ABO Antibody Production in Mice

by

Ibrahim Sulieman Yahia Adam

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Immunology

Department of Medical Microbiology and Immunology
University of Alberta

Thesis Abstract

Introduction: ABO-incompatible organ transplants (ABOi-Tx) recipients are at high-risk of rapid rejection mediated by ABO antibodies (ABO Abs). To manage and expand ABOi-Tx, it is necessary to understand the mechanism(s) by which natural and induced ABO Abs develop. Prior studies have used chemically-synthesized carbohydrate (CHO) structures or mouse models with various limitations to study induction of ABO Abs production. 'Natural' (spontaneous) ABO Abs are thought to be produced in humans and mice without exposure to A/B-Ags by mechanisms that remain poorly understood, although early studies suggested a role for the participation of bacterial flora. West and colleagues found that infant recipients of ABOi heart Tx (HTx) develop B cell tolerance to donor A/B-Ags and that ABO Abs are mainly produced by $CD27^+$ IgM^+ B cells. CD22 participates in B cell tolerance and West research group found that $CD27^+$ IgM^+ B cells express high-levels of CD22 in human and this expression decreases with age. Chemically-synthesized CHO-Ags have also been used to study Ab response in *CD22 knock-out* (*KO*) mice, but various outcomes have been reported that could be attributed to the nature of these Ags and/or lack of consideration for natural Ab production. Herein, I investigated the role of CD4+ T cell participation in induction of ABO Abs production, the impact of CD4+ T cell, sex, and bacterial flora on development of natural ABO Abs, and the impact of CD22 modulatory molecules in natural and induced ABO Abs production.

Results: Wild-type (*WT*) mice were stimulated by A-Ag in the context of *syngeneic*, *allogeneic*, and *xenogeneic* stimulation. Injection of *syngeneic* A-transgenic blood cell membranes (A-Tg BCM) did not induce anti-A Ab production, but human blood group A BCM (Hu A-BCM) and *allogeneic* A-Tg BCM induced abundant anti-A Abs. However, Hu A-BCM failed to elicit anti-A Ab following CD4+ T cell depletion. In *CD4KO* mice, injection of Hu A-BCM did not induce anti-A Ab production, but reconstitution with sex-matched *WT* CD4+ T cells restored the ability of the *CD4KO* mice to produce anti-

A Ab to Hu A-BCM. In *CD4, MHC-II,* and *αβ/γδ T cell receptor KO* mice, females produced significantly higher natural anti-A Ab than males and Hu A-RBC injection did not induce more anti-A Ab beyond that naturally produced. Treatment of *CD4KO* mice with broad-spectrum antibiotics significantly reduced natural anti-A Ab production, but did not abolish it. Importantly, germ-free mice produced natural ABO Abs without exposure to A/B-Ags. *CD22KO* mice developed higher natural anti-A Ab than *WT* mice, with higher Abs production in female *CD22KO* than male mice. Furthermore, injection of Hu A-BCM induced massive amount of anti-A Ab production. In contrast to *WT* mice, anti-A Ab was elicited by *syngeneic* A-Tg BCM in *CD22KO* mice, or following CD4+ T cell depletion and Hu A-BCM injection.

Conclusion: Induction of ABO Abs production requires exposure to non-self A/B-Ags and foreign protein, consistent with T-dependent Ab response. In contrast, natural ABO Abs developed in the absence of CD4+ T cells, suggesting a T-independent Abs response. Production of dramatically higher natural ABO Abs in *CD4, MHC-II,* and *TCR KO* female mice than in males suggests a sex-linked role in natural ABO Abs production. In contrast to the common paradigm, bacterial flora is not absolutely required for natural ABO Abs production, suggesting that nAbs are spontaneously produced. In *CD22KO* mice, occurrence of higher natural and induced ABO Abs than *WT* mice and induction of ABO Abs production without CD4+ T cell participation suggests a modulatory role for CD22 signaling in ABO Abs production. **In summary, my findings indicate that:** *A*) Induction of ABO Abs production requires CD4+ T cell participation (T-dependent), *B*) Natural ABO Abs production occurs in complete absence of bacterial flora or CD4+ T cells (T-independent), *C*) Without CD22, induction of ABO Abs production no longer requires CD4+ T cells (T-independent), but CD4+ T cell participation result in more ABO Abs (T-dependent), and *D*) Sex hugely impacts natural ABO Abs level.

Dedication

I dedicate this thesis to my family,

To all transplant donors and recipients

Acknowledgements

I would like to express my deepest appreciation to my supervisor and mentor, Dr. Lori West. Her guidance shaped me into the scientist I am today, and her passion for the pursuit of knowledge opened my eyes to the wonders of the immune system.

Dr. Lori West has been an incredible mentor for me. I would like to thank her for giving me guidance and encouragement throughout my research training and the freedom to explore beyond the confines of our discipline. I am very thankful for your patience, understanding, kindness, friendship, and importantly, your support during difficult times.

It would have been impossible to stay on track all these years and to finish this doctoral thesis without your support. Your kindness and generous help was absolutely indispensable and immensely appreciated. It has truly been a pleasure to work under the supervision of Dr. Lori West.

I would like to thank my supervisory committee members, Dr. Robert Ingham, and Dr. Colin Anderson, for their patience, understanding, invaluable intellectual input and guidance throughout my PhD training. My gratitude goes out as well to my exam committee members, Dr Bart Hazes, Dr Frans Claas, and Dr James Smiley whom provided me with various advices during my training.

Thank you to all the people in the department of Medical Microbiology and Immunology for administrative support. My sincere thanks go to Dr. Edan Foley for his help and support. Thank you so much for welcoming me in your office and for providing me with the guidance and advices to complete my Ph.D. thesis. I also would like to thank Ms. Tabitha Vasquez for her technical and administrative help, not to mention her compassion and support for students during difficult times.

I am very thankful to Dr. Bruce Motyka, for contributing his time reviewing my writing and providing his feedback throughout my training period. Thank you for all your patience, motivation, enthusiasm, and your guidance to help me to improve my theoretical and analytical skills. Moreover, I am very grateful for the opportunity to interact and discuss with many colleges in the lab, especially Dr. Kesheng Tao, who has been a very good friend, and Dr. Kunimasa Suzuki, who provided help whenever I asked him.

I also want to thank many past and present members of the West Research Group, including Kim Biffis, Anne Halpin, Ingrid Larsen, Dr. Esme Dijke, Dr. Mylvaganam Jeyakanthan, Carrie Andrews, Dr. Roger Bascom, Tess Ellis, Jean Pearcey, and Dr. Simon Urschel, for their friendship, and for their invaluable support. Many thanks go to many other people who helped me through my degree both in the University of Alberta and in Edmonton. I am sincerely thankful for their generosity

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 Awatif Hamed for love and unwavering support through difficult times; my father, brothers and sisters in Sudan, who always supported me.

Table of contents **Page**

List of figures **Page**

Chapter 1

Chapter 2

Chapter 3

Chapter 4

List of tables **Page**

Abbreviations

%: Percent

°C: Degree Celsius

A/B: A or B blood group

Ab: Antibody

A/B-Ags: A, B, and O blood group antigens

ABO: A, B, and O blood groups

ABOi: ABO-incompatible

ABOi-HTx: ABO-incompatible Heart Transplantation

Abs: Antibodies

Ag: Antigen

α-gal: Gala1-3Galß1-4GlcNAc-R

A-Tg BCM: A-Tg blood cell membranes

A-Tg: A-transgeneic

BALB: BALB/c mouse strain

Bcl-2: B-cell lymphoma 2

BCM: blood cell membranes

BCR: B cell receptor

BGB^{+}: blood group B-positive

BLNK: B-cell_linker

Btk: Bruton protein-tyrosin

C3H: C3H/HeJ mouse strain

CD22L: CD22 ligand

CD40L: CD40 ligand

CD4-deficient mice: Mice deficient for CD4+ T cell, including CD4, MHC-IIK, and/or TCR *KO* strains

CFA: Complete Freund's adjuvant

CGG: Chicken Gamma Globulin

CHO: Carbohydrates

CHO-Ag: Carbohydrate antigens

DNP: 2,4-Dinitrophenyl

E. coli O86: Escherichia coli serotype O86

Grb2: Growth factor receptor-bound protein 2

HTx: Heart Transplantation

Hu A-BCM: Human group A blood cell membranes

Hu B-BCM: Human group B blood cell membranes

Hu O-BCM: Human group O blood cell membranes

ICAM: Intercellular Adhesion Molecule

IFA: Incomplete Freund's adjuvant

IL: interleukin

ITIM: Immunoreceptor tyrosine-based inhibitory motif

KLH: Keyhole limpet hemocyanin

KO: Knock-out

KTX: Kidney Transplantation

LPS: Lipopolysaccharide

Lyn: a Tyrosine-protein kinase

MHC: Major Histocompatibility Complex

NAbs: natural antibodies

ND: Not determined

NKT cells: natural Killer T cell

nu/nu: Nude/Nude

NZB/W: New Zealand Black/New Zealand White mouse strain

OVA: Ovalbumin

PAMP: Pathogen-associated molecular pattern

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate-buffered saline

Pi3K: Phosphoinositide 3-kinase

PLCγ: Phospholipase C

pMHC: Peptide-Major Histocompatibility Complex

PPS: Pneumococcal capsular polysaccharide

Rag2: Recombination-activating genes 2

RT: room temperature

SH2: Src Homology 2

SHIP: Src homology 2 (SH2) domain containing inositol polyphosphate 5-phosphatase 1

SHP-1: Src homology region 2 domain-containing phosphatase-1

Sigelc-deficient: Deficient for CD22, Siglec-g, or CD22 and Siglec-g

Siglec: Sialic acid-binding immunoglobulin-type lectins

Siglec-g: Sialic acid-binding immunoglobulin-type lectins-g

SiglecgL: Siglec-g ligand

Siglecs: CD22 and Siglec-g

SLE: Systemic lupus erythematosus

Syk: Spleen tyrosine kinase

TCR: T cell receptor

TD: Thymus-dependentTI

Thymus-independent

TI-1: Thymus-independent type 1

TI-2: Thymus-independent type 2

TLR: Toll-like receptor

TNP: 2,4,6-Trinitrophenyl

Tx: transplantation

WT: Wild-type

xid: X-linked immune deficiency

Glossary (only for the purpose of this thesis):

Natural (spontaneous) ABO Abs: Abs produced without exposure to A/B-blood product

Induced ABO Abs: Abs induced by intentional immunization or exposure to A/B-blood product

ABO Abs produced by bacteria: ABO Abs produced in presence of bacterial flora, such as by *E. coli* O_{86}

Mono-contamination: Germ-free animal exposed to one type of microorganism, e.g.: *E. coli* O_{86}

Conventional mice: Mice colonized with a diverse and largely undefined microbiome within the gut

Probiotic bacteria: Nutritional supplement of live bacteria, including bacteria expressing "A/B-like Ags"

Blood group B-positive bacteria (BGB^{+}): Bacteria expressing Ag similar to blood group B-Ag "B-like Ag"

Non-A/B anti-RBC: Antibody against erythrocytes protein, but not against A or B antigen

Regulatory role of CD4+ T cells: CD4+ T cells involved in enhancing or preventing Ab production

Chemically-synthesized structures: Synthetic CHO Ags, such as 2,4-Dinitrophenyl & 2,4,6-Trinitrophenyl

Naturally-occurring carbohydrates: Natural Ags, such as ABO, α-gal, and capsular polysaccharides.

Chapter 1:

Introduction

This thesis will shed light by increasing our understanding of how important is the role of carbohydrates (CHO) in health and disease. How the immune system responds to CHO antigens (CHO-Ags) will open new avenues in the development of strategies to induce immunity against infections. Similarly, understanding how antibodies (Abs) are produced against CHO-Ags expressed in tissues, such as ABO and α-gal, will lead to development of strategies to utilize these tissues for treatment, such as organ transplantation (Tx). Moreover, my findings will also be relevant in future work in areas such as vaccine development and blood transfusion.

In this regard, ABO-incompatible organ Tx (ABOi-Tx) has expanded the donor pool and therefore has saved many lives [1-4]. In order to optimize safe ABO-mismatched organ Tx, I explored how ABO Abs are produced and which stimulation is required. This work is built on the previous work done in Dr. West's lab and utilized a humanized mouse model.

1.1. Carbohydrates and antibody production

Polysaccharides and glycoconjugates, such as glycoproteins, glycolipids, and sialic acids, are crucial to leukocyte trafficking, cell-to-cell contact, self/non-self binding, and modulating the immune response [5]. For instance, the glycoprotein L-selectin (CD62 ligand) is very important in inflammation, because it regulates lymphocyte-endothelial cell adhesion, rolling, extravasation, and migration toward inflammatory bodies [6].

Similarly, many pathogens express CHO-Ags, including virulence factors like capsular polysaccharide Ags that facilitate pathogen invasion and infection. Immunity against these types of pathogens is

facilitated by the production of antibodies (Abs) that specifically react with these capsular polysaccharides. Glycoconjugate vaccines can stimulate antibody (Ab) production, providing immunity against such encapsulated pathogens. The vaccine to prevent meningitis caused by *Neisseria meningitides* is an example of such glycoconjugate vaccines [7].

Oligosaccharide cores of glycoproteins and glycolipids expressed on endothelium or erythrocytes can be immunological barriers for blood transfusion and organ Tx. For instance, the major *xenogeneic* Galα1-3Galβ1-4GlcNAc-R (α-gal) antigen (Ag) expressed on the endothelium of most mammalians (including mice and New World monkeys, but not Old World monkeys, apes, and humans) is an important immunological barrier in xenotransplantation due to spontaneous (natural) production of anti-gal Ab in humans, apes, and Old World monkeys [8, 9].

Similarly, A/B histo-blood group Ags (A/B-Ags) expressed on endothelium, erythrocytes, and other tissues, constitute an immunological barrier to blood transfusion and organ Tx, because the binding of the recipient's pre-existing non-self ABO Abs to cognate A/B-Ags expressed on donor erythrocytes and vascular endothelium [10-12] would typically induce severe hemolysis or hyperacute rejection, respectively [13, 14]. Therefore, studying the mechanisms of Abs development stimulated by naturally-occurring CHO-Ags is paramount to our understanding of health and disease.

1.2. Immune responses to protein and CHO-Ags

In 1912, László Detre (a Hungarian scientist who worked with Élie Metchnikoff) was the first to use the term "*Antigen*"[15]. At that early stage in immunological research, all Ags that had been found to induce Ab production were thought to be proteins: "*Kein Antigen ohne Eiweiss*" (no Antigen without protein)[16], despite a 1907 report suggesting that fungal glycosides can induce Ab production and therefore non-protein Ags could also exist [17].

Numerous studies (reviewed in [18]) investigated the nature and structure of Ags and today it is understood that the general order of antigenic structures that induce Ab production, according to their immunogenicity (from highest to lowest), are proteins, polysaccharides, lipids, and nucleic acids [19]. The immunity to Ags capable of inducing Abs production is classified based on the requirement of cells from the thymus (T cells), as thymus-dependent (TD) and thymus-independent (TI) immune response, respectively [20, 21](**Figure 1.1.**).

1.2.1. TD Ags

To produce Abs against a protein Ag, a B cell (as an antigen-presenting cell (APC)) endocytoses Ags that are bound to B cell receptor (BCR), or clathrin-mediated endocytosis, into clathrin-coated vesicle [22]. In the cytosol, the B cell processes and degrades the protein into peptide fragments in the B cell's early and late endosomes, loads the resulting antigenic peptides on the B cell's major histocompatibility complex-II (MHC)-II in the form of a peptide-MHC (pMHC) complex, and presents to a helper CD4+ T cell that recognizes the pMHC complex via its T cell receptors (TCR) [23]. The T cell help leads to B cell activation [24-29], differentiation to Ab-secreting cells [30], as well as formation of Ag-specific memory B cells [31] (**Figure 1.1. A**).

Importantly, cognate binding of pMHC/CD40 on B cell with TCR/CD40 ligand (CD40L) on activated CD4+ T cell, respectively, generates a T-dependent (TD) B cell response [32-34] in which the protein Ag is called the TD-Ag. In this regard, the co-stimulatory CD40 protein (expressed on APC, including B cells and dendritic cells) binds to the co-stimulatory CD40L protein (expressed on activated CD4+ T cells) [32] to stimulate an optimal B cell response. Additionally, T cell cytokines, including interleukin (IL)-2 [35-37], IL-4 [38], and IL-6 [39, 40] are also required for B cell proliferation and isotype switching.

Importantly, TD Ab responses are also associated with B cell somatic hypermutation [41, 42] and immunoglobulin (Ig) isotype switching (IgM to IgG, IgA, and IgE classes) [43].

1.2.2. TI Ags

In contrast to protein Ags, some Ags can induce a B cell response that is generally thought to occur without T cell participation [44-50]. However, B cell activation in the absence of T cell participation may not necessarily exclude further B cell interactions and activation in the presence of T cell. For instance, B cell activation by lipopolysaccharides (LPS, which could occur in the absence of T cell participation) was reported to induce memory B cell response that normally requires T cell participation [51]. Furthermore, studies showed that CD40-CD40L interaction [52-54] or infusion of T cells into *athymic* mouse [46, 55] would enhance Ab response to CHO-Ags. These data led to confusion regarding the concept of "T independence" immune response to whether B cell response to CHO-Ags always proceeds without T cell participation (truly always T-independent), or rather that T cell participation is not always required, and when it participates under some conditions, T cell would enhance the Ab response to CHO-Ags.

Without regard to these caveats about the requirement of T cell participation, the stimulation that induce B cell responses in the absence of T cell help is called "T-independent" (TI) and classified as either TI type 1 (TI-1) or TI type 2 (TI-2) immune response. In TI-1 Ab production, complex Ags such as LPS derived from Gram-negative microorganisms, can engage the BCR and co-engage toll-like receptors (TLR) to stimulate a B cell response [20, 47, 55-59]. The B cell response triggered by such Ags usually requires a second signaling provided by TLR [60-62]. At high concentration, LPS is considered B cell mitogens and can non-specifically activate both naïve and mature B cells [63, 64]. The TI-1 pathway is generally presumed to be rapid, does not require T cell participation, and can provide long-lasting immunity against bacterial infection [65, 66] (**Figure 1.1. B**).

In TI-2 pathway, an extensive cross-linking of BCR by CHO-structures containing repetitive multivalent epitopes is thought to provide strong signaling that is sufficient to stimulate a CHO-specific B cell response [50, 67]. The stimulation capable of inducing a B cell response in this manner are called TI-2 pathway [47, 68] and include stimulation by high-molecular-weight polysaccharides such as capsular polysaccharides expressed on bacteria [47, 50, 68, 69] (**Figure 1.1. C**).

A/B histo-blood group Ags are naturally-occurring complex polysaccharides composed of multivalent repeating glyco-epitope structures [70, 71]. They were thought to engage sufficient BCRs to stimulate the B cell without T cell help, resulting in Ab production to non-self A/B-Ags [72, 73]. Therefore, ABO Abs responses in general have been termed TI-2, without careful discrimination between ABO Abs induced by A/B-Ags (i.e. A/B-Ags are prepared from human type A/B-erythrocytes), from that spontaneously produced without stimulation by A/B-Ags.

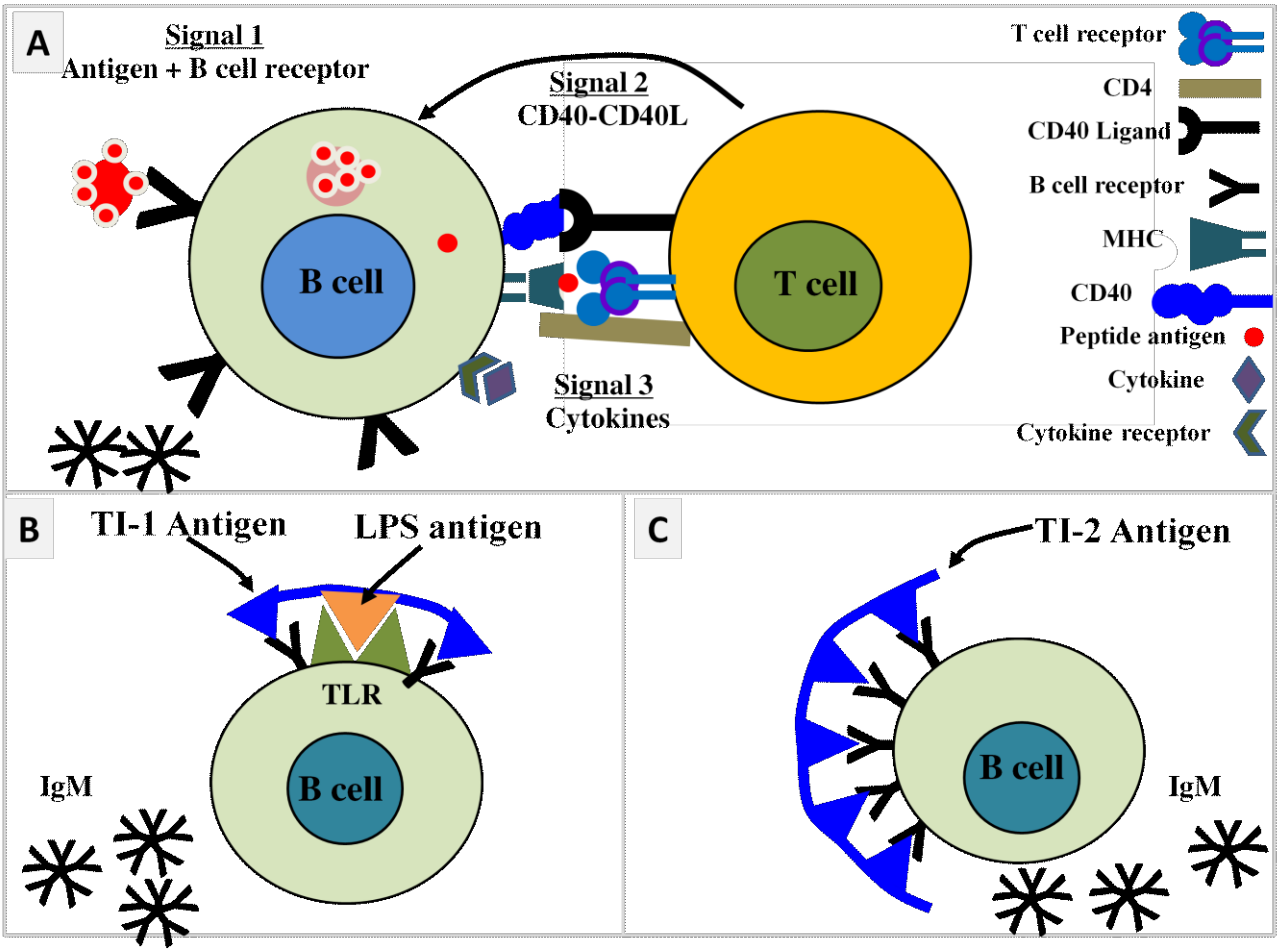

Fig. 1.1: Thymus-dependent (TD) and thymus-independent (TI) Ab response [45] (**A**) BCR recognizes a protein Ag, degrades that Ag and presents its antigenic peptides on the B cell's MHC-II as a peptide-MHC (pMHC) complex. The TCR of an activated CD4+ T cell (helper T cell) can recognize the pMHC complex, and via CD40-CD40L interaction and cytokines production by CD4+ T cell, B cell is stimulated to respond to a T-dependent protein Ag. (**B**) Some mitogens, such as LPS, can simultaneously engage a BCR and a TLR and stimulate both naive and mature B cells without CD4+ T cell participation. (**C**) Some CHO structures with repetitive multivalent epitopes, such as capsular polysaccharides, can extensively cross-link multiple BCR to provide strong signaling sufficient to stimulate T-independent B cell response. BCR: B cell receptor, MHC: major histocompatibility complex, TCR: T cell receptor, Ags: antigens, LPS: lipopolysaccharides, TLR: toll-like receptor. *Pictures were made by I. Adam.*

1.3. Biochemistry and tissue expression of A/B-Ags

The synthesis of A/B structures is mediated by glycosyltransferases that sequentially add a terminal A or B-trisaccharide to the glycolipids and glycoproteins core chain during embryonic life [74-77]. Expression of only the H chain on the glycolipids and glycoproteins core chain defines individuals of the O blood group, whereas addition of the A, B, or A and B terminal trisaccharide sugar, catalyzed by genetically-determined glycosyltransferases, defines individuals of A, B, and AB blood groups, respectively [10] (**Figure 1.2. A, B**).

A/B-Ags are expressed on many tissues of embryonic mesodermal origin, including erythrocytes, vascular endothelium, and other tissues such as epithelium [10, 11, 78-80]. Humans express at least four A/B precursor chains (type I–IV) and alpha-2-fucosyltransferases that could lead to different A/B(H)-subtypes expression in tissues [71]. West's research group showed that, unlike erythrocytes, the cardiac endothelium in humans expresses only subtype II A/B(H)-Ags, indicating that immunity and/or tolerance to A/B-Ags expressed on endothelial cells of heart transplants (HTx) does not necessarily correspond to immune response to A/B-Ags expressed on erythrocytes [81]. Currently, assessment of ABO Abs in the setting of blood transfusion and ABOi-HTx is detected with the isohemagglutinin assay, using reagent erythrocytes. However, detection subtype-specificity of ABO Abs, such as Abs against A/B-subtype Ags expressed in the transplanted organs, is essential for clinical decision-making and management of ABO-incompatible transplant recipients.

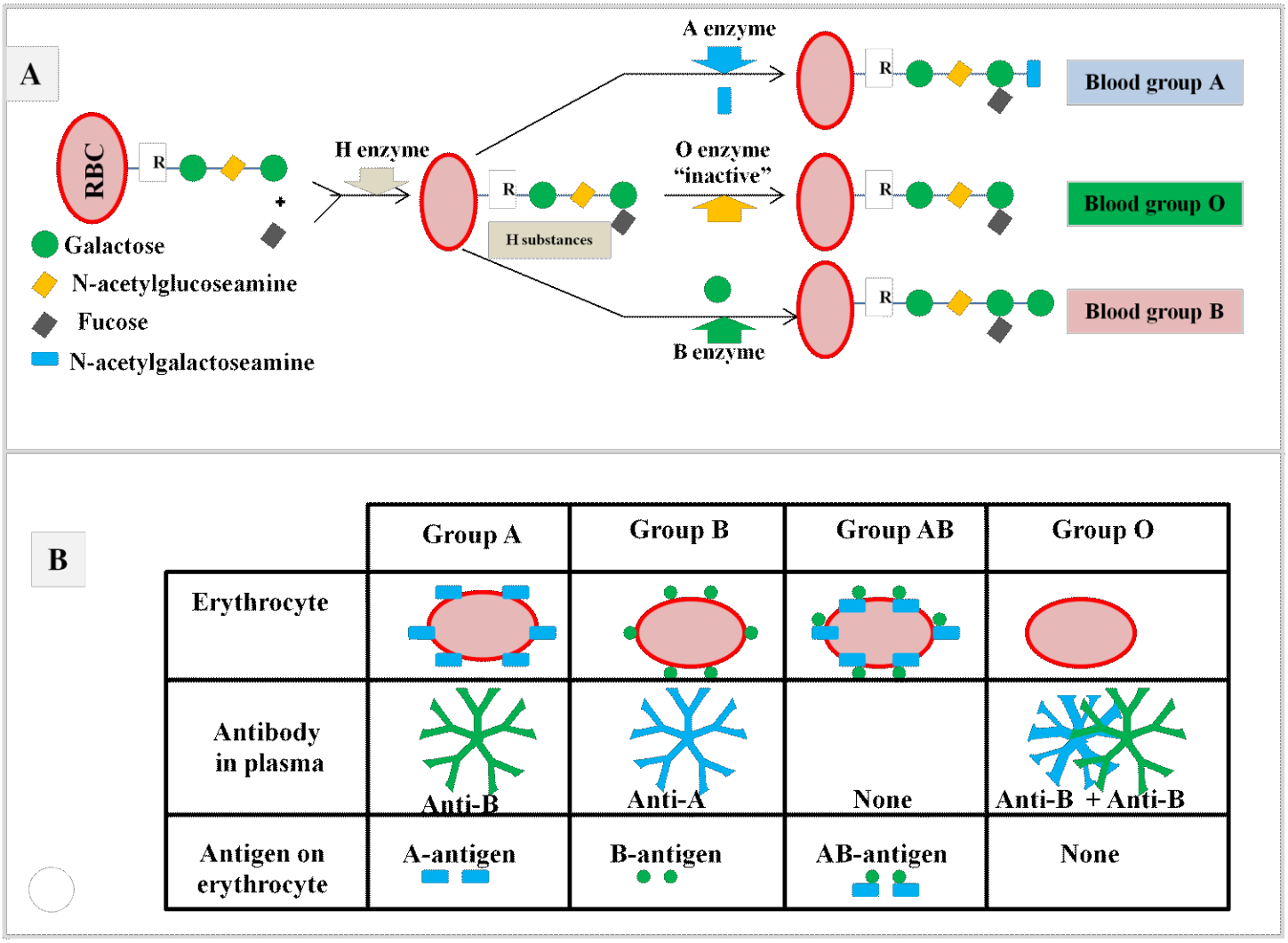

Fig. 1.2: A/B blood biosynthesis and principle of ABO-Abs as an immunological barrier [74-77]. (**A**) Glycosyltransferase enzymes mediate the A/B synthesis by sequentially adding a terminal monosaccharide A (galactose) or B (N-acetylgalactosamine) to the terminal galactose sugar on the glycolipids and glycoproteins core chain. (**B**) A and/or B-Ag are expressed on many tissues, including erythrocytes, and vascular endothelium. *Pictures were made by I. Adam.*

1.4. Importance of A/B-Ags and Abs to ABOi-organ Transplantation (ABOi-Tx)

Tx is a highly successful therapy for organ failure that would otherwise be lethal and that Kidney transplantation (KTx) has saved many patients with end-stage renal disease. However, due to overwhelming need for kidney donors, including whom are ABO-incompatible with their intended recipients, early attempts have been made to perform KTx across the ABO barrier [2, 3, 82-84]. Due to aggressive interventions to remove pre-formed ABO Abs (such as splenectomy, plasmapheresis, and B cell depletion) and feasibility of hemodialysis for KTx recipients who might experience graft failure due to hyperacute rejection, it was possible to perform ABOi-KTx without high risk of death. However, potential recipient of ABOi-HTx would have high risk of death due to susceptibility of heart grafts to hyperacute rejection [85], without saving therapy such as hemodialysis in the case of hyperacute rejection after ABOi-KTx.

Infants enlisted for heart Tx have the highest waiting list mortality; more than 30% died during the long wait times without finding a suitable donor [86], but also they have the best heart Tx outcomes relative to any other age group. Because of a developmental lag in ABO Abs production in infants, they can receive a safe ABOi-Tx, including heart Tx [87, 88], without risk of hyperacute rejection that would otherwise normally occurs in adults due to pre-existed ABO Abs [82, 89]. In this regard, binding of pre-existed spontaneously-produced ABO Abs in adults to cognate non-self A/B-Ags expressed on graft endothelium would typically lead to complement activation and eventually to graft loss [90](**Fig. 1.3).**

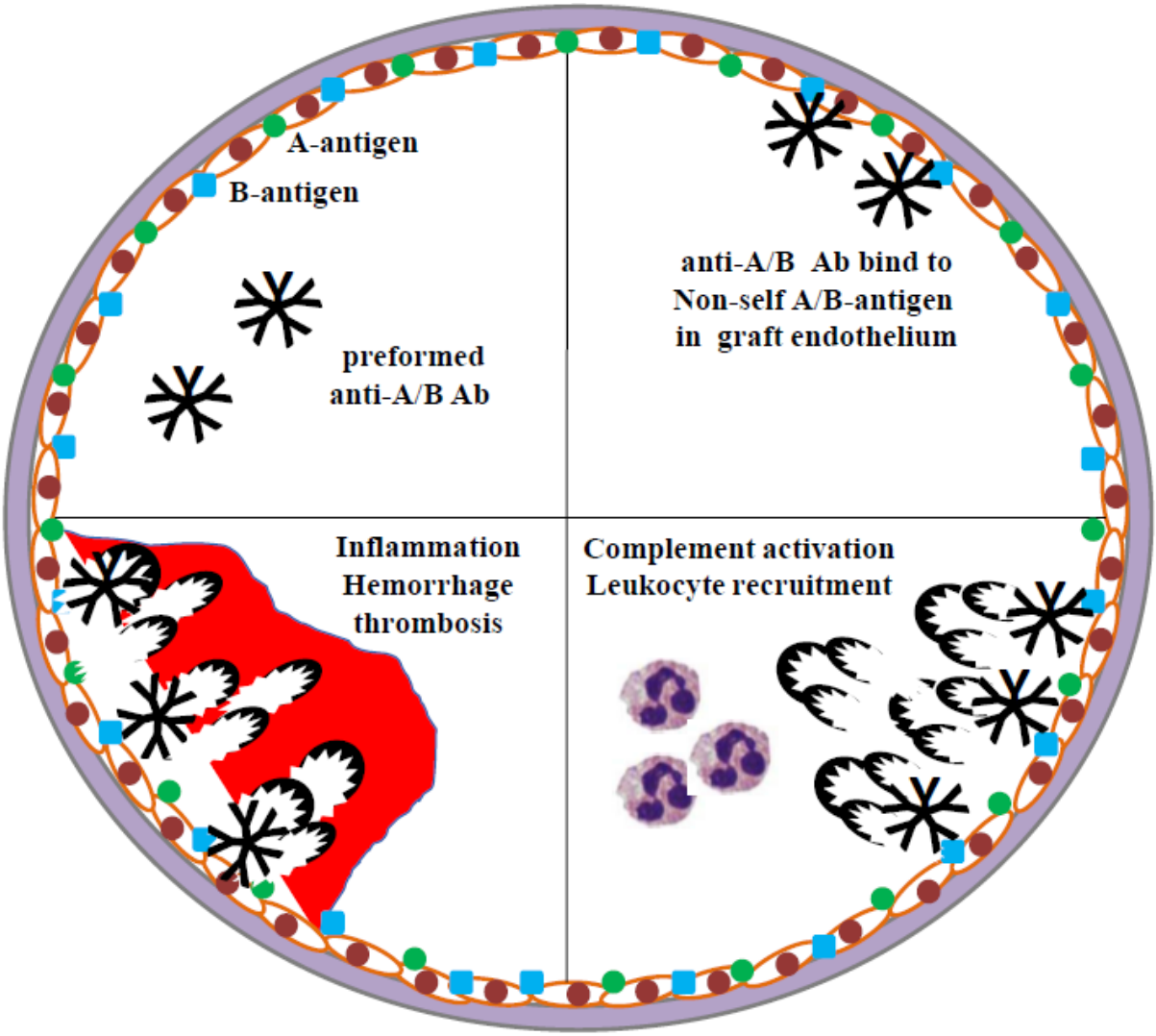

Fig. 1.3: Binding of endothelium A/B-Ags with circulating ABO-antibodies during ABOi-Tx. Binding of pre-formed anti-A and/or anti-B Ab to cognate A and/or B-Ag, respectively, on a vascularised organ would typically fix and activate complement pathway. This usually results in recruitment of leukocytes, inflammation, hemorrhage, vascular thrombosis and hyperacute rejection of the ABOi-graft. *Picture was made by I. Adam.*

1.4.1. Crossing the ABO barrier in infant HTx is safe and leads to development of B cell tolerance

Due to the overwhelming need for donor organs, attempts to across the ABO barrier have been made, particularly in kidney Tx [2, 3, 82-84]. Therefore, recognition of the delayed production of ABO Abs during normal infancy **(Figure 1.4 A)**, combined with the very high mortality for infant patients awaiting HTx, led West's research group to start the first intentional ABOi-HTx in infants between 1996 and 2000 and was demonstrated to be safe [87]. This has reduced the high mortality for infant patients awaiting heart transplant and also resulted in increasing in donor organs being utilized [91].

West *et al*. showed that not only was ABOi-HTx safe in infants [87, 92, 93], as noted above, but also that B cell tolerance developed to donor A/B-Ags expressed on the heart allograft [88]**(Figure 1.4 B-D)**. This was the first study showing that acquired neonatal transplant tolerance can occur in humans [92], and by mechanisms that in preliminary studies appeared similar to those demonstrated previously in mouse models [94].

In order to expand ABOi-KTx effectively, such as developing ABO Abs removal strategies to prepare kidney Tx or managing ABO Abs produced after the kidney Tx, it is critical to attain a precise understanding of ABO immunobiology and the mechanisms of how B cell immunity and tolerance develop to A/B-Ags.

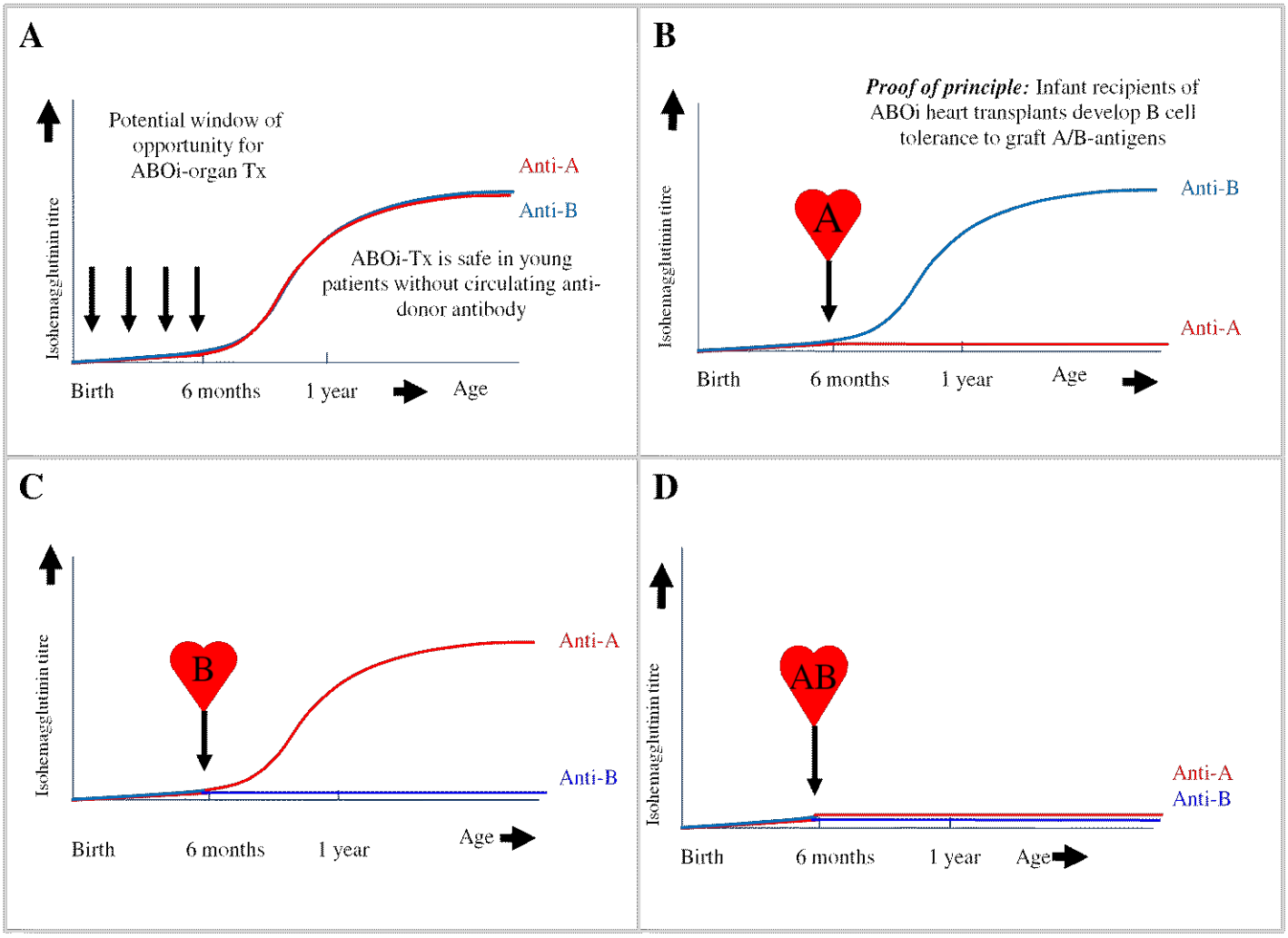

Fig. 1.4: Isohemagglutinins ontogeny in normal human infants and after ABO-incompatible heart transplantation (ABOi-HTx)[87, 88]**.** (**A**) ABO Abs development during normal infancy is delayed, potentially allowing safe ABOi-HTx with minimal risk of hyperacute rejection. (**B, C, D**) West's research group showed that not only is ABOi-HTx safe before development of ABO Abs, but also that infants develop B cell tolerance to donor A/B-Ags expressed on the heart allograft. *Courtesy of Dr. L. West*

1.4.2. Transgenic mouse model to study immunity and tolerance to ABOi-Tx

Despite the availability of biological specimens, there are major limitations to studying mechanisms of B cell tolerance in humans. Therefore, most of immunity or tolerance studies are generally limited to animal models [95, 96]. Moreover, there are also limitations in the use of animal models for ABO studies, such as that pigs express AO system [97], but not expressed in pig vascular endothelium [98]. There is no clinically relevant small animal model to study ABOi-organ Tx because wild-type mice do not express A/B-Ags [99, 100].

To study immunity to A/B-Ags and tolerance following ABOi-Tx, together with collaborators, West's research group generated A-antigen transgenic (A-Tg) mice that express human A1- and H-transferase under the control of the intercellular adhesion molecule 2 (ICAM-2) promoters on C57BL/6 and BALB/c backgrounds [101]. We showed that these mice express A-antigen (A-Ag) on the vascular endothelium of solid organs, erythrocytes, leukocytes, and epithelial tissues [101]. Our research group showed similar expression of A-Ag on human and A-Tg mouse erythrocytes [101]. The availability of this transgenic mouse allows us to study of immunologic features of ABOi-Tx in the context of *syngeneic* and *allogeneic* stimulation.

1.5. α-gal and ABO natural antibodies are immunological barrier for Tx

Naturally-occurring antibodies (nAbs) are commonly defined as the spontaneous presence of Abs without intentional immunization [102] and have been long attributed to B1 cell subsets [103]. Prior to Ab production during adaptive immunity, nAbs are able to react with many bacterial Ags and therefore provide an immediate protection against some bacterial infections [104-106]. On the other hand, nAbs cross-reactivity constitutes a significant immunological barrier in blood transfusion and ABOi-Tx. Similar to nAbs produced against the *xenogeneic* α-gal that is an immunological barrier in xenotransplantation [8,

9], natural ABO Abs (ABO nAbs) reacting with A/B-Ags, as previously mentioned, can pose a significant risk to ABOi-Tx [8, 9]. Therefore, this known immunological barrier is particularly important, because these ABO and α-gal nAbs occur without prior exposure to A/B or α-gal Ags, respectively [13, 14].

As noted above, due to the overwhelming need for donor organs, attempts have been made to perform organ Tx across the ABO Abs barrier [2, 3, 82-84]. Therefore, aggressive interventions to remove pre-formed ABO nAbs (splenectomy, plasmapheresis, and B cells depletion) were required for successful ABOi-Tx (kidney), but often these ABO nAbs return due to B cell memory, long-lived plasma cells, and newly generated B cells [107, 108]. How B cells are stimulated to produce ABO nAbs is unknown, but it is generally thought that bacteria could play an important role in ABO nAbs production [109].

1.6. Participation of bacterial flora in the development of ABO nAbs

The observation that bacteria can induce production of Abs against erythrocytes dates back to 1923 when Iijima demonstrated that injection of heat-killed *Shigella dysenteriae* into rabbits induced Abs that hemolysed sheep erythrocytes [110]. Further studies in the 1930s found that the majority of *Streptococcus pneumoniae* strains (encapsulated by a polysaccharide capsule) injected into rabbits can induce a marked rise of hemolytic Abs against sheep erythrocytes [111, 112]. Interestingly, serum obtained from horses for use as passive immunization after stimulation with *Streptococcus pneumoniae* contained massive amounts of hemagglutinin (i.e. Ab or Ag that causes erythrocytes to agglutinate) against human ABO red blood cells (RBCs), with Ab titer range from "1/80 to 1/2560" [113]. Furthermore, another report showed that when the same *Streptococcus pneumoniae* strain was injected into rabbits, the bacteria induced production of Abs that agglutinated only human A and AB erythrocytes (presumably anti-A Ab)[114].

In humans, studies in the 1980s showed that pneumococcal vaccination can generate IgM, IgG, and IgA anti-A Abs, consistent with data obtained in animals [115, 116]. Further studies reported that during some

infections, bacteria can induce expression of an acquired Ag similar to blood group B-Ag on human type A erythrocytes [117], suggesting that bacteria may be able to synthesize a blood group-like Ag (this Ag expressed on blood group-like bacteria was recently called "blood group B-positive (BGB^+) bacterial Ag [118] and hereafter referred to as BGB^+). Springer's work on development of ABO nAbs following exposure to BGB^+ Ag expressed in *E. coli* O_{86} bacteria [119-121] is important in the setting of organ Tx and blood transfusion, because humans produce ABO nAbs against these "A/B-like" epitopes expressed on bacterial flora. How ABO Abs develop following exposure to "bacterial stimulus" Ag will be analyzed in detail.

1.6.1. Springer's hypothesis on the bacterial origin of ABO Abs development

To challenge a prevailing theory that human ABO Abs are genetically inherited [122, 123], Springer studied the formation of ABO Abs in germ-free *White Leghorn* chicks [119]. Springer hypothesized that the development of ABO Abs is due to stimulation by "A/B-like Ags" present in exogenous stimuli located in gut, as it had been previously proposed by Wiener and Kabat [124, 125]. To evaluate this, germ-free *White Leghorn* chicks were left untreated or mono-contaminated by *E. coli* O_{86} expressing "B-like" Ag in their food. In contrast to germ-free chicks raised on a germ-free diet (i.e. no "B-like" bacterial Ags), germ-free chicks produced anti-B Ab when their food was contaminated by the *E. coli* O_{86} meconium [119]. However, Springer noted that some of the germ-free chicks fed on germ-free diet also developed low anti-B Ab titers in amount of 10% of that found in non-germ-free chicks [119], indicating that the anti-B Ab development in germ-free chicks intentionally immunized with the "B-like" bacterial Ags in diet is quantitative [119].

In his next experiment in humans, Springer administered via the gut killed *E. coli* O_{86} to blood group A or O individuals, with ages ranging from 1 week to 70 years old [120]. Springer found that the increase in

anti-B Abs (up to 4-fold in infants) was accompanied by an increase in specific Abs against *E. coli* O_{86}. Moreover, the neutralization of these specific Abs by the bacterial "B-like Ag" would inhibit the hemagglutination of the blood group B reagent erythrocytes [120]. As expected, administration of the bacteria via the gut induced anti-B Ab in blood group A individuals, but not in group B ones [120].

Importantly, once the chemical structure of blood group B-Ag was revealed [126-128], it was found to be similar to "B-like" Ags of the *E. coli* O_{86} [129]. Springer's hypothesis about the origin of ABO Abs was supported by 1980s studies showed that Abs to CHO-Ags, including ABO Abs, were greatly reduced in germ-free mice [130, 131]. Moreover, germ-free mice exposed to non-sterile food or to gavage of digested food of conventional mice developed ABO Abs [132]. Without such exposure, the germ-free mice did not produce detectable ABO Abs [132].

In humans, infants generally do not produce ABO nAbs until the age of 3-9 months [133, 134], and ingestion of *E. coli* O_{86} bacteria was shown to raise the anti-B Ab titer in 2-17 weeks old infants [120]. In further agreement with the bacterial stimulus hypothesis, ingestion of probiotic bacteria was associated with extremely high titers of anti-B hemagglutinin (up to 1/16,384) in blood group A individuals [135]. Taken together, these data supported Springer's hypothesis that ABO Abs are induced following gut exposure to exogenous "A/B-like" Ags, consistent with other studies suggesting that "A/B-like" Ags present in food and environment can affect ABO Abs levels among blood donors [136-138].

1.6.2. Limitations of the hypothesis on the bacterial origin for ABO nAbs development

In contrast to Springer's hypothesis about the gut bacterial origin of the stimulation that elicits ABO Abs production, other studies showed that the total IgM level in serum is relatively stable and similar in both germ-free and conventional mice [130, 131]. Early in the 1950s, germ-free chickens were reported to produce natural hemagglutinins against various bacterial strains [139]. These studies also reported that,

following intravenous injection of bacterial Ags, the germ-free chickens produced Ab titers comparable to conventional chickens [139].

Moreover, it was shown that in germ-free mice the reactivity of serum IgM against bacterial extracts was not significantly different from the reactivity observed in conventionally housed mice [140]. Additionally, it was also noted that induction of ABO Abs does not usually correspond with the expression of "A/B-like" Ags on the bacteria used to stimulate the germ-free mice [132]. In particular, molecular mimicry, in which a foreign Ag shares epitope similar with self-Ag [141]("A/B-like Ags"?), was thought to explain Springer's hypothesis [132].

In short, Springer's hypothesis does not explain why some germ-free mice produce low titer of ABO nAbs or other IgM nAbs against bacterial CHO-Ags [130, 131, 140]. Knowing that "A/B-Ags" are ubiquitously distributed in food and other environmental factors [136, 142, 143], it remains unclear whether mice and human spontaneously produce ABO nAbs, or whether these nAbs are only produced after exposure of naïve B cells to a bacterial stimulus. However, whether participation of bacterial flora is required for production of *de novo* ABO Abs by naïve B cells, or merely augment pre-formed ABO nAbs, is unknown.

1.7. B1 cells and nAbs production

NAbs have an important function in immunity and tolerance, such as protection against infection [104-106], cancer [144], autoimmune and [145] chronic diseases [146] (**Figure 1.5**). Nabs repertoire contains immunoglobulin of class M (IgM), IgG, and IgA and could play important roles in B cell functions, such as development of B cell tolerance [147, 148], repertoire selection [149], and Ab production [150].

In mice, nAbs are thought to be produced mainly by the B1 cell population [106, 151-153]. These B1 cells reside in many locations, including peritoneal and pleural cavities, spleen, peripheral blood and bone marrow (reviewed in [154]). B1 cells are defined by the surface markers $CD19^{hi}$, $CD1d^{mid}$, $CD23^{-}$, $CD43^{+}$,

IgM^{hi}, IgD^{low} and $CD5^{+}$ (B1a) or $CD5^{-}$ (B1b) [153, 154]. In humans, it is not clear whether cells equivalent to mouse B1 cells exist. Some studies reported the existence of B1 cells population in human blood [155-158], however, it is thought that they are activated B cells or plasmaplast [159, 160]. In this regard, human MZ B cells are the main innate-like IgM-producer B cells with specificity for conserved microbial Ags (i.e. a pathogen-associated molecular patterns such as LPS or peptidoglycan [161, 162]) and therefore may respond in lieu of mouse B1 cells.

Mouse B1 cells spontaneously produce nAbs by mechanisms that are yet to be fully understood [163]. The abundant nAbs produced by B1 cells can bind to various naturally-occurring CHO structures, including A/B-Ags, glycolipids, glycoproteins, and microbial polysaccharides [164-166]. Although nAbs recognize various bacterial Ags, thereby providing protection against many bacterial pathogens [167-171], it is not fully understood whether exposure to such bacteria is required to induce nAbs production. For instance, it was reported that germ-free mice can produce anti-fungal β-glucan and chitin nAbs [171], suggesting that B1 cells do not require external stimulation to produce nAbs [171-173].

Recently, the Kubes' group showed that female mice produce higher anti-*E. coli* nAbs than male mice and that these nAbs are produced independent of exposure to bacteria [174]. They also showed that germ-free female mice were able to produce higher anti-*E. coli* nAbs titers than males and that these titers are comparable to those in conventionally-housed female mice [174]. Similar to nAbs produced during puberty in rabbits [175], sex hormones produced during puberty might be key factor eliciting these anti-*E. coli* nAbs produced in conventional and germ-free mice [174].

In general, the repertoire of nAbs (which includes IgM, IgG, IgE, and IgA) is characterized by low affinity and broad reactivity. These nAbs are encoded by germline Ig sequences and were found to specifically react to conserved epitopes expressed on microorganisms [105, 169, 176, 177] or on endogenous epitopes

expressed on host cells [178, 179](**Figure 1.5**). Therefore, two pathways have been proposed, based on the stimulus that leads to nAbs production [180-182].

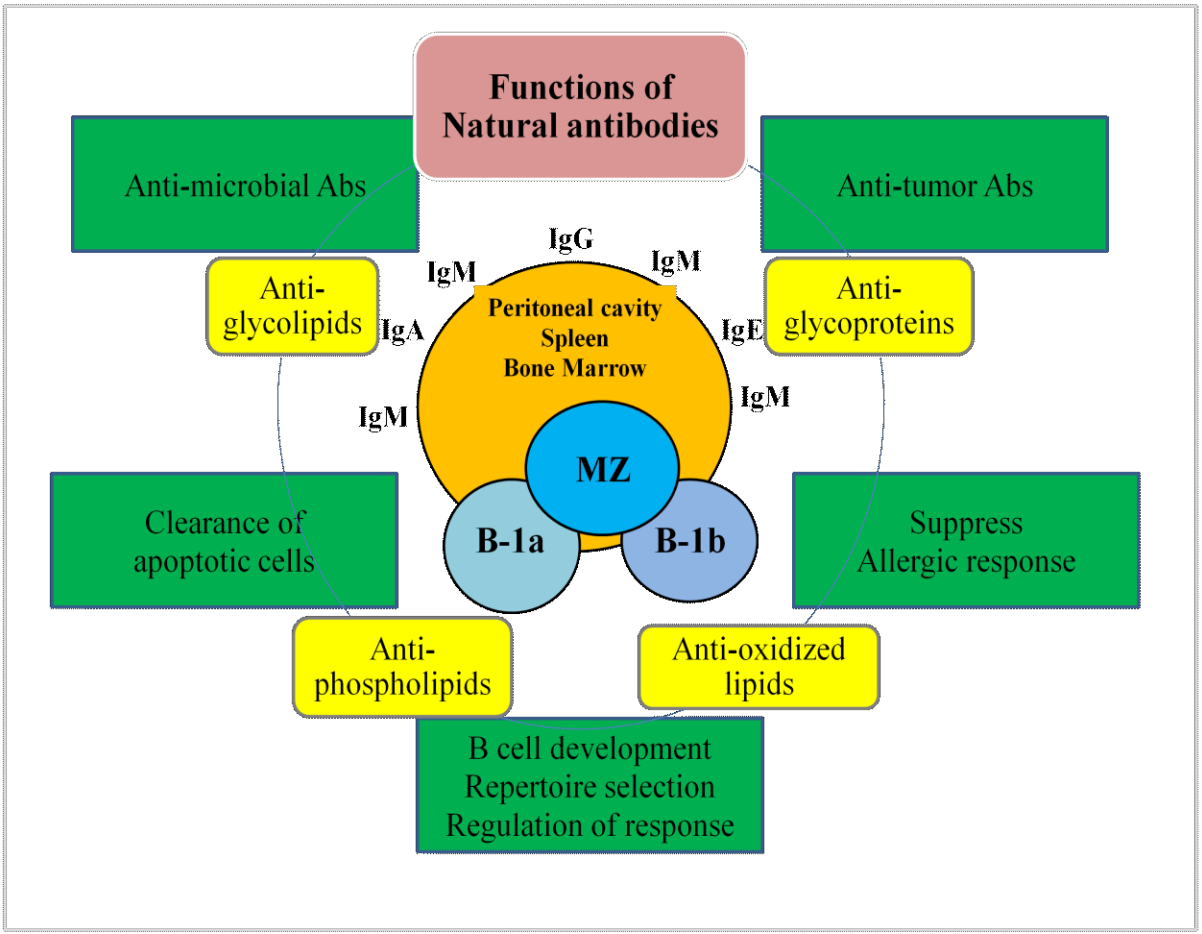

Figure 1.5: Natural Abs: production, function, isotypes, and antigen recognition. An overview of nAbs location (orange circle), function (green boxes), targeted epitopes (yellow boxes), Immunoglobulin subclasses, and cells producing these nAbs (blue circles). *Picture is modified from Holodick et al.* [106].

1.7.1. Stimulation of nAbs production

There are data indicating that healthy people ubiquitously produce nAbs that can bind to self-Ags [183-185]. It was hypothesized that nAbs are a result of endogenous stimulation by waste material and cellular by-products, such as apoptotic and senescent cells [186-189], tumor-associated CHO-Ags [144, 190], as well as normal tissue by-products [178, 179, 191, 192]. Accordingly, it was suggested that nAbs play an important role in maintaining an "internal homeostasis", by phagocytosing the nAbs-covered dead and apoptotic cells [193-195].

NAbs is also considered as a first line of defense against microbial invasions, such as bacteria [167, 196], spirochetes [168, 197], viruses [169, 198, 199], parasites [200, 201], and fungi [202, 203]. The function of nAbs with specificity for bacteria is of special interest, as the intestine is colonized by bacteria within days after birth [204]. The role of intestinal bacteria during stimulation of nAbs, such as ABO nAbs, is particularly important, because ABO nAbs are important immunological barrier in the setting of ABOi-Tx and in blood transfusion.

1.7.2. Spontaneous production of ABO nAbs

Despite data supporting Springer's hypothesis that ABO Abs are induced by exposure to "A/B-like Ags" on bacteria [119, 120, 130-132], it cannot be concluded that bacterial flora is absolutely required during production of ABO nAbs. Other nAbs were reported to be induced by "non-bacterial stimuli" such as food allergens [143, 182], and self-Ags [183-185]. Furthermore, there are data, as noted above, showing that the expression of "A/B-like Ags" on bacteria does not usually correspond with ABO Abs production [119, 132] or with IgM levels in germ-free and conventional mice [131, 140]. For instance, Arend's data in 1970s suggested that some GalNAc glycan-bearing hydrophilic ovarian glycolipids may endogenously stimulate anti-A Ab in female mice [205-207]. He showed that ovariectomised female mice exhibited

lower anti-A Ab production than that in non-ovariectomised mice [205-207]. Interestingly, mice [174] and rabbits [175] showed an elevation of nAbs during puberty (in both sexes), suggesting that sexual or physiological maturation corresponds with nAbs production.

In summary, current data suggest that the origin of nAbs can be attributed to three factors. First, it is due to exposure to exogenous pathogens [167-169, 196-203]. Second, it is a result of spontaneous production by B cells in the absence of known stimulation [140, 172, 208]. Third, it is a result of stimulation by endogenous host epitopes [183-185], such as stimulation by cell products of normal tissues [178, 179, 186-189, 191, 192] or tumor cells [144, 190]. Thus, mechanisms of nAbs production, including ABO Abs, remain unclear and require further investigation.

1.8. Mechanisms of B cell tolerance

This section will explore how B cells with specificity to self-Ags are regulated, with emphasis in the role of Siglecs in autoimmunity, tolerance, and Ab production against CHO-Ags. Studying B cell tolerance is important, because one of the major obstacles in organ Tx is the need for life-long immunosuppressive drug therapy with its many side-effects, and preventing Ab-mediated rejection would save the use of insufficient donor organs for Tx. Moreover, studying B cell tolerance to A/B-Ags is also important, because our studies showed that B cell tolerance develops in infant recipients of ABOi-HTx [88] by poorly understood mechanisms that require further investigations.

Paul Ehrlich (1854–1915) rejected the idea that the immune system can attack and destroy self-tissue, "*horror autotoxicus*", in a healthy individual and he postulated that immunological mechanism(s), “certain contrivances”, can prevent such immunological attacks [209]. Currently, it is understood that lymphocytes undergo several processes (both central and peripheral) to eliminate newly developed self-reactive cells. For B cell tolerance, a number of intrinsic and extrinsic mechanisms have been described to control self-

reactive B cells, including receptor editing [210], deletion [210], anergy [211], and active suppression by regulatory cells [212]. B cell receptor editing occurs in the bone marrow (central tolerance), anergy and suppression occur in the periphery (peripheral tolerance), and deletion occurs in both locations.

However, these mechanisms of tolerance are not "perfect" [213, 214]. For instance, some B cells residing in peripheral blood are in a quiescent state and therefore might react with self-Ags upon appropriate stimulation [215-218]. In addition, some B cells in the periphery (reacting to TD-Ags) can develop autoreactive Abs against self-Ag following somatic hypermutation [219-222].

Expression of co-receptor molecules, such as CD22 and other sialic acid-binding immunoglobulin-like lectins (Siglec, eg: Siglec-g), on mouse B cells can serve as "rheostats" to maintain activation or down-regulation of B cells and therefore maintain immunity while preventing autoimmunity in the periphery [223, 224]. Interestingly, deficiency of CD22 or Siglec-g in older mice (C57BL/6 and BALB/c strains) does not automatically result in autoimmunity, but the lack of both CD22 and Siglec-g can lead in old mice to spontaneous development of high level of IgG auto-Abs, glomerulonephritis, as well as lupus-like autoimmune disease [224]. These findings suggest that CD22 and Siglec-g have compensatory rather than redundant functions.

1.9. Siglecs and B cell immunity to CHO Ags

CD22 and Siglec-g are important B cell regulators of immune response to TI-2 CHO-Ags [225-229], where CD22 and Siglec-g involvement could result in B cell inhibition [227, 229, 230] or tolerance [225, 228]. CD22 and Siglec-g are members of the Siglec family of adhesion molecules involved in regulation of BCR signaling [231-233], and in autoimmunity and infection (reviewed in [234-237]). CD22 is predominantly expressed on mature conventional B2 cells in the marginal zone and mantle zone of the spleen [238, 239], suggesting that CD22 may regulate nAbs produced by marginal zone B cells. Siglec-g

expression is restricted to B cells, with higher expression on $CD5^+$ B1a cells and lower expression on B1b and conventional B2 B cells [238, 239].

1.9.1. Siglecs signaling pathway

During B cell activation via BCR cross-linking, the activation of the BCR-associated kinase Lyn results in phosphorylation of the immunoreceptor tyrosine-based inhibition motifs (ITIM) or ITIM-like molecules in the cytoplasmic domain (tail) of CD22 [231, 233, 239-246]. The phosphorylation and activation of ITIM in the CD22 tail leads to recruitment of the Src homology 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1) or the SH2 domain-containing inositol 5-phosphatase (SHIP, via the adapter molecules Grb2) into the BCR complex, followed by dephosphorylation of the B-cell linker protein (BLNK) complex and thereby inhibiting or dampening signaling downstream from the BCR complex [231, 233, 239, 241, 243-248] (**Figure 1.6 A**).

Similar to CD22, Siglec-g (Siglec-10 in humans) was shown to be involved in the regulation of BCR signaling [231, 239]. The exact pathway of Siglec-g signaling is less understood, but may involve phosphorylation of ITIM motif and recruitment of SHIP downstream of Siglec-g [231, 249]. Interestingly, Siglec-g knock-out (*KO*) mice show higher Ca^{++} mobilization in B1 cells, but not splenic B2 cells [249](**Figure 1.6 B**).

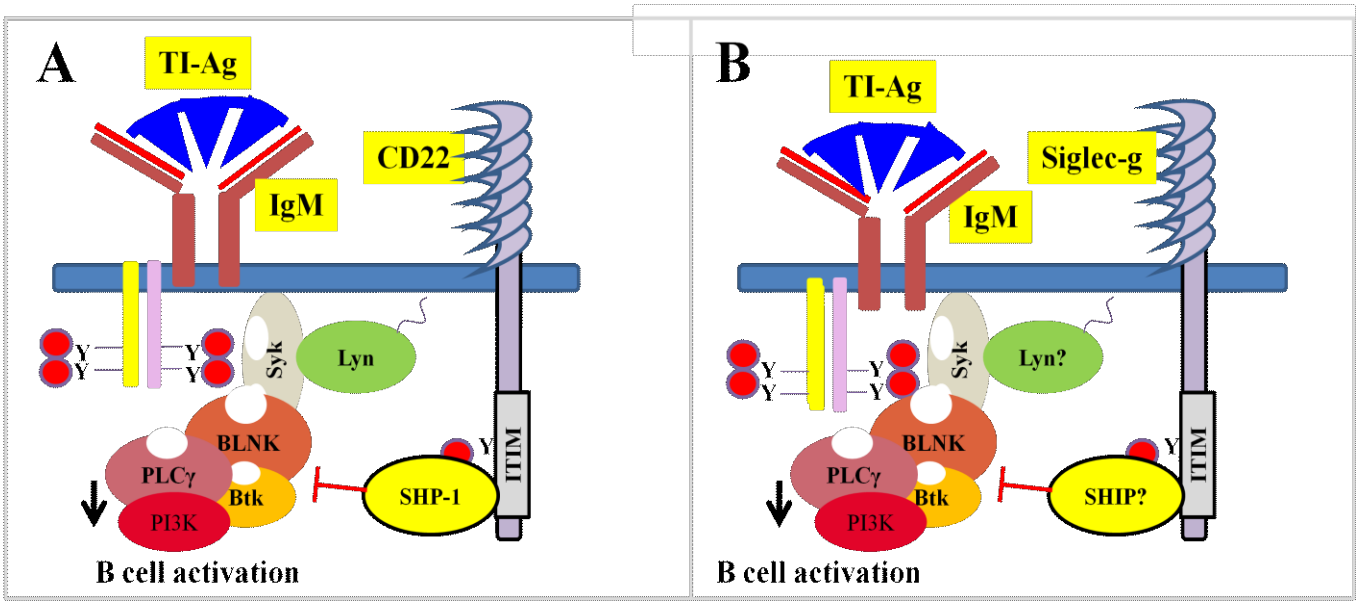

Fig. 1.6: Mechanisms of CD22 and Siglec-g signaling. (**A**) During BCR cross-linking, the BCR-associated kinase Lyn is activated and result in phosphorylation of ITIM or ITIM-like molecules down of CD22 tail. Next, the activated ITIM in the CD22 tail would lead to recruitment of SHP-1 and SHIP (via Grb2) into the BCR complex, resulting in dephosphorylation of BLNK and inhibiting /dampening the BCR downstream signaling. (**B**) The exact pathway of Siglec-g is not completely understood, but may involve phosphorylation of ITIM motif and recruitment of SHIP linked to Siglec-g tail [231]. Siglec: sialic acid-binding immunoglobulin-like lectin, BCR: B cell receptor, ITIM: immunoreceptor tyrosine-based inhibition motifs, SHP: Src homology 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1), SHIP: SH2 domain containing inositol phosphatase, BLNK: B-cell linker protein. *Picture is modified from Nitschke et al.* [231].

1.9.2. Impact of Siglecs-deficiency on B cell expansion, survival, and Ab production

In *Siglec-gKO* mice, there is expansion of B1a cells in the bone marrow, spleen, and peritoneal cavity [249, 250]. Additionally, mice deficient for both CD22 and Siglec-g (*CD22xSiglecgKO*) have a greater expansion of B1 cells accompanied by a 5-8 fold increase in IgM nAbs production compared to wild-type (*WT*) mice. This B1 cell expansion is significantly higher than in either *CD22* or *Siglec-g* single *KO* mice [224](**Table 1.1**).

Despite the discovery of CD22 molecule in the early 1990s [251], the exact impact of CD22 deficiency on Ab production remains controversial. Some studies reported that in *CD22KO* mice the lack of CD22 expression on B cells promotes B cell hyper-responsiveness to protein and CHO-Ags [252-254]. In contrast, other studies reported that *CD22KO* mice have impaired Ab production [224, 255, 256] and that CD22 deficiency does not impact anti-CHO Ab production [257-259]. Similar conflicting results to CD22 have also been reported in *Siglec-gKO* mice [224, 249, 250](**Table 1.2)**. An explanation for these widely varying conclusions may be that these studies did not examine the occurrence of nAbs production against these chemical structures (i.e. dinitrophenyl/trinitrophenyl (DNP/TNP)) used to immunize the mice **(Table 1.3)**.

In contrast to both *CD22* [252-254] and *Siglec-gKO* mice [249], *CD22xSiglecgKO* mice developed weaker Ab production in response to immunization by TNP-based Ags [224]. It was speculated that the weak Ab production in *CD22xSiglecgKO* mice is due to stimulation by TNP that would induce hyper-cross linkage of BCR and therefore an excessive stimulation of B cells that leads to apoptosis and impairment of TNP Ab production [254, 260]. These studies further speculated that CD4+ T cell help (eg: via CD40-CD40L [245, 260]) could rescue this excessive B cell stimulation by TNP, thus preventing B cell apoptosis [254, 258, 260].

However, the exact role of CD4+ T cells in Ab production in *CD22xSiglecgKO* mice remains unclear. For instance, whether CD4+ T cells are required to rescue B cells that would otherwise undergo apoptosis (and therefore allowing Ab to CHO-Ags to be produced) has not been studied in *Siglecs-deficient* mice. Moreover, whether B cell hyper-stimulation and apoptosis are uniquely induced by DNP/TNP, but not by naturally-occurring CHO, is unknown. Nevertheless, it was reported that stimulation of *CD22xSiglecgKO* mice by LPS, R848, or CpG (TLR4, TLR7, and TLR9 agonist, respectively) would induce B1 cell hyper-proliferation [224], suggesting that TLR and CD4+ T cells could play a role in autoimmunity in *CD22xSiglecgKO* mice [261].

Table 1.1: Expansion of B1 cells: Effect of Siglecs deficiency on the expansion of peritoneal B1 cells and IgM nAbs production in *CD22, Siglec-g,* and *CD22xSiglecgKO* mice. The table shows a summary from various studies.

Mice	**Peritoneal B1 cell expansion**	**IgM nAbs**	**Ref.**
CD22KO	Higher (1.5x B1a, 0.7x B1b)	Higher (1.4x)	[258]
CD22KO	Higher (2x B1)	Higher	[252]
Siglec-gKO	Higher	Higher	[262]
Siglec-gKO	Higher (5-9x B1a, 2.5x B1b)	Higher (5-7x)	[249]
Siglec-gKO	Higher (5x B1a, normal B1b)	Higher (5-10x)	[250]
Siglec-gKO	Higher (10x B1a, normal B1b)	Higher (10x)	[226]
CD22KO	Normal	Higher	[224]
Siglec-gKO	Higher (6x B1a, 2x B1b)	Higher (5x)	[224]
CD22xSiglecgKO	Higher (10x B1a)	Higher (8x)	[224]

Table 1.2: Impact of Siglecs-deficiency on induced Ab production: The effect of Siglecs-deficiency on Ab production, compared to *WT* mice. The table summarizes data from various studies. TD: T-dependent, TI-2: T-independent type 2, ND: not determined, DNP: 2,4-dinitrophenyl, TNP: 2,4,6-trinitrophenyl, CGG: chicken gamma globulin, KLH: Keyhole limpet hemocyanin, PPS: pneumococcal polysaccharides.

Mice	**TD-Ag**	**anti-D/TNP**	**TI-2 Ag**	**anti-D/TNP**	**Ref.**
CD22KO	DNP-KLH	Augmented	TNP-Ficoll, PPS-3	Impaired	[254]
CD22KO	OVA-Alum	Impaired	DNP-Ficoll	Impaired	[256]
CD22KO	NP-CGG	Normal	N/A	ND	[257]
CD22xSiglecgKO	TNP-Ficoll	Impaired	NP-OVA	Impaired	[224]
CD22KO	TNP-CGG	Augmented	N/A	ND	[253]
Siglec-gKO	TNP-OVA	Normal/Augmented	Dextran, TNP-Ficoll	Augmented	[249]
Siglec-gKO	OVA/CFA	Normal	N/A	ND	[250]
CD22KO	N/A	ND	NP-Ficoll	Normal	[259]
CD22KO	N/A	ND	TNP-Ficoll	Impaired	[255]
CD22KO	NP-OVA	Normal	TNP-Ficoll	Impaired	[260]
CD22KO	DNP-KLH	Normal	DNP-Ficoll	Impaired	[263]
CD22KO	DNP-KLH	Normal	DNP-Ficoll	Normal	[258]
CD22KO	TNP-CGG	Augmented	ND	ND	[252]

Table 1.3: Anti-TNP nAbs: IgM and IgG nAbs anti-TNP in humans and animals, summary from various studies. ND: not determined, TNP: 2,4,6-trinitrophenyl.

Animal	**IgM anti-TNP nAbs**	**IgG anti-TNP nAbs**	**Ref**
Mice	0.15% of B cells, Ab is ND	ND	[264]
Monkeys	0.17% of B cells, Ab is low	Low	[265]
Pigs	High	High	[266]
Humans	High	High	[267]
SLE patients	High	High	[268]
Mice	High	High	[269]
Mice	High	ND	[270]
Rats	High (5% of B cell hybridoma)	ND	[271]
Mice	High (6.25% of B cells)	ND	[272]
Mice	High	ND	[273]

1.9.3. Impact of Siglecs/Siglecs-ligand interaction in Ab production

CD22 and Siglec-g are stimulated by binding to sialic acid-bearing glycoproteins, such as the binding of CD22 to α2,6-linked sialic acid-bearing glycans, and Siglec-g to both α2,6- and α2,3-linked sialic acid–bearing glycans [225, 231]. The binding of CD22/Siglec-g to their ligands (CD22L/SiglecgL) can occur through binding in *cis* on the same cell surface structures, such as to the BCR-CD22L complex [226, 227, 274-276]. Alternatively, CD22 and Siglec-g can bind to their ligands expressed on other cell surfaces, such as binding in *trans* to T cells [275, 277], dendritic cells [278], endothelial cells [279, 280], or to synthetic chemical structures [225, 228, 229, 281].

Interestingly, studies showed that CD22 and Siglec-g expressed on B cells can regulate the immune response to sialylated TI-2 glycans, such as the DNP-CD22L-polymer [229], in the setting of B cell self/non-self-discrimination [225-229](**Figure 1.7**). The outcome of *trans* interactions of CD22/Siglec-g with CD22L/SiglecgL resulted in B cell inhibition [226, 227, 229] or tolerance [225, 228]. In our studies following the development of B cell tolerance to donor ABH-Ags post ABOi-HTx during infancy [88], West's research group found that not only does the $CD27^{+}IgM^{+}$ B cell population express higher levels of CD22 in infants than in adults [282, 283], but also that ABO Abs are mainly produced by this $CD27^{+}IgM^{+}$ B cell population [282, 283]. It was suggested that the high-level of CD22 expression on B cells in infants may play a role in the development of B cell tolerance to donor A/B-Ags following ABOi-HTx in infants [282, 283].

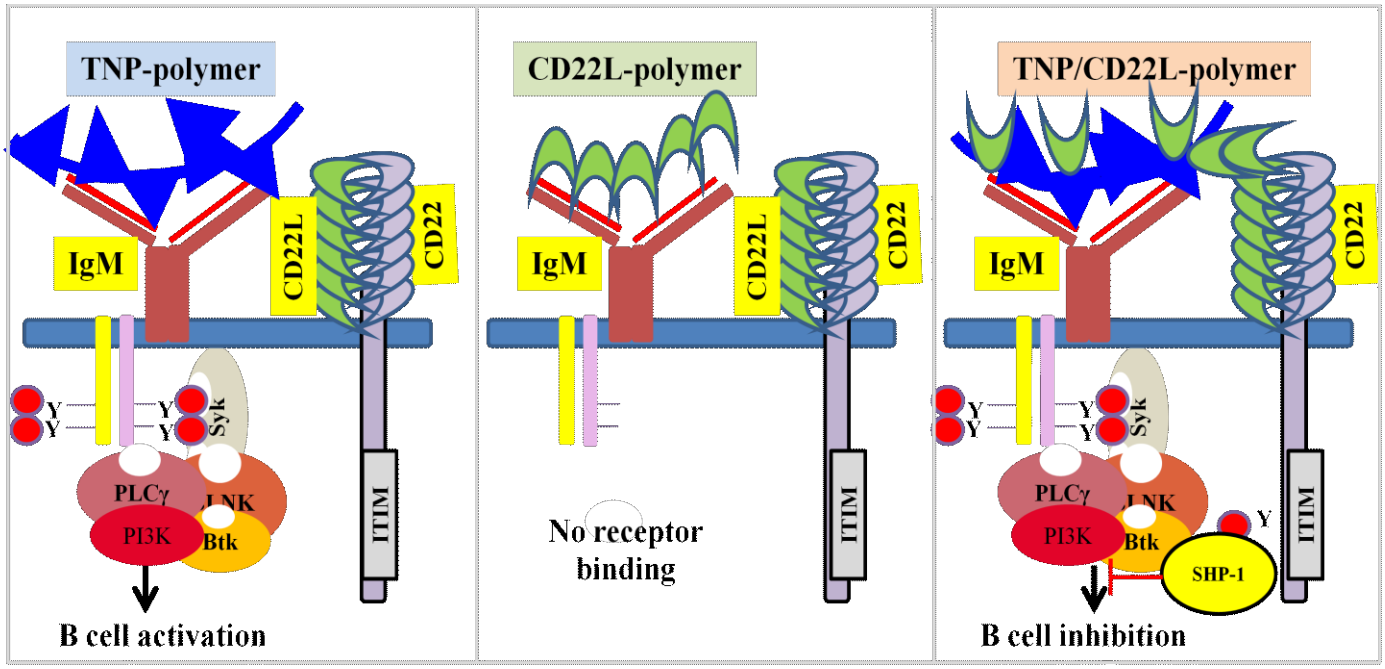

Fig. 1.7: Impact of CD22-CD22L interaction on the B cell response to TI Ags. Without CD22/CD22L interaction, antigenic polymers displaying DNP can initiate BCR downstream signaling and therefore induce B cell response. However, binding of BCR/CD22, to displayed DNP/CD22L co-polymer, respectively, can attenuate BCR signalling and inhibit B cell response to DNP-Ag. TI: thymus-independent, DNP: 2,4-dinitrophenyl, BCR: B cell receptor. *Picture is modified from Courtney et al.*[229].

1.10. Sex and nAbs

Recently, sex has received increased attention as an important biological factor and the importance of sex in immune responses is becoming increasingly understood in both human and mouse (reviewed in [284-286]). For instance, leukocytes show higher reconstitution rate in female than male mice, such as B cells reconstitution in the bone marrow of humanized mouse [287] or B and T cells reconstitution in peritoneal and pleural cavities of $Rag2^{-/-}$ mice [288]. However, whether Ab production is generally impacted by sex is not clear and requires further investigation.

Early observations showed that *WT* mice develop variable levels of ABO nAbs [72, 101, 289] with increasing age in a similar fashion to humans [133, 134]. However, it is not clear whether there is also variation in natural anti-A and anti-B Abs levels associated with sex. Our preliminary observation in BALB/c mice suggested that old female mice tend to produce more ABO nAbs than old males (unpublished observations). Intriguingly, early reports noted that female mice produced higher anti-A nAbs than males, which was explained by endogenous stimulation by "A-like Ag" produced by ovarian tissues during puberty [205-207]. As noted above, similar results in mice were recently reported for nAbs production during puberty [174]. In this report, female mice produced more anti-*E. coli* nAbs that allowed capturing and clearing bacterial infections in offspring [174].

1.11. Background to induced ABO Abs production (This work will be described in Chapter 3)

1.11.1. Gap of knowledge related to induction of ABO Abs production

Induction of Ab production to CHO-Ags with repetitive epitopes has generally been considered to occur without T cell participation, thus "T cell-independent" [47, 50, 68, 69]. Although A/B-Ags are naturally-occurring CHO-Ags, there is no consensus regarding the requirement of T cell participation for the production of anti-A and anti-B Abs in response to stimulation by A and B-Ags, respectively. Some

reports concluded that CD4+ T cell participation is required for anti-A [269] or anti-B [290] Ab production while other studies reported that it is not for anti-A [291] or anti-A and anti-B Abs production [72]. A third possibility is that CD4+ T cells participation is not required for anti-A and anti-B Abs production but nonetheless impacts Abs level. To evaluate the gap of knowledge regarding T cell involvement in the induction of anti-A/B Abs in mice, I briefly analyzed the outcome of these studies.

1.11.1.1. Induction of ABO Abs production does not require T cell participation

One study used *athymic* and *CBA/xid* mice and concluded that induction of ABO Abs production in mice does not require T cell participation [72]. However, *athymic* mouse produces extrathymic T cells [292-294] that may have participation in induction of ABO Abs production. Moreover, mutation in Bruton's tyrosine kinase signaling was reported to impair B cell maturation and response in *CBA/xid* mice [295-297]. These conclusions were based on inappropriate mouse models and therefore might be flawed.

NAbs develop in the absence of known exposure to cognate Ag and this study [72] did not investigate whether *athymic* mice spontaneously develop ABO nAbs without exposure to human A/B-RBC. Therefore, investigating the spontaneous development of ABO Abs is paramount to confirm whether induction of ABO Abs was due exposure to A/B-Ags or they occurred naturally without stimulation by the A/B-Ags, and whether ABO Abs could be induced beyond the natural ABO Abs production.

1.11.1.2. Induction of ABO Abs production requires natural killer T cell participation

It was also concluded that interaction of NKT cells with CD1d is required for induction of ABO Abs production, by demonstrating that human A-RBC injection can induce anti-A Ab production in *MHC-IIKO* mice, but not in *athymic* or in *CD1dKO* mice [291].

Similar to the previous study [72], this study [291] did not demonstrate whether ABO Abs develop without exposure to human A-RBC in these strains. Interestingly, this study showed development of low level of anti-A Ab in the *CD1dKO* mice, suggesting that NKT cell may not be an absolute requirement for ABO Abs production [291]. Furthermore, spontaneous production of anti-A Ab prior to immunization by A/B-Ags in *athymic* and *MHC-IIKO* mice [291] brings into question whether human A-RBC injection did induce anti-A Ab production, or whether the mice spontaneously produce natural anti-A Ab (anti-A nAbs) regardless of exposure to human A-RBC.

The similarity between the study that reported that T cell is not required for induction of ABO Abs [72] and the requirement of NKT cell in this study [291] is that they both concluded that ABO Abs production does not require CD4+ T cell participation. However, the two groups did not show whether ABO Abs production was induced or occurred independent of the human A/B-RBC injection. Interestingly, the difference in these two studies [72, 291] is that the induction of ABO Abs in *athymic* mice in this study [72] was in direct contrast to the data in *athymic* mice in the other study [291].

1.11.1.3. Induced ABO Abs production requires CD4+ T cell participation

A third group concluded that CD4+ T cell participation is required to induce ABO Abs production [290], similar to unpublished observations from our research group suggesting that CD40-CD40L interaction is required for induction of ABO Abs production [269]. Similar to previous studies [72, 291], whether *CD40* or *CD40LKO* mice are induced with injection of human A/B-RBC to produce ABO Abs, without CD40-CD40L interaction that suggest CD4+ T cell involvement, or whether these mice spontaneously produce ABO nAbs regardless of human A/B-BCM injection, or whether both processes occur, was not investigated.

Moreover, following depletion of CD3+, CD4+, and CD40L+ T cells and injection of A/B-RBC or rat thymocytes, respectively, they detected production of ABO or α-gal (in *α-galKO* mice) Abs [290]. However, this study, like the previously mentioned studies, also did not investigate whether development of ABO and α-gal nAbs were induced by A/B and α-gal Ags, respectively [290].

1.11.1.4. Biological factors related to nature of CHO-Ags, injection route, and mouse model

The outcome of the BCR engagement with CHO-Ags is thought to depend on the dose and route of administration of CHO-Ags [196]. For instance, in mice, peritoneal exposure to CHO-Ags is thought to recruit B1 cells, whereas intravenous exposure to the same CHO-Ags is thought to recruit both B1 and marginal zone B cells [196]. Accordingly, exposure to non-self A/B-Ags in the setting of organ Tx or blood transfusion might result in different stimulation pathway than an exposure via intraperitoneal injection of A/B-Ags.

Furthermore, those studies that concluded that Ab production to CHO-Ags is T-independent relied on chemically synthesized CHO structures, such as DNP/TNP-Ficoll [298-302]. These synthetic CHO-structures may not function as surrogates for naturally-occurring CHO-Ags, such as A/B or α-gal Ags. Moreover, reported discrepancies in ABO Abs production in response to ABO Ags may be due to use of inappropriate mouse models with particular limitations, such as *athymic* mice [72].

1.11.2. Rationale to study the role of T cells in induction of ABO Abs production

For expanding ABOi-HTx effectively into patients beyond infancy and managing ABO Abs pre and post ABOi organ Tx in adults, it is necessary to have a clear understanding of the mechanism(s) by which ABO Abs develop after exposure to non-self A/B-Ags. Previous studies have used chemically synthesized CHO structures [303-308] or mouse models with various limitations, such as *athymic* and *CBA/N xid* mice [72, 291], to conclude that Ab production to CHO-Ags occurs without T cell participation. Moreover, the role

of T cells on induction of ABO Abs to A/B-Ags remains unclear as those studies did not differentiate development of ABO nAbs from those induced by exposure to A/B-RBC [72, 290, 291].

1.11.3. Hypothesis

Based on preliminary findings from our research group suggesting a role for CD40 in the induction of ABO Abs production [269], I hypothesize that A/B-Ags will not stimulate ABO Abs production without CD4+ T cell participation. For instance, A/B-Ags expressed on erythrocytes (blood group type A/B) may be immunogenic in *WT* mice provided that additional elements (such as foreign proteins that are known to engage CD4+ T cells) are physically/chemically linked to that A/B-Ags. Accordingly, I expect that in the presence of CD4+ T cell participation, exposure to A-Ag in the context of foreign proteins would induce anti-A Ab production. Thus, I will use our *syngeneic* and *allogeneic* A-Tg blood cells or heart graft, in comparison with *xenogeneic* Hu A-BCM, to test the requirement for CD4+ T cell participation in my experiments.

1.11.4. Goals and objectives to study induced ABO Abs production

My goal is to investigate the role of CD4+ T cell participation on induction of ABO Abs production. Therefore, I will determine whether anti-A Ab is induced following immunization by A-Ag in the context of *syngeneic*, *allogeneic*, or *xenogeneic* stimulation (Chapter 3 will discuss this details).

1.12. Background to natural ABO Abs (ABO nAbs) production (This work is described in Chapter 4)

1.12.1 Gap of knowledge related to ABO nAbs production

Mice produce nAbs to various polysaccharide Ags (including ABO nAbs [101, 290, 291, 309]) that make mice are a good model to study ABO nAbs development without exposure to A/B-Ags. It is generally accepted that formation of these ABO nAbs in animals or in humans are induced by “A/B-like Ags”

expressed on bacterial flora [130-132], such as *blood group B +ve E. coli O_{86}* (*BGB^+ E. coli O_{86}*)[119]. This theory (Springer's theory) was supported by data showing that the chemical structure of the blood group B-epitope is similar to epitope expressed in BGB^+ *E. coli O_{86}* [126, 129]. Herein, I summarize the conclusions related to the mechanisms causing nAbs production against polysaccharide Ags, with special interest on ABO nAbs production.

1.12.1.1. ABO nAbs development and requirement of T cells participation

Studies demonstrated that polysaccharide Ags, such as dextran and bacterial capsular polysaccharides can stimulate strong BCR signaling causing production of nAbs without CD4+ T cell participation (T-independent) [47, 50, 68, 69]. ABO Abs production in *WT* mice has also been regarded as CD4+ T-independent [72, 291] in a manner similar to the production of α-gal nAbs in *α-gal KO* mice following T cell depletion [310]. Due to the close similarity of α-gal and B-Ags [311, 312], it has been assumed that CD4+ T cell participation is not required for production of ABO Abs in this setting.

However, my data [313, 314] and others [290] showed that CD4+ T cell participation is necessary for production of ABO Abs following immunization of mice with human A/B-RBC, consistent with a CD4+ T-dependent Abs production. A confounding factor related to the CD4+ T cell role in these different findings may have been the lack of differentiation between ABO nAbs production and that induced by intentional immunization. The mechanism(s) causing ABO nAbs production may be different from those causing an induced Abs following injection with human ABO-RBCs, prompting me to study the requirement of CD4+ T cell participation in ABO nAbs production.

1.12.1.2. CD4+ T cells and regulation of ABO nAbs production

Despite the fact that the induction of α-gal Ab production following intraperitoneal injection of *xenogeneic* pig kidney membrane in *α-galKO* mice appears to require CD4+ T cell participation [290, 315-317],

depletion of T cells was shown to enhance α-gal nAbs production without prior sensitization [310]. This later study suggested that the absence of CD4+ T cell participation enhances α-gal nAbs development, possibly due to lack of regulatory role of CD4+ T cell on B cells producing α-gal nAbs [310]. Therefore, the mechanisms involved in α-gal nAbs production may not be similar to induction of α-gal Ab production upon intentional stimulation. Based on preliminary findings in our lab suggesting an important role for CD4+ T cells in the induction of ABO Abs production (Chapter 3), I expect that CD4+ T cells would also play an important role in spontaneous production and regulation of ABO nAbs.

1.12.1.3. Gut bacteria and ABO nAbs development

Aside from the data supporting Springer's theory regarding the role of bacteria in the formation of ABO nAbs in germ-free animals and in humans [119, 126, 129-132], there are also data that may not fit with Springer's theory [130-132, 140]. For instance, Springer noted that some germ-free chicks developed very low anti-B Ab without exposure to *E. coli* O_{86} [119]. Moreover, it was also noted that after exposure of germ-free mice to bacterial stimulus from gavage of conventionally-housed mice, the specificity of Abs developed in those germ-free mice does not usually correspond to the bacterial Ags in that gavage [132]. Furthermore, studies found that in serum of mice, the overall repertoire of IgM reacting to bacterial extracts, or the abundance of total IgM against natural CHO-Ags, are generally stable in both germ-free and in conventional mice [130, 140].

Knowing that substances in food and environment affect ABO nAbs levels in human blood donors [136-138], it remains unresolved question as to whether bacterial flora is absolutely required for production of ABO nAbs, or whether the bacterial flora partially participate in the production of ABO nAbs. Therefore, I will explore the exact contribution of bacterial flora in ABO nAbs production, by assessing ABO nAbs

development in *WT* mice treated with antibiotics following standard protocol [318, 319] and in germ-free housed-mice.

1.12.1.4. Sex as a biological variable in nAbs production.

Sex is an important biological factor in immune responses in mice and humans [284, 285]. Recent data in mice [174] found that there was a significant sex-biased increase in nAbs reactive to pathogenic *E. coli* and that these Abs were predominantly produced in female mice, where sex hormones (such as estrogen) play important role in production of anti-*E. coli* nAbs [174]. Interestingly, these anti-*E coli* nAbs (IgG) were found to react to capsular oligosaccharides of the bacteria and cross the placenta to provide protection to the mice offspring [174]. Importantly, both germ-free and conventionally-housed female mice produced similar level of anti-*E. coli* nAbs [174].

Despite data showing that older *WT* mice develop ABO nAbs with variation in titer [72, 101, 289] in a similar fashion to humans [133, 134], it is not clear whether this variation in titer is linked to sex *per se*. As the impact of sex as a biological variable is becoming increasingly understood, I will investigate the impact of sex on ABO nAbs development in mice and potentially in humans (human study is beyond the scope of this thesis).

1.12.2. Rationale to study impact of T cells, bacterial flora, and sex in ABO nAbs production

Individuals normally produce ABO nAbs reactive to non-self A/B-Ags, presumably due to immunologic cross-reaction to "A/B-like" epitopes expressed on gut bacterial flora [120, 133]. Binding of those pre-formed ABO nAbs to cognate non-self A/B-Ags expressed on graft endothelium is a potential barrier for ABOi-organ Tx, because those nAbs would trigger a cascade of complement reactions that would typically lead to hyperacute rejection. Therefore, mechanism involved in the spontaneous production of ABO nAbs

is clinically important and may not be identical to ABO Abs induced by intentional immunization by A/B-Ag expressed on RBC and graft endothelium.

As noted above, a confounding factor related to whether CD4+ T cell is required [269, 290] or not required [72, 291] for induction of ABO Abs production may have been the lack of differentiation of ABO nAbs production from that induced by intentional exposure to A/B-Ags. Importantly, regulation of ABO nAbs production may be different from that regulate the induction of ABO Abs production. Additionally, it has been reported that bacterial gut flora expressing highly repetitive "B-like" epitopes participate in stimulation of anti-B Ab production [119, 120], but whether the bacterial flora is an absolute requirement for ABO nAbs production is not clear.

1.12.3. Hypotheses for the role of CD4+ T cell, bacterial flora, and sex in ABO nAbs production

1.12.3.1. The role of CD4+ T cells in ABO nAbs production

Previous work showed that depletion of CD4+ T cells enhances the α-gal nAbs production [310]. Therefore, I hypothesize that the process of spontaneous production of ABO nAbs occurs in the absence of CD4+ T cell participation. Alternatively, it is possible that the participation of CD4+ T cell during ABO nAbs production is not absolutely required but nonetheless CD4+ T cell plays an important regulatory role.

1.12.3.2. The role of gut bacterial flora in ABO nAbs production

Based on the formation of ABO Abs in germ-free chicks [119] and mice [130-132] following exposure to bacterial flora, I hypothesize that gut bacterial flora could play important role in the formation of ABO nAbs, but is not absolutely required for ABO nAbs production.

1.12.3.3. The impact of sex as a biological variable in ABO nAbs production

The impact of sex as a biological variable in immunity and tolerance is becoming increasingly understood. Recently [308], it was shown that female mice produced significantly higher anti-*E. coli* nAbs than males, suggesting that sex is important in nAbs production. Herein, I hypothesize that sex as a biological variable would also play important role in ABO nAbs production in mice.

1.12.4. Goals and objectives to study ABO nAbs production

My goal is to investigate the requirement of CD4+ T cells, bacterial flora, and the impact of sex as a biological variable on spontaneous production of ABO nAbs in mice (Chapter 4).

1.13. Background to role of B cell Siglecs in ABO Abs production (Will be described in Chapter 5)

1.13.1 Gap of knowledge related to the role of B cell Siglecs in ABO Abs production

CD22 and Siglec-g are expressed at high levels on mouse marginal zone and B1 B cell subsets [239, 278, 320], and were shown to regulate the immune response to T-independent sialylated CHO-Ags [154, 196, 225-229, 321, 322]. These data suggest that CD22 and Siglec-g may play a role in the production of Abs against naturally-occurring CHO-Ags, such as A/B-Ags.

1.13.1.1. Impact of B cell Siglecs in production of ABO nAbs

NAbs are thought to have specificities for epitopes expressed on microorganisms [105, 169, 176, 177] or self-tissues [178, 179], and to be mainly produced by B1 cells in mice [103, 323]. Compared with *WT* mice, there are higher numbers of B1 cell populations in the peritoneal cavity, with increased production of IgM nAbs in *CD22KO* [252, 258], *Siglec-gKO* [226, 249, 250, 262] and *CD22xSiglecgKO* mice [224](**Table 1.1)**.

Although these studies looked at the impact of Siglecs-deficiency in production of nAbs, the role of Siglecs-deficiency in production of ABO nAbs has not been investigated. Therefore, I will investigate the impacts of Siglecs-deficiency, sex, and age in development of ABO nAbs. I predict that in the absence of Siglec, higher levels of natural and induced ABO Abs are produced.

1.13.1.2. Impact of B cell Siglecs in induction of ABO Abs production

As previously mentioned, there is a discrepancy among research groups regarding the role of B cell Siglecs in induction of anti-CHO Abs production (**Table 1.2**). These groups have used chemically synthesized CHO structures, such as DNP/TNP-conjugated Ags, to study the impact of Siglecs-deficiency in induction of anti-CHO Abs production in *Siglecs-deficient* mice. However, these studies had various conclusions regarding the impact of Siglecs-deficiency on anti-DNP/TNP Abs production. For instance, some studies reported that CD22/Siglecg-deficiency promoted B cell "hyper-response" to stimulation by DNP/TNP-Ags [249, 252-254]. In contrast, others studies concluded that CD22/Siglecg-deficiency results in impaired anti-CHO Abs production [224, 254-256, 260, 263], or has no significant impact at all [249, 250, 257-260, 263](**Table 1.2**).

A confounding factor in these various conclusions with regard to the impact of CD22/Siglecg-deficiency on induction of anti-DNP/TNP Ab production may have been the lack of differentiation between natural vs. induced anti-DNP/TNP Abs. For instance, anti-DNP/TNP nAbs have been reported to be spontaneously produced in mice [264, 269, 270, 272, 273], rats [271], humans [267, 268], pigs [266], monkeys [265], and fish [154, 231, 324](**Table 3**). Therefore, the requirements for development of DNP/TNP nAbs may differ from that induced by DNP/TNP-Ags stimulation.

1.13.1.3. Impact of B cell Siglecs-deficiency in the T cell requirement for induction of ABO Abs

In Chapter 3, I showed a requirement for CD4+ T cell participation for induction of ABO Abs. However, the impact of Siglecs effect on the requirement of CD4+ T cell participation for induction of ABO Abs production (or other protein Ags) has not been investigated. It is generally accepted that B cells from *Siglecs-deficient* mice require less stimulation for induction of B cell activation [260, 325]. Therefore, CD4+ T cell participation may not be required for induction of ABO Abs production.

Using *Siglecs-deficient* mice, induction of ABO Abs production in the context of *syngeneic* and *xenogeneic* stimulation, compared with *WT* mice, may reveal a mechanism by which CD22/Siglecg-inhibitory signaling regulates immunity and tolerance to naturally-occurring CHO-Ags in the context of self/non-self discrimination.

1.13.2. Rationale to study the role of B cell Siglecs in ABO Abs production

West's lab data using human samples showed that ABO Abs are mainly produced by $CD27^+$ IgM^+ B cells and that this B cell subset in spleen expressed higher levels of CD22 in infants than adults [282, 283]. Moreover, our research group found that high level expression of CD22 on these B cells corresponds to the development of ABO tolerance following ABOi-HTx during infancy.

Although B cell Siglecs were described more than 25 years ago [251], the precise impact of Siglecs-deficiency in Ab production to CHO-Ags remains controversial (**Table 1.2)**. These studies did not determine the occurrence of nAbs production against the chemically synthesized CHO structures used in their studies (**Table 1.3**). Moreover, these studies did not use naturally-occurring CHO-Ags (such as A/B and α-gal Ags) than may extensively cross-link the BCR [254, 260] and therefore induce B cell apoptosis rather than Ab production [245, 246, 326, 327].

1.13.3. Hypotheses on the role of Siglecs in natural and induced ABO Abs production

1.13.3.1. Siglecs and natural ABO Abs production

Expansion of B1 cells population [224, 252, 258] in *Siglecs-deficient* mice could play important role in ABO nAbs production in these mice. Accordingly, I hypothesize that Siglecs-deficiency will lead to production of high ABO nAbs. Therefore, I will use *CD22KO* mice to examine the impact of Siglecs-deficiency in ABO nAbs production.

1.13.3.2. Siglecs and induction of ABO Abs production

I also hypothesize that without Siglecs-mediated inhibitory effects on B cells, induction of ABO Abs production by A/B-Ags in *Siglecs-deficient* mice will not require CD4+ T cell help. Therefore, I expect that in *CD22KO* mice, exposure to A-Ag alone (using *syngeneic* A-Tg blood) will induce anti-A Ab production.

1.13.4. Goals and objectives to study impact of B cell Siglecs in ABO Abs production

My goal is to define the impact of B cell Siglecs on ABO nAbs production, and define the impact of B cell Siglecs-mediated inhibitory effect on the requirement of CD4+ T cells participation for induction of ABO Abs production.

Chapter 2:

Materials and Methods

2.1. Mice:

2.1.2. Wild-type and germ-free mice: C57BL/6 (B6, H-2^b), BALB/c (BALB, H-2^d), and C3H/He (C3H, H-2^k) mice were purchased from Charles River Laboratories (Quebec, Canada). Serum of germ-free mice were kindly obtained from various sources, including University of Calgary (B6 and BALB serum from Dr. Paul Kubes' Lab), University of Chicago (B6 serum from Dr. Marisa Alegre's Lab), and University of Alberta (Swiss Webster (outbreed) serum from Dr. Ben Willings' Lab).

2.1.3. Knock-out mice: Mice homozygous for B6.129S2-Cd4^{tm1Mak} (Stock No: 002663) targeted mutation (*CD4KO*) on B6 backgrounds were purchased from Jackson Laboratory (USA). B6.129-H2-Ab1^{tm1Gru}N12 (Model #ABBN12-F) MHC class II genes targeted mutation (*MHC-IIKO*) and B6.129P2-Tcrβ^{tm1Mom}/Tcrδ^{tm1Mom} (Stock No: 002122) targeted mutation *(TCRKO)* mice on B6 backgrounds were purchased from Taconic or The Jackson Laboratory, USA, respectively. *CD22KO* (B6, H-2^b) mice were kindly provided by Dr. L. Nitschke (Erlangen, Germany).

2.1.4. Transgenic mice for A-antigen: Using B6 and BALB background mice, our group (with our collaborators) have developed transgenic mice expressing human A-Ag, by linking human H-glycosyltransferase and A-glycosyltransferase, under control of the ICAM-2 promoter [99-101]. The expression of A-Ag was confirmed on various tissues, such as erythrocytes, leukocytes, vascular endothelial cells, heart, kidney and lung [101]. Our research group also generated *A-Tg* BALB mice by backcrossing of the *A-Tg* B6 line to BALB mice for 12 generations.

2.1.5. Animal care: Protocols were approved by the University of Alberta Health Sciences Animal Care and Use Committee; animals were cared for according to the guidelines of the Canadian Council on Animal Care. All mice were used at 6-12 weeks of age unless otherwise noted.

2.2 Mice treatments:

2.2.1. Antibiotic treatment: Drinking water of some mice was supplemented with 1g/L each of neomycin, ampicillin, streptomycin, metronidazole and 0.25 g/L of vancomycin. The antibiotic administration was started at 7 days of age and discontinued at the end of experiment (maximum at 12 weeks of age). Antibiotics were replaced twice per week. All drugs except vancomycin (Alfa Aesar, USA) were purchased from (Sigma-Aldrich, Canada). Following antibiotics treatment, mouse feces were collected and diluted in sterile Phosphate-buffered saline (PBS, dilution factor for one pellet was 1:100,000-1,000,000). Feces suspension was swapped and platted in Blood agar plates and incubated both aerobically and an aerobically for 18-24hr at 37 °C. Next, bacterial colonies were counted to evaluate the impact of antibiotics treatment on growth of the bacterial flora (**Fig. 2.1**)

2.2.2. Blood cell membranes preparation for injection: BCM were prepared from human reagent (Immucor Inc., USA) A_1, B, or O-blood cell membranes (Hu-BCM) and from whole blood from *A-Tg* mice (A-Tg BCM). Membranes preparation has been described in detail elsewhere [328]. Briefly, cells were washed in PBS, lysed in hypotonic buffer, and membranes isolated following multiple centrifugations at 20,000 *g*. Membranes were suspended in PBS at 10% (v/v) and stored at -30 °C until the time of injection.

2.2.3. Immunization: Mice were injected intra-peritoneal (i.p.) with 100-150 µL of 10%v/v, with or without complete/incomplete Freund's adjuvant (CFA/IFA, 1:1 mixture), of human type A_1, B, O, or A-Tg BCM prepared from A-Tg blood. After the initial injection (week 7), mice received weekly two injections on week 8 and 9 (3 injections in total). In some experiments, mice were first injected with *syngeneic* A-Tg

BCM (3 injections at weeks 7, 8, 9). Four-weeks following the injection with A-Tg BCM, the mice were injected again with Hu A-BCM (3 injections at weeks 15, 16, 17) and anti-A Ab production was assessed again.

2.2.4. Heart Transplantation: Adult *WT* B6, BALB or C3H received age-and sex-matched *syngeneic* or *allogeneic* A-Tg B6 hearts heterotopically. After mouse was anesthetized, heart was heterotopically transplanted into abdomin of recipient mouse, as described previously [329, 330]. Briefly, the donor aortic root was anastomosed end-to-side to the recipient abdominal aorta and the donor pulmonary artery trunk end-to-side to the inferior vena cava of the recipient mouse. [329, 330]. Graft survival was assessed by abdominal palpation and scored as 4 is strongest palpation and 0 as no pulsation. The graft was also grossly and microscopically visualized and examined upon harvest.

2.2.5. T cell depletion *in vivo*: *WT* mice received intra-peritoneal injections (7-9 injections on day -2, -1, 0, and thereafter twice per week) with 150-200 µg of purified anti-mouse CD4 Ab (clone GK1.5) or anti-mouse CD8 Ab (2.43), X-Biocell, USA. T cells depletion was confirmed and monitored by flow cytometry staining of peripheral blood mononuclear cells (PBMC) on week 7, 8, 9 and 10.

2.2.6. CD4+ T cell isolation and adoptive transfer: CD4+ T cells were isolated and purified from adult spleens of *WT* or *A-Tg* mice, using mouse EasySep™ CD4+ T cell isolation kits (Stem Cell Technologies, Canada). The purity of CD4+ T cells was examined by flow-cytometry. The cells were injected via tail vein into 4 weeks old *CD4KO* mice at $8\text{-}12 \times 10^6$ CD4+ T cells per mouse in 140-200 µL of 0.9% PBS, using a modified protocol established in our lab [331]. Two weeks post-adoptive transfer, CD4+ T cell reconstitution in peripheral blood was assessed by flow-cytometry.

2.2.7. Flow-cytometry: Mouse peripheral blood mononuclear cells (PBMC) or splenocytes were labeled with rat monoclonal FITC anti-CD3 (cat: 555274), Alexa Fluor 647 anti-CD4 (cat: 557681)), and Pacific

Blue anti-CD8 (cat: 558106), BD Biosciences, Canada, and/or PE-anti-CD19 (clone: MB19-1), eBioscience, USA. The Ab-labelled cells were incubated for 30-60 minutes at 4°C and cells were analyzed using a MACSQuant flow cytometer (Miltenyi Biotech, Germany). The acquired data were analyzed using FlowJo 7.6.4 software (Tree Star, Inc., USA).

2.3. Assessment of ABO Abs production:

2.3.1. Hemagglutination assay: To measure anti-A/B Abs (mainly IgM) production, plasma was obtained from tail bleeds on week 7, 8, 9 and 10 following injection with A-Tg BCM or Hu A/B-BCM. A hemagglutination assay was used to assess anti-A/B Abs titer by incubating serially diluted serum samples (starting at 1:2 - 1:8) with 1-2% of A-Tg, human type A, or type B reagent erythrocytes in a 96-well micro-well plate. Hemagglutination plate was read after incubation at room temperature for one hour, and re-read after incubation at 4°C overnight. The Ab titer was assessed as the highest dilution at which agglutination was detected [328](**Fig. 2.2**).

2.3.2. ELISA: By modifying a method previously published elsewhere [81, 332], transparent flat-bottom Greiner medium-binding ELISA 96-well plates (Sigma-Aldrich, Germany) were coated with 50 µL of standard anti-mouse IgM and IgG (Bethyl Laboratories Inc., USA), or with synthetic A-Ag or B-Ag (A or B-trisaccharides polyacrylamides (A-PAA or B-PAA)) at 5 µg/mL in 0.1M Na_2CO_3 (pH 9.6). The plate was incubated at room temperature for one hour, and then at 4°C overnight. The next day, 5% normal goat serum in PBS was used to block non-specific Ab. Next, 10% diluted mouse serum/plasma, or serially diluted standard mouse reference serum was incubated at room temperature (RT) for 60-90 min. To detect the bound Ab, goat anti-mouse IgM or IgG conjugated with alkaline phosphatase (Bethyl Lab. Inc., USA) was applied for 60 min at RT to detect anti-A/B Abs. Next, p-nitrophenyl phosphate (Sigma-Aldrich, Germany) was used to visualize the phosphatase activity on the secondary Ab at 450 nm.

2.3.3. Microarray for detection ABH-subtype Abs: The microarray technique has been previously described [332]. Herein, ABH-subtypes were printed into cassettes and cassettes were loaded into the 96-well format microarray slide module (ACH4x24; Arrayit Corp., Sunnyvale, CA) and blocked with 1% BSA in PBS. Serum samples were spun and diluted (6 μL at 1:50) in 1% BSA/ PBS blocking buffer and incubated at 37°C in plate warmer for 30-60 min. Bound ABH-subtypes Abs were detected using AF647 goat anti-mouse IgM or Cyanine3 goat anti-mouse IgG Abs in blocking buffer (Jackson ImmunoResearch Inc., West Grove, PA). Microarray slides were scanned using GenePix 4000B scanner (Molecular Devices, San Jose, CA) at 635 nm at a PMT gain of 470 (IgM) and 532 nm at a PMT gain of 350 (IgG) and analyzed using ImaGene software (BioDiscovery, Hawthorne, CA). Normalized mean fluorescent intensities (MFIs) were calculated (using Global Sub-grid based normalization) by subtracting local background fluorescence for individual spots and BSA-only spots; averages of triplicates were reported.

2.3.4. Statistical analysis: Each experimental group contains at least 3 mice. Data analysis was performed using GraphPad (GraphPad Software Inc, USA). Data are presented as indicated. Student's T-test or Two-Way ANOVA post-hoc analyses were used to compare groups for ABO Abs production. Graft survival post-HTx was calculated using Kaplan–Meier analysis.

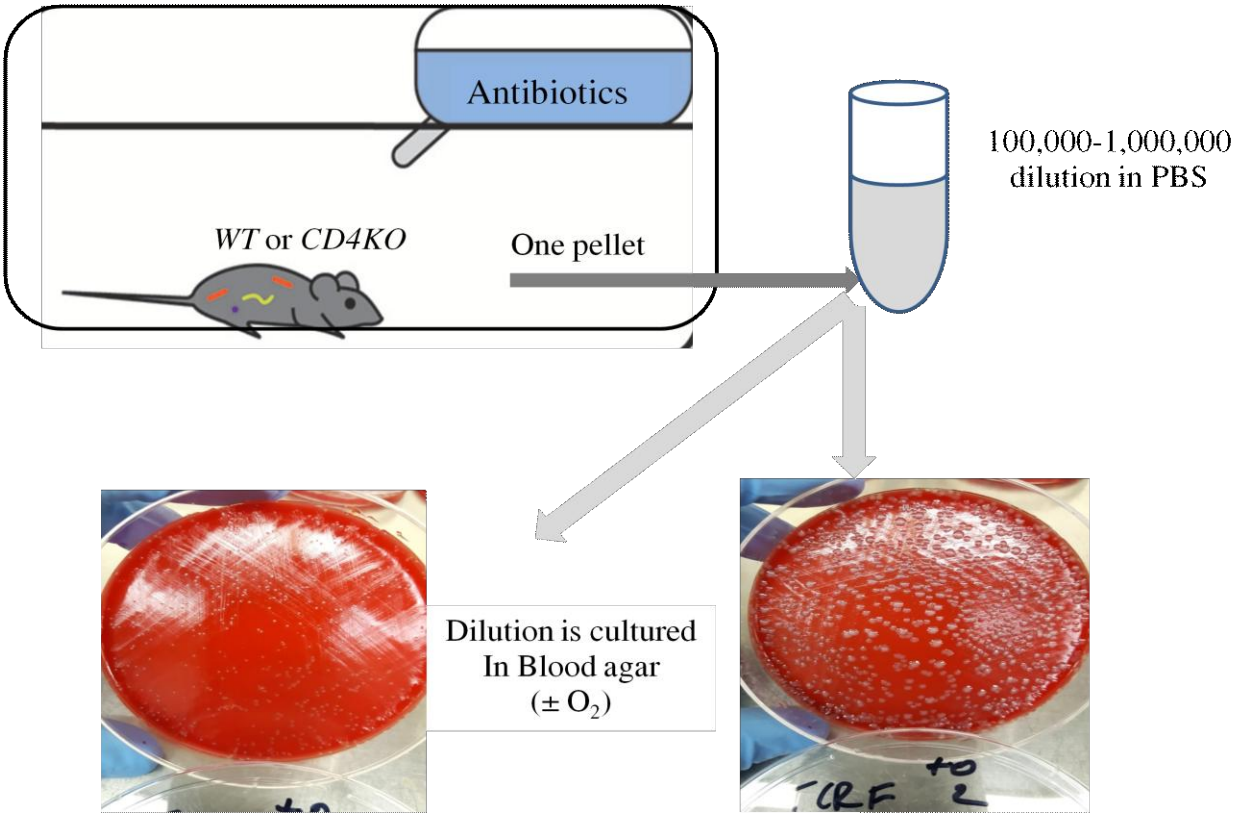

Fig. 2.1 Antibiotic treatment: Drinking water of *CD4KO* mice was supplemented with antibiotics started at 7 days of age and discontinued at the end of experiment at11-12 weeks. Following antibiotics treatment, feces were collected and diluted in sterile PBS, and then the dilution was streaked in Blood agar plates. Next, blood agar plates were incubated (aerobic and an aerobic) for 18-24hr at 37 °C. Finally, bacterial colonies were counted to evaluate the effect of antibiotics treatment. *Picture was made by I. Adam*

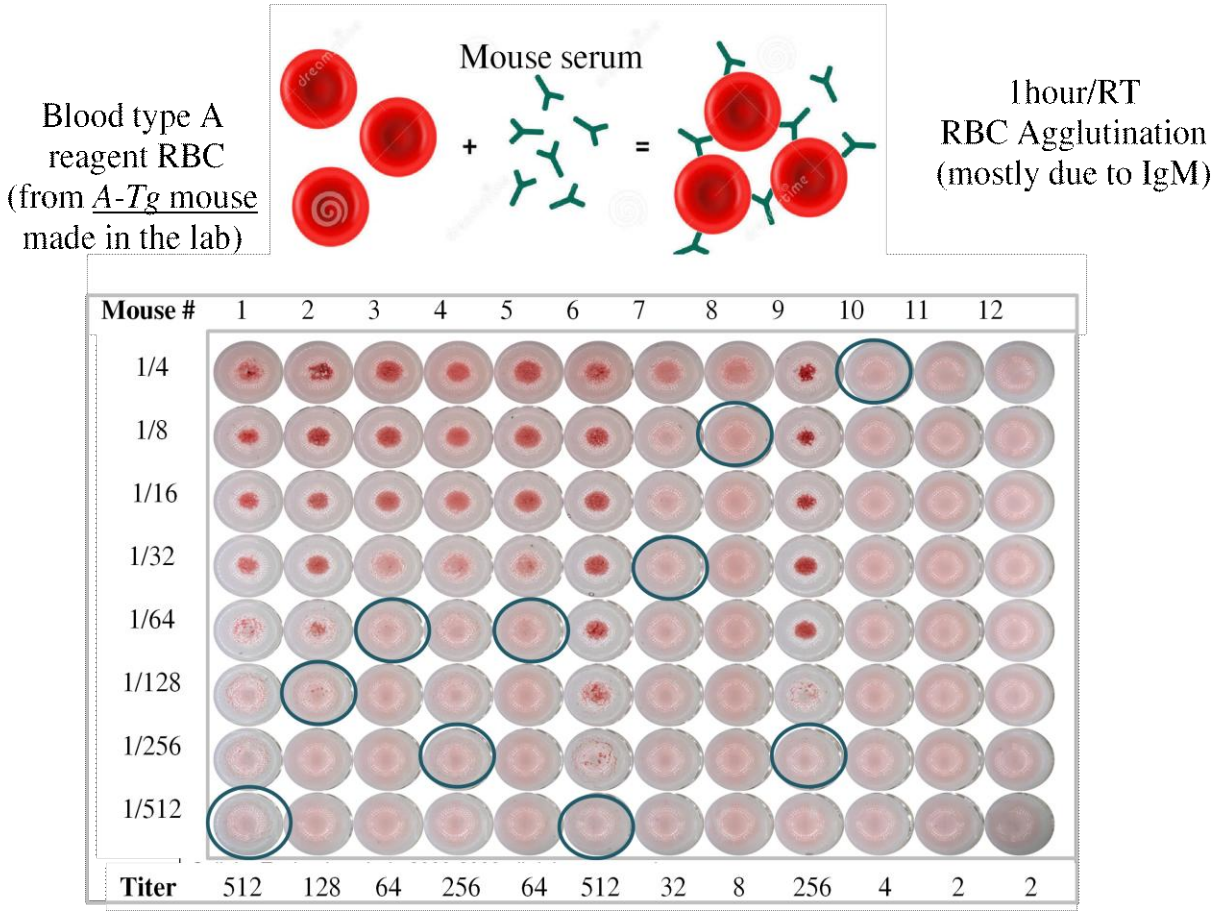

Fig. 2.2 Hemagglutination assay: Plasma obtained from tail bleeds of immunized mice was incubated in two-fold serial dilution (starting at 1:2, 1:4, or 1:8) with 1-2% of A-Tg (or human type A or B) reagent red blood cells in a 96-well micro-well plate. Hemagglutination plate was read after 1hr incubation at room temperature. Next, the plate is re-read after overnight incubation at 4°C overnight and the Ab titer is assessed as the highest dilution at which agglutination was detected. *Picture was made by I. Adam*

Chapter 3

Antibody response to non-self A-antigen is dependent on CD4+ T cell participation and foreign protein

3.1. Introduction:

ABO-incompatible organ Tx (ABOi-Tx) recipients are at high risk of rapid rejection mediated by preformed ABO antibodies (Abs), except during infancy when these Abs are naturally absent, and during which ABOi-heart Tx leads (ABOi-HTx) to B cell tolerance [87, 88]. Precise understanding of Ab production to A/B-Ags is essential in order to optimize safe ABOi transplantation. How B cell produces Ab against Ags is described in detail in 1.2.1 in Chapter 1. Briefly, a B cell, similar to other APCs, degrades the Ag and presents its antigenic peptides on the B cell's MHC-II as a peptide/MHC (pMHC) complex, to a helper CD4+ T cell that recognizes the pMHC complex via its TCR, and therefore stimulates a B cell response [23]. The CD4+ T cell participation would induce B cell activation [24, 25], differentiation [30], and formation of Ag-specific memory B cell [31].

In contrast to protein Ags, carbohydrate-antigens (CHO-Ags) induce B cell response that have been thought to occur without CD4+ T cell participation [46, 333]. These T cell-independent (TI) immune response is classified as TI type 1 (TI-1) or TI type 2 (TI-2) [334]. Extensive cross-linking of BCR by CHO-Ag containing repetitive epitopes is thought to provide strong and extensive BCR signaling that is sufficient to stimulate the carbohydrates (CHO) specific B cell response [47, 50, 68, 69]. The immune response induced by A/B-Ags have been classified as TI-2 [72] and generally thought to stimulate B cells for Ab production to non-self A/B-Ags without CD4+ T cell help [73, 291] and hence B cell activation by repetitive CHO-Ag (such as A/B-Ags) is classified as a TI-2 B cell response [46, 335].

Previous experiments that concluded immune responses to CHO-Ags are TI relied on chemically synthesized CHO structures, such as TNP-Ficoll [303-307]. These chemical structures may not function as surrogates for naturally-occurring CHO Ags, such as α-gal or A/B-Ags. Moreover, the conclusion that Ab response to A/B-Ags is TI was based on mice with limitations, such as *nu/nu* [72] that produces extrathymic T cells [292-294], or *CBA/xid*, that have an impaired B cell response [336, 337].

To study tolerance to self-A/B-Ags and immunity to non-self A/B-Ags in both *syngeneic* and *allogeneic* stimulation in a more relevant setting (this is not possible in humans), our research group previously generated an A-Ag-transgenic (*A-Tg*) mouse model on *B6* and *BALB* background mice [101]. In *A-Tg* mice, A-Ag is expressed on various tissues and cells, including vascular endothelium, erythrocytes and leukocytes [101]. Herein, I investigated anti-A Ab production to stimulation by A-Ag and the role of CD4+ T cells on inducing anti-A Ab production, aiming to gain a better understanding of the mechanisms of induced ABO Abs production to A/B-Ags.

3.2. Results:

3.2.1. *WT* mice produced natural anti-A Ab and responded to stimulation by A/B-Ags, *A-Tg* mice did not produce Ab to self A-Ag.

Mice are an appropriate small animal model to study questions related to ABO Abs production [72, 290, 291, 338]. However, there is inconsistency in the literature as to whether mice produce ABO antibodies in similar fashion to humans [289, 339]. Therefore, I first investigated whether *WT* B6 mice (H-2^b) develop anti-A natural antibodies (nAbs) without stimulation. I collected blood from mice at different ages housed in standard housing and measured anti-A Ab titer by hemagglutination assay using A-Tg reagent RBC. I found that *WT B6* mice do produce anti-A nAb, low in early life (4 weeks old) and widely variable in Ab titer range in later life (>12 weeks old, **Fig.3.1A**). I also found that *WT* mice developed increasing natural

anti-A Ab as they age over time without intentional stimulation, and injection with human type A blood cell membranes (Hu A-BCM) induced anti-A Ab production (**Fig.3.1B**). At 7 – 12 weeks old, I did not find a sex difference in natural or induced anti-A Ab production.

Using our *A-Tg* mice [101], I tested whether B cells in *A-Tg* mice produce Ab to self A-Ag. I injected *A-Tg* and *WT* B6 mice with mixed human type A and B BCM (Hu A-BCM and B-BCM). Given that *A-Tg* mice express A-Ag as self Ag, I predicted that they would not produce anti-A Ab. As expected, the *WT* mice produced both anti-A and anti-B Abs in response to Hu A-BCM and B-BCM, whilst the *A-Tg* mice produced only anti-B in response to Hu B-BCM (**Fig.3.1C, table 3.2 and table 3.3**).

In summary, I showed that *WT* B6 mice spontaneously produce anti-A Ab over time in similar fashion to humans and they can also be induced to produce ABO Abs. Furthermore, I showed that B cells in *A-Tg* mice do not produce Ab to stimulation by A-Ag, suggesting B cell self-tolerance in *A-Tg* mice. Therefore mice are good animal model to study tolerance and immunity to A/B-Ags, because *A-Tg* mice were tolerant to self A-Ag and *WT* mice responded to stimulation by non-self A/B-Ags.

3.2.2. *Syngeneic* A-Tg BCM injection did not induce anti-A Ab production in *WT* mice whereas *xenogeneic* Hu A-BCM injection induced abundant anti-A Ab production.

The α-gal epitope is a naturally-occurring CHO structure expressed in non-primate mammals and New World monkeys, but not in humans in which α-gal nAbs are produced [8, 9]. Galili's studies with α-gal Ags showed that induction of anti-gal Ab production in α-gal knock-out *(α-galKO)* mice requires CD4+ T cell participation [315-317, 340]. Due to the close similarity of α-gal and B-Ag [311, 312], I expected that CD4+ T cells are required for induction of both anti-B and anti-A Abs production. Furthermore, because our preliminary data suggested that CD40/CD40L interaction is required to induce anti-A Ab production [269], I expected that CD4+ T cells are also important in induction of anti-A Ab production.

To examine the required stimulation for induction of anti-A Ab in *WT* mice, I tested whether A-Ag in the context of *syngeneic* vs. *xenogeneic* proteins induce anti-A Ab production. I found that *WT* mice injected with *syngeneic* A-Tg BCM did not produce anti-A Ab production, but *xenogeneic* injection with Hu A-BCM induced significantly more anti-A Ab compared to untreated *WT* mice or mice injected with *syngeneic* A-Tg BCM (**Fig. 3.2A, table 3.2 and table 3.3**). I repeated this experiment in BALB (H-2^{d}) mice with injection of *xenogeneic* Hu A or *syngeneic* A-Tg BALB BCM into *WT* BALB mice. I found that, in contrast to *syngeneic* A-Tg BALB BCM, Hu A-BCM induced abundant anti-A Ab production (**Fig. 3.2A**). I also analyzed my results by sex and I did not observe sex differences in anti-A Ab production following stimulation by *syngeneic* A-Tg BCM or *xenogeneic* Hu A-BCM (**table 3.2 and table 3.3**).

Failure to produce anti-A Ab to *syngeneic* A-Tg BCM in *WT* mice, compared to high anti-A Ab stimulated by *xenogeneic* Hu A-BCM, may indicate that *xenogeneic* proteins expressed on Hu-BCM participated in induction of anti-A Ab production. Accordingly, I tested whether *xenogeneic* proteins in human type O-BCM mixed with A-Ag from *syngeneic* A-Tg BCM, can induce anti-A Ab production. I injected Hu O-BCM and A-Tg BCM (1:1 mixture) into *WT* B6 mice. Compared to injection of Hu A-BCM that induced abundant anti-A Ab, *WT* B6 mice injected with Hu O-BCM/A-Tg mixture induced significantly lower anti-A Ab (**Fig. 3.2B, table 3.2 and table 3.3**), suggesting that foreign protein stimulates additional immune response, but not sufficient to make *syngeneic* A-Tg BCM immunogenic, similar to *xenogeneic* Hu A-BCM.

3.2.3. Hu A-BCM injection induced anti-A Ab production following unsuccessful stimulation by *syngeneic* A-Tg BCM.

Failure to produce anti-A Ab to *syngeneic* A-Tg BCM in *WT* mice also triggered me to further examine whether this lack of Ab response to *syngeneic* A-Tg BCM in *WT* mice reflected induction of a state that

could prevent anti-A Ab production (deletion/tolerance?) to subsequent stimulation by *xenogeneic* Hu A-BCM. By using a modified protocol adopted from Gallili's studies in *α-galKO* mice [317], I first injected *syngeneic* A-Tg B6 BCM into *WT* B6 and evaluated anti-A Ab production. Four weeks following the A-Tg BCM injection, the mice were injected with Hu A-BCM and anti-A Ab production was assessed again. Following the unsuccessful immunization with A-Tg B6 BCM in *WT* B6 mice, injection of Hu A-BCM induced abundant anti-A Ab production (**Fig. 3.3A**).

Absence of tolerance to subsequent immunization with *xenogeneic* Hu-A-BCM in *WT* B6 mice above might be attributed due to use of complete Freund's adjuvant that known to break tolerance [341-343]. To exclude any potential effect of the adjuvant, I injected A-Tg BCM and Hu A-BCM without the adjuvant. I found that in the absence of the adjuvant, the *WT* B6 mice did not produce anti-A Ab in response to *syngeneic* A-Tg B6 BCM, whereas responded to subsequent immunization by Hu A-BCM (**Fig. 3.3B**).

I also examined whether introduction of A-Ag in mouse's early life would induce a subsequent unresponsiveness to immunization by Hu A-BCM. Therefore, I injected *WT* mice at 1, 2, and 3 weeks old "juvenile mice" with Hu A-BCM or A-Tg BCM without adjuvant. Four weeks following unsuccessful stimulation by A-Tg BCM, Hu A-BCM injection induced abundant anti-A Ab production comparable to that Ab produced in *WT* mice injected with Hu A-BCM (**Fig.3.3C**).

These data indicated that production of anti-A Ab in response to Hu A-BCM, following initial failure to respond to A-Tg BCM, is due insufficient stimulation by *syngeneic* A-Tg BCM rather than the *syngeneic* A-Tg BCM being non-immunogenic.

3.2.4. Induced anti-A Ab production required CD4+ T cells participation.

My results so far showed that exposure to A-Ag alone (in the context of *syngeneic* A-Tg BCM) did not stimulate anti-A Ab production, but A-Ag and foreign proteins (in the context of *xenogeneic* Hu A-BCM)

stimulated abundant anti-A Ab production. This indicated that stimulation of anti-A Ab production required exposure to A-Ag physically linked with non-self protein Ag, which suggests T cells participation recruited by *xenogeneic* proteins.

Previous studies indicated that CD4+ T cells were required for the response to α-gal [316, 317, 340, 344] and other glycolipids/glycoproteins-borne CHO-Ags [290]. Since our preliminary data suggested that CD40/CD40L interaction was required to induce anti-A Ab production and that mice responded to *xenogeneic* Hu A-BCM but not to *syngeneic* A-Tg BCM, I hypothesized that *xenogeneic* protein Ags were required to induce anti-A Ab production and that CD4+ T cells played important role in induction of anti-A Ab production. Therefore, I first depleted CD4+ T cells [345] and tested the effect of the CD4+ T cell depletion in induction of anti-A Ab production. In contrast to induction of anti-A Ab production in *WT* B6, CD4-depleted *B6* mice did not produce anti-A Ab following Hu A-BCM injection (**Fig. 3.4A, table 3.2 and table 3.3**), indicating that CD4+ T cells were indeed required to induce anti-A Ab production.

The CD4+ T cell dependency of anti-A Ab production prompted me to test whether *allogeneic* proteins expressed on A-Tg BCM would also induce anti-A Ab production similar to stimulation by *xenogeneic* Hu A-BCM. This test is valuable because *allogeneic* proteins are expected to engage CD4+ T cell participation. Therefore, I speculated that foreign proteins (such as MHC) and A-Ag expressed on *allogeneic* A-Tg BCM [101] could be presented to CD4+ T cell and therefore would recruit CD4+ T cell participation to induce anti-A Ab production by B cells. When I injected *allogeneic* A-Tg B6 BCM into either *WT* BALB or C3H mice, significantly more anti-A Ab was produced in the injected mice than in untreated BALB or C3H mice (**Fig. 3.4B, table 3.2 and table 3.3**).

B cell stimulation by *allogeneic* A-Tg BCM prompted me to further examine whether *allogeneic* A-Tg heart graft can also induce anti-A Ab production. As expected, A-Tg B6 heart allograft heterotopically

transplanted into *WT* BALB (A-Tg B6 HTx→BALB) or C3H (A-Tg B6 HTx→C3H) recipients were rejected (no pulsation by abdominal palpation, scored 0) in BALB and C3H by day 8-11 after transplantation (*syngeneic* graft survived for 100 days). More importantly, when the A-Tg B6 heart allograft was left *in situ* after rejection, anti-A Ab was produced significantly more in both BALB and C3H recipients compared to non-transplanted mice and *WT* recipient of *syngeneic* A-Tg graft (**Fig. 3.4.C, table 3.2 and table 3.3**).

These results suggested that CD4+ T cells are necessary for anti-A Ab production to be induced and that CD4+ T cells, presumably recruited and engaged by foreign proteins expressed in *allogeneic* blood or heart graft, contribute to induction of anti-A Ab production. In this regard, A-Tg mouse is valuable tool to examine immunity and tolerance of A-Ag in the context of *syngeneic* and *allogeneic* stimulation that is impossible in humans.

3.2.5. Adoptive transfer of CD4+ T cells into male *CD4KO* mice restored their ability to produce anti-A Ab in response to Hu A-BCM injection.

CD4+ T cells were shown to play important role in B cells response to glycoconjugate Ags, such as conjugated vaccine against bacterial infections [34, 346-349]. My finding that CD4+ T cell depletion abolished induction of anti-A Ab production in response to exposure to *xenogeneic* Hu A-BCM urged me to test whether CD4+ T cells do provide help for induction of anti-A Ab production using *CD4KO* mice. Using these mice, a clear sex difference was emerged (will be discussed in details in the next chapter). Moreover, I found that *CD4KO* male mice produced significantly higher natural anti-A Ab than *WT* mice (natural Abs will be discussed in details in the upcoming Chapter 4). After *CD4KO* male mice were reconstituted by *WT* male CD4+ T cells, Hu A-BCM was injected to stimulate anti-A Ab production (**Fig. 3.5A**). I found that the adoptive transfer of CD4+ T cells from *WT* mice into *syngeneic CD4KO* male mice

restored their ability to produce anti-A Ab in response to *xenogeneic* Hu A-BCM injection (**Fig. 3.5B, table 3.2 and table 3.3**). Interestingly, CD4+ T cell from *A-Tg* mice also restored the ability of *CD4KO* male mice to produce anti-A Ab following *xenogeneic* Hu A-BCM injection, bringing up interesting issues of CD4+ T cell and immunity to self CHO-Ags.

Thus, in agreement with my other evidence for T cell requirement for induction of anti-A Ab production, adoptive transfer of CD4+ T cells into *CD4KO* mice restored their ability to respond to stimulation by *xenogeneic* Hu A-BCM, providing further evidence that induced ABO Abs production is CD4+ T cell dependent.

3.3. Discussion:

In summary, my experiments described in this chapter showed that *WT* mice produced both anti-A and anti-B Abs in abundance in response to stimulation by *xenogeneic* Hu A/B-BCM, whereas *syngeneic* A-Tg BCM alone failed to induce anti-A Ab production. Co-injection of *syngeneic* A-Tg and *xenogeneic* Hu O-BCM did not induce abundant anti-A Ab production similar to stimulation by *xenogeneic* Hu A-BCM. Stimulation of *WT* mice by *allogeneic* A-Tg BCM or heart graft also induced abundant anti-A Ab production. Following CD4+ T cell depletion, induction of anti-A Ab production by *xenogeneic* Hu A-BCM injection was significantly diminished and ability to respond to stimulation was re-established after reconstitution of *CD4KO* mice with CD4+ T cells. I did not observe sex difference in *WT* mice for both natural and induced ABO Abs production. However, I separated the mice by sex in the summary table (**Table 3.1**).

I found that mice only develop ABO Abs to non-self A/B-Ags, as in other mammals [109, 182]. Unlike *WT* mice who could be induced to produce both anti-A and B Abs, *A-Tg* mice produced only anti-B Ab following stimulation by *xenogeneic* Hu A/B-BCM. I observed in *WT* mice that anti-B Ab response was

lower than anti-A Ab; this low anti-B Ab titer may be due to expression of "B-like" α-gal Ag in mice [350, 351]. This α-gal Ag has a galactose in the terminal sugar structure similar to B-Ag [312] and it was estimated that 85% of anti-B Abs were reactive to α-gal [13].

My finding that *syngeneic* A-Tg BCM did not induce anti-A Ab production is consistent with our research group previous observations that *syngeneic* A-Tg B6 heart grafts did not induce anti-A Ab in *WT* B6 mice [101]. Moreover, my finding that *syngeneic* A-Tg BCM mixed with *xenogeneic* Hu O-BCM induced significantly higher anti-A Ab production than mice injected with *syngeneic* A-Tg BCM, but significantly lower than mice injected with *xenogeneic* Hu A-BCM, was consistent with the requirement for chemical and/or physical conjugation of protein/CHO (as in glycoconjugate vaccines [352, 353]) for optimal stimulation of anti-CHO Ab production.

Data on α-gal Ag (in the context of *syngeneic* and *xenogeneic* stimulation) showed that T-independent stimulation of *α-galKO* mice induces gal-specific B cell tolerance [317, 344]. However, the lack of response to *syngeneic* A-Tg BCM did not seem to reflect induction of tolerance to A-Ag, because subsequent *xenogeneic* Hu A-BCM injection induced anti-A Ab production. Rather, the production of anti-A Ab to Hu A-BCM following the initial failure to respond to A-Tg BCM indicates that *syngeneic* A-Tg BCM challenge was not sufficiently immunogenic to stimulate B cell response, because: *A*) In a setting where the stimulation by A-Tg BCM did induce anti-A Ab production, Hu A-BCM injection stimulated CD4+ T cell help for B cells to produce anti-A Ab, and *B*) the lack of B cell response to A-Tg BCM did not lead to inhibition (deletion?) of the A-Ag specific B cells response, as anti-A Ab developed following the second subsequent challenge by Hu A-BCM injection. Interestingly, our lab previously observed that after transplantation of A-Tg graft in juvenile mice (4-weeks old), Hu A-BCM injection in adulthood (>8 weeks old) did not induce strong anti-A Ab production [354], suggesting that persistent exposure of B cell to A-

Ag (ie, *in situ* graft or intact erythrocytes that live longer in circulation than erythrocyte membranes) may be required to induce tolerance.

B cell response to A-Ag on Hu A-BCM suggested an involvement of other immune cells (i.e., CD4+ T cell) due to the presence of additional foreign proteins on *xenogeneic* Hu-BCM. Therefore, and in contrast to general belief [20, 47, 72, 355], I found that anti-A Ab production (and presumably anti-B Ab) required CD4+ T cell participation. I have shown a requirement for T cell help to induce Ab production to A-Ag, as CD4+ T cell depletion prevented induction of anti-A Ab production and that CD4+ T cell reconstitution restored it. Anti-A Ab production following stimulation by *allogeneic* A-Tg BCM and/or graft confirmed the T-cell dependency, because it indicated that immune response to *allogeneic* proteins on A-Tg BCM/graft recruited CD4+ T cell participation.

Similar to studies that showed CD4+ T cell was required to stimulate Ab production to glycoconjugate vaccines [348, 349, 356, 357], I showed that the ability of *xenogeneic* Hu A-BCM to stimulate anti-A Ab production in *WT* mice required participation of CD4+ T cell of *WT* (or *A-Tg*) mice. The ability of CD4+ T cell from *A-Tg* mouse to restore the ability of induction of anti-A Ab production has brought up interesting issues of the role of CD4+ T cell in immunity to self CHO-Ag. In this regard, it was suggested that a TCR can recognize CHO epitope of a glycoconjugate when presented on MHC of a B cell [347, 358, 359]. However, whether CD4+ T cells directly interact (or "recognize") A-Ag in *allogeneic* A-Tg BCM and graft, or in *xenogeneic* Hu A-BCM, are unknown and require further investigations in future. In addition, future studies should investigate whether injection of intact *syngeneic* A-Tg erythrocytes in adult *WT* mice would induce tolerance to subsequent stimulation with *xenogeneic* Hu A-BCM. Moreover, I did not investigate which B cell subset responded to stimulation by A/B-Ags and it is important to tease out whether B1 cell or marginal zone B cells participated in production of ABO Abs.

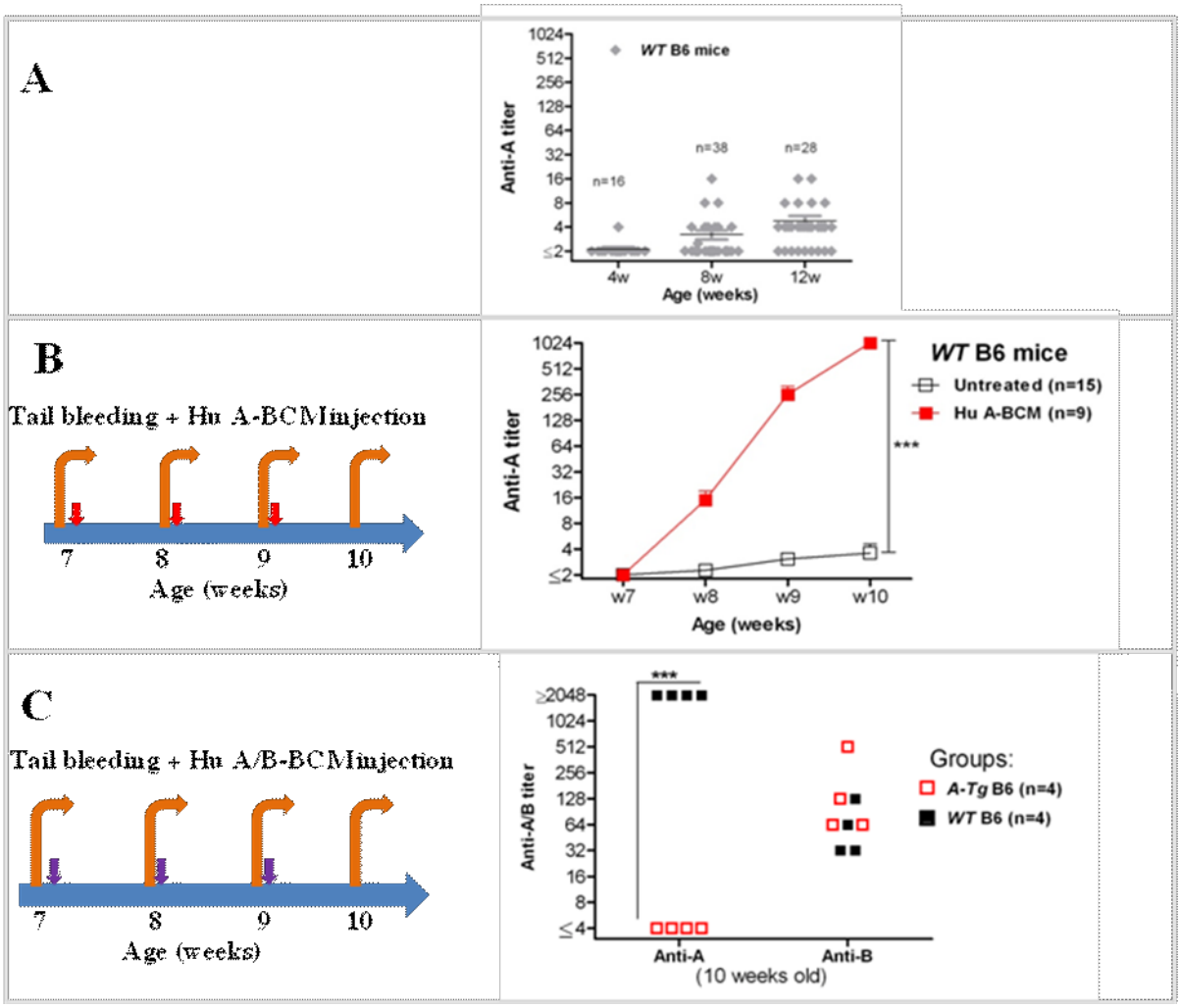

Fig.3.1. *WT* mice produced anti-A nAbs and responded to stimulation by A/B-Ags, *A-Tg* mice did not produce Ab to self A-Ag. A) Historical sera collected in the lab from mice of different ages and assessed for anti-A Ab showed that *WT* B6 mice produced low Ab in early life and widely variable Ab range in later life. **B)** Tail blood was collected from untreated mice (4 tail bleeding in total, one week apart; *orange long arrows*) or mice injected with Hu A-BCM at 7-10 weeks old (*red short arrows*). Sera were longitudinally assessed for Ab production and showed that *WT* B6 mice develop *natural* (without any blood injection) and *induced* (with blood injection) anti-A Ab. **C)** *WT* and *A-Tg* B6 mice received weekly injections (3 injections in total; *pink short arrows*) of *xenogeneic* Hu A-BCM and B-BCM and assessed for anti-A/B Abs production (shown Ab titer at week 10). I used hemagglutination to measure anti-A/B Abs, using human type A/B reagent RBC to measure anti-A/B Abs, respectively. Data are presented using standard error of mean (Mean + SEM) in T-Test (*** = $P \leq 0.001$, see table 3.2 and 3.3 for comparison between groups).

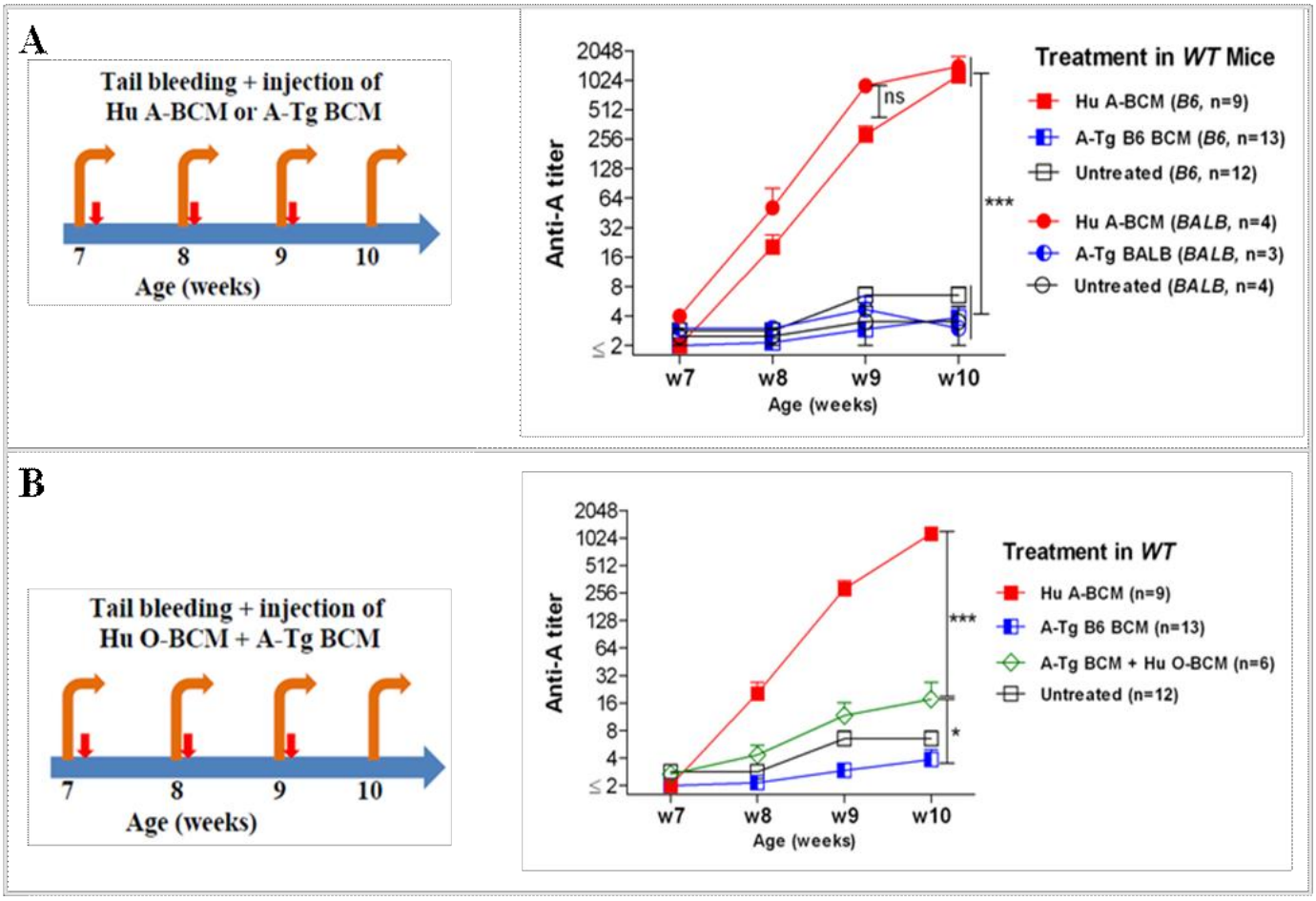

Fig.3.2. *Syngeneic* A-Tg BCM injection did not induce anti-A Ab production in *WT* mice; in contrast Hu A-BCM induced abundant anti-A Ab production. A) *WT* B6 mice received weekly injections (3 injections in total, *red short arrows*) of *xenogeneic* Hu A-BCM, *syngeneic* A-Tg BCM, or left untreated. Tail blood was collected before each injection at week 7, 8, 10, and 10 (*orange long arrows*). *WT* BALB mice were also injected with A-Tg BALB BCM or Hu A-BCM. **B)** *WT* B6 mice received weekly injections of *xenogeneic* Hu O-BCM mixed with *syngeneic* A-Tg BCM and tail blood was collected before each injection. I used hemagglutination to measure anti-A Ab, using A-Tg reagent RBC. Data are presented using standard error of mean (Mean + SEM) in T-Test (ns=non-significant, * = $P \leq 0.05$, *** = $P \leq 0.001$, see table 3.2 and 3.3 for comparison between groups).

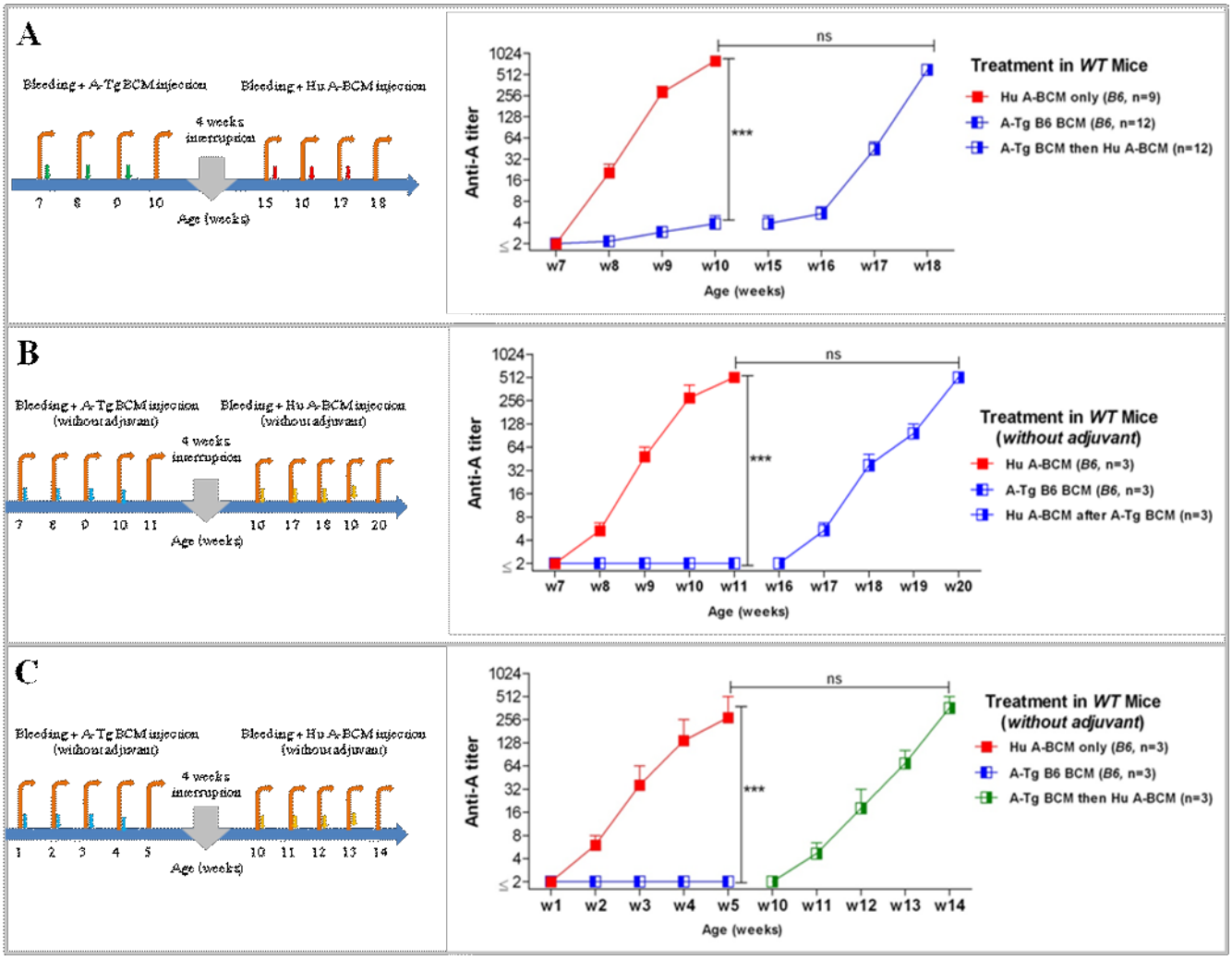

Fig.3.3. Hu A-BCM injection induced anti-A Ab production following unsuccessful stimulation by *syngeneic* A-Tg BCM. A) In my experimental timeline and immunization procedure, some mice were first injected with A-Tg BCM and adjuvant (3 injections at weeks 7, 8, 9, *green triangles*). Four-weeks following the initial A-Tg BCM injection (*gray thick arrow*), the mice were injected again (3 injections at weeks 15, 16, 17) with Hu A-BCM and adjuvant (*red short arrows*). **B)** Without adjuvant, *WT* B6 were injected with Hu A-BCM (*yellow short arrows*) following unsuccessful stimulation by A-Tg BCM (*blue triangles*). **C)** Without adjuvant, one-week old "juvenile" mice were first injected with A-Tg BCM (at weeks 1, 2, 3, and 4 (*blue triangles*). Four-weeks following the initial A-Tg BCM injection (*gray thick arrow*), the mice were injected again (at weeks 10, 11, 12, and 13 (*yellow short arrows*) with Hu A-BCM. I used hemagglutination to measure anti-A Ab, using A-Tg reagent RBC. Data are presented using standard error of mean (Mean + SEM) in T-Test (ns=non-significant, *** = $P \leq 0.001$).

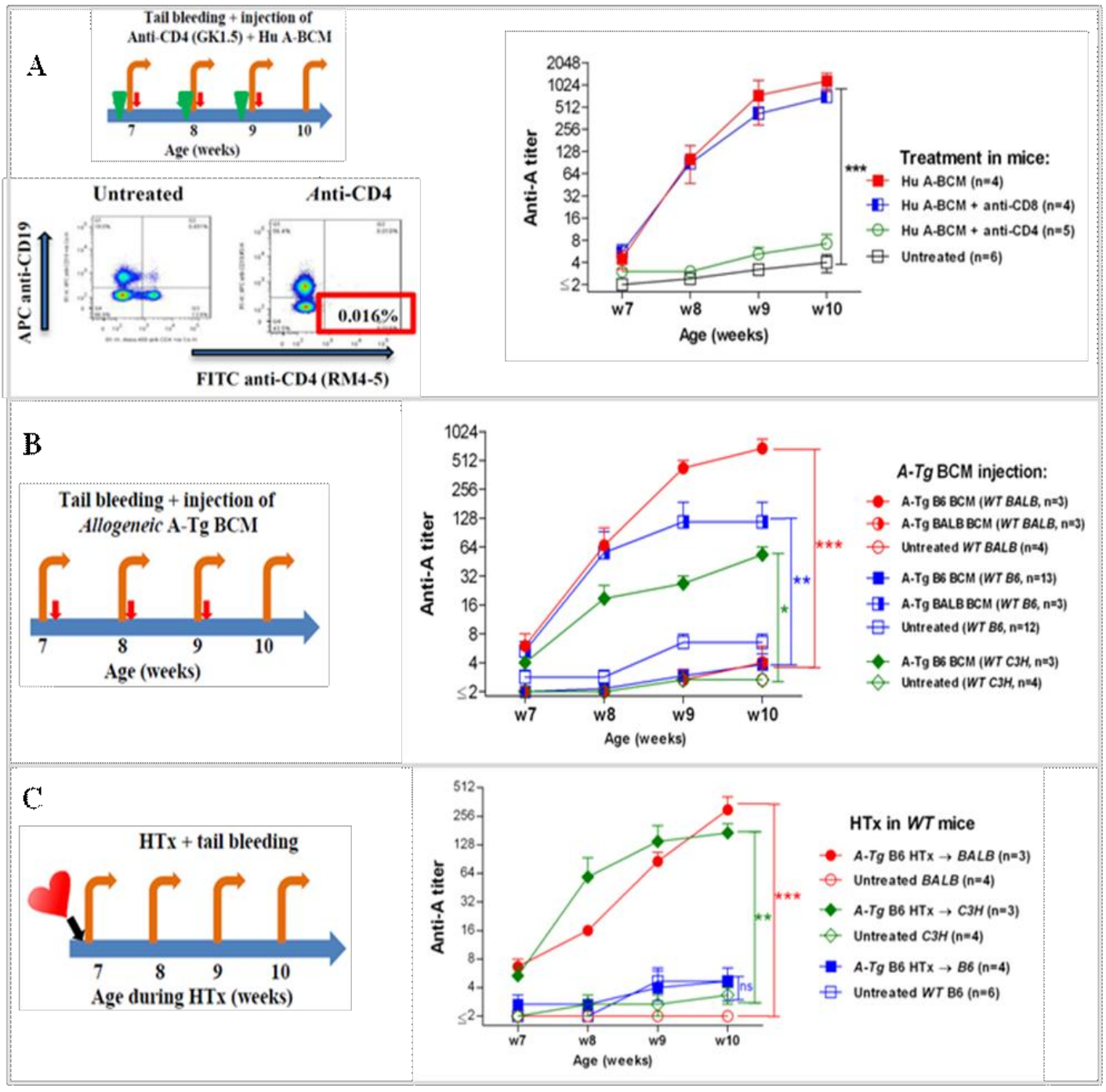

Fig.3.4. Induction of anti-A Ab production requires participation of CD4+ T cells and *allogeneic* antigens. A) *WT* mice received injections of GK1.5 anti-CD4 (250 µg i.p inj., twice per week for 4 weeks, *green triangles*) and Hu A-BCM (weekly, 3 injections in total, *red short arrows*). Tail blood was collected before each injection (*orange long arrows*). Effective depletion of CD4+ T cells in *WT* mice is demonstrated by flow cytometry of PBMC on week 10. CD4+ T cells depletion

abolished anti-A Ab production whereas CD8+ T cells depletion did not. **B)** *Allogeneic WT* B6, BALB, and *C3H* mice received weekly injections (3 injections, *red short arrows*) with BCM (prepared from A-TgB6 or BALB blood) and tail blood was collected before each injection (*orange long arrows)*. **C)** *Allogeneic WT* BALB and C3H were transplanted with age-and sex-matched A-Tg B6 hearts and the beating was monitored and assessed by abdominal palpation. All allografts were rejected with day 8-11 post-Tx but left *in situ*. Anti-A Ab was measured in tail blood (*orange long arrows*) by hemagglutination assay using A-Tg reagent RBC. Data are presented using standard error of the mean (Mean + SEM) in paired T-Test (ns=non-significant, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, see table 3.2 and 3.3 for comparison between groups).

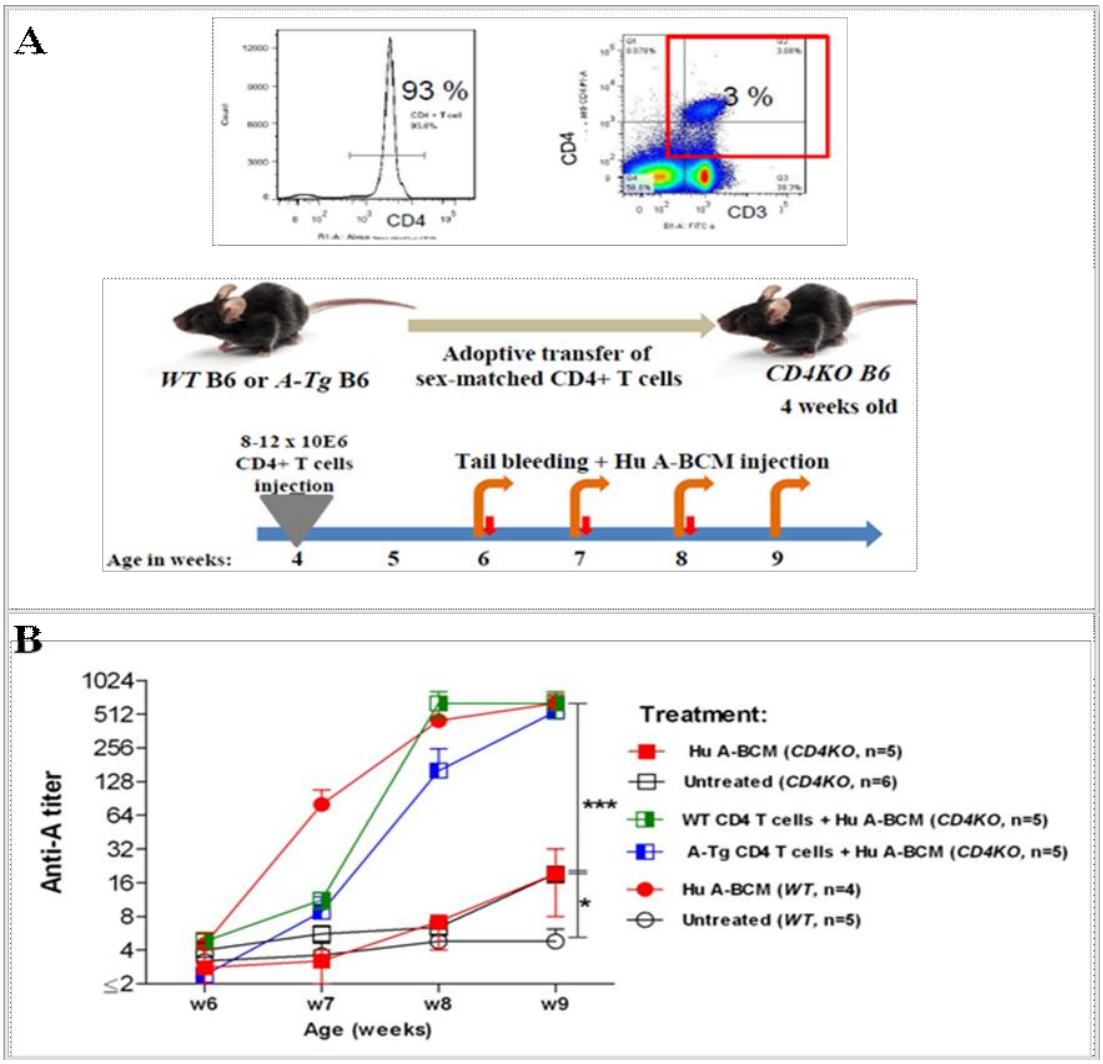

Fig.3.5. Adoptive transfer of CD4+ T cells into male *CD4KO* mice restored their ability to produce anti-A Ab in response to Hu A-BCM injection. A) CD4+ T cells isolated from splenocytes obtained from *WT* or *A-Tg* mice were examined by flow cytometry (purity is 93%) and injected into 4 weeks old *CD4KO* mice (8-12x10^6 cells, *gray triangle*). Two weeks later, CD4+ T cell reconstitution was assessed by flow cytometry and showed that 3% of PBMCs are CD4+ T cells. Mice were then injected with Hu A-BCM (3 injections at week 6, 7, and 8, *red short arrows*) and tail bleed was used to measure anti-A Ab production. **B)** CD4+ T cells isolated from *WT* or *A-Tg* splenocytes participated (or helped) in induction of anti-A Ab production in *CD4KO* male mice. Anti-A Ab titer was measured by hemagglutination assay using A-Tg reagent RBC. Data presented using standard error of mean (Mean + SEM) in T-Test (* = $P \leq 0.05$, *** = $P \leq 0.001$, see table 3.2 and 3.3 for comparison between groups).

Table 3.1: Summarized data of Chapter 3

Treatment	Recipient	No	Age in weeks	Anti-A titer
Untreated				
Untreated	*WT B6* males	7	7 - 10	<4
Untreated	*WT B6* females	8	7 - 10	<8
Untreated	*WT BALB* females	4	7 – 10	<8
Untreated	*WT C3H* males	4	7 – 10	<4
***syngeneic* stimulation**				
A-Tg B6 BCM	*WT B6*, 6 males, 7 females	13	7 – 10	<8
A-Tg B6 heart	*WT B6* males	4	7 – 10	<4
A-Tg BALB BCM	*WT BALB*, 3 males, 1 female	4	7 – 10	<8
syngeneic* + *xenogeneic				
Hu O-BCM + *A-Tg B6* BCM	*WT B6* males	6	7 – 10	<16
***allogeneic* stimulation**				
A-Tg B6 BCM	*WT BALB* females	3	7 – 10	512
A-Tg B6 BCM	*WT C3H* males	3	7 – 10	64
A-Tg BALB BCM	*WT B6* females	3	7 – 10	128
A-Tg B6 heart	*WT BALB* males	3	7 – 10	128
A-Tg B6 heart	*WT C3H* males	3	7 – 10	128
***xenogeneic* stimulation**				
Hu A-BCM	*A-Tg B6* females	4	7 – 10	<2
Hu A-BCM	*WT B6*, 6 males, 3 females	9	7 – 10	512
Hu A-BCM	*WT BALB*, 3 males, 1 female	4	7 - 10	1024
Hu O-BCM	*WT B6* males	3	7 – 10	<4
Hu A-BCM + anti-CD4 Ab	*WT B6* males	6	7 – 10	<8
Hu A-BCM + anti-CD8 Ab	*WT B6* males	6	7 – 10	512
CD4KO				
Untreated (nAbs)	*CD4KO B6* males	6	6 – 9	16
Hu A-BCM	*CD4KO B6* males	5	6 – 9	16
Hu A-BCM + CD4+ T cells	*CD4KO B6* males	5	4 – 9	512

Notes:

- Pre-titer: anti-A Ab level in untreated mice at 7 weeks old
- Post-titer: anti-A Ab level in mice at 10 weeks old, after several treatment
- Human A/B-BCM from pooled human blood donors
- *A-Tg* BCM was prepared from whole blood of A-Tg B6 or BALB mice
- I did not observe sex differences in terms of anti-A Ab titer in *WT* mice, with or without human A-BCM injection in either pre or post-treatment (**Table 3.2**).

Table 3.2: Comparison tables of groups (between 7 - 10 weeks old)

Groups	Treatment	Recipient	No	Anti-A	7 *vs.* 10 weeks Ab titer
#1	Untreated	*WT B6*	15	<4	ns
#2	Untreated	*WT BALB*	4	<8	ns
#3	Untreated	*WT C3H*	4	<4	ns
#4	Untreated	*CD4KO B6*	6	16	*
#5	*A-Tg B6* BCM	*WT B6*	13	<8	ns
#6	*A-Tg BALB* BCM	*WT BALB*	4	<8	ns
#7	*A-Tg B6* BCM	*WT BALB*	3	512	***
#8	*A-Tg B6* BCM	*WT C3H*	3	64	**
#9	*A-Tg BALB* BCM	*WT B6*	3	128	**
#10	*A-Tg B6* heart	*WT B6*	4	<4	ns
#11	*A-Tg B6* heart	*WT BALB*	3	128	**
#12	*A-Tg B6* heart	*WT C3H*	3	128	**
#13	Hu A-BCM	*A-Tg B6*	4	<2	ns
#14	Hu A-BCM	*WT B6*	9	512	***
#15	Hu A-BCM	*WT BALB*	4	1024	***
#16	Hu O-BCM	*WT B6*	3	<4	ns
#17	Hu O-BCM + *A-Tg B6* BCM	*WT B6*	6	<16	*
#18	Hu A-BCM + anti-CD4 Ab	*WT B6*	6	<8	ns
#19	Hu A-BCM + anti-CD8 Ab	*WT B6*	6	512	***
#20	Hu A-BCM	*CD4KO B6*	5	16	*
#21	Hu A-BCM + CD4+ T cells	*CD4KO B6*	5	512	***

- Data are presented using standard error of mean (Mean + SEM) in T-Test.
- ns = ≥ 0.5, * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001

Table 3.3: Comparisons of group's #

Groups	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16	#17	#18	#19	#20	#21
#1		ns	ns	***	ns				***	ns				***		ns	*	ns	***		
#2	ns		ns	ns		ns	***				***				***						
#3	ns	ns		ns				***				***									
#4	***	***	***																	ns	***
#5	ns				ns	ns		***	***	ns						ns	*	ns	***		
#6		ns			ns		***				***				***						
#7		***				***		***	**		ns				**						
#8			***				ns		**			**									
#9	***				***		**	ns		ns				***		ns	ns	ns	*		
#10	ns				ns				ns		**			***		ns	ns				
#11		**				**	ns			**		ns			**						
#12			ns					ns		*	ns										
#13														***	***					**	
#14	***				***				***	***					*	***	***	***	**	***	***
#15		***				**	ns				**			*						***	
#16	ns				ns				ns	ns				***			ns	ns	**		
#17	*				*				ns	ns				***		ns		ns	***		
#18	ns				ns				ns	ns				***		ns	ns		***		
#19	***				***				*	***				**		**	***	***			
#20				ns									**	***	***						***
#21				***									**	***	ns					***	

- Data are presented using standard error of mean (Mean + SEM) in Two-way ANOVA.
- ns = ≥ 0.5, * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001

Chapter 4

Bacterial flora, sex, and CD4+ T cells play important roles in natural ABO Abs production in mice

4.1. Introduction:

Naturally-occurring Abs (nAbs) are defined as spontaneous production of Abs in the absence of known stimulation by mechanisms that are not fully understood [163]. In mice, nAbs are mainly produced by the B1 cell population located in peritoneal and pleural cavities [106, 151-153]. In humans, ABO nAbs develop spontaneously without known exposure to ABO antigens (Ags) and posing high risk of hyper-acute rejection of transplants from ABO-incompatible (ABOi) donors [82, 89]. To expand the ABOi-Tx or to develop strategies to manage the ABO nAbs that make ABOi-Tx risky, a clear understanding of how ABO nAbs develop is vital.

It has been hypothesized that the development of ABO nAbs occurs due to stimulation by *"*A/B-like*"* Ags present in an exogenous stimulus, particularly via the gut [124, 125]. Springer's studies showed that feeding of germ-free chicks on blood group B +ve (BGB^{+}) *E. coli* O_{86} stimulated anti-B Ab in those chicks [119]. However, Springer's data also showed that exposure to gut bacteria is not an absolute requirement for anti-B Ab development in the germ-free chicks [119]. Since Springer's experiments in germ-free chicks were done in 1959, there could be limitations on whether those chicks were truly germ-free (they tested germ-free conditions by bacterial culture [119]) and therefore the so-called 'germ-free' may not have actually been germ-free. Data in the 1980s showed that natural Abs were greatly reduced (but not absent) in germ-free BALB mice compared to conventionally-housed mice [130], suggesting that bacterial flora probably have positive impact in ABO Abs production, but are not an absolute requirement. Importantly, recent data [132] showed that exposure of germ-free mice to bacterial stimulus in non-sterile

food or to gavage of conventionally-housed mice can stimulate ABO Abs development in those germ-free mice.

Despite an impact of gut bacterial flora in the formation of ABO Abs in germ-free mice [132], the specificity of the ABO Abs produced in the mice does not usually correspond with the chemical structures of the carbohydrates expressed on those gut bacterial flora [132]. Furthermore, studies found that the overall repertoire of IgM specific for bacteria [140] or the abundance of total IgM are generally stable in both germ-free and conventionally housed mice [131, 140]. Therefore, it is unclear whether the gut "bacterial stimulus" initiates the ABO Abs production in naïve B cells, or whether the "bacterial stimulus" is only required to augment the spontaneous production of ABO Abs. Interestingly, exposure to A/B-Ags in food was reported to stimulate ABO Abs production among blood donors and that eating processing food has decreased ABO Abs level among blood donors [135, 360].

As discussed in Chapter 3, one study concluded that induction of ABO Abs production in mice does not require T cell participation [72], but this study was limited because it was based on an *athymic* mouse model that produces extrathymic T cells [292-294]. Another study concluded that NKT cells, but not CD4+ T cells, are required to induce ABO Abs production [291], but this study did not investigate whether CD4+ T cells are required for development of ABO nAbs [291]. Although both studies concluded that induction of ABO Abs production does not require CD4+ T cell participation, neither study showed whether ABO Abs can develop spontaneously independent of exposure to A/B-Ags [72, 291].

A confounding factor related to the role of CD4+ T cells [72, 290, 291] may have been lack of differentiation of ABO nAbs production from that induced by intentional immunization and therefore the CD4+ T cell involvement in ABO nAbs production requires further investigation. Additionally, the reports showed that gut bacterial flora expressing highly-repetitive "A/B-like" epitopes participate in ABO nAbs

production [119, 120] did not prove whether a bacterial stimulus is absolutely required for production of those ABO nAbs.

Recently, sex as a biologic variable has been more recognized and increasingly being studied when investigating immune responses in humans and mice (reviewed in [284, 285]). Although data showed that *WT* mice develop low ABO nAbs titer in early life and widely variable in Abs titer range in later life [72, 101, 289] in a similar fashion to humans [133, 134], it is not clear whether sex as a biological variable plays a role in ABO nAbs production. As the effect of sex as a biological variable is becoming increasingly understood, I considered the impact of sex in ABO nAbs development. In this chapter, I investigated the roles of sex, gut bacterial flora, and requirement of CD4+ T cells for the production of ABO nAbs in mice.

4.2. Results:

4.2.1. *CD4KO* mice developed T-independent anti-A nAbs production with a striking sex difference.

Previous studies showed that the induction of anti-gal Abs requires T cell participation [290, 315-317], similar to induction of anti-A Ab production (Chapters 3 and others [290]). However, T cell depletion has also been shown to enhance the anti-gal nAbs production [310], suggesting that T cells may play a regulatory role in anti-gal natural Abs (nAbs) development. In humans, some X-linked hyper-IgM patients who have defective CD40 signaling [361] develop normal level of ABO and anti-gal nAbs [362], suggesting that T cells are also not required for ABO nAbs production. This is also similar to infants receiving T cell-directed immunosuppressant drugs who nonetheless go on produce ABO nAbs [88]. Therefore, I investigated whether the process of spontaneous production of anti-A nAb requires T cells.

I used *WT* and *CD4KO* mice on a *C57BL/6* (*B6*) background, aged 6-8 weeks old, to investigate the production of anti-A nAbs longitudinally over time. As suggested by preliminary data shown in Figure 3.1A, I found that *WT B6* mice do produce anti-A nAbs over time, with low Ab production in early life at

7-10 weeks old (**Fig. 4.1A, table 4.2 and table 4.3**). I noted that in 9 and 10 weeks old mice, *WT* females tend to produce more anti-A nAbs than males, but the difference was not significant. Therefore, sex seems to not play important role in anti-A nAbs production in *WT* mice, at least in early life (7-10 weeks).

Surprisingly, when I examined both natural and induced anti-A Ab production in *CD4KO* mice, I found that *CD4KO* mice not only developed significantly higher anti-A nAbs titers than *WT* mice, but there was also a striking difference in Ab production between sexes, with much higher anti-A nAbs production in female *CD4KO* mice than males (**Fig. 4.1A, table 4.2 and table 4.3**). To exclude environmental variations between cages, I co-housed males and females *CD4KO* from day 0 to the end of my experiment (week 10), but the sex difference remained of similar magnitude to that observed in cages separated by sex. Furthermore, to investigate whether this substantial sex difference is related to age, I monitored the *CD4KO* mice for a longer period and found that males still produce significantly lower anti-A nAbs levels compared to females independent of age (**Fig. 4.1B**).

My Chapter 3 data showed a requirement for CD4+ T cells for induction of anti-A Ab production, consistent with a "T-dependent" Ab response. Unlike in *WT* mice [101, 290, 291, 309], injection of Hu A-BCM in *CD4KO* mice did not further augment anti-A Ab levels beyond those naturally produced (**Fig. 4.1C, table 4.2 and table 4.3**). Altogether, these data indicate that high level of anti-A nAbs occurs in the absence of CD4+ T cells (in *CD4KO* mice), whereas that induction of anti-A Ab production requires CD4+ T cell participation (in *WT* mice).

4.2.2. *MHC-IIKO* and *TCRKO* mice produced anti-A nAbs with a similar striking sex difference.

Studies showed that C*D4KO* mice have CD8+ T cells that can respond to MHC-II restricted epitopes [363]. MHC-II is required for the normal function of CD4+ T cells [364] therefore I investigated whether expression of MHC-II is required for anti-A nAbs development. Male and female *MHC-IIKO* mice (aged

6-8 weeks) were left untreated or injected with Hu A-BCM to investigate the natural and induced anti-A Ab production, respectively. Similar to *CD4KO*, I found that *MHC-IIKO* mice produced significantly higher anti-A nAbs than *WT* mice without exposure to Hu A-BCM. Moreover, I found that *MHC-IIKO* female mice produced significantly higher anti-A nAbs than *MHC-IIKO* male mice, with sex-biased difference similar to that in *CD4KO* (**Fig. 4.2A, table 4.2 and table 4.3**). Unlike *WT* mice, I found that injection of Hu A-BCM in *MHC-IIKO* mice did not induce anti-A Abs beyond the anti-A nAbs (**Fig. 4.2B, table 4.2 and table 4.3**), indicating that CD4+ T cells are required.

Studies in mice showed that γδ-intraepithelial lymphocytes can develop in the absence of thymus and lymph nodes [365] which could complicate the interpretation of T cell requirement. Therefore, I investigated whether anti-A nAbs develop without any T cell participation. I used *αβ/γδ T cell receptor KO* (*TCRKO*) mice to investigate the development of anti-A nAbs in the absence of T cell participation. As I expected, female *TCRKO* mice produced significantly higher anti-A nAbs levels than male *TCRKO* or *WT* mice, similar to sex-biased difference observed in *CD4KO* and *MHC-IIKO* female mice (**Fig. 4.2C, table 4.2 and table 4.3**). Moreover, I found that injection of Hu A-BCM in *TCRKO* mice did not induce more anti-A nAbs beyond that naturally produced, unlike *WT* mice that responded to the blood injection (**Fig. 4.2D, table 4.2 and table 4.3**).

Taken together, my data showed that not only production of anti-A nAbs occur in the absence of T cells, but female *CD4, MHC-II,* and *TCR KO* mice beyond juvenile age produce much higher quantity of anti-A nAbs than males. Furthermore, in contrast to induction of anti-A Ab production which requires CD4+ T cells help for Ab production (Chapter 3), I showed that injection of Hu A-BCM in *CD4, MHC-II,* and *TCR KO* mice did not induce additional anti-A Ab beyond that naturally produced.

4.2.3. CD4+ T cells were involved in regulation of anti-A nAbs production.

Despite the fact that the induction of anti-A [290] and anti-gal Abs [316, 317, 340] production appears to require T cell participation, depletion of T cells was shown actually to enhance the anti-gal nAbs production in *α-galKO* mice (sex was not reported)[310]. Moreover, generation of high anti-A nAbs in *CD4, MHC-II* and *TCR KO* mice described above, especially in females, stimulated me to investigate whether the presence of CD4+ T cells will result in down-regulation of ABO nAbs production. I hypothesized that although T cells are not absolutely required for production of ABO nAbs, they nonetheless may play a "regulatory role" in the setting of nAbs production.

To address this, I used a modified protocol [331] to perform adoptive transfer of sex-matched *WT* CD4+ T cells into 4 week old *CD4KO* mice. Two weeks following the transfer of the *WT* CD4+ T cells, I detected CD4+ T cells in peripheral blood of the *CD4KO* mice (**Fig. 4.3A**). Importantly, the adoptive transfer of the *WT* CD4+ T cells resulted in significantly reduced anti-A nAbs production in treated *CD4KO* mice than in untreated mice (in both sexes, **Fig. 4.3B, table 4.2 and table 4.3**).

My data showed that CD4+ T cells not only required for induction of anti-A Ab production (demonstrated in **Chapter 3**), but they also involved in the down-regulation of anti-A nAbs production.

4.2.4. Mice produced anti-A nAbs despite treatment with broad-spectrum antibiotics.

Although the formation of anti-B Ab in germ-free white leghorn chickens required an exposure to "bacterial B-like" Ag [119] and the chemical structure of blood group B-Ag is similar to that "bacterial Ag" [126, 129], germ-free animals can nevertheless develop ABO nAbs without exposure to bacterial "A/B-like" Ags [119, 132]. Given that the overall repertoire of IgM reacting to bacteria [140] and the abundance of total IgM are generally stable in both germ-free and conventionally-housed mice [131], I became interested in investigating whether gut bacterial flora is absolutely required for ABO nAbs

production. I predicted that bacterial flora in mouse gut could play an important role in ABO nAbs production, but may not be absolutely required.

To study the role of gut bacteria in anti-A nAbs production in mice, I supplemented the drinking water of *CD4KO* and *WT* mice, starting at 7 days of age, with 1g/L each of neomycin, ampicillin, streptomycin, metronidazole and 0.25 g/L of vancomycin. As I predicted, antibiotic treatment significantly reduced anti-A nAbs production in antibiotics-treated *CD4KO* mice than in antibiotics-untreated mice, but did not completely abolish production of the Abs (**Fig. 4.4A, table 4.2 and table 4.3**). The magnitude of the anti-A nAbs reduction after antibiotics treatment was visibly appreciated in females than in males *CD4KO* mice (**Fig. 4.4A, table 4.2 and table 4.3**).

To test whether gut bacterial flora participates in ABO Abs production, I injected *WT* and *CD4KO* mice with Hu A-BCM during the antibiotic treatment. Hu A-BCM injection induced anti-A Ab production in *WT* mice during antibiotic treatment, however, the Ab titers were significantly lower than antibiotics-untreated *WT* mice, suggesting that gut flora contributes in ABO Abs production (**Fig. 4.4B, table 4.2 and table 4.3**). As expected, *CD4KO* mice produced significantly less anti-A nAbs during antibiotics treatment and that injection of Hu A-BCM did not induce anti-A Abs production beyond that naturally produced (**Fig. 4.4C, table 4.2 and table 4.3**), indicating that bacterial flora could play important role for ABO nAbs production but are not absolutely required and that CD4+ T cell is required to induce ABO Abs production.

My data showed that antibiotic treatment reduced but did not prevent either natural or induced anti-A Ab production. This suggests that gut bacterial flora plays an important role in anti-A Abs production (greater impact in nAbs than in induced Abs) and that the impact of bacterial flora is greater for anti-A nAbs

production in females *CD4KO* mice than male mice. However, the exact contribution of bacterial flora in natural and induced ABO Abs production is not completely clear.

4.2.5. Germ-free mice produced ABO nAbs without exposure to A/B-blood product.

Reduction of anti-A nAbs in *CD4KO* mice following antibiotic treatment raised the question whether bacterial flora is absolutely required to produce anti-A nAbs. Since antibiotics use is limited by emergence of drug-resistant bacterial strains, I used germ-free mice to determine the ABO nAbs development without exposure to bacterial flora. With the help of our research group collaborators, I investigated ABO Abs development in serum obtained from *WT* B6 (from Universities of Calgary and Chicago), *WT* BALB (from University of Calgary), and *outbred* Swiss Webster mice (from University of Alberta).

Contrary to general belief, germ-free mice produced both anti-A and B nAbs with age in a similar fashion to conventionally housed mice (**Fig. 4.5A-D**). Interestingly, I also found significant sex difference in germ-free strains, with female mice older than 4 weeks generally producing significantly higher anti-A and anti-B nAb than males (with some exceptions, such as *B6* and *BALB* at 8 weeks old). I also noted that conventional Swiss Webster female mice (25 weeks old) produced comparable anti-A nAbs level to germ-free females (16 weeks old), but whether germ-free *Swiss Webster* females produce higher anti-A nAbs that conventional females at similar age is currently unknown. Interestingly, germ-free mice produced lower anti-B nAbs than anti-A nAbs may be due to expression of α-gal Ag as self-Ag [350, 351] which has a galactose in the terminal sugar similar to B-Ag [312]. Hence, the close related structure of α-gal Ag to B-Ag results in partial tolerance to B-Ag in *WT* mice [366].

My novel data showed that ABO nAbs spontaneously develop in germ-free mice despite absence of bacterial flora. Interestingly, unlike conventionally-housed mice, germ-free mice showed a significant sex

difference in ABO nAbs development (higher Abs in females) that is not limited to *inbred* B6 and BALB, but also occurred in *outbred* Swiss Webster mice.

4.3. Discussion:

In summary, my data showed that *CD4, MHC-II,* and *TCR KO* mice not only produced anti-A nAbs without CD4+ T cell help, but produced significantly more anti-A nAbs than *WT* mice. Thus, CD4+ T cell participation appears to down-regulate the production of the anti-A nAbs. Moreover, sex is an important biological variable in ABO nAbs production in *CD4, MHC-II, TCR KO* mice, and in germ-free mice, with females produced markedly higher levels of ABO nAbs than males. In addition, my experiments showed that bacterial flora was not absolutely required for either natural or for induction of ABO Abs production. Nonetheless, bacterial flora could still play a role in both natural and induced ABO Abs production.

Mice spontaneously produce ABO nAbs over time in similar fashion to humans but whether T cells participate or required for ABO nAbs production has not been clear. While Neron's study [72] concluded that ABO Abs production in mice is *"T-independent",* Christiansen's study [290] showed a requirement for CD4+ T cells participation, consistent with a *T-dependent* process. Additionally, Tazawa's study [291] concluded that NKT cells, but not CD4+ T cells, are required for the ABO Abs production. However, none of these studies considered the development of ABO nAbs over course of their experiments in the absence of immunization to conclude whether ABO Abs production is induced or developed spontaneously.

In my longitudinal studies, I found that *WT B6* mice do produce anti-A nAbs over time, with lower Ab production in early life (7-10 weeks old), with a tendency for a higher anti-A nAbs production in female *WT* mice compared to male mice. Nevertheless, sex seems to not play an important role in anti-A nAbs production in *WT* mice in early life (7-10 weeks old) or in induction of anti-A Ab at that time period. Surprisingly, *CD4, MHC-II,* and *TCR KO* mice not only developed higher anti-A nAbs levels than *WT*

mice, but there was also a striking sex difference, with much higher anti-A nAbs produced in females. Furthermore, I found that this sex difference in anti-A nAbs production was not impacted by co-housing male and female *CD4KO* mice, indicating that the difference is due to sex and not to age or environmental factors, such as food and allergens [143, 182]. The failure to induce anti-A Ab production with injection of Hu A-BCM in *CD4, MHC-II,* and *TCR KO* mice, unlike *WT* mice [101, 290, 291, 309], together with the failure of Hu A-BCM to augment anti-A Ab production beyond the naturally produced anti-A Ab in these mice, indicated that CD4+ T cell participation is required to induce anti-A Ab production. Therefore, these data suggested that anti-A nAbs production occurs without T cell help, in contrast to induction of anti-A Ab which requires CD4+ T cell participation (Chapter 3).

Although the intentional induction of anti-A/B and anti-gal Abs production appears to require T cell participation [290, 315-317], depletion of T cells was shown to enhance anti-gal nAbs production [310], suggesting that absence of T cell participation might also enhance anti-A nAbs development. In my experiments, adoptive transfer of sex-matched *WT* CD4+ T cells indeed resulted in reduced anti-A nAbs production in *CD4KO* mice, indicating that the presence of CD4+ T cells would result in lower ABO nAbs production. In **Chapter 3**, I showed that *CD4KO* mice require CD4+ T cell participation in order to induce anti-A Ab as adoptive transfer of *WT* CD4+ T cells followed with injection of Hu A-BCM induced abundant anti-A Ab in *CD4KO* mice. Therefore, these data in (**Chapter 3** and **Chapter 4**) suggest that CD4+ T cells may play two roles: 1) regulation of the ABO nAbs production (unknown which CD4+ T cell subset is involved in this regulation), and 2) providing help for B cells of *WT* mice to produce ABO Abs. It will be interesting to examine whether CD4+CD25+FoxP3+ regulatory T cells are involved in ABO nAbs regulation.

Springer's studies showed that formation of anti-B Ab in germ-free chicks was induced by blood group B +ve (BGB^+) *E. coli* O_{86} [119]. Additional data in 1980s showed that ABO Abs were greatly reduced in

germ-free mice [130] and that exposure of germ-free mice to bacterial contamination would lead to ABO Abs development [132]. In agreement with the bacterial stimulus hypothesis, ingestion of probiotic bacteria was associated with extremely high ABO Abs titers [135] and that feeding of humans on *E. coli* O_{86} resulted in stimulation of anti-B Ab production [120]. In agreement with Springer's hypothesis that ABO Abs are induced by exposure to "A/B-like" Ags on bacteria [119, 120, 130-132], antibiotic treatment in my experiments reduced both the induced and anti-A nAbs production in *WT* and *CD4KO* female mice, respectively, suggesting that bacterial flora plays an important role in ABO Abs production. However, I cannot conclude from these experiments that bacterial flora is absolutely required during stimulation of ABO Abs production, because antibiotic treatment cannot eliminate all bacterial floras. In addition, the difference in bacterial flora in the vagina or due to difference in bacterial composition in females [367, 368] would necessitate the use of germ-free mice in lieu of antibiotics [369].

Detection of ABO nAbs in three germ-free mice strains (*WT* B6, BALB, and *outbred* Swiss Webster) and from three different facilities (Edmonton, Calgary, and Chicago) indicated that a gut bacterial stimulus in mice is not absolutely required for ABO nAbs production. Detection of ABO Abs in germ-free mice brings the question whether B cells spontaneously produce nAbs [140, 172, 208], or require an endogenous stimulus [183-185], such as cellular by-products [186-189] of normal tissues [178, 179, 191, 192] and tumors [144, 190]. One possible explanation for the fact that different strains of germ-free mice from different locations produced ABO nAbs is that the Abs might be induced by a "non-bacterial stimulus", possibly food and allergens [143, 182], or other self-antigens [183-185], including ovarian glycolipids [205, 206]. Therefore, this possibility may explain why specificity of serum ABO Abs produced, after feeding on bacterial flora, does not correspond with the Ags expressed on that bacterial flora [119, 132] or why total IgM in both germ-free and conventionally housed mice are stable [131, 140]. Interestingly, mice

[174] and rabbits [175] showed an elevation of nAbs during puberty, where estrogen was reported to be important for nAbs production [174].

Although *WT* mice older than 3 months develop high level of ABO nAbs [72, 101, 289] in a similar fashion to humans [133, 134], sex does not seem to play a significant role in ABO nAbs in *WT* mice. The striking sex difference in ABO nAbs production in *CD4, MHC-II,* and *TCR KO* mice, but not in *WT* mice, showed that the absence of a regulatory role of CD4+ T cell could result in higher ABO nAbs in *CD4KO* mice than in *WT* mice. I speculated that a lack of suppressive influence, such as biased CD4+CD25+FoxP3+ regulatory T cell function [370], or a sex-related internal stimulus, such as estrogen [174] or glycolipids [205], may participate in the production of ABO nAbs in *CD4, MHC-II,* and *TCR KO* female mice. Exclusion of environmental variance, by co-housing the *CD4KO* male and female mice, did not affect this sex difference, suggesting that the stimulus is endogenous.

In summary, in contrast to the requirement for CD4+ T cell help for induction of ABO Abs production (Chapter 3), CD4+ T cell help is not required for ABO nAbs production; CD4+ T cells (possibly subsets) play a different role in this process. Therefore, future studies should consider the possibly that CD4+ T cell subset, such as CD25+FoxP3+ regulatory T cell, could down-regulate ABO nAbs.

Female *CD4, MHC-II,* and *TCR KO* mice develop significantly higher ABO nAbs than male mice. This could be due to differences in sex hormones and/or hormonal receptors, similar to stimulation of anti-*E.coli* nAbs production in female mice by estrogen regardless of colonization by bacterial flora [174]. Future studies could study the contribution of sex hormone, such as estrogen, in induction of ABO nAbs. Other future studies should distinguish between natural and induced ABO Abs produced. In this regard, ABH glycan microarray could possibly be very useful for the distinction of these antibodies.

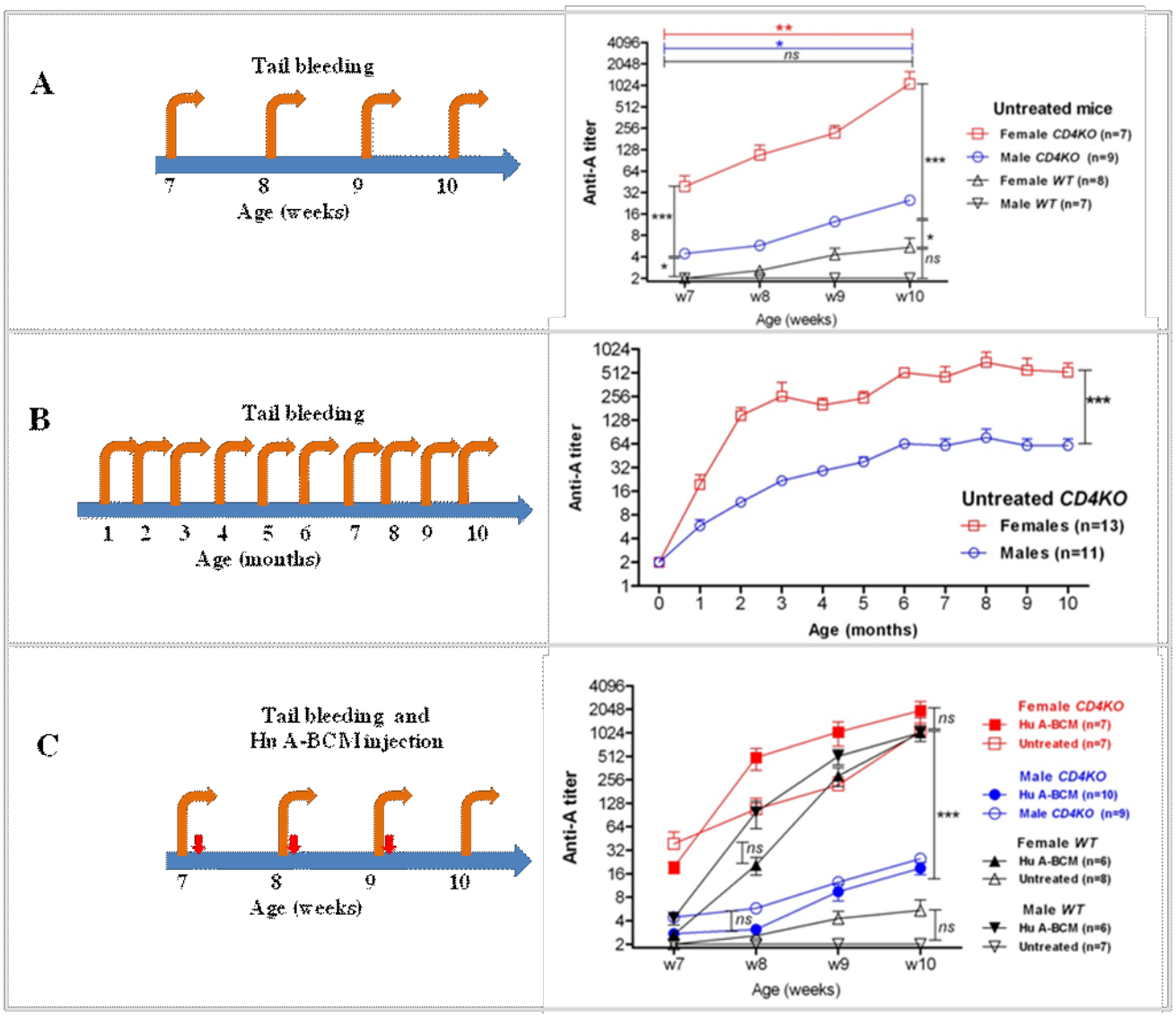

Fig. 4.1: Female *CD4KO* mice developed significantly higher anti-A nAbs than male *CD4KO* mice and female *WT* mice. (**A**) Serial sera collected from untreated mice (*orange long arrows*) showed that female and male *WT* B6 mice produce similar anti-A nAbs titer in early weeks but female mice produce slightly higher Ab titer in later weeks (non-significant sex difference). Female *CD4KO* mice produced significantly higher anti-A nAbs than male *CD4KO* (ie, without CD4+ T cell help, see table 4.2 for comparison between groups). (**B**) Anti-A nAbs production remains lower in older *CD4KO* male mice compared to females over time. (**C**) Hu A-BCM injection (*red short arrows*) does not augment anti-A Ab production in *CD4KO* mice. Hemagglutination was used to measure anti-A Ab titer, using A-Tg reagent RBC. Mean of anti-A Ab was used in paired T-Test (ns=non significant, * = $P \leq 0.05$, *** = $P \leq 0.001$, see table 4.2 and 4.3 for comparison between groups).

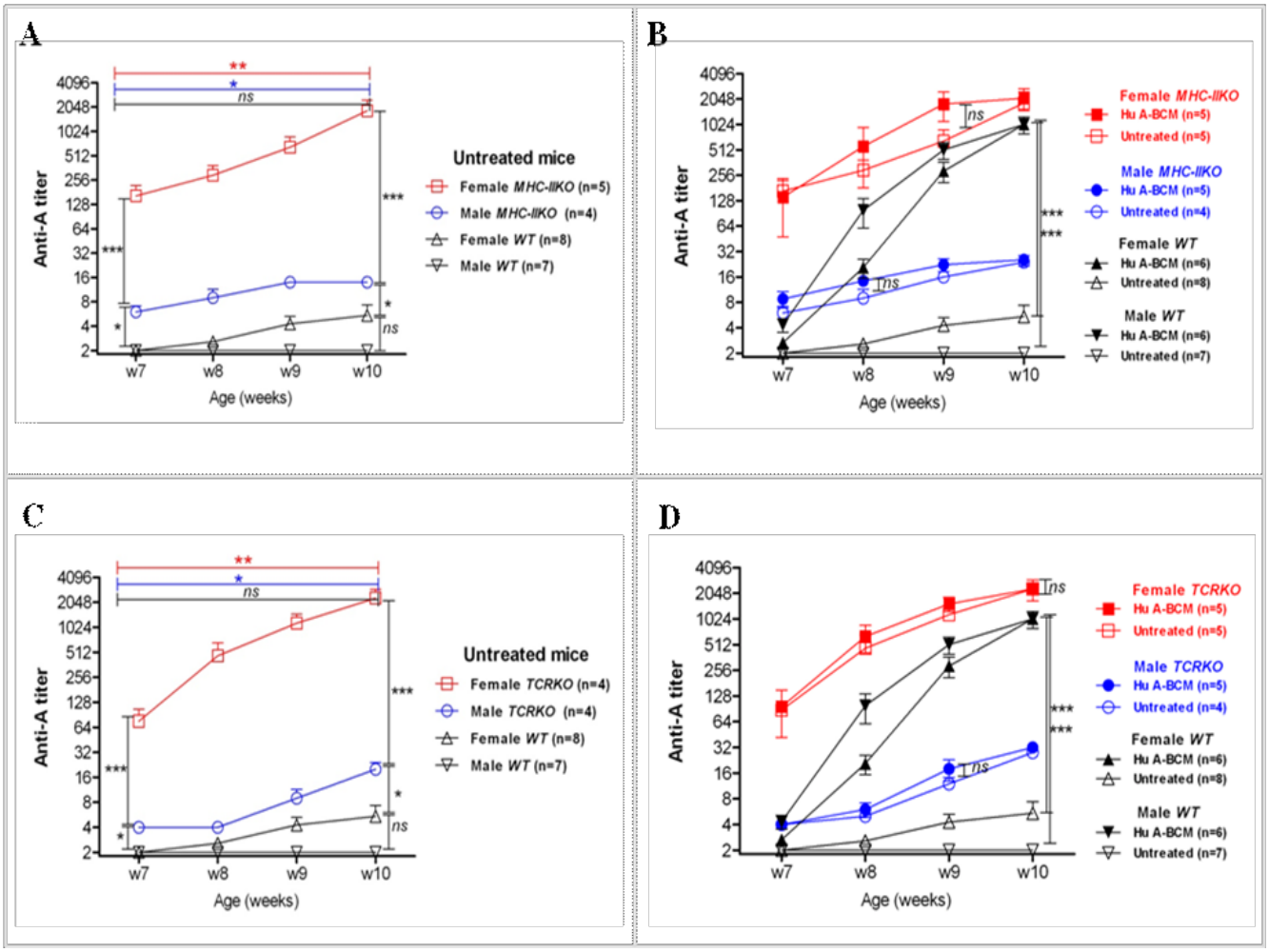

Fig. 4.2: *T cell-deficient* mice produced anti-A nAbs with a similar striking sex difference. **(A)** Untreated *MHC-IIKO* mice produce anti-A nAbs, with a similar sex difference to *CD4KO* mice. Anti-A nAbs remained low in *WT* male and female mice **(B)** The anti-A nAbs developed in *MHC-IIKO* mice are not further augmented with injection of Hu A-BCM. **(C)** Untreated *TCRKO* mice produce anti-A nAbs with similar sex difference to *CD4KO* and *MHC-IIKO* mice. **(D)** *TCRKO* mice produce anti-A nAbs that are not further augmented with injection of Hu A-BCM. Hemagglutination was used to measure anti-A Ab titer, using A-Tg reagent RBC. Mean of anti-A Ab was used in paired T-Test (ns=non significant, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, see table 4.2 and 4.3 for comparison between groups).

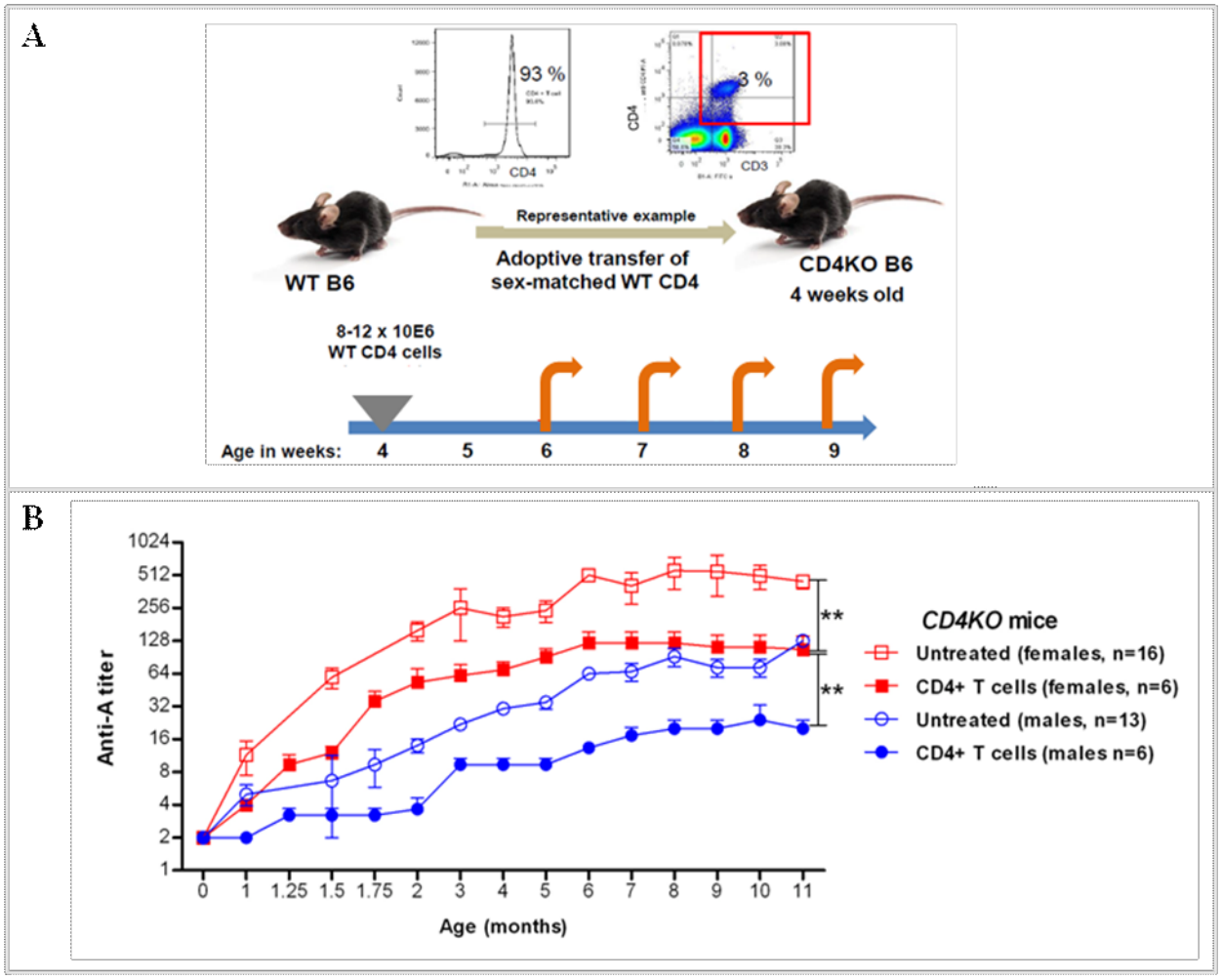

Fig. 4.3: CD4+ T cells were involved in down-regulation of the anti-A nAbs production. (A CD4+ T cells were isolated (left histogram) from *WT* mice and adoptively transferred [331] into sex-matched *CD4KO* mice at 4 weeks of age (*gray triangle*). Two weeks after the adoptive transfer, CD4+ T cells were detected in peripheral blood of *CD4KO* mice, a representative example of CD4+ T cell reconstitution is shown (right dot plot). Starting from 1 month old, blood was collected for anti-A Ab detection (*orange long arrows*). **(B)** Adoptive transfer of CD4+ T cells into *CD4KO* mice at age 1 month resulted in significantly reduced anti-A nAbs production than untreated mice. Hemagglutination was used to measure anti-A Ab titer, using A-Tg reagent RBC. Standard error of mean (Mean + SEM) of anti-A Ab was used in paired T-Test (** = $P \leq 0.01$).

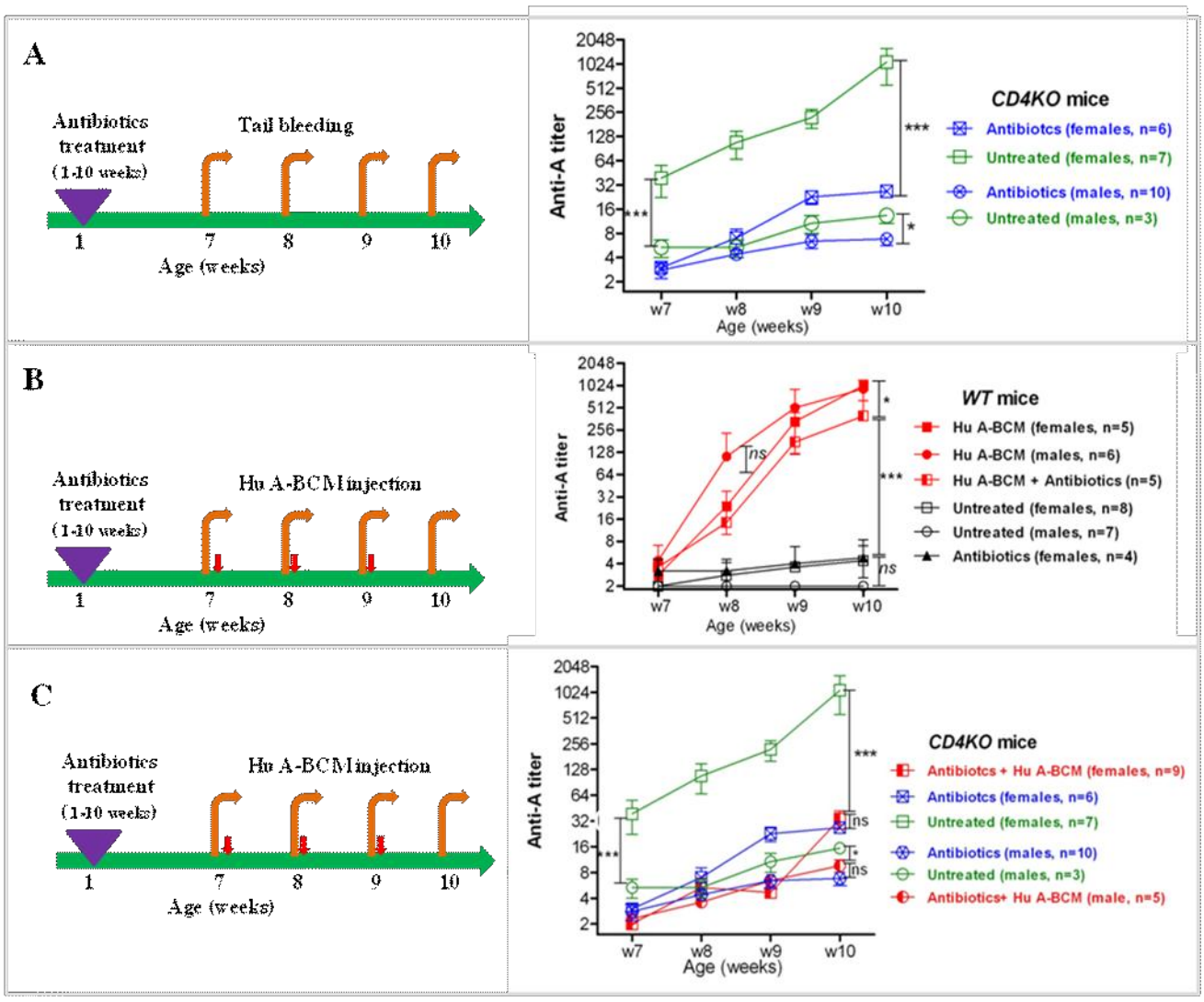

Fig. 4.4: Despite treatment with broad-spectrum antibiotics, anti-A Ab was produced in *CD4KO* mice. (A) After broad-spectrum antibiotic treatment, started at week one (*pink triangle*) and ended at week 10, anti-A nAbs production in *CD4KO* mice was significantly reduced (dramatic reduction was in females). **(B)** Hu A-BCM injection (*red short arrows*) induced anti-A Ab in *WT* mice. However, antibiotics treatment resulted in significantly lower induced anti-A Ab than in antibiotics-untreated *WT* mice. **(C)** *CD4KO* mice treated with antibiotics (*pink triangle*) and injected with Hu A-BCM (*red short arrows*) did not induce anti-A Ab beyond that naturally produced. Hemagglutination was used to measure anti-A Ab titer using A-Tg reagent RBC. Standard error of mean (Mean + SEM) of anti-A Ab was used in paired T-Test (ns=non significant, * = $P \leq 0.05$, *** = $P \leq 0.001$, see table 4.2 and 4.3 for comparison between groups).

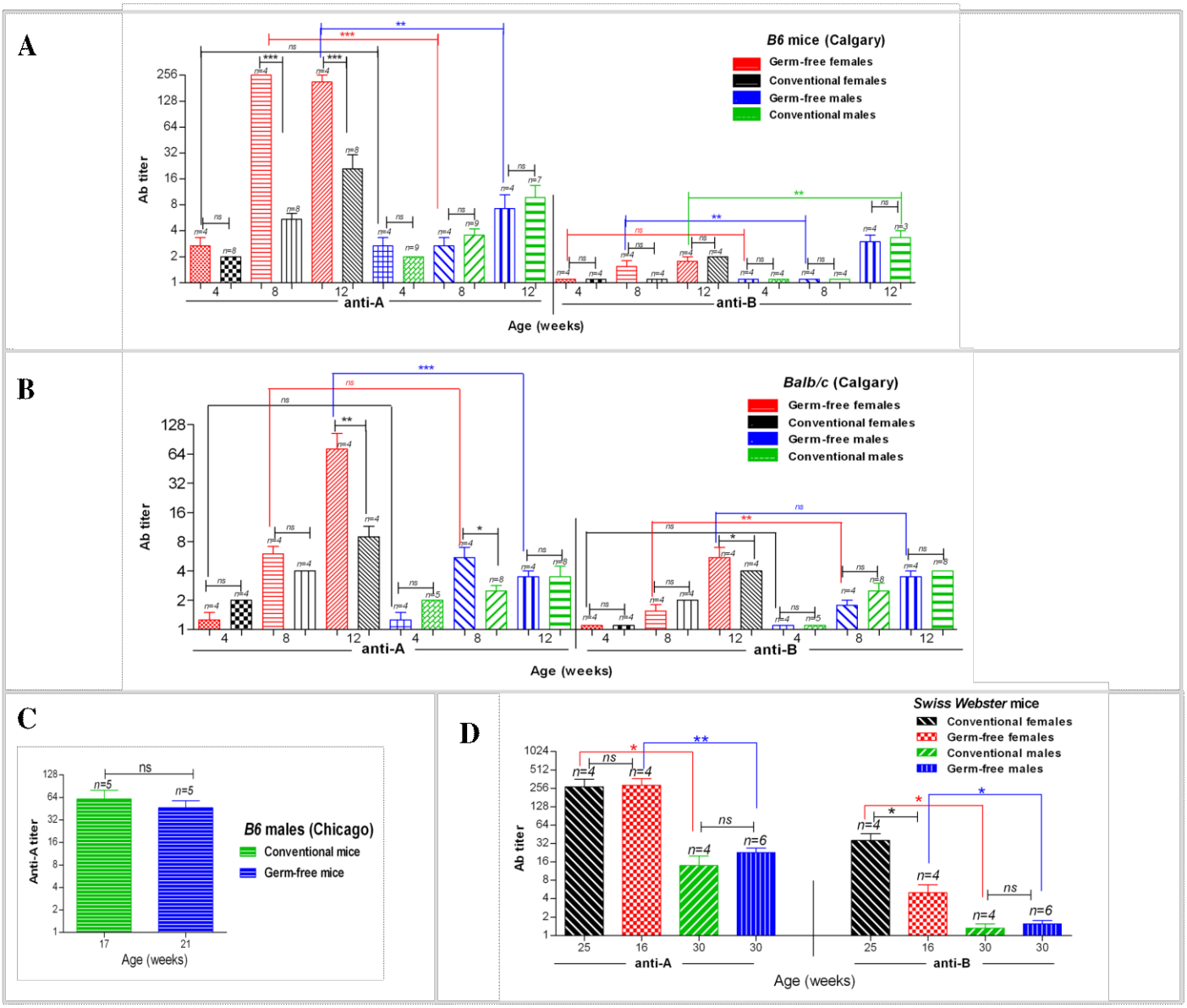

Fig. 4.5: Germ-free mice produced ABO nAbs without exposure to A/B-Ags or bacterial flora. Serum collected from *B6* **(A)** and BALB **(B)** mice housed in germ-free condition at University of Calgary developed both anti-A (left) and anti-B (right) nAbs without exposure to bacterial flora or A/B-Ags. Moreover, serum obtained from germ-free B6 male mice housed at University of Chicago (**C**) showed germ-free male mice produced comparable anti-A Ab level to conventional housed male mice. Similarly, serum obtained from *outbreed* Swiss Webster mice housed in germ-free condition at University of Alberta (**D**) showed that both the mice produce anti-A (left) and anti-B Abs (right) without exposure to bacterial flora or A/B-Ags. Hemagglutination was used to measure anti-A and B Ab titer using A-Tg or human type A reagent RBC, respectively. Standard error of mean (Mean + SEM) of anti-A/B Ab was used in paired T-Test (ns=non significant, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$).

Table 4.1: Summarized data of Chapter 4

Treatment	Recipients	No	Age in weeks	anti-A	anti-B
Untreated	*WT* B6 females	8	6 – 10	4	ND
Untreated	*WT* B6 males	7	6 – 10	2	ND
Hu A-RBC injection	*WT* B6 females	3	6 – 10	1024	ND
Hu A-RBC injection	*WT* B6 males	6	6 – 10	1024	ND
Untreated	*CD4KO* females	7	6 – 10	1024 - 4096	ND
Untreated	*CD4KO* males	3	6 – 10	16	ND
Hu A-RBC injection	*CD4KO* females	7	6 – 10	1024 - 4096	ND
Hu A-RBC injection	*CD4KO* males	5	6 – 10	16	ND
Untreated	*MHC-iiKO* females	5	6 – 10	1024 - 4096	ND
Untreated	*MHC-iiKO* males	3	6 – 10	16	ND
Hu A-RBC injection	*MHC-iiKO* females	3	6 – 10	1024 - 4096	ND
Hu A-RBC injection	*MHC-iiKO* males	4	6 – 10	16- 32	ND
Untreated	*T cells KO* females	3	6 – 10	1024 - 4096	ND
Untreated	*T cells KO* males	3	6 – 10	16- 32	ND
Hu A-RBC injection	*T cells KO* females	5	6 – 10	1024 - 4096	ND
Hu A-RBC injection	*T cells KO* males	5	6 – 10	16- 32	ND
Antibiotics	*WT* B6	5	6 – 10	4	ND
Antibiotics + Hu A-RBC injection	*WT* B6	5	6 – 10	256 - 512	ND
Antibiotics	*CD4KO* females	6	6 – 10	32	ND
Antibiotics	*CD4KO* males	10	6 – 10	4 - 8	ND
Antibiotics + Hu A-RBC injection	*CD4KO* females	9	6 – 10	32	ND
Antibiotics + Hu A-RBC injection	*CD4KO* males	5	6 – 10	8 – 16	ND
Germ-free condition (Calgary)	*WT* B6 females	4	4	2 - 4	1
Germ-free condition (Calgary)	*WT* B6 males	4	4	2 - 4	1
Germ-free condition (Calgary)	*WT* B6 females	4	8	256	2
Germ-free condition (Calgary)	*WT* B6 males	4	8	2 - 4	1
Germ-free condition (Calgary)	*WT* B6 females	4	12	256	2
Germ-free condition (Calgary)	*WT* B6 males	4	12	8	4
Germ-free condition (Calgary)	*WT* BALB females	4	4	1	1
Germ-free condition (Calgary)	*WT* BALB males	4	4	1	1
Germ-free condition (Calgary)	*WT* BALB females	4	8	8	2
Germ-free condition (Calgary)	*WT* BALB males	4	8	4 - 8	2
Germ-free condition (Calgary)	*WT* B6 females	4	12	256	4-8
Germ-free condition (Calgary)	*WT* B6 males	4	12	8	4
Conventional condition (Chicago)	*WT* B6 males	5	17	64	ND
Germ-free condition (Chicago)	*WT* B6 males	5	21	32 – 64	ND
Germ-free condition (Edmonton)	*Swiss Webster* females	4	16	256	4-8
Germ-free condition (Edmonton)	*Swiss Webster* males	4	30	16 – 32	1-2

Table 4.2: Comparison tables of groups (between 6 - 10 weeks old)

Groups	Treatment	Recipients	No	Anti-A	6 vs. 10 weeks titer
#1	Untreated	*WT* B6 females	8	4	ns
#2	Untreated	*WT* B6 males	7	2	ns
#3	Untreated	*CD4KO* females	7	1024-4096	**
#4	Untreated	*CD4KO* males	3	16	*
#5	Untreated	*MHC-iiKO* females	5	1024-4096	**
#6	Untreated	*MHC-iiKO* males	3	16	*
#7	Untreated	*αβ/γδ T cells KO* females	3	1024-4096	**
#8	Untreated	*αβ/γδ T cells KO* males	3	16-32	*
#9	Hu A-RBC	*WT* B6 females	3	1024	***
#10	Hu A-RBC	*WT* B6 males	6	1024	***
#11	Hu A-RBC	*CD4KO* females	7	1024-4096	**
#12	Hu A-RBC	*CD4KO* males	5	16	*
#13	Hu A-RBC	*MHC-iiKO* females	3	1024-4096	**
#14	Hu A-RBC	*MHC-iiKO* males	4	16-32	*
#15	Hu A-RBC	*αβ/γδ T cells KO* females	5	1024-4096	**
#16	Hu A-RBC	*αβ/γδ T cells KO* males	5	16-32	*
#17	Antibiotics	*WT* B6	5	4	ns
#18	Antibiotics	*CD4KO* females	6	32	**
#19	Antibiotics	*CD4KO* males	10	4-8	*
#20	Antibiotics + Hu A-RBC	*WT* B6	5	256-512	***
#21	Antibiotics + Hu A-RBC	*CD4KO* females	9	32	*
#22	Antibiotics + Hu A-RBC	*CD4KO* males	5	8-16	ns

- Standard error of mean (Mean + SEM) of anti-A Ab was used in T-Test.
- ns = ≥ 0.5, * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001

Table 4.3: Comparisons of group's #

Groups	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16	#17	#18	#19	#20	#21	#22
#1		ns	*		***		***		**								ns			**		
#2	ns			*		ns		***		***							ns			**		
#3	*			*	ns		ns				ns							ns			**	
#4		***	*			ns		ns				ns							**			**
#5	ns		ns			ns	ns						ns									
#6		***		ns	***			*						ns								
#7	*		ns		ns			ns							ns							
#8		***		ns		ns	**									ns						
#9	**									ns	ns		ns		ns			**			***	
#10		***							ns			***		***		***	***		***	***		
#11			ns									***		*		ns						
#12				ns						***	***			ns		ns			***			ns
#13					ns				ns		ns			*	ns							
#14						ns				***		ns	*			ns						
#15							ns		ns		ns		ns			**						
#16								ns		***		ns		ns	**							
#17	ns	*							*	***								***	ns	*		
#18			ns								**								***		*	
#19				**								***					ns	***				ns
#20	**	**							ns	***							*				**	*
#21			**								***							*		**		**
#22				**								ns							***	ns	**	

- Standard error of mean (Mean + SEM) of anti-A Ab was used in Two-way ANOVA
- ns = ≥ 0.5, * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001

Chapter 5:

Siglecs play important role in modulation of both natural and induced anti-A Ab production

5.1. Introduction:

Heart transplantation (HTx) during infancy is successful long-term therapy for congenital heart disease; however there remains a lack of sufficient donors. Use of ABO-incompatible (ABOi) donors [87] expands the donor pool and results in immunologic tolerance to donor A/B-antigens (Ags) by mechanisms that remain not well-defined [88]. West's research group previously found that ABO Abs are mainly produced by $CD27^+$ IgM^+ B cells and that this B cell subset in spleen expressed significantly higher levels of CD22 in infants than adults [282, 283]. High level expression of CD22 on these B cells corresponds to the time of ABO tolerance development following ABOi-HTx during infancy.

CD22 and Siglec-g (Siglec 10 in humans) are members of a family of cell surface proteins that bind sialic acid, sialic acid-binding immunoglobulin-like lectins (Siglecs). CD22 and Siglec-g are involved in regulation of B cell receptor (BCR) signaling [231-233], autoimmunity, and immunity and infection (reviewed in [234-237]). In mice, CD22 and Siglec-g are expressed at high levels on B1 and marginal zone B cells [239, 278, 320] and these B cell subsets are thought to produce Abs against T-independent (TI) Ags [154, 196, 321, 322]. CD22 and Siglec-g expressed on B cells can inhibit the immune response to TI type 2 (TI-2) sialylated glycan Ags in the setting of B cell self/non-self-discrimination [225-229] where the *trans* interaction of CD22 and Siglec-g with their ligands (i.e. sialylated multivalent chemical structures) results in B cell inhibition [226, 227, 229] or tolerance [225, 228].

Despite discovery of CD22 molecule in 1980s [371, 372], the impact of CD22 molecule on Ab production to carbohydrates (CHO) remained controversial [224, 252-259](**Table 1.2**). Some of these studies reported that in *CD22 knock-out (KO)* mice the lack of CD22 expression on B cells resulted in B cell hyper-responsiveness to CHO Ags [252-254]. In contrast, other studies reported that Ab production to CHO-Ags is impaired in *CD22KO* mice [224, 255, 256]. Moreover, others reported that CD22-deficiency did not impact Ab production to CHO-Ags [257-259]. Similar controversies were also reported with *Siglec-gKO* mice [224, 249, 250](**Table 1.2**). However, these studies investigated the role of Siglecs in Ab production stimulated by limited set of artificial CHO structures rather than naturally-occurring CHO-Ags (such as ABO or α-gal Ags). In addition, these studies did not determine the occurrence of natural Abs (nAbs) produced against these CHO structures used to immunize the mice **(Table 1.3)**.

Despite that Siglecs have been identified as important inhibitory molecule for B cells, the potential role of Siglecs in modulating the ABO Abs response has not been studied. In addition, CD22 could play important role in ABO tolerance following ABOi-HTx in infants [282, 283]. Herein, I used *CD22KO*, *Siglec-gKO*, and *CD22xSiglecgKO* mice as tools to explore the role of Siglecs in natural (without exposure to A/B-Ags) and induced ABO Abs production. I speculated that in the absence of inhibitory CD22/Siglecg inhibitory molecule, *CD22/Siglecg-deficient* mice would produce higher levels of ABO Abs than in *WT* mice. Based on expansion of B1 cells (**Table 1.1**) and hyper-responsiveness of B cells observed in *CD22/Siglecg-deficient* mice [249, 252-254], I hypothesized that Siglecs are involved in the regulation of both natural and induced ABO Abs production.

5.2. Results:

5.2.1. Siglecs played important role in production of ABO nAbs.

As I discussed in Chapter 4, nAbs, mostly of the IgM isotype, are thought to be produced spontaneously by B1 cells [103, 323] and have specificity for epitopes expressed on microorganisms [105, 169, 176, 177] or host cells [178, 179]. I demonstrated that *CD4, MHC-II,* and *TCR KO* B6 mice spontaneously produce higher anti-A nAbs than *WT* B6 mice (both sexes, but especially females; Chapter 4), suggesting that CD4+ T cells may be involved in regulation of ABO nAbs production. I also showed that beyond the anti-A nAbs, *CD4, MHC-II,* and *TCR KO* mice did not produce anti-A Ab following stimulation by A-Ag in the form of Hu A-RBC, suggesting that CD4+ T cell participation is required for *induced* anti-A Ab production (**Chapter 3**).

It was shown that compared with *WT* mice, there were higher numbers of B1 cells in peritoneal cavity and increased production of nAbs IgM in serum of *CD22KO* [252, 258], *Siglec-gKO* [226, 249, 250, 262] and *CD22*x*SiglecgKO* mice [224](**Table 1.1**). However, it is unknown whether *CD22KO* mice produce higher ABO nAbs than *WT* mice. Furthermore, whether sex impacts anti-A nAbs production in *CD22KO* mice, similar to *CD4, MHC-II,* and *TCR KO* mice, is unknown.

Using age-matched historical sera collected from *CD22KO* and *WT* B6 mice, I measured anti-A nAbs and I found that *CD22KO* mice develop significantly higher anti-A nAbs than *WT* mice at different age groups (**Fig. 5.1A, table 5.1 and table 5.2**).

I also studied anti-A nAb development longitudinally in untreated *WT* and *CD22KO* males and in females (*CD22KO* mice used in Chapter 5 were housed in HSLAS facility and therefore might be exposed to different bacterial flora). I found that at early age (6-10 weeks old) female *CD22KO* mice produced significantly higher anti-A nAbs than males. Similarly, female *WT* mice during

adulthood (8-12 weeks old) produced non-significantly higher anti-A nAbs than male mice (**Fig. 5.1B, table 5.1 and table 5.2)**. In comparison of *CD22KO* with *WT* mice data at early age, I found that not only female *CD22KO* mice developed significantly higher anti-A nAbs than male *CD22O* and female *WT* mice, but also that male *CD22KO* mice developed significantly higher anti-A nAbs than female and male *WT* mice (see the **table 5.1** and **table 5.2** for group comparisons).

I longitudinally followed-up anti-A nAbs production in some of the mice previously presented in (**Fig. 5.1B**). I found that in later life, females *WT* mice continued to produce significantly higher anti-A nAbs than male *WT* mice, suggesting that sex plays important role in ABO nAbs in adult and old *WT* mice (**Fig. 5.1C**). In contrast, sex does not play important role in anti-A nAbs production in *CD22KO* mice at later age (>3 months), because both male and female *CD22KO* mice produced similar level of anti-A nAbs in longitudinal follow-up (**Fig. 5.1C**). Starting at 3 months old, I observed a wide variation in anti-A nAbs titer, with some male *CD22KO* mice producing higher titer than female *CD22* mice that could be attributed to smaller number of *CD22KO* mice that I followed-up their anti-A nAbs titer over time (4 females and 5 males).

Taken together, my results showed that in early months, *CD22KO* mice produce higher anti-A nAbs than in *WT* mice and that *CD22KO* female mice produced higher titer than males, suggesting that lack of CD22-mediated inhibition results in high ABO nAbs development and that sex plays an important role in production of ABO nAbs.

5.2.2. B cell Siglecs were important for induction of ABO Abs production.

There is a discrepancy among research groups regarding the role of Siglecs (CD22 and Siglec-g) in Ab production following stimulation of *Siglecs-deficient* mice with chemically-conjugated Ags, such as 2,4-dinitrophenyl (DNP) or 2,4,6-trinitrophenyl (TNP) (**Table 1.2**). For instance, as noted

above, some studies reported that *CD22/Siglecg*-deficiency promoted B cell hyper-responsiveness to stimulation and augmented anti-DNP/TNP Ab production [249, 252-254]. In contrast, other studies reported that *CD22/SiglecgKO* mice developed impaired Ab production following stimulation by DNP/TNP Ags [224, 254-256, 263]. Interestingly, other groups reported that CD22/Siglecg-deficiency had no significant impact on anti-DNP/TNP Ab production [249, 250, 257-260, 263].

A confounding factor in these studies in regard to the impact of CD22/Siglecg-deficiency on anti-DNP/TNP Abs production may have been lack of differentiation between natural vs. induced anti-DNP/TNP Abs. For instance, DNP/TNP Abs have been shown to be produced spontaneously in mice [264, 269, 270, 272, 273], rats [271], humans [267, 268], pigs [266], monkeys [265], and fish [154, 231, 324](**Table 1.3**). The requirements for spontaneous/natural DNP/TNP Abs development may differ from Ab development induced by exposure to DNP/TNP Ags. In addition, these studies all relied on chemically-synthesized structures that may not represent the *in vivo* production of Ab against naturally-occurring CHO-Ags.

First, I examined anti-A Ab production following injecting human types A blood cell membranes (Hu A-BCM) in age and sex-matched *WT* and *CD22KO* B6 mice (**Fig. 5.2A**). I found that male and female responded equally to the Hu A-BCM injection (**Fig. 5.2B,C**), suggesting that sex does not play important role in induced anti-A Ab production in *WT* mice. Here, in *CD22KO* mice, injection of Hu A-BCM induced massive anti-A Ab production (1/4096 - 1/16,384), in both females (**Fig. 5.2B, table 5.1 and 5.2**) and males (**Fig. 5.2C, table 5.1 and 5.2**) mice, showing that induced anti-A Ab production is higher in *CD22KO* than in *WT* mice and in female *CD22KO* mice than in *CD22KO* males. Altogether, these data indicated that in the absence of inhibitory CD22-signaling, higher levels of anti-A Ab is produced following stimulation by *xenogeneic* Hu A-BCM.

5.2.3. Without B cell Siglecs, induction of ABO Abs production did not require CD4+ T cells

Previous studies concluded that induced ABO Abs production in mice did not require CD4+ T cell participation [72, 291], whereas other studies showed that CD4+ T cells were necessary for induced anti-B Ab in *WT* mice [290]. My experiments demonstrated that induction of ABO Abs required CD4+ T cell involvement (Chapter 3). The role of Siglecs signaling in the context of CD4+ T cell help in induction of ABO Abs production is not clear, but it is generally accepted that B cells from *CD22KO* mice require less stimulation than *WT* mice to induce B cell activation [260, 325]. Therefore, I examined induction of ABO Abs production in the context of *syngeneic* or *xenogeneic* stimulation in *CD22KO* mice compared with *WT* mice. I predicted that in the absence of CD22 co-inhibitory signaling, CD4+ T cell help might not be required for induction of ABO Abs production in *CD22KO* mice, similar to previous study showed that a reduced need for T cell help when a B cell FcγR co-inhibitory signaling was blocked [373],

In order to address this prediction, I examined induction of anti-A Ab production in *CD22KO* mice without *allogeneic* or *xenogeneic* stimulation (without CD4+ T cell participation that usually recruited by foreign protein). Unlike my results demonstrating that induction of anti-A Ab production in *WT* mice required CD4+ T cell participation (Chapter 3), I found that injection of *syngeneic* A-Tg BCM (**Fig. 5.3A**) induced significantly higher level of anti-A Ab production in *CD22KO* mice, but not in untreated mice (**Fig. 5.3B, C, table 5.1 and table 5.2**). This result demonstrated that stimulation by A-Ag alone without foreign protein known to engage CD4+ T cell help would induce Ab production in the absence of CD22 inhibitory signaling. Therefore, I specifically examined whether CD4+ T cell participation is required for induction of anti-A Ab production in *CD22KO* mice. First, I induced CD4+ T cell depletion with injection of GK1.5 anti-CD4 Ab in *CD22KO* mice and assessed the depletion efficacy by flow cytometry (**Fig. 5.3.D**). In

contrast to *WT* mice following CD4+ T cell depletion (Chapter 3), Hu A-BCM injection induced significant amount of anti-A Ab production independent of CD4+ T cell participation (**Fig. 5.3E, F, table 5.1 and table 5.2**), indicating that CD4+ T cells may not be required for induction of anti-A Ab production when CD22 inhibition is absent. I observed that *syngeneic* A-Tg BCM injection induced comparable anti-A Ab to Hu A-BCM injection.

In summary, these data indicated that, in the absence of CD22 inhibitory signaling, A-antigen alone on A-Tg BCM is sufficient to induce anti-A Ab production in *syngeneic* mice and that induced anti-A Ab production following Hu A-BCM injection in mice without CD22-mediated B cell inhibition is CD4+ T cell-independent.

5.3. Discussion:

Compared to *WT* mice, *CD22KO* mice spontaneously produced higher anti-A nAbs without exposure to known source of A/B-Ags. The exact stimulus for production of anti-A Ab remains unknown, but may be "A/B-like" Ags present in environment, such as food or bacterial flora [360, 374]. The reason for increased anti-A Ab production in *CD22KO* mice vs. *WT* is unknown, but may be due to B cell hyper-responsiveness without CD22-mediated inhibition, or due to B1a cell expansion in *CD22/Siglecg-deficient* mice (**Table 1.1**).

Sex is an important biological factor in immune responses in mice and humans (reviewed in [284, 285]). Recently, Kubes group showed that nAbs produced in female mice provided an innate immunity against bacterial infection [174] and these nAbs were produced during puberty in response to estrogen, independent of bacterial colonization [174]. Similar to *CD4, MHC-II,* and *TCR KO* (Chapter 4), sex may also play an important role in ABO nAbs production in *CD22KO* mice. In addition, it was shown that *Lactobacillus* can disrupt normal bacterial flora colonization in

vagina of female mice [375] and therefore female mice might have exposed to different "A/B-like" bacterial Ags. Although my data demonstrated that female mice in general produce higher ABO nAbs than males, the interaction between sex hormones, CD22, and bacterial flora is important as-yet-unknown and requires further investigation.

I showed that stimulation of *CD22KO* mice with injection of Hu A-BCM induced massive anti-A Ab production. This "hyper-response" to stimulation by *xenogeneic* Hu A-BCM is consistent with the requirement of CD4+ T cell participation in induced ABO Abs production [290]. As I showed in Chapter 3 that induction of anti-A Ab in *WT* mice required the presence of CD4+ T cells (presumably recruited by foreign proteins expressed in *xenogeneic* Hu A-BCM), therefore, it might be that massive anti-A Ab production following *xenogeneic* Hu A-BCM injection in *CD22KO* mice was a result of combination of CD4+ T cell help and B cell "hyper-responsiveness".

In addition to the massive level anti-A Ab induced with injection of *xenogeneic* Hu A-BCM, injection of *CD22KO* mice with *syngeneic* A-Tg BCM (with no foreign protein that could engage CD4+ T cells help) induced anti-A Ab production, suggesting that "hyper-responsive" B cells in the *CD22KO* mice could respond to A-Ag without CD4+ T cell participation. In this regard it is noteworthy that CD4+ T cell depletion in *CD22KO* mice followed with injection of Hu A-BCM induced comparable anti-A Ab production to that of *syngeneic* A-Tg BCM. These results are consistent with the CD22 providing an inhibitory signal for induction of ABO Abs production that could be overcome with CD4+ T cell help, similar to previous study showed that blocking of B cell FcγR co-inhibitory receptor would reduce the need for T cell help [373]. Therefore, my speculation is that in the absence of CD22 inhibition, CD4+ T cell help is not required for induced ABO Abs production.

My results demonstrated that unlike *WT* mice, in the absence of Siglecs (*CD22KO* mice) there was no requirement for foreign protein or CD4+ T cells for anti-A Ab induced by stimulation with A-Ag. Thus in the absence of Siglecs, induction of anti-A Ab production is "T-independent". These results suggest that Siglecs play an important role in both natural and induced ABO Abs production. Future experiments should study whether B cell "hyper-responsiveness" and/or expansion of B1 cell population resulted in higher production of ABO Abs in *CD22KO* mice than in *WT* mice. Future studies should also investigate whether X-linked specific genes, hormones, and/or hormonal receptors would impact both natural and induced ABO Abs production in female mice.

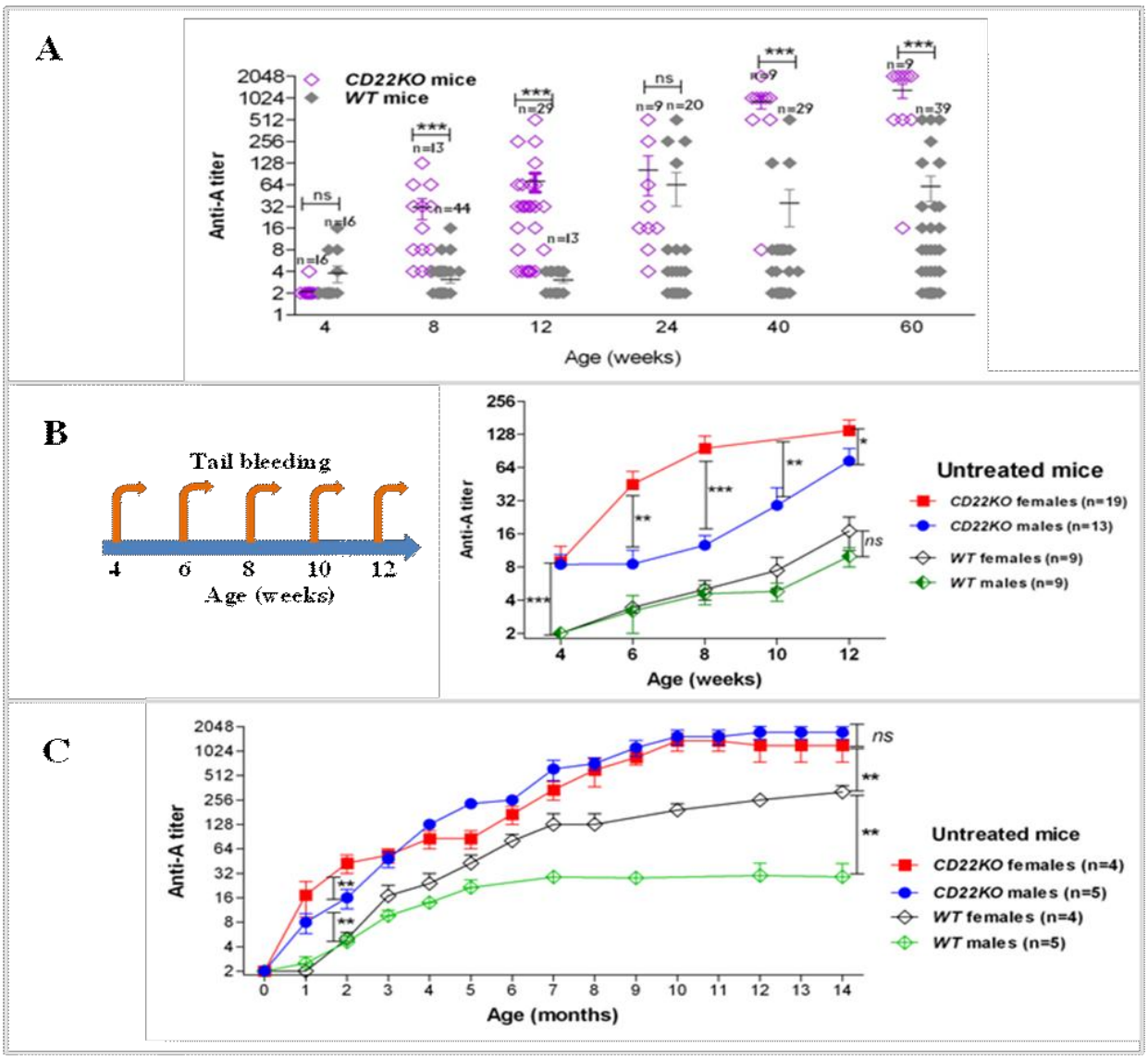

Fig. 5.1: *C22KO* B6 mice developed higher anti-A nAbs than *WT* mice. A) At different age groups, *CD22KO* (mixed sex, housed in HSLAS) developed significantly higher anti-A nAbs than *WT* mice (mixed sex, housed in satellite room). **B)** Anti-A nAbs production in *CD22KO* mice and *WT* mice at 4-12 weeks of age were longitudinally monitored (*orange long arrows*). *WT* and *CD22KO* female mice developed significantly higher anti-A nAbs titer than *WT* and *CD22KO* male mice, respectively. **C)** Using some mice shown in **B)** for a longitudinal follow-up, I showed that *WT* females and *CD22KO* male and female mice developed significantly higher anti-A nAbs titer than male *WT* mice. Hemagglutination was used to measure anti-A Ab titer, using A-Tg reagent RBC. Data are presented using standard error mean (Mean + SEM) in T-Test (ns=non-significant, * = ≥0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, see table 5.1 and 5.2 for all the comparisons).

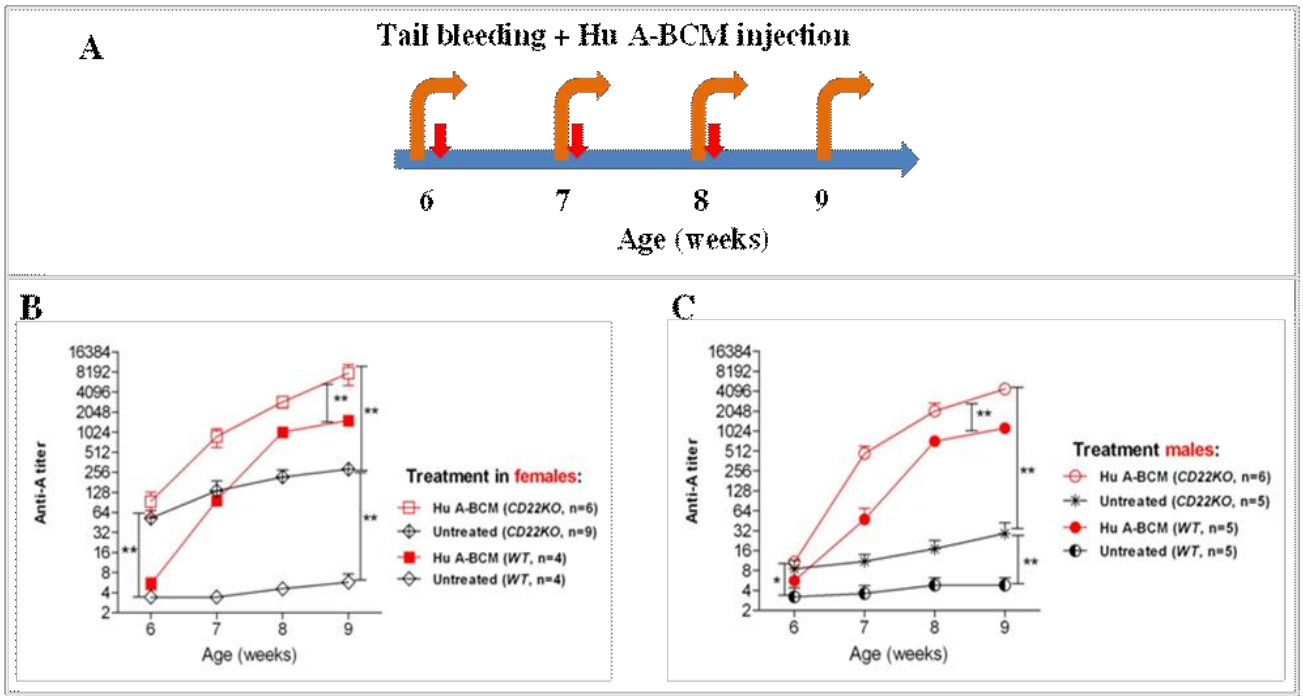

Fig. 5.2: Hu A-BCM injection induced massive anti-A Ab production in *CD22KO* B6 mice. **A)** My experimental design for Hu A-BCM injections (*red short arrows*) and tail bleeding (*orange long arrows*). **B)** Hu A-BCM injection induced massive anti-A Ab production in female *CD22KO* mice, much higher than induction of anti-A Ab in female *WT* mice by Hu A-BCM. **C)** Similarly, Hu A-BCM injection induced massive anti-A Ab production in male *CD22KO* mice. Hemagglutination was used to measure anti-A Ab titer, using A-Tg reagent RBC. Data are presented using standard error of mean (Mean + SEM) in T-Test (* = ≥0.05, ** = P ≤ 0.01, see table 5.1 and 5.2 for all the comparisons).

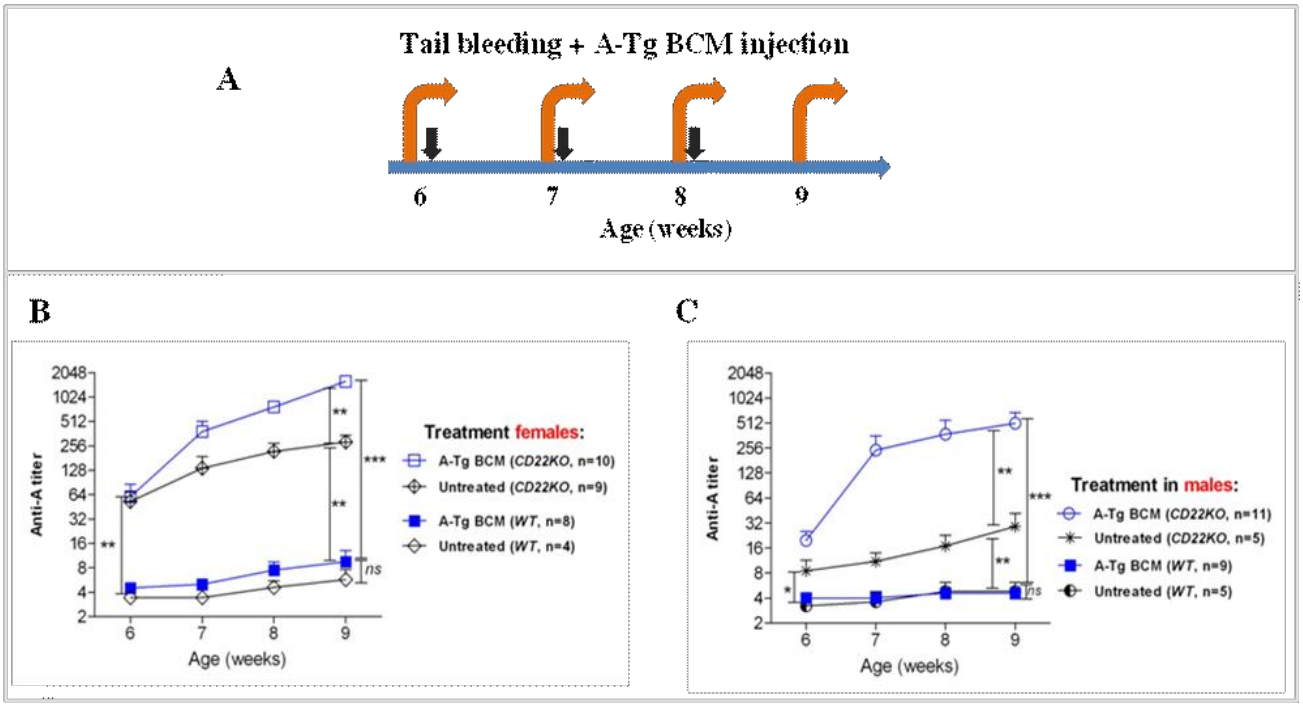

Fig. 5.3: *Syngeneic* A-Tg BCM injection induced anti-A Ab production in *CD22KO* B6 mice. **A)** My experimental design for sensitization with injection of *syngeneic* A-Tg BCM (*black short arrows*) and tail bleeding (*orange long arrows*). **B)** Injection of *syngeneic* A-Tg BCM induced anti-A Ab production in female *CD22KO* mice. **C)** Similarly, *syngeneic* A-Tg B6 BCM injection also induced anti-A Ab production in male *CD22KO* mice. I used hemagglutination to measure anti-A Ab titer, using A-Tg reagent RBC. Data are presented using standard error of mean (Mean + SEM) in T-Test (ns=non-significant, * = ≥0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, see table 5.1 and 5.2 for all the comparisons).

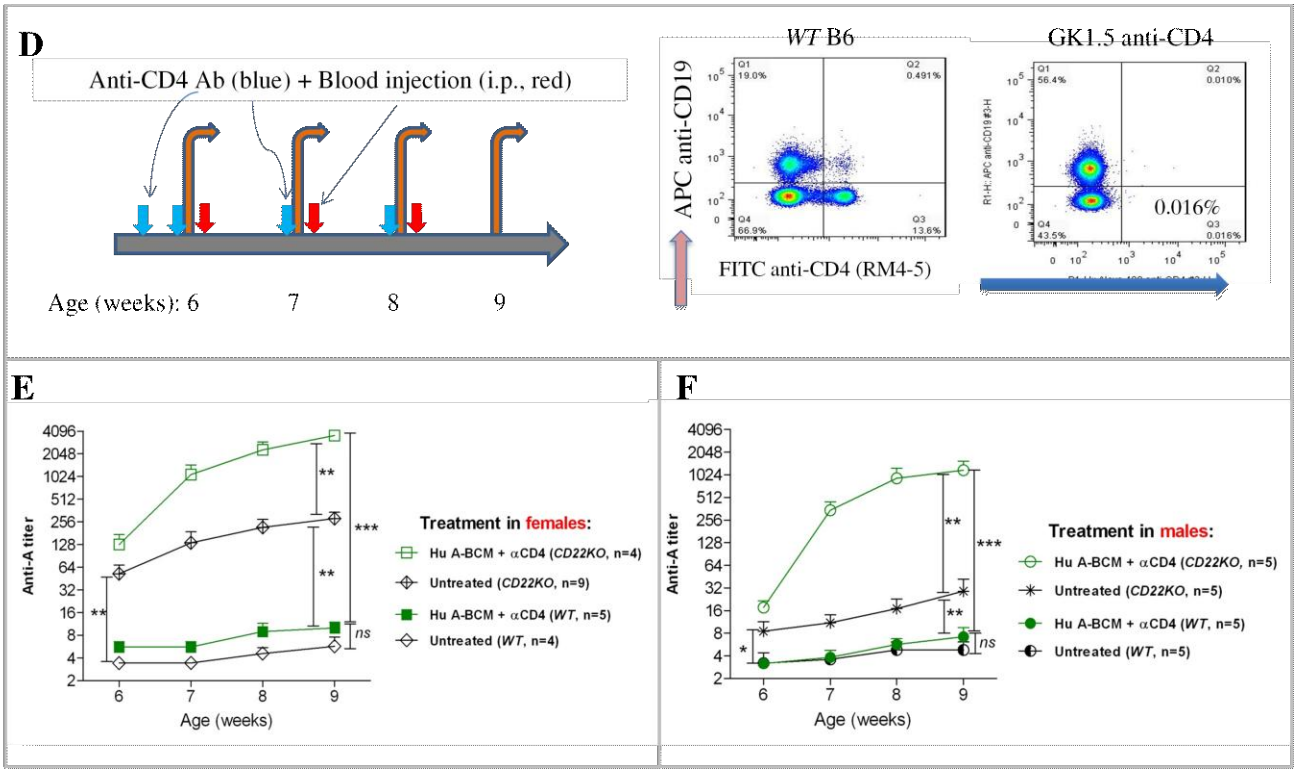

Fig. 5.3: Hu A-BCM injection induced anti-A Ab in *CD22KO* mice, independent of CD4+ T cell participation. D) In my experimental design, CD4+ T cell depletion was induced with injection of GK1.5 anti-CD4 Ab (250 ug i.p inj., twice per week, (*blue arrows*), effectiveness of T cell depletion is shown) followed with injection of Hu A-BCM (*red short arrows*). **E)** Following T cell depletion, Hu A-BCM injection induced anti-A Ab in female *CD22KO* mice, compared to untreated mice. **F)** Similarly, T cell depletion and Hu A-BCM injection induced anti-A Ab in male *CD22KO* mice, compared to untreated mice. Anti-A Ab titer was measure by hemagglutination, using A-Tg reagent RBC. Data are presented using standard error of mean (Mean + SEM) in T-Test (ns=non-significant, * = ≥0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, see table 5.1 and 5.2 for all comparisons).

Table 5.1 for summary of immunization in *B6* mice (between 6 - 12 weeks old):

Groups	Treatment	Recipients	No	Anti-A	6 *vs.* 12 weeks
#1	Untreated	*WT* females	4	<4	ns
#2	Untreated	*WT* males	5	<4	ns
#3	Untreated	*CD22KO* females	9	128	**
#4	Untreated	*CD22KO* males	5	64	**
#5	Hu A-BCM	*WT* females	4	1024	***
#6	Hu A-BCM	*WT* males	4	1024	***
#7	Hu A-BCM	*CD22KO* females	6	8192	***
#8	Hu A-BCM	*CD22KO* males	6	4096	***
#9	A-Tg BCM	*WT* females	8	<4	ns
#10	A-Tg BCM	*WT* males	9	<4	ns
#11	A-Tg BCM	*CD22KO* females	10	2048	**
#12	A-Tg BCM	*CD22KO* males	11	512	**
#13	Hu A-BCM + anti-CD4	*WT* females	5	<4	ns
#14	Hu A-BCM + anti-CD4	*WT* males	5	<4	ns
#15	Hu A-BCM + anti-CD4	*CD22KO* females	4	4096	**
#16	Hu A-BCM + anti-CD4	*CD22KO* males	5	2048	**

- Standard error of mean (Mean + SEM) of anti-A Ab titer was used in T-Test
- ns = ≥ 0.5, * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001

Table 5.2 of: Comparisons of group's #

Groups	#1	# 2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12	#13	#14	#15	#16
#1		ns	**	**	***				ns				ns			
#2	ns		**	**		***				ns				ns		
#3	**	**		ns			**				**				**	
#4	**	**	ns					**				**				**
#5	***						**		***				***			
#6		***						**		***				***		
#7			**		**			ns	**		**				**	
#8				**		**	ns					**				**
#9	ns				***		**			ns	**		ns			
#10		ns				***			ns			**		ns		
#11			**				**		**			ns			ns	
#12				**				**		**	ns					ns
#13	ns				***				ns					ns	**	**
#14		ns				****				ns			ns		**	**
#15			**				**				ns		**	**		ns
#16				**				**				ns	**	**	ns	

- Standard error of mean (Mean + SEM) of anti-A Ab titer was used in Two-way ANOVA
- ns = ≥ 0.5, * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001
-

Chapter 6:

Summary, discussion and future directions

6.1. Overall summary

My data (**Chapter 3)** showed that injection of *xenogeneic* Hu A-BCM (A-Ag plus foreign glycoproteins/glycolipids) induced abundant anti-A Ab production, similar to exposure to *allogeneic* A-Tg BCM or heart graft (A-Ag plus foreign proteins). Following CD4+ T cell depletion, stimulation of anti-A Ab production by *xenogeneic* Hu A-BCM injection was abolished, indicating that the response was CD4+ T cell-dependent.

Despite comparable expression of A-Ag, *syngeneic* A-Tg BCM alone (without foreign proteins) did not induce anti-A Ab production. Mice co-injected with *syngeneic* A-Tg BCM and *xenogeneic* Hu O-BCM (source of foreign proteins) produced significantly lower anti-A Ab production than mice stimulated by *xenogeneic* Hu A-BCM, consistent with a requirement for a chemical linkage of foreign protein with CHO-Ag [376, 377]. Interestingly, injection of *xenogeneic* Hu A-BCM 4 weeks after *syngeneic* A-Tg BCM injection induced anti-A Ab, indicating that A-Tg BCM was neither sufficiently immunogenic nor tolerogenic.

My data (**Chapter 4)** showed that *WT* mice of both sexes produced increasing amounts of anti-A natural Abs (nAbs) with age. In *CD4, MHC-II, and αβ/γδ T cell KO* mice, not only females produced significantly higher anti-A nAbs than *KO* males, but also than *WT* males and females. These data suggesting both sex-linked role and CD4+ T cells role are important in production and regulation of ABO nAbs.

Injection of *xenogeneic* Hu A-RBC in *CD4, MHC-II,* or *αβ/γδ T cell KO* mice did not produce additional anti-A Ab beyond what was already present (i.e. nAbs), indicating that CD4+ T cells were required for

induction of anti-A Ab, but not for spontaneous production of anti-A Ab. Furthermore, *CD4KO* mice reconstituted with *WT* CD4+ T cells produced anti-A Ab in response to stimulation by Hu A-BCM, comparable to induction of anti-A Ab produced in *WT* mice. Interestingly, adoptive transfer of sex-matched *WT* CD4+ T cells resulted in reduced anti-A nAbs production in *CD4KO* mice, indicating that the presence of CD4+ T cells would result in dampening the ABO nAbs production in mice.

I showed that antibiotics treatment significantly reduced anti-A nAbs production in *CD4KO* mice (dramatic reduction in females). In *WT* mice, antibiotic treatment also resulted in lower induction of anti-A Ab than in antibiotic-untreated mice. However, both male and female germ-free mice produced anti-A/B nAbs without exposure to known A/B-Ags. Taken together, my findings suggested that bacterial flora could play role during production of ABO nAbs (as antibiotic treatment significantly reduced induction of anti-A Ab in *WT* mice) but are not absolutely required (as germ-free mice produced abundant anti-A/B Abs).

My data **(Chapter 5)** demonstrated a potential role for CD22 signaling in down regulation of natural and induced anti-A Ab production. I found at different ages, *CD22KO* mice developed significantly higher anti-A nAbs than *WT* mice (both sexes) and that female *CD22KO* mice developed significantly higher anti-A nAbs than male *CD22KO* mice at early age.

In contrast to *WT* mice, injection of *xenogeneic* Hu A-BCM induced massive anti-A Ab in *CD22KO* mice (in both sexes), suggested an important role of CD22 signaling for the regulation of B cell response to A/B-Ags. However, anti-A Ab was also elicited in *CD22KO* mice by *syngeneic* A-Tg BCM injection, or by *xenogeneic* Hu A-RBC with CD4+ T cell depletion, unlike *WT* mice. This is consistent with foreign protein or CD4+ T cells being not required (T-independent) for induction of anti-A Ab production in the absence of CD22 signaling.

6.2. General discussion

6.2.1. Gut bacterial flora was not absolutely required for production of ABO nAbs

It has generally been accepted that ABO nAbs are produced following exposure to microbial "A/B-like" Ags [124, 125]. For instance, anti-A Ab could be stimulated by α-D-N-galactosamine Ag determinant derived from influenza virus vaccination [182, 378], similar to data that showed that anti-B Ab were produced in chicks and humans after exposure to "B-like" Ag expressed on *E. coli* O_{86} [119, 120]. The "A/B-like" Ags are ubiquitously distributed in food and environment (plants, allergens of house dust, etc) [136, 142, 143] and therefore may play role in production of ABO Abs in humans [135-138]. However, the exact mechanism causing ABO nAbs production remains speculative.

6.2.1.1. Antibiotic treatment resulted in reduction of ABO Abs production in mice

Spontaneous production of ABO nAbs, without apparent exposure to blood-derived A/B-Ags, prompted me to investigate whether bacterial flora was involved in the mechanism causing ABO Abs production in mice. In agreement with the hypothesis that ABO nAbs were produced following exposure to bacterial "A/B-like" Ags [119, 120, 130-132], I found that antibiotic administration resulted in reduced production of anti-A nAbs in *WT* and *CD4KO* mice. These data suggested that bacterial flora may be important for ABO nAbs production, but other factors, such as "A/B-like" substances in food [143, 182], could also have an important role during the ABO nAbs production.

Antibiotics use in mice has major limitations, including a difficulty to eliminate all bacteria flora and emergence of drug resistant bacterial strains. In addition, a difference in bacterial flora composition in mice, such as vaginal flora in female mice, makes it difficult to interpret why female mice produced significantly higher ABO nAbs than males. Therefore, I overcame these hurdles by using germ-free housed mice and re-evaluated the exact contribution of bacterial flora in ABO nAbs production.

6.2.1.2. Gut bacterial flora was not absolutely required for ABO nAbs production

I examined serum obtained from different strains of germ-free mice housed at three different germ-free facilities for spontaneous development of ABO nAbs. Surprisingly, germ-free mice produced abundant ABO Abs, indicating that bacterial flora was not absolutely required for production of ABO nAbs in mice. This is unlike the common paradigm that participation of bacterial flora is required for production of ABO nAbs in germ-free chicks and humans [119, 120].

The exact stimulus for spontaneous production of nAbs in general is not clear. Some studies suggested that B1 cell can spontaneously produce nAbs, without any known stimulation [140, 172, 208]. However, other studies suggested that an endogenous by-product Ags derived from normal [178, 179, 191, 192] or tumor tissues [144, 190] were the main stimulus for B1 cell to produce nAbs [183-189]. Moreover, some finding in mice also suggested that hormones [174] or substances released from ovary were further stimulated the production of nAbs, including anti-A nAbs [205, 206].

Accordingly, it could be speculated that following antibiotic treatment, production of ABO nAbs was caused by exposure to endogenous "A/B-like" self-Ags [183-185]. This hypothesis might explain why the specificity of ABO nAbs produced in mice does not usually correlate with expression of "A/B-like" Ags on bacterial flora [132]. Moreover, this speculation might also explain that production of equal level of anti-bacterial nAbs occurs in germ-free and conventionally housed mice [130, 131, 140]. However, this hypothesis is limited, because complete elimination of bacterial traces in sterile food of germ-free housed mice is difficult. Furthermore, I cannot rule out that production of ABO nAbs in germ-free housed mice was due to exposure to "A/B-like" bacterial Ags exist in the sterile food of germ-free mice. However, it could also be that ABO nAbs are spontaneously produced without any known stimulation (i.e. B1 cells are evolved to produced nAbs without any stimulations).

6.2.2. ABO nAbs production occurred without CD4+ T cell participation.

6.2.2.1. *CD4KO* mice developed ABO nAbs independent of CD4+ T cell participation.

Production of nAbs is considered to be protective against bacterial infection [104-106]. I observed that *WT* mice did produce ABO nAbs over time, low Ab production in early life and widely variable in later life. This is consistent with spontaneous production of nAbs by B1 cell [163-166]. However, production of high ABO nAbs in early life (pre-bleeding age at 6 weeks old) of *CD4KO* mice (highest titer was in females) was surprising, because *WT* mice at comparable age did not produce such high ABO nAbs titer.

Production of ABO nAbs without CD4+ T cell participation in *CD4KO* mice was consistent with a study concluded that induction of ABO Abs production in mice was "T-independent" [72]. However, that study did not evaluate whether ABO Abs were spontaneously produced (natural) or it only occurred after intentional sensitization by A/B-Ags [72]. My data showed that, unlike *WT* mice [101, 290, 291, 309] or *CD4KO* mice reconstituted by CD4+ T cells (**Fig. 3.5**), injection of Hu A-BCM in *CD4KO* mice did not induce anti-A Ab beyond the Abs that naturally produced, consistent with a requirement for CD4+ T cell participation for induction of ABO Abs production.

6.2.2.2. *MHC-II* and *TCRKO* mice produced ABO nAbs without any T cell participation.

Unlike *MHC-IIKO* mice [364], *CD4KO* mice might develop MHC-II-restricted T cells that can function in lieu of CD4+ T cell [363]. However, my data in *MHC-IIKO* mice showed similar high ABO nAbs was produced in *MHC-IIKO* mice, similar to that produced in *CD4KO* mice. Additional results showed that in *αβ/γδ T cell KO* mice (no T cells, including γδ-intraepithelial T cells [365]), high ABO nAbs titer was also produced; suggesting that ABO nAbs could develop in the complete absence of T cell participation. Taken together, my data showed that natural production of ABO Abs were T cell-independent. This is in contrast to my data in chapter 3 showed that induced ABO Abs in *WT* mice that required CD4+ T cell participation.

It remains possible that in *CD4, MHC-II,* and *TCR KO* mice, the males might have class-switched their ABO nAbs from IgM to IgG, resulted in potentially undetectable ABO nAbs by hemagglutination assay, since agglutination is mainly attributed to IgM. Notably, IgM-to-IgG class switching has been shown to occur in the absence of CD4+ T cell help [379, 380]. Moreover, the which B cell subtype (such as B1a, B1b, marginal zone, or other B2 B cells) is required for production of ABO nAbs in *CD4, MHC-II,* and *TCR KO* mice is currently unknown and requires investigation.

6.2.2.3. CD4+ T cells are involved in down-regulation of ABO nAbs production.

My demonstration that *CD4* and *MHC-II KO* female mice developed significantly higher ABO nAbs compared to *WT* females suggested that CD4+ T cell participation might have a role related to down-regulation of nAbs production. In agreement with this, my experiments showed that adoptive transfer of sex-matched *WT* CD4+ T cells resulted in a significantly reduced ABO nAbs production in *CD4KO* mice than the untreated mice. Thus, presence of CD4+ T cells (i.e. in *WT* mice) may down-regulate ABO nAbs production, consistent with my observations that young *WT* mice (similar age to *CD4KO* mice, at 6 weeks old) do not normally produce ABO nAbs (minimal titer, usually <4).

My data are consistent with other studies showed an enhanced production of IgM nAbs following CD4+ T cell depletion [381]. In that study, CD4+ T cell depletion in *NZB/W* mouse model for autoimmune disease showed an enhanced production of serum IgM nAbs by unknown mechanism [381]. Similarly, *α-galKO* mice produced significantly higher anti-α-gal nAbs following T cells depletion [310], supporting that nAbs are down-regulated in the presence of CD4+ T cells. In humans, it was suggested that defective expression of CD40 ligand on CD4+ T cells impedes the IgM-to-IgG class-switching, causing hyper-IgM syndrome [382].

6.2.3. Induction of ABO Abs production required CD4+ T cell participation.

6.2.3.1. Exposure to non-self A-Ag alone did not induce anti-A Ab production.

Mice can be used as a good model to study ABO immunity and tolerance as *WT* mice produce ABO Abs following exposure to foreign A/B-Ags [101, 290, 291], but not to self Ags, such as production of Ab reactive to α-gal Ag [8, 9]. Studies in *α-galKO* mice showed that stimulation by *syngeneic* lymphocytes expressing α-gal Ag induced tolerance to subsequent stimulation by *xenogeneic* pig kidney membranes (source of α-gal Ag) [317, 344, 383]. However, I showed that *WT* mice injected with *syngeneic* A-Tg BCM did not develop tolerance to subsequent stimulation by *xenogeneic* Hu A-BCM, as the mice produced abundant anti-A Ab following the re-challenge by Hu A-BCM. My findings showed that *syngeneic* A-Tg BCM was not sufficiently immunogenic to stimulate abundant anti-A Ab production in *WT* mice, in contrast to stimulation by *xenogeneic* Hu A-BCM.

Since these α-gal studies were conducted by adoptive transfer of viable lymphocytes expressing α-gal Ag into irradiated *α-galKO* mice and therefore there was a persistence engraftment of the lymphocytes in the mice [383], it is possible that injection of *syngeneic* A-Tg BCM (blood cell membrane Ags) did not last long enough in the mice, hence a persistent exposure to *syngeneic* A-Tg erythrocytes (intact erythrocyte lives longer in circulation) would induce B cell tolerance. In agreement with this possibility, our research group studies related to tolerance in mice [384, 385] observed that following exposure to *syngeneic* A-Tg heart graft in juvenile mice (4 weeks old), injection of *xenogeneic* Hu A-BCM in adulthood (8-9 weeks old) would not induce anti-A Ab production (tolerance to A-Ag?) [354]. In addition, exposure of neonatal *WT* mice to A-Tg erythrocytes (via i.p. injection of intact cells) resulted in development of tolerance to A-Ag, demonstrated by lack of anti-A Ab production following re-challenge by *xenogeneic* Hu A-BCM [384].

Failure of *syngeneic* A-Tg BCM to induce anti-A Ab production might be explained if the A-Ag expression was lower on A-Tg erythrocytes than on Hu A-erythrocytes. However, our lab showed a comparable expression levels of A-Ag on Hu A-erythrocytes and on A-Tg erythrocytes [101]. Moreover, it is also possible that lack of anti-A Ab production in response to stimulation by A-Tg erythrocytes might be due to A-Ag on A-Tg mouse being insufficiently immunogenic since the sugar structures of A-Ag were not identically shaped between Hu A-BCM and A-Tg BCM. However, my finding that anti-A Ab could be produced following stimulation of *WT* mice by *allogeneic* A-Tg blood (ie, A-Tg BALB blood injected into B6 *WT* mice) indicated that the A-Tg Ag was immunogenic, but that the immune response to A-Ag on A-Tg erythrocytes required non-self *xenogeneic* or *allogeneic* proteins.

6.2.3.2. CD4+ T cell participation was required for induction of ABO Abs production.

Mice can be induced to produce ABO Abs following intraperitoneal injection of Hu A/B-RBC [101, 290, 291, 309]. However, my data showing that stimulation of anti-A Ab production by *xenogeneic* Hu A-BCM or *allogeneic* A-Tg BCM (but not by *syngeneic* A-Tg BCM) suggested that CD4+ T cell participation was recruited by the additional foreign protein Ags expressed on *xenogeneic* or *allogeneic* blood group type A erythrocytes. Additionally, the presence of class-switched IgG isotype Ab nonetheless suggested the important role of CD4+ T cell participation, although the T cell is not absolutely required for class-switching [386, 387]. In agreement with CD4+ T cell requirement, my results showed that CD4+ T cell depletion abolished the formation of anti-A Ab in response to stimulation by Hu A-BCM, indicating that induction of ABO Abs production was CD4+ T cell-dependent. However, I cannot exclude that some CD4+ T cells, such as tissue resident CD4+ T cells, remained inaccessible to depleting Abs [388] and therefore they might have participated in induction of ABO Abs production.

6.2.3.3. Induction of ABO Abs production required carbohydrates-protein linkage.

Studies showed that strong immunity against CHO-Ags expressed in capsulated bacteria required CD4+ T cell participation that would be recruited by peptide in glycoconjugate vaccines [348, 349, 356, 357]. My finding that CD4+ T cell participation was required to induce ABO Abs production suggested that the presence of *xenogeneic* proteins expressed in Hu A-RBC played important role in development of the Abs. However, failure of *syngeneic* A-Tg BCM mixed with *xenogeneic* Hu O-BCM to induce high anti-A Ab level (similar to Ab level stimulated by *xenogeneic* Hu A-BCM) suggested that a conjugation of CHO with protein Ags was required. This suggestion is consistent with other studies showed that chemical/physical union of glycoconjugate vaccines was required for effective immunization [376, 377, 389, 390].

In contrast to my results showed that production of ABO nAbs did not require CD4+ T cell participation (consistent with T-independent Ab production), induction of ABO Abs production appeared to depend not only on exposure to A-Ag but also co-engagement with foreign protein and a requirement for CD4+ T cell participation (consistent with T-dependent Ab production). However, there were some limitations on the role of CD4+ T cell participation for induction of ABO Abs production. For instance, A-Ag concentration, route of exposure to A-Ag, and the setting of exposure to that Ag (i.e. erythrocytes membranes vs. intact cells vs. organ graft) could have resulted in different outcomes. In addition, I did not explore the role of other antigen presenting cells (i.e. dendritic cells and macrophages) in induction of ABO Abs production.

6.2.3.4. Mechanisms of CD4+ T cell participation for induction of ABO Abs production.

A possible mechanism explaining how B cells produce anti-A Ab to stimulation by *allogeneic* A-Tg BCM or graft is that it occurs due to simultaneous (or sequential?) stimulation of CD4+ T cells by the *allogeneic* protein Ags, and stimulation of B cells by A-Ag. This could be possible because in *allogeneic* A-Tg BCM or graft, the A-Ag is expressed on A-Tg blood cells (erythrocytes and leukocytes) and on graft endothelium

[101], whilst the protein Ags (i.e. foreign MHC and proteins) are also expressed on *allogeneic* leukocytes and on graft endothelium, respectively.

The close proximity of A-Ag/foreign MHC could allow the B cell to first interacts with A-Ag in *allogeneic* A-Tg BCM and then to present the foreign peptides (derived from *allogeneic*/*xenogeneic* proteins) to CD4+ T cell (**Figure 6.2.1**). This CD4+ T cell-B cell interaction would be in a similar fashion to CD4+ T cell-B cell interaction during immune response to glycoconjugate vaccines [391]. Therefore, I suggest that when CHO-protein epitopes of a glycoconjugate Ags are located in close proximity (such as in Hu-BCM or in *allogeneic* A-Tg BCM and graft), CD4+ T cell would be recruited by foreign protein Ags and therefore CD4+ T cell participates by providing help for the B cell to produce anti-A Ab.

An alternative mechanism on the importance of CD4+ T cell participation for induction of Ab production to glycoconjugate Ags is that the CHO epitope is presented on MHC-II of B cell, for the TCR, then the TCR would interact with the CHO epitope and subsequently respond to that CHO-epitopes in a glycoconjugate Ags [347, 358, 359](**Figure 6.2.2**). Because this model suggests a BCR recognizes the foreign CHO-Ag expressed in glycoconjugate Ags, I speculate that a TCR would interact and recognizes the A-Ag only when epitopes of *allogeneic* A-Tg BCM or *xenogeneic* Hu A-BCM are presented on MHC-II of a CHO-specific B cell (**Figure 6.2.2**).

According to this model [347, 358, 359], it could be hypothesize that a TCR in CD4+ T cell would interact with self A-Ag only in the presence of BCR that could interact and present that A-Ag/foreign protein complex. Therefore, I would speculate that in *WT* mice, some BCR can interact with A-Ag, process and present CHO-epitope of the A-Ag, to some TCR in that *WT* mice. Moreover, I would further speculate that such BCR that interacts with A-Ag is absent in *A-Tg* mouse, because B cell is tolerant to self A-Ag in such

A-Tg mouse since my data showed that the *A-Tg* mouse did not produce anti-A Ab in response to stimulation by Hu A-BCM.

However, this model requires further validations, because 1) the research group that proposed a CHO-TCR interaction have also reported that some CHO-Ags (such as Ags extracted from *Neisseria meningitides*) nevertheless require a peptide-TCR interaction [392] and that 2) it is possible that other antigen presenting cells, such as dendritic cells and macrophages, would also present glycopeptides Ags to CD4+ T cells and therefore, there will be no requirement for cognate interaction between Ags presented on MHC-II of a B cell, with TCR of CD4+ T cell.

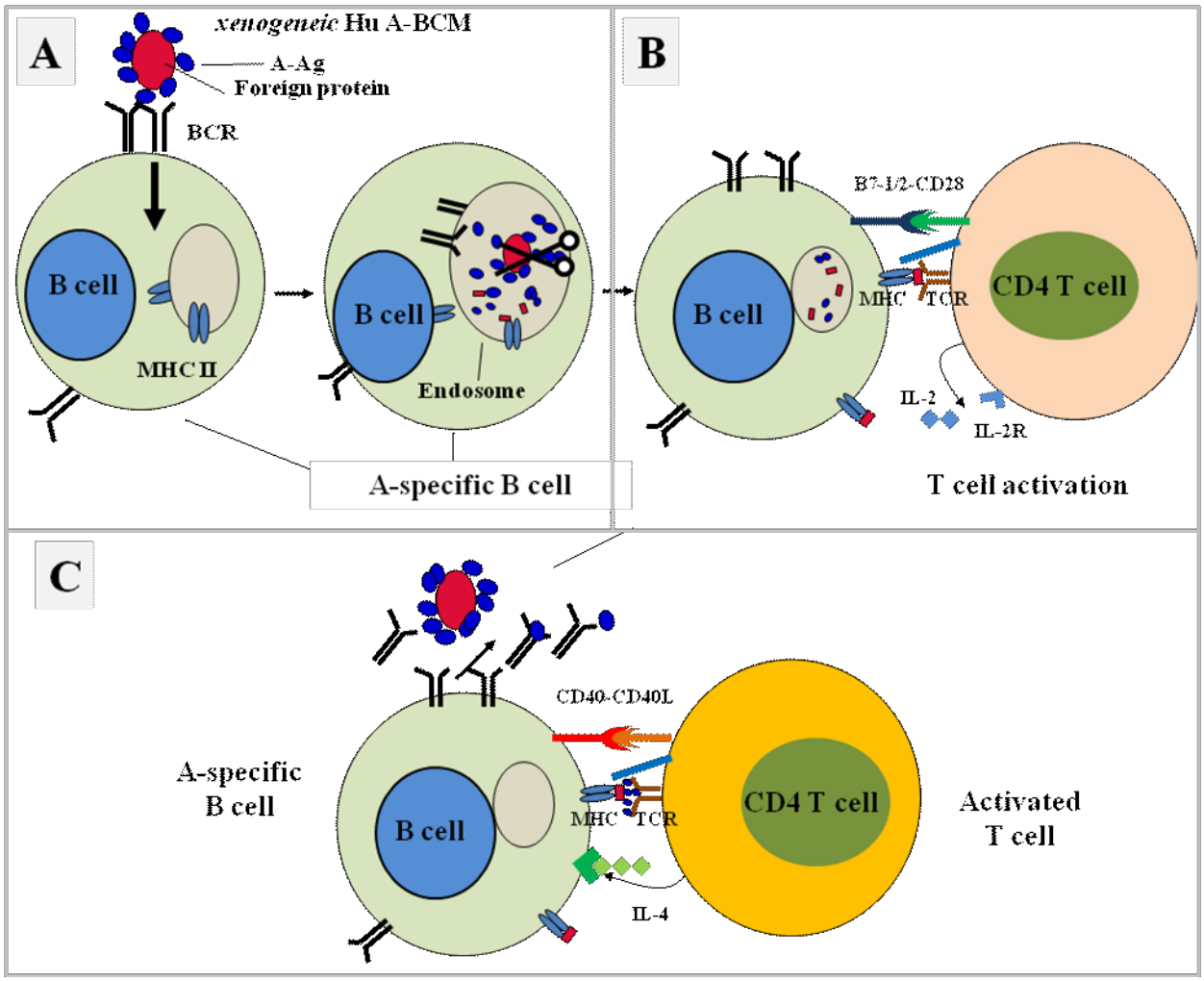

Fig. 6.2.1: Foreign peptide-TCR interaction (Model 1):

This model suggests that **A)** *xenogeneic* Hu A-BCM fragments are endocytosed via the BCR and the *xenogeneic* protein/A-Ag complex processed in endosomes into peptides and CHO-epitopes. **B)** The peptide epitope loaded into the MHC groove would be presented to the TCR resulting in T cell optimal activation via TCR-pMHC and B7-CD28 interactions. **C)** Following CD4+ T cell activation, CD40-CD40L interaction and cytokine production by the activated CD4+ T cell, such as IL-4, has an important role in B cell activation "T-dependent Ab response" [391, 393]. *Picture was made by I. Adam.*

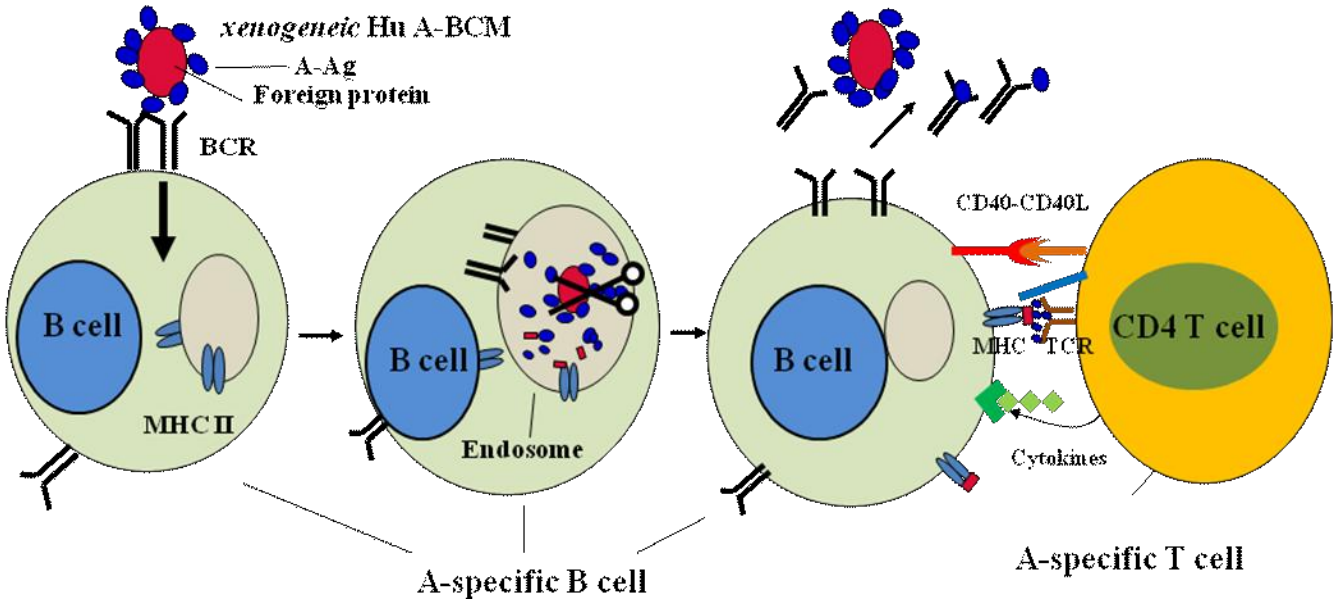

Fig. 6.2.2: Carbohydrate epitope-TCR interaction (Model 2):

In this model, *xenogeneic* Hu A-BCM fragments are endocytosed via cognate BCRs and the *xenogeneic* protein/A-Ag complex are processed into peptides-polysaccharide epitopes. The peptide (anchored into the MHC groove) would allow the zwitterionic polysaccharides portion to be presented to cognate TCRs. The TCR binds and responds to this zwitterionic polysaccharides epitope and result in CD4+ T cell engagement (cytokines and CD40L) that leads to B cell activation [347, 358, 359]. *Picture was made by I. Adam.*

6.2.4. B cell CD22 modulates ABO Abs production.

6.2.4.1. CD22 modulates natural and induced ABO Abs production.

Production of higher anti-A nAbs in *CD22KO* female mice than in males was limited to the early life stage, because *CD22KO* male and female mice older than 12 weeks old produced equivalent amount of anti-A nAbs (**Fig. 5.1 A,C**). The exact reason that young *CD22KO* female mice, compared to males, produced higher anti-A nAbs is currently unknown. My speculation is that this could be due sex-related hormone, hormonal receptor [174, 205], or biased CD4+CD25+FoxP3+ Tregs function [370], similar to my finding in *CD4, MHC-II,* and *TCR KO* female mice, or to other observations regarding nAbs production in general [175]. It is likely that the reason why *CD22KO* mice generally produce higher anti-A nAbs than *WT* mice is attributed to 1) B cell "hyper-responsiveness" in the absence of CD22-mediated inhibition, and/or 2) due to B1 cell expansion in *CD22KO* mice (**Table 1**).

In contrast to *WT* mice, I showed that stimulation of *CD22KO* mice by *xenogeneic* Hu A-BCM induced extremely high anti-A Abs that had never been reported in mice. I predict that, in addition to B cell "hyper-responsiveness" and/or B1 cell expansion, CD4+ T cell participation was also engaged by *xenogeneic* Ags contained in human blood. Therefore, CD4+ T cell participation would provide additional help for the "hyper-responsive" B cell of the *CD22KO* mice, leading to augmentation of anti-A Ab production beyond that naturally produced in the mice (**Figure 6.2.3**). Similar to *WT* mice, my data showed that sex did not seem to be crucial in inducing anti-A Ab production during stimulation by *xenogeneic* Hu A-BCM, because the difference in the anti-A Ab level between female and male *CD22KO* mice was due to the difference in the natural anti-A Ab level before the stimulation by Hu A-BCM.

6.2.4.2. Without CD22-inhibitory effect, CD4+ T cell help was not required to induce ABO Abs.

Syngeneic A-Tg BCM injection induced anti-A Ab in *CD22KO* mice, although at levels much lower than that induced by *xenogeneic* Hu A-BCM. However, my finding that *syngeneic* A-Tg BCM have induced anti-A Ab in *CD22KO* mice, but not in *WT* mice, suggested that in the absence of CD22 signaling (i.e. in *CD22KO* mice), A-Ag alone can induce anti-A Ab production without a requirement for CD4+ T cell participation (**Figure 6.2.4**), similar to previous study showed that blocking of B cell FcγR co-inhibitory receptor would reduce the need for T cell help [373]. However, perhaps the CD4+ T cells are required and they are responding to CD22 foreign peptide presented by B cell MHC-II.

In this regard, it is noteworthy that CD4+ T cell depletion followed by Hu A-BCM injection in *CD22KO* mice induced comparable anti-A Ab production to that of *syngeneic* A-Tg BCM injection in the *CD22KO* mice. These results were consistent with CD22 providing an inhibitory signal for B cell during stimulation of the ABO Abs production in *WT* mice, but that inhibitory signal in the B cell of the *WT* mice could be overcome with CD4+ T cell participation/help for the B cell as have been previously suggested [373]. In contrast, I showed that in the absence of B cell CD22 inhibitory signaling (in *CD22KO* mice), induction of ABO Abs production by B cell did not require CD4+ T cell participation/help (T-independent). Therefore, CD22 plays an important role in the modulation of B cell activation/inhibition in response to stimulation by A/B-Ags.

Although the use of *CD22KO* mice does have caveats (eg: expansion of B1 cells population), they are good tools to investigate the contribution of CD4+ T cells and CD22 in both natural and induced ABO Abs production. My data in *CD22KO* mice showed that B cell response to A/B-Ags involves both pathways: 1) T-dependent and 2) T-independent mechanisms.

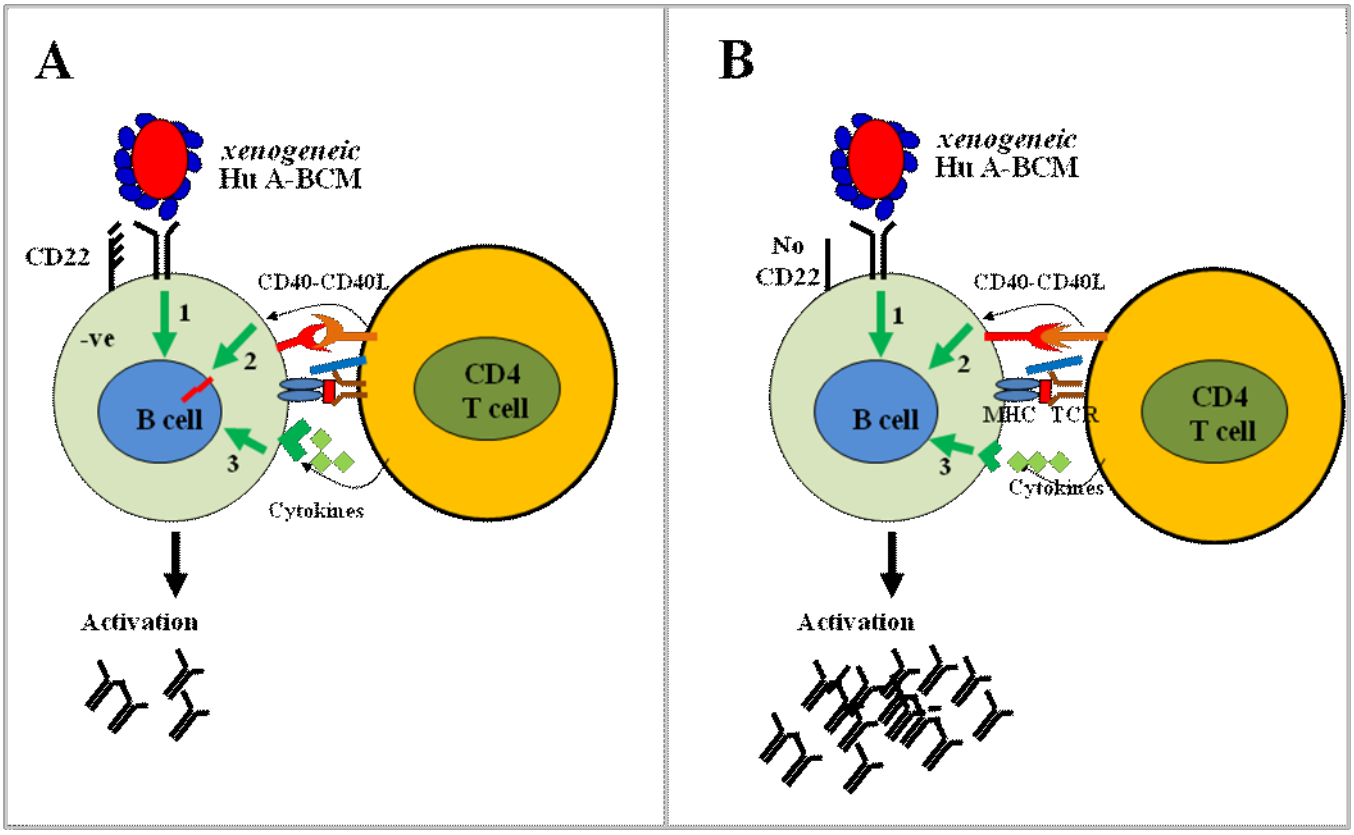

Fig. 6.2.3: Impact of CD22 and CD4+ T cell during induction of ABO Abs production (Model 3):

A) In a *WT* mouse, B cell stimulation by *xenogeneic* Hu A-BCM induces anti-A Ab production due to engagement of CD4+ T cell help that is presumably induced by foreign proteins expressed in *xenogeneic* Hu A-BCM which is known to engage CD4+ T cell. This CD4+ T cell help can overcome the inhibitory signals provided by CD22 molecules expressed on the B cell in *WT* mice. **B)** In *CD22-deficient* mouse, B cell is "hyper-responsiveness" to stimulation. Therefore, injection of *xenogeneic* Hu A-BCM induces "hyper" anti-A Ab production due to 1) induction of BCR signaling by A-Ag, 2) lack of CD22-inhibitory signaling, and 3) CD4+ T cell participation presumably induced by *xenogeneic* proteins in Hu A-BCM. *Picture was made by I. Adam.*

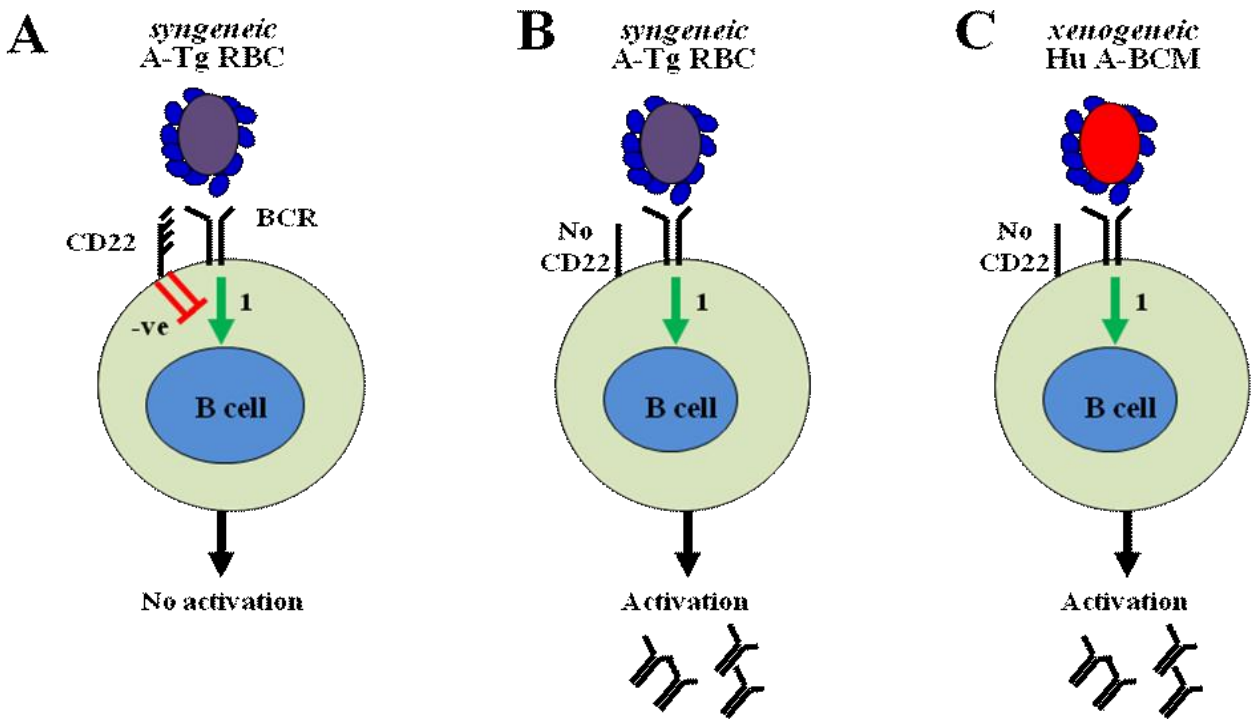

Fig. 6.2.4: Impact of CD22-deficiency during stimulation by *syngeneic* A-Tg blood (Model 4):

A) *Syngeneic* A-Tg BCM expresses A-Ag that known to engage BCR, but not CD4+ T cell participation, due to absence of foreign protein. Therefore, injection of *syngeneic* A-Tg BCM does not induce anti-A Ab production, because CD22 expressed on B cell in *WT* mouse provides an inhibitory signaling that prevent B cell activation. **B)** Injection of *syngeneic* A-Tg BCM (no foreign protein that could recruit CD4+ T cells participation) induces anti-A Ab production, because of B cells "hyper-responsive" in *CD22KO* mouse. Therefore, anti-A Ab is stimulated by *syngeneic* A-Tg BCM without any CD4+ T cell participation. **C)** In the absence of CD4+ T cell help (by depleting CD4+ T cells), injection of Hu A-BCM in *CD22KO* mouse induces comparable anti-A Ab production to that of *syngeneic* A-Tg BCM, because A-Ag in A-ATg or Hu A-BCM would only BCR stimulated the BCR. *Picture was made by I. Adam.*

6.2.5. Sex as biologic variable plays an important role in ABO nAbs production in mice.

Sex is an important biological factor in immune responses in both mice and humans [284, 285] and recently shown that nAbs were higher produced in female than male mice [174]. My data showed that female mice produced higher ABO nAbs than males. Since the impact of sex in immunity and tolerance is becoming increasingly understood, it is crucial to analyze all data in consideration to the impacts of sex.

6.2.5.1. ABO nAbs were produced in *CD4* and *CD22KO* mice with striking sex difference.

My data showed a striking sex difference in ABO nAbs production in *CD4, MHC-II,* and *TCR KO* mice, with higher Abs produced in female mice compared to males, which could be attributed due to lack of CD4+ T cell participation. Although *WT B6* mice did produce ABO nAbs over time [72, 101, 289], sex did not seem to play a significant role in both natural and induced ABO Abs in young *WT* mice, in contrast to *CD22, CD4, MHC-II,* and *TCR KO* mice.

One possibility for the sex variation in ABO nAbs development is that it is due to difference in composition of the bacterial flora in female mice [367, 368]. Some strains of bacterial flora in female lower urinary tract can disrupt bacterial flora colonization in vagina [375]. Therefore, I co-housed male and females mice starting at birth till the end of experiments (at 10 weeks old) but I did not observe any change in ABO nAbs production, suggesting that the sex difference in ABO nAbs production was not due to difference in co-housing environment (such as food and bacterial flora). Therefore, I predict that the difference in ABO nAbs production was linked to the mice sex.

6.2.5.2. Sex hormones and/or receptors might impact ABO nAbs production in female mice.

Although my data showed that sex of the mice is an important biological factor in ABO nAbs production in *CD4, MHC-II, TCR* and *CD22 KO* mice, with females producing markedly higher levels of ABO nAbs

than males, the exact role of sex in ABO nAbs production is currently unknown. I speculate that X-linked specific genes involved in females susceptibility to autoimmune disease [394], biased CD4+CD25+FoxP3+ regulatory T cells function [370], sex-related steroid hormone, or hormone-receptors, were deviating the immune response in the female mice. For instance, it was reported that estrogen can affect not only B cell survival and Abs production [395-397], but also immunity and tolerance [396, 398-401]. Accordingly, I predict that a sex-related factor(s), such as estrogen, could drive a higher ABO nAbs production in *CD22*, *CD4, MHC-II,* and *TCR KO* female mice, than in males.

The impact of estrogen in immunity is consistent with recent data showed that female mice produce higher *anti-E. coli* nAbs during puberty, independent of exposure to *E. coli*. [174]. In that study, germ-free female mice produced higher *anti-E. coli* nAbs than male mice, indicating that external factor, such as exposure to bacterial Ags, were not important for nAbs production [174]. However, whether estrogen also participated in production of a higher ABO nAbs in female mice than in male is currently unknown and worth further investigations.

Interestingly, an old study (published in 1977) suggested that ovarian glycolipids (GalNAc glycan-bearing hydrophilic ovarian glycolipids) were responsible for stimulating the anti-A Abs in female mice [205]. Although the study did not investigate anti-A Abs production in germ-free condition, the study observed that the spontaneous production of anti-A Abs increased during puberty in female mice, but not in ovariectomised mice [205]. Unfortunately, this group did not also exclude the impact of bacterial flora in the mechanism involved in production of anti-A nAbs in those ovariectomised female mice.

Similar to *CD4, MHC-II,* and *TCR KO* mice, ABO nAbs production was higher in female *CD22KO* than male mice, suggesting that "sex-related factor(s)" may also be important player(s) in development of ABO Abs in *CD22KO* mice. However, the exact interplay between that "sex-related factor(s)" and CD22-

mediated inhibition in not clear and requires further investigations. Interestingly, it was reported that estrogen produced in female mice could up regulate the expression of CD22, SHP-1, and B-cell lymphoma 2(Bcl-2) on B cells [402]. This study suggested that CD22, SHP-1, and Bcl-2 signaling would impair B cell apoptosis and therefore enhances the B cell capacity to produce Abs with specificity to self-Ags [402]. Although estrogen might explain why *CD22KO* female mice produced higher ABO nAbs than *WT* females, a mechanism by which nAbs were higher produced in *CD4KO* females, but not in *WT* female mice, is currently unknown.

Interestingly, the poor Ab production in *CD22xSiglecgKO* mice was attributed to strong B cell stimulation by DNP/TNP-Ficoll that would excessively stimulate B cells in the *CD22xSiglecgKO* mice and therefore would induce B cell apoptosis rather than activation and Ab production [224]. However, induction of B cell apoptosis by DNP/TNP-Ficoll was not reported in *CD22* or *Siglec-g* single *KO* mice [249, 252-254]. Therefore, it is unknown whether the higher ABO nAbs produced in female *CD22KO* than in female *WT* mice is due decrease in B cell apoptosis caused by higher level of estrogen in the *CD22KO* mice.

6.3. Overall conclusions

6.3.1. The impact of CD4+ T cell participation in induction of ABO Abs production.

In **Chapter 3** I investigated the role of CD4+ T cell participation in induction of ABO Abs production, with the overall aim to gain a better understanding of the mechanisms of induced ABO Abs production. I showed that exposure to non-self A-Ag in the context of self-protein was insufficient to induce anti-A Ab production. This is consistent with induction of ABO Abs depends not only on exposure to the foreign A/B-Ags, but requires a co-engagement with non-self protein and CD4+ T cell participation.

6.3.2. The role sex, CD4+ T cell, and bacterial flora in ABO nAbs production.

My results in **Chapter 4** showed that ABO nAbs could develop in the absence of CD4+ T cell participation, in contrast to induced ABO Abs production, suggesting that both T-dependent and T-independent immune mechanisms were involved in ABO Abs production. Production of dramatically increased anti-A nAbs in *CD4, MHC-II,* and *TCR KO* female mice than males, and in *CD4, MHC-II,* and *TCR KO* mice than *WT*, indicated that a sex-linked factor(s) and absence of CD4+ T cell participation, respectively, were involved in regulation of ABO nAbs production. I showed that CD4+ T cells were involved in down-regulation of ABO nAbs production in *CD4KO* mice by unknown mechanism(s).

Studying ABO nAbs development is important, because the ABO nAbs pose high risk of hyper-acute rejection of transplants from ABOi-donors [82, 89]. Early studies showed that production of ABO nAbs required a purposeful administration of *E. coli* [119]. However, my data showed that there might be a small role for bacterial flora in modulation the production of ABO Abs, suggested by a reduction of ABO nAbs following antibiotic treatment. However unlike the common paradigm [119, 120], my findings indicated that bacterial flora was not absolutely required for production of ABO nAbs.

6.3.3. The impact of Siglecs in modulation ABO Abs production.

The impact of CD22 [224, 252-259] and Siglec-g [224, 249, 250] on anti-CHO Abs production was controversial. In **Chapter 5** I explored the role of Siglecs in natural and induced ABO Abs production, using naturally occurring CHO-Ags (A/B-Ags). I showed that *CD22KO* mice produced significantly higher ABO nAbs than *WT* mice and that sex of *CD22KO* mice played a significant role in ABO nAbs production. Unlike *WT* mice, production of anti-A Ab following injection of *CD22KO* mice with Hu A-BCM did not require foreign protein or CD4+ T cell participation; consistent with induction of ABO Abs being T-independent in the absence of CD22 inhibitory signaling.

Overall, my findings are consistent with ABO Abs production in response to stimulation by A/B-Ags (but other naturally-occurring CHO-Ags, such as α-gal Ag, are unknown) involved both T-dependent and T-independent pathways/mechanisms, and that **A**) induced ABO Abs production requires CD4+ T cell participation (T-dependent pathway), **B**) spontaneous production of ABO Abs does not require CD4+ T cell participation (T-independent pathway), and **C**) production of ABO Abs in the absence of CD22-mediated inhibition does not require CD4+ T cell help, but CD4+ T cell participation/help results in more ABO Abs (both T-dependent and T-independent pathways). **D)** In *CD4, MHCII, TCR,* and *CD22 KO* mice, females produced significantly higher ABO nAbs than males and compared to *WT* females and therefore the impact of sex as a biologic variable in ABO Abs (and other nAbs) production should be further studied.

6.4. Future directions

6.4.1. CD4+ T cell participation in induction of ABO Abs production

Abundant data have shown that high affinity and long-lasting Ab production against CHO-Ags expressed on encapsulated bacteria requires CD4+ T cell participation and that the T cell participation could be recruited by foreign protein Ag in glycoconjugate vaccines [348, 349, 356, 357]. Accordingly, in my studies it is possibly that *xenogeneic* Hu A-BCM would provide sufficient stimulation for B cell and CD4+ T cell engagement, due to expression of A-Ag and foreign proteins, respectively (**Figure 6.2.1**). However, the alternative explanation for the role of CD4+ T cell participation in inducing an immune response to Hu A-BCM could be via interaction of TCR with CHO-Ag [347, 358, 359](**Figure 6.2.2**). Therefore, future studies should investigate whether A/B-Ags are presented on MHC-II on B cell so that TCR on CD4+ T cell can directly interact with the presented A/B-Ags and results in ABO Abs production.

Related to mechanisms of induced ABO Abs production, marginal zone B cells were shown to be recruited and activated during CHO-Ags entrance into blood circulation [196]. In my studies, I did not investigate

which antigen presenting cells (i.e. macrophage, dendritic cell) or B cell subset (B1a, B1b, or marginal zone B cells) in mice are required for production of ABO Abs. Therefore, future studies could investigate which B cells subset and/or antigen presenting cells are involved in natural and induced ABO Abs production.

6.4.2. ABO nAbs production in the absence of CD4+ T cell participation.

Production of ABO nAbs in *CD4, MHC-II,* and *TCR KO* mice is consistent with spontaneous production of nAbs by B1 cell [163-166]. However, the distinction between nAbs produced without exposure to A/B-Ags, from that developed following immunization of *WT* mice with Hu A/B-BCM, is unknown. Our newly developed ABH glycan microarray (A/B subtype Ags) could be very useful for characterization of natural vs. induced ABO Abs.

Although hemagglutination assays using reagent RBC is clinically relevant and useful for detection of ABO Abs (mainly IgM Abs), a major limitation is the limited ability to detect IgG Ab isotypes (without special treatment by dithiothreitol [403]). Future plans are to use the ABH glycan microarray to study the fine-specificity of isotypes of ABO Abs reacting with ABH-subtype Ags.

A significant drop in anti-A nAb titer following reconstitution of *CD4KO* mice by sex-matched *WT* CD4+ T cells suggested that CD4+ T cells were down-regulating the production of ABO nAbs. However, the mechanism by which this down-regulation occurred requires further investigations. Future studies should examine whether CD25+FoxP3+ regulatory T cells would down-regulate nAbs, including ABO nAbs.

6.4.3. Participation of gut bacterial flora in ABO Abs production.

I found that gut bacterial flora played important role in ABO Abs production, by demonstrating that antibiotic treatment in mice resulted in reduction of both natural and induced anti-A Ab production.

However, my finding that germ-free mice produced ABO nAbs opened future directions to explore whether B cells spontaneously produce ABO nAbs in complete absence of A/B-Ags, similar to other nAbs in mice [140, 172, 208], or whether unknown "endogenous" Ag was required to induce ABO Abs production. Beyond the scope of this thesis, I'm planning to study 1) the kinetic of natural and induced ABO Abs production in germ-free condition; and 2) the contribution of different species of bacterial flora in ABO nAbs production (i.e. *Lactobacillus, E. coli*).

6.4.4. Siglecs and modulation of ABO Abs production.

This was the first study to investigate the role of B cell Siglecs on ABO Abs production (using *CD22KO* mice). However, my data did not investigate whether production of higher ABO nAbs in *CD22KO* mice than *WT* was due to B cell "hyper-responsiveness" to CHO-Ags [252-254], and/or due to expansion of B1 cell population [249, 250]. Therefore, future experiments should examine the exact contribution of B1 cell expansion and/or B cell "hyper-responsiveness" in ABO Abs production.

6.4.5. Impact of sex as biologic variable in ABO nAbs production.

I showed that *CD4. MHC-II, TCR* and *CD22-deficient* female mice produced markedly higher ABO nAbs than male counterparts. Some of sex-related differences that could impact immunity in females are X-linked specific genes [394]. Other differences could be sex hormones, and/or hormone receptors that have been shown to impact B cell survival and Abs production [174, 395-397]. Therefore, future experiments should examine whether exposure to female hormones (such as estrogen) would induce ABO nAbs in male mice.

Among these experiments are the use of steroid hormones and/or hormones receptor antagonist [404] as well as ovariectomised mice [405]. Although these experiments could possible reveal the impact sex hormones in ABO nAbs production, the interpretation of these experiments could be challenging, because

they might require a demonstration of the mechanism by which hormones and/or hormone receptors could impact B cell survival and ABO nAbs production. Moreover, future experiments should tease out the mechanisms/pathways involved in induction of ABO Abs production by intentional stimulation by A/B-Ags from that involved in natural production of ABO Abs or that induced by exposure to sex hormones.

6.5. Concluding remark.

ABOi organ Tx has expanded the potential donor pool and therefore has saved many lives [2, 3, 406, 407]. To optimize safe ABOi organ Tx, it is paramount to understand how natural and induced ABO Abs are developed. This study is anticipated to help us to develop strategies to manage and expand ABOi organ Tx and therefore to improve the lives of transplant recipients. Moreover, understanding how B cells respond to CHO-Ags will open new avenues, not only to develop vaccines that can stimulate immunity against infection, but also to boost immunity against tumor-associated carbohydrate antigens.

References:

1. Osorio, *ABO-mismatched renal transplantation in children: a report of the North American Pediatric Renal Transplant Cooperative Study (NAPRTCS) and the Midwest Organ Bank (MOB).* Pediatrics Transplantation, 1998. **2**(1): p. 26-29.
2. Gugenheim, J., et al., *Liver-Transplantation across Abo Blood-Group Barriers.* Lancet, 1990. **336**(8714): p. 519-523.
3. Alexandre, G.P.J., et al., *Present Experiences in a Series of 26 ABO-Incompatible Living Donor Renal-Allografts.* Transplantation Proceedings, 1987. **19**(6): p. 4538-4542.
4. Yandza, T., et al., *Outcome of Abo-Incompatible Liver-Transplantation in Children with No Specific Alloantibodies at the Time of Transplantation.* Transplantation, 1994. **58**(1): p. 46-50.
5. Zhou, J.Y., et al., *The Glycoscience of Immunity.* Trends in Immunology, 2018. **39**(7): p. 523-535.
6. Ivetic, A., H.L.H. Green, and S.J. Hart, *L-selectin: A Major Regulator of Leukocyte Adhesion, Migration and Signaling.* Frontiers in Immunology, 2019. **10**(Article 1068).
7. Gasparini, R. and D. Panatto, *Meningococcal glycoconjugate vaccines.* Human Vaccines, 2011. **7**(2): p. 170-182.
8. Galili, U., et al., *Evolutionary Relationship between the Natural Anti-Gal Antibody and the Gal-Alpha-1-]3gal Epitope in Primates.* Proceedings of the National Academy of Sciences of the United States of America, 1987. **84**(5): p. 1369-1373.
9. Galili, U., et al., *Man, Apes, and Old-World Monkeys Differ from Other Mammals in the Expression of Alpha-Galactosyl Epitopes on Nucleated Cells.* Journal of Biological Chemistry, 1988. **263**(33): p. 17755-17762.
10. Yamamoto, F., et al., *Molecular Genetic-Basis of the Histo-Blood Group Abo System.* Nature, 1990. **345**(6272): p. 229-233.
11. Oriol, R., et al., *Genetic-Regulation of the Expression of Abh and Lewis Antigens in Tissues.* Apmis, 1992. **100**: p. 28-38.
12. Szulman, A.E., *The Histological Distribution of Blood Group Substances-a and Substances-B in Man.* Journal of Experimental Medicine, 1960. **111**(6): p. 785-800.
13. Galili, U., *Xenotransplantation and ABO incompatible transplantation: The similarities they share.* Transfusion and Apheresis Science, 2006. **35**(1): p. 45-58.
14. Rydberg, L., *ABO-incompatibility in solid organ transplantation.* Transfusion Medicine, 2001. **11**(4): p. 325-342.
15. Lindenmann, J., *Origin of the Terms Antibody and Antigen.* Scandinavian Journal of Immunology, 1984. **19**(4): p. 281-285.
16. Mazumdar, *The antigen-antibody reaction and the physics and chemistry of life.* Bulletin of the History of Medicine, 1974. **48**(1): p. 1-21.
17. Abel JJ, F.W., *On the poisons of Amanita phalloides.* journal of biological chemistry, 1907. **2**: p. 273–288.
18. Eisen, H.N. and J.H. Pearce, *Nature of Antibodies and Antigens.* Annual Review of Microbiology, 1962. **16**: p. 101-126.
19. Liljeroos, L., et al., *Structural and Computational Biology in the Design of Immunogenic Vaccine Antigens.* Journal of Immunology Research, 2015.
20. Vos, Q., et al., *B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms.* Immunological Reviews, 2000. **176**: p. 154-170.
21. MacLennan, I.C.M., et al., *The changing preference of T and B cells for partners as T-dependent antibody responses develop.* Immunological Reviews, 1997. **156**: p. 53-66.

22. Kaksonen, M. and A. Roux, *Mechanisms of clathrin-mediated endocytosis.* Nature Reviews Molecular Cell Biology, 2018. **19**(5): p. 313-326.
23. Vidard, L., et al., *Analysis of MHC class II presentation of particulate antigens by B lymphocytes.* Journal of Immunology, 1996. **156**(8): p. 2809-2818.
24. Armitage, R.J., et al., *Molecular and Biological Characterization of a Murine Ligand for Cd40.* Nature, 1992. **357**(6373): p. 80-82.
25. Lane, P., et al., *Activated Human T-Cells Express a Ligand for the Human-B Cell-Associated Antigen Cd40 Which Participates in T-Cell-Dependent Activation of Lymphocytes-B.* European Journal of Immunology, 1992. **22**(10): p. 2573-2578.
26. Miller, J.F.A. and G.F. Mitchell, *Cell to Cell Interaction in Immune Response .I. Hemolysin-Forming Cells in Neonatally Thymectomized Mice Reconstituted with Thymus of Thoracic Duct Lymphocytes.* Journal of Experimental Medicine, 1968. **128**(4): p. 801-820.
27. Mitchell, G.F. and J.F.A. Miller, *Cell to Cell Interaction in Immune Response .2. Source of Hemolysin-Forming Cells in Irradiated Mice Given Bone Marrow and Thymus or Thoracic Duct Lymphocytes.* Journal of Experimental Medicine, 1968. **128**(4): p. 821-837.
28. Nossal, G.J.V., et al., *Cell to Cell Interaction in Immune Response .3. Chromosomal Marker Analysis of Single Antibody-Forming Cells in Reconstituted Irradiated or Thymectomized Mice.* Journal of Experimental Medicine, 1968. **128**(4): p. 839-853.
29. Katz, B., *The Regulatory Influence of Activated T Cells on B Cell Responses to Antigen.* Advances in Immunology, 1972. **15**: p. 1-94.
30. Fairfax, K.A., et al., *Plasma cell development: From B-cell subsets to long-term survival niches.* Seminars in Immunology, 2008. **20**(1): p. 49-58.
31. Foy, T.M., et al., *Gp39-Cd40 Interactions Are Essential for Germinal Center Formation and the Development of B-Cell Memory.* Journal of Experimental Medicine, 1994. **180**(1): p. 157-163.
32. Banchereau, J., et al., *The Cd40 Antigen and Its Ligand.* Annual Review of Immunology, 1994. **12**: p. 881-922.
33. Elgueta, R., et al., *Molecular mechanism and function of CD40/CD40L engagement in the immune system.* Immunological Reviews, 2009. **229**: p. 152-172.
34. Mitchison, N.A., *Landmark - T-cell-B-cell cooperation.* Nature Reviews Immunology, 2004. **4**(4): p. 308-312.
35. Mingari, M.C., et al., *Human Interleukin-2 Promotes Proliferation of Activated B-Cells Via Surface-Receptors Similar to Those of Activated T-Cells.* Nature, 1984. **312**(5995): p. 641-643.
36. Falkoff, R.J.M., L.P. Zhu, and A.S. Fauci, *Separate Signals for Human B-Cell Proliferation and Differentiation in Response to Staphylococcus-Aureus - Evidence for a 2-Signal Model of B-Cell Activation.* Journal of Immunology, 1982. **129**(1): p. 97-102.
37. Lagoo, A., C.K. Tseng, and S. Sell, *Molecular Signals in B-Cell Activation .2. Il-2-Mediated Signals Are Required in Late G1 for Transition to S-Phase after Ionomycin and Pma Treatment.* Cellular Immunology, 1990. **127**(2): p. 497-505.
38. Noelle, R.J., et al., *Cognate Interactions between Helper T-Cells and B-Cells .3. Contact-Dependent, Lymphokine-Independent Induction of B-Cell Cycle Entry by Activated Helper T-Cells.* Journal of Immunology, 1989. **143**(6): p. 1807-1814.
39. Jego, G., R. Bataille, and C. Pellat-Deceunynck, *Interleukin-6 is a growth factor for nonmalignant human plasmablasts.* Blood, 2001. **97**(6): p. 1817-22.
40. Abos, B., et al., *Distinct Differentiation Programs Triggered by IL-6 and LPS in Teleost IgM(+) B Cells in The Absence of Germinal Centers.* Scientific Reports, 2016. **6**.
41. Teng, G. and E.N. Papavasiliou, *Immunoglobulin somatic hypermutation.* Annual Review of Genetics, 2007. **41**: p. 107-120.

42. Neuberger, M.S. and C. Milstein, *Somatic Hypermutation.* Current Opinion in Immunology, 1995. **7**(2): p. 248-254.
43. Stavnezer, J., *Antibody class switching.* Advances in Immunology, Vol 61, 1996. **61**: p. 79-146.
44. Feldmann, M. and A. Easten, *The relationship between antigenic structure and the requirement for thymus-derived cells in the immune response.* Journal of Experimental Medicine, 1971. **134**(1): p. 103 - 119.
45. Murphy, K. and C. Weaver, *Janeway's Immunobiology, 9th Edition.* Janeway's Immunobiology, 9th Edition, 2017: p. 1-904.
46. Mosier, D.E., J.J. Mond, and E.A. Goldings, *Ontogeny of Thymic Independent Antibody-Responses Invitro in Normal Mice and Mice with an X-Linked B Cell Defect.* Journal of Immunology, 1977. **119**(6): p. 1874-1878.
47. Mond, J.J., A. Lees, and C.M. Snapper, *T-Cell-Independent Antigens Type-2.* Annual Review of Immunology, 1995. **13**: p. 655-692.
48. Sharon, R., et al., *Dnp-Lys-Ficoll - T-Independent Antigen Which Elicits Both Igm and Igg Anti-Dnp Antibody-Secreting Cells.* Journal of Immunology, 1975. **114**(5): p. 1585-1589.
49. Dintzis, R.Z., M.H. Middleton, and H.M. Dintzis, *Studies on the Immunogenicity and Tolerogenicity of T-Independent Antigens.* Journal of Immunology, 1983. **131**(5): p. 2196-2203.
50. Dintzis, R.Z., et al., *The Immunogenicity of Soluble Haptenated Polymers Is Determined by Molecular Mass and Hapten Valence.* Journal of Immunology, 1989. **143**(4): p. 1239-1244.
51. Zhang, J., et al., *B-Cell Memory to Thymus-Independent Antigens Type-1 and Type-2 - the Role of Lipopolysaccharide in B-Memory Induction.* European Journal of Immunology, 1988. **18**(9): p. 1417-1424.
52. Jeurissen, A., et al., *Essential role for CD40 ligand interactions in T lymphocyte-mediated modulation of the murine immune response to pneumococcal capsular polysaccharides.* Journal of Immunology, 2002. **168**(6): p. 2773-2781.
53. Jeurissen, A., et al., *The human antibody response to pneumococcal capsular polysaccharides is dependent on the CD40-CD40 ligand interaction.* European Journal of Immunology, 2004. **34**(3): p. 850-858.
54. Dullforce, P., D.C. Sutton, and A.W. Heath, *Enhancement of T cell-independent immune responses in vivo by CD40 antibodies.* Nature Medicine, 1998. **4**(1): p. 88-91.
55. Akira, S., *Toll-like receptor signaling.* Journal of Biological Chemistry, 2003. **278**(40): p. 38105-38108.
56. Snapper, C.M. and J.J. Mond, *A model for induction of T cell-independent humoral immunity in response to polysaccharide antigens.* Journal of Immunology, 1996. **157**(6): p. 2229-2233.
57. Pone, E.J., et al., *Toll-Like Receptors and B-Cell Receptors Synergize to Induce Immunoglobulin Class-Switch DNA Recombination: Relevance to Microbial Antibody Responses.* Critical Reviews in Immunology, 2010. **30**(1): p. 1-29.
58. Minguet, S., et al., *Enhanced B-cell activation mediated by TLR4 and BCR crosstalk.* European Journal of Immunology, 2008. **38**(9): p. 2475-2487.
59. Quintana, F.J., et al., *Induction of IgG3 to LPS via Toll-Like Receptor 4 Co-Stimulation.* Plos One, 2008. **3**(10).
60. Herlands, R.A., et al., *T cell-independent and toll-like receptor-dependent antigen-driven activation of autoreactive B cells.* Immunity, 2008. **29**(2): p. 249-260.
61. Hua, Z.L. and B.D. Hou, *TLR signaling in B-cell development and activation.* Cellular & Molecular Immunology, 2013. **10**(2): p. 103-106.
62. Coutinho, A. and G. Moller, *B-Cell Mitogenic Properties of Thymus-Independent Antigens.* Nature-New Biology, 1973. **245**(140): p. 12-14.

63. Andersson, J., O. Sjoberg, and G. Moller, *Selective Induction of DNA-Synthesis in T and B Lymphocytes.* Cellular Immunology, 1972. **4**(4): p. 381-393.
64. Gery, I., S.Z. Spiesel, and J. Kruger, *Stimulation of B-Lymphocytes by Endotoxin - Reactions of Thymus-Deprived Mice and Karyotypic Analysis of Dividing Cells in Mice Bearing T6t6 Thymus Grafts.* Journal of Immunology, 1972. **108**(4): p. 1088-1091.
65. Alugupalli, K.R., et al., *B1b lymphocytes confer T cell-independent long-lasting immunity.* Immunity, 2004. **21**(3): p. 379-390.
66. Defrance, T., M. Taillardet, and L. Genestier, *T cell-independent B cell memory.* Current Opinion in Immunology, 2011. **23**(3): p. 330-336.
67. Feldmann, *The relationship between antigenic structure and the requirement for thymus-derived cells in the immune response.* Journal of Experimental Medicine, 1971. **134**(1): p. 103-119.
68. Mond, J.J., et al., *T-Cell Independent Antigens.* Current Opinion in Immunology, 1995. **7**(3): p. 349-354.
69. Feldmann, M. and A. Basten, *Relationship between Antigenic Structure and Requirement for Thymus-Derived Cells in Immune Response.* Journal of Experimental Medicine, 1971. **134**(1): p. 103-119.
70. Risley, J.M., *Structures for the ABO(H) blood group: Which textbook is correct?* Journal of Chemical Education, 2007. **84**(9): p. 1546-1547.
71. Oriol, R., *Genetic-Control of the Fucosylation of Abh Precursor Chains - Evidence for New Epistatic Interactions in Different Cells and Tissues.* Journal of Immunogenetics, 1990. **17**(4-5): p. 235-245.
72. Neron, S. and R. Lemieux, *Type-2 T-Cell-Independent Murine Immune-Response to the Human Abo Blood-Group Antigens.* Vox Sanguinis, 1994. **67**(1): p. 68-74.
73. Kay, L.A., *Cellular Basis of Immune-Response to Antigens of Abo Blood-Group System.* Lancet, 1984. **2**(8416): p. 1369-1371.
74. Watkins, W.M., *Genetics and biochemistry of some human blood groups.* Proceedings of the Royal Society B: Biological Sciences, 1978. **202**(1146): p. 31-53.
75. Watkins, W.M. and W.T. Morgan, *Possible genetical pathways for the biosynthesis of blood group mucopolysaccharides.* Vox Sanguinis, 1959. **4**(2): p. 97-119.
76. Morgan, W.T. and W.M. Watkins, *Some aspects of the biochemistry of the human blood-group substances. British Medical Bulletin*, 1959. **15**(2): p. 109-13.
77. Kobata, A., E.F. Grollman, and V. Ginsburg, *An enzymatic basis for blood type B in humans. Biochemical and Biophysical Research Communications*, 1968. **32**(2): p. 272-7.
78. Watkins, W.M., *Genetics and Biochemistry of Some Human-Blood Groups.* Proceedings of the Royal Society Series B-Biological Sciences, 1978. **202**(1146): p. 31-53.
79. Watkins, W.M. and W.T.J. Morgan, *Possible Genetic Pathways for the Biosynthesis of Blood-Group Mucopolysaccharides - Commentary.* Vox Sanguinis, 1993. **64**(4): p. 245-245.
80. Morgan, W.T.J. and W.M. Watkins, *Some Aspects of the Biochemistry of the Human Blood-Group Substances.* British Medical Bulletin, 1959. **15**(2): p. 109-113.
81. Jeyakanthan, M., et al., *Chemical Basis for Qualitative and Quantitative Differences Between ABO Blood Groups and Subgroups: Implications for Organ Transplantation.* American Journal of Transplantation, 2015. **15**(10): p. 2602-2615.
82. Slapak, M., R.B. Naik, and H.A. Lee, *Renal-Transplant in a Patient with Major Donor-Recipient Blood-Group Incompatibility - Reversal of Acute Rejection by the Use of Modified Plasmapheresis.* Transplantation, 1981. **31**(1): p. 4-7.
83. Tanabe, K., et al., *Abo-Incompatible Living Kidney Donor Transplantation - Results and Immunological Aspects.* Transplantation Proceedings, 1995. **27**(1): p. 1020-1023.

84. Fuchinoue, S., et al., *The 5-Year Outcome of ABO-Incompatible Kidney Transplantation With Rituximab Induction.* Transplantation, 2011. **91**(8): p. 853-857.
85. Cooper, D.K.C., *Clinical Survey of Heart-Transplantation between Abo Blood-Group Incompatible Recipients and Donors.* Journal of Heart Transplantation, 1990. **9**(4): p. 376-381.
86. Morrow, W.R., et al., *Outcome of listing for heart transplantation in infants younger than six months: Predictors of death and interval to transplantation.* Journal of Heart and Lung Transplantation, 1997. **16**(12): p. 1255-1266.
87. West, L.J., et al., *ABO-incompatible heart transplantation in infants.* New England Journal of Medicine, 2001. **344**(11): p. 793-800.
88. Fan, X.H., et al., *Donor-specific B-cell tolerance after ABO-incompatible infant heart transplantation.* Nature Medicine, 2004. **10**(11): p. 1227-1233.
89. Starzl, T.E., et al., *Progress in and Deterrents to Orthotopic Liver-Transplantation, with Special Reference to Survival, Resistance to Hyperacute Rejection, and Biliary Duct Reconstruction.* Transplantation Proceedings, 1974. **6**(4): p. 129-139.
90. Biglarnia, A.R., et al., *The multifaceted role of complement in kidney transplantation.* Nature Reviews Nephrology, 2018. **14**(12): p. 767-781.
91. Pollock-BarZiv, S.M., et al., *Competing outcomes after neonatal and infant wait-listing for heart transplantation.* Journal of Heart and Lung Transplantation, 2007. **26**(10): p. 980-985.
92. West, L.J., et al., *Adoption of a strategy to accept an ABO blood group incompatible donor heart improves outcomes after listing with no adverse impact on outcomes after cardiac transplantation in neonates and infants.* Circulation, 2003. **108**(17): p. 375-376.
93. Dipchand, A.I., et al., *Equivalent Outcomes for Pediatric Heart Transplantation Recipients: ABO-Blood Group Incompatible versus ABO-Compatible.* American Journal of Transplantation, 2010. **10**(2): p. 389-397.
94. Billingham, R.E., L. Brent, and P.B. Medawar, *Actively Acquired Tolerance of Foreign Cells.* Nature, 1953. **172**(4379): p. 603-606.
95. Tao, L.L. and T.A. Reese, *Making Mouse Models That Reflect Human Immune Responses.* Trends in Immunology, 2017. **38**(3): p. 181-193.
96. Miyagawa, F., et al., *The use of mouse models to better understand mechanisms of autoimmunity and tolerance.* Journal of Autoimmunity, 2010. **35**(3): p. 192-198.
97. Smith, D.M., et al., *Blood groups and transfusions in pigs.* Xenotransplantation, 2006. **13**(3): p. 186-194.
98. Jeyakanthan, M., et al., *Failure of Neonatal B-Cell Tolerance Induction by ABO-Incompatible Kidney Grafts in Piglets.* Transplantation, 2013. **96**(6): p. 519-528.
99. Reibel, J., et al., *The Distribution of Blood-Group Antigens in Rodent Epithelia.* Cell and Tissue Research, 1984. **237**(1): p. 111-116.
100. Fan, X.H., et al., *Induction of Human Blood Group A Antigen Expression on Mouse Cells, Using Lentiviral Gene Transduction.* Human Gene Therapy, 2010. **21**(7): p. 877-890.
101. Motyka, B., et al., *Antibody-Mediated Rejection in a Blood Group A-Transgenic Mouse Model of ABO-Incompatible Heart Transplantation.* Transplantation, 2016. **100**(6): p. 1228-1237.
102. Bos, N.A., et al., *The Influence of Exogenous Antigenic-Stimulation on the Specificity Repertoire of Background Immunoglobulin-Secreting Cells of Different Isotypes.* Cellular Immunology, 1988. **112**(2): p. 371-380.
103. Hayakawa, K., et al., *Ly-1 B-Cells - Functionally Distinct Lymphocytes That Secrete Igm Autoantibodies.* Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences, 1984. **81**(8): p. 2494-2498.
104. Panda, S. and J.L. Ding, *Natural Antibodies Bridge Innate and Adaptive Immunity.* Journal of Immunology, 2015. **194**(1): p. 13-20.

105. Zhou, Z.H., et al., *The broad antibacterial activity of the natural antibody repertoire is due to polyreactive antibodies.* Cell Host & Microbe, 2007. **1**(1): p. 51-61.
106. Holodick, N.E., N. Rodriguez-Zhurbenko, and A.M. Hernandez, *Defining Natural Antibodies.* Frontiers in Immunology, 2017. **8**(Article 872).
107. Genberg, H., et al., *Isoagglutinin adsorption in ABC-incompatible transplantation.* Transfusion and Apheresis Science, 2010. **43**(2): p. 231-235.
108. Crew, R.J. and L.E. Ratner, *ABO-incompatible kidney transplantation: current practice and the decade ahead.* Current Opinion in Organ Transplantation, 2010. **15**(4): p. 526-530.
109. Springer, G.F., R.E. Horton, and M. Forbes, *Origin of Anti-Human Blood Group-B Agglutinins in White Leghorn Chicks.* Journal of Experimental Medicine, 1959. **110**(2): p. 221-244.
110. Iijima, T., *The presence of heterophile antigen in bacteria, with special reference to Shiga's dysentery bacillus - A contribution to the study of Forssman antigen and antibody.* Journal of Pathology and Bacteriology, 1923. **26**(4): p. 519-534.
111. Bailey, C.H. and M.S. Shorb, *Heterophile antigen in pneumococci.* American Journal of Hygiene, 1931. **13**(3): p. 831-856.
112. Bailey, G.H. and M.S. Shorb, *Immunological relationships of pneumococci and other heterophile antigens and biological significance in pneumococcus infections.* American Journal of Hygiene, 1933. **17**(2): p. 358-411.
113. Finland, M. and E.C. Curnen, *Agglutinins for human erythrocytes in type XIV Anti-pneumococcic horse serums.* Science, 1938. **87**: p. 417-418.
114. Stuart, C.A., et al., *Group-specific agglutinins in rabbit serums for human cells II. Immune group-specific A agglutinins.* Journal of Immunology, 1936. **31**(1): p. 31-36.
115. Siber, G.R., D.M. Ambrosino, and B.C. Gorgone, *Blood-Group-a-Like Substance in a Preparation of Pneumococcal Vaccine.* Annals of Internal Medicine, 1982. **96**(5): p. 580-586.
116. Koskela, P., T. Nurmi, and V.M. Haiva, *Iga, Igg and Igm Anti-Blood Group-a Antibodies Induced by Pneumococcal Vaccine.* Vaccine, 1988. **6**(3): p. 221-222.
117. Stayboldt, C., A. Rearden, and T.A. Lane, *B-Antigen Acquired by Normal A1 Red-Cells Exposed to a Patients Serum.* Transfusion, 1987. **27**(1): p. 41-44.
118. Stowell, S.R., et al., *Innate immune lectins kill bacteria expressing blood group antigen.* Nature Medicine, 2010. **16**(3): p. 295-301.
119. Springer, G.F., R.E. Horton, and M. Forbes, *Origin of Antihuman Blood Group-B Agglutinins in Germfree Chicks.* Annals of the New York Academy of Sciences, 1959. **78**(1): p. 272-275.
120. Springer, G.F. and R.E. Horton, *Blood Group Isoantibody Stimulation in Man by Feeding Blood Group-Active Bacteria.* Journal of Clinical Investigation, 1969. **48**(7): p. 1280-1291.
121. Springer, G.F., *Blood-Group and Forssman Antigenic Determinants Shared between Microbes and Mammalian Cells.* Progress in Allergy, 1971. **15**: p. 9-77.
122. Thomsen, O., *The research on the heredity of blood isoagglutinates (Serological groups).* Comptes Rendus Des Seances De La Societe De Biologie Et De Ses Filiales, 1927. **96**: p. 1469-1471.
123. Furuhata, T., *A Summarized Review on the Gen-Hypothesis of the Blood Groups.* American Journal of Physical Anthropology, 1929. **13**(1): p. 109-130.
124. Wiener, A.S., *Origin of Naturally Occurring Hemagglutinins and Hemolysins - a Review.* Journal of Immunology, 1951. **66**(2): p. 287-295.
125. Kabat, E.A., *Immunochemistry of the Blood Group Substances.* Harvey Lectures, 1951. **46**: p. 252-275.
126. Yi, W., et al., *Escherichia coli O86O-antigen biosynthetic gene cluster and stepwise enzymatic synthesis of human blood group B antigen tetrasaccharide.* Journal of the American Chemical Society, 2005. **127**(7): p. 2040-2041.

127. Lloyd, K.O. and E.A. Kabat, *Immunochemical studies on blood groups. XLI. Proposed structures for the carbohydrate portions of blood group A, B, H, Lewis-a, and Lewis-b substances.* Proceedings of the National Academy of Sciences of the United States of America, 1968. **61**(4): p. 1470-7.
128. Morgan, W.T. and W.M. Watkins, *Genetic and biochemical aspects of human blood-group A-, B-, H-, Le-a- and Le-b-specificity.* British Medical Bulletin, 1969. **25**(1): p. 30-34.
129. Williamson, P. and G.F. Springer, *Blood-Group-B Active Somatic Antigen of E-Coli 086-B7.* Federation Proceedings, 1959. **18**(1): p. 604-604.
130. Bos, N.A., et al., *Serum Immunoglobulin Levels and Naturally-Occurring Antibodies against Carbohydrate Antigens in Germ-Free Balb/C Mice Fed Chemically Defined Ultrafiltered Diet.* European Journal of Immunology, 1989. **19**(12): p. 2335-2339.
131. Hooijkaas, H., et al., *Isotypes and Specificities of Immunoglobulins Produced by Germ-Free Mice Fed Chemically Defined Ultrafiltered Antigen-Free Diet.* European Journal of Immunology, 1984. **14**(12): p. 1127-1130.
132. Khasbiullina, N.R., et al., *Repertoire of Abs primed by bacteria in gnotobiotic mice.* Innate Immunity, 2018. **24**(3): p. 180-187.
133. Fong, S.W., B.Y. Qaqundah, and W.F. Taylor, *Developmental Patterns of Abo Isoagglutinins in Normal Children Correlated with Effects of Age, Sex, and Maternal Isoagglutinins.* Transfusion, 1974. **14**(6): p. 551-559.
134. Maur, C.A.D., et al., *Age Dependency of Abo Histo-Blood Group Antibodies - Reexamination of an Old Dogma.* Transfusion, 1993. **33**(11): p. 915-918.
135. Daniel-Johnson, J., et al., *Probiotic-associated high-titer anti-B in a group A platelet donor as a cause of severe hemolytic transfusion reactions.* Transfusion, 2009. **49**(9): p. 1845-1849.
136. *Differences in ABO antibody levels among blood donors: A comparison between present and past Japanese, Lao and Thai populations.* Vox Sanguinis, 2005. **89**: p. 30-30.
137. Clarke, C. and R.M. Hussey, *Decline in Deaths from Rhesus Hemolytic-Disease of the Newborn.* Journal of the Royal College of Physicians of London, 1994. **28**(4): p. 310-311.
138. Clarke, C. and N.C. Hughesjones, *Hemolytic-Disease of the Newborn.* Saudi Medical Journal, 1986. **7**(1): p. 5-13.
139. Wagner, *Germfree research: a basic study in host-contaminant relationship. II. Serologic observations in germfree animals. Bulletin of the New York Academy of Medicine*, 1955. **3**: p. 236-239.
140. Haury, M., et al., *The repertoire of serum IgM in normal mice is largely independent of external antigenic contact.* European Journal of Immunology, 1997. **27**(6): p. 1557-1563.
141. Cusick, M.F., J.E. Libbey, and R.S. Fujinami, *Molecular Mimicry as a Mechanism of Autoimmune Disease.* Clinical Reviews in Allergy & Immunology, 2012. **42**(1): p. 102-111.
142. Springer, G.F., *Inhibition of Blood-Group Agglutinins by Substances Occurring in Plants.* Journal of Immunology, 1956. **76**(6): p. 399-407.
143. Rimington, C., D.E. Stillwell, and K. Maunsell, *The Allergen(S) of House Dust - Purification and Chemical Nature of Active Constituents.* British Journal of Experimental Pathology, 1947. **28**(5): p. 309-325.
144. Vollmers, H.P. and S. Brandlein, *Natural antibodies and cancer.* New Biotechnology, 2009. **25**(5): p. 294-298.
145. Kearney, J.F., et al., *Natural Antibody Repertoires: Development and Functional Role in Inhibiting Allergic Airway Disease.* Annual Review of Immunology Vol 33, 2015. **33**: p. 475-504.
146. Binder, C.J. and G.J. Silverman, *Natural antibodies and the autoimmunity of atherosclerosis.* Springer Seminars in Immunopathology, 2005. **26**(4): p. 385-404.

147. Nguyen, T.T.T., et al., *The IgM receptor Fc mu R limits tonic BCR signaling by regulating expression of the IgM BCR.* Nature Immunology, 2017. **18**(3): p. 321-333.
148. Nguyen, T.T.T., R.A. Elsner, and N. Baumgarth, *Natural IgM Prevents Autoimmunity by Enforcing B Cell Central Tolerance Induction.* Journal of Immunology, 2015. **194**(4): p. 1489-1502.
149. Freitas, A.A., et al., *Normal Serum Immunoglobulins Participate in the Selection of Peripheral B-Cell Repertoires.* Proceedings of the National Academy of Sciences of the United States of America, 1991. **88**(13): p. 5640-5644.
150. Boes, M., et al., *Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM.* Journal of Immunology, 1998. **160**(10): p. 4776-4787.
151. Kantor, A.B. and L.A. Herzenberg, *Origin of Murine B-Cell Lineages.* Annual Review of Immunology, 1993. **11**: p. 501-538.
152. Baumgarth, N., *B-1 Cell Heterogeneity and the Regulation of Natural and Antigen-induced IgM Production.* Frontiers in Immunology, 2016. **7**(Article 324).
153. Hayakawa, K., et al., *Progenitors for Ly-1 B-Cells Are Distinct from Progenitors for Other B-Cells.* Journal of Experimental Medicine, 1985. **161**(6): p. 1554-1568.
154. Baumgarth, N., *The double life of a B-1 cell: self-reactivity selects for protective effector functions.* Nature Reviews Immunology, 2011. **11**(1): p. 34-46.
155. Quach, T.D., et al., *Distinctions among Circulating Antibody-Secreting Cell Populations, Including B-1 Cells, in Human Adult Peripheral Blood.* Journal of Immunology, 2016. **196**(3): p. 1060-1069.
156. Griffin, D.O., N.E. Holodick, and T.L. Rothstein, *Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20(+)CD27(+)TD43(+)CD70(-) (vol 208, pg 67, 2011).* Journal of Experimental Medicine, 2011. **208**(4).
157. Rothstein, T.L., et al., *Human B-1 cells take the stage.* Year in Immunology, 2013. **1285**: p. 97-114.
158. Griffin, D.O. and T.L. Rothstein, *Human B1 cell frequency: isolation and analysis of human B1 cells.* Frontiers in Immunology, 2012. **3**(Article 122).
159. Griffin, D.O., N.E. Holodick, and T.L. Rothstein, *Human B1 cells are CD3(-): A reply to "A human equivalent of mouse B-1 cells?" and "The nature of circulating CD27(+)CD43(+) B cells".* Journal of Experimental Medicine, 2011. **208**(13): p. 2566-2569.
160. Covens, K., et al., *Characterization of proposed human B-1 cells reveals pre-plasmablast phenotype.* Blood, 2013. **121**(26): p. 5176-5183.
161. Appelgren, D., et al., *Marginal-Zone B-Cells Are Main Producers of IgM in Humans, and Are Reduced in Patients With Autoimmune Vasculitis.* Frontiers in Immunology, 2018. **9**(Article 2242).
162. Cerutti, A., M. Cols, and I. Puga, *Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes.* Nature Reviews Immunology, 2013. **13**(2): p. 118-132.
163. Tumang, J.R., et al., *Cutting edge: Spontaneously Ig-secreting B-1 cells violate the accepted paradigm for expression of differentiation-associated transcription factors.* Journal of Immunology, 2005. **174**(6): p. 3173-3177.
164. Notkins, A.L., *Polyreactivity of antibody molecules.* Trends in Immunology, 2004. **25**(4): p. 174-179.
165. Dai, H., et al., *A Study on the Glycan Specificity of Natural Antibody Repertoires in Rodents.* Cellular & Molecular Immunology, 2009. **6**(6): p. 453-459.
166. Huflejt, M. and N.V. Bovin, *Anti-Carbohydrate Antibodies of Normal Sera: Findings, Surprises and Challenges.* Glycobiology, 2009. **19**(11): p. 1292-1292.
167. Haas, K.M., et al., *B-1a and b-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S-pneumoniae.* Immunity, 2005. **23**(1): p. 7-18.

168. Alugupalli, K.R., et al., *The resolution of relapsing fever borreliosis requires IgM and is concurrent with expansion of B1b lymphocytes.* Journal of Immunology, 2003. **170**(7): p. 3819-3827.
169. Ochsenbein, A.F., et al., *Control of early viral and bacterial distribution and disease by natural antibodies.* Science, 1999. **286**(5447): p. 2156-9.
170. Subramaniam, K.S., et al., *The Absence of Serum IgM Enhances the Susceptibility of Mice to Pulmonary Challenge with Cryptococcus neoformans.* Journal of Immunology, 2010. **184**(10): p. 5755-5767.
171. Rapaka, R.R., et al., *Conserved natural IgM antibodies mediate innate and adaptive immunity against the opportunistic fungus Pneumocystis murina.* Journal of Experimental Medicine, 2010. **207**(13): p. 2907-2919.
172. Choi, Y.S., et al., *B-1 cells in the bone marrow are a significant source of natural IgM.* European Journal of Immunology, 2012. **42**(1): p. 120-129.
173. Holodick, N.E., J.R. Tumang, and T.L. Rothstein, *Immunoglobulin secretion by B1 cells: Differential intensity and IRF4-dependence of spontaneous IgM secretion by peritoneal and splenic B1 cells.* European Journal of Immunology, 2010. **40**(11): p. 3007-3016.
174. Zeng, Z.T., et al., *Sex-hormone-driven innate antibodies protect females and infants against EPEC infection.* Nature Immunology, 2018. **19**(10): p. 1100-1111.
175. Hook, W.A., Toussain.Aj, and L.A. Simonton, *Appearance of Natural Antibodies in Young Rabbits.* Nature, 1966. **210**(5035): p. 543-544.
176. Casali, P. and A.L. Notkins, *Probing the Human B-Cell Repertoire with Ebv - Polyreactive Antibodies and Cd5+ Lymphocyte-B.* Annual Review of Immunology, 1989. **7**: p. 513-535.
177. Ehrenstein, M.R. and C.A. Notley, *The importance of natural IgM: scavenger, protector and regulator.* Nature Reviews Immunology, 2010. **10**(11): p. 778-786.
178. Mouthon, L., et al., *Analysis of the Normal Human Igg Antibody Repertoire - Evidence That Igg Autoantibodies of Healthy-Adults Recognize a Limited and Conserved Set of Protein Antigens in Homologous Tissues.* Journal of Immunology, 1995. **154**(11): p. 5769-5778.
179. Lacroix-Desmazes, S., et al., *Self-reactive antibodies (natural autoantibodies) in healthy individuals.* Journal of Immunological Methods, 1998. **216**(1-2): p. 117-137.
180. Khasbiullina, N.R. and N.V. Bovin, *Hypotheses of the origin of natural antibodies: A glycobiologist's opinion.* Biochemistry-Moscow, 2015. **80**(7): p. 820-835.
181. Adelman, M.K., S.F. Schluter, and J.J. Marchalonis, *''Natural'' versus regular antibodies - Response.* Protein Journal, 2004. **23**(6): p. 359-360.
182. van Oss, C.J., *''Natural'' versus regular antibodies.* Protein Journal, 2004. **23**(6): p. 357-357.
183. Nagele, E.P., et al., *Natural IgG Autoantibodies Are Abundant and Ubiquitous in Human Sera, and Their Number Is Influenced By Age, Gender, and Disease.* Plos One, 2013. **8**(4).
184. Spalter, S.H., et al., *Normal human serum contains natural antibodies reactive with autologous ABO blood group antigens.* Blood, 1999. **93**(12): p. 4418-4424.
185. Vale, A.M., et al., *The Global Self-Reactivity Profile of the Natural Antibody Repertoire Is Largely Independent of Germline D-H Sequence.* Frontiers in Immunology, 2016. **7**(Article 296).
186. Kulik, L., et al., *Pathogenic Natural Antibodies Recognizing Annexin IV Are Required to Develop Intestinal Ischemia-Reperfusion Injury.* Journal of Immunology, 2009. **182**(9): p. 5363-5373.
187. Shaw, P.X., et al., *Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity.* Journal of Clinical Investigation, 2000. **105**(12): p. 1731-1740.
188. Chen, Y.F., et al., *IgM Antibodies to Apoptosis-Associated Determinants Recruit C1q and Enhance Dendritic Cell Phagocytosis of Apoptotic Cells.* Journal of Immunology, 2009. **182**(10): p. 6031-6043.

189. Hardy, R.R. and K. Hayakawa, *Development of B cells producing natural autoantibodies to thymocytes and senescent erythrocytes.* Springer Seminars in Immunopathology, 2005. **26**(4): p. 363-375.
190. Danussi, C., et al., *A newly generated functional antibody identifies Tn antigen as a novel determinant in the cancer cell-lymphatic endothelium interaction.* Glycobiology, 2009. **19**(10): p. 1056-1067.
191. Nores, G.A., et al., *Anti-GM1 antibodies as a model of the immune response to self-glycans.* Biochimica Et Biophysica Acta-General Subjects, 2008. **1780**(3): p. 538-545.
192. Servettaz, A., et al., *Natural anti-endothelial cell antibodies.* Autoimmunity Reviews, 2008. **7**(6): p. 426-430.
193. deCathelineau, A.M. and P.M. Henson, *The final step in programmed cell death: phagocytes carry apoptotic cells to the grave.* Programmed Cell Death, 2003. **39**: p. 105-117.
194. Gronwall, C., J. Vas, and G.J. Silverman, *Protective roles of natural IgM antibodies.* Frontiers in Immunology, 2012. **3**(Article 66).
195. Devitt, A. and L.J. Marshall, *The innate immune system and the clearance of apoptotic cells.* Journal of Leukocyte Biology, 2011. **90**(3): p. 447-457.
196. Martin, F., A.M. Oliver, and J.F. Kearney, *Marginal zone and B1B cells unite in the early response against T-independent blood-borne particulate antigens.* Immunity, 2001. **14**(5): p. 617-629.
197. Connolly, S.E. and J.L. Benach, *Cutting edge: The spirochetemia of murine relapsing fever is cleared by complement-independent bactericidal antibodies.* Journal of Immunology, 2001. **167**(6): p. 3029-3032.
198. Gobet, R., et al., *The Role of Antibodies in Natural and Acquired-Resistance of Mice to Vesicular Stomatitis-Virus.* Experimental Cell Biology, 1988. **56**(4): p. 175-180.
199. Jayasekera, J.P., E.A. Moseman, and M.C. Carroll, *Natural antibody and complement mediate neutralization of influenza virus in the absence of prior immunity.* Journal of Virology, 2007. **81**(7): p. 3487-3494.
200. Paciorkowski, N., et al., *B1B lymphocytes play a critical role in host protection against lymphatic filarial parasites.* Journal of Experimental Medicine, 2000. **191**(4): p. 731-735.
201. Couper, K.N., et al., *Parasite-specific IgM plays a significant role in the protective immune response to asexual erythrocytic stage Plasmodium chabaudi AS infection.* Parasite Immunology, 2005. **27**(5): p. 171-180.
202. Mukherjee, J., et al., *Protective and Nonprotective Monoclonal-Antibodies to Cryptococcus-Neoformans Originating from One B-Cell.* Journal of Experimental Medicine, 1995. **181**(1): p. 405-409.
203. Salinas-Carmona, M.C. and I. Perez-Rivera, *Humoral immunity through immunoglobulin M protects mice from an experimental actinomycetoma infection by Nocardia brasiliensis.* Infection and Immunity, 2004. **72**(10): p. 5597-5604.
204. Dethlefsen, L., et al., *Assembly of the human intestinal microbiota.* Trends in Ecology & Evolution, 2006. **21**(9): p. 517-523.
205. Arend, P. and J. Nijssen, *A-Specific Auto-Antigenic Ovarian Glycolipids Inducing Production of Natural Anti-a Antibody.* Nature, 1977. **269**(5625): p. 255-257.
206. Arend, P., *"Natural" antibodies and histo-blood groups in biological development with respect to histo-blood group A. A perspective review.* Immunobiology, 2011. **216**(12): p. 1318-1321.
207. Arend, P., *Early ovariectomy reveals the germline encoding of natural anti-A- and Tn-cross-reactive immunoglobulin M (IgM) arising from developmental O-GalNAc glycosylations. (Germline-encoded natural anti-A/Tn cross-reactive IgM).* Cancer Medicine, 2017. **6**(7): p. 1601-1613.

208. Holodick, N.E., T. Vizconde, and T.L. Rothstein, *Splenic B-1a cells expressing CD138 spontaneously secrete large amounts of immunoglobulin in naive mice.* Frontiers in Immunology, 2014. **5**(Article 129).
209. Silverstein, A.M., *Autoimmunity versus horror autotoxicus: The struggle for recognition.* Nature Immunology, 2001. **2**(4): p. 279-281.
210. Sandel, P.C. and J.G. Monroe, *Negative selection of immature B cells by receptor editing or deletion is determined by site of antigen encounter.* Faseb Journal, 1999. **13**(4): p. A330-A330.
211. Gauld, S.B., et al., *Maintenance of B cell anergy requires constant antigen receptor occupancy and signaling.* Nature Immunology, 2005. **6**(11): p. 1160-1167.
212. Iikuni, N., et al., *Cutting Edge: Regulatory T Cells Directly Suppress B Cells in Systemic Lupus Erythematosus.* Journal of Immunology, 2009. **183**(3): p. 1518-1522.
213. Goodnow, C.C., et al., *Induction of Self-Tolerance in Mature Peripheral Lymphocytes-B.* Nature, 1989. **342**(6248): p. 385-391.
214. Klinman, N.R., *The ''clonal selection hypothesis'' and current concepts of B cell tolerance.* Immunity, 1996. **5**(3): p. 189-195.
215. Merrell, K.T., et al., *Identification of anergic B cells within a wild-type repertoire.* Immunity, 2006. **25**(6): p. 953-962.
216. Rosenspire, A.J. and K. Chen, *Anergic B Cells: Precarious On-Call Warriors at the Nexus of Autoimmunity and False-Flagged Pathogens.* Frontiers in Immunology, 2015. **6**: p. 2695-2699.
217. Lyubchenko, T., et al., *Cutting edge: Complement (C3d)-Linked antigens break B cell anergy.* Journal of Immunology, 2007. **179**(5): p. 2695-2699.
218. Wardemann, H., et al., *Predominant autoantibody production by early human B cell precursors.* Science, 2003. **301**(5638): p. 1374-1377.
219. Schroeder, K., M. Herrmann, and T.H. Winkler, *The role of somatic hypermutation in the generation of pathogenic antibodies in SLE.* Autoimmunity, 2013. **46**(2): p. 121-127.
220. Dorner, T., C. Giesecke, and P.E. Lipsky, *Mechanisms of B cell autoimmunity in SLE.* Arthritis Research & Therapy, 2011. **13**(5).
221. Detanico, T., et al., *Somatic mutagenesis in autoimmunity.* Autoimmunity, 2013. **46**(2): p. 102-114.
222. Guo, W.Z., et al., *Somatic hypermutation as a generator of antinuclear antibodies in a murine model of systemic autoimmunity.* Journal of Experimental Medicine, 2010. **207**(10): p. 2225-2237.
223. O'Keefe, T.L., et al., *Deficiency in CD22, a B cell-specific inhibitory receptor, is sufficient to predispose to development of high affinity autoantibodies.* Journal of Experimental Medicine, 1999. **189**(8): p. 1307-1313.
224. Jellusova, J., et al., *CD22 x Siglec-G Double-Deficient Mice Have Massively Increased B1 Cell Numbers and Develop Systemic Autoimmunity.* Journal of Immunology, 2010. **184**(7): p. 3618-3627.
225. Duong, B.H., et al., *Decoration of T-independent antigen with ligands for CD22 and Siglec-G can suppress immunity and induce B cell tolerance in vivo.* Journal of Experimental Medicine, 2010. **207**(2)444.
226. Hutzler, S., et al., *The Ligand-Binding Domain of Siglec-G Is Crucial for Its Selective Inhibitory Function on B1 Cells.* Journal of Immunology, 2014. **192**(11): p. 5406-5414.
227. Muller, J., et al., *CD22 ligand-binding and signaling domains reciprocally regulate B-cell Ca2+ signaling.* Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(30): p. 12402-12407.
228. Pfrengle, F., et al., *Copresentation of Antigen and Ligands of Siglec-G Induces B Cell Tolerance Independent of CD22.* Journal of Immunology, 2013. **191**(4): p. 1724-1731.

229. Courtney, A.H., et al., *Sialylated multivalent antigens engage CD22 in trans and inhibit B cell activation.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(8): p. 2500-2505.
230. Duong, B.H., et al., *Decoration of T-independent antigen with ligands for CD22 and Siglec-G can suppress immunity and induce B cell tolerance in vivo.* Journal of Experimental Medicine, 2010. **207**(1): p. 173-187.
231. Nitschke, L., *CD22 and Siglec-G: B-cell inhibitory receptors with distinct functions.* Immunological Reviews, 2009. **230**: p. 128-143.
232. Clark, E.A. and N.V. Giltiay, *CD22: A Regulator of Innate and Adaptive B Cell Responses and Autoimmunity.* Frontiers in Immunology, 2018. **9**(Article 2235).
233. Sgroi, D., et al., *Cd22, a B-Cell-Specific Immunoglobulin Superfamily Member, Is a Sialic Acid-Binding Lectin.* Journal of Biological Chemistry, 1993. **268**(10): p. 7011-7018.
234. Macauley, M.S., P.R. Crocker, and J.C. Paulson, *Siglec-mediated regulation of immune cell function in disease.* Nature Reviews Immunology, 2014. **14**(10): p. 653-666.
235. Mahajan, V.S. and S. Pillai, *Sialic acids and autoimmune disease.* Immunological Reviews, 2016. **269**(1): p. 145-161.
236. Varki, A. and P. Gagneux, *Multifarious roles of sialic acids in immunity.* Glycobiology of the Immune Response, 2012. **1253**: p. 16-36.
237. Bochner, B.S. and N. Zimmermann, *Role of siglecs and related glycan-binding proteins in immune responses and immunoregulation.* Journal of Allergy and Clinical Immunology, 2015. **135**(3): p. 598-608.
238. Crocker, P.R., J.C. Paulson, and A. Varki, *Siglecs and their roles in the immune system.* Nature Reviews Immunology, 2007. **7**(4): p. 255-266.
239. Poe, J.C. and T.F. Tedder, *CD22 and Siglec-G in B cell function and tolerance.* Trends in Immunology, 2012. **33**(8): p. 413-420.
240. Leprince, C., et al., *Cd22 Associates with the Human Surface Igm-B-Cell Antigen Receptor Complex.* Proceedings of the National Academy of Sciences of the United States of America, 1993. **90**(8): p. 3236-3240.
241. Cornall, R.J., et al., *Polygenic autoimmune traits: Lyn, CD22, and SHP-1 are limiting elements of a biochemical pathway regulating BCR signaling and selection.* Immunity, 1998. **8**(4): p. 497-508.
242. Smith, K.G.C., et al., *Inhibition of the B cell by CD22: A requirement for Lyn.* Journal of Experimental Medicine, 1998. **187**(5): p. 807-811.
243. Pillai, S., et al., *Siglecs and Immune Regulation.* Annual Review of Immunology, Vol 30, 2012. **30**: p. 357-392.
244. Chen, G.Y., et al., *Siglec-G/10 in self-nonself discrimination of innate and adaptive immunity.* Glycobiology, 2014. **24**(9): p. 800-806.
245. Parry, S.L., et al., *Hypercross-Linking Surface Igm or Igd Receptors on Mature B-Cells Induces Apoptosis That Is Reversed by Costimulation with Il-4 and Anti-Cd40.* Journal of Immunology, 1994. **152**(6): p. 2821-2829.
246. Mattson, M.P. and S.L. Chan, *Calcium orchestrates apoptosis.* Nature Cell Biology, 2003. **5**(12): p. 1041-1043.
247. Leprince, C., et al., *CD22 associates with the human surface IgM-B-cell antigen receptor complex.* Proceedings of the National Academy of Sciences of the United States of America, 1993. **90**(8): p. 3236-40.
248. Smith, K.G., et al., *Inhibition of the B cell by CD22: a requirement for Lyn.* Journal of Experimental Medicine, 1998. **187**(5): p. 807-11.
249. Hoffmann, A., et al., *Siglec-G is a B1 cell-inhibitory receptor that controls expansion and calcium signaling of the B1 cell population.* Nature Immunology, 2007. **8**(7): p. 694-704.

250. Ding, C., et al., *Siglecg Limits the Size of B1a B Cell Lineage by Down-Regulating NF kappa B Activation.* Plos One, 2007. **2**(10).
251. Schwartzalbiez, R., et al., *Cd22 Antigen - Biosynthesis, Glycosylation and Surface Expression of a B-Lymphocyte Protein Involved in B-Cell Activation and Adhesion.* International Immunology, 1991. **3**(7): p. 623-633.
252. OKeefe, T.L., et al., *Hyperresponsive B cells in CD22-Deficient mice.* Science, 1996. **274**(5288): p. 798-801.
253. Onodera, T., et al., *CD22 regulates time course of both B cell division and antibody response.* Journal of Immunology, 2008. **180**(2): p. 907-913.
254. Haas, K.M., et al., *CD22 Promotes B-1b Cell Responses to T Cell-Independent Type 2 Antigens.* Journal of Immunology, 2018. **200**(5): p. 1671-1681.
255. Samardzic, T., et al., *Reduction of marginal zone B cells in CD22-deficient mice.* European Journal of Immunology, 2002. **32**(2): p. 561-567.
256. Bednar, K.J., et al., *Human CD22 Inhibits Murine B Cell Receptor Activation in a Human CD22 Transgenic Mouse Model.* Journal of Immunology, 2017. **199**(9): p. 3116-3128.
257. Chappell, C.P., K.E. Draves, and E.A. Clark, *CD22 is required for formation of memory B cell precursors within germinal centers.* Plos One, 2017. **12**(3).
258. Sato, S., et al., *CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: Altered signaling in CD22-deficient mice.* Immunity, 1996. **5**(6): p. 551-562.
259. Shih, T.A.Y., et al., *Role of BCR affinity in T cell-dependent antibody responses in vivo.* Nature Immunology, 2002. **3**(6): p. 570-575.
260. Nitschke, L., et al., *CD22 is a negative regulator of B-cell receptor signalling.* Current Biology, 1997. **7**(2): p. 133-143.
261. Viglianti, G.A., et al., *Activation of autoreactive B cells by CpG dsDNA.* Immunity, 2003. **19**(6): p. 837-847.
262. Jellusova, J., et al., *Siglec-G Regulates B1 Cell Survival and Selection.* Journal of Immunology, 2010. **185**(6): p. 3277-3284.
263. Otipoby, K.L., et al., *CD22 regulates thymus-independent responses and the lifespan of B cells.* Nature, 1996. **384**(6610): p. 634-637.
264. Haas, K.M., *B-1 lymphocytes in mice and nonhuman primates.* B-1 Cell Development and Function, 2015. **1362**: p. 98-109.
265. Yammani, R.D. and K.M. Haas, *Primate B-1 Cells Generate Antigen-Specific B Cell Responses to T Cell-Independent Type 2 Antigens.* Journal of Immunology, 2013. **190**(7): p. 3100-3108.
266. Butler, J.E., et al., *Antibody repertoire development in fetal and neonatal piglets. XVI. Influenza stimulates adaptive immunity, class switch and diversification of the IgG repertoire encoded by downstream C gamma genes.* Immunology, 2013. **138**(2): p. 134-144.
267. Matsiota, P., et al., *Detection of Natural Autoantibodies in the Serum of Anti-Hiv-Positive Individuals.* Annales De L Institut Pasteur-Immunology, 1987. **138**(2): p. 223-233.
268. Benfadhel, F., et al., *Natural Antibodies in Sera of Patients with Systemic Lupus-Erythematosus.* Journal of Clinical Laboratory Analysis, 1987. **1**(3): p. 275-279.
269. Jeyakanthan, M., et al., *T-Cell Dependency of T-Independent (TI) Antigens: Antibody Response to Blood Group A-Antigen Requires CD4 T-Cells and CD40/CD40L Interaction.* Transplantation, 2012. **94**(10): p. 458-458.
270. Mahana, W., P. Matsiota, and S. Avrameas, *Both Natural and Induced Murine Anti-Tnp Antibodies Possess Anti-DNA Activity.* Annales De L Institut Pasteur-Immunology, 1987. **138**(6): p. 805-814.
271. Haas, K.M., et al., *CD22 ligand binding regulates normal and malignant B lymphocyte survival in vivo.* Journal of Immunology, 2006. **177**(5): p. 3063-3073.

272. Dighiero, G., et al., *High-Frequency of Natural Autoantibodies in Normal Newborn Mice.* Journal of Immunology, 1985. **134**(2): p. 765-771.
273. Holmberg, D., et al., *Idiotypic Determinants of Natural Igm Antibodies That Resemble Self Ia Antigens.* Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences, 1984. **81**(10): p. 3175-3179.
274. Lanoue, A., et al., *Interaction of CD22 with alpha 2,6-linked sialoglycoconjugates: innate recognition of self to dampen B cell autoreactivity?* European Journal of Immunology, 2002. **32**(2): p. 348-355.
275. Collins, B.E., et al., *Masking of CD22 by cis ligands does not prevent redistribution of CD22 to sites of cell contact.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(16): p. 6104-6109.
276. Razi, N. and A. Varki, *Masking and unmasking of the sialic acid-binding lectin activity of CD22 (Siglec-2) on B lymphocytes.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(13): p. 7469-7474.
277. Lajaunias, F., et al., *Differential control of CD22 ligand expression on B and T lymphocytes, and enhanced expression in murine systemic lupus.* Arthritis and Rheumatism, 2003. **48**(6): p. 1612-1621.
278. Santos, L., et al., *Dendritic cell-dependent inhibition of B cell proliferation requires CD22.* Journal of Immunology, 2008. **180**(7): p. 4561-4569.
279. Lee, M., et al., *Transcriptional programs of the lymphoid tissue capillary and high endothelium reveal control mechanisms for lymphocyte homing (vol 15, pg 982, 2014).* Nature Immunology, 2015. **16**(2).
280. Nitschke, L., et al., *Identification of CD22 ligands on bone marrow sinusoidal endothelium implicated in CD22-dependent homing of recirculating B cells.* Journal of Experimental Medicine, 1999. **189**(9): p. 1513-1518.
281. Macauley, M.S., et al., *Antigenic liposomes displaying CD22 ligands induce antigen-specific B cell apoptosis.* Journal of Clinical Investigation, 2013. **123**(7): p. 3074-3083.
282. Derkatz, K., et al., *Increased Expression of the Inhibitory B-Cell Molecule CD22 on CD27(+)IgM(+) B Cells Containing ABO-Antibody-Secreting Cells: A Role in Susceptibility to B-Cell Tolerance after ABO-Incompatible Infant Heart Transplantation?* Transplantation, 2012. **94**(10): p. 486-486.
283. Derkatz, K., et al., *Infant B-Cell Signaling after ABO-Incompatible Heart Transplantation (ABOi HTx): Defining the Role of the Inhibitory Molecule CD22.* Journal of Heart and Lung Transplantation, 2013. **32**(4): p. S298-S299.
284. Bouman, A., M.J. Heineman, and M.M. Faas, *Sex hormones and the immune response in humans.* Human Reproduction Update, 2005. **11**(4): p. 411-423.
285. Klein, S.L., I. Marriott, and E.N. Fish, *Sex-based differences in immune function and responses to vaccination.* Transactions of the Royal Society of Tropical Medicine and Hygiene, 2015. **109**(1): p. 9-15.
286. Moulton, V.R., *Sex Hormones in Acquired Immunity and Autoimmune Disease.* Frontiers in Immunology, 2018. **9**(Article 2279).
287. Borsotti, C., et al., *HSC extrinsic sex-related and intrinsic autoimmune disease-related human B-cell variation is recapitulated in humanized mice.* Blood Advances, 2017. **1**(23): p. 2007-2018.
288. Scotland, R.S., et al., *Sex differences in resident immune cell phenotype underlie more efficient acute inflammatory responses in female mice.* Blood, 2011. **118**(22): p. 5918-5927.
289. Huppes, W., et al., *The Role of Natural Antibodies and Abo (H) Blood-Groups in Transplantation of Human Lymphoid-Cells into Mice.* European Journal of Immunology, 1993. **23**(1): p. 26-32.

290. Christiansen, D., et al., *Antibody responses to glycolipid-borne carbohydrates require CD4(+) T cells but not CD1 or NKT cells (vol 89, pg 502, 2011).* Immunology and Cell Biology, 2011. **89**(4): p. 574-574.
291. Tazawa, H., et al., *Blockade of invariant TCR-CD1d interaction specifically inhibits antibody production against blood group A carbohydrates.* Blood, 2013. **122**(15): p. 2582-2590.
292. Ikehara, S., et al., *Functional T-Cells in Athymic Nude-Mice.* Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences, 1984. **81**(3): p. 886-888.
293. Poussier, P., et al., *Thymus-Independent Development and Negative Selection of T-Cells Expressing T-Cell Receptor-Alpha/Beta in the Intestinal Epithelium - Evidence for Distinct Circulation Patterns of Gut-Derived and Thymus-Derived Lymphocytes-T.* Journal of Experimental Medicine, 1992. **176**(1): p. 187-199.
294. Rocha, B., P. Vassalli, and D. Guygrand, *The V-Beta Repertoire of Mouse Gut Homodimeric-Alpha Cd8+ Intraepithelial T-Cell Receptor Alpha/Beta+ Lymphocytes Reveals a Major Extrathymic Pathway of T-Cell Differentiation.* Journal of Experimental Medicine, 1991. **173**(2): p. 483-486.
295. Amsbaugh, D.F., et al., *Genetic control of the antibody response to type 3 pneumococcal polysaccharide in mice. I. Evidence that an X-linked gene plays a decisive role in determining responsiveness.* Journal of Experimental Medicine, 1972. **136**(4): p. 931 - 949.
296. Tanwar, S., et al., *Mediation of transitional B cell maturation in the absence of functional Bruton's tyrosine kinase.* Scientific Reports, 2017. **7**: p. 46029.
297. Khan, W.N., et al., *Impaired B cell maturation in mice lacking Bruton's tyrosine kinase (Btk) and CD40.* International Immunology, 1997. **9**(3): p. 395-405.
298. Lewis, *Activation of B cell subsets by T-dependent and T-independent antigens.* Advances in Experimental Medicine and Biology, 1978. **98**.
299. Reed, S.G., et al., *Immune-Responses to T-Dependent and T-Independent Antigens during Visceral Leishmaniasis in Mice - Evidence for Altered T-Cell Regulation of Immune-Responses to Non-Parasite Antigens.* Cellular Immunology, 1985. **96**(1): p. 12.
300. Brodeur, P.H. and H.H. Wortis, *Regulation of Thymus-Independent Responses - Unresponsiveness to a 2nd Challenge of Tnp-Ficoll Is Mediated by Hapten-Specific Antibodies.* Journal of Immunology, 1980. **125**(4): p. 1499.
301. Goud, S.N., A.M. Kaplan, and B. Subbarao, *Primary Antibody-Responses to Thymus-Independent Antigens in the Lungs and Hilar Lymph-Nodes of Mice.* Infection and Immunity, 1990. **58**(7): p. 2035-2041.
302. Chused, *Macrophage requirement for the in vitro response to TNP Ficoll: a thymic independent antigen.* Journal of Immunology, 1976. **116**(6): p. 1579-1581.
303. Lewis, G.K., J.W. Goodman, and R. Ranken, *Activation of B cell subsets by T-dependent and T-independent antigens. Advances in Experimental Medicine and Biology*, 1978. **98**: p. 339-56.
304. Reed, S.G., et al., *Immune responses to T-dependent and T-independent antigens during visceral leishmaniasis in mice: evidence for altered T-cell regulation of immune responses to non-parasite antigens.* Cell Immunology, 1985. **96**(1): p. 12-25.
305. Brodeur, P.H. and H.H. Wortis, *Regulation of thymus-independent responses: unresponsiveness to a second challenge of TNP-Ficoll is mediated by hapten-specific antibodies.* Journal of Immunology, 1980. **125**(4): p. 1499-505.
306. Goud, S.N., A.M. Kaplan, and B. Subbarao, *Primary antibody responses to thymus-independent antigens in the lungs and hilar lymph nodes of mice.* Infection and Immunity, 1990. **58**(7): p. 2035-41.
307. Chused, T.M., S.S. Kassan, and D.E. Mosier, *Macrophage requirement for the in vitro response to TNP Ficoll: a thymic independent antigen.* Journal of Immunology, 1976. **116**(6): p. 1579-81.

308. Betts, M., et al., *Lipopolysaccharide from Brucella-Abortus Behaves as a T-Cell-Independent Type-1 Carrier in Murine Antigen-Specific Antibody-Responses.* Infection and Immunity, 1993. **61**(5): p. 1722-1729.
309. Irei, T., et al., *The persistent elimination of B cells responding to blood group A carbohydrates by synthetic group A carbohydrates and B-1 cell differentiation blockade: novel concept in preventing antibody-mediated rejection in ABO-incompatible transplantation.* Blood, 2007. **110**(13): p. 4567-4575.
310. Ohdan, H., et al., *In vivo T-cell depletion enhances production of anti-Gal alpha 1,3Gal natural antibodies in alpha 1,3-galactosyltransferase-deficient mice.* Transplantation, 2000. **69**(5): p. 910-913.
311. Basu, M. and S. Basu, *Enzymatic-Synthesis of a Blood-Group B-Related Pentaglycosylceramide by an Alpha-Galactosyltransferase from Rabbit Bone-Marrow.* Journal of Biological Chemistry, 1973. **248**(5): p. 1700-1706.
312. Macher, B.A. and U. Galili, *The Gal alpha 1,3Gal beta 1,4GlcNAc-R (alpha-Gal) epitope: A carbohydrate of unique evolution and clinical relevance.* Biochimica Et Biophysica Acta-General Subjects, 2008. **1780**(2): p. 75-88.
313. Adam, I., et al., *Antibody Response to Non-Self Blood Group A-Antigen Is Dependent on CD4 T Cells and Co-Engagement with Foreign Protein.* Journal of Heart and Lung Transplantation, 2017. **36**(4): p. S134-S134.
314. Adam, I., et al., *Anti-A Antibody Response is Dependent on CD4 T Cells and Co-Engagement With Foreign Protein.* Transplantation, 2017. **101**(5): p. S4-S4.
315. Tanemura, M., et al., *Differential immune response to carbohydrate epitopes on allo- and xenografts: Implications for accommodation.* Transplantation Proceedings, 2000. **32**(5): p. 991-993.
316. Cretin, N., et al., *The role of T cell help in the production of antibodies specific for Gal alpha 1-3Gal.* Journal of Immunology, 2002. **168**(3): p. 1479-1483.
317. Galili, U., *Immune response, accommodation, and tolerance to transplantation carbohydrate antigens.* Transplantation, 2004. **78**(8): p. 1093-1098.
318. Rakoff-Nahoum, S., et al., *Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis.* Cell, 2004. **118**(2): p. 229-241.
319. Morgun, A., et al., *Uncovering effects of antibiotics on the host and microbiota using transkingdom gene networks.* Gut, 2015. **64**(11): p. 1732-1743.
320. Erickson, L.D., et al., *Differential expression of CD22 (Lyb8) on murine B cells.* International Immunology, 1996. **8**(7): p. 1121-1129.
321. Fagarasan, S. and T. Honjo, *T-independent immune response: New aspects of B cell biology.* Science, 2000. **290**(5489): p. 89-92.
322. Balazs, M., et al., *Blood dendritic cells interact with splenic marginal zone B cells to initiate T-Independent immune responses.* Immunity, 2002. **17**(3): p. 341-352.
323. Hayakawa, K., et al., *The Ly-1-B Cell Subpopulation in Normal, Immunodefective, and Autoimmune Mice.* Journal of Experimental Medicine, 1983. **157**(1): p. 202-218.
324. Grewal, P.K., et al., *ST6Gal-I restrains CD22-dependent antigen receptor endocytosis and Shp-1 recruitment in normal and pathogenic immune signaling.* Molecular and Cellular Biology, 2006. **26**(13): p. 4970-4981.
325. Doody, G.M., et al., *A Role in B-Cell Activation for Cd22 and the Protein-Tyrosine-Phosphatase Shp.* Science, 1995. **269**(5221): p. 242-244.
326. Parry, S.L., et al., *Plastic-Immobilized Anti-Mu or Anti-Delta Antibodies Induce Apoptosis in Mature Murine B-Lymphocytes.* European Journal of Immunology, 1994. **24**(4): p. 974-979.

327. Demaurex, N. and C. Distelhorst, *Apoptosis - the calcium connection.* Science, 2003. **300**(5616): p. 65-67.
328. Jeyakanthan, M., et al., *Failure of neonatal B-cell tolerance induction by ABO-incompatible kidney grafts in piglets.* Transplantation, 2013. **96**(6): p. 519-28.
329. West, L.J. and K.S. Tao, *Acceptance of third-party cardiac but not skin allografts induced by neonatal exposure to semi-allogeneic lymphohematopoietic cells.* American Journal of Transplantation, 2002. **2**(8): p. 733-744.
330. Corry, R.J., H.J. Winn, and P.S. Russell, *Primarily Vascularized Allografts of Hearts in Mice - Role of H-2d, H-2k, and Non-H-2 Antigens in Rejection.* Transplantation, 1973. **16**(4): p. 343-350.
331. Jeyakanthan, *Immunity and Tolerance to Carbohydrate Antigens.* University of Alberta Library, 2014. **PhD Thesis**: p. 52.
332. Jeyakanthan, M., et al., *ABH-Glycan Microarray Characterizes ABO Subtype Antibodies: Fine Specificity of Immune Tolerance After ABO-Incompatible Transplantation.* American Journal of Transplantation, 2016. **16**(5): p. 1548-1558.
333. Murphy, K. and C. Weaver, *Janeway's Immunobiology, 9th Edition.* 2017. **Part 2**(Chapter 10): p. 419
334. Mond, J.J., A. Lees, and C.M. Snapper, *T cell-independent antigens type 2.* Annual Review Immunology, 1995. **13**: p. 655-92.
335. Mond, J.J., et al., *T-Independent Responses in B Cell-Defective Cba-N Mice to Brucella-Abortus and to Trinitrophenyl (Tnp) Conjugates of Brucella-Abortus.* European Journal of Immunology, 1978. **8**(7): p. 459-463.
336. Amsbaugh, D.F., et al., *Genetic-Control of Antibody-Response to Type Iii Pneumococcal Polysaccharide in Mice .1. Evidence That an X-Linked Gene Plays a Decisive Role in Determining Responsiveness.* Journal of Experimental Medicine, 1972. **136**(4): p. 931-949.
337. Tanwar, S., et al., *Mediation of transitional B cell maturation in the absence of functional Bruton's tyrosine kinase.* Scientific Reports, 2017. **7**.
338. Motyka, B., et al., *Antibody-Mediated Rejection in a Blood Group A-Transgenic Mouse Model of ABO-Incompatible Heart Transplantation.* Transplantation, 2016. **100**(6): p. 1228-37.
339. Larkin, J.M.G. and C.D. Porter, *Mice are unsuitable for modelling ABO discordance despite strain-specific A cross-reactive natural IgM.* British Journal of Haematology, 2005. **130**(2): p. 310-317.
340. Tanemura, M., et al., *Differential immune responses to alpha-gal epitopes on xenografts and allografts: implications for accommodation in xenotransplantation.* Journal of Clinical Investigation, 2000. **105**(3): p. 301-310.
341. McLachlan, S.M., et al., *Breaking Tolerance in Transgenic Mice Expressing the Human TSH Receptor A-Subunit: Thyroiditis, Epitope Spreading and Adjuvant as a 'Double Edged Sword'.* Plos One, 2012. **7**(9).
342. Pichurin, P.N., et al., *Targeted expression of the human thyrotropin receptor A-subunit to the mouse thyroid: Insight into overcoming the lack of response to A-subunit adenovirus immunization.* Journal of Immunology, 2006. **176**(1): p. 668-676.
343. Terplan, K.L., et al., *Experimental Thyroiditis in Rabbits, Guinea Pigs and Dogs, Following Immunization with Thyroid Extracts of Their Own and of Heterologous Species.* American Journal of Pathology, 1960. **36**(2): p. 213-239.
344. Tanemura, M., et al., *Differential immune responses to alpha-gal epitopes on xenografts and allografts: implications for accommodation in xenotransplantation.* Journal of Clinical Investigation, 2000. **105**(3): p. 301-10.
345. Moreland, L.W., et al., *Use of a Chimeric Monoclonal Anti-Cd4 Antibody in Patients with Refractory Rheumatoid-Arthritis.* Arthritis and Rheumatism, 1993. **36**(3): p. 307-318.

346. Avci, F.Y. and D.L. Kasper, *How Bacterial Carbohydrates Influence the Adaptive Immune System.* Annual Review of Immunology, Vol 28, 2010. **28**: p. 107-130.
347. Avci, F.Y., et al., *Carbohydrates and T cells: A sweet twosome.* Seminars in Immunology, 2013. **25**(2): p. 146-151.
348. Beuvery, E.C., F. Vanrossum, and J. Nagel, *Comparison of the Induction of Immunoglobulin-M and Immunoglobulin-G Antibodies in Mice with Purified Pneumococcal Type-3 and Meningococcal Group-C Polysaccharides and Their Protein Conjugates.* Infection and Immunity, 1982. **37**(1): p. 15-22.
349. Weintraub, A., *Immunology of bacterial polysaccharide antigens.* Carbohydrate Research, 2003. **338**(23): p. 2539-2547.
350. Landsteiner, K. and J. van der Scheer, *On the Specificity of Serological Reactions with Simple Chemical Compounds (Inhibition Reactions).* Journal of Experimental Medicine, 1931. **54**(3): p. 295-305.
351. Eckhardt, A.E. and I.J. Goldstein, *Isolation and characterization of a family of alpha-D-galactosyl-containing glycopeptides from Ehrlich ascites tumor cells.* Biochemistry, 1983. **22**(23): p. 5290-7.
352. Avery, O.T. and W.F. Goebel, *Chemo-Immunological Studies on Conjugated Carbohydrate-Proteins : V. The Immunological Specifity of an Antigen Prepared by Combining the Capsular Polysaccharide of Type Iii Pneumococcus with Foreign Protein.* Journal of Experimental Medicine, 1931. **54**(3): p. 437-47.
353. Micoli, F., R. Adamo, and P. Costantino, *Protein Carriers for Glycoconjugate Vaccines: History, Selection Criteria, Characterization and New Trends.* Molecules, 2018. **23**(6).
354. Motyka, B., et al., *Blood Group A-Antigen Specific Tolerance Following Exposure to A-Antigen in Infant Mice.* Journal of Heart and Lung Transplantation, 2017. **36**(4): p. S49-S50.
355. Snapper, C.M., et al., *An in-Vitro Model for T-Cell-Independent Induction of Humoral Immunity - a Requirement for Nk Cells.* Journal of Immunology, 1994. **152**(10): p. 4884-4892.
356. Schneerson, R., et al., *Preparation, Characterization, and Immunogenicity of Hemophilus-Influenzae Type-B Polysaccharide-Protein Conjugates.* Journal of Experimental Medicine, 1980. **152**(2): p. 361-376.
357. Wessels, M.R., et al., *Stimulation of Protective Antibodies against Type-Ia and Type-Ib Group-B Streptococci by a Type-Ia Polysaccharide-Tetanus Toxoid Conjugate Vaccine.* Infection and Immunity, 1993. **61**(11): p. 4760-4766.
358. Avci, F.Y., et al., *A mechanism for glycoconjugate vaccine activation of the adaptive immune system and its implications for vaccine design.* Nature Medicine, 2011. **17**(12): p. 1602-1609.
359. Avci, F.Y., et al., *Isolation of carbohydrate-specific CD4(+) T cell clones from mice after stimulation by two model glycoconjugate vaccines.* Nature Protocols, 2012. **7**(12): p. 2180-2192.
360. Mazda, T., et al., *Differences in ABO antibody levels among blood donors: a comparison between past and present Japanese, Laotian, and Thai populations.* Immunohematology, 2007. **23**(1): p. 38-41.
361. Conley, M.E., et al., *Hyper Igm Syndrome-Associated with Defective Cd40-Mediated B-Cell Activation.* Journal of Clinical Investigation, 1994. **94**(4): p. 1404-1409.
362. Parker, W., et al., *Specificity and function of ''natural'' antibodies in immunodeficient subjects: Clues to B cell lineage and development.* Journal of Clinical Immunology, 1997. **17**(4): p. 311-321.
363. Tyznik, A.J., J.C. Sun, and M.J. Bevan, *The CD8 population in CD4-deficient mice is heavily contaminated with MHC class II-restricted T cells.* Journal of Experimental Medicine, 2004. **199**(4): p. 559-565.
364. Grusby, M.J., et al., *Depletion of Cd4+ T-Cells in Major Histocompatibility Complex Class-Ii Deficient Mice.* Science, 1991. **253**(5026): p. 1417-1420.

365. Nonaka, S., et al., *Intestinal gamma delta T cells develop in mice lacking thymus, all lymph nodes, Peyer's patches, and isolated lymphoid follicles.* Journal of Immunology, 2005. **174**(4): p. 1906-1912.
366. Galili, U., et al., *The human natural anti-Gal IgG. III. The subtlety of immune tolerance in man as demonstrated by crossreactivity between natural anti-Gal and anti-B antibodies.* Journal of Experimental Medicine, 1987. **165**(3): p. 693-704.
367. Markle, J.G.M., et al., *Sex Differences in the Gut Microbiome Drive Hormone-Dependent Regulation of Autoimmunity.* Science, 2013. **339**(6123): p. 1084-1088.
368. Yurkovetskiy, L., et al., *Gender Bias in Autoimmunity Is Influenced by Microbiota.* Immunity, 2013. **39**(2): p. 400-412.
369. Kennedy, E.A., K.Y. King, and M.T. Baldridge, *Mouse Microbiota Models: Comparing Germ-Free Mice and Antibiotics Treatment as Tools for Modifying Gut Bacteria.* Frontiers in Physiology, 2018. **9**(Article 1534).
370. Nie, J., et al., *FOXP3(+) Treg cells and gender bias in autoimmune diseases.* Frontiers in Immunology, 2015. **6**(Article 493).
371. Dorken, B., et al., *Hd39 (B3), a B-Lineage-Restricted Antigen Whose Cell-Surface Expression Is Limited to Resting and Activated Human Lymphocytes-B.* Journal of Immunology, 1986. **136**(12): p. 4470-4479.
372. Boue, D.R. and T.W. Lebien, *Structural Characterization of the Human Lymphocyte-B-Restricted Differentiation Antigen Cd22 - Comparison with Cd21 (Complement Receptor Type-2 Epstein-Barr Virus Receptor).* Journal of Immunology, 1988. **140**(1): p. 192-199.
373. Panoskaltsis, A. and N.R.S. Sinclair, *Rheumatoid-Factor Blocks Regulatory Fc Signals.* Cellular Immunology, 1989. **123**(1): p. 177-188.
374. Tovey, L.A., J.M. Taverner, and G.H. Longster, *The effect of environment on ABO antibodies.* Vox Sanguinis, 1970. **19**(1): p. 64-72.
375. Skarin, A. and J. Sylwan, *Vaginal Lactobacilli Inhibiting Growth of Gardnerella-Vaginalis, Mobiluncus and Other Bacterial Species Cultured from Vaginal Content of Women with Bacterial Vaginosis.* Acta Pathologica Microbiologica Et Immunologica Scandinavica Section B-Microbiology, 1986. **94**(6): p. 399-403.
376. Seid, R.C., et al., *Chemical Evidence for Covalent Linkages of a Semi-Synthetic Glycoconjugate Vaccine for Hemophilus-Influenzae Type-B Disease.* Glycoconjugate Journal, 1989. **6**(4): p. 489-498.
377. Pillot, A., et al., *Site-Specific Conjugation for Fully Controlled Glycoconjugate Vaccine Preparation.* Frontiers in Chemistry, 2019. **7**(Article 726).
378. Springer, G.F., *Influenza Virus Vaccine and Blood Group a-Like Substances.* Transfusion, 1963. **3**(3): p. 233-236.
379. Wen, *Germinal center formation, immunoglobulin class switching, and autoantibody production driven by non alpha/beta T cells (vol 183, pg 2271, 1996).* Journal of Experimental Medicine, 1996. **184**(2): p. 795-796.
380. Dianda, L., et al., *Germinal center formation in mice lacking alpha beta T cells.* European Journal of Immunology, 1996. **26**(7): p. 1603-1607.
381. Cowdery, J.S., *Chronic Invivo Depletion of Cd4 T-Cells Increases Both Serum Igm Levels and the Expressed Frequency of Anti-Ssdna Precursors in Both Nzb and Dba/2 Mice.* Clinical Immunology and Immunopathology, 1990. **56**(3): p. 360-372.
382. Leman, J.D., E. Claassen, and R.J. Noelle, *Functions of CD40 and Its Ligand, gp39 (CD40L).* Critical Reviews in Immunology, 2017. **37**(2-6): p. 371-420.

383. Mohiuddin, M.M., et al., *Tolerance induction to a mammalian blood group-like carbohydrate antigen by syngeneic lymphocytes expressing the antigen, II: tolerance induction on memory B cells.* Blood, 2003. **102**(1): p. 229-236.
384. Motyka, B., et al., *MHC-Matched A-Expressing Blood Cells Induce ABO Tolerance in Infant and Adult Mice.* Transplantation, 2018. **102**: p. S292-S292.
385. Motyka, B., et al., *Tolerance to Graft A-Antigens Following ABO-Incompatible Heart Transplantation (HTx) in a Blood Group A-Transgenic Mouse Model.* American Journal of Transplantation, 2015. **15**.
386. Ueda, Y., et al., *T-independent activation-induced cytidine deaminase expression, class-switch recombination, and antibody production by immature/transitional 1 B cells.* Journal of Immunology, 2007. **178**(6): p. 3593-3601.
387. Snapper, C.M., et al., *Induction of Igg3 Secretion by Interferon Gamma - a Model for T-Cell Independent Class Switching in Response to T-Cell Independent Type-2 Antigens.* Journal of Experimental Medicine, 1992. **175**(5): p. 1367-1371.
388. Rice, J.C. and R.P. Bucy, *Differences in the Degree of Depletion, Rate of Recovery, and the Preferential Elimination of Naive Cd4(+) T-Cells by Anti-Cd4 Monoclonal-Antibody (Gk1.5) in Young and Aged Mice.* Journal of Immunology, 1995. **154**(12): p. 6644-6654.
389. Avery, O.T. and W.F. Goebel, *Chemo-Immunological Studies on Conjugated Carbohydrate-Proteins : V. The Immunological Specifity of an Antigen Prepared by Combining the Capsular Polysaccharide of Type Iii Pneumococcus with Foreign Protein.* Journal of Experimental Medicine, 1931. **54**(3): p. 437-47.
390. Astronomo, R.D. and D.R. Burton, *Carbohydrate vaccines: developing sweet solutions to sticky situations?* Nature Reviews Drug Discovery, 2010. **9**(4): p. 308-324.
391. Guttormsen, H.K., et al., *Cognate stimulatory B-cell-T-cell interactions are critical for T-cell help recruited by glycoconjugate vaccines.* Infection and Immunity, 1999. **67**(12): p. 6375-6384.
392. Sun, X.M., et al., *Polysaccharide structure dictates mechanism of adaptive immune response to glycoconjugate vaccines.* Proceedings of the National Academy of Sciences of the United States of America, 2019. **116**(1): p. 193-198.
393. Crotty, S., *A brief history of T cell help to B cells.* Nature Reviews Immunology, 2015. **15**(3): p. 185-189.
394. Smith-Bouvier, D.L., et al., *A role for sex chromosome complement in the female bias in autoimmune disease.* Journal of Experimental Medicine, 2008. **205**(5): p. 1099-1108.
395. Verthelyi, D.I. and S.A. Ahmed, *Estrogen increases the number of plasma cells and enhances their autoantibody production in nonautoimmune C57BL/6 mice.* Cellular Immunology, 1998. **189**(2): p. 125-134.
396. Medina, K.L., A. Strasser, and P.W. Kincade, *Estrogen influences the differentiation, proliferation, and survival of early B-lineage precursors.* Blood, 2000. **95**(6): p. 2059-2067.
397. Grimaldi, C.M., V. Jeganathan, and B. Diamond, *Hormonal regulation of B cell development: 17 beta-estradiol impairs negative selection of high-affinity DNA-reactive B cells at more than one developmental checkpoint.* Journal of Immunology, 2006. **176**(5): p. 2703-2710.
398. Cunningham, M.A., et al., *Estrogen receptor alpha modulates toll-like receptor signaling in murine lupus.* Clinical Immunology, 2012. **144**(1): p. 1-12.
399. Nalbandian, G., et al., *The selective estrogen receptor modulators, tamoxifen and raloxifene, impair dendritic cell differentiation and activation.* Journal of Immunology, 2005. **175**(4): p. 2666-2675.
400. Rettew, J.A., Y.M. Huet, and I. Marriott, *Estrogens Augment Cell Surface TLR4 Expression on Murine Macrophages and Regulate Sepsis Susceptibility in Vivo.* Endocrinology, 2009. **150**(8): p. 3877-3884.

401. Calippe, B., et al., *17 beta-Estradiol Promotes TLR4-Triggered Proinflammatory Mediator Production through Direct Estrogen Receptor alpha Signaling in Macrophages In Vivo.* Journal of Immunology, 2010. **185**(2): p. 1169-1176.
402. Grimaldi, C.M., et al., *Estrogen alters thresholds for B cell apoptosis and activation.* Journal of Clinical Investigation, 2002. **109**(12): p. 1625-1633.
403. Khodadadi, L., M. Adib, and A. Pourazar, *Lmmunoglobulin class (IgG, IgM) determination by dithiothreitol in sensitized kidney transplant candidates.* Transplantation Proceedings, 2006. **38**(9): p. 2813-2815.
404. Peeva, E., J. Venkatesh, and B. Diamond, *Tamoxifen blocks estrogen-induced B cell maturation but not survival.* Journal of Immunology, 2005. **175**(3): p. 1415-1423.
405. Sarvari, M., et al., *Ovariectomy and Subsequent Treatment with Estrogen Receptor Agonists Tune the Innate Immune System of the Hippocampus in Middle-Aged Female Rats.* Plos One, 2014. **9**(2).
406. Osorio, A.V., et al., *ABO-mismatched renal transplantation in children: a report of the North American Pediatric Renal Transplant Cooperative Study (NAPRTCS) and the Midwest Organ Bank (MOB).* Pediatric Transplantation, 1998. **2**(1): p. 26-9.
407. Yandza, T., et al., *Outcome of ABO-incompatible liver transplantation in children with no specific alloantibodies at the time of transplantation.* Transplantation, 1994. **58**(1): p. 46-50.