Immunity and Tolerance to Carbohydrate Antigens

by

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Abstract

Access to ABO-incompatible (ABOi) organ transplantation has expanded donor availability for patients with end-stage organ failure. Determination of eligibility for safe ABOi transplantation and effective post-transplant management to deal with potential ongoing antibody production are dependent on accurate measurement of serum ABO antibodies and an understanding of ABO immunobiology.

Thus, clear understanding of the structural nature of these carbohydrate antigens and immune mechanisms can help to improve patient care and devise new strategies for tolerance induction to such antigens. Immunity to carbohydrate antigens is one of the less well understood features of immunology. This class of antigens is thought to induce antibody responses by a mechanism of extensive cross-linking of B cell receptors, without the help of T cells. Antibodies produced in this fashion are considered to have low affinity and high cross-reactivity to other antigens of this class.

The work in this thesis seeks to fully understand the mechanisms of immune responses to carbohydrate antigens by using clinically relevant model systems. The main objectives were to, i) study mechanisms of antibody responses to carbohydrate antigens; ii) to understand how the adaptive immune system discriminates such self and foreign antigens; iii) to characterize blood group antibodies and their specificities to minor structural differences in ABO antigens; iv) to devise a tool for the accurate determination of such antibodies in ABOi transplant patients and patients being assessed for possible ABOi transplantation; v) to establish a suitable animal model that may be used to study blood group antibodies and various strategies to induce neonatal tolerance.

Studies described in chapter 2 reveal a mechanism for antibody responses to carbohydrates and, in contrast to prevailing theory, demonstrate their requirement for T cell help. The findings also provide a basis for proposing a model for self-nonself discrimination of carbohydrates. ABH subtype antigens and the differences in their expression on erythrocytes

and organs are presented in chapter 3. Based on these findings, a glycan microarray for detecting antibodies against ABH subtype antigens actually expressed in the donor graft rather than in reagent erythrocytes (*ie*., 'donor-specific' antibodies) was developed and validated in an international study of heart transplant patients. Results presented in chapters 3 and 4 also reveal fundamental flaws in the 'gold standard' agglutination assay. Development of a large animal model of neonatal tolerance is described in chapter 5. Although naturally delayed isohemagglutinin production in piglets is analogous to the developmental kinetics in human infants, tolerance surprisingly did not develop in ABOi kidney transplant piglet recipients, despite graft A-antigen persistence long after transplant. Our studies demonstrated that in the pig, A-antigens are not expressed in vascular endothelium, in contrast to previously reported studies. These findings suggest that the impact on the host immune system of exposure to non-self ABH antigens during early life in human heart vs porcine kidney grafts may depend on expression in vascular endothelium. This model may still be valuable for studying the effects of ABO antibodies on kidney tubules where abundant expression of blood group A antigen was observed.

In summary, studies presented in this thesis challenge some of the current theories related to the immune response to natural carbohydrate antigens. In addition to characterizing blood group antigens and antibodies this work also offers solutions to improve patient management in the setting of ABOi transplantation. Furthermore, these findings are also relevant to infection, autoimmunity, cancer and vaccine design.

Preface

This thesis is an original work by Mylvaganam Jeyakanthan. The research project, of which this thesis is a part, received ethics approval from the University of Alberta Research Ethics Board, Cardiac transplantation in infancy, ID. Pro00001408, January 29, 2014.

Chapter 2 of this thesis has been submitted for publication in *Nature Immunology* as Mylvaganam Jeyakanthan, Stephanie Tollenaar, KeSheng Tao, Lu Zou, Peter Meloncelli, Todd L. Lowary, Colin Anderson, and Lori J. West, Self-Nonself Discrimination of Polysaccharide Antigens. L.Z., P.M. and T.L.L. synthesized and characterized glycan and protein/peptide conjugates; S.T. and K.T. assisted in performing experiments in mice; C.A. and L.J.W. assisted in designing experiments.

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transplantation by ABO-glycan microarray. L.Z., P.M. and T.L.L. synthesized and characterized glycan and protein/peptide conjugates; P.M., T.L.L., C.W.C. and J.M.B. assisted in the development of ABO-microarray; I.L., S.T., M.F., K.T., J.R., R.C., N.S., M.B., K.B., L.A., W.Z., E.P., J.R., D.R., I.R., K.R.K and L.J.W. recruited patients, obtained blood and tissue samples or assisted in processing and storage of plasma samples.

Chapter 5 of this thesis has been published as Jeyakanthan M, Zhou X, Tao K, Mengel M, Rajotte RV, Larsen I, West LJ. Failure of neonatal B-cell tolerance induction by ABO-incompatible kidney grafts in piglets. *Transplantation*. 2013;96:519-528. X.Z., K.T., R.V.R. and I.L. assisted in transplant surgery and sample collection; M.M. provided pathology reports on kidney biopsies.

I dedicate this thesis to my family

&

To all transplant patients

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Words cannot express how grateful I am to my family. I would like to express my appreciation to many special people in my life; my wife Priya for love and unwavering support through difficult times; my daughters Anita and Tamaraa who always bring smile to my face; my parents, brother and sister for their unconditional love.

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Abbreviations

ABO	Blood group ABO system
ABOc	ABO-compatible
ABOi	ABO-incompatible
ABH(O)	Blood group A, B and H antigens
ABMR	Antibody mediated rejection
AcHN/NHAc	N-acetylaminogroup
BCR	B cell receptor
BM	Bone marrow
BSA	Bovine serum albumin
C4d	Complement component 4d
C57BL/6	mouse strain C57BL/6
CBA	mouse strain CBA
CBA/N	X-linked immune-deficient mouse strain
CFA	Complete Freund's adjuvant
CST	Clonal Selection Theory
DNP	2,4-dinitrophenol
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescent associated cell sorting
Fuc	Fucose
Gal	Galactose
GalNac	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
HAR	Hyperacute rejection
HEL	Hen egg lysozyme
HLA	Human leukocyte antigen
IFTA	Interstitial fibrosis and tubular atrophy
Le	Lewis
LEAF	Low endotoxin, Azide-free

LPS	Lipopolysaccharide
MACS	Magnetic associated cell sorting
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation primary response gene (88)
NP	Nitrophenol
NGL	Neoglycolipid
NGP	Neoglycoprotein
OVA	Ovalbumin
PAA	Polyacrylamide
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RAG-2	Recombination-activating gene
RBC	Red blood cell
Se	Secretor
SMH	Somatic hypermutation
SNS	Self-nonself
TCMR	T cell mediated rejection
TCR	T cell receptor
TD	Thymus dependent or T cell dependent
TI	Thymus independent or T cell independent
TLR	Toll-like receptor
TNP-	2,4,6-trinitrophenyl-
Tri	Trisaccharide

Chapter 1

General Introduction

Carbohydrate antigens are an immunological barrier to transplantation which can be a life-saving therapy for patients with end-stage organ failure. Such antigens include blood group ABO and αGal, a blood group-like xeno-antigen. Overcoming this barrier has many advantages including, but not limited to, efficient donor organ distribution and shorter waiting time thereby improving survival of patients. Immunity to carbohydrate antigens is one of the less well understood features of immunology. This class of antigens is thought to induce antibody responses by a mechanism of extensive cross-linking of B cell receptors, without the help of T cells. Antibodies produced in this fashion are considered to have low affinity and high cross-reactivity to other antigens of this class. This paradigm presents an apparent exception to many current theories of antibody production and self-nonself discrimination by the adaptive immune system. A clear understanding of the structural nature of carbohydrate antigens and immune mechanisms is necessary to optimize transplant strategies that can help improve patient care and develop approaches for tolerance induction to such antigens.

1.1 Immunity and tolerance: historical insights

Early theories of immunity are as important as the 'Clonal Selection Theory' (CST)[1] that revolutionized modern immunology. Several theories about antibody formation laid the groundwork for the advent of CST and helped to shape our understanding of immunity and tolerance. Instructive theories suppose that the antigen conveys the instructions for the specificity of the globulin synthesized under its governance (Pauling). Elective theories postulate that the information required to synthesize a given antibody is already inherent to the organism before the antigenic stimulus is received (Ehrlich, Jerne, Talmage and Burnet)[2]. Some important theories and experiments that are relevant to the studies described in this thesis are highlighted. ***PLEASE NOTE: In order to avoid misrepresentation of core proposals, excerpts below are presented as written by the authors in their original communications.***

1.1.1 Side-chain theory

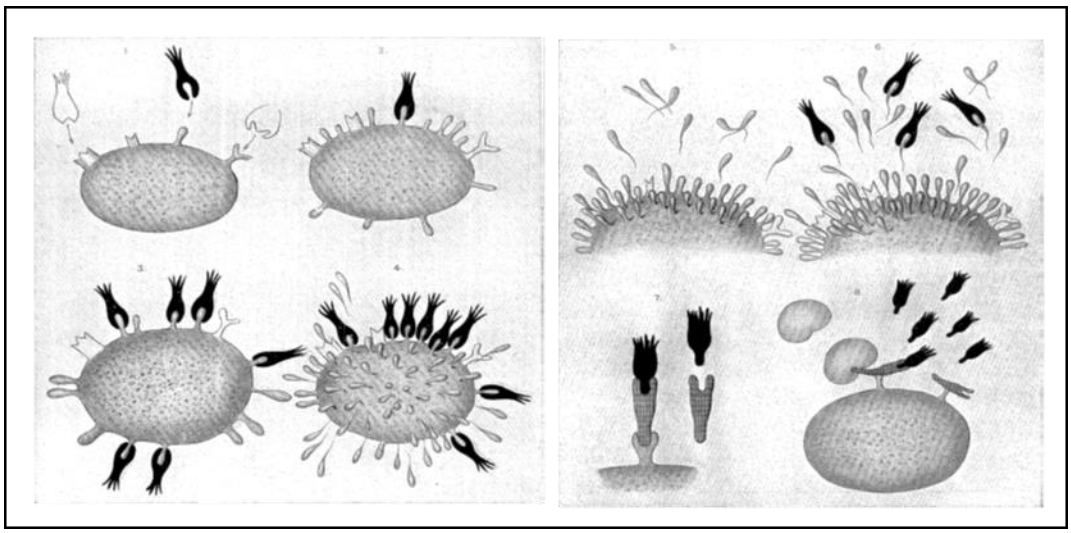


Illustration of 'side-chain' theory by Ehrlich [adapted from Paul Ehrlich (1900). Croonian Lecture - On immunity with special reference to cell life. *P R Soc London* 66, 424-448]

Paul Ehrlich introduced the concept of antibody formation in his 'side-chain' theory. He proposed that blood cells possess side chains (receptors) for toxines (antigens) and their interaction results in excess production of antitoxines (antibodies)[3].

> "The possession of a toxophile group by the cell is the necessary preliminary and cause of the poisonous action of the toxine. …..If the cells of these organs lack side-chains fitted to unite with them, the toxophore group cannot become fixed to the cell, which therefore suffers no injury, i.e. the organism is naturally immune. ……Regarded in accordance with this conception, the *antitoxines represent nothing more than side-chains reproduced in excess during regeneration, and therefore pushed off from the protoplasm, and so coming to exist in a free state* [my italics]. ……I have now laid before you the fundamental facts which up to the present constitute our knowledge in the field pertaining to immunity, and which can be most easily and successfully explained through the agency of 'the side-chain theory'."
>
> - Paul Ehrlich, 1900[3]

1.1.2 Antigen template theory

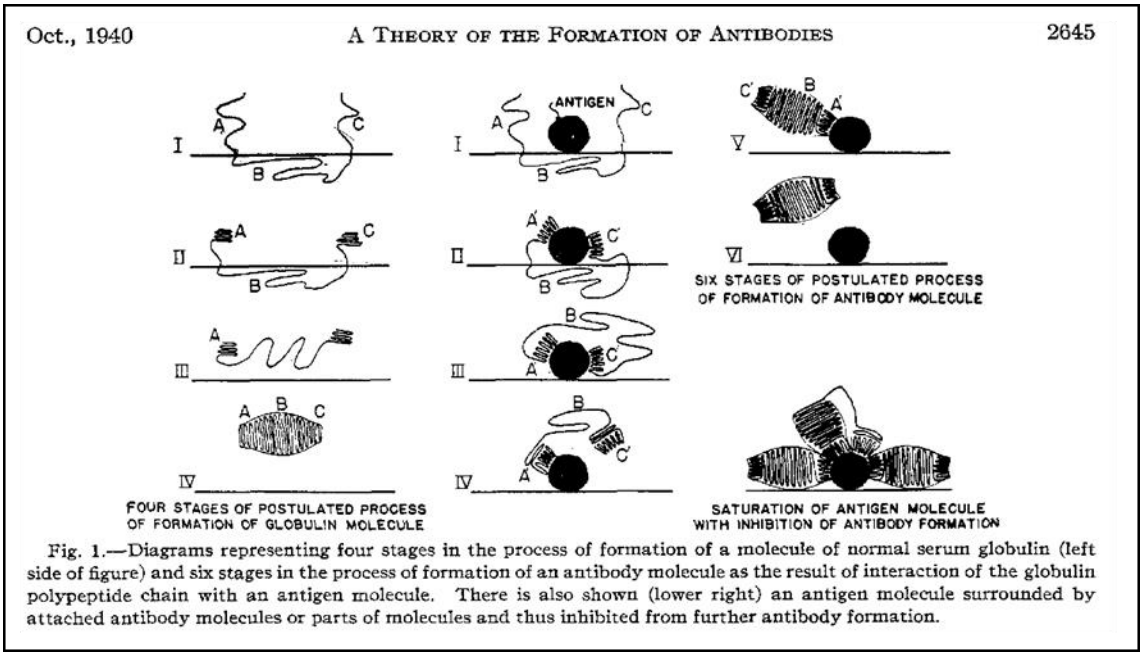


Fig. 1.—Diagrams representing four stages in the process of formation of a molecule of normal serum globulin (left side of figure) and six stages in the process of formation of an antibody molecule as the result of interaction of the globulin polypeptide chain with an antigen molecule. There is also shown (lower right) an antigen molecule surrounded by attached antibody molecules or parts of molecules and thus inhibited from further antibody formation.

Illustration of the 'antigen template theory' by Pauling [adapted from Pauling, L. A. (1940). Theory of the Structure and Process of Formation of Antibodies. *J Am Chem Soc* **62**, 2643-2657]

Pauling proposed that the antigen directs the response rather than antibody. He suggested that antigens provide a template to which antibodies modify themselves[4].

> "It is assumed that antibodies differ from normal serum globulin only in the way in which the two end parts of the globulin polypeptide chain are coiled, these parts, as a result of their amino-acid composition and order, having accessible a very great many configurations with nearly the same stability; under the influence of an antigen molecule they assume configurations complementary to surface regions of the antigen, thus forming two active ends. After the freeing of one end and the liberation of the central part of the chain this part of the chain folds up to form the central part of the antibody molecule, with two oppositely-directed ends able to attach themselves to two antigen molecules."
>
> - Linus Pauling, 1940[4]

1.1.3 Natural selection theory

Jerne postulated that the spontaneous presence of small number of antibodies (natural antibodies) against all antigens to which animal can respond, and that the antigen has the sole responsibility for carrying specific antibody from the circulation into cells which produce more of their kind[5].

> "The 'natural-selection' theory, proposed in the present paper, may be stated as follows: The role of the antigen is neither that of a template nor that of an enzyme modifier. *The antigen is solely a selective carrier of spontaneously circulating antibody to a system of cells which can reproduce this antibody* [my italics]. Globulin molecules are continuously being synthesized in an enormous variety of different configurations. Among the population of circulating globulin molecules there will, spontaneously, be fractions possessing affinity toward any antigen to which the animal can respond. These are the so-called 'natural' antibodies."
>
> - Niels Jerne, 1955[5]

1.1.4 Clonal selection theory

Based on Jerne's natural selection theory Talmage hypothesized that it is replicating cells, not antibodies, responsible for effective immune response[6].

> "The process of natural selection requires the selective multiplication of a few species out of a diverse population. As a working hypothesis *it is tempting to consider that one of the multiplying units in the antibody response is the cell itself* [my italics]. According to this hypothesis, only those cells are selected for multiplication whose synthesized product has affinity for the antigen injected. This would have the disadvantage of requiring a different species of cell for each species of protein produced, but would not increase the total amount of configurational

information required of the hereditary process."

- David Talmage, 1957[6]

Burnet also proposed a theory of antibody production based on Jerne's theory, called it the 'clonal selection hypothesis'. He suggested that each lymphocyte possesses receptors with a single specificity and that clonal expansion occurs upon binding of an antigen resulting in the production of antibodies with the same specificity[1,7]. Burnet's 'Clonal Selection Theory' became one of the most important concepts in immunology. Burnet, with Peter Medawar, was awarded Nobel Prize in 1960 for the discovery of acquired immunological tolerance.

"Before receiving Talmage's review we had adopted virtually the same approach but had developed it from what might be called a 'clonal' point of view. ……..It is assumed that when an antigen enters the blood or tissue fluids it will attach to the surface of any lymphocyte carrying reactive sites which correspond to one of its antigenic determinants. …….It is postulated that when antigen-natural antibody contact takes place on the surface of a lymphocyte the cell is activated to settle in an appropriate tissue, spleen, lymph node or local inflammatory accumulation, and there undergo proliferation to produce a variety of descendants. In this way preferential proliferation will be initiated of all those clones whose reactive sites correspond to the antigenic determinants on the antigen used. The descendants will include plasmacytoid forms capable of active liberation of soluble antibody and lymphocytes which can fulfill the same functions as the parental forms. ….. Despite the speculative character of much of the detail of this modification of Jerne's theory-which might be referred to as the 'clonal selection hypothesis' -it has so many implications calling for experimental inquiry that it has been thought justifiable to submit this preliminary account for publication."

- Frank Burnet, 1957[1]

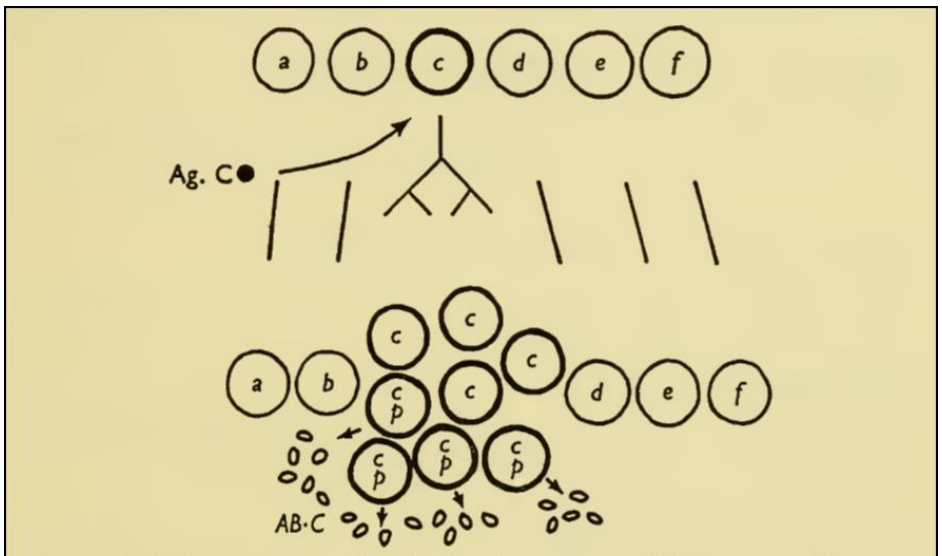


Fig. 7. To illustrate the clonal selection theory of immunity. Contact of the corresponding antigenic determinant *Ag.C* with cells of clone *c* stimulates proliferation to antibody-producing plasma cells *cp* and non-antibody producing types *c*.

Burnet's illustration of the clonal selection theory [adapted from Frank M. Burnet (1959). The Clonal Selection Theory of Acquired Immunity[7], page 59: Cambridge University Press]

Key points of the clonal selection theory (CST)[8]

- The entire repertoire (clones of cells) develops in the host and is subject to selection process.
- Each clone of lymphocyte expresses, on the cell surface, receptors with reactive sites specific to antigenic determinants.
- Antigen binding to reactive site on the surface of lymphocyte activates cell to undergo proliferation and differentiation.
- Descendants include plasma cells that produce clones of antibodies and circulating clones of lymphocytes that participate in rapid secondary response.

Nossal and Lederberg were the first to validate the CST by demonstrating the monoclonal nature of antibody produced by single cells despite immunizing mice with two contrasting antigens[9]. Many experiments subsequently supported the CST[10] and it remains one of the fundamental principles of modern immunology.

1.1.5 Self-nonself discrimination (SNS)

"How can an immunized animal recognize the difference between an injected material like insulin or serum albumin from another species and its own corresponding substance?"

- Frank M. Burnet, Nobel Lecture, 1960[11]

This question dominated early years of immunology and formed the basis for immunological theorizing. Immunology has been called the science of self-non-self discrimination before[8,12]. Ehrlich introduced self-nonself discrimination in his concept of '*horror autotoxicus'*, which indicated that the body could not produce antibodies against itself, since this would lead to self-destruction[13]. Burnet and Fenner[14] introduced 'self-marker' hypothesis (…expendable body cells carry 'self marker' components which allow recognition of their 'self' character…[14]), based on Owen's finding of chimeric red cells in dizygotic twins[15]. They claimed that tolerance to foreign entities can only be induced during embryonic life. Although the 'Clonal Selection Theory' did not address self-nonself discrimination in its original description, Burnet went on to address this in his subsequent communications[7], including in his Nobel lecture titled "Immunological recognition of self" [11]. He reinforced the Burnet-Fenner theory in his book[7] "The clonal selection theory of acquired immunity", citing Medawar's work on the antenatal induction of tolerance and natural chimeras in animals[15] and human[16] as supportive evidence for his hypothesis. Burnet concluded that "Self-not-self recognition means simply that all those clones which would recognize (that is, produce antibody against) a self component have been eliminated in embryonic life. All the rest are retained" [7]. However, further experiments by Billingham and Brent suggested that tolerance to foreign antigens can be induced beyond embryonic life[17].

Lederberg postulated nine propositions[2] in an effort to formulate a genetic basis for antibody specificity. He emphasized (i) that antibody-forming cells are specialized, (ii) that their diversity arises from some random process, and (iii) that the diversification of these cells continues, in company with their proliferation, *throughout the life of the animal*. Additionally,

he proposed that the immature antibody-forming cell is hypersensitive to an antigen-antibody combination; it will be suppressed if it encounters the homologous antigen at this time. Many studies later supported Lederberg's proposals[18-21] and provided a basis for understanding central tolerance of B and T cells.

Bretscher and Cohn proposed a two-signal model for lymphocyte activation and antibody production[22] that provided a basis for understanding peripheral tolerance and SNS discrimination. They proposed that lymphocyte activation requires the associative recognition of linked epitopes of the antigen and that first signal alone results in paralysis.

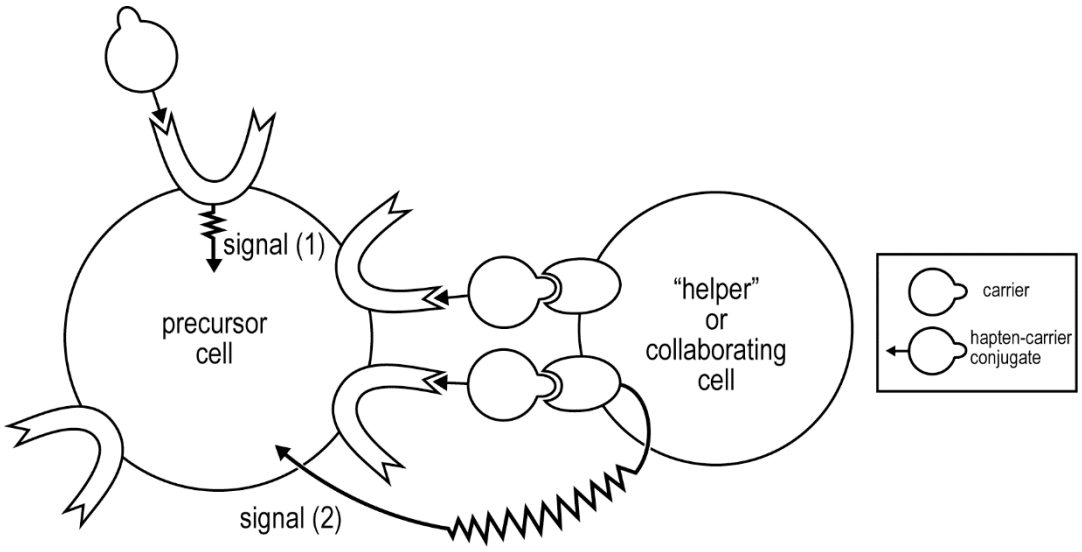
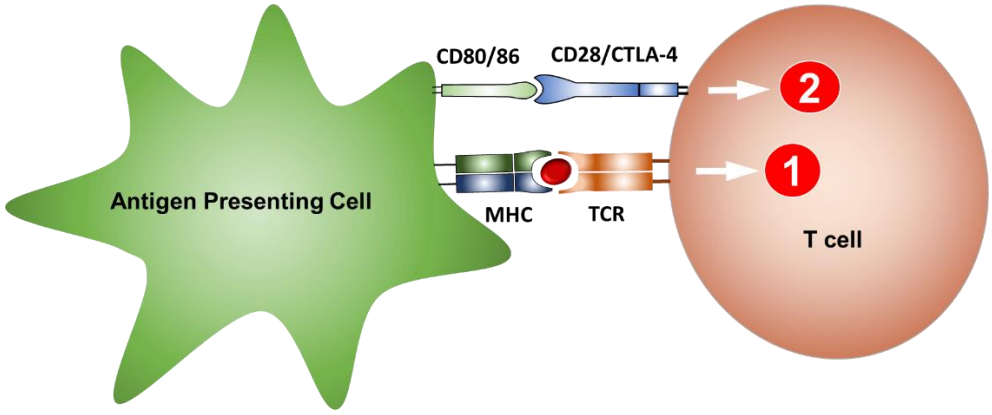


Illustration of the two-signal model proposed by Bretscher and Cohn [figure courtesy of Dr. Peter Bretscher; art work by J Duebner]

This proposal has been refined and updated over the years taking into account new findings[23-28]. In this model, signal 2 determines the fate of the antigen sensitive cell and is delivered by effector T helper cells (eTh). But the origin of eTh cells remained unclear, referred to as the primer question[29]. Based on Bretscher-Cohn scheme of cell interaction, Lafferty and Cunningham[30] proposed that a non-specific signal-2 can be provided by stimulator cells (later became known as antigen presenting cells). The nature of the second signal became clear with the advent of co-stimulatory receptor interactions such as CD28/B7[31] and CD40/CD40L[32]

and their role in T and B cell activation[33]. Activated T cells can provide the second signal to B cells through CD40/CD40L interaction resulting in B cell activation and differentiation[34].



A simplified schematic diagram illustrating the two-signal model for T cell activation.

Lafferty and Cunningham's two-signal model shares many similarities to the two-signal model proposed by Bretscher and Cohn[22] but the non-antigen specificity of the second signal does not provide a rationale for the maintenance of immunological tolerance[28]. Janeway proposed that it is the infectious agent, not unique qualities of its protein constituents, that determines immunogenicity and that receptors and response mechanisms of the innate immune system, selected over evolutionary time to recognize and induce responses to infectious agents, also initiate adaptive immune responses to infectious nonself[35]. Adding another layer to this, Matzinger proposed that antigen presenting cells are activated by danger/alarm from injured cells, such as those exposed to pathogens, toxins and mechanical damage, termed 'danger model' [36]. She argues that immunity is controlled by the tissues, not by the cells of the adaptive or innate immune system; tissues induce immunity when distressed, tolerance when healthy.

In proposing various theories for lymphocyte activation and SNS discrimination the protein or carbohydrate nature of antigens was generally not discussed in their original proposals. Thus, the two signal model fits with antibody responses to protein antigens, 'T-dependent' antigens, but so called 'T-independent' antigens (hapten-polymer conjugates, polysaccharides etc.) can

be an exception to this rule as they are thought to be capable of inducing antibody responses by a mechanism of extensive cross-linking of B cell receptors. Bretscher argues that this class of antigens is T-dependent but likely require several orders of ten less associative antigen recognition due to their polymeric nature[23,37]. Cohn suggests that the interaction of appropriately spaced repetitive antigens generates a single signal, replacing both signal 1 and 2, resulting in eTh-independent activation, and that SNS discrimination of these antigens are germline selected[29].

1.2 Antibody response to T-dependent and T-independent antigens

Interaction of B cell receptor with a foreign antigen induces B cells to proliferate and differentiate into antibody secreting cells (plasma cells) or memory cells[38,39]. This process requires B cells to interact with thymus-dependent T cells[40-43]. However some antigens were found to be able to induce antibody responses in the absence of such cells[43,44]. Based on this observation, antigens were originally grouped into thymus-dependent (TD) and thymus-independent (TI) classes. This classification was largely based on how various antigens induced antibody production in thymectomized or congenitally athymic mice[40-47]. These experiments were performed during the 1960s and early 1970s when T and B cells were referred to as thymus-dependent and thymus-independent populations[48]. With more understanding of antigen presentation and mechanisms of T cell activation[49] it became clear how T-B cell interaction leads to antibody production against protein antigens[34,41].

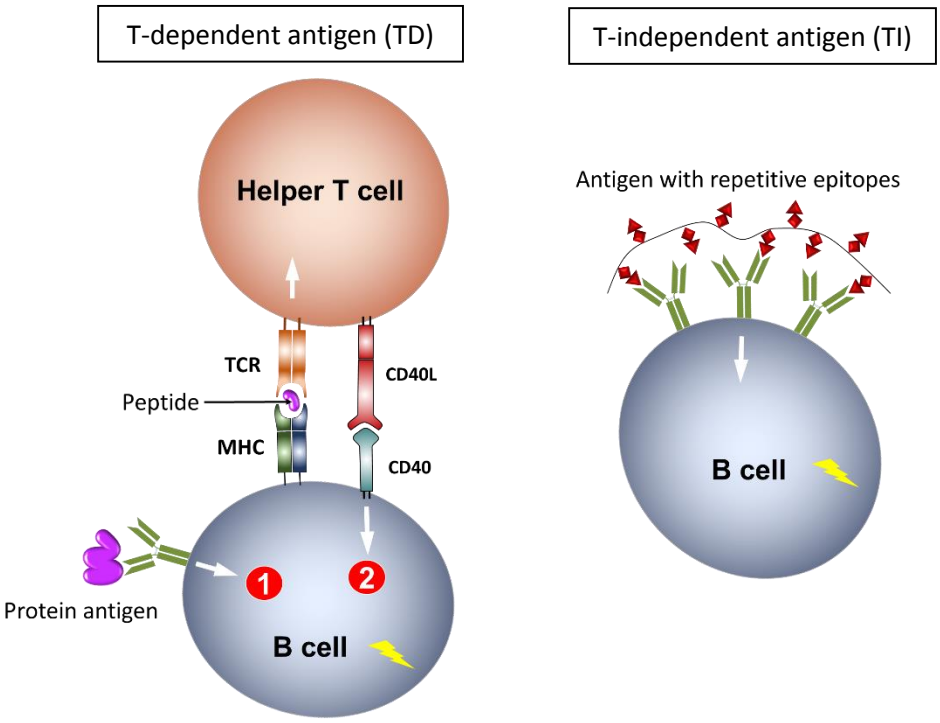
For TD antigens to induce antibody production, they must first be processed into peptides and presented on MHC class II by antigen presenting cells, which can themselves be B cells[50], and activate antigen-specific T helper cells which in turn interact with B cells that display the same processed peptide-MHC class II complex. Activated T cells transiently express ligand for CD40, the receptor for which is constitutively expressed on all B cells[51]. Crosslinking of CD40 as well as other co-receptors and cytokine secretion result in proliferation and differentiation of B

cells to secrete antibodies[32,34]. With continued T cell-dependent B cell stimulation, B cells undergo somatic hypermutation with affinity maturation, as well as immunoglobulin class switching, resulting in secretion of IgG antibodies with increased avidity for the antigen[34,52]. T cells and CD40L-CD40 interactions are thought to be essential for TD B cell activation[53] and required for germinal centre formation and immunoglobulin class switching although not required for some IgM response[34,54]. This scheme fits with the two-signal model for antibody production and provides a basis for self-nonself discrimination.

In contrast to TD antigens, TI antigens induce antibody production without the help of T cells and the mechanism was thought to be somewhat less complex than TD antibody responses[52]. Antigens that consists of repetitive structures are generally allocated to this class. For example, haptenated derivatives of polymers such as trinitrophenyl-ficoll (TNP-ficoll) and dinitrophenyl-ficoll (DNP-ficoll) have been widely used as model TI antigens. Bacterial polysaccharides such as pneumococcal polysaccharide have also been considered TI antigens[55]. TI antigens are further classified into TI type-1 and TI type-2 based on their (haptenated derivatives) ability to stimulate neonatal or CBA/N[56], X-linked immune deficient[57], B cells. Under this classification, TNP-lipopolysaccharide (TNP-LPS) is considered a TI type I antigen that can stimulate neonatal B cells or B cells from CBA/N mice. TNP-ficoll, considered a TI type-2 antigen, failed to induce neonatal or CBA/N B cells. Bacterial polysaccharides and carbohydrates in general have also been considered TI type-2 antigens[58]. These include clinically relevant antigens such as pneumococcus polysaccharides and ABO blood group carbohydrate antigens[59].

TI type-1 antigens, such as LPS, were found to be B cell mitogens that can stimulate B cells through Toll-like receptors (TLRs) to proliferate and differentiate into antibody secreting cells[60,61]. In contrast, TI type-2 antigens with repetitive structures induce antibody production by a mechanism of cross-linking B cell receptors without the second signal[62-64] that is required in TD responses. Importantly, this proposed mechanism does not explain how foreign antigens

with repetitive structures can be distinguished from self-antigens with similar structures. Furthermore, many studies carried out on this subject used haptenated (TNP, DNP or NP) derivatives of polymers as model antigens. Although the T cell independence of these antigens has been questioned[65-68], they continue to be used as model antigens[69,70].



A simplified diagram showing mechanisms of B cell stimulation by TD and TI antigens.

More studies are needed to better understand primary antibody responses to naturally occurring polysaccharides or carbohydrates in naïve animals. This may lead to a plausible mechanism to explain how the immune system discriminates self from foreign polysaccharides and maintains peripheral tolerance to such antigens.

1.3 B-cell tolerance

Productive rearrangement of immunoglobulin heavy (IgH) and light (IgL) chain gene segments in B lymphocyte precursor cells results in the generation and expression of a mature B cell antigen receptor (BCR)[71] on the cell surface. The B-cell repertoire is generated in two

stages: in the first, V(D)J recombination occurs within the bone marrow (BM) to create the 'pre-immune repertoire', while the second involves somatic hypermutation (SMH) of immunoglobulin variable region genes within germinal centres following antigen stimulation and provision of co-stimulatory signals from T-cells and/or external pathogens[72]. The random nature of the process can potentially result in self-reactive B cells. Therefore multiple mechanisms of tolerance at several checkpoints exist to deal with self-reactive B cells.

Central tolerance: Selection in the bone marrow is mediated by deletion and receptor editing of auto-reactive B cells[72]. Early studies of B cell selection suggested elimination of auto-reactive B-cells by clonal deletion as the mechanism of B cell tolerance to self-antigens[20,21,73], supporting Lederberg's proposal[2]. However, further studies suggested receptor editing as the main mechanism of B cell tolerance, and that clonal deletion is a default pathway that functions only when receptor editing has been exhausted[74-77].

Peripheral tolerance: peripheral tolerance mechanisms act in secondary lymphoid organs by modulating the responsiveness of mostly mature B cells. Only 10% of newly generated immature B cells emerge from BM as transitional B cells which may encounter peripheral self-antigens not present in BM. These immature B cells are susceptible to BCR-induced apoptosis. Relative avidity of the antigen-BCR interaction leads to either deletion or anergy[72]. Support for clonal anergy emerged from experiments using a murine double-transgenic strategy[78]. Mice were rendered transgenic for monoclonal B cell IgM and IgD receptors with high affinity for hen egg lysozyme (HEL) and were mated with mice transgenic for HEL itself. Antigen concentration-dependent B cell tolerance was observed when challenged with either soluble (s-HEL) or membrane anchored HEL (m-HEL); high concentration of s-HEL resulted in clonal anergy whereas strong cross-linking of BCR with m-HEL lead to deletion of HEL reactive B cells[78,79].

Immature B cells differ from mature B cells in that they are particularly susceptible to BCR-induced apoptosis. Immature B cells are the first B lineage cells to express surface BCRs,

displaying surface IgM but little or no IgD. B cells remain in the immature compartment for an average of 3.5 days, and it is in this compartment that self-reactive B cells failing to edit their receptors are deleted or anergized[80]. Peripheral tolerance mechanisms for mature B cells conform to Bretscher and Cohn's two-signal model[22] with the exception of certain T-independent antigens.

1.4 Blood group ABO system

Karl Landsteiner discovered human blood groups in 1900[81] by carrying out a simple experiment using the serum and red cells of six of his colleagues working in the Pathological Anatomy Institute of the University of Vienna[82]. He showed that when serum and erythrocytes were mixed together agglutination of erythrocytes frequently took place, and that in some mixtures no agglutination occurred[83]. According to his definition cells either had A or B antigens or neither A nor B (group C, later to be renamed O). He showed that a person's serum does not contain antibody for the antigens present on his/her own red cells and that both anti-A and anti-B are present in group O serum when the antigens are missing from the red cells[82]. The groups are designated according to the agglutinogens (antigens) contained in the cells as shown in the table below [adapted from Landsteiner, K (1931). Individual Differences in Human Blood. *Science* 73, 403-409; sign + indicates agglutination].

Serum of group	Agglutinins in serum (antibody)	Erythrocytes of groups			
		O	**A**	**B**	**AB**
O	$\alpha\beta$ (anti-A, Anti-B)	-	+	+	+
A	β (Anti-B)	-	-	+	+
B	α (anti-A)	-	+	-	+
AB	-	-	-	-	-

These findings laid the foundation for the clinical practice of blood transfusion. Since Landsteiner's description of ABO blood groups, a total of 328 red cell antigens have been recognized, 284 of which are clustered in 30 blood group systems according to the classification by the International Society of Blood Transfusion[84,85] and this list continues to increase with new antigens. The ABO system is one of the most important blood group systems in transfusion and transplantation.

1.4.1 Chemical structure and biosynthesis of ABO antigens

The chemical nature of ABO blood group substances and the enzymatic basis for synthesis of these antigens have been clarified during the 1950s and 1960s[86-90]. These studies found that group A individuals express α1,3-N-acetylgalactosaminyltransferase (A transferase) activity and group B individuals express α1,3-Galactosyltransferase (B transferase) activity. Group AB individuals express both enzymatic activities and group O individuals express neither. The discovery of Bombay O_h phenotype, in which individuals lacked A, B and O antigens on their red cells and had anti-A, anti-B and anti-H antibodies in their serum led to identifying a genetically independent blood group system Hh[82]. The antigen on O red cells is the product of the H transferase gene giving rise to a structure which is the immediate precursor of the A and B structures. Hence in blood group O individuals the H structure does not undergo further change; the gene h is a silent allele[91]. The A, B, and H blood group molecules are carbohydrate antigens that are synthesized by the sequential action of specific glycosyltransferase products of ABO genes. Each enzyme is encoded by a distinct genetic locus, and catalyses the addition of a specific monosaccharide to a specific position on the oligosaccharide precursor, or 'acceptor' molecule[92].

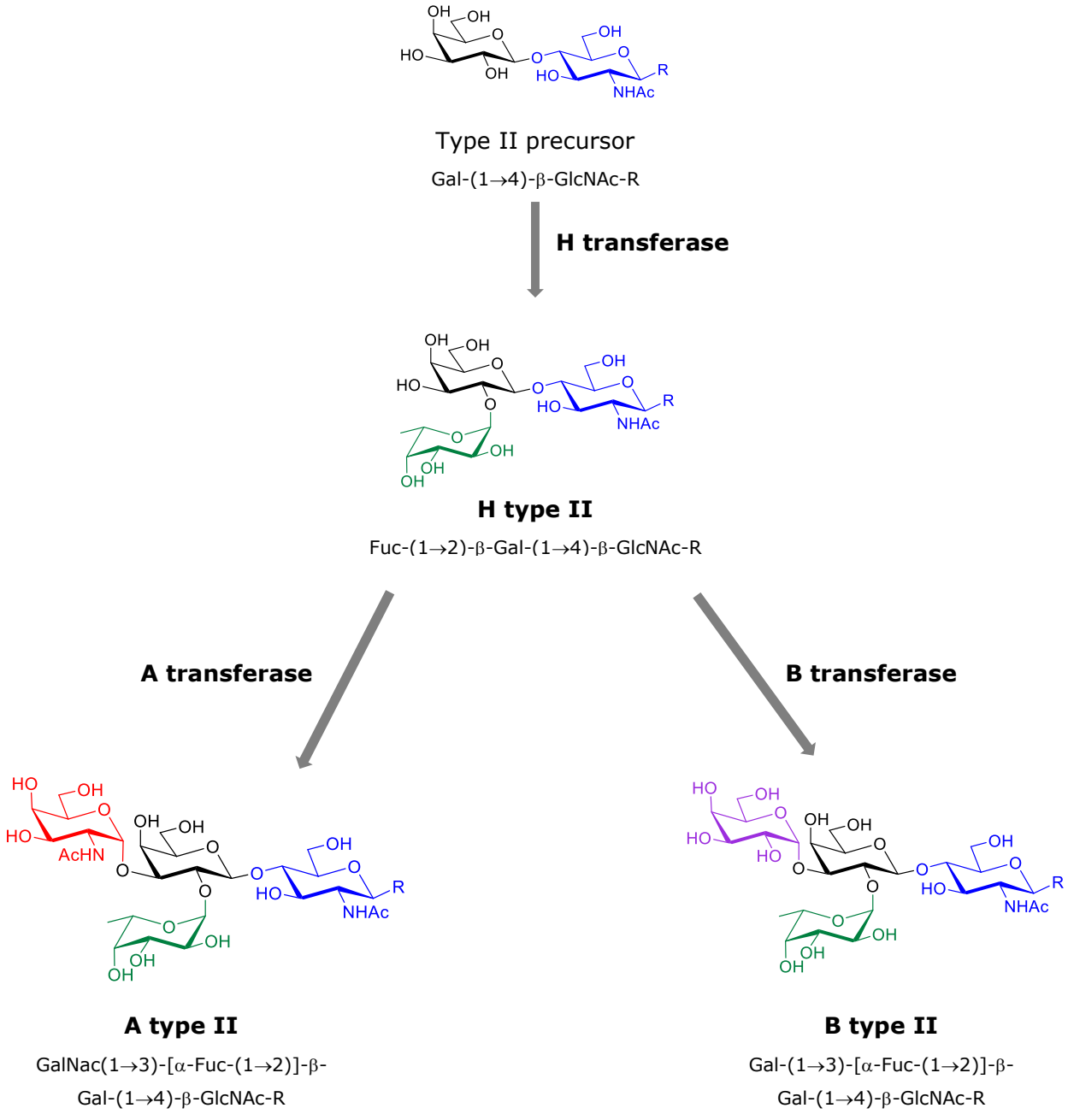
The ABO blood group system (adapted from Watkins, W.M. (2001). The ABO blood group system: historical background. *Transfusion medicine* 11, 243-265)

Red cells		Serum	
Genotypes	**Phenotypes[a]**	**Antibodies**	**Glycosyltransferases**
A^1A^1, A^1A^2, A^1O	A_1	Anti-B	α-3-N-acetylgalactosaminyl
A^2A^2, A^2O	A_2	Anti-B[b]	α-3-N-acetylgalactosaminyl[c]
BB, BO	B	Anti-A	α-3-galactosyl
A^1B	A_1B	-	α-3-N-acetylgalactosaminyl and α-3-galactosyl
A^2B	A_2B	-[b]	α-3-N-acetylgalactosaminyl and α-3-galactosyl
OO	O	Anti-A and Anti-B	-

[a]Defined by anti-sera, anti-A, anti-B and anti-A_1. [b]Anti-A_1 sometimes present in sera of group A_2 and A_2B individuals. [c]N-acetylgalactosaminylransferases in group A_2 sera differ in kinetic properties from enzymes in A_1 sera. Sub-groups are discussed in section 1.3.2.

The first step in the biosynthesis of ABH antigens is the addition of L-fucose to a common precursor by H transferase to form H antigen. α1,3-N-acetylgalactosaminyltransferase (A transferase) and α1,3-galactosyltransferase (B transferase) catalyze the addition of specific sugars, N-acetylgalactosamine (GalNAc) and galactose (Gal) residue, respectively, to H antigen to form A and B antigens. Since O allele encodes proteins without glycosyltransferase (O transferase) function, H antigen is the only ABO structure present in blood type O[93]. These terminal sugars become the antigenic determinants of H, A and B antigens.

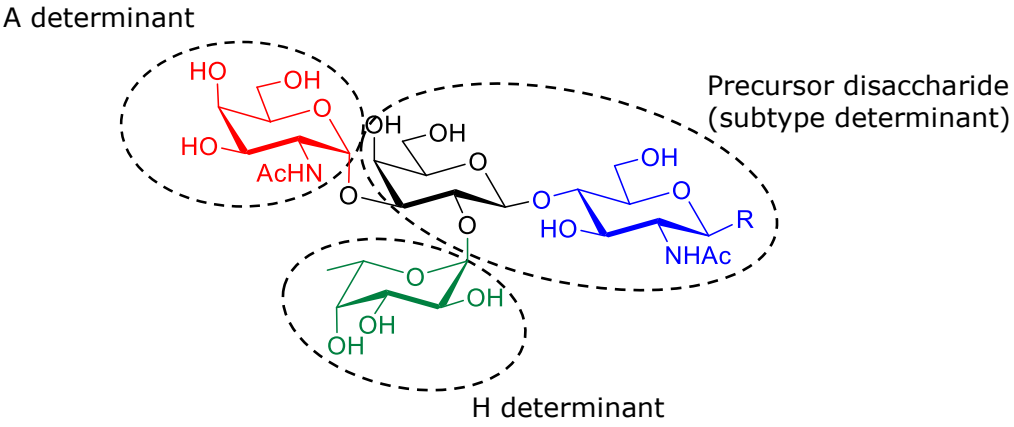
A simplified diagram below showing biosynthetic pathway of ABO antigens



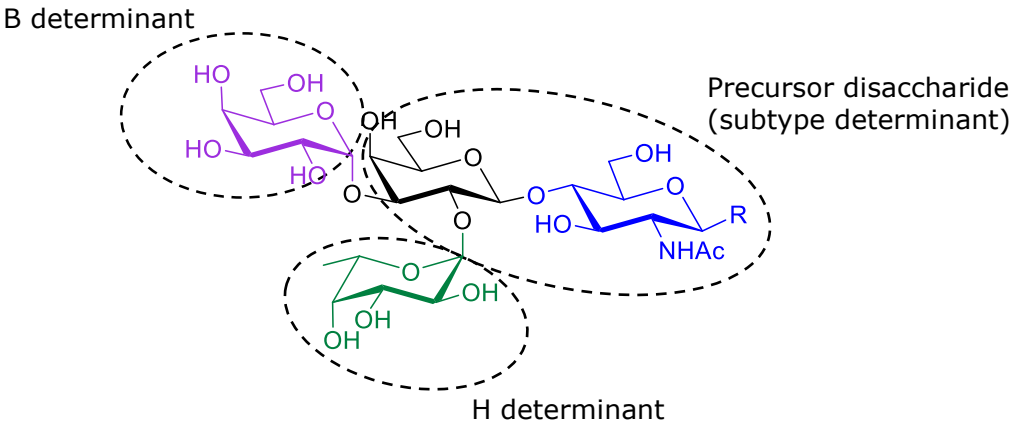
(R: glycoprotein or glycolipid, Fuc: fucose, Gal: galactose, GalNac: N-acetylgalactosamine, GlcNAc: N-acetylglucosamine)

Chemical structures of A and B are shown below; A, B and H determinants are highlighted.

A type II antigen



B type II antigen



There are at least six types of possible precursor chains that define the subtypes of ABH antigens. These have been classified as follows[94].

Type I: …….β-Gal-(1→3)-β-GlcNAc1→R

Type II: ……β-Gal-(1→4)-β-GlcNAc1→R

Type III: …….β-Gal-(1→3)-α-GalNAc1→R

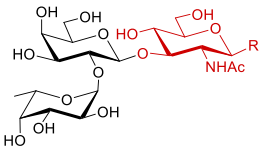
Type IV: ……β-Gal-(1→3)-β- GalNAc1→R

Type V: …….β-Gal-(1→3)-β-Gal1→R

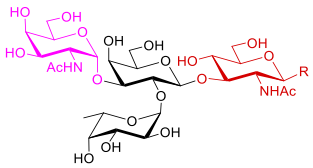
Type VI: ……β-Gal-(1→4)-β-Glc1→R

Type I-IV based ABH structures have been found on human red cells or secretions. A type I-VI, B type I-VI and H type I-VI antigens possess shared or common monosaccharide units that can potentially create common or cross-reactive antigen epitopes. Chemical structures[95,96] of type I-VI A, B and H antigens are as follows; common monosaccharide structures are depicted in the same color.

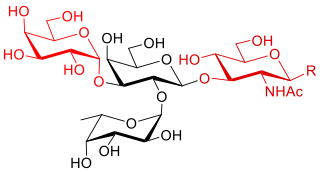
H type I



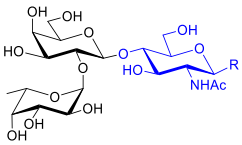
A type I



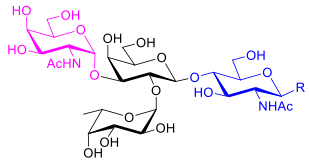
B type I



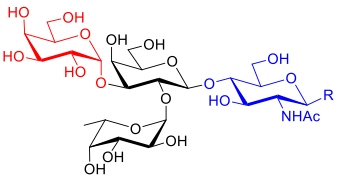
H type II



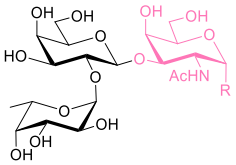
A type II



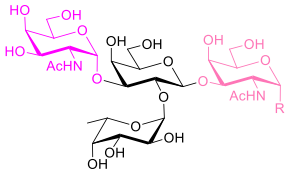
B type II



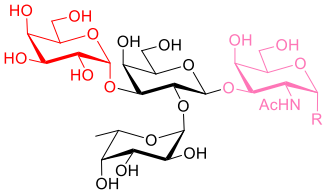
H type III



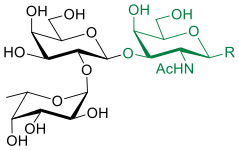
A type III



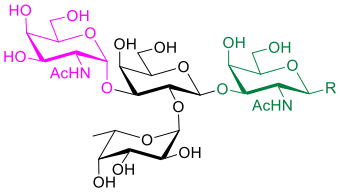
B type III



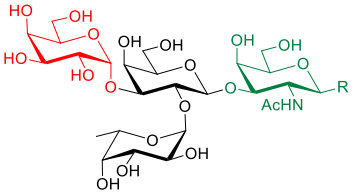
H type IV



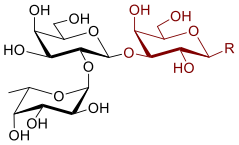
A type IV



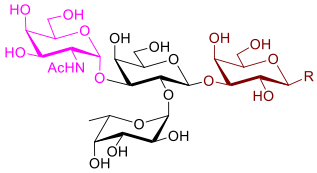
B type IV



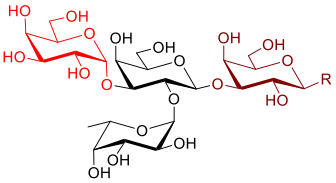
H type V



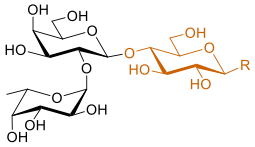
A type V



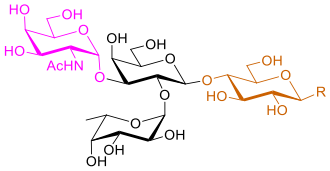
B type V



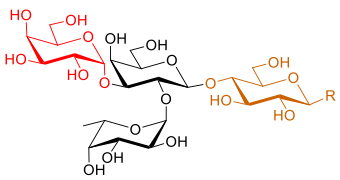
H type VI



A type VI



B type VI



The regulatory gene Se (secretor) with other regulatory genes controls the activity of the H gene in cells synthesizing ABH antigens, hence their expression on cells and in secretions[91]. The Lewis blood group system, a closely related system, is inherited independently but interacts with the product of ABO, H and Se genes at the phenotypic level[82]. Interaction between Lewis (Le) gene and secretor (Se) gene results in further modification of ABH antigens to express Lewis antigens[82,91].

Much work has been done on the expression of ABH antigens on red cells and as free saccharides in body secretions and these areas are well documented[97,98]. However, ABH subtypes and closely related Lewis antigen expression in tissues and major organs has not been completely elucidated. Although it has been shown that ABH antigens carried by type I precursors are expressed on cells and body fluids and excretions in a secretor (Se) dependent manner[98], the influence of secretor (Se) and Lewis (Le) genes on the expression of other subtypes in different organs and tissues is not fully understood. Clarifying differences in the expression of A type I-VI, B type I-VI and H type I-VI on red cells and on cells of major organs and tissues can be very useful in the proper assessment of donor antigens and antibodies directed against them in the setting of ABO-incompatible transplantation.

1.4.2 Subgroups of A, B, and AB

Subgroups and/or variants of blood group ABO are distinguished by decreased amounts of A, B and H antigens on red cells. Subgroups of A and B are most common. Blood group A is thought to have the most variation and classified by the quantity of A antigen on red cells in decreasing order A_1, A_2, A_3, A_x, A_{end}, A_m, A_{el}. Similarly, B subgroups are classified by the quantity of B antigen on red cells in decreasing order B, B_3, B_x, B_m, B_{el}. Blood group AB is classified into subgroups based on the quantity of A and B antigens as A_xB, A_1B_x, A_mB, A_1B_m, $A_{el}B$, A_1B_{el}, cisA_2B_3, cisA_2B, cisA_1B_3[93].

Among the many subgroups the most common A_1 and A_2 have been studied in some detail. It has been reported that approximately 20% of blood group A individuals belong to subgroup A_2[93]. Generally, distinction of A_1 and A_2 is based on agglutination of red cells by the lectin, *Dolichos biflorus*[99]. Identification of a single mutation which characterizes A_2 alleles made it possible to genetically analyze A_2[100] and activities of A_1 and A_2 transferases. Varying enzyme kinetics of A_1 and A_2 transferases causes both quantitative and qualitative differences between A_1 and A_2 antigens[101-108]. Many studies have focused on the A antigen expression on A_1 and A_2 red cells. Further studies are needed to address both qualitative and quantitative differences in A antigen expression in other cells and organs as well as the similarities and differences in subtype I-VI specificities.

1.4.3 Blood group antigens as barriers in transplantation

Individuals develop 'natural' antibodies against ABH antigens they lack. Blood group O individuals develop both anti-A and anti-B antibodies; A and B individuals develop anti-B and anti-A respectively[83]. Individuals who are Bombay phenotype, lacking A, B and H, develop anti-A, anti-B and anti-H antibodies[82]. 'Natural' antibodies are thought to be induced by environmental stimuli that share similar ABH carbohydrate epitopes[109-111].

Blood group ABH and related antigen expression is not limited to red blood cells but is also found in cells, tissues, body fluids and excretions as glycolipids, glycoproteins or as free oligosaccharides[98]. ABH antigens are also present in all major organs and abundantly expressed on vascular endothelium of the vascular tree[112-117]. The expression of ABH antigens in organs and the presence of circulating 'natural' antibodies directed against these antigens creates a barrier for solid organ transplantation. Crossing this barrier increases the risk of hyperacute rejection[118] leading to graft loss and, in case of heart transplantation, patient death[119]. Hence, ABO compatibility between recipients and donors was thought to be critical for successful transplantation. The requirement for ABO compatibility coupled with severe

shortage of donor organs restricts patients with end-stage organ failure from receiving transplant in a timely manner. Crossing the ABO barrier safely has the potential to expand the deceased and living donor pool and improve efficient donor allocation.

1.4.3 ABO-incompatible (ABOi) transplantation

Early experience with ABOi kidney[50,51], liver[120] and heart[119] transplantation reported poor outcomes in terms of graft and patient survival. However, in these early reports ABOi transplants were performed either by accident (heart) or without pre-emptive strategies to deal with natural ABO antibodies and antibody producing cells. ABOi transplantation has re-emerged following the first successful large-scale trial of intentional ABOi kidney transplantation using a protocol that included splenectomy[121]. Further refinements in immunosuppression and patient management continue to improve both short- and long-term graft survival[122,123]. Although there is no standardized protocol for performing ABOi transplant, a common approach has been the removal of anti-A and anti-B antibodies by either plasmapheresis or A/B antigen-specific immunoadsorption and reduction of B cells by splenectomy or B cell depleting agents[124-126]. Monitoring of antibodies directed against donor blood group antigens has been performed using isohemagglutination assay, the only method available in clinical laboratories. The effectiveness of therapeutic interventions to reduce ABO antibodies is solely based on agglutination titre. In ABOi kidney transplant recipients, antibody titres against donor blood group ABO antigens have been reported to return and persist long after transplant in the absence of humoral rejection of the graft, a phenomenon called 'accommodation'[127,128]. Although described many decades ago, the mechanism of this phenomenon remains elusive. It is possible that the antibodies measured by agglutination testing are, in fact, not donor-specific. Thus, A and B subtypes may be differentially expressed on red cells, vascular endothelium and tubular epithelium of the kidney, and the antibodies detected by red cell agglutination may not be specific to subtype antigens expressed in the capillaries or tubules of the kidney. Further characterization of recipient agglutinins and donor

antigens would be valuable in understanding the phenomenon of graft accommodation, if indeed it truly exists.

Endogenous production of 'natural' antibodies directed against ABO antigens does not occur until late in the infancy[129-131]. This window of antibody deficiency, and presumed deficiency of antibody secreting and memory B cells, may allow ABOi transplantation without aggressive therapies that adults require. West *et al.* exploited this natural lag in the antibody development and demonstrated that ABOi heart transplantation can be performed safely in infants without the aggressive interventions required in older patients[132]. This protocol has been adapted by many centers demonstrating that short- and long-term outcomes were comparable to ABO-compatible heart transplants[133-135] and pediatric cardiac transplant waiting list mortality was reduced by increasing the likelihood of transplant[136,137]. Similar experience has been reported in young children receiving ABOi liver transplantation[138], suggesting that the immature immune system creates a favourable environment to receive such transplants. West *et al.* also observed that ABOi heart transplant recipients failed to develop antibodies against the donor blood group with time after transplant, yet produced antibodies against third-party non-self ABO antigens[117]. Others reported low level antibody titres against the donor blood group in some patients[135,139]. Further studies are needed to clarify the nature of these antibodies detected by red cell agglutination and their specificity to ABO antigens expressed in the heart graft.

1.5 Acquired immune tolerance

1.5.1 Nature's experiments on acquired tolerance

In 1916, in dizygotic opposite sex bovine twins, Lillie[140] demonstrated reproductive abnormalities of the female twin embryos, called freemartins, due to the union of circulation. He focused on sexual development due to mixing of hormones. In 1945, Owen demonstrated the existence of a mixture of two distinct types of erythrocytes in certain twins[141]. He

concluded that interchange of cells between bovine twin embryos occurs as a result of vascular anastomoses in the shared placenta. Chimerism of red cells has also been observed in human twins[16].

1.5.2 Medawar's experiments on acquired tolerance

Unaware of Owen's findings, Medawar performed skin transplant experiments in order to distinguish monozygotic and dizygotic twins in cattle[142,143]. Unexpectedly, the majority of dizygotic cattle twins were tolerant of each other's skin grafts. Owen's findings provided a rationale for this observation that led to the famous study in mice that recapitulated nature's experiment. In 1953, Billingham, Brent and Medawar showed that exposure to foreign antigens prenatally induces tolerance to those antigens that persists in mature mice[144]. In this experiment, fetuses of a CBA female in the 15-16th day of pregnancy by a CBA male were injected intra-embryonically with suspensions of adult tissue cells. The inoculum consisted of testis, kidney and spleen tissue clumps and cells from an adult A-line mouse. Eight weeks after birth, the surviving five mice were challenged with skin grafts from an A-line donor. Three of the five grafts survived long-term with no further treatment, yet the injected mice retained their ability to reject third-party skin grafts. Medawar and colleagues carried out similar studies using different strain combinations and even different species, confirming their original findings. These subsequent studies demonstrated that tolerance could be induced in neonatal mice also, although the efficiency decreased rapidly over time[17,145,146]. Medawar shared the Nobel Prize with Burnet in 1960 for demonstrating immunologic tolerance. In 1953, Hasek had also reported similar findings by producing parabiosis between two chicken embryos of different breeds and lack of antibody responses to red cells in adult life. But he interpreted his findings in terms of metabolic effects rather than immunological mechanism[147]. Despite many animal models describing tolerance induction, achieving this in the setting of transplantation in human remains a challenging task.

1.5.3 Transplantation tolerance in human

Studies by Medawar and his group suggested that inducing tolerance during the fetal and neonatal period was more likely to be successful than in mature adults. Cooper and Lusher reported tolerance to ABO antigens following ABO-incompatible transfusion in three infants[148]. Absence of isoagglutinins against the red cell donor blood group was transient and did not persist beyond survival time of the donor red cells. As noted above, Fan *et al.* demonstrated B cell tolerance to donor ABO antigens in children who received ABOi heart transplants as infants[117]. Deficiency of isoagglutinins against donor blood group antigens was observed long after transplant, yet normal antibody titres were detected against third-party ABO antigens. Peripheral blood mononuclear cells did not produce antibodies against donor blood group antigens when stimulated in *in vitro* cultures. They concluded that the absence of donor-specific antibodies and donor-reactive B cells was indicative of neonatal B cell tolerance.

Attempts have also been made to induce tolerance to donor HLA molecules in adult kidney transplant patients and withdraw immunosuppression. Kawai *et al*.[149] reported successful withdrawal of immunosuppression in four of five patients who received combined bone marrow and kidney transplants from HLA single-haplotype mismatched living related donors using a conditioning regimen that included cyclophosphamide, anti-CD2 monoclonal antibody, cyclosporine A and thymic irradiation. They observed transient chimerism in all patients and showed donor-specific unresponsiveness of T cells from these patients in *in vitro* cultures. Four patients in this series remained immunosuppression-free for a period of 4.5-11.4 years[150]. Scandling *et al*.[151] also reported a similar case of HLA-matched kidney and hematopoietic-cell transplants using a conditioning regimen of total lymphoid irradiation (TLI) and anti-thymocyte globulin (ATG). In a follow-up study of 16 patients, immunosuppressive drugs were withdrawn from 11 patients[152]. Leventhal *et al*.[153,154] used hematopoietic stem cells and graft-facilitating cells (composed of plasmacytoid precursor dendritic cells) in conjunction with nonmyeloablative conditioning to induce mixed chimerism and tolerance in

HLA-mismatched kidney transplant recipients. Successful immunosuppression withdrawal has also been reported in liver transplant recipients[155,156].

Many strategies used to induce tolerance in animal models are not feasible in human. There are a few patients who have stopped their immunosuppression and continue to have good graft function. Because of the difficulties in studying these patients due to non-compliance, there is little reported evidence for spontaneous development of transplant tolerance to donor HLA molecules following transplantation. Moreover, there is no agreed definition of tolerance in this setting and demonstration of it may not meet all the criteria used to define tolerance in animal models.

1.6 Thesis overview

Carbohydrates and polysaccharides with repetitive epitopes, including blood group ABH antigens, have been classified as TI antigens and are thought to induce antibody responses by a mechanism of extensive cross-linking of B cell receptors, without the help of T cells. This proposed mechanism does not explain how foreign antigens with repetitive structures can be distinguished from self-antigens with similar structures by the adaptive immune system. Blood group ABH antigens and related structures, like many other carbohydrate structures, are abundantly expressed in human and other entities in the environment. The expression of these antigens and the circulating 'natural' antibodies, thought to be induced by environmental stimuli, create a barrier for solid organ transplantation. Dealing with pre-existing 'natural' antibodies, memory cells and antibody-secreting plasma cells as well as the ongoing immune response is critical for the success of ABOi transplantation. Thus, clear understanding of the structural nature of these carbohydrate antigens and immune mechanisms will help to improve patient care and encourage new strategies for tolerance induction to such antigens.

The overall objectives of this thesis were to study mechanisms of immune response to naturally-occurring carbohydrates such as blood group ABH antigens and to characterize ABO antibodies and their specificities to minor structural differences in ABH antigens.

Experiments described in chapter 2 were designed to answer two fundamental questions: 1) what are the mechanisms of antibody responses to polysaccharide antigens? and 2) how does the adaptive immune system discriminate self from non-self polysaccharide antigens? Primary antibody responses to blood group antigens and other polysaccharides were assessed in naïve mice using a standardized immunization protocol. *In vivo* CD4 T cell depleted mice were used to re-evaluate the role of T cells; the roles of TLR and CD40/CD40L pathways were explored using knockout mouse strains and adoptive transfer experiments; chemically synthesized blood group antigens and various conjugates were used to assess the nature of antigens that are capable of inducing antibody responses. We used T cell deficient mice reconstituted with monoclonal T cells and their response to specific glycopeptides to determine the nature of T-B cell interactions. Based on the findings, we propose a model for antibody responses to polysaccharide antigens within the framework of the two-signal model, thereby revealing a mechanism for self-nonself discrimination of such antigens.

The aims of studies described in chapter 3 and chapter 4 were: 1) to study important differences in blood group ABH subtype antigen expression between human erythrocytes and cardiovascular tissues from individuals of different blood groups, 2) to determine fine specificities of 'natural' ABO antibodies in human plasma, and 3) to determine 'donor-specific' blood group antibodies in ABOi heart transplant recipients. We generated monoclonal antibodies that can differentiate ABH subtypes known to be expressed in humans; using flow cytometry and immunohistochemistry, important differences in the expression of ABH subtypes and closely related antigens on erythrocytes, heart and spleen tissues from individuals of blood groups A, B, AB, O and other subgroups were determined. We developed an ABO-glycan microarray to determine fine specificities of 'natural' antibodies. An

international study of specimens from heart transplant patients was conducted to assess the accurate measurement of 'donor-specific' antibodies using the glycan microarray.

Studies described thus far were focused on characteristics of blood group ABH antigens, ABO antibodies and mechanisms of immune response to these antigens. Further studies are needed to understand mechanisms of acquired B cell tolerance to ABH antigens in infants. A suitable animal model would allow investigation of mechanisms and conditions that facilitate B cell tolerance induction. Development of such an animal model is described in chapter 5. Aims were: 1) to study the ontogeny of ABO antibodies in piglets, and 2) to assess whether the pig is suitable to model acquired neonatal B cell tolerance as observed in human ABOi infant heart transplant recipients. We studied 'natural' antibodies against ABH antigens in group O pigs from birth to adulthood and identified a window of antibody deficiency analogous to human infants. Immunohistochemistry studies revealed abundant expression of AH antigens in kidneys but expression was absent in the heart and vascular endothelium. AO-incompatible and AO-compatible kidney transplants were performed in neonatal piglets; evidence for B cell tolerance induction was assessed by monitoring anti-A antibody development and histological studies of kidney biopsies post-transplant.

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Chapter 2

T cell dependence of antibody responses to polysaccharide antigens: implications for the mechanisms of self-nonself discrimination

A version of this chapter has been submitted for publication in ***Nature Immunology***

2.1 Introduction

Self-nonself discrimination by the immune system remains one of its major but not well-understood features. In 1957, Burnet proposed the Clonal Selection Theory of antibody formation[1], which became the cornerstone of modern immunology. Although immunological tolerance was, strictly speaking, not central to the Clonal Selection Theory, Burnet postulated that self-tolerance results from the deletion of self-reactive clones during embryonic life[2]. In contrast, Lederberg envisaged that lymphocytes are continuously produced in individuals throughout their life and that those with receptors specific for self-antigens are eliminated[3]. Many subsequent observations[4-7] were congruent with Lederberg's proposals, and Lederberg's basic mechanism came to be accepted as underlying central tolerance of both B and T cells. Bretscher and Cohn[8] proposed a theory of peripheral self-nonself discrimination in the context of Lederberg's idea that lymphocytes are generated throughout life. They proposed that the activation of lymphocytes requires the associative recognition of linked epitopes of the antigen, reflecting a requirement for lymphocyte cooperation in the activation of lymphocytes. An antigen-specific B cell receives the first signal through recognition of an antigenic determinant by the B cell receptor (BCR) and is induced to form antibodies against that antigen only if it receives a second signal. The second signal is delivered to the B cell following the recognition of a peptide/class II MHC complex, expressed on the B cell surface, by the T cell receptor (TCR) of an activated CD4 T cell. This scheme provides a useful description of how B cells are activated by protein, 'T-dependent' (TD) antigens. A plausible exception to this scheme is the antibody response to carbohydrate or 'T-independent' (TI) antigens that, according to the current paradigm, are capable of inducing an antibody response in the absence of T helper cells.

Such TI antigens are recognized as belonging to one of two types[9]. TI type-1 antigens, such as lipopolysaccharides (LPS), are B cell mitogens that can stimulate B cells, for example, through Toll-like receptors (TLRs), to proliferate and, in the context of receiving signal one,

to give rise to antibody-secreting cells[10,11]. It has been suggested, in contrast, that TI type-2 antigens such as polysaccharides can induce antibody production by extensive cross-linking of B cell receptors without T cell help[12-14]. A wealth of information on B cell responses to TI type-2 antigens has been generated using DNP (2,4-dinitrophenol) or TNP (2,4,6-trinitrophenol) conjugated to ficoll or other polymers[15-18]. However, how foreign antigens having such structures induce antibody responses, whereas self-antigens with similar structures do not, remains an enigma.

Histo-blood group ABH carbohydrate structures are abundantly expressed on human red blood cells (RBCs) and other tissues, and have been shown to induce TI type-2 immune responses in mice, based on the generally accepted criteria[19] described above. We chose these clinically-relevant naturally-occurring antigens, in addition to other carbohydrates, to study how polysaccharide antigens exert their biological effect on B cells to produce antibodies and how the immune system may discriminate self from non-self polysaccharide antigens.

2.2 Materials and Methods

2.2.1 Mice

C57BL/6 (B6) mice were purchased from Charles River Laboratories, Canada. T cell-deficient (B6.129P2-Tcrb^{tm1Mom} Tcrd^{tm1Mom}/J; TCR $\gamma\delta$ KO), B cell-deficient (B6.129S2-Ighm^{tm1Cgn}/J; μMT), CD40 ligand knockout (B6.129S2-Cd40lg^{tm1Imx}/J; CD40L KO), CD40 knockout (B6.129P2-Cd40^{tm1Kik}/J; CD40 KO), and MyD88 knockout (B6.129P2(SJL)-Myd88$^{tm1.1Defr}$/J; MyD88 KO) mice were purchased from Jackson Lab, USA. Rag2/OT-II (B6.129S6-Rag2^{tm1Fwa} Tg(TcraTcrb)425Cbn; OT-II) mice were purchase from Taconic Farms, Inc., USA. All mice were on B6 background and used at 6-8 weeks of age. Each experimental group contained 5 or more mice. Protocols were approved by the University of Alberta Health Sciences Animal

Care Committee; animals were cared for under guidelines of the Canadian Council of Animal Care.

2.2.2 Red cell membranes

Red blood cell (RBC) membranes for immunization were prepared as described before[20] with some modifications. Briefly, reagent RBCs A_1 and B (Immucor Inc., USA) were washed in PBS and rested for 30 minutes at 4°C. RBCs were subsequently added to hypotonic solution (50 mM NaH_2PO_4, 50 mM Na_2HPO_4, pH 7.4). After the mixture was gently shaken for 30 min at 4°C, membranes were washed in hypotonic solution by successive centrifugations at 20,000 x g for 30 min until supernatant became clear. Membranes were re-suspended in PBS and stored at -30°C.

2.2.3 Reagents and antibodies

The following reagents from different suppliers were used in this study: Ovalbumin (OVA) and $OVA_{257-264}$ (SINFEKL, InvivoGen, USA), bovine serum albumin (BSA, Jackson Immuno Research Laboratories, Inc.), purified pneumococcal capsular polysaccharide serotype 3 (PPS-3, ATCC, USA and Statens Serum Institut, Denmark), blood group B trisaccharide-polyacrylamide conjugate (B-PAA, Glycotech, USA), complete Freund's adjuvant (CFA) and myoglobin from sperm whale skeletal muscle (SPWM, Sigma-Aldrich, USA). Blood group-A type III tetrasaccharide-lipid conjugate (A-NGL), blood group-B trisaccharide-BSA conjugate (B-BSA), B trisaccharide- human serum albumin conjugate (B-HSA), and α-Gal-(1$\rightarrow$3)-β-Gal-(1$\rightarrow$4)-β-GlcNAc-BSA conjugate (αGal-BSA) were purchased from V-Labs, Inc., USA. $OVA_{323-339}$ peptides with extra Lysine (KISQAVHAAHAEINEAGR) were synthesized by IBD, University of Alberta, Canada. Anti-CD3 (17A2), anti-CD8 (53-6.7), anti-CD4 (RM4-5), anti-CD19 (ID3), anti-βTCR, and anti-$\gamma\delta$TCR (GL3) were purchased from BD Biosciences, USA and anti-CD4 (GK1.5) was purchased from eBioscience, USA.

2.2.4 Synthesis and characterization of blood group-A antigen and conjugates (performed by Dr. Todd Lowary group, Department of Chemistry, University of Alberta)

Chemical synthesis and characterization of blood group-A type III tetrasaccharide has been published elsewhere[21]. Conjugation of blood group-A antigen to BSA, mouse serum albumin (MSA), $OVA_{323-339,}$ and $OVA_{257-264}$ was performed as described before[22]. The degree of conjugation of A type III tetrasaccharide to proteins and peptides was characterized by MALDI mass spectrometry.

2.2.5 Immunization

Mice were injected subcutaneously on the back with 300 μl of A_1 or B RBC membranes (in PBS; 30-40 μl packed red cells equivalent per mouse) in CFA (1:1 mixture). Mice received two further intra-peritoneal injections of A_1 or B RBC membranes in PBS on days 7 and 14 after the initial injection. For all other polysaccharides, proteins, and conjugates, an antigen dose of 50-75 μg per mouse was used. The same standardized protocol was used for the entire study.

2.2.6 *In vivo* depletion of CD4 T cells

Mice received intra-peritoneal injections of 200 μg of LEAF (Low Endotoxin, Azide-free) purified anti-mouse CD4 antibody (clone GK1.5, BioLegend, Inc., USA) on days -1, 0, and +1. Control groups received rat IgG2b,k isotype control antibody. The first injection of immunization was given on day 0. Depletion of CD4 T cells in all mice was confirmed by flow cytometry staining of peripheral blood on day 14.

2.2.7 Flow cytometry

Peripheral blood mononuclear cells (PBMC) or spleen cells were first incubated with Fc receptor blocking antibody (anti-mouse CD16/CD32, eBioscience, USA). Then cells were labelled with relevant antibodies by incubating with either fluorophore conjugated monoclonal antibody or isotype-matched control antibody at a pre-determined dilution for 30 minutes at 4°C. Antibody labelled cells were analyzed using MACSquant flowcytometer (Miltenyi Biotech, Germany); 30,000 gated events were collected and analyzed using FlowJo 7.6.4 (Tree Star, Inc., USA).

2.2.8 Enzyme linked immunosorbent assay (ELISA)

Medium-binding ELISA plates (Costar, Cambridge, MA) were incubated overnight with relevant antigen (50 μL volume, concentration 5 μg/ml in 0.1M Na_2CO_3 [pH 9.6]). For standards, anti-mouse IgM, IgG or IgA at 10 μg/ml in PBS was added in triplicate wells. After blocking with 1% BSA/PBS, diluted plasma or serially diluted mouse reference serum as standards (Bethyl Laboratories Inc., USA) was incubated in triplicate wells. Bound antibodies were detected using alkaline phosphatase-conjugated goat anti-mouse IgM, IgG or IgA antibodies (Bethyl Laboratories Inc., USA) and p-nitrophenyl phosphate (Sigma) and read at 405 nm. Concentration of antigen-specific serum antibodies was determined from the standard curves generated using mouse reference serum. Since PPS-3 used for immunization had some cell wall polysaccharides, for detection of PPS-3-specific antibodies, serum samples were pre-incubated with pneumococcal cell wall polysaccharide mixture (Statens Serum Institut, Denmark) in order to neutralize pre-existing or induced serum antibodies against cell wall polysaccharides.

2.2.9 Hemagglutination assay

Anti-A and anti-B agglutination titres were assessed by incubating two-fold serially diluted serum samples with blood group-A_1 or B reagent RBCs (Immucor, Inc., USA) in a microplate haemagglutination assay. Titres were expressed as the highest dilution at which agglutination was detected. Starting dilution for all serum samples was 1:8, a negative agglutinations at this dilution were expressed as ≤1:4.

2.2.10 Magnetic associated cell sorting (MACS) and adoptive transfer

Spleens were harvested from 6-8 week old mice and single cell suspensions of splenocytes were prepared. CD4+ T cells were purified by labelling with anti-CD4 micro beads followed by positive selection using AutoMACS (Miltenyi Biotech, Germany). B cells were sorted using a B cell isolation kit and negative selection using AutoMACS according to manufacturer's protocol. Purity of CD4+ T cells and CD19+ B cells was more than 95% in all experiments. For adoptive transfer experiments, each mouse received an intravenous injection of 10-20 million purified CD4+ T cells or B cells. In all adoptive transfer experiments, survival of transferred cells *in vivo* was confirmed by analyzing PBMC using flow cytometry two weeks following the transfer.

2.3 Results and Discussion

The primary antibody response to the blood group-A antigen (Figure 2.1) was characterized by immunizing C57BL/6 (B6) mice with blood group-A RBC membranes in complete Freund's adjuvant (CFA) and assessing serum anti-A antibodies pre- and post-immunization. CFA was used as an adjuvant in order to elicit a potent and consistent antibody response and to assess the degree of T cell dependency in the presence of other accessary pathways (for example, TLR pathways, danger signals etc.). The immunization protocol was optimized and a standardized protocol used throughout the study. The requirement for T cell help for the antibody response to natural polysaccharides including blood group-A and group-B antigens

was assessed by depleting CD4 T cells with anti-CD4 antibody (GK1.5) in naïve B6 mice. Selective depletion of CD4 T cells was achieved and CD4 T cells remained deficient at least until the time of final antigen injection on day 14 (Figure 2.2). CD4 T cell-depleted mice failed to produce anti-A and anti-B IgM, IgG, and IgA antibodies, whereas isotype control antibody-treated mice that remained CD4 T cell replete showed a normal antibody response (Figure 2.3 and Figure 2.4). Effectiveness of CD4 T cell depletion was confirmed by immunizing with a protein antigen, sperm whale myoglobin (SPWM). Both anti-CD4 treated and control groups produced comparable amounts of IgM antibodies against SPWM on day 21 but only the former failed to produce IgG and IgA antibodies (Figure 2.5a-c). The antibody response returned to normal when mice were re-challenged with SPWM on day 42 (Figure 2.5d-f), indicating that T cell depletion did not result in long-term tolerance to SPWM. Together, these observations indicate a high degree of CD4 T cell dependency for the antibody response against naturally-occurring carbohydrate antigens.

The CD40/CD40L pathway has been shown to play a critical role in the antibody response to TD antigens, but not to TI antigens, when TNP-ficoll conjugates were used as the TI type-2 antigen in mice deficient for CD40 or CD40 ligand (CD40L)[23,24]. A potential role for a CD40/CD40L interaction or TLR pathways in antibody responses to polysaccharide antigens was re-evaluated by immunizing mice deficient for CD40 ($Cd40^{tm1Kik}$), CD40L ($Cd40lg^{tm1Imx}$), and MyD88 (a TLR signaling adaptor protein; $Myd88^{tm1.1Defr}$) with blood group-A RBC membranes. Anti-A antibody responses were markedly reduced in both CD40 and CD40L knockout (KO) mice as compared to wild-type B6 (Figure 2.6). A modest reduction in anti-A antibody production was also seen in MyD88KO mice indicating the role of TLRs and their adjuvant effect. Given that CD40, CD40L, and TLRs are expressed in cells other than T and B cells, it is possible that the observed deficiency of antibody production was not entirely due to their absence in T and B cells. Therefore we performed adoptive transfer experiments (Figure 2.7) to assess the contribution of CD40/CD40L and TLR pathways on CD4 T cell help

to B cells. Purity of sorted cells (Figure 2.8) and survival of transferred B and T cells in peripheral blood were confirmed two weeks after transfer (Figure 2.9). Normal anti-A antibody production was seen in T cell-deficient ($Tcrb^{tm1Mom}$ $Tcrd^{tm1Mom}$) mice injected with purified wild-type B6 CD4 T cells. In contrast, anti-A antibodies were absent in T cell-deficient groups injected with CD4 T cells from CD40LKO mice, comparable to mice that did not receive CD4 T cells (Figure 2.10a-d). Similarly, the anti-A antibody response was minimal in B cell-deficient ($Ighm^{tm1Cgn}$) mice injected with purified B cells from CD40KO mice, comparable to those not receiving B cells. Normal antibody production was seen in B cell-deficient mice that received B cells from wild-type B6 mice with the exception of anti-A IgG which was absent (Figure 2.10e-h). Anti-A IgM and IgA antibodies were reduced in B cell-deficient mice injected with MyD88 KO B cells, and IgG was minimal. In contrast to previous findings using TNP-ficoll as a model TI type-2 antigen, our results, using a naturally-occurring carbohydrate antigen, suggest that the CD40/CD40L pathway plays a critical role during T and B cell interactions, leading to antibody production against carbohydrate antigens, and that TLRs enhance this response. The role of TLRs in B cells in antibody responses to TD antigens is controversial[25,26].

Blood group carbohydrate antigens are expressed on RBCs and other cells as glycoproteins and glycolipids[27]. To determine whether both glycoproteins and glycolipids are capable of eliciting antibodies against the glycan antigen, we tested chemically synthesized blood group-A antigens as neoglycolipid conjugates (A-NGL), protein conjugates (bovine serum albumin; A-BSA), or unconjugated A antigens mixed with BSA. Only A-BSA conjugates induced anti-A antibody production in B6 mice (Figure 2.11). To re-evaluate whether pure polysaccharides or carbohydrates on a polymer backbone can elicit a primary antibody response, B6 mice were immunized with purified pneumococcus polysaccharide serotype 3 (PPS3), chemically synthesized blood group-B antigen conjugated to polyacrylamide polymer (B-PAA), B antigen conjugated to human serum albumin (B-HSA), unconjugated synthesized blood group-A antigen, and A-BSA as the positive control. Antibodies were produced only when protein

conjugates were used (Figure 2.12). These results indicate that pure polysaccharides, carbohydrates as glycolipids, and carbohydrates on a polymer backbone are incapable of eliciting a carbohydrate-specific antibody response.

Our results so far suggest that induction of antibody responses to polysaccharide antigens requires presentation of carbohydrates as glycoproteins as well as activated CD4 T cells expressing CD40L that in turn interacts with CD40 on B cells. We next examined the nature of the CD4 T cells that can help B cells respond to carbohydrate antigens. To test for the involvement of peptide-specific CD4 T cell help we set up a model system in which monoclonal T cells from Rag2/OT-II mice, recognizing a peptide corresponding to amino acids 328–338 of chicken ovalbumin ($OVA_{323-339}$) when presented on MHC class II, were transferred into T cell-deficient mice (Figure 2.13). Recipient mice were then immunized with synthesized blood group-A antigen conjugated to $OVA_{323-339}$; control mice were immunized with blood group-A antigen conjugated to $OVA_{257-264}$ that is not recognized by OT-II CD4 T cells. Mice immunized with A-$OVA_{323-339}$ produced anti-A antibodies of all isotypes whereas those immunized with A-$OVA_{257-264}$ did not produce anti-A antibodies (Figure 2.14), indicating that peptide-specific T cell responses mediate help for carbohydrate antigens.

Based on data presented thus far, we propose that the two-signal model for antibody responses to protein antigens also governs the response to polysaccharide antigens, thereby revealing a mechanism that might provide a basis for self-nonself discrimination against such antigens (Figure 2.15). We have already established that antibody production to polysaccharide antigens occurs only when presented as glycoproteins. In order to test how this model would explain antibody production to a foreign or non-self glycan of a glycoprotein, in contrast to the self-glycan of a self-glycoprotein, we immunized B6 mice with synthesized blood group-A antigen conjugated to a self-protein, mouse serum albumin (MSA), or to a foreign or non-self protein BSA. Mice immunized with A-MSA did not produce anti-A antibodies whereas those immunized with A-BSA produced anti-A antibodies (Figure 2.16a-d). This

implies that antibody production to foreign polysaccharides depends on the foreign nature of the attached protein. We tested this proposition further by immunizing B6 mice with different glycoproteins: A-BSA, BSA or BSA conjugated to α-Gal (α-Gal-(1→3)-β-Gal-(1→4)-β-GlcNAc-BSA), a blood group-like carbohydrate antigen expressed on cells and tissues in mice to which they are thus tolerant. Anti-BSA responses were comparable in all groups (Figure 2.16e-g), whereas self-tolerance to α-Gal was maintained in mice immunized with α-Gal-BSA (Figure 2.16h-g). In this setting, although it was possible for any existing B cells to receive a second signal from activated T cells, self-tolerance to α-Gal was maintained. Furthermore, the comparable anti-BSA response in all groups suggests that the influence of glycan on antibody responses to protein was insignificant.

Taken together, our data suggest that an immune response is induced to polysaccharide antigens when presented as glycoproteins if the protein component is foreign and activates CD4 T cells, and that the absence of such CD4 T cells results in a relative deficiency of polysaccharide-specific antibody responses. Unravelling of this mechanism has significant implications in many areas including crossing carbohydrate barriers in transplantation and designing of effective carbohydrate vaccines against infectious agents and cancer. For example, expression by cancer cells of altered self and non-self glycan structures is an important feature mediating survival and progression of cancer[28,29]. According to our findings, the expression of such glycan structures on self-proteins likely plays a role in evading the adaptive immune system. We anticipate that this will spur further investigations on this mechanism that may help find new therapeutic approaches and design effective carbohydrate-based vaccines.

2.4 Acknowledgments:

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2.5 Figures

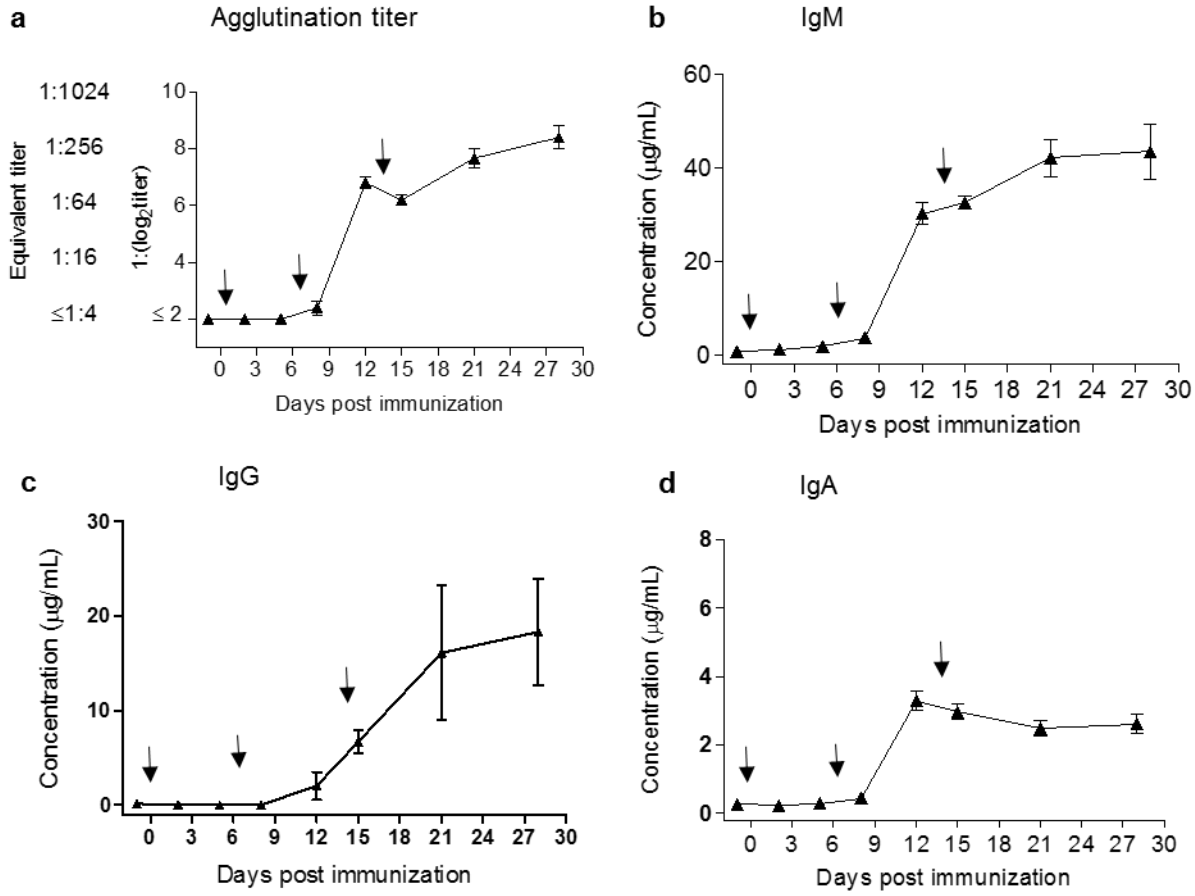


Figure 2.1. Anti-A antibody production following immunization. Naïve B6 mice were immunized with blood group-A_1 RBC membranes in CFA on day 0 followed by two further

injections in PBS on days 7 and 14 (arrows). Blood samples were collected pre- and post-injection at defined times. **a**, Serum anti-A antibody titres were measured using blood group-A_1 reagent RBCs in an agglutination assay. Serum anti-A antibody concentrations (**b-d**) of indicated immunoglobulin isotypes were measured by ABO-ELISA. Data points are presented as mean ± SEM; n=6.

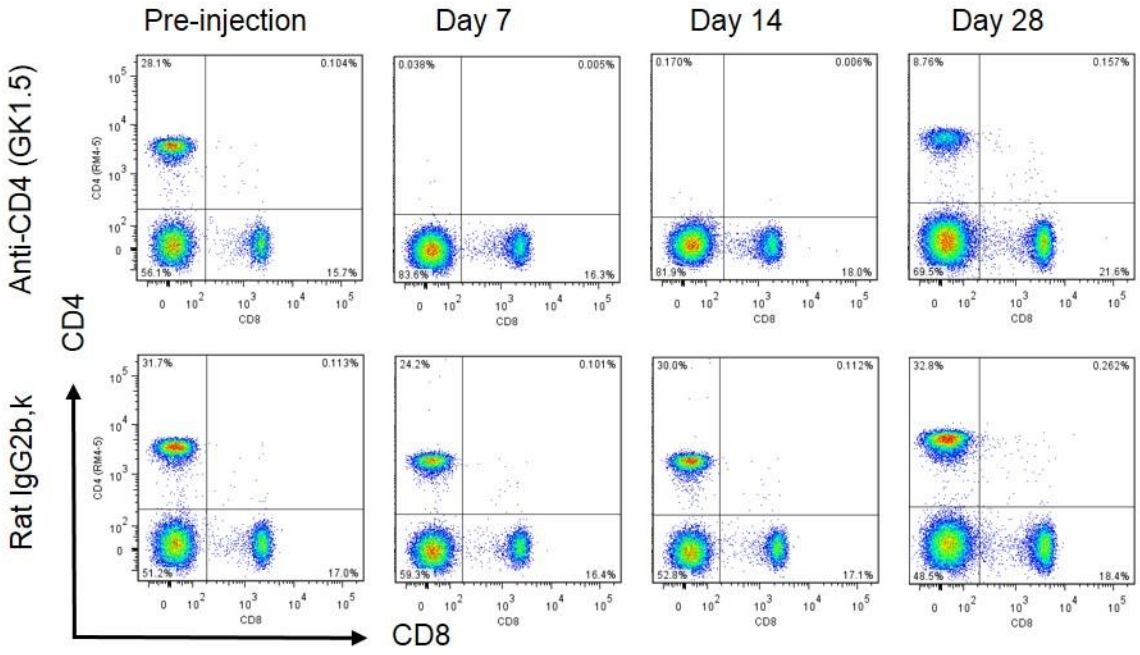


Figure 2.2. CD4 T cell depletion *in vivo*. Fluorescence-activated cell sorting (FACS) data showing selective depletion of CD4 T cells with anti-CD4 (GK1.5) antibody compared to isotype control antibody (rat IgG2b,k). Blood samples were taken pre- and post-injection at indicated times and stained with anti-CD3, anti-CD8, and anti-CD4 (RM4-5). CD4+ and CD8+ populations within lymphocyte-gated events are shown. Representative FACS plots are shown from one of six mice in each group. Different specificities of anti-CD4 antibodies GK1.5 and RM4-5 were confirmed.

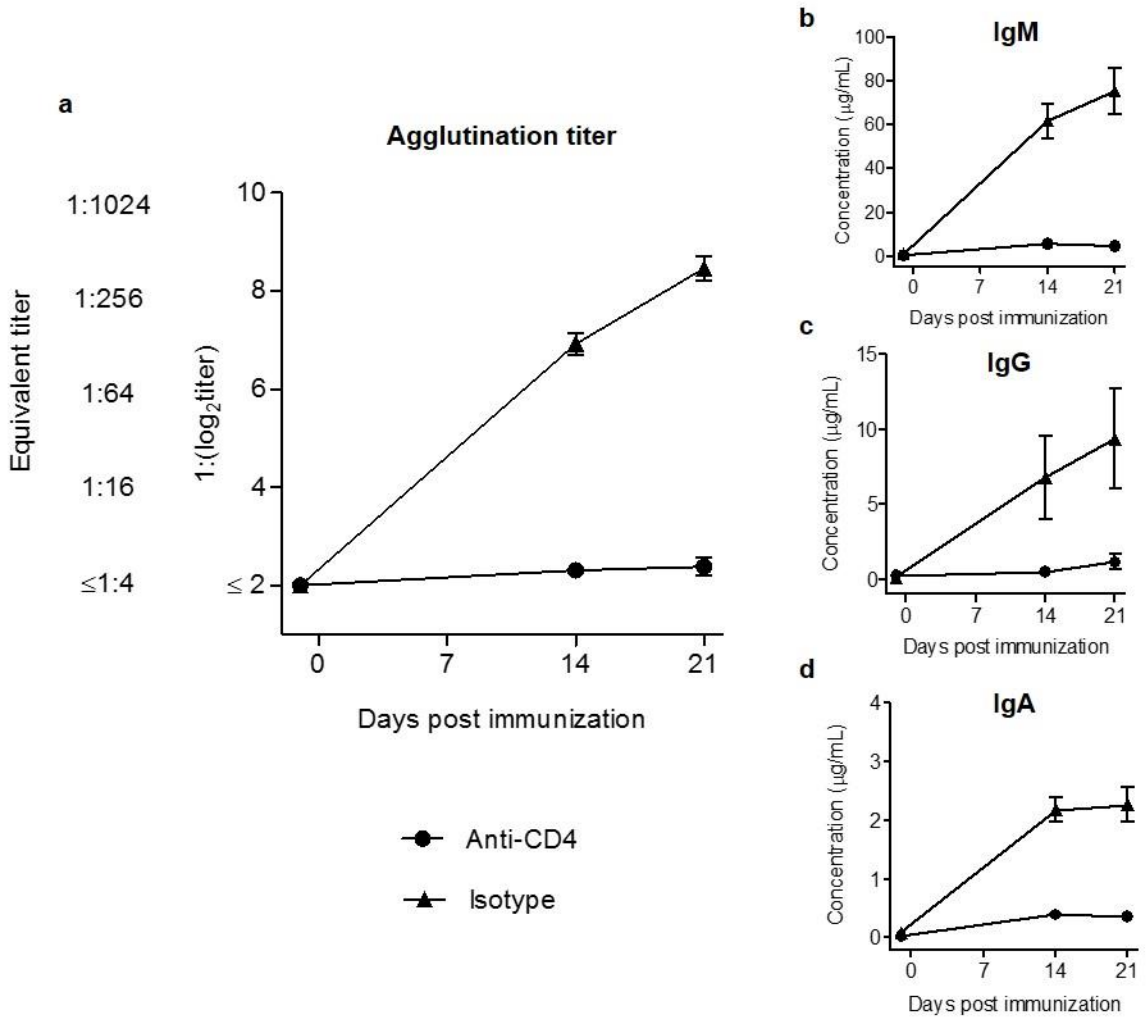


Figure 2.3. Antibody production to blood group-A antigen is CD4 T cell-dependent. **a-d,** Mice were injected with either anti-CD4 (GK1.5) or isotype control (rat IgG2b,k) antibody. Both groups were immunized with blood group-A_1 red blood cell (RBC) membranes on days 0, 7 and 14. Serum anti-A RBC agglutination titres (**a**) and anti-A antibody concentrations (**b-d**) were measured on days -1, 14, and 21.

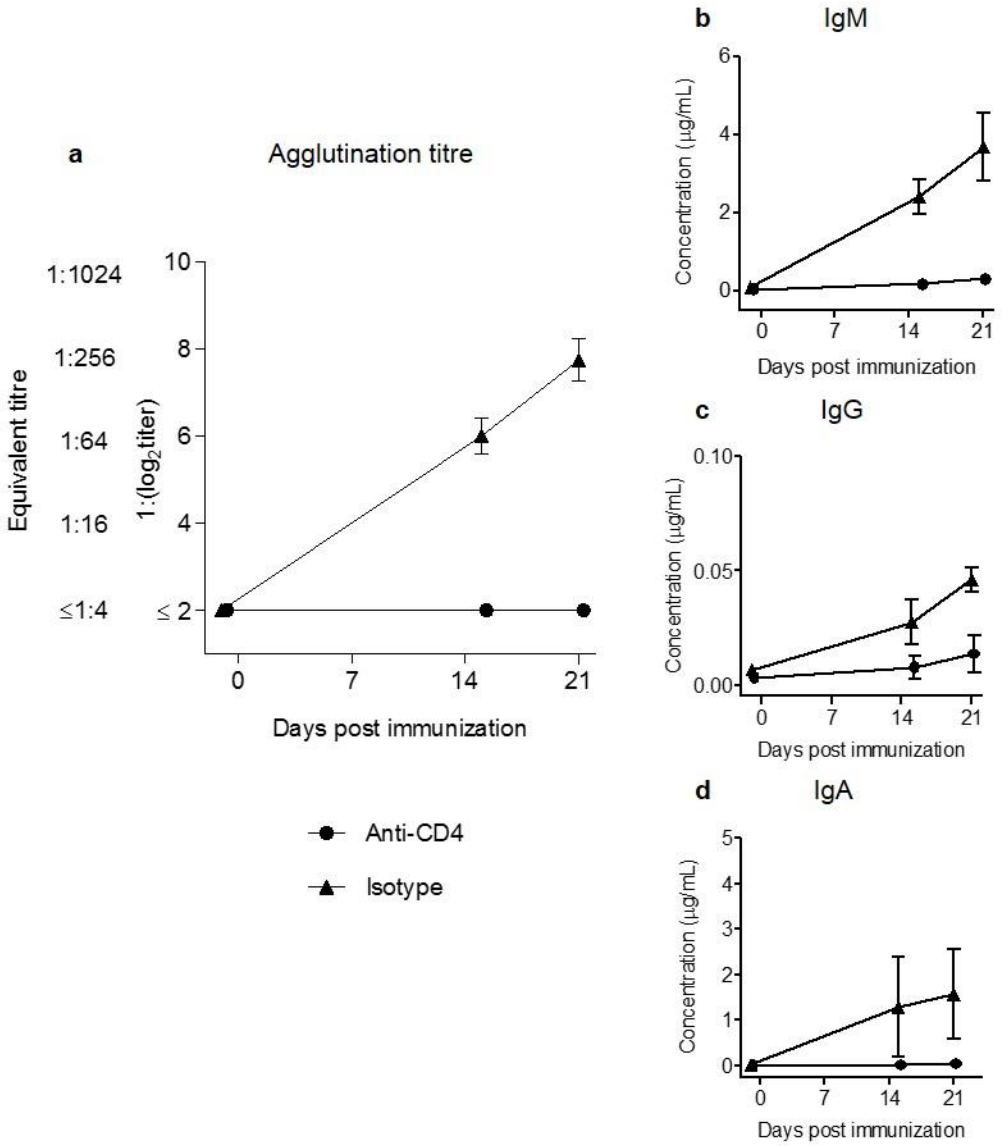


Figure 2.4. Antibody response to blood group-B antigen is CD4 T cell-dependent. Mice were injected with either anti-CD4 (GK1.5) or isotype control antibody. Both groups were immunized with blood group-B RBC membranes in complete Freund's adjuvant (CFA). Serum anti-B antibodies were measured on days -1, 15, and 21. Anti-B antibody titres (**a**) were measured using blood group-B reagent RBCs in an agglutination assay. Serum anti-B antibody concentrations (**b-d**) of indicated immunoglobulin isotypes were measured by ABO-ELISA. Data points are presented as mean ± SEM. n≥5.

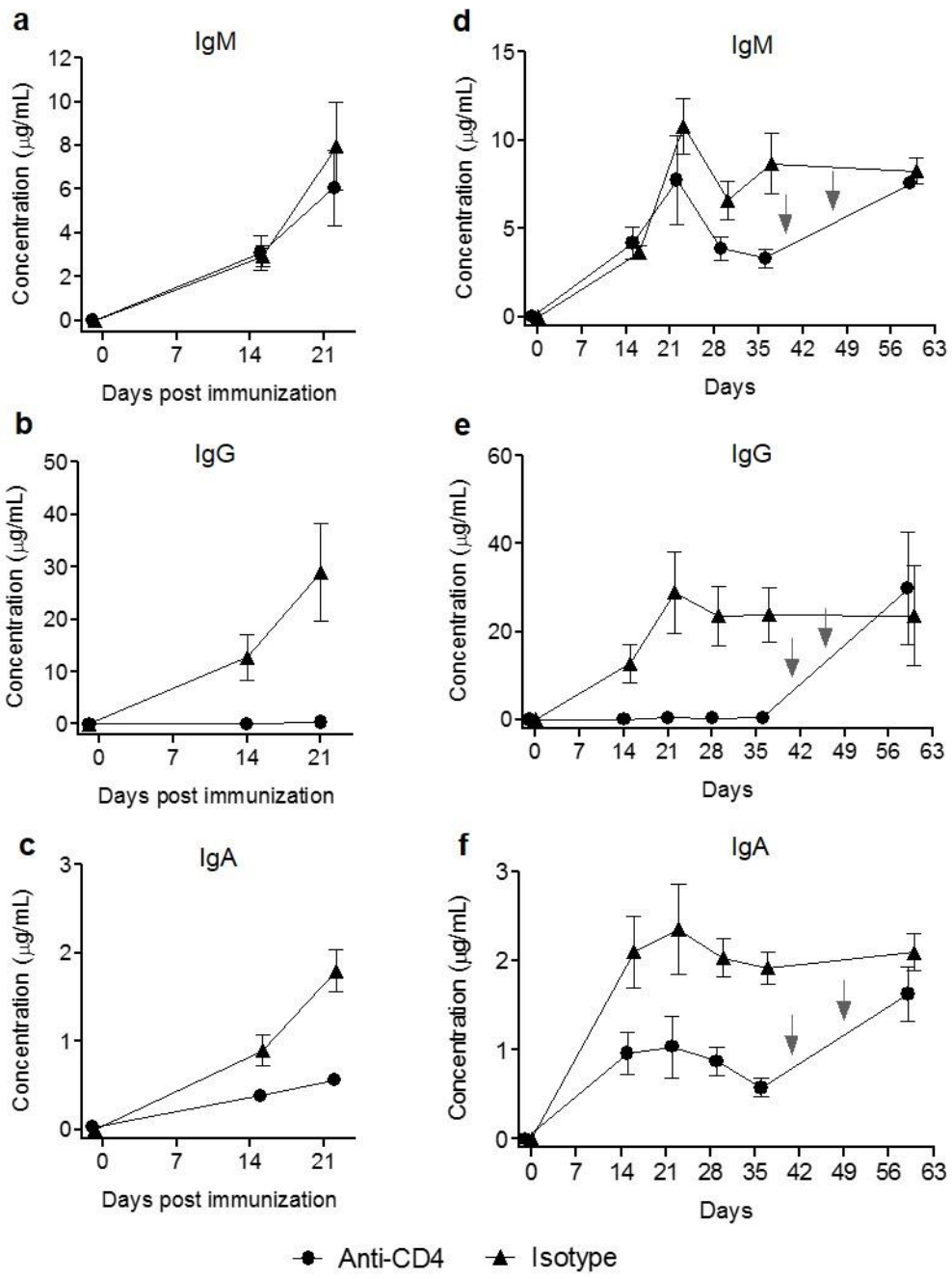


Figure 2.5. T cell depletion by anti-CD4 antibody does not result in tolerance. a-c, Naïve B6 mice were injected with either anti-CD4 (GK1.5) or isotype control (rat IgG2b,k) antibody. Both groups were immunized with sperm whale myoglobin (SPWM) in CFA; serum

SPWM-specific antibodies were measured by ELISA. **d-f**, Mice that received anti-CD4 were re-challenged with two further injections of SPWM at indicated timepoints (arrows) when CD4+ T cells returned to normal levels. Data presented as mean ± SEM. n≥5.

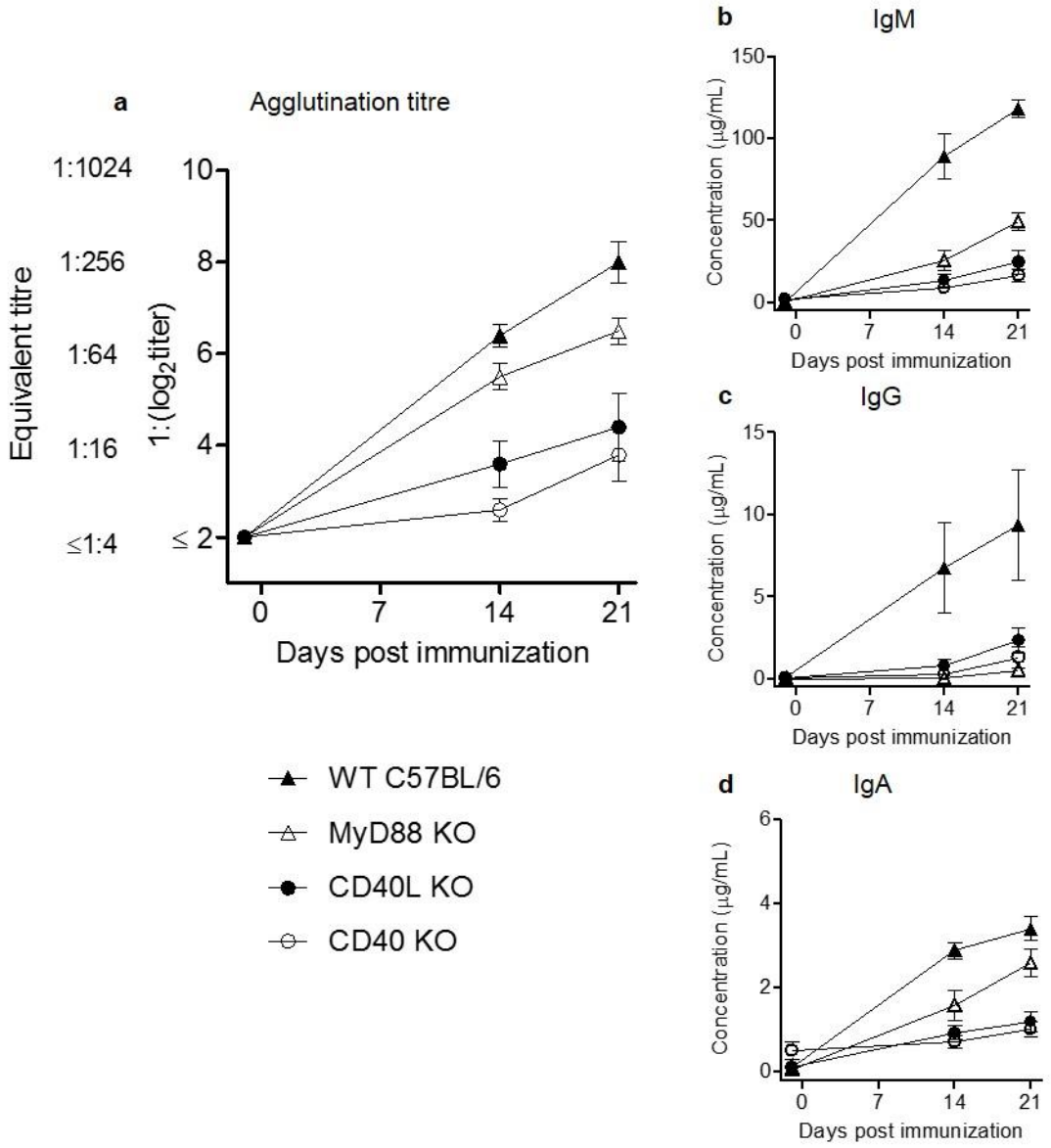


Figure 2.6. Importance of CD40/CD40L and TLR pathways. CD40 knockout (KO), CD40L KO, MyD88 KO, and wild-type (WT) B6 mice were immunized with blood group-A_1 red

cell membranes. All mice were B6 background. Anti-A RBC agglutination titres (**a**) and anti-A antibody concentrations (**b-d**) are shown. Data presented as mean ± SEM. n≥5.

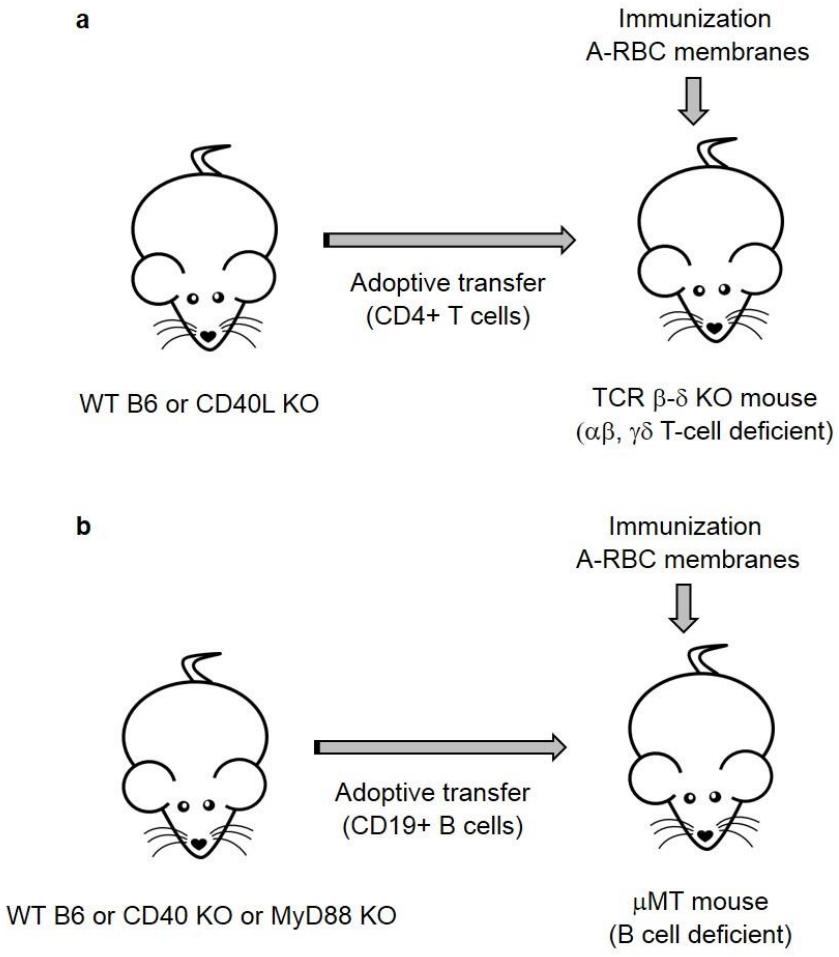


Figure 2.7. Illustration of adoptive transfer experiments. a, T cell-deficient mice (TCR β–δ KO) were reconstituted with purified CD4+ T cells from either WT B6 or CD40 ligand knockout (CD40L KO) mice. All groups, including a group of T cell-deficient mice that did not receive CD4+ T cells, were immunized with blood group-A_1 RBC membranes. **b**, B cell-deficient mice (μMT) were reconstituted with purified splenic B cells from WT B6, CD40 KO or

MyD88 KO mice. All groups, including a control group of B cell-deficient mice that did not receive B cells, were immunized with A_1 RBC membranes.

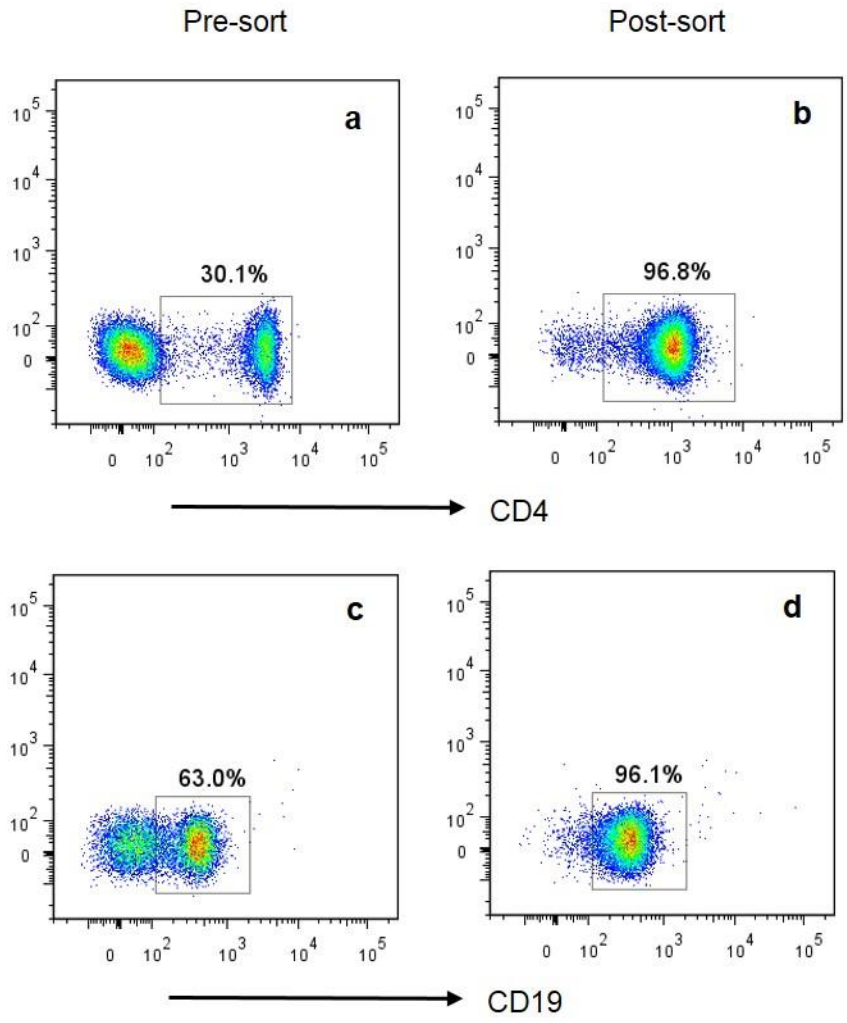


Figure 2.8. Purification of T and B cells. Representative FACS dot plots showing purity of splenic CD4+ T cells (**a, b**) and CD19+ B cells (**c, d**) pre- and post-sorting by magnetic associated cell sorting (MACS).

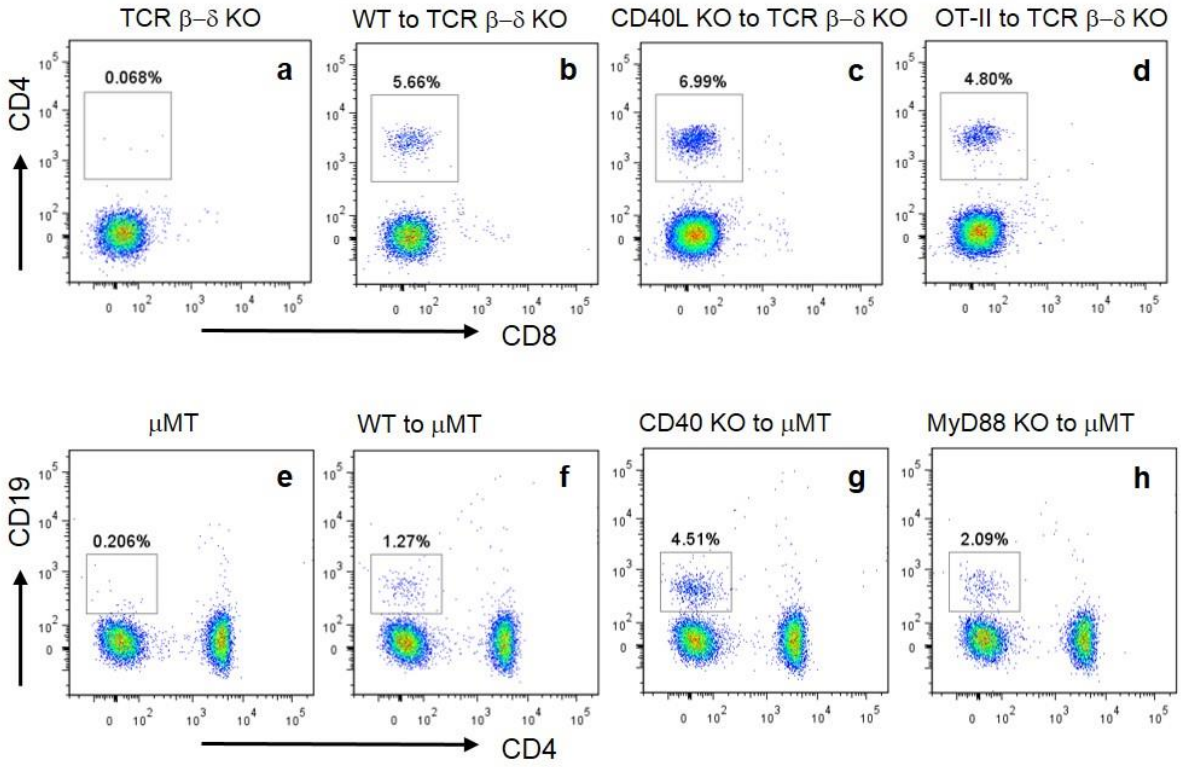


Figure 2.9. Adoptive transfer and cell survival. Purified CD4+ T cells from WT B6, CD40L KO, and Rag2/OT-II mice were injected into T cell-deficient (TCR β–δ KO) mice. Peripheral blood samples were analysed 2 weeks after cell transfer for circulating CD4+ T cells (**a-d**). Purified B cells from WT B6, CD40 KO, and MyD88 KO mice were injected into B cell-deficient (μMT) mice. Peripheral blood samples were analysed 2 weeks after cell transfer for circulating CD19+ B cells (**e-h**). Representative FACS dot plots are shown.

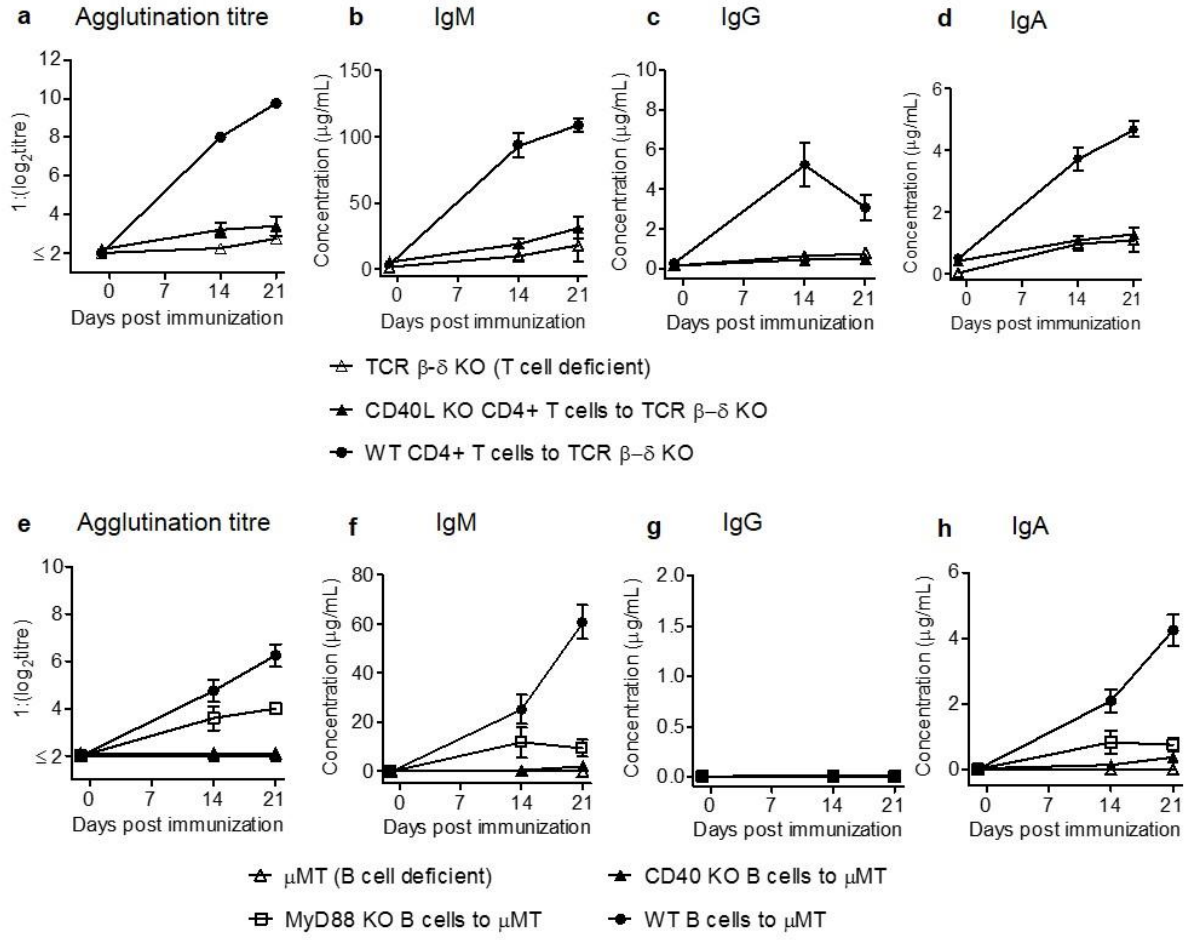


Figure 2.10. Role of CD40/CD40L pathway in antibody production to blood group-A antigen. a-d, T cell-deficient mice (TCR β–δ KO) were reconstituted with purified CD4+ T cells from either CD40 ligand (CD40L) knockout (KO) or wild-type (WT) B6 mice. All groups, including a group of T cell-deficient mice that did not receive CD4+ T cells, were immunized with A_1 RBC membranes. **e-h,** B cell-deficient mice (μMT) were reconstituted with purified splenic B cells from MyD88 KO, CD40 KO or WT B6 mice. All groups, including a control group of B cell-deficient mice that did not receive B cells, were immunized with A_1 RBC membranes. Data points are presented as mean ± SEM. n≥5.

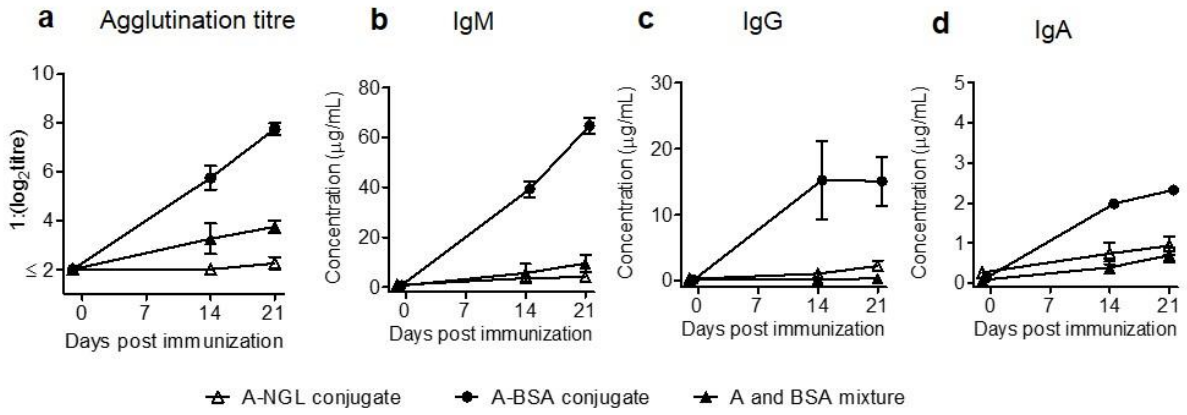


Figure 2.11. Antibody production to glyco-conjugates. Naïve B6 mice were immunized with blood group-A as neoglycolipid (A-NGL), glycoprotein (A-BSA) or A + BSA mixture without chemical conjugation. Anti-A RBC agglutination titres (**a**) and anti-A antibody concentrations (**b-d**) are shown. Data presented as mean ± SEM. n≥5.

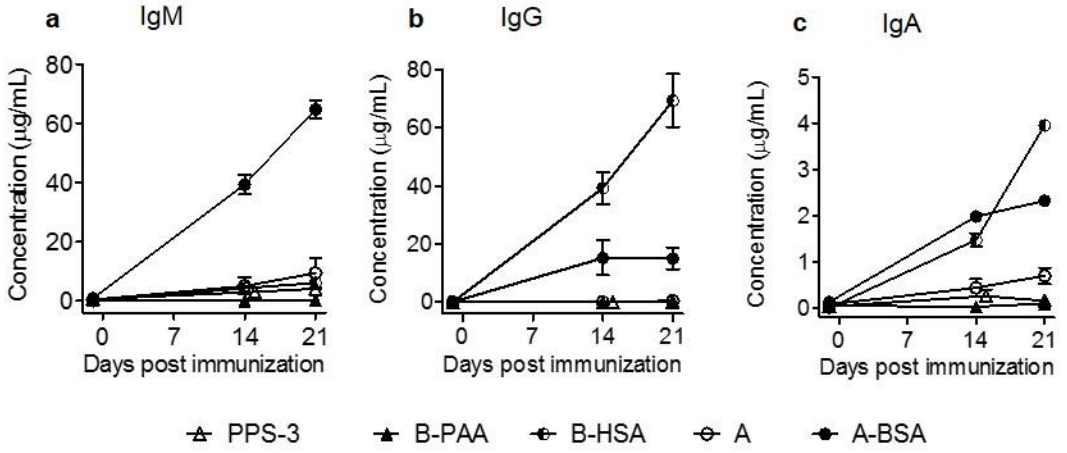


Figure 2.12. Antibody production to glycoproteins. a-c, Naïve B6 mice were immunized with purified pneumococcus serotype 3 (PPS-3), chemically synthesized blood group-B antigens conjugated to polyacrylamide (B-PAA), B antigens conjugated to human serum albumin (B-HSA), blood group-A antigens (A), or A conjugated to bovine serum albumin (A-

BSA). Antigen-specific antibodies (anti-PPS-3, anti-B, and anti-A) of indicated immunoglobulin isotypes are presented. Data points are presented as mean ± SEM. n≥5.

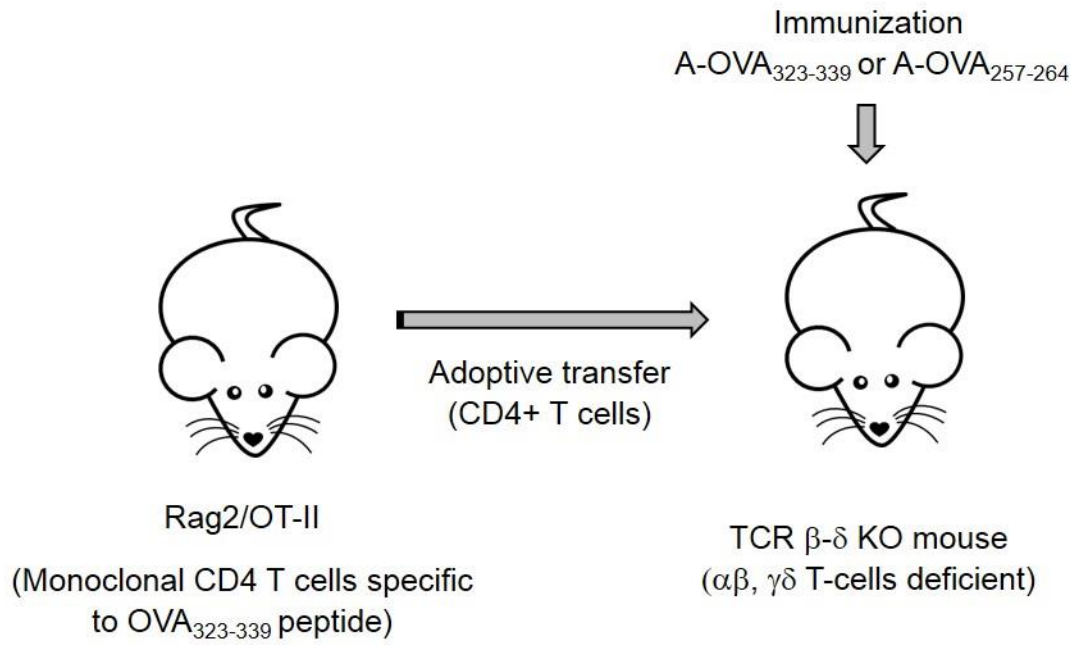


Figure 2.13. Illustration of adoptive transfer experiments. T cell-deficient mice (TCR β–δ KO) were reconstituted with CD4+ T cells from Rag2/OT-II ($Rag2^{tm1Fwa}$ Tg(TcraTcrb)425Cbn) mice and immunized with A antigens conjugated to either $OVA_{323-339}$ or $OVA_{257-264}$.

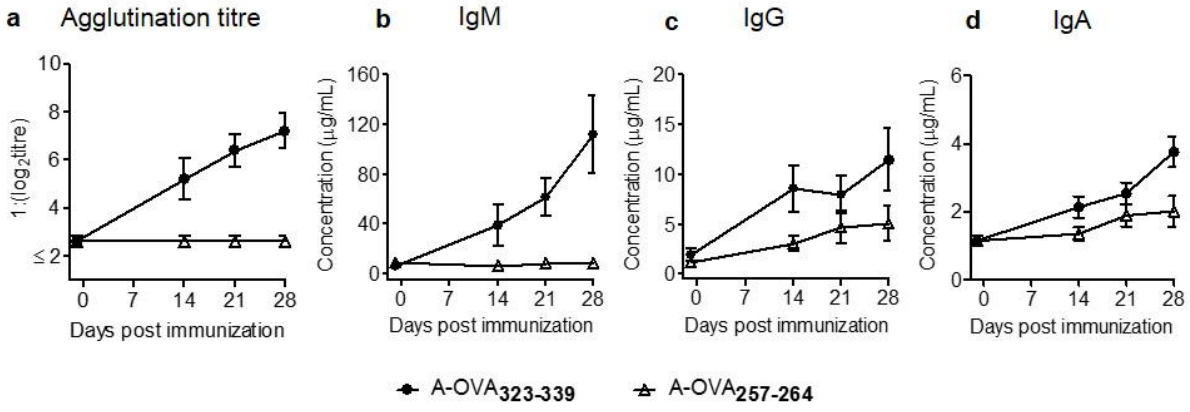
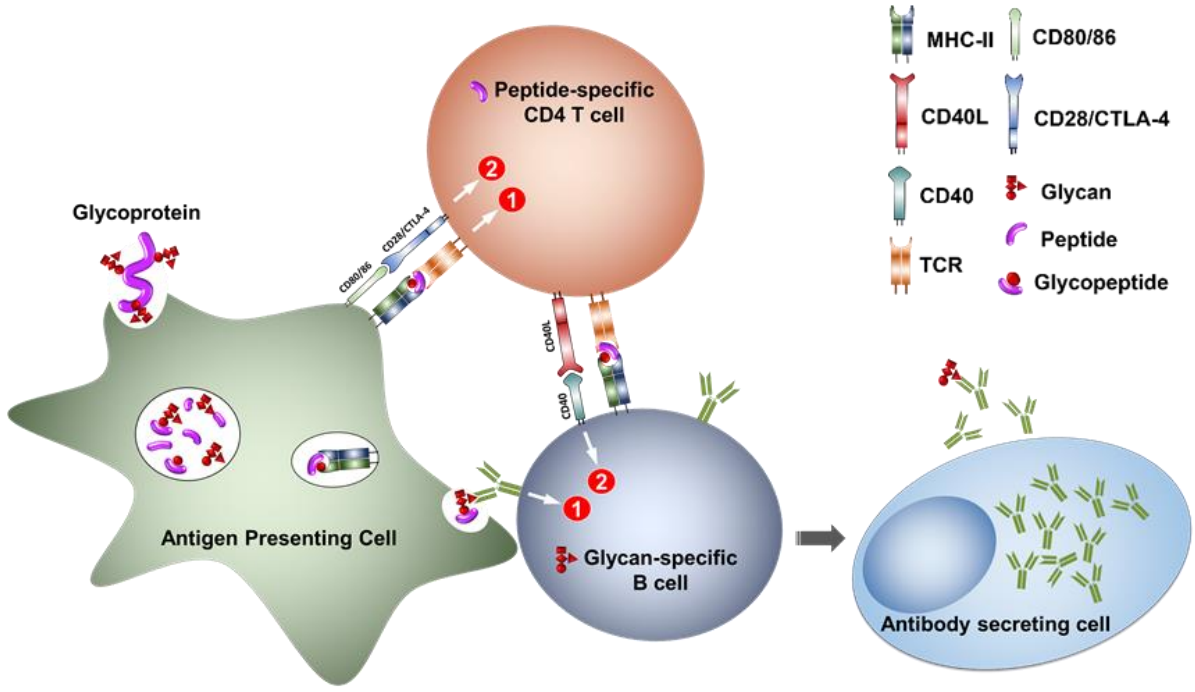


Figure 2.14. Antibody production to glycoproteins. a-c, T cell-deficient mice (TCR β–δ KO) were reconstituted with CD4+ T cells from Rag2/OT-II ($Rag2^{tm1Fwa}$ Tg(TcraTcrb)425Cbn) mice and immunized with blood group-A antigens conjugated to either $OVA_{323-339}$ or $OVA_{257-264}$. Development of serum anti-A RBC agglutination titres and anti-A antibody concentration of indicated immunoglobulin isotypes over time are presented. Data points are presented as mean ± SEM. n≥5.

a



b

Glycoprotein GP (**G**=Glycan; **P**=Protein)	Signal-1 to B cell	Signal-2 to B cell	Anti-G	Anti-P
Self-protein, non-self glycan	Yes	No	No	No
Non-self protein, non-self glycan	Yes	Yes	Yes	Yes
Non-self protein, self-glycan	No	Yes	No	Yes

Figure 2.15. A two-signal model for antibody responses to polysaccharide antigens and self-nonself discrimination. **a,** Schematic diagram depicts the proposed mechanism for B cell induction and antibody secretion to polysaccharide antigens. Glycan-specific B cell receives signal-1 by recognizing glycan component of glycoprotein or glycopeptide through B cell receptor (BCR) engagement. Peptide-specific CD4 T cell can be activated or primed by T cell receptor (TCR) binding to the peptide, not the glycan, of glycopeptide presented on MHC class II by the antigen presenting cell. Activated T cell expresses CD40 ligand that in turn interacts with CD40 on B cell generating signal-2. Upon receiving signal-2, B cells proliferate

to become antibody-secreting cells that produce glycan-specific antibodies. **b,** Mechanisms of self-nonself discrimination of polysaccharide antigens based on the proposed two-signal model. Both signal-1 and signal-2 described are for glycan-specific B cells interacting with peptide-specific T cells. Anti-P antibody secretion resulting from a similar interaction between peptide-specific B cell and peptide-specific T cell is also shown. Experimental data testing these scenarios are presented in fig. 2.16.

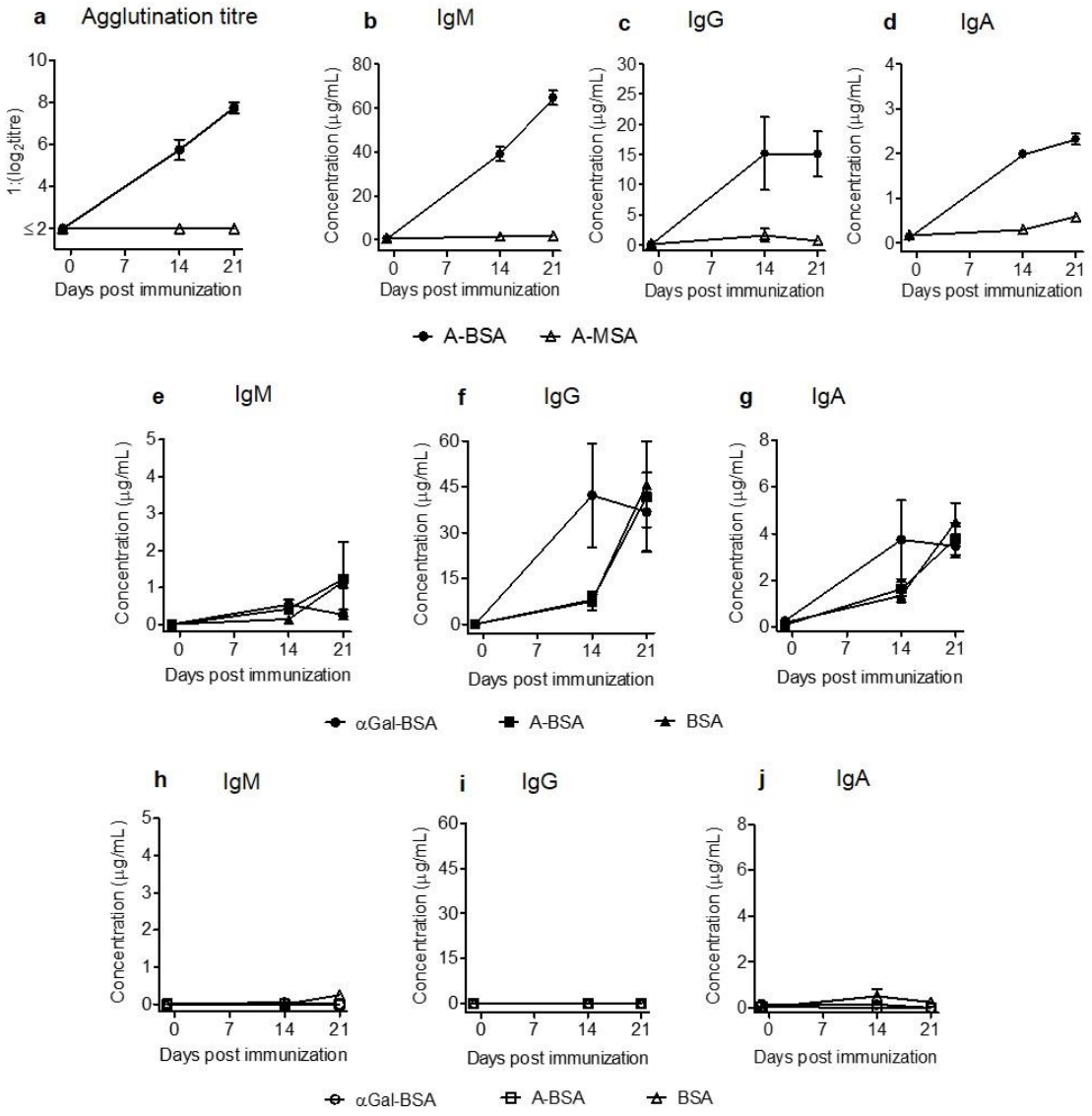


Figure 2.16. Antibody production to non-self polysaccharides requires conjugation to non-self protein. a-d, B6 mice were immunized with blood group-A conjugated to BSA (A-BSA) or to mouse serum albumin (A-MSA). Development of anti-A RBC agglutination titres and anti-A antibody concentrations are shown. **e-j**, B6 mice were immunized with either a self-glycan, α-Gal-(1→3)-β-Gal-(1→4)-β-GlcNAc, conjugated to a non-self protein, BSA, (α-Gal-BSA) or non-self glycan-non-self protein (A-BSA) conjugates. A control group was immunized with BSA alone. Development of anti-BSA (**e-g**) and anti-α-Gal (**h-j**) antibodies is shown. All data are presented as mean ± SEM. n≥5.

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Chapter 3

Differential expression of blood group ABH type I-IV structures on human erythrocytes and tissues: implications for ABO-incompatible transplantation

A version of this chapter has been submitted for publication in ***Proceedings of the National Academy of Sciences***

3.1 Introduction

The ABH(O) blood group antigens on red blood cells were discovered by Karl Landsteiner in 1900[1] and are the most clinically important among the blood group systems[2]. ABH antigens are oligosaccharides synthesized by the action of genetically determined glycosyltransferases that sequentially add specific monosaccharides to precursor chains to form ABH glycoproteins and glycolipids[3, 4]. There are at least six types of possible precursor chains[3]; four of these are known to carry ABH and Lewis antigens in different human tissues and cells have been classified as follows[3, 5, 6]:

Type-I: ABH-β-(1→3)-β-GlcNAc1→R

Type-II: ABH-β-(1→4)-β-GlcNAc1→R

Type-III: ABH-β-(1→3)-α-GalNAc1→R

Type-IV: ABH-β-(1→3)-β- GalNAc1→R

R= glycoprotein or glycolipid

These variations in precursor structures that carry ABH and Lewis blood group structures create unique antigen epitopes[5, 7-9]. Genetic and biochemical evidence indicates that at least two different α-(1, 2)-fucosyltransferases can participate in H antigen biosynthesis, and that these enzymes correspond to the products of the H and the Se (secretor) blood group loci. Type-I-IV H determinants are subsequently used by co-dominant glycosyltransferases encoded by the ABO blood group locus to form A or B antigens[10]. Mutations in A and B transferase genes may result in diminished antigen expression; these subgroups or variants of A, B, and AB have been classified by the quantity of A or B antigens on erythrocytes, A_2 being the major A subgroup[11, 12].

Anti-A and/or anti-B 'natural' antibodies in individuals lacking expression of the corresponding antigens[1] are presumed to be produced as an immunologic cross-reaction to environmental stimuli such as gut flora[13]. Thus, blood group A individuals express A antigen on their tissues

and produce anti-B antibodies in their plasma. Similarly, blood group B individuals produce anti-A antibodies, group O individuals produce both anti-A and anti-B antibodies and group AB individuals produce neither. The existence of these antibodies and the abundant expression of ABH antigens on erythrocytes and other tissues have important clinical implications. ABO-incompatible (ABOi) organ transplantation may result in hyperacute antibody-mediated rejection leading to graft loss or death. Although ABOi heart transplantation can be performed safely in children due to a normal developmental lag in production of natural antibodies, determination of suitability for ABOi heart transplantation and effective post-transplant management depend on accurate detection and quantification of ABO antibodies. In clinical laboratories the most commonly used technique for measuring ABO antibodies is the isohemagglutination assay using reagent erythrocytes. Based on Landsteiner's observations more than 100 years ago, this assay assigns a semi-quantitative 'titre' to the final dilution of serially diluted patient serum in which an observer can visually detect agglutination of erythrocytes from donors of known blood groups.

Cellular expression of ABH Type-I-IV structures differs amongst organs and tissues[6]. In the setting of ABOi heart transplantation, detection of antibodies with specificity for ABO antigens expressed in the donor heart is critical. In order to define the antigen specificity of agglutinating antibodies, ABO and closely related glycans expressed on erythrocytes must be characterized precisely and compared to those in the heart and blood vessels. To accomplish this, we generated and characterized monoclonal antibodies that differentiate Types-I, II, III, and IV precursor structures of ABH antigens. This allowed us to demonstrate, using flow cytometry and immunohistochemistry, important differences in ABH and related blood group antigen expression between erythrocytes and cardiovascular tissues from blood group A_1, A_2, B, O, A_1B, and A_2B donors. This gives rise to immediate impact on clinical interpretation of results of isohemagglutination assays.

3.2 Materials and methods

3.2.1 Preparation of erythrocyte ghosts

Red cell ghosts for immunization were prepared as described before[14, 15] with some modifications. Briefly, reagent erythrocytes A_1 and A_2 (Immucor Inc., USA) were washed in PBS and rested for 30 minutes at 4°C. Erythrocytes were subsequently added to the hypotonic solution (50mM NaH_2PO_4, 50 mM Na_2HPO_4, pH 7.4). After the mixture was gently shaken for 30 min at 4°C, ghosts were washed in hypotonic solution by successive centrifugations at 20 000 x g for 30 min until supernatant became clear. Ghosts were re-suspended in PBS and stored at -30°C.

3.2.2 ABH and other glycan-specific monoclonal antibodies

JTL series monoclonal antibody production was carried out using methods previously described[16, 17]. Briefly, BALB/c mice were injected intra-peritoneally with 150 µl of blood group A_1 and A_2 red cell ghosts (30-40 µl packed cells equivalent) mixed with Complete Freund's Adjuvant (CFA) initially, with one subsequent injection using the same ghosts mixed with Incomplete Freund's Adjuvant (IFA). Three to four days after the second injection, spleens were harvested and spleen cells were fused with the myeloma cell line Sp2/mIL6. The fused cells were plated into 96-well culture plates and when colonies formed, the supernatants screened by agglutination assay and ELISA to detect positive clones. The selected clones were then sub-cloned by limiting dilution. JTL-1, JTL-2, JTL-4, JTL-5, and JTL-6 monoclonal antibodies were selected for this study.

Antibody purification: Cells were grown in 8% FBS/high glucose DMEM with antibiotics until cells were confluent and medium had turned yellow. Ammonium sulfate was added to the antibody containing tissue culture supernatant to give a final concentration of 50%, and the solution was stirred overnight at room temperature. The IgM pellet obtained by centrifugation

was dissolved in PBS, pH 7.4. After extensive dialysis against PBS, pH 7.4, IgM containing solution was run over anti-IgM column; IgM fractions were run on 10% SDS-PAGE with an IgM standard as a control. Further quantitation was performed using IgM ELISA.

Additional monoclonal antibodies used in this study were TH-1 and AH-21 (kindly provided by Professor Henrik Clausen and Dr. Ulla Mandel, Copenhagen Center for Glycomics, University of Copenhagen, Denmark); Z2B-1, 89-F, 89/8 (Virogen corporation, USA); 86-N, LWB01, MEM-158 (Pierce Biotechnology, USA); A46-B/B10 (Santa Cruz Biotechnoloy, inc., USA); T174, F3 (GeneTex, USA).

3.2.3 Erythrocytes and tissue samples

Reagent erythrocytes of blood groups A_1, A_2, B, and O used in this study were as follows: Referencells® A_1, A_2, B, O (Immucor Inc., USA), Reverse-Cyte® Cells A_1, A_2, B (Median Grifols Diagnostics AG, Switzerland), and Biotestcell® A_1, A_2, B, O (Bio-Rad Medical Diagnostics GmbH, Germany). A_1B and A_2B erythrocytes were obtained from individual healthy donors whose blood group was confirmed by ABO-genotyping. Endomyocardial biopsies were obtained from patients who underwent heart transplants (42 biopsy samples from 31 patients; age 4.5 months to 17.5 years). Spleen tissue samples were obtained from organ donors (n=11). The study was approved by institutional review committees from all centers; informed consent was obtained from all volunteers, patients and family members of organ donors.

3.2.4 Genotyping of the ABO-locus

ABO genotypes of donors of blood groups A_2 and A_2B subgroups were determined by analyzing complete sequences of Exons 6 and 7 of ABH(O) glycosyltransferase gene[18]. Genomic DNA of donors was prepared from peripheral blood mononuclear cells (PBMC) by Tissue DNA kit (OMEGA bio-tek). Partial ABH glycosyltransferase gene containing Intron5, Exon6, Intron6,

and Exon7 was amplified by polymerase chain reaction (PCR) using a high fidelity KAPA HiFi DNA polymerase (KAPA Biosystems, USA). To isolate a single allele of an individual donor, the PCR fragment was cloned into pUC19 plasmid using the Xba-I linker in the PCR primers. The pUC19 plasmid containing the PCR fragment was identified by Blue/White screening[19] and a plasmid was prepared from a single white colony. The DNA sequences of PCR fragments and the pUC19 plasmids were analyzed using the specific primers to read Exons 6 and 7 sequences, Big Dye Terminator Cycle Sequencing kit and a 3130 Genetic Analyzer (Life Technologies, USA). The DNA sequences of both alleles for an individual donor were determined by comparing the DNA sequences of the mixed alleles in PCR fragment and a single allele in pUC19 plasmid. The DNA sequence of each allele was submitted to NCBI Blast search to identify the ABO genotype.

3.2.5 ABH and other related glycan antigens

Chemical synthesis and characterization of blood group antigens A Type-I-IV, B Type-I-IV and H Type-I-IV (ID 1-12, Table 3.1 and Figure 3.1) have been published elsewhere[20, 21]. Conjugation of ABH Type-I-IV structures to bovine serum albumin (BSA) was performed as described before[22]. Polyacrylamide (PAA) or BSA conjugates of glycan structures (ID 13-27, Table 3.1, 3.2) were purchased from GlycoTech, USA and V-labs, Inc., USA as follows: 13. β-GlcNAc-PAA, 14. α-GalNAc-PAA, 15. β-GalNAc-PAA, 16. β-Gal-(1→3)-β-GlcNAc-PAA, 17. β-Gal-(1→4)-β-GlcNAc-PAA, 18. β-Gal-(1→3)-α-GalNAc-PAA, 19. β-Gal-(1→3)-β-GalNAc-PAA, 20. α-Fuc-(1→2)-β-Gal-PAA, 21. Le^a: β-Gal-(1→3)-[α-Fuc-(1→4)]-β-GlcNAc-PAA, 22. Le^b: α-Fuc-(1→2)-β-Gal-(1→3)-[α-Fuc-(1→4)]-β-GlcNAc-PAA, 24. Le^y: α-Fuc-(1→2)-β-Gal-(1→4)-[α-Fuc-(1→3)-]β-GlcNAc-PAA, 27. α-Gal: α-Gal-(1→3)-β-Gal-(1→4)-β-GlcNAc-PAA (GlycoTech). 23. Le^x: β-Gal-(1→4)-[α-Fuc-(1→3)]-β-GlcNAc-BSA, 25. Atri: α-GalNAc-(1→3)-α-Fuc-(1→2)-β-Gal-BSA, 26. Btri: α-Gal-(1→3)-[α-Fuc-(1→2)]-β-Gal-BSA (V-labs).

3.2.6 ELISA characterization of antibody specificity

Medium-binding ELISA plates (Costar, Cambridge, MA) were incubated for 2 hours with BSA or PAA conjugated glycan (1-27, Table 3.1, 3.2), PAA, and BSA (50 μL volume, concentration 5 μg/ml in 0.1M Na_2CO_3 [pH 9.6]). After blocking with 1% BSA/PBS, diluted monoclonal antibodies were incubated in duplicate wells. Bound antibodies were detected using alkaline phosphatase-conjugated goat anti-mouse IgM or IgG antibody (Bethyl Laboratories Inc., USA) and p-nitrophenyl phosphate (Sigma) and read at 405nm (Tecan Infinite M200, Tecan Group Ltd, USA). Antigen-specific binding was determined by subtracting background optical density (OD_{405nm}) of PAA or BSA alone from the OD_{405nm} readings for the PAA or BSA conjugated glycan.

3.2.7 Removal of sialic acids on erythrocytes

Sialidase treatment of erythrocytes was performed according to a previously described method [23] with minor modifications. Briefly, group A_1, A_2, B, O, A_1B, and A_2B erythrocytes were washed in phosphate buffered saline (PBS) and re-suspended in Hepes buffered saline (HeBS) at $2x10^6$ cells/ml. Sialidase, α(2→3,6,8,9) neuraminidase from *Arthrobacter ureafaciens* (Sigma, USA) was added at 100 mU/ml and incubated one hour at 37°C. Control cells were incubated in the same buffer without neuraminidase. Cells were washed 3 times in PBS to remove residual neuraminidase and re-suspended in PBS for further antibody staining and analysis by fluorescent activated cell sorting (FACS).

3.2.8 Flow cytometric analysis of ABH and related antigens on erythrocytes

Antibody labelling of 10^6 erythrocytes was performed by incubating erythrocytes with either antigen-specific monoclonal antibody or isotype-matched control antibody at a pre-

determined dilution in a final volume of 100 µl PBS for 30 minutes at 4°C. Bound primary antibody was detected with appropriate secondary anti-mouse antibody labelled with either Dylight-649 or APC (anti-mouse IgG-APC; anti-mouse IgM-Dylight-649, Jackson ImmunoResearch Laboratories, Inc., PA, USA). Cells were washed and re-suspended in PBS; samples were run using MACSquant (Miltenyi Biotech, USA); 30,000 gated events were collected and analyzed using FlowJo 7.6.4 (Tree Star, Inc., USA) analysis software. Staining and washing steps were performed using PBS without adding human or animal serum components in order to avoid interaction of glycan structures and antibodies in serum with detection antibodies and erythrocytes respectively.

3.2.9 Immunohistochemistry

Paraffin sections were processed for staining by deparaffinization (3 x 5 minutes in Toluene) and rehydration (3 x 100%, 1 x 95%, 1 x 70% ethanol, and distilled water). After quenching peroxidase activity, slides were blocked with 3%BSA/PBS then incubated with monoclonal antibodies at predetermined dilutions in blocking buffer. Secondary staining was performed with biotinylated anti-mouse IgM or IgG (Bethyl Laboratories, TX, USA) and streptavidin-HRP (Jackson ImmunoResearch Labs, Inc.) in blocking buffer; color development was performed using ImmPACT DAB (Vector Laboratories, CA, USA). Slides were counterstained with Mayer's hematoxylin (Sigma-Aldrich), dehydrated and mounted with Entellen mounting media (Electron Microscopy Sciences, PA, USA). Bright-field microscopy images were obtained using Nikon Eclipse E400 microscope fitted with Spot idea camera (SPOT imaging Solutions, Michigan, USA) and processed using SPOT software.

3.3 Results

3.3.1 JTL series monoclonal antibodies (mAb) distinguishes type II and III/IV based ABH structures

To investigate Type-I-IV ABH antigen expression on erythrocytes, it was necessary to generate mAbs and define their specificities to particular antigen epitopes. We generated mAbs that detect sub-type-specific precursor structures of A, B and H (O) antigens. Since many glycan structures including ABH antigens are composed of common monosaccharide units we characterized these antibodies against Type-I-IV ABH and other related glycan structures 1-27 listed in Table 3.1 and 3.2, conjugated to BSA or PAA, using ELISA. Hereafter, sub-type ABH antigens will be referred to as A-I-IV, B-I-IV, and H-I-IV. Clone JTL-1 antibody reacted with A-III, A-IV, B-III, and H-III structures (Figure 3.2a); JTL-2 reacted with A-III, A-IV, B-III, B-IV, H-III, and H-IV structures (Figure 3.2b); JTL-4 and JTL-5 had similar specificities to A-II, B-II, and A trisaccharide (Figures 3-2c, d); JTL-6 reacted with A-II and B-II structures (Figure 3.2e). Additionally, we screened mAbs from other sources (Figure 3.3a-k) described to recognize ABH and related antigens. These mAbs enabled us to differentiate Type-I-IV precursor structures and their A/B glycosylation status as well as Lewis antigens generated by further fucosylation of H antigens.

3.3.2 Relative expression of A and B antigens varies among erythrocytes from group A_1, A_2, B, O, A_1B, and A_2B donors

Blood group A and/or B antigen densities may vary among red cells from different subgroups. On order to compare A and B antigen expression, red cells from different blood groups and sub-groups were stained with MAbs Z2B-1 (Figure 3.3a) and 89-F (Figure 3.3b), specifically recognized terminal sugars (on Type-I, II, III, or IV precursor chains) that define blood group-A and B antigens respectively. Figure 3.4 depicts relative expression of A and B antigens on erythrocytes from A_1, A_2, B, O, A_1B, and A_2B donors. Blood group-A antigen expression levels

were similar on A_1 and A_1B cells, but lower on A_2 cells. Differences in A antigen expression were also seen between A_2 and A_2B cells; expression of A antigen on A_2B erythrocytes was much lower than A_2 (Figure 3.4a). Expression of blood group-B antigens, on the other hand, was relatively higher on A_2B than A_1B erythrocytes; strong expression was seen on A_2B and B cells (Figure 3.4b).

3.3.3 Expression of ABH type I-IV and related antigen expression on erythrocytes

JTL-1 and JTL-2 clones specifically recognize ABH Type-III/IV precursor structures (Figure 3.2a, b) and these structures were detected on A_1, A_2, A_1B, and to a lesser extent on A_2B erythrocytes but were absent on B and O cells (Figure 3.5a, b and Figure 3.6a, b). Although the expression level of H Type-III/IV precursor chains on A_2 cells was similar to that of A_1 cells, only a trace amount was detected by TH-1[24] antibody that is specific to A Type-III antigens (Figure 3.5c). This implies that H Type-III/IV precursor chains on A_2 erythrocytes are largely un-glycosylated with the terminal sugar, α-GalNAc, that defines blood group A antigen. Expression of A Type-III antigen on A_1 and A_1B were similar. A Type-II and B Type-II antigens were detected on A_1, A_2, B, A_1B, and A_2B cells by A/B Type-II specific clones JTL-4, JTL-5, and JTL-6 (Figure 3.5d-f and Figure 3.6c-e). A and H Type-I structures were not detected (Figure 3.6k, l); expression for B Type-I was not tested. Thus, A antigens on A_1 and A_1B cells are Type-II, III, and IV; A antigens on A_2 and A_2B cells are predominantly Type-II; B antigens on B and A_2B cells are Type-II. These findings were confirmed on reagent erythrocytes from three different clinical lab suppliers from North America and Europe (Figure 3.7a-f). Further, there were no clear differences in the level of ABH Type-I-IV antigen expression on reagent A_1, A_2, B, and O erythrocytes from different suppliers.

Erythrocyte and salivary Le^a and Le^b determinants are built on Type-I precursor structures. In individuals lacking the secretor gene, only Le^a can be made by the Lewis enzyme. In individuals with both the Lewis and secretor genes, Le^b is made[25, 26]. Le^x and Le^y antigens are

the Type-II isomers of Le^a and Le^b respectively[25]. High levels of Le^y antigen were detected in A_2 and O erythrocytes. Variable amounts of Le^y antigen were also detected in A_1, B, A_1B, and A_2B erythrocytes (Figure 3.5i and Figure 3.6p). Therefore it is likely that all erythrocytes used in this study were from secretor positive individuals.

In humans, sialyltransferases may add sialic acid structures at the terminal positions of sugar chains[5, 27] thereby preventing ABH and precursor-specific antibody binding. We used sialidase to remove sialic acid residues on erythrocytes and confirmed sialidase activity by labelling for one of the human sialic acids Neu5Ac (Figure 3.8). Erythrocytes treated with either sialidase or buffer alone were analysed for ABH Type-I-IV and related structures. Although more Type-II, III, and IV ABH epitopes were unmasked by sialidase (Figure 3.9a-g and Figure 3.10a-g), ABH Type-III/IV structures were still not detected on B and O erythrocytes (Figure 3.9a, b). Unmasking of H disaccharide epitopes by sialidase was most prominent in A_2 and O cells. H antigen was detected by anti-H antibodies in all O and A_2 cells following sialidase treatment (Figure 3.9g and Figure 3.10a).

3.3.4 Only ABH Type-II antigens are expressed in the heart and blood vessels

Endomyocardial biopsies (n=42 from 31 patients) consisted of cardiomyocytes and microvasculature including capillaries. We also stained spleen tissues (n=11) alongside cardiac biopsies to confirm similar antigen expression in blood vessels. Blood group A antigen was detected in capillaries of cardiac biopsies from A_1 (n=12), A_2 (n=5), and A_1B (n=3) donors by anti-A antibody (Z2B-1) that recognizes A Type-I-IV (Figure 3.11a and Figure 3.12a). Similarly, blood group-B antigen was detected in capillaries of cardiac biopsies from B (n=9), A_1B (n=3), and A_2B (n=3) donors by anti-B antibody (89F) that recognizes B Type-I-IV (Figure 3.11b and Figure 3.12b). Despite confirmation of A antigen expression in group A_2B erythrocytes, A antigen was not detected in A_2B hearts (Figure 3.12a). ABH Type-III/IV structures were not detected in any of the cardiac tissues (n=42) stained with JTL-1, JTL-2

or TH-1 antibodies (Figure 3.11c, 3.13a, 3.13c, 3.12c, 3.14a, 3.14c). On closer inspection of endothelial cells, vesicles of Type-III/IV structures were present in the cytoplasm in close proximity to the nucleus of some endothelial cells of A_1 heart and spleen. This may represent artifact however it is also possible that these structures are synthesized but not expressed on the cell surface. All A_1, A_2, B, A_1B, and A_2B biopsies examined expressed A or B Type-II structures that were detected by JTL-4 and JTL-5 (Figure 3.11d, 3.13b, 3.12d, 3.14b). A and H Type-I structures were not detected in A and O biopsies respectively. Lewis^y and weak expression of H disaccharides was observed only in blood group O endomyocardial biopsies. ABH Type-I-IV and related antigen expression in blood vessels of spleen (Figure 3.15, 3-16, 3-17) was similar to that seen in cardiac biopsies. In all spleens tested, Lewis^y antigen was detected only on red cells in the red pulp, not on vascular endothelium, and the expression was stronger in those of blood group O and B than A_1 and A_1B (Figure 3.17c). Antigen expression on erythrocytes and cardiovascular tissues is summarized in Table 3.3.

3.4 Discussion

Discovery of ABO(H) blood group antigens on human red blood cells by Karl Landsteiner[1] laid the foundation for the safe clinical practice of transfusion and eventually transplantation. He demonstrated that individuals lacking the A and/or B antigen on their erythrocytes had natural serum antibodies that agglutinated erythrocytes expressing that antigen[1, 28]. It became apparent later that the ABH antigens are not confined to erythrocytes but are also present on tissues and in secretions[29, 30]. Expression of ABH antigens on circulating cells and on tissues, and the corresponding presence of natural agglutinins against these antigens, creates a barrier for organ transplantation and transfusion. Therefore matching for ABO compatibility and measuring agglutinins is important in these clinical settings. Although methods of isohemagglutinin assessment are not well-standardized, most clinical laboratories rely on using reagent erythrocytes from commercially available sources, or from known blood group donors in cases of uncommon blood groups, for measuring antibody titres. In order to understand the specificity of these hemagglutinins it is important to characterize the ABH antigens expressed on erythrocytes.

Early studies on isolation and chemical characterization of ABH and Lewis antigens added much to the understanding of their polysaccharide structures[31, 32]. Advancement in techniques allowed more detailed characterization and revealed the presence of Type-I-IV ABH structures attached to lipids and proteins on erythrocytes and tissues[3, 5, 7, 33-38]. Studies by us (data not shown) and others[39] recognized a problem in the stated specificity of many commercially available blood group antibodies. Since there are common precursor structures amongst ABH (Figure 3.1) and Lewis[5, 20, 21] antigens, it is expected that antibodies may cross-react with more than one of these antigens. Synthesis of A Type-I-IV, B Type-I-IV, and H Type-I-IV structures enabled us to generate and characterize mAbs that can reliably detect precursor structures of the native antigens expressed on erythrocytes using flow cytometry. Labelling the same number of erythrocytes from different blood group donors with the same pre-

determined amount of mAbs allowed us not only to study the presence or absence of antigens but also to compare expression levels on the erythrocyte surfaces relative to each other.

Although Type-I-IV precursor structures were previously isolated and characterized from erythrocyte membranes, most of these studies were performed on A_1 and A_2 erythrocytes[7, 9, 24, 37]. To our knowledge, detailed studies comparing the expression of Type-I-IV ABH antigens on erythrocytes, hearts, and spleens from various blood group donors have not been performed. We detected A and B antigens based on Type-II precursor structures on group A_1 and B erythrocytes respectively, by three different mAbs specific to A and B Type-II structures. Type-II structures were also detected on erythrocytes from A_2, A_1B, and A_2B donors. Expression of Lewis^y antigen on group O erythrocytes also implies the presence of H Type-II structures on O cells. In contrast, Type-III/IV structures were not detected on group B and O erythrocytes by two different mAbs specific to ABH Type-III/IV precursor structures. The absence of Type-III/IV structures on B and O cells was confirmed on reagent erythrocytes from three different suppliers. Sialidase treatment did not alter the staining pattern. Type-I ABH antigens are influenced by the Lewis and secretor systems[40]; erythrocytes do not synthesize Type-I based structures, but they adsorb circulating glycolipids from plasma[5, 25]. Type-I based ABH structures were not detected on any erythrocytes tested despite the fact that these cells were likely to be from secretors, based on expression of Lewis^y antigen. ABH and Lewis structures may be modified further by sialylation of terminal sugars[5, 40]. Unmasking of antigen epitopes with sialidase treatment was more pronounced on A_2 and O than any other erythrocytes indicating that most, if not all, H antigens are sialylated. Taken together, our results demonstrate that all erythrocytes express Type-II ABH structures but Type-III/IV structures are expressed only on A_1/A_2, not on B and O erythrocytes. H antigen epitopes on A_2 and O erythrocytes are further modified with sialic acids. Our studies of cardiac biopsies and spleen tissues revealed that expression of blood group antigens on vascular endothelium is restricted to Type-II based structures regardless of the blood groups or subgroups. The

expression observed in cardiac and spleen tissues is likely independent of secretor status as there were no differences amongst tissues from individuals of the same blood groups.

There is ongoing debate as to whether there is a quantitative or qualitative difference, or both, between blood group A_1 and A_2 antigens[9, 24, 33, 41-45]. Our study supports the existence of both quantitative and qualitative differences. Labelling of A_1 and A_2 erythrocytes with anti-A mAb specific to terminal A-trisaccharide showed positive yet different MFI values (Fig. 3.4a). This suggests that the same A antigen is present on both A_1 and A_2 erythrocytes but the number or density on the surface of A_2 cells is lower than A_1, as previously described[44]. Furthermore, expression of Type-II, III and IV precursor structures on A_1 and A_2 cells was similar but A antigen sites on A_2 cells were almost exclusively carried by Type-II precursors with only a small fraction of A-III structures. The presence of Lewis^y antigens on A_2 similar to O erythrocytes also suggests that glycosylation of Type-II precursors on A_2 is incomplete compared to A_1, causing lower A antigen density on A_2. Different antigen epitopes created by these A precursor structural differences are likely responsible for the qualitative differences. Interestingly, quantitative and qualitative differences of A antigen expression were also seen between A_2 and A_2B cells. This qualitative difference is in agreement with the observation that some blood group-A_2 and A_2B individuals produce natural anti-A antibodies[46-48]. Similarly, there were quantitative differences of B antigen expression on B, A_1B and A_2B cells. One explanation for the quantitative differences is that A_1, A_2, and B transferases have different enzyme kinetics[43] and compete for the same Type-II precursor substrates. It can be suggested that the efficiency of transferases in decreasing order is likely to be A_1 (N-acetylgalactosaminyltransferase) >B (Galactosyltransferase) >A_2 (N-acetylgalactosaminyltransferase).

Our study highlights a major problem with current methods of determining ABO antibody titres using erythrocytes. This can be illustrated with an example of ABOi heart transplantation. Although ABOi heart transplantation can be safely performed in infants with

low levels of isohemagglutinins[49-51], it is critical to monitor potential production of donor-specific antibodies after transplant (*ie,* antibodies against ABH antigens expressed in the heart graft). After a blood group-O infant receives a group-A donor heart, development of anti-A antibodies is monitored over time by measuring antibody titres, typically using reagent A_1 erythrocytes. Yet, because only Type-II-based A structures are expressed on cardiac vascular endothelium, only a portion of anti-A antibodies, or perhaps none, detected by agglutination of A erythrocytes (expressing A Type-I-IV) would be 'donor-specific', with specificity to the antigens expressed in the heart graft. This may result in unnecessary antibody-removal interventions such as plasmapheresis. Furthermore, a blood group-O infant awaiting heart transplantation may be deemed unsuitable to receive a group-A donor heart based on an arbitrary threshold anti-A agglutination titre (1:4 in current UNOS regulation, policy 3.7.8). The patient's serum may contain little or no antibodies against A Type-II, thus would theoretically be safe to receive a group-A donor heart. This may unnecessarily prevent the patient from being transplanted in a timely manner. Similarly, anti-B antibodies detected by B erythrocytes may also include antibodies against $Lewis^y$ structures expressed on erythrocytes but not on cardiac vascular endothelium. Similar issues may arise in other ABOi solid organ (*ie*, kidney) and hematopoietic stem cell transplants, and in fetal/maternal ABO-related issues. Detailed characterization of ABH Type-I-IV expression in other organs and hematopoietic stem cells will help define the extent of this problem and allow assessment of clinically relevant antibodies. In conclusion, interpretation of ABO antibody specificities for clinical decision-making must consider characteristics of ABH antigen sub-types as well as their possible modification by different transferases.

3.5 Acknowledgments

This work was supported by grants from the Natural Sciences and Engineering Research Council (NSERC) and the Canadian Institutes of Health Research (CIHR) CHRP (Collaborative Health Research Projects) program, Canadian Institutes of Health Research (CIHR) Emerging Team Grant, and Alberta Glycomics Centre. We like to thank Professor Henrik Clausen and Dr. Ulla Mandel for providing monoclonal antibodies TH-1 and AH-21 and those who donated red cells for the study.

3.6 Tables

Table 3.1. Summary of monoclonal antibody specificity to glycan antigens

ID	Glycan	Antibody															
		JTL-1	JTL-2	JTL-4	JTL-5	JTL-6	Anti-A (Z2B-1)	Anti-B (89-F)	Anti-A (TH-1)	Anti-H (89/8)	Anti-H (86-N)	Anti-A (AH-21)	Anti-H (A46-B/B10)	Lewis a (T174)	Lewis b (LWB01)	Lewis x (MEM-158)	Lewis y (F3)
1	A Type I						+					+					
2	A Type II			+	+	+	+										
3	A Type III	+	+				+		+								
4	A Type IV	+	+				+										
5	B Type I							+									
6	B Type II			+	+	+		+									
7	B Type III	+	+														
8	B Type IV		+					+									
9	H Type I												+				
10	H Type II																
11	H Type III	+	+														
12	H Type IV		+														
13	β-GlcNAc-R																
14	α-GalNAc-R																
15	β-GalNAc-R																
16	β-Gal-(1→3)-β -GlcNAc-R																
17	β-Gal-(1→4)-β-GlcNAc-R																
18	β-Gal-(1→3)-α-GalNAc-R																
19	β-Gal-(1→3)-β-GalNAc-R																

Table 3.2. Summary of monoclonal antibody specificity to glycan antigens

		JTL-1	JTL-2	JTL-4	JTL-5	JTL-6	Anti-A (Z2B-1)	Anti-B (89-F)	Anti-A (TH-1)	Anti-H (89/8)	Anti-H (86-N)	Anti-A (AH-21)	Anti-H (A46-B/B10)	Lewis a (T174)	Lewis b (LWB01)	Lewis x (MEM-158)	Lewis y (F3)
20	H disaccharide									+	+						
21	Le^a													+			
22	Le^b												+		+		
23	Le^x															+	
24	Le^y									+	+						+
25	A trisaccharide			+	+		+					+					
26	B trisaccharide							+									
27	α-Gal																

A Type I: α-GalNAc-(1→3)-α-Fuc-(1→2)-β-Gal-(1→3)-β-GlcNAc-R; **A Type II**: α-GalNAc-(1→3)-α-Fuc-(1→2)-β-Gal-(1→4)-β-GlcNAc-R; A Type III: α-GalNAc-(1→3)-α-Fuc-(1→2)-β-Gal-(1→3)-α-GalNAc-R; **A Type IV**: α-GalNAc-(1→3)-α-Fuc-(1→2)-β-Gal-(1→3)-β-GalNAc-R; **B Type I**: α-Gal-(1→3)-[α-Fuc(1→2)]-β-Gal-(1→3)-β-GlcNAc-R; **B Type II**: α-Gal-(1→3)-[α-Fuc-(1→2)]-β-Gal-(1→4)-β-GlcNAc-R; **B Type III**: α-Gal-(1→3)-[α-Fuc-(1→2)]-β-Gal-(1→3)-α-GalNAc-R; **B Type IV**: α-Gal-(1→3)-[α-Fuc-(1→2)]-β-Gal-(1→3)-β-GalNAc-R; **H Type I**: α-Fuc-(1→2)-β-Gal-(1→3)-β-GlcNAc-R; **H Type II**: α-Fuc-(1→2)-β-Gal-(1→4)-β-GlcNAc-R; **H Type III**: α-Fuc-(1→2)-β-Gal-(1→3)-α-GalNAc-R; **H Type IV**: α-Fuc-(1→2)-β-Gal-(1→3)-β-GalNAc-R; **H-disaccharide**: α-Fuc-(1→2)-β-Gal-R; **Le^a**: β-Gal-(1→3)-[α-Fuc(1→4)]-β-GlcNAc-R; **Le^b**: α-Fuc-(1→2)-β-Gal-(1→3)-[α-Fuc(1→4)]-β-GlcNAc-R; **Le^x**: β-Gal-(1→4)-[α-Fuc-(1→3)]-β-GlcNAc-R; **Le^y**: α-Fuc-(1→2)-β-Gal-(1→4)-[α-Fuc-(1→3)]-β-GlcNAc-R; **A-trisaccharide**: α-GalNAc-(1→3)-α-Fuc-(1→2)-β-Gal-R; **B-trisaccharide**: α-Gal-(1→3)-[α-Fuc-(1→2)]-β-Gal-R; **α-Gal**: α-Gal-(1→3)-β-Gal-(1→4)-β-GlcNAc-R; Fuc: L-Fucose, Gal: D-Galactose, GalNAc: N-acetylgalactosamine, GlcNAc: N-acetylglucosamine, Le: Lewis, tri: Trisaccharide, R: Bovine serum albumin or Polyacrylamide

Table 3.3. Summary of ABH sub-types and related glycan expression.

Blood group	ABH precursor and related glycan								
	Type I	Type II	Type III/IV	Lewis a	Lewis b	Lewis x	Lewis y	Sialic acid	H disaccharide
<u>Erythrocytes</u>									
A_1	-	+	+	-	-	-	+/-	+	-
B	ND	+	-	-	-	-	+	+	-
O	-	ND	-	-	-	-	+	+	+
A_2	-	+	+	-	-	-	+	+	-
A_1B	-	+	+	-	-	-	+/-	+	-
A_2B	-	+	+	-	-	-	+	+	-
<u>Heart</u>									
A_1	-	+	-	-	-	-	-	-	-
B	ND	+	-	-	-	-	-	-	-
O	-	ND	-	-	-	-	+	-	+/-
A_2	-	+	-	-	-	-	-	-	-
A_1B	-	+	-	-	-	-	-	-	-
A_2B	-	+	-	-	-	-	-	-	-
<u>Spleen (blood vessels)</u>									
A_1	-	+	-	-	-	-	-	-	-
B	ND	+	-	-	-	-	-	-	-
O	-	ND	-	-	-	-	+	-	+/-
A_1B	-	+	-	-	-	-	-	-	-

(+) positive; (-) negative; (+/-) weakly positive; ND not done

3.7 Figures

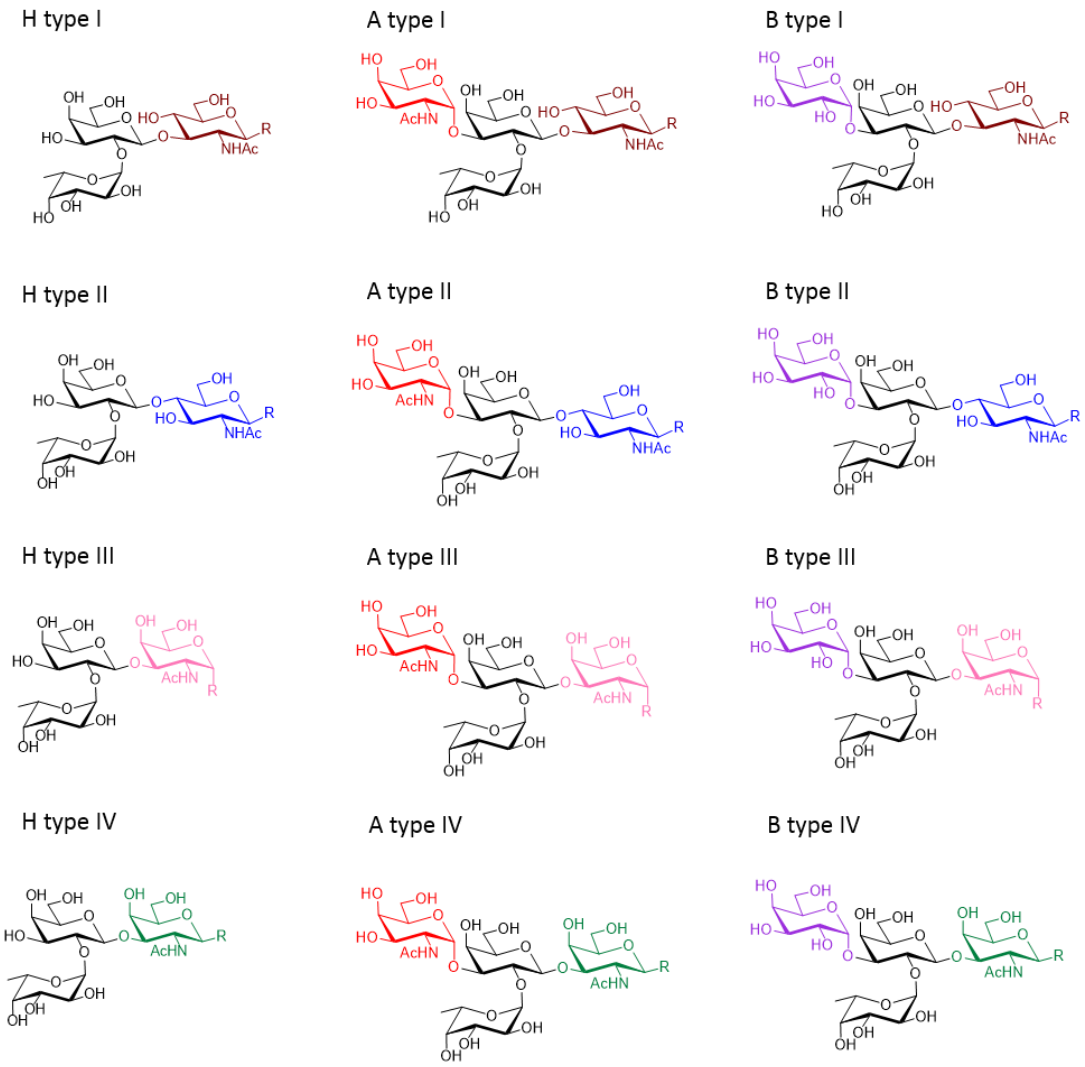
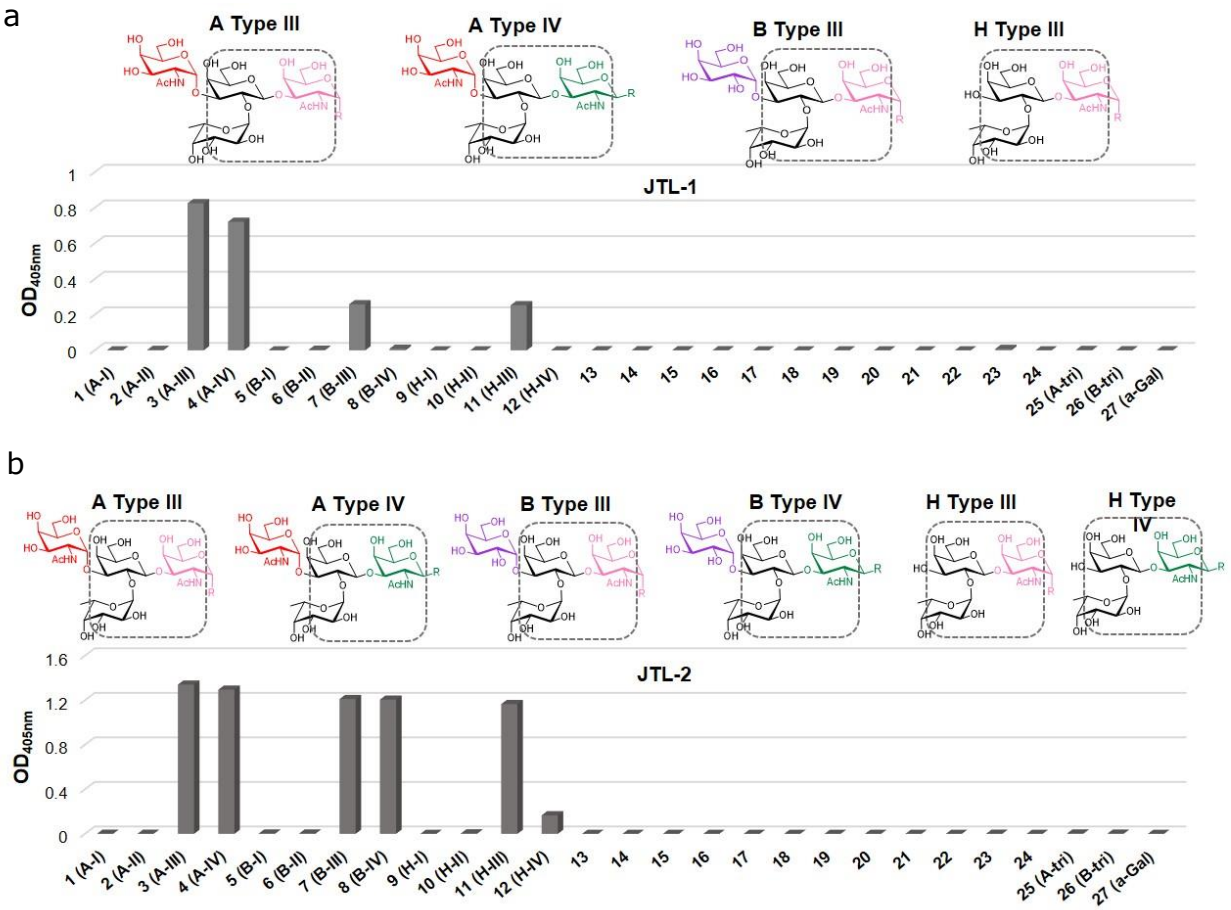


Figure 3.1. Chemical structures of blood group ABH type I-IV determinants. Common structures are depicted in the same color. R, glycoprotein or glycolipid; AcHN/NHAc, N-acetylaminogroup.


a
A Type III
A Type IV
B Type III
H Type III
JTL-1
OD405nm
1
0.8
0.6
0.4
0.2
0
1 (A-I)
2 (A-II)
3 (A-III)
4 (A-IV)
5 (B-I)
6 (B-II)
7 (B-III)
8 (B-IV)
9 (H-I)
10 (H-II)
11 (H-III)
12 (H-IV)
13
14
15
16
17
18
19
20
21
22
23
24
25 (A-tri)
26 (B-tri)
27 (a-Gal)
b
A Type III
A Type IV
B Type III
B Type IV
H Type III
H Type IV
JTL-2
OD405nm
1.6
1.2
0.8
0.4
0
1 (A-I)
2 (A-II)
3 (A-III)
4 (A-IV)
5 (B-I)
6 (B-II)
7 (B-III)
8 (B-IV)
9 (H-I)
10 (H-II)
11 (H-III)
12 (H-IV)
13
14
15
16
17
18
19
20
21
22
23
24
25 (A-tri)
26 (B-tri)
27 (a-Gal)

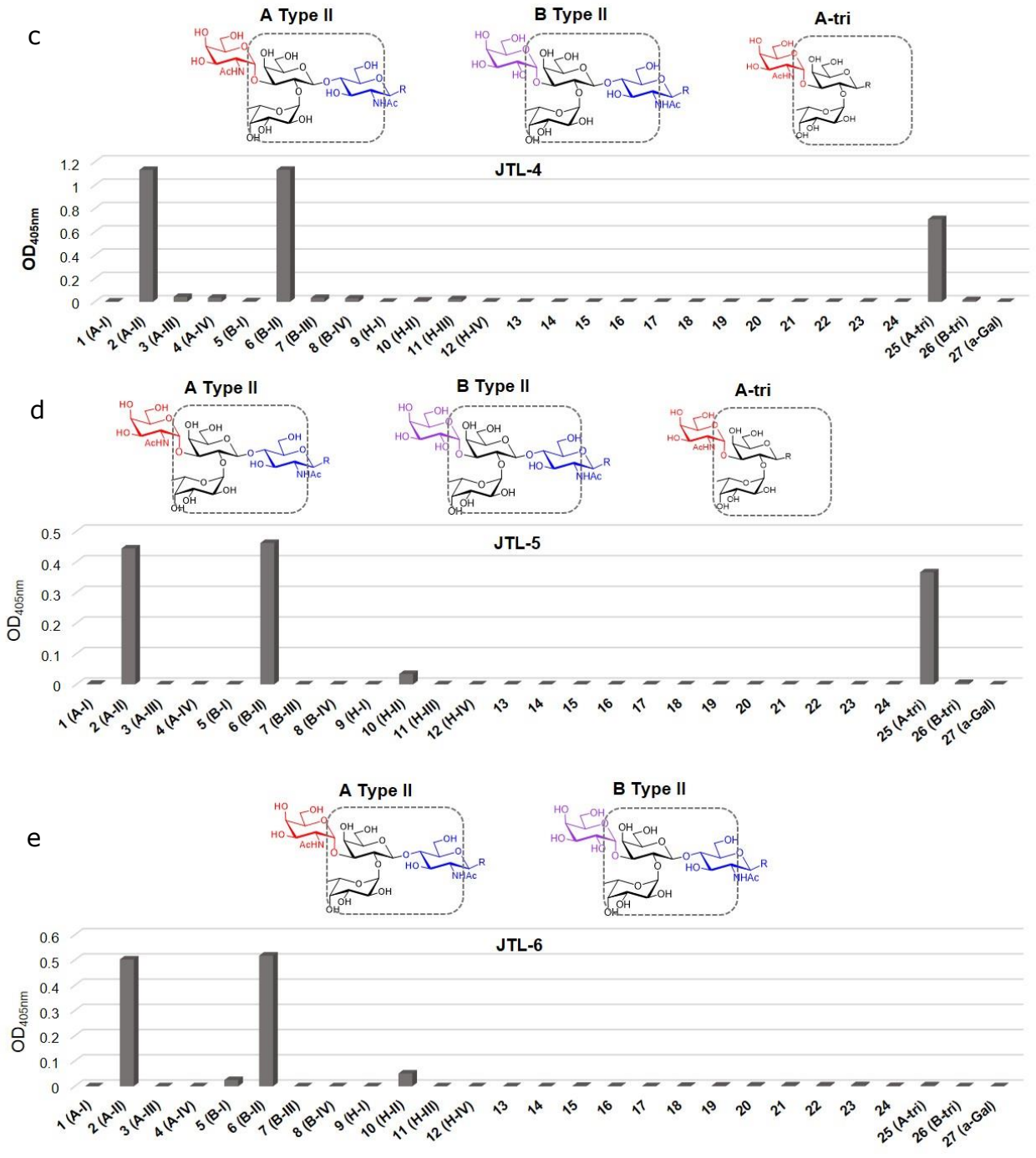
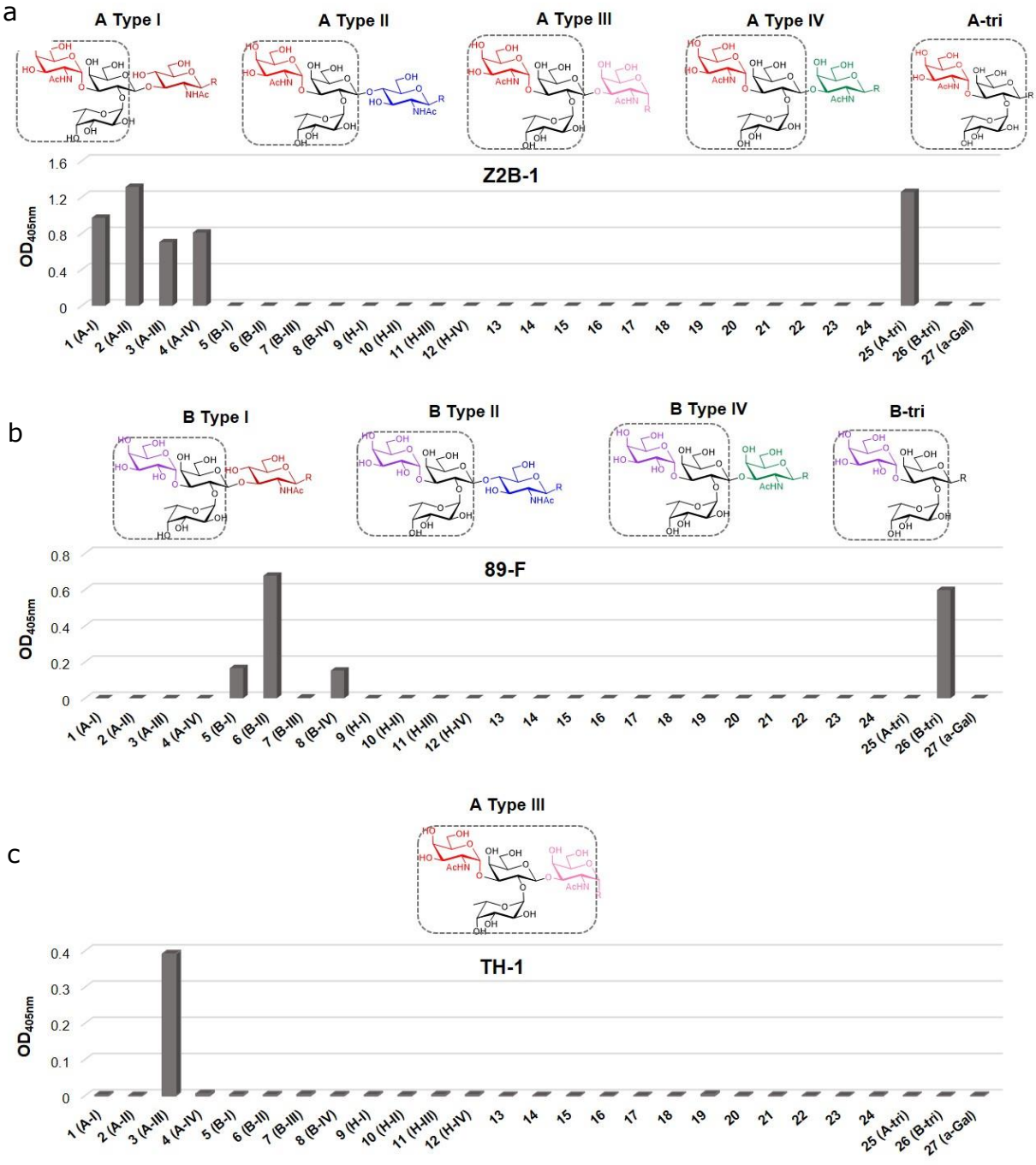
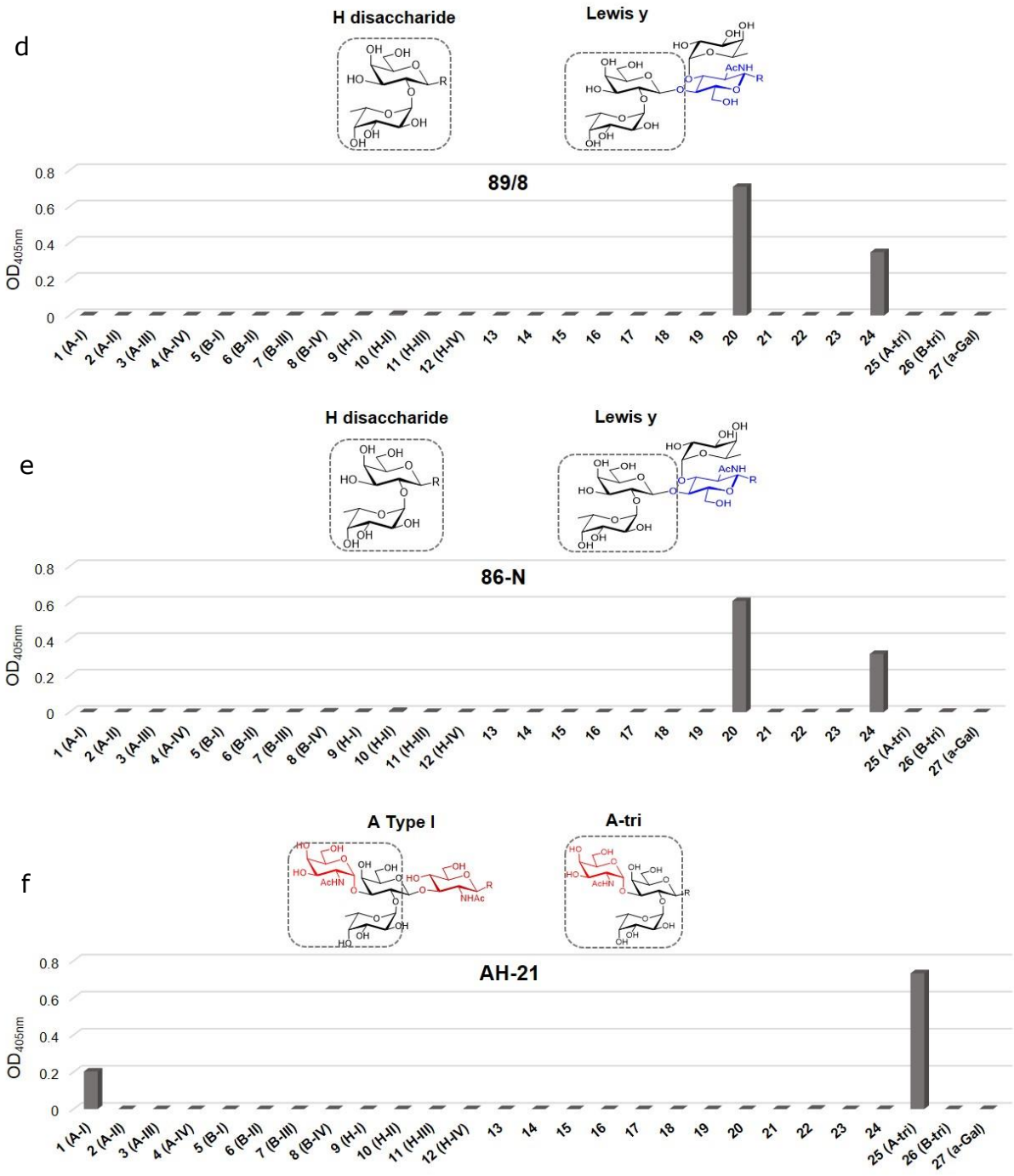


Figure 3.2. Monoclonal antibody specificity to blood group ABH and related antigens. Antigen-specific binding of monoclonal antibodies JTL-1 (**a**), JTL-2 (**b**), JTL-4 (**c**), JTL-5 (**d**) and JTL-6 (**e**) to Type-I-IV histo-blood group ABH and related antigens are shown. ELISA results are presented as background-subtracted optical densities at 405 nm (OD value for

antigen conjugate – OD value for PAA or BSA) versus glycan ID (Table 3.1, 3.2). Chemical structures of all antigens recognized by each monoclonal antibody are shown; potential epitopes or common structures between antigens recognized by the monoclonal antibody are highlighted (dotted line).




d
H disaccharide
Lewis y
89/8
OD405nm
0.8
0.6
0.4
0.2
0
1 (A-I)
2 (A-II)
3 (A-III)
4 (A-IV)
5 (B-I)
6 (B-II)
7 (B-III)
8 (B-IV)
9 (H-I)
10 (H-II)
11 (H-III)
12 (H-IV)
13
14
15
16
17
18
19
20
21
22
23
24
25 (A-tri)
26 (B-tri)
27 (a-Gal)
e
H disaccharide
Lewis y
86-N
OD405nm
0.8
0.6
0.4
0.2
0
1 (A-I)
2 (A-II)
3 (A-III)
4 (A-IV)
5 (B-I)
6 (B-II)
7 (B-III)
8 (B-IV)
9 (H-I)
10 (H-II)
11 (H-III)
12 (H-IV)
13
14
15
16
17
18
19
20
21
22
23
24
25 (A-tri)
26 (B-tri)
27 (a-Gal)
f
A Type I
A-tri
AH-21
OD405nm
0.8
0.6
0.4
0.2
0
1 (A-I)
2 (A-II)
3 (A-III)
4 (A-IV)
5 (B-I)
6 (B-II)
7 (B-III)
8 (B-IV)
9 (H-I)
10 (H-II)
11 (H-III)
12 (H-IV)
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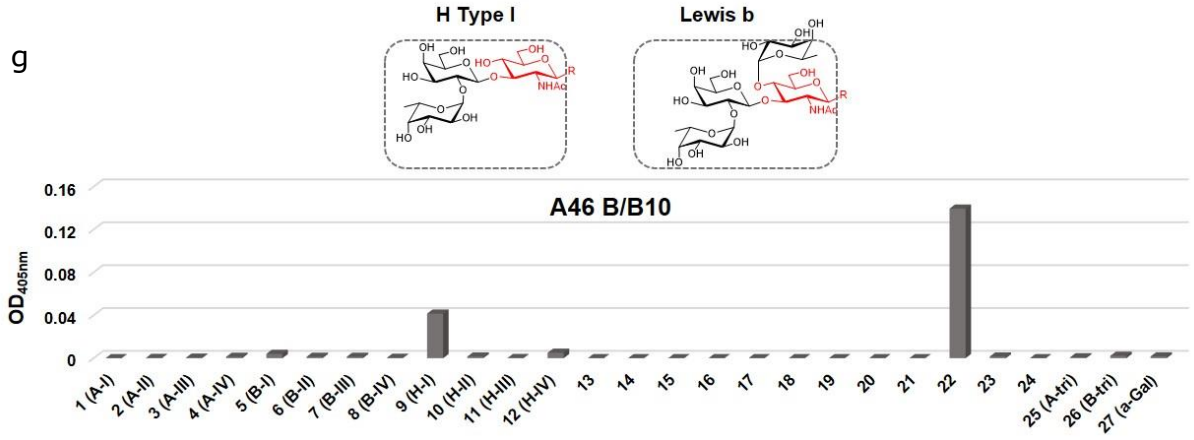
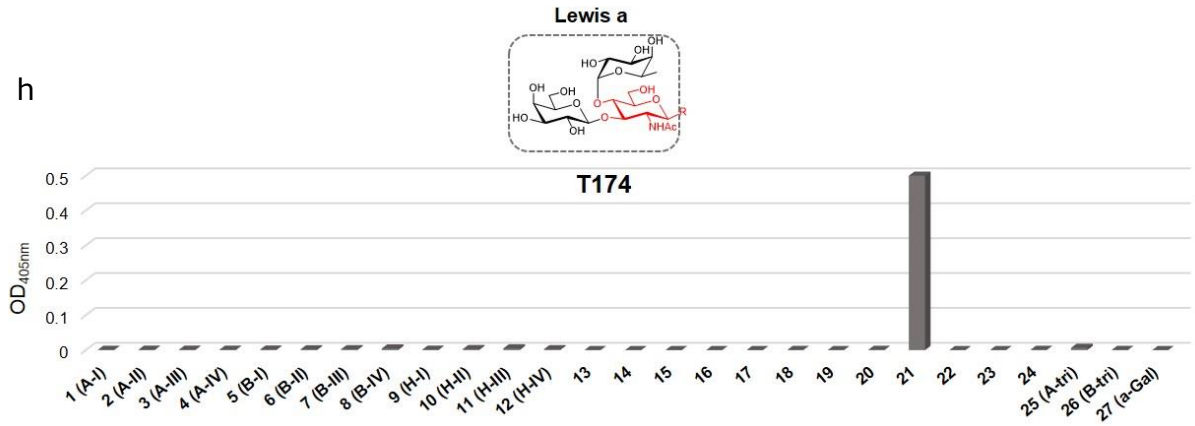
24
25 (A-tri)
26 (B-tri)
27 (a-Gal)

g


H Type I
Lewis b
A46 B/B10
OD405nm
0.16
0.12
0.08
0.04
0
1 (A-I)
2 (A-II)
3 (A-III)
4 (A-IV)
5 (B-I)
6 (B-II)
7 (B-III)
8 (B-IV)
9 (H-I)
10 (H-II)
11 (H-III)
12 (H-IV)
13
14
15
16
17
18
19
20
21
22
23
24
25 (A-tri)
26 (B-tri)
27 (a-Gal)

h


Lewis a
T174
OD405nm
0.5
0.4
0.3
0.2
0.1
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4 (A-IV)
5 (B-I)
6 (B-II)
7 (B-III)
8 (B-IV)
9 (H-I)
10 (H-II)
11 (H-III)
12 (H-IV)
13
14
15
16
17
18
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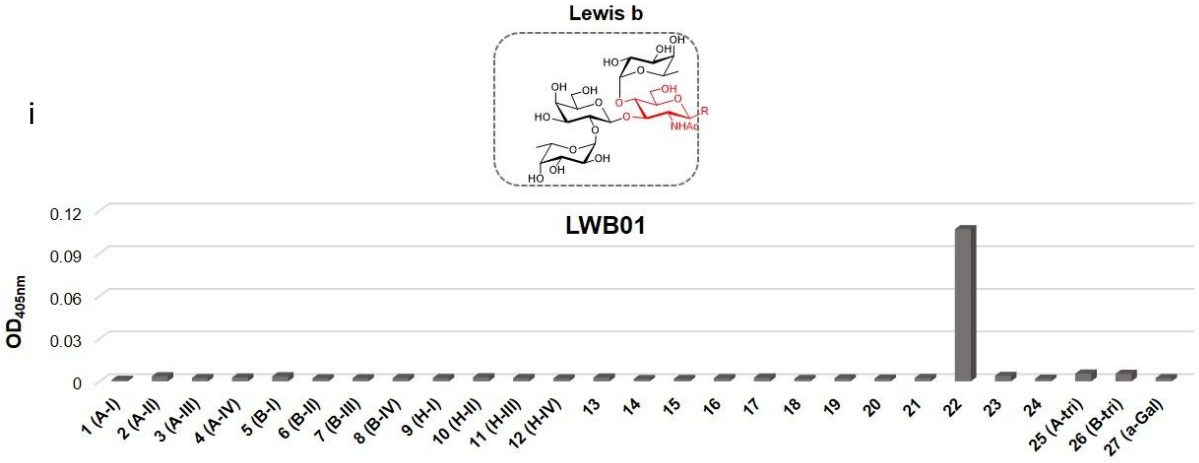
21
22
23
24
25 (A-tri)
26 (B-tri)
27 (a-Gal)

i


Lewis b
LWB01
OD405nm
0.12
0.09
0.06
0.03
0
1 (A-I)
2 (A-II)
3 (A-III)
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5 (B-I)
6 (B-II)
7 (B-III)
8 (B-IV)
9 (H-I)
10 (H-II)
11 (H-III)
12 (H-IV)
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25 (A-tri)
26 (B-tri)
27 (a-Gal)

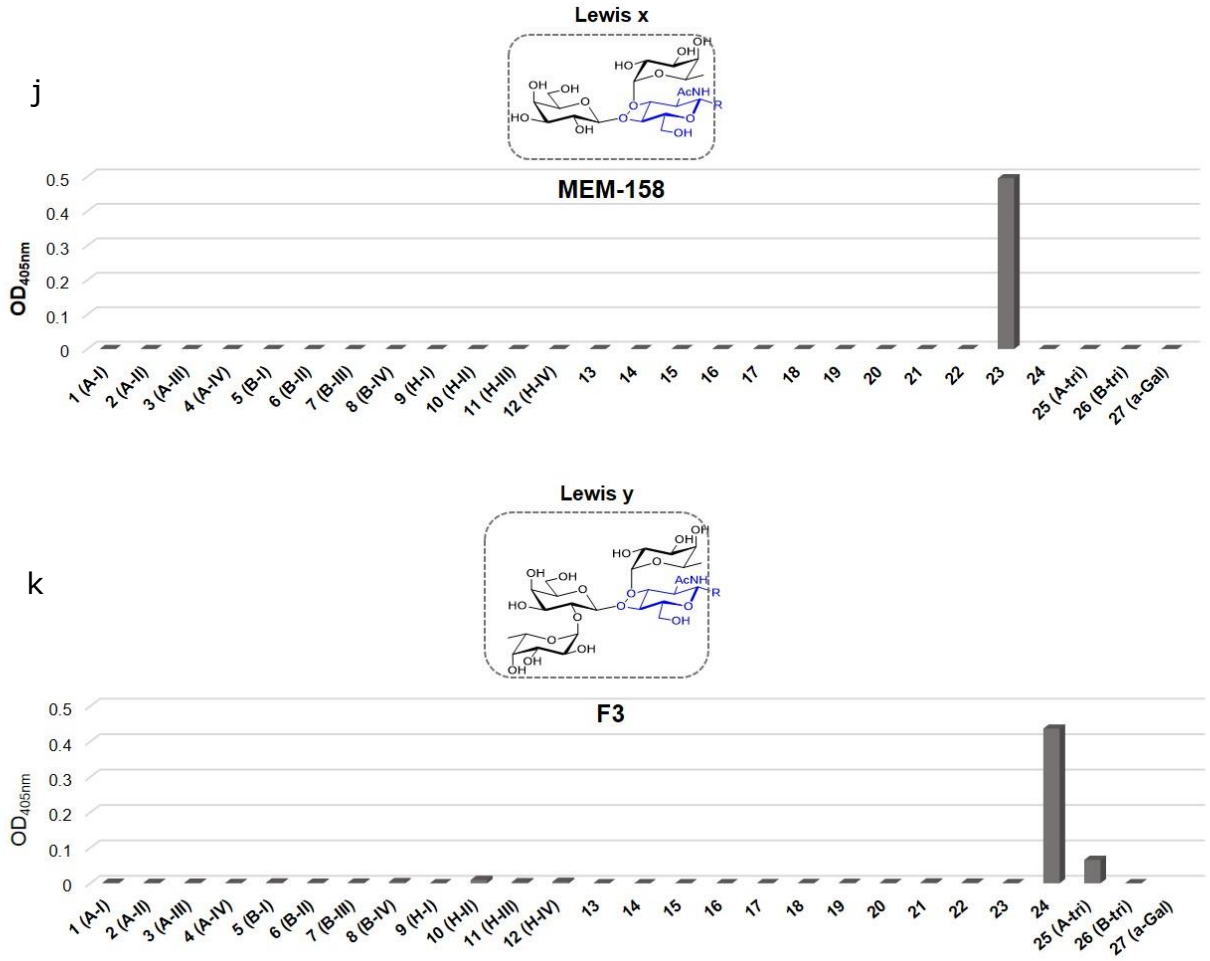


Figure 3.3. Monoclonal antibody specificity to blood group ABH and related antigens. (**a-k**) Antigen-specific binding of monoclonal antibodies to Type-I-IV histo-blood group ABH antigens and related glycan structures are shown. ELISA results are presented as background-subtracted optical densities at 405 nm (OD value for antigen conjugate – OD value for PAA or BSA) versus glycan ID (Table 3.1, 3.2). Chemical structures of all antigens recognized by each monoclonal antibody are shown; potential epitopes or common structures between antigens recognized by the monoclonal antibody are highlighted (dotted line).

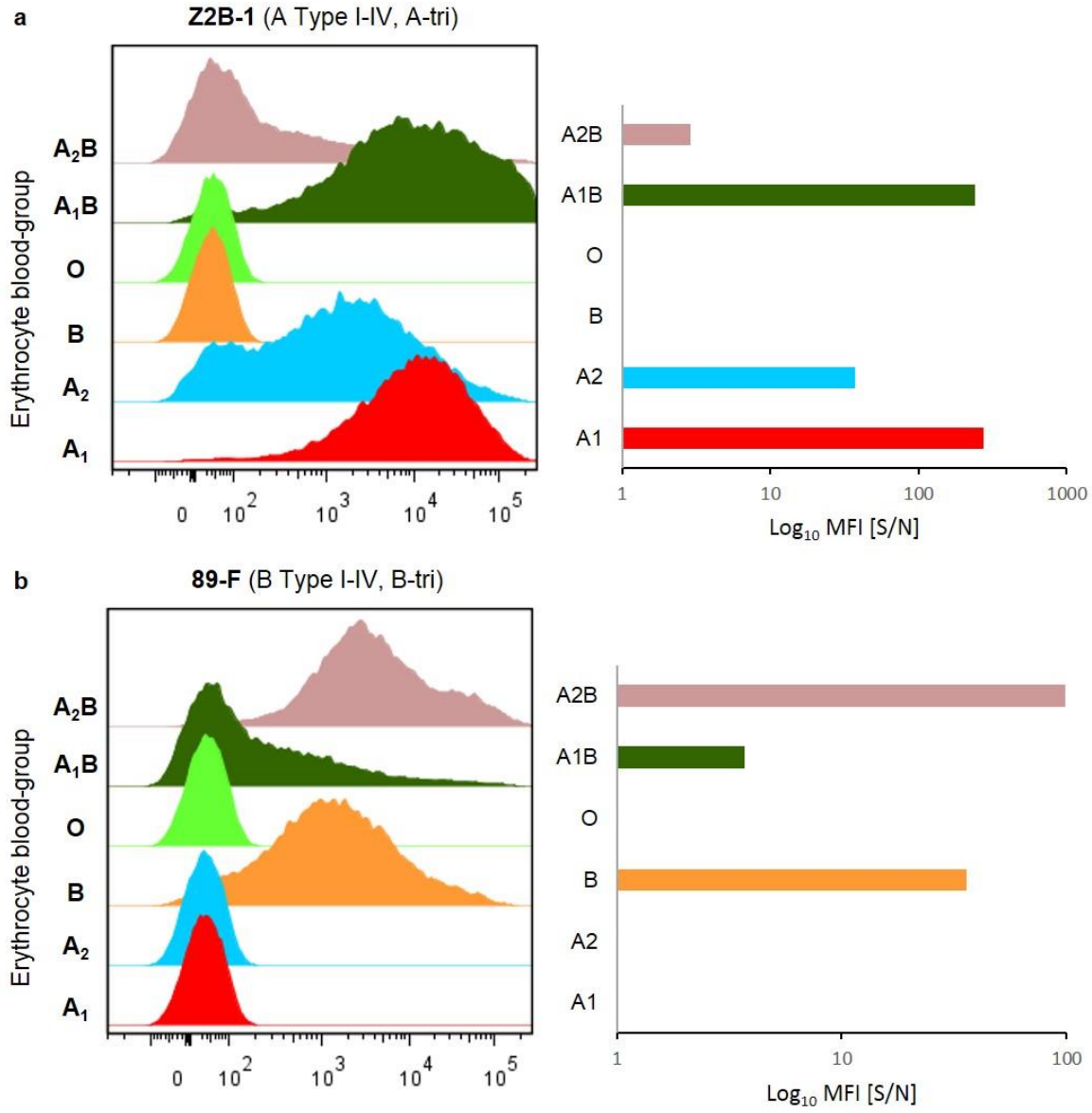


Figure 3.4. Comparison of blood group A and B antigen expression on erythrocytes of different blood groups. Erythrocytes from individuals of different blood groups (A_1, A_2, B, O, A_1B and A_2B) were analyzed for expression of (**a**) blood group-A antigens using anti-A/Z2B-1 and (**b**) blood group-B antigens using anti-B/89-F monoclonal antibodies. Fluorescent activated cell sorting (FACS) data are presented as overlaid histograms showing relative expression of A and B antigens on erythrocytes. Median fluorescent intensities (MFI) are presented as signal/noise ratio (MFI anti-A/B antibody/MFI isotype control antibody).

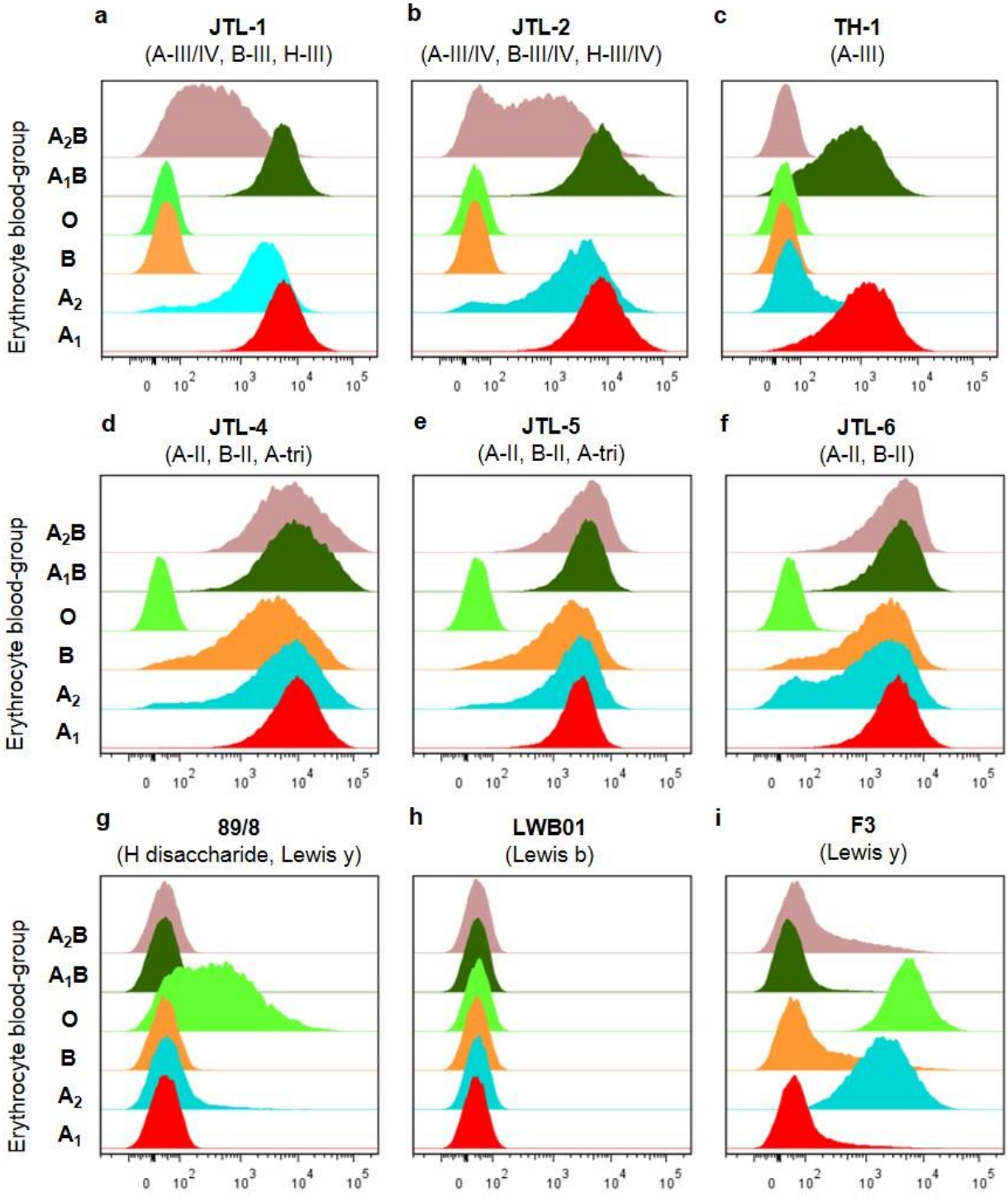
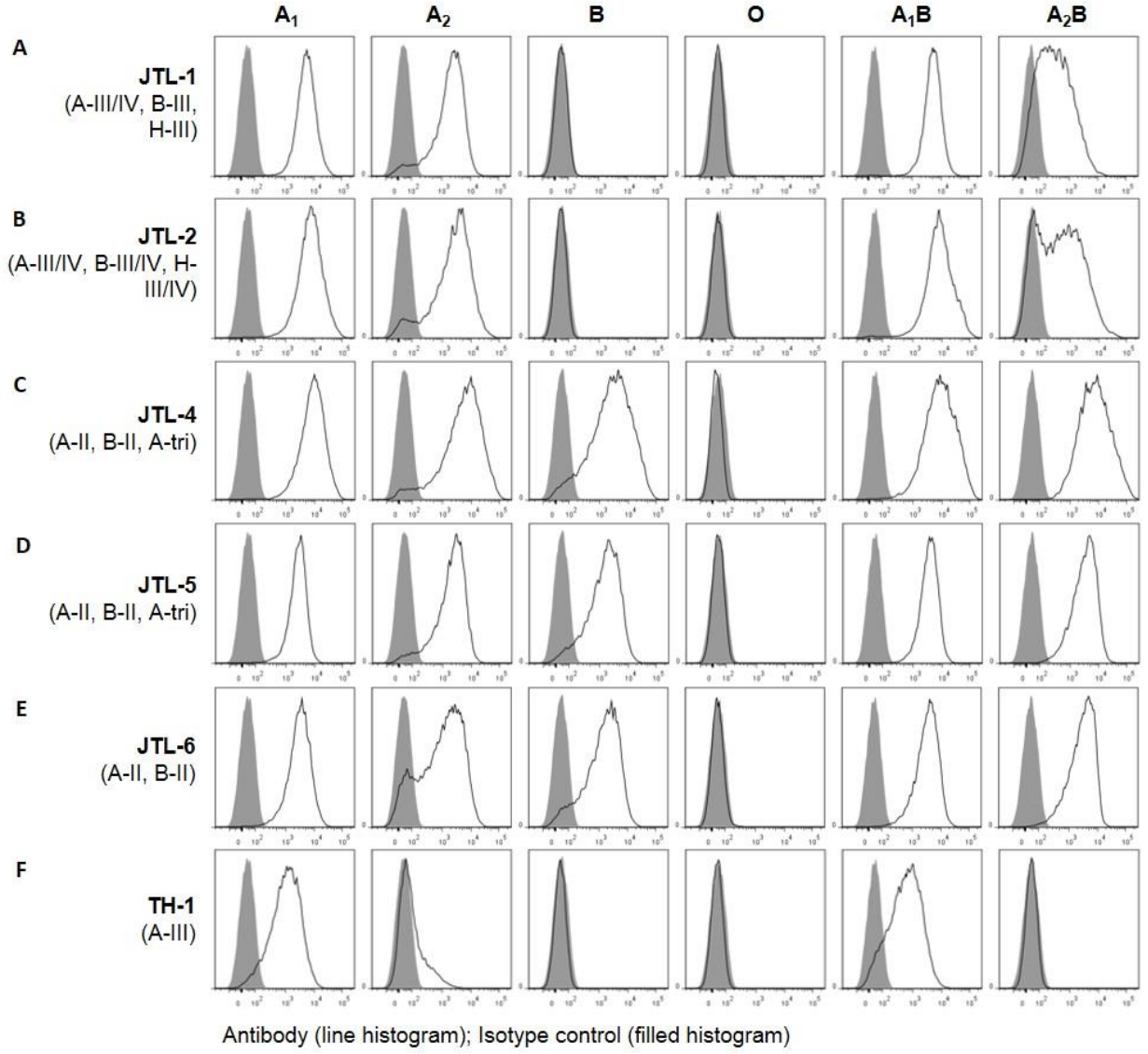
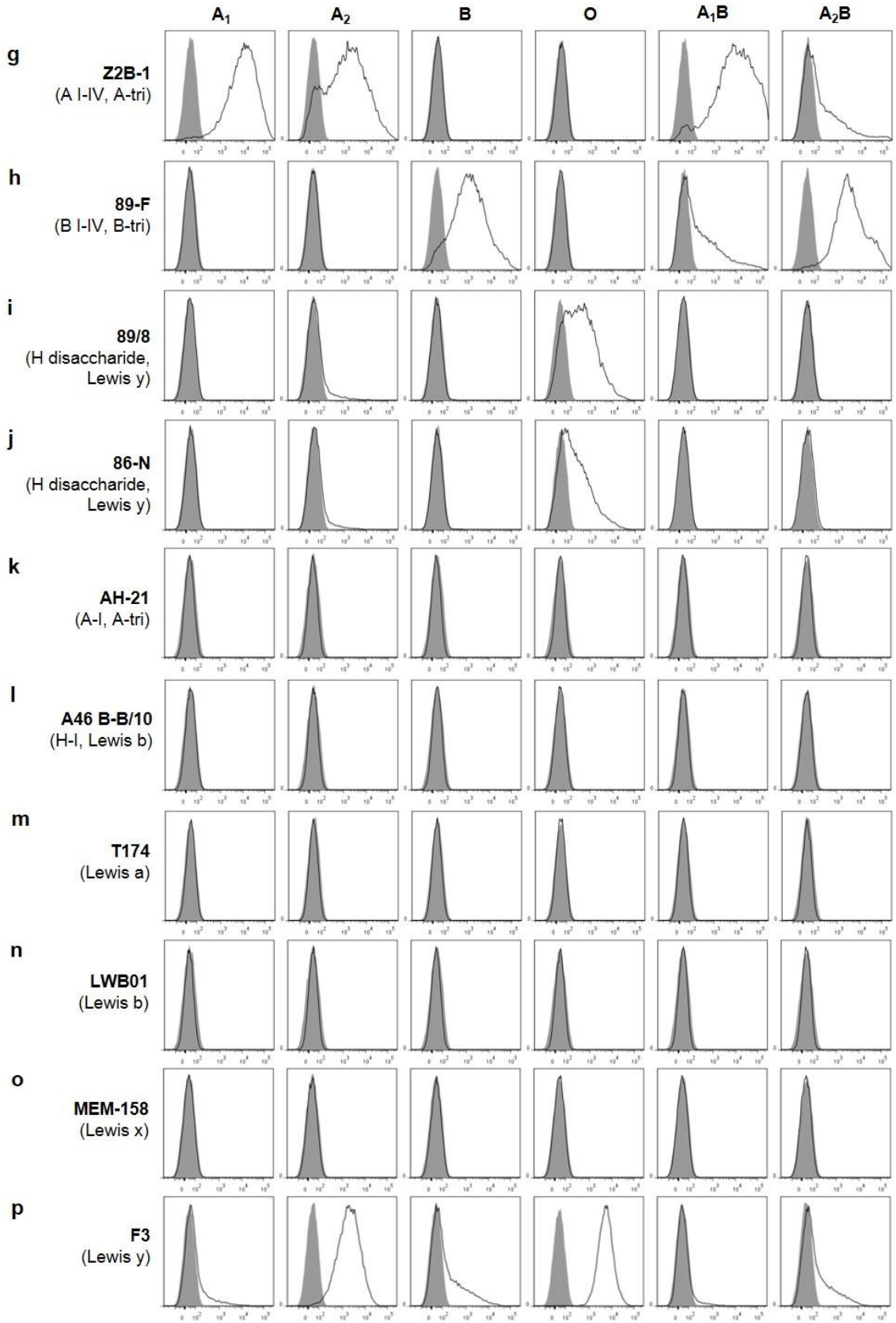


Figure 3.5. Characterization of ABH Type-I-IV expression on erythrocytes of different blood groups. Differential expression of Type-I-IV ABH precursor structures on erythrocytes from different blood groups (A_1, A_2, B, O, A_1B and A_2B) as detected by JTL-1 (**a**)

and JTL-2 (**b**). (**c**) Expression of Type-III precursor structures glycosylated with A antigen is detected with TH-1 antibody. Expression of Type-II precursor structures is detected using JTL-4 (**d**), JTL-5 (**e**) and JTL-6 (**f**). Characterization of blood group H antigen expression using antibodies recognizing H disaccharide (**g**), fucosylated Type-I H/Lewis^{b} (**h**) and fucosylated Type-II H/Lewis^{y} (**i**) antigens.





Antibody (line histogram); Isotype control (filled histogram)

Figure 3.6. Characterization of ABH Type-I-IV expression on erythrocytes of different blood groups. (**a-p**) Differential expression of Type-I-IV ABH and related blood group structures on erythrocytes from individuals of different blood groups (A_1, A_2, B, O, A_1B and A_2B). Flow cytometry data are presented as histograms for A_1, A_2, B, O, A_1B and A_2B erythrocytes stained with monoclonal antibody (line histogram) and isotype-control antibody (filled histogram).

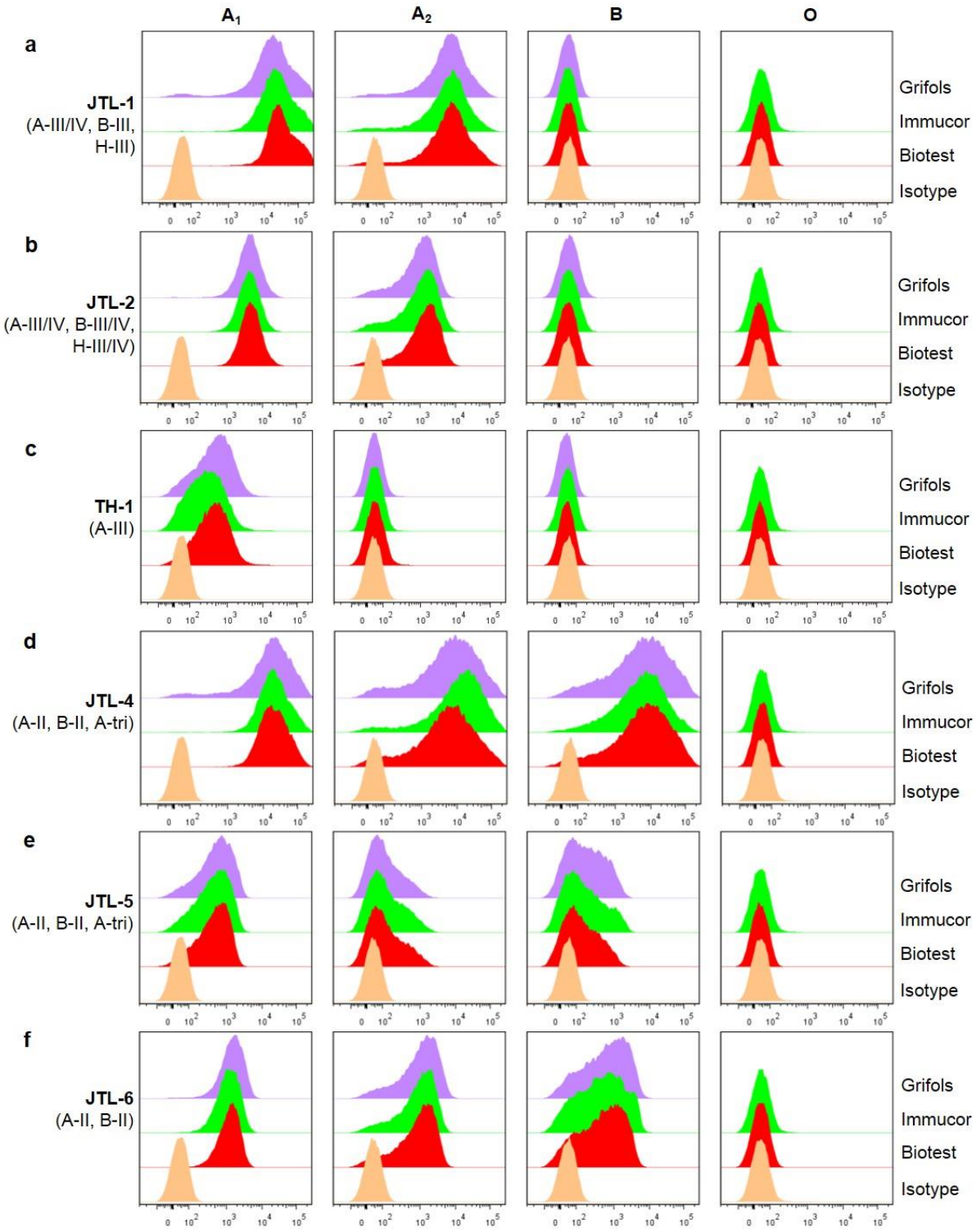


Figure 3.7. Expression of ABH Type-I-IV antigens on reagent erythrocytes from different suppliers. (**a-f**) Comparison of Type-I-IV ABH structures on reagent erythrocytes

from three different suppliers (Biotest, Immucor, and Grifols). Flow cytometry data are presented as overlaid histograms, including isotype control antibody stained erythrocytes from Immucor.

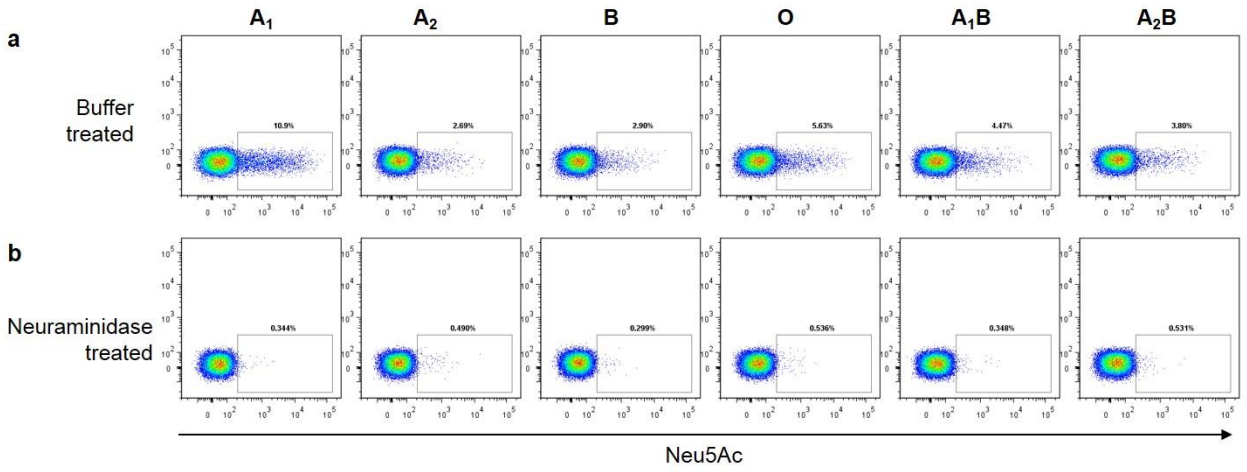


Figure 3.8. Sialidase treatment of erythrocytes. Removal of sialic acids on glycan structures by neuraminidase was confirmed by the expression of one of the sialic acids, Neu5Ac, on buffer-treated (**a**) versus neuraminidase-treated (**b**) erythrocytes from different blood groups A_1, A_2, B, O, A_1B and A_2B individuals.

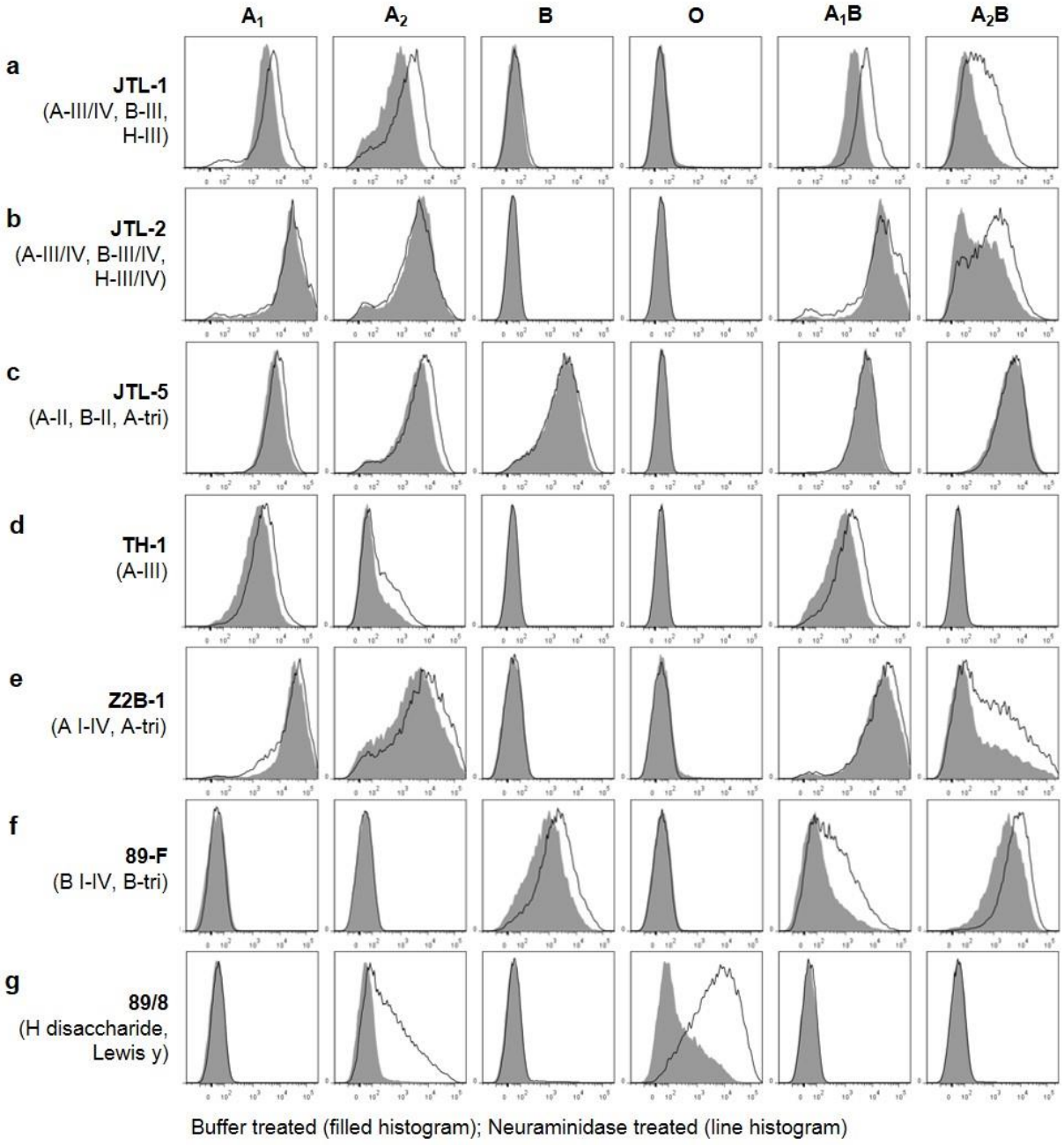


Figure 3.9. Blood group ABH antigen expression following sialidase treatment. (**a-g**) Expression of Type-I-IV ABH structures on neuraminidase-treated (line histogram) and buffer-treated (filled histogram) erythrocytes from blood groups A_1, A_2, B, O, A_1B and A_2B individuals.

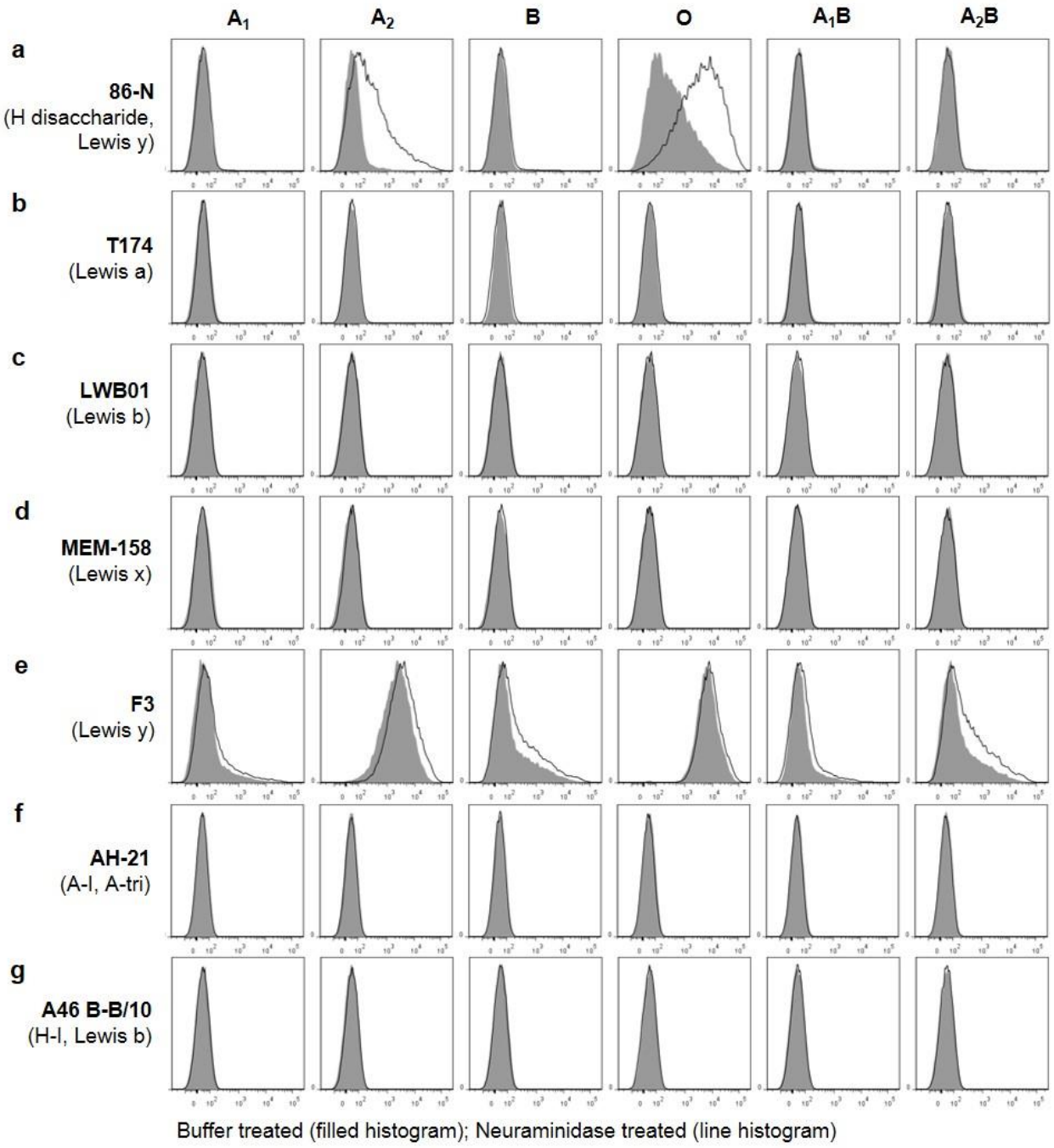


Figure 3.10. Blood group ABH antigen expression following sialidase treatment. (**a-g**) Expression of Type-I-IV ABH and related blood group structures on neuraminidase-treated (line histogram) and buffer-treated (filled histogram) erythrocytes from A_1, A_2, B, O, A_1B and A_2B individuals.

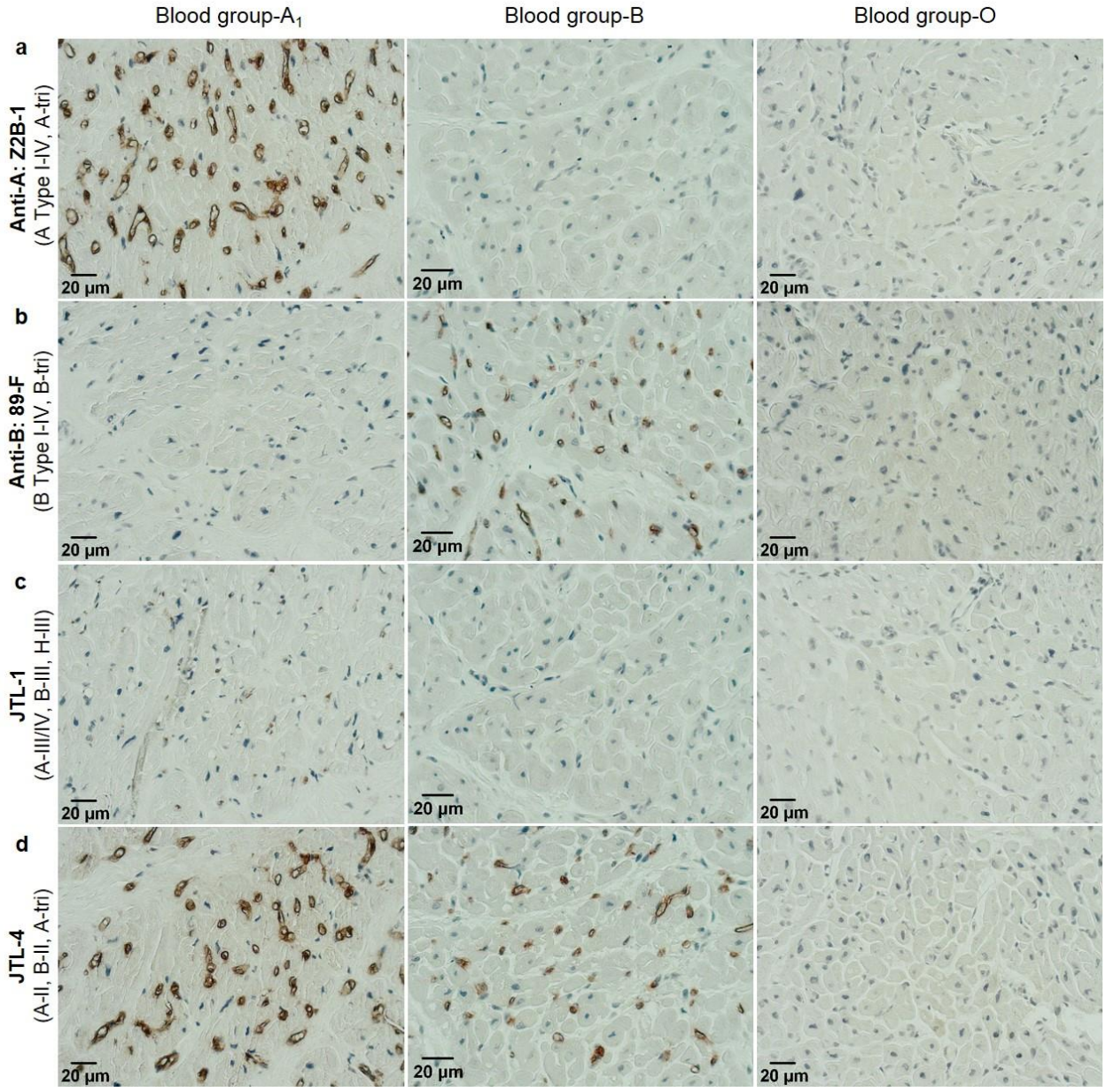


Figure 3.11. Blood group ABH antigen expression in cardiac tissues of different blood groups. Endomyocardial biopsies from blood group A_1, B, and O donors were stained with anti-A/Z2B-1 (**a**), anti-B/89-F (**b**), JTL-1 (**c**), and JTL-4 (**d**) antibodies. Data shown are representative immunohistochemistry images from blood group A_1 (n=12), B (n=9), and O (n=10) donors. Dark brown represents positive staining (e.g. positive staining of capillaries of A_1 biopsy with anti-A); Blue represents nuclear staining with hematoxylin.

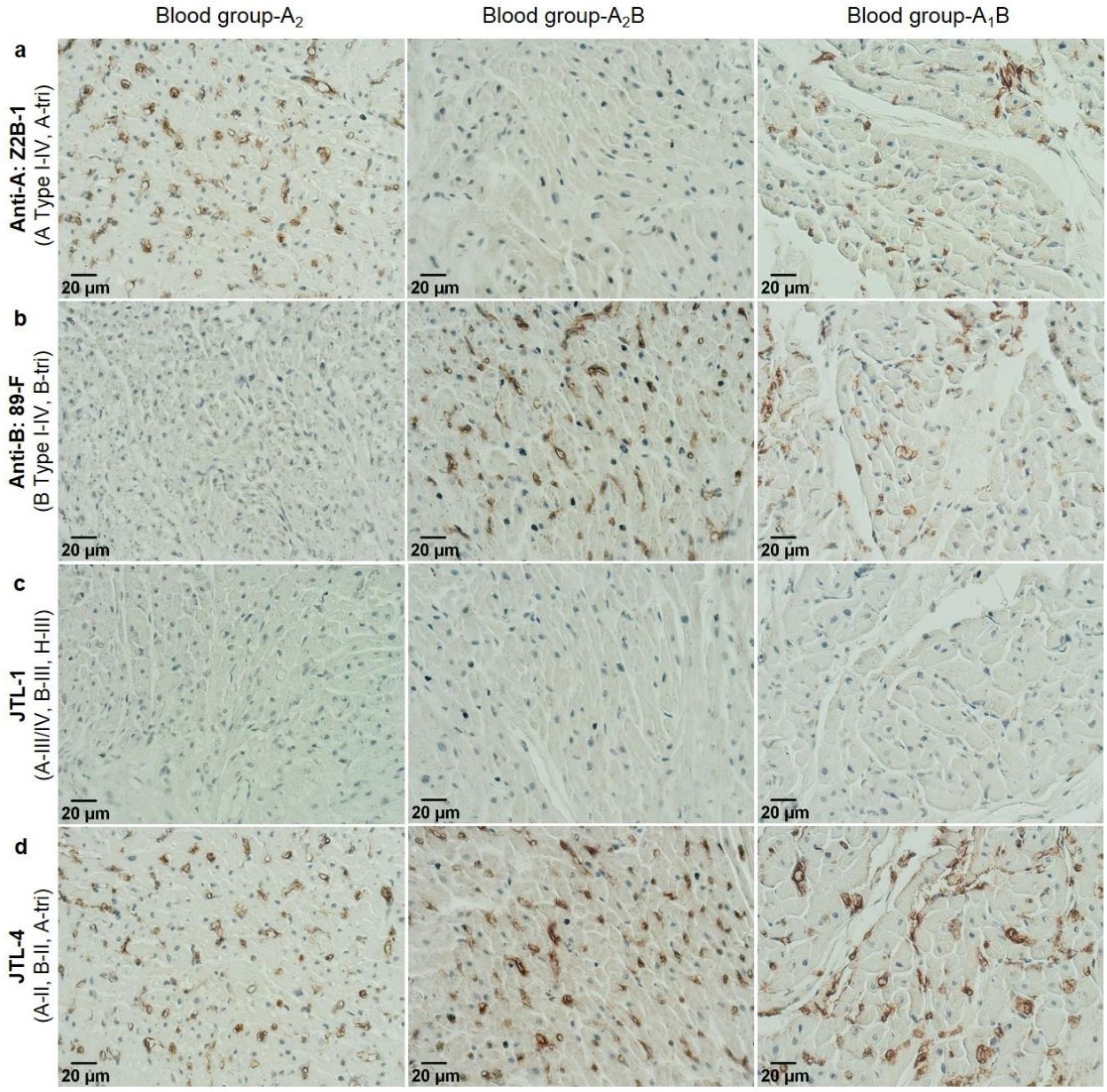


Figure 3.12. Blood group ABH antigen expression in cardiac tissues of different blood groups. Endomyocardial biopsies from blood group A_2, A_2B, and A_1B donors were stained with anti-A/Z2B-1 (**a**), anti-B/89-F (**b**), JTL-1 (**c**), and JTL-4 (**d**) antibodies. Data shown are representative immunohistochemistry images from blood group A_2 (n=5), A_2B (n=3), and A_1B (n=3) donors. Dark brown represents positive staining (e.g. positive staining of capillaries of A_2 biopsy with anti-A); Blue represents nuclear staining by hematoxylin.

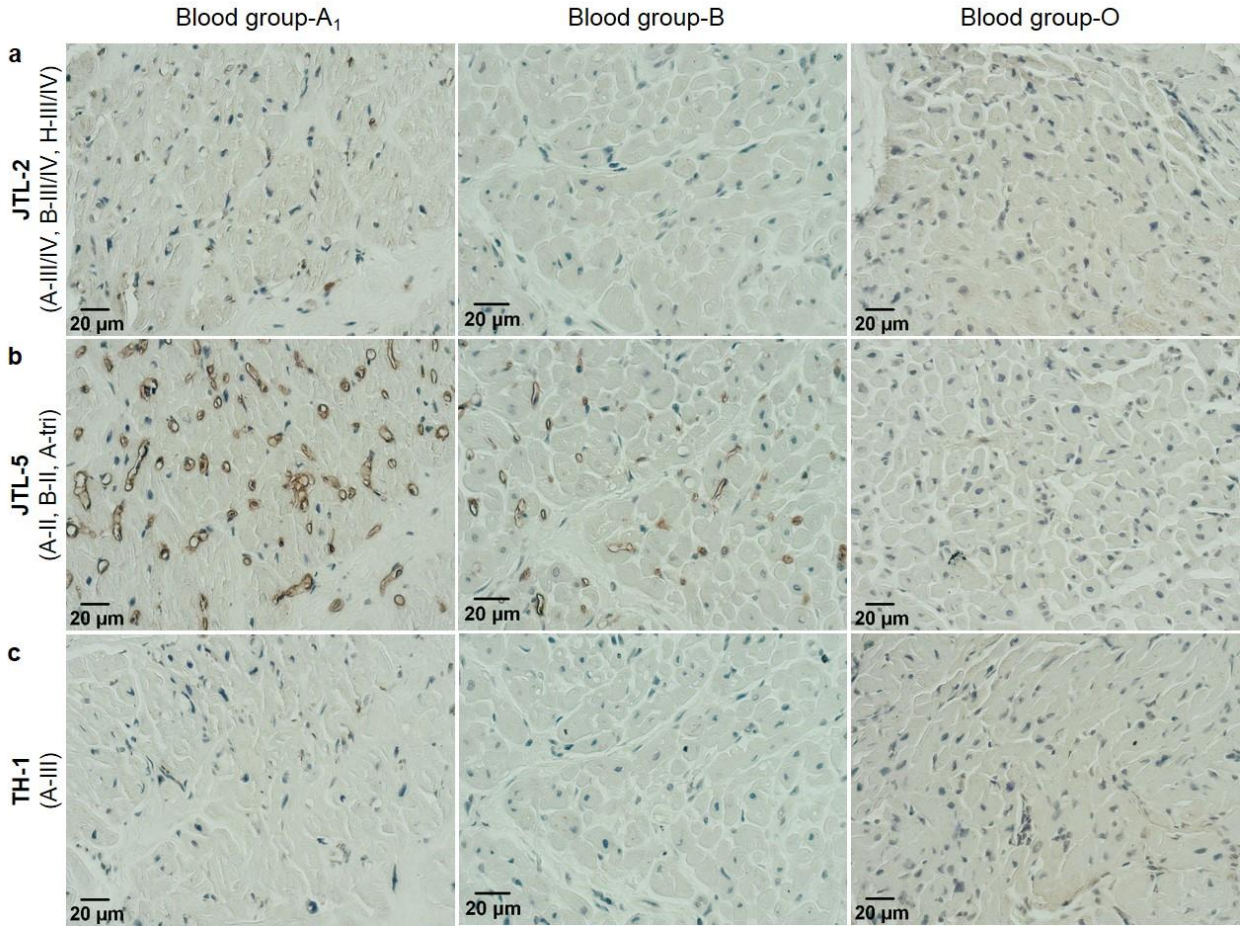


Figure 3.13. Blood group ABH antigen expression in cardiac tissues of different blood groups. Endomyocardial biopsies from blood group A_1, B, and O donors were stained with JTL-2 (**a**), JTL-5 (**b**), and TH-1 (**c**) antibodies. Data shown are representative immunohistochemistry images from blood group A_1 (n=12), B (n=9), and O (n=10) donors. Dark brown represents positive staining (e.g. positive staining of capillaries of A_1 biopsy with JTL-5); Blue represents nuclear staining by hematoxylin.

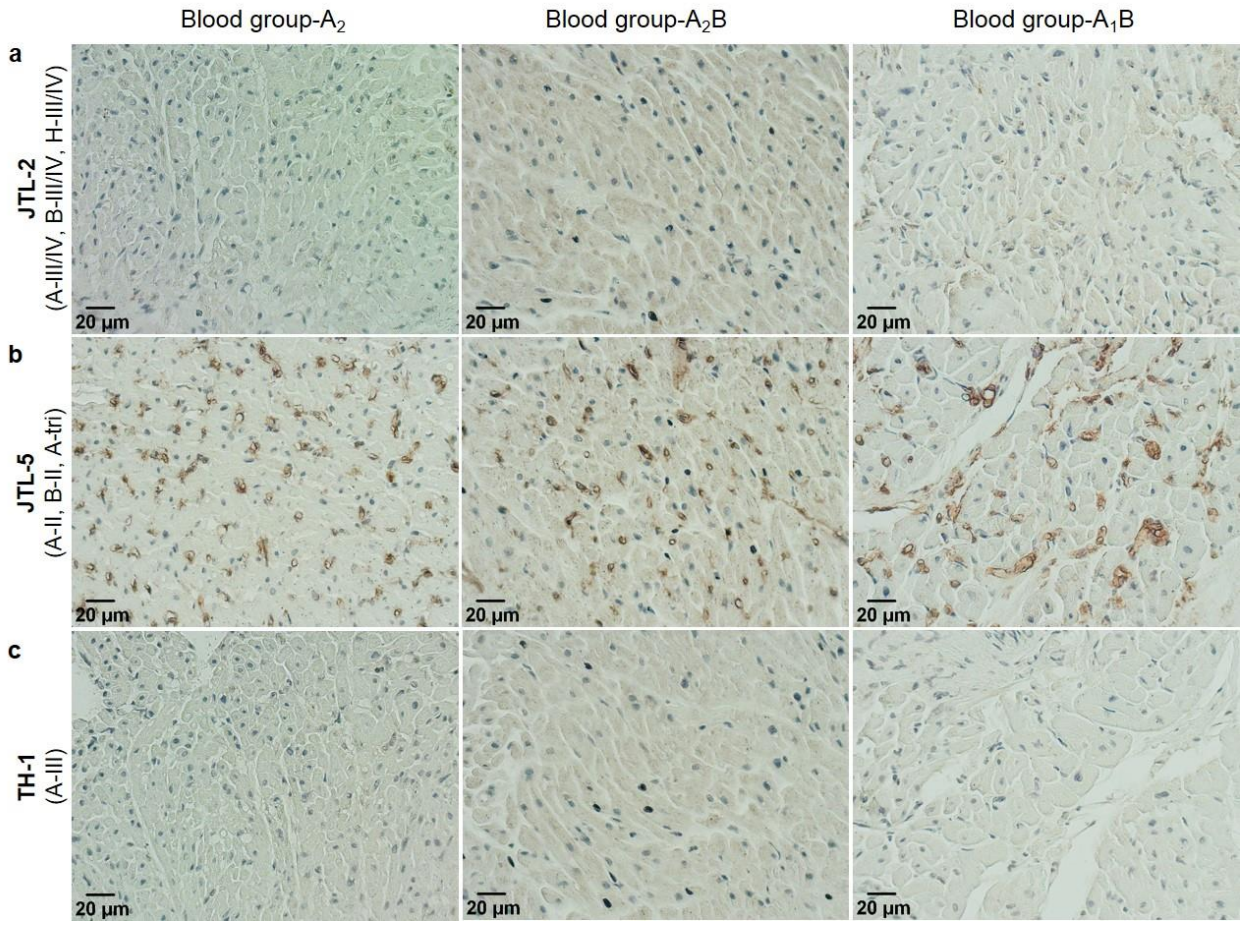


Figure 3.14. Blood group ABH antigen expression in cardiac tissues of different blood groups. Endomyocardial biopsies from blood group A_2, A_2B, and A_1B donors were stained with JTL-2 (**a**), JTL-5 (**b**), and TH-1 (**c**) antibodies. Data shown are representative immunohistochemistry images from blood group A_2 (n=5), A_2B (n=3), and A_1B (n=3) donors. Dark brown represents positive staining (e.g. positive staining of capillaries of A_2 biopsy with JTL-5); Blue represents nuclear staining by hematoxylin.

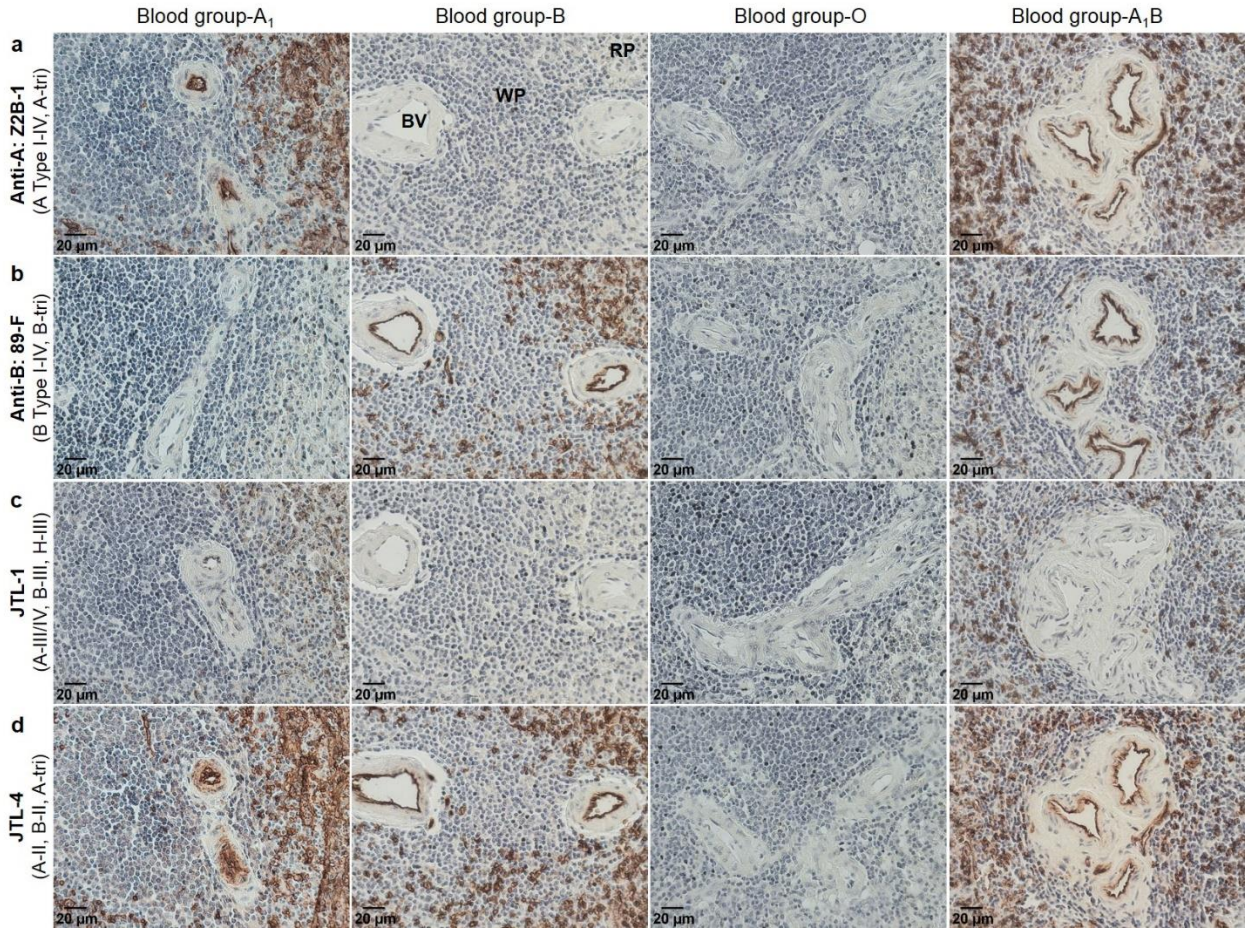


Figure 3.15. Blood group ABH antigen expression in spleen of different blood groups. Spleen tissues from blood group A_1, B, O, and A_1B donors were stained with anti-A/Z2B-1 (**a**), anti-B/89-F (**b**), JTL-1 (**c**), and JTL-4 (**d**) antibodies. Data shown are representative immunohistochemistry images from blood group A_1 (n=5), B (n=2), O (n=3), and A_1B (n=1) donors. Dark brown represents positive staining (e.g. positive staining of vascular endothelium and erythrocytes in the red pulp of blood group A_1 spleen with anti-A); Blue represents nuclear staining with hematoxylin. Only Type-II structures are seen in vascular endothelium as stained by JTL-4 (**d**); vascular endothelial cells are negative for Type-III/IV structures (**c**) but red cells are positive (as seen with flow cytometry in Figure 3.5) in A_1 and A_1B spleens. **BV** blood vessel; **WP** white pulp; **RP** red pulp.

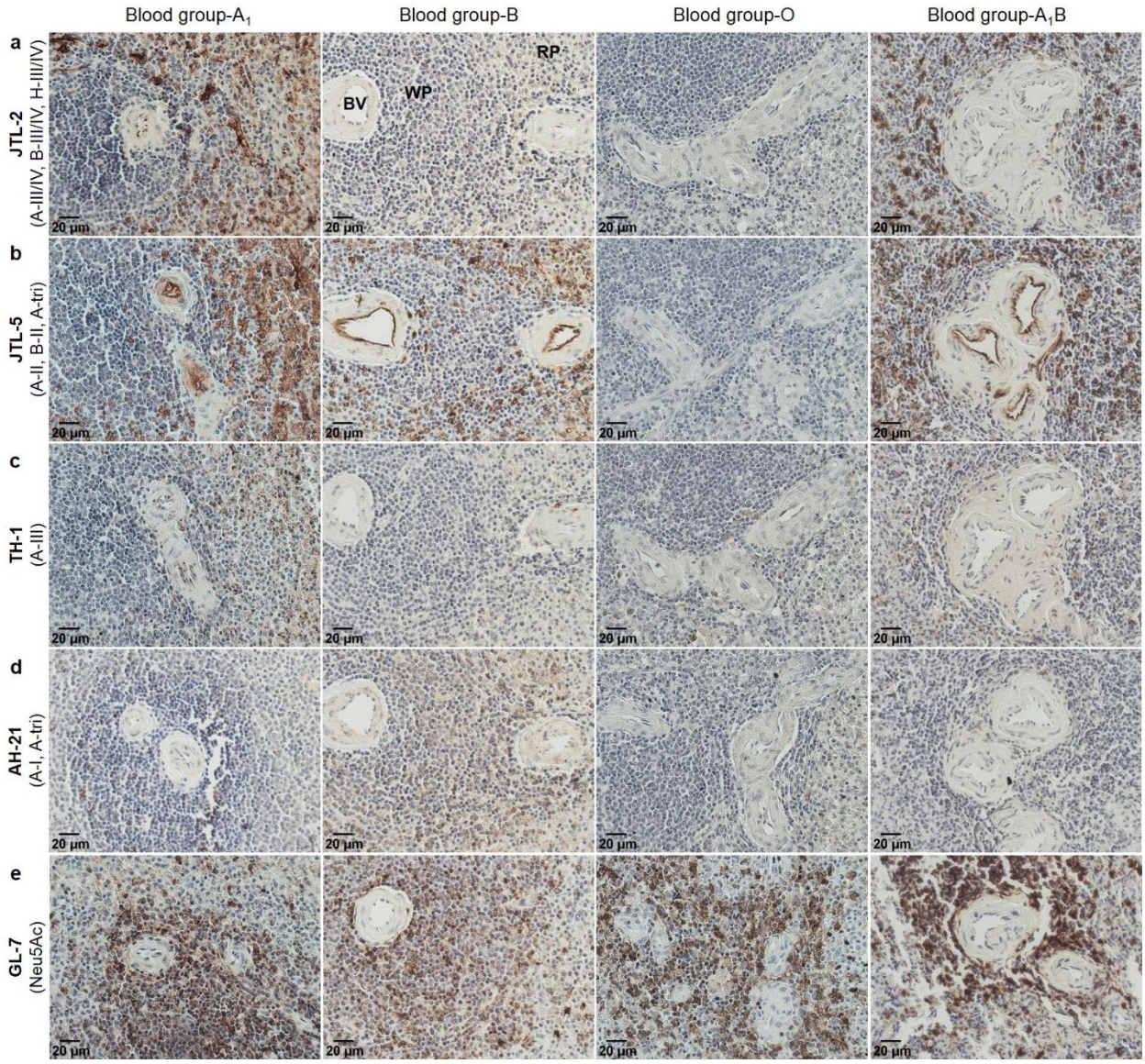


Figure 3.16. Blood group ABH antigen expression in spleen of different blood groups. Spleen tissues from blood group A_1, B, O, and A_1B donors were stained with JTL-2 (**a**), JTL-5 (**b**), TH-1 (**c**), AH-21 (**d**), and GL-7 (**e**) antibodies. Data shown are representative immunohistochemistry images from blood group A_1 (n=5), B (n=2), O (n=3), and A_1B (n=1) donors. Dark brown represents positive staining; Blue represents nuclear staining by hematoxylin. Only Type-II structures are seen in vascular endothelium as stained by JTL-5

(**b**); vascular endothelial cells are negative for Type-III/IV structures (**a, c**) but red cells are positive (as seen with flow cytometry in Figure 3.5) in A_1 and A_1B spleens. Similarly, positive staining of lymphocytes in white pulp with GL-7 (**e**) confirms the presence of sialic acid (Neu5Ac) on lymphocytes, not on vascular endothelium. **BV** blood vessel; **WP** white pulp; **RP** red pulp.

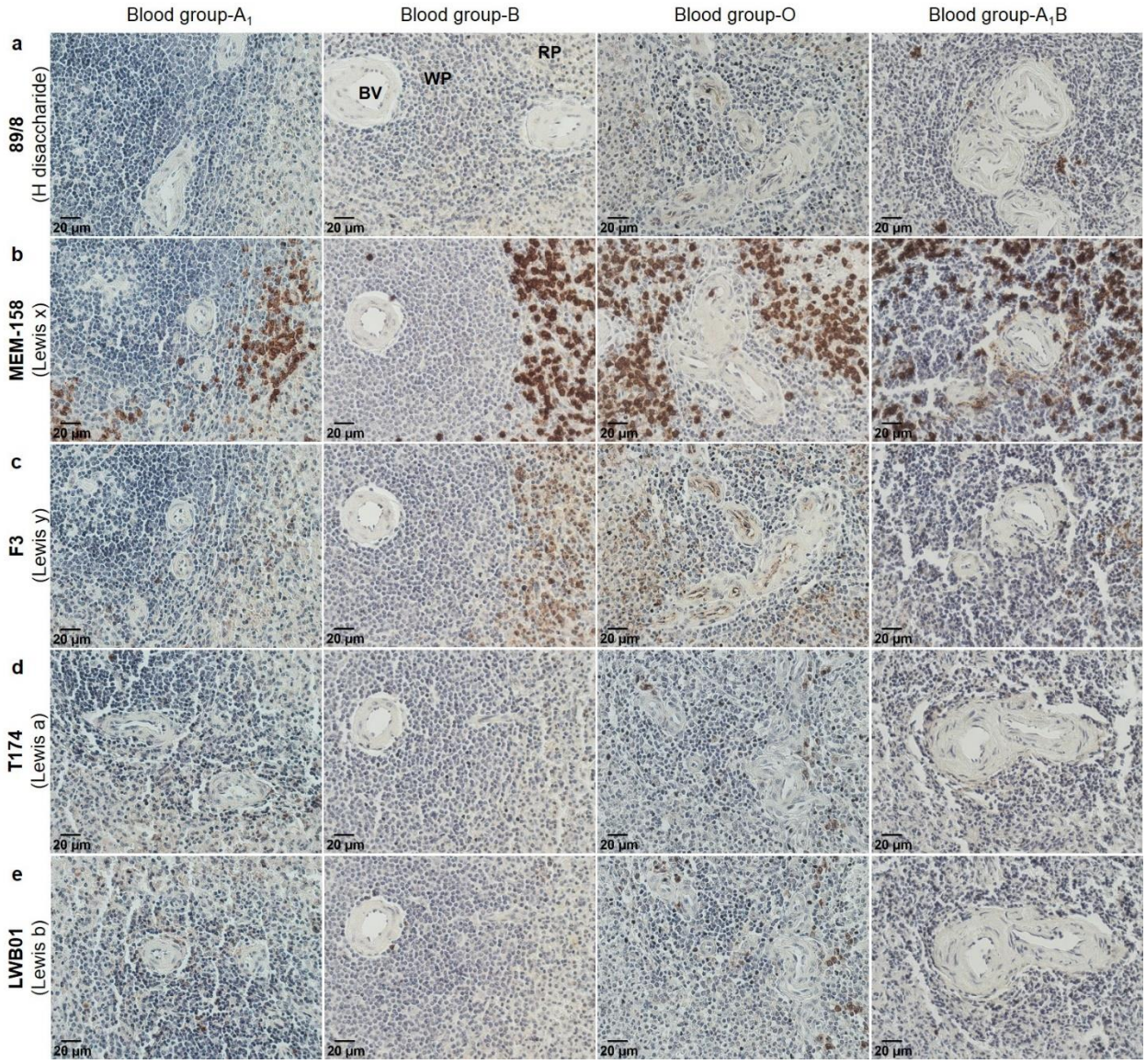


Figure 3.17. Blood group H and Lewis antigen expression in spleen of different blood groups. Spleen tissues from blood group A_1, B, O, and A_1B donors were stained with anti-H (**a**), Lewis^x (**b**), Lewis^y (**c**), Lewis^a (**d**), and Lewis^b (**e**) antibodies. Data shown are representative immunohistochemistry images from blood group A_1 (n=5), B (n=2), O (n=3), and A_1B (n=1) donors. Dark brown represents positive staining; Blue represents nuclear staining by hematoxylin. Lewis^x antigens are constitutively expressed in granulocytes and

monocytes as can be seen in all spleen sections (**b**). Lewis^{y} antigen expression can be seen in vascular endothelium of blood group O spleen (**c**) but red cells are positive in O and B spleens (as seen with flow cytometry in Figure 3.5). **BV** blood vessel; **WP** white pulp; **RP** red pulp.

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Chapter 4

Fine Specificity of B Cell Tolerance to Blood Group Antigens Following ABO-incompatible Heart Transplantation in Children: Implications for Donor-Specific Antibody Monitoring

A version of this chapter has been submitted for publication in ***Nature Medicine***

4.1 Introduction

Transplantation can be a life-saving therapy for patients with end-stage organ failure and greatly improves quality of life however there are a number of potential barriers. One such barrier is the ABO blood group system, discovered by Karl Landsteiner[1] based on expression of these antigens on red cells. A, B and H(0) antigens are expressed on many cells and tissues; individuals lacking A or B antigens develop anti-A or anti-B 'natural' antibodies respectively, and blood group O individuals produce both anti-A and anti-B antibodies[2]. Crossing the ABO barrier in solid organ transplantation may result in hyperacute or acute antibody-mediated rejection[3] due to preformed antibodies in recipients and abundant expression of ABH antigens in donor organs, leading to graft loss or death[4]. However, with aggressive antibody removal strategies and depletion of antibody-producing cells, ABO-incompatible (ABOi) transplantation has gained success in recent years[5], aided by advances in immunosuppressive therapies and better patient management. ABOi solid organ transplants are increasingly performed in an effort to distribute donor organs efficiently and reduce waiting time, thereby improving survival of patients needing transplants[6, 7].

ABH antigens are oligosaccharides expressed as glycoproteins or glycolipids on cells and tissues and are synthesized by glycosyltransferases that sequentially add monosaccharides to different precursor chains[8, 9]. Based on the carbohydrate moieties of these precursor structures ABH antigens can be classified as A type I-VI, B type I-VI and H type I-VI[8]. At least four of these subtype chains have been known to carry ABH antigens in humans[8, 10, 11]. The variations in precursor structures that carry ABH antigens create unique antigen epitopes[5, 7-9]. Biochemical and immunohistochemical studies suggest that subtype antigens are differentially expressed in red cells and tissues or organs[11]. Therefore erythrocyte agglutination, the current method used to assess ABO antibodies, is inadequate for effectively managing patients undergoing ABOi organ transplantation or for determining eligibility for safe ABOi transplantation. In this setting, assessment of 'donor-specific' antibodies requires

measurement of antibodies against ABH-subtype antigens expressed in the donor organ or tissue.

We present here a method for determining ABH-subtype antigen-specific antibodies in plasma. The aims of this international multi-center study were: 1) to determine fine specificities of 'natural' ABO antibodies in human plasma, and 2) to assess the clinical application of this assay for detecting 'donor-specific' antibodies (*ie*, against ABH-subtype antigens expressed in the donor organ) in the setting of ABOi transplantation. The study cohorts consisted of children receiving ABOi heart transplants and a control group of children of similar age receiving ABO-compatible (ABOc) heart transplants under similar immunosuppressive therapies, and healthy adult volunteers.

4.2 Materials and Methods

4.2.1 Synthesis of type I-VI ABH and fabrication of glycan microarray

Chemical synthesis and characterization of blood group antigens A type I-VI, B type I-VI, and H type I-VI has been described before[12, 13].

A type I α-D-Gal*p*NAc-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-R,

A type II α-D-Gal*p*NAc-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-R,

A type III α-D-Gal*p*NAc-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-R,

A type IV α-D-Gal*p*NAc-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-R,

A type V α-D-Gal*p*NAc-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→3)-β-D-Gal*p*-R,

A type VI α-D-Gal*p*NAc-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-R,

B type I α-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-R,

B type II α-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-R,

B type III α-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-R,

B type IV α-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-R,

B type V α-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→3)-β-D-Gal*p*-R,

B type VI α-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-R,

H type I α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-R,

H type II α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-R,

H type III α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-R,

H type IV α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-R,

H type V α-L-Fuc*p*-(1→2) -β-D-Gal*p*-(1→3)-β-D-Gal*p*-R, and

H type VI α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-R, wherein R is a linker group for covalently attaching the antigen subtypes to bovine serum albumin (BSA). Conjugation of ABH type I-VI structures to BSA was performed using methods described before[14]. Briefly, BSA (2.5 mg) was dissolved in sodium phosphate buffer pH 7.5 (0.5 ml), and the PNP ester of a glycan at a selected molar ratio was dissolved in dry DMF (25 μl). The resulting the solution was then injected into the reaction medium drop-wise and the reaction was left rotating for 24 hours at room temperature. The mixture was then dialyzed in a 4 L beaker against five changes of deionized water, with each change lasting for at least 4 hours. The solution was then lyophilized to obtain a white solid. MALDI mass spectrometry was performed to determine the antigen loading on BSA. The αGal-BSA [α-Gal-(1→3)-β-Gal-(1→4)-β-GlcNAc-BSA] was purchased from V-labs, Inc., USA.

Microarray slides were dispensed at Engineering Arts LLC (Phoenix, AZ, USA). BSA conjugates were non-contact printed on Schott type E (SCHOTT North America, Inc., USA) slides using

Engineering Arts au301-Rainmaker microarray printer. Printed slides were stored at -30°C until used.

4.2.2 Study Patients and sample collection

Children undergoing heart transplantation were recruited from seven transplant centers in North America and the United Kingdom (University of Alberta, Edmonton; Loma Linda University Medical Center, Loma Linda; Great Ormond Street Hospital, London; Hospital for Sick Children, Toronto; Columbia University, New York; Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago; Emory University School of Medicine, Atlanta). Blood samples were collected during routine follow-up. Heart biopsies were obtained from patients (n=21) undergoing protocol endomyocardial biopsy for rejection surveillance. The study was approved by institutional review committees from all centers; informed consent was obtained from all patients and/or family members.

4.2.3 Blood group

Standard blood-bank techniques were used to determine blood type of transplant recipients. Briefly, patient's red cells are tested with anti-A and anti-B antibodies to identify A and/or B antigens on red cells. Reverse grouping is also performed using reagent red cells to identify the presence of anti-A and/or anti-B in patient's serum. Donor blood type was provided by the referring centre or organ procurement agency.

4.2.4 Sub-type specific antibody detection assay and data analysis

Glycan microarray slides (up to four slides per cassette) were loaded into the 96-well format slide module (ACH4x24, Arrayit Cooperation, USA) and blocked with 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Plasma samples were diluted (100 µl at 1:100) in blocking buffer and incubated for 30 minutes at 37°C. Bound antibodies were

detected using fluorochrome (DyLight 549™ or Dylight 649™) conjugated goat anti-human IgM, IgG or IgA antibodies (Jackson Immunoresearch, USA) at predetermined dilutions in blocking buffer. Microarray slides were scanned using Nimblegen MS200 (Roche) at 5 μm resolution and analysed using ImaGene software (Biodiscovery, USA). Normalized mean fluorescent intensities (MFI) were derived by subtracting local background fluorescence and averages of triplicates were reported.

4.2.5 Immunohistochemistry

Paraffin sections were processed for staining by deparaffinization in toluene and rehydration with alcohol and water. After quenching peroxidase activity, slides were blocked with 3%BSA/PBS then incubated with monoclonal antibodies at predetermined dilutions in blocking buffer. Secondary staining was performed with biotinylated anti-mouse IgM (Bethyl Laboratories, TX, USA) and streptavidin-HRP (Jackson ImmunoResearch Labs, Inc., USA) in blocking buffer; color development was performed using ImmPACT DAB (Vector Laboratories, USA). Slides were counterstained with Mayer's hematoxylin (Sigma-Aldrich), dehydrated and mounted with Entellen mounting media (Electron Microscopy Sciences, USA). Bright-field microscopy images were obtained using Nikon Eclipse E400 microscope fitted with Spot idea camera (SPOT imaging Solutions, USA) and processed using SPOT software.

JTL-1 and JTL-4 monoclonal antibodies were generated using methods previously described[15, 16] and their specificity was confirmed using microarray. Anti-A (Z2B-1) and anti-B (89-F) antibodies were purchased from Virogen corporation, USA.

4.3 Results

4.3.1 Glycan microarray development and determination of ABH subtype-specific antibodies in healthy adults

Microarray printing protocols for dispensing BSA conjugates of ABH type I-VI and αGal antigens were optimized based on spot morphology and antigen density using monoclonal anti-A and anti-B antibodies. Printing of microarrays was uniform and spot morphology was consistent (Figure 4.1). The coefficient of variation within triplicate spots for any particular antigen was less than 10%. A schema for ABH subtype-specific antibody detection is presented in Figure 4.2. The plasma antibody detection assay was optimized to achieve minimal or no background issues and was tested using plasma samples from adult volunteers of known blood group (Figure 4.3, 4.4). In healthy volunteers of blood group O (n=17), IgM, IgG and IgA isotype antibodies against A type I-VI, B type I-VI and H type I, III-VI were detected. Antibodies against H-type II were largely absent with the exception of one individual with low level IgG antibodies. As expected, antibodies against 'self' A-type I-IV and B-type I-II, known to be expressed in cells and tissues, were largely absent in blood group A (n=15) and B (n=9) individuals respectively.

4.3.2 Patient demographics

Children who received ABOi heart transplants (n=53) and a control group of children who received ABOc heart transplants (n=60) were recruited from 6 sites in Canada, US and UK. Patient characteristics and age distribution are presented in Table 4.1 and Figure 4.5. Age at sample date and interval from transplantation to sample date were comparable between ABOi and ABOc groups; median age at transplant was identical between the two groups. Immunosuppressive strategies were determined by individual institutional protocols and, although not identical across patients or institutions, were similar in scope between ABOi and ABOc groups, as previously reported[17].

4.3.3 Selective absence of ABH type II-specific antibodies in ABOi heart transplant recipients

Antibodies against A-type II antigens were not detected in blood group O or B recipients of heart grafts from group A donors (n=25; Figure 4.6a-c), whereas antibodies against all other ABH antigen subtypes and αGal were abundant. Similarly, antibodies against B-type II antigens were absent in blood group O or A recipients of group B donor hearts (n=19; Figure 4.7a-c), and antibodies against both A-type II and B-type II antigens were absent in group O or A recipients of group AB donor hearts (n=9; Figure 4.7d-f). In order to assess the influence of transplantation and immunosuppressive therapy on the development of natural antibodies against ABH antigens in children we included a comparable group of children who received ABOc transplants under similar immunosuppressive therapy. In contrast to ABOi recipients, group O recipients of heart grafts from group O donors produced abundant antibodies against A-type II antigens as well as other A-subtypes (n=30); Figure 4.6d-f). Antibodies against ABH subtype antigens in blood group A (n=21, Figure 4.8a-c), B (n=14, Figure 4.8d-f) and AB (n=6, Figure 4.9a-c) recipients of ABOc heart transplant are also shown for comparison.

4.3.4 A type II and B type II antigen expression in the heart

Figure 4.10 shows a representative image of immunohistochemical staining of endomyocardial biopsies from a blood group A donor heart (a-d; age 3.3 years, 2.7 years post-transplant) and a blood group B heart (e-h; age 11.6 years, 11.2 years post-transplant). Blood group A and B antigen expression on vascular endothelium was confirmed by staining with anti-A (a, e) and anti-B (b, f) antibodies that recognise A-type I-VI and B-type I-VI respectively. However, staining with subtype-specific antibodies demonstrated only type II A and B antigen structures in cardiac tissue (c, g), while type III/IV-based structures were not detected (d, h). Tissue expression of A and B subtype antigens was similar in all blood group A (n=12) and B (n=9) cardiac biopsies examined.

4.4 Discussion

Transplantation of organs into ABOi recipients typically leads to a cascade of immunological events initiated by the interaction between circulating 'natural' antibodies in the recipient and vascular endothelial cells in the donor organ expressing target ABH antigens[3]. This can lead to hyperacute or acute antibody-mediated rejection resulting in graft loss and, in some cases, patient death[4]. Persistence of antibodies against donor graft antigens may also result in chronic injury with detrimental impact on long-term outcomes. Organ transplantation across the ABO barrier has the potential to increase donor availability, decrease waitlist mortality and decrease organ wastage. However, ABOi transplantation carries high immunologic risk in which antibody-mediated graft damage in the setting of heart or lung transplantation can result in patient death. Therefore, precise monitoring of antibodies against ABO antigens is critical for guiding treatment and for assessing patient suitability for ABOi transplantation. The only method currently used to measure ABO antibodies in clinical laboratories is the isohemagglutination assay, a semi-quantitative titre measurement obtained by mixing patient serum with erythrocytes from donors of known blood group. This assay, developed more than a century ago, has been used reliably in transfusion medicine in which compatibility between recipient serum and donor red cells is assessed. When this crude method is used in the setting of ABOi organ transplantation, it is generally assumed that antigens expressed on reagent red cells are identical to those in the donor organ. However, biochemical and immunohiostochemical studies have suggested that ABH subtype antigens are expressed differentially in cells and tissues[11]. Moreover, approximately 328 red cell antigens belonging to 30 blood group systems have been identified[18] and can potentially interact with plasma antibodies. Therefore a quantitative assay that can provide information about fine specificities of ABO antibodies of all isotypes is much needed for reliable clinical assessment. In addition to detecting the presence of donor-specific antibodies in serum (*ie*, antibodies against ABH subtype antigens expressed in the donor organ), the assay must also be reliable enough to

confirm absence of donor-specific antibodies so that unnecessary interventions or inappropriate denial of transplant eligibility can be avoided.

Natural antibodies against non-self ABH antigens ('isohemagglutinins') are produced in most people and are thought to be induced by environmental stimuli that share similar carbohydrate epitopes[19]. Of six described ABH antigen subtypes, at least four have been characterized in human red cells[8, 10]. Type I-based structures are known to exist only in secretions in a secretor (Se) dependent manner[20,10, 21]. Type I- and II-based structures have been characterized from blood group A, B and O individuals[22-26] whereas studies on type III/IV structures have been reported only in blood group A individuals[27-29]. Our studies of red cells and tissues by immunofluorescence staining also found type II, III, and IV precursor structures in blood group A individuals but only type II structures in O and B individuals (data not shown). This is reflected in the ABH subtype-specific antibody profiles in healthy adult volunteers. Antibodies against H-type II, a self- antigen in O individuals, were not detected, with the exception of one individual who had low level of IgG against H-type II. Individuals of Bombay blood group, a rare phenotype, lack H antigen and are able to produce antibodies against H antigen; this individual may belong to this or another variant. Subtype-specific antibody profiles of blood group A and B individuals presented in this report are also consistent with the previous findings of A and B subtype antigen expression respectively. In the general population approximately 20% are non-secretors[8] and it is likely that this cohort includes some non-secretors lacking ABH type I, hence develop antibodies against ABH type I.

In contrast to adults, infants do not produce natural antibodies to ABH antigens, with isohemagglutinin ontogeny generally beginning only after about 4-6 months of age[30]. This developmental lag allows infants the opportunity to undergo safe ABOi heart transplantation[17, 31] without the need for aggressive therapeutic interventions that are usually required for ABOi (kidney) transplants in adults, or additional immunosuppressive therapies compared to infants receiving ABOc heart transplants. Early studies of a small cohort of infants suggested B cell

tolerance to graft A/B antigens developing spontaneously after ABOi infant heart transplantation, manifest as selective and persistent absence or major deficiency of antibodies to the donor blood group[32]. Subsequently, however, recent case studies reported antibodies against the donor blood group in some children as measured by hemagglutination assay[33, 34]. Yet no histological and/or functional features of antibody-mediated graft rejection were observed or requirements for augmented immunosuppression. This raises the possibility that graft 'accommodation' occurred, or that antibodies detected by the red cell agglutination test may not be specific to ABH antigens expressed in the donor heart tissue (*ie*, not 'donor-specific'). Hence, children receiving ABOi heart transplants as infants are well suited for studying fine specificities of plasma antibodies against ABH antigens and validating the glycan microarray for detecting donor-specific antibodies.

Blood group O or B infants receiving A donor hearts as infants had no detectable IgM, IgG or IgA antibodies against A-type II antigens, yet developed antibodies against all other A antigen subtypes as well as B antigens. In contrast, antibodies to all A-type I-VI antigens were clearly seen in blood group O and B children who received ABOc transplants. Similarly, antibodies against B-type II or A- and B-type II antigens were not detected in children who received B or AB donor hearts respectively. This antibody profile, *ie*, selective absence of antibodies against A- and/or B-type II antigens, was uniformly observed in ABOi heart recipients and was distinctly different from that of age-matched ABOc recipients receiving similar immunosuppressive therapy. Given that immunohistochemical staining of tissue from donor hearts revealed that only type II A/B antigens were expressed on cardiac endothelium, only antibodies against A or B type II antigens can be considered donor-specific in this clinical setting. Absence of these antibodies years after ABOi heart transplantation confirms that donor-specific tolerance has developed with regard to ABOi graft antigens. The results show clearly the fine specificities of ABO blood group antibodies and the importance of determining ABH subtype specific antibodies in ABOi transplantation.

In summary, this international multi-center study demonstrates that the glycan microarray can be used for accurate determination of donor-specific ABO antibodies in the setting of ABOi transplantation. Characterization of ABH subtype antigen expression in other organs such as kidney and liver will be valuable for its wider application in ABOi organ transplantation. This can help with the reliable assessment of patients for their suitability to receive an ABOi transplant as well as for appropriate pre- and post-transplant clinical management. Furthermore, by accurately assessing the absence of donor-specific antibodies, unnecessary interventions can be avoided such as antibody removal by plasmapheresis or aggressive immunosuppressive therapies.

4.5 Acknowledgements

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4.6 Tables

Table 4.1. Characteristics of ABO-incompatible and ABO-compatible heart transplant recipients.

Recipient's blood group	Donor's blood group	Number of patients	Number of samples	Age at transplantation Median, years (range)	Age at sample date Median, years (range)	Interval from transplantation to sample date Median, years (range)
ABO-incompatible transplants		**53**	**53**	**0.5** (2 days – 4.1 yr)	**3.4** (0.4 – 11.4 yr)	**2.8** (0.1 – 11.1 yr)
O or B	A	25	25	0.4 (8 days – 2.7 yr)	2.7 (0.4 – 11.4 yr)	2.0 (0.1 – 11.1 yr)
O or A	B	19	19	0.5 (2 days – 4.1 yr)	3.4 (0.1 – 10.3 yr)	2.8 (0.1 – 8.4 yr)
O or A	AB	9	9	0.7 (0.1 – 3.6 yr)	5.7 (1.7 – 10.1 yr)	4.3 (1 - 10 yr)
ABO-compatible transplants		**60**	**71**	**0.5** (0 – 8.8 yr)	**3.8** (0.1 – 12.1 yr)	**1.9** (2 wk – 11.8 yr)
O	O	24	30	0.5 (1 day – 8.1yr)	3.9 (0.5 – 11.4 yr)	2.0 (0.3 – 11.3 yr)
A	O or A	19	21	0.5 (0.1 – 4.8 yr)	3.0 (0.2 – 12.0 yr)	1.9 (0.1 – 11.8 yr)
B	O or B	12	14	0.7 (0 day – 8.8 yr)	6.1 (0.1 – 12.1 yr)	3.2 (2 wk – 10.7 yr)
AB	A, B, AB or O	5	6	0.1 (1 day – 7.9 yr)	1.2 (0.2 – 8.7 yr)	0.27 (0.1 – 8.6 yr)

4.7 Figures

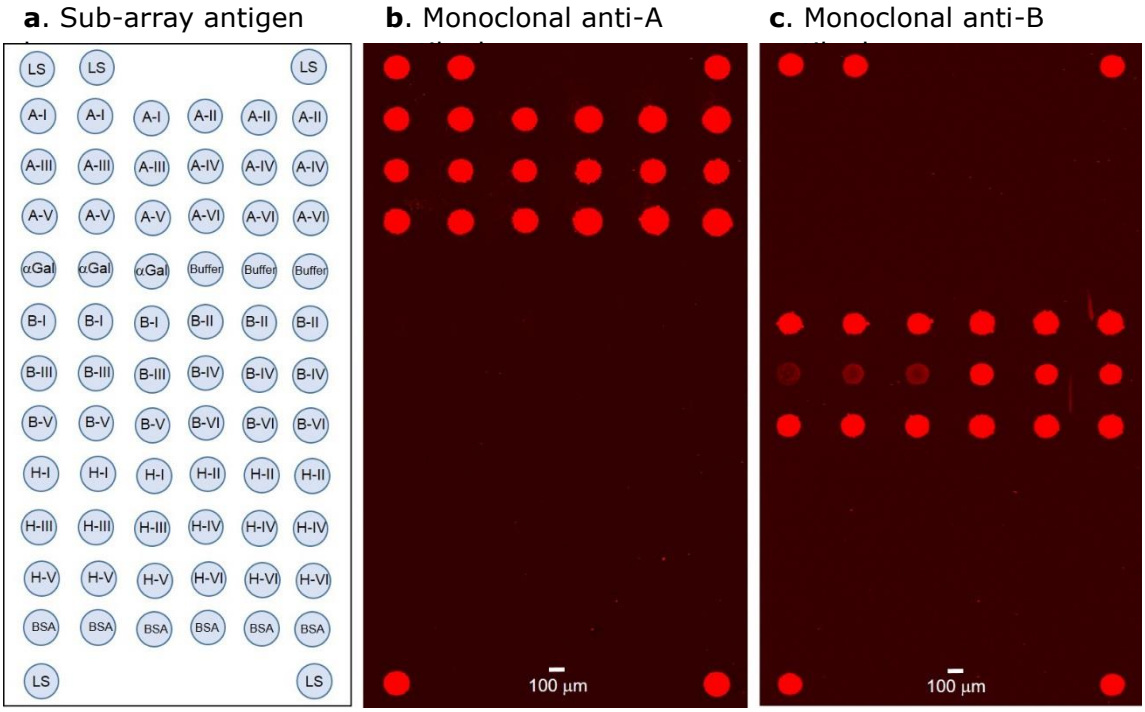

Figure 4.1. Printing layout of sub-arrays and spot morphology. (**a**) Bovine serum albumin (BSA) conjugates of blood group A type I-VI, B type I-VI, H type I-VI, αGal antigens are printed in triplicates. BSA alone or printing buffer were also printed as negative control spots. Landing spots (LS) were printed with a mixture of BSA conjugated Alexa Fluor® 555 and Alexa Fluor® 647. A type I-VI (**b**) and B type I-VI (**c**) spots were assessed by hybridizing with anti-A and anti-B monoclonal antibodies respectively; fluorescence was developed using DyLight 649™ conjugated secondary antibody.

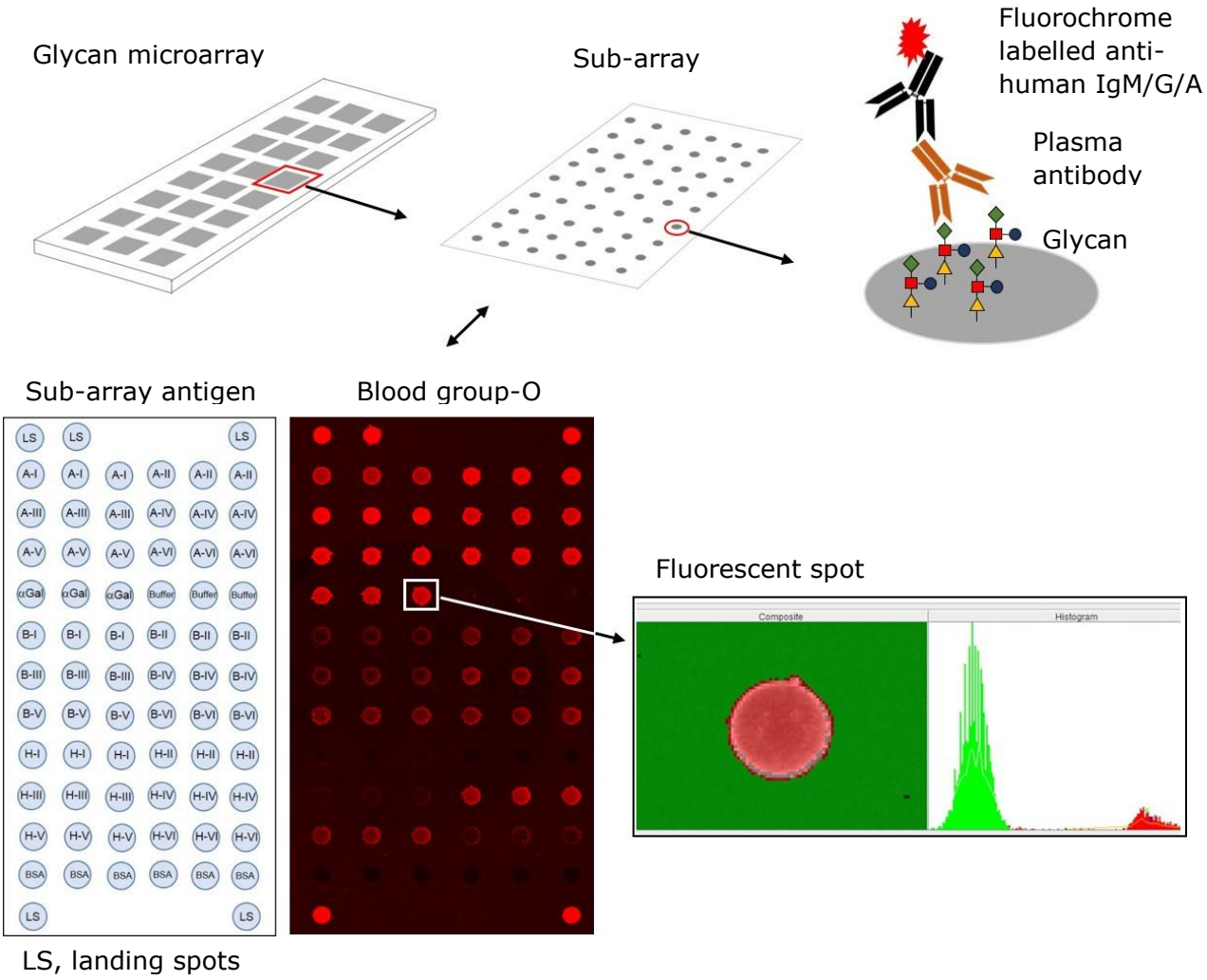

Figure 4.2. Schematics of 'donor-specific' antibody detection methodology. Glycan microarray slides were printed with 24 (3 x 8) sub-arrays. In each sub-array, BSA conjugates of A type I-VI, B type I-VI, H type I-VI and αGal antigens were printed in triplicates. Hybridization steps were performed using a 96-well format slide module loaded with up to four microarray slides. Bound plasma antibodies were detected using fluorochrome (DyLight 549™ or Dylight 649™) conjugated anti-human IgM, IgG or IgA antibodies. Slides were scanned at 5μm resolution and fluorescent intensities were analyzed using ImaGene software

and reported as average of normalized mean fluorescent intensities from triplicate spots (online methods).

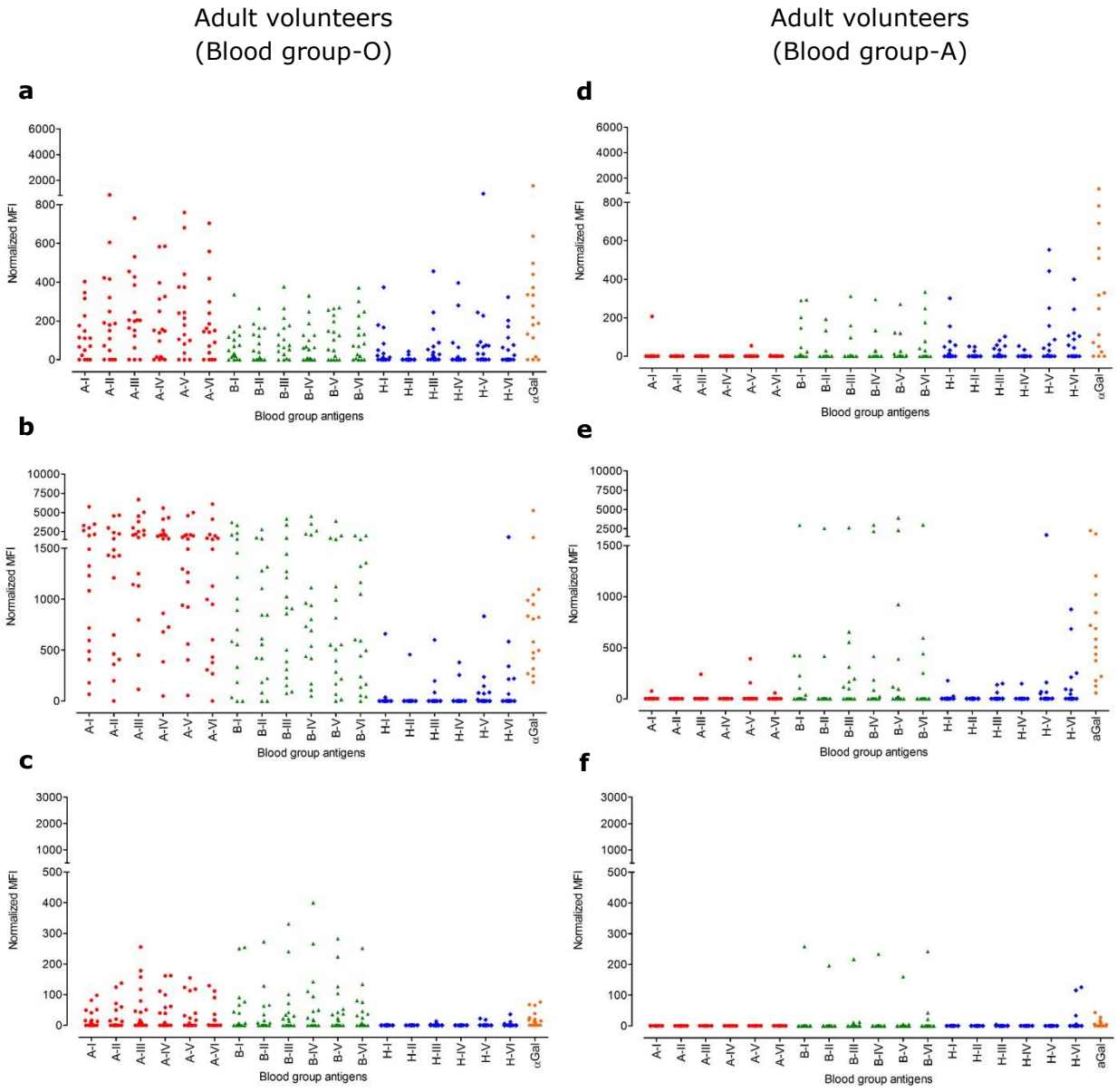

Figure 4.3. Subtype-specific ABH antibody profile of healthy adult volunteers. (**a-c**) Blood group O individuals (n=17); (**d-f**) Blood group A individuals (n=15). Anti-A/B IgM (**a, d**), IgG (**b, e**), and IgA (**c, f**) antibodies against type I-VI ABH and αGal antigens are shown.

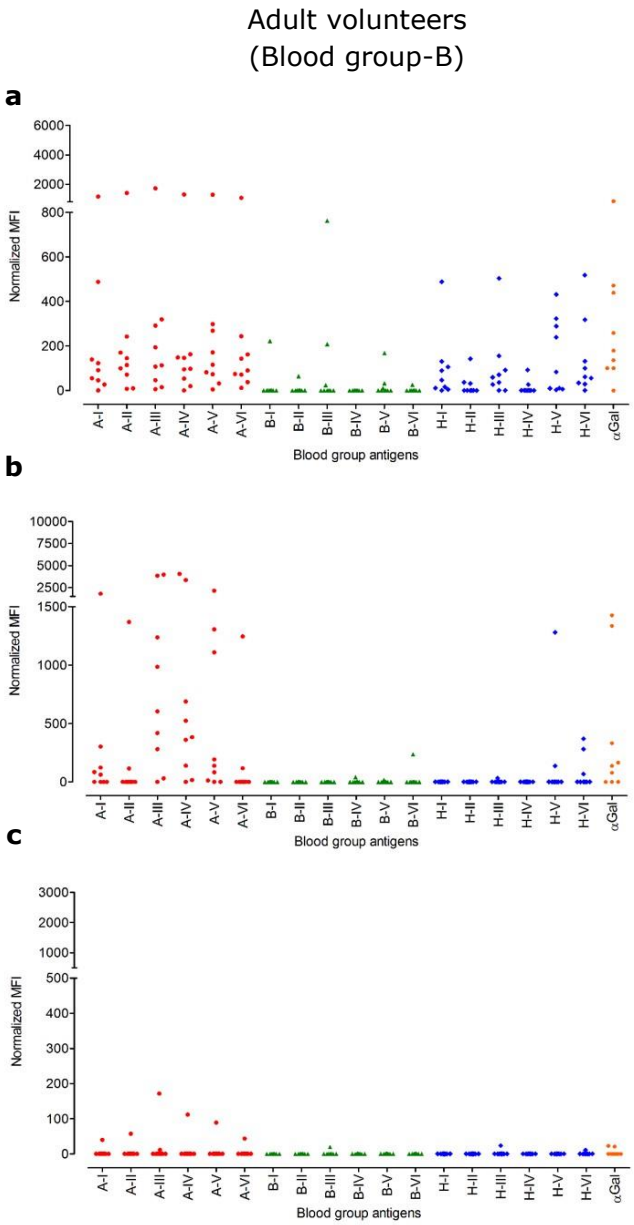

Figure 4.4. Subtype-specific ABH antibody profile of healthy adult volunteers. Anti-A/B IgM (**a**), IgG (**b**), and IgA (**c**) antibodies against type I-VI ABH and αGal antigens in blood group-B (n=9) individuals are shown.

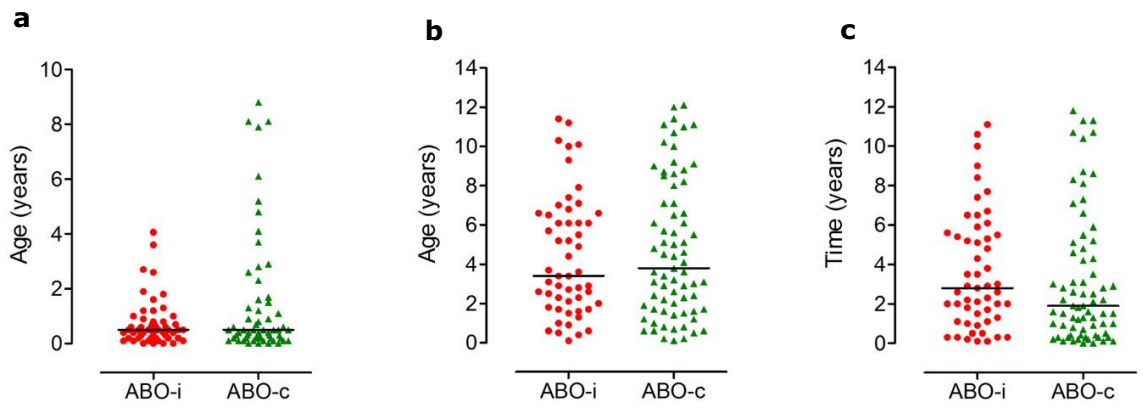

Figure 4.5. Patient characteristics. Scatter plot comparing (**a**) age at transplantation, (**b**) age at sample date and (**c**) interval from transplant to sample date between patients receiving ABO-I (n=53) and ABO-c (n=71) heart transplants. Line represents median years.

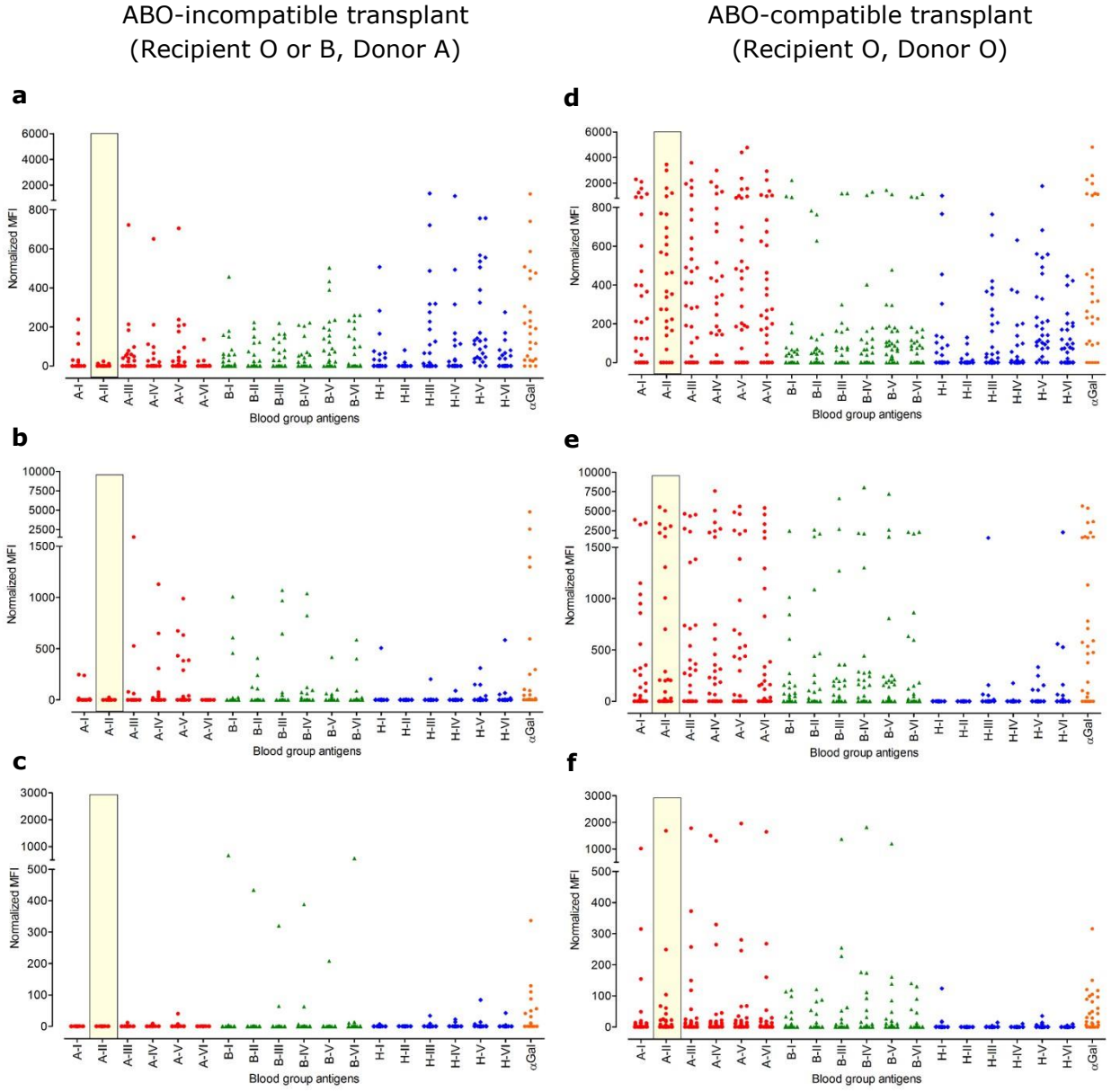

Figure 4.6. Subtype-specific ABH antibody profile of patients who received ABO-incompatible and ABO-compatible heart transplants. (**a-c**) Blood group O or B recipients who received A donor hearts (ABOi), n=25. (**d-f**) Blood group O recipients who received O donor hearts (ABOc), n=30. Anti-A/B IgM (**a, d**), IgG (**b, e**), and IgA (**c,f**) antibodies against type I-VI ABH and αGal antigens are shown. Antibodies against A-type II antigens are highlighted (*ie,* expressed in heart tissue thus 'donor-specific' antibodies in ABOi patients).

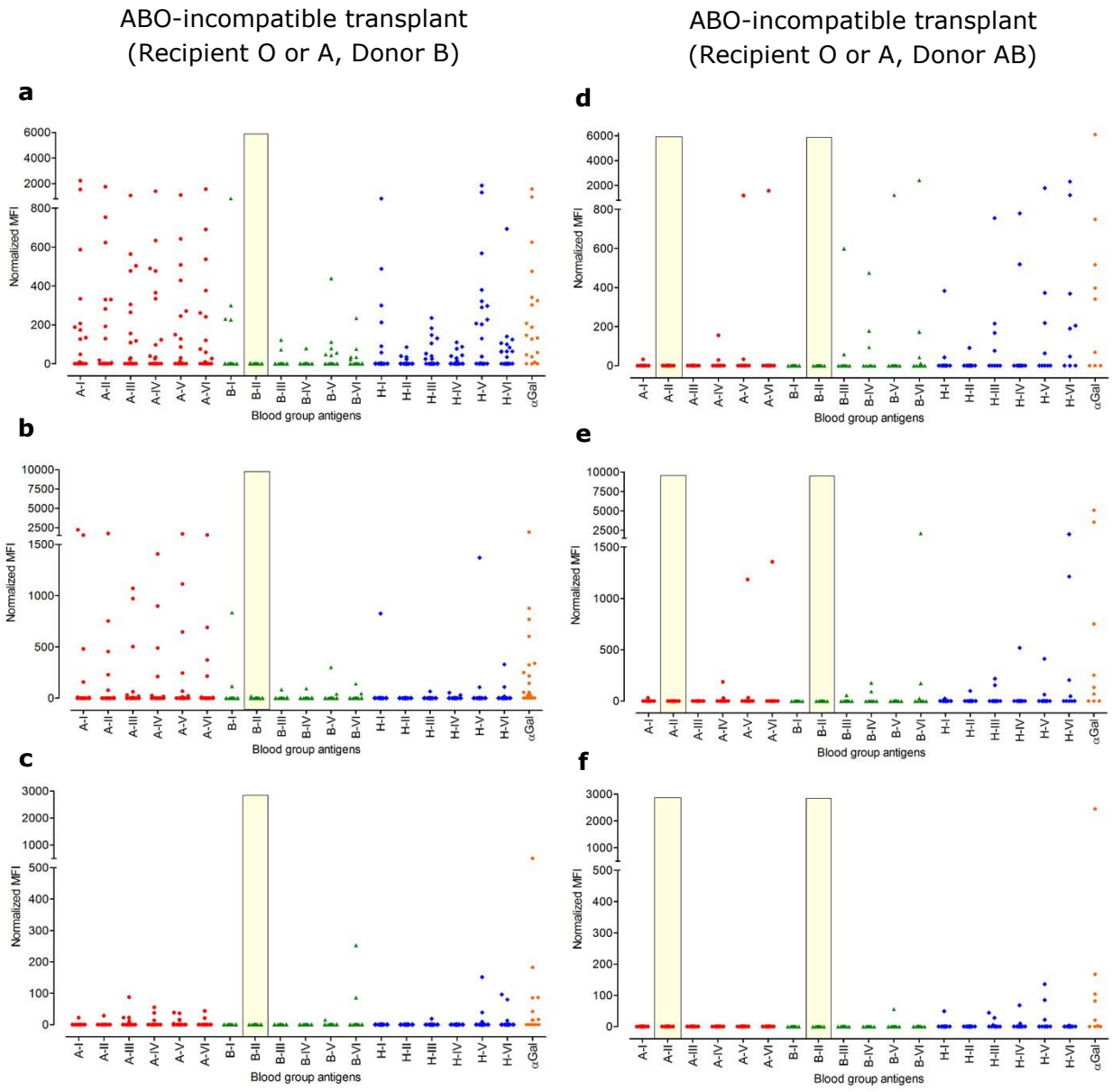

Figure 4.7. Subtype-specific ABH antibody profile of patients who received ABO-incompatible heart transplants. Blood group O or A recipients who received B donor hearts (**a-c**), n=19. Blood group O or A recipients who received AB donor hearts (**d-f**), n=9. Anti-A/B IgM (**a, d**), IgG (**b, e**), and IgA (**c, f**) antibodies against type I-VI ABH and αGal antigens are shown. Antibodies against A type II and/or B type II antigens that are expressed in the heart, (*ie,* 'donor-specific' antibodies) are highlighted.

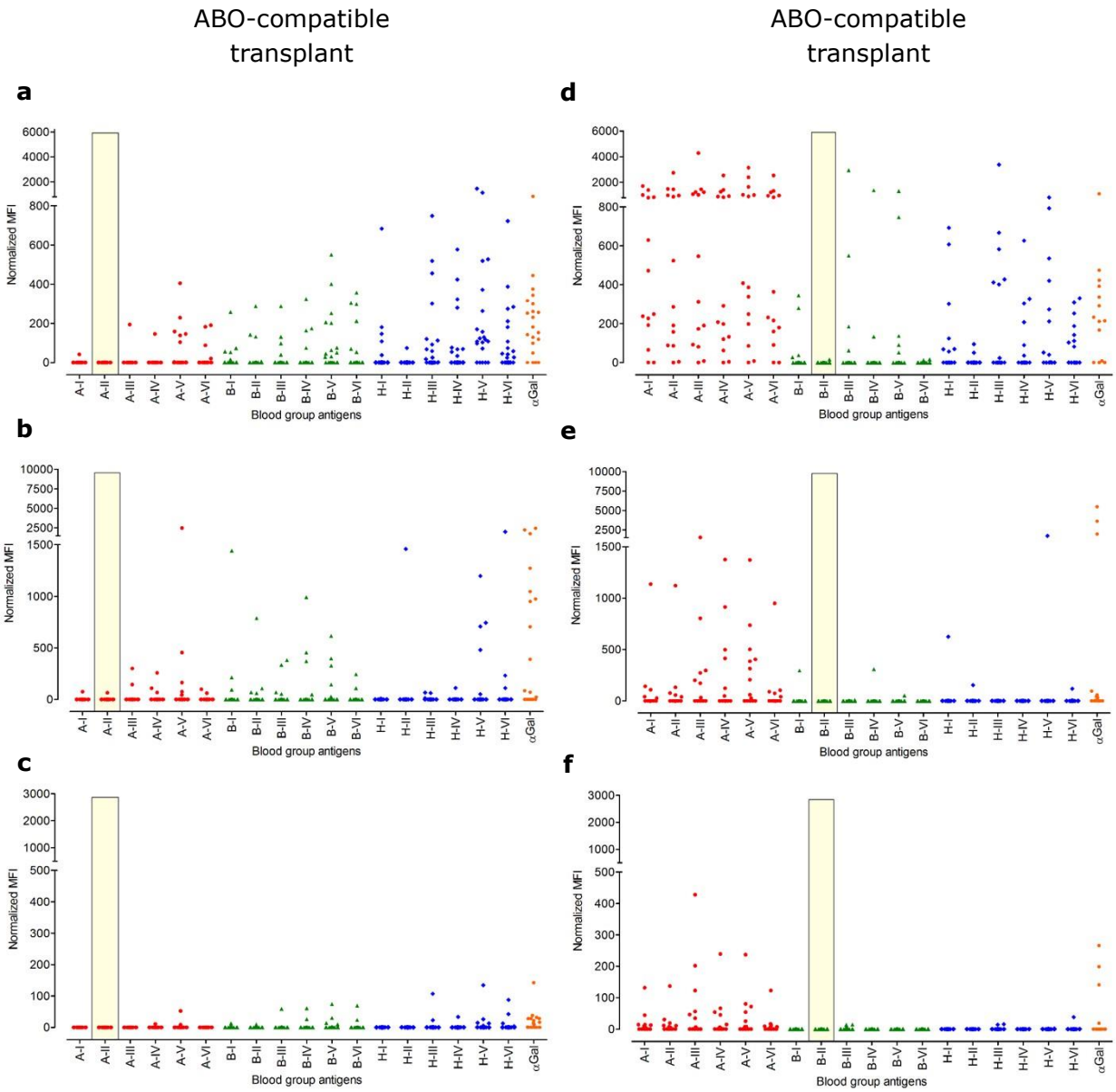

Figure 4.8. Subtype-specific ABH antibody profile of patients who received ABO-compatible heart transplants. Group A recipients of A or O donor hearts (**a-c**), n=21; group B recipients of B or O donor hearts (**d-f**), n=14. Anti-A/B IgM (**a, d**), IgG (**b, e**), and IgA (**c, f**) antibodies against type I-VI ABH and αGal antigens are shown. Antibodies against A-type II or B-type II are highlighted.

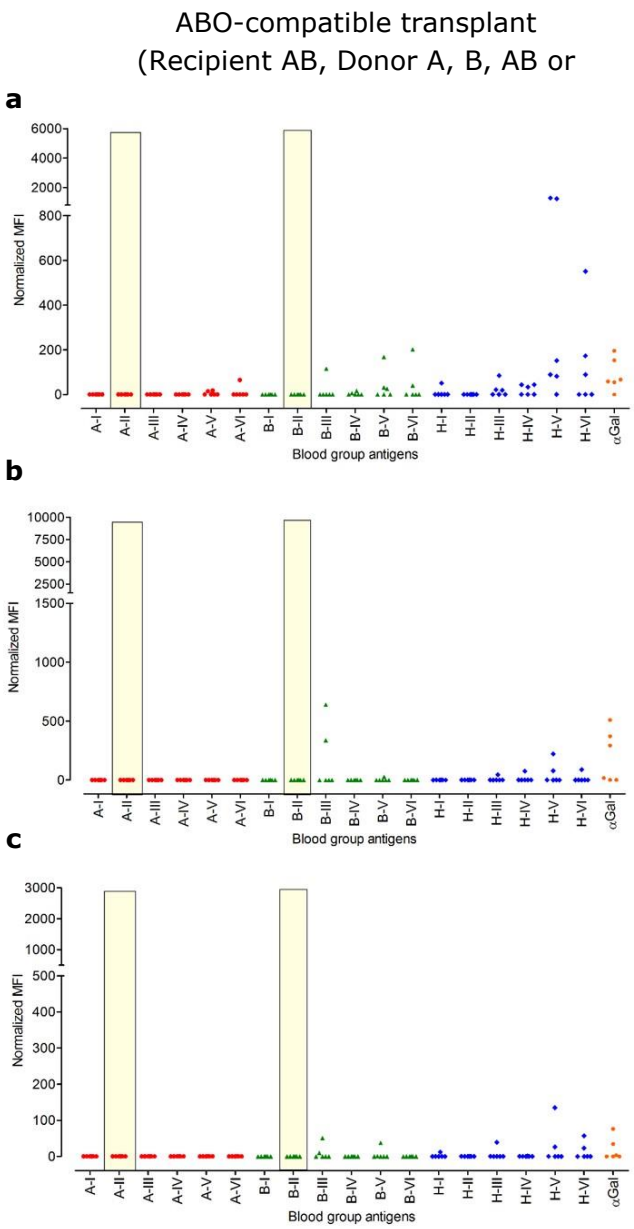

Figure 4.9. Subtype-specific ABH antibody profile of patients who received ABO-compatible heart transplants. (**a-c**) Blood group AB recipients who received A, B, AB or O donor hearts (ABOc), n=6. Anti-A/B IgM (**a**), IgG (**b**), and IgA (**c**) antibodies against type I-VI ABH and αGal antigens are shown. Antibodies against A- and B-type II antigens are highlighted.

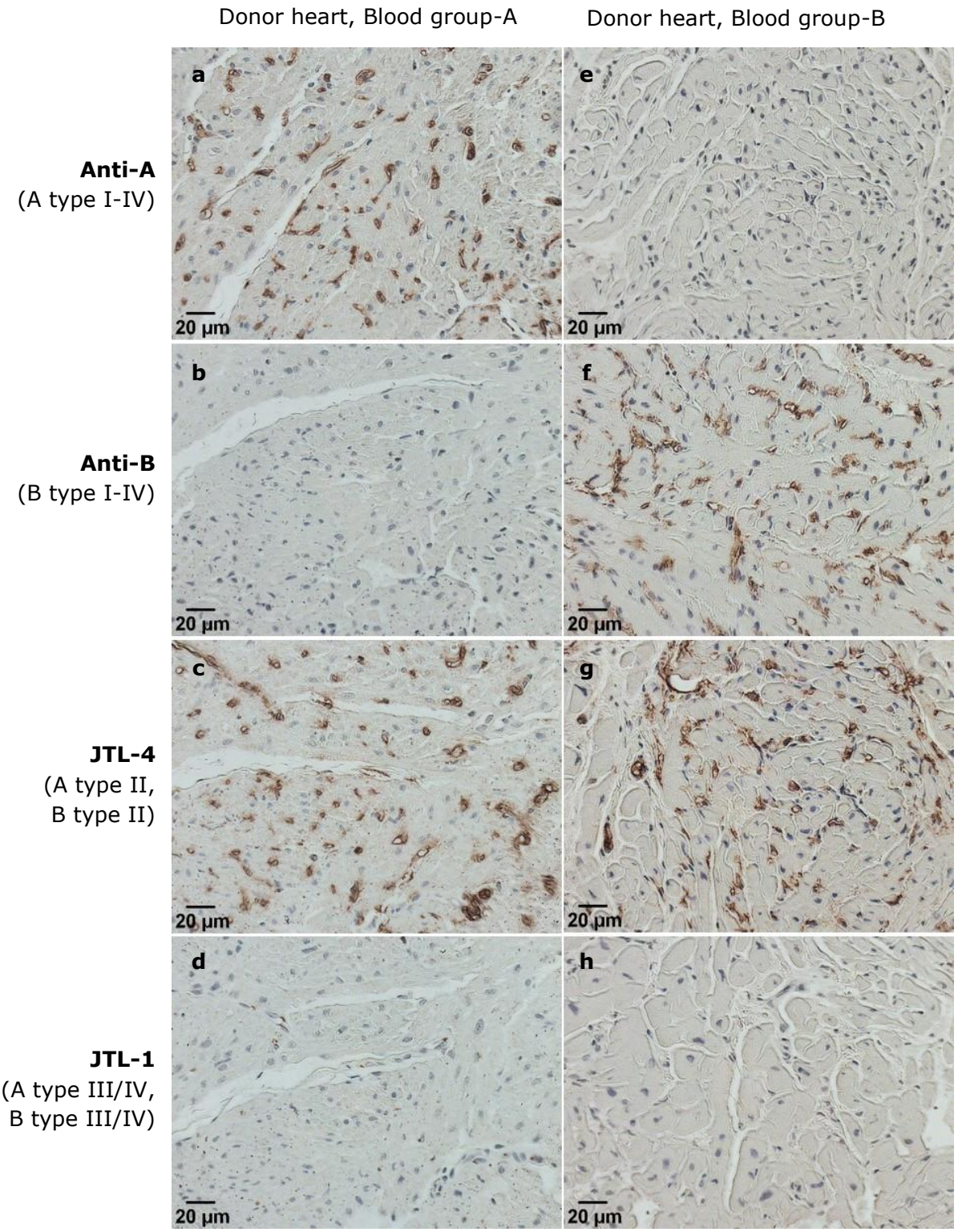
Donor heart, Blood group-A
Donor heart, Blood group-B
Anti-A
(A type I-IV)
Anti-B
(B type I-IV)
JTL-4
(A type II,
B type II)
JTL-1
(A type III/IV,
B type III/IV)
a
b
c
d
e
f
g
h
20 µm

Figure 4.10. Only type II A/B structures are expressed in cardiac tissue. Endomyocardial biopsies from blood group A **(a-d)** and B donors **(e-g)** were stained with anti-A (**a, e**; A type I-IV), anti-B (**b, f**; B type I-IV), JTL-1 (**c, g**; A type II/IV and B type III/IV) and JTL-4 (**d, h**; A type II and B type II). Shown are representative bright field images from blood group A (n=12) and B (n=9) donors. Dark brown represents positive staining; blue represents nuclear staining with hematoxylin.

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Chapter 5

Failure of neonatal B-cell tolerance induction by ABO-incompatible kidney grafts in piglets

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5.1 Introduction

The ABO histo-blood group system is of major importance in solid organ transplantation due to ABO(H) antigen expression on cells of transplantable organs[1] and the presence of 'natural' antibodies against those antigens in potential transplant recipients[2, 3]. These antibodies confer a high risk of hyperacute rejection (HAR) of organ allografts expressing non-self A/B antigens[4, 5]. Although ABO-incompatible (ABOi) kidney and liver transplants are now successful, reports of ABOi cardiac transplants are rare, typically performed inadvertently as a result of errors in determining or reporting the donor blood group. In a global survey, Cooper (1990) described eight such cases, all in adults, in whom HAR occurred in five and only one survived beyond one year[6]. In contradistinction, we showed that ABOi heart transplantation in infants was safe[7] due to lack of isohemagglutinins in early infancy[8-10]. Our group further demonstrated that the surprising short and long-term success of ABOi infant heart transplantation[11] is due to i) minimal anti-A/B antibodies at the time of transplant, ii) persistence of graft A/B antigens and iii) neonatally-acquired tolerance through donor-specific B-cell elimination[12]. A suitable animal model would allow investigation of detailed mechanisms of tolerance in this setting. Given that neonatal organ transplantation in mice is logistically impossible, a large animal model is needed to transplant vascularized organ grafts in neonates.

Pigs have been reported to have a blood group AO system comparable to the human ABO system[13], with genetically-defined specific glycosyltransferases controlling A and H antigen expression in various tissues[14, 15]. Similar to humans, pigs produce 'natural' antibodies against non-self ABH antigens, *i.e.,* group-A pigs produce anti-B antibodies and group-O pigs produce both anti-A and anti-B antibodies[16]. Our objectives were: 1) to study isohemagglutinin ontogeny in piglets and identify whether there is a neonatal window of opportunity for safe AO-incompatible (AOi) transplantation defined by the absence of antibodies; 2) to assess whether the juvenile pig is suitable to model ABO B-cell tolerance as observed in human ABOi infant heart transplant recipients.

5.2 Materials and Methods

5.2.1 Experimental design

Anti-A and anti-B antibody production was measured at serial time points in peripheral blood from group-O piglets from birth to adulthood. Tissue samples were studied for A- and H-antigen expression and distribution. In a separate study, group-O piglets were transplanted with kidney allografts from group-O or group-A donors under cyclosporine immunosuppression. Plasma was monitored for anti-A antibody development and graft biopsies were studied for A/H-antigen persistence and graft rejection.

5.2.2 Animals

All pigs were domestic type (Duroc x F1 [Landrace x Large White]) and housed at the University of Alberta Swine Research Centre or Health Sciences Laboratory Animal Services facility. Piglets were nursed by the sow until weaned at age three weeks. For anti-A/B antibody testing, peripheral blood was serially sampled from age 1 - 124 days. Recipients and donors used in transplant studies were age 21-35 days. Pigs used for the antibody development study received routine live oral vaccines (Suvaxyn E-oral and Enterisol Ileitis) at age 45-63 days, whereas pigs used in transplant studies did not. Protocols were approved by the University of Alberta Health Sciences Animal Care Committee; animals were cared for under guidelines of the Canadian Council of Animal Care.

5.2.3 Blood typing

Blood group was determined by immunohistochemical staining of buccal mucosa smears, as previously described with modifications[16]. Briefly, buccal mucosa cells were obtained from newborn piglets. Samples were applied onto polysine microscope slides (Thermo Scientific),

air-dried and stored at room temperature (RT). Positive controls were buccal mucosa smears from human volunteers of known blood group. Slides were fixed in cold acetone (Sigma, ON, Canada), air-dried and rehydrated in PBS. After quenching peroxidase activity, slides were blocked with normal goat serum/PBS then incubated with mouse anti-A, anti-H monoclonal antibodies (M-ab) (Virogen Corporation, USA) at pre-determined dilutions in blocking buffer. Secondary staining and colour development were performed using Envision+ system according to manufacturer's instructions (Dako North America, Inc). Slides were counterstained with Mayer's hematoxylin, dehydrated and mounted with Entellen media (Electron Microscopy Sciences, USA).

5.2.4 ABO-ELISA

Medium-binding ELISA plates (Costar, Cambridge, MA) were incubated overnight with A-PAA, B-PAA trisaccharides and PAA (Glycotech, USA) (50 μL volume, concentration 5 μg/ml in 0.1M Na_2CO_3 [pH 9.6]). For standards, anti-pig IgM, IgG or IgA at 10 μg/ml in PBS was added in triplicate wells. After blocking with normal goat serum/PBS, diluted plasma or colostrum (50 μL at 1:4 – 1:20 in blocking buffer) or serially diluted pig reference serum as standards (Bethyl Laboratories Inc., USA;1ug/mL-15.625ng/mL IgM, IgG or IgA) was incubated in triplicate wells. Bound antibodies were detected using alkaline phosphatase-conjugated goat anti-pig IgM, IgG or IgA antibody (Bethyl Laboratories Inc., USA) and p-nitrophenyl phosphate (Sigma) and read at 405 nm. Concentration of antigen-specific plasma antibodies was determined from the standard curves generated using pig reference serum as described previously [17]. Positive control plasma samples were from a group-O pig immunized with human blood group A and B erythrocytes.

5.2.5 Kidney Transplantation

Anesthesia: Surgical procedures were performed under general anesthesia. Anesthesia in piglets was induced with isoflurane and maintained on a mixture of 1% isoflurane, nitrous oxide and oxygen. Adult pigs were pre-medicated with intramuscular injection of Ketamine (20 mg/kg): Xylazine (2mg/kg).

Donor operation: Donor pigs received 50 units/kg of heparin intravenously. The kidneys, aorta and vena cava were exposed through a midline laparotomy; an infra-renal aortic cannula was placed and the kidneys were perfused with cold Custodiol-HTK solution (Methapharm, Canada). Kidneys were excised with the renal artery, containing an aortic cuff, renal vein and ureter. Donor kidneys were stored in cold HTK solution until transplanted. Cold ischemic time was minimized by preparing recipient and donor simultaneously.

Transplantation: The left kidney of the recipient was exposed by an extra-peritoneal approach through a flank incision. When aorta, left renal artery and ureter were exposed a side-biting vascular clamp was applied to the aorta encasing the left renal artery leaving hind limb blood circulation intact. Clamps were also applied to renal vein and ureter. Intravenous heparin (30 units/kg) was given at least 10 minutes before aortic clamping. Care was taken to preserve ureteric blood supply. Left nephrectomy was performed with excision of the ureter at the uretero-pelvic junction; the native right kidney was left *in situ*. The donor renal artery and vein were anastomosed to the aorta and left renal vein using standard vascular techniques. Spatulated ureteric ends were anastomosed end-to-end, avoiding anastomotic stricture. The incision was closed in layers using absorbable sutures. Animals were mobile within hours of surgery and resumed good intake of food and water.

5.2.6 Immunosuppression and Rejection Monitoring

Immunosuppression: Transplant recipients received intravenous cyclosporine (Sandimmune, 15 mg/kg) (kindly donated by Novartis Pharmaceuticals Canada) on the day

of transplant. For maintenance immunosuppression recipients received one daily oral dose of cyclosporine (20-50 mg/kg)[18, 19] (Apotex Fermentation Inc., Winnipeg, Canada) beginning three days pre-transplant and continuing post-transplant until the study end-point when animals were euthanized. Blood cyclosporine trough levels were maintained at 200-500 μg/L[20], measured by the clinical laboratory using tandem mass spectrometry.

Biopsy and Histology: Rejection was assessed by protocol biopsy at three time-points (11-24 days, 49-56 days and 65-98 days post-transplant). Needle core biopsies were obtained under direct visualization through a left flank incision. Specimens were either fixed in 4% paraformaldehyde (Polysciences Inc., USA), embedded in paraffin or snap frozen in O.C.T. compound (Electron Microscopy Sciences, USA). Sections were stained using hematoxylin-eosin and periodic acid-Schiff stains. Biopsies were interpreted according to the Banff classification for kidney allograft rejection[21]. Banff classification was used to assess the respective common lesions, but if additional pathology was seen that might be specific to the animal model, i.e. beyond the human, clinical setting, the features are described in Table 5.1. Additionally, A- and H-antigen expression was assessed by staining with anti-A and anti-H M-abs as described above.

Immunofluorescence detection of C4d: Frozen sections were stained with anti-human C4d that is known to cross-react with pig (clone 12D11, Cedarlane, Canada) followed by detection with Alexa-Fluor 488 labelled secondary antibody as described previously[22]. Positive control was a post-transplant kidney biopsy from an adult pig recipient of AOi and MHC-mismatched kidney graft without immunosuppression.

5.3 Results

5.3.1 Developmental profile of anti-A and anti-B antibody production in blood group-O piglets

Plasma from group-O pigs (n=7) was analyzed for anti-A and anti-B antibodies of IgM, IgG, and IgA isotypes from birth to adulthood. Figure 5.1 shows the trend in antibody development from age 1-124 days. Anti-A IgM detected on day one disappeared by day 15-21; endogenous production of anti-A IgM began to rise at 35 days of age. Similarly, both IgG and IgA anti-A were present at birth; the window during which these isotypes were absent was longer than observed for IgM, with endogenous production of IgG and IgA detected after age 51 days. A large spike in anti-A of all three isotypes was observed following routine oral vaccinations (Suvaxyn E-oral- an avirulent live culture of *Erysipelothrix rhusiopathiae*; Enterisol Ileitis- live attenuated *Lawsonia intracellularis* vaccine). Anti-B IgM and IgG were not detected until age 92-103 days and remained at lower concentration than anti-A; anti-B IgA was not detected throughout the sampling period.

5.3.2 Tissue expression of blood group antigens

A-antigen or H-antigen expression can be seen in buccal mucosal cells used to determine the piglet blood group (Figure 5.2a). In a four week-old blood group-A piglet (Figures 5-2b, c, d, e), A-antigen is expressed in a number of tissues: Hassall's corpuscles of thymus, ductal epithelial cells of liver and pancreas, mucous-secreting cells of terminal bronchioles in lung, and strong expression in the small bowel goblet cells. In kidney, A-antigen was detected in distal tubules and collecting ducts but was absent in glomerulus and capillary endothelial cells. A-antigen was not detected in heart, spleen, lymph nodes or bone marrow cells, or vascular endothelial cells of any tissues tested, including aortic endothelial cells.

5.3.3 Post-transplant anti-A antibody production

To assess whether B cell tolerance to donor blood group antigens was induced, plasma anti-A antibody production pre- and post-transplant in AOi ('A into O') and AOc ('O into O') groups was measured (Fig. 5.3). IgM (a and b) was undetectable or detected at low concentration pre-transplant and gradually increased in both AOi and AOc groups, similar to that observed in naïve animals. IgG concentration (c and d) remained low in both AOi and AOc groups throughout the follow-up period. IgA (e and f) in the AOi group was relatively higher than the AOc group after 70 days post-transplant but this was not statistically significant.

5.3.4 Persistence of donor antigens after transplant

Blood group antigen expression after transplant was examined in all graft biopsies in both groups. A-antigen expression in the AOi group and H-antigen expression in the AOc group are shown in Figure 5.4 (final biopsy from each pig except M3 and M5, for which first biopsies are presented). Antigen expression was clearly persistent long-term post-transplant. Tissue distribution of A and H antigen expression (Figure 5.4) was comparable to naïve kidneys (Figure 5.2b).

5.3.5 Allograft rejection studies of AOi and AOc kidney transplants

Banff classification rejection profiles of both transplant groups are presented in Table 5.1 and 5.2. All surviving donor kidneys were well-perfused and of normal size compared to native kidneys at study end-point.

AOi group: No deposition of complement component C4d or histological signs of antibody-mediated rejection (ABMR; endothelial injury with vasculitis, glomerular or small vessel thrombosis, glomerulitis, marginating cells in peritubular capillaries) were observed in any biopsies at any timepoint. No T-cell-mediated rejection (TCMR) or borderline minimal rejection was seen in early biopsies (days 11-24 days post-transplant); sample from pig M1 was

inadequate. In late biopsies, neither TCMR nor chronic changes were seen in pig M16. In pig M1, mild (grade IA/IB) TCMR was seen at 57 and 98 days post-transplant respectively, and grade I chronic changes at day 98. In two recipients (M3 and M5) the grafts were resorbed despite minimal evidence of early TCMR, leaving only nodular remnants at 56 and 54 days post-transplant respectively; no identifiable graft tissue was present at 90 days when final biopsy was attempted.

AOc group: In 3 of 4 piglets, no TCMR or borderline minimal TCMR was seen in early biopsies. In pig M8, mild TCMR (grade IA/IB) occurred early, as well as grade I chronic changes. Late TCMR (grade III) and grade I chronic changes occurred at 90 days in pig M19. No signs of ABMR were observed in any biopsies.

5.4 Discussion

An animal model of ABO-i organ transplantation is needed to study mechanisms of rejection and tolerance that are impossible to investigate in humans, particularly in children. In piglets, abundant expression of blood group antigens in kidney was reported by Rydberg *et al*[15] and is confirmed by our results. However, in contrast to earlier reports of A-antigen expression in pig endothelium and endocardium[23], we did not detect A-antigen in porcine cardiac tissue or vascular endothelium, consistent with later studies[13]. This may be attributed to differences in techniques and antibodies used. It is also important to note the inconsistencies in the study by Bravery et al.[23] that reported positive findings with only one of the two immunofluorescence techniques used in addition to observing differences among primary antibodies, particularly negative staining of pig heart tissues with anti-ABH antibodies despite positive staining of human heart tissues of the same blood group.

Our study shows that the developmental profile of endogenous anti-A antibody production in neonatal pigs is largely analogous to human infants, including a window in early life during

which anti-A is naturally absent. Accordingly, all kidney transplants in this study were performed at age 21-35 days when anti-A antibodies were not detected. Production of 'natural' antibodies against non-self A/B antigens is thought to be an immunologic cross-reaction stimulated by exposure to non-pathogenic gut flora carrying structurally similar polysaccharide epitopes[24-27]. In humans, anti-A/B IgG antibodies may be as high at birth as maternal levels due to trans-placental transfer, tending to disappear within weeks; endogenous isohemagglutinin production generally starts mid-infancy[8]. Anti-A/B antibodies of IgM and IgA isotypes remain low or undetectable in the first 3-6 months before gradually increasing to adult levels by 10-12 years of age[8-10]. In contrast to humans, trans-placental transfer of antibodies of any isotype into the porcine fetal circulation does not occur[28, 29]. Therefore, anti-A/B antibodies detected in newborn piglets were likely transferred through colostrum that is rich in antibodies of all isotypes[30] (data not shown).

The spike in anti-A antibodies observed in the antibody development study was likely stimulated by blood group-A-like substances we detected in the Suvaxyn E-oral vaccine preparation (data not shown); blood group-B-like substances were not detected in the vaccines. The transplant groups (AOc and AOi) did not receive vaccines and did not exhibit an anti-A spike. Stimulation of anti-A production by vaccines has also been observed in humans after pneumococcal vaccination[31-33]. Since vaccine was administered after the onset of endogenous anti-A antibody production, this did not prevent us from characterizing the onset of natural antibody development in piglets.

ABOi heart transplantation in human infants induces specific B-cell tolerance to donor blood group antigens[12]. ABH antigens are known to be expressed in vascular endothelium in humans[1], and our previous studies confirmed donor A/B antigen persistence in graft biopsies more than 4 years after ABOi infant heart transplantation[12]. However, precise mechanisms of graft A/B-antigen and host cellular interactions that lead to establishment of B-cell tolerance are unclear, including whether transient antigen exposure during immunological immaturity

is adequate or whether persistent antigen exposure is required. In this study, two AOi kidney grafts survived at least two weeks while others survived to 100 days, with clear A-antigen expression evident in all biopsies. The development of anti-A antibodies in both AOi and AOc groups, in comparable quantity and time-frame to naïve piglets, not suppressed by cyclosporine, indicates that neither specific B-cell tolerance nor sensitization to A-antigen occurred despite long-term persistence of A-antigen in the donor kidneys.

The outcome of antigen exposure is likely to depend not only on antigen persistence but also on antigen load and location of antigen encounter by the host immune system[34, 35]. Despite abundant expression of A-antigen in pig kidney tubules, absence of ABH antigen expression on vascular endothelium may have precluded neonatal tolerance in this model. However, other factors may also contribute, including but not limited to, differences in the immune system between human infants and young piglets and the effects of cyclosporine. Certain aspects of the swine immune system are characteristic, if not unique, to the species. An important example of this difference is the manner of providing passive immunity and the precosial nature of its offspring. The porcine fetal immune system develops in an environment separated from maternal regulatory antibodies and lymphocytes by an epitheliochorial placenta[26]. In addition, phenotypic characteristics of T and B cells such as the existence of unique CD4+8+ T cells in porcine peripheral blood[36] and the presence of thymic B cells that spontaneously secrete antibodies in fetal and neonatal pigs[37] may be influential.

Graft 'accommodation' was first described in the context of clinical ABOi kidney transplants in which graft damage did not occur despite return of circulating antibodies specific for donor blood groups and persistent graft A/B antigen expression[38, 39]. In the current study, the presence of anti-A antibodies and persistence of graft A-antigens in the absence of antibody-mediated damage in AOi recipients whose grafts survived long-term suggests accommodation occurred. However, we are limited in this interpretation because histological assessment of

ABMR according to Banff classification is largely based on changes in the vascular compartment[21, 40] where blood group antigens are expressed in human kidneys.

Although short-term kidney transplant studies in adult pigs have been described previously[41-43], models of kidney transplants in young piglets have not been reported. The goal of this study was to expose piglets in early life to non-self A/B antigens in a vascularized graft to attempt to model ABO B-cell tolerance as observed in young human ABOi heart transplant recipients. Maintenance of physiologic graft function was not a goal, therefore one native kidney was left *in situ* and kidney function was not monitored. Nonetheless, biopsy evidence suggests that grafts survived up to three months in 75% (6/8) recipients with minimal rejection using oral cyclosporine monotherapy. The finding that two grafts in the AOi group were completely scarred despite normal biopsies at 11 and 13 days post-transplant is likely due to late vascular complications (*e.g.* thrombus); nonetheless, graft destruction due to TCMR, ABMR or both cannot be ruled out. One animal showed at a late time point (day 98) numerous dense nodular, follicular lymphatic aggregates in the renal cortex, mostly in peri-vascular areas. Such infiltrates have been described in human renal allografts, in increasing frequency with time post-transplantation[44]. Their role in the rejection process still needs to be determined. The biopsy did not show histologic features suggestive of an infectious process; neither were viral inclusions observed or clusters of neutrophils as a sign of a bacterial infection.

The absence of natural antibodies, as well as immaturity of other key components of the immune response to carbohydrate antigens including memory B-cells and/or plasma cells, are likely important in the propensity of young children to develop B-cell tolerance upon introduction of an ABOi heart graft[12]. Although age boundaries have been pushed somewhat beyond infancy[45, 46], the age at which ABOi heart transplantation can be performed safely without aggressive interventions remains to be defined. Due to improved early survival after palliative cardiac surgery, some children with congenital heart disease will require a heart

transplant only in later childhood when susceptibility to immune tolerance has diminished. Delineation of the mechanisms by which B-cell tolerance occurs in this setting may allow development of novel strategies for intentional induction of ABO tolerance and continued extension of the window of opportunity for safe ABOi transplantation. Furthermore, the impact of variable antigen expression in different tissues (heart vs kidney) clearly needs further investigation. Thus, studies should continue toward elucidation of an appropriate animal model of ABOi transplantation.

5.5 Acknowledgements

We thank the staff at the University of Alberta Swine Research Centre, Health Sciences Lab Animal Services and the Surgical Medical Research Institute for their contribution to this study. This work was supported by a Fellowship Grant from the International Society for Heart and Lung Transplantation.

5.6 Tables

Table 5.1. Post-transplant rejection history in AO-incompatible (A to O) transplant groups according to the Banff classification

Pig ID	Time of biopsy (days post op)	Banff grade acute TCMR	Banff grade chronic damage / IFTA	Histological signs of ABMR	C4d	Comments
A to O						
M1	14	n/a	n/a	No	Neg	Inadequate biopsy
	57	IA (i2 t2 v0)	0	No	Neg	
	98	IB (i2 t3 v0)	1 (ci1 ct1 cv0)	No	Neg	Numerous dense nodular, follicular lymphatic aggregates
M3	13	Borderline (i1 t1 v0)	0	No	Neg	
	56					Scarring
	97					Scarring
M5	11	Borderline (i2 t1 v0)	0	No	Neg	
	54					Scarring
	96					Scarring
M16	21	0	0	No	Neg	
	50	0	0	No	Neg	
	85	0	0	No	Neg	

Neg, Negative; IFTA, Interstitial fibrosis and tubular atrophy

Table 5.2. Post-transplant rejection history in AO-compatible (O to O) transplant groups according to the Banff classification

Pig ID	Time of biopsy (days post op)	Banff grade acute TCMR	Banff grade chronic damage / IFTA	Histological signs of ABMR	C4d	Comments
O to O						
M7	24	0	0	No	Neg	
	53	0	0	No	Neg	
	88	0	0	No	Neg	
M8	23	IA (i2 t2 v0)	0	No	Neg	
	53	IB (i3 t3 v0)	1 (ci1 ct1 cv0)	No	Neg	
	65	IB (i2 t3 v0)	1 (ci1 ct1 cv0)	No	Neg	
M18	20	Borderline (i1 t1 v0)	0	No	Neg	
	49	Borderline (i1 t2 v0)	0	No	Neg	
	85	Borderline (i1 t3 v0)	0	No	Neg	
M19	20	Borderline (i1 t1 v0)	0	No	Neg	
	49	Borderline (i1 t2 v0)	0	No	Neg	
	90	III (i2 t3 v3)	1 (ci1 ct1 cv1)	No	Neg	

Neg, Negative; IFTA, Interstitial fibrosis and tubular atrophy

5.7 Figures

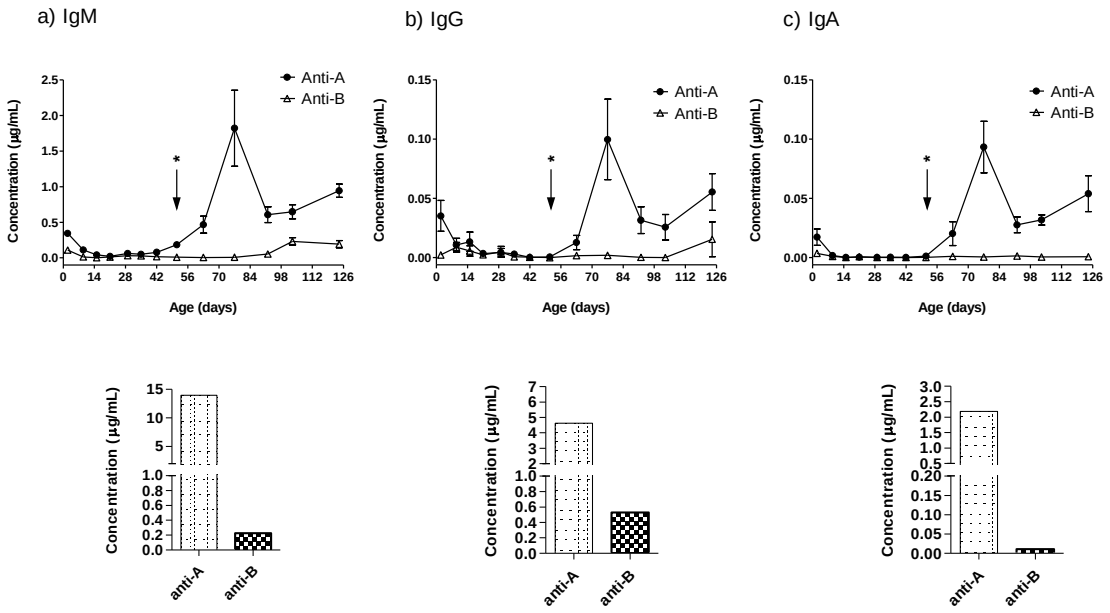
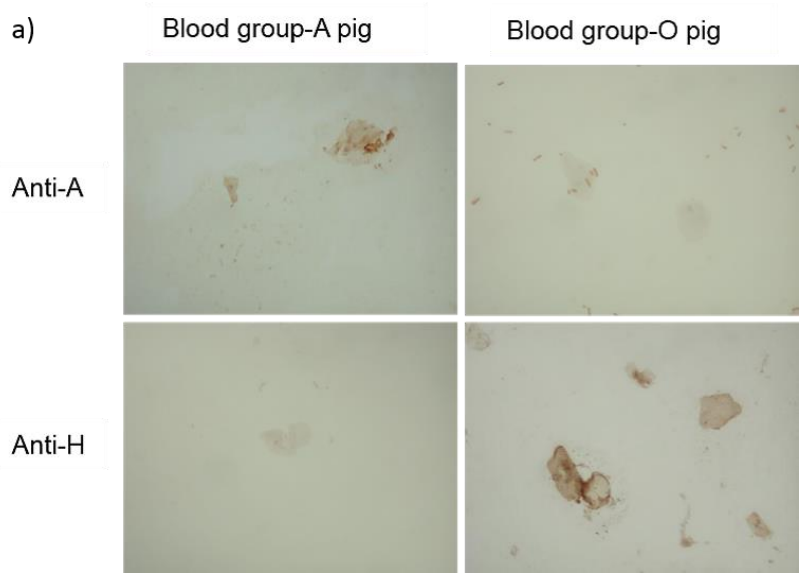
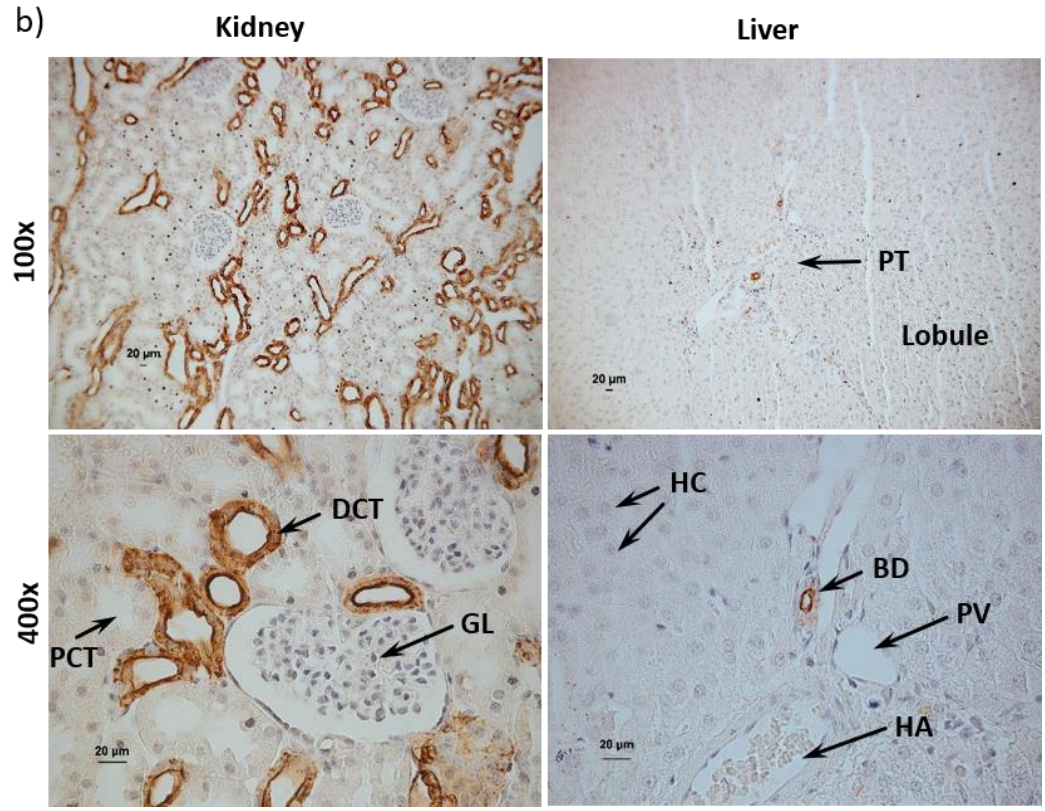
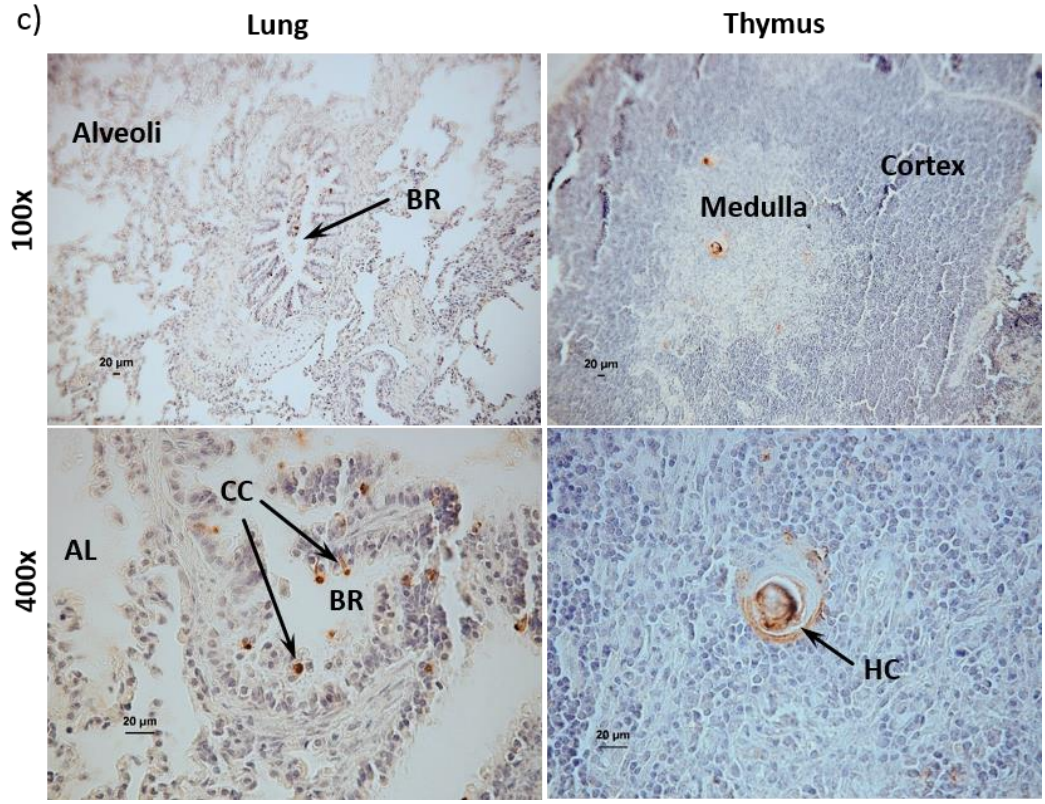


Figure 5.1. Anti-A and anti-B antibody development in blood group-O pigs (n=7). Plasma concentrations (µg/ml) of anti-A/B **a**) IgM, **b**) IgG and **c**) IgA antibody from birth to 124 days are shown. Values represent mean (±SEM). Positive controls for corresponding IgM, IgG and IgA antibodies in plasma from an immunized pig are shown. * Vaccination

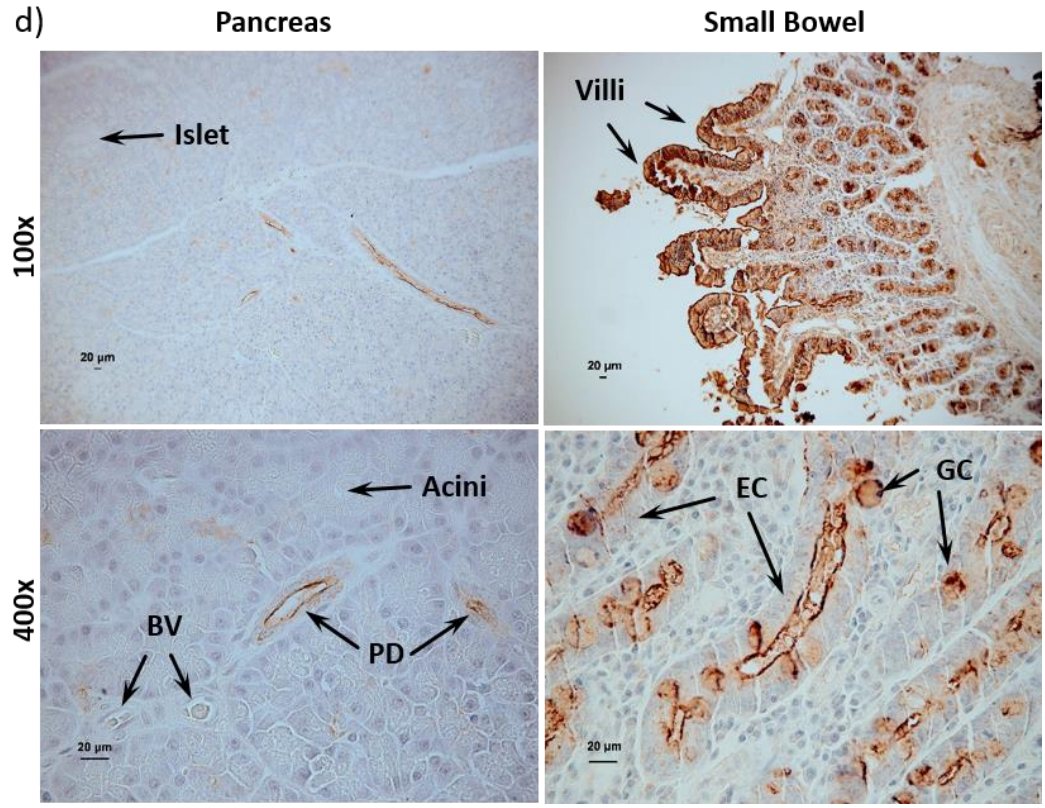




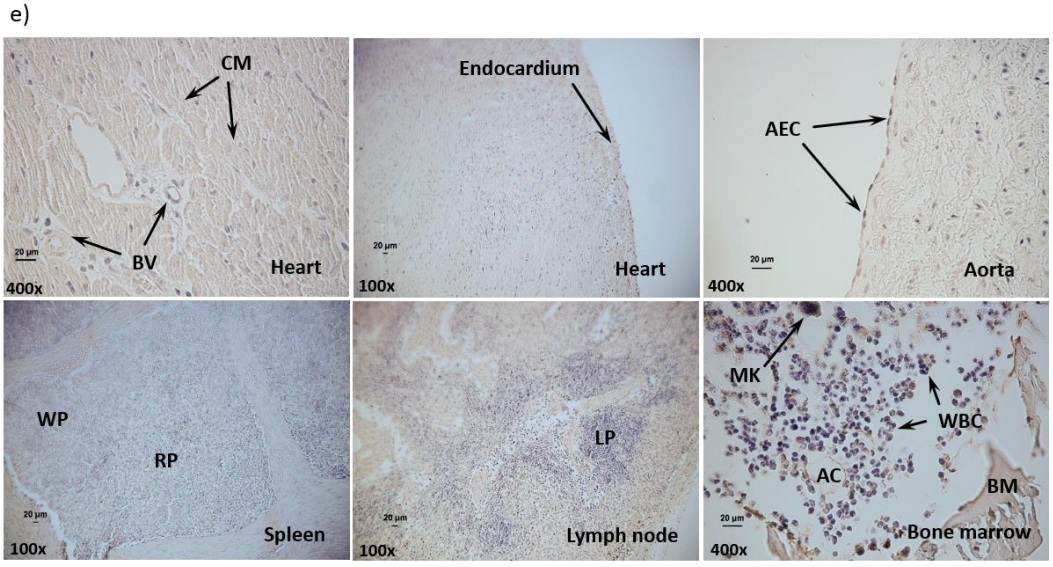
GL-Glomerulus; **DCT**-Distal Convoluted Tubule; **PCT**- Proximal Convoluted Tubule; **PT**- Portal Tract; **HC**- Hepatocytes; **BD**- Bile Duct; **PV**- Branch of Portal Vein; **HA**- Branch of Hepatic Artery.



BR- Bronchiole; **AL**- Alveoli; **CC**- Clara Cells; **HC**- Hassall's Corpuscle.



BV- Blood Vessels; **PD**- Pancreatic Ducts; **EC**- Enterocytes; **GC**- Goblet Cells.



BV- Blood Vessels; **CM**- Cardiomyocytes; **AEC**- Aortic Endothelial Cells; **WP**- White pulp; **RP**: Red pulp; **LP**- Lymphoid Follicle; **MK**- Megakaryocytes; **BM**- Bone Matrix; **AC**- Adipose Cell; **WBC**- White Blood Cells.

Figure 5.2. Light microscopy images (100x – 400x) of A-antigen and H-antigen expression in tissues and organs of blood group A and O pigs. **a,** Example of blood group determination by buccal cell immunohistochemistry. A-antigen expression was clearly seen in buccal cells from blood group-A pigs whereas cells from blood group-O pigs expressed H-antigen and lacked A-antigen. **b-d,** Tissue expression of A-antigen in various tissues and organs from 4 week old blood group-A pig. A-antigen expression was detected in distal tubules and collecting ducts of kidney and in other tissues including mucous-secreting cells of terminal bronchioles in lung, ductal epithelial cells of liver and pancreas, Hassall's corpuscles of thymus and strong expression in small bowel goblet cells. **e,** A-antigen expression was not detected in vascular endothelium, heart, spleen, lymph nodes or bone marrow cells. Tissue structures are labelled and are similar to that of human with the exception of lymph node which has an inverted structure in pigs[47].

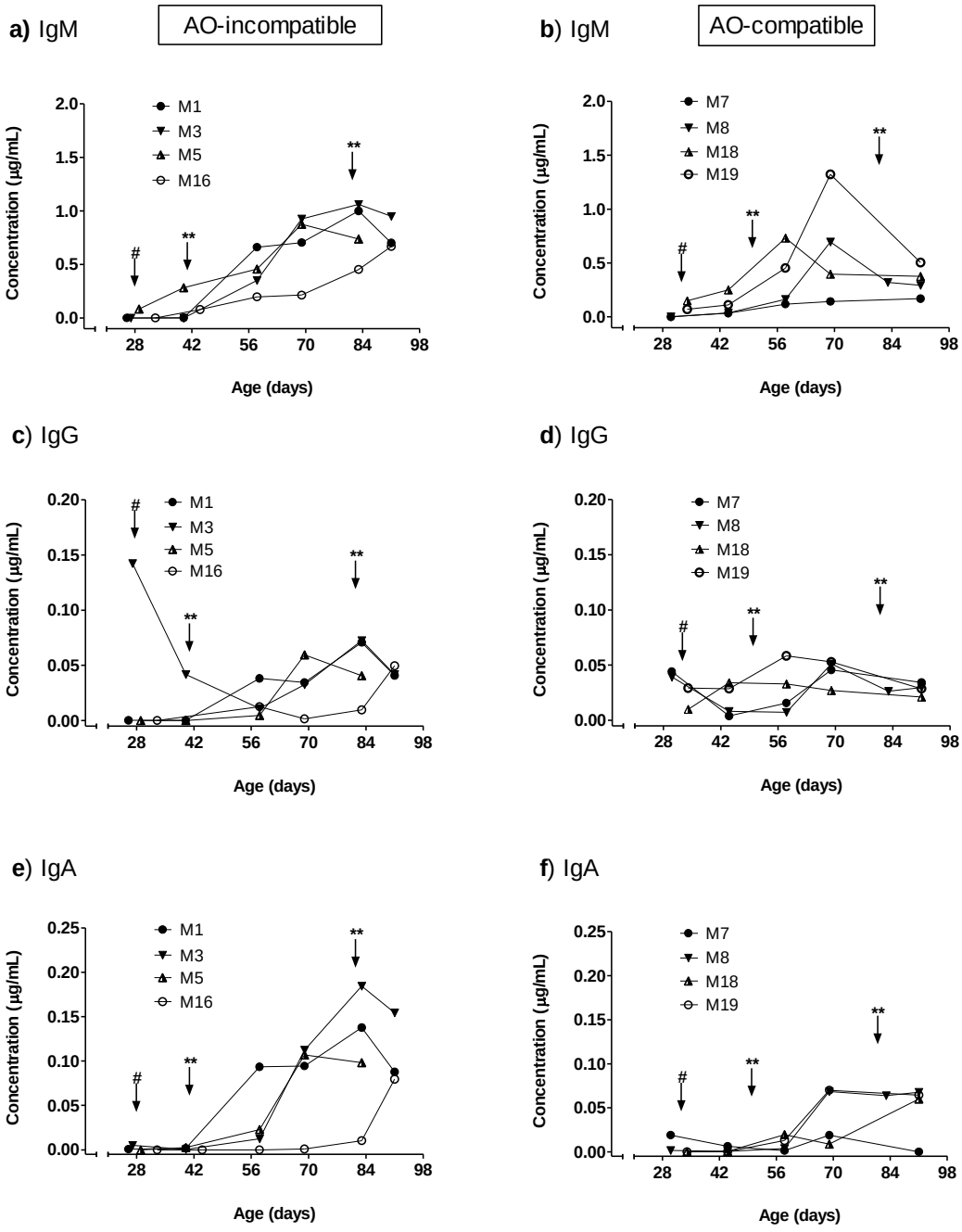
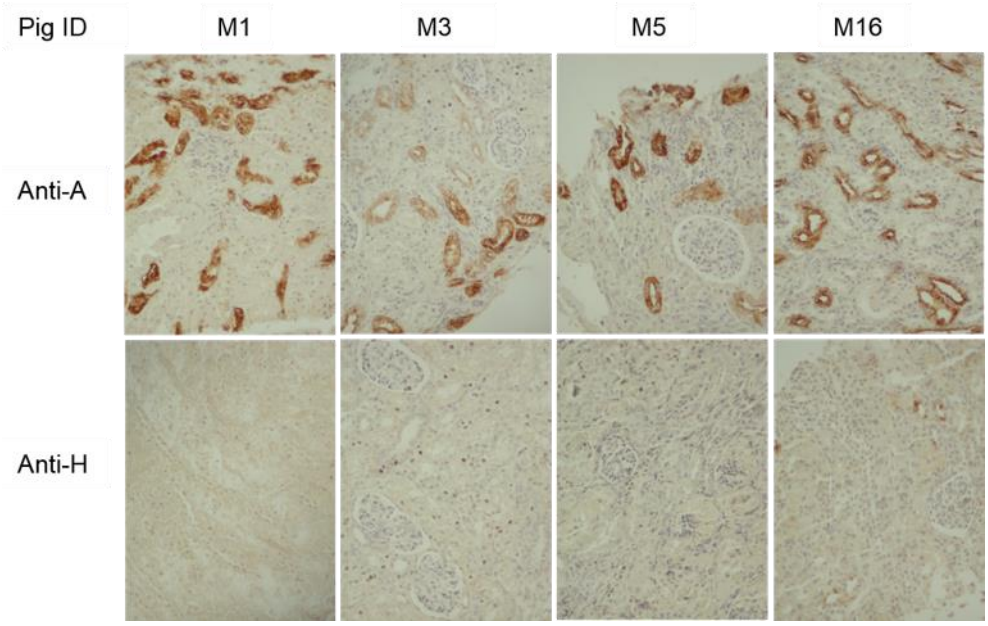


Figure 5.3. Anti-A antibody levels pre-and post-transplant. Plasma concentrations (µg/ml) are shown of anti-A IgM (**a and b**), IgG (**c and d**) and IgA (**e and f**) in AO-incompatible (n=4; panels a,c,e) up to day 91 and AO-compatible recipients (n=4; panels b,d,f) up to day 91. **#** Transplant; ****** Biopsy; M1-19 are pig ID's.

a)



b)

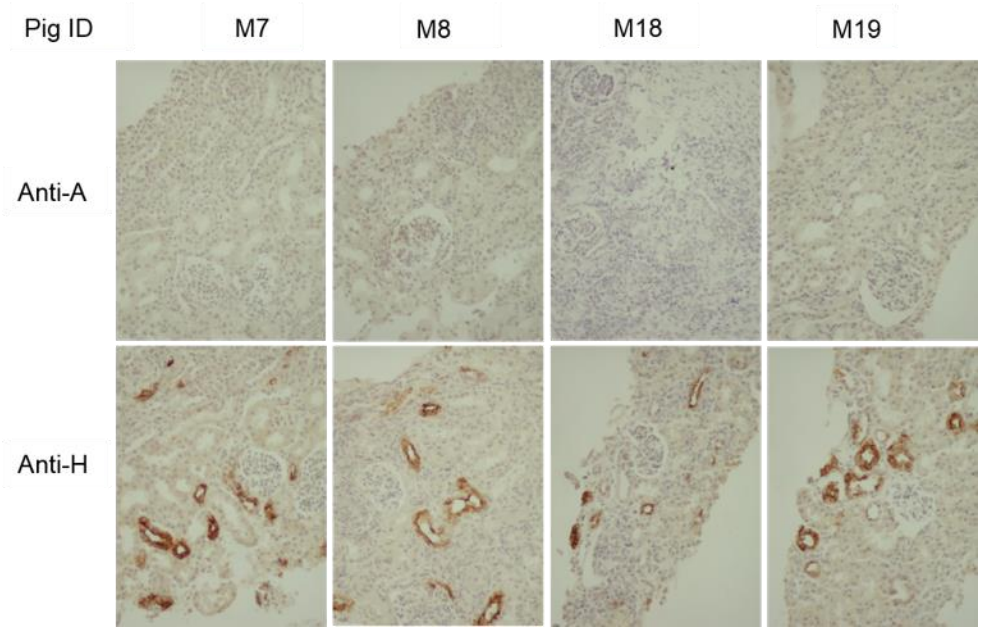


Figure 5.4: Light microscopy images (x100) of donor blood group antigen expression in post-transplant biopsies. **a,** A and H-antigen expression in kidney graft biopsies from pigs M1, M3, M5 and M16 at 98, 13, 11 and 85 days post-transplant respectively. **b,** A and H-antigen expression in kidney graft biopsies from pigs M7, M8, M18 and M19 at 88, 65, 85 and 90 days post-transplant respectively.

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Chapter 6

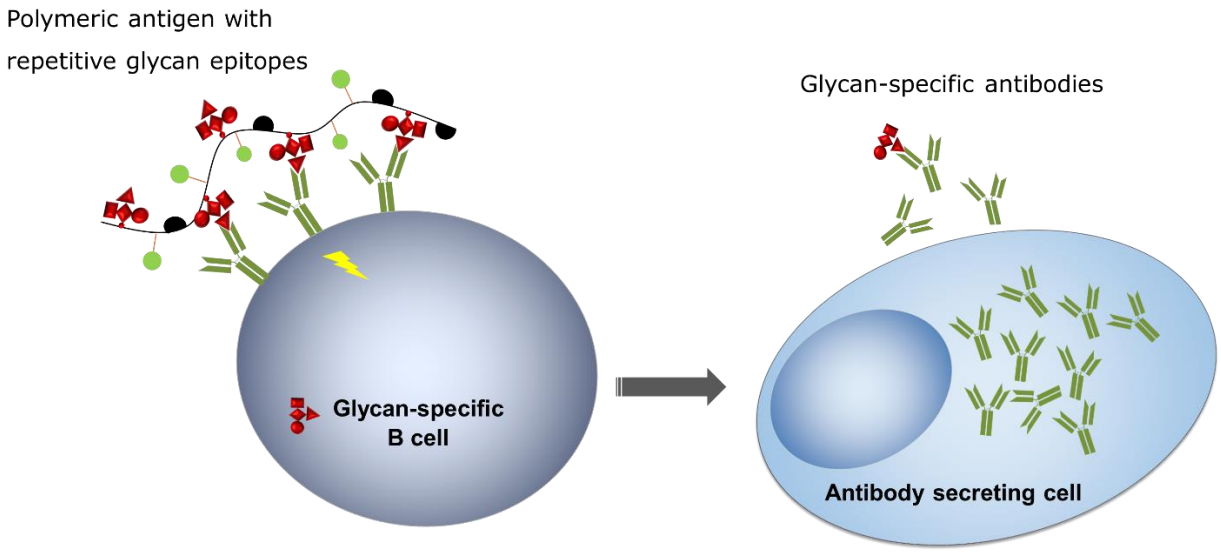
General Discussion

In this thesis, I have attempted to clarify some fundamental issues in immunology and transplantation that are not well understood. Since each chapter includes a detailed discussion on the relevant subject I shall keep this section brief. Some remarks are based on additional experiments that are not presented here while others may be speculative in nature.

6.1 Immune response to carbohydrates and self-nonself (SNS) discrimination

Unlike proteins, carbohydrates are made up of simple monosaccharide units that exist abundantly in nature. Most polysaccharides are made up of a limited number of monosaccharide units and possess antigen epitopes that are identical. Chronic exposure to these antigens may induce natural antibodies in animals and humans. This causes difficulties when studying primary antibody responses to such antigens, particularly when polysaccharides from pathogens that colonize animals are used. These pre-existing antibodies (as well as memory and plasma cells) make the interpretation of primary antibody responses more difficult and misleading at times.

According to the current paradigm, cross-linking of B cell receptors by antigens with repetitive epitopes induces B cells to produce antibodies against that antigen[2] without the participation of T cells (as shown below).

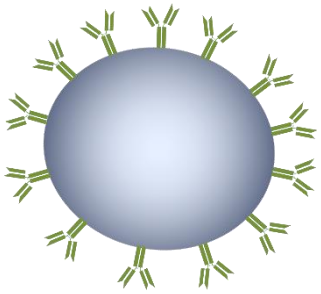


Evidence for this rule is not overwhelming and was constructed from the generalization of early experiments with trinitrophol (TNP) derivatives. Although we have not shown direct evidence for BCR cross-linking by the antigens used in this study, these (ABH antigens) have been previously classified as TI type-2 antigens[3]. In addition to their polymeric nature, blood group ABH antigens are densely (approximately three million blood group A antigens on A red cells[4]) packed in the glycocalyx of the red cell surface.

Red cell

Blood group-A antigens
~ 3 million

B-cell



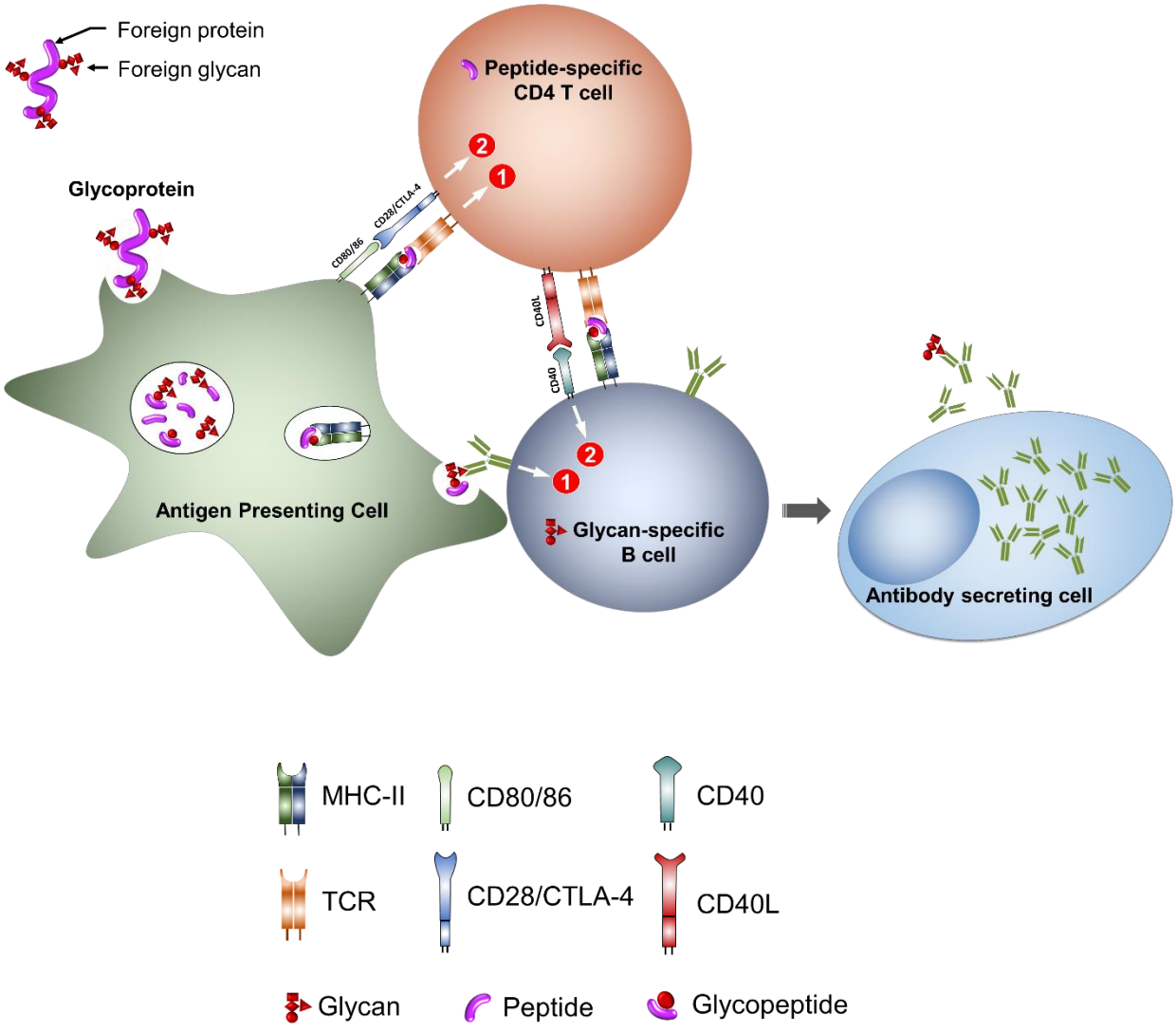
B-cell receptors
~ 120 000[1]

Despite having all the features of TI type-2 antigens, blood group A and B red cells did not induce anti-A/B antibody production in CD4 T cell-depleted mice. This indicates that the interaction between B cells and polymeric antigen epitopes alone may not be adequate for differentiation of B cells to antibody producing cells.

This model and the classification of TI antigens leave many unanswered questions; examples include i) how does the adaptive immune system distinguish foreign polymeric antigens from self and control autoimmunity? ii) what are the mechanisms for memory formation and isotype switching? iii) what are the mechanisms of antibody responses to carbohydrates that are not polymeric in nature. The existing model in fact does not address any of these issues. A plausible model should accommodate many features that have been established in the context of antibody responses to protein antigens. The model described in chapter 2 within

the framework of the two-signal model provides a basis for addressing such issues. Based on experiments described in chapter 2, a number of hypothetical scenarios are presented below (a requirement for this model is that the carbohydrate antigens are presented as glycoproteins).

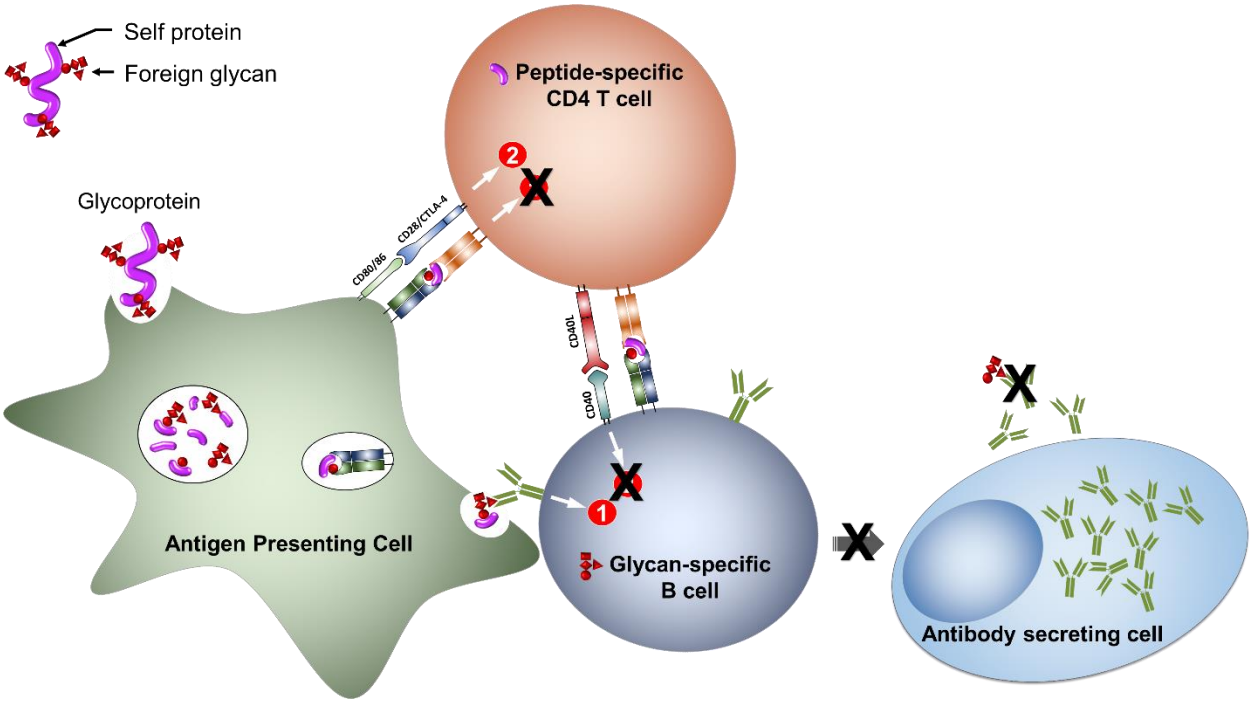
Scenario 1. Response to a glycoprotein containing foreign protein and glycan



In Scenario 1 the antigen-presenting cell (APC) can itself be a B cell. However participation of other professional APC is likely due to their ability to process or transport antigens from peripheral tissues to lymphoid organs and activate T cells far more efficiently. Interaction

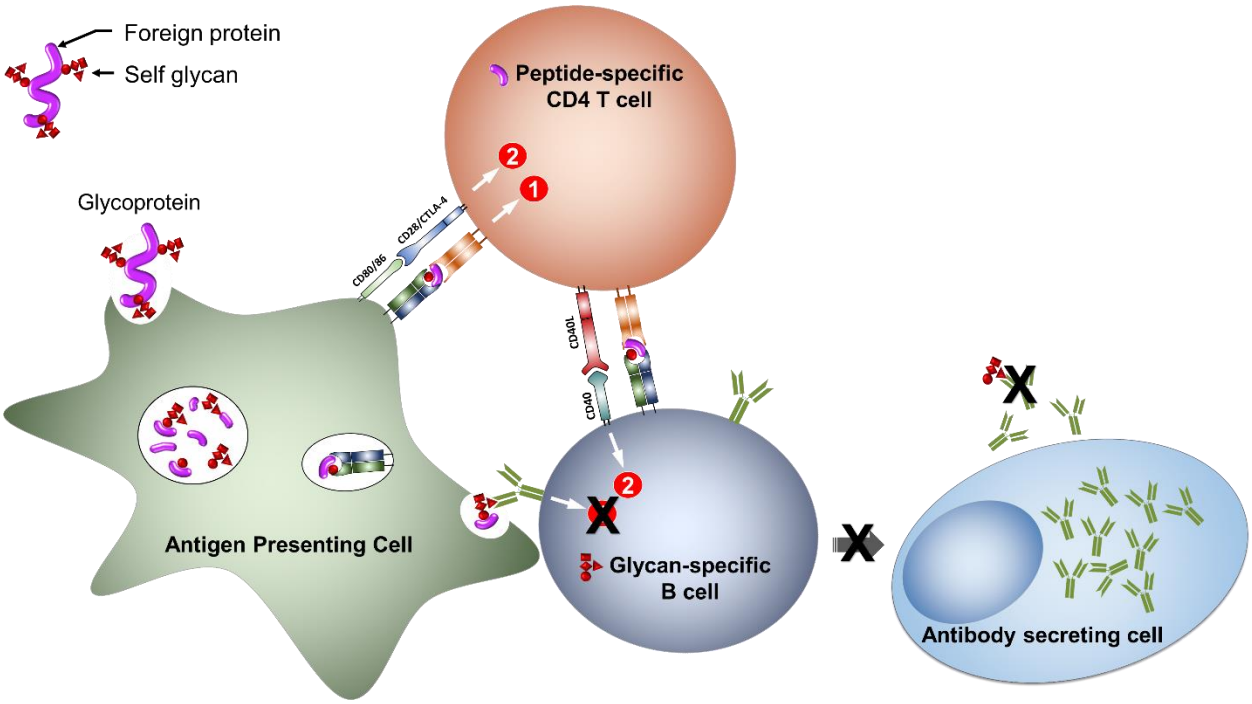
between unprocessed or processed glycan and a glycan-specific B cell receptor generates signal-1. The B cell receives signal-2 from an activated CD4 T cell, recognizing the peptide component of the glycopeptide presented on MHC class II, through CD40/CD40L interaction. This interactions would result in anti-glycan antibody production. A similar encounter between peptide-specific B cell and peptide-specific T cell may result in anti-peptide antibody production.

Scenario 2. Response to glycoprotein containing self-protein and foreign glycan



In Scenario 2, anti-glycan antibody production is not expected despite the fact that the glycan is foreign. Although signal-1 is generated from the interaction between the foreign glycan and a glycan-specific B cell, the B cell does not receive signal-2 due to mechanisms that exist to maintain central and peripheral tolerance to self-protein antigens in T cells.

Scenario 3. Response to glycoprotein containing foreign protein and self-glycan



Experiments indicated that tolerance to self-glycan structures cannot be broken even if attached to a foreign protein. In Scenario 3, central tolerance to self-glycan structures is expected to be maintained at B cell level (*ie*, absence of self-glycan-reactive B cells or through receptor editing). Encounter between peptide-specific B cell and peptide-specific CD4 T cell may still result in anti-peptide antibody production and glycation of the protein may not interfere with this process. The observation that individuals only generate antibodies against blood group antigens they lack supports the above interpretation.

Many other observations in animals and human are supportive of the two-signal model for responses to carbohydrate antigens. As shown in chapter 2, anti-A antibody production can be elicited in mice using blood group A red cells from humans. However, the antibody response was deficient when A red cells from blood group A transgenic mice were used (data not shown). Eliciting an anti-αGal antibody response in Gal knockout (do not express αGal) mice required immunization with porcine cells. In mice, proteins that are glycosylated with

the blood group A or αGal carbohydrate antigens are likely to be the same (*ie*, self-protein for mice). Therefore, elicitation of a response required cells from a different species having the same glycan but attached to a foreign protein. Similarly, ABO-mismatched vascular tissues are often used in children undergoing surgery for their congenital heart defects. They become sensitized to HLA antigens[5,6] but neither sensitization nor tolerance to graft ABO antigens has been observed[6].

The mechanism described above applies to robust primary antibody responses to carbohydrate antigens. It may be possible that chronic priming with polysaccharides results in non-specific (or bystander activation) signals to glycan-specific B cells, eliciting low level of IgM antibody production. Unravelling of the two-signal model for antibody responses to carbohydrates has implications in many areas including infection, autoimmunity, allo- and xeno-transplantation, cancer and polysaccharide vaccine design and this will likely spur more research in these areas.

6.2 Declassification of T-dependent (TD) and T-independent (TI) antigens

Classification of immune responses can help to clarify experimental observations and advance knowledge translation. However, misinterpretation of experimental results due to some classifications may impede progress. Perhaps TD/TI classification falls into such a category. Nearly four decades have passed since the first description of TNP derivatives as model antigens and these are still widely used in the study of immunobiology[7-10]. Contrary to protein antigens, decades of research has not resulted in fully understanding the mechanism of immune response to TI antigens. This raises doubts about the use of polymer-TNP conjugates as model antigens and generalization of their characteristics to all natural carbohydrates or polysaccharides. It is known now that TI antigens such as lipopolysaccharide (LPS) can bind to specific toll-like receptors that are expressed in memory B cells and induce proliferation and differentiation to antibody-secreting cells[11,12]. Serum from naïve mice contained anti-TNP

antibody titre in the range of 1:10,000 to 1:100,000 without prior active immunization. The antibody response to immunization with TNP-ficoll far exceeded that of any natural antigens (data not shown). This indicates that either TNP is abundantly expressed in the environment and induces 'natural' antibodies in mice or that TNP interacts with antibodies and immune cells by a mechanism that differs from other natural antigens. The pre-existing TNP binding antibodies and secondary immune responses may produce results that are misleading. The likelihood of this highly volatile chemical interacting with the immune system by a mechanism other than antigen-receptor interaction (similar to that seen with super antigens) has not been studied.

Studies presented in chapter 2 suggest that both proteins and carbohydrates are T cell dependent but the nature and degree of T cell help may vary between the two types of antigens. Therefore re-assessment of the classification based on genetically defective B cells and their response to certain haptens[13] can help to clarify the immunobiology concerning TI antigens.

6.3 Acquired B cell tolerance to ABO antigens

In 1953, Billingham, Brent and Medawar described acquired tolerance in mice[14]. The acquired tolerance described in their original and subsequent series of studies were focused on different strains of mice (*ie*, MHC protein antigens). Fan *et al*. described acquired tolerance to blood group carbohydrate antigens in children who received ABOi heart transplants as infants[15]. These children acquired tolerance to ABO (carbohydrates) antigens but not to HLA (proteins) antigens. The question is whether the observed deficiency of antibodies against donor blood group antigens is due to tolerance or lack of immune response due to some form of immune suppression. It can be argued, according to the model for antibody response to carbohydrates described in this thesis, that ABO incompatibility across the same species is not expected to elicit an antibody response against the incompatible antigen. The lack of sensitization

following ABO-incompatible tissue or organ transplantation in children and adults supports this. Dealing with pre-existing antibodies and antibody-producing cells, rather than continued immune responses long after transplant, has been most challenging in these patients. However, the observed phenomenon may not be completely due to suppression of immune response. The argument for tolerance induction can be made with an example: a blood group O recipient of an A heart failed to produce anti-A (donor-specific) antibodies long after transplant, yet produced anti-B antibodies. A blood group O recipient of similar age receiving ABOc heart transplant produced both anti-A and anti-B antibodies under similar T cell-directed immunosuppressive therapy. In both of these patients, nature performs its own experiment to break tolerance in the form of environmental stimuli (such as colonizing bacteria and other pathogens) with glycoproteins that contain blood group A- and B-like carbohydrate antigens on foreign proteins. Donor-specific antibodies remained deficient but the immune system retained the ability to respond to third-party antigens. Therefore mechanism(s) of central tolerance at B cell level are likely to exist in these patients. Induction of tolerance to blood group and other carbohydrate antigens can be valuable in allo- and xeno-transplantation. Perhaps, introducing the antigen in the right place at the right time and keeping it there is all that is required for tolerance induction to carbohydrate antigens.

6.4 Donor-specific antibody monitoring

Significant technological advances have been made in the field of histocompatibility and sensitive assays for detecting donor-specific antibodies to HLA antigens continue to evolve[16]. In contrast, ABOi transplant patients are managed using a 'shotgun' approach of antibody titre monitoring that has not evolved in 100 years. The erythrocyte agglutination test is the only method currently used in clinical laboratories for assessing antibodies against blood group antigens. Patients are assessed for their suitability to receive ABOi transplants using the agglutination titre. Pre- and post-transplant antibody removal maneuvers are also guided by this titre. The glycan microarray provides a solution to this problem. It can be used to

measure IgM and other important isotypes against blood group antigens expressed in the organ.

As demonstrated in chapter 3, only antibodies against ABH type II subtypes can be considered 'donor-specific' in ABOi heart transplant recipients. This method can also be useful in the larger numbers of patients undergoing ABOi kidney transplantation. In the human kidney, ABH type II structures are expressed on endothelial cells of blood vessels, including glomerular capillaries. Blood group A individuals additionally express A type III/IV structures in the tubular epithelium (data not shown). Studying antibodies against ABH subtype antigens in these patients can help us understand the importance of commonly reported agglutination titres post-transplant. Furthermore, the existence of the phenomenon 'transplant accommodation' can be clarified.

6.5 Future directions

Data presented in this thesis suggest that immune responses to carbohydrates are more T-dependent than protein antigens when the IgM response is considered. However, there is a need for further clarifying experiments before generalizing the described mechanism to all carbohydrate antigens. The current paradigm of antibody responses to carbohydrate antigens was deducted from studies using mostly TNP-polymer conjugates as model antigens. The exact nature of the interaction between TNP-polymer conjugates and lymphocytes needs to be elucidated. Experiments should be designed and carried out to determine whether it is only the antigen-BCR interaction that leads to an anti-TNP antibody response or non-specific stimulation of B cells and/or other cells leading to proliferation and differentiation of existing memory B cells or plasma cells to secrete excess antibodies. Primary antibody responses to a variety of bacterial polysaccharides should also be studied. Achieving this objective can be tricky because most animals and humans develop natural antibodies against many common polysaccharides that are expressed on microflora. Using germ-free animals may not provide

a solution due to their abnormal immune responses[17]. So it may be necessary to restrict these studies to a limited number of different polysaccharides that are unique to certain pathogens that do not colonize mucous membranes. Furthermore, requirements for isotype switching in antibody responses to carbohydrates likely differ from protein antigens. The proximity of the sugar epitope to the protein in a glycoprotein as well as the immunogenicity of the protein likely determine the degree of isotype switching to IgG. This postulate need to be tested in experiments using glycopeptides containing a peptide and a sugar epitope separated by different number of monosaccharide units. The information obtained from such experiments would be a valuable tool in modulating antibody responses to polysaccharide vaccines.

Acquired B cell tolerance to blood group antigens following ABOi heart transplantation in infants suggests that one or more features of the developing immune system facilitate this process. It may be proposed that predominantly immature or transitional B cells in neonates are susceptible to tolerance, and that diminished CD40 ligand expression on T cells[18-20] contributes to this. Such mechanisms may also be responsible for tolerance induction to blood group antigens in adults following ABOi transplantation once antigen experienced or when memory B cells are eliminated. Persistent expression of blood group antigens in the donor graft and the lack of T cell help to B cells specific for non-self blood group antigens (explained in section 6.3) likely cause newly emerging immature and mature B cells to become tolerant. This implies that some or most of ABOi kidney transplant recipients are more likely to have established tolerance to donor blood group antigens rather than accommodation. Assessment of truly 'donor-specific' blood group antibodies in these patients by ABO-glycan microarray may reveal the existence of tolerance in these patients.

The proposed model for antibody responses to carbohydrate antigens also provides a basis for understanding the challenges in xeno-transplantation. The expression of non-self carbohydrate antigens on foreign proteins in the xenograft likely induces effector T helper cells that can provide second signal to B cells resulting in vigorous antibody production and

isotype switching. Therefore, xeno-transplantation in adults requires extreme measures to deal with ongoing antibody responses to carbohydrate antigens expressed in the xenograft long after transplant. Tolerance induction strategies may help to overcome some of these hurdles. Such strategies to induce B cell tolerance to foreign carbohydrate antigens are more likely to succeed in neonates that in adults.

6.6 Summary

Carbohydrates are a major class of naturally-occurring organic compounds that exert diverse biological functions. Yet, mechanisms of immune response to these antigens remain unclear. Data presented in this thesis demonstrate that primary antibody responses to naturally-occurring glycoproteins occur only if the responding B cells receive a second signal from CD4 T cells through the CD40/CD40L pathway. A robust antibody response to the carbohydrate portion of such a glycoprotein thus usually requires recognition of the protein component by CD4 T cells, which more readily occurs if this component is foreign than self. Contrary to the current paradigm, our study demonstrates that most natural polysaccharide antigens are clearly T cell dependent in terms of generating an antibody response, and that the activation of their associated B cells involves a two-signal pathway, thereby revealing a mechanism for self-nonself discrimination of such antigens by the adaptive immune system.

Blood group ABH antigens are carbohydrates that are relevant to transfusion and transplantation. Based on carbohydrate moieties of precursor structures ABH antigens can be classified as subtype I-VI. These subtype ABH antigens are differentially expressed on cells and in major organs. In an international study of heart transplant patients, we demonstrate accurate assessment of antibodies against the ABH subtype antigens expressed in the donor heart (*ie*, 'donor-specific') using an ABO-glycan microarray. Our study also demonstrates the fine specificity of B cell tolerance to blood group antigen following ABOi heart transplantation

in infants. Accurate detection of truly 'donor-specific' blood group antibodies using the glycan microarray will be valuable in the management of patients undergoing ABOi transplantation.

Studies presented in this thesis challenge some of the current theories related to the immune response to natural carbohydrate antigens. In addition to characterizing blood group antigens and antibodies this work also offers solutions to improve patient management in the setting of ABOi transplantation. I anticipate that these findings will have significant implications in many areas including, but not limited to, immune responses to infection, cancer, transplantation, autoimmunity and carbohydrate vaccine design.

6.5 References

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