Expression and Distribution of Endoglycan on B cells

by

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B.Sc. (H), The University of British Columbia, 2002

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Genetics)

THE UNIVERSITY OF BRITISH COLUMBIA

July 2005

ABSTRACT

Endoglycan is the third and newest member of the CD34-family (CD34, Podocalyxin and Endoglycan) of sialomucins. Although all three molecules are markers of early hematopoietic progenitors and vascular associated tissues, they also have additional unique distribution patterns. In most tissues, CD34 and Podocalyxin have been shown to act as anti-adhesives, most likely due to the strong negative charges conferred by terminal sialic acid residues on their highly glycosylated mucin domains (Doyonnas et al., 2001 and unpublished data). We have demonstrated that ectopic expression of CD34 on murine mast cells, or Podocalyxin in breast cancer cells, induces a profound reduction in homotypic aggregation/adhesion. Surprisingly, ectopic expression of Endoglycan in similar assays does not decrease aggregation, suggesting that it may have a different function. In addition, most mucins (including CD34 and Podocalyxin) do not show a high degree of interspecific sequence conservation. However, Endoglycan shows a strikingly high degree of conservation across species. Therefore, the conservation in the Endoglycan sequence may reflect the maintenance of a specific binding domain for extracellular ligand(s). Hence, we propose that Endoglycan has a pro-adhesive function and act as an antagonist of Podocalyxin and CD34.

Using an anti-Endoglycan monoclonal antibody (F4B10) generated in our lab, along with RT-PCR data, we determined the general distribution of Endoglycan. In addition to early hematopoietic progenitors and vascular associated tissues, Endoglycan is expressed on macrophages, thymocytes and lipopolysaccharide (LPS)-stimulated B cells. A survey of splenic B cells and immature B cells showed that Endoglycan is expressed on $IgM^{med/hi}IgD^{lo}CD11b^{med}CD21^{med/hi}CD22^{hi}CD23^{lo}$ subset of splenocytes. These cells are characteristic of marginal zone B cells, which have the phenotype: IgM^{hi}, IgD^{lo}, $CD21^{hi}$, $CD22^{hi}$, $CD23^{lo}$ and $CD1^{hi}$. Furthermore, I did not detect Endoglycan expression on immature or pre-B cells. My results show that Endoglycan is up-regulated on LPS-stimulated B220-positive splenocytes. In these cultures, Endoglycan is expressed on a subset of plasma cells (as indicated by the marker syndecan-1 (SDC1)), and all Endoglycan-positive cells express β1-integrin. We have evaluated the time course of Endoglycan induction by LPS using FACS and RT-PCR analysis, cell surface expression peaks at 48hr and Endoglycan transcript levels peaks at 24hr. The induction of

Endoglycan is specific to TLR-stimulations and is not induced by other B cell activators, such as anti-IgM, anti-CD40 or BAFF.

Since T cell cytokines play a large role in the regulation of B cell activation and maturation, I examined the effect of T cell secreted cytokines on Endoglycan expression. I found that IL-2, IL-4, IL-5 and TGFβ reduce the up-regulation of Endolgycan by LPS on splenic B cells. On the other hand, IL-1β increases Endoglycan expression. The regulated expression of Endoglycan suggests that this molecule may serve an important role on activated B cells.

The data in this thesis provide the first description of Endoglycan expression and regulation on B cells. Endoglycan is a highly specific marker for TLR-activated B cells. Expression of β1-integrin on these Endoglycan-positive plasma cells suggests that these cells may home to specific niches for the establishment of long-term antibody repertoire. Therefore, Endoglycan may have a role in homing and migration of these cells.

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β-Me	beta-mercaptoethanol
Ab	antibody
BAFF	B cell activating factor belonging to the TNF family
BCR	B cell receptor
Blimp-1	B-lymphocyte-induced maturation protein 1
BM	bone marrow
bp	base pair
C2GnTI	core2 β(1,6)-N-acetylglucosaminyltransferase I
CD40L	clustered domain 40 ligand
cDNA	complement deoxyribonucleic acid
CK2	casein kinase II
CLP	common lymphoid progenitor
Co-Smad	common mediator Smad
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DTHL	aspartic acid, threonine, histidine, leucine
ELISA	enzyme-linked immunosorbent assay
ERM	ezrin/radixin/moesin
EST	expressed sequence tag
FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
FDC	follicular dendritic cell
FITC	fluorescein isothiocyanate
FO	follicular
Fr.	fractions
FTVII	α(1,3)-fucosyltransferase VII
GADD45	growth arrest and DNA-damage-inducible 45
HAT	hypoxanthine, aminopterin, thymidine
HBSS	Hank's balanced saline solution
HSA	heat-stable antigen
HSC	hematopoietic stem cell
ICOS	inducible T-cell co-stimulator
IFNγ	interferon γ

Ig	immunoglobulin
IgH	immunoglobulin heavy
IL-1β	interleukin 1β
IL-10	interleukin 10
IL-13	interleukin 13
IL-2	interleukin 2
IL-3	interleukin 3
IL-4	interleukin 4
IL-5	interleukin 5
IL-6	interleukin 6
IL-7Rα	IL-7 receptor α chain
IRAK	IL-1 receptor-associated kinases
IRF	interferon-regulatory factor
kb	kilobase
kDa	kiloDalton
KO	knockout
LN	lymph node
LPS	lipopolysaccharide
MARCO	macrophage receptor with collagenous structure
MFI	mean fluorescence intensity
MLP	multilineage progenitor
MR	mature recirculating
MyD	myeloid differentiation protein
MZ	marginal zone
MZM	marginal zone macrophage
NF	newly formed
NHERF-1	Na+/H+ exchanger regulatory factor-1
NK	natural killer
OD	optical density
PALS	periarteriolar lymphoid sheath
PAMPS	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDZ	PSD-95/Drosophila disk large (Dlg)-1/ZO-1

PE	phycoerythrin
PKC	protein kinase C
PRR	pattern recognition receptors
PSGL-1	P-selectin glycoprotein ligand-1
PW	peritoneal wash
RAG	recombination activating gene
RHIM	RIP homotypic interaction motif
RPMI	Roswell Park Memorial Institute
R-Smad	receptor-activated Smad
RT-PCR	reverse transcriptase polymerase chain reaction
S1P	lysophospholipid sphingosine-1 phosphate
SCID	severe combined immunodeficiency
SDC1	syndecan-1
SLC	surrogate light chain
STAT	signal transducers and activators of transcription
TβRI	type I TGFβ receptor
TβRII	type II TGFβ receptor
T1	transitional stage 1
T2	transitional stage 2
T3	transitional stage 3
TAK1	TGFβ activated kinase 1
TGFβ	transforming growth factor β
TI-2	T-cell independent type 2
TIR	Toll-interleukin 1 receptor
TLR	toll-like receptor
TNFα	tumour necrosis factor α
TRAF	TNF-receptor associated factor
VCAM-1	vascular cell-adhesion molecule 1

ACKNOWLEDGEMENTS

I would like to thank all the individuals who have provided me guidance and assistance throughout the course of my studies. My supervisor, Dr. Kelly McNagny, provided constant support, encouragement and mentorship. I am grateful to him for giving me the opportunity to study in his lab. I would like to thank members of the McNagny lab, both past and present, especially Helen Merkens, Shierley Chelliah, Erin Drew, Sebastian Furness and Poh Tan. They have not only provided training and technical assistance, but also for their support, stimulating discussions and friendship. In addition, thanks to the members of my committee, Dr. Michael Gold and Dr. Fabio Rossi, for their suggestions and mentorship at multiple committee meetings.

Over the years, I have had the opportunity to be involved in various collaborations. I would like to thank Muriel David (Schrader Lab), Kevin Lin (Gold Lab) and Arthur Sampaio (Underhill Lab) for their help and guidance; Dr. Mansoor Husain (University of Toronto) for providing the MOVAS cell line; and Dr. Dietmar Vestweber (University of Munster) for providing the anti-PSGL-1 supernatant. As well, I have received advice or reagents from various people both inside and outside of the Biomedical Research Centre (BRC). I would like to acknowledge Nelson Wong (Johnson Lab), Jason Grant (Jefferies Lab), Caylib Durand (Gold Lab), Jeff Duenas (Rossi Lab), Doug Carlow (Ziltener Lab), Jasmeen Merzaban (Ziltener lab), Jennifer Bishop (Finlay Lab) and Brian Ruffell (Johnson Lab).

The BRC provided an enriching environment that fostered cooperation, interaction and scientific discussions and I would like to thank everyone for creating such a wonderful learning environment. However, this superb environment would not have been possible without core staffs of the BRC. I would like to thank Lea Wong and Sam Hoium for their management of the animal facility. As well, I would like to thank Nicole Voglmaier and Les Rollins for their preparation of various reagents for this institute; Takahide Murakami for managing the BRC Genotyping Facility; and Andy Johnson for managing the BRC FACS Facility.

I would like to extend thanks to my family and friends who have been so understanding and encouraging. In particular, I would like to thank Florence Lam, Samantha Mak, David Wong, Eric Yee, Yanni Wang, Jacqueline Lai, Nina Maeshima, Eunice Yao, Dennis Lefevbre and Sie-Lung Tjew for their continuous support. Lastly, research was made possible through funding from Heart and Stroke Foundation of Canada, Stem Cell Network and CIHR/MSFHR Transplantation Research.

1. INTRODUCTION

1.1 CD34 Family

The CD34 family includes three molecules, CD34, Podocalyxin and Endoglycan. The term was coined by Sassetti and colleagues (Sassetti et al., 2000) when they identified human Endoglycan as the third member of this family. These molecules are classified by similar domain structures, as well as similar exon and intron organization (Doyonnas et al., 2001). The most studied member of the CD34-family is CD34. Although little is known about the function of this molecule, the use of CD34 as a marker for the enrichment of hematopoietic stem cells (HSC) began shortly after its discovery in 1988 (Berenson et al., 1988; Sutherland et al., 1996). The other two members, Podocalyxin and Endoglycan, are relatively new in comparison to CD34. However, despite their later discovery, much progress has been made to understanding the function of these molecules in recent years.

Members of the CD34 family are type-I transmembrane sialomucins (Fig. 1.1). Their extracellular region contains a mucin-like domain that is decorated by sialylated O-linked carbohydrate chains. The densely glycosylated domain not only provides an area of high negative charge; it also promotes an extended rod-like structure, allowing these molecules to protrude pass most cell surface molecules. In the extracellular membrane proximal region is a cysteine-rich domain, presumed to fold into immunoglobulin-like structures through disulfide bonds of paired cysteines. The cytoplasmic domain of these molecules is 73-80 amino acids long and is highly conserved through evolution. Moreover, this is also the region with the highest homology between the three family members. The cytoplasmic tail contains potential phosphorylation sites, as well as a potential docking site at the C-terminus for PDZ-containing proteins. All three members are encoded by eight exons, which are alternatively spliced to produce transcripts encoding a protein with either a full length or a cytoplasmically truncated tail (Fig. 1.2, (Doyonnas et al., 2001; Suda et al., 1992). These proteins are expressed on early hematopoietic progenitors and vascular associated tissues; however, each protein also has additional unique distribution patterns. The following two sections will present a brief review of the family members.

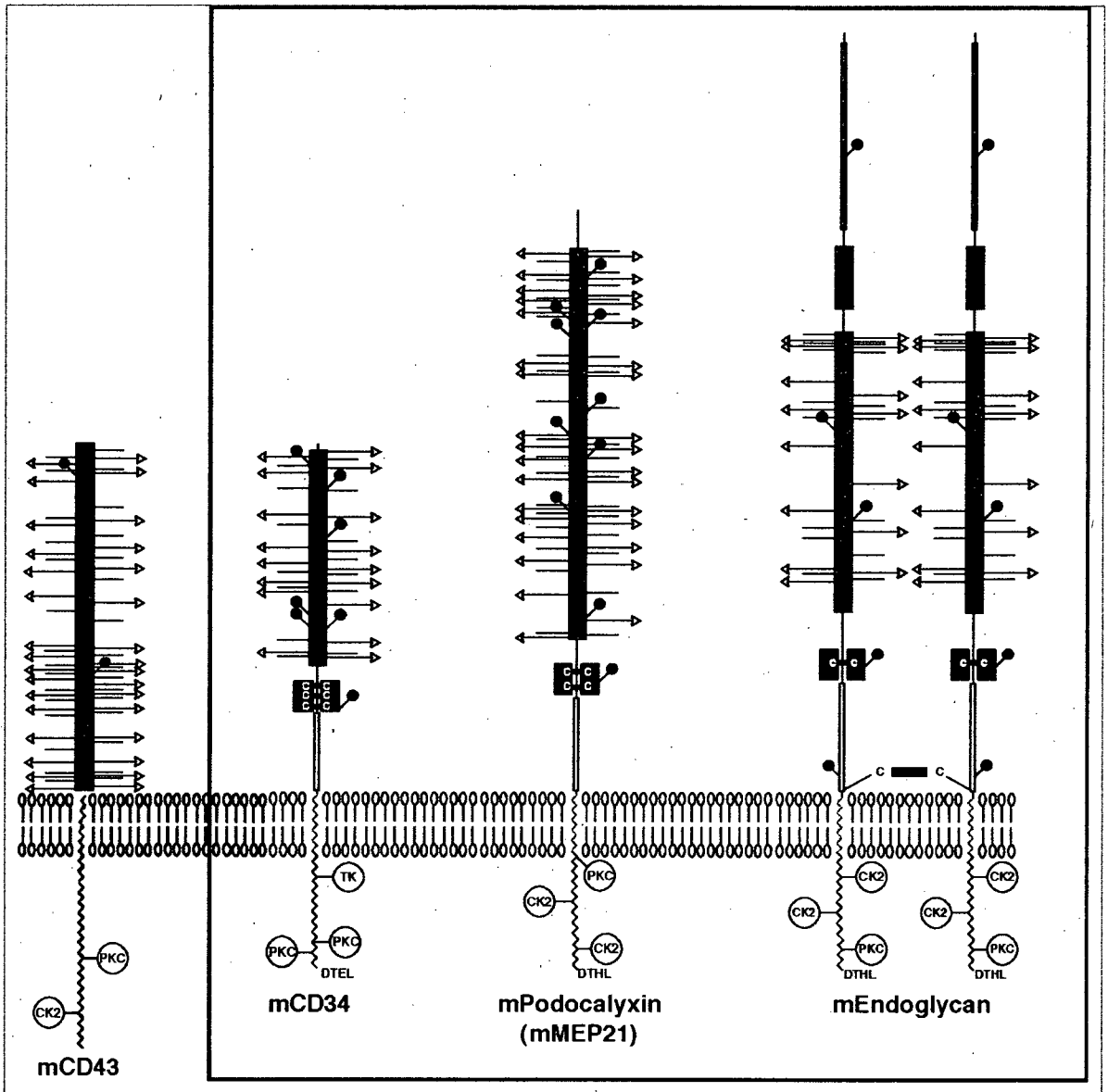


Fig. 1.1: CD34 family. Illustration of the predicted murine structure of CD43 and members of the CD34 family (indicated by black box). Bars, putative O-linked carbohydrates; Black circles, putative N-linked carbohydrates; Triangles, potential sialic acid motifs; Blue boxes; mucin domains; Green boxes, cysteine-rich domain; Pink boxes, glutamate-rich region; PKC, CK2 and TK, potential phosphorylation sites; DTEL or DTHL, potential PDZ-docking sites. Adapted from Doyonnas et al., 2001.

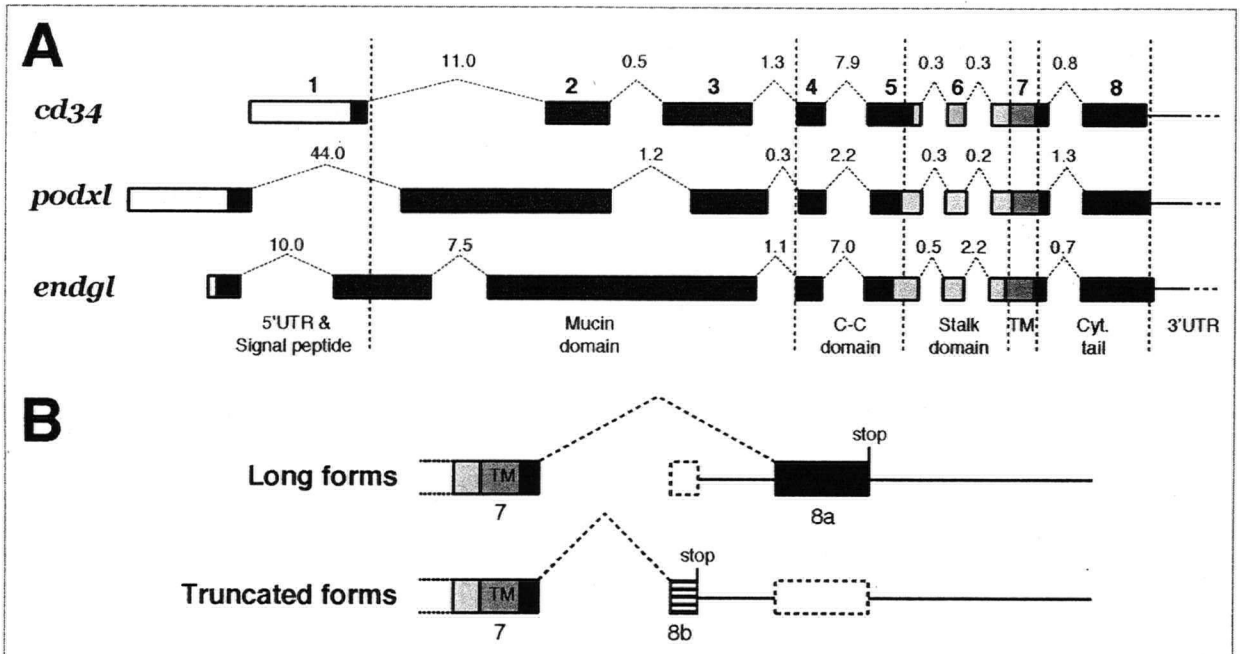


Fig. 1.2: CD34 family domain structure and alternative splicing. A. Genomic organization of murine *cd34*, podocalyxin (*podxl*) and endoglycan (*endgl*). Boxes indicate the eight exons and dotted regions indicate introns, the size of each intron is indicated in kbs. Light purple boxes, 5' UTR; Purple boxes, signal peptide; Blue boxes, mucin domain; Pink box, glutamate-rich region; Green boxes, cysteine-rich dromain; Yellow boxes, stalk domain; Orange boxes, transmembrane (TM) domain; Red boxes, cytoplasmic tail. B. Schematic of the naturally occurring alternative splicing of the cytoplasmic tail. Adapted from Doyonnas et al., 2001.

1.1.1 Overview of CD34 and Podocalyxin

Expression of the CD34-related molecules on hematopoietic progenitors has led to the clinical use of CD34 in the isolation of HSC; however, these molecules are also expressed on distinct mature cell populations (Table 1.1). CD34, for example, is found on murine mast cells (Drew et al., 2002) while Podocalyxin is expressed by platelets (McNagny et al., 1997; Miettinen et al., 1999), embryonic and anemic erythroid cells (Doyonnas et al., 2005; McNagny et al., 1992) and by non-hematopoietic cells like podocytes of the kidney glomerulus (Kershaw et al., 1995). Due to the bulky, glycosylated extracellular domain of these molecules, it has been proposed that they have anti-adhesive properties due to charge repulsion. It has been demonstrated that the lack of CD34 on murine mast cells leads to homotypic aggregation, and that, this molecule is required for the repopulation of peritoneal mast cells (Drew et al., 2005). Similarly, Podocalyxin-deficient mice die shortly after birth from renal defect due to the lost of filtration slits on foot processes of podocytes (Doyonnas et al., 2001).

Both molecules, when expressed on high endothelial venules (HEV) function as L-selectin ligands (Baumhueter et al., 1994; Sassetti et al., 1998). Thus, there is evidence for both pro- and anti-adhesion functions for these molecules. Interestingly, CD34-deficient mice show no difference in neutrophil extravasation in inflammatory response (Cheng et al., 1996) but reduction in eosinophil migration to the lungs in allergic response (Suzuki et al., 1996). These opposing results may be explained by functional compensation between members of the CD34 family.

1.1.2 Overview of Endoglycan

Endoglycan is the third and newest member of the CD34 family. The human form of this molecule was isolated by searching for homology to the cytoplasmic domain of Podocalyxin using GenBank expressed sequence tag (EST). The full length human cDNA was then cloned via PCR using primers designed based on the available mouse sequence (Fig. 1.3, Sassetti et al., 2000). The mouse form of Endoglycan was cloned at the same time as the human form in our laboratory. Through homology to Podocalyxin cytoplasmic tail, an EST clone (accession # W13047) was identified and obtained from IMAGE consortium. This 348bp sequence was labelled with ^{32}P for screening of a 16d mouse cDNA λExlox phage library (Novagen). Clones were isolated, sequenced and aligned resulting in the identification of a 2092bp (605 amino acids) mouse full length Endoglycan sequence.

Despite similar domain structures to CD34 and Podocalyxin, Endoglycan possesses a unique N-terminal acidic domain of 161 amino acids. This region contains three polyglutamate tracts of 5-11 residues each. Moreover, the cysteine-rich domain of Endoglycan contains three cysteines, whereas CD34 and Podocalyxin have six and four cysteines respectively, therefore the unpaired cysteine in Endoglycan permits homodimerization (Sassetti et al., 2000).

The cytoplasmic domain consists of several potential phosphorylation sites for casein kinase II (CK2) and protein kinase C (PKC). The C-terminal amino acid sequence of Endoglycan is DTHL (aspartic acid, threonine, histidine, leucine), which is a potential PDZ-domain docking sequence. By using a λphage screen, our laboratory has isolated Na+/H+ exchanger regulatory factor-1 (NHERF-1) as a potential intracellular binding

partner of Endoglycan. NHERF-1 contains tandem PDZ-domains and an ERM-domain. ERM-domains have been shown to associate with the actin cytoskeleton, therefore, this molecule is an important adaptor for the targeting and trafficking of PDZ-binding proteins (Fanning and Anderson, 1998; Shenolikar and Weinman, 2001).

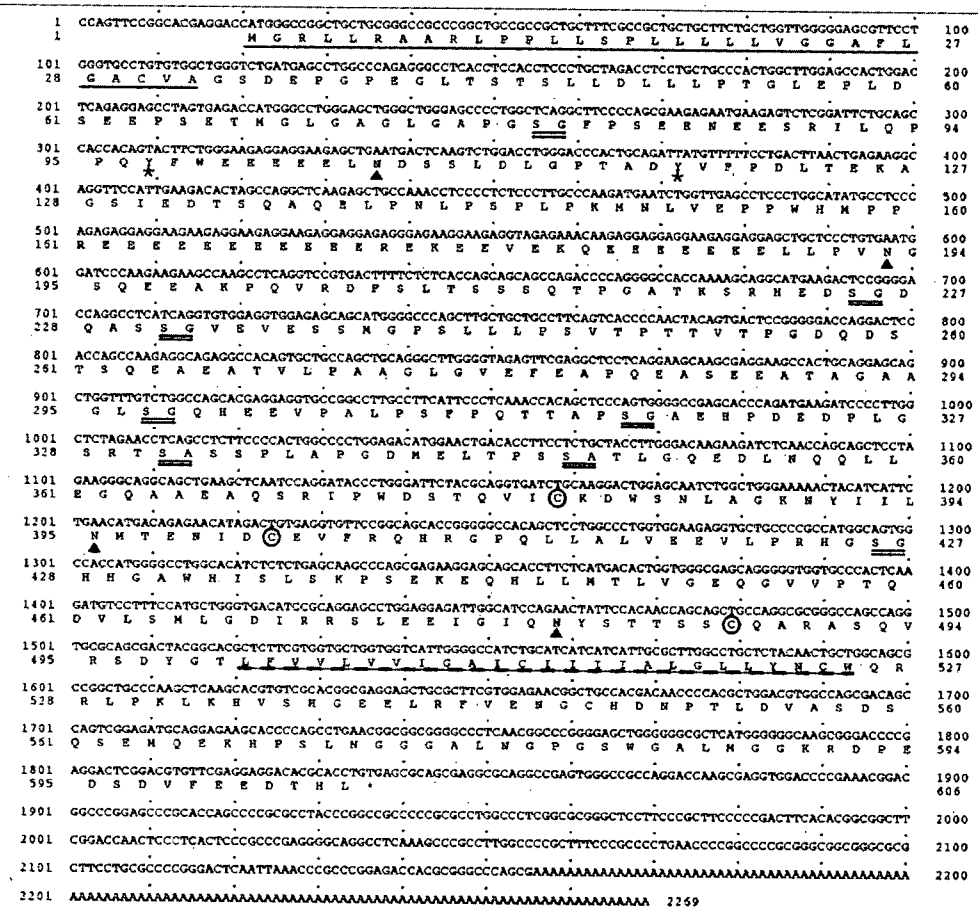
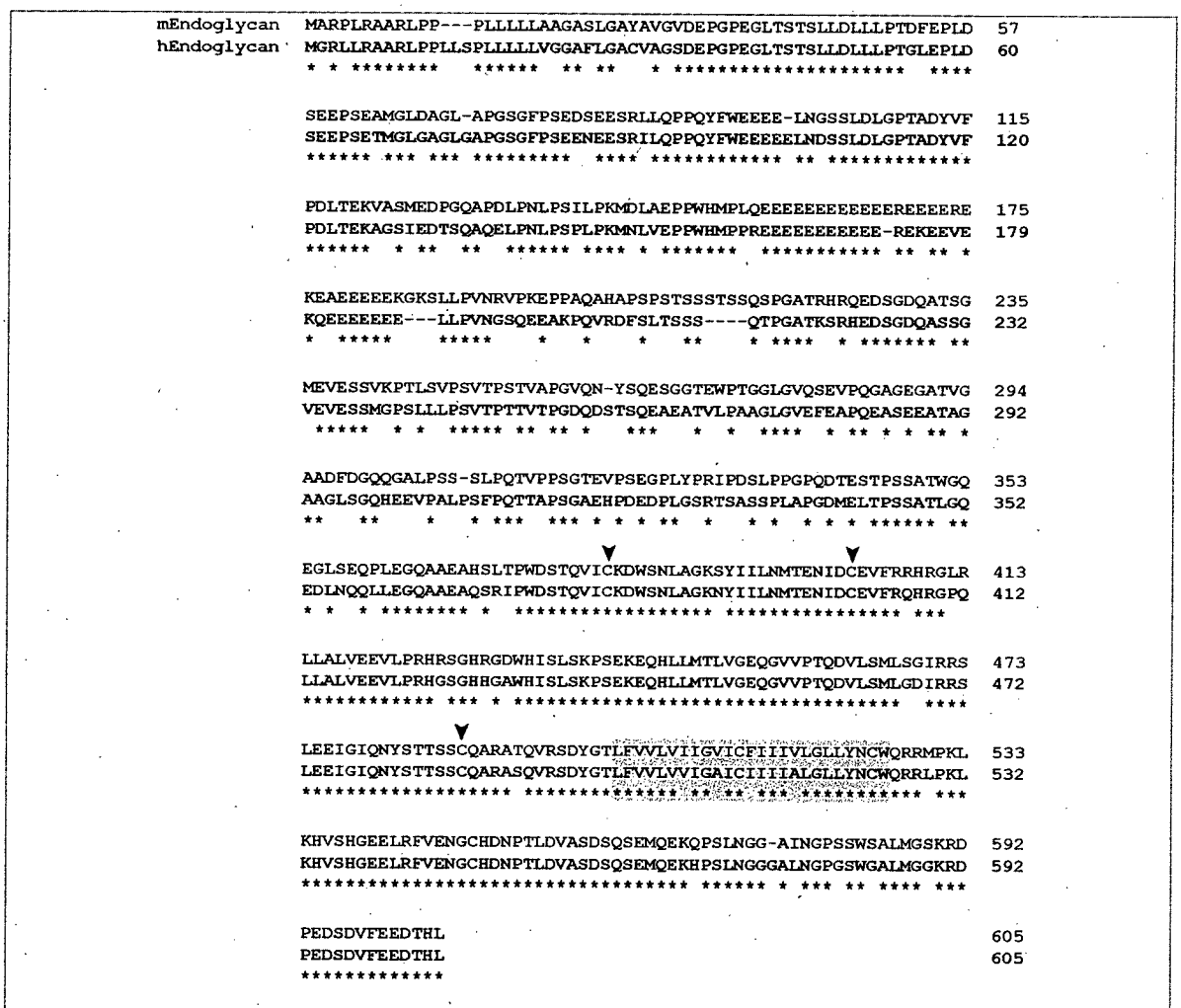


Fig. 1.3: Amino acid sequence of human Endoglycan. Underline, signal peptide; Double underline, potential sites for glycosaminoglycan addition; Triangle, potential N-linked carbohydrate addition site; Asterisk, potential tyrosine sulfation site; Circle, cysteines in cysteine-rich region; Dotted underline, predicted transmembrane domain. Adapted from Sassetti et al., 2000.

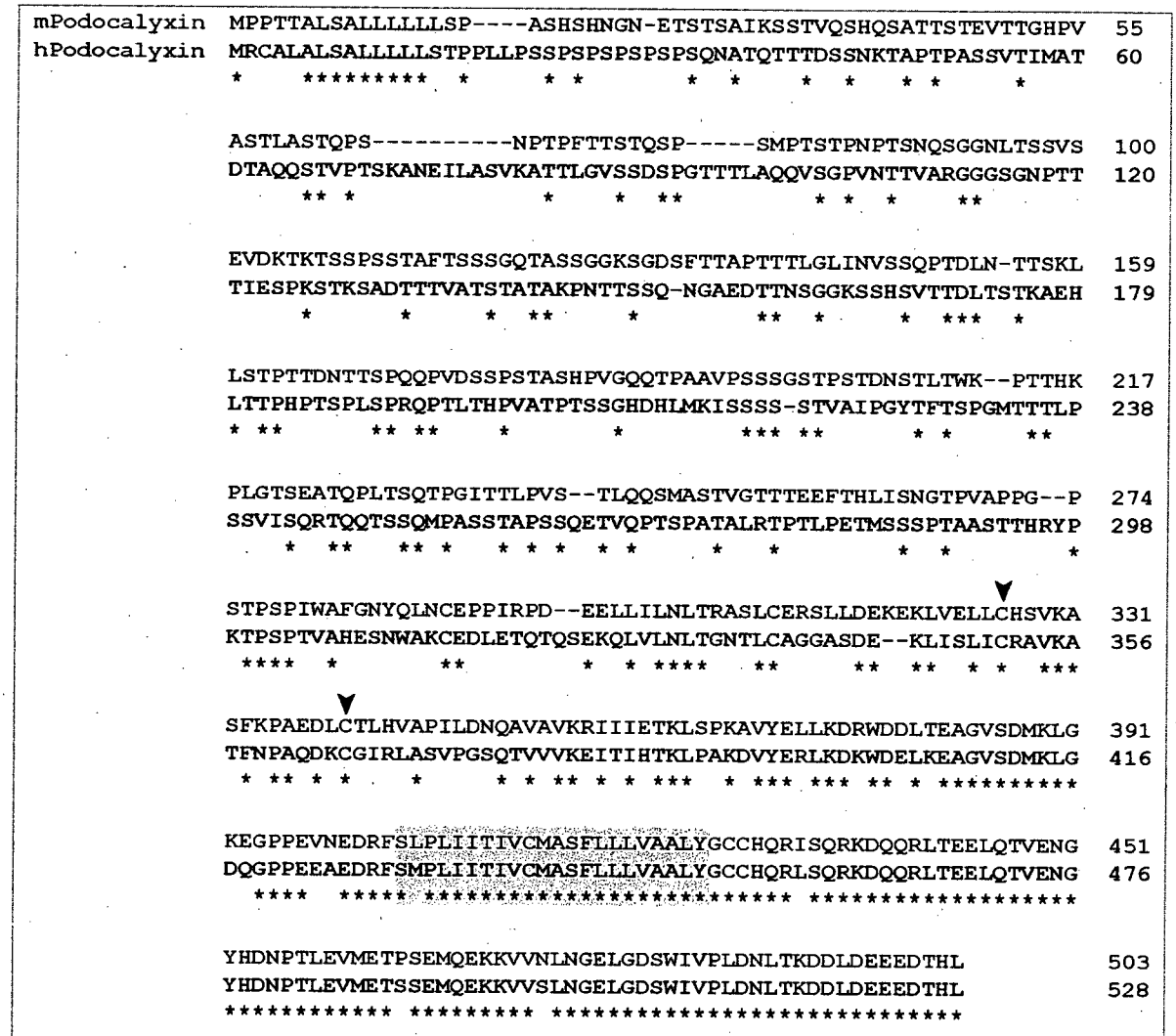
Alignment of the mouse and human orthologs of the CD34 family members shows that Endoglycan contains high amino acid conservation in the extracellular domain (73%) as compared to CD34 and Podocalyxin (55% and 30% respectively, Fig. 1.4A-C). Such a high degree of identity in mucin domains is rare. Glycosylations are added on serine, threonine or asparagine residues by glycotransferases. These transferases recognize short consensus sequences; therefore, if the purpose of the mucin domain is to provide

general bulk and charge to a molecule, conservation of these short consensus sequences is not necessary. Therefore, the conservation of this extracellular domain across species suggests that there maybe an extracellular binding partner for Endoglycan.



% Amino Acid Identity	
Overall	76%
Extracellular Domain	72%
Cytoplasmic Domain	92%

Fig. 1.4A: Mouse and human amino acid alignment of Endoglycan. Amino acid sequence alignment of the mouse and human forms of Endoglycan using SIM Binary Alignment from ExPASy Proteomics Tools (www.expasy.org, 2003). Stars indicate identical amino acids. Triangles indicate cysteines for disulfide bonding. The shaded region indicate the transmembrane domain. The overall sequence identity is 76%, the extracellular and cytoplasmic regions contain 72% and 92% identity respectively.



% Amino Acid Identity	
Overall	43%
Extracellular Domain	30%
Cytoplasmic Domain	96%

Fig. 1.4B: Mouse and human amino acid alignment of Podocalyxin. Amino acid sequence alignment of the mouse and human forms of Endoglycan using SIM Binary Alignment from ExPASy Proteomics Tools (www.expasy.org, 2003). Stars indicate identical amino acids. Triangles indicate cysteines for disulfide bonding. The shaded region indicate the transmembrane domain. The overall sequence identity is 43%, the extracellular and cytoplasmic regions contain 30% and 96% identity respectively.

% Amino Acid Identity	
Overall	65%
Extracellular Domain	55%
Cytoplasmic Domain	93%

Fig. 1.4C: Mouse and human amino acid alignment of CD34. Amino acid sequence alignment of the mouse and human forms of Endoglycan using SIM Binary Alignment from ExPASy Proteomics Tools (www.expasy.org, 2003). Stars indicate identical amino acids. Triangles indicate cysteines for disulfide bonding. The shaded region indicate the transmembrane domain. The overall sequence identity is 65%, the extracellular and cytoplasmic regions contain 55% and 93% identity respectively.

In a recent study (Fieger et al., 2003), similarities were drawn between the structure and sequence of Endoglycan and P-selectin glycoprotein ligand-1 (PSGL-1). These two proteins have homologies in residues important for PSGL-1 ligand function, such as sulfated tyrosine residues and sLex modified threonine residues. In addition, both

PSGL-1 and Endoglycan have an unpaired cysteine in the membrane proximal region that permits dimerization. Through the use of recombinant Endoglycan chimeras and exogeneous post-translational modification enzymes, such as α(1,3)-fucosyltransferase VII (FTVII), Endoglycan was shown to bind L-selectin under flow conditions.

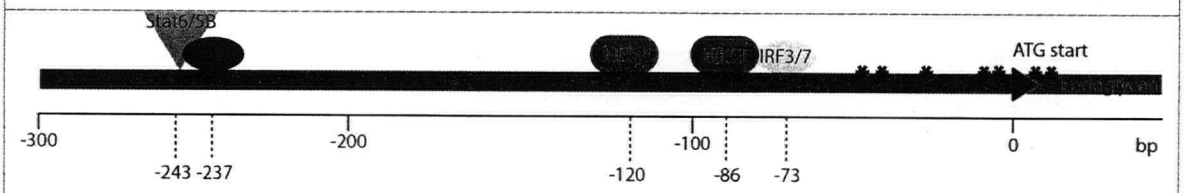
Initial analysis of Endoglycan mRNA expression in various human tissues by Sassetti and colleagues showed that there is broad expression pattern of the transcript, which includes brain, pancreas, kidney, liver and all hematopoietic tissues (Sassetti et al., 2000). This broad distribution of Endoglycan is likely to reflect its vascular endothelial expression. In sharp contrast, both monoclonal rat anti-mEndoglycan antibody staining (F4B10, generated in our laboratory), as well as RT-PCR analysis, failed to show the endothelial expression of mouse Endoglycan in primary tissue or cell lines. However, we did detect expression of Endoglycan on closely-associated vascular smooth muscle, and in macrophages, thymocytes and activated B cells (Table 1.1).

To get a hint at the transcriptional regulation of Endoglycan, the databases TSSG, TSSW and Neural Network Promoter Prediction was used to screen the promoter of Endoglycan for potential transcriptional factor binding sites. Our analysis predicts that there is an overlapping Stat5b/6 and NF-κB site, 247bp and 243bp from the transcriptional start site respectively. This may indicate competitive regulation of Endoglycan expression whereby NF-κB enhances transcription and Stat5b/6 represses. Moreover, potential binding site for the transcriptional activator, IRF, was found (Fig. 1.5). The signaling pathways leading to the activation of these potential transcription factors will be described in later sections.

Tissue/Cells	Endoglycan	Podocalyxin	CD34
Multipotent hematopoietic precursors			
Adult	+	+	+
Embryo	+	+	+
Monopotent precursors			
Erythroid	+	+	-
Thrombocytic	?	+	+
Myeloid	+/-	-	+
Lymphoid (subset of thymocytes)	+	+	+
Mature hematopoietic cells			
B Cells (LPS activated)	+	-	-
T Cells	-	-	-
Macrophages	+	-	-
Granulocytes	-	-	-
Eosinophils	-	-	-
Mast Cells	-	-	+
Erythrocytes	+*	+*	-
Platelets	?	+	-
Vessels			
Vascular endothelial	-	+	+
Vascular smooth muscle	+	-	-
Intestinal Epithelial	+	-	-
Podocytes	+/-	+	-
Brain (Neurons)	+	+**	?
Boundary Elements (mesothelial)	-	+	-

* embryonic erythrocytes only
** ependymal layer only

Table 1.1: Tissue distribution of the CD34 family. Table indicating th edistribution of Endoglycan, Podocalyxin and CD34 in murine tissue. Undetermined expression indicated by (?); Controversy between human and mouse data indicated by (+/-). Data courtesy of Helen Merkens.



✱ TSS = Transcription Start Site
Promoter predicted using TSSG, TSSW and Neural Network Promoter Prediction, February 2005.

Fig. 1.5: Predicted transcription factor binding sites in the Endoglycan promoter. Schematic of potential transcription factor binding sites on the Endoglycan promoter. The binding sites for Stat6/5B overlaps with NFκB. Stars, potential transcription start sites; Black arrowhead, ATG start site.

1.2 B cell Development in Bone Marrow

B cells are generated in the liver during mid- to late-stage fetal development and in the bone marrow (BM) thereafter. These cells are derivative of the common lymphoid progenitors (CLP, Fig. 1.6). There are five stages in B cell development in the bone marrow and they are characterised by various cell surface markers (Fig. 1.7) and rearrangement and expression of immunoglobulin (Ig) genes. B cell lineage commitment is distinguished by the expression of the B220 isoform of CD45 (CD45R). Through the use of surface markers, six different factions (Fr.) representing various stages of B cell development have been identified (Fr. A, Fr. B/C, Fr. C', Fr. D and Fr. E) in the bone marrow. There are two different nomenclatures, Philadelphia and Basel, for naming these various B cell fractions and this is summarised in Fig. 1.7. For simplicity, the following description of B cell development will use the Philadelphia nomenclature (Hardy and Hayakawa, 2001).

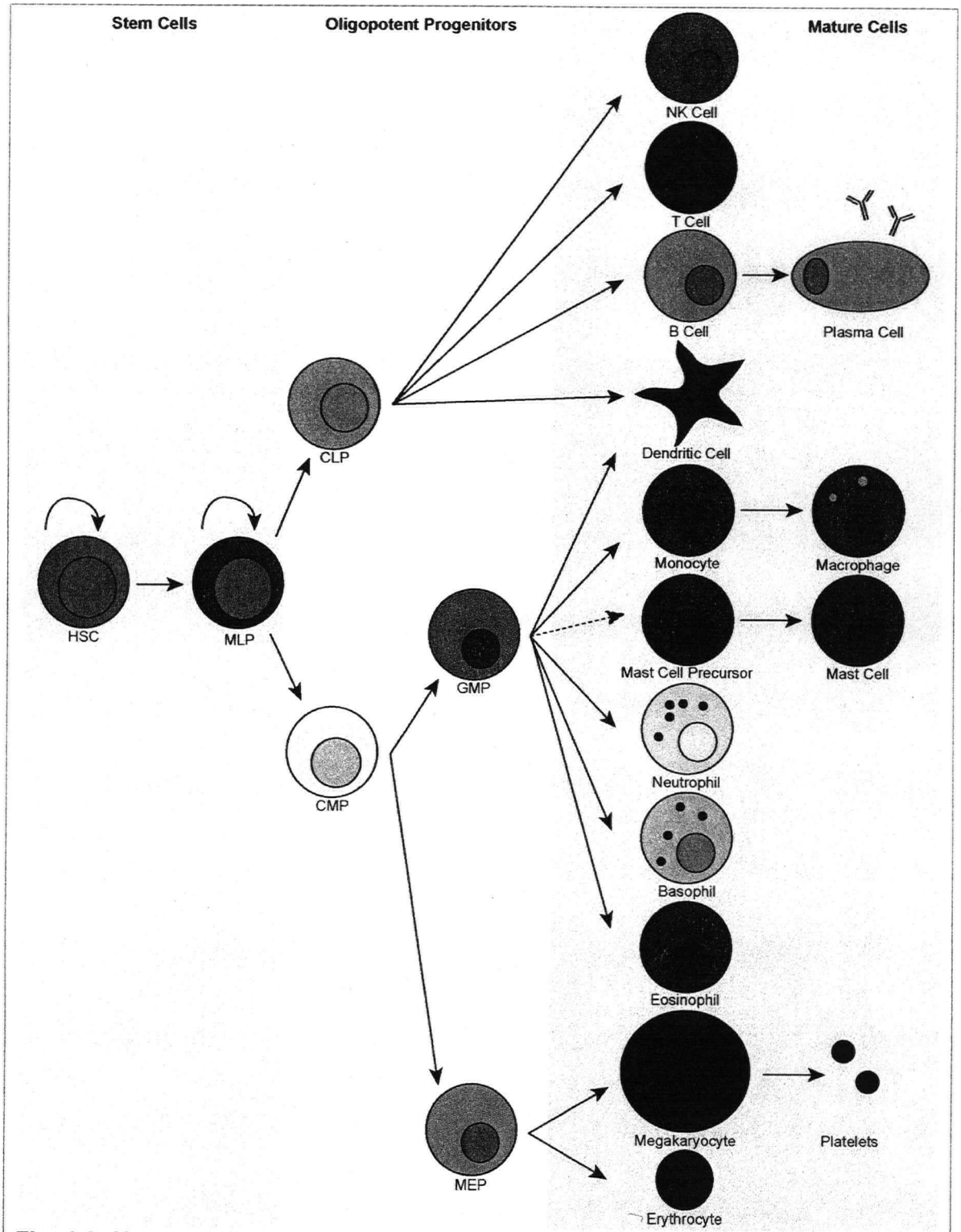


Fig. 1.6: Hematopoiesis. Schematic of hematopoiesis. HSC, hematopoietic stem cell; MLP, multi-lineage progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor; MEP, megakaryocyte/eryhroid progenitor. Adapted from (Wagers et al., 2002).

Fig. 1.7: Molecular markers and B cell lymphopoiesis. Table showing markers expressed through B cell lymphopoiesis. Adapted from Hardy and Hayakawa, 2001.

CLP are cells that give rise to B, T and natural killer (NK) cells. They lack the expression of lineage markers including B220/CD45R (B lineage marker), CD11b/Mac-1 and Gr-1 (myeloid/granulocyte/macrophage lineage markers) and Ter119 (erythroid lineage marker). However, they are characterised by the expression of c-kit and IL-7 receptor α chain (IL-7Rα).

Immediately following CLP stage and B lineage commitment is the pre-pro-B stage characterised by the expression of B220/CD45R, AA4.1 and low levels of heat-stable antigen (CD24/HSA). AA4.1 is an antigen expressed on cells from early hematopoietic progenitors to immature B cell stage. Pre-pro-B cells have little or no Ig rearrangement and low levels of IL-7Rα. Although the low level of IL-7Rα expression has been under controversy in recent years (Tudor et al., 2000), it has been suggested through propidium iodide analysis of DNA content that this diminished expression is due, in part, to cell arrest in G_o/G_1 stage (Hardy and Hayakawa, 2001). Therefore, suggesting that these cells are quiescent and IL-7Rα has a higher turnover rate than other surface molecules such as B220 or AA4.1. Hence, the use of IL-7Rα as an identifier and molecular handle for the isolation of B cells should be used with caution as the proliferative phase of cells can alter its expression.

Pro-B cells are defined as a stage when immunoglobulin heavy (IgH) locus is undergoing rearrangement for production of the immunoglobulin μ heavy chain. These cells are characterised by CD19 and high level of IL-7Rα expression. Moreover, Pro-B cells express the surrogate light chain (SLC), as well as the B cell receptor (BCR)-associated molecules Igα and Igβ. The SLC (comprised of two proteins, VpreB and λ5) together with Igμ and the signaling heterodimer Igα/Igβ will form the pre-BCR in early pre-B cells. Transgenic mice have been generated that targeted proteins responsible for Ig rearrangement, such as the severe combined immunodeficiency (SCID) and recombination activating gene (RAG)-deficient mice. These mice fail to generate the pre-BCR leading to a block of B cell development at the early pro-B stage (Blunt et al., 1995; Oettinger et al., 1990; Reichman-Fried et al., 1990; Shinkai et al., 1992). Similarly, elimination of components of the pre-BCR, such as λ5 or Igβ also blocks development at this stage (Gong and Nussenzweig, 1996; Kitamura et al., 1992).

Early pre-B cells are marked by a rapid proliferation and are characterised by the expression of the pre-BCR, BP-1 and CD25. A conceptual model for this proliferative burst is that it is an Igμ-dependent quality assurance step for the future production of stable BCR. The SLC acts as a template and failure of the Igμ to interact with the SLC to form a properly signaling pre-BCR will be negatively selected (Karasuyama et al., 1996; Meffre and Nussenzweig, 2002). Upon exiting this proliferative phase, cells enter the late pre-B stage. At this stage, cells are undergoing immunoglobulin light chain (IgL) locus rearrangement for the production of the immunoglobulin κ or λ light chain. Late pre-B cells no longer express c-kit, CD43 or SLC.

Cell surface expression of the BCR (which consists of Igμ heavy chain, κ or λ light chain and Igα/Igβ) is a hallmark of new or immature B cells. Cells at this stage of maturation no longer express CD25, IL-7Rα or BP-1, and also at this stage, selection against self-reactive B cells occurs. One of three events may follow during this selection process: 1. Apoptosis (clonal deletion); 2. Anergy; or 3. Receptor editing. Ligation of an antigen with a BCR that produces a strong signal leads to apoptosis. This is a negative selection process to prevent autoreactive B cells from leaving the bone marrow. Of the ~$2x10^7$ immature B cells produced in the BM of mice daily, only ~10% exit to the periphery (Chung et al., 2003; Freitas et al., 1986). Lower affinity interactions will result in anergy, which are nonresponsive short-lived B cells, or editing, whereby cells revise their BCR to eliminate self-reactivity (Benschop et al., 1999; Goodnow et al., 1990; Hartley et al., 1991; Hartley and Goodnow, 1994).

1.3 Peripheral B cells

There are seven subsets of peripheral B cells and they differ by expression of cell surface markers (Fig. 1.8), reactivity and location. However, it is important to note that the use of surface markers for characterisation of peripheral B cells must be interpreted with caution as maturation may lead to transient expression of precursor-like molecules, and activation may alter the expression of these molecules. Transitional B cells mark the link between immature and mature B cells; these cells express AA4.1 and high levels of CD24/HSA. The ability for these cells to edit their receptor is still a matter of controversy with studies of RAG expression in these cells. Suggesting that selection and receptor editing may take place in the periphery prior to B cell activation (Monroe et

al., 1999; Yu et al., 1999). Transitional B cells migrate to the spleen upon entering the open circulation from the BM, however, they are excluded from the lymph nodes.

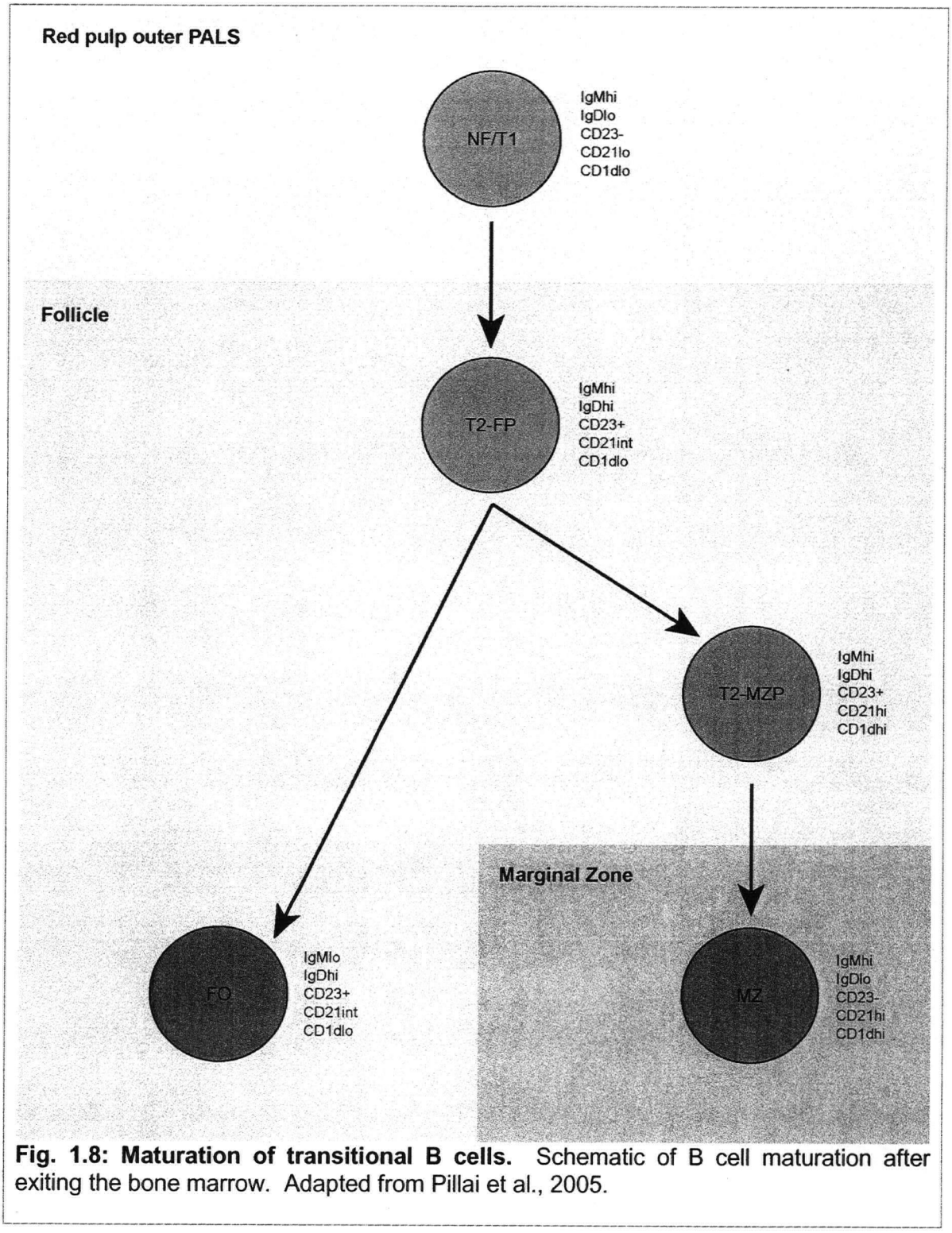


Fig. 1.8: Maturation of transitional B cells. Schematic of B cell maturation after exiting the bone marrow. Adapted from Pillai et al., 2005.

The tissue specific homing phenomenon may be due, in part, to the lack of CD62L or fibronectin expression on these cells (Flaishon et al., 2000; Loder et al., 1999). There are three types of transitional B cells, Transitional stage 1 (T1), transitional stage 2 (T2) and transitional stage 3 (T3) B cells. T1 B cells, also known as newly formed (NF) B cells, phenotypically resemble immature B cells in their cell surface markers and susceptibility to negative selection (Allman et al., 1992; Norvell and Monroe, 1996). They are $IgM^{hi}IgD^{lo}CD21^{lo}CD23^{-}CD1d^{lo}$ and are found in the red pulp outer periarteriolar lymphoid sheath (PALS, Liu, 1997). T2 B cells can be delineated from T1 B cells by their expression of CD23, CD21 and IgD. These cells are detected in splenic B cell follicles (Chung et al., 2002; Loder et al., 1999; Schiemann et al., 2001), a migrational process that is dependent on the expression of the chemokine receptor CXCR5 (Forster et al., 1996). There are two types of T2 B cells: T2 follicular precursors (T2-FP) and T2 marginal zone precursor (T2-MZP). T2-FP B cells are recirculating B cells that reside primarily in follicles and are $CD1d^{lo}$ (Pillai et al., 2005). T2-MZP B cells are $CD1d^{hi}$ cycling cells found only in the spleen and not in the BM, blood or lymph nodes (Loder et al., 1999; Saito et al., 2003). The third transitional subset, T3 B cells, are newly matured follicular (FO) B cells found in the spleen or lymph node that are phenotypically similar to T2 B cells with the exception of lower surface IgM expression (Allman et al., 2001). The sequential maturation of transitional B cells is summarised in Fig. 1.8.

Accumulating evidence has demonstrated the role of positive selection on the recruitment of transitional B cells to the mature B cell pool. In germ-free mice, the number of mature B cells is reduced without a reduction in the number of T2 B cells (Loder et al., 1999). Moreover, in *in vitro* analyses, BCR engagement has been shown to positively select T2 B cells for differentiation into mature B cells (Su and Rawlings, 2002). Although other studies have also shown that T1 and T2 B cells are sensitive to deletion by antigen stimulation, apoptosis in these cells can be rescued by helper T cells (Allman et al., 2001; Chung et al., 2002). Aside from antigen-dependent selection on transitional B cells, studies are beginning to show the importance of non-antigen receptor-mediated selection processes. The absence of BAFF (B cell activating factor belonging to the tumour necrosis factor family) will block the maturation progression from T1 to T2 B cells (Gross et al., 2001; Schiemann et al., 2001).

There are five mature B cell subsets. FO B cells are $IgM^{lo}IgD^{hi}CD21^{int}CD23^{+}CD1d^{lo}$ and are found in follicles of spleen and lymph nodes. Marginal zone (MZ) B cells carry an activated phenotype ($IgM^{hi}IgD^{lo}CD21^{hi}CD1d^{hi}$). They are found in the marginal zone of the spleen and represent ~5% of the splenic B cell population. Mature recirculating (MR) or B-2 B cells lack AA4.1 and CD5 expression. B-1a B cells also lack AA4.1 expression, but do express CD5. B-1b B cells are similar to B-1a B cells with the exception of no CD5 expression. Both B-1 B cell subpopulations are self-renewing and they have preferential localisation in the peritoneal, pleural cavities and gut lamina propria. These cells express CD11b/Mac-1 in peritoneal or pleural cavities, however, CD11b/Mac-1 expression is absent in the spleen.

1.4 Mechanisms for Localisation and Migration of B cells in Lymphoid Organs

As described previously, different subsets of mature B cells exhibit preferential localisation in different regions within lymphoid organs (Fig. 1.9). For example, the only subsets found in the follicles of spleen are FO, T2-FP and T2-MZP B cells. Conversely, MZ B cells are only found in the marginal zone. The following will explore some of the mechanisms that play a role in retaining MZ B cells in the marginal zone.

The entry of lymphocytes into the spleen is mediated by the interaction between LFA-1 and $\alpha4\beta1$ integrins on lymphocytes and ICAM-1 and vascular cell-adhesion molecule 1 (VCAM-1) on splenic radioresistant cells, respectively (Lo et al., 2003). This adhesion event is likely to be dependent on a chemokine that is yet to be defined. Once in the spleen, B cells may be drawn into the follicles by a CXCL13 chemokine gradient. CXCR5 is the receptor found on B cells for CXCL13. However, MZ B cells are able to evade this gradient via high expression of $S1P_1$ and $S1P_3$, which are receptors for lysophospholipid sphingosine-1 phosphate (S1P). S1P-deficiency represents the only genetic model in which MZ B cells are formed but are mislocalised in follicles (Cinamon et al., 2004). Therefore, CXCL13 and S1P act in an opposing manner to manage the localisation of MZ B cells. Activation of MZ B cells by antigen or LPS leads to a downregulation of $S1P_1$, thus allowing for MZ B cells to migrate into follicles.

The mechanism by which MZ B cells are retained in the marginal zone is poorly understood. It has been postulated that interaction between MZ B cells and marginal zone macrophages (MZM) plays an important role in this phenomenon. One possible

molecular player for the interaction between MZ B cells and MZMs is the scavenger receptor, macrophage receptor with collagenous structure (MARCO), on MZMs. However, the biochemical basis of this interaction remains unknown (Karlsson et al., 2003; Kraal, 1992). Another possible mechanism in the retention of MZ B cells is the interaction between integrins on B cells and integrin ligands on stromal cells. Both integrins, LFA-1 and $\alpha 4\beta 1$ integrin, are expressed at higher levels on MZ B cells than FO B cells. Interactions between LFA-1 with ICAM-1, and $\alpha 4\beta 1$ integrin with VCAM-1 and fibronectin may be responsible for the adhesion of MZ B cells in marginal zone. Similar to $S1P_1$, these integrins are downregulated upon activation (Lu and Cyster, 2002).

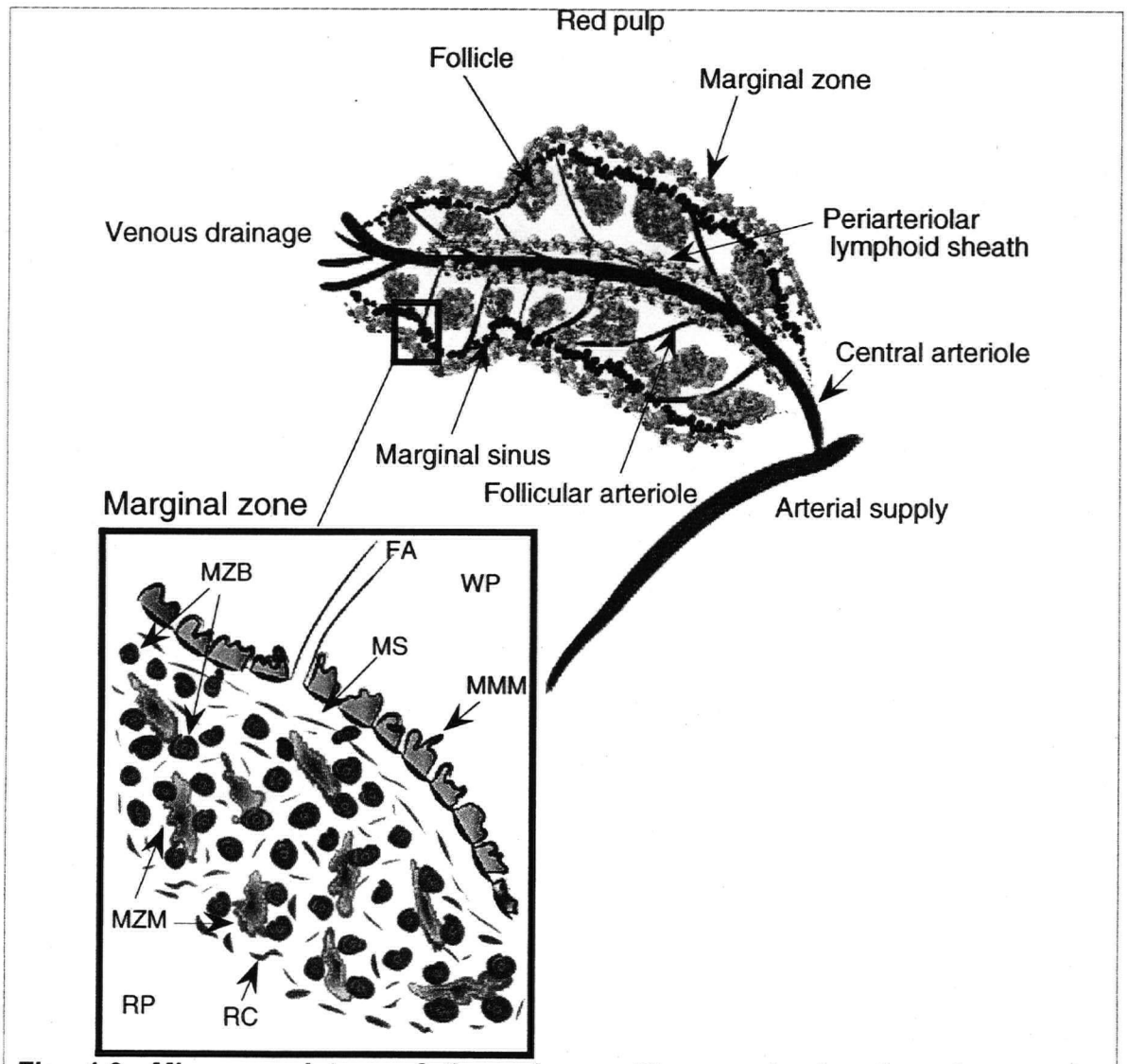


Fig. 1.9: Microvasculature of the spleen. Diagram showing the microvascular organisation of the spleen. Newly-formed B cells exit the artery into the periarteriolar lymphoid sheath (PALS) where they migrate to the follicle and marginal zone as they mature. Pillai et al., 2005.

1.5 B cell Activation and Inflammatory Response

1.5.1 T cell-Dependent Activation

Upon binding of the BCR on mature recirculating (MR) or FO B cells to an antigen, these cells migrate to the T cell zone where they receive co-stimulation by CD40 ligand (CD40L) and cytokines, primarily IL-4, from helper T cells (Cyster et al., 1999; Gold and Matsuuchi, 1995). If B cells fail to receive co-stimulatory signals, they will become anergic and undergo apoptosis (Gold, 2002). On the other hand, if both BCR and co-stimulatory signals are present, B cells become activated, proliferate and migrate to the lymphoid follicles. Germinal centres (GC) are established in the follicles, and these are specialised compartments where activated B cells undergo a proliferative burst, affinity maturation and immunoglobulin class-switch recombination. Affinity maturation is a process where somatic hypermutation of Ig genes will refine the BCR to bind the stimulating antigen at the highest affinity, whereas class-switch recombination is a process where the Ig heavy chain (usually μ) is replaced by a 3' gene segment (γ, ε or α). Both antigen-specific T helper cells and follicular dendritic cells (FDC) are crucial for this response in the GC (Manser, 2004). Helper T cells provide co-stimulation to the activated B cells by the interaction of CD40L and inducible T-cell co-stimulator (ICOS) on T cells with CD40 and ICOS ligand on B cells respectively. FDCs are responsible for sequestering antigen in germinal centres. Activity in germinal centres peak at 10 to 14 days post-immunization, when plasma cells and memory B cells exit the germinal centre.

1.5.2 T cell-Independent Activation

B-1 and MZ B cells have been described as cells with "natural memory" because they have a repertoire that recognises T-cell independent type 2 (TI-2) antigens. TI-2 antigens contain multiple identical epitopes that are capable of cross-linking BCRs. B-1 and MZ B cells are the first response to foreign antigens. Upon activation, these cells migrate to the red pulp of the spleen and form foci of plasmablasts (Lopes-Carvalho and Kearney, 2004), and mature into antibody-secreting plasma cells (Bikah et al., 1996; Hippen et al., 2000; Oliver et al., 1997).

The ability for these subsets of B cells to respond rapidly to multivalent antigens is due, in part, to the high expression of complement receptor 2 (CD21) that assists in the capture of complement coated polysaccharides (Guinamard et al., 2000). Furthermore,

these cells have a lower threshold for activation in response to BCR or toll-like receptors (TLR) than MR and FO B cells. One reason for this lowered threshold is due to higher resting levels of B-lymphocyte-induced maturation protein 1 (Blimp-1), hence allowing these cells to differentiate quickly into plasma cells upon activation (Martin et al., 2001). Similar to T cell-dependent activation, B-1 and MZ B cells also require a second signal in addition to BCR ligation. This signal is most likely provided by TLRs, however, differentiation of these cells into plasma cells appears to require signals from blood-borne dendritic cells (Pillai et al., 2005).

1.5.3 TLRs and B cell Activation

TLRs are a family of pattern recognition receptors (PRRs) that recognise pathogen-associated molecular patterns (PAMPS). PAMPS are conserved molecular patterns that are unique to microorganisms (Janeway and Medzhitov, 1998). The number of TLRs varies between mammalian species; however, most have 10 to 15 TLRs. Different TLRs recognise different PAMPS (Table 1.2) and their specificity is regulated by location of expression. For example, TLR4 recgonises LPS, TLR2 detects bacterial lipoproteins and lipoteichoic acids and TLR9 detects unmethylated CpG DNA of bacteria and viruses. As shown in Table 1.4, TLRs 1, 2, 4, 5 and 6 are specific for the recognition of PAMPS unique to microorganisms. However, this self-non-self discrimination is not as evident in the ligands of TLRs 3, 7, 8 and 9. Therefore, specificity of these TLRs is controlled by intracellular localisation where they detect viral nucleic acids in late endosomes-lysosomes (Diebold et al., 2004; Heil et al., 2003; Lee et al., 2003; Lund et al., 2004; Matsumoto et al., 2003).

B-1 and MZ B cells are the most sensitive of all the B cells subsets to TLR stimulation in their TI-1 response. TLR stimulation in these cells leads to polyclonal activation and production of low affinity IgM antibodies. In the absence of BCR ligation, LPS alone is sufficient to induce expression of Blimp-1 and differentiation of B cells into plasma cells in murine splenocytes (Lin et al., 2002; Schliephake and Schimpl, 1996; Shaffer et al., 2002; Shaffer et al., 2004). Moreover, NF-κB-binding sites are found upstream of the gene encoding Blimp-1, *Prdm1*. The murine 3T3 fibroblasts, which lack the p50 and p65

Receptor	Ligand	Origin of ligand
TLR1	Triacyl lipopeptides	Bacteria and mycobacteria
	Soluble factors	*Neisseria meningitidis*
TLR2	Lipoprotein/lipopeptides	Various pathogens
	Peptidoglycan	Gram-positive bacteria
	Lipoteichoic acid	Gram-positive bacteria
	Lipoarabinomannan	Mycobacteria
	Phenol-soluble modulin	*Staphylococcus epidermidis*
	Glycoinositolphospholipids	*Trypanosoma cruzi*
	Glycolipids	*Treponema maltophilum*
	Porins	*Neisseria*
	Atypical lipopolysaccharide	*Leptospira interrogans*
	Atypical lipopolysaccharide	*Porphyromonas gingivalis*
	Zymosan	Fungi
	Heat-shock protein 70*	Host
TLR3	Double-stranded RNA	Viruses
TLR4	Lipopolysaccharide	Gram-negative bacteria
	Taxol	Plants
	Fusion protein	Respiratory syncytial virus
	Envelope protein	Mouse mammary-tumour virus
	Heat-shock protein 60*	*Chlamydia pneumoniae*
	Heat-shock protein 70*	Host
	Type III repeat extra domain A of fibronectin*	Host
	Oligosaccharides of hyaluronic acid*	Host
	Polysaccharide fragments of heparan sulphate*	Host
	Fibrinogen*	Host
TLR5	Flagellin	Bacteria
TLR6	Diacyl lipopeptides	*Mycoplasma*
	Lipoteichoic acid	Gram-positive bacteria
	Zymosan	Fungi
TLR7	Imidazoquinoline	Synthetic compounds
	Loxoribine	Synthetic compounds
	Bropirimine	Synthetic compounds
	Single-stranded RNA	Viruses
TLR8	Imidazoquinoline	Synthetic compounds
	Single-stranded RNA	Viruses
TLR9	CpG-containing DNA	Bacteria and viruses
TLR10	N.D.	N.D.
TLR11	N.D.	Uropathogenic bacteria

Table. 1.2: Toll-like receptors and their ligands. Table of various TLR, the potential ligands and the origins of those ligands. N.D., not determined; TLR, toll-like receptor. Adapted from Akira and Takeda, 2004.

subunits of NF-κB, fail to elicit an induction of Blimp-1 upon TLR stimulation (Shapiro-Shelef and Calame, 2005). These results are consistent with the observation that TLR stimulation leads to B cell activation and plasma cell differentiation. In order to prevent autoreactivity, B cells expressing self-reactive BCR have induced tolerogenic signaling pathways that inhibit TLR-induced activation (Rui et al., 2003). However, in memory B

cells where specificity for antigens has already been selected for, TLR stimulation can induce polyclonal activation with cognate T cell help (Bernasconi et al., 2002).

1.5.4 Memory B and Plasma cells Formation

Memory B and plasma cells are products of germinal centres. Memory B cells retain their high affinity BCR and have an intrinsic ability to respond more quickly upon antigen stimulation than naïve B cells (Tangye et al., 2003). These are Igβ$^+$Syndecan-1(SDC1)$^-$ cells that provide surveillance against secondary encounter of antigen and are not antibody-secreting cells. As described previously, TLR stimulation combined with T cell help is suffice to induce the activation of memory B cells (Bernasconi et al., 2002). There are two subpopulations of memory B cells and they are delineated by their B220 expression. Through adoptive transfer and antigen recall experiments, it is believed that these two subsets are in a linear developmental path (Driver et al., 2001). The post-germinal centre memory B cells are B220$^+$ and they differentiate into 'pre-plasma' memory cells which are B220$^-$. However, the existence of this B220$^-$ subpopulation has been challenged recently by the suggestion that these are non-B cells that captured BCRs via surface Fc receptors (Bell and Gray, 2003).

Plasma cells are SDC1$^+$, terminally differentiated, non-dividing, antibody-secreting B cells. Their derivation is driven by the transcription factor Blimp-1 (Turner et al., 1994). Blimp-1 represses the expression of anti-apoptotic BCL-2 family protein A1 and induces the expression of pro-apoptotic genes, such as growth arrest and DNA-damage-inducible 45 (GADD45) and C/EBP homologous protein (CHOP, Knodel et al., 1999; Shaffer et al., 2002). It has long been understood that, due, in part, to the expression of Blimp-1, plasma cells are short-lived. In cultures, plasma cells stop dividing and die within a few days of differentiation (Shapiro-Shelef and Calame, 2005). However, it has been demonstrated that adoptive transfer of virus-specific plasma cells into mice depleted of memory B cells leads to survival and antibody production for more than a year (Slifka et al., 1998). Therefore, although memory B cells have a role in long-term immunity, there is evidence for a new group of antibody-secreting B cells that provide persistent antibodies independent of antigen; these are called long-lived plasma cells. Long-lived plasma cells reside mainly in the BM; however, some have been found to reside in the spleen (Sze et al., 2000). The mechanism by which these two types of plasma cells are derived is unknown. One possibility is that long-lived plasma cells arise

depending on the ability of plasma cells to find a niche in the BM for survival signals provided by BM stromal cells (Manz and Radbruch, 2002).

The relationship between memory B cells and plasma B cells is yet to be defined. There are two alternative hypotheses for these late stages of B cell differentiation: 1. Plasma cells differentiate from memory B cells in a linear pathway (Shapiro-Shelef et al., 2003), or 2. memory B cells and plasma cells are separate cell fates derived from germinal centres.

1.6 Mechanism for Localisation and Migration of Memory and Plasma cells

As described above, the migration and localisation of B cells in the marginal zone of lymphoid organs is highly specific and requires chemokine receptors, integrins and carbohydrates that bind selectins. The homing and migration of memory and plasma cells is similar and requires participation from each of these molecular players (Fig. 1.10).

By altering the expression levels of chemokine receptors, B cells are directed to different lymphoid regions and organs. Downregulation of CXCR5 on post-germinal centre cells reduces signals from its ligand CXCL13, and allows these cells to exit from follicles (Hopken et al., 2004). Expression of CXCR4 is critical for the migration of plasma cells to regions that express its ligand, CXCL12, such as splenic red pulp, lymph-node medullary cords and BM (Hargreaves et al., 2001). It has been shown that CXCR3 and CXCR6 with their ligands, CXCL9 and CXCL16 respectively, are important for plasma cell homing.

Similar to the expression of chemokine receptors, the expression of different integrins can alter homing of plasma cells. BM plasma cells express $\alpha4\beta1$ integrin, which binds VCAM-1 at the surface of BM epithelial cells. However, plasma cells that express $\alpha4\beta7$ integrin will bind mucosal vascular addressin cell-adhesion molecule 1 (MADCAM-1) on intestinal epithelial cells and thereby reside in mucosal sites (Kunkel and Butcher, 2003). The localisation of plasma cells in BM also involves interactions between endothelial-cell selectin (E-selectin) on BM epithelial cells and its polysaccharide ligand on plasma cells (Underhill et al., 2002).

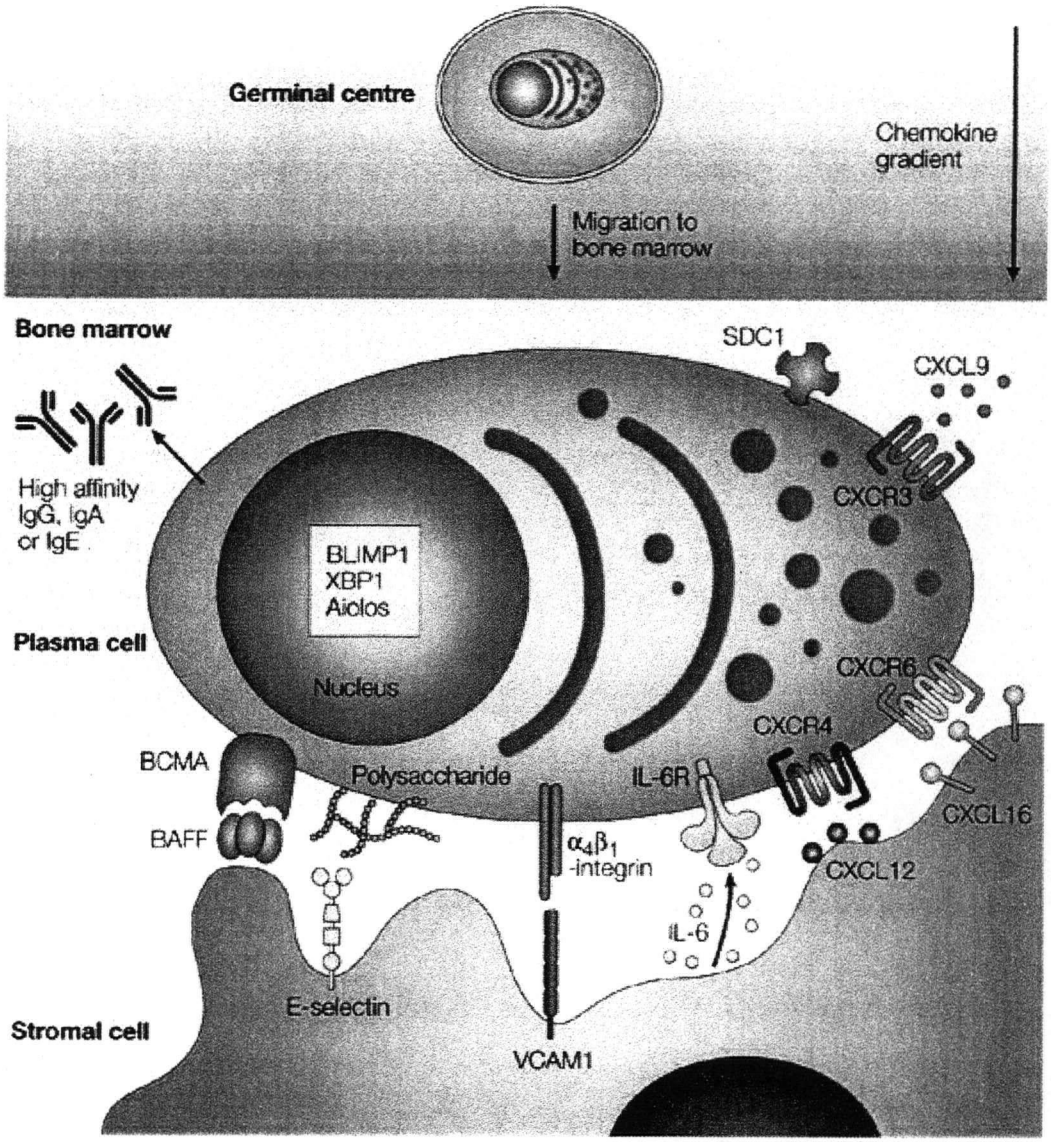


Fig. 1.10: Molecular interaction for retention of plasma cells in BM. Illustration of the molecular interactions involved in retaining plasma cells in the BM niche. Adapted from Shapiro-Shelef and Calame, 2005.

Blimp-1, as described previously, is important in the differentiation of B cells into plasma cells. This transcription factor has been shown to repress expression of CXCR5 (Shaffer et al., 2002) and induces expression of α4 integrin and CXCR4 (Sciammas and Davis, 2004). Although Blimp-1 is partially responsible for changes in expression levels of

these chemokine receptors and adhesion molecules, the regulatory mechanisms by which plasma cells determine their localisation is yet to be defined.

1.7 Cytokines and Their Effect on B cells

Cytokines secreted by dendritic cells or T cells have many roles in modulating B cell activation, development and survival. Cytokine receptors are heterodimers and usually consist of a long signaling chain and a shorter common chain. Different combinations of the various chains allow for cross-reactivity of cytokines. The common chain, γ_c, is shared in the receptor of IL-2, IL-4 IL-9, IL-7, IL-15 and IL-21. However, receptor combination is not restricted to the γ_c chain. IL-4 and IL-13 share a common signaling chain, IL-4Rα. It was shown that IgM-specific antibodies inhibit the differentiation of splenic B cells into plasma cells by LPS (Schliephake and Schimpl, 1996). However, the presence of IL-2 and IL-5 can overcome the effects of IgM-specific antibodies and drive plasma cell differentiation (Schliephake and Schimpl, 1996). Moreover, CD40L, IL-4 and IL-5 along with multivalent BCR signals lead to generation of a much more pronounced plasma cell population than Ig-specific antibodies alone (Snapper et al., 1995). In studies with cell lines, IL-2 and IL-5 (Turner et al., 1994) or IL-6 alone (Piskurich et al., 2000) can induce Blimp-1 expression. In human centrocyte cultures, IL-10 was also able to induce Blimp-1 expression (Shapiro-Shelef and Calame, 2005). Similarly, IL-21 can lead to plasma cell formation in primary B cells (Ozaki et al., 2004). The signaling downstream of many interleukin receptors involves the signal transducers and activators of transcription (STAT) family of transcription factors. IL-4 and IL-5 induces the activation of STAT6 and STAT5b respectively. Hence, showing the importance of these transcription factors in the regulation of B cell development.

IL-1β is a unique cytokine because its receptor is a part of the IL-1β receptor/toll-like receptor superfamily. These receptors share similar signaling cytoplasmic domains with conserved regions called toll-interleukin 1 receptor (TIR) domain. Stimulation of the IL-1β receptor will lead to expression of many pro-inflammatory genes; hence IL-1β is an important mediator of the inflammatory response (O'Neill, 2000). The downstream signaling from the receptor of this pro-inflammatory cytokine is similar to that of the TLR pathway which is described in detail below.

Conversely, transforming growth factor β (TGFβ) is an anti-inflammatory cytokine. The role of this cytokine in early hematopoiesis has been shown to influence proliferation and differentiation (Jacobsen et al., 1991; Keller et al., 1991). The ability of this cytokine to inhibit cell cycle progression, along with endogenous production of TGFβ by HSC, serves as a means for maintaining quiescence in these cells (Ducos et al., 2000; Hatzfeld et al., 1991; Pierelli et al., 2000). This is further demonstrated by studies using TGFβ-deficient mice, where their number of circulating granulocytes, monocytes and platelets is significantly increased (Kulkarni et al., 1993; Shull et al., 1992). Moreover, the transcription of the stem cell antigen, CD34, is upregulated by TGFβ (Batard et al., 2000; Marone et al., 2002).

In B cell lymphopoiesis, TGFβ was shown to inhibit the proliferation and the expression of κ light chain in B cell precursors (Lee et al., 1987). TGFβ also inhibits proliferation of activated mature B cells (Armitage et al., 1993; Bouchard et al., 1994). Furthermore, the downmodulation of NF-κB activity by TGFβ has been implicated in the induction of apoptosis of resting B cells (Lomo et al., 1995) and certain B cell lines (Arsura et al., 1996; Coffey et al., 1988; Fischer et al., 1994; Pietenpol et al., 1990). Aside from its antiproliferative and proapoptotic role, TGFβ can also influence isotype switch in activated B cells. In contrast to IL-4, which promotes the secretion of IgE antibodies (Lebman and Coffman, 1988), TGFβ induces isotype switching to secreted IgA antibodies (Kim and Kagnoff, 1990a). It has also been observed that IL-2 and IL-5 increase this IgA-specificity effect (Coffman et al., 1989; Kim and Kagnoff, 1990a; Kim and Kagnoff, 1990b; Lebman et al., 1990; Sonoda et al., 1989).

TGFβ signals through a two-part receptor, type I (TβRI) and type II (TβRII). Upon binding of TGFβ to TβRII, TβRI is recruited and phosphorylated, forming the activated TGFβ-receptor complex. TβRI then phosphorylates Smad2 and Smad3, both of which are receptor-activated Smad (R-Smad) proteins. Activated R-Smad forms a heteromeric complex with common mediator Smad (co-Smad) 4 and translocate into the nucleus where they act, directly or with coactivators or corepressors, to regulate transcription (Hata, 2001; Wotton et al., 1999). This pathway can be modulated through inhibitory-Smads, such as Smad6 and Smad7, which binds activated R-Smad to form a stable complex. TGFβ can also activate other signaling pathways including the PI3K and

MAPK pathways (Hartsough and Mulder, 1995; Kretzschmar et al., 1999; Sano et al., 1999; Shibuya et al., 1996; Yamaguchi et al., 1995; Yue et al., 1999).

Signaling of these various cytokines is interwoven in a network and much crosstalk exists between the pathways. Proinflammatory cytokines, such as IFNγ, TNFα and IL-1β, inhibit the TGFβ signaling pathway through expression of Smad7 (Bitzer et al., 2000; Topper et al., 1997; Ulloa et al., 1999). Similarly, the induction of proliferation by IL-1β-induced cytokines in HSC is inhibited by TGFβ (Ruscetti et al., 1992). Moreover, the MAPKKK homologue, TGFβ activated kinase 1 (TAK1, (Yamaguchi et al., 1995), is a mediator of both TGFβ and IL-1β signaling cascades (Jiang et al., 2002; Ninomiya-Tsuji et al., 1999; Takaesu et al., 2000).

1.8 TLR Signaling

All TLR share a common signaling cytoplasmic TIR domain. The best-characterised signaling pathway is the myeloid differentiation protein (MyD) 88-dependent pathway (Fig. 1.11). MyD88 consists of two domains, a C-terminal TIR-binding domain that interacts with TLR and an N-terminal death domain which recruits members of the IL-1 receptor-associated kinases, IRAK-1 and IRAK-4. These kinases undergo autophosphorylation and associate with the TNF-receptor associated factor, TRAF6. Through unknown mechanisms, TRAF6 activates MAP kinases, as well as the IκB kinases, IKKα and IKKβ. Activation of these kinases leads to the activation of the transcription factors AP-1 and NF-κB, and expression of proinflammatory genes.

However, emerging evidence is beginning to reveal the importance of another TLR signaling pathway, the MyD88-independent pathway (Fig. 1.12). Studies have shown that signals from TLR3 and TLR4 are not abolished in MyD88-deficient mice (Hoshino et al., 2002; Kawai et al., 2001). This phenonmenon has been attributed to another TIR adaptor molecule, Trif. Trif can associate directly with TLR3 or indirectly with TLR4 through another TIR adaptor molecule, TRAM. The importance of Trif in TLR signaling was demonstrated through Trif-MyD88 double-deficient cells, whereby all TLR4 responses were abrogated (Yamamoto et al., 2003). Trif associates with both the transcription factor interferon regulatory factor 3 (IRF-3) and its activating kinase, TBK-1 (Fitzgerald et al., 2003; Sato et al., 2003; Sharma et al., 2003). Activation of IRF-3 will

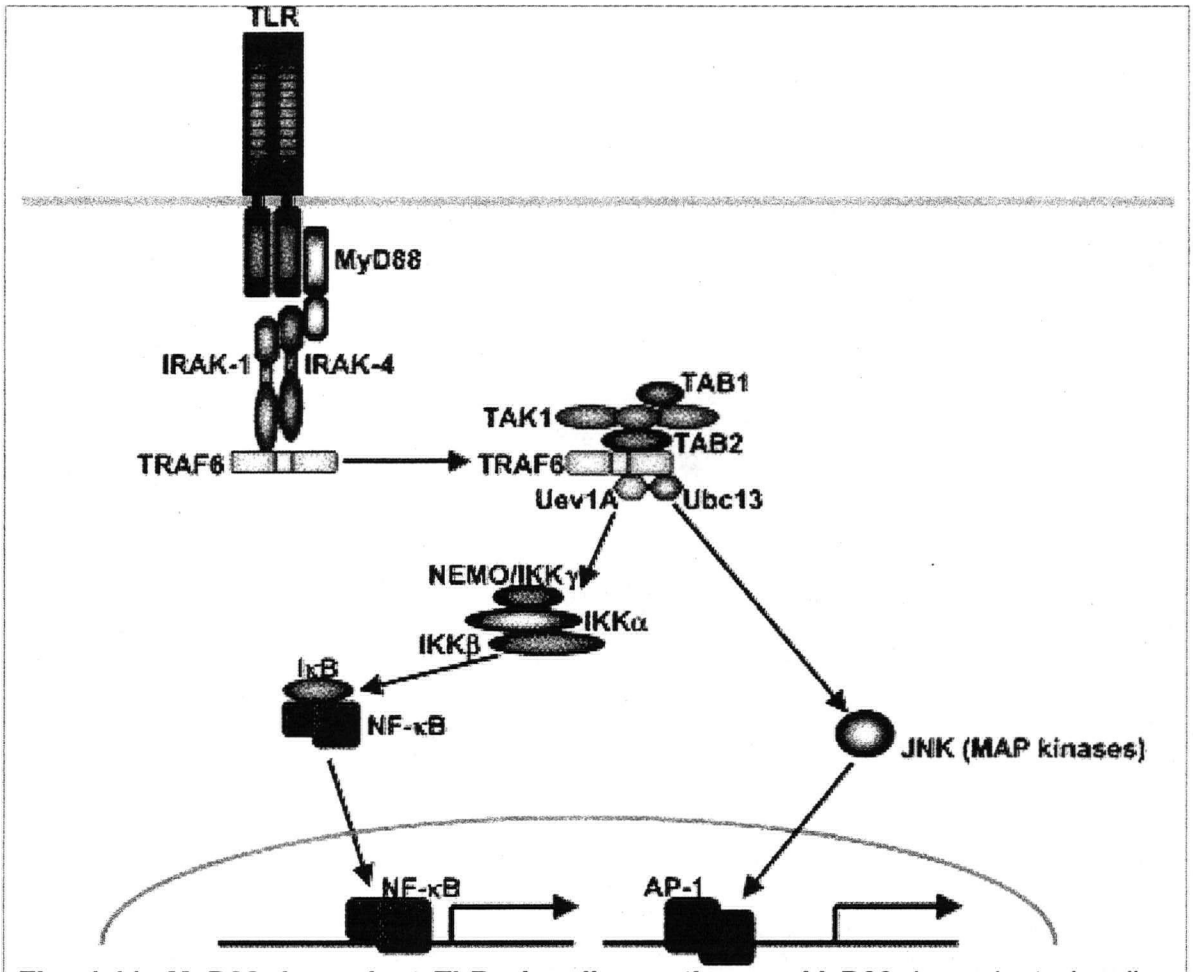


Fig. 1.11: MyD88-dependent TLR signaling pathway. MyD88-dependent signaling pathway upon TLR activation. Takeda and Akira, 2004.

lead to expression of type I interferons, such as IFNβ. Trif also associates with TRAF6, hence leading to downstream activation of MAP kinases and NF-κB, similar to the MyD88-dependent pathway. More recently, Trif was shown to also associate with RIP1 and RIP3 through RIP homotypic interaction motif (RHIM) in the C-terminal region of Trif. Unlike RIP3, RIP1 contains a death domain capable of initiating downstream signaling to activate NF-κB. Conversely, RIP3 acts as a negative regulator to RIP1 signaling by preventing the association of RIP1 with Trif (Meylan et al., 2004).

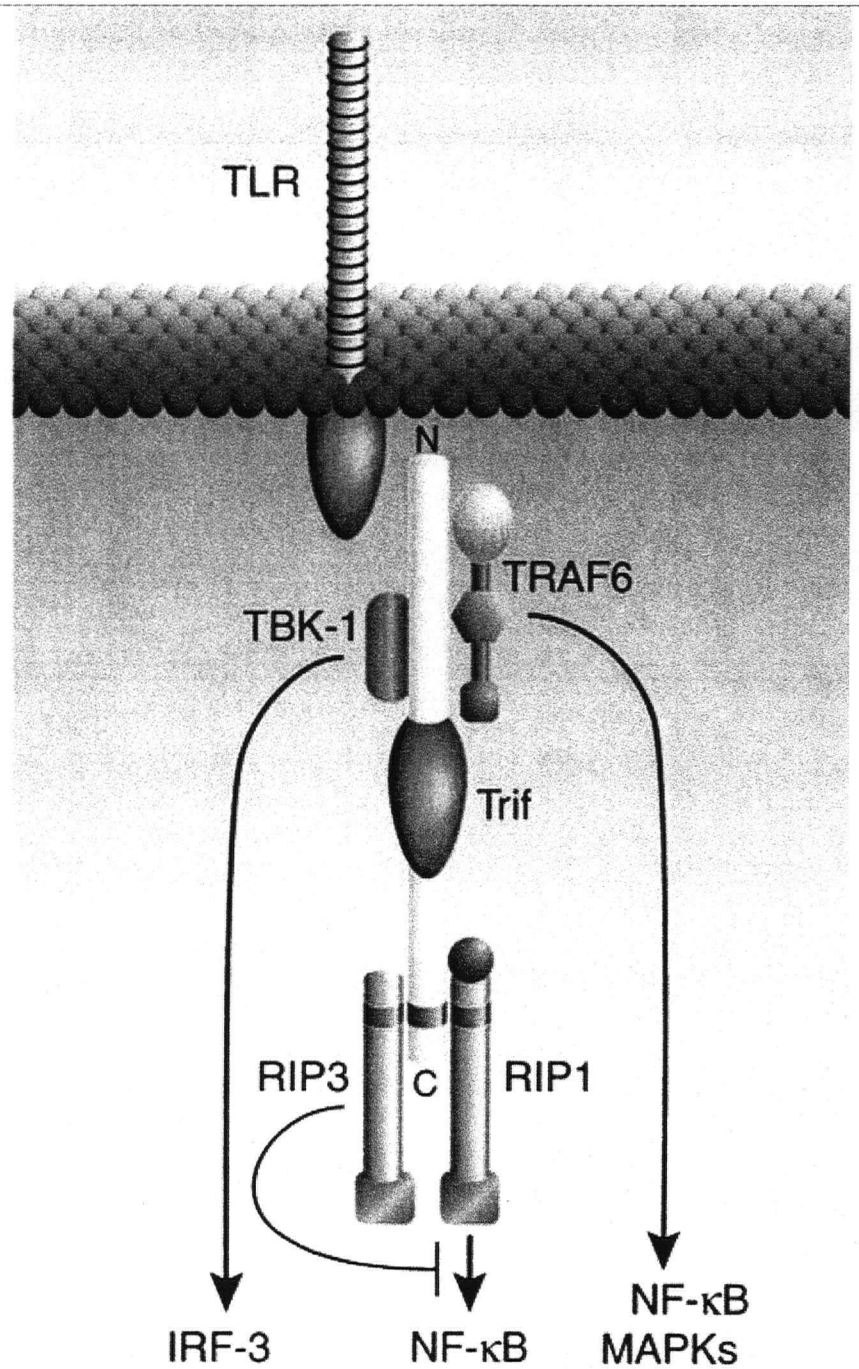


Fig. 1.12: MyD88-independent TLR signaling pathway. MyD88-independent signaling pathway upon TLR activation. Barton and Medzhitov, 2004.

1.9 Thesis Objectives

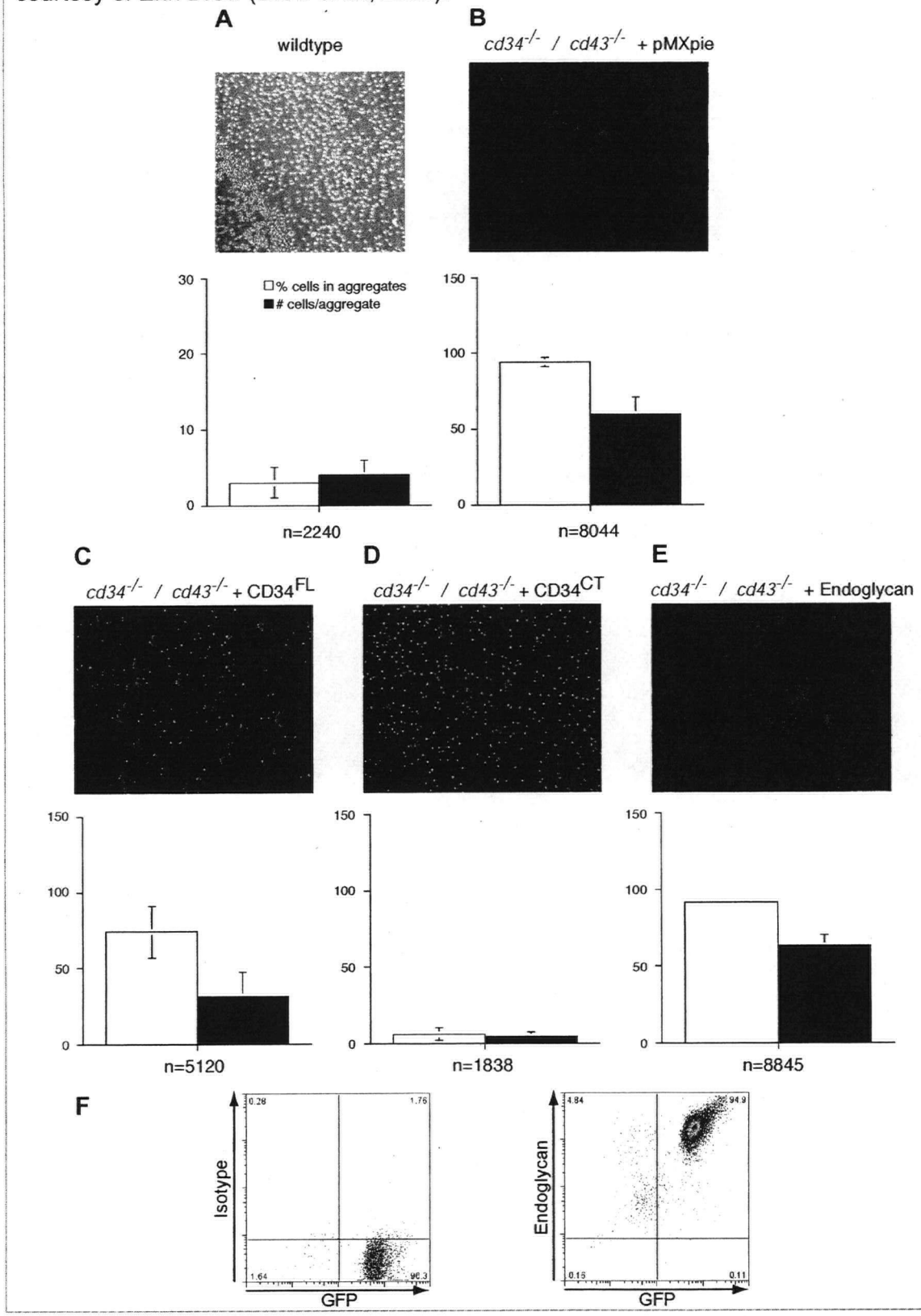
Since the discovery of human Endoglycan by Sassetti and colleagues in 2000 and murine Endoglycan in our laboratory, little has been revealed about the regulation and function of this novel molecule.

Our initial attempt to determine the function of Endoglycan was to compare this molecule to its family members, CD34 and Podocalyxin. Our laboratory has demonstrated that ectopic expression of CD34 in CD34-deficient murine mast cells (Drew et al. 2005), or Podocalyxin in breast cancer cells, induces a profound reduction in homotypic aggregation/adhesion. Surprisingly, ectopic expression of Endoglycan in CD34/CD43-deficient BM-derived mast cells did not decrease aggregation (Fig. 1.13). Moreover, higher levels of Endoglycan expression were observed in well-behaved highly polarized breast cancer cell lines (such as T47D and MCF-7) compared to aggressive lines (such as MDA-231), whereas the opposite is true of the anti-adhesin Podocalyxin (Fig. 1.14). These data suggest that Endoglycan may have a different function than CD34 and Podocalyxin. Rather than a global anti-adhesive molecule, we propose that the highly conserved extracellular domain of Endoglycan confers a pro-adhesive function in this molecule.

Although Endoglycan has been demonstrated to bind L-selectin, these assays were performed in an in vitro and artificial environment (Fieger et al. 2003). Therefore, the function of Endoglycan on primary cells remains largely unknown. Furthermore, the tools available to analyse this novel molecule are also limited. Our laboratory has generated a rat monoclonal IgM antibody against the region N-terminal to the extracellular acidic domain of Endoglycan using the sequence N-VASMEDPGQAPDLPNLPSILPKMDLAEPPWHMPLQGGC-C. This antibody recognises both the mouse and human forms of Endoglycan; however, its utility is limited to flow cytometry.

Fig. 1.13: Bone marrow-derived mast cell aggregation assay. Aggregation assay using BM-derived mast cells. A. Mast cells cultured from wild-type C57Bl/6 BM. 3 ± 2% of these cells are in aggregates and the average number of cells per aggregate was 4 ± 2. B. Mast cells cultured from CD34 and CD43-deficient C57Bl/6 BM and infected with empty pMXpie vector. 94 ± 3% of these cells were in aggregates and the average number of cells per aggregate was 63 ± 8 cells. C. Mast cells cultured from CD34 and CD43-deficient C57Bl/6 BM and infected with pMXpie plasmid containing the full-length CD34. 41 ± 19% of these cells were in aggregates and the average number of cells per aggregate was 14 ± 5 cells. D. Mast cells cultured from CD34 and CD43-deficient C57Bl/6 BM and infected w/ cytoplasmically truncated CD34. 27 ± 2% of these cells were in aggregates and the average number of cells per aggregate was 14 ± 6. E. Mast cells cultured from CD34 and CD43-deficient C57Bl/6 BM and infected with pMXpie plasmid containing full-length Endoglycan. 92 ±1% of these cells were in aggregates and the average number of cells per aggregate was 63 ± 8 cells. F. FACS profiles

showing the expression of GFP and Endoglycan, represented by the x- and y-axis respectively, by the Endoglycan-expressing mast cells. Wildtype and CD34 data courtesy of Erin Drew (Drew et al., 2005).



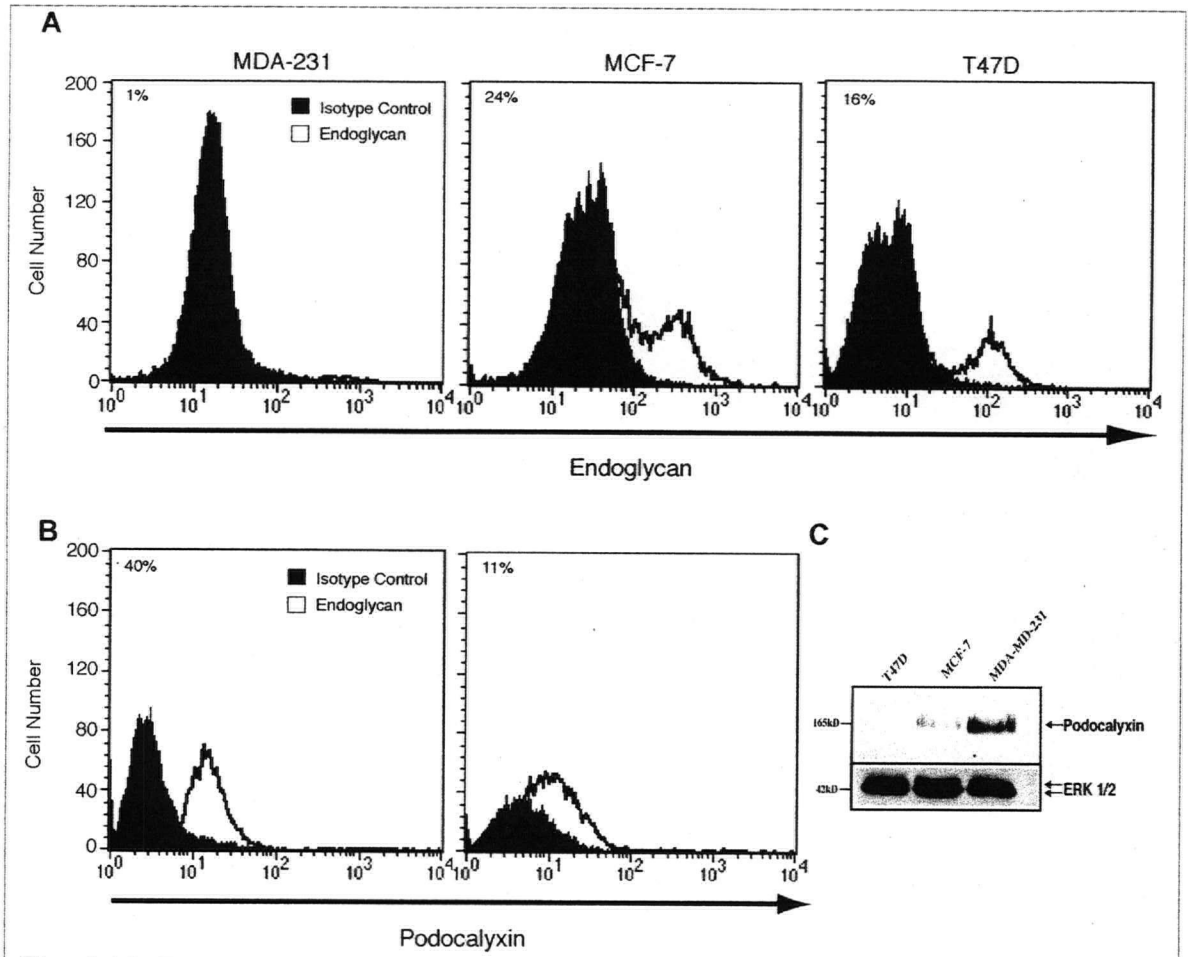


Fig. 1.14: Expression levels of Podocalyxin and Endoglycan on breast cancer cell lines. Expression of Endoglycan and Podocalyxin on three human breast carcinoma lines: MDA-231, MCF-7 and T47D. A. FACS profiles of Endoglycan expression on the three breast cancer cell lines. Endoglycan was not expressed on MDA-231. However, expression was found on both MCF-7 and T47D. B. FACS profiles of Podocalyxin on the breast cancer lines. Podocalyxin was highly expressed on MDA-231 and less on MCF-7. C. Western analysis showing the lack of Podocalyxin on T47D. Podocalyxin data courtesy of Julie Nielsen and (Doyonnas et al., 2001).

In order to study the function of Endoglycan in a natural setting, we looked to the naturally inducible expression of Endoglycan on primary splenocytes. This regulated expression of Endoglycan on activated B cells may be indicative of a specific function for Endoglycan in the inflammatory response. The results obtained in this thesis will provide understanding of the expression of Endoglycan on B cells and the regulation of its expression. The function of Endoglycan still needs further exploration, however, by understanding the regulation of this molecule in B cells, we provide here an *in vivo* functional model, as well as a system to further explore this field.

2. MATERIALS AND METHODS

2.1 Mouse Strains

All mice were maintained at the Biomedical Research Centre (BRC) animal core facility. C57Bl/6, C57Bl/10, CBA/N, CBA/N-xid (Berning et al., 1980) and C57Bl/10ScN (Poltorak et al., 2001) were all purchased originally from Jackson Laboratory (Bar Harbor, ME). Balb/c, Balb/c-nu and CD1-nu were obtained from Charles River Laboratory (Wilmington, MA). CD1 mice were purchased from UBC Animal Care Centre (Vancouver, BC). P-sel KO Bl/6 mice (Dr. Richard Hynes, Massachusetts Institute of Technology) and PSGL-1 KO Bl/6 mice (Bruce Furie, Harvard Medical School/Beth Israel Deaconess) were a kind gifts from Dr. Hermann Ziltener (Rossi et al., 2005). C2GnTI KO mice and PSGL-1/C2GnTI KO mice were a kind gifts from Dr. Hermann Ziltener (Merzaban et al., 2005). CD34/CD43 KO mice were generated in Ziltener laboratory as described previously (Drew et al., 2005).

2.2 Primary Cell Culture

Primary BM cells were obtained by flushing the femur with HBSS supplemented with 2% heat-inactivated fetal bovine serum (FBS) using a $26_G1/2$ needle and homogenizing with a 22_G1 1/2 needle. Red cells were lysed by treatment with 0.1M NH_4Cl for 3 min at 37°C and washed with equal volume of supplemented HBSS. BM cells were cultured in RPMI 1640 (Invitrogen Gibco, Burlington, ON) supplemented with 15% heat-inactivated FBS, 2mM glutamine, 2% IL-3 condition media (JWW_3, BRC, Vancouver, BC), 15% stem cell factor (SCF) condition media (BRC, Vancouver, BC), 10ng/ml recombinant mouse IL-6 (R&D Systems, Minneapolis, MN), penicillin and streptomycin. BM mast cells were derived by culturing BM cells in RPMI 1640 supplemented with 15% heat-inactivated FBS, 2mM glutamine, 4% JWW_3, penicillin and streptomycin.

Primary splenocytes were obtained through homogenizing whole spleen through 70µm nylon cell strainers (Becton Dickinson (BD) Biosciences Discovery Labware, Bedford, MA) in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2mM glutamine, penicillin and streptomycin. Red cells were lysed with treated 0.1M NH_4Cl for 3 min at 37°C and washed with equal volume of media. Primary cells were plated at $2x10^6$ cells per ml and cultured at 37°C. In lipopolysaccharide (LPS) stimulations, cells were stimulated with 15µg/ml of LPS, E. *coli* J5 (EMD Biosciences, Inc., La Jolla, CA). Other TLR agonists tested include 5-15µg/ml LPS-EB ultrapure from E. *coli* 0111:B4

(Invivogen, San Diego, CA), 1μM CpG DNA (Sigma-Genosys, The Woodlands, TX) containing the sequence 5'-TTCATGACpGTTCCTGATGCT-3' and 5μg/ml Pam3CSK4 (Invivogen, San Diego, CA).

In cytokine stimulations, cells were stimulated with either 4% condition media (BRC, Vancouver, BC) or 10ng/ml of recombinant cytokines, these includes: rmIL-1β, rmIL-3, rmIL-4, rmIL-6, rmIL-7 and rmIL-13 all purchased from R&D Systems (Minneapolis, MN), rhuIL-5 (Intergen, Purchase, NY) and rmIL-10 (StemCell Technologies, Vancouver, BC). TNFα (used at 10ng/ml), IFNγ (used at 100ng/ml) and rmSDF-1 (used at 100ng/ml) were obtained from R&D Systems (Minneapolis, MN). hTGFβ-1 (used at 1ng/ml) was purchased from StemCell Technologies (Vancouver, BCV) and S1P (used at 30μM) was obtained from Biomol (Plymouth Meeting, PA). Rat anti-mIgM-FITC antibody (BD Pharmingen, Mississauga, ON), at 5μg/ml, was used to co-treat splenocytes stimulated with either 1μg/ml anti-mCD40 Ab (a gift from Dr. Michael Gold, University of British Columbia) or 200ng/ml BAFF (Alexis Biochemicals, San Diego, CA).

2.3 Cell Lines

All cells were originally obtained from American Typed Culture Collection (ATCC). Y3 rat B myeloma cells (Galfre et al., 1979) and their stable transfectants expressing Endoglycan were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2mM glutamine, 50μM β-ME, penicillin and streptomycin. A20 cells (Kim et al., 1979) were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2mM glutamine, 50μM β-ME, 1mM pyruvate, penicillin and streptomycin. BOSC-1 cells (Sena-Esteves et al., 1999) were grown in DMEM (Invitrogen Gibco, Burlington, ON) supplemented with 10% heat-inactivated FBS, 2mM glutamine, penicillin and streptomycin.

2.4 Expression of mouse Endoglycan (mEndoglycan) by Retroviral Infection

Full-length cDNA encoding mEndoglycan was cloned into TOPO vector (Invitrogen Life Technologies, Carlsbad, CA) and subsequently into pMXpie retroviral vector using BamHI and XhoI sites. BOSC-1 cells were transfected with 7μg of pMXpie vector or pMXpie containing Endoglycan (pMXpie Endoglycan), along with 1μg of pclECO packaging plasmid using Lipofectamine PLUS (Invitrogen, Burlington, ON). Transfected

BOSC-1 cells were cultured for two days and irradiated at 5000 rads. Infection of A20 cells or BM cells was achieved by co-culturing with transfected and irradiated BOSC-1 cells supplemented with 6μg/ml polybrene (Drew et al., 2005). A20 cells were selected in 0.25μg/ml puromycin (McLeod et al., 2004) and BM mast cells were selected in 0.8μg/ml puromycin (Drew et al., 2005). Cells were sorted through FACSVantage (BRC, Vancouver, BC) after at least one week of puromycin-selection.

2.6 Anti-mEndoglycan Anitbody (F4B10) Production

Anti-mEndoglycan antibody was established in our laboratory by immunizing 10-12 weeks-old Wistar female rats with a synthetic peptide (KM-5: N-VASMEDPGQAPDLPNLPSILPKMDLAEPPWHMPLQGGC-C) encoding the non-glycosylated extracellular region of this molecule. Hybridomas were established by cell fusion of splenocytes from immunized rats and mouse X63-Ag8.653 myeloma according to Milstein et al. Hybridomas were selected with HAT medium and cultured in RPMI 1640 supplemented with 20% heat-inactivated FBS, 2% 3T3, 2mM glutamine, penicillin and streptomycin. Antibodies produced by hybridoma clones were isotyped and screened by reaction to KM-5 in enzyme-linked immunosorbent assay (ELISA) and to Y3 rat myeloma expressing Endoglycan in flow cytometry. The anti-mEndoglycan antibody selected is the F4B10 hybridoma.

2.7 Antibodies (Ab)

Anti-mouse -B220/CD45R-PE (used at 0.7μg/ml), -CD138/SDC1 (used at 5μg/ml), -CD29/β1-integrin (used at 5μg/ml), -IgM-PE (used at 5μg/ml), -CD11b-PE (used at 5μg/ml), -CD21-FITC (used at 5μg/ml), -CD23-FITC (used at 5μg/ml), -CD19-FITC (used at 5μg/ml), -B220-PECy7 (used at 5μg/ml), -IgM-FITC (used at 5μg/ml) and -IgD-FITC (used at 5μg/ml) were all purchased from BD Pharmingen (Mississauga, ON). Mouse anti-hamster-FITC (used at 5μg/ml), purified hamster IgG2 (used at 5μg/ml), biotinylated anti-rat IgM (used at 5μg/ml), streptavidin-PE (used at 2.5μg/ml) and streptavidin-FITC (used at 2.5μg/ml) were all purchased from BD Pharmingen (Mississauga, ON). Goat anti-rIgG-AlexaFlur488 used at 4μg/ml was purchased from Invitrogen Molecular Probes (Burlington, ON). Anti-mouse IgD-FITC used at 5μg/ml was purchased at Southern Biotechnology (Birmingham, AL). Anti-mouse CD22-FITC used at 5μg/ml was purchased from Chemicon (Temecula, CA). Anti-mIgG-FITC (used at 5μg/ml) and

purified rat IgM (used at 1μg/ml) were purchased from Cedarlane Laboratory (Hornby, ON). Anti-mouse Endoglycan (F4B10, generated in our laboratory) supernatant was used undiluted.

2.8 Flow Cytometry

FACS buffer used was PBS supplemented with 5% heat-inactivated FBS and 0.05% sodium azide. Mouse serum and goat serum were purchased from Cedarlane (Hornby, ON) and rat serum was purchased from StemCell Technology (Vancouver, BC). Serum was used at 2% diluted in FACS buffer as blocking reagent prior to staining. Cells were harvested at 1200rpm for 3 minutes and stained in 96-well V-bottom plates (Nalge Nunc, Rochester, NY). Staining of cells with antibodies was performed on ice for at least 10 minutes and washed with 150μl FACS buffer in between each staining interval. FACSCalibur or FACScan (BD Biosciences, Franklin Lakes, NJ) was used to collect samples. FlowJo version 4.5.5 (Tree Star, Ashland, OR) was used to analyse and create all FACS profiles. Relative fluorescence intensity was calculated by subtracting the mean fluorescence intensity of the background (isotype control) from staining. Secondary antibody used to detect F4B10, biotinylated anti-rat IgM, was pre-cleared before staining by incubating with 10% mouse serum on ice and centrifuged at 13000rpm for 10 minutes before use.

2.9 Bone Marrow-Derived Mast Cell Aggregation Assays

BM mast cells from wild-type C57Bl/6 and CD34-/- CD43-/- C57Bl/6 overexpressing either CD34 or mEndoglycan were derived as follows (Drew et al., 2005): Cells were plated at $5x10^5$ cells/ml, 1ml in each well of a 6-well plate (BD Labware, Franklin Lakes, NJ) and incubated at 37°C overnight. Cells in groups of 3 or more were scored as aggregates. The total cell number per field was estimated by determining the number of single suspension cells added to average number of cells in aggregates (counting the number of cells in three aggregates multiplied by the number of aggregates). Percent of cells in aggregates per field was calculated by determining the number of cells in aggregates and divided by the total number of cells.

2.10 LPS Administration

Mice were administered 0.5mg of LPS per kg intraperitoneally in 200µl volume with phosphate buffer saline (PBS). Endoglycan expression on hematopoietic tissues were analysed by FACS at 6hr and 24hr.

2.11 Semi-quantitative Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Cells were lysed in TRIzol (Invitrogen, Burlington, ON) reagent, and total RNA was precipitated with chloroform and isopropanol, followed by 75% ethanol wash. Extracted RNA was quantitated at 260nm. Equal amounts of RNA was used to generate cDNA using oligo$(dT)_{20}$ primers from Thermoscript RT-PCR System (Invitrogen, Burlington, ON). Primers specific for Endoglycan transcript (forward: 5'-ACCTGGCCGGGAAGAGCTACATC-3'; reverse: 5'-AACACGTCCGAGTCCTCAG-3') was used for the PCR reaction. PCR for Endoglycan was performed for 50 cycles of 94°C for 1min, 54°C for 1min and 72°C for 2min. Primers specific for HPRT (forward: 5'-CTCGAAGTGTTGGATACAGG-3'; reverse: 5'-TGGCCTATAGGCTCATAGTG-3') was used as loading control. PCR for HPRT was performed for 30 cycles of 94°C for 1min, 55°C for 1min and 72°C for 2min. A20 cells overexpressing Endoglycan along with the vector control cells were used as positive and negative Endoglycan controls respectively. RT-PCR products were visualised in 2% agarose gel containing ethidium bromide. Total RNA integrity was determined by running extracted RNA on 1% agarose gel containing ethidium bromide. Densitometric analysis to determine the intensities of the ethidium bromide stained PCR products was carried out using the Alpha Imager System (Alpha Innotech Corporation, San Leandro, CA).

3. RESULTS

3.1 Endoglycan Expression by B cells

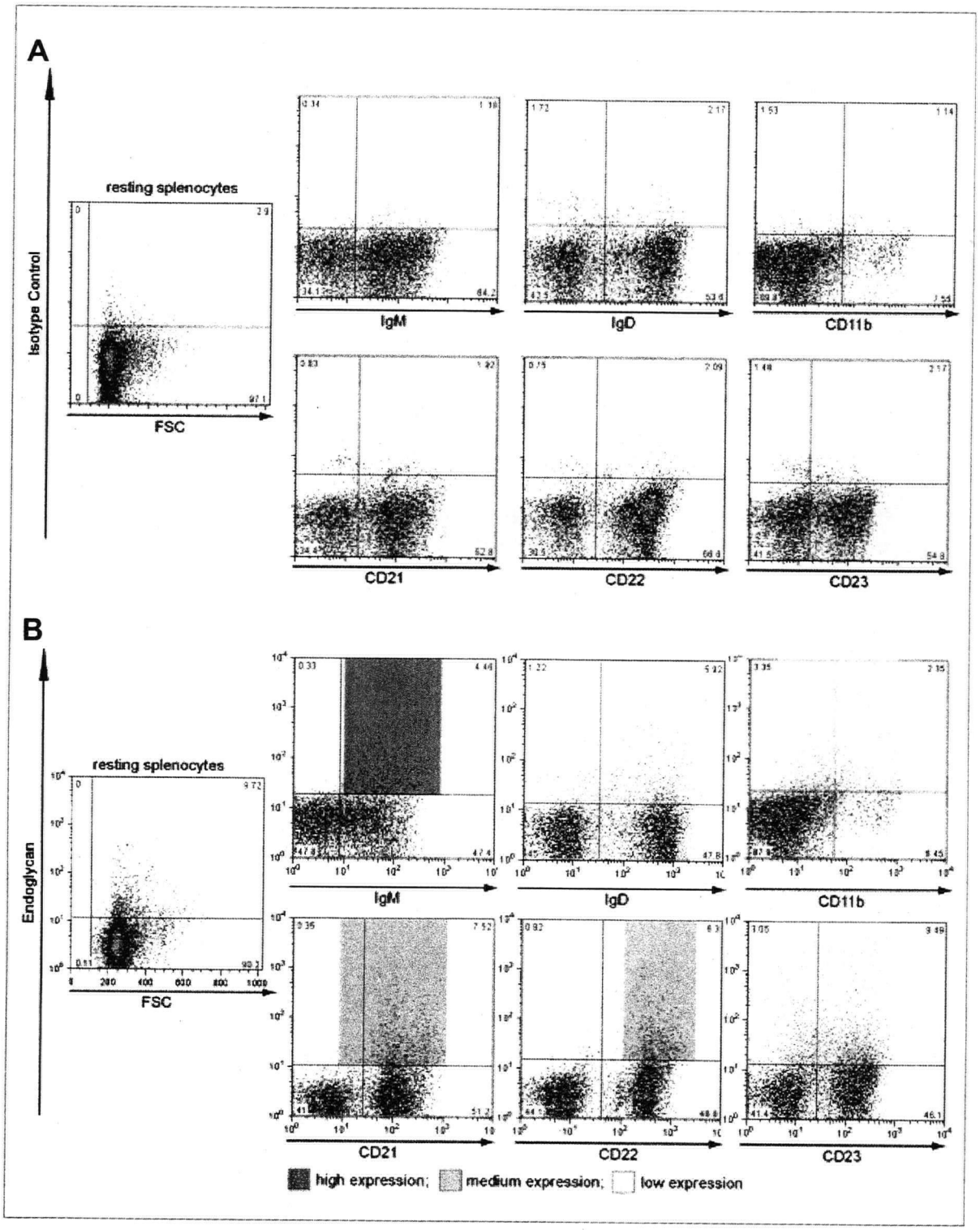
3.1.1 Endoglycan is selectively expressed on splenic B cells with a marginal zone B phenotype and is not expressed on immature B cells

The expression of Endoglycan on resting splenocytes was investigated. In order to determine the subset of B cells that Endoglycan is expressed by these cells, we costained for various molecular markers indicative of discrete B cell subsets. As described previously, B cells can be categorised by the level of expression of surface IgM, IgD, CD21, CD22, CD23 and CD11b. Endoglycan-positive B cells were found to be $IgM^{med/hi}IgD^{lo}CD11b^{med}CD21^{med/hi}CD22^{hi}CD23^{lo}$. This is characteristic of MZ B cells, which are described as IgM^{hi}, IgD^{lo}, $CD21^{hi}$, $CD22^{hi}$, $CD23^{lo}$ and $CD1^{hi}$ (Fig. 3.1). To further confirm that Endoglycan is not expressed by the B-1a subset of B cells, we examined CBA/N-xid mice carrying a defect for the development of B-1a subset of B cells. We observed no decrease in Endoglycan expression levels or the frequency of Endoglycan-positive cells in the spleen relative to B220 expression, suggesting that Endoglycan is not expressed on B-1a B cells (Fig. 3.2).

To further characterise the distribution of Endoglycan on B lineage cells, the expression of this molecule was analysed on bone marrow cells, which contains the earliest committed B cell precursors, pre-B cells and immature, newly-formed B cells. Co-staining of bone marrow cells for Endoglycan and various early B cell markers (CD19, mIgM, B220) showed that Endoglycan is not expressed on immature B cells or pre-B cells (Fig. 3.3).

In summary, Endoglycan is expressed on a small population of primary splenocytes, and this population of cells are similar to marginal zone B cells. There was no evidence for the expression of this molecule on pre-B or immature B cells. Moreover, analysis of CBA/N-xid mice showed that Endoglycan expression is not compromised in these mice, hence suggesting that this molecule is not expressed on the B-1a subset of B cells.

Fig. 3.1: Endoglycan is expressed on cells with a MZ B phenotype. Endoglycan is expressed by ~10% of the resting splenocyte population. A. FACS profile of staining of various B cell marker (x-axis) and the isotype control for Endoglycan (y-axis). B. Double staining of Endoglycan and B cell markers is represented by the y- and x-axis respectively. The levels of B cell marker expression by Endoglycan-positive cells are represented by coloured boxes. Endoglycan-positive resting splenocytes are $IgM^{med/hi}IgD^{lo}CD11b^{med}CD21^{med/hi}CD22^{hi}CD23^{lo}$.


A
Isotype Control
resting splenocytes
FSC
IgM
IgD
CD11b
CD21
CD22
CD23
B
Endoglycan
resting splenocytes
FSC
IgM
IgD
CD11b
CD21
CD22
CD23
high expression;
medium expression;
low expression

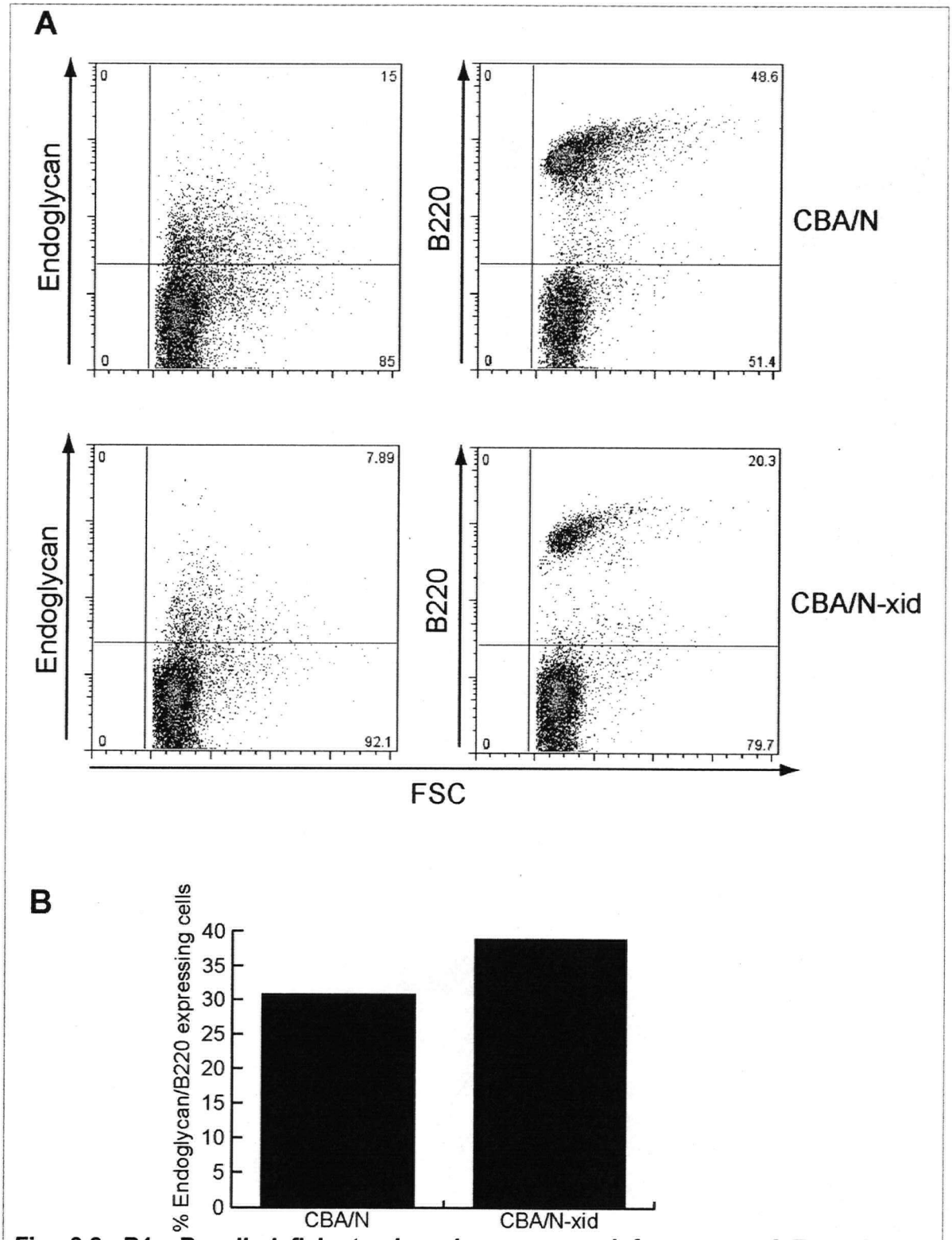


Fig. 3.2: B1a B cell deficient mice show a normal frequency of Endoglycan-positive B cells. A. FACS profiles showing Endoglycan and B220 expression on 48hr LPS-stimulated splenocytes from CBA/N or CBA/N-xid mice. Gates are set with 2% background. B. Frequency of Endoglycan-positive B cells in CBA/N and CBA/N-xid mice relative to B220 expression. Representative of at least three experiments.

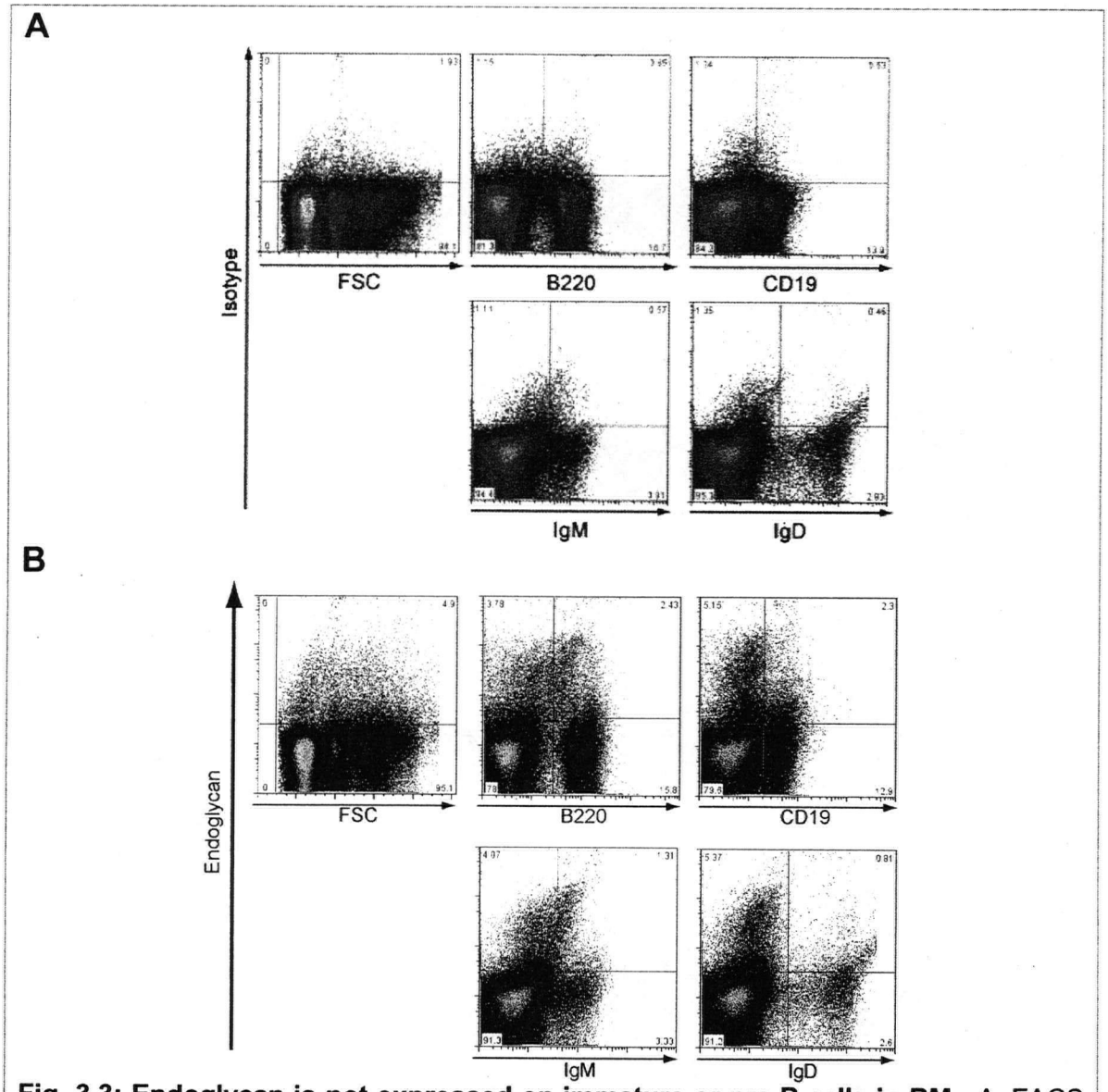


Fig. 3.3: Endoglycan is not expressed on immature or pre-B cells in BM. A. FACS profile of Endoglycan isotype control staining with B cell markers. B. Co-staining of BM cells with Endoglycan and B cell markers represented by the y- and x-axis respectively. Endoglycan-positive cells are not on immature or pre-B cells. Representative of at least three experiments.

3.2 Endoglycan Expression on Activated B cells

3.2.1 Endoglycan surface expression and transcript levels increase upon LPS-stimulation

Endoglycan expression on unstimulated splenocytes constitutes ~10% of the entire splenic population (Fig. 3.1). To test whether this reflected an activated subset of B cells, splenocytes were stimulated with 5-15μg/ml LPS over 48hrs in culture. As shown

in Fig. 3.5, LPS treatment led to a dramatic increase in Endoglycan expression in terms of both the level (mean fluorescence intensity (MFI) 3.8±3.0 before and 21±5.3 after treatment for 48hrs) and in terms of frequency (5.6±1.9% before and 35±2.0% after). The effect of LPS on splenocytes is specific as shown by LPS treatment on splenocyte cultures from C57Bl/10ScN, which lacks the LPS receptor TLR4 (Poltorak et al., 2001). C57Bl/10ScN failed to show expression of Endoglycan in responses to LPS (MFI of 1.49±1.41; frequency of 4.17±2.12%). We conclude that the induction of Endoglycan by LPS is TLR4 dependent. The expression of Endoglycan is observed only on the $B220^{+}$ (a pan-B cell marker) population (Fig. 3.4). Time course experiments revealed that Endoglycan expression peaked at 48hrs and sustained thereafter (Fig. 3.5).

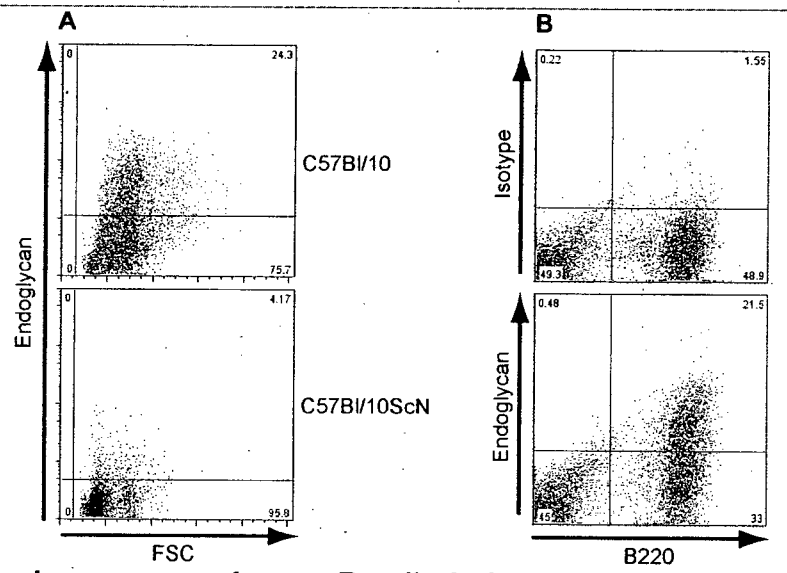


Fig. 3.4: Endoglycan expression on B cells is induced by LPS treatment and is TLR4 dependent. A. FACS profiles showing Endoglycan expression after 48hr of LPS-stimulation on splenocytes from C57Bl/10 or C57Bl/10ScN. The specificity of LPS-induced Endoglycan upregulation is shown by the lack of LPS-effect on splenocytes from C57Bl/10ScN, TLR4-deficient mice. B. FACS profiles showing that Endoglycan is expressed by B220-positive splenocytes after 48hr of LPS-stimulation. Representative of at least three experiments.

In order to correlate the observed increase in surface expression of Endoglycan to transcriptional upregulation, a semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) was performed. 2μg of whole RNA extracts from LPS-stimulated splenocytes at 0, 6, 12, 24, 48 and 72 hours post-stimulation was used in the RT-PCR reaction. The "house keeping" transcript, hypoxanthine phosphoribosyl transferase (HPRT), was used as a loading control. A20 cells transfected with pMXpie or pMXpie

containing Endoglycan were used as negative and positive controls for Endoglycan respectively. These cells express low levels of endogenous Endoglycan. As shown in Fig. 3.5B, the band for the Endoglycan-positive control was higher than the other samples, this is an anomaly due to overloading of DNA. Results by dot densitometry analysis revealed an increase in Endoglycan transcript over time that peaked at 48h (Fig. 3.5).

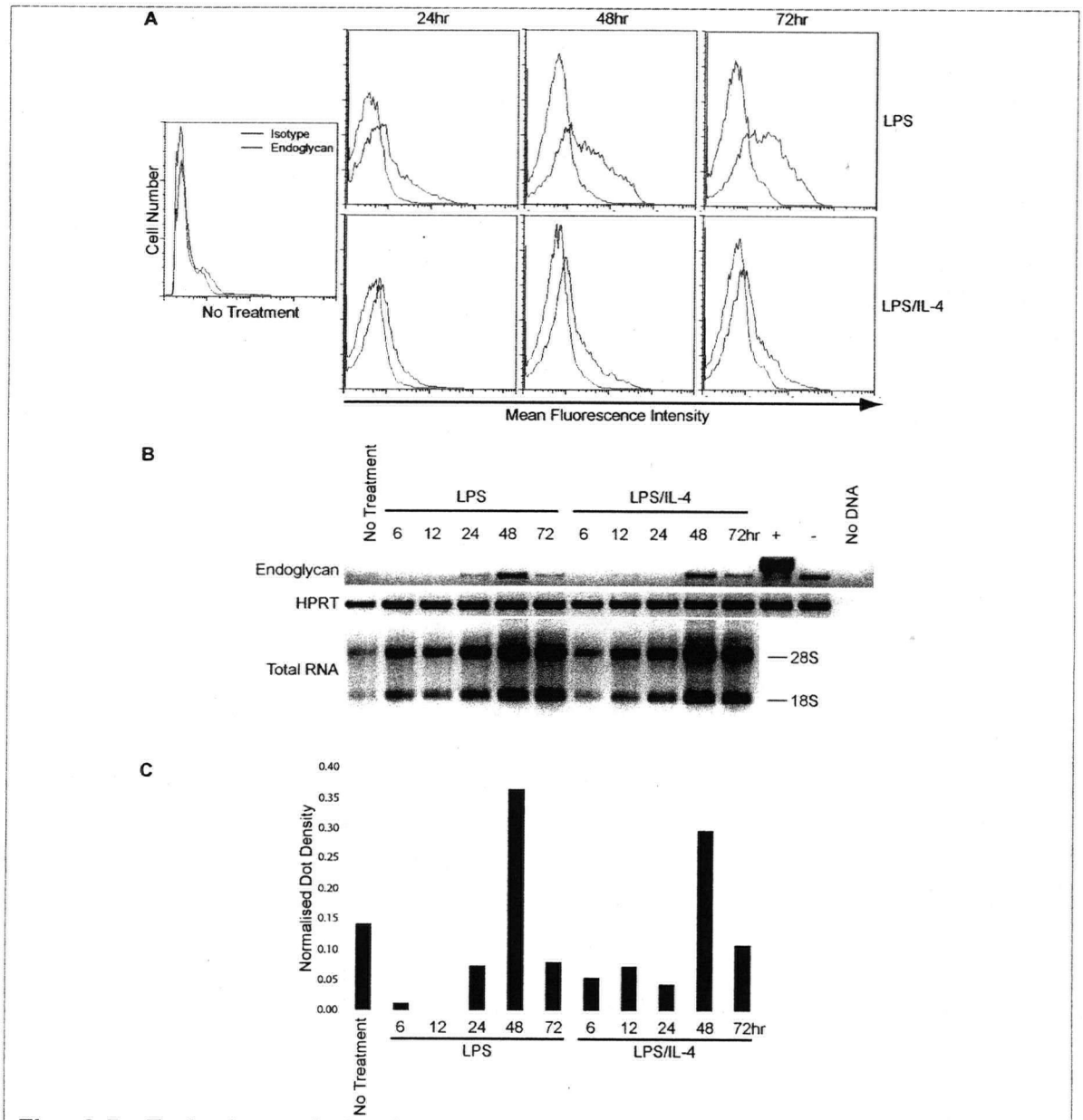


Fig. 3.5: Endoglycan induction by LPS is blocked by IL-4 treatment and is regulated by transcriptional and post-transcriptional mechanisms. A. FACS profiles showing Endoglycan expression on splenocytes over 72hr during LPS or LPS/IL-4 stimulation. LPS-induced Endoglycan expression peaks at 48hr and IL-4 suppresses surface expression of Endoglycan as detected by F4B10 Ab. B. RT-PCR of RNA extracted from splenocytes stimulated with LPS or LPS/IL-4 over 72hrs. HPRT was

used as loading control and total RNA show the integrity of extracted RNA samples. C. Dot densitometry of Endoglycan mRNA normalised against HPRT mRNA. Representative of at least three experiments. RT-PCR data courtesy of Takahide Murakami.

3.2.2 CD1 mice are defective for LPS-induced Endoglycan expression on B cells

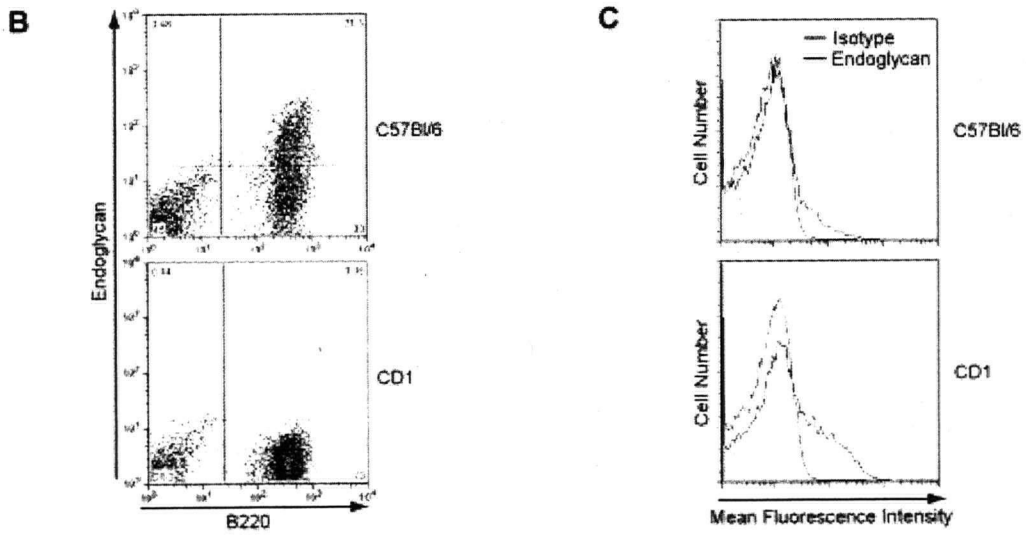
The LPS-induced Endoglycan upregulation was not mouse-strain specific since we found that C57Bl/6, C57Bl/10, Balb/c, and CBA/N all responded with similar increases. As well, splenocytes from various mutant and transgenic mice tested (Balb/c-nu CBA/N-xid, P-sel KO, PSGL-1 KO, C2GnTI KO and PSGL-1/C2GnTI KO) responded to LPS with increase in Endoglycan expression. The exception to this was CD1 mice. The CD1 strain seems to have a lower level of Endoglycan upregulation upon LPS-stimulation on splenocytes in culture. Splenocytes from CD1 mice respond to C57Bl/6 upon LPS-stimulation with increase in B220 population. However, CD1 splenocytes did not upregulate Endoglycan upon LPS stimulation. The possibility that this is a natural occuring Endoglycan-deficient strain was eliminated since the thymic expression of Endoglycan in these mice was not compromised. Interestingly, Balb/c nude mice did not show a reduced Endoglycan upregulation by LPS stimulation on splenocytes. Therefore, suggesting that Endoglycan expression is not T-cell dependent. Moreover, CBA/N-xid mice also did not show decreased level of Endoglycan expression upon LPS-stimulation, further confirming Endoglycan is not expressed on B1a B cells (Fig. 3.6).

Endoglycan expression on LPS-treated splenocytes is T cell independent and is not on the B-1a subset of B cells. Moreover, there are minimal strain differences in the levels of Endoglycan upregulation. The variability observed in Endoglycan expression on CD1 splenocytes may explain differences in inflammatory response between these strains.

Fig. 3.6: Endoglycan expression by various inbred and KO mouse strains. A. Table of the various mouse strains tested for LPS-induced Endoglycan expression on splenocytes. B. FACS profiles showing Endoglycan and B220 expression, as represented by the y- and x-axis respectively, on 48hr LPS-stimulated splenocytes from CD1 and C57Bl/6 mice. Gates were set with 2% background staining. C. FACS profiles showing Endoglycan expression on resting thymocytes from CD1 and C57Bl/6 mice. Representative of at least three experiments.

A

Strain	Phenotype	Splenic Endoglycan Expression with LPS
C57Bl/6		+
C57Bl/10		+
Balb/c		+
Balb/c-nu	athymic; lack mature T cells	+
CD1		-
CD1-nu	athymic; lack mature T cells	-
CBA/N		+
CBA/N-xid	X-linked immune-deficient mice (reduced B-1a B cell subset)	+
P-sel KO	lack P-selectin	+
PSGL-1 KO	lack PSGL-1	+
C2GnTI KO	lack C2GnTI enzyme	+
PSGL-1/C2GnTI-KO	lack PSGL-1 and C2GnTI enzyme	+
C57Bl/10ScN	lack TLR4	-



3.2.3 Endoglycan is expressed on a subset of plasma cells and these are β1-integrin positive

It has been shown that LPS stimulation induces plasma cell differentiation in splenic B cells (Lin et al., 2002; Schliephake and Schimpl, 1996; Shaffer et al., 2002; Shaffer et al., 2004). Therefore, to confirm that Endolgycan is expressed on plasma cells, the plasma cell marker, CD138/SDC1, was used to co-stain LPS induced plasma cells. SDC1 and Endoglycan co-staining shows that Endoglycan expressing cells are all SDC1-positive. However, not all SDC1-positive cells express Endolgycan. Therefore, Endoglycan is expressed on a subset of plasma cells. Moreover, co-staining of 48hr LPS-stimulated splenocytes for Endoglycan and β1-integrin show that these cultures are mostly β1-

integrin positive and that all Endoglycan-expressing cells express β1-integrin as well (Fig. 3.7).

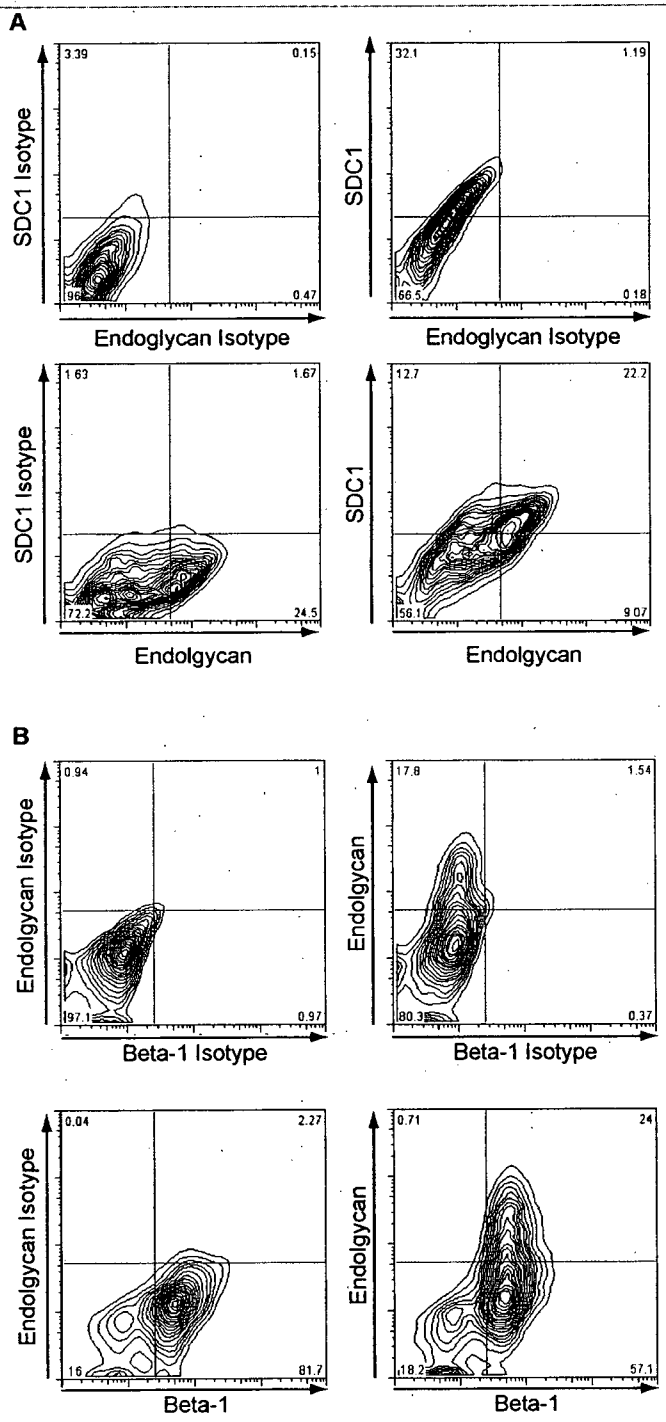


Fig. 3.7: Endoglycan is upregulated on a subset of plasma cells expessing β1-integrin. A. FACS profiles of 48hr LPS-stimulated C57Bl/6 splenocytes stained with isotype controls, SDC1 and/or Endoglycan. B. FACS profile of 48hr LPS-stimulated C57Bl/6 splenocytes stained with isotype controls, β1-integrin and/or Endoglycan.

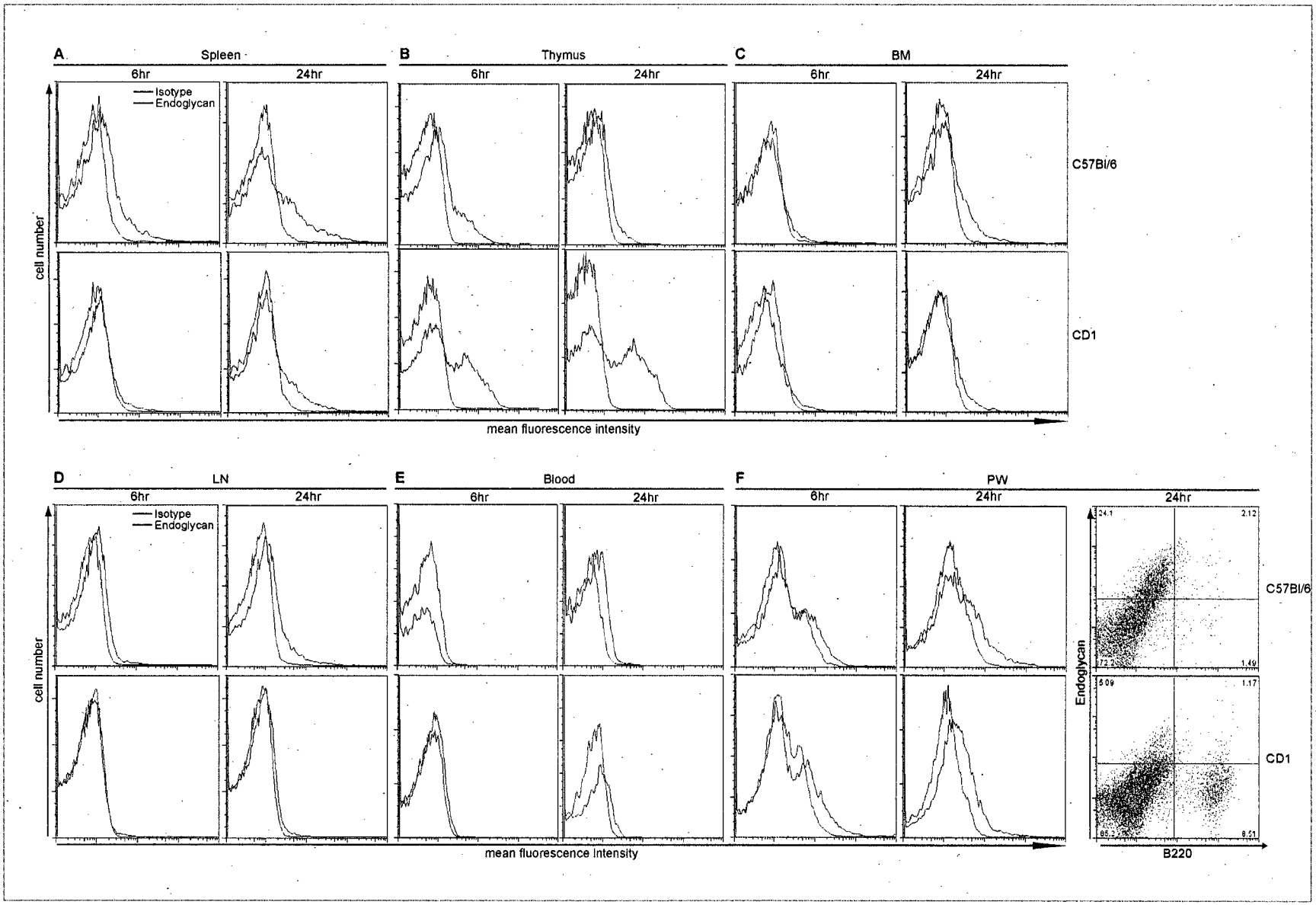
3.2.4 Endoglycan expression by B splenocytes is increased upon LPS-administration *in vivo*

In order to show that the LPS-induced upregulation of Endoglycan was not selective to *in vitro* cultured B cells, the expression of this molecule upon LPS stimulation was analysed *in vivo*. LPS was administered intraperitoneally into C57Bl/6 or CD1 mice and hematopoietic tissues were analysed at 6 and 24hr. Since splenocytes from CD1 mice showed a reduced induction of Endoglycan expression by LPS *in vitro*, this strain of mice was used as a negative control for LPS-administration. Although Endoglycan expression on CD1 splenocytes increases when activated *in vivo*, the intensity of upregulation is less than C57Bl/6 splenocytes. Endoglycan was upregulated in the spleen as well as cells of the peritoneum of C57Bl/6 mice after 6 and 24hr of LPS administration. However, the CD1 strain, in response to LPS *in vivo*, showed little or no increased induction of Endoglycan expression, similar to that observed in *in vitro* cultures (Fig. 3.8).

The thymic expression of Endoglycan from CD1 mice is inherently higher than that of the C57Bl/6. This difference in expression was shown using unstimulated thymocytes (Fig. 3.6C), as well, on thymocytes after LPS-administration from C57Bl/6 and CD1 (Fig. 3.8B).

24hr after LPS administration in C57Bl/6, there was a slight increase in Endoglycan expression in the BM. Further analysis is needed to determine if these are long-term plasma cells that have migrated to the BM niche or if these are BM cells that have induced Endoglycan expression upon LPS challenge.

Fig. 3.8: Expression of Endoglycan on hematopoietic tissues after LPS-administration at 6 and 24hr. FACS profiles of Endoglycan expression on hematopoietic tissues/cells after LPS-administration. A. Endoglycan expression on C57Bl/6 and CD1 splenocytes increases from 6hr to 24hr post LPS administration. B. Thymic expression of Endoglycan in C57Bl/6 decreases from 6hr to 24hr post LPS administration. However, the Endoglycan expression on CD1 thymocytes increases from 6hr to 24hr post administration. C, D, E. Endoglycan expression in C57Bl/6 BM (C), mesenteric lymph node (LN) (D) and blood (E) increases from 6hr to 24hr post LPS administration. The Endoglycan expression in CD1 of these tissues does not vary or increase to a slighter extent from 6hr to 24hr as compared to C57Bl/6. F. Peritoneal wash (PW) show an increase in Endoglycan expressing cells from 6hr to 24hr post LPS administration in both mouse strains. However, whereas all B220 cells in C57Bl/6 PW is Endoglycan-positive, B220 cells in CD1 PW are Endoglycan-negative.


A
Spleen
B
Thymus
C
BM
6hr
24hr
Isotype
Endoglycan
cell number
C57Bl/6
CD1
mean fluorescence intensity
D
LN
E
Blood
F
PW
Endoglycan
B220
24.1
2.12
73.2
1.49
5.09
1.17
85.3
9.51

3.2.5 Endoglycan expression is selectively upregulated by TLR agonists

The expression of Endoglycan on splenocytes through TLR4 stimulation was consistently observed as described above. Therefore, to investigate if this upregulation is TLR4-specific, other TLR-agonists were surveyed for their ability to upregulate this molecule. Stimulation of primary splenocyte cultures with 5μg/ml Pam3CSK4 (TLR2 agonist) and 1μM CpG (TLR9 agonist), over 48hrs all induced the upregulation of Endoglycan. The lack of Endoglycan upregulation by LPS on C57Bl/10ScN splenocytes demonstrates the specificity of LPS. Therefore, stimulation of TLR2, 4 and 9 enhances the expression of Endoglycan on splenocytes (Fig. 3.9).

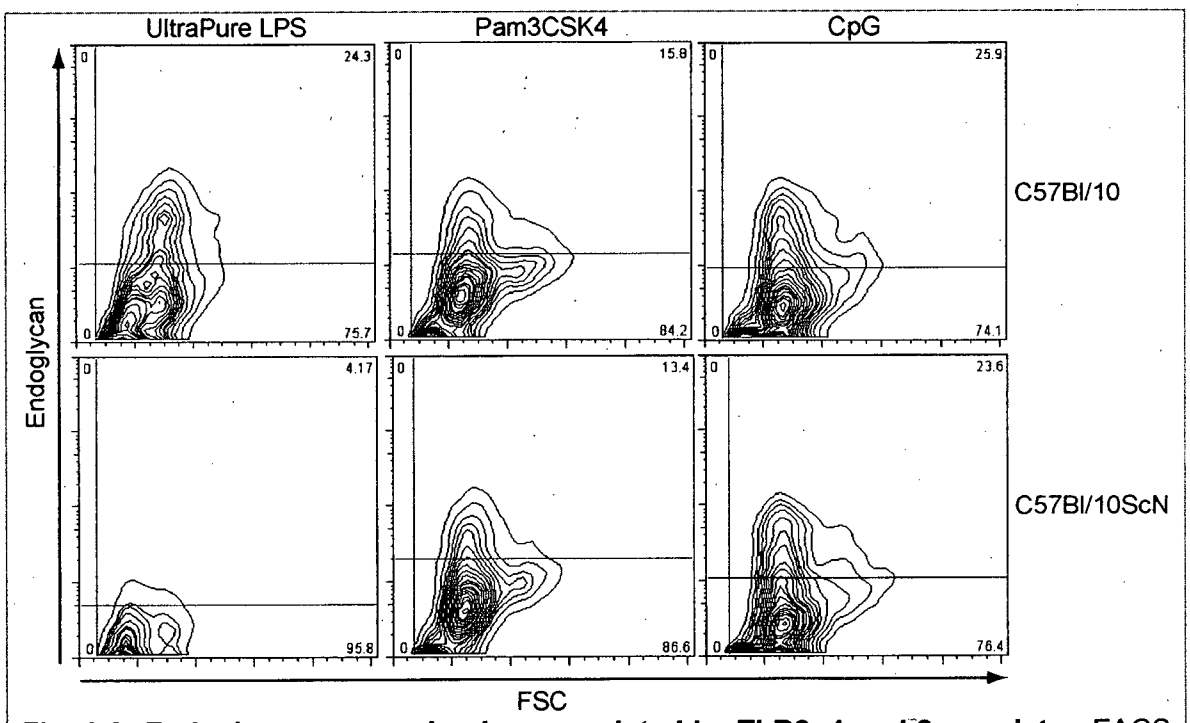


Fig. 3.9: Endoglycan expression is upregulated by TLR2, 4 and 9 agonists. FACS profiles showing Endoglycan expression on splenocytes from C57Bl/10 or C57Bl/10ScN induced by stimulation with UltraPure LPS, Pam3CSK4 or CpG. Endoglycan expression on splenocytes was upregulated by stimulation with TLR2 agonist, Pam3CSK4, TLR4 agonist, UltraPure LPS, and TLR9 agonist, CpG.

To test whether the increased Endoglycan expression on splenocytes was specific to TLR agonists or could be induced by other B cell activators or mitogens, we treated splenic B cells with anti-mIgM Ab (5μg/ml), BAFF (200ng/ml, with or without anti-mIgM costimulation), anti-mCD40 Ab (1μg/ml, with or without anti-mIgM costimulation), TNFα (10ng/ml) and IFNγ (300ng/ml). Although these activatiors stimulated proliferation and

formation of blasts in culture, none led to the upregulation of Endoglycan on splenocytes over 48hr (Fig. 3.10).

We conclude that the upregulation of Endoglycan on activated B cells is highly specific to TLR-stimulation and not achievable through other B cell signaling pathways.

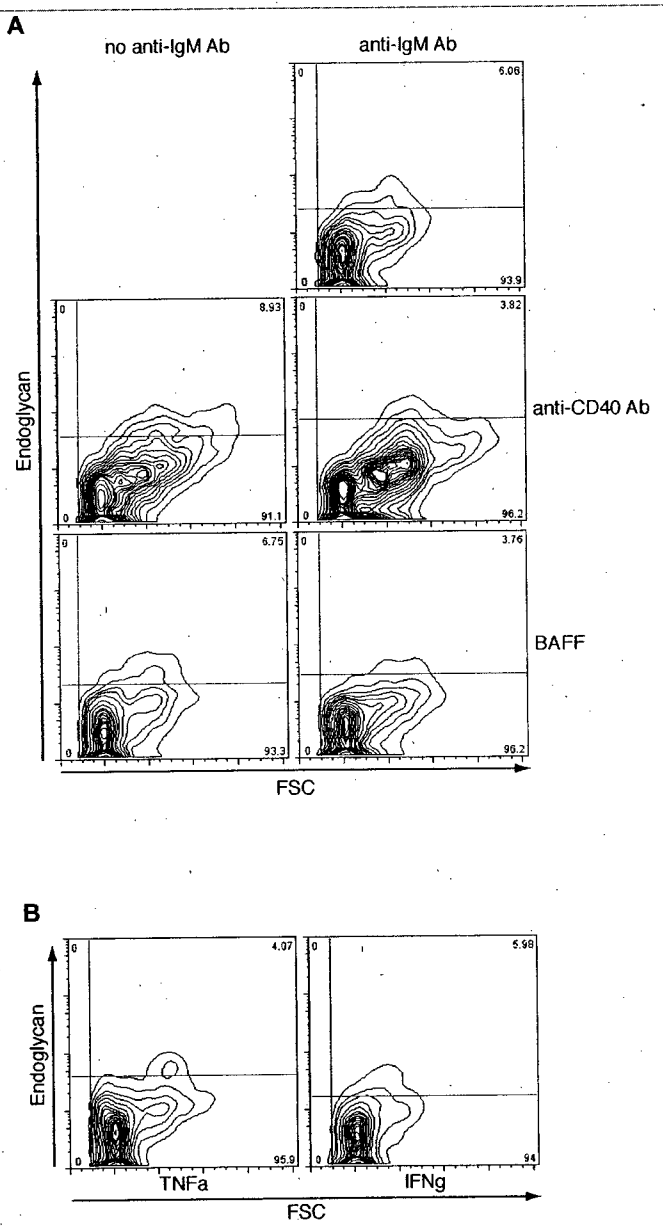
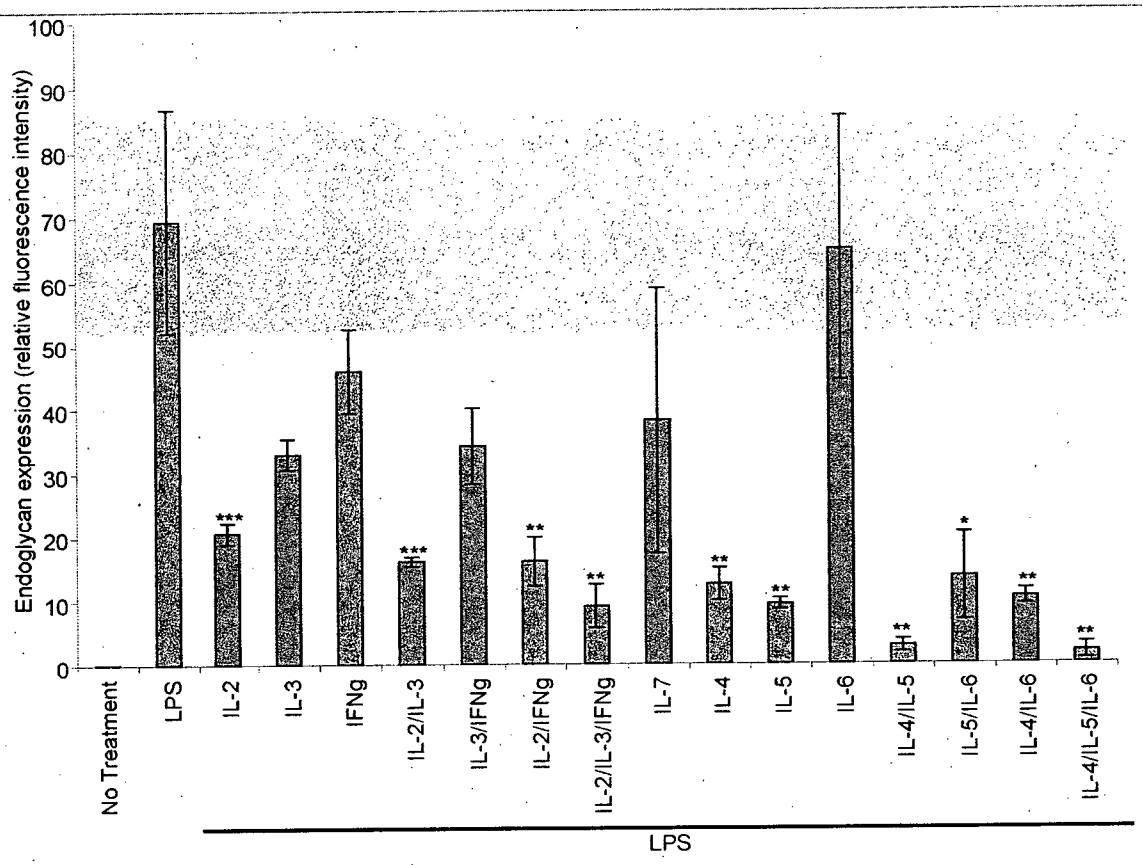


Fig. 3.10: Endoglycan expression on splenocytes is not affected by stimulation with anti-IgM, anti-CD40, BAFF, TNFα and IFNγ. A. FACS profiles showing Endoglycan expression on splenocytes after 48hr stimulation with anti-CD40 Ab or BAFF, both with or without anti-mIgM Ab. Gates are set with 2% background. B. FACS profiles showing Endoglycan expression on splenocytes after stimulation with TNFα or IFNγ for 48hr. Gates are set with 2% background.

3.3 LPS-induced Endoglycan Expression is Suppressed by T_H2-type Cytokines and the Anti-Inflammatory Factor, TGFβ

Cytokines have long been known to have a regulatory role in B cell development (Armitage et al., 1993; Bouchard et al., 1994; Ford et al., 1999; Ozaki et al., 2004). Therefore, the regulatory role of cytokines on Endoglycan expression induced by LPS-stimulation was explored. 10ng/ml or 4% condition media containing IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10 or IL-13 were used to co-stimulate splenic B cells with LPS and Endoglycan expression was analysed by FACS. IL-2, IL-4 and IL-5 suppressed the LPS-induced Endoglycan upregulation. Although not statistically significant, the data suggest that combining IL-4 and IL-5 may have an additive affect (Fig. 3.11). The IL-4 suppressive effect was also observed when this cytokine was added at 24hr post LPS-stimulation (at a time when Endoglycan mRNA levels have normally increase), suggesting the potential for IL-4 to suppress Endoglycan expression is a post-transcriptional-event. The addition of IL-4 at 24hr suppressed the LPS-induced upregulation of Endoglycan (Fig. 3.12: right panel), similar to that observed when both reagents LPS and IL-4 were added at time 0hr (Fig. 3.12: centre panel). However, this data does not rule out the possibility that IL-4 may also repress Endoglycan expression (Fig. 3.12).



* P = 0.05; ** P = 0.06; *** 0.07 ≤ P < 0.10

Fig. 3.11: Effect of T_H1 and T_H2 cytokines on Endoglycan expression. Graph showing the expression of Endoglycan on 48hr-stimulated splenocytes. Endoglycan expression is represented by relative fluorescence intensity (after reduction of bacground fluorescence). Gates are set with 2% background. The shaded area represent the expression of Endoglycan induced by LPS. LPS treatment with IL-2, IL-4 and IL-5 (or these cytokines in combination with other cytokines) reduced Endoglycan expression compared to LPS treatment alone, resulting in P values below 0.10 in unpaired single-tail T-test.

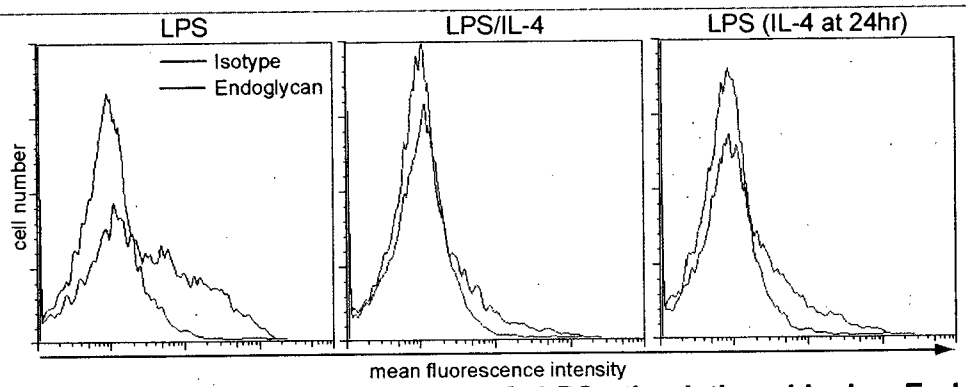


Fig. 3.12: IL-4 treatment after 24hr of LPS-stimulation blocks Endoglycan expression on splenocytes. FACS profiles showing the expression of Endoglycan on splenocytes cultured for 48hrs. LPS-induced expression of Endoglycan on splenocytes is shown in the left panel. Addition of IL-4 after 24hr of LPS-stimulation (right panel). Stimulation with both LPS and IL-4 at time 0hr (centre panel).

Since receptors for IL-4 and IL-13 share the same IL-4Rα signaling chain (Rolling et al., 1996; Umeshita-Suyama et al., 2000), it was expected that IL-13 should have the same affect on LPS-stimulated splenocytes as IL-4. Strikingly, IL-13 was unable to suppress Enodglycan expression.

Both IL-4 and IL-5 are secreted by T_h2 cells and promote the development of IgG, IgE or IgA-secreting plasma cells. Therefore, the suppressive effect of these cytokines on LPS-induced Endoglycan expression suggests that Endoglycan is expressed on a distinct population of activated B cells.

Thus far, Endoglycan has been shown to be a pro-inflammatory marker on B cells in response to TLR stimulation, however, its expression as an activation marker remains to be verified with other TLR agonists. Co-stimulation of splenocytes with LPS and the anti-inflammatory factor, TGFβ, suppresses the expression of Endoglycan. Moreover, co-stimulation of splenocytes with the pro-inflammatory cytokine, IL-1β, induces a slight increase in Endoglycan expression (Fig. 3.13). Therefore, Endoglycan is a marker on B cell during inflammatory responses.

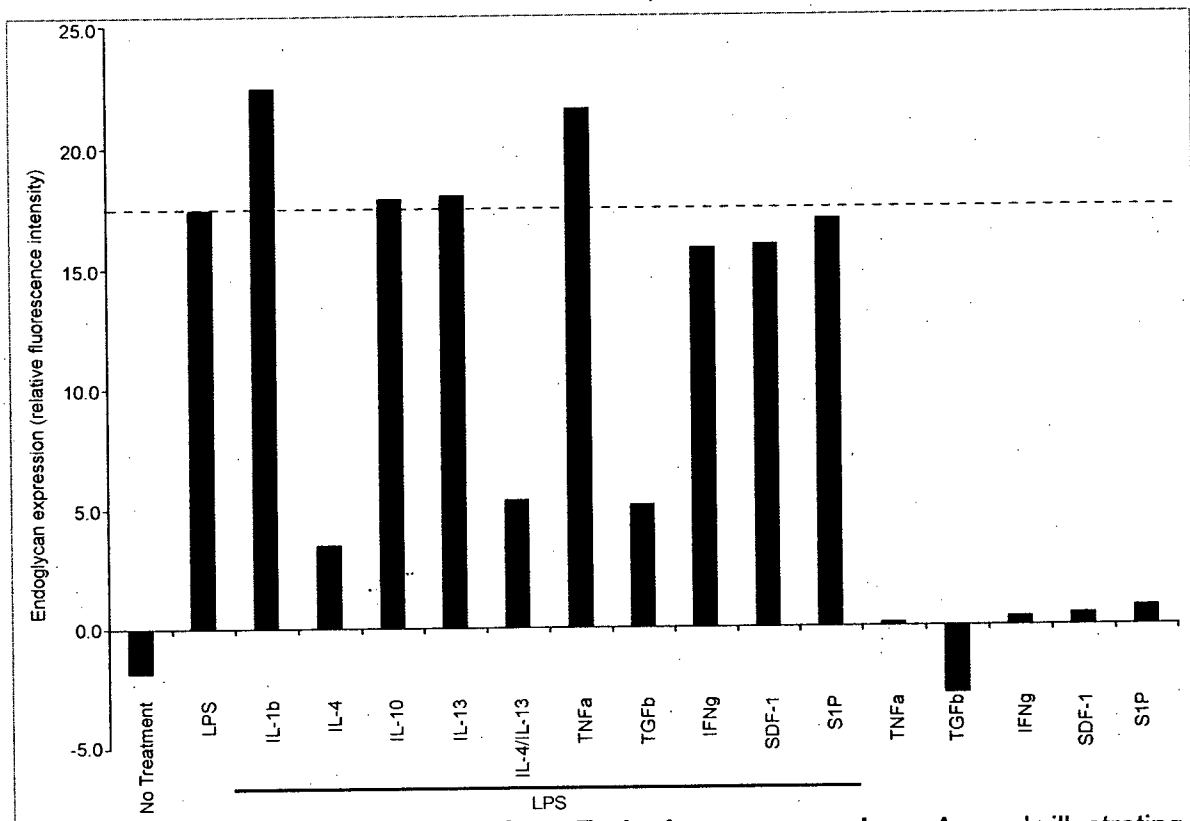


Fig. 3.13: Effect of IL-1β and TGFβ on Endoglycan expression. A graph illustrating Endoglycan expression on 48hr-stimulated splenocytes. Endoglycan expression is represented by the relative fluorescent intensity (after reduction of background fluorescence). Reduced Endoglycan expression with IL-4 treatment is consistent with previous observations. In addition, IL-1β seem to enhance Endoglycan expression where as TGFβ reduces Endoglycan expression. None of the stimulations in the absence of LPS induces Endoglycan expression. Representative of two individual data sets.

4. DISCUSSION

4.1 Endoglycan is on splenic B cells with a MZ B phenotype

In naïve splenocytes, only 2-10% of the cells expresses Endoglycan. By delineating this small population using various B cell markers, it was observed that these cells are $IgM^{med/hi}IgD^{lo}CD11b^{med}CD21^{med/hi}CD22^{hi}CD23^{lo}$; a phenotype characteristic of MZ B cells. Moreover, Endoglycan was not observed on immature B cells and B cell precursors in the BM by co-staining for Endoglycan and various early B cell markers. These findings suggest that Endoglycan is a marker on a specific population of mature B cells.

Several transgenic mice have been reported to have lower or higher levels of or lack MZ B cells (Table 4.1). Analysing various transgenics and the expression of Endoglycan in the spleens of these mice may confirm the expression of Endoglycan on MZ B cells.

Knockout and transgenic mice with alterations in MZ B cells

Mouse System	Observations
L5-/-, IL7-/-, IL7ra-/-, Rag-/- conditional	Larger proportion of MZ and B1 B cells and reduced follicular B cells
CD19-/-	Lack of MZ B cells; defects in TD and TI responses
Baff-/-, Baffr-/-	Lack of B cells beyond NF/TR stage
Baff-transgenic	Increase in TR and MZ B cells, in particular; B-cell autoimmune manisfestation
Pyk2-/-	Lack of MZ B cells; normal B1 cells; hyporesponsivity to chemokines

Table 4.1: Transgenic mice with alterations in MZ B cells. Adapted from Martin and Kearney, 2002.

4.2 Endoglycan is expressed on B cells activated via TLR

Endoglycan is a specific marker on B cells activated via TLR. The expression of this molecule was analysed through FACS and confirmed by RT-PCR. A time course studying the expression of Endoglycan on LPS-activated splenocytes over 72hrs showed increase in transcript levels for Endoglycan with increasing surface expression of this molecule as detected by the rat monoclonal antibody, F4B10. The level of Endoglycan protein expression post-stimulation peaked at 48hr. The specificity of this LPS effect was tested by stimulation of a TLR4-deficient mouse, C57Bl/10ScN, where Endoglycan expression was not observed. Agonists of TLR2, 4 and 9 were able to induce the upregulation of Endoglycan on splenocytes. Moreover, IL-1β slightly enhances the LPS-induced upregulation, therefore, suggesting that the signaling pathway is through TIR-domains since both TLR and IL-1β receptors are TIR-containing receptors. Consistent with this hypothesis, *in silico* promoter analysis revealed potential

binding sites for NF-κB and IRF, TIR domains activate downstream signaling that lead to the activation of these transcriptional activators. The specificity of Endoglycan upregulation to this TLR-downstream event is shown by stimulation of splenocytes with various activating factors such as anti-IgM, BAFF (in the presence and absence of anti-IgM) and CD40L (in the presence and absence of anti-IgM). The distribution of Endoglycan suggests that this molecule serve a specific funciton on plasma cells (see below).

In order to confirm the effect of NF-κB and IRF on Endoglycan expression, promoter deletion experiments need to be performed. The binding sites for these transcription factors can be mutated or altered, and changes in Endoglycan expression upon LPS-stimulation can be analysed. Hence, the upregulation of Endoglycan by TLR agonists should be reduced or abolished by dysfunctioning either NF-κB and/or IRF binding sites.

4.3 LPS-induced Endoglycan expression on a subset of plasma cells and this expression is downregulated by T_H2-type cytokines and TGFβ

LPS has been shown to induce the formation of plasma cells from B cells (Lin et al., 2002; Schliephake and Schimpl, 1996; Shaffer et al., 2002; Shaffer et al., 2004). To confirm that the LPS-stimulated splenocyte cultures used in this thesis produced plasma cells, these cultures were stained with the plasma cell marker, SDC1. We found that majority of the cells were $SDC1^+$.

The co-stimulation of splenocytes with LPS and IL-4 has been shown to promote the differentiation of B cells into IgG- and IgE-secreting plasma cells (Hasbold et al., 1998; Tangye and Hodgkin, 2004) and co-stimulation with IL-5 leds to IgA-secreting plasma cells (Sonoda et al., 1989). However, data presented here show that co-stimulation of LPS with IL-4 and/or IL-5 reduces the LPS-induced Endoglycan expression. A possible explanation for this effect is suggested by transcription factor binding sites in the Endoglycan promoter. The overlapping binding sites for STAT and NF-κB may suggest that NF-κB competes for binding site with the transcriptional repressors, Stat5 and Stat6. The anti-inflammatory factor, TGFβ, also suppressed the LPS-induced upregulation of Endoglycan.

These observations, together with the specificity for the induction of this antigen, suggest that Endoglycan expression is highly regulated and its expression marks a subset of plasma cells. The expression of Endoglycan may allow cells to exit follicles of lymphoid organs, either through chemotactic signals, adhesive or anti-adhesive mechanisms. Therefore, by downregulating this molecule in the presence of cytokines produced by T cells, B cells may be retained in the follicles or these cells may migrate to sites different from Endoglycan-positive cells.

The signaling capabilities of Endoglycan have not been explored. However, the potential for this molecule to dimerize, as well as, be phosphorylated in the cytoplasmic domain, does not rule out a possible signaling function. Moreover, the high amino acid identity across species suggests that this molecule have an unidentified extracellular ligand. As suggested by studies using human recombinant Endoglycan proteins, this molecule has the potential to bind L-selectin (Fieger et al., 2003). Since Endoglycan-expressing cells in the LPS-stimulated splenocyte cultures all express β1-integrin, these cells may be long-lived plasma cells that home to BM. Long-lived plasma cells are retained in the BM niche by various adhesion molecules, including E-selectin (Underhill et al., 2002). Therefore, it may be possible that Endoglycan is an E-selectin ligand when expressed on activated B cells and thereby aid retention of plasma cells in BM. Due to the similarity between Endoglycan and its family members, CD34 and Podocalyxin, Endoglycan may also act as an anti-adhesive molecule through its highly negative-charged extracellular domain (Doyonnas et al., 2001; Drew et al., 2005). Thereby allowing plasma cells to migrate through the endosteum to their BM niche.

Similar to the promoter mutational analyses that are needed to confirm of the effect of NF-κB and IRF, mutagenesis experiments are also needed to confirm the downregulatory effect of Stats and Smads. Moreover, in order to test the homing effect of Endoglycan, *ex vivo* short-term homing assays can be performed. By using LPS-stimulated splenocyte cultures and sorting for $B220^{+}Endoglycan^{+}$ and $B220^{+}Endoglycan^{-}$ populations, we may label these cells with fluorescencent dyes such as Tracker Orange or CSFE and analyse the differential homing of these cells over 24hrs by FACS (Drew et al., 2005). To test the possibility that Endolgycan acts as a selectin ligand, static selectin-binding can be performed using recombinant selectin proteins and staining LPS-stimulated splenocytes with or without IL-4. However, in order to verify that the binding

is due to Endoglycan, anti-Endo F_{ab} fragments must be created to block selectin-binding interactions. As shown in chapter 1, full-length Endoglycan when expressed in CD34/CD43-deficient BM-derived mast cells did not decrease homotypic aggregation of these cells (Fig. 1.13). However, BM mast cells also express NHERF-1. Since NHERF-1 has been shown to associate with actin cytoskeleton (Shenolikar and Weinman, 2001), it is possible that the lack of anti-adhesive effect is due to capping of Endoglycan by NHERF-1. Therefore, to confirm that this phenomenon is not due to capping of Enodglycan, a naturally truncated form of Endoglycan needs to be cloned and expressed in the BM mast cell assay.

4.4 Differences in the effect of IL-4 and IL-13 on Endoglycan expression

Since the IL-13 receptor share a common signaling chain, IL-4Rα, as IL-4 receptor, it was expected that these two cytokines would have similar effect on Endoglycan expression by LPS-stimulation. However, it was observed that IL-13 had no effect on Endoglycan expression, whereas IL-4 suppressed the LPS-induced Endoglycan expression. Early studies in transgenic mouse models have demonstrated that IL-4 and IL-13 have nonreduntant roles in immunity (Barner et al., 1998; Kaplan et al., 1996; McKenzie et al., 1999; Noben-Trauth et al., 1997; Takeda et al., 1996; Urban et al., 1998). Therefore, the effect of these cytokines on Endoglycan expression may be attributed to these nonredundant functions. IL-13 can bind receptors consisting of the IL-13Rα and IL-4Rα, or IL-13Rα alone, and IL-4 can bind receptors consisting of IL-4Rα and γ_c, or IL-13Rα and IL-4Rα, or IL-4Rα alone. Therefore, the suppression of Endoglycan in LPS-stimulated splenocytes by IL-4, but not IL-13, may be due to signaling from the γ_c common chain. Moreover, it has been demonstrated in human lymphocytes that IL-13 responses decrease with maturation (Ford et al., 1999). Therefore, the difference in the effect of these cytokines may also be due to differences in the expression levels or the complement of receptor chains for these cytokine receptors.

To confirm the suppressive effect of IL-4 on Endoglycan expression, IL-4 treatment on LPS-stimulated splenocyte cultures from IL-4Rα-deficient mice should show no effect. It is difficult to test the signalling effect of the γ_c chain in the IL-4 receptor because mice with mutations in the *IL2RG* gene, which encodes the γ_c chain, exhibit severe combined immunodeficiency (SCID) mice and are deficient in B cells. In order to investigate the

expression levels of IL-4 and IL-13 receptors, FACS staining of for these receptors on LPS-stimulated splenocytes should be carried out. Moreover, testing various dilutions of these cytokines will show if the suppressive effect is due to different levels of IL-4 receptor compared to IL-13 receptor.

4.5 Differences in LPS-induced expression of Endoglycan between CD1 and other mouse strains

LPS was found to induced the expression of Endoglycan by B cells in virtually every strain of mice tested. This includes: CBA/N-xid, which have reduced B-1a subsets of B cells; C2GnTI-deficient mice, which lack the enzyme for adding O-linked glycosylations to glycoproteins; and Balb/c nude mice, which are athymic and lack mature T cells. These observations suggest that Endoglycan is not found on B-1a subsets and its upregulation is independent of T cells. Moreover, data from the C2GnTI-deficient mice confirmed that reactivity of the anti-mouse Endoglycan monoclonal antibody is not dependent on glycosylation added by C2GnTI.

The exceptions were CD1 and CD1 nude mice and data suggest that CD1 strains have an intrinsic deficiency in upregulating this molecule. Analyses of thymocytes from CD1 mice show an elevated Endoglycan expression profile. These mice do not lack the ability to express the molecule *per se*, but rather, have a defect in the ability to express it on B cells. Hence, eliminating the possibility of a natural occurring Endoglycan-deficient strain. Moreover, LPS administration intraperitoneally into these mice showed lower levels of Endoglycan upregulation in the spleen after 24hr as compared to C57Bl/6 mice.

Studies have shown that there is variability in the level of lymphocyte recruitment between different inbred mouse strains during inflammatory response. It was demonstrated that under uniform stimulus, of the four strains tested, C57Bl/6 has the highest level of cell recruitment, then Balb/c, followed by CD1 and lastly 129x1/SvJ (White et al., 2002). It is possible that the lack of Endoglycan upregulation in response to LPS in CD1 mice is related to their low levels of cell recruitment compared to C57Bl/6 and Balb/c strains. Experiments using RT-PCR will alert us to possible differences in the transcriptional regulation of Endoglycan between these two strains of mice. As well, looking at the differences in the Endoglycan promoter between these strains of mice

may provide an explanation for the differences in Endoglycan expression upon LPS stimulation between C57Bl/6 and CD1.

To investigate the possible role of Endoglycan in B cell recruitment, the LPS-induced upregulation of Endoglycan on 129x1/SvJ splenocytes can be analysed for a correlation between the observed levels of Endoglycan and the levels of cell recruitment. HSC transplants between C57Bl/6 and CD1 strains or C57Bl/6 and 129x1/SvJ strains will determine if the differences in Endoglycan expression on B cells is due to intrinsic or extrinsic factors.

4.4 Endoglycan expression increases upon LPS-administration in the spleen and BM

The observed *in vitro* upregulation of Endoglycan by LPS was also observed *in vivo* when LPS was administered intraperitoneally into C57Bl/6. There was increased Endoglycan expression in the spleen, peritoneal cavity and BM, after 24hr of LPS administration. The increased expression in the spleen was observed on the B cell population, as seen in *in vitro* splenocyte cultures. The increase expression on peritoneal cavity was on B220$^-$, suggesting that these are most likely activated macrophages. The increased level of expression in the BM may be attributed to the induction of Endoglycan expression on BM cells during inflammatory response, or these may be Endoglycan-expressing plasma cells from lymphoid organs that have migrated to the BM.

To test whether the increased Endoglycan expression by BM is due to migration of Endoglycan-expressing plasma cells, we will survey BM cells after LPS administration for B220 and Endoglycan co-expression. Moreover, the *ex vivo* short-term homing assay described above should answer questions regarding the possibility of Endoglycan-positive plasma cell homing to BM.

5. CONCLUSION

5.1 Endoglycan on MZ B cells

MZ B cells are the first line of defense against pathogens, they have a repertoire that recognizes TI-2 antigens. This is a T cell independent event where MZ B cells proliferate and mature into antibody-secreting cells upon antigen encounter. MZ B cells are retained in a specific region in the spleen (peripheral to the periarteriolar lymphoid sheath) that allows these cells to be exposed to blood-borne antigens for quick immune response. Transgenic mice that lack MZ B cells are more susceptible to bacterial infections (Tanigaki et al., 2002). As well, it has been shown that MZ B cells proliferate prior to pathogenic T cells in lupus-prone NZB/W mice (Wither et al., 2000). Studying the importance of MZ B cells in immunity and autoimmunity would be greatly facilitated by a molecule that can be used to identify and isolate these cells. We propose that Endoglycan is such a marker for MZ B cells that it may be important as a target for therapeutic interventions.

5.2 A possible role for Endoglycan in homing and migration during inflammation?

Long-lived plasma cells have been found to home to BM, mucosa or inflammatory sites (Kunkel and Butcher, 2003), however, the mechanism by which this homing occurs remains largely unknown. The fluctuations in the expression of chemokine receptors (Hargreaves et al., 2001; Hopken et al., 2004) and adhesion molecules (Kunkel and Butcher, 2003), such as integrins, has been shown to be important in this migratory process. It has been demonstrated that plasma cells that reside in the BM expresses $\alpha4\beta1$-integrin, whereas mucosal plasma cells express $\alpha4\beta7$-integrin. Moreover, BM plasma cells express E-selectin ligands that aid in their retention in the BM niche (Underhill et al., 2002). The difference in the expression of these surface molecules helps plasma cells determine their site of retention. By understanding the expression of other homing and/or adhesion molecules, we may be able to delineate the different subsets of long-lived plasma cells and determine their migratory destination. Endoglycan, as shown in the results from this thesis, is expressed on a subset of plasma cells upon activation and all Endoglycan-expressing cells also express $\beta1$-integrin. The expression of Endoglycan is modulated by cytokines that promote certain paths of plasma cell differentiation. Therefore, this molecule may mark unique plasma cells that are destined to home to specific tissues, such as BM or other specific sites of

inflammation. Endoglycan maybe a new homing and/or adhesion molecule that will allow the delineation of plasma cells that are programmed to migrate to different niches. Therefore, it will be important to study the differences in homing of Endoglycan-positive and negative plasma cells.

5.3 Concluding Remark

Endoglycan is the newest member of the CD34 family of sialomucins. Since its discovery five years ago, little has been known about its regulation and function. The results presented in this thesis are key to understanding the regulation of this molecule in B cells. Moreover, this thesis proposes possible function for this novel molecule on B cells. There is much still to clarify about Endoglycan, including its: 1. transcriptional regulation (specifically promoter mutation analysis); 2. function, in terms of homing, adhesion or anti-adhesion; and 3. biochemical properties, such as extracellular and intracellular binding partners. As well, due to its novelty, only limited analytical tools are available. Therefore, molecular, genetic and biochemical tools are needed to further the study this molecule. Ultimately, the production of an Endoglycan-deficient mouse should be highly informative for determining the role of this novel CD34-family member.

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