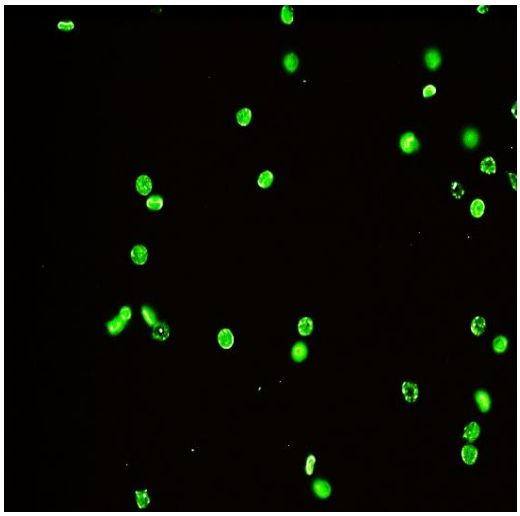
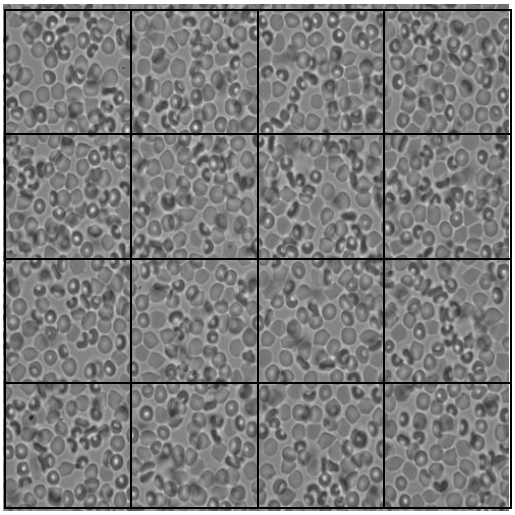
# INSIGHTS INTO THE MANIPULATION OF THE IMMUNE RESPONSE TO CARBOHYDRATE ANTIGENS WITH BLOOD GROUP FUNCTIONAL-SPACER-LIPID CONSTRUCTS





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**ATTESTATION OF AUTHOURSHIP**

"I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning."

Caroline Ann Oliver

Auckland

2012

**ACKNOWLEDGMENTS**

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I believe my passion for transplant immunology was fuelled after I constantly read the plaque near the Laboratory elevator at Waikato Hospital, dedicated to Edward Jenner the "father of immunology" who vaccinated himself with small pox (1796) and whose work is said to have "saved more lives than the work of any other man".

**Edward Jenner vaccinating a boy. Oil painting by E. E. Hillemacher, 1884**

**Caroline Oliver, AUT 2012**

## ETHICAL APPROVAL

All procedures were approved by The University of Auckland Animal Ethics Committee- AEC#C500 and AEC#R629.

## ABSTRACT

The immune system is extremely complex and critical to the survival of all species as it protects them from attack by foreign micro-organisms and viruses. However the immune system can also be a foe in that it can sometimes turn against the organism it is designed to protect resulting in a range of autoimmune diseases. Additionally, its design to protect against invasion of foreign antigens, creates a major hurdle for transfusion and transplantation. If the immune system could be manipulated to allow for transfusion and transplantation of incompatible tissues, or turned off against specific targets in the case of autoimmune disease, all the while maintaining normal protective functions, then major advances in human health and well-being could be made. A variety of approaches have been used to try to manipulate the immune system, but with no one approach being the panacea, thus leaving open the opportunity for novel approaches to try and manipulate it towards creating beneficial outcomes. The recent development of a technology using novel function-spacer-lipid constructs (FSLs) has the ability to modify cell surfaces. As some of these FSLs are related in structure to glycolipids, they have the potential to inhibit antibodies.

This research, using mice, set out to determine if FSL constructs could be used to manipulate the immune system by modifying membrane antigens and inhibiting/neutralizing antibodies. By using KODE™ technology, carbohydrate antigens were introduced to red cells (kodecytes) and visualization molecules provided a novel method with which to study and manipulate the immune response. This research involved a series of experiments in mice to investigate antibody stimulation, cell survival measurement and recovery, antibody neutralization and tolerance induction using various FSL constructs.

Four hypotheses concerning the use of Functional-Spacer-Lipid (FSL) constructs were developed to investigate their potential for altering the immune response in mice.

The hypothesis that FSLs might induce antibody production was tested with FSL constructs to determine the immunogenicity of the molecules with direct infusion into the circulation and subcutaneous immunization. FSL constructs injected by subcutaneous and intravenous routes were not immunogenic, with no anti-A production detected.

The hypothesis that kodecytes could be used safely to determine cell survival after transfusion was tested with kodecyte transfusion in both compatible and incompatible mice. Incompatible mice were created by immunization with salivary blood group

substance A with the successful production of anti-A and transfusion of A kodecytes. The FSL construct representing blood group A antigen (FSL-A) was intravenously infused into naive and anti-A-positive mice to determine *in vivo* antigen transformation and cell survival after A antigen-compatible and incompatible (A+biotin kodecyte) transfusions. Other FSL kodecytes were used to create non-lethal, compatible transfusion control mouse models. The FSL-biotin construct provided a label allowing *in vitro* binding with avidin/Alexafluor 488 providing a fluorescent marker to track kodecyte survival. A method was also developed to recover transfused, circulating A+biotin kodecytes from a whole blood sample with a use of avidin-agarose beads in gel cards.

The hypothesis that FSLs could be used to neutralize circulating antibody was tested by infusing FSL constructs to neutralize antibody and then infusing antibody incompatible kodecytes. It was demonstrated that FSL-A was able to neutralize circulating anti-A, mitigating the consequences of kodecyte-incompatible red blood cell transfusion, and allowing for normal cell survival for up to 72 hours.

The hypothesis that FSL constructs could potentially induce tolerance was tested by measuring the consequences of infusing FSL-A by direct circulatory infusion into mice, pre-immunization with salivary blood group A substance. Primary immunization with FSL-A did not induce tolerance to the A epitope since anti-A was produced after a secondary immunization of salivary A substance. However, there was some evidence of a partial down-regulation of the immune response in terms of tolerance induction after infusion of FSL-A.

With further research, the potential for FSLs to be used safely in humans as a new methodology to determine 24-hour transfused cell survival, or to recover the transfused cells or to neutralize circulating antibody without subsequent antibody stimulation, is now possible.

## LIST OF FREQUENTLY USED ABBREVIATIONS

| | |
|---|---|
| APC | Antigen Presenting Cell |
| BCR | B cell receptor |
| BSA | Bovine Serum Albumin |
| DC | Dendritic cells |
| DIC | Differential interference contrast |
| EIA | Enzyme immunoassay |
| FSL | Functional-Spacer-Lipid KODE™ Construct |
| HLA | Human Leucocyte Antigen |
| Ig | Total immunoglobulin including IgA, IgM, IgG, IgD and IgE |
| ILL | Innate-like lymphocyte |
| IV | Intravenous |
| MHC | Major Histocompatiblility Complex |
| NK | Natural Killer cell |
| NKT | Natural Killer T cell |
| PBS | Phosphate Buffered Saline |
| PCV | Packed cell volume |
| RBC | Red blood cell (erythrocyte) |
| TCR | T cell receptor |
| TD | T dependent antigen |
| TI | T independent antigen |
| Tc | T cytotoxic cell |
| Th | T helper cell |
| Treg | T regulatory cell |
| Tx | Transfusion or transplant |

# CHAPTER 1 - INTRODUCTION

## 1.1 Overview

Manipulation and deliberate control of the powerful immune response is useful and necessary to both suppress unwanted immune responses in graft rejection, allergy and autoimmunity or to stimulate protective immune responses. It would be useful to be able to deploy the immune response to attack and destroy tumours and improve vaccine strategies or to inhibit antibody production. The use of biological therapy, with controlled administration of antigen using synthetic peptides or carbohydrates, or constructs such as FSLs, has the potential to manipulate the nature of an immunological response. This is useful because the manner in which the antigen is presented to the immune system affects the nature of the response (Melief et al., 1996; Liblau et al., 1997).

Four hypotheses concerning the use of Functional-Spacer-Lipid (FSL) constructs were developed to investigate their potential for altering the immune response in mice.

1. The hypothesis that FSLs might induce antibody production was tested with FSL constructs to determine the immunogenicity of the molecules with direct infusion into the circulation and subcutaneous immunization.
2. The hypothesis that kodecytes could be used safely to determine cell survival after transfusion was tested with kodecyte transfusion in both incompatible anti-A positive and compatible anti-A negative mice.
3. The hypothesis that FSLs could be used to neutralize circulating antibody was tested by infusing FSL constructs to neutralize antibody and then infusing antibody incompatible kodecytes.
4. The hypothesis that FSL constructs could potentially induce tolerance was tested by measuring the consequences of infusing FSL-A by direct circulatory infusion into animals, pre-immunization with salivary blood group A substance.

In order to be able to measure the immunological outcomes of using FSL constructs, a range of novel tools had to be developed, including the creation of incompatible cells (kodecytes) using FSLs and applications for measuring immune consequences *in vivo*; specifically the ability to label and track at specified time points, and recover manipulated

kodecytes. Each of these new methodologies are discussed within the framework of the hypotheses being explored. *In vitro* experiments with human blood and blood group A Function-Spacer-Lipid constructs (FSL-A) were used to determine baseline rates and concentrations that caused antigen transformation, anti-A neutralization and incompatiblity. Other FSL constructs were used as controls to provide baseline data. FSL-biotin was used as the label to track the kodecyte survival.

The toxicity of the molecules is also investigated as this could impact on the interpretation of the results and therefore affect the ability for the molecules to be eventually used as therapeutic protocols.

This chapter discusses the immune response, with antibody production or the induction of tolerance, the type of cells involved in the response, the response to particular antigens, the response to immunization, the clinical relevance of circulating antibodies and the manipulation of the immune response with current therapies. The concept of KODE ™ cell surface modification technology using synthetic glycolipid-like Function-Spacer-Lipids is introduced.

## 1.2 The Innate and Adaptive Immune Response

The immune system has evolved to counteract assault on the body by non-self entities or antigens and is the central player in the maintenance of health and disease. It is a coordinated action by numerous cellular and soluble components in a network of tissues and circulatory systems that recognizes, attacks and destroys that which is foreign to the body. Processing and presentation of the antigen for elimination is affected by many factors, including the physical form of the antigen, the site, dose and method of delivery, the adjuvant or inflammatory status and the antigen presenting cell (APC) that first meets the antigen (Harrison and Hafler., 2000; Zinkernagel and Hengartner, 2001). The route of administration of the antigen also determines the response - subcutaneous invokes antibody production (Kirkley, 1999); oral or intravenous can induce tolerance (Fowler and Weiner, 1997). Binding of the antibody protein marks the antigen for elimination by one of several destruction mechanisms depending on what cell type captures it or whether it activates immune components such as the complement cascade. The reaction of the immune system to antigen also depends on the relative frequencies of responding T and B cells and on the thresholds of binding avidity their receptors displayed.

The immune response can be broken down into two stages: recognition of antigen being non-self and the effector response leading to the elimination of the antigen. Vertebrates are capable of two types of immune responses, innate and adaptive. Both of these distinguish between self and non-self antigens but the degree of specificity and the mechanisms of recognition of non-self are very different. The innate response is non-specific compared to the adaptive response which recognizes specific epitopes on an antigen. It greets an antigen the same way each time it enters the host whereas the adaptive response can remember that it has 'seen' the antigen before and has adapted and acquired the ability to exclude this antigen. Immunological memory is produced as a result of the primary immunization. Each of the two responses is enhanced by or dependent on the other (Janeway, 2005).

The innate system takes effect within the first 6 hours of antigen detection providing defence with phagocytes, other leukocytes, natural killer cells, proteases and cytokines until the adaptive response can be engaged (Figure 1). This system uses cell receptors, including CD1d molecules, to recognize antigen presence to directly stimulate phagocytosis, secretion of effector molecules or signalling to other cells including Dendritic Cells (DC), Natural Killer cells (NK) and Innate-Like Lymphocytes (ILLs). Carbohydrate antigens, including glycolipids are detected by CD1d molecules on NK and ILL cells.

Within 4-5 days, the adaptive response is initiated by recognition and effector actions that are highly specific to the antigen that has triggered the response when circulating T cells encounter the corresponding antigen in draining lymphoid tissues and become activated. This response is concerned with effector action against the antigen and depends on the cooperative interaction of the two types of lymphocytes, B and T cells.

The capture and processing of antigens is very much the role of DCs which are designated professional antigen-presenting cells (APCs), (Trombetta and Mellman, 2005). APCs must migrate from the site of antigen uptake to the lymphoid organs and tissues to interact with the antigen specific lymphocytes. Once in the lymphoid tissue, DCs deliver intact antigen to B lymphocytes and processed antigenic-peptide complexes to T lymphocytes to stimulate an antibody response from B cells, thereby playing a major role in linking the innate with the adaptive response. This is compared to cell mediated immunity which involves cytotoxic T lymphocytes and macrophage activation. The location of the antigen, extra or intracellular, and its chemical nature, determines which effector lymphocyte cell of the adaptive response will be activated.

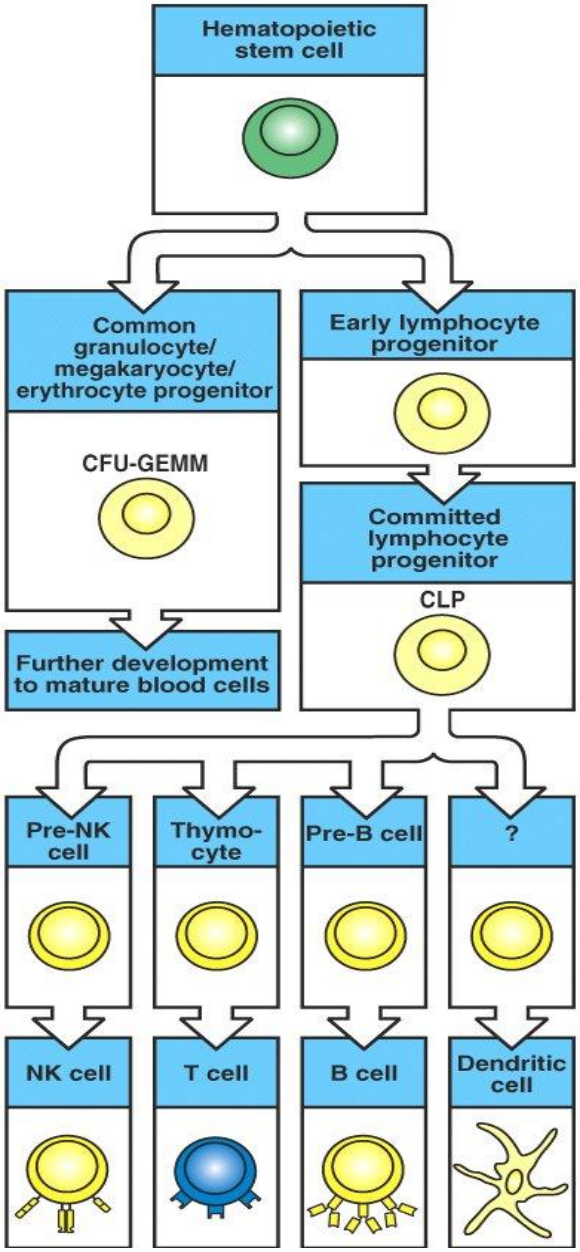


Figure 1.Immune response cells derived from pluripotent haematopoietic stem cells.

These give rise to myeloid, erythroid and lymphoid cells from which come monocytes, granulocytes, macrophages, dendrititc cells; NK cells T and B cells (reproduced from Janeway, Immunobiology, 2005).

T and B cells circulate in blood as naive cells bearing antigen receptors of single specificity. This is determined by the generation of millions of genetic variations encoding the receptor molecules in the marrow and thymus. There are billions of lymphocytes, each clone with its unique epitope receptor specificity - only those that encounter the antigen to their specific receptor will undergo proliferation. This is called clonal selection. On antigen binding, the activated B cell is sequestered in the lymph nodes and stimulated to

differentiate and produce many identical antibody-producing progeny and memory cells – this is called clonal expansion (Figure 2). This is also called the humoral response - mediated by antibodies found in blood plasma and extracellular body fluids which were once known as humors.

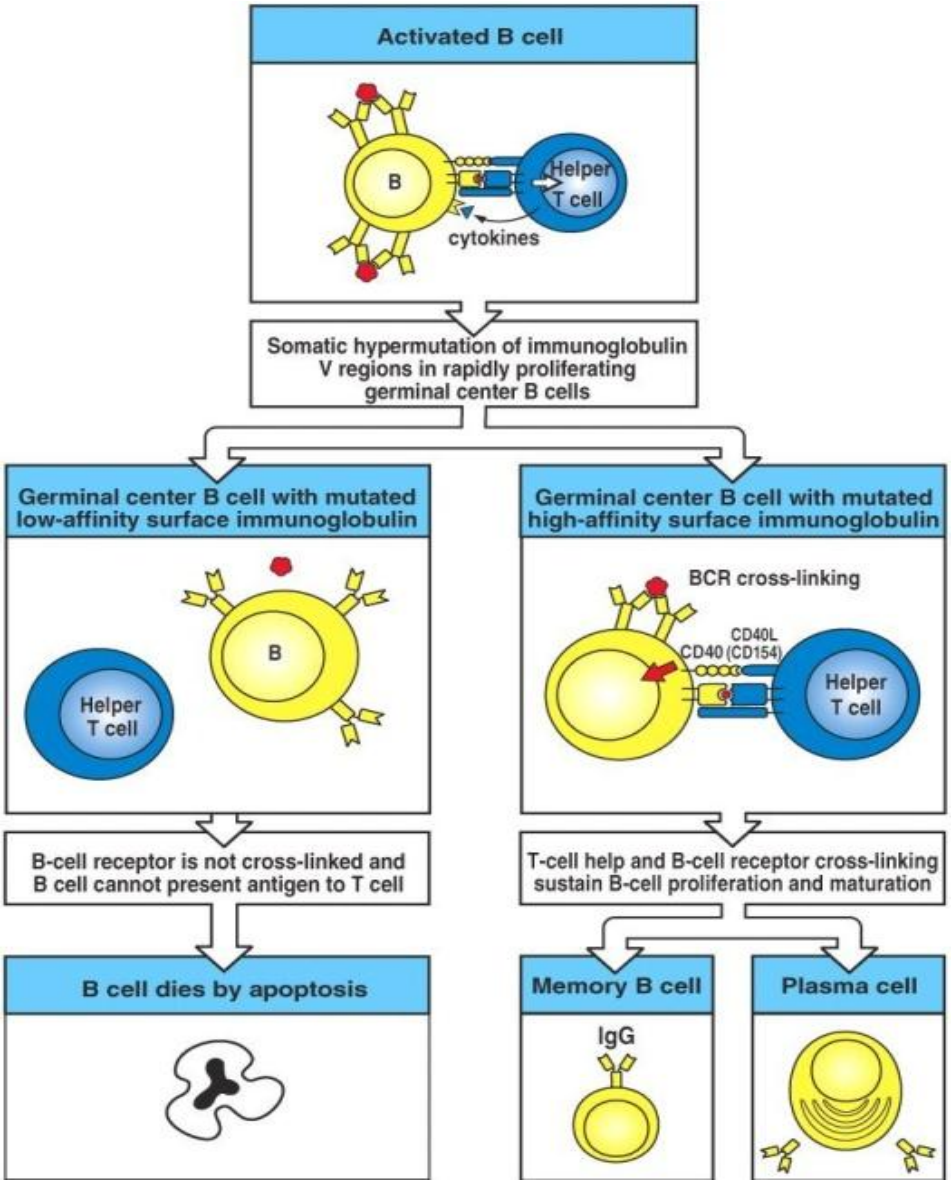


Figure 2. Activated B cells result in antibody producing cells and memory cells.

B cells are activated by Tcells and antigen and migrate to germinal centres. Low affinity antigen binding with BCR result in apoptosis whereas high affinity binding leads to IgG and memory cell production (reproduced from Janeway, Immunobiology, 2005).

The response is then generated in lymphoid tissues such as bone marrow, thymus, tonsils, and most importantly lymph nodes - filled with B and T lymphocytes (McCullough and Summerfield, 2005). Equally important to the type of response generated are the levels of antigen present and the period during which the antigen remains in secondary organized lymphatic tissues, where primary immune responses are initiated (Zinkernagel and Hengartner, 2001).

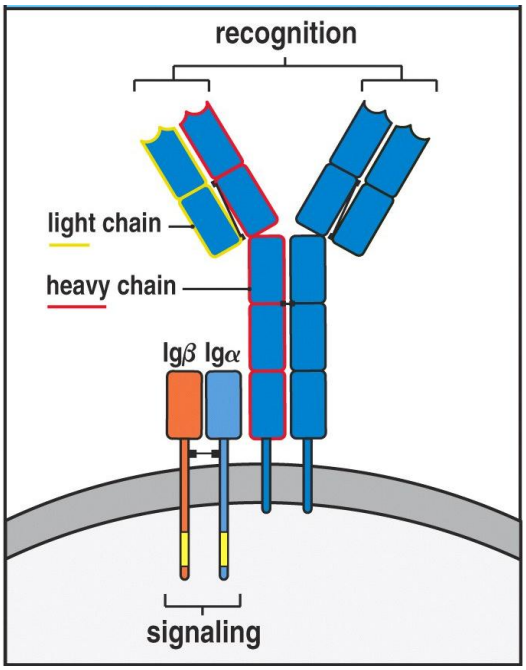


Figure 3.The membrane bound B cell receptor, BCR or membrane bound IgM (IgM).
The immunoglobulin recognises and binds antigen and relies on amino acid membrane complexes to signal that binding has occurred in order that resting genes are turned on (adapted from Janeway, Immunobiology, 2005).

### 1.2.1 Cells involved in the immune response

B and T cell lymphocytes are mediators of immunity under the control of immature DCs which ingest antigens and provide the link between the innate and adaptive immune systems. The relationship between three cell types - dendritic cells, T cell lymphocytes; subgroups cytotoxic (Tc), helper, (Th) and regulatory (Treg); and B cell lymphocytes, influences the precise control and regulation of the immune response and depends on cognate ligand-receptor recognition between the T helper and B cells (McCullough and Summerfield, 2005; Takeuchi and Akira, 2010).

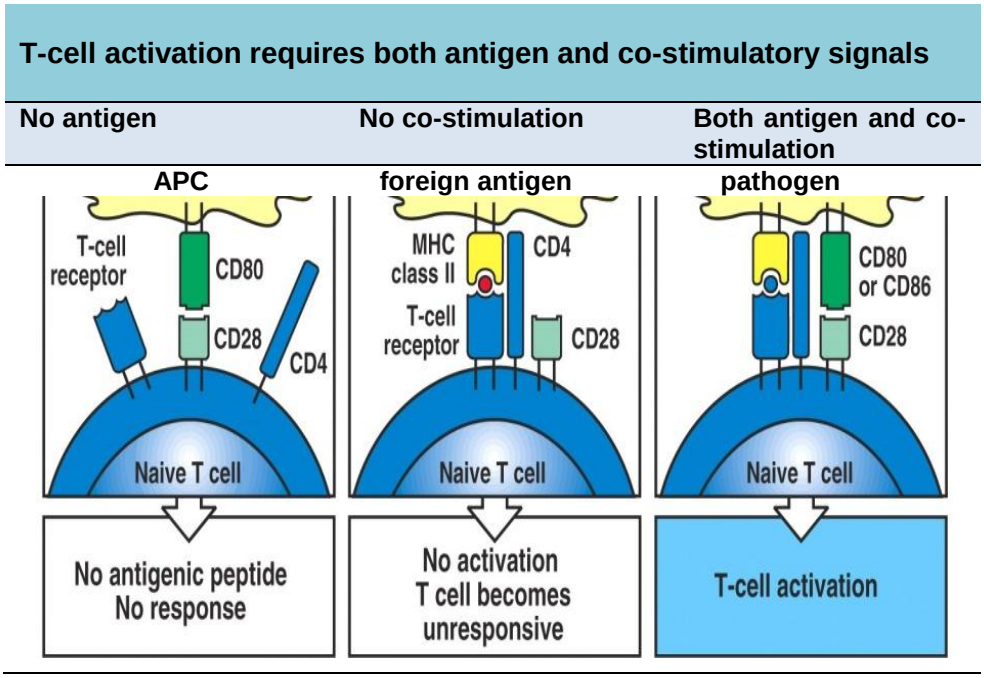


Figure 4. Effect of antigen presentation by APC such as DCs.
Antigen must be presented by APC that also expresses co-stimulatory molecules (Adapted from Janeway, Immunobiology, 2005).

The numbers of adaptive immune cell clones generated - the lymphocyte repertoire- guarantee that there will be a lymphocyte clone with a receptor sequence that can bind any antigen encountered during a host's lifetime. Only those lymphocytes that encounter the antigen corresponding to their own receptor will be activated to proliferate into effector cells. Once there are sufficient antigen-B cell receptor pairs on the surface of a B cell, and with T help cell when needed, the lymphocyte becomes fully activated. B cells differ from T cells in that they have membrane-bound immunoglobulins (mIgM) or B cell receptors (BCR), of the same specificity as antibodies produced by plasma cells (Figure 3), while T cells have membrane antigen receptors (TCR) which are similar to the Fab fragment of immunoglobulin (Figure 4).

*Antigen Presenting Cells – APCs*

Professional antigen-presenting cells capture and internalize antigens either by phagocytosis or endocytosis then display a fragment of the antigen to Major Histocompatability Complex (MHC) molecules, Class I and II. This complex is then

recognised by T cells which are activated by co-stimulatory molecules secreted by the APCs. There are three main types of professional antigen-presenting cells - dendritic cells (DCs), macrophages and B cells.

*Dendritic cells*

Dendritic cells are professional antigen-presenting cells initiating T cell responses, inducing macrophages to take up particulate material and inducing B cells to internalise specific antigen by receptor-mediated endocytosis of the antigen bound to their surface immunoglobulin. Once stimulated, DCs express co-stimulatory molecules, migrate to lymphoid organs for delivery to B cells; secrete cytokines to initiate the immune responses and present processed antigen to T cells. DCs continuously sample the environment for the presence of antigens, which can be either invading microbes/foreign antigens or self-antigens present in tissues (Unger and van Kooyk, 2011). DCs have a dual function - initiating an immune response in the presence of pathogens and tolerance in the absence of infection (Adams et al., 2008).

They are equipped with different immune sensors. The initial sensing of antigen is by pattern recognition receptors (PRRs), which include: CD molecules, Toll-like receptors (TLR), retinoic-acid inducible gene (RIG)-like receptors (RLR) that recognize pathogen related structures and certain C-type lectin receptors (CLR) that recognize carbohydrate structures present on self-antigens or pathogens (García-Vallejo and Van Kooyk, 2009; Takeuchi and Akira, 2010). Recent evidence shows that CLRs are not merely specialized in antigen uptake but they also signal pathways upon interactions with glycans or oligosaccharides (Unger and van Kooyk, 2011). When antigenic molecules bind to these glycan receptors on DCs, specific downstream signaling pathways are activated resulting in innate immune activation as well as tight control of adaptive responses (Bonifaz et al., 2004). DCs with CD1 capture glycolipid antigens and with CD 103+ capture luminal carbohydrate antigens in the intestine (Bollyky and Wilson, 2004; McDole et al., 2012).

Antigenic proteins are endocytosed by DCs, transported into endocytic compartments for loading onto MHC molecules (Figure 4) creating peptide major histocompatibility complexes (pMHC) which makes them available on the surface for perusal by passing T helper and Natural Killer T cells with co- receptor surface proteins including CD1+, CD4+ or CD8+.

The DC migration to lymph nodes can be slow (1-3 days) which enhances both tolerance and immunogenic responses (Delamarre et al., 2005). They accumulate in lymphoid tissue where macrophages and B cells are generally excluded and in areas where naive T cells are activated and have surveillance and migratory properties carrying antigens to lymphoid tissue.

*Macrophages*

Macrophages are part of innate response but by secretion can also help initiate specific defense mechanisms of adaptive immunity of vertebrate animals. Their role is to phagocytose cellular antigens. They also present carbohydrate antigens to lymphocytes and other immune cells (Gorczynski, 1979) however they are poor presenters of antigen due to the lack of class II HLA antigens on their surfaces (Mollison, 2005). Macrophages have receptors for IgG immunoglobuin and the complement fragment C3b but not for its stable breadkdown product C3dg. This fact explains why some antibody/complement coated red cells are not removed by phagocytosis.

*B cells*

The primary function of B cells is to secrete immunoglobulins (Ig) or antibodies and express co-stimulatory and adhesion molecules for T cell interaction, each with unique specificity. Naive B cells have B Cell Receptors which have membrane bound IgM - the first antibody type produced in the immune response. Switching to IgG production occurs after further stimulation by antigen (Mond et al., 1995). B cell responses are classified as T-dependent (TD) or T-independent (TI) based on their requirement for T cell help in antibody production - divided into groups 1 and 2, (Mond et al., 1995; Obukhanych and Nussenzweig, 2006). They are induced very efficiently without T cell help when an antigen is presented in a repetitive rigid form or when linked to polyclonal B cell activators such as lipopolysaccharides. Other antigen configurations, including multimeric antigens present on flexible backbones (e.g., flagellae of bacteria) or those inserted into infected cell membranes, as well as monomeric or oligomeric protein antigens, induce B cells (with the production of IgM) only if helped by specific CD4+ T cells (Zinkernagel and Hengartner, 2001). TD antigens elicit memory B cells with various Ig isotopes, (McHeyzer-Williams and McHeyzer-Williams, 2005).

B cells stimulated by TI-1 antigens result in a polyclonal antibody response via Toll-like receptors while TI-2 antigens stimulate long lasting IgM and, with T cell help, IgG antibody production (Obukhanych and Nussenzweig, 2006). The recognition of TI antigens by B cells involves binding of intact antigen with the Fab fragment (V region) of the secreted immunoglobulin or by binding with a membrane BCR.

B cells have CD1d molecule receptors which are specific to glycolipid antigens. CD1d mediated interaction between activated T and B cells was shown to be essential for B-cell proliferation in α-Gal-antibody production (Liu et al., 2009).

*Innate-like lymphocytes (ILLS)*

B-1 cells

B-1 cells predominate in peritoneal and pleural cavities. The B-1 response is TI and occurs within 48 hours of antigenic exposure. These cells have surface CD5+ molecules and recognise polysaccharide, carbohydrate antigens. Immunological memory is not generated as they only secrete IgM antibodies in blood without T cell help. The presence of CD5+ molecule on B-1 cells serves to mitigate activating signals from the BCR so that these cells can be activated only by very strong stimuli (such as bacterial proteins) and not by normal tissue proteins.

B-2 cells

B-2 cells refer to the majority of the B cells in spleen and lymph nodes. These cells are small, long-lived resting cells that express low levels of surface IgM and high amounts of IgD. They are stimulated by TD antigens.

NK T cells

Natural KillerT cells exist in thymus and lymphoid organs and appear to recognise glycolipid antigen mediated by secretion of cytokines. They constitute only 0.2% of all peripheral blood T cells. Upon stimulation, NK T cells produce significant quantities of interleukin (IL)-4 and interferon-gamma, and exhibit enhanced cytolytic activity. They can either up-or down-regulate immune responses by promoting the secretion of Th1, Th2, or immune regulatory cytokines (Godfrey and Kronenberg, 2004). Specific NK T cells recognize glycolipid antigens associated with the molecules of the CD1 family present on the membrane of APCs (Leadbetter et al., 2008; Mori and De Libero, 2008). These CD1-restricted T cells are involved in anti-microbial responses, anti-tumor immunity, and in

regulating the balance between tolerance and autoimmunity (Brigl and Brenner, 2004). They have receptors with a repertoire specific to binding lipid-containing antigens which, upon activation, can rapidly produce T helper cell (Th1 and Th2) cytokines (Moody and Porcelli, 2003). CD1-restricted T cells carry out effector, helper, and adjuvant-like functions and interact with other cell types including macrophages, dendritic cells, NK cells, T cells, and B cells, thereby contributing to both innate and adaptive immune responses (Brigl and Brenner, 2004).

Humans have 5 different CD complexes (a-e) on APCs and on other cells including red cells. Mice only have CD1d found on APCs and most haematopoetic cells (Bollyky and Wilson, 2004; Lockridge et al., 2011). CD1a, b, and c molecules and specific NK T cells recognize microbial and glycolipid self-antigens complexed to both MHC Class I and II (Liu et al., 2009) – also microbial ligands and other self-antigens as yet undefined (Brigl and Brenner, 2004; Borg et al., 2007; De Libero and Mori, 2010). CD1d presents lipid based antigens to NK T cells (Christiansen et al., 2011) and membrane -associated oligosaccharides are known to modulate NK cell activity via CD1 receptors (Kovalenko et al., 2007; Liu et al., 2009).

*Natural Killer Cells*

Natural killer (NK) cells are cytotoxic lymphocytes activated by macrophage cytokines early in the innate response but are also able to kill non-self cells without secondary activation since they require no preliminary sensitization and can independently recognize damaged cells to be eliminated in a similar way to cytotoxic T cells. These cells regulate responses to antigens that do not require T cell help to stimulate antibodies (Mond et al., 1995a) and do not have T cell receptors or B cell membrane immunoglobulin. They lack antigen specificity but have receptors on the cell surface that are modulated by signals from the target cell and distinguish between self and non-self by the changes in MHC expression or changes in cell membrane glycoproteins. They have two types of receptors which appear to control cytotoxic activity. Binding of one receptor Ly-49D enables a cytotoxic response and binding of Ly-49A inhibits the response; that is, one reaction activates the killer cell behaviour and the other prevents killing of normal host cells (Shimizu et al., 2005). The identification of the inhibitor NK cell receptor provided the molecular basis for the “missing-self” hypothesis that proposed that expression of self-MHC class I antigens on cells leads to NK tolerance of the antigen but alteration of self-

MHC I antigens leads to removal of the antigen by NK cells (Nakamura and Seaman, 2001). Both mice and humans have these receptors (Shimizu et al., 2005).

Carbohydrates may be ligands for C-type lectin-like protein NK cell receptors which are associated with the activation or inhibition of the cell effector functions and carbohydrate recognizing receptors also play a part in certain pathways of the adaptive immune response (Crocker and Feizi, 1996).

Tumour-associated carbohydrate antigens, of which gastrointestinal, pancreatic, liver, colorectal cancer are representative, are generally not recognized by the immune system because DC activating non-self signals are missing. DCs are classically defined as the sentinels of the immune system, capable of recognizing foreign antigens and specialized in the initiation of the adaptive response against potentially harmful agents. However, NK-cell activation by tumor cells has been shown to initiate DC activation and thus induce protective T-cell responses due to the presence of NK receptors that allow them to detect "missing self" and / or "induced-self" antigens (Mocikat et al., 2003; Kalinski et al., 2005).

*T cells*

The T cell can be thought of as the conductor of the immunological orchestra (McCullough and Summerfield, 2005). It serves as an overseer of the immune response. Without its action, no major aspect of the immune response occurs or is made tolerant. The T cell decides, on the basis of the energetics of the binding of its receptors with antigen, whether to activate other cells on or not. The degree to which it does either or both is dependent on the nature of the TCR:antigen bond and feedback from other cells (B cells, macrophages, eosinophils, basophils and other T cell classes). T cells distinguish between cytosolic and internalised antigen by the type of major histocompatibility complex (MHC) molecules that are bound to the antigen at presentation.

There are different types of effector T cells: Cytotoxic T cells which are mature cells with CD8+ co-receptor that recognize MHC Class I bound peptides derived from proteins in the cytosol; Helper T cells,Th1 which are mature cells with CD4+co-receptor that recognize MHC Class II bound peptides degraded in endocytic vesicles and which also activate macrophages and B cells to produce IgG antibodies; Helper T cells,Th2 which are mature cells with CD4+coreceptor that also recognize MHC Class II bound peptides degraded in

endocytic vesicles and stimulate naive B cells to secrete IgM and to differentiate into other antibody isotypes.

Recent studies indicate the existence of a previously unrecognised population of glycolipid antigen-specific CD4(+) T cells that are CD1-independent and do not require peptide help for antibody stimulation (Christiansen et al., 2011). The response to carbohydrate antigens is generally T cell independent (Wong and Arsequell, 2003).

The decision for T cells to be stimulated or silenced depends on antigen; the dose, time of expression, environment of antigen encounter and T cell frequency. T cells only recognize foreign antigens that have been internalized by endocytosis from extracellular fluid and displayed on surfaces of body's own cells. They do not bind soluble or native unbound antigens. They are related to immunoglobulin both in their protein structure with both V and C portions and their genetic ability to produce great variation in specificity. Membrane bound T Cell Receptor molecules (TCR) function to signal T cells for activation recognising only the short peptide fragments displayed on the APC cell surface bound to MHC I or MHC II glycoproteins. Any T cell receptor is specific for a unique combination of peptide fragments and a particular MHC molecule (with typically small amounts of this complex present on cells - one hundred or less per cell) and this complex must be recognized by specific T cell clones thought to be present at a frequency of 1/100,000 T cells (Banchereau and Steinman, 1998). The recognition of antigen by the MHC complex to which it is attached is called MHC restriction (Klein et al., 1982). TCRs bind to short amino acid sequences in proteins often buried within the native protein which are not recognized unless some processing and unfolding has occurred. If a TCR site recognizes the shape of the pMHC it can be triggered to activate, proliferate, differentiate and take effector action that will ultimately mean removal of the antigen. This results in the secretion of cytokines to promote further activation of cytolytic T cells and B cells.

*Haematopoeitic Stem Cells (HSCs)*

Haematpoetic stems cells are multipotent stem cells that give rise to all the myeloid and lymphocytic blood cell lineages. All stem cells have three general properties: they are capable of dividing and renewing themselves for long periods; they are unspecialized; and they can give rise to specialized cell types. They are found in the bone marrow in adults

and HSCs can usually be obtained directly from the iliac crest part of the pelvic bone, using a special needle and a syringe. These cells morphologically resemble lymphocytes.

Introduction of antigen-encoding genes into the HSCs allows effective delivery of antigen to the DC progenitors. The potential of genetically antigen-modified stem cells to sustain cell lineage resulting in corrections of various diseases has been well-demonstrated (Cui et al., 2003; Nienhuis, 2008; Snowden et al., 2011). Stem cells expressing specific antigens transplanted in a mouse model in low numbers resulted in induction and prolonged maintenance of functional effector T cells killing target cells thus using the ability of cytotoxic T cells to eradicate tumor or infected cells (Denning et al., 2011).

*Goblet cells*

Goblet cells in the intestine function as passages to deliver soluble antigen to DC in the lumen inducing tolerance- shown with *in vivo* imaging in a recent paper (McDole et al., 2012).

### 1.2.2 The complement system and its role in the immune response

The complement system evolved as part of the innate system and it 'complements' the anti-bacterial activity of antibodies. Activated complement causes a cascade of reaction pathways; one resulting in the formation of the terminal complement components forming the membrane attack complex (MAC) that can act directly on cells causing lysis. Another pathway allows activated complement to act indirectly via C3b forming C3a and C5a which mediate phagocytosis and inflammation. The other pathway forms C3b which binds to the complement receptors on phagocytes which lead to the removal of the immune complex. Three complement activation pathways have evolved to label pathogens for recognition and elimination; the classical pathway with components named C1-9; and the alternate and lectin pathways with components called factors (Figure 5).

Complement activation is mainly confined to the surface on which it is initiated and the system is controlled by inhibitors and enzymatic degradation to prevent uncontrolled activation and cell lysis. Enzymatic cleavage of C3 results in C3b and C4b which are rapidly inactivated if there is no immediate binding with proteins or carbohydrates in the immediate vicinity.

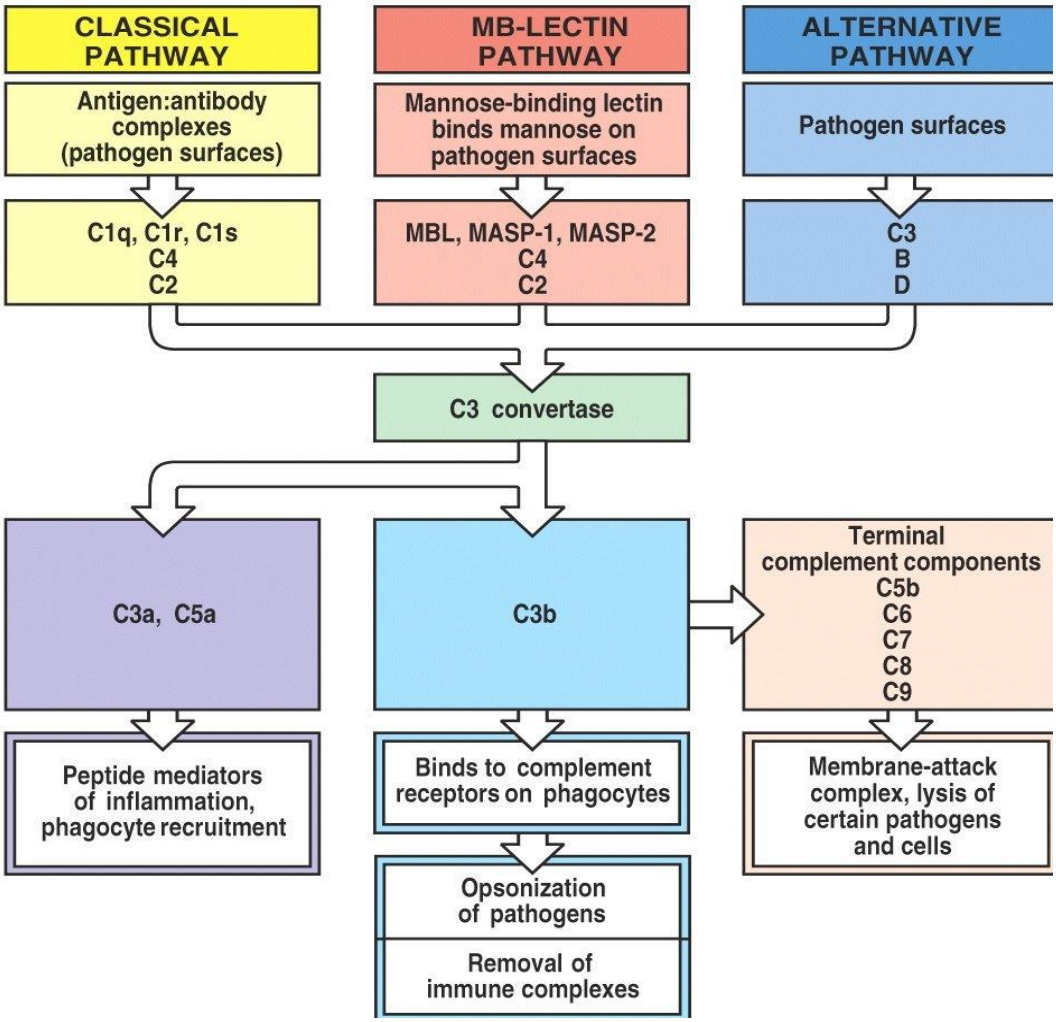


Figure 5. Overview of complement components and effector pathways.

The early events involve cleavage reactions culminating in the formation of C3 convertase enzyme which cleaves complement component C3 to C3b and C3a which encourage phagocyte involvement. The formation of C5a and C5b trigger inflammation and the events leading to the M-AC complex respectively (reproduced from Janeway, Immunobiology, 2005).

Macrophages have complement receptors for the C3 breakdown products C3b and iC3b but not for the next products, C3dg or C3d, so cells coated with these fragments acquire resistance to complement-mediated destruction (phagocytosis) allowing normal survival (Ehlenberger and Nussenzweig, 1977; Nielsen et al., 2002).

Anticoagulants such as heparin and $Na_2EDTA$ are anti-complementary since complement activation requires the presence of calcium and magnesium ions which are chelated by EDTA and heparin inhibits cleavage of C4 (Mollison, 2005).

*The classical pathway*

The classical pathway links to the adaptive immune system and is initiated by antibodies bound to the antigen on the surface of the target cell. Surface bound IgG and IgM antibodies activate complement via this pathway, with the Fc region of antibody binding with the first component of the complement system - the head group of C1(C1q). This molecule has six Fc binding sites and at least two of them must be bound to the Fc portion of the antibody for C1 activation. In the case of IgG, two antigen-bound IgG molecules must be present on the antigen surface within about 20-30nm of each other for C1q to bind - this being the maximum span width of the molecule. However, C1q can bind two sites on one-antigen bound IgM molecule thus making IgM antibodies more efficient in complement activation (Mollison, 2005). Binding results in a conformational change in the C1q molecule and initiation of the cascade to produce fragments C3a and C3b. The C3a fragment mediates inflammation and phagocyte recruitment while the C3b molecule binds to the complement receptors on phagocytes, opsonising the cells to encourage removal of the immune complexes. When C3b is generated rapidly enough and in sufficient quantity, the cascade proceeds to the C5-9 stage leading to the formation of the MAC complex which results in cell lysis. The lesions in the cell membrane produced by the MAC appear as 10nm holes when seen by electron microscopy (Mollison, 2005).

*The lectin and alternate pathways*

The lectin and alternate pathways are independent of antibody initiation. The lectin pathway is initiated after binding of the mannose-binding lectin (MBL) to the mannose residues present on pathogen surfaces. The alternative (to the classical) pathway is activated due to spontaneous hydrolysis of C3 by the C3b binding of hydroxyl and amine groups and can bind to surfaces of pathogens or host cells.

### 1.2.3 Major Histocompatiblity Complex

The Major Histocompatiblity Complex (MHC) is part of the immune response in that it co-ordinates cell-cell interactions in the immune response mechanisms. It is comprised of two types of molecules. Class I molecules, which recognize peptides derived in the cytosol and display these fragments on the cell surface and Class II molecules which bind peptides derived from proteins in the intracellular vesicles. MHC I and II molecules form complexes

with glycolipids that are recognized by CD1d receptors with a repertoire specific to glycolipid antigens (Moody and Besra, 2001; Bollyky and Wilson, 2004).

### 1.2.4 Immune complexes

Immune complexes can inhibit or augment the immune response. Once complement is activated, antigen-immune complexes bound to the Fc component of IgM or IgG antibody become localized via binding with receptors on dendritic cells in follicles. Maintenance of the antigen presence in this way influences the immune response since the antigen is made available longer for B cell uptake or T cells. If the immune complex is removed by phagocytosis the immune response is stopped.

### 1.2.5 Summary of cells involved with immune response particularly to glycolipids

This thesis concerns the murine immune response to the infusion of synthetic glycolipid FSL constructs so the cells and complexes involved in the immune response to glycolipids are of particular interest.

Macrophages present carbohydrate antigens to lymphocytes and other immune cells (Gorczynski, 1979). Dendritic cells are equipped with different immune sensors which include: CD molecules, and certain C-type lectin receptors (CLR) that recognize carbohydrate structures present on self-antigens or pathogens (García-Vallejo and Van Kooyk, 2009; Takeuchi and Akira, 2010). Recent evidence shows that CLRs are not merely specialized in antigen uptake but on interaction with glycans or oligosaccharides, (Unger and van Kooyk, 2011) they signal pathways downstream activating innate immune activation as well as initiating regulatory control of adaptive responses (Bonifaz et al., 2004). Specific NK T cell receptors recognize glycolipid antigens and membrane associated oligosaccharides are presented to DCs, macrophages and B cells by molecules of the CD1 family (Crocker and Feizi, 1996; Moody and Besra, 2001; Kovalenko et al., 2007; Mori and De Libero, 2008; Liu et al., 2009). These receptors, CD1a, b, c and d also recognize microbial and glycolipid self-antigens complexed to both MHC Class I and II molecules (Liu et al., 2009) - the glycolipid antigens are presented to the T cells in the hydrophobic antigen binding cleft of the MHC molecule (Bollyky and Wilson, 2004). Mice APCs and most haematopoetic cells have CD1d receptor molecules (Bollyky and Wilson,

2004; Lockridge et al., 2011). Recent studies showed the existence of CD4 + T cells which are glycolipid antigen-specific (Christiansen et al., 2011).

It should be appreciated that in the experiments described later, when FSL constructs are infused into the circulation, all the circulating cells, including red cells, B cells, T cells, NK cells, haematopeotic stem cells, dendritic cells and macrophages described above would all acquire FSL antigens.

## 1.3 Stimulation of the Immune Response

Stimulation of the immune response due to the presence of antigen normally results in recognition of the antigen as foreign and its removal by antibody production. If it is not recognized as foreign there is ignorance, tolerance or accommodation of the antigen with no humoral response. These terms are discussed later (1.5).

An antigen is a substance that binds to specific antigen receptors on T cells (TCR) and B cells (BCR) and can neutralize antibody. However, not all antigens can induce an immune response with lymphocyte activation. An immunogen is a substance that can stimulate an antigenic immune response on its own, with the production of antibodies, and is said to be immunogenic. So, all immunogens are antigens, but not all antigens are immunogenic. Immunogenicity is determined by many factors, including antigenic molecular size, chemical composition and complexity, dose, susceptibility to MHC genes for antigen processing and presentation, the route of administration, and the requirement for adjuvants (Delamarre et al., 2005).

B and T cells bear thousands of copies of a single type of specialized antigen receptor on their cell surfaces that recognizes only one antigen or more precisely, molecules with closely related shapes or amino acid sequences. The interaction of these multiple receptors with multiple copies of the same antigenic determinant is required to trigger the activation of the lymphocyte and the generation of a particular effector response that ultimately eliminates the antigen (Delamarre et al., 2005). Suppression of the immune response down-modulates the response for example, by the deletion of B cells or cytokine networks, and generally once the antigen is eliminated the response is halted (Zinkernagel and Hengartner, 2001).

Antigen presence in the organised lymphatic tissues for 3-5 days will effect an immune response (Zinkernagel and Hengartner, 2001) and the route of administration and form of antigen determines the type of that immune response. Antigens presented in soluble forms are captured by APCs including macrophages and immature dendritic cells with macropinocytic activity. These are the main cell types found to carry injected soluble antigens to lymph nodes after intravenous, intraperitoneal or intradermal injections. The trapping of antigens by APC in the lymphoid tissues and the continuous recirculation of naive T cells ensure that rare antigen specific T-cells will encounter their specific antigen on the APC surface due to the architecture of the lymph nodes, spleen or mucosal lymphoid tissues.

Antigens administered in the skin are processed by dendritic cells which stimulate a full immune response by T cells (Kirkley, 1999). Antigens administered orally, or by intraperitoneal or intranasal routes and antigens introduced by intravascular routes may be recognized by macophages or B cells which do not induce a T cell response due to lack of costimulatory interactions (Liblau et al., 1997; Kirkley, 1999).

Both T and B cells must see an epitope on the same antigen although it need not be the same epitope. Thus, when there is a complex macromolecule, there is plenty of scope for both T and B cell epitopes, so the antigen is immunogenic. However, when the antigen is a small chemical group or peptide or a carbohydrate not conjugated to MHC molecules, it is not recognized by T cells so is poorly immunogenic (Slovin et al., 2005). Aggregated proteins are immunogenic compared with peptides or monomeric antigens which are tolerogens (Liblau et al., 1997; Kirkley, 1999). There is a threshold of sufficient antigen-receptor multimers on a cell surface, which once attained, encourages proliferation and differentiation of B and T cells into effector and memory cells. The binding of a single antigen molecule to a single receptor in a membrane is insufficient to motivate lymphocyte activation.

The concentration of the immunogen greatly affects the immune response and acts as a control determining the level of help appropriated from T cells. The lesser the amount of antigen in absolute and local concentrations, the more directly the B cell response is reliant on T cell help. The efficient switching from short-lived IgM immunoglobulin production (IgM half life is about 24 hours) to other classes, in particular long-lived IgG immunoglobulin (IgG half life is about 20 days) requires conventional T cell helper activity (Zinkernagel and Hengartner, 2001). Once the number of antigen-receptor complexes

drops the activation threshold is not reached and the response subsides (Zinkernagel, 1996). Antigens are classified as Thymus Dependent (TD) or Independent (TI) and TI antigens are further classified as type 1 and type 2.

### 1.3.1 Thymus dependent antigens

Thymus Dependent (TD) antigens require the help of T cells to deliver activating signals to B cells to elicit memory B cells (Figures 6 and 7) and IgG isotype antibodies (McHeyzer-Williams and McHeyzer-Williams, 2005). TD antigens must be first bound by dendritic cells, processed in endocytic compartments and then presented as MHC II peptide complexed to antigen to activate specific T cells (Mond et al., 1995b; Trombetta and Mellman, 2005).

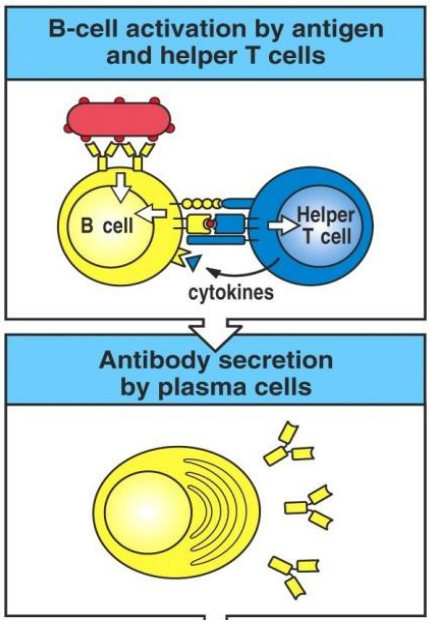


Figure 6. TD antigens stimulate the humoral response with the production of antibody molecules.

These are secreted by plasma cells. Antigens bind to the BCR and are internally processed into peptides that activate Th cells. Signals from the bound antigen and from the Th cells induce B cell proliferation and differentiation into plasma cells which secrete specific antibodies (reproduced from Janeway, Immunobiology, 2005).

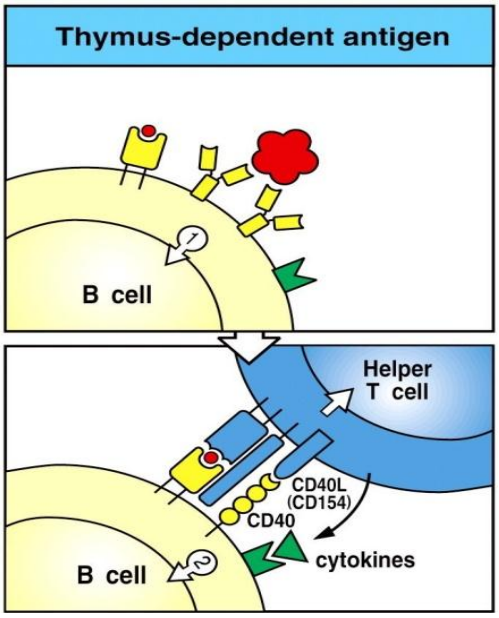


Figure 7. TD antigens require two signals to activate B cells.

The first antigen (red) signal is delivered through its antigen receptor (1) and the second signal is delivered by a helper T cell that recognises degraded fragments of the antigen as peptide bound to MHC class II molecules on the B cell surface (2). The CD molecules and cytokines contribute an essential part of the signal (reproduced from Janeway, Immunobiology, 2005).

The first B cell signal is delivered through the antigen receptor and the second by a T cell helper that recognizes the MHC degraded fragments. TD antigens can also activate B cells via CD 40 (the B cell activating membrane protein). Activation leads to IgM production and subsequent Ig class switching, with secretion of IgG antibody dependent on cytokines. It is widely accepted that memory B cells are derived from TD responses resulting in long lived high-affinity IgG antibodies (Mond et al., 1995b; Astronomo and Burton, 2010).

### 1.3.2 Thymus independent antigens

Thymus independent (TI) antigens, TI-1 and TI-2 (Figure 8) are large multivalent molecules with long half-lives when injected in vivo (Mond et al., 1995b). The multivalency of these antigens enables them to induce domains of cross-linked membrane Ig inducing B cell stimulation at a low antigen concentration (Mond et al., 1995b). Many microbial constituents and lipopolysaccharide antigens are TI (Wong and Arsequell, 2003; Janeway, 2005). Glycolipid antigens are generally regarded as TI antigens (Wong and Arsequell, 2003), but are also recognized by T cells when complexed with another molecule – the glycoprotein receptor CD1 on APCs (De Libero and Mori, 2006).

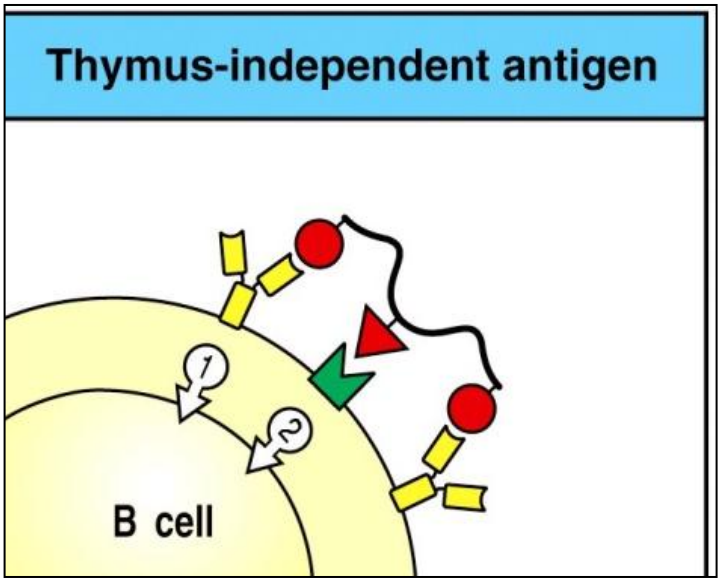


Figure 8. Thymus independent antigens.

These antigens firstly bind to the BCR (1) and then can provide a second signal through direct binding of the antigen (red) to a receptor (2) such as Toll like receptor (green) or by cross-linking (not shown) (reproduced from Janeway, Immunobiology, 2005).

*TI-1 antigens*

TI-1 antigens are mitogenic stimuli that elicit polyclonal B cell activation via Toll-like receptors. They have intrinsic activity that at high concentration can directly induce cell division of mature and immature B cells even without binding to surface immunoglobulin- thus all B cells respond with polyclonal, nonspecific antibody production (Stein, 1992; Mond et al., 1995a). At low concentration, TI-1 antigens induce a response from only those B cells specific for the antigen epitope (Zinkernagel and Hengartner, 2001) and at high concentration stimulate non-specific antibody production from B cells (Figure 9).

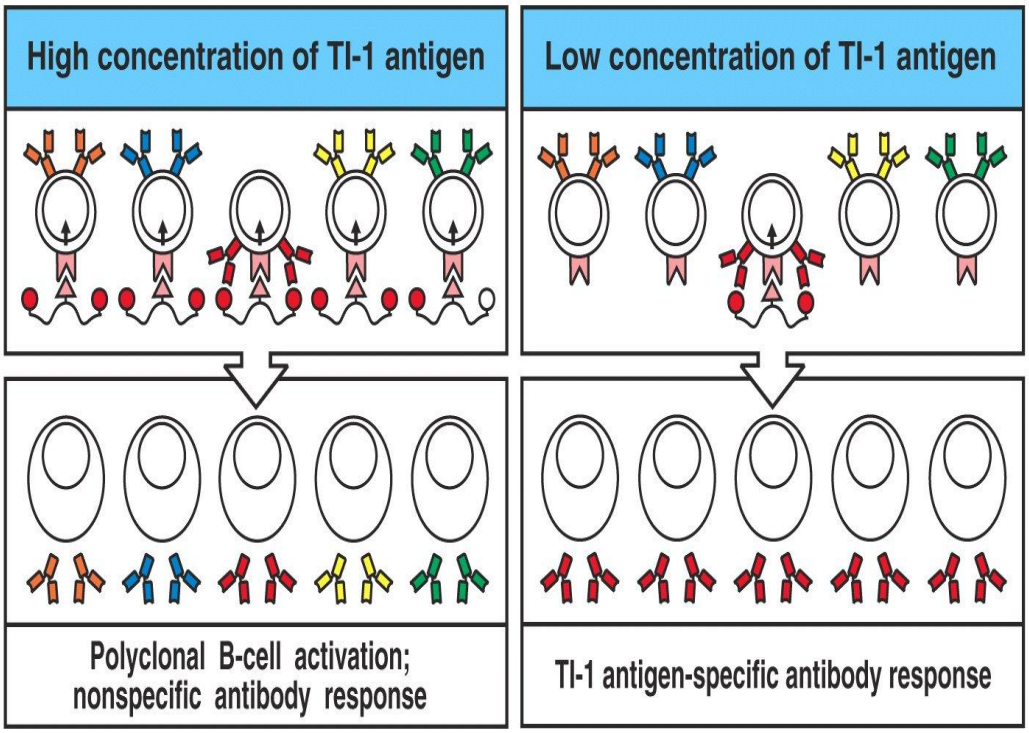


Figure 9. Thymus independent antigens type 1 (TI-1).

These antigens stimulate different immune response depending on antigen concentration. At high concentration, the signal is sufficient to induce polyclonal B cell proliferation and antibody secretion in the absence of specific antigen binding to surface Ig. At low concentration only B cells specific for the TI-1 antigen bind enough antigen to induce an antigen-specific antigen body response (reproduced from Janeway, Immunobiology, 2005).

*TI-2 antigens*

TI-2 antigens consist of non-protein molecules such as polysaccharides with highly repetitive structures that can activate only mature B-1 cells as immature B cells are inactivated by repetitive epitopes and cannot elicit a humoral response (Mond et al., 1995a). TI-2 antigens include glycolipid ABO determinants which are highly repetitive on mammalian cell surfaces and are recognised by CD1 molecules on NK T cells (Neron and Lemieux, 1994). The oligosaccharide human blood group A antigen was shown to stimulate a murine TI-2, IgM immune response (Neron and Lemieux, 1994).

Studies show that TI- 2 antigens can elicit robust and long-lasting primary antibody responses in mice using other than T cell stimulation to induce B cell differentiation and isotope switching (Garcia de Vinuesa et al., 1999). TI-2 carbohydrate antigens can activate NK cell carbohydrate receptors directly or with the help of cytokines (Daniels et al., 1994;

Mond et al., 1995a). B cell activation by TI-2 antigens such as glycolipid antigens can lead to IgM antibody production due to multiple cross-linking of BCR receptors by polyvalent antigen.TI-2 antigens require or are enhanced by cytokines (green) which leads to isotope switching (Figure 10).

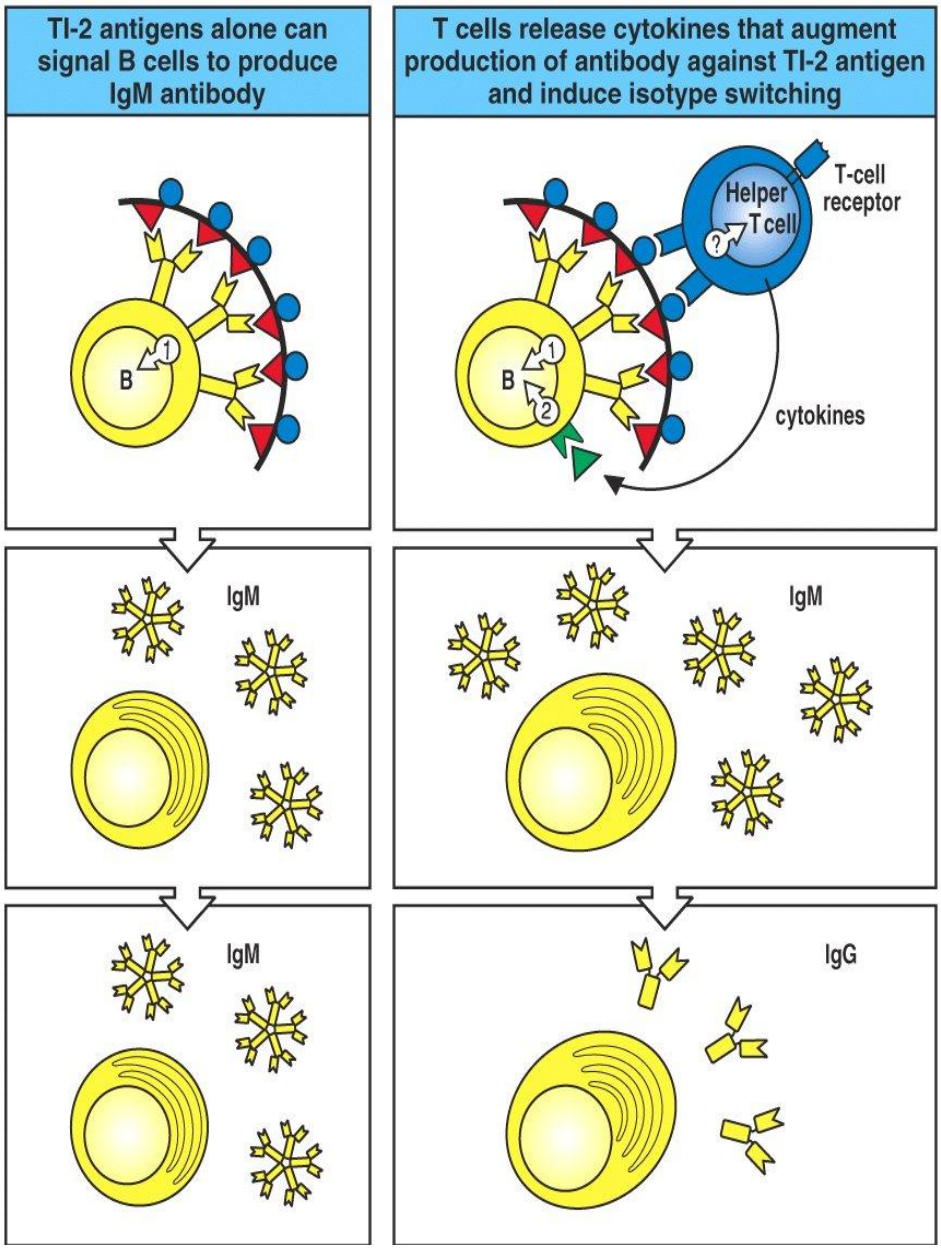


Figure 10. Thymus Independent antigens type 2 (TI-2).

B cell activation by TI-2 antigens such as glycolipid antigens can lead to IgM antibody production due to multiple cross-linking of BCR receptors by polyvalent antigen.TI-2 antigens require or are enhanced by cytokines (green) which leads to isotope switching . Specific T cells can also recognise these antigens when they are bound to MHC molecules such as CD molecules (not shown) (reproduced from Janeway, Immunobiology, 2005).

### 1.3.3 Carbohydrate antigens

Research into the activation of the immune response by small carbohydrate antigens presented as FSL constructs is the basis of this thesis. Previous research in carbohydrate biology has shown that carbohydrate recognizing receptors are an integral part of the innate immune system (Crocker and Feizi, 1996; Feizi, 2000). Membrane-associated oligosaccharides are known to take part in interactions between natural killer (NK) cells and their targets and modulate NK cell activity via CD1 receptors including CDd1, (Crocker and Feizi, 1996; Kovalenko et al., 2007; Liu et al., 2009). It has also been shown that carbohydrate antigens can be presented to specific T cells by APCs which are not restricted to MHC or CD1 molecule binding (Corinti et al., 1997; Christiansen et al., 2011) and TI-2 carbohydrate antigens can activate cytokine production which then influence Th cells (Mond et al., 1995b).

Recent evidence has shown that several distinct zwitterions carbohydrate polysaccharides found on the surface of commensal bacteria in the gut promote the development and function of adaptive immunity. There is increasing evidence that the absence of these bacterial molecules may allow the development of autoimmune disease (Lee and Mazmanian, 2010). In particular, the polysaccharide A molecule on *Bacteroides fragilis* stimulates a CD4+ T cell response and T cell regulation and also elicits cytokine production (Eynon et al., 2005, Mazmanian et al., 2005). Purified PSA, given orally, treated colitis in mice and was able to prevent and cure autoimmune disease in an experimental animal model for multiple sclerosis and rheumatoid arthritis (Lee and Mazmanian, 2010).

However, carbohydrate antigens are generally thought to be T cell independent and therefore initiate a different immune response to proteins and peptides. They stimulate B cells after binding to BCR resulting in the stimulation of IgM antibody production only (Buskas et al., 2005). This is because the switch to IgG antibody appears to require antigen presentation by APCs to T cells (Guo and Wang, 2009). Crystallography studies show that carbohydrate antigens endocytosed in the APCs protrude from the cell in such a way that prevents APC interaction with the T cell receptor accessory molecules which are necessary for inducing T cell activation.

Carbohydrate antigen-antibody reactions require the presence of several monosaccharide moieties presented in the correct conformation in order to bind to the receptor with high affinity. Sugars attached to self are not normally presented in the appropriate homogenous

geometrical array needed to activate the immune response (Rudd et al., 2001). Carbohydrate antigens are covalently linked to protein or lipid to form glycoproteins or glycolipids. To elicit an immune response, glycoproteins are processed to glycopeptides which are presented by classical MHC molecules. Glycolipid based antigens are presented by MHC- like protein CD1 molecules (Christiansen et al., 2011). Glycolipids and glycoproteins in APCs are cleaved into non-immunogenic monosaccharides (Galili, 2004) and only saccharides in polymeric, or clustered, but not monomeric glycoconjugates resulted in alteration of cytotoxicity suggesting that appropriate presentation is critical for carbohydrate recognition and subsequent biological effects (Kovalenko et al., 2007).

Carbohydrates or saccharides alone cannot activate T-CD8+ (MHC I) cells nor stimulate B cell proliferation and so, in the absence of this T cell help, anti-carbohydrate B cells exposed to incompatible carbohydrate antigen gradually become tolerised (Ogawa et al., 2004).

*Glycolipid antigens*

Glycolipids are carbohydrates attached to lipids which serve to help maintain the stability of the cell membrane and to serve as markers for cellular recognition. The antigenicity of glycolipids is dependent on their concentration and accessibility to receptor molecules. The concentration required for glycolipid antigen T cell activation is provided by the formation of lipid–protein complex aggregates in cells and membranes (Kannagi et al., 1983; Marcus, 1984; De Libero and Mori, 2006). A large body of evidence has shown that the hydrophobic parts of lipids participate in immunogenicity and the length and the structure of the ceramide tail contributes to TCR recognition serving as a ruler to orientate the hydrophobic head, with the length of the acyl chain important for providing binding sites for CD1 cells (De Libero and Mori, 2006). The hydrophobic nature affects how they remain in biological fluids and are they are perceived. The structure of the ceramide moieties of the glycolipid oligosaccharide greatly influence the antigen conformation and therefore its recognition by T cells - although the antibody response is mainly directed to the oligosaccharide (Portoukalian, 2000).

Glycolipids are immunologically processed very differently to glycoproteins (De Libero, 2004) and are generally regarded as T independent antigens, and research has shown that specific NK T cells recognize glycolipids by their CD1d molecules (Borg et al., 2007). However, α-gal glycolipid produced a T dependent Th1 response with CD1d, B cell

receptors on NKT cells (Liu et al., 2009) and CD1d receptors with a repertoire specific to glycolipid antigens recognised both TD and TI antigens complexed to both MHC I and II molecules (Moody and Besra, 2001; Bollyky and Wilson, 2004).

Lipid immunogenicity is determined by the mode of uptake, membrane trafficking abilities, and capability to form stable complexes with CD1 molecules (De Libero and Mori, 2010). CD1 molecules (CD1d in particular) found on DCs, monocytes and thymocytes, splenic B cells and on specific NKT cells (Koch et al., 2005; De Libero and Mori, 2006) bind and present lipid antigens, including foreign lipids, self-glycosphingolipids and phospholipids to T cells (Moody et al., 1999). The glycolipid antigens bind to CD1 molecules with their chains buried in the hydrophobic antigen binding cleft and the polar head-group is recognized by the NK T cell which then reacts with macrophages DCs, NK cells and B cells (Bollyky and Wilson, 2004; Brigl and Brenner, 2004). The human antigen CD1b binding groove has 4 pockets and the capacity to bind large lipid chains (Moody and Porcelli, 2003). The similar CD1d structure of the binding groove in the mouse has 2 pockets with amino acid residues that react with amphipathic lipids (Koch et al., 2005). This interaction exposes the polar head of the antigen to recognition by NKT killer and T cells as was demonstrated when CD1d was loaded with glycolipid α-Gal-Cer (Koch et al., 2005). It may be that human NKT cells do not have as potent immunological effects as mice and that they have different antigen trafficking and presentations from each other (Borg et al., 2007).

Although mice have shown to be useful as an in vivo model, the success of glycolipids as therapeutic agents to activate human NKT cells by CD receptors requires a system to examine the human CD1d pathway (Lockridge et al., 2011). New studies have shown glycolipid antigens can also react with specific CD4(+) T cells stimulating antibodies without CD1d , NKT cells or peptide help (Christiansen et al., 2011).

However, it should be noted that the presence of the spacer on a synthetic construct makes this molecule differ significantly from glycolipids and thus it may interact very differently with the immune system. The structure of the glycolipid affects its function and where it partitions within the cell membrane (Holthuis et al., 2003).

*ABO histo-blood group antigens*

Landsteiner concluded that ABO antigens of red cells consist of two parts: one part protein which stimulates antibodies and the other is alcohol-soluble, perhaps a lipoid and contains

the specific group but is not antigenic until combined with proteins (Landsteiner and Simms, 1923). Blood group carbohydrate structures, such as ABO, Lewis, I, P and a few other systems, are found in blood and tissues so are best described as histo-blood group antigens (Samuelsson and Breimer,1987). ABH and Lewis blood group glycoconjugates of the skin, urinary and gastrointestinal tract may serve as body armour against invading environmental microorganisms (Springer and Horton, 1969; Pittiglio, 1986).

ABO histo-blood group antigens on vascular endothelial cells differ from those on the red cell membrane both in terms of structure and of antigenicity depending on the different proteins bound to the carbohydrate chain of each antigen. In a recent paper, antigens have been distinguished as ABO blood group antigens on red cells and ABO histo group antigens on vascular cells (Takahashi and Saito, 2012). There are also ABO histo-blood group associated antigens found on bacterial surfaces which can result in cross reactive activity against the graft in cases where sepsis develops post-transplant (Takahashi and Saito, 2012) and results in antibody-mediated rejection (AMR).

The blood group antigens A and B are major risk factors in transplantation and blood transfusion. When expressed on grafts but absent on recipients, the histo-blood group antigens can bind natural antibodies or elicit antibodies and cause immediate hyperacute graft rejection. Such rejection is observed in most patients receiving ABO incompatible kidneys or hearts. Transplantation across ABO barriers may result in rejection due to antigen/antibody binding; or accommodation if the antibodies do not reject the graft even though present; or tolerance where no blood group antibodies are produced and the graft is accepted (Galili, 2004). It seems accomodation can be established if the graft can survive for the first 1-2 weeks without stimulation of production of anti-graft antibody (Takahashi and Saito, 2012).

Blood group saccharide antigens are found on red cells linked to proteins (glycoproteins) and linked to sphingolipid (glycosphingolipids) at the end of longer linear and or branched backbone glycan chains. Most individuals also have the glycoprotein forms of the ABO antigens in secretions. There are different types of glycan chains, labelled depending on the sugar-carbon linkage. Type 1 chain oligosaccharides contribute A, B and H activity as glycoproteins in body fluids and secretions; in milk and urine (Lemieux, Baker, & Bundle, 1977), in gut tissue, membranes and blood plasma bound to lipids as glycolipids or glycosphingolipids (Marcus and Cass, 1969; Marcus, 1984; Breimer et al., 1988; Koscielak, 2001). Type 1 and 2 glycoproteins provide A, B and H antigens found in saliva

(Vengelen-Tyler, 1999). The dominant red cell ABO antigens are type 2 (Obukhova et al., 2011). The number of potential A, B and H sites on red cells is thought to be up to an excess of two million (Berneman et al., 1991), but this number is still uncertain.

To elicit an anti-carbohydrate immune response, glycoproteins are processed to glycopeptides and presented by the classical antigen-presenting molecules, the major histocompatibility complex (MHC) Class I and II. Blood group A and B antigens and others stimulate production of complement-binding antibodies. Once these antibodies bind to these antigens on red cell membranes, complement is activated via the classical pathway with the Fc region of the antibody binding with the first component of the complement system, Cq1. The C3b molecule then formed from cleavage of this molecule binds to complement receptors on phagocytes resulting in phagocytosis or the formation of the membrane attack complex on the cell surface which results in cell lysis.

*Blood group substance antigens in saliva*

Yamakami first discovered the presence of A antigen blood-group substance in his saliva (Yamakami, 1926). The most potent source of the group-specific substance was found to be in gastric juice and saliva, with saliva secretion containing up to 100 milligrams of the group substance per litre. The injection of saliva containing blood group A substance produces an immune response with anti-A production (Mollison, 2005). About eighty per cent of the white human population has ABO blood-group substance present in saliva.

### 1.3.4 Summary of the immune response especially to glycolipid antigens

Lipid immunogenicity is determined by the mode of uptake, their membrane trafficking abilities, and their capability to form stable complexes with CD1 molecules (Kannagi et al., 1983; Marcus, 1984; De Libero and Mori, 2006, 2010), when complexed to both MHC I and II (Moody and Besra, 2001; Bollyky and Wilson, 2004) and to specific CD4(+) T cells (Christiansen et al., 2011).The structure of the glycolipid affects its function and where it partitions within the cell membrane (Holthuis et al., 2003).

Glycolipid ABO determinants are T-2 antigens which are highly repetitive on mammalian cell surfaces and recognised by CD1 molecules on NK T cells (Neron and Lemieux, 1994; Daniels et al., 2003). The oligosaccharide glycolipid, human blood group A antigen stimulated a murine TI-2, IgM immune response (Neron and Lemieux, 1994). Hydrophobic parts of lipids participate in immunogenicity and the length and the structure of the

ceramide tail contributes to TCR recognition providing binding sites for CD1 cells (De Libero and Mori, 2006). It is noted that the tail in the synthetic FSL glycolipid constructs used in this research is DOPE (a phosphatidyl-ethanolamine structure) not ceramide and this may affect the immune response. Also the presence of the spacer on an FSL construct makes it differ significantly from glycolipids and it may affect the antigen/APC binding or CD1d receptor binding and thus it may interact very differently with the immune system as compared with CD1 interaction of natural glycolipid (Koch et al., 2005).

## 1.4 Stimulation of antibody production by immunisation

Immunisation is a process by which an individual's immune system reacts to the exposure of foreign or non-self immunogenic molecules. B cells are stimulated, with or without T cell help, to produce immunoglobulins (Ig) or antibodies. In this way, the body learns to protect itself in a controlled way and eliminate any non-self antigens.

### 1.4.1 Immunisation of mice

Mice are frequently used in immunisation and carbohydrate studies to test for proof of concept with immune response data and antibody production (Voak et al., 1980; Hansson et al., 1983; Neron and Lemieux, 1994; Love et al., 2000; Levy et al., 2001). It has been shown that mice can make antibody to the blood group A antigen and to $P^K$ antigen after immunisation with these immunogens (Kasai et al., 1985; Miyazaki et al., 1991). Mice have CD1d receptors which stimulate a TI-2 independent response to glycolipids (Bollyky and Wilson, 2004). However, it is likely that FSLs have different immunological attributes to natural glycolipids and so the result of the immune response to these constructs in immunized mice is part of this research.

The fact that the oligosaccharide glycolipid human blood group A antigen was shown to stimulate a murine TI-2 immune response with production of IgM anti-A (Neron and Lemieux, 1994; Liu et al., 2009) provides a basis for investigation of the response to a synthetic glycolipid immunization. Studies have shown that TI- 2 antigens can elicit robust and long-lasting primary antibody responses in mice (Garcia de Vinuesa et al., 1999) and immune responses stimulated by polysaccharides can generate memory B cells with antigen-specific IgG antibodies (Obukhanych and Nussenzweig, 2006).

### 1.4.2 Factors that affect immunisation

The efficacy of the immunization process is usually measured by the degree of humoral (antibody) response. The details of the administration of the immunogen, including the route, dose, form, addition of adjuvant, and duration of antigen exposure affect the outcome of immunization. The resultant antibodies can be used to neutralize cell surface antigens, peptides and proteins. Animal species, age, adjuvants, route and volume of injection, number of injection sites and boosters are factors to be considered in the immunization protocols (Leenaars and Hendriksen., 2005; Stills HF, 2005). If IgM antibodies are required an immunisation regimen of twice is advised and for IgG production a minimum of three is required (Ritter and Ladyman, 1995).

*Route of administration*

Different responses can be elicited since the route of antigen administration affects the immune response - oral or intravenous routes can induce tolerance while subcutaneous injection invokes antibody production (Liblau et al., 1997; Kirkley, 1999). However, immunization using a high dose soluble peptide delivered by intravenous and subcutaneous routes induces clonal deletion or anergy (Harrison and Hafler., 2000). Various immunization methods are safe, convenient and have been shown to induce systemic responses in mice (Edelman et al., 1984; Miyazaki et al., 1991; Schunk and Macallum, 2005) by subcutaneous routes (TitremaxGold™; Barrie et al., 1983; Chen et al., 2002) and by intraveous routes (Voak et al., 1982).

*Dose and duration of exposure*

T and B cells respond to antigens that become transiently localised within organised lymphatic tissues for at least 3-5 days (Zinkernagel & Hengartner, 2001). Antigen concentration determines the level of response from T cells. The smaller the amount of antigen the more directly the B cell activation is dependent on T cell response as is the switching from IgM production to IgG (Zinkernagel and Hengartner, 2001). The constant presence of antigen is also necessary to switch isotope production with the T independent α-gal glycolipid (Liu et al., 2009).

*Use of adjuvant*

In experimental animals, the induction of effective T and B cell responses usually requires the administration of an adjuvant with the antigen. Since most proteins and carbohydrates and purified antigens are poorly immunogenic when administered alone an adjuvant is often used to enhance the immunogenicity of such substances by converting soluble antigens into particulate material which is more easily ingested by APCs (Bennett et al., 1992). It also provides an *in vivo* depot from which antigen is slowly released with the induction of an inflammatory response tricking the immune system into thinking there is an active infection, requiring a response. Inflammation enhances the consumption and presentation of transfused RBC antigens by dendritic cells (Hendrickson. et al., 2007).

Water-in-oil adjuvants maintain the antigen in the aqueous phase using a surfactant to aid distribution of the antigen to the surface of the aqueous micelles. An adjuvant must maintain the conformational integrity of the antigen to present it to the appropriate effector cells (Stills HF, 2005). TiterMax™Gold (CytRx, Norcross, GA) was used in this and in other studies, (Bennett et al., 1992; Stills HF, 2005) to stimulate antibody production to the blood group antigen A. It consists of a copolymer CRL8300, bonded to silica microparticulate stabilizer and an emulsifier, sorbitan monooleate 80 and squalene oil. Some studies suggest the use of Titermax Gold™ adjuvant resulted in low antibody response (Leenaars et al., 1994) but others reported less toxicity than with other adjuvants and better antibody production (Jennings, 1995). The University of Auckland Ethics committee rules precluded the use of Freund's complete and incomplete adjuvant although these are well established as being able to produce immune responses to antigen (Sacks and Lennox, 1981). The use of adjuvants with glycolipid immunisation produces specific antibodies directed mostly against the oligosaccharide moiety (Portoukalian, 2000).

### 1.4.3 Antibody production

Antibodies are a diverse class of glycoproteins that bind specifically with antigen and initiate a variety of secondary responses such as complement fixation and histamine release from mast cells. Antibodies are collectively named immunoglobins and there are five classes IgM, IgD, IgG, IgA, IgE distinguished by their C region. They consist mainly of polypeptide and carbohydrate oligosaccharides O'Shannessy and Quarles, 1987). Antibodies are the secreted form of the B Cell Receptor molecules (BCR) which are produced by terminally differentiated B cells or plasma cells, in response to the

corresponding antigen determinant or epitope. Their specificity is identical to that of the membrane bound BCR except for a small portion of the carboxy terminus of the heavy chain C region. They have two functions: one is to bind specifically to the molecules (antigens) that elicit an immune response and the other to recruit immune response molecules to eliminate the antigen. Once the antigen has been eliminated the immune response ceases.

An antibody molecule has a unique protein structure that enables it to bind specifically to a corresponding antigen. Each molecule is made up of four polypeptide chains composed of two identical heavy and two identical light chains and each chain has two distinct regions. One is the constant region (Fc) which takes one of five distinguishable forms and is the isotope recognised by effector molecules which determines how the antigen will be disposed of once it is bound. The other is the variable region (Fab) with sequences forming different structures, which allow binding to a vast variety of antigens- this structure determines the antigen-antibody specificity.

Antibodies recognize conformational determinants on proteins, carbohydrates or antigen particles on mucosal membranes and in blood. They cannot enter solid tissues except through lesions (Zinkernagel, 1996). Antibody induction is different for T and B cells. B cells respond to rigid form, repetitive structures or lipopolysaccharides with IgM antibody production (Zinkernagel and Hengartner, 2001). Helper T cells, Th1, CD4+co-receptor activate macrophages and B cells to produce IgG antibodies on recognition of MHC class II bound peptides degraded in endocytic vesicles (Janeway, 2005). Helper T cells, Th2, CD4+co-receptor stimulate naive B cells to secrete IgM antibodies and to differentiate and produce other antibody isotypes on recognition of MHC class II bound peptides degraded in endocytic vesicles (Janeway, 2005). NK T cells induce antibody production on interaction with CD1 molecules (Brigl and Brenner, 2004; Borg et al., 2007; Liu et al., 2009).

*Antibodies induced by carbohydrates*

Carbohydrates are T cell independent and therefore initiate a different immune response to proteins and peptides. They cannot activate T helper cells alone and therefore have limited immunogenicity forming only low affinity IgM antibodies with no memory cells and, the absence of IgG antibodies (Mond et al., 1995a; Buskas et al., 2005). Most of the known high-titre natural antibodies seem to have anti-carbohydrate activity including anti-

blood group A and anti-B (Galili et al., 1985). The majority of antibodies produced against carbohydrate antigens such as glycoproteins (Buskas et al., 2005), glycosphingolipids (Marcus, 1984) and lipopolysaccharides are IgM (Zinkernagel and Hengartner, 2001). The immune response to blood group substance in serum is usually reached 10-15 days post blood transfusion (Mollison, 2005).

Most individuals develop antibodies to specificities of the ABO system without previous sensitization by red cells. This occurs through coincidental contact with identical epitopes to A and B glycoprotein antigens found on many and various microbes, plants and organisms (Race and Sanger, 1975). Anti-A is hypothesized to originate from immune response towards the influenza virus, whose epitopes are similar enough to the α-D-N-galactosamine on the A glycoprotein to be able to elicit a cross-reaction. Anti-B antibodies are hypothesized to originate from antibodies produced against Gram- negative bacteria such as E coli, cross-reacting with the α-D-galactose on the B glycoprotein. Individuals who are blood group A produce anti-B, group B produce anti-A and group O produce both anti-A and anti-B and anti-A,B (Levy et al., 2001). Anti-A and anti-B may be IgM, IgG or IgA (Oriol et al., 1990; Rieben et al., 1991).

The host immune system probably recognizes the saccharide and binding proteins as a whole and proceeds to make antibodies to the whole molecule (Takahashi and Saito, 2012). Antibodies are made to the ABO blood group antigens and to the ABO histo blood group antigens. Serum natural anti-A/anti-B antibodies might react with the red cell membrane ABO antigens but not necessarily with the the ABO histo group antigens on vascular endothelial cells with their different structure.

Recent data has shown that the anti-A/B antibodies that elicit acute antibody-mediated reaction (AMR) after transplantation are actually newly synthesised antibodies produced after transplantation as a result of stimulation and sensitization by the ABO histo group antigens on the graft and also by ABO histo blood group associated (bacterial) antigens. There is a silent period of about 2 days post transplant while the host mounts the anti-histo group antibody response. These findings are compared with the current theory that AMR is caused by natural anti-A/B antibodies that are present in the pre-transplant recipient (Takahashi and Saito, 2012).

*Complement-binding antibodies*

Complement-binding antibodies include IgM and IgG anti-A, anti-B, anti-A, B, anti-PP1Pk, anti-Vel, anti-Lewis and anti-Kidd (Mollison, 2005; Poole and Daniels, 2007). Antibodies that bind complement can cause red cell destruction due to the activation of the complement cascade. IgM antibody/complement activation results in the membrane attack complex with the formation of a large molecule that punctures the red cell membrane causing rapid intravascular lysis. Extravascular destruction then continues at a slower rate in the presence of IgG antibody (Mollison, 1989) and is slowed abruptly once the cells are coated with C3dg, the breakdown component of iC3b. This occurs within a few hours after its production, (Mollison, 2005) and these cells then have normal cell survival (Garratty, 2008a). This is due to the fact that macrophages have complement receptors for C3b and iC3b breakdown products from C3 but not for the next products, C3dg or C3d. It was shown that complement-binding antibodies (IgM or IgG) in the mouse bring about red cell destruction with a rapid rate of clearance in the liver (Schreiber and Frank, 1972) although mouse complement is less effective than human complement at causing *in vivo* haemolysis (Ong and Mattes, 1989). Antibodies such as anti-$Le^a$ and anti-Kidd also activate the complement pathway but only to the formation of the complement breakdown products C3b, iC3b. Red cells, coated with antibody bound to these C3 fragments are phagocytosed.

*Non-complement-binding antibodies*

Extracellular destruction of antibody-sensitised red cells occurs when the antibodies do not bind sufficient complement to initiate the haemolytic pathway. These cells are removed by the Kupffer cells of the liver or the spleen due to specific receptors for immunoglobulins and the C3b component of complement.

*Antibodies induced by glycolipid antigens*

The antibody response to glycolipids first depends on the structure of the oligosaccharide and the ceramide moiety. Studies in mice showed that the presence of a trisaccharide sequence was more important than a disaccharide sequence in terms of immunogenic potency and the structure of the ceramide moiety influences the antigen conformation of the oligosaccharide moiety (Portoukalian, 2000). Lipopolysaccharides induce a strong immune response and activate T cells with production of IgG antibodies (Yuki et al., 1993). The oligosaccharide glycolipid human blood group A antigen was shown to stimulate a

murine TI-2 immune response with production of IgM anti-A (Clausen et al., 1988; Neron and Lemieux, 1994; Liu et al., 2009). Lipids do not elicit an antibody (Rapport and Graf, 1969) response by themselves but are regarded as haptens (Prescott et al., 1970). This was seen when native antigens gave a weak response in rabbits but once bound to protein were highly immunogenic suggesting the structure contributed to immunogenicity (Razin et al., 1970; Liu et al., 2009). It appears that lipases assist with CD1 molecule binding by removal of lipid moieties. Synthetic disaccharide glycolipid and lipid isogloboside only became immunogenic once the terminal sugar was removed (De Libero and Mori, 2006).

### 1.4.4 The clinical significance of circulating ABO antibody

The presence of circulating A or B antibody reactive with red cell surface antigen, in the presence of complement, results in intravascular destruction of antibody-sensitised red cells with haemolysis and rapid liberation of haemoglobin into the plasma. When only small amounts of red cells are affected (less than 30mL), the cells can be cleared by the reticuloendothelial system or phagocytosis before haemolysis occurs (Mollison, 2005).

The majority of the population has antibodies directed against ABO antigens and accidental incompatible transfusions although rare, still do occur. Breaching the ABO-barrier can be met with significant and sometimes fatal consequences. The reaction to transfused ABO incompatible blood can range from no observable reaction through to renal failure, shock, and disseminated intravascular coagulation causing death. The outcome is not usually fatal. Usually prompt clinical intervention, particularly discontinuing the transfusion, will minimize morbidity and mortality (Janatpour et al., 2008) although the death rate is higher if more than 50mL are transfused (Mollison, 2005; Janatpour et al., 2008). Clinical management usually includes prompt recognition, preventing hypotension, promoting urine output, improving renal blood flow, and vasopressive medication (Janatpour et al., 2008). No therapy to halt further antibody-antigen–mediated destruction is currently used. The clearance of ABO incompatible red cells is clearly mediated by the actions of antibody and complement (Mollison, 2005), and incompatible blood once transfused is usually destined to a reduced lifespan. The rate of vascular clearance has been shown to be dependent on antibody levels (Chaplin, 1959; Mollison, 2005).

Not all transfused incompatible blood will be rapidly and intravascularly haemolysed, because the incompatible transfusion itself effectively reduces antibody and complement levels thereby temporarily mitigating the reaction (Chaplin, 1959; Mollison, 2005), antibody can induce antigen loss from the red cells (Zimring. et al., 2005; Zimring et al., 2009) and some cells are more robust (Liepkalns and Zimring, 2009).

There is substantial evidence that red cells can also survive in the presence of low levels of ABO antibodies, including antibody sensitization, as seen by the practice of group O whole blood transfusions into group A and B individuals (Lefebre et al., 1987; Mollison, 2005), and the occasional tolerated accidental transfusions of ABO incompatible blood (Mollison, 2005; Janatpour et al., 2008).

More exemplary is the deliberate use of ABO-incompatible blood transfusions to reduce antibody titres as a preparative treatment for marrow transplants has been safely achieved in patients with normal renal function (Nussbaumer et al., 1995; Scholl et al., 2005), but it is not an alternative transfusion regime.

*Effects of antibody binding on red cells*

Antibodies bound to red cells cause damage by activating complement. IgM, IgG1 or IgG3 antibody-binding results in activation of complement to C8/9 stage leading to intravascular cell lysis. Antibodies also cause damage to red cells by interaction with macrophages and other phagocytic cell receptors. Cells sensitized with IgG, IgA, C3b or iC3b interact with the macrophage Fc receptor in the spleen or liver resulting in extravascular lysis in the reticuloendothelial system (Garratty, 2008a). There is also evidence that there may be an antibody-mediated effect independent of complement or macrophages but due to antibody destablisation of the phospholipid bilayer of the membrane (Brain et al., 2002). Macrophages can shorten red cell survival completely by phagocytosing or partially with the release of spherocytes.

The antibody-antigen reaction causing red cell agglutination is due to the position of the antigens relative to the bilipid layer of the plasma membrane – A and B antigens are located at the outer edge of the red cell glycocalyx and so the distance between these antigens on two red cells is close enough for IgM anti-A and anti-B and some IgG anti-A to bridge the gap. There is a minimum number of IgM antibody molecules per red cell required for agglutination in saline - about 50 for anti-A, and centrifugation enhances

agglutination with antibody bonding due to electrostatic forces. The distance between the molecules of IgG on separate red cells is too great to allow agglutination due to the zeta potential and ionic cloud around each red cell. Antiglobulin is therefore used to bridge the gap between the red cells and allow agglutination.

The pathogenicity of antibodies depends on the class type, the quantity bound to RBCs, the levels and type of complement and its activating efficiency, the activity of the macrophage system and in the case of carbohydrate immunoglobulins, the amount of galactose present on the Fc portion (Walker et al., 1989; Garratty, 2008a*).* IgM antibody is more efficient than IgG as a complement activator with IgG3 being the most active in the IgG subclasses*.* NK cells, dendritic cells and cytotoxic T cells also interact with sensitized red cells via various receptor molecules and cytokines. There is some suggestion that sensitized RBCs generate hydrogen peroxide in the presence of granulocytes and monocytes resulting in cell lysis (Garratty, 2008a).

*Effects of antibody binding on transplants*

Humoral rejection in ABO incompatible transplants is initiated by recipient antibody binding of donor antigens on the graft epithelium with subsequent initiation of complement and blood clotting cascades. Production of antibodies after transplant causes graft rejection due to antibody binding to the blood group antigens on the endothelial cells of the graft which activate complement and result in rejection of the graft (Galili, 2004; Takahashi and Saito, 2012).

## 1.5 Stimulation of immune tolerance

Tolerance can be defined as the absence of an antigen-specific immune response in the presence of intact immunity to other antigens (West, 2006). The ability to manipulate the immune response and create a perception of self by inducing tolerance is highly desirable for the treatment and prevention of autoimmunity, allergy and transplant rejection. Normally self- tolerance mechanisms prevent autoimmune disease but destruction of self occurs as a result of breakdown in central and peripheral tolerance. If the system reacts inappropriately to a harmless antigen and the response is strong, it is said to be autoimmune.

Immune responses to incompatible ABO antigens on allografts include rejection, accomodation or immune tolerance (Galili, 2004; Ogawa et al., 2004, Takahasi and Saito, 2012). When the immune system recognizes a transplanted organ as foreign and harms a life-preserving transplant then rejection is said to have occurred. Accommodation refers to the condition in which an organ transplant functions normally by acquiring resistance to immune-mediated injury (especially), despite the presence of anti-transplant antibodies in the recipient.

The vascular endothelial cells in the graft have ABO antigens on their surface and the blood of the recipient contains antibodies but if there is no antigen-antibody reaction there is no acute antibody-mediated rejection (AMR). Takahashi refers to it as a “lock and key” mismatch (Takahashi and Saito, 2012). This status requires several modifications in the recipient as well as in the graft, such as previous depletion of anti-graft antibodies (and their slow return once the graft is placed); expression of several protective genes in the graft; a Th2 immune response in the recipient; and inhibition of the membrane attack complex of complement (Dehoux and Gianello, 2009).

Immune tolerance is brought about in many ways. Clonal deletion, named Burnet’s theory, where self-reactive lymphoid cells are destroyed during the development of the immune system in an individual. Burnet and Medawar were awarded the 1960 Nobel Prize in Physiology or Medicine "for discovery of acquired immunological tolerance"; clonal anergy where self-reactive T- or B-cells become inactivated in the normal individual and cannot amplify the immune response (Pike et al., 1982); the presence of an idiotypic network, wherein a network of antibodies capable of neutralizing self-reactive antibodies exists naturally within the body (Jerne, 1974); clonal ignorance where host immune responses are directed to ignore self-antigens (Perales et al., 2002), and regulatory T cells where regulatory T-lymphocytes (commonly $CD4^{+}FoxP3^{+}$ cells, among others) function to prevent, downregulate, or limit autoaggressive immune responses in the immune system Hess, 2006).

The ultimate goal in organ transplantation would be to induce donor-specific tolerance by modification of either the donor organ or of the host immune response. This could allow the transplant of partially mismatched or xenogenic organs and thus eliminate the need for immunosuppression and its adverse side effects (Lechler et al., 2005).

The immune system is finely balanced to distinguish between foreign and self antigens. When functioning properly, the adaptive immune response has tolerance for host antigens and therefore does not attack self antigens. Self/non-self discrimination is controlled by both the innate and adaptive response with several tolerance mechanisms working in parallel under physiological conditions (Banchereau and Steinman, 1998). If inflammation is present, mature lymphocytes interpret signals through antigen receptors and the presence of cytokines which then lead to proliferation of effector functions. If inflammation is not present the cells respond to the lack of cytokine signals by apoptosis or anergy. The idea that the immune system evolved to simply react to “something foreign” is now seen to be an oversimplification. The foreign antigen needs to evoke many immune response mechanisms to activate T cells and foreign antigens lacking these features are likely to be ignored by the response system.

The nature of the antigen presenting cells (APC) first presenting the antigen may determine whether an immune response is stimulatory or tolerant. Immune unresponsiveness results if an antigen is presented to T cells by a non-professional APC that cannot provide co-stimulation (Klinman et al., 1981). Dendritic cells continuously sample the environment for the presence of antigens and depending on the antigen encountered, triggering of receptors may lead to tolerance rather than immunity (Bonifaz et al., 2004). The presence of antigens in the lympho-haemopoietic system at some level will eventually deactivate and delete all T cells specific for that antigen. Antigens correlate with self when expressed in strong and constant concentrations and those that are continuously present in blood and lymphoid organs generally do not activate T cells, do not induce an immune response, and are effectively ignored by the immune system (Zinkernagel, 1996). Antigen administration that fails to induce activation and or migration of DC leads to tolerance (Banchereau and Steinman, 1998).

Full tolerance has yet been developed in only two circumstances: when the recipient is not immunologically competent or mature and when there is full replacement of host lymphocytes by donor lymphocytes accomplished by immunosuppression and stem cell transplantation (Touraine and Sanhadji, 2011).

### 1.5.1 Cells involved in tolerance

The innate system uses pattern recognition receptors to distinguish non-infectious self from pathogenic non-self whilst the adaptive system recognises receptors and molecular details on various target cells and is affected by the innate response (Medzhitov and Janeway, 2000; Schenten and Medzhitov, 2011). The fate of the many lymphoid types involved especially self-reactive lymphocytes depends on the maturity of the cell, the avidity of its receptor for, and the nature and concentration of the antigen, and where the antigen is found (Klinman et al., 1981). Immature B cells are susceptible to tolerance induction but this tolerance can be reversed if a second signal induces stimulation (Klinman, 1996). Studies show that regulatory T cells (Tregs) play a vital role in the facilitation of donor tolerance as demonstrated in animal models (Hess, 2006) and in neonatal infants with graft transplants (Touraine and Sanhadji, 2011).

Classical theories of tolerance state that non-recognition of self is ensured by the elimination of potential self-reactive clones by clonal abortion of B cells in an early stage of maturation, with tolerance primarily dependent on the maturational status of the B cell (Klinman, 1996). However, there is strong evidence for other tolerance mechanisms such as anergy and suppression (Waldmann et al., 2001). Co-stimulatory molecules reacting between T cells and APCs are also required for activation and thus mechanisms of central and peripheral tolerance ensure that antigens or peptides derived from self do not initiate an immune response.

Central tolerance results from deletion of T cells in the thymus and peripheral tolerance results from clonal deletion or anergy of T or B lymphocytes in circulation. When the T cell receptor (TCR) of a mature naive T cell in a lymphoid organ is ligated by an antigen-peptide displayed by an APC, it is activated to proliferate and produce effector T cells. In contrast, if a developing thymocyte is ligated by a self-antigen on cells derived in the thymus it dies by apoptosis. Deletion appears to be the default pathway for immature B cells that encounter specific antigen emerging from bone marrow and when antigens are membrane bound (West, 2006). During the course of memory cell generation after antigenic stimulation, the initial repertoire of specificities can be destabilised by generation of novel anti-self specificities. This requires a second window of tolerance susceptibility for these newly generated memory cells (Linton et al., 1991).

In the 1950's, Burnet proposed the original model of clonal deletion suggesting that self-reactive lymphocytes were eliminated upon contact with self-antigens and this was later expanded to state that as a lymphocyte develops immunological competence there is a stage at which contact with self results in elimination (Teale et al., 1979). It has now been shown that these self-lymphocytes must be removed by apoptosis or cell death before they can mature to ensure there is no activation by self antigens (Arnold, 2002, Klinman et al., 1981).

The antigenic specificity of each lymphocyte receptor is randomly generated early in development by somatic gene rearrangement and so by chance some protein sequences will recognize self tissues. Tolerance depends on those lymphocytes with receptors recognizing self-antigens being eliminated either in the thymus, bone marrow or periphery and thus absent from the repertoire of mature lymphocytes. Lymphocyte maturation and survival depends on signals received through their receptors. Antigens that have not been presented to immature lymphocytes during this critical period may be later recognized as non-self, or foreign, by the immune system.

*Dendritic cells*

Antigens and lymphocytes have long been known as the focus of immunology but processing and presentation of self antigens by steady-state dendritic cells (DCs) are now thought to be major components of the establishment of tolerance in the periphery (Banchereau and Steinman, 1998; Mukhopadhaya et al., 2008). DCs maintain peripheral tolerance by regulating the numbers and states of self-reactive T cells (Adams et al., 2008). Immature dendritic cells readily take up antigen but can only present antigen to T cells once they have been activated to maturity. Studies show that immature DCs may present antigen in a way that favours Treg induction suggesting that tolerance may be induced if the antigen presentation can be altered to attract immature rather than mature DC binding (Waldmann et al., 2001). Without antigenic stimulus, DC maturation does not occur and the induced immune response will be ineffective and may result in tolerising T cells as occurs with self-antigens (Banchereau and Steinman, 1998).

In the absence of infection or inflammation, DCs present antigens in a tolerogenic manner and cause naive CD8+ cells to proliferate but then these are deleted (Mukhopadhaya et al., 2008). These lymphocytes respond to the receptor signals by undergoing deletion by apoptosis or anergy (Janeway, 2005). The presentation of antigen by DCs with

accompanying inflammation and infection sends signals from cytokines through their receptor sites to mature lymphocytes. If that signal is positive, it leads to proliferation and stimulation of effector functions.

*B cells*

Self-reactive B cells have four possible fates depending on what antigen binds to the membrane-bound antibody. These are cell death; a change in the receptor site; induction of anergy where they are unresponsive; and an ignorant response if the antigen is very weakly bound. When the self-antigen is multivalent, clonal deletion occurs. If the self-antigen is of low valence, such as small soluble proteins, the self-reactive B cells become anergic or non-responsive.

The long-standing major premise of tolerance is that the signal cascade sent to immature B cells induces tolerance (Klinman, 1996) and the absence of T cell help in the stimulation of immature B cells by antigen results in clonal abortion (Teale et al., 1979). However, lymphocyte survival in inactive form or anergy and dominant regulation or suppression by Treg lymphocytes can also induce tolerance (Waldmann et al., 2001). Susceptibility to tolerance induction during immaturity and in animals has been well documented (Zinkernagel and Hengartner, 2001). Several studies indicate that immunoglobulin receptors appear on the membrane of neonatal lymphoid cells before the animal is able to synthesise antibody in response to antigens (Bruyns et al., 1976). The interaction of antigens with the receptors at this time leads to the inactivation and subsequent non-re-expression of the receptors of those lymphocytes that bind the ligand. (Mature cells can re-express the receptor after antigen binding and endocytosis). Immature B cells reach a maturational stage where they can be both tolerized and stimulated depending on the signal received. Newly formed, immature lymphocytes are especially sensitive to inactivation by strong signals from self antigens - MHC molecules that would otherwise activate mature lymphocytes (Janeway, 2005). Infants do not make antibodies against carbohydrates because they have mainly immature B cells - the development of anti-A and anti-B does not occur in humans until 5-6 months of age. This means they could be rendered tolerant to antigens of any ABO type without genetic manipulation, in order that they could receive future transfusions or transplants without risk of rejection (Griffiths, 2005; West, 2006). Tolerance is readily achieved by antigen injection in neonates and this non-reexpression of surface immunoglobulins could lead to establishment of natural

tolerance (Bruyns et al.1976). Immunological immaturity of donor T cells can allow successful T cell engraftment and reconstitution of immunocompetence without induction of graft-versus-host disease in immunodeficient patients showing partial identity with donor major histocompatibility complex (MHC) genes. This allows persistent chimerism and the removal of newly-formed alloreactive lymphocytes (Do Canto et al., 2008). Transgenic mouse experiments indicate that auto-reactive B cells are eliminated on encounter with membrane self–antigen (Hartley et al., 1991; Lang and Nemazee, 2000) and B cells that react with membrane antigens expressed in the bone marrow are deleted from the peripheral lymphocyte pool (Russell et al., 1991).

*T cells*

T cells distinguish between self and non-self by T cell receptors. T cells are not only effector cells but also regulators or suppressors as they participate in the immune response. Self-reactive T cells are deleted at certain developmental stages or rendered anergic and may ignore self-antigens if they are sequestered in tissues and not in circulation. Self-antigens are also ignored in privileged sites such as the anterior chamber of the eye, brain and testes.
The presence of antigens in the lympho-haemopoietic system at some level will eventually deactivate and delete all T cells specific for that antigen. Antigens correlate with self when expressed in strong and constant concentrations and those that are continuously present in blood and lymphoid organs even at low levels, generally do not activate T cells, do not induce an immune response, and are effectively ignored by the immune system (Zinkernagel, 1996).

Regulatory T cells (Tregs) act as regulators in certain conditions acting on activated APC or T and B cells to suppress activity. The T-cells that bind to self antigens and escape elimination are usually held in check by Tregs by various mechanisms: clonal anergy, deletion or ignorance (Miller et al., 2007). It has been shown that Tregs play a role in transplantation tolerance and can prevent graft rejection in addition to delaying Graft Versus Host Disease (Hess, 2006). The ability of these cells to exert antigen non-specific ‘bystander suppression’ removes the need to identify and tolerise the primary pathogenic antigen. This is useful for immunotherapy when considering the multiple autoantigens involved in human autoimmune disease (Harrison,1992).

The thymic central tolerance process eliminates high affinity self-antigen specific T cells and those T cells that fail to recognise self-antigens at all. It spares T cells that recognise self-antigens of intermediate affinity (Bundle, 2007). This has been suggested to be an extrathymic mechanism for maintaining self-tolerance (Markham et al., 1988). Intrathymic negative selection is not restricted to those self-peptides made in the thymus: there are other mechanisms in the periphery that also prevent mature lymphocytes from responding to tissue-specific self-antigens. T helper cells appear to be more sensitive to tolerance in the presence of high dose antigen or by repeated stimulation resulting in the appearance of regulatory Tregs (Harrison., 1992).

Autoimmunity and organ transplant rejection are inextricably linked to T cell activation and differentiation, meaning T cells are a target for tolerance induction strategies. Self-reactive T cells are involved in most organ-specific autoimmune processes. For example, Type 1 diabetes is an autoimmune disease resulting in T cell mediated destruction of islet β-cells due to defects in peripheral cell tolerance (Mukhopadhaya et al., 2008).

### 1.5.2 Tolerance to carbohydrate antigens

Individuals make antibodies against those A or B carbohydrate antigens which they do not have. Human self-tolerance to A and B histo-blood group (which are TI antigens) is thought to be due to B cell deletion (Rieben. et al., 1992) or inactivation when antigens are membrane-bound (Hartley et al., 1993; Klinman, 1996; West, 2006). Carbohydrates or saccharides alone cannot activate T -CD8+ (MHC I) cells nor stimulate B cell proliferation. So, in the absence of T cell help, anti-carbohydrate B cells exposed to incompatible carbohydrate antigen gradually become tolerised (Ogawa et al., 2004).

Carbohydrate inactivation of B cells is more likely when a soluble antigen is present (Lang and Nemazee, 2000; Galili, 2004) and constantly present (Griffiths, 2005) and tolerance induction was shown to be a time-dependent process of at least 10 days (Mohiuddin et al., 2003). Since B cell receptors appear to be more specific for the repetitive structure of carbohydrate antigens, B cell tolerance could be important for control of anti-carbohydrate self-activity (Zinkernagel and Hengartner, 2001; Mohiuddin et al., 2003).

### 1.5.3 Tolerance to glycolipid antigens

Glycoproteins such as those in blood group A substance would be expected to be good immunogens and induce a significant T cell-stimulated antibody response, while glycolipids are poor immunogens since they must be recognised by CD1d receptors to activate an immune response (Lingwood et al., 1980; De Libero, 2004; Koch et al., 2005; De Libero and Mori, 2006). NK T cells have CD1d receptors which, when engaged with glycolipid antigens, rapidly produce T helper cell (Th1 and Th2) cytokines. They can either up-or down-regulate immune responses by promoting the secretion of these and other immune regulatory cytokines (Godfrey and Kronenberg, 2004) and induce tolerance (Mocikat et al., 2003).

### 1.5.4 Tolerance to fetus in pregnancy

The fetus represents a foreign entity to the maternal immune system but it is not rejected. Successful reproduction depends on mechanisms that control the activation of the potentially hostile maternal immune system against the fetus. Fifty years ago, Medawar proposed mechanisms that enable this to occur and his theories remain valid to this day (Billington, 2003). One mechanism is an anatomical barrier between mother and fetus that prevents access of maternal immune cells to fetal antigens. This appears to be due to the presence of trophoblast cells of the placenta at the maternal-fetal interface which resist immune attack (Guleria and Sayegh, 2007). The second mechanism is the prevention of an immune response to the maternal cells. Fetal antigens cause the induction of anergy or tolerance and T cell apoptosis of maternal cells at the implantation site with Treg involvement (Guleria and Sayegh, 2007). Fetal trophoblasts express an enzyme which degrades tryptophan, an essential amino acid required by T cells, and so inhibits T cell activation (Koch and Platt, 2007). A third mechanism is the suppression of fetal antigen expression. The trophoblasts express non-classical MHC molecules and may suppress the expression of alloantigens to evade detection (Koch and Platt, 2007). There is a recent theory that the fetus generates site-specific immune suppression where the effector functions of maternal immune cells are blocked at the maternal-fetal interface only and peripheral maternal immune responses continue (Kochand Platt, 2007). It seems the suppression of this cell -mediated immune response is accompanied by the activation of the innate immune system and this activation then causes many of the complications of pregnancy such as pre eclampsia (Veenstra van Nieuwenhoven et al., 2003).

### 1.5.5 Factors that affect tolerance

The decision for T cells to be stimulated or silenced depends on the antigen dose, site and time of expression, T cell frequency and environment in which the antigen is encountered. The route, dose and nature of the antigen appear to be crucial with intraperitoneal or intravenous routes more effective than subcutaneous in inducing tolerance (Liblau et al., 1997; Arnold, 2002).

*Route of administration*

The route of administration of antigen influences the immune response. Antigens given subcutaneously or intradermally may invoke an immune response but given intravenously or orally it may cause tolerance by anergy or deletion (Arnold, 2002; Liblau et al., 1997; Kirkley, 1999). Oral auto-antigens were used to suppress autoimmune disease (Fowler and Weiner, 1997). The introduction of soluble antigen through the blood has long been established as a model for studying the mechanism of T-cell tolerance induction. This route of antigen presentation allows almost immediate access of antigen to B cells, DCs and macrophages in the spleen (Gutgemann et al., 2002)**.** Experiments have shown that both immature and mature B cells are inactivated when constantly exposed to high dose soluble antigen (Harrison and Hafler, 2000; Janeway, 2005). Soluble peptide antigens given intravenously bind to MHC class II on B cells and tend to induce anergy (Magee and Sayegh, 1997) and systemic administration of soluble protein antigen was shown to inhibit the immune response to that antigen (Liblau et al., 1997).

The delivery of antigens to the thymus by T cells is important in overcoming transplant rejection and establishing self/non-self recognition in autoimmunity. Immune tolerance in mice was induced by intrathymic gene delivery (Chu et al., 2010). Direct injection of foreign antigen into the adult thymus is a potent route of antigen delivery for the induction of tolerance in vivo by deletion of thymocytes (Jones et al., 1997).

*Duration of antigen exposure*

Research with anti-Gal epitopes has shown that the type of elicited response depended on the amount of time antigens were exposed to the B cells in the absence of T- cell help. Immediate T-cell help produced cytolytic antibodies and delayed T cell help induced production of non-cytolytic accommodating antibodies. However, in the absence of T cell

help for prolonged periods, anti-carbohydrate B cells exposed to incompatible carbohydrate antigens differentiated first into cells making accommodating antibodies without graft rejection, and gradually were deleted resulting in tolerance (Galili, 2004). T cells do not respond to antigens that are continuously present at some level in blood and lymphoid organs (Zinkernagel and Hengartner, 2001). Persistance of low levels of antigen throughout the body leads to deletion of T cells (Zinkernagel, 1996). Continued tolerance appears to require the continued exposure to the tolerized antigen as is the case in solid organ transplants but not with blood transfusion (Chen et al., 1996).

*Concentration of antigen*

The amount of antigen administered determines the mechanism of tolerance. High doses favour clonal deletion and anergy and low doses triggers regulatory T cell induction secreting cytokines to suppress imflammation. High dose soluble peptide given subcutaneously invoked clonal deletion (Harrison, 1992). High dose soluble peptide or monomeric protein given by intraperitoneal subcutaneous, intravenous or systemic delivery induced clonal deletion or anergy in response to the individual antigens (Liblau et al., 1997; Harrison and Hafler, 2000).

Avidity-based elimination is intrinsic to the T cell. Immunisation with strong antigens led to elimination of T cells bearing high affinity receptors while immunisation with weak antigens led to clonal expansion of these T cells (Arnold, 2002). Very large doses of T-independent polysaccharide antigens were shown to generate tolerance in B cells (Male et al., 2006).

The high and constant concentration of self antigen provides strong signals for inactivation through the antigen receptors and the absence of costimulatory molecules on the APC tends to give a negative signal (Zinkernagel, 1996). Tolerance can be induced in transplants when the graft represents a sufficiently large source of antigen even in the presence of evolving antibody production suggesting repeated intentional antigenic exposure induces tolerance but antigen presence needs to be persistent to be long lasting (Galili, 2004).

When a glycolipid antigen was present at high concentration in human tissues, there was immune tolerance but in mice, where the antigen was present at low tissue concentrations, it was immunogenic (Portoukalian, 2000).

*Antigen form*

Immunisation with soluble peptides and monomeric antigens at a high dose resulted in tolerance whereas clustered peptides tend to be immunogenic and intravenous administration of native or denatured ovalbumin resulted in tolerance to both forms of the antigen (Endres and Grey, 1980). Soluble peptide-MHC complexes also induce tolerance (Harrison, 1992). Antigens presenting multiple epitopes providing cross-linking sites are capable of inducing tolerance (Klinman et al., 1981).
Tolerance can be induced with the use of molecular chimeras if the retrovirally transduced allogeneic MHC /antigen complex is expressed on the surface of mature lymphocytes that populate the host thymus (Tian et al., 2004). Non-recognized carriers such as copolymers of D-amino acids or soluble monomeric immunoglobulins appear able to induce tolerance and in general, soluble antigens do not induce tolerance in mature B cells *in vivo* or *in vitro* (Klinman et al., 1981).

Murine red blood cells were inserted with hapten-linked antigens and shown to tolerise mature B cells (Hamilton and Miller, 1974). Nicolau and co workers were able to insert human glycophorin A into mice red cells by electroinsertion and these cells failed to induce an antibody response once in circulation but the same purified antigen injected intramuscularly gave an excellent immune response with antibody production (Nicolau et al., 1993).

### 1.5.6 Summary of immune tolerance induced by glycolipids

Tolerance to A and B histo-blood group antigens (which are TI antigens) is thought to be due to B cell deletion (Rieben. et al., 1992) or inactivation when antigens are membrane bound (Hartley et al., 1993; Klinman, 1996; West, 2006). Tolerance induction on memory anti-Gal B cells was shown to be a time dependent process of at least 10 days (Mohiuddin et al., 2003). Glycolipids are generally poor immunogens and they must first be recognised by CD1d receptors (Lingwood et al., 1980; De Libero, 2004; Koch et al., 2005) or specific CD4(+) T cells (Christiansen et al., 2011) to activate an immune response. CD1d receptors on NK T cells engage with glycolipid antigens producing T helper cell (Th1 and Th2) cytokines which either up-or down-regulate immune responses by promoting the secretion of other immune regulatory cytokines (Godfrey and Kronenberg, 2004) and induce tolerance (Mocikat et al., 2003).

## 1.6 Current methods for manipulation of the immune response

The ability to manipulate the immune response to an antigen or immunogen has huge potential benefits for therapeutic use. The ultimate goal of immunotherapy for autoimmune disease and transplantation is specific intervention to promote tolerance and prevent the immune system from causing an undesired pathological response. The carbohydrate antigens, blood group A and B, are major risk factors in blood transfusion and in transplantation involving the use of incompatible red cells or donor transplant tissues. The provision of blood units for transfusion or the ability to transplant organs that are free of immunologic dangers and consequences is the “Holy Grail” of transfusion medicine (Wilson and Spitalnik, 1994). Manipulation can involve stimulation of antibody production, removal of antigen or tolerising the recipient to the offending antigen. These procedures include vaccination, neonatal tolerising, chemotherapeutic immunosuppression, plasmapheresis, blood group substance neutralization and manipulation by insertion of antigen into cell membranes.

The administration of antigen to stimulate the immune system to develop antibodies is described as vaccination. It is believed that the first vaccination (by inoculation) involving the injection of infective fluid taken from a smallpox victim, began in India, 1000 AD, and in Turkey using powdered smallpox scabs. The description of immunization was submitted to the Royal Society in London in 1724 by a doctor from Istanbul and this encouraged vaccination after the Turkish tradition was introduced in England and Germany. The Englishman, Edward Jenner demonstrated his famous smallpox vaccine in 1796 and later, Louis Pasteur vaccinated with “treated agents of disease”. He offered the word ‘vaccine’ as a generic term to honour Jenner. Current vaccines include those made against human papillomavirus, hepatitis B, diptheria, tetanus, whooping cough, polio, hepatitis B, Haemophilus influenzae type B, measles, mumps, and rubella to name but a few. Carbohydrate vaccines are discussed below.

Current therapies to manipulate the immune system to tolerance include the use of immunosuppressive drugs which interfere with DNA synthesis of dividing lymphocytes, antibodies to deplete immune response cells, gene therapy, with molecular chimeras and systemic administration of soluble protein antigen. These have all been shown to induce tolerance. Full tolerance has been developed only in immunologically incompetent recipients when there is full replacement of host lymphocytes by donor lymphocytes

accomplished by immunosuppression and stem cell transplantation (Touraine and Sanhadji, 2011).

Immunosuppressive drugs are helpful in preventing graft rejection for the first few years post transplantation but are less effective in preventing problems of chronic rejection which limit long-term graft survival (Wood, 1991). They limit T cell response (Hale et al., 1998) but are relatively unselective and have unwanted side effects including increased susceptibility to infectious disease, cancer, diabetes and also liver, kidney and neurological toxicity (Rossi et al., 1993; Lechler et al., 2005).

### 1.6.1 Manipulation by immunization with carbohydrate antigens as vaccines

Carbohydrates antigens are commonly tested and developed for vaccines against infectious disease (Guo and Wang, 2009) and tumors (Toyokuni et al., 1994). However, a serious problem with their use as vaccines is their poor immunogenicity (Slovin et al., 2005). They elicit only humoral response with low affinity IgM although when conjugated to a carrier, become immunogenic and induce T cell help (Stein, 1992; Buskas et al., 2005). The use of carbohydrate structures as an alternate to the antibody-based targeting of DCs offers the possibility to modulate DC signalling networks providing a way to enhance antigen processing presentation of selected antigens, drugs or nucleic acids (Rudd et al., 2001). Many tumor antigens are carbohydrate self-antigens lacking immunogenicity and immunological recognition. However, when coupled to proteins, tumour antigens activate T cells, become T cell dependent (TD) and induce memory cells and produce antibodies which then attack tumour cells (Slovin et al., 2005). Tumour-associated carbohydrate self-antigens expressed on tumour surfaces allow immune tolerance but if these can be manipulated into different antigenic structures they can serve as targets for immune recognition (Slovin et al., 2003).

### 1.6.2 Manipulation by immunization with blood group glycolipids

Mice have been shown to produce the corresponding appropriate antibodies after immunisation with the following blood group glycolipid antigens: human blood group A red cells (Kabat et al., 1947; Neron and Lemieux, 1994; Levy et al., 2001); blood group B red cells (Rouger et al., 1983); blood group Acquired B cells (Janvier et al., 1990), and $P^k$ antigen positive cancer cells (Kasai et al., 1985).

### 1.6.3 Manipulation by immunization with blood group glycoproteins

Blood group specific A and B substances (glycoproteins as opposed to glycolipids) have been used to neutralize and increase titres of anti-A in humans when given intravenously (Mond et al., 1995b). Plasma containing $Le^a$ substance has been used to neutralize anti-$Le^a$ and this produced a subsequent increase in antibody titre (Mollison et al., 1963; Rickard and Worlledge, 1968; Pelosi et al., 1974). Blood group A substance was used to make anti-A in mice (Fletcher et al., 1984; Chen. et al., 1987; Miyazaki et al., 1991; Nickerson et al., 1995) and anti-A,B in mice (Miyazaki et al., 1991). B group substance was used to make monoclonal anti-B in mice (Sacks and Lennox, 1981; Voak et al., 1982; Barrie et al., 1983; Fletcher et al., 1984; Moore et al., 1984).

### 1.6.4 Manipulation by immunization with synthetic conjugates

The pioneering work of Goebel and Avery showed that antibody specific for carbohydrate structures could be generated in experimental animals immunized with synthetic antigenic carbohydrate-protein structures (Avery and Goebel, 1929; Landsteiner, 1945). Synthetic carbohydrate vaccines against malaria, cancer, melanoma, AIDS, fungus, shigella, cholera, bacteria and enteric pathogens with surface liposaccharides have been made or are in research (Borman, 2004). These vaccines are often formulated as liposomes as vaccines in this form are highly effective at direct in vivo antigen loading and activation of DC leading to protective antiviral and anti-tumor immune responses (Schwendener et al., 2010).

Carbohydrate antigen-linked with an amino acid peptide sequence (T cell epitope) and a lipopeptide immunoadjuvant (for dendritic cell activation) induced a T cell dependent (TD) immune response and resulted in the production of IgG antibody against the carbohydrate antigen (Buskas et al., 2005). Synthetic carbohydrate-protein conjugates have also been used to change an antigenic response from T cell independent (TI) to T cell dependent (TD) stimulating immunological memory (Obukhanych and Nussenzweig, 2006). Synthetic clustered sugars have been used to increase the concentration of antigen provided to initiate immune response but they may affect the specificity of response (Irazoqui et al., 2005). Synthetic multivalent carbohydrate ligands can compensate for the weak affinities of individual carbohydrate moieties – polymeric backbone constructions including the use of mannoside, galactoside and Sialyl Lewis X moieties have shown increases in binding potency valuable for potential in treating infectious diseases or cell targeting agents

(Gardiner, 1998). Dendrimers, polymers, micelles, vesicles and nanoparticles are currently produced to enhance therapeutic potency of multivalent carbohydrates (Pieters, 2009). Carbohydrate-antigen interaction with DC-expressed lectins leads to enhanced presentation to T cells (Rudd et al., 2001; Geijtenbeek and Gringhuis, 2009).

The use of synthetic oligosaccharides in a form ready for attachment to polymeric material for the preparation of target cells, such as red blood cells, has been used to study the immunological responses in animals immunized with the synthetic terminal disaccharide unit of the human blood group A, B, O and $Le^a$ determinant (Lemieux et al., 1975). The trisaccharide Lewis a-hapten to BSA was used to make carbohydrate antibody in mice (Lemiuex et al., 1977). Synthetic B oligosaccharide carbohydrate coupled to O red cells was used to vaccinate mice to make anti-B (Bundle et al., 1982; Chen. et al., 1987). Synthetic carbohydrates coupled to OVA antigen can be used to target DCs and enhance antigen presentation via class I and class II molecules due to carbohydrate-lectin interactions (Adams et al., 2008). Dendritic cell-specific ICAM-3-grabbing non-integrin 1 (DC-SIGN) is known to bind high-mannose and fucose structures. Modification of ovalbumin (OVA) with fucose-containing Lewis B-structures not only improved targeting of OVA to DC-SIGN as shown by enhanced antigen uptake by DC from DC-SIGN Tg mice, but also increased cross-presentation to OVA-specific CD8+ and CD4+ T-cells (Singh et al., 2009).

The development of specific antigens found on human tumors to enhance immunogenicity offers promise for cancer therapy (Melief et al., 1996) with the use of synthetic carbohydrate-conjugates to produce IgM and IgG antibodies in comparison to carbohydrate antigens which produce only IgM (Toyokuni et al., 1994; Bundle, 2007). A lipopeptide conjugate using a di or tri-meric tumour-associated carbohydrate antigen produced IgM and, unusually for carbohydrate antigens, also IgG (Toyokuni et al., 1994) with the monomeric form having a weaker response.

Cytotoxic T cells have been shown to recognize tumour peptides displayed on the surface of tumour cells and immunization against these antigens is clinically valuable (Boon et al., 1997; Janeway, 2005). A tumour-associated glycopeptide (B cell epitope) from human mucin, with a lipopeptide Toll-like receptor ligand (T epitope), when presented in a liposome format produced high titre IgG antibody (Bundle, 2007). The use of glycolipids and carbohydrate antigens using carriers such as keyhole limpet haemocyanin and saponin adjuvant conjugated with a monosaccharide found on most epithelial cancers,

stimulated high titre IgM and IgG antibodies (Slovin et al., 2003; Slovin et al., 2005). The use of a potential carrier molecule and built-in adjuvant for the construction of structurally defined fully synthetic glycoconjugate vaccine with a monophosphorylated derivative of *Neisseria meningitidis* lipid, A (MPLA)-TACA (Tumour Associated Carbohydrate Antigen) conjugate elicited robust IgG antibody responses binding selectively to target tumor cells (Wang et al., 2011).

Red cell expression of blood group antigens can be generated by gene transfer. Cord blood cells were genetically manipulated with a Kidd blood group antigen using lentiviral gene transfer which resulted in the production of cells exhibiting the chosen phenotype (Bagnis et al., 2009) to be used for immunologic testing and antibody detection. Transgenic mice expressing the human KEL1 or KEL2 antigens have been generated using vectors were created with cDNAs encoding either KEL1 or KEL2 to investigate anti-Kell antibodies (Smith et al., 2012).

### 1.6.5 Neutralizing circulating antibody with red cell antigens

Deliberately transfusing ABO incompatible red blood cell antigens has been used to reduce antibodies levels prior to bone marrow transplantation (Nussbaumer et al., 1995; Scholl et al., 2005; Crew and Ratner, 2010). However, the antibody titres must be bought down to a steady level of <1:8 before transplantation is attempted (Tyden et al., 2005; Tyden et al., 2007).

### 1.6.6 Neutralizing circulating antibody with synthetic carbohydrate antigen

Intravenous carbohydrate therapy used with continuous infusion of synthetic A or B trisaccharide was used neutralize circulating antibodies (Romano et al., 1987a) prolonging transplant survival and preventing antibody-mediated graft failure (Cooper et al., 1993). These synthetic antigens have also shown to be useful in clinical situations such as Haemolytic Disease of the Newborn by enabling dissociation of maternal antibody bound to red cells (Romano et al., 1994).

### 1.6.7 Neutralizing circulating antibody with glycolipid antigen

The neutralization of a circulating antibody by transfusion of glycolipid antigen in plasma (Mollison et al., 1963; Rickard and Worlledge, 1968; Pelosi et al., 1974) can allow

incompatible red cell survival. Historically, Lewis incompatible red cells have been deliberately transfused without adverse consequences into patients with complement-binding hemolytic antibodies. Such was a reported practice last century to transfuse Lewis positive plasma into recipients with Lewis antibodies and then safely follow this with incompatible Lewis-positive blood (Mollison et al., 1963; Hossaini, 1972; Pelosi et al., 1974). The underlying principle was that the Lewis substances present in the plasma were able to neutralize the circulating Lewis antibodies, and thus allow Lewis-positive blood to be immediately tolerated. The subsequent elution of the Lewis antigens from the transfused cells allowed for their prolonged survival.

### 1.6.8 Elimination of ABO antibody by plasmapheresis

Strategies to allow transplant of incompatible organs involve pre-transplant plasmapheresis, splenectomy, immunosuppressive drugs, monoclonal antibody infusion, and intravenous immunoglobulin (KumLien et al., 2006, Rieben et al., 1991; Rydberg, 2001; Sassi et al., 2011). However, some of these therapies are not ABO specific so remove other protective antibodies and clotting factors (Tyden et al., 2005; Tyden et al., 2007). Immunoadsorbent columns of immobilized A and B trisaccharides are used to remove anti-A and anti-B from plasma before bone marrow and renal transplant (Blomberg et al., 1993). Plasmapharesis of plasma using apheresis filters such as one containing synthetic terminal trisaccharide A or B blood group antigen linked to a Sepharose matrix enabled successful ABO-mismatched kidney transplantations without splenectomy. ABO antibodies were effectively and safely depleted with the Glycosorb ABO column (KumLien et al., 2006). An IgG antibody titre <1:8 at the time of transplantation is associated with an almost nonexistent risk of antibody-mediated rejection (Winters et al., 2004).

### 1.6.9 Manipulation to induce tolerance by depletion or inhibition of antibody-producing lymphocytes

Pre-transplant immunosuppressive treatment to eliminate B cell immunity to allow ABO incompatible transplant has become more important than antibody removal and splenectomy (Takahashi and Saito, 2012). Antibodies such as anti-lymphocyte globulin eliminate specific lymphocyte subsets and inhibit cell function to induce tolerance (Cobbold et al., 1992). Tolerance can also be induced by passive antibody-mediated immune suppression due to down regulation of antigen-specific B cells (Kumpel, 2002; Clatworthy, 2011). Monoclonal antibodies that destroy B lymphocytes or non-depleting monoclonal

antibodies that block the function of B cells are used to inhibit antibody production which causes autoimmune disease (Edwards et al., 2002). Rituximab, an antibody to protein CD20 found on B cell surfaces reduces B cell numbers (Clatworthy, 2011; Yin et al., 2011). Synthetic group A carbohydrates conjugated to bovine serum albumin along with cyclosporine blocked B cell differentiation allowing successful ABO incompatible transplantation (Irei et al., 2007).

Depletion of antigen presenting cells using antibodies to prevent graft rejection has been successful in animals (Kirk et al., 1999). Transplantation tolerance-inducing protocols using monoclonal antibodies against CD4 or CD8 or costimulatory molecules have been shown to induce regulatory T cells (Bilsborough et al., 2003). Lymphocyte populations depleted of self-reactive cells ignore tumourous cancers with altered self-antigens - allowing tolerance. This phenomenon could be exploited for therapeutic use with the development of strategies to alter self-antigens in autoimmune disease (Jones et al., 1997; Caspi, 2008).

Current treatment of autoimmune disease involves manipulation therapy by interference with co-stimulatory immune response pathways (Aruffo and Hollenbaugh, 2001; Taylor et al., 2001); by using powerful anti-inflammatory and cytotoxic drugs and drugs that interfere with T cell signalling since auto-reactive T cells are involved in most organ-specific autoimmune processes (Mukhopadhaya et al., 2008).

Synthetic peptides have been used to develop tolerance to a particular epitope rather than intact antigens. For example, high dose intravenous transfusions of antigenic peptides, known to cause multiple sclerosis, have been used in mice to activate T cells which are then deleted on subsequent encounter with administered disease proteins (Liblau et al., 1997).

### 1.6.10 Manipulation of response by inhibition of complement

Eculizumab, a complement protein C5 antibody (Borowitz et al., 2010), is used to block the action of complement after antibody-binding to the cell membrane which thus prevents the initiation of tissue injury (Hasegawa et al., 2008; Crew and Ratner, 2010).

### 1.6.11 Manipulation of tolerance using haematopoietic stem cells

Immune tolerance of organ transplants has been induced in animals using haematopoietic stem cells from the donor to maintain a mixed immune cell chimerism; chimeric conditions providing tolerance to donor by T cell clonal deletion and tolerance to host by clonal anergy (Waldmann et al., 2001; Fairchild et al., 2004; Beyth et al., 2005; Scandling et al., 2011; Touraine and Sanhadji, 2011). Stem cells from fetal livers induced tolerance when transplanted in neonatal patients who then developed full tolerance to both donor and host antigens (Touraine and Sanhadji, 2011). Stem cells have been used to induce tolerance in kidney transplant patients (Miller et al., 2007; Scandling et al., 2011).

Long-term tolerance in bone marrow using gene therapy with molecular chimerism to induce modifications of host response has been successful in establishing donor as "self" (Bagley et al., 2002). The continued presence of the donor's immune cells circulating through the thymus and lymphoid tissues promotes immune tolerance by eliminating reactive T cells (Chan et al., 2008; Scandling et al., 2008).

However, stem cell membranes inserted with antigen can be either immunogenic or tolerising. When low level numbers of antigen specific modified stem cells are transplanted, cytotoxic T cells are induced but if high levels are used the result is T cell anergy and the subsequent absence of T cell killing (Denning et al., 2011).

### 1.6.12 Manipulation of tolerance using manipulated antigens

The creation of designer red cells could simplify the practice of transfusion medicine (Wilson and Spitalnik, 1994; Bagnis et al., 2011). Stealth donor red cells or antigen masking systems have been proposed as possible future options to allow transfusion across the ABO barrier, but as yet these techniques are not in current use due to shortened red cell survival (Garratty, 2008b) or antibody formation to the chemicals used such as polyethylene glycol (PEG) derivative of phosphatidyethanolamine (PE) (Sroda et al., 2005) or the efficacy of target delivery issues (Cruz et al., 2011). Antigens on red cells were "immuno-camouflaged" and made unavailable to antibody by the use of PEG and when inserted into red cell membranes, PEG blocked most red cell antigen binding including A and B (Chen and Scott., 2001; Garratty, 2008b; Cruz et al., 2011).

The use of anti-A (produced in mice with blood group A substance in saliva) to mask the A antigen on antibody-coated hybridoma or red blood cells pre-infusion, showed no

antibody-antigen interaction and there was subsequently a significantly reduced antibody production. These experiments showed that the antibody blocked the antigen allowing transfused cell survival even in the presence of anti-A (Hasegawa et al., 2008).

Several proteins have been inserted into the lipid bilayers and biomembranes by electroinsertion. Antigenic molecules such as fluorescent-labelled glycophorin and human CD4 antigen can be inserted into cell membranes by electroinsertion reacting with circulating antibody without causing subsequent stimulation of antibody (Mouneimne et al., 1991; Zeira et al., 1991). This insertion of antigen into cell membranes was described as “protein paint” (Wilson and Spitalnik, 1994), for example, when CD4 was inserted into the red cell membrane (Nicolau et al., 1993) and when the protein CD 8 antigen coupled to a glycophosphatidyl inositol anchor was inserted into APCs (Tykocinski and Kaplan, 1993).

Bacterial glycosidase enzymes are used to degrade A and B antigens to develop universal red blood cells, by the removal of the terminal sugars from carbohydrate chains to create the precursor O antigen (Goldstein et al., 1982; Liu. et al., 2007; Garratty, 2008b). Blood group B antigens can be enzymatically converted to a compatible H antigen status thus failing to provoke an immune response in the presence of anti-B (Lenny et al., 1994; Wilson and Spitalnik, 1994; Olsson and Clausen, 2007). Research has also been undertaken for the conversion of A antigens using bacterial enzymes (Falk et al., 1991; Izumi et al., 1992).

## 1.7 KODE™ Technology

The development of suitable methodology with animal models for research into neutralising antibodies and subsequent cell survival of transfused cells or tissue would greatly assist the field of organ transplant and transfusion. However, traditional techniques are generally limited to genetic engineering (Bagnis et al., 2009) or covalent attachment of chemical molecules to the surface membrane (Strable and Finn, 2009), which expose the cell chemical reactants or require manipulation (Wilson and Spitalnik, 1994) and may inadvertently alter the function of the modified cells (Nicolai et al., 1993). KODE ™ technology, using synthetic glycolipid-like Function-Spacer-Lipids allows for the artificial attachment of blood group antigens onto red blood cells (Frame et al., 2007), plus visualization and recovery of the constructs, making them potentially suitable to study both transfusion reactions and determine in vivo cell survival (Blake et al., 2011).

KODE ™ FSL constructs can be likened to the structure of a sunflower (Figure 11), where the head of the flower is the functional part (F) containing carbohydrates, biotin, proteins, peptides, fluorescent labels, variations in the mass and charge. The stem provides the spacer (S) with length, rigidity, substitutions for charge and polarity, linear or branched branches for spacing and remains inert. Finally, the roots are the lipid for membrane insertion (L): either diacyl e.g. DOPE or sterol e.g. cholesterol or ceramide.

The choice of functional head group is limited by chemical and solubility requirements with synthetic glycolipids designed to have specific advantages over their natural counterparts. Synthetic analogues can be manufactured to be any structure including those that may not occur naturally. For example, the FSL-A and B molecules are lacking in the fourth sugar which designates chain type and so they are both generic for all A and B types. The incorporation of a linker (spacer) introduced a water solubility characteristic which lowers the insertion temperature requirement and makes insertion more efficient.

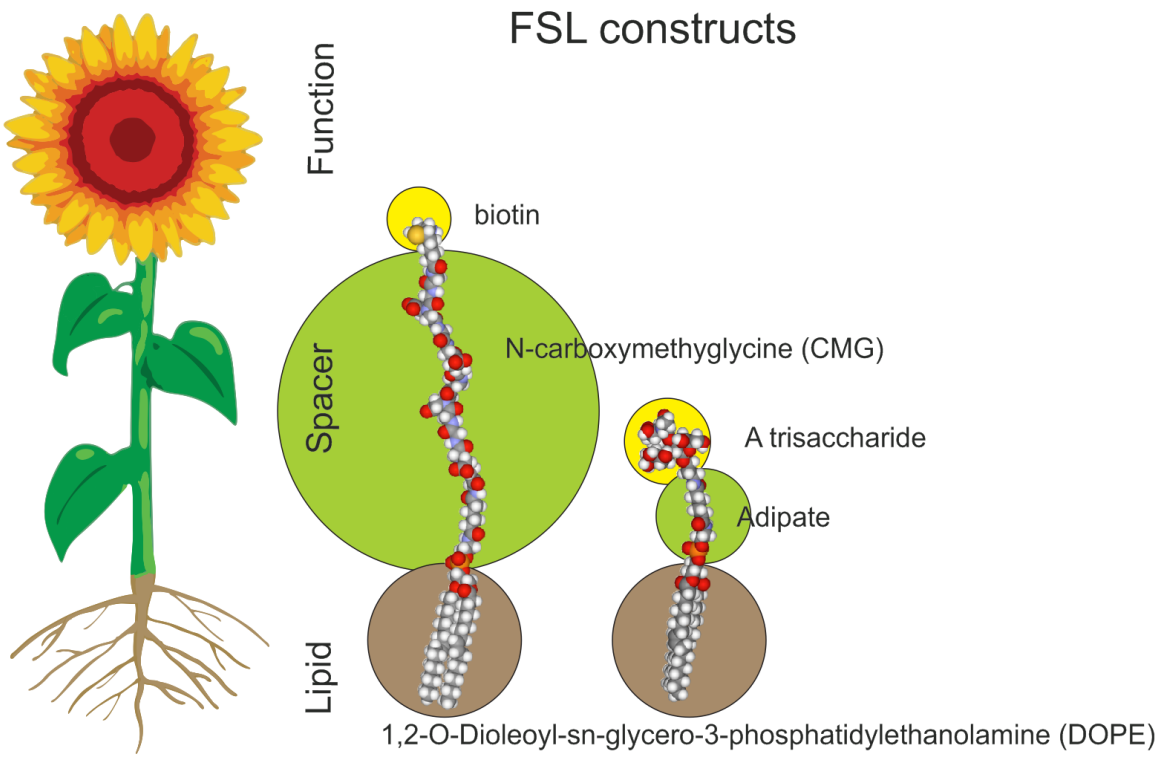


Figure 11. Structural analogy of a sunflower to model of selected FSL constructs.

### 1.7.1 FSL Constructs

KODE ™ technology represents a platform of novel constructs, termed FSL's, which consist of three functional components: a bioactive functional head group (F), a spacer (S) that contributes to water dispersibility and a diacyl-lipid tail (L) which causes the construct to either insert into a membrane, or attach to surfaces, or to form micelles due to the amphilic nature of some of the constructs. The process of applying an FSL KODE ™ construct to a surface is terming "koding", while the FSL modified surface is "koded"(KODE Biotech Ltd, Technology overview) and the modified cell or virus is termed a kodecyte (Henry,2009) or kodeviron (Hadac et al., 2011) respectively. Koded cells so far include blood cells, epithelial, endometrial, cultured cells, spermatozoa, and embryos (Blake et al., 2011, Flower et al., 2008, Heathcote et al., 2008). Koding inserts FSLs into all cells non-specifically and untargetted although cell types have different lipid composition and thus potentially different propensity for labelling. Live cells lose constructs over time but dead ones remain labeled. FSL constructs do not react non-specifically - the unexpected positivity rate ("false positive") of a benign FSL in serum is less than 0.5%.

The generic koding process involves simply contacting cells, virions, or surfaces with a solution of FSL's, incubating them for 30-120 minutes within the temperature range of 4-37°C, and the constructs spontaneously incorporate. The koded surface now displays the F group. The creation of kodecytes/ kodevirions does not harm the cell/virus, and they retain their normal in vivo and in vitro vitality and functionality. FSLs as solutions can be also be used to inhibit toxins and cell/virus binding (Lund et al., 2006; Harrison et al., 2010). The construct remains in the membrane of red cells for the life of the cells in lipid free media and are lost in circulation at the rate of about 1% per hour (Oliver et al., 2010). The exact period of time the FSL constructs will remain in plasma is uncertain, but studies with radiolabeled FSLs injected into rats suggested they are cleared from the circulation within 24 hours (Hadac et al., 2011).

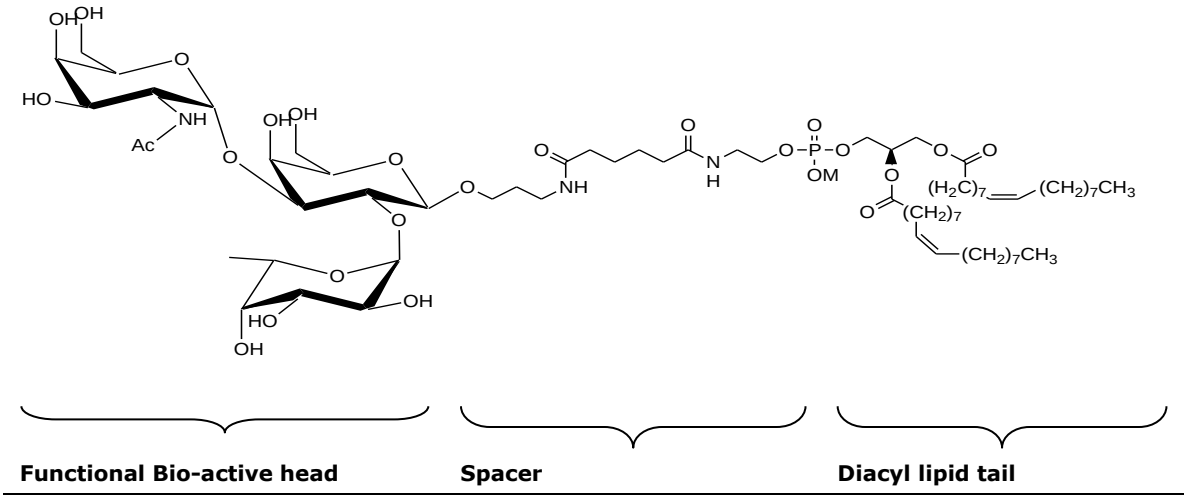


Figure 12. Typical structure of synthetic KODE molecule.

In this example A trisaccharide, GalNAc α3(Fuc α2) is the bioactive head, $O(CH_2)_3NH$ is the spacer and 1, 2-O-dioleoylphospatidylethanolamine, DOPE is the tail .

*Functional Head*

As the functional (F) head of FSLs can be user-selected (Figure 12), constructs have been made to a large range of carbohydrates including A, B, H, Lewis, Galili, GB3, sialic acids, hyaluronic acid polymers, plus a range of peptides representative of blood group antigens and infectious markers, as well as fluorophores, radioisotopes, PEG, and biotin. FSLs can

be used to create live kodecytes and infective kodevirions expressing investigator-controlled levels of bioactives/labels. Specificity will increase with correctly chosen FSLs although maybe more than one construct may be required to cover the assay range with the FSL using a more discrete peptide part of the antigen rather than protein giving a more restricted antibody profile (KODE™ Biotech, 2012).

*Spacer*

The spacer is selected to provide a construct that is dispersible in water and will spontaneously incorporate into a red cell membrane (Figure 12). Consideration is taken of the distance from the membrane after insertion (Korchagina, 2012). Spacers required for use with undiluted serum must not only be chemically unreactive but also devoid of any affinity towards the serum and cellular components (Henry et al., 2011).

The presence of the spacer may be the factor that differentiates the immune response of FSLs from that with glycolipids. The presentation of the antigen some distance away from the surface usually increases sensitivity – for example increasing the spacer from 1.9 nm to 7.2 nm increased red cell sensitivity two fold. The spacer is semi-rigid and designed to ensure accessibility for target binding and proper presentation of functional antigens at the cell surface and also solubility. Electrostatic forces due to the spacer's anionic groups probably favour uniform distribution of the incorporated constructs on the cell membrane.

*Lipid Tail*

The tail is used to insert and anchor the construct to the cell membrane or to create micelles. For red cell insertion, 1, 2-O-dioleoylphospatidylethanolamine, DOPE, lipid is the most suitable. The DOPE tail has a *cis*-double bond, which creates a structural kink in each lipid tail (Figure 12). The kink is believed to prevent the molecules from being packed tightly together, inferring different physicochemical properties in the cell membrane, possibly affecting insertion and fluidity in the membrane. The phosphatidyl-ethanolamine structure that forms the base of the tail structure in the synthetic molecule is similar to one of four major phospholipids that predominate in the plasma membrane of many mammalian cells.

However, the bi-lipid tail portion of the synthetic glycolipid is different to the lipid portion of the natural glycolipid because it lacks the long-chain base of the sphingolipid, having

instead two fatty acid chains. The diacyl nature of the tail – a glyceride consisting of two fatty acid chains covalently bonded to a glycerol molecule by esters, is important for maintaining normal cell retention. The structure is actually more similar to that of glycerophospholipids than it is to the lipid moiety of natural glycolipids.

Kodecytes differ from natural cells in that, due to composition of the lipid tail, they are laterally mobile and may cluster due to the functional head group and the lipid tail (Korchagina et al., 2011). FSLs do not actively pass through the plasma membrane but may enter the cell via endocytosis (Blake et al., 2010).

### 1.7.2 FSL constructs used in this study

FSLs were used to create compatible and incompatible red cells that could be identified in the circulation of mice. FSL-A, FSL-B, FSL-GB3 and FSL-biotin molecules were attached to the red cells of laboratory mice to provide a source of compatible but distinctly identifiable red cells (kodecytes) to obtain baseline and control data. FSL-A was used to create incompatible murine kodecytes to measure cell survival after transfusion into anti-A positive mice. The presence of kodecytes were determined by serology with gel cards and monoclonal anti-A reagent and the presence of anti-A in mice detected by inkjet enzyme immunoassay with FSL-A construct (Barr et al., 2010). FSL-biotin was used as label and once bound with avidin-Alexafluor 488 was tracked with fluorescent microscopy. FSL-A was also used to neutralize circulating anti-A before an incompatible transfusion of A+biotin kodecytes and to attempt to induce tolerance. The toxicity of the FSLs was also examined.

*FSL-A and FSL-B*

FSL-A (tri) and FSL-B (tri) are KODE™ technology constructs (Figure 13 and 14) designed to label hydrophobic surfaces including living cells, with the blood group A or B trisaccharide. FSL-A (tri) is constructed with the functional group F being the blood group A trisaccharide GalNAc$\alpha$3(Fuc$\alpha$2) Gal$\beta$, and FSL-B (GALa3 [Fuca2] GAL$\beta$)-SA1-L1 is constructed with the functional group F being blood group B trisaccharide Galα3 (Fucα2) Galα. Both constructs are conjugated via an O(CH2)3NH spacer (SA1) to an activated adipate derivative of dioleoyl phosphatidyl ethanolamine (L1).They have been specifically designed to insert into membranes of live cells and modify other hydrophobic surfaces

including fixed cells and solid phase surfaces (Frame et al., 2007; Henry, 2009). It is of note that higher concentrations of FSL-B (tri) are required than FSLA (tri) to achieve similar serology – this is believed to be due to the nature of antibodies to B antigen (Frame et al., 2007; Henry, 2009).

*FSL-GB3*

FSL-GB3 is a KODE™ technology construct (Figure 15) designed to label cells with GB3 trisaccharide (also known as Pk) comprised of the blood group GB3 (Pk) trisaccharide Galα4Galβ4Glcβ representing F, conjugated via an $O(CH_2)_3NH$ spacer (SA1) to an activated adipate derivative of dioleoylphosphatidylethanolamine (L1). This product has been used *in vitro* to inhibit HIV virions and to bind to *Shigella* veratoxin *(*Harrison et al., 2010).

*FSL-biotin*

FSL-biotin R&D-CONJ(1biotin)-SC2-L1 (Figure 16) is comprised of a monomer of biotin (vitamin B7) representing F, conjugated to a maleimide-bearing carboxy-methylglycine based linker (SC2) in turn conjugated to an activated adipate derivative of dioleoylphosphatidyl ethanolamine (L1). FSL-biotin has been specifically designed to create biotinylated live cells (kodecytes) but can also be used to modify other hydrophobic surfaces including fixed cells and solid phase surfaces. Biotinylated kodecytes can be reacted with a variety of biotin binding protein constructs including fluorescent labeled avidin and avidinylated beads/solid surfaces (Frame et al., 2007; Blake et al., 2011).

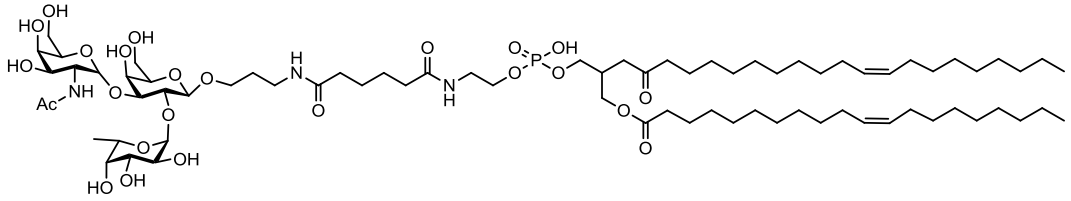

Figure 13. Schematic diagram of FSL-A

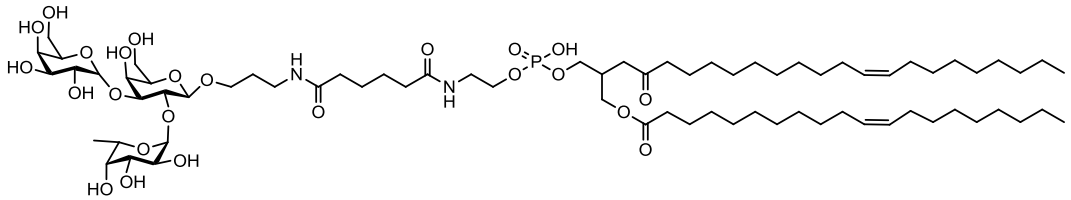

Figure 14. Schematic diagram of FSL-B

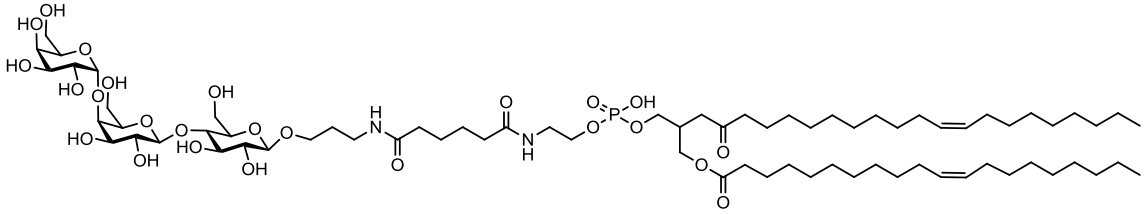

Figure 15. Schematic diagram of FSL-GB3

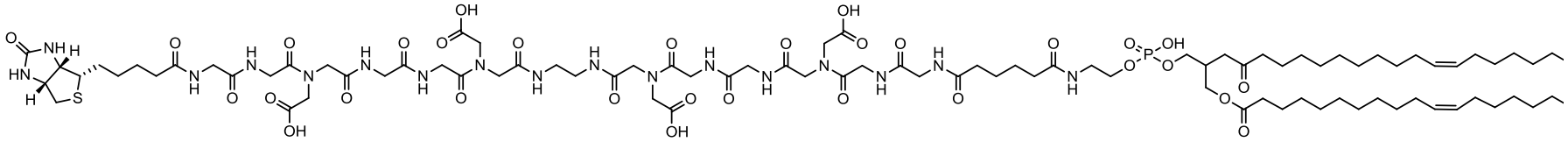

Figure 16.Schematic diagram of FSL-biotin

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## 1.8 Aims of the research

The aim of this thesis was to attempt to manipulate the immune response with Functional-Spacer-Lipids (FSLs) constructs to test four hypotheses. The toxicity of the FSLs was established in baseline studies.

Hypothesis 1: FSLs stimulate antibody production.

Hypothesis 2: FSLs be used for measurement of cell survival and cell recovery.

Hypothesis 3: FSL neutralize antibody and allow for incompatible transfusion.

Hypothesis 4: FSLs induce tolerance in mice.

# CHAPTER 2 - BASELINE FSL EXPERIMENTS AND IMMUNOGENICITY

This chapter investigates whether the toxicity of FSL constructs present safety concerns with their use *in vivo*. All procedures were approved by The University of Auckland Animal Ethics Committee-AEC#C500 and AEC#R629.

## 2.1 Consequences of FSL intravenous infusion

### *Method Overview*

FSL-A with the blood group A trisaccharide epitope, or FSL-GB3 with the $P^k$ epitope were used to establish the baseline toxicity status of FSLs in C57BL/6 mice. Anaesthetized, (Protocol 1) 6 week old mice were given FSL-A or FSL-GB3 infusions of 3mM or 1.5mM (200 or 100 mg/kg body mass) through the pectoral muscle (which ensures closure of the puncture site) directly into the surgically exposed subclavian vessel (Protocol 3 V1). Untreated control animals were tested in parallel. The health and behavior of the mice was monitored and recorded at the following intervals: immediately post infusion, 24 hours, 48 hours, one week, one month, 2 months and 3 months. Mice were autopsied at 3 months and histological investigations were conducted on their organs (liver, spleen, hepatic lymph nodes, lung, and kidney). Histologic samples taken were blinded and performed independently by Gribbles Veterinary Pathology Ltd. Blood films were prepared, Giemsa stained, and white cell differentials conducted to determine if there were any differences between the controls and the FSL treated animals. Thin Layer Chromatography was performed to detect anti-A or anti-GB3 production, with the FSL used in the assay being the same FSL-A and GB3 as used for infusion (Protocol 5).

### *Results*

*Health and Weight*

Mice receiving FSL-A or FSL-GB3 did not show any adverse reaction to the subclavian vessel intravascular infusion. They were weighed and observed for signs of normal behavior. Mice were then evaluated at various time points - 1, 2 and 3 months and throughout the study showed no overt signs of toxicity.

The values for the control mice in the separate trials were combined as they were all housed in identical conditions. There was no statistical difference in weight gain over the three months between the groups of infused and control animals with either FSL-A (p-value 0.6) or with FSL-GB3 (p-value 0.4) (Table 1). Three mice were found to have been pregnant at the time of infusion and the delivered pups were housed with the experimental animals. The litters had normal health over the three months. The pups were not autopsied or serologically tested.

Table 1. Comparison of the weight of mice infused with FSL and naive control mice.

There was no significant difference between the weight of mice with FSL-A molecule and controls p-value =0.6; or in the weight of mice with FSL-GB3 and controls p-value = 0.4. Naive controls are grouped together.

| **Comparison of weights after FSL infusion** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Mouse** | | **Intravenous injection** | | **Bodyweight (g) Months post FSL infusion** | | | | |
| Infusion | ID | Sex | Dose mM | | 0 | 1 | 2 | 3 | Weight change over 3 months |
| FSL-A | A | F | 3 | | 25 | 26 | 27 | 28 | 3 |
| | B | F | 3 | | 24 | 27 | 25 | 26 | 2 |
| | C | F | 3 | | 23 | 24 | 25 | 25 | 2 |
| | D | F | 3 | | 26 | 27 | 26 | 27 | 1 |
| **Average weight gain** | | | | | | | | | **+2** |
| FSL-GB3 | E | F | 0 | 3 | 23 | 24 | 25 | 25 | 2 |
| | G | F | 0 | 3 | 23 | 24 | 25 | 25 | 2 |
| | G | M | 0 | 3 | 23 | 26 | 27 | 26 | 3 |
| | H | M | 0 | 3 | 20 | 25 | 28 | 29 | 9 |
| | I | M | 0 | 3 | 27 | 28 | 30 | 29 | 2 |
| | J | F | 0 | 1.5 | 19 | 22 | 24 | 24 | 5 |
| | K | M | 0 | 1.5 | 24 | 26 | 28 | 28 | 4 |
| **Average weight gain** | | | | | | | | | **+3** |
| | *L | F | 0 | 3 | 18 | 26 | 24 | 24 | 6 |
| | *M | F | 0 | 1.5 | 18 | 31 | 27 | 27 | 9 |
| | *N | F | 0 | 1.5 | 19 | 27 | 24 | 25 | 6 |
| No infusion (controls) | O | F | 0 | 0 | 24 | 25 | 25 | 26 | 2 |
| | P | F | 0 | 0 | 23 | 23 | 25 | 24 | 1 |
| | Q | F | 0 | 0 | 24 | 24 | 26 | 26 | 2 |
| | R | F | 0 | 0 | 22 | 23 | 24 | 24 | 2 |
| | S | F | 0 | 0 | 25 | 28 | 27 | 26 | 1 |
| | T | F | 0 | 0 | 21 | 22 | 23 | 23 | 2 |
| | U | F | 0 | 0 | 21 | 22 | 23 | 26 | 5 |
| | V | F | 0 | 0 | 26 | 26 | 26 | 26 | 0 |
| **Average weight gain** | | | | | | | | | **+2** |

* these mice were pregnant and were not included in average weight data since weight gain was influenced by pregnancy

*Blood smears*

One hundred white cells were counted for each sample and differentials of cell types were performed. Hematological examination of Giemsa stained blood smears found no significant differences in the differential white counts between FSL infused and naive mice. For the three types of cells counted in the FSL infused animals and naive controls there appeared to be no significant FSL affect on the differential cell counts with p values: neutrophils p = 0.3; lymphocytes p = 1; and for monocytes p = 0.9.

*Antibody analysis*

Five mice had FSL-A and 10 had FSL-GB3 by intravenous administration. Five control naive mice were housed with each series. Antibody production was assigned following enzyme immunoassay (EIA) (Protocol 5). Antibody was not detected to either the FSL-A or the FSL-GB3 antigen after intravenous infusion or in the naive mice (Table 2).

Table 2. Detection of antibody production in naive C57BL6 mice after intravenous FSL infusion.
FSL-A and FSL-GB3 did not stimulate antibody production.

| **Antibody Status after FSL infusion** | | | | | |
|---|---|---|---|---|---|
| | Intravenous FSL infusion 100 µL/200mg/Kg body weight | | Antibody detected | | |
| No. mice | FSL-A | FSL-GB3 | Anti-A | Anti-GB3 | Total Antibody |
| 5 | + | 0 | 0 | NT | 0/5 |
| 10 | 0 | + | 0 | 0 | 0/10 |
| 10 | 0 | 0 | 0 | 0 | 0/10 |

NT = not tested

## *Conclusion*

Mice evaluated at various time points to investigate the effects of FSL infusion showed no overt signs of FSL toxicity to either 3mM or 1.5mM FSL-GB3 or 3mM FSL-A. Histological examination of spleen, liver, hepatic lymph nodes, kidney and lung showed no differences between test and control animals. There was no evidence of antibody stimulation to FSL-A or FSL-GB3 as determined by thin layer chromatography antibody overlay analysis. Overall the results can be interpreted that FSL constructs are non-toxic or of very low toxicity.

## 2.2 Immune stimulation

This chapter investigates the hypothesis that FSL constructs could cause an immune response resulting in the stimulation of specific antibody in mice.

**Overview**

Balb/C mice were originally chosen due to the high level of inter-breeding and the lack of genetic diversity. The tail vein was also easily distinguished. This strain of mice had natural levels of anti-A (Huppes et al., 1993, Neron and Lemieux, 1994; Larkin and Porter, 2005) which it was hoped could be stimulated to higher levels. However, it was found we were unable to stimulate increased antibody levels with either FSLs or glycoproteins, which is in agreement with Larkin and Porter, (Larkin and Porter, 2005), who showed that the specificity of this IgM anti-A was different from human anti-A and that immunization with red cell A antigen did not increase the reactive IgM titre.

C57 black 6 laboratory mice (C57BL/6) were then chosen for their good immune response to blood group antigens (Rouger et al., 1983; Edelman et al., 1984; Kunzendorf et al., 1996; Halverson et al., 2001; Cretin and Iacomini, 2002), and have been used for measurement of antibody-mediated haemolysis using monoclonal antibody and in transgenic mice (Schirmer et al., 2007). Mice used in this research were aged between 6 and 20 weeks and the gender was not considered a factor (Schunk and Macallum, 2005). The naive mice were regarded as genetically similar due to a high level of inter-breeding. Genetic variability within inbred strains is negligible and thus offspring can be considered as clonal (Taft et al., 2006). All mice were housed, fed and monitored in the same manner for the total time period according to University of Auckland animal welfare procedure protocols. Protocols (Protocol 1), were developed for the use of adjuvant, route of immunization, blood collection (Hoff, 2000; Leenaars and Hendriksen., 2005; Research Animal Resources, 2007).

### 2.2.1 Baseline studies: naive mice antigen/antibody status

***Method Overview***

Blood was obtained by tail vein collect from anaesthetized naive mice (Protocol 1.4), and tested for the presence of A and B antigens on red cells (Protocol 2.3). Serum antibodies,

if present, were measured by EIA using inkjet printing (Protocol 5). Packed cell volumes were measured on some of the mice when blood was available (Protocol 1 V2). Some mice sera were also tested in baseline studies for anti-$Le^a$ or anti-$Le^b$ and for the presence of red cell A and B antigens (Protocol 2.4).

### *Results*

Blood was collected from cardiac puncture or tail vein collects into heparinised or plain capillary tubes. Naive C57 BL/6 mice red cells were found to be both antigen A and B negative. Anti-A was not detected in any of the 317 naive mice tested. Some of the naive mice sera was also tested for IgG anti-A (5), anti-biotin (5), anti-B (5) anti-GB3 (5) or anti-$Le^a$ or $Le^b$ (5) (Table 3).

Table 3. Summary of antigens and antibodies detected in naive mice pre transfusion.
No A or B antigens or antibodies were detected in naive mice.

| **Antigen and antibody status of naive C56Bl/6 mice** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Naive Mice | Red cell antigens detected | | Antibody detected by EIA | | | | | | | |
| Total | A | B | anti-A Ig | anti-A IgG | anti-B | anti-biotin | anti-GB3 | anti-$Le^a$ | anti-$Le^b$ |
| 5 | 0 | 0 | | | | | | | |
| 317 | | | 0 | | | | | | |
| 5 | | | | 0 | | | | | |
| 5 | | | | | 0 | | | | |
| 5 | | | | | | 0 | | | |
| 5 | | | | | | | 0 | | |
| 5 | | | | | | | | 0 | 0 |

### *Conclusion*

Naive C57 BL/6 mice did not have natural red cell A or B antigens or anti-A, anti-biotin, anti-GB3, anti-$Le^a$ or anti-$Le^b$ and were therefore suitable for use to investigate the immune response to FSL constructs, for compatible kodecyte survival measurement and for immunization with A substance to provide incompatible serum for the anti-A neutralization experiments.

### 2.2.2 Subcutaneous immunization of naive mice with salivary A glycoproteins

Blood group A substance in saliva was used to produce anti-A in naive mice. These anti-A positive mice were then used to create models for incompatible red cell transfusion, to demonstrate neutralization with FSL-A and to provide controls for the tolerance experiments.

***Method Overview***

Saliva was collected from an ALe(a-b+) human donor (Protocol 1.6 and 1.7) and the titre of the A substance was determined. Anti-A was stimulated in anti-A negative animals by subcutaneous injection of ALe(a-b+) saliva mixed 1:1 with Titermax™Gold adjuvant (Protocol 1.8). All mice were tested pre and post experimentation for anti-A status (Protocol 5) and antibody levels of some serum samples (16) were determined by titre. Some mice sera (5) were also tested for anti-$Le^a$ or anti-$Le^b$.

***Results***

Immunisation with A substance from ALe(a-b+) saliva resulted in the production of anti-A immunoglobulin in 221 mice out of total of 222 (>99%). The antigens $Le^a$ and $Le^b$ would also have been present in the saliva but none of the immunized, anti-A positive mice tested (5) produced detectable anti-$Le^a$ or anti-$Le^b$ (Table 4).

Table 4. Effects of immunisation or infusion in C57BL6 mice.

All but one saliva immunisation resulted in anti-A production and none of the FSL infusions resulted in antibody production.

| Production of antibody after infusion | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Manipulation by infusion | | | | | Analysis by EIA | | | | | |
| | Subcut injection | | Intravenous infusion | | Serum antibody | | | | | |
| Total | * A saliva | † FSL-A | † FSL-A | †† FSL-GB3 | anti-A Ig | anti-A IgG | anti-GB3 | anti-Le[a] or anti- Le[b] | Anti-A I titre ≥128 | % Ig anti-A pos |
| 1 | + | | | | 0 | 0 | | | | 0 |
| 221 | + | | | | 221 | | | | 16 | 100 |
| 5 | + | | | | 5 | | | 0 | | 100 |
| 5 | + | | | | 5 | 0 | | | | 100 |
| 14 | + | | | | 14 | 14 | | | | 100 |
| 11 | | + | | | 0 | | | | | 0 |
| 12 | | | + | | 0 | | | | | 0 |
| 10 | | | | + | 0 | | 0 | | | 0 |

*A substance saliva or FSL-A 1:1TiterMax™Gold (100 µL) given 3 X over a period of 10 weeks.
† FSL-A in solution (20, 10, 5 or 4 mg/mL)
†† FSL-GB3 in solution (20mg / µL)

### *Conclusion*

All naive mice tested (222) were anti-A negative pre-immunization and immunisation with A substance from ALe(a-b+) saliva resulted in the production of IgG anti-A in all the mice tested but one. The level and specificity of this anti-A was considered appropriate for the planned experiments. Mice given subcutaneous FSL-A or FSL-GB3 did not result in anti-A or anti-GB3.

### 2.2.3 Subcutaneous immunization of naive mice with FSL-A in solution

***Method Overview***

The immunogenicity of FSL was tested to establish if the constructs would stimulate anti-A production. Eleven naive, anti-A negative mice were given subcutaneous immunisations using 100 µL FSL-A solution 4mg/mL mixed 1:1 with TiterMax™ Gold adjuvant 3 occasions at intervals of 3 weeks (Protocol 1.8).

***Results***

Subcutaneous immunization of 11 mice with FSL-A in solution, 4mg/mL with TiterMax™Gold adjuvant did not result in anti-A production (Table 4).

***Conclusion***

FSL-A was not immunogenic when administered by subcutaneous injection in solution with adjuvant and there was no stimulation of antibody production.

### 2.2.4 Intravenous immunization of naive mice with FSL-A and FSL-GB3

***Method Overview***

Twelve anaesthetised naive C57BL/6 laboratory mice were transfused with FSL-A at concentrations between 50-200 mg/Kg body weight and another 10 mice with FSL-GB3 at 200 mg/Kg. All constructs were prepared in sterile saline and administered through the pectoral muscle directly into the subclavian vessel (Protocols 1 and 3). Antibody production was then determined by TLC at exsanguination at least 6 weeks later (Protocol 5). Naive mice were tested as controls.

***Results***

None of the mice given FSL-A or FSL-GB3 by intravenous injection produced anti-A or anti-GB3 (Table 4).

***Conclusion***

FSL-A or FSL-GB3 molecule in solution (50-200 mg/Kg), when injected intravenously into anti-A negative mice, does not stimulate antibody production. FSL molecules in solution do

not appear to be immunogenic (although they are shown later (5.2.2) to be antigenic and react strongly with anti-A).

### 2.2.5 Immunogenicity of infused compatible kodecytes in anti-A negative mice

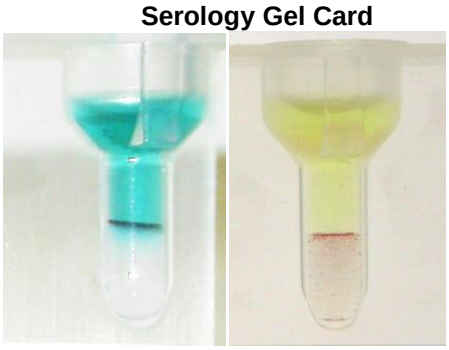
***Method Overview***

Four FSL constructs, FSL-A, FSL-B, FSL-GB3 and FSL-biotin were used to prepare kodecytes using red cells from naive, anti-A negative mice (Protocol 2). Anaesthetised, (Protocol 1), naive, anti-A negative, C57 BL/6 laboratory mice were transfused with 200 µL kodecyte suspensions of A+biotin, B+biotin, GB3 +biotin and FSL-biotin - 20 µL kodecytes in saline representing 3.2% of the total circulating red cell mass in mice (Protocol 3). At exsanguination, at least 3 weeks post infusion, all sera was tested for anti-A, 5 tested for anti-B, 8 tested for anti-biotin and 3 tested for anti-GB3. This series of infused mice was also used for compatible cell survival studies at 0.1, 2, 8, 24, 48, 72, and 96 hour intervals (see 3.2). A 50 µL blood sample was collected into a heparinized capillary tube (Protocol 1.3 and 1.4). Plain capillary tube samples were also collected from selected animals to obtain serum to determine antibody status (Protocol 5).

***Results***

Naive mouse red cells were used to create FSL-A+biotin, FSL-B+biotin, and FSL-GB3 +biotin kodecytes. A and B positive antigen status of the kodecytes was confirmed by serology (Figure 17). GB3 antigen was not tested due to the unavailability of anti-GB3 reagent. Serology was in NaCl (saline only) gel cards. Scoring was recorded as follows: 0 = no visible agglutination; + = many small clumps, turbid reddish background; ++ = medium-sized clumps, clear background; +++ = several large clumps, clear background; ++++ = one large clump.

FSL-biotin status of red cells was confirmed as positive with avidin/Alexa Fluor 488® (Invitrogen, CA, USA), under fluorescent microscopy and differential interference contrast microscopy (Olympus BX51) (Figure 18). No antibodies (0/41 anti-A, 0/5 anti-B, 0/8 anti-biotin and 0/3 for anti-GB3) were detected on inkjet printed TLC wells tested against FSL molecules at 1mg/mL (Table 5). All infused animals negative for anti-A pre infusion remained negative.




I II

Figure 17. Confirmation of the presence of A or B antigen kodecytes.

Image I: A+biotin kodecytes reacted with monoclonal anti-A reagent forming a single band indicating that all the kodecytes have A antigen. Image II: B+biotin kodecytes reacted with monoclonal anti-B reagent forming a single band indicating that all the kodecytes have B antigen.

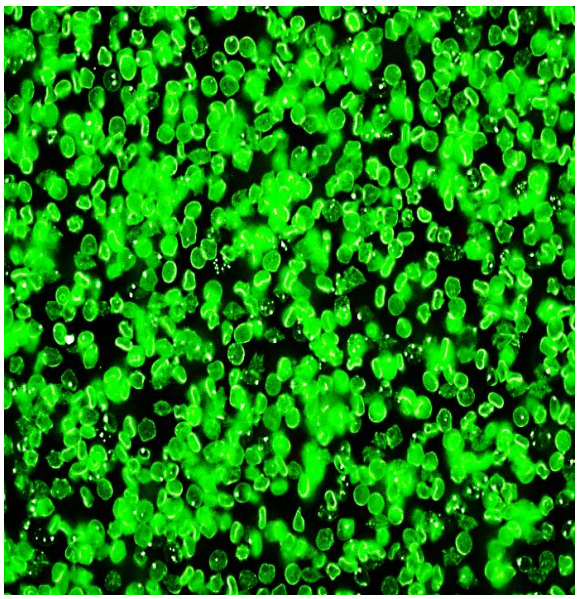


Figure 18. Confirmation of the presence of FSL-biotin kodecytes.

Figure seen under fluorescence microscope with avidin/Alexafluor conjugate and 488nm excitation using Olympus BX51, 200 x magnification. Red cells had been transformed with 100 µg/mL FSL-A and 50 µg/mL FSL-biotin.

Table 5. Effects of infusion of kodecytes in mice used for all series
None of the kodecyte infusions in mice resulted in antibody production.

| Production of antibody after infusion | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Manipulation by infusion | | | | | | Analysis by EIA | | | | | | | |
| | Subcut injection | Intravenous infusion | | | | Serum antibody | | | | | | | |
| Total | *A saliva | A+biotin kodecytes | B+biotin kodecytes | biotin-kodecytes | GB3+biotin kodecytes | anti-A Ig | anti-A IgG | anti-B | anti-biotin | anti-GB3 | Anti-A Ig titre ≥128 | % Ig anti-A pos |
| 1 | | + | | | | 0 | | | | | | 0 |
| 28 | | + | | | | 0 | | | | | | 0 |
| 12 | | + | | | | | | | 0 | | | 0 |
| 6 | + | +** | | | | 6 | 6 | | | | 6 | 100 |
| 5 | | | + | | | 0 | | 0 | | | | 0 |
| 4 | + | | + | | | 4 | 2 | 0 | | | 2 | 100 |
| 8 | | | | + | | 0 | | | 0 | | | 0 |
| 8 | + | | | + | | 8 | | | 0 | | 4 | 100 |
| 2 | | | | | + | 0 | | | | 0 | | 0 |
| 4 | + | | | | + | 4 | 4 | | | 0 | 2 | 100 |

*A substance saliva 1:1 TiterMax™Gold (100 µL) given 3 X over a period of 10 weeks.
** 2 kodecyte transfusions were given

### *Conclusion*

The compatible transfusion of A+biotin, B+biotin, GB3+biotin or biotin-only kodecytes into compatible mice did not stimulate antibody production against the functional head group of the FSL construct or to the spacer. FSL-A, B, GB3 or biotin constructs injected as kodecytes into naive mice were therefore considered non-immunogenic.

## 2.2.6 Immunogenicity of infused compatible kodecytes in anti-A positive mice

### *Method Overview*

Anaesthetised C57 BL/6 mice, previously immunized with A saliva and found to be anti-A positive (2.2.2), were transfused with 100 µL or 200 µL suspensions - 63 or 20 µL kodecytes in saline (representing 10% or 3.2% of the total circulating red cell mass in mice

respectively) of B+biotin, GB3 +biotin or biotin kodecytes (Protocols 2 and 3). This series of infused mice was also used for compatible cell survival studies at 0.1, 2, 8, 24, 48, 72, and 96 hour intervals (see 3.2). A 50 µL blood sample was collected into a heparinized capillary tube from the tail vein. Plain capillary tube samples were also collected from selected animals to obtain serum to determine antibody status (Protocol 5).

### *Results*

None of the anti-A positive mice transfused with compatible B+biotin, GB3+biotin or biotin-only kodecytes produced anti-B (0/4), anti-GB3 (0/4), or anti-biotin (0/8) post transfusion. Serum was tested >10 weeks post the primary subcutaneous immunisation and at least 2 weeks post secondary immunisation. All anti-A positive mice (16) given compatible kodecyte transfusions, remained anti-A positive at exsanguinations (Table 6).

Table 6. Antibodies measured in anti-A positive mice after exposure to compatible B+biotin, GB3 +biotin or biotin kodecytes. Infusions of compatible kodecytes did not result in antibody production.

**Antibodies in anti-A positive mice after compatible kodecyte infusion**

| Intravenous injection | | | | Analysis | | | | |
|---|---|---|---|---|---|---|---|---|
| Mice | Kodecytes | | | serum antibodies post kodecyte infusion | | | | |
| anti-A positive | B+biotin | Biotin | GB3+biotin | Anti-A Ig | Anti-B | Anti-biotin | Anti-GB3 | % Total Anti-A Ig |
| 4 | + | | | 4 | 0 | | | 100 |
| 8 | | + | | 8 | | 0 | | 100 |
| 4 | | | + | 4 | | | 0 | 100 |

### *Conclusion*

B+biotin, GB3+biotin and biotin-kodecytes were not immunogenic when administered by subclavian injection to compatible anti-A positive mice. The FSL-A antigen/anti-A incompatibility was chosen as the model as B antigen/antibodies in animals are complex due to the similarity with that of the animal Galili antigen (Galili et al, 1985). B+biotin kodecytes were used as a source of compatible transfusion for baseline experiments but were not used in further experiments.

# CHAPTER 3 - MEASUREMENT OF TRANSFUSED RED CELL SURVIVAL IN MICE

The suitability of animal models in research on transfusion reactions is always limited by the dissimilar biological/clinical equivalence to antigen-antibody responses of man, and a lack of similar human-like antigen-antibody systems. The presence of haemoglobinuria due to intravascular red cell lysis and the release of haemoglobin into the plasma can be used as a measure of transfused red cell survival. However, red cells can be removed from the circulation and cleared by the reticuloendothelial system or phagocytosis before haemolysis occurs (Mollison, 2005) and measurement of incompatible red cell transfusion reactions in small anaethetised animals is difficult. The detection of haemoglobinuria was evaluated as a potential method for determining acute intravascular haemolysis. The use of FSL constructs was also evaluated as an alternative method to measure cell survival.

## 3.1 Determination of red cell survival by detection of haemoglobinuria

***Method Overview***

In order to detect haemoglobinuria after an incompatible transfusion reaction, a trial was undertaken by injecting mice with a pre-haemolysed suspension of mouse cells to determine the level of haemolysis required for visual detection (Protocol 3 V2).

Haemolysed (frozen-thawed) packed red cells were infused into 6 anaesthetized mice. Five mice were sacrificed at 5, 15, 30, 45 and 60 min and bladders inspected. The 6$^{th}$ mouse was allowed to urinate on the filter paper before necropsy and urine colour was noted. The volume of blood and urine obtainable from mice was very limited and so optical measurements were not done.

***Results***

Bladder urine samples of the first 5 animals showed haemoglobinuria ranging in color from pink at 5 min to deep red after 15 minutes. Red urine was clearly observed on the filter paper of the 6$^{th}$ mouse. As 40 µL of haemolysed red cells represents 2/3rds of a 100 µL transfusion of 60% kodecytes, the detection of pink/red colored urine on filter paper for up to 2 hours post transfusion was considered indicative of an acute intravascular hemolytic

transfusion reaction. The detection of haemoglobinuria, although a method for determining acute intravascular haemolysis, was unreliable and non-quantitative in this study.

***Conclusion***

Although haemoglobinuria was a detectable endpoint for massive haemolysis, it was not accurate enough for measuring kodecyte survival in mice and required sacrificing the mice or trying to quantify the passed urine on filter paper. In later experiments (4.3), with intact A+biotin kodecytes or FSL-A infusions in mice, there were no observable symptoms, including hemoglobinuria as detected as passed urine on filter paper, regardless of the presence or absence of anti-A. The failure to detect haemoglobinuria in the kodecyte transfusions, despite their rapid clearance (within 6 minutes) may have been due to rapid clearance of the released haemoglobin. This is in contrast to the experiment described above, which used free haemoglobin. This method to detect haemoglobinuria was not an appropriate assay for red cell (kodecyte) destruction and was abandoned.

## 3.2 Red cell survival studies using biotin kodecytes

The use of FSL-biotin was investigated as a method to determine cell survival with the creation of biotin-kodecytes for cell survival measurement by fluorescent microscopy with avidin/Alexaflor 488 binding. Once attached to the red cells of laboratory animals, the biotin kodecytes provided a source of distinctly identifiable red cells to obtain baseline and control data. Other FSL constructs, such as FSL-A, FSL-B and FSL-GB3, can also be inserted with FSL-biotin, and used to create compatible and incompatible kodecytes. In all cases, the kodecytes were prepared from the same in-bred mouse red cells, by the same process, and therefore the only foreign structures on these kodecytes were those introduced by the FSL construct.

***Method Overview***

Based on an estimated blood volume of 1.4 mL and hemocrit of 0.45 the transfusion of 20μL of kodecytes was estimated to represent 3.2% of circulating red cells (Hoff, 2000).

FSL-biotin kodecytes were prepared for transfusion (Protocol 2) and transfused into anti-A negative (8) and anti-A positive mice (8) (Protocol 3). Cell survival was measured at time periods 0.1h-96hr (Protocol 2 V4). The blood sampling time at 6 minutes (0.1h), post-

transfusion or infusion was chosen as it has been established that within 6 minutes, blood has had sufficient time to be evenly distributed in the circulation (Strumia et al., 1958; Mollison, 1989). The animal was then surgically closed and monitored. At 2, 8, 24, 48, 72, and 96 h intervals, a 50 µL blood sample was collected into a heparinized (and sometimes an extra plain sample) capillary tube from the tail vein. The presence of A+kodecytes was determined with gel card serology and monoclonal anti-A reagent. Kodecyte survival was determined by the detection of kodecytes via their biotin label and *in vitro* staining with an avidin fluorophore and fluorescent microscopy.

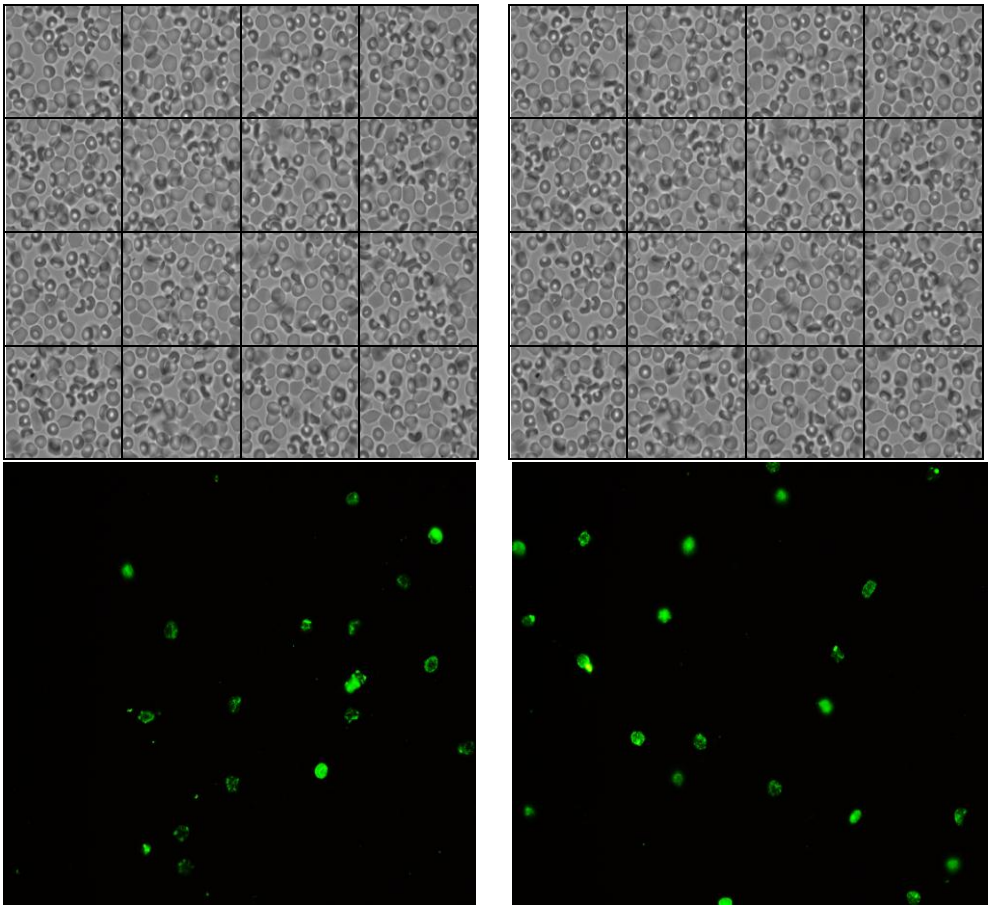
***Results***

Biotin kodecytes from compatible mouse red cells were transfused into naive and anti-A positive mice, and their *in vivo* survival was determined with microsampling and subsequent fluorescence visualization of the kodecytes in blood films. Ten random fields (approx 6000 cells) examined where the cells were in a monolayer, were photographed under both differential interference contrast, then 488 nm excitation fluorescence microscopy at 200x magnification (Figure 19). Blinded paired photographs were then used to count fluorescent kodecytes and non-fluorescent red cells in order to calculate the percentage of kodecytes present. The red cell count was obtained using a grid. The cells in four corner squares were counted and the number multiplied by four. All the squares were examined to get the total fluorescent cell number present. Ten photographs were examined in this way. The total number of flurorescent cells was divided by the total number of red cells and multiplied by 100 to get a percentage.

The fluorescence subsequent to *in vitro* fluorescence labeling of biotin kodecytes gradually diminished in intensity over time, and after day 5 became unreliable to recognize. At the labeling concentration used, and assuming a straight-line loss, this represents about a 20% loss per day of FSL constructs from the kodecyte. The elution of FSL-biotin constructs to the surroundings does not affect the kodecyte count, because the kodecytes are still identifiable, albeit with decreased strength in their fluorescence signal.

The averaged survival count for compatible biotin-kodecytes was at 0.1h (count 2.1%), at 2h (count 1.9%), at 8h (count 1.8%),at 24h (count 1.5%), at 48h (count 1.6%), at 72h (count 1.1%) and at 96h (count 1.0%) while in anti-A positive mice survival count was at 0.1h (count 2.3%), at 2h (count 1.9%), at 8h (count 1.9%), at 24h (count 1.8%), at 48h (count 1.6%), at 72h (count 1.1%) and at 96h (count 1.1%). Comparisons of survival

percentages in naive and anti-A positive mice infused with compatible biotin-kodecytes were made (Appendix B-1). There was no significant statistical difference between the results of the biotin-only kodecytes transfused into anti-A positive mice compared with the same kodecytes transfused into anti-A negative mice, p = 0.46.



**Anti-A negative (mouse 6414) transfused with compatible biotin kodecytes**

**Anti-A positive (mouse 6352) transfused with compatible biotin kodecytes**

Figure 19. Example of the survival assay photographs and the grid used to count cells.

This figure shows surviving biotin-kodecytes at 0.1hr in anti-A negative and positive animals. Upper images are (DIC) light microscopy while the lower image is the same frame but viewed under fluorescence to visualize the biotin-kodecytes as labeled with avidin Alexa Fluor® 488 (200 x magnification). In the anti-A negative mouse (6414) 19 surviving kodecytes can be seen by fluorescence while approximately 960 cells can be seen by DIC. The percentage of surviving kodecytes is therefore 19/960 which is 2.0%. 10 similar images were counted for each sample and the totals combined to give % kodecyte survival.

***Conclusion***

The use of FSL biotin kodecytes to measure cell survival proved to be a successful new method for estimation of cell survival in a compatible transfusion. In the majority of compatible animals there was an apparent immediate drop in kodecyte count as measured at time point 0.1h (6 minutes) versus the maximum theoretical count (3.2%). This either represents an immediate loss of kodecytes and/or errors in blood volume estimation and/or differences between animals. Kodecytes are still identifiable regardless of elution of FSL-biotin constructs into the surroundings, although with less intense fluorescence, but they probably should only be used to determine cell survival within the first 24 hours. From a survival perspective this is adequate, as Mollison states, “In practice, knowledge of the percentage survival at 24 hours makes it possible to predict how the whole population will survive and there is therefore little reason to continue assays beyond this time” (Mollison, 2005).

## 3.3 Retrieval of surviving transfused biotin kodecytes and detection of A antigen on kodecytes

Transfused kodecytes were recovered from the circulation via their biotin label and analysed for the presence of A antigen. This was done in order to establish that the A antigen label remained in the membrane post transfusion.

***Method Overview***

To determine method sensitivity, an *in vitro* experiment using a series of suspensions created by spking whole blood with A+biotin kodecytes. For *in vivo* experiments, about 50 µL whole blood was collected from mice by tail vein nick (Protocol 1.4) taken at 24h and 48h post kodecyte transfusion. Empty gel cards were refilled with PBS-washed avidin agarose beads and a sample of the whole blood was directly applied to the top of the gel. Following centrifugation, the kodecytes were separated from non-kodecytes – the bound kodecytes being contained in the upper two-thirds of the supernatant and the unbound cells from the bottom of the gel. The kodecytes were then dislodged from the avidin beads by vortex. All cells were resuspended in saline and tested for the presence of A antigen (Protocol 6).

***Results***

*In vitro spiked samples*

Avidin agarose beads in gel cards were used to demonstrate recovery of A+biotin kodecytes from blood samples with measured cell survival. Passage of 5 µL of whole blood sample containing between 1% and 5% A+biotin kodecytes through an avidin agarose column was shown to be able to isolate the kodecytes from the endogenous cells (Figure 20). Kodecytes when recovered and dislodged from the avidin gel could be shown as blood group A antigen positive, while the endogenous non-kodecyte population recovered from the bottom of the gel were A antigen negative. The FSL construct was physically ripped from the cell membrane, without disrupting the membrane (there was no evidence of haemolysis). It would be expected that the avidin-biotin bond would be unbroken. The isolated kodecytes and non-kodecytes when analysed in the anti-A column, (Figure 20), produced only single bands, indicating that the A+biotin kodecytes were all A antigen positive, and that the non-FSL biotin modified population were all A antigen negative, that is the two populations could be separated completely.

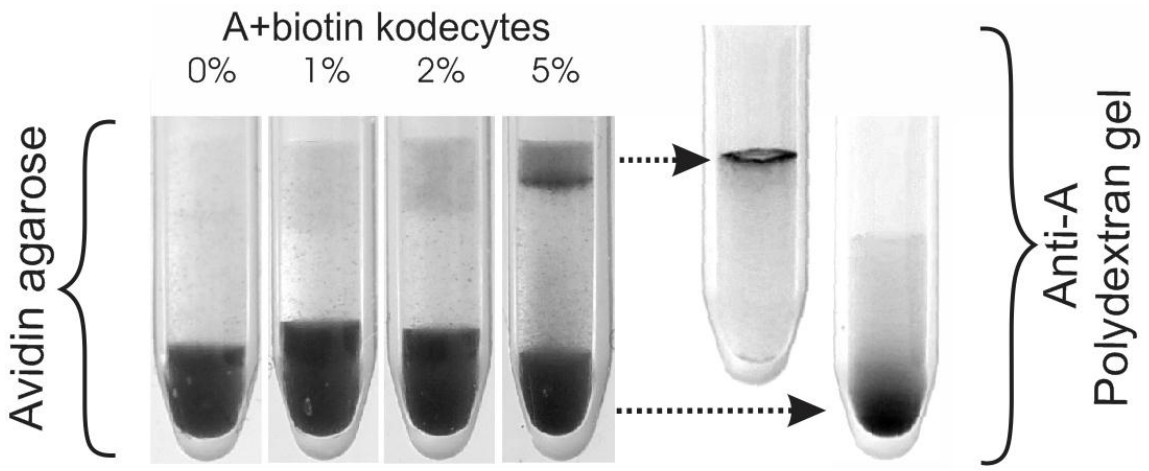



Figure 20. The recovery of A+biotin kodecytes taken from spiked whole blood on avidin agarose gel.

5 µl of whole blood containing A+biotin kodecytes was separated by centrifugation in avidin agarose cards resulting in the capture of A+biotin kodecytes via FSL-biotin in the upper of the avidin gel. If both cell populations are recovered from the avidin gel and then reacted in a blood grouping card containing anti-A, then the A+biotin kodecytes are captured via FSL-A

*In vivo circulating compatible kodecytes*

About 50 µl whole blood was taken by tail vein collect at 24h and 48h post transfusion from four mice transfused with A+ biotin kodecytes (Protocol 1.4). The whole blood was layered into agarose gel as for *in vitro* method described above and when released from the avidin beads could be shown to be A antigen when reacted on with monoclonal anti-A in a gel card (Figure 21).

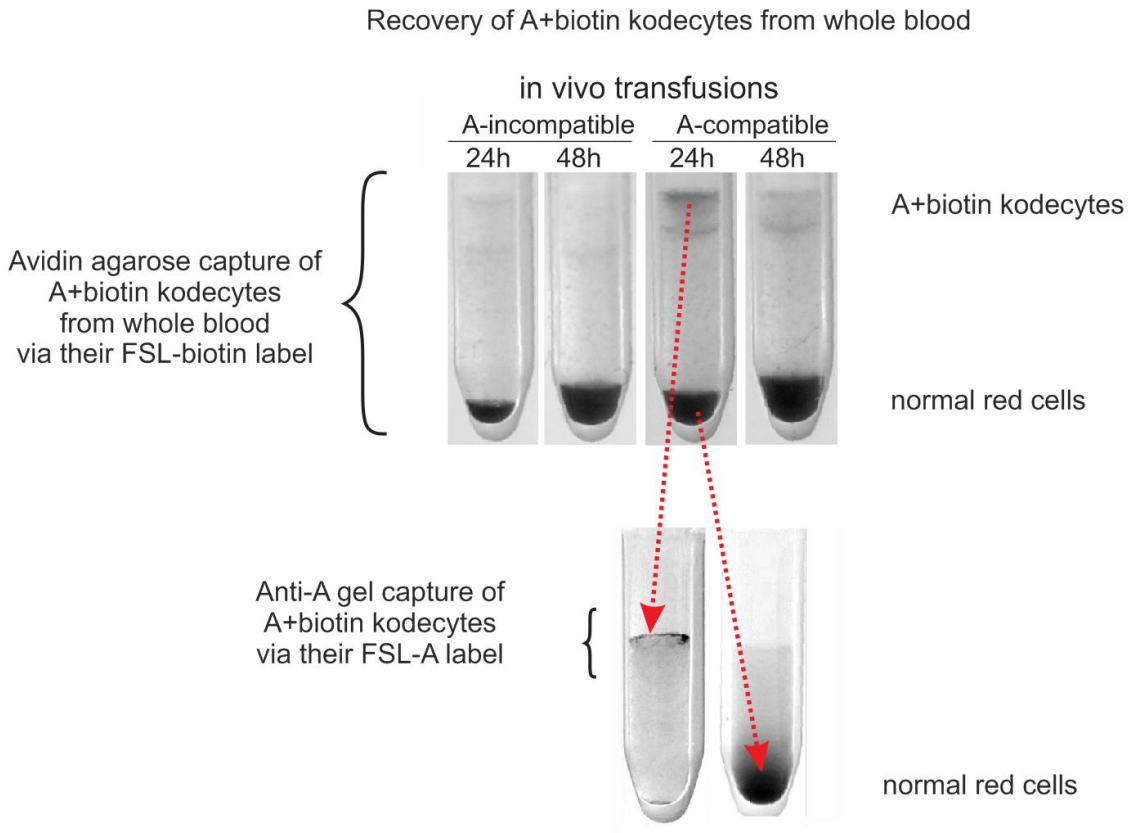



Figure 21.The recovery of A+biotin kodecytes from post transfusion whole blood sample, separated on avidin agarose gel

***Conclusion***

*In vitro* and *in vivo* kodecytes recovered and dislodged from the avidin gel could be shown to be blood group A antigen positive, while the endogenous non-kodecyte population recovered from the bottom of the gel were A antigen negative. This method had the potential to be used for analyzing recovered kodecytes but was not used further in this work.

# CHAPTER 4 - KODECYTE TRANSFUSION AND SURVIVAL

This chapter investigates the use of kodecytes for cell survival measurement in antibody compatible and incompatible mice. In this study, two series of mice, one immunized to be anti-A positive and the other naive (no anti-A), were tested with both compatible and incompatible kodecytes. Studies of red cell survival currently depend on methods adding a traceable marker to an aliquot of cells. According to Mollison (Mollison, 2005), the perfect marker would label only the red cell, adhere tightly and unchanged for the duration of the study, prove non-toxic to the cell and recipient, lack immunogenicity, be of no radioactive risk to the patient, inexpensive and easy methodology. Incompatablitily was created with FSL-A constructs while the ability to measure cell survival was via the biotin construct as previously described (3.2).

## 4.1 Survival of 3.2% kodecyte transfusion in compatible mice

***Method Overview***

Baseline experiments with compatible kodecytes was undertaken to establish the baseline for normal kodecyte survival. Kodecytes were prepared for transfusion (Protocol 2 V1 and 3). GB3+biotin, and biotin kodecytes were transfused into both anti-A negative and anti-A positive mice. A+biotin kodecytes were only transfused into anti-A negative mice (Protocol 3 V7). Based on an estimated mouse blood volume of 1.4 mL and hemocrit of 0.45 the transfusion of 20μL of packed kodecytes was estimated to represent 3.2% of circulating red cells (Hoff, 2000). Anaesthetized mice were given transfusions of 200 μL 20% suspension A+biotin kodecytes, (3.2 % of red cell mass). Cell survival was measured at time periods from 6 minutes to 96hr (Protocol 2 V4). All mice had anti-A status confirmed before experimentation commenced.). Standard deviations and one-way ANOVA p values were calculated using the SPSS Statistics v17 package.

***Results***

Twenty-nine different compatible kodecyte transfusions were tested to establish the baseline of kodecyte survival. Using fluorescence microscopy of blood films, biotin only and GB3+biotin compatible kodecyte transfusions showed similar survival rates and were present in the circulation at >72 hours in naive and anti-A positive mice (Figure 22).

Kodecyte survival stabilized in all controls to a similar constant reduction over the time period. The similar curves of the survival of biotin-only kodecytes with the GB3+biotin kodecytes transfused into anti-A positive and negative animals clearly showed that dual kodecyte labeling did not influence survival outcomes (Figure 22).

There was no significant difference between the survival results for the compatible biotin-only kodecytes (n = 16) compared with dual labeled biotin+GB3 kodecytes (n = 5) in anti-A positive ($p = 0.7$) and negative mice ($p = 0.9$). Percentage survivals in anti-A negative mice with compatible biotin/GB3+biotin kodecytes having a 0.1h count of 2.3% reducing to 91% at 2h (count 2.1%), 71% at 24h (count 1.8%), 68% (count 1.8%), at 48h (count 1.4%), and 50% at 72h (count 1.4%) (Appendix B-1, 2) while the values for biotin/GB3+biotin in anti-A positive mice at 0.1h had a count of 2.4% reducing to 86% at 2h (count 2.1%), 71% at 24h (count 1.7%), 65% at 48h (count 1.7%) and 61% at 72h (count 1.3%) (Appendix B-1, 2).

Using the averaged results from A+biotin kodecytes transfused to compatible anti-A negative mice there was an initial drop in kodecytes from the 0.1h count of 3.1% to about 88% at 2 h (count 2.7%), then a further reduction to 71% at 24h (count 2.2%), 61% at 48h (count 1.9%) and 52% at 72h (count 1.6%) (Appendix B-4). The survival curve is compared to the curve for the biotin+GB3 kodecytes (Figure 22).

A+biotin kodecytes transfused into anti-A negative animals gave similar outcomes to the benign FSL-GB3/ biotin antigen controls (Appendix B-5), indicating that the blood group A antigen *per se* does not result in reduced cell survival. However, comparison of the biotin-only and GB3+biotin kodecytes (n=21) with the A+biotin kodecytes (n=8) transfused to the compatible anti-A negative mice resulted in a significant difference ($p = 0.05$) was believed to be due to differences in post transfusion hematocrits in the A+biotin transfusions (Figure 22).

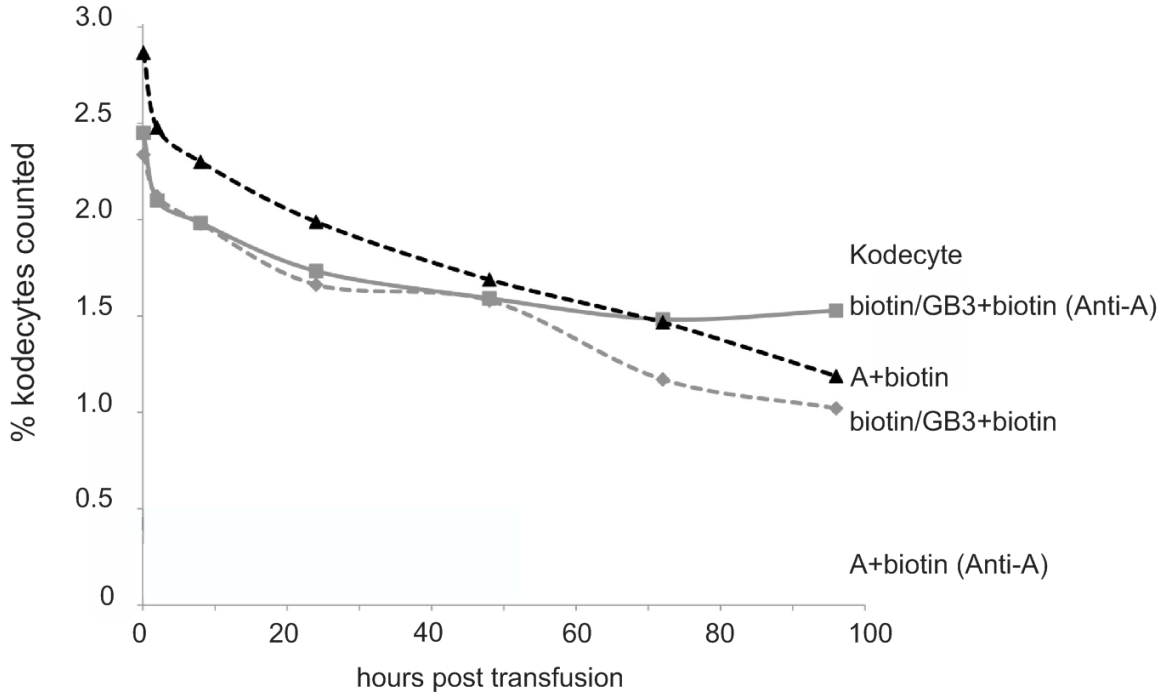



Figure 22. Average kodecyte survival percentages post compatible transfusion.

Line diagram compares trends over time. Compatible A+biotin kodecytes transfused into anti-A negative animals (n= 7,dashed black line) gave similar results to biotin/GB3+biotin control kodecytes in the presence of anti-A (n = 8, solid grey line) or absence of anti-A (n = 8, dashed grey line).

### *Conclusion*

FSL constructs used to make kodecytes with which to measure cell survival was shown to be a novel way to visualise cell survival after compatible transfusion. The similarity between all curves suggests that dual FSL labeling is of no consequence to kodecyte survival in antibody compatible animals. In the majority of compatible animals there was an apparent immediate drop in kodecyte count as measured at time point 0.1h (6 minutes) versus the maximum theoretical count. This either represents an immediate loss of kodecytes and/or errors in blood volume estimation and/or differences between animals.

The shape of these compatible cell survival curves is in concordance with the literature but compatible kodecytes were lost at a rate of approximately 20% per day. It is possible this increased loss was due to the use of an in vitro cell storage solution for storing cells for transfusion and/or changes in their shape - a significant factor in determining in vivo RBC survival. It remains to be established if kodecytes would have a better survival if stored in transfusion media or if they would have a reduced survival - only a comparison of two populations, one labeled with FSL-biotin and the other with $^{51}$Cr would be able to establish the comparative merits.

## 4.2 Survival of 3.2% kodecyte transfusion in incompatible mice

***Method Overview***

A+biotin kodecytes were prepared for transfusion (Protocol 2 V1 and 3) and twelve anaesthetized, immunized anti-A positive mice were given transfusions of 200 µL 20% suspension A+biotin kodecytes, (3.2 % of red cell mass) (Protocol 3 V7). Cell survival was measured by fluorescence over a time period of 0.1h-96 h (Protocol 2 V4).

***Results***

Twelve confirmed anti-A positive mice were given transfusions of A+ biotin incompatible kodecytes. Using fluorescence microscopy of blood films, A+biotin kodecytes were shown to be present in the circulation at >72 hours in naive mice but were mostly cleared within 6 minutes in anti-A immunized mice (Appendix B-3). Two animals with either weak or no response to immunization with A to saliva immunization were given transfusions but their survival rate data was not included in the survival curve data. Mouse ID 4998, with a weak anti-A response, appeared to have an intermediate survival rate between anti-A negative and positive animals; while the animal that was negative, ID 0232 showed an anti-A compatible survival profile (Appendix B-3).

Recovery of a blood sample at 0.1h indicated an 86% loss of kodecytes compared with that expected from the same A+ biotin kodecytes (about 12%) transfused into antibody compatible animals (0.4% compared with 3.1%, $p < 0.001$) showing a very significant difference in cell survival (Figure 23). Data for compatible animals is found 4.1.

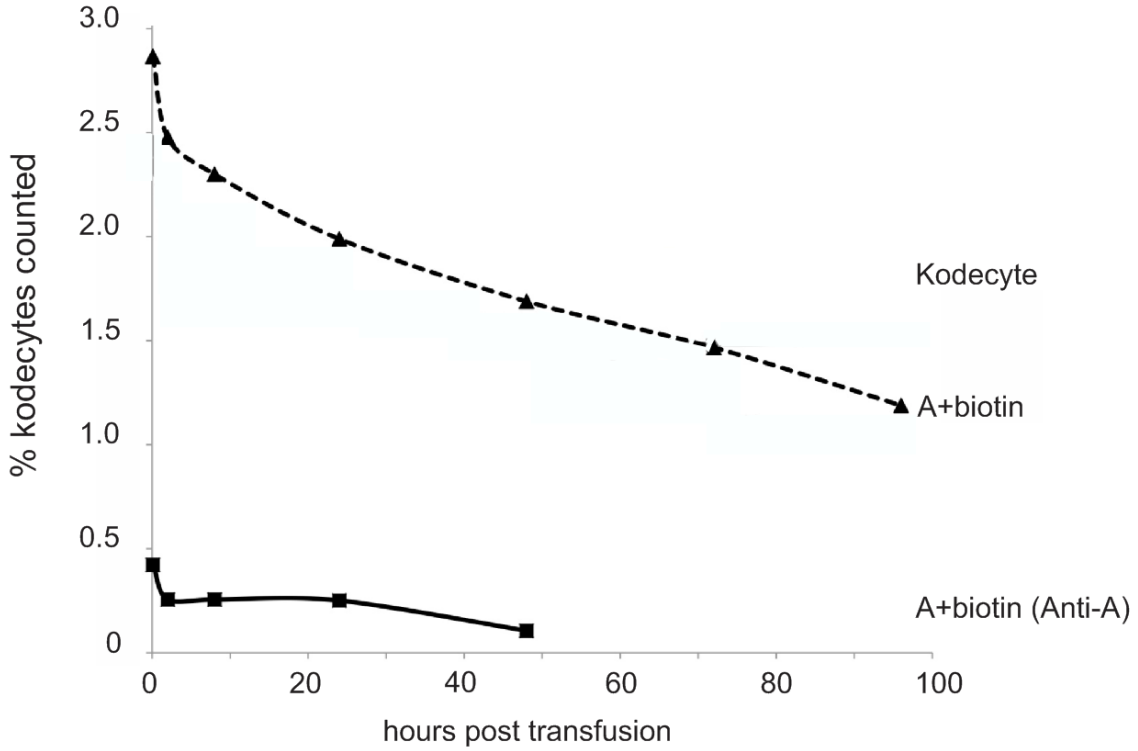



Figure 23. Average kodecyte survival percentages post compatible and incompatible transfusion.

Line diagram compares trends over time. Compatible A+biotin kodecytes transfused into anti-A negative animals (n= 7, dashed black line) compared results with A+ biotin transfused into anti-A positive animals (n = 12, solid black line).

When A+biotin kodecytes were transfused into anti-A positive animals, a rapid reduction in circulating kodecyte count occurred within 6 minutes, appeared to stabilize, and then reduced at the same rate as compatible kodecytes. There was a statistically significant difference, $p < 0.001$, between the survival of the 3.2% transfusion of compatible A+biotin kodecytes in naive (Figure 24 - white bars) and incompatible anti-A positive mice (Figure 24 - black bars). There was no visible evidence of free hemoglobin in the plasma or urine.

Subsequent to the initial dramatic loss of kodecytes the survival rate of non-eliminated kodecytes paralleled that of compatible kodecytes, although there were no detectable kodecytes at 96 hours. It is established that the amount of FSL on the transfused kodecytes in this setting is inadequate to cause any significant anti-A neutralization since anti-A positive animals tested remained anti-A positive after transfusion.

The comparison of the kodecyte survival in compatible and incompatible mice is shown (Figure 25).

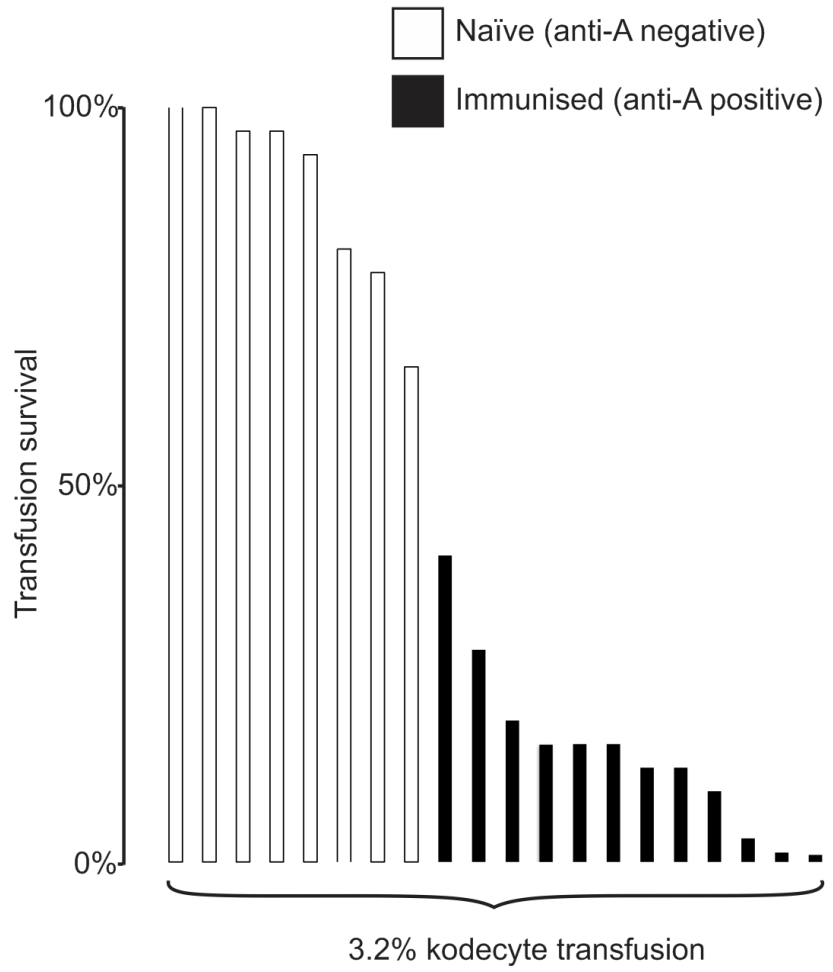



Figure 24. Comparison of cell survival in transfusions of 20 µL (3.2 % of the total red cell mass), with compatible and incompatible A+biotin kodecyte in individual mice.

Murine A+biotin kodecytes were transfused to mice without anti-A (white bars) or with anti-A (black bars)

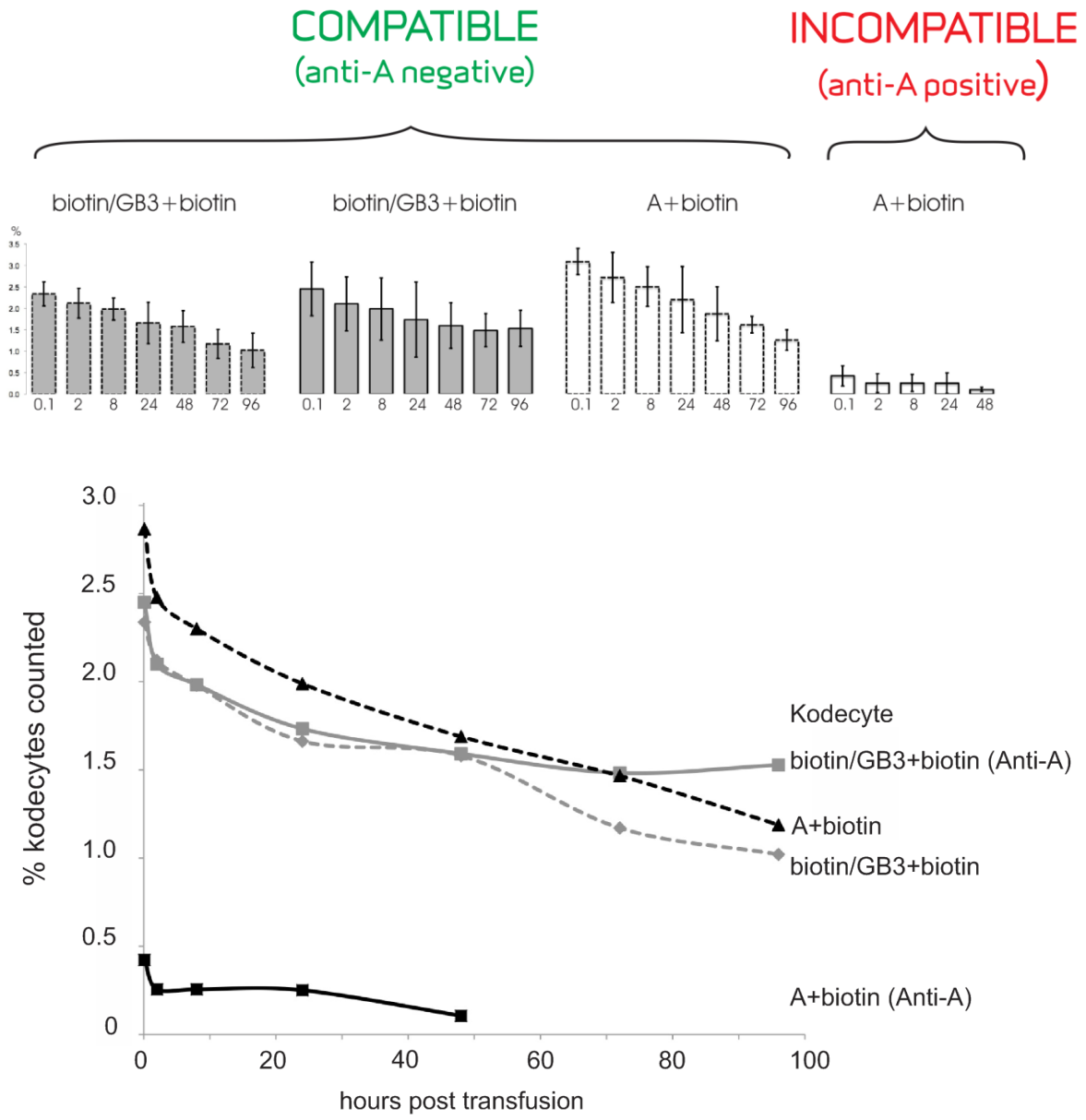
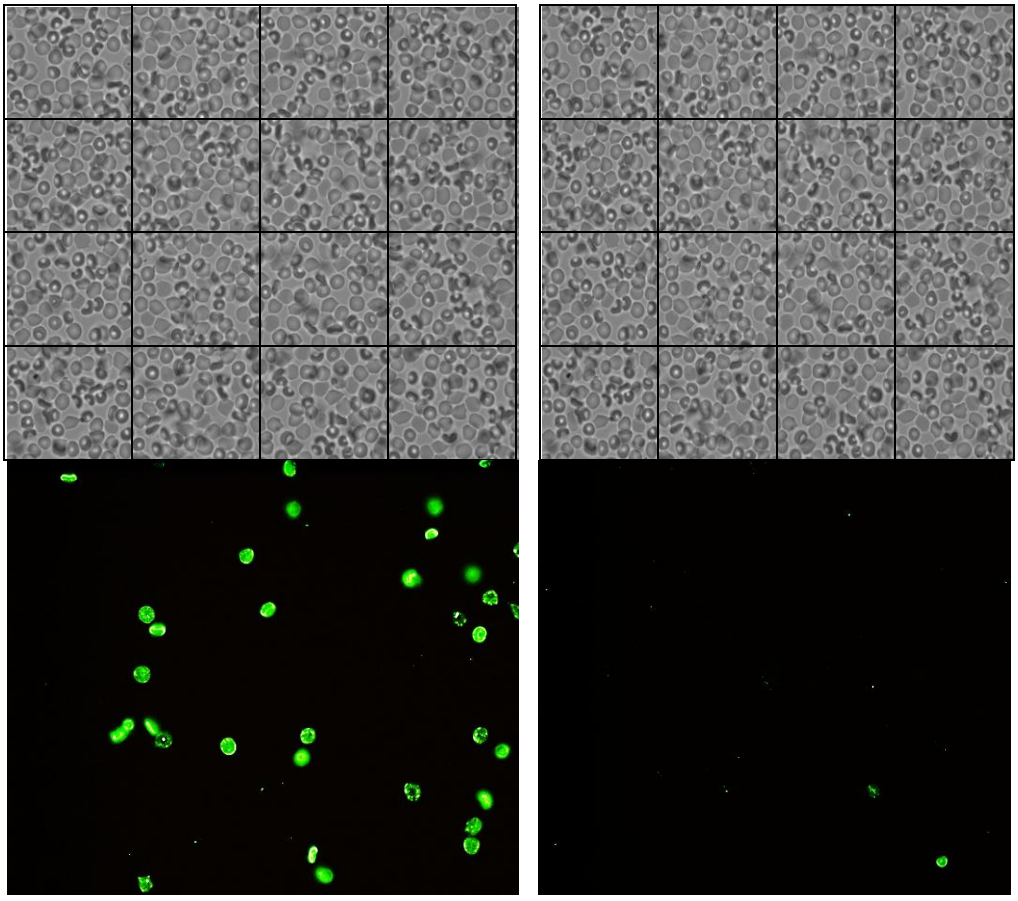



Figure 25. Average kodecyte survival percentages post transfusion.

Upper bar graphs indicate average results (with 1 SD error bars) obtained at each sampling time point. Line diagram compares trends over time. Compatible A+biotin kodecytes transfused into anti-A negative animals (n= 7, dashed black line) gave similar results to the biotin/GB3+biotin control kodecytes in the presence (n = 12, solid grey line) or absence of anti-A (n = 8, dashed grey line). In contrast incompatible A+biotin kodecytes transfused to anti-A positive animals immediately dropped to near baseline values (n = 12, solid black lines)

Photographs (Figure 26) show 30 fluorescent kodecytes visible in the field in compatible anti-A negative mice (ID#5811) but only 2 kodecytes are present in incompatible anti-A positive mice (ID#3809) at 0.1hr post transfusion.



**Anti-A negative (ID 5811)transfused compatible A+biotin kodecytes**

**Anti-A positive (ID 3809)transfused incompatible A+biotin kodecytes**

Figure 26. Example of A+biotin kodecyte survival assay photographs.

Figure shows surviving A+biotin kodecytes at 0.1hr in anti-A negative and positive animals. Upper images are (DIC) light microscopy while the lower image is the same frame but viewed under fluorescence to visualize the A+biotin kodecytes as labeled with avidin Alexa Fluor® 488 (200 × magnification). In the anti-A negative mouse (ID 5811) 30 surviving kodecytes can be seen while in the anti-A negative mouse (ID 3809) only 2 kodecytes are seen. 10 similar images were obtained for each sample and total kodecytes and red cells present are counted in the four corner cells of a grid as shown in Figure 20, and the count was then multiplied by 4 and calculated as a percentage of kodecytes / total red cells counted.

***Conclusion***

Anti-A positive mice were transfused with incompatible A+biotin kodecytes. Most of the transfusions showed an immediate reduction, probably representing a loss of the “fragile tail”, but the incompatible transfusions showed rapid destruction not seen with compatible transfusions. Data collected at additional time points up to 48 hours was no more informative than that derived at 6 minutes, a time period sufficient to allow for full circulatory distribution. The survival curve of the A+biotin kodecyte incompatible transfusion compared with that of the anti-A negative compatible transfusion was significant with clearly rapid kodecyte removal in the former and in accord with literature predictions of incompatible survival curves. The use of A+biotin kodecytes proved to be novel way to create incompatibility and to measure cell survival in anti-A positive mice.

## 4.3 Antibody status post kodecyte transfusion

***Method Overview***

Twenty-eight anti-A positive mice and eighteen anti-A negative mice were used in the cell survival studies. Two immunized with weak or no response to immunization were also transfused. All were tested for anti-A; some for anti-B, anti-GB3 and anti-biotin against the appropriate FSL antigens (Protocol 5). 13/27 anti-A positive samples were also tested for IgG anti-A and 10/27 samples also had Ig anti-A level measured by titre (Protocol 5 V1).

***Results***

All 18 naive, un-immunised mice were anti-A negative while 28 immunised mice were Ig anti-A positive. There were two immunized animals whose sera were found to be anti-A negative or very weak positive were included in the kodecyte survival study but not used in the calculation of averages for subsequent data. All naive animals negative for anti-A with the anti-Ig conjugate if tested were also negative with the anti-IgG conjugate and 14 of those positive for Ig anti-A were also IgG anti-A positive. Five were negative suggesting these animals did not have IgG anti-A as detected on inkjet wells. However, A+biotin kodecyte survival at 0.1h in these animals without detectable IgG anti-A was no different to that of IgG anti-A positive animals (Appendix B-3).

All animals, including compatibly transfused animals, were retested for anti-A status between 3 and 15 days post transfusion and in all instances the anti-A status was

unchanged from the pre-transfusion result. Serum from 10/28 anti-A positive, kodecyte transfused mice was tested to find the titre of anti-A produced. All were >128.

Twenty of the twenty-eight biotin-kodecyte transfused mice were tested for anti-biotin and were all negative. Five of the B+biotin kodecyte transfused mice were tested for anti-B and five of the GB3+biotin kodecyte transfused mice were tested for anti-GB3. There was no antibody produced to any of the FSL constructs (Table 7).

Table 7. Antibodies measured in anti-A positive and anti-A negative mice after exposure to kodecytes.
No antibodies were produced in response to kodecyte infusion. All sera anti-A positive pre transfusion remained positive and titres were >128 in the serum tested. IgG was present in 14/19 anti-A Ig sera tested.

**Antibodies detected in mice after kodecyte infusion**

| | | Intravenous injection | | | | Analysis | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mice | | Kodecytes * | | | | serum antibodies post kodecyte infusion | | | | | | |
| Anti-A positive | Anti-A negative | A+biotin transfusion | B+biotin | Biotin | GB3+biotin | Anti-A Ig | Anti-A IgG | Anti-A Ig titre >128 | Anti-B | Anti-biotin | Anti-GB3 | % Total Anti-A Ig |
| 5 | | + | | | | 5 | 0 | 4 | | | | 100 |
| 6 | | + | | | | 6 | 6 | 6 | | | | 100 |
| 1 | | + | | | | 1 | | | | | | 100 |
| 4 | | | + | | | 4 | 2 | | 0 | | | 100 |
| 8 | | | | + | | 8 | 4 | | | 0 | | 100 |
| 4 | | | | | + | 4 | 2 | | | | 0 | 100 |
| | 8 | + | | | | 0 | | | | 0 | | 0 |
| | 1 | | + | | | 0 | | | 0 | 0 | | 0 |
| | 8 | | | + | | 0 | | | | 0 | | 0 |
| | 1 | | | | + | 0 | | | | 0 | 0 | 0 |

*20 µL A+biotin, B+biotin, GB3+biotin or biotin kodecytes.

***Conclusion***

Infusion of A+biotin kodecytes did not initiate the production of anti-A in anti-A negative mice, and infusion of B+biotin or GB3+biotin, or biotin-kodecytes did not initiate anti-B, anti-GB3 or anti-biotin production in either anti-A positive or anti-A negative mice. Kodecytes used in this study do not appear to be immunogenic.

# CHAPTER 5 - NEUTRALIZATION OF ANTI-A WITH FSL-A

This chapter explores the possibility that intravenous FSL construct (FSL-A) can neutralize anti-A in the circulation of mice, thus allowing incompatible transfused cells (A kodecytes) to survive. A two-pronged experimental approach was taken firstly to obtain *in vitro* neutralization data anti-A in human serum and the second to neutralize anti-A *in vivo* in mice with FSL-A.

## 5.1 The use of FSL-A *in vitro* to provide baseline data for neutralization studies

The first series was designed to generate *in vitro* data with human samples that could be extrapolated to a human setting and guide the murine *in vivo* experimentation. Three human samples (volunteers) were used as a source of red cells and plasma/serum. Red cells were obtained from an ALe(a-b+) human donor. The group O serum sample was a "high titre IgG" anti-A, with activity of 1024, while the B serum sample had an IgG anti-A titre of 256. Both O and B samples had IgM anti-A titres of 64.

As only blood group A incompatibility was studied, antibody activity against A antigen is defined simply as anti-A, which, in the case of the O serum, also encompasses anti-A,B.

This series investigated five uses of FSL-A.

1. The ability of FSL-A to insert into cell membranes at varying concentrations in the presence of anti-A in serum was compared with its ability to insert in the absence of serum or anti-A.
2. The possibility that anti-A present in the O serum may sensitize the A kodecytes during transformation was evaluated.
3. The ability of FSL-A to inhibit anti-A activity against $A_1$ red cells.
4. The ability of FSL-A to dissociate anti-A from the cells was evaluated over time after blood group $A_1$ red blood cells were sensitized with serial dilutions of group O serum containing anti-A.

### 5.1.1 *In vitro* kodecyte formation in serum versus PBS.

***Method Overview***

Washed, packed group O human red cells (200μL) were mixed with an equal volume of either blood group O serum, A serum, or PBS, each of which had been spiked with decreasing concentrations of FSL-A (final concentration range 4.0 – 0.025 mg/mL) (Protocol 4 V1 ). Transformation in serum was compared with PBS (the usual *in vitro* FSL transformation diluent) in order to determine the amount of FSL-A which would probably insert into red cells *in vivo*. Mice red cells were also tested in PBS. Serology was in NaCl (saline only) gel cards.

***Results***

FSL-A in PBS could be shown to have inserted into red cells, within 6 min, with concentrations as low as 0.10 mg/mL (Table 8). Increasing the incubation time up to 2 hours increased the amount of construct inserted, but after that no further transformation was evident. Based on these and other unpublished results >95% of FSL-A in PBS has inserted into the red cells at 2 hours (Table 8). The results were identical when tested with mice red cells in PBS.

The transformation of red cells with FSL-A in serum was substantially less and slower. In contrast to FSL-A in PBS, which at 6 minutes and a concentration of 0.1 mg/mL had achieved 4+ serology, serum with a 40 × higher concentration of FSL-A (4 mg/mL) could only achieve a 1+ reaction. At 1 hour FSL-A in serum at the 4.0 and 2.0 mg/mL levels had resulted in strong A antigen expressing kodecytes, and by 8h could be seen down to 1.0 mg/mL concentration.

Table 8.*in vitro* transformation of human red cells into A kodecytes with FSL-A dilutions in PBS or O serum.

| | **A kodecyte serology using monoclonal anti-A (level of transformation)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | RBC contact time (h) with FSL-A in PBS | | | | | RBC contact time (h) with FSL-A in O serum | | | | |
| FSL-A (mg/mL) | 0.1 | 1 | 2 | 4 | 8 | 0.1 | 1 | 2 | 4 | 8 |
| 4.0 | ++++ | ++++ | ++++ | ++++ | ++++ | + | ++++ | ++++ | ++++ | ++++ |
| 2.0 | ++++ | ++++ | ++++ | ++++ | ++++ | + | +++ | +++ | +++ | ++++ |
| 1.0 | ++++ | ++++ | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | +++ |
| 0.5 | ++++ | ++++ | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 |
| 0.25 | ++++ | ++++ | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 |
| 0.10 | ++++ | +++ | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 |
| 0.05 | 0 | + | +++ | +++ | ++++ | 0 | 0 | 0 | 0 | 0 |
| 0.025 | 0 | 0 | +++ | +++ | +++ | 0 | 0 | 0 | 0 | 0 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

### *Conclusion*

Transformation in serum was substantially less and slower than in PBS. Assuming the FSL-A in serum result achieved with 1.0 mg of FSL-A at 8 hours is at completion and equivalent to the 0.025 mg/mL of FSL-A in PBS result, these results suggest that only 2.5% of the FSL-A in serum inserts into cells compared with >95% when the FSL-A is in PBS. If the O serum was substituted for A serum then the results were identical, indicating that the presence of anti-A does not influence the transformation process.

### 5.1.2 *In vitro* antibody sensitization of A kodecytes during transformation

***Method Overview***

*In vitro* kodecytes were prepared with group O serum spiked with 4.0, 2.0, 1.0 and 0 mg/mL of FSL-A. Kodecyte formation was confirmed with monoclonal anti-A. Sensitization of the A kodecytes with IgM or IgG anti-A, which occurred during transformation, was measured at the various time intervals in saline IgM (detection) and anti-IgG+C3d gel cards, immediately following the washing step. In addition the washed A kodecytes from each time point were subsequently incubated for 15 minutes at 37°C with unspiked group O serum, to determine their ability to be sensitized with IgG and/or IgM anti-A present in the untreated O serum (Protocol 4 V2). Serology was in LISS/Coombs (low ionic strength saline with anti-IgG+anti-C3d) and NaCl (saline only) gel cards.

***Results***

The possibility that anti-A present in the O serum may sensitize the A kodecytes as they formed was evaluated (Table 9). When FSL-A concentrations in serum were greater than 1.0 mg/mL then A kodecytes resulted, in agreement with previous results (Table 8). At all time points during the transformation in group O serum (1-8 hours) at no stage could either IgM or IgG be detected on the A kodecytes. To ensure the kodecytes formed were reactive with the anti-A in the O serum, untreated serum (without FSL-A) was tested against the washed A kodecytes. Positive serology due to IgG was present on all A kodecytes. Despite the IgM monoclonal anti-A reagent reacting with the A kodecytes, the IgM anti-A activity of the untreated serum (titre 64 at 37°C) was negative. This is probably due to the relative low density of FSL-A antigens on *in vitro* kodecytes when created with FSL-A in serum (it can be shown that this O serum will react strongly with *in vitro* kodecytes if formed in PBS).

***Conclusion***

These results show that IgG anti-A is capable of reacting with the A kodecytes, but the high level FSL-A in serum required to induce the creation of kodecytes was more than adequate to also neutralize the serum IgG anti-A thereby preventing it from interacting with the kodecytes.

Table 9. Anti-A sensitization of human A kodecytes during *in vitro* formation with FSL-A spiked O serum.

| | **Serological results obtained after contact hours of FSL-A spiked group O serum with O red cells** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| FSL-A serum mg/mL | *In vitro kodecyte formation* | | | | Sensitization during transformation | Indirect sensitization *(15' 37°C)* against post transformation A kodecytes | | | | |
| | Anti-A (monoclonal) | | | | IgM or IgG | IgM | IgG | | | |
| | 1 | 2 | 4 | 8 | 1, 2, 4, 8 | 1, 2, 4, 8 | 1 | 2 | 4 | 8 |
| 4.0 | ++++ | ++++ | ++++ | ++++ | 0 | 0 | ++ | ++ | ++ | +++ |
| 2.0 | ++ | +++ | ++++ | ++++ | 0 | 0 | ++ | ++ | ++ | +++ |
| 1.0 | 0 | 0 | ++ | ++ | 0 | 0 | 0 | | + | + |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 0 |

### 5.1.3 *in vitro* inhibition of anti-A activity with FSL-A against $A_1$ cells

***Method Overview***

Based on the same 2:7 volume ratio of the FSL-A solution to plasma later given *in vivo* to mice, a series of *in vitro* experiments with fresh human serum were conducted to determine the amount of FSL-A that could be required to prevent polyclonal anti-A activity against blood group $A_1$ red cells. $A_1$ red cells are the strongest known expression of A antigen positive cells. Fresh group O and B serum containing anti-A was neutralized with FSL-A solutions. Without consideration to the volume contribution of the FSL-A, the treated group O serum was incubated with packed human $A_1$ cells at a ratio of 1:1, and the level of IgM and IgG/C3d sensitization of the red cells determined (Protocol 4 V3). FSL-A, FSL-B and FSL-A+B solution, each containing a total of 20, 10, 5, 2, 1, and 0.5 mg/mL of FSL were used. FSL-A+B had a total of 20 mg/mL as 10mg/mL of FSL-A and 10 mg/mL of FSL-B).

***Results***

There was no evidence of hemolysis in any tubes. In the absence of FSL-A (0 mg/mL tube) positive reactivity was observed with both IgM and IgG anti-A. FSL-A at all concentrations was able to completely neutralize all 64 titre IgM anti-A activity in both the group B and O sera (Table 10). FSL-B was able to partially neutralize the IgM anti-A, presumably due to neutralization of some anti-A, B. The 256 titre IgG anti-A of the group B serum was neutralized by FSL-A at concentrations as low as 2 mg/mL but the 1024 titre IgG anti-A of the group O sample was more difficult to neutralize. The FSL-A 20 mg/mL solution was able to reduce the 4+ serological activity score to 1+ while the 10 mg/mL solution reduced it to 2+. The FSL-A+B combination appeared to have no advantages in neutralization of anti-A in O serum and the FSL-B only had little effect.

Table 10. FSL-A *in vitro* inhibition of anti-A activity against A cells
FSL-A at various concentrations neutralized anti-A (IgM and IgG) in both B and O serum

| **FSL-A inhibition of anti-A** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | FSL concentration (mg/mL serum) Serological activity against $A_1$ cells – human | | | | | | |
| ABO serum | Anti-A activity (titre) | Neutralizer | 20 | 10 | 5 | 2 | 1 | 0.5 | 0 |
| B | | | | | | | | | |
| | IgM (1:64) | FSL-A | 0 | 0 | 0 | 0 | 0 | 0 | ++++ |
| | IgG (1:256) | FSL-A | 0 | 0 | 0 | 0 | + | + | ++++ |
| O | IgM (1:64) | FSL-A | 0 | 0 | 0 | 0 | 0 | 0 | ++++ |
| | IgG (1:1024) | FSL-A | + | ++ | +++ | +++ | +++ | +++ | ++++ |
| O | IgM (1:64) | FSL-A+B | 0 | 0 | 0 | 0 | 0 | 0 | ++++ |
| | IgG (1:1024) | FSL-A+B | ++ | ++ | +++ | +++ | +++ | ++++ | ++++ |
| O | IgM (1:64) | FSL-B | ++ | ++ | ++ | ++ | ++ | ++ | ++++ |
| | IgG (1:1024) | FSL-B | +++ | +++ | ++++ | ++++ | ++++ | ++++ | ++++ |

### *Conclusion*

FSL-A at all concentrations was able to completely neutralize all 64 titre IgM anti-A activity in both the group B and O sera. The 256 titre IgG anti-A of the group B serum was neutralized by FSL-A at concentrations as low as 2 mg/mL but the 1024 titre IgG anti-A of the group O sample was more difficult to neutralize. The FSL-A 20 mg/mL solution was able to reduce the 4+ serological activity score to 1+ while the 10 mg/mL solution reduced it to 2+. The FSL-B and FSL-A+B combination appeared to have no advantages over the use of FSL-A alone in neutralization of anti-A in O serum.

### 5.1.4 *In vitro* disassociation of anti-A from A cells with FSL-A

#### *Method Overview*

Anti-A sensitized cells were prepared by mixing serial dilutions of group O serum (400 µL) with an equal volume of washed packed $A_1$ red cells and incubating at 37°C for 30 minutes. From this mix, 175 µL was transferred in two new tubes to which was added either 35 µL of FSL-A (20 mg/mL to give a final concentration of 4 mg/mL) or PBS (Protocol 4 V4).

#### *Results*

The ability of FSL-A to dissociate bound anti-A from $A_1$ red cells pre-sensitized with serial dilutions of group O serum was evaluated over time (Table 11). The PBS reaction represents the sensitization score obtained in the absence of FSL-A. It was clear that the cell bound IgM anti-A was completely dissociated from the red cells within 30 minutes. In contrast, IgG anti-A was more resilient and only resulted in a minor reduction in score. Identical results were obtained at each 30-minute interval up to 120 minutes indicating no increased elution occurred after 30 minutes.

Table 11. FSL-A *in vitro* dissociation of anti-A from sensitized A1 red cells.

Cell bound IgM anti-A was completely dissociated from the red cells in the presence of FSL-A within 30 minutes. In contrast, IgG anti-A was more resilient and only resulted in a minor reduction in score.

| FSL-A dissociation of anti-A | | | | | | | |
|---|---|---|---|---|---|---|---|
| Antibody (detection system) | Neutralizer | Anti-A sensitization (created by serial dilution) 1 | 2 | 4 | 8 | 16 | 32 |
| IgM anti-A (direct agglutination) | PBS | +++ | ++ | 0 | | | |
| | FSL-A (4 mg/mL) | 0 | 0 | 0 | | | |
| IgG anti-A (anti-IgG) | PBS | ++++ | ++++ | ++ | ++ | + | 0 |
| | FSL-A (4 mg/mL) | +++ | ++ | + | 0 | 0 | 0 |

***Conclusion***

Cell bound IgM anti-A was completely dissociated from red cells within 30 minutes with FSL-A 4 mg/mL. IgG anti-A is capable of reacting with the A kodecytes, but the high level FSL-A in serum required to induce the creation of kodecytes was more than adequate to also neutralize the serum IgG anti-A thereby preventing it from interacting with the kodecytes. This has the added advantageous effect that, even if antibody did get a chance to combine with a cell, there is a good chance that it can also be eluted from the cell allowing for incompatible survival.

## 5.2 The use of FSL-A for *in vivo* neutralization of anti-A

The second line of experimentation was to model the process *in vivo* in mice using kodecytes. Since the immunized mice have produced anti-A, neutralization by FSL-A *in vivo*, should allow the subsequent survival of transfused incompatible red blood cells, A+Biotin kodecytes. It was previously established that A+biotin kodecytes transfused into antibody A positive mice without prior FSL neutralization may have a reduced cell survival (4.2).

### 5.2.1 Survival of 10% kodecyte transfusion in compatible and incompatible mice

***Method Overview***

Anaesthetized mice (with anti-A present or absent) were given transfusion of 100 µL 60% suspension A+biotin kodecytes (10% of total red cell mass). Transfusions were usually done as two pairs of anti-A positive and anti-A negative animals per day using A+biotin kodecytes (Protocol 2 V1). 10% was transfused in contrast to the 3.2% used in the survival studies (4.2). The 10% suspension was chosen to represent the same volume of cells given in a human packed red cell transfusion (about 220 mL). At 2, 8, 24, 48, 72, and 96 h intervals a 50 µL blood sample was collected into a heparinized capillary tube from the tail vein for survival studies (Protocol 1.4). Plain capillary tube samples were also collected from selected animals to obtain serum to determine antibody status by TLC (Protocol 4). The presence of kodecytes in circulation was measured using fluorescent microscopy (Protocol 2 V4).

***Results***

A+biotin kodecytes were transfused into compatible anti-A negative (naive) mice and incompatible anti-A positive mice. The individual mice kodecyte survival results were sorted into related clusters, Clusters I to II (Table 12). These Cluster I and II mice were used as controls, given 10% transfusion of A+biotin kodecytes. The transfusion of 100 µL A+biotin kodecytes, 60% suspension, (estimated to be 10% of total mouse red cell mass), into three mice that were negative for anti-A (Cluster I) did not result in any observable symptoms. Starting from the theoretical 10% point (the contribution of the kodecyte transfusion to cell count), the kodecytes count of these animals reduced by about 10% at the first sampling point (0.1 h) before reducing further and then stabilizing into a gradual decline at about 8 hours (Table 12).

Table 12. Comparison of 10% A+biotin kodecyte transfusions in control mice.

Clusters I-II, surviving mouse kodecytes and anti-A presence or absence at time intervals. Serum was tested for Ig anti-A against FSL-A at 1mg/mL in TLC assays using inkjet wells.

| | | | | **Percentage surviving A+biotin kodecytes and anti-A presence [+] or absence [-]** Hours post 1st transfusion (10%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Cluster | ID | pre 1st anti-A Ig | FSL-A mg/mL plasma | 0.1 | 2 | 8 | 24 | 48 | *Anti-A Ig |
| I | 0086 | [-] | 0 | 9.5 | 9.6 | 7.9 | 7.6 | 6.6 | - |
| I | 1240 | [-] | 0 | 8.9 | 7.5 | 5.6 | 5.4 | 4.5 | - |
| II | 0506 | [+] | 0 | 5.2 | 3.6 | | 2.3 | | + |
| II | 4862 | [+] | 0 | 4.7 | 3.9 | | 3.0 | | + |
| II | 4467 | [+] | 0 | 3.5 | 3.4 | 3.3 | | | + |
| II | 3397 | [+] | 0 | 3.2 | 1.9 | | 1.4 | | + |
| II | 0954 | [+] | 0 | 2.2 | 1.3 | | | | + |
| II | 6161 | [+] | 0 | 2.1 [+] | 2.1 | 2.1 | 0.4 | | + |
| II | 3226 | [+] | 0 | 1.5 [+] | 1.2 [+] | 1.2 | 1.1 | 0.7 | + |

*Serum obtained at necroscopy

The transfusion of a 100 µL 60% suspension of A+biotin kodecytes into eight mice that were positive for anti-A (Cluster II) and were not given FS L-A pre transfusion also did not result in any observable symptoms. There was no evidence of haemoglobinuria.

Starting from the theoretical 10% point, the kodecytes count of all animals reduced by about 50-85% at the first sampling point (0.1 h) then stabilized into a gradual decline (Table 12).

Most transfusions showed an immediate reduction, probably representing a loss of the “fragile tail”, but none of the compatible transfusions showed the rapid destruction seen with incompatible transfusions where results showed a rapid reduction in surviving kodecytes within 6 minutes. Data collected at additional time points up to 48 hours was no more informative than that derived at 6 minutes, a time period sufficient to allow for full circulatory distribution.

It was noted that there was a statistically significant difference ($p<0.001$), between the survival of the 3.2% incompatible transfused kodecytes and the 10% incompatible A+biotin kodecytes in anti-A positive mice (Figure 27 - black bars).

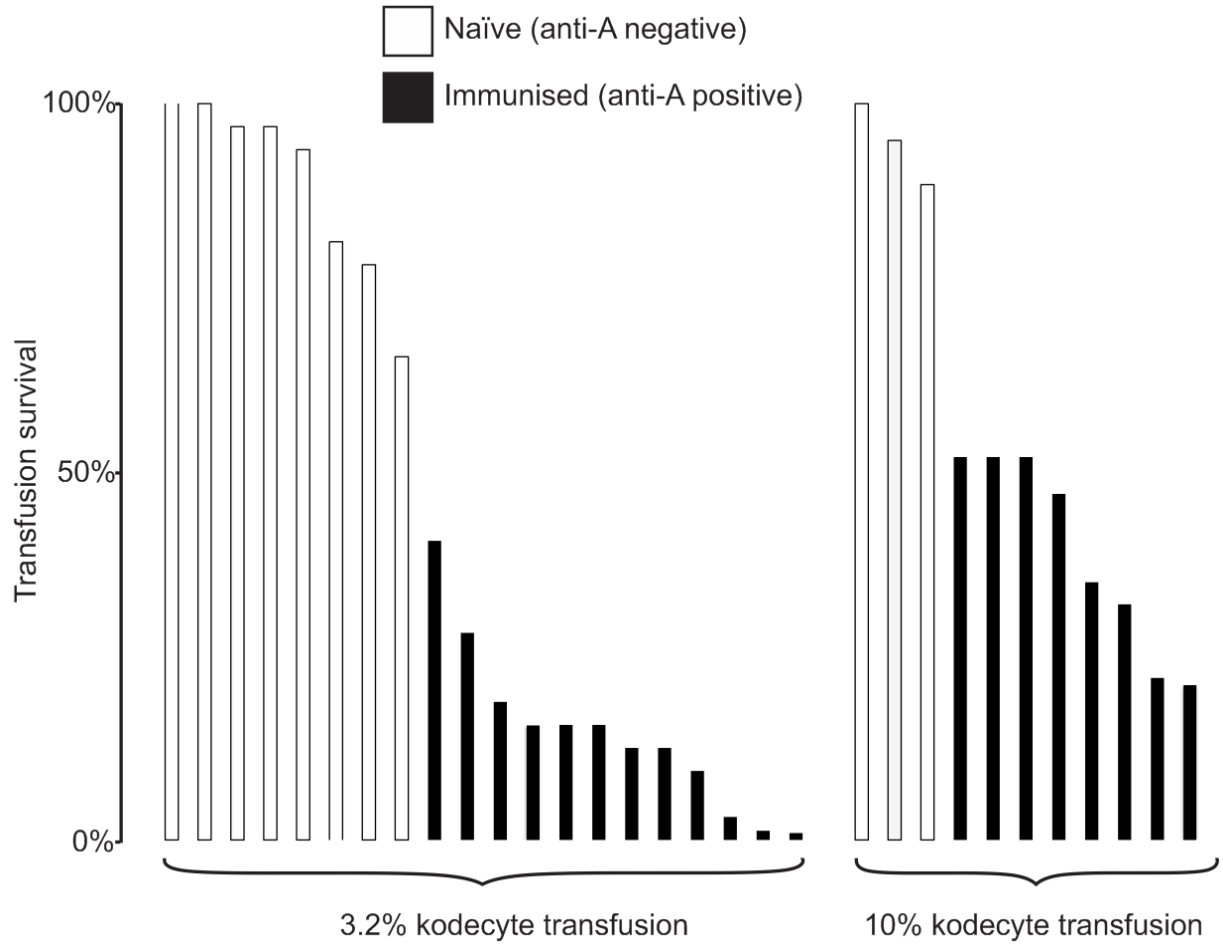



Figure 27. Comparison of kodecyte cell survival in transfusions of 3.2 % and 10 % of the total red cell mass.
Murine A+biotin kodecytes were transfused to mice without anti-A (white bars) or with anti-A (black bars). The difference in volume of cell survival may be due to the increased volume of the kodecytes transfused.

***Conclusion***

The transfusion of a A+biotin kodecytes, (at 10% of total mouse red cell mass), into mice that were negative or positive for anti-A did not result in any observable symptoms. The kodecyte counts of the anti-A negative animals reduced by about 10% at the first sampling point (0.1 h) before reducing further and then stabilizing into a gradual decline at about 8 hours. This reduction probably represents a loss of fragile cells, potential differences between animals, and experimental error. The kodecyte counts of all anti-A positive animals reduced by about 50-85% at the first sampling point (0.1 h) then stabilized into a gradual decline. The statistically significant difference between the survival of the 10% and 3.2% incompatible transfusions in anti-A positive mice is probably due to reduction of antibody and complement levels by the incompatible cells due to the increased number of kodecytes transfused. There was no evidence of haemoglobinuria; possibly due to the fact that when only small amounts of red cells are infused (less than 2% of the total red cell mass) the cells can be cleared by the reticuloendothelial system or phagocytosis before haemolysis occurs (Mollison, 1959, 2005).

### 5.2.2 Survival of 10% incompatible A+kodecytes in FSL-A treated mice

Anaesthetized mice (with anti-A present or absent) were given 200 µL of FSL-A infusions (2:7 volume ratio to serum and dose range of 50 to 200 mg FSL-A/Kg body mass) and a transfusion of 100 µL 60% suspension A+biotin kodecytes, (10% of red cell mass), via the subclavian vein (Protocol 3). The FSL-A infusion was given followed at 2 minutes by the kodecyte transfusion.

***Method Overview***

Six anaesthetized anti-A positive mice and one anti-A negative control mouse were given 200µL FSL-A, (200 mg/Kg body mass) followed 2 minutes later by the A+biotin kodecyte transfusion (Protocol 3 V3 and 3 V4). The administered volume of 200 µL equates with a ratio of about 2:7 of FSL-A infusion volume to the estimated plasma volume (Hoff, 2000). Then at 6 minutes post-transfusion, using new equipment, a blood sample was taken from the subclavian, vein at a new site. At 2, 8, 24, 48, 72, and 96 h intervals a 50 µL blood sample was collected into a heparinized capillary tube from the tail vein for survival studies. Plain capillary tube samples were also collected from selected animals to obtain

serum to determine antibody status. The presence of FSL-A+biotin insertion into red cells thus forming kodecytes was demonstrated with using gel cards and anti-A monoclonal reagent (Protocol 2.3). Six other anaesthetised mice used for the tolerance induction series (6.1.1), were given either 50, 100 or 200 mg/Kg body mass and included in Table 13.

***Results***

Two phenomena were observed. Firstly, the FSL-A transformed the circulating mouse red cells, *in vivo,* into A antigen positive cells thus creating incompatible A+ biotin kodecytes, as detected on gel cards (Figure 28), without consequence in animals either with or without circulating anti-A. Secondly, the incompatible kodeyctes were able to survive despite the transfusion into anti-A positive mice due to the neutralization of the anti-A. The individual results are sorted into related clusters. Clusters IV - VIII are discussed while Clusters I - III were described above in 5.2.1.

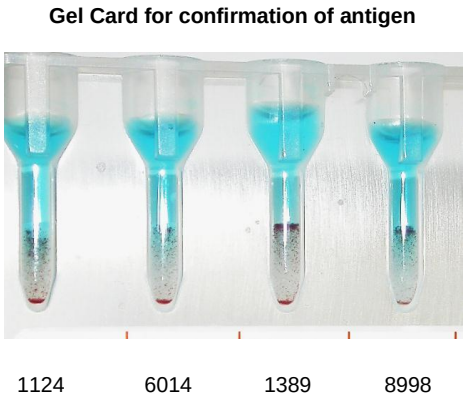



Figure 28. A+biotin kodecytes from FSL-A infused mice.

A+biotin kodecytes taken from anti-A positive mice 2 h post FSL-A infusion detected by agglutination with monoclonal anti-A reagent. This result showed evidence of A antigen and thus kodecyte formation in vivo.

Clusters VI - VIII demonstrated FSL-A infusion and kodecyte formation but did not then have kodecyte transfusions. Although not unambiguous, there is some suggestion that the level of FSL-A detected at 24h on the *in vivo* kodecytes in the anti-A positive animals (Clusters IV and V) was slightly less than that in the anti-A negative animals (Cluster VI, VII ) (Table 13). It is possible that less FSL-A was available for *in vivo* modification, due to its prior interaction with anti-A and possible systemic clearance.

The *in vivo* A kodecyte status was transient and generally detectable for < 48 hours. The similarity between the clearance rate of the 200 mg/Kg and 100 mg/Kg *in vivo* created A kodecytes, suggests that clearance maybe more strongly influenced by time than concentration (Table 13).

The time FSL-A construct remained in circulatory plasma, or its concentration was not specifically established. However, post 8 hours the levels of plasma FSL-A must have decreased below 1 mg/mL (the minimum level at which *in vivo* transformation can occur) thereby allowing the kodecytes to begin to lose constructs from their membranes (Table 13).

Table 13. *In vivo* transformation of mouse red cells with FSL-A infusions

FSL-A was able to *in vivo* transform circulating mouse cells into A antigen positive cells (*in vivo* A kodecytes) in the presence of anti-A. The *in vivo* A kodecyte status was transient and generally detectable for < 48 hours. The results were sorted into related clusters.

| ***In vivo* transformation with FSL-A** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | **FSL-A dose** | | **Anti-A serology hours post FSL-A infusion (*in vivo* A kodecytes)** | | | | | |
| **Cluster** | **ID** | **Pre Anti-A** | **mg / Kg body mass** | **mg / mL plasma** | **0.1** | **2** | **8** | **24** | **48** | **72** |
| III | 7994 | 0 | 200 | 5.7 | ++++ | ++++ | ++++ | ++ | 0 | |
| IV(i) | 1124 | + | 200 | 5.7 | ++++ | ++++ | ++++ | 0 | 0 | |
| IV(i) | 1389* | + | 200 | 5.7 | ++++ | (+) | (+) | | | |
| IV(i) | 9239 | + | 200 | 5.7 | ++++ | ++++ | ++++ | +++ | 0 | |
| IV(ii) | 8998 | + | 200 | 5.7 | ++++ | ++++ | +++ | ++ | 0 | |
| IV(ii) | 6014 | + | 200 | 5.7 | ++++ | ++++ | ++++ | ++ | 0 | |
| V | 3649 | + | 200 | 5.7 | ++++ | ++++ | ++++ | +++ | 0 | |
| VI | 1091 | 0 | 200 | 5.7 | ++++ | ++++ | ++++ | ++++ | ++ | 0 |
| VI | 7058 | 0 | 200 | 5.7 | ++++ | ++++ | +++ | ++++ | 0 | 0 |
| VII | 4424 | 0 | 100 | 2.9 | +++ | ++++ | +++ | +++ | 0 | |
| VII | 9570 | 0 | 100 | 2.9 | +++ | ++++ | ++++ | +++ | 0 | |
| VIII | 4310 | 0 | 50 | 1.4 | 0 | 0 | 0 | 0 | 0 | |
| VIII | 0343 | 0 | 50 | 1.4 | 0 | 0 | 0 | 0 | 0 | |

1389 * due to difficult tail vein collect, only very small blood sample was obtained

The transfusion of a 100 µL 60% suspension of A+biotin kodecytes into the anti-A negative mouse and to the mice that were positive for anti-A (Clusters III, IV & V) and which had

been given 5.7 mg of FSL-A per mL of plasma pre transfusion, did not produce in any observable symptoms. Four anti-A positive mice tested immediately post transfusion had a negative anti-A antibody status (symbol [-] (Table 14). Post FSL-A infusion, anti-A did not become detectable in plasma until after 8 hours suggesting that the FSL-A levels of plasma had fallen below that capable of causing total anti-A neutralization. At 24 hours when the anti-A had started again to become detectable, (symbol [+] (Table 14), the *in vivo* A kodecyte status was also changing to negative (Table 13). It needs to be noted that the level of FSL-A in the transfused A+biotin kodecytes is at least 10 × higher than that in the *in vivo* formed kodecytes, and remains detectable in the surviving kodecytes for at least 3 days as previously described (4.1).

The average shape of the kodecyte survival curve for the five anti-A positive FSL-A neutralized Cluster IV animals was very dissimilar to the untreated anti-A positive animals (Cluster II), $p<0.001$ for all points (Figure 29,Table 14). In fact, the curve for the treated anti-A positive animals (Cluster IV) was not significantly different to that of anti-A negative animals (Cluster I) $p=0.3$ (Figure 29). This result clearly indicates that circulating anti-A had been neutralized (Figure 30). A similar pattern to these neutralized mice, albeit less abrupt over the first 2 hours, was observed in the single naive (anti-A negative) animal #7994 (Cluster III) who was also given FSL-A (Figure 29).

Table 14. Comparison of incompatible kodecyte survival after FSL-A neutralization; individual results are sorted into related clusters.

The results were sorted into related Clusters. Clusters III -V mice were given FSL-A to neutralize anti-A then an incompatible transfusion. Serum was tested for Ig anti-A; [+] denotes presence and [-] denotes absence of anti-A at various time periods.

| **Percentage surviving A+biotin kodecytes and anti-A presence [+] or absence [-]** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Hours post 1st transfusion (10%) | | | | | Inkjet TLC |
| Cluster | ID | pre 1st anti-A Ig | FSL-A mg/mL plasma | 0.1 | 2 | 8 | 24 | 48 | *anti-A IgG |
| III | 7994 | [-] | 5.7 | 10.0 [-] | 9.6 | 7.8 | 7.0 | 5.6 | - |
| IV(i) | 1124 | [+] | 5.7 | 10.0 [-] | 9.4 [-] | 7.6 | 7.2 [+] | 6.2 [+] | + |
| IV(i) | 1389 | [+] | 5.7 | 9.5 [-] | 6.3 [-] | 6.4 | 5.4 [+] | 5.2 | + |
| IV(i) | 9239 | [+] | 5.7 | 8.8 | 6.4 | 6.4 | 6.0 | 4.4 [+] | + |
| IV(ii) | 8998 | [+] | 5.7 | 9.4 [-] | 7.4 | 5.4 [-] | 5.6 | 4.9 [+] | + |
| IV(ii) | 6014 | [+] | 5.7 | 8.4 [-] | 8.3 | 8.2 [-] | 7.3 [+] | 5.4 [+] | + |
| V | 3649 | [+] | 5.7 | 7.6 | 7.5 | 3.4 | 0.5[+] | 0.5 | + |

*Serum obtained 7-14d post FSL-A infusion

One animal #3649 (Cluster V) had an unusual survival profile (Table 14) and was therefore split into its own cluster. In this animal, the rate of A+biotin kodecyte loss was markedly greater than the others, and by 24 hours had crashed to 0.5%. At first glance it appears that the reappearance of anti-A at 24 hours (symbol [+] Table 14) may have been responsible, however anti-A was also detectable in other Cluster IV animals who did not respond so adversely. At this time-point, this animal would be expected to be losing its *in vivo* A kodecyte status, and there was no evidence of a hemolytic event involving its *in vivo* A kodecytes. It was considered that FSL-biotin may have induced anti-Biotin production. However, anti-A levels and isotype were similar with other anti-A positive animals, and there was no immunological ELISA evidence that this animal, or any others, had an antibody to either biotin or the FSL construct *per se*.

Mice were then rested for seven days before their anti-A status was tested again.

Anti-A positive mice were first given an infusion of FSL-A and two minutes later transfused with incompatible A+biotin kodecytes (Figure 30 - grey bars). This time the incompatible kodecytes showed normal survival ($p = 0.3$) clearly indicating that circulating anti-A had been neutralized. The comparison is seen with the survival of the incompatible kodecytes (Figure 30 black bars).

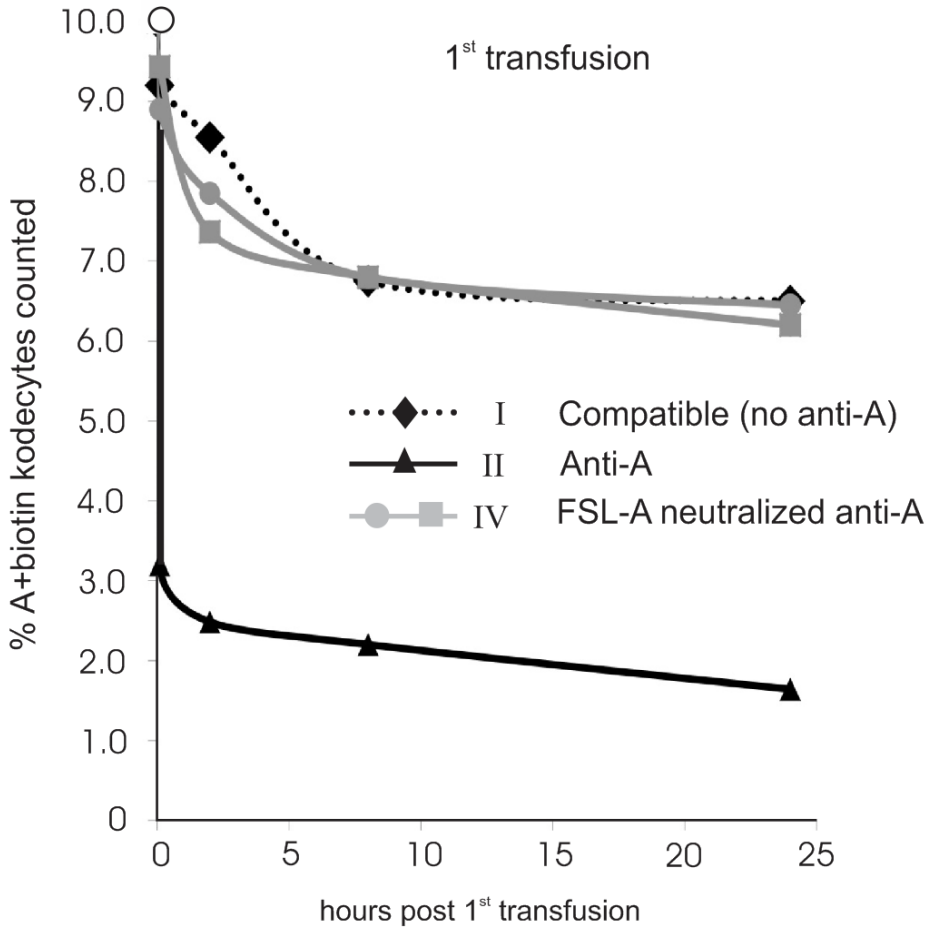



Figure 29. Average cluster survival curves of A+biotin kodecytes transfused into animals with and without anti-A and with and without FSL-A infusions.

Cluster II (anti-A) and Cluster IV (FSL-A neutralized anti-A) animals. Line diagram compares trends over time. Control animals are Cluster I, which did not have anti-A and showed normal survival, while Cluster II animals with anti-A showed antibody-mediated kodecyte clearance. FSL-A neutralized anti-A in Cluster IV animals showed normal survival of their first kodecyte transfusion. All curves are means of the data in their clusters and are plotted from the theoretical 10% transfusion starting point, indicated by an open circle.

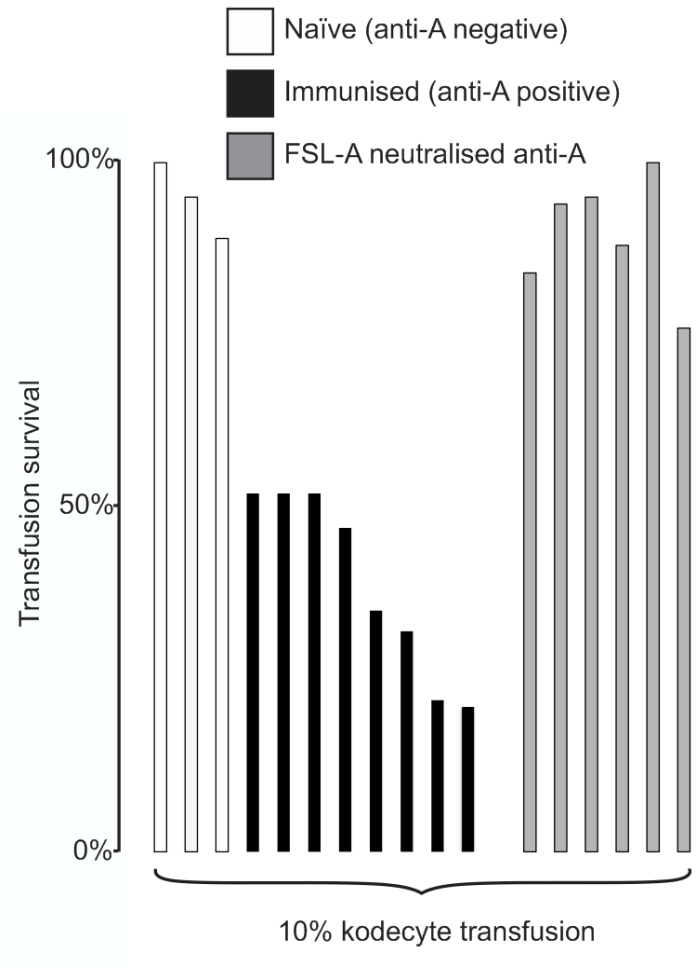



Figure 30. Comparison of incompatible kodecyte survival with anti-A and with neutralized anti-A.

Comparison of the survival of 10% incompatible kodecyte transfusion in anti-A positive mice with the survival of incompatible kodecytes in mice which have had pre-transfusion FSL-A neutralization *in vivo* of anti-A (grey bars).

### *Conclusion*

These results demonstrated that the infusion of FSL-A had neutralized circulating anti-A antibody *in vivo and* allowed normal cell survival of “incompatible” A kodecytes. The FSL-A molecule was thus shown to be antigenic when anti-A was neutralized at this point and anti-A remained neutralized in these animals for at least 8 hours.

### 5.2.3 Rechallenge with incompatible kodecyte transfusion in mice given FSL-A infusion once FSL-A is depleted

***Method Overview***

One or two weeks after the infusion of FSL-A and the first kodecyte transfusion, when the FSL-A had cleared from the circulation and anti-A was detectable, the same animals were given a re-challenge transfusion of incompatible A+biotin kodecytes. This series was carried out to demonstrate the difference in kodecyte survival after incompatible transfusion once the neutralising effect of the FSL-A was absent - with reduced survival of transfused kodecytes due to the presence of circulating anti-A.

The six anaesthetized anti-A positive mice (cluster IV and V animals), that had been given 200 µL FSL-A, (20mg/mL) were given a second incompatible A+biotin kodecyte transfusion (Protocol 3 V6). These mice had tolerated an incompatible A+biotin kodecyte transfusion either one or two weeks previously.

***Results***

The second (rechallenge) transfusion with A kodecytes without FSL-A anti-A neutralization in the cluster IV animals, whose serum could be demonstrated to have anti-A (symbol [+] Table 15), did not result in any observable symptoms. This rechallenge demonstrated that the animals were actually still capable of destroying the incompatible kodecytes when the antibodies were not first neutralized as seen previously in Cluster II animals (Table 12, Figure 27). However, this time surprisingly, there appeared to be two different outcomes. As expected most animals (4/6) destroyed the incompatible kodecytes but surprisingly one animal showed normal kodecyte survival and another showed moderate survival. There was no evidence of haemoglobinuria.

Starting from the theoretical 10% point, the kodecyte count of three animals (cluster IV (i)) reduced by about 50-85% at the first sampling point (0.1 h) then stabilized into a gradual decline (Figure 31, Table 15). The average shape of the kodecyte survival curve (Figure 31) for these three animals classified as Cluster IV(i) was very similar to that of anti-A positive, Cluster II animals not receiving FSL-A anti-A neutralization and was evidence that in the absence of FSL-A protection, A+kodecytes would not survive normally in these specific animals. Unexpectedly, and despite being ELISA anti-A positive, two animals #8998 and #6014, classified as cluster IV (ii), had second kodecyte transfusion survival curves very similar to that of antibody negative (or neutralized) animals (Figure 31). The

reason for this apparent normal survival in animal #8998 can perhaps be explained by a low anti-A titre (8), and although the 128 titre of animal #6014 was high, it was not >128 as observed in the other 14 animals tested. It is not known whether this reduced level of antibody in these two animals was an indirect consequence of FSL-A treatment, or a natural variation.

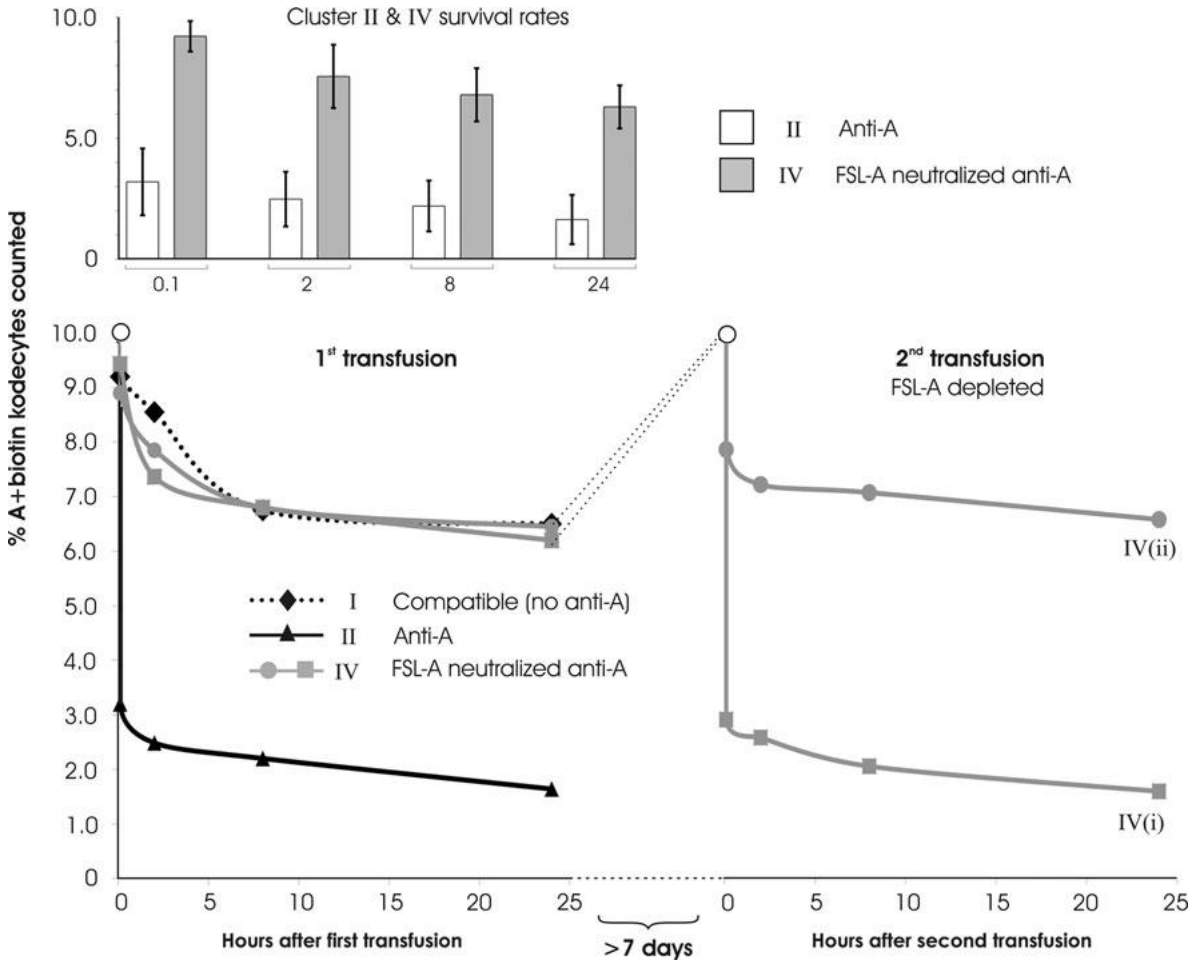



Figure 31. Average cluster survival curves of A+biotin kodecytes transfused into animals with and without anti-A and with and without FSL-A infusions.

Upper bar graph indicates average kodecyte first transfusion survival (with 1 SD error bars) obtained at each sampling time point for Cluster II (anti-A) and Cluster IV (FSL-A neutralized anti-A) animals. Line diagram compares trends over time. Control animals are Cluster I, which did not have anti-A and showed normal survival, while Cluster II animals with anti-A showed antibody-mediated kodecyte clearance. FSL-A neutralized anti-A in Cluster IV animals showed normal survival of their first kodecyte transfusion. The second transfusion into these Cluster IV animals, when their plasma FSL-A was absent, resulted in two different patterns. Cluster IV (i) animals resulted in the expected antibody-mediated kodecyte clearance pattern, while Cluster IV(ii) animals showed a normal survival pattern. All curves are means of the data in their clusters and are plotted from the theoretical 10% transfusion starting point, indicated by an open circle.

Table 15. Comparison of all surviving kodecytes and anti-A presence or absence at time intervals, sorted into related clusters.

Clusters I-II mice were used as controls, given 10% transfusion of A+biotin kodecytes.Clusters III -V mice were given FSL-A to neutralize anti-A then an incompatible transfusion. A second incompatible transfusion was given 1 or 2 weeks later.

| | | | | **Percentage surviving A+biotin kodecytes and anti-A presence [+] or absence [-]at time intervals (hours)** | | | | | | | | | | | Inkjet TLC against FSL-A Necroscopy | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Hours post 1st transfusion (10%) | | | | | | Hours post 2nd transfusion (10%) | | | | | | |
| Cluster | ID | pre 1st anti-A Ig | FSL-A mg/mL plasma | 0.1 | 2 | 8 | 24 | 48 | Pre 2nd Anti-A | 0.1 | 2 | 8 | 24 | 48 | Anti-A IgG | Anti-A Ig Titre |
| I | 0086 | [-] | 0 | 9.5 | 9.6 | 7.9 | 7.6 | 6.6 | | | | | | | - | |
| I | 1240 | [-] | 0 | 8.9 | 7.5 | 5.6 | 5.4 | 4.5 | | | | | | | - | |
| II | 0506 | [+] | 0 | 5.2 | 3.6 | | 2.3 | | | | | | | | + | |
| II | 4862 | [+] | 0 | 4.7 | 3.9 | | 3.0 | | | | | | | | + | |
| II | 4467 | [+] | 0 | 3.5 | 3.4 | 3.3 | | | | | | | | | + | |
| II | 3397 | [+] | 0 | 3.2 | 1.9 | | 1.4 | | | | | | | | + | |
| II | 0954 | [+] | 0 | 2.2 | 1.3 | | | | | | | | | | + | |
| II | 6161 | [+] | 0 | 2.1 [+] | 2.1 | 2.1 | 0.4 | | | | | | | | + | |
| II | 3226 | [+] | 0 | 1.5 [+] | 1.2 [+] | 1.2 | 1.1 | 0.7 | | | | | | | + | |
| III | 7994 | [-] | 5.7 | 10.0 [-] | 9.6 | 7.8 | 7.0 | 5.6 | | | | | | | - | |
| IV(i) | 1124 | [+] | 5.7 | 10.0 [-] | 9.4 [-] | 7.6 | 7.2 [+] | 6.2 [+] | [+] | 1.7 [+] | 1.6 | 1.3 | 1.4 | 1.4 | + | [+] >128 |
| IV(i) | 1389 | [+] | 5.7 | 9.5 [-] | 6.3 [-] | 6.4 | 5.4 [+] | 5.2 | | 4.5 | 3.7 [+] | 2.3 | 1.6 | 1.3 | + | [+] >128 |
| IV(i) | 9239 | [+] | 5.7 | 8.8 | 6.4 | 6.4 | 6.0 | 4.4 [+] | [+] | 2.6 | 2.5 | 2.6 | 1.8 | | + | [+] >128 |
| IV(ii) | 8998 | [+] | 5.7 | 9.4 [-] | 7.4 | 5.4 [-] | 5.6 | 4.9 [+] | [+] | 6.4 | 6.3 | 6.1 | 5.9 | 5.8 | + | [+] = 8 |
| IV(ii) | 6014* | [+] | 5.7 | 8.4 [-] | 8.3 | 8.2 [-] | 7.3 [+] | 5.4 [+] | [+] | 9.5 | 8.3 | 8.2 | 7.4 | 7.2 | + | [+] = 128 |
| V | 3649* | [+] | 5.7 | 7.6 | 7.5 | 3.4 | 0.5 | 0.5 | | 0.2 | 0.1 | 0.1 | | | + | [+] >128 |

* 2nd transfusion was given 1-week post 1st transfusion, all others were 2 weeks post 1st transfusion

The results of the second transfusion of incompatible kodecytes are compared with kodecyte survival seen after anti-A neutralization by FSL-A before the first incompatible transfusion (Figure 32 – black bars, 2nd transfusion).

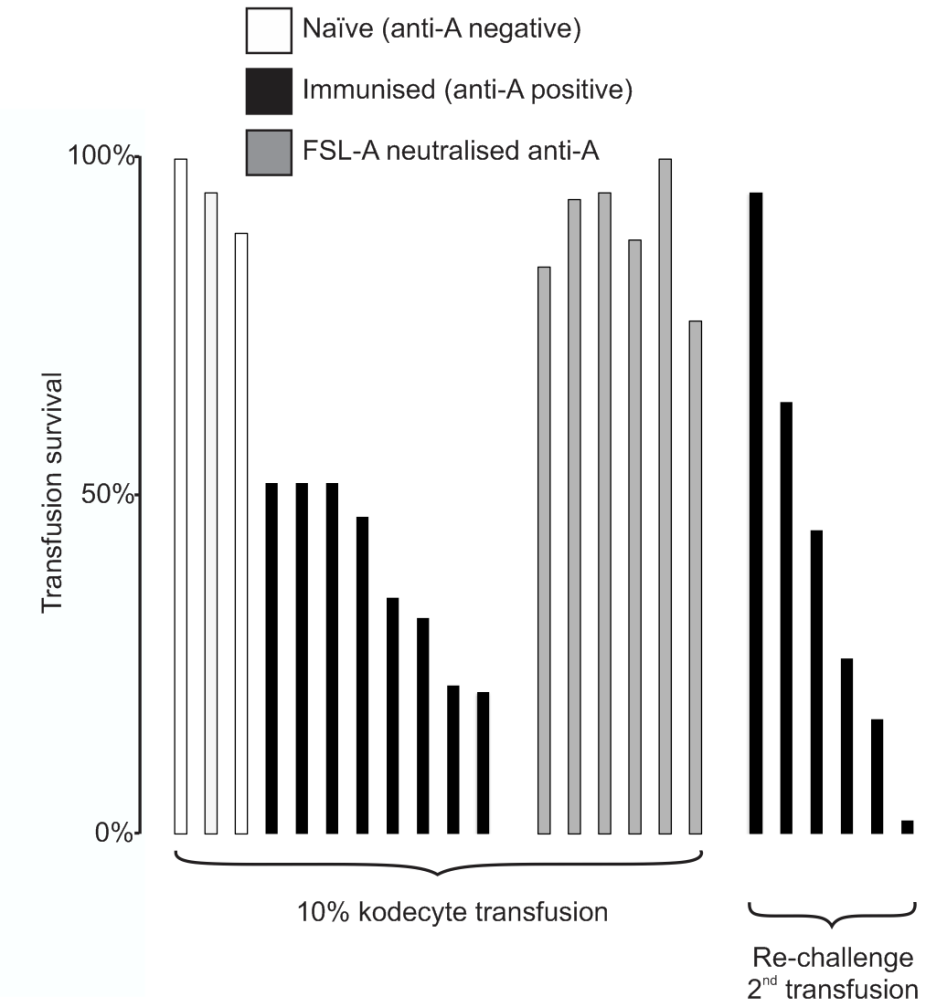



Figure 32. Comparison of kodecyyte survival in transfusions of 10% incompatible A+biotin kodecytes with neutralized anti-A and anti-A.

Mice without anti-A (white bars), with anti-A (black bars), with FSL-A neutralized anti-A (grey bars) and the same mice later given a rechallenge 2nd transfusion when the FSL had cleared their circulation and anti-A was detectable (black bars ) Individual animal results after the second transfusion (black bars) are in the same order for the grey bars).

Examples of various different scenarios with transfused A+biotin kodecytes in anti-A negative mice, anti-A positive mice pre and post FSL-A neutralization and finally after the depletion of FSL-A are shown in photographs taken for kodecyte survival measurement (Figure 33). Ten similar images of both fluorescence and light microscopy were obtained for each sample to calculate kodecyte survival.

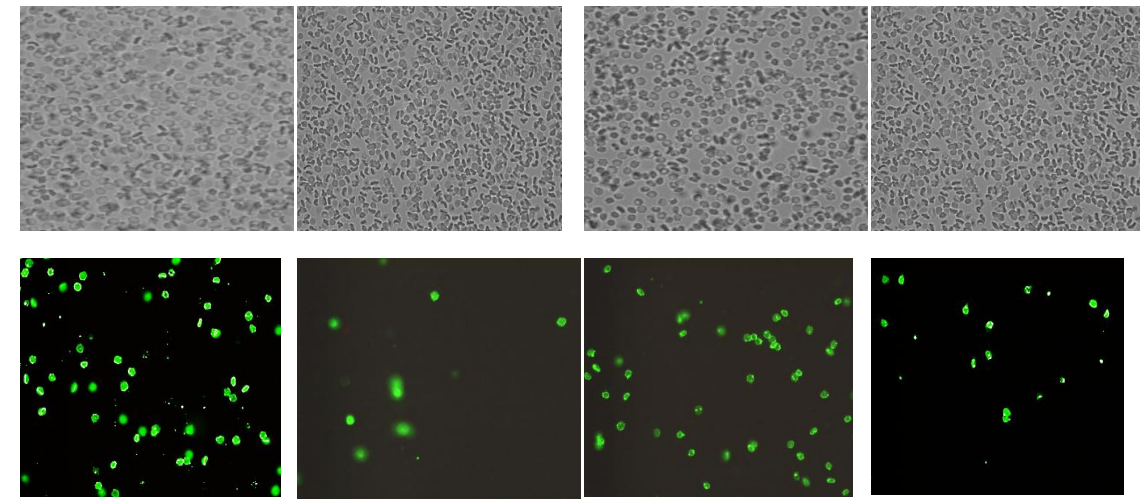



Figure 33. Examples of photographs of A+biotin kodecyte survivals 6 minutes after transfusion into animals with and without anti-A.

Cluster I Animal1240 has no anti-A and shows normal 8% to 10% kodecyte survival, while Cluster II animal with anti-A (Animal 3226) shows antibody-mediated kodecyte reduction (<5%). Animal with anti-A (Animal 1124) is given FSL-A and a kodecyte transfusion, then normal kodecyte survival results (Cluster IV), but when retransfused and circulating FSL-A is no longer present, antibody-mediated kodecyte clearance occurs (Cluster IV(i) <5% survival). Images are fluorescence of A+biotin kodecytes visualized with avidin Alexa Fluor 488 (200x magnification). Also shown are the corresponding differential interference contrast light microscopy images of the same frames showing equivalent cell counts between samples.

***Conclusion***

This rechallenge demonstrated that the anti-A positive animals (that had been previously infused with FSL-A) were still capable of destroying the incompatible kodecytes once FSL-A was depleted. As expected most animals destroyed the incompatible kodecytes although one animal showed normal kodecyte survival and another showed moderate survival. Kodecyte destruction was extremely rapid in four of the anti-A positive mice Cluster IV (i) reaching 0.2% within 6 minutes when the second A+biotin kodecyte transfusion was given without pre FSL-A anti-A neutralization.

Although still unproven, it was speculated that possibly tolerance, to some extent, may have been induced, either by causing a reduction in available antibody (as a consequence of clearance) or through a down-regulation of the immune response (potentially as a consequence of clonal deletion). Cell survival in the other two animals, Cluster IV (ii), possibly showed this phenomenon since their sera anti-A titres were lower than the other animals tested. The other explanation was that the cells survived due to the presence of a lowered level of antibody or complement subsequent to the first transfusion since an incompatible transfusion itself also effectively reduces antibody and complement levels thereby temporarily mitigating the reaction (Chaplin, 1959; Mollison, 2005).

# CHAPTER 6 - ATTEMPTED INDUCTION OF TOLERANCE USING FSL-CONSTRUCTS

This chapter discusses preliminary attempts to induce tolerance with KODE™ technology using FSL constructs.

## 6.1 Overview

It is possible that FSL constructs may also be able to down-regulate the immune system by inducing auto regulation of specific antibody production. The underlying concept was that if all the cells exposed to the circulation (including lymphatic and thymus), became antigen positive due to the infused FSL construct, then the immune response to the antigen in the future may be affected. This effect is supported by the literature, where prolonged immunizations with glycolipids resulted in decreased specific antibody titres (Alving and Richards, 1977; Lingwood et al., 1980).

Such a phenomenon has been noted in pregnancy when Lewis glycolipids were infused to neutralize anti-Lewis antibody pre-transfusion and the patient's Lewis antigen positive red cells became Lewis negative. The patient then made anti-Lewis antibodies to the infused glycolipids but the antibody titre lowered once the antigens reappeared on the red cells (Hossaini 1972, Mollison 2005, Pelosi et al., 1974).

Studies show the route of antigen administration affects the immune response - oral or intravenous routes induce tolerance and subcutaneous injection invokes antibody production (Liblau et al., 1997; Kirkley, 1999). This study only investigated the immune response to administration of antigen by the intravenous route.

### 6.1.1 Consequences of intravenous injection of FSL-A on tolerance induction

Antigens correlate with self when expressed in strong and constant concentrations and those that are continuously present in blood and lymphoid organs generally do not activate T cells, do not induce an immune response, and are effectively ignored by the immune system (Zinkernagel, 1996). The underlying concept for these experiments was that if infusion of FSL-A caused all the cells and tissues exposed to circulating FSL-A to become A antigen positive, then the immune response would be compromised in its ability to produce circulatory antibodies to glycoprotein after vaccination.

C57 black 6 laboratory mice (C57BL/6) naive mice were given an intravenous injection, via the subclavian vein, of FSL-A solution at various concentrations. They were then immunized one week later with A substance in saliva with Titermax™ Gold adjuvant subcutaneously, three times at three weekly intervals to induce an immune response. Previous results showed that naive mice usually responded to this immunogen with the production of anti-A.

***Method Overview***

Six naive mice were given an intravenous injection of FSL-A solution at concentrations 4, 2, and 1 mg/mL (Protocol 3 V8). A tail vein blood sample (Protocol 1.4), was taken at the time points 0.1 hr to 72 hours to show the continued presence of circulating FSL-A on red cells (A kodecytes) (Protocol 2.3).

One week post FSL-A infusion, the “tolerised” mice and other naive mice were immunized subcutaneously at one site with saliva from an ALe(a-b+) donor (Protocol 1.8). Plain capillary tube samples were collected from naive animals by tail vein nick method and serum obtained was used to determine pre-immunisation antibody status (Protocol 5).

***Results***

Mice that were given 4 mg/mL FSL-A formed kodecytes *in vivo,* which were detected in circulation for up to 72 hours. Mice that were given 2 mg/mL FSL-A formed kodecytes detected in circulation between 48h and 72h and mice given 1 mg/mL did not form detectable A kodecytes (Table 16). None of the six FSL-A infused mice produced anti-A in agreement with previous results in 2.2.4 (Table 4). All produced anti-A antibody after secondary subcutaneous immunisation with A substance saliva (Figure 27, Table 16),

implying that the FSL-A at the concentration given did not induce tolerance to the A antigen. Naive mice given the same A saliva developed anti-A antibody (Table 16). Due to ethical approval and time constraints, these mice were not challenged subsequently with salivary A substance antigen as a secondary immunization and so it is not known if such a challenge would have been tolerated or would have stimulated anti-A.

Table 16. Effect of FSL-A infusion before vaccination with A glycoprotein.
All mice, whether tolerised with FSL-A or not, made anti-A when immunised with saliva

| **Effect of infusion on vaccination with adjuvant** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Mice | | Infusion | | Analysis | | | | | | | |
| | | Manipulation and Dose | | Serology with monoclonal A | | | | | | | |
| Number | Pre Anti-A | IV FSL-A | Sub-cut A glyco-protein with adjuvant | *In vivo* kodecytes at various time points (h) | | | | | | Post Anti-A | |
| | Ig | mg/mL | | 0.1 | 2 | 8 | 24 | 48 | 72 | Ig | IgG |
| 1091 | 0 | 4 | 3x | ++++ | ++++ | ++++ | ++++ | ++ | 0 | + | + |
| 7058 | 0 | 4 | 3x | ++++ | ++++ | +++ | +++ | 0 | 0 | + | + |
| 4424 | 0 | 2 | 3x | +++ | ++++ | +++ | +++ | 0 | 0 | + | + |
| 9570 | 0 | 2 | 3x | +++ | ++++ | ++++ | +++ | 0 | 0 | + | + |
| 4310 | 0 | 1 | 3x | 0 | 0 | 0 | 0 | 0 | 0 | + | + |
| 0343 | 0 | 1 | 3x | 0 | 0 | 0 | 0 | 0 | 0 | + | + |
| Naive 1 | 0 | 0 | 3x | 0 | | | | | | + | + |
| Naive 2 | 0 | 0 | 3x | 0 | | | | | | + | + |
| Naive 3 | 0 | 0 | 3x | 0 | | | | | | + | + |
| Naive 4 | 0 | 0 | 3x | 0 | | | | | | + | + |
| Naive 5 | 0 | 0 | 3x | 0 | | | | | | + | + |
| Naive (x317) | 0 | 0 | 0 | | | | | | | 0 | 0 |

### *Conclusion*

Despite the lack of positive results from this preliminary experiment, where the FSL-A infused mice made anti-A and were not tolerised, there is a possibility that FSL constructs may in some circumstances, be able to down regulate the immune system by inducing auto regulation, thereby causing down-regulation of specific antibody production. The Cluster IV (ii) mice potentially showed this effect (Table 12). It could be that tolerance was induced by clonal deletion. Further experimentation in this area is warranted.

# CHAPTER 7 - DISCUSSION

This thesis focused on various uses of Functional-Spacer-Lipid constructs, (FSLs), to establish opportunities to manipulate the immune response system. The investigation of the hypotheses required the use of FSLs to try to stimulate antibody; to create incompatible cells and measure their survival in an incompatible transfusion with the use of the FSL-biotin label; to neutralize anti-A both *in vivo* and *in vitro*, and to try to induce tolerance to the A blood group antigen in mice. The FSL-biotin label also allowed recovery of circulating kodecytes.

The ABO system was chosen as a model since the immune response to the blood group antigens A and B are major risk factors in transplantation and blood transfusion and the ability to manipulate the immune response to these antigens would be of valuable therapeutic benefit. It was elected to study only the response to the A blood group antigen since animals have a restricted antibody response to the linear blood group B antigen, due to its similarity with a natural antigen present in animals (Galili et al., 1985).

In light of the inability to directly experiment on humans, animal models remain the best alternative to study interactions of the immune system. There are two prerequisites for creating a red cell/antibody incompatible transfusion animal model. The animal must have, or be able to make, an appropriate antibody against the incompatible antigen and there must be incompatible antigen-positive red cells available for transfusion. C57BL/6 mice were immunised subcutaneously with salivary A blood group glycoprotein, and TiterMax™ Gold as the adjuvant, and they effectively mounted an immune response to the human A antigen with the production of IgM and IgG anti-A in > 99% mice. The presence of anti-A in mice created an incompatible transfusion model when A+biotin kodecytes were transfused.

The FSL antigen constructs, synthetic blood group A antigen epitope (FSL-A), synthetic blood group B antigen epitope (FSL-B), and synthetic GB3 antigen epitope (FSL-GB3), once inserted into red cell membranes, created murine kodecytes both *in vivo* and *in vitro.* These kodecytes provided the source of compatible or incompatible antigen-positive red cells for transfusion. It was necessary to make incompatible red cells (kodecytes) since ABO antigens are not present on the red cells of small laboratory animals (Oriol et al., 1986). Transfusing human red cells into laboratory animals is not usually an option due to anti-species activity, substantial antigenic and physical differences. For example, the

human red cell is 2-times larger in diameter than the mouse red cell, and is known to have reduced survival in mice (Hod et al., 2008). Thus, artificial ABO incompatible mouse red cells were created using FSL-A construct to insert blood group A antigens into cell membranes, creating *in vivo and in vitro* A kodecytes where the only foreign antigen(s) are those introduced by the FSL construct(s). FSL-biotin was added simultaneously to provide a label to enable cell survival measurement using fluorescence microscopy after conjugation with avidin-fluorophore.

GB3+biotin and biotin-only kodecyte transfusions were given to compatible anti-A positive mice and these kodecytes, along with A+biotin kodecytes, were also transfused into anti-A negative mice as baseline experiments for kodecyte survival measurements. A+biotin kodecytes were also used to demonstrate kodecyte recovery from whole blood post-compatible transfusion and for demonstration of the FSL-A neutralization of anti-A - allowing red cell survival in an incompatible transfusion.

There are many factors that influence the interpretation of the models' results and their extrapolation into human (Hod et al., 2008). Modelling "human-like" transfusion reactions in animal models requires a series of artificial interventions and cross-species extrapolations. As a consequence, an animal model will not necessarily have the same reaction that would occur in humans due to the dissimilar biological/clinical equivalence to antigen-antibody responses of man, and a lack of similar human-like antigen-antibody systems. For example, mice complement is less effective than human complement at causing in vivo haemolysis (Ong and Mattes, 1989), mice have higher lymphocyte counts and they have different CD1 genes (Haley, 2003; Hod et al., 2010, Mestas and Hughes, 2004). The CD receptors, which bind lipid-based antigens such as glycolipids, have 5 variations (a-e) in human while mice only have CD1d (Bollyky and Wilson, 2004; Lockridge et al., 2011).

A variety of animal models have been used to investigate intravascular reduced cell survival and each has its own advantages and disadvantages. Non-human primates are probably the most appropriate for human comparison and rabbits are easy to manipulate and inexpensive but neither of these are as easily studied as mice with their small size, relatively low cost, short gestation, fecundity and less significant ethical issues (Hod et al., 2008; Hod et al., 2010) and genetic variability within inbred strains is negligible (Taft et al., 2006). Murine models are suitable for the study of the mechanisms and consequences of red cell clearance, despite some molecular and immunological response differences to

humans (Haley, 2003; Mestas and Hughes, 2004; Hod et al., 2010, Schirmer et al., 2007). It is important to appreciate that strains may have different antibody responses and so red cells from another species should not be used for transfusion as there may be significant structural and antigenic differences which will complicate the interpretation of the outcomes (Hod et al., 2008; Hod et al., 2010). Red cell survival studies done previously in mice involved the use of green fluorescent protein expressed on red cells and measured by flow cytometry (Gilson et al., 2009) and the use of human glycophorin A transgenic mice red cells transfused into passively immunised incompatible mice to investigate cell survival and IgM and IgG mediated haemolysis (Schirmer et al., 2007).

Four hypotheses concerning the use of Functional-Spacer-Lipid (FSL) constructs were tested. The first hypothesis, that FSLs might induce antibody production, was tested with FSL constructs to determine the immunogenicity of the molecules with direct infusion into the circulation and subcutaneous immunization.

Since the route of administration of the antigen determines the immune response pathway (Liblau et al., 1997; Kirkley, 1999) different routes of antigen delivery were investigated. Subcutaneous injection, between the epidermis and dermal layers, usually elicits a strong response due to the uptake of antigens by Langerhan's cells or DCs and subsequent delivery to lymph nodes and recognition by T cells (TitremaxGold™; Schunk and Macallum, 2005). Subcutaneous immunisation of mice with synthetic glycolipid FSL (A or GB3) mixed with the same adjuvant used successfully with glycoprotein, did not stimulate anti-A or anti-GB3 production.

Intravenous antigen administration allows almost immediate access of the antigen to B cells, DCs and macrophages in the spleen (Gutgemann et al., 2002) but tends to induce tolerance or unresponsiveness unless antigens bind to host cells or are in the form of aggregates that are readily seen by APCs. When human glycophorin A was inserted by electroinsertion into membranes, the modified red cells had normal survival in mice but failed to provoke an immune response whereas the intramascular injection of purified antigen produced an immune response with antibody production (Nicolau et al., 1993). Lymphoid cell membranes altered with CD8 were antigenic (Tykocinski and Kaplan, 1993) but did not stimulate antibody production. T and B cells respond to antigens that become transiently localised within organised lymphatic tissues for at least 3 - 5 days (Zinkernagel and Hengartner, 2001).

Intravenous FSL constructs (50-200mg/Kg body mass) given to immunologically mature mice by subclavian vein injection as kodecytes or in solution did not evoke an immune response even though it was demonstrated that the compatible kodecytes and FSL-A remained detectable in the circulation up to 72 hours which is a reasonable time to allow for a B cell response (Zinkernagel and Hengartner 2001).

The form and concentration of the antigen also affects the type of immune response. Glycoproteins would be expected to induce a significant antibody response, while glycolipids are poor immunogens and probably would not (De Libero, 2004) since they are immunologically processed differently to glycoproteins. Glycoproteins are T dependent antigens but glycolipids are generally regarded as T independent (TI 1 or 2) antigens. Previous studies in mice have shown that TI- 2 glycolipid antigens, can elicit robust and long-lasting primary antibody responses (Garcia de Vinuesa et al., 1999) and polysaccharides, also TI antigens, are able to generate memory B cells with antigen-specific IgG antibodies (Obukhanych and Nussenzweig, 2006).

Although FSL constructs are glycolipid-like they have a spacer and a different lipid tail – and as a consequence, may have different immunological attributes to glycolipids. The presence of the spacer may be the factor that differentiates the immune response of FSLs from that with glycolipids. Based on natural glycolipid data, the infused FSL constructs, will be expected to predominately associate with plasma lipids (e.g. HDLs and LDLs) where they will be mobile and flexible, and become multivalent neutralizing lipid particles. Multivalent carbohydrates have been previously shown to have therapeutic potential in compensating for the weak affinity of carbohydrate-ligand moieties (Gardiner, 1998, Pieters, 2009). The FSL-A blood group antigen is a monomeric trisaccharide and in membranes it may occur as clusters making it multivalent. Only saccharides in polymeric or clustered glycoconjugates, and not monomeric structures, result in alteration of cytotoxicity suggesting that the appropriate presentation is critical for carbohydrate recognition and subsequent biological effects (Kovalenko et al., 2007).

Mice have the B-1, CD1d receptors that recognize glycolipids (De Libero, 2004) and can stimulate a TI-2 independent response (Bollyky and Wilson, 2004). These TI-2 antigens engage both B-1 cell and NKT cell CD1d receptors. The B-1, CD1d receptors recognize polysaccharide or carbohydrate antigens and secrete IgM antibodies. NKT CD1d receptors rapidly produce T helper cell (Th1 and Th2) cytokine responses with the secretion of cytokines promoting further activation of cytolytic T cells and antibody-producing B cells.

NKT cells can either up-or-down regulate immune responses (antibody production or tolerance) by promoting the secretion of the immune regulatory cytokines.

Based on the crystal structure of the CDd1 structure (Koch et al., 2005) it appears that the spacer of the FSL construct would probably impair its ability to bind within the appropriate grooves in the CD1d molecule, and glycolipids are only immunogenic when bound to these molecules (De Libero, 2004). This was suggested when the glycolipid Gal-α-ceramide powerfully activated human invariant NKT cells (iNKT) after binding to CD1d. (Shimizu et al., 2007) but a synthetic FSL-Gal α construct showed no ability to stimulate human iNKT cell clones (Oliver et al., 2011a).

The infusion of FSL constructs in solution or as kodecytes into compatible or incompatible mice did not stimulate antibody production against the functional head group of the FSL construct or the spacer and were therefore considered non-immunogenic. This is potentially an important factor when considering the beneficial uses of FSL constructs.

The second hypothesis that FSLs can be used to study *in vivo* red cell survival in compatible and incompatible transfusion (using a clinical equivalent to a human transfusion scenario) was tested in mice using compatible and incompatible kodecytes and an FSL-biotin label subsequently conjugated *in vitro* with avidin/Alexafluor 488. This enabled differentiation of endogenous cells from transfused cells using fluorescence microscopy and measurement of kodecyte survival. The transfused cells were also recovered using the FSL-biotin label. The ability to modify red cells with foreign antigens was already established (Frame et al., 2007, Heathcote et al., 2010, Henry 2009, Henry et al., 2011).

In a transfusion setting, immune-mediated transfusion reactions due to the presence of circulating antibody and autoimmunity are the dominant causes of reduced cell survival. Research into human transfusion reactions is generally limited by the risks associated with deliberately causing reduced cell survival and potential sequelae such as disseminated intravascular coagulation, renal tubular necrosis or antibody stimulation (Mollison, 2005), hence most data in humans is derived after the event. To understand the process of immune mediated transfusion reactions, the variables ideally need to be deliberate, controlled, and relatively safe. Estimates of red cell survival can be helpful when serologically incompatible red cells are involved in a transfusion reaction or when only incompatible blood is the only blood available in an emergency.

The normal life span of human red cells newly released into the circulation is about 115 days (Franco, 2009). Transfused cells are usually removed by the natural course of ageing with less than 1% destroyed each day (Mollison, 2005). However, red cell survival curves are approximately linear and show a small variation-yielding a tail at the end of the curve. The standard deviation of the red cell lifespan can be deduced and suggest it may even be as short as 6 days in normal subjects (Mollison, 2005). Most techniques require that a label be placed on the red cells that can then be followed while the cells age using flow cytometry. Exposure of recipients or blood to chemicals (or radiation), and ensuring the modified cells are functionally unaffected by the modification (Mollison, 2005), together with specialized and laborious techniques, are potential reasons why cell survival studies are not more routinely used.

Methods currently used to label red cells for survival measurement depend on the purpose for which survival studies are required and include radioisotopes $^{51}$Chromium ($^{51}$Cr), di-isopropyl phosphofluoridate (DF$^{32}$P), $^{14}$C-labelled compounds, $^{99m}$ Technetium, $^{111}$Indium; non-radioactive isotopes such as $^{15}$ N-glycine and $^{52}$Cr (Heaton et al., 1989), labeling with a fluorescent, lyphophilic probe (Kleusch et al., 2012, Slezak and Horan, 1989) and biotinylation (Franco 2009). Other methods include *in vitro* differential agglutination with anti-sera for a quantitative estimation of surviving cells in a sample, rosetting techniques and separation of cells according to age or volume (Mollison, 2005).

Radioactive $^{51}$Cr labeling of red cells (Gray and Sterling, 1950, Morel et al., 1978, Mollison, 2005) has remained the benchmark for labeling cells for determining *in vivo* survival and is still seen as a reliable method to predict the intravascular success of an incompatible transfusion (Mollison, 2005). However, a serious disadvantage of the use of $^{51}$Cr is that radiation is dangerous and particularly so in vulnerable patients such as fetuses or pregnant women and children and therefore is unethical for experimental purposes. Other disadvantages in its use for survival or sequestration studies include the fact that it elutes from cells at various individual rates, about 2% per day (Garby and Mollison, 1971, Christian et al., 1996); there is the so called early loss (Mollison and Veall, 1955) so the 10 minute value is 98% and the 24 hour value is 96%; the rate is affected by the technique of labelling (Szymanski and Valeri, 1970) and high doses are required.

Covalent biotinylation of red cells has established the utility and principles that biotinylation of red cells *per se* does not appear to significantly affect the survival of the red cell and show equivalent cell survivals to the $^{51}$Cr method and that biotin attached to red cells is

*prima facie* safe; utilized both in animals (Suzuki and Dale, 1987; Hoffmann-Fezer et al., 1993; Christian et al., 1996; Mock et al., 2009) and in humans (Cavill et al., 1988; Hoffmann-Fezer et al., 1997; Mock et al., 1999; Franco, 2009; Mock et al., 2009). Mice red cells have been used previously for *in vivo* direct biotinylation experiments (Hoffmann-Fezer et al., 1991).

However, there are perceived risks associated with the use of biotinylated cells with untargeted chemically modified surfaces (biotin reacts with available epilson amino and similar residues) and risks of any residual chemical reactants. Also survivals may be slightly reduced or affected by avidin (egg) intake (Cavill et al., 1988; Hoffmann-Fezer et al., 1997). Two succinimide esters, biotin-N-hydroxysuccinimide and caproylamidobiotin-N-hydroxysuccinimide were used for *in vivo* biotin labeling of mice erythrocytes (Hoffmann-Fezer et al., 1991). However, these esters must first be dissolved in an organic solvent, with the most commonly solvents used for this purpose being dimethyl sulfoxide (DMSO) and dimethyl formamide (DMF) but these chemicals have shown some toxicity issues for use with humans (Hanslick et al., 2009*;* Redlich et al., 1988). FSL biotin construct used here was shown to be non-toxic and dispersable in normal saline.

The U.S. Food and Drug Administration are assessing the use of biotin-labelled RBCs for *in vivo* use and current information indicates that *in vitro* biotinylated RBCs may be safely used in humans. It is believed by some that biotin labelling is reaching its promise and will be the cell survival reference method in the future since it is safe, inexpensive and simple to use with flow cytometry (Franco, 2009, Mock et al., 1999a; Mock et al., 2011) (Franco, 2009; Mock et al., 2011; Mock et al., 2012). Labelling with biotin has the following advantages over the use of $^{51}$Cr: radiation exposure is avoided and can be used in pregnant women and children; there is no label loss; and multiple populations can be studied with different biotin densities (Mock et al., 2011). Recent studies with diabetic patients used a biotin label (labeled *in vitro*) and magnetic isolation of cells to monitor red cells containing glycated haemoglobin (HbA1c), the presence of which depends on blood glucose levels and is dependent in part on RBC lifespan (Franco, 2009). Biotin labeling is used for multicolor flow cytometric analysis of labeled cells to follow a number of properties as red cells age in the circulation, including with Sickle Cell Disease and Haemoglobin F (HbF) content. Magnetic isolation allows additional haemoglobin analysis by HPLC as the labeled cells age, including time-dependent changes in HbF. Biotinylation and streptavidin conjugated magnetic beads have been used to measure and recover 80% transfused cells

in rabbits (Russo et al., 1992). Another important use of biotinylation is in the evaluation of RBC storage media over twenty-four hours since survival over that time is indictative of long term survival (Franco, 2009).

There is no evidence for toxicity from biotin (Mock, 2004), although the development of transient antibodies to biotin-labelled RBCs developed in 15% of adults who had received 50 mL biotinylated red cells (Cordle et al., 1999). These antibodies had no effect on the survival of the biotin-labeled cells (Mock 2012). However, in this study there was no production of anti-biotin in any of the FSL-biotin infused mice.

Biotinylation for murine red cell survival measurement and cell recovery in this research involved the use of the KODE™ Technology construct FSL-biotin with the diacyl nature of the lipid tail allowing cell membrane retention. This construct has previously been shown to create kodecytes by spontaneously simply and harmlessly, inserting into cell membranes of a variety of cells including blood cells (Blake et al., 2010) with the cells retaining normal vitality and functionality while gaining a new function due to the insertion of biotin. The FSL-biotin label method offers advantages over the radioactive $^{51}Cr$ method and other biotinylation methodology in that the construct is simple, non radioactive, safe to use, dissolves in water or saline, requires very little technology or technician time and FSL-biotin does not invoke antibody production.

A method using the biotin label to enable recovery of the transfused surviving cells was developed. In a proof-of-concept experiment, a series of biotin kodecyte suspensions was created to study the ability to recover circulating biotinylated kodecytes from whole blood samples. Using avidin-agarose columns and following centrifugation of a sample of whole blood directly applied to the top of the gel, the kodecytes could be separated from the sample. Unlike previous reports, where the biotinylated red cells were strongly bound to a matrix, and when released were coated with avidin and linker residues (Suzuki and Dale, 1987; Christian et al., 1996), the kodecytes were simply dissociated from the avidin resin by shear forces created by vortexing. The inserted FSL construct was physically extracted from the cell membrane, without disrupting the membrane. The ability to recover the surviving biotinylated A+biotin kodecytes from mice whole blood samples, post-transfusion, was demonstrated. The recovered kodecytes could be separated from the other red cells by the FSL-biotin label and could be shown to be A antigen positive against monoclonal anti-A reagent. This label thus proved to be a useful tool for isolating recovered kodecytes.

Before starting the trials for *in vivo* red cell survival, consideration had to be given to the volume of the red cell transfusion required for cell measurement. For the initial survival measurement experiments, it was decided to give a transfusion representing about 3.2% of the estimated circulating red cells, which involved transfusing 20 μL of kodecytes. This equates with a transfusion of about 70 mL of packed red cells to a 70 Kg human. This volume was chosen because it is adequate to determine intravascular haemolysis and unlikely to kill the recipient (Mollison, 2005) yet sufficient to avoid the problem of rapid reticulo-endothelial clearance of very small infusions (Mollison, 1959; Mollison, 1986; Mollison, 1989; Mollison, 2005).

In humans, 6 minutes is sufficient time for infused cells to be evenly distributed in circulation (Strumia et al., 1958; Mollison, 1989) so this time was taken as the initial time point for cell survival measurement. In the compatible controls it could be seen that starting from the estimated 3.2% transfusion there was an apparent immediate reduction to 2.3 - 2.9% within 6 minutes. This reduction probably represents a loss of fragile cells, described as the “fragile tail” (Mollison, 2005), potential differences between animals, and experimental error. Following the initial drop, kodecyte survival stabilized in all controls to a similar constant reduction over the following days. The shape of these compatible cell survival curves are in concordance with the literature (Mollison, 1986), albeit more rapid. It would be normally expected that 2% of mouse red cells would be lost per day (Hod et al., 2008); however compatible kodecytes were lost a rate of about 20% per day. It is possible this increased loss was due to the use of an *in vitro* cell storage solution for storing cells for transfusion and/or changes in their shape - a significant factor in determining *in vivo* red cell survival (Murdock et al., 2000). It remains to be established if kodecytes would have a better survival if stored in transfusion media.

Compatible murine biotin-only kodecytes were transfused into naive and anti-A positive mice, and their *in vivo* survival was determined with microsampling and subsequent fluorescence visualization of the kodecytes in blood films. Blinded paired photographs were then used to count fluorescent kodecytes and non-fluorescent red cells in order to calculate the percentage of kodecytes present.

The fluorescence subsequent to *in vitro* fluorescence labeling of biotin kodecytes gradually diminished in intensity over time, and after day 5 became unreliable to recognize. At the labeling concentration used, and assuming a straight-line loss, this represented about 20% loss per day of FSL constructs from the kodecyte. The elution of FSL-biotin constructs to

the surroundings does not affect the kodecyte count, because the kodecytes are still identifiable, albeit with decreased strength in their fluorescence signal. FSL constructs, with a similar construction to glycolipids, were slowly lost to the plasma as they exchange from the cell into the lipid compartment when present in a cell in a lipid environment such as plasma – as do Lewis glycolipids (Henry et al., 1995). This loss of incompatible antigens will eventually render the kodecytes back into normal cells. However, it should be appreciated that even intrinsic incompatible antigens are lost in the presence of incompatible polyclonal antibodies (Zimring et al., 2007). From this perspective kodecytes differ significantly from covalently biotinylated or $^{51}$Cr -labelled cells with intrinsic antigens, which are able to monitor cell survival for weeks. The FSLs lost from the cell membranes will label circulating lipid particles – they will not preferentially relabel cells (Blake et al., 2011). The amount of FSL on the transfused kodecytes in this setting was inadequate to cause any significant antibody neutralization demonstrated when comparing the survival rates of A+ kodecytes after incompatible transfusions with rates after compatible A+ kodecyte transfusions in mice having had FSL-A neutralization of anti-A (Figure 23 and 30).

A comparison of the survival rates of biotin-only kodecytes with the GB3+biotin kodecytes transfused into anti-A positive and negative animals were very similar, showing that dual kodecyte labeling did not influence survival outcomes. Similarly A+biotin kodecytes transfused into anti-A negative animals gave similar outcomes to the benign GB3 +biotin antigen controls, indicating that the blood group A antigen *per se* does not result in reduced cell survival. When A+biotin kodecytes were transfused into anti-A positive animals a rapid reduction in expected circulating kodecyte count occurred within 6 minutes, appeared to stabilize, and then reduce at the same rate as compatible kodecytes. This result is as predicted by the literature (Mollison, 1989), where a rapid antibody-mediated removal of incompatible cells occurs. The post 6 minute surviving kodecytes, potentially C3d coated (Mollison, 1989), were then able to survive at a rate similar to compatible kodecytes (Atkinson and Frank, 1974; Lachmann et al., 1983).

These results clearly show the principles that incompatible blood group A murine kodecytes artificially created by KODE™ Technology appear to follow the expected antibody-mediated *in vivo* survival profiles when monitored by the inclusion of a secondary FSL-biotin label in the same cell, followed by subsequent visualization of the kodecytes by fluorescence microscopy. Kodecytes probably should only be used to determine cell

survival within the first 24 hours - from a survival perspective, this time is sufficient (Mollison, 2005).

The third hypothesis that FSL-A might neutralize anti-A to allow incompatible cell survival after transfusion was demonstrated. FSL constructs have properties like glycolipids, a propensity to associate with plasma (Rohr et al., 1980; Schwarzmann, 2001), and an ability to inhibit viruses and toxins (Harrison et al., 2010), and so it was considered a possibility that these constructs could also be used to inhibit ABO antibodies and allow incompatible transfusions. Attempting to neutralize ABO antibodies *in vivo* has been experimentally attempted previously, where intravenous trisaccharide infusions allowed for experimental incompatible transplants in baboons (Cooper et al., 1993) and were used in treatment of ABO hemolytic disease of the newborn (Romano et al., 1994). There are also reports of attempting to neutralize recipient ABO antibodies *in vivo* using red cell antigens and then giving a deliberate ABO incompatible transplant (Nussbaumer et al., 1995; Scholl et al., 2005). However, the antibody titres must be bought down to a steady level of <1:8 before transplantation is attempted (Sassi et al., 2011, Tyden et al., 2007). Pre-transplant reduction of the titre of circulating antibodies (<1:8) in the plasma of those patients receiving ABO incompatible renal transplants, at the time of transplant, appears to allow an accommodation-type survival of the graft with a reduction of the need for immunosuppressive drug treatment and almost no risk of rejection (Winters et al., 2004).

Historical evidence has been established for neutralizing the complement-activating antibodies of the Lewis blood group system with glycolipids or blood group substances and then successfully transfusing incompatible red cells (Hossaini, 1972; Mollison et al., 1963; Pelosi et al., 1974). Lewis positive plasma, which contains only about 2 mg/mL of Lewis glycolipids (Rohr et al., 1980; Hammar et al., 1981), will effectively neutralize circulating Lewis antibodies and allow for the subsequent transfusion of Lewis positive red cells (Hossaini,1972; Mollison et al., 1963; Pelosi et al., 1974). A significant concern using the Lewis blood group system is that the neutralization process can induce a secondary antibody response (Mollison et al., 1963). In the case of Lewis incompatibility, this is not a problem as the transfused cells become compatible through antigen loss (Mollison et al., 1963). But for cell-bound antigens such as ABO, this secondary response would pose a significant issue (despite the fact that cell-bound antigens may undergo antigenic modulation (Zimring et al., 2005; Zimring et al., 2009). However, the material historically used to neutralize Lewis antibodies was plasma containing glycolipids and glycoproteins

(Andorka et al., 1974; Pelosi et al., 1974) or Lewis blood group substance glycoprotein (Mollison et al., 1963), and not purified glycolipids. Plasma alone (glycolipids plus glycoproteins) appeared not to change the antibody titre substantially (Andorka et al., 1974; Pelosi et al., 1974) but the use of purified glycoprotein Lewis substance neutralization resulted in a substantial secondary antibody response (Mollison et al., 1963).

Anti-A positive mice provided the model for demonstration *in vivo* of the neutralization of circulating anti-A after infusion of FSL-A and the subsequent measurement of the survival of transfused incompatible A+biotin kodecytes. These results were compared to incompatible A+biotin kodecyte survival in anti-A mice that did not receive FSL-A neutralization and to kodecyte survival in compatible, anti-A negative mice.

Baseline *in vitro* experiments determined the concentration of FSL required for neutralization of circulating anti-A. The preferred association of injected FSL-A with plasma rather than for cells was demonstrated both *in vitro* (Table 8) and *in vivo* (Table 13) where about 40x more FSL-A was required in serum to bring about the same level of transformation as seen with cells modified in PBS. It was estimated that only 2.5% of the FSLs in plasma will associate with red cells, and as a consequence, FSLs which dissociate from the kodecytes are unlikely to detectably relabel other circulating cells. The association with cells, so-called *in vivo* transformation, occurred regardless of the presence or absence of anti-A, and always where the FSL-A dose was greater than 2 mg/mL of plasma (Table 13). This is similar to the phenomenon described when O cells took up B antigens in the circulation of B patients or when Lewis positive plasma is transfused to Lewis negative individuals (Sneath and Sneath, 1955; Renton and Hancock, 1962).

The *in vitro* experiments suggest that at least 5-10mg of FSL-A per mL of plasma would be required to ensure significant antibody neutralization of human group O plasma with high titre anti-A (Table 10). Survival of incompatible A kodecytes in the *in vivo* mouse model suggested doses as low as 4 mg/mL of plasma could be adequate (Table 13). Similarly, *in vivo* kodecytes spontaneously forming in anti-A positive animals with levels of FSL-A greater than 1 mg/mL, were tolerated without any evidence of cell destruction or sensitization. These *in vivo* formed kodecytes are probably tolerated because of their much lower antigen density than *in vitro* kodecytes. In contrast, natural blood group cells appeared to be less *in vitro* tolerant of FSL-A neutralized antibody (Table 10) probably

because they have more A antigen than A kodecytes, and the natural antigens may also present in a bivalent configurations making them more susceptible to antibody binding.

However, results from *in vitro* neutralization assays, where the antibody is still present but neutralized by FSL-A, may not necessarily reflect *in vivo* neutralization dynamics. As a consequence, the speculated actual dose that could prevent an incompatible transfusion reaction in humans is probably somewhere between 1 and 10 mg per mL of plasma. If the dose was 5 mg/mL of plasma (or 0.2 g/Kg body mass) then this would equate with a 13.5 g FSL-A infusion for a 70 Kg human. This dose is compatible with intravenous infusion practice where for example 0.7 g/Kg body mass of saccharides in the form of glucose (Moreno et al., 2001) or 2.5 g/Kg body mass of lipid in the form of intralipid (Josephson, 2004) can be safely given to a human. Doses of A and B trisaccharides at 1-2 g per Kg body mass have been safely given to baboons to prevent incompatible transplant rejection (Cooper et al., 1993) and about 0.025 mg/Kg body mass to human newborns (Romano et al., 1987a).

The antigenic and neutralizing effect of FSL-A on anti-A was demonstrated in six anti-A positive mice when A +biotin kodecytes transfused by subclavian injection had a normal cell survival (and the anti-A status was negative for up to 24 hours after the FSL-A infusion (Table 15) compared to the much reduced cell survival rate in non-neutralized mice due to anti-A presence (Figure 30). Studies with related $^{125}$I radiolabeled FSLs injected into mice show the constructs are cleared from the circulation within 24 hours (Hadac et al., 2011) and this appears to correlate with the *in vivo* antibody neutralization data which shows reappearance of detectable antibody at about 24 hours (Table 15). Simple trisaccharides are excreted rapidly (after 8 hours) in urine (Romano et al., 1987b), and the FSL construct neutralant appears to be efficient up to 24 hours (Table 14).

The *in vivo* experiments showed the neutralizing effects of administration of the FSL construct immediately prior to the transfusion of incompatible blood. This of course, would only be of value if a deliberate transfusion of incompatible blood (or organs) was anticipated.

One or two weeks after the infusion of FSL-A and the first incompatible kodecyte transfusion, when the FSL-A had cleared from the circulation and anti-A was detectable, the same animals were given a re-challenge transfusion of incompatible A+biotin kodecytes. This series was designed to demonstrate that the mice, whose neutralized

anti-A allowed normal cell survival, actually did still have an antibody capable of causing destruction of the infused incompatible cells. The fact that it had been previously established that FSLs did not cause immune stimulation of antibody meant that antibody present was not stimulated by the infusion itself.

As expected, most animals destroyed the incompatible kodecytes although one animal showed normal kodecyte survival and another showed moderate survival. Kodecyte destruction was rapid in four of the anti-A positive mice Cluster IV (i) reaching 0.2% within 6 minutes when the second A+biotin kodecyte transfusion was given without prior FSL-A anti-A neutralization.

Although still unproven, it was speculated that possibly, to some extent, tolerance may have been induced in those animals with normal and moderate survival; either by a reduction in available antibody as a consequence of clearance or through a down-regulation of the immune response (potentially as a consequence of clonal deletion). The other explanation was that the cells survived due to the presence of a lowered level of antibody or complement subsequent to the first transfusion since an incompatible transfusion itself also effectively reduces antibody and complement levels thereby temporarily mitigating the reaction (Chaplin, 1959; Mollison, 2005).

The fourth hypothesis that FSL constructs could potentially induce tolerance was tested by measuring the consequences of infusing FSL-A by direct circulatory infusion into animals one week before immunization with salivary blood group A substance.

B-cell tolerance to T cell independent carbohydrate antigens, such as the A and B histo-blood group antigens, occurs by clonal deletion (Rieben. et al., 1992) and inactivation when the antigens are membrane-bound, (Hartley et al., 1993; Klinman, 1996, West, 2006); when soluble antigen is present (Lang and Nemazee, 2000; Galili, 2004) in high dose (Janeway, 2005); and when concentrated in tissues (Portoukalian, 2000). Since B cell receptors appear to be more specific for the repetitive structure of carbohydrate antigens, B cell tolerance could be the important mechanism for control of anti-carbohydrate self-activity (Zinkernagel and Hengartner, 2001; Mohiuddin et al., 2003). Current regimes rate pre-transplant immunosuppressive treatment to eliminate B cell immunity as more important than antibody removal and splenectomy to enable ABO incompatible kidney transplant (Takahashi and Saito, 2012). Since glycolipid antigens are generally regarded as T independent antigens, B cell tolerization may be of clinical

significance for the induction of tolerance to ABO-incompatible blood group antigens on allografts or to gal epitopes on xenografts.

Certain conditions need to be met before antigenic tolerance can develop. Antigen administration that fails to induce activation and or migration of DC leads to tolerance (Banchereau and Steinman, 1998). Current research suggests full tolerance has only been achieved when the recipient is immunologically incompetent or immature and when there is full replacement of host lymphocytes by donor lymphocytes accomplished by immunosuppression and stem cell transplantation (Touraine and Sanhadji, 2011). Immature cells may not have yet have receptors and so cannot recognize antigen (Pike et al., 1982). Treatment with Heat-Aggregated Human Gamma Globulin, a form of Human Gamma Globulin HGG that is highly immunogenic in euthymic adult mice, is capable of inducing specific unresponsiveness when injected into neonatal animals resulting in the induction of tolerance in T as well as B cells (Etlinger and Chiller, 1979).

The route, dose and nature of the antigen appear to be crucial with intraperitoneal or intravenous routes more effective than subcutaneous routes in inducing tolerance (Liblau et al., 1997; Arnold, 2002) although high dose soluble peptide given subcutaneously can invoke clonal deletion (Harrison, 1992). Research has shown that both immature and mature B cells can be inactivated when constantly exposed to soluble antigen in high dose (Janeway, 2005) and with glycolipids in persistently high concentration in tissues (Griffiths, 2005, Portoukalian, 2000). Tolerance induction on memory carbohydrate anti-Gal B cells was shown to be a time dependent process of at least 10 days (Zinkernagel, 1996). Continued tolerance appears to require the continued exposure to the tolerized antigen in solid organ transplants (Chen et al., 1996). The FSL-A molecule introduced by an intravenous route remained detectable on red cells in the circulation for between 2 and 3 days (Table 13), but the exact period of time the FSL-A constructs will remain in plasma is uncertain.

FSLs were used at various concentrations; 50, 100 and 200 mg/Kg body mass, introduced into immunologically competent, six week old mice by both subcutaneous (with adjuvant) and intravenous injection. Intravenous infusion of the FSL-A molecule did not induce tolerance to the A antigen epitope since production of anti-A occurred after the challenge of a secondary immunization with salivary A antigen.

The FSL construct administered subcutaneously was not immunogenic, as discussed previously, and may have been ignored by the immune system. Glycolipids must be first recognised by CD1d receptors (Lingwood et al., 1980; De Libero, 2004; Koch et al., 2005) or specific CD4 (+) T cells (Christiansen et al., 2011) to activate an immune response. CD1d receptors on NK T cells engage with glycolipid antigens producing T helper cell (Th1 and Th2) cytokines which either up-or down-regulate immune responses by promoting the secretion of other immune regulatory cytokines (Godfrey and Kronenberg, 2004) or inducing tolerance (Mocikat et al., 2003). If glycolipid antigens cannot bind to host cells or are not available in the form of aggregates that are readily seen by APCs and the CD1 receptors (Kovalenko et al., 2007), inactivation of these membrane receptors leads to tolerance (Banchereau and Steinman, 1998) or ignorance of apparent self antigens (De Libero, 2004). It is speculated that glycolipids cause down-regulation due to B cell clonal deletion (Harrison and Hafler., 2000) after prolonged immunization with glycolipids has resulted in decreased specific antibody titres (Alving and Richards, 1977; Lingwood et al., 1980).

As previously stated, the crystal structure of the CDd1 (Koch et al., 2005) showed that the spacer of the FSL construct would probably impair its ability to bind within the appropriate grooves in the CD1d molecule and the antigens would therefore be ignored by the immune response system and could thus be deemed to be tolerated through ignorance.

Despite the lack of positive results from this preliminary experiment, where the FSL-A infused mice made anti-A and were not tolerised, there is a possibility that FSL constructs may in some circumstances, be able to down regulate the immune system by inducing auto-regulation, thereby causing down-regulation of specific antibody production potentially as a consequence of clonal deletion. The two Cluster IV (ii) mice in the incompatible transfusion re-challenge experiments (Figure 32, Table 15) potentially showed this effect with anti-A titres 8 and < 128. This compared to all other titre results (16) being >128 experiments (Figure 32, Table 15). It is not known whether this reduced level of antibody in these two animals, post incompatible transfusion, was an indirect consequence of FSL-A treatment, or a natural variation. This area requires further investigation. (All serum anti-A titres measured were >128 except in these two animals). However unfortunately, these particular animals did not have pre-transfusion anti-A titres measured.

Future research using FSLs would include clinical trials to show they are non-hazardous in humans. While the molecules are apparently safe *in vivo* in mice and do not alter red cell function or viability, they have only been studied with human cells *in vitro*. Research similar to that carried out in this thesis but using human trials, *in vivo*, with FSLs and kodecytes, is required to maximize the benefits of the technology.

The ability to simply and safely modify a population of blood cells with both a specific antigen of interest and an identification-recovery label with KODE™ constructs could provide the diagnostic potential for tracking and tracing cells *in vivo* and *in vitro.* FSL-biotin could be easily developed into a simple cell survival assay, based solely on the ability to recover the expected number of biotin kodecytes post transfusion. Survival measurement with FSL-biotin visualization could be improved by utilizing the high performance features of a flow cytometer other than microscopy including taking advantage of its sorting features, particularly if the FSL construct used was a FSL-fluorophore (Hult et al., 2008). A comparison of two populations, one labeled with FSL-biotin and the other with $^{51}Cr$ would be able to establish the comparative merits of both techniques and the simplicity of using FSLs. Equally possible, is the use of radiolabeled FSL constructs and real-time bioimaging for example, to measure phagocytosis to visualize cell removal. The use of different solutions for the storage of kodecytes for transfusion and their effect on the survival rate of kodecytes needs to be compared to ensure optimum conditions for kodecyte survival.

There is the potential to use FSLs clinically in man, to simply determine cell survival pre-incompatible transfusion and also to neutralize circulating antibodies to allow incompatible cell survival. The neutralizing effects of administration of the FSL construct immediately prior to the transfusion of incompatible blood would be of value if a deliberate transfusion of incompatible blood (or organs) was anticipated.

If an incompatible transfusion was accidentally given, then FSL intervention to mitigate the reaction would be of value if surviving incompatible cells were present and pending destruction could be prevented, particularly if they can also dissociate bound antibody as suggested by the *in vitro* data (Table 11) and the literature (Romano et al., 1994). This is often the case, as the ABO incompatiblity is to some extent self-limiting, due to the consumption of antibody and/or complement (Mollison, 2005).

The ultimate goal in organ transplantation would be to induce donor-specific tolerance by modification of either the donor organ or of the host immune response. In this scenario,

FSL constructs could be used to eliminate B cell clones (by clonal deletion) pre-transplant thus preventing antibody stimulation from the donor antigens. This may manipulate accomodation tolerance to allow the transplant of partially mismatched or xenogenic organs eliminating the need for immunosuppression and its adverse side effects (Lechler et al., 2005) to counter acute allograft rejection. If the graft can survive for the first 1-2 weeks without anti-graft antibody production it appears that antibody-mediated rejection (AMR) is prevented and accomodation tolerance occurs (Takahashi and Saito, 2012). Autologous human blood lymphocytes could be manipulated with non-immunogenic FSLs to express the carbohydrate antigens needed to induce tolerance (Mohiuddin et al., 2003).

It has been demonstrated that B cells can be made immunologically tolerant by multivalent antigens *in vivo* and *in vitro* (Nossal, 1993), if they are subjected to antigen at the first emergence of the BCR membrane receptors (Pike et al., 1982). The use of FSLs with immature B cells in neonates and infants genetically or familiarly destined to require future transplant, could render them tolerant to antigens of any ABO type. This could mean they could receive future transfusions or transplants without risk of rejection (Griffiths, 2005; West, 2006) and would be a major immunological achievement.

Immunotherapy using FSL constructs could be designed to manipulate the cell surface phenotype of antigen-presenting cells to change the immune response, for example, to convert antigens from T cell activators into inhibitors or vice versa. FSLs could provide immunotherapeutic possibilities with chimeric polypeptides which have been shown to induce tolerance with renal and other organ transplantation (Tykocinski and Kaplan 1993) and with haematopoeitic stem cells which have been used to express specific antigens resulting in induction and prolonged maintenance of functional effector T cells (Denning et al., 2011). Since HSCs can settle in the thymus to become T cells they could be used with FSLs to induce antigen tolerance. FSL constructs could also be used with cells in the same way that goblet cells in the lumen were used to deliver soluble antigen and then measured with *in vivo* imaging (McDole et al., 2012). Both mice and humans have NK cell receptors (Shimizu et al., 2005) that recognise self-MHC class I glycolipid antigens on cells which lead to NK tolerance and the detection of an altered self-MHC I antigen can lead to removal of that antigen (Nakamura and Seaman, 2001). Antigenic FSLs could be used in this way to encourage deletion of targeted cells.

FSL-constructs could be used to manipulate cell membranes to potentially encourage immune maturation and recognition. The presence of antigenic sugars on the surface of

commensal bacteria in the gut, such as Polysaccharide A on *Bacteroides fragilis,* has been shown to be critical in directing the maturation of the immune response. The presence of this antigen introduced orally into the gut of patients with autoimmune disease has been shown to be beneficial in reducing the effect of multiple sclerosis and rheumatoid arthritis (Eynon et al., 2005, Mazmanian et al., 2005). More than 150 diseases are either accepted or suspected as autoimmune – diseases where the body makes antibody to self such as lupus erythematosis, alopecia aereata, diabetes type 1, ulcerative colitis, rheumatoid arthritis, celiac disease, multiple sclerosis. There may be a potential future treatment for autoimmune disease using surface-inserted FSL constructs in symbiotic microbes to affect the immune reactions in a manner much more simply than genomic manipulation.

There are many opportunities to use FSL constructs in an *in vitro* setting. They can be made to detect rare ABO incompatible reagent antibodies and used for cell recovery. FSL constructs mimic the bioactive components present on biological surfaces, and present them in novel ways. The potential to change the functional head (F) of KODE™ constructs allows a virtually unlimited range of molecules including lipids, peptides, proteins, polymer, or other chemical grouping, with a range of spacers (S) and different lipid tails (L) and the spacer has been specifically designed to be inert with serum. FSLs can function as liposomes, micelles and lipid particles and can be attached to many surfaces such as hydrophobic or hydrophilic membranes/fibres, paper, cotton, silk, glass, Teflon, silica, avidin-coated beads, and living organisms in biological solutions. FSL peptides would be expected to have a different immune response and further research using these would perhaps allow these constructs to be used as vaccines since proteins are more immunogenic than glycolipids.

## Conclusion

The research in this thesis provided insights into the use of carbohydrate FSLs to manipulate the immune response. In particular, the technology has the potential to:

- provide an alternative red cell survival assay with a label that can be measured with fluorescent microscopy or flow cytometry for *in vivo or in vitro* research.
- easily and safely manipulate red cell membranes with specific antigen insertion *in vivo or in vitro,* allowing cell survival measurement after compatible or incompatible transfusion, cell tracking, and to recover of the manipulated cells from circulation.
- neutralize circulating antibody to allow incompatible transfusion or mitigate the effects of autoimmune disease.
- investigate the possibility of tolerance induction for use in transplantation or autoimmune disease.

# REFERENCES


Adams EW, Ratner DM, Seeberger PH, Hacohen N. 2008. Carbohydrate-mediated targeting of antigen to dendritic cells leads to enhanced presentation of antigen to T cells. Chembiochem 9:294-303.

Alving CR, Richards RL. 1977. Immune reactivities of antibodies against glycolipids--II comparative properties, using liposomes, of purified antibodies against mono-, di- and thihexosyl ceramide haptens. Immunochemistry 14:383-389.

Andorka DW, Arosemena A, Harris JL. 1974. Neutralization in vivo of Lewis antibodies. Report of two cases. Am J Clin Pathol 62:47-51.

Arnold B. 2002. Levels of peripheral T cell tolerance. Transpl Immunol 10:109-114.

Aruffo A, Hollenbaugh D. 2001. Therapeutic intervention with inhibitors of co-stimulatory pathways in autoimmune disease. Curr Opin Immunol 13:683-686.

Astronomo RD, Burton DR. 2010. Carbohydrate vaccines: developing sweet solutions to sticky situations? Nat Rev Drug Discov 9:308-324.

Atkinson JP, Frank MM. 1974. Studies on the in vivo effects of antibody. Interaction of IgM antibody and complement in the immune clearance and destruction of erythrocytes in man. J Clin Invest 54:339-348.

Avery OT, Goebel WF. 1929. I.Chemo-immunological studies on congugated carbohydrate proteins:II. Immunological specificity of synthetic sugar-protein antigens. J Exp Med 50:533-550.

Bagley J, Bracy JL, Tian C, Kang ES, Iacomini J. 2002. Establishing immunological tolerance through the induction of molecular chimerism. Front Biosci 7:d1331-1337.

Bagnis C, Chapel S, Chiaroni J, Bailly P. 2009. A genetic strategy to control expression of human blood group antigens in red blood cells generated in vitro. Transfusion 49:967-976.

Bagnis C, Chiaroni J, Bailly P. 2011. Elimination of blood group antigens: hope and reality. British Journal of Haematology 152:392-400.

Banchereau J, Steinman RM. 1998. Dendritic cells and the control of immunity. Nature 392:245-252.

Barr K, Diegel O, Parker S, Bovin N, Henry S. 2010. Function-Spacer-Lipid (FSL) constructs enable inkjet printing of blood group antigens. FEBS 277:235.

Barrie EK, Fraser RH, Munro AC, Williamson AR, Hamilton EA, Mitchell R. 1983. Monoclonal anti-B produced by the immunization of mice with soluble salivary glycoproteins. J Immunogenet 10:41-44.

Bennett B, Check IJ, Olsen MR, Hunter RL. 1992. A comparison of commercially available adjuvants for use in research. J Immunol Methods 153:31-40.

Berneman ZN, van Bockstaele DR, Uyttenbroeck WM, Van Zaelen C, Cole-Dergent J, Muylle L, Peetermans ME. 1991. Flow-cytometric analysis of erythrocytic blood group A antigen density profile. Vox Sang 61:265-274.

Beyth S, Borovsky Z, Mevorach D, Liebergall M, Gazit Z, Aslan H, Galun E, Rachmilewitz J. 2005. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. Blood 105:2214-2219.

Billington WD. 2003. The immunological problem of pregnancy: 50 years with the hope of progress. A tribute to Peter Medawar. J Reprod Immunol 60:1-11.

Bilsborough J, George TC, Norment A, Viney JL. 2003. Mucosal CD8alpha+ DC, with a plasmacytoid phenotype, induce differentiation and support function of T cells with regulatory properties. Immunology 108:481-492.

Blake D, Lan A, Love D, Bovin N, Henry S. 2010. Fluorophore-kodecytes-fluorescent function-spacer -lipid (FSL) modified cells for in vitro and in vivo analyses FEBS journal abstract C3.12:199.

Blake DA, Bovin NV, Bess D, Henry SM. 2011. FSL Constructs: A Simple Method for Modifying Cell/Virion Surfaces with a Range of Biological Markers Without Affecting their Viability. J Vis Exp:e3289.

Blomberg L, Bratt T, Chester MA, Hansson B. 1993. Improved removal of anti-A and anti-B antibodies from plasma using blood-group-active haptens. Vox Sang 65:126-135.

Bollyky PL, Wilson SB. 2004. CD1d-restricted T-cell subsets and dendritic cell function in autoimmunity. Immunol Cell Biol 82:307-314.

Bonifaz LC, Bonnyay DP, Charalambous A, Darguste DI, Fujii S-I, Soares H, Brimnes MK, Moltedo B, Moran TM, Steinman RM. 2004. In Vivo Targeting of Antigens to Maturing Dendritic Cells via the DEC-205 Receptor Improves T Cell Vaccination. The Journal of Experimental Medicine 199:815-824.

Boon T, Coulie PG, Van den Eynde B. 1997. Tumor antigens recognized by T cells. Immunol Today 18:267-268.

Borg NA, Wun KS, Kjer-Nielsen L, Wilce MCJ, Pellicci DG, Koh R, Besra GS, Bharadwaj M, Godfrey DI, McCluskey J, Rossjohn J. 2007. CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor. Nature 448:44-49.

Borman S. 2004. Chemical and Engineering News 82:31-35.

Borowitz MJ, Craig FE, Digiuseppe JA, Illingworth AJ, Rosse W, Sutherland DR, Wittwer CT, Richards SJ. 2010. Guidelines for the diagnosis and monitoring of paroxysmal nocturnal hemoglobinuria and related disorders by flow cytometry. Cytometry B Clin Cytom 78:211-230.

Brain MC, Prevost JM, Pihl CE, Brown CB. 2002. Glycophorin A-mediated haemolysis of normal human erythrocytes: evidence for antigen aggregation in the pathogenesis of immune haemolysis. Br J Haematol 118:899-908.

Breimer ME, Karlsson H, Karlsson KA, Nilson K, Samuelsson BE, Stromberg N. 1988. Structures of the eight- to nine-sugar glycolipids of human blood group A erythrocytes. Carbohydr Res 178:111-120.

Brigl M, Brenner MB. 2004. CD1: antigen presentation and T cell function. Annu Rev Immunol 22:817-890.

Bruyns C, Urbain-Vansanten G, Planard C, Vos-Cloetens C, Urbain J. 1976. Ontogeny of mouse B lymphocytes and inactivation by antigen of early B lymphocytes. Proc Natl Acad Sci U S A 73:2462-2466.

Bundle DR. 2007. A carbohydrate vaccine exceeds the sum of its parts. Nat Chem Biol 3:605-606.

Bundle DR, Gidney MA, Kassam N, Rahman AF. 1982. Hybridomas specific for carbohydrates; synthetic human blood group antigens for the production, selection, and characterization of monoclonal typing reagents. J Immunol 129:678-672.

Buskas T, Ingale S, Boons GJ. 2005. Towards a fully synthetic carbohydrate-based anticancer vaccine: synthesis and immunological evaluation of a lipidated glycopeptide containing the tumor-associated tn antigen. Angew Chem Int Ed Engl 44:5985-5988.

Caspi RR. 2008. Immunotherapy of autoimmunity and cancer: the penalty for success. Nat Rev Immunol 8:970-976.

Cavill I, Trevett D, Fisher J, Hoy T. 1988. The measurement of the total volume of red cells in man: a non-radioactive approach using biotin. Br J Haematol 70:491-493.

Chan J, Ban EJ, Chun KH, Wang S, Bäckström BT, Bernard CCA, Toh B-H, Alderuccio F. 2008. Transplantation of Bone Marrow Transduced to Express Self-Antigen Establishes Deletional Tolerance and Permanently Remits Autoimmune Disease. The Journal of Immunology 181:7571-7580.

Chaplin H, Jr. 1959. Studies on the survival of incompatible cells in patients with hypogammaglobulinemia. Blood 14:24-36.

Chen, Colditz IG, Glenn GM, Tsonis CG. 2002. Effect of transcutaneous immunization with co-administered antigen and cholera toxin on systemic and mucosal antibody responses in sheep. Vet Immunol Immunopathol 86:177-182.

Chen ZK, Cobbold SP, Waldmann H, Metcalfe S. 1996. Amplification of natural regulatory immune mechanisms for transplantation tolerance. Transplantation 62:1200-1206.

Chen HT, Kabat E, Lundblad A, Ratcliffe R. 1987. Nucleotide and translated amino acid sequences of cDNA coding for the variable regions of the light and heavy chains of mouse hybridoma antibodies to blood group A and B substances. J Biol Chem 262:13579-13583.

Chen AM, Scott MD. 2001. Current and future applications of immunological attenuation via pegylation of cells and tissue. BioDrugs 15:833-847.

Christian JA, Rebar AH, Boon GD, Low PS. 1996. Methodologic considerations for the use of canine in vivo aged biotinylated erythrocytes to study RBC senescence. Exp Hematol 24:82-88.

Christiansen D, Vaughan HA, Milland J, Dodge N, Mouhtouris E, Smyth MJ, Godfrey DI, Sandrin MS. 2011. Antibody responses to glycolipid-borne carbohydrates require CD4+ T cells but not CD1 or NKT cells. Immunol Cell Biol 89:502-510.

Chu Q, Moreland RJ, Gao L, Taylor KM, Meyers E, Cheng SH, Scheule RK. 2010. Induction of immune tolerance to a therapeutic protein by intrathymic gene delivery. Mol Ther 18:2146-2154.

Clatworthy MR. 2011. Targeting B cells and antibody in transplantation. Am J Transplant 11:1359-1367.

Clausen H, Stroud M, Parker J, Springer G, Hakomori S. 1988. Monoclonal antibodies directed to the blood group A associated structure, galactosyl-A: specificity and relation to the Thomsen-Friedenreich antigen. Mol Immunol 25:199-204.

Cobbold SP, Qin S, Leong LY, Martin G, Waldmann H. 1992. Reprogramming the immune system for peripheral tolerance with CD4 and CD8 monoclonal antibodies. Immunol Rev 129:165-201.

Cooper DK, Ye Y, Niekrasz M, Kehoe M, Martin M, Neethling FA, Kosanke S, DeBault LE, Worsley G, Zuhdi N, et al. 1993. Specific intravenous carbohydrate therapy. A new concept in inhibiting antibody-mediated rejection--experience with ABO-incompatible cardiac allografting in the baboon. Transplantation 56:769-777.

Cordle DG, Strauss RG, Lankford G, Mock DM. 1999. Antibodies provoked by the transfusion of biotin-labeled red cells. Transfusion 39:1065-1069.

Corinti S, Palma RD, Fontana A, Gagliardi MC, Pini C, Sallusto F. 1997. Major Histocompatibility Complex-independent Recognition of a Distinctive Pollen Antigen, Most Likely a Carbohydrate, by Human CD8+ alpha /beta T Cells. J Exp Med 186:899-908.

Cretin N, Iacomini J. 2002. Immunoglobulin heavy chain transgenic mice expressing Galalpha(1,3)Gal-reactive antibodies. Transplantation 73:1558-1564.

Crew RJ, Ratner LE. 2010. ABO-incompatible kidney transplantation: current practice and the decade ahead. Curr Opin Organ Transplant 15:526-530.

Crocker PR, Feizi T. 1996. Carbohydrate recognition systems: functional triads in cell-cell interactions. Curr Opin Struct Biol 6:679-691.

Cruz LJ, Tacken PJ, Fokkink R, Figdor CG. 2011. The influence of PEG chain length and targeting moiety on antibody-mediated delivery of nanoparticle vaccines to human dendritic cells. Biomaterials 32:6791-6803.

Cui Y, Kelleher E, Straley E, Fuchs E, Gorski K, Levitsky H, Borrello I, Civin CI, Schoenberger SP, Cheng L, Pardoll DM, Whartenby KA. 2003. Immunotherapy of established tumors using bone marrow transplantation with antigen gene-modified hematopoietic stem cells. Nature Medicine 9:952.

Daniels BF, Nakamura MC, Rosen SD, Yokoyama WM, Seaman WE. 1994. Ly-49A, a receptor for H-2Dd, has a functional carbohydrate recognition domain. Immunity 1:785-792.

Dehoux JP, Gianello P. 2009. Accommodation and antibodies. Transpl Immunol 21:106-110.

De Libero G. 2004. Immunology. The Robin Hood of antigen presentation. Science 303:485-487.

De Libero G, Mori L. 2006. How T lymphocytes recognize lipid antigens. FEBS Lett 580:5580-5587.

De Libero G, Mori L. 2010. How the immune system detects lipid antigens. Prog Lipid Res 49:120-127.

Delamarre L, Pack M, Chang H, Mellman I, Trombetta ES. 2005. Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. Science 307:1630-1634.

Denning WL, Xu J, Guo S, Klug CA, Hel Z. 2011. Limited transplantation of antigen-expressing hematopoietic stem cells induces long-lasting cytotoxic T cell responses. PLoS ONE 6:e168

Do Canto FB, Junior CL, Teixeira IA, Bellio M, Nóbrega A, Fucs R. 2008. Susceptibility of neonatal T cells and adult thymocytes to peripheral tolerance to allogeneic stimuli. Immunology 125:387-396.

Edelman L, Bach JF, Reviron J. 1984. Monoclonal antibodies against blood group antigens. Dev Biol Stand 57:43-47.

Edwards JC, Leandro MJ, Cambridge G. 2002. B-lymphocyte depletion therapy in rheumatoid arthritis and other autoimmune disorders. Biochem Soc Trans 30:824-828.

Ehlenberger AG, Nussenzweig V. 1977. The role of membrane receptors for C3b and C3d in phagocytosis. J Exp Med 145:357-371.

Endres RO, Grey HM. 1980. Antigen recognition by T cells. II. Intravenous administration of native or denatured ovalbumin results in tolerance to both forms of the antigen. J Immunol 125:1521-1525.

Etlinger HM, Chiller JM. 1979. Maturation of the lymphoid system. I. Induction of tolerance in neonates with a T-dependent antigen that is an obligate immunogen in adults. J Immunol 122:2558-2563.

Eynon EE, Zenewicz LA, Flavell RA. 2005. Sugar-Coated Regulation of T Cells. Cell 122:2-4.

Fairchild PJ, Cartland S, Nolan KF, Waldmann H. 2004. Embryonic stem cells and the challenge of transplantation tolerance. Trends in Immunology 25:465-470.

Falk P, Hoskins LC, Lindstedt R, Svanborg C, Larson G. 1991. Deantigenation of human erythrocytes by bacterial glycosidases--evidence for the noninvolvement of medium-sized glycosphingolipids in the Dolichos biflorus lectin hemagglutination. Arch Biochem Biophys 290:312-319.

Feizi T. 2000. Carbohydrate-mediated recognition systems in innate immunity. Immunol Rev 173:79-88.

Fletcher A, Harbour C, De Zwart R. 1984. monoclonal antiodies specific for blood groups A and B. AustJExpBiolMed Sci 62:421-428.

Flower R, Lin P-H, Heathcote D, Chan M, Teo D, Selkirk A, Shepherd R, Henry S (2008). "Insertion of KODE peptide constructs into red cell membranes: Creating artificial variant MNS blood group antigens. ISBT Regional Congress, Macao SAR China, 2008". (P-396) *Vox Sanguinis*; 95:Suppl 1, 203–204

Fowler E, Weiner HL. 1997. Oral tolerance: Elucidation of mechanisms and application to treatment of autoimmune diseases. Peptide Science 43:323-335.

Frame T, Carroll T, Korchagina E, Bovin N, Henry S. 2007. Synthetic glycolipid modification of red blood cell membranes. Transfusion 47:876-882. Frame T, Carroll T, Korchagina E, Bovin N, Henry S. 2007. Synthetic glycolipid modification of red blood cell membranes. Transfusion 47:876-882.

Franco RS. 2009. The measurement and importance of red cell survival. American Journal of Hematology 84:109-114.

Galili U. 2004. Immune response, accommodation, and tolerance to transplantation carbohydrate antigens. Transplantation 78:1093-1098.

Galili U, Macher BA, Buehler J, Shohet SB. 1985. Human natural anti-alpha-galactosyl IgG. II. The specific recognition of alpha (1----3)-linked galactose residues. J Exp Med 162:573-582.

Garby L, Mollison PL. 1971. Deduction of mean red-cell life-span from 51Cr survival curves. Br J Haematol 20:527-536.

García-Vallejo JJ, Van Kooyk Y. 2009. Endogenous ligands for C-type lectin receptors: the true regulators of immune homeostasis. Immunological Reviews 230:22-37.

Garcia de Vinuesa C, O'Leary P, Sze DM, Toellner KM, MacLennan IC. 1999. T-independent type 2 antigens induce B cell proliferation in multiple splenic sites, but exponential growth is confined to extrafollicular foci. Eur J Immunol 29:1314-1323.

Gardiner JM. 1998. The therapeutic potential of synthetic multivalent carbohydrates. Expert Opin Investig Drugs 7:405-411.

Garratty G. 2008a. The James Blundell Award Lecture 2007: Do we really understand immune red cell destruction? Transfusion Medicine 18:321-334.

Garratty G. 2008b. Modulating the red cell membrane to produce universal/stealth donor red cells suitable for transfusion. Vox sanguinis 94:87-95.

Geijtenbeek TB, Gringhuis SI. 2009. Signalling through C-type lectin receptors: shaping immune responses. Nat Rev Immunol 9:465-479.

Gilson CR, Kraus TS, Hod EA, Hendrickson JE, Spitalnik SL, Hillyer CD, Shaz BH, Zimring JC. 2009. A novel mouse model of red blood cell storage and posttransfusion in vivo survival. Transfusion 49:1546-1553.

Godfrey DI, Kronenberg M. 2004. Going both ways: immune regulation via CD1d-dependent NKT cells. J Clin Invest 114:1379-1388.

Goldstein J, Siviglia G, Hurst R, Lenny L, Reich L. 1982. Group B erythrocytes enzymatically converted to group O survive normally in A, B, and O individuals. Science 215:168-170.

Gray SJ, Sterling K. 1950. The tagging of red cells and plasma proteins with radioactive chromium. J Clin Invest 29:1604-1613.

Griffiths PD. 2005. Induced tolerance to isohaemagglutinins. Rev Med Virol 15:145-147.

Guleria I, Sayegh MH. 2007. Maternal Acceptance of the Fetus: True Human Tolerance. The Journal of Immunology 178:3345-3351.

Guo Z, Wang Q. 2009. Recent development in carbohydrate-based cancer vaccines. Curr Opin Chem Biol 13:608-617.

Gutgemann I, Darling JM, Greenberg HB, Davis MM, Chien YH. 2002. A blood-borne antigen induces rapid T-B cell contact: a potential mechanism for tolerance induction. Immunology 107:420-425.

Hadac EM, Federspiel MJ, Chernyy E, Tuzikov A, Korchagina E, Bovin NV, Russell S, Henry SM. 2011. Fluorescein and radiolabeled Function-Spacer-Lipid constructs allow for simple in vitro and in vivo bioimaging of enveloped virions. J Virol Methods 176:78-84.

Hale G, Zhang MJ, Bunjes D, Prentice HG, Spence D, Horowitz MM, Barrett AJ, Waldmann H. 1998. Improving the outcome of bone marrow transplantation by using CD52 monoclonal antibodies to prevent graft-versus-host disease and graft rejection. Blood 92:4581-4590.

Haley PJ. 2003. Species differences in the structure and function of the immune system. Toxicology 188:49-71.

Halverson GR, Chaudhuri A, Huang T, Yazdanbakhsh K, Reid ME. 2001. Immunization of transgenic mice for production of MoAbs directed at polymorphic blood group antigens. Transfusion 41:1393-1396.

Hamilton JA, Miller JF. 1974. Hapten-specific tolerance in mice. I. Induction with hapten-coupled syngeneic erythrocytes. Eur J Immunol 4:261-268.

Hammar L, Mansson S, Rohr T, Chester MA, Ginsburg V, Lundblad A, Zopf D. 1981. Lewis phenotype of erythrocytes and Leb-active glycolipid in serum of pregnant women. Vox Sang 40:27-33.

Hanslick JL, Lau K, Noguchi KK, Olney JW, Zorumski CF, Mennerick S, Farber NB. 2009. Dimethyl sulfoxide (DMSO) produces widespread apoptosis in the developing central nervous system. Neurobiology of Disease 34:1-10.

Hansson GC, Karlsson KA, Larson G, McKibbin JM, Blaszczyk M, Herlyn M, Steplewski Z, Koprowski H. 1983. Mouse monoclonal antibodies against human cancer cell lines with specificities for blood group and related antigens. Characterization by antibody binding to glycosphingolipids in a chromatogram binding assay. J Biol Chem 258:4091-4097.

Harrison AL, Olsson ML, Jones RB, Ramkumar S, Sakac D, Binnington B, Henry S, Lingwood CA, Branch DR. 2010. A synthetic globotriaosylceramide analogue inhibits HIV-1 infection in vitro by two mechanisms. Glycoconj J 27:515-524.

Harrison LC. 1992. Islet cell antigens in insulin-dependent diabetes: Pandora's box revisited. Immunology Today 13:348-352.

Harrison LC, Hafler DA. 2000. Antigen-specific therapy for autoimmune disease. Current Opinion in Immunology 12:704-711.

Hartley SB, Cooke MP, Fulcher DA, Harris AW, Cory S, Basten A, Goodnow CC. 1993. Elimination of self-reactive B lymphocytes proceeds in two stages: arrested development and cell death. Cell 72:325-335.

Hartley SB, Crosbie J, Brink R, Kantor AB, Basten A, Goodnow CC. 1991. Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. Nature 353:765-769.

Hasegawa Y, Kato Y, Kaneko MK, Ogasawara S, Shimazu M, Tanabe M, Kawachi S, Obara H, Shinoda M, Kitagawa Y, Narimatsu H, Kitajima M. 2008. Neutralization of blood group A-antigen by a novel anti-A antibody: overcoming ABO-incompatible solid-organ transplantation. Transplantation 85:378-385.

Heathcote D, Carroll T, Wang JJ, Flower R, Rodionov I, Tuzikov A, Bovin N, Henry S. 2010. Novel antibody screening cells, MUT+Mur kodecytes, created by attaching peptides onto red blood cells. Transfusion 50.

Heathcote D, Flower R, Henry S (2008). "Development of novel alloantibody screening cells – the first example of the addition of peptide antigens to human red cells using KODE technology. ISBT Regional Congress, Macao SAR China, 2008". (P-303) *Vox Sanguinis* 2008; 95:Suppl 1, 174

Heaton WA, Keegan T, Hanbury CM, Holme S, Pleban P. 1989. Studies with nonradioisotopic sodium chromate. II. Single- and double-label 52Cr/51Cr posttransfusion recovery estimations. Transfusion 29:703-707.

Hendrickson JE,Chadwick T, E., Roback J, D., Hillyer C, D., Zimring C. 2007. Inflammation enhances consumption and presentation of transfused RBC antigens by dendritic cells. Blood 110:2736-2743.

Henry. 2009. Modification of red blood cells for laboratory quality control use. Current Opinion in Hematology 16:467-472.

Henry SM, Barr KL, Oliver CA. 2012. Modeling transfusion reactions with kodecytes and enabling ABO-incompatible transfusion with Function-Spacer-Lipid constructs. ISBT Science Series in press.

Henry S, Komarraju S, Heathcote D, Rodionov IL. 2011. Designing peptide-based FSL constructs to create Miltenberger kodecytes. ISBT Science Series 6:306-312.

Henry SM, Oliver CA. 2011. Method of modifying the immune response.Publication Number: US 2011/0229511 A1 www.google.com/patents/US20110229511.pdf

Henry S, Oriol R, Samuelsson B. 1995. Lewis histo-blood group system and associated secretory phenotypes. Vox Sang 69:166-182.

Henry S, Perry H. 2010. Technical Bulletin:FSL-A+B (tri) Serologic Teaching Kit. Scholarly Commons. http://hdl.handle.net/10292/2827.

Hess AD. 2006. Modulation of graft-versus-host disease: role of regulatory T lymphocytes. Biol Blood Marrow Transplant 12:13-21. Hod EA, Arinsburg SA, Francis RO, Hendrickson JE, Zimring JC, Spitalnik SL. 2010. Use of mouse models to study the mechanisms and consequences of RBC clearance. Vox sanguinis 99:99-111.

Hod EA, Arinsburg SA, Francis RO, Hendrickson JE, Zimring JC, Spitalnik SL. 2010. Use of mouse models to study the mechanisms and consequences of RBC clearance. Vox sanguinis 99:99-111.

Hod EA, Zimring JC, Spitalnik SL. 2008. Lessons learned from mouse models of hemolytic transfusion reactions. Curr Opin Hematol 15:601-605.

Hoff J. 2000. Methods of Blood Collection in the Mouse. Lab Animal 29:48-53.

Hoffmann-Fezer G, Maschke H, Zeitler HJ, Gais P, Heger W, Ellwart J, Thierfelder S. 1991. Direct in vivo biotinylation of erythrocytes as an assay for red cell survival studies. Ann Hematol 63:214-217.

Hoffmann-Fezer G, Mysliwietz J, Mortlbauer W, Zeitler HJ, Eberle E, Honle U, Thierfelder S. 1993. Biotin labeling as an alternative nonradioactive approach to determination of red cell survival. Ann Hematol 67:81-87.

Hoffmann-Fezer G, Trastl C, Beisker W, Berg D, Obermaier J, Kessler W, Mysliwietz J, Schumm M, Filser J, Thierfelder S. 1997. Preclinical evaluation of biotin labeling for red cell survival testing. Ann Hematol 74:231-238.

Holthuis JC, van Meer G, Huitema K. 2003. Lipid microdomains, lipid translocation and the organization of intracellular membrane transport (Review). Mol Membr Biol 20:231-241.

Hossaini AA. 1972. Neutralization of Lewis antibodies in vivo and transfusion of Lewis incompatible blood. Am J Clin Pathol 57:489-493.

Hult A, Frame T, Henry S, Olsson M. 2008. Flow cytometry evaluation of red blood cells transformed with variable amounts of synthetic A and B glycolipids. . Vox Sang 95:180.

Huppes W, Paulonis J, Dijk H, Van Rooijen N, Van Bekkum DW. 1993. The role of natural antibodies and ABO (H) blood groups in transplantation of human lymphoid cells into mice. Eur J Immunol 23:26-32.

Irazoqui FJ, Sendra VG, Lardone RD, Nores GA. 2005. Immune response to Thomsen-Friedenreich disaccharide and glycan engineering. Immunol Cell Biol 83:405-412.

Irei T, Ohdan H, Zhou W, Ishiyama K, Tanaka Y, Ide K, Asahara T. 2007. The persistent elimination of B cells responding to blood group A carbohydrates by synthetic group A carbohydrates and B-1 cell differentiation blockade: novel concept in preventing antibody-mediated rejection in ABO-incompatible transplantation. Blood 110:4567-4575.

Izumi K, Yamamoto K, Tochikura T, Hirabayashi Y. 1992. Serological study using alpha-N-acetylgalactosaminidase from Acremonium sp. Biochim Biophys Acta 1116:72-74.

Janatpour KA, Kalmin ND, Jensen HM, Holland PV. 2008. Clinical outcomes of ABO-incompatible RBC transfusions. Am J Clin Pathol 129:276-281.

Janeway C. 2005. Immunobiology: the immune system in health and disease. New York: Garland Science.

Janvier D, Veaux S, Reviron M, Guignier F, Benbunan M. 1990. Serological characterization of murine monoclonal antibodies directed against acquired B red cells. Vox Sang 59:92-95.

Jennings V. 1995. Review of selected adjuvants used in antibody production. ILAR J 37:119-125.

Jerne NK. 1974. Towards a network theory of the immune system. Ann Immunol (Paris) 125C:373-389.

Jones ND, Fluck NC, Roelen DL, Mellor AL, Morris PJ, Wood KJ. 1997. Deletion of alloantigen-reactive thymocytes as a mechanism of adult tolerance induction following intrathymic antigen administration. Eur J Immunol 27:1591-1600.

Josephson DL. 2004. Intravenous infusion therapy for nurses. In: Josephon D L, editor. Intravenous infusion therapy for nurses, 2nd ed. New York: Thomson Delmar Learning. p 399.

Kabat EA, Bendich A, Bezer AE, Beiser SM. 1947. Immunochemical studies on blood groups:IV. Preparation of blood group A substances from human sources and a comparison of their chemical and immunochemical properties with those of the blood group A substance from hog stomach J Exp Med 85:685-699.

Kalinski P, Mailliard RB, Giermasz A, Zeh HJ, Basse P, Bartlett DL, Kirkwood JM, Lotze MT, Herberman RB. 2005. Natural killer-dendritic cell cross-talk in cancer immunotherapy. Expert Opin Biol Ther 5:1303-1315.

Kannagi R, Stroup R, Cochran NA, Urdal DL, Young WW, Jr., Hakomori S. 1983. Factors affecting expression of glycolipid tumor antigens: influence of ceramide composition and coexisting glycolipid on the antigenicity of gangliotriaosylceramide in murine lymphoma cells. Cancer Res 43:4997-5005.

Kasai K, Galton J, Terasaki PI, Wakisaka A, Kawahara M, Root T, Hakomori SI. 1985. Tissue distribution of the Pk antigen as determined by a monoclonal antibody. J Immunogenet 12:213-220.

Kirk AD, Burkly LC, Batty DS, Baumgartner RE, Berning JD, Buchanan K, Fechner JH, Jr., Germond RL, Kampen RL, Patterson NB, Swanson SJ, Tadaki DK, TenHoor CN, White L, Knechtle SJ, Harlan DM. 1999. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. Nat Med 5:686-693.

Kirkley SA. 1999. Proposed mechanisms of transfusion-induced immunomodulation. Clin Diagn Lab Immunol 6:652-657.

Klein J, Marusic M, Nagy ZA. 1982. The seven rules of MHC restriction. Transplant Proc 14:581-583.

Kleusch C, Hersch N, Hoffmann B, Merkel R, Csiszar A. 2012. Fluorescent lipids: functional parts of fusogenic liposomes and tools for cell membrane labeling and visualization. Molecules 17:1055-1073.

Klinman NR. 1996. The "clonal selection hypothesis" and current concepts of B cell tolerance. Immunity 5:189-195.

Klinman NR, Schrater AF, Katz DH. 1981. Immature B cells as the target for in vivo tolerance induction. J Immunol 126:1970-1973.

Koch CA, Platt JL. 2007. T cell recognition and immunity in the fetus and mother. Cell Immunol 248:12.

Koch M, Stronge VS, Shepherd D, Gadola SD, Mathew B, Ritter G, Fersht AR, Besra GS, Schmidt RR, Jones EY, Cerundolo V. 2005. The crystal structure of human CD1d with and without α-galactosylceramide. Nature Immunology 6:819-826.

KODE Biotech Ltd. 2012. http://www.kodebiotech.com

KODE Biotech Ltd Technology Overview. http://kodebiotech.com/kode-technology-overview.pdf

Korchagina E; Tuzikov A; Formanovsky A; Popova I; Henry S; Bovin N (In Press). Toward creating cell membrane glycolandscapes with glycan lipid constructs. Carbohydrate Research 2012, doi:10.1016/j.carres.2012.03.044

Koscielak J. 2001. ABH blood group active glycoconjugates from human red cells. Transfus Med 11:267-279.

Kovalenko E, Abakushina E, Telford W, Kapoor V, Korchagina E, Khaidukov S, Molotkovskaya I, Sapozhnikov A, Vlaskin P, Bovin N. 2007. Clustered carbohydrates as a target for natural killer cells: a model system. Histochem Cell Biol 127:313-326.

Kumlien G, Ullstrom L, Losvall A, Persson LG, Tyden G. 2006. Clinical experience with a new apheresis filter that specifically depletes ABO blood group antibodies. Transfusion 46:1568-1575.

Kumpel BM. 2002. In vivo studies of monoclonal anti-D and the mechanism of immune suppression. Transfus Clin Biol 9:9-14.

Kunzendorf U, Pohl T, Bulfone-Paus S, Krause H, Notter M, Onu A, Walz G, Diamantstein T. 1996. Suppression of cell-mediated and humoral immune responses by an interleukin-2-immunoglobulin fusion protein in mice. J Clin Invest 97:1204-1210.

Lachmann PJ, Voak D, Oldroyd RG, Downie DM, Bevan PC. 1983. Use of monoclonal anti-C3 antibodies to characterise the fragments of C3 that are found on erythrocytes. Vox Sang 45:367-372.

Landsteiner K. 1945. The Specificity of Serological Reactions. Studies on sensitisation of animals with simple chemical compounds; Sections VII and IX.Cambridge, MA: Harvard University Press.

Landsteiner K, Simms S. 1923. Production of heterogenitc antibodies with mixtures of the binding part of the antigen and protein. J Exp med 38:127-138.

Lang J, Nemazee D. 2000. B cell clonal elimination induced by membrane-bound self-antigen may require repeated antigen encounter or cell competition. Eur J Immunol 30:689-696.

Larkin JM, Porter CD. 2005. Mice are unsuitable for modelling ABO discordance despite strain-specific A cross-reactive natural IgM. Br J Haematol 130:310-317.

Leadbetter EA, Brigl M, Illarionov P, Cohen N, Luteran MC, Pillai S, Besra GS, Brenner MB. 2008. NK T cells provide lipid antigen-specific cognate help for B cells. Proceedings of the National Academy of Sciences 105:8339-8344.

Lechler RI, Sykes M, Thomson AW, Turka LA. 2005. Organ transplantation -how much of the promise has been realized? Nat Med 11:605-613.

Lee YK, Mazmanian SK. 2010. Has the microbia played a critical role in the evolution of the adaptive immune system? Science 330(6012): 1768-73. Doi.10.1126/science.1195568.

Leenaars, Hendriksen. 2005. Critical steps in the production of polyclonal and monoclonal antibodies: evaluation and recommendations. ILAR J 46:269-279.

Leenaars PP, Hendriksen CF, Angulo AF, Koedam MA, Claassen E. 1994. Evaluation of several adjuvants as alternatives to the use of Freund's adjuvant in rabbits. Vet Immunol Immunopathol 40:225-241.

Lefebre J, McLellan BA, Coovadia AS. 1987. Seven years experience with group O unmatched packed red blood cells in a regional trauma unit. Ann Emerg Med 16:1344-1349.

Lemiuex R, Baker DA, Bundle D. 1977. A methodology for the production of carbohydrate-specific antibody. Canadian Journal of Biochemistry 55:507-512.

Lemieux RU, Bundle DR, Baker DA. 1975. The properties of a "synthetic" antigen related to the human blood-group Lewis a. J Am Chem Soc 97:4076-4083.

Lenny LL, Hurst R, Goldstein J, Galbraith RA. 1994. Transfusions to group O subjects of 2 units of red cells enzymatically converted from group B to group O. Transfusion 34:209-214.

Levy M, Edelman L, Dighiero G. 2001. Molecular characterisation of a monoclonal murine anti-blood group A antibody. Immunology Letters 76:15-23.

Liblau R, Tisch R, Bercovici N, McDevitt HO. 1997. Systemic antigen in the treatment of T-cell-mediated autoimmune diseases. Immunology Today 18:599-604.

Liepkalns, Zimring. 2009. Scientific Section. Transfusion 49:1A-305A.

Lingwood CA, Murray RK, Schachter H. 1980. The preparation of rabbit antiserum specific for mammalian testicular sulfogalactoglycerolipid. J Immunol 124:769-774.

Linton PJ, Rudie A, Klinman NR. 1991. Tolerance susceptibility of newly generating memory B cells. J Immunol 146:4099-4104.

Liu S, Kandeva T, Tchervenkov J. 2009. CD1d-mediated interaction between activated T cells and B cells is essential to B-cell proliferation and anti-alpha-Gal antibody production. Transplant Proc 41:398-402.

Liu.S, Sulzenbacher G, Yuan H, Bennett EP, Pietz G, Saunders K, Spence J, Nudelman E, Levery SB, White T, Neveu JM, Lane WS, Bourne Y, Olsson ML, Henrissat B, Clausen H. 2007. Bacterial glycosidases for the production of universal red blood cells. Nat Biotechnol 25:454-464.

Lockridge JL, Chen X, Zhou Y, Rajesh D, Roenneburg DA, Hegde S, Gerdts S, Cheng T-Y, Anderson RJ, Painter GF, Moody DB, Burlingham WJ, Gumperz JE. 2011. Analysis of the CD1 Antigen Presenting System in Humanized SCID Mice. PLoS ONE 6:e21701.

Love SD, Lee W, Nakamura YC, Platt JL, Bollinger RR, Parker W. 2000. Natural anti-carbohydrate IgM in mice: dependence on age and strain. J Immunol Methods 246:61-68.

Lund N, Branch DR, Mylvaganam M, Chark D, Ma X-Z, Sakac D, Binnington B, Fantini J, Puri A, Blumenthal R, Lingwood CA. 2006. A novel soluble mimic of the glycolipid, globotriaosyl ceramide inhibits HIV infection. AIDS 20:333-343 310.1097/1001.aids.0000206499.0000278664.0000206458.

Magee CC, Sayegh MH. 1997. Peptide-mediated immunosuppression. Current Opinion in Immunology 9:669-675.

Male DK, Brostoff J, Roth DB, Roitt I. 2006. Immunology, 7th ed. Philadelphia: Mosby.

Marcus DM. 1984. A review of the immunogenic and immuno-modulatory properties of glycosphingolipids. Mol Immunol 21:1083-1091.

Marcus DM, Cass LE. 1969. Glycosphingolipids with Lewis blood group activity: uptake by human erythrocytes. Science 164:553-555.

Markham RB, Pier GB, Powderly WG. 1988. Suppressor T cells regulating the cell-mediated immune response to Pseudomonas aeruginosa can be generated by immunization with anti-bacterial T cells. J Immunol 141:3975-3979.

Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. 2005. An Immunomodulatory Molecule of Symbiotic Bacteria Directs Maturation of the Host Immune System. Cell 122:107-118.

McCullough K, Summerfield A. 2005. Basic concepts of immune response and defense development. ILAR J 46:230-240.

McDole JR, Wheeler LW, McDonald KG, Wang B, Konjufca V, Knoop KA, Newberry RD, Miller MJ. 2012. Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. Nature 483:345-349.

McHeyzer-Williams LJ, McHeyzer-Williams MG. 2005. Antigen-specific memory B cell development. Annu Rev Immunol 23:487-513.

Medzhitov R, Janeway CA, Jr. 2000. How does the immune system distinguish self from nonself? Semin Immunol 12:185-188; discussion 257-344.

Melief CJ, Offringa R, Toes RE, Kast WM. 1996. Peptide-based cancer vaccines. Curr Opin Immunol 8:651-657.

Mestas J, Hughes CCW. 2004. Of Mice and Not Men: Differences between Mouse and Human Immunology. The Journal of Immunology 172:2731-2738.

Miller SD, Turley DM, Podojil JR. 2007. Antigen-specific tolerance strategies for the prevention and treatment of autoimmune disease. Nat Rev Immunol 7:665-677.

Miyazaki S, Nakajima T, Furukawa K. 1991. Monoclonal anti-A and anti-A,B antibodies from a mouse immunized with A secretor saliva. Exp Clin Immunogenet 8:16-23.

Mocikat R,Braumuller H, Gumy A, Egeter O, Ziegler H, Reusch U, Bubeck A, Louis J, Mailhammer R, Riethmuller G, Koszinowski U, Rocken M. 2003. Natural killer cells activated by MHC class I(low) targets prime dendritic cells to induce protective CD8 T cell responses. Immunity 19:561-569.

Mock D. Biotin: Physiology, dietary sources and requirements. In: Caballero B, Allen L, Prentice A, editors. Encyclopedia of human nutrition. London: Academic Press; 2004.

Mock DM, Lankford GL, Widness JA, Burmeister LF, Kahn D, Strauss RG. 1999. Measurement of red cell survival using biotin-labeled red cells: validation against 51Cr-labeled red cells. Transfusion 39:156-162.

Mock DM, Lankford GL, Widness JA, Burmeister LF, Kahn D, Strauss RG. 1999a. Measurement of circulating red cell mass using biotin-labeled red cells: validation against 51Cr-labeled red cells. Transfusion 39:149-155.

Mock DM, Matthews NI, Strauss RG, Burmeister LF, Schmidt R, Widness JA. 2009. Red blood cell volume can be independently determined in vitro using sheep and human red blood cells labeled at different densities of biotin. Transfusion 49:1178-1185.

Mock DM, Matthews NI, Zhu S, Strauss RG, Schmidt RL, Nalbant D, Cress GA, Widness JA. 2011. Red blood cell (RBC) survival determined in humans using RBCs labeled at multiple biotin densities. Transfusion 51:1047-1057.

Mock DM, Widness JA, Strauss RG, Franco RS. 2012. Posttransfusion red blood cell (RBC) survival determined using biotin-labeled RBCs has distinct advantages over labeling with 51Cr. Transfusion 52:1596-1598.

Mohiuddin MM, Ogawa H, Yin DP, Galili U. 2003. Tolerance induction to a mammalian blood group-like carbohydrate antigen by syngeneic lymphocytes expressing the antigen, II: tolerance induction on memory B cells. Blood 102:229-236.

Mollison PL. 1959. Blood-group antibodies and red-cell destruction. Br Med J 2:1035-1041.

Mollison PL. 1986. Survival curves of incompatible red cells. An analytical review. Transfusion 26:43-50.

Mollison PL. 1989. Further observations on the patterns of clearance of incompatible red cells. Transfusion 29:347-354.

Mollison PL. 2005. Blood Transfusion in Clinical Medicine, 11 ed. Oxford: Blackwell Scientific Publications.

Mollison PL. 2005. Survival of red cells. In: Anstee HKD, editor. Blood Transfusion in Clinical Medicine, eleventh edition ed: Blackwell Publishing. p 352-360.

Mollison PL, Polley MJ, Crome P. 1963. Temporary suppression of Lewis blood-group antibodies to permit incompatible transfusion. Lancet 1:909-912.

Mollison PL, Veall N. 1955. The use of the isotope 51Cr as a label for red cells. Br J Haematol 1:62-74.

Mond JJ, Lees A, Snapper CM. 1995a. T cell-independent antigens type 2. Annu Rev Immunol 13:655-692.

Mond JJ, Vos Q, Lees A, Snapper CM. 1995b. T cell independent antigens. Curr Opin Immunol 7:349-354.

Moody, Besra. 2001. Glycolipid targets of CD1-mediated T-cell responses. Immunology 104:243-251.

Moody, Besra GS, Wilson IA, Porceili SA. 1999. The molecular basis of CD 1 -mediated presentation of Iipid antigens. Immunological Reviews 172:285-296.

Moody, Porcelli. 2003. Intracellular pathways of CD1 antigen presentation. Nat Rev Immunol 3:11-22.

Moore S, McCardle A, Micklem L, Scott A, James K, McClelland B. 1984. A monoclonal antibody to human blood group B. Performance, evaluation and optimisation. Dev Biol Stand 57:55-59.

Morel PA, Garratty G, Perkins HA. 1978. Clinically significant and insignificant antibodies in blood transfusion. Am J Med Technol 44:122-129.

Moreno A, BlümL S, Hwang J-H, Ross BD. 2001. Alternative 1-13C glucose infusion protocols for clinical 13C MRS examinations of the brain. Magnetic Resonance in Medicine 46:39-48.

Mori L, De Libero G. 2008. Presentation of lipid antigens to T cells. Immunol Lett 117:1-8.

Mouneimne Y, Tosi PF, Barhoumi R, Nicolau C. 1991. Electroinsertion of xeno proteins in red blood cell membranes yields a long lived protein carrier in circulation. Biochim Biophys Acta 1066:83-89.

Mukhopadhaya A, Hanafusa T, Jarchum I, Chen Y-G, Iwai Y, Serreze DV, Steinman RM, Tarbell KV, DiLorenzo TP. 2008. Selective delivery of β cell antigen to dendritic cells in vivo leads to deletion and tolerance of autoreactive CD8+ T cells in NOD mice. Proceedings of the National Academy of Sciences 105:6374-6379.

Murdock RC, Reynolds C, Sarelius IH, Waugh RE. 2000. Adaptation and survival of surface-deprived red blood cells in mice. Am J Physiol Cell Physiol 279:C970-980.

Nakamura MC, Seaman WE. 2001. Ligand interactions by activating and inhibitory Ly-49 receptors. Immunological Reviews 181:138-148.

Neron S, Lemieux R. 1994. Type 2 T-cell-independent murine immune response to the human AB0 blood group antigens. Vox Sang 67:68-74.

Nickerson KG, Tao MH, Chen HT, Larrick J, Kabat EA. 1995. Human and mouse monoclonal antibodies to blood group A substance, which are nearly identical immunochemically, use radically different primary sequences. J Biol Chem 270:12457-12465.

Nicolau C, Mouneimne Y, Tosi PF. 1993. Electroinsertion of proteins in the plasma membrane of red blood cells. Anal Biochem 214:1-10.

Nielsen CH, Pedersen ML, Marquart HV, Prodinger WM, Leslie RG. 2002. The role of complement receptors type 1 (CR1, CD35) and 2 (CR2, CD21) in promoting C3 fragment deposition and membrane attack complex formation on normal peripheral human B cells. Eur J Immunol 32:1359-1367.

Nienhuis AW. 2008. Development of gene therapy for blood disorders. Blood 111:4431-4444.

Nossal GJV. 1993. Tolerance and Ways to Break Ita. Annals of the New York Academy of Sciences 690:34-41.

Nussbaumer W, Schwaighofer H, Gratwohl A, Kilga S, Schonitzer D, Nachbaur D, Niederwieser D. 1995. Transfusion of donor-type red cells as a single preparative treatment for bone marrow transplants with major ABO incompatibility. Transfusion 35:592-595.

Obukhanych TV, Nussenzweig MC. 2006. T-independent type II immune responses generate memory B cells. J Exp Med 203:305-310.

Obukhova P, Korchagina E, Henry S, Bovin N. 2011. Natural anti-A and anti-B of the ABO system: allo- and autoantibodies have different epitope specificity. Transfusion:no-no.

Ogawa H, Mohiuddin MM, Yin DP, Shen J, Chong AS, Galili U. 2004. Mouse-heart grafts expressing an incompatible carbohydrate antigen. II. Transition from accommodation to tolerance. Transplantation 77:366-373.

Oliver C, Blake D, Ferguson S, Bovin N,Henry, S. 2010. Poster Presentations. FEBS Journal 277(Suppl. 1) 37-271 (2010). A2.31 p50-1

Oliver C, Blake D, Henry S. 2011a. In vivo neutralization of anti-A and successful transfusion of A antigen-incompatible red blood cells in an animal model. Transfusion 51:2664-2675.

Oliver C, Blake D, Henry S. 2011b. Modeling transfusion reactions and predicting in vivo cell survival with kodecytes. Transfusion 51:1723-1730.

Olsson ML, Clausen H. 2007. Modifying the red cell surface: towards an ABO-universal blood supply. Br J Haematol 140:3-12.

Ong GL, Mattes MJ. 1989. Mouse strains with typical mammalian levels of complement activity. Journal of Immunological Methods 125:147-158.

Oriol R, Le Pendu J, Mollicone R. 1986. Genetics of ABO, H, Lewis, X and related antigens. Vox Sang 51:161-171.

Oriol R, Samuelsson BE, Messeter L. 1990. ABO antibodies--serological behaviour and immuno-chemical characterization. J Immunogenet 17:279-299.

O'Shannessy DJ, Quarles RH. 1987. Labeling of the oligosaccharide moieties of immunoglobulins. J Immunol Methods 99:153-161.

Pelosi MA, Bauer JL, Langer A, Hung CT. 1974. Transfusion of incompatible blood after neutralization of Lewis antibodies. Obstet Gynecol 44:590-593.

Perales MA, Blachere NE, Engelhorn ME, Ferrone CR, Gold JS, Gregor PD, Noffz G, Wolchok JD, Houghton AN. 2002. Strategies to overcome immune ignorance and tolerance. Semin Cancer Biol 12:63-71.

Pieters RJ. 2009. Maximising multivalency effects in protein-carbohydrate interactions. Org Biomol Chem 7:2013-2025.

Pike BL, Boyd AW, Nossal GJ. 1982. Clonal anergy: the universally anergic B lymphocyte. Proc Natl Acad Sci U S A 79:2013-2017.

Pittiglio D. 1986. Genetics and Biochemistry of A,B,H, and Lewis antigens. In: Blood Group systems: ABH and Lewis. Wallace ME and Gibbs FL.: American Association of Blood Banks. Arlington, VA p1-56.

Poole J, Daniels G. 2007. Blood group antibodies and their significance in transfusion medicine. Transfus Med Rev 21:58-71.

Portoukalian J. 2000. Immunogenicity of glycolipids. Clin Rev Allergy Immunol 19:73-78.

Prescott B, Chernick SS, James WD, Caldes G, Barker D, Sloan HR, Chanock RM. 1970. Mycoplasma pneumoniae phosphatidyl glycerol. Proc Soc Exp Biol Med 134:711-719.

Race R, Sanger R. 1975. Blood Groups in Man. Oxford: Blackwell Scientific.

Rapport MM, Graf L. 1969. Immunochemical reactions of lipids. Prog Allergy 13:273-331.

Razin S, Prescott B, Chanock RM. 1970. Immunogenicity of Mycoplasma pneumoniae glycolipids: a novel approach to the production of antisera to membrane lipids. Proc Natl Acad Sci, U S A 67:590-597.

Redlich CA, Beckett WS, Sparer J, Barwick KW, Riely CA, Miller H, Sigal SL, Shalat SL, Cullen MR. 1988. Liver disease associated with occupational exposure to the solvent dimethylformamide. Ann Intern Med 108:680-686.

Renton PH, Hancock JA. 1962. Uptake of A and B Antigens by Transfused Group O Erythrocytes. Vox sanguinis 7:33-38.

Research Animal Resources. 2007. Guidelines for collection of blood from experimental animals. University of Minnesota RAR.

Rickard KA, Worlledge SM. 1968. Temporary suppression of Lewis blood-group antibodies. Lancet 2:456.

Rieben R, Buchs JP, Fluckiger E, Nydegger UE. 1991. Antibodies to histo-blood group substances A and B: agglutination titres, Ig class, and IgG subclasses in healthy persons of different age categories. Transfusion 31:607-615.

Rieben. R, Tucci. A, Nydegger. UE, Zubler. RH. 1992. Self tolerance to human A and B histo-blood group antigens exists at the B cell level and cannot be broken by potent polyclonal B cell activation in vitro. European Journal of Immunology 22:2713-2717.

Ritter M, Ladyman H. 1995. Production of Monoclonal antibodies. In: Mary A. Ritter HML, editor. Monoclonal antibodies: production, engineering, and clinical application Cambridge: University Press Cambridge. p 9-15.

Rohr TE, Smith DF, Zopf DA, Ginsburg V. 1980. Leb-active glycolipid in human plasma: Measurement by radioimmunoassay. Archives of Biochemistry and Biophysics 199:265-269.

Romano EL, Soyano A, Linares J, Lauzon GJ. 1987a. Neutralization of ABO blood group antibodies by specific oligosaccharides. Transplant Proc 19:4426-4430.

Romano EL, Soyano A, Linares J. 1987b. Preliminary human study of synthetic trisaccharide representing blood substance A. Transplant Proc 19:4475-4478.

Romano EL, Soyano A, Montaño RF, Ratcliffe M, Olson M, Suarez G, Martínez N, Worstey G. 1994. Treatment of ABO Hemolytic Disease with Synthetic Blood Group Trisaccharides. Vox Sang 66:194-199.

Rossi SJ, Schroeder TJ, Hariharan S, First MR. 1993. Prevention and management of the adverse effects associated with immunosuppressive therapy. Drug Saf 9:104-131.

Rouger P, Edelman L, Doinel C, Reviron J, Salmon C, Bach JF. 1983. Study of blood group B antigen with a specific monoclonal antibody (anti-B, b-183). Immunology 49:77-82.

Rudd PM, Elliott T, Cresswell P, Wilson IA, Dwek RA. 2001. Glycosylation and the immune system. Science 291:2370-2376.

Russell DM, Dembic Z, Morahan G, Miller JF, Burki K, Nemazee D. 1991. Peripheral deletion of self-reactive B cells. Nature 354:308-311.

Russo V, Barker-Gear R, Gates R, Franco R. 1992. Studies with biotinylated RBC: (1) use of flow cytometry to determine posttransfusion survival and (2) isolation using streptavidin conjugated magnetic beads. Adv Exp Med Biol 326:101-107.

Rydberg L. 2001. ABO-incompatibility in solid organ transplantation. Transfus Med 11:325-342.

Sacks SH, Lennox ES. 1981. Monoclonal anti-B as a new blood-typing reagent. Vox Sang 40:99-104.

Samuelsson BE, Breimer ME. 1987. ABH antigens: some basic aspects. Transplant Proc 19:4401-4407.

Sassi M, Maggiore U, Buzio C, Franchini M. 2011. Immunohaematological and apheretic aspects of the first kidney transplant from a living, ABO-incompatible donor carried out in Italy. Blood Transfus 9:218-224.

Scandling JD, Busque S, Dejbakhsh-Jones S, Benike C, Millan MT, Shizuru JA, Hoppe RT, Lowsky R, Engleman EG, Strober S. 2008. Tolerance and Chimerism after Renal and Hematopoietic-Cell Transplantation. New England Journal of Medicine 358:362-368.

Scandling JD, Busque S, Shizuru JA, Engleman EG, Strober S. 2011. Induced Immune Tolerance for Kidney Transplantation. New England Journal of Medicine 365:1359-1360.

Schenten D, Medzhitov R. 2011. The control of adaptive immune responses by the innate immune system. Adv Immunol 109:87-124.

Schirmer DA, Song SC, Baliff JP, Harbers SO, Clynes RA, Krop-Watorek A, Halverson GR, Czerwinski M, Spitalnik SL. 2007. Mouse models of IgG- and IgM-mediated hemolysis. Blood 109:3099-3107.

Scholl S, Klink A, Mügge L-O, Schilling K, Höffken K, Sayer HG. 2005. Safety and impact of donor-type red blood cell transfusion before allogeneic peripheral blood progenitor cell transplantation with major ABO mismatch. Transfusion 45:1676-1683.

Schreiber AD, Frank MM. 1972. Role of antibody and complement in the immune clearance and destruction of erythrocytes. I. In vivo effects of IgG and IgM complement-fixing sites. J Clin Invest 51:575-582.

Schunk MK, Macallum GE. 2005. Applications and optimization of immunization procedures. ILAR J 46:241-257.

Schwarzmann G. 2001. Uptake and metabolism of exogenous glycosphingolipids by cultured cells. Semin Cell Dev Biol 12:163-171.

Schwendener RA, Ludewig B, Cerny A, Engler O. 2010. Liposome-based vaccines. Methods Mol Biol 605:163-175.

Shehata N, Tinmouth A, Naglie G, Freedman J, Wilson K. 2009. ABO-identical versus nonidentical platelet transfusion: a systematic review. Transfusion 49:2442-2453.

Shimizu I, Tomita Y, Iwai T, Zhang QW, Matsuzaki G, Nomoto K, Yasui H. 2005. The regulatory functions of Ly-49A, Ly-49D and Ly-49G2 on NK cells in the recognition and rejection of the alloantigen in vivo. Transpl Int 18:1090-1099.

Shimizu K, Goto A, Fukui M, Taniguchi M, Fujii S-i. 2007. Tumor Cells Loaded with α-Galactosylceramide Induce Innate NKT and NK Cell-Dependent Resistance to Tumor Implantation in Mice. The Journal of Immunology 178:2853-2861.

Sigma. Sigma Catalogue; http://www.sigmaaldrich.com/new-zealand.htmL

Singh SK, Stephani J, Schaefer M, Kalay H, Garcia-Vallejo JJ, den Haan J, Saeland E, Sparwasser T, van Kooyk Y. 2009. Targeting glycan modified OVA to murine DC-SIGN transgenic dendritic cells enhances MHC class I and II presentation. Mol Immunol 47:164-174.

Slezak SE, Horan PK. 1989. Fluorescent in vivo tracking of hematopoietic cells. Part I. Technical considerations. Blood 74:2172-2177.

Slovin, Keding SJ, Ragupathi G. 2005. Carbohydrate vaccines as immunotherapy for cancer. Immunol Cell Biol 83:418-428.

Slovin, Ragupathi G, Musselli C, Olkiewicz K, Verbel D, Kuduk SD, Schwarz JB, Sames D, Danishefsky S, Livingston PO, Scher HI. 2003. Fully synthetic carbohydrate-based vaccines in biochemically relapsed prostate cancer: clinical trial results with alpha-N-acetylgalactosamine-O-serine/threonine conjugate vaccine. J Clin Oncol 21:4292-4298.

Smith NH, Henry KL, Cadwell CM, Bennett A, Hendrickson JE, Frame T, Zimring JC. 2012. Generation of transgenic mice with antithetical KEL1 and KEL2 human blood group antigens on red blood cells. Transfusion.

Sneath JS, Sneath PH. 1955. Transformation of the Lewis groups of human red cells. Nature 176:172.

Snowden JA, Saccardi R, Allez M, Ardizzone S, Arnold R, Cervera R, Denton C, Hawkey C, Labopin M, Mancardi G, Martin R, Moore JJ, Passweg J, Peters C, Rabusin M, Rovira M, van Laar JM, Farge D. 2011. Haematopoietic SCT in severe autoimmune diseases: updated guidelines of the European Group for Blood and Marrow Transplantation. Bone Marrow Transplant.

Springer GF, Horton RE. 1969. Blood group isoantibody stimulation in man by feeding blood group-active bacteria. J Clin Invest 48:1280-1291.

Sroda K, Rydlewski J, Langner M, Kozubek A, Grzybek M, Sikorski AF. 2005. Repeated injections of PEG-PE liposomes generate anti-PEG antibodies. Cell Mol Biol Lett 10:37-47.

Stein KE. 1992. Thymus-independent and thymus-dependent responses to polysaccharide antigens. J Infect Dis 165 Suppl 1:S49-52.

Stills HF. 2005. Adjuvants and Antibody Production: Dispelling the Myths Associated with Freund's Complete and Other Adjuvants. ILAR Journal 46:280-293.

Strable E, Finn MG. 2009. Chemical modification of viruses and virus-like particles. Curr Top Microbiol Immunol 327:1-21.

Strumia MM, Colwell LS, Dugan A. 1958. The measure of erythropoiesis in anemias. I. The mixing time and the immediate post-transfusion dissappearance of T-1824 dye and of Cr-51-tagged erythrocytes in relation to blood volume determination. Blood 13:128-145.

Suzuki T, Dale GL. 1987. Biotinylated erythrocytes: in vivo survival and in vitro recovery. Blood 70:791-795.

Szymanski IO, Valeri CR. 1970. Factors influencing chromium elution from labelled red cells in vivo and the effect of elution on red-cell survival measurements. Br J Haematol 19:397-409.

Taft RA, Davisson M, Wiles MV. 2006. Know thy mouse. Trends in Genetics 22:649-653.

Takahashi K, Saito K. 2012. ABO-incompatible kidney transplantation. Transplant Rev (Orlando) in press http://dx.doi.org/10.1016/j.trre.2012.07.003

Takeuchi O, Akira S. 2010. Pattern recognition receptors and inflammation. Cell 140:805-820.

Taylor, Williams RO, Maini RN. 2001. Immunotherapy for rheumatoid arthritis. Curr Opin Immunol 13:611-616.

Teale JM, Layton JE, Nossal GJ. 1979. In vitro model for natural tolerance to self-antigens. Inhibition of the development of surface-immunoglobulin-negative lymphocytes into T-dependent responsive B cells by antigen. J Exp Med 150:205-217.

Tian C, Bagley J, Forman D, Iacomini J. 2004. Induction of central tolerance by mature T cells. J Immunol 173:7217-7222.

TitermaxGold™. http://www.titermax.com/i_tech.htm

Touraine JL, Sanhadji K. 2011. Transplantation tolerance induced in humans at the fetal or the neonatal stage. J Transplant 2011:760319.

Toyokuni T, Hakomori S, Singhal AK. 1994. Synthetic carbohydrate vaccines: synthesis and immunogenicity of Tn antigen conjugates. Bioorg Med Chem 2:1119-1132.

Trombetta ES, Mellman I. 2005. Cell biology of antigen processing in vitro and in vivo. Annu Rev Immunol 23:975-1028.

Tyden G, KumLien G, Efvergren M. 2007. Present techniques for antibody removal. Transplantation 84:S27-29.

Tyden G, KumLien G, Genberg H, Sandberg J, Lundgren T, Fehrman I. 2005. ABO-incompatible kidney transplantation and rituximab. Transplant Proc 37:3286-3287.

Tykocinski ML, Kaplan DR. 1993. Prospects for anti-rejection therapies based upon CD8-dependent immunoregulation. Kidney Int Suppl 39:S120-123.

Unger WW, van Kooyk Y. 2011. 'Dressed for success' C-type lectin receptors for the delivery of glyco-vaccines to dendritic cells. Curr Opin Immunol 23:131-137.

Veenstra van Nieuwenhoven AL, Heineman MJ, Faas MM. 2003. The immunology of successful pregnancy. Human Reproduction Update 9:347-357.

Vengelen-Tyler V. 1999. aaBB Technical Manual, 13th ed. Bethesda,MD: American Association of Blood Banks.

Voak D, Lennox E, Sacks S, Milstein C, Darnborough J. 1982. Monoclonal anti-A and anti-B: development as cost-effective reagents. Med Lab Sci 39:109-122.

Voak D, Sacks S, Alderson T, Takei F, Lennox E, Jarvis J, Milstein C, Darnborough J. 1980. Monoclonal anti-A from a hybrid-myeloma: evaluating as a blood grouping reagent. Vox Sang 39:134-140.

Waldmann H, Cobbold SP, Fairchild P, Adams E. 2001. Therapeutic aspects of tolerance. Current Opinion in Pharmacology 1:392-397.

Walker MR, Lund J, Thompson KM, Jefferis R. 1989. Aglycosylation of human IgG1 and IgG3 monoclonal antibodies can eliminate recognition by human cells expressing Fc gamma RI and/or Fc gamma RII receptors. Biochem J 259:347-353.

Wang Q, Zhou Z, Tang S, Guo Z. 2011. Carbohydrate-Monophosphoryl Lipid A Conjugates Are Fully Synthetic Self-Adjuvanting Cancer Vaccines Eliciting Robust Immune Responses in the Mouse. ACS Chem Biol.

West LJ. 2006. B-cell tolerance following ABO-incompatible infant heart transplantation. Transplantation 81:301-307.

Wilson RB, Spitalnik SL. 1994. Designer red cells. Transfusion 34:189-191.

Winters JL, Gloor JM, Pineda AA, Stegall MD, Moore SB. 2004. Plasma exchange conditioning for ABO-incompatible renal transplantation. J Clin Apher 19:79-85.

Wong S, Arsequell G. 2003. Immunobiology of Carbohydrates. New York: Kluwer.

Wood KJ. 1991. Alternative approaches for the induction of transplantation tolerance. Immunol Lett 29:133-137.

Yamakami K. 1926. The Individuality of semen, with reference to its property of Inhibiting specifically Isohemoagglutination. J Immunol 12:185-189.

Yin H, Wan H, Hu XP, Li XB, Wang W, Liu H, Ren L, Zhang XD. 2011. Rituximab induction therapy in highly sensitized kidney transplant recipients. Chin Med J (Engl) 124:1928-1932.

Yuki N, Yamada M, Sato S, Ohama E, Kawase Y, Ikuta F, Miyatake T. 1993. Association of IgG anti-GD1a antibody with severe Guillain-Barre syndrome. Muscle Nerve 16:642-647.

Zeira M, Tosi PF, Mouneimne Y, Lazarte J, Sneed L, Volsky DJ, Nicolau C. 1991. Full-length CD4 electroinserted in the erythrocyte membrane as a long-lived inhibitor of infection by human immunodeficiency virus. Proceedings of the National Academy of Sciences 88:4409-4413.

Zimring JC, Cadwell CM, Chadwick TE, Spitalnik SL, Schirmer DA, Wu T, Parkos CA, Hillyer CD. 2007. Nonhemolytic antigen loss from red blood cells requires cooperative binding of multiple antibodies recognizing different epitopes. Blood 110:2201-2208.

Zimring JC, Cadwell CM, Spitalnik SL. 2009. Antigen loss from antibody-coated red blood cells. Transfus Med Rev 23:189-204.

Zimring JC, Hair GA, Chadwick TE, Deshpande SS, Anderson KM, Hillyer CD, Roback JD. 2005. Nonhemolytic antibody-induced loss of erythrocyte surface antigen. Blood 106:1105-1112.

Zinkernagel RM. 1996. Immunology Taught by Viruses. Science 271:173-178.

Zinkernagel RM, Hengartner H. 2001. Regulation of the Immune Response by Antigen. Science 293:251-253.

# EXPERIMENTAL PROTOCOLS

## 1: Manipulations with mice

**OBJECTIVE, RATIONALE**

To anaesthetize mice using isoflurane gas or Emla cream for the insertion of identification tags, subclavian vein collect, tail vein collect ,cardiac puncture, and subcutaneous immunization with saliva of known A substance titre or FSL in solution.

**SAMPLES, REAGENTS AND EQUIPMENT**

**Reagents**

- Attane isoflurane (Bomac Laboratories Ltd, Auckland, NZ)
- Emla anaesthetic cream - Eutectic Mixture of Local Anaesthetics -Lidocaine/prilocaine 5 emulsion (http://www.nz-online-pharmacy.com/products)
- Human Saliva from $A_1$Le(a-b+) donor
- Celpresol (CSL, Australia)
- Anti-A monoclonal reagent (Epiclone, Australia)
- PBS
- TiterMax™Gold 1mL vial (TiterMax USA, Georgia, USA)
- 1mL A Le(a-b+) saliva saliva freeze-dried and reconstituted in MQ water
- FSL-A 4 mg/mL in sterile saline (FSL-Acat # 421604) http://www.sigmaaldrich.com/new-zealand.htmL)
- Group A human blood red cells

**Equipment**

- Anaesthetic equipment-Fluotech vaporizer and mask
- Oxygen gas
- Allflex Permanent Identification System, EI-002-025 Allflex NZ Ltd, (Palmerston North, NZ)
- Tissues
- Heparinised and plain capillary tubes and caps
- Heat lamp
- Eppindorf microcentrifuge tubes 1.5mL (3445 Raylab, Auckland)
- Scalpel
- Holding block for tail vein collect
- Hitachi hematocrit centrifuge
- Centrifuge (Sorvall MC-12V, Du Pont, USA)
- 3 mL syringe
- 22 g needle
- 23 g needle
- Plastic 5 mL syringe with no rubber pistons
- Beaker of water
- Scales
- Double sided tape
- Clock
- Heating block (Wealtec HB-2)
- Sterile centrifuge tubes
- Centrifuge (Hitachi 05PR-22)
- Balance (Mettler Toledo PB1502-S)
- Glass freeze dry vials (B02168, Bonnet Equipment)
- Freeze drier (Heto Lylolab 3000)
- Micropipettes (1-1000 µL)
- -85°C freezer
- Parafilm (PM-996, Pechiney Plastic Packaging)
- Magnifying eye-glass 10 X

## METHODS

### 1.1 Surgical anaesthesia

- Weigh mice.
- Mix the volatile anaesthetic Isofluorane (Attane) with oxygen and introduce into an airtight plastic anaesthetic chamber into which the mice are placed.
- Set oxygen at a flow rate of 1-1.5 L per minute and a high concentration of isoflurane-vaporizer setting (4).
- Anaesthetize mice one at a time and monitor their level of anaesthesia carefully.
- Surgical anaesthesia is indicated by lack of pedal reflexes, lack of voluntary movement, lack of consciousness and a respiration rate of about 60 breaths per minute.
- Reduce the concentration of the isofluorane to a maintenance level (vaporizer setting of 2-3) and move the animal from the chamber; place its head in an anaesthetic mask assuring excellent control of the depth of anaesthesia until the mouse is allowed to wake up at the end of the procedure.

### 1.2 Electronic Tagging

- Attach Transponder needle to the syringe.
- Insert the needle under the skin on the back.
- Check the number is readable with the reader unit.

### 1.3 Blood collect from subclavian vein

- Weigh mouse and place in chamber breathing Attane isoflurane.
- The subclavian vein of anaesthetized mice is surgically exposed, with forearms taped to table as in photo below.
- After infusion, at pre-determined time, prick subclavian vein and collect blood into a heparinised haematocrit tube.
- If a PCV is required a plain tube is collected.
- Surgically close the wound with 3 sutures.
- Monitor mice for respiration and haemorrhage throughout the procedure and for up to 2 hours post surgery.
- Centrifuge sample to obtain serum and cells. 5,000 rpm for 10 min at 5°C.
- Store serum in fridge; cells in celpresol or saline.

### 1.4 Anaesthesia and tail vein prick

- Apply Emla cream to the top third of the tail, 15 mins prior to incision.
- Warm the mouse tail under the heat lamp for up to 2 min ensuring the mouse does not overheat.
- Restrain mouse in box with tail extended through opening slit.
- Nick tail once with scalpel and collect blood into heparinised and or plain capillary tube to obtain plasma and cells - about 50-75 µL.
- Collect plain capillary tube 50 µL samples from selected animals.
- Press on wound firmly to stop bleeding, once finished.
- Record collect details.
- Centrifuge sample to obtain serum and cells. 5,000 rpm for 10 min at 5°C.
- Store serum in fridge; cells in celpresol or saline in fridge.

### 1.5 Cardiac Puncture for Exsanguination Blood Collection

- Mice are anaesthetised as for surgery.
- Open chest and expose heart.
- Remove whole blood as cleanly and efficiently as possible using 3 mL syringe and 22 g needle.
- Put sample into plain eppindorf tube and allow to clot for at least one hour.
- Centrifuge sample to obtain serum and cells. 5,000 rpm for 10 min at 5°C.
- Store serum in fridge; cells in celpresol or saline in fridge.

### 1.6 Preparation of saliva for immunization with blood group substance mice

- Collect saliva from clean mouth ensuring free of conntaminant such as lipstick or gloss.
- Centrifuge 3,000rpm for 10 min.
- Transfer supernatant to clean glass vials and place in heating block for 10 min at 100° C to inactivate salivary enzymes.
- Recentrifuge as above to remove opaque or semisolid material.
- Record supernatant volume.
- Transfer small aliquots to preweighed glass vials and cover with parafilm – make small perforations in this.
- Freeze in -85°C.
- Transfer frozen to freeze drier to freeze-dry.
- Reweigh concentrate to obtain grams saliva present if required.

- Resuspend saliva to 4 X less original volume with deionised water.
- Set aside about 65 µL for testing..
- Aliquot into eppindorf tubes in useful amounts (1mL).
- Place in heating block for 10 min at 100°C to sterilise pre-immunisation.

**1.7 Titre of blood group substance in saliva by the inhibition of antibody activity**

*Anti-A dilution*

- Prepare serial dilutions of monoclonal anti-A monoclonal reagent neat to 8192 in PBS.
- Take 30 µL from each dilution into a glass tube.
- Make a 5 % (A1, Le(a-b+) red cell suspension with PBS.
- Add 30 µL 5 % red cells to each antibody dilution.
- Incubate RT 5 min.
- Spin and read for agglutination using magnifying eye-glass.
- Use the antisera dilution giving the last 2+ agglutination for subsequent saliva antigen testing.

*Saliva A substance titre*

- Prepare serial dilutions of saliva neat to 8192 in PBS.
- Add 30 µL of each dilution to 30 µL diluted antiserum at the pre-determinded concentration.
- Mix and incubate 30 min RT.
- Add 30 µL 5 % indicator red cells.
- Mix and incubate RT 5 min.
- Spin and read.
- Record agglutination titre: agglutination at > 32 is suitable for iimmunisation use

**1.8 Subcutaneous immunization**

*Preparation of antigen/adjuvant suspension*

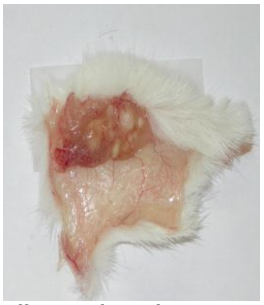
- Prepare 4 mg/mL FSL-A in saline, well mixed.
- TiterMax™Gold suspension is vortexed to mix thoroughly.
- 500 µL saliva or 4 mg/mL FSL-A is drawn into a rubber free syringe using 22 g needle
- Expel saliva or FSL-A into the 1 mL vial of Titermax™ Gold.
- The suspension is drawn up and down to mix until it begins to thicken.
- The remaining 500 µL saliva or FSL-A is drawn into a syringe and expelled into the vial of mixture.
- A tiny sample is squirted into beaker of water to test for worm formation as per Titermax protocol

*Injection of adjuvant/antigen mixture*

- Weigh mice.
- Place under anaesthetic with Attane chamber to light surgical anaesthesia.
- Implant electronic tag or check ID.
- Take pre immunization blood sample by tail vein prick for anti-A status.
- Using 23 g needle subcutaneously inject 100 µL antigen / titremax 1:1 suspension behind the head.
- The needle is then withdrawn and the area sealed with two fingers with gently rubbing.
- The mouse is placed back in its cage and monitored for behavioral changes.
- Details are recorded on the blue card attached to the cage.
- Control naive mice are housed alongside immunized mice.

**Mouse in mask**

**Cardiac puncture**



**Adjuvant deposit at autopsy**

## METHOD VARIATION

**1V 1. Sample collection for cell survival measurement**

- At 2, 8, 24, 48, 72, and 96 h intervals, collect a blood sample from the tail vein.
- To minimise damage to the tail veins only a single attempt to obtain blood is made.
  Plain capillary tube samples are also collected from selected animals to obtain serum to determine
- the presence or absence of anti-A pre and post-transfusion.
- Centrifuge samples to obtain serum and cells. 5,000 rpm for 10 min at 5°C.
- Store serum in fridge; cells in celpresol or saline.

**1V2. Packed cell volume**

- Spin haematocrit in centrifuge 5 min.
- Measure volume of serum and volume of packed red cells with a ruler.
- Divide packed cell cm by serum cm to get percentage or packed cell volume.
- Cardiac puncture exsanguinations gave an average collect of 900-1000 µL while a tail vein collect provided up to 75 µL whole blood. Serum obtained by centrifugation of the whole blood provided an average 400 µL. Serum or plasma obtained after centrifugation of a hematocrit tube averaged 40 µL with an average packed cell volume 45 %.

# 2: Creation of kodecytes with FSL constructs and confirmation of transformation

## OBJECTIVE, RATIONALE

To create kodecytes by transforming red blood cells using KODE™ FSL constructs and the confirmation of FSL-A and FSL-biotin status.

## SAMPLES, REAGENTS AND EQUIPMENT

**Samples**

- C57 mice red blood cells, washed and packed
- Human red blood cells, washed and packed

**Reagents**

All FSL molecules were obtained from KODE Biotech Materials Ltd, Auckland, New Zealand

- FSL-A (cat # 421604)
- FSL-B (cat #199283)
- FSL-GB3 (cat #352439)
- FSL-biotin (cat#187786)
- Sterile saline ( Gelman,DEMO S.A. Athens, Greece)
- Phosphate Buffered Saline pH 7.4
- Celpresol (CSL, Australia)
- d-biotin (cat # 47868 Sigma, Auckland, New Zealand)
- avidin/Alexafluor 488 Molecular Probes A-2901 Lot 84C1-1
- Appropriate anti-sera for antigen detection

**Equipment**

- Waterbath (Julabo F18)
- Glass Kimble tubes (KIM45048-18150,Biolab)
- Eppindorf microcentrifuge tubes,1.5 mL (3445,RayLab)
- Vortex (Thermolyne Maximix Plus M63210-26)
- Diamed gel cards (NaCl enzyme and cold agglutininsDiamed AG,Cressier-Sur Morat, Switzerland)
- Centrifuge Immufuge II, American Dade, USA
- Diamed centrifuge (Diamed AG,Cressier-Sur Morat, Switzerland)
- Micropipettes (1-1000 µL)
- Plastic transfer pipettes
- Olympus Fluorescence Microscope BX51

## METHOD

### 2.1 Preparation of FSLs

- Dissolve FSL construct at required concentration (less than 2 mg/mL) in sterile saline, celpresol or PBS or diluents as required.
- Vortex to mix and dissolve before use

### 2.2 Transformation of red cells to create kodecytes

- Add equal volumes of packed washed red cells to FSL solution and to a saline negative control.
- Mix well.
- Incubate 2h at 37 ° C.
- Wash cells 3X in Celpresol.
- Store in fridge 4° C.
- Check cells are transformed by agglutination with monoclonal anti-A reagent and or avidin/Alexafluor 488 binding.

### 2.3 Detection of red cell antigen

- Tube method; Reference::Vengelen-Tyler V. (1999). Technical manual (13th ed.) Bethesda ML 20814: American Association of Blood Banks.
- Diamed Gel card method; Refer: Diamed gel card package insert.
- Using anti-A or anti-B monoclonal reagentor anti-$Le^a$ or anti-$Le^b$ antisera.

**2.4 Detection of kodecytes inserted with FSL-biotin using avidin-Alexafluor 488 by fluorescence**

- Add 5 µL washed packed kodecytes or post transfusion kodecytes to 5 µL avidin/ Alexafluor 488 0.1mg/mL in PBS.
- Mix well.
- Incubate at 37°C for 30 min in the dark.
- Add 5 µL biotin (1 mg/mL in PBS) to prevent clumping of cells due to unbound avidin.
- Mix well.
- Incubate at 37°C for 1 min in the dark.
- Wash 3 x with saline and pellet packed cells in about 10 µL saline to make a suspension for microscopic detection of fluorescence
- Keep in dark as fluorescence fades with time.
- Make a wet film on glass slide and cover with coverslip to detect FSL-biotin presence by fluorescence.
- Use fluorescent microscope at 488nm excitation wavelength, 200 X magnification and differential interference contrast light (DIC) to examine cells for fluorescence.
- Take photograph of both fields at setting where fluorescence is strong and clear.

## METHOD VARIATIONS

**2V 1. FSL and murine red cell preparation for murine kodeyctes for cell survival measurement**

*FSL-A, -B, -GB3 are each mixed with FSL- biotin 0.1mg/mL in equal volumes*

- FSL-A 0.2 mg/mL in sterile saline
- FSL-B 0.2 mg/mL in sterile saline
- FSL-GB3 0.2 mg/mL in sterile saline
- FSL-biotin 0.1 mg/mL in sterile saline
- FSL-biotin 0.05 mg/mL in sterile saline
- Wash and pack murine red cells.

**2V 2. FSL and red cell preparation for human kodecytes**

- Prepare FSL at concentration; usually1- 2 mg/mL ( Human red cells lyse in > 2 mg/mL).
- Wash and pack human blood group O red cells.

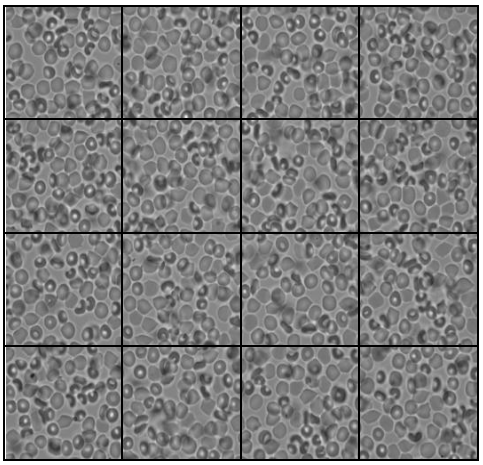
**2V 3. Murine kodecyte suspension for mice transfusions**

- For 3.2 % transfusion: 20 µL packed cells are added to 180 µL sterile saline.
- For 10 % transfusion:: 63 µL packed cells are added to 37 µL sterile saline.
- Store in fridge overnight in celpresol and resuspend in saline before use.

**2V 4. Red cell survival measurement from 0.1-96 h using FSL-biotin and fluorescence**

- Ten random fields (approx 6000 cells) where the cells, in a monolayer, are photographed under both differential interference contrast and 488 nm excitation fluorescence microscopy. Repeat blood collect, measuring cell count daily until cells cannot be seen, up to 96 h.
- Blinded paired photographs are then used to count fluorescent kodecytes and non-fluorescent red cells in order to calculate the percentage of kodecytes present.
- Using a 16 square grid placed over the photograph, calculate the number of red cells present in ten fields by counting 4 corner squares and multiplying the count by 4.Repeat with fluorescent cells but count all cells. Add red cell totals and fluorescent totals for the 10 slides and get % by division.
- Kodecyte survivals are expressed as percentages of total cells counted following the viewing of approximately 6000 cells for each sample.(3.2% or 10% being 100%).
- Use naive and pre-transfusion cells as controls for fluorescence.

**Image of grid used for counting cells**



**2V 5. To transform human RBCs to create A kodecytes by the insertion of FSL-A molecules using different concentrations of FSL-A dissolved in either PBS, Group O or A serum.**

*Make FSL-A concentrations at 4, 2, 1, 0.5, 0.25, 0.1, 0.05, 0.025 mg/mL*

- Dilute the FSL-A to the desired concentration in O serum, A serum and PBS.

*Red cell transformation at 0.1-8 h.*

- Protocol as for 2.2
- At 0.1hr remove 50 µL cell suspension. Immediately place in ice to stop reaction.
- At 1, 2, 4, 8 hr remove 50 µL cell suspension.
- Wash transformed cells 3 x in Celpresol.
- Pack cells removing as much diluent as possible.
- Test for antigen presence with Diamed cards as per technical manual
- Test for antibody presence using Diamed Liss Coombs cards as per technical manual

**2V 6. To detect sensitisation of A kodecytes with various concentrations of FSLA solution in vitro at varying time points using Diamed gel cards.**

*FSL-A concentrations*

- Make 4, 2, 1 mg/mL FSL-A, each dissolved in O serum.
- Controls using serum and 2 % BSA/PBS, without FSL-A are run in parallel.

*FSL-A transformation and sensitisation*

- Mix washed, packed O red cells with equal volume of FSL-A in O serum (200 µL) for each concentration.
- Incubate 37°C with intermittent mixing- 0.1 - 8h.

*Detection of FSL-A insertion at various time points*

- Take 50 uL from cell mixture in water-bath 37°C at 0.1h, 2, 4, 8h.
- Wash 4x with celpresol.
- Make 0.8% cell suspension with celpresol.
- Test for presence of A using monoclonal anti-A reagent.

Diamed serology:

*Direct Antibody agglutination, IgM*

- Take 50 µL of each FSL-A concentration mixture, 0.8%, into NaCl enzyme gel card.
- Spin in Diamed centrifuge.
- Read for agglutination as per manual.

*Indirect Antibody agglutination, IgM*

- Take 50 µL of each FSL-A concentration mixture, 0.8%, in to NaCl enzyme gel card.
- Add 25 µL Human O serum anti-A or monoclonal anti-A reagent for control.
- Spin in Diamed centrifuge.
- Read for agglutination as per manual.

*Indirect Antibody agglutination, IgG*

- Take 50 µL of each FSL-A concentration mixture, 0.8%, in to Liss Coombs gel card.
- Add 25 µL Human O serum anti-A
- Incubate in Diamed incubator
- Spin in Diamed centrifuge
- Read for agglutination using Diamed chart for column analysis.

---

**Agglutination Scoring**

Insertion of blood group molecules is assessed by visually by agglutination of the relevant IgM or IgG antibodies. The methods used include manual tube serology and Diamed ID Micro Typing column method. The Diamed system is not pre-loaded with antiserum and thus allows for detection of various antibodies.

The relationship between two scoring methods is listed below. The system used in this research is based on the 4 point score system (++++, +++, ++,+, w+, vw+,0).

Manual tube serology involves the addition of 30 µL 3% cell suspension in PBS to 30 µL antisera in a glass tube. The suspension is mixed and centrifuged and spun 2200rpm for 15 seconds. Scores are assigned according to the appearance of the cells in the bottom of the tube.

The column technique involves the use of a gel-card containing microtubes pre-filled with buffered sephadex gel. 50 µL of 0/8% cell suspension is added to 50 µL antisera in the well above the gel. The card is centrifuged in the Diamed centrifuge at a pre-programmed speed for 10 min. Scores are assigned according to the pattern and position of the cells in the gel.

**The 4 point agglutination scoring system**

| Score | Glass Tube | Diamed column |
|---|---|---|
| ++++ | Single firm disc | Single sharp band at top of column |
| +++ | Single disc that breaks into large clumps | More diffuse band at base of column |
| ++ | Several soft clumps | Cells spread out from top to bottom of column |
| + | Many small clumps | Diffuse band at base of column |
| w+ | Most cells negative, but some evidence of minor agglutination | Some cells above the band at base of column |
| vw | Most cells negative but some evidence of stickiness | A few cells above the band of the column |
| 0 | No clumps | Single sharp band at base of column |
| ± | Cells unreadable-impossible to score | |

Variation of one score level is considered to be within the margin of error of this technique and is not considered significant. Significant score variances are those of more than one score level.

**Haemolysis Scoring in GlassTtubes**

| Score | Results |
|---|---|
| 4+ | Severely haemolysed |
| 3+ | Very haemolysed |
| 2+ | Moderately haemolysed |
| 1+ | Mildy haemolysed |
| w+ | Faintly haemolysed |
| 0 | No haemolysis seen |
| NR | Cells unreadable-solution brown |

# 3: Subclavian vein infusion in mouse

**OBJECTIVE, RATIONALE**

To transfuse kodecytes or FSL molecules in solution into anaesthetised mice via subclavian injection.

**SAMPLES, REAGENTS AND EQUIPMENT**

**Reagents**

All FSL molecules were obtained from KODE Biotech Materials Ltd ,Auckland, New Zealand

- FSL-A (cat # 421604)
- FSL-GB3 (cat #352439)
- FSL-biotin (cat#187786)
- Sterile saline (Gelman,DEMO S.A. Athens, Greece)
- Phosphate Buffered Saline pH 7.4
- A+biotin kodecytes
- B+biotin kodecytes
- GB3+biotin kodecytes
- biotin kodecytes

**Equipment**

- 23 g needle
- 22 g needle
- Stopwatch
- Anaesthetic equipment
- Scales
- Surgical suture DS-18
- 1mL tuberculin syringe
- Vortex (Thermolyne Maximix Plus M63210-26)
- Plain capillary tubes and caps
- Heparinised capillary tubes and caps
- Eppindorf microcentrifuge tubes 1.5mL (3445 Raylab, Auckland)
- Hitachi hematocrit centrifuge
- Centrifuge (Sorvall MC-12V, Du Pont, USA)

**METHOD**

**3.1 Preparation of FSLs in solution**

As per protocol 2.1

**3.2 Preparation of FSL- kodecytes**

As per protocol 2.2

**3.3 Transfusion by subclavian vein injection**

- Weigh mice and read electronic tag.
- Place in chamber with Attane gas to anaesthetise mice. Protocol 1.
- Transfer mouse to surgical slab and place on its back with mouth inside rubber mask for anaesthetic gas.
- Use double sided tape to hold mouse in place and tape its forearms back (This allowed the vein to be more prominent)see photo below.
- Open mouse chest cavity to expose the subclavian vein.
- Inject required amount of FSL or kodecyte suspension into the vein and record time.
- Take blood samples at 0.1hr from subclavian vein. The wound is sutured.
- Make tail vein collects at 2, 8, 24,48,72,96 h (Protocol1 4).
- Monitor respiration rate and for signs of haemolytic transfusion reaction: haemoglobinuria. Observe for posture, activity level, coat appearance, breathing rate/type, dehydration, diarrhoea, vocalisation, CNS signs, wound appearance (bleeding, healing, discharge). Details are recorded on behavioural monitoring sheet.

## METHOD VARIATIONS

**3V 1. FSL-A or GB3 infusion to determine whether subclavian vein infusion of FSL molecules (3mM) has any toxic effect on mice.**

P*repare 15mM FSL in sterile saline ( using semi-sterile technique)*

- Weigh 21.2 mg FSL-A or FSL-GB3 to give 3mM.
- Suspend in1 mL sterile saline and vortex to mix.
- Mix carefully and dissolve at RT for about 2 hours.
- Store at 4° C and bring to RT before use (for about 2 hours).

*Injection of FSL*

- Inject as protocol 3.3
- Observe health and welfare immediately and at 2h, 24 h, 48 h, 1 week, 1 month, 2 months and 3 months.
- Weigh mice at 1 week, 1 month, 2 months and 3 months.

*After three months:*

- Exsanguinate by cardiac puncture (Protocol1.5).
- Weigh immunised and control animals.
- Make 3 blood smears/mouse for haematological study using Giemsa stain 1:3 (Ref: Dacie and Lewis Practical Haematology).
- Collect serum for antibody serology (Protocol 1.4).
- Harvest hepatic lymphatic nodes, lungs, kidney, liver, spleen at autopsy.
- Fix one half of organ tissue in formalin.
- Examine histology sections and gross morphology of all organs.

Note: In the event of an early death above procedures will be carried out on the mouse

**3V 2. Infusion of red cell haemolysate for observation of hemoglobinuria after an incompatible cell transfusion**.

- Collect naive mice red cells.
- Add 40 µL packed washed cells to100 µL deionised water.
- Transfer small aliquots into eppindorf centrifuge tubes.
- Freeze for about one hour.
- Make transfusion volumes 100 µL.

*Injection of haemolysate*

- Inject as protocol 3.3
- Monitor mouse and collect urine.
- Exsanguinate mice at 15, 30, 45 and 60 min post transfusion.
- Examine organs at autopsy for signs of incompatible transfusion.
- Examine bladder for haemoglobinuria.

**3V 3. Infusion of FSL-A for neutralization of anti-A**

*FSL-A for neutralization*

- FSL-A 20 mg/mL in sterile saline (Protocol 2 V.1).

*Neutralization of anti-A*

- Tagged, immunised, anti-A positive and naive mice are weighed.
- Mice are placed in anaesthetic chamber to a level of surgical anaesthesia.
- 200 µL FSL-A (20 mg/mL) is injected into the surgically exposed subclavian vein and time noted.

**3V 4. Preparation of kodecytes for transfusion**

- 10 % A+Biotin kodecytes are prepared Protocol 2 V3.

**3V 5. Infusion of 60 µL (10 %) A+biotin kodecyte suspension at 2 min**

- At 2 minutes post FSL-A infusion, 100 µL kodecyte suspension, 10 % in sterile saline, is injected into the subclavian vein.
- At 6 min a blood sample is collected from subclavian vein.
- Mouse is sutured and monitored.
- Blood collects are made by tail vein nick for kodecyte cell survival experiments Protocol 1 V.1.

**3V 6. Infusion of 60 µL (10 % )A+biotin kodecytes for measurement of cell survival one or two weeks post FSL-A infusion**

- 100 µL kodecyte suspension, 10 % in sterile saline, is injected into the subclavian vein.
- At 6 min a blood sample is collected from subclavian vein.
- Mouse is sutured and monitored.
- Blood collects are made by tail vein nick for kodecyte cell survival experiments Protocol 1.V.1.

**3V 7. Infusion of 20 µL (3.2%) A,GB3 and biotin kodecytes for measurement of cell survival**

- Create kodecytes Protocol 2 V. 1.
- 200 µL kodecyte suspension, 3.2 % in sterile saline, is injected into the subclavian vein.
- At 6 min a blood sample is collected from subclavian vein.
- Mouse is sutured and monitored.
- Blood collects are made by tail vein nick for kodecyte cell survival experiments Protocol 1.V.1.

**3V 8. FSL-A infusion for tolerance series**

- Prepare FSL-A at 4, 2,1mg/mL in sterile saline.
- Inject Protocol 3.3
- At 0.1hr take subclavian blood collect (Protocol 1.3).
- Repeat at 2,8,24,36,48,72 until FSL-A not detected on cells
- Determine presence of FSL-A on cells Protocol 2.3

**Infusion of FSL via subclavian vein**

# 4*:* Serology Procedures

## *In vitro* sensitisation of human red cells, creation of kodecytes, inhibition and dissociation of anti-A with FSL-A

**OBJECTIVE, RATIONALE**

To study the sensitisation of human red cells, creation of kodecytes, *in vitro* inhibition and dissociation of anti-A with FSL molecules with varying concentrations of FSLs.

**SAMPLES, REAGENTS AND EQUIPMENT**

**Samples**

- Human blood group O serum (anti-A, anti-B, anti A,B)
- Human blood group B serum (anti-A)
- Human blood group $A_1$ red cells-washed and packed

**Reagents**

All FSL molecules were obtained from KODE Biotech Materials Ltd, Auckland, New Zealand

- FSL-A (cat # 421604)
- FSL-B (cat #199283)
- MQ water
- Celpresol (CSL, Australia)
- 2 %BSA/PBS
- Monoclonal anti-A reagent (Epiclone CSL, Australia)
- Blank gel-cards (NaCl Enzyme and cold agglutinins Diamed AG,Cressier-Sur Morat, Switzerland)
- Blank Liss Coombs gel-card (Diamed AG,Cressier-Sur Morat, Switzerland)
- PBS

**Equipment**

- Micropipettes (1-1000 µL)
- Eppindorf microcentrifuge tubes,1.5 mL (3445,RayLab)
- Glass Kimble tubes (KIM45048-18150,Biolab)
- Waterbath (Julabo F18)
- Centrifuge (Immufuge II, American Dade, USA)
- Plastic transfer pipettes
- Diamed centrifuge (Diamed AG,Cressier-Sur Morat, Switzerland)
- Diamed incubator (Diamed AG,Cressier-Sur Morat, Switzerland)

**PROCEDURES**

**4V1. *In vitro* transformation of human RBCs to create kodecytes with FSL-A dilutions in PBS or serum using different concentrations of FSL-A dissolved in either PBS, Group O or A serum over 0.1-8h.**

*Make FSL-A concentrations at 4, 2, 1, 0.5, 0.25, 0.1, 0.05, 0.025 mg/mL*

- Dilute the FSL-A to the desired concentration in Oserum, A serum and PBS.

*Red cell transformation*

- Wash the RBC's three times in PBS by centrifugation at 2700 rpm for 3 minutes by immufuge
- Add equal volumes of FSL solution and packed RBC to eppindorf. Mix well
- Place cells in a 37$^{o}$C waterbath for 2hr, resuspending the cells by intermittent gentle mixing.
- At 0.1hr remove 50 µL cell suspension. Immediately place in ice to stop reaction
- At 0.1,1, 2, 4, 8 h remove 50 µL cell suspension.
- Wash cells 3 x in Celpresol

*Diamed gel-card NaCl to test for presence of A antigen on red cell*

- Suspend washed red cells to 0.8% in PBS.
- Add 50 µL sample to well in the Diamed gel card.
- Add 25 µL anti-A monoclonal reagent to the well.
- Spin in Diamed centrifuge.
- Record the agglutination score.

*Indirect Antibody agglutination, IgG*

- Take 50 µL of each FSL-A concentration mixture, 0.8%, in to Liss Coombs gel card.
- Add 25µL Human O serum anti-A
- Incubate in Diamed incubator
- Spin in Diamed centrifuge
- Read for agglutination using Diamed chart for column analysis

**4V.2 *In vitro* antibody sensitization of A kodecytes during transformation due to presence of anti-A in O serum with various concentrations of FSLA solution measured at varying time points using Diamed gel cards**.

*FSL-A concentrations*

- Make 4, 2, 1 mg/mL FSL-A, each dissolved in O serum
- Controls using serum and 2% BSA/PBS, without FSL-A are run in parallel

*FSL-A transformation and sensitization*

- Mix washed, packed O red cells with equal volume of FSL-A in O serum (200uL) for each concentration
- Incubate 37°C with intermittent mixing- 0.1 - 8hr

*Detection of FSL-A insertion at various time points*

- Take 50uL from cell mixture in water-bath 37°C at 0.1hr, 2, 4, 8hr
- Wash 4X with celpresol
- Make 0.8% cell suspension with celpresol
- Test for presence of A using monoclonal anti-A reagent using Diamed technology.

*Direct Antibody agglutination, IgM*

- Take 50 µL of each FSL-A concentration mixture, 0.8%, into NaCl enzyme gel card
- Spin in Diamed centrifuge
- Read for agglutination.

*Indirect Antibody agglutination, IgM*

- Take 50µL of each FSL-A concentration mixture, 0.8%, in to NaCl enzyme gel card.
- Add 25µL Human O serum anti-A or monoclonal anti-A reagent for control.
- Spin in Diamed centrifuge.
- Read for agglutination.

*Indirect Antibody agglutination, IgG*

- Take 50 µL of each FSL-A concentration mixture, 0.8%, in to Liss Coombs gel card.
- Add 25 µL Human O serum anti-A.
- Incubate in Diamed incubator.
- Spin in Diamed centrifuge.
- Read for agglutination

**4V3. *In vitro* inhibition of human anti-A, A,B of known titre in human O and B serum with FSL-A at various concentrations. (FSL-A volumes based on same 2:7 ratio of FSL-A / plasma given to mice in vivo).**

*Preparation of FSL concentrations*

- Dissolve FSL-A, FSL-B and FSLA+B; 20, 10, 5, 2, 1, 0.5 mg/mL in PBS.
- Controls using 2%BSA/PBS, without FSL-A are run in parallel

*Inhibition/neutralization of anti-A by FSL-A*

- Label three sets of tubes for O and B serum of known titre.
- Add 10 µL of each FSL concentration or PBS for control tube.
- Add 35 µL of O or B serum to appropriate FSL-A concentration.
- Mix and immediately add 45 µL of washed, packed $A_1$ red blood cells to each tube.
- Mix and incubate 37°C for 2h.

*Detection of antibody presence at various time points – 30min, 60 min, 90min, 120min.*

- Take 20 µL sample from each tube and examine for haemolysis and agglutination.
- Wash each mixture 4 x in Celpresol.

*Direct Antibody Test–for anti-A, Ig*

- Make 0.8% cell suspension with Celpresol.
- Take 50 µL of FSL-A concentration mixture and control suspension into NaCl enzyme gel

*Indirect Antibody Test–for anti-A, IgG*

- Take 50 µL of FSL-A mixture and control at 0.8% suspension into a Liss Coombs gel card.
- Incubate in Diamed incubator.
- Spin in Diamed centrifuge and read for agglutination.

**4V 4. In vitro dissociation over time of anti-A from sensitized red cells due to the presence of FSL-A. The cells are pre-sensitised with various dilutions of group O serum containing anti-A.**

- O serum serially diluted to 1:512 in PBS.
- Make 4 mg/mL FSL-A dissolved in O serum.
- Controls, using serum and 2%BSA/PBS, without FSL-A are run in parallel.

*Sensitisation of A red cells*

- Mix equal volume of washed, packed $A_1$ red cells with each dilution of O serum.
- Incubate 37°C with intermittent mixing for 30 min. This marks time zero.

*Neutralization of anti-A by addition of FSL-A*

- To tube A add 35 µL FSL-A.
- To tube B add 35 µL PBS.
- Incubate 37°C with intermittent mixing for time periods 30 - 120 min.
- Take 40µL from cell mixture in water-bath 37°C at 30, 60, 90 and 120 min.

*Direct Antibody Test - anti-A, Ig*

- Wash 4 x with celpresol.
- Make 0.8% cell suspension with celpresol.
- Take 50 µL of FSL-A concentration mixture and from control at 0.8% suspension into NaCL enzyme gel card.
- Spin in Diamed centrifuge and read for agglutination.

*Indirect Antibody Test - anti-A, IgG/C3d*

- Take 50 µL FSL-A concentration mixture and control at 0.8% suspension into a Liss/Coombs gel card.
- Incubate in Diamed incubator.
- Spin in Diamed centrifuge and read for agglutination.

# 5: Antibody detection by Enzyme Immuno-Assay using Inkjet wells

## OBJECTIVE, RATIONALE

To test mice serum or plasma for anti-A (Ig or IgG) by thin layer chromatography using inkjet wells containing 1mg/mL FSL – detected with alkaline phosphate conjugated Ig or IgG and immunostained NBT/BCIP dye.

## SAMPLES, REAGENTS AND EQUIPMENT

**Reagents**

- Mouse plasma or serum
- TLC silica plates Alugram Nano-Sil G,Magarey-Nagel, Germany #818141
- Deionised water
- Bovine Serum Albumin 2% in PBS
- Monoclonal anti-A, or anti-B reagent (Epiclone, Australia)
- Anti-mouse IgG alkaline phosphatase conjugate Sigma A3438
- Anti-mouse Ig alkaline phosphatase conjugate Chemicon, Australia AP 326 A
- NBT/BCIP substrate (Cat No 1 681 451 Roche Diagnostics GmbH, Penzberg, Germany)
- Tris buffer substrate pH 9.5
- PBS pH 7.4
- Sterile saline(Gelman,DEMO S.A. Athens, Greece)
- Celpresol (CSL Australia)
- FSL 1 mg/mL in saline or PBS, as appropriate to antibody detection required

**Equipment**

- Inkjet wells prepared with FSL-A, B, GB3, Biotin at 1 mg/mL (http://www.sigmaaldrich.com/new-zealand.htmL )
- Plastic transfer pipettes
- Centrifuge Immufuge II, American Dade, USA)
- Micropipettes (10-1000 µL)
- Eppindorf microcentrifuge tubes,1.5 mL (3445,RayLab)
- Clock
- Tissues

## METHOD

**5.1 Reagent Preparation**

**1. Blocking reagent 2% BSA/PBS**

- 8g Bovine Serum Albumin
- 400 mL PBS pH7.4
- 4 mL 10% sodium azide Na N3

**2.Primary antibody mouse serum**

- Make 1:3 dilution of the primary antibody (serum or plasma) in 2% BSA in PBS (require at least 90 µL in well).

**3.Secondary antibody**

- Using appropriate anti-mouse Ig or IgG alkaline phosphatase conjugate make a 2.5 µL/1mL dilution of the conjugate antibody in 2% BSA/PBS.

**4.Tris Substrate Buffer**

- Tris 12.11 g SCR CC75-
- $MgCl_2$ 10.15 g SCR C43-1
- NaCl 5.84 g SCR C401-3-2
- Add 950 mL DI water
- Adjust pH to 9.5 with conc HCl
- Make up to 1L with DI water

**5.NBT/BCIP**

- Nitroblue tetrazolium chloride/5-bromo-4chloro-3 indolyl phosphate, toluidine dye conjugate (NBT/CBIP) toluidine salt in DMSO.
- Make a 20 µL/mL dilution of the NBT/BCIP substrate in Tris substrate buffer

**6. TLC in inket printed wells**

**6.1 Block non-specific antibodies**

- Cover pre-prepared FSL inkjet well plate with 2% BSA for at least one hour to bind all the unspecific ligands to prevent or reduce excessive background staining.

**6.2 Primary antibody Immunised mice serum**

- Remove 2% BSA in PBS from the TLC plate, drain off well.
- Fill well with 90 µL primary antibody solution.
- Cover so wells to prevent drying, for 90 min -3 h.

**6.3 Secondary Antibody Mouse anti- Ig or IgG antibody conjugate with alkaline phosphatise**

- Remove and keep the primary antibody solution using a transfer pipette if the serum to be kept.
- Wash the plate several times with PBS.
- Flick dry and blot plastic surface to remove any excess PBS from plate.
- Fill the wells with the conjugate antibody solution and leave covered for an hour.

**6.4 Immunostain NBT/BCIP dye substrate**

- Remove the conjugate antibody solution from the wells by washing several times in PBS.
- Wash the wells several times with the substrate buffer and flick off solution to drain.
- Fill the wells with the dye substrate and leave until letters have developed - with minimal background staining (15-20 min) and remove substrate under gentle stream of deionised water.
- Shake off excess water and stand to drip dry overnight.
- TLC reactivity was assigned following enzyme immunoassay by the appearance of the letter A or B, GB3 for example in Figure- I, or the letters IgG in the case of specific anti-IgG conjugate for example in Figure II, and its co-ordinates in the microwell. Titres were carried out on selected serum for example in Figure III.(Figure 34).

## METHOD VARIATION

**5V 1. Antibody Titre**

- Dilute mice serum 1:2 to1:128 in 2% BSA/PBS.
- Perform TLC as above.

**5V.2 To create inkjet wells (Barr et al., 2010).**

*Reagents*

- 0.5% polyisobutylmethacrylate in n-hexane and diethyl eyther
- plexigum p28
- Alugram Nano-SIL G silica TCL plate, 0.2mm Nano silica gel 60, Macherey-Nagel
- 0.05% Bromophenol blue in PBS, pH7.2
- 2% BSA in PBS
- Monoclonal Anti-A (Epiclone, CSL, Australia)
- Sheep Anti-Mouse Ig, Alkaline phosphatase conjugated, Chemicon
- NBT/BCIP stock solution, 18.75 mg/mL NBT (Nitro blue tetrazolium chloride) and 9.4 mg/mL BCIP (5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt) in 67% DMSO (v/v),
- Substrate buffer (100mM Tris, 100mM NaCl, 50mM $MgCl_2$), pH9.5
- Deionised water (DI)

*Equipment*

- 3 mm Acrylic
- 3M Super 77 multipurpose adhesive
- Epson stylus colour T21 piezoelectric printer

*Laser cutting of 3mm acrylic*

- Cut acrylic using a laser cutter and a template drawn in Illustrator. The plate measures 85 mm by 64 mm, and the wells are a 6 mm diameter circle
- Cut acrylic in a 8x4 arrangement
- Numbers, letters and logos engraved on the different wells

*Printing of FSL*

- The same template used to laser cut the acrylic is used to print the FSL construct onto a silica TLC plate, enabling perfect alignment of the printed numbers and the wells.
- FSL 1 mg/mL with 0.05% Bromophenol blue (to visualise the printed area) in PBS, pH7,2, is loaded into a modified, refillable inkjet cartridge and printed using an Epson stylus colour T21 piezoelectric printer onto the TLC plate in the letter ‘A’ for example.

**Adhesion of the acrylic and silica**

*Washing step*

- Place the silica plate in a beaker of DI water for 20 min.
- Wash the plate to remove salts and dye.
- Air dry.

*Plasticising Step*

- The silica plate is then dropped into 0.5% polyisobutylmethacrylate for 1 min vertically
- Remove and air dry vertically

*Adhesion*

- The back of the acrylic is sprayed with multipurpose adhesive, then stuck to the silica and left to dry for 20 min.

---

## Examples of Inkjet wells used for antibody detection

**I** **II** **III**

Figure 34. FSL-A constructs printed as alphanumeric characters onto membranes.

They were then made into microplates by adhering laser-cut Perspex templates (6mm diameter wells) onto the membranes. Printed plates are blank but when incubated with serum containing antibody, alphanumeric characters appear (following appropriate washing and incubation with alkaline phosphatise labelled secondary anti-murine immunoglobulin reagents and development wth a precipitating chromogenic substrate. Image I shows a complete microplate stained with anti-Ig; The appearance of the printed letter A and its microwell co-ordinates e.g. A1, B1, C1, D1 in the microwell following EIA development defines the presence of anti-A. C1, D2 are negative. D7 contained BSA as a negative control. D8 contained monoclonal anti-A positive control. FSLs-GB3,B and biotin were also printed into other microwells using respective letters with negative results (not shown). Image II shows a titre of Ig anti-A. It is noted that the letter fades as the titre reaches its endpoint in serum. Row 1 had a titre to 32, row 2 and 3 titre >128 and row 4 contained monoclonal anti-A reagent. A1 is a negative control, BSA. Image III shows a microwell format to detect Ig and IgG. Ig Anti-A in rows 1 and 3 and IgG anti-A in rows 2 and 4, present in immunised mice serum.

# 6: Recovery of kodecytes from circulation using agarose - avidin gel

## OBJECTIVE, RATIONALE

To recover biotinylated kodecytes from whole blood in an agarose-avidin gel and remove them from the gel to determine the presence of FSL-A or FSL-B by agglutination with anti-A or anti-B monoclonal reagent. Avidin Alexafluor 488 is used to detect the presence of FSL-biotin with fluorescent microscopy. The 20uL volume transfused represented 3% of the estimated total red cells whole blood in circulation (63 µL represented 10%).

## SAMPLES, REAGENTS AND EQUIPMENT

**Samples**

- Mouse whole blood, post kodecyte transfusion

**Reagents**

- FSL-A+biotin murine kodecytes
- FSL-B+biotin murine kodecytes
- FSL-GB3+biotin murine kodecytes
- Pierce ®avidin agarose resin (Cat 20219 Pierce www.thermo.com/pierce)
- Anti-A monoclonal reagent Epiclone, CSL, Australia)
- Anti-B monoclonal reagent Epiclone, CSL, Australia
- avidin/Alexafluor 488 (Molecular Probes A-2901 Lot 84C1-1)
- Celpresol (Epiclone, Australia)
- 2% BSA in PBS
- Diamed gel cards (NaCl enzyme and cold agglutininsDiamed AG,Cressier-Sur Morat, Switzerland)

**Equipment**

- Centrifuge Immufuge II, American Dade, USA
- Diamed centrifuge (Diamed AG,Cressier-Sur Morat, Switzerland)
- Fluorescent microscope Olympus BX51
- Eppindorf microcentrifuge tubes,1.5 mL (3445,RayLab)

## PROCEDURE

**6.1 Transfusion of kodecytes prepared as per Protocol 2**

- Inject 100 µL or 200 µL kodecyte suspension, (20 µL and 180 µL or 63 µL cells to 37 µL saline), into the subclavian vein.

**6.2 Blood sampling as per Protocol 1.4**

- Blood sample, 50 µL, was taken by prick to tail vein and collected into heparinised haematocrit tube at 0.1, 2, 8, 24, 48 and 72 h

**6.3 Agarose-avidin separation**

- Wash agarose-avidin gel in celpresol to remove sodium azide.
- Resuspend in equal volumes with celpresol.
- Fill Diamed gel cards with about 40 µL agarose – avidin suspension.
- Add 5 µL whole blood to the gel in a Diamed card.
- Centrifuge card 3 x in Diamed centrifuge and examine for separation into bands.
- Make a set of standard set of controls for comparison using 100, 10, 5, 2, 1, and 0% FSL-A/FSL-biotin kodecytes or FSL-B/FSL-biotin kodecytes in naive cells.

**6.4 Testing for FSL-A or FSL-B presence on red cells**

- Remove the bands of cells from the top and bottom of the gel card.
- Resuspend cells in celpresol (0.5 mL) and leave to stand for about 10 min to allow gel to settle.
- Remove the supernatant and centrifuge these cells for one minute on high immufuge.
- Resuspend the red cell pellet in as little solution as needed to make a suspension.
- Add all the suspension obtained to a Diamed gel card and add 25 uL anti-A or anti-B reagent. A stronger concentration, about 15 %, ensured the band was visible when few cells retrieved.

# APPENDIX A: PUBLICATIONS

Oliver C, Blake D, Henry S. 2011a. In vivo neutralization of anti-A and successful transfusion of A antigen-incompatible red blood cells in an animal model. Transfusion 51:2664-2675.

Oliver C, Blake D, Henry S. 2011b. Modeling transfusion reactions and predicting in vivo cell survival with kodecytes. Transfusion 51:1723-1730.

Henry SM, Barr KL, Oliver CA. 2012. Modeling transfusion reactions with kodecytes and enabling ABO-incompatible transfusion with Function-Spacer-Lipid constructs. ISBT Science Series 7.

Oliver C, Blake D, Ferguson S, Bovin N,Henry, S. 2010. Poster Presentations. FEBS Journal 277(Suppl. 1) 37-271; A2.31 p50-1

# APPENDIX B: TABLES

Table B-1. Comparison of % survival of biotin kodecytes in naive and anti-A positive mice after 20 µL (3.2% transfusion) over 96 h.

| Infusion of 20 µL biotin kodecytes | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Mice | | Anti-A status | % Survival (h) | | | | | | | Ig Anti-A status |
| Number | ID | Pre tx | 0.1 | 2 | 8 | 24 | 48 | 72 | 96 | Post Tx |
| 1 | 9925 | 0 | 2.0 | NT | 1.3 | 1.0 | 1.2 | 0.6 | 0.9 | 0 |
| 2 | 8740 | 0 | 2.7 | NT | 1.9 | 2.0 | 1.7 | 1.0 | 1.2 | 0 |
| 3 | 4895 | 0 | 2.3 | 2.2 | 2.4 | 2.0 | 2.5 | NT | NT | 0 |
| 4 | 1850 | 0 | 2.1 | 2.1 | 1.8 | 2.0 | 1.4 | NT | NT | 0 |
| 5 | 8779 | 0 | 2.5 | 2.3 | 2.2 | 1.0 | 1.4 | 1.3 | 1.0 | 0 |
| 6 | 3198 | 0 | 2.3 | 1.9 | 1.8 | 1.5 | 1.6 | 1.2 | 1.0 | 0 |
| 7 | 6414 | 0 | 2.0 | 1.5 | 1.8 | 1.5 | 1.4 | 1.4 | 1.0 | 0 |
| 8 | 7736 | 0 | 1.2 | 1.3 | 1.3 | 1.3 | 1.5 | 1.1 | 1.0 | 0 |
| **Ave** | | | **2.1** | **1.9** | **1.8** | **1.5** | **1.6** | **1.1** | **1.0** | |
| 1 | 2313 | + | 1.9 | 1.8 | 1.5 | 1.4 | 1.4 | NT | NT | W+ |
| 2 | 9930 | + | 3.1 | 2.3 | 3.8 | 3.7 | 2.8 | NT | NT | W+ |
| 3 | 1416 | + | 3.6 | 3.4 | 2.5 | 3.2 | 2.0 | NT | NT | W+ |
| 4 | 2719 | + | 1.5 | 1.6 | 1.4 | 1.5 | 1.3 | 1.2 | 1.0 | + |
| 5 | 6242 | + | 2.1 | 2.0 | 2.0 | 1.0 | 1.0 | 1.0 | 1.1 | + |
| 6 | 3264 | + | 2.7 | 2.0 | 1.8 | 1.5 | 1.5 | 1.1 | 1.1 | + |
| 7 | 8565 | + | 1.9 | 1.6 | 1.5 | 1.3 | 1.4 | 1.2 | 1.2 | + |
| 8 | 6352 | + | 2.1 | 1.1 | 1.1 | 1.0 | 1.3 | 1.0 | 1.1 | + |
| **Ave** | | | **2.3** | **1.9** | **1.9** | **1.8** | **1.6** | **1.1** | **1.1** | |

NT=not tested

Tx =transfusion

Table B-2. Comparison of % survival of GB3+biotin kodecytes in naive and anti-A positive mice after 20 µL (3.2% transfusion) over 96 h.

| Infusion of 20 µL GB3+biotin kodecytes | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Mice | | Anti-A status | % Survival (h) | | | | | | | Anti-A Ig Status |
| Number | ID | Pre tx | 0.1 | 2 | 8 | 24 | 48 | 72 | 96 | Post tx |
| 1 | 2731 | 0 | 2.5 | 2.3 | 2.3 | 2.1 | 1.9 | 1.9 | 2.1 | 0 |
| **Ave** | | | **2.5** | **2.3** | **2.3** | **2.1** | **1.9** | **1.9** | **2.1** | |
| 1 | 3708 | + | 2.8 | 2.6 | 2.0 | 2.1 | 2.1 | 1.9 | 2.1 | + |
| 2 | 4793 | + | 2.1 | 2.0 | 1.8 | 1.1 | 1.3 | 1.0 | 1.3 | + |
| 3 | 9881 | + | 2.5 | 2.0 | 2.1 | 1.2 | 1.6 | 1.7 | 1.7 | + |
| 4 | 7674 | + | 2.5 | 2.5 | 2.3 | 1.9 | 2.0 | 1.9 | 1.9 | + |
| **Ave** | | | **2.5** | **2.3** | **2.1** | **1.6** | **1.7** | **1.6** | **1.7** | |

Table B-3. Comparison of survival of A+biotin kodecytes in naive and anti-A positive mice at 0.1h. 20 µL tx (transfused) represents 3.2% (and 63 µL represents 10%) of total circulating red cells.

| **Comparison of A+biotin kodecytes in anti-A positive and negative mice at 0.1h post infusion** | | | | | | |
|---|---|---|---|---|---|---|
| Mice | | Pre transfusion antibody status | % kodecyte survival measured | | Post transfusion antibody status | |
| No. | ID | Anti-A Ig | 20 µL tx | 63 µL tx | Anti-A Ig | Anti-A IgG |
| 1 | 5848 | - | 2.5 | | - | - |
| 2 | 4861 | - | 2.1 | | - | - |
| 3 | 1669 | - | 3.1 | | - | - |
| 4 | 5811 | - | 2.6 | | - | - |
| 5 | 4722 | - | 3.0 | | - | - |
| 6 | 7738 | - | 3.2 | | - | - |
| 7 | 4636 | - | 3.1 | | - | - |
| 8 | 9048 | - | 3.5 | | - | - |
| 9 | 7994 | - | | 10 | - | - |
| 1 | 4822 | + | 0.4 | | + | - |
| 2 | 9135 | + | 0.1 | | + | + |
| 3 | 9819 | + | 0.5 | | + | + |
| 4 | 2473 | + | 0.5 | | + | + |
| 5 | 3747 | + | 0.3 | | + | + |
| 6 | 2543 | + | 0.5 | | + | - |
| 7 | 3844 | + | 0.4 | | + | - |
| 8 | 1326 | + | 0.03 | | + | - |
| 9 | 9686 | + | 0.9 | | + | - |
| 10 | 4913 | + | 0.04 | | + | + |
| 11 | 3809 | + | 0.6 | | + | + |
| 12 | 0954 | + | | 2.2 | + | + |
| 13 | 4862 | + | | 4.7 | + | + |
| 14 | 1124 | + | | 1.7 | + | + |
| 15 | 1389 | + | | 4.5 | + | + |
| 16 | 8998 | + | | 6.4 | + | + |
| 17 | 1389 | + | | 4.5 | + | + |
| 18 | 3649 | + | | 0.2 | + | + |
| 19 | 0232 | - | 1.7 | | - | - |
| 20 | 4998 | *w+ | | 2.5 | **vw+ | vw+ |

* w= weak result; ** vw= very weak positive result

Table B-4. Comparison of % survival of A+biotin kodecytes (20 µL) in naive and anti-A positive mice after 20 µL 3.2%transfusion, or 10% transfusion (*) over 96 h.

**Infusion of 20 µL (** 63 µL) A+biotin kodecytes**

| Mice | | Anti-A Ig status | % Survival (h) | | | | | | | Anti-A Ig | Anti-A IgG |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Number | ID | Pre tx | 0.1 | 2 | 8 | 24 | 48 | 72 | 96 | Post tx | |
| 1 | 5848 | 0 | 2.5 | 3.4 | 3.0 | 2.1 | 1.3 | | | 0 | 0 |
| 2 | 4861 | 0 | 2.1 | 2.9 | 3.4 | 2.4 | 2.1 | | | 0 | 0 |
| 3 | 1669 | 0 | 3.1 | 3.1 | 2.3 | 1.9 | 1.5 | 1.4 | 1.0 | 0 | 0 |
| 4 | 5811 | 0 | 2.6 | 2.9 | 2.9 | 2.3 | 1.3 | 1.4 | 1.4 | 0 | 0 |
| 5 | 4722 | 0 | 3.0 | 2.6 | 2.6 | 1.6 | 1.8 | 2.0 | 1.3 | 0 | 0 |
| 6 | 7738 | 0 | 3.2 | 1.9 | 2.1 | 1.7 | 1.6 | 1.6 | 1.0 | 0 | 0 |
| 7 | 4636 | 0 | 3.1 | 2.4 | 1.8 | 1.7 | 1.9 | 2.3 | 1.7 | 0 | 0 |
| 8 | 9048 | 0 | 3.5 | 3.7 | 3.2 | 3.8 | 3.2 | 1.5 | 1.2 | 0 | 0 |
| **Ave** | | | **2.9** | **2.8** | **2.6** | **2.2** | **1.8** | **1.7** | **1.3** | | |
| 9 | 7994* | | 10 | 9.6 | 7.8 | 6.9 | 5.6 | 5.3 | 5.3 | 0 | 0 |
| **Ave** | | | **10** | **9.6** | **7.8** | **6.9** | **5.6** | **5.3** | **5.3** | | |
| 1 | 4822 | + | 0.4 | 0.2 | 0.6 | 0.5 | 0.3 | | | + | + |
| 2 | 9135 | + | 0.1 | 0.05 | 0.04 | 0.01 | 0 | 0 | | + | + |
| 3 | 9819 | + | 0.5 | 0.8 | 0.4 | 0.5 | 0.2 | | | + | + |
| 4 | 2473 | + | 0.5 | 0.3 | 0.5 | 0.5 | 0.05 | | | + | + |
| 5 | 3747 | + | 0.3 | 0.3 | 0.1 | 0.02 | | | | + | + |
| 6 | 2543 | + | 0.5 | 0.6 | 0.4 | 0.1 | | | | + | + |
| 7 | 3844 | + | 0.4 | 0.1 | 0 | 0 | 0.06 | 0.04 | 0 | + | + |
| 8 | 1326 | + | 0.03 | 0 | 0 | 0 | | | | + | + |
| 9 | 9686 | + | 0.9 | 0.9 | 1.0 | 1.0 | 0.8 | 0.6 | 0.4 | + | + |
| 10 | 4913 | + | 0.04 | 0.04 | 0.1 | 0.1 | 0.1 | 0.5 | 0.4 | + | + |
| 11 | 3809 | + | 0.6 | 0.1 | 0.2 | 0.1 | 0.1 | 0.02 | | + | + |
| **Ave** | | | **0.4** | **0.3** | **0.2** | **0.2** | **0.1** | **0.2** | | | |
| 12 | 3226* | + | 1.3 | 1.2 | 1.2 | 1.1 | 0.7 | | | + | + |
| 13 | 0954* | + | 2.2 | 1.3 | | | | | | + | + |
| 14 | 4467* | + | 3.5 | 3.4 | 3.3 | | | | | + | + |
| 15 | 6161* | + | 2.1 | 2.1 | 2.1 | 0.4 | | | | + | + |
| 16 | 0506* | + | 5.2 | 2.3 | | 2.3 | | | | + | + |
| 17 | 3397* | + | 3.2 | 1.3 | | 1.4 | | | | + | + |
| 28 | 4862* | + | 4.7 | 2.9 | | 3.0 | | | | + | + |
| 19 | 2837* | + | 5.2 | 4.2 | | 4.8 | | | | + | + |
| **Ave** | | | **3.4** | **2.3** | **2.2** | **2.2** | **0.7** | | | | |
| 20 | 0232† | 0 | 1.7 | 1.7 | 1.4 | 1.6 | 1.4 | 0.7 | 1.1 | - | - |
| 21 | 4998† | w+ | 2.5 | 1.6 | 1.8 | 0.9 | 0.9 | 1.1 | 0.8 | - | vw+ |

† 0232 had no anti-A and 4998 had very weak (vw) anti-A result and thus were not included in the subsequent data

Table B-5.Comparison of cell survival % averages between various series after transfusion (20 µL with FSL constructs.

| Comparison of %survival averages with various FSL constructs | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| FSL | Anti-A status Pre tx | Average % Survival (h) | | | | | | |
| | | 0.1 | 2 | 8 | 24 | 48 | 72 | 96 |
| biotin | 0 | 2.1 | 1.9 | 1.8 | 1.5 | 1.6 | 1.1 | 1.0 |
| GB3 | 0 | 2.5 | 2.3 | 2.3 | 2.1 | 1.9 | 1.9 | 2.1 |
| biotin/GB3 | 0 | 2.3 | 2.1 | 2.0 | 1.8 | 1.8 | 1.4 | 1.5 |
| A+biotin | 0 | 3.1 | 2.7 | 2.6 | 2.2 | 1.9 | 1.6 | 1.3 |
| biotin | + | 2.3 | 1.9 | 1.9 | 1.8 | 1.6 | 1.1 | 1.1 |
| GB3 | + | 2.5 | 2.3 | 2.1 | 1.6 | 1.7 | 1.6 | 1.7 |
| biotin/GB3 | + | 2.4 | 2.1 | 2.0 | 1.7 | 1.7 | 1.3 | 1.4 |
| A+biotin | + | 0.4 | 0.3 | 0.2 | 0.2 | 0.1 | 0.2 | 0.4 |
| biotin/GB3 | 0 | 2.3 | 2.1 | 2.0 | 1.8 | 1.8 | 1.4 | 1.5 |
| biotin/GB3 | + | 2.5 | 2.3 | 2.1 | 1.6 | 1.7 | 1.6 | 1.7 |
| A+biotin | 0 | 3.1 | 2.7 | 2.6 | 2.2 | 1.9 | 1.6 | 1.3 |
| A+biotin | + | 0.4 | 0.3 | 0.2 | 0.2 | 0.1 | 0.2 | 0.4 |