Ab	Standard	Detection Ab
IL-6	eBioscience Rat anti-mouse IL-6, 1 µg/mL (Cat# 14-7061; clone MP5-20F3)	eBioscience, 32-0.5 ng/mL (Cat# 14-8061)	eBioscience Biotinylated rat anti-mouse IL-6, 2 µg/mL (Cat# 13-7326; clone MP5-32C11)
IL-10	eBioscience Rat anti-mouse IL-10, 1 µg/mL (Cat# 14-7101; clone JES5-16E3)	eBioscience, 32-0.5 ng/mL (Cat# 14-8101)	eBioscience Biotinylated rat anti-mouse IL-10, 2 µg/mL (Cat# 13-7102; clone JES5-2A5)
IL-12	BioLegend Rat anti-mouse IL-12 p70, 2 µg/mL (Cat# 511002; clone 7B12)	BioLegend, 20-0.3 ng/mL (Cat# 573009)	eBioscience Biotinylated rat anti-mouse IL-12, 4 µg/mL (Cat# 511802; clone C18.2)
TNFα	eBioscience Rat anti-mouse TNFα, 2 µg/mL (Cat# 14-7325; clone 1F3F3D4)	eBioscience, 32-0.5 ng/mL (Cat# 14-8321)	eBioscience Biotinylated rat anti-mouse TNFα, 2 µg/mL (Cat# 13-7326; clone XT3/XT22)

Serum samples were diluted 1:2 in PBS/0.05% Tween 20/1% BSA for cytokine detection. Cell culture supernatants were assayed neat for IL-10, IL-12 and TNFα and diluted 1:4 for IL-6 detection.

2.7.3. HMGB1 quantitation by ELISA

Protein HMGB1 was quantified in human plasma, mouse or sheep serum by commercial ELISA. Human plasma samples were analysed by Shino-test (IBL-International), using a 1 in 2 sample dilution factor for high sensitivity detection and a standard curve from 80-0.3 ng/mL according to the manufacturer's instructions. Sheep serum samples were analysed by a human HMG1 ELISA kit (Cloud-Clone Corp) with a standard curve range of 4,000 – 63 pg/mL according to the manufacturer's instructions. Mouse serum samples were analysed by the murine HMG1 ELISA kit (Cloud-Clone Corp) using a standard curve ranging from 6,000 – 49 pg/mL according to the manufacturer's instructions.

2.7.4. Multiplex cytokine analysis

Cytokines present in human plasma and mouse serum were analysed via BioPlex multiplex platform (Bio-Rad MagPix). Custom cytokine kits were purchased from Bio-Rad and samples assayed according to the manufacturer's instructions. Human cytokines measured included IL-1β, IL-1RA, IL-4, IL-5, IL-6, IL-8, IL-10, interferon gamma (IFNγ), monocyte chemotactic protein 1 (MCP-1) and TNFα. Measured murine cytokines included IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IFNγ, GM-CSF and TNFα. All samples were run in duplicate using a one-in-five sample dilution and concentrations calculated from a standard curve using Bio-Rad Manager MP Software.

2.8 Flow cytometry

2.8.1. Cell staining and FACS analysis

For single cell splenocyte suspensions, spleens were dissected from humanely killed mice and placed in 2 mL cold iRPMI. Spleens were homogenised between two frosted glass slides before passage through a 70 μm cell strainer and centrifugation (400 xg, 5 min). Pellets were resuspended in RBC lysis buffer (1 mL, 5 min) followed by 2 x 1 mL washes with cold FACS buffer. Cells were counted and 1 x 10^6 cells were pipetted into FACS tubes for staining. Cells from peritoneal lavage fluid (0.5 – 1 x 10^6 cells) were similarly subjected to RBC lysis and washed prior to staining.

For cell staining, cells were incubated with murine Fc block (antiCD16/CD32) for 15 min and washed with 1 mL FACS buffer. Resultant cell suspensions were stained with the appropriate Ab panel for 30 min in the dark on ice. If biotinylated Abs were used cells were washed with 1 mL FACS buffer and subsequently stained with streptavidin-fluorophore conjugates for 15 min. After 2 x 1 mL washes, cells were resuspended in FACS buffer for same-day analysis or FACS Fix solution for short-term storage at 4 °C. Cells were analysed on a three-laser BD FacsCANTO II Flow Cytometer. Single-stained controls were used to set voltages and multicolour compensation before samples were analysed. Doublets were excluded by gating on FSC-A/FSC-H, and viable cells were used for analysis.

2.8.2. Antibodies

Reagents for flow cytometric analysis of cells were purchased from BD Biosciences or eBioscience. Abs were diluted in FACS buffer for cell staining. Single stained controls were stained in parallel to sample tubes for voltage and compensation setup. Viability dyes (DAPI, 1:100, Sigma; or FVS510, BD Biosciences, 1:1000) were used after Ab staining.

For dendritic cell analysis of splenocytes, cells were stained with Fc Block (anti-CD16/CD32) (1:100), anti-CD3 PE (1:100), anti-CD19 PE (1:100), anti-CD86-FITC (1:50), anti-CD11b PE-Cy7 (1:100), anti-CD11c APC-Cy7 (1:100) and anti-MHCII-biotin with streptavidin-APC.

For macrophage analysis of peritoneal lavage cells and splenoctyes, cells were stained with Fc Block (anti-CD16/CD32) (1:100), anti-F4/80 PE (1:100), anti-CD11b AF488 (1:100) and anti-Gr-1-biotin (1:100) with streptavidin-APC.

2.9 Murine influenza model

2.9.1. Intranasal influenza infection

Purified influenza viral strains were provided by Dr Mohammed Alsharifi, The University of Adelaide. Groups of mice were administered purified pAbs (25 mg/kg) or PBS either via IP injection (500 μL) or intranasal (IN) inhalation (32 μL) under anaesthetic. This dose was selected empirically from a pilot study (data not shown). For severe influenza infection, mice received IN challenge (32 μL) with PR8 H1N1 (500 $TCID_{50}$) or A/PC H3N2 (3000 $TCID_{50}$) influenza virus that had been purified from hen egg amniotic fluid as previously described [349]. Viral inocula were diluted on the day of use from freshly thawed viral stocks and untreated controls were run in parallel with all treated groups to ensure that the dose of virus administered caused an infection requiring euthanasia within 6-10 days. All challenged mice were provided with soaked food and sunflower seeds to support them through the associated pain and distress of infection, and were assessed daily for clinical symptoms of infection which included weight loss, a hunched posture, ruffled coat, an abnormal respiratory rate or reluctance to move. A clinical score of 5 or 20% weight loss was used to identify those reaching predetermined humane endpoints requiring euthanasia. Weight loss was the primary indicator for euthanasia in this study as most mice did not reach clinical thresholds.

2.9.2. In vivo cytotoxic lymphocyte (CTL) assay

The ability of mice to mount a cell-mediated response to influenza infection was assessed using an *in vivo* cytotoxicity assay. Splenocytes from a donor mouse were harvested and RBC subsequently lysed. Washed splenocytes were pulsed with Kd-restricted influenza nucleoprotein-derived peptide TYQRTRALV (NPP) before two washes in endotoxin-free PBS followed by CellTrace™ Far Red staining (0.1 μM) for 20 minutes at 37˚C. Cells were subjected to two more washes and mixed at a 1:1 ratio with unpulsed control cells stained with CFSE (3μM; 10 minutes at 37˚C). Optimal staining was confirmed by flow cytometry (BD FACSCanto II) before transfer into infected or control mice via tail vein injection (8-10 million cells per mouse). Spleens from recipient mice were harvested 18 hours later and single cell preparations were assessed for percentage of FarRed and CFSE cells remaining via flow cytometry and FACSDiva software. Percent cell specific lysis was calculated using the following equation:

% specific lysis = [1- (infected mouse $\%CFSE^{+}$:$\%FarRed^{+}$ / control mouse $\%CFSE^{+}$:$\%FarRed^{+}$)] x 100

2.10 Murine sepsis models

2.10.1. Endotoxaemia

Endotoxaemia was induced in 6-8 week old C57Bl/6 mice by IP injection of 20mg/kg 0111:B4 LPS (Sigma). Animals were monitored for signs of toxicity which included hunched posture, ruffled coat, diarrhoea, dehydrated eyes, reduced temperature and reluctance to move. Ovine pAbs or PBS were administered via IP injection either one hour before (prophylactic) or one hour after (therapeutic) LPS administration. Analgesia was not provided during this protocol, as the administration of analgesics can render the interpretation of acute sickness behaviours difficult and alter the immune response to endotoxin [350] and pathogens [351].

2.10.2. Caecal ligation and puncture (CLP) sepsis model

Aged male 10-12 week old C57Bl/6 mice were weighed, anaesthetised with isoflurane and their abdomen shaved and disinfected with Betadine solution. Mice were positioned ventral-side-up on a sterile bench coat and their forelimbs and tail secured with masking tape to expose the abdomen. A 1.5 cm medial incision in the outer skin was made with a surgical blade and surgical scissors were used to incise the inner abdominal wall (1 cm). The caecum was located and exteriorised using sterile forceps and saline-moistened cotton tips (Figure 2.3 A).

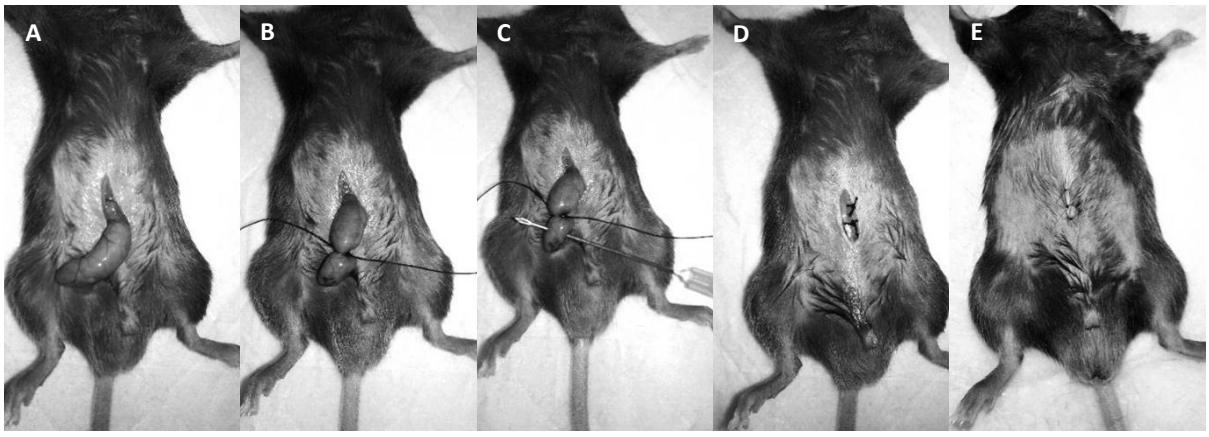


Figure 2.3 CLP surgery procedure

Mice were anesthetised and their abdomen shaved and disinfected. (A) The outer and inner abdominal walls were incised to expose the caeum. (B) After ensuring the caecal tip was filled, a 4.0 silk suture was used to ligate a 7 mm section. (C) The ligated portion was punctured either twice with a 21G needle (depicted) or once with a 23G needle for the double-hit model and the ligated portion gently squeezed to extrude a small amount of caecal contents. (D) The ligating suture was trimmed, the caecum returned to the abdomen and the inner abdominal wall closed with 4.0 silk sutures. (E) The outer abdominal wall was closed with 6.0 prolene sutures and the mouse was administered Buprenorphine, Enrofloxacin and saline and allowed to recover from anaesthesia in a warmed cage.

Two moistened sterile cotton tips were used to roll the caecum and fill the end section with material, with a length of 4.0 silk suture used to ligate the caecum ~7mm from of the end (Figure 2.3 B). The ligated end section was pierced twice with a 21G needle and gently squeezed to extrude a small amount (~1mm) of caecal contents (Figure 2.3 C). Sutures were trimmed and the caecum was returned to the abdomen using moistened cotton tips. The inner abdominal wall was sutured with 4.0 silk sutures (Figure 2.3 D) as required and the outer abdominal wall was sutured with 6.0 prolene sutures (Figure 2.3 E). Mice were removed from the anaesthetic mask and immediately administered SC Buprenorphine (0.05 mg/kg), Enrofloxacin (5 mg/kg) and saline (1 mL). Mice were allowed to recover from anaesthetic in a pre-warmed cage (1 hour) before transfer to individual IVCs. Mice were monitored 1-3 times daily for signs of clinical disease and euthanised upon reaching pre-determined humane endpoints. Saline (1mL) was injected SC every morning, Buprenorphine (0.05 mg/kg; SC) was injected every 12 hours as required and mice were provided with moistened food daily throughout the study. This protocol resulted in >60% of mice reaching humane endpoints requiring euthanasia within four days and is considered a septic syndrome of moderate severity. Blood samples for cytokine analysis were obtained by lancing the facial vein under anaesthesia or as a terminal cardiac bleed as appropriate.

2.10.3. The 'double hit' sepsis model

P. aeruginosa (ATCC 27853) was streaked from a frozen stock onto TSB agar and incubated at 37°C (O/N). Fresh TSB (100 mL) was inoculated with a single colony and grown O/N at 35°C with constant shaking. Bacteria were harvested by centrifugation (3,000 xg, 10 min) and the resultant pellet washed twice with sterile EF- PBS. The washed pellet was resuspended in EF-PBS and the opacity of the suspension was measured on a spectrophotometer and adjusted to an absorbance of 0.5 $A_{600\ nm}$. A colony count of ~1 x 10^9 CFU/mL was confirmed by serial dilution onto TSB agar. Aged male 10-12 week old C57Bl/6 mice were subjected to sham surgery or a modified CLP procedure, where a 5 mm section of caecum was ligated and one puncture was performed with a 23G needle, resulting in over 90% survival. Supportive medications were administered and monitoring performed as described above. On day 4, 96 hours after modified CLP surgery, *P. aeruginosa* inoculum (25 µL, corresponding to 2.5 x 10^7 CFU) was administered via IN inhalation under anaesthesia. Mice were monitored as above and euthanised according to pre-determined endpoints.

2.11 Ovine septic shock model

A model of ovine sepsis with intensive care supports (OSICS) was performed on six sheep as part of this study in collaboration with Dr Matthew Maiden and Dr Timothy Kuchel using protocols previously described in published works [347, 348]. Data generated within this study were compared to results obtained from untreated or placebo (saline) administered sheep in previous studies.

2.11.1. Sheep preparation

Sheep were fasted O/N one day prior to each study for one night after which a blood sample (8 mL) was collected via the jugular vein by trained facility staff. Anaesthesia was induced by intravenous (IV) injection of Thiopentone (1 g) and the sheep subsequently transferred to a surgical theatre. The trachea was intubated and anaesthesia was maintained throughout surgical procedures with spontaneous ventilation of isoflurane (2%) in oxygen. Surgical procedures were performed by a trained veterinary surgeon with assistance from facility staff.

For the placement of vascular catheters, the right side of the neck was shaved and disinfected with Betadine solution and an incision was made to expose the carotid sheath. An arterial catheter (Angiocath 1.7 x 133 mm, Becton Dickinson) was placed into the right carotid artery, secured by silk ligature and connected to a pressure monitoring line filled with heparinised saline. A quad-lumen central venous catheter (8.5 Fr x 20 cm, Arrow International Inc.) and a large-bore single lumen catheter (8.5 Fr x 10 cm, Arrow International Inc.) were inserted into the right jugular vein and secured with silk sutures.

The large-bore single lumen catheter was used for the placement of a pulmonary artery catheter (Swan Ganz CCOmbo 7.5 Fr x 110 cm, Edwards Lifesciences) performed under real-time radiology imaging. After placement into the right ventricle, the attached balloon was inflated to ‘float’ the catheter tip into a branch of the pulmonary artery. Once characteristic pulmonary artery pressure waveforms were detected, the balloon was deflated and the catheter secured and connected to a pressure monitoring line containing heparinised saline.

A tracheostomy tube was inserted into the proximal trachea (Portex, ID 9.0, 24 cm, Smith’s Medical) and a rumen tube (16 gauge, Bard Medical) was inserted via the nose.

An indwelling urethral catheter (14Fr, Covington, Bard Medical) was placed into the bladder and attached to a burette to allow hourly measurement of urine output.

2.11.2. Monitoring and care protocol

Once catheters were placed, sheep were moved onto a surgical table, lying prone with head and neck supported in an upright position to prevent oedemous fluid accumulation in the lower limbs. The tracheostomy tube was connected to a ventilator (Puritan Bennett 7200) and ventilation was initiated.

Venous and arterial catheters were attached to a pressure monitor (Intellivue MP 50, Phillips) and a cardiac output monitor (Vigilance Monitor, Edwards Lifesciences). Cardiac output was continuously monitored and indexed to sheep body surface area according to the calculation (weight (kg)$^{0.67}$ x 0.0842) for recording.

Once all monitors were connected, sheep were allowed to reach a stable state before induction of sepsis. All sheep were monitored by a dedicated ICU research nurse throughout the study according to parameters listed in Table 2.4, and mean arterial pressure (MAP) was maintained according to the protocol described in Table 2.5.

Table 2.4 Sheep monitoring and maintenance

Parameter	Monitoring	Maintenance
Sedation	Depth of sedation continuously monitored; medication dose recorded hourly	IV infusion of midazolam (0.1 – 0.5 mg/kg/h) and ketamine (1 – 5 mg/kg/h)
Ventilation	Respiratory rate, tidal volume, peak and plateau pressures and O_2 and CO_2 saturation recorded hourly. Tracheal suctioning was performed hourly or as required.	Synchronised intermittent mandatory ventilation with 10 mL/kg tidal volume and positive end expiratory pressure of 5 cmH_2O. Respiration adjusted to maintain end tidal CO_2 of 30 mmHg. Fraction of inspired oxygen was adjusted to maintain pulse O_2 saturation >95%
Haemodynamics	HR, CVP and pulmonary arterial pressure were recorded hourly. MAP and CO recorded every 10 min.	Parenteral fluid was administered at 3 mL/kg/h via peristaltic pump. CVP was maintained ≥5 mmHg via fluid bolus administration (250 mL saline) MAP was maintained ≥75 mm/Hg (see Table 2.5)
Temperature	Recorded every 1 hour	Temperature was maintained <42 °C with the application of cool damp drapes to the back
Blood analysis	-	Samples taken every 2 hours for cytokine and lactate analysis, every 4 hours for blood gas analysis and every 6 hours for pathology analysis including coagulation, haematological and biochemical tests
Fluid balance	Fluids administered and urine output recorded hourly	Total urine collected at 0, 12 and 26 hours was sampled for biochemical testing

HR = heart rate, CVP = central venous pressure, MAP = mean arterial pressure, CO = cardiac output

Table 2.5 Ovine model haemodynamic management protocol

Blood pressure parameters	**Action**
MAP <75 mmHg and CVP <5 mmHg	250 mL saline bolus
75 mmHg and CVP ≥ 5 mmHg	Noradrenaline (NA) infusion as below; adjust every 2 min
MAP < 55 mmHg	↑ 10 μg/min NA
MAP 55-59 mmHg	↑ 5 μg/min NA
MAP 60-64 mmHg	↑ 3 μg/min NA
MAP 65-69 mmHg	↑ 2 μg/min NA
MAP 70-74 mmHg	↑ 1 μg/min NA
MAP 75-80 mmHg	No change to NA dose
MAP 81-85 mmHg	↓ 1 μg/min NA
MAP 86-90 mmHg	↓ 2 μg/min NA
MAP 91-95 mmHg	↓ 3 μg/min NA
MAP 96-00 mmHg	↓ 5 μg/min NA
MAP > 100 mmHg	↓ 10 μg/min NA

MAP = mean arterial pressure, CVP = central venous pressure, NA = noradrenaline

2.11.3. Preparation and administration of E. Coli

E. coli (ATCC 25922) was prepared from a fresh Columbia horse blood agar plate (bioMérieux Australia) streaked for single colonies and incubated O/N. Colonies (8-10) were emulsified in 100 mL sterile saline and the optical density of the suspension at 550 nm was determined via spectrophotometer (Shimadzu UV-160A). Empirical assessment prior to the study revealed an OD of 0.25 would yield 1.5 x 10^8 Colony Forming Units (CFU)/mL. The inoculum was kept chilled (4°C) before use.

The number of CFUs/mL of each inoculum was determined by plating 100 μL of a 10^{-5} dilution in sterile saline onto three separate Columbia Horse Blood agar plates. Plates were incubated at 35ºC ± 2ºC O/N and the mean colony count was multiplied by the dilution factor to calculate CFUs/mL for the inoculum. The inoculum (1 mL/kg) was administered to prepared, anaesthetised sheep as an IV infusion via over one hour to achieve 1.5 x 10^8 CFU/kg.

2.11.4. Study termination

Sheep were euthanised under anaesthesia at 26 hours post-*E. coli* administration by IV injection of a lethal dose of pentobarbitone (6.5 g). Death was apparent by absence of respiration and detectable pulse, and confirmed by a trained ICU nurse. Sheep were subjected to post mortem assessment of the abdominal and thoracic cavities and samples of skeletal muscle, liver, kidney, spleen, lung tissue and cardiac muscle were excised and fixed in formalin for future analysis.

2.12 Data analysis and statistics

Analysis was performed using GraphPad Prism V3.0 software. Survival analysis was conducted using Mantel-Cox testing. Sequential measurements over time (Ab titres, HMGB1 content, cytokine profiles, weight loss and clinical score) were compared between groups using Two-Way ANOVA. Cytokine measurements at individual time points were compared using Two-tailed t-tests or Mann-Whitney analysis.

PART A:

HAEMAGGLUTININ-SPECIFIC OVINE POLYCLONAL ANTIBODIES FOR THE TREATMENT OF INFLUENZA INFECTION

CHAPTER 3.

An empirical approach towards the efficient and optimal production of influenza-neutralizing ovine polyclonal antibodies

The significant outcomes presented in this chapter have contributed to one candidate first author publication:

N.E. Stevens, C.K. Fraser, M. Alsharifi, M.P. Brown, K.R. Diener, and J.D. Hayball (2013). An empirical approach towards the efficient and optimal production of influenza-neutralizing ovine polyclonal antibodies demonstrates that the novel adjuvant CoVaccine HT™ is functionally superior to Freund's adjuvant. PlosONE, 8: e68895

3.1 Introduction

Antigen-specific polyclonal antibodies are generated for a wide array of purposes which range from fundamental laboratory studies and protocols to passive immunotherapy for life threatening conditions including snake envenomation [203] and drug toxicity [352]. Sheep are particularly attractive for the generation of passive polyclonal immunotherapeutics as ovine antibody fragments have demonstrated reduced immunogenicity and more consistent biological function than those derived from other animals [353]. Furthermore, large quantities of serum can be repeatedly obtained from sheep, with reduced maintenance costs and lower immune boosting demands than other large animals such as horses [201]. Specifically, polyclonal ovine antibodies in the form of antigen-binding antibody fragments or 'Fab' underlie the use of antibodies in critical care situations such as snake envenomation and digoxin toxicity [100]. Potential hypersensitivity reactions often associated with administration of whole antibody are considerably reduced by using these Fab fragments or their divalent counterpart $F(ab)_2$. Hence, this type of treatment has the potential to be readily transferable to infectious disease management, particularly in light of the increased incidence of drug resistance to circulating pathogens [354, 355] and the medley of undesirable side effects often associated with conventional drug treatments [356-358]. Of particular interest here is the applicability of this approach to infections with viral pathogens such as influenza, as natural immunity to many such viruses is facilitated through the action of neutralising antibodies [353, 359, 360].

Whilst traditional vaccination reduces influenza-associated mortality [361], it is least efficacious in the immunocompromised individuals who are most susceptible to complications and increased mortality [129, 131], and who include pregnant women [362]. Consequently, immunocompromised individuals make up the majority of the many thousands of annual influenza-related deaths [131, 363]. This provides the rationale for development of passive immunotherapy regimens for influenza prophylaxis or treatment in these individuals as additional time is not needed to generate an efficient vaccine-induced adaptive immune response [364]. Indeed, passively administered influenza-specific antibody has been shown to inhibit influenza-induced mortality in rodents [365], although the selection of a suitable clinically applicable passive immunotherapeutic will be determined by its inherent neutralisation capacity, its safety as well as its commercial scalability and overall cost effectiveness [182]. These factors highlight the requirement for optimal efficiency at every stage of the production process.

Whilst downstream processing methods for existing commercial ovine polyclonal antibody preparations have been methodically optimised [366-368], there has been limited investigation into the best way to generate maximal antibody titres and overall yield of effective antibody from the sheep themselves. Indeed, there are few reports in the published literature directly comparing the parameters that can influence humoral immune responses in sheep [369]. This is particularly important considering that route of administration, antigen dose and adjuvant are well recognised as critical parameters in antibody production from other species [370, 371].

The route of immunisation can influence the induction of the humoral immune responses [372] by dictating which population of dendritic cells (DCs) interacts with antigen [373, 374]. For instance, subcutaneous (SC) immunisation is routinely applied commercially to produce hyperimmune ovine sera [367, 368] and facilitates antigen interaction with skin-associated DCs, including Langerhans cells, conventional DCs and macrophage-derived DCs [375]. Alternatively, intraperitoneal (IP) immunisation promotes antigen interaction with conventional DCs, macrophages, and plasmacytoid DC's, which may be beneficial depending on the antigen type [376, 377]. The functionality of site-specific DC subsets in sheep has not been well studied and thus empirical assessment is required to determine an optimal immunisation route. Antigen dose can also influence the outcome of immunisation; too little antigen may elicit inefficient responses [378], and too much antigen can promote adverse effects and immunotolerance [369]. The standard antigen dose used in generating ovine antisera varies widely from micrograms to as much as five grams per animal [369] and this uncertainty necessitates investigation of appropriate antigen dosage for optimal antibody output. The choice of adjuvant is another key factor in dictating both the quality and quantity of the humoral immune response [370, 379]. Adjuvants prolong and augment the effects of vaccination through various mechanisms which include increasing antigen persistence, stimulation of local inflammation, upregulation of cytokines and immunomodulatory factors, activation of phagocytic cells and promotion of antigen presentation [380] [108]. The current gold-standard for generation of a humoral immune responses in animals is Freund's adjuvant (FA), a water-in-oil emulsion which is available in 'complete' or 'incomplete' formulations, that is, with or without heat-killed *Mycobacterium tuberculosis* respectively [381]. This adjuvant primarily works by increasing antigen persistence in the tissues [382] and stimulating a foreign-body reaction which results in local inflammation and recruitment of innate and adaptive immune cells [383]. It is however often associated with the formation of painful granulomas and can also result in tuberculin-type hypersensitivity [384]. It is not approved for human use and concerns about animal welfare have prompted investigation into

alternative adjuvants for use in animals [383].

The current study applies empirical methods to optimise anti-A/Puerto Rico/8/1934 H1N1 (PR8) haemagglutinin (HA) ovine polyclonal antibody production using FA and a proprietary experimental adjuvant, CoVaccine HT™ (CV) [108, 385-387]. CoVaccine HT™ is in clinical development and consists of sucrose fatty acid sulphate esters (SFASE) immobilised on the oil droplets of a submicron emulsion of squalane in water [385]. We show that the age of sheep, the prime immunisation route, and dose has minimal impact on the antibody titre, although CV adjuvant induced superior anti-HA antibody titres than immunisation regimens incorporating FA. The influenza-neutralisation capacity of the anti-HA antibody was subsequently assessed by haemagglutination-inhibition (HAI) assays and was found to correlate closely with the antibody titres measured for each of the variables. Finally, it was demonstrated that high titre ovine hyperimmune serum can be successfully applied in a lethal *in vivo* murine influenza challenge model to prevent mortality in both a prophylactic and therapeutic context.

3.2 Results

3.2.1. Intraperitoneal or subcutaneous prime immunisation induces similar peak anti-haemagglutinin antibody titres

Generally, antigen emulsified with FA is administered via subcutaneous (SC) injection over multiple sites. However, it has been previously demonstrated that IP prime immunisation can yield significantly higher antibody titres [388, 389]. Therefore, to directly compare these routes of prime immunisation, groups of sheep were immunised either SC (four sites) or IP (bolus injection) with HA emulsified in complete FA. Both groups were boosted SC every two weeks with HA in incomplete FA for a total of five boosts. Serum samples were collected fortnightly and the titre of anti-HA immunoglobulin G (IgG) was analysed by ELISA. Assessment of results by two-way repeated-measures ANOVA indicated no significant difference between antibody titres during the induction phase (weeks 2-12) of the immune response (Figure 3.1A). However when ELISA data from all time points including twelve weeks post-immunisation were analysed, a significant trend for higher antibody titres was observed in the SC primed group (Figure 3.1A; $P<0.05$), which may indicate a slower decline in antibody titre for this group. Importantly, neither group exhibited a significant difference in the ability of various serum dilutions to inhibit haemagglutination of PR8 in an HAI assay (Figure 3.1B). Based on these results, the SC prime immunisation was selected for all subsequent comparisons.

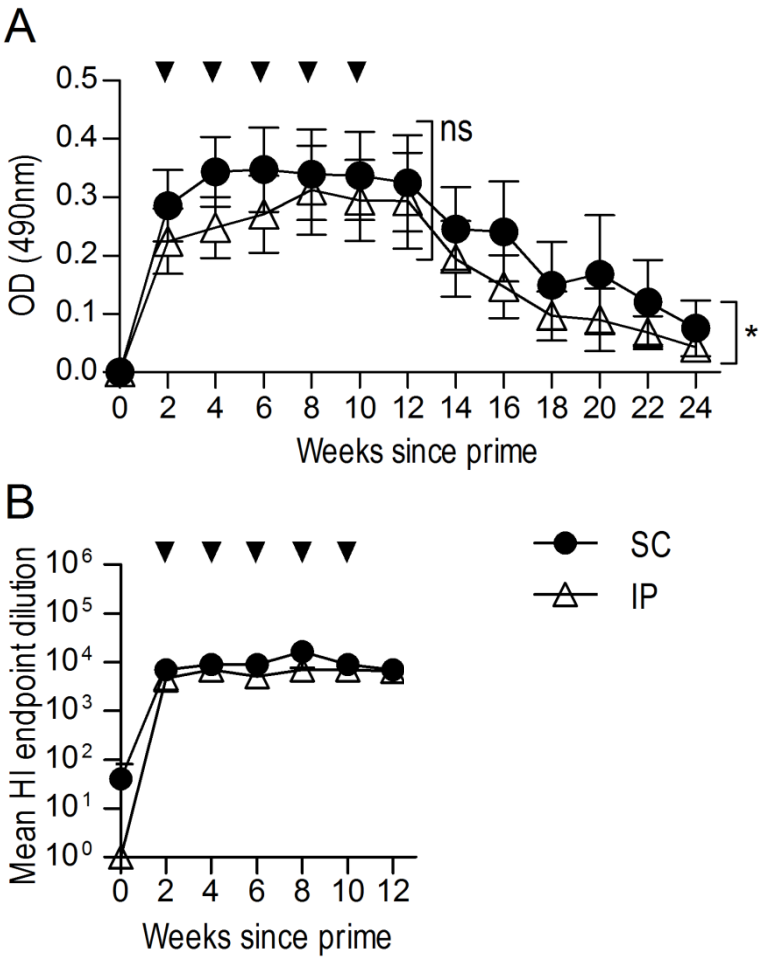


Figure 3.1 Different routes of prime immunisation yield similar anti-HA antibody titres

Sheep (n=5) were immunised with 200 µg HA SC or IP in complete FA. Sheep were boosted SC every two weeks to a total of five boosts in incomplete FA (indicated by arrows). Pre-immune (time 0) or hyperimmune serum samples were analysed for anti-HA IgG via ELISA (1/50, 000 dilution) (A), and HAI (B). Data are expressed as the mean ± SEM. Two-way repeated-measures ANOVA was applied to evaluate significance which is denoted as thus: * = $P<0.05$, ns = not significant.

3.2.2. CoVaccine HT™ elicits significantly higher ovine anti-haemagglutinin antibody titres than complete Freund's adjuvant

The experimental adjuvant CoVaccine HT™ is an oil-in-water emulsion, which is designed with synthetic carbohydrate structures on squalane microdroplets and which produces proinflammatory responses through interaction with innate immune receptors, including TLR-4 [385, 390]. This adjuvant offers the advantage of presenting amphipathic membrane target antigens in native formation due to the squalane-in-water formulation. It has shown efficacy in combination with a range of antigens including malarial antigens [391], influenza glycoproteins [390] and gonadatropin-releasing hormone [392]. In order to determine if CV is capable of inducing anti-HA antibody responses comparable to those generated with FA, sheep were primed with HA either traditionally emulsified with complete FA or gently mixed with CV and boosted fortnightly with HA in incomplete FA or CV respectively. Serum samples were collected fortnightly and analysed via ELISA and HAI assay. Two-way repeated-measures ANOVA and post-test analysis indicated that CV elicited significantly higher antibody titres overall ($P<0.01$) and at multiple time-points post prime ($P<0.05$) compared to FA (Figure 3.2A). Consistent with the ELISA results, the CV group sera demonstrated significantly higher HAI capacity than those from the FA group ($P<0.05$; Figure 3.2B), suggesting that CV may be a good alternative to FA in future immunisation regimens.

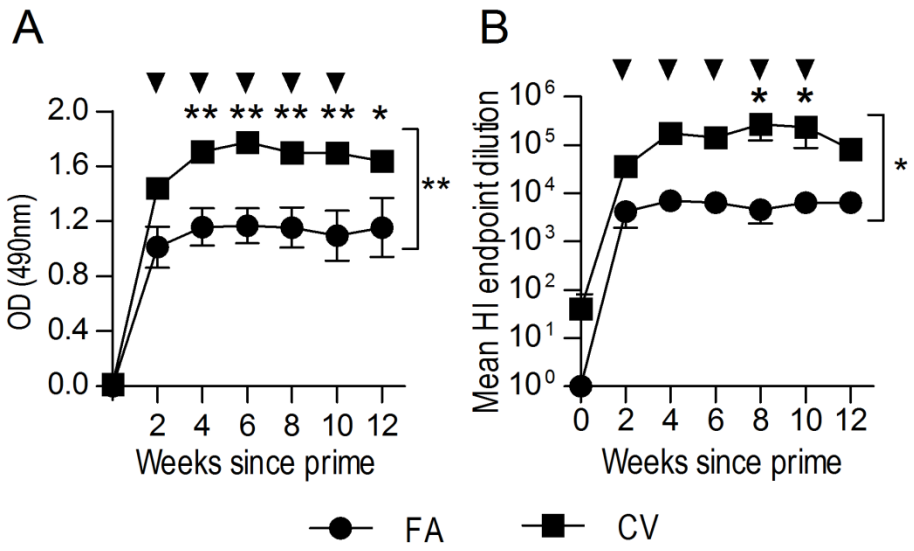


Figure 3.2 CoVaccine HT™ adjuvant elicits significantly higher anti-HA antibody titres than Freund's adjuvant

Sheep (n=5) were immunised SC with HA (200 µg) in complete FA or CoVaccine HT^{TM} (CV). Sheep were boosted similarly every two weeks (five boosts indicated by arrows) with HA in incomplete FA or CV. Pre-immune (time 0) or hyperimmune serum samples were analysed for anti-rHA IgG via ELISA (1/50,000 dilution) (A) and HAI (B). Data are represented as the mean ± SEM. Two-way repeated-measures ANOVA with Bonferroni post-test was applied to evaluate significance which is denoted as thus: * = P<0.05, ** = P<0.01.

3.2.3. Age of sheep did not significantly alter the ability to generate high anti-haemagglutinin antibody titres

It was hypothesised that younger sheep may produce higher antibody titres than older sheep due to potential age-related decline in immunity in aged sheep. To assess the effect of sheep age on antibody output, sheep at nine months or three years of age were immunised SC with HA either in complete/incomplete FA or CV as described above. Serum samples taken every two weeks were assessed by ELISA and HAI assays (Figure 3.3). Analysis of ELISA results by two-way repeated-measures ANOVA with Bonferroni post-tests revealed no significant difference between antibody titres overall, or at individual time points for serum produced in young or old sheep by administration of HA emulsified in FA (Figure 3.3 Ai) or CV (Figure 3.3 Bi). Similarly, there was no significant difference in the neutralising activity of anti-HA serum from different aged sheep in HAI assays in either the FA-immunised (Figure 3.3Aii) or CV-immunised groups (Figure 3.3Bii). Consistent with previous results (Figure 3.2), HAI titres for either old or young sheep immunised with HA-FA emulsion were significantly lower ($P=0.001$) than the corresponding CV group (Figure 3.3Aii and Figure 3.3Bii respectively). These results suggest that age does not impact on the quality of the antigen-induced antibody response.

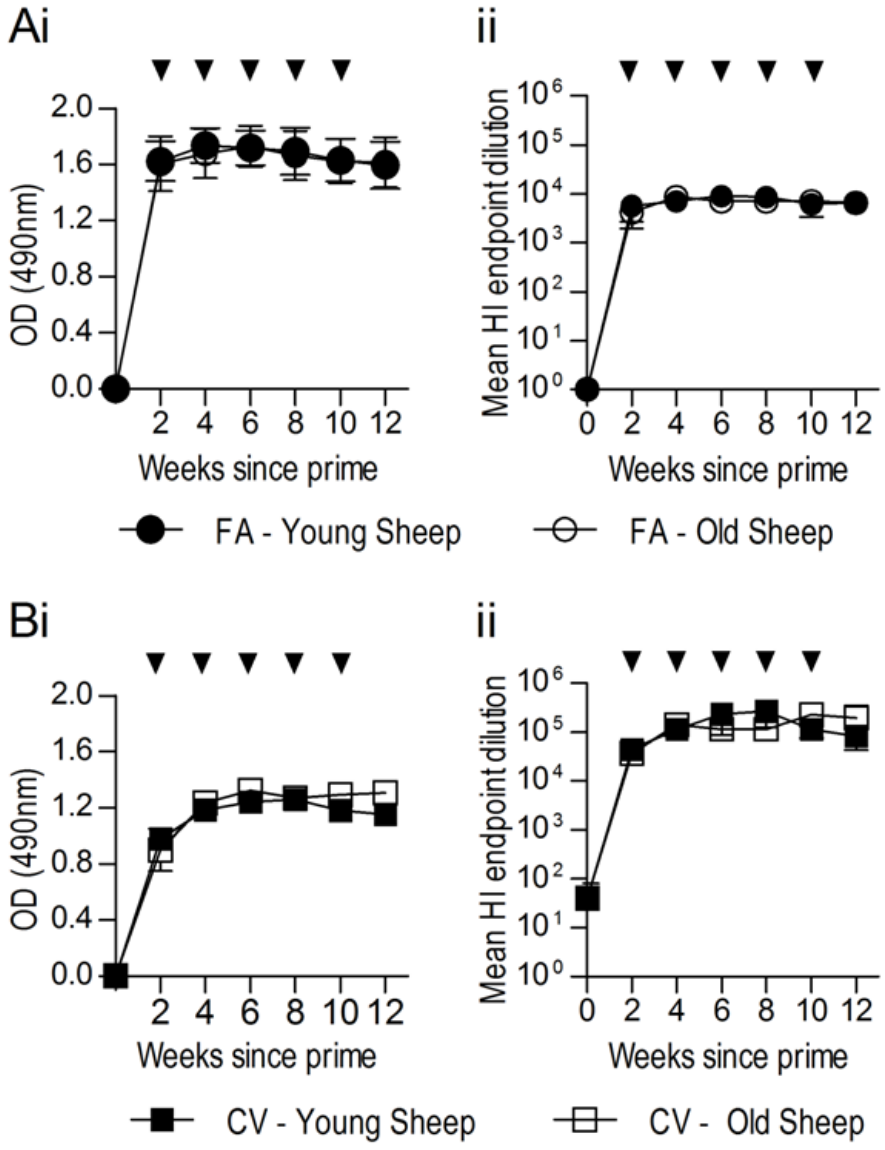


Figure 3.3 Nine-month old and three-year old sheep produce similar anti-HA antibody titres

Sheep (n=5) at either nine months (young) or three years (old) were immunised SC with 200 µg of HA in complete FA (A) or CV (B). Sheep were subsequently boosted SC every two weeks to a total of five boosts in incomplete FA or CV (indicated by arrows). Pre-immune (time 0) or hyperimmune serum samples were analysed for anti-rHA IgG via ELISA (1/50,000 dilution) (Ai, Bi) and HAI (Aii, Bii). Data are expressed as the mean ± SEM.

3.2.4. A ten-fold lower dosage of antigen did not yield significantly lower anti-haemagglutinin antibody titres

Cost of antigen is an important factor in any immunisation regimen, therefore to determine whether economic gains may be made by immunisation with a lower dose of antigen, sheep were immunised SC with 200μg or 20 μg HA either in FA or CV as previously described. Serum samples taken every two weeks were subsequently analysed by ELISA and HAI assays (Figure 3.4). Statistical scrutiny revealed no significant difference in antibody titre between high-dose and low-dose FA groups as indicated by ELISA (Figure 3.4Ai) and HAI results (Figure 3.4Aii). In comparison, significant differences were observed within the CV groups in both the ELISA assay (Figure 3.4Bi; $P<0.05$) and HAI assay (Figure 3.4Bii; $P<0.05$), however the magnitude of the decrease in the low-dose group was comparatively small. Indeed, analysis of individual time points by Bonferroni post-test comparisons revealed a significant difference in HAI titre at only one time point. Importantly, HAI results of CV group sera again revealed a more than ten-fold increase in mean endpoint serum dilution for both the high and low dose HA over that observed with FA-induced sera (Figure 3.4Bii vs Figure 3.4Aii). These data supports the use of a lower antigen dose to elicit comparably high quantities of anti-HA antibody whilst reducing costs associated with antigen production.

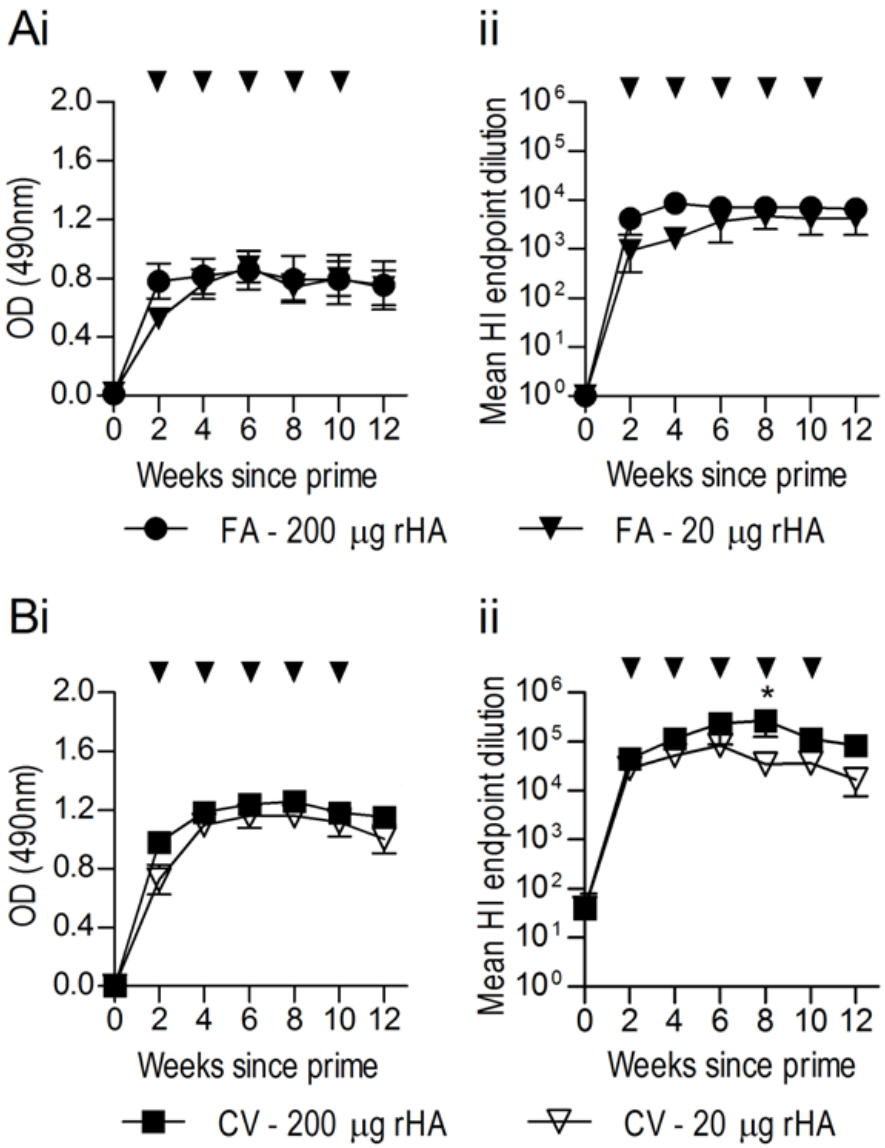


Figure 3.4: Low antigen dose produces similar anti-HA antibody titres

Sheep (n=5) were immunised SC with 200 or 20 µg of HA in complete FA (A) or CV (B). Sheep were then boosted SC every two weeks to a total of five boosts in incomplete FA or CV (indicated by arrows). Pre-immune (time 0) or hyperimmune serum samples at a 1/50,000 dilution were analysed for anti-HA IgG via ELISA (Ai, Bi) and HAI (Aii, Bii). Data are represented as the mean ± SEM endpoint dilution. Data of both assays were analysed by two-way repeated-measures ANOVA with Bonferroni post-tests; significance is denoted as thus: * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$, ns = not significant

3.2.5. CoVaccine HT™ induced fewer adverse site reactions as compared to Freund's adjuvant

As FA immunisation often induces site-specific reactions, it was necessary to determine whether CV similarly induced local inflammatory reactions at the immunisation site. Observational data were collected twelve weeks post-prime where the number and size of palpable SC lumps at each site was recorded, and sites were given a rating on a graded system (denoted in Figure 3.5). No significant differences in size or number of reactive sites were observed within adjuvant groups in the age and antigen dose experiments (data not shown); therefore observational data from all sheep receiving each SC immunisation regimen were pooled and compared. No sheep receiving either adjuvant exhibited abscess formation or signs of distress requiring veterinary attention within this study. Statistical analysis by Mann-Whitney rank test revealed a significant difference between the number of reactive sites, with sheep receiving HA in FA exhibiting significantly more reactive sites per animal, many at all injection sites, than those sheep receiving antigen mixed with CV (Figure 3.5A; $P>0.05$). Interestingly, there was a trend toward lower reactivity scores of CV sites than of FA sites (Figure 3.5B), although altogether, the variation of CV reactivity scores was greater (SD = 1.750) than that of FA scores (SD = 1.136).

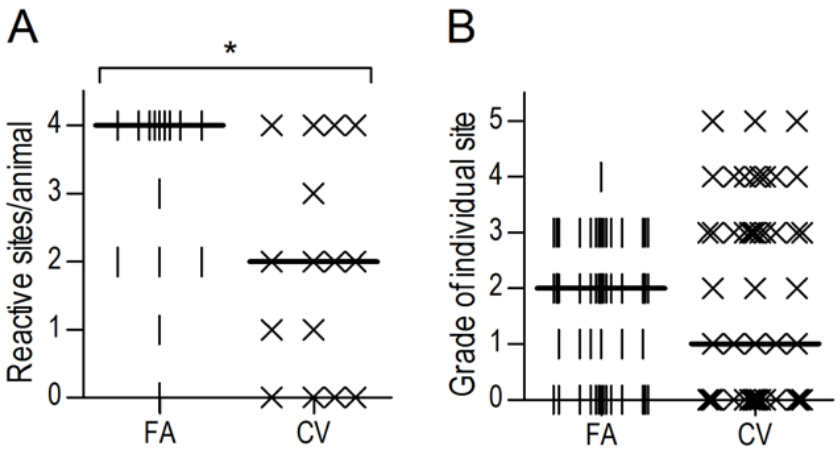


Figure 3.5 Repeated subcutaneous immunisation with CoVaccine HT™ elicits fewer reactive immunisation sites than Freund's adjuvant

Sheep (n=15) were immunised SC with HA antigen either in complete/incomplete FA or CV. Two weeks following the final boost immunisation, injection sites were examined and palpable lumps were enumerated (A) and graded (B). A scoring system was devised based on the size and characteristics of the reaction sites to rank the level of reactivity of each individual site. Grades of site reactivity: 0 – no reaction; 1 – slight skin irregularity; 2 – lump <10mm diameter or larger skin irregularity; 3 – multiple small lumps/single lump <40mm; 4 – lump <80mm; 5 - ≥80mm lump. The data was analysed by Mann-Whitney rank test; significance is denoted as thus: * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$, ns = not significant

3.2.6. Prophylactic and therapeutic administration of anti-haemagglutinin hyperimmune serum protected mice from a lethal influenza challenge

Even though hyperimmune serum consistently prevented influenza-induced haemagglutination of RBC *in vitro*, we wished to determine the *in vivo* potential of this serum to prevent lethal influenza infection. To assess the protective ability of polyclonal anti-HA antibodies, mice received pooled hyperimmune serum (1 mL, IP injection) from sheep immunised with HA emulsified in CV or FA, or pooled non-immune serum or PBS diluent as controls. Twenty-four hours later, mice were challenged with 500 50% tissue-culture infective doses ($TCID_{50}$) of live PR8 by intranasal (IN) administration and the clinical course was closely monitored (Figure 3.6 A). Results indicated that mice which received prophylactic hyperimmune serum from either CV- or FA-immunised sheep exhibited some weight loss immediately after influenza infection, although this loss was quickly regained (Figure 3.6 Ai and Aii respectively). In contrast, those mice that received non-immune serum or PBS had sustained weight loss and all had reached their pre-determined clinical endpoints by day 10 (Figure 3.6 Aiii and Aiv respectively). Mantel-Cox survival analysis showed that both CV and FA hyperimmune serum prevented the lethal consequences of influenza infection in contrast to the effects of administration of non-immune serum or PBS (Figure 3.6 Av; $P<0.001$).

To determine whether these polyclonal antibodies could treat an active influenza infection, hyperimmune or non-immune serum or PBS was administered twenty-four hours after viral challenge with 500 $TCID_{50}$ PR8 (Figure 3.6 B). Similarly, results indicated that infected mice which received hyperimmune serum therapy exhibited some weight loss before recovery (Figure 3.6 Bi, Bii), whereas most mice that received non-immune serum or PBS had sustained weight loss and were euthanased by day 10 (Figure 3.6 Biii, Biv). Similar to the effects of prophylactic administration of hyperimmune serum, Mantel-Cox survival analysis revealed a significant improvement in survival of the mice that received hyperimmune serum compared to control mice (Figure 3.6 Bv; $P<0.05$). Taken together, it is clear that ovine hyperimmune serum can protect against and treat lethal influenza infections in mice.

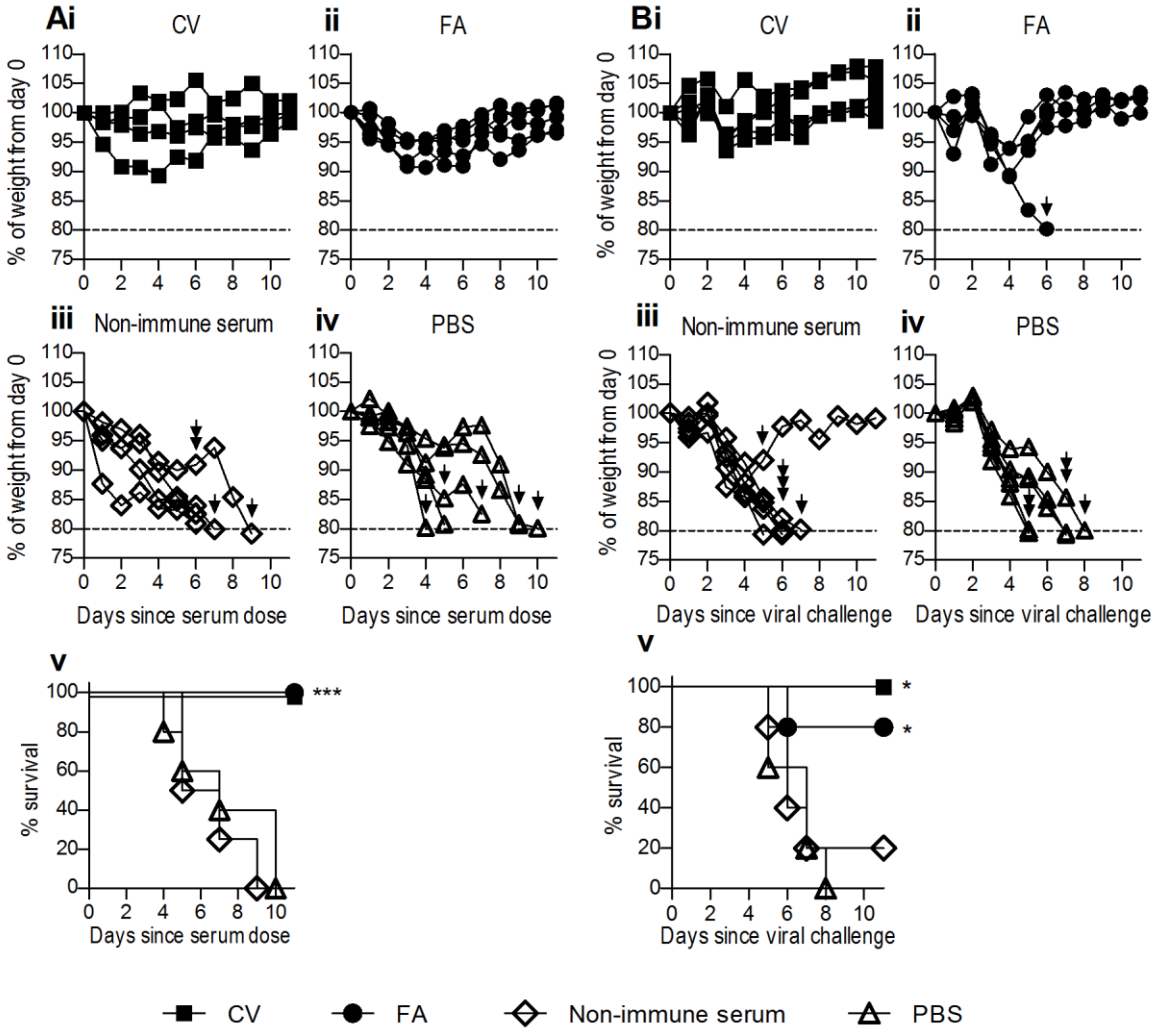


Figure 3.6 Prophylactic or therapeutic administration of ovine anti-HA serum is protective against lethal influenza challenge

Mice (n=5) were prophylactically administered pooled serum (1 mL, IP) from either young sheep receiving 200 µg HA SC in CV (Ai) or FA (Aii), day zero pre-bleeds from corresponding sheep from both groups (Aiii) or PBS as a control (Aiv). Twenty-four hours later mice were challenged with a lethal dose of PR8 (500 $TCID_{50}$). Mice reaching a predetermined endpoint of 20% weight loss (dotted line) were euthanised as indicated by arrows. Mice (n=5) were challenged with 500 $TCID_{50}$ PR8 and twenty-four hours later therapeutically administered serum or PBS control as above (Bi-iv). In each panel, data show percentage weight loss of individual mice. Survival curves of mice are also shown (Av, Bv). Mantel-Cox survival analysis was performed on survival curves; significance between all curves is denoted as thus: * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

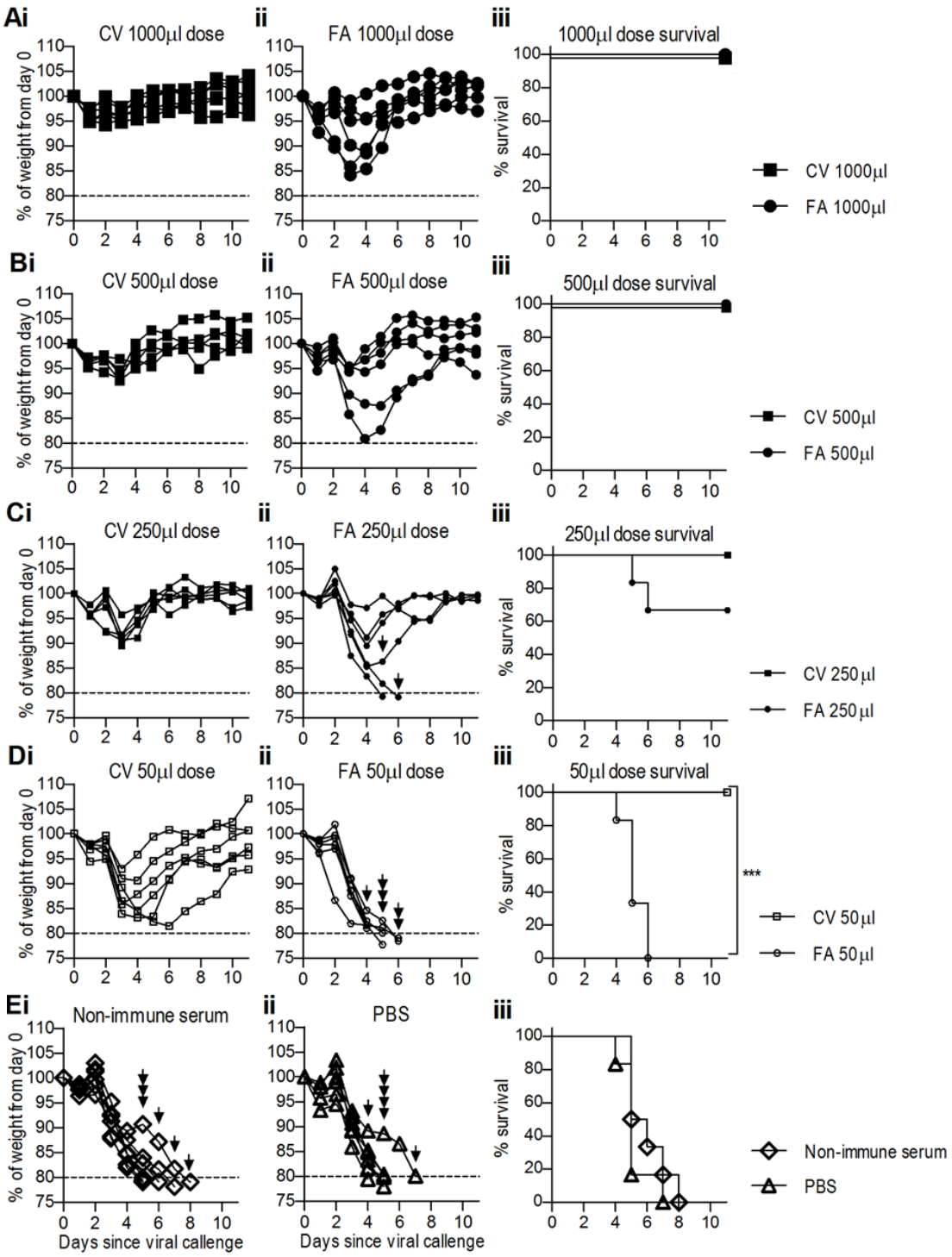
3.2.7. Hyperimmune anti-haemagglutinin serum elicited with CoVaccine HT™ exhibits greater potency in vivo as compared to that elicited by Freund's adjuvant

Anti-HA serum elicited with CV as an adjuvant had significantly better potency *in vitro* (Figure 3.2); to determine if this translated to increased potency *in vivo*, mice (n=6) were inoculated with 500 $TCID_{50}$ PR8 influenza (IN) and twenty-four hours later treated with different doses of pooled hyperimmune serum (IP, equivalent to 1000, 500, 250 or 50 µl serum in a 1 mL injection) elicited with either CV or FA. Groups of mice received PBS or non-immune sheep serum as controls. Clinical disease course was monitored and euthanasia performed according to endpoints described above.

Most notably, mice that had therapeutically received CV serum were completely protected from lethality from infection (Figure 3.7 Ai-Di), regardless of dose, whereas the lower doses of 250 µl to 50 µl of FA serum were not completely protective (Figure 3.7 Cii-Dii). Furthermore, although the 500 and 1000 µl FA serum dose protected mice from lethality of infection (Figure 3.7 Aii-Bii) weight loss in these mice was more severe than that observed in mice receiving equivalent doses of CV serum (Figure 3.7 Ai-Bi). Mantel-Cox survival analysis revealed a significant difference between survival curves of all of the CV serum mice and controls (data not shown). Comparison of the survival curves of FA and CV serum mice revealed a significant survival advantage of CV serum at a 50µl dose (Figure 3.7 Diii; $P<0.001$). A minimum dose of 500 µl of FA serum was required for complete protection whereas a ten-fold lower dose of CV serum also offered complete protection. These data are consistent with the HAI results that demonstrate an approximate ten-fold higher neutralising antibody titre in serum from sheep immunised with antigen in CV as compared to FA. Taken together, these data suggest that lower doses of CV-elicited sera provide protection against influenza and that implementation of this adjuvant in ovine polyclonal antibody production could dramatically increase the efficiency of eliciting functional neutralising antibodies in sheep.

Figure 3.7 Ovine anti-HA serum elicited with CoVaccine HT™ adjuvant show greater *in vivo* potency as compared to that by Freund's adjuvant

Mice (n=6) were challenged with 500 $TCID_{50}$ PR8 and twenty-four hours later therapeutically administered dose equivalents of 1000 (A), 500 (B), 250 (C) or 50 µl (D) of pooled hyperimmune serum (1mL, IP) from sheep that received HA antigen in CV (i) or FA (ii). Control groups of mice (n=6) received non-immune serum (Ei) or PBS (Eii) twenty-four hours following viral challenge. Mice reaching a predetermined endpoint of 20% weight loss (dotted line) were euthanized as indicated by arrows. In each panel, data show percentage weight loss of individual mice. Survival curves of mice are also shown (iii). Mantel-Cox survival analysis was performed on survival curves; significance between survival curves is denoted as thus: * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.


Ai
CV 1000µl dose
ii
FA 1000µl dose
iii
1000µl dose survival
% of weight from day 0
% survival
CV 1000µl
FA 1000µl
Bi
CV 500µl dose
FA 500µl dose
500µl dose survival
CV 500µl
FA 500µl
Ci
CV 250µl dose
FA 250µl dose
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***
CV 50µl
FA 50µl
Ei
Non-immune serum
PBS
Days since viral callenge
Non-immune serum
PBS

3.3 Discussion

Ovine polyclonal antibodies are frequently generated for commercial and small laboratory scale applications, yet there has been limited investigation into maximising the amount of antibody generated through optimised ovine immunisation regimens. Within this study, methods to improve ovine antibody production were empirically investigated and demonstrated that such ovine polyclonal antibodies effectively bound influenza virus *in vitro* and prevented the death of mice infected with influenza virus.

The route of immunisation has been shown to influence the nature and intensity of the immune response generated to an antigen [374, 388, 389]. In the current study, SC prime immunisation with antigen in FA yielded significantly higher overall anti-HA antibody titres when compared to IP prime. These findings are in contrast to an earlier study which demonstrated that anti-*C. parvum* antibody titres at parturition [388] were sixteen-fold higher in the serum when sheep received IP immunisation compared to intramuscular immunisation. Furthermore, at twenty days post-lambing, detectable anti-*C. parvum* IgG had stabilised to approximately two-fold higher in the IP-administered group. This discrepancy may be a consequence of altered immunological function in the sheep during pregnancy, or the differing nature of the antigens used [389]. It is possible that the HA antigen is more efficiently presented by cells of the SC tissues than those in the peritoneal environment [373, 374], as $CD14^+$ DC's present in the dermis are directly involved in the differentiation of antibody-producing plasma cells [55, 393]. Furthermore, the hydrophobic properties of FA may enable the stable deposition of the antigen-adjuvant mixture at immunisation sites which is likely to be more favourable for long term immunity [394]. That is, the sheep primed via SC injection may have had more local and stabilised antigen depots than the IP-primed sheep as comparatively the peritoneal environment allows substances to disperse more freely.

The use of a novel experimental adjuvant CV was compared to FA, the adjuvant routinely used for the commercial and experimental production of polyclonal ovine antibodies. Interestingly, CV was found to significantly raise higher specific antibody titres than FA. This may be partially attributable to the integrity of the antigen once mixed with this particular adjuvant. Protein antigens may be fully or partially denatured in water-in-oil adjuvants such as FA, which may lead to the deformation of native epitopes [395] resulting in antibodies with a poor affinity for the native protein. In contrast, CV is an oil-in-water emulsion of hydrophobic and negatively-charged sucrose fatty acid sulphate esters formulated with

submicron emulsions of squalane that is combined with aqueous antigen and thus would result in less protein denaturation than FA [385]. The accessibility of the antigen to immune cells may also have contributed to increased efficacy of CV compared to FA. While the tendency of FA to form viscous deposits of antigen-adjuvant mixture in the tissues results in sustained slow-release of antigen, it may also result in limited antigen exposure to the cellular environment. In contrast, antigen mixed with CV is in an aqueous environment and this may facilitate dispersal into the tissues and increase the amount of antigen immediately accessible to antigen processing immune cells, thus increasing antibody titres. Indeed, recent preclinical studies have also shown that this adjuvant can also enhance vaccine responses in not only sheep as described here but also in pigs [385], mice [390] [386], ferrets [108] and macaques [387]. The likely enhanced dispersal of CV as compared to FA may also explain why CV induced significantly fewer reactive sites that FA, which importantly also suggests a superior safety profile to FA. These observations highlight CV as a safer and highly effective potential alternative to FA for adjuvanting antigens, particularly when factoring in the time-consuming and often hazardous emulsification periods needed with FA and other water-in-oil adjuvants prior to administration. Furthermore, these advantages combined with the ability to elicit a high titre IgG antibody response in a broad range of other animal species, favours the future practical application of CV to the rapid and large scale production of passive immunotherapeutics for veterinary and clinical use.

Within this study, nine-month old sheep were compared to three year old sheep for antibody production. At nine months old, sheep are approaching sexual maturity and previous investigations have indicated that some aspects of the immune response, including CD4 and CD8 T cell numbers, may be immature at this age [396]. In contrast, 3 year old sheep are considered mature and generally immunocompetent [397] however age-related decline in sheep at 3 years of age has been observed [396]. There were no significant age-related differences in the anti-HA antibody titres achieved after immunisation with either CV- or FA-rHA. Depending on size and growth characteristics, the quantity of blood routinely obtained from younger sheep may be upwards of a third less than that obtained from older sheep [398]. However, the ability to utilise younger sheep for commercial polyclonal antibody production means that significant increases in antibody output over the lifespan of the animal would be possible thus offsetting agistment costs much sooner. This would be particularly important if younger sheep were employed together with adjuvants such as CV that increase overall antibody titre. The costs associated with ovine antibody production could be minimised further if high titre antibody titres could be maintained with reduced amounts of costly antigen. Here, we showed that a tenfold lower dose of antigen than that used conventionally

resulted in only slightly lower (albeit significantly lower with CV adjuvant) anti-HA antibody titres in sheep. However, the magnitude of this difference is unlikely to matter particularly when compared to the difference in the amount of antigen used and instead reflects the complex relationship between antigen dosage and antibody titre [378]. Furthermore, the optimal antigen dosage will be affected by different adjuvants and antigens with correspondingly different pathways used for antigen processing, presentation and antibody responses [399]. Our results clearly indicate that the lower HA antigen dose induced anti-HA ovine antibody titres similar to the high titres induced with the higher antigen dose. Moreover, we expect that this reduced need for antigen will have the added benefit of markedly reducing production costs. Importantly, all immunisation regimens tested here resulted in the production of potent anti-HA antibodies that were able to treat and prevent lethal influenza infection in mice.

Antigenic variability among influenza strains remains one of the major challenges facing the development of an influenza-specific passive immunotherapeutic. However, it has already been shown that it is possible to elicit strain cross-reactive anti-influenza hyperimmune serum by administering different subtypes of influenza HA [400, 401]. In addition, administration of an antigen (whether it be a whole pathogen or parts thereof) to a live animal benefits from 'natural selection' by the host for the most immunogenic targets and mimics the natural immune response to infection or vaccination [87]. Neutralisation capacity against PR8 and other influenza strains could be assessed by *in vitro* microneutralisation assay and further *in vivo* trails. The widespread application of passive immunotherapy to treat influenza may be of particular benefit during pandemic outbreaks of the infection, such as the 2009 H1N1 pandemic [191]. During an influenza pandemic, a premium is attached to the timely and large-scale production and distribution of an effective therapy. Knowing best how to rapidly make at least cost large quantities of potent ovine neutralising antibodies against influenza virus or other potential pandemic pathogens could be an important contribution to reducing the disease burden and its associated societal and economic costs.

CHAPTER 4.

Preserved antiviral adaptive immunity following polyclonal antibody immunotherapy for severe murine influenza infection

The significant outcomes presented in this chapter have contributed to one candidate first author publication:

N.E. Stevens, A. Hatjopolous, C.K. Fraser, M. Alsharifi, K.R. Diener, and J.D. Hayball. (2016) Preserved antiviral adaptive immunity following neutralising polyclonal antibody immunotherapy for severe murine influenza infection. Sci Reports, http://dx.doi.org/10.1038/srep29154

4.1 Introduction

Influenza infection claims approximately 250,000 lives worldwide annually and remains a significant burden on public health systems [132]. The majority of deaths from influenza infection occur in at-risk populations which include the immunocompromised, infants and the elderly, all which exhibit suboptimal responses to vaccination [129, 130] and are at higher risk of 'severe' influenza infection [131]. Severe influenza is defined as influenza infection accompanied by complications that require hospitalisation, and is attributed to over 3.4% of all critical hospitalisations during influenza season [132]. Current treatment for severe influenza generally incorporates supportive care and antiviral medications such as oseltamivir (Tamiflu^{TM}) or zanamivir (Relenza^{TM}) [168]; however viral resistance to such medications is increasing, and is most often acquired by infective viruses during hospitalisation and treatment [170], which is a significant risk for immunocompromised patients hospitalised for extended durations [171].

Vaccination is the most widely-used strategy to combat the morbidity and economic burden of influenza, with severe infection generally associated with failure to vaccinate [402] or reduced efficacy of vaccination in immunocompromised populations [129, 403]. Effective seasonal influenza vaccines elicit neutralising antibodies (Abs) against strain-specific glycoproteins, in particular haemagglutinin (HA) which is considered the major neutralising determinant of influenza [404]. However differences between the predicted HA incorporated into seasonal vaccines and the actual HA expressed by circulating strains can impact vaccine efficacy resulting in increased transmission and a higher burden of severe influenza infections [130, 405]. It is clear therefore, that strategies beyond seasonal vaccination and antiviral medications should be explored to combat the morbidity and mortality of influenza infection, particularly during critical hospitalisation periods [406].

Passive immunotherapy using neutralising Ab may be the ideal rapid treatment strategy for influenza infection that functions independently of the individual's immunocompetency [406] as highlighted by previous animal and human studies [184, 407, 408]. Furthermore, a recent multicentre double-blind randomised controlled trial has found that hyperimmune intravenous immunoglobulin (IVIG) prepared from donors exposed to pandemic H1N1 influenza significantly reduced viral load in infected individuals compared to IVIG prepared from donations received before the 2009 pandemic [190]. In a similar manner, further studies have shown reduced mortality in patients treated with convalescent plasma [409]. Animal studies have shown efficacy of anti-influenza enriched IVIG preparations in protecting

immunodeficient mice from morbidity and mortality following influenza infection [185], which indicates that this may be an effective strategy for use in immunocompromised patients. Despite clinical efficacy, the use of human-derived Ab therapies have significant logistical challenges including lengthy time periods for identification and screening of potential serum donors, and validation of sera and subsequent Ab products [410]. Consequently, current guidelines do not recommend IVIG or convalescent sera as a therapy for severe influenza [168]. Alternatively, passive immunotherapies utilising humanised monoclonal Abs (mAbs) have been developed for influenza prophylaxis and treatment, and whilst mAbs targeting HA have potent neutralising capacity, they are often not cross-reactive against multiple strains and may prompt the development of resistant mutants [193]. In contrast, mAb strategies that target highly conserved residues on the M2 ion channel protein are cross-reactive and demonstrate *in vivo* efficacy in experimental models [192, 411] however these are generally thought to function through the instigation of antibody-mediated cell cytotoxicity rather than direct viral neutralisation [193]. As such, the efficacy of these mAbs in immunocompromised individuals may be reduced.

Polyclonal Abs (pAbs) can overcome these deficiencies by their intrinsic ability to target multiple epitopes, which can increase cross-reactivity and reduce the development of resistant strains [87, 114]. Approaches using pAbs sourced from animal sera are advantageous as large amounts of pAb can be collected and batched for reduced variability. Furthermore, pAbs have simplified screening procedures compared to human-derived Abs. Ovine pAbs are particularly efficient and cost-effective to produce, and exhibit reduced immunogenicity compared to those sourced from horses or other large mammals [201]. Though ovine-origin products carry a risk of contamination with transmissible prion proteins, sourcing of ovine serum from countries free from known prion disease such as Australia and New Zealand can remove this risk, and thus commercially available ovine pAb products are commonly sourced from regions such as these. Ovine pAb fragments have been used with success in the clinic, and are currently available as critical care therapies for life-threatening digoxin toxicity (DigiFab™, DIGIBIND™) [202] and crotalid snake envenomation (CroFab™) [203].

We have previously shown that ovine serum pAbs elicited against HA have potent neutralising capacity *in vitro* and are protective in an *in vivo* murine influenza model [184]. However, any Ab administered to prevent or treat influenza must not prevent the formation of adaptive immunity necessary for enduring protection against reinfection with future circulating strains, and this has not been explored in the context of foreign pAb delivery. Therefore the aims of this study were to explore the protective role of neutralising ovine anti-

HA pAb therapy in a mouse model of severe influenza and to assess the effect on subsequent influenza-specific adaptive responses. It was demonstrated that ovine purified pAb treatment can effectively reduce mortality in the murine model and does not impede the development of protective humoral or cell-mediated responses to influenza.

4.2 Results

4.2.1. Prophylactic administration of ovine anti-HA pAbs protects mice against lethal influenza infection

Previous studies have established the neutralisation capacity of generated whole ovine anti-HA antisera in vitro and *in vivo* [184], however it remained necessary to confirm the potent neutralising capacity of purified IgG *in vivo*. To assess this, anti-HA pAbs from sheep immunised with recombinant HA in FA[1] were purified by Protein G chromatography. Groups of mice were intraperitoneally administered purified ovine anti-HA pAbs, control pAbs, or PBS, and twenty-four hours later challenged intranasally with a lethal dose of PR8 influenza. Mice that had received anti-HA pAbs exhibited reduced weight loss (Figure 4.1 Ai) and mild signs of infection from days 2-6 (Figure 4.1 B), and variable weight loss after day 7. However, all resumed weight gain after day 11, and none required euthanasia. In contrast, administration of control ovine pAbs or PBS could not control influenza infection which led to rapid weight loss and increased clinical scores from day 3 and required euthanasia by day 6 (Figure 4.1 Aii and 4.1 Aiii respectively). Thus a statistically significant survival advantage was conferred to the group of mice that received anti-HA ovine pAbs (Figure 4.1 C). Comparison of clinical score revealed a statistically significant reduction in clinical symptoms in anti-HA treated mice compared to control groups on days 5 and 6 (Figure 4.1 B). These results clearly demonstrated the capacity of prophylactically-administered ovine anti-HA pAbs to completely protect against the fatal effects of a lethal influenza virus infection *in vivo*.

[1] CoVaccine HT™ adjuvant comparison studies (described in Chapter 3) were performed in commercial collaboration with BTG plc. Subsequent antibody studies described in this chapter were not part of this collaboration and were therefore performed using antibodies generated in sheep immunised with HA in Freund's adjuvant.

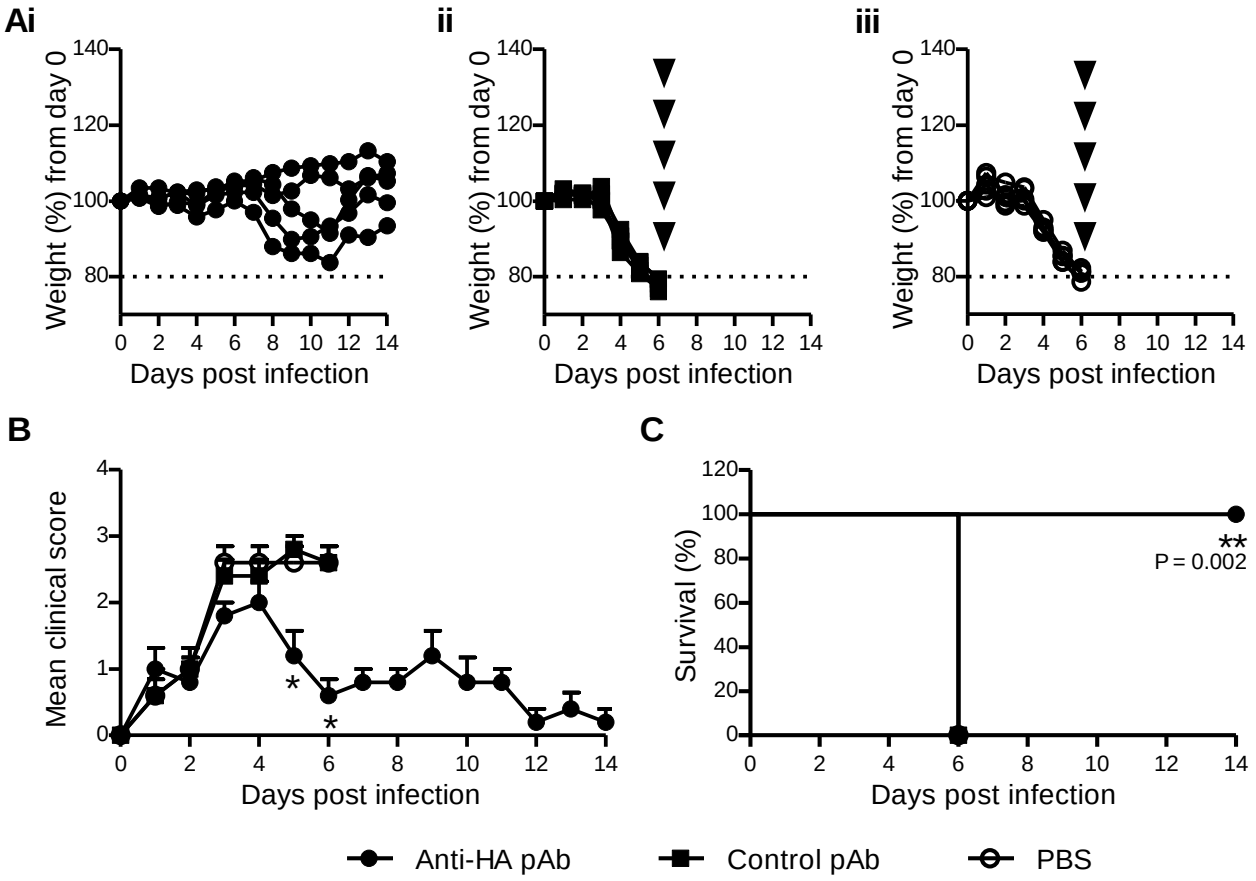


Figure 4.1 Administration of ovine anti-HA pAbs protects against subsequent lethal influenza infection

Mice (n = 5) were prophylactically administered (25 mg/kg in 500 µL) ovine anti-HA pAbs (Ai), control pAbs (Aii) or PBS (Aiii) via the intraperitoneal route. Twenty-four hours later mice were challenged with 500 $TCID_{50}$ (32 µL) PR8 influenza. The mice were closely monitored for weight loss (Ai-iii) and euthanised according to predetermined humane endpoints (indicated by arrowheads). Data is presented as the weight loss of individual mice from infection day (A), with mean clinical score compared via two-tailed t tests in (B). Corresponding survival curves (C) were analysed using the Mantel-Cox test to compare anti-HA treated to PBS treated and control treated mice. Significance is denoted as thus: *=P<0.05, ** = P<0.01.

4.2.2. Ovine anti-HA pAbs can reduce mortality in an established influenza infection

The efficacy of passive immunotherapy in reducing morbidity from influenza infection has been linked to the timing of Ab administration, both in animal [365, 412] and human [190, 413] studies. Ideally, passive immune therapies would be of greatest benefit if administered as soon as possible after exposure, however in practice a diagnosis would not normally be made until after clinical symptoms were observed. Viral shedding in this murine model peaks two days after infection [414, 415] which correlates with an established infection in humans triggering symptom development [146] and marks the timepoint at which clinical interventions may be provided. Therefore, the therapeutic capacity of ovine anti-HA pAbs were assessed 48 hours after influenza challenge. Furthermore, as the route of administration can impact the effectiveness of Ab therapies [416] two different routes were assessed in this study. Whilst therapeutic administration of pAbs did not completely protect mice from reaching humane endpoints requiring euthanasia after infection, those mice that received ovine anti-HA pAbs via IP injection (Figure 4.2 Ai) or IN administration (Figure 4.2 Aii) exhibited reduced weight loss compared to controls that received control pAbs delivered systemically (Figure 4.2 Aiii) or via nasal delivery (Figure 4.2 Aiv). Interestingly, while variable weight loss and clinical symptoms were present in the IP treatment group, IN administration of pAbs resulted in a significant reduction of clinical scores 4-5 days post infection (Figure 4.2 B). Of note, an unusual pattern of weight loss was observed in one mouse administered Ab via IP injection (Figure 4.2 Ai), however no physiological anomaly was detected in this mouse while normal signs of infection were present and thus all the data pertaining to this mouse has been included. Analysis of overall survival (Figure 4.2 C) indicated significantly increased survival in IN-treated mice compared to the IP administered control pAb group ($P=0.022$), although a survival benefit was also suggested in the IP anti-HA pAb treated mice ($P=0.142$). These results support the development of a passive immunotherapeutic to treat established influenza infection, with treatment potentially more potent when administered intranasally.

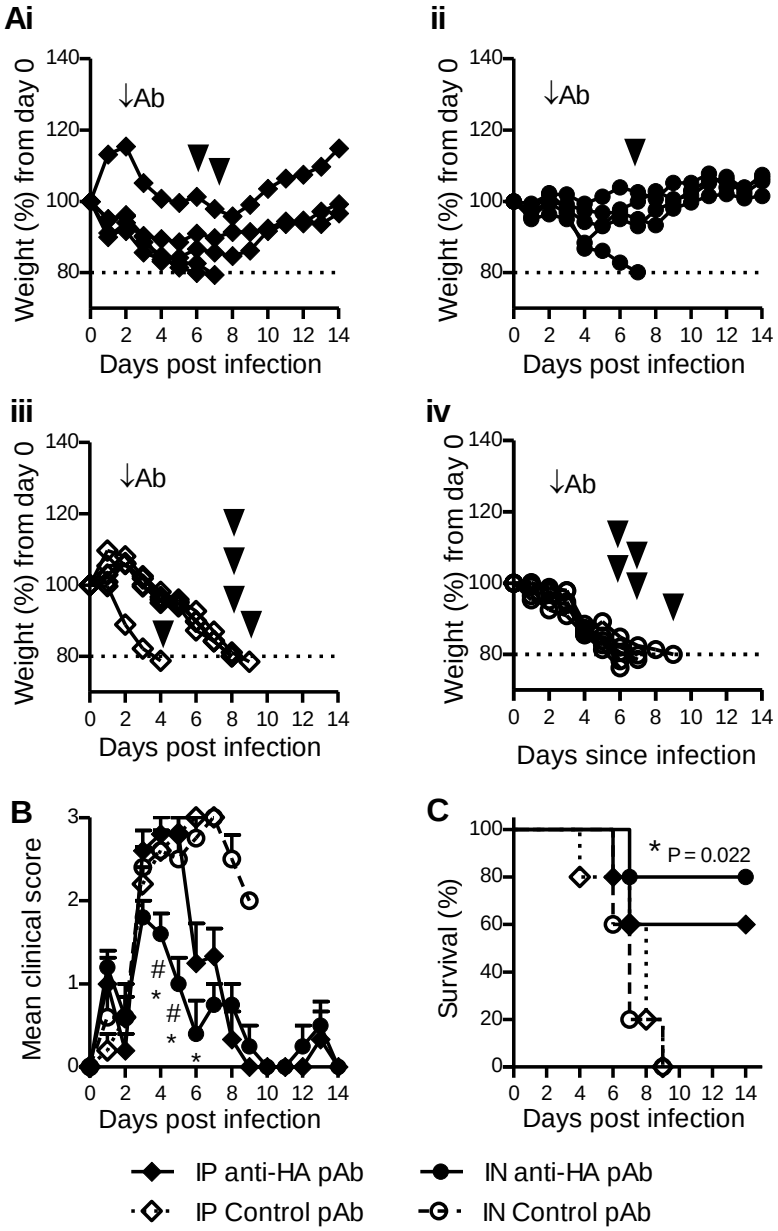


Figure 4.2 Ovine anti-HA pAbs can treat established influenza infection

Mice (*n* = 5) were infected with 500 $TCID_{50}$ (32 μL) PR8 Influenza. Forty-eight hours later mice were administered ovine anti-HA pAbs (25 mg/kg) by IP injection (Ai), or intranasal (IN) administration (Aii), or control ovine pAbs by IP (Aiii) or IN (Aiv) routes. The mice were monitored for weight loss (Ai-iv) and clinical score (B) and euthanized according to predetermined humane endpoints (indicated by arrowheads). Data is representative of the percentage weight loss of individual mice from infection day or the mean clinical score of each group. Clinical scores of treatment groups were compared via Mann-Whitney analysis, where * = $p>0.05$ compared to IP control group, # = $p>0.05$ compared to IP anti-HA group. Survival curves (C) of treated groups were compared to control groups using Mantel-Cox survival analysis; significance compared to IP control pAb group is denoted as thus: * = $P<0.05$.

4.2.3. Anti-HA pAb treatment does not inhibit the formation of adaptive immune responses

A passive immunotherapeutic approach to treat severe influenza infection should not prevent the formation of endogenous antigen-specific immune responses against the infective strain. To investigate whether treatment with ovine anti-HA pAbs impacts on the development of long-term protective immunity, a rechallenge model was developed. Mice were prophylactically administered anti-HA pAbs twenty-four hours before administration of a lethal dose of PR8, while a second group received a sub-lethal dose of PR8 with no pAb treatment. All mice recovered from infection and were rechallenged on day 28 with a lethal dose of PR8. Mice that had received initial protection by ovine anti-HA pAb administration survived a second lethal challenge without exhibiting the clinical signs associated with infection (Figure 4.3 A). The sub-lethal control group also survived a second lethal challenge without any significant morbidity (Figure 4.3 B). To ascertain the adaptive immune responses that may have contributed to this sustained protection, samples collected fourteen days post-infection from anti-HA pAb and PBS administered mice were assessed for PR8-reactive murine IgG via ELISA. Equivalent Ab responses were evident in anti-HA and PBS treated groups and were significantly higher than in uninfected animals (Figure 4.3 C). To measure cytotoxic T lymphocyte (CTL) responses, mice administered anti-HA pAbs, control pAbs or PBS were infected with a sub-lethal dose of PR8 influenza and an *in vivo* CTL performed ten days later. CTL responses to HA-specific targets from anti-HA pAb, control pAb and PBS treated groups were all of similar intensity, and all significantly higher than uninfected controls (Figure 4.3 D). However, as previous studies have shown that the formation of adaptive immunity to influenza is influenced by the number of viral particles [415] and as anti-HA pAbs act to directly neutralise virions, responses between pAb-treated and untreated mice may have been confounded by this difference in viral load. To investigate this possibility, an alternate influenza strain with a disparate HA residue was employed. Mice were prophylactically administered anti-HA pAb or PBS and challenged with a sublethal dose of A/PC (H3N2) influenza with analysis of CTL responses performed ten days later. No significant difference in *in vivo* killing capacity between the groups was observed (Figure 4.3 E), indicating that the reduction in influenza symptoms, and thus viral load, did not impact on the generation of CTL responses in this model. Further confirmation was obtained by analysing serum samples from preliminary studies in which different doses of anti-HA pAb were administered prior to influenza infection for the development of murine anti-PR8 Abs (Figure 4.4). There was no trend towards increased or decreased Ab responses in mice that received higher quantities of anti-HA Abs.

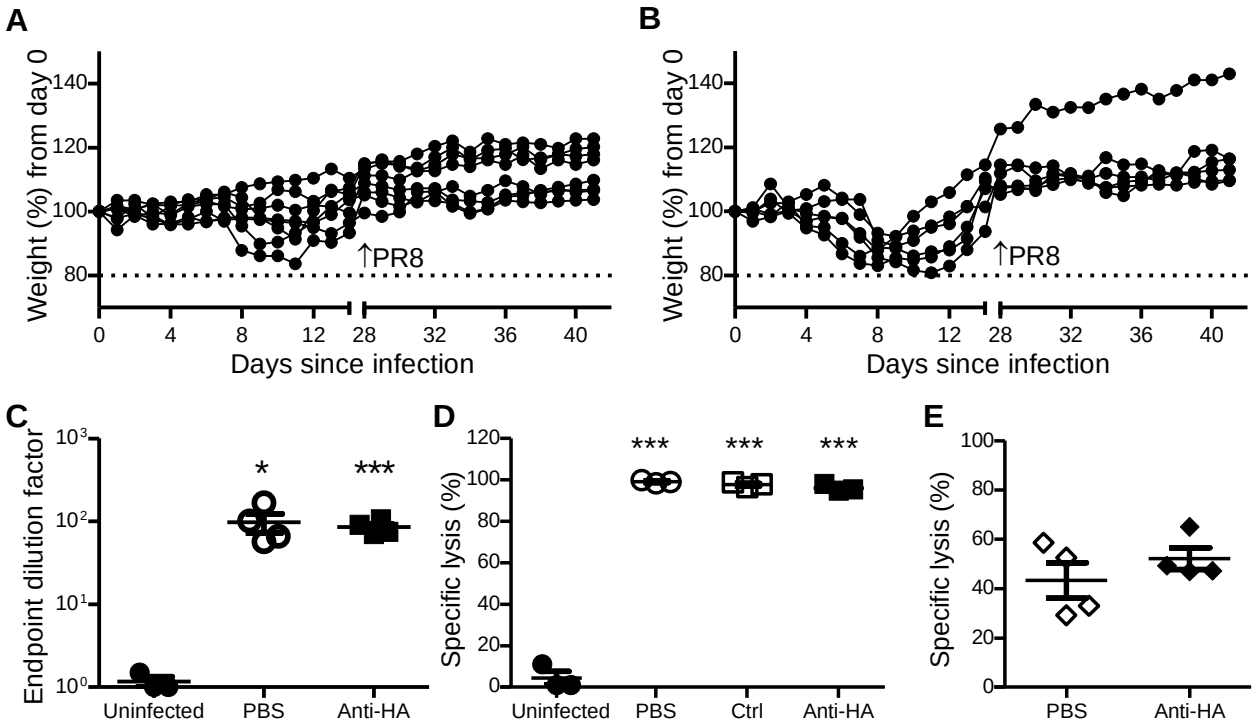


Figure 4.3 Prophylactic administration of anti-HA pAb does not inhibit the formation of protective adaptive immune responses

Mice that had survived influenza challenge via prophylactic intraperitoneal administration of anti-HA pAbs (25 mg/kg, n=8; A) or survived a sub-lethal challenge of 50 TCID50 PR8 influenza (n=6; B), were intranasally administered a lethal influenza dose of 500 $TCID_{50}$ (32 µL) PR8 Influenza four weeks after their initial challenge. Data is presented as individual percent weight loss (A-B) depicting combined data from two separate experiments. For assessment of adaptive responses, serum samples from challenged mice prophylactically administered anti-HA pAbs or saline, or uninfected mice, were analysed by ELISA for PR8-specific IgG (C). For CTL responses, mice were prophylactically administered ovine anti-HA or control pAbs, or PBS and twenty four hours later infected with 250 $TCID_{50}$ PR8 influenza. Nine days later mice received 8-10 million CTL target cells IV and were assessed for specific killing capacity 18 hours later (D). Mice infected with A/PC influenza were also subjected to *in vivo* CTL as described (E). Data are representative of mean ± SEM (C-E). Ab titre and CTL data were compared using two-tailed Student's t test; experimental groups were compared to uninfected controls. Significance is denoted as thus: * = $P<0.05$, *** = $P<0.001$.

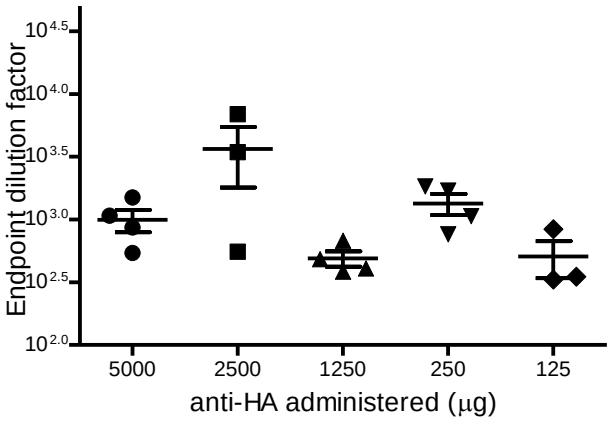


Figure 4.4 Passively administered anti-HA pAbs do not inhibit the generation of murine anti-PR8 Abs upon influenza infection

Mice (*n* = 3-4) were administered ovine serum containing anti-HA antibodies (IP) to the indicated dose, and twenty-four hours later were infected with 500 TCID50 PR8 Influenza (32 μL; IN). Mice were bled 14 days following infection and anti-PR8 IgG titres were evaluated via endpoint ELISA. Data are presented as mean ± SEM.

4.2.4. Ovine anti-HA pAbs are immunogenic and are more efficiently cleared following a second administration

In the event of an influenza epidemic, a passive immunotherapeutic approach targeting influenza would ideally provide protection for several weeks. In order to evaluate the longevity of ovine anti-HA passive immunotherapy, the half-life of ovine pAbs in the circulation was determined. Two groups of mice received anti-HA pAbs via IP injection with a second dose administered eight weeks later. No observable morbidity was associated with the initial dose of the ovine pAb or subsequent dose. Serum samples were collected every 14 days on a staggered timeline to achieve weekly measurements and samples were subsequently analysed for the presence of ovine Abs as well as the induction of murine anti-ovine IgG Abs as a measure of anti-treatment responses. Seven days after Ab administration, circulating ovine Abs (Figure 4.5 A) were detected at a maximum mean concentration of 700 pg/mL, which slowly declined over 5 weeks with a calculated half-life of 2.44 weeks. After a second pAb administration, circulating ovine pAb levels declined more rapidly with the calculated half-life decreasing to 1.5 weeks. The development of murine anti-ovine IgG Abs was shown to occur 1-2 weeks after ovine pAb administration (Figure 4.5 B) with levels plateaued after three weeks. A second ovine pAb dose further increased murine anti-ovine IgG Ab levels, which suggests that the anti-treatment response contributed to the decreased half-life of circulating pAbs following a second administration.

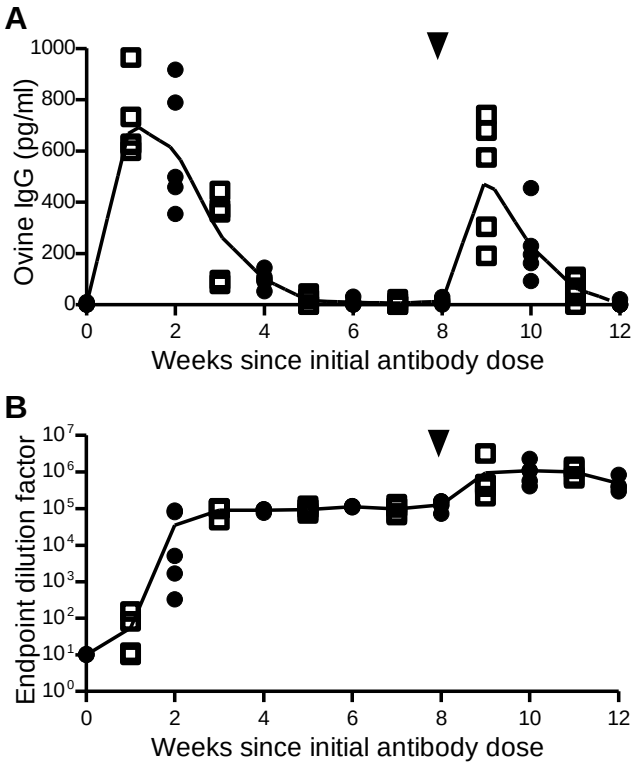


Figure 4.5 Kinetics of ovine pAb clearance and development of anti-treatment response.

Two groups of BALB/C mice (n=5) were administered ovine anti-HA pAbs (25 mg/kg) at day zero and eight weeks later (indicated by arrowheads), and bled every 14 days in two staggered timelines. Circulating ovine IgG was quantified via ELISA (individual values and mean depicted; A) and mouse anti-ovine IgG response was quantified via endpoint ELISA (individual values and mean depicted; B). Data is representative of two independent experiments.

4.2.5. Ovine anti-HA pAbs cross-react with an antigenically distinct influenza strain in vitro, but are unable to effectively protect against infection in vivo

A polyclonal approach towards influenza-specific passive immunotherapy has the potential for cross-protective capacity of the administered pAbs against circulating variants of the infective strain, and in some cases, different strains of influenza [198]. Here, the ovine anti-HA pAbs were generated against full length PR8 (H1N1) HA, and cross-reactivity against an antigenically distinct H3N2 strain (A/PC) was assessed *in vitro* and *in vivo*. Purified anti-HA pAbs were shown to be capable of binding both viral strains via ELISA (Figure 4.6 A), suggesting that the production of ovine anti-HA pAb by immunisation of sheep with PR8 yielded some Abs capable of binding to A/PC. However, a haemagglutination-inhibition assay performed as a measure of direct neutralisation capacity against each strain clearly indicated that whilst the generated Abs were able to inhibit PR8-induced agglutination at a maximum of 10^{13} dilution, they were not able to inhibit agglutination by the A/PC viral strain at relatively low dilution of eight (Figure 4.6 B). In order to explore these conflicting results further, an *in vivo* infection model of A/PC influenza was employed where mice were administered anti-HA or control pAbs twenty-four hours before a lethal dose of A/PC. The mice administered control pAb exhibited sustained weight loss and succumbed to infection (Figure 4.6 Ci), as did those administered anti-HA pAb (Figure 4.6 Cii), thus indicating a lack of protection of anti-HA pAbs against A/PC influenza infection (Figure 4.6 Ciii). These results taken together indicate that whilst the generated anti-HA pAbs are capable of binding to the A/PC strain *in vitro*, they are likely to have bound to sites on the A/PC HA that are not involved in host cell binding and entry, and therefore does not translate into protective capacity.

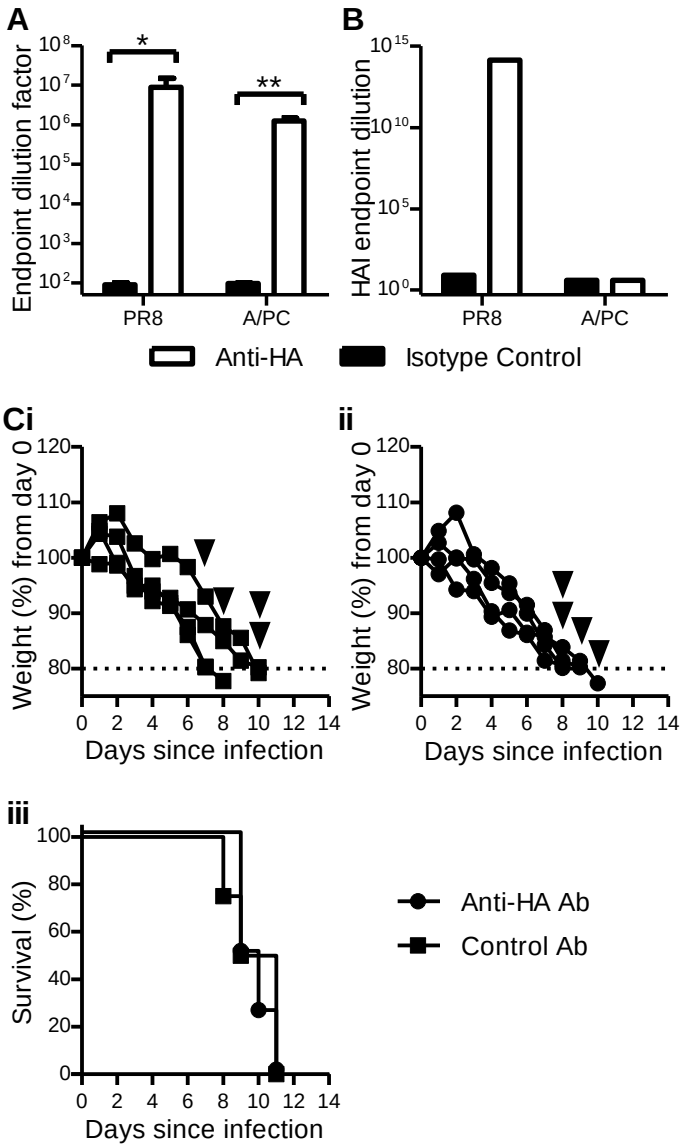


Figure 4.6 Cross-reactive ovine anti-HA pAbs do not exhibit neutralising capacity *in vitro* or protective capacity *in vivo* against a different influenza strain

Purified ovine anti-HA pAbs were analysed for reactivity against PR8 H1N1 and A/PC H3N2 influenza strains via ELISA and haemagglutination inhibition (HAI) assays. Absorbance values from ELISA results (mean ± SEM; A) were analysed via Mann-Whitney analysis. Endpoint HAI titres are depicted in (B); data are representative of one assay performed in duplicate. To determine *in vivo* protective capacity against A/PC influenza, mice were administered either control pAb (Ci) or anti-HA ovine pAb (Cii) IP (25 mg/kg) and 24 hours later infected (32 µl; IN) with A/PC (3000 $TCID_{50}$). The mice were monitored closely and euthanized if 20% weight loss or a clinical score of 5 was achieved (indicated by arrowheads). Weight loss of individual mice (Ci-ii) and group survival (Ciii) are presented. Significance is denoted as thus: * = $P<0.05$, ** = $P<0.01$.

4.3 Discussion

The number of people at higher risk of developing severe influenza complications is increasing due to the ageing population and expanding global burden of chronic disease. While vaccination and antiviral medications remain vital tools for the reduction of influenza mortality, vaccination in particular exhibits reduced efficacy in populations most vulnerable to severe infection leaving a significant number of deaths still attributed to influenza [131, 417]. Therefore additional strategies to manage influenza infections are required. An influenza specific pAb with potent neutralising capacity may be particularly useful for severe influenza therapy as they exhibit rapid tissue penetration[114] and could provide rapid specific protection to immunocompromised individuals who are at high risk of mortality. Additionally, polyclonal Abs boast straightforward and cost effective preparation methods [87] and a good safety profile when administered to individuals with decreased immune capabilities [418]. Within this study, a potential role for passive immunotherapy via administration of influenza-neutralising ovine pAbs for the treatment of severe influenza infection was explored.

The efficacy of neutralising anti-HA pAb of human origin for protection against or treatment of influenza infection has been demonstrated in both animal [185, 365, 408, 419] and clinical [190, 407, 409] studies. Previous studies, including our own, have demonstrated that hyperimmune anti-HA of ovine origin is efficacious in reducing mortality in a murine influenza model [184, 408] and this study confirmed that purified IgG from hyperimmune ovine serum retained protective and therapeutic capacity *in vivo* and *in vitro* (Figures 4.1 & 4.6). Administration of ovine pAb prior to infection reduced the severity of infection and reduced mortality compared to control mice. Importantly, administration of pAbs at 48 hours after challenge when peak viral shedding is observed was able to reduce mortality, with the IN route potentially more effective (Figure 4.2). As viral shedding is higher in patients with comorbidities [420], this was an important time point to assess. Mice that received anti-HA pAb via intraperitoneal injection 48 hours post infection exhibited an increased survival rate compared to control groups. While this difference was not statistically significant, a larger sample size may enable statistically significant differences to be detected. The ability to neutralise an established infection is crucial for a potential passive immunotherapeutic, as influenza-specific medication can only commence after the first symptoms of infection or positive blood test. In the therapeutic study, only those mice that received pAb intranasally exhibited a significant difference in clinical symptoms and overall survival from control

groups. This is consistent with previous studies which showed that intranasal administration of anti-influenza Abs provided a survival advantage by significantly reducing clinical symptoms, weight loss and lung viral titres [365, 408, 421]. As this route of administration was also associated with higher Ab concentrations within the lung, it was postulated that a higher proportion of the delivered Abs were able to directly interact with virions in the nasal passages, as opposed to being distributed systemically via intraperitoneal injection before reaching the lungs [421]. Furthermore, previous studies, including our own with hyperimmune serum, have indicated that intranasal Ab therapy requires less delivered Ab than systemic methods for the same outcome and can increase the longevity of protection [421, 422].

These findings have important implications for the cost effectiveness of pAb products, which are often prohibitively expensive [423], and may allow lower effective doses to be used and more affordable treatments. Administration via the IN route is also compatible with whole Ab and Ab fragments, which has been demonstrated experimentally for influenza [183]. Clinically, IN delivery of pAb products has been well tolerated and can provide adequate prophylaxis against respiratory infection [424] though only IV administered human mAbs [192] and IVIG [190] have been effectively used for influenza treatment. Studies using an experimental human influenza model found that IV administration of anti-M2e Mab TCN-032 reduced symptom development and viral load when administered twenty-four hours following inoculation [192], though whether IN administration may increase efficacy of treatments such as these remains to be investigated. Intransally administered pooled immune globulin has been used unsuccessfully in the treatment of other respiratory infections [425] however no targeted Ab products have been trialled clinically for IN treatment of respiratory infections, including influenza, and this remains to be investigated.

The current study also explored the effect of influenza-specific passive immunotherapy on the development of adaptive immune responses, which are important for protection against reinfection in at-risk populations. Previous studies have demonstrated that the presence of circulating influenza-specific Abs can inhibit humoral responses to a subsequent influenza vaccination in piglets [426] and ferrets [419]. In the context of this study where ovine pAbs were assessed in murine influenza, the presence of foreign Abs might have favourable or non-favourable impacts on the generation of virus-specific responses. For example, Ab may effectively ‘mask’ available antigen from the immune system and prevent the generation of adaptive responses, as observed with the prevention of haemolytic disease of the newborn when pregnant mothers are prophylactically administered anti-D before sensitisation to the

foetal D antigen can take place [427]. Alternatively, immune complexes formed *in situ* following administered foreign Ab may have an adjuvant-like function, as observed with historical use of antigen-antibody complexes to stimulate adaptive immunity [428]. In this study, mice surviving infection via ovine anti-HA pAb administration exhibited sustained protection against influenza (Figure 4.3) and symptoms of infection were largely absent upon reinfection. Influenza-specific antibody and cell mediated responses in mice administered pAb before infection were also assessed and both responses were found to be consistent with untreated groups. As symptoms of infection upon initial challenge were still evident even if administered anti-HA pAbs, it is postulated that in this setting passive immunotherapy served to neutralise some of the viable virions, effectively reducing but not eliminating viral load. Administration of passive pAb therapies clinically would occur only in an established infection, with a high viral load, thus viral-specific Abs would serve to attenuate rather than completely neutralise the infection. However, with a comparatively low viral load an alternate outcome may occur, and indeed studies have demonstrated that immunosuppressive effects are often present when Ab is administered in excess of antigen [429]. In the context of influenza, the use of ovine pAb in a sublethal model of PR8 infection (with a five-fold lower viral dose than the current study) was associated with reduced adaptive responses when infection was prevented via pAb administration one hour prior to viral challenge. However unlike our study where pAb were administered twenty-four hours prior to or forty-eight hours post challenge, in this scenario, virions were likely neutralised before attachment to host cells and antigen engagement by the immune system and thus the formation of an endogenous anti-PR8 immune response was prevented.

As part of the evaluation of an ovine pAb approach towards severe influenza treatment, within the current study the longevity and immunogenicity of passively administered pAbs in a murine model were assessed. The observed half-life of administered pAb was over two weeks and decreased after a second pAb administration. Correspondingly, anti-ovine responses which were evident from 1-2 weeks increased after re-administration and these likely contributed to the increased pAb clearance following re-administration. Though the administered ovine pAb were immunogenic, no hypersensitivity reactions were observed after pAb administration in this study. However, such reactions following administration of pAb of animal origin have been observed in the clinic [201] . Formulation of therapies as Fab or $F(ab)_2$ fragments, thus removing the F_c portion, serve to reduce this risk but do not eliminate it. As such, administration of ovine pAb fragment therapies is closely monitored and appropriate precautions, including readiness with IV epinephrine and antihistamines, are taken in patients with a history of sensitivity to sheep proteins or previous administration of

ovine pAbs [430]. Administration of ovine pAb also did not impact on the development of influenza-specific immunity in this model. Other studies have indicated that passive immunotherapies can perform an immunogenic 'adjuvant-like' function and increase adaptive immune responses to their targets. For instance, the clinical efficacy of IV immunoglobulin (IVIG) against a wide variety of conditions has been linked to immunomodulatory properties potentially via engagement of Fc-gamma immunoglobulin receptors [431]. An immunomodulatory adjuvant effect has also been observed in candidate anti-D mAbs developed for prevention of hemolytic disease of the newborn which enhanced rather than prevented endogenous anti-D immune responses [432]. It was proposed that Ab glycosylation may be the key to these adjuvant functions and this is supported by studies examining carbohydrate-dependent effector function of Abs [433]. In the context of influenza, these effector-functions could be harnessed to develop passive Ab therapies that not only neutralise influenza virions but enhance host anti-viral immunity, potentially via the formation of immunogenic immune complexes and engagement with Fc-gamma receptors. While no enhancement of adaptive responses was associated with ovine pAb administration in this study, whether whole ovine pAb are capable of instigating immunomodulatory effects through interaction with human Fc receptors remains to be explored.

While influenza-specific mAb therapies have been shown to be efficacious in animal [66, 189, 421] and human [192] studies, these monoclonal therapies may not be neutralising against a diverse range of viral strains [411] and may prompt escape mutants to be generated [354, 404]. A major advantage of a polyclonal approach to influenza treatment is the ability to target multiple epitopes to discourage escape mutants and engineer therapies that neutralise multiple strains of influenza. Within this study, pAbs were generated against a single recombinant HA from PR8, H1N1, and shown to have *in vitro* binding capacity against a H3N2 strain (A/PC) via ELISA analysis. However the pAbs did not exhibit functional neutralisation capacity against this strain (Figure 4.6) and thus were not cross protective. It may be that the H3N2-reactive pAbs evident by ELISA are targeted towards epitopes distant from the sialic-acid binding site responsible for host cell binding *in vivo* and haemagglutination *in vitro.* As pAbs in this study were elicited against full length monomeric HA, and fragmented or denatured virions may have been present in the ELISA method, it is possible that cross-reactive Abs were binding to stem region epitopes that were exposed in ELISA but are not exposed in trimerised HA on whole virus [434]. These non-neutralising Ab therefore did not impact on the course of infection in an *in vivo* H3N2 model. While cross-protective capacity between two strains was not evident within this study, strategies to increase cross-protective capacity could be employed for the generation of anti-influenza

polyclonals. Such strategies could include immunisation of sheep with multiple influenza strains, repeated boosting and the application of superior adjuvants not approved for human use or simply combination of antisera elicited against different strains [114, 435, 436].

The current study provides proof-of-principle for development of a passive immunotherapeutic based on ovine pAb for the treatment of established severe influenza infection. It also provides evidence that pAb therapies will not reduce adaptive anti-influenza responses which are crucial to ongoing protection from influenza infection. Further studies are however required to assess the *in vivo* viral neutralisation capacity of the therapy, particularly in the context of pAb fragmentation for clinical use. Through further development, strategies to decrease Ab immunogenicity, increase cross-protective capacity or increase immunomodulatory functions of the Abs could be employed to produce a purpose-built passive pAb therapy capable of controlling life-threatening influenza infections in immunologically-deficient populations.

PART B:

FEASIBILTY AND EVALUATION OF OVINE ANTI-HIGH MOBILITY GROUP BOX 1 POLYCLONAL ANTIBODIES FOR THE TREATMENT OF BACTERIAL SEPSIS AND SEPTIC SHOCK

CHAPTER 5.

Clinical investigations into HMGB1 as a potential target for passive immunotherapy in sepsis

5.1 Introduction

Sepsis remains a significant burden on the healthcare system, as 51 million sepsis cases occur annually [437] with mortality rates ranging from 30% in developed countries to 70-80% in resource-poor countries [438]. Sepsis is triggered by an overwhelming inflammatory response to infection termed systemic inflammatory response syndrome (SIRS), which if left uncontrolled instigates multiple organ damage and death. Current therapies for sepsis are primarily limited to broad-spectrum antibiotics to control infection and supportive medications which aim to maintain blood volume and haemostasis. Early sepsis identification and recent improvements in supportive care algorithms have reduced mortality from sepsis in intensive care units (ICU) in developed countries [227], however mortality is still high and moreover increasing due to the ageing population and increased prevalence of nosocomial infections [206].

The pathogenesis of sepsis as an over-exuberant response to infection suggests that global blockade of proinflammatory mediators could reduce the severity of sepsis, alleviate organ damage and prevent lethality. However, the simplistic understanding of sepsis pathogenesis as a direct result of the massive release of proinflammatory mediators has diminished in favour of the hypothesis that sepsis pathology is a result of 'immunologic dissonance' triggered by a systemic innate immune response which is unable to effectively self-regulate and balance pro- and anti-inflammatory mediators [439, 440]. It is now understood that following the pro-inflammatory SIRS phase of sepsis, a compensatory anti-inflammatory response syndrome (CARS) can dominate which is also a significant cause of sepsis mortality. Both SIRS and CARS phases can cause pathology in their own right [441] and can indeed occur simultaneously in a pattern termed mixed antagonist response syndrome (MARS) [442]. As a patient may oscillate between these phases through their clinical course [443], these complex patterns render the reversal of pathogenic inflammatory processes difficult in the clinic.

It is perhaps then unsurprising that while a number of immune-modulating therapies have shown promise in preclinical models, thus far none have provided significant survival benefits in controlled clinical trials [206, 444]. This failure has been attributed to the use of preclinical models that are unable to replicate the complexity of human sepsis [325, 326, 329] and potential flaws in the design and execution of clinical trials, including selection of appropriate patient populations [264, 445, 446]. The variability in septic severity, comorbidities, pathogenic factors and patient genetic backgrounds each contribute to the clinical

heterogeneity of sepsis and septic shock and this heterogeneity has complicated the interpretation of clinical trial results [447]. Therapies for sepsis can only be delivered after clinical symptoms are apparent however as the innate inflammatory response can progress rapidly in SIRS [448], approaches targeting early inflammatory mediators may be initiated too late [264]. Targeting immune mediators that act later in sepsis, with a wider therapeutic window may thus be a superior approach for achieving immune modulation in sepsis.

As introduced in Chapter 1, high mobility group box 1 (HMGB1) has been identified as a potential therapeutic target in sepsis as it is released later than many early pro-inflammatory mediators. Normally located within the nucleus, HMGB1 is released during sepsis by activated immune cells and passively by necrotic tissues [449] where it promotes inflammation as a damage associated molecular pattern (DAMP). Extracellular HMGB1 interacts with toll-like receptor 4 (TLR4) and the receptor for advanced glycation endproducts (RAGE) in addition to other receptors to promote chemotaxis and NF-κB signalling. Increases in HMGB1 are also evident in trauma patients who develop SIRS [443] and can induce sterile inflammation [272], thus targeting HMGB1 may be a useful approach, particularly when the infective organism is not known. Preclinical modelling has shown that methods used to neutralise or inhibit HMGB1 activity via molecular inhibitors [317, 319] or upstream inhibition [318] have a wide therapeutic window and reduced mortality in these models [272]. Importantly, HMGB1 has also been implicated in the CARS phase of sepsis, as differential oxidation states can alter HMGB1 function to suppress inflammation and adaptive responses [309] and thus HMGB1 may be considered a biological ‘switch’ to instigate inflammation resolution following damage. Consequently, an ideal approach to sepsis treatment may be to control inflammatory damage during SIRS without exacerbating CARS by targeting HMGB1. Application of HMGB1-neutralising pAb may be particularly beneficial as targeting multiple sites on the protein may prevent interactions with circulating binding partners which synergistically increase HMGB1 potency [450-453].

It is clear that part of a successful approach to sepsis treatment involves identifying the correct population likely to benefit from therapy in a similar manner to current personalised cancer therapies. Previous observational studies on septic patients have demonstrated that circulating HMGB1 levels are dependent on the severity and foci of infection [454], with marked HMGB1 increases present in patients presenting with abdominal, urinary and reproductive infections [455]. Associations between elevated plasma HMGB1 and sepsis complications, including disseminated intravascular coagulation (DIC), shock and organ failure [456] have also been revealed, and together these broad clinical indications could

potentially identify which patients are likely to benefit from HMGB1 targeting. As the inflammatory profile in sepsis can influence the efficacy of immune-modulating therapies [457], establishing whether a SIRS, CARS or MARS phenotype is predominant via analysis of multiple immune markers may be crucial to directing treatment.

In order to establish the efficacy of an approach using HMGB1-specific neutralising pAbs as a therapy for sepsis, it was necessary to understand the role of HMGB1 in the septic inflammatory milieu. Within this chapter, a study of HMGB1 levels and inflammatory markers in a cohort of septic shock patients is described. Kinetic plasma samples from septic shock patients were analysed to determine concentrations of HMGB1 and selected pro- and anti-inflammatory cytokines. It was revealed that HMGB1 was significantly increased in non-surviving patients, and correlated with disease severity. Several individual cytokines were differentially expressed between survivors and non-survivors and a cytokine profile skewed towards proinflammatory markers was associated with mortality. While HMGB1 did not correlate with any individual cytokine, increased HMGB1 levels were associated with an increased proinflammatory phenotype.

5.2 Results

5.2.1. Elevated plasma HMGB1 is associated with morbidity and mortality in a cohort of septic shock patients

Previous studies have demonstrated elevated HMGB1 in patients currently with, or recovered from sepsis, however measurements were performed a minimum of 24 hours apart [323]. To more closely profile the presence of serum HMGB1 in patients during the early phase of septic shock, seventeen patients were enrolled in an observational study where arterial blood was sampled twice daily for the duration of septic shock. Patient characteristics are summarised in Table 5.1; patients had a median age of 61 and the majority presented with respiratory (35%) or gastrointestinal (29%) sepsis. The age of surviving and non-surviving patients was not significantly different.

Patients were assigned an acute physiology and chronic health evaluation (APACHE) II sickness severity score within 24 hours of admission to ICU. Parameters used to calculate APACHE II score are detailed in Appendix A. Non-surviving patients had significantly higher APACHE II scores than surviving patients (Figure 5.1 A) indicating that these patients presented to ICU with more severe illness. Plasma HMGB1 content was assessed, and HMGB1 levels at 24 hours were positively correlated with APACHE II score (Figure 5.1 B). Comparison of HMGB1 levels over time revealed significantly elevated HMGB1 concentrations overall in non-surviving patients, which was most pronounced 24-48 hours after admission, where HMGB1 levels were threefold higher in non-surviving patients (Figure 5.1 C). Together, these results suggest that HMGB1 is associated with an enhanced pro-inflammatory state in septic shock and therefore has the potential to be a candidate therapeutic target with a wide intervention window, where inhibition may increase survival, particularly in those patients that present with more severe illness.

Table 5.1 Patient cohort characteristics

Patients in the Intensive Care Unit with diagnoses consistent with infection and hypoperfusion requiring haemodynamic support were recruited by qualified nursing staff. Arterial plasma samples (5mL) were taken from enrolled patients twice daily (morning and afternoon) for as long as haemodynamic intervention was required, the length of ICU stay, or a maximum of 7 days. Exclusion criteria included the use of steroids or enrolment in clinical trials. Patient non-survival is defined as death within ICU stay or a 14 day period.

Characteristic	**Total (n = 17)**	**Survivors (n = 9)**	**Non Survivors (n = 8)**
Age[a]	61 (47-68)	55 (45-65)	64.5 (49-77)
Gender	11 female (64%)	7 female (77%)	4 female (50%)
APACHE II Score[a]	18 (15-26.5)	18 (13.5-18.5)	26.5 (16.25-34.75)
Site of infection			
Lung	6 (35.3%)	3 (33.3%)	3 (37.5%)
Gastrointestinal	5 (29.4%)	3 (33.3%)	2 (25%)
Skin	4 (23.5%)	2 (22.2%)	2 (25%)
Other	2 (11.8%)	1 (11.1%)	1 (12.5%)
Microbiological culture	12 (70.5%)	6 (66.6%)	6 (75%)
G+	3 (17.6%)	1 (11.1%)	2 (25%)
G-	3 (17.6%)	2 (22.2%)	1 (12.5%)
Fungal	1 (5.8%)	0	1 (12.5%)
Mixed	5 (29.4%)	3 (33.3%)	2 (25%)

Unless otherwise stated, data has been represented as: number of patients (percentage of *n*). [a] Data represents median (interquartile range)

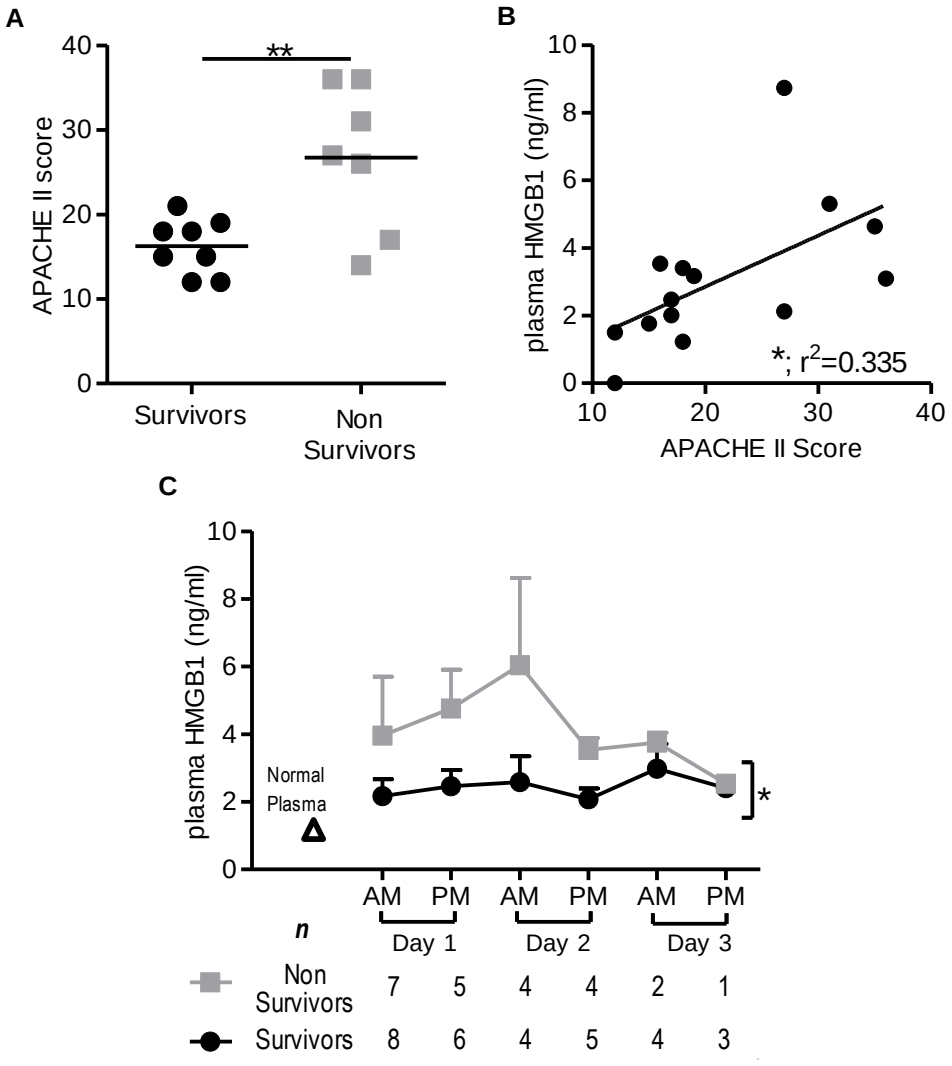


Figure 5.1 Elevated plasma HMGB1 is associated with increased disease severity upon ICU admission and mortality in septic shock patients

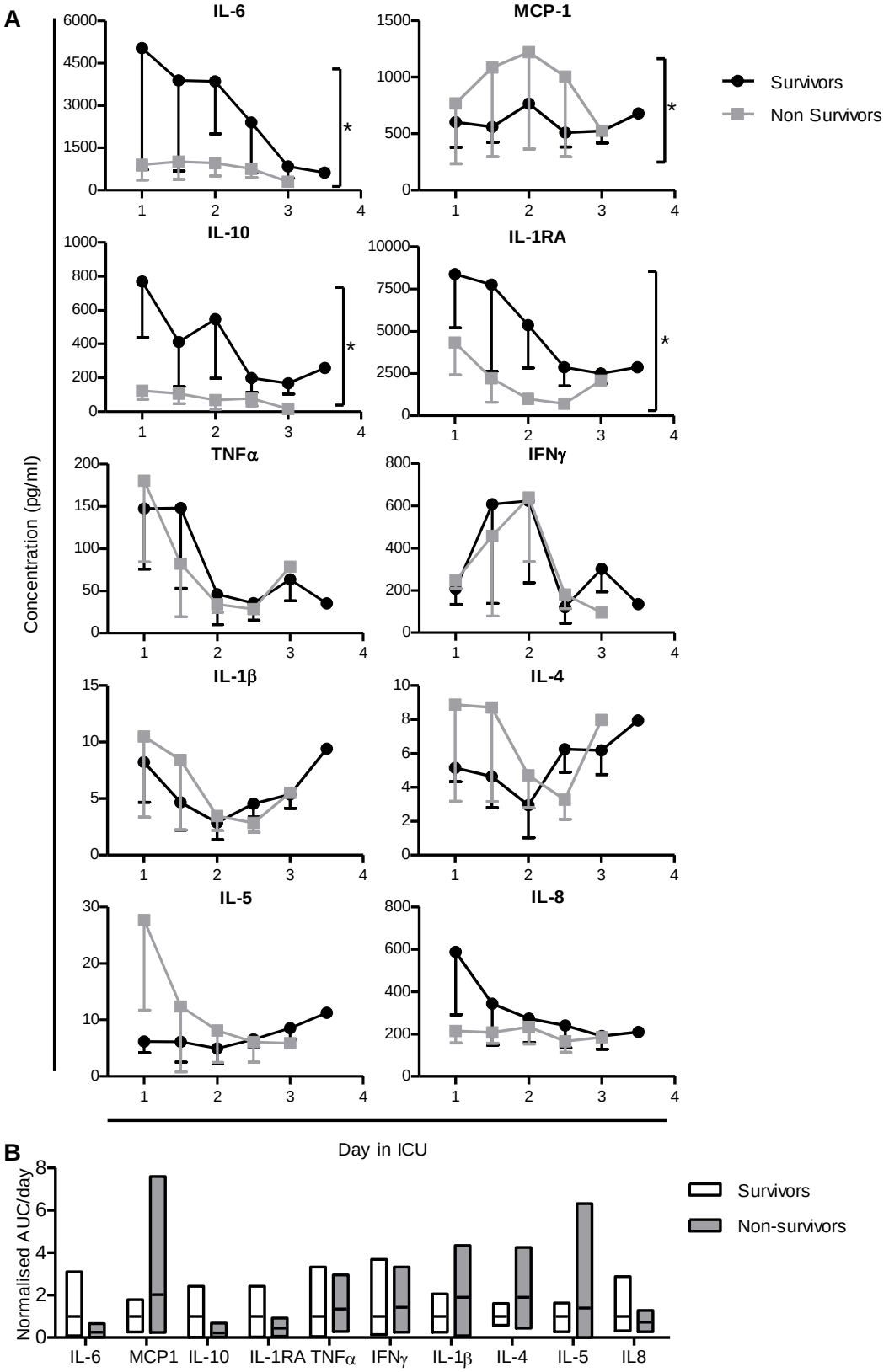
Plasma samples from ICU patients with diagnoses consistent with septic shock were taken twice daily (morning and afternoon) for the length of ICU stay. (A) APACHE II scores in survivors and non-survivors were compared via two-tailed t test. (B) HMGB1 was quantified in patient samples by ELISA and regression analyses performed to compare plasma HMGB1 at 24 hours post ICU admission and APACHE II score. (C) Mean kinetic plasma HMGB1 levels in survivors versus non-survivors were compared by paired two-way t-test. Significance is denoted as thus: * = $P<0.05$, ** = $P<0.01$.

5.2.2. Differential cytokine expression in surviving and non-surviving septic shock patients

In order to define the cytokine profiles within the septic shock cohort, individual plasma samples were subjected to multiplex cytokine analysis by measurement of IL-1β, IL-1RA, IL-4, IL-5, IL-6, IL-8, IL-10, IFNγ, MCP-1 and TNFα. Comparison between survivors and non-surviving patients within the cohort revealed significant differences in four individual cytokines where non-survivors had lower levels of IL-1RA, IL-6 and IL-10, and increased MCP-1 levels compared to surviving patients (Figure 5.2). Anti-inflammatory IL-1RA was almost twofold higher in survivors as compared to non-survivors over the first two days of sepsis, with IL-10 concentration also consistently increased in the surviving group. Notably, IL-6 was fivefold higher in surviving patients on day 1 compared to non-survivors, and levels reduced over time to the same level as non-survivors by day 3. Chemotactic cytokine MCP-1 was increased up to twofold in non-surviving patients in the first 2 days of septic shock, and peak concentrations were observed on day 2 for both groups. Consistent with previous studies, concentrations of pro-inflammatory cytokines TNFα and IL-1β were highest on the first day of septic shock in both groups before declining. Kinetics of IFNγ and IL-4 were similar between survivors and non-survivors. Levels of IL-5 and IL-8 were not significantly different between the groups; however, different release patterns where observed where IL-5 levels decreased over time in non-survivors compared to survivors whereas IL-8 levels decreased over time in survivors but remained stable in non-survivors. Plasma IL-1RA, IL-6, IL-8 and MCP-1 concentrations were also consistently elevated in samples from the septic shock cohort than in previously published healthy cohorts [458, 459]. In order to assess overall cytokine signalling between survivors and non-survivors, cytokine measurements over tie were integrated into area under the curve (AUC) measurements per day in ICU and normalised to mean AUC in surviving patients. Consistent with the time-course data, IL-6, IL-10 and IL-1RA AUC values were decreased in non-survivors whereas MCP-1 AUC was increased. However, no significant differences were detected in integrated cytokine values between survivors and non-survivors.

Figure 5.2 Plasma from septic shock non-survivors exhibited significantly increased levels of MCP-1 and significantly reduced levels of IL-1RA, IL-6 and IL-10 compared to survivors

Plasma samples from septic shock patients were stored and analysed via multiplex analysis (Bio-Rad). Ten cytokines (IL-6, MCP-1, IL-10, IFNγ, TNFα, IL-1β, IL-4, IL-5, IL-8 and IL-1ra) were assessed and concentrations calculated from samples run in duplicate. (A) Plasma concentrations between survivors (black) and non-survivors (grey) were compared via paired t-test. (B) Cytokine values over time were integrated into area under the curve (AUC) per day in ICU. Mean, minimum and maximum cytokine AUC for survivors (clear bars) and non-survivors (grey bars) are represented. Significance is denoted as thus: * = $P<0.05$.


A
IL-6
MCP-1
IL-10
IL-1RA
TNFα
IFNγ
IL-1β
IL-4
IL-5
IL-8
Concentration (pg/ml)
Day in ICU
Survivors
Non Survivors
B
Normalised AUC/day
IL-6
MCP1
IL-10
IL-1RA
TNFα
IFNγ
IL-1β
IL-4
IL-5
IL8
Survivors
Non-survivors

5.2.3. An exacerbated pro-inflammatory cytokine profile is associated with increased plasma HMGB1 and non-survival during septic shock

The variable efficacy of previously trialled immune modulating therapies such as anti-TNFα has been linked to variation in inflammatory profiles within patients [460]. Thus, an approach targeting HMGB1 in the clinic should be informed by analysis of HMGB1 in the context of the inflammatory state within sepsis. In order to evaluate overall inflammatory profiles within the septic shock cohort, composite cytokine scores (CCS) expressing the ratio of pro-inflammatory to anti-inflammatory cytokines were calculated based on a previously published method [442]. For each sample, cytokine concentrations were first normalised to the median concentration in the cohort and the CCS was then calculated by dividing the sum of normalised pro-inflammatory cytokine values (IL-6, MCP-1, IFNγ, TNFα, IL-1β, IL-5 and IL-8) by the sum of normalised anti-inflammatory cytokine values (IL-10, IL-4 and IL-1RA).

Analysis of normalised CCS revealed a significant decrease in the ratio of pro-inflammatory to anti-inflammatory cytokines in surviving patients compared with non-survivors (Figure 5.3 A). Furthermore, pro-inflammatory:anti-inflammatory CCS ratios were significantly increased in patient plasma samples with elevated HMGB1 (>2.5 ng/mL, the median HMGB1 value in analysed samples) compared to samples with lower HMGB1 content (Figure 5.3 B), though no individual cytokine significantly correlated with HMGB1 content. Normalised cytokine scores for each sample were summed (total CCS) to indicate the magnitude of cytokine signalling. Interestingly, surviving patients exhibited significantly higher cytokine magnitude scores than non-survivors (Figure 5.3 C). Cytokine magnitude also decreased over time in survivors, whereas non-survivors exhibited low total CSS levels throughout the sampling period. No association was observed between HMGB1 content and total CCS in samples from this cohort (Figure 5.3 D). Integration of pro-inflammatory:anti-inflammmatory signalling into AUC values revealed no statistically significant differences between survivors and non-survivors (data not shown).

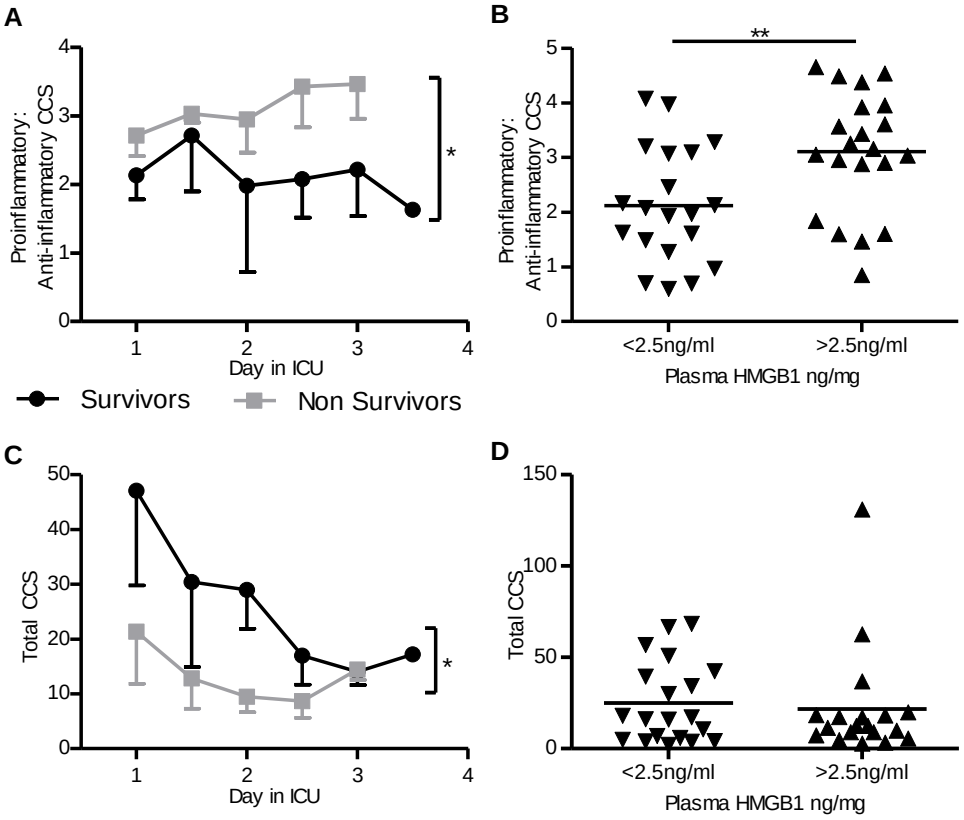


Figure 5.3 Mortality and elevated HMGB1 are associated with a proinflammatory serum cytokine profile in a cohort of septic shock patients

Composite cytokine scores (CCS) were determined from normalised plasma cytokine values, where the sum of proinflammatory values (IL-6, MCP-1, IFNγ, TNFα, IL-1β, IL-5 and IL-8) was divided by the sum of anti-inflammatory values (IL-10, IL-4 and IL-1RA) to generate a pro:anti-inflammatory CCS for each sample. Calculated pro:anti-inflammatory CCS were compared between survivors and non-survivors via paired two-tailed t-test (A) and between samples with low and high HMGB1 content via two-tailed t-test (B). Total CCS indicating the total magnitude of cytokine levels were calculated by summation of all ten normalised cytokine scores and compared between survivors and non-survivors (C) and against HMGB1 content (D). Data are representative of mean ± SEM with significance denoted as thus: * = P<0.05, ** = P<0.01.

5.3 Discussion

Within this chapter, HMGB1 release and inflammatory profiles were examined in a cohort of septic shock patients. While only a small number of patients were included within this study, differences in circulating HMGB1 levels were apparent between survivors and non-survivors. As sampling did not continue if inotrope therapy was ceased (considered recovered from shock) or if patients recovered sufficiently to warrant transfer out of ICU, measures within this study are more representative of the acute phase of septic shock in critically ill patients than chronic sepsis which manifests in a range of clinical severities.

Measurement of plasma HMGB1 in the early phase of septic shock in this cohort revealed a significant correlation of HMGB1 concentration with both morbidity and death. This finding corroborates with previous studies that have demonstrated similar associations with HMGB1 expression in critically ill patients [323, 454, 461]. Increased plasma HMGB1 was also associated with an enhanced pro-inflammatory profile which supports the notion of HMGB1 as a pro-inflammatory cytokine in SIRS. Plasma HMGB1 has been demonstrated to have some prognostic value in septic shock patients [462], with a day 3 HMGB1 concentration >4 ng/mL associated with a 6.5-fold increase in the risk of death. However, individual plasma cytokine levels are generally not considered good prognostic indicators in the clinic [463-465], as variability in the type and foci of infection, immune status, presence of comorbidities and illness severity can alter their plasma levels and make interpretation of cytokine profiles difficult [466, 467]. Likewise, cytokine levels in the patient cohort here were also highly variable (Figure 5.2), however overall cytokine profiles and the levels of some particular cytokines were altered between survivors and non-survivors. In particular, IL-6 was significantly higher in surviving patients, which disagrees with previous studies where IL-6 has been associated with non-survival in sepsis [468] or not significantly different between survivors and non-survivors [469]. However, within this study only septic shock patients were sampled, which comprise a unique sub-population within sepsis patients [470] and may account for differences with studies using whole sepsis cohorts. A 2005 study that revealed no association of IL-6 levels with survival in a septic cohort demonstrated similar release kinetics to those of the current study, with elevated IL-6 apparent in the first 48 hours from sepsis onset which rapidly declined in survivors, while remaining more stable in non-survivors [469]. All measured anti-inflammatory cytokines IL-10, IL-4 and IL-1RA plateaued or increased in the cohort by day 3. In particular, non-survivors exhibited rapid increases in IL-4 and IL-1RA from day 2, and concurrent increases in TNFα and IL-1β. This observation

correlates with recent clinical and experimental findings that increased expression of both pro-inflammatory and anti-inflammatory cytokines, referred to as MARS, frequently precedes death from sepsis [442, 471]. Integration of cytokine expression over time into AUC measures did not yield any statistically significant differences between survivors and non-survivors, though trends towards decreases in IL-1, IL-10 and IL-1RA and an increase in MCP-1 were observed in non-survivors. However, AUC integration may not accurately reflect expression in patients were values were missing, and could not include one patient from whom only one sample was obtained. However, this approach towards the analysis of cytokine profiles may be useful when used for larger cohorts.

These findings highlight the importance of measuring a panel of cytokines to determine sepsis status in addition to canonical sepsis cytokines including TNFα and IL-1. It may be proposed that the kinetics of cytokine release from sequential measurements is a superior indicator of immune regulation and is a more valuable measurement than defined concentrations, which can vary widely with the specific infective and damage burden of the patient [443]. Within this study, measurements of ten individual cytokines were used to calculate pro:anti-inflammatory CCS for each sample, and it was found that non-surviving patients had a significantly increased pro-inflammatory profile in their plasma which also increased over time compared to survivors. Total CCS signifying the magnitude of cytokine signalling patterns were higher in survivors than non-survivors, though survivors did exhibit a decrease in total CSS with time. Together these cytokine patterns may indicate that non-survivors were unable to mount effective anti-inflammatory responses to efficiently control the pro-inflammatory cascade in this acute timeframe, ultimately leading to increased pathology and lethality. Additionally, measured concentrations of anti-inflammatory cytokines IL-4, IL-10, and IL-1RA increased at later time points in the surviving cohort, which also supports the notion that pro-inflammatory responses during SIRS were more efficiently regulated in these patients. The timeframe of sampling in this study was too short to deduce long-term signalling patterns, however it can be speculated that assessment of HMGB1 concentration and pro-inflammatory:anti-inflammatory cytokine ratios may aid in monitoring the clinical course or direct treatment options towards subpopulations that are more likely to show benefits. In support of this, clinical treatment of sepsis with anti-TNFα has been demonstrated to be more effective in patients with a plasma IL-6 concentration >1000 pg/mL [264]. With broader availability of multiplexed plasma analysis technology [472, 473], and the increased speed at which such analyses can be performed, these parameters can be more easily monitored in a clinical setting and may enable the use of more personalised treatment options.

The kinetic analysis of plasma HMGB1 together with functionally relevant cytokines has shed light onto release kinetics of HMGB1 in the context of the inflammatory response in human septic shock. Even within a relatively small cohort, HMGB1 levels were significantly correlated with sickness severity and mortality and elevated HMGB1 was associated with a pro-inflammatory phenotype. The non-surviving cohort in this study exhibited reduced magnitude of cytokine signalling, which may indicate that these patients are poor candidates for immunomodulatory therapies that target classical pro-inflammatory cytokines. Consequently, HMGB1 blockade therapy may be a viable treatment for critically ill septic shock patients that exhibit immunoparalysis.

CHAPTER 6.

Assessment of ovine anti-HMGB1 polyclonal antibody administration in murine sepsis models

6.1 Introduction

Sepsis remains a significant healthcare burden, and thus an ongoing search exists for new approaches to the treatment of sepsis [444]. The observations from the patient study (Chapter 5) support development of a septic shock treatment targeting high mobility group box 1 (HMGB1). It was hypothesised that HMGB1 blockade may be more efficacious in the clinic than targeting proinflammatory cytokines, as HMGB1 has been demonstrated to have both proinflammatory and anti-inflammatory activities, and a wider therapeutic window [309, 449] as HMGB1 release occurs later and is more sustained than many key proinflammatory cytokines. Passive immunotherapy utilising polyclonal antibodies (pAbs) may be an ideal approach for HMGB1 blockade in sepsis, as HMGB1 contains multiple binding sites [474] and targeting multiple epitopes may prevent interactions with circulating binding partners which synergistically increase HMGB1 potency [450-453]. Passive immunotherapies utilising ovine polyclonal Fab fragments have been demonstrated as safe during clinical trials involving critical illness [258, 475] and thus this existing technology may be amenable to the development of interventions targeting HMGB1 in sepsis.

The impact of HMGB1 blockade on the complex immunological signalling events that take place in sepsis is not well understood. Previous studies that targeted HMGB1 in murine sepsis have linked HMGB1 blockade to reduced expression of early proinflammatory cytokines, including TNFα, IL-6 and MIP-1α [476-478], though impacts on anti-inflammatory cytokines have not been demonstrated. An ideal immunomodulatory therapy for sepsis would reduce inflammatory organ damage in the acute phase of sepsis without eliciting a CARS phenotype where patients are at increased risk of mortality following secondary infection. However, previous studies that have assessed the impact of HMGB1 blockade in sepsis models have focused on morbidity and mortality following sepsis rather than susceptibility to secondary infection [479]. Recently, HMGB1 blockade has been demonstrated as effective in reducing immunosuppression following tissue trauma [480], however the impact of this strategy in sepsis remains to be elucidated.

In order to assess whether blockade of HMGB1 via ovine anti-HMGB1 pAbs fulfils these criteria, high titre Abs were first generated in sheep against recombinant human HMGB1 and used for all studies owing to the high homology (99%) between human and rodent HMGB1 sequences [481]. Total IgG was purified from anti-HMGB1 serum to yield anti-HMGB1 pAbs which were subsequently assessed in three murine sepsis models of escalating

complexity. A simple model of endotoxaemia was initially employed to confirm *in vivo* neutralisation capacity of the generated anti-HMGB1 pAbs and demonstrated reduced morbidity and mortality. The gold-standard polymicrobial sepsis model, caecal ligation and puncture, was subsequently employed to determine the effects of anti-HMGB1 pAb administration on sepsis survival and the generated inflammatory profile. Importantly, alterations in cytokine levels evident in anti-HMGB1 pAb treated mice also correlated with those associated with survival in the patient cohort. Administration of anti-HMGB1 pAb was also protective against secondary respiratory infection following sepsis in a double-hit sepsis model and induced changes in the inflammatory signalling profile of innate immune cell types. Together, these results demonstrate that anti-HMGB1 pAb therapies can prevent lethality from acute and chronic sepsis, do not support immunosuppression following SIRS, and are able to alter inflammatory cytokine signalling associated with clinical lethality.

6.2 Results

6.2.1. Ovine anti-HMGB1 pAb therapy improves survival in an in vivo model of murine endotoxaemia

As elevated HMGB1 levels were associated with poor clinical outcomes and dysregulated proinflammatory signalling in the patient cohort, an approach to block HMGB1 in a murine sepsis model was developed and assessed for impact on survival. To achieve this, anti-HMGB1 pAbs were generated in sheep and applied to a simple model of sepsis, lethal endotoxaemia. Purified ovine anti-HMGB1 pAbs were administered prophylactically or therapeutically to mice administered a lethal dose of LPS (Figure 6.1). Mice that received anti-HMGB1 pAbs one hour prior to LPS administration exhibited a significant survival advantage compared to controls, with a survival rate greater than 70% (Figure 6.1 A). Control groups that received either control antibody or PBS one hour prior to LPS administration displayed signs of endotoxaemia including ruffled fur, hunched posture, reduced temperature, diarrhoea and reluctance to move and typically required euthanasia within twenty four hours. This was reflected in the mean clinical score, where the mice that received anti-HMGB1 pAbs prophylactically exhibited significantly less symptoms than controls, including the surviving mice in the PBS control group (Fig 6.1C). Whilst not significant, a trend towards increased survival was also observed in mice treated with anti-HMGB1 pAbs one hour after LPS administration, with 30% not requiring euthanasia within the experimental period (Figure 6.1 B). This lower survival rate was evidenced by an increase in the mean clinical score compared to those that received prophylactic administration of pAbs (Figure 6.1 D). These results suggest that ovine pAb directed against HMGB1 can increase survival from acute and rapid systemic inflammation caused by administration of a bolus of LPS in this very lethal and artificial model of sepsis.

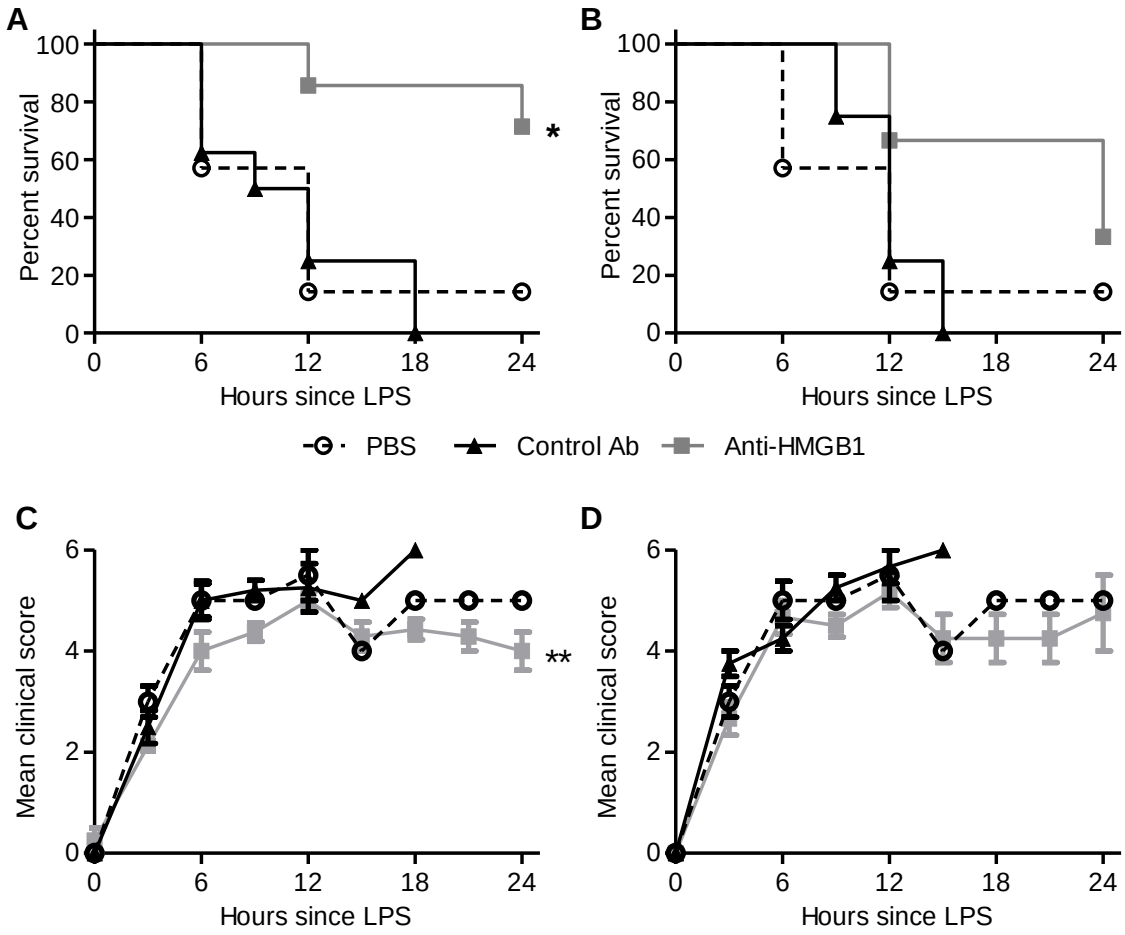


Figure 6.1 Ovine anti-HMGB1 pAb therapy improves survival in an *in vivo* model of murine endotoxaemia

Mice (n = 6-8) were administered 20mg/kg anti-HMGB1 pAb via IP injection one hour before (A & C) or after (B & D) IP injection of 20 mg/kg LPS. Control mice were administered ovine pAb (n = 4-6) before or after LPS administration or PBS (n = 7). Experimental mice were monitored over twenty-four hours for clinical score and euthanised if predetermined humane endpoints were reached. Survival (A-B) and mean clinical score (C-D) of groups is represented. Survival curves were compared to controls using the Mantel-Cox test and clinical score data compared to PBS control via two-way ANOVA, with significance denoted as: * = P>0.05, ** = P>0.01.

6.2.2. Therapeutic administration of ovine anti-HMGB1 pAb improves survival from CLP-induced sepsis.

While sudden endotoxaemia is a useful model for acute systemic inflammation, sepsis induced by caecal ligation and puncture (CLP) is considered the gold-standard rodent sepsis model (35). The perforated portion of the caecum forms a necrotic mass within the peritoneal cavity which intermittently challenges the body with bacteria and results in a prolonged period of inflammatory cytokine release. Together this more accurately mimics human sepsis which is generally caused by a central focus of infection. A CLP protocol that generated 60-70% mortality was developed to assess whether elevated HMGB1 levels were also associated with illness in this model of sepsis. Analysis of serum obtained from mice subjected to CLP or sham surgery indicated that the CLP procedure significantly elevated circulating HMGB1 levels over that of controls at 24hrs, with levels declining (Figure 6.2 A). Groups of mice administered PBS or control pAb eight hours following surgery displayed symptoms of ill health such as hunched posture, ruffled fur, a reluctance to move and weight loss in the first four days of sepsis, symptoms which were not exhibited by mice subjected to sham surgery (Figure 6.2 B). Mice treated with anti-HMGB1 pAbs eight hours after CLP surgery exhibited consistently lower mean clinical scores compared to PBS-treated mice, and surviving anti-HMGB1 treated mice improved in clinical condition from days 5-7. Overall weight loss was significantly less severe in anti-HMGB1 treated mice compared to control Ab treated mice (Figure 6.2 C), though not when compared to mice administered PBS. Overall, whilst therapeutic treatment with anti-HMGB1 pAbs only resulted in a mild reduction of clinical symptoms, it was enough to prevent mice from reaching humane endpoints resulting in a significantly increased rate of survival compared to untreated controls (Figure 6.2 D). These results indicate that HMGB1-neutralising pAb can be used to treat septic mice, and promote recovery from critical symptoms to increase survival.

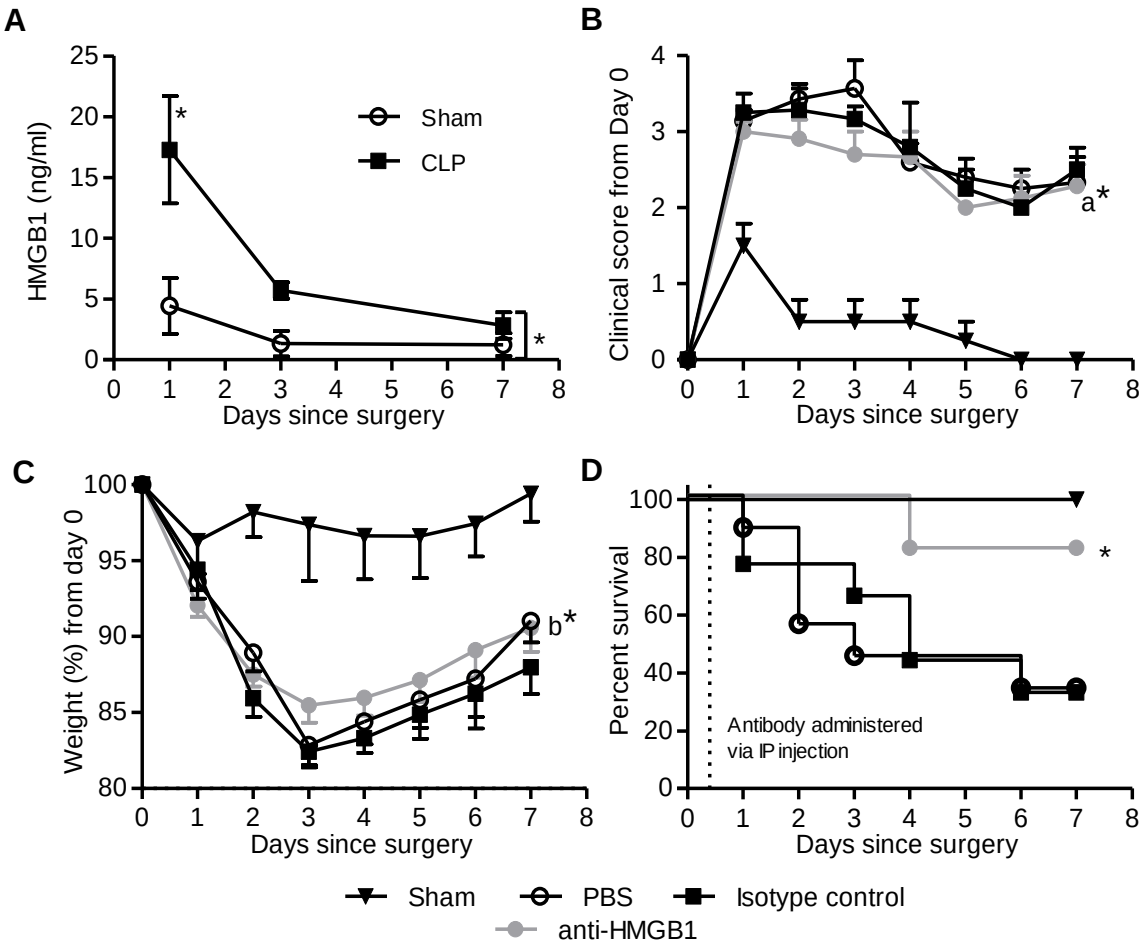


Figure 6.2 Therapeutic administration of ovine anti-HMGB1 pAb improves survival CLP-induced sepsis

Mice were subjected to sham surgery alone (n = 4) or CLP surgery (n = 4) and serum samples from sham or CLP mice were assayed for HMGB1 via ELISA (A). CLP mice (n = 9-11) were administered PBS, control antibody or anti-HMGB1 pAb (25 mg/kg) via IP injection eight hours following surgery. Mice were monitored and euthanised if predetermined humane endpoints were reached. Mean ± SEM of clinical score (B) and weight (C) and survival curves of the groups (D) are depicted. Sequential measurements were compared via two-way ANOVA and survival of anti-HMGB1 treated mice was compared to control groups using the Mantel Cox test with significance denoted as: * = P>0.05, a* = P>0.05 compared against PBS control, b* = P>0.05 compared against control Ab.

6.2.3. Ovine anti-HMGB1 pAb-treated mice display cytokine profiles associated with improved clinical outcomes in the early phase of CLP-induced sepsis

In order to determine the effect of HMGB1 neutralisation on the inflammatory profile in CLP-induced sepsis, serum samples were taken from mice on day 1, 3 and 7 post surgery and assessed for levels of pro- and anti-inflammatory cytokines via multiplex cytokine array, specifically IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IFNγ, GM-CSF and TNFα. Cytokine values for CLP groups were normalised to control mice that underwent 'sham' surgery at each time point (Figure 6.3 A). Sham control mice exhibited increases in IL-2, IL-5 and IL-10 on day 1 compared to later time points, likely the result of transient post-surgery inflammation caused by the performed laparotomy (Table 6.1). The CLP surgery caused an early twenty-fold increase in IL-6 and a 1.5-fold increase in TNFα over that of sham controls, with cytokine profiles tended towards normal as inflammation resolved over time. Treatment with anti-HMGB1 pAbs altered several individual cytokines 24 hours after CLP surgery. Most notably, comparison of cytokine levels at day 1 revealed statistically significant cytokine alterations, with a five-fold increase in IL-6 and a four-fold increase in IL-10 in anti-HMGB1 mice compared to the PBS control group. Additionally, levels of IL-2 IL-4, IL-12 and IL-13 at day 1 were diminished in anti-HMGB1 mice compared to controls although these differences did not reach statistical significance. Different patterns of cytokine release over time were apparent in anti-HMGB1 treated mice compared to control Ab and PBS treated mice. In particular, a delayed IL-12 response was associated with treatment, where anti-HMGB1 mice had peak IL-12 on day 3 compared to day 1 in controls. This delayed pattern was also observed for proinflammatory cytokine IL-2 and anti-inflammatory IL-4, indicating that HMGB1 blockade could mediate downstream effects several days after peak HMGB1 levels on day 1. Proinflammatory:anti-inflammatory composite cytokine scores (CCS) were calculated for the panel of cytokines tested and ratios were not significantly altered in anti-HMGB1 treated mice compared to controls (Figure 6.3 B). While cytokine profiles in sepsis are highly complex, these data suggest that the application of anti-HMGB1 pAb alters the early-phase inflammatory response in moderate severity sepsis generated by CLP, which ultimately improves survival.

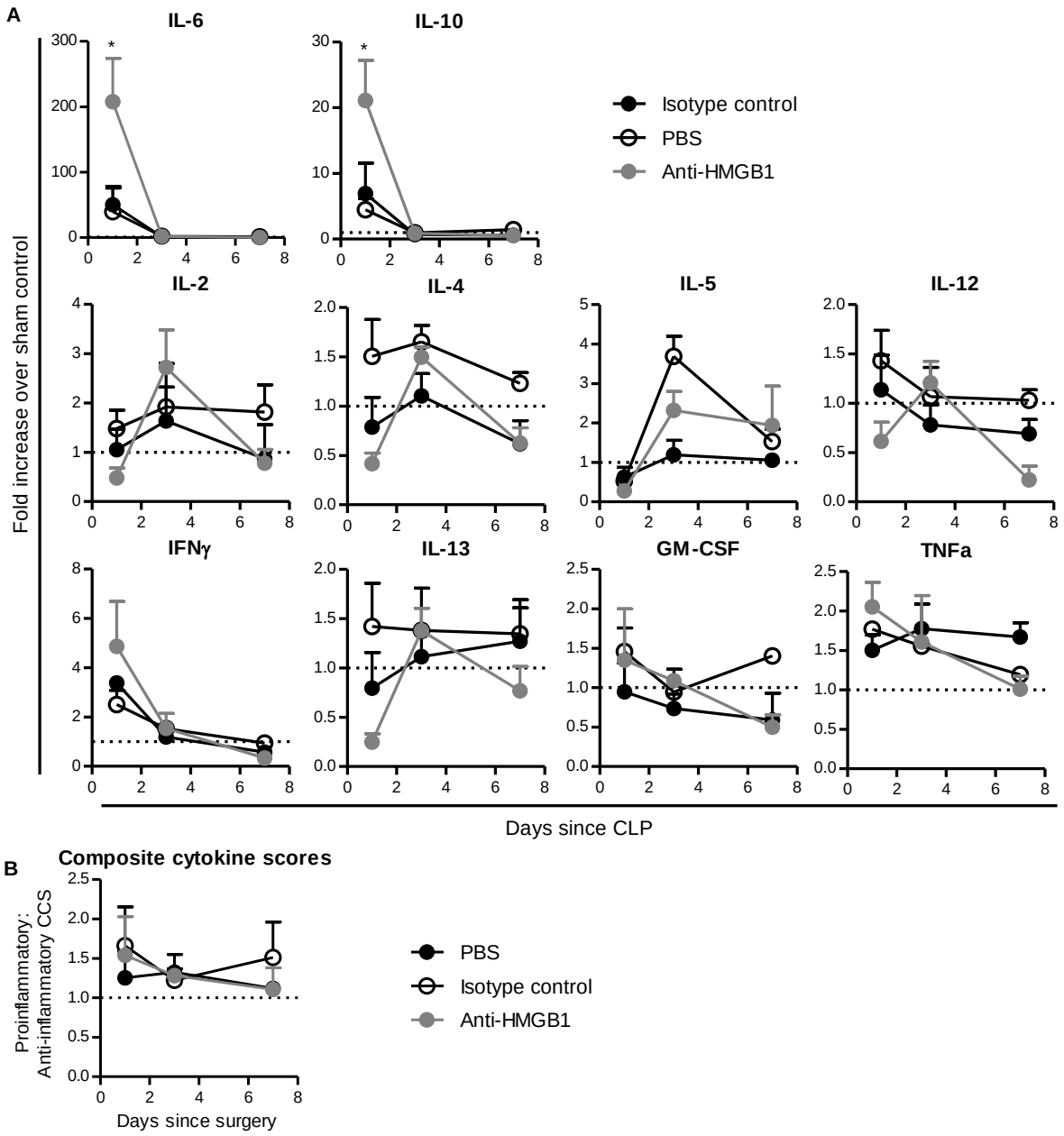


Figure 6.3 Ovine anti-HMGB1 pAb-treated mice display cytokine profiles associated with improved clinical outcomes in the early phase of CLP-induced sepsis

Blood samples from mice (n = 3-5) subjected to sham surgery or CLP-induced sepsis and treated with PBS (unfilled symbols), control pAb (25 mg/kg; black symbols) or anti-HMGB1 pAb (25 mg/kg; grey symbols) were analysed for cytokine content using a multiplex platform. Mean + SEM of individual cytokines normalised to the mean of sham surgery mice at each timepoint are depicted. (A). Proinflammatory (IL-2, IL-5, IL-6, IL-12, IFNγ, GM-CSF and TNFα) to anti-inflammatory (IL-4, IL-10 & IL-13) CCS were calculated for each sample, mean + SEM group CCS normalised to sham group are depicted in (B). Levels of cytokines were compared using two-tailed T-tests, with significance denoted as thus: * = P>0.05

Table 6.1 Cytokine values from mice that underwent sham-surgery

	Mean (SEM) pg/mL		
Measured Cytokine	**Day 1 (*n* = 3)**	**Day 3 (*n* = 4)**	**Day 7 (*n* = 5)**
IL-2	28.4 (7.15)	15.1 (3.26)	9.84 (2.44)
IL-4	18.2 (2.08)	14.4 (2.09)	10.4 (1.72)
IL-5	37.8 (9.63)	13.7 (3.86)	7.59 (0.638)
IL-6	61.15 (19.5)	100 (10.7)	58.4 (10.9)
IL-10	93.6 (17.3)	73.4 (17.3)	28.1 (3.10)
IL-12 (p70)	400 (113)	466 (87.0)	450 (53.9)
IL-13	176 (26.9)	176 (25.9)	144 (21.9)
GM-CSF	107 (24.8)	140 (10.6)	82.8 (13.8)
IFNγ	25.7 (8.41)	41.0 (16.8)	77.8 (13.7)
TNFα	3257 (159)	2687 (564)	3782 (603)

6.2.4. Ovine anti-HMGB1 pAb treatment during CLP-induced sepsis prevents susceptibility to secondary P. aeruginosa infection

While anti-HMGB1 pAb treatment increased survival from SIRS in a short term CLP-induced sepsis model, it was necessary to determine if treated mice exhibited reduced post-septic immunosuppression, indicative of CARS. To assess this, mice were subjected to a less severe one-puncture CLP surgery, which resulted in approximately 30% lethality without intervention, before intransasal challenge with live *P. aeruginosa*. Control mice subjected to initial sham surgery exhibited mild symptoms of infection, and transient weight loss after surgery and secondary bacterial challenge (Figure 6.4 A). Though one out of six mice succumbed to infection in this group, 80% did not require euthanasia (Figure 6.4 B). Septic mice treated with PBS eight hours following surgery exhibited sharp clinical decline after bacterial instillation, and the majority required euthanasia 12-48 hours after the challenge. Treatment with control pAb did not alter survival or weight loss compared to PBS-treated mice. In contrast, anti-HMGB1 treated mice exhibited significantly higher survival rates than either PBS- or control pAb-treated groups (Figure 6.4 B). Weight loss in this group was not significantly different from controls; anti-HMGB1 treated mice appeared to gain weight more rapidly after secondary bacterial infection, suggestive of a faster time to recovery (Figure 6.4 A). To assess the effect of anti-HMGB1 pAb on systemic cytokine profiles in this double-hit model, blood samples were collected immediately prior to challenge with *P. aeruginosa* on day 4, and then 24 hours later following secondary challenge by terminal bleed. The concentrations of IL-6, IL-10, IL-12 and TNFα in pre- and post-secondary challenge samples were evaluated by ELISA (Figure 6.4 C). Paired t-test analysis revealed no significant difference in IL-6, IL-10 or TNFα concentrations between pre- and post- challenge samples in either pAb treated group, however mice that received anti-HMGB1 pAb exhibited a significant increase in IL-12 serum concentration after challenge, a result not observed in control pAb treated mice. Analysis of the change in cytokine concentrations pre- and post-challenge between groups via two-way matched-pairs ANOVA revealed that secondary bacterial challenge had significantly different impacts on IL-6 and IL-12 concentration between the treatment groups. Taken together, these results suggest that anti-HMGB1 pAb treatment improves survival from secondary infection following CLP-induced sepsis, potentially via altering the immune cytokine response to secondary challenge.

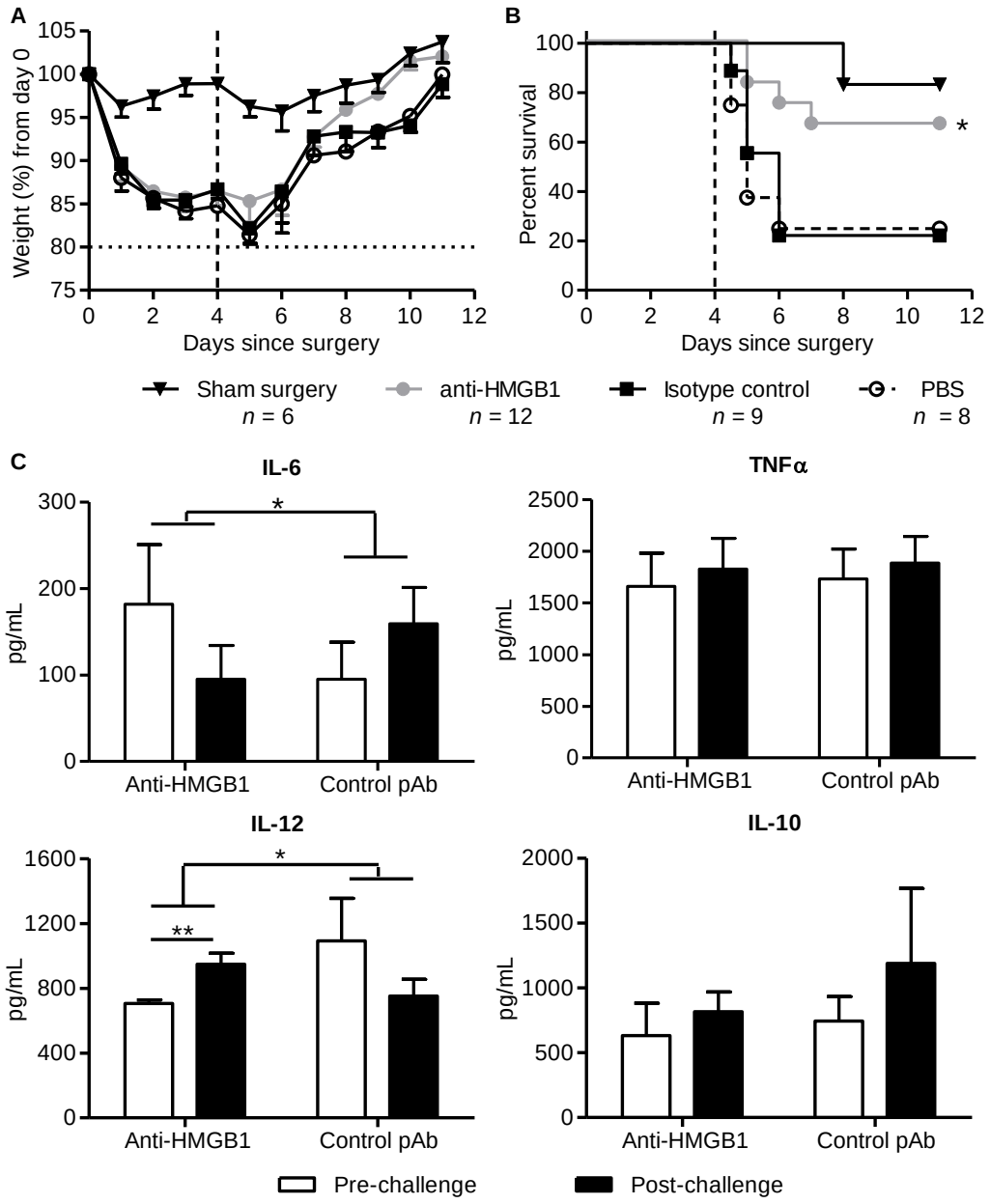


Figure 6.4 Prior anti-HMGB1 pAb treatment alters the inflammatory response and survival to secondary bacterial challenge in mice that have survived CLP-induced sepsis

Mice (n = 6-12) were subjected to sham surgery or mild CLP and allowed to recover before IN instillation of *P. aeruginosa* (2.5 x 10^7 CFU) or PBS on Day 4 (indicated by dashed line). Mice were treated 8 hours following surgery with anti-HMGB1 or control pAbs (20mg/kg) or PBS and administered maintenance pAb doses (10mg/kg) or PBS on days 3 and 6. Mice were monitored for disease symptoms and euthanised at predetermined humane endpoints. Mean - SEM weight loss (A) and survival (B) and of mice are depicted. Serum samples collected from mice (*n* = 4-5) immediately prior to bacterial challenge and 24 hours after challenge were analysed for IL-6, IL-10, IL-12 or TNFα content via ELISA (C). Survival curves were compared via Mantel-Cox analysis, and changes in cytokines before and after treatment were compared via paired t-test. Cytokine changes between groups were compared via two-way matched pairs ANOVA, with significance denoted as thus: * = P<0.05, ** = P<0.01.

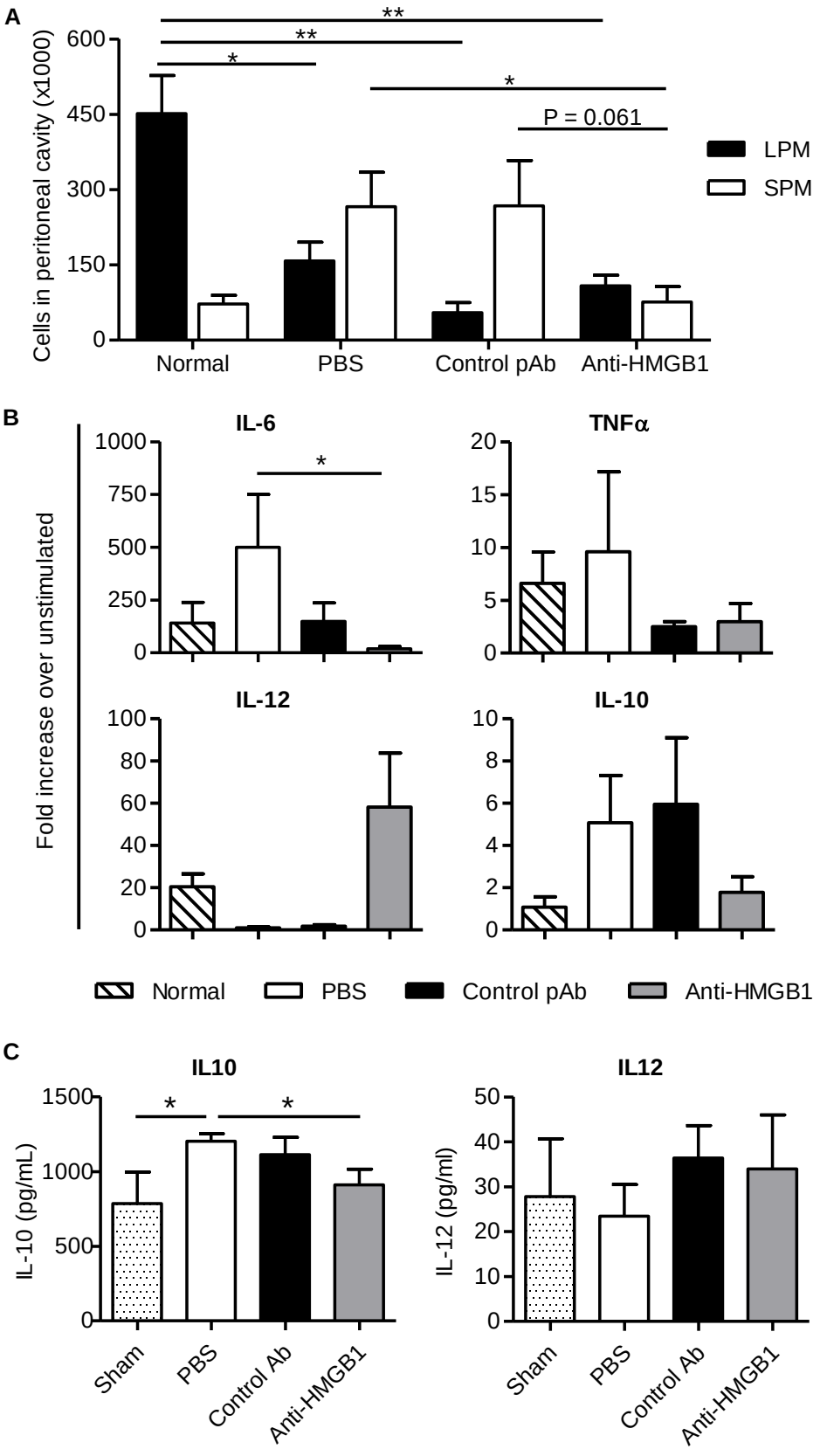
6.2.5. Anti-HMGB1 pAb treatment during CLP-induced sepsis alters macrophage and dendritic cell cytokine expression profiles in surviving mice

The increased susceptibility of sepsis survivors to secondary infection has been linked to an immunosuppressive CARS state that is triggered in response to excessive proinflammatory signalling. A number of mechanisms have been described that contribute to immunosuppression following murine sepsis, including functional exhaustion of lymphocytes, generalised immune cell apoptosis and epigenetic changes within immune cells, including macrophages [482, 483]. Previously, two distinct subsets of macrophages within the peritoneal compartment have been described with differential capacity for responding to stimuli [484]. Large peritoneal macrophages (LPM) are most abundant at resting state and perform roles in homeostasis and maintenance. Small peritoneal macrophages (SPM) are normally in low abundance but rapidly accumulate in the peritoneum following an inflammatory stimulus, such as endotoxin. Recent studies have implicated SPM in conferring inflammatory function in disease states [485, 486]. To investigate the effect of anti-HMGB1 pAb treatment in altering peritoneal macrophage function in CLP-induced sepsis, mice subjected to one-puncture CLP and treated with control pAb, anti-HMGB1 pAb or PBS were humanely killed and peritoneal cells harvested by lavage. Flow cytometric analysis using CD11b-, F4/80- and Gr-1-specific antibodies was performed to determine the frequency of LPM ($CD11b^{hi}$, $F4/80^{hi}$, $Gr\text{-}1^{+}$) and SPM ($CD11b^{lo}$, $F4/80^{lo}$, $Gr\text{-}1^{-}$) in the peritoneal compartment (Figure 6.5 A). The frequency of LPM was highest in normal mice not subjected to surgery, with all CLP mice regardless of treatment exhibiting significantly lower total numbers of LPM. An increase in SPM numbers was observed in CLP mice treated with PBS or control pAb, however anti-HMGB1 pAb treated mice failed to exhibit this same increase, with numbers of SPM remaining equivalent to that of normal mice. In order to determine if the macrophage population within the peritoneal compartment had altered responses to a pathogenic stimulus, adherent peritoneal monocytes and macrophages from septic mice were stimulated *in vitro* with LPS and cytokine release measured by ELISA. Anti-HMGB1 pAb treatment was associated with altered cytokine responses (Figure 6.5 B), most notably, peritoneal cells from anti-HMGB1 pAb treated mice secreted less IL-6 and more IL-12 in response to LPS than those harvested from PBS or control pAb treated mice. While reduced IL-10 secretion was observed in the anti-HMGB1 pAb treatment group this was not statistically significant. Secretion of TNFα was not significantly different between sham surgery mice and CLP-induced sepsis mice in any group.

Susceptibility to *P. aeruginosa* following CLP-induced sepsis has been linked to dysregulated immune cytokine signalling by dendritic cells (DCs), where reduced IL-12 and increased IL-10 is secreted in response to infection in immunosuppressed individuals. In order to determine if anti-HMGB1 pAb treatment impacted the inflammatory response generated following infection, bone marrow derived dendritic cells (BMDCs) were generated from bone marrows harvested from sham surgery, PBS treated, control pAb treated or anti-HMGB1 treated mice four days after CLP surgery. After 7 days culture in the presence of GM-CSF, semi-adherent BMDCs were harvested and stimulated with LPS and supernatants were collected after 18 hours and assayed for IL-10 and IL-12 content. It was found that BMDC generated from anti-HMGB1 mice secreted significantly less IL-10 in response to LPS stimulation than those from PBS-treated CLP mice (Figure 6.5 C). Secretion of IL-12 was not significantly different for any treatment group. These results suggest that anti-HMGB1 treatment alters DC function and the post-septic inflammatory response which may contribute to reduced susceptibility to *P. aeruginosa* challenge following survival from sepsis.

Figure 6.5 Anti-HMGB1 pAb treatment alters macrophage and DC cytokine expression post survival from CLP-induced sepsis

Peritoneal cells were harvested four days following CLP surgery and treatment with either PBS (n = 4), control ovine pAb (n = 4) or anti-HMGB1 pAb (n = 5) and evaluated via flow cytometry. Frequency of large peritoneal macrophages (LPM; $CD11b^{hi}$, $F4/80^{hi}$, $Gr\text{-}1^{+}$) and small peritoneal macrophages (SPM; $CD11b^{lo}$, $F4/80^{lo}$, $Gr\text{-}1^{-}$) compared to normal mice (n =2) are expressed (A; Mean + SEM). Adherent macrophages from peritoneal lavages were stimulated with LPS (100 ng/mL) for 18 hours and IL-6, IL-10, IL-12 and TNFα content in the supernatant was evaluated via ELISA. Mean + SEM expression for each treatment relative to unstimulated wells is expressed (B). Bone marrow cells harvested from Day 4 septic or sham surgery mice were cultured in the presence of GM-CSF to generate bone-marrow derived dendritic cells (BMDCs). Harvested BMDC's were plated and cultured in the presence of LPS (100 ng/mL); Mean + SEM IL-10 and IL-12 cytokine content of resulting supernatants is expressed (C). Mean values of individual groups were compared via two-tailed t tests, with significance denoted as thus: ** $P < 0.01$, * $P < 0.05$.


A
Cells in peritoneal cavity (x1000)
600
450
300
150
0
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*
*
P = 0.061
LPM
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Normal
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Control pAb
Anti-HMGB1
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Fold increase over unstimulated
IL-6
1000
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250
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TNFα
20
15
10
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Normal
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Anti-HMGB1
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Anti-HMGB1

6.3 Discussion

Clinical and preclinical studies have implicated HMGB1 as a mediator of sepsis pathogenesis and mortality, and thus HMGB1 is a candidate for passive immune targeting in sepsis. Within this chapter, a role for ovine anti-HMGB1 pAbs for the treatment of sepsis has been explored using preclinical animal models. In a short-term model of murine endotoxaemia, anti-HMGB1 pAb administration increased survival and was most effective when administered 1 hour before LPS, however did not significantly increase survival when given 1 hour after LPS administration. This is likely due to the nature of the model itself where a lethal dose of LPS is administered as a single bolus to rapidly instigate inflammation and tissue damage over twenty four hours, and therefore does not accurately replicate clinical sepsis [325]. A previous study which utilised anti-HMGB1 antisera in a longer term endotoxaemia model required repeated prophylactic dosing of antisera to increase survival. When pAb treatment was given therapeutically, survival benefit was reduced in agreement with this current study [316]. However the endotoxaemia model was used here to provide proof-of-principle that ovine HMGB1-neutralising pAb can reduce lethality from inflammation.

The CLP-induced sepsis model employed in this study was able to recapitulate several of the hallmarks of clinical septic disease including sustained systemic inflammation and bacteraemia. Multiple innate signalling pathways are activated in this model by the presence of both polymicrobial infection and extensive abdominal necrosis, unlike endotoxaemia which is largely dependent on LPS signalling through TLR4. Consequently, LPS-resistant C3H/HeJ mice, which harbour a mutation in the TLR4 gene, do not succumb to endotoxaemia but are still susceptible to mortality following CLP surgery to induce sepsis [487]. Here, mice subjected to CLP surgery had significantly higher serum HMGB1 levels than sham surgery controls, and treatment with anti-HMGB1 pAb increased survival. Treated mice also had fewer clinical signs of disease and reduced weight loss than control groups, indicating that HMGB1 blockade effectively reduced the morbidity and mortality associated with CLP-induced sepsis, an outcome in agreement with previous studies [488, 489]. Analysis of cytokine profiles following treatment revealed significant elevations in circulating IL-6 and IL-10 in anti-HMGB1 pAb treated mice 24 hours following surgery, results which were also associated with survival in the septic shock patient cohort. Previous studies have demonstrated that IL-10 may be an important regulator of early inflammation and may potentially delay the onset of lethal septic shock [267]. In the CLP model, IL-10-deficient mice exhibited higher mortality with rescue surgery (removal of the ligated portion of the

caecum) only effective in reducing lethality when performed early after CLP-induced sepsis in comparison to wild type mice [490]. In the context of our findings, the observed increase in IL-10 in surviving patients and anti-HMGB1 pAb treated mice may be associated with modulating early proinflammatory pathways to reduce lethality. When later time points were examined, altered cytokine expression was also apparent for anti-inflammatory cytokines IL-4 and IL-13, and proinflammatory cytokines IL-2 and IL-12 which peaked later than in untreated controls. Taken together, these results suggest that anti-HMGB1 pAb treatment reduces SIRS in experimental sepsis via inhibiting uncontrolled proinflammatory signalling and supporting the induction of anti-inflammatory signals.

In the clinic, patients that survive the acute SIRS phase of sepsis are at increased risk of secondary infection as CARS reduces the capacity for protective immune responses to be generated. While anti-HMGB1 pAb therapy increased survival in simple endotoxaemia and short-term sepsis models, it was unknown whether this survival could potentiate immunosuppression and potentially result in increased mortality from secondary infection. To investigate this, a 'double-hit' model of CLP-induced sepsis followed by *P. aeruginosa* pneumonia was employed, where murine survivors of sepsis exhibit increased susceptibility to *Pseudomonas* challenge compared to those undergoing sham surgery [491]. Anti-HMGB1 pAb treated septic mice were less susceptible to secondary infection than control mice and exhibited a more rapid recovery. Additionally, serum cytokine expression following challenge was altered in anti-HMGB1 pAb treated mice, where an increase in IL-12 and a decrease in IL-6 was apparent.

As the CARS phase of sepsis and susceptibility to secondary infection has been linked to altered function of innate immune cell types [492, 493], macrophages in the peritoneal cavity from CLP mice were evaluated at day 4, when the 'second hit' was administered. Anti-HMGB1 pAb treated mice exhibited decreased numbers of SPMs within the peritoneal cavity, which may be a result of a less severe disease course as SPMs migrate to the peritoneal cavity in response to inflammatory challenge [484, 494]. Importantly, peritoneal macrophages from anti-HMGB1 pAb treated mice displayed differential cytokine responses after *in vitro* LPS stimulation, including significantly decreased IL-6 and increased IL-12 expression. Production of IL-12 has been strongly linked to susceptibility to *P. aeruginosa* challenge following CLP, as epigenetic programming of DCs results in decreased production of protective IL-12 [495], and increased production of IL-10. Stimulation of BMDC generated from anti-HMGB1 pAb treated CLP mice generated lower quantities of the anti-inflammatory cytokine IL-10, when compared to those generated from septic control mice, indicating that

the immunosuppressive phenotype of dendritic cells following survival from sepsis was altered by HMGB1 inhibition, however no such differences were observed for IL-12.

While the role of HMGB1 in the development of immunosuppression and immunoparalysis in sepsis is poorly understood, several potential mechanisms have been described that may contribute to the results observed in this study. The observed phenotype of peritoneal macrophages from septic mice likely represents a partial tolerance to LPS, as while IL-12 production was decreased and IL-10 production increased in response to *ex vivo* stimulation compared to macrophages from non-septic mice, no marked differences in TNFα or IL-6 were observed. Anti-HMGB1 pAb therapy following CLP reduced numbers of SPM which are often attributed with proinflammatory functions, however adherent peritoneal cells from anti-HMGB1 pAb treated mice exhibited increased expression of proinflammatory IL-12 and reduced IL-6 following stimulation. Initial exposure of macrophages to HMGB1 induces a proinflammatory response [305, 452, 496] however studies have also highlighted the contribution of HMGB1 to immunosuppressive macrophage function. It has been demonstrated that incubation of macrophages with HMGB1 *in vitro* reduces macrophage phagocytic capacity [497] and induces tolerance to LPS or lipoteichoic acid stimulation [498, 499]. Macrophage tolerance to LPS stimulation can also be induced by proinflammatory cytokines [500] and other TLR ligands and thus it has been postulated that *in vivo* tolerance is induced by cumulative signals through TLRs rather than a single pathway [501]. Consequently, the effect of anti-HMGB1 pAb treatment on the induction of macrophage LPS-induced tolerance may be mediated by both direct blockade of HMGB1 signalling on macrophages and by an altered inflammatory milieu.

Impaired DC function is considered central to the development of increased susceptibility to secondary infection following sepsis [502], as animal models have demonstrated that administration of normal ‘naïve’ DCs can reverse disease susceptibility in post-septic mice [503]. In the current study, anti-HMGB1 pAb treatment altered the response of DCs to LPS stimulation as BMDC generated from treated septic mice secreted less IL-10 after LPS stimulation than those derived from untreated septic mice. Previous studies have demonstrated that DCs secrete HMGB1 upon stimulation, which signals in an autocrine and paracrine manner to promote migration [504] and that HMGB1 is necessary for DC maturation. However, excessive HMGB1 release may contribute to abnormal maturation of DCs to promote Th2 responses, as demonstrated in a rat model of SIRS following thermal injury [505]. Sepsis results in DC apoptosis and repopulation by progenitors which exhibit modified gene expression, thus epigenetic mechanisms occurring in the bone marrow are

thought to contribute to post-septic immunosuppression [495]. Evidence suggests that the local environment within the bone marrow influences epigenetic regulation of DC [506], thus circulating inflammatory cytokines and HMGB1 that access the bone marrow via the circulation may impact on this process. Together, these results indicate that HMGB1 blockade can reduce the severity of SIRS and partially reverse the development of CARS in murine sepsis. However, it is unknown whether the major contribution of HMGB1 blockade in this model is to prevent direct immunosuppressive functions of HMGB1 [309] or to neutralise HMGB1 during SIRS and thereby reduce the cumulative proinflammatory signalling that drives CARS.

It has been demonstrated here, through the use of three preclinical models, that anti-HMGB1 pAb therapies are efficacious in experimental acute and chronic SIRS, do not promote immunosuppression following SIRS and are able to alter inflammatory profiles that are associated with clinical lethality. Though multiple immune-modulating therapies have been trialled in sepsis, few are able to provide significant clinical benefit over current treatment paradigms in placebo-controlled studies [444]. Combined therapies targeting multiple redundant immune pathways may have increased efficacy than a single treatment and therapies that target master immune regulators could serve to first reduce the severity of SIRS in early sepsis before targeting specific pro- or anti- inflammatory cytokines to reverse pathogenic immune signalling. As such, anti-HMGB1 pAb therapy may serve an important role in dampening the magnitude of signalling in SIRS or MARS sepsis and also leave patients more responsive to further immune-modulating approaches. The findings of the current study indicate that anti-HMGB1 blockade via ovine pAb administration may be a valid and efficacious approach to treating sepsis and septic shock and should be considered in conjunction with other immune modulating therapies to ultimately reduce the burden of disease and mortality from sepsis.

CHAPTER 7.

Development of an ovine sepsis model for the assessment of anti-HMGB1 pAb passive immunotherapy

7.1 Introduction

Anti-HMGB1 therapy has demonstrated efficacy in rodent sepsis models however it remains necessary to further examine the effect of therapy on clinically relevant parameters in a large animal model before translation into human studies. As discussed in Chapter 1, large animal models permit the measurement of haemodynamic and respiratory parameters and can also be modified to incorporate clinically-relevant treatment paradigms. This is particularly important for sepsis research, as the failure of the vast majority of sepsis therapies to provide benefits in clinical trials is often attributed to the pervasive use of inappropriate preclinical models [325]. An ideal preclinical sepsis model would incorporate relevant clinical endpoints to enable translation into humans. Indeed, clinical endpoints for randomised controlled trials in sepsis have been under scrutiny in recent years [447, 507] and the failure of multiple sepsis-specific therapies to generate meaningful differences in study endpoints highlights the need for the careful selection of endpoints at all stages of the process.

Ideal endpoint measures are not only robust and clinically meaningful but also sensitive enough to detect more subtle alterations in clinical course. Additionally, timespans of monitoring endpoints must be long enough to appropriately measure outcomes of the intervention, though as timespan is extended the effects of the intervention become more difficult to separate from other causes of mortality [507]. This is particularly true in clinical sepsis, where patients frequently suffer from co-morbidities [204, 208]. As the majority of sepsis related deaths occur within the first month, 28-day all-cause mortality has been the most common endpoint used in clinical trials [444, 507]. However, is has been speculated that a single therapeutic approach is unlikely to have a clinical effect strong enough to overcome the influence of other factors that contribute to all-cause mortality in the clinic [508]. Efforts to determine cause-specific mortality may simultaneously allow improved detection of therapeutic effects and identification of patient sub-populations most likely to benefit from the intervention. In preclinical studies, short-term mortality is used as an endpoint in severe sepsis; however for more moderate forms of sepsis, extending the duration to more accurately assess long-term survival is considered unethical. Thus, surrogate measures of disease morbidity are often employed as humane alternatives in these cases and therefore the validity of such endpoints being translated into clinical studies are debatable [509].

Mortality is unquestionably an important outcome to assess in the clinic, as the sepsis mortality rate remains as high as 30% in developed countries. However, as the overall

incidence of sepsis increases [510], the total number of sepsis survivors is also increasing potentially due to advances in diagnosis and intensive care support protocols. As sepsis is associated with the development of long-term physiological abnormalities, increased 5-year mortality rates and reduced quality of life [511, 512], interventions that reduce morbidity warrant further investigation. Measures of morbidity during ICU stay are increasingly useful, and frequently employed as secondary outcomes in randomised controlled trials [513]. Duration of the requirement for support therapies, such as ventilation or dialysis, are often used as indexes of morbidity. An ideal treatment would reduce both morbidity and mortality, though as treatments may have divergent effects, it is important both morbidity and mortality are effectively assessed in both clinical studies and preclinical sepsis models.

Sheep are generally selected for large animal sepsis models due to their size, ease of handling and haemodynamic similarity to humans. It has been demonstrated that sheep are comparable to humans for the majority of common coagulation tests [514], and exhibit similar metabolic and haemodynamic changes in response to sepsis [515]. Recently, ovine sepsis with intensive care supports (OSICS) has been used as a model to investigate renal pathology [348] and thyroid hormone therapy [347] in sepsis. Within the OSICS model, the development of shock indicated by the need for vasopressor therapy to maintain adequate blood pressure was used as a primary endpoint, with other morbidity measures employed as secondary endpoints. As the duration of sepsis in the OSICS model was short (26 hours), morbidity endpoints were used in favour of mortality, however an extended experimental period may allow reliable data to be generated regarding short-term mortality in sepsis.

To determine suitability of HMGB1-blockade therapy in ovine sepsis, HMGB1 levels were first assessed in banked samples from septic and non-septic sheep, which demonstrated that plasma HMGB1 elevation observed in the OSICS model was comparable to the levels observed in human sepsis. In order to assess how the OSICS model may be employed for the evaluation of anti-HMGB1 pAb therapy in sepsis, preliminary studies were performed on six sheep and compared to previously collected data from sheep administered placebo (normal saline) in a study evaluating the effect of thyroid hormone therapy in sepsis [347]. Sheep were administered *E. coli* via intravenous (IV) infusion to generate a sepsis and subsequently septic shock identified by reduced blood pressure and vascular resistance, leucocytosis and coagulative and metabolic acidosis characteristic of the SIRS phase of sepsis. Anti-HMGB1 pAbs were administered to three sheep in this study two hours following bacterial challenge. Sheep treated with anti-HMGB1 did not exhibit improved haemodynamics, coagulation or metabolic abnormalities compared to control animals, and had an increased tendency towards

the development of acidosis than control sheep. These results indicate that a more mild sepsis phenotype with a longer duration may be necessary to ascertain the true physiological intervention outcomes of anti-HMGB1 pAb therapy in this model.

7.2 Results

7.2.1. Ovine sepsis induces significant elevation of plasma HMGB1

Previous findings indicated that anti-HMGB1 pAb therapy is efficacious in reducing mortality in short-term murine endotoxaemia, moderate CLP-induced sepsis and CLP-induced sepsis followed by bacterial challenge; nevertheless further analysis of the impact of anti-HMGB1 therapy on the physiological manifestations of sepsis are required. Recently, the ovine sepsis model has been adapted with clinically-relevant supportive care protocols and used for the study of renal pathology and thyroid hormone therapy in sepsis and septic shock [347, 348], however the utility of this model for studying the physiologic effects of immune-modulating therapies has not been thoroughly examined. In support of the use of an ovine model to recapitulate clinical sepsis, previous studies have uncovered high degrees of similarity between ovine and human coagulation [514], haemodynamics [516] and renal function [517] and recent genetic analyses of ovine and human leucocytes stimulated *in vitro* with TLR agonists revealed similar expression patterns of inflammation-related genes [518]. In order to establish whether the ovine sepsis model is amenable to the study of anti-HMGB1 therapy in sepsis, it was necessary to determine whether HMGB1 release kinetics were similar between sheep and humans. To achieve this, banked plasma samples from previous OSICS subjects were obtained and analysed for HMGB1 content by ELISA.

Analysis of results showed that elevated HMGB1was detectable post *E. coli* infusion, with a rapid increase in levels observed from 12 hours (Figure 7.1 A). Non-septic animals (no *E. coli* administered) did not exhibit elevations in HMGB1 over time. Significantly, plasma HMGB1 levels at 26 hours were three-fold higher in septic sheep than non-septic sheep, or those measured at the onset of sepsis (Figure 7.1 B). Septic sheep exhibited HMGB1 levels between 2-6 ng/mL, which was comparable to patient plasma HMGB1 within the first 24 hours post ICU admission (1-10 ng/mL; Chapter 5). Levels at 26 hours were used to generate a receiver-operator characteristic (ROC) curve to define the HMGB1 concentration indicative of sepsis within this model. Based on these data, a HMGB1 concentration greater than 1.252 ng/mL would distinguish septic from non-septic sheep, with 100% sensitivity (95% confidence interval 39.8-100%) and 100% specificity (95% confidence interval 29.2-100%). These results support the use of the ovine sepsis model for the study of anti-HMGB1 therapy in sepsis.

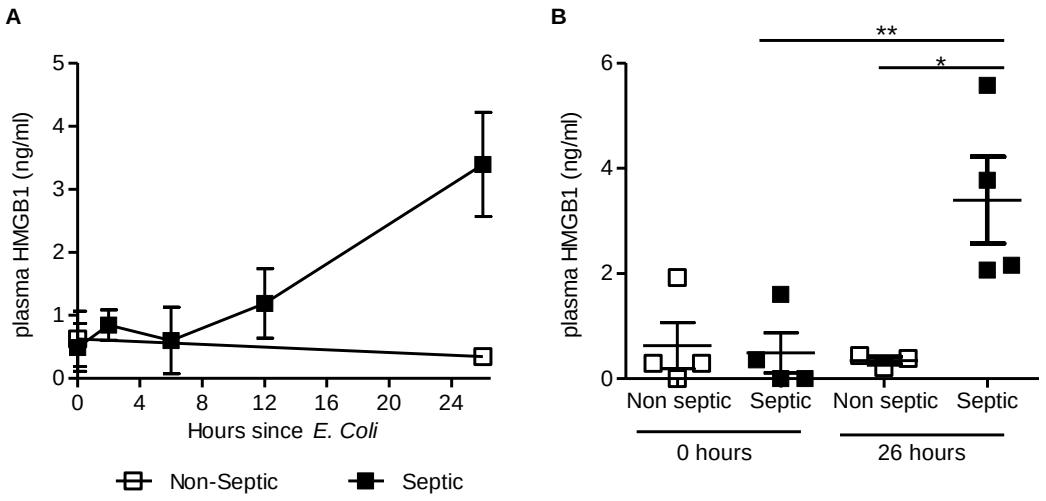


Figure 7.1 Significant elevation of plasma HMGB1 is detectable at 26 hours in septic sheep

Kinetic plasma samples from septic (n = 4) and non-septic (n = 4) sheep from previous studies were assayed for HMGB1 content via ELISA. Mean ± SEM of plasma HMGB1 over time (A) and comparison between individual time points (B) via two-tailed student's t-test are represented, with significance denoted as thus: * P = <0.05, ** P = <0.01.

7.2.2. Anti-HMGB1 pAb therapy did not reduce the development of shock or requirement for noradrenaline in an ovine sepsis model

Hypoperfusion accompanying sepsis is a hallmark of septic shock which contributes to tissue hypoxia, organ damage and death. The requirement for vasopressor therapy in the form of noradrenaline (NorA) to maintain adequate blood pressure has previously been used as the primary outcome measure in the OSICS model. Within this study, sepsis was generated in six sheep and compared to data from previous studies utilising the same protocol. Characteristics of study sheep and outcomes are depicted in Table 7.1. Initially, two sheep (#1 and #2) were administered the same *E. coli* dose as used previously, however the sheep required little or no NorA to maintain blood pressure and did not progress to prolonged septic shock. In order to replicate a degree of sickness severity observed in previous studies, an increased *E. coli* dose was employed for the remaining sheep within the study (#3–6). Three sheep were administered anti-HMGB1 pAbs (10 mg/kg; sheep 4-6) two hours after the induction of sepsis.

Haemodynamic parameters and NorA infusion were evaluated in sheep for 1 hour before the induction of sepsis via IV infusion of live *E. coli* and for 26 hours thereafter (Figure 7.2). Mean arterial pressure (MAP) was continuously monitored and used to determine the required dose of NorA, with a target MAP of 75 mmHg (Materials & Methods, Table 2.5). Placebo-treated sheep from previous studies (n =8; hereafter referred to as historical data) displayed a rapid decrease in MAP within 2 hours of bacterial infusion before increasing to a stable level within 4 hours of infusion (Figure 7.2 A). This was accompanied by a similar decrease and gradual increase in systemic vascular resistance index (SVRI; Figure 7.2 B), which may be indicative of rapid vasodilation in response to bolus bacterial challenge. Between 6-8 hours after infusion, MAP and SVRI gradually declined until a MAP <75 mmHg was achieved and NorA therapy was initiated (Figure 7.2 C). Two sheep within the current study were administered 1 x 10^8 CFU/kg *E. coli* and these sheep displayed an overall reduction in MAP accompanied by a markedly reduced need for NorA compared to historical data. One sheep with an increased bacterial dose (1.5 x 10^8 CFU/kg) exhibited consistently low MAP and SVRI and had high NorA requirements.

Infusion of anti-HMGB1 pAbs two hours post-challenge in three sheep that received the increased bacterial dose of 1.5 x 10^8 CFU/kg did not appear to impact on NorA requirements compared to the equivalently-dosed control sheep, however cumulative NorA dosages are not comparable within this cohort as one sheep died 12 hours after challenge. Area under the

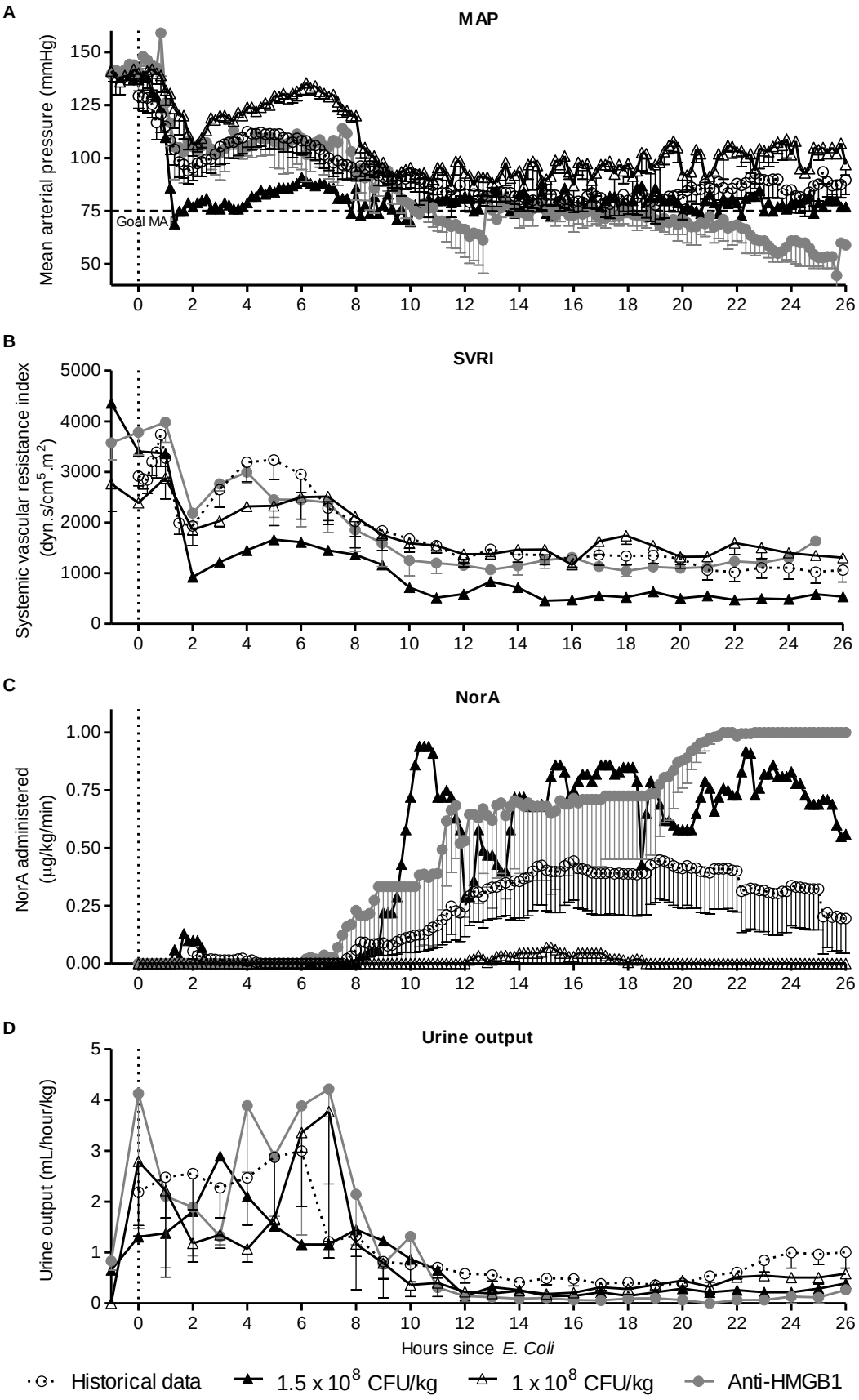
curve (AUC) of mean NorA dosages over time (Table 7.1) is a more accurate measure of NorA requirement within the groups considering variable survival time, and indicates that total NorA infusion was marginally greater in the anti-HMGB1 group. Urine output (Figure 7.2 D) in the OSICS model appeared consistent between historical data and the current study. Measured urine output was between 1-4 mL/hour/kg and subsequently fell to less than 1mL/hour/kg at 8 hours post-challenge which coincided with the reduction in MAP and SVRI and the initiation of NorA therapy.

Table 7.1 Characteristics of sheep and survival outcomes within the study as compared to historical data

Sheep	**Weight**	***E. coli* dose**	**Treatment**	**Survival time**	**Cumulative NorA administered**	**NorA dose rate mean AUC**
1	68 kg	1.08×10^8 CFU/kg	Placebo (normal saline)	26 hours	26.6 μg/kg	13.3 μg/kg
2	74 kg	0.94×10^8 CFU/kg	Placebo (normal saline)	26 hours	0 μg/kg	
3	69 kg	1.47×10^8 CFU/kg	Placebo (normal saline)	26 hours	718.5 μg/kg	715 μg/kg
4	58 kg	1.62×10^8 CFU/kg	10 mg/kg anti-HMGB1 pAbs	26 hours	556.2 μg/kg	794 μg/kg
5	71 kg	1.50×10^8 CFU/kg	10 mg/kg anti-HMGB1 pAbs	12 hours	234.4 μg/kg	
6	76 kg	1.58×10^8 CFU/kg	10 mg/kg anti-HMGB1 pAbs	25 hours	869.4 μg/kg	
Historical data (n = 8; mean ±SD)	63 ± 8 kg	$1.08 \pm 0.5 \times 10^8$ CFU/kg	Placebo (normal saline)	25 ± 2.1 hours	330 ± 403 μg/kg	340 μg/kg

Figure 7.2 Measured haemodynamic parameters in an intensive care support ovine sepsis model

Sheep were anesthetised and catheterised to enable measurement of vascular pressure and urine output. After one hour, sepsis was induced (indicated by dotted vertical line) by the IV infusion of 1 x 10^8 CFU/kg *E. coli* (*n* = 2), or 1.5 x 10^8 CFU/kg *E. coli* with no treatment (*n* = 1) or 10mg/kg anti-HMGB1 pAb (*n* = 3). Mean arterial pressure (MAP; A) and noradrenaline (NorA) requirements (C) were recorded at 10 minute intervals while systemic vascular resistance indexed to body surface area (SVRI; B) and urine output (D) were recorded hourly. Data represented as mean – SEM of measurements for each group with historical data from placebo-administered sheep (*n* = 8) included for comparison.


A
MAP
Mean arterial pressure (mmHg)
150
125
100
75
50
Goal MA
0
2
4
6
8
10
12
14
16
18
20
22
24
26
B
SVRI
Systemic vascular resistance index
(dyn.s/cm5.m2)
5000
4000
3000
2000
1000
0
C
NorA
NorA administered
(µg/kg/min)
1.00
0.75
0.50
0.25
0.00
D
Urine output
Urine output (mL/hour/kg)
5
4
3
2
1
0
Hours since E. Coli
Historical data
1.5 x 10^8 CFU/kg
1 x 10^8 CFU/kg
Anti-HMGB1

7.2.3. Administration of anti-HMGB1 pAb did not alter HMGB1 levels in septic sheep plasma

The administered dose of an immune-modulating therapy undoubtedly impacts its function, and for Ab based therapies the dose rate also impacts the clearance, accessibility to target antigens and observed off-target effects [519]. In this preclinical model, a dose rate of 10mg/kg anti-HMGB1 pAb was selected based on doses used in murine studies, and known concentrations of HMGB1 present within the circulation of septic sheep. In order to establish whether sufficient anti-HMGB1 pAb was administered in this study to observe effects, kinetic serum samples from the three Ab dosed sheep were subjected to specific ELISA to detect anti-HMGB1 ovine IgG (Figure 7.3 A) and to commercial human HMGB1 ELISA for circulating HMGB1 (Figure 7.3 B). The administered pAbs were detectable two hours following Ab infusion at a mean concentration of 18 μg/mL (18 mg/L), and decreased thereafter. One sheep died 11 hours following *E. coli* infusion and thus measurements at later timepoints are the mean values between the two remaining sheep. These sheep exhibited vastly different kinetics of pAb clearance, as the detected concentration decreased rapidly in one sheep and remained constant in another. These differences may be due to haemodynamic variability in the sheep following the induction of sepsis.

Homology of HMGB1 between humans and sheep is predicted to be over 98% [520] thus a commercial ELISA manufactured for measurement of human HMGB1 was able to measure plasma HMGB1 in septic sheep. Measured HMGB1 levels in septic sheep within this study were higher than those observed in historical samples, however this may be attributed to sample storage, as samples from previous studies were stored >24 months prior to assay. Levels of HMGB1 in sheep that received a lower *E. coli* dose were lower than that observed in sheep that received an increased dose. While measured HMGB1 were lower in anti-HMGB1 pAb treated sheep than the same-dose control at earlier timepoints, after 12 hours HMGB1 levels were equivalent. Importantly, measured HMGB1 levels did not exceed 15 ng/mL, and thus in treated sheep pAb was circulating at 1000-fold higher concentration than HMGB1, which represents a 180-fold molar excess.

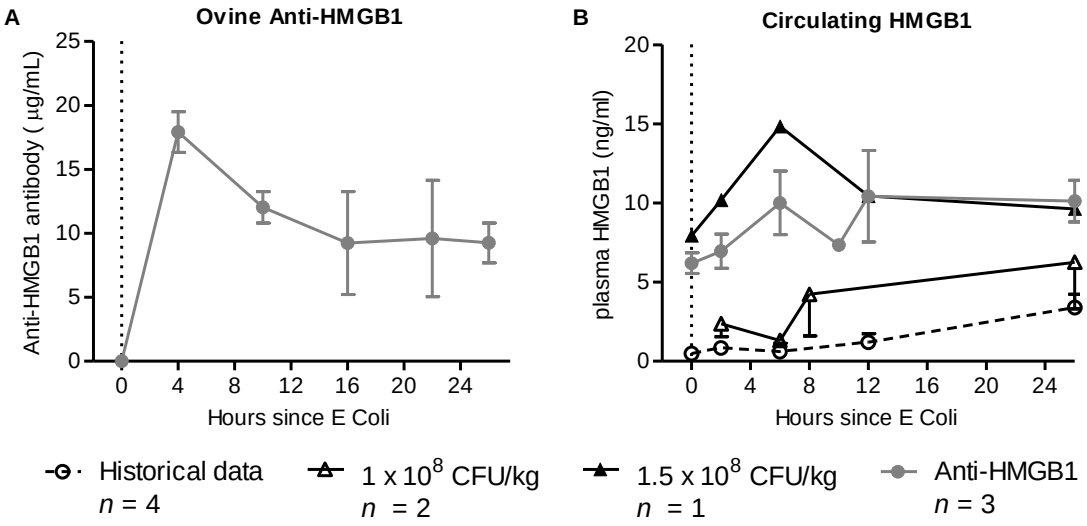


Figure 7.3 Kinetics of circulating anti-HMGB1 pAb and impact on plasma HMGB1 in the OSICS model

Sheep were anesthetised and sepsis was induced (indicated by dotted vertical line) by the IV infusion of 1 x 10^8 CFU/kg *E. coli* (n = 2), or 1.5 x 10^8 CFU/kg *E. coli* with no treatment (n = 1) or 10mg/kg anti-HMGB1 pAb (n = 3) administered two hours following bacterial challenge. Levels of circulating ovine anti-HMGB1 pAb (A) and HMGB1 (B) were determined by ELISA analysis.

7.2.4. Ovine sepsis is associated with the formation of progressive anaemia and leucocytosis

Multiple haematological parameters are monitored during sepsis management as alterations in cellular component of the blood are useful diagnostic and prognostic indicators [212]. In the clinic, septic patients often develop anaemia progressively throughout the course of illness. Persistent anaemia in chronic sepsis can also be the result of decreased production of red cell progenitors in the bone marrow, however in the short term alterations in vascular tone, oedema, inflammation and the formation of microthrombi all contribute to red blood cell (RBC) membrane changes and subsequent destruction [521]. Excessive blood loss may also precipitate anaemia, and in severe cases this is treated in the clinic with transfusion. The development of anaemia reduces the oxygen carrying capacity of the blood and thus contributes to organ damage and mortality in sepsis [522]. Infection and sepsis are both associated with a phase of profound leucocytosis, however in the later immunosuppressive phase of sepsis, white blood cell (WBC) count may be decreased to below normal ranges. As such, WBC count changes over time are usually considered in relation to the duration of sepsis within the patient or in conjunction with other markers as prognostic indicators [523].

In the clinic, the first hours of sepsis following initiation of infection are rarely recorded, as sepsis is identified in patients only after the development of SIRS symptoms. However, experimental sepsis allows these very early time-points to be recorded. Here, sheep were monitored for 26 hours following IV *E. coli* challenge which corresponds to the early phase of sepsis. Historical data indicated that ovine sepsis was associated with the progressive decline in packed cell volume (PCV) and haemoglobin (Figure 7.4 A & 7.4 B), however as decreases were mild in severity and did not decrease below baseline (0 hours post challenge), these observations may not be indicative of the presence of clinical anaemia. Additional pre-operative samples were taken within the current study which showed increased PCV and haemoglobin compared to baseline samples, which is likely the result of haemodilution following fluid administration during preparation of the sheep. Measured haemoglobin and PCV sharply increased in all groups 2 hours post challenge and declined similarly for all groups for the duration of the study. While the study duration was short, a trend towards the development of anaemia was observed; however further analysis of blood plasma is required to distinguish between true anaemia and haemodilution. The development of anaemia, evidenced by reduced PCV and haemoglobin concentration, appeared less pronounced in anti-HMGB1 treated sheep compared to untreated high and low *E. coli* dose groups.

Conversely to RBC data, leucocyte counts increased from pre-operative to baseline time points, which may be due to transient inflammation caused by surgical placement of catheters or the administration of isoflurane during surgery. Kinetic analysis of WBC counts during sepsis revealed an 80% decrease in WBC counts 2 hours post challenge (Figure 7.4 C) followed by gradual increase over the duration of sepsis. Differential analyses of WBC types in the current study revealed that this decrease was due to marked depletion of neutrophils, which decreased by 98% from baseline values (Figure 7.4 D), whereas lymphocytes (Figure 7.4 E) and other granulocytes (data not shown) did not exhibit marked changes throughout the septic period.

Interestingly, the development of leucocytosis after 12 hours appeared to have an inverse relationship with sepsis severity, as the low *E. coli* dose sheep exhibited the greatest increase in total WBC and neutrophil counts compared to pre-operative samples. Treated and untreated high *E. coli* dose sheep had the highest NorA requirements yet only exhibited mild increases in WBC and neutrophil counts comparable to pre-operative counts.

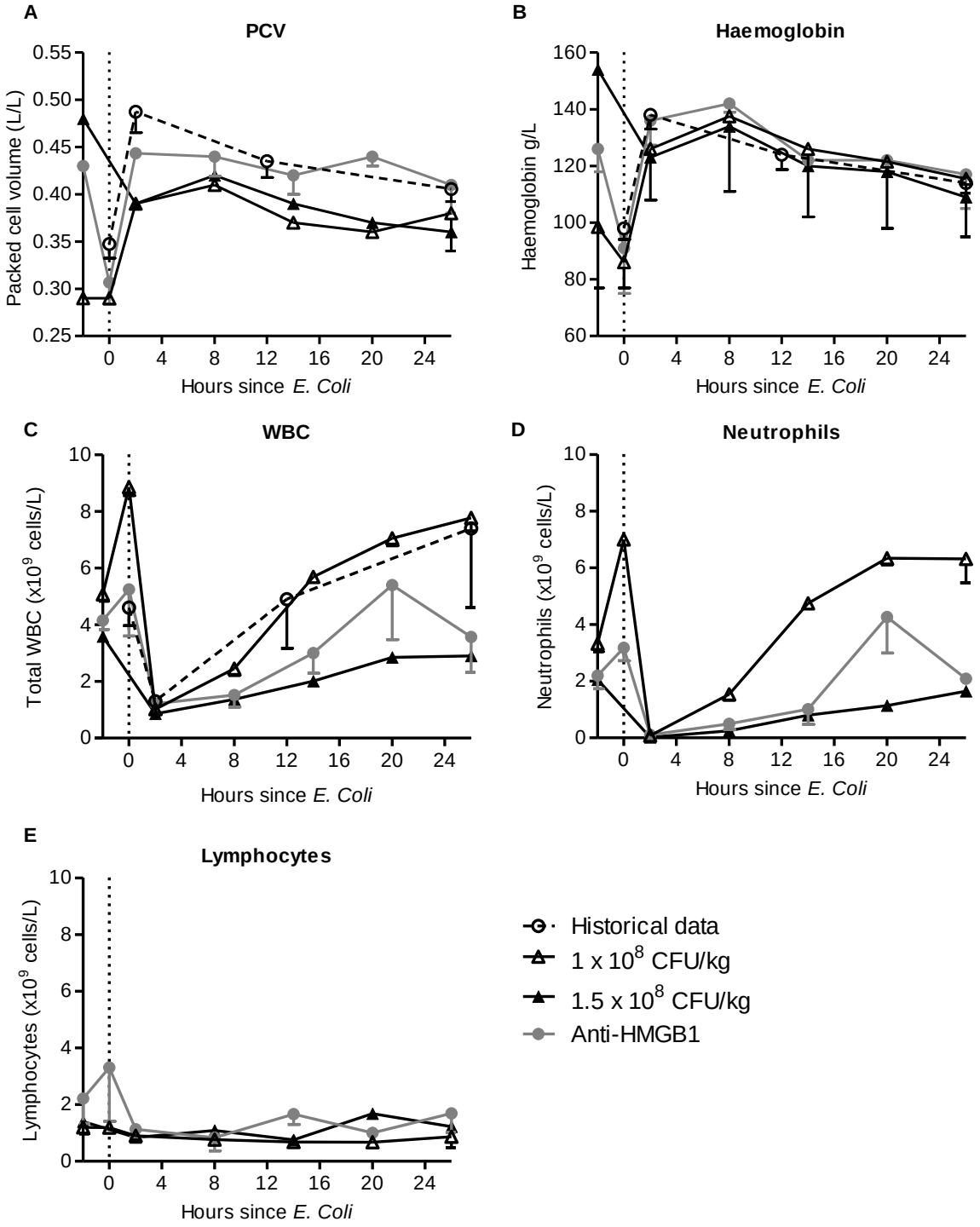


Figure 7.4 Alterations in erythrocytes and leucocytes in the OSICS model

Sheep were administered 1.5 x10^8 CFU/mL *E. coli* (IV) to induce sepsis and either treated with anti-HMGB1 two hours post challenge (n = 3) or untreated (n = 1). Two sheep were administered a lower *E. coli* dose (1 x10^8 CFU/mL). Whole blood samples from septic sheep were subjected to full blood exam. Packed cell volume (PCV; A) and haemoglobin (B) were measured to assess the development of anaemia. Total white blood cell (WBC; C) counts and differential counts of neutrophils (D) and lymphocytes (E) are represented. Data represents mean – SEM of treatment groups.

7.2.5. Septic sheep exhibit marked coagulative deficiency

The processes of coagulation and inflammation are intricately linked, thus deregulated inflammation in sepsis is associated with varying degrees of coagulopathy [210]. Inflammatory mediators such as IL-6 interact with endothelial cells to increase expression of pro-coagulant tissue factor leading to downstream generation of thrombin [524]. The subsequent development of disseminated intravascular coagulation (DIC), characterised by formation of intravascular emboli and microthrombi, is a significant cause of organ damage as tissues are deprived of vital oxygen and nutrients. Persistent bleeding following DIC is also common, as the formation of clots deplete circulating stores of fibrinogen and other tissue factors. Clinically, such depletions are managed by infusion of fresh frozen plasma, cryoprecipitate or platelets to prevent blood loss and subsequent tissue hypoxia. In the absence of bleeding, heparin may also be administered for prevention of venous thrombi formation in an effort to stave off organ damage. Clinical monitoring of coagulative capacity is thus important for determining whether patients are at risk of blood loss or organ damage and to direct therapies. Previously, it has been demonstrated that sheep have similar coagulation kinetics to humans for the majority of common coagulation tests [514]. However, sheep have increased clot firmness and a reduced capacity for clot lysis than humans, which may indicate that sheep may be predisposed to increased organ damage following DIC in sepsis. Baseline data from normal sheep indicate a longer time to clot formation than humans in both prothrombin time (PT) and activated partial thromboplastin time (APTT) tests.

Within this study, coagulation tests were performed on serial blood samples from septic sheep administered either a low or high *E. coli* dose and compared to historical data. Administration of anti-HMGB1 pAbs was performed in three sheep that received a higher *E. coli* dose. Sheep that received the lower *E. coli* dose (1×10^8 CFU/kg) exhibited mild coagulopathy, with only a modest increase in PT (Figure 7.5 A) and APTT (Figure 7.5 B) from baseline values compared to historical data. Fibrinogen depletion was also mild in these sheep, indicating that there may be reduced tendency for intravascular clotting. Sheep that received a high *E. coli* dose exhibited increased time to clot formation and marked fibrinogen depletion. Interestingly, the three sheep treated with anti-HMGB1 pAbs exhibited more profound clotting dysfunction than an untreated sheep given the same *E. coli* dose, however these did not appear altered from historical data. As anti-HMGB1 pAb treated sheep also exhibited the highest mortality compared to other groups, these results support the notion of coagulation function tests as a marker of disease severity in ovine sepsis, however further investigation is required to determine if anti-HMGB1 therapy can promote clotting dysfunction.

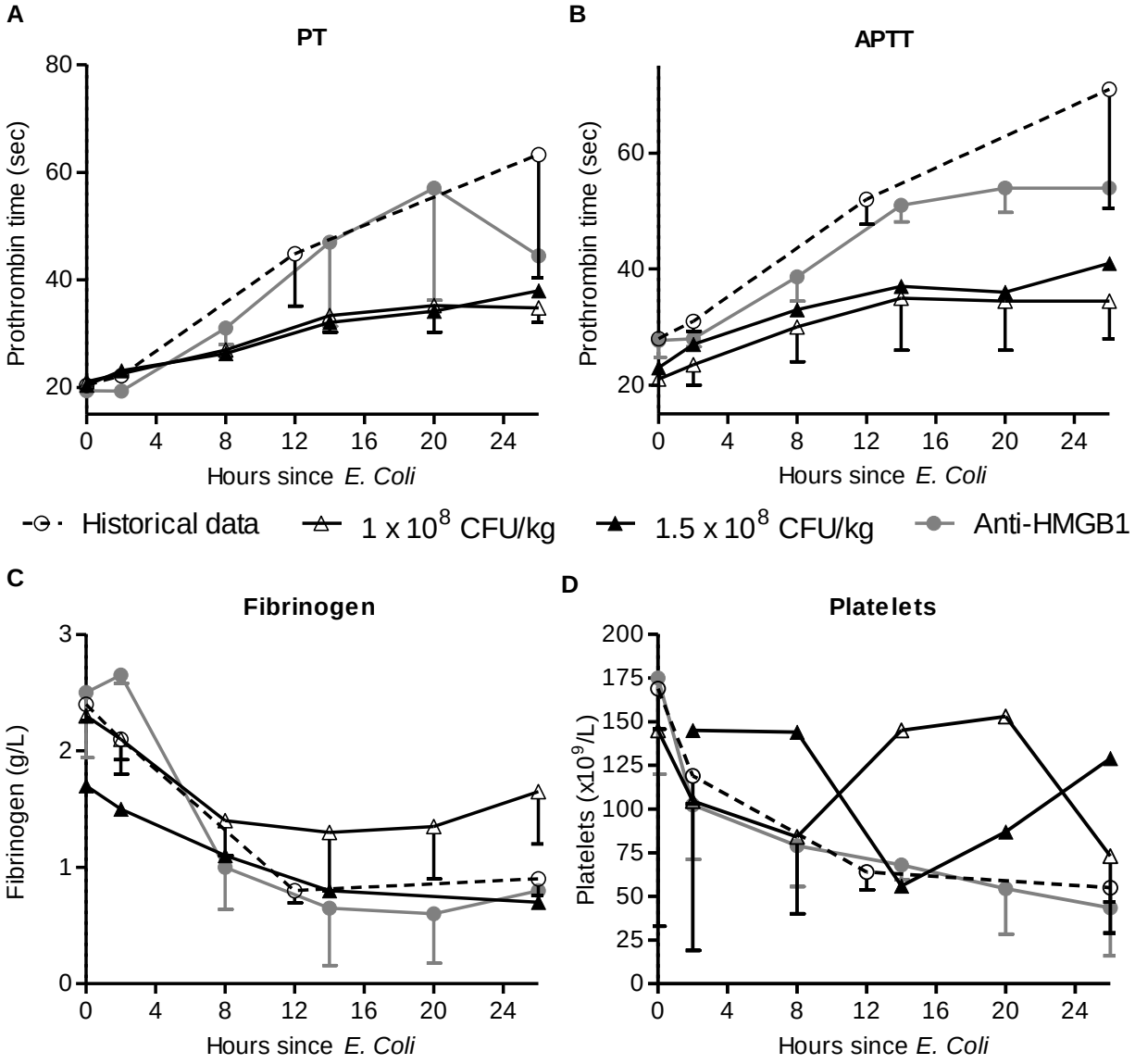


Figure 7.5 Coagulation tests performed on plasma from septic sheep

Sheep were administered 1.5 x10^8 CFU/mL *E. coli* (IV) to induce sepsis and either treated with anti-HMGB1 two hours post challenge (n = 3) or untreated (n = 1). Two sheep were administered a lower *E. coli* dose (1 x10^8 CFU/mL). Coagulation studies were performed on plasma samples collected at 0 and 2 hours post challenge and every 6 hours thereafter. Group (mean - SEM) values of prothrombin time (A), activated partial thromboplastin time (B), plasma fibrinogen (C) and platelet count (D) are depicted and compared to historical data from placebo treated animals (n = 8).

7.2.6. Metabolic parameters are altered in ovine sepsis corresponding to illness severity

In addition to survival and organ damage scores, metabolic measures are increasingly employed as clinical trial endpoints. Alterations in metabolic markers have been associated with illness severity and mortality [525-528], and long-term tissue metabolic abnormalities have also been described in sepsis survivors [529] and animal models [530-532]. Consequently, metabolic pathways have been considered as potential targets for the treatment of sepsis [533]. Plasma creatinine levels are commonly used to indicate renal function, as doubling of serum creatinine over a period of time indicates renal insufficiency and has been used as the threshold for the initiation of dialysis therapy in renal disease patients [534]. Plasma lactate is a marker for cellular hypoxia and in the context of sepsis may indicate inadequate blood perfusion and the development of shock. Metabolic acidosis in sepsis may result as a product of either cellular hypoxia and increased lactate levels or reduced kidney function. The development of acute metabolic acidosis is thought to interfere with haemodynamics [535] and myocardial contractility [536, 537] and contribute to organ damage in sepsis. Severe acidosis (pH < 6.8) is considered incompatible with life in all but the most extreme cases [538, 539].

Within the current study, metabolic measures were performed on serial blood samples from septic sheep, including creatinine, lactate and pH measurements. Plasma creatinine (Figure 7.6 A) increased in all groups from 2 hours post challenge, however a marked increase was observed in sheep treated with anti-HMGB1 pAbs. Similarly, the anti-HMGB1 pAb group exhibited the most elevated lactate (Figure 7.6 B) measurements and the highest degree of acidosis (Figure 7.6 C). The group that received a low *E. coli* dose exhibited mild increases in creatinine, and no increase in plasma lactate. Correspondingly, the development of acidosis was largely absent in this group, as pH only decreased a maximum of 0.06 units below baseline. These results support the notion that metabolic parameters are useful clinical tests in ovine sepsis that mimic the changes observed in humans.

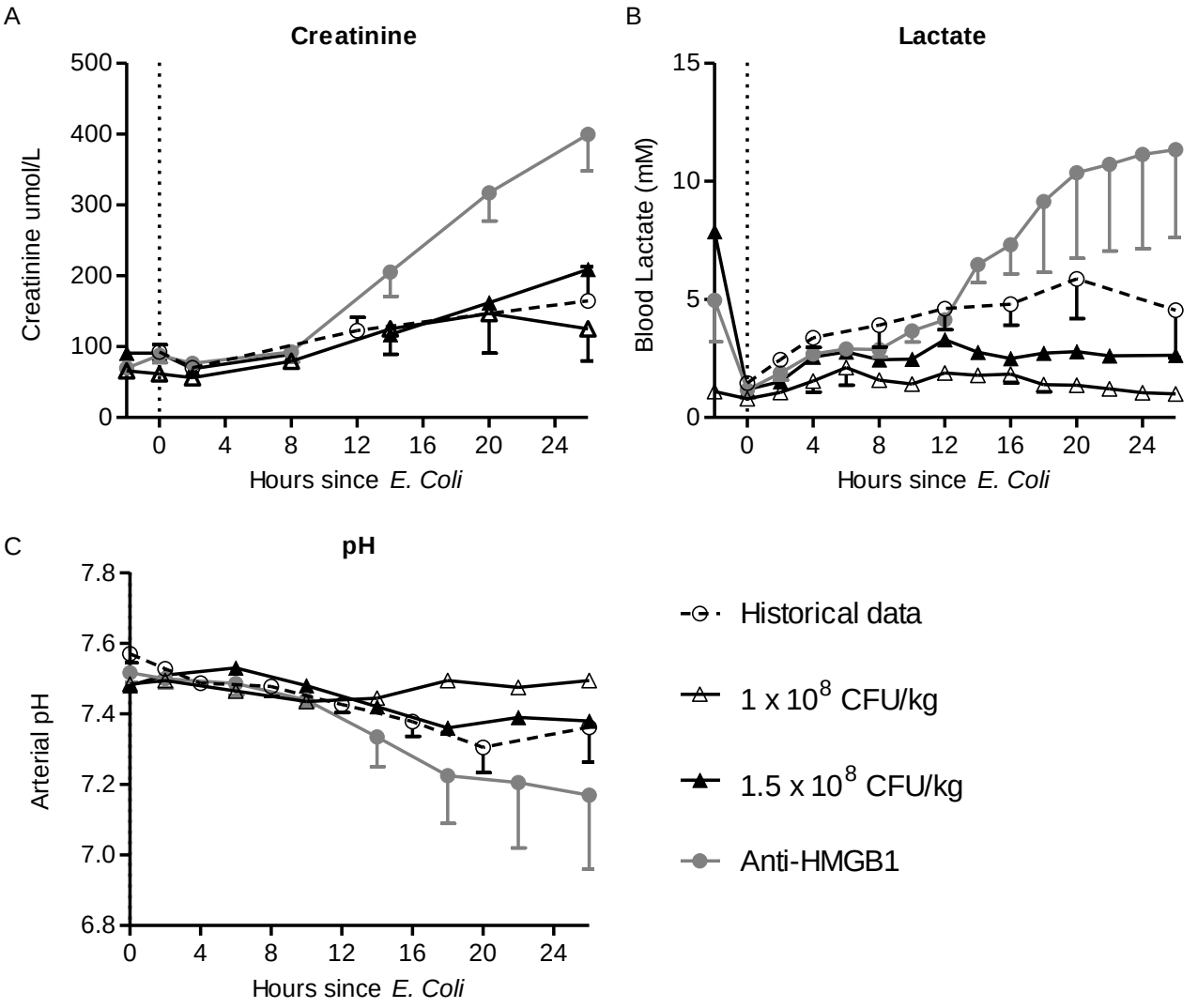


Figure 7.6 Ovine experimental sepsis is associated with elevated plasma creatinine and lactate and the development of metabolic acidosis

Sheep were administered 1.5 x10^8 CFU/mL *E. coli* (IV) to induce sepsis and either treated with anti-HMGB1 two hours post challenge (n = 3) or untreated (n = 1). Two sheep were administered a lower *E. coli* dose (1 x10^8 CFU/mL). Plasma samples collected at 0 and 2 hours post *E. coli* infusion and every 6 hours thereafter were assessed for creatinine content (A) while plasma samples collected every 2 hours were immediately frozen for lactate analysis (B). Plasma pH was determined in samples collected every 4 hours by bedside blood gas analysis (C). Data are representative of mean – SEM.

7.3 Discussion

Previous studies indicated that anti-HMGB1 pAbs conveyed a survival benefit in three murine models of sepsis: basic endotoxaemia, polymicrobial CLP-induced sepsis and CLP-induced sepsis followed by respiratory bacterial challenge. While the broad effects of anti-HMGB1 pAb therapy on the immune phenotype of sepsis could be assessed in the murine model, little could be ascertained about the impact of therapy on sepsis physiology. To address this question, it was necessary to assess anti-HMGB pAb therapy in a large animal model that allows the monitoring of sepsis morbidity and the use of clinically relevant endpoints. Banked plasma samples collected from septic and non-septic OSICS sheep were analysed and found to have similar HMGB1 content to that found in septic humans. Ovine HMGB1 has been demonstrated to have inflammatory effects and release kinetics that mirror those found in humans in studies of chorioamnionitis [540, 541], cell migration [542] and foetal brain injury [543]. A relatively high HMGB1 level is observed in the murine CLP model, which generates a sepsis syndrome of moderate severity with the bulk of mortalities occurring 2-6 days post-surgery. Thus, the effects of HMGB1 neutralisation in the CLP model may be amplified compared to human sepsis, and the OSICS model may be more suitable for assessing the subtle effects of anti-HMGB1 pAb therapy.

Within this study, the feasibility of using the OSICS model for evaluating the impact of anti-HMGB1 on physiological manifestations of sepsis was assessed in six sheep. While only a short experimental period of 26 hours following challenge was selected, anti-HMGB1 pAbs were efficacious in a 24-hour endotoxin murine model and thus a 26 hour timeframe was considered sufficient to detect effects of the therapy on early morbidity measures. Overall, anti-HMGB1 treated sheep did not exhibit reduced morbidity than control sheep, however a high degree of variation in this group and an early death complicated the interpretation of results. As no physiological phenotype associated with treatment was observed, analysis of ovine inflammatory markers was not performed. Initial trials aimed to reproduce a sickness severity achieved in previous studies, however administration of the same *E. coli* dose (1 x 10^8 CFU/kg) resulted in decreased morbidity with a low requirement for NorA compared to historical data. This discrepancy may be due to differences in the preparation of *E. coli* – though the same strain was used between studies, different personnel prepared the inoculum in the current study and a greater period between preparation and infusion of the inoculum may have decreased viable bacteria. Additionally, differences in the flock from which studied sheep were selected may impact their ability to respond to infection. It was subsequently

hypothesised that a higher *E. coli* dose (1.5 x 10^8 CFU/kg) may increase the sickness severity to a level observed in previous studies, thus the remainder of trials were performed with a higher *E. coli* dose. While the degree of illness achieved with a higher *E. coli* dose was greater, a very rapid disease course was observed which culminated in two early deaths. Consequently, for future studies an *E. coli* dose in the mid-range between 1-1.5 x 10^8 CFU/kg should be employed to generate a moderate septic phenotype.

Comparison of haemodynamic variables in sheep over the time period revealed differences between subgroups administered different *E. coli* doses, where a lower *E. coli* dose resulted in reduced requirement for NorA to maintain blood pressure. Anti-HMGB1 therapy was associated with no improvement to MAP, NorA requirement or vascular resistance in anti-HMGB1 pAb treated sheep compared to controls, however a high degree of variation did exist within this group and an early death prevented the collection of more data. As the NorA requirements of the control sheep administered an increased bacterial dose were higher than historical data, and anti-HMGB1 sheep exhibited a wide degree of variation, a reduced *E. coli* dose may allow more sensitive detection of any physiological benefit associated with treatment and avoid premature death that could confound interpretation of these endpoints.

Kinetic analysis of administered anti-HMGB1 pAb in treated sheep revealed that circulating pAb concentration decreased over the study period. The reasons for this are unknown, however fluctuations in mAb concentration in the context of tumour therapy have been attributed to several mechanisms, including Ab deposition, FcR binding or consumption within immune complexes [544]. Potential aggregation of therapeutic pAbs was minimised by passage through a 0.22 μm membrane filter prior to administration. Within this model, sheep exhibited marked vasodilation and developed a positive fluid balance throughout the study period as fluids were administered to maintain blood pressure while urinary production was simultaneously inhibited. Thus, significant loss of administered pAb into oedematous tissues and peritoneal fluid accumulations may have occurred.

The administration of anti-HMGB1 pAb did not appear to reduce the detectable plasma HMGB1 significantly, as measured by human HMGB1 ELISA. However, it has not been confirmed as to whether generated anti-HMGB1 pAbs are capable of blocking the epitope necessary for binding to the monoclonal coating Ab used in the commercial assay. Alternatively, generated anti-HMGB1 pAb may bind to HMGB1 with low affinity in comparison to the mAb assay reagents used [545]. As HMGB1 exhibits such high homology between species [300] and the pAbs generated against human HMGB1 exhibited

neutralisation capacity against murine HMGB1, it is likely that ovine HMGB1 can be similarly neutralised by the pAb preparation, however this remains to be confirmed in a functional assay.

Analysis of haematological parameters within this study supported previous evidence that progressive anaemia and leucocytosis develop within the OSICS model. The reduction in PCV and haemoglobin observed may be a result of true anaemia from RBC destruction or haemodilution due to increased blood volume from parenteral fluid administration. As fluids were continuously administered while urine output was severely impaired in this model, it is likely that some degree of haemodilution occurred. Nevertheless, following rapid declines in WBC and neutrophil counts upon challenge, a progressive leucocytosis characteristic of an acute phase response was observed [546]. Marked leucocytosis equivalent to historical data was apparent in subjects that received a lower *E. coli* dose, and an increase *E. coli* dose with or without anti-HMGB1 treatment was not associated with increased leucocytosis. While the magnitude of leucocytosis and neutrophilia have been correlated with the dose of endotoxin in human challenge studies [547], leucopoenia has been associated with mortality in gram-negative sepsis in humans [548]. Thus in this model, delayed leucocyte responses may indicate failure to control bacteraemia resulting in a more severe septic phenotype. In clinical sepsis, patients often develop lymphopenia due to apoptosis of circulating lymphocytes [549, 550] and this can be apparent as little as 12 hours following admission. No marked alterations in lymphocyte counts were observed in the current study within the 26 hour timeframe, however no specific assay for the detection of apoptosis was performed on these samples. In several sheep the presence of ‘atypical lymphocytes’ or ‘blast forms’ were noted in blood samples from later timepoints, however these were not consistent observations. Consequently, it remains to be investigated as to whether lymphocyte apoptosis was absent in these subjects or if circulating lymphocytes were undergoing apoptosis.

The sepsis generated within the OSICS model caused marked coagulopathy in study sheep. Of the groups within the current study, anti-HMGB1 pAb treated sheep exhibited the most profound clotting deficiencies, with longer clotting time recorded in APTT and PT assays which measure intrinsic and extrinsic coagulation pathways respectively. These clotting deficiencies could be a direct result of marked fibrinogen depletion, which was apparent in anti-HMGB1 treated sheep from 2-4 hours following challenge. Type III hypersensitivity reactions are associated with platelet activation and the formation of clotting-factor depleting microthrombi [551], however in the context of sepsis, IVIG infusion has been used to treat sepsis-associated coagulopathy [552]. Comparison of the current study to OSICS sheep

administered non-immune ovine pAbs may provide answers as to the potential relationship between coagulopathy and anti-HMGB1 pAb therapy. Interestingly, the non-treated sheep within this study exhibited less severe coagulopathy than historical data. This may be the result of genetic variation between sheep, as the sheep used in the current study were sourced from different flocks at a much later date.

Anti-HMGB1 pAb treated sheep also exhibited increased acidosis, with mean serum creatinine and mean serum lactate increased compared to control sheep and historical data. However, only two sheep were alive for the latter half of the study and one exhibited creatinine, lactate and pH levels comparable to controls, whereas the second sheep exhibited marked metabolic abnormities that were still within the variation observed in eight historical sheep. Thus, an increased number of sheep are required to determine whether this effect is real or just observed by chance due to the high variation in this model. The high degree of variation in the OSICS model compared to murine models does however allow greater comparison to heterogeneous human sepsis, but as with human studies large numbers must be included for reliable conclusions to be drawn. Though the source and nature of infection were controlled and sheep were selected from a single flock, variation was observed in the response to the septic challenge in this model, likely due to the outbred nature of the sheep. Analysis of WBC counts in pre-operative samples revealed that some sheep had higher relative counts of neutrophils compared to lymphocytes, and this may impact the nature of the innate response mounted after bacterial challenge.

The results of this study support the use of the OSICS model where multiple physiological parameters can be monitored in sepsis. With refinement, the model could be defined to include a morbidity score similar to the sequential organ failure assessment (SOFA) score used clinically and appropriate reference ranges for physiological criteria. These preliminary studies can however be used to further develop a large animal model for the assessment of anti-HMGB1 pAb therapy in sepsis. As the highest bacterial dose used in this study caused a high level of morbidity and early mortality in two sheep, a lower bacterial dose may benefit the performance of this model. Reduced bacterial dosing may generate a reduced risk of death to allow high quality data to be generated for the analysis of morbidity endpoints, an increasingly common outcome in clinical trials. A lower bacterial dose may also allow the study of mortality endpoints, though this will require a longer study period. Antibiotics were not administered in the current study as previously the OSICS model had been validated without antibiotic therapy. However, the administration of antibiotics in addition to supportive therapies may serve to reduce the mortality rate of the model and achieve a septic

syndrome more comparable to human sepsis. A more clinical approach to the monitoring of morbidity would significantly add to the power of the OSICS model. Additional analyses above those conducted within this study may improve the interpretation of morbidity, such as analysis of blood plasma protein content to determine whether the observed anaemia is a consequence of haemodilution or red blood cell destruction. Additionally, D-dimer assessment may yield more conclusive evidence of DIC occurring in ovine sepsis [553, 554]. An establishment of an ovine composite morbidity endpoint, similar to the SOFA score in humans, may also aid the monitoring of the disease course [555-557]. This may include the monitoring of bilirubin, creatinine and platelets according to standardised ovine sepsis reference ranges.

With the incorporation of such refinements, the OSICS model could become a robust tool in assessing sepsis therapies in late preclinical stages and allow translation into the clinic using clinically relevant endpoints. The intelligent optimisation of such models may reduce the discrepancy seen in results between preclinical and clinical studies and enable the development of effective host-specific therapeutics targeting sepsis.

CHAPTER 8.
GENERAL DISCUSSION & SUMMARY

As the number of elderly, immune-compromised and chronically ill persons within the population grows, there are more people at higher risk of severe complications following infections [558-562]. While preventative measures for diseases are undoubtedly important public health approaches, there remains a need for additional therapies for severe infectious disease where patients are dependent on supportive care for survival [563, 564]. Therapies that act against a broad range of pathogens or impact multiple biological pathways are favoured as in critical cases therapies need to be administered as soon as possible, often before detailed pathology results can be obtained [563, 565-568]. Passive immunotherapies based on polyclonal antibodies (pAbs) are thus excellent platforms for the development of critical care therapies, as the good safety profile and broad reactive capacity of pAbs enable neutralisation of multiple targets. Within this thesis, the application of ovine pAbs to the clinical scenarios of severe influenza infection and bacterial sepsis has been explored using clinical data and animal models. In the influenza study, pAbs were generated with specificity to the major neutralising determinant of influenza, haemagglutinin (HA); however, the application to bacterial sepsis required a different approach as sepsis may present with a diverse range of pathogens. Consequently, pAbs were generated against high mobility group box 1 (HMGB1) protein, a damage associated molecular pattern (DAMP) which is implicated as a mediator of the inflammatory pathogenesis of sepsis [449, 451, 454, 476] in addition to other inflammatory conditions [569-571].

Recent reclassification of sepsis has led to an increased focus on organ dysfunction in the identification of sepsis and septic shock [225]. The severe influenza classification signifies an influenza infection presenting with a complication, commonly pneumonia, respiratory failure, seizures or encephalopathy [132, 168, 572]. Thus, influenza may be a cause of sepsis or potentially the source of a nosocomial infection, a common occurrence in septic patients hospitalised for extended durations [573-575]. Both severe influenza and sepsis are consequences of immunological failure to control a local infection, thus groups considered to be at increased risk of both diseases have reduced immune function. The elderly, the immunosuppressed and those with chronic illness are at increased risk of both contracting infections [576-579] and developing complications including SIRS and organ damage once infected [580-583]. These groups all have some form of compromised immune defence, be they physical barriers, innate or adaptive immune defences, which increases the risk of contracting life-threatening infection.

The genesis of microbial infection involves four phases: attachment to the host (commonly at a mucous surface), growth within the host, immune evasion and host damage [584]. Gut

barrier dysfunction is a common consequence of many chronic inflammatory diseases [585-588], a side effect of chemotherapeutic [589] or anti-inflammatory [590] medications and is associated with cancer [591] and ageing [592, 593] while changes in the lipid content and fragility of skin that occur with age increase the risk of skin infection [594]. Deficiencies in innate immune functions can increase infection rate, with decreased neutrophil [595], DC [596] and NK cell [597, 598] function and an increased proinflammatory response to endotoxin [599, 600] predisposing elderly patients to infectious disease. While immunosuppressive medications administered to transplant recipients aim to reduce adaptive immune function to facilitate graft acceptance, medicated transplant recipients often exhibit innate immune defects including altered NK cell [601], macrophage [602] and neutrophil function [603, 604]. Patients hospitalised for extended durations are also at increased risk of contracting a nosocomial infection [605, 606], and the increased circulation of drug-resistant strains within hospitals engenders difficulty in controlling these infections [607, 608]. Chronically ill patients and those with organ transplants have frequent hospital stays [609-611] and the elderly are more likely than the general population to be hospitalised even for conditions unrelated to infection [612, 613]. Consequently, people most at risk of contracting infections often have increased exposure to difficult-to-treat pathogens in hospitals.

Adaptive immune deficiencies that reduce the resolution of established infections are well documented in immunosuppressed patients [614, 615], those with chronic illness [616-618] and in the elderly. The decline of immune function with age is known as immunosenescence, and chiefly manifests as reduced Ab responses following vaccination [619, 620], reduced naïve CD8 and CD4 T cell populations [621, 622] and increased regulatory T cell populations [623, 624] in addition to innate immune system defects. Wound repair mechanisms that are integral to the resolution of infection and the maintenance of mucosal barriers are also dysfunctional in the elderly [625, 626] and certain chronic inflammatory conditions [627]. Together, these factors result in unique populations of people that are frequently exposed to pathogens, more likely to form infections at compromised mucosal barriers, less capable of controlling infection through innate or adaptive mechanisms and more likely to suffer sustained organ damage without efficient healing mechanisms following infection. It is thus unsurprising that the elderly, chronically ill and immunosuppressed comprise over 80% of hospitalised sepsis cases [579, 628] and approximately 50% of severe influenza cases [132, 629] in developed countries. The need for therapies which are able to rapidly impact the course of disease is vital to survival from these life threatening conditions, particularly in at-risk populations.

The ability to generate Abs with a high degree of specificity is one of the major advantages of passive immunotherapy methods; however pAbs can also be generated to target a broad range of epitopes [630, 631]. This is particularly advantageous in the setting of influenza as circulating viral strains are subject to antigenic drift and can potentially escape immunity conferred from previous strains [632]. As mAb therapies target a single epitope, there is increased selective pressure for the formation of viral escape mutants, which is reduced with a polyclonal or combination approach [633-635]. Severe influenza is common in populations hospitalised for extended durations and the prolonged time to viral clearance in these patients promotes the generation of mutant strains resistant to therapies, such as antiviral drugs [636] or potentially mAb therapies [637-640]. Thus, in the absence of a universal vaccine or an approved broadly neutralising mAb, pAb based therapies are a logical therapeutic option for life-threatening severe influenza infection. Passive therapies are used to provide immediate viral neutralisation, which is particularly relevant to immunocompromised hosts who do not mount effective adaptive responses to influenza, and do not efficiently clear influenza virus [636]. Clinically, IVIG prepared from influenza-exposed donors can reduce the severity of infection in severe cases [10, 168, 413]. The results obtained from the studies presented in this thesis have supported the use of pAb products as therapies for severe influenza infection. Within Part A, the generation of ovine pAbs against a known antigenic determinant of influenza was empirically optimised and the efficacy of the generated pAbs were assessed in a murine model of PR8 influenza infection. It was found that proprietary adjuvant CoVaccine HT^{TM} (CV) induced tenfold higher anti-HA pAb titres than Freund's Adjuvant (FA). Additionally, the use of CV enabled the administration of a tenfold lower dose of HA antigen to achieve equivalent pAb titres to those achieved by FA regimens. No significant effects on peak pAb titres were observed when older sheep or an IP immunisation route were used. The procurement of antigen is a significant cost in the production of pAb therapies, thus reducing the amount of antigen required to elicit neutralising Ab responses may reduce the overall cost of therapeutics [641]. A reduced need for antigen may have additional benefits where antigen is in scarce supply, or may pose some health risk to the animal [642, 643].

The increased efficacy of CV compared to FA observed in this study may be due to its physical properties as an oil-in-water emulsion which does not promote denaturation of protein antigens [644]; alternatively the efficacy of CV as an adjuvant may be entirely dependent on its immunomodulatory properties due to its formulation including sucrose fatty-acid sulphate esters which signal through TLR4 [385]. These results highlight the potential role for novel immunisation strategies for the production of pAb therapeutics as well as effective vaccines. Animal pAb production involves an inherent cost to the animal, however

the potential for the development of painful immunisation sites can be minimised to uphold ethical standards in the use of animals for the production of human therapeutics. Comparison of immunisation sites between CV and FA revealed significantly fewer apparent 'reactive' sites associated with the novel adjuvant, and thus the use of CV adjuvant may serve not only to increase pAb titres but decrease negative impacts within sheep. While cross reactivity of pAbs generated by the two adjuvants was not investigated, this could be a future avenue of exploration, as some adjuvants have been associated with improved Ab diversity and affinity maturation in preclinical [645-647] and clinical studies [648, 649]. In particular, the use of TLR agonists as adjuvants has been demonstrated to elicit diverse Ab responses after vaccination [650], thus it is postulated that CoVaccine HT^{TM} may enhance the quality of Ab responses.

Within the second part of the study, the efficacy of purified anti-HA pAbs in a murine model of severe PR8 influenza was explored and it was found that administration of anti-HA pAbs prevented lethal infection and also reduced lethality from an established infection within the model. Notably, intranasal (IN) administration demonstrated higher efficacy for the treatment of established infection, which supports previous studies [421, 422]. Direct administration to the respiratory tract facilitates immediate interaction with virions for rapid neutralisation [421]. In the clinic, convalescent sera administered to infected patients is delivered through an intravenous (IV) route [186, 407], which may delay viral neutralisation. An IN route may allow more rapid viral neutralisation from pAb therapies and reduce the dose required, and thus this route of administration should be investigated in future clinical trials. In the current study, mice administered anti-HA pAbs which protected from initial infection were still able to mount protective adaptive responses against the virus, as Ab titres and CTL responses were no different between treated and untreated mice. This finding may have implications to the clinical application of this therapy, as developing long lasting adaptive responses will exert protection against re-infection with the same strain. As immunocompromised and elderly patients may exhibit poor responses to influenza infection [631], and are thus at risk of re-infection, repeated administration of anti-HA pAbs may be required in these cases. Within this study, no adverse effects were associated with re-administration of ovine pAbs to mice, though anti-treatment responses were evident which may have contributed to accelerated clearance of the pAbs following readministration. Administered pAbs also exhibited a half-life over two weeks, which may contribute to the observed therapeutic efficacy. Examination of the half-life of pAbs in the respiratory tract following IN administration as compared to systemic administration would be invaluable in confirming the efficacy of this approach. The murine model used in this study may not be directly applicable to the clinic [651, 652], as the

contribution of Fc-mediated functions to the efficacy of anti-HA ovine pAbs in mice is not known. Potentially, different binding capacity of human Fc receptors to ovine pAbs may alter the efficacy of this approach in the clinic, and this should be explored [653, 654]. A therapy developed for human use based on ovine pAbs is likely to utilise fragmented pAbs, which are less likely to induce hypersensitivity reactions [117]. Fragmented pAbs do not partake in Fc-mediated effector functions, and thus ovine Fab or $F(ab)_2$ fragments would rely on direct viral neutralisation to control infection. A polyclonal approach towards passive immunotherapy allows the development of broadly cross-reactive Abs to confer protection against antigenic drift [82, 87, 114, 632]. Within this study, ovine pAbs generated against a single HA from H1N1 exhibited some cross-reactivity to whole H3N2 virus *in vitro* however this did not translate to *in vivo* protective capacity against the different subtype. It is postulated that the pAbs generated bound to shared stalk epitopes on the H3N2 HA that are not required for binding to sialic acid receptors and entry into host cells. Previous studies that have utilised recombinant HA as an antigen have reported the generation of stalk-specific pAbs that contribute to protective efficacy between variant HA within the same subtype, but are not protective between subtypes [655]. Anti-stalk Abs are capable of direct pathogen neutralisation, however higher doses are often required. Efficacy of some anti-stalk mAbs requires FcR-mediated mechanisms for protection at low doses however effective neutralisation can be achieved in the absence of FcR binding using high mAb doses [189]. Thus, the generation of stalk specific Abs within ovine polyclonal products may enhance rather than reduce efficacy of the treatment, particularly for mutated variants. Immunisation studies utilising subunit vaccines have demonstrated increased formation of stalk-specific pAbs following immunisation with recombinant HA including a hyperglycosylated head domain [656] which selectively shifts immuno-dominance of epitopes away from this region. Furthermore, stalk specific pAbs generated in this way demonstrated cross-protective capacity against different HA subtypes. Previous studies have confirmed that baculovirus expression, as used in the current study for the HA antigen, produces recombinant HA with similar glycosylation patterns to that present in whole virions [657]. Thus, while stalk-specific pAbs may have been present within the generated polyclonals in the current study, a large proportion of pAbs would be specific to dominant head-region epitopes, which may explain why cross-protective capacity was not observed. The results presented herein describe a valuable role for anti-HA pAbs in the treatment of severe influenza, however broad cross protective capacity would encourage applicability to clinical influenza infection. Cross protective capacity of anti-HA pAb therapies may be increased by sequential immunisation with variant HA types [400], the use of modified HA antigen [656] or combination of potent antisera generated against different HA subtypes [658] and development of a broadly acting

severe influenza treatment based on ovine anti-HA pAbs should explore these options.

Within Part B of this thesis, the potential role of anti-HMGB1 ovine pAbs for the treatment of sepsis is explored. Previously, HMGB1 has been identified as a key late mediator of sepsis pathogenesis [316, 449], which contributes to organ damage and death in animal models and in the clinic. The view of sepsis as a disease with two different inflammatory phases of SIRS and CARS has been challenged in favour of the concept of 'immunologic dissonance' whereby both pro- and anti-inflammatory responses are uncontrolled [440, 442, 659, 660] and can potentially contribute to organ damage in sepsis [443, 661]. Recently, experts have proposed new recommendations for the diagnosis and classification of sepsis, termed Sepsis-3, which focus on organ damage endpoints over the presence of systemic inflammation [225-226]. While this new framework for sepsis diagnosis may impact the conduct of clinical trials, it is unlikely to affect the validity of an anti-HMGB1 and other immunomodulatory approaches to the treatment of patients considered septic. This thesis explores the effect of HMGB1 blockade on cytokine responses in murine sepsis and presents a model system for evaluating the effect of treatment on physiological parameters and organ damage in sepsis.

To better understand the role of HMGB1 in the SIRS/CARS/MARS paradigm of sepsis, HMGB1 levels and inflammatory profiles were evaluated in the serum of septic shock patients. Non-surviving patients had higher plasma HMGB1 and their circulating cytokine profiles were skewed towards proinflammatory signalling. Measured HMGB1 was highest at early time points and also associated with proinflammatory cytokine profiles. Interestingly, comparative analysis of the magnitude of cytokine signalling over time revealed that survivors had greater total cytokine release which was most pronounced in the first 24 hours following admission. Non survival was also associated with reduced expression of IL-10, which is considered the archetypal anti-inflammatory cytokine [662], and also reduced expression of IL-6 and IL-1RA, particularly at early time points. Taken together, these results suggest that cytokine profiles associated with non-survival in this cohort constitute a failure to mount effective anti-inflammatory signalling for the control of proinflammatory responses, rather than an over-exuberant proinflammatory response.

Previous studies that have examined circulating cytokines in sepsis patients have found varied results. A single-centre study of a cohort of septic patients in Greece found that non-survivors exhibited higher levels of IL-10 [663] compared to survivors. However, as all sepsis was included within the study, the surviving cohort represented may not have exhibited the same level of illness as in the septic shock survivors within this study. Increased IL-6

concentrations in the surviving septic shock cohort were observed in this study; in contrast, previous studies have associated elevated IL-6 with sepsis non-survival [468, 664]. Importantly, as sequential samples were analysed in the current study, a pattern could be observed where survivors had increased IL-6 that decreased over time and non-survivors maintained IL-6 levels throughout the course of sepsis, which agrees with previous kinetic analysis of IL-6 in sepsis patients [469]. Elevated MCP-1 was observed in non-survivors which agrees with a previous multiplex study where MCP-1 correlated with illness severity, SOFA score and mortality [473].

These results demonstrated an association between HMGB1 release and increased proinflammatory signalling with dampened anti-inflammatory responses in sepsis. However, the use of a composite cytokine score (CCS) to assess the inflammatory profile of septic shock patients is a potential limitation of this study, and may have limited prognostic value in the clinic. This method has been employed previously in a paper examining the cytokine profiles in murine CLP-induced sepsis [442], which demonstrated divergent patterns in surviving versus non-surviving mice. Within the current study, the CCS was calculated using the measurement of ten cytokines that have previously been shown to be differentially expressed between survivors and non-survivors [473]. Cumulative scores were calculated from normalised values, however the calculation used gives equal weight to all cytokines when they may not have the same pro- or anti-inflammatory capacity. Additionally, as values were expressed in fold-changes, the assumption exists that the impact of a doubled cytokine concentration is equivalent for all cytokines, however the functional relevance of a doubled cytokine concentration may not be the same between cytokines. A more accurate approach would be to use comprehensive data from normal and septic patients to establish low, normal and elevated cytokine ranges and assign points to each scenario, which could be used to compute total pro- and anti-inflammatory scores, however such detailed analysis of cytokine responses in sepsis of varying severities has not been performed. Nevertheless, it is anticipated that the findings in this study may assist our understanding of the aetiology of sepsis and the role of HMGB1 in the inflammatory milieu. To assess the effect of anti-HMGB1 pAbs on sepsis survival and cytokine responses, purified pAbs were administered to mice in three models of systemic inflammation and sepsis. Prophylactic administration of anti-HMGB1 ovine pAbs protected mice from lethality following short-term endotoxeamia; these data provided evidence that these pAbs were functional *in vivo*. However, to examine the effect of anti-HMGB1 pAbs on dynamic cytokine responses, a model of sepsis with a longer duration was employed. The murine CLP model recapitulates severe peritonitis followed by bacteraemia and sepsis, and is considered the gold-standard rodent model for

sepsis [325, 329, 665]. Anti-HMGB1 pAb effectively prevented lethality from CLP-induced sepsis when administered before or eight hours following surgery. Sequential analysis of pro- and anti-inflammatory cytokines revealed alterations in cytokine responses 24 hours following surgery in anti-HMGB1 pAb treated mice. Consistent with the surviving septic shock patient cohort, anti-HMGB1 pAb treated mice had increased IL-6 and IL-10 at 24 hours compared to PBS or non-immune pAb treated control animals. Despite morbidity and survival benefits, anti-HMGB1 pAb treated mice did not exhibit significant alterations in proinflammatory CCS compared to controls. However, these mice did exhibit reduced susceptibility to secondary *P. aeruginosa* infection following sepsis in a double-hit model [666]. Examination of inflammatory profiles in this model revealed that anti-HMGB1 pAb treated mice secreted significantly more IL-12 and less IL-6 in response to secondary infection than control mice. Functional analysis of peritoneal macrophages from septic mice revealed a similar pattern of increased IL-12 production and decreased IL-6 production following LPS stimulation in the anti-HMGB1 pAb treated group compared to controls. The role of macrophages in the overall inflammatory response in sepsis has been well established [667, 668] and their dysfunction has been associated with reduced pathogen clearance [669] and reduced ability to prompt adaptive responses [670-672]. In particular, dampened IL-12 secretion from macrophages has been associated with increased susceptibility to secondary infections in sepsis [673, 674]. The observed increase in IL-12 production in macrophages from anti-HMGB1 pAb treated mice implies that these mice are more capable of responding to a secondary infection, as IL-12 release promotes CTL responses and pathogen clearance [675]. The role of IL-6 in this setting is less clear, as IL-6 is often associated with sepsis mortality in murine [442, 676] and human sepsis [468], however IL-6 deficient mice do not appear to be protected from sepsis mortality [677], and IL-6 has been demonstrated to have both pro- and anti-inflammatory effects [678]. In light of the survival advantage of anti-HMGB1 pAb treated mice in the double hit model, reduced IL-6 production by macrophages from post-septic anti-HMGB1 pAb treated mice may indicate reduced immunomodulatory function, however this remains unclear. Stimulation of DCs generated from septic mice revealed reduced IL-10 and a trend towards increased IL-12 production, but no alteration in IL-6 production. Increased IL-10 expression has been observed in alveolar DCs during post-sepsis immunosuppression [503], and it has been proposed that the ratio of IL-12 to IL-10 release impacts the ability of DCs to drive neutrophil-mediated clearance of respiratory pathogens [679-681]. While the experiments here were performed on DCs derived *in vitro* from the bone marrow of septic mice, alterations in lung DC function *in vivo* have been linked to the apoptosis of DCs and repopulation by DCs that epigenetically reprogrammed [495] and thus these DCs may be somewhat representative of lung DC function. Taken together, these

results suggest that anti-HMGB1 pAb treatment improves survival and partially reverses the innate immune cell reprogramming observed following sepsis, which may contribute to a heightened ability to mount effective immune responses to control secondary challenge [682]. These results may also indicate a role for anti-HMGB1 treated in other difficult-to-manage infections with an inflammatory component.

The final chapter of this thesis describes a pilot study in which the application of anti-HMGB1 pAbs to a model of ovine sepsis with intensive care supports (OSICS) was explored. While several approaches that aim to modulate the inflammatory response have been trialled in sepsis patients, none have exhibited significant survival benefit over supportive care alone [444]. This has been attributed to both the poor selection of preclinical models in which therapies are developed and tested, and also the conduct of clinical trials which focus primarily on survival endpoints [444, 683, 684]. Therefore, in order to provide strong evidence for the use of anti-HMGB1 pAbs as a therapy for clinical sepsis, a large animal model must be employed that recapitulates many of the physiological hallmarks of human sepsis [333, 685].

The findings within the pilot study did not demonstrate any survival or morbidity benefit to anti-HMGB1 pAb therapy in the OSICS model, however with alterations the model could be adjusted to result in less severe septic disease and increase the opportunity for morbidity benefits to be observed. The primary endpoint in the model was the requirement for NorA to maintain adequate blood pressure, which appeared to be sensitive to the bacterial dose used to generate sepsis. Animals that received a reduced *E. coli* dose had a very low requirement for NorA, however a 50% increase in bacterial dose generated a severe level of illness where sheep required close to maximum NorA dosage. Importantly, this alteration in bacterial dose altered the development of systemic vasodilation, metabolic acidosis and clotting abnormalities in the model. While HMGB1 release was observed as early as 12 hours following bacterial challenge in the OSICS model, the degree of sickness observed in sheep administered at the higher *E. coli* dose was extremely severe within the experimental period. These sheep progressed to a physiological state clinically associated with long-term organ damage, including reduced perfusion and oxygenation, almost totally deficient clotting capacity, and severe metabolic acidosis. Reduction of the *E. coli* dose may prompt a level of sickness where sheep reach endpoints over a longer timeframe, and this would more appropriately facilitate the assessment of anti-HMGB1 pAb therapy in sepsis. Co-administration of antibiotics may also alter the course of disease to enable the assessment of immunomodulatory therapy in the OSICS model over a longer timeframe. As broad-spectrum

antibiotic administration is a common approach utilised in the clinic [227], the addition of antibiotic therapy to the model may alter the balance between viable and dead bacteria to better recapitulate clinical sepsis. While no conclusions regarding the effects of anti-HMGB1 pAb in the OSICS model could be ascertained from this pilot study, these results provide a framework for the conduct of future studies using the OSICS model.

In summary, this thesis details two potential applications for ovine pAb therapies in infectious diseases: as potent antiviral agents in severe influenza infection and as immunomodulatory agents in bacterial sepsis and septic shock. The results of these studies provide insight into the development of ovine pAbs as therapies which may be of particular value in at-risk populations that form the majority of infectious critical illness hospitalisations. While the protective effects of anti-HA pAbs in influenza have been described previously, the impact of pAb therapy to the development of adaptive antiviral responses has to date not been described. Evaluation of anti-HMGB1 pAb therapy in preclinical sepsis models supported this approach to sepsis treatment. Cytokine analysis in murine and human sepsis explored the role of HMGB1 in the inflammatory milieu in sepsis and shed light on its contribution to post septic immunosuppression. It is anticipated that the findings of this thesis will assist in the development of passive approaches to the prevention and treatment of influenza and HMGB1-blockade therapies for the treatment of bacterial sepsis and septic shock.

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APPENDIX A

APACHE II calculation APACHE II scores are calculated from the sum of the 12-parameter acute physiology score (including coma score), age, chronic health points and bicarbonate status. Maximum possible score is 74.

ACUTE PHYSIOLOGY SCORE											
PHYSIOLOGICAL VARIABLE	**4**	**3**	**2**	**1**	**0**	**1**	**2**	**3**	**4**
TEMPERATURE (°C)	> 41	39-40.8		35.5-38.9	36-38.4	34-35.8	32-33.9	30-31.9	<29.9
MEAN ARTERIAL PRESSURE (mm Hg)	> 160	130 - 159			70 - 109		50-69		< 49
HEART RATE	> 180	140 - 178			70 - 109		55-69		< 39
RESPIRATORY RATE (non ventilated or ventilated)	> 50	35 - 49			12-24		6-9		< 5
OXYGENATION a) $FiO_2 \geq 0.5$ use A-aDO_2 b) $FiO_2 < 0.5$ use PAO_2	> 500	350-498	200-349		< 200 > 70	61 - 70		55-60	< 55
ARTERIAL pH	> 7.7	7.6-7.69		7.5-7.59	7.33-7.49		7.15-7.32	7.15-7.24	< 7.15
SERUM SODIUM (meg/dl)	> 180	160-179	155-159	150-154	130-149		120-129	111-119	< 110
SERUM POTASSIUM (meg/dl)	> 7	6 - 6.9		5.5 - 5.9	3.5 - 5.4	3 - 3.4	2.5 - 2.9		< 2.5
SERUM CREATININE (mg/100 ml)	> 3.5	2 - 3.4	1.5 - 1.9	0.6 - 1.4			< 0.6		
HEMATOCRIT (%)	> 60		50 - 59.9	48 - 49.9	30 - 45.9		20-29.9		< 20
WHITE BLOOD COUNT (/mL) (in 1000s)	> 40		20 - 39.9	15 - 19.9	3 - 14.9		1-2.9		< 1
GLASGOW COMA SCORE (score 15 minus actual GCS)				**1**	**2**	**3**	**4**	**5**	**6**
	Eye opening			No response	Response to pain	Response to speech	Spontaneous		
	Verbal response			None	Incomprehensible	Inappropriate	Confused	Orientated	
	Best motor response			Nil	Extensor response	Abnormal Flexion	Withdraws to pain	Localises to pain	Obeys commands
ADDITIONAL SCORES									
SERUM HCO_3	**4**	**3**	**1**	**0**	**2**	**3**	**4**		
	> 52	41 - 51.9	32 - 40.9	22 - 31.9	18 - 21.9	15 - 17.9	< 15		
AGE SCORE	**0**	**2**	**3**	**5**	**6**				
	<44	45-54	55-64	65-74	>75				
CHRONIC HEALTH POINTS (Severe organ system deficiency or immune compromised)	Post-operative patient =2			Non-operative or emergency post-operative patient = 5					

APPENDIX B

Supporting Publication: Published

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An Empirical Approach towards the Efficient and Optimal Production of Influenza-Neutralizing Ovine Polyclonal Antibodies Demonstrates That the Novel Adjuvant CoVaccine HT™ Is Functionally Superior to Freund's Adjuvant

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Abstract

Passive immunotherapies utilising polyclonal antibodies could have a valuable role in preventing and treating infectious diseases such as influenza, particularly in pandemic situations but also in immunocompromised populations such as the elderly, the chronically immunosuppressed, pregnant women, infants and those with chronic diseases. The aim of this study was to optimise current methods used to generate ovine polyclonal antibodies. Polyclonal antibodies to baculovirus-expressed recombinant influenza haemagglutinin from A/Puerto Rico/8/1934 H1N1 (PR8) were elicited in sheep using various immunisation regimens designed to investigate the priming immunisation route, adjuvant formulation, sheep age, and antigen dose, and to empirically ascertain which combination maximised antibody output. The novel adjuvant CoVaccine HT™ was compared to Freund's adjuvant which is currently the adjuvant of choice for commercial production of ovine polyclonal Fab therapies. CoVaccine HT™ induced significantly higher titres of functional ovine anti-haemagglutinin IgG than Freund's adjuvant but with fewer side effects, including reduced site reactions. Polyclonal hyperimmune sheep sera effectively neutralised influenza virus *in vitro* and, when given before or after influenza virus challenge, prevented the death of infected mice. Neither the age of the sheep nor the route of antigen administration appeared to influence antibody titre. Moreover, reducing the administrated dose of haemagglutinin antigen minimally affected antibody titre. Together, these results suggest a cost effective way of producing high and sustained yields of functional ovine polyclonal antibodies specifically for the prevention and treatment of globally significant diseases.

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Introduction

Antigen-specific polyclonal antibodies are generated for a wide array of purposes which range from fundamental laboratory studies and protocols to passive immunotherapy for life threatening conditions including snake envenomation [1] and drug toxicity [2]. Sheep are particularly attractive for the generation of passive polyclonal immunotherapeutics as ovine antibody fragments have demonstrated reduced immunogenicity and more consistent biological function than those derived from other animals [3]. Furthermore, large quantities of serum can be repeatedly obtained from sheep, with reduced maintenance costs and lower immune boosting demands than other large animals such as horses [4]. Specifically, polyclonal ovine antibodies in the form of antigen-binding antibody fragments or 'Fab' underlie the use of antibodies in critical care situations such as snake envenomation and digoxin toxicity [5]. Potential hypersensitivity reactions often associated with administration of whole antibody are considerably reduced by using these Fab fragments or their divalent counterpart $F(ab)_2$. Hence, this type of treatment has the potential to be readily transferable to infectious disease management, particularly in light of the increased incidence of drug resistance to circulating

pathogens [6,7] and the medley of undesirable side effects often associated with conventional drug treatments [8,9,10]. Of particular interest here is the applicability of this approach to infections with viral pathogens such as influenza, as natural immunity to many such viruses is facilitated through the action of neutralising antibodies [3,11,12].

Whilst traditional vaccination reduces influenza-associated mortality [13], it is least efficacious in the immunocompromised individuals who are most susceptible to complications and increased mortality [14,15], and who include pregnant women [16]. Consequently, immunocompromised individuals make up the majority of the many thousands of annual influenza-related deaths [14,17], which provides the rationale for passive immunotherapy as influenza prophylaxis or treatment in these individuals because additional time is not needed to generate an efficient vaccine-induced adaptive immune response [18]. Indeed, passively administered influenza-specific antibody has been shown to inhibit influenza-induced mortality in rodents [19], although the selection of a suitable clinically applicable passive immunotherapeutic will be determined by its inherent neutralisation capacity, its safety as well as its commercial scalability and overall cost effectiveness [20]. These factors highlight the requirement for optimal efficiency at every stage of the production process.

Whilst downstream processing methods for existing commercial ovine polyclonal antibody preparations have been methodically optimised [21,22,23], there has been limited investigation into the best way to generate maximal antibody titres and overall yield of effective antibody from the sheep themselves. Indeed, there are few reports in the published literature directly comparing the parameters that can influence humoral immune responses in sheep [24]. This is particularly important considering that route of administration, antigen dose and adjuvant are well recognised as critical parameters in antibody production from other species [25,26].

The route of immunisation can influence the induction of the humoral immune responses [27] by dictating which population of dendritic cells (DCs) interacts with antigen [28,29]. For instance, subcutaneous immunisation is routinely applied commercially to produce hyperimmune ovine sera [22,23] and facilitates antigen interaction with skin-associated DCs, including Langerhans cells, conventional DCs and macrophage-derived DCs [30]. Alternatively, intraperitoneal immunisation promotes antigen interaction with conventional DCs macrophages and plasmacytoid DCs, which may be beneficial depending on the antigen type [31,32]. The functionality of site-specific DC subsets in sheep has not been well studied and thus empirical assessment is required to determine an optimal immunisation route. Antigen dose can also influence the outcome of immunisation; too little antigen may elicit inefficient responses [33], and too much antigen can promote adverse effects and immunotolerance [24]. The standard antigen dose used in generating ovine antisera varies widely (from micrograms to as much as five grams per animal [24]) and this uncertainty necessitates investigation of appropriate antigen dosage for optimal antibody output. The choice of adjuvant is another key factor in dictating both the quality and quantity of the humoral immune response [25,34]. Adjuvants prolong and augment the effects of vaccination through various mechanisms which include increasing antigen persistence, stimulation of local inflammation, upregulation of cytokines and immunomodulatory factors, activation of phagocytic cells and promotion of antigen presentation [35,36]. The current gold-standard for generation of a humoral immune responses in animals is Freund's adjuvant (FA), a water-in-oil emulsion which is available in 'complete' or 'incomplete' formulations, that is, with or without heat-killed *Mycobacterium tuberculosis* respectively [37]. This adjuvant primarily works by increasing antigen persistence in the tissues [38] and stimulating a foreign-body reaction which results in local inflammation and recruitment of innate and adaptive immune cells [39]. It is however often associated with the formation of painful granulomas and can also result in tuberculin-type hypersensitivity [40]. It is not approved for human use and concerns with animal welfare have prompted investigation into alternative adjuvants for use in animals [39].

The current study applies empirical methods to optimise anti-A/Puerto Rico/8/1934 H1N1 (PR8) haemagglutinin (HA) ovine polyclonal antibody production using FA and a proprietary experimental adjuvant, CoVaccine HT^{TM} (CV) [36,41,42,43]. CoVaccine HT^{TM} is in clinical development and consists of sucrose fatty acid sulphate esters (SFASE) immobilised on the oil droplets of a submicron emulsion of squalane-in-water [41]. We show that the age of sheep, the prime immunisation route, and dose has minimal impact on the antibody titre, although CV adjuvant induced superior anti-HA antibody titres than immunisation regimens incorporating FA. The influenza-neutralisation capacity of the anti-HA antibody was subsequently assessed by haemagglutination-inhibition (HAI) assays and was found to correlate closely with the antibody titres measured for each of the variables. Finally, it was demonstrated that high titre ovine hyperimmune serum can be successfully applied in a lethal *in vivo* murine influenza challenge model to prevent mortality in both a prophylactic and therapeutic context.

Materials and Methods

Recombinant Haemagglutinin Production

The HA sequence of A/Puerto Rico/8/1934 H1N1 (PR8) H1N1 (NCBI accession: AF389118) from PR8-transfected AB1 malignant melanoma cells (AB1-HA) was PCR cloned into pGEM-T Easy Vector system (Promega) using primers with appropriate restriction sites and a C-terminal 6x-His tag. Confirmed HA sequence was subsequently used to produce recombinant baculovirus using the Bac-to-Bac expression system (Invitrogen). Insect cells (Sf21) were maintained in sf900 SFM III (Invitrogen) supplemented with L-glutamine (100 μg/ml), penicillin (100 U/ml), gentamycin (100 μg/ml) and HEPES (10 mM, pH 7.2) in roller bottle flasks at 27°C. Protein was produced by infection of Sf21 insect cells 96 hours prior to protein harvest, and cultures were supplemented with 100 μg/ml L-glutamine 24 hours prior to harvest. Expressed protein was purified from cell lysate using Ni-NTA Agarose beads (Qiagen) according to the manufacturer's instructions before dialysis with PBS. Protein purity was analysed by SDS-PAGE and anti-HA Western Blot and concentration determined with BCA assay.

Immunisation and Sampling

Purified recombinant HA (rHA) antigen was diluted in PBS and emulsified with an equal volume of complete FA for prime immunisation or incomplete FA for boost immunisation (Gibco). Groups of five 9-month old or 3-year old Border Leicester x Merino ewes were immunised with 200 or 20 μg rHA in 4 ml emulsion, either subcutaneously (SC) at 4 axillary sites or as a bolus intraperitoneal (IP) injection (prime only). Alternatively, antigen in PBS was gently mixed with an equal quantity of CV suspension (Protherics Medicines Ltd) and sheep were immunised with 2 ml emulsion SC at 4 axillary sites. Sheep were administered a prime immunisation and a boost dose every 14 days for a total of five boosts. Serum was sampled prior to each immunisation and stored at −20°C.

Anti-HA Antibody ELISA

Briefly, EIA/RIA high-binding ELISA plates (Costar) were coated with 10 μg/ml rHA overnight at 4°C. Plates were blocked with 2% (w/v) BSA (1 hour, 37°C) and pre-immune or hyperimmune sheep serum diluted as indicated in Figure S1 was added to duplicate wells and incubated for 2 hours (37°C). Bound ovine antibodies were detected with HRP-linked anti-ovine IgG antibody (Sigma; 1 hour, 37°C). The plates were developed with OPD substrate (Sigma), the reaction stopped with 3 M HCl and the absorbance read at 490 nm. Absorbance readings from negative control wells were subtracted from all readings. ELISA analysis of serially diluted samples indicated that a dilution of 1/50,000 could enable accurate comparison of a range of samples in the linear portion of the curve. A representative figure has been included in Figure S1.

Haemagglutination-inhibition (HAI) Assay

Diluted pre-immune or hyperimmune serum samples (1/400) were pre-treated with chicken red blood cells (cRBC) to remove non-specific agglutinins (2 hours, room temperature) and the HAI assay was performed using a previously described method [44]. Briefly, treated serum (10 μl) was serially diluted 1/4 in duplicate wells of a round-bottom 96-well plate before the addition of PR8 influenza virus (5 haemagglutination units in 30 μl). After 30 minutes incubation at room temperature, 0.5% (v/v) cRBC in PBS (30 μl) was added to each well and gently mixed. Plates were visualised over a light box after 45 minutes. The endpoint HAI titre was recorded as the highest dilution of serum that was able to completely inhibit the agglutination of cRBC by virus in duplicate wells. Potential non-specific inhibition was discounted by the use of receptor-destroying enzyme (Table S1).

Murine Model of Influenza Infection

Female 6–8 week old BALB/c mice were housed in PC2 defined pathogen-free conditions following institutional guidelines. Groups of 5 mice were administered high titre anti-HA ovine serum (1 ml) or PBS intraperitoneally, and challenged intranasally 24 hours later with 500 $TCID_{50}$ PR8 influenza virus (32 μl). In the treatment models, mice were challenged with virus twenty-four hours prior to intraperitoneal administration of whole or diluted serum or control PBS as a 1 ml injection. Weight and clinical score of the mice were monitored and mice were euthanased upon achieving 20% (w/w) weight loss.

Statistical Analysis

All statistical analysis was performed with GraphPad Prism V4.00 software.

Ethics Statement

All animal experiments were approved by the SA Pathology/CHN and, where appropriate, Primary Industries and Resources South Australia animal ethics committees. All experiments were conducted in accordance with National and Institutional ethical guidelines.

Results

Intraperitoneal or Subcutaneous Prime Immunisation Induces Similar Peak Anti-Haemagglutinin Antibody Titres

Generally, antigen emulsified with FA is administered subcutaneously over multiple sites. However, it has been previously demonstrated that intraperitoneal prime immunisation can yield significantly higher antibody titres [45,46]. Therefore, to directly compare these routes of prime immunisation, groups of sheep were immunised either subcutaneously (four sites) or intraperitoneally (bolus injection) with rHA emulsified in complete FA. Both groups were boosted subcutaneously every two weeks with rHA in incomplete FA for a total of five boosts.

Serum samples were collected fortnightly and the titre of anti-HA immunoglobulin G (IgG) was analysed by ELISA. Assessment of results by two-way repeated-measures ANOVA indicated no significant difference between antibody titres during the induction phase (weeks 2–12) of the immune response (Figure 1A). However when ELISA data from all time points including twelve weeks post-immunisation were analysed, a significant trend for higher antibody titres was observed in the subcutaneously primed group (Figure 1A; $P<0.05$), which may indicate a slower decline in antibody titre for this group. Importantly, neither group exhibited a significant difference in the ability of various serum dilutions to inhibit haemagglutination of PR8 in an HAI assay (Figure 1B). Based on these results, the subcutaneous prime immunisation was selected for all subsequent comparisons.

CoVaccine HT^{TM} Elicits Significantly Higher Ovine Anti-haemagglutinin Antibody Titres than Freund's Adjuvant

The experimental adjuvant CoVaccine HT^{TM} is an oil-in-water emulsion, which is designed with synthetic carbohydrate structures on squalane microdroplets and which produces proinflammatory responses through interaction with innate immune receptors, including TLR-4 [41,47]. This adjuvant offers the advantage of presenting amphipathic membrane target antigens in native formation due to the squalane-in-water formulation. It has shown efficacy in combination with a range of antigens including malarial antigens [48], influenza glycoproteins [47] and gonadatropin-releasing hormone [49]. In order to determine if CV is capable of inducing anti-HA antibody responses comparable to those generated with FA, sheep were primed with rHA either traditionally emulsified with complete FA or gently mixed with CV and boosted fortnightly with rHA in incomplete FA or CV respectively. Serum samples were collected fortnightly and analysed via ELISA and HAI assay.

Two-way repeated-measures ANOVA and post-test analysis indicated that CV elicited significantly higher antibody titres overall ($P<0.01$) and at multiple time-points post prime ($P<0.05$) compared to FA (Figure 2A). Consistent with the ELISA results, the CV group sera demonstrated significantly higher HAI capacity than those from the FA group ($P<0.05$; Figure 2B), suggesting that CV may be a good alternative to FA in future immunisation regimens.

Age of Sheep did not Significantly Alter the Ability to Generate High Anti- haemagglutinin Antibody Titres

It was hypothesised that younger sheep may produce higher antibody titres than older sheep due to a potential age-related decline in immunity in aged sheep. To assess the effect of sheep age on antibody output, sheep at nine months or three years of age were immunised subcutaneously with rHA either in complete/incomplete FA or CV as described above. Serum samples taken every two weeks were assessed by ELISA and HAI assays (Figure 3). Analysis of ELISA results by two-way repeated-measures ANOVA with Bonferroni post-tests revealed no significant difference between antibody titres overall, or at individual time points for serum produced in young or old sheep by administration of rHA emulsified in FA (Figure 3Ai) or CV (Figure 3Bi). Similarly, there was no significant difference in the

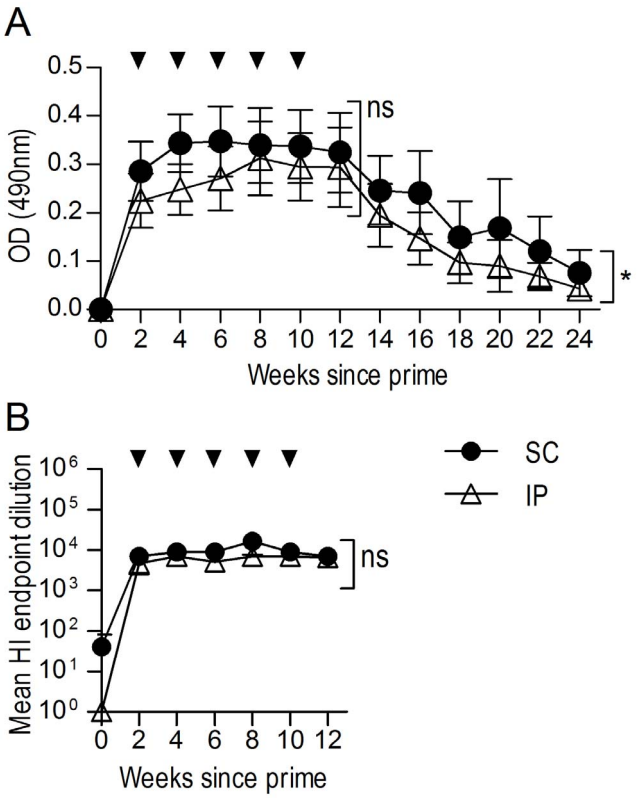

Figure 1. Different routes of prime immunisation yield similar anti-HA antibody titres. Sheep (n = 5) were immunised with 200 μg rHA SC or IP in complete FA. Sheep were boosted SC every two weeks to a total of five boosts in incomplete FA (indicated by arrows). Pre-immune (time 0) or hyperimmune serum samples were analysed for anti-HA IgG via ELISA (1/50, 000 dilution) (A), and HAI(B). Data are expressed as the mean ± SEM. Two-way repeated-measures ANOVA was applied to evaluate significance which is denoted as thus: * = P<0.05, ** = P<0.01, *** = P<0.001, ns = not significant.
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neutralising activity of anti-HA serum from different aged sheep in HAI assays in either the FA-immunised (Figure 3Aii) or CV-immunised groups (Figure 3Bii). Consistent with previous results, HAI titres for either old or young sheep immunised with rHA-FA emulsion were significantly lower (P = 0.001) than the corresponding CV group (Figure 3Aii and Figure 3Bii respectively). These results suggest that age does not impact on the quality of the antigen-induced antibody response.

A Ten-fold Lower Dosage of Antigen did not Yield Significantly Lower Anti-haemagglutinin Antibody Titres

Cost of antigen is an important factor in any immunisation regimen, therefore to determine whether economic gains may be made by immunisation with a lower dose of antigen, sheep were immunised subcutaneously with 200 μg or 20 μg rHA either in FA or CV as previously described. Serum samples taken every two weeks were subsequently analysed by ELISA and HAI assays (Figure 4). Statistical scrutiny revealed no significant difference in antibody titre between high-dose and low-dose FA groups as indicated by ELISA (Figure 4Ai) and HAI results (Figure 4Aii). In comparison, significant differences were observed within the CV groups in both the ELISA assay (Figure 4Bi; P<0.05) and HAI assay (Figure 4Bii; P<0.05), however the magnitude of the decrease in the low-dose group was comparatively small. Indeed,

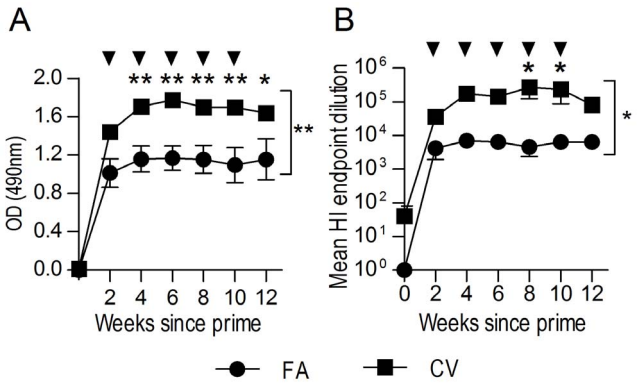

Figure 2. CoVaccine HT^{TM} adjuvant elicits significantly higher anti-HA antibody titres than Freund's adjuvant. Sheep (n = 5) were immunised SC with rHA (200 μg) in complete FA or CoVaccine HT^{TM} (CV). Sheep were boosted similarly every two weeks (five boosts indicated by arrows) with rHA in incomplete FA or CV. Pre-immune (time 0) or hyperimmune serum samples were analysed for anti-rHA IgG via ELISA (1/50, 000 dilution) (A) and HAI (B). Data are represented as the mean ± SEM. Two-way repeated-measures ANOVA with Bonferroni post-test was applied to evaluate significance which is denoted as thus: * = P<0.05, ** = P<0.01, *** = P<0.001, ns = not significant.
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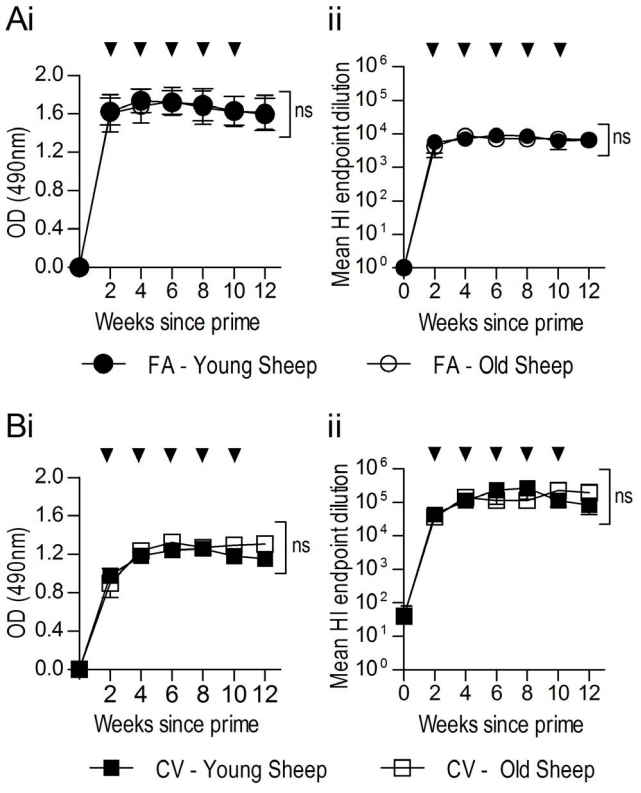

Figure 3. Nine-month old and three-year old sheep produce similar anti-HA antibody titres. Sheep (n = 5) at either nine months (young) or three years (old) were immunised SC with 200 μg of rHA in complete FA (A) or CV (B). Sheep were subsequently boosted SC every two weeks to a total of five boosts in incomplete FA or CV (indicated by arrows). Pre-immune (time 0) or hyperimmune serum samples were analysed for anti-rHA IgG via ELISA (1/50, 000 dilution) (Ai, Bi) and HAI (Aii, Bii). Data are expressed as the mean ± SEM. Two-way repeated-measures ANOVA with Bonferroni post-test was applied to evaluate significance; ns = not significant.
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analysis of individual time points by Bonferroni post-test comparisons revealed a significant difference in HAI titre at only one time point. Importantly, HAI results of CV group sera again revealed a more than ten-fold increase in mean endpoint serum dilution for both the high and low dose rHA over that observed with FA-induced sera (Figure 4Bii vs Figure 4Aii). These data supports the use of a lower antigen dose to elicit comparably high quantities of anti-HA antibody whilst reducing costs associated with antigen production.

CoVaccine HT^{TM} Induced Fewer Adverse Site Reactions as Compared to Freund's Adjuvant

As FA immunisation often induces site-specific reactions, it was necessary to determine whether CV similarly induced local inflammatory reactions at the immunisation site. Observational data were collected twelve weeks post-prime where the number and size of palpable subcutaneous lumps at each site was recorded, and sites were given a rating on a graded system (denoted in Figure 5). No significant differences in size or number of reactive sites were observed within adjuvant groups in the age and antigen dose experiments (data not shown); therefore observational data from all sheep receiving each subcutaneous immunisation regimen were pooled and compared. Statistical analysis by Mann-Whitney rank test revealed a significant difference between the number of reactive sites, with sheep receiving rHA in FA exhibiting significantly more reactive sites per animal, many at all injection sites, than those sheep receiving antigen mixed with CV (Figure 5A; $P<0.05$). Interestingly, there was a trend toward lower reactivity scores of CV sites than of FA sites (Figure 5B), although altogether, the variation of CV reactivity scores was greater than (SD = 1.750) than that of FA scores (SD = 1.136).

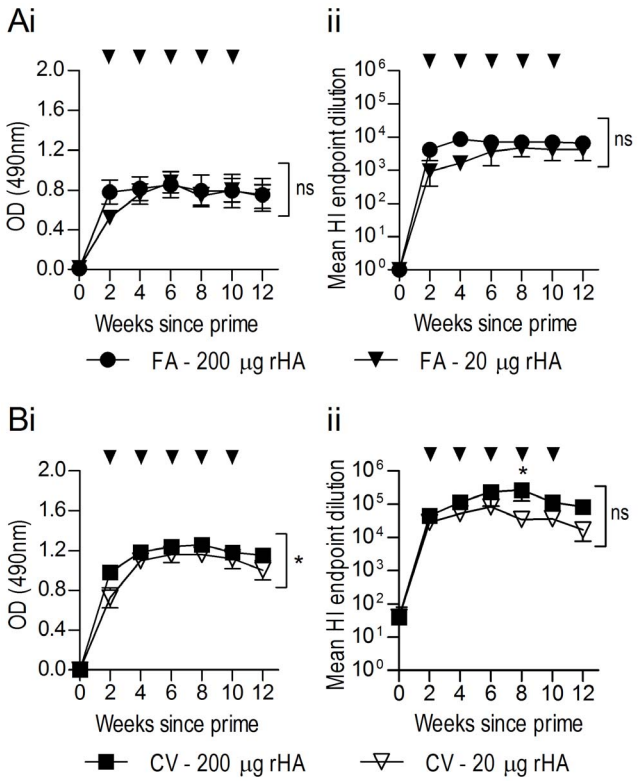

Figure 4. Low antigen dose produces similar anti-HA antibody titres. Sheep (n = 5) were immunised SC with 200 or 20 μg of rHA in complete FA (A) or CV (B). Sheep were then boosted SC every two weeks to a total of five boosts in incomplete FA or CV (indicated by arrows). Pre-immune (time 0) or hyperimmune serum samples at a 1/50,000 dilution were analysed for anti-HA IgG via ELISA (Ai, Bi) and HAI (Aii, Bii). Data are represented as the mean ± SEM endpoint dilution. Data of both assays were analysed by two-way repeated-measures ANOVA with Bonferroni post-tests; significance is denoted as thus: * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$, ns = not significant.
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Prophylactic and Therapeutic Administration of Anti-haemagglutinin Hyperimmune Serum Protected Mice from a Lethal Influenza Challenge

Even though hyperimmune serum consistently prevented influenza-induced haemagglutination of RBC *in vitro*, we wished to determine the *in vivo* potential of this serum to prevent lethal influenza infection. To assess the protective ability of polyclonal anti-HA antibodies, mice received pooled hyperimmune serum from sheep immunised with rHA emulsified in CV or FA, or pooled non-immune serum or PBS diluent as controls. Twenty-four hours later, mice were challenged with 500 50% tissue-culture infective doses ($TCID_{50}$) of live PR8 by intranasal administration and the clinical course was closely monitored (Figure 6A). Results indicated that mice which received prophylactic hyperimmune serum from either CV- or FA-immunised sheep exhibited some weight loss immediately after influenza infection, although this loss was quickly regained (Figure 6Ai and 6Aii respectively). In contrast, those mice that received non-immune serum or PBS had sustained weight loss and all had reached their pre-determined clinical endpoints by day 10 (Figure 6Aiii and 6Aiv respectively). Mantel-Cox survival analysis showed that both CV and FA hyperimmune serum prevented the lethal consequences of influenza infection in contrast to the effects of administration of non-immune serum or PBS (Figure 6Av; $P<0.001$).

To determine whether these polyclonal antibodies could treat an active influenza infection, hyperimmune or non-immune serum or PBS was administered twenty-four hours after viral challenge with 500 $TCID_{50}$ PR8 (Figure 6B). Similarly, results indicated that infected mice which received hyperimmune serum therapy

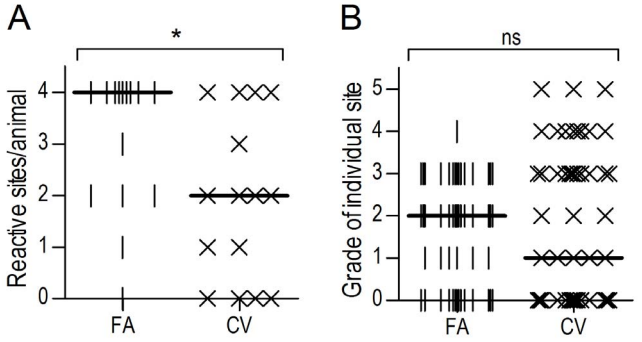

Figure 5. Repeated subcutaneous immunisation with CoVaccine HT^{TM} elicits fewer reactive immunisation sites than Freund's adjuvant. Sheep (n = 15) were immunised SC with rHA antigen either in complete/incomplete FA or CV. Two weeks following the final boost immunisation, injection sites were examined and palpable lumps were enumerated (A) and graded (B). A scoring system was devised based on the size and characteristics of the reaction sites to rank the level of reactivity of each individual site. Grades of site reactivity: 0– no reaction; 1– slight skin irregularity; 2– lump<10 mm diameter or larger skin irregularity; 3– multiple small lumps/single lump<40 mm; 4– lump<80 mm; 5–≥80 mm lump. The data was analysed by Mann-Whitney rank test; significance is denoted as thus: * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$, ns = not significant.
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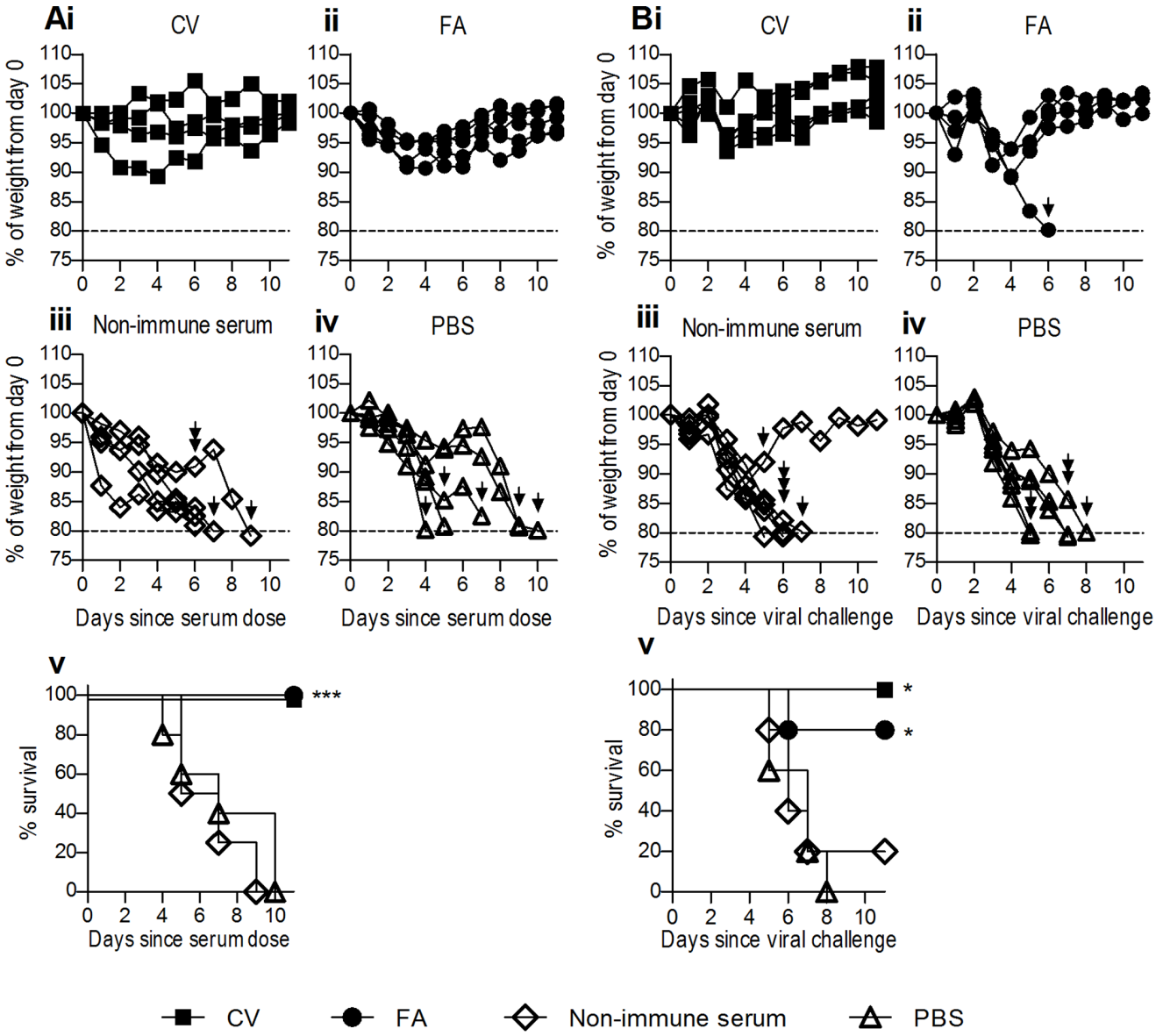

Figure 6. Prophylactic or therapeutic administration of ovine anti-HA serum is protective against lethal influenza challenge. Mice (n = 5) were prophylactically administered pooled serum (1 ml, IP) from either young sheep receiving 200 µg rHA SC in CV (Ai) or FA (Aii), day zero pre-bleeds from corresponding sheep from both groups (Aiii) or PBS as a control (Aiv). Twenty-four hours later mice were challenged with a lethal dose of PR8 (500 $TCID_{50}$). Mice reaching a predetermined endpoint of 20% weight loss (dotted line) were euthanased as indicated by arrows. Mice (n = 5) were challenged with 500 $TCID_{50}$ PR8 and twenty-four hours later therapeutically administered serum or PBS control as above (Bi–iv). In each panel, data show percentage weight loss of individual mice. Survival curves of mice are also shown (Av, Bv). Mantel-Cox survival analysis was performed on survival curves; significance between all curves is denoted as thus: * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$, ns = not significant.
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exhibited some weight loss before recovery (Figure 6Bi, 6Bii), whereas most mice that received non-immune serum or PBS had sustained weight loss and were euthanased by day 10 (Figure 6Biii, 6Biv). Similar to the effects of prophylactic administration of hyperimmune serum, Mantel-Cox survival analysis revealed a significant improvement in survival of the mice that received hyperimmune serum compared to control mice (Figure 6Bv; $P<0.05$). Taken together, it is clear that ovine hyperimmune serum can protect against and treat lethal influenza infections in mice.

Hyperimmune Anti-haemagglutinin Serum Elicited with CoVaccine HT™ Exhibits Greater Potency in vivo as Compared to that Elicited by Freund's Adjuvant

Anti-HA serum elicited with CV as an adjuvant had significantly better potency *in vitro* (Figure 2); to determine if this translated to increased potency *in vivo*, mice (n = 6) were intranasally inoculated with 500 $TCID_{50}$ PR8 influenza and twenty-four hours later treated with different doses of high titre pooled serum (IP, equivalent to 1000, 500, 250 or 50 µl serum in a 1 ml injection) elicited with either CV or FA. Groups of mice received PBS or non-immune sheep serum as controls. Clinical disease

course was monitored and euthanasia performed according to endpoints described above.

Most notably, mice that had therapeutically received CV serum were completely protected from lethality from infection (Figure 7Ai–Di), regardless of dose, whereas the lower doses of 250 µl to 50 µl of FA serum were not completely protective (Figure 7Cii–Dii). Furthermore, although the 500 and 1000 µl FA serum dose protected mice from lethality of infection (Figure 7Aii–Bii) weight loss in these mice was more severe than that observed in mice receiving equivalent doses of CV serum (Figure 7Ai–Bi).

Mantel-Cox survival analysis revealed a significant difference between survival curves of all of the CV serum mice and controls (data not shown). Comparison of the survival curves of FA and CV serum mice revealed a significant survival advantage of CV serum at a 50 µl dose (Figure 7Diii; $P<0.001$). A minimum dose of 500 µl of FA serum was required for complete protection whereas a ten-fold lower dose of CV serum also offered complete protection. These data are consistent with the HI results that demonstrate an approximate ten-fold higher neutralising antibody titre in serum from sheep immunised with antigen in CV as compared to FA.

Taken together, these data suggest that lower doses of CV-elicited sera provide protection against influenza and that implementation of this adjuvant in ovine polyclonal antibody production could dramatically increase the efficiency of eliciting functional neutralising antibodies in sheep.

Discussion

Ovine polyclonal antibodies are frequently generated for commercial and small laboratory scale applications, yet there has been limited investigation into maximising the amount of antibody generated through optimised ovine immunisation regimens. Within this study, methods to improve ovine antibody production were empirically investigated and demonstrated that such ovine polyclonal antibodies effectively bound influenza virus *in vitro* and prevented the death of mice infected with influenza virus.

The route of immunisation has been shown to influence the nature and intensity of the immune response generated to an antigen [29,45,46]. In the current study, subcutaneous prime immunisation with antigen in FA yielded significantly higher overall anti-HA antibody titres when compared to intraperitoneal prime. These findings are in contrast to an earlier study which demonstrated that anti-*C. parvum* antibody titres at parturition [45] were sixteen-fold higher in the serum when sheep received intraperitoneal immunisation compared to intramuscular immunisation. Furthermore, at twenty days post-lambing, detectable anti-*C. parvum* IgG had stabilised to approximately two-fold higher in the intraperitoneal-administered group. This discrepancy may be a consequence of altered immunological function in the sheep during pregnancy, or the differing nature of the antigens used [46]. It is possible that the rHA antigen is more efficiently presented by cells of the subcutaneous tissues than those in the peritoneal environment [28,29], as CD14-positive DCs present in the dermis are directly involved in the differentiation of antibody-producing plasma cells [50,51]. Furthermore, the hydrophobic properties of FA may enable the stable deposition of the antigen-adjuvant mixture at immunisation sites which is likely to be more favourable for long term immunity [52]. That is, the sheep subcutaneously primed may have had more local and stabilised antigen depots than the intraperitoneally-primed sheep as comparatively the peritoneal environment allows substances to disperse more freely.

The use of a novel experimental adjuvant CV was compared to FA, the adjuvant routinely used for the commercial and experimental production of polyclonal ovine antibodies. Interestingly, CV was found to raise significantly higher specific serum antibody titres than FA. The CV serum was also confirmed to exhibit higher potency *in vivo*. The enhanced efficacy of CV to induce high functional antibody titres may be partially attributable to the integrity of the antigen once mixed with this particular adjuvant. Protein antigens may be fully or partially denatured in water-in-oil adjuvants such as FA, which may lead to the deformation of native epitopes [53] resulting in antibodies with a poor affinity for the native protein. In contrast, CV is an oil-in-water emulsion of hydrophobic and negatively-charged sucrose fatty acid sulphate esters formulated with submicron emulsions of squalane that is combined with aqueous antigen and thus would result in less protein denaturation than FA [41]. The accessibility of the antigen to immune cells may also have contributed to increased efficacy of CV compared to FA. While the tendency of FA to form viscous deposits of antigen-adjuvant mixture in the tissues results in sustained slow-release of antigen, it may also result in limited antigen exposure to the cellular environment. In contrast, antigen mixed with CV is in an aqueous environment and this may facilitate dispersal into the tissues and increase the amount of antigen immediately accessible to antigen processing immune cells, thus increasing antibody titres. Indeed, recent preclinical studies have also shown that this adjuvant can enhance vaccine responses in not only sheep as described here but also in pigs [41], mice [42,47], ferrets [36] and macaques [43]. The likely enhanced dispersal of CV as compared to FA may also explain why CV induced significantly fewer reactive sites that FA, which importantly suggests a superior safety profile to FA. These observations highlight CV as a safer and highly effective potential alternative to FA for adjuvanting antigens, particularly when factoring in the time-consuming and often hazardous emulsification periods needed with FA and other water-in-oil adjuvants prior to administration. Furthermore, these advantages combined with the ability to elicit a high titre IgG antibody response in a broad range of other animal species, favours the future practical application of CV to the rapid and large scale production of passive immunotherapeutics for veterinary and clinical use.

There were no significant age-related differences in the anti-HA antibody titres achieved after immunisation with either CV- or FA-rHA. Depending on size and growth characteristics, the quantity of blood routinely obtained from younger sheep may be upwards of a third less than that obtained from adult sheep [54]. However, the ability to utilise younger sheep for commercial polyclonal antibody production means that significant increases in antibody output over the lifespan of the animal would be possible, thus offsetting agistment costs much sooner. This would be particularly important if younger sheep were employed together with adjuvants such as CV that increase overall antibody titre. The costs associated with ovine antibody production could be minimised further if high antibody titres could be maintained with reduced amounts of costly antigen. Here, we showed that a ten-fold lower dose of antigen than that used conventionally resulted in only slightly lower (albeit significantly lower with CV adjuvant) anti-HA antibody titres in sheep. However, the magnitude of this difference is unlikely to matter particularly when compared to the difference in the amount of antigen used and instead reflects the complex relationship between antigen dosage and antibody titre [33]. Furthermore, the optimal antigen dosage will be affected by different adjuvants and antigens with correspondingly different pathways used for antigen processing, presentation and antibody responses [55]. Our results clearly

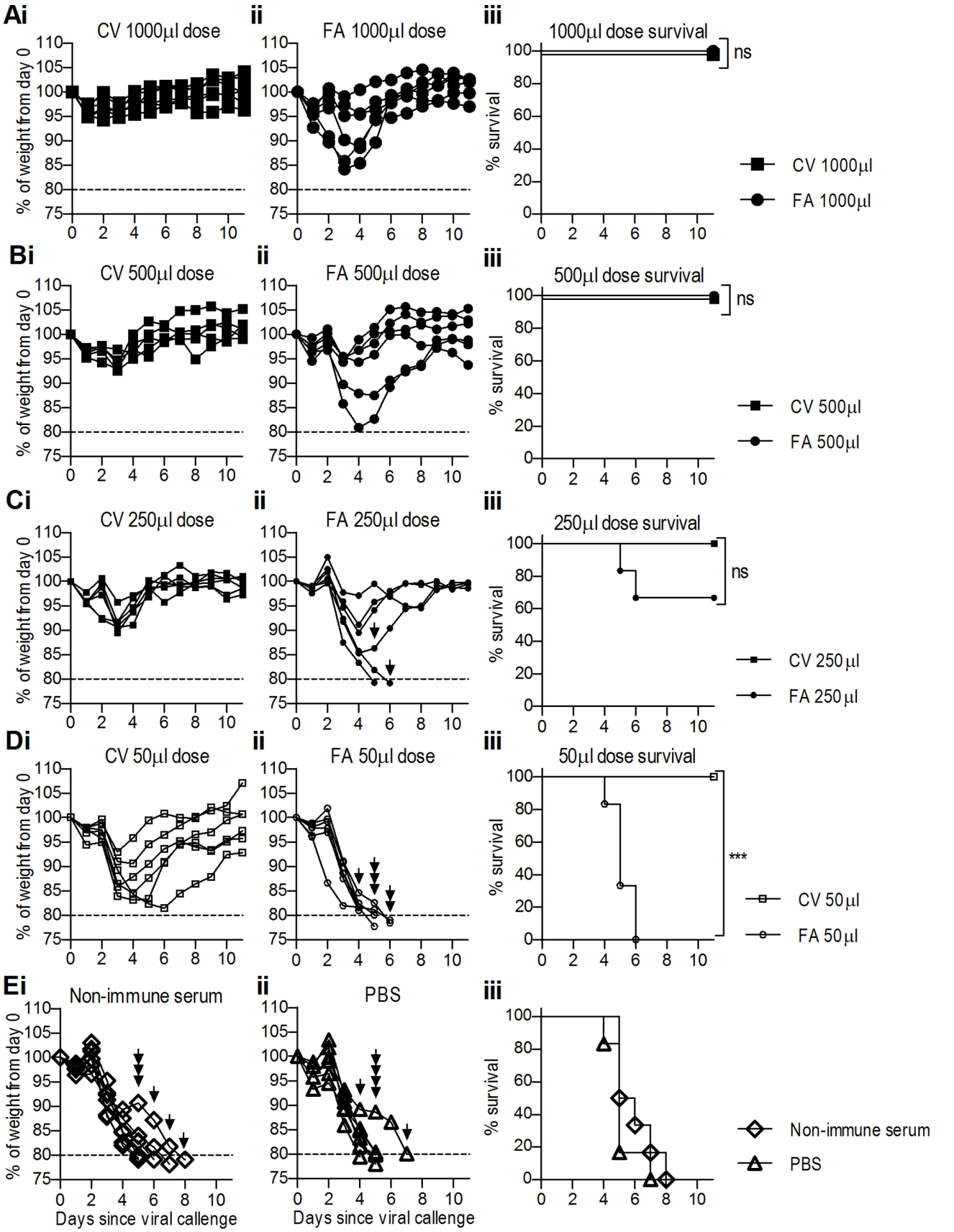
Ai
CV 1000µl dose
ii
FA 1000µl dose
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Figure 7. Ovine anti-HA serum elicited with CoVaccine HT^{TM} adjuvant show greater *in vivo* potency as compared to that by Freund's adjuvant. Mice (n = 6) were challenged with 500 $TCID_{50}$ PR8 and twenty-four hours later therapeutically administered dose equivalents of 1000 (A), 500 (B), 250 (C) or 50 µl (D) of pooled hyperimmune serum (1 ml, IP) from sheep that received rHA antigen in CV (i) or FA (ii). Control groups of mice (n = 6) received non-immune serum (Ei) or PBS (Eii) twenty-four hours following viral challenge. Mice reaching a predetermined endpoint of 20% weight loss (dotted line) were euthanased as indicated by arrows. In each panel, data show percentage weight loss of individual mice. Survival curves of mice are also shown (iii). Mantel-Cox survival analysis was performed on survival curves; significance between survival curves is denoted as thus: * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$, ns = not significant.
doi:10.1371/journal.pone.0068895.g007

indicate that the lower HA antigen dose induced anti-HA ovine antibody titres similar to the high titres induced with the higher antigen dose. Moreover, we expect that this reduced need for antigen will have the added benefit of markedly reducing production costs. Importantly, all immunisation regimens tested here resulted in the production of potent anti-HA antibodies that were able to treat and prevent lethal influenza infection in mice.

Antigenic variability among influenza strains remains one of the major challenges facing the development of an influenza-specific passive immunotherapeutic. However, it has been already been shown that it is possible to elicit strain cross-reactive anti-influenza hyperimmune serum by administering different subtypes of influenza haemagglutinin [56,57]. In addition, administration of an antigen (whether it be a whole pathogen or parts thereof) to a live animal benefits from 'natural selection' by the host for the most immunogenic targets and mimics the natural immune response to infection or vaccination [58]. The widespread application of passive immunotherapy to treat influenza may be of particular benefit during pandemic outbreaks of the infection, such as the recent 2009 H1N1 pandemic [59]. During an influenza pandemic, a premium is attached to the timely and large-scale production and distribution of an effective therapy. Knowing best how to rapidly make at least cost large quantities of potent ovine neutralising antibodies against influenza virus or other potential pandemic pathogens could be an important contribution to reducing the disease burden and its associated societal and economic costs.

Supporting Information

Figure S1 Preliminary ELISA of anti-HA ovine serum samples. An ELISA was performed on selected samples for assay development. Serial dilutions of pre-immune or hyperimmune sheep serum were added to duplicate wells of a rHA-coated ELISA plate and specific antibody was detected with HRP-linked anti-ovine IgG antibody. Signal was developed with OPD substrate until colour was visible in serum-free wells. Absorbance readings from blank wells were subtracted from all readings. The results indicated that a 1/50,000 serum dilution gave OD readings within the linear portion of the generated curve for hyperimmune samples. Consequently a 1/50,000 dilution was used in subsequent assays to assess experimental samples.
(TIF)

Table S1 Assessment of the effects of Receptor Destroying Enzyme on Haemagglutination-inhibition endpoint titres. In order to determine the effects of receptor-destroying enzyme (RDE) on endpoint HAI titres; selected serum samples were assayed with or without treatment with RDE (Sigma, 37°C, O/N). Samples were then treated with chicken red blood cells and assayed as described. Endpoint HAI titres were identical for all samples tested.
(DOCX)

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Author Contributions

Conceived and designed the experiments: JDH KRD. Performed the experiments: NES CKF. Analyzed the data: NES CKF KRD MA MPB JDH. Contributed reagents/materials/analysis tools: JDH MA. Wrote the paper: NES CKF MPB KRD JDH.

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SUPPLEMENTARY INFORMATION

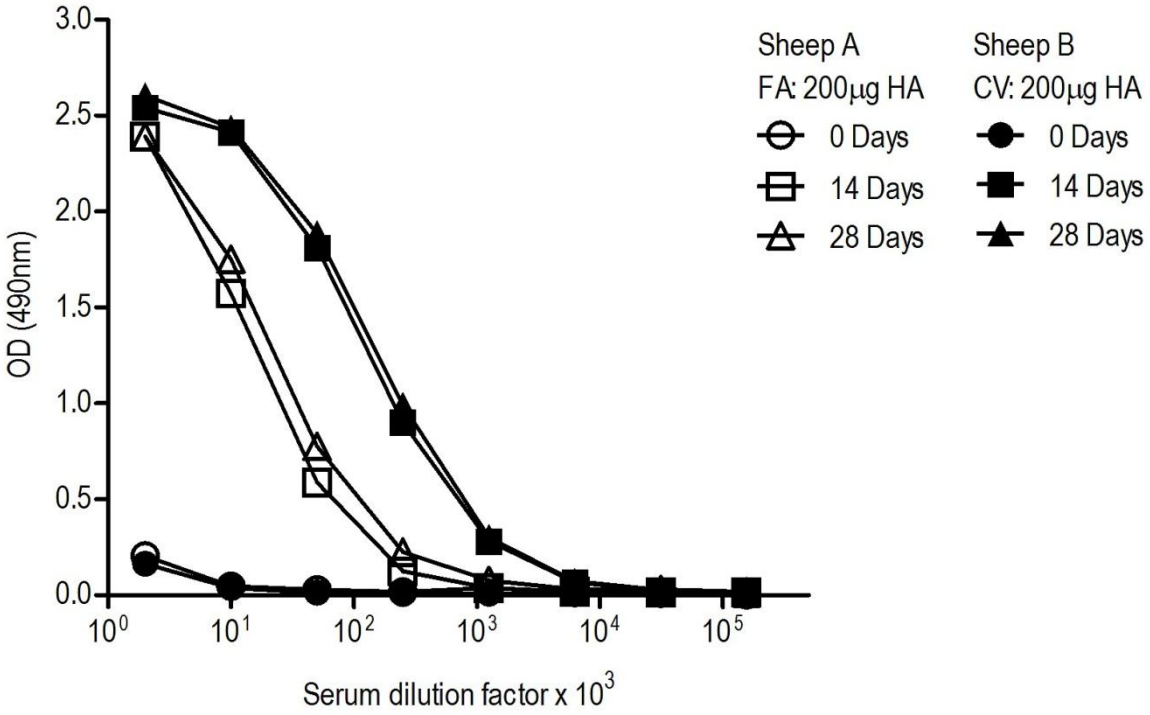


Figure S1: Preliminary ELISA of anti-HA ovine serum samples. An ELISA was performed on selected samples for assay development. Serial dilutions of pre-immune or hyperimmune sheep serum were added to duplicate wells of a rHA-coated ELISA plate and specific antibody was detected with HRP-linked anti-ovine IgG antibody. Signal was developed with OPD substrate until colour was visible in serum-free wells. Absorbance readings from blank wells were subtracted from all readings. The results indicated that a 1/50,000 serum dilution gave OD readings within the linear portion of the generated curve for hyperimmune samples. Consequently a 1/50,000 dilution was used in subsequent assays to assess experimental samples.

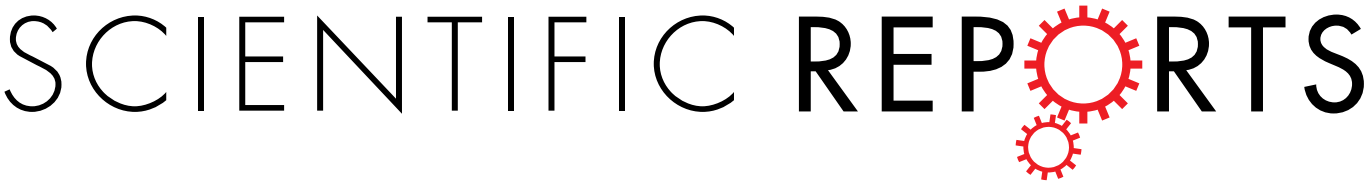
Table S1: Comparison of Haemagglutination-Inhibition endpoints with and without the use of receptor destroying enzyme

		+ RDE	- RDE
A – FA, 200µg HA	Day 0	1	1
	Day 14	3200	3200
	Day 42	3200	3200
B – CV, 200µg HA	Day 0	1	1
	Day 14	3200	3200
	Day 42	12800	12800
C – CV, 20µg HA	Day 0	200	200
	Day 14	800	800
	Day 42	12800	12800

APPENDIX C

Supporting Publication: Published

N.E. Stevens, A. Hatjopolous, C.K. Fraser, M. Alsharifi, K.R. Diener, and J.D. Hayball. (2016) Preserved antiviral adaptive immunity following neutralising polyclonal antibody immunotherapy for severe murine influenza infection. Scientific Reports. http://dx.doi.org/10.1038/srep29154



OPEN

Preserved antiviral adaptive immunity following polyclonal antibody immunotherapy for severe murine influenza infection

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Passive immunotherapy may have particular benefits for the treatment of severe influenza infection in at-risk populations, however little is known of the impact of passive immunotherapy on the formation of memory responses to the virus. Ideally, passive immunotherapy should attenuate the severity of infection while still allowing the formation of adaptive responses to confer protection from future exposure. In this study, we sought to determine if administration of influenza-specific ovine polyclonal antibodies could inhibit adaptive immune responses in a murine model of lethal influenza infection. Ovine polyclonal antibodies generated against recombinant PR8 (H1N1) hemagglutinin exhibited potent prophylactic capacity and reduced lethality in an established influenza infection, particularly when administered intranasally. Surviving mice were also protected against reinfection and generated normal antibody and cytotoxic T lymphocyte responses to the virus. The longevity of ovine polyclonal antibodies was explored with a half-life of over two weeks following a single antibody administration. These findings support the development of an ovine passive polyclonal antibody therapy for treatment of severe influenza infection which does not affect the formation of subsequent acquired immunity to the virus.

Influenza infection claims approximately 250,000 lives worldwide annually and remains a significant burden on public health systems[1]. The majority of deaths from influenza infection occur in at-risk populations which include the immunocompromised, infants and the elderly, all which exhibit suboptimal responses to vaccination[2,3] and are at higher risk of 'severe' influenza infection[4]. Severe influenza is defined as influenza infection accompanied by complications that require hospitalisation, and is attributed to over 3.4% of all critical hospitalisations during influenza season[1]. Current treatment for severe influenza generally incorporates supportive care and antiviral medications such as oseltamivir (Tamiflu™) or zanamivir (Relenza™)[5], however viral resistance to such medications is increasing, and is most often acquired by infective viruses during hospitalisation and treatment[6], which is a significant risk for immunocompromised patients hospitalised for extended durations[7].

Vaccination is the most widely-used strategy to combat the morbidity and economic burden of influenza, with severe infection generally associated with failure to vaccinate[8] or reduced efficacy of vaccination in immunocompromised populations[2,9]. Effective seasonal influenza vaccines elicit neutralising antibodies (Abs) against strain-specific glycoproteins, in particular haemagglutinin (HA) which is considered the major neutralising determinant of influenza[10]. However differences between the predicted HA incorporated into seasonal vaccines and the actual HA expressed by circulating strains can impact vaccine efficacy resulting in increased transmission and a higher burden of severe influenza infections[3,11]. It is clear therefore, that strategies beyond seasonal

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vaccination and antiviral medications should be explored to combat the morbidity and mortality of influenza infection, particularly during critical hospitalisation periods[12].

Passive immunotherapy using neutralising Ab may be the ideal rapid treatment strategy for influenza infection that functions independently of the individual's immunocompetency[12] as highlighted by previous animal and human studies[13–15]. Furthermore, a recent multicentre double-blind randomised controlled trial has found that hyperimmune intravenous immunoglobulin (IVIG) prepared from donors exposed to pandemic H1N1 influenza significantly reduced viral load in infected individuals compared to IVIG prepared from donations received before the 2009 pandemic[16]. In a similar manner, further studies have shown reduced mortality in patients treated with convalescent plasma[17]. Animal studies have shown efficacy of anti-influenza enriched IVIG preparations in protecting immunodeficient mice from morbidity and mortality following influenza infection[18], which indicates that this may be an effective strategy for use in immunocompromised patients. Despite clinical efficacy, the use of human-derived Ab therapies have significant logistical challenges including lengthy time periods for identification and screening of potential serum donors, and validation of sera and subsequent Ab products[19]. Consequently, current guidelines do not recommend IVIG or convalescent sera as a therapy for severe influenza[5]. Alternatively, passive immunotherapies utilising humanised monoclonal Abs (mAbs) have been developed for influenza prophylaxis and treatment, and whilst mAbs targeting HA have potent neutralising capacity, they are often not cross-reactive against multiple strains and may prompt the development of resistant mutants[20]. In contrast, mAb strategies that target highly conserved residues on the M2 ion channel protein are cross-reactive and demonstrate *in vivo* efficacy in experimental models[21] however these are generally thought to function through the instigation of antibody-mediated cell cytotoxicity rather than direct viral neutralisation[20,22]. As such, the efficacy of these mAbs in immunocompromised individuals may be reduced.

Polyclonal Abs (pAbs) can overcome these deficiencies by their intrinsic ability to target multiple epitopes, which can increase cross-reactivity and reduce the development of resistant strains[23,24]. Approaches using pAbs sourced from animal sera are advantageous as large amounts of pAb can be collected and batched for reduced variability. Furthermore, pAbs have simplified screening procedures compared to human-derived Abs. Ovine pAbs are particularly efficient and cost-effective to produce, and exhibit reduced immunogenicity compared to those sourced from horses or other large mammals[25]. Though ovine-origin products carry a risk of contamination with transmissible prion proteins, sourcing of ovine serum from countries free from known prion disease such as Australia and New Zealand can remove this risk, and thus commercially available ovine pAb products are commonly sourced from regions such as these. Ovine pAb fragments have been used with success in the clinic, and are currently available as critical care therapies for life-threatening digoxin toxicity (DigiFab™, DIGIBIND™)[26] and crotalid snake envenomation (CroFab™)[27].

We have previously shown that ovine serum pAbs elicited against HA have potent neutralising capacity *in vitro* and are protective in an *in vivo* murine influenza model[13]. However, any Ab administered to prevent or treat influenza must not prevent the formation of adaptive immunity necessary for enduring protection against reinfection with future circulating strains, and this has not been explored in the context of foreign pAb delivery. Therefore the aims of this study were to explore the role of ovine anti-HA pAb therapy in a mouse model of severe influenza and to assess the effect on subsequent influenza-specific adaptive responses. It was demonstrated that ovine purified pAb treatment can effectively reduce mortality in the murine model and does not impede the development of protective humoral or cell-mediated responses to influenza.

Methods

Animals. Female 6–8 week old BALB/c mice were purchased from Laboratory Animal Services, The University of Adelaide and housed in IVCs under specific pathogen-free facility conditions with food and water provided *ad libitum*. All animal experiments were approved by the University of South Australia Animal Ethics Committee, and conducted in accordance with National and Institutional ethical and regulatory guidelines.

Purification of ovine polyclonal anti-HA from serum. Sheep were immunised with baculovirus-produced recombinant HA (rHA) derived from PR8 influenza with serum stored at −20 °C as previously described[13]. Thawed serum was filtered through glass wool and pAb precipitated by addition of saturated ammonium sulfate solution to 45% (v/v). After centrifugation (20 min, 10,000 × g) pelleted protein precipitate was dissolved in milliQ water and dialysed into PBS before application to a Protein G agarose column (Thermo Fisher Scientific). Bound IgG was eluted with 0.1 M glycine (pH 2.5) and dialysed into PBS before concentration determination via BCA assay (Thermo Fisher Scientific) according to manufacturer's instructions. Concentrated IgG was subsequently analysed for purity via SDS-PAGE. Control serum was sourced from non-immunised sheep and total IgG purified as described.

Murine model of influenza infection. Groups of mice were administered purified pAbs (25 mg/kg) or PBS either via intraperitoneal (IP) injection (500 μL) or intranasal (IN) inhalation (32 μL) under anaesthetic. This dose was selected empirically from a pilot study (data not shown). For severe influenza infection, mice received IN challenge (32 μL) with PR8 H1N1 (500 $TCID_{50}$) or A/PC H3N2 (3000 $TCID_{50}$) influenza virus that had been purified from hen egg amniotic fluid as previously described[28]. Viral inocula were diluted on the day of use from freshly thawed viral stocks and untreated controls were run in parallel with all treated groups to ensure that the dose of virus administered caused an infection requiring euthanasia within 6–10 days. All challenged mice were provided with soaked food and sunflower seeds to support them through the associated pain and distress of infection, and were assessed daily for clinical symptoms of infection which included weight loss, ruffled coat, wheezing or abnormal respiration, hunched posture and reluctance to move. A clinical score of 5 or 20% weight loss was used to identify those mice reaching predetermined endpoints requiring euthanasia. Weight loss was the primary indicator for euthanasia in this study as most mice did not reach clinical thresholds.

Detected Analyte	Sample	Coating protein	Coating Concentration	Detection Ab	Concentration	Depicted in
Murine anti-PR8 IgG	Murine sera	PR8 virus	500 $TCID_{50}$/mL in 100 mM $NaHCO_3$	HRP-linked rabbit anti-murine IgG (Sigma; Cat #A9044)	1:10,000 in 1% BSA PBS-T	Figure 3c
Ovine IgG in circulation	Murine sera	Purified donkey anti-sheep IgG (Sigma; Cat # SAB3700721)	5 μg/mL in 100 mM $NaHCO_3$	HRP-linked anti-sheep IgG (Sigma; Cat # A3415)	1:15,000 in 1% BAS PBS-T	Figure 4a
Murine anti-ovine IgG	Murine sera	Ovine pAb	10 μg/mL in 100 mM $NaHCO_3$	HRP-linked rabbit anti-murine IgG (Sigma; Cat # A9044)	1:10,000 in 1% BSA PBS-T	Figure 4b
Ovine anti-PR8 IgG	Ovine pAb	PR8 virus	500 $TCID_{50}$/mL in 100 mM $NaHCO_3$	HRP-linked anti-sheep IgG (Sigma; Cat # A3415)	1:15,000 in 1% BAS PBS-T	Figure 5a
Ovine anti-A/PC IgG	Ovine pAb	A/PC virus	500 $TCID_{50}$/mL in 100 mM $NaHCO_3$	HRP-linked anti-sheep IgG (Sigma; Cat # A3415)	1:15,000 in 1% BAS PBS-T	Figure 5a

Table 1. Antibodies used for ELISA assays. ELISA protocols were developed to assess the levels of murine anti-PR8 IgG, circulating ovine IgG or murine anti-ovine IgG in sera from infected or treated mice, or to assess the ability of ovine IgG to bind to PR8 and A/PC viruses.

Haemagglutination-inhibition assay (HAI). All HAI assays were performed as previously described[13]. Briefly, purified pAb (500 μg/mL; 30 μL) was pipetted into duplicate wells of a round-bottom 96-well plate and serially diluted two-fold in PBS before the addition of either PR8 influenza virus (5 haemagglutination (HA) units in 30 μL) to all wells. After 30 minutes incubation at room temperature, 0.5% (v/v) chicken red blood cells (cRBC) in PBS (30 μL) was added to each well and gently mixed. Plates were visualised over a light box after 45 minutes. The endpoint HAI titre was recorded as the highest dilution of test sample that was able to completely inhibit the agglutination of cRBC by virus in duplicate wells.

ELISA analysis. Specific ELISA methods were employed to measure binding of anti-HA Abs against PR8 or A/PC virus and to quantify circulating anti-influenza Abs, anti-ovine Abs or ovine pAbs in mouse sera. Combinations of coating and detection Ab are summarised in Table 1. Briefly, EIA/RIA high-binding ELISA plates (Corning) were coated with 100 μL of the relevant coating protein diluted in 100 mM $NaHCO_3$ buffer (pH9) and incubated overnight at 4 °C. Plates were blocked with 2% (w/v) BSA in PBS (1 hour, 37 °C) and the relevant samples diluted 1:2 for Ab quantification, or serially diluted for endpoint analysis in 1% (w/v) BSA in PBS with 0.05% Tween (PBS-T). After 3 washes with PBS-T (200 μL), diluted samples or serially diluted ovine pAb standards (32,000–2 ng/mL in PBS-T) were added to duplicate wells (100 μL) before incubation (2 hour, 37 °C). Plates were washed as before and bound serum Abs were detected by addition of the relevant detection Ab (100 μL) followed by incubation for 1 hour (37 °C). The plates were subsequently developed with OPD substrate (Sigma; 100 μL), the reaction stopped with 3 M HCl (50 μL), and the absorbance read at 490 nm. For endpoint ELISA methods, endpoint absorbance values were defined as the mean absorbance plus two standard deviations of negative control samples diluted 1:100.

***In vivo* cytotoxic lymphocyte (CTL) assay.** The ability of mice to mount a cell-mediated response to influenza infection was assessed using an *in vivo* cytotoxicity assay. Splenocytes from a donor mouse were harvested and RBC subsequently lysed. Washed splenocytes were pulsed with K^d-restricted influenza nucleoprotein-derived peptide TYQRTRALV (NPP) before two washes in endotoxin-free PBS followed by CellTrace™ Far Red staining (0.1 μM) for 20 minutes at 37 °C. Cells were subjected to two more washes and mixed at a 1:1 ratio with unpulsed control cells stained with CFSE (3 μM; 10 minutes at 37 °C). Optimal staining was confirmed by flow cytometry (BD FACSCanto II) before transfer into infected or control mice via tail vein injection (8–10 million cells per mouse). Spleens from recipient mice were harvested 18 hours later and single cell preparations were assessed for percentage of FarRed and CFSE cells remaining via flow cytometry and FACSDiva software. Percent cell specific lysis was calculated using the following equation: % specific lysis = [1−(infected mouse $\%CFSE^{pos}$: $\%FarRed^{pos}$/ control mouse $\%CFSE^{pos}$: $\%FarRed^{pos}$)] × 100.

Statistical analyses. Statistical analyses were performed using GraphPad Prism V5 software. Survival curves were compared using the Mantel-Cox (log-rank) test. ELISA endpoints and *in vivo* CTL data were compared using two-tailed unpaired T-tests. Statistical significance was defined as $P \leq 0.05$.

Results

Prophylactic administration of ovine anti-HA pAbs protects mice against lethal influenza infection. Previous studies have established the neutralisation capacity of generated whole ovine anti-HA antisera *in vitro* and *in vivo*[13], however it remained necessary to confirm the potent neutralising capacity of the now purified IgG *in vivo.* To assess this, groups of mice were intraperitoneally administered purified ovine anti-HA pAbs, control pAbs, or PBS, and twenty-four hours later challenged intranasally with a lethal dose of PR8 influenza. Mice that had received anti-HA pAbs exhibited reduced weight loss (Fig. 1a) and mild signs of infection from days 2–6 (Supplementary Figure S1) and variable weight loss after day 7. However, all resumed weight gain after day 11, and none required euthanasia. In contrast, administration of control ovine pAbs or PBS could not control influenza infection which led to rapid weight loss from day 3 and required euthanasia by day 6 (Fig. 1b,c respectively). Thus a statistically significant survival advantage was conferred to the group of mice that received anti-HA ovine

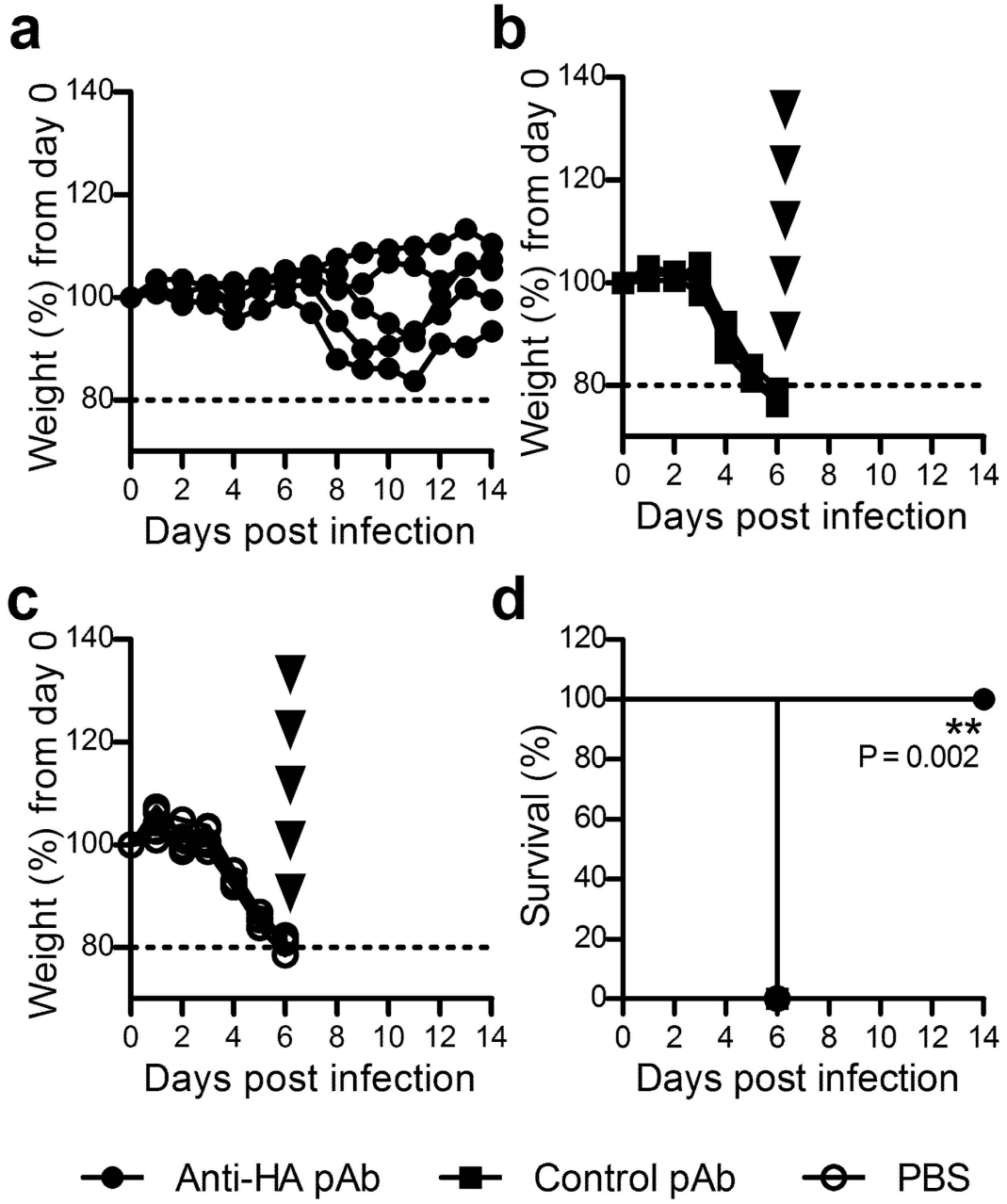

Figure 1. Administration of ovine anti-HA pAbs protects against subsequent lethal influenza infection. Mice were prophylactically administered (25 mg/kg in 500 μL) ovine anti-HA pAbs (**a**), control pAbs (**b**) or PBS (**c**) via the intraperitoneal route. Twenty-four hours later mice were challenged with 500 $TCID_{50}$ (32 μL) PR8 influenza. The mice were closely monitored for weight loss (**a–c**) and euthanized according to predetermined humane endpoints (indicated by arrowheads). Data is presented as the weight loss of individual mice from infection day (n = 5). Corresponding survival curves (**d**) were analysed using the Mantel-Cox test to compare anti-HA treated to PBS treated and control treated mice. Significance is denoted as thus: ** = $P < 0.01$.

pAbs (Fig. 1d). These results clearly demonstrated the capacity of prophylactically-administered ovine anti-HA pAbs to completely protect against the fatal effects of a lethal influenza virus infection *in vivo*.

Ovine anti-HA pAbs can reduce mortality in an established influenza infection. The use of passive Ab immunotherapy to treat influenza infection would be of greatest benefit if administered as soon as possible after exposure, however in practice a diagnosis would not normally be made until after clinical symptoms were observed. Viral shedding in this murine model peaks two days after infection[29,30] which correlates with an established infection in humans triggering symptom development[31]. Therefore, the therapeutic capacity of ovine anti-HA pAbs were assessed 48 hours after influenza challenge. Furthermore, as the route of administration can impact the effectiveness of Ab therapies[32], two different routes were employed for this study. Whilst therapeutic administration of pAbs did not completely protect mice from reaching humane endpoints requiring euthanasia

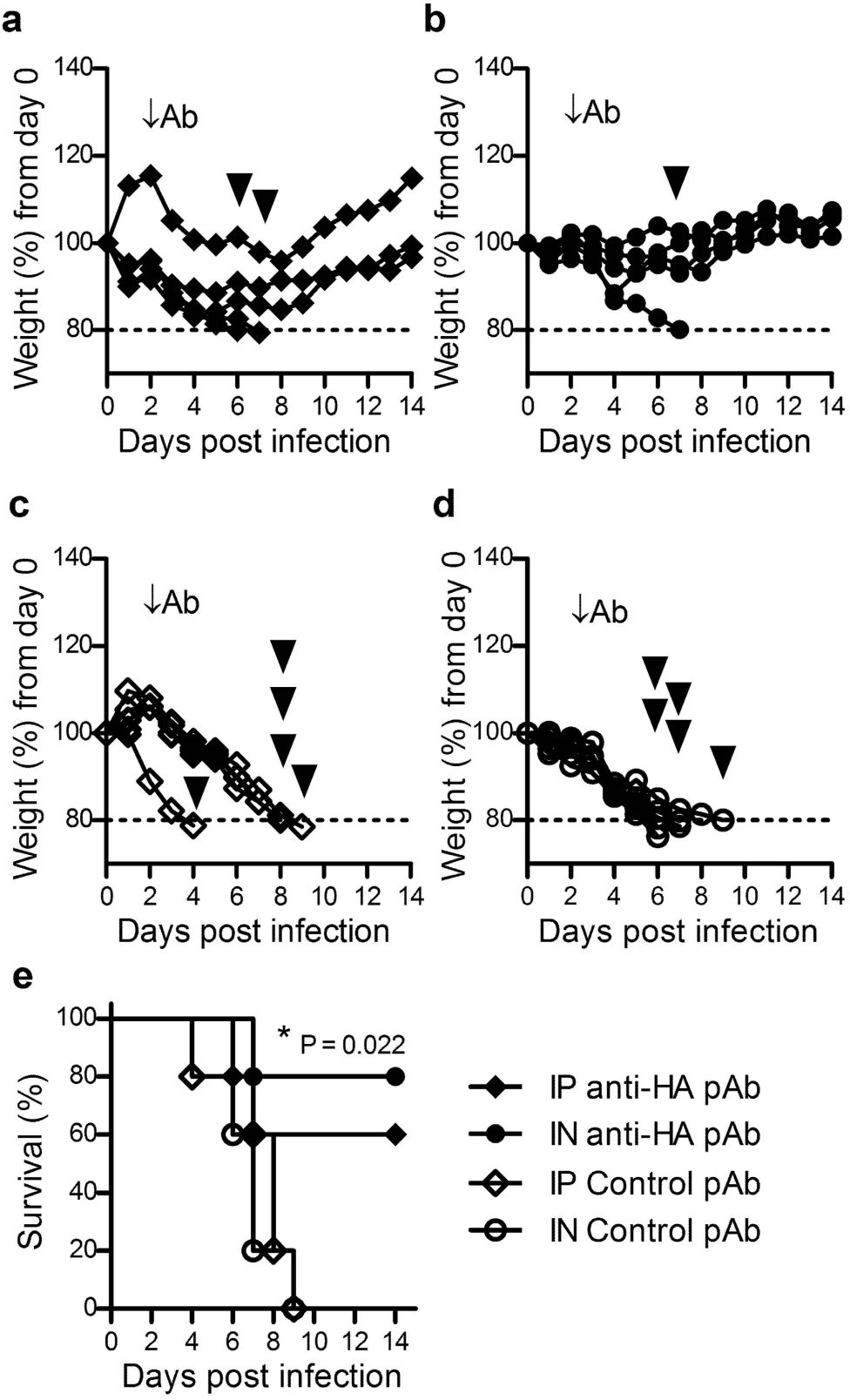

Figure 2. Ovine anti-HA pAb can treat established influenza infection. Mice were intranasally infected with 500 $TCID_{50}$ (32 uL) PR8 influenza. Forty-eight hours later mice were administered anti-HA pAbs (25 mg/kg) via intraperitoneal injection (**a**), or intranasal administration (**b**). Control groups were intraperitoneally (**c**) or intranasally (**d**) administered control pAb. The mice were closely monitored for weight loss (**a–d**) and euthanized according to predetermined humane endpoints (indicated by arrowheads). Data is presented as weight loss of individual mice from infection day (n = 5). Survival curves (**e**) of treated groups were compared to control groups using the Mantel-Cox test. Significance is denoted as thus: * = $P < 0.05$.

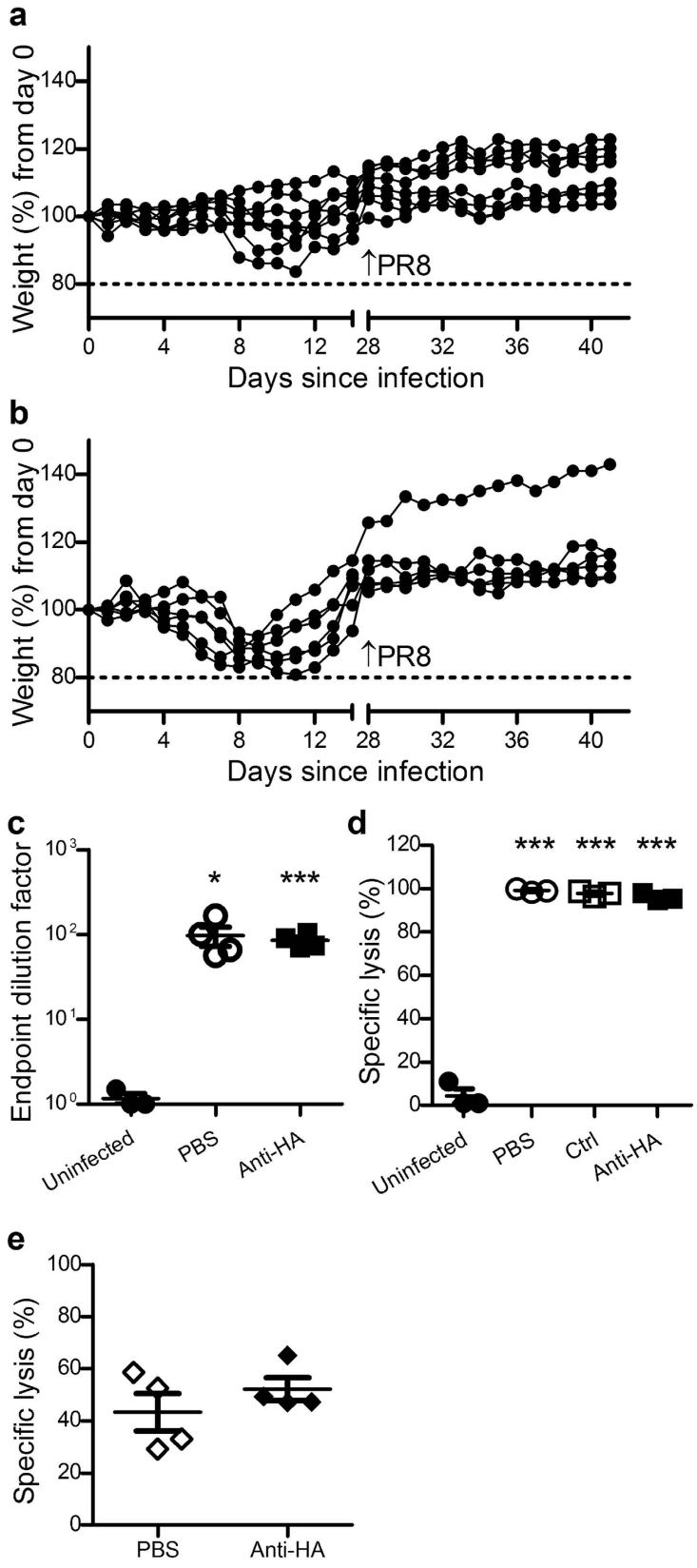

Figure 3. Prophylactic administration of anti-HA pAb does not inhibit the formation of protective adaptive immune responses. Mice that had survived influenza challenge via prophylactic intraperitoneal administration of anti-HA pAbs (25 mg/kg, n = 8; (**a**) or survived a sub-lethal challenge of 50 $TCID_{50}$ PR8 influenza (n = 6; (**b**), were intranasally administered a lethal influenza dose of 500 $TCID_{50}$ (32 μL) PR8 Influenza four weeks after their initial challenge. Data is presented as individual percent weight loss (**a–b**) depicting combined data from two separate experiments. For assessment of adaptive responses, serum samples from

challenged mice prophylactically administered anti-HA pAbs or saline, or uninfected mice, were analysed by ELISA for PR8-specific IgG (**c**). For CTL responses, mice were prophylactically administered ovine anti-HA or control pAbs, or PBS and twenty four hours later infected with $250TCID_{50}$ PR8 influenza. Nine days later mice received 8–10 million CTL target cells IV and were assessed for specific killing capacity 18 hours later (**d**). Mice infected with A/PC influenza were also subjected to *in vivo* CTL as described (**e**). Data are representative of mean ± SEM (c–e). Ab titre and CTL data were compared using two-tailed Student's t test; experimental groups were compared to uninfected controls. Significance is denoted as thus: * = $P < 0.05$, *** = $P < 0.001$.

after infection, those mice that received ovine anti-HA pAbs via intraperitoneal injection (Fig. 2a) or intranasal administration (Fig. 2b) exhibited reduced weight loss compared to control mice that received control pAbs delivered systemically (Fig. 2c) or intranasally (Fig. 2d). These observations were also reflected in the corresponding clinical scores (Supplementary Figure S2). Of note, an unusual pattern of weight loss was observed in one mouse administered Ab via intraperitoneal injection (Fig. 2a), however no physiological anomaly was detected in this mouse while normal signs of infection were present and thus all the data pertaining to this mouse has been included. Analysis of overall survival (Fig. 2e) indicated significantly increased survival in intranasally-treated mice compared to the control groups ($P = 0.022$), although a survival benefit was also suggested in the intraperitoneal anti-HA pAb treated mice ($P = 0.142$). In a similar manner, mice that received antibodies by the intranasal route exhibited reduced severity in clinical symptoms of infection compared to antibodies delivered intraperitoneally (Supplementary Figure S2). These results support the development of a passive immunotherapeutic to treat established influenza infection, with treatment potentially more potent when administered intranasally.

Anti-HA pAb treatment does not inhibit the formation of adaptive immune responses. A passive immunotherapeutic approach to treat severe influenza infection should not prevent the formation of endogenous antigen-specific immune responses against the infective strain. To investigate whether treatment with ovine anti-HA pAbs impacts on the development of long-term protective immunity, a rechallenge model was developed. Mice were prophylactically administered anti-HA pAbs twenty-four hours before administration of a lethal dose of PR8, while a second experimental group received a sub-lethal dose of PR8 with no pAb treatment. All mice recovered from infection and were rechallenged on day 28 with a lethal dose of PR8. Mice that had received initial protection by ovine anti-HA pAbs administration, survived a second lethal challenge without exhibiting the clinical signs associated with infection (Fig. 3a). The sub-lethal control group also survived a second lethal challenge without any significant morbidity (Fig. 3b). To ascertain the adaptive immune responses that may have contributed to this sustained protection, samples collected fourteen days post-infection from anti-HA pAb and PBS administered mice were assessed for PR8-reactive murine IgG via ELISA. Equivalent Ab responses were evident in anti-HA and PBS treated groups and were significantly higher than in uninfected animals (Fig. 3c). To measure cytotoxic T lymphocyte (CTL) responses, mice administered anti-HA pAbs, control pAbs or PBS were infected with a sub-lethal dose of PR8 influenza and an *in vivo* CTL performed ten days later. CTL responses to HA-specific targets from anti-HA pAb, control pAb and PBS treated groups were all of similar intensity, and all significantly higher than uninfected controls (Fig. 3d). However, since previous studies have shown that the formation of adaptive immunity to influenza is influenced by the number of viral particles[30], and since anti-HA pAbs act to directly neutralise virions, responses between pAb-treated and untreated mice may have been confounded by this difference in viral load. To investigate this possibility, an alternate influenza strain with a disparate HA residue was employed. Mice were prophylactically administered anti-HA pAb or PBS and challenged with a sublethal dose of A/PC (H3N2) influenza with analysis of CTL responses performed ten days later. No significant difference in *in vivo* killing capacity between the groups was observed (Fig. 3e), indicating that the reduction in influenza symptoms, and thus viral load, did not impact on the generation of CTL responses in this model. Further confirmation was obtained by analysing serum samples from preliminary studies in which different doses of anti-HA pAb were administered prior to influenza infection for the development of murine anti-PR8 Abs (Supplementary Figure S3). There was no trend towards increased or decreased Ab responses in mice that received higher quantities of anti-HA Abs.

Ovine anti-HA pAbs are immunogenic and are more efficiently cleared following a second administration. In the event of an influenza epidemic, a passive immunotherapeutic approach targeting influenza would ideally provide protection for several weeks. In order to evaluate the longevity of ovine anti-HA passive immunotherapy, the half-life of circulating ovine pAbs was determined. Two groups of mice were dosed with anti-HA pAbs via intraperitoneal injection and administered a second dose eight weeks later. No observable morbidity was associated with the initial dose of the ovine pAb or re-dosing. Serum samples were collected every 14 days on a staggered timeline to achieve weekly measurements and analysed for the presence of ovine Abs, as well as the induction of murine anti-ovine IgG Abs as a measure of anti-treatment response. Seven days after the Ab administration, circulating ovine Abs (Fig. 4a) were detected at a maximum mean concentration of 700 pg/mL, which slowly declined over 5 weeks with a calculated half-life of 2.44 weeks. After a second pAb administration, ovine Abs were cleared more rapidly with the calculated half-life decreasing to 1.5 weeks. The development of murine anti-ovine IgG Abs was shown to occur 1–2 weeks after ovine pAb administration (Fig. 4b), with levels plateaued after three weeks. A second ovine pAb dose further increased murine anti-ovine IgG Ab levels, which suggested that the anti-treatment response contributed to the decreased half-life of circulating pAbs following a second administration.

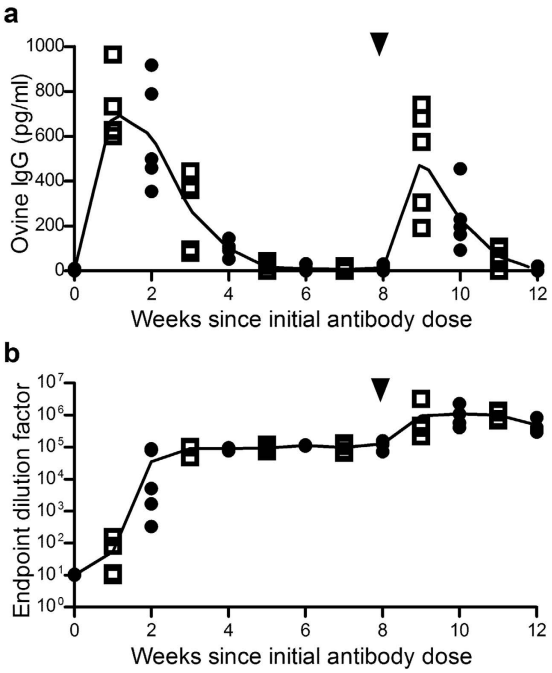

Figure 4. Kinetics of ovine pAb clearance and development of anti-treatment response. Two groups of BALB/C mice (n = 5) were administered ovine anti-HA pAbs (25 mg/kg) at day zero and eight weeks later (indicated by arrowheads), and bled every 14 days in two staggered timelines. Circulating ovine IgG was quantified via ELISA (individual values and mean depicted; (**a**) and mouse anti-ovine IgG response was quantified via endpoint ELISA (individual values and mean depicted; (**b**). Data is representative of two independent experiments.

Ovine anti-HA pAbs cross-react with an antigenically distinct influenza strain *in vitro*, but are unable to effectively protect against infection *in vivo*. A polyclonal approach towards influenza-specific passive immunotherapy has the potential for cross-protective capacity of the administered pAbs against circulating variants of the infective strain, and in some cases, different strains of influenza[33]. Here, the ovine anti-HA pAbs were generated against full length PR8 (H1N1) HA, and cross-reactivity against an antigenically distinct H3N2 strain (A/PC) was assessed *in vitro* and *in vivo*. Purified anti-HA pAbs were shown to be capable of binding both viral strains via ELISA (Fig. 5a), suggesting that the production of ovine anti-HA pAb by immunisation of sheep with PR8 yielded some Abs capable of binding to A/PC. However, a haemagglutination-inhibition assay performed as a measure of direct neutralisation capacity against each strain clearly indicated that whilst the generated Abs were able to inhibit PR8-induced agglutination at a maximum of 10^{13} dilution, they were not able to inhibit agglutination by the A/PC viral strain at relatively low dilution of eight (Fig. 5b). In order to explore these conflicting results further, an *in vivo* infection model of A/PC influenza was employed where mice were administered anti-HA or control pAbs twenty-four hours before a lethal dose of A/PC. The mice administered control pAb exhibited sustained weight loss and succumbed to infection (Fig. 5c), as did those administered anti-HA pAb (Fig. 5d), thus indicating a lack of protection of anti-HA pAbs against A/PC influenza infection (Fig. 5e). These results taken together indicate that whilst the generated anti-HA pAbs are capable of binding to the A/PC strain *in vitro*, they are likely to have bound to sites on the A/PC HA that are not involved in host cell binding and entry, and therefore does not translate into protective capacity.

Discussion

The number of people at higher risk of developing severe influenza complications is increasing due to the ageing population and expanding global burden of chronic disease. While vaccination and antiviral medications remain vital tools for the reduction of influenza mortality, vaccination in particular exhibits reduced efficacy in populations most vulnerable to severe infection leaving a significant number of deaths still attributed to influenza[4,34]. Therefore additional strategies to manage influenza infections are required. An influenza specific pAb with potent neutralising capacity may be particularly useful for severe influenza therapy as they exhibit rapid tissue penetration[23] and could provide rapid specific protection to immunocompromised individuals who are at high risk of mortality. Additionally, polyclonal Abs boast straightforward and cost effective preparation methods[24] and a good safety profile when administered to individuals with decreased immune capabilities[35]. Within this study, a potential role for passive immunotherapy via administration of influenza-neutralising ovine pAbs for the treatment of severe influenza infection was explored.

The efficacy of neutralising anti-HA pAb of human origin for protection against or treatment of influenza infection has been demonstrated in both animal[15,18,36,37] and clinical[14,16,17] studies. Previous studies, including our own, have demonstrated that hyperimmune anti-HA of ovine origin is efficacious in reducing mortality in a

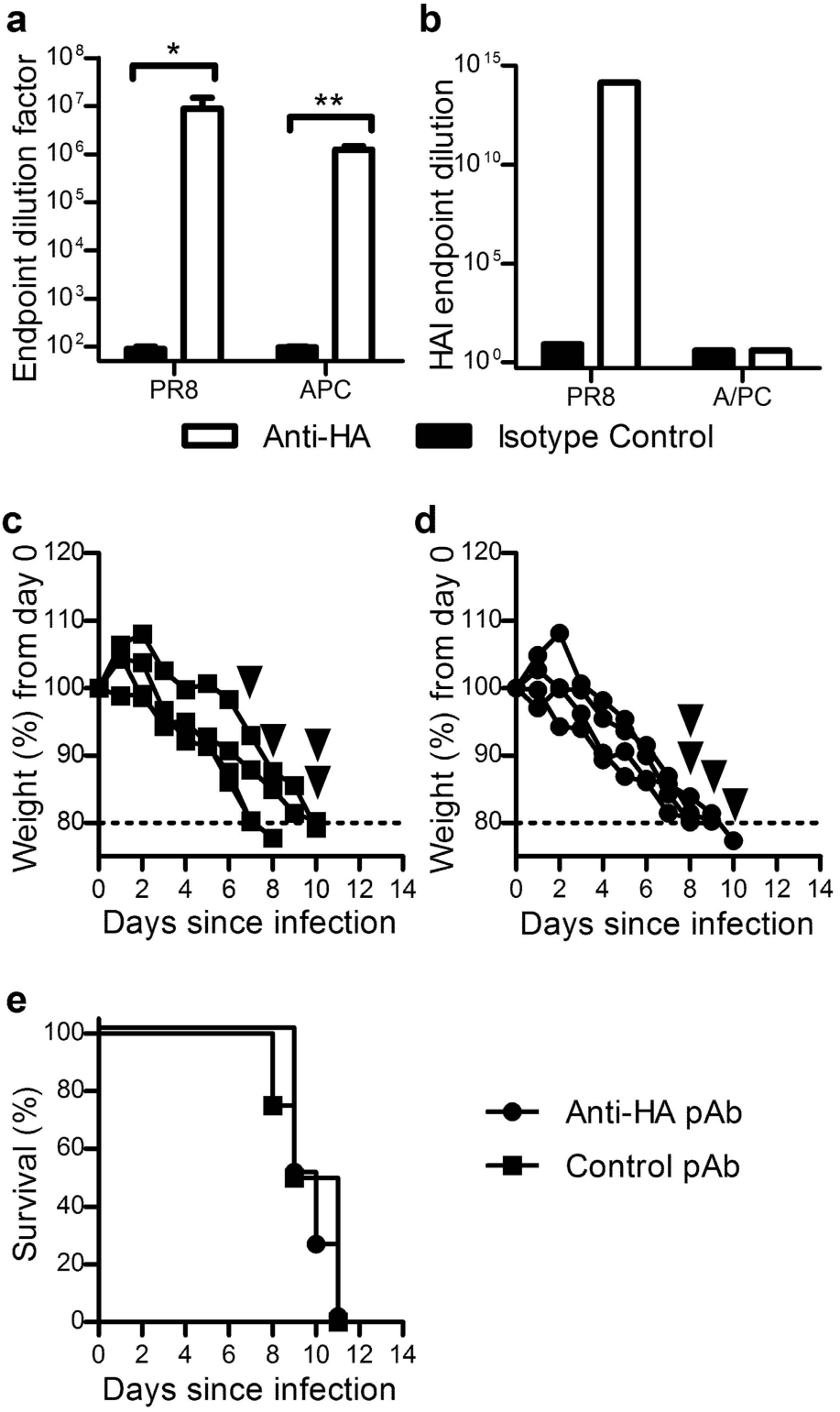

Figure 5. Cross-reactive ovine anti-HA pAbs do not exhibit neutralising capacity *in vitro* or protective capacity *in vivo* against a different influenza strain. Purified ovine anti-HA pAbs were analysed for reactivity against PR8 H1N1 and A/PC H3N2 influenza strains via ELISA and haemagglutination inhibition (HAI) assays. Absorbance values from ELISA results (mean ± SEM; (**a**) were analysed via Mann-Whitney analysis. Endpoint HAI titres are depicted in (**b**); data are representative of one assay performed in duplicate. To determine *in vivo* protective capacity against A/PC influenza, mice were administered either control pAb (**c**) or anti-HA ovine pAb (**d**) IP (25 mg/kg) and 24 hours later infected intranasally (32 μl) with A/PC (3000 $TCID_{50}$). The mice were monitored closely and euthanized if 20% weight loss or a clinical score of 5 was achieved (indicated by arrowheads). Weight loss of individual mice (**c,d**) and group survival (**e**) are presented. Significance is denoted as thus: * = $P < 0.05$, ** = $P < 0.01$.

murine influenza model[13,15] and this study confirmed that purified IgG from hyperimmune ovine serum retained protective and therapeutic capacity *in vivo* and *in vitro* (Figs 1 and 5). Administration of ovine pAb prior to infection reduced the severity of infection and reduced mortality compared to control mice. Importantly, administration of pAbs at 48 hours after challenge when peak viral shedding is observed was able to reduce mortality, with the intranasal route potentially more effective (Fig. 2). As viral shedding is higher in patients with comorbidities[38], this was an important time point to assess. The ability to neutralise an established infection is crucial for a potential passive immunotherapeutic, as influenza-specific medication can only commence after the first symptoms of infection or positive blood test. In this therapeutic study, only those mice that received pAb intranasally exhibited a significant difference in clinical symptoms and overall survival from control groups. This is consistent with previous studies which showed that intranasal administration of anti-influenza Abs provided a survival advantage by significantly reducing clinical symptoms, weight loss and lung viral titres[15,37,39]. As this route of administration was also associated with higher Ab concentrations within the lung, it was postulated that a higher proportion of the delivered Abs were able to directly interact with virions in the nasal passages, as opposed to being distributed systemically via intraperitoneal injection before reaching the lungs[39]. Furthermore, previous studies, including our own with hyperimmune serum (Supplementary Figure S4), have indicated that intranasal Ab therapy requires less delivered Ab than systemic methods for the same outcome and can increase the longevity of protection[39,40]. These findings have important implications for the cost effectiveness of pAb products, which are often prohibitively expensive[41], and may allow lower effective doses to be use and more affordable treatments. Intranasal pAb administration is also compatible with whole Ab and Ab fragments, which has been demonstrated experimentally for influenza[42]. Clinically, intranasal delivery of pAb products has been well tolerated and can provide adequate prophylaxis against respiratory infection[43] though only intravenously administered human mAbs[21,44] and IVIG[16] have been effectively used for influenza treatment. Studies using an experimental human influenza model found that intravenous administration of anti-M2e Mab TCN-032 reduced symptom development and viral load when administered twenty-four hours following inoculation[21], though whether intranasal administration may increase efficacy of treatments such as these remains to be investigated. Whilst intransally administered pooled immune globulin has been used unsuccessfully in the treatment of other respiratory infections[45], no targeted Ab products have been trialled clinically for intranasal treatment of respiratory infections, including influenza, and remains to be investigated.

The current study also explored the effect of influenza-specific passive immunotherapy on the development of adaptive immune responses, which are important for protection against reinfection in at-risk populations. Previous studies have demonstrated that the presence of circulating influenza-specific Abs can inhibit humoral responses to a subsequent influenza vaccination in piglets[46] and ferrets[36]. In the context of this study where ovine pAbs were assessed in murine influenza, the presence of foreign Abs might have favourable or non-favourable impacts on the generation of virus-specific responses. For example, Ab may effectively 'mask' available antigen from the immune system and prevent the generation of adaptive responses, as observed with the prevention of haemolytic disease of the newborn when pregnant mothers are prophylactically administered anti-D before sensitisation to the foetal D antigen can take place[47]. Alternatively, immune complexes formed *in situ* following administered foreign Ab may have an adjuvant-like function, as observed with historical use of antigen-antibody complexes to stimulate adaptive immunity[48]. In the current study, mice surviving infection via ovine anti-HA pAb administration exhibited sustained protection against influenza (Fig. 3) and symptoms of infection were largely absent upon reinfection. Influenza-specific antibody and cell mediated responses in mice administered pAb before infection were also assessed and both responses were found to be consistent with untreated groups. As symptoms of infection upon initial challenge were still evident even if administered anti-HA pAbs, it is postulated that in this setting passive immunotherapy served to neutralise some of the viable virions, effectively reducing but not eliminating viral load. Administration of passive pAb therapies clinically would occur only in an established infection, with a high viral load, thus viral-specific Abs would serve to attenuate rather than completely neutralise the infection. However, with a comparatively low viral load an alternate outcome may occur, and indeed studies have demonstrated that immunosuppressive effects are often present when Ab is administered in excess of antigen[49]. In the context of influenza, the use of ovine pAb in a sublethal model of PR8 infection (with a five-fold lower viral dose than the current study) was associated with reduced adaptive responses when infection was prevented via pAb administration one hour prior to viral challenge. However unlike our study where pAb were administered twenty-four hours prior to or forty-eight hours post challenge, in this scenario, virions were likely neutralised before attachment to host cells and antigen engagement by the immune system and thus the formation of an endogenous anti-PR8 immune response was prevented.

As part of the evaluation of an ovine pAb approach towards severe influenza treatment, within the current study the longevity and immunogenicity of passively administered pAbs in a murine model were assessed. The observed half-life of administered pAb was over two weeks and decreased after a second pAb administration. Correspondingly, anti-ovine responses which were evident from 1–2 weeks increased after re-administration and these likely contributed to the increased pAb clearance following re-administration. Though the administered ovine pAb were immunogenic, no hypersensitivity reactions were observed after pAb administration in this study. However, such reactions following administration of pAb of animal origin have been observed in the clinic[25]. Formulation of therapies as Fab or $F(ab)_2$ fragments, thus removing the F_c portion, serve to reduce this risk but do not eliminate it. As such, administration of ovine pAb fragment therapies is closely monitored and appropriate precautions, including readiness with intravenous epinephrine and antihistamines, are taken in patients with a history of sensitivity to sheep proteins or previous administration of ovine pAbs[50]. Administration of ovine pAb also did not impact on the development of influenza-specific immunity in this model. Other studies have indicated that passive immunotherapies can perform an immunogenic 'adjuvant-like' function and increase adaptive immune responses to their targets. For instance, the clinical efficacy of intravenous immunoglobulin (IVIG) against a wide variety of conditions has been linked to immunomodulatory properties potentially via engagement

of Fc-gamma immunoglobulin receptors[51]. An immunomodulatory adjuvant effect has also been observed in candidate anti-D mAbs developed for prevention of hemolytic disease of the newborn which enhanced rather than prevented endogenous anti-D immune responses[52]. It was proposed that Ab glycosylation may be the key to these adjuvant functions and this is supported by studies examining carbohydrate-dependent effector function of Abs[53]. In the context of influenza, these effector-functions could be harnessed to develop passive Ab therapies that not only neutralise influenza virions but enhance host anti-viral immunity, potentially via the formation of immunogenic immune complexes and engagement with Fc-gamma receptors. While no enhancement of adaptive responses was associated with ovine pAb administration in this study, whether whole ovine pAb are capable of instigating immunomodulatory effects through interaction with human Fc receptors remains to be explored.

While influenza-specific mAb therapies have been shown to be efficacious in animal[32,39,54] and human[21] studies, these monoclonal therapies may not be neutralising against a diverse range of viral strains[22] and may prompt escape mutants to be generated[10,55]. A major advantage of a polyclonal approach to influenza treatment is the ability to target multiple epitopes to discourage escape mutants and engineer therapies that neutralise multiple strains of influenza. Within this study, pAbs were generated against a single recombinant HA from PR8, H1N1, and shown to have *in vitro* binding capacity against a H3N2 strain (A/PC) via ELISA analysis. However the pAbs did not exhibit functional neutralisation capacity against this strain (Fig. 5b) and thus were not cross protective. It may be that the H3N2-reactive pAbs evident by ELISA are targeted towards epitopes distant from the sialic-acid binding site responsible for host cell binding *in vivo* and haemagglutination *in vitro.* As pAbs in this study were elicited against full length monomeric HA, and fragmented or denatured virions may have been present in the ELISA method, it is possible that cross-reactive Abs were binding to stem region epitopes that were exposed in ELISA but are not exposed in trimerised HA on whole virus[56]. These non-neutralising Ab therefore did not impact on the course of infection in an *in vivo* H3N2 model (Fig. 5c–e). While cross-protective capacity between two strains was not evident within this study, strategies to increase cross-protective capacity could be employed for the generation of anti-influenza polyclonals. Such strategies could include immunisation of sheep with multiple influenza strains, repeated boosting and the application of superior adjuvants not approved for human use or simply combination of antisera elicited against different strains[23,57,58].

The current study provides proof-of-principle for development of a passive immunotherapeutic based on ovine pAb for the treatment of established severe influenza infection. It also provides evidence that pAb therapies will not reduce adaptive anti-influenza responses which are crucial to ongoing protection from influenza infection. Further studies are however required to assess the *in vivo* viral neutralisation capacity of the therapy, particularly in the context of pAb fragmentation for clinical use. Through further development, strategies to decrease Ab immunogenicity, increase cross-protective capacity or increased immunomodulatory functions of the Abs could be employed to produce a purpose-built passive pAb therapy capable of controlling life-threatening influenza infections in immunologically-deficient populations.

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Author Contributions

N.E.S. performed experiments, analysed the data and wrote the main manuscript text. A.H. performed experiments and analysed data. C.K.F. provided intellectual input for the project. M.A. provided reagents and intellectual input for the project. K.R.D. edited and contributed to the main manuscript text and provided intellectual input. J.D.H. supervised the project, provided intellectual input, edited and contributed to the main manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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SUPPLEMENTARY INFORMATION

Preserved antiviral adaptive immunity following polyclonal antibody immunotherapy for severe murine influenza infection

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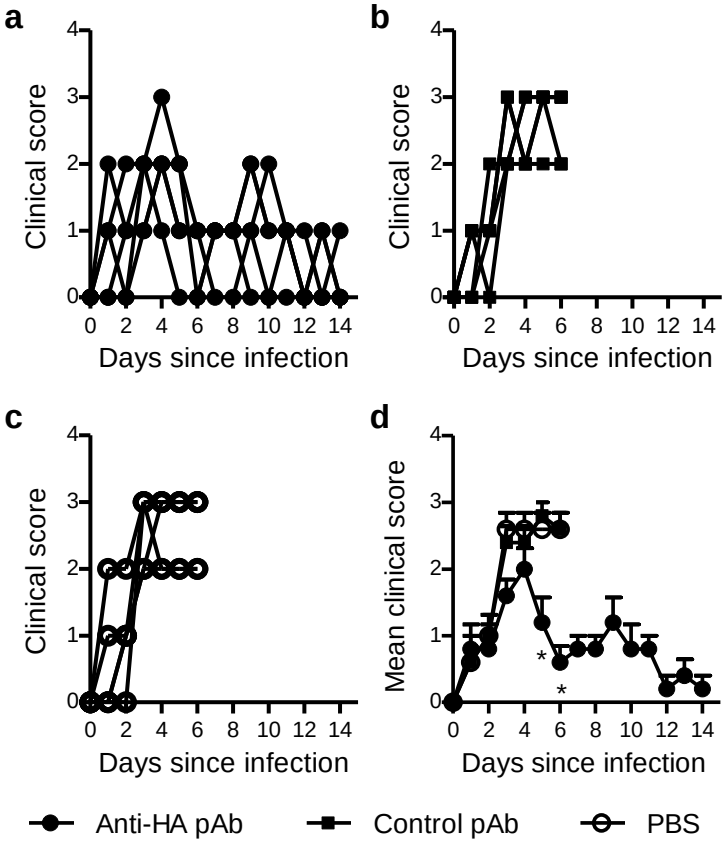
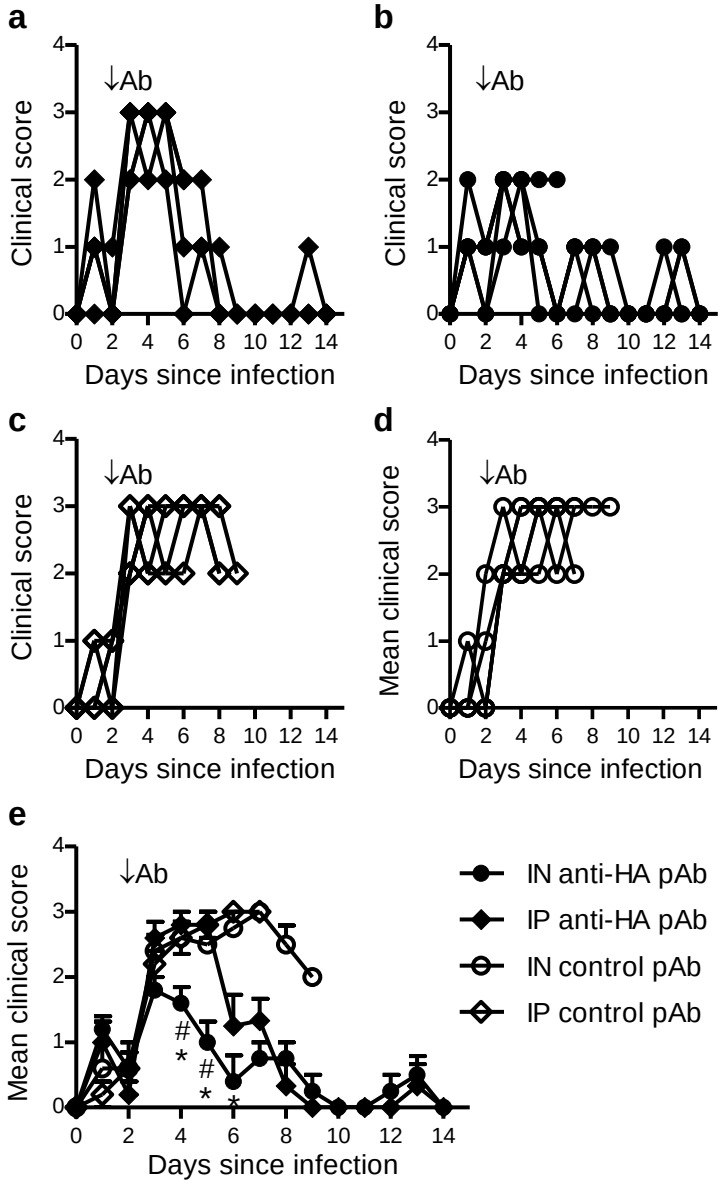
SUPPLEMENTARY FIGURE S1



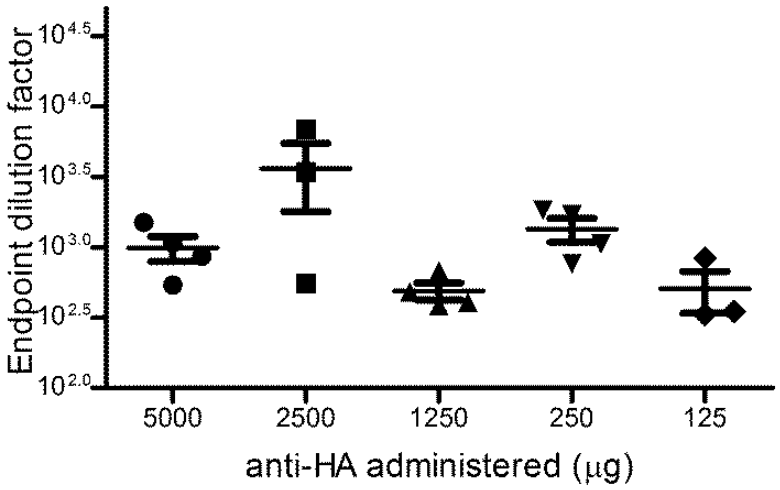
Supplementary Figure S1: Prophylactic anti-HA pAb intraperitoneal administration reduces symptoms of influenza infection. Mice (*n* = 5) were administered (a) ovine anti-HA or (b) control pAbs (25 mg/kg), or (c) PBS, via intraperitoneal injection twenty four hours prior to infection with 500 $TCID_{50}$ PR8 influenza. Mice were monitored for disease progression and attributed a clinical score daily. Scores of individual mice (a-c) and mean group scores (d) are depicted. Mean scores of anti-HA pAb administration group on days 5 and 6 were compared to control groups via Mann-Whitney analysis, where * = p>0.05.

SUPPLEMENTARY FIGURE S2



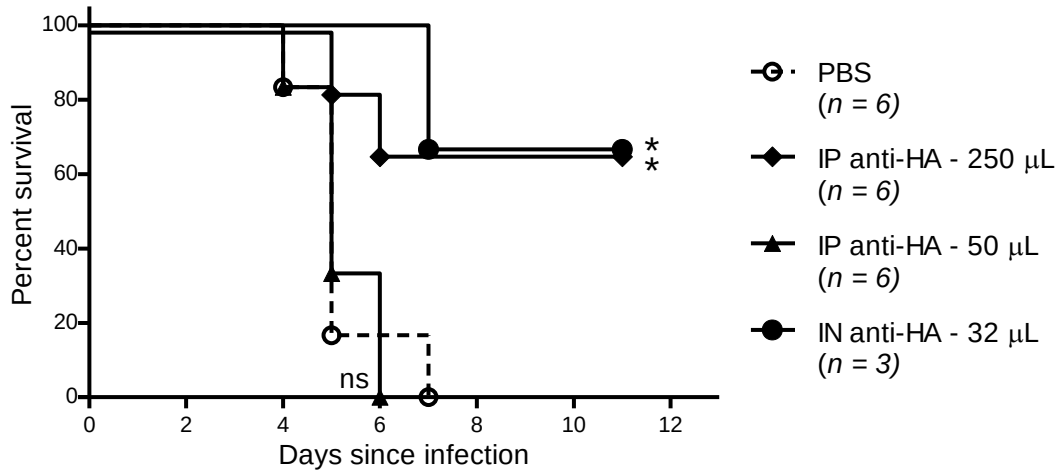
Supplementary Figure S2: Intranasal administration of anti-HA pAbs significantly reduce the clinical symptoms of influenza infection. Mice (*n* = 5) were infected with 500 $TCID_{50}$ PR8 influenza and forty-eight hours later were administered ovine anti-HA pAbs (a, b) or control pAbs (c, d) (25 mg/kg) via intraperitoneal injection (a, c) or intranasal instillation (b, d). Mice were monitored for disease progression and attributed a clinical score daily. (e) Group scores of the intranasal administration group on days 4, 5 and 6 were compared to control Ab administration and intraperitoneal anti-HA administration via Mann-Whitney analysis, where * = p>0.05 compared to control groups, # = p>0.05 compared to IP anti-HA group.

SUPPLEMENTARY FIGURE S3



Supplementary Figure S3: Passively administered anti-HA pAbs do not inhibit the generation of murine anti-PR8 Abs upon influenza infection. Mice were intraperitoneally administered ovine serum containing anti-HA antibodies to the indicated dose, and twenty-four hours later were infected intranasally (32 μL) with 500 $TCID_{50}$ PR8 Influenza. Mice were bled 14 days following infection and anti-PR8 IgG titres were evaluated via endpoint ELISA. Data are presented as mean ± SEM.

SUPPLEMENTARY FIGURE S4



Supplementary Figure S4: Effective influenza treatment can be achieved from a lower dose of anti-HA hyperimmune serum administered through an intranasal route compared to an intraperitoneal route in a murine influenza model Mice (*n* = 3-6) were infected with 500 $TCID_{50}$ PR8 influenza and twenty four hours later were intraperitoneally administered anti-HA hyperimmune serum or PBS. Mice were monitored and those reaching a predetermined endpoint of 20% weight loss were euthanized. Survival curves of treatment groups are depicted. Mantel-Cox survival analysis was performed to compare treatment groups to PBS administration group; significance between survival curves is denoted as thus: * = P<0.05, ns = not significant.

APPENDIX D

Supporting Publication: Accepted

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Research paper

CoVaccine HT™ adjuvant is superior to Freund's adjuvants in eliciting antibodies against the endogenous alarmin HMGB1

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ABSTRACT

Adjuvants are used to enhance the immune response against specific antigens for the production of antibodies, with the choice of adjuvant most critical for poorly immunogenic and self-antigens. This study quantitatively and qualitatively evaluated CoVaccine HT™ and Freund's adjuvants for eliciting therapeutic ovine polyclonal antibodies targeting the endogenous alarmin, high mobility group box-1 (HMGB1). Sheep were immunised with HMGB1 protein in CoVaccine HT™ or Freund's adjuvants, with injection site reactions and antibody titres periodically assessed. The binding affinity of antibodies for HMGB1 and their neutralisation activity was determined *in-vitro*, with *in vivo* activity confirmed using a murine model of endotoxemia. Results indicated that CoVaccine HT™ elicited significantly higher antibody tires with stronger affinity and more functional potency than antibodies induced with Freund's adjuvants. These studies provide evidence that CoVaccine HT™ is superior to Freund's adjuvants for the production of antibodies to antigens with low immunogenicity and supports the use of this alternative adjuvant for clinical and experimental use antibodies.

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1. Introduction

The passive administration of antibodies (Abs) has been effective in rapidly targeting foreign antigens and inflammatory mediators to limit the progression of potentially life threatening and/or debilitating infections and diseases. This practice has extended to direct pathogen and toxin neutralisation, reversal of drug toxicity, depletion of immune effector cells or molecules as well as the inhibition of host cell function in cancer therapy (Tolstrup et al., 2006; Casadevall, 1996, 1999; Tallman, 2002; de Cassan et al., 2011; Buonaguro et al., 2011). This approach is also widely applicable to complex disease scenarios such as autoimmune disease (Harrison, 2008; Keijzer et al., 2013), inflammatory diseases (Kazatchkine & Kaveri, 2001), neurodegenerative disorders (Brody & Holtzman, 2008; Schwartz & Kipnis, 2001; Fisher et al., 2001) as well as traumatic stress or injury (Schwartz & Kipnis, 2001; Hauben et al., 2001; Hauben & Schwartz, 2003) in which therapies are targeted to self-antigens.

Antibodies are generated by the immune system's T helper and B cells responding to the presentation of normally foreign antigen epitopes which causes B cell maturation, antigen-specific antibody (Ab) production and the induction of memory cells. This classical immune response enables an enhanced reaction upon rechallenge with the same foreign antigen and is a mechanism exploited for the rapid production of Abs for clinical or experimental purposes. However, the generation of Abs against self-antigens is much more difficult as exposure of the immune system to self-antigens instigates immune tolerance, a response which operates to prevent auto-Ab production and autoimmune disease in the host. These tolerogenic mechanisms need to be interrupted to be able to produce Abs for passive immunotherapeutic approaches (Bachmann et al., 1993).

Adjuvants are commonly used in vaccine production to enhance the immune response to the often weakly immunogenic immunising antigen (Jennings, 1995; Fraser et al., 2007), which include self-antigens, but are also used to increase the overall yield (Petrovsky & Aguilar, 2004). The use of Freund's adjuvants (FA) for experimental purposes has remained the 'gold standard' for producing potent Abs against soluble antigens. Complete Freund's adjuvant (CFA), commonly used for the first immunisation, consists of heat killed *mycobacterium tuberculosis* and mannose monooleates in paraffin oil. This elicits a Th-1 dominated inflammatory response (Billiau & Matthys, 2001) and often results in granuloma formation at the site of administration. Incomplete Freund's adjuvant (IFA), which lacks the mycobacterial components, is the adjuvant of choice for subsequent immunisations as it induces a Th-2

Abbreviations: CV, CoVaccine^{HT}; FA, Freund's adjuvant; CFA, Complete Freund's adjuvant; IFA, Incomplete Freund's adjuvant; SFASE, sucrose fatty acid sulphate ester; HMGB1, high mobility group box-1; Ab, antibody; Abs, antibodies.

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dominated response. The intense inflammatory response and localised granuloma formation of FA have been associated with the formation of distant sub-pleural hepatic and renal granulomas (Broderson, 1989; Johnston et al., 1991; Leenaars et al., 1994), necrotising-dermatitis (Steiner et al., 1960), as well as spinal cord compression from an injection site granuloma (Kleinman et al., 1993). The extent of inflammation, necrosis, pain and distress associated with FA limits its use to experimental purposes only (Petrovsky & Aguilar, 2004; Bodewes et al., 2010). Consequently, the use of alternative adjuvants is highly encouraged, particularly those that can improve the welfare of animals used for immunisation regimens (Petrovsky & Aguilar, 2004). One such adjuvant is CoVaccine HT™ (CV), an oil-in-water adjuvant which consists of sucrose fatty acid sulphate ester (SFASE) immobilised on oil droplets of a submicron emulsion of squalane-in-water (Bodewes et al., 2010). The effectiveness of the aqueous formulation is similar to water-in-oil based adjuvants with no severe or persistent side effects at the site of injection, thus making it safe for even human use. Indeed, we and others have shown that CV employed in production of Abs against viral antigens is strongly immunogenic (Stevens et al., 2013) and safe (Kusi et al., 2011) while it also produces cell mediated and intense humoral immunity to poor immunogens (de Cassan et al., 2011) in large, non-rodent species (Stevens et al., 2013; Blom & Hilgers, 2004; Draper et al., 2010; Hilgers & Blom, 2006).

In this study the use of CV and FA were evaluated for the production of therapeutic polyclonal Abs targeting the endogenous alarmin, High Mobility Group Box-1 (HMGB1) protein. High Mobility Group Box-1 is a highly conserved nuclear protein involved in DNA replication, transcription, and tissue repair (Diener et al., 2013; Landsman & Bustin, 1993), however it has also emerged as a key mediator in the regulation of immune responses to infection and injury (Schaper et al., 2014). Furthermore HMGB1 influences the development of auto-immune disorders (Tang et al., 1799), chronic inflammatory diseases (Abdulahad et al., 2011) and cellular processes that can dictate disease progression in cancer (Tang et al., 1799; Sims et al., 2010). As extracellular HMGB1 has recently been implicated as a key late inflammatory mediator in sepsis, where it regulates multiple pathways associated with pathogenesis of the disease, it is therefore considered an important potential therapeutic target for clinical evaluation (Diener et al., 2013; Yang et al., 2006). In this report, we show that CV induced significantly higher Ab tires, with a stronger affinity than Abs induced with FA. These Abs also proved to provide potent protection in a murine model of endotoxemia. Most importantly, the negative side effects of immunisation were minimal, thus increasing the welfare of host animals without compromising Ab yield. Therefore these results provide evidence that CV is superior to FA for the production of Abs against poor immunogen rHMGB1, highlighting the prospective advantages of using CV in the production of Abs for experimental and/or clinical use.

2. Materials and Methods

2.1. Recombinant HMGB1 production

The HMGB1 mRNA sequence was cloned from cDNA prepared from healthy human donor PBMC's using custom primers with appropriate restriction sites and a C-terminal 6× His-tag. The sequence was cloned into the pFastBac™1 vector from the Bac-to-Bac expression system (Invitrogen) and the construct used to produce recombinant baculovirus according to the manufacturer's instructions. Briefly; insect cells (sf21) were maintained in sf900 SFM III (Invitrogen) supplemented with L-Glutamine (100 μg/mL), penicillin (100 U/mL), gentamycin (100 μg/ml) and HEPES (10 mM, pH 7.2) in roller bottle flasks at 27 °C. Insect cells at 2×10^6 cells/mL were infected for 96 h prior to protein harvest. Cultures were supplemented with L-glutamine 100 μg/mL 24 h prior to protein harvest to enable maximum protein yield. Expressed protein was purified from cell lysate using Ni-NTA agarose beads (Qiagen) according to the manufacturer's instructions before dialysis in PBS. Protein purity was analysed by SDS-PAGE and anti-HMGB1 western blot. Concentration was determined by BCA assay (Thermo Fisher Scientific).

2.2. Immunisation and sample collection

For FA administration purified recombinant HMGB1 (rHMGB1) antigen (200 μg) was diluted in PBS and emulsified with an equal volume of CFA (Sigma.) for prime immunisation and IFA (Sigma.) for boost immunisation 14 and 28 days later. A sample of the resulting emulsion (10ul) was extruded into PBS and stored at room temperature to assess stability of the emulsion. The droplet was inspected daily for signs of dissolution. Groups of 2 year old Border Leister x Merino ewes were immunised with the 2 mL emulsion subcutaneously, at 4 axillary sites (500 μL at each site). The CFA and IFA used for the immunisations were commercially sourced from Sigma Aldrich (catalogue number: F5881 and F5506 respectively), and are comprised of 15% mannide monooleate in paraffin oil with the addition of 1 mg/mL heat-killed and dried Mycobacterium tuberculosis (ATCC 25177) for complete Freund's adjuvant. For CV (kindly provided by BTG Plc (UK)) administration 200 μg or 20 μg rHMGB1 in PBS was gently mixed with an equal quantity of CV (40 mg/mL) and sheep were immunised with 2 mL emulsions subcutaneously at 4 axillary sites (500 μL at each site). Sheep were administered a prime immunisation and boosted every 14 days for a total of 2 boosts at sites adjacent to prime immunisations. Serum was taken prior to each immunisation and stored at −20 °C for later analysis. Sheep that received either 200 μg or 20 μg of rHMGB1 in CV were routinely monitored for immunisation site reactions by trained veterinary staff for bleeding and ulceration consistent with animal welfare requirements and in addition, size of the local reaction measured with callipers and site surface temperature was measured with a laser thermometer (FLUKE IR thermometer 561). At the end of the immunisation period sheep were anaesthetised with 5% Isoflurane in oxygen, exsanguinated via the jugular vein, then after the right atrium/anterior vena cava stops bleeding, sheep are humanely killed under anaesthesia with 35 ml of 4 M KCl and serum collected stored at −20 °C for IgG purification.

2.3. Endpoint anti-HMGB1 Ab ELISA

Stored serum samples from immunised sheep were used for Ab endpoint analysis as previously desscribed (Frey et al., 1998). Briefly, high-binding EIA/RIA ELISA plates (Costar) were coated with100μL rHMGB1 (5 μg/mL) overnight at 4 °C in a bicarbonate buffer (pH 9). Plates were washed (3 times) with PBS, blocked with 2% (*w*/*v*) BSA (Sigma) in PBS for 1 h at 37 °C, and then washed with PBS-T (PBS + 0.05% Tween). Thawed pre-immune and hyper-immune serum was diluted 1:50,000 and 100 μL added to duplicate wells and further serially diluted 1:4, with plates subsequently incubated for 2 h at 37 °C. Plates were washed 3 times with PBS-T before bound ovine Abs were detected with HRP conjugated anti-ovine IgG (Sigma); 1:10,000 at 37 °C for 1 h. Plates were washed with PBS-T three times and developed using SIGMAFAST ™ OPD substrate (Sigma: A3415-1ML). The reaction was stopped with 3 M HCL and the OD read at 490 nm by absorbance spectroscopy. The endpoint titre of the samples are the reciprocal of the highest dilution that has a reading above the cut off which is calculated as mean + 2 standard deviations of negative serum samples.

2.4. Purification of ovine polyclonal anti-HMGB1 from serum

Stored serum from CV and FA immunised sheep was thawed and filtered through glass wool. The Abs were precipitated by addition of saturated ammonium sulfate solution to 45% (*v*/*v*). After centrifugation (20 min, 10,000 x g) pelleted protein precipitate was dissolved in milliQ water and dialysed into PBS before application to a Protein G agarose column (Thermo Fisher Scientific). Bound IgG was eluted with 0.1 M

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glycine (pH 2.5) and dialysed into PBS. Concentration was determined via BCA assay (Thermo Fisher Scientific) according to manufacturer's instructions. Concentrated IgG was subsequently analysed for purity via SDS-PAGE.

2.5. Indirect ELISA for affinity determinations

An ELISA was conducted using the same protocol for blocking, washing, and detection as above. High binding EIA/RIA ELISA plates (Costar) were coated with 100 μL rHMGB1 (10 μg/mL) overnight at 4 °C. Purified CoVaccine elicited anti-HMGB1 (CV-anti-HMGB1) and FA elicited anti-HMGB1 (FA-anti-HMGB1) were serially diluted 1:2 and added to duplicate wells. Resultant absorbance was calculated as a function of Ab concentration and the determination of dissociation and association constants were calculated using methods previously described (Orosz & Ovádi, 2002; Liliom et al., 1991) by the following formulas:

$$\frac{1}{1-i}=\frac{\frac{Ab_{tot}}{i}}{\frac{K_d^{Ab}}{p}}-\frac{fqS_{Ag}}{K_d^{Ab}}=\frac{\frac{Ab_{tot}}{i}}{K_d^{app}}-b \tag{1}$$

$$i=\frac{S_{Ab}}{S_{Ab}^{max}}\ then\ i=\frac{A_{490nm}}{A_{490nm}^{max}} \tag{2}$$

$$K_a=\frac{1}{K_d} \tag{3}$$

where i is the relative saturation of the antigen (Ag), Ab, S_{Ag} and S_{Ab} the surface concentration of Ag and Ab, with Ab_{tot} the total concentration of Ab. q Is the number of epitopes per Ag molecule, p is the number of blocked epitopes per Ab molecule, f is the quotient of the reaction area and volume. Using the indirect ELISA methods and the Eqs. (1) and (2), the plot of data points is represented in a weighted linear regression with concentration of Ab on the surface (S_{Ab}) proportional to absorbance (A_{490nm}) (Eq. (2)). The plot of $\frac{1}{1-i}$ versus $\frac{Ab_{tot}}{i}$ gives a linear regression and the parameters of this plot is then substituted into the formulas (1), (2) and (3). Apparent equilibrium constant ($K_d^{app}=\frac{K_d^{Ab}}{p}$), is the reciprocal of straight line ($\frac{1}{1-i}$ versus $\frac{Ab_{tot}}{i}$ ($slope=\frac{1}{K_d^{app}}$)) and is calculated with the intercept representing $-b=-\frac{fqS_{Ag}}{K_d^{Ab}}$.

2.6. Inhibition of HMGB1 stimulation of murine macrophages

Murine RAW 264.7 (ATCC® TIB-71™) cells (a macrophage cell line) were cultured in complete DMEM (Sigma) until 50% confluent in a T75 tissue culture flask (Sigma). Cells were scraped off the flask, spun at 300 g for 5 min before being resuspended in DMEM and counted. Cells were plated at 1.65×10^5 cells/mL with 200 μL/well in a 96 well plate and left to culture for a further 24 h. Recombinant HMGB1 (2 μg/mL) (Abcam) in complete DMEM was added to purified CV-anti-HMGB1 (0.2 μg/mL) and FA-anti-HMGB1 (0.2 μg/mL) and samples were allowed to incubate at 37 °C for 1 h. Samples of rHMGB1 (2 μg/mL) alone as well as rHMGB1 incubated with Abs (0.2 μg/mL) was added (200 μL) in duplicate wells. Supernatant was harvested at 24 h post stimulation, spun down and diluted 1:2. Cytokine capture ELISA methods was used to measure the concentration of TNFα. Briefly High binding EIA/RIA ELISA plates (Costar) were coated with 100 μL purified anti-mouse TNFα (1 μg/mL) (eBioscience 14–7325-81) overnight at 4 °C. Plates were washed and blocked as above. Supernatant was added in duplicate (100 μL), with recombinant mouse TNFα (eBioscience 14–8321–62) ranging from 10,000 to 0.305 ng/mL as a standard and incubated for 2 h at 37 °C. Plates were washed as above. Secondary antibody (100 μL), anti-mouse TNFα-Biotin (eBioscience 13–7326-85) (1 μg/mL) added and incubated for 1 h at 37 °C. Plates were washed again. HRP-Streptavidin (Sigma) (1:20,000) was added and incubated for a further 1 h at 37 °C. Plates were washed and developed as above using OPD substrate, the reaction was stopped with 3 M HCL and the OD read at 490 nm by absorbance spectroscopy.

2.7. Inhibition of murine endotoxemia

Six week old C57BL/6 mice were housed in specific pathogen-free conditions. Groups of 3–5 mice were administered purified CV or FA-anti-HMGB1 (5-50 mg/kg) 1 h prior to endotoxin (LPS) administration of 50 mg/kg. Mice were then monitored every 3 h for surface temperature (using a laser thermometer at the nape of the neck) and clinical signs that included ruffled fur, diarrhoea, hunching and reduced movement. Mice were scored based on the degree of change in clinical signs as indicated in Table 1. Mice reaching a total clinical threshold score of 6 or more were humanely killed after recording threshold scores.

2.8. Statistical analysis

All statistical analyses were performed with GraphPad Prism V6.00 software.

2.9. Ethics statement

All animal experiments were approved by the University of South Australia and SAHMRI (South Australian Health and Medical Research Institute) Animal Ethics Committees for mouse and sheep studies respectively. All experiments were conducted in accordance with National and Institutional ethical guidelines.

3. Results

3.1. CoVaccine HT™ elicits significantly higher anti-HMGB1 Ab titres with a stronger affinity than Freund's adjuvant

Anti-HMGB1 Ab titres were assessed in sheep that were immunised with 20 μg or 200 μg of rHMGB1 in CV or 200 μg rHMGB1 in FA and were boosted fortnightly. Serum samples taken before each immunisation were tested in an end-point ELISA to determine Ab titres. Significantly higher anti-HMGB1 titres were induced when rHMGB1 was administered with CV adjuvant compared to FA (Fig. 1a(i)). Titres in the CV high antigen dose (200 μg) group rose exponentially after initial immunisation and after each boost. In contrast, rHMGB1 (200 μg) delivered in FA induced anti-HMGB1 Abs that gradually rose after each boost, but an approximate 16,000-fold lower rise than the CV group. Sheep immunised with a ten-fold lower antigen dose (20 μg) in CV produced significantly lower anti-HMGB1 Ab titres than the 200 μg dose in CV, though final titres were still 100-fold higher than the FA group that received a tenfold higher antigen dose. To investigate if the site reactions following CV administration were related to the administration of rHMGB1, reactive sites of boost immunisations were assessed 4 weeks after prime. No evidence of ulceration or bleeding was detected and in

Table 1
Clinical scouring rubric for acute endotoxemia studies.

Symptom	Score 0	Score 1	Score 2
Coat	Appears normal	Dull	Very ruffled
Eyes	No exudate/dryness	Dry/slight exudate	Significant exudate
Stool	No changes	Soft	Marked diarrhoea
Mobility	Mobile when cage is moved/opened	Reluctant to move	Poor response to stimulation
Posture	Appears normal	Hunched	
Weight	Equal to or greater than previous measurement	Lower weight than previous day	

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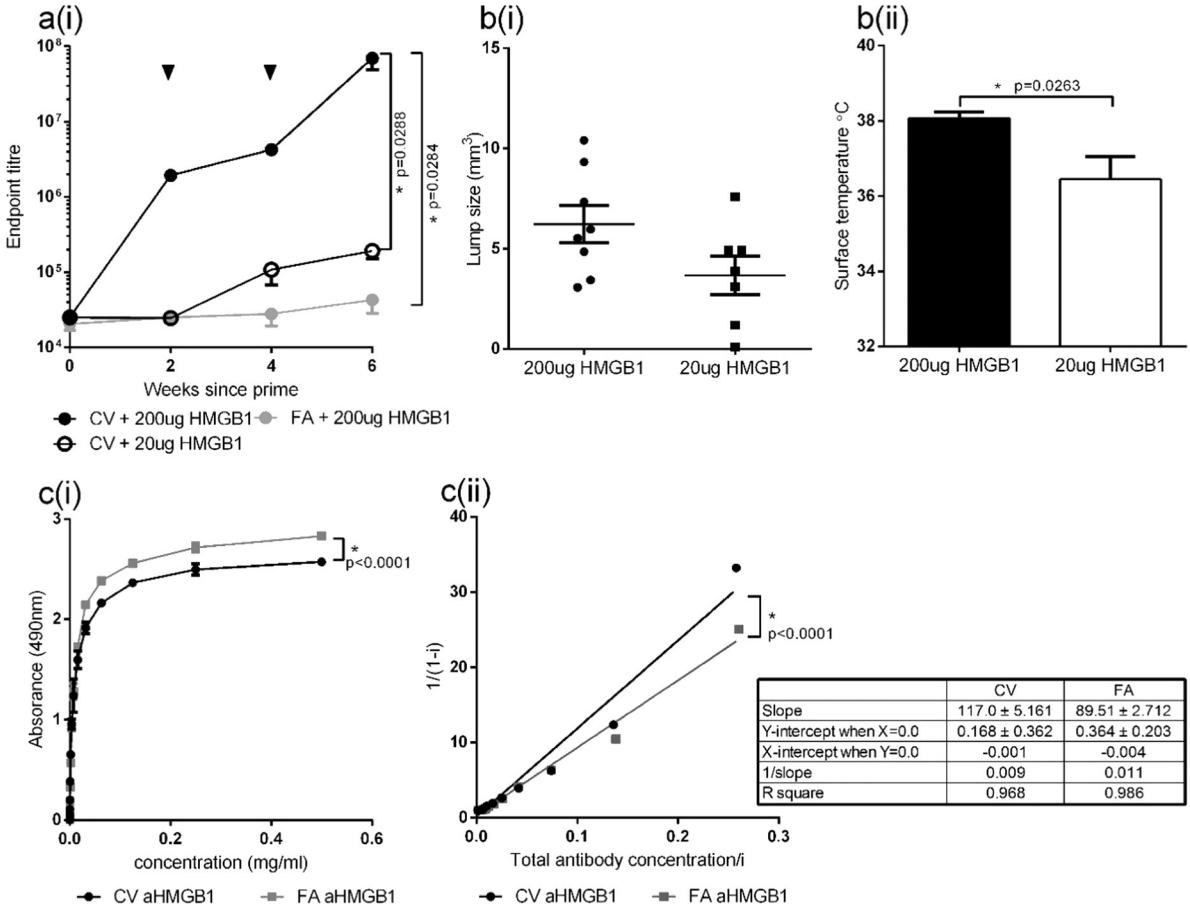

	CV	FA
Slope	117.0 ± 5.161	89.51 ± 2.712
Y-intercept when X=0.0	0.168 ± 0.362	0.364 ± 0.203
X-intercept when Y=0.0	-0.001	-0.004
1/slope	0.009	0.011
R square	0.968	0.986

Fig. 1. CoVaccine HT™ adjuvant elicits significantly higher anti-HMGB1 Ab titres with a stronger affinity than Freund's adjuvant, whilst HMGB1 dose significantly affects end-point titres and the severity of site reactions. Sheep ($n = 2$) were immunised with 200 μg HMGB1 in FA and 200 μg or 20 μg HMGB1 in CV and boosted fortnightly (indicated by arrows). Blood samples were taken fortnightly and end point titres determined (a(i)) with data expressed as mean ± SEM and analysed via two-way ANOVA. Reactive sites from CV immunised sheep (four weeks following prime) were measured with callipers and expressed as mm^3 (b(i)) surface temperature was also recorded (b(ii)). Data is represented as mean ± SEM and were compared by two-way ANOVA. Antibody affinity was calculated by indirect, non-competitive ELISA with absorbance at 490 nm presented as a function of Ab concentration (c(i)). Data is expressed as mean ± SEM and representative of one other, with analysis performed via two-way ANOVA. The functions obtained from (c(i)) were substituted into formula 1 and 2 for each experimental point to express relative saturation for each adjuvant and association constants sought by weighted linear regression (c(ii)). The parameters of the straight lines (correlation coefficient, slope, intercepts) are described in the legend. Data were analysed using software slope comparison (two tailed). Significance for all statistical tests is denoted * $P < 0.05$.

many cases reactive sites from prime immunisation had resolved. The group ($n = 2$) that received a higher antigen dose exhibited a larger site reaction size (Fig. 1b(i)), approximately $5mm^3$, whereas the group that received a tenfold lower antigen dose had a mean reaction size of $3mm^3$. Volume of site reactions were not found to be significantly different between the doses of antigen ($p = 0.076$). The local surface temperature of immunisation sites was also measured (Fig. 1b (ii)) and sites from the higher antigen dose group were significantly warmer than those from the lower group ($p = 0.0263$). While the size and surface temperature of reactive sites on sheep immunised with HMGB1 and FA were unable to be measured, it was noted by veterinary staff that the lumps formed at reaction sites of CV immunised sheep resolved faster than lumps observed in FA sheep. Veterinary staff monitoring sheep compared site reactions to simultaneous immunisation regimens that used FA within the facility. To investigate the strength of complexes formed with Abs elicited with different adjuvants, methods (Liliom et al., 1991) to assess association and dissociation values were adapted using formulas 1, 2 and 3. Absorbance values and concentrations of respective Abs from Fig. 1c(i) were substituted into formula (2). This resulted in the linear regression (Fig. 1c (ii)) and parameters of the slopes were substituted into formula (1) to calculate apparent dissociation constants. Apparent dissociation constants calculated for CV-anti-HMGB1 was $K_d^{app} = 0.009$ and for FA-anti-HMGB1 $K_d^{app} = 0.011$ and were significantly different ($p < 0.0001$). As dissociation constants are inversely proportional to association constants from formula (3), CV-anti-HMGB1 (slope = 117.0) had a significantly higher association constant than FA-anti-HMGB1 (slope = 89.51) ($p < 0.0001$).

3.2. CoVaccine HT™ elicited Abs are more functionally potent and provide greater protection from lethality in a murine endotoxemia model

Potency was evaluated by the Ab's ability to neutralise HMGB1. Murine macrophage cells stimulated with HMBG1 produced TNFα (Fig. 2a (i)). Addition of FA-anti-HMGB1 with rHMGB1 reduced TNFα production by 14% which was not found to be significantly different from rHMGB1 alone. However, the same concentration of CV-anti-HMGB1 and rHMGB1 resulted in a 61% reduction of TNFα production compared to rHMGB1 alone and this was shown to be significantly different ($p < 0.0001$). After observing significant differences *in-vitro* between Abs against rHMGB1 elicited with CV as compared to FA, the murine endotoxemia model was used to compare inhibitory activity *in-vivo.* Mice were administered CV or FA-anti-HMGB1 one hour prior to LPS administration and survival for each antibody was compared to baseline (saline group). Administration of 50 mg/kg anti-HMGB1 Abs (Fig. 2b(i)) elicited with either CV or FA resulted in significant survival improvements to 60% ($p = 0.0281$) and 30% ($p = 0.0334$) respectively compared to saline control cohort. Administration of 20 mg/kg (Fig. 2b (ii)) of FA-anti-HMGB1 resulted in a 20% increase in survival, CV-anti-

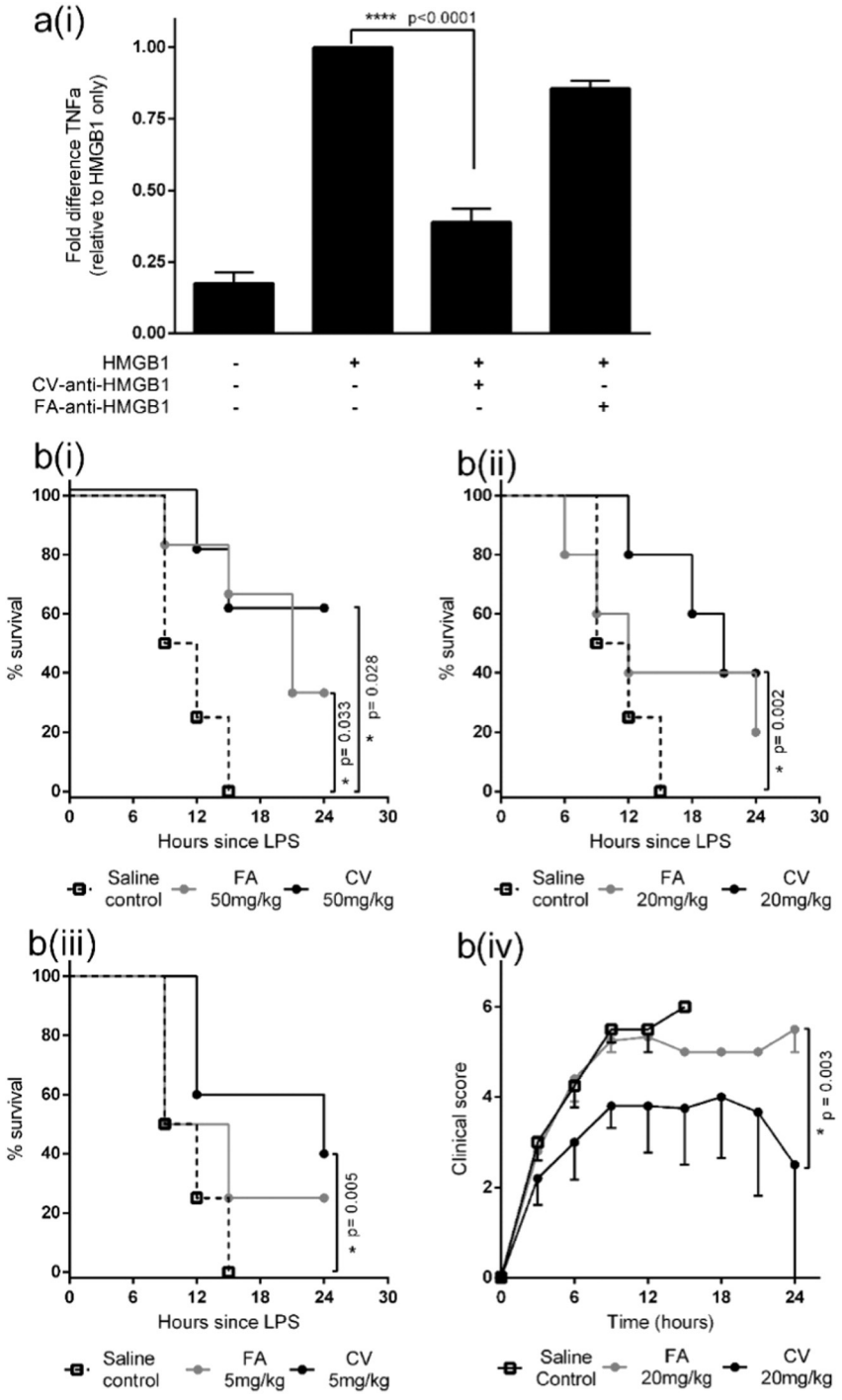

Fig. 2. CoVaccine HT™ adjuvant elicits anti-HMGB1 Abs that are more functionally potent than those from Freund's adjuvant immunisations and provide enhanced protection in a murine endotoxemia model. RAW 264.7 cells were stimulated with rHMGB1 alone, rHMGB1 + CV-anti-HMGB1 and rHMGB1 + FA-anti-HMGB1 in duplicate. Supernatant was harvested 24 h post-stimulation and TNF-α concentration measured (a(i)). Data are expressed as the mean ± SEM and groups were compared, relative to HMGB1, with analysis performed via one-way ANOVA. Mice were administered saline or CV-anti-HMGB1 or FA-anti-HMGB1 (5-50 mg/kg) one hour prior to endotoxemia induction and monitored for clinical sickness signs. Mice reaching clinical thresholds were humanely killed and survival is presented for each dose of Ab (b(i):50 mg/kg, b(ii):20 mg/kg and b(iii):5 mg/kg). Average clinical score over time is presented (b(iv)) as mean ± SEM for mice in 20 mg/kg group. Data were analysed using Mantel-Cox survival analysis and two-way ANOVA respectively. Significance for all statistical tests is denoted as * $P \leq 0.05$.

HMGB1 resulted in a significant increase in survival, 40%, compared to mice in the saline control cohort ($p = 0.00154$). Administration of 5 mg/kg (Fig. 2b(iii)) of FA-anti-HMGB1 resulted in 20% increased survival, however, mice that received CV-anti-HMGB1 had a significant increase in survival, 40%, compared to saline control cohort ($p = 0.048$). While survival in the CV-anti-HMGB1 groups was increased compared to FA-anti-HMGB1 for each dose, survival between Ab groups was not found to be statistically significant (50 mg/kg $p = 0.5448$, 20 mg/kg $p = 0.382$ and 5 mg/kg $p = 0.4116$). Nevertheless, there was a significant difference in the average clinical score over time for cohorts that

received 20 mg/kg of Ab (Fig. 2b(iv)). Clinical scores were obtained prior to humane killing of mice obtaining a score of 6 or more. Mice in the saline group rapidly reached a clinical score of 6 and above, all mice in this groups were required to be humanely killed after 15 h. Mice that received FA-anti-HMGB1 had an average clinical score that rapidly increased above 5 at 9 h post-LPS administration, similar to the saline control group. After 12 h, remaining mice in FA-anti-HMGB1 group continued a clinical score of 5. In contrast surviving mice that received CV-anti-HMGB1 obtained a highest average clinical score of 3. Mice that received CV-anti-HMGB1 had a significantly lower clinical score over time compared to mice that received FA-anti-HMGB1 ($p = 0.0034$). In summary our results show that mice pre-treated with CV elicited Abs exhibit a lower level of morbidity and mortality than mice administrated FA elicited Abs.

4. Discussion

Passive polyclonal Ab therapies have become an effective approach in treating diseases with high morbidity and mortality (Tolstrup et al., 2006) especially in complex disease scenarios where therapies are needed to target poorly immunogenic self-antigens (Harrison, 2008; Keijzer et al., 2013; Kazatchkine & Kaveri, 2001; Brody & Holtzman, 2008; Schwartz & Kipnis, 2001; Fisher et al., 2001; Hauben et al., 2001; Hauben & Schwartz, 2003). While immunisation strategies that induce potent Ab responses are continuously undergoing development, they still require the use of an adjuvant to make large stocks of high affinity Abs (Fraser et al., 2007; Hermeling et al., 2004). For a long time FA has remained the 'gold standard' adjuvant for use in animals, however, it can be associated with significant pain and distress thus compromising the welfare of the host (Stevens et al., 2013; Stils, 2005).

We and others have approached the need to consider new adjuvants to improve vaccination regimens for therapeutic Ab production (Jennings, 1995; Fraser et al., 2007; Johnston et al., 1991; Leenaars et al., 1994). Our group has previously shown that CV produced higher Ab titres compared to FA toward highly immunogenic influenza hemagglutinin (Stevens et al., 2013). The results of the current study support our previous findings and demonstrate that CV produces significantly higher Ab titres than FA, even for a poor immunogen like endogenous HMGB1. It has been speculated that the increased efficacy of CV could be attributed to the emulsion composition of the two adjuvants and the subsequent impact on antigen integrity (Jennings, 1995; Billiau & Matthys, 2001; Stils, 2005). Proprietary adjuvant CV is a pre-formed oil-in-water emulsion (Blom & Hilgers, 2004) which is combined with aqueous antigen and FA is a water-in-oil emulsion where aqueous antigen is emulsified with mineral oil, potentially disrupting secondary and tertiary protein structures (Leenaars et al., 1994). Additionally FA forms viscous deposits that release antigen over a long period of time whereas CV allows quick dispersion of antigen to surrounding tissues at site of immunisation. The SFASE in CV contains engineered synthetic carbohydrates that mimic lipopolysaccharides of gram negative bacteria, acting as a strong immunostimulant (Blom & Hilgers, 2004). The binding of SFASE on CV to Toll-like receptor-4, a fundamental pathogen recognition receptor, rapidly escalates the release of pro-inflammatory cytokines, thus increasing local inflammation and upregulating co-stimulatory molecules on antigen presenting cells (Bodewes et al., 2009) via MyD88 or Trif dependant pathways. Toll-like receptor ligands, such as SFASE, that mimic pathogens are effective for overcoming tolerance as the activation of TLR's initiate dendritic cells maturation, T cell priming and B cell proliferation (Pasare & Medzhitov, 2004). This may facilitate a local environment whereby potent antibody responses may be generated against a self-antigen.

In our previous work (Stevens et al., 2013) we found that a lower antigen dose of the highly immunogenic antigen used there did not significantly impact Ab titres. In contrast we show here that using self-antigen HMGB1 at a lower dose resulted in significantly lower Abs titres. Furthermore the current study shows that a reduced antigen dose elicited with CV still produced significantly higher antigen titres than a tenfold higher antigen dose elicited with FA. This supports the use of CV as an ideal adjuvant in the production of Abs by reducing costs associated with high antigen doses usually required for low immunogenic proteins.

Previously, we demonstrated that CV produced fewer inflammatory site reactions than FA with viral protein hemagglutinin (Stevens et al., 2013). In the current study, with self-antigen, CV was associated with the formation of reactive sites which appeared smaller in sheep that received a lower antigen dose. While the size of sites was not significantly different between the two antigen doses, measured surface temperature of the site reactions was significantly lower with reduced antigen dose than the higher antigen dose. This difference may be attributed to the potent pro-inflammatory DAMP function of HMGB1 (Diener et al., 2013). It has also been previously suggested that the ratio of antigen and CV can affect the associated adversities of immunisation in the host (Bodewes et al., 2010). Here we demonstrate that negative outcomes can be minimised with a lower antigen dose thereby maintaining the welfare of the host animal without compromising the ability to obtain high yields of Abs. It must be noted that while immunisation and antibody production data discussed above is distinctly different between FA and CV, the sample size in each adjuvant group is indicative of promising preliminary results that provide the basis for large scale investigation to be undertaken.

The superiority of CV is further reflected in the quality of Abs produced. This study demonstrates that CV Abs have significantly higher affinity for HMGB1 than FA Abs *in-vitro*. Protein oxidation, altered during the storage phase of in-house produced HMGB1, results in changes in function (Diener et al., 2013). Therefore, in order to achieve consistency commercially available HMGB1 was used to demonstrate differences in neutralising capacity *in-vitro*. These differences are further supported by the increased potency of the Abs observed *in-vivo*, where protection against endotoxemia with CV elicited Abs significantly improved survival compared to protection with FA elicited Abs when used at the same concentration. Furthermore protection with CV Abs resulted in a lower overall level of sickness highlighting their superior neutralising activity. These differences in Ab affinity may be attributed to the preserved antigen integrity associated with water-in-oil adjuvants such as CV (Stevens et al., 2013). Antigen integrity and the absence of denaturation is important for the development of high affinity Ab, as antigen at the immunisation site can be endocytosed and presented to specific B cells in native formation (Aguzzi et al., 2014). These results suggest that CV may be a superior adjuvant for inducing high affinity Abs against self-antigens.

5. Conclusion

In conclusion, we have demonstrated that CV produces higher Ab titres than FA and that CV Abs have increased antigen affinity. The use of CV also allows a reduced antigen dose to be employed for generation of high Ab titres while minimising adverse site reactions associated with self-antigen administration. These observations highlight the potential value that new and superior adjuvants, such as CV (CoVaccine HT™), can have in the production of Abs.

Funding and competing financial interest

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Conflict of interest

The CoVaccine HT™ used in this study was kindly provided by BTG Plc (UK).

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Acknowledgments and author contributions

N.L performed experiments, analysed the data and wrote the main manuscript text. N.E.S performed experiments, edited and provided technical support. K.R.D. edited and provided intellectual input. J.D.H. supervised the project, provided intellectual input, edited and contributed to the main manuscript.

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