EXPRESSION AND FUNCTION OF TOLL-LIKE RECEPTORS IN LYMPHOCYTES FROM HUMAN NEONATES

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A thesis submitted for the degree of

Doctor of Philosophy

February 2011

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SUMMARY

Neonates have high global rates of morbidity and mortality due to infectious diseases; this susceptibility is attributed to the immaturity of the neonatal immune response. The immune system of neonates, compared to adults, has reduced function in several aspects of immunity and lacks the long-term memory response.

Toll-like receptors (TLR) are a family of pattern recognition receptors which bind various microbial components and alert the immune system to invading pathogens. Comparing TLR expression and function in neonatal lymphocytes and adult lymphocytes may reveal how TLR influence these immune cells in early life. Due to the immaturity of their immune responses, neonates may be more reliant on TLR for protection against infection.

The extracellular and intracellular expression of TLR1, TLR2, TLR3, TLR4, TLR6, TLR8 and TLR9 was examined on non-stimulated and stimulated T lymphocytes and B lymphocytes from cord blood, adult peripheral blood and tonsils from human subjects. B lymphocytes were categorised into subsets to see if differentiation stage of B lymphocytes affects TLR expression. The responses of purified B lymphocytes, from neonates, adults and tonsils, to ligands of TLR3, TLR4, TLR8 and TLR9 were compared. The functions measured were proliferation, levels of total Ig, IgG and IgM, and levels of IL-6 and IL-8. Tonsil B lymphocytes were tested for expression of activation markers (CD23, CD25, CD69 and HLA-DR), co-stimulatory molecules (CD40, CD80 and CD86), and CD21 and CD210.

The TLR expression patterns by T lymphocytes and B lymphocytes were similar between neonates and adults, and stimulation of lymphocytes had little effect on TLR expression. T lymphocytes from neonates and adults expressed TLR2, TLR3, TLR4, TLR8 and TLR9. B lymphocytes from neonates and adults expressed TLR1, TLR3, TLR4, TLR8 and TLR9. B lymphocytes from tonsils expressed TLR3, TLR4, TLR8 and TLR9. Cellular location of TLR was mostly consistent with the literature, except for detection of TLR4 in permeabilised cells and TLR8 on non-permeabilised cells. CpG ODN, TLR9 ligand, induced strong proliferation, secretion of total Ig, IgG, IgM and IL-6 from B lymphocytes from neonates, adults and tonsils. Adult B lymphocytes produced higher levels of total Ig, IgM and IL-6 in response to the other TLR ligands compared to neonatal and tonsil B lymphocytes. IL-8 levels were unaffected by TLR ligands in neonates, adults and tonsils.

Neonatal lymphocytes have adult-like capacity to "innately" recognise foreign pathogens. Neonatal B lymphocytes have reduced responses to TLR ligands compared to adult B lymphocytes. However, neonatal B lymphocytes respond to TLR ligands, especially CpG ODN, with increased functions. TLR agonists, particularly CpG ODN, are potentially strong candidates for future research in neonatal vaccinology.

DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Pallave Dasari

February 2011

ACKNOWLEDGEMENTS

I wish to thank Flinders University of South Australia for awarding the Flinders University Research Scholarship for my PhD and I am grateful to the Faculty of Health Sciences and Flinders University Library for all their support.

I would like to thank my supervisors, Professor Heddy Zola and Dr Ian Nicholson, for their invaluable advice and support throughout this project, from its inception through to its progress and completion. I am deeply grateful for their encouragement and support for the various endeavours I was fortunate to experience during my PhD. Their guidance and patience over the years have allowed me to immeasurably develop my abilities as a research scientist from the novice that I was. I wish to thank Heddy Zola especially for all his time, effort and patience during the trials associated with this project.

Dr Doreen Krumbiegel, Dr Randall Grose, Christos Mavrangelos, Debbie Millard, Daniel Bird, Dr Alice Beare and Naomi Knoblauch of Leucocyte Biology, Women's and Children's Health Research Institute (WCHRI) deserve an enormous thanks for all the help they provided through my project. In particular, I would like to express my gratitude to Doreen Krumbiegel for the onerous duty she had of reading through the drafts of my thesis.

I also wish to thank all the past and present staff and students at WCHRI, especially the PhD students, for their assistance and support throughout my studies. I am particularly grateful to the past and present PhD students for their empathy, support

and friendship during the difficult phases of my studies. The presence of everyone at WCHRI made it an enjoyable place to be.

I am grateful to Dr Greg Hodge, Dr Judi Nairn and Lyn Morelli from Dept of Haematology, Women's and Children's Hospital (WCH), for their advice on flow cytometry and ELISA. I also wish to thank Dr Peter MacArdle of Flinders University for advice on cell activation protocols, Dr Hilary Warren of Australian National University for advice on cell purification, Dr Ashley Mansell of Monash Institute of Medical Research for advice on TLR ligands and staff at WCH Dept of Immunopathology for assistance with the proliferation assays. I am thankful to Dr Sally Plush of University of South Australia for her advice and assistance with the spectrophotometric studies. I wish to thank Dr Richard Woodman of Flinders University for his guidance and assistance on biostatistics. I am deeply grateful to Andrew Chittleborough of Jomar Bioscience for all his assistance with tracking down the numerous antibodies and other reagents over the years. I wish to thank Prof Kevin Forsyth of Flinders University for his advice on my project.

A deep thank you is offered to the numerous volunteers and staff that have facilitated samples for this project. I am grateful to WCHRI staff and students for the numerous blood donations over the year and WCH Phlebotomy staff for their patience for the endless collections. I am very grateful to the staff of WCH Delivery Suite for collecting cord blood and the mothers that kindly participated in this study. I also wish to thank the parents and children who donated tonsils and to the staff of WCH Ear, Nose and Throat Department for tonsil collections. I thank the Australian Red Cross for providing buffy coats for this project.

Lastly, I want to thank my family and friends for all their support and encouragement. Thank you to my friends for encouraging me and being patient with me over the years. Of course, I owe an enormous debt of gratitude to my parents, Niranjan and Usha Dasari, and brother, Snehal Dasari. Unfortunately they were passengers on the emotional rollercoaster I was on during the years and I thank them for their patience, encouragement and help. I could not have persisted with and completed my studies without them.

PUBLICATIONS ARISING FROM THIS PROJECT

(See Appendix)

PAPER FROM PRELIMINARY STUDY PRECEDING PHD STUDY

Dasari P, Nicholson IC, Hodge G, Dandie GW, Zola H. Expression of Toll-like receptors on B lymphocytes. Cell Immunol 2005;236(1-2):140-45.

PAPERS ARISING FROM PHD STUDY

Dasari P, Zola H, Nicholson IC. Expression of Toll-like receptors by neonatal leukocytes. Pediatr Allergy Immunol 2010 (in press)

Dasari P, Nicholson IC, Zola H. Toll-like receptors. J Biol Reg Homeost Agents 2008;22(1):17-26.

ABBREVIATIONS

7AAD:	7-amino-actinomycin
AF647:	Alexa Fluor® 647
AP-1:	Activating protein-1
APC:	Antigen-presenting cells
B1 cells:	$CD5^+$ B lymphocytes
BCR:	B cell receptor
BSA:	Bovine Serum Albumin
CBA:	Cytometric Bead Array
CD:	Clusters of differentiation
CL075:	A thiazoloquinolone compound – 3M002
CLR:	C-type lectin receptors
CpG ODN:	Oligodeoxynucleotides with unmethylated CpG dinucleotides
CRP:	C-reactive protein
DAPI:	4',6-diamidino-2-phenylindole
DC:	Dendritic cell
DDA-PE:	PE-conjugated anti-mouse Ig $F(ab)_2$
EC:	Extracellular
ELISA:	Enzyme-linked immunosorbent assay
ERK1:	Extracellular regulated kinase 1
Fab:	antibody fragment of single antigen-binding region
$F(ab)_2$:	antibody fragment of both antigen-binding regions
FBS:	Foetal bovine serum
Fc:	crystallisable antibody fragment with no antigen-binding region

FITC:	Fluorescein isothiocyanate
FSC:	Forward scatter
GC:	Germinal centre
HLA-DR:	Human leucocyte antigen DR-1
HLDA:	Human leucocyte differentiation antigen
HRP:	Horseradish peroxidase
HαMBi:	Biotinylated horse anti-mouse IgG antibody
IC:	Intracellular
IF:	Immunofluorescence
IFN:	Interferon
Ig:	Immunoglobulin
IgA:	Immunoglobulin with α heavy chains
IgD:	Immunoglobulin with δ heavy chains
IgE:	Immunoglobulin with ε heavy chains
IgG:	Immunoglobulin with γ heavy chains
IgM:	Immunoglobulin with μ heavy chains
IHC:	Immunohistochemistry
IL:	Interleukin
IRF:	Interferon-regulatory factor
LPS:	Lipopolysaccharide
LRR:	Leucine-rich repeats
LTA:	Lipotechoic acid
mAb:	Monoclonal antibody
MBL:	Mannan-binding lectin
MFI:	Median fluorescence intensity
MHC:	Major histocompatibility complex

mRNA:	messenger ribonucleic acid
MyD88:	Myeloid differentiation primary-response protein 88
NAIP:	Neuronal apoptosis inhibitor proteins
NALP:	NACHT-LRR-PYD containing proteins
NF-κB:	Nuclear factor-kappaB
NK:	Natural killer
NLR:	Nod-like receptor, NACHT proteins or NAIP receptors
NOD:	Nucleotide-binding oligodimerisation domain
N-S:	Non-Stimulated
OPD:	o-phenylenediamine dihydrochloride
p38-MAPK:	p38 Mitogen-activated protein kinase
PAMP:	Pathogen-associated molecular patterns
PBS/Azide:	PBS/0.02% Sodium azide
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PE:	Phyco-erythrin
PerCP-Cy5.5:	Peridinin chlorophyll protein cyanine 5.5
PFA:	Paraformaldehyde
PolyI:C:	Polyinosinic-polycytidylic acid sodium salt
PRR:	Pathogen recognition receptors
RF10:	RF10 cell culture media
RNA:	Ribonucleic acid
RT-PCR:	Reverse transcriptase polymerase chain reaction
SA:	Streptavidin
SAP:	Serum amyloid protein
SSC:	Side scatter

T_C cell:	Cytotoxic T cell
TCR:	T cell receptor
T-D:	T cell-dependent response
T_H cell:	T helper cell
T_H1:	T helper cells type 1
T_H2:	T helper cells type 2
T-I:	T cell-independent response
T-I1:	T cell-independent type 1 response
T-I2:	T cell-independent type 2 response
TIR:	Toll/IL-1R
TIRAP:	TIR domain-containing adaptor protein
TLR:	Toll-like receptors
TNF:	Tumour necrosis factor
T_R cell:	Regulatory T cell
TRAM:	TRIF-related adaptor protein
TRIF:	TIR domain-containing adaptor protein inducing IFN-β
UV:	Ultraviolet
V_H region:	Variable region of antibody heavy chains
V_L region:	Variable region of antibody light chains
WCH:	Women's and Children's Hospital

CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

The immune system, composed of various cells, organs, immune receptors and chemicals, defends the host from morbidity and mortality due to infection. In humans, the immune system differs between adults and neonates. The neonatal immune system undergoes a gradual maturation which eventually develops the ability to mount an effective response against infection. Toll-like receptors (TLR) are a family of immune receptors which alert the host to the presence of micro-organisms. As discussed further in Section 1.3, TLR have numerous influences on the immune system, but this study is focussed on how TLR affect neonatal immune responses; particularly the role of TLR in neonatal lymphocytes. This review will detail the immune system with special focus on the neonatal immune system. Further information on T lymphocytes and, particularly, B lymphocytes will be presented. The role of TLR in the immune system and how they affect the neonatal immune response will be examined.

1.2 THE IMMUNE SYSTEM

1.2.1 Neonatal Susceptibility to Infectious Diseases

The World Health Report 2005 on Maternal and Infant Health states that approximately 4 million neonates die every year, predominantly in Africa and Asia, with neonatal mortality contributing 30% of all deaths in children under five (1). Over the past century modern health care has reduced global rates of neonatal

mortality, especially in developed nations; but approximately a third of neonatal deaths were due to infectious diseases (1). The susceptibility of neonates indicates there are weaknesses in their immune response, perhaps due to the immaturity of neonatal immunity, which fails to provide sufficient protection against infection.

1.2.2 Barrier System

The first protective mechanism for humans against infection is the physical barrier of skin and mucosal surfaces. It is a non-specific, mechanical, obstacle which is the first immunological defence to protect humans. In adults, skin and mucosal surfaces are coated with enzymes, anti-microbial peptides and commensal bacterial flora which resist pathogens (2). Post-partum, neonates are exposed to a non-sterile environment after growing in the sterile uterine surroundings. As reviewed (3), neonatal skin is coated with various anti-microbial peptides that have greater activity in neonates than adults which can aid in the transition to the non-sterile environment.

1.2.3 Innate Immune System

The second immunological barrier is the innate immune system; a germ-line encoded, non-clonal, defence system capable of immediate reaction. The innate immune system consists of several processes and components, including inflammation and complement, which cooperate for maximal immune responses against infection.

1.2.3.1 Inflammatory Response

Inflammation is an early response which counters, and often eliminates, infection via a cascade of non-specific events involving soluble and cellular components. In

addition to the innate immune responses, inflammation also plays a role in cellular and humoral functions of adaptive immunity. Infected cells initiate inflammation by secreting cytokines and other mediators which alert the immune system and commence the cascade. The cascade of events escalates inflammation via a varied cellular response and a humoral response comprised of soluble proteins. An influx of neutrophils and macrophages migrate to the site of infection, secrete inflammatory mediators and anti-microbial compounds, phagocytose microbes, and clear dead or dying host cells. The humoral response, composed of cytokines, acute-phase proteins, antibodies, and other soluble components eliminates microbes via opsonisation or instigation of leucocytes to engage with pathogens. Extravasation, the movement of phagocytes and leakage of various humoral components from capillaries to infected cells, is part of the inflammatory response. Inflammation also primes the adaptive immune response to the infectious agent through lymphocytes and antigen-presenting cells (APC). Inflammation is an effective process for early elimination of infection and priming of adaptive immune responses.

The inflammatory response of neonates is weak, compared to adults, which makes them susceptible to infection. *In utero*, the maternal and foetal levels of pro-inflammatory cytokines, interleukin (IL)-1β, interferon (IFN)-γ and tumour necrosis factor (TNF), are reduced; elevated levels of pro-inflammatory cytokines are associated with premature labour and foetal loss (3-4). This anti-inflammatory bias in the foetus continues in the neonate leading to the lack of an effective inflammatory response to infection.

As reviewed, neonates are biased to secrete anti-inflammatory and helper T (T_H) type 2 cell-mediated cytokines (3-4). Despite one report of neonatal leucocytes producing pro-inflammatory cytokines in response to certain bacterial species (5), several studies show neonatal leucocytes have an anti-inflammatory bias with poor production of pro-inflammatory cytokines (3, 6). When neonatal leucocytes are treated with bacterial products there is little production of pro-inflammatory cytokines (6), but one study reports neonatal leucocyte production of pro-inflammatory cytokines in response to bacteria (5). Plasma levels of acute-phase proteins, anti-microbial proteins produced by the liver, are significantly lower in neonates than adults (3). Neonatal neutrophils, despite transient neutrophilia at birth, and macrophages have impaired functions including migratory ability, phagocytic ability and anti-microbial cellular mechanisms (3-4, 6-7). The inflammatory response, an early part of the immune response, is ineffective in neonates because of the immaturity of the humoral and cellular components, leaving neonates less able to eliminate infection.

1.2.3.2 Complement

Complement is a group of immune proteins which, in tandem with inflammation, eliminates infection. Complement has several anti-microbial functions including direct lysis of bacteria, fungi and infected cells, opsonisation of microbes for phagocytosis by leucocytes, activating inflammation via granulocytes, clearance of immune complexes, leucocyte activation and chemotaxis. It is a collection of over 40 proteins, mostly produced in the liver, found either in plasma or bound to cells (7). Depending on the initial event, complement can be triggered through three different pathways; classical, alternative or lectin pathways. The classical pathway commences from an antibody-antigen complex, the alternative pathway from

complement protein C3b binding directly to microbes and the lectin pathway after mannose-binding ligand binds to mannose on microbes. The detection of microbes initiates one of the three cascades of complement proteins which results in the formation of membrane-attack complex to lyse cells. Aside from the complement cascade, individual complement components induce cytokine secretion and engage with lymphocytes.

Neonates have lower levels of complement than adults, which gradually increase to adult levels between 6 – 18 months of age (7). Though complement levels are low in neonates, premature neonates have high levels of complement protein activity (7). The expression of complement regulation receptors by neonatal leucocytes is variable; there are low levels of CD21 (8), but high levels of CD35 and CD55 (9). Despite the presence of complement in neonates, the low levels may not offer sufficient protection.

1.2.4 Adaptive Immune System

The last developed, in evolutionary terms, defence of vertebrates against pathogens is the adaptive immune system; a clonal, acquired immune response system which provides a delayed, but specific, reaction with long-term memory. In the adaptive immune system, the diversity of antigen recognition is due to gene rearrangement of lymphocyte receptors. Lymphocyte clones which recognise self-antigens are eliminated by negative selection, and lymphocytes which recognise foreign antigens are positively selected for activation. The adaptive immune system liaises with the innate immune system to counter infection. The adaptive immune response is particular to an individual and their exposure to infection; it cannot be inherited by the descendents of the individual.

1.2.4.1 Cellular Response

Cell-mediated immunity in the adaptive immune system involves T lymphocytes and dendritic cells (DC) which produces an antigen-specific response against infections. T lymphocytes and DC mediate the immune response either by direct engagement or indirectly through secreted cytokines.

DC are professional APC which can activate T lymphocytes. As sentinel cells located in surface epithelia, when DC detect and phagocytose pathogens they mature and migrate to the nearest lymph node. In the lymph node, the DC presents peptides of the processed pathogen to high numbers of T lymphocytes until recognition by an antigen-specific T lymphocyte. The DC and T lymphocyte trigger secondary co-stimulatory signals, CD80 or CD86 on DC engages CD28 on T lymphocytes, and cytokines secreted by the DC promote T lymphocyte activation and proliferation, and polarise T lymphocytes into subsets which enhance inflammatory or antibody responses.

1.2.4.1.1 T lymphocyte Subsets and Function

T lymphocytes are $CD3^+$ and categorised further into several subsets on the basis of cell surface markers and functional attributes. T cell subsets include helper T (T_H) cells, cytotoxic T (T_C) cells, natural killer T (NKT) cells and γδ T cells.

Each T lymphocyte expresses a T cell receptor (TCR) which is antigen-specific, but for recognition they require an APC to present peptides of the processed antigen on major histocompatibility complex (MHC) class I or class II receptors. Depending on the pathogen, DC activate and direct T lymphocytes to generate specific

responses from T lymphocytes subsets. DC activate T_H cells via MHC class II receptors and polarise them to T_H type 1 (T_H1) or T_H type 2 (T_H2) responses on the basis of pathogen recognition and the cytokine pattern secreted. T_H1 cells secrete mostly pro-inflammatory cytokines which influence surrounding leucocytes and other cells, while T_H2 cells induce stronger antibody responses. T_C cells require activation via MHC class I, and then destroy infected cells through the release of perforins, granzymes and cytokines. A smaller subset of T lymphocytes, the regulatory T (T_R) cells, controls the immune response and regulates the immune balance. The cell-mediated branch of the adaptive immune system is necessary for the regulated, specific, response to infection.

1.2.4.1.1.1 T helper cells

T_H cells are $CD4^+$, interact with MHC class II, and mediate the cellular and antibody branches of adaptive immunity. The cellular, humoral and immunoregulatory effects of T_H cells occur through its numerous subsets, further information is reviewed by Zhu (10). Its importance for immunity can be observed in AIDS patients who succumb to numerous, previously harmless, microbes; HIV replicates in and subsequently destroys $CD4^+$ T cells. T_H cells activated by cell-to-cell interactions with APC require two signals; the first is antigen-specific TCR engagement by peptide presentation by MHC class II, the second is co-stimulatory signals with CD28 on T_H cells engaged by CD80 or CD86 on the APC, with secretion of IL-2 and IL-4 to support T_H cell proliferation and activation. T_H cells can also be polyclonally activated through the influences of cytokines or microbial antigens, though this will not produce an antigen-specific, adaptive response.

T_H cells can be divided into several subsets; the main subsets include T_H1, T_H2, T_{REG}, T_H17 cells and follicular T_H (T_HF) cells. The different T_H subsets produce different patterns of cytokines and influence different aspects of immunity. DC direct T_H cell development to T_H1 or T_H2 cells depending on the microbial stimulation of DC. T_H1 cells direct responses against intracellular, mycobacterial and viral, infections and have anti-viral effects by induction of IFN-γ. The cytokines produced by T_H1 cells enhance inflammatory responses and support cytotoxic and memory activity of T_C cells. T_H2 cells skew responses to anti-parasitic effects, influence the humoral response and often induce asthma and allergic diseases. T_H2-derived IL-10 suppresses induction of T_H1 polarisation and activity by DC. Several cytokines, in particular IL-4, secreted by T_H2 cells, affect eosinophils and mast cells, and IL-4 induces B lymphocytes to class-switch from IgG to IgE. The function of T_H17 cells isn't completely elucidated, but they are effective against extracellular bacterial infections, secretes IL-17 and inflammatory cytokines, and are involved in autoimmune conditions.

T_{REG} cells, $CD4^+CD25^+FoxP3^+$, as regulatory cells (11) which balance the immune system, preventing excessive responses. Some T_{REG} cells act through cytokines TGF-β, IL-10 and IL-35 and there is evidence that direct cell-to-cell interactions control the responses of other leucocytes.

T_HF cells, located mainly in the GC of secondary lymphoid organs, are non-T_H1/T_H2 polarised cells and mediate the antigen-specific activation of B lymphocytes (12). A naïve T_H cell in the GC follicles is antigenically primed by follicular DC and then migrates to the periphery of the B lymphocyte zone where it encounters antigen-primed B lymphocytes. The T_HF cell engages the antigen-

specific B lymphocyte via TCR and three separate co-stimulatory signals which help B lymphocytes develop an antigen-specific, adaptive response with affinity-matured antibodies

1.2.4.1.1.2 Cytotoxic T cells

T_C cells (13), $CD8^+$, are important for countering intracellular infections and controlling tumour cells. They recognise target cells either by engagement of their TCR to antigen presented by MHC class I on target cells or through presentation of lipid antigens by CD1. Upon target recognition T_C cells produce cytokines and eliminate cells by secretion of various proteins including perforins, granzymes and granulysins.

1.2.4.1.1.3 Natural killer T cells

NKT cells (14), $CD161^+$, express CD3 and several natural killer lymphocyte markers and are mainly located in secondary lymphoid organs and the liver, very few are found in the circulation. The ligands of NKT cells are not yet established but they secrete IFN-γ and other T_H1 cytokines and effectively clear bacterial infections.

1.2.4.1.1.4 Gamma-Delta T cells

The γδ T cells (12) are found in peripheral blood and tissues and produce growth factors which may have a role in skin growth and repair. They differ from αβ T cells in the structure of their TCR which is made of γ and δ chains rather than α and β chains. Unlike other T lymphocytes, γδ T cells can recognise bacterial and parasitic antigens directly and do not require antigen processing and presentation. The γδ T cells secrete an antimicrobial peptide and, depending on the stimulus, T_H2

cytokines. Upon encountering infected cells or tumour cells, γδ T cells display cytotoxic activity via the same mechanisms as T_C cells.

1.2.4.1.2 Neonatal Cellular Response

The cell-mediated adaptive immune response allows neonates to develop their own specific response to pathogens they encounter. However, neonates lack the ability to develop an effective antigen-specific response with long-term memory, as seen in the poor responses to vaccination during early infancy (15). This inability to mount an effective cell-mediated response may be attributed to the immaturity of the cells as elaborated in the following paragraphs.

Compared to adults, neonates have lower numbers of DC in blood with reduced expression of MHC class II (16-18), thereby reducing neonatal DC antigen-presenting capacity. Lower expression of co-stimulatory molecules, CD40, CD80 and CD86, was reported (16, 19) but other studies show comparable expression (17-18). Neonatal DC secrete lower levels of IFN-γ (18) and IL-12p70 (19), which reduces the ability of DC to influence other leucocytes, leading to a reduced T_H1 response.

Though neonatal T lymphocytes recognise a wide range of antigen peptides via their TCR (20), their response is controlled by the cytokines in their microenvironment (20) which polarise neonatal T lymphocytes to the T_H2 bias. The immaturity of cell signalling between DC and T lymphocytes leads to poor activation of T lymphocytes which reduces their influence on other leucocytes. Neonatal T lymphocytes secrete low levels of IFN-γ (21) and moderate levels of IL-4 (22) compared to adult T lymphocytes. Neonatal T lymphocyte expression of

CD154, the ligand for CD40, is uncertain with conflicting reports about higher or lower expression than adult T lymphocytes as reviewed by Marchant (21). The T_H2 polarisation of neonatal T lymphocytes doesn't induce IgG class switching of B lymphocytes, an inflammatory response, or cellular immune function. Neonatal T_C cells also have poor function with low expression of granzyme B and poor secretion of IFN-γ and TNF-α (23), therefore affecting their cytotoxicity.

In summary, the cell-mediated adaptive immune response in neonates is poor due to their immaturity and reduced function of T lymphocytes and DC. Neonatal DC fail to effectively activate and tailor T lymphocytes, partially due to lack of pro-inflammatory cytokines in neonates. In turn, neonatal T lymphocytes function poorly, and consequently they do not support several immune functions leading to an overall reduction in immunity, both innate and adaptive.

1.2.4.2 Antibody Response

Antibodies, also known as immunoglobulins (Ig), are plasma proteins which form part of the humoral immune response. An antibody can be either anchored to B lymphocytes as part of their cell membrane receptor (BCR) or secreted in plasma.

An antibody is composed of four polypeptide chains, a pair of heavy chains and a pair of light chains, linked together with disulphide bonds (Figure 1.1). The $F(ab)_2$ region binds to antigen; a single antibody can bind to two identical antigens. The Fc region, the effector region, engages immune cells by binding to Fc receptors for immune responses. The antigen-binding sites of the $F(ab)_2$ fragment consist of the variable regions of the heavy chain (V_H) and light chain (V_L); these are highly variable regions which provide the diversity and specificity of the antibody binding

to antigen. The variability of antibody binding is increased further by somatic hypermutation; the genes encoding the V_H and V_L regions undergo very high rates of mutation and increase the diversity of antibody binding.

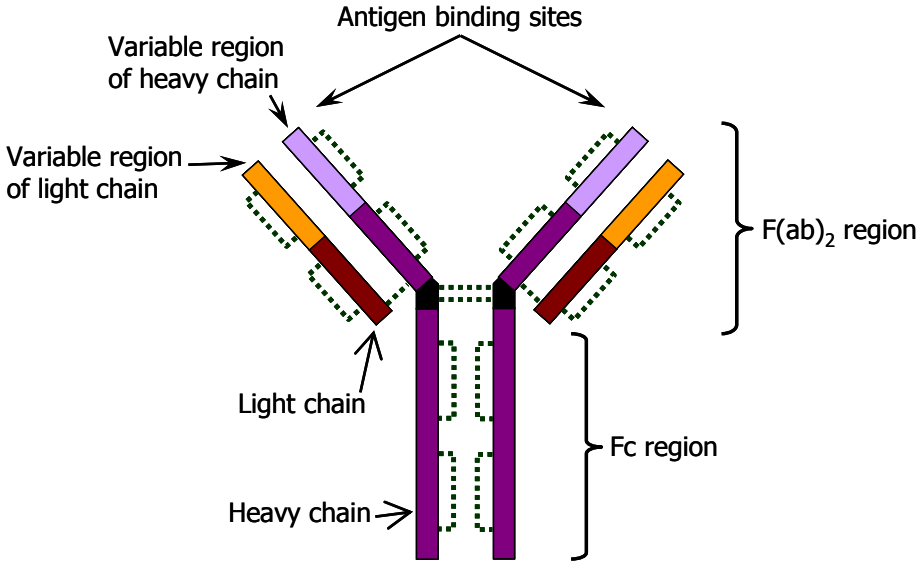
Antibodies are divided into five classes on the basis of the heavy chain, α, δ, ε, γ and μ chains; IgA (α), IgD (δ), IgE (ε), IgG (γ) and IgM (μ). Individual antibody classes have different roles; IgA is a secretory antibody in breast milk and mucosal lining of the gut and respiratory tract, IgD is mostly found on B cell surfaces, IgE is a plasma protein targeting parasitic infections, IgG is the main Ig in plasma and participates extensively in immunity, and IgM is the first antibody class in early life and often considered the "natural" antibody.

The stage of B cell maturation affects the antibody class secreted; naive B lymphocytes secrete IgM while memory B lymphocytes secrete IgG or IgA. Antibodies can eliminate infection directly through neutralisation of microbes or indirectly by binding to microbes for phagocytosis or to infected cells for elimination via antibody-dependent cell-mediated cytotoxicity via leucocytes such as neutrophils, macrophages or natural killer lymphocytes.

1.2.4.2.1 Subsets of B Lymphocytes

B lymphocytes are $CD19^+$ and can be classified into subsets on the basis of cell surface markers and functionality. B lymphocytes are found in peripheral blood and secondary lymphoid organs, like the spleen and lymph nodes. They react to three types of antigens; T cell-independent type 1 (T-I1), T cell-independent type 2 (T-I2) and T cell-dependent (T-D) antigens. T-I1 antigens are microbial components and are polyclonal activators of B lymphocytes. T-I2 antigens are

A. Antibody Structure



B. Antibody Classes

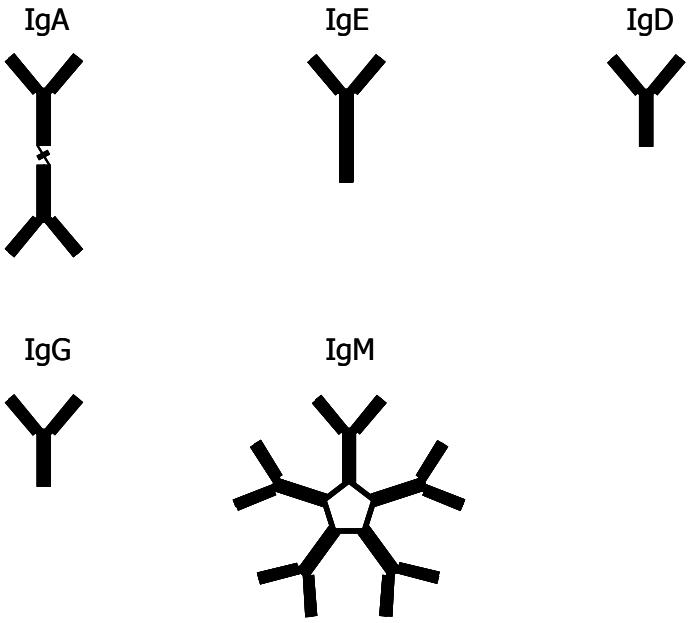


Figure 1.1 Antibody Structure and Classes
A. Schematic diagram of monomer antibody structure. Heavy chains in purple with its variable region in light purple. Light chains in red with its variable region in light red. Disulfide bonds in dark green dashed lines. Antigen binding, $F(ab)_2$ and Fc regions are marked. **B.** Schematic diagrams of the five antibody classes.

microbial proteins, like flagellin, with repeating antigenic domains which cross link several BCR on B lymphocytes. T-I1 and T-I2 antigens do not require T_H cells for B lymphocyte activation and they induce low-affinity antibodies from B lymphocytes. T-D antigens are soluble proteins which require T_H cell interactions with B lymphocytes for development of antigen-specific, affinity-matured, memory responses.

1.2.4.2.1.1 Peripheral Blood

The B cell subsets in peripheral blood are B1 and B2 B cells, and B2 B cells are differentiated into naïve, memory and plasma B cells according to antigenic stimulation. These subsets function in the innate and adaptive immune branches.

1.2.4.2.1.1.1 B1 B cells

B1 B cells (24-25) are $CD5^+$, the main source of IgM and a long-lived, self-renewing population of lymphocytes. They are larger and more granular than B2 B cells, located mainly in the circulation but also found in the gut and spleen. B1 B cells can form plasma B cells for antibody secretion. B1 B cells are an "innate"-like population of cells which is required for early antibody defences against infection through T-I2 antigens.

1.2.4.2.1.1.2 B2 B cells

B2 B cells (25) are $CD5^-$ and may be the equivalent of follicular B cells in secondary lymphoid organs. They constantly recirculate between peripheral blood and secondary lymphoid organs, react to T-D antigens and are responsible for the adaptive antibody response. The subsets of B2 B cells, naïve, memory and plasma B cells, reflect degrees of antigen exposure and maturation. Naïve B cells, $CD27^-$,

have not been exposed to specific antigen, express surface IgM and lack maturity. Memory B cells, $CD27^+$, are antigenically-exposed cells which are immune matured in secondary lymphoid organs, class-switched to high-affinity IgG isotypes, and can differentiate into plasma B cells. Plasma B cells are terminally differentiated, antibody-secreting, cells descended from memory B cells.

1.2.4.2.1.2 Secondary Lymphoid Tissue

The secondary lymphoid organs include lymph nodes and spleen which are the sites of B lymphocyte maturation to T-D antigens. Several B cell subsets, including naïve and memory B cells, are found in peripheral circulation and the follicles of the organs.

1.2.4.2.1.2.1 Germinal Centre Subsets

GC (26) are the site for a highly developed adaptive response for antibody development against antigen. In secondary lymphoid organs, such as lymph nodes, the cortex contains several follicles where GC form. The interfollicular zone in the cortex is rich with T lymphocytes. The follicle has a mantle zone, containing resting B lymphocytes, and in the centre of the follicle is the GC. The GC is divided into the dark zone, where activated B lymphocytes proliferate as centroblasts, and the light zone, where centroblasts migrate and differentiate to centrocytes and encounter follicular DC and T_HF cells for T-D antibody responses (Figure 1.2). The B cell subsets in the GC are naïve, pre-GC, GC and memory B cells, distinguished through their expression of IgD and CD38 (27), have different stages of maturation and antigen experience. Naïve B cells, IgD^+CD38^-, are antigenically-naïve but travel to the GC during recirculation. Pre-GC B cells, IgD^+CD38^+, are antigenically-exposed and enter the GC. GC B cells, IgD^-CD38^+,

with follicular DC and T_H cell assistance undergoes somatic hypermutation and selection for affinity maturation. The selected cells which can produce high-affinity antibody differentiate into memory B cells, IgD^-CD38^-, or plasma B cells.

1.2.4.2.1.2.2 Marginal Zone B cells

Marginal zone (MZ) B cells (25) are non-circulating, resident, cells in the marginal zone of the spleen which respond to T-I antigens. They are $CD5^-IgM^+$, secrete IgM and, like B1 B cells, play a more "innate" role in response to infection. The MZ B cells travel within the spleen from the marginal zone to the white pulp and encounter antigen-specific presentation on follicular DC which activates them.

1.2.4.2.2 B Lymphocyte Function

B lymphocytes are the source of antibodies; T-I1, T-I2 and T-D antigens induce different classes of antibodies. The antibody response, described in Section 1.2.4.2, engages several arms of the immune responses which via antibodies can neutralise microbes or their toxins, opsonise microbes and induce complement for lysis or phagocytosis, form multi-linked immune complexes with antigens for clearance by leucocytes, and eliminate antibody-labelled infected cells through antibody-dependent cellular cytoxicity. B lymphocytes also have cellular effects on the immune response through secretion of cytokines and cognate interactions with other cells to tailor immune responses.

1.2.4.2.2.1 Role in Antibody Responses

B lymphocytes are activated by microbes or their components which fall into three types of antigens, T-I1, T-I2 and T-D antigens. T-I1 antigens polyclonally activate most B lymphocytes, regardless of B cell subsets. T-I1 antigens are microbial

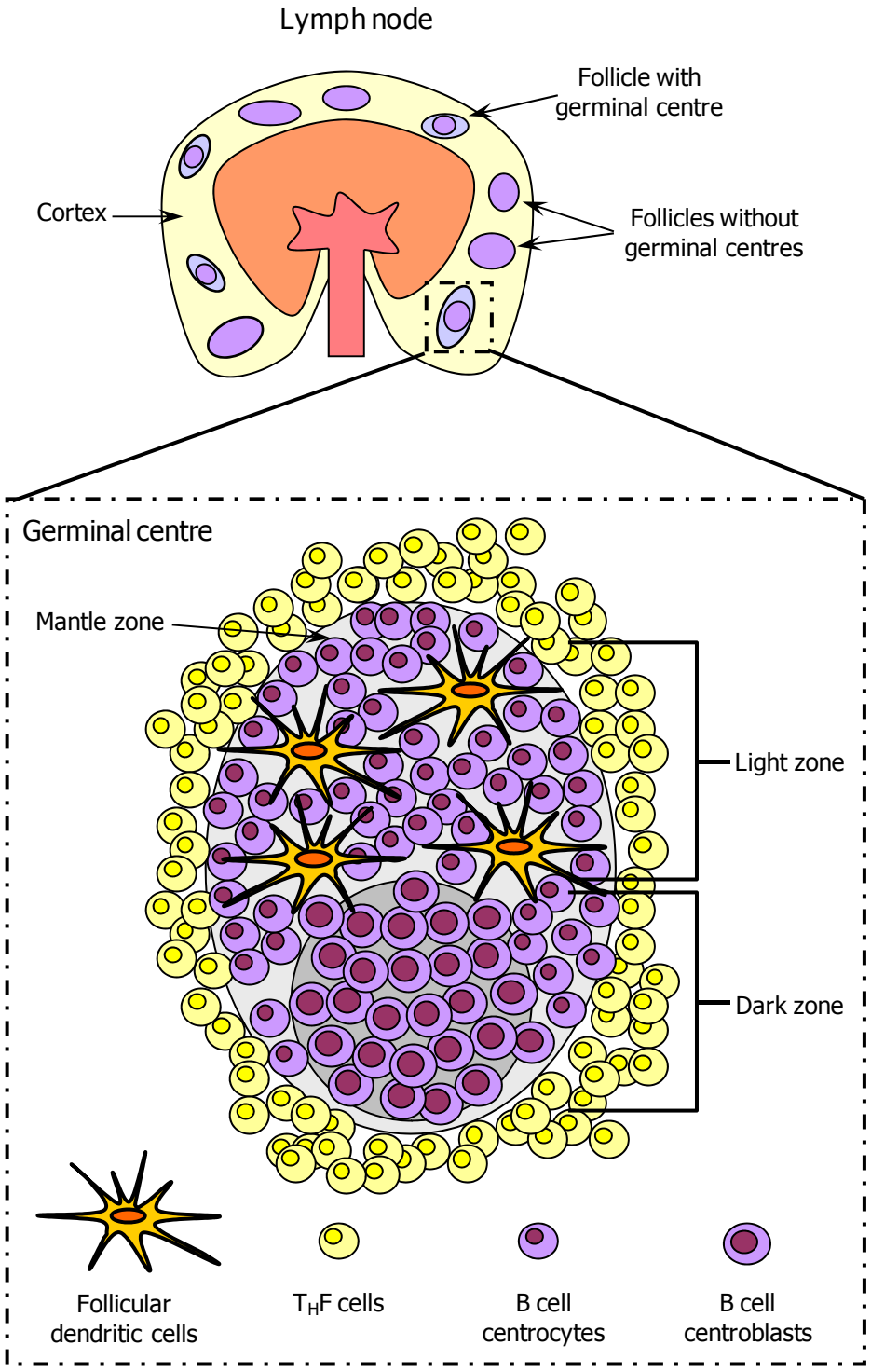


Figure 1.2 Germinal Centres in Lymph Nodes
Schematic diagram of a germinal centre from a lymph node. Cortex of lymph node contains follicles, with and without germinal centres. The germinal centre is the site for development of T-D antibody responses. It comprises follicular DC, T_HF cells and B cells at various stages of differentiation.

components, many are TLR ligands like LPS, which trigger B lymphocytes independently of the BCR. They instigate a non-specific, polyclonal, antibody response with no memory. As most B cell subsets are activated, the antibody response will include IgM from naïve B and B1 B cells, affinity-matured IgG isotypes from memory B cells and the various antibody classes from other B cell subsets.

T-I2 antigens have repeating antigenic domains which cross-link several BCR and activate B lymphocytes through strong BCR signalling. In response, B1 and MZ B cell subsets secrete IgM, with B1 B cells in blood secreting IgM and IgG3, and in the gut and peritoneal cavity secreting IgA. The low-affinity reactivity of B1 B cells has also been implicated in the development of autoimmune conditions like rheumatoid arthritis (24). MZ B cells, unlike B1 B cells, can provide limited long-term vaccine responses with T-I2 antigens, as seen in mouse models with *Streptococcus pneumoniae* (24).

T-D antigens activate B2 B cells and develop an adaptive, memory response (28). Initial activation of naïve B cells occurs when the antigen is processed, the antigen-primed B lymphocyte interacts with an antigen-specific primed T_H cells to induce an antibody class switch to IgG1. At this stage the B lymphocyte clonally expands and differentiates to memory and short-lived plasma B cells. The B lymphocytes, activated by specific antigen and T_H cell, travels to the GC where they clonally expand and the V_H and V_L genes hypermutate to produce antibodies of varying affinities to the antigen. Follicular DC in the GC present antigen to the GC B cells to select for high-affinity antibody B cells; non-selected cells undergo apoptosis. The surviving high-affinity antibody B cells receive selection signals from T_HF cells

and then differentiate into long-lived memory and plasma B cells (Figure 1.3). The T-D antigen response produces a high-affinity, class switched antibody response with memory, but earlier stages of B lymphocyte responses also produce antibody.

The antibody responses to various antigens provide a range of responses in terms of specificity and efficiency of antibodies. The T-I antibody responses produce polyclonal, low-affinity antibodies while the T-D responses have high-affinity, high-specificity antibodies with memory capacity.

1.2.4.2.2.2 Role in Cellular Responses

B lymphocytes are not the principal cytokine-secreting cells, but they do produce low titres of cytokines which affect the microenvironment (29). Depending on the antigen, the primed B2 B cells secrete either T_H1 cytokines, IL-12, IFN-α and TNF-α, or T_H2 cytokines, IL-2, IL-4, IL-6 and TNF-α. The antigens to influence B2 B cell cytokine secretions can include TLR ligands like LPS. The influence of the B cell derived T_H1 or T_H2 cytokine patterns would tailor or amplify the polarisation of T_H cells to T_H1 or T_H2. B lymphocytes can also secrete immunosuppressive cytokines; B1 and MZ B cells produce IL-10 which can block inflammation. Cytokines secreted by B lymphocytes can either direct or restrict immune responses.

Though DC are the professional APC of the immune system, B lymphocytes possess some ability to present antigen on MHC class II to antigen-specific T_H cells (30). There is some suggestion that B lymphocytes can process antigen, present it to antigen-specific T_H cells, and provide necessary co-stimulatory and cytokine signals to prime and tailor their responses to T_H1 or T_H2 (30). The MZ B cells also process bacteria and transport them to follicular T_H cells for presentation and priming.

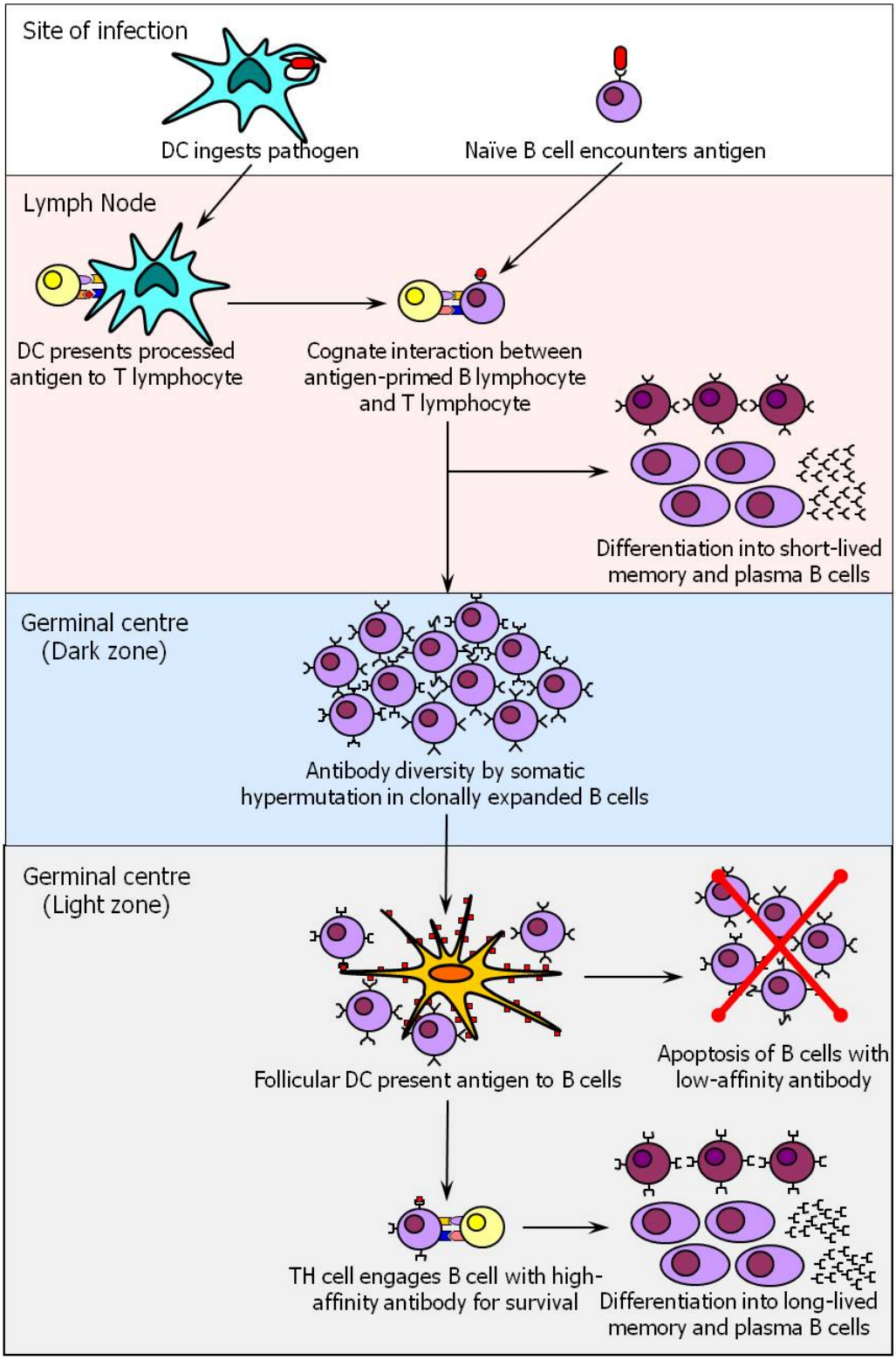


Figure 1.3 Development of T-D Antibody Response
The process in the germinal centre for developing and selecting affinity-matured antibodies against pathogens. Multiple interactions between DC, T lymphocytes and B lymphocytes produce a high-affinity, class switched antibody response.

These roles permit B lymphocytes to participate in immunity other than the antibody response.

1.2.4.2.3 Neonatal Antibody Response

Antibody titres in neonates are lower than in adults. IgM, the main antibody in neonates, is secreted by B1 B cells, the main neonatal B cell subset, and naïve B cells. B1 B cells are easily activated by T cell-independent (T-I) antigens, but they secrete low-affinity IgM in response and are unable to switch to IgG or produce memory cells.

As reviewed, neonatal B lymphocytes have limited antibody responses, in titres and duration, to T cell-dependent (T-D) (15) and T-I type 2 (T-I2) antigens (8). However neonatal B lymphocytes have elevated levels of surface IgM present (31) which can facilitate heightened sensitivity to very low levels of antigen (32). Complement plays an important role in the response of B lymphocytes to T-I type 2 antigens; complement protein C3d opsonises T-I type 2 antigens for enhanced immunogenicity (33). A factor for the poor response to T-I type 2 antigens by neonates is the low levels of complement protein C3 (34) and lower expression of C3d receptor, CD21, by neonatal B lymphocytes (31). This poor opsonisation of T-1 type 2 antigens and the low CD21 expression leads to reduced engagement of T-I type 2 antigens by neonatal B lymphocytes.

Human (35) and murine (36) neonates lack germinal centres (GC) in the spleen, a important site to facilitate lymphocyte and dendritic cell interactions to develop humoral and cell-mediated immunity. Moreover, neonatal B lymphocytes have reduced expression of co-stimulatory molecules, CD40, CD80 and CD86 (34),

needed for T-D antigen interactions with T_H cells and DC. The limited ability of the neonatal leucocytes reduces cognate interactions required for antibody class-switching to IgG, selection of high-affinity antibodies and development of memory B cells. Somatic hypermutation is decreased in neonates compared to adults (37) meaning that high-affinity antibodies are not produced.

Several factors in neonates combine to reduce the effectiveness of the antibody response; higher proportion of B1 B cells, impaired functions of adaptive immune cells, the lack of GC and reduced rates of somatic hypermutation. These weaknesses result in neonates being unable to mount a robust, mature antibody response, as seen in the vaccine regimens of infants which require repeated doses in the first year before they develop a long-term, adaptive response.

1.2.5 Special Features of the Neonatal Immune System

1.2.5.1 Passive Immunity of Neonates

The cells and organs of the neonatal immune system are developed, but not functioning to their full potential; therefore they are susceptible to infection. This weakness is partially compensated by the passive immunity neonates receive via placental transfer of maternal IgG and breast milk passing on the mature maternal immunity. *In utero*, the placental transfer of maternal IgG to the foetus increases during gestation from low levels in the first trimester to very high levels at term (38). At term the neonatal IgG levels exceed maternal IgG levels, and the neonate is protected by the maternal IgG for approximately two months (39). Despite the protective effects of maternal IgG, a disadvantage for neonatal immunity is the inhibition of the neonatal vaccine response by maternal IgG (40). For example, with neonatal polio vaccination maternal IgG binds to vaccine epitopes which

limits the binding of antigen-specific neonatal B lymphocytes (39-40). This inhibition by maternal IgG does not allow neonates to develop their own immune response to foreign antigens. However, the immune protectiveness of breast milk can be seen in the higher rates of morbidity and mortality due to infectious diseases experienced by non-breastfed infants versus breastfed infants (41-42). Exclusive breastfeeding is important for reducing neonatal mortality rates (43) as seen in the reduced infant mortality rates due to acute respiratory and diarrhoeal infections (44).

The protective immune effects of breast milk on the neonate occur primarily in the gastrointestinal mucosa as the milk is ingested. A complex mixture of proteins from the mother's developed immune system is transferred to the neonate. This transfer results in passive immunity for the neonate; the neonate does not adopt the maternal immune effects into a memory response of its own. The main antibody class in breast milk is IgA, a secretory antibody, which is approximately 80% of total Ig in colostrum and milk (41). After ingestion, IgA passes to the mucosal surfaces of the gastrointestinal tract where invasion of the mucosa is prevented by IgA binding to and neutralising microbes (41); as seen with antigen-specific, maternal IgA in breast milk neutralising rotavirus (45) and *Helicobacter pylori* (46). Although IgG is present in breast milk, it is at low levels and is less active than IgA in the infant compared to *in utero* when the foetus is protected by maternal IgG crossing the placental barrier (41).

Breast milk can have immunomodulatory functions on neonates via cytokines and enzymes. Pro-inflammatory cytokines, IL-1β, IL-2, IL-6, IL-8 and TNF-α, which are present in colostrum and breast milk (47-48) can assist in the development of

neonatal immunity and perhaps to overcome the anti-inflammatory bias. Lactoferrin, the second most abundant protein in breast milk, also found in neutrophils, has extensive antibacterial and antifungal activity (41, 49). Lactoferrin binds to and neutralises numerous viral, bacterial and parasitic enteric pathogens and reduces severity of gastrointestinal illnesses (49). Breast milk contains large numbers of carbohydrate components including various oligosaccharides, glycoproteins and glycolipids which all block microbes from binding to mucosal membranes (41). Individual oligosaccharides and glycoproteins from breast milk reduce infant vulnerability to respiratory and diarrhoeal infections (50). The immune effects of breast milk glycans are a significant component of the protectiveness of breast milk and the extent of their function is still being investigated. The numerous immune effects of breast milk have a highly protective effect for infants in early life and reduce their susceptibility to infectious diseases.

1.2.5.2 Neonatal Immune System Maturation

At birth, the immune system of neonates does not have adult-like functions. There is gradual maturation of the neonatal immune response, for which the principal trigger is microbial stimulation (51). This process commences at birth with ingestion of maternal vaginal flora during vaginal delivery leading to colonisation of the neonatal gastrointestinal tract with probiotic bacterial flora which reduces the inflammatory response in the gut (41). The production of pro-inflammatory cytokines by neonatal leucocytes in response to bacteria is conflicted in the literature, with some reporting adult-like levels of cytokines (5) and other reports stating low levels of cytokines (4, 6, 17). The low levels of acute-phase proteins in neonates at birth start to rise in the first week of life, triggered by microbial exposure (3). The activation of the acute-phase response after birth provides some immune

protection for neonates. Continual exposure to microbes, preferably probiotics, is necessary for the development of a consistent inflammatory response from neonatal cells to adult-like responses.

For the cell-mediated branch of the adaptive immune system, the T_H2 bias of neonates can be transformed gradually to T_H1 responses by vaccination (40), but very low antigenic doses are required for effective T_H1 and cytotoxic T lymphocyte activity (20). Low levels of IFN-γ in neonates increase gradually and reach adult levels by 5 years of age (40, 51). The neonatal bias for T_H2 cells may be attributed to its microenvironment and the deficiencies of neonatal APC (40) which may be altered. For neonatal DC, bacterial stimulus can instigate secretion of T_H1 cytokines, in particular IFN-γ and IL-12, and increase expression of CD80 and CD86 (8, 19, 40, 52). This allows a gradual development of the neonatal T_H1 immune response.

The lack of GC in the secondary lymphoid organs of neonates impairs cell-mediated and antibody responses of their adaptive immune system. Murine models reveal the importance of GC formation for adaptive immunity (53) and their development in neonates (36). As GC form in neonates, the B lymphocytes develop the ability to antibody class-switch and produce high affinity antibodies through somatic hypermutation (34). In the first 10 months there is an increase in somatic hypermutation of Ig genes in neonates and infants (54) indicating a gradual maturation in the antibody response in early life.

The process of maturation of the immune system takes several years before a child can launch adult-like responses by their innate and adaptive immunity. As

mentioned above, it appears that microbial stimulation is critical to trigger the maturation of the neonatal immune response. Lack of exposure to microbes leading to insufficient generation of immune responses in early life is the basis of the "Hygiene Hypothesis" which is elaborated in the review by Garn (55). Without exposure there is little inflammation and the T_H2 bias remains, leading to an increase in allergic diseases. Neonates require their immune systems to develop in response to the non-sterile extra-uterine environment.

1.2.5.3 Cord Blood

Cord blood is part of the neonate's continuous circulation until the moment the umbilical cord is cut. The cord blood which remains in the placenta and umbilical cord is frequently harvested for research which can include neonatal studies and haemopoietic stem cell research. Cord blood is used in neonatal immunology studies due to its high availability and large volumes which can be obtained. There may be differences in the leucocytes between the collected cord blood and the blood circulating in the neonate, but there is no practical way of assessing these potential differences. It would be unethical to collect large volumes of blood from neonates for comparative studies with cord blood.

Another consideration for using cord blood in neonatal immunology studies is the variability that exists in human subjects. Collecting cord blood at birth allows for a consistent point for comparing subjects.

1.3 TOLL-LIKE RECEPTORS

1.3.1 Pattern Recognition Receptors

Pattern recognition receptors (PRR) are a part of the innate immune system that recognises pathogen-associated molecular patterns (PAMP) associated with foreign organisms (56). PRR are germline encoded, include families of receptors or individual receptors, and can be located on the cell surface, internally or secreted extracellularly (56). The microbial components of PAMP are critical to the organisms' function, hence evolutionarily conserved with little modification, and allowing the innate immune system to discriminate between the host and microbes (57-58). Families of receptors include TLR and C-lectin receptors (CLR). Upon engagement of PRR, several immune responses are initiated including inflammation, opsonisation, phagocytosis of microbes, apoptosis of infected cells and induction of complement. PRR innately recognise pathogens and launch early immune responses against infection.

The recognition of PAMP by PRR permits self/non-self discrimination by the immune system followed by appropriate immune responses. The individual PRR has a broad specificity of low-affinity binding to a wide repertoire of microbial ligands. Individual PRR can increase their range of PAMP by forming heterodimers with TLR or other cell molecules. Upon recognition, PRR alert the immune system and initial responses are launched. Immune responses can be tailored further by the synergy of two or more PRR engaged by pathogens.

The most studied PRR are TLR, a family of transmembrane receptors. In 1985 the Toll receptor was first identified in Drosophila larvae with a role in embryological development (59) and, later, immune function (60), then its human homologue,

TLR, was identified (58). Although hundreds of TLR have been identified in the genome of sea urchins (61), in humans ten TLR, TLR1 to TLR10, have been identified (62-65) and potentially another two TLR.

CLR, also transmembrane PRR, are found on dendritic cells and macrophages, which endocytose and present glycosylated carbohydrate antigens to modulate immune responses. Dectin 1, one CLR, recognises a fungal component, β-glucan, and initiates an inflammatory response.

NLR (66) are a family of approximately 20 cytosolic proteins that can be categorised into 3 subfamilies depending on the domain at the N-terminus; the nucleotide-binding oligodimerisation domain (NOD) proteins, NACHT-LRR-PYD-containing (NALP) proteins and neuronal apoptosis inhibitor proteins (NAIP). The different domains instigate different signalling pathways for each NLR subfamily, but MyD88 is one of the signalling proteins of NLR. The particular PAMP recognised by NLR are unknown, but NOD1 and NOD2 proteins bind to peptidoglycan from Gram-negative bacteria. NLR, upon engagement, instigate antimicrobial effects including an inflammatory response. Other cytosolic PRR include the RNA helicase family and protein kinase PKR which also recognise viral nucleic acids and induce anti-viral inflammation and apoptosis of infected cells.

PRR can also be secreted by cells, these include acute-phase proteins secreted by the liver which include C-reactive protein (CRP), serum amyloid protein (SAP) and mannan-binding lectin (MBL). CRP, SAP and MBL all bind to and opsonise pathogens for clearance via complement and phagocytosis.

1.3.2 Structure of TLR

TLR are Type I integral membrane glycoproteins with molecular weights ranging 90-115kDa (67). The ten TLR found in humans are phylogenetically classified into five subfamilies on the basis of genomic sequencing: three sub-families have a single member, TLR3, TLR4 and TLR5; the TLR2 sub-family has four members, TLR1, TLR2, TLR6 and TLR10; and the TLR9 sub-family has three members, TLR7, TLR8 and TLR9 (68). The TLR proteins share a conserved cytoplasmic domain, known as Toll/IL-1R (TIR) domain, with the interleukin-1 receptor family (IL-1R) (58). The ectodomain of TLR and IL-1R differ in that TLR have several leucine-rich repeat (LRR) motifs, while IL-1R have three immunoglobulin-like domains (58). TLR can be located either on cell surfaces or on endosomal surfaces (67) (See Figure 1.4).

Depending on the TLR, the ectodomains of TLR contain 19-25 copies of LRR which is capped with a 31-amino acid long N-flanking region and a cysteine-rich domain on the C-terminal end (58, 67). An individual LRR is made of approximately 24 residues and forms a loop, with the first 10 residues forming a β-hairpin (67). The LRR motifs all form a coil with a large β-sheet on the concave surface formed by β-strands of each motif (67). Similar receptors bind to their ligands on the concave β-sheet of the LRR motifs with new binding sites created by inserting or deleting residues in the LRR motifs, suggesting that similar flexibility for binding to ligands may occur for TLR (67).

The TIR domain of TLR has a sequence conservation of approximately 20-30% with most of the conserved residues located in the hydrophobic core of the

structure, the core approximately 130-165 residues in length (69). The TIR domain has five β-strands, parallel, surrounded by five α-helices, with secondary structures all connected by loops (69). Three regions in the conserved sequence identified as boxes 1, 2 and 3 appear to have a role in signalling (70). Mutations in boxes 1 and 2 affected signalling and mutations in box 3 reduced cell surface expression, therefore these three regions all play essential roles in receptor localisation and signalling (70).

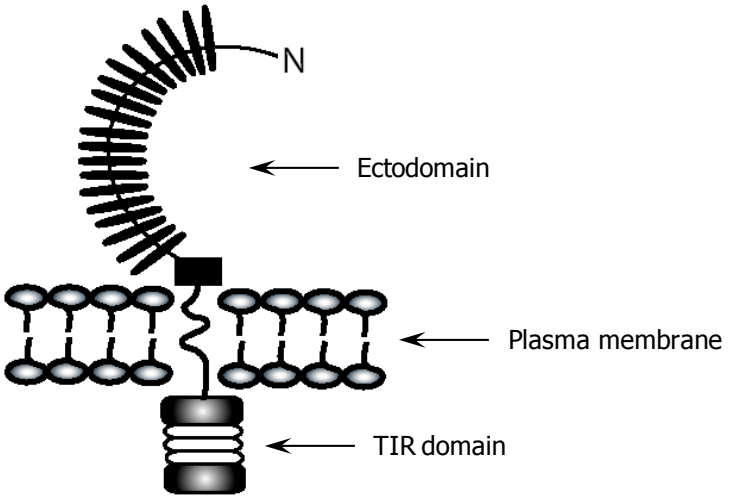


Figure 1.4 Schematic Structure of TLR1
TLR are transmembrane receptors which consists of an ectodomain and a cytoplasmic TIR domain. The specificity of each TLR is derived from the particular number of LRR motifs in the ectodomain.

1.3.3 Cell and Tissue Distribution

TLR are expressed by a wide range of cells and tissues, including endothelia and epithelia (71), as well as leucocytes (see Table 1.1). This section on TLR expression will focus exclusively on studies done on human cells to assess the current knowledge of TLR expression in the human immune system.

Early studies examining expression of TLR in cells focussed on detecting mRNA with PCR-based methods. Then with increasing availability of commercial antibodies against TLR, the protein expression was examined. As shown in Table 1.1, the mRNA and protein expression studies show almost the full range of TLR expressed by several leucocyte populations. However, discrepancies between mRNA and protein studies are present, such as TLR8 in neutrophils and TLR3 in monocytes. These discrepancies may be explained by lack of mRNA translation in protein, or studies not testing for TLR protein expression due to early unavailability of commercial TLR antibodies.

1.3.4 TLR Ligands

TLR identify pathogens through recognition of PAMP with self-recognition of some host molecular components such as heat shock proteins and fibrinogen. TLR1 - TLR10 recognise PAMP from the entire spectrum of microorganisms which includes bacteria, fungi, parasites and viruses. The structural flexibility of the TLR ectodomain permits an individual TLR to bind, albeit at low-affinity, to multiple ligands. As shown in Table 1.2, TLR2 and TLR4 have the largest numbers of ligands identified. The ligands of individual TLR appear to all come from a particular domain of microorganisms, for example the ligands of TLR2 and TLR4 are mostly bacterial while TLR3 recognise mainly viral ligands.

The cellular location of TLR correlates to its ligands with cell surface TLR detecting bacterial ligands and intracellular TLR detecting viral ligands. The TLR2 and TLR5 sub-families recognise bacterial ligands and expressed on the cell membrane,

Table 1.1 Expression of TLR on Human Immune Cells (References in brackets)

CELL TYPE	POLYMERASE CHAIN REACTION	FLOW CYTOMETRY	IMMUNOHISTO-CHEMISTRY
Peripheral Blood			
Neutrophils	1, 2, 3, 4, 5, 6, 7, 8, 9, 10 (72-73)	1, 2, 3, 4, 5, 6, 7, 9 (72, 74-77)	NR*
Monocytes	1, 2, 4, 5, 6, 7, 8, 9 (78-80)	1, 2, 3, 4, 6, 8, 9 (74-76, 78, 80-82)	9 (81)
Eosinophils	1, 4, 6, 7, 9, 10 (73, 75)	1, 2, 4, 6, 7, 9 (77, 83)	NR
Basophils	2, 4 (75)	2, 4 (75)	NR
T lymphocytes	1, 2, 3, 4, 5, 6, 8, 9 (79, 84-86)	2, 3, 4, 5, 6, 8, 9, 10 (84, 86-89)	2, 3, 4 (84, 86)
B lymphocytes	1, 2, 4, 6, 7, 9, 10 (79, 90-91)	1, 2, 3, 4, 8, 9 (81-82, 92-94)	NR
Natural killer lymphocytes	1, 2, 3, 4, 5, 6, 8, 9, 10 (79, 85, 95)	1, 2, 3, 4 (96-97)	NR
Tonsil T lymphocytes	1, 2, 3, 4, 5, 7, 9, 10 (98)	1, 3, 4, 9 (98)	NR
Tonsil B lymphocytes	1, 6, 7, 8, 9, 10 (91, 99)	1, 2, 7, 9, 10 (91, 100)	2, 3 (74, 101)
Macrophages	NR	4 (80, 102)	2, 4 (74, 102)
Dendritic cells	1, 2, 3, 4, 6, 7, 8, 9, 10 (78-79, 90, 103)	1, 2, 3, 4, 8, 9 (78, 82, 100)	NR

*NR – Not reported

and the TLR3 and TLR9 sub-family recognise viral ligands and expressed in intracellular endosomes. Studies report cell surface expression of TLR1, TLR2, TLR4, TLR6, TLR9 and TLR10 (72, 76, 90), and intracellular endosomal expression of TLR3 (86), TLR8 (82) and TLR9 (104). Cells also vary TLR expression according to their activation state, activated T lymphocytes upregulate surface expression of TLR2 and TLR4 (84). Activation of cells which alters TLR expression can occur by several mechanisms including *in vitro* activation (84), microbial infections (74, 81) and cytokines (74).

TLR can discriminate between similar ligands to produce differing immune responses; various immune cell responses are seen when different cell wall proteins engage TLR2 (105) or CpG ODN engages TLR9 (106). For example, two types of CpG ODN which engage TLR9 on plasmacytoid DC induce differing responses; CpG ODN A limits DC maturation and induces secretion of high levels of IFN-α, and CpG ODN B induces maturation and survival of DC with increased secretion of TNF-α and IL-8 (106). Table 1.2 lists some of the ligands for individual TLR, but TLR10 is an orphan receptor.

1.3.5 TLR Signalling

TLR signalling involves a highly complex network of proteins that form several signalling cascades resulting in distinct cellular responses. A family of adaptor proteins, each containing a TIR domain, are the first proteins to interact with an engaged TLR by dimerising the TIR domains of the TLR and adaptor protein. The adaptor proteins, myeloid differentiation primary-response protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP), TIR-domain-containing adaptor protein inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM), are

Table 1.2 Ligands of TLR

TLR	Ligands
1	Lipoprotein (*Mycobacterium sp.*) (107) LPS (108) Soluble factors (*Neisseria meningitidis*) (108)
2	Lipopeptides/lipoproteins (including Mycoplasma and *Mycobacterium tuberculosis*) (76, 84, 109) Glycolipids (76, 84) LPS (74, 108) Soluble factors (*Neisseria meningitidis*) (108) Zymosan (84) Peptidoglycan (76, 84) Porin (110) Bacterial fimbrae (110) Haemagglutinin protein (110) Cytomegalovirus virions (110) Human Heat shock protein (HSP) 60 and HSP70 (110-111)
3	dsRNA (112)
4	LPS (64, 72) Respiratory syncytial virus (113) Chlamydial HSP60 (110) Mycobacterial HSP65 (110) Fibrinogen (110, 113) Human HSP60 and HSP70 (110-111) Human fibronectin (110) Human hyaluronic acid, etc (110)
5	Bacterial flagellin (114)
6	Lipopeptide/lipoprotein (Mycoplasma) (76) Peptidoglycan (76) Zymosan (72)
7	ssRNA (110)
8	ssRNA (110)
9	Unmethylated CpG DNA motifs (115)
10	Not known

important for directing TLR signals to different signalling cascades to activate particular transcription factors required for specific responses.

MyD88, the principal adaptor for nearly all TLR, separates the signalling pathways into MyD88-dependent and MyD88-independent pathways. When engaged, several TLR activate the MyD88-dependent signalling by associating with MyD88 to initiate a signalling cascade, described further by Akira (116), which culminates in early-phase activation of transcription factor, nuclear factor-kappaB (NF-κB). Early-phase activation of NF-κB results in instigation of an inflammatory response (116-117); one of the main effects of TLR engagement. TLR2 and TLR4 requires TIRAP to act upstream of MyD88 for MyD88-dependent signalling to activate NF-κB (118).

MyD88-independent signalling dimerises TIR regions of other adaptors, TRAM and TRIF, with engaged TLR to initiate late-phase activation of NF-κB which induces expression of co-stimulatory molecules and IFN-associated products (116). Other MyD88-independent signalling pathways use TRIF to trigger activation interferon-regulatory factor 3 (IRF3) to directly induce expression of type 1 IFN genes (116). LPS-activated TLR4 utilises both MyD88-dependent and MyD88-independent pathways for inflammatory cytokine production and TRAM is required by TLR4 for the MyD88-independent pathway (119).

The various TLR signalling pathways can activate several transcription factors which include NF-κB, activating protein-1 (AP-1) and the family of interferon regulatory factors (IRF). These transcription factors can induce inflammatory responses, type I interferon responses and upregulation of co-stimulatory

expression. The numerous genes transcribed in response to TLR signalling contribute to inflammation and various other immune mechanisms.

1.3.6 TLR Function

TLR have a profound impact on both innate and adaptive immune responses through direct and indirect influences. The number of TLR and the complexity of signalling pathways allow a varied and nuanced response to TLR ligation. The main effects of TLR are inflammation, innate anti-microbial effects, apoptosis of infected cells, influencing the adaptive response and, on occasion, autoimmune effects. Further discussion on the effects of TLR on the immune system for this section will be based on studies on human and mouse systems.

Inflammation is an early response against infection that is comprised of an intricate network of cellular and humoral responses that operate to clear pathogens. TLR engagement can induce secretion of pro-inflammatory cytokines (108) as seen in neutrophils (72), monocytes (120), macrophages (107, 121), mononuclear immune cells (120, 122-123), dendritic cells (123-124), mast cells (112) and epithelial cells (125). Knockout murine models for TLR2 or MyD88 both showed decreases in streptococcal cell wall-induced joint inflammation (126). The effects of several TLR on neutrophils include increased phagocytosis, superoxide production and chemokine expression with decreased chemotaxis and prolonged survival of neutrophils (72, 75, 127).

TLR activate various anti-microbial mechanisms in neutrophils (72), macrophages (107), eosinophils (73) and natural killer lymphocytes (79, 128) to resist infection. TLR2 is necessary for effective clearance of *Mycobacterium tuberculosis* respiratory

infections in mice otherwise chronic pneumonia develops (129). Apoptosis can be TLR-mediated; apoptosis of cells via TLR2 has been observed with neutrophils of TB-infected patients (130) and murine microglia in herpes simplex viral-infected mice (131). TLR contribute to the development of anti-microbial activity.

The link of TLR between the innate and adaptive immune responses is important as the two branches have been studied independently of each other. The influences of TLR on the adaptive responses of lymphocytes will be discussed in the next paragraph; this paragraph will focus on the sentinel cells of the adaptive immune response, DC. DC maturation by TLR engagement results in up-regulation of co-stimulatory molecules including CD80 and CD86, altered expression of chemokines and secretion of pro-inflammatory cytokines allowing them to increase their antigen presenting ability and migrate to lymph nodes (71, 128). Different TLR trigger different pathways; subsets of DC shape T cell differentiation through differential cytokine release and co-stimulatory molecule expression, thereby influencing the adaptive response (71, 128). TLR8 ligand, CL075, induces IL-12 and TNF-α from myeloid DC and minimal levels of IFN-γ and TNF-α from plasmacytoid DC (123). In response to CpG ODN, murine DC secrete IL-12 and elevate expression of CD40, CD80 and CD86, and murine macrophages secrete IL-6, TNF-a, IL-12 (115), while human DC secrete IFN-γ and low levels of TNF-α in response to CpG2216 (123). DC can also be stimulated by agonists for TLR3 and TLR4 acting synergistically with each other or with TLR8, with synergy enhanced further by IFN-γ or CD40L, which induces DC to secrete IL-12p70 and activate T cells to secrete IFN-γ (132). A recent study has found TLR-activated DC secrete IL-6 which suppresses regulatory T cells from interfering with activated $CD4^+$ T cells (133).

The influence which TLR exert over the adaptive response and their ability to recognise endogenous ligands has implications for development of systemic autoimmune diseases. In autoreactive murine B cells the B cell receptor engages with chromatin released from apoptotic cells; the engaged B cell receptor then cross-links with TLR9 which is stimulated by the few CpG motifs present in the mammalian chromatin and the two receptors act synergistically to induce activation and proliferation of the B cell (134). TLR9 is also implicated in breaking T cell self tolerance via TLR9-engaged APC and inducing murine experimental autoimmune encephalomyelitis, the animal model of multiple sclerosis (135). Inflammatory bowel disease, a localised autoimmune condition, has been linked to Asp299Gly TLR4 polymorphism which has an impaired signalling pathway for lipopolysaccharide (LPS) (136-137). TLR dysregulation or impaired signalling pathways can lead to systemic or localised autoimmune conditions.

1.3.7 TLR and Lymphocytes

T lymphocytes are activated by TLR ligands directly or indirectly through TLR-engaged DC and mast cells (112, 138-139), can proliferate (84, 140) and upregulate expression of T lymphocyte activation markers (138, 141). TLR engagement also instigates T lymphocytes to secrete cytokine patterns differentially depending on the TLR ligand (84, 140-141). TLR ligands can have co-stimulatory effects on TCR-engaged T lymphocytes to enhance proliferation and cytokine secretion (84, 141). One instance of TLR influencing adaptive responses is the polarisation of T helper cells to T_H1 subset by cytokine signals from TLR-engaged accessory cells (142-143). The functions of cytotoxic T lymphocytes can be affected by TLR either directly

(141) or indirectly (104, 139, 144). Regulatory T lymphocytes are suppressed upon TLR engagement which prevents dampening of the immune response (133, 145).

B lymphocytes proliferate, upregulate expression of co-stimulatory molecules including CD40, CD80, CD86 and MHC class II, and secrete antibodies in response to TLR ligands (146-149). CpG oligodeoxynucleotides (ODN), a TLR9 ligand, is a well-known mitogen of B lymphocytes that initiates the TLR signalling cascade and induces proliferation, upregulation of co-stimulatory molecules, differentiation of memory B cells into plasma cells and secretion of IgG and IgM (147, 150-153). Additional effects of CpG ODN on B lymphocytes include enhanced functions after co-stimulatory engagement with BCR (152, 154), reduced apoptosis (153) and secretion of cytokines (115). The differentiation stage of B lymphocyte can affect their responses to TLR ligands; memory B cells secrete IgG (154) whereas naïve B cells secrete IgM and activate T lymphocytes (153) and murine splenic B lymphocyte subsets secrete variable cytokine patterns (155). Studies also indicate the possible requirement of TLR engagement for antibody class-switching, isotype-switching and somatic hypermutation (156-159). TLR have an important role in the function of B lymphocytes.

1.3.8 TLR and Neonatal Immune Response

As discussed in Section 1.1, the neonatal immune system is under developed and unable to launch effective responses against infection. Nonetheless, neonates receive protection from passive immunity passed from the mother via breast milk. Another potential source of protection are PRR; the neonatal immune system may be able to discriminate between self and nonself to distinguish invading pathogens. The potential ability of neonates to recognise infections is confirmed by cord blood

leucocytes responding to Gram-positive and Gram-negative bacteria (5) and microbial components, Poly I:C and LPS (17, 160). In the neonate, TLR ligands can enhance certain immune functions, but not the neonatal inflammatory response. TLR ligands induce secretion of pro-inflammatory cytokines by cord blood mononuclear leucocytes (6, 17, 122), but levels vary when compared to adult blood mononuclear leucocytes. When compared to adults, levels of IFN-gamma and IL-12p70 by whole cord blood (17, 122), and IL-1, IL-6, IL-12 and TNF by neonatal murine macrophages (6) in response to TLR ligands are lower. However, one study showed elevated IL-6 and TNF-alpha by TLR-engaged cord blood mononuclear leucocytes when compared to mononuclear leucocytes from one year old infants (160). The current knowledge indicates that several components of the neonatal inflammatory response are deficient compared to adults, as discussed in Section 1.2.3.1. The literature shows that neonatal leucocytes have a partial inflammatory response to TLR ligands, but not at adult levels. The report of higher levels of pro-inflammatory cytokines in cord blood leucocytes was compared to infants; infants' immune systems are still developing when compared to adults, therefore this study does not contradict the earlier studies.

Adaptive immune responses of neonates are affected by TLR as seen with multiple TLR engagement of neonatal DC resulting in increased levels of pro-inflammatory cytokines and co-stimulatory molecule expression (52, 161-162). TLR ligands, acting as co-stimulatory signals, induced cord blood T lymphocytes to secrete IFN-α, TNF-α, IL-2 and IL-10 with increased expression of CD25 and intracellular granzyme B in $CD8^+$ T cells (23, 84). CpG ODN stimulates cord blood B lymphocytes to secrete IgM (163) but there is little in the literature looking at TLR and neonatal B lymphocytes. However, neonatal murine B1 B cells stimulated by

TLR ligands may have an anti-inflammatory effect and tailor a T_H2 response by secreting high levels of IL-10 which suppresses adult murine DC from secreting pro-inflammatory cytokine (164-166). The immaturity of the neonatal adaptive immune cells and subsequent responses may be overcome with treatment by TLR ligands, at least for DC and T lymphocytes.

Despite enhanced responses of DC and T lymphocytes to TLR engagement, TLR signalling in cord blood is impaired, which may explain the functional discrepancies between adult blood and cord blood (17). Cord blood leucocytes, compared to adult blood leucocytes, treated with TLR ligands have reduced expression of MyD88 and IRF5, decreased activation of signalling proteins p38-MAPK and ERK1/2 and reduced binding of transcription factor IRF3 which results in decreased secretion of pro-inflammatory cytokines (167-169).

1.3.9 TLR Ligands as Vaccine Adjuvants

Vaccines are highly successful in minimising several infectious diseases, to the point that scourges such as smallpox, polio and measles have diminished. Ideally, an effective vaccine requires a single-dose administration to produce a specific, memory immune response. However, many infectious diseases lack effective vaccines which produce a robust immune response, so the use of vaccine adjuvants can enhance the immunogenicity of vaccines. Vaccine adjuvants are compounds which enhance the immune response to the vaccine antigen. For an effective immune adaptive response, DC, T lymphocytes and B lymphocytes need to interact with each other via immunological synapses for antigen presentation and co-stimulation signals for activation followed by paracrine cytokine influence. These cellular interactions occur in GC which are located in secondary lymphoid tissue.

The immaturity of neonatal immunity includes a relative lack of GC formation in secondary lymphoid tissue and reduced functions of DC, T lymphocytes and B lymphocytes; these weaknesses do not allow neonates to develop a specific, long-term, memory response. TLR ligands influence and enhance immune responses thereby indicating a potential role as vaccine adjuvants for neonatal vaccines.

TLR ligands enhance the systemic adaptive immune response indicating their potential as vaccine adjuvants. An example would be the Yellow fever vaccine 17-D, in use for 65 years, it was recently found to activate several TLR pathways to produce multiple responses (144). Murine studies indicate the importance of MyD88 in long-term, antibody, response (170) and necessity of TLR for the development of GC (170-171). Vaccine studies trialling CpG ODN as an adjuvant found enhanced IgG titres and avidity in adults (172), increased IgG titres and proliferation of murine B lymphocytes (173-174), and adult-like T_H1 and T_H17 responses with DC activation in neonatal mice (175). There is further evidence for TLR ligands to counter the T_H2 response in neonatal and adult mice (176-177). The observations of enhanced antibody responses to TLR ligands are challenged by one study showing elevated antibody titres in response to Freund's adjuvant and a TLR2 ligand in $MyD88^{-/-}TRIF^{-/-}$ mice (178), but it does not contradict the data on CpG ODN. So far, most of the evidence demonstrates that TLR ligands, in particular CpG ODN, may be effective adjuvants for vaccines to produce an increased adaptive response with specific memory against infection. However there must be caution when considering TLR ligands as vaccine adjuvants in neonates. As described at the end of Section 1.3.6, TLR ligands have been implicated in the development of autoimmune conditions in mouse models (135, 179). TLR ligands

should definitely be researched as potential adjuvants in neonatal vaccines, but all their potential effects require further examination.

1.4 PROJECT PLAN

Neonates have poorer immunity when compared to adults and are vulnerable to infection. The poor function of T lymphocytes and B lymphocytes in neonates leads to deficient innate and adaptive immunity against infectious diseases. The deficiencies of the neonatal immune response may be compensated for by immune protection from innate immunity like TLR which are present through germ-line encoding and do not need to be acquired.

At the commencement of this study there was very little in the literature about the role of TLR on lymphocytes. Much of the information was on expression of TLR mRNA, there were few TLR antibodies commercially available to determine expression of TLR proteins. With flow cytometry, the literature showed cord blood T lymphocytes expressed TLR2 and TLR4 (84), and adult blood B lymphocytes expressed TLR9 (81). In the intervening years published reports on TLR protein expression have increased due to availability of antibodies (Table 1.1), but there is still little information about expression by neonatal lymphocytes.

Initial data (92) showed that TLR1, TLR2, TLR3, TLR4 and TLR9 were not detected on adult blood T lymphocytes, but surface expression of TLR1 and TLR9 on B lymphocytes was detected. TLR9 is considered an intracellular marker and the detected surface expression initially contradicted the literature, but other studies supported this observation (81). Much of the literature used knock-out models of

TLR and TLR signalling proteins, but very few studies indicated whether the cell of interest expressed the TLR. As seen in Table 1.1, the mRNA and protein expression of TLR are often not consistent, for example TLR3 and TLR8 expression by B lymphocytes was not detected as mRNA, but detected as protein. For this reason expression of TLR protein by lymphocytes from adults and neonates is of interest.

In the preliminary study (92) it was found that, when distinguishing B lymphocytes into B1 and B2 subsets, mean fluorescence intensities of TLR1 and TLR9 were greater on B1 B cells. It is hypothesised that early lineage B lymphocytes may express higher levels of TLR than the mature, differentiated B lymphocytes.

The mitogenic nature of many TLR ligands, like LPS and CpG ODN, which stimulate the function of adult lymphocytes, may also stimulate neonatal lymphocytes. This has been shown in the adult-like functions of neonatal DC upon stimulation with combined TLR ligands (52, 162). It is possible that TLR may play a greater role in the immature immune system of neonates compared to the mature immune system of adults thus compensating for the inadequate immunity of neonates. It is hypothesised that the degree of TLR expression and function is greater in neonatal lymphocytes than adult lymphocytes.

1.4.1 Research Question

What potential role do TLR have in the expression and function of neonatal lymphocytes?

1.4.2 Research Hypotheses

That the early lineage B lymphocytes may express higher levels of TLR than the mature differentiated B lymphocytes.

That the degree of TLR expression and function is greater in neonatal lymphocytes than adult lymphocytes.

1.4.3 Aims

To determine TLR expression by T lymphocytes from neonates and adults.

To determine TLR expression by B lymphocytes from neonatal blood, adult blood, and tonsils.

To examine functions in response to TLR ligands of B lymphocytes from neonatal blood, adult blood and tonsils.

1.4.4 Research Plan

This study comparing the immune systems of neonates and adults will focus on human subjects. The research samples will be cord blood for neonates, buffy coats from the Australian Red Cross for adults and tonsils as lymph nodes.

As far as possible the entire TLR panel will be tested and the commercial TLR antibodies available for this study are TLR1, TLR2, TLR3, TLR4, TLR6, TLR8 and TLR9. Due to discrepancies in TLR expression between mRNA and protein, TLR protein expression will be detected through flow cytometry. TLR expression according to the activation state of lymphocytes, and the extracellular and intracellular expression are also of interest. Subsets of B lymphocytes from cord blood, adult blood and tonsils will be examined for patterns of TLR expression.

Once the TLR expressed by B lymphocytes are identified, B lymphocytes from the three subject groups will be treated with the relevant ligands and the functions will be studied. The functions of interest are B lymphocyte proliferation, quantification of total antibody and individual antibody classes, and quantification of cytokines IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF.

In this study a broad examination of TLR expression will be performed, with the intention of identifying differences which will merit further comparative analysis (beyond the scope of this thesis).

CHAPTER TWO

MATERIALS AND METHODS

2.1 MONOCLONAL ANTIBODIES AND IMMUNOFLUORESCENCE STAINING REAGENTS

All monoclonal antibodies (mAb) and immunofluorescence (IF) staining reagents were titrated for optimal labelling of cells. Table 2.1 lists all mAb sourced from hybridoma cell cultures and the Human Leucocyte Differentiation Antigens (HLDA) Workshop 8. MAb from hybridoma cell cultures were used in culture supernatant form or purified mAb. Table 2.2 lists all commercial reagents used for IF staining and Table 2.3 lists all commercial fluorescent mAb. All monoclonal antibodies targeting human proteins were raised in mice unless otherwise stated.

2.2 BUFFERS AND SOLUTIONS

All reagents used were of analytical standard.

2.2.1 Phosphate Buffered Saline

Phosphate buffered saline (PBS) was sourced commercially and made in the laboratory. Commercial, sterile, Dulbecco's PBS (SAFC Biosciences, Sigma Aldrich Inc.; catalogue no. 59321C) was used in all cell separation and culture protocols.

Laboratory PBS (pH 7.2-7.6; osmolality 281-297) was produced by dissolving 160 g NaCl, 4 g KCl, 23 g Na_2HPO_4 and 4 g KH_2PO_4 in 20 L deionised water. Sodium

Table 2.1 MAb from Hybridoma Cultures and HLDA Workshop 8

mAb	Isotype	Clone	Reference
Erythrocyte Marker			
CD235a	IgG1, κ	10F7MN	ATCC
Activation Stimuli			
CD3	IgG2a	OKT3	ATCC
CD40	IgG1	G28/5	ATCC
Anti-IgM	IgG1	HB57	ATCC
Controls			
IgG1 Negative Control	IgG1	x63	IgG1 myeloma protein
IgG2a Negative Control	IgG2a	SAL5	IgG2a anti-Salmonella mAb
CD25	IgG2a	7G7B6	(180)
Co-Stimulatory Molecules and Cytokine Receptors			
CD21	IgG1	B98	HLDA Workshop 8
CD80	IgG1	L307.4	HLDA Workshop 8
CD86	IgG1	FUN-1	HLDA Workshop 8
CD210	Rat IgG2a, κ	3F9	HLDA Workshop 8

Table 2.2 Immunofluorescence Staining Commercial Reagents

Reagent	Company	Cat. No.
Anti-Mouse Ig $F(ab)_2$ – PE Conjugated (DDA-PE)	Chemicon	AQ326H
Biotinylated Anti-Mouse IgG (H+L) – Made in Horse (HαMBi)	Vector Laboratories	BA-2000
Mouse Serum (Normal)	Dako	091001
Streptavidin/RPE (SA-PE)	Dako	R0438
Streptavidin, Alexa Fluor 647 Conjugate (SA-AF647)	Molecular Probes, Invitrogen	S-21374

Table 2.3 Commercial Antibodies

mAb	Format	Isotype	Clone	Company	Cat. No.
Lineage Markers					
CD3	FITC	IgG2a, κ	HIT3a	BD Pharmingen	555339
CD3	PE	IgG2a, κ	HIT3a	BD Pharmingen	555340
CD5	FITC	IgG1, κ	UCHT2	BD Pharmingen	555352
CD5	PE	IgG1, κ	UCHT2	BD Pharmingen	555353
CD19	PE	IgG1, κ	HIB19	BD Pharmingen	555413
CD19	PerCP-Cy5.5	IgG1, κ	SJ25C1	BD Pharmingen	340951
CD27	FITC	IgG1, κ	M-T271	BD Pharmingen	555440
CD38	FITC	IgG1, κ	HIT2	BD Pharmingen	555459
CD45	FITC	IgG1, κ	HI30	BD Pharmingen	555482
IgD	Biotin	IgG2a, κ	IA6-2	BD Pharmingen	555777
Activation Markers					
CD23	PE	IgG1, κ	M-L233	BD Pharmingen	555711
CD25	PE	IgG1, κ	M-A251	BD Pharmingen	555432
CD69	PE	IgG1, κ	FN50	BD Pharmingen	555531
CD95	PE	IgG1, κ	DX2	BD Pharmingen	555674
HLA-DR	PE	IgG2a, κ	L243	BD Pharmingen	347367
Negative Isotype Controls and TLR Markers					
IgG1	PE	IgG1, κ	MOPC-21	BD Pharmingen	555749
IgG2a	PE	IgG2a, κ	G155-178	BD Pharmingen	555574
TLR1	PE	IgG1, κ	GD2.F4	eBioscience	12-9911
TLR2	PE	IgG2a, κ	TL2.1	eBioscience	12-9922
TLR3	PE	IgG1, κ	TLR3.7	eBioscience	12-9039
TLR4	PE	IgG2a, κ	HTA125	eBioscience	12-9917
TLR6	Biotin	Rat IgG2a, κ	hPer6	eBioscience	13-9069
TLR8	Purified	IgG1	44C143	Imgenex	IMG-321A
TLR8	PE	IgG1	44C143	Imgenex	IMG-321D
TLR9	Purified	Rat IgG2a, κ	eB72-1665	eBioscience	14-9099
TLR9	PE	Rat IgG2a, κ	eB72-1665	eBioscience	12-9099

azide (Sigma-Aldrich Inc.; catalogue no. S-8032) was added to laboratory PBS at 0.02% w/v (PBS/Azide) and used for phenotyping protocols. For intracellular phenotyping assay, saponin (Sigma-Aldrich Inc; catalogue no. S-2149) was added to PBS/Azide at 0.1% w/v (Current Protocols in Immunology, Section 6.24.9).

For 20x PBS, dissolved 160 g NaCl, 4 g KCl, 23 g Na_2HPO_4 and 4 g KH_2PO_4 in 1 L deionised water.

2.2.2 Leucocyte Stain

Azure II (0.1 g; Sigma Aldrich Inc.; catalogue no. 861065) was dissolved in 50 mL deionised water and 0.25 mL Triton X100 (Sigma Aldrich Inc.; catalogue no. X100) added, then stored for one week and filtered. The stock was diluted 1/100 with deionised water for erythrocyte lysis and leucocyte counts, and cell counts were read on Improved Neubauer Haemocytometer.

2.2.3 FACS Permeabilisation Solution

BD FACS Permeabilizing Solution 2 (BD Biosciences; catalogue no. 340973), as per manufacturer's instructions, was diluted 1/10 with deionised water for permeabilisation of cells.

2.2.4 Intracellular Skim Milk Block

To prepare skim milk block for intracellular staining, skim milk powder (Black & Gold, Australia) was added to Saponin/PBS/Azide at 5% w/v, mixed on a rotary mixer for 15 minutes, then centrifuged for 30 minutes at 15, 000 x g before retaining the supernatant and storing at 4ºC for up to 6 months (Current Protocols in Immunology, Section 6.24.9).

Skim milk block for cell surface staining was prepared as described above, except PBS/Azide was used in lieu of Saponin/PBS/Azide.

2.2.5 2% Paraformaldehyde Fixative

Paraformaldehyde (PFA) fixative (181) was prepared by dissolving 10 g of PFA powder (BDH Laboratory Services, UK; catalogue no. 294474L) in 475 mL deionised water on a hot plate for one hour in a fume hood. The temperature was not allowed to exceed 70°C. Two drops of 5 M NaOH were added to turn the solution clear and cooled to room temperature. Then 25 mL of 20x PBS was added to the solution, pH adjusted to 7.3, filtered, aliquoted and stored in freezer for use.

2.2.6 RF10 Cell Culture Medium

Foetal bovine serum (FBS) (JRH Bioscience, Sigma-Aldrich Inc.; catalogue no. 12003C) was heat-inactivated in a 56°C water bath for 30 minutes, aliquoted and stored in freezer.

RF10 cell culture media was, aseptically, prepared by addition of FBS at 10% v/v and Penicillin-Streptomycin-Glutamine x100 at 1% v/v (Invitrogen Pty Ltd; catalogue no. 10378-16) to RPMI 1640 Medium (Modified) (SAFC Biosciences, Sigma Aldrich Inc.; catalogue no. 51502C). RF10 was stored for up to two weeks before discarding due to degradation of glutamine.

2.2.7 Buffers and Reagents for ELISA Assays

All ELISA buffers were made with laboratory PBS free of sodium azide.

2.2.7.1 ELISA Antibodies

Table 2.4 lists capture and detection antibodies used in protocols. The detection antibodies were conjugated with horseradish peroxidase (HRP) and all antibodies were purchased from Chemicon International, Millipore Corp.

Table 2.4 ELISA Capture and Detection Antibodies

Antibody	Host	Reactivity	Cat. No.
Capture Antibody			
Ig Capture	Goat	Human IgG, IgM, IgA	AQ503
Detection Antibodies			
Ig Detection – HRP	Sheep	Human IgG, IgM, IgA, IgD	AP301P
IgG Detection – HRP	Sheep	Human IgG	AP316P
IgM Detection – HRP	Sheep	Human IgM	AP320P

2.2.7.2 ELISA Buffers

ELISA coating buffer was prepared with 45.3 mL of 1 M $NaHCO_3$, 18.2 mL of 1 M Na_2CO_3 and added deionised water to 1 L before adjusting pH to 9.6 with sodium bicarbonate or sodium carbonate solutions.

ELISA wash buffer consisted of Tween 20 (Sigma Aldrich Inc.; catalogue no. P-5927) added to PBS at 0.05% v/v.

ELISA blocking buffer consisted of Bovine Serum Albumin (Sigma Aldrich Inc.; catalogue no. A-7906) dissolved in ELISA wash buffer at 5% v/v.

ELISA diluent buffer had BSA dissolved in ELISA wash buffer at 1% v/v.

2.2.7.3 ELISA Substrate Solution

The substrate was made fresh for each assay. Phosphate citrate buffer, 0.05 M pH 5.0, was prepared by dissolving one tablet in 100 mL deionised water (Sigma Aldrich Inc.; catalogue no. P-4809), then filter-sterilised and aliquoted. Two tablets of 5 mg o-Phenylenediamine dihydrochloride (OPD) (Sigma Aldrich Inc.; catalogue no. P-3804) and 10 μL of 30% w/w H2O2 (Sigma Aldrich Inc.; catalogue no. H-1009) were added to 20 mL phosphate citrate buffer.

2.3 CRYOPRESERVATION

The protocols for storing and thawing cells from cryopreserved cell stocks were the same for hybridoma cell lines and clinical tonsil cells. Aseptic technique is required at all times.

2.3.1 Storage

Two solutions, A and B, were prepared:

- Solution A – 50% FBS and 50% RF10
- Solution B – 70% RF10 and 30% dimethyl sulfoxide (Sigma Aldrich Inc.; catalogue no. D-5879)

Cell viability was checked by live cell exclusion of Trypan Blue (Sigma Aldrich Inc.; catalogue no. T-8154) with storage proceeding only if viability was greater than 80%.

Cells were centrifuged at 300 x g for 5 minutes, supernatant discarded and cells were resuspended in solution A at 10^8 cells/mL. Over ten minutes an equal volume of solution B was added drop by drop with simultaneous gentle shaking of the cell suspension. The final suspension had a cell count of 5 x 10^7 cells/mL and 1 mL aliquots were transferred into labelled polypropylene cryopreservation tubes (Greiner Bio-One; catalogue no. 121-263).

The tubes were placed in a thick polystyrene container that was sealed and stored in -80ºC freezer overnight. The following day the tubes were transferred to liquid nitrogen storage facilities.

2.3.2 Thawing

A tube of frozen cells was thawed in a 37ºC water bath until the ice just melted, then the contents quickly transferred to a 30 mL tube. Slowly, 2 mL of warm RF10 was added drop by drop to the cells with simultaneous gentle shaking for 10 minutes, then the suspension was stood for another 10 minutes. This procedure was repeated for 6 ml of RF10, and then 10 mL. Cells were centrifuged at 300 x g for 5 minutes, the supernatant discarded and cells resuspended in RF10. Cell viability was checked and dead cells removed with Lymphoprep separation, described in Section 2.8.

2.4 Production of Monoclonal Antibodies from Hybridoma Cell Lines

Four hybridoma cell lines were cultured for antibody production; OKT3 (CD3), G28/5 (CD40), 10F7MN (CD235a) and HB57 (anti-IgM).

The cell lines were thawed and their growth was established in T25 (Nunc™; catalogue no. 156367) and T75 (Nunc™; catalogue no. 156499) flasks before antibody was harvested. During cell growth there is antibody secretion and a pH shift seen in RF10 turning yellow. Over 2 months, antibody was collected from the flasks every 3 to 5 days by harvesting 90% of media and replacing with fresh RF10. The harvested culture media was centrifuged, supernatant collected and stored at 4ºC until antibody purification at a later stage.

2.5 PURIFICATION AND QUANTIFICATION OF MONOCLONAL ANTIBODIES

Protein G Sepharose 4 Fast Flow beads (GE Healthcare; catalogue no. 17-0618) were used for purification of IgG antibodies from OKT3, G28/5 and HB57 culture supernatants. Laboratory PBS was used for antibody purification.

2.5.1 Antibody Purification

- Clean column of old beads and add 2 mL of fresh Protein G Sepharose 4 Fast Flow beads.
- Flush air bubbles from peristaltic pump tubing with PBS and attach column to pump.
- Fill column with PBS, carefully open column tap for a steady drip and wash beads with 30 mL PBS at a flow rate of 4 mL/minute. This will wash beads free of ethanol and pack them at the bottom of the column. The column must be upright so the interface of beads is flat.

- Reduce PBS level to about 1 cm above the beads. The column must not run dry or beads will be damaged.
- Feed culture supernatant into the column and reduce the flow rate to 2.5 mL/minute. After the culture supernatant, wash beads with 30 mL PBS.
- Elute antibody from beads with 3 mL 0.1M glycine pH 2.5-3.0, collect in 6 x 1 mL aliquots and neutralise glycine by adding 100 μL 1 M Tris pH 9.0.

2.5.2 Quantification of IgG

- Pool aliquots of purified antibody and dialyse overnight with PBS at 4ºC using "Slide-A-Lyzer" 7 kDalton molecular weight cut-off cassettes (Thermo Fisher Scientific Inc.; catalogue no. 66370).
- Filter-sterilise antibody with 0.22 μm syringe filters (Millipore Corp.; catalogue no. SLGV033RS)
- Measure antibody concentration with spectrophotometric reading at 280 nm. Determine the antibody quantity with this equation; Antibody concentration = Absorbance x 0.769 mg/cm/mL.

Antibodies against CD3, CD40 and IgM were purified from the hybridoma supernatants and after purification, dialysis and sterilisation, the antibody yields were; 5.76 mg of OKT3 IgG, 5.3 mg of G28/5 IgG and 3.63 mg of HB57 IgG.

2.6 SUBJECT GROUPS

From healthy adult volunteers, the Australian Red Cross provided leucocyte-rich buffy coats from blood that was processed within 18 hours of collection.

Umbilical cord blood was collected from healthy patients undergoing vaginal delivery of infants at the Delivery Suite of WCH. The gestational ages of the collected cord bloods were not made available to researchers; the variation and interpretation of results may be affected by the gestational age of cord blood. Cord blood was collected via drainage of severed umbilical cord into CPDA-1 Blood-Pack Units (Fenwal Blood Technologies; catalogue no. FBR7110) and processed within 18 hours.

Tonsils were collected from patients admitted for elective surgical removal of tonsils and adenoids due to sleep apnoea or recurrent tonsillitis. All subjects were under 12 years of age and parental consent was obtained for participation in the study. For TLR phenotyping studies tonsils were processed within 12 hours of collection. For TLR functional studies, tonsil cells were immediately extracted from the tissue and cryopreserved until use.

TLR phenotyping studies used three subjects per group; adult blood subjects 1, 2 and 3; cord blood subjects 1, 2 and 3; and tonsil subjects 1, 2 and 3. Mononuclear leucocytes from adult blood and cord blood were split into three aliquots to examine TLR expression; non-stimulated lymphocytes, stimulated T lymphocytes and stimulated B lymphocytes. Mononuclear leucocytes from tonsils were split into two aliquots, one for non-stimulated B lymphocyte and the other for stimulated B lymphocytes. TLR functional studies used three subjects per group; adult blood subjects 4, 5 and 6; cord blood subjects 4, 5 and 6; and tonsil subjects 4, 5 and 6.

Ethics approval for this study was part of a larger study, "The Human Cell Differentiation Molecule (HCDM) study; An international study on white blood

cells" (REC 1804/2/2009), approved by Children Youth and Women's Health Service Research Ethics Committee at the Women's and Children's Hospital, Adelaide. The Flinders Clinical Research Ethics Committee of Flinders University, Adelaide, approved the existing ethics from Women's and Children's Hospital (FCREC 06/09/2007).

2.7 Extraction of Cells from Tonsil Tissue

For flow cytometry, tonsil tissue was separated into a single-cell suspension as follows:

- Use aseptic technique.
- Dip tonsils into Riodine (10% Povidone Iodine; Orion Laboratories; catalogue no. RIO00802F) briefly, then wash in commercial PBS to completely remove iodine.
- With sterile scissors, cut tonsil tissue into pieces and place on a sterile sieve resting in a Petri dish containing RF10.
- Grind tonsil pieces through the sieve using a disposable syringe plunger, RF10 will become cloudy as cells pass through. Discard waste tissue in sieve.
- Treat the tonsil cell suspension to separate mononuclear leucocytes, as described below.

2.8 Separation of Mononuclear Leucocytes

Mononuclear leucocytes are separated from erythrocytes and other leucocytes using density centrifugation with Lymphoprep (Axis Shield PoC AS; catalogue no.

1114545). The presence of nucleated erythrocytes in cord blood affects its separation protocol. Commercial, sterile, PBS was used and the Lymphoprep separation protocol is as follows:

- Dilute blood samples with PBS and tonsil cells with RF10; for adult buffy coat and tonsils 1:1, and cord blood 1:3.
- Carefully, without disturbing the Lymphoprep interface, layer diluted sample over Lymphoprep (one volume of Lymphoprep for two volumes of diluted sample).
- Centrifuge tubes with no brake; for adult blood and tonsil at 700 x g for 20 minutes, and for cord blood at 350 x g for 30 minutes.
- Carefully harvest the mononuclear cell layer on top of the Lymphoprep interface and transfer to a separate tube.
- Dilute harvested cells with PBS at least 1:4, then wash and centrifuge cells twice for 5 minutes each time; first at 700 x g and second at 300 x g.
- Resuspend cells in appropriate solutions to desired cell count for later assays.

2.9 Removal of Nucleated Erythrocytes from Cord Blood Mononuclear Cells

Mononuclear cells isolated from cord blood contain high proportions of nucleated erythrocytes that interfere in studies examining mononuclear leucocytes, hence removal of nucleated erythrocytes is required. Modifying an in-house protocol (182), cord blood erythrocytes were labelled with a monoclonal antibody (10F7MN) targeting CD235a, an erythroid cell lineage marker (182), and removed via magnetic selection with MACS Goat Anti-Mouse IgG Microbeads (Miltenyi

Biotec, Germany; catalogue no. 130-048). Nucleated erythrocytes were removed for TLR phenotyping studies, this was not necessary for TLR functional studies.

10F7MN supernatant and MACS Microbeads were titrated previously for optimal conditions. MACS Buffer was prepared by addition of BSA at 0.5% w/v and EDTA at 2 mM (Sigma Aldrich Inc.; catalogue no. E6758) to commercial PBS. The protocol is as follows:

- Use aseptic technique
- Suspend cord blood cells to 10^7 cells/mL in commercial PBS and add 10 μL 10F7MN supernatant per 10^7 cells.
- Mix on rotary mixer for 30 minutes at room temperature, then wash cells with cold MACS buffer twice at 300 x g for 5 minutes.
- Follow manufacturer's instructions for removal of nucleated erythrocytes with MACS Microbeads. Changes include using 5 μL Microbeads per 10^7 cells and separation with the CS column on VarioMACS in the biohazard cabinet.
- After obtaining the 10F7MN-depleted cell fraction, wash cells once and resuspend to desired cell count in appropriate solution.

Aliquots of cells were removed post-separation to measure any nucleated erythrocyte contamination using anti-CD45 antibody, a pan-leucocyte marker, and 10F7MN. The nucleated erythrocyte contamination after separation was fewer than 7% cells.

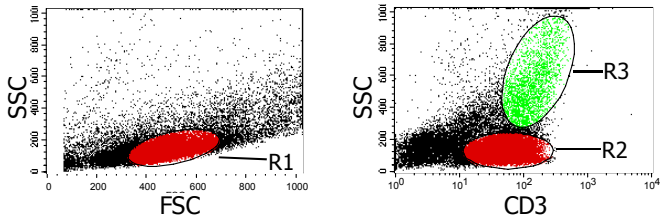
2.10 STIMULATION OF T LYMPHOCYTES

For TLR phenotyping studies, one aliquot of mononuclear leucocytes from adult blood and cord blood were treated to stimulate T lymphocytes. T lymphocytes were stimulated with dual signals, the CD3 antibody to engage the TCR and co-stimulation via interactions with monocytes, in the presence of IL-2 to mimic physiological activation of T lymphocytes. The stimulation protocol is as follows:

- Coat 4 wells in a 6-well tissue culture plate (Nunc™; catalogue no. 140675) with 10 μg/mL purified OKT3, diluted in PBS, overnight at 37ºC in the 5% CO_2 incubator. Wash OKT3-coated wells thrice with sterile PBS.
- Suspend mononuclear leucocytes to 5 x 10^6 cells/mL in warm RF10, and add 10^7 cells and 160 pg of IL-2 to each well coated with OKT3.
- Incubate tissue culture plate at 37ºC in 5% CO_2 incubator for 24 hours and harvest cells. Wash harvested cells twice (300 x g, 5 minutes) and resuspend in PBS/Azide at 10^7 cells/mL.

T lymphocytes were identified by CD3 expression, then categorised into T cells and T blasts on the basis of forward scatter (FSC) and side scatter (SSC) which are indicators of cell size and granularity respectively; T cells are smaller and less granular than T blasts (Figure 2.1). Activation of T lymphocytes was measured by upregulation of T lymphocyte activation markers, CD25, CD69 and CD95 (183-184) (Table 2.1). T cells and T blasts after stimulation expressed higher levels of activation markers than before stimulation demonstrating the effectiveness of the T lymphocyte stimulation protocol.

A. Adult Blood Mononuclear Leucocytes



B. Cord Blood Mononuclear Leucocytes

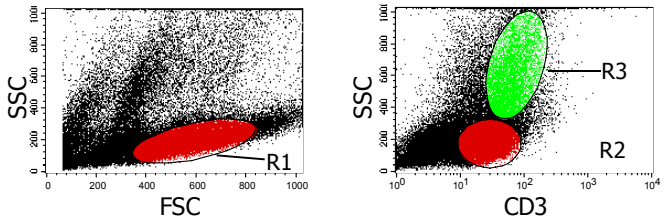


Figure 2.1 Flow Cytometry Gating Strategy for T Lymphocytes
Identification of T lymphocytes from mononuclear leucocytes of **A.** Adult blood and **B.** Cord blood. Region one (R1) gated for lymphocytes based on FSC and SSC. CD3 identified T lymphocytes with region two (R2) gating for T cells and region three (R3) gating for T blasts.

Table 2.1 Expression of Activation Markers by T Lymphocytes (% positive cells)

		Non-stimulated			Stimulated		
		CD25	CD69	CD95	CD25	CD69	CD95
Adult Blood							
1	**T cells**	10.3	1.1	57.1	75.7	82.2	96.3
	T blasts	32.5	82.7	98.9	95.7	93.8	99.3
2	**T cells**	11.3	0.9	62.5	85.5	87.2	99.9
	T blasts	39.3	60.9	98.8	95.3	95.3	99.9
3	**T cells**	4.6	1.7	77.3	78.9	85.8	99.8
	T blasts	9.6	36.8	98.9	96.1	91.7	99.9
Cord Blood							
1	**T cells**	9.8	0.7	59.0	97.7	98.5	99.9
	T blasts	15.6	7.1	100.0	24.5	37.4	99.5
2	**T cells**	5.3	0.4	11.5	98.2	99.7	97.7
	T blasts	8.3	0.1	96.2	88.4	95.1	98.6
3	**T cells**	6.6	0.5	39.5	97.9	98.6	99.2
	T blasts	8.0	4.1	99.2	42.9	62.2	99.3

Activation of T lymphocytes from each subject in the adult blood and cord blood groups, in response to stimulation, was measured as percentage of cells with positive expression of CD25, CD69 and CD95.

2.11 STIMULATION OF B LYMPHOCYTES

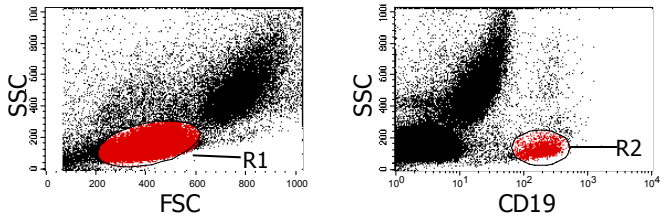
For TLR phenotyping studies, one aliquot of mononuclear leucocytes from adult blood, cord blood and tonsils was treated to stimulate B lymphocytes. To mimic physiological T-D activation of B lymphocytes, leucocytes were treated with antibodies for dual activation signals via BCR and CD40 in the presence of IL-2 and IL-4. The B lymphocyte stimulation protocol was modified from an in-house

method and cytokines were titrated for optimal concentration. The protocol is as follows:

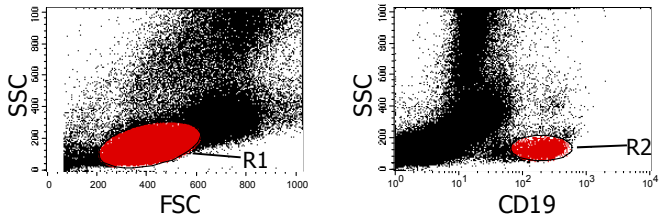
- Coat six wells in a 12-well tissue culture plate (Nunc™; catalogue no. 150628) with 2 mL of 2 μg/mL purified HB57, diluted in ELISA coating buffer, for 1 hour at 37ºC in 5% CO_2 incubator. Wash HB57-coated wells thrice with sterile PBS.
- Suspend mononuclear leucocytes in warm RF10 at 2 x 10^7 cells/mL and add 10^7 cells to each HB57-coated well. To each well, add 1 μg of purified anti-CD40 (G28/5), 50 U IL-2 and 100 U IL-4 and extra RF10 to bring the well volume to 1 mL.
- Incubate tissue culture plate at 37ºC in 5% CO_2 incubator for 24 hours and harvest cells. Wash harvested cells twice (300 x g, 5 minutes) and resuspend in PBS/Azide at 10^7 cells/mL.

B lymphocytes were identified by CD19 expression. Adult blood and cord blood B lymphocytes were mainly smaller B cells which did not develop into B blasts, whereas tonsil B lymphocytes were divided into B cells and B blasts on the basis of FSC and SSC for cell size and granularity (Figure 2.2). Activation of B lymphocytes was measured by upregulation of activation markers, CD23, CD25 and HLA-DR (185-186), with HLA-DR expression measured by median fluorescence intensity (MFI) (Table 2.2). B cells expressed higher levels of activation markers after stimulation of B lymphocytes compared to before stimulation.

A. Adult Blood Mononuclear Leucocytes



B. Cord Blood Mononuclear Leucocytes



C. Tonsil Mononuclear Leucocytes

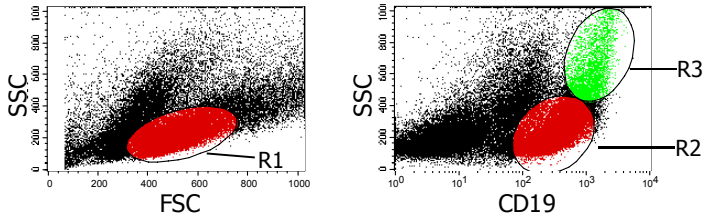


Figure 2.2 Flow Cytometry Gating Strategy for B Lymphocytes
Identification of B lymphocytes from mononuclear leucocytes of **A.** Adult blood, **B.** Cord blood and **C.** Tonsils. Region one (R1) gated for lymphocytes based on FSC and SSC. CD19 identified B lymphocytes with region two (R2) gating for B cells from all three groups and region three (R3) gating for B blasts from tonsils.

Table 2.2 Expression of Activation Markers by B Lymphocytes

		Non-stimulated			Stimulated		
		CD23	CD25	HLA-DR	CD23	CD25	HLA-DR
Adult Blood							
1	**B cells**	84.0	29.9	1778.3	44.1	74.1	3959.6
2	**B cells**	78.2	27.1	1654.8	61.7	83.2	3491.2
3	**B cells**	51.9	52.8	1459.0	30.3	84.9	4782.9
Cord Blood							
1	**B cells**	59.5	5.7	1945.6	63.0	49.8	3586.6
2	**B cells**	74.4	1.0	1134.2	69.1	26.6	4740.0
3	**B cells**	44.7	3.5	991.1	54.6	54.6	3522.7
Tonsils							
1	**B cells**	52.3	22.6	2350.1	57.6	53.5	4740.0
	B blasts	47.3	47.5	2350.1	37.4	84.4	9910.5
2	**B cells**	71.1	12.8	2287.6	86.5	42.8	5376.1
	B blasts	57.6	17.2	7704.0	79.0	64.5	9910.5
3	**B cells**	69.0	13.5	1945.6	75.2	39.6	3651.7
	B blasts	73.7	44.1	7169.2	64.8	73.7	9910.5

Activation of B lymphocytes from each subject in the adult blood, cord blood and tonsil groups, in response to stimulation, was measured. Expression of CD23 and CD25 was calculated as percentage of cells with positive expression, and HLA-DR expression by B lymphocytes was measured as MFI.

2.12 SUBSETS OF B LYMPHOCYTES

Subsets of B cells, not B blasts, were identified from the groups with lineage markers. After their identification, B cells from adult blood using CD5 and CD27 were categorised into; B1 ($CD5^+$), B2 ($CD5^-$), naïve ($CD27^-$) and memory ($CD27^+$)

B cells. Cord blood B cell subsets were divided into B1 and B2 B cell subsets. Tonsil B cells were categorised into four subsets with IgD and CD38; Naive (IgD^+CD38^-), pre-GC (IgD^+CD38^+), GC (IgD^-CD38^+) and memory (IgD^-CD38^-) subsets (27). (Figure 2.3)

2.13 IMMUNOFLUORESCENCE LABELLING FOR FLOW CYTOMETRY

For cell staining, cells from each subject were suspended in PBS/Azide at 10^7 cells/mL. For TLR phenotyping studies and the expression of activation markers by tonsil B lymphocytes, 5 x 10^5 cells were aliquoted into individual tubes for direct IF cell staining. For indirect IF assays on co-stimulatory and cytokine receptor expression by tonsil B lymphocytes, aliquots of 10^6 cells were stained.

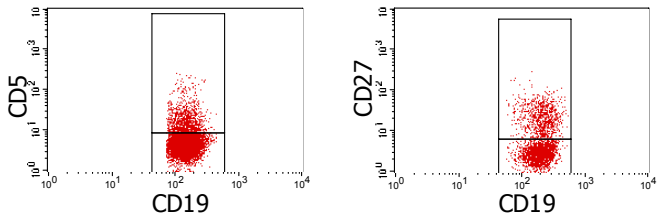
TLR antibodies, DDA-PE, HαMBi, SA-PE and SA-AF647 were titrated for optimal staining. All staining experiments:

- Use appropriate negative isotype controls.
- Incubate cells with antibodies for 30 minutes on melting ice in the dark.
- For washes, centrifuge cells with brake at 300 x g for 5 minutes at 4ºC.
- Fix cells at the end with 50 µL PFA fixative, store in the dark at 4ºC and read on the flow cytometer the following day.

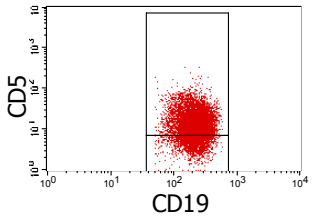
2.13.1 Extracellular Labelling

Direct cell surface staining was done with fluorescently-conjugated antibodies for TLR phenotyping studies, and activation markers by tonsil B lymphocytes.

A. Adult Blood B Lymphocyte Subsets



B. Cord Blood B Lymphocyte Subsets



C. Tonsil B Lymphocytes Subsets

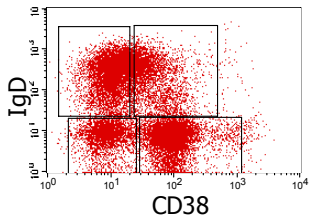


Figure 2.3 Flow Cytometry Gating Strategy for B Lymphocyte Subsets
Identification of B lymphocyte subsets from **A.** Adult blood, **B.** Cord blood and **C.** Tonsils. Adult blood B lymphocytes subsets are: B1 ($CD5^+$), B2 ($CD5^-$), naïve ($CD27^-$) and memory ($CD27^+$) B cells. Cord blood B lymphocyte subsets are: B1 ($CD5^+$) and B2 ($CD5^-$) B cells. Tonsil B lymphocyte subsets are: naive (IgD^+CD38^-), pre-germinal centre (IgD^+CD38^+), germinal centre (IgD^-CD38^+) and memory (IgD^-CD38^-) B cells.

PBS/Azide was the wash buffer for extracellular labelling of cells.

For TLR phenotyping studies, a panel of 20 fluorescent antibodies identified T lymphocytes, B lymphocytes, their respective activation status, the different subsets of B lymphocytes and whether TLR are expressed by these different cells. Antibodies were added in various combinations to individual tubes, and then incubated before cells were washed twice and fixed. The only changes were for cells labelled with anti-IgD or TLR6, as they were biotinylated antibodies. No commercial fluorescently-conjugated anti-TLR6 antibody was available, only a biotinylated anti-TLR6 antibody was found. For TLR6, after the cells were labelled with TLR6 and other markers, SA-PE was added, incubated, washed twice, and fixed. For IgD, after the tonsil cells were labelled with anti-IgD and other markers, SA-AF647 was added, incubated, washed twice, and fixed. It was not possible to simultaneously label tonsil cells with the two biotinylated antibodies, TLR6 and anti-IgD, and obtain distinct, non-interfering, fluorescent signals, despite extensive experimentation. For this reason, TLR6 expression was examined on tonsil B lymphocytes, but tonsil B cell subsets were not identified.

For TLR functional studies, expression of activation markers by tonsil B lymphocytes was determined after treatment with TLR ligands. The cells were incubated with CD19, CD23, CD25, CD69 and HLA-DR antibodies, washed twice, and fixed.

2.13.2 Intracellular Labelling

TLR phenotyping studies included intracellular staining with fluorescently-conjugated antibodies for adult blood and cord blood cells as follows:

- Stain for surface expression of lineage markers by incubation with antibodies, and then wash twice with PBS/Azide.
- Permeabilise cells with 500 μL of 1x BD FACS Permeabilization solution, incubate in the dark for 10 minutes at room temperature, centrifuge cells and carefully remove supernatant.
- Add 50 μL skim milk intracellular block to permeabilised cells and incubate for 30 minutes on melting ice in the dark.
- Add negative isotype control and TLR antibodies, incubate, wash twice using 0.1% Saponin/PBS/Azide, and fix.
- For TLR6, label cells with surface markers, permeabilise, block, label with intracellular TLR6 antibody, wash, add SA-PE, incubate, wash twice with 0.1% Saponin/PBS/Azide, and fix.

For tonsils, intracellular staining for TLR is as follows:

- Stain for surface expression of lineage markers of tonsil cells by incubation with antibodies and wash twice with PBS/Azide.
- Add SA-AF647, to bind to IgD-biotin, incubate and wash twice with PBS/Azide.
- Permeabilise cells and block as described earlier, add negative isotype controls and TLR antibodies, incubate, wash twice using 0.1% Saponin/PBS/Azide, and fix.
- For TLR6, follow with additional staining with SA-PE, as described earlier.

For intracellular staining of TLR in permeabilised cells, there was no blocking of TLR expression on the cell surface. Consequently, detection of TLR expression by permeabilised cells includes intracellular TLR expression and surface TLR expression present on the permeabilised cell membrane.

2.13.3 Indirect Immunofluorescence Labelling

A three-step high sensitivity staining protocol (187) was modified slightly and used to detect expression of co-stimulatory markers and cytokine receptors of tonsil B lymphocytes. Purified antibodies against CD21, CD40 (G28/5), CD80, CD86 and CD210, and IgG1 isotype (x63) hybridoma culture supernatant were used. For reducing non-specific binding, earlier experiments showed skim milk was more effective than human serum for blocking. The protocol follows:

- Add antibodies to 10^6 cell aliquots, incubate and wash twice. To block non-specific binding on B lymphocytes, add 100 μL skim milk block (no saponin) and incubate on ice for 30 minutes in the dark.
- Add HαMBi, incubate and wash twice before addition of 10 μL mouse serum block for 10 minutes incubation.
- Add SA-PE and CD19 antibody, and then incubate, wash twice and fix.

2.13.4 Flow Cytometry

The studies were processed on the BD FACSCalibur System (BD Biosciences). To acquire at least 3 x 10^3 B lymphocytes from blood mononuclear leucocytes, a minimum number of 10^5 total events was collected during data acquisition. For tonsil mononuclear leucocytes, 5 x 10^4 total events were acquired as tonsils are rich with B lymphocytes. As per standard practice in flow cytometry, all measurements of marker expression by flow cytometry were tested once and each expression

measurement is calculated from a sample size of thousands of cells. The collective fluorescence intensity of the cell population, such as median fluorescent intensity, will account for the variability of staining by individual cells, as seen by histograms of TLR expression showing cells with a range in fluorescence intensities.

Data was analysed with BD CellQuest Pro software (BD Biosciences). The gating strategy to identify T lymphocytes and B lymphocytes first gated the lymphocyte region, based on cell size and granularity using FSC and SSC, then fluorescent tagging of CD3 for T lymphocytes and CD19 for B lymphocytes (Refer Figures 2.1 and 2.2). Lymphoblasts were identified by CD3 or CD19 expression and SSC of cells. The subsets of B lymphocytes from each subject group were identified by lineage markers (Refer Figure 2.3). Though cellular viability was not examined with a marker, cells were labelled and fixed to minimise loss and the gating strategy (Refer Figures 2.1 and 2.2) excluded dead cells which are FSC^{lo} SSC^{hi} (188).

2.13.5 Analysis of Expression of TLR and Other Markers

Expression of TLR was unimodal so the MFI of cell populations was used; calculating percentage of positive cells was not appropriate as TLR expression was not bimodal. Kolmogorov-Smirnov (KS) statistics (189) is a non-parametric test that can be used to compare two datasets in flow cytometry; in this instance it allows comparisons of fluorescent histograms between a control and test. However, KS statistics are highly sensitive, especially for large sample sizes, and calculate highly significant differences between two histograms which are virtually identical. In these instances the biological significance of two virtually identical histograms would be dubious. Therefore, to calculate TLR expression, percentages and K-S statistics were not used and MFI ratios were preferred.

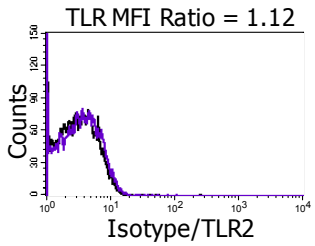
To detect whether TLR were expressed, the ratio of TLR MFI to the MFI of the matched negative isotype control was calculated. This method of measurement has been reported elsewhere (190), though mean fluorescence intensity was used. Based on earlier experiments and advice by expert flow cytometrists, a MFI ratio value of 1.3 was arbitrarily selected as the value for positive expression of TLR. A minimum of a 30% increase on the MFI of the negative isotype control for the TLR MFI was considered sufficient to support positive expression of TLR and account for any background fluorescence. A MFI ratio of 1.3 or greater exhibited a shift in PE fluorescence large enough to confirm positive expression of TLR. (Figure 2.4)

For expression of activation markers, co-stimulatory molecules and cytokine receptors by tonsil B lymphocytes, the distribution was either unimodal or bimodal. HLA-DR, CD21, CD40, CD80, CD86 and CD210 were all expressed as a unimodal distribution, so MFI ratio values were used to assess expression levels. CD23, CD25, CD69 and CD95 expression was bimodal so differences in expression were determined by the proportion of positive or negative cells. MFI ratio or percent positive cells assessed the expression of cell markers by tonsil B lymphocytes in response to TLR ligands.

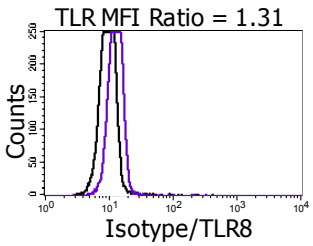
2.13.6 Statistical Analysis of Expression of TLR and Other Markers

Statistical analysis on TLR expression was not possible because as TLR expression was assessed as positive or negative based on the TLR MFI ratio of 1.3, the results were qualitative data. Though quantitative information from TLR MFI ratios was available, this study was interested only in assessing the presence or absence of TLR. Therefore statistical comparisons of group means would be confusing as the

A. TLR2



B. TLR8



C. TLR9

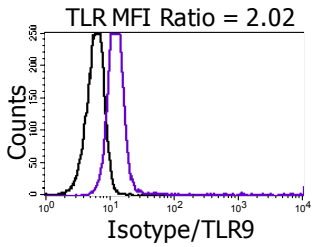


Figure 2.4 TLR MFI Ratio
TLR MFI ratio calculated by TLR MFI divided by negative isotype control MFI. TLR MFI ratio greater than 1.3 is designated as positive for TLR expression. TLR MFI ratios below, at and above 1.3 is represented by **A.** TLR2, **B.** TLR8 and **C.** TLR9, respectively. (TLR = purple, negative isotype control = black)

results are qualitative, not quantitative. If all subjects in both groups express TLR, statistical analysis won't reveal further information. For this reason, only descriptive statistics for TLR expression are presented as numbers of subjects out of three that were positive for TLR expression.

Expression of activation markers, co-stimulatory molecules and cytokine receptors by tonsil B lymphocytes in response to TLR ligands was statistically analysed using Univariate analysis-of-variance (ANOVA) (191) and values of $p < 0.05$ were considered statistically significant. Raw data for CD21, CD80, CD86 and HLA-DR was transformed by logarithm function to obtain normal frequency distribution of data; for other molecules, raw data was normally distributed and did not require transformation. For data analysis, there was a Simple Contrast of "Between-Subjects Factors" which compared timepoint zero and TLR ligands to the RF10 media control. Although the tonsil group has three subjects, all three subjects are all treated with the same TLR ligands under the same conditions, therefore it is possible to compare the differences in levels of marker expression.

2.14 Isolation of B Lymphocytes

For TLR functional studies, purified B lymphocytes were required to determine the direct effects of TLR engagement on B lymphocytes, not the indirect effects of TLR-engaged T lymphocytes or monocytes on B lymphocyte function. As erythrocytes are immunologically inert cells, their presence was not expected to interfere with TLR effects on B lymphocyte. A commercial kit, RosetteSep™ Human B cell Enrichment (Stemcell Technologies; catalogue no. 15064), was used for B cell purification from adult blood, cord blood and tonsil cells. The RosetteSep™

tetramer bi-specific antibody reagent contains multiple antibody specificities to react with erythrocytes and leucocytes so erythrocytes encircle and bind to all leucocytes, except B lymphocytes, and then are removed via density centrifugation leaving negatively selected B lymphocytes (Figure 2.5). The protocol was modified slightly for efficiency and economy as follows:

- Add FBS to commercial, sterile, PBS at 2% v/v (FBS/PBS).
- For erythrocytes, collect whole blood in Acid Citrate Dextrose Whole Blood Tubes (BD Vacutainer, BD Biosciences; catalogue no. 364606) and store at 4°C. For use, remove blood from the tube, wash thrice (700 x g, 10 minutes, no brake) with FBS/PBS and skim the blood interface after each centrifugation to remove leucocytes for washed erythrocytes.
- Extract mononuclear leucocytes from sample with Lymphoprep separation, calculate cell count and add appropriate number of washed erythrocytes for leucocyte:erythrocyte ratio of 1:50.
- Centrifuge cells (700 x g, 10 minutes, no brake), carefully remove supernatant with sterile pipette and resuspend in sufficient volume of FBS/PBS to obtain 5 x 10^7 leucocytes/mL.
- Add 50 µL of RosetteSep™ per 5 x 10^7 leucocytes. Mix carefully with gentle inversion to avoid air bubble formation. Incubate at room temperature for 20 minutes with gentle mixing every 5 minutes.
- Dilute cell suspension 1:4 with FBS/PBS. Using a large bore pipette to preserve rosettes, carefully layer cell suspension over Lymphoprep (one volume of Lymphoprep for two volumes of diluted samples).
- Follow adult blood procedure for Lymphoprep separation. Use FBS/PBS as diluent.

- After collection of purified B lymphocytes, remove an aliquot to examine for B cell purity with CD19, CD45 and 10F7 antibodies via flow cytometry.
- Resuspend purified B lymphocytes in RF10 to desired cell count for later assays.

The purity of enriched B lymphocytes in the final cell fraction was examined by flow cytometry studies identifying numbers of $CD45^+$ leucocytes, a pan-leucocyte marker (192), and $CD19^+$ B lymphocytes, and then calculating the percentage of $CD19^+$ B lymphocytes to $CD45^+$ leucocytes. The purity of B lymphocytes, to other leucocytes, in the cell fraction was greater than 97% for tonsil subjects, 83.9% – 94.8% for adults, and 50% - 74.5% for cord blood (Table 2.3).

The purity of B lymphocytes enriched from cord blood was poorer compared to B lymphocytes enriched from adult blood and tonsils. The B lymphocytes enriched from cord blood contained a large proportion of erythrocyte precursors. An explanation for this is erythrocyte precursors, but not mature erythrocytes, express CD45 (193); this contamination would interfere with the assessment of B cell purity in cord blood. The poorer purity of cord blood B lymphocytes may be due to cord blood erythrocytes precursors being labelled with CD45 and falsely elevating the leucocyte count when calculating B cell purity.

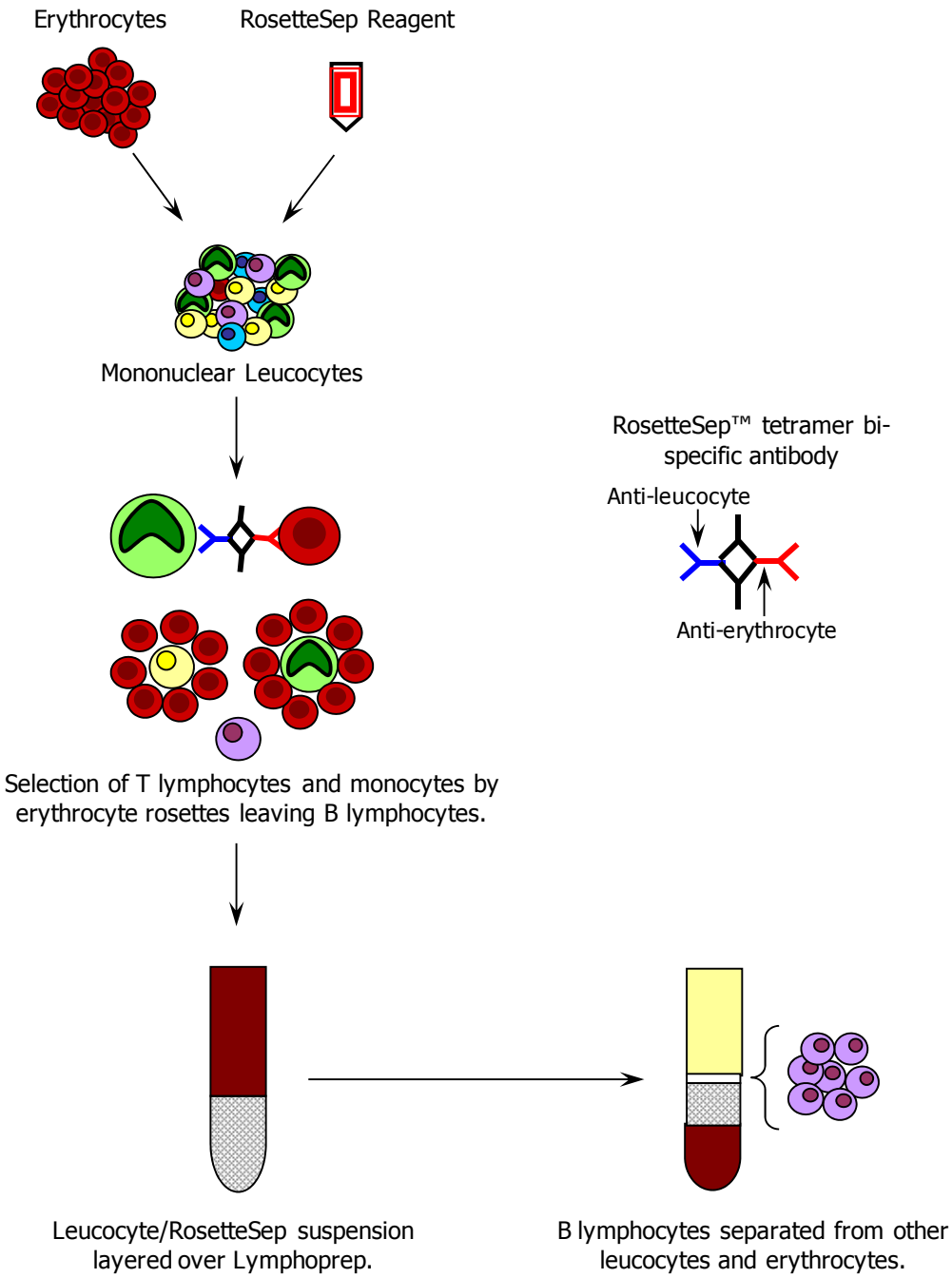


Figure 2.5 Protocol for StemCell RosetteSep™ Human B cell Enrichment Mononuclear leucocytes incubated with erythrocytes and RosetteSep™ bi-specific tetramer antibody reagent. Erythrocytes encircle all leucocytes, excluding B lymphocytes, for negative selection of B lymphocytes. Cell suspension layered over Lymphoprep for density separation of cells by centrifugation. Purified B lymphocytes isolated.

Table 2.3 Purity of Enriched B Lymphocytes (percentage $CD19^+$ cells/$CD45^+$ cells)

	Individual Group Subject		
	4	**5**	**6**
Adult Blood	94.4	83.9	94.8
Cord Blood	50.0	74.5	66.6
Tonsils	97.7	98.5	98.7

2.15 TREATMENT OF B LYMPHOCYTES WITH TLR LIGANDS

Ligands of detected TLR from phenotyping studies were used to treat B lymphocytes isolated from adult blood, cord blood and tonsils for TLR functional studies. TLR ligands were selected on the advice of Dr Ashley Mansell, Monash Institute of Medical Research, Melbourne. The ligands were:

- TLR3 ligand - Poly(I):Poly(C) (Amersham Biosciences, GE Healthcare; catalogue no. 27-4729) (Poly I:C)
- TLR4 ligand – Lipopolysaccharide (Sigma Aldrich Inc; catalogue no. L-2143) (LPS)
- TLR8 ligand – CL075 (InvivoGen; catalogue no. tlrl-c75)
- TLR9 ligand – CpG B ODN 2006 motifs; sequence T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T (145) (Geneworks Pty Ltd, Australia) (CpG ODN)

Poly I:C, LPS and CL075 were reconstituted according to manufacturer's instructions and CpG ODN was dissolved in sterile, deionised water. All ligands were titrated for optimal concentration to activate B lymphocytes. TLR ligand

treatment of purified B lymphocytes from the three groups was carried out as follows:

- For the proliferation assay, resuspend B lymphocytes in warm RF10 at 10^6 cells/mL and aliquot 10^5 B cells per well into 5 rows of triplicate wells in a round-bottomed 96-well tissue culture plate (Greiner Bio-One; catalogue no. 650-180).
- Dilute ligands in RF10 and aliquot into an individual row of triplicate wells, the final well volume is 200 μL with final ligand concentrations; first row is RF10, second is 10 μg/mL Poly I:C, third is 100 ng/mL LPS, fourth is 10 μM CL075, and fifth is 100 nM CpG ODN.
- Incubate plate for 3 days at 37ºC in 5% CO_2 incubator, then measure proliferation with incorporation of [^{3}H] thymidine.
- Set up second plate like the proliferation assay, except prepare five rows of two sets of triplicate wells (total of six), incubate this plate for five days, then harvest supernatant and store at -20ºC until performing assays to measure antibody and cytokine levels.

B lymphocytes were purified in large numbers from tonsils allowing further investigation into its expression of co-stimulatory marker and cytokine receptors after TLR ligand treatment. The very low numbers of B lymphocytes from adult blood and cord blood did not permit similar investigations. Tonsil B lymphocytes were treated with TLR ligands as follows:

- Allocate an aliquot of tonsil B lymphocytes for marker expression at timepoint zero.
- Suspend remaining tonsil B lymphocytes in RF10 at 2 x 10^6 cells/mL. Aliquot 5 x 10^6 B cells per well into 5 wells in 5 plates of 6-well tissue culture

plates. Assign each plate a TLR ligand, and after addition of TLR ligand the final well volumes are 5 mL.

- Treat B lymphocytes with; RF10 in the first plate, 10 μg/mL Poly I:C in the second, 100 ng/mL LPS in the third, 10 μM CL075 in the fourth and 100 nM CpG ODN in the fifth plate.
- Incubate plates for 24 hours at 37°C in 5% CO_2 incubator, then harvest cells with a rubber policeman.
- Pool the cells treated with the same reagent, then wash twice and resuspend in PBS/Azide at 10^7 cells/mL before IF staining for flow cytometric analysis of activation, co-stimulatory markers and cytokine receptor expression.

2.16 PROLIFERATION ASSAY

Proliferation was determined by incorporation of [^{3}H] thymidine by B lymphocytes on the third day of treatment with TLR ligands. B lymphocytes were pulsed with 1 μCi [^{3}H] thymidine (GE Healthcare; catalogue no. TRK120) per well for 6 hours prior to cell washing and harvesting by FilterMate Harvester (PerkinElmer Inc.). Betaplate Scint (PerkinElmer Inc.; catalogue no. 1250-440) was added to the harvested cells that were then read and analysed by 1450 MicroBeta Jet: LSC & Luminescence Counter (PerkinElmer Inc.) to determine rate of proliferation by B lymphocytes. Proliferation from triplicate cultures was in counts per minute (c.p.m.) and expressed as mean with standard error.

2.17 ELISA FOR IG, IGG AND IGM LEVELS IN CELL CULTURE SUPERNATANTS

Supernatants from B lymphocytes after five days of treatment with TLR ligands were thawed just before ELISA assays were performed. The groups were tested simultaneously and ELISA assays for total Ig, IgG and IgM quantification were performed on consecutive days. ELISA were performed in triplicate and, if necessary, repeated with different dilutions of supernatant. ELISA antibodies were optimised at 1/500 for Ig Capture antibody and 1/2000 for all three detection antibodies using ELISA diluent buffer. Standards for Ig, IgG and IgM were pooled human sera. The ELISA protocol was identical for each Ig, IgG and IgM assay as follows:

- Coat three 96-well plates of Polysorb ELISA wells (Nunc™; catalogue no. 469922) with Ig Capture antibody overnight at 4ºC and wash x 5 with ELISA wash buffer the following day.
- Block wells with ELISA blocking buffer for one hour at room temperature and wash x 5.
- Dilute standards, internal controls and test samples with ELISA diluent buffer, aliquot into the wells, incubate at room temperature for two hours, and then wash x 5.
- Aliquot the appropriate HRP-conjugated Detection antibody into wells, incubate at room temperature for two hours, wash x 5, add the OPD substrate, and incubate for 30 minutes at room temperature in the dark.
- Using Sunrise™ microplate absorbance reader (Tecan Group Ltd), read the plates for absorbance at 450 nm.

ELISA results were analysed by MultiCalc software (PerkinElmer Inc.) and values (ng/mL) were presented as mean with standard error.

2.18 CBA FOR HUMAN INFLAMMATORY CYTOKINES IN CELL CULTURE SUPERNATANT

A commercial kit, BD Cytometric Bead Array (CBA) Human Inflammation Kit (BD Biosciences; catalogue no. 551811), measured IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF levels in B lymphocyte culture supernatant. These kits were provided by BD Biosciences, courtesy of Dr Homero Sepulveda and Dr Robert Balderas. Capture beads, with six levels of fluorescence intensities to correspond to a different cytokine, bind to cytokines present in culture supernatant. PE-conjugated antibodies against the six cytokines were added to form an antibody-sandwich complex with the cytokine in the centre. This antibody-sandwich complex is determines which cytokines are present and the PE intensity allows quantification of the detected cytokine (Figure 2.6). Supernatants were thawed just before testing, used undiluted and assayed in triplicate. The nine subjects were tested over three days; three subjects, one from each group, were tested each day. The CBA kit was used as per manufacturer's instructions. Briefly, capture beads and PE detection reagent were incubated with standards, internal controls and test samples for 3 hours, then washed and analysed on FACSCalibur where 5000 events in region 1 were collected. Results were analysed on CellQuest software using the template for CBA analysis.

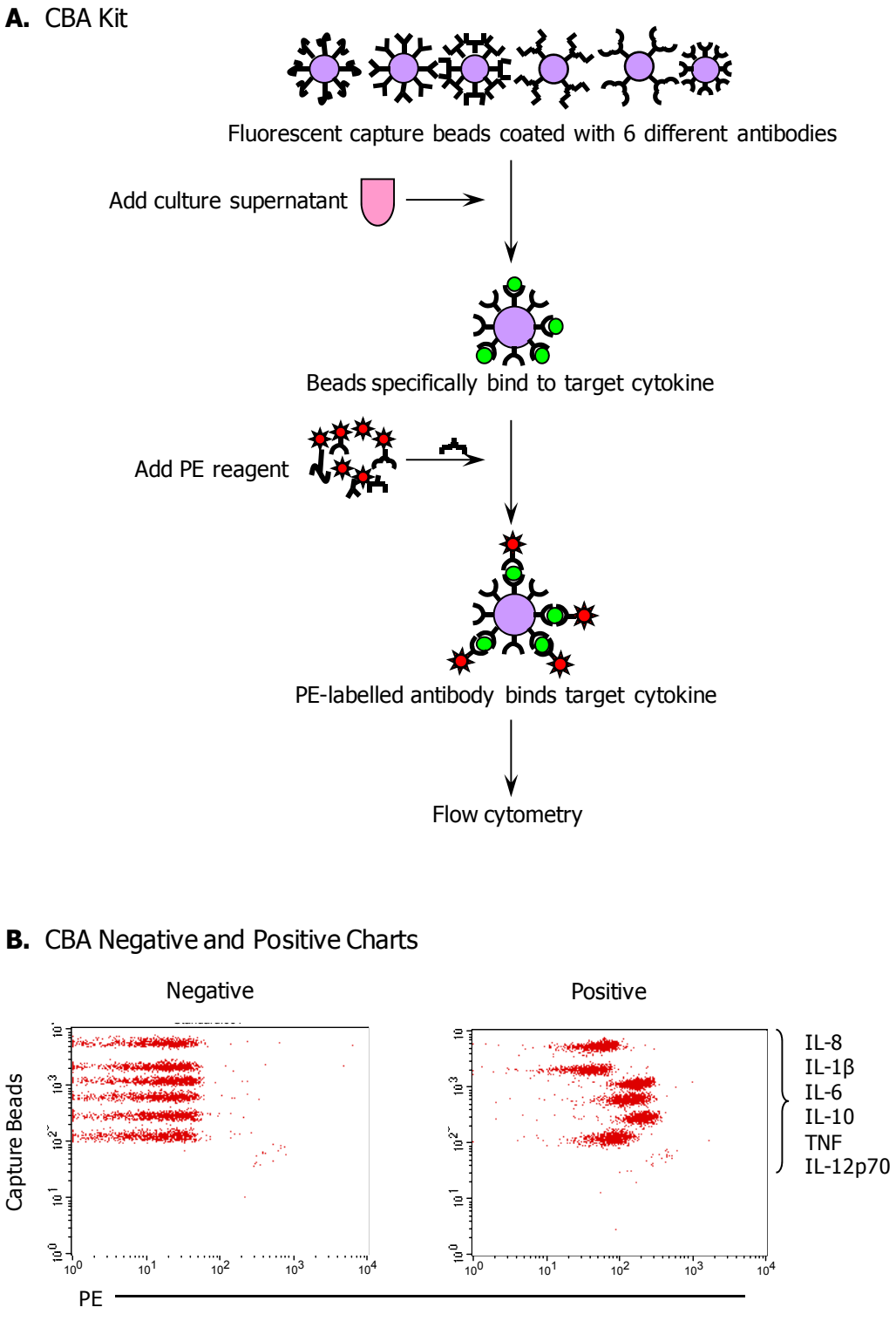


Figure 2.6 Protocol for CBA Kit
A. Flow cytometry procedure for quantitation of cytokines from culture supernatant using antibody-labelled fluorescent beads and PE-conjugated antibodies. **B.** Charts of negative and positive standards illustrating the different fluorescent intensities of the beads and their individual specificities to particular cytokines, and the detection of cytokines through the PE antibodies.

2.19 Statistical Analysis

For statistical analysis of proliferation, antibody and cytokine data, Repeated-measures ANOVA (191) was applied to compare within TLR ligands, and Bonferroni post hoc analysis compared results between the adult, neonate and tonsil groups. Values of $p < 0.05$ were considered statistically significant. All raw data was transformed by logarithm function to obtain normal frequency distribution of data prior to testing. For Repeated-measures ANOVA to analyse within TLR ligands, the replicates were assessed by "Within-Subjects Factors", and Simple Contrast of "Between-Subjects Factors" compared TLR ligands to the RF10 media control. For Bonferroni post hoc analysis between the groups, the two sets of "Within-Subjects Factors" were the replicates and TLR ligands, and the "Between-Subjects Factors" were the groups of adults, neonates and tonsils.

CHAPTER THREE

EXPRESSION OF TOLL-LIKE RECEPTORS BY T LYMPHOCYTES FROM ADULT BLOOD AND CORD BLOOD

3.1 INTRODUCTION

As described in Chapter One, the lack of development in the immune system of neonates impairs their ability to mount a response against infection, hence the greater reliance of neonates on innate immunity. Neonatal T lymphocytes, compared to adult T lymphocytes, have reduced adaptive immune functions. TLR, directly and indirectly, activate T lymphocytes and instigate several responses including tailored cytokine secretion and induction of T_H1 responses and regulation of immune activity. In this chapter the potential role of TLR in neonatal immunity will be explored by comparing TLR expression patterns of non-stimulated and stimulated T lymphocytes from adult blood and cord blood. As most of the TLR research literature is on adult subjects, this study is intended to provide data on TLR expression at the early stage of immune development.

At the start of this study, the literature on TLR expression had little information about TLR protein because few TLR antibodies were available, the bulk of expression studies were on TLR mRNA. As described in Table 1.2, RT-PCR studies on TLR mRNA showed all TLR, except TLR7 and TLR10, were detected in adult blood T lymphocytes. With increasing antibody availability, TLR expression by adult T lymphocytes was reported for TLR1, TLR2, TLR3, TLR4, TLR5 and TLR9 (86-89) and TLR6, TLR8 and TLR10 by T regulatory cells (89).

According to these studies, there was surface expression of TLR1, TLR2, TLR4, TLR5, TLR6, TLR8 and TLR10, and internal expression of TLR3, TLR5 and TLR9. A literature search revealed only one report on TLR expression by cord blood cells, which showed internal expression of TLR2 and TLR4 by T cells (84). Further data about TLR expression by cord blood T lymphocytes may clarify whether TLR can engage and activate neonatal T lymphocytes and affect their function.

Literature on TLR expression in relation to activation states of lymphocytes is scant, but some studies examine how diseases affect TLR expression. Activation by TCR-engagement can stimulate expression of TLR2 and TLR4 by cord blood T_H cells (84), TLR5 by adult blood T_H cells (87) and TLR10 by adult blood T_{REG} cells (89). With disease, tonsillitis induces higher levels of TLR2, TLR3 and TLR5 expression by tonsil T_C cells (98), and lymphatic filariasis decreases TLR1, TLR2 and TLR4 expression and increases TLR9 expression by T lymphocytes (88). Ascertaining how TLR expression by T lymphocytes can be affected by the cell activation state, or by disease, is necessary to comprehend TLR influence on immune responses.

For TLR phenotyping of T lymphocytes from adult blood and cord blood, several parameters were examined to provide a comprehensive picture. For this study, the following TLR: TLR1, TLR2, TLR3, TLR4, TLR6, TLR8 and TLR9, were tested on non-stimulated and stimulated lymphocytes to detect expression. To reflect activation states, T lymphocytes were categorised into T cells or T blasts and TLR expression was assessed. Extracellular and intracellular expression was examined to determine the surface and endosomal locations of TLR.

3.2 RESULTS

3.2.1 TLR Expression by T Lymphocytes

The extracellular and intracellular expression of TLR1, TLR2, TLR3, TLR4, TLR6, TLR8 and TLR9 by non-stimulated and stimulated T lymphocytes from adult blood (n = 3) and cord blood (n = 3) were examined. As described earlier (Refer Chapter Two, Section 2.8), after enrichment of mononuclear cells from blood each cell suspension was divided into three for TLR phenotyping studies; one aliquot for non-stimulated lymphocytes, a second aliquot for stimulating T lymphocytes for 24 hours, and the third aliquot for B lymphocyte studies (Refer Chapter Four). Using anti-CD3 antibody and cell-to-cell interactions with monocytes, T lymphocytes were activated via engagement of CD3 and co-stimulation with monocytes in the presence of IL-2 and IL-4. T lymphocytes were identified with FITC-conjugated CD3 antibody, followed by detection of TLR with PE-conjugated antibodies. T lymphocytes were classed as T cells or T blasts based on cell size and granularity (Refer Chapter Two, Figure 2.1). TLR were detected on non-permeabilised cells for extracellular expression, and in permeabilised cells for intracellular expression. TLR expression by permeabilised cells is the sum total of intracellular expression and surface TLR expression on permeabilised cell membranes (Refer Chapter Two, Section 2.13.2). TLR expression was compared with matched negative isotype controls and expression levels greater than MFI ratio value 1.3 were deemed positive for presence of TLR (Refer Chapter Two, Figure 2.4). Statistical comparison of TLR expression between adult blood and cord blood was not done as results were deemed positive or negative for TLR expression (Refer Chapter Two, Section 2.13.6). Instead, TLR expression was presented as descriptive data in Table 3.1.

3.2.1.1 TLR1

Adult blood T lymphocytes:

- Non-stimulated T cells: one subject expressed TLR1 in permeabilised cells.
- Non-stimulated T blasts: two subjects expressed TLR1 on non-permeabilised cells and all three subjects expressed TLR1 in permeabilised cells.
- Stimulated T cells: two subjects expressed TLR1 in permeabilised cells.
- Stimulated T blasts: one subject expressed TLR1 in permeabilised cells and another subject expressed TLR1 by non-permeabilised and permeabilised cells.

(Figures 3.1, 3.2 and Table 3.1)

Cord blood T lymphocytes:

- Non-stimulated T cells: one subject expressed TLR1 in permeabilised cells.
- Non-stimulated T blasts: two subjects expressed TLR1 by non-permeabilised and permeabilised cells.
- Stimulated T cells: no TLR1 expression was detected.
- Stimulated T blasts: one subject expressed TLR1 in permeabilised cells and another subject expressed TLR1 by non-permeabilised and permeabilised cells.

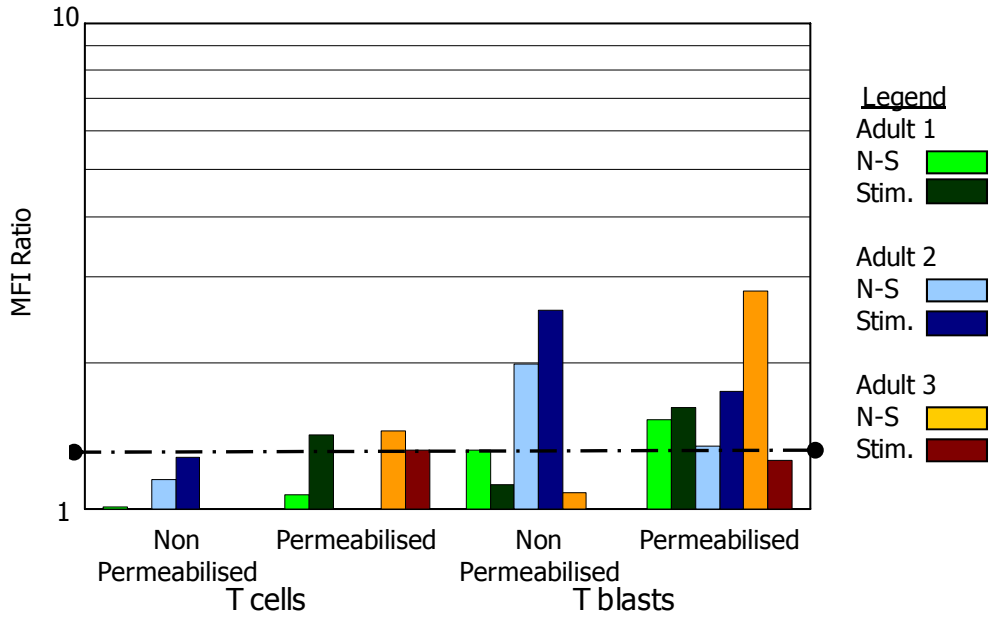
(Figure 3.1, 3.2 and Table 3.1)

3.2.1.2 TLR2

Adult blood T lymphocytes:

- Non-stimulated T cells: one subject expressed TLR2 in permeabilised cells.

A. Adult Blood T Lymphocytes



B. Cord Blood T Lymphocytes

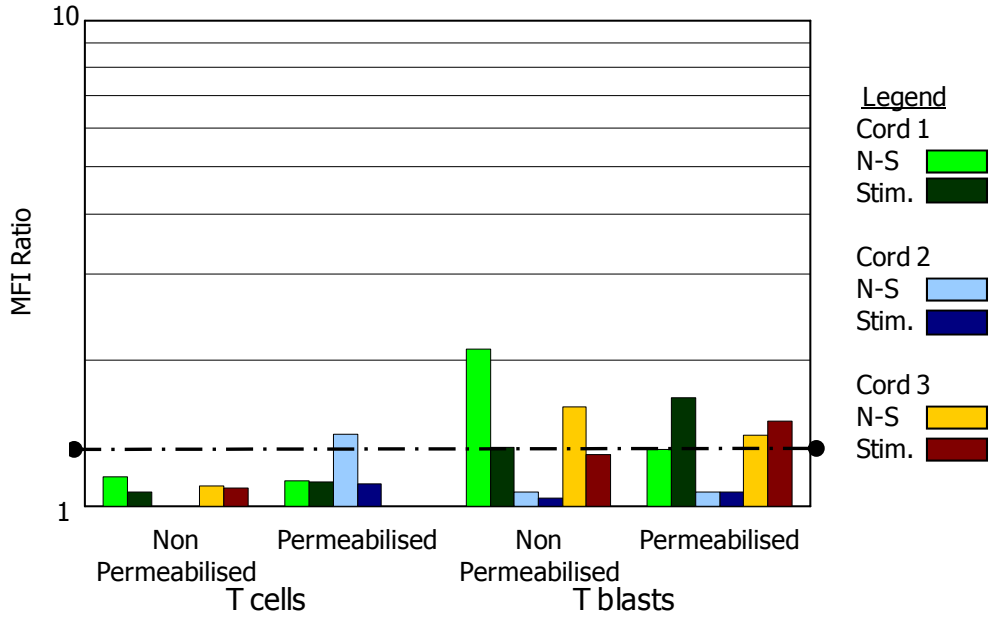
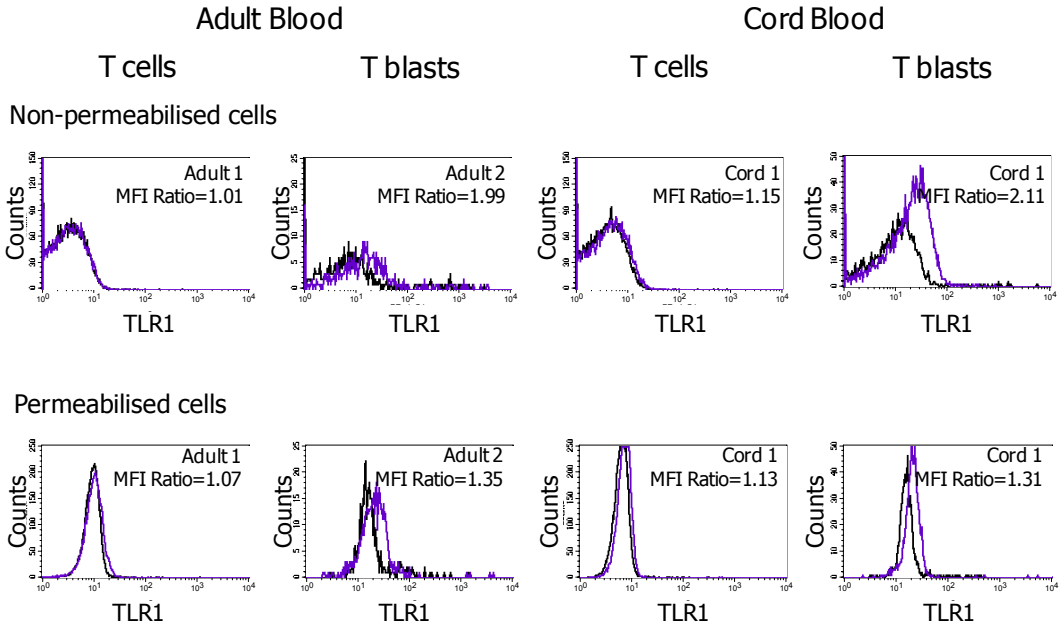


Figure 3.1 TLR1 Expression by T Lymphocytes
MFI ratio of TLR1 expression by non-stimulated (N-S) and stimulated (Stim.) T lymphocytes, both non-permeabilised and permeabilised, from **A.** Adult blood (n = 3) and **B.** Cord blood (n = 3). Dotted black line corresponds to MFI ratio of 1.3.

A. Non-Stimulated T Lymphocytes



B. Stimulated T Lymphocytes

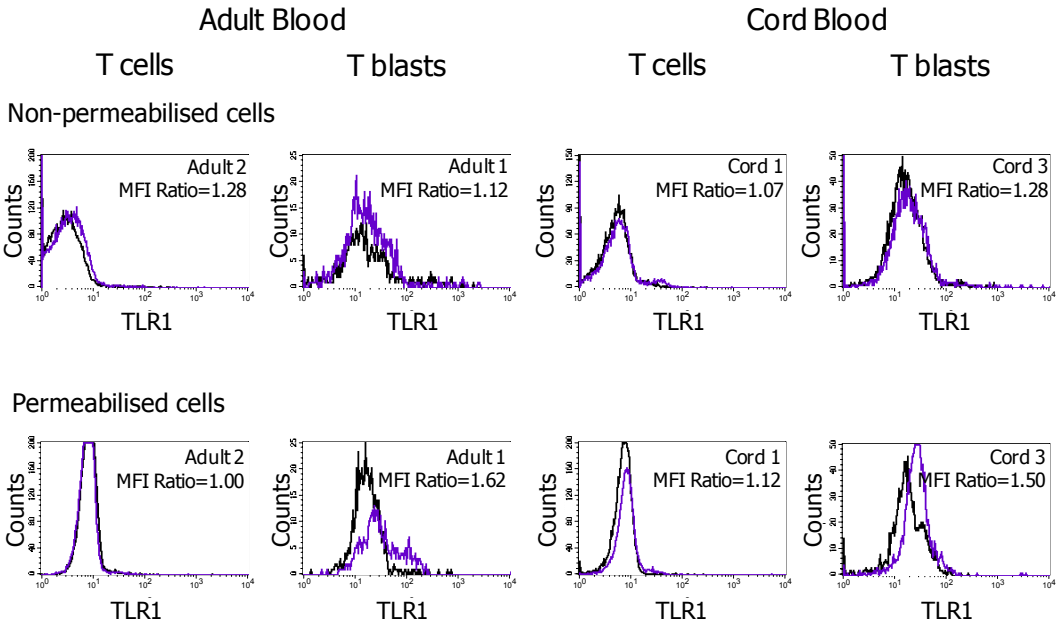


Figure 3.2 TLR1 Expression by T Lymphocytes
Representative example of TLR1 expression by T cells and T blasts, both non-permeabilised and permeabilised, from **A.** Non-stimulated adult blood and cord blood and **B.** Stimulated adult and cord blood. (TLR1 = purple, negative isotype control = black)

- Non-stimulated T blasts: all three subjects expressed high levels of TLR2 on non-permeabilised cells and low levels of TLR2 in permeabilised cells.
- Stimulated T cells: all three subjects expressed TLR2 on non-permeabilised cells and one subject expressed TLR2 in permeabilised cells.
- Stimulated T blasts: all three subjects expressed TLR2 on non-permeabilised cells and two subjects expressed TLR2 in permeabilised cells.

(Figures 3.3, 3.4 and Table 3.1)

Cord blood T lymphocytes:

- Non-stimulated T cells: no TLR2 was detected.
- Non-stimulated T blasts: all three subjects expressed high levels of TLR2 on non-permeabilised cells and low levels of TLR2 in permeabilised cells.
- Stimulated T cells: all three subjects expressed TLR2 on non-permeabilised cells.
- Stimulated T blasts: all three subjects expressed TLR2 on non-permeabilised cells and two subjects expressed TLR2 in permeabilised cells.

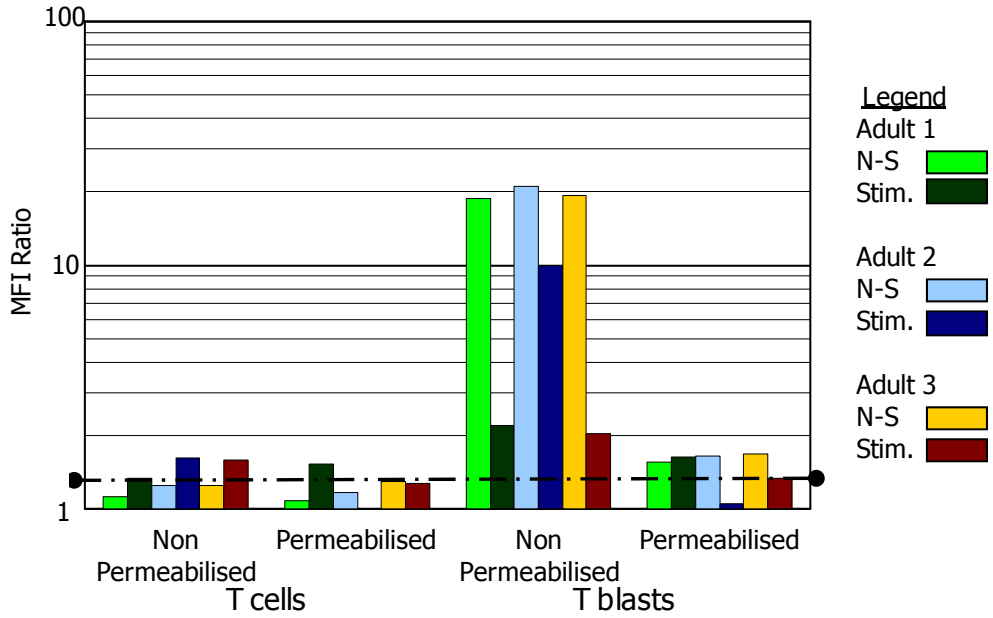
(Figures 3.3, 3.4 and Table 3.1)

3.2.1.3 TLR3

Adult blood T lymphocytes:

- Non-stimulated T cells: all three subjects expressed TLR3 in permeabilised cells.
- Non-stimulated T blasts: all three subjects expressed TLR3 on non-permeabilised cells and two subjects expressed TLR3 in permeabilised cells.
- Stimulated T cells: all three subjects expressed bright TLR3 in permeabilised cells.

A. Adult Blood T Lymphocytes



B. Cord Blood T Lymphocytes

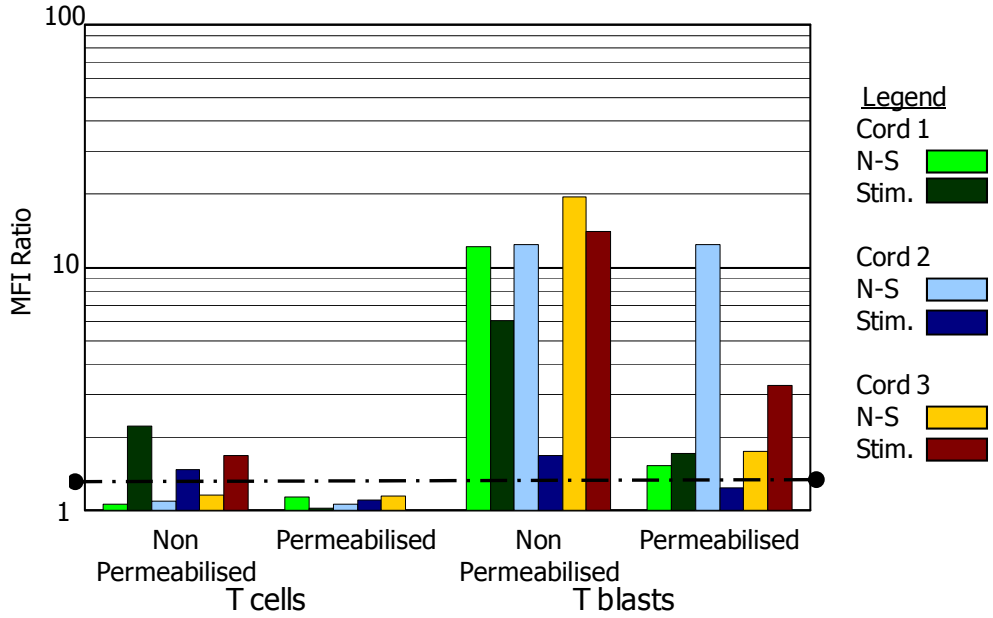


Figure 3.3 TLR2 Expression by T Lymphocytes
MFI ratio of TLR2 expression by non-stimulated (N-S) and stimulated (Stim.) T lymphocytes, both non-permeabilised and permeabilised, from **A.** Adult blood (n = 3) and **B.** Cord blood (n = 3). Dotted black line corresponds to MFI ratio of 1.3.

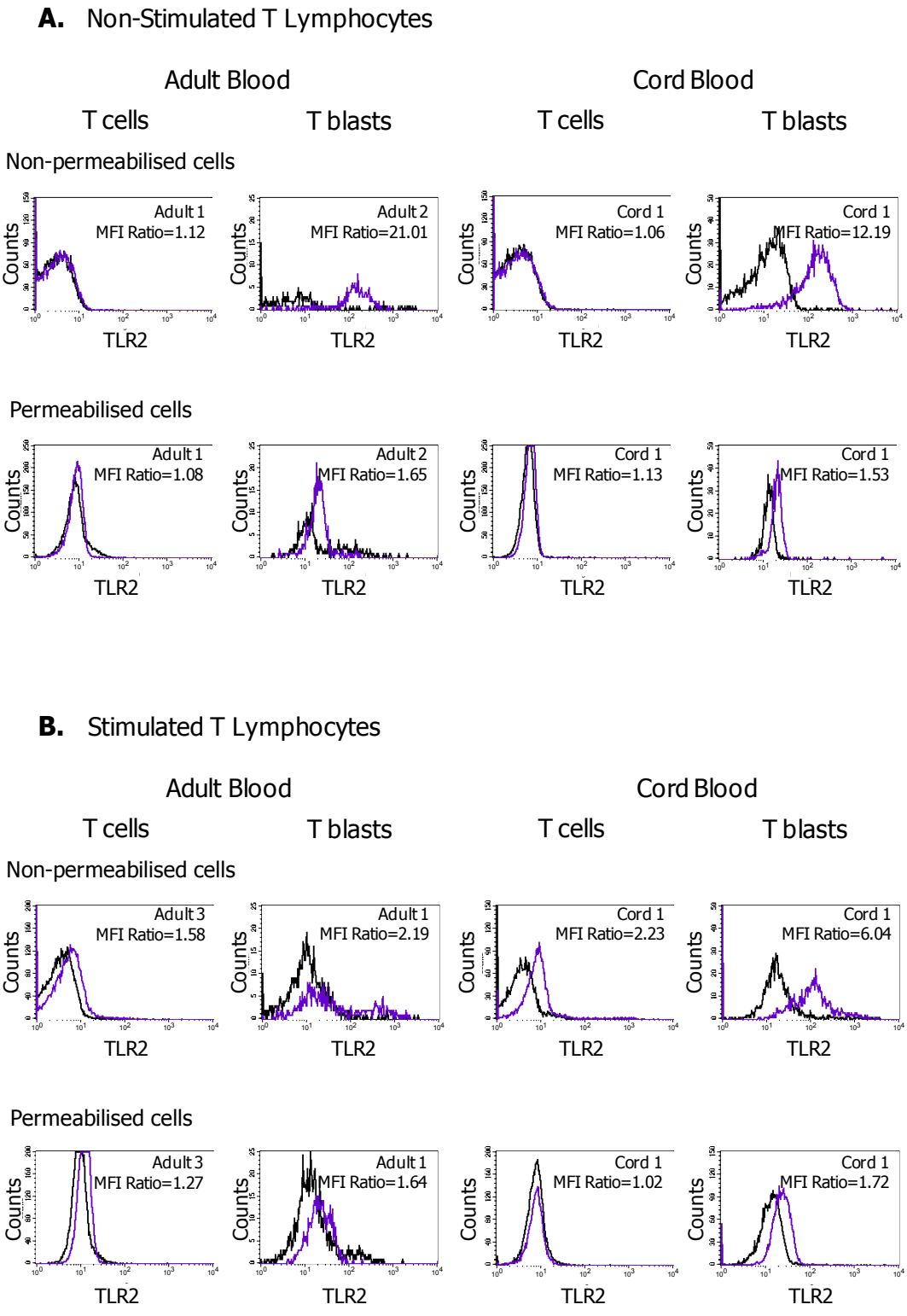


Figure 3.4 TLR2 Expression by T Lymphocytes
Representative example of TLR2 expression by T cells and T blasts, both non-permeabilised and permeabilised, from **A.** Non-stimulated adult blood and cord blood and **B.** Stimulated adult and cord blood. (TLR2 = purple, negative isotype control = black)

- Stimulated T blasts: all three subjects expressed high levels of TLR3 on non-permeabilised cells and two subjects expressed TLR3 in permeabilised cells.

(Figure 3.5, 3.6 and Table 3.1)

Cord blood T lymphocytes:

- Non-stimulated T cells: all three subjects expressed high levels of TLR3 in permeabilised cells.
- Non-stimulated T blasts: all three subjects expressed TLR3 by non-permeabilised and permeabilised cells.
- Stimulated T cells: one subject expressed TLR3 on non-permeabilised cells and all three subjects expressed high levels of TLR3 in permeabilised cells.
- Stimulated T blasts: two subjects expressed TLR3 on non-permeabilised cells and all three subjects expressed TLR3 in permeabilised cells.

(Figure 3.5, 3.6 and Table 3.1)

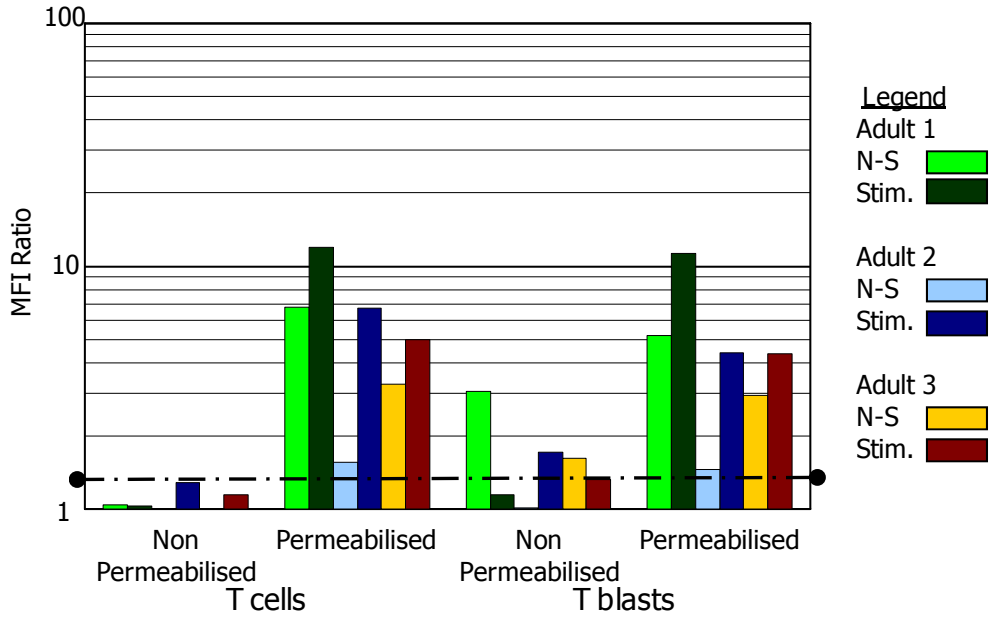
3.2.1.4 TLR4

Adult blood T lymphocytes:

- Non-stimulated T cells: all three subjects expressed TLR4 in permeabilised cells.
- Non-stimulated T blasts: all three subjects expressed high levels of TLR4 on non-permeabilised cells and low levels of TLR4 in permeabilised cells.
- Stimulated T cells: two subjects expressed TLR4 in permeabilised cells.
- Stimulated T blasts: two subjects expressed TLR4 on non-permeabilised cells and all three subjects expressed TLR4 in permeabilised cells.

(Figure 3.7, 3.8 and Table 3.1)

A. Adult Blood T Lymphocytes



B. Cord Blood T Lymphocytes

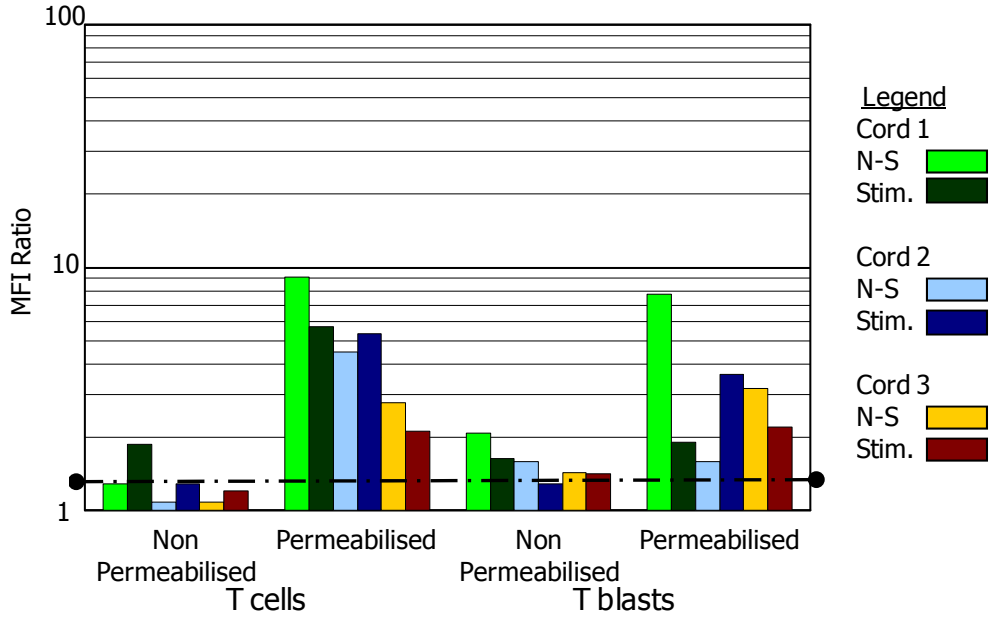


Figure 3.5 TLR3 Expression by T Lymphocytes
MFI ratio of TLR3 expression by non-stimulated (N-S) and stimulated (Stim.) T lymphocytes, both non-permeabilised and permeabilised, from **A.** Adult blood (n = 3) and **B.** Cord blood (n = 3). Dotted black line corresponds to MFI ratio of 1.3.

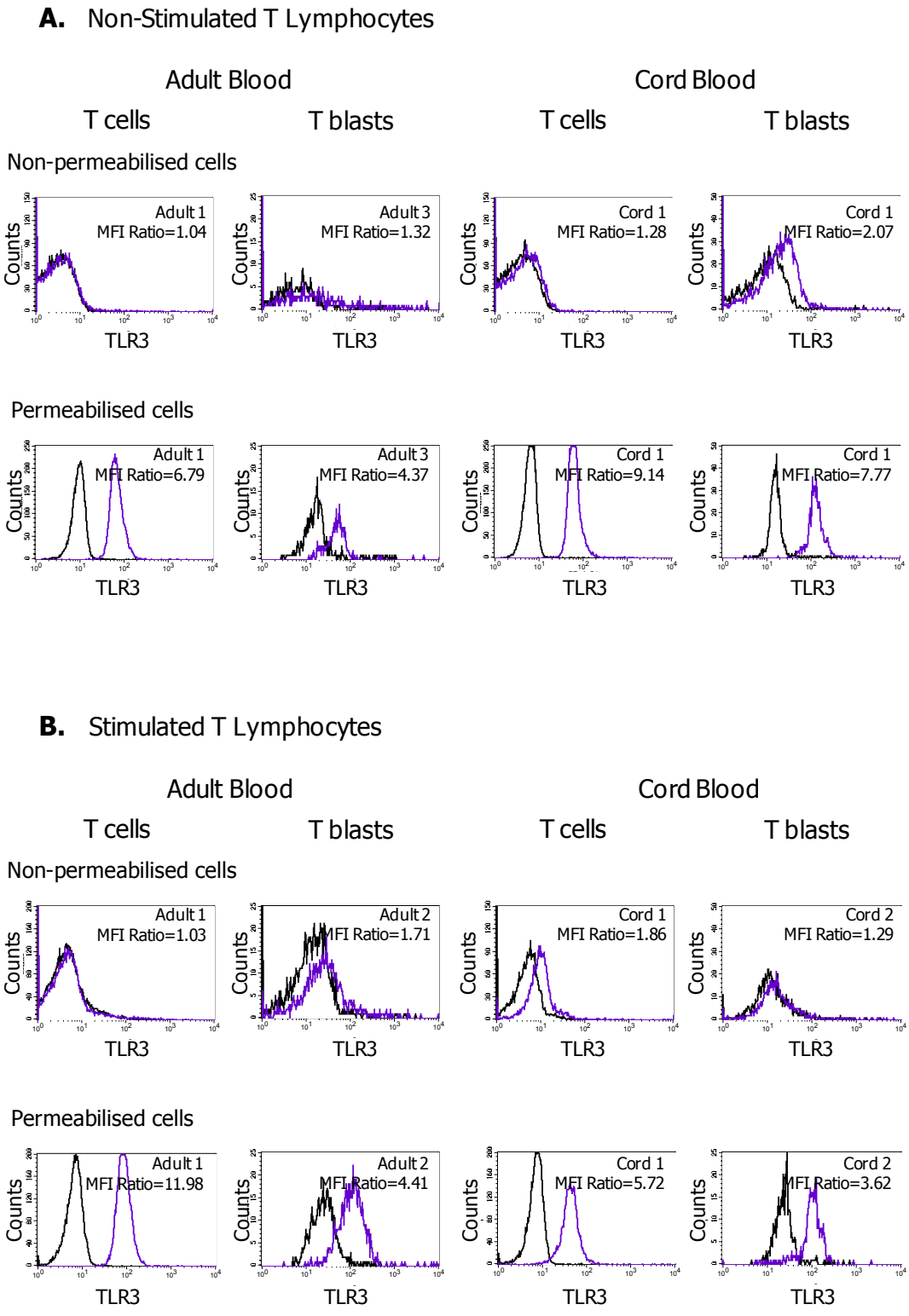


Figure 3.6 TLR3 Expression by T Lymphocytes
Representative example of TLR3 expression by T cells and T blasts, both non-permeabilised and permeabilised, from **A.** Non-stimulated adult blood and cord blood and **B.** Stimulated adult and cord blood. (TLR3 = purple, negative isotype control = black)

Cord blood T lymphocytes:

- Non-stimulated T cells: two subjects expressed TLR4 in permeabilised cells.
- Non-stimulated T blasts: all three subjects expressed high levels of TLR4 on non-permeabilised cells and lower levels of TLR4 in permeabilised cells.
- Stimulated T cells: all three subjects expressed TLR4 on non-permeabilised cells and one subject expressed TLR4 in permeabilised cells.
- Stimulated T blasts: all three subjects expressed TLR4 by non-permeabilised and permeabilised cells.

(Figure 3.7, 3.8 and Table 3.1)

3.2.1.5 TLR6

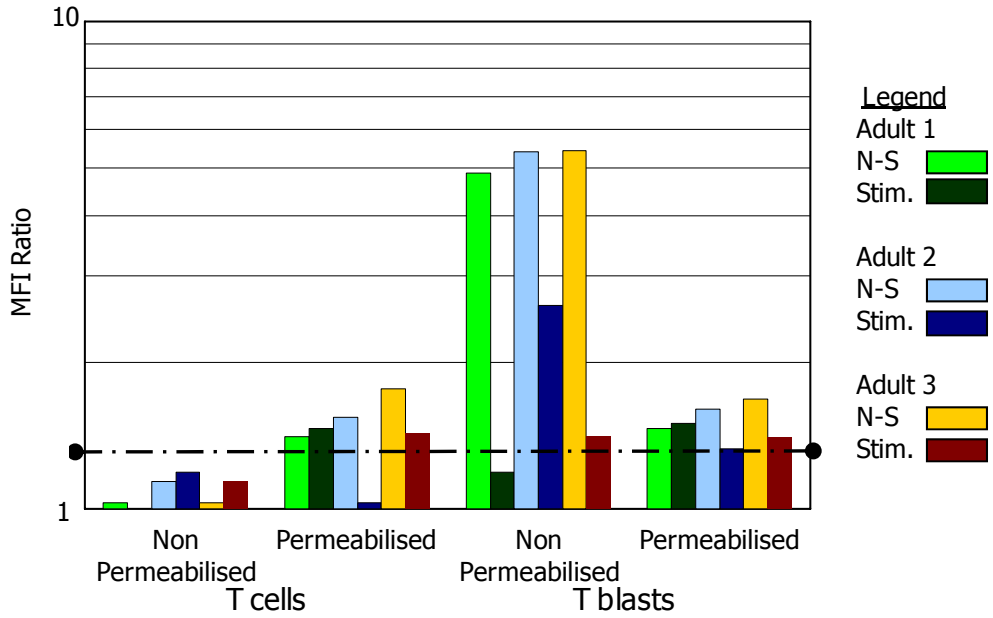
Appropriate controls with intracellular TLR6 expression in non-stimulated T lymphocytes from adult and cord blood were omitted except for one cord blood subject, as a consequence it was not possible to calculate MFI ratios for TLR6 expression by non-stimulated T cells and T blasts from adult blood and cord blood.

Adult blood T lymphocytes:

- Non-stimulated T cells: no extracellular TLR6 was expressed.
- Non-stimulated T blasts: two subjects expressed TLR6 on non-permeabilised cells.
- Stimulated T cells: no TLR6 was detected.
- Stimulated T blasts: no TLR6 was detected.

(Figure 3.9, 3.10 and Table 3.1)

A. Adult Blood T Lymphocytes



B. Cord Blood T Lymphocytes

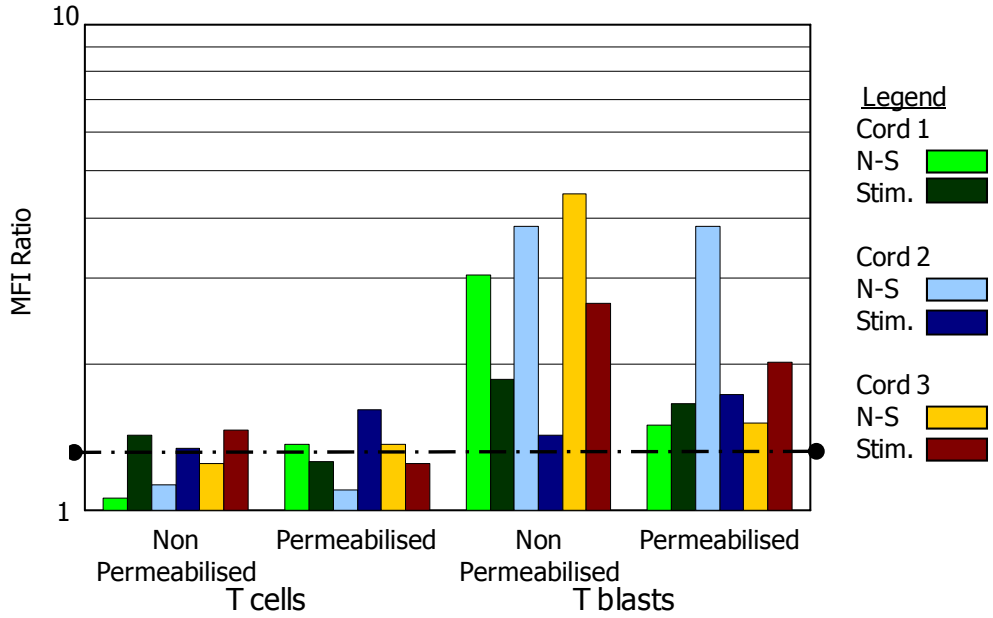
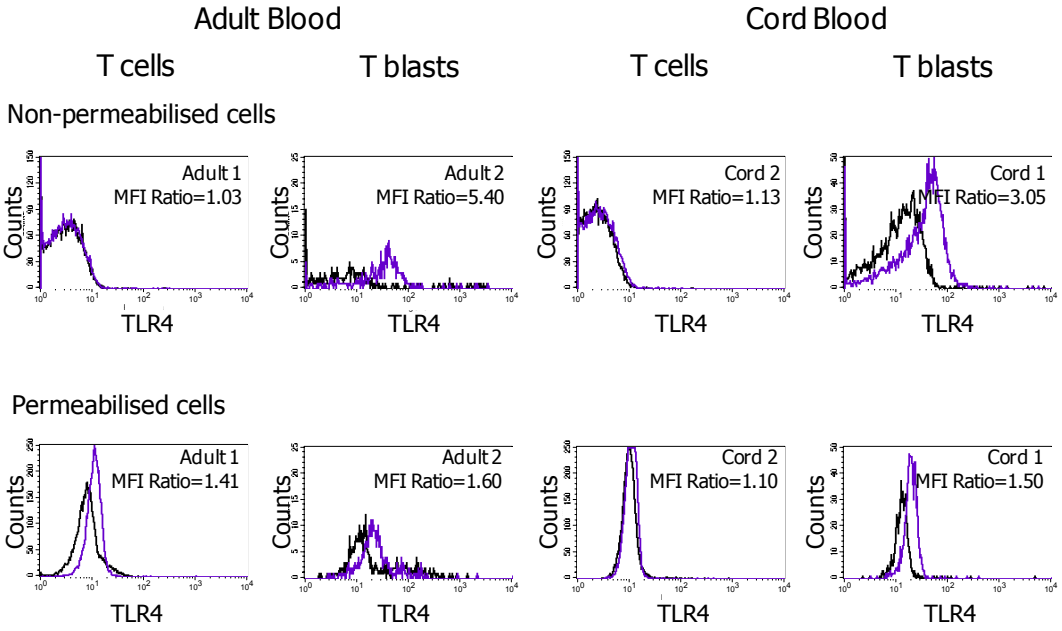


Figure 3.7 TLR4 Expression by T Lymphocytes
MFI ratio of TLR4 expression by non-stimulated (N-S) and stimulated (Stim.) T lymphocytes, both non-permeabilised and permeabilised, from **A.** Adult blood (n = 3) and **B.** Cord blood (n = 3). Dotted black line corresponds to MFI ratio of 1.3.

A. Non-Stimulated T Lymphocytes



B. Stimulated T Lymphocytes

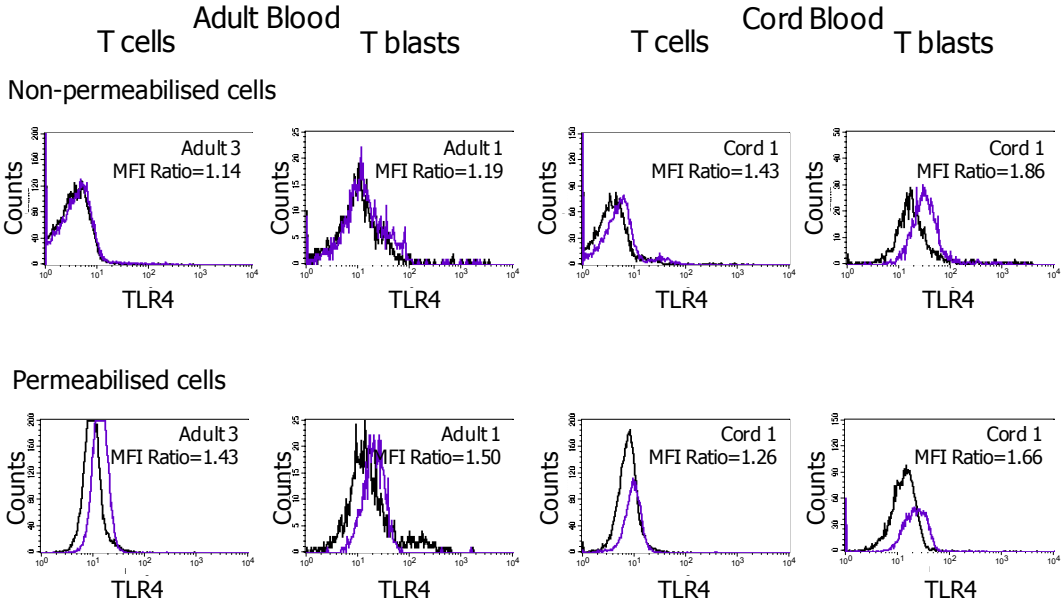


Figure 3.8 TLR4 Expression by T Lymphocytes
Representative example of TLR4 expression by T cells and T blasts, both non-permeabilised and permeabilised, from **A.** Non-stimulated adult blood and cord blood and **B.** Stimulated adult and cord blood. (TLR4 = purple, negative isotype control = black)

Cord blood T lymphocytes:

- Non-stimulated T cells: two subjects expressed TLR6 on non-permeabilised cells.
- Non-stimulated T blasts: two subjects expressed TLR6 on non-permeabilised cells.
- Stimulated T cells: no TLR6 was detected.
- Stimulated T blasts: no TLR6 was detected

(Figure 3.9, 3.10 and Table 3.1)

3.2.1.6 TLR8

Adult blood T lymphocytes:

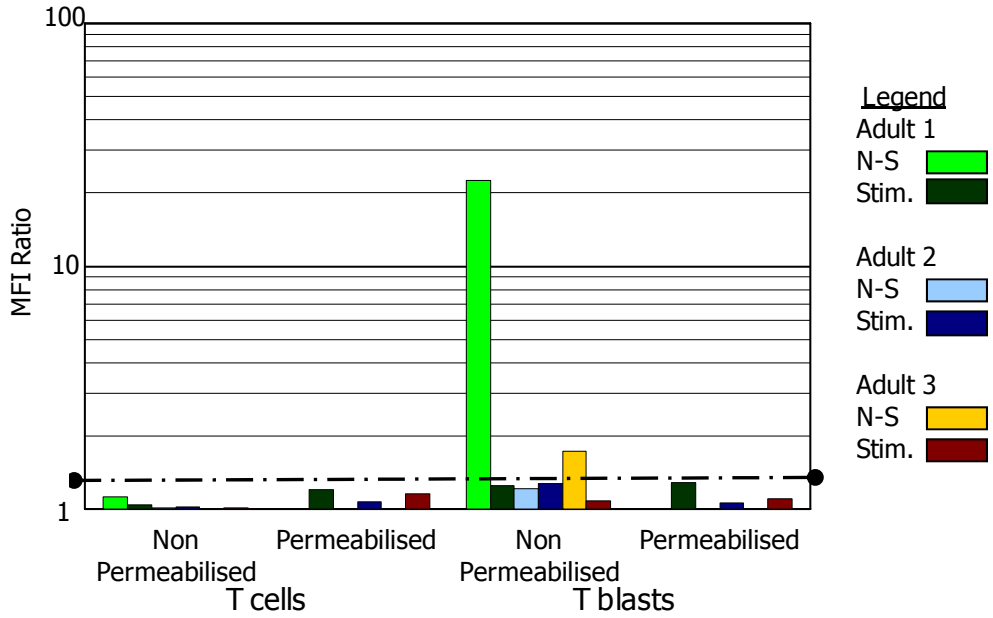
- Non-stimulated T cells: one subject expressed TLR8 on non-permeabilised cells and all three subjects expressed TLR8 in permeabilised cells.
- Non-stimulated T blasts: all three subjects expressed TLR8 by non-permeabilised and permeabilised cells.
- Stimulated T cells: all three subjects expressed TLR8 by non-permeabilised and permeabilised cells.
- Stimulated T blasts: all three subjects expressed TLR8 by non-permeabilised and permeabilised cells.

(Figure 3.11, 3.12 and Table 3.1)

Cord blood T lymphocytes:

- Non-stimulated T cells: all three subjects expressed TLR8 on non-permeabilised cells and two subjects expressed TLR8 in permeabilised cells.
- Non-stimulated T blasts: all three subjects expressed TLR8 on non-permeabilised cells and two subjects expressed TLR8 in permeabilised cells.

A. Adult Blood T Lymphocytes



B. Cord Blood T Lymphocytes

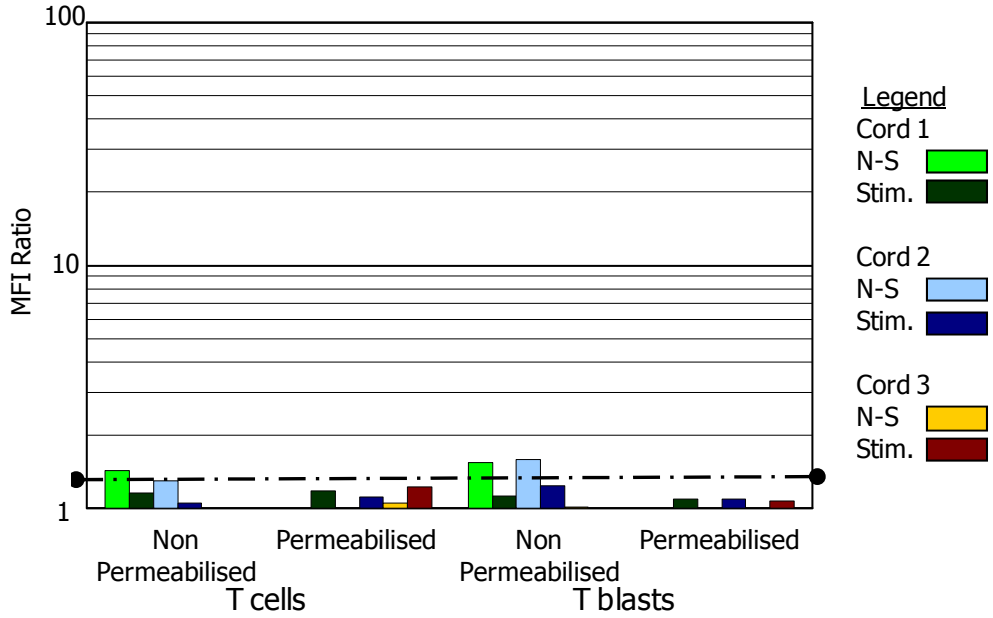


Figure 3.9 TLR6 Expression by T Lymphocytes
MFI ratio of TLR6 expression by non-permeabilised and non-stimulated (N-S) T lymphocytes, and by non-permeabilised and permeabilised stimulated (Stim.) T lymphocytes from **A.** Adult blood (n = 3) and **B.** Cord blood (n = 3). Dotted black line corresponds to MFI ratio of 1.3.

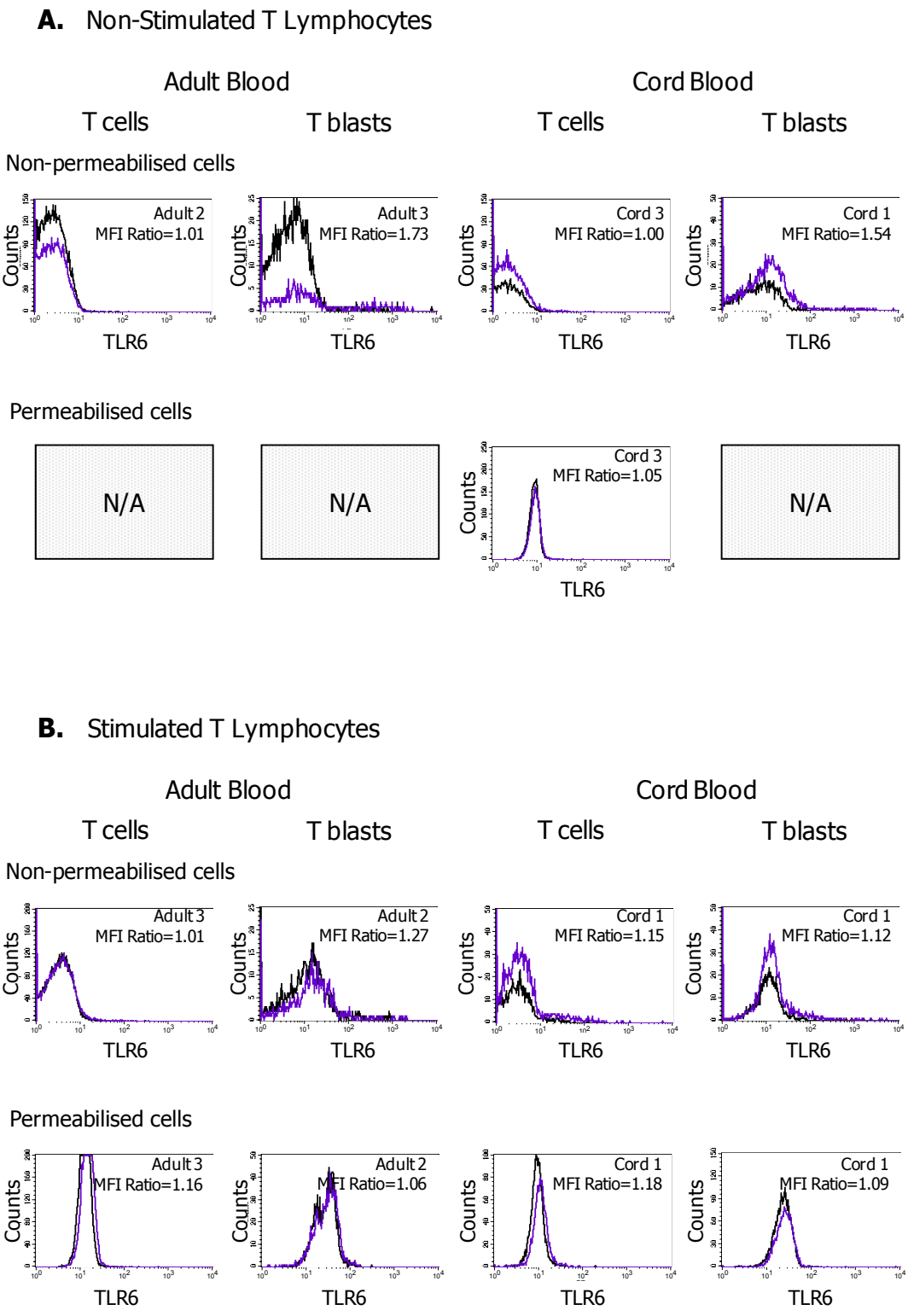


Figure 3.10 TLR6 Expression by T Lymphocytes
Representative example of TLR6 expression by T cells and T blasts, both non-permeabilised and permeabilised, from **A.** Non-stimulated adult blood and cord blood and **B.** Stimulated adult and cord blood. (TLR6 = purple, negative isotype control = black)

- Stimulated T cells: all three subjects expressed TLR8 on non-permeabilised cells and two subjects expressed TLR8 in permeabilised cells.
- Stimulated T blasts: all three subjects expressed TLR8 on non-permeabilised cells and two subjects expressed TLR8 in permeabilised cells.

(Figure 3.11, 3.12 and Table 3.1)

3.2.1.7 TLR9

Adult blood T lymphocytes:

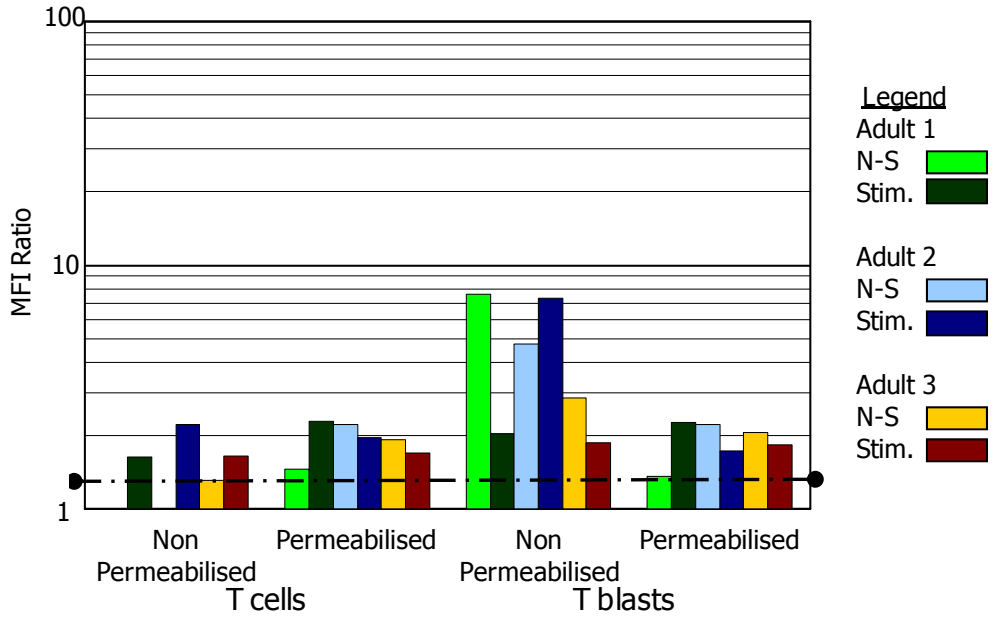
- Non-stimulated T cells: all three subjects expressed bright TLR9 in permeabilised cells.
- Non-stimulated T blasts: two subjects expressed TLR9 on non-permeabilised cells and all three subjects expressed bright TLR9 in permeabilised cells.
- Stimulated T cells: one subject expressed TLR9 on non-permeabilised cells and all three subjects expressed bright TLR9 in permeabilised cells.
- Stimulated T blasts: all three subjects expressed dim TLR9 on non-permeabilised cells and bright TLR9 in permeabilised cells.

(Figure 3.13, 3.14 and Table 3.1)

Cord blood T lymphocytes:

- Non-stimulated T cells: all three subjects expressed TLR9 in permeabilised cells.
- Non-stimulated T blasts: two subjects expressed TLR9 on non-permeabilised cells and all three subjects expressed TLR9 in permeabilised cells.

A. Adult Blood T Lymphocytes



B. Cord Blood T Lymphocytes

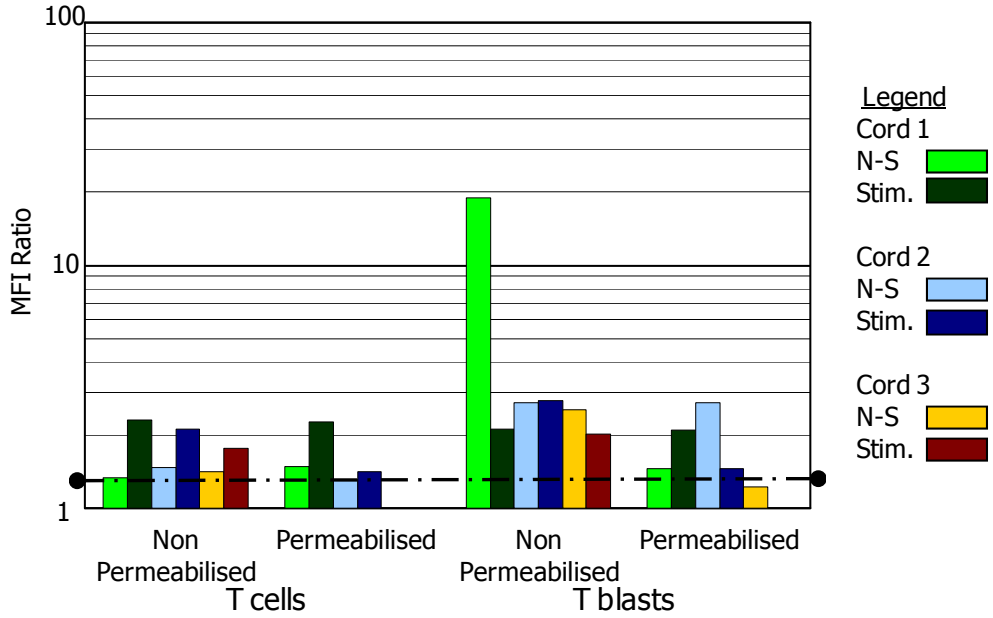


Figure 3.11 TLR8 Expression by T Lymphocytes
MFI ratio of TLR8 expression by non-stimulated (N-S) and stimulated (Stim.) T lymphocytes, both non-permeabilised and permeabilised, from **A.** Adult blood (n = 3) and **B.** Cord blood (n = 3). Dotted black line corresponds to MFI ratio of 1.3.

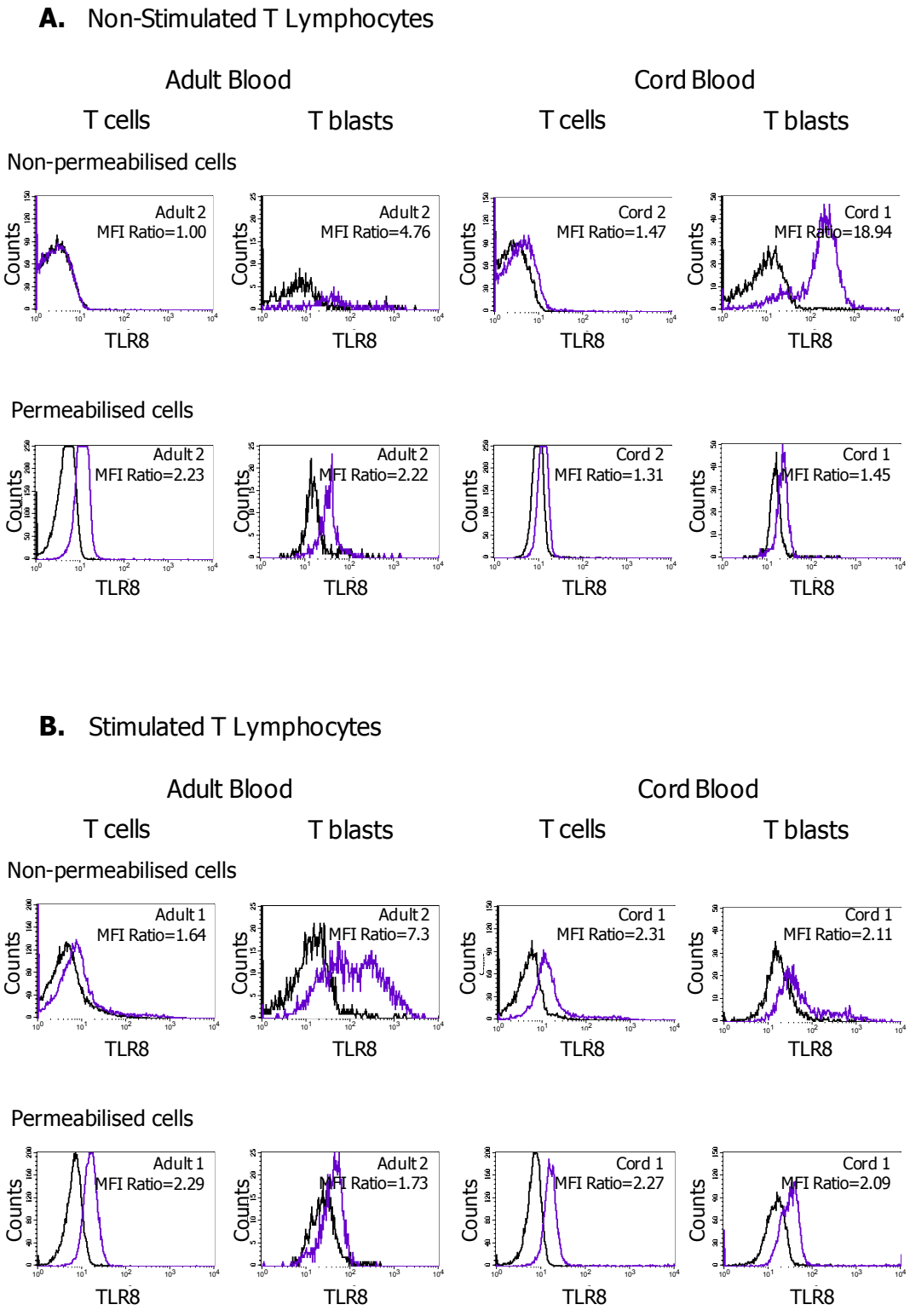


Figure 3.12 TLR8 Expression by T Lymphocytes
Representative example of TLR8 expression by T cells and T blasts, both non-permeabilised and permeabilised, from **A.** Non-stimulated adult blood and cord blood and **B.** Stimulated adult and cord blood. (TLR8 = purple, negative isotype control = black)

- Stimulated T cells: all three subjects expressed low levels of TLR9 on non-permeabilised cells and high levels of TLR9 in permeabilised cells.
- Stimulated T blasts: one subject expressed TLR9 on non-permeabilised cells and all three subjects expressed TLR9 in permeabilised cells.

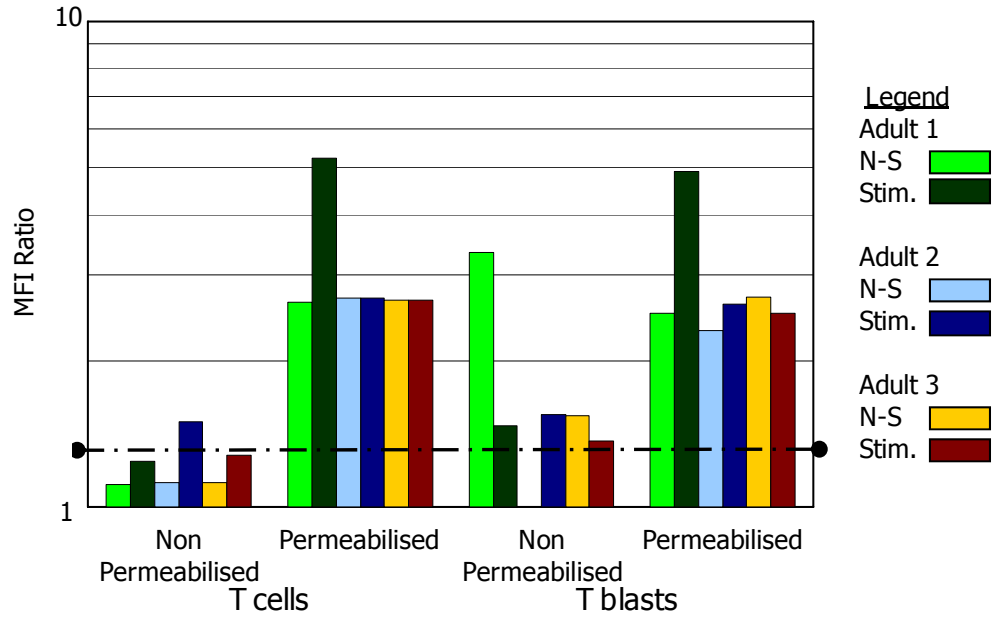
(Figure 3.13, 3.14 and Table 3.1)

Table 3.1 Summary of TLR Expression Patterns by T Lymphocytes

TLR	Group (n = 3)	Non-Stim.				Stim.			
		Cells		Blasts		Cells		Blasts	
		N-P	P	N-P	P	N-P	P	N-P	P
1	Adult blood	0	1	2	3	0	2	1	2
	Cord blood	0	1	2	2	0	0	1	2
2	Adult blood	0	1	3	3	3	1	3	2
	Cord blood	0	0	3	3	3	0	3	2
3	Adult blood	0	3	2	3	0	3	2	3
	Cord blood	0	3	3	3	1	3	2	3
4	Adult blood	0	3	3	3	0	2	2	3
	Cord blood	0	2	3	3	3	1	3	3
6	Adult blood	0	NA	2	NA	0	0	0	0
	Cord blood	2	NA	2	NA	0	0	0	0
8	Adult blood	1	3	3	3	3	3	3	3
	Cord blood	3	2	3	2	3	2	3	2
9	Adult blood	0	3	2	3	1	3	3	3
	Cord blood	0	3	2	3	3	3	3	3

The two groups, adult blood and cord blood, had three subjects per group. The numbers of subjects in the table are the number of subjects out of three that had TLR detected.
Non-Stim. = Non-stimulated, Stim. = Stimulated, cells = T cells, blasts = T blasts, N-P = Non-permeabilised cells, P = Permeabilised cells, NA = Not available

A. Adult Blood T Lymphocytes



B. Cord Blood T Lymphocytes

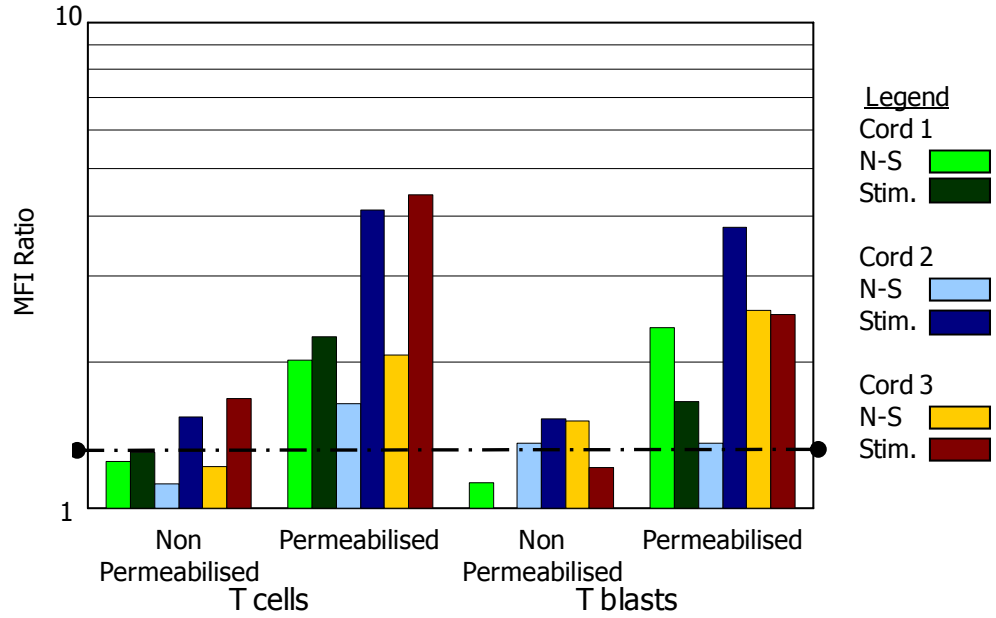


Figure 3.13 TLR9 Expression by T Lymphocytes
MFI ratio of TLR9 expression by non-stimulated (N-S) and stimulated (Stim.) T lymphocytes, both non-permeabilised and permeabilised, from **A.** Adult blood (n = 3) and **B.** Cord blood (n = 3). Dotted black line corresponds to MFI ratio of 1.3.

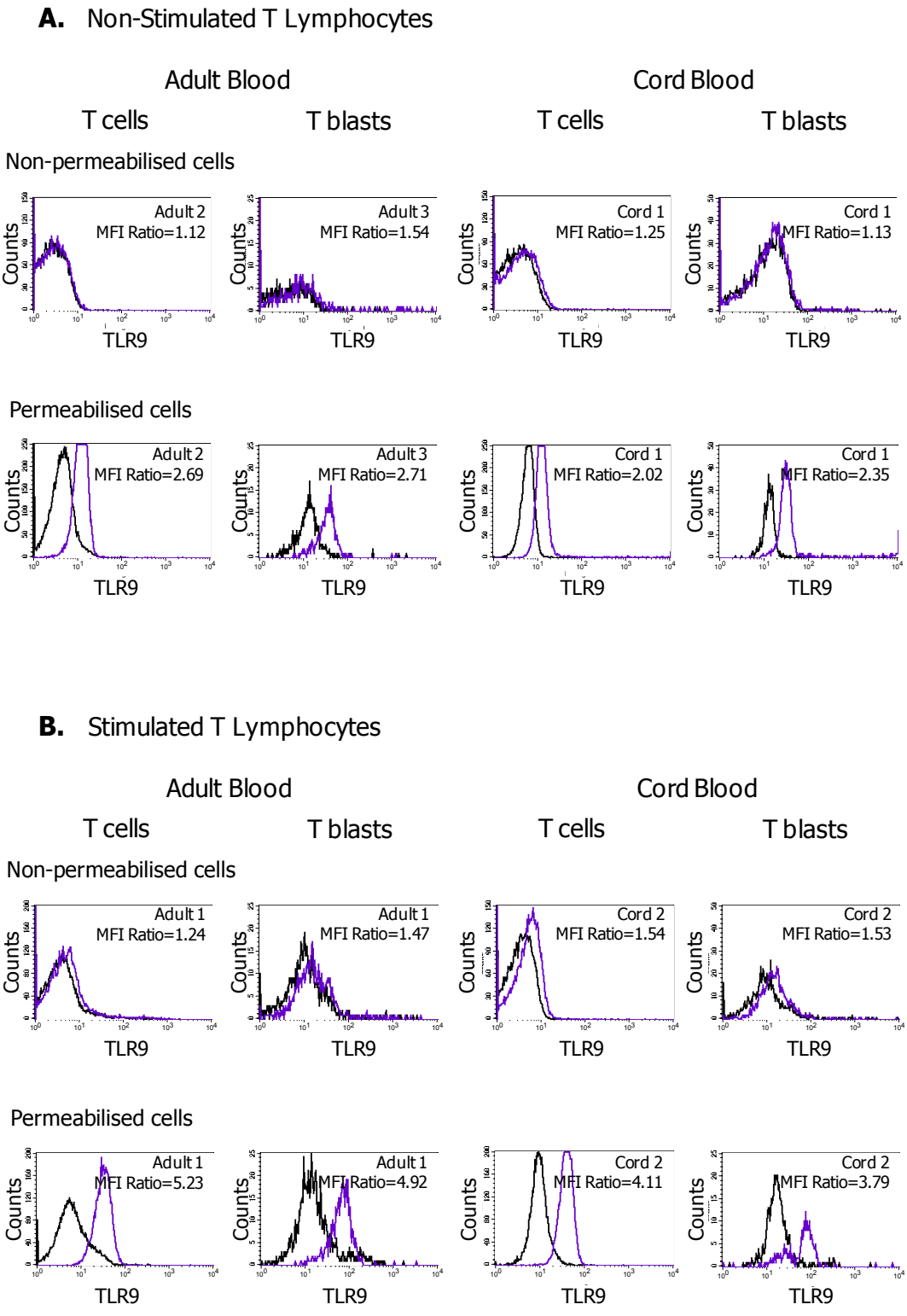


Figure 3.14 TLR9 Expression by T Lymphocytes
Representative example of TLR9 expression by T cells and T blasts, both non-permeabilised and permeabilised, from **A.** Non-stimulated adult blood and cord blood and **B.** Stimulated adult and cord blood. (TLR9 = purple, negative isotype control = black)

3.3 DISCUSSION

From the results there are three main observations. First, adult blood and cord blood T lymphocytes expressed similar patterns of most TLR. Second, stimulation of T lymphocytes did not alter most TLR expression patterns, and third T cells expressed fewer TLR than T blasts.

These studies showed T lymphocytes from adult blood and cord blood consistently expressed TLR2, TLR3, TLR4, TLR8 and TLR9. In contrast, TLR1 and TLR6 expression by T lymphocytes was inconsistent within the two groups.

The first observation that cord blood T lymphocytes express most of the TLR that adult blood T lymphocytes express, and indeed the additional expression of TLR4 by stimulated cord blood T cells, suggests that neonates possess the ability to "innately" recognise pathogens. Despite this, the functional capability of neonatal T lymphocytes after TLR engagement needs investigation to determine if they have adult-like abilities. One study showed that neonatal cord blood leucocytes, compared to adult blood leucocytes, have reduced mRNA levels of TLR signalling proteins MyD88 and IRF5, and post-stimulation with lipoteichoic acid there is decreased phosphorylation of p38-MAPK and ERK1 with reduced cytokine secretion (167). Though TLR expression is present in cord blood T lymphocytes, the integrity of TLR signalling pathways and functional ability of neonatal T lymphocytes need further examination to clarify their role.

The second observation from the results is that stimulation of T lymphocytes did not affect the expression of most TLR, excluding TLR2, TLR4 and TLR6. The upregulation of TLR2 and TLR4 by stimulated T cells from cord blood was

supported by a similar study (84). T lymphocytes were activated by anti-CD3 antibody and co-stimulation by monocytes, these are signals required for adaptive immune activation. TLR expression may not be as responsive to adaptive immune signals as TLR are part of the innate recognition of infection, and instead activation via other mechanisms may be more effective to alter TLR expression. Disease does affect TLR expression by increasing or decreasing particular TLR in T lymphocytes, as seen for patients with tonsillitis (98) or lymphatic filarial infection (88). Despite the inertia in expression of several TLR to T lymphocyte stimulation, the cited studies and results for TLR2, TLR4 and TLR6 show that T lymphocytes are capable of regulating TLR expression in response to appropriate stimuli.

TLR expression was affected by the activation state of T lymphocytes. T blasts from adult blood and cord blood, both non-stimulated and activated, expressed all tested TLR except TLR6. In contrast, T cells from adult blood and cord blood had fewer TLR detected; TLR2, TLR3, TLR4, TLR8 and TLR9. T blasts expressed a broader range of TLR than T cells. Most literature examining TLR expression by T lymphocytes focus on either T lymphocytes or subsets like T_H cells or T_C cells, there is little published on T blasts.

The cell activation state of T lymphocytes has an effect on TLR expression, as seen with non-stimulated and stimulated T blasts expressing more TLR than non-stimulated and stimulated T cells. As discussed previously, the stimulation of T lymphocytes increases expression of activation markers (Refer Chapter Two, Table 2.1) and numbers of T blasts, but does not affect TLR expression patterns. The activation cell status of T lymphocytes, as T cells or T blasts, is more relevant for TLR expression than whether there was *in vitro* stimulation of T lymphocytes. This

can be seen in the lower TLR expression by stimulated T cells with higher activation compared to non-stimulated T blasts with lower activation. Stimulation of T lymphocytes may not increase expression of TLR unless they develop into T blasts.

The broader range of TLR expressed shows that T blasts will be more responsive to infection as most tested TLR were constitutively expressed. Further study on untested TLR would reveal if all TLR are expressed by T blasts. There is little in the literature about TLR5, TLR7 and TLR10 expression by T blasts, though TLR5 and TLR10 are expressed by T lymphocytes (87, 89). The TLR expressed by cord blood T lymphocytes and adult blood T lymphocytes indicates that neonates are capable of altering TLR expression and detecting infection. The ability of blasts to respond to infection should be enabled by its broad TLR expression.

An experimental oversight for intracellular TLR expression by lymphocytes occurred when the surface TLR was not blocked, therefore the intracellular detection using permeabilised cells includes surface TLR expression on the perforated cell membrane and the intracellular TLR expression. Not blocking surface TLR that is brightly expressed may lead to confusion between extracellular and intracellular TLR. From the results, TLR2 and TLR4 have bright surface expression and dim internal expression, and therefore they are inconclusive.

In some instances, there is brighter surface expression than the sum total of intracellular staining and extracellular staining on perforated cell membranes. This may occur when TLR expression is primarily on the surface with minimal intracellular expression, such as for TLR2 or TLR8. Another explanation for the

discrepancy in expression between non-permeabilised versus permeabilised cells is that TLR epitopes may be sensitive to the conditions used for cell permeabilisation, possibly damaged, and subsequently not detected by antibodies.

The results show variation in TLR expression within subjects, for example TLR1 and TLR6 was expressed by one or two subjects in the groups of three. This high variability of TLR expression can be expected from human samples. Numerous parameters were measured to assess TLR expression by human lymphocytes; these parameters include non-stimulated versus stimulated cells, cell permeabilisation and other parameters. TLR expression will vary considerably between these different parameters; for example, a surface marker will be poorly expressed intracellularly or expression may vary according to the cell activation state, i.e. T blasts or T cells. Though the groups were too small to be representative of an entire population, if TLR were consistently expressed in all three subjects per group in the same parameter, it was deemed that the subject group expressed that particular TLR in that particular parameter. If there was variability in TLR expression in the three subjects per group, no conclusions were made. Expression studies on larger groups are desirable, but it is expected that they also will show considerable variability in human subjects.

Though the group size was too small to provide statistically significant data, preliminary information on individual TLR expression can be gleaned from the results to contribute further knowledge about TLR and form the basis for a larger study, as elaborated in the following paragraphs.

The presence of TLR2 expression adds to existing literature which reports surface TLR2 on non-stimulated adult blood T lymphocytes (88) and TLR2 expression by non-stimulated and stimulated T lymphocytes from cord blood (84). It was not possible to consider TLR2 expression by T blasts as literature on T lymphocytes do not differentiate T cells from T blasts.

TLR4 was detected in permeabilised T cells and T blasts with elevated surface expression on T blasts. Though one study confirmed absence of surface TLR4 by adult T cells (89) other studies report surface expression on T lymphocytes (88, 98) and, internal and external TLR4 expression by non-stimulated and stimulated cord blood T lymphocytes (84). The lack of TLR4 surface expression on T cells, excluding stimulated T cells from cord blood, contradicts the cell surface location of TLR4 in the literature (76, 84).

The TLR8 expression using permeabilised T blasts and adult T cells is expected as TLR8 is described as an internal marker (82), but external TLR8 expression by T blasts and cord blood T cells, though seen on adult T regulatory cells (89), was surprising. The surface location of TLR8 by T lymphocytes was unexpected and indicates TLR8 may have additional functions to being an internal receptor for detecting viral pathogens.

The expression of TLR1 and TLR6, co-receptors of TLR2, was variable. TLR1 expression was mostly internal, which contradicts a previous report of surface TLR1 on tonsil T lymphocytes (98). TLR6 expression was external as reported earlier (89), but it was not possible to determine internal TLR6 expression due to the lack of appropriate controls. The inconsistent TLR1 and TLR6 expression by T

lymphocytes may be due to experimental variation due to the small sample size of the groups. If the inconsistency of expression is confirmed with a larger sample size, it may reflect a mechanism to regulate formation of TLR2/TLR1 or TLR2/TLR6 heterodimers.

TLR3 and TLR9 were expressed by T lymphocytes from adult blood and cord blood, and both TLR are internal receptors as reported previously (86, 88, 98). External expression of TLR3 and TLR9 were seen on T blasts, and for TLR9 on activated cord blood T cells. The results show the similarities of these TLR by T lymphocytes from adult blood and cord blood.

As discussed in Chapter One, neonates are vulnerable to infection due to an immature immune system which has T lymphocytes that are impaired in several functions. Research into the neonatal immune system will expand knowledge and assist in discovering how to accelerate immune maturation of neonates and decrease susceptibility to infection. A neonatal murine study showed CpG ODN overcame the neonatal T_H2 bias and induced T_H1 responses including increased T_C lymphocyte activity (176); an effective response against infection which supports research into TLR ligands as potential vaccine adjuvants. However, another study reported different TLR ligands injected into neonatal mice induced an excessive inflammatory response only controlled with adoptive transfer of adult mouse T lymphocytes compensating for low numbers of neonatal T lymphocytes (194). Comprehending the various effects of TLR on neonatal T lymphocyte functions and how it serves in the overall immune response is necessary.

CHAPTER FOUR

EXPRESSION OF TOLL-LIKE RECEPTORS BY B LYMPHOCYTES FROM ADULT BLOOD, CORD BLOOD AND TONSILS

4.1 INTRODUCTION

B lymphocytes can be activated by TLR engagement and secrete antibodies and cytokines in response. As discussed previously in Chapter One, neonatal B lymphocytes have reduced antibody class-switching, minimal antibody affinity maturation and a poor memory response compared to adult B lymphocytes. In lymph nodes from adults and children, B lymphocytes can undergo a T-D immune response to produce an effective antibody response with memory. B lymphocytes have different functions according to their location and the degree of immune maturation. In this chapter the potential role of TLR in neonatal immunity will be explored by examining TLR expression patterns of non-stimulated and stimulated B lymphocytes from adult blood, cord blood and tonsils.

TLR expression by B lymphocytes has been studied in adult blood and tonsils, but no studies in cord blood were available. RT-PCR studies of TLR mRNA (Refer Chapter One, Table 1.1) showed adult B lymphocytes expressed all TLR (79, 90-91) except TLR3, TLR5 and TLR8, and tonsil B lymphocytes expressed TLR1, TLR6, TLR7, TLR8, TLR9 and TLR10 (91, 99). In studies examining TLR proteins, adult B lymphocytes expressed extracellular TLR1, TLR2, TLR4 and TLR9 (81, 92-94, 100) and intracellular TLR9 (195), and tonsil B lymphocytes expressed extracellular TLR2 and TLR10 (74, 100) and intracellular TLR1, TLR2, TLR7 and

TLR9 (91). The literature shows little correlation in the expression between TLR mRNA and TLR protein.

Activation of B lymphocytes occurs via receptors which include BCR and MHC class II, and may influence expression of TLR. B lymphocytes activated via BCR upregulated mRNA expression of TLR4, TLR7, TLR9 and TLR10 (99, 154) and in response to LPS upregulated TLR9 (100). In disease, HIV patients have diminished TLR9 mRNA levels in B lymphocytes (196), for tonsillitis TLR mRNA expression by B lymphocytes is increased for TLR1, TLR9 and TLR10, and decreased for TLR7 (91), and for lymphatic filariasis B lymphocytes have decreased protein expression of TLR1, TLR2, TLR4 and TLR9 (93). Whether B lymphocytes regulate TLR protein expression upon activation requires further study.

As previously discussed (Refer Chapter One, Section 1.2.4.2.1), B cell subsets from adult blood and cord blood differ to subsets from tonsils. Neonatal B lymphocytes are predominantly B1 B cells that are self-renewing and provide a more rapid, innate response to infection compared to B2 B cell, and tonsillar B cell subsets develop according to immune maturation to T-D immune interactions. Earlier work showed greater TLR1 and TLR9 protein expression by adult B1 B cells (92), and memory B cells upregulated TLR4, TLR9 and TLR10 mRNA levels (154, 197). It is hypothesised that the stage of B cell development may affect the patterns of expressed TLR with earlier, immature, B cell subsets expressing more TLR than mature, developed B cell subsets.

As with Chapter Three, several parameters were examined to assess TLR expression by B lymphocytes. The following TLR: TLR1, TLR2, TLR3, TLR4,

TLR6, TLR8 and TLR9, were tested to detect extracellular and intracellular expression by non-stimulated and stimulated B lymphocytes from adult blood, cord blood and tonsils. Tonsil B lymphocytes were categorised into B cells or B blasts on the basis of cell size and granularity. B lymphocytes were further delineated into subsets according to their sources; B1, B2, naïve and memory B cells from adult blood; B1 and B2 B cells from cord blood; and naïve, pre-GC, GC and memory B cells from tonsils.

4.2 RESULTS

4.2.1 TLR Expression by B Lymphocytes

Non-stimulated and stimulated B lymphocytes from adult blood (n = 3), cord blood (n = 3) and tonsils (n = 3) were examined for extracellular and intracellular expression of TLR1, TLR2, TLR3, TLR4, TLR6, TLR8 and TLR9. TLR phenotyping studies were on enriched mononuclear cells for non-stimulated and stimulated B lymphocytes. For stimulation, B lymphocytes were treated for 24 hours with anti-IgM and anti-CD40 antibodies in the presence of IL-2 and IL-4. B lymphocytes were identified with PerCP-Cy5.5-conjugated anti-CD19 antibody and TLR were detected with PE-conjugated antibodies. Tonsil B lymphocytes were sub-categorised as B cells or B blasts based on cell size and granularity (Refer Chapter Two, Figure 2.2). TLR were detected on non-permeabilised cells for extracellular expression, and in permeabilised cells for intracellular expression. TLR expression is the sum total of intracellular expression and surface TLR expression on permeabilised cell membranes (Refer Chapter Two, Section 2.13.2). TLR expression was compared with matched negative isotype controls and expression levels greater than MFI ratio value 1.3 were deemed positive for

presence of TLR (Refer Chapter Two, Figure 2.4). Statistical comparisons of TLR expression between adult blood, cord blood and tonsil were not done as results were deemed positive or negative for TLR expression (Refer Chapter Two, Section 2.13.6). Instead, TLR expression was presented as descriptive data in Table 4.1.

4.2.1.1 TLR1

Adult blood B lymphocytes:

- Non-stimulated B cells: two subjects expressed TLR1 on non-permeabilised cells and the third subject expressed TLR1 in permeabilised cells.
- Stimulated B cells: two subjects expressed TLR1 on non-permeabilised cells and all three subjects expressed TLR1 in permeabilised cells.

(Figures 4.1, 4.2 and Table 4.1)

Cord blood B lymphocytes:

- Non-stimulated B cells: all three subjects expressed TLR1 on non-permeabilised cells.
- Stimulated B cells: two subjects expressed TLR1 on non-permeabilised cells and the third subject expressed TLR1 in permeabilised cells.

(Refer Figures 4.1, 4.2 and Table 4.1)

Tonsil B lymphocytes:

- Non-stimulated B cells: one subject expressed TLR1 on non-permeabilised cells and another subject expressed TLR1 in permeabilised cells.
- Non-stimulated B blasts: one subject expressed TLR1 on non-permeabilised cells and another subject expressed TLR1 in permeabilised cells.

- Stimulated B cells: one subject expressed TLR1 on non-permeabilised cells and another subject expressed TLR1 in permeabilised cells.
- Stimulated B blasts: one subject expressed TLR1 on non-permeabilised cells.

(Figures 4.1, 4.2 and Table 4.1)

4.2.1.2 TLR2

Adult blood B lymphocytes:

- Non-stimulated B cells: no TLR2 was detected.
- Stimulated B cells: two subjects expressed TLR2 on non-permeabilised cells and the third subject expressed TLR2 in permeabilised cells.

(Figures 4.3, 4.4 and Table 4.1)

Cord blood B lymphocytes:

- Non-stimulated B cells: no TLR2 was detected.
- Stimulated B cells: all three subjects expressed TLR2 on non-permeabilised cells.

(Figures 4.3, 4.4 and Table 4.1)

Tonsil B lymphocytes:

- Non-stimulated B cells: no TLR2 was detected.
- Non-stimulated B blasts: one subject expressed TLR2 on non-permeabilised cells and another subject expressed TLR2 in permeabilised cells.
- Stimulated B cells: no TLR2 was detected.
- Stimulated B blasts: one subject expressed TLR2 in permeabilised cells.

(Figures 4.3, 4.4 and Table 4.1)

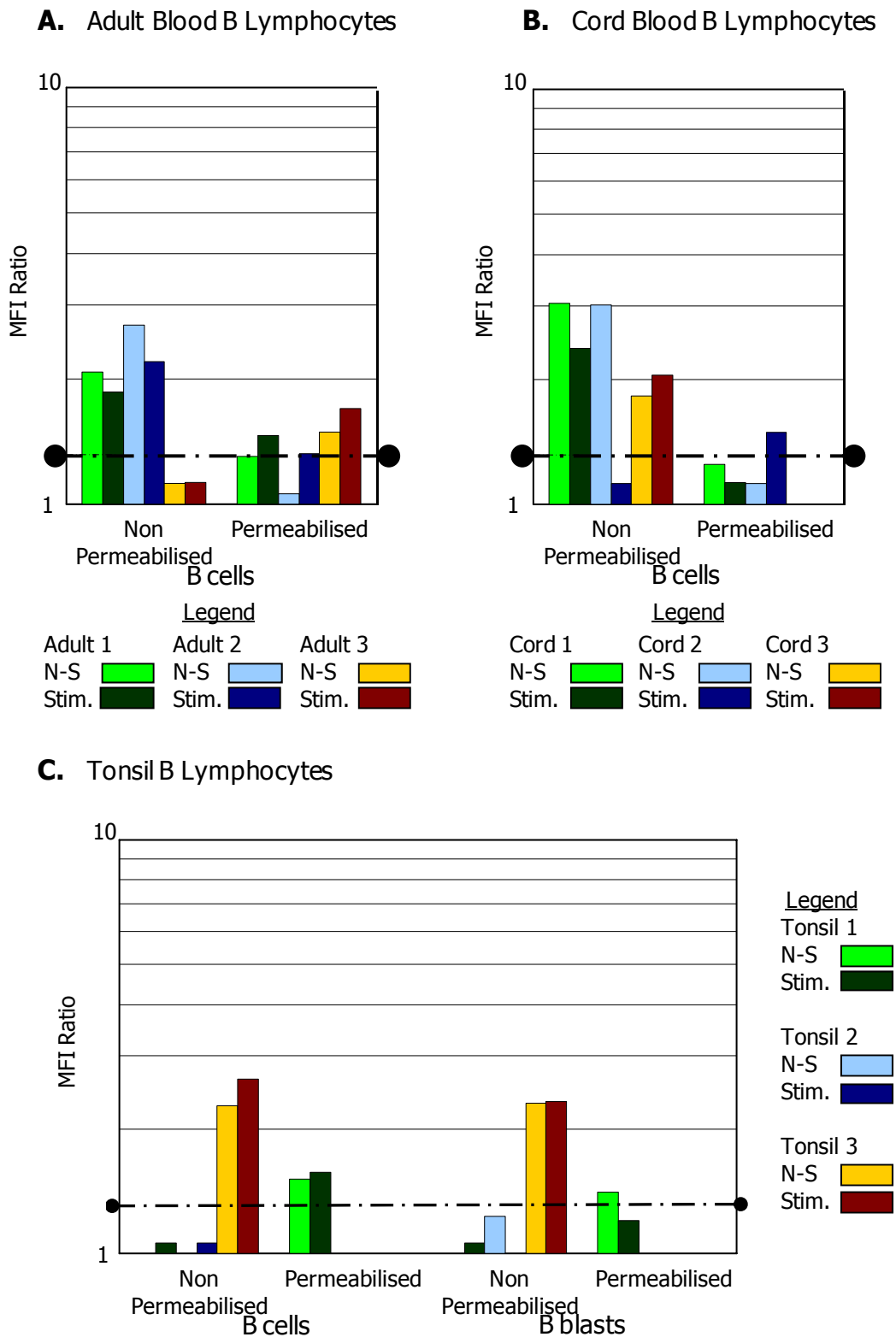


Figure 4.1 TLR1 Expression by B Lymphocytes
MFI ratio of TLR1 expression by non-stimulated (N-S) and stimulated (Stim.) B lymphocytes, both non-permeabilised and permeabilised, from **A.** Adult blood (n = 3), **B.** Cord blood (n = 3) and **C.** Tonsils (n = 3). Dotted black line corresponds to MFI ratio of 1.3.

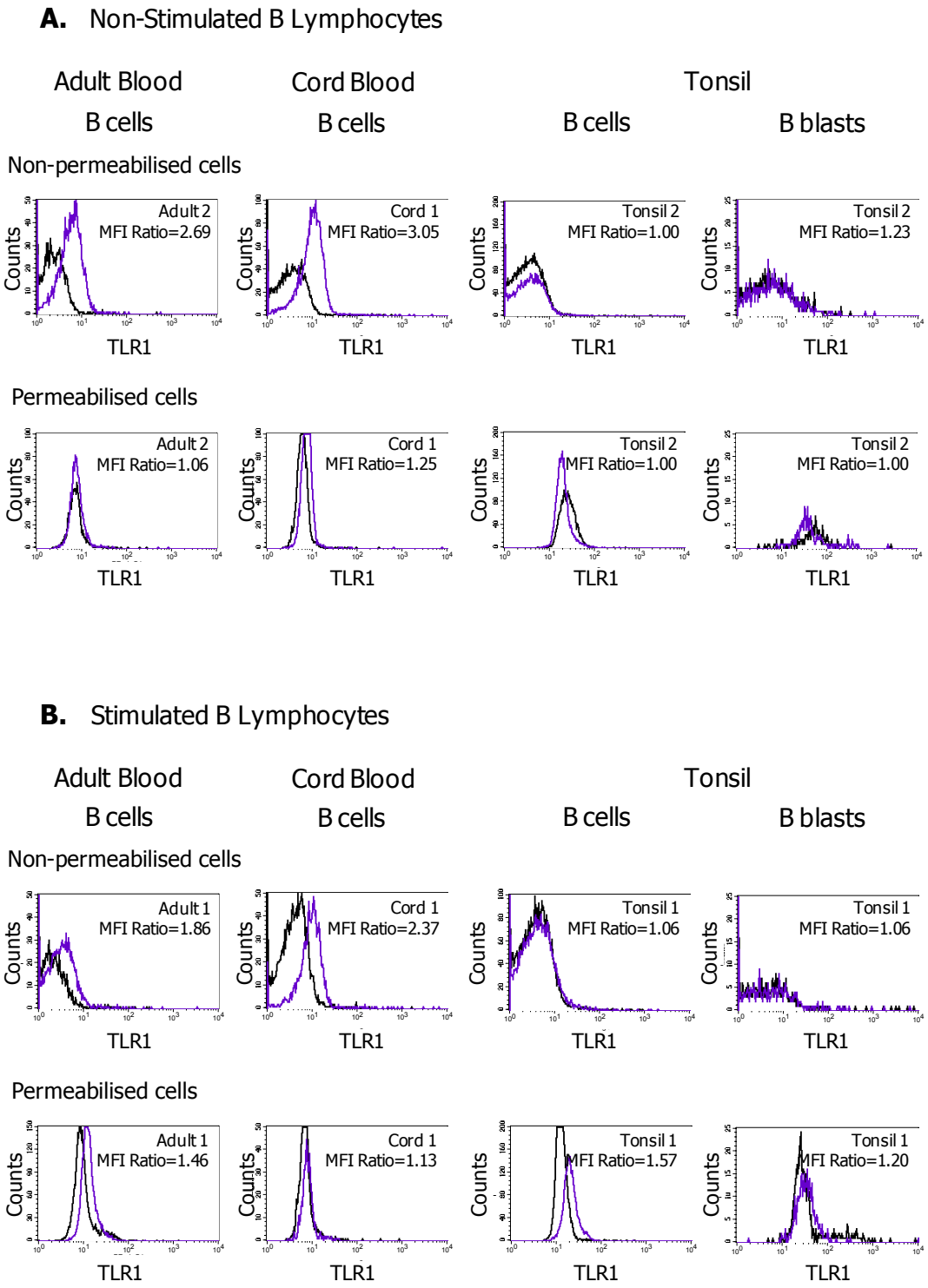


Figure 4.2 TLR1 Expression by B Lymphocytes
Representative example of TLR1 expression by B cells and B blasts, both non-permeabilised and permeabilised, from **A.** Non-stimulated adult blood, cord blood and tonsil and **B.** Stimulated adult blood, cord blood and tonsils. (TLR1 = purple, negative isotype control = black)

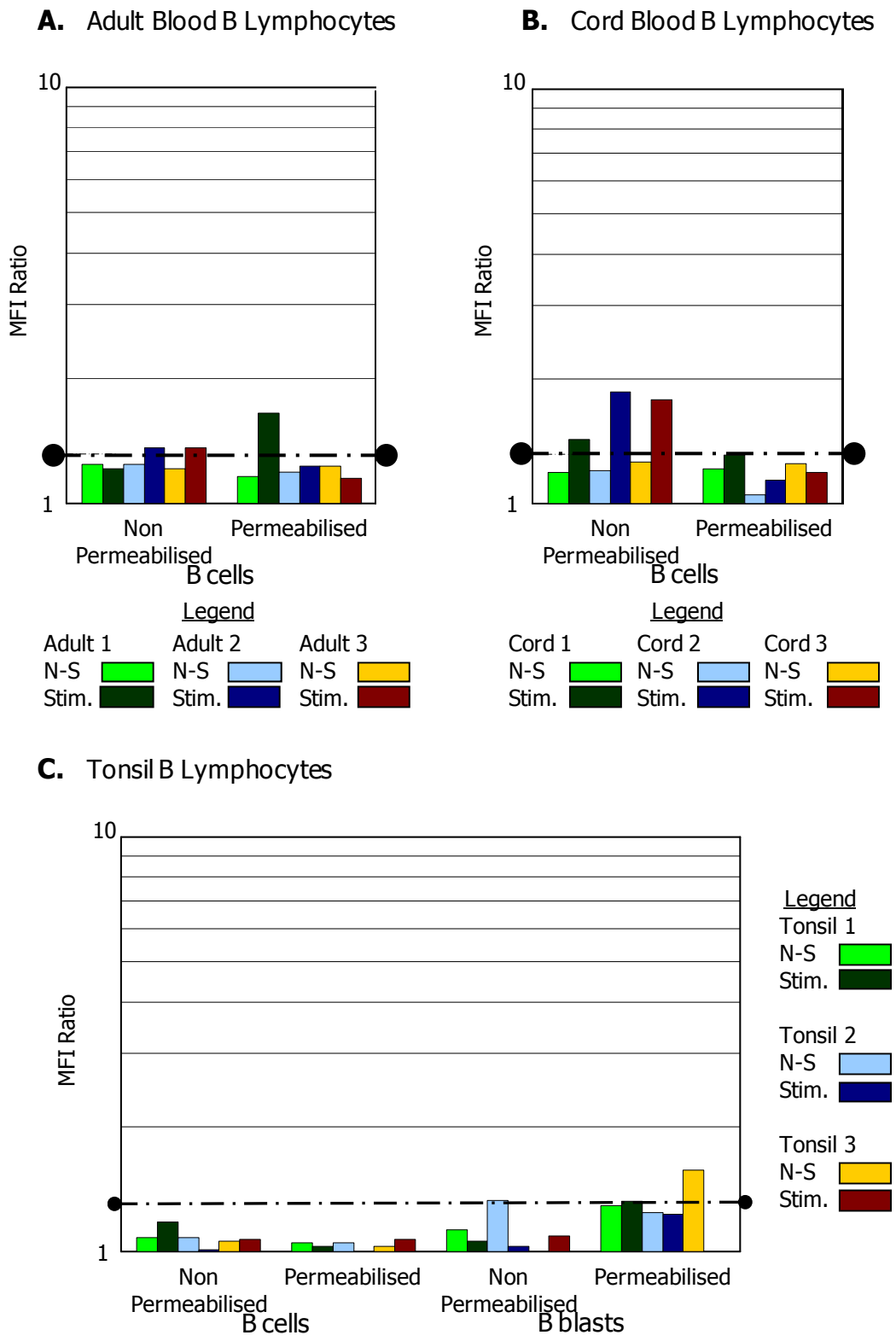


Figure 4.3 TLR2 Expression by B Lymphocytes
MFI ratio of TLR2 expression by non-stimulated (N-S) and stimulated (Stim.) B lymphocytes, both non-permeabilised and permeabilised, from **A.** Adult blood (n = 3), **B.** Cord blood (n = 3) and **C.** Tonsils (n = 3). Dotted black line corresponds to MFI ratio of 1.3.

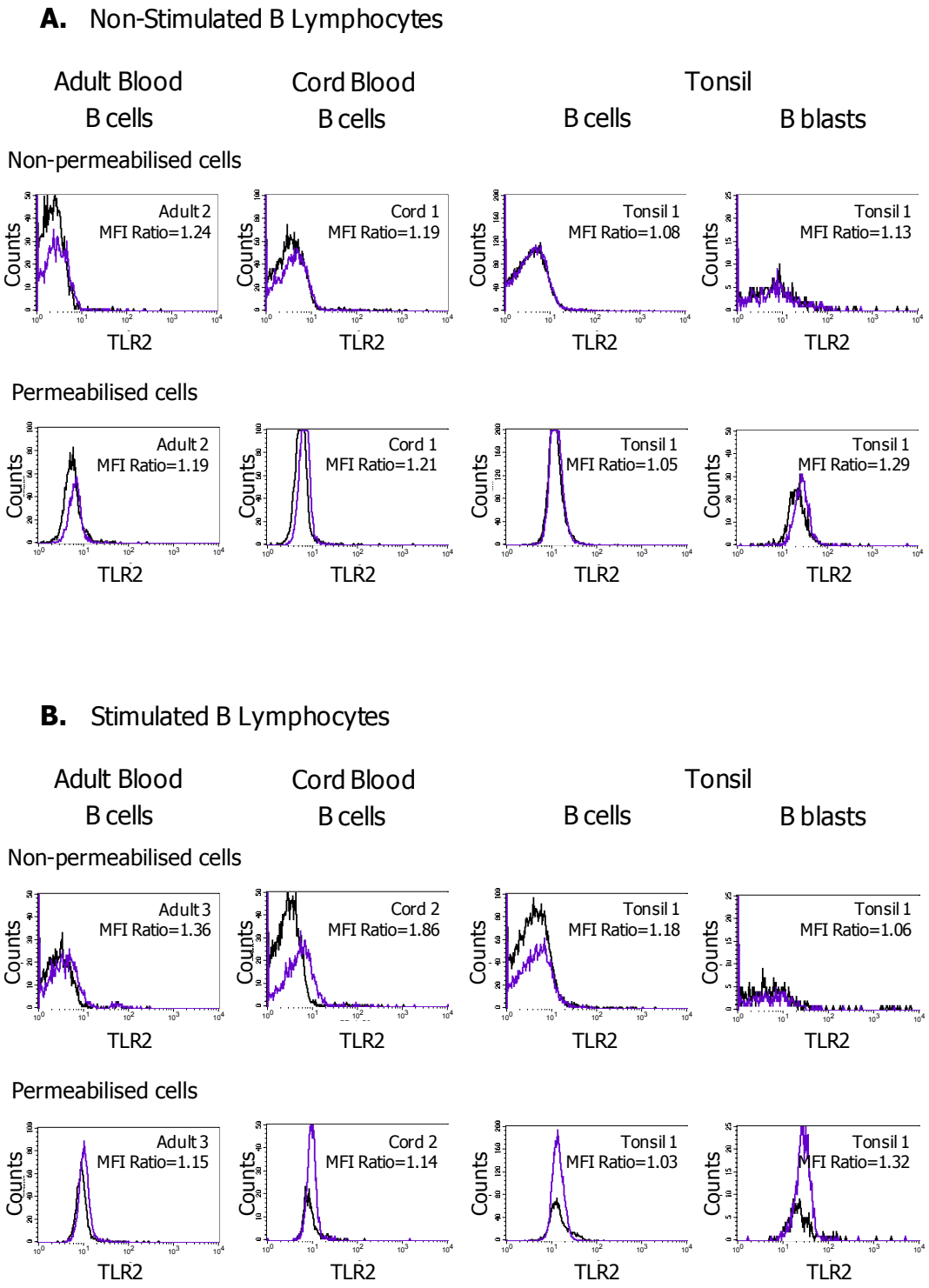


Figure 4.4 TLR2 Expression by B Lymphocytes
Representative example of TLR2 expression by B cells and B blasts, both non-permeabilised and permeabilised, from **A.** Non-stimulated adult blood, cord blood and tonsil and **B.** Stimulated adult blood, cord blood and tonsils. (TLR2 = purple, negative isotype control = black)

4.2.1.3 TLR3

Adult B lymphocytes:

- Non-stimulated B cells: one subject expressed TLR3 on non-permeabilised cells and all three subjects expressed high levels of TLR3 in permeabilised cells.
- Stimulated B cells: one subject expressed TLR3 on non-permeabilised cells and all three subjects expressed high levels of TLR3 in permeabilised cells.

(Figures 4.5, 4.6 and Table 4.1)

Cord blood B lymphocytes:

- Non-stimulated B cells: all three subjects expressed low levels of TLR3 on non-permeabilised cells and high levels of TLR3 in permeabilised cells.
- Stimulated B cells: one subject expressed TLR3 on non-permeabilised cells and all three subjects expressed high levels of TLR3 in permeabilised cells.

(Figures 4.5, 4.6 and Table 4.1)

Tonsil B lymphocytes:

- Non-stimulated B cells: all three subjects expressed bright TLR3 in permeabilised cells.
- Non-stimulated B blasts: one subject expressed TLR3 on non-permeabilised cells and all three subjects expressed bright TLR3 in permeabilised cells.
- Stimulated B cells: all three subjects expressed bright TLR3 in permeabilised cells.
- Stimulated B blasts: two subjects expressed TLR3 on non-permeabilised cells and all three subjects expressed bright TLR3 in permeabilised cells.

(Figures 4.5, 4.6 and Table 4.1)

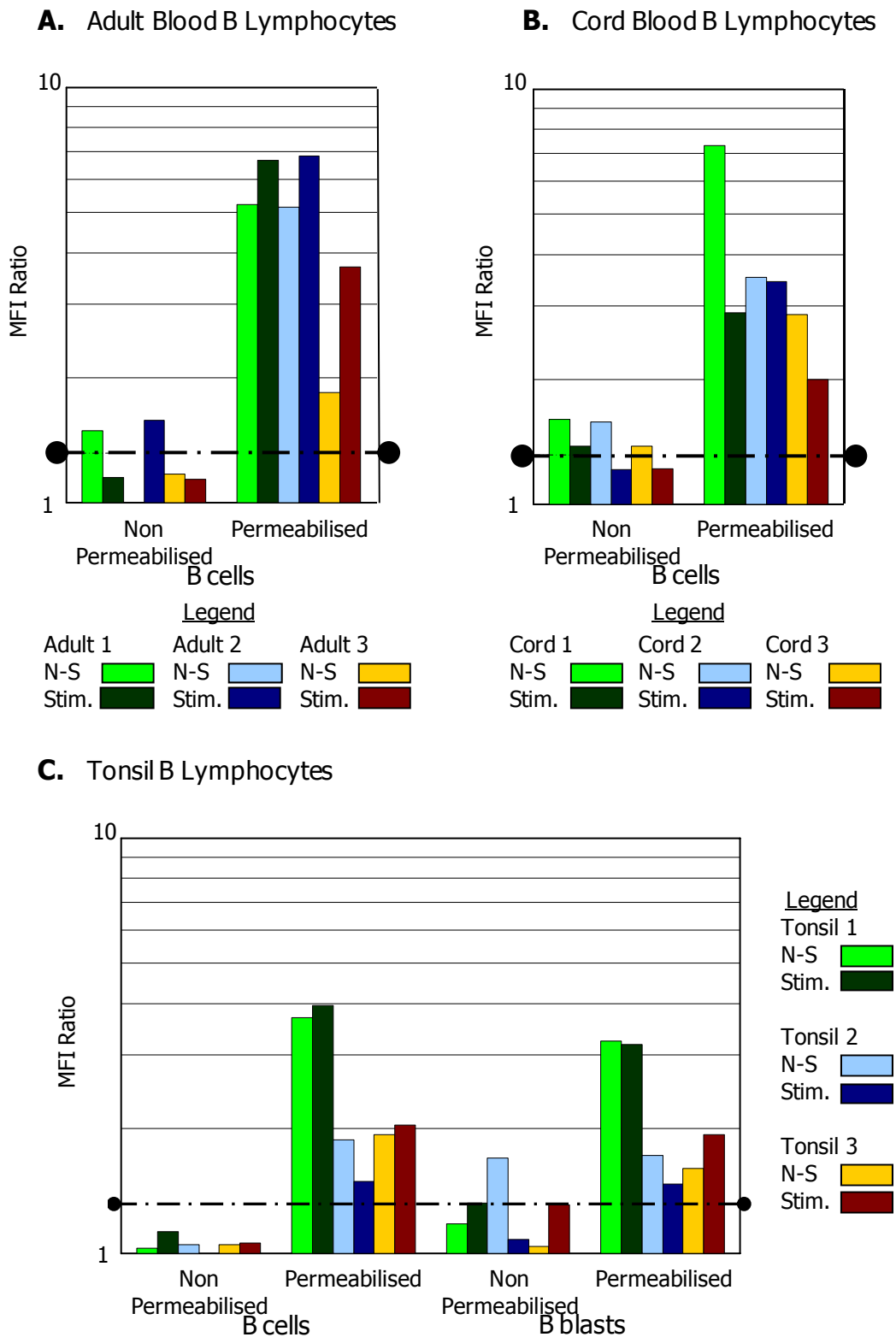


Figure 4.5 TLR3 Expression by B Lymphocytes
MFI ratio of TLR3 expression by non-stimulated (N-S) and stimulated (Stim.) B lymphocytes, both non-permeabilised and permeabilised, from **A.** Adult blood (n = 3), **B.** Cord blood (n = 3) and **C.** Tonsils (n = 3). Dotted black line corresponds to MFI ratio of 1.3.

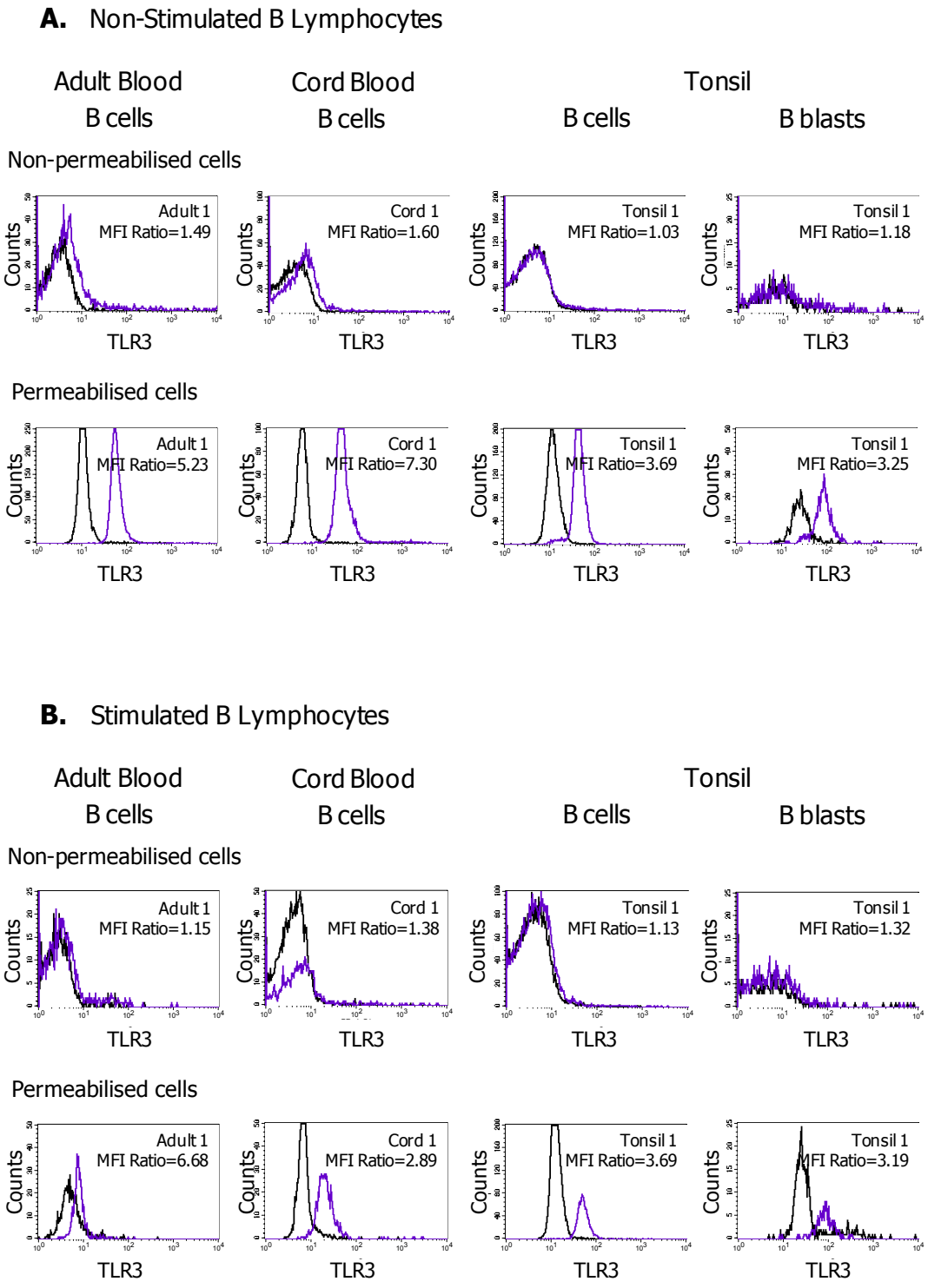


Figure 4.6 TLR3 Expression by B Lymphocytes
Representative example of TLR3 expression by B cells and B blasts, both non-permeabilised and permeabilised, from **A.** Non-stimulated adult blood, cord blood and tonsil and **B.** Stimulated adult blood, cord blood and tonsils. (TLR3 = purple, negative isotype control = black)

4.2.1.4 TLR4

Adult blood B lymphocytes:

- Non-stimulated B cells: two subjects expressed TLR4 on non-permeabilised cells and all three subjects expressed TLR4 in permeabilised cells.
- Stimulated B cells: all three subjects expressed TLR4 in permeabilised cells.

(Figures 4.7, 4.8 and Table 4.1)

Cord blood B lymphocytes:

- Non-stimulated B cells: all three subjects expressed TLR4 by non-permeabilised and permeabilised cells.
- Stimulated B cells: one subject expressed TLR4 on non-permeabilised cells and all three subjects expressed TLR4 in permeabilised cells.

(Figures 4.7, 4.8 and Table 4.1)

Tonsil B lymphocytes:

- Non-stimulated B cells: all three subjects expressed TLR4 in permeabilised cells.
- Non-stimulated B blasts: one subject expressed TLR4 on non-permeabilised cells and all three subjects expressed TLR4 in permeabilised cells.
- Stimulated B cells: no TLR4 was detected.
- Stimulated B blasts: two subjects expressed TLR4 in permeabilised cells.

(Figures 4.7, 4.8 and Table 4.1)

4.2.1.5 TLR6

Adult blood B lymphocytes:

- Non-stimulated B cells: no TLR6 was detected.

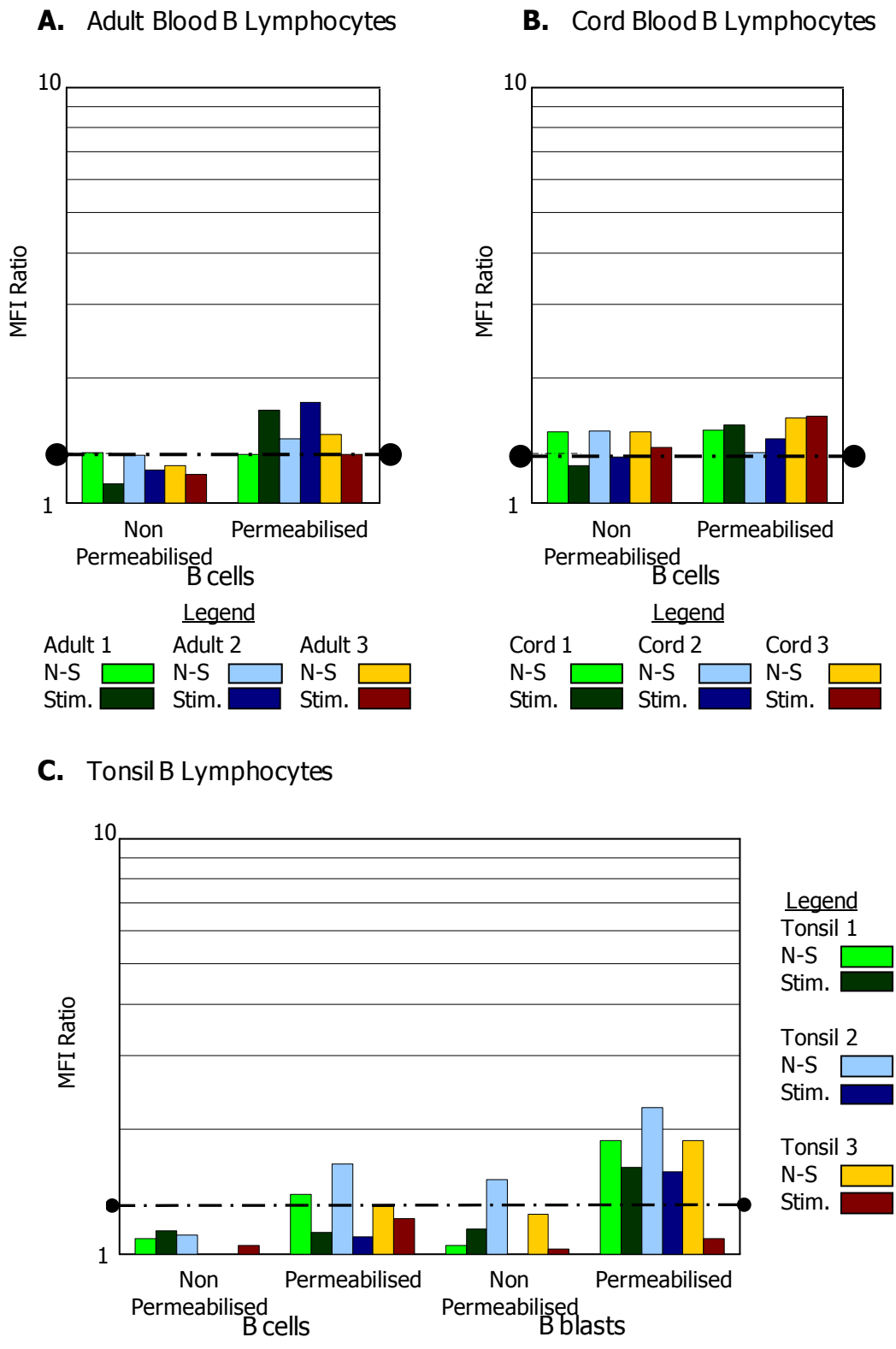


Figure 4.7 TLR4 Expression by B Lymphocytes
MFI ratio of TLR4 expression by non-stimulated (N-S) and stimulated (Stim.) B lymphocytes, both non-permeabilised and permeabilised, from **A.** Adult blood (n = 3), **B.** Cord blood (n = 3) and **C.** Tonsils (n = 3). Dotted black line corresponds to MFI ratio of 1.3.

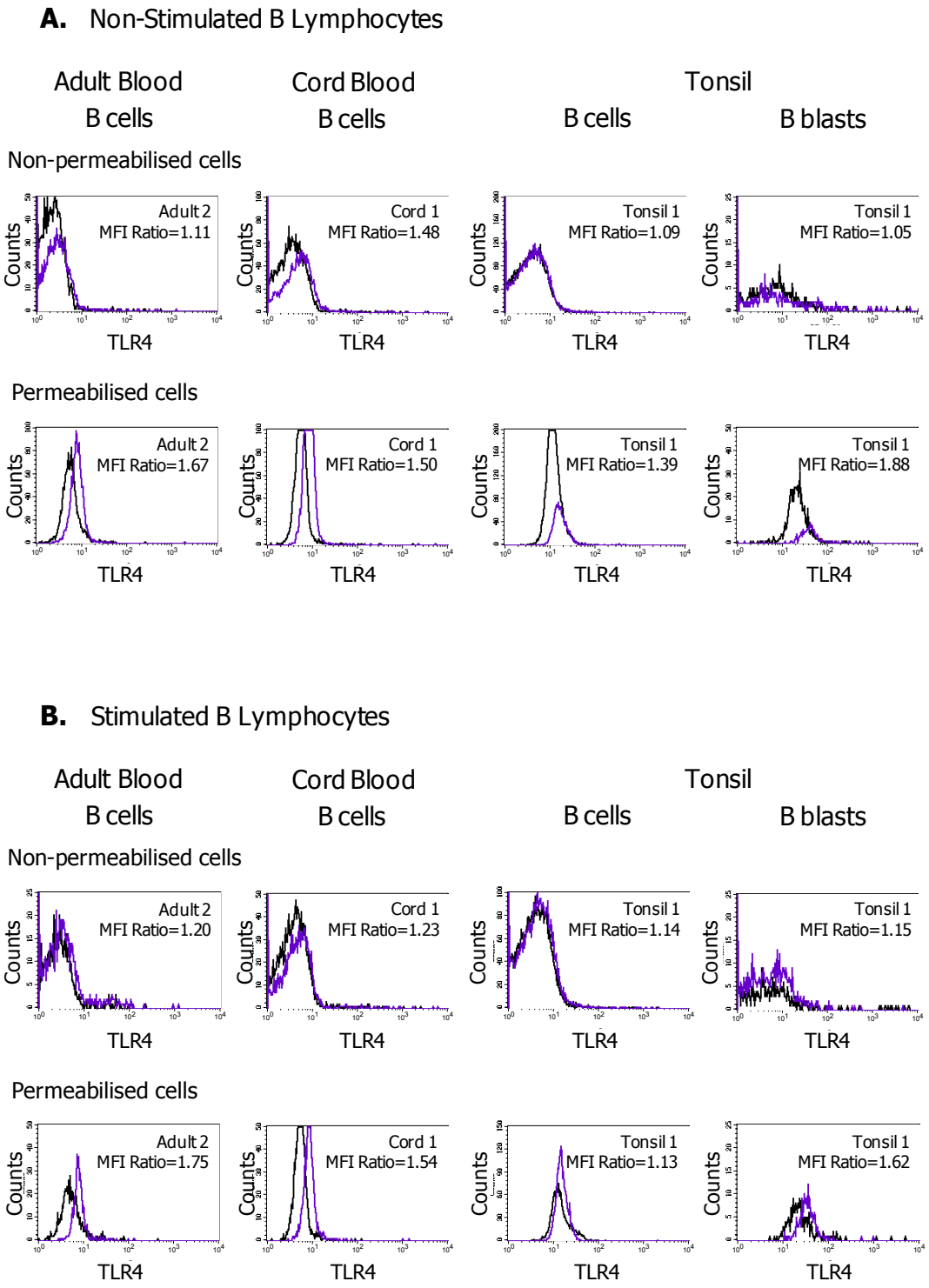


Figure 4.8 TLR4 Expression by B Lymphocytes
Representative example of TLR4 expression by B cells and B blasts, both non-permeabilised and permeabilised, from **A.** Non-stimulated adult blood, cord blood and tonsil and **B.** Stimulated adult blood, cord blood and tonsils. (TLR4 = purple, negative isotype control = black)

- Stimulated B cells: two subjects expressed TLR6 in permeabilised cells.

(Figures 4.9, 4.10 and Table 4.1)

Cord blood B lymphocytes:

- Non-stimulated B cells: one subject expressed TLR6 on non-permeabilised cells.
- Stimulated B cells: no TLR6 was detected.

(Figures 4.9, 4.10 and Table 4.1)

Tonsil B lymphocytes:

- Non-stimulated B cells: one subject expressed TLR6 in permeabilised cells.
- Non-stimulated B blasts: two subjects expressed TLR6 in permeabilised cells.
- Stimulated B cells: one subject expressed TLR6 on non-permeabilised cells and another subject expressed TLR6 in permeabilised cells.
- Stimulated B blasts: one subject expressed TLR6 on non-permeabilised cells and another subject expressed TLR6 by non-permeabilised and permeabilised cells.

(Figures 4.9, 4.10 and Table 4.1)

4.2.1.6 TLR8

Adult blood B lymphocytes:

- Non-stimulated B cells: all three subjects expressed high levels of TLR8 on non-permeabilised cells and low levels of TLR8 in permeabilised cells.
- Stimulated B cells: all three subjects expressed high levels of TLR8 on non-permeabilised cells and low levels of TLR8 in permeabilised cells.

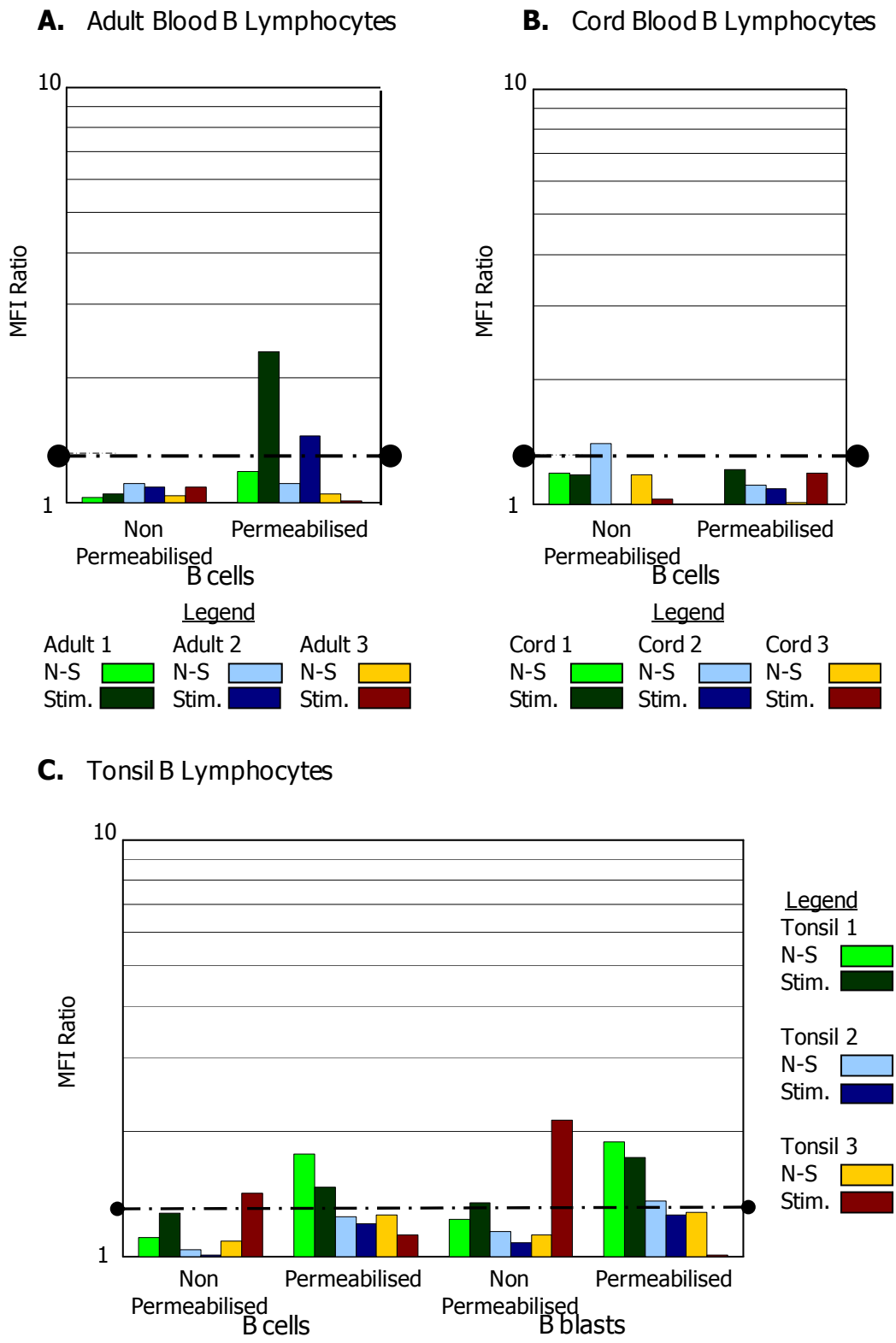


Figure 4.9 TLR6 Expression by B Lymphocytes
MFI ratio of TLR6 expression by non-stimulated (N-S) and stimulated (Stim.) B lymphocytes, both non-permeabilised and permeabilised, from **A.** Adult blood (n = 3), **B.** Cord blood (n = 3) and **C.** Tonsils (n = 3). Dotted black line corresponds to MFI ratio of 1.3.

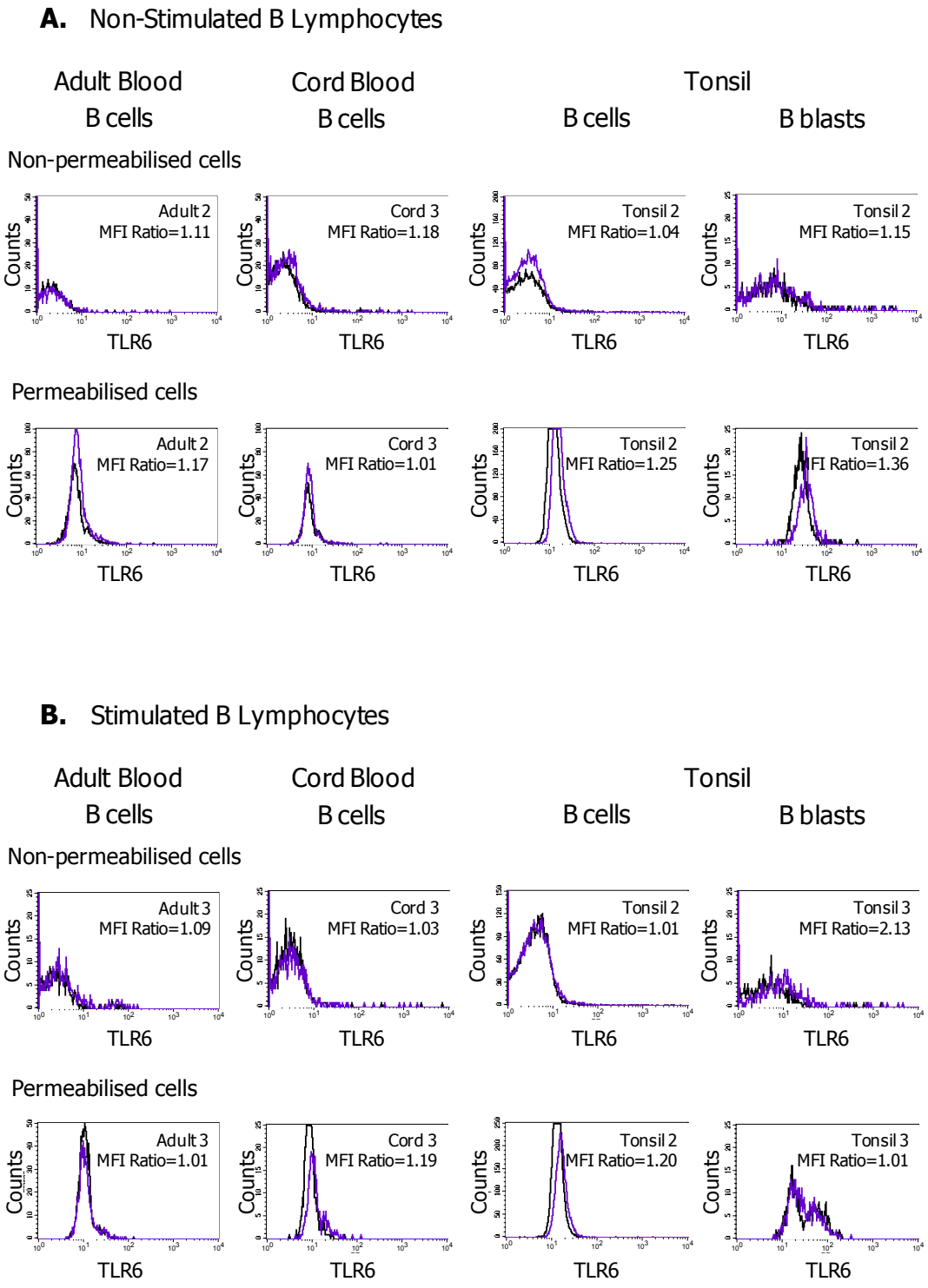


Figure 4.10 TLR6 Expression by B Lymphocytes
Representative example of TLR6 expression by B cells and B blasts, both non-permeabilised and permeabilised, from **A.** Non-stimulated adult blood, cord blood and tonsil and **B.** Stimulated adult blood, cord blood and tonsils. (TLR6 = purple, negative isotype control = black)

(Figures 4.11, 4.12 and Table 4.1)

Cord blood B lymphocytes:

- Non-stimulated B cells: all three subjects expressed high levels of TLR8 on non-permeabilised cells and one subject expressed TLR8 in permeabilised cells.
- Stimulated B cells: all three subjects expressed TLR8 on non-permeabilised cells and two subjects expressed TLR8 in permeabilised cells.

(Figures 4.11, 4.12 and Table 4.1)

Tonsil B lymphocytes:

- Non-stimulated B cells: one subject expressed TLR8 on non-permeabilised cells and another subject expressed TLR8 by non-permeabilised and permeabilised cells.
- Non-stimulated B blasts: all three subjects expressed TLR8 on non-permeabilised cells and one subject expressed TLR8 in permeabilised cells.
- Stimulated B cells: all three subjects expressed TLR8 on non-permeabilised cells and one subject expressed TLR8 in permeabilised cells.
- Stimulated B blasts: all three subjects expressed TLR8 on non-permeabilised cells and one subject expressed TLR8 in permeabilised cells.

(Figures 4.11, 4.12 and Table 4.1)

4.2.1.7 TLR9

Adult blood B lymphocytes:

- Non-stimulated B cells: two subjects expressed TLR9 on non-permeabilised cells and all three subjects expressed bright TLR9 in permeabilised cells.

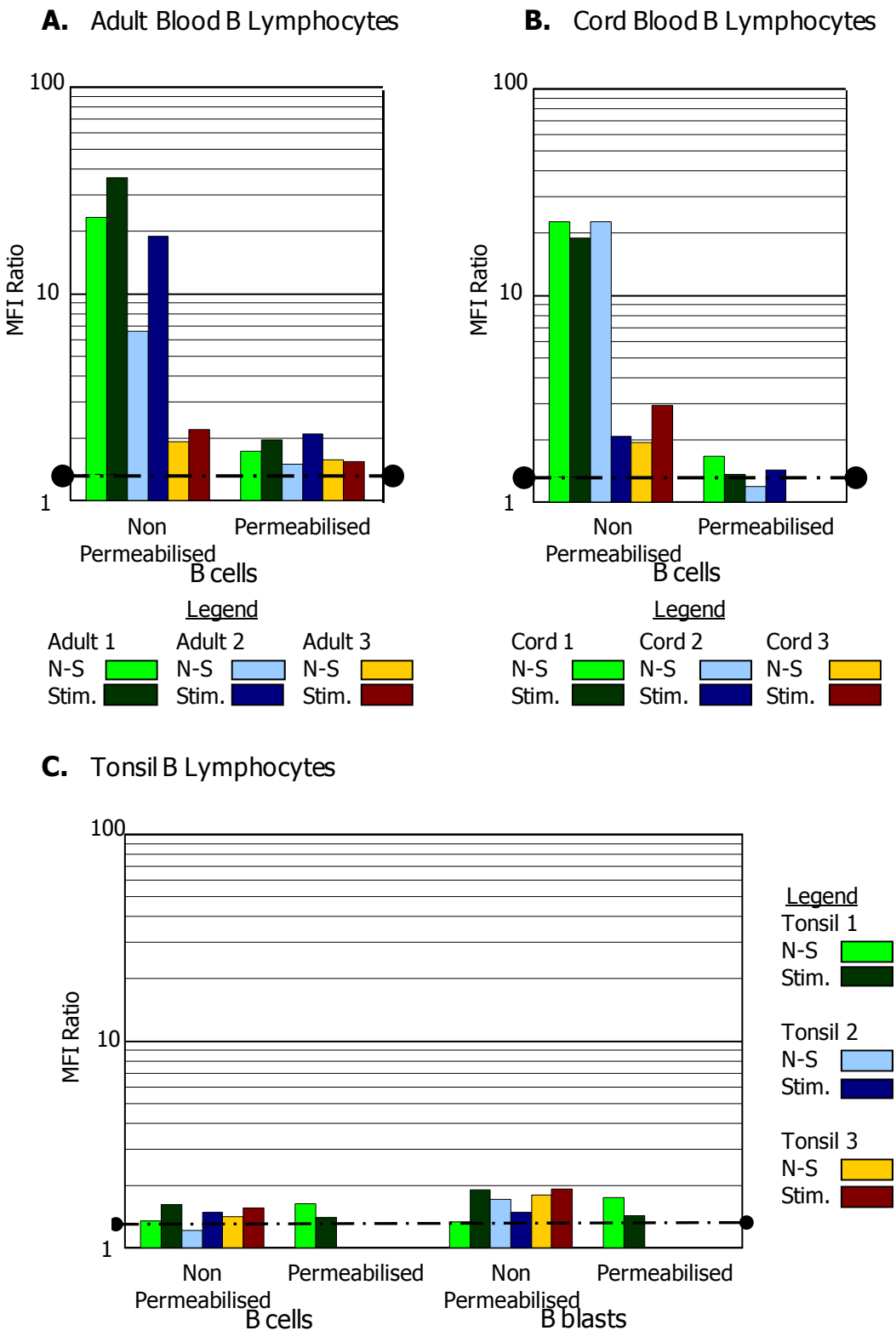


Figure 4.11 TLR8 Expression by B Lymphocytes
MFI ratio of TLR8 expression by non-stimulated (N-S) and stimulated (Stim.) B lymphocytes, both non-permeabilised and permeabilised, from **A.** Adult blood (n = 3), **B.** Cord blood (n = 3) and **C.** Tonsils (n = 3). Dotted black line corresponds to MFI ratio of 1.3.

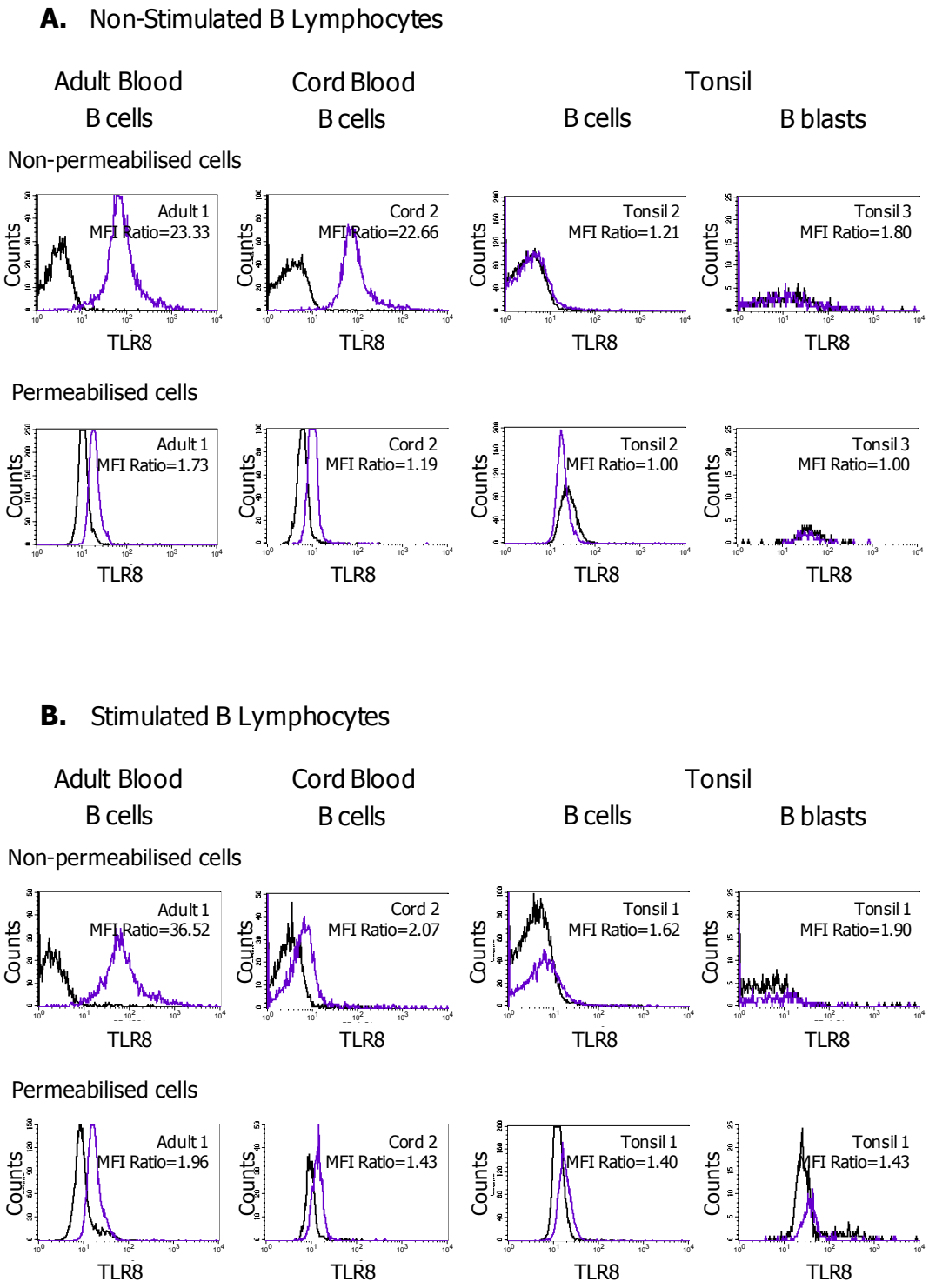


Figure 4.12 TLR8 Expression by B Lymphocytes
Representative example of TLR8 expression by B cells and B blasts, both non-permeabilised and permeabilised, from **A.** Non-stimulated adult blood, cord blood and tonsil and **B.** Stimulated adult blood, cord blood and tonsils. (TLR8 = purple, negative isotype control = black)

- Stimulated B cells: two subjects expressed TLR9 on non-permeabilised cells and all three subjects expressed bright TLR9 in permeabilised cells.

(Figures 4.13, 4.14 and Table 4.1)

Cord blood B lymphocytes:

- Non-stimulated B cells: one subject expressed TLR9 on non-permeabilised cells and all three subjects expressed TLR9 in permeabilised cells.
- Stimulated B cells: all three subjects expressed TLR9 by non-permeabilised and permeabilised cells.

(Figures 4.13, 4.14 and Table 4.1)

Tonsil B lymphocytes:

- Non-stimulated B cells: all three subjects expressed TLR9 in permeabilised cells.
- Non-stimulated B blasts: one subject expressed TLR9 on non-permeabilised cells and all three subjects expressed TLR9 in permeabilised cells.
- Stimulated B cells: all three subjects expressed TLR9 in permeabilised cells.
- Stimulated B blasts: all three subjects expressed TLR9 in permeabilised cells.

(Figures 4.13, 4.14 and Table 4.1)

4.2.2 TLR Expression by B cell Subsets

To observe if B cell differentiation affects TLR expression, B cell subsets of adult blood, cord blood and tonsils had TLR expression examined. With fluorescently-labelled antibodies against CD5 and CD27, B lymphocytes from blood were categorised into the following subsets: B1 ($CD5^+$), B2 ($CD5^-$), naïve ($CD27^-$) and memory ($CD27^+$) B cells. Adult blood B lymphocytes have all four subsets, but

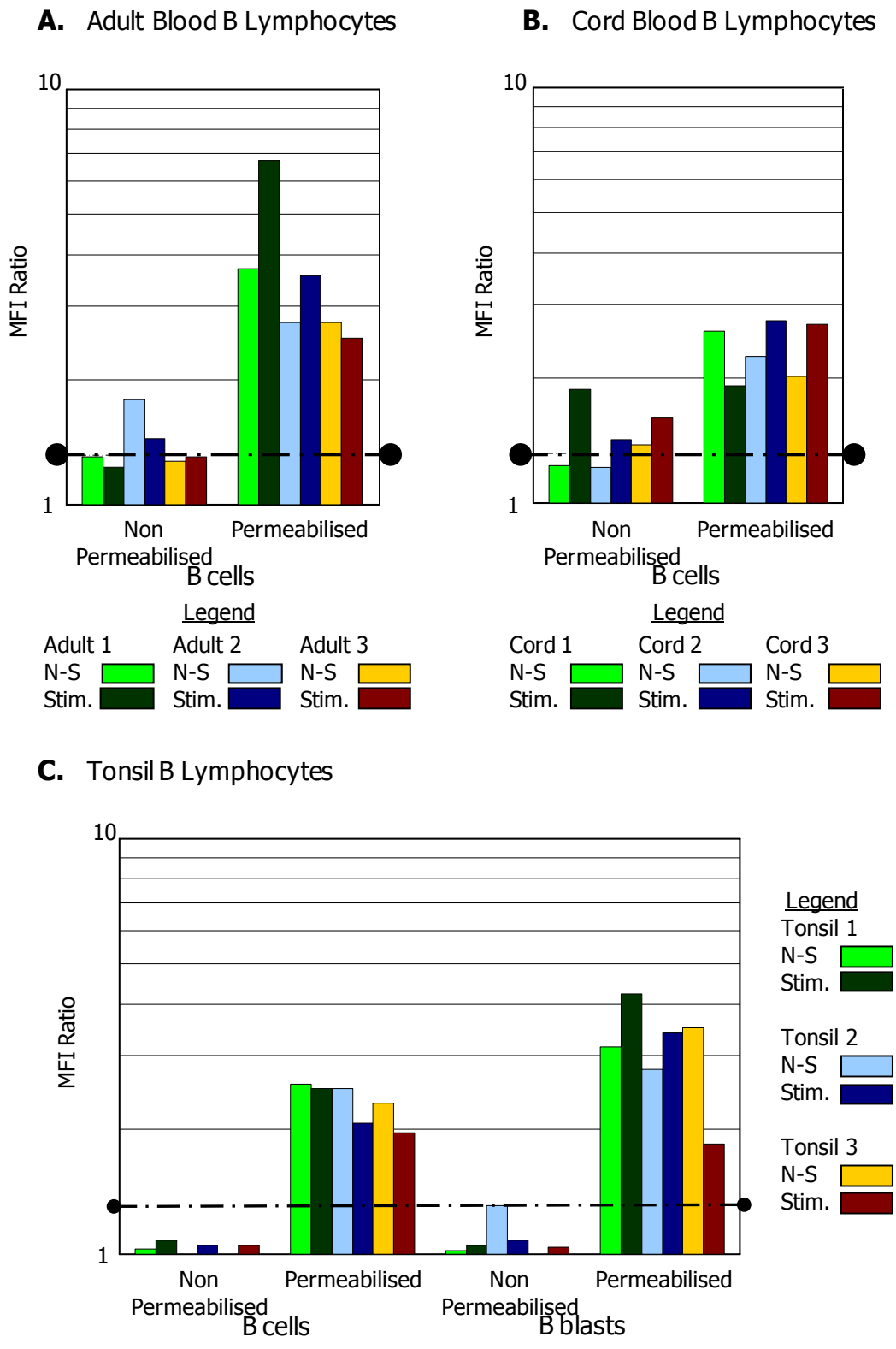


Figure 4.13 TLR9 Expression by B Lymphocytes
MFI ratio of TLR9 expression by non-stimulated (N-S) and stimulated (Stim.) B lymphocytes, both non-permeabilised and permeabilised, from **A.** Adult blood (n = 3), **B.** Cord blood (n = 3) and **C.** Tonsils (n = 3). Dotted black line corresponds to MFI ratio of 1.3.

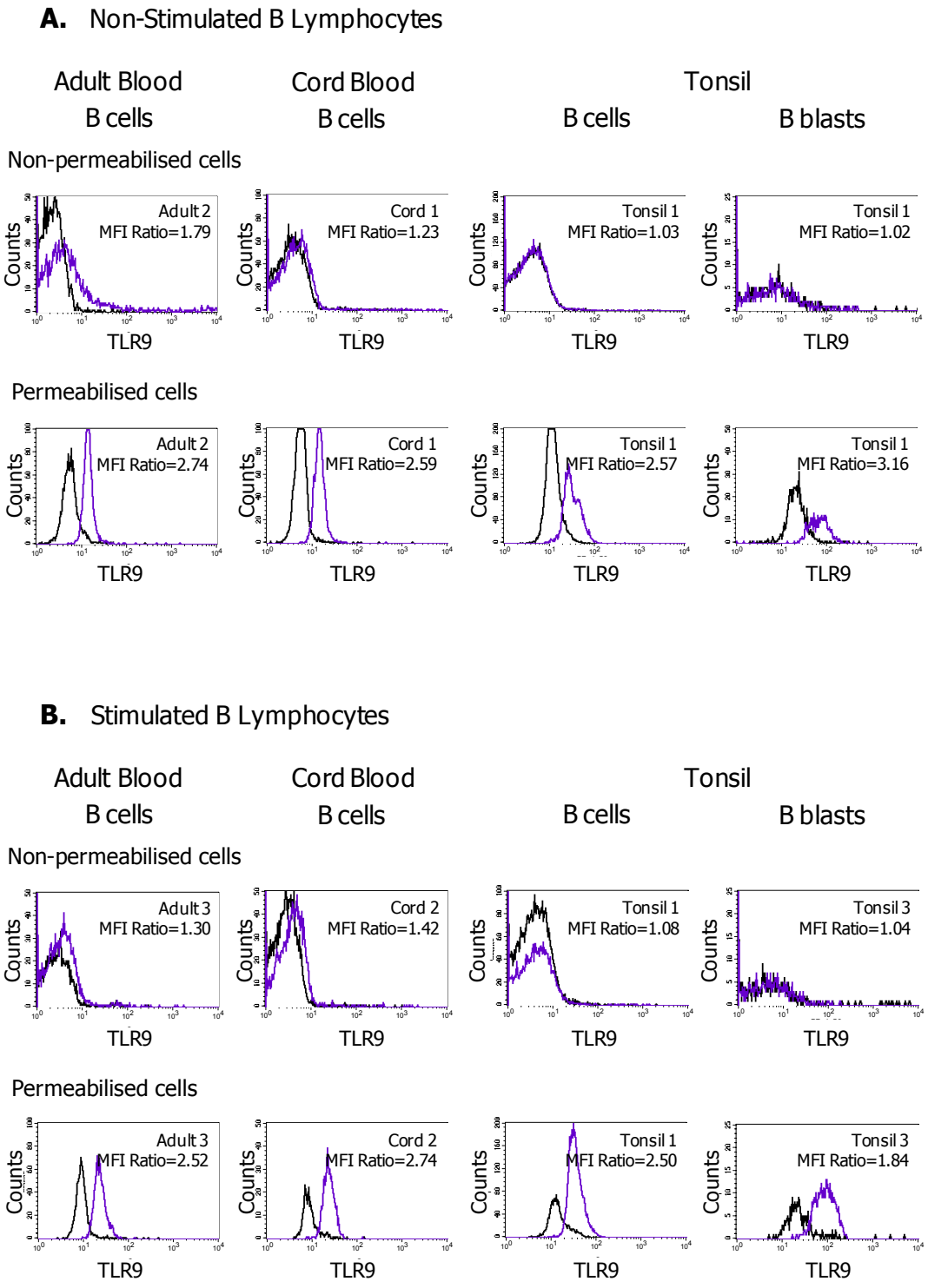


Figure 4.14 TLR9 Expression by B Lymphocytes
Representative example of TLR9 expression by B cells and B blasts, both non-permeabilised and permeabilised, from **A.** Non-stimulated adult blood, cord blood and tonsil and **B.** Stimulated adult blood, cord blood and tonsils. (TLR9 = purple, negative isotype control = black)

Table 4.1 Summary of TLR Expression Patterns by B Lymphocytes

TLR	Group (n = 3)	Cells				Blasts			
		Non-Stim.		Stim.		Non-Stim.		Stim.	
		N-P	P	N-P	P	N-P	P	N-P	P
1	Adult blood	2	2	2	3	NA		NA	
	Cord blood	3	0	2	1				
	Tonsil	1	1	1	1	1	1	1	0
2	Adult blood	0	0	2	1	NA		NA	
	Cord blood	0	0	3	1				
	Tonsil	0	0	0	0	1	1	0	1
3	Adult blood	1	3	1	3	NA		NA	
	Cord blood	3	3	1	3				
	Tonsil	0	3	0	3	1	3	2	3
4	Adult blood	2	3	0	3	NA		NA	
	Cord blood	3	3	1	3				
	Tonsil	0	3	0	0	1	3	0	2
6	Adult blood	0	0	0	2	NA		NA	
	Cord blood	1	0	0	0				
	Tonsil	0	1	1	1	0	2	2	1
8	Adult blood	3	3	3	3	NA		NA	
	Cord blood	3	1	3	2				
	Tonsil	2	1	3	1	3	1	3	1
9	Adult blood	2	3	2	3	NA		NA	
	Cord blood	1	3	3	3				
	Tonsil	0	3	0	3	1	3	0	3

The three groups, adult blood, cord blood and tonsils, had three subjects per group. The numbers of subjects in the table are the number of subjects out of three that had TLR detected.

Non-Stim. = Non-stimulated, Stim. = Stimulated, cells = B cells, blast = B blasts, N-P = Non-permeabilised cells, P = Permeabilised cells, NA = Not available.

cord blood B lymphocytes are comprised of B1 and B2 B cell subsets. Fluorescent antibody markers against IgD and CD38 tagged tonsil B lymphocytes into the following four subsets: naïve (IgD^+CD38^-), pre-GC (IgD^+CD38^+), GC (IgD^-CD38^+) and memory (IgD^-CD38^-) B cells. As before, with matched negative isotype controls, TLR expression was measured with the MFI ratio.

4.2.2.1 TLR Expression by Adult Blood B cell Subsets

Non-stimulated B cells from adult blood expressed TLR8 on non-permeabilised cells, and TLR3, TLR4 and TLR9 in permeabilised cells. Levels of TLR3 and TLR9 expression are similar between B1 and B2 subsets, and between naïve and memory subsets. TLR4 and TLR8 have slightly lower levels in expression ranges for the B2 B cell subset compared to the other three subsets. (Refer Figure 4.15)

Stimulated B cells from adult blood expressed TLR8 on non-permeabilised cells, and TLR1, TLR3, TLR4 and TLR9 in permeabilised cells. Between B1 and B2 subsets, the TLR1 expression range was lower for the B1 subset. Between naïve and memory subsets, the expression ranges of TLR1 and TLR4 were lower in the naïve subset, and for TLR3 and TLR9 the ranges were higher in the naïve subset. (Refer Figure 4.16)

Adult B cell subsets have slight variations in the levels of TLR expression between the B1/B2 subsets and between the naïve/memory subsets.

4.2.2.2 TLR Expression by Cord Blood B cell Subsets

Non-stimulated B cells from cord blood expressed TLR1, TLR4 and TLR8 on non-permeabilised cells, and TLR3 and TLR9 in permeabilised cells. The levels of

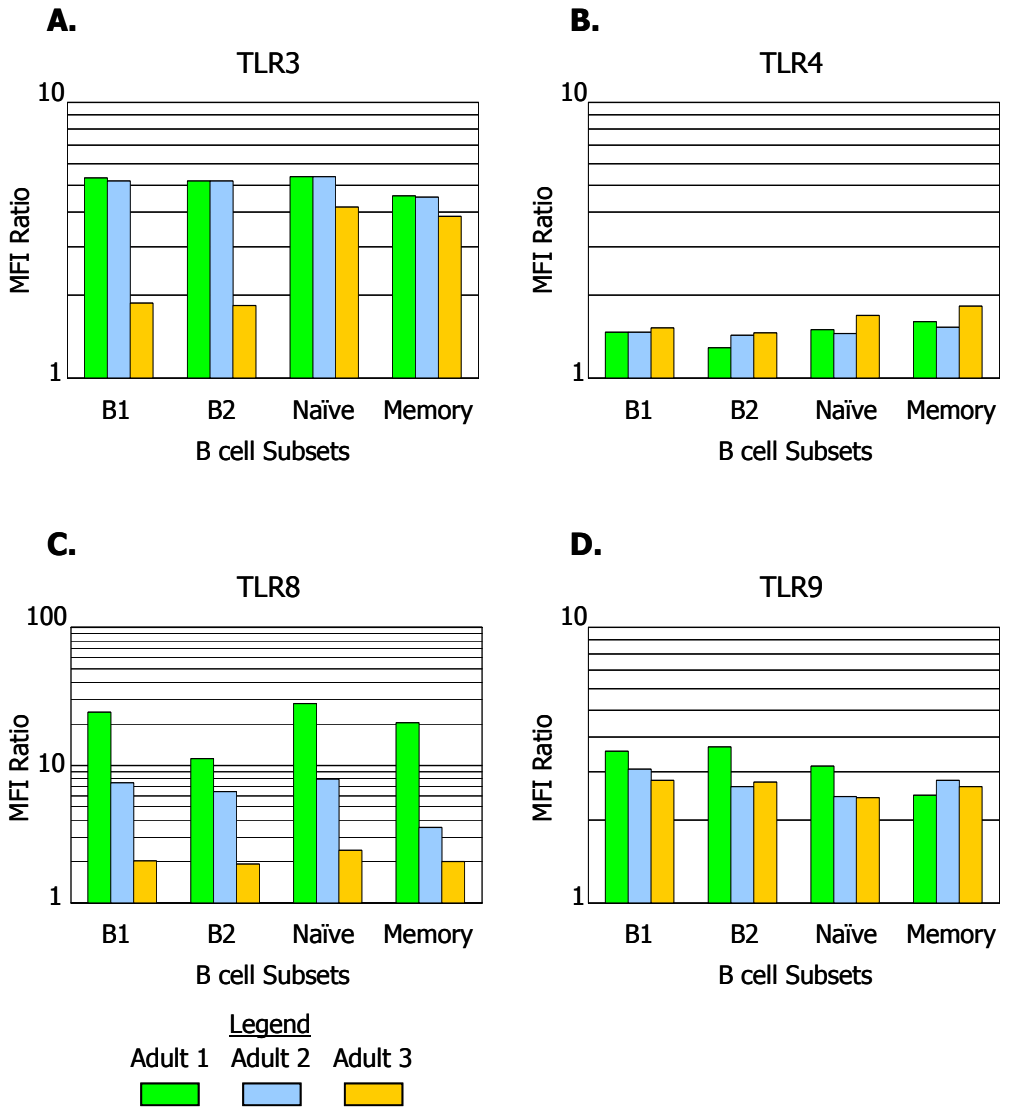


Figure 4.15 TLR Expression by Non-Stimulated Adult B cell Subsets
Non-stimulated B cells from adult blood (n = 3) with detected TLR were categorised into adult blood B cells subsets; B1, B2, naive and memory B cells. Non-stimulated adult B cells expressed **A.** Intracellular TLR3, **B.** Intracellular TLR4, **C.** Extracellular TLR8 and **D.** Intracellular TLR9.

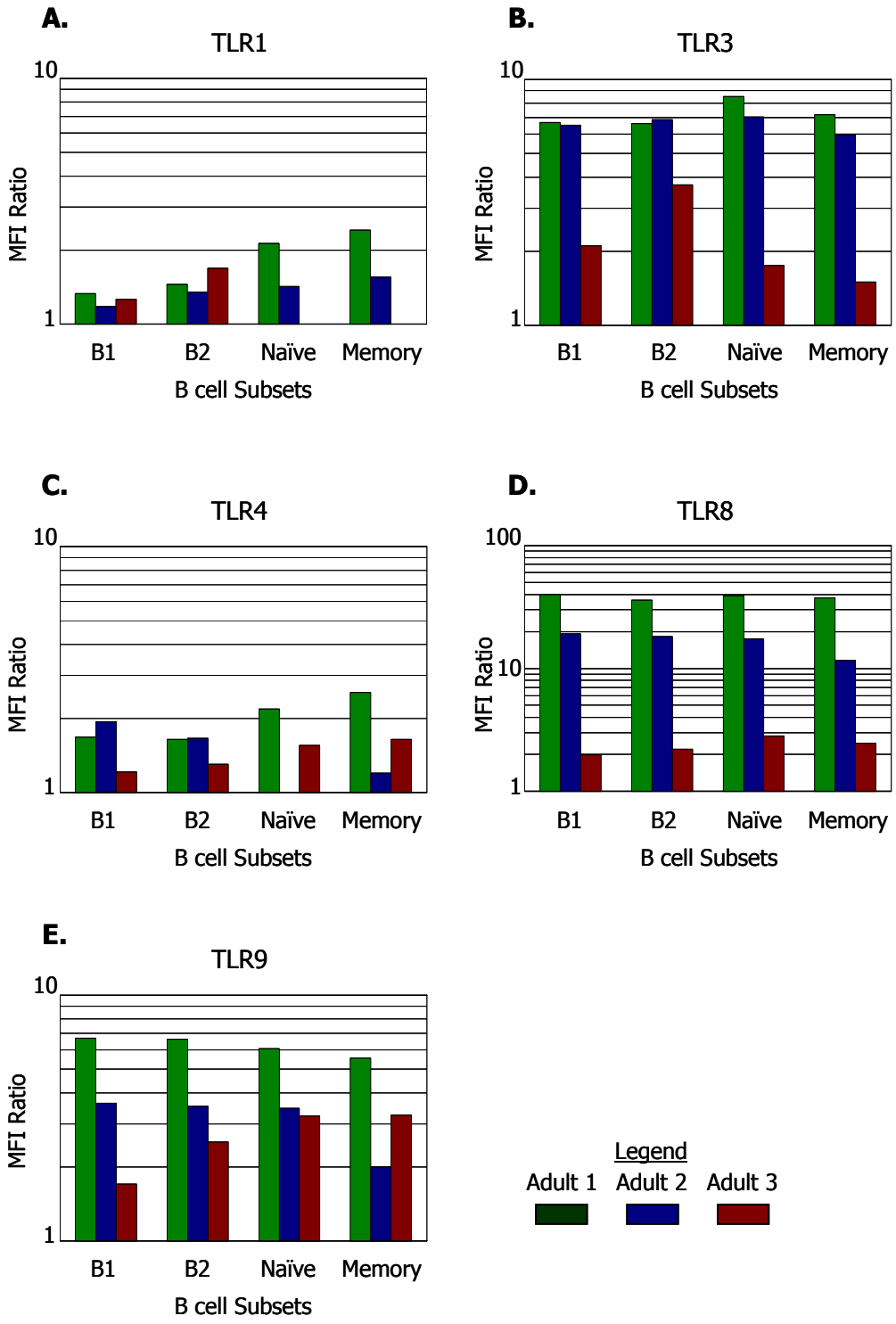


Figure 4.16 TLR Expression by Stimulated Adult B cell Subsets
Stimulated B cells from adult blood (n = 3) with detected TLR were categorised into adult blood B cells subsets; B1, B2, naive and memory B cells. Stimulated adult B cells expressed **A.** Intracellular TLR1, **B.** Intracellular TLR3, **C.** Intracellular TLR4, **D.** Extracellular TLR8 and **E.** Intracellular TLR9.

TLR1, TLR3, TLR4, TLR8 and TLR9 expression ranges were similar between B1 and B2 B cell subsets. (Refer Figure 4.17)

Stimulated B cells expressed TLR2 and TLR8 on non-permeabilised cells, and TLR3, TLR4 and TLR9 in permeabilised cells. The levels of of TLR2, TLR3, TLR4, TLR8 and TLR9 expression were similar between B1 and B2 B cell subsets. (Refer Figure 4.18)

B cell subsets from cord blood do not differ in TLR expression.

4.2.2.3 TLR Expression by Tonsil B cell Subsets

Non-stimulated B cells from tonsils expressed TLR3, TLR4 and TLR9 in permeabilised cells. The GC B cell subset, compared to other subsets, appears to have slightly lower levels of TLR3 expression and higher levels of TLR4 and, especially, TLR9 expression. (Refer Figure 4.19)

Stimulated B cells from tonsils expressed TLR8 on non-permeabilised cells, and TLR3 and TLR9 in permeabilised cells. There is little difference for TLR3, but TLR8 has lower expression by memory B cells and GC B cells express TLR9 at a higher range. (Refer Figure 4.20)

Tonsil B cell subsets had differences in TLR expression, especially the GC B cell subset.

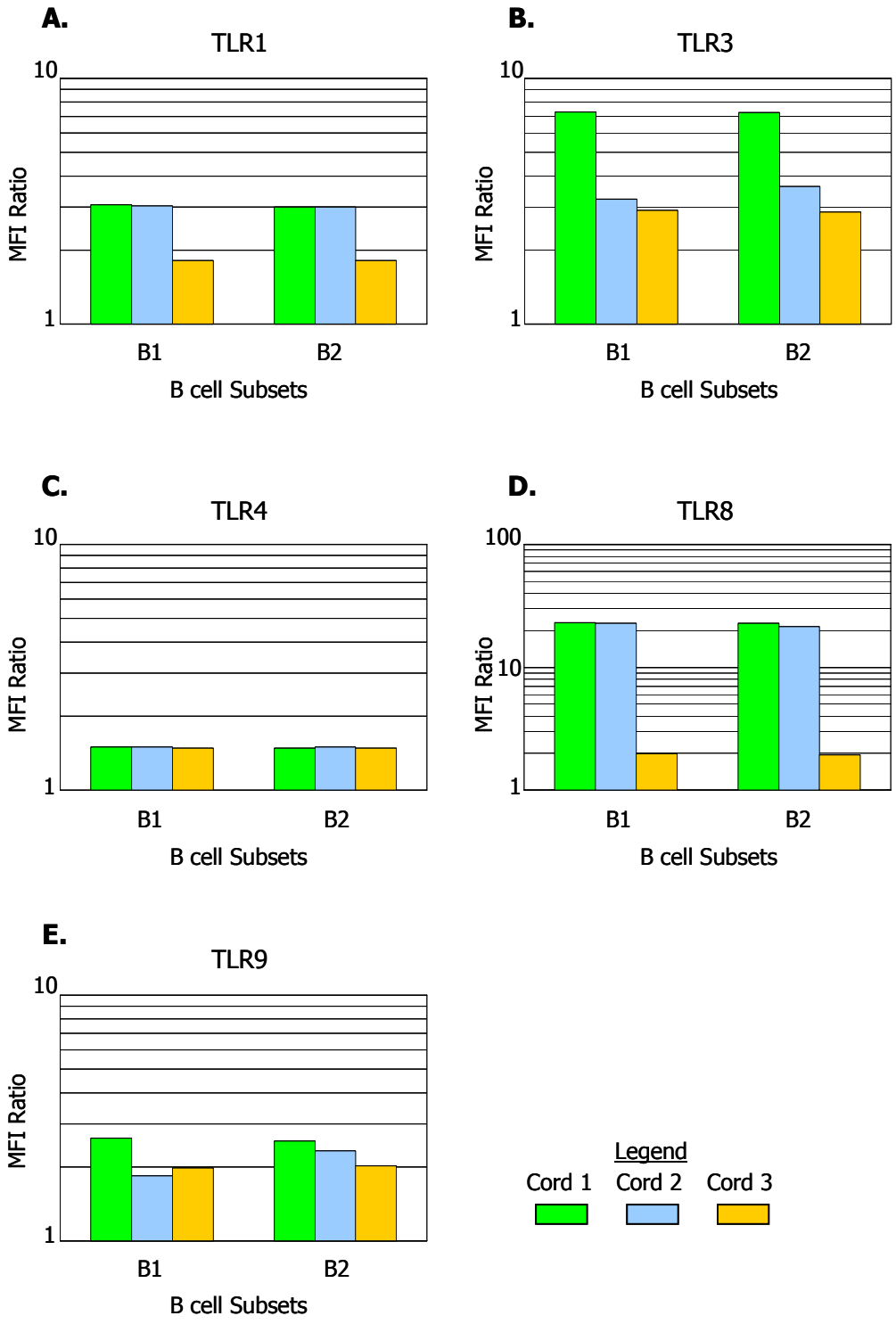


Figure 4.17 TLR Expression by Non-Stimulated Cord Blood B cell Subsets
Non-stimulated B cells from cord blood (n = 3) with detected TLR were categorised into cord blood B cells subsets, B1 and B2 B cells. Cord blood B cells expressed **A.** Extracellular TLR1, **B.** Intracellular TLR3, **C.** Extracellular TLR4, **D.** Extracellular TLR8 and **E.** Intracellular TLR9.

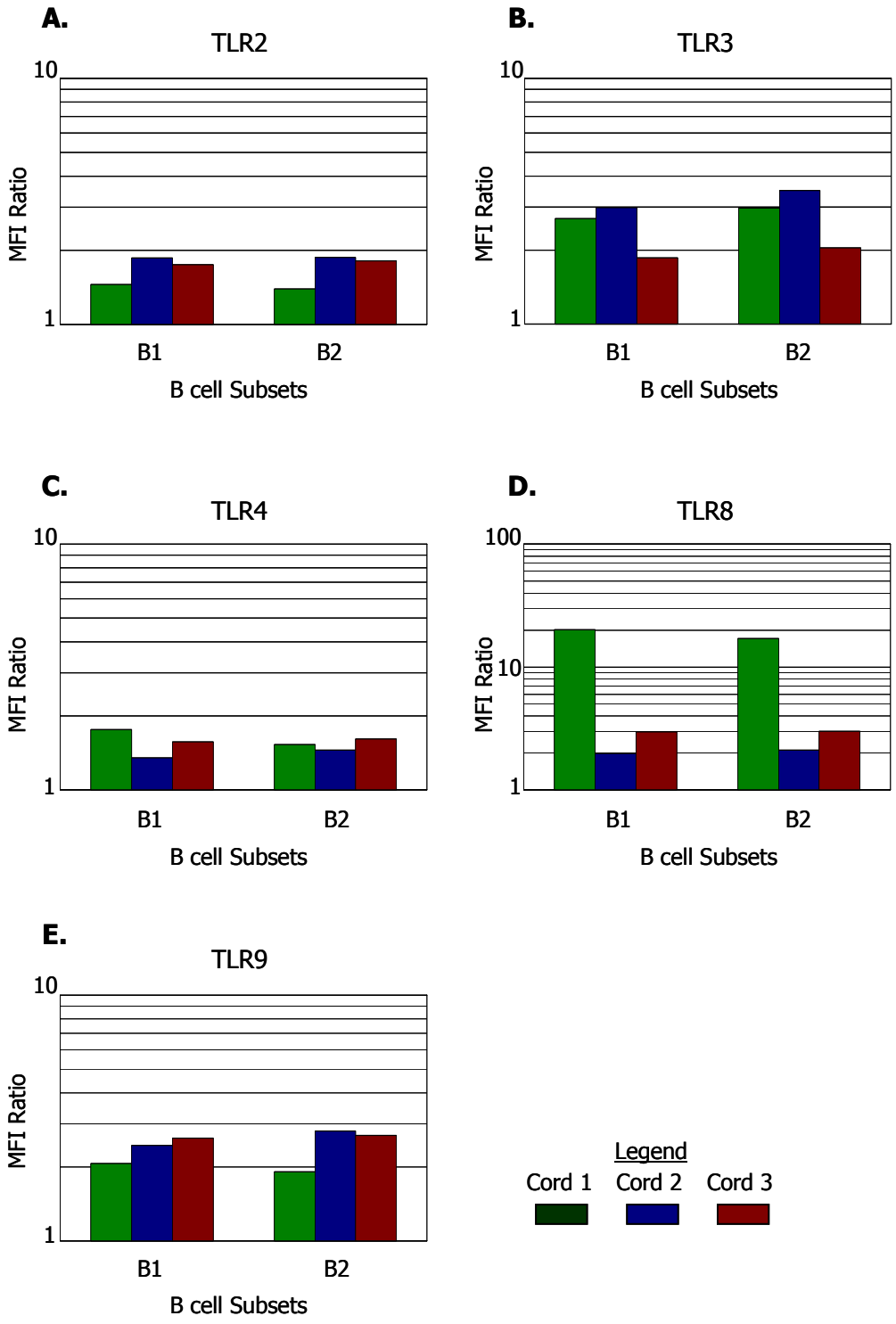


Figure 4.18 TLR Expression by Stimulated Cord Blood B cell Subsets
Stimulated B cells from cord blood (n = 3) with detected TLR were categorised into cord blood B cells subsets, B1 and B2 B cells. Cord blood B cells expressed **A.** Extracellular TLR2, **B.** Intracellular TLR3, **C.** Intracellular TLR4, **D.** Extracellular TLR8 and **E.** Intracellular TLR9.

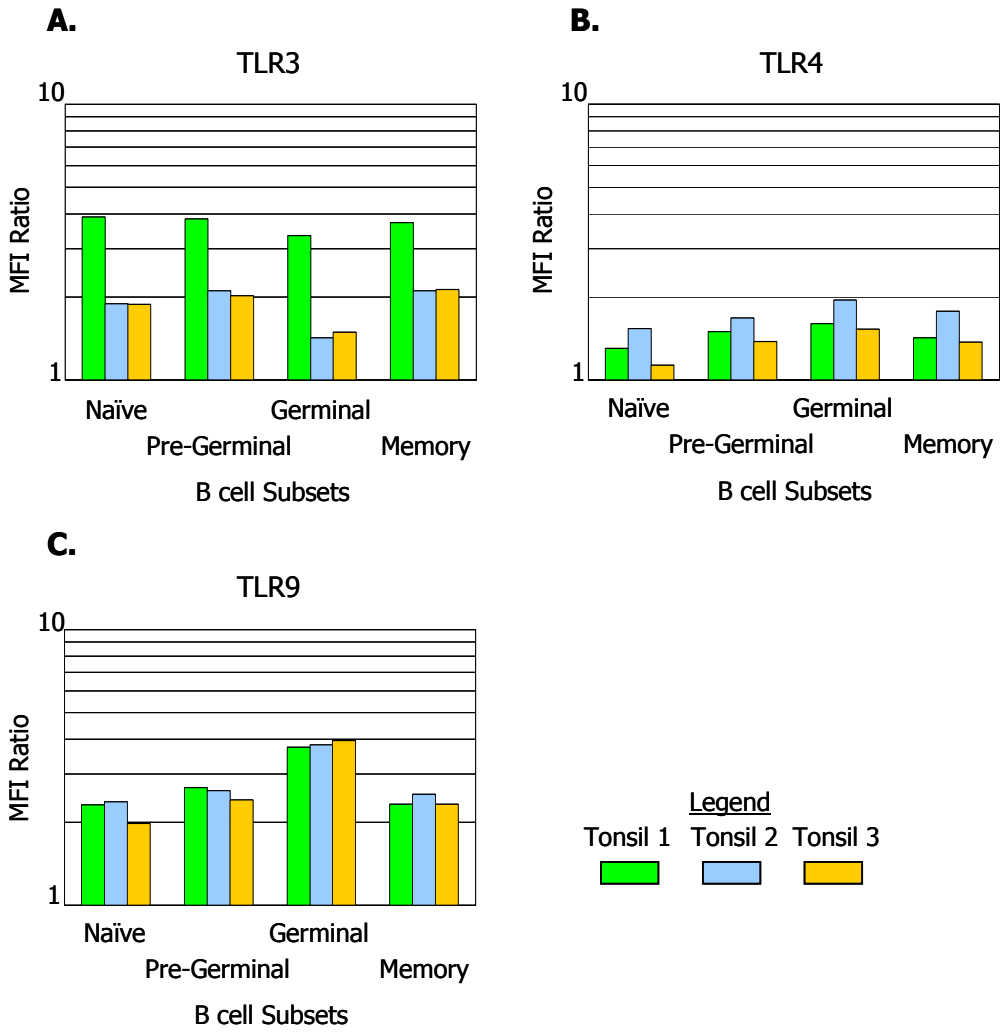


Figure 4.19 TLR Expression by Non-Stimulated Tonsil B cell Subsets
Non-stimulated B cells from tonsils (n = 3) with detected TLR were categorised into tonsil B cells subsets; naïve, pre-germinal, germinal and memory B cells. Tonsil B cells expressed **A.** Intracellular TLR3, **B.** Intracellular TLR4 and **C.** Intracellular TLR9.

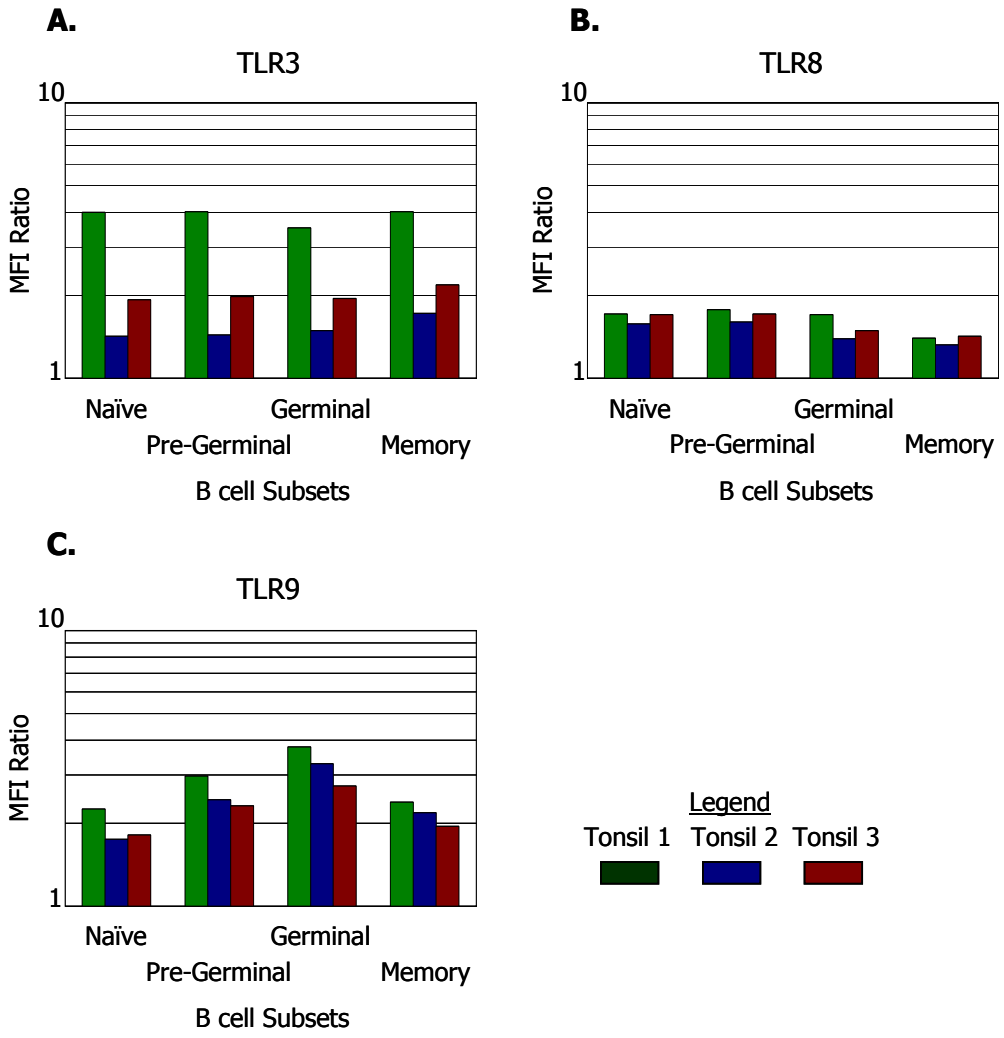


Figure 4.20 TLR Expression by Stimulated Tonsil B cell Subsets
Stimulated B cells from tonsils (n = 3) with detected TLR were categorised into tonsil B cells subsets; naïve, pre-germinal, germinal and memory B cells. Tonsil B cells expressed **A.** Intracellular TLR3, **B.** Extracellular TLR8 and **C.** Intracellular TLR9.

4.3 DISCUSSION

The main observations from the results are, firstly B lymphocytes from adult blood and cord blood expressed similar patterns of TLR. Secondly, B lymphocytes from tonsils expressed fewer TLR than B lymphocytes from adult blood and cord blood. Thirdly, stimulation of B lymphocytes did not alter most TLR expression patterns. Lastly, differences in levels of TLR expression within B cell subsets were observed in tonsils.

The results showed that B lymphocytes from adult blood and cord blood consistently expressed TLR1, TLR2, TLR3, TLR4, TLR8 and TLR9, but expression of TLR6 by B lymphocytes was inconsistent within the two groups. The consistency of TLR expression by B lymphocytes from adult blood and cord blood was such that in both groups TLR2 expression was upregulated to positive expression by stimulated B cells.

The observation that cord blood B lymphocytes express TLR that adult blood B lymphocytes express, which was also seen in T lymphocytes, suggests that B lymphocytes from neonates are capable of adult-like "innate" recognition of pathogens. However, cord blood B lymphocytes may not have responses like adult blood B lymphocytes if TLR signalling, as discussed in Chapter Three, is impaired. The reduced TLR signalling in neonatal leucocytes coupled with the lowered function of B lymphocytes from neonates, discussed in Chapter One, may affect TLR-induced responses of neonatal B lymphocytes compared to adult B lymphocytes. To establish the capacity of cord blood B lymphocytes in their response to TLR engagement further study into their functional abilities is required.

The expectation that TLR may be expressed highly by under developed neonatal B lymphocytes has not been supported with these expression studies.

The second observation was that B lymphocytes from tonsils expressed fewer TLR than B lymphocytes from adult blood and cord blood. Tonsil B lymphocytes expressed TLR3, TLR4, TLR8 and TLR9, but the expression was inconsistent compared to blood B lymphocytes. From these results it appears that the site of B lymphocytes, instead of age-related immune development, may have a greater influence on TLR expression.

B cells and B blasts from tonsils expressed TLR3, TLR4, TLR8 and TLR9, in contrast to Chapter Three which showed more TLR expressed by T blasts than T cells. It wasn't possible to compare with B blasts from adult blood and cord blood as there were too few in the blood for a defined sub-population (Refer Chapter Two, Figure 2.2).

B lymphocytes from blood express more TLR than tonsil B lymphocytes. The greater expression of TLR by B lymphocytes from blood may be due to regulation as B lymphocytes circulate between blood and secondary lymphoid organs. Blood B lymphocytes with their greater TLR expression can facilitate T-I responses to TLR ligands; several T-I antigens are TLR ligands, LPS and CpG ODN can act through TLR4 and TLR9 respectively. However, differences in expression by B lymphocytes from blood and tonsils require further investigation to establish if circulatory B lymphocytes are regulating TLR or if the TLR expression is constitutive to the particular populations of B lymphocytes.

In tonsils, B lymphocytes participate in T-D immune responses and produce an adaptive, specific, high-affinity, memory response. Although tonsil B lymphocytes expressed fewer TLR, there are murine (158) and human (157) studies that postulate B lymphocytes require TLR engagement in addition to BCR engagement and cognate interaction for effective T-D immune responses. The reduced expression of TLR by tonsil B lymphocytes appears to contradict these recent studies, but TLR expression may be carefully regulated to fulfil this particular role in T-D responses.

The third observation was that *in vitro* stimulation of B lymphocytes did not affect expression of most TLR; non-stimulated and stimulated B lymphocytes expressed the same TLR. The TLR expression affected by *in vitro* activation was TLR2 and TLR6 for adult blood and cord blood, and TLR4 for tonsils. As with T lymphocytes, the method of B lymphocyte activation, which mimicked T-D activation, appeared to be ineffective for altering TLR expression. B lymphocytes have increased or decreased expression of TLR in response to BCR engagement (99, 154) and to diseases like tonsillitis (91), lymphatic filariasis (93) and HIV (196). However, most of these published reports are in disease states and they focus on mRNA expression rather than protein expression. B lymphocytes can regulate TLR expression in response to appropriate stimuli as seen in the literature and the results of TLR2, TLR4 and TLR6 expression.

The final observation was that levels in TLR expression were different in B cell subsets from tonsils. For B cell subsets from tonsils, GC B cells differed from other subsets in levels of TLR expression. In particular, TLR9 expression by GC B cells was considerably higher than in other tonsil subsets. One report showed tonsil GC

B cells, compared to other subsets, had elevated levels of TLR10 mRNA, but similar mRNA levels of TLR1, TLR2, TLR7 and TLR9 (91). Murine (158) and human (157) studies show that TLR engagement may be a third signal required for activation of B lymphocytes by T-D antigen, but the human study used naïve B cells. Also, the studies didn't clarify if TLR had a direct or indirect effect on B lymphocytes, an indirect effect may not require GC B cells to express TLR for the third signal. This preliminary data supports the idea that stages of B cell development in tonsils can influence TLR expression.

B1 and B2 B cell subsets from cord blood and adult blood did not differ in levels of TLR expression. This result contradicts our previous report of greater mean fluorescence intensity in TLR expression by B1 B cells than B2 B cells (92). The difference between the current results and the report is due to two reasons. The first reason is as B1 B cells are larger than B2 B cells the fluorescent signal will be brighter, thereby increasing the mean fluorescence intensity of TLR on B1 B cells. The second reason is in the report, B1 B cells had a small population of extremely bright TLR positive cells which can be seen in the TLR fluorescence histogram. This population of very bright cells skewed the TLR mean fluorescence intensity to a higher value compared to B2 B cells which lacked the very bright cells. Using the median fluorescence intensity to measure TLR expression, as done in these studies, gives a more representative average of TLR expression in a cell population compared to using mean fluorescence intensity. There is little evidence from the literature and results to support differing TLR expression between B1 and B2 B cell subsets.

The different subsets of B lymphocytes can have different responses to TLR. B1 B cells can proliferate and secrete IgM, IgA and IgG (148, 198), and assist in anti-inflammatory responses in neonates (164, 166) in response to TLR ligands. From blood and in response to TLR9 ligation, naïve B cells can be activated, proliferate, secrete IgM and upregulate MHC class II, CD40 and CD80 (153), and memory B cells are polyclonally activated and secrete IgG (152). From tonsils, there are differences in TLR expression in GC B cells, but not in the earlier lineage B cells as previously thought. GC B cells, compared to other subsets respond to TLR engagement by proliferation and increased signalling (199). Unlike blood, the development of B cell subsets in tonsils influences the patterns of TLR expression.

As with T lymphocytes, B lymphocytes exhibited considerable variability of TLR expression. For example, one adult blood subject had surface TLR3 expression on stimulated B lymphocytes and one cord blood subject expressed TLR8 in permeabilised, non-stimulated, B lymphocytes. As the donors are human subjects, considerable variability has to be expected in the expression studies. There may be consistency in certain TLR expression patterns, such as intracellular TLR9 expression by B lymphocytes, but other expressions of TLR may vary considerably. The causes of variation may be due to genetics, environment or a combination. A subject who has suffered recent infection may have elevated expression of TLR compared to a healthy, non-infected, subject. There are many unknown factors which influence TLR expression which need to be accounted for. Studies on larger groups of human subjects will provide a clearer indication of the degree of variability of TLR expression among humans.

Though the group size was too small to provide statistically significant data, preliminary information on individual TLR expression can be gleaned from the results to contribute to further knowledge, as elaborated in the following paragraphs.

TLR2 absence from non-stimulated B lymphocytes from blood and tonsils contradicts several report (74, 91, 93-94). This discrepancy is difficult to explain as surface TLR2 was detected on adult blood B lymphocytes with flow cytometry (93-94) and on activated B lymphocytes from tonsils with immunohistochemistry (74). TLR2 was detected on stimulated blood B lymphocytes, but there is no literature available to support the results.

TLR4 was detected at low levels in permeabilised B lymphocytes from all three groups with variable expression of surface TLR4. The internal TLR4 expression was unexpected (200) and adds to reports of external expression by adult B cells (93). B lymphocytes can detect invasive, intracellular, pathogens such as *Francisella tularensis* (201) with internal TLR4. The internal expression of TLR4 by B lymphocytes, also seen with T lymphocytes, may reduce immune sensitivity to LPS, a ligand of TLR4 that also is a potent mitogen that can induce septic shock (202).

B lymphocytes from all three groups expressed extracellular TLR8, although TLR8 is reported as an intracellular receptor (203). In the adult blood and cord blood groups, the extracellular expression of TLR8 was very bright compared to the permeabilised cells. As discussed in Chapter Three, the surface TLR8 was not blocked prior to intracellular labelling, the low level intracellular TLR8 may be

attributed to surface TLR8 on the perforated lymphocyte membrane. There is minimal literature about TLR8 expression by human B lymphocytes, but this will be explored further in Chapter Six.

B lymphocytes from each group expressed TLR1, but expression was highly variable. B lymphocytes from each group expressed very little TLR6. TLR1 expression has been reported on surfaces of adult B lymphocytes (92-93) and internally in tonsil B lymphocytes (91). There are few reports about TLR6 expression by B lymphocytes.

Permeabilised B lymphocytes from the three groups expressed TLR3 and TLR9, and extracellular expression was variable. Extracellular and intracellular TLR9 expression by B lymphocytes from adult blood (81, 92-93) and tonsils (91, 100) has been reported. A literature search showed no reports of TLR3 expression by B lymphocytes.

As with T lymphocytes, B lymphocytes from adult blood and cord blood expressed the same pattern of TLR. Stages of B cell development in blood, both adult and cord, did not influence levels of TLR expression; earlier B cell subsets, such as B1 or naïve B cells, did not express higher levels of TLR. The previous hypothesis that to compensate for an immature immune system, neonates will have greater protection with TLR is not supported by the results. B lymphocytes from adults and neonates express similar patterns of TLR, but the functional capacity of neonatal B lymphocytes requires investigation.

From Chapters Three and Four, TLR expression patterns by T lymphocytes and B lymphocytes are similar between adults and neonates. This observation implies that neonates have an "innate" ability to discriminate between the self and non-self to detect the presence of pathogens. Neonates can express TLR at birth; unlike the adaptive immune response which the neonate has to acquire. Infection is responsible for a large proportion of neonatal deaths (1), therefore neonates require an "emergency" immune response. TLR expression allows neonates to recognise the presence of pathogens which can instigate an early, defensive response against infection. TLR expression by neonatal lymphocytes, and subsequent activation of lymphocytes upon TLR engagement, may have a role in early immunity. TLR-engaged neonatal B lymphocytes, which are mainly B1 B cells, may secrete low affinity IgM which can provide neonates with some protection by neutralising infection. The pattern of TLR expression by neonatal lymphocytes also allows neonates to discriminate between bacteria, viruses and fungi. This discrimination may instigate differing immune responses by the neonate to counter the pathogens. TLR expression is unaffected by stages of B cell development in adult and neonates which indicates TLR have a consistent role in the function of B lymphocytes. TLR expression by neonatal lymphocytes plays a role in early neonatal immunity by recognition of infection and, possibly, instigation of activity by T lymphocytes and B lymphocytes.

B lymphocytes from tonsils expressed fewer TLR than B lymphocytes from blood. Of the subsets from tonsil B lymphocytes, GC B cells express TLR at different levels to the other B cell subsets. The decreased expression of TLR by tonsil B lymphocytes may belie their importance in the secondary lymphoid organs. Despite the role of TLR in the early immune response, from the results and recent

literature, TLR may have a role in the adaptive, humoral, response of B lymphocytes.

In the next chapter, the functional abilities of B lymphocytes from adult blood, cord blood and tonsils in response to TLR ligand stimulation is tested. Knowledge about the capacity of neonatal B lymphocytes will be obtained to further understanding about the role of TLR in early immunity.

CHAPTER FIVE

RESPONSES OF B LYMPHOCYTES TO LIGANDS OF TOLL-LIKE RECEPTORS

5.1 INTRODUCTION

As discussed in Chapter One, B lymphocytes are the effector cells of the humoral response and have different functions in innate and adaptive immunity. Neonatal B lymphocytes have impaired function which includes reduced proliferation, poor antibody class-switching and poor responses to T-D antigens with minimal somatic hypermutation. Neonatal B lymphocytes may have increased or decreased responses to TLR engagement compared to adult B lymphocytes, but neonatal function can be enhanced to adult levels as seen in multiple TLR ligand treatment of neonatal dendritic cells (52, 161-162). In lymphoid tissue, B lymphocytes respond to T-D antigens to produce mature cells with high-affinity antibodies and memory. The study of tonsil B lymphocyte responses to TLR engagement will examine whether TLR have a potential role in lymphoid tissue. As TLR are important components of the immune system, when engaged on B lymphocytes, their effects may reveal the interplay of the innate immunity with differences between neonatal and adult humoral responses, and with adaptive responses of tonsil B lymphocytes.

Most studies into TLR effects on functions of B lymphocytes focus on murine models or human adult studies. TLR9 is engaged by its ligand CpG ODN (115), and it induces proliferation (154, 157, 204-205), antibody secretion by memory B

cells (152) and differentiation of human adult B lymphocytes into plasma B cells (163, 205). Engagement of TLR9 and CD40 on B lymphocytes induces secretion of IgM, IL-6, IL-10, IL-12 and TNF-α (197), and may be required for naïve B cells to mature and switch antibody classes (157). B lymphocytes did not proliferate in response to other TLR ligands for TLR2, TLR4, TLR6 and TLR7/8 (204). Indirect effects on B lymphocytes include enhanced proliferation and secretion of IgM, IgG and IL-6 by naïve and memory B cells in the presence of TLR7-engaged plasmacytoid dendritic cells (204) and increased IgG titres and avidity from Hepatitis B vaccines using CpG ODN motifs as an adjuvant (172). TLR, through direct and indirect engagement, have a broad influence on function of adult B lymphocytes on both innate and adaptive immune systems.

Little research has been done into the effects of TLR ligands on neonatal human B lymphocytes. Cord blood B lymphocytes secrete IgM and IgG in response to CpG ODN (163). As neonatal dendritic cell function is enhanced to adult levels in response to TLR engagement (52, 161-162), perhaps neonatal B lymphocytes can overcome their immaturity to provide stronger immune responses using TLR ligands.

As described earlier (Refer Chapter One, Section 1.2.4.2.1), B lymphocytes in peripheral blood consist of B1 and B2 B cell subsets, and B2 B cells which circulate between blood and secondary lymphoid organs are further divided into naïve or memory B cell subsets. B cell subsets isolated from peripheral blood can secrete antibodies upon activation by either T-I antigens or BCR engagement, but peripheral blood B lymphocytes are not able to undergo maturation through antigen

exposure *in vitro*. TLR engagement of B1 B cells induces IL-10 secretion (164), and for naïve and memory B cells it induces antibody secretion (152-153).

B lymphocytes in lymphoid tissue function differently to peripheral blood, in lymphoid tissue antigen-exposed B lymphocytes mature. This defined role may be reflected in an altered response of tonsil B lymphocytes to TLR ligands. Human tonsil B lymphocytes respond to CpG ODN by upregulating CD23, CD25 and CD86 and co-stimulation of TLR9 and CD40 induces IgM, IgG, IL-6 and IL-10 production (206). IgM is produced in response to TLR2 ligand (94) and B lymphocytes upregulate HLA-DR and IL-6 for TLR4, TLR7 and TLR9 ligands (91). How TLR activation of B lymphocytes influences the function of tonsils requires further investigation.

For these studies, the functions of B lymphocytes from adult blood, cord blood and tonsils in response to TLR ligands were compared. The tonsils in this study were collected from children under ten years old and undergoing tonsillectomies. The scarcity of B lymphocytes in blood did not permit studies on B cell subsets so focus was kept on B lymphocytes as a whole. The earlier work on TLR phenotyping of B lymphocytes detected several TLR; specifically TLR3, TLR4, TLR8 and TLR9. Purified B lymphocytes from the three groups were treated with ligands of the detected TLR. The direct effects of TLR engagement on B lymphocytes were the target of these experiments, not the indirect effects of TLR engaged leucocytes influencing B lymphocytes. The B lymphocyte functions examined were proliferation, levels of total Ig, IgG and IgM, and levels of six cytokines, IL-1, IL-6, IL-8, IL-10, IL-12p70 and TNF. For tonsils, sufficient numbers of B lymphocytes

allowed examination of activation, co-stimulation and other markers in response to TLR ligands.

5.2 RESULTS

The TLR3, TLR4, TLR8 and TLR9 ligands, Poly I:C, LPS, CL075 and CpG ODN respectively, were cultured with B lymphocytes purified from adult blood (n = 3), cord blood (n = 3) and tonsils (n = 3) to examine functional responses. In the tonsil group, the age and reason for tonsillectomies were: subject four was six years old with chronic tonsillitis, subject five was two years old with sleep apnoea, and subject six was three years old with sleep apnoea. As described earlier (Refer Chapter Two, Section 2.14), B lymphocytes were negatively selected using Human B cell Enrichment Cocktail RosetteSep reagent to remove other leucocytes. B lymphocytes from the groups were cultured with RF10 media or TLR ligands, three days for proliferation studies and five days for antibody and cytokine levels. B lymphocyte proliferation was measured via incorporation of [^{3}H] thymidine. Antibodies from culture supernatant were quantified for levels of total Ig, IgG and IgM using the sandwich ELISA. The cytokines from culture supernatant were measured with the Human Inflammation CBA kit from BD Biosciences. The large yields of B lymphocytes from tonsils allowed additional flow cytometry studies to examine the expression of various markers by tonsil B lymphocytes when treated with TLR ligands for 24 hours. For statistical analysis of proliferation, antibody and cytokine data, Repeated-measures ANOVA was applied to compare within TLR ligands, and Bonferroni post hoc analysis compared results between the adult, neonate and tonsil groups. For statistical analysis of the expression of various markers by B lymphocytes, Univariate ANOVA was applied to compare the

responses to TLR ligands. Values of $p < 0.05$ were considered statistically significant (Refer Chp 2, Sections 2.13.6 and 2.18).

5.2.1 Proliferation of B Lymphocytes

Adult blood B lymphocytes significantly increased proliferation in response to each TLR ligand, especially CL075 and CpG ODN (Figure 5.1). Cord blood B lymphocytes proliferated significantly in response to all TLR ligands, especially CpG ODN (Figure 5.1). Post hoc analysis of proliferation rates of B lymphocytes between adult blood and cord blood showed no significant difference ($p = 0.374$).

Tonsil B lymphocytes proliferated significantly in response to CpG ODN, but there were minimal responses to other TLR ligands (Figure 5.1). Post hoc analysis of proliferation rates of B lymphocytes showed significant differences between tonsils and adult blood ($p < 0.001$), and tonsils and cord blood ($p < 0.001$). The proliferation rates of tonsil B lymphocytes were significantly lower compared to B lymphocytes from adult blood or cord blood.

5.2.2 Antibody Secretion by B Lymphocytes

5.2.2.1 Total Ig Levels

Adult blood B lymphocytes secreted significant levels of Ig in response to CL075 and CpG ODN (Figure 5.2). Cord blood B lymphocytes secreted significant levels of Ig, slightly in response to Poly I:C and CL075, and strongly in response to CpG ODN (Figure 5.2). Post hoc analysis showed a significant difference between Ig levels from adult blood and cord blood B lymphocytes ($p = 0.007$) in response to TLR ligands; therefore, in response to TLR ligands, cord blood B lymphocytes secreted lower levels of Ig than adult blood B lymphocytes.

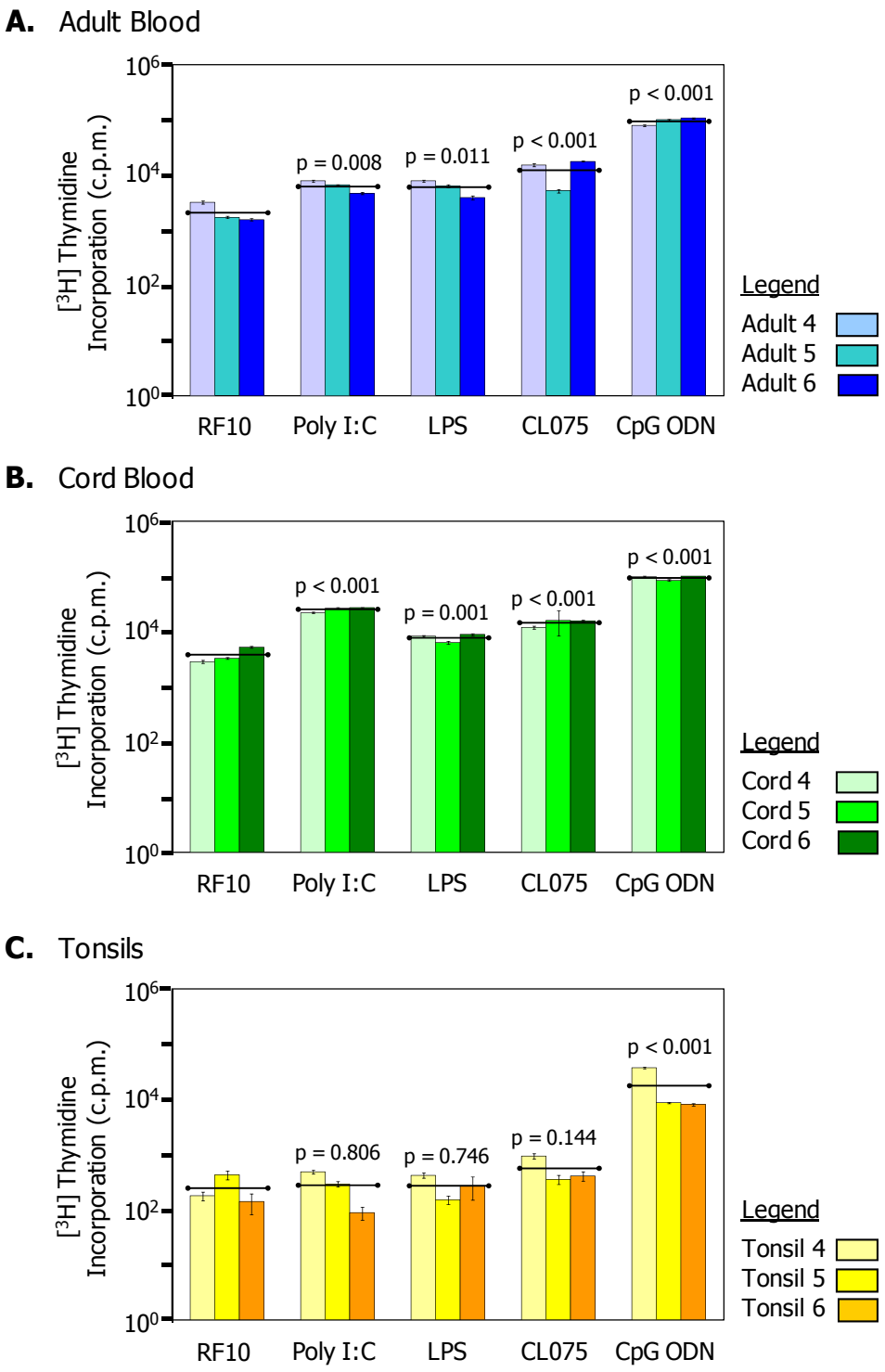


Figure 5.1 Proliferation of B Lymphocytes Treated with TLR Ligands
B lymphocytes from **A.** Adult blood (n = 3), **B.** Cord blood (n = 3) and **C.** Tonsils (n = 3), were treated in triplicate cultures with RF10 control or TLR ligands for three days. Proliferation was measured with [^{3}H] thymidine incorporation (c.p.m. = counts per minute) and results presented as mean with standard error; horizontal bar represents mean of three subjects. Differences in proliferation between RF10 control and TLR ligands were compared using Repeated-measures ANOVA and $p>0.05$ was considered not significant.

Tonsil B lymphocytes secreted Ig significantly in response to CpG ODN, but there were minimal responses to other TLR ligands (Figure 5.2). Post hoc analysis of Ig secretion by B lymphocytes in response to TLR ligand showed a significant difference between tonsils and adult blood ($p = 0.005$), and no difference between tonsils and cord blood ($p = 1.000$). Tonsil B lymphocytes were less responsive and secreted lower levels of total Ig compared to adult blood B lymphocytes.

5.2.2.2 IgG Levels

Adult blood B lymphocytes secreted IgG at significantly high levels in response to CL075 and, especially, CpG ODN (Figure 5.3). Cord blood B lymphocytes secreted IgG significantly in response to CpG ODN, but not to other TLR ligands (Figure 5.3). Post hoc analysis showed a significant difference in IgG levels by B lymphocytes between adult blood and cord blood ($p < 0.001$); cord blood B lymphocytes were far less responsive to most TLR ligands, excluding CpG ODN, compared to adult blood B lymphocytes.

Tonsil B lymphocytes secreted IgG significantly in response to CpG ODN, but not to other TLR ligands (Figure 5.3). Post hoc analysis of IgG secretion by B lymphocytes in response to TLR ligands showed no significant difference between tonsils and adult blood ($p = 0.144$), and a significant difference between tonsils and cord blood ($p < 0.001$). Tonsil B lymphocytes secreted IgG at comparable levels to adult blood B lymphocytes and at higher levels than cord blood B lymphocytes.

5.2.2.3 IgM Levels

Adult blood B lymphocytes secreted significant levels of IgM in response to LPS, CL075 and CpG ODN (Figure 5.4). Cord blood B lymphocytes secreted significant

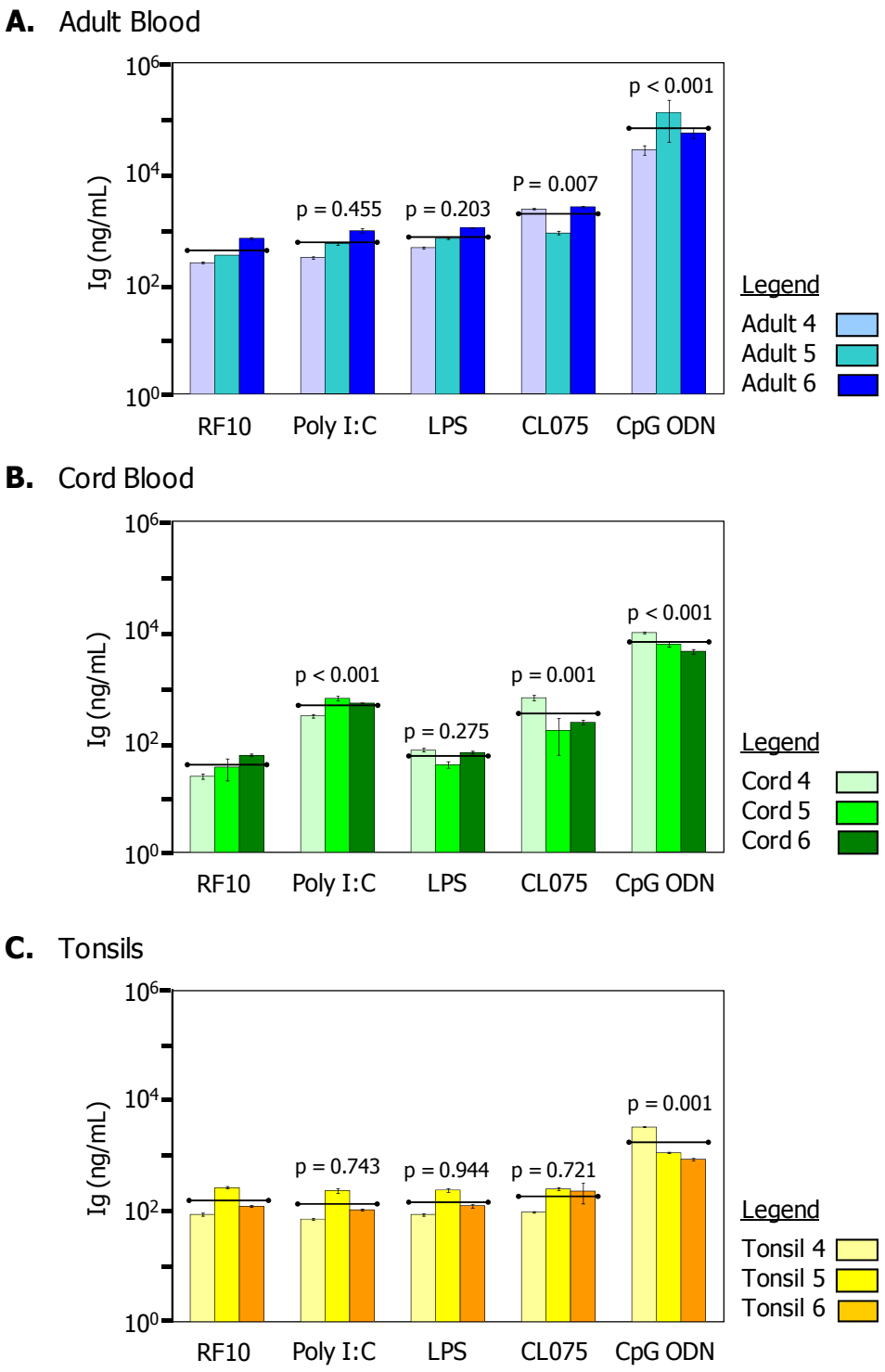


Figure 5.2 Ig Secretion by B Lymphocytes Treated with TLR Ligands
B lymphocytes from **A.** Adult blood (n = 3), **B.** Cord blood (n = 3) and **C.** Tonsils (n = 3), were treated in triplicate cultures with RF10 control or TLR ligands for five days. Ig levels in culture supernatant was measured using ELISA and results presented as mean with standard error; horizontal bar represents mean of three subjects. Differences in Ig levels between RF10 control and TLR ligands were compared using Repeated-measure ANOVA and $p>0.05$ was considered not significant.

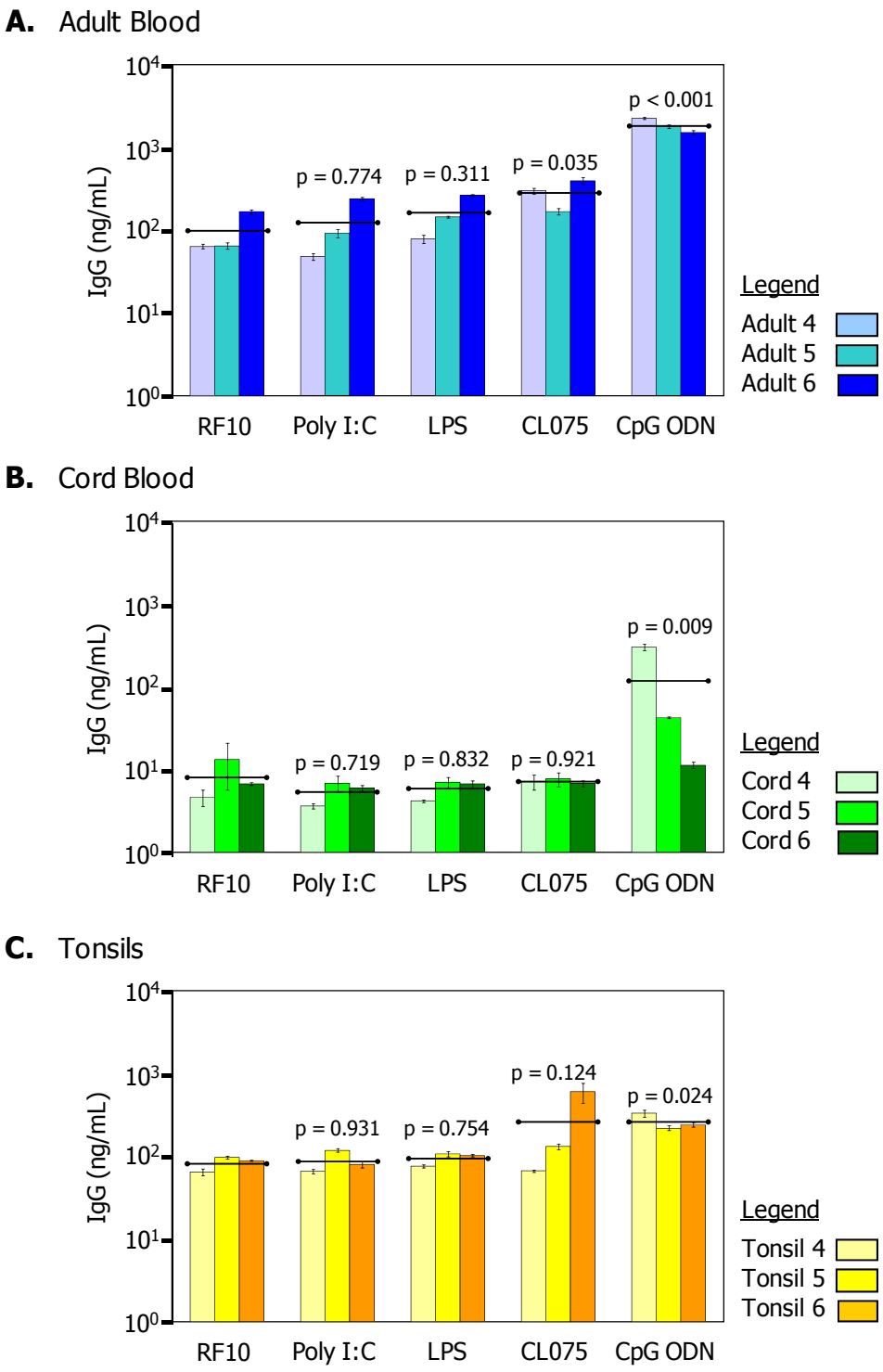


Figure 5.3 IgG Secretion by B Lymphocytes Treated with TLR Ligands
B lymphocytes from **A.** Adult blood (n = 3), **B.** Cord blood (n = 3) and **C.** Tonsils (n = 3), were treated in triplicate cultures with RF10 control or TLR ligands for five days. IgG levels in culture supernatant was measured using ELISA and results presented as mean with standard error; horizontal bar represents mean of three subjects. Differences in IgG levels between RF10 control and TLR ligands were compared using Repeated-measures ANOVA and $p>0.05$ was considered not significant.

levels of IgM in response to Poly I:C, CL075 and CpG ODN (Figure 5.4). Post hoc analysis of IgM secretion by B lymphocytes in response to TLR ligands showed no difference between adult blood and cord blood ($p = 0.224$).

Tonsil B lymphocytes secreted IgM significantly in response to CL075 and, strongly, CpG ODN. Post hoc analysis of IgM secretion by B lymphocytes in response to TLR ligands showed significant differences between tonsils and adult blood ($p = 0.001$), and tonsils and cord blood ($p = 0.003$). Tonsil B lymphocytes secreted lower levels of IgM compared to B lymphocytes from adult blood and cord blood.

5.2.3 Cytokine Secretion by B Lymphocytes

Problems with the Human Inflammation CBA Kit prevented accurate measurements of several cytokines from culture supernatants. Data on the level of cytokines secreted by B lymphocytes following treatment with TLR ligands could only be obtained for IL-6 and IL-8. No useful data could be obtained for the other cytokines, IL-1β, IL-10, IL-12p70 and TNF, as the internal controls were not reproducible between experiments.

5.2.3.1 IL-6 Levels

B lymphocytes from adult blood secreted significant levels of IL-6 in response to LPS, CL075 and CpG ODN (Figure 5.5). Cord blood B lymphocytes secreted IL-6 significantly in response to all TLR ligands, especially Poly I:C, CL075 and CpG ODN (Figure 5.5). Post hoc analysis of IL-6 secretion by B lymphocytes in response to TLR ligands showed no difference between adult blood and cord blood ($p = 1.000$).

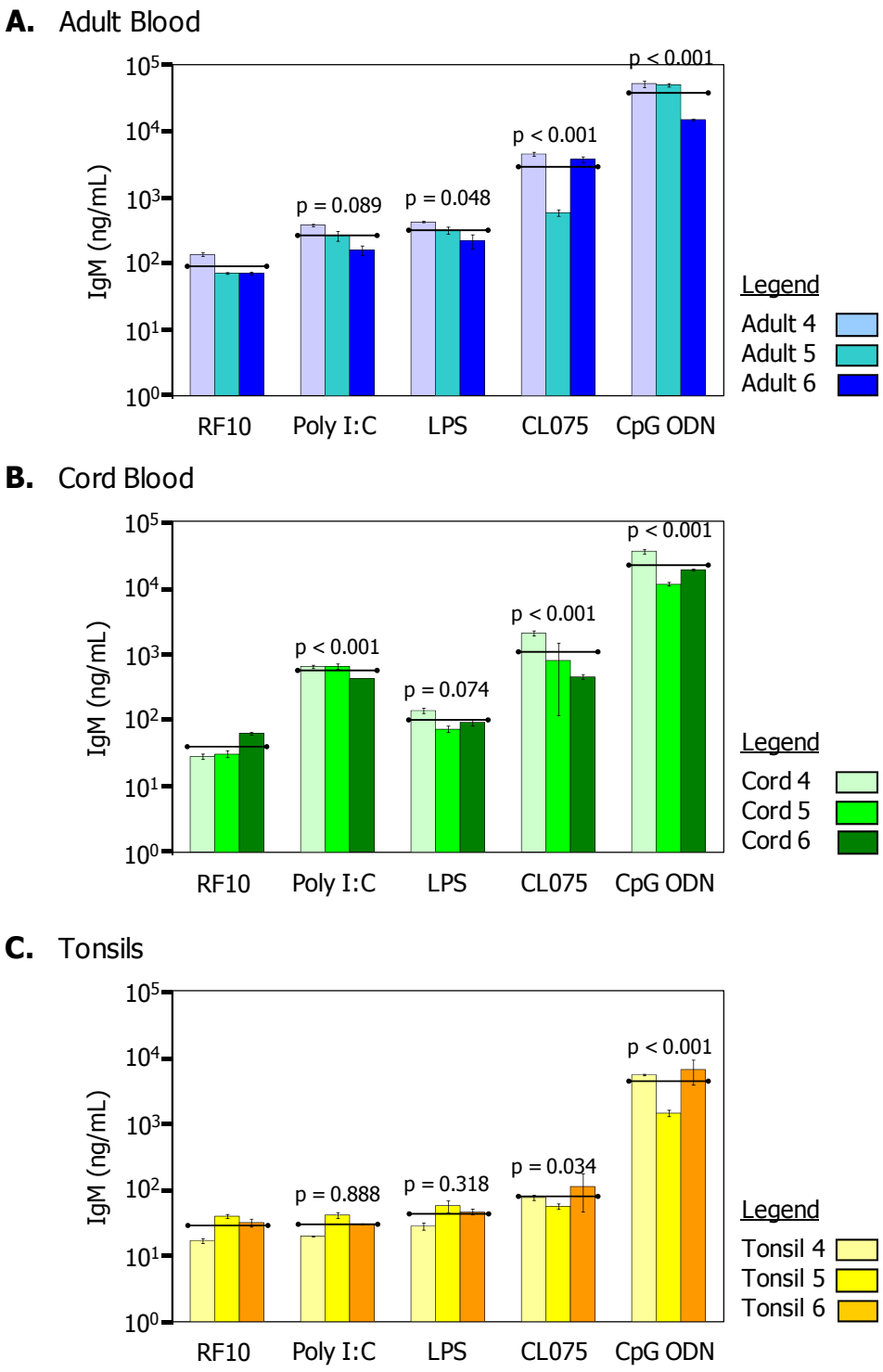


Figure 5.4 IgM Secretion by B Lymphocytes Treated with TLR Ligands
B lymphocytes from **A.** Adult blood (n = 3), **B.** Cord blood (n = 3) and **C.** Tonsils (n = 3), were treated in triplicate cultures with RF10 control or TLR ligands for five days. IgM levels in culture supernatant was measured using ELISA and results presented as mean with standard error; horizontal bar represents mean of three subjects. Differences in IgM levels between RF10 control and TLR ligands were compared using Repeated-measures ANOVA and $p>0.05$ was considered not significant.

Tonsil B lymphocytes secreted IL-6 at significant levels in response to CpG ODN (Figure 5.5). Post hoc analysis of IL-6 secretion by B lymphocytes in response to TLR ligands showed no significant differences between tonsils and adult blood ($p = 0.952$), and tonsils and cord blood ($p = 0.596$).

5.2.3.2 IL-8 Levels

Adult blood B lymphocytes secreted IL-8, but with no significant response to TLR ligands (Figure 5.6). Cord blood B lymphocytes secreted high levels of IL-8 with slight, but significant, increases in response to LPS and CL075 (Figure 5.6). Post hoc analysis of IL-8 secreted by B lymphocytes in response to TLR ligands showed a significant difference between adult blood and cord blood ($p = 0.034$). Cord blood B lymphocytes secreted higher levels of IL-8 than adult blood B lymphocytes.

Tonsil B lymphocytes secreted IL-8, but with no significant response to TLR ligands, except for Poly I:C which had significantly decreased secretion of IL-8 (Figure 5.6). Post hoc analysis of IL-8 secreted by B lymphocytes in response to TLR showed no significant difference between tonsils and adult blood ($p = 1.000$), and a significant difference between tonsils and cord blood ($p = 0.040$). Tonsil B lymphocytes secreted IL-8 at lower levels than cord blood B lymphocytes.

5.2.4 Activation Markers of Tonsil B Lymphocytes

At timepoint zero and after 24 hour treatment with TLR ligands, tonsil B lymphocytes were identified with PerCp-Cy5.5-conjugated CD19 antibody, then activation measured with PE-conjugated antibodies to CD23, CD25, CD69 and HLA-DR, before reading on flow cytometry. Expression levels of CD23, CD25

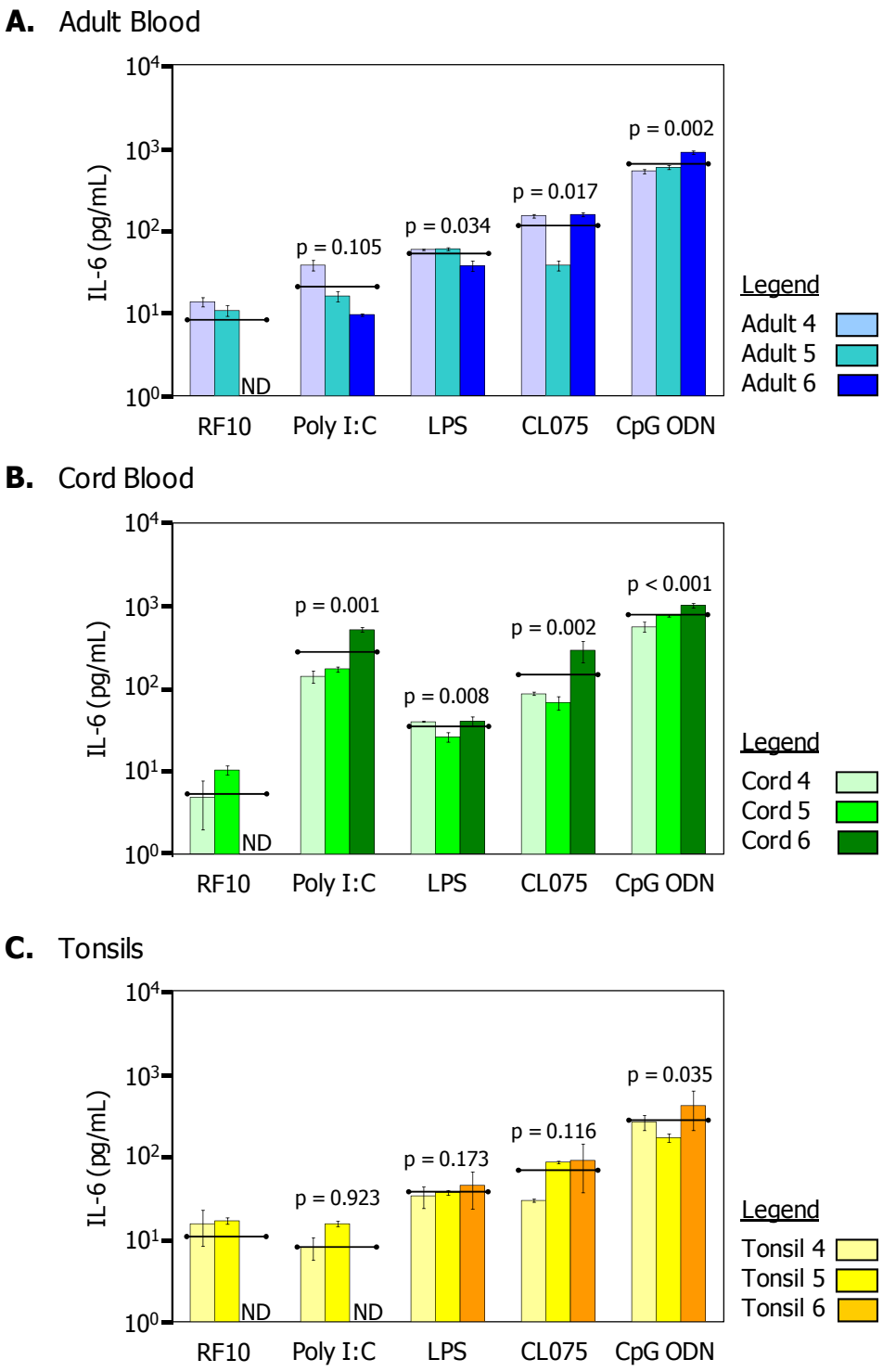


Figure 5.5 IL-6 Secretion by B Lymphocytes Treated with TLR Ligands
B lymphocytes from **A.** Adult blood (n = 3), **B.** Cord blood (n = 3) and **C.** Tonsils (n = 3), were treated in triplicate cultures with RF10 control or TLR ligands for five days. IL-6 levels in culture supernatant was measured using Human Inflammation CBA Kit and results presented as mean with standard error; horizontal bar represents mean of three subjects. Differences in IL-6 levels between RF10 control and TLR ligands were compared using Repeated-measures ANOVA and $p>0.05$ was considered not significant. ND = Not Detected

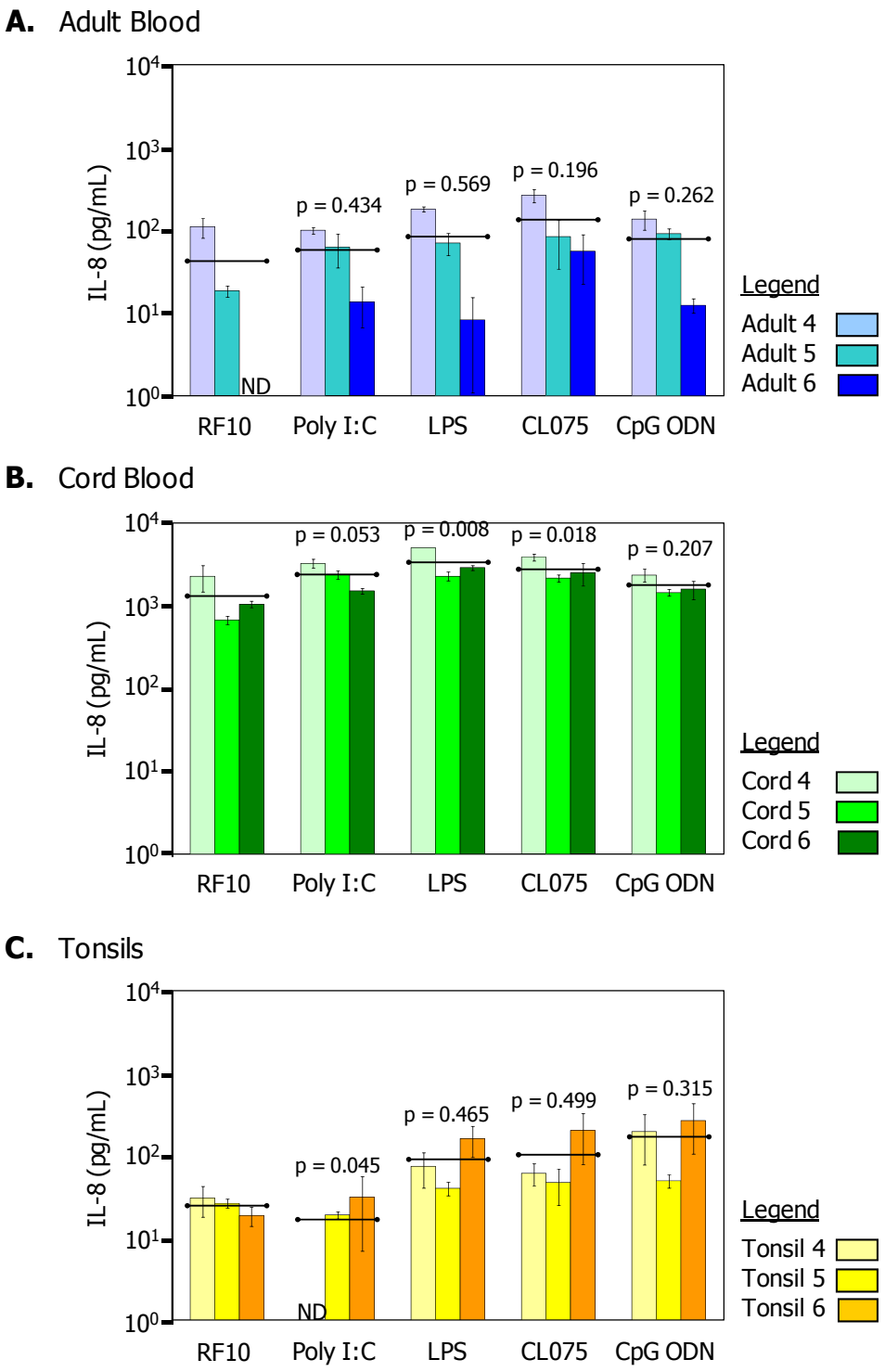


Figure 5.6 IL-8 Secretion by B Lymphocytes Treated with TLR Ligands
B lymphocytes from **A.** Adult blood (n = 3), **B.** Cord blood (n = 3) and **C.** Tonsils (n = 3), were treated in triplicate cultures with RF10 control or TLR ligands for five days. IL-8 levels in culture supernatant was measured using Human Inflammation CBA Kit and results presented as mean with standard error; horizontal bar represents mean of three subjects. Differences in IL-8 levels between RF10 control and TLR ligands were compared using Repeated-measures ANOVA and $p>0.05$ was considered not significant. ND = Not Detected

and CD69 were measured as percentage of positive cells, and HLA-DR was measured as changes in MFI as all B lymphocytes express HLA-DR.

5.2.4.1 CD23

The proportion of $CD23^+$ tonsil B lymphocytes was not significantly affected by the 24 hour cell culture or treatment by TLR ligands. (Figure 5.7)

5.2.4.2 CD25

The proportion of $CD25^+$ tonsil B lymphocytes increased significantly during the 24 hours cell culture, and this proportion was significantly increased in response to CpG ODN. (Figure 5.7)

5.2.4.3 CD69

The proportion of $CD69^+$ tonsil B lymphocytes increased significantly during 24 hour cell culture, and there were no significant increases in the proportion upon treatment with TLR ligands. (Figure 5.7)

5.2.4.4 HLA-DR

The expression of HLA-DR by tonsil B lymphocytes was not significantly affected by the 24 hour cell culture or the treatment by TLR ligands. (Figure 5.7)

5.2.5 Co-Stimulatory Molecules of Tonsil B Lymphocytes

At timepoint zero and after 24 hours of treatment with TLR ligands, tonsil B lymphocytes were tested for co-stimulatory markers, CD40, CD80 and CD86. The indirect immunofluorescence labelling method (Refer Chapter Two, Section 2.13.3) was used to identify B lymphocytes and expression of co-stimulatory molecules for

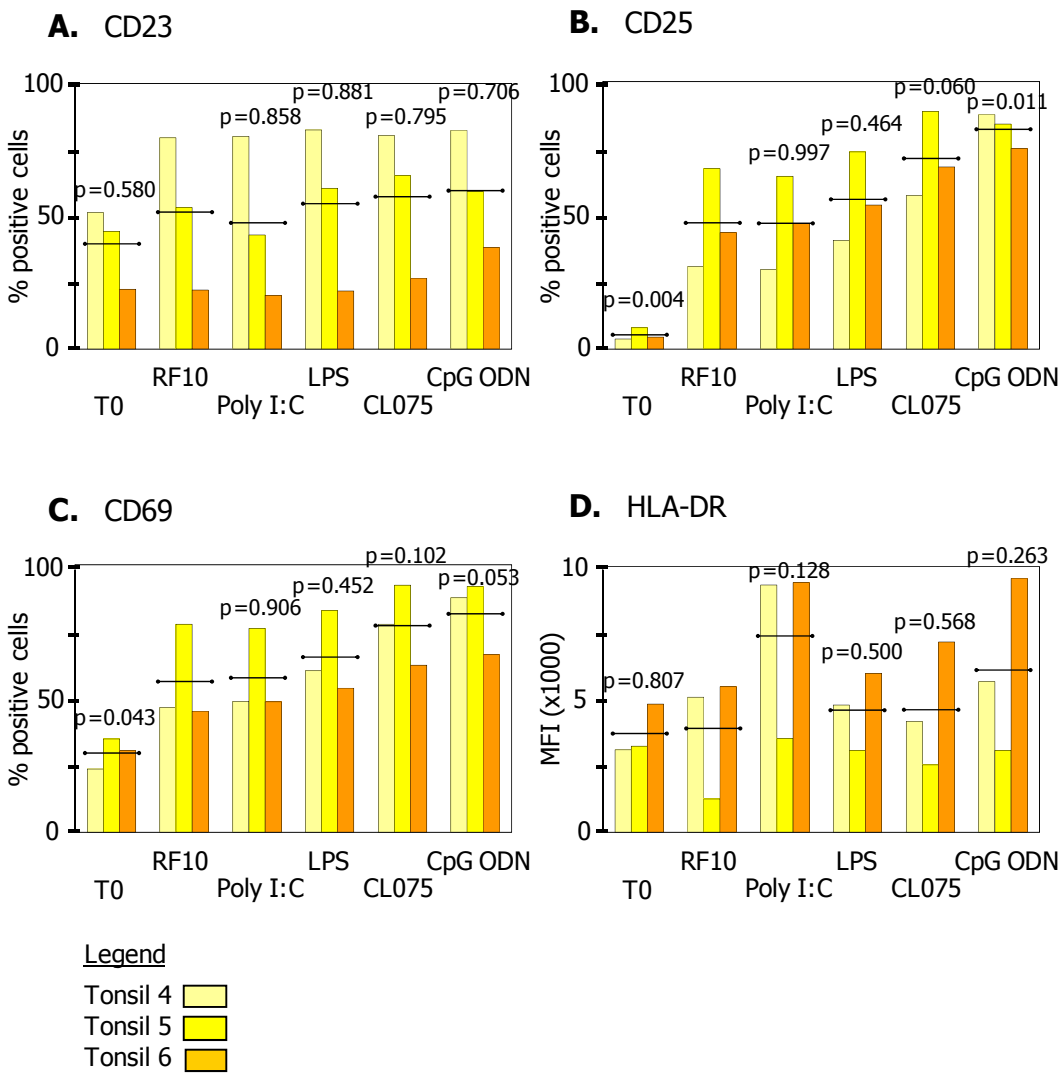


Figure 5.7 Activation of Tonsil B Lymphocytes
Tonsil B lymphocytes were treated with RF10 control or TLR ligands for 24 hours and expression of activation markers was detected by flow cytometry. Percentage of positive cells for **A.** CD23, **B.** CD25 and **C.** CD69, and MFI of **D.** HLA-DR, from B lymphocytes from time-point 0 hour (T0) and 24 hour treated cells is shown. Horizontal bar represents mean of three subjects. Differences in expression between RF10 control and T0/TLR ligands was compared using Univariate ANOVA and $p>0.05$ was considered not significant.

flow cytometry. As for TLR expression, the expression of the molecules were calculated as MFI ratio using the MFI values of markers to matched negative isotype controls.

5.2.5.1 CD40

One tonsil subject did not express CD40 at any point. B lymphocytes from the other tonsil subjects were not significantly affected by the 24 hour cell culture or treatment by TLR ligands. (Refer Figure 5.8)

5.2.5.2 CD80

The expression of CD80 by tonsil B lymphocytes was not significantly affected by the 24 hour cell culture or treatment by TLR ligands, except for a significant increase in response to CpG ODN. (Refer Figure 5.8)

5.2.5.3 CD86

The expression of CD86 by tonsil B lymphocytes was not significantly affected by the 24 hour cell culture or treatment by TLR ligands. (Figure 5.8)

5.2.6 Additional Markers of Tonsil B Lymphocytes

Expression of CD21, a receptor for complement, and CD210, the IL-10 receptor, by tonsil B lymphocytes in response to TLR ligands was detected as described for co-stimulatory molecules.

5.2.6.1 CD21

Expression of CD21 by tonsil B lymphocytes was not significantly affected by the 24 hour cell culture or treatment by TLR ligands. (Figure 5.9)

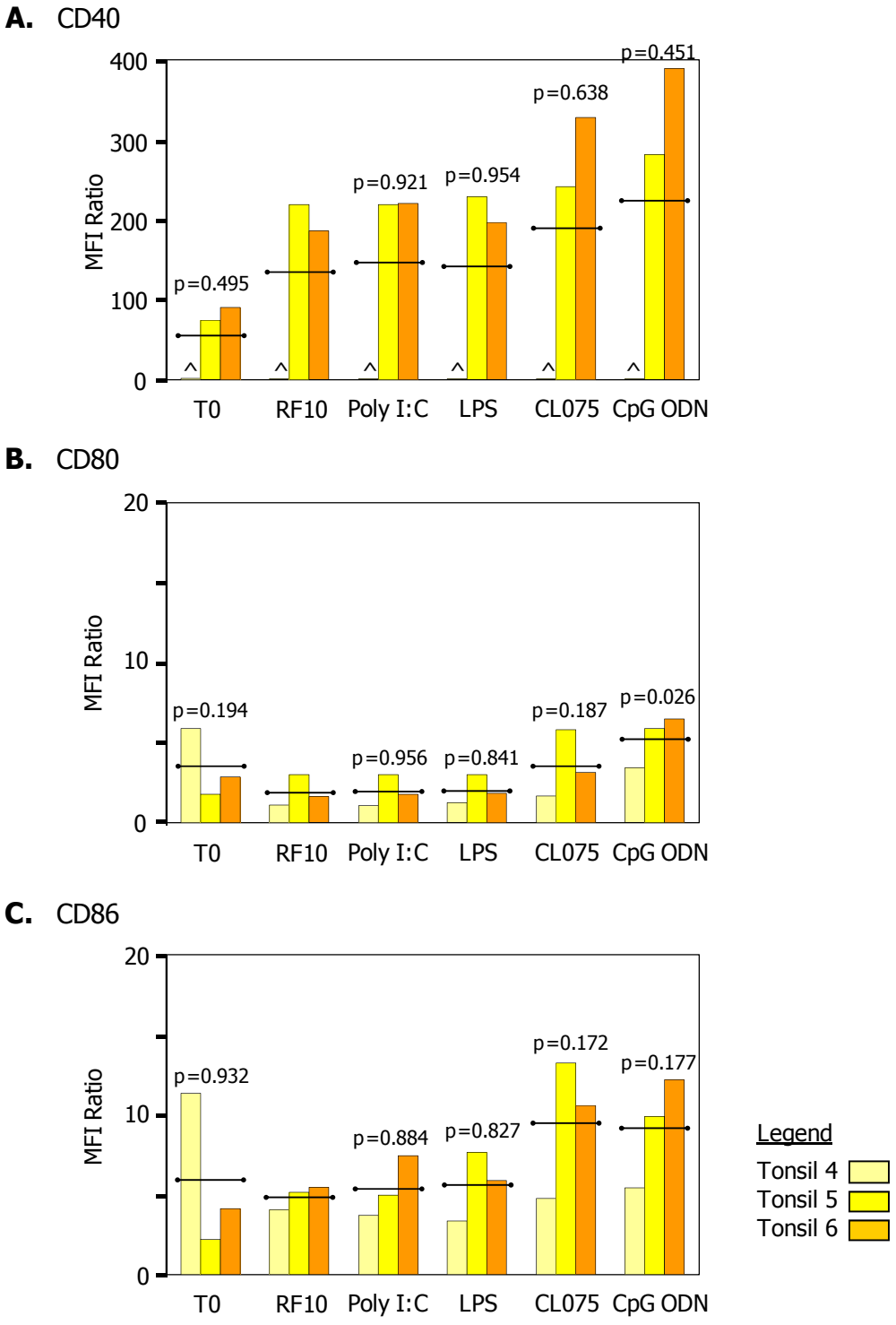


Figure 5.8 Co-Stimulatory Molecule Expression by Tonsil B Lymphocytes
Tonsil B lymphocytes were treated with RF10 control or TLR ligands for 24 hours and expression of co-stimulatory markers was detected by flow cytometry. MFI ratio of **A.** CD40, **B.** CD80 and **C.** CD86, from B lymphocytes from time-point 0 hour (T0) and 24 hour treated cells is shown. Horizontal bar represents mean of three subjects. Differences in expression between RF10 control and T0/TLR ligands was compared using Univariate ANOVA and $p>0.05$ was considered not significant. ^ = MFI ratio < 2

5.2.6.2 CD210

Expression of CD210 by tonsil B lymphocytes was not significantly affected by the 24 hour cell culture or treatment by TLR ligands. (Figure 5.9)

5.3 DISCUSSION

The main observations from the results are, firstly B lymphocytes from adult blood and cord blood have similar rates of proliferation and IL-6 secretion, and reduced antibody levels from neonatal B lymphocytes. Secondly, TLR ligands, especially CpG ODN, enhance functions of B lymphocytes from adult blood and cord blood. Thirdly, certain functions of tonsil B lymphocytes are reduced compared with adult blood B lymphocytes. Lastly, tonsil B lymphocytes respond poorly to most TLR ligands.

B lymphocytes enriched from cord blood were contaminated, despite best efforts, with up to 50% of erythrocyte precursors. Published methods (182, 207) were used for positive selection and removal of erythrocyte precursors. Due to the immunologically "inert" nature of erythrocytes, their presence was not expected to influence the responses of cord blood B lymphocytes. As the direct effects of TLR ligands on B lymphocytes were of interest, it was critical that other leucocytes were removed from the B lymphocyte fraction. As described in Chapter One, T lymphocyte and monocytes stimulated by TLR ligands release various cytokines which may affect B lymphocyte function. Despite the contaminating erythrocytes, the TLR expressed by B lymphocytes can engage with TLR ligands and induce various responses. Cell-to-cell contact was not

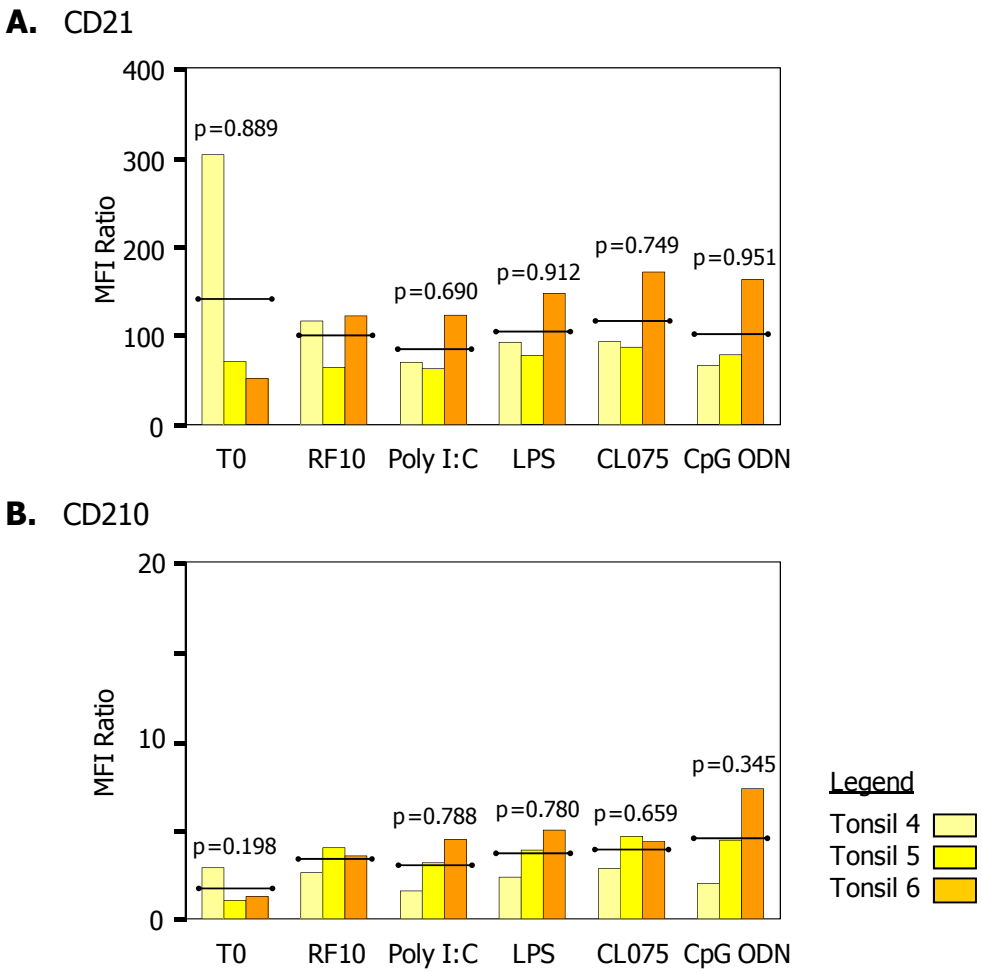


Figure 5.9 CD21 and CD210 Expression by Tonsil B Lymphocytes
Tonsil B lymphocytes were treated with RF10 control or TLR ligands for 24 hours and expression of **A.** CD21 and **B.** CD210 was detected by flow cytometry. MFI ratio of CD21 and CD210 from B lymphocytes from time-point 0 hour (T0) and 24 hour treated cells is shown. Horizontal bar represents mean of three subjects. Differences in expression between RF10 control and T0/TLR ligands was compared using Univariate ANOVA and $p>0.05$ was considered not significant.

required in these assays, therefore the contaminating erythrocyte would have little effect on B lymphocytes.

The first observation showed that in direct response to TLR ligands, B lymphocytes from adult blood and cord blood had, statistically, similar proliferation rates and IL-6 levels, and antibody levels from cord blood are reduced. IL-8 levels from cord blood B lymphocytes were high with slight increases to particular TLR ligands. Despite the similarity of IL-6 levels from adults and neonates to TLR ligands, Poly I:C did stimulate IL-6 secretion by cord blood B lymphocytes. Adult blood B lymphocytes do secrete IL-6 in response to ligands of TLR7 (204) and TLR9 (195), but not TLR4 (197). However, the similarity of IL-6 levels was not observed in whole blood treated with TLR2 ligand from adults and neonates (167). This may be explained by a failure of other neonatal immune cells secreting IL-6 (8). TLR ligands' induction of IL-6 may then influence inflammatory responses (208) and differentiate B lymphocytes into antibody-secreting cells (209).

Neonatal and adult B lymphocytes proliferated at similar rates in response to TLR ligands, with greater proliferation of cord blood B lymphocyte in response to Poly I:C. Published reports confirm the stronger response to CpG ODN (154, 205) compared to other TLR ligands (204). The adult-like proliferation of cord blood B lymphocytes to *in vitro* stimulation with TLR ligands, particularly Poly I:C and CpG ODN, indicates that neonates can, under certain circumstances, initiate a vigorous antibody response. However, the antibody response will not be the specific, high-affinity IgG antibodies; instead the high avidity, low affinity IgM secreted by the B1 B cell subset.

Neonatal B lymphocytes secreted significantly lower levels of total Ig and IgG, and similar IgM levels, in response to TLR ligands compared to adult B lymphocytes. Cord blood B lymphocytes secreted Ig and IgM significantly in response to Poly I:C, CL075 and CpG ODN, and IgG was secreted at significantly increased levels in response to CpG ODN. Published reports confirm that CpG ODN induces secretion of IgG and IgM by B lymphocytes from adult blood (152, 154, 197) and cord blood (163). The poor IgG response by cord blood B lymphocytes treated with other TLR ligands may be due to intrinsic neonatal defects for antibody class-switching. The ability of CpG ODN to overcome this defect and induce IgG production points to CpG ODN as a potential agent to promote an effective antibody response in neonates. Overall, antibody responses from neonatal B lymphocytes are lower than adult B lymphocytes, but TLR ligands enhance cord blood responses to adult basal levels. This improvement of cord blood antibody responses, while not affinity-matured IgG, can still provide protection for the vulnerable neonate.

Discrepancies in the levels of total Ig to IgG and IgM were observed in certain instances. For adult blood B lymphocytes, Poly I:C had little effect on total Ig, IgG and IgM levels compared to the control. LPS induced a slight, but statistically insignificant, increase of IgM from cord blood B lymphocytes, but this increase was not reflected in levels of total Ig by cord blood B lymphocytes stimulated with LPS. Discrepancies in levels of antibody classes are difficult to interpret, but it may be that comparisons between the different antibody classes are limited due to the different capture antibodies used for the ELISA. The different ELISA may have differing sensitivities for measuring total Ig, IgG and IgM, which would account for the observed discrepancies.

The significant effect of LPS on increasing IL-8 levels by cord blood B lymphocytes has been also reported in neutrophils and monocytes from adult blood (210). There is also a report of IL-8 secretion by cord blood treated with a ligand of TLR2 (167); TLR ligands can induce IL-8 secretion by leucocytes in adults and neonates. IL-8 is a pro-inflammatory cytokine which mainly affects neutrophils (3) and also has an important role during parturition (3).

When comparing the functions of B lymphocytes from neonates and adults, proliferation and IL-6 responses were comparable, but antibody responses were reduced. While cytokine levels can be affected by many other immune cells, the antibody response is derived entirely from B lymphocytes. For the neonatal antibody response, IgM levels were increased by all TLR ligands tested and IgG levels were enhanced by CpG ODN. The ability of TLR ligands to enhance neonatal immune function to adult basal levels, as seen for B lymphocytes in the results, has been reported in dendritic cells (52, 162).

The second observation was that TLR ligands, especially CpG ODN, increased functions of B lymphocytes from adult blood and cord blood. Responses of B lymphocytes to cytokines or CD40 ligation were enhanced further with TLR ligands (154, 204). CpG ODN stimulated functions of B lymphocytes from adult blood and cord blood. CpG ODN is a well-known mitogen of B lymphocytes (150) that induces proliferation (115), upregulates co-stimulatory markers (211), drives differentiation of B lymphocytes (163) and induces antibody secretion (151). The stimulatory effect of TLR ligands on B lymphocytes supports them as possible

candidates for vaccine adjuvants in neonates and adults, particularly CpG ODN which has produced enhanced antibody responses in vaccine adjuvant trials (172).

The third observation was that functions of tonsil B lymphocytes, excluding IgG and cytokine secretion, were lower than adult blood B lymphocytes. Proliferative responses of tonsil B lymphocytes to TLR ligands were limited, but CpG ODN induced significant proliferation, as reported for tonsil B lymphocytes (206) and murine splenic B lymphocytes (148, 198). The proliferation rates of B lymphocytes were significantly lower in tonsils compared to adult blood and cord blood. However, CpG ODN did induce significant proliferation of tonsil B lymphocytes. The lack of proliferation by tonsil B lymphocytes in response to TLR ligands may reflect the particular role B lymphocytes have in tonsils. Tonsil B lymphocytes undergo maturation upon antigenic exposure to produce a tailored antibody response, polyclonal stimulation of tonsil B lymphocytes for proliferation may not be as useful for this role.

Tonsil B lymphocytes secreted IL-6 in response to CpG ODN, but had no response for IL-8 secretion. Reports show tonsil B lymphocytes, co-stimulated with anti-CD40 antibody, secreted IL-6 in response to ligands against TLR2, TLR4, TLR8 and TLR9 (91, 155, 206). In tonsils the adaptive immune effects of IL-6 involve differentiation of B lymphocytes into IgG-secreting plasma B cells. The results show that tonsil B lymphocytes did not secrete IL-8 in response to TLR ligands. As IL-8 has little effect on B lymphocytes it is harder to evaluate the secretion of IL-8 by tonsil B lymphocytes in response to TLR ligands, but production of IL-8 by tonsil B lymphocytes may induce an inflammatory response upon signs of infection.

B lymphocytes from tonsils, compared to adult blood, secreted significantly lower levels of Ig and IgM, and similar levels of IgG. In one study tonsil B lymphocytes secreted IgG and IgM in response to CpG ODN and CD40 ligand (206). Most studies were in murine models and showed splenic B lymphocytes secreting various Ig (198, 212) including IgG (148, 213) and IgM (146, 148, 213) in response to TLR2, TLR4, TLR2/6, TLR7 and TLR9 ligands. In this study, CL075 induced tonsil B lymphocytes to secrete significantly increased levels of IgM, but had insignificant effects on total Ig and IgG. The selective enhancement of IgM by CL075 in tonsils indicates that IgM secreting B cells are responsive, but class-switched B cells are not either because they lack TLR8 or because down-stream response elements are switched off. The similar levels of IgG secreted by B lymphocytes between blood and tonsils is because tonsils are one site for B lymphocytes to mature into affinity-enhanced, class-switched, IgG-secreting cells. TLR engagement of tonsil B lymphocytes may influence cells to secrete antibodies according to the stage of differentiation, and assist in antibody class-switching.

The last observation was that tonsil B lymphocytes responded poorly to TLR ligands in most functions tested. Tonsil B lymphocytes were activated with the appropriate concentrations of TLR ligands, as shown in the results, but they had minimal effect on B lymphocyte proliferation and secretion of antibodies and cytokines. An interesting observation was that B lymphocytes were activated, as indicated by upregulation of CD25 and CD69 expression, after culturing with RF10 media from timepoint zero to 24 hour treatment.

Tonsil B lymphocytes, in response to CL075 and CpG ODN, had statistically insignificant increases in the expression of co-stimulatory molecules, CD40, CD80

and CD86. Published reports confirm increased expression of co-stimulatory molecules in response to TLR engagement in B lymphocytes from blood (214) and tonsils (91, 206), and murine splenic B lymphocytes (146, 155). TLR engagement and subsequent upregulation of co-stimulatory markers prepares tonsil B lymphocytes for cognate interactions with T lymphocytes.

CD40 expression was absent in tonsil B lymphocytes from tonsil subject four who suffered chronic tonsillitis. This lack of CD40 expression by the tonsil B lymphocytes remained unaffected by treatment with various TLR ligands, which did upregulate the activation markers. The lack of CD40 expression by B lymphocytes has been associated with Hyper-IgM Syndrome, a condition in which B lymphocytes fail to mature and class-switch from IgM to IgG, and undergo somatic hypermutation (215). This condition is unlikely in this patient as the cells are producing comparable levels of IgG and IgM levels are not excessive. The specific absence of CD40 in this chronic tonsillitis patient is surprising, but a literature search revealed no reports of an association between an absence of CD40 expression and chronic tonsillitis.

CD21 and CD210 expression was unaffected by TLR ligand treatment of tonsil B lymphocytes. Expression of CD21, a complement receptor, by tonsil B lymphocytes is present, but is not regulated by TLR engagement. Expression of CD210, the IL-10 receptor, by tonsil B lymphocytes is present, but not regulated by TLR engagement. As with CD21, the constitutive expression of CD210 by tonsil B lymphocytes indicates that regulation by TLR engagement may not be required, and IL-10 can still induce B lymphocytes to differentiate and secrete antibodies

(209). The expression of CD21 and CD210 by tonsil B lymphocytes may be sufficient without further regulation to early signs of infection.

Tonsil B lymphocytes in response to CL075 and CpG ODN increased antibody levels and expression of co-stimulatory molecules. These enhanced functions are consistent with the role of lymphoid tissue as a site for B lymphocytes to mature and develop a specific, adaptive, humoral response. As the results show, some TLR ligands have the capacity to assist B lymphocyte adaptive responses in tonsils.

The functions of B lymphocytes in response to TLR ligands were mostly reduced in neonates compared to adults. Although the responses of neonatal B lymphocytes were improved by TLR ligands, they only reached the basal levels of adult blood B lymphocytes; neonatal B lymphocyte function rarely reached the ligand-treated levels of adult B lymphocytes. Chapter Four demonstrated similar patterns of TLR expression by B lymphocytes from cord blood and adult blood, but the reduced function of neonatal B lymphocytes may be due to impaired TLR signalling reported previously in neonatal leucocytes (167). Nonetheless, the function of B lymphocytes from neonates was still noticeably enhanced by TLR ligands, particularly by CpG ODN. The most interesting observation was the increased IgG secretion by cord blood B lymphocytes after treatment with CpG ODN. This demonstrates that CpG may induce adaptive responses from neonates, especially as CpG ODN induces B lymphocytes to secrete IgG (151) and enhances the antibody response of adults during vaccination (172). Neonatal B2 B cells secrete minimal IgG, but CpG ODN may have overcome this intrinsic defect to instigate the cells to secrete IgG. This ability of CpG ODN requires further investigation and has implications for developing effective neonatal vaccines.

CHAPTER SIX

INVESTIGATIONS INTO EXTRACELLULAR EXPRESSION OF TLR8 BY B LYMPHOCYTES

6.1 INTRODUCTION

As previously discussed (Refer Chapter One, Section 1.3.3), TLR differ in their cell locations according to the microbial nature of their ligands, extracellular TLR recognise bacteria and intracellular TLR recognise viruses. Most TLR expression studies using flow cytometry directly examine the expected location of TLR, for example staining for surface expression of TLR2 or TLR4 and intracellular expression for TLR3 or TLR9. Chapters Three and Four examined extracellular and intracellular expression of TLR, and most TLR tested had expression patterns that were consistent with published studies, such as dim extracellular expression and bright intracellular expression of TLR9. However, the results in Chapter Four showed that B lymphocytes expressed TLR8 at high levels on the surface and low levels inside cells. As this particular result differs from the literature which describes TLR8 as an intracellular receptor (82), further investigations into TLR8 expression by B lymphocytes were done.

Despite TLR8 being considered an internal marker, there is little evidence in the literature to support this and the impression of TLR8 as an internal marker may be due to inferences from several studies. TLR7, TLR8 and TLR9 were identified using genomic sequencing, and due to their high homology they were phylogenetically classified as a TLR sub-family (64, 216). TLR7 and TLR8 bind to

single-stranded RNA (217) and TLR9 binds to CpG ODN (115), and these ligands require endosomal uptake (150, 218). TLR7, TLR8 and TLR9 require endosomal maturation for activity (219-220), but one report states that TLR8 does not require endosomal maturation (219). Intracellular expression of TLR7 (221) and TLR9 (88, 195) was detected, but until recently no TLR8 expression studies were available. For TLR8, a chimera created with the TLR4 ectodomain and TLR8 cytoplasmic domain was transfected into leucocytes and its expression indicated that it may be an internal marker (203). There is only one recent study (82) demonstrating intracellular expression of TLR8, but this study did not block any surface expression of TLR8 prior to intracellular labelling. Unlike TLR7 or TLR9, there was insufficient literature to clearly state TLR8 was mainly expressed internally. The phylogenetic classification of TLR8 with TLR7 and TLR9, coupled with the possible requirement of endosomal maturation for TLR8 activity, seemed to indicate that TLR8 was an internal marker.

The new finding in Chapter Four indicates that TLR8 is a surface marker on B lymphocytes and not an internal marker. This is supported by one report of surface TLR8 expression by regulatory T lymphocytes (89). TLR8 may actually be expressed on cell surfaces before being internalised with its ligand for endosomal maturation and immune responses. In light of this new finding, the experimental result required validation before drawing any final conclusions about TLR8 expression. Confirmation of surface TLR8 expression with independent antibodies was not possible as other anti-TLR8 monoclonal antibodies bind to the same antigenic peptide of TLR8. The following studies were done to examine surface expression of TLR8:

Viability of B lymphocytes was assessed: integrity of the cell membrane was examined to ensure TLR8 antibody was not entering the cell.

TLR8 expression was examined with indirect immunofluorescence labelling: to ensure PE dye was not binding non-specifically to B lymphocytes.

PE-conjugated TLR8 antibody was tested: its effectiveness in labelling was assessed through competitive binding with an unconjugated TLR8 antibody.

Spectrophotometric absorption and emission of CL075: CL075, a ligand for TLR8, may have fluorescent properties as it contains aromatic rings in its chemical structure. (Figure 6.1)

Cell culture studies with CL075: to examine the localisation of CL075, a potentially fluorescent TLR8 ligand, on the surface of leucocytes.

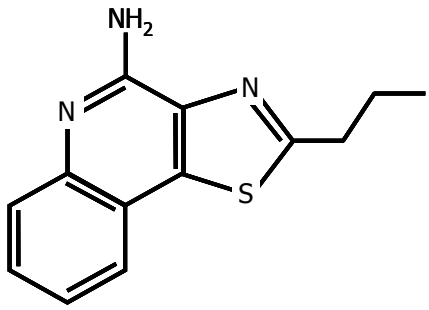


Figure 6.1 Chemical Structure of CL075
CL075, a thiazoloquinolone derivative, is a ligand for TLR8. Its chemical formula is $C_{13}H_{13}N_3S$ and molecular weight is 243.33 Daltons.

6.2 MATERIALS AND METHODS

Most buffers and reagents used are listed in Chapter Two, but additional reagents and protocols for Chapter Six are listed here.

6.2.1 Monoclonal Antibodies and Immunofluorescence Staining Reagents

Table 6.1 lists additional immunofluorescence staining reagents.

Table 6.1 Immunofluorescence Staining Commercial Reagents

Reagent	Company	Cat. No.
CD19 – FITC conjugated (Mouse IgG1 isotype; Clone HIB19)	BD Pharmingen	555412
7-amino-actinomycin D (7AAD)	BD Pharmingen	559925
Annexin V – FITC conjugated	BD Pharmingen	556419
Cytochrome C – FITC conjugated (Mouse IgG1 isotype)	eBioscience	11-6601
4′,6-diamidino-2-phenylindole (DAPI)	Sigma Aldrich Inc.	32670

6.2.2 Immunofluorescence Labelling for Flow Cytometry

Viability of B lymphocytes before and after permeabilisation was tested with two markers, Annexin V is a marker for apoptosis and 7AAD is a marker for cell viability. Mononuclear leucocytes from adult blood were not permeabilised or permeabilised, then stained for extracellular and intracellular expression of TLR8 and TLR9 using PE-conjugated antibodies (Refer Chapter Two, Sections 2.13.1 and 2.13.2). Mononuclear leucocytes were incubated with either Annexin V for 15 minutes, or 7AAD added just prior, then the cells were read on FACSCalibur. For

flow cytometric studies, viable lymphocytes were selected by gating on forward and side scatter parameters.

The detection reagents for indirect labelling of TLR8 and TLR9 on lymphocytes were SA-PE and SA-AF647. Adult blood mononuclear leucocytes were labelled with indirect immunofluorescence protocol (Refer Chapter Two, Section 2.13.2).

For competitive TLR8 staining on B lymphocytes, adult blood mononuclear leucocytes were incubated with unconjugated x63 or TLR8 antibodies, washed twice, incubated with PE-conjugated TLR8 antibody and washed twice. The x63 antibody is a negative control for IgG1 isotype antibodies. The cells were also stained for TLR8 expression using direct PE-conjugated antibodies and indirect immunofluorescence labelling.

6.2.3 Spectrophotometric Analysis of CL075

Spectrophotometric analysis determined the absorption and emission properties of CL075 and adult blood mononuclear leucocytes in RF10. The samples analysed were sterile, distilled water for the blank, CL075 dissolved in water at 10^{-5} M, and mononuclear leucocytes suspended in RF10 at 1 x 10^6 cells/mL. For the UV-visible absorption spectra of CL075 and cell culture were determined with the CARY 300 UV-Vis Spectrophotometer (Varian Inc.). Samples were tested in matched 1.0 cm path length quartz cells over the wavelength range 200 to 600 nm at 1 nm intervals, with a scan rate of 600 nm/min. The fluorescence of CL075 and cell culture in response to excitation by CL075 absorption bands were measured by the LS50B Fluorescence Spectrometer (PerkinElmer Inc.). Samples were tested in matched 1.0 cm path length quartz cells over the wavelength range 290 to 600 nm

with excitation and emission slit widths of 10 nm each, by means of a 1% transmittance emission filter, scanning occurred at 0.5 nm intervals, with a scan rate of 200 nm/min. Dr Sally Plush, University of South Australia, provided expert assistance for these experiments.

6.2.4 Immunofluorescence Microscopy

Immunofluorescence microscopy studies on B lymphocytes and CL075 using Leica SP5 spectral scanning confocal microscope (Adelaide Microscopy). The protocol was as follows:

- Suspend mononuclear leucocytes to 1 x 10^7 cells/mL in warm RF10 and add 10^6 cells per well to 3 wells in a 12-well tissue culture plate. Add CL075 to third well for a final concentration of 10μM, and add RF10 to all wells for final volume of 1 mL.
- Incubate plate at 37ºC in 5% CO_2 incubator for 2 hours, then harvest cells using a rubber policeman and wash cells once.
- Transfer cells from each well to separate tubes for immunofluorescence staining with FITC-conjuated CD19 antibody (Refer Chapter Two, Section 2.13.1). Add DAPI stain for one minute, then wash cells twice.
- Drain excess supernatant, add 10 μL Glycerol (Sigma Aldrich Inc.; catalogue no. G8773), resuspend cells, transfer cells to glass slides and mount coverslips.
- Capture images with confocal microscope and examine localisation of CL075 in B lymphocytes.

6.3 RESULTS

6.3.1 Viability of B Lymphocytes

TLR8 was detected on non-permeabilised B lymphocytes. In permeabilised B lymphocytes, high non-specific binding precluded a conclusion for TLR8 expression. TLR9 was detected at low levels on non-permeabilised B lymphocytes and at high levels in permeabilised B lymphocytes. CD45RO was detected on a proportion of non-permeabilised T lymphocytes, and it was detected in a larger proportion of permeabilised T lymphocytes. Cytochrome C was not detected on non-permeabilised B lymphocytes, and detected at high levels in permeabilised B lymphocytes. Annexin V was detected at extremely low levels on non-permeabilised and permeabilised B lymphocytes. 7AAD was detected at extremely low levels in non-permeabilised lymphocytes, and detected in almost all permeabilised mononuclear leucocytes. (Figures 6.2 and 6.3)

6.3.2 TLR8 Indirect Labelling of Lymphocytes

Surface TLR8 on B lymphocytes was not detected using SA-AF647, and for SA-PE high non-specific binding precluded a conclusion. Surface TLR9 was detected on B lymphocytes with both SA-PE and SA-AF647. Surface TLR8 and TLR9 on T lymphocytes were not detected with SA-PE or SA-AF647. (Figure 6.4 and 6.5)

6.3.3 Competitive TLR8 Antibody Labelling

TLR8 surface expression by B lymphocytes was detected with PE-conjugated TLR8 antibody, but not with the indirect immunofluorescence labelling with unconjugated TLR8 antibody. For competitive binding of PE-conjugated TLR8 antibody, there was a reduction in TLR8 staining in two subjects when comparing addition of x63 to unconjugated TLR8 antibody. (Figures 6.6 and 6.7)

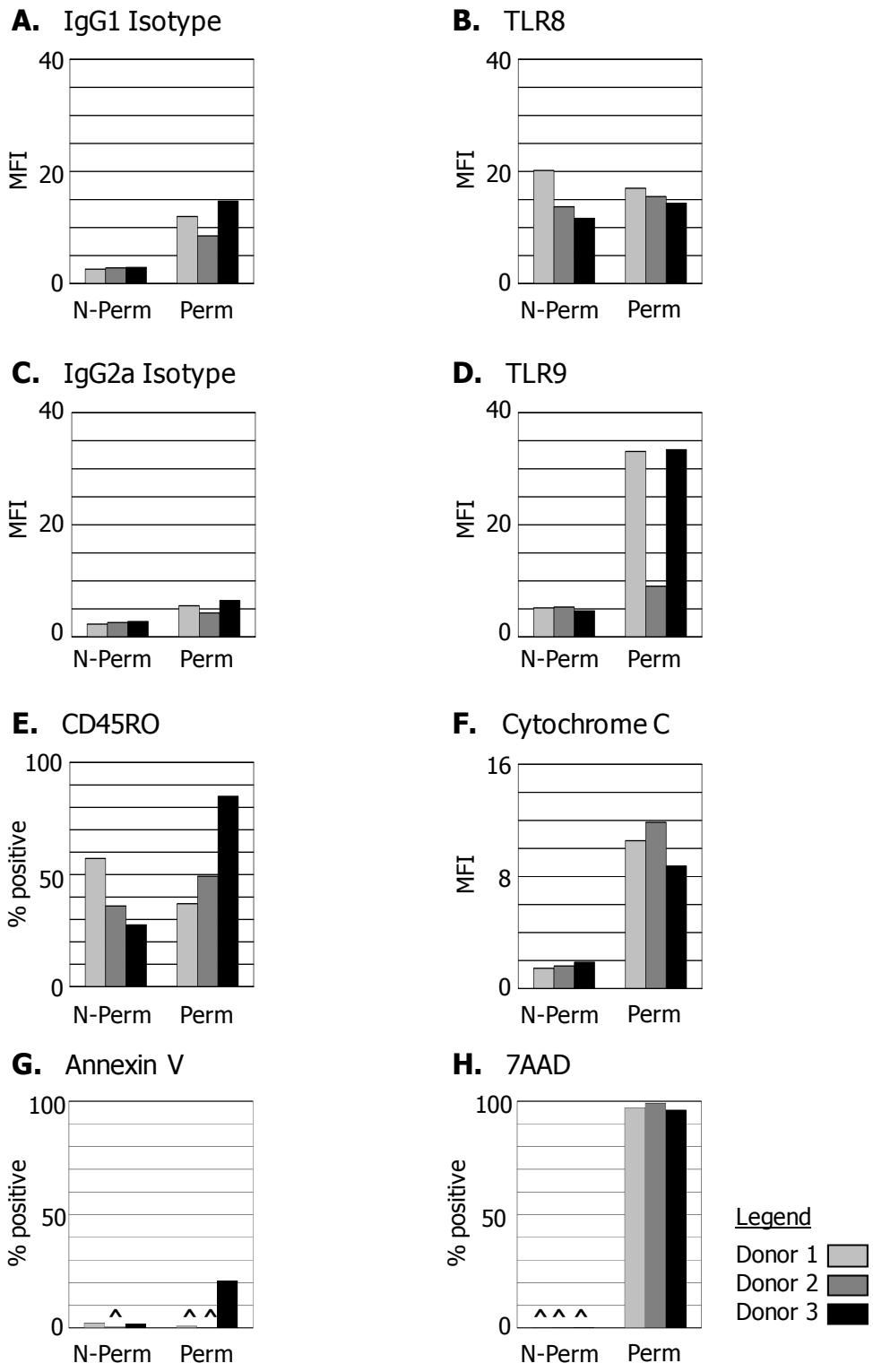


Figure 6.2 Viability of B Lymphocytes
Expression of molecules by non-permeabilised (N-Perm) and permeabilised (Perm) cells from adult blood. Detected **A.** IgG1 isotype, **B.** TLR8, **C.** IgG2a isotype and **D.** TLR9 by B lymphocytes; **E.** CD45RO expression of T lymphocytes; **F.** Cytochrome C and **G.** Annexin V by B lymphocytes; and **H.** 7AAD by lymphocytes. Results from three experiments. ^ = < 1%

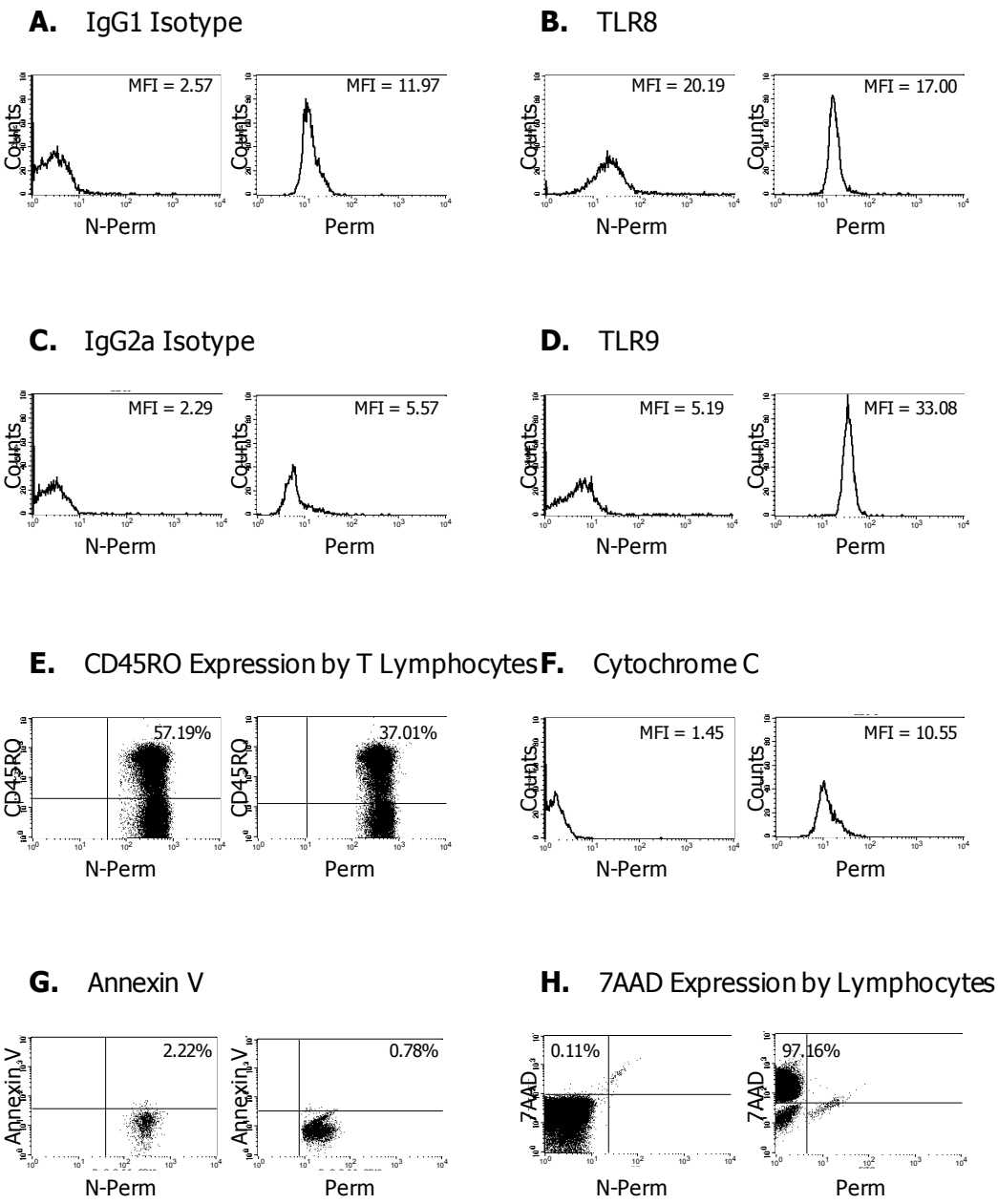


Figure 6.3 Viability of B Lymphocytes
Representative example (Donor 1) of molecule expression by non-permeabilised (N-Perm) and permeabilised (Perm) cells from adult blood. Molecules detected are **A.** IgG1 isotype, **B.** TLR8, **C.** IgG2a isotype and **D.** TLR9 by B lymphocytes; **E.** CD45RO expression of T lymphocytes; **F.** Cytochrome C and **G.** Annexin V by B lymphocytes; and **H.** 7AAD by lymphocytes.

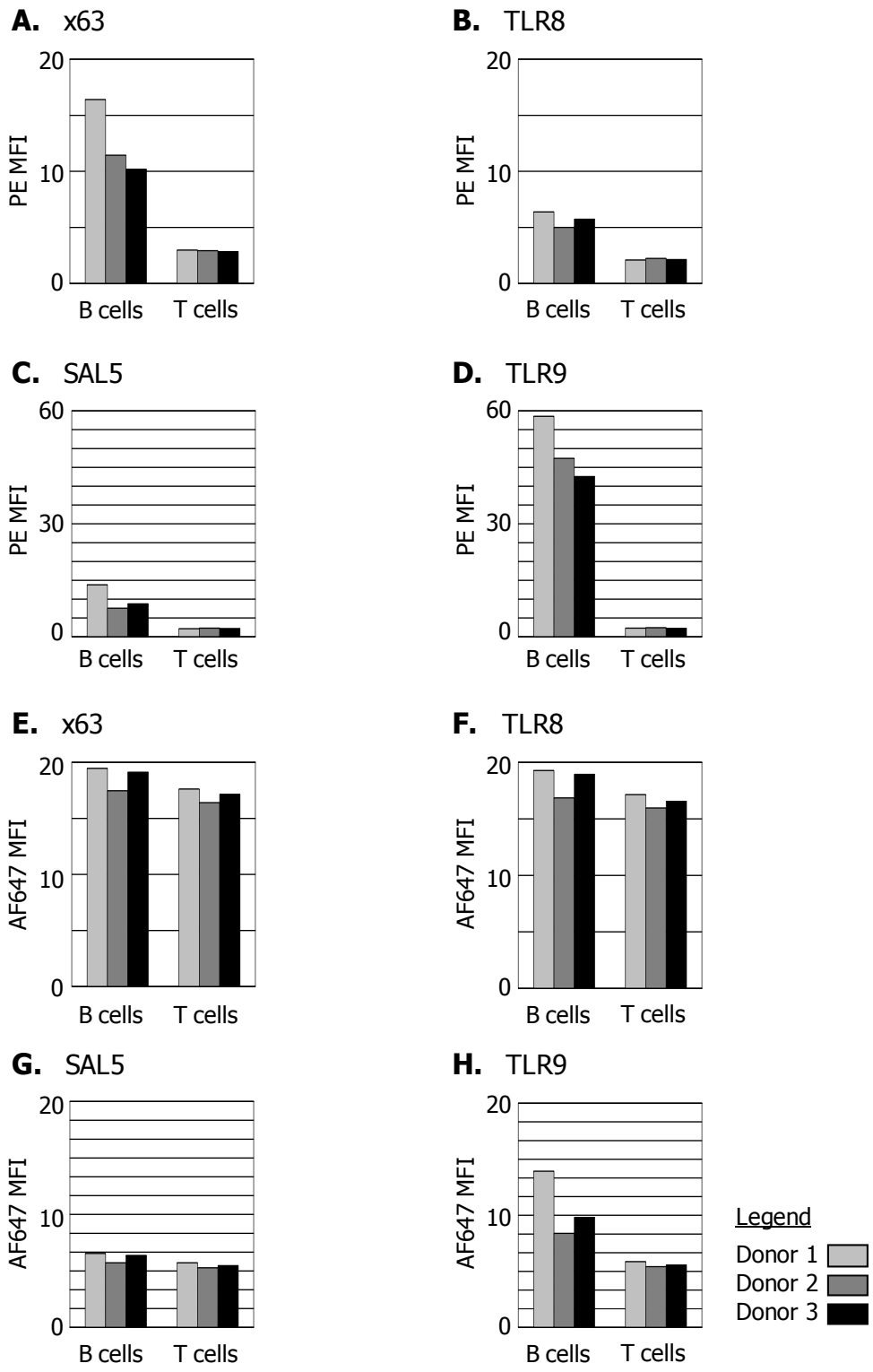


Figure 6.4 TLR8 Indirect Labelling of Lymphocytes
Extracellular TLR8 (**B**, **F**) and TLR9 (**D**, **H**) with matched negative isotype controls (x63 – **A**, **E**; SAL5 – **C**, **G**) were detected on B lymphocytes and T lymphocytes using high-sensitivity three-step immunofluorescence labelling with fluorescent dyes. Results from three experiments.

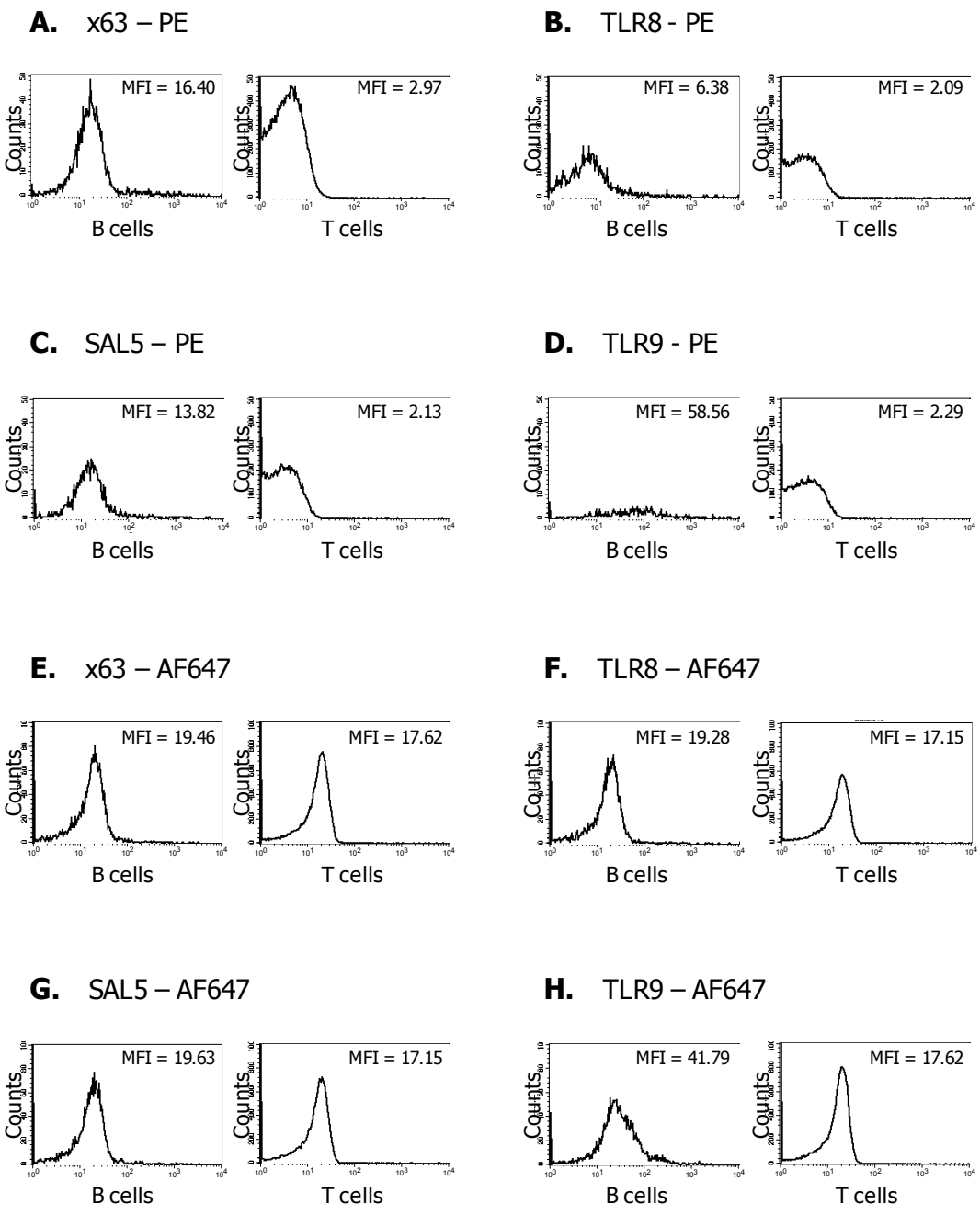


Figure 6.5 TLR8 Indirect Labelling of Lymphocytes
Representative example (Donor 1) of detection of extracellular TLR8 (**B**, **F**) and TLR9 (**D**, **H**) with matched negative isotype controls (x63 – **A**, **E**; SAL5 – **C**, **G**) on B lymphocytes and T lymphocytes using high-sensitivity three-step immunofluorescence labelling with fluorescent dyes.

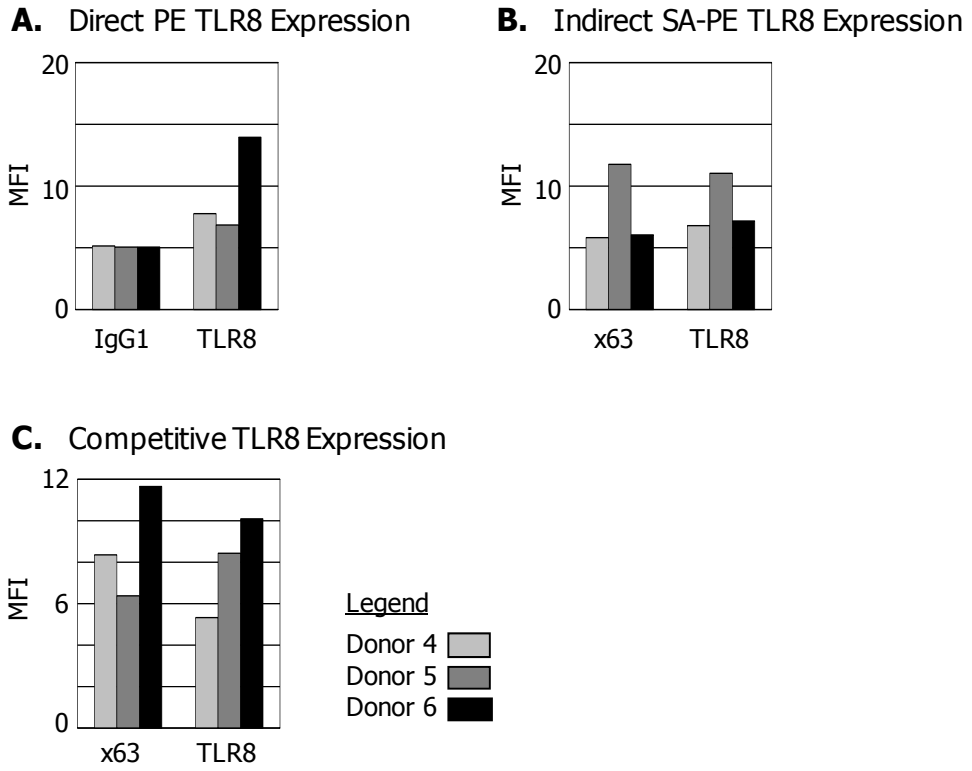


Figure 6.6 Competitive TLR8 Antibody Labelling
TLR8 expression with negative isotype controls by B lymphocytes was detected with **A.** PE-conjugated TLR8 and **B.** Indirect PE-labelling of TLR8. Competitive binding of PE-conjugated TLR8 antibody was assessed with **C.** Unconjugated x63 and TLR8 antibodies. Results from three experiments.

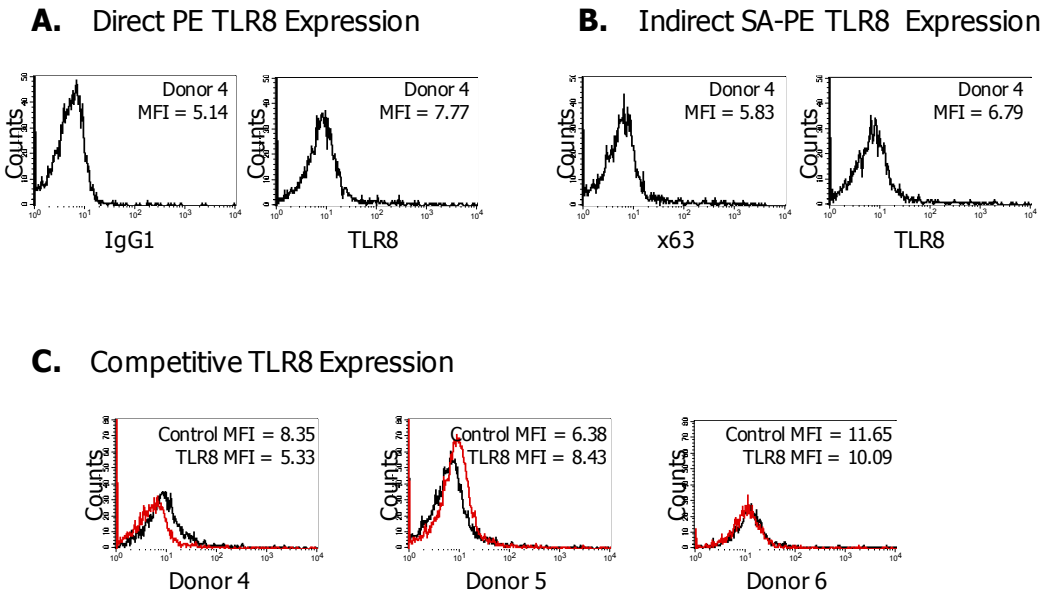


Figure 6.7 Competitive TLR8 Antibody Labelling
Representative example of TLR8 expression with negative isotype controls by B lymphocytes detected with **A.** PE-conjugated TLR8 and **B.** Indirect PE-labelling of TLR8. Competitive binding of PE-conjugated TLR8 antibody was assessed with **C.** Unconjugated x63 and TLR8 antibodies (x63 antibody = black, TLR8 antibody = red).

Spectrophotometric Absorption and Emission of CLO75

Four absorption bands for CL075 were recorded at 236, 253, 323 and 337 nm. Five absorption bands for cell culture in RF10 were recorded at 202, 212-216, 222, 236 and 280 nm. (Figure 6.8)

When CL075 was excited at 236, 253, 323 or 337 nm, large emission signals were detected, the largest emission maxima resulted from excitation at 236 nm. Emission properties of cell culture in RF10 when excited at 236, 253, 323 or 337 nm had appreciably lower quantum yield when compared with excitation of CL075 at 236 and 253 nm. (Figure 6.9)

6.3.4 Immunofluorescence Microscopy of Leucocyte Culture with CL075

Though B lymphocytes were identified, there were no fluorescent signals by CL075 in response to immunofluorescence imaging. (Figure 6.10)

6.4 DISCUSSION

The surface expression of TLR8 on B lymphocytes from Chapter Four results was unexpected as the literature indicates that TLR8 is an internal receptor. In the introduction, the literature on internal TLR8 expression was examined and found to be lacking. It is conjecture on the basis of the relationship of TLR8 to TLR7 and TLR9 (64, 216), internal expression of TLR7 and TLR9 (88, 221), and possible endosomal maturation required for TLR8 activity (219-220). TLR8 expression from Chapters Three and Four shows low level expression inside T lymphocytes

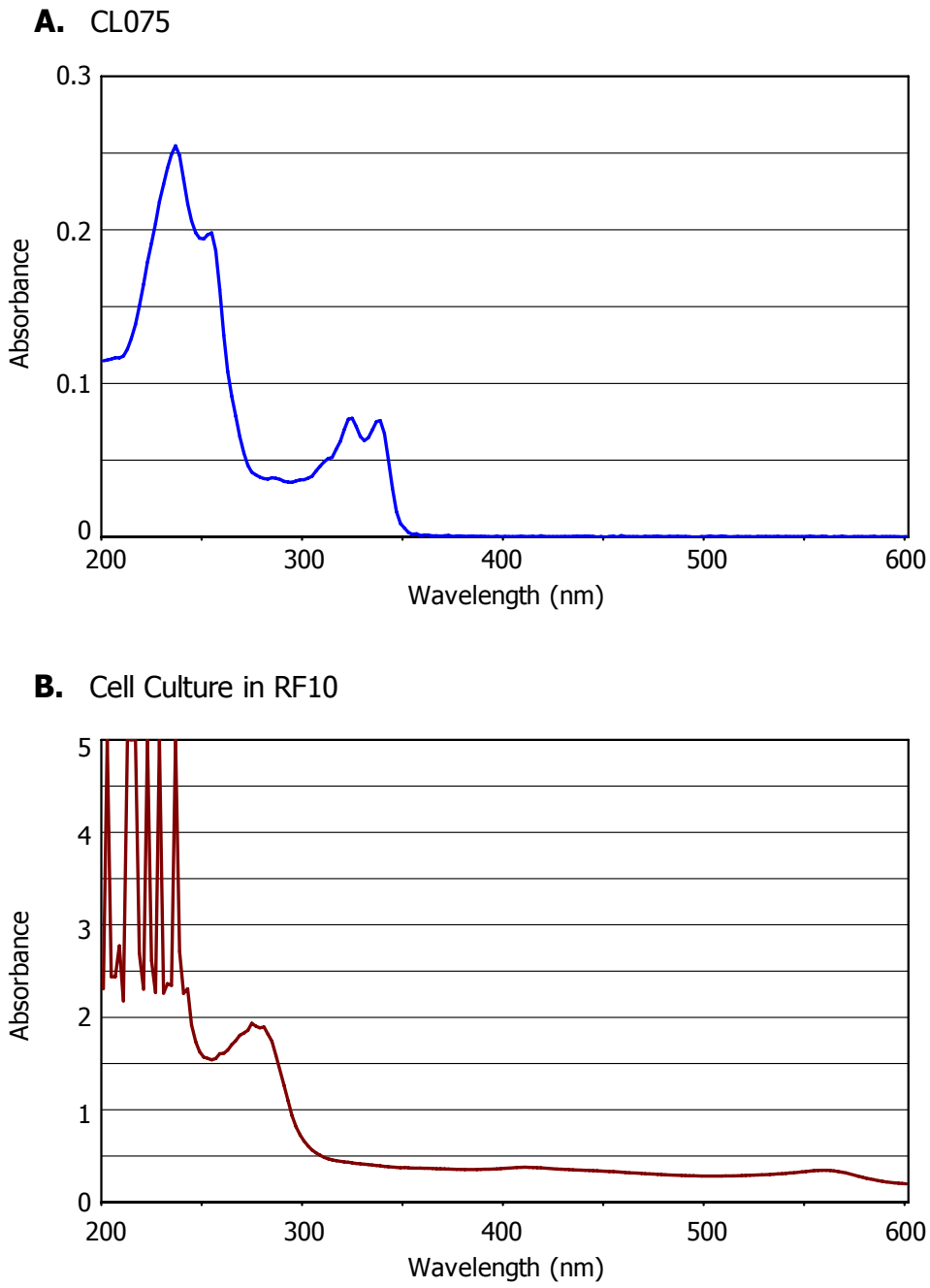


Figure 6.8 Spectrophotometric Absorption of CL075 and Cell Culture Absorption bands of **A.** CL075 and **B.** Cell culture in RF10 media.

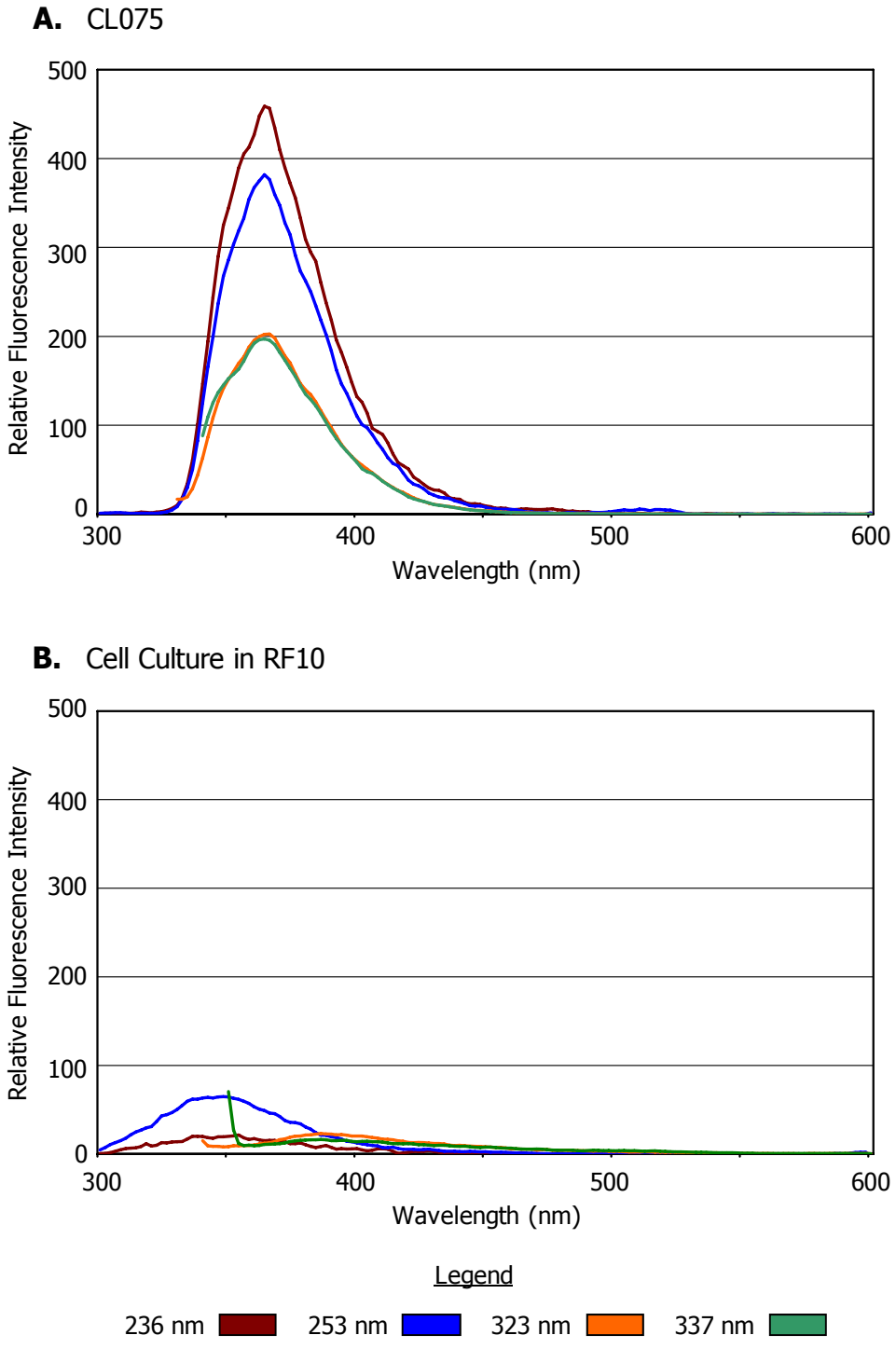


Figure 6.9 Spectrophotometric Emission of CL075 and Cell Culture
Emission spectra of **A.** CL075 and **B.** Cell culture in RF10 media upon excitation with CL075 excitation wavelengths; 236 nm, 235 nm, 323 nm and 337 nm.

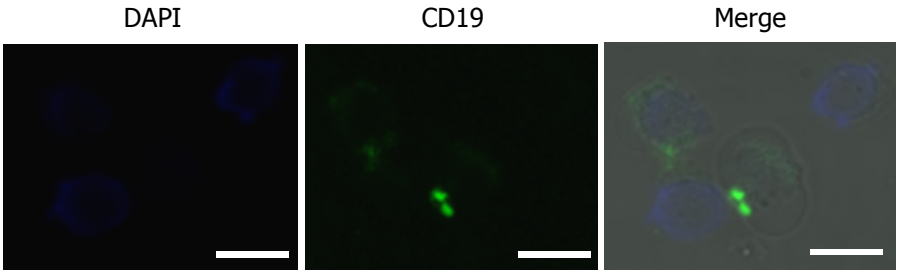


Figure 6.10 Immunofluorescence Microscopy of Leucocyte Culture with CL075
Confocal images of PBMC treated with CL075 and labelled with DAPI and FITC-conjugated CD19. Merged image shows $CD19^+$ leucocytes. CL075 did not emit any detected fluorescent signals. Magnification x 100. Scale bar = 10 µm.

and B lymphocytes, but surface expression of TLR8 is greater. The result of surface TLR8 expression needed to be verified with further experimentation to ensure the validity of the observation.

The surface TLR8 expression result could not be confirmed with another anti-TLR8 monoclonal antibody. There were no appropriate monoclonal antibodies available and polyclonal antibodies were not acceptable due to their potential lack of specificity. The Imgenex antibody used in the TLR phenotyping studies was a mouse anti-human TLR8 monoclonal antibody, clone 44C143, developed against a synthetic peptide of human TLR8 within amino acids 750-850. Four anti-TLR8 monoclonal antibodies of different clones were identified from three companies, but were not suitable because they were all raised against the same synthetic peptide of TLR8 as Imgenex clone 44C143. As the result could not be confirmed independently, the Imgenex anti-TLR8 antibody binding was examined further.

The lower intracellular expression of TLR8 by lymphocytes compared to the surface TLR8 expression was unexpected. A possible explanation is the anti-TLR8 antibody is internalised by cells, the antibody is damaged by proteolysis in

endosomes and therefore has reduced detection of intracellular TLR8. However, bright internal expression of TLR3 and TLR9 by lymphocytes has been detected.

Irrespective of intracellular expression of TLR8, bright surface expression of TLR8 was observed.

Cell viability of B lymphocytes was examined to ensure that the integrity of the cell membrane was maintained and that the surface expression of TLR8 was not due to antibodies detecting intracellular TLR8. TLR8 and TLR9 expression by B lymphocytes, detected with PE-conjugated antibodies, were consistent with the results of Chapter Four. Cytochrome C, an intracellular protein (222), was detected internally with no detection on non-permeabilised cells. CD45RO expression was measured on T lymphocytes as they were part of the mononuclear leucocytes fraction and underwent identical conditions to B lymphocytes which don't express CD45RO. CD45RO expression, an extracellular marker, was detected on non-permeabilised and permeabilised T lymphocytes. An explanation for the greater presence of CD45RO on permeabilised cells is that extracellular and intracellular CD45RO was detected on permeabilised cells, whereas only extracellular staining is detected on non -permeabilised cells.

Apoptosis and membrane integrity of non-permeabilised and permeabilised cells was examined by expression of Annexin V, a marker of apoptosis, and 7AAD, which binds to DNA and is therefore a marker of membrane permeability. The gating strategy excludes apoptotic cells with FSC^{lo} SSC^{hi} scatter, and viability of gated lymphocytes was examined. Very low levels of Annexin V were expressed by non-permeabilised and permeabilised B lymphocytes, indicating that B lymphocytes

within the lymphocyte gate have minimal apoptosis. Cells outside the gate would include apoptotic cells, but the analysis of TLR8 expression was based on the same gating strategy. The membrane integrity of non-permeabilised lymphocytes was excellent, as shown by the low level of 7AAD staining. Finally, no cytochrome C was detected on the non-permeabilised cells. These experiments rule out the possibility that TLR8 detection on non-permeabilised cells was because the antibody could enter the cells and stain intracellular TLR8.

Another possibility for the surface staining of TLR8 was that PE dye may be binding non-specifically. Unconjugated TLR8 antibody, the same clone as the PE-conjugated antibody, was used for indirect immunofluorescence labelling. Surface expression of TLR8 was not detected on B lymphocytes or T lymphocytes. Surface TLR9 expression was detected with indirect immunofluorescence labelling with both fluorescent dyes. The PE conjugation of TLR8 antibody may result in greater adherence to cells as the process of PE conjugation may affect the antibody structure and binding. Another problem is that the negative isotype control, x63 antibody, has high levels of binding to B lymphocytes, thereby affecting the interpretation of TLR8 binding to B lymphocytes. The high levels of binding may be due to Fc receptors present on B lymphocytes; inclusion of human sera in the experiments to block Fc receptors may have mitigated the non-specific binding. It is not possible to interpret whether the PE dye is binding non-specifically to B lymphocytes due to the inconclusive results of this experiment.

The PE-conjugated TLR8 antibody may be binding non-specifically to other cell surface proteins and not binding specifically to TLR8. Therefore the specificity of PE-conjugated TLR8 antibody binding was tested through competitive binding with

either unconjugated TLR8 antibody or x63 antibody. The unconjugated TLR8 antibody, unlike the x63 antibody, will block binding of the PE-conjugated TLR8 antibody. This was seen in two out of three subjects tested, but one subject did not have inhibited TLR8 expression after addition of unconjugated TLR8 antibody. There is some evidence that PE-conjugated TLR8 antibody binds to TLR8 on the surface of B lymphocytes, but as this was not consistently observed for all subjects tested, no definite conclusions can be made.

Another alternative is cell culture studies to examine co-localisation of TLR8 with its ligand on cell surfaces by using fluorescently-conjugated TLR8 ligands. The commercial unavailability of fluorescently-conjugated ligands of TLR8 led to investigations of the fluorescent properties of CL075, the TLR8 ligand used in Chapter Five.

The absorption bands of CL075 and cell culture in RF10 were determined and CL075 had some bands that did not overlap with absorption bands of cell cultures in RF10. Excitation of CL075 in cell cultures resulted in emission of a bright fluorescent signal with minimal interference by the cell culture or RF10. This permits studies into localisation of CL075 in B lymphocytes using fluorescence microscopy to determine whether CL075 can bind to TLR8 with subsequent internalisation. Initial studies with confocal microscopy were not successful as no lasers had the appropriate wavelengths for excitation of CL075. With the confocal microscope the laser with the shortest wavelength was 405 nm and the longest wavelength for exciting CL075 was 337 nm. Though the excitation and emission spectra of CL075 was determined, the lack of appropriate technology precluded

investigations into CL075 interactions with B lymphocytes. Lasers with shorter wavelengths are required for fluorescent studies using CL075.

The verification of external TLR8 expression requires investigations which were not possible due to the constraints of time, so potential experiments are discussed further. The use of labelled TLR8 ligands would be very useful to clarify the site and function of TLR8, as described earlier for CL075. Using confocal microscopy, co-localisation studies with an effective anti-TLR8 antibody will reveal whether TLR8 binds to its ligand on the cell surface of B lymphocytes which is then internalised for endosomal maturation to instigate TLR8 activation. Another option for investigation is to extract cell membranes of purified B lymphocytes and probe for TLR8 expression in the membrane preparation, although purity is important to ensure there is little contamination with intracellular proteins. Other experimental avenues are available to support or refute the observation of external TLR8 expression on B lymphocytes, but additional time and resources are required.

If the surface expression of TLR8 is confirmed, the functionality of the receptor would be of interest. Endosomal maturation is required for TLR8 activity (220) which indicates any surface TLR8 which is engaged with its ligand would have to be internalised for activity. Confocal microscopy studies examining engagement of surface TLR8 and possible internalisation would be useful. Further cell culture studies examining any functional responses of TLR8 would clarify the functionality of surface TLR8 expression.

The surface expression of TLR8 detected on B lymphocytes was observed because of simultaneous examination of extracellular and intracellular TLR studies.

Although the results of the Imgenex PE-conjugated anti-TLR8 antibody were inconclusive, further analysis showed non-permeabilised B lymphocytes were viable with no aberrant rearrangement of external and internal molecules. Although further studies are required, this study demonstrated surface expression of TLR8 on B lymphocytes. Surface TLR8 expression indicates that TLR expression is more flexible than previously realised and that variable cellular locations of TLR may be a regulatory mechanism of immune responses.

CHAPTER SEVEN

GENERAL DISCUSSION

7.1 DISCUSSION

TLR expression and function by lymphocytes was compared between adults, neonates and secondary lymphoid tissue. With the immaturity of the neonatal acquired immune system and the innate role of TLR, it was hypothesised that TLR expression and function would be greater in neonates compared to adults. The hypothesis was negated by the results. TLR expression by cord blood and adult blood lymphocytes was similar, and responses of B lymphocytes to TLR ligands were not greater in cord blood compared to adult blood. The second hypothesis tested was that early lineage B cells express higher levels of TLR than mature, differentiated, B cells. This hypothesis was also negated by the results. Levels of TLR expression in blood and tonsils were similar between subsets of B lymphocytes, excluding the GC B cell subset from tonsils.

These two hypotheses were tested to see if the immaturity of the neonatal acquired immune response was in some way compensated for by an enhanced response to TLR. The results negated the hypotheses, but showed clearly that neonatal lymphocytes express TLR and are able to respond to TLR ligands, observations with important implications for vaccination of neonates.

T lymphocytes and B lymphocytes from adult blood and cord blood expressed several TLR. When particular TLR are present, it is expected that TLR function will correlate with expression. The functions of B lymphocytes from adult blood

and cord blood were examined after treatment with ligands of TLR3, TLR4, TLR8 and TLR9. B lymphocytes proliferated in response to TLR ligands, but the effects on downstream functions were less. Several factors may contribute to the discrepancy between TLR expression and the poor downstream functions of TLR-engaged B lymphocytes. Different ligands to the same TLR can produce differing responses (Refer Chp 1, Section 1.3.4); TLR9 discriminates between CpG ODN A and CpG ODN B, and produces different patterns of cytokines (106). Cell types may affect TLR responses; as cord blood B lymphocytes are predominantly IgM secreting B1 B cells, TLR-engaged B lymphocytes from cord blood secreted minimal IgG. Recognition of ligands by TLR is necessary for responses; for some ligands, recognition requires the TLR to form a heterodimer with another cellular receptor. As an example, LPS is recognised by TLR4/MD-2 heterodimer, without MD-2 there are minimal response to LPS (200). For neonates, poor TLR signalling in neonatal leucocytes (167, 169) may explain the discrepancy between TLR expression and downstream function in neonatal B lymphocytes. Correlation between TLR expression and TLR function can not be assumed; several factors are involved in TLR functionality.

TLR expression by T lymphocytes and B lymphocytes was similar between adult blood and cord blood demonstrating that neonates can detect infection; the first step to counter infection. T blasts expressed almost all tested TLR, compared to T cells with fewer TLR, which suggests they can respond vigorously to a variety of infectious agents. Unlike the development of TCR and Ig expression by lymphocytes, germ-line encoding of TLR makes innate TLR expression by neonatal lymphocytes possible. As discussed in Chapter Four, if neonates have partial immune function coupled with TLR expression, this will help mitigate the

vulnerability of neonates to infectious diseases. TLR ligation induces T lymphocytes (23, 84) and mononuclear leucocytes (5, 160) from cord blood to secrete moderate levels of pro-inflammatory cytokines. TLR ligands can shift the neonatal bias of T_H2 immune responses towards T_H1 responses (176) and increase activity of neonatal T_C cells (23, 176). In adults, TLR-engaged T lymphocytes instigate inflammatory response, destruction of infected cells and regulation of adaptive responses. Likewise, TLR ligands can boost the impaired response of neonatal T lymphocytes against infection via induction of the inflammatory response, shifting neonatal T_H2 polarisation and T_C activity. This enhancement of neonatal T lymphocyte activity contributes to the critical role of T lymphocytes in cell-mediated and humoral immune defences.

The expression of TLR by neonatal lymphocytes adds to the particular immune features that neonates possess, including sensitivity of their B lymphocytes to very low levels of antigens (32) and elevated surface expression of IgM and IgD (31). The proliferative response of B lymphocytes to TLR ligands was similar between adults and neonates; therefore neonatal B lymphocytes recognise infection, are activated and proliferate. For antibody functions, neonates have lower titres of total Ig and IgG which may lead to reduced antibody-mediated elimination of infection. IgG titres in neonates are limited for several reasons including reduced expression of co-stimulatory molecules by neonatal B lymphocytes, immaturity of T lymphocytes and DC, and lack of GC in secondary lymphoid tissue (34). Neonatal B lymphocytes, in response to all TLR ligands tested, secreted IgM; IgM has high avidity which can partially compensate for the low affinity binding. The high levels of IgM provide the neonate with an "emergency" response to neutralise infection.

Though neonates lack the mature IgG response, the sensitivity of neonatal B lymphocytes to TLR ligands offers some antibody protection with high IgM titres.

Neonatal B lymphocytes, compared to adult B lymphocytes, secreted similar levels of IL-6 and higher levels of IL-8. Though B lymphocytes are not the main cytokine secreting cells, they can influence their micro-environment. Murine neonatal B1 B cells, upon TLR engagement, secrete IL-10 which prevents DC from polarising to T_H1 immune responses and limits IL-12 production (164-165). IL-6 secretion by TLR-engaged neonatal macrophages enhances antibody secretion by neonatal B lymphocytes in response to T-I antigens (6). Cytokine secretion by neonatal B lymphocytes upon TLR ligation affects the microenvironment and modulates immune responses.

For secondary lymphoid tissue, tonsil B lymphocytes expressed fewer TLR and TLR ligands had less effect on cell function compared to adult blood B lymphocytes. In response to most TLR ligands, tonsil B lymphocytes did not proliferate and low levels of antibodies and cytokines were secreted. This discrepancy between TLR expression and poor TLR functionality is puzzling. TLR ligands are reported to have some effect on tonsil B lymphocytes; TLR-engaged tonsil B lymphocytes upregulate expression of CXCR5 and CD77 which assist in GC formation (94), several co-stimulatory molecules (206), and TLR signalling may be the third signal required for cognate interactions between B lymphocyte and T_H cells (157-158). However, these reports do not reflect the tested responses in this study. One suggestion is that, as secondary lymphoid tissue is the site for maturation of B lymphocytes, the environment may not be conducive for B lymphocytes to respond to TLR ligands. In lymphoid tissue T-D immune reactions

occur; B lymphocyte responses to T-I antigens, which include several TLR ligands, may not be critical in this location. If so, B lymphocytes may respond to TLR ligation in a different manner, most likely features of T-D immunity will be increased.

GC B cells expressed TLR at different levels compared to other B cell subsets of tonsils; thus the differentiation of tonsil B lymphocytes affects TLR expression. As discussed in Chapter Four, this result adds to suggestions that TLR may be a third signal required for B lymphocyte interactions with T-D antigens (157-158). Despite reports of statistically unverified upregulation of co-stimulatory molecules by TLR-engaged B lymphocytes (91, 155, 206), the results did not show significant upregulation of CD40, CD80 and CD86 by tonsil B lymphocytes in response to most TLR ligands. It may be that TLR-engaged B lymphocytes in tonsils also require other signals for upregulation of co-stimulatory molecules, which can then facilitate cognate interactions. GC B cells expressed higher levels of TLR9 compared to other B cell subsets, and tonsil B lymphocytes did proliferate and secrete high levels of antibodies and cytokines in response to CpG ODN. The results suggest that TLR9 and CpG ODN affect functions of B lymphocytes in tonsils.

TLR recognition of infection by B lymphocytes will induce a rapid, polyclonal antibody-mediated, early defence. TLR engagement induces differentiation of naive, B1 and marginal zone B cell subsets into plasma B cells for an antibody response (148, 153). B cell subsets respond differently to TLR engagement; low affinity IgM is secreted by naive B cells (153, 205), and high affinity IgG is secreted by marginal zone splenic B cells (198) and memory B cells (212). Murine studies

reveal that marginal zone B cells upregulate CD86 expression to TLR ligands (155) and GC B cells have greater levels of proliferation and higher expression of TLR signalling proteins compared to splenic B cell subsets (199). Sources of B lymphocytes also affect TLR responses; as seen in the results, B lymphocytes from blood have a higher antibody response compared to tonsil B lymphocytes. TLR-engaged B lymphocytes will participate in T-I and T-D antigen responses depending on the B cell subset and the particular TLR signalling pathway.

CpG ODN is a potent mitogen of B lymphocytes (115), the proliferative and antibody responses of blood and tonsil B lymphocytes to CpG ODN is supported by the literature (154, 204, 206). A particularly interesting result was that purified cord blood B lymphocytes secreted high levels of IgG in response to CpG ODN. B1 B cells do not antibody class-switch to IgG (25); therefore it is likely that cord blood B2 B cells switched from IgM to IgG, with no cognate interactions with T lymphocytes and DC, in response to CpG ODN. There is some evidence in the literature linking TLR9 signalling to antibody class-switch recombination. CpG ODN and IL-10 stimulate naive B cells to trigger early T-I antibody class-switch recombination to IgG, and additional IgG secretion requires BCR and B cell activating factor signals (156). Expression of AID and Blimp-1, genes for somatic hypermutation and class-switch recombination, increased in cord blood B lymphocytes treated with CpG ODN (156, 163). CpG ODN can act on B lymphocytes, either solely or as a co-stimulatory signal, and increase proliferation, expression of co-stimulatory molecules (CD40, CD80, CD86 and HLA-DR), secretion of antibodies and cytokines (IL-6, IL-10, TNF-α and IL-12), and terminally differentiate to plasma cells (153, 155, 163, 197, 206). B lymphocytes, stimulated by CpG ODN, can modulate immune responses; as an APC, B

lymphocytes stimulate T lymphocytes to proliferate and secrete IFN-γ (153, 197). CpG ODN has extensive effects on the innate and adaptive roles of B lymphocytes; it interacts with the T-I and T-D antigens and can influence immune responses. The broad range of functions which CpG ODN has makes it a strong candidate as a vaccine adjuvant.

TLR ligands have potential as vaccine adjuvants; the Yellow Fever vaccine, in use for 65 years, activates DC via TLR2, TLR7, TLR8 and TLR9 to produce a broad range of immune responses (144). Human (157) and murine (158) studies report that TLR engagement on B lymphocytes is the third signal required for T-D antibody responses. This is disputed by studies showing MyD88 knock-out mice with robust IgG responses (178, 199, 223), but another study with MyD88 knock-out mice showed that though germinal centres formed, and class-switch recombination and somatic hypermutation occurred, the long-term antibody responses with plasma cell differentiation did not develop (170). Nonetheless, TLR ligands as adjuvants in murine models induced germinal centre formation, selectively primed antigen-specific memory B cells, and induced proliferation of antigen-specific B lymphocytes and elevated titres of IgG, IgG2a and IgG2b (146, 171, 174, 223). Some vaccine studies trialling CpG ODN as an adjuvant show increased IgG titres and affinity (172), and increased proliferation and cytotoxicity of antigen-specific T_C (224). The broad immune effects which TLR exert contribute to vaccine immunisation; TLR assist the development of a robust antibody response, instigate inflammation, prime the immune system, and skew DC to T_H1 bias. There is strong evidence to support TLR ligands as vaccine adjuvants, but further research is needed to clarify contradictions in the literature.

Neonates and infants undergo repeated vaccinations to compensate for their poor immunological memory to vaccine immunogens. Adjuvants that can enhance neonatal immunity would reduce the pressure on the vaccination regimen in early life. In neonates, TLR ligands, separately or synergistically, induce DC and monocytes to produce IL-12 and TNF-α, and upregulate expression of co-stimulatory molecules (52, 161-162). This encourages neonatal APC to overcome their anti-inflammatory bias and skew towards the T_H1 paradigm. However, use of TLR ligands needs to account for peculiarities in neonatal immunity. TLR ligation of neonatal murine B1 B cells induces high levels of IL-10 which limits DC from producing IL-12 and encourages a T_H2 bias (164-165). Antibody production by neonatal murine B lymphocytes is impeded by high levels of IL-10, though this can be partially countered by IL-6 from LPS-engaged B lymphocytes (6). Neonatal vaccines can benefit from TLR ligands; Hepatitis B vaccination with CpG ODN in neonatal mice produced T_H1 responses with high titres of IgG1 and IgG2a (176), TB vaccination with a TLR9 ligand in neonatal mice induced an inflammatory response with T_H1 bias and maturation of antigen-specific DC, and TLR ligands enhanced human neonatal T_C cell cytotoxic activity (23). TLR ligands enhance several aspects of the neonatal immune system and accelerate immune development; exploiting this ability may reduce the immune vulnerability of neonates. The long-term effects of TLR ligands as adjuvants need to be examined to avoid the potential of autoimmunity. Nonetheless, TLR ligands as vaccine adjuvants are promising and further research is required to complete understanding of the neonatal immune system.

The present study contributed to our understanding of the effects of TLR in vaccine responses by providing direct evidence of TLR protein expression by neonatal cells, and functional responses through TLR.

This study tested the expression of TLR proteins by non-permeabilised and permeabilised cells, regardless of expected cellular locations. Most of the results supported existing literature, for example TLR2 is a surface receptor and TLR3 is an intracellular receptor. However, the use of flow cytometry yielded new observations on the cellular locations of TLR expression. Early on, this approach bore new information about the surface expression of TLR9 (81, 92, 100). Another unexpected result was the surface expression of TLR8 by B lymphocytes. As discussed in the Introduction of Chapter 6, TLR8 was considered an internal receptor because of its classification with TLR7 and TLR9, but external TLR8 expression has not been reported in the literature so far. This new observation indicates that TLR expression may be more flexible than previously thought. Flexibility of TLR expression may be an immune regulatory mechanism or may allow adaptability of the host to interact with pathogens under different conditions. Further work is required to confirm surface TLR8 expression, but this is an informative result. It is important to note that unexpected results need to be considered, even if, and arguably *especially* if, they differ from accepted dogma.

There are several directions from this study for further investigation. The nature of IgG produced by CpG ODN-treated cord blood B lymphocytes needs more research. The isotype, affinity and antigen-specificity of IgG needs to be examined to determine the effectiveness of the antibody. The IgG needs to bind to infectious agents and contribute to an effective immune response; non-specific IgG will not

offer any protection for neonates. Another direction is to study the effects of TLR ligands on tonsil B lymphocytes. Greater understanding of how TLR can affect cognate interactions between T lymphocytes and B lymphocytes to produce the mature antibody response is required. The question of whether TLR ligands enhance long-term antibody responses is important for their role as vaccine adjuvants. The potential role of TLR to induce autoimmune diseases must be examined. TLR dysfunction has been implicated in the pathogenesis of autoimmune diseases in humans (136-137) and mice (135). TLR ligands as adjuvants in neonatal vaccines may induce autoimmunity; it is imperative to investigate the possibility and avoid potentially adverse vaccine sequelae.

In conclusion, neonatal lymphocytes have the ability to recognise pathogens to the same extent as adult lymphocytes, and TLR ligands increase the functions of neonatal B lymphocytes. The enhanced functions of neonatal immunity upon treatment by TLR ligands strongly support further investigations of TLR ligands as adjuvants in neonatal vaccines. TLR may reduce immune vulnerability in early life and provide neonates with partial protection against infectious diseases.

Appendix

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Cellular Immunology 236 (2005) 140–145
Cellular Immunology
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Expression of toll-like receptors on B lymphocytes

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Received 18 February 2005; accepted 23 May 2005
Available online 26 September 2005

Abstract

Toll-like receptors (TLRs) are a family of trans-membrane receptors that play an important role in the innate immune system. Most studies examining the cellular expression of TLRs on immune cells have focussed on neutrophils, monocytes and dendritic cells, but there is little evidence of TLRs being expressed on lymphocytes. Using 3-colour flow cytometry, expression of TLR-1, TLR-2, TLR-3, TLR-4, and TLR-9 on peripheral blood lymphocyte populations was determined. Further examination of TLRs on $CD5^-$ and $CD5^+$ $CD19^+$ B cell subsets was performed. The binding of TLR1 and TLR9 antibodies was detected on 15-90% of resting B cells, but not on resting T-cells. The higher expression of TLR1 and TLR9 on $CD5^+$ B cells compared to $CD5^-$ B cells may reflect the role of B1 cells in more primitive, less specific antibody responses.

Keywords: Lymphocytes; Toll-like receptors

1. Introduction

Toll-like receptors (TLRs) are a family of trans-membrane receptors responsible for recognition and the initiation of a response to invading microbes by the immune system [1]. As part of the innate immune system, TLRs recognise pathogen-associated molecular patterns (PAMPs), highly conserved components essential to microbial function [2]. Ten TLRs have been identified in humans with some TLRs able to recognise several PAMPs [2–4]. Numerous studies have identified TLRs on various immune cells through PCR-based molecular screening and flow cytometric analysis (Table 1).

Ligand recognition by TLRs can trigger a host of responses in both the innate and adaptive immune systems. The importance of TLRs in the adaptive response has been suggested in light of observations showing reduced antibody production in humans and mice with reduced or deficient TLR1 and TLR2 expression [5]. Many immune cells can differentially regulate TLR expression when cells are activated with cytokines or TLR ligands, as observed in stimulated lymphocytes and dendritic cells (DCs) [6–10]. Upon activation, different TLRs trigger different signalling pathways, leading to a variety of cellular responses [11]. Engagement of TLRs expressed on antigen-presenting cells (APCs) including DCs and macrophages with their ligand(s) will result in chemokine and cytokine production, increased antigen presentation and the expression of co-stimulatory molecules [4,11]. These events can initiate an inflammatory reaction through chemokine secretion and cellular recruitment [4,11,12]. TLR recognition by DCs can also enhance the adaptive response that is initiated by DCs presenting antigen to naïve T cells [13]. TLR ligands such as lipopolysaccharide and double-stranded RNA are known to act as adjuvants, enhancing the adaptive immune response [13].

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0008-8749/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellimm.2005.08.020

TLR engagement mediates the maturation and migration of DCs to lymph nodes that facilitates interaction with T lymphocytes [4,14]. DC interactions with naïve T lymphocytes differentiate them into T_H1 or T_H2 lymphocytes or T regulatory lymphocytes [15].

B lymphocytes can generally be divided into B-1 and B-2 B cells on the basis of positive or negative expression of CD5, respectively [16]. B-1 B cells, which appear before B-2 B cells during embryonic development, are self-renewing cells that can secrete natural IgM against many common pathogen-associated carbohydrate antigens [16]. B-1 B cells also secrete IgA in gut mucosa where their interactions with commensal flora may be a mechanism bridging the innate and adaptive immune responses [17]. B1-B cells are responsive to various antigens, lipopolysaccharide and cytokines [17], but the response of B-1 B cells to thymus independent antigens suggests participation in a natural, innate response [18]. TLR expression on B cells can be up regulated when B cells are activated [7], however, it has not been determined whether this increase is on B-1 or B-2 B cells. Given the role of B-1 B cells in innate immunity, the expression of TLR on this subset would be anticipated.

TLR expression by immune cells has mainly been detected through presence of mRNA using PCR-based methods (Table 1), but there is less information available about cell surface expression due to the limited availability of monoclonal antibodies directed against TLRs. The generation of a number of monoclonal antibodies with specificity for TLRs that were being tested as a part of the 8th International Workshop on Human Leucocyte Differentiation Antigens (HLDA8), provided us with an opportunity to confirm the results of a number of these earlier PCR-based studies by fluorescence staining and flow cytometry. We therefore set out to examine the cell surface expression of TLRs on resting T and B lymphocytes from peripheral blood. It also provided an opportunity to determine whether TLRs are present on B-1 B cells and to consider the role these receptors may play in the response of B-1 B cells to bacterial antigens.

We are interested in what TLRs are expressed on the surface of peripheral blood T and B lymphocytes and as part of this, we examined whether the TLR^+ B cells are preferentially B-1 cells.

2. Materials and methods

Three healthy adult donors each provided 20 mL of blood. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Isopaque-Ficoll density gradient centrifugation (Lymphoprep™, Axis-Shield, Oslo, Norway), concentrated to 1×10^7 cells/mL in PBS/0.02% Sodium Azide (PBS/Azide) and stored on ice.

The purified monoclonal TLR antibodies (Suppliers clone number; HLDA8 Workshop code given in parentheses) available for study were TLR1 (GD2.F4; HLDA8 code 80675), TLR2 (TLR2.1; 80676 and TLR2.3; 80677), TLR3 (TLR3.7; 80678), TLR4 (HTA125; 80679), and TLR9 (eB72-1665; 80680) (eBiosciences, CA, USA).

TLRs were detected by flow cytometry using a three-step immunofluorescence technique with mouse monoclonal anti-human TLR antibodies, biotinylated horse anti-mouse immunoglobulin (Hα Mbi) (Vector Laboratories, CA, USA) and streptavidin-PeCy5 (SA-QR) (Sigma-Aldrich, St Louis, USA). TLR expression on peripheral blood lymphocytes (PBLs) was examined using a combination of antibodies:

- FITC-anti-CD3 (BD Biosciences, CA, USA), PE-anti-CD19 (BD Biosciences, CA, USA) and TLR/HαMbi/SA-QR

Table 1
Literature reports of the detection of TLRs in human immune cells

Cell type	TLRs	PCR	Flow cytometry	Fluorescent microscopy
Peripheral blood				
Neutrophils	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	Yes [26,27]	Yes [28,29]	NR[a]
Monocytes	1, 2, 4, 5, 6, 7, 8, 9	Yes [8,9]	Yes [9,28–30]	Yes [30]
Eosinophils	1, 4, 6, 7, 9, 10	Yes [27,29]	NR	NR
Basophils	2, 4	Yes [29]	Yes [29]	NR
T cells	1, 2, 3, 4, 5, 6, 8, 9	Yes [6,8,31]	Yes [6]	NR
B cells	1, 2, 4, 6, 7, 9, 10	Yes [8]	Yes [30]	NR
NK cells	1, 2, 3, 4, 5, 6, 8, 9	Yes [8,31]	NR	NR
Tonsillar B lymphocytes	1, 2, 6, 7, 8, 9, 10	Yes [7]	Yes [25]	Yes [28]
Macrophages	2, 4	Yes [32]	Yes [32]	Yes [28,33]
Dendritic cells				
Immature DCs	1, 2, 3, 4, 6	Yes [9]	Yes [9]	NR
Mature DCs	1, 6	Yes [9]	Yes [25]	NR
Plasmacytoid DCs	1, 6, 7, 9, 10	Yes [7]	Yes [25]	NR

[a] NR = Not Reported

- FITC-anti-CD5 (Immunotech, Marseille, France), PE-anti-CD19 and TLR/HαMbi/SA-QR

PBMCs were aliquoted in 50 μL volumes and incubated with TLR monoclonal antibodies or matched isotype controls, X63 and SAL5 [19], on ice for 30 min, then washed in ice-cold PBS/Azide and pelleted by centrifugation (270*g* for 5 min at 4 °C). Appropriate tubes had 50 μL of 1/50 dilution of HαMbi added and incubated on ice for 30 min before cells were washed and pelleted. Five microlitres of mouse serum (DakoCytomation, Glostrup, Denmark) was added to tubes and incubated on ice for 10 min as a blocking step against non-specific binding. Fifty microlitres of 1/20 dilution of SA-QR and 5 μL of the commercial antibodies, PE-anti-CD19, FITC-anti-CD3 or FITC-anti-CD5 was added to the appropriate tubes and incubated on ice for 30 min. Cells were washed, pelleted and resuspended in PBS/Azide for data acquisition using an FACSCalibur™ (BD Biosciences, CA, USA). Data were analysed using a lymphocyte forward scatter versus side scatter gate using CellQuest™ software (BD Biosciences, CA, USA).

3. Results

PBLs were identified as either T lymphocytes or B lymphocytes by positive staining with CD3 or CD19 specific antibodies, respectively. The B lymphocytes were further categorised on the basis of CD5 expression into B-1 ($CD5^+$) cells or B-2 ($CD5^-$) cells.

The TLR staining of T and B lymphocytes for three donors are shown in Tables 2 and 3. While expression of TLRs was not detected in T lymphocytes, as shown in Table 2, the potential for very low level, or inducible TLR expression cannot be ruled out. Higher numbers of B lymphocytes expressing surface TLRs were detected compared to T lymphocytes. This was particularly evident in the case of TLR1 and TLR9, where increased numbers of TLR^+ cells were observed (Table 3 and Fig. 1). Other TLRs were detected on a small proportion of B lymphocytes.

Table 2
Proportion $CD3^+$ cells that are TLR^+ (expressed as percentage)

Donor	Negative isotypes and TLRs						
	X63	TLR1 (80675)	TLR2 (80676)	TLR2 (80677)	TLR3 (80678)	TLR4 (80679)	TLR9 (80680)
1	NA	0.7	0.7	0.5	0.7	0.5	0.5
2	0.3	0.2	0.3	0.1	0.1	0.1	0.2
3	0.4	0.2	0.3	0.2	0.2	0.2	0.2

Table 3
Proportion $CD19^+$ cells that are TLR^+ (expressed as percentage)

Donor	Negative isotypes and TLRs							
	X63	SAL5	TLR1 (80675)	TLR2 (80676)	TLR2 (80677)	TLR3 (80678)	TLR4 (80679)	TLR9 (80680)
1	7.6	7.7	84.3	13.6	9.1	8.7	7.8	51.5
2	10.1	11.1	68.2	14.4	12.8	12.0	11.6	89.9
3	13.1	14.9	15.6	31.3	15.3	16.8	15.1	68.0

Both the B-1 and B-2 lymphocyte subsets express TLR1 and TLR9 however analysis of B-1 B cells demonstrated brighter staining for TLR expression compared with B-2 B cells (Fig. 2).

4. Discussion

Surface TLR1 and TLR9 were detected on the majority of resting B lymphocytes, but other TLRs were found on smaller proportions of resting B lymphocytes. It can therefore be stated that our analysis of TLR expression on lymphocytes partially supports observations made in other studies that have reported detection of TLR encoding mRNA in lymphocytes. In this study TLR expression was detected in B lymphocytes, but not T lymphocytes.

This does not mean that TLRs do not play any role in T cell pathway as TLRs can influence the differentiation of naïve T lymphocyte development through the engagement

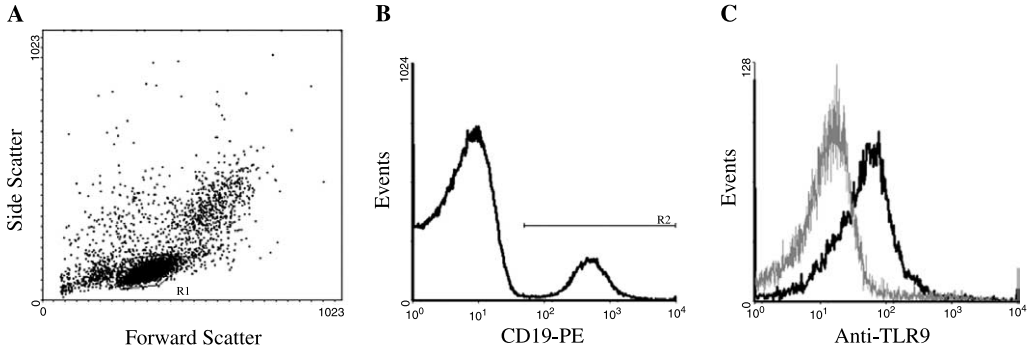

Fig. 1. TLR9 expression on B cells of Donor 1. (A) Region 1 is gated around the lymphocyte population. (B) Region 2 selects for $CD19^+$ cells to distinguish B cells. (C) B cells stained with isotype control (grey outline) or TLR9 (black outline) to show detection of TLR9 on B cells.

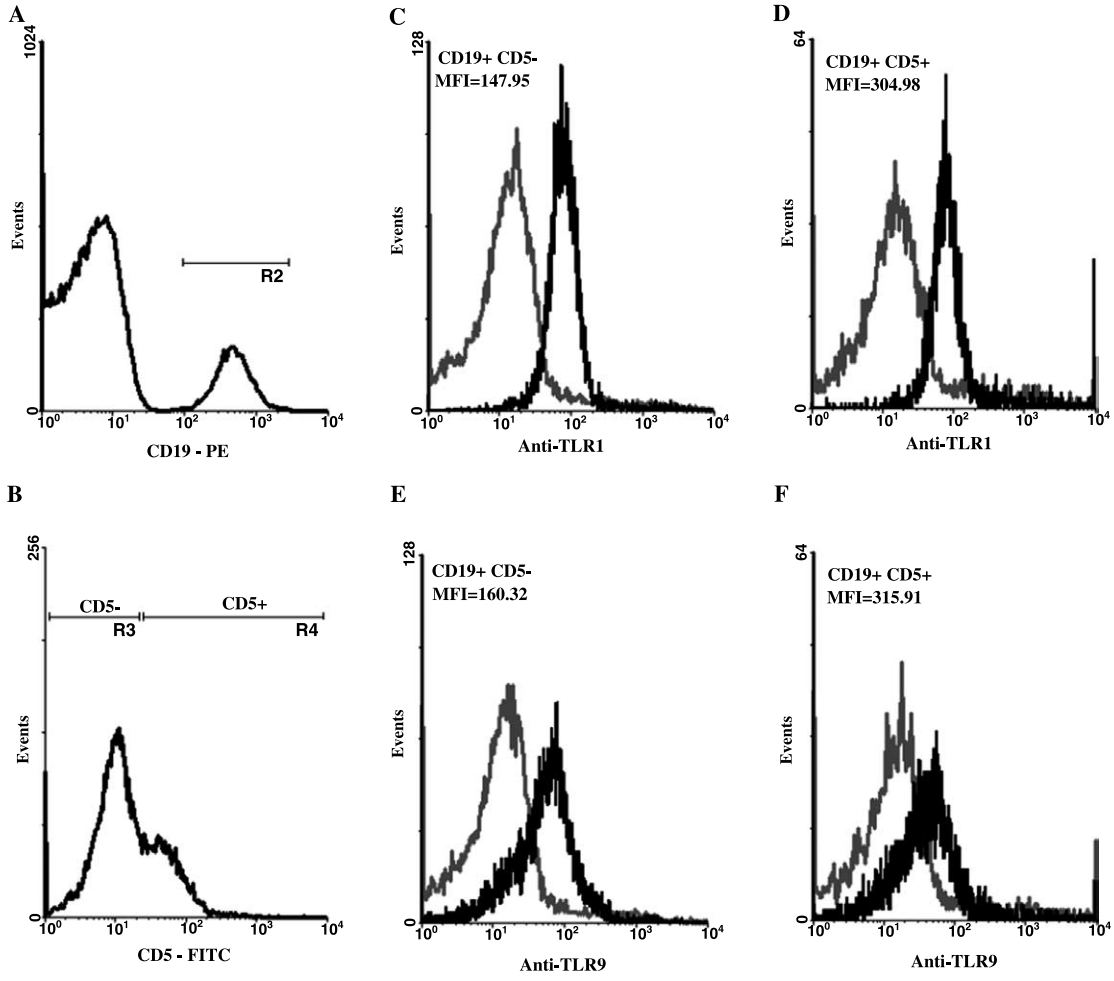

Fig. 2. Staining of TLR1 and TLR9 on B-1 and B-2 B cells from Donor 1. Lymphocytes were selected with a lymphocyte gate (R1) as in Fig. 1. (A) Staining of lymphocytes with CD19 antibody to show R2 region for selecting B lymphocytes. (B) Staining of B lymphocytes (gated R1 AND R2) with CD5 antibody to show R3 and R4 regions used to distinguish CD5- and $CD5^+$ B cells, respectively. (C)–(F) Staining of CD5-negative (B2) and CD5-positive (B1) B cells with isotype control antibodies (grey outline) or TLR 1 or TLR9 antibodies (black outline). The intensity of TLR staining is measured by mean fluorescent intensity (MFI). (C) and (D) Staining with TLR1 antibody. (E) and (F) Staining with TLR9 antibody.

by TLR activated APCs. TLR expression on T lymphocytic cell surfaces can influence T lymphocytes behaviour more directly and enable them to participate in a modified manner in the adaptive immune response if APCs are ineffective, as suggested by Gelman et al. [10]. TLRs have been found in T regulatory cells by rtPCR analysis and are believed to play a role in cell function by monitoring the inflammatory response [20].

While the PCR techniques used by other groups to demonstrate the expression of TLRs in T lymphocytes are very sensitive, it should be remembered that such findings are not definitive proof of TLR expression on the surface of such cells. Expression at the cell surface may be required for these receptors to play an active role in cellular activity or immune function. While this study did not detect TLR cell surface expression on resting T lymphocytes, this does not discount the possibility of TLRs being present on activated T lymphocytes. This would be consistent with the earlier findings of Komai-Koma et al. 6, who demonstrated elevated TLR2 and TLR4 expression associated with activated T lymphocytes compared to naïve T lymphocytes by RT-PCR and flow cytometry [6].

TLR expression was detected on the surface of B lymphocytes, particularly TLR1 and TLR9. While B lymphocytes play an active role in the adaptive immune system, they are also prominent in the innate immune response [16–18]. Consequently the high proportion of TLR^+ B lymphocytes is consistent with their dual roles. The greater proportion of TLR expression on resting B lymphocytes may indicate a bigger role for B lymphocytes in innate responses.

The importance of TLRs in the adaptive response has been suggested in light of observations showing reduced antibody production in humans and mice with reduced or deficient TLR1 and TLR2 expression [5]. TLR9 engages with its ligand, bacterial DNA in CpG motifs [21,22]. B cells stimulated with DNA CpG motifs show

an increased expression of co-stimulatory molecules, IL-6 synthesis, sustained induction of NF-kB activity and B cell proliferation which indicates the importance of this receptor in the innate immune system [23]. TLR9 has been found on endoplasmic reticulum and endocytic vesicles where they engage with CpG motifs [22,24]. These studies used cell lines for examining TLR9 expression and Leifer et al. found no cell surface expression of TLR9 [24]. Our study did not examine for intracellular expression of TLRs, but did reveal TLR9 expression on the majority of circulating peripheral B cell surfaces (Table 3 and Fig. 1), which is consistent with earlier reports [25].

Other TLRs investigated in this study were not found to be as highly expressed as TLR1 and TLR9 and this may be due to either functional differences, differences in expression pattern or a need for activation of B lymphocytes in order to induce the expression of other TLR types. Further studies investigating the presence and role of TLRs in activated B lymphocytes are needed.

It has been reported previously that B cells are able to respond to bacterial DNA [23]. We observed that TLR1 and TLR9 were both expressed at high levels on the majority of B cells. The expression of each was higher on the $CD5^+$ B-1 B cells than on $CD5^-$ B-2 B cells, which again is consistent with earlier suggestions that B-1 B cells exhibit a more primitive and less specific antibody response [16]. However, the high levels expression on B-2 B cells may indicate a stronger role for these cells in the innate immune response than previously believed, as well as their role in the adaptive response.

While the staining of B cells with TLR1 and TLR9 antibodies clearly demonstrated high levels of expression, an unexpectedly high level of background staining may have masked low-level expression of other TLR on B cells. The levels of individual TLR may also be affected by the different exposure of individuals to various immunological stimuli.

In conclusion, we have found that TLRs were not detectable on resting peripheral T lymphocytes. This study also confirms that TLRs are expressed on the surface of peripheral B lymphocytes. We found that these receptors were mainly TLR1 and TLR9, although other TLRs were found to be present at lower levels. The high expression of TLR1 and TLR9 on B1 cells may reflect their role in the innate immune response, as it provides a means by which the cells can recognise bacterial antigens.

References

[1] D.M. Underhill, Toll-like receptors and microbes take aim at each other, Curr. Opin. Immunol. 16 (2004) 483–487.
[2] S. Janssens, R. Beyaert, Role of toll-like receptors in pathogen recognition, Clin. Microbiol. Rev. 16 (2003) 637–646.
[3] M.T. Abreu, M. Arditi, Innate immunity and toll-like receptors: clinical implications of basic science research, J. Pediatr. 144 (2004) 421–429.
[4] A. Iwasaki, R. Medzhitov, Toll-like receptor control of the adaptive immune responses, Nat. Immunol. 5 (2004) 987–995.
[5] L. Alexopoulou, V. Thomas, M. Schnare, Y. Lobet, J. Anguita, R.T. Schoen, R. Medzhitov, E. Fikrig, R.A. Flavell, Hyporesponsiveness to vaccination with Borrelia burgdorferi OspA in humans and in TLR1- and TLR2-deficient mice, Nat. Med. 8 (2002) 878–884.
[6] M. Komai-Koma, L. Jones, G.S. Ogg, D. Xu, F.Y. Liew, TLR2 is expressed on activated T cells as a costimulatory receptor, Proc. Natl. Acad. Sci. USA 101 (2004) 3029–3034.
[7] E. Bourke, D. Bosisio, J. Golay, N. Polentarutti, A. Mantovani, The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells, Blood 102 (2003) 956–963.
[8] V. Hornung, S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, G. Hartmann, Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides, J. Immunol. 168 (2002) 4531–4537.
[9] A. Visintin, A. Mazzoni, J.H. Spitzer, D.H. Wyllie, S.K. Dower, D.M. Segal, Regulation of toll-like receptors in human monocytes and dendritic cells, J. Immunol. 166 (2001) 249–255.
[10] A.E. Gelman, J. Zhang, Y. Choi, L.A. Turka, Toll-like receptor ligands directly promote activated CD4+ T cell survival, J. Immunol. 172 (2004) 6065–6073.
[11] S. Akira, K. Takeda, T. Kaisho, Toll-like receptors: critical proteins linking innate and acquired immunity, Nat. Immunol. 2 (2001) 675–680.
[12] S. Akira, Mammalian toll-like receptors, Curr. Opin. Immunol. 15 (2003) 5–11.
[13] K. Hoebe, E. Janssen, B. Beutler, The interface between innate and adaptive immunity, Nat. Immunol. 5 (2004) 971–974.
[14] T. Kaisho, O. Takeuchi, T. Kawai, K. Hoshino, S. Akira, Endotoxin-induced maturation of MyD88-deficient dendritic cells, J. Immunol. 166 (2001) 5688–5694.
[15] A. Mazzoni, D.M. Segal, Controlling the toll road to dendritic cell polarization, J. Leukoc. Biol. 75 (2004) 721–730.
[16] R. Berland, H.H. Wortis, Origins and functions of B-1 cells with notes on the role of CD5, Annu. Rev. Immunol. 20 (2002) 253–300.
[17] F. Martin, J.F. Kearney, B1 cells: similarities and differences with other B cell subsets, Curr. Opin. Immunol. 13 (2001) 195–201.
[18] M.G. McHeyzer-Williams, B cells as effectors, Curr. Opin. Immunol. 15 (2003) 354–361.
[19] H. Zola, P.J. Macardle, L. Flego, J. Webster, The expression of subpopulation markers on B cells: a re-evaluation using high-sensitivity fluorescence flow cytometry, Dis. Markers 9 (1991) 103–118.
[20] I. Caramalho, T. Lopes-Carvalho, D. Ostler, S. Zelenay, M. Haury, J. Demengeot, Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide, J. Exp. Med. 197 (2003) 403–411.
[21] S. Bauer, C.J. Kirschning, H. Hacker, V. Redecke, S. Hausmann, S. Akira, H. Wagner, G.B. Lipford, Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition, Proc. Natl. Acad. Sci. USA 98 (2001) 9237–9242.
[22] F. Takeshita, C.A. Leifer, I. Gursel, K.J. Ishii, S. Takeshita, M. Gursel, D.M. Klinman, Cutting edge: role of toll-like receptor 9 in CpG DNA-induced activation of human cells, J. Immunol. 167 (2001) 3555–3558.
[23] G. Hartmann, A.M. Krieg, Mechanism and function of a newly identified CpG DNA motif in human primary B cells, J. Immunol. 164 (2000) 944–953.
[24] C.A. Leifer, M.N. Kennedy, A. Mazzoni, C. Lee, M.J. Kruhlak, D.M. Segal, TLR9 is localized in the endoplasmic reticulum prior to stimulation, J. Immunol. 173 (2004) 1179–1183.

[25] A. Eaton-Bassiri, S.B. Dillon, M. Cunningham, M.A. Rycyzyn, J. Mills, R.T. Sarisky, M.L. Mbow, Toll-like receptor 9 can be expressed at the cell surface of distinct populations of tonsils and human peripheral blood mononuclear cells, Infect. Immunol. 72 (2004) 7202–7211.

[26] F. Hayashi, T.K. Means, A.D. Luster, Toll-like receptors stimulate human neutrophil function, Blood 102 (2003) 2660–2669.

[27] H. Nagase, S. Okugawa, Y. Ota, M. Yamaguchi, H. Tomizawa, K. Matsushima, K. Ohta, K. Yamamoto, K. Hirai, Expression and function of toll-like receptors in eosinophils: activation by toll-like receptor 7 ligand, J. Immunol. 171 (2003) 3977–3982.

[28] T.H. Flo, O. Halaas, S. Torp, L. Ryan, E. Lien, B. Dybdahl, A. Sundan, T. Espevik, Differential expression of toll-like receptor 2 in human cells, J. Leukoc. Biol. 69 (2001) 474–481.

[29] I. Sabroe, E.C. Jones, L.R. Usher, M.K. Whyte, S.K. Dower, Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: a critical role for monocytes in leukocyte lipopolysaccharide responses, J. Immunol. 168 (2002) 4701–4710.

[30] K.U. Saikh, T.L. Kissner, A. Sultana, G. Ruthel, R.G. Ulrich, Human monocytes infected with Yersinia pestis express cell surface TLR9 and differentiate into dendritic cells, J. Immunol. 173 (2004) 7426–7434.

[31] K.U. Saikh, J.S. Lee, T.L. Kissner, B. Dyas, R.G. Ulrich, Toll-like receptor and cytokine expression patterns of CD56+ T cells are similar to natural killer cells in response to infection with Venezuelan equine encephalitis virus replicons, J. Infect. Dis. 188 (2003) 1562–1570.

[32] D. Bosisio, N. Polentarutti, M. Sironi, S. Bernasconi, K. Miyake, G.R. Webb, M.U. Martin, A. Mantovani, M. Muzio, Stimulation of toll-like receptor 4 expression in human mononuclear phagocytes by interferon-gamma: a molecular basis for priming and synergism with bacterial lipopolysaccharide, Blood 99 (2002) 3427–3431.

[33] M. Hausmann, S. Kiessling, S. Mestermann, G. Webb, T. Spottl, T. Andus, J. Scholmerich, H. Herfarth, K. Ray, W. Falk, G. Rogler, Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation, Gastroenterology 122 (2002) 1987–2000.

JOURNAL OF BIOLOGICAL REGULATORS & HOMEOSTATIC AGENTS Vol. 22, no. 1, 17-26 (2008)

EDITORIAL

TOLL-LIKE RECEPTORS

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Received January 30, 2006 – Accepted March 2, 2007

Toll-like receptors are a family of transmembrane receptors responsible for recognition and initiation of a response to invading microbes by the immune system. As part of the innate immune system, toll-like receptors recognise pathogen-associated molecular patterns, highly conserved components that are essential to microbial function. Some of ten toll-like receptors identified in humans are able to recognise several pathogen-associated molecular patterns.

Toll-like receptors (TLR) are a family of transmembrane receptors responsible for recognition and the initiation of a response to invading microbes by the immune system (1). As part of the innate immune system, TLR recognise pathogen-associated molecular patterns (PAMP), highly conserved components that are essential to microbial function (2, 3). Ten TLR have been identified in humans with some TLR able to recognise several PAMPs (4-7).

Structure

TLR are Type I integral membrane glycoproteins with molecular weights ranging 90-115kDa (8). The protein chains range from 780-1,000 amino acids (5, 9) and they share a conserved cytoplasmic domain, known as Toll/IL-1R (TIR) domain, with the interleukin-1 receptor family (IL-1R) (3). The extracellular domains of TLR and IL-1R differ in that TLR have several leucine-rich repeat (LRR) motifs, while IL-1R have three immunoglobulin domains (3). TLR can be located either on cell surfaces or on endosomal surfaces (8) (See Fig. 1 and Table I).

The ectodomain of TLR contain 19-25 copies of LRR capped with a 31-amino acid long N-flanking region and a cysteine-rich domain on the C-terminal end (3, 8). An individual LRR is made of approximately 24 residues and forms a loop, with the first 10 residues forming a β-hairpin (8). The LRR motifs all form a coil with a large β-sheet on the concave surface formed by β-strands of each motif (8). Other, similar, receptors bind to their ligands on the concave β-sheet of the LRR motifs with new binding sites created by inserting or deleting residues in the LRR motifs, suggesting that similar binding sites for ligands may occur in TLR (8).

The TIR domain has a sequence conservation of approximately 20-30% with most of the conserved residues located in the hydrophobic core of the structure, the core approximately 130-165 residues in length (10). The TIR domain has five β-strands, parallel, surrounded by five α-helices, with secondary structures all connected by loops (10). Three regions in the conserved sequence identified as boxes 1, 2 and 3 appear to have a role in signalling as deletions in each box eliminated signalling (11). Mutations in boxes 1 and 2 affected signalling and mutations

Key words: CD281 - TLR1, CD282 - TLR2, CD283 - TLR3, CD284 - TLR4, CD289 - TLR9, CD290 - TLR10

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0393-974X (2008)

in box 3 reduced cell surface expression (11). The three regions in the TIR domain all play essential roles in receptor localisation and signalling (11).

Cell and tissue distribution

TLR are expressed by a wide range of cells and tissues, including endothelia and epithelia (1), as well as being present on immune cells (see Table II). TLR can be expressed either on the cell surface, such as TLR 1, 2, 4, 6, 9 and 10 (12-14), or in the case of TLR3, on cell endosomes (15). Both the functions of the cells and the TLR type affect location of TLR expression, for instance, TLR with viral ligands are more likely to be expressed in dendritic cell (DC) endosomes. Cells also vary TLR expression according to whether they are resting or activated. As observed by Komai-Koma, activated T cells upregulate surface expression of TLR2 and TLR4 (16). Activation of cells which alters TLR expression can occur by several mechanisms including direct activation (16), microbial infections (17-18) and cytokines (18). TLR expression can be differentially regulated from resting cells to activated cells through inflammation or disease.

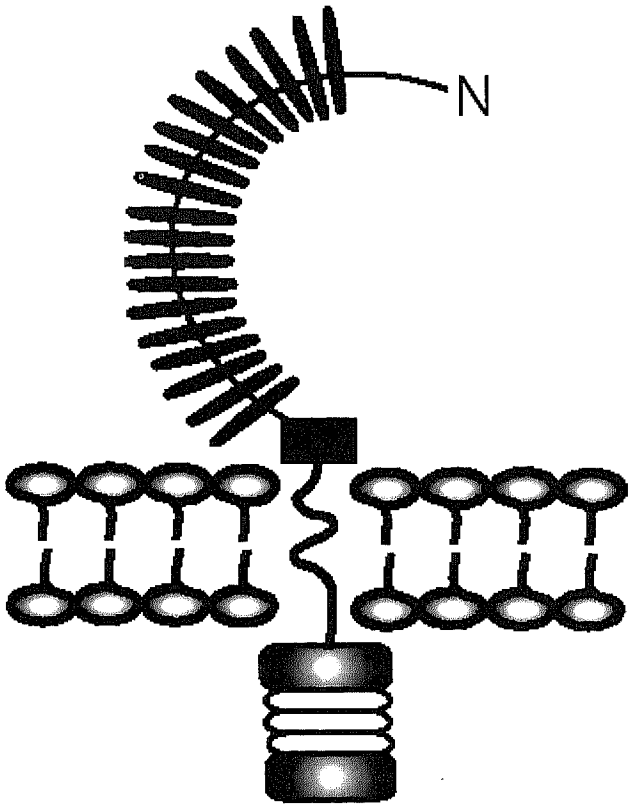

Fig. 1. *Schematic structure of Toll-like Receptor 1. Other TLR have similar structure, except for differences in numbers of LRR motifs.*

Signalling

TLR engagement initiates signalling cascades eventuating in activation of nuclear factor-κB (NF-κB) to express target genes encoding inflammatory cytokines, costimulatory molecules and interferon (IFN)-inducible products (19). A family of adaptor proteins, each containing a TIR domain, directs TLR signals to different signalling cascades resulting in distinct outcomes. The adaptor proteins, myeloid differentiation primary-response protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP), TIR-domain-containing adaptor protein inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM), interact with TLR to trigger signalling pathways (19).

Engaged TLR 1, 2, 4, 5, 6, 7 or 9 can activate NF-κB via MyD88-dependent pathway by dimerising TIR domains with MyD88 in the cytoplasm to initiate a signalling cascade as described by Akira (19). This cascade culminates in early-phase activation of NF-κB which transcribes expression of inflammatory cytokines (19-20). TIRAP acts upstream of MyD88 in the pathway and is essential for TLR 2 and 4 to induce cytokine production (21).

Late-phase activation of NF-κB to induce expression of co-stimulatory molecules and IFN-associated products is triggered by MyD88-independent signalling pathway (19). This pathway induces DC maturation via TLR4 (22) and also triggers activation of interferon-regulatory factor (IRF3) to directly induce expression of type 1 IFN genes (19). The MyD88-independent pathway has not yet been elucidated, but TLR 3 and 4 require TRIF for signalling through the MyD88-independent pathway (22-23).

LPS-activated TLR4 utilises both MyD88-dependent and MyD88-independent pathways for inflammatory cytokine production and TRAM is required by TLR4 for the MyD88-independent pathway (24). TLR initiate several signalling

cascades for NF-κB and IRF3 activation, but further work is required to elucidate the steps in these cascades completely.

Function

TLR have a profound impact on both innate and adaptive immune responses by either directly activating immune cells or indirectly influencing them through cytokine signals from TLR-engaged cells. TLR can interact with ligands individually or increase their range of ligands through dimerisation with other TLR or cell receptors (see Table III). This section gives a brief overview of the impact each TLR has on immune cells.

TLR1. Heterodimers of TLR1 and TLR2 recognise lipopeptides or lipopolysaccharide (LPS) and activate cells to secrete proinflammatory cytokines (25-26). The importance of TLR1 as an accessory molecule is observed in TLR2-activated macrophages with enhanced responses in the presence of TLR1 against mycobacterial components (26).

TLR2. Heterodimers of TLR2 with either TLR1 or TLR6, can recognise a wide repertoire of ligands from bacterial and viral products to endogenous ligands (16, 25, 27).

TLR2-activated neutrophils display several anti-microbial functions including phagocytosis and recruitment of immune cells through increased proinflammatory cytokine and chemokine expression (12). TLR2 ligands also activate B lymphocytes to proliferate and secrete IgG or IgM antibodies (28) and T lymphocytes to proliferate and secrete cytokines which augment the adaptive response (16).

TLR3. Engagement of TLR3 activates macrophages to secrete IFN (29) and stimulates mast cells to activate lymphocytes through chemokines and co-stimulatory molecule signals (30, 31). TLR3 can activate T lymphocytes directly (32) or activate and influence CD8+ T cell function indirectly through TLR3-stimulated mast cells and DCs (15, 30).

TLR4. As discussed above, LPS-activated TLR4, with co-receptor CD14 (27), initiates several signalling cascades, thereby amplifying the signals leading to cellular responses (24).

The stimulation of neutrophils, the primary innate immune leucocytes, by LPS via TLR4 increases antimicrobial activities through increased phagocytosis and superoxide production, decreased chemotaxis and increased chemokine and cytokine expression (12, 33). TLR4 engagement can activate T lymphocytes directly (32) or indirectly through cytokine secretions of TLR4-stimulated DCs (34). TLR4 stimulation of B lymphocytes causes maturation and proliferation in the adaptive response (35).

Table I. *TLR differ in structure through variable numbers of LRR motifs.*

TLR	Numbers of LRR
1	19
2	19
3	23
4	21
5	20
6	19
7	25
8	25
9	25
10	19

TLR5. TLR5-activated neutrophils display increased phagocytosis, superoxide production and immune cell recruitment through increased expression of proinflammatory cytokines and chemokines (12).

TLR6. TLR6 can form heterodimers with TLR1 or TLR2 to increase specificity of ligand recognition and enhance cellular activation with subsequent cytokine induction (13).

TLR7. As has been noted with the other TLR, TLR7 engagement is effective for activating antimicrobial functions in neutrophils (12). TLR7-

Table II. *Expression of TLR on human immune cells (references in brackets).*

Cell Type	Polymerase Chain Reaction	Flow Cytometry	Fluorescent Microscopy
Peripheral Blood			
• Neutrophils	1, 2, 3, 4, 5, 6, 7, 8, 9, 10 (12, 36)	1, 2, 4, 6, 9 (12, 13, 18, 33)	NR*
• Monocytes	1, 2, 4, 5, 6, 7, 8, 9 (42, 56, 57)	1, 2, 4, 6, 9 (18, 33, 56), (13, 17, 57)	9 (17)
• Eosinophils	1, 4, 6, 7, 9, 10 (33, 36)	NR	NR
• Basophils	2, 4 (33)	2, 4 (33)	NR
• T cells	1, 2, 3, 4, 5, 6, 8, 9 (16, 42, 58)	2, 4 (16)	2, 4 (16)
• B cells	1, 2, 4, 6, 7, 9, 10 (14, 42)	1, 9 (17, 59)	NR
• Natural Killer cells	1, 2, 3, 4, 5, 6, 8, 9 (42, 58)	NR	NR
Tonsillar B lymphocytes	1, 6, 7, 8, 9, 10 (41)	9 (60)	2 (18)
Macrophages	NR	4 (57, 61)	2, 4 (18, 61)
Dendritic Cells			
• Immature DCs	1, 2, 3, 4, 6 (56)	1 (56)	NR
• Mature DCs	1, 6 (56)	9 (60)	NR
• Plasmacytoid DCs	1, 6, 7, 9, 10 (14, 42)	9 (60)	NR

**NR – Not reported*

Table III. *Ligands of TLR.*

TLR	Ligands
1	Lipoprotein (*Mycobacterium sp.*) (26) LPS (25) Soluble factors (*Neisseria meningitidis*) (25)
2	Lipopeptides/lipoproteins (including Mycoplasma and *Mycobacterium tuberculosis*) (13, 16, 51) Glycolipids (13, 16) LPS (18, 25) Soluble factors (*Neisseria meningitidis*) (25) Zymosan (16) Peptidoglycan (13, 16) Porin (27) Bacterial fimbrae (27) Haemagglutinin protein (27) Cytomegalovirus virions (27) Human Heat shock protein (HSP) 60 and HSP70 (27, 62)
3	dsRNA (31)
4	LPS (6, 12) Respiratory syncytial virus (53) Chlamydial HSP60 (27) Mycobacterial HSP65 (27) Fibrinogen (27, 53)
	Human HSP60 and HSP70 (27, 62) Human fibronectin (27) Human hyaluronic acid, etc (27)
5	Bacterial flagellin (52)
6	Lipopeptide/lipoprotein (Mycoplasma) (13) Peptidoglycan (13) Zymosan (12)
7	ssRNA (27)
8	ssRNA (27)
9	Unmethylated CpG DNA motifs (9)
10	Not known

engaged eosinophils have prolonged survival and induce superoxide production (36). TLR7 ligands activates T lymphocytes directly (32) and induce IFN-α and IFN-regulated cytokines in PBMCs (34).

TLR8. TLR8 ligands are effective for inducing proinflammatory cytokine expression from peripheral blood mononuclear cells (PBMCs) and differential cytokine signals from plasmacytoid DCs and myeloid DCs (34). A recent study has revealed

Table IV. *Mouse and rat anti-human TLR monoclonal antibodies.*

TLR	Format	Source
1	P, B, PE	eBiosciences, BD Biosciences, Imgenex, RnD Systems
2	P, B, F, PE, Pe-Cy7, APC, AF405, AF488, AF647	eBiosciences, BD Biosciences, Imgenex, RnD Systems
3	P, B, F, PE	eBiosciences, BD Biosciences, Imgenex
4	P, B, F, PE, Pe-Cy5, Pe-Cy7, APC, AF405, AF488, AF647	eBiosciences, BD Biosciences, Imgenex
5	P, F, PE	Imgenex
6	P*, B*, F	eBiosciences, Imgenex
7	-	
8	P, B, F, PE	Imgenex
9	P*, B, F, PE*	eBiosciences, Imgenex
10	P	Imgenex

P – Purified; B – Biotinylated; F – Fluorescein; PE – Phycoerythrin; Pe-Cy5; Pe-Cy7; APC – Allophycocyanin; AF – Alexa Fluor
**Available as rat antibodies from eBiosciences*

a direct, DC-independent, role for TLR8 ligand suppressing T regulatory cells and reversing their effects (37).

TLR9. CpG DNA is a strong immune adjuvant (38) which stimulates B lymphocytes directly through TLR9, inducing polyclonal activation and proliferation, low affinity antibody production, co-stimulatory molecular expression and cytokine secretion (39-41). Independently of T lymphocytes, TLR9-engaged memory B cells can secrete antibodies allowing innate immunity to induce an adaptive response (39). Macrophages and DCs activated by TLR9 ligands elevate co-stimulatory molecule expression and secrete cytokines for differential immune responses (9, 34). TLR9 engagement can activate T lymphocytes directly (32) or indirectly through cytokine signals from TLR9-engaged DCs (32). TLR9 ligands activate natural killer (NK) lymphocytes to secrete cytokines and clear pathogens (39, 42) and neutrophils to induce antimicrobial functions (12).

TLR10. TLR10 forms heterodimers with TLR1 or TLR2 (14), but its ligand and function are still unknown.

General Functions

DC maturation by TLR engagement results in up-regulation of co-stimulatory molecules,

Table V. *Genebank accession numbers.*

		NCBI Reference Sequence	
TLR	**Species**	**Protein**	**mRNA**
1	Mouse	NP_109607	NM_030683
	Human	NP_003254	NM_003263
2	Mouse	NP_036035	NM_011905
	Human	NP_003255	NM_003264
3	Mouse	NP_569054	NM_126166
	Human	NP_003256	NM_003265
4	Mouse	NP_067272	NM_021297
	Human	NP_612564	NM_138554
5	Mouse	XP_622092	XM_622092
	Human	NP_003259	NM_003268
6	Mouse	NP_035734	NM_011604
	Human	NP_006059	NM_006068
7	Mouse	NP_573474	NM_133211
	Human	NP_057646	NM_016562
8	Mouse	NP_573475	NM_133212
	Human	NP_057694	NM_016610
9	Mouse	NP_112455	NM_031178
	Human	NP_059138	NM_017442
10	Rat	XP_223422	XM_223422
	Human	NP_112218	NM_030956

altered expression of chemokines and secretion of proinflammatory cytokines allowing them to increase their antigen presenting ability and migrate to lymph nodes (1, 39). Different TLR, when engaged, trigger different pathways with different cellular outcomes. Differential cytokine release and co-stimulatory molecule expression by subsets of DCs shapes T cell differentiation, therefore influencing adaptive response (1, 39). DCs can also be stimulated by agonists for TLR3 and TLR4 acting synergistically with each other or with TLR8, with synergy enhanced further by IFN-γ or CD40L. The cytokine signals expressed in this instance activate T cells to secrete IFN-γ (43). A recent study has found TLR-activated DCs secrete IL-6 to suppress regulatory T cells from interfering with activated CD4+ T cells (44).

Disease

Autoimmunity. The influence which TLR exert over the adaptive response and their ability to recognise endogenous ligands has implications for development of systemic autoimmune diseases. TLR9 and B cell receptor on autoreactive B cells engaged with murine chromatin from apoptotic cells, triggering activation and proliferation (45). TLR9 is also implicated in breaking T cell self tolerance via TLR9-engaged antigen-presenting cells and inducing murine experimental autoimmune encephalomyelitis, the animal model of multiple sclerosis (46). Inflammatory bowel disease, a localised autoimmune condition, has been linked to Asp299Gly TLR4 polymorphism which has an impaired signalling pathway for LPS (47, 48). In summary, TLR dysregulation or impaired signalling pathways can lead to systemic or localised autoimmune conditions.

Infectious Diseases. Since TLR launch an immune response against invading pathogens, TLR dysfunction may be related to pathology of infectious diseases. With focus on polymorphisms, the Asp299Gly TLR4 polymorphism, known to have lowered response to LPS (47), has been associated with patient cohorts suffering Gram-negative septic shock (49) and systemic inflammatory response syndrome (50) indicating predisposition of this population carrying this polymorphism to these illnesses. Other polymorphisms in TLR2 and TLR5 may be associated with increased susceptibility to tuberculosis (51) and Legionnaire's Disease (52). TLR activity can aggravate effects of microbes further as seen in infants with the Asp299Gly TLR4 polymorphism suffering severe respiratory syncytial viral infections (53). The effects of excessive presence of inflammatory cytokines from TLR activation can be damaging as is demonstrated by patients developing septic shock (54) or in murine models where TLR2 activity induced streptococcal arthritis in joints (55). New therapies may need to target TLR or their signalling pathways to reduce pathology of certain diseases.

CONCLUSIONS

In conclusion, interest in TLR has increased considerably in recent years as their significance in the innate and adaptive immune systems has been revealed. The discovery of TLR has renewed interest in the innate immune system by showing, through cell activation and inflammatory responses, the importance of the early innate immune response against invading pathogens. The influence the innate system has on adaptive immunity is illustrated by how TLR tailor the adaptive response through cytokine signals, directly activating lymphocytes and indirectly influencing T cell differentiation. These known functions have linked the two branches of immunity, innate and adaptive, previously considered to have separate, isolated roles from each other. TLR have extensive effects in immunity and the full extent of TLR activities on the immune system requires further research.

Available Reagents: See Table IV
Genebank Accession Numbers: See Table V

REFERENCES

1. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2001; 2:675-80.
2. Medzhitov R, Janeway CA, Jr. Innate immunity: the virtues of a non-clonal system of recognition. Cell 1997; 91:295-8.
3. Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature

1997; 388:394-7.
4. Rock FL, Hardiman G, Timans JC et al. A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci USA 1998; 95:588-93.
5. Takeuchi O, Kawai T, Sanjo H et al. TLR6: A novel member of an expanding toll-like receptor family. Gene 1999; 231:59-65.
6. Du X, Poltorak A, Wei Y, Beutler B. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. Eur Cytokine Netw 2000; 11:362-71.
7. Chuang T, Ulevitch RJ. Identification of hTLR10: a novel human Toll-like receptor preferentially expressed in immune cells. Biochim Biophys Acta 2001; 1518:157-61.
8. Bell JK, Mullen GE, Leifer CA et al. Leucine-rich repeats and pathogen recognition in Toll-like receptors. Trends Immunol 2003; 24:528-33.
9. Hemmi H, Takeuchi O, Kawai T et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000; 408:740-5.
10. Xu Y, Tao X, Shen B et al. Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. Nature 2000; 408:111-5.
11. Slack JL, Schooley K, Bonnert TP et al. Identification of two major sites in the type I interleukin-1 receptor cytoplasmic region responsible for coupling to pro-inflammatory signaling pathways. J Biol Chem 2000; 275:4670-8.
12. Hayashi F, Means TK, Luster AD. Toll-like receptors stimulate human neutrophil function. Blood 2003; 102:2660-9.
13. Nakao Y, Funami K, Kikkawa S et al. Surface-expressed TLR6 participates in the recognition of diacylated lipopeptide and peptidoglycan in human cells. J Immunol 2005; 174:1566-73.
14. Hasan U, Chaffois C, Gaillard C et al. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. J Immunol 2005; 174:2942-50.
15. Schulz O, Diebold SS, Chen M et al. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature 2005; 433:887-92.
16. Komai-Koma M, Jones L, Ogg GS et al. TLR2 is expressed on activated T cells as a costimulatory receptor. Proc Natl Acad Sci USA 2004; 101:3029-34.
17. Saikh KU, Kissner TL, Sultana A et al. Human monocytes infected with Yersinia pestis express cell surface TLR9 and differentiate into dendritic cells. J Immunol 2004; 173:7426-34.
18. Flo TH, Halaas O, Torp S et al. Differential expression of Toll-like receptor 2 in human cells. J Leukoc Biol 2001; 69:474-81.
19. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004; 4:499-511.
20. Chen F, Sun SC, Kuh DC et al. Essential role of NF-kappa B activation in silica-induced inflammatory mediator production in macrophages. Biochem Biophys Res Commun 1995; 214:985-92.
21. Yamamoto M, Sato S, Hemmi H et al. Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. Nature 2002; 420:324-9.
22. Horng T, Barton GM, Medzhitov R. TIRAP: an adapter molecule in the Toll signaling pathway. Nat Immunol 2001; 2:835-41.
23. Yamamoto M, Sato S, Hemmi H et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 2003; 301:640-3.
24. Fitzgerald KA, Rowe DC, Barnes BJ et al. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J Exp Med 2003; 198:1043-55.
25. Wyllie DH, Kiss-Toth E, Visintin A et al. Evidence for an accessory protein function for Toll-like receptor 1 in anti-bacterial responses. J Immunol 2000; 165:7125-32.
26. Takeuchi O, Sato S, Horiuchi T et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol 2002; 169:10-4.
27. Abreu MT, Arditi M. Innate immunity and toll-like receptors: clinical implications of basic science research. J Pediatr 2004; 144:421-9.
28. Borsutzky S, Kretschmer K, Becker PD et al. The mucosal adjuvant macrophage-activating lipopeptide-2 directly stimulates B lymphocytes via the TLR2 without the need of accessory cells. J Immunol 2005; 174:6308-13.
29. Doyle SE, O'Connell R, Vaidya SA et al. Toll-like receptor 3 mediates a more potent antiviral response

than Toll-like receptor 4. J Immunol 2003; 170:3565-71.
30. Orinska Z, Bulanova E, Budagian V et al. TLR3-induced activation of mast cells modulates CD8+ T-cell recruitment. Blood 2005; 106:978-87.
31. Kulka M, Alexopoulou L, Flavell RA, Metcalfe DD. Activation of mast cells by double-stranded RNA: evidence for activation through Toll-like receptor 3. J Allergy Clin Immunol 2004; 114:174-82.
32. Kamath AT, Sheasby CE, Tough DF. Dendritic cells and NK cells stimulate bystander T cell activation in response to TLR agonists through secretion of IFN-alpha beta and IFN-gamma. J Immunol 2005; 174: 767-76.
33. Sabroe I, Jones EC, Usher LR et al. Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: a critical role for monocytes in leukocyte lipopolysaccharide responses. J Immunol 2002; 168:4701-10.
34. Gorden KB, Gorski KS, Gibson SJ et al. Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8. J Immunol 2005; 174:1259-68.
35. Hayashi EA, Akira S, Nobrega A. Role of TLR in B cell development: signaling through TLR4 promotes B cell maturation and is inhibited by TLR2. J Immunol 2005; 174:6639-47.
36. Nagase H, Okugawa S, Ota Y et al. Expression and function of Toll-like receptors in eosinophils: activation by Toll-like receptor 7 ligand. J Immunol 2003; 171:3977-82.
37. Peng G, Guo Z, Kiniwa Y et al. Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. Science 2005; 309:1380-4.
38. Krieg AM, Yi AK, Matson S et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995; 374:546-9.
39. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol 2004; 5:987-95.
40. Hartmann G, Krieg AM. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. J Immunol 2000; 164:944-53.
41. Bourke E, Bosisio D, Golay J et al. The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. Blood 2003; 102:956-63.
42. Hornung V, Rothenfusser S, Britsch S et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol 2002; 168:4531-7.
43. Napolitani G, Rinaldi A, Bertoni F et al. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. Nat Immunol 2005; 6:769-76.
44. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science 2003; 299: 1033-6.
45. Viglianti GA, Lau CM, Hanley TM et al. Activation of autoreactive B cells by CpG dsDNA. Immunity 2003; 19:837-47.
46. Waldner H, Collins M, Kuchroo VK. Activation of antigen-presenting cells by microbial products breaks self tolerance and induces autoimmune disease. J Clin Invest 2004; 113:990-7.
47. Arbour NC, Lorenz E, Schutte BC et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet 2000; 25: 187-91.
48. Franchimont D, Vermeire S, El Housni H et al. Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. Gut 2004; 53:987-92.
49. Lorenz E, Mira JP, Frees KL, Schwartz DA. Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. Arch Intern Med 2002; 162:1028-32.
50. Child NJ, Yang IA, Pulletz MC et al. Polymorphisms in Toll-like receptor 4 and the systemic inflammatory response syndrome. Biochem Soc Trans 2003; 31: 652-3.
51. Ogus AC, Yoldas B, Ozdemir T et al. The Arg753GLn polymorphism of the human toll-like receptor 2 gene in tuberculosis disease. Eur Respir J 2004; 23:219-23.
52. Hawn TR, Verbon A, Lettinga KD et al. A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with

susceptibility to legionnaires' disease. J Exp Med 2003; 198:1563-72.
53. Tal G, Mandelberg A, Dalal I et al. Association between common Toll-like receptor 4 mutations and severe respiratory syncytial virus disease. J Infect Dis 2004; 189:2057-63.
54. Brown MA, Jones WK. NF-kappaB action in sepsis: the innate immune system and the heart. Front Biosci 2004; 9:1201-17.
55. Joosten LA, Koenders MI, Smeets RL et al. Toll-like receptor 2 pathway drives streptococcal cell wall-induced joint inflammation: critical role of myeloid differentiation factor 88. J Immunol 2003; 171:6145-53.
56. Visintin A, Mazzoni A, Spitzer JH et al. Regulation of Toll-like receptors in human monocytes and dendritic cells. J Immunol 2001; 166:249-55.
57. Bosisio D, Polentarutti N, Sironi M et al. Stimulation of toll-like receptor 4 expression in human mononuclear phagocytes by interferon-gamma: a molecular basis for priming and synergism with bacterial lipopolysaccharide. Blood 2002; 99:3427-31.
58. Saikh KU, Lee JS, Kissner TL et al. Toll-like receptor and cytokine expression patterns of CD56+ T cells are similar to natural killer cells in response to infection with Venezuelan equine encephalitis virus replicons. J Infect Dis 2003; 188:1562-70.
59. Dasari P, Nicholson IC, Hodge G et al. Expression of toll-like receptors on B lymphocytes. Cell Immunol 2005; 236:140-5.
60. Eaton-Bassiri A, Dillon SB, Cunningham M et al. Toll-like receptor 9 can be expressed at the cell surface of distinct populations of tonsils and human peripheral blood mononuclear cells. Infect Immun 2004; 72:7202-11.
61. Hausmann M, Kiessling S, Mestermann S et al. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. Gastroenterology 2002; 122:1987-2000.
62. Zanin-Zhorov A, Nussbaum G, Franitza S et al. T cells respond to heat shock protein 60 via TLR2: activation of adhesion and inhibition of chemokine receptors. Faseb J 2003; 17:1567-9.

Pediatr Allergy Immunol 2010
DOI: 10.1111/j.1399-3038.2010.01091.x

PEDIATRIC ALLERGY AND IMMUNOLOGY

Expression of Toll-like receptors by neonatal leukocytes

Dasari P, Zola H, Nicholson IC. Expression of Toll-like receptors by neonatal leukocytes.
Pediatr Allergy Immunol 2010.

The immune system of neonates is poorly developed; this increases the susceptibility of neonates to infection. For neonates to counter infection effectively, they first need to recognize the presence of pathogens. Toll-like receptors (TLR) are a family of pattern recognition receptors that alert the host to the presence of invading pathogens. To determine whether differences in TLR expression by leukocytes compensate for immunologic immaturity in neonates, TLR expression by monocytes and T lymphocytes from adults and neonates was compared. Expression of TLR1, TLR2, TLR3, TLR4, TLR8 and TLR9 by monocytes and T lymphocytes was detected with antibodies by flow cytometry. TLR1, TLR2, TLR3, TLR4, TLR8 and TLR9 expression by monocytes was detected in adults and neonates. TLR2, TLR3, TLR4, TLR8 and TLR9 expression by T lymphocytes was detected in adults and neonates. Monocytes and T lymphocytes from neonates are capable, like adults, of recognizing the presence of pathogens through TLR.

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Key words: Toll-like receptors; neonates; leukocytes; monocytes; T lymphocytes

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Accepted 14 June 2010

Neonates have an immature immune system, with several components of the innate (1) and adaptive immunity (2, 3) having impaired functions. This weakness of the neonatal immune response may explain the susceptibility of neonates to infection: approximately one-third of neonatal deaths, globally, were because of infectious diseases (4). The deficient cellular and humoral components of innate and adaptive immunity in neonates may account for its poor function. Neonatal leukocytes function poorly; monocytes and neutrophils have reduced inflammatory responses (1), and lymphocytes and dendritic cells have poor function in cell-mediated immunity (3).

Monocytes are precursor cells for macrophages and dendritic cells, which differentiate and participate in inflammatory and cell-mediated responses. Neonatal monocytes have several functions impaired, which include an inability to secrete cytokines or differentiate into macrophages, reviewed by Velilla (2). The poor functions of neonatal monocytes adversely affect the early immune responses to infection. The cell-mediated adaptive immune response develops pathogen-specific long-term memory, but neonates lack this ability as seen in the poor responses to vaccination during early infancy (3). The impairment of cognate interactions between dendritic cells and T lymphocytes leads to poor activation of T lymphocytes which, in turn, affects T cell-mediated activation of other leukocytes. The polarization of neonatal T lymphocytes to type 2 T helper (T_H) cells leads to reduced type 1 T_H responses. The reduced function of neonatal T lymphocytes adversely affects the adaptive immune response.

Toll-like receptors (TLR) recognize microbial pathogen-associated molecular patterns and alert the host's immune system to the presence of

Abbreviations: FITC, Fluorescein isothiocyanate; IL, Interleukin; MFI, Median fluorescence intensity; mRNA, messenger ribonucleic acid; PE, Phycoerythrin; PerCpCy5.5, Peridinin chlorophyll protein cyanine 5.5; RT-PCR, Reverse transcriptase polymerase chain reaction; T_H cell, T helper lymphocyte; TLR, Toll-like receptor; TNF, Tumor necrosis factor.

invading microbes (5). The molecules containing the pathogen-associated molecular patterns are critical to the function of microbes; hence, the innate immune system can use their presence to determine that microbes are present (6). In humans, ten TLR, TLR1 to TLR10, have been identified (7–10). Toll-like receptors can be located on the cell surface or internally and are expressed by various cells and tissues in the body including leukocytes (5, 11).

Toll-like receptors instigate a diverse range of immune responses against pathogens. For the inflammatory response, TLR engagement activates monocytes, inducing secretion of pro-inflammatory cytokines and upregulation of chemokine receptors (12). For the adaptive immune response, TLR can have an influence on T lymphocyte responses. T lymphocytes activated by TLR ligands can proliferate (13), increase cytotoxic activity (14) and, in response to different ligands, secrete different patterns of cytokines (13, 14), which can tailor subsequent immune responses to type 1 T_H responses (15). Toll-like receptors can regulate the immune system by suppressing regulatory T lymphocytes (16).

As neonates have impaired immunity, we hypothesized that they may be more reliant on TLR for protection against infection. Little is known about TLR expression by neonatal leukocytes with much of the literature focused on adult leukocytes. For TLR expression by adult blood monocytes, RT-PCR studies on TLR mRNA showed all TLR, except TLR3 and TLR10, were detected (17, 18), and using antibodies, TLR1, TLR2, TLR3, TLR4, TLR6, TLR8 and TLR9 were detected (17, 19–21). The antibody studies showed surface expression of TLR1, TLR2, TLR4 and TLR6, intracellular expression of TLR3 and TLR8, and TLR9 detected at both locations. For neonatal monocytes, using flow cytometry, TLR2, TLR4 (12, 22, 23) and TLR8 (24) were detected.

RT-PCR studies on adult blood T lymphocytes showed all TLR, except for TLR7 and TLR10 (18, 25, 26). Using antibodies, all TLR, except TLR7, (26–29) were detected on adult blood T lymphocytes. These studies showed most detected TLR were expressed on the cell surface, except for TLR3, TLR5 and TLR9, which were detected internally. One study showed internal expression of TLR2 and TLR4 by cord blood T cells (13).

Our hypothesis is that TLR will have a greater presence in the neonatal immune system compared to the adult immune system. As there is very little known about expression of TLR by neonatal leukocytes, the aim of this study is to compare the range of TLR expression by monocytes and T lymphocytes from adults and neonates. TLR expression was examined on monocytes and T lymphocytes from adult blood and cord blood. Toll-like receptors' expression by non-stimulated and stimulated T lymphocytes was assessed as TLR expression varies according to the activation state of T lymphocytes (13, 29). The extracellular and intracellular TLR expression was measured on non-permeabilized and permeabilized cells.

Methods

Subjects

Umbilical cord blood was collected from three healthy patients undergoing vaginal delivery of healthy term neonates at the Women's and Children's Hospital, Adelaide, Australia. From three healthy adult volunteers, the Australian Red Cross provided leukocyte-rich buffy coats from blood. All blood samples were processed within 18 h of collection. This study received ethics approval from the Children Youth and Women's Health Service Research Ethics Committee.

Monoclonal antibodies and reagents

The following monoclonal antibodies were used: mouse anti-human CD3-FITC (clone HIT3a), IgG1 isotype control-PE (clone MOPC-21), IgG2a isotype control-PE (clone G155–178) (all from BD Pharmingen, San Diego, CA, USA), TLR1-PE (clone Gd2.F4), TLR2-PE (clone TL2.1), TLR3-PE (clone TLR3.7), TLR4-PE (clone HTA125), rat anti-human TLR9 (clone eB72-1665) (all from eBioscience, San Diego, CA, USA), mouse anti-human TLR8-PE (clone 44C143) (Imgenex, San Diego, CA, USA) and mouse anti-human antibodies from OKT3 (anti-CD3) and 10F7MN (anti-CD235a) cell lines (all from ATCC). OKT3 antibodies were purified from cell culture supernatant using Protein G Sepharose 4 Fast Flow beads (GE Healthcare, Little Chalfont, UK) as per manufacturer's instructions.

Cells and cultures

Mononuclear leukocytes from adult blood and cord blood were enriched using density centrifugation with Lymphoprep (Nycomed, Oslo, Norway) as per manufacturer's instructions. Cord blood mononuclear leukocytes were

contaminated by excessive numbers of erythrocyte precursors that could not be removed by density centrifugation. To avoid interference in leukocyte assays, erythrocyte precursors were eliminated using a previously described method (30, 31) that was modified. Briefly, enriched mononuclear leukocytes from cord blood were treated with anti-CD235a antibody to label erythrocytes that were then removed with MACS Goat anti-Mouse IgG Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Mononuclear leukocytes were cultured in RPMI 1640 medium (SAFC Biosciences, Lenexa, KS, USA) supplemented with 10% fetal bovine serum (SAFC Bioscience) and 1% penicillin–streptomycin–glutamine (Invitrogen, Carlsbad, CA, USA) (RF10 media) at 1×10^7 cells/well in 6-well flat-bottom tissue culture plate (Nunc™, Roskilde, Denmark). T lymphocytes in the culture were stimulated with plate-bound purified OKT3 and 160 pg IL-2 per well for 24 h in a 37°C 5% CO_2 incubator. For plate-bound OKT3, purified OKT3 (10 μg/ml) diluted in phosphate-buffered saline (SAFC Biosciences) was added to wells, incubated overnight at 37°C, and then the wells were washed thrice with PBS before use.

Immunofluorescence staining and flow cytometry

Toll-like receptors' expression by mononuclear leukocytes from adult blood and cord blood was detected by immunofluorescence staining. For TLR expression by non-stimulated T lymphocytes, staining was performed on mononuclear leukocytes immediately after enrichment and erythrocyte removal. For TLR expression by stimulated T lymphocytes, staining was performed on harvested cells from T lymphocyte stimulation assays. Experiments for extracellular and intracellular expression of TLR by individual specimens were conducted simultaneously, and monocytes, non-stimulated T lymphocytes and stimulated T lymphocytes were examined. For extracellular TLR expression, cells were stained with fluorescent antibodies against CD3, TLR and negative isotype controls, incubated at 4°C in the dark for 30 min, washed twice with PBS containing 0.02% sodium azide (Sigma-Aldrich, St Louis, MO, USA) and fixed with 2% paraformaldehyde. For intracellular TLR expression, cells were first stained for surface expression of CD3, and then cells were permeabilized with BD FACS Permeabilization Solution 2 (BD Biosciences, San Diego, CA, USA) according to manufacturer's instructions. Permeabilized cells were incubated with skim milk block at 4°C in the dark for 30 min, fluorescent antibodies against TLR and negative isotype controls were added and incubated at 4°C for 30 min. Cells were washed twice with 0.1% saponin (Sigma Aldrich) dissolved in PBS containing 0.02% sodium azide, and fixed as above. For skim milk block, 5% w/v skim milk powder (Black & Gold, Silverwater, Australia) was mixed for 15 min in PBS containing 0.1% w/v saponin and 0.02% w/v sodium azide, centrifuged for 30 min at 15,000 ***g***, the supernatant harvested and stored at 4°C. All cells were analyzed on the flow cytometer the next day.

Data analysis

Flow cytometric studies on the cells were carried out with the BD FACSCalibur System and data analyzed with BD CellQuest Pro software (BD Biosciences) (Fig. 1). T lymphocytes were identified using CD3 expression, and small T lymphocytes and T lymphoblasts were differentiated by side scatter properties. Monocytes were identified by forward and side scatter properties, and negative expression of CD3. Toll-like receptors' expression was calculated as the ratio of TLR median fluorescence intensity (MFI) to the MFI of the matched negative isotype control. Median fluorescence intensity ratio values greater than 1.3 were identified as positive for expression of TLR. Statistical comparison of data was not possible because of the small size of subject groups.

Results

Expression of TLR by non-stimulated monocytes

The expression of TLR1, TLR2, TLR3, TLR4, TLR8 and TLR9 by non-permeabilized and permeabilized monocytes from adult blood and cord blood was compared (Fig. 2). TLR1, TLR2, TLR3, TLR4, TLR8 and TLR9 expression by monocytes was detected in adult blood and cord blood. TLR2, TLR4 and TLR8 had greater expression on non-permeabilized monocytes compared to permeabilized monocytes. TLR1, TLR3 and TLR9 were detected at higher levels in permeabilized monocytes compared to non-permeabilized monocytes. The pattern of TLR expression by monocytes was similar between adult blood and cord blood.

Expression of TLR by small T lymphocytes

The expression of TLR1, TLR2, TLR3, TLR4, TLR8 and TLR9 by non-permeabilized and

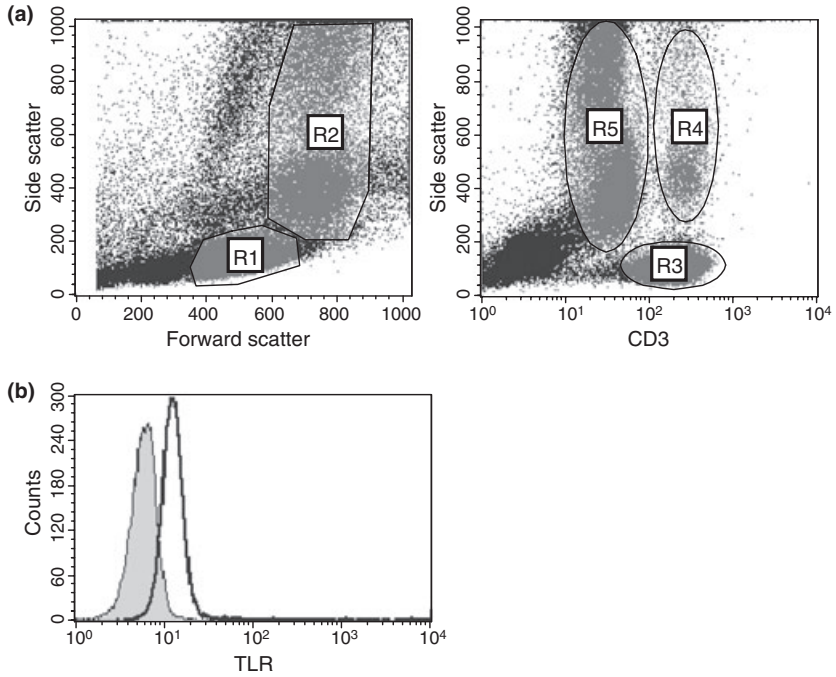

Fig. 1. Identification of monocytes and T lymphocytes for TLR expression studies. (a) A representative example of identification of monocytes and T lymphocytes by forward scatter, side scatter and CD3 expression; monocytes gated on R2 and R5, small T lymphocytes gated on R1 and R3, and T lymphoblasts gated on R4. (b) An example of positive TLR expression with a TLR MFI ratio of 2.02. Toll-like receptor expression was considered positive if the MFI ratio of the TLR (black lined histogram) to its matched negative isotype (gray filled histogram) was greater than 1.3.

permeabilized small T lymphocytes, either non-stimulated or stimulated, from adult blood and cord blood was compared (Fig. 3). The pattern of TLR2, TLR3, TLR8 and TLR9 expression by non-stimulated and stimulated small T lymphocytes was similar between adult blood and cord blood. However, TLR4 expression was detected in non-stimulated small T lymphocytes from adult blood and stimulated small T lymphocytes from cord blood. Stimulation of small T lymphocytes did not upregulate or downregulate expression of TLR1, TLR3, TLR8 and TLR9. However, stimulation of small T lymphocytes did affect TLR2 and TLR4; upon stimulation, TLR2 was upregulated, and TLR4 expression was downregulated in adults and upregulated in neonates. For TLR cellular location, TLR3 and TLR9 expression was higher in permeabilized small T lymphocytes, and TLR2 expression was higher for non-permeabilized cells. TLR4 and TLR8 were detected in permeabilized cells from adult blood and on non-permeabilized cells from cord blood.

Expression of TLR by T lymphoblasts

The expression of TLR1, TLR2, TLR3, TLR4, TLR8 and TLR9 by non-permeabilized and permeabilized T lymphoblasts, either non-stimulated or stimulated, from adult blood and cord blood was compared (Fig. 4). The pattern of TLR2, TLR3, TLR4, TLR8 and TLR9 expression by non-stimulated and stimulated T lymphoblasts was similar between adult blood and cord blood. The stimulation of T lymphoblasts did not upregulate or downregulate expression of TLR2, TLR3, TLR4, TLR8 and TLR9. For TLR cellular location, TLR2, TLR4 and TLR8 expression was higher in non-permeabilized T lymphoblasts, and TLR3 and TLR9 expression was higher in permeabilized T lymphoblasts. The pattern of TLR expression by T lymphoblasts is similar between adult blood and cord blood.

Discussion

From the results, there are three main observations. First, monocytes from adults and neonates expressed similar patterns of TLR. Second, T lymphocytes from adults and neonates expressed similar patterns of most TLR. Third, stimulation of T lymphocytes did not alter TLR expression patterns by small T lymphocytes or T lymphoblasts.

Fractions of enriched cord blood mononuclear leukocytes can contain up to 50% of erythrocyte precursors; these precursors can interfere in assays unless they are removed. Published methods (30,

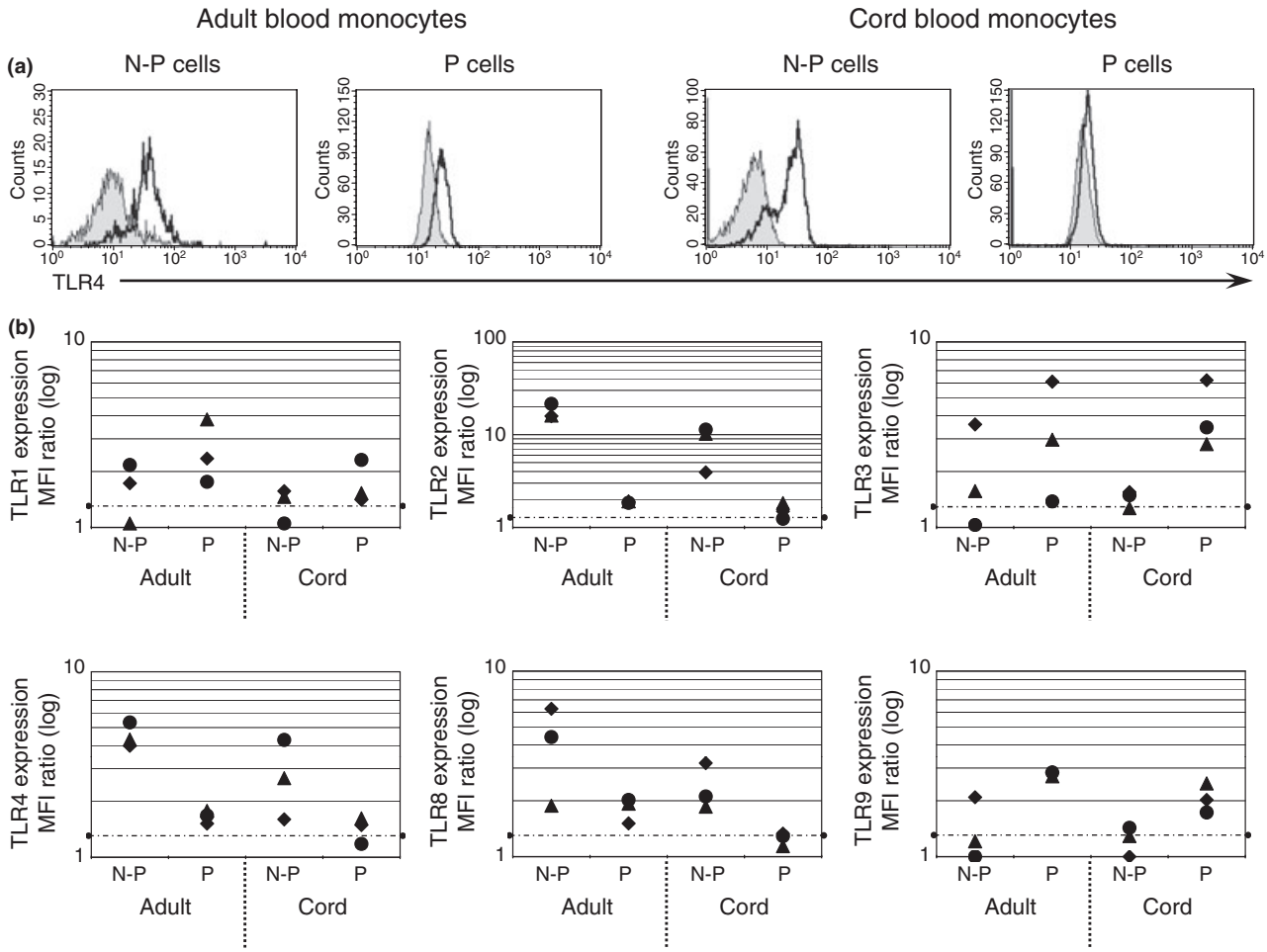

Fig. 2. Toll-like receptor expression by monocytes from adult blood and cord blood. Immunofluorescence staining of non-permeabilized (N-P) and permeabilized (P) cells for expression of TLR1, TLR2, TLR3, TLR4, TLR8 and TLR9 by monocytes from adult blood (n = 3) and cord blood (n = 3) was examined by flow cytometry. (a) Example of TLR4 expression by monocytes from one adult blood subject and one cord blood subject; TLR4 expression (black lined histogram) against its matched negative isotype (gray filled histogram). (b) TLR expression as TLR MFI ratios were plotted on a log scale, and the dashed line represents TLR MFI ratio value of 1.3. Each point represents an individual specimen.

31) were used for positive selection and removal of erythrocyte precursors. The additional step of removing erythrocyte precursors from cord blood mononuclear leukocytes, which was not required for adult blood mononuclear leukocytes, does not seem to affect subsequent TLR expression, as shown in the similarity of TLR expression between adult blood and cord blood leukocytes.

For the first observation, the similarity of TLR expression indicates that neonatal monocytes are capable of recognizing infection. As part of the early immune response to infection, neonatal monocytes should be able to engage with the microbes upon recognition through TLR. However, there is evidence that neonatal monocytes, compared to adult monocytes, have decreased mRNA levels of TLR signaling proteins MyD88 and IRF5, and stimulation with lipoteichoic acid leads to decreased phosphorylation of p38-MAPK and ERK1 with reduced cytokine secretion (22). This is seen with cord blood monocytes that, in response to TLR ligands, secrete variable levels of IL-12 (24) or lower levels of IL-1β, IL-6, IL-12 and tumor necrosis factor (TNF)-α (12). Despite the capacity of neonatal monocytes to recognize infection through TLR, impaired TLR signaling pathways may affect their function.

The second observation from these studies showed that T lymphocytes from adults and neonates expressed similar patterns of TLR2, TLR3, TLR4, TLR8 and TLR9. The similarity of TLR expression by T lymphocytes from adults and neonates, and expression of TLR4 by stimulated cord blood T cells, suggests that neonates possess the ability to 'innately'

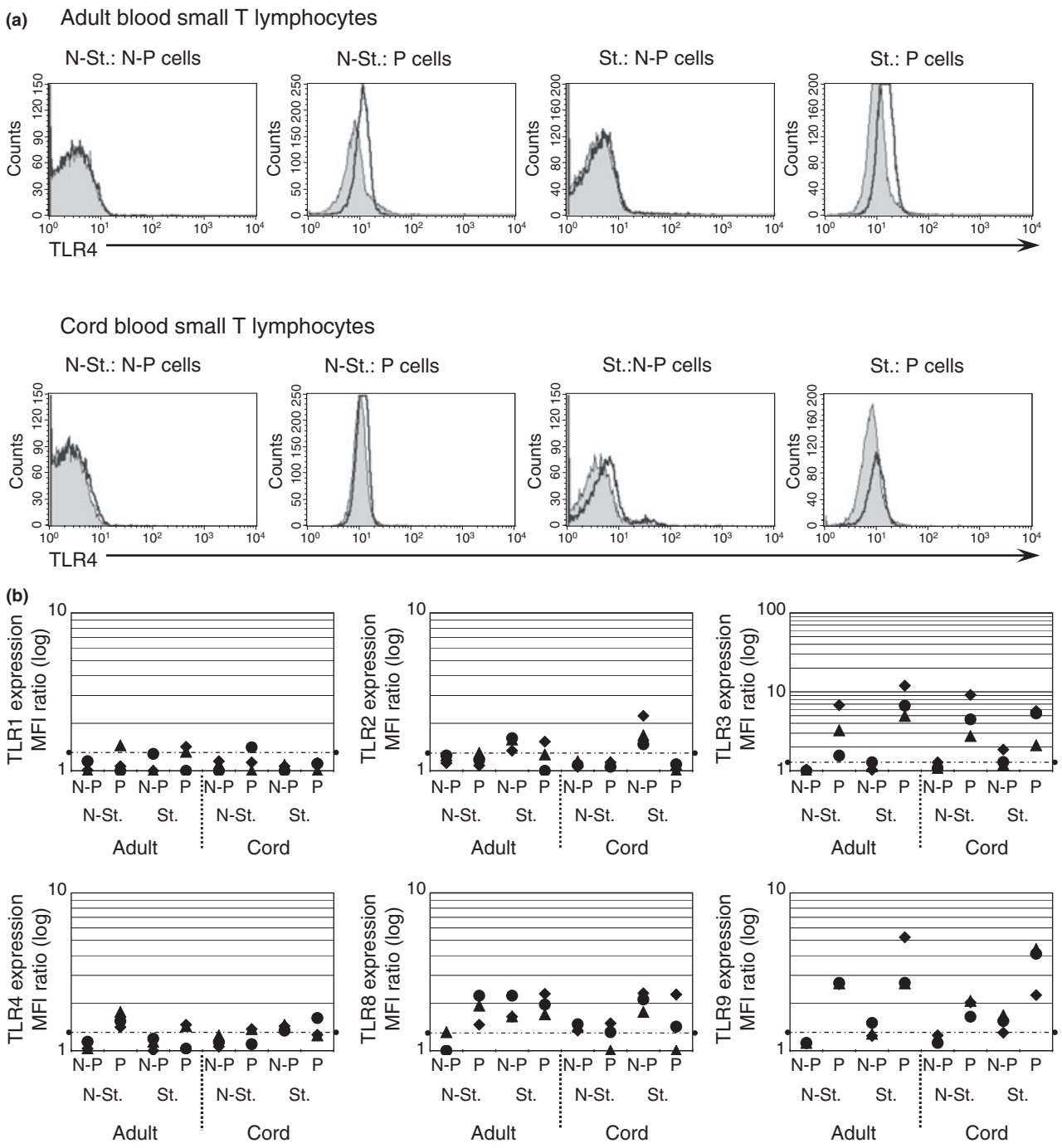

Fig. 3 Toll-like receptor expression by small T lymphocytes from adult blood and cord blood. Immunofluorescence staining of non-permeabilized (N-P) and permeabilized (P) cells for expression of TLR1, TLR2, TLR3, TLR4, TLR8 and TLR9 by small T lymphocytes from adult blood (n = 3) and cord blood (n = 3) was examined by flow cytometry. Toll-like receptor expression was examined on non-stimulated (N-St.) and stimulated (St.) T lymphocytes. For TLR expression by N-St. T lymphocytes, mononuclear leukocytes were enriched from blood and immediately stained. For TLR expression by St. T lymphocytes, enriched mononuclear leukocytes were stimulated for 24 h by anti-CD3 antibody in the presence of IL-2. (a) Example of TLR4 expression by small T lymphocytes from one adult blood subject and one cord blood subject; TLR4 expression (black lined histogram) against its matched negative isotype (gray filled histogram). (b) Toll-like receptor expression as TLR MFI ratios were plotted on a log scale, and the dashed line represents MFI ratio value of 1.3. Each point represents an individual specimen.

recognize pathogens. Classifying T lymphocytes into cell activation states of small T lymphocytes and T lymphoblasts revealed the different patterns of TLR expression; T lymphoblasts expressed a greater range of TLR than small T lymphocytes. T lymphoblasts expressed all tested

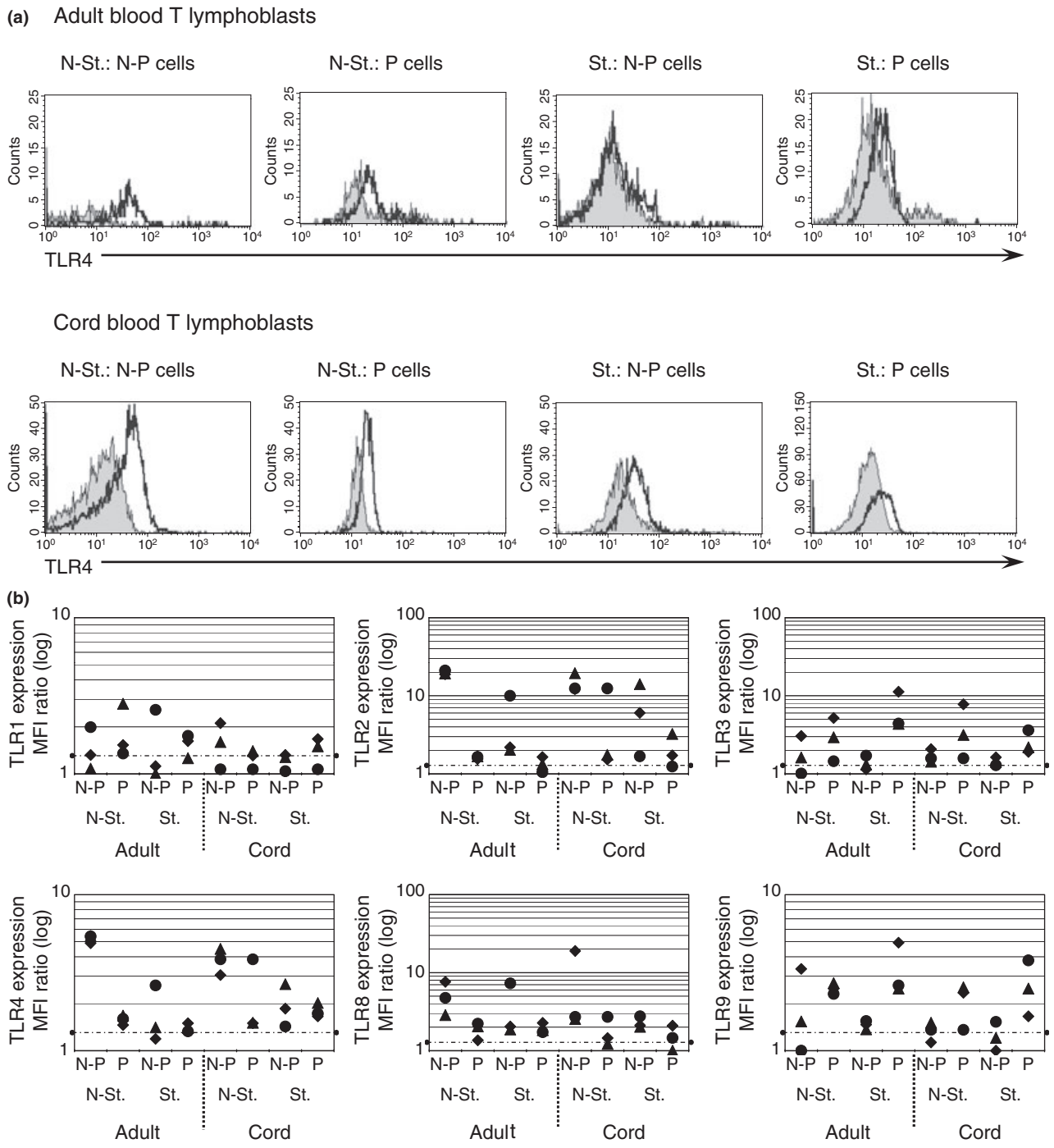

Fig. 4. Toll-like receptor expression by T lymphoblasts from adult blood and cord blood. Immunofluorescence staining of non-permeabilized (N-P) and permeabilized (P) cells for expression of TLR1, TLR2, TLR3, TLR4, TLR8 and TLR9 by T lymphoblasts from adult blood (n = 3) and cord blood (n = 3) was examined by flow cytometry. Toll-like receptor expression was examined on non-stimulated (N-St.) and stimulated (St.) T lymphoblasts. For TLR expression by N-St. T lymphoblasts, mononuclear leukocytes were enriched from blood and immediately stained. For TLR expression by St. T lymphoblasts, enriched mononuclear leukocytes were stimulated for 24 h by anti-CD3 antibody in the presence of IL-2. (a) Example of TLR4 expression by T lymphoblasts from one adult blood subject and one cord blood subject; TLR4 expression (black lined histogram) against its matched negative isotype (gray filled histogram). (b) Toll-like receptor expression as TLR MFI ratios were plotted on a log scale, and the dashed line represents MFI ratio value of 1.3. Each point represents an individual specimen.

TLR, while small T lymphocytes expressed fewer TLR: TLR2, TLR3, TLR4, TLR8 and TLR9. The greater expression of TLR shows that T blasts may be more responsive to infection as most tested TLR were constitutively expressed. Toll-like receptor ligands, acting as costimulatory signals, induced cord blood T lymphocytes to secrete interferon-α, TNF-α, IL-2 and IL-10 (13). Toll-like receptors ligands may be able to assist in overcoming the immune deficiencies of the neonatal adaptive immune response. The similarity in expression between neonates and adults indicates that neonates are capable of altering TLR expression and detecting infection.

The third observation from the results is that stimulation of T lymphocytes did not affect the expression of most TLR, excluding TLR2 and TLR4. Upregulation of TLR2 and TLR4 by stimulated T lymphocytes from cord blood was supported by a similar study (13). The non-responsiveness of TLR expression upon stimulation of T lymphocytes may be because of the stimulation assay; stimulation via CD3 and CD28 are signals for adaptive immune activation. Toll-like receptors' expression may not be as responsive to adaptive immune signals as TLR are part of the innate recognition of infection; activation via other mechanisms may be more effective to alter TLR expression. Disease does affect TLR expression by increasing or decreasing particular TLR in T lymphocytes, as seen for patients with lymphatic filarial infection (28). Despite the inertia in expression of several TLR to T lymphocyte stimulation in this study, the cited studies and results for TLR2 and TLR4 show that T lymphocytes are capable of regulating TLR expression in response to appropriate stimuli. Furthermore, *in vivo*-stimulated T lymphocytes from the cited studies and T lymphoblasts in this study did show significant differences in TLR expression compared with small T lymphocytes.

This is a pilot study intended to provide data on the role of TLR in the neonatal immune system; little is known about the ability of neonates to recognize pathogens through TLR. The results show similar expression of most TLR by monocytes and T lymphocytes from adult blood and cord blood; to date, these data are not available in the literature. The range of individual TLR expression is variable within each subject group; the high variability of TLR expression can be expected from human samples. Expression studies on larger groups will also show considerable variability in human subjects.

A surprising observation from this study is the expression of TLR8 on the surface of adult blood and cord blood leukocytes, despite TLR8 being known as an internal receptor (20). The results are inconclusive for internal TLR8 expression. Although expression by permeabilized cells is expected to show the sum of surface and internal expression, TLR8 expression is lower in permeabilized cells compared to non-permeabilized, intact cells, suggesting the epitope is sensitive to the conditions used for permeabilization. Nevertheless, the staining of intact cells for TLR8 is unequivocal. Like TLR8, TLR9 is also considered an internal marker, but later studies show TLR9 expression on immune cell surfaces (32, 33). This pilot study, although small in numbers, has produced interesting results that merit further investigations.

The hypothesis underlying this study was that TLR would have a greater presence in the neonatal immune system compared to the adult immune system. This was not supported by the results; the patterns of TLR expression by monocytes and T lymphocytes were similar between neonates and adults. The immaturity of the neonatal immune system is not reflected in TLR expression patterns by monocytes and T lymphocytes. Despite the similarity of TLR expression, deficiencies in TLR signaling in neonates may affect TLR responses as previously discussed; TLR functional studies will clarify their role in neonatal immunity. The results demonstrate that neonatal monocytes and T lymphocytes, whether non-stimulated or stimulated, possess adult-like ability for innate recognition of pathogens through TLR expression.

Acknowledgments

The authors thank the staff of the Delivery Suite, Women's and Children's Hospital, Adelaide, and the Australian Red Cross for the collection of cord blood and buffy coats, respectively. We also acknowledge assistance from Dr Greg Hodge for his expert advice on flow cytometry.

References

1. Levy O. Innate immunity of the newborn: basic mechanisms and clinical correlates. Nat Rev Immunol 2007: 7: 379–90.
2. Velilla PA, Rugeles MT, Chougnet CA. Defective antigen-presenting cell function in human neonates. Clin Immunol 2006: 121: 251–9.
3. Siegrist CA. The challenges of vaccine responses in early life: selected examples. J Comp Pathol 2007: 137 (Suppl 1): S4–9.
4. The World Health Report 2005. Make every mother and child count. World Health Organisation. Geneva: WHO Press 2005.
5. Janeway CA Jr, Medzhitov R. Innate immune recognition. Annu Rev Immunol 2002: 20: 197–216.
6. Medzhitov R, Janeway CA Jr. Innate immunity: the virtues of a nonclonal system of recognition. Cell 1997: 91: 295–8.

7. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci USA 1998: 95: 588–93.
8. Takeuchi O, Kawai T, Sanjo H, et al. TLR6: A novel member of an expanding toll-like receptor family. Gene 1999: 231: 59–65.
9. Du X, Poltorak A, Wei Y, Beutler B. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. Eur Cytokine Netw 2000: 11: 362–71.
10. Chuang T, Ulevitch RJ. Identification of hTLR10: a novel human Toll-like receptor preferentially expressed in immune cells. Biochim Biophys Acta 2001: 1518: 157–61.
11. Dasari P, Nicholson IC, Zola H. Toll-like receptors. J Biol Regul Homeost Agents 2008: 22: 17–26.
12. Morgado JM, Pratas R, Laranjeira P, et al. The phenotypical and functional characteristics of cord blood monocytes and CD14(-/low)/CD16(+) dendritic cells can be relevant to the development of cellular immune responses after transplantation. Transpl Immunol 2008: 19: 55–63.
13. Komai-Koma M, Jones L, Ogg GS, Xu D, Liew FY. TLR2 is expressed on activated T cells as a costimulatory receptor. Proc Natl Acad Sci U S A 2004: 101: 3029–34.
14. Cottalorda A, Verschelde C, Marcais A, et al. TLR2 engagement on CD8 T cells lowers the threshold for optimal antigen-induced T cell activation. Eur J Immunol 2006: 36: 1684–93.
15. Mazzoni A, Segal DM. Controlling the Toll road to dendritic cell polarization. J Leukoc Biol 2004: 75: 721–30.
16. Peng G, Guo Z, Kiniwa Y, et al. Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. Science 2005: 309: 1380–4.
17. Visintin A, Mazzoni A, Spitzer JH, Wyllie DH, Dower SK, Segal DM. Regulation of Toll-like receptors in human monocytes and dendritic cells. J Immunol 2001: 166: 249–55.
18. Hornung V, Rothenfusser S, Britsch S, et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol 2002: 168: 4531–7.
19. Nakao Y, Funami K, Kikkawa S, et al. Surface-expressed TLR6 participates in the recognition of diacylated lipopeptide and peptidoglycan in human cells. J Immunol 2005: 174: 1566–73.
20. Lun SW, Wong CK, Ko FW, Hui DS, Lam CW. Expression and functional analysis of Toll-like receptors of peripheral Blood cells in asthmatic patients: implication for immunopathological mechanism in asthma. J Clin Immunol 2008: 29: 330–42.
21. Saikh KU, Kissner TL, Sultana A, Ruthel G, Ulrich RG. Human monocytes infected with Yersinia pestis express cell surface TLR9 and differentiate into dendritic cells. J Immunol 2004: 173: 7426–34.
22. Sadeghi K, Berger A, Langgartner M, et al. Immaturity of infection control in preterm and term newborns is associated with impaired toll-like receptor signaling. J Infect Dis 2007: 195: 296–302.
23. Viemann D, Dubbel G, Schleifenbaum S, Harms E, Sorg C, Roth J. Expression of toll-like receptors in neonatal sepsis. Pediatr Res 2005: 58: 654–9.
24. Levy O, Suter EE, Miller RL, Wessels MR. Unique efficacy of Toll-like receptor 8 agonists in activating human neonatal antigen-presenting cells. Blood 2006: 108: 1284–90.
25. Saikh KU, Lee JS, Kissner TL, Dyas B, Ulrich RG. Toll-like receptor and cytokine expression patterns of CD56 + T cells are similar to natural killer cells in response to infection with Venezuelan equine encephalitis virus replicons. J Infect Dis 2003: 188: 1562–70.
26. Wesch D, Beetz S, Oberg HH, Marget M, Krengel K, Kabelitz D. Direct costimulatory effect of TLR3 ligand poly(I:C) on human gamma delta T lymphocytes. J Immunol 2006: 176: 1348–54.
27. Bell MP, Svingen PA, Rahman MK, Xiong Y, Faubion WA Jr. FOXP3 regulates TLR10 expression in human T regulatory cells. J Immunol 2007: 179: 1893–900.
28. Babu S, Blauvelt CP, Kumaraswami V, Nutman TB. Cutting edge: diminished T cell TLR expression and function modulates the immune response in human filarial infection. J Immunol 2006: 176: 3885–9.
29. Crellin NK, Garcia RV, Hadisfar O, Allan SE, Steiner TS, Levings MK. Human CD4 + T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4 + CD25 + T regulatory cells. J Immunol 2005: 175: 8051–9.
30. Elliott SR, Macardle PJ, Zola H. Removal of erythroid cells from umbilical cord blood mononuclear cell preparations using magnetic beads and a monoclonal antibody against glycophorin A. J Immunol Methods 1998: 217: 121–30.
31. Fuss IJ, Kanof ME, Smith PD, Zola H. Isolation of whole mononuclear cells from peripheral blood and cord blood. Curr Protoc Immunol 2009: 1–7. Chapter 7: Unit7 1.
32. Eaton-Bassiri A, Dillon SB, Cunningham M, et al. Toll-like receptor 9 can be expressed at the cell surface of distinct populations of tonsils and human peripheral blood mononuclear cells. Infect Immun 2004: 72: 7202–11.
33. Dasari P, Nicholson IC, Hodge G, Dandie GW, Zola H. Expression of toll-like receptors on B lymphocytes. Cell Immunol 2005: 236: 140–5.

Bibliography

1. The World Health Report 2005: Make every mother and child count. World Health Organisation: WHO Press; 2005.

2. Tosi MF. Innate immune responses to infection. J Allergy Clin Immunol. 2005 Aug;116(2):241-9.

3. Levy O. Innate immunity of the newborn: basic mechanisms and clinical correlates. Nat Rev Immunol. 2007 May;7(5):379-90.

4. Marodi L. Innate cellular immune responses in newborns. Clin Immunol. 2006 Feb-Mar;118(2-3):137-44.

5. Karlsson H, Hessle C, Rudin A. Innate immune responses of human neonatal cells to bacteria from the normal gastrointestinal flora. Infect Immun. 2002 Dec;70(12):6688-96.

6. Chelvarajan RL, Collins SM, Doubinskaia IE, Goes S, Van Willigen J, Flanagan D, et al. Defective macrophage function in neonates and its impact on unresponsiveness of neonates to polysaccharide antigens. J Leukoc Biol. 2004 Jun;75(6):982-94.

7. Petrova A, Mehta R. Dysfunction of innate immunity and associated pathology in neonates. Indian J Pediatr. 2007 Feb;74(2):185-91.

8. Landers CD, Chelvarajan RL, Bondada S. The role of B cells and accessory cells in the neonatal response to TI-2 antigens. Immunol Res. 2005;31(1):25-36.

9. Lothian C, Dahlgren C, Lagercrantz H, Lundahl J. Different expression and mobilisation of the complement regulatory proteins CD35, CD55 and CD59 in neonatal and adult neutrophils. Biol Neonate. 1997;72(1):15-21.

10. Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. Blood. 2008 Sep 1;112(5):1557-69.

11. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol. 2004;22:531-62.

12. King C, Tangye SG, Mackay CR. T follicular helper (TFH) cells in normal and dysregulated immune responses. Annu Rev Immunol. 2008;26:741-66.

13. Chavez-Galan L, Arenas-Del Angel MC, Zenteno E, Chavez R, Lascurain R. Cell death mechanisms induced by cytotoxic lymphocytes. Cell Mol Immunol. 2009 Feb;6(1):15-25.

14. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. Annu Rev Immunol. 2007;25:297-336.

15. Siegrist CA. The challenges of vaccine responses in early life: selected examples. J Comp Pathol. 2007 Jul;137 Suppl 1:S4-9.

16. Crespo I, Paiva A, Couceiro A, Pimentel P, Orfao A, Regateiro F. Immunophenotypic and functional characterization of cord blood dendritic cells. Stem Cells Dev. 2004 Feb;13(1):63-70.

17. De Wit D, Tonon S, Olislagers V, Goriely S, Boutriaux M, Goldman M, et al. Impaired responses to toll-like receptor 4 and toll-like receptor 3 ligands in human cord blood. J Autoimmun. 2003 Nov;21(3):277-81.

18. De Wit D, Olislagers V, Goriely S, Vermeulen F, Wagner H, Goldman M, et al. Blood plasmacytoid dendritic cell responses to CpG oligodeoxynucleotides are impaired in human newborns. Blood. 2004 Feb 1;103(3):1030-2.

19. Willems F, Vollstedt S, Suter M. Phenotype and function of neonatal DC. Eur J Immunol. 2009 Jan;39(1):26-35.

20. Garcia AM, Fadel SA, Cao S, Sarzotti M. T cell immunity in neonates. Immunol Res. 2000;22(2-3):177-90.

21. Marchant A, Goldman M. T cell-mediated immune responses in human newborns: ready to learn? Clin Exp Immunol. 2005 Jul;141(1):10-8.

22. Delespesse G, Yang LP, Ohshima Y, Demeure C, Shu U, Byun DG, et al. Maturation of human neonatal CD4+ and CD8+ T lymphocytes into Th1/Th2 effectors. Vaccine. 1998 Aug-Sep;16(14-15):1415-9.

23. McCarron M, Reen DJ. Activated human neonatal CD8+ T cells are subject to immunomodulation by direct TLR2 or TLR5 stimulation. J Immunol. 2009 Jan 1;182(1):55-62.

24. Berland R, Wortis HH. Origins and functions of B-1 cells with notes on the role of CD5. Annu Rev Immunol. 2002;20:253-300.

25. Martin F, Kearney JF. B1 cells: similarities and differences with other B cell subsets. Curr Opin Immunol. 2001 Apr;13(2):195-201.

26. MacLennan IC. Germinal centers. Annu Rev Immunol. 1994;12:117-39.

27. Liu YJ, Arpin C. Germinal center development. Immunol Rev. 1997 Apr;156:111-26.

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

29. Lund FE. Cytokine-producing B lymphocytes-key regulators of immunity. Curr Opin Immunol. 2008 Jun;20(3):332-8.

30. Gray D, Gray M, Barr T. Innate responses of B cells. Eur J Immunol. 2007 Dec;37(12):3304-10.

31. Macardle PJ, Weedon H, Fusco M, Nobbs S, Ridings J, Flego L, et al. The antigen receptor complex on cord B lymphocytes. Immunology. 1997 Mar;90(3):376-82.

32. Zola H. The development of antibody responses in the infant. Immunol Cell Biol. 1997 Dec;75(6):587-90.

33. Zandvoort A, Timens W. The dual function of the splenic marginal zone: essential for initiation of anti-TI-2 responses but also vital in the general first-line defense against blood-borne antigens. Clin Exp Immunol. 2002 Oct;130(1):4-11.

34. Siegrist CA, Aspinall R. B-cell responses to vaccination at the extremes of age. Nat Rev Immunol. 2009 Mar;9(3):185-94.

35. Kruschinski C, Zidan M, Debertin AS, von Horsten S, Pabst R. Age-dependent development of the splenic marginal zone in human infants is associated with different causes of death. Hum Pathol. 2004 Jan;35(1):113-21.

36. Pihlgren M, Tougne C, Bozzotti P, Fulurija A, Duchosal MA, Lambert PH, et al. Unresponsiveness to lymphoid-mediated signals at the neonatal follicular dendritic cell precursor level contributes to delayed germinal center induction and limitations of neonatal antibody responses to T-dependent antigens. J Immunol. 2003 Mar 15;170(6):2824-32.

37. Ridings J, Nicholson IC, Goldsworthy W, Haslam R, Roberton DM, Zola H. Somatic hypermutation of immunoglobulin genes in human neonates. Clin Exp Immunol. 1997 May;108(2):366-74.

38. Simister NE. Placental transport of immunoglobulin G. Vaccine. 2003 Jul 28;21(24):3365-9.

39. Glezen WP. Effect of maternal antibodies on the infant immune response. Vaccine. 2003 Jul 28;21(24):3389-92.

40. Siegrist CA. Neonatal and early life vaccinology. Vaccine. 2001 May 14;19(25-26):3331-46.

41. Hanson LA. Session 1: Feeding and infant development breast-feeding and immune function. Proc Nutr Soc. 2007 Aug;66(3):384-96.

42. Bahl R, Frost C, Kirkwood BR, Edmond K, Martines J, Bhandari N, et al. Infant feeding patterns and risks of death and hospitalization in the first half of infancy: multicentre cohort study. Bull World Health Organ. 2005 Jun;83(6):418-26.

43. Edmond KM, Zandoh C, Quigley MA, Amenga-Etego S, Owusu-Agyei S, Kirkwood BR. Delayed breastfeeding initiation increases risk of neonatal mortality. Pediatrics. 2006 Mar;117(3):e380-6.

44. Arifeen S, Black RE, Antelman G, Baqui A, Caulfield L, Becker S. Exclusive breastfeeding reduces acute respiratory infection and diarrhea deaths among infants in Dhaka slums. Pediatrics. 2001 Oct;108(4):E67.

45. Asensi MT, Martinez-Costa C, Buesa J. Anti-rotavirus antibodies in human milk: quantification and neutralizing activity. J Pediatr Gastroenterol Nutr. 2006 May;42(5):560-7.

46. Thomas JE, Bunn JE, Kleanthous H, Monath TP, Harding M, Coward WA, et al. Specific immunoglobulin A antibodies in maternal milk and delayed Helicobacter pylori colonization in Gambian infants. Clin Infect Dis. 2004 Oct 15;39(8):1155-60.

47. Hawkes JS, Bryan DL, James MJ, Gibson RA. Cytokines (IL-1beta, IL-6, TNF-alpha, TGF-beta1, and TGF-beta2) and prostaglandin E2 in human milk during the first three months postpartum. Pediatr Res. 1999 Aug;46(2):194-9.

48. Ustundag B, Yilmaz E, Dogan Y, Akarsu S, Canatan H, Halifeoglu I, et al. Levels of cytokines (IL-1beta, IL-2, IL-6, IL-8, TNF-alpha) and trace elements (Zn, Cu) in breast milk from mothers of preterm and term infants. Mediators Inflamm. 2005 Dec 14;2005(6):331-6.

49. Ochoa TJ, Cleary TG. Effect of lactoferrin on enteric pathogens. Biochimie. 2009 Jan;91(1):30-4.

50. Newburg DS. Neonatal protection by an innate immune system of human milk consisting of oligosaccharides and glycans. J Anim Sci. 2009 Apr;87(13 Suppl):26-34.

51. Holt PG, Jones CA. The development of the immune system during pregnancy and early life. Allergy. 2000 Aug;55(8):688-97.

52. Krumbiegel D, Zepp F, Meyer CU. Combined Toll-like receptor agonists synergistically increase production of inflammatory cytokines in human neonatal dendritic cells. Hum Immunol. 2007 Oct;68(10):813-22.

53. Traggiai E, Chicha L, Mazzucchelli L, Bronz L, Piffaretti JC, Lanzavecchia A, et al. Development of a human adaptive immune system in cord blood cell-transplanted mice. Science. 2004 Apr 2;304(5667):104-7.

54. Ridings J, Dinan L, Williams R, Roberton D, Zola H. Somatic mutation of immunoglobulin V(H)6 genes in human infants. Clin Exp Immunol. 1998 Oct;114(1):33-9.

55. Garn H, Renz H. Epidemiological and immunological evidence for the hygiene hypothesis. Immunobiology. 2007;212(6):441-52.

56. Janeway CA, Jr., Medzhitov R. Innate immune recognition. Annu Rev Immunol. 2002;20:197-216.

57. Medzhitov R, Janeway CA, Jr. Innate immunity: the virtues of a nonclonal system of recognition. Cell. 1997 Oct 31;91(3):295-8.

58. Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature. 1997 Jul 24;388(6640):394-7.

59. Anderson KV, Jurgens G, Nusslein-Volhard C. Establishment of dorsal-ventral polarity in the Drosophila embryo: genetic studies on the role of the Toll gene product. Cell. 1985 Oct;42(3):779-89.

60. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell. 1996 Sep 20;86(6):973-83.

61. Rast JP, Smith LC, Loza-Coll M, Hibino T, Litman GW. Genomic insights into the immune system of the sea urchin. Science. 2006 Nov 10;314(5801):952-6.

62. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci U S A. 1998 Jan 20;95(2):588-93.

63. Takeuchi O, Kawai T, Sanjo H, Copeland NG, Gilbert DJ, Jenkins NA, et al. TLR6: A novel member of an expanding toll-like receptor family. Gene. 1999 Apr 29;231(1-2):59-65.

64. Du X, Poltorak A, Wei Y, Beutler B. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. Eur Cytokine Netw. 2000 Sep;11(3):362-71.

65. Chuang T, Ulevitch RJ. Identification of hTLR10: a novel human Toll-like receptor preferentially expressed in immune cells. Biochim Biophys Acta. 2001 Mar 19;1518(1-2):157-61.

66. Kufer TA, Fritz JH, Philpott DJ. NACHT-LRR proteins (NLRs) in bacterial infection and immunity. Trends Microbiol. 2005 Aug;13(8):381-8.

67. Bell JK, Mullen GE, Leifer CA, Mazzoni A, Davies DR, Segal DM. Leucine-rich repeats and pathogen recognition in Toll-like receptors. Trends Immunol. 2003 Oct;24(10):528-33.

68. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol. 2003;21:335-76.

69. Xu Y, Tao X, Shen B, Horng T, Medzhitov R, Manley JL, et al. Structural basis for signal transduction by the Toll/interleukin-1 receptor domains. Nature. 2000 Nov 2;408(6808):111-5.

70. Slack JL, Schooley K, Bonnert TP, Mitcham JL, Qwarnstrom EE, Sims JE, et al. Identification of two major sites in the type I interleukin-1 receptor cytoplasmic region responsible for coupling to pro-inflammatory signaling pathways. J Biol Chem. 2000 Feb 18;275(7):4670-8.

71. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol. 2001 Aug;2(8):675-80.

72. Hayashi F, Means TK, Luster AD. Toll-like receptors stimulate human neutrophil function. Blood. 2003 Oct 1;102(7):2660-9.

73. Nagase H, Okugawa S, Ota Y, Yamaguchi M, Tomizawa H, Matsushima K, et al. Expression and function of Toll-like receptors in eosinophils: activation by Toll-like receptor 7 ligand. J Immunol. 2003 Oct 15;171(8):3977-82.

74. Flo TH, Halaas O, Torp S, Ryan L, Lien E, Dybdahl B, et al. Differential expression of Toll-like receptor 2 in human cells. J Leukoc Biol. 2001 Mar;69(3):474-81.

75. Sabroe I, Jones EC, Usher LR, Whyte MK, Dower SK. Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: a critical role for monocytes in leukocyte lipopolysaccharide responses. J Immunol. 2002 May 1;168(9):4701-10.

76. Nakao Y, Funami K, Kikkawa S, Taniguchi M, Nishiguchi M, Fukumori Y, et al. Surface-expressed TLR6 participates in the recognition of diacylated lipopeptide and peptidoglycan in human cells. J Immunol. 2005 Feb 1;174(3):1566-73.

77. Wong CK, Cheung PF, Ip WK, Lam CW. Intracellular signaling mechanisms regulating toll-like receptor-mediated activation of eosinophils. Am J Respir Cell Mol Biol. 2007 Jul;37(1):85-96.

78. Visintin A, Mazzoni A, Spitzer JH, Wyllie DH, Dower SK, Segal DM. Regulation of Toll-like receptors in human monocytes and dendritic cells. J Immunol. 2001 Jan 1;166(1):249-55.

79. Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdorfer B, Giese T, et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets

of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J Immunol. 2002 May 1;168(9):4531-7.

80. Bosisio D, Polentarutti N, Sironi M, Bernasconi S, Miyake K, Webb GR, et al. Stimulation of toll-like receptor 4 expression in human mononuclear phagocytes by interferon-gamma: a molecular basis for priming and synergism with bacterial lipopolysaccharide. Blood. 2002 May 1;99(9):3427-31.

81. Saikh KU, Kissner TL, Sultana A, Ruthel G, Ulrich RG. Human monocytes infected with Yersinia pestis express cell surface TLR9 and differentiate into dendritic cells. J Immunol. 2004 Dec 15;173(12):7426-34.

82. Lun SW, Wong CK, Ko FW, Hui DS, Lam CW. Expression and Functional Analysis of Toll-Like Receptors of Peripheral Blood Cells in Asthmatic Patients: Implication for Immunopathological Mechanism in Asthma. J Clin Immunol. 2008 Dec 6;29(3):330-42.

83. Mansson A, Cardell LO. Role of atopic status in Toll-like receptor (TLR)7- and TLR9-mediated activation of human eosinophils. J Leukoc Biol. 2009 Apr;85(4):719-27.

84. Komai-Koma M, Jones L, Ogg GS, Xu D, Liew FY. TLR2 is expressed on activated T cells as a costimulatory receptor. Proc Natl Acad Sci U S A. 2004 Mar 2;101(9):3029-34.

85. Saikh KU, Lee JS, Kissner TL, Dyas B, Ulrich RG. Toll-like receptor and cytokine expression patterns of CD56+ T cells are similar to natural killer cells in response to infection with Venezuelan equine encephalitis virus replicons. J Infect Dis. 2003 Nov 15;188(10):1562-70.

86. Wesch D, Beetz S, Oberg HH, Marget M, Krengel K, Kabelitz D. Direct costimulatory effect of TLR3 ligand poly(I:C) on human gamma delta T lymphocytes. J Immunol. 2006 Feb 1;176(3):1348-54.

87. Crellin NK, Garcia RV, Hadisfar O, Allan SE, Steiner TS, Levings MK. Human CD4+ T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4+CD25+ T regulatory cells. J Immunol. 2005 Dec 15;175(12):8051-9.

88. Babu S, Blauvelt CP, Kumaraswami V, Nutman TB. Cutting edge: diminished T cell TLR expression and function modulates the immune response in human filarial infection. J Immunol. 2006 Apr 1;176(7):3885-9.

89. Bell MP, Svingen PA, Rahman MK, Xiong Y, Faubion WA, Jr. FOXP3 regulates TLR10 expression in human T regulatory cells. J Immunol. 2007 Aug 1;179(3):1893-900.

90. Hasan U, Chaffois C, Gaillard C, Saulnier V, Merck E, Tancredi S, et al. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. J Immunol. 2005 Mar 1;174(5):2942-50.

91. Mansson A, Adner M, Hockerfelt U, Cardell LO. A distinct Toll-like receptor repertoire in human tonsillar B cells, directly activated by PamCSK, R-837 and CpG-2006 stimulation. Immunology. 2006 Aug;118(4):539-48.

92. Dasari P, Nicholson IC, Hodge G, Dandie GW, Zola H. Expression of toll-like receptors on B lymphocytes. Cell Immunol. 2005 July - August;236(1-2):140-5.

93. Babu S, Blauvelt CP, Kumaraswami V, Nutman TB. Diminished expression and function of TLR in lymphatic filariasis: a novel mechanism of immune dysregulation. J Immunol. 2005 Jul 15;175(2):1170-6.

94. Ganley-Leal LM, Liu X, Wetzler LM. Toll-like receptor 2-mediated human B cell differentiation. Clin Immunol. 2006 Jun 9.

95. Alter G, Suscovich TJ, Teigen N, Meier A, Streeck H, Brander C, et al. Single-stranded RNA derived from HIV-1 serves as a potent activator of NK cells. J Immunol. 2007 Jun 15;178(12):7658-66.

96. Prabha C, Rajashree P, Sulochana DD. TLR2 and TLR4 expression on the immune cells of tuberculous pleural fluid. Immunol Lett. 2008 Apr 15;117(1):26-34.

97. Comin F, Speziali E, Martins-Filho OA, Caldas IR, Moura V, Gazzinelli A, et al. Ageing and Toll-like receptor expression by innate immune cells in chronic human schistosomiasis. Clin Exp Immunol. 2007 Aug;149(2):274-84.

98. Mansson A, Adner M, Cardell LO. Toll-like receptors in cellular subsets of human tonsil T cells: altered expression during recurrent tonsillitis. Respir Res. 2006;7:36.

99. Bourke E, Bosisio D, Golay J, Polentarutti N, Mantovani A. The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. Blood. 2003 Aug 1;102(3):956-63.

100. Eaton-Bassiri A, Dillon SB, Cunningham M, Rycyzyn MA, Mills J, Sarisky RT, et al. Toll-like receptor 9 can be expressed at the cell surface of distinct populations of tonsils and human peripheral blood mononuclear cells. Infect Immun. 2004 Dec;72(12):7202-11.

101. Xu W, Santini PA, Matthews AJ, Chiu A, Plebani A, He B, et al. Viral double-stranded RNA triggers Ig class switching by activating upper respiratory mucosa B cells through an innate TLR3 pathway involving BAFF. J Immunol. 2008 Jul 1;181(1):276-87.

102. Hausmann M, Kiessling S, Mestermann S, Webb G, Spottl T, Andus T, et al. Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. Gastroenterology. 2002 Jun;122(7):1987-2000.

103. Lombardi V, Van Overtvelt L, Horiot S, Moingeon P. Human dendritic cells stimulated via TLR7 and/or TLR8 induce the sequential production of Il-10, IFN-gamma, and IL-17A by naive CD4+ T cells. J Immunol. 2009 Mar 15;182(6):3372-9.

104. Schulz O, Diebold SS, Chen M, Naslund TI, Nolte MA, Alexopoulou L, et al. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature. 2005 Feb 24;433(7028):887-92.

105. Lien E, Sellati TJ, Yoshimura A, Flo TH, Rawadi G, Finberg RW, et al. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. J Biol Chem. 1999 Nov 19;274(47):33419-25.

106. Verthelyi D, Zeuner RA. Differential signaling by CpG DNA in DCs and B cells: not just TLR9. Trends Immunol. 2003 Oct;24(10):519-22.

107. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol. 2002 Jul 1;169(1):10-4.

108. Wyllie DH, Kiss-Toth E, Visintin A, Smith SC, Boussouf S, Segal DM, et al. Evidence for an accessory protein function for Toll-like receptor 1 in anti-bacterial responses. J Immunol. 2000 Dec 15;165(12):7125-32.

109. Ogus AC, Yoldas B, Ozdemir T, Uguz A, Olcen S, Keser I, et al. The Arg753GLn polymorphism of the human toll-like receptor 2 gene in tuberculosis disease. Eur Respir J. 2004 Feb;23(2):219-23.

110. Abreu MT, Arditi M. Innate immunity and toll-like receptors: clinical implications of basic science research. J Pediatr. 2004 Apr;144(4):421-9.

111. Zanin-Zhorov A, Nussbaum G, Franitza S, Cohen IR, Lider O. T cells respond to heat shock protein 60 via TLR2: activation of adhesion and inhibition of chemokine receptors. Faseb J. 2003 Aug;17(11):1567-9.

112. Kulka M, Alexopoulou L, Flavell RA, Metcalfe DD. Activation of mast cells by double-stranded RNA: evidence for activation through Toll-like receptor 3. J Allergy Clin Immunol. 2004 Jul;114(1):174-82.

113. Tal G, Mandelberg A, Dalal I, Cesar K, Somekh E, Tal A, et al. Association between common Toll-like receptor 4 mutations and severe respiratory syncytial virus disease. J Infect Dis. 2004 Jun 1;189(11):2057-63.

114. Hawn TR, Verbon A, Lettinga KD, Zhao LP, Li SS, Laws RJ, et al. A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to legionnaires' disease. J Exp Med. 2003 Nov 17;198(10):1563-72.

115. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. Nature. 2000 Dec 7;408(6813):740-5.

116. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004 Jul;4(7):499-511.

117. Chen F, Sun SC, Kuh DC, Gaydos LJ, Demers LM. Essential role of NF-kappa B activation in silica-induced inflammatory mediator production in macrophages. Biochem Biophys Res Commun. 1995 Sep 25;214(3):985-92.

118. Yamamoto M, Sato S, Hemmi H, Sanjo H, Uematsu S, Kaisho T, et al. Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. Nature. 2002 Nov 21;420(6913):324-9.

119. Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, et al. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J Exp Med. 2003 Oct 6;198(7):1043-55.

120. Kurt-Jones EA, Popova L, Kwinn L, Haynes LM, Jones LP, Tripp RA, et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol. 2000 Nov;1(5):398-401.

121. Doyle SE, O'Connell R, Vaidya SA, Chow EK, Yee K, Cheng G. Toll-like receptor 3 mediates a more potent antiviral response than Toll-like receptor 4. J Immunol. 2003 Apr 1;170(7):3565-71.

122. Schaub B, Bellou A, Gibbons FK, Velasco G, Campo M, He H, et al. TLR2 and TLR4 stimulation differentially induce cytokine secretion in human neonatal, adult, and murine mononuclear cells. J Interferon Cytokine Res. 2004 Sep;24(9):543-52.

123. Gorden KB, Gorski KS, Gibson SJ, Kedl RM, Kieper WC, Qiu X, et al. Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8. J Immunol. 2005 Feb 1;174(3):1259-68.

124. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science. 2004 Mar 5;303(5663):1529-31.

125. Monick MM, Yarovinsky TO, Powers LS, Butler NS, Carter AB, Gudmundsson G, et al. Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. J Biol Chem. 2003 Dec 26;278(52):53035-44.

126. Joosten LA, Koenders MI, Smeets RL, Heuvelmans-Jacobs M, Helsen MM, Takeda K, et al. Toll-like receptor 2 pathway drives streptococcal cell wall-induced joint inflammation: critical role of myeloid differentiation factor 88. J Immunol. 2003 Dec 1;171(11):6145-53.

127. Sabroe I, Prince LR, Jones EC, Horsburgh MJ, Foster SJ, Vogel SN, et al. Selective roles for Toll-like receptor (TLR)2 and TLR4 in the regulation of neutrophil activation and life span. J Immunol. 2003 May 15;170(10):5268-75.

128. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol. 2004 Oct;5(10):987-95.

129. Drennan MB, Nicolle D, Quesniaux VJ, Jacobs M, Allie N, Mpagi J, et al. Toll-like receptor 2-deficient mice succumb to Mycobacterium tuberculosis infection. Am J Pathol. 2004 Jan;164(1):49-57.

130. Aleman M, Schierloh P, de la Barrera SS, Musella RM, Saab MA, Baldini M, et al. Mycobacterium tuberculosis triggers apoptosis in peripheral neutrophils involving toll-like receptor 2 and p38 mitogen protein kinase in tuberculosis patients. Infect Immun. 2004 Sep;72(9):5150-8.

131. Aravalli RN, Hu S, Lokensgard JR. Toll-like receptor 2 signaling is a mediator of apoptosis in herpes simplex virus-infected microglia. J Neuroinflammation. 2007;4:11.

132. Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T

helper type 1-polarizing program in dendritic cells. Nat Immunol. 2005 Aug;6(8):769-76.

133. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science. 2003 Feb 14;299(5609):1033-6.

134. Viglianti GA, Lau CM, Hanley TM, Miko BA, Shlomchik MJ, Marshak-Rothstein A. Activation of autoreactive B cells by CpG dsDNA. Immunity. 2003 Dec;19(6):837-47.

135. Waldner H, Collins M, Kuchroo VK. Activation of antigen-presenting cells by microbial products breaks self tolerance and induces autoimmune disease. J Clin Invest. 2004 Apr;113(7):990-7.

136. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet. 2000 Jun;25(2):187-91.

137. Franchimont D, Vermeire S, El Housni H, Pierik M, Van Steen K, Gustot T, et al. Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. Gut. 2004 Jul;53(7):987-92.

138. Kamath AT, Sheasby CE, Tough DF. Dendritic cells and NK cells stimulate bystander T cell activation in response to TLR agonists through secretion of IFN-alpha beta and IFN-gamma. J Immunol. 2005 Jan 15;174(2):767-76.

139. Orinska Z, Bulanova E, Budagian V, Metz M, Maurer M, Bulfone-Paus S. TLR3-induced activation of mast cells modulates CD8+ T-cell recruitment. Blood. 2005 Aug 1;106(3):978-87.

140. Imanishi T, Hara H, Suzuki S, Suzuki N, Akira S, Saito T. Cutting Edge: TLR2 Directly Triggers Th1 Effector Functions. J Immunol. 2007 Jun 1;178(11):6715-9.

141. Cottalorda A, Verschelde C, Marcais A, Tomkowiak M, Musette P, Uematsu S, et al. TLR2 engagement on CD8 T cells lowers the threshold for optimal antigen-induced T cell activation. Eur J Immunol. 2006 Jul;36(7):1684-93.

142. Vollmer J, Weeratna RD, Jurk M, Samulowitz U, McCluskie MJ, Payette P, et al. Oligodeoxynucleotides lacking CpG dinucleotides mediate Toll-like receptor 9 dependent T helper type 2 biased immune stimulation. Immunology. 2004 Oct;113(2):212-23.

143. Mazzoni A, Segal DM. Controlling the Toll road to dendritic cell polarization. J Leukoc Biol. 2004 May;75(5):721-30.

144. Querec T, Bennouna S, Alkan S, Laouar Y, Gorden K, Flavell R, et al. Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. J Exp Med. 2006 Feb 20;203(2):413-24.

145. Peng G, Guo Z, Kiniwa Y, Voo KS, Peng W, Fu T, et al. Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. Science. 2005 Aug 26;309(5739):1380-4.

146. Borsutzky S, Kretschmer K, Becker PD, Muhlradt PF, Kirschning CJ, Weiss S, et al. The mucosal adjuvant macrophage-activating lipopeptide-2 directly stimulates B lymphocytes via the TLR2 without the need of accessory cells. J Immunol. 2005 May 15;174(10):6308-13.

147. Hartmann G, Krieg AM. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. J Immunol. 2000 Jan 15;164(2):944-53.

148. Genestier L, Taillardet M, Mondiere P, Gheit H, Bella C, Defrance T. TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. J Immunol. 2007 Jun 15;178(12):7779-86.

149. Hayashi EA, Akira S, Nobrega A. Role of TLR in B cell development: signaling through TLR4 promotes B cell maturation and is inhibited by TLR2. J Immunol. 2005 Jun 1;174(11):6639-47.

150. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature. 1995 Apr 6;374(6522):546-9.

151. Krieg AM. CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol. 2002;20:709-60.

152. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science. 2002 Dec 13;298(5601):2199-202.

153. Jiang W, Lederman MM, Harding CV, Rodriguez B, Mohner RJ, Sieg SF. TLR9 stimulation drives naive B cells to proliferate and to attain enhanced antigen presenting function. Eur J Immunol. 2007 Jul 10;37(8):2205-13.

154. Bernasconi NL, Onai N, Lanzavecchia A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. Blood. 2003 Jun 1;101(11):4500-4.

155. Barr TA, Brown S, Ryan G, Zhao J, Gray D. TLR-mediated stimulation of APC: Distinct cytokine responses of B cells and dendritic cells. Eur J Immunol. 2007 Nov;37(11):3040-53.

156. He B, Qiao X, Cerutti A. CpG DNA induces IgG class switch DNA recombination by activating human B cells through an innate pathway that requires TLR9 and cooperates with IL-10. J Immunol. 2004 Oct 1;173(7):4479-91.

157. Ruprecht CR, Lanzavecchia A. Toll-like receptor stimulation as a third signal required for activation of human naive B cells. Eur J Immunol. 2006 Apr;36(4):810-6.

158. Pasare C, Medzhitov R. Control of B-cell responses by Toll-like receptors. Nature. 2005 Nov 17;438(7066):364-8.

159. Han JH, Akira S, Calame K, Beutler B, Selsing E, Imanishi-Kari T. Class switch recombination and somatic hypermutation in early mouse B cells are mediated by B cell and Toll-like receptors. Immunity. 2007 Jul;27(1):64-75.

160. Yerkovich ST, Wikstrom ME, Suriyaarachchi D, Prescott SL, Upham JW, Holt PG. Postnatal development of monocyte cytokine responses to bacterial lipopolysaccharide. Pediatr Res. 2007 Nov;62(5):547-52.

161. Gold MC, Donnelly E, Cook MS, Leclair CM, Lewinsohn DA. Purified neonatal plasmacytoid dendritic cells overcome intrinsic maturation defect with TLR agonist stimulation. Pediatr Res. 2006 Jul;60(1):34-7.

162. Levy O, Suter EE, Miller RL, Wessels MR. Unique efficacy of Toll-like receptor 8 agonists in activating human neonatal antigen-presenting cells. Blood. 2006 Apr 25.

163. Capolunghi F, Cascioli S, Giorda E, Rosado MM, Plebani A, Auriti C, et al. CpG drives human transitional B cells to terminal differentiation and production of natural antibodies. J Immunol. 2008 Jan 15;180(2):800-8.

164. Sun CM, Deriaud E, Leclerc C, Lo-Man R. Upon TLR9 signaling, CD5+ B cells control the IL-12-dependent Th1-priming capacity of neonatal DCs. Immunity. 2005 Apr;22(4):467-77.

165. Walker WE, Goldstein DR. Neonatal B cells suppress innate toll-like receptor immune responses and modulate alloimmunity. J Immunol. 2007 Aug 1;179(3):1700-10.

166. Zhang X, Deriaud E, Jiao X, Braun D, Leclerc C, Lo-Man R. Type I interferons protect neonates from acute inflammation through interleukin 10-producing B cells. J Exp Med. 2007 May 14;204(5):1107-18.

167. Sadeghi K, Berger A, Langgartner M, Prusa AR, Hayde M, Herkner K, et al. Immaturity of infection control in preterm and term newborns is associated with impaired toll-like receptor signaling. J Infect Dis. 2007 Jan 15;195(2):296-302.

168. Al-Hertani W, Yan SR, Byers DM, Bortolussi R. Human newborn polymorphonuclear neutrophils exhibit decreased levels of MyD88 and attenuated p38 phosphorylation in response to lipopolysaccharide. Clin Invest Med. 2007;30(2):E44-53.

169. Aksoy E, Albarani V, Nguyen M, Laes JF, Ruelle JL, De Wit D, et al. Interferon regulatory factor 3-dependent responses to lipopolysaccharide are selectively blunted in cord blood cells. Blood. 2007 Apr 1;109(7):2887-93.

170. Guay HM, Andreyeva TA, Garcea RL, Welsh RM, Szomolanyi-Tsuda E. MyD88 is required for the formation of long-term humoral immunity to virus infection. J Immunol. 2007 Apr 15;178(8):5124-31.

171. Raman VS, Lind EF, Benson MJ, Noelle RJ. Strategies for selective priming of memory B cells. Immunol Lett. 2007 Apr 15;109(2):93-100.

172. Siegrist CA, Pihlgren M, Tougne C, Efler SM, Morris ML, AlAdhami MJ, et al. Co-administration of CpG oligonucleotides enhances the late affinity maturation process of human anti-hepatitis B vaccine response. Vaccine. 2004 Dec 16;23(5):615-22.

173. Weeratna RD, Makinen SR, McCluskie MJ, Davis HL. TLR agonists as vaccine adjuvants: comparison of CpG ODN and Resiquimod (R-848). Vaccine. 2005 Nov 1;23(45):5263-70.

174. Tudor D, Dubuquoy C, Gaboriau V, Lefevre F, Charley B, Riffault S. TLR9 pathway is involved in adjuvant effects of plasmid DNA-based vaccines. Vaccine. 2005 Jan 26;23(10):1258-64.

175. Kamath AT, Rochat AF, Valenti MP, Agger EM, Lingnau K, Andersen P, et al. Adult-like anti-mycobacterial T cell and in vivo dendritic cell responses following neonatal immunization with Ag85B-ESAT-6 in the IC31 adjuvant. PLoS ONE. 2008;3(11):e3683.

176. Weeratna RD, Brazolot Millan CL, McCluskie MJ, Davis HL. CpG ODN can re-direct the Th bias of established Th2 immune responses in adult and young mice. FEMS Immunol Med Microbiol. 2001 Dec;32(1):65-71.

177. Hancock GE, Heers KM, Pryharski KS, Smith JD, Tiberio L. Adjuvants recognized by toll-like receptors inhibit the induction of polarized type 2 T cell

responses by natural attachment (G) protein of respiratory syncytial virus. Vaccine. 2003 Oct 1;21(27-30):4348-58.

178. Gavin AL, Hoebe K, Duong B, Ota T, Martin C, Beutler B, et al. Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. Science. 2006 Dec 22;314(5807):1936-8.

179. Goriely S, Goldman M. From tolerance to autoimmunity: is there a risk in early life vaccination? J Comp Pathol. 2007 Jul;137 Suppl 1:S57-61.

180. Rubin LA, Kurman CC, Biddison WE, Goldman ND, Nelson DL. A monoclonal antibody 7G7/B6, binds to an epitope on the human interleukin-2 (IL-2) receptor that is distinct from that recognized by IL-2 or anti-Tac. Hybridoma. 1985 Summer;4(2):91-102.

181. Lanier LL, Warner NL. Paraformaldehyde fixation of hematopoietic cells for quantitative flow cytometry (FACS) analysis. J Immunol Methods. 1981;47(1):25-30.

182. Elliott SR, Macardle PJ, Zola H. Removal of erythroid cells from umbilical cord blood mononuclear cell preparations using magnetic beads and a monoclonal antibody against glycophorin A. J Immunol Methods. 1998 Aug 1;217(1-2):121-30.

183. Pires V, Harab RC, Olej B, Rumjanek VM. Ouabain effects on activated lymphocytes: augmentation of CD25 expression on TPA-stimulated cells and of CD69 on PHA-and TPA-stimulated cells. Int J Immunopharmacol. 1997 Mar;19(3):143-8.

184. Brunner T, Mogil RJ, LaFace D, Yoo NJ, Mahboubi A, Echeverri F, et al. Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. Nature. 1995 Feb 2;373(6513):441-4.

185. Crow MK, Jover JA, Friedman SM. Direct T helper-B cell interactions induce an early B cell activation antigen. J Exp Med. 1986 Nov 1;164(5):1760-72.

186. Nashar TO, Hirst TR, Williams NA. Modulation of B-cell activation by the B subunit of Escherichia coli enterotoxin: receptor interaction up-regulates MHC class II, B7, CD40, CD25 and ICAM-1. Immunology. 1997 Aug;91(4):572-8.

187. Zola H, Neoh SH, Mantzioris BX, Webster J, Loughnan MS. Detection by immunofluorescence of surface molecules present in low copy numbers. High sensitivity staining and calibration of flow cytometer. J Immunol Methods. 1990 Dec 31;135(1-2):247-55.

188. Schmid I, Ferbas J, Uittenbogaart CH, Giorgi JV. Flow cytometric analysis of live cell proliferation and phenotype in populations with low viability. Cytometry. 1999 Jan 1;35(1):64-74.

189. Lampariello F. On the use of the Kolmogorov-Smirnov statistical test for immunofluorescence histogram comparison. Cytometry. 2000 Mar 1;39(3):179-88.

190. Viemann D, Dubbel G, Schleifenbaum S, Harms E, Sorg C, Roth J. Expression of toll-like receptors in neonatal sepsis. Pediatr Res. 2005 Oct;58(4):654-9.

191. McCullagh P, Nelder JA. Generalized Linear Models. 2nd Edition ed. Boca Raton, Florida: Chapman and Hall/CRC; 1989.

192. Shah VO, Civin CI, Loken MR. Flow cytometric analysis of human bone marrow. IV. Differential quantitative expression of T-200 common

leukocyte antigen during normal hemopoiesis. J Immunol. 1988 Mar 15;140(6):1861-7.

193. Loken MR, Shah VO, Dattilio KL, Civin CI. Flow cytometric analysis of human bone marrow: I. Normal erythroid development. Blood. 1987 Jan;69(1):255-63.

194. Zhao J, Kim KD, Yang X, Auh S, Fu YX, Tang H. Hyper innate responses in neonates lead to increased morbidity and mortality after infection. Proc Natl Acad Sci U S A. 2008 May 27;105(21):7528-33.

195. Cognasse F, Hamzeh-Cognasse H, Lafarge S, Chavarin P, Pozzetto B, Richard Y, et al. Identification of two subpopulations of purified human blood B cells, CD27(-) CD23(+) and CD27(high) CD80(+), that strongly express cell surface Toll-like receptor 9 and secrete high levels of interleukin-6. Immunology. 2008 Apr 28.

196. Jiang W, Lederman MM, Mohner RJ, Rodriguez B, Nedrich TM, Harding CV, et al. Impaired naive and memory B-cell responsiveness to TLR9 stimulation in human immunodeficiency virus infection. J Virol. 2008 Aug;82(16):7837-45.

197. Wagner M, Poeck H, Jahrsdoerfer B, Rothenfusser S, Prell D, Bohle B, et al. IL-12p70-dependent Th1 induction by human B cells requires combined activation with CD40 ligand and CpG DNA. J Immunol. 2004 Jan 15;172(2):954-63.

198. Gururajan M, Jacob J, Pulendran B. Toll-like receptor expression and responsiveness of distinct murine splenic and mucosal B-cell subsets. PLoS ONE. 2007;2(9):e863.

199. Meyer-Bahlburg A, Khim S, Rawlings DJ. B cell intrinsic TLR signals amplify but are not required for humoral immunity. J Exp Med. 2007 Dec 24;204(13):3095-101.

200. Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, et al. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med. 1999 Jun 7;189(11):1777-82.

201. Krocova Z, Hartlova A, Souckova D, Zivna L, Kroca M, Rudolf E, et al. Interaction of B cells with intracellular pathogen Francisella tularensis. Microb Pathog. 2008 Aug;45(2):79-85.

202. Faure E, Thomas L, Xu H, Medvedev A, Equils O, Arditi M. Bacterial lipopolysaccharide and IFN-gamma induce Toll-like receptor 2 and Toll-like receptor 4 expression in human endothelial cells: role of NF-kappa B activation. J Immunol. 2001 Feb 1;166(3):2018-24.

203. Nishiya T, DeFranco AL. Ligand-regulated chimeric receptor approach reveals distinctive subcellular localization and signaling properties of the Toll-like receptors. J Biol Chem. 2004 Apr 30;279(18):19008-17.

204. Bekeredjian-Ding IB, Wagner M, Hornung V, Giese T, Schnurr M, Endres S, et al. Plasmacytoid dendritic cells control TLR7 sensitivity of naive B cells via type I IFN. J Immunol. 2005 Apr 1;174(7):4043-50.

205. Huggins J, Pellegrin T, Felgar RE, Wei C, Brown M, Zheng B, et al. CpG DNA activation and plasma-cell differentiation of CD27- naive human B cells. Blood. 2007 Feb 15;109(4):1611-9.

206. Gantner F, Hermann P, Nakashima K, Matsukawa S, Sakai K, Bacon KB. CD40-dependent and -independent activation of human tonsil B cells by CpG oligodeoxynucleotides. Eur J Immunol. 2003 Jun;33(6):1576-85.

207. Fuss IJ, Kanof ME, Smith PD, Zola H. Isolation of whole mononuclear cells from peripheral blood and cord blood. Curr Protoc Immunol. 2009 Apr;Chapter 7:Unit7 1.

208. Dollner H, Vatten L, Austgulen R. Early diagnostic markers for neonatal sepsis: comparing C-reactive protein, interleukin-6, soluble tumour necrosis factor receptors and soluble adhesion molecules. J Clin Epidemiol. 2001 Dec;54(12):1251-7.

209. Agematsu K, Hokibara S, Nagumo H, Shinozaki K, Yamada S, Komiyama A. Plasma cell generation from B-lymphocytes via CD27/CD70 interaction. Leuk Lymphoma. 1999 Oct;35(3-4):219-25.

210. Harter L, Mica L, Stocker R, Trentz O, Keel M. Increased expression of toll-like receptor-2 and -4 on leukocytes from patients with sepsis. Shock. 2004 Nov;22(5):403-9.

211. Marshall JD, Fearon K, Abbate C, Subramanian S, Yee P, Gregorio J, et al. Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. J Leukoc Biol. 2003 Jun;73(6):781-92.

212. Richard K, Pierce SK, Song W. The agonists of TLR4 and 9 are sufficient to activate memory B cells to differentiate into plasma cells in vitro but not in vivo. J Immunol. 2008 Aug 1;181(3):1746-52.

213. Ueda Y, Liao D, Yang K, Patel A, Kelsoe G. T-independent activation-induced cytidine deaminase expression, class-switch recombination, and antibody production by immature/transitional 1 B cells. J Immunol. 2007 Mar 15;178(6):3593-601.

214. Reid GS, She K, Terrett L, Food MR, Trudeau JD, Schultz KR. CpG stimulation of precursor B-lineage acute lymphoblastic leukemia induces a distinct change in costimulatory molecule expression and shifts allogeneic T cells toward a Th1 response. Blood. 2005 May 1;105(9):3641-7.

215. Lougaris V, Badolato R, Ferrari S, Plebani A. Hyper immunoglobulin M syndrome due to CD40 deficiency: clinical, molecular, and immunological features. Immunol Rev. 2005 Feb;203:48-66.

216. Chuang TH, Ulevitch RJ. Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. Eur Cytokine Netw. 2000 Sep;11(3):372-8.

217. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science. 2004 Mar 5;303(5663):1526-9.

218. Hacker H, Mischak H, Miethke T, Liptay S, Schmid R, Sparwasser T, et al. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. Embo J. 1998 Nov 2;17(21):6230-40.

219. Lee J, Chuang TH, Redecke V, She L, Pitha PM, Carson DA, et al. Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: activation of Toll-like receptor 7. Proc Natl Acad Sci U S A. 2003 May 27;100(11):6646-51.

220. Heil F, Ahmad-Nejad P, Hemmi H, Hochrein H, Ampenberger F, Gellert T, et al. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. Eur J Immunol. 2003 Nov;33(11):2987-97.

221. Nishiya T, Kajita E, Miwa S, Defranco AL. TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements. J Biol Chem. 2005 Nov 4;280(44):37107-17.

222. Tehranchi R, Fadeel B, Forsblom AM, Christensson B, Samuelsson J, Zhivotovsky B, et al. Granulocyte colony-stimulating factor inhibits spontaneous cytochrome c release and mitochondria-dependent apoptosis of myelodysplastic syndrome hematopoietic progenitors. Blood. 2003 Feb 1;101(3):1080-6.

223. Jegerlehner A, Maurer P, Bessa J, Hinton HJ, Kopf M, Bachmann MF. TLR9 Signaling in B Cells Determines Class Switch Recombination to IgG2a. J Immunol. 2007 Feb 15;178(4):2415-20.

224. Speiser DE, Lienard D, Rufer N, Rubio-Godoy V, Rimoldi D, Lejeune F, et al. Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. J Clin Invest. 2005 Mar;115(3):739-46.