AUTOREACTIVE B CELL DEVELOPMENT IN THE PERIPHERY

By

Emily Jean Woodward

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Microbiology and Immunology

December, 2006

Nashville, Tennessee

Approved:

James W. Thomas

Wasif N. Khan

Eugene M. Oltz

Mark R. Boothby

P. Anthony Weil

ACKNOWLEDGEMENTS

In the completion of this degree, I have one person to thank above all others – James W. "Tom" Thomas. I knew that I wanted to join Tom's lab from the day of my entrance interview, and my mind never changed. He is a teacher, mentor and friend not only of science but also of life. I am forever indebted to his patience – above all other qualities – for if not for that, I would not be the scientist and person I am today.

The other members of the Thomas Lab are very important to me. My fellow graduate students, Carlos Ariel Acevedo Suárez and Rachel Anne Henry are older brother and younger sister to me now. Carlos is part of the reason I joined the lab and is a mentor, friend, and reference manual to me. Through mentoring Rachel, I have learned as much about myself as about science. Our lab manager, Chrys Hulbert, is truly a kindred spirit and has been a teacher, ally and colleague. She reminds me to remain true to the important aspects of life. Peggy Kendall is a worthy colleague. I have enjoyed taking on the non-B cell diabetes world with her. My thanks also go out to past members of the lab who have taught me much – Mauricio and Andres Rojas. In joining Tom's lab you become a step child of the Miller laboratory, and I am forever indebted to both Geraldine (Gerry) Miller and Martha B. (Marty) Reich.

I am also particularly obliged to my dissertation committee, Chairman Wasif N. Khan and members Eugene (Gene) M. Oltz, Mark R. Boothby, and P. Anthony (Tony) Weil. They are indeed much wiser than I likely will ever be, and I am very grateful for their guidance, constructive criticism, and friendship in all arenas.

My departmental family has become very dear to me. The graduate students of the Khan laboratory, especially Iris Castro and Nicolas Shinners – who are members of my class

– as well as Joan Llanes are dear friends to me as well as valuable colleagues. The Oltz lab, particularly Steven Pierce, is invaluable as friend, teacher, and co-worker. Shreevrat Goenka and Rachel Graham are two of my dearest friends and associates. I would like to thank the personnel of the Department of Microbiology and Immunology as well as the Division of Rheumatology for all their support throughout this time.

The studies reported in this work were supported in part by grants from the National Institutes of Health (AI47763 and AI051448). Personally, I have been supported by a GAANN Fellowship (Middle Tennessee State University through the Federal Department of Education) and the Immunobiology of Blood and Vascular Systems Training Program (Vanderbilt University – 5 T32 HL069765). The Vanderbilt DNA Sequencing Facility (chapter II) is supported by NIH grants CA68485 (Vanderbilt-Ingram Cancer Center), DK20593 (Vanderbilt Diabetes Research and Training Center) and HL65962 (Vanderbilt Pharmacogenomics Research Center). The VAMC Flow Cytometry Core is supported through the Veteran's Authority, and the technical assistance of Catherine Alford at this facility has been particularly noteworthy. The statistical advice of Dr. S. Haneuse (chapter II) is greatly appreciated. I would like to thank T. Dermody (Vanderbilt University) and C. Klug (University of Alabama, Birmingham) for generous mouse donations (chapter III).

Two people are more important to me than all others – my fiancé, Eric Shane Davis and my mother, Elizabeth Jean Russell Woodward. Eric is an infinite source of stability, love, and support that I would rather not do without. My mother is the singular reason that I am at all successful. From the age of three, she has raised me single-handedly and instilled in me perseverance, self-reliance, and at least a modicum of rational thought. She molded me into the woman I am today. There are no words with which I can adequately describe my deep and sincere gratitude toward my mother.

TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS ii

LIST OF TABLES vii

LIST OF FIGURES viii

LIST OF ABBREVIATIONS x

Chapter

I. BACKGROUND AND RESEARCH OBJECTIVES 1

A Brief History of B Cells 1
B Lymphocyte Development 1
Developmental Stages and Surface Phenotypes of B cells in the Bone Marrow 1
B cell Receptor Editing in the Bone Marrow 3
Developmental Stages and Surface Phenotypes of B cells in the Spleen 5
Transcriptional Control of B Cell Development 5
Transcription Factors Involved in Central B cell Development 7
PU.1 7
E Proteins 7
Early B cell Factor 8
Pax5 8
Notch Family Members are Critical to Peripheral B cell Development 9
The Notch-Notch Ligand Superfamily 9
Notch Signal Transduction 9
Co-regulators of Notch Signaling 10
Deletions of Notch Genes Pertinent to B cell Development 11
Other Transcription Factors Critical to Peripheral B cell Development 12
E2A 12
Aiolos 12
NF-κB 14
BOB.1 15
Plasma Cell Differentiation 16
Peripheral, Mature B Cell Populations 16
B1 B Cells 16
Follicular B Cells 18
Marginal Zone B Cells 19
Localization 19
Antigen Receptor Specificity 19

Immune Functions 20
Autoreactivity and Autoimmune Disease 20
Targeted Gene Deletions Impacting MZ B Cell Development 23
Mature, Peripheral B Cell Phenotype is Dictated by BCR Signals 23
Basal BCR Signaling 25
Antigen Characteristics Impact Signaling 25
Signaling Throughout B cell Development 27
B Cell Tolerance 28
The Controversial T3 B cell Subset 29
The 125Tg, Anti-insulin, Models 29
Research Objectives 31

II. MULTIPLE GERMLINE KAPPA LIGHT CHAINS GENERATE ANTI-INSULIN B CELLS IN NON-OBESE DIABETIC MICE 33

Abstract 33
Introduction 34
Materials and Methods 35
Results 38
Discussion 52

III. MARGINAL ZONE B CELL DEFECTS IMPOSED BY NOTCH2 HAPLOINSUFFICIENCY ARE AMELIORATED BY AN AUTOREACTIVE B CELL RECEPTOR 56

Abstract 56
Introduction 57
Materials and Methods 59
Results and Discussion 61
Concluding Remarks 76

IV. THE TRANSCRIPTIONAL PROFILE OF 125Tg MARGINAL ZONE B CELLS: ZINC FINGER PROTEIN 532 78

Abstract 78
Introduction 79
Materials and Methods 82
Results 87
Discussion 108

V. DISCUSSION AND FUTURE DIRECTIONS 115

BCR specificity can be fine-tuned by IgL structure and further impacted by polymorphisms 115
BCR specificity impacts B cell differentiation into MZ or FO subsets 119
BCR specificity can override Notch2 haploinsufficiency to generate MZ B cells 121

Mature B cell phenotypes are maintained by global gene expression programs 123
Concluding Remarks 125

Appendix

A. NUCLEOTIDE SEQUENCES OF ZFP532 126

B. LIST OF PUBLICATIONS 133

REFERENCES 135

LIST OF TABLES

Table Page

1-1. Surface Immunophenotypes of Mature Peripheral B Cell Populations 17

1-2. Immunoglobulin Transgenic Murine Models Exhibiting an Enhanced Marginal Zone B Cell Population..21

1-3. Targeted Gene Deletions Resulting in an Altered Marginal Zone B Cell Population..24

2-1. Amino Acid changes due to Polymorphic Residues in NOD mice..................................50

3-1. Absolute Numbers of Total B cells and B cell Subsets...73

4-1. Known Marginal Zone B cell Gene Expression Patterns Confirmed by Microarray Analysis..92

4-2. Known and Putative Transcription Factors and Transcriptional Cofactors Increased in Marginal Zone B cells..94

4-3. Known and Putative Transcription Factors and Transcriptional Cofactors Increased in Follicular B cells..95

4-4. ZFP532 Expression by RT-PCR and Real Time PCR Analysis in Cell Lines and Primary Tissues...102

LIST OF FIGURES

Figure Page

1-1. B cell development proceeds in continuous developmental stages in the bone marrow and is dictated by immunoglobulin gene rearrangement status....................2

1-2. Splenic B cell development is characterized by unique surface phenotypes and localization..6

2-1. Identification of splenic anti-insulin B cells in V_H125Tg/NOD mice 40

2-2. The Vκ gene families expressed by insulin binding B cells from V_H125Tg/NOD are heterogeneous...41

2-3. Specific Vκ gene segments are expressed by insulin binding B cells from V_H125Tg/NOD mice 43

2-4. Anti-insulin Vκ1 genes expressed in V_H125Tg/NOD mice are germline encoded, clonally independent, and polymorphic to non-autoimmune strains 45

2-5. NOD Vκ9 genes exhibit germline polymorphisms 47

2-6. Polymorphisms are not limited to insulin binding Vκ genes...48

2-7. Identification of anti-insulin B cells in the bone marrow of V_H125Tg/NOD mice...51

3-1. The MZ B cell population is augmented in anti-insulin, 125Tg, mice 62

3-2. The anti-insulin 125Tg potentiates MZ B cell development in *Notch2*$^{+/-}$ mice..64

3-3. The anti-insulin, 125Tg, BCR increases Notch2 expression on MZ B cells from 125Tg/*Notch2*$^{+/-}$ mice 67

3-4. Anti-insulin, 125Tg, MZ B cells localize to the appropriate areas in the spleen...............69

3-5. The 125Tg enforces allelic exclusion in B6 and *Notch2*$^{+/-}$ B cells 71

3-6. CD1d and CD9 are differentially regulated by BCR and Notch2......................................75

4-1. 125Tg splenocytes can be sorted by flow cytometry to generate phenotypically homogenous B cell populations 89

4-2. A small cohort of genes is over-expressed greater than two fold by 125Tg MZ and FO B cells 91

4-3. ZFP532 is a large gene that likely encodes a zinc finger transcription factor.................... 97

4-4. Murine ZFP532 has one human ortholog, ZNF532.. 99

4-5. ZFP532 over-expression is validated by semi-quantitative RT-PCR and real time PCR.. 100

4-6. Expressed sequence tag (EST) analysis demonstrates wide tissue distribution for ZFP532 transcripts.. 103

4-7. The 3' UTR of ZFP532 encoded by 125Tg/C57BL/6 B cells is distinct from other full-length transcripts... 105

4-8. ZFP532 protein exhibits a predominantly perinuclear pattern in transfected NIH-3T3 fibroblasts.. 107

4-9. ZFP532 has been targeted for germline disruption in embryonic stem cells................. 109

LIST OF ABBREVIATIONS

125Tg	anti-insulin BCR composed of IgH and IgL Tg
Ab	antibody
AID	activation induced cytidine deaminase
autoAb	autoreactive (self reactive) Ab
B6	C57BL/6 mouse
BAFF	B cell activating factor of the TNF family
BCR	B cell receptor for antigen (surface immunoglobulin)
CDR	complementarity determining region of IgH or IgL
CLP	common lymphoid progenitor
CSR	class switch recombination
D	diversity region of IgH
ds	double-stranded
DSL	delta-serrate like (Notch ligands)
EST	expressed sequence tag
FACS	fluorescence activated cell sorting (flow cytometry)
FcR	fragment crystallizable (of an Ig) receptor
FO	follicular
fwd	forward, or 5', primer in PCR
FWR	framework region of Ig
HEL	hen egg lysozyme
helTg	anti-hen egg lysozyme BCR transgene (MD4)
HSC	hematopoietic stem cell

ICN	intracellular notch
Ig	immunoglobulin
IgH	Ig heavy chain
IgL	Ig light chain
IMGT	ImMunoGeneTics Foundation
J_H	joining region of IgH
$J\kappa$	joining region of kappa IgL
LPS	lipopolysaccharide (endotoxin)
mAb	monoclonal Ab
MFI	mean fluorescence intensity
MZ	marginal zone
$N2^{+/-}$ or *Notch2*$^{+/-}$	*Notch2* heterozygous (haploinsufficient) mouse
NOD	non-obese diabetic mouse
nonTg	non transgenic
NS	not statistically significant
PC	phosphorylcholine
RE	receptor editing
rev	reverse, or 3', primer in PCR
sHEL	soluble HEL expressed as a neo-antigen from a Tg
SHM	somatic hypermutation
ss	single-stranded
T1	transitional type 1
T1DM	autoimmune, type 1 diabetes mellitus
T2	transitional type 2

TF	transcription factor
Tg	transgene or transgenic
TI	T cell-independent
TLR	Toll-like receptor
V_H	variable region of IgH
V_H125Tg	IgH Tg from anti-insulin mAb125
Vκ	variable region of kappa IgL
WT	wild type
ZF	zinc finger – zinc ion coordinating protein domain

CHAPTER I

BACKGROUND AND RESEARCH OBJECTIVES

A Brief History of B cells

Between 1956 and 1966, Bruce Glick, Max Cooper, and Robert Good discovered and confirmed that, in birds, the bursa of Fabricius was responsible for antibody (Ab) and B cell production. It wasn't until the 1970s that B cell generation was pinpointed to the bone marrow in mammals (1). Many diverse immune system roles for B cells have been discovered since that time. Indeed, this class of lymphocytes has functions in both the innate and adaptive branches of the immune system and interacts with many other cell types. An overview of many of these functions will be covered in this chapter to establish how integral B lymphocytes are to mammalian host defense.

B Lymphocyte Development

Developmental Stages and Surface Phenotypes of B cells in the Bone Marrow

In mammals, B lymphocytes begin their development in the bone marrow (figure 1-1) and complete their maturation in the spleen. This developmental progression is marked by changes in surface phenotype, gene expression, and immunoglobulin (Ig) rearrangement. Hematopoietic stem cells (HSC) differentiate into common lymphoid progenitors (CLP) and then into a B lineage progenitor that is referred to as either fraction A, CLP2, pre-pro B cell, or germline pro B cell [reviewed in (2)]. That progenitor, is pluripotent *in vivo* and *vitro* (3, 4), and gives rise to the earliest identifiable B cell, the pro B cell (fraction B). This marks the

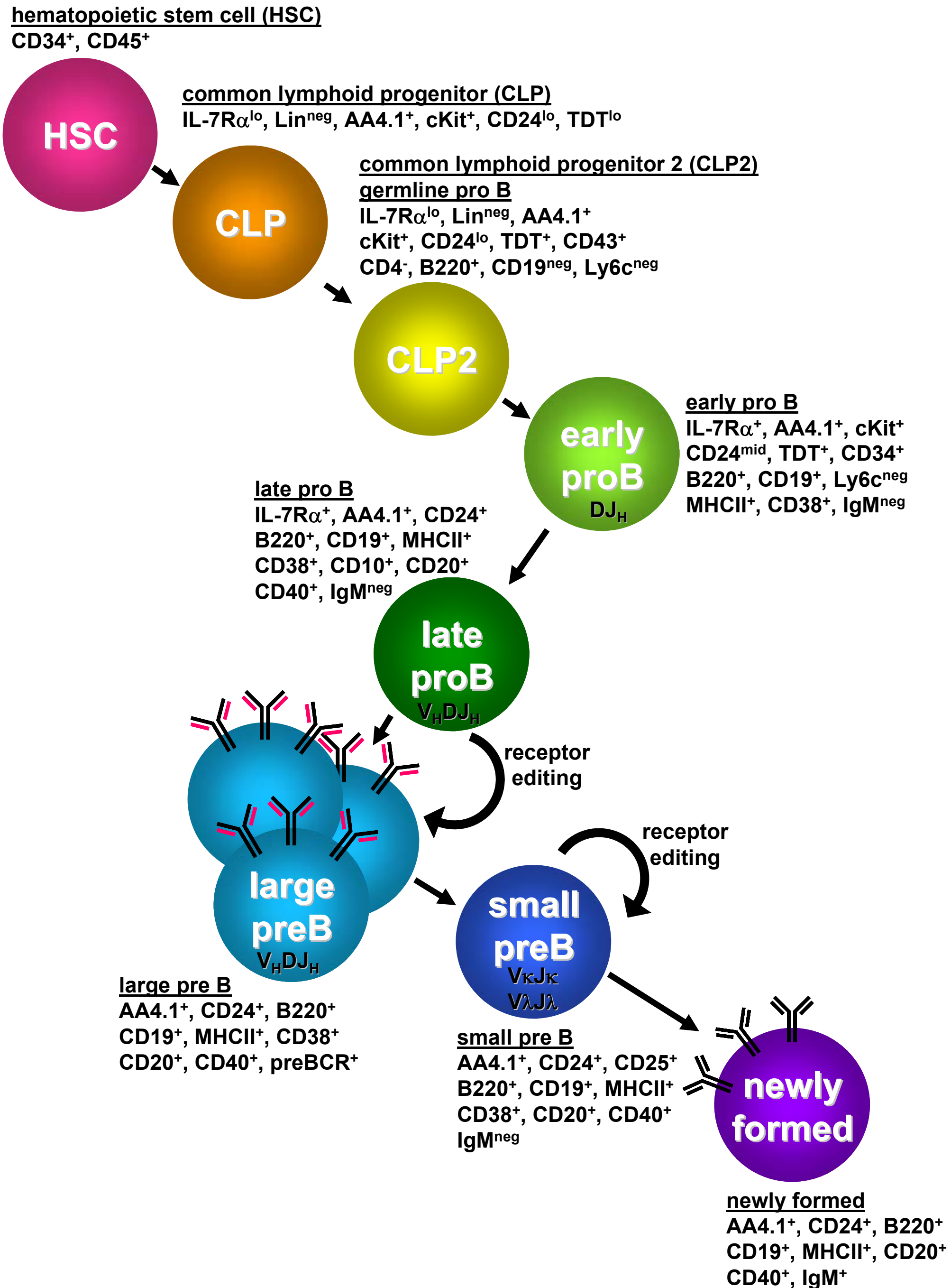


Figure 1-1. B cell development proceeds in continuous developmental stages in the bone marrow and is dictated by immunoglobulin gene rearrangement status. B lymphopoiesis begins with HSC and completes when newly formed B cells aggress from the bone marrow into circulation. Pertinent surface proteins are listed under each developmental stage. The relevant status of IgH or IgL recombination is noted in the early pro through small pre B cell stages. Receptor editing occurs between the late pro and small pre B cell stages [compiled from (5-8)].

point in B cell development when the stages are classified in terms of B cell receptor (BCR or surface Ig) rearrangement and surface protein expression. Early pro B cells initiate rearrangement of their heavy chain (IgH) diversity (D) and joining (J_H) segments. The IgH variable (V_H) and all Ig light chain (IgL) gene segments remain germline (unrearranged) in configuration. Late pro B cells undergo rearrangement of their V_H gene segments to the previously rearranged DJ_H gene segments to form a functional IgH gene. At this point, they are considered large pre B cells and express, at their surface, the rearranged IgH paired with two transiently expressed proteins, λ5 and VpreB, which compose the surrogate light chain. This complex is known as the pre-BCR and signals large, pre B cells to undergo several rounds of replication. Proliferation at the large pre B cell stage aids in diversification of the repertoire as each daughter, small pre B cell will independently rearrange its own IgL. Small pre B cells (preB-II) begin to rearrange V and J segments to form a functional IgL while internalizing their pre-BCR. The surrogate light chain is replaced by the newly rearranged IgL, and the complete Ig-expressing immature, or newly formed, B cell emigrates from the bone marrow to the periphery.

B cell Receptor Editing in the Bone Marrow

It is between the late pro B and small pre B cell stages (circular arrows, figure 1-1) that a process termed "receptor editing" (RE) occurs (6, 9-15). In its broadest definition, RE refers to the replacement of rearranged IgH or IgL chains by novel rearrangements. For example, if a newly rearranged IgH (V_HDJ_H) is either out of frame or unable to pair effectively with surrogate light chain, the B cell will pause at the pro to pre B cell transition and will continue to rearrange its IgH genes. If the new rearrangement takes place on the same allele, the previously used V_H must contain a cryptic recombination signal sequence so

that the new V_H may join the old DJ_H – as the intervening D segments were deleted upon rearrangement of the first V_H. Otherwise, V_HDJ_H recombination will occur on the second allele.

Later, at the pre B cell stage, IgL which are either untranslatable, pair poorly with IgH, or are autoreactive may also be replaced by RE (12, 16, 17). The inappropriate IgL (IgL-κ or IgL-λ) is deleted or inverted when a new VκJκ join is made using an upstream, 5', Vκ and a downstream, 3', Jκ. This process will continue on the same allele until all available Vκ and Jκ gene segments are used and then proceeds on the other allele until those gene segments are also spent. After both Igκ alleles are exhausted, the process repeats on the IgL-λ alleles. In mice, IgL-κ alleles generally rearrange before IgL-λ alleles, but it is possible that IgL-λ may rearrange concurrently. During the course of IgL RE, the generation of a productive (translatable) rearrangement that is not overtly autoreactive and pairs well with IgH will cease this process.

To summarize, RE allows B cell clones to eliminate Ig that are either untranslatable, ineffective, or autoreactive. It is important to note that an estimated 50-75% of B cells that emerge in the periphery have undergone RE (9-11). Thus, this process is important in reclaiming otherwise ineffectual or dangerous B cells that the organism has spent energy producing. The relevance of RE to the generation of marginal zone B cells will be discussed later in this chapter.

Developmental Stages and Surface Phenotypes of B cells in the Spleen

Once immature B cells enter the spleen, they progress through a series of transitional stages before they are considered mature (summarized in figure 1-2) (18-20). Transitional type 1 (T1) B cells are the new émigrés from circulation. If challenged with antigen at this juncture, they will either undergo apoptosis (21) or become functionally silenced (anergized) (22). Apoptosis or anergy induction is generally regarded as a function of strength of antigen binding by the BCR. Transitional type 2 (T2) B cells are a heterogeneous population which, contrary to their predecessors, will proliferate when challenged with antigen (18). T2 B cells further subdivide into – follicular precursors (T2-FP or T3 in some nomenclatures) and marginal zone precursors (T2-MZP) (23). Both are generally considered the immediate antecedents of their respective mature counterparts. At this point, two mature B cell populations are generated, marginal zone (MZ) or follicular (FO). Another mature subset, B1 B cells, develop from precursors in the fetal liver or bone marrow and may represent a separate lineage from MZ and FO B cells (24, 25). The functional characteristics of all three mature B cell subsets will be covered in depth later in this chapter.

Transcriptional Control of B cell Development

A major part of the work presented herein deals with the role of transcription factors (TF) and transcriptional cofactors in MZ B cell development. Thus, an overview of how different TF govern B cell development from bone marrow to periphery will be given here. These are important paradigms for understanding lineage restriction and binomial cell fate determination. For clarity's sake, I will consider them chronologically in terms of development. All TF classifications are from the most current version of the TransFac

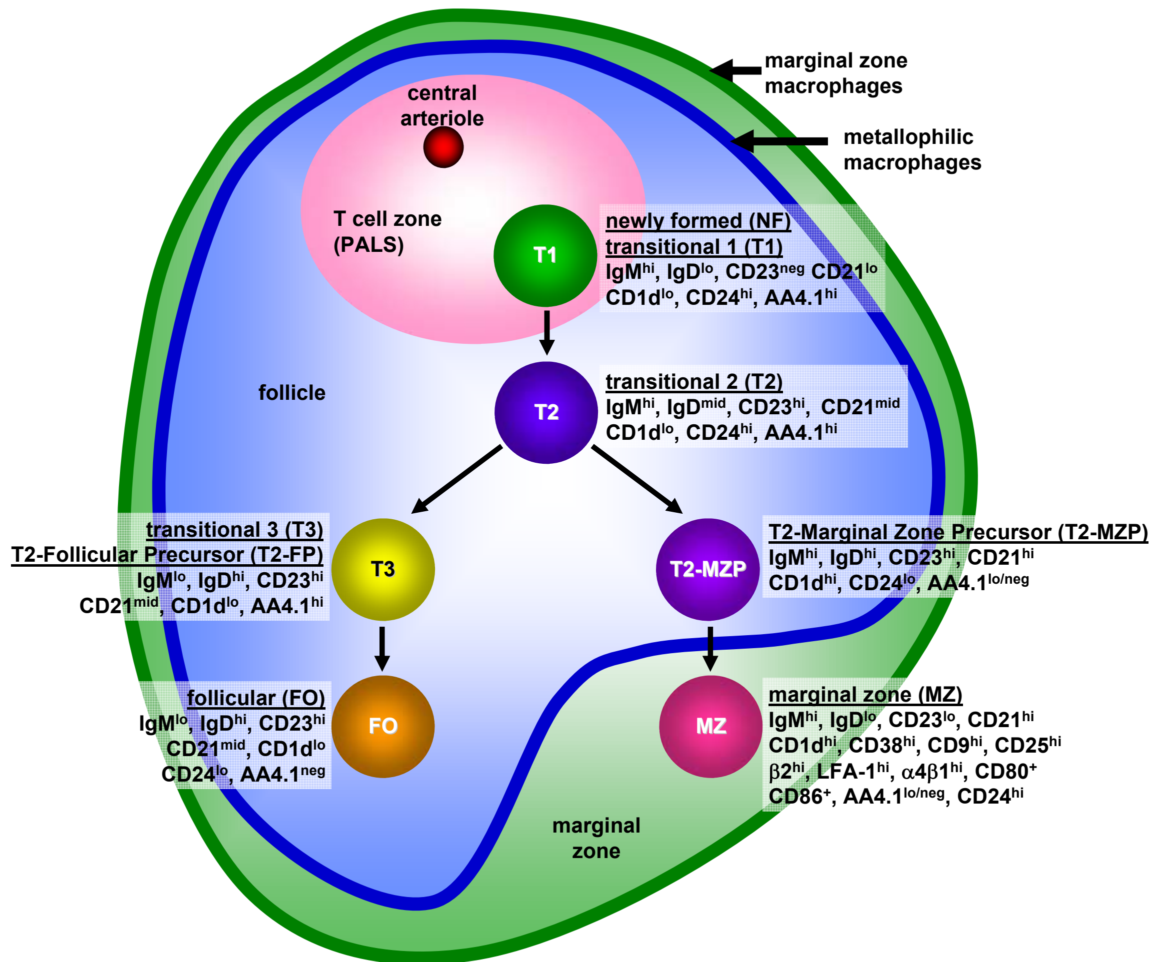


Figure 1-2. Splenic B cell development is characterized by unique surface phenotypes and localization. Newly formed B cells enter the spleen through the central arteriole as T1 B cells. Immature B cells pass through a series of developmental stages in different areas of the spleen on their way to becoming mature MZ or FO B cells. The surface proteins that characterize each stage are listed [compiled from (18-20)].

Database (www.gene-regulation.com/pub/databases/transfac). Aliases are noted in parentheses and the official gene symbol of each TF is in italics.

Transcription Factors Involved in Central B cell Development

PU.1. PU.1 (Spi-1, *sfpi-1*) is an Ets family TF that is required for the earliest stages of B cell development (Superclass: helix-turn-helix, Class: tryptophan clusters, Family: Ets-type). Its effects are concentration dependent as high levels of PU.1 expression potentiate myeloid development whereas low levels favor B cell development (26). PU.1 null ($sfpi\text{-}1^{-/-}$) mice either completely lack B, T, and myeloid cells (27, 28) or develop B-like cells that are unable to form Ig rearrangements (29). This indicates that PU.1 functions upstream of lineage commitment. Indeed, the most critical function of PU.1 in B cell development is up-regulation of IL-7Rα. IL-7 signaling increases pro B cell survival and is responsible for the up-regulation of two other TF, paired box gene 5 (Pax5) and early B cell factor (EBF) that are required for B cell development (30, 31). It is the coordinated efforts of E2A, Pax5, and EBF that mediate changes in B cell specific gene transcription.

E Proteins. Early B lineage commitment requires E2A, a heterodimer of two differentially spliced *Tcfe2a* gene products, E12 and E47, that bind promoters containing E-box consensus sequences (Superclass: Basic domains, Class: helix-loop-helix, Family: Ubiquitous, class A, factors) (32). E2A is expressed in HSC and CLP progenitors, and it is at these stages that *Rag* (recombinase activating gene) expression can first be detected. A loss of *Rag* expression in $Tcfe2a^{-/-}$ mice results in a block in B cell development at the germline pro to pro B cell transition (33-36). These data highlight the importance of Ig gene recombination in B cell development. Other E proteins such as E2-2 and HEB are also involved in B cell development but are most likely redundant to E2A as deletions of these

genes exhibit less severe phenotypes (37). In addition to controlling *Rag* expression, E2A (E12) up-regulates the expression of another TF, EBF (38).

Early B cell Factor. One of the most important target genes of E2A is EBF (*Ebf1*, Olf-1, or COE – Superclass: Basic Domains, Class: helix-loop-helix, Family: HLH domain only, Subfamily: Olf-1) (31, 39). While PU.1 and E2A null mice both lack mature B cells, enforced expression of EBF in progenitors from either of these models will restore B cell development (40). This implies that a main goal of PU.1 and E2A expression is the up-regulation of EBF which may be able to mediate B cell specific gene transcription on its own. Additionally, E2A works coordinately with EBF to control *Rag1/2*, *VpreB*, *λ5*, and *mb-1* (Igα) gene expression (41-43).

Pax5. The cascade continues with up-regulation of Pax5 by EBF (*Pax5*, B cell specific activator protein/BSAP, EBB-1, or KLP – Superclass: helix-turn-helix, Class: Paired box, Family: Paired domain only) (33). True B lineage commitment at the pro to pre B cell transition requires the coordinated efforts of E2A, EBF, and Pax5. These proteins synergistically activate the transcription of several B cell specific genes including, but not limited to, those involved in preBCR assembly – *λ5* and *VpreB*, BCR signaling – *CD19* and *Igα*, and Ig rearrangement – *Rag1/2* (32, 44, 45). Pax5 is also responsible for repressing other lymphoid and myeloid lineages (3, 4). Pax5 expression continues through all B cell stages until terminal differentiation into Ig secreting plasma cells. This continued expression is critical to the maintenance of B cell identity (46, 47).

After the formation of a complete surface Ig protein, immature B cells maintain high E2A (E47) expression facilitating RE in autoreactive clones (41, 43, 48). This function is consistent with E2A's ability to increase chromatin accessibility around Ig gene segments

facilitate *Rag1/2* expression. The process of RE as well as its role in MZ B cell formation will be discussed later.

Notch Family Members are Critical to Peripheral B cell Development

The Notch-Notch Ligand Superfamily. Nine members of the Notch-Notch ligand system have been identified in mammals. There are four Notch receptor genes (*Notch1-4*) and five Notch ligands [reviewed in (49, 50)]. The ligands include three homologs of the *Drosophila* Delta gene (*delta-like 1*, *3*, and *4*) and two homologs of the *Drosophila* Serrate gene (*jagged 1* and *2*). These ligands are collectively referred to as DSL proteins (delta-serrate like ligands). The distribution of Notch receptors and DSL ligands is highly regulated at many different levels. Expression of these genes is controlled temporally and by cell type (51-64) although the specific transcription factors that control Notch expression are not well characterized. Notch transcript stability is regulated by endogenous micro RNAs *in vivo* (65) and translation can be repressed or promoted in *C. elegans* by two distinct elements in the 3' untranslated region of the mRNA (66). The presence of Notch and DSL at the cell's surface is also regulated at the protein level by proteolytic processing in the secretory pathway (67, 68) as well as endocytosis and degradation (69-78). Because Notch mRNA can be degraded or remain untranslated and surface display of protein is dependent on prior processing, determination of cell surface Notch protein level provides more biologically useful information than assessing Notch transcripts alone. This reasoning is employed in chapter III where I determine surface Notch protein levels on splenic B cell subsets by FACS (fluorescence activated cell sorting).

Notch Signal Transduction. Upon interaction with one of the DSL proteins, two cleavage events occur in the Notch receptor. Proteolysis at the S2 cleavage site by ADAM

family metalloproteinases (Kuzbanian/ADAM10 or TNFα converting enzyme/ADAM17) (79, 80) releases the extracellular domain which may be internalized by the ligand bearing cell (81-84). The intracellular portion (ICN) is then cleaved from the plasma membrane at the S3 site by presenilins in a γ-secretase complex (81, 85-87). Once liberated into the cytoplasm, ICN may interact with Numb (*Numb*) (88) or Deltex (*Dtx1-4*) (89) which function as negative and positive regulators respectively. Reports of Deltex suppressing Notch activity also exist, and its function is most likely cell type specific (90, 91). ICN translocates to the nucleus where it activates the transcription of various target genes. Transcriptional activation occurs at genes that are constitutively repressed by RBP-Jκ (recombination signal sequence binding protein of Jκ, *Rbpsuh*, SUH, CBF-1, CSL, Lag-1 – Superclass: basic domains, Class: helix-loop-helix factors, Family: other bHLH factors) (92, 93). ICN interacts directly with RBP-Jκ and displaces a repressor complex that contains SMRT and HDAC-1 (67, 94). ICN also recruits co-activators such as Mastermind-like (*Maml1-3*), pCAF, and p300 which aid in activating transcription (95-99). Activation of transcription by ICN is self-limiting owing to its PEST domain and degradation via SEL-10 (*Fbxw7*) family proteins (100). Clearly, the regulation of Notch and DSL ligands and the signaling that proceeds from Notch receptors is highly complex. Several of these proteins exhibit cell type specific effects, and thus conclusions drawn from one model system cannot necessarily be applied to another. Collectively, these data demonstrate the need of studying Notch and DSL proteins in context. It is for this reason that chapter III focuses on the relationship of Notch to the BCR in the context of MZ B cell development *in vivo*.

Co-regulators of Notch Signaling. Notch signaling is modulated even further by additional proteins. There is extensive evidence that despite structural homology, the four

Notch receptors deliver different signals and activate the transcription of different target genes (101-109). Furthermore, ligation of the same Notch receptor by different ligands in some cases perpetuates divergent signals (104-108, 110-114). Indeed the presentation of the ligand (soluble versus membrane-bound) can also alter the outcome of Notch signaling (112, 115). The family of fringe glucosyltransferases can modify the Notch receptors themselves to limit their interaction with certain DSL proteins (116). Of relevance to the data presented in chapter III, expression of the ligand, Delta-like1 (*Dll-1*) is required on a non-B cell population for the development of MZ B cells (117). Dendritic cells in the marginal sinus commonly express *Dll-1* and therefore may be the instructive cell type.

Deletions of Notch Genes Pertinent to B cell Development. Of the four receptors, only Notch1 and 2 are involved in B cell development. Notch1 functions early in HSC maintenance (118) and later potentiates T cell development while inhibiting that of B cells (119, 120). Notch2 is expressed by all mature B cells and functions in the periphery during the binary fate decision between FO and MZ B cells (121, 122). Two groups have generated specific deletions of the *Notch2* gene. In 1999, Hamada *et al.* replaced the intracellular ankyrin repeats with β-galactosidase (123). In the homozygous condition ($N2^{-/-}$), this mutation is embryonic lethal at E11.5. Later analysis of heterozygous mice ($N2^{+/-}$) revealed a specific defect in MZ B cell development (122). These mice are used in chapter III. Later, Saito *et al.* generated a B cell specific Notch2 deletion ($N2^{-/cre}$) which removes the sequences encoding the extracellular domains via cre recombinase under the control of the CD19 promoter (121). The phenotypes of the $N2^{+/-}$ (heterozygous) and $N2^{-/cre}$ (null in B cells) mice are virtually identical – both lacking MZ B cells with little alteration in other lymphocyte populations. Thus, a particular quantity of Notch2 protein, or more likely of Notch2 signal, must be attained to generate the MZ population. In a

polyclonal B cell repertoire, only the homozygous wild type Notch2 locus is sufficient to mediate MZ B cell generation. In chapter III, I demonstrate that a monoclonal, autoreactive BCR repertoire can partially overcome a heterozygous Notch2 locus.

Importantly, RBP-Jκ itself is necessary for MZ B cell differentiation indicating the importance of the interaction between RBP-Jκ and ICN (124). In the absence of ICN, RBP-Jκ is repressive so one might assume that a lack of this repressor would facilitate MZ B cell production. However, it is only the action of ICN that recruits chromatin modifying complexes to RBP-Jκ-bound loci, and ICN itself does not bind DNA. Thus, RBP-Jκ may merely be a docking site or genomic "flag" and thus repressive by default. In this scenario, absence of RBP-Jκ would confer a constitutively repressed chromatin structure which the presence of ICN alone could not alleviate.

Other Transcription Factors Critical to Peripheral B cell Development

E2A. The roles of E2A are not limited to bone marrow development but extend to peripheral B cell differentiation as well. High concentrations of E2A promote the FO rather than MZ B cell fate decision (48). Stimulation with various mitogens – LPS, anti-IgM, or anti-CD40 and IL-4 – increase E2A transcript levels (125) leading to class switch recombination (CSR) and somatic hypermutation (SHM) via the E2A target gene, AID (activation induced cytidine deaminase) (126). These functions again take advantage of E2A's role in enhancing Ig gene accessibility via the recruitment of chromatin modifying proteins and augmenting *Rag1/2* expression.

Aiolos. C_2H_2 (Krueppel-like) zinc finger (ZF) TF are common players in cell fate determination throughout the mammalian system. Members of the Ikaros (*Zfpn1a1*) family

include Ikaros, Aiolos, Helios, Eos, Pegasus, and Daedalus (Superclass: zinc-coordinating DNA binding domains, Class: Cys2His2 zinc finger domains, Family: developmental/cell cycle regulators, Subfamily: Krueppel-like). These C_2H_2 TF exemplify how ZF proteins control cell fate decisions (127). All family members encode multiple isoforms via alternative splicing, and all isoforms are capable of homo- and heterodimerizing with one another to generate a very complex transcriptional control system. In early B cell development, it is clear that Ikaros gene products are essential for B, T, and NK cell development. However, because the Ikaros gene is capable of eleven alternative splice forms, the direct role(s) of Ikaros have not been determined (128). Data regarding the functions of Helios, Eos, Pegasus, and Daedalus are limiting. These genes appear highly redundant with Ikaros and Aiolos gene products.

In peripheral B cell ontogeny, the most important member of this family is Aiolos (*Zfpn1a3*) (127, 129-131). Aiolos null (*Zfpn1a3*$^{-/-}$) mice lack MZ B cells and exhibit decreased CD21 expression on FO B cells (132). Later analysis indicated that this phenotype is dependent upon Btk (Bruton's agammaglobulinemia tyrosine kinase) as *Btk*$^{-/-}$/*Zfpn1a3*$^{-/-}$ (double deficient) mice exhibit a phenotype similar to *Btk*$^{-/-}$ mice (133). In that study, Cariappa *et al.* conclude that Aiolos is epistatic to Btk and that *Zfpn1a3*$^{-/-}$ B cells display unfettered Btk signaling that predisposes their differentiation into FO B cells. However, caution must be exercised when interpreting these data because the seven isoforms of Aiolos control the expression of multiple target genes by either activating or repressing transcription (127). Thus, it may be that a lack of gene products downstream of Aiolos is responsible for the phenotype of *Zfpn1a3*$^{-/-}$ mice. Interestingly, *Btk*$^{-/-}$ animals are capable of generating MZ B cells (134-137) while FO B cell survival is markedly reduced (138-143). Thus, Btk signaling is most likely required for enhancement of MZ populations instead of

initial recruitment into the MZ (144, 145). Clearly, further research as to how the target genes of Aiolos interact with the Btk signaling axis is needed.

NF-κB. Nuclear factor of κ IgL gene enhancer in B cells, NF-κB, is a 5-member (RelA/p65, Rel/cRel, RelB, NF-κB1/p50, NF-κB2/p52) TF family homologous to the Relish proteins discovered in *Drosophila* (Superclass: β-scaffold factors with minor groove contacts, Class: rel homology region, Family: Rel/ankyrin) (146). These proteins can homo- and heterodimerize with one another to activate and repress a wide array of target genes. The functions of some family members are redundant, necessitating analysis of double knockout models in some cases. Each of the five Rel proteins have functions in B cell development, differentiation, function and survival [reviewed in (147)]. The *Rela*$^{-/-}$ mutation is embryonic lethal and must be analyzed in bone marrow chimera experiments (148). Both *Rela*$^{-/-}$/*Nfkb1*$^{-/-}$ (149) and *Rela*$^{-/-}$/*Rel*$^{-/-}$ mice (150) have no lymphocytes due most likely to excessive TNF production and apoptosis. As indicated by their name, NF-κB subunits have roles in Igκ rearrangement and germline transcription and are thus important for immature B cell development (151). *Nfkb1*$^{-/-}$/*Nfkb2*$^{-/-}$ mice exhibit a block at the T1 stage in the spleen whereas *Rela*$^{-/-}$/*Rel*$^{-/-}$ mice are capable of forming T2 cells *in vitro* (152).

The most relevant models to this dissertation are the single knockouts, all of which impact MZ B cell generation in some manner. *Nfkb1*$^{-/-}$, *Nfkb2*$^{-/-}$, and *Relb*$^{-/-}$ mice specifically lack MZ B cells, while *Rel*$^{-/-}$ and *Rela*$^{-/-}$ mice demonstrate decreases in this population (153-155). NF-κB activation is downstream of receptors other than the BCR such as BAFF-R (153, 156-160) and Notch (161). Since MZ B cells are particularly long lived and dependent on survival signals delivered through BAFF-R and differentiation signals delivered through Notch, it is not surprising that NF-κB members figure heavily in MZ B cell differentiation.

Interestingly, by 12-15 weeks of age, *Nfkb1*$^{-/-}$ mice do accumulate MZ B cells [(162) and my unpublished observations]. In this model, other NF-κB subunits may compensate for the loss of p50. Further investigation into the nature of aged *Nfkb1*$^{-/-}$ MZ B cells is needed to clarify this discrepancy.

BOB.1. The *Pou2af1* gene encodes two translational isoforms, p34 and p35, which are known as BOB.1 (OBF.1, OCA-B) (163). BOB.1 is a transcriptional co-activator which interacts with the POU domain of the ubiquitous, Oct-1 and B cell specific, Oct-2 TF (Superclass: helix-turn-helix, Class: homeo domain, Family: POU domain factors, Subfamily: II) (164, 165). BOB.1 protein is induced by anti-IgM, CD40L/IL-4 and TLR4 stimulation (166-170). Initially, B cell development in *Pou2af1*$^{-/-}$ mice seemed overtly normal, however these mice do exhibit reduced numbers of transitional (T1) B cells in the spleen (171, 172). Samardzic *et al.* (173) analyzed developing and mature B cell compartments in *Pou2af1*$^{-/-}$ mice and found that these animals generate FO and B1 B cells normally but almost entirely lack MZ B cells. The role of BOB.1 in B1 development is controversial. Different groups report either no changes (173, 174), elevated (175), or reduced (176) B1 numbers. It is interesting to note that BCR repertoire skewing occurs in *Pou2af1*$^{-/-}$ B cells via the IgL-κ locus (177) because changes in BCR specificity may influence B cell selection into B1 and MZ compartments. *Pou2af1*$^{-/-}$ B cells exhibit decreased tyrosine phosphorylation and calcium mobilization, a phenotype concomitant with increased CD22 expression (178, 179). The interplay between BCR specificity, signal strength, and B cell differentiation into mature subsets will be discussed below.

BOB.1 is also critical for germinal center (GC) formation and the expression of isotype switched Ig (174, 180, 181). To date, the target genes of BOB.1 important to GC formation are unknown (182) which likely indicates a complex control system.

Plasma Cell Differentiation. B lymphocyte induced maturation protein 1 (Blimp1, ZNFPR1A1, *Prdm1*, unclassified) is a zinc finger transcriptional repressor responsible for plasma cell differentiation (183-185). In addition to potentiating the transcription of several target genes, Blimp1 inhibits Pax5 expression. This effectively ceases the mature B cell transcription profile allowing terminal differentiation into Ig secreting plasma cells. X box binding protein 1 (*Xbp1*, TREB5 – Superclass: basic domains, Class: leucine zipper factors (bZIP), Family: AP-1-like components, Subfamily: Jun), a potential target gene of Blimp1 (184), is also required for plasma cell differentiation and Ig secretion (186).

Peripheral, Mature B cell Populations

Three mature B cell populations exist in higher mammals – B1, conventional follicular (FO or B2), and marginal zone (MZ) (7, 19, 20, 25, 187-189). A major focus of the research presented here focuses on the generation of MZ B cells, and thus a significant portion of this section will be devoted to characterizing that population. For completeness, B1 and FO B cells are also covered. Figure 1-2 and table 1-1 [compiled from (19, 20, 189, 190)] detail the surface immunophenotypes of each population.

B1 B cells

B1 B cells can be divided into two subpopulations based on surface expression of the glycosylated scavenger receptor, CD5 (24). B1a B cells are $CD5^+$ and are derived from precursors in the fetal liver. B1b B cells are generated from bone marrow precursors and lack CD5 [reviewed in (7)]. Whereas B1a B cells represent a separate lineage, it is unclear whether B1b cells are separate as well or instead pass through the same developmental program as MZ and FO B cells (191). Both B1a and b cells reside primarily in the peritoneal

Table 1-1. Surface Immunophenotypes of Mature Peripheral B Cell Populations

marker	FO	MZ	B1
IgM	low	high	high
IgD	high	low	low/negative
AA4.1 (493)	negative	negative	negative
CD21 (CR1/2)	intermediate	high	low
CD23 (FcεRII)	high	low	positive or negative
CD22	high	high	unknown
CD24 (HSA)	low	high	high
CD1d	low	high	negative
CD9	low	high	negative
CD5	negative	negative	positive or negative
CD11b (Mac-1)	negative	negative	positive

and pleural cavities but are also found in the spleen. B1 differentiation is dependent upon positive selection by antigen (189, 192, 193). These cells are commonly autoreactive and exhibit a restricted BCR repertoire (24). The Ig genes encoding their BCR are generally unmutated and recognize repetitive epitopes such as lipopolysaccharide (LPS), phosphocholine (PC), and self certain antigens (194, 195). In conjunction with MZ B cells, they are responsible for the majority of "natural" IgM, a polyreactive, low affinity IgM present in serum that is involved in the opsinization and neutralization of pathogens. B1 and MZ B cells share other characteristics such as a pre-activated phenotype and certain BCR specificities that suggest similar developmental requirements (24, 25, 196).

Follicular B cells

The major B cell population in circulation and secondary lymphoid organs – spleen and lymph nodes – is composed of follicular (FO) B cells. After emigrating from the bone marrow as immature (T1) cells and completing their maturation in the spleen (figure 1-2), FO B cells recirculate through the blood and home to the B cell follicles for which they are named. The most important function of these cells is to generate the highly somatically mutated and class switched Ig characteristic of GC reactions. Their antigen receptors are highly diverse, and they are attuned to antigen stimulation via the BCR and to T cell help. Many of the functions classically attributed to B cells in general are hallmarks of FO B cells in particular – the anti-IgM response, SHM, CSR, and memory B cell formation. But other characteristics, such as stimulation by LPS and the early wave of IgM secretion during an immune response, are more accurately attributable to MZ and B1 B cells. Thus, canonical/historical B cell functions are actually an amalgamation of the independent and somewhat unique functions of different B cell subsets (7, 19, 20, 25, 187-189).

Marginal Zone B cells

Localization. Marginal zone (MZ) B cells are unique in several respects. Once differentiated in the spleen, they do not recirculate but are localized to the marginal sinus which borders the white and red pulps (197, 198). By immunohistochemistry, MZ B cells can be identified as a ring between the MZ macrophages (SIGN-R1$^+$, MARCO$^+$, and ERTR-9$^+$) and metallophilic macrophages (CD169$^+$ and MOMA-1$^+$) (199, 200). Being proximal to the marginal sinus, an area of sluggish blood flow, brings MZ B cells into close association with antigens circulating in the blood. MZ B cells only leave this area upon activation, as discussed below.

Antigen Receptor Specificity. MZ B cells exhibit a somewhat restricted repertoire in terms of Ig gene usage and specificity. The IgH and IgL genes that encode their BCR are often germline in configuration indicating that they have not undergone SHM or GC reactions. Indeed, it is generally thought that MZ B cells do not enter into GC reactions, although evidence to the contrary has been suggested (201). Their Ig genes are either characteristic of those found in fetal B cells (24, 196), a quality similar to B1 B cells, or exhibit evidence of RE due to autoreactivity (194, 202-207). In some cases, a single MZ B cell expresses two IgL paired with the same IgH at its surface. This effectively results in two BCR, each having a different antigen specificity. This phenomenon is referred to as receptor inclusion or dilution and is usually the result of the cell's attempt to abrogate an autoreactive receptor via RE (194, 205-209). This is also evidence of a lack of allelic exclusion at the IgL locus – a phenomenon that also occurs in some T cell receptor alpha genes. Often, MZ BCR are polyreactive, or cross-reactive, recognizing multiple antigens with a low affinity often including self proteins. This may be a result of more than one BCR at the surface (as described above) or the same BCR exhibiting low affinity for multiple antigens that share

physiochemical properties. For example, the PO_4 groups of dsDNA and cardiolipin are similarly spaced, and B cells exhibiting this dual specificity are commonplace (210). A propensity toward autoreactivity in MZ B cells is evidenced by many transgenic (Tg) Ig murine models (table 1-2, compiled from (144, 203, 207, 211-217) as noted in table) as well as naturally occurring autoimmune mouse strains – NOD (218, 219) and NZB/W_{F1} (220-224) – which demonstrate an enlarged MZ B cell population when compared to non-Tg or non-autoimmune controls respectively.

Immune Functions. MZ B cells are a lymphocytic bridge between the innate and adaptive immune systems. They are one of the earliest lines of defense against blood-borne pathogens and serve several functions during the course of a host response (25, 196, 225-228). Due to their anatomical location, MZ B cells are one of the first cell types to encounter antigen and pathogens in circulation. Stimulation can result in at least two divergent outcomes. MZ B cells that encounter a combination of antigen and complement via dual engagement of BCR and CD21/CD35 (CR2/CR1) will travel to the T-B border of the lymphoid follicle. Here, the captured antigen is transferred to follicular dendritic cells (FDC) via a proteolytic cleavage of CD21/CD35. The FDC will then participate in downstream T cell activation (229). Alternatively, MZ B cells that are stimulated through a combination of BCR and toll like receptors (TLR) or TLR alone migrate to the T-B border where they can present antigen to $CD4^+$ T cells (230). Afterwards, these MZ B cells will migrate into the red pulp via the bridging channels where they differentiate into plasma cells and secrete large amounts of IgM (196). This activity is largely responsible for the initial wave of low affinity IgM during the first few days of an immune challenge.

Autoreactivity and Autoimmune Disease. Low-level autoreactivity is likely concomitant with the ability to recognize a wide range of bacterial and viral antigens due to

Table 1-2. Immunoglobulin Transgenic Murine Models Exhibiting an Enhanced Marginal Zone B Cell Population

model	Heavy Chain	Light Chain (5)	background	specificity	idiotypic Ab	%id+ (6)	% MZ (7)	% MZ of id+ (8)	reference(s)
81X	VH7183/DFL16.1/JH1	endogenous Vκ1c/Jκ5 (1)	C57BL/6 or BALB/c	undefined cytoplasmic Ag, PC	35-1	7.2%	40%	94%	144, 207
M167	VHS107/DFL16.1/JH1	endogenous Vκ24 (2)	C57BL/6	phosphorylcholine	M167	3.1%	28%	83%	144
3-32	VHQ52/DSP2.5/JH1	endogenous Vκfl12/Jκ1 (3)	C57BL/6	ss and dsDNA	U33	8-10%	13%	40%	211
3-32	VHQ52/DSP2.5/JH1	endogenous Vκfl12/Jκ1	NZB/W F1	ss and dsDNA	U33	4-6%	24%	78%	211
MD2	VH36-60/DQ52/JH3	endogenous Vκ23 (4)	C57BL/6	hen egg lysozyme	none (labeled HEL)	0.35%	30%	17%	144
VH125Tg	VH9-2-1/DFL16.1/JH1	endogenous various	C57BL/6 or NOD	insulin	none (labeled insulin)	1-2%	30%	71%	216 and EJW
125Tg	VH9-2-1/DFL16.1/JH1	Tg - Vκ4-74/Jκ5	C57BL/6 or NOD	insulin	none (labeled insulin)	>98%	30-50%	30-50%	ch. III
HK171	J558/D?/JH? (IgH included) (9)	endogenous various	C57BL/6	arsonate	none (IgMa+)	11.60%	40%	40%	212
M54	?	Vλ	C57BL/6	nitrophenyl-acetyl	17.2.25	28%	<15%	?	213
3H9	J558/D?/JH?	Vκ3, 4, 8, 9, 12/13, 21, 23 and Vλ1	BALB/c	ss, dsDNA, and cardiolipin	none (antigen)	various	various	various	203, 217
3H9/56R	J558/D?/JH?	Vκ/Vλ allelically included (10)	BALB/c or MRL/lpr	ssDNA	1.209	19%	13-35%	6-26%	214, 215

(1) Vκ1C = Vk1-117
(2) Vκ24 = Vk2-112
(3) Vκfl12 = Vk12-89
(4) Vκ23 = ?
(5) conferring antigen specificity or idiotype positivity
(6) % of total B cells that are either idiotype positive or bind the antigen in question
(7) % of total B cells that bear the MZ phenotype
(8) % of idiotype positive or antigen binding cells that bear the MZ phenotype
(9) B cells of interest express two IgH - Tg and endogenous
(10) B cells of interest express two IgL - one Vκ and one Vλ

overlap in structural or chemical properties (231-233). Thus, reactivity with self-antigen may aid the selection of B cells with a MZ phenotype because these specificities are highly favorable to survival of the species. Maintaining the benefits of this potentially harmful subset is accomplished by physically segregating them in the MZ of the spleen. In this location, MZ B cells are spatially separated from T cells that could provide help in generating high affinity Ig via GC reactions and cytokine stimulation. This high-risk strategy appears to break down in autoimmune disorders. In both the NZB/W_{F1} model of lupus and the NOD model of type 1 diabetes mellitus (T1DM), expansion of the MZ subset precedes proliferation of pathogenic T cells, insulitis, and nephritis respectively (234-236). In murine models of lupus and T1DM, B cells play a key role in priming pathogenic T cells. The enhanced antigen presenting function of the MZ B cell subset may help trigger overt T-cell dependent disease (227, 230). Consistent with this postulate is the observation that autoAb producing B cells in lupus mice do not arise in GC, rather they are observed in the bridging channels between the white and red pulps and at the T-B interface (237). MZ B cell-derived plasma cells normally migrate to the T-B border and then expand through the bridging channels into the red pulp (196). Thus, MZ B cells may fuel breaches in tolerance either by driving autoaggressive T cells or by differentiation into autoAb producing plasma cells. MZ B cells also govern the response of iNKT cells, an important regulatory subset in several autoimmune disorders (238). The relative contribution of MZ B cells likely differs depending on the underlying disorder. In lupus, Ig mediated tissue damage is significant while in T1DM, MZ B cells may amplify T cell responses. In autoimmune diseases, MZ B cells are not limited to the spleen as exemplified by ectopic MZ B cell development in a model of Sjögren's Syndrome that accompanies BAFF over expression (239). To summarize, multiple characteristics make MZ B cells a liability for the development of

autoimmune disease. **1)** Localization in the marginal sinuses facilitates exposure to circulating autoantigen. **2)** Heightened responses to innate immune receptors (e.g. TLR and CR) drive rapid relocalization and differentiation. **3)** Efficient antigen presentation to naïve $CD4^{+}$ T cells could promote autoaggressive T cell responses. **4)** High CD1d expression can promote iNKT cell responses.

Targeted Gene Deletions Impacting MZ B cell Development. The TF and cofactors critical to MZ B cell development (Aiolos, NF-κB members, Notch2, and BOB.1) are covered above. Table 1-3 details other knockout models that impact the MZ B cell population [compiled from (133, 144, 225, 240-254), as noted in table]. These can be divided into the following broad categories: survival, localization, and signaling. In summary, MZ B cells are long lived and are particularly susceptible to alterations in genes governing lymphocyte survival. Intrinsic to the phenotype of MZ B cells is their splenic localization. Correct positioning requires input from certain chemokines and other receptors. Finally, differentiation into FO, MZ, or B1 subsets is dictated by BCR mediated signals. Therefore, deletions in genes that either augment or deplete BCR signals concomitantly alter mature B cell fate. This last feature will be discussed below and in chapter V.

Mature, Peripheral B Cell Phenotype is Dictated by BCR Signals

Several lines of experimentation have clearly demonstrated that BCR signals, mediated by Igα and Igβ, are not only required for B cell development but are also required for the maintenance of B cell populations in the periphery (242, 255-258). Conditional ablation of these proteins in mature B cells causes them to gradually die. Thus, BCR signals of some sort are required. The question remains, what manner of signals are required?

Table 1-3. Targeted Gene Deletions Resulting in an Altered MZ B Cell Population

Process	Gene	Phenotype	references
Development			
	λ5-/-	increased MZ proportion of B cells that are produced	(225)
	IL-7-/- and IL-7Rα-/-	increased MZ proportion of B cells that are produced	(240)
	conditional Rag-/-	Rag deletion at birth causes increased MZ B cell population	(241)
Signaling			
	CD79a-/- ITAM	MZ B cells are reduced to < 1% of B cells	(242)
	CD19-/-	reduced MZ B cells and TI responses	(243, 144)
	CD21-/-	small increase in MZ	(133)
	Btk-/- or Xid	impaired MZ B cell enrichment	(144)
Survival			
	BAFFTg	increased MZ numbers, autoimmune manifestations	(244-246)
Localization and Microenvironment			
	LTα-/-, LTβ-/-, LT-R-/-	disorganized spleen, decreased MZ B cells	(247-250)
	Pyk2-/-	no MZ and decreased responsiveness to chemokines	(251)
	Lsc-/-	decreased MZ due to mislocalization	(252)
	Dock2-/-	decreased MZ B cells	(253)
	vav1/2/3-/-	decreased MZ B cells	(254)

Additionally, what is the impact of signals delivered through other surface proteins including cytokine receptors, TLR, integrins, and other micro-environmental receptors (Edg1-3/S1P-R, scavenger receptors, etc.) (259-263)? For the moment, I will focus on the BCR and its co-receptors as this complex is generally regarded as the master regulator of B cell maturation.

Basal BCR Signaling

As the plasma membrane is fluid and dynamic, the molecules housed therein are in constant motion. This random shuffling may lead to the transient association and subsequent phosphorylation of BCR signaling components (Igα/β). This is generally referred to as "tonic" or "basal" signaling and should be considered the lowest level of stimulation a B cell might experience (256). It is important to note that B cells likely never experience a complete lack of signal because even if the ITAM of all the Igα/β molecules were separated, the other receptors mentioned above would still be in place. It is basal signaling that is required for progression past the pre B cell stage as well as for survival in the periphery (257, 258, 264-266).

Antigen Characteristics Impact Signaling

BCR stimulation via antigen must take into account several different parameters. Affinity is defined as the sum of the attractive and repulsive forces at work between the antigen and the surface Ig. While the strength of binding, or affinity, is important in Ig specificity for antigen, most antigens are multivalent – exhibiting the same epitope many times. As there are many Ig molecules per B cell, this means a single B cell can bind a multivalent antigen with more than one Ig molecule. The total of these interactions is avidity. Efficient stimulation of a B cell requires BCR cross-linking to allow cross-

phosphorylation of Igα/β tyrosines (267). Thus, the avidity of an antigen-Ig interaction is likely more important than the affinity of that reaction. This being said, an Ig with a high affinity for cognate antigen will retain that antigen at its surface longer, thus potentiating more phosphorylation downstream. Therefore avidity and affinity are inextricable factors impacting BCR signal strength.

While some antigens are composed only of protein, others are not. For example, dsDNA can is often wrapped around histones and bacteria often display LPS as well as organism-specific surface proteins. In these instances, B cells which express BCRs specific for the proteinaceous component of these antigens can also simultaneously engage them with receptors that recognize repetitive patterns such as TLR. dsDNA may interact with TLR9 (268-271) while LPS binds TLR4 (272-275). An entire family of pattern recognition receptors exists that can bind a range of non-protein antigen.

Another factor that may augment BCR signaling is complement (260). Three pathways lead to the activation of complement proteins in the serum – classical, lectin, and alternative (276-278). Classical complement activation is accomplished when immune complexes are formed between antigen and secreted IgM or IgG. The Fc domain of these Ig binds the complement component C1q. Through a cascade of proteolytic events, C3b and C3d complement fragments are generated which bind CD35 (CR1) and CD21 (CR2) respectively (279-286). In mice, CD21 and CD35 exist as a fusion protein and thus, immune complexes decorated with C3 components will bind these receptors as well. CD21 comprises, with CD81 and CD19, a co-receptor complex that lowers the activation threshold for B cells (287, 288). The lectin pathway comes into play when serum lectins bind carbohydrates on bacteria and viruses. This also leads to the deposition of C3b on the pathogen's surface again recruiting CD35 and the co-receptor complex. Finally, the

alternative pathway directly deposits activated C3 fragments again recruiting CD21/35. Thus, whole pathogens or simple antigen can augment B cell responses via complement proteins. This is particularly important for MZ B cells which exhibit increased CD21/CD35 at their surface.

Signaling Throughout B cell Development

In the bone marrow, basal signals delivered through the pre-BCR (IgH with surrogate light chain) and newly formed BCR are required for the appropriate progression of developing B cells. Insufficient signals inform the cell that a functioning IgH or BCR has not been formed properly and too strong signals – specifically self antigen ligation at the immature stage – induce RE or apoptosis. A similar situation occurs in the peripheral splenic stages of development. If T1 B cells encounter antigen, they will be removed from the repertoire by apoptosis (21) or become anergized (22). Conversely, T2 B cells proliferate and mature when they encounter antigen (21). What happens after this stage is a point of controversy.

It is generally accepted that strong signals delivered through the BCR mediate the development of B1 B cells as this population depends on antigen for its differentiation (24, 289). But, whether all B1 B cells are generated from the same precursors as MZ and FO B cells is up for debate. Additionally, there is some dispute over whether stronger or weaker signals are needed for MZ B cell differentiation over that of FO B cells. Pillai and colleagues favor a model wherein stronger signals dictate FO cell fate (133, 189, 290). Evidence for this schema is derived from studies of knockout models that lack FO B cells when BCR signaling is negatively impacted. Conversely, I and others favor the opposite model, specifically that stronger signals favor MZ B cell fate (19, 242, 291, 292). Evidence stems from many

different lines of investigation primarily hinging on BCR specificity. Specifically, autoreactive specificities augment the MZ B cell population (refer to table 1-2) whereas naïve specificities favor the FO compartment. These data suggest that some BCR engagement is necessary for MZ B cell commitment. Further discussion of these models and the evidence for and against will be covered in the discussion as chapter III deals with these issues.

B cell Tolerance

As B cells express antigen specific receptors (BCR or surface Ig) that may recognize self proteins, a system must be in place to keep them in check. There are three main processes that keep autoreactive B cells at bay, and they are collectively referred to as tolerance mechanisms (293-297). The first, and most frequently employed, mechanism is RE (covered in depth in the section concerning B cell development in the bone marrow). The majority of B cells that emerge in the periphery are thought to have undergone RE (9). As this process can be stimulated by ineffective BCR assembly as well as autoreactivity, it cannot be determined how many clones were at first autoreactive (6, 9, 11, 13-15, 298). B cells which cannot effectively rearrange a new IgL to ameliorate their self reactive nature and are sufficiently autoreactive to deliver a strong signal via that BCR may be removed from the repertoire via apoptosis. This mechanism of tolerance is known as clonal deletion. Finally, some autoreactive clones that are not deleted are maintained in a state known as anergy (22, 218, 299-303). The common feature of all anergic B cells is a functional quiescence wherein the B cells are refractory to stimulation via their BCR. This typically results in a lack of plasma cell differentiation and secreted Ig. Apart from being a narrowly defined state, anergic phenotypes exist in a range. Some anergic cells, e.g. anti-HEL, are arrested in their

development at a relatively immature stage (22) while others, e.g. anti-insulin, fully differentiate into mature MZ and FO B cell subsets (218).

The Controversial T3 B cell Subset

At this point, I will consider the splenic T3 developmental subset. Debate exists over whether T3 cells are a normal developmental stage or represent an anergized population. If the surface marker profile is carefully considered (IgM^{lo}, IgD^{hi}, $CD23^{hi}$, $CD21^{mid}$, $CD1d^{lo}$, $AA4.1^{hi}$), it appears that this subset most closely resembles mature FO B cells (IgM^{lo}, IgD^{hi}, $CD23^{hi}$, $CD21^{mid}$, $CD1d^{lo}$, $AA4.1^{neg}$). The persistence of the surface marker AA4.1 indicates that T3 cells are most likely a FO B cell precursor (more accurately T2-FP) (23). That is, the AA4.1 mAb identifies C1q-R, a complement receptor (304-308) that is associated with immature B cell stages [figure 1-2 and (135, 309, 310)]. Taking into account that a state of anergy could theoretically be induced at any developing B cell stage, it is reasonable to conclude that some cells at the T3 stage may become anergized. The T3 subset may be a bona fide maturational stage, but for an anergized cell to maintain the expression of AA4.1, the state of anergy would have to be implemented at an $AA4.1^{+}$ stage. 125Tg, anti-insulin, B cells are anergic and $AA4.1^{neg}$ (J. Cambier, personal communication). Therefore, not all anergic B cells necessarily express this surface marker.

The 125Tg, Anti-insulin, Models

To study B cell development in the context of a physiological autoantigen, Hulbert and Thomas developed a murine model that encodes BCR IgH and IgL Tg which are reactive to insulin, 125Tg (311). The B cells in these mice are functionally unresponsive (anergic) to signals delivered through BCR and TLR4 but are capable of proliferating to a

combination of T_H-mimicking signals, anti-CD40 and IL4 (218, 299). Unlike other models of anergy (22), anti-insulin B cells are not developmentally arrested and generate mature MZ and FO B cell populations. These B cells also retain some antigen presentation capabilities (P. Kendall and H. K. Huston, unpublished results). Thus, anti-insulin B cells maintain some critical features of anergy while bypassing others.

125Tg mice embody an ideal system in which to study MZ B cell development as they have a greatly augmented MZ B cell compartment. However, as described above, these cells do not response to BCR or TLR4 stimulation. Therefore, conclusions drawn from this model may be indicative of MZ B cells in general or anergic MZ B cells in particular. This feature is not a hindrance but instead provides at least two unique opportunities. First, the MZ population in 125Tg mice is more easily managed as it attains 30-50% of total splenic B cells as compared to a mere 5-10% in wild type C57BL/6 mice. Furthermore, these MZ B cells exhibit a consistent specificity making them a homogenous population for investigation. Second, no other experimental model has demonstrated anergic MZ B cells. Most autoreactive Tg impose early developmental arrest or allelic inclusion as a result of RE. It is likely that anergized MZ B cells are a part of normal polyclonal repertoires. Understanding whether anergy in the MZ compartment limits an individual's ability to clear bacterial and viral infections is an important undertaking. Implications for studying anergic MZ B cells will be discussed further in chapters IV and V.

A second line of mice that only express the IgH Tg (V_H125Tg) are used in experiments detailed in chapter II. The IgH Tg alone allows the generation of a pauciclonal B cell repertoire via the usage of endogenous IgL. This repertoire contains a subpopulation (1-2% of $B220^+$ lymphocytes) of insulin reactive B cells that can be tracked by FACS and isolated by magnetic sorting or binding to immobilized insulin. When this Tg is expressed

on the non-obese diabetic (NOD) background, the penetrance and kinetics of diabetes progression are accelerated compared to wild type NOD (311). Understanding how repertoire influences autoimmune disease progression is important for both early diagnosis and therapy. The IgL identity of the anti-insulin B cells in V_H125Tg/NOD mice is the subject of chapter II (216).

Research Objectives

The overall goal of the research reported in this dissertation is to understand how autoreactivity influences B cell maturation in the periphery. These projects are all concerned with different aspects anti-insulin B cell development. As mature B cell phenotype – MZ, FO, and B1 – is ultimately governed by BCR mediated signals that are a consequence of antigen specificity, lines of investigation which seem divergent are, in reality, related.

In chapter II, I sought to define the IgL repertoire that governs insulin binding when paired with a Tg IgH (V_H125Tg). There exists an accelerated disease phenotype in V_H125Tg/NOD (IgH Tg only) as compared to WT or 125Tg/NOD (IgH and IgL Tg). Only a subset of splenic B cells in V_H125Tg/NOD is capable of binding insulin. Thus, in this model, the IgH does not dictate insulin specificity. Therefore, determination of the total endogenous IgL repertoire that paired with the IgH Tg as well as which IgL facilitated insulin binding was an important next step in elucidating how the V_H125Tg could accelerate diabetes. Accessory goals of this project included defining the extent of clonality or individuality present in the anti-insulin B cell sub-repertoire and delineation of whether insulin specific B cells were generated in both NOD and C57BL/6 bone marrow (216).

The goal of chapter III was to determine whether the 125Tg could generate a MZ B cell phenotype in the absence of a Notch2 allele. The phenotype of anti-insulin 125Tg B

cells is exceptional in several respects including overpopulation of the MZ. Two strong lines of evidence demonstrate that heterozygosity or deletion of the Notch2 gene is sufficient to inhibit MZ B cell development. Reasoning that BCR signals can activate and repress particular TF and that BCR signals interconnect with other signaling pathways, the question of whether the 125Tg BCR could override or rescue the Notch2 defect arose.

The experiments reported in chapter IV began the ambitious task of characterizing 125Tg MZ B cells in contrast to their FO B cell counterparts. These studies were initiated with a global perspective using microarrays. Known expression profiles for certain MZ and FO specific genes were confirmed, and a panel of differentially expressed known TF was established for each B cell population. One novel gene stood out among the microarray data, zinc finger protein 532 (ZFP532). ZFP532 was over expressed twelve fold in the MZ B cell population. The remaining data in chapter IV aim to characterize the transcript structure and tissue distribution of this novel gene.

Taken together, these studies expand our understanding of how autoreactivity impacts B lymphocyte maturation and maintenance. Anti-insulin B cells are not removed from the repertoire by deletion or receptor editing. They are instead maintained in a state of incomplete anergy in the periphery and are capable of allowing the progression of T1DM. How autoreactivity, anergy, and the functions of MZ B cells intersect are important lines of study for the future.

CHAPTER II

MULTIPLE GERMLINE KAPPA LIGHT CHAINS GENERATE ANTI-INSULIN B CELLS IN NON-OBESE DIABETIC MICE

Abstract

The highly selective nature of organ specific autoimmune disease is consistent with a critical role for adaptive immune responses against specific autoantigens. In type 1 diabetes mellitus (T1DM), autoantibodies to insulin are important markers of the disease process in humans and non-obese diabetic (NOD) mice; however the antigen specific receptors responsible for these autoantibodies (autoAb) are obscured by the polyclonal repertoire. NOD mice that harbor an anti-insulin transgene (Tg) (V_H125Tg/NOD) circumvent this problem by generating a tractable population of insulin binding B cells. The nucleotide structure and genetic origin of the endogenous kappa light chain (Vκ or IgL) repertoire that pairs with the V_H125Tg was analyzed. In contrast to oligoclonal expansion observed in systemic autoimmune disease models, insulin binding B cells from V_H125Tg/NOD mice employ specific Vκ genes that are clonally independent and germline encoded. When compared to homologous IgL genes from non-autoimmune strains, Vκ genes from NOD mice are polymorphic. Analysis of the most frequently expressed Vκ1 and Vκ9 genes indicates these are shared with lupus-prone NZB/BINJ mice (e.g. Vκ1-110*02 and 9-124) and suggests that NOD mice use the infrequent b haplotype. These findings show that a diverse repertoire of anti-insulin B cells is part of the autoimmune process in NOD mice and structural or regulatory elements within the kappa locus maybe shared with a systemic autoimmune disease.

Introduction

T1DM is an organ specific autoimmune disease in which T cells mediate the destruction of insulin producing pancreatic beta cells. Directly, T cell involvement is well established by adoptive transfer experiments in animal models (312-317) and indirectly in humans by strong genetic linkage to specific class II MHC alleles (318-324). The role of B cells in this disease is both necessary (325, 326) and complex. Experiments in the NOD murine model of autoimmune diabetes indicate that B cells function in an antigen presentation capacity that is essential for disease progression (327-330). In this model, B cells specific for islet antigen capture and process autoantigen resulting in presentation of peptides to cognate T cells. These T-B interactions likely result in two outcomes. One, autoaggressive T cells would undergo clonal expansion and ultimately target pancreatic islets, and two, activated B cells would produce class-switched (IgG), islet specific autoAb. Consistent with this process, islet specific autoAb, particularly ones reactive with insulin, are recognized as sensitive indicators of disease (331-337). To date, tolerance inducing therapies initiated after the appearance of IgG anti-insulin autoAb fail to halt disease progression (338, 339). Thus, earlier predictive methods are required to maintain or restore lymphocyte tolerance prior to beta cell destruction.

The presence of IgG autoAb to insulin and other islet antigen in the prodrome of T1DM are assumed to be the product of clonal expansion via interaction with autoreactive T cells (340). However, this has not been definitively shown. Additionally, initial interaction of the B cell antigen receptor (surface Ig or BCR) with islet antigen necessitates the existence of identifiable molecular characteristics inherent in that receptor that promote antigen binding. To address these gaps, we generated NOD mice harboring an Ig heavy chain Tg (V_H125Tg/NOD) derived from an anti-insulin mAb. These mice are unique among Ig

transgenic NOD, in that not only do they support the development of diabetes, but it may be accelerated (311). Other Ig transgenic NOD mice which harbor specificities for non-islet antigen (329, 341) exhibit protection from diabetes further supporting the importance of BCR specificity to diabetogenesis. V_H125Tg/NOD mice generate a polyclonal B cell repertoire by utilizing endogenous IgL. This polyclonal repertoire contains a subset (1-2%) of highly insulin reactive B cells that are not observed in V_H125Tg/C57BL/6 mice. By analyzing the nucleotide sequences of the IgL that pair with the V_H125Tg, we are able to draw conclusions about a model B cell population that recognizes a key diabetes-associated antigen, insulin. In contrast to expectations, our data demonstrate that anti-insulin B cells are not the product of oligoclonal expansion. Instead, they are independently seeded into the peripheral repertoire. Additionally they exhibit no evidence of antigen driven selection or hypermutation. In addition, IgL genes from NOD encode multiple germline polymorphisms distinct from non-autoimmune prone mouse strains such as C57BL/6, C3H, and BALB/c. Rather, certain NOD Vκ genes, such as Vκ1-110*02 and Vκ9-124, are identical to those present in autoimmune-prone NZB/BINJ mice which are of the rare Vκ b haplotype. Thus, the polymorphic residues in structural, coding regions and intervening regulatory elements characteristic of the b haplotype of NZB/BINJ may contribute to autoimmune features in both systemic and organ specific autoimmune disease.

Materials and Methods

Animals

V_H125Tg/NOD mice were described previously (311). Lines were maintained as heterozygotes by backcrosses to wild type (WT) NOD (>20 generations). All mice were

housed under pathogen-free conditions and all experiments were approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

Flow Cytometry

Splenocytes and bone marrow cells were analyzed on a FACSCalibur™ flow cytometer (BD Biosciences, Mountain View, CA). mAb (BD Pharmingen) used were reactive with: IgM^a (DS-1), IgM^b (AF6-78), CD23 (B3B4), and B220 (RA3-6B2). Biotinylated insulin (50ng/mL – NOVO, Copenhagen, Denmark) was used to detect insulin binding B cells with PerCP-streptavidin. Specificity was confirmed by inhibition with excess human insulin (299). WinMDI 2.8 software (Dr. J. Trotter, Scripps Institute, San Diego, CA) was used for data analysis.

Selection of Anti-insulin B cells

V_H125Tg/NOD spleens were depleted of T cells via anti-thy1.1 and complement. Insulin binding B cells were selected by MACS or adherence to insulin coated plates. MACS: T cell-depleted splenocytes (10^7 cells/90 μl) were incubated with biotinylated insulin (50 ng/mL/10^6 cells) in buffer (2 mM EDTA, 0.5% BSA in 1X PBS) for 10 min at 4 °C, washed and incubated with streptavidin conjugated magnetic beads (20 μl beads/10^7 cells – Miltenyi Biotec, Auburn, CA) for 15 min at 4 °C. Cells were resuspended (10^8 cells/500 μl) and passed over an LS column (Miltenyi Biotec). Insulin-binding cells were eluted from the column and lysed in TRI Reagent (Molecular Research Center, Inc, Cincinnati, OH). Plate Binding: Dishes (Corning Incorporated, Corning, NY) were pre-coated with human insulin (1 μg/ml in PBS overnight at 4 °C) and blocked with BSA. Unbound cells were thoroughly washed from the plate with PBS. Cells were removed by scraping in TRI reagent.

Vκ Amplification and Analysis

To analyze expressed Vκ genes, RNA was isolated from T cell-depleted splenocytes (total Vκ) or from insulin selected B cells. First strand cDNA was generated from total RNA using Superscript II RT (Invitrogen, Carlsbad, CA) and 0.67 μg oligo-dT primer (Amersham Biosciences, Piscataway, NJ) in a standard cDNA synthesis protocol. Vκ sequences were amplified from first strand cDNA using the following primers: murine κ constant region primer - 5' GGA TAC AGT TGG TGC AGC ATC 3', murine VκA - 5' ATT GTK MTS ACM CAR TCT CCA 3', murine VκB - 5' GAT RTT KTG RTR ACB CAR RM 3', murine VκC - 5' AYA TYN WGM TGA CHC ARW CTM M 3'. Vκ sequences were amplified using AmpliTAQ DNA Polymerase (2U/reaction) (Applied Biosystems, Foster City, CA) and the following: 200 nM dNTP, 1.25 mM $MgCl_2$, 13.35 mM constant primer and 13.35 mM of one of the three Vκ primers. The PCR protocol was 94 °C/1 min, 42 °C/1 min, and 72 °C/2 min for 35 cycles. PCR product was ligated into pGEM-T easy plasmid (pGEM-T Easy Vector System I, Promega, Madison, WI). Positive clones were sequenced using an Applied Biosystems 3730xl DNA Analyzer (Vanderbilt-Ingram Cancer Center). Analysis, homologies, and germline gene segment assignment were accomplished with blastn (www.ncbi.nlm.nih.gov/BLAST), the ImMunoGeneTics (IMGT) database (imgt.cines.fr:8104/), and BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html). Statistical significance for Vκ families and individual genes in the insulin selected and unselected groups (Figures 2 and 3) are derived from a Chi Square (χ^2) test of independence.

NOD Germline Vκ Genes

Germline Vκ gene segments were amplified from NOD tail DNA. Primers were designed based on known C57BL/6 germline genes. Vκ9-120/4FWD 5' ATG GAC ATG AGG GYT CCT GC 3', Vκ9-120FWD2 5' GGG CTC CTG CAC AGA TTT TTG 3', Vκ9-120iFWD 5' GGG GGA TGT CCT CTT TTC TC 3', Vκ9-120/4REV 5' CAC TGT GGG AGG AKA ACT AG 3', Vκ1-132/3FWD 5' ATG ATG AGT CCT GTC CAG TTC C 3', Vκ1-110FWD 5' ATG AAG TTG CCT GTT AGG CTG TTG G 3', Vκ1-132/3REV 5' CAC TGT GTG AGG AWA ATR TGT ACC 3', and Vκ1-110REV 5' CAC TGT GGG AGG AAC ATG TGT AC 3'. Germline Vκ sequences were amplified using AmpliTAQ DNA Polymerase (2 U/reaction) and the following: 500 ng genomic DNA, 2.5 mM $MgCl_2$, 250 nM each primer, and 200 nM dNTP. Reactions were cycled at 94 °C/1min, 53 °C (Vκ9) 55 °C (Vκ1-132/3) 58 °C (Vκ1-110)/1 min, 72 °C/1 min for 40 cycles. PCR products were cloned, sequenced and analyzed as described above.

GenBank Accession Numbers

Accession numbers for novel sequences presented in this paper: AY675526 through AY675540 and AY731701 through AY731709.

Results

V_H125Tg/NOD mice generate a detectable population of anti-insulin B cells.

V_H125Tg/NOD mice develop diabetes at an accelerated rate compared to their WT littermates (311). In these mice, 1-2% of B cells bind biotinylated insulin with a mean

fluorescence intensity (MFI) >200 (Figure 2-1, panel B). The specificity of this population is confirmed by inhibition with soluble, unlabeled insulin (299). Insulin specific B cells are undetectable in C57BL/6 mice expressing V_H125Tg (Figure 2-1, panel D) as well as in WT NOD and C57BL/6 (Figure 2-1, panels A and C respectively) by this method. These findings are consistent with the low frequency (10^{-5}) of anti-insulin B cells predicted by T cell independent responses to insulin (342). The presence of insulin autoAb in WT NOD indicates that anti-insulin B cells are present in these animals, and the V_H125Tg increases the frequency of this population.

The Vκ gene families expressed by insulin binding B cells in V_H125Tg/NOD mice are heterogeneous.

To understand their molecular origins, we isolated insulin binding B cells from V_H125Tg/NOD mice and examined their expressed Vκ genes. Vκ genes were cloned and sequenced from B cells either selected for insulin binding or unselected (total). Vκ identity was assigned based on nucleotide homology to germline reference sequences in the IMGT database. The findings do not differ for B cells captured on insulin-coated plates or MACS columns, and the results of six independent experiments are pooled. Histograms show the frequency of Vκ families used by anti-insulin V_H125Tg/NOD B cells (Figure 2-2, panel A) compared to unselected V_H125Tg/NOD B cells (Figure 2-2, panel B). The most frequently used family in this repertoire was Vκ1, representing 45% of isolates from insulin binding V_H125Tg/NOD B cells and 31% of the unselected population. The high frequency of Vκ1 here is consistent with the representation of this family in other primary repertoires (343). In addition to Vκ1, insulin binding B cells also use genes from Vκ families 9, 10 and 19 while unselected B cells also use Vκ2, 3, and 6 (compare Figure 2-2, panel A to B).

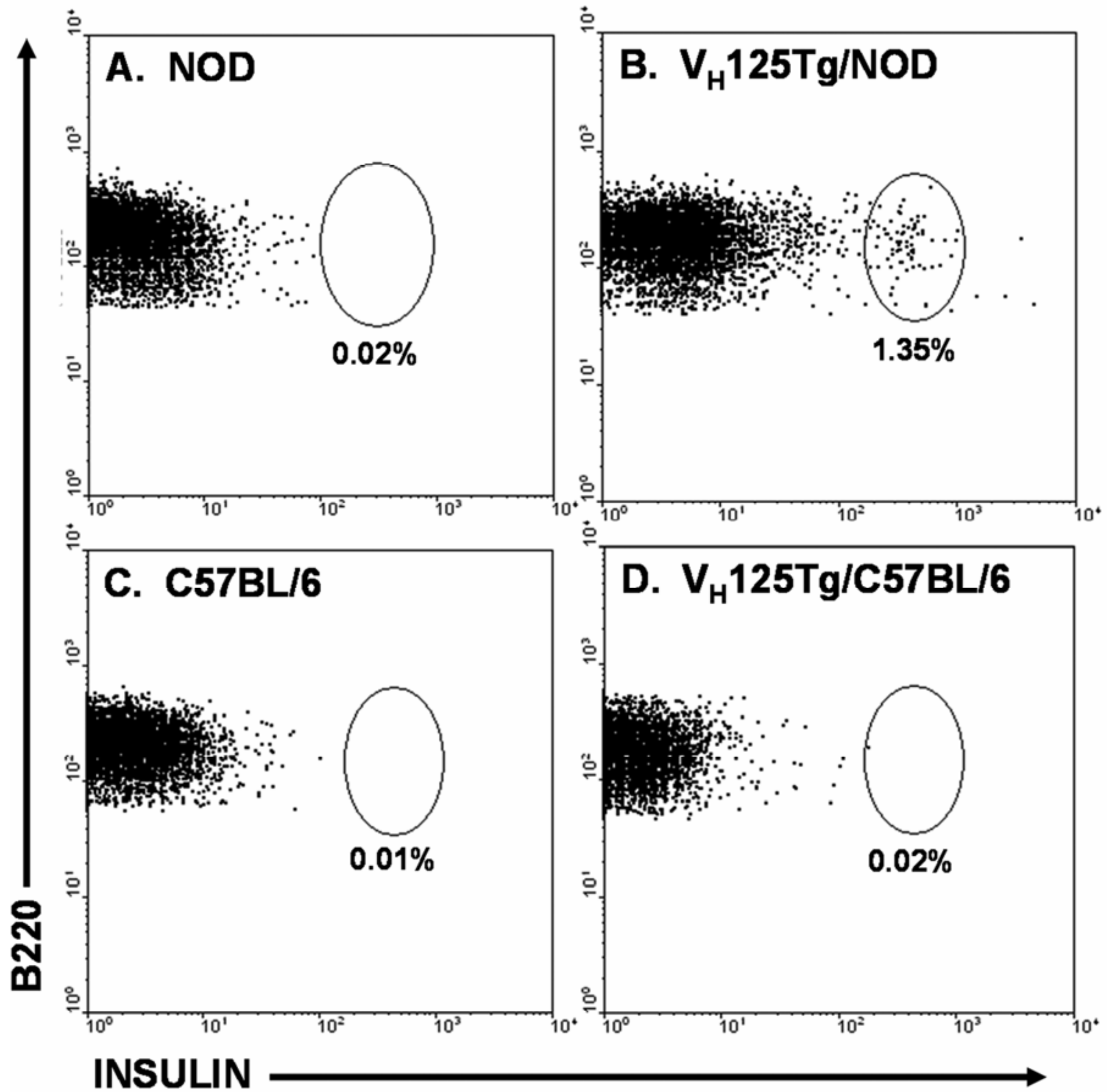


Figure 2-1. Identification of splenic anti-insulin B cells in V_H125Tg/NOD mice. Flow cytometry on splenic B cells ($B220^+$) that bind insulin (biotin-insulin/streptavidin-PerCP) from WT NOD (A), V_H125TgNOD (B), WT B6 (C), and V_H125Tg/B6 (D). Insulin specific B cells bind insulin with a MFI $\geq$ 200 (B, ellipse).

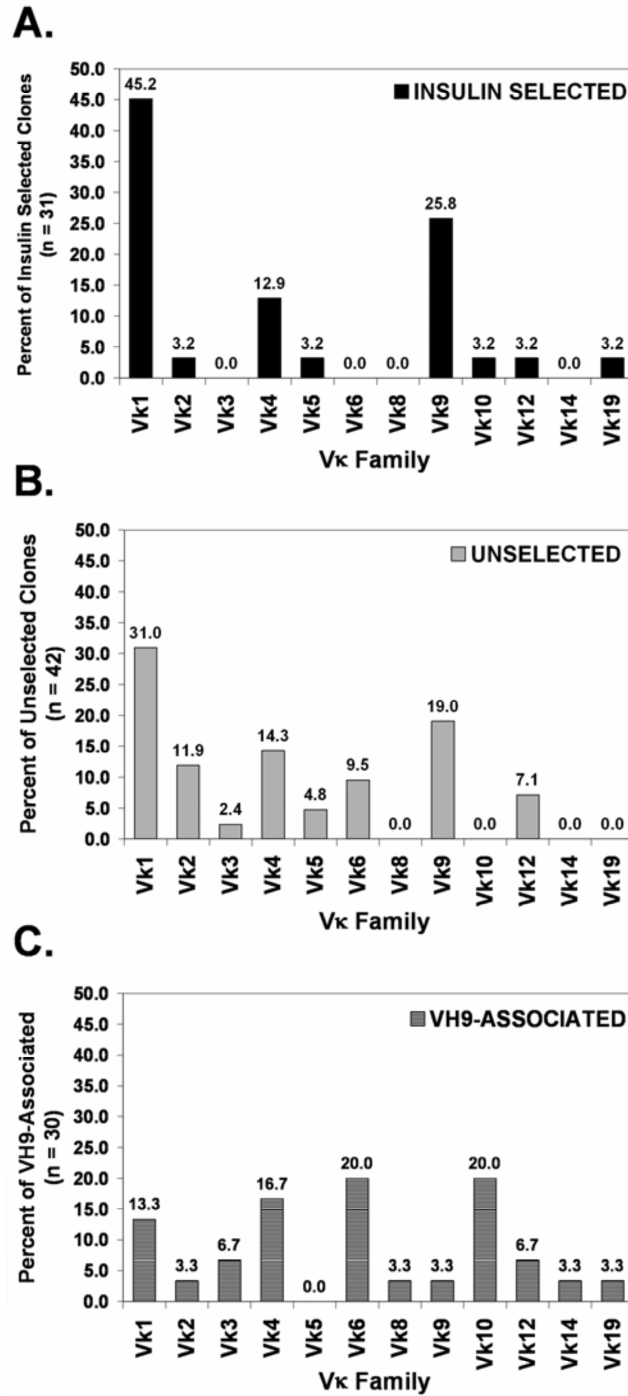


Figure 2-2. The Vκ gene families expressed by insulin binding B cells from V_H125Tg/NOD are heterogeneous. Vκ genes isolated from V_H125Tg/NOD B cells were assigned to a Vκ family based on nucleotide sequence. The frequency of Vκ family use by insulin selected B cells (A) is compared to that of the unselected repertoire (B). For comparison, the Vκ repertoire paired with VH9 IgH homologous (>95%) to V_H125Tg is also shown (C). The distributions in A and B do not differ from those expected by a χ^2 test.

Surprisingly, Vκ4 usage was not dramatically increased in the insulin binding population even though the original partner of V_H125 is Vκ4-74*01 (344). These findings demonstrate that multiple IgL families contribute to the insulin binding repertoire. The distribution of Vκ families used by the insulin selected and unselected groups did not differ from those expected based on a Chi Square test for independence. To corroborate the IgL heterogeneity exhibited by V_H125Tg/NOD B cells, we surveyed Vκ gene usage in a panel of VH9-containing mAb collected from GenBank. The variety of IgL used by these V_H9 heavy chains, as shown in Figure 2-2, panel C, suggests that the heterogeneity exhibited by the V_H125Tg is representative of similar IgH.

Specific Vκ genes are expressed by insulin binding B cells in V_H125Tg/NOD mice.

The previous analysis demonstrates that a variety of Vκ families can pair with the V_H125Tg to generate anti-insulin BCR but does not indicate how any single gene is utilized. Therefore, we examined the frequency of specific Vκ genes. Using this approach, marked differences in Vκ gene usage between insulin binding and unselected B cells were revealed (Figure 2-3). For example, within the Vκ1 family, the Vκ1-110*02 gene is significantly overrepresented in sequences derived from insulin selected B cells ($p=0.0044$ by χ^2) while Vκ1-135 is preferred by the unselected population (NS, $p=0.086$). Likewise, the Vκ9-120 gene is more frequently associated with insulin selected B cells than the Vκ9-124 gene. Thus, within the diverse family repertoire, a subset of Vκ genes preferentially generates anti-insulin BCR. Therefore, insulin binding is not governed by a single Vκ family but is determined by unique features of individual genes from different families.

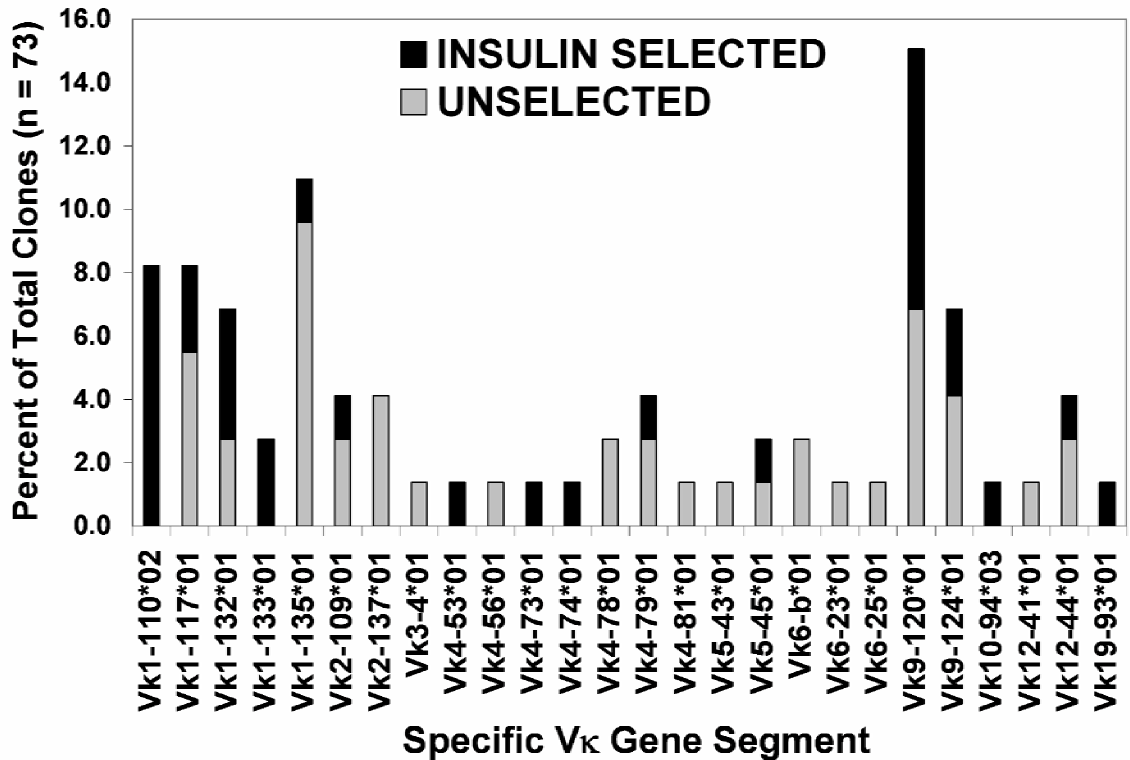


Figure 2-3. Specific Vκ gene segments are expressed by insulin binding B cells from V_H125Tg/NOD mice. Histograms show the frequency of individual Vκ genes used by B cells selected for insulin binding or unselected. Specific Vκ gene identity is based on nucleotide sequences from the IMGT database. Only the Vκ1-110*02 gene is statistically overrepresented (p=0.0044).

Anti-insulin Vκ1 genes expressed in V_H125Tg/NOD mice are germline encoded, clonally independent, and polymorphic to non-autoimmune strains.

As shown in Figure 2-3, Vκ1-110*02 ("V1B") is exclusively expressed by insulin binding V_H125Tg/NOD B cells (p=0.0044). To investigate the roles of antigen driven clonal expansion and somatic mutation in the generation of anti-insulin B cells, we compared the nucleotide sequences of expressed anti-insulin genes to known germline Vκ1 genes (Figure 2-4). The *02 allele from lupus prone NZB mice (345), haplotype b, (346) is allelic to Vκ1-110*01 germline genes from C57BL/6 and BALB/c mice (haplotype c). Polymorphic residues associated with the *02 allele are located principally in the complementarity determining regions (CDR) and account for most of the structural differences exhibited by NOD Vκ1-110. The frequency of the other nucleotide differences does not exceed that which is anticipated from errors in amplification and sequencing. Because V_H125Tg/C57BL/6 mice do not exhibit anti-insulin B cells in the periphery, this suggests that the Vκ1-110*02 allele may favor insulin binding. Additionally, Vκ1 genes expressed by anti-insulin V_H125Tg/NOD B cells are germline encoded and clonally independent. Lack of clonality is deduced from the nucleotide sequence of each VκJκ joining site in CDR3 (Figure 2-4). Clones 39 and 92 use the same joining sequence but were derived from different donor mice. These data demonstrate that anti-insulin Vκ1 genes in V_H125Tg/NOD mice are principally derived from independent clones rather than recurrent expansion of the same clone. In 58 independently derived isolates, only one pair exhibited evidence of potential clonality.

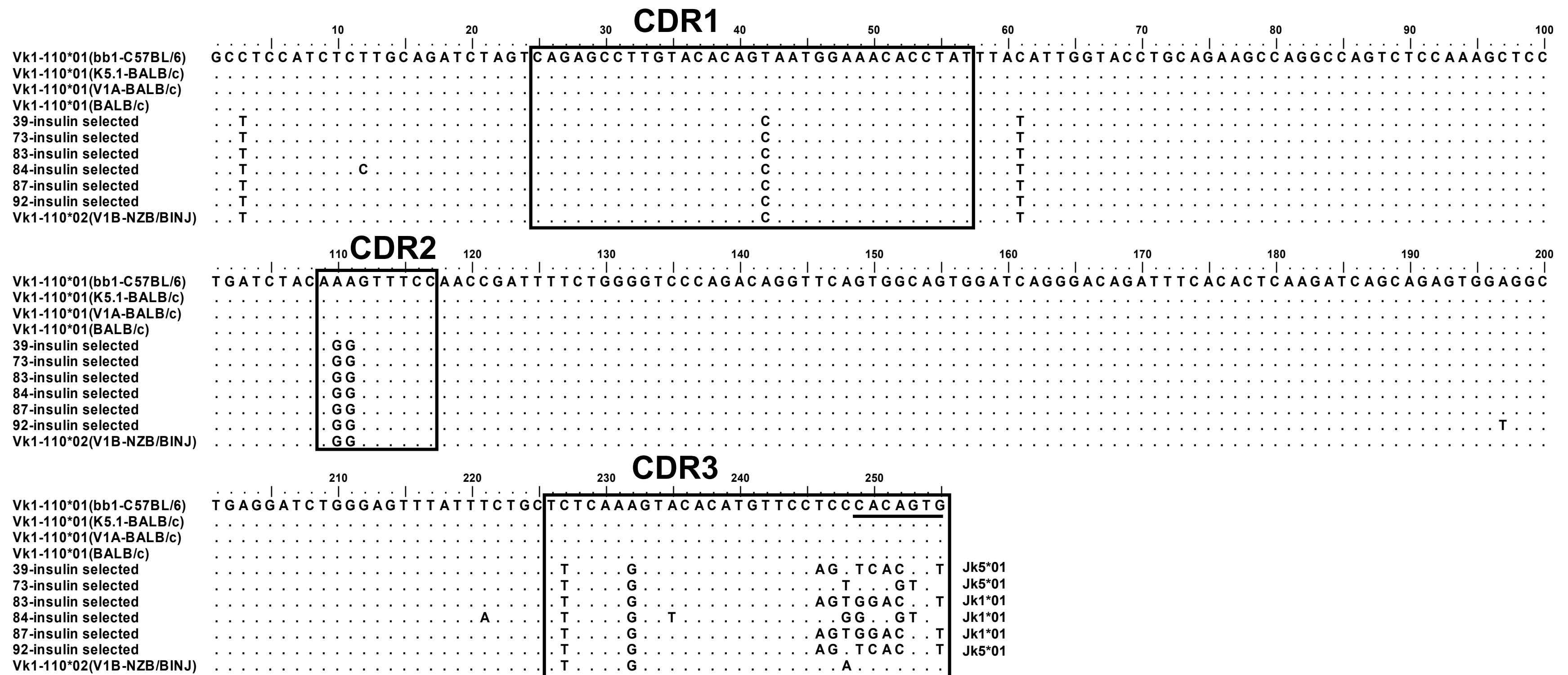


Figure 2-4. Anti-insulin Vκ1 genes expressed in V_H125Tg/NOD mice are germline encoded, clonally independent, and polymorphic to non-autoimmune strains. Nucleotide sequences of anti-insulin, expressed Vκ genes from V_H125Tg/NOD B cells (sequences 73, 83, 84, 87, 92, and 39) were compared to homologous reference sequences (Vκ1-110*01 – C57BL/6 and BALB/c) and a germline gene from NZB mice (Vκ1-110*02). Boxes indicate CDR. The recombination signal sequence heptamer (CACAGTG) is underlined. The Jκ gene used by each expressed IgL is indicated.

Vκ9 genes expressed in V_H125Tg/NOD mice are also polymorphic and germline encoded.

The preceding data on Vκ1 genes suggest that polymorphisms in the IgL locus may contribute to B cell autoreactivity in NOD mice. Therefore, we extended our analysis to Vκ9 genes (Figure 2-5). A subset of Vκ9 sequences from V_H125Tg/NOD are most closely related to Vκ9-120*01 (C3H and C57BL/6 strains). Vκ9-120 clones from NOD differ at 11 nucleotides from the reference sequences (*01). To confirm these polymorphisms, we amplified genomic DNA from NOD mice (Materials and Methods). Ten independent isolates from four separate experiments identified a Vκ9 gene that is polymorphic to the known Vκ9-120*01 germline sequences. When compared to the Vκ9-120 genes expressed in V_H125Tg/NOD B cells, the germline polymorphisms account for all the nucleotide differences between expressed NOD Vκ9-120 and the reference Vκ9-120*01 sequence. Thus, the Vκ9 gene used in V_H125Tg/NOD mice is a novel Vκ9-120 allele. These data support the conclusion that germline NOD Vκ are highly polymorphic when compared to those in other strains.

Polymorphisms are not limited to insulin binding Vκ genes.

As shown in Figure 2-3, Vκ9-124 is highly expressed in the unselected V_H125Tg/NOD B cell population. The sequences in Figure 2-5 suggest that numerous polymorphisms occur in genes used by the unselected population. To further cement this observation, we analyzed the nucleotide sequence of expressed and germline Vκ9-124 genes (Figure 2-6). The six isolates (A4 – genomic DNA; 17, 59, 226, 227, and 244 – expressed) exhibit limited polymorphisms in CDR 2 and 3. The A4 group represents allelic

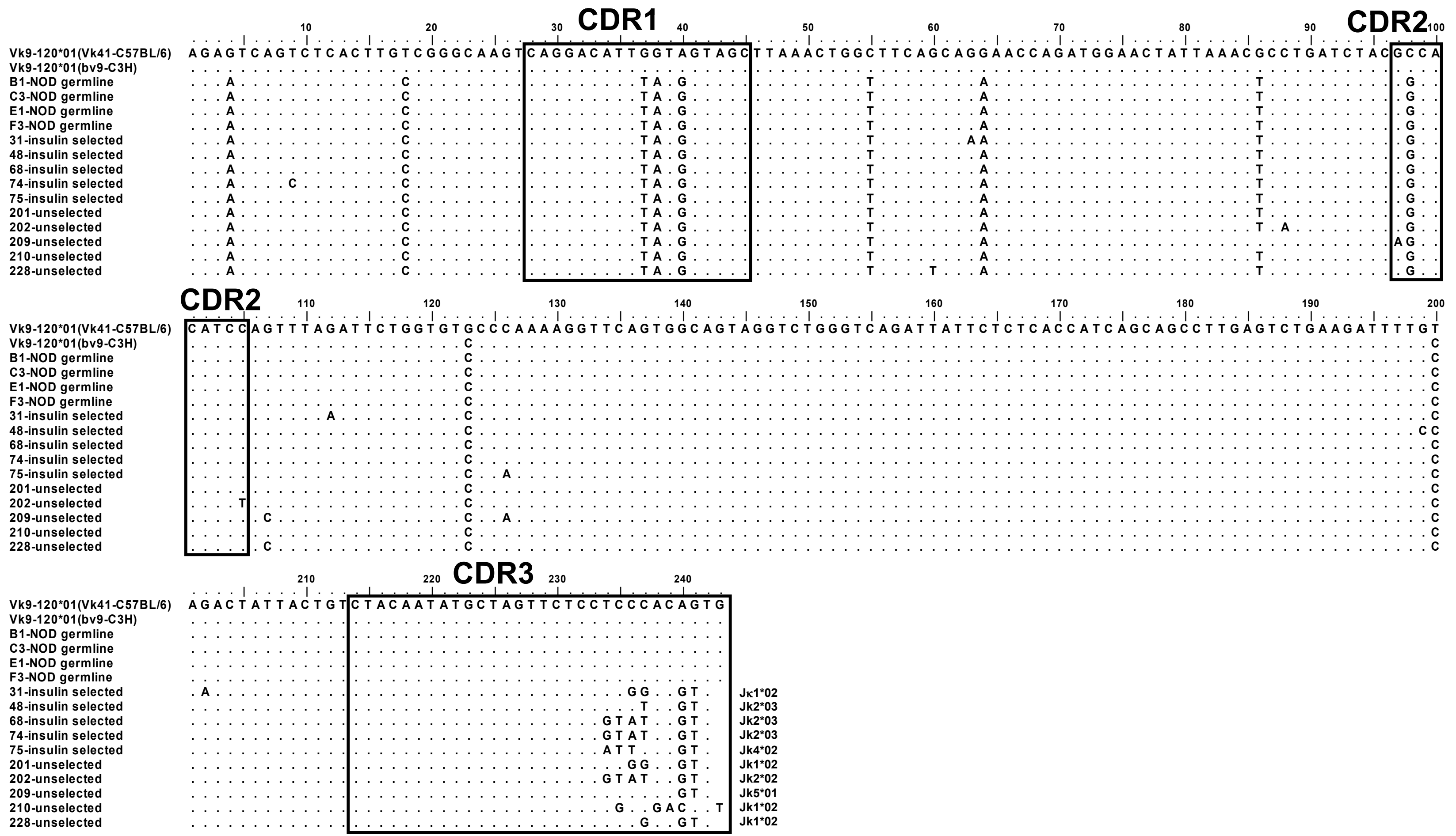


Figure 2-5. NOD Vκ9 genes exhibit germline polymorphisms. Nucleotide sequence from NOD Vκ9-120 genes (expressed, insulin selected: 31, 48, 68, 74, 75; expressed unselected: 201, 202, 209, 210, and 228; genomic: B1, C3, E1, F3) are compared to the reference Vκ9-120*01 (C57BL/6 and C3H). CDR, recombination signal sequence heptamer, and Jκ partners are indicated as in Figure 2-4.

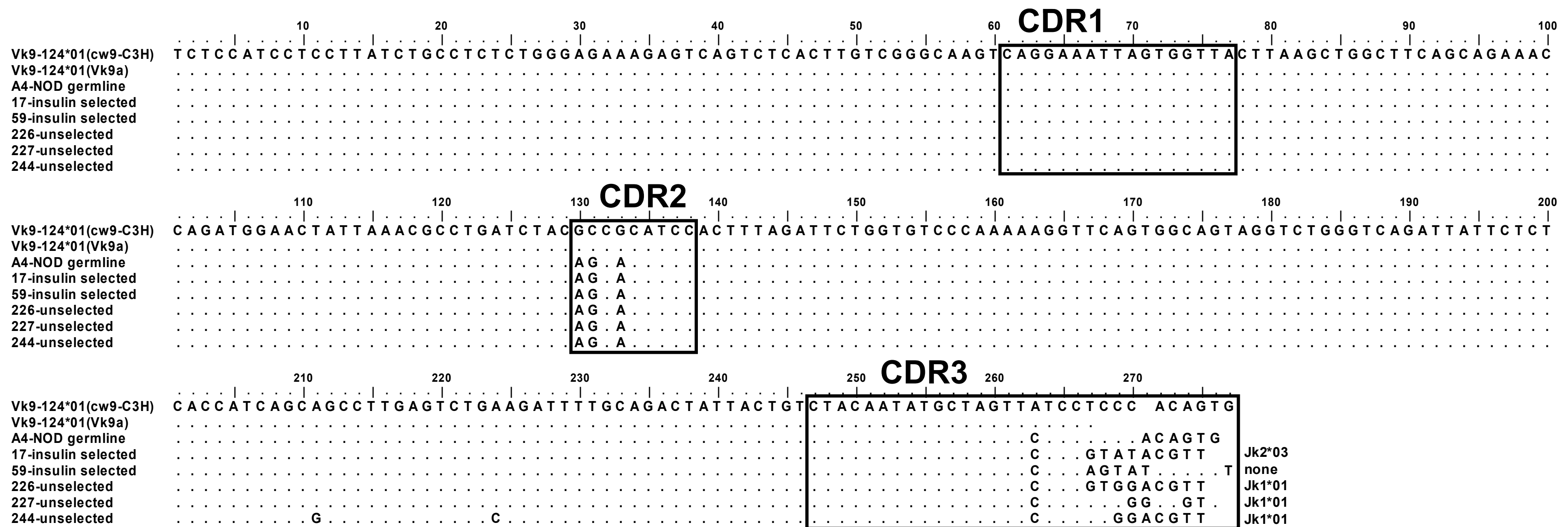


Figure 2-6. Polymorphisms are not limited to insulin binding Vκ genes. Nucleotide sequence of five expressed Vκ genes (17, 59, 226, 227, and 244) from V_H125Tg/NOD is compared with the cognate NOD germline sequences (A4) and the reference Vκ9-124*01 gene. These clones identify a novel Vκ9-124 allele (A4, 17, 59, 226, 227, and 244). CDR, recombination signal sequence heptamer, and Jκ partners are indicated as in Figure 2-4.

homologues of Vκ9-124*01, and further exhibits germline polymorphisms in both the insulin specific and nonspecific repertoires. A search of the public database has revealed that germline NOD Vκ9-124 is identical to two expressed mAb IgL from NZB (gb: AF321948 and Z22118).

Germline nucleotide polymorphisms in NOD Vκ confer amino acid changes.

To determine if the polymorphic nucleotide residues seen in NOD expressed and germline Vκ genes could have functional implications, their predicted amino acid sequences were analyzed (Table 2-1). Polymorphisms conferring no amino acid change are not detailed. The total number of polymorphisms and their segregation into CDR and FWR are indicated. Only half the amino acid changes are conservative while the rest constitute alterations in size and/or charge. These unique NOD polymorphisms could thus influence antigen specificity and BCR assembly consequently governing the primary B cell repertoire.

Anti-insulin B cells are generated in the bone marrow of V_H125Tg/NOD mice.

The presence of anti-insulin B cells in the spleens of V_H125Tg/NOD but not in V_H125Tg/C57BL/6 mice could result from two processes. First, anti-insulin B cells could be generated on both backgrounds but only be removed (by deletion or receptor editing) from the repertoire of C57BL/6 mice. Alternatively, germline polymorphisms unique to NOD could favor the production of B cells that recognize insulin. To address these possibilities, we analyzed the bone marrow of V_H125Tg/C57BL/6 and V_H125Tg/NOD for insulin binding B cells. As shown in Figure 2-7, anti-insulin B cells (indicated by M1) are only detected in the bone marrow of V_H125Tg/NOD mice. The cells shown are $B220^{+}$, IgM^{a+}, and $CD23^{neg}$ representing immature and newly formed bone marrow B cells and not

Table 2-1. Amino acid changes are due to polymorphic residues in NOD mice.

NOD Vκ Gene	Polymorphisms		Non-silent changes*
Vκ1-110	total	7	
	CDR	5	K110/111R, S227F, S232G
	FWR	2	H61Y
Vκ9-120	total	11	
	CDR	4	G37/38Y, S40G, A98G
	FWR	7	V4I, L55F, E64K, R86L, V200A
Vκ9-124	total	4	
	CDR	4	A130/131S, A133T, Y263S
	FWR	0	

*Silent amino acid changes are not detailed. Numbers indicate nucleotide positions (not amino acids) as indicated in Figures 2-4, 2-5, and 2-6.

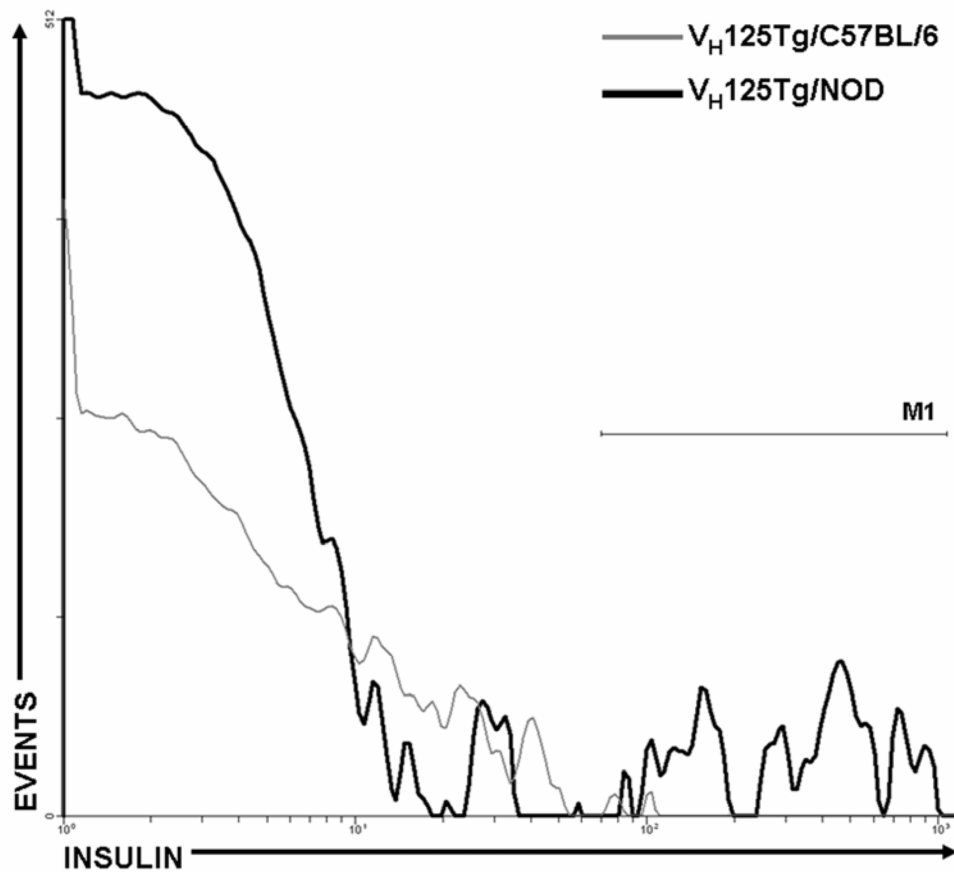


Figure 2-7. Identification of anti-insulin B cells in the bone marrow of V_H125Tg/NOD mice. Flow cytometry of bone marrow cells shown are immature and newly formed B cells ($IgM^{a+}/B220^{+}/CD23^{neg}$) from V_H125Tg/C57BL/6 (grey line) and V_H125Tg/NOD (heavy black line) mice. The M1 marker denotes insulin specific B cells.

mature recirculating B cells from the spleen. The specificity of this population was confirmed by inhibition with excess unlabeled insulin (for V_H125Tg/NOD B cells in the M1 gate: uninhibited MFI = 293, inhibited MFI = 151 – data not shown). These data demonstrate that germline Ig polymorphisms in NOD mice favor the production of autoantigen specific B cells. Anti-insulin B cells are generated in the bone marrow of NOD mice in the absence of peripheral positive selection. Additionally, anti-insulin B cells are not generated in V_H125Tg/C57BL/6 bone marrow and then negatively selected.

Discussion

NOD mice that carry the IgH chain of anti-insulin mAb125 (V_H125Tg/NOD) generate a small population of insulin binding B cells that are not observed in V_H125Tg/B6 mice. In this study, we have isolated these anti-insulin B cells from V_H125Tg/NOD and demonstrate that their BCRs are independently generated by the recombination of several different Vκ and Jκ gene families. Genes from the Vκ1 and Vκ9 families are found in the majority of anti-insulin B cells and their nucleotide sequences do not show evidence of somatic hypermutation. In depth analysis of germline and expressed Vκ genes isolated from NOD mice demonstrates germline encoded polymorphisms that are allelic (e.g. Vκ1-110*02) to non-autoimmune prone strains of the c haplotype (C57BL/6, C3H, and BALB/c). Since anti-insulin B cells are not observed in the bone marrow or spleen of V_H125Tg/C57BL/6 (Figures 2-1 and 2-7), these data support the hypothesis that the kappa locus of the NOD strain facilitates skewing of the primary repertoire toward this autoantigen.

In systemic autoimmune disease, such as lupus, oligoclonal expansion and somatic mutation typify the anti-DNA response even when IgH Tg are present (237, 347-349). Because IgG insulin autoAb are documented in the prodrome of autoimmune diabetes

(333), we anticipated that anti-insulin B cells in adult NOD mice would reflect oligoclonal expansion and somatic mutation as a consequence of T cell help. However, the data advocate that anti-insulin B cells in adult V_H125Tg/NOD mice are not derived from this process. Rather, seeding of a diverse group of anti-insulin B cells into the repertoire may provide an important source of antigen presenting cells that capture insulin-related antigen and contribute to expansion of the autoreactive T cell pool. An important issue for future studies is to understand the relationship between the primary repertoire of anti-insulin B cells and that which differentiates to produce IgG autoAb. This will require the production of NOD mice in which fully functional Tg are targeted into the IgH locus.

In NOD mice, multiple genes, particularly from the Vκ1 and Vκ9 families, are capable of forming anti-insulin BCRs (Figures 2-2 and 2-3). Although multiple families contribute to insulin binding, specific genes within each family preferentially segregate into the insulin binding pool. For example, Vκ1-110 segregates exclusively with insulin binding whereas Vκ1-135 is associated with the unselected population. Comparison of Vκ1-110 to published germline counterparts clearly demonstrates that the NOD and NZB/BINJ strains share this allele (Figure 2-4). To our knowledge, Vκ1-110*02 (345) is the only germline NZB Vκ gene published in the database. We therefore compared our germline NOD Vκ9-120 and Vκ9-124 genes to NZB Vκ from published mAb. NOD Vκ9-124 (clone A4) is identical to an anti-peptide Vκ (gb: AF321948) and differs by 1 nucleotide from an anti-DNA Vκ (gb: Z22118) both from NZB x NZW_{F1} mice. Earlier work in our lab has demonstrated substantial (>99%) identity between spontaneous mAb generated from NOD and NZB hybridomas (350). In 1988, D'Hoostelaere and coworkers (346) used RFLP analysis to deduce that NZB/BINJ mice have the Igκ b haplotype whereas C3H, BALB/c,

and C57BL/6 all share the c haplotype. Although NOD was not included in that study, our data strongly support the hypothesis that NOD and NZB share the Igκ b haplotype. This is not the case for IgH, as studies clearly indicate that the IgH loci of NOD and C57BL/6 mice are highly similar if not identical (351, 352). Interestingly, in the D'Hoostelaere study, NZB was the only strain of the 55 investigated that exhibited the b haplotype. This observation raises the possibility that the Igκ b allelic group may predispose susceptible strains to autoreactivity. A number of genetic features are shared by systemic and organ specific autoimmune diseases (353) and our findings suggest that the Igκ b haplotype may be included among these. It is of note that two diabetes susceptibility loci, *Idd6* and *Idd19*, map to chromosome 6 distal to the Igκ locus, but the relationship of these loci to the b haplotype is not known.

The finding that V_H125Tg/NOD, and not V_H125Tg/C57BL/6 mice, have anti-insulin B cell populations in both their immature bone marrow and splenic repertoires suggests intrinsic differences in B cell generation between NOD and C57BL/6 strains. Since the polymorphisms observed in NOD Vκ alleles encode alterations in primary structure, it is possible that these structures may skew the repertoire of antigen recognized by NOD B cells. However, the original partner of V_H125 is a member of the largest family, Vκ4, recombined with Jκ5 (344). The Vκ4-Jκ5 configuration is considered an indicator of receptor editing (354, 355). It is possible that Vκ1 and Vκ9 genes are rapidly edited in V_H125Tg/C57BL/6 mice and that this process is less efficient in NOD. Since antigen driven receptor editing takes place chiefly at the IgL loci, it is also possible that polymorphisms in NOD Vκ alleles extend to regulatory elements that impact IgL replacement. Recent studies using fixed Ig Tg also reveal a selection defect in NOD mice

that permits autoreactive B cells to enter the peripheral repertoire (329). Thus, the Vκ haplotype may synergize with other defects in NOD B cell generation (356, 357) resulting in the maintenance of autoreactive specificities in the repertoire. Further studies confirming the Vκ haplotype of NOD as well as directly assessing the role of the b haplotype in predisposition to autoimmunity are clearly required.

CHAPTER III

MARGINAL ZONE B CELL DEFECTS IMPOSED BY NOTCH2 HAPLOINSUFFICIENCY ARE AMELIORATED BY AN AUTOREACTIVE B CELL RECEPTOR

Abstract

The marginal zone subset of splenic B cells plays an important role in innate and adaptive immunity. In addition, the marginal zone may contribute to autoimmune disease by serving as a reservoir for autoreactive B cells. This characteristic is exemplified by anti-insulin transgenic (125Tg) B cells which preferentially populate the marginal zone compartment. The Notch family of receptors regulates cell fate decisions in many lineages, and haploinsufficiency of the Notch2 gene ($Notch2^{+/-}$) results in specific marginal zone B cell defects. In this study, we have investigated how B cell specificity impacts marginal zone B cell development in the context of Notch2 haploinsufficiency using mice that harbor anti-hel (naïve) or anti-insulin (autoreactive) B cell receptor transgenes. We find that the anti-insulin B cell receptor is capable of generating marginal zone B cells despite a mutant Notch2 allele whereas the naïve, anti-hel receptor (MD4) fails to restore the marginal zone compartment. The anti-insulin transgene is also associated with increased surface expression of Notch2 and CD9 but not CD1d on rescued marginal zone B cells. Collectively, these data provide in vivo evidence that an anti-insulin B cell receptor can affect marginal zone B cell differentiation despite Notch2 deficiency.

Introduction

The spleen is populated by three mature B cell subsets, follicular (FO), B1, and marginal zone (MZ). In normal C57BL/6 (B6) mice, the MZ subset displays a specific surface phenotype (IgM^{hi}, IgD^{lo}, $CD21^{hi}$, $CD23^{lo}$, $CD1d^{hi}$, $CD9^{hi}$) and represents 5-10% of mature, splenic B cells (18, 225, 358). They reside at the border of the B cell follicle and red pulp between MZ macrophages and metallophilic macrophages ($MOMA-1^{+}$) (199, 200). The B cell receptor (BCR or surface Ig) repertoire of MZ B cells is prone to recognizing bacterial epitopes but is often reactive to autoantigens as well (207, 359-361). Multiple lines of evidence support the segregation of autoreactive B cells into the MZ. Murine models of autoimmune diseases have specific increases in the MZ B cell population including systemic lupus (220-224), type 1 diabetes (218, 219), and Sjögren's Syndrome (239, 244, 245, 362). Some autoreactive BCR Tg models undergo receptor editing and allelic inclusion with affected B cells preferentially populating the MZ (194, 215). In these models, the autoreactive BCR is diluted by a second light (L) chain that allows the B cell to survive in the peripheral repertoire (209, 363, 364). Combined with the observation that certain BCR specificities are preferentially selected into the MZ B cell population (e.g. 81X and M167 (207, 215)), these findings indicate that the BCR contributes to the genesis of MZ B cells.

Deletion of the transcriptional co-activator, Notch2, results in specific defects in MZ B cell maturation. Hamada et al. generated Notch2 mutant mice (*Notch2*$^{+/-}$ or $N2^{+/-}$) in which the cytoplasmic ankyrin repeats are replaced with β-galactosidase (123). The targeted allele encodes an intact extracellular portion, but cannot signal due to the disrupted intracellular domain. In the homozygous state (*Notch2*$^{-/-}$), this mutation is embryonic lethal. However, heterozygous, *Notch2*$^{+/-}$, mice exhibit specific defects in MZ B cell generation (122). Conditional Notch2 deletion, specific to the B cell lineage, recapitulates the findings

of the mutant model and illustrates that the requirement for Notch2 in MZ B cell development is B cell intrinsic (121). In both models, B cell maturation is halted at the transitional 2–marginal zone precursor stage. Real time PCR analysis illustrates that the differential requirement for Notch2 by MZ B cells is likely post-transcriptional as follicular (FO) and MZ B cells exhibit similar levels of Notch2 mRNA (121). Additionally, studies in C. elegans demonstrate translational control of Notch mRNA by specific RNA binding proteins that interact with the 3' untranslated region (66). Studies such as these illustrate the complexity of the Notch-Notch ligand system.

Mice bearing heavy (H) and L chain Ig transgenes (Tg) reactive to the protein hormone, insulin (125Tg), provide a unique opportunity to investigate several aspects of MZ B cells. While other Tg models also augment the MZ compartment, those models express only an Ig H chain Tg that combines with endogenous Ig L chains to generate a diverse repertoire. In contrast to these, the 125Tg BCR encodes both Ig H and L chains generating a monospecific B cell population with a uniform affinity for cognate antigen. Unlike other commonly studied antigens recognized by MZ B cells, such as DNA and phosphorylcholine, insulin is a small globular protein that lacks nucleotide, lipid, and carbohydrate moieties (365-367). Thus, there should be no interaction with toll-like receptors, and single insulin molecules are not expected to extensively crosslink BCR. The concentration of insulin in the serum is metabolically regulated such that the presence of the Tg does alter antigen availability. Additionally, insulin is recognized as a tissue-specific autoantigen involved in type 1 diabetes pathogenesis (368-372), and MZ B cells play an important role in the non-obese diabetic (NOD) murine model of this disease (234). All of these factors emphasize the uniqueness of the 125Tg, anti-insulin BCR model.

Autoreactive specificities are selected into the MZ compartment, but can they bypass the block to MZ B cell differentiation in *Notch2*$^{+/-}$ mice? In this report, we have investigated whether the anti-insulin BCR can generate MZ B cells in *Notch2*$^{+/-}$ mice. We report that the anti-insulin, 125Tg, BCR can ameliorate MZ B cell deficiency in Notch2$^{+/-}$ animals. In contrast, the naïve, anti-hel, BCR is not capable of generating MZ B cells in *Notch2*$^{+/-}$ mice. Furthermore, only MZ B cells from 125Tg/*Notch2*$^{+/-}$ mice exhibit a specific increase of surface Notch2 protein. These data support the hypothesis that in vivo BCR engagement is vital during MZ B cell differentiation despite Notch2 haploinsufficiency that would otherwise impair MZ B cell development.

Materials and Methods

Mice

All mice have been backcrossed onto the B6 background for >20 generations. Anti-insulin, 125Tg, mice were generated in our lab as described previously (299). Notch2$^{+/-}$ (*Notch2*$^{+/-}$ or N2$^{+/-}$) mice were a gift of C. Klug, University of Alabama at Birmingham (generated by Hamada *et al.* (123)). MD4 (anti-hel Tg) mice (22) were obtained from The Jackson Laboratory (Bar Harbor, ME). Anti-insulin 125Tg, anti-hel Tg, and Notch2$^{+/-}$ genotyping was performed on tail biopsy DNA as previously described. The six genotypes of mice used in this paper are abbreviated as follows. C57BL/6 mice are non-transgenic (nonTg B6), anti-hel (helTg/B6), or anti-insulin (125Tg/B6). Notch2 haploinsufficient mice (*Notch2*$^{+/-}$ or N2$^{+/-}$) are either non-transgenic (nonTg *Notch2*$^{+/-}$), anti-hel (helTg/*Notch2*$^{+/-}$), or anti-insulin (125Tg/*Notch2*$^{+/-}$). All procedures were approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

Flow Cytometry

Single cell suspensions were made from spleens using a 70 μm screen (Fisher Scientific) and tris ammonium chloride to lyse red blood cells. Cells were stained with antibodies reactive to the following molecules: B220 (RA3-6B2), CD19 (1D3), IgMa (DS-1), IgMb (AF6-78), CD21 (7G6), CD23 (B3B4), CD24 (M1/69), CD9 (KMC8), CD1d (1B1) (BD PharMingen, San Diego, CA), Notch2 (extracellular sc-5545, Santa Cruz Biotechnology, Santa Cruz, CA), and donkey-anti-rabbit-PE (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA). Insulin specificity was assessed by binding to biotinylated human insulin (50ng/ml, NOVO) (299). Antibodies that were conjugated to biotin were revealed by counter staining with streptavidin-PerCP or streptavidin-APC (BD PharMingen). Data was collected on a 4-color FACSCalibur flow cytometer (BD Biosciences). All plots were gated for live, $B220^+$ lymphocytes. In figure 3-6, CD1d and CD9 levels were normalized to the MFI of B220 in the same fluorochrome on aliquots of the same cells. Post analysis was conducted with WinMDI2.8 (J. Trotter, Scripps Institute, San Diego, CA).

Statistical Analysis

All pair wise comparisons were made using Student's t-test accommodating 2-tailed outcomes and heteroscedastic variance using SPSS software (Systat Software Inc., Point Richmond, CA). p values of ≤ 0.05 were considered statistically significant.

Immunohistochemistry

Spleens were perfused with 30% sucrose for ≥ 24 h prior to immobilization in OCT Compound (Sakura, Torrance, CA) on dry ice. Eight μm sections were cut on a Leica CM1850 cyrostat (Leica Microsystems, Nussloch, Germany) and adhered to Superfrost/Plus

slides (Fisher Scientific, Pittsburgh, PA). After fixation in 1% paraformaldehyde for 5 min, slides were blocked with 5% normal goat serum in 1% BSA for ≥ 30 min. Slides were serially stained with anti-MOMA-1 (CL89149 Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) and goat-anti-rat IgG-Texas Red (Southern Biotechnology Associates, Birmingham, AL) for 1 h each followed by another fixation step in 1% paraformaldehyde. B cells were stained with anti-B220-FITC (RA3-6B2) for 1 h. Finished slides were mounted with Dako Cytomation Fluorescent Mounting Medium (Dako Cytomation Inc., Carpinteria, CA) and premium cover glasses (Fisher Scientific) and visualized on an Olympus BX60 (Olympus America Inc., Melville, NY). All pictures were taken on a 10X objective (100X total magnification) using Magnafire Software (Optronics, Goleta, CA).

Results and Discussion

Anti-insulin, 125Tg, B cells are preferentially diverted into the marginal zone.

Anti-insulin, 125Tg mice (299) were used to investigate the development of MZ B cells specific for a physiologically regulated protein. Splenocytes from nonTg and 125Tg C57BL/6 (B6) mice were analyzed by FACS using Ab specific for CD21, CD23, and B220 to define three B cell ($B220^{+}$) populations: MZ ($CD21^{hi}$, $CD23^{lo/neg}$), FO ($CD21^{lo}$, $CD23^{hi}$), and T1 ($CD21^{neg}$, $CD23^{neg}$). Adult nonTg B6 mice exhibit a normal MZ B cell compartment as expected (figure 3-1, panel A, 15 wk B6 – 10.8%). In contrast, adult 125Tg/B6 mice exhibit a markedly increased MZ B cell population at the same age (figure 3-1, panel B, 15 wk 125Tg/B6 – 36.7%).

The anti-insulin specificity also accelerates MZ B cell maturation at 4 wk of age, a time at which the MZ B cell compartment is not fully developed (figure 3-1, panel C, 4 wk

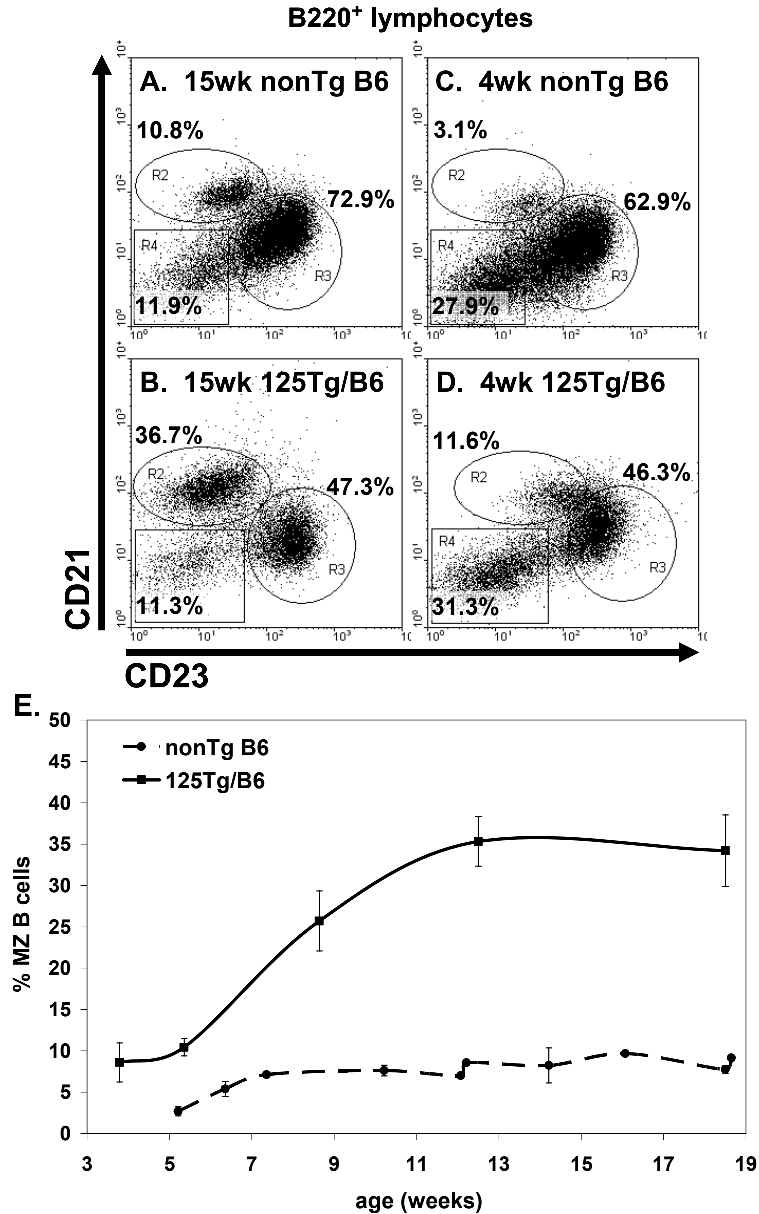


Figure 3-1. The MZ B cell population is augmented in anti-insulin, 125Tg, mice. Splenocytes from 15 wk old (A and B) and 4 wk old (C and D) mice with (B and D) and without (A and C) 125Tgs were stained with Ab to B220, CD21, and CD23 and analyzed by FACS. Dot plots are gated on live, $B220^{+}$ lymphocytes. B cells subsets are gated as MZ ($CD21^{hi}$, $CD23^{lo}$ – R2), FO ($CD21^{lo}$, $CD23^{hi}$ – R3) and T1 ($CD21^{neg}$, $CD23^{neg}$ – R4). (E) NonTg B6 (circle, dashed line) and 125Tg/B6 (squares, solid line) mice from 3 wk to 20 wk of age were analyzed as in A-D to determine the percentage MZ B cells of the total live, $B220^{+}$ lymphocyte population. Error bars represent SD from the mean (n = 2-15 mice per time point).

nonTg B6 – 3.1% vs. panel D, 4 wk 125Tg/B6 – 11.6%). In light of this acceleration, we assessed the extent of MZ B cell development at various ages post-partum beginning at 3 wk. Panel E of figure 3-1 illustrates the accumulation of MZ B cells over time in both nonTg B6 and 125Tg/B6 mice. In both genotypes, the MZ compartment stabilizes at 12-13 wk of age. When aged further (25-35 wk), MZ B cell percentages can reach up to 50% of $B220^{+}$ lymphocytes in 125Tg/B6 (data not shown). The appearance of MZ B cells as early as 3 wk in 125Tg/B6 mice suggests that mice of this age are not incapable of producing MZ B cells. In a wild type animal, MZ colonization may be the result of a gradual accumulation of appropriate specificities over time. This anti-insulin BCR is one such appropriate specificity.

The anti-insulin BCR restores MZ B cell development in Notch2$^{+/-}$ mice.

In Notch2 haploinsufficient mice (*Notch2*$^{+/-}$ or N2$^{+/-}$), the intracellular ankyrin repeats of one allele are replaced with the β-galactosidase gene rendering that allele non-functional (123). The extracellular domain remains intact, but the mutant protein cannot signal via its intracellular domain. This murine model exhibits specific defects in MZ B cell differentiation (122). As shown in figure 3-1, the MZ B cell compartment is amplified in 125Tg/B6 B cells. This observation led us to question whether the 125Tg could generate MZ B cells in *Notch2*$^{+/-}$ mice. Accordingly, we intercrossed the 125Tg onto the *Notch2*$^{+/-}$ background (125Tg/N2$^{+/-}$, figure 3-2). At 12 wk of age, nonTg *Notch2*$^{+/-}$ mice exhibit the expected, profound reduction in MZ B cells (figure 3-2, panel B, 1.08% and panel G, 1.49 ± 0.47%, n = 10) as compared to nonTg B6 mice (figure 3-2, panel A, 6.01% and panel G, 6.14 ± 1.64%, n = 13). Surprisingly, 125Tg/*Notch2*$^{+/-}$ mice produce a significant population of MZ B cells (figure 3-2, panel D, 11.55% and panel G, 9.28 ± 5.32%, n = 9). The MZ

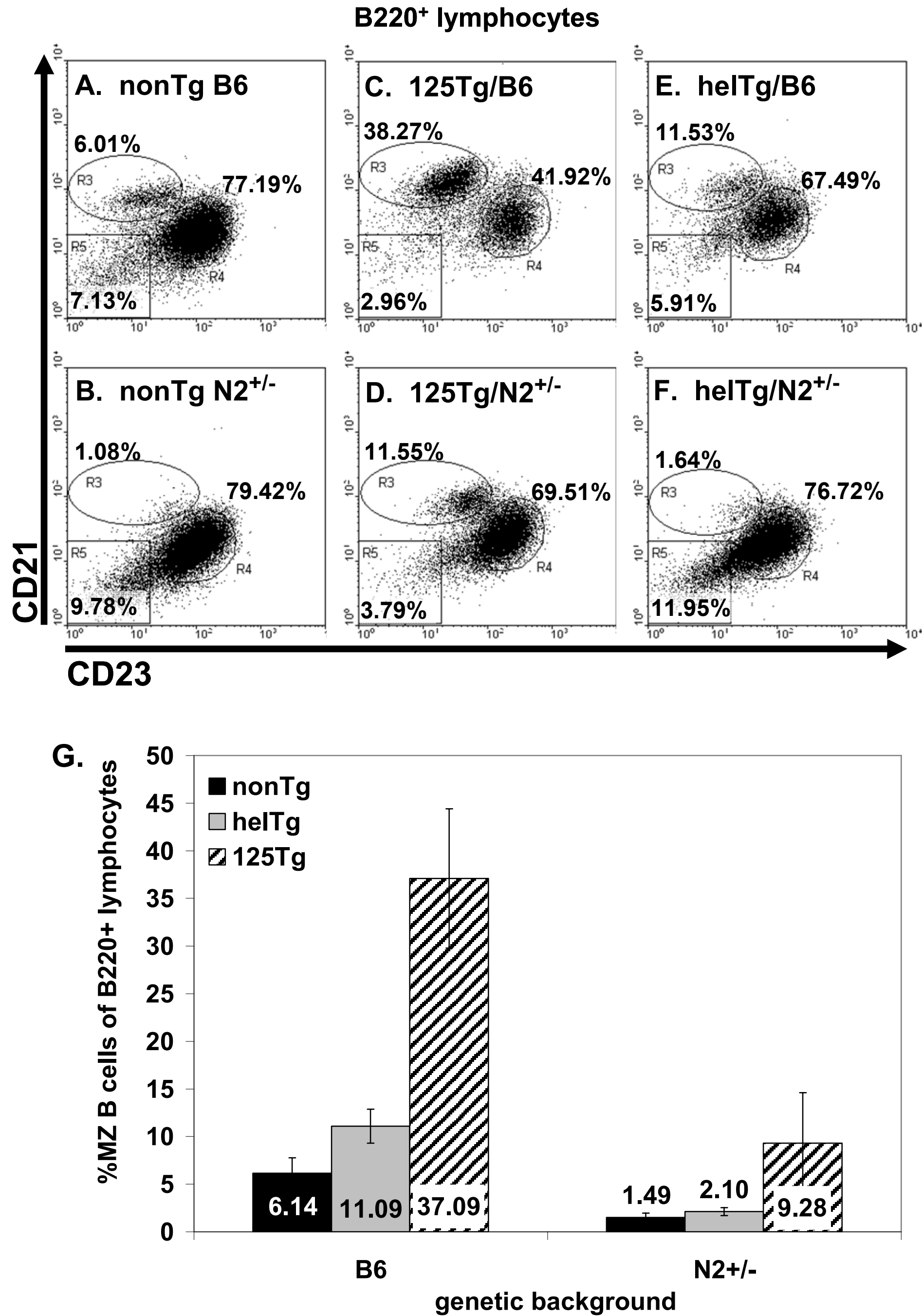


Figure 3-2. The anti-insulin 125Tg potentiates MZ B cell development in *Notch2*$^{+/-}$ mice. Splenocytes from 12-13 wk old mice were analyzed by FACS as in figure 3-1. Panels A, C, and E are representative of B6 mice whereas B, D, and E are *Notch2*$^{+/-}$. NonTg (A and B), anti-insulin (125Tg, C and D), and hel specific B cells (helTg, E and F) are shown. Dot plots and percentages are of live, B220^{+} lymphocytes. The mean percentage of MZ B cells from each genotype ± SD is summarized in panel G ($n \geq 7$ for each genotype, refer to table 3-1).

population in 125Tg/*Notch2*$^{+/-}$ is significantly increased compared to nonTg *Notch2*$^{+/-}$ (p = 0.0023) and does not differ statistically from that of nonTg B6 (p = 0.120, NS). However, the proportion of MZ B cells in 125Tg/*Notch2*$^{+/-}$ mice does not reach that which is seen in 125Tg/B6 (figure 3-2, panel C, 38.27% and panel G, 37.09 ± 7.32%, *n* = 7). These data demonstrate that the 125Tg specificity can generate MZ B cells in *Notch2*$^{+/-}$ mice but that this BCR cannot fully overcome the haploinsufficiency of Notch2. However, the fact that 125Tg/*Notch2*$^{+/-}$ mice are capable of generating MZ B cells at all suggests that the BCR may be able to modulate the requirement for Notch2. Absolute numbers of each B cell subset are discussed below and shown in Table 3-1.

The accumulation of the MZ compartment in 125Tg/B6 and 125Tg/*Notch2*$^{+/-}$ peripheral B cells could be attributable to non-specific Tg effects such as enforced Ig expression. To address this issue, we introgressed the anti-hen egg lysozyme BCR Tg (anti-hel or helTg) onto the *Notch2*$^{+/-}$ background. In a non-hel expressing environment, anti-hel B cells are naïve (22). B6 mice expressing the helTg (helTg/B6 – figure 3-2, panel E, 11.53% and panel G, 11.09 ± 1.78%, *n* = 10) do not accumulate MZ B cells above the range that is seen in nonTg B6 mice (panel A). HelTg/*Notch2*$^{+/-}$ mice (figure 3-2, panel F, 1.64%, and panel G, 2.10 ± 1.42%) are indistinguishable from nonTg *Notch2*$^{+/-}$. HelTg/*Notch2*$^{+/-}$ B cells do not encounter antigen in this model and are not capable of generating MZ B cells. In 125Tg/*Notch2*$^{+/-}$ B cells, *in vivo* BCR engagement by insulin may enable the generation of a B cell subset which otherwise would not develop. To date, no other studies demonstrate the development of MZ B cells in *Notch2*$^{+/-}$ mice. Together, these data suggest that the anti-insulin BCR is capable of facilitating MZ B cell development despite a specific genetic defect.

The anti-insulin, 125Tg, BCR enhances Notch2 expression on MZ B cells.

The Notch2 model used in these studies is heterozygous, maintaining one intact allele. This enables the study of Notch2 protein levels *in vivo*. Accordingly, we analyzed Notch2 surface levels by FACS on splenocytes from both nonTg and 125Tg *Notch2*$^{+/-}$ and B6 mice. Initially, we compared only gated MZ B cells from nonTg and 125Tg B6 and *Notch2*$^{+/-}$ mice. Cells falling into the MZ B cell gate from nonTg *Notch2*$^{+/-}$ (1.49 ± 0.47%) express very low levels of Notch2 surface protein (figure 3-3, panel A, nonTg *Notch2*$^{+/-}$ MFI (mean fluorescence intensity) – 9.98). Alternatively, MZ B cells from nonTg B6 (MFI – 25.94), 125Tg/B6 (MFI – 23.12), and 125Tg/*Notch2*$^{+/-}$ (MFI – 26.70) exhibit similar, elevated, surface levels of Notch2 (figure 3-3, panel A). Thus, the 125Tg BCR is associated with increased Notch2 protein on the *Notch2*$^{+/-}$ background in MZ B cells.

We next compared surface levels of Notch2 on MZ, FO, and T1 subsets of each genotype. In nonTg B6 (figure 3-3, panel B) and 125Tg/*Notch2*$^{+/-}$ (panel D) B cells, only the MZ subset exhibits increased surface Notch2. Conversely, all B cell populations, from nonTg *Notch2*$^{+/-}$ mice exhibit similar, decreased levels of Notch2 (figure 3-3, panel C). Collectively, these results demonstrate that Notch2 is preferentially expressed by MZ B cells and that the 125Tg is associated with increased Notch2 levels specifically on rescued MZ B cells in 125Tg/*Notch2*$^{+/-}$ mice. An important feature of *Notch2*$^{+/-}$ mice is that extracellular measures of Notch2 protein do not differentiate between wild type and mutant (intracellular β-galactosidase) receptors. Accordingly, the increased levels of Notch2 likely represent a mixed population of functional and nonfunctional molecules. This situation potentially explains why the number of MZ B cells in 125Tg/*Notch2*$^{+/-}$ mice does not reach that seen in 125Tg/B6 mice.

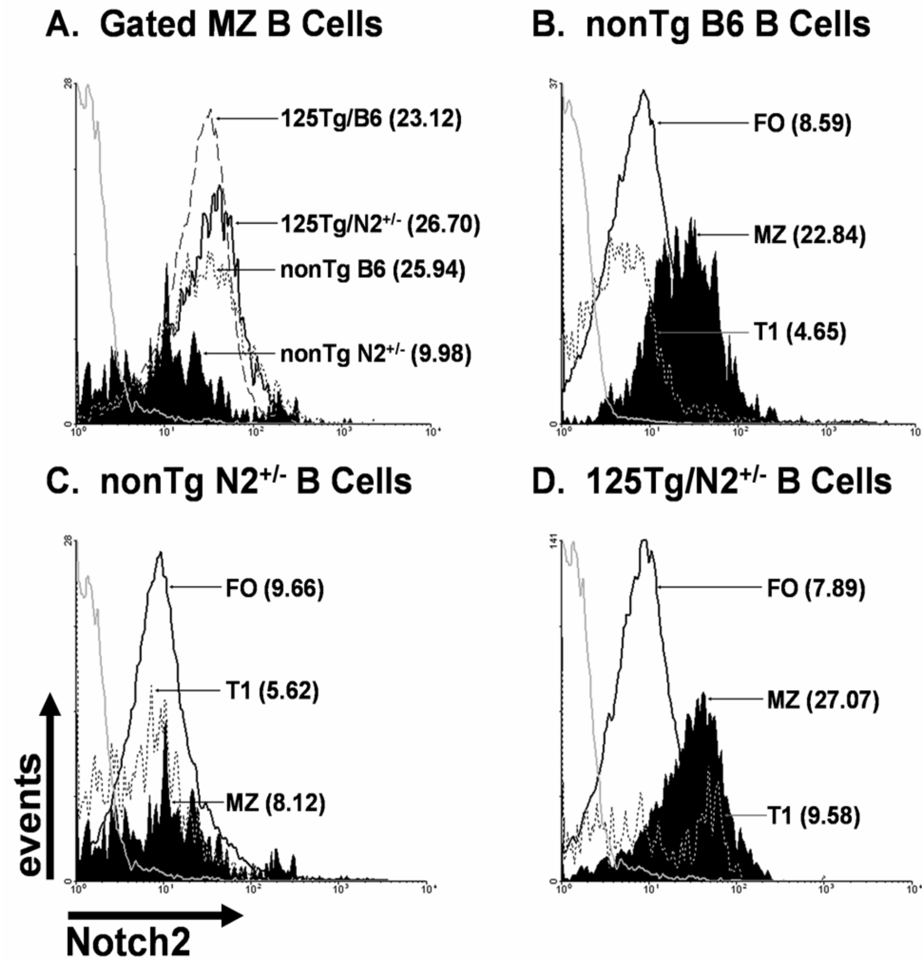


Figure 3-3. The anti-insulin, 125Tg, BCR increases Notch2 expression on MZ B cells from 125Tg/*Notch2*$^{+/-}$ mice. Splenocytes from nonTg and 125Tg B6 and *Notch2*$^{+/-}$ (N2$^{+/-}$) mice were analyzed by FACS using Ab to CD21, CD23, B220, and Notch2 (sc-5545). The MFI of Notch2 on gated MZ B cells is shown in A. MZ, FO, and T1 B cell fractions from nonTg B6 mice (B), nonTg *Notch2*$^{+/-}$ (C), and 125Tg/*Notch2*$^{+/-}$ mice (D) are also shown. The MFI of each population is given in parentheses.

Very little is known about the transcriptional control of the Notch2 gene itself. Previous studies illustrate that wild type MZ and FO B cells possess roughly equivalent levels of Notch2 mRNA (121) and that Notch translation is tightly regulated (66). Thus, differences in Notch2 protein levels at the MZ B cell's surface may be the result of a complex post transcriptional mechanism that may be related to the BCR. Further studies are needed to clarify the mechanisms responsible for Notch2 protein regulation.

MZ B cells generated in 125Tg mice localize to the appropriate splenic microenvironments.

Homing to the splenic marginal sinus is characteristic of mature MZ B cells. This area is demarcated by metallophilic macrophages (MOMA-1^{+}) on the FO side and MZ macrophages (ERTR-9^{+}) on the red pulp face (199, 200). To assess whether MZ B cells, as defined by FACS analysis (figures 3-1 and 3-2), were in fact populating the MZ, we stained spleen cryosections with Ab specific to B cells (B220, green) and metallophilic macrophages (MOMA-1, red – figure 3-4). In this assay, MZ B cells are identified as B220^{+} staining outside the MOMA-1 ring (figure 3-4, arrows – panel A and D, brackets – panel B). As expected, the MZ B cell area is only 1-2 cell layers thick in genotypes exhibiting a normal complement (5-10%) of MZ B cells (arrows – panel A, nonTg B6 and panel D, 125Tg/*Notch2*$^{+/-}$). Patches of B220^{+} cells dispersed among the MOMA-1^{+} cells in the red pulp can also be seen (arrowheads – panel D, 125Tg/*Notch2*$^{+/-}$). These may represent ectopic patches of MZ B cells that mislocalize due to widespread MOMA-1 expression. 125Tg/B6 mice which exhibit 37.09 ± 7.32% MZ B cells by FACS have an observable accumulation of B220^{+} cells in the MZ (brackets – panel B, 125Tg/B6). Finally, nonTg *Notch2*$^{+/-}$ mice having greatly impaired MZ B cell production (panel C, *Notch2*$^{+/-}$) exhibit no discernable B220^{+} population in the MZ. Further FACS analyses of additional markers

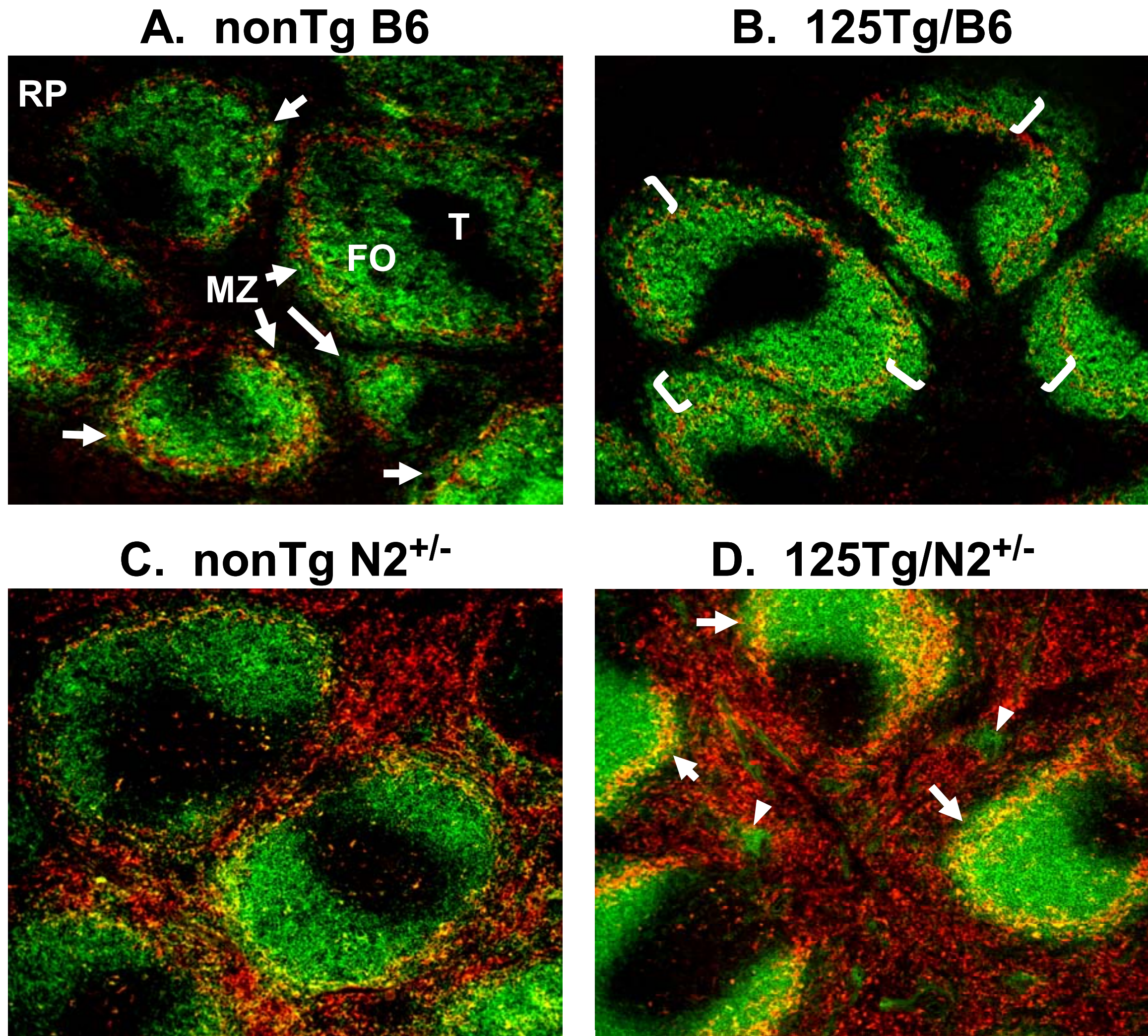


Figure 3-4. Anti-insulin, 125Tg, MZ B cells localize to the appropriate areas in the spleen. Eight μm splenic cryosections were stained with Ab to B220 (green) and MOMA-1 antigen (red). The MZ (arrows – A and D, brackets – B) is represented by the B220^{+} area outside the MOMA-1 ring. The T cell zone (T) and B cell follicle (FO) are representatively labeled in A. Ectopic patches of B cells are indicated by arrowheads in D. total magnification = 100X

including AA4.1, CD24 (data not shown), CD1d, and CD9 (figure 3-6) confirmed that 125Tg B cells from B6 and *Notch2*$^{+/-}$ mice are bona fide, mature MZ B cells and not T2-MZ precursors.

The deregulated MOMA-1 expression in the red pulp of *Notch2*$^{+/-}$ spleens (panels C and D) is consistent with an earlier report (122). Organization of the MZ requires input from both B cell and macrophage populations (373-376). Thus, widespread MOMA-1 staining in *Notch2*$^{+/-}$ spleens could be due to misplaced MOMA-1^{+} macrophages. However, deregulated MOMA-1 expression is observed in 125Tg/*Notch2*$^{+/-}$ (figure 3-4, panel D) despite MZ B cell production. Therefore, Notch2 itself may regulate, either directly or indirectly, the Antigen recognized by the MOMA-1 mAb and this model may reveal additional roles for the MOMA-1 Antigen in MZ organization.

The 125Tgs efficiently allelically exclude and maintain insulin reactivity on the Notch2$^{+/-}$ background.

Many BCR Tg models exhibit a lack of allelic exclusion, particularly in the case of autoreactive specificities. This may result from Ig L chain gene replacement (receptor editing) or inclusion (receptor dilution), Ig H chain gene replacement, or expansion of endogenous B cells in the periphery concomitant with Tg B cell deletion. Edited or allelically included B cells typically accumulate in the MZ (194, 215). To determine if these events were occurring in 125Tg B cells, we stained splenocytes from anti-insulin mice with reagents specific for IgM_a (125Tg allotype), IgM_b (endogenous, B6 allotype) and insulin. Figure 3-5 illustrates that in nonTg B6 mice, ≥ 97.45% of the B cells are IgM_b^{+} and do not cross react with insulin (figure 3-5, panels A and B). In 125Tg mice (B6 – C and D or *Notch2*$^{+/-}$ – E and F), < 5% are endogenous (IgM_b^{+}) B cells (figure 3-5, panels C and E). Whereas, > 96% of the B cells are IgM_a^{+} and bind insulin (figure 3-5, panels D and F –

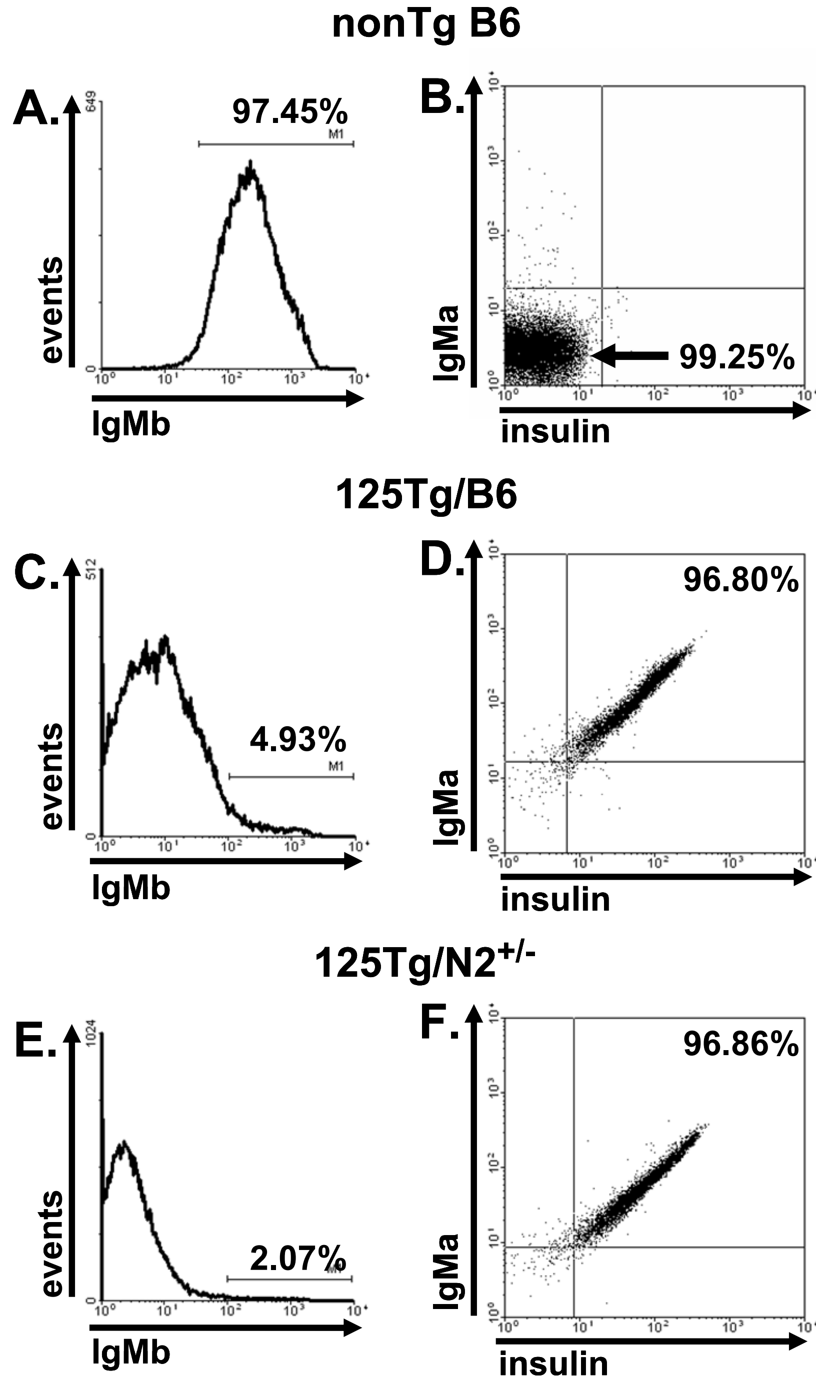


Figure 3-5. The 125Tg enforces allelic exclusion in B6 and *Notch2*$^{+/-}$ B cells. Splenocytes from 12-13 wk old mice were stained with Ab to IgM_a (Tg allotype), IgM_b (endogenous allotype), B220, and biotinylated insulin. Histograms and dot plots are gated on live, B220$^+$ lymphocytes. Panels A, C, and E illustrate the percentage of B cells expressing the endogenous IgM_b allele. Panels B, D, and F depict the percentage of B cells that express the 125Tg (IgM_a) and bind insulin.

diagonal, 1:1 relationship). These data confirm that expression of the 125Tg are maintained in peripheral, mature B cells and that Notch2 haploinsufficiency does not alter allelic exclusion or B cell specificity.

Lymphopenia is not a direct cause of MZ B cell accumulation in 125Tg animals.

A B cell lymphopenic environment has been proposed to cause a preferential accumulation of MZ B cells (225). As most BCR Tg models exhibit some degree of lymphopenia, we analyzed the absolute numbers of total B cells in nonTg, 125Tg and helTg B6 and *Notch2*$^{+/-}$ mice. At 12 wk of age, both 125Tg and helTg mice, are B lymphopenic compared to their nonTg counterparts (Table 3-1). Either Tg results in decreased total B cell numbers to 30-39% of the nonTg controls. These findings are consistent with observations of reduced B cell production in Tg models in general. Absolute numbers of MZ, FO and T1 B cells from nonTg, helTg, and 125Tg mice are detailed in Table 3-1. The number of MZ B cells is either unchanged or significantly decreased in helTg mice compared to nonTg controls (nonTg B6, $2.16 \times 10^6 \pm 0.76$ vs. helTg/B6, $1.72 \times 10^6 \pm 0.83$ – $p = 0.215$; nonTg *Notch2*$^{+/-}$, $0.55 \times 10^6 \pm 0.23$ vs. helTg/*Notch2*$^{+/-}$, $0.30 \times 10^6 \pm 0.13$ – $p = 0.011$) indicating that a lack of antigen engagement does not favor MZ B cell differentiation. Despite overall lymphopenia, MZ B cell augmentation only occurs in 125Tg mice (nonTg B6, $2.16 \times 10^6 \pm 0.76$ vs. 125Tg/B6, $4.86 \times 10^6 \pm 0.78$ – $p = 0.018$; nonTg *Notch2*$^{+/-}$, $0.55 \times 10^6 \pm 0.23$ vs. 125Tg/*Notch2*$^{+/-}$, $1.12 \times 10^6 \pm 0.60$ – $p = 0.022$). Thus, lymphopenia alone does not account for the specific increase in MZ B cells in 125Tg mice.

Table 3-1. Absolute Numbers of Total B Cells and B Cell Subsets

		absolute number of cells (x 10^6 ± SD)			
genotype	***n***	**total B cells**	**MZ B cells**	**FO B cells**	**T1 B cells**
B6	13	36.45 ± 13.21	2.16 ± 0.76	28.65 ± 10.38	2.91 ± 1.38
Notch2$^{+/-}$	10	36.33 ± 13.10	0.55 ± 0.23	30.50 ± 10.67	3.27 ± 1.73
helTg/B6	10	13.50 ± 7.16	1.72 ± 0.83	9.19 ± 4.76	1.48 ± 0.87
helTg/Notch2$^{+/-}$	10	13.95 ± 3.76	0.30 ± 0.13	11.12 ± 3.12	1.70 ± 0.54
125Tg/B6	7	14.09 ± 4.66	4.86 ± 0.78	6.38 ± 1.65	1.08 ± 0.49
125Tg/Notch2$^{+/-}$	9	12.69 ± 4.14	1.12 ± 0.60	9.71 ± 3.68	0.62 ± 0.34

CD9 and CD1d are differentially regulated by the 125Tg and Notch2.

Fully mature MZ B cells express other surface proteins that mediate their functions and anatomical location. These include CD1d and CD9. CD1d is a non-classical MHC class I-like molecule which presents lipid Antigen to iNKT cells (377-379). CD9 is a tetraspanin glycoprotein that organizes cell surface proteins for multiple purposes (380-383). As both proteins are specifically up regulated by MZ B cells (134, 384-386), we analyzed their expression by FACS on splenocytes isolated from helTg, 125Tg, and nonTg mice. In all cases, the levels of CD1d and CD9 were higher on MZ B cells than on FO and T1 B cells from the same spleen, consistent with their status as MZ B cell markers (data not shown). Therefore, we examined CD1d and CD9 levels specifically on MZ B cell populations (figure 3-6, Methods). B cells falling into the MZ gate from nonTg *Notch2*$^{+/-}$ mice (1.49 ± 0.47% of B220^{+} lymphocytes) exhibit lower levels of CD1d (figure 3-6, panel A, *Notch2*$^{+/-}$ MFI = 34.12) upon comparison with nonTg B6 MZ B cells (figure 3-6, panel A, B6 MFI = 70.60). While the level of CD1d is slightly increased on 125Tg/*Notch2*$^{+/-}$ MZ B cells (figure 3-6, panel A, 125Tg/*Notch2*$^{+/-}$ MFI = 57.41), it does not reach the level expressed on 125Tg/B6 MZ B cells (figure 3-6, panel A, 125Tg/B6 MFI = 105.51). When the raw MFI of CD1d on each MZ B cell population is normalized to the level of B220 on the same cells, neither the anti-insulin nor anti-hel Tg significantly alters CD1d expression (figure 3-6, panel B). Additionally, MZ B cells from all lines of *Notch2*$^{+/-}$ mice expressed significantly decreased levels of CD1d as compared to all lines of B6 mice ($p \leq 0.037$). This suggests that CD1d expression is influenced, at least in part, by events downstream of Notch2 activation and that these BCR Tg have little effect on CD1d expression.

The regulation of CD9 appeared more complex. The anti-insulin specificity was consistently associated with increased expression of CD9 on MZ B cells (figure 3-6, panel C,

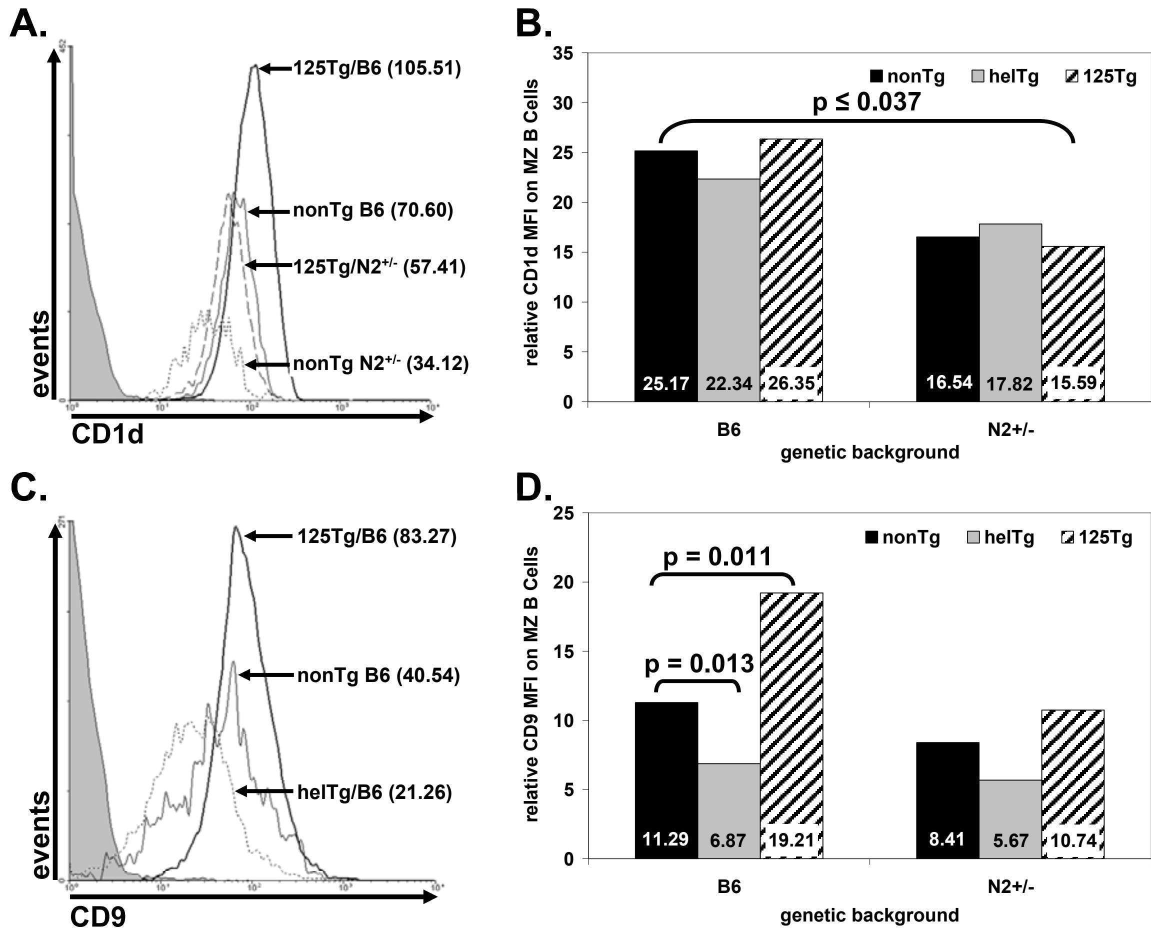


Figure 3-6. CD1d and CD9 are differentially regulated by BCR and Notch2. Splenocytes were analyzed as in figure 3-1 with the addition of either CD1d or CD9 mAb. The MFI of CD1d and CD9 on MZ B cells is depicted in raw form (histograms, A and C, MFI in parentheses) or as a normalized mean (B and D – normalized MFI = MFI CD1d or CD9/MFI B220). Sample size for each group is noted in table 3-1. Significantly different population means (B and D) were determined using Student's t-test (2-tailed, heteroscedastic variance).

125Tg/B6 MFI = 83.27 and panel D, nonTg B6 vs. 125Tg/B6 p = 0.011) while the anti-hel BCR was associated with decreased CD9 expression (figure 3-6, panel C, helTg/B6 MFI = 21.26 and panel D, nonTg B6 vs. helTg/B6 p = 0.013). A similar trend is observed in *Notch2*$^{+/-}$ mice even though all *Notch2*$^{+/-}$ animals exhibit lower CD9 levels. These data suggest that CD9 expression is influenced by BCR but only when Notch2 signaling is intact.

Concluding Remarks

This study reveals potential interactions between the BCR and Notch2 pathways. The anti-insulin BCR is capable of generating a significant population of MZ B cells, despite a mutant Notch2 allele, whereas the naïve, anti-hel BCR cannot. The impact of the mutant Notch2 protein is still evident because the number and percentage of MZ B cells in 125Tg/*Notch2*$^{+/-}$ spleens does not equal that which is seen in 125Tg/B6. At least two issues potentially explain this incomplete rescue. First, insulin concentrations fluctuate in response to physiologic demands such that antigen encounter by individual B cells is variable. Second, *Notch2*$^{+/-}$ animals possess one mutant (intracellular β-galactosidase) and one wild type allele. Therefore, despite similar surface levels of Notch2 protein in 125Tg/*Notch2*$^{+/-}$ and control MZ B cells, the intracellular portion of the molecule may be defective. Thus, these studies reiterate the importance of Notch2 in MZ B cell development and establish the potential for certain BCR specificities to partially correct Notch2 deficiency. Data showing differential expression of CD1d and CD9 support a paradigm in which MZ B cell phenotype is modulated by inputs from multiple receptors. That is, in *Notch2*$^{+/-}$ mice, the 125Tg BCR cannot rescue CD1d expression but does affect CD9 levels. Clearly, a balance of signals delivered through the BCR and Notch2 exists. In this study, the 125Tg tips that balance in favor of MZ B cell production. Further studies are required to define the molecular contacts

between the BCR and Notch2 pathways to thoroughly define how B cell specificity can ameliorate Notch2 deficiency.

CHAPTER IV

THE TRANSCRIPTIONAL PROFILE OF 125Tg MARGINAL ZONE B CELLS: ZINC FINGER PROTEIN 532

Abstract

Marginal zone (MZ) B cells are a unique subset that is phenotypically and anatomically positioned to initiate rapid immune responses to blood borne pathogens. This population is also recognized as a reservoir for autoreactive B cells that survive negative selection and are maintained in the peripheral repertoire. While an absence of the MZ subset in gene targeted models infers a specific requirement for the ablated genes in question, little data exist on gene usage in extant MZ B cells. Previously we have demonstrated that anti-insulin, 125Tg, B cells are targeted to the MZ in increased numbers. These B cells are unique in that they are globally anergic to stimulation through the B cell receptor (anti-IgM or insulin), TLR4 (LPS) and CD40. Thus, mature autoreactive MZ B cells can be subjected to mechanisms of functional inactivation. To identify known and novel genes important to MZ B cell maintenance and anergy, we have explored global gene expression in anti-insulin, 125Tg, B cells using microarray technology. Collectively, these experiments **1)** confirm the differential expression of mRNA encoding proteins known to be regulated in MZ B cells, **2)** describe the transcriptome of intact anergic MZ B cells, and **3)** identify a novel gene (ZFP532) that is highly over-expressed by these cells. The data presented here lay the groundwork for further investigations into MZ B cell development and anergy maintenance.

Introduction

Marginal zone (MZ) B cells are a long lived subset of splenic B lymphocytes that exhibit unique phenotypic and functional characteristics. They are distinguished from follicular (FO) B cells by their proximal location to the splenic marginal sinuses and a unique constellation of surface molecules including high levels of complement receptors (CR1/2 or CD21/CD35) and the non-classical MHC-like molecule, CD1d [reviewed in (225) and table 1-1]. Most splenic blood flow empties into the marginal sinuses continuously exposing MZ B cells to blood-borne antigens. This subset of B cells displays a partially activated phenotype that allows them to differentiate rapidly into plasma cells. Additionally, they are highly effective at antigen presentation to $CD4^{+}$ T cells *in vitro* (230).

MZ B cells develop late in ontogeny (4 weeks in rodents and 2 years in humans). This developmental hiatus corresponds to an infant's inability to mount humoral responses to bacterial and viral pathogens. In addition to microbial defense, the MZ is also a reservoir for B cells displaying autoimmune antigen receptor specificities. MZ B cell autoreactivity comes in two varieties. First, weakly cross-reactive specificities that recognize both self and heterologous antigens select cells into the MZ compartment (207, 225). Second, strongly autoreactive specificities that recognize pathological autoantigens are also housed in the MZ. These include cells specific for dsDNA that have undergone receptor editing (214, 215), rheumatoid factors from RA patients (387), precursors of plasma cells in murine lupus (388), and anti-insulin B cells that are permissive of T1DM (218, 311). These observations imply that the MZ compartment integrates rapid immune responses to common pathogens with autoreactivity. Data on how these two seemingly conflicting processes are regulated will fill important gaps in our understanding of the relationship between infection and autoimmune disease progression.

Mechanisms that generate and maintain the MZ B cell phenotype include genetic programs that govern development, such as transcription factor (TF) induction (50, 91, 127, 130, 389, 390), and signals from the microenvironment mediated by integrins, chemokines (391-393), and sphingolipids (394, 395). Among these various inputs, BCR signaling is the major determinant of MZ B cell differentiation [chapter III, (18, 189, 396)]. Signal transduction from most receptors culminates in the induction of certain TF and hence transcriptional profiles (397-400). Thus, we have chosen to focus on these genes as opposed to others required for MZ B cell development. As detailed in chapter III, BCR specificity can partly overcome Notch2 deficiency, which is itself a complex transcriptional regulator. Therefore, the question as to which TF are activated or themselves up-regulated downstream of an autoreactive BCR arises. While gene ablation studies have identified several TF and transcriptional co-activators that control MZ B cell differentiation – Notch2, Aiolos, BOB.1, and NF-κB1 – these models have limited utility in describing the transcriptional cohorts present in intact MZ B cells (reviewed in chapter I). Additionally, autoreactive MZ B cells which are also anergic (125Tg) may utilize different TF than fully functioning MZ B cells (401). It is important to note that, prior to studies conducted on 125Tg anti-insulin B cells, the concept of "anergic MZ B cells" was never discussed because in most models of anergy, B cells are developmentally arrested. Thus, 125Tg MZ B cells uniquely permit the study of differentiation and anergy in this compartment.

Some gene knockout models have proven useful in demonstrating specific transcriptional requirements in MZ B cell maturation. A prime example of this process is Notch2 (reviewed in chapter I). After interaction with the ligands, Delta and Serrate/Jagged (50, 402, 403) the cleaved, intracellular portion of Notch translocates to the nucleus and displaces repressor complexes from RBP-Jκ to activate transcription. Both conditional (121)

and traditional (122) knockouts of Notch2 demonstrate its requirement in MZ B cell development. Other proteins in the Notch2 signaling axis regulate MZ B cell differentiation. Deltex-1 and Numb are cytoplasmic proteins which potentiate and repress Notch2 signaling respectively (72, 76). In the nucleus, another RBP-Jκ binding protein, MINT (Msx2-interacting nuclear target protein), suppresses Notch2 transcriptional activity thereby favoring the development of FO B cells (404). Deltex-1 is specifically up-regulated at the mRNA level in MZ B cells while MINT is decreased (121, 404). There appears to be no statistical difference in Notch2 transcripts between MZ and FO B cells (121). Thus, gene ablation models can generate useful information, but transcriptional profiling of intact MZ B cells may also be a useful starting point for determining which genes are important to MZ B cell development.

Our studies (chapter III) demonstrate that MZ B cells develop in anti-insulin, 125Tg *Notch2* heterozygous (*Notch2*$^{+/-}$) mice. These data suggest that BCR mediated signals can compensate for the developmental defect characteristic of *Notch2*$^{+/-}$ mice. Evidence for an altered signaling program in 125Tg B cells has been previously demonstrated (218, 299, 405). Western blots reveal levels of tyrosine phosphorylation higher than that seen in controls, and as previously mentioned, 125Tg/B6 B cells are functionally anergic to exogenous stimulation through the BCR or TLR4 (218). Anti-insulin B cells also demonstrate altered Ca^{2+} and NF-ATc1 mobilization (405). These phenotypic changes may be due to continual stimulation via insulin *in vivo* (299). Collectively, these data demonstrate that signaling events downstream of the 125Tg antigen receptor are altered when compared to non-transgenic C57BL/6. The effects of antigen receptor signaling are transduced to the nucleus by a specific cohort of TF which mediate gene expression and thus effector function (397-400). We hypothesize that

the TF cohort of autoreactive MZ B cells will be fundamentally different from autoreactive FO B cells as well as WT MZ B cells.

Materials and Methods

Mice

Anti-insulin, 125Tg/C57BL/6 mice have been described previously (311). These mice have been maintained in a heterozygous state by backcrossing to the C57BL/6 background for > 20 generations. All experiments were approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

B Cell Sorting by Flow Cytometry

Splenocytes from 125Tg/C57BL/6 mice were isolated and pooled. Erythrocytes were lysed by incubation with tris-ammonium chloride and T cells were lysed by incubation with anti-Thy1.1 mAb and rabbit complement. The resulting suspension was centrifuged over a Ficoll cushion and the resulting B cell enriched population was stained with mAb specific for B220, CD21, CD23, and CD5. Cells were left unfixed for sorting as paraformaldehyde will crosslink cellular proteins to RNA thereby destroying it for the purposes of microarray (406). Stained cells are sorted in the VAMC Flow Cytometry facility on a FACSaria flow cytometer (Beckton-Dickson). $B220^{+}$ cells exhibiting the characteristic CD21/CD23 profile for MZ ($CD21^{hi}$, $CD23^{lo/neg}$) or FO ($CD21^{lo}$, $CD23^{hi}$) B cells were collected for the microarray. $B220^{neg}$, $CD5^{+}$ lymphocytes (T cells) and $B220^{+}$, $CD21^{neg}$, $CD23^{neg}$ lymphocytes (T1 B cells) were also collected for real time PCR analysis. Samples of the collected cells were re-analyzed for purity by flow cytometry.

Microarray

Total RNA was isolated in a standard protocol using tri-reagent (Molecular Research Center, Inc.). 5μg of total RNA from each population was supplied to the Vanderbilt Microarray Shared Resource (VMSR - Shawn Levy, Director). This core facility carried out labeling, hybridization, and preliminary analysis of the samples according to the protocols available on their website (array.mc.vanderbilt.edu). Labeled RNA samples were hybridized to the Affymetrix, Mouse Genome 430 plus 2.0 array (Santa Clara, CA). Data were analyzed by GCOS software as well as publicly available programs (www.ncbi.nlm.nih.gov, genome.ucsc.edu, smart.embl-heidelberg.de, elm.eu.org, and www.informatics.jax.org). A summary of the microarray data is available at http://array.mc.vanderbilt.edu/project/project_info.vmsr?project_id=vmsr04JWT61.

Generation of First Strand cDNA

For real time and RT-PCR protocols, first strand cDNA was generated from 1-5μg total RNA using Superscript II RT (Invitrogen, Carlsbad, CA) and 0.67 μg oligo-dT primer (Amersham Biosciences, Piscataway, NJ) in a standard cDNA synthesis protocol.

cDNA Cloning and Real Time PCR

All oligonucleotides listed below have T_m between 59-62 ºC (Integrated DNA Technologies, Coralville, IA). Unless otherwise noted, primers were used at a final concentration of 250 nM in a 50 μl total volume containing 5 μl NaCl. PCR reactions were cycled at 94 ºC/1 min, 60 ºC/1 min, and 72 ºC/1 min 35-40 times. PCR products of the appropriate size were gel purified and T/A cloned into pGEM-T easy vectors (pGEM-T Easy Vector System I, Promega, Madison, WI) and sequenced using an Applied Biosystems

3730xl DNA Analyzer (Vanderbilt-Ingram Cancer Center). For real time PCR, 200 ng cDNA per well was combined with the appropriate primers and SYBR green I dye (Applied Biosystems – Molecular Probes, Foster City, CA) according to the manufacturer's instructions (Platinum qPCR Supermix-UDG – Invitrogen, Carlsbad, CA).

HPRT fwd (#240): 5' AGG TTG CAA GCT TGC TGG T 3'

HPRT rev (#241): 5' TGA AGT ACT CAT TAT AGT CAA GGG CA 3'

ZnF BAC 1 fwd (#213): 5' AGG ACG CCA AGG TCA GAC ATC TC 3'

ZnF cDNA 1 fwd (#212): 5' CAG AGC TGA CCC CCA AAC AGG 3'

ZnF cDNA 2 rev (#219): 5' GGT AAC GTG ATC CCT GCG TTG G 3'

ZnF cDNA 2 fwd (#214): 5' CCA ACG CAG GGA TCA CGT TAC C 3'

ZnF BAC 2 rev (#220): 5' TTG AGA AAC TGT GTC AGC CTC TTC TAG AC 3'

ZnF BAC 2 fwd (#215): 5' GTC TAG AAG AGG CTG ACA CAG TTT CTC AA 3'

ZnF BAC/cDNA4 rev (#221): 5' GGC TCC TCC AAC TTC CGC TT 3'

ZnF BAC/cDNA4 fwd (#216): 5' AAG CGG AAG TTG GAG GAG CC 3'

ZnF BAC/cDNA6 rev (#222): 5' GGT TCG AAT TCA GGA ACT GCC GC 3'

ZnF BAC/cDNA6 fwd (#217): 5' GCG GCA GTT CCT GAA TTC GAA CC 3'

ZnFBAC/cDNA7rev (#223): 5'CCA ACT GTG TAG ACA GCA AAG GGT TAA GTC3'

ZnF BAC/cDNA7fwd (#218): 5'GAC TTA ACC CTT TGC TGT CTA CAC AGT TGG3'

ZnF BAC/cDNA8rev (#224): 5' TTG AAT ACA GTC GCG GCG GTG 3'

MZnFox12 10fwd (#248): 5' AAG CTC TCC TCG TGC ATA GCG G 3'

MZnFox12 10 rev (#249): 5' CCG CTA TGC ACG AGG AGA GCT T 3'

MZnFox12 5'UTR fwd (#250): 5' TTG GCT TCA AGA TCC TGG GTA GAG AGG3'

ZFP532 ATG fwd (#264): 5' ATG ACC ATG GGG GAT ATG AAG ACC C 3'

ZFP532 5'ATG fwd (#263): 5' GCA ACT GTG TGA CAG TAA CTG AAC ACT GG 3'

ZFP532 212 rev (#265): 5' CCT GTT TGG GGG TCA GCT CTG 3'

ZFP532 exon 6 rev (#293): 5' TCCTCA GGG CTG GGC TTG T 3'

5' RACE PCR

5' race PCR was accomplished according to the manufacturer's instructions using the GeneRacer PCR kit and the primers listed below (Invitrogen, Carlsbad, CA).

GeneRacer 5' (#231): 5' CGA CTG GAG CAC GGAG GAC ACT GA 3'

REV 5' ZnF Race (#232): 5' TGT GGC GGC ACA GGC TGT GGG AGG AGC TG 3'

FWD 3' ZnF Race (#233): 5' CAG CTC CTC CCA CAG CCT GTG CCG CCA CA 3'

***In situ* Hybridization**

In situ hybridization on spleen sections from WT and 125Tg/C57BL/6 mice was accomplished using either the oligonucleotides detailed below and/or a PCR-generated (primers 218 and 224, above) probe that was either biotinylated (EZ-link Photoactivatable Biotin kit – Pierce, Rockford, IL) or digoxigenin labeled according to the manufacturer's instructions. Hybridization was carried out overnight at 42 ºC. Biotinylated probes were counterstained with streptavidin conjugated digoxigenin and all were developed with BCP substrate.

5'biotin MZnFox12C (#255): 5'/5Bio/GCA CCT TAC ACC GCC GCG ACT GTA TTC3'

5'biotin MZnFox12B (#254): 5'/5Bio/CCC TCT CAA GGT CAC CTG ACT GGA GC 3'

5'biotin MZnFox12A (#253): 5'/5Bio/GGG TTC CTG TTC TGG GTG CTA CTG CC 3'

Targeted ES Cell Validation

Southern Blotting. Genomic DNA from WT and targeted (RRS823) ES clones was prepared by standard phenol-chloroform extraction (S. Pierce) and digested overnight with a combination of SacII and XhoI or SacII and AvrII (5.3 units/μg DNA for each enzyme – New England Biolabs, Beverly, MA). 20 μg of each digest were electrophoresed for 36 h on a 0.8% agarose gel at 35 volts. Blots were hybridized (R. Henry) to a ^{32}P-labeled 1122 bp β-geo specific probe digested from pGTϕLFX (targeting vector from MMRRC) using SacI and XmaI.

RT-PCR of fusion mRNA. Integration of the targeting vector into an intron results in a fusion mRNA consisting of the 5' exon of the targeted gene, the splice acceptor sequence of engrailed 2 and β-galactosidase-neomycin coding sequence. Thus, validation of successful targeting at the RNA level can be accomplished by RT-PCR using a 5' primer specific to the gene of interest (#263, above) and a 3' primer specific to β-geo (#289, below). The expected size of the appropriate PCR product is 501 bp.

β-geo rev (#289): 5' GGA TTC TCC GTG GGA ACA AAC GG 3'

Expression Vector

The complete coding sequence of ZFP532 was T/A cloned from the original sequencing vector into pGEM-T easy using a highly processive and error-proof polymerase (Herculase – Stratagene, La Jolla, CA) and primers 277 and 278 (below). Primer 277 adds a Kozac consensus sequence (underlined, below) 5' of the start codon (bold, below) and primer 278 terminate just 5' of the endogenous stop codon. This insert was subcloned into pcDNA3.1-C-myc/his using the EcoRI and NotI sites from the pGEM-T easy multiple

cloning cassette. The final construct was sequenced and two point mutations were fixed using primers 302-304 detailed below.

Kozac fwd (#277): 5' GCC ACC **ATG** ACC ATG GGG GAT ATG AAG A 3'

no stop codon rev (#278): 5' TTT TTC AGC TGA ACT CAT TCT TTT GGA TTT 3'

EcoRV rev (#304): 5' GTG CTG GAT ATC TGC AGA ATT GCC GCG TG 3'

BbsI fwd (#302): 5' CGG GGC TGG GGA AGA CAG CCA GCA GG 3'

BbsI rev (#303): 5' CCT GCT GGC TGT CTT CCC CAG CCC CG 3'

Retroviral Vector

The coding sequence of ZFP532 and the in-frame myc and his tags were subcloned from the pcDNA3.1 construct into the MSCV-ires-GFP retrovector (gift of M. Boothby, Vanderbilt University) using PmeI and BglII.

Results

125Tg/B6 splenocytes can be sorted by flow cytometry to generate phenotypically homogenous B cell populations.

The microarray and real time PCR data presented in this chapter depend upon highly purified MZ and FO B cell populations. For the microarray, a combination of B cell enrichment and flow cytometry was used (flow cytometric sorting only for real time samples). Briefly, single cell suspensions of splenocytes from 125Tg/B6 animals were made and enriched for B cells by complement mediated T cell lysis (anti-Thy1.1 and guinea pig complement). The resulting sample was centrifuged over a ficoll cushion to remove cell debris, dead cells, and residual erythrocytes. Cells were stained with fluorochrome-conjugated antibodies to B220, CD21, CD23, and CD5. For the microarray, $B220^+$

lymphocytes matching the CD21/CD23 profile of MZ ($CD21^{hi}$, $CD23^{lo}$) or FO ($CD21^{lo}$, $CD23^{hi}$) B cells were collected and re-analyzed for purity. For real time PCR, T1 B cells (live lymphocyte gate, $CD21^{neg}$, $CD23^{neg}$, $B220^{+}$) and T cells (live lymphocyte gate, $CD5^{+}$, $B220^{neg}$) were also collected. As shown in figure 4-1, this protocol generates T1, MZ, FO, and T cell populations of high purity (> 98%). Cells from multiple sorts were combined for the microarray (17.5×10^6 MZ cells and 14.2×10^6 FO cells) and individual sorts ($1\text{-}5 \times 10^6$ cells each) were used for real time analysis. For the microarray, RNA from combined, sorted cells was extracted and DNAse treated (6.4 μg of MZ RNA and 5.0 μg of FO) and transferred to the VMSR microarray core facility for labeling and hybridization to an Affymetrix 430 2.0 mouse gene chip. For the purposes of data analysis, the FO B cell RNA was used as baseline. Thus, all statements noting over expression of a gene by the MZ B cell population are in comparison to the FO sample.

Two cohorts of differentially expressed genes are maintained by 125Tg MZ and FO B cells.

To determine the global gene expression profile of anti-insulin MZ and FO B cells, I used an Affymetrix 430 2.0A/B gene chip that analyzes 45,101 murine genes. Analysis of the hybridization signal of the fluorescently labeled RNA to each probeset allows the user to determine if a particular RNA is present, absent, increased or decreased. "Present" indicates that a particular transcript was detectable in the sample and thus expressed by the cells in question. Present transcripts may be either unchanged, increased or decreased compared to the baseline sample (FO B cells). Probesets that exhibit no hybridization to the labeled transcripts are deemed "absent". It is important to note that absent transcripts may either be undetectable or unexpressed by the cells of interest. In the MZ B cell sample, 16,333 genes (36% of total) were "present" and 1,619 (3.6% of total) of these were increased relative to

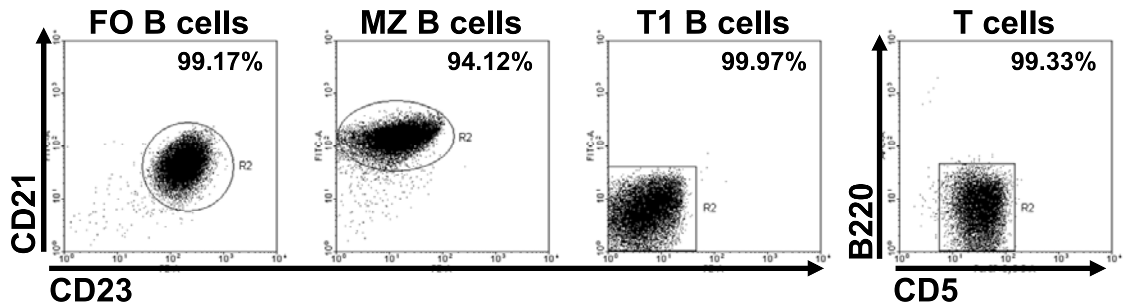


Figure 4-1. 125Tg splenocytes can be sorted by flow cytometry to generate phenotypically homogenous B cell populations. Splenocytes from 125Tg mice were stained with antibodies to CD21, CD23, CD5, and B220 and sorted by flow cytometry. Single, live cells matching the immunophenotypic properties of each population were collected and reanalyzed for purity. All samples exhibited > 94% purity at the time of RNA isolation. These results are typical of those obtained from WT C57BL/6 splenocytes that were used in real time PCR.

the FO B cell sample. Of those, 554 genes (1.2% of total) were increased ≥ 2 fold. Conversely, 17,114 genes (38%) were present in the FO B cell sample and 2,397 (5.3%) of these were over expressed compared to the MZ B cell sample. 574 genes (1.3%) were increased ≥ 2 fold. The study of differentially expressed genes is facilitated by at least a two fold difference between the populations of interest. The expression levels of the 554 and 574 genes meeting this criterion were broken down into fold over expression categories as depicted in figure 4-2. Of the genes differentially expressed at least 2 fold or more, most were in the 2-3.8 fold range for both cell types. Only a very small number of genes (19 in MZ and 5 in FO) were differentially expressed 8 fold or more. It is important to note that a difference less than two fold is difficult to study but may be very biologically important as in the case of Notch2. Collectively, these data indicate that the phenotypic differences between MZ and FO B cells are governed by two sets of differentially expressed genes. The first is a large cohort that exhibits small differences in expression. The second is small group of genes that are greatly differentially expressed.

The microarray data confirm the differential expression of genes known to be regulated in WT C57BL/6 MZ and FO B cells.

The gene expression pattern of anergic MZ B cells likely differs from that of fully functional MZ B cells. Thus, it was important to confirm that certain genes were regulated in 125Tg MZ B cells as would be expected from WT MZ B cells (table 4-1). Several genes that are specific to MZ B cells at the protein level in WT C57BL/6 MZ B cells (CD21 and CD1d) are upregulated at the RNA level. Conversely, genes that are characteristic of FO B cells such as CD62L and CD23 are decreased in the MZ B cell population. While not all differentially expressed proteins can be explained by modulated RNA expression, these data nevertheless serve as a reliable internal control for the microarray itself. Also, these data

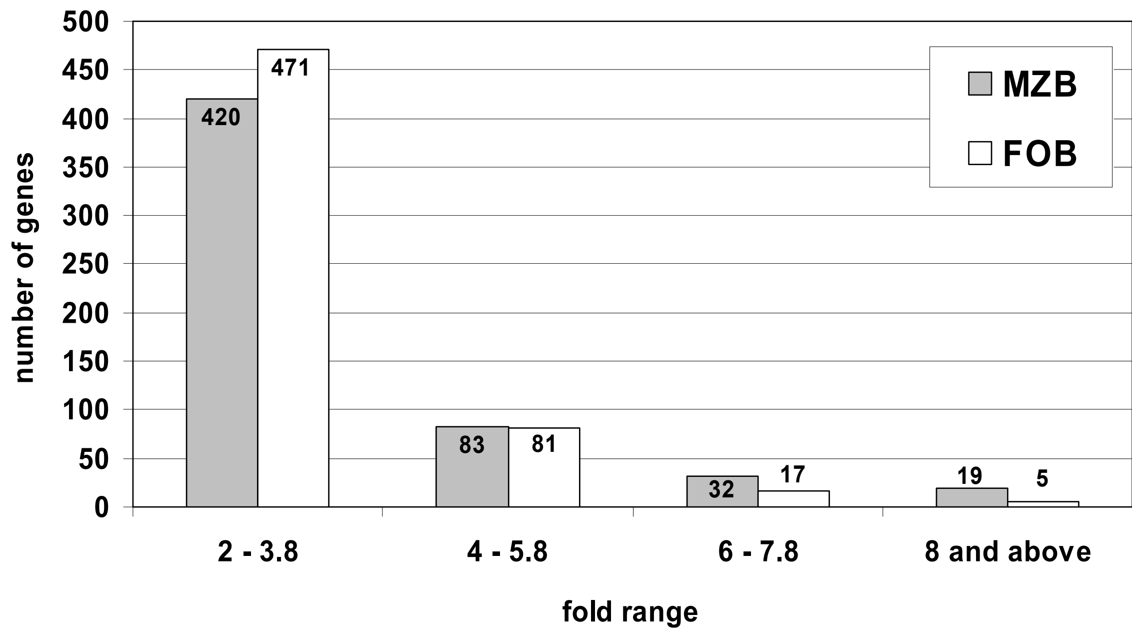


Figure 4-2. A small cohort of genes is over-expressed greater than two fold by 125Tg MZ and FO B cells. 554 and 574 genes were over-expressed at least two fold or greater by the MZ and FO B cell populations, respectively. These genes were delimited into 2 fold increment categories. The majority of over expressed genes fall into the 2-3.8 fold range. The genes over-expressed eight fold or greater (19 MZ and 5 FO) are largely unidentified transcripts.

Table 4-1. Known Marginal Zone B Cell Gene Expression Patterns Confirmed by Microarray Analysis

Increased		Decreased	
FOLD	**GENE**	**FOLD**	**GENE**
2.6	CD86	2	CD62L (LN homing R)
3.4	CD21 (CR2)	2.2	IL4Rα
5.8	Deltex (Notch2 signaling)	5.2	CD93 (Ly68, AA4.1, C1qR)
6.4	CD1d	6.2	CD23 (FcεRII)
9.6	Edg3 (S1P3-R)		

suggest that information gained from 125Tg MZ B cells may provide useful in the study of WT MZ B cells.

Different transcription factors are up-regulated at the RNA level by MZ and FO B cells.

A central hypothesis in the field of mature B cell fate determination centers on BCR signaling as the determining factor of mature B cell phenotype. One major outcome of different receptor signaling pathways is to activate certain TF that ultimately alter gene expression (401, 407, 408). Thus, the microarray data were specifically analyzed for known and putative TF expression levels. Putative TF were defined as having sufficient sequence homology to known TF or specific domains characteristic of TF. As transcriptional co-activators are often as important as the TF itself in determining cell specificity, these were also included. Sixteen TF or transcriptional co-activators were identified as over expressed by MZ B cells and 20 by FO B cells ≥ 2 fold. Tables 4-2 and 4-3 detail these factors as well as their classification and the specific fold expression. Many genes on this array are represented by more than one probeset. Accordingly, some genes were identified multiple times and this is noted under the fold expression column where applicable. This finding also serves as internal validation for the efficacy of the array. Many zinc finger (ZF) containing proteins were identified in both populations. These types of TF are often involved in lineage restriction. Specifically, GKLF, IKLF, and Egr1 are known to regulate fate decisions in other tissues (401, 409-416). Another TF, HES1 is itself a transcriptional target of Notch activation and was specifically up-regulated by MZ B cells (417). The B cell specific functions of most of the TF identified are not known and may be implicated in either cell fate determination and/or anergy maintenance. These data are a useful starting point for

Table 4-2. Known and Putative Transcription Factors and Transcriptional Cofactors Increased in Marginal Zone B Cells

FOLD	GENE	Subfamily	Family	Class	Superclass
2	HES1		Hairy	bHLH	Basic Domains
2	GKLF (KLF4)	Krueppel-like	Developmental and Cell Cycle	C_2H_2 ZnF	Zn Coordinating
2.2	Mlf1			cofactor	
2.2	Ddit3			leucine zipper (bzip)	Basic Domains
2.2, 2.4	Egr1 (Krox20)	Egr/Krox	Developmental and Cell Cycle	C_2H_2 ZnF	Zn Coordinating
2.4	Trps1		GATA factors	diverse cys4 ZnF	Zn Coordinating
2.4	ZFP40 (NTfin12)		Developmental and Cell Cycle	C_2H_2 ZnF	Zn Coordinating
2.4	ZFP73			C_2H_2 ZnF (KRAB)	Zn Coordinating
2.4, 2.6	SpiC		Ets-type	Tryptophan Cluster	Helix-Turn-Helix
2.6	FosB	Fos	AP1-like components	leucine zipper (bzip)	Basic Domains
2.8	Cbfa2t3h		Heteromeric CCAAT factors	Heteromeric CCAAT factors	β-scaffold with minor groove contacts
3.4	Atf3	CRE-BP/ATF	AP1-like components	leucine zipper (bzip)	Basic Domains
3.6	IKLF (KLF5)	Krueppel-like	Developmental and Cell Cycle	C_2H_2 ZnF	Zn Coordinating
4.8	Trim27			ZnF (cofactor)	Zn Coordinating
5.8	Dtx1			ZnF (intermediate)	Zn Coordinating
12	ZFP532			C_2H_2 ZnF	Zn Coordinating

Table 4-3. Known and Putative Transcription Factors and Transcriptional Cofactors Increased in Follicular B Cells

FOLD	GENE	Subfamily	Family	Class	Superclass
2	C/EBP		C/EBP-like factors	leucine zipper (bzip)	Basic Domains
2	ZFP207			C_2H_2 ZnF	Zn Coordinating
2	Mynn (SBBIZ1)			C_2H_2 ZnF (BTB/POZ)	Zn Coordinating
2, 3.4	Crem		CREB	leucine zipper (bzip)	Basic Domains
2.2	Rnf2 (Ring1b)			ZnF (ring)	Zn Coordinating
2.2	ZFP58 (Mfg1)			C_2H_2 ZnF	Zn Coordinating
2.2	Bcl6			C_2H_2 ZnF (BTB/POZ)	Zn Coordinating
2.2	Runx1		Runt	Runt	β-scaffold with minor groove contacts
2.4	Fkh		Developmental Regulators	Forkhead/Winged helix	Helix-Turn-Helix
2.6	Mrg1			Homeo Domain	Helix-Turn-Helix
2.8	Pbx1	PBC	Homeo Domain Only	Homeo Domain	Helix-Turn-Helix
2.8	Mxi1	Mad/Max	Cell-Cycle Controlling factors	Helix loop helix/leucine zipper factors	Basic Domains
2.8	ZFP218			ZnF	Zn Coordinating
3	Fli1		Ets-type	Tryptophan Cluster	Helix-Turn-Helix
3.2, 5	ZFP318 (TZF-L)			C_2H_2 ZnF	Zn Coordinating
3.2	ZFP118 (ZFP53)			C_2H_2 ZnF (KRAB)	Zn Coordinating
3.6	ZFP36l2			C_3H	Zn Coordinating
3.6	Pknox1			Homeo Domain	Helix-Turn-Helix
3.6, 6.4	Maf2	Maf	AP1-like components	leucine zipper (bzip)	Basic Domains
5.4	Bach2	Jun	AP1-like components	leucine zipper (bzip)	Basic Domains

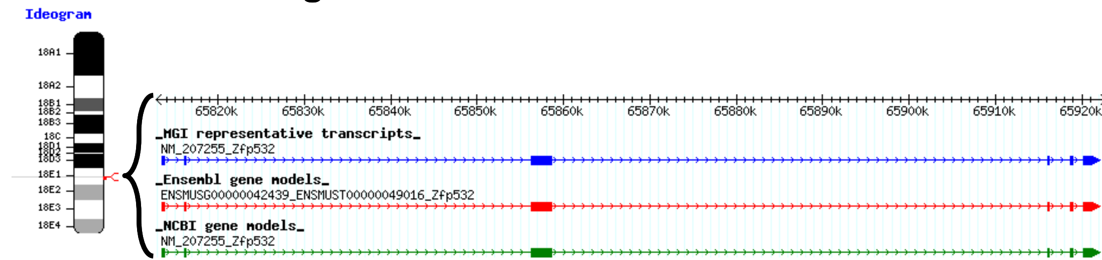
further investigations into the transcriptional regulation of mature B cell development and anergy.

A novel transcription factor is over-expressed 12 fold by 125Tg MZ B cells.

Of striking note in the MZ B cell cohort of genes was a transcript identified as a RIKEN cDNA, C530030I18Rik, which was over-expressed 12 fold when compared to the FO cohort (later termed ZFP532, gene ID: 328977, MGI: 3036282). Three published transcripts were identical to this cDNA – IMAGE: 30618961 (gb: BC094671), IMAGE: 6841127 (gb: BC067032), and IMAGE: 4012958 (gb: BC046409). When analyzed, the cDNA predicted a protein structure encoding ZF domains of the C_2H_2 type. Domains of this kind are typical of TF of the Krueppel ZF family. As Krueppel-like factors are common in binary cell fate decisions, this gene necessitated further characterization.

ZFP532: In silico data – Genomic, Transcript, and Predicted Protein Structures. The entire gene encoding ZFP532 spans 108,703 bp on murine chromosome 18E1 (gb: NT_039674) (figure 4-3, panel A). Comparison of the published cDNA to the genomic sequence indicates eight exons (exon 1: 282 bp, 2: 109 bp, 3: 2352 bp, 4: 113 bp, 5: 167 bp, 6: 777 bp, 7: 112 bp, and 8: 1006 bp). The bulk of the gene is made up of seven introns of greatly divergent sizes (intron 1: 2,776 bp, 2: 40,085 bp, 3: undetermined, 4: 2,517 bp, 5: 1,267 bp, 6: 55 bp, 7: 24 bp). The full length cDNA is 5,028 bp (figure 4-3, panel B). Interestingly, this gene encodes a 405 bp 5' untranslated region (UTR) and a very long 3' UTR of 1,512 bp. A 3' UTR of this size is most likely involved in transcript regulation (stability or degradation) and data regarding this region will be discussed below (418, 419). The remaining 3,111 bp is predicted to encode a protein of 1,036 amino acids or 110.9 KDa. No post-translational modifications are predicted by the primary amino acid structure.

A. Genomic Organization



B. mRNA – 5,028bp

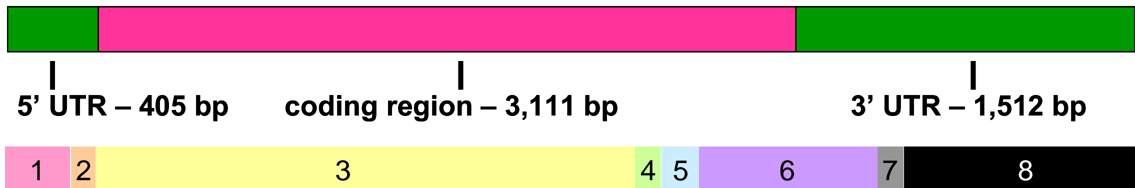


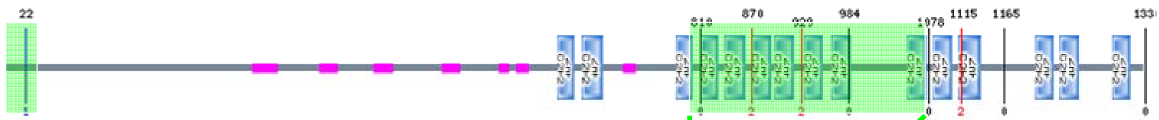
Figure 4-3. ZFP532 is a large gene that likely encodes a zinc finger transcription factor. The position of ZFP532 on murine chromosome 18 and the intron/exon structure of the gene (108,703 bp) are depicted in A. The full length mRNA including 5' and 3' UTR (green) is 5,028 bp (B). The contributions of the eight exons are shown in proportion to the mRNA and predicted protein structure (C). Exons 2-6 contribute to the final protein. Low complexity domains are in pink, and C_2H_2 zinc finger domains are in blue (C).

ZFP532 is expected to have eight C_2H_2 ZF domains and several regions of low complexity (figure 4-3, panel C). No traditional transactivation domains are noted, and thus, ZFP532 might be itself, intrinsically inhibitory. No nuclear localization sequence is indicated. As ZF domains are commonly employed in nucleic acid (DNA and RNA) binding as well as protein-protein interactions, ZFP532 likely homodimerizes with itself or heterodimerizes with other ZF proteins. This is a common characteristic of Krueppel-like TF. Such an interaction could also allow entrance into the nucleus.

An Evolutionary Perspective on ZFP532. Based on sequence homologies to predicted proteins in other species, ZFP532 is conserved in Class Amniota only. There are two orthologs – ZNF532 in *H. sapiens* (gb: NM_018181, protein: Q7L7Z7 – figure 4-4) located on chromosome 18q21.32 and ZNF532 in *R. norvegicus* (gb: NM_225923, protein 1311719) located on chromosome 18q12.1. Only one homolog is predicted in *G. gallus* (gb: XP_424459, locus: 426851) encoded on an undetermined chromosome. A lack of conservation in lower order animals (e.g. *D. melanogaster* and *C. elegans*) likely indicates a specialized function for ZFP532 in higher order organisms. Regulating the differentiation of mature B cell subsets or maintaining anergy maintenance would fit with this evolutionary perspective.

Validation of ZFP532 Expression in Lymphocytes. Before delving further into the biology of ZFP532, I aimed to validate it's over expression by MZ B cells. Primers were designed that spanned the entire published cDNA sequence (see Materials and Methods). These primers were used in a standard semi-quantitative RT-PCR assay on RNA from sorted 125Tg MZ and FO B cells (figure 4-5, panel A). Serial 2-fold dilutions of the cDNA were used as template, such that by this method, ZFP532 appears to be over expressed 8-10 fold by 125Tg MZ B cells. ZFP532 expression was also analyzed by real time PCR on RNA

ZNF532 - human ortholog (Q7L7Z7)
1301 aa
15 - C_2H_2 ZnF



ZFP532 - murine (NP_997138)
1036 aa
8 - C_2H_2 ZnF

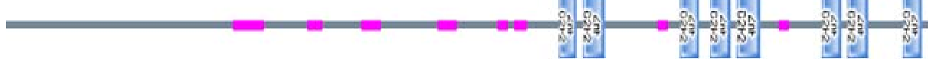


Figure 4-4. Murine ZFP532 has one human ortholog, ZNF532. The predicted protein structures of ZNF532 (human, above) and ZFP532 (murine, below) are shown in proportion to one another. The human protein encodes seven additional C_2H_2 zinc finger domains (blue). The areas not shaded in green exhibit > 80% amino acid identity.

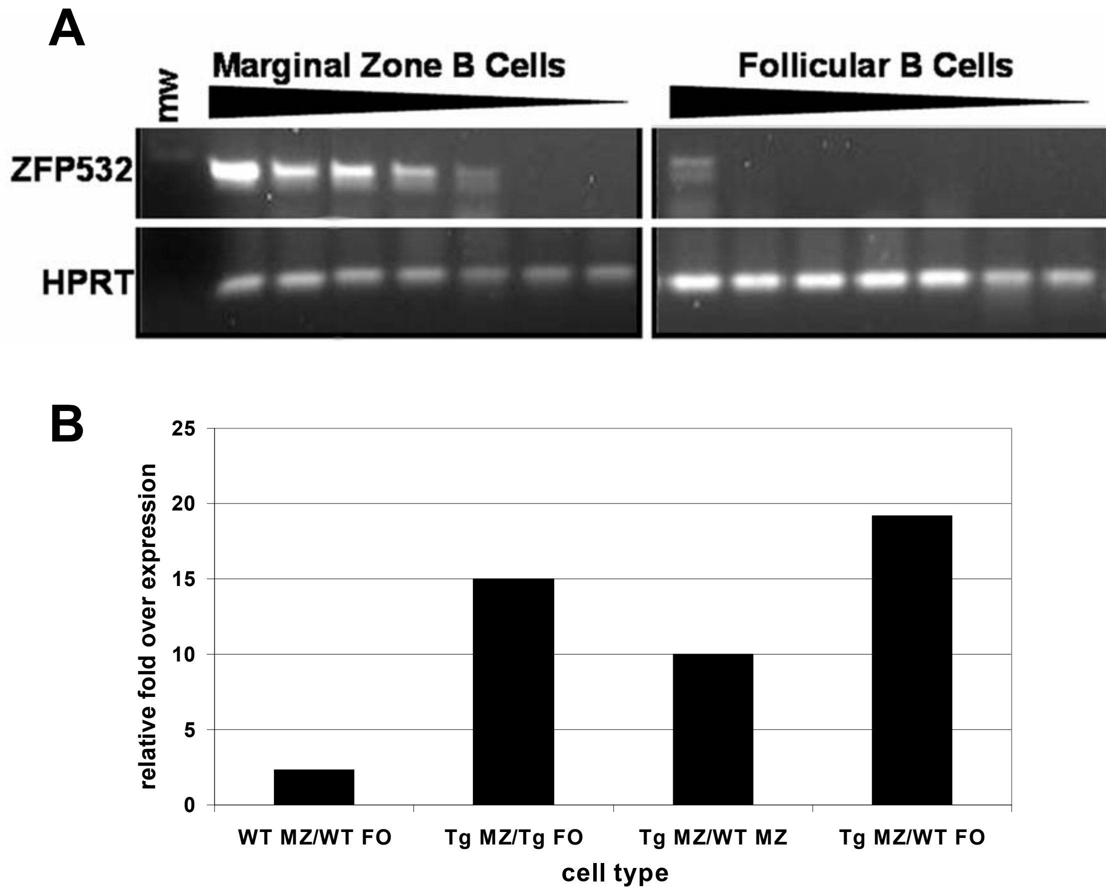


Figure 4-5. ZFP532 over-expression is validated by semi-quantitative RT-PCR and real time PCR. RNA from sorted 125Tg/C57BL/6 MZ and FO B cells was used as template for semi-quantitative RT-PCR employing 2 fold serial dilutions (A). Data are representative of three FACS sorts and four RT-PCR experiments. RNA from sorted MZ, FO, and T1 B cells as well as T cells from 125Tg and WT C57BL/6 mice was used as template in real time PCR (B). Data are representative of four FACS sorts (distinct from those in A) and five to seven individual real time experiments. T1 B cells and T cells do not express ZFP532 transcript above background levels (not shown). Absolute amounts of PCR product vary between experiments such that ZFP532 expression is normalized to a housekeeping gene (HPRT) in each experiment. The values are reported as relative fold over-expression = [cell 1 (pg ZFP532/input)/(pg HPRT/input)]/[cell 2 (pg ZFP532/input)/(pg HPRT/input)].

samples derived from WT and 125Tg MZ, FO, and T1 B cells and T cells. By both RT-PCR and real time PCR, neither T cells nor T1 B cells expressed ZFP532 above background levels (data not shown). The relative levels of ZFP532 transcript among WT and 125Tg MZ and FO B cells are depicted in panel B of figure 4-5. WT FO B cells express the least ZFP532 while 125Tg MZ B cell express the most. Surprisingly, the level of ZFP532 is increased in 125Tg MZ and FO B cells over their WT counterparts, but is always highest in the MZ subset. Expression can be summarized thusly: 125Tg MZ > WT MZ > 125Tg FO > WT FO. This data supports three conclusions. ZFP532 is not expressed by T cells, increases with maturation in B cells (MZ and FO > T1), and increases in anergic cells (125Tg > WT).

ZFP532 Expression Profiling in Other Cell Types. The expression of ZFP532 in other tissues, both primary cells and cell lines, was established using RT-PCR (primary data not shown). The results of these experiments are summarized in table 4-4. Expressed sequence tag (EST) data from the national database (NCBI) have also been compiled and demonstrate a very wide tissue distribution outside the lymphoid lineage. In figure 4-6, relative transcript abundance is indicated by the intensity of the dot. Prior to these studies, ZFP532 transcripts had not been demonstrated in the spleen or in B or T cells. This observation tangentially supports the hypothesis that ZFP532 is upregulated by anergic B cells which would be infrequent in a normal polyclonal repertoire.

Primary, murine B Cells express the full length ZFP532 transcript typical of neuronal tissue.

At the beginning of this study, the only published full length cDNA sequences for ZFP532 were from whole brain (C57BL/6, BC094671) and embryonic spinal cord (C57BL/6, AK083001). A second, shorter transcript had been isolated from a metastasized

Table 4-4. ZFP532 Expression by RT-PCR and Real Time PCR Analysis in Cell Lines and Primary Tissues

Cell Lines

cell line	cell type	ZFP532	# of experiments real time	# of experiments RT-PCR
WEHI-231	murine immature B cell lymphoma	high	6	12
EL4	mature T cell lymphoma	high	0	1
jurkat	human T cell lymphoma	negative	6	3
38-B9	murine pro-B cell	negative	2	3
RAW	macrophage	negative	0	3
S107	plasma cell	negative	0	2
S194	plasma cell	negative	0	2
J558L	plasma cell	negative	0	2
P5424	pro T cell	negative	0	2
LN-EBV	human peripheral B cell, immortalized	negative	0	3
M12	mature B cell lymphoma	negative	0	2
RRS823	ZFP532 targeted ES cells (heterozygous)	positive	4	1
TTS	wild type ES cells	positive	4	1

Primary Cells

tissue	source	ZFP532	# of experiments real time	# of experiments RT-PCR
whole brain	WT/C57BL/6	high	0	6
splenic B cells	WT/C57BL/6	positive	0	7
T1	WT/C57BL/6	negative	7	0
FO	WT/C57BL/6	low	7	0
MZ	WT/C57BL/6	high	7	0
total B cells	NF-κB1$^{-/-}$ C57BL/6	positive	0	3
splenic B cells	125Tg/C57BL/6	positive	1	8
T1	125Tg/C57BL/6	negative	5	0
FO	125Tg/C57BL/6	low	5	5
MZ	125Tg/C57BL/6	high	5	6
total B cells	NF-κB1$^{-/-}$ 125Tg/C57BL/6	positive	0	2
lymph node	WT/C57BL/6	positive	0	2
bone marrow	WT/C57BL/6	positive	0	2
thymus	WT/C57BL/6	positive	0	2
splenic T cells	WT/C57BL/6	negative	7	3
splenic T cells	NF-κB1$^{-/-}$ C57BL/6	positive	0	3
splenic T cells	125Tg/C57BL/6	negative	0	3
splenic T cells	NF-κB1$^{-/-}$ 125Tg/C57BL/6	positive	0	3

Breakdown by Tissue				
Pool name	**transcripts per 10^6**		**gene EST**	**total EST**
bone	77		3	/ 38907
bone marrow	26		1	/ 37513
brain	126		62	/ 488469
colon	0		0	/ 52042
eye	158		27	/ 170141
heart	0		0	/ 53201
kidney	17		2	/ 117081
liver	38		4	/ 104513
lung	0		0	/ 43546
lymph node	39		1	/ 25585
mammary gland	54		19	/ 348702
muscle	0		0	/ 19385
ovary	0		0	/ 14892
pancreas	123		10	/ 81176
placenta	0		0	/ 32705
pituitary g...	22		1	/ 44225
skin	35		3	/ 83617
spleen	0		0	/ 69426
stomach	63		2	/ 31521
testis	9		1	/ 103166
thymus	20		2	/ 99751
uterus	0		0	/ 6585
Breakdown by Developmental Stage				
egg	41		1	/ 23867
pre-implantation	166		26	/ 155959
post-implantation	32		2	/ 61174
mid-gestation	153		65	/ 424206
late-gestation	99		22	/ 220025
neonate	0		0	/ 57255
post natal	43		3	/ 68716
adult	42		36	/ 850802

Figure 4-6. Expressed sequence tag (EST) analysis demonstrates wide tissue distribution for ZFP532 transcripts. These data are summarized from GenBank accessions. Each tissue ("pool name") has a total number of EST associated with it ("total EST"). Of these, a certain number were identical to portions of the full length ZFP532 mRNA ("gene EST"). The proportion of ZFP532 transcripts out of the total for each tissue is indicated graphically by the opacity of the dot. The number of ZFP532 transcripts per million in each tissue is also given.

mammary tumor in another mouse strain (Czech II, BC046409). As depicted in figure 4-6, there was no EST data from whole spleen or purified B cells in the public database. To rule out alternative splicing and determine the transcript structure in B lymphocytes, the entire ZFP532 transcript from both WT/C57BL/6 and 125Tg/C57BL/6 purified B cells was cloned and sequenced. Primers were designed according to the published full-length cDNA (see Materials and Methods) and used to sequence the cDNA from first strand, oligo-dT-primed mRNA. The coding sequence matched the two full length clones previously described in neuronal tissue. However, differences in the 3' UTR of ZFP532 transcript from 125Tg B cells were discovered (figure 4-7). A 35 bp repeat and a GG dinucleotide were found in the 125Tg B cells that matched the previously published mammary tumor sequence but were absent from the WT B cell, brain, and embryonic stem cell sequences. These differences were confirmed in three subsequent cloning experiments. The 35 bp repeat and GG dinucleotide were analyzed by UTRscan (www.ba.itb.cnr.it/BIG/UTRScan). No significant homologies to known regulatory sequences were found. The 3' UTR of many transcripts serves to regulate RNA stability and efficiency of translation. The observation that the anergic, 125Tg, ZFP532 sequence shares 3' UTR motifs with one isolated from a tumor is very intriguing and may require further investigation once the biological role of ZFP532 is determined.

Expression cloning defines the subcellular localization of ZFP532 in fibroblasts.

As ZFP532 is a novel gene, no anti-sera or mAb exist that recognize this protein. To study the subcellular localization of ZFP532, the coding region was cloned by PCR from a previously generated sequencing vector (BC094671, Open Biosystems, Birmingham, AL). A consensus Kozac sequence was added 5' of the ATG start codon and the entire coding

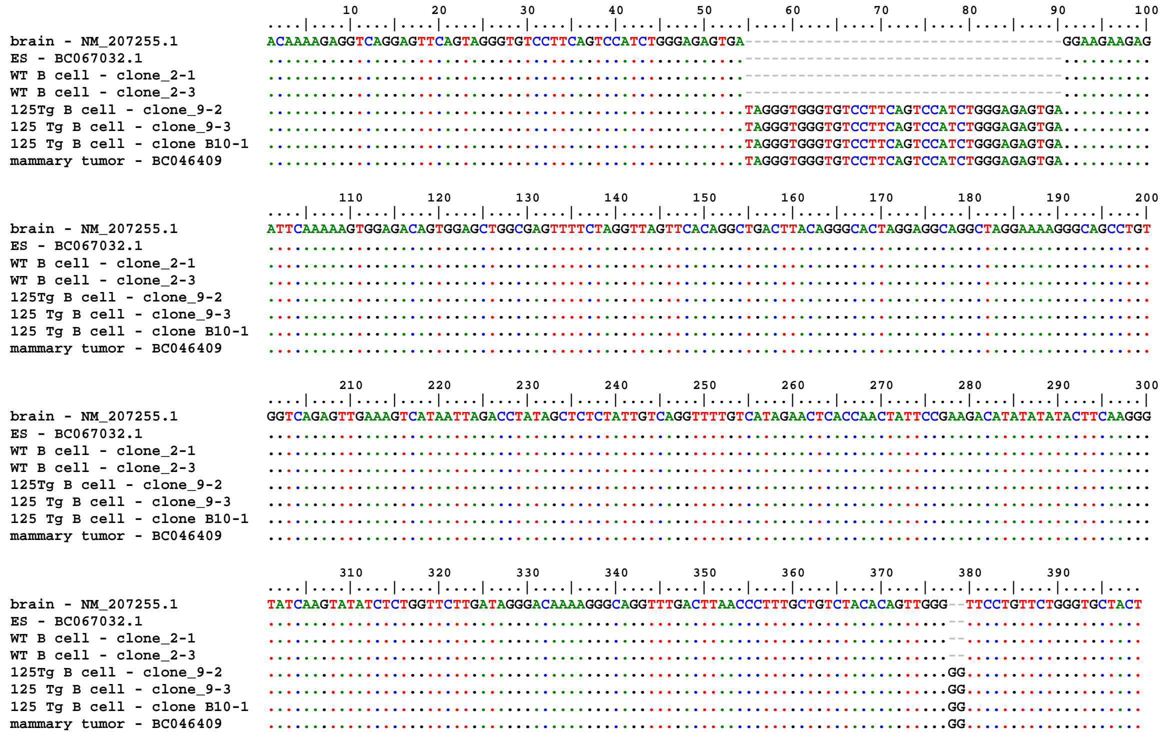


Figure 4-7. The 3' UTR of ZFP532 encoded by 125Tg/C57BL/6 B cells is distinct from other full-length transcripts. RNA was isolated from 125Tg and WT C57BL/6 purified B cells. The full length mRNA were cloned by PCR and subsequently sequenced. Those sequences were compared to published sequences from brain (NM_207255), embryonic stem cells (BC067032), and a mammary tumor (BC046409). The 3' UTR (shown) from the 125Tg B cells shares a 35 bp repeat and GG dinucleotide with the mammary tumor sequence but not with ES, brain, or WT B cell sequences. The last nucleotide shown (#399) is approximately 700 bp 5' of the polyA tail.

sequence was inserted into pcDNA3.1 encoding in-frame myc and 6X-histadine epitope tags. This vector was transfected into NIH-3T3 fibroblasts and ZFP532 expression was monitored by staining with anti-myc mAb (9E10). Confocal microscopy (figure 4-8) depicts ZFP532-myc/his protein (green) in the cytoplasm and nuclei (red by propidium iodide, panel A) of transfected NIH-3T3 cells but not in NIH-3T3 transfected with an empty, control vector (panel B). An alternate counter-stain that outlines the actin cytoskeleton (red by phalloidin-TRITC) is shown in panel C. From these data, it is apparent that ZFP532 can be found in the nucleus. However, the majority of the protein localizes in a perinuclear pattern. This could be due to over-expression of ZFP532 by the vector or a lack of an appropriate dimerization partner in NIH-3T3 cells. As mentioned previously, ZFP532 does not encode a canonical nuclear localization sequence and likely needs to pair with another TF which has this domain to efficiently enter the nucleus. As C_2H_2 ZF proteins can also bind dsRNA, ZFP532 might interact with rRNA molecules. This hypothesis correlates well with the observed localization pattern in NIH-3T3 cells. Clearly, further experiments using different cell types as well as a ZFP532 specific mAb are required.

ZFP532 has been targeted for germline disruption in embryonic stem cells.

Gene trapping strategies (targeting by randomly integrated retroviral vectors) have progressed in leaps over the last few years. The Mutant Mouse Regional Resource Center (MMRC, www.mmrrc.org) is an NCRR-NIH funded foundation that provides targeted ES cells at a minimal cost to researches in academia. Their database of targeted ES clones was searched for homologies to ZFP532, and two appropriate clones were obtained (special thanks to E. Oltz, S. Pierce, and The Functional Genomics of Inflammation program project grant). Sequence analysis of clone RRS823 indicated that the trapping vector had integrated

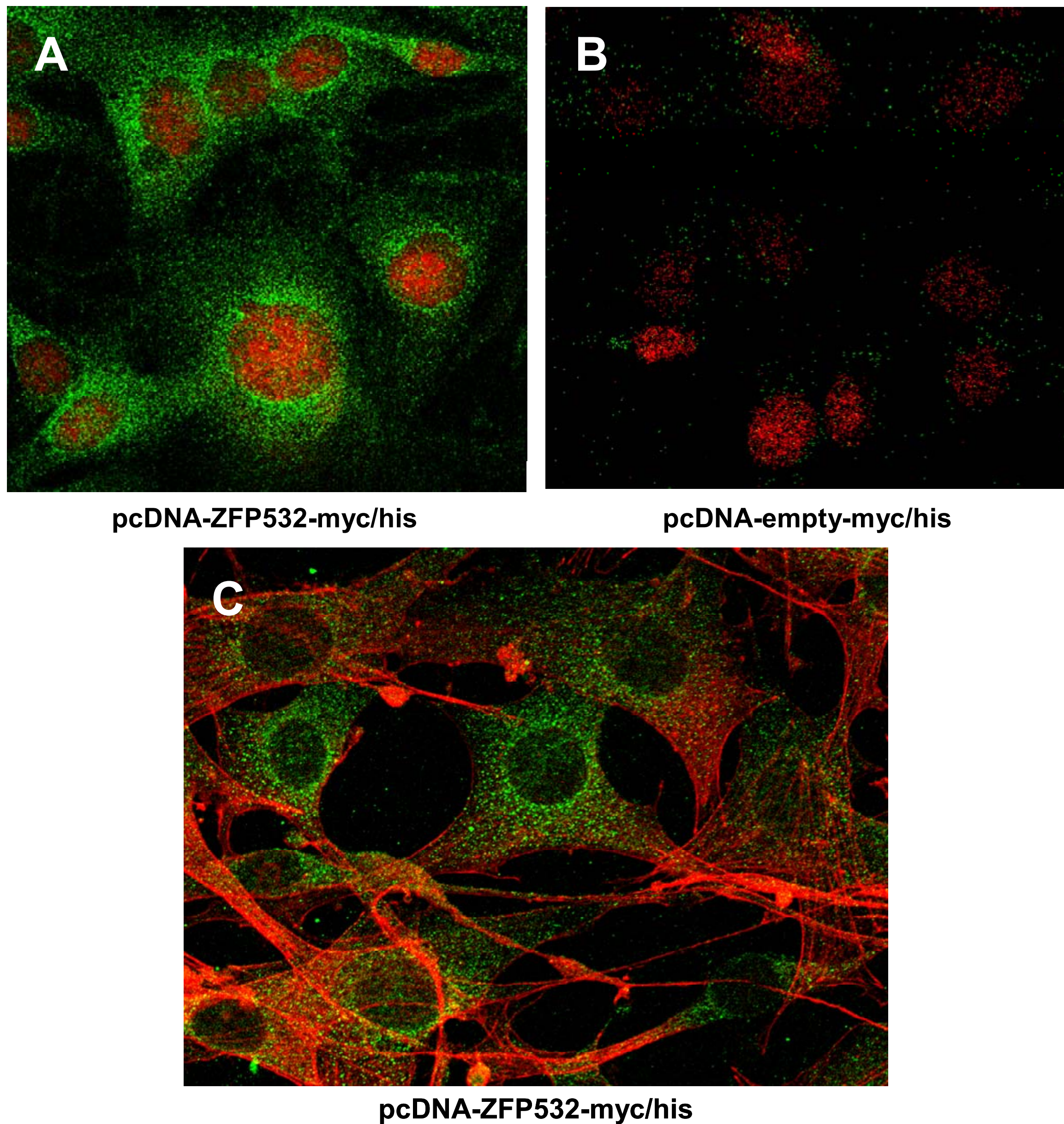


Figure 4-8. ZFP532 protein exhibits a predominantly perinuclear pattern in transfected NIH-3T3 fibroblasts. The entire coding region of ZFP532 was cloned into a eukaryotic expression vector (pcDNA3.1) encoding in-frame 3' myc and 6XHIS epitope tags. This vector was transfected into NIH-3T3 fibroblasts and ZFP532 expression was revealed by anti-myc mAb (green, A-C) and confocal microscopy. In A (ZFP532 vector) and B (empty vector), propidium iodide was used to stain nucleic acids (red). In C, TRITC conjugated phalloidin was used to stain the cytoskeleton (red). total magnification = 400X

into intron 2 which is 40,085 bp in length (figure 4-9). Preliminary Southern blots using a probe specific to the β-galactosidase-neomycin insertion sequence confirm that the vector has recombined into the 3' region of intron 2 (figure 4-9, panel A and B). RT-PCR analysis of targeted, RRS823, and parental stem cells confirms that a fusion transcript is being generated from the targeted locus (figure 4-9, panel C). These stem cells have been injected into blastocysts and the resulting agouti pups are currently being backcrossed onto the C57BL/6 background.

Discussion

MZ B cells are critical for host defense and represent a reservoir for autoreactivity, thus the mechanisms that contribute to their maturation and maintenance in the periphery are a compelling topic for investigation. As BCR signaling is critical for mature B lymphocyte differentiation likely via the induction of a particular set of TF, I have investigated the TF profiles of autoreactive, anergic MZ and FO B cells isolated from 125Tg/C57BL/6 mice. These studies are unique in at least two ways. First, intact lymphocytes are used as the starting point. Second, the cells are autoreactive and functionally unresponsive to BCR and TLR stimulation. In other models, anergized cells do not mature into MZ and FO B cell subsets, and thus singular information can be garnered from this model. Despite a lack of proliferation to LPS challenge, 125Tg, anti-insulin, MZ B cells exhibit the appropriate surface markers and reside proximal to the marginal sinus (chapter III). Thus, I feel that these cells are best described as bona fide MZ B cells that have been anergized. Furthermore, microarray analysis demonstrates that known gene expression patterns are appropriately maintained in 125Tg MZ B cells as is expected from WT MZ B cells (table 4-1). While protein levels do not always correlate with transcript

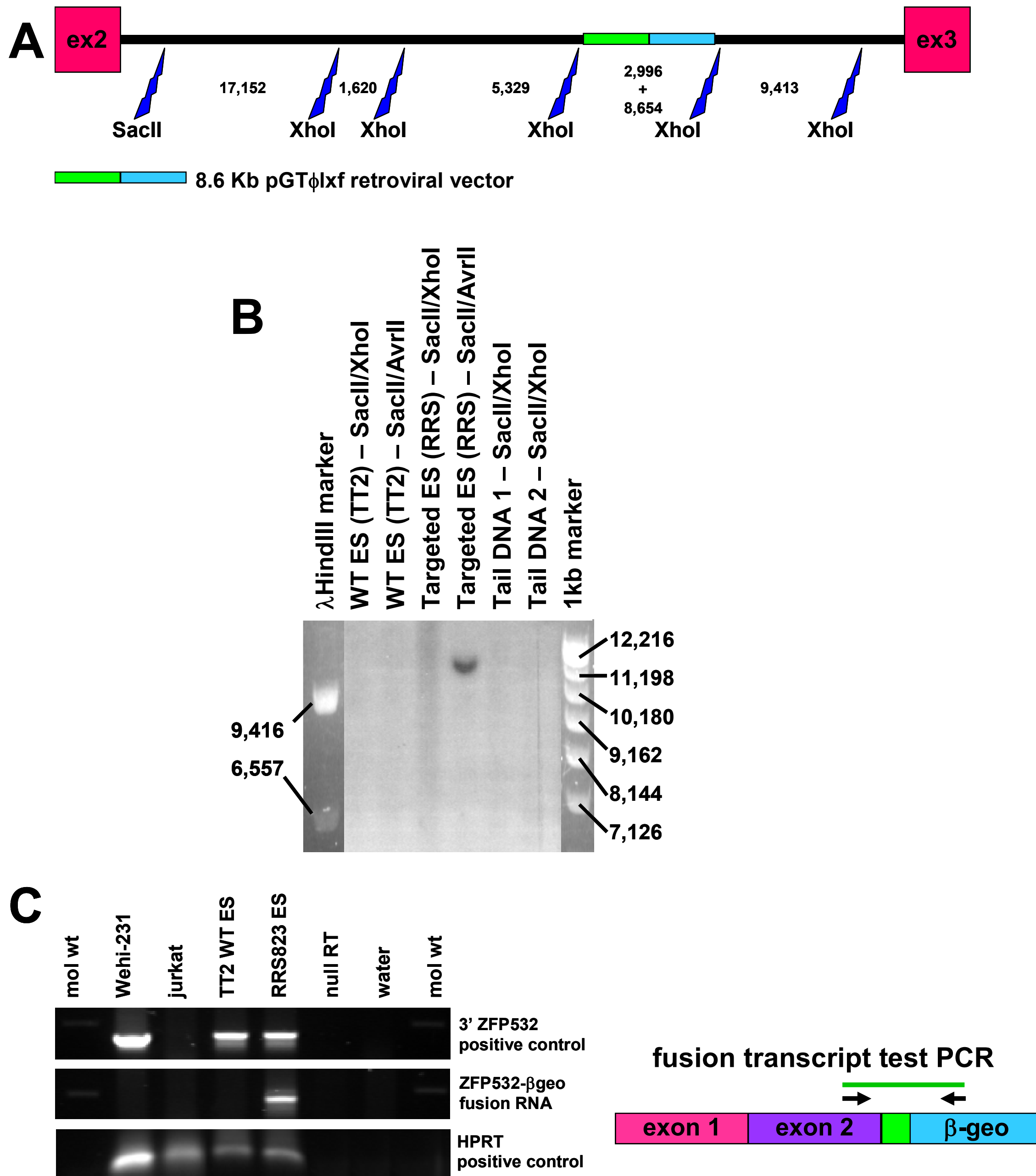


Figure 4-9. ZFP532 has been targeted for germline disruption in embryonic stem cells. Strategic digests of genomic DNA focusing on intron 2 (A) allowed pinpointing of the retroviral integration site to the 3' end of that intron by Southern blot analysis (B) using a β-geo specific probe. ZFP532-β-geo fusion RNA was detected by RT-PCR using ZFP532 and β-geo specific primer pairs (C).

levels, these data nonetheless serve as a sensible internal control that authenticates the MZ phenotype.

Microarray data is useful in several respects. In the widest, most general sense, it allows the researcher to obtain a synopsis of gene expression in a particular cell type. On the whole, more genes are being actively transcribed ("present") in the FO population than in the MZ – 17,114 (38%) vs. 16,333 (36%), respectively. Thus, far from being quiescent, mature, anergized FO and MZ B cells are actively maintaining their phenotype and anergic state. Interestingly, of the genes present in each sample, only a small percentage are increased in either subset relative to the other (FO – 2,397 genes or 14% of present/5.3% of total array vs. MZ – 1,619 genes or 9.9% of present/3.6% of total array). One might infer that the majority of genes transcribed are responsible for general B cell characteristics and that the 10-15% that are differentially expressed are responsible for the differences in phenotype. While small changes in gene expression are almost certainly biologically significant, they are typically hard to assay by normal laboratory methods. Thus, the genes that are upregulated at least 2 fold or greater are, in actuality, the only ones amenable to study (figure 4-2). In both populations, this is a very small percentage of the total array (FO – 574 genes/1.3% vs. MZ – 554 genes 1.2%). Taken together, these data imply that relatively large differences in phenotype (MZ vs. FO) are maintained by a few genes that exhibit minimal differences in expression. Thus, while gene targeted models are sometimes useful in elucidating gene function, global gene profiling will be more valuable to define how developmental programs are ultimately executed.

Among genes that are up-regulated in either MZ or FO B cells at least two fold or greater exists a particular contingent of TF. As transcription is often controlled by large multimolecular complexes, it is useful to look at these profiles as a whole. Inferences can be

made about some of the known genes returned by the microarray. For example, *Hes1* (hairy enhancer of split 1, increased 2 fold) is a target gene of Notch family members. As Notch signaling is required by MZ B cells, it is reasonable to assume that Notch target genes would be up-regulated by this population. *Deltex1* (increased 5.8 fold), while not a TF itself, is a critical facilitator of Notch2 signaling. Preferential expression of *Deltex1* by MZ B cells has been reported earlier (121). Both findings are consistent with the known requirement for Notch2 in MZ B cell generation. Interestingly, *Notch2* itself was not up-regulated two fold or more. This may indicate that facilitators of Notch2 signaling are more critical to MZ B cell development than the gene itself. Alternatively, the regulation of Notch protein may be more highly regulated than Notch transcripts (68, 72, 74, 78). In both cell types many ZF containing known and putative TF are up-regulated. TF of this type are common in binary cell fate decisions (e.g. *Klf4*/GKLF, *Zfpn1a1*/Ikaros, *Zfpn1a3*/Aiolos, and *Gata3*)(409-416). A function for GKLF and IKLF in B cells has not been reported previously and require further investigation.

The most striking observation in this microarray is that a novel gene, *Zfp532*, is up-regulated 12 fold by the MZ B cell population. The abundant transcript levels were validated independently by semi-quantitative RT-PCR and real time PCR on sorted MZ, FO, and T1 B cells as well as in T cells. Intensive molecular characterization of this gene was done using traditional laboratory techniques and resources available through public databases (www.ncbi.nlm.nih.gov). *Zfp532* encodes a predicted protein of 110 KDa. The structural features of this protein are consistent with eight C_2H_2 ZF domains, no nuclear localization sequence, and no traditional transactivation domains. Thus, ZFP532 may dimerize with other TF and is most likely repressive. The function of the ZF domains could be in protein-protein interactions, nucleic acid binding, or both. As consensus sequence binding sites are

often dictated by both subunits in a TF complex, the physical spacing and overall charge of the ZF domains in ZFP532 could alter the target genes recognized by its binding partner(s) (127, 129, 130, 146, 420). Alternatively, ZFP532 could be an RNA binding protein. This would be consistent with its subcellular localization (perinuclear) observed in preliminary expression experiments (figure 4-8). However, these experiments were done in NIH-3T3 cells, a fibroblast line, which may lack the appropriate binding partner(s) to shuttle ZFP532 into the nucleus. Unfortunately, rationalizing the role of an RNA binding protein in MZ B cell differentiation is somewhat more difficult than doing so for a TF. However, the wide transcript distribution of ZFP532 observed in non-immune system tissues (figure 4-6) is consistent with RNA binding activity or some other more generalized function. This too is not definite. For example, targeted deletions of Notch2, which is necessary for MZ B cell development, are embryonic lethal (121). Thus, a specific function in a particular cell type does not exclude the possibility of different functions in other tissues.

A common feature of ZF TF families (e.g. Ikaros, Aiolos, Helios, Eos, and Pegasus) is extensive alternative splicing to generate protein isoforms performing different functions (127, 128, 130, 131). In ZFP532, the majority of the protein, and four of the eight ZF domains, are encoded by exon 3. Exon 4 encodes one ZF; exon 5 encodes none; and exon 6 encodes the remaining three ZF (figure 4-3). Thus, differential splicing could be a mechanism employed by *Zfp532* to generate at least four functioning isoforms. As Northern blotting experiments have been unsatisfying, we cannot establish how many transcript isoforms are expressed by B lymphocytes, at this time.

Sequence homologies indicate that *Zfp532* is only conserved in higher order animals (Class Amniota and above). Genes required for basic cellular functions and even rudimentary immune systems are typically conserved across classes. A lack of conservation

may imply a specialized function. The differentiation of MZ B cells and/or the maintenance of anergy are two possible roles which comply with this evolutionary perspective. The maintenance of anergy only in MZ B cells would constitute an even more specific function. As animals evolve more sophisticated immune systems, with more subsets of uniquely functioning cells, it becomes ever more critical to regulate those elements. This speculation is, in part, supported by the real time PCR data which compared WT and anergic (125Tg) MZ and FO B cells. *Zfp532* expression is greatest in anergic MZ B cells and least in WT FO cells. Hence, its expression level is directly proportional to the MZ and anergic phenotypes. Furthermore, prior to these studies, *Zfp532* expression had not been demonstrated in splenic tissue (figure 4-6). Collectively, these observations imply that the unique characteristics of 125Tg MZ B cells are correlated with ZFP532 expression. As mentioned earlier, anergic MZ B cells had not been described prior to studies on 125Tg mice. In a broader context, one could hypothesize that "normal" MZ B cells in a polyclonal repertoire are partially anergic. That is, they do not proliferate in response to BCR stimulation (anti-IgM) as FO B cells do but maintain a vigorous response to LPS (226). Perhaps the anti-insulin specificity augments an already partially anergic MZ B cell state to include unresponsiveness to other forms of stimulation (LPS)? Clearly, further investigations as to the nature of anergy in mature B cell subsets are needed.

MZ B cells are a developmentally regulated subset that occupies a critical and somewhat unique interface between the adaptive and innate immune responses. This B cell subset is challenged by the dangerous task of maintaining active host defense while holding autoimmunity in check. The ontogenetic delay in MZ B cell development has serious consequences for susceptibility of neonates to infections, yet this developmental hiatus is evolutionarily conserved across species. The association of different autoimmune diseases

with an expanding MZ suggests that postponement of MZ B cell development is important for immune tolerance. However, there are no studies that directly address how tolerance is maintained for this key B cell subset. Conversely, enhanced tolerance, or functional anergy, in the MZ compartment may impair host defense and thwart attempts at vaccination. Enhancing immune function without increasing the risk of autoimmune disease is an important benefit gained from understanding the mechanisms of tolerance in MZ B cells. Identifying genetic elements that reside downstream of common signaling pathways in MZ B cells will provide viable targets for modifying their fate and function in the future. Infections are recognized as an essential component in the progression of certain autoimmune diseases. MZ B cells are uniquely responsive to bacterial and viral challenges. Thus, understanding how the autoimmune potential of MZ B cells is held in check is critical to understanding how environmental signals or infections trigger autoimmunity.

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

The research summarized in this dissertation focus on two major questions. How is an autoreactive B cell preserved in the peripheral repertoire and what effect does specificity have on gene expression and mature fate determination? BCR specificity is inexorably linked with BCR signal strength and quality. The characteristics of an antigen, dictate the extent of BCR cross-linking, the duration of interaction, and whether co-receptors are engaged. The sum of all these inputs determines the type of signal delivered to the nucleus which in turn controls gene expression profiles and ultimately phenotype.

BCR specificity can be fine-tuned by IgL structure and further impacted by polymorphisms.

BCR specificity is determined by the unique three dimensional structure of the combined CDRs of the IgH and IgL chains. In some cases, the majority of antigen binding is imposed by the IgH alone allowing the usage of several IgL (421, 422). In other instances, the IgH and IgL chains both contribute to the specificity, and only a few IgL with a particular amino acid structure will mediate binding to a particular antigen (144, 211). In the case of V_H125, insulin binding is dominated by members of both the Vκ1 and Vκ9 families; however, only certain IgL genes from each of these families can interact with insulin. This is intriguing given that families of IgL genes share a certain amount of amino acid homology. One might expect an entire Vκ family to mediate binding to an antigen when paired with a particular IgH. Clearly, this is not the case for insulin. Thus, antigen specificity can be fine-

tuned within a Vκ family by using different gene segments.

Of particular interest is the generation of anti-insulin B cells in the bone marrow of autoimmune, V_H125Tg/NOD mice but not normal, V_H125Tg/C57BL/6 mice. Absence of anti-insulin B cells in the spleen of V_H125Tg/C57BL/6 mice could be explained by effective negative selection of these specificities early in development. However, because they do not appear in V_H125Tg/C57BL/6 mice at any stage, it can be surmised that C57BL/6 mice are not capable of generating anti-insulin B cells using this IgH Tg. As suggested by the data in chapter II, the NOD mouse strain shares the IgL-b haplotype (group of related alleles polymorphic to another set of the same alleles) with the NZB strain. This strain is also a naturally occurring autoimmune disease model, but develops systemic lupus instead of T1DM. Of 56 strains surveyed, only NOD and NZB exhibit the IgL b haplotype (423). Indeed, the IgL that V_H125Tg/NOD B cells use for insulin the most frequently, Vκ1-110*02, is the same one that NZB mice use for anti-dsDNA Ig. Collectively, these data suggest an intriguing model wherein IgL haplotype may predispose an individual to producing autoreactive B lymphocytes. In a normal, non-autoimmune, individual, these cells would be eliminated via negative selection. However, if a patient exhibited the correct Ig haplotype as well as an autoimmune-permissive background (i.e. a group of genes that predisposed that individual to the development of autoimmune disease), autoreactive B cells may not be eliminated and might instead emerge into the periphery. These cells might then contribute to the development of disease.

Several lines of investigation could be followed to better define the role of Ig haplotype in autoimmune disease progression. First, does exchange of the IgL haplotypes confer reduced disease penetrance in V_H125Tg/NOD mice? This experiment would require generating NOD mice bearing the IgL-c locus and C57BL/6 mice bearing the IgL-b locus

either by successive intercrosses or artificial chromosome Tg. If haplotype is indeed important for the generation of anti-insulin B cells by V_H125Tg/NOD mice, then IgL-c/NOD mice should be incapable of producing insulin binding B cells. Conversely, IgL-b/C57BL/6 mice may acquire the ability to generate anti-insulin B cells. Likely these would be confined to the bone marrow due to negative selection. The long-term experiment would monitor IgL-c/NOD mice for the appearance of T1DM. Since B cell specificity is known to be important in diabetes progression, IgL-c/NOD mice would not be expected to develop diabetes at a rate similar to IgL-b (WT) NOD mice.

Second, do NZB mice carrying the V_H125Tg develop an anti-insulin B cell population similar to that seen in V_H125Tg/NOD mice? As NZB mice share the same haplotype as NOD mice, simply introgressing the V_H125Tg onto the NZB background would allow assessment of insulin specificity. While this approach carries with it the caveats of strain differences impacting B cell generation, it would be a preliminary way to assess whether all IgL-b haplotypes are capable of generating anti-insulin B cells in combination with the V_H125Tg.

Third, does IgL haplotype impact MZ B cell development? One common characteristic of the NOD and NZB strains is an augmentation of the MZ B cell compartment (218-224). The association between autoreactive BCR specificities, autoimmune disease, and the MZ B cell population makes it tempting to draw a relationship between IgL haplotype and MZ B cell generation. Reviewing the current data, it is difficult to ascertain whether MZ enlargement is a general strain characteristic or due to a particular gene(s). Using congenic IgL-b/C57BL/6 mice, one could begin to examine this question. If haplotype did impact BCR specificity, could it also alter mature B cell differentiation? Conversely, IgL-c/NOD mice would be expected to generate a more normal (~10%) MZ B

cell population.

Fourth, are all anti-insulin B cells from V_H125Tg/NOD mice capable of antigen presentation to T cells and Ig secretion? Anti-insulin IgG in the serum is a hallmark of the pre-diabetic state (335, 368, 424-426). This presupposes that B cells have been in contact with cognate T cells to facilitate CSR prior to the development of overt diabetes. While T cells are the end state mediator of diabetes (β cell destruction), the early stages of the disease are thought to involve cross-talk between antigen-specific B cells and potentially pathogenic T cells (427, 428). V_H125Tg/NOD mice exhibit an accelerated disease phenotype compared to WT/NOD (311). Accumulated evidence suggests that this alteration is due to changes in the B cell repertoire mediated by the IgH Tg. In chapter II, I demonstrate that the proportion of anti-insulin B cells is increased in V_H125Tg/NOD mice and that these BCRs are encoded by a somewhat heterogeneous group of IgL genes (216). Thus, it becomes important to understand whether all V_H125Tg/NOD B cells are capable of interacting with diabetogenic T cells, or only a subset. Others in our lab are currently working on antigen presentation using anti-insulin B cells from 125Tg/NOD. These mice carry IgH and IgL Tg. The IgL Tg, Vκ125, is a member of the Vκ4 family and not representative of the majority of anti-insulin B cells isolated from V_H125Tg/NOD mice (216). Thus, potentially more pathogenic specificities may exist in this repertoire. Similar antigen presentation experiments need to be conducted using V_H125Tg/NOD B cells. I expect V_H125Tg/NOD B cells to be more capable of T cell stimulation than either WT/NOD or 125Tg/NOD B cells as the IgH Tg only mice exhibit the most severe disease. As mentioned earlier, one outcome of these T-B cell interactions is CSR and IgG secretion. The Ig itself is not deemed pathogenic, but is instead an indicator of the emerging disease process (335, 368, 424-426). While the current V_H125Tg is fixed (IgM only), it will become increasingly important to

generate V_H125Tg/NOD mice that are class switch competent by targeting the V_H125Tg to the endogenous IgH locus. In these mice, secreted V_H125Tg IgG would serve as a measure of and further corroborate T-B cell communication. V_H125Tg knock-in mice would also allow assessment of how important anti-insulin Ig is to the disease process.

BCR specificity impacts B cell differentiation into MZ or FO subsets.

The studies in chapter II allude to a connection between BCR specificity and mature B cell phenotype. Some authors speculate that BCR specificity *per se* does not influence differentiation but that it is only signal strength which determines fate (18, 189, 396). I believe this is an artificial distinction because BCR specificity inherently determines BCR signal strength due to the character of the antigen. This hypothesis is supported by the data presented in chapter III. B cells specific for insulin, which bind insulin *in vivo* continually, and thus are subject to continual stimulation, are preferentially diverted into the MZ B cell compartment. Conversely, B cells specific for hen egg lysozyme – a protein not present in mice – receive no antigen ligation or signal, and populate all mature subsets in normal proportions.

If mature B cell phenotype is ultimately dictated by BCR specificity and signaling, a conundrum arises. Why aren't all anti-insulin B cells diverted into the MZ? Three possibilities exist. First, antigen concentration – insulin is a physiologically regulated hormone, the levels of which fluctuate throughout a 24 h period concomitant with feeding. Thus, newly formed B cells (T1) emerging from the bone marrow into circulation will encounter different levels of insulin at different times of the day. Second, co-receptor ligation – low level anti-insulin Ig is a normal component of serum and these Ab may form immune complexes that would co-engage BCR and FcR altering the quality of signal

delivered to the 125Tg B cell. Alternatively, some B cells may encounter bacterial antigen (e.g. nucleic acids) during their development leading to TLR engagement subsequent to or concurrent with insulin ligation. Can insulin or anti-insulin Ig bind complement proteins? If so, these would co-engage CD21/CD35 with BCR ligation. Third, space filling – while the width of the MZ can vary in parallel to the proportion of MZ B cells observed by FACS, no murine models demonstrate a splenic enlargement over that which is seen in 125Tg mice. Thus, there may be a physical limit to the capacity of the MZ. As microenvironment is critical to phenotype in this context (signals delivered via chemokines, integrins, S1P, Notch2, etc.), a B cell that has the capacity to develop a MZ phenotype – due to specificity – but is excluded from this locality may not be able to upregulate the surface markers characteristic of mature MZ B cells. This hypothesis is supported by two lines of data presented in chapter III. First, MZ B cell production in 125Tg/C57BL/6 mice begins earlier than in WT (figure 3-1). Thus, the MZ may "fill up" earlier in these mice than in WT animals in which MZ B cell development is delayed. Second, CD1d and CD9 expression is diminished in 125Tg/N2$^{+/-}$ mice (figure 3-6). MZ B cells are present but cannot upregulate accessory surface markers appropriately due in part to decreased Notch2 signals. Thus, BCR signals are likely the impetus for MZ B cell differentiation but are not capable of maintaining that phenotype in the absence of microenvironmental signals.

Various lines of experimentation are suggested by the observations made in chapter III. First, can insulin deprived 125Tg/C57BL/6 mice produce MZ B cells similar to insulin replete 125Tg/C57BL/6? Unfortunately, mice which were engineered to not express insulin from birth would exhibit severe diabetes and reduced survival to an acceptable experimental age. So alternatives to insulin knock-outs have to be conceived. The 125Tgs have the highest affinity for human insulin and reduced affinities for other species, including mouse

(344). Knock-in mice could be generated where the murine insulin loci are replaced by human or bovine insulin. These insulins are still hormonally active but would not impact the 125Tg BCR as murine insulin does. One may expect altered mature B cell populations in this type of model. Similar approaches using siRNA Tg to knock down insulin expression levels could also be used.

Second, are co-receptors involved in MZ B cell maturation? Crossing the 125Tg/C57BL/6 mice onto preexisting FcR (immune complex), TLR (microbial co-infection), and CD21 (complement deposition) knock-outs would permit the dissection of co-receptor input into MZ B cell development. If these co-receptors did exhibit an impact, then altered MZ B cell production would be expected from co-receptor knock-out, 125Tg mice.

BCR specificity can override Notch2 haploinsufficiency to generate MZ B cells.

Generation of MZ B cells requires input from many different sources, and lacking any one of these often leads to a diminished if not absent MZ B cell population (introduction and table 1-3). In chapter III, I demonstrate that MZ B cell deficiency imposed by heterozygosity of the *Notch2* gene (*Notch2*$^{+/-}$) can be overcome by an autoreactive BCR specificity (anti-insulin) but not by a naïve BCR (anti-hel). The ability of the 125Tg to override the Notch2 defect is complex. Prior to the studies in chapter III, a link between BCR signals and Notch2 signals had not been made. There was evidence that both pathways are required in conjunction for MZ B cell development (291), but interaction between the two pathways had not been demonstrated. A possible mechanism is suggested by figure 3-3. I show that B cells in nonTg/N2$^{+/-}$ mice are not capable of modulating Notch2 surface expression. However, MZ B cells from nonTg/C57BL/6, 125Tg/C57BL/6,

and 125Tg/N2$^{+/-}$ mice display higher levels of Notch2 than their FO or T1 counterparts. These data strongly support the concept that the quality of signal delivered by the autoreactive BCR causes accumulation of Notch2 at the cell's surface. Whether this effect is mediated at the transcriptional or post-transcriptional levels is unknown and the subject of future research. As reviewed in chapter I, Notch protein is regulated by transcript stability (65), translation efficiency (66), protein processing (82, 84, 87, 429), and turnover (70-72, 76, 100, 430, 431). Signals derived from the BCR could impact any of these regulatory points. For example, BCR mediated signals could down-regulate the endogenous microRNAs that degrade Notch transcripts or could alter the efficiency with which surface Notch is ubiquitinated and recycled. The level of complexity involved in Notch and DSL protein regulation necessitates an in depth investigation as to how BCR signals interact with the Notch pathway. The 125Tg versus helTg/*Notch2*$^{+/-}$ model system is ideal for these types of experiments. As there are many candidates for BCR-Notch interaction, it may be advisable to begin with a transcriptome or proteome-wide scan of differences between 125Tg and helTg/*Notch2*$^{+/-}$ B cells. Studies such as these would yield insight into how the adaptive immune system (BCR) coordinates with a developmental regulator (Notch2) to alter B cell fate.

Extending the findings of chapter III to other MZ (table 1-2) or B1 enriched specificities would be useful in determining the level of BCR engagement required for MZ B cell maturation. That is, while B1 and MZ repertoires exhibit some overlapping specificities (24, 25, 291), certain others are more specific to one lineage or the other (218, 311, 432, 433). It may be that a certain level of BCR signaling neither too strong (B1) nor too weak (FO) is required for MZ B cell generation in *Notch2*$^{+/-}$ mice.

Extending the observations of chapter II suggests other experiments. That is, if the

IgL-b haplotype characteristic of the NOD and NZB strains is responsible for altering the B cell repertoire (towards autoreactivity) and expanding the MZ, then NOD and NZB mice heterozygous for Notch2 should be capable of generating minor MZ B cell populations in the absence of any Tg. As NOD and NZB mice exhibit greater MZ B cells populations than IgL-c haplotype strains (e.g. C57BL/6, BALB/c, and C3H), one might expect the $N2^{+/-}$ phenotype to be less severe in these mice.

In a polyclonal (WT) repertoire, why do only the MZ B cells up-regulate Notch2 on their surface? This is the phenotype I see at steady state and doesn't illustrate what happens in transitional (T1 and T2) stages. It is likely that all 125Tg B cells destined to become MZ transiently up-regulate Notch2. However, it may be that sustained up-regulation requires orientation in the MZ itself. This could be explained by an autoregulatory loop wherein intracellular, activated Notch2 causes its own transcription, translation, or surface stability. Thus, being located in the MZ where Notch2 ligands are more prevalent would facilitate this proposed mechanism. Alternatively, input from other receptors such as integrins or S1P-R (Egr1/3) may control Notch2 expression. All of these possibilities are material for future experimentation.

Mature B cell phenotypes are maintained by global gene expression programs.

Ligation of BCR by antigen causes an intracellular cascade of events which ultimately culminates in the mobilization of TF in the nucleus (397-400). Different degrees of BCR interaction and co-receptor involvement modify which TF are recruited and silenced. The sum total of these inputs results in mature B cell phenotype. As MZ and FO B cells exhibit different surface proteins and mediate different functions in the immune system, it is expected that they would present differing gene expression profiles. As demonstrated in

chapter IV, the phenotypes of these two populations are maintained by a large group of genes that are only minimally differentially expressed and a smaller group of genes whose expression levels are markedly altered (figure 4-2). All of these genes could be investigated in future studies, however, the most intriguing, ZFP532, undoubtedly requires further consideration.

There are several characteristics that make ZFP532 an attractive candidate for future studies. An increased expression level of 12 times that which is seen in FO B cells should simplify its examination. Its predicted amino acid sequence encodes eight potential zinc coordinating domains (ZF). These domains are common in nucleic acid binding to RNA and DNA (434-436), and ZF containing TF are commonly utilized in binary cell fate decisions (127, 129, 130, 132, 409, 411, 437, 438). Thus, it is logical that ZFP532 might direct MZ fate determination. As 125Tg B cells are anergic, it may also impose functional quiescence in the MZ subset.

The initial stages of ZFP532 characterization proved difficult. The transcript is over 5 Kb in length, which complicates cloning and sequencing. However, now that the coding region is cloned into a tagged expression vector, subsequent biochemical studies are on the horizon. Determining how ZFP532 interacts with DNA (or RNA) and at what consensus sequence, what the binding partner(s) are, and whether it is inhibitory or activating are all important questions. Identifying potential target genes is also a worthy endeavor. Currently, in conjunction with the Oltz laboratory, we are developing a ZFP532 null mouse. If these mice are fertile and not embryonic lethal, they will be very useful in determining the function of ZFP532. However, this gene seems to be expressed at most stages of embryonic development (figure 4-6) and expectations for ZFP532$^{-/-}$ survival are not high. As mentioned previously, the function of ZFP532 may be in maintaining anergy and not simply

in MZ B cell development. Thus, ZFP532$^{-/-}$ B cells may not exhibit much of a phenotype unless challenged with antigen. In this case, crossing the ZFP532$^{-/-}$ line onto the 125Tg line may result in anti-insulin B cells that are not functionally silenced. These cells would be capable of reacting to insulin *in vivo* and may exhibit autoimmune manifestations such as Ig secretion, hyperglycemia, and immune complex formation. I have also constructed a ZFP532 containing retroviral vector (MiG, see Materials and Methods, chapter IV). This vector could be used to generate bone marrow chimeras that over-express ZFP532. I would expect ZFP532 over-expressing cells to exhibit either a MZ B cell phenotype, be anergic, or both. Currently, we are optimizing the use of this vector to initiate these types of experiments.

Concluding Remarks

B cells differentiate into B1, MZ and FO phenotypes as a result of BCR signal strength, quality and timing. In this context, BCR signaling is dependent on several features of the antigen – developmental stage at which antigen is first encountered, binding strength (affinity), antigen character (soluble vs. membrane-bound, monomeric vs. multimeric, opsonized vs. naked, co-receptor engagement vs. BCR alone), and duration. The sum of these factors induces a particular signal that causes B cells to differentiate into mature subsets and determines whether they are anergized. Thus, considering the maintenance of anergy in terms of the MZ/FO fate decision is a unique and valuable endeavor which may be approached from several angles. Understanding how autoreactivity, anergy and differentiation are manifest in terms of one another may enable the manipulation of B cell populations to diminish the impact of autoimmune disorders and enhance the ability of the immune system in combating microbial infections.

APPENDIX A

NUCLEOTIDE SEQUENCES OF ZFP532

KEY:
bold and underlined – **PRIMERS(###fwd OR rev)**
putative start codon – **ATG**
putative stop codon – **TAG**
5' and 3' untranslated regions – YELLOW HIGHLIGHT
polyA tail, bold and blue – **AAA**
sequences not in chromosomal clone, but present in cDNAs – **RED BOLD**

>NM_207255 (full length mRNA from C57BL/6 brain and ES cells)
(exon1→)TTCTCCCCACCCCAGGGGTGTCTTCCATTCTTTTGTGGCTCAGTTTAA
GGCGAAAAGGGCTCCAAACCACTAACTAACAGAAGGGAGCCCTTTCTTCCACC
TCCTGGGAGAATCTCAGATTGAATTTATCTGAAGATAGCGTGCTCTCTTCTTAC
TTATTGCCACCATTACGAGGAGGACAGCACAACCACCACC**TTGGCTTCAAGAT
CCTGGGTAGAGAGG(250fwd)**CTCACGGGCATTTTTTTTCAACCATCTTTGGCG
AGGCCTTGCATCCTTCCACTCCAG**(exon2→)**CCTGGTGACTGGGGCTGCTTTTA
ACCCTTTCCTATTTGCAGAGAAT**GCAACTGTGTGACAGTAACTGAACACTGG(2
63fwd)**GCCAAAGTCTTTTCAAAAGGTCAAGGTTCACAAG**(exon3→)**AACTGATCA
AATTC**ATGACCATGGGGGATATGAAGACCC(264fwd)**CAGACTTTGATGACCTC
TTGGCAGCATTTGACATACCAGATATGGTCGATCCCAAAGCAGCGATTGAGTC
CGGACACGATGACCATGAGAGCCACATTAAGCAGAATGCTCACGTGGATGAC
GACTCTCACACCCCATCATCCTCAGACGTCGGCGTCAGTGTGATTGTGAAGAA
TGTCCGCAACATCGACTCCTCCGAGGGGGTGGAAAAAGATGGCCACAATCCC
ACAGGCAATGGTTTGCATAATGGGTTCCTCACGGCATCCTCTCTTGACAGCTA
TGGTAAGGATGGAGCCAAGTCCTTAAAAGGAGACACACCTGCCTCGGAGGTG
ACTCTTAAGGACCCGGCATTCAGCCAGTTCAGCCCCATCTCCAGCGCCGAGGA
GTTTGAGGACGATGAGAAGATAGAGGTGGACGACCCGCCTGATAAGGAGGA
GGCGCGGGCCGGTTTCAGATCGAATGTGCTGACGGGCTCAGCACCCCAGCAG
GACTTCGACAAACTGAAGGCACTTGGAGGGGAAAACTCCAGCAAGACTGGAG
TCTCTACATCAGGCCACACGGATAAAAACAAGGTCAAGAGGGAGGCAGAAAG
CAATTCTATAACCCTGAGTGTTTATGAGCCATTTAAGGTCAGAAAAGCAGAGG
ATAAGTTGAAGGAGAACTCTGAGAAGATGCTTGAGAGCAGGGTCCTTGACGG
GAAGCCGAGCTCCGAGAAGAGCGACTCCGGCATCGCTGCTGCCGCATCTTCC
AAAACGAAGCCGTCCTCC**AAGCTCTCCTCGTGCATAGCGG(248fwd/249rev)**CC
ATTGCGGCGCTCAGCGCTAAAAAGGCTGCGTCCGACTCCTGCAAAGAGCCTG
TGGCCAACTCCAGGGAAGCCTCCCCGTTACCAAAAGAAGTGAATGACAGTCCC
AAAGCTGCCGACAAGTCTCCCGAGTCCCAGAATCTCATCGATGGCACCAAGAA
GGCCTCCCTGAAGCCATCAGACAGTCCCAGGAGCGTATCCAGTGAGAACAGC
AGCAAAGGGTCACCATCCTCACCCGTGGGCTCTACCCCAGCCATCCCCAAAGT

CCGCATCAAGACCATCAAGACATCGTCTGGGGAGATCAAGAGGACTGTGACC
AGAGTGCTGCCAGAAGTGGACCTGGACTCTGGAAAGAAGCCTTCTGAGCAGG
CAGCGTCCGTGATGGCGTCTGTGACATCACTCCTGTCATCTTCAGCATCAGCC
ACGGTCCTCTCCTCCCCGCCCAGGGCACCTCTGCAGACGGCCATGGTTACAAG
TGCAGTTTCCTCTG**CAGAGCTGACCCCCAAACAGG(212fwd/265rev)**TCACCATC
AAGCCCGTGGCGACAGCTTTTCTTCCCGTGTCTGCCGTCAAGACGGCAGGGTC
TCAAGTCATCAATCTGAAGCTCGCCAACAACACAACGGTGAAAGCCACGGTCA
TATCCGCCGCCTCTGTTCAGAGTGCCAGTAGCGCCATCATCAAAGCTGCCAAT
GCCATCCAGCAGCAAACCGTTGTGGTGCCGGCATCCAGCCTGGCCAATGCCAA
ACTCGTGCCAAAGACTGTGCACCTTGCCAACCTTAACCTTCTGCCTCAGGGTGC
CCAGGCCACCTCTGAACTCCGCCAAGTGCTCACCAAACCTCAGCAGCAAATAA
AGCAGGCAATAATCAATGCAGCGGCCTCGCAGCCACCTAAGAAGGTGTCTCG
GGTCCAGGTGGTGTCGTCCTTGCAGAGTTCTGTGGTGGAAGCTTTCAACAAG
GTGCTGAGCAGCGTCAACCCAGTCCCGGTTTACACCCCCAACCTCAGTCCTCCT
G**CCAACGCAGGGATCACGTTACC(214fwd/219rev)**GATGCGTGGGTACAAGTGC
TTGGAGTGCGGGGACGCCTTTGCCCTGGAGAAGAGCCTGAGCCAGCACTACG
ACAGGCGAAGCGTGCGCATCGAAGTGACGTGCAACCACTGTACCAAGAACCT
TGTTTTTTACAACAAATGCAGCCTCCTTTCTCACGCCCGCGGGCATAAGGAGA
AAGGCGTGGTGATGCAGTGCTCCCACCTGATCCTAAAGCCGGTCCCGGCAGA
CCAGATGATAGTTCCTCCATCCAGCAATACTGCTGCTTCCACTCTGCAGAGCTC
TGTGGGAGCTGCCACACACACTGTCCCAAAAGTCCAGCCTGGCATAGCCGGG
GCAGTTATCTCAGCTCCGGCAAGCACACCCATGAGCCCAGCCATGCCCCTAGA
CGAAGACCCCTCCAAGCTCTGTAGACACAGTCTCAAGTGTTTGGAGTGTAATG
AAGTCTTCCAGGATGAGCCGTCCCTGGCCACACATTTCCAGCACGCTGCAGAC
ACCAGTGGACAA**(exon4→)**CAAATGAAGAAGCACCCGTGCCGCCAGTGTGACA
AGTCTTT**CAGCTCCTCCCACAGCCTGTGCCGCC(233fwd/232rev)**ACAATCGCAT
CAAGCACAAAGGCATCAGGAAAGTTTACGCCTGCTCG**(exon5→)**CACTGCCCAG
ACTCCCGGCGGACCTTCACCAAGCGGCTGATGCTGGAGAGGCACATACAGCT
GATGCACGGGATCAAGGACCCTGATGTAAAAGAGCTGAGTGATGACGCTGGT
GATGTTACCAACGATGAGGAGGAGGAGGCGGAGATAAAGGAGGACGCCAAG
GT**(exon6→)**TCCCAGTCCC**AAGCGGAAGTTGGAGGAGCC(216fwd/221rev)**GGTT
TTAGAGTTCAGGCCTCCCAGAGGAGCCATCACTCAGCCACTGAAGAAACTGAA
AATCAATGTCTTTAAGGTCCACAAGTGTGCCGTGTGTGGCTTCACCACCGAGA
ACCTGCTGCAGTTCCACGAACACATCCCACAGCACAGGTCGGACGGCTCCTCC
CACCAGTGCCGGGAGTGTGGCCTGTGCTACACGTCCCACGGCTCCCTGGCCA
GGCACCTCTTCATCGTGCACAAGCTGAAGGAGCCTCAGCCCGTGTCCAAGCAG
AACGGGGCTGGGGAAGACAGCCAGCAGGAGAACAAGCCCAGCCCTGAGGAC
GAGGCCGCCGAGGGGGCAGCATCAGACAGGAAGTGCAAAGTGTGCGCCAAG
ACTTTTGAAACGGAAGCTGCCTTAAACACACACATGCGGACACATGGCATGGC
CTTCATCAAATCCAAAAGAATGAGTTCAGCTGAAAAA**TAG**CCACAGAACTTCC
AGGAGGACAACCCCTATCCACATAGGAATGGAGAATAAGACGTTTTTGTTACC
AAAAGTTGGCAGTATAACAAGAGTTACCAGTACCGTCTAGGCTGTCGCCACAG
ACTCCCTGTCCTGCCCTCTCACCTCTGCAGATGTGTCCCTTCCCATAAGTGTTA
AGGCAGTATTTGAGTTTTAAAGAGTTTTGTATATATTTAAATAACGTTTTATAC
TCTTTGTTACATGTTTGTATCAGTATTTGGTGGAAAATGTTTTGAGGTTTCTT**T**
GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGATTAGGATTCTCTTTTTT
TT(exon7→)GTACTGTTTCTTTAAAATGGAGTTCTTAGTAACA**GCGGCAGTTCC**

TGAATTCGAACC(217fwd/222rev)AACCATTTTGTATGTTACAAATTTGAATGAG
TTCAATAATAACAGGCTATCATGCC**TTTTTTTAGTGTTTTTTAATTTTT(exon8
→)**AGAACTCGCCACATAAATTGTAAGTGATTGTGGGTCTCACAACACTAGCAA
CTTTTAAGTGTCTTAGCACCACACGCAGCGTGCCTGCTCCTAGCAACCGAGGG
CTCCAAGGACAACATCACCCAGGTGAGGATGTGGCCTGAGCCACCCCAGACA
GCGTCAGCCTTCCAGGCCCTACCTCTGTCCACAGTGGACAAAAGAGGTCAGGA
GTTCAGTAGGGTGTCCTTCAGTCCATCTGGGAGAGTGAGGAAGAAGAGATTC
AAAAAGTGGAGACAGTGGAGCTGGCGAGTTTTCTAGGTTAGTTCACAGGCTG
ACTTACAGGGCACTAGGAGGCAGGCTAGGAAAAGGGCAGCCTGTGGTCAGA
GTTGAAAGTCATAATTAGACCTATAGCTCTCTATTGTCAGGTTTTGTCATAGAA
CTCACCAACTATTCCGAAGACATATATATACTTCAAGGGTATCAAGTATATCTC
TGGTTCTTGATAGGGACAAAAGGGCAGGTTT**GACTTAACCCTTTGCTGTCTA
CACAGTTGG(218fwd/223rev)**GTTCCTGTTCTGGGTGCTACTGCCAAATGTTCTG
GTACTTAAGTGTCGAGACGCCCAGCCTCACCACCGACTTAGCACTGCAGCAGC
CTGTACTCTGCAACTGGCCATAGACGAGCCACCAGGCTTCTAGAGTCGTCTCA
GCACCCTCTCAAGGTCACCTGACTGGAGCACTGCCGACGACGTGCTCTTGGTC
ACATCCCTGTATAGTTCTCTGGGAAAGCTATAAATATATATATTTTGGTTATTG
TTTTGTGTTTTCCGTTACATTTTATATCTTGTATTTATCGCCCGATATGTTTTGT
ACTTTTGTTTTCCTAAAACAAAGAAATCCATGTGTGTGTATATAGAGACTTGCA
TGCTAGACTGTAGTCAATGTTCAGTTCCTTGAAAAGTCTTGCTGCTGTCGGGT
GTGCACCTTA**CACCGCCGCGACTGTATTCAA**(224rev)CCCATTTCACATGTAAA
TAAATGAGGAACGTCTG**AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**

KEY:
pcDNA3.1 vector sequence – UNDERLINED
CMV promoter – **BLUE BOLD**
T7 promoter and primer for sequencing – **RED BOLD**
remaining pGEM-t easy sequences – **BOLD, GREY HIGHLIGHT**
added Kozac sequence – **GREEN BOLD**
putative start codon – **ATG**
nucleotides ("g" and stop codons) fixed from earlier clone – lowercase green highlight
new point mutation – lowercase yellow highlight
last codon of ZFP532 – **AAA**
3' PCR added nucleotide – **A**
myc and his epitope tags – **RED BOLD**

>fixed pcDNA3.1C-ZFP532 (eukaryotic expression vector)
GACGGATCGGGAGATCTCCCGATCCCCTATGGTGCACTCTCAGTACAATCTGC
TCTGATGCCGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGT
CGCTGAGTAGTGCGCGAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACC
GACAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCG**CGA
TGTACGGGCCAGATATACGCGTTGACATTGATTATTGACTAGTTATTAATAG
TAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTA
CATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCC
ATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTC**

CATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTAC
ATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAA
ATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACT
TGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTT
GGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAA
GTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACG
GGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGT
AGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAG
AACCCACTGCTTACTGGCTTATCGAAATT(CMV.promoter)AATACGACTCACT
ATAGGG(T7.promoter/primer)AGACCCAAGCTGGCTAGTTAAGCTTGGTACCG
AGCTCGGATCCACTAGTCCAGTGTGGTGGAATTC**GATTGCCACC(added.kozac)**
ATGACCATGGGGGATATGAAGACCCCAGACTTTGATGACCTCTTGGCAGCATT
TGACATACCAGATATGGTCGATCCCAAAGCAGCGATTGAGTCCGGACACGAT
GACCATGAGAGCCACATTAAGCAGAATGCTCACGTGGATGACGACTCTCACAC
CCCATCATCCTCAGACGTCGGCGTCAGTGTGATTGTGAAGAATGTCCGCAACA
TCGACTCCTCCGAGGGGGTGGAAAAAGATGGCCACAATCCCACAGGCAATGG
TTTGCATAATGGGTTCCTCACGGCATCCTCTCTTGACAGCTATGGTAAGGATG
GAGCCAAGTCCTTAAAAGGAGACACACCTGCCTCGGAGGTGACTCTTAAGGA
CCCGGCATTCAGCCAGTTCAGCCCCATCTCCAGCGCCGAGGAGTTTGAGGACG
ATGAGAAGATAGAGGTGGACGACCCGCCTGATAAGGAGGAGGCGCGGGCCG
GTTTCAGATCGAATGTGCTGACGGGCTCAGCACCCCAGCAGGACTTCGACAAA
CTGAAGGCACTTGGAGGGGAAAACTCCAGCAAGACTGGAGTCTCTACATCAG
GCCACACGGATAAAAACAAGGTCAAGAGGGAGGCAGAAAGCAATTCTATAAC
CCTGAGTGTTTATGAGCCATTTAAGGTCAGAAAAGCAGAGGATAAGTTGAAG
GAGAACTCTGAGAAGATGCTTGAGAGCAGGGTCCTTGACGGGAAGCCGAGC
TCCGAGAAGAGCGACTCCGGCATCGCTGCTGCCGCATCTTCCAAAACGAAGCC
GTCCTCCAAGCTCTCCTCGTGCATAGCGGCCATTGCGGCGCTCAGCGCTAAAA
AGGCTGCGTCCGACTCCTGCAAAGAGCCTGTGGCCAACTCCAGGGAAGCCTC
CCCGTTACCAAAAGAAGTGAATGACAGTCCCAAAGCTGCCGACAAGTCTCCCG
AGTCCCAGAATCTCATCGATGGCACCAAGAAGGCCTCCCTGAAGCCATCAGAC
AGTCCCAGGAGCGTATCCAGTGAGAACAGCAGCAAAGGGTCACCATCCTCACC
CGTGGGCTCTACCCCAGCCATCCCCAAAGTCCGCATCAAGACCATCAAGACATC
GTCTGGGGAGATCAAGAGGACTGTGACCAGAGTGCTGCCAGAAGTGGACCT
GGACTCTGGAAAGAAGCCTTCTGAGCAGGCAGCGTCCGTGATGGCGTCTGTG
ACATCACTCCTGTCATCTTCAGCATCAGCCACGGTCCTCTCCTCCCCGCCCAGG
GCACCTCTGCAGACGGCCATGGTTACAAGTGCAGTTTCCTCTGCAGAGCTGAC
CCCCAAACAGGTCACCATCAAGCCCGTGGCGACAGCTTTTCTTCCCGTGTCTGC
CGTCAAGACGGCAGGGTCTCAAGTCATCAATCTGAAGCTCGCCAACAACACAA
CGGTGAAAGCCACGGTCATATCCGCCGCCTCTGTTCAGAGTGCCAGTAGCGCC
ATCATCAAAGCTGCCAATGCCATCCAGCAGCAAACCGTTGTGGTGCCGGCATC
CAGCCTGGCCAATGCCAAACTCGTGCCAAAGACTGTGCACCTTGCCAACCTTA
ACCTTCTGCCTCAGGGTGCCCAGGCCACCTCTGAACTCCGCCAAGTGCTCACC
AAACCTCAGCAGCAAATAAAGCAGGCAATAATCAATGCAGCGGCCTCGCAGCC
ACCTAAGAAGGTGTCTCGGGTCCAGGTGGTGTCGTCCTTGCAGAGTTCTGTG
GTGGAAGCTTTCAACAAGGTGCTGAGCAGCGTCAACCCAGTCCCGGTTTACAC
CCCCAACCTCAGTCCTCCTGCCAACGCAGGGATCACGTTACCGATGCGTGGGT
ACAAGTGCTTGGAGTGCGGGGACGCCTTTGCCCTGGAGAAGAGCCTGAGCCA
GCACTACGACAGGCGAAGCGTGCGCATCGAAGTGACGTGCAACCACTGTACC

AAGAACCTTGTTTTTACAACAAATGCAGCCTCCTTTCTCACGCCCGCGGGCAT
AAGGAGAAAGGCGTGGTGATGCAGTGCTCCCACCTGATCCTAAAGCCGGTCC
CGGCAGACCAGATGATAGTTCCTCCATCCAGCAATACTGCTGCTTCCACTCTGC
AGAGCTCTGTGGGAGCTGCCACACACACTGTCCCAAAAGTCCAGCCTGGCATA
GCCGGGGCAGTTATCTCAGCTCCGGCAAGCACACCCATGAGCCCAGCCATGCC
CCTAGACGAAGACCCCTCCAAGCTCTGTAGACACAGTCTCAAGTGTTTGGAGT
GTAATGAAGTCTTCCAGGATGAGCCGTCCCTGGCCACACATTTCCAGCACGCT
GCAGACACCAGTGGACAACAAATGAAGAAGCACCCGTGCCGCCAGTGTGACA
AGTCTTTCAGCTCCTCCCACAGCCTGTGCCGCCACAATCGCATCAAGCACAAAG
GCATCAGGAAAGTTTACGCCTGCTCGCACTGCCCAGACTCCCGGCGGACCTTC
ACCAAGCGGCTGATGCTGGAGAGGCACATACAGCTGATGCACGGGATCAAGG
ACCCTGATGTAAAAGAGCTGAGTGATGACGCTGGTGATGTTACCAACGATGA
GGAGGAGGAGGCGGAGATAAAGGAGGACGCCAAGGTTCCCAGTCCCAAGCG
GAAGTTGGAGGAGCCGGTTTTAGAGTTCAGGCCTCCCAGAGGAGCCATCACT
CAGCCACTGAAGAAACTGAAAATCAATGTCTTTAAGGTCCACAAGTGTGCCGT
GTGTGGCTTCACCACCGAGAACCTGCTGCAGTTCCACGAACACATCCCACAGC
ACAGGTCGGACGGCTCCTCCCACCAGTGCCGGGAGTGTGGCCTGTGCTACAC
GTCCCACGGCTCCCTGGCCAGGCACCTCTTCATCGTGCACAAGCTGAAGGAGC
CTCAGCCCGTGTCCAAGCAGAACGGGgCTGGGGAAGACAGCCAGCAGGAGAA
CAAGCCCAGCCCTGAGGACGAGGCCGCCGAGGGGGCAGCATCAGACAGGAA
GTGCAAAGTGTGCGCCAAGACTTTTGAAACGGAAGCTGCCTTAAACACACACA
TGCGGACACATGGCATGGCCTTCATCAAATCCAAAAGAATGAGcTCAGCTGAA
AAAAATCACgcGgcAATTCTGCAGATATCCAGCACAGTGGCGGCCGCTCGAGG
TCACCCATTC**GAACAAAAACTCATCTCAGAAGAGGATCTG(MYC.tag)**AATATGC
ATACCGGT**CATCATCACCATCACCAT(6X.HIS.tag)**TGAGTTTAAACCCGCTGAT
CAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCG
TGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATG
AGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGG
GGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATG
CTGGGGATGCGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGCTGGG
GCTCTAGGGGGTATCCCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGG
TGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCC
GCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTC
AAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCAC
CTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCC
CTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTG
GACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTG
ATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATT
TAACAAAAATTTAACGCGAATTAATTCTGTGGAATGTGTGTCAGTTAGGGTGT
GGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCA
ATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAG
TATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCC
GCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTG
ACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCTGCCTCTGAGCTATT
CCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTC
CCGGGAGCTTGTATATCCATTTTCGGATCTGATCAAGAGACAGGATGAGGAT
CGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGG
GTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTG

ATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAA
GACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTA
TCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCA
CTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATC
TCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAA
TGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCG
AAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATC
AGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGC
CAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGC
GATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCAT
CGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCT
ACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCG
TGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTC
TTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGCGAAATGACCGACCAAG
CGACGCCCAACCTGCCATCACGAGATTTCGATTCCACCGCCGCCTTCTATGAAA
GGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCG
CGGGGATCTCATGCTGGAGTTCTTCGCCCACCCCAACTTGTTTATTGCAGCTTA
TAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTT
TTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCATGT
CTGTATACCGTCGACCTCTAGCTAGAGCTTGGCGTAATCATGGTCATAGCTGT
TTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAA
GCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATT
GCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCA
TTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCT
TCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGC
GGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGAT
AACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTA
AAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCAT
CACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAA
GATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCC
TGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTT
TCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAA
GCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCG
GTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCA
GCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACA
GAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTG
GTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCT
TGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCA
GCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTA
CGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCAT
GAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTT
TAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTT
AATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGC
CTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCC
CCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCA
GCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTT
TATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGT
TCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGT

GTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAA
GGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGT
CCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTAT
GGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGT
GACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCG
AGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAAC
TTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGA
TCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGAT
CTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGG
CAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCA
TACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGA
GCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGC
ACATTTCCCCGAAAAGTGCCACCTGACGTC

APPENDIX B

LIST OF PUBLICATIONS

I. **Woodward EJ**, Thomas JW. Marginal Zone B Cell Defects Imposed by Notch2 Haploinsufficiency are Ameliorated by an Autoreactive B Cell Receptor. 2006. Submitted.

II. Kendall PL, **Woodward EJ**, Yu G, Thomas JW. Selection of B lymphocytes in prediabetic pancreatic islets implies a unique role for tertiary lymphoid structures. 2006. In Preparation.

III. **Woodward EJ**, Thomas JW. Multiple germline kappa light chains generate anti-insulin B cells in nonobese diabetic mice. J Immunol. 2005 Jul 15;175(2):1073-9. PMID: 16002708

IV. Acevedo-Suarez CA, Hulbert C, **Woodward EJ**, Thomas JW. Uncoupling of anergy from developmental arrest in anti-insulin B cells supports the development of autoimmune diabetes. J Immunol. 2005 Jan 15;174(2):827-33. PMID: 15634904

V. Stephenson L, Johns MH, **Woodward E**, Mora AL, Boothby M. An IL-4R alpha allelic variant, I50, acts as a gain-of-function variant relative to V50 for Stat6, but not Th2 differentiation. J Immunol. 2004 Oct 1;173(7):4523-8.

PMID: 15383584

VI. Kendall PL, **Woodward EJ**, Hulbert C, Thomas JW. Peritoneal B cells govern the outcome of diabetes in non-obese diabetic mice. Eur J Immunol. 2004 Sep;34(9):2387-95.

PMID: 15307171

REFERENCES

1. Glick, B. 1991. Historical perspective: the bursa of Fabricius and its influence on B-cell development, past and present. *Vet.Immunol.Immunopathol.* 30:3-12.

2. Hardy, R. R. 2003. B-cell commitment: deciding on the players. *Curr.Opin.Immunol.* 15:158-165.

3. Nutt, S. L., B. Heavey, A. G. Rolink, and M. Busslinger. 1999. Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature* 401:556-562.

4. Rolink, A. G., S. L. Nutt, F. Melchers, and M. Busslinger. 1999. Long-term in vivo reconstitution of T-cell development by Pax5-deficient B-cell progenitors. *Nature* 401:603-606.

5. Melchers, F., D. Haasner, U. Grawunder, C. Kalberer, H. Karasuyama, T. Winkler, and A. G. Rolink. 1994. Roles of IgH and L chains and of surrogate H and L chains in the development of cells of the B lymphocyte lineage. *Annu.Rev.Immunol.* 12:209-225.

6. Hertz, M. and D. Nemazee. 1998. Receptor editing and commitment in B lymphocytes. *Curr.Opin.Immunol.* 10:208-213.

7. Hardy, R. R. and K. Hayakawa. 2001. B cell development pathways. *Annu.Rev.Immunol.* 19:595-621.

8. Busslinger, M., S. L. Nutt, and A. G. Rolink. 2000. Lineage commitment in lymphopoiesis. *Curr.Opin.Immunol.* 12:151-158.

9. Pelanda, R. and R. M. Torres. 2006. Receptor editing for better or for worse. *Curr.Opin.Immunol.* 18:184-190.

10. Verkoczy, L. K., A. S. Martensson, and D. Nemazee. 2004. The scope of receptor editing and its association with autoimmunity. *Curr.Opin.Immunol.* 16:808-814.

11. Edry, E. and D. Melamed. 2004. Receptor editing in positive and negative selection of B lymphopoiesis. *J.Immunol.* 173:4265-4271.

12. Ait-Azzouzene, D., P. Skog, M. Retter, V. Kouskoff, M. Hertz, J. Lang, J. Kench, M. Chumley, D. Melamed, J. Sudaria, A. Gavin, A. Martensson, L. Verkoczy, B. Duong, J. Vela, and D. Nemazee. 2004. Tolerance-induced receptor selection: scope, sensitivity, locus specificity, and relationship to lymphocyte-positive selection. *Immunol.Rev.* 197:219-230.

13. Retter, M. W. and D. Nemazee. 1999. Receptor editing: genetic reprogramming of autoreactive lymphocytes. *Cell Biochem.Biophys.* 31:81-88.

14. Nussenzweig, M. C. 1998. Immune receptor editing: revise and select. *Cell* 95:875-878.

15. Fanning, L., F. E. Bertrand, C. Steinberg, and G. E. Wu. 1998. Molecular mechanisms involved in receptor editing at the Ig heavy chain locus. *Int.Immunol.* 10:241-246.

16. Nemazee, D. 1996. Can receptor editing play an important role in normal B-cell development? *J.Autoimmun.* 9:259-261.

17. Nemazee, D. 2000. Role of B cell antigen receptor in regulation of V(D)J recombination and cell survival. *Immunol.Res.* 21:259-263.

18. Pillai, S., A. Cariappa, and S. T. Moran. 2004. Marginal Zone B Cells. *Annu.Rev.Immunol.* 23:161-196.

19. Allman, D., B. Srivastava, and R. C. Lindsley. 2004. Alternative routes to maturity: branch points and pathways for generating follicular and marginal zone B cells. *Immunol.Rev.* 197:147-160.

20. Srivastava, B., R. C. Lindsley, N. Nikbakht, and D. Allman. 2005. Models for peripheral B cell development and homeostasis. *Semin.Immunol.* 17:175-182.

21. Petro, J. B., R. M. Gerstein, J. Lowe, R. S. Carter, N. Shinners, and W. N. Khan. 2002. Transitional type 1 and 2 B lymphocyte subsets are differentially responsive to antigen receptor signaling. *J.Biol.Chem.* 277:48009-48019.

22. Goodnow, C. C., J. Crosbie, S. Adelstein, T. B. Lavoie, S. J. Smith-Gill, R. A. Brink, H. Pritchard-Briscoe, J. S. Wotherspoon, R. H. Loblay, K. Raphael, and . 1988. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature* 334:676-682.

23. Srivastava, B., W. J. Quinn, III, K. Hazard, J. Erikson, and D. Allman. 2005. Characterization of marginal zone B cell precursors. *J.Exp.Med.* 202:1225-1234.

24. Martin, F. and J. F. Kearney. 2001. B1 cells: similarities and differences with other B cell subsets. *Curr.Opin.Immunol.* 13:195-201.

25. Martin, F. and J. F. Kearney. 2000. B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a "natural immune memory". *Immunol.Rev.* 175:70-79.

26. DeKoter, R. P., H. J. Lee, and H. Singh. 2002. PU.1 regulates expression of the interleukin-7 receptor in lymphoid progenitors. *Immunity.* 16:297-309.

27. Scott, E. W., M. C. Simon, J. Anastasi, and H. Singh. 1994. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* 265:1573-1577.

28. Scott, E. W., R. C. Fisher, M. C. Olson, E. W. Kehrli, M. C. Simon, and H. Singh. 1997. PU.1 functions in a cell-autonomous manner to control the differentiation of multipotential lymphoid-myeloid progenitors. *Immunity.* 6:437-447.

29. McKercher, S. R., B. E. Torbett, K. L. Anderson, G. W. Henkel, D. J. Vestal, H. Baribault, M. Klemsz, A. J. Feeney, G. E. Wu, C. J. Paige, and R. A. Maki. 1996. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO J.* 15:5647-5658.

30. Zou, G. M., J. J. Chen, M. C. Yoder, W. Wu, and J. D. Rowley. 2005. Knockdown of Pu.1 by small interfering RNA in CD34+ embryoid body cells derived from mouse ES cells turns cell fate determination to pro-B cells. *Proc.Natl.Acad.Sci.U.S.A* 102:13236-13241.

31. Greenbaum, S. and Y. Zhuang. 2002. Identification of E2A target genes in B lymphocyte development by using a gene tagging-based chromatin immunoprecipitation system. *Proc.Natl.Acad.Sci.U.S.A* 99:15030-15035.

32. Murre, C. 2005. Helix-loop-helix proteins and lymphocyte development. *Nat.Immunol.* 6:1079-1086.

33. Ikawa, T., H. Kawamoto, L. Y. Wright, and C. Murre. 2004. Long-term cultured E2A-deficient hematopoietic progenitor cells are pluripotent. *Immunity.* 20:349-360.

34. Bain, G., E. C. Maandag, D. J. Izon, D. Amsen, A. M. Kruisbeek, B. C. Weintraub, I. Krop, M. S. Schlissel, A. J. Feeney, M. van Roon, and . 1994. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell* 79:885-892.

35. Zhuang, Y., P. Soriano, and H. Weintraub. 1994. The helix-loop-helix gene E2A is required for B cell formation. *Cell* 79:875-884.

36. Sun, X. H. 1994. Constitutive expression of the Id1 gene impairs mouse B cell development. *Cell* 79:893-900.

37. Johnson, K. and K. Calame. 2003. Transcription factors controlling the beginning and end of B-cell differentiation. *Curr.Opin.Genet.Dev.* 13:522-528.

38. Kee, B. L. and C. Murre. 1998. Induction of early B cell factor (EBF) and multiple B lineage genes by the basic helix-loop-helix transcription factor E12. *J.Exp.Med.* 188:699-713.

39. Greenbaum, S. and Y. Zhuang. 2002. Regulation of early lymphocyte development by E2A family proteins. *Semin.Immunol.* 14:405-414.

40. Hagman, J. and K. Lukin. 2005. Early B-cell factor 'pioneers' the way for B-cell development. *Trends Immunol.* 26:455-461.

41. Romanow, W. J., A. W. Langerak, P. Goebel, I. L. Wolvers-Tettero, J. J. van Dongen, A. J. Feeney, and C. Murre. 2000. E2A and EBF act in synergy with the V(D)J recombinase to generate a diverse immunoglobulin repertoire in nonlymphoid cells. *Mol.Cell* 5:343-353.

42. Goebel, P., N. Janney, J. R. Valenzuela, W. J. Romanow, C. Murre, and A. J. Feeney. 2001. Localized gene-specific induction of accessibility to V(D)J recombination induced by E2A and early B cell factor in nonlymphoid cells. *J.Exp.Med.* 194:645-656.

43. Hsu, L. Y., J. Lauring, H. E. Liang, S. Greenbaum, D. Cado, Y. Zhuang, and M. S. Schlissel. 2003. A conserved transcriptional enhancer regulates RAG gene expression in developing B cells. *Immunity.* 19:105-117.

44. Hagman, J. and K. Lukin. 2006. Transcription factors drive B cell development. *Curr.Opin.Immunol.* 18:127-134.

45. Busslinger, M. 2004. Transcriptional control of early B cell development. *Annu.Rev.Immunol.* 22:55-79.

46. Mikkola, I., B. Heavey, M. Horcher, and M. Busslinger. 2002. Reversion of B cell commitment upon loss of Pax5 expression. *Science* 297:110-113.

47. Horcher, M., A. Souabni, and M. Busslinger. 2001. Pax5/BSAP maintains the identity of B cells in late B lymphopoiesis. *Immunity.* 14:779-790.

48. Quong, M. W., A. Martensson, A. W. Langerak, R. R. Rivera, D. Nemazee, and C. Murre. 2004. Receptor editing and marginal zone B cell development are regulated by the helix-loop-helix protein, E2A. *J.Exp.Med.* 199:1101-1112.

49. Maillard, I., T. Fang, and W. S. Pear. 2005. Regulation of lymphoid development, differentiation, and function by the Notch pathway. *Annu.Rev.Immunol.* 23:945-974.

50. Hoyne, G. F. 2003. Notch signaling in the immune system. *J.Leukoc.Biol.* 74:971-981.

51. Bertrand, F. E., C. E. Eckfeldt, A. S. Lysholm, and T. W. LeBien. 2000. Notch-1 and Notch-2 exhibit unique patterns of expression in human B-lineage cells. *Leukemia* 14:2095-2102.

52. Baldi, A., M. De Falco, L. De Luca, G. Cottone, M. G. Paggi, B. J. Nickoloff, L. Miele, and A. De Luca. 2004. Characterization of tissue specific expression of Notch-1 in human tissues. *Biol.Cell* 96:303-311.

53. Caprioli, A., R. Goitsuka, C. Pouget, D. Dunon, and T. Jaffredo. 2002. Expression of Notch genes and their ligands during gastrulation in the chicken embryo. *Mech.Dev.* 116:161-164.

54. Chen, C. W., H. S. Jung, T. X. Jiang, and C. M. Chuong. 1997. Asymmetric expression of Notch/Delta/Serrate is associated with the anterior-posterior axis of feather buds. *Dev.Biol.* 188:181-187.

55. Cormier, S., S. Vandormael-Pournin, C. Babinet, and M. Cohen-Tannoudji. 2004. Developmental expression of the Notch signaling pathway genes during mouse preimplantation development. *Gene Expr.Patterns.* 4:713-717.

56. Dirami, G., N. Ravindranath, M. V. Achi, and M. Dym. 2001. Expression of Notch pathway components in spermatogonia and Sertoli cells of neonatal mice. *J.Androl* 22:944-952.

57. Fehon, R. G., K. Johansen, I. Rebay, and S. Artavanis-Tsakonas. 1991. Complex cellular and subcellular regulation of notch expression during embryonic and imaginal development of Drosophila: implications for notch function. *J.Cell Biol.* 113:657-669.

58. Irvin, D. K., S. D. Zurcher, T. Nguyen, G. Weinmaster, and H. I. Kornblum. 2001. Expression patterns of Notch1, Notch2, and Notch3 suggest multiple functional roles for the Notch-DSL signaling system during brain development. *J.Comp Neurol.* 436:167-181.

59. Jonsson, J. I., Z. Xiang, M. Pettersson, M. Lardelli, and G. Nilsson. 2001. Distinct and regulated expression of Notch receptors in hematopoietic lineages and during myeloid differentiation. *Eur.J.Immunol.* 31:3240-3247.

60. Kooh, P. J., R. G. Fehon, and M. A. Muskavitch. 1993. Implications of dynamic patterns of Delta and Notch expression for cellular interactions during Drosophila development. *Development* 117:493-507.

61. Lammert, E., J. Brown, and D. A. Melton. 2000. Notch gene expression during pancreatic organogenesis. *Mech.Dev.* 94:199-203.

62. Singh, N., R. A. Phillips, N. N. Iscove, and S. E. Egan. 2000. Expression of notch receptors, notch ligands, and fringe genes in hematopoiesis. *Exp.Hematol.* 28:527-534.

63. Williams, R., U. Lendahl, and M. Lardelli. 1995. Complementary and combinatorial patterns of Notch gene family expression during early mouse development. *Mech.Dev.* 53:357-368.

64. Yamaguchi, E., S. Chiba, K. Kumano, A. Kunisato, T. Takahashi, T. Takahashi, and H. Hirai. 2002. Expression of Notch ligands, Jagged1, 2 and Delta1 in antigen presenting cells in mice. *Immunol.Lett.* 81:59-64.

65. Kwon, C., Z. Han, E. N. Olson, and D. Srivastava. 2005. MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling. *Proc.Natl.Acad.Sci.U.S.A* 102:18986-18991.

66. Marin, V. A. and T. C. Evans. 2003. Translational repression of a C. elegans Notch mRNA by the STAR/KH domain protein GLD-1. *Development* 130:2623-2632.

67. Mumm, J. S. and R. Kopan. 2000. Notch signaling: from the outside in. *Dev.Biol.* 228:151-165.

68. Baron, M., H. Aslam, M. Flasza, M. Fostier, J. E. Higgs, S. L. Mazaleyrat, and M. B. Wilkin. 2002. Multiple levels of Notch signal regulation (review). *Mol.Membr.Biol.* 19:27-38.

69. Le Borgne, R. and F. Schweisguth. 2003. Notch signaling: endocytosis makes delta signal better. *Curr.Biol.* 13:R273-R275.

70. Le Borgne, R., A. Bardin, and F. Schweisguth. 2005. The roles of receptor and ligand endocytosis in regulating Notch signaling. *Development* 132:1751-1762.

71. Le Borgne, R. 2006. Regulation of Notch signalling by endocytosis and endosomal sorting. *Curr.Opin.Cell Biol.* 18:213-222.

72. Wilkin, M. B. and M. Baron. 2005. Endocytic regulation of Notch activation and down-regulation (review). *Mol.Membr.Biol.* 22:279-289.

73. Weber, U., C. Eroglu, and M. Mlodzik. 2003. Phospholipid membrane composition affects EGF receptor and Notch signaling through effects on endocytosis during Drosophila development. *Dev.Cell* 5:559-570.

74. Shaye, D. D. and I. Greenwald. 2005. LIN-12/Notch trafficking and regulation of DSL ligand activity during vulval induction in Caenorhabditis elegans. *Development* 132:5081-5092.

75. Shaye, D. D. and I. Greenwald. 2002. Endocytosis-mediated downregulation of LIN-12/Notch upon Ras activation in Caenorhabditis elegans. *Nature* 420:686-690.

76. Jafar-Nejad, H., K. Norga, and H. Bellen. 2002. Numb: "Adapting" notch for endocytosis. *Dev.Cell* 3:155-156.

77. Sakata, T., H. Sakaguchi, L. Tsuda, A. Higashitani, T. Aigaki, K. Matsuno, and S. Hayashi. 2004. Drosophila Nedd4 regulates endocytosis of notch and suppresses its ligand-independent activation. *Curr.Biol.* 14:2228-2236.

78. Wilkin, M. B., A. M. Carbery, M. Fostier, H. Aslam, S. L. Mazaleyrat, J. Higgs, A. Myat, D. A. Evans, M. Cornell, and M. Baron. 2004. Regulation of notch endosomal sorting and signaling by Drosophila Nedd4 family proteins. *Curr.Biol.* 14:2237-2244.

79. Rooke, J., D. Pan, T. Xu, and G. M. Rubin. 1996. KUZ, a conserved metalloprotease-disintegrin protein with two roles in Drosophila neurogenesis. *Science* 273:1227-1231.

80. Pan, D. and G. M. Rubin. 1997. Kuzbanian controls proteolytic processing of Notch and mediates lateral inhibition during Drosophila and vertebrate neurogenesis. *Cell* 90:271-280.

81. Baron, M. 2003. An overview of the Notch signalling pathway. *Semin.Cell Dev.Biol.* 14:113-119.

82. Brou, C., F. Logeat, N. Gupta, C. Bessia, O. LeBail, J. R. Doedens, A. Cumano, P. Roux, R. A. Black, and A. Israel. 2000. A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. *Mol.Cell* 5:207-216.

83. Klein, T. 2002. kuzbanian is required cell autonomously during Notch signalling in the Drosophila wing. *Dev.Genes Evol.* 212:251-255.

84. Lieber, T., S. Kidd, and M. W. Young. 2002. kuzbanian-mediated cleavage of Drosophila Notch. *Genes Dev.* 16:209-221.

85. Schroeter, E. H., J. A. Kisslinger, and R. Kopan. 1998. Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* 393:382-386.

86. Levitan, D. and I. Greenwald. 1995. Facilitation of lin-12-mediated signalling by sel-12, a Caenorhabditis elegans S182 Alzheimer's disease gene. *Nature* 377:351-354.

87. Struhl, G. and I. Greenwald. 1999. Presenilin is required for activity and nuclear access of Notch in Drosophila. *Nature* 398:522-525.

88. Berdnik, D., T. Torok, M. Gonzalez-Gaitan, and J. A. Knoblich. 2002. The endocytic protein alpha-Adaptin is required for numb-mediated asymmetric cell division in Drosophila. *Dev.Cell* 3:221-231.

89. Matsuno, K., R. J. Diederich, M. J. Go, C. M. Blaumueller, and S. Artavanis-Tsakonas. 1995. Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. *Development* 121:2633-2644.

90. Izon, D. J., J. C. Aster, Y. He, A. Weng, F. G. Karnell, V. Patriub, L. Xu, S. Bakkour, C. Rodriguez, D. Allman, and W. S. Pear. 2002. Deltex1 redirects lymphoid progenitors to the B cell lineage by antagonizing Notch1. *Immunity.* 16:231-243.

91. Izon, D. J., J. A. Punt, and W. S. Pear. 2002. Deciphering the role of Notch signaling in lymphopoiesis. *Curr.Opin.Immunol.* 14:192-199.

92. Bailey, A. M. and J. W. Posakony. 1995. Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity. *Genes Dev.* 9:2609-2622.

93. Lecourtois, M. and F. Schweisguth. 1995. The neurogenic suppressor of hairless DNA-binding protein mediates the transcriptional activation of the enhancer of split complex genes triggered by Notch signaling. *Genes Dev.* 9:2598-2608.

94. Kao, H. Y., P. Ordentlich, N. Koyano-Nakagawa, Z. Tang, M. Downes, C. R. Kintner, R. M. Evans, and T. Kadesch. 1998. A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. *Genes Dev.* 12:2269-2277.

95. Maillard, I., A. P. Weng, A. C. Carpenter, C. G. Rodriguez, H. Sai, L. Xu, D. Allman, J. C. Aster, and W. S. Pear. 2004. Mastermind critically regulates Notch-mediated lymphoid cell fate decisions. *Blood* 104:1696-1702.

96. Zhou, S., M. Fujimuro, J. J. Hsieh, L. Chen, A. Miyamoto, G. Weinmaster, and S. D. Hayward. 2000. SKIP, a CBF1-associated protein, interacts with the ankyrin repeat domain of NotchIC To facilitate NotchIC function. *Mol.Cell Biol.* 20:2400-2410.

97. Kurooka, H. and T. Honjo. 2000. Functional interaction between the mouse notch1 intracellular region and histone acetyltransferases PCAF and GCN5. *J.Biol.Chem.* 275:17211-17220.

98. Wu, L., J. C. Aster, S. C. Blacklow, R. Lake, S. Artavanis-Tsakonas, and J. D. Griffin. 2000. MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. *Nat.Genet.* 26:484-489.

99. Wallberg, A. E., K. Pedersen, U. Lendahl, and R. G. Roeder. 2002. p300 and PCAF act cooperatively to mediate transcriptional activation from chromatin templates by notch intracellular domains in vitro. *Mol.Cell Biol.* 22:7812-7819.

100. Lai, E. C. 2002. Protein degradation: four E3s for the notch pathway. *Curr.Biol.* 12:R74-R78.

101. Wilson, A. and F. Radtke. 2006. Multiple functions of Notch signaling in self-renewing organs and cancer. *FEBS Lett.* 580:2860-2868.

102. Maillard, I., Y. He, and W. S. Pear. 2003. From the yolk sac to the spleen: New roles for Notch in regulating hematopoiesis. *Immunity.* 18:587-589.

103. Maillard, I., S. H. Adler, and W. S. Pear. 2003. Notch and the immune system. *Immunity.* 19:781-791.

104. Robey, E. A. and J. A. Bluestone. 2004. Notch signaling in lymphocyte development and function. *Curr.Opin.Immunol.* 16:360-366.

105. Kong, Y., J. Glickman, M. Subramaniam, A. Shahsafaei, K. P. Allamneni, J. C. Aster, J. Sklar, and M. E. Sunday. 2004. Functional diversity of notch family genes in fetal lung development. *Am.J.Physiol Lung Cell Mol.Physiol* 286:L1075-L1083.

106. Radtke, F., A. Wilson, B. Ernst, and H. R. MacDonald. 2002. The role of Notch signaling during hematopoietic lineage commitment. *Immunol.Rev.* 187:65-74.

107. Radtke, F., A. Wilson, and H. R. MacDonald. 2004. Notch signaling in T- and B-cell development. *Curr.Opin.Immunol.* 16:174-179.

108. Radtke, F., A. Wilson, S. J. Mancini, and H. R. MacDonald. 2004. Notch regulation of lymphocyte development and function. *Nat.Immunol.* 5:247-253.

109. Shimizu, K., S. Chiba, T. Saito, K. Kumano, Y. Hamada, and H. Hirai. 2002. Functional diversity among Notch1, Notch2, and Notch3 receptors. *Biochem.Biophys.Res.Commun.* 291:775-779.

110. Brooker, R., K. Hozumi, and J. Lewis. 2006. Notch ligands with contrasting functions: Jagged1 and Delta1 in the mouse inner ear. *Development* 133:1277-1286.

111. de La, C. A. and A. A. Freitas. 2006. Notch signaling: distinct ligands induce specific signals during lymphocyte development and maturation. *Immunol.Lett.* 102:1-9.

112. Jaleco, A. C., H. Neves, E. Hooijberg, P. Gameiro, N. Clode, M. Haury, D. Henrique, and L. Parreira. 2001. Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. *J.Exp.Med.* 194:991-1002.

113. Crowe, R., D. Henrique, D. Ish-Horowicz, and L. Niswander. 1998. A new role for Notch and Delta in cell fate decisions: patterning the feather array. *Development* 125:767-775.

114. Lehar, S. M., J. Dooley, A. G. Farr, and M. J. Bevan. 2004. Notch ligands Delta1 and Jagged1 transmit distinct signals to T cell precursors. *Blood.*

115. Hicks, C., E. Ladi, C. Lindsell, J. J. Hsieh, S. D. Hayward, A. Collazo, and G. Weinmaster. 2002. A secreted Delta1-Fc fusion protein functions both as an activator and inhibitor of Notch1 signaling. *J.Neurosci.Res.* 68:655-667.

116. Moloney, D. J., V. M. Panin, S. H. Johnston, J. Chen, L. Shao, R. Wilson, Y. Wang, P. Stanley, K. D. Irvine, R. S. Haltiwanger, and T. F. Vogt. 2000. Fringe is a glycosyltransferase that modifies Notch. *Nature* 406:369-375.

117. Hozumi, K., N. Negishi, D. Suzuki, N. Abe, Y. Sotomaru, N. Tamaoki, C. Mailhos, D. Ish-Horowicz, S. Habu, and M. J. Owen. 2004. Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells in vivo. *Nat.Immunol.* 5:638-644.

118. Kumano, K., S. Chiba, A. Kunisato, M. Sata, T. Saito, E. Nakagami-Yamaguchi, T. Yamaguchi, S. Masuda, K. Shimizu, T. Takahashi, S. Ogawa, Y. Hamada, and H. Hirai. 2003. Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity.* 18:699-711.

119. Radtke, F., A. Wilson, G. Stark, M. Bauer, J. van Meerwijk, H. R. MacDonald, and M. Aguet. 1999. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity.* 10:547-558.

120. Wolfer, A., A. Wilson, M. Nemir, H. R. MacDonald, and F. Radtke. 2002. Inactivation of Notch1 impairs VDJbeta rearrangement and allows pre-TCR-independent survival of early alpha beta Lineage Thymocytes. *Immunity.* 16:869-879.

121. Saito, T., S. Chiba, M. Ichikawa, A. Kunisato, T. Asai, K. Shimizu, T. Yamaguchi, G. Yamamoto, S. Seo, K. Kumano, E. Nakagami-Yamaguchi, Y. Hamada, S. Aizawa, and H. Hirai. 2003. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. *Immunity.* 18:675-685.

122. Witt, C. M., W. J. Won, V. Hurez, and C. A. Klug. 2003. Notch2 haploinsufficiency results in diminished B1 B cells and a severe reduction in marginal zone B cells. *J.Immunol.* 171:2783-2788.

123. Hamada, Y., Y. Kadokawa, M. Okabe, M. Ikawa, J. R. Coleman, and Y. Tsujimoto. 1999. Mutation in ankyrin repeats of the mouse Notch2 gene induces early embryonic lethality. *Development* 126:3415-3424.

124. Tanigaki, K., H. Han, N. Yamamoto, K. Tashiro, M. Ikegawa, K. Kuroda, A. Suzuki, T. Nakano, and T. Honjo. 2002. Notch-RBP-J signaling is involved in cell fate determination of marginal zone B cells. *Nat.Immunol.* 3:443-450.

125. Quong, M. W., D. P. Harris, S. L. Swain, and C. Murre. 1999. E2A activity is induced during B-cell activation to promote immunoglobulin class switch recombination. *EMBO J.* 18:6307-6318.

126. Sayegh, C. E., M. W. Quong, Y. Agata, and C. Murre. 2003. E-proteins directly regulate expression of activation-induced deaminase in mature B cells. *Nat.Immunol.* 4:586-593.

127. Rebollo, A. and C. Schmitt. 2003. Ikaros, Aiolos and Helios: transcription regulators and lymphoid malignancies. *Immunol.Cell Biol.* 81:171-175.

128. Georgopoulos, K. 2002. Haematopoietic cell-fate decisions, chromatin regulation and ikaros. *Nat.Rev.Immunol.* 2:162-174.

129. Morgan, B., L. Sun, N. Avitahl, K. Andrikopoulos, T. Ikeda, E. Gonzales, P. Wu, S. Neben, and K. Georgopoulos. 1997. Aiolos, a lymphoid restricted transcription factor that interacts with Ikaros to regulate lymphocyte differentiation. *EMBO J.* 16:2004-2013.

130. Georgopoulos, K., S. Winandy, and N. Avitahl. 1997. The role of the Ikaros gene in lymphocyte development and homeostasis. *Annu.Rev.Immunol.* 15:155-176.

131. Georgopoulos, K. 1997. Transcription factors required for lymphoid lineage commitment. *Curr.Opin.Immunol.* 9:222-227.

132. Wang, J. H., N. Avitahl, A. Cariappa, C. Friedrich, T. Ikeda, A. Renold, K. Andrikopoulos, L. Liang, S. Pillai, B. A. Morgan, and K. Georgopoulos. 1998. Aiolos regulates B cell activation and maturation to effector state. *Immunity.* 9:543-553.

133. Cariappa, A., M. Tang, C. Parng, E. Nebelitskiy, M. Carroll, K. Georgopoulos, and S. Pillai. 2001. The follicular versus marginal zone B lymphocyte cell fate decision is regulated by Aiolos, Btk, and CD21. *Immunity.* 14:603-615.

134. Makowska, A., N. N. Faizunnessa, P. Anderson, T. Midtvedt, and S. Cardell. 1999. CD1high B cells: a population of mixed origin. *Eur.J.Immunol.* 29:3285-3294.

135. Loder, F., B. Mutschler, R. J. Ray, C. J. Paige, P. Sideras, R. Torres, M. C. Lamers, and R. Carsetti. 1999. B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. *J.Exp.Med.* 190:75-89.

136. Kraal, G., K. Hoeben, and M. Janse. 1988. Splenic microenvironment of the CBA/N mouse: immunohistochemical analysis using monoclonal antibodies against lymphocytes and nonlymphoid cells. *Am.J.Anat.* 182:148-154.

137. Liu, Y. J., J. E. Lortan, S. Oldfield, and I. C. Maclennan. 1988. CBA/N mice have marginal zone B cells with normal surface immunoglobulin phenotype. *Adv.Exp.Med.Biol.* 237:105-111.

138. Hardy, R. R., K. Hayakawa, J. Haaijman, and L. A. Herzenberg. 1982. B-cell subpopulations identified by two-colour fluorescence analysis. *Nature* 297:589-591.

139. Hardy, R. R., K. Hayakawa, J. Haaijman, and L. A. Herzenberg. 1982. B-cell subpopulations identifiable by two-color fluorescence analysis using a dual-laser FACS. *Ann.N.Y.Acad.Sci.* 399:112-121.

140. Khan, W. N., F. W. Alt, R. M. Gerstein, B. A. Malynn, I. Larsson, G. Rathbun, L. Davidson, S. Muller, A. B. Kantor, L. A. Herzenberg, and . 1995. Defective B cell development and function in Btk-deficient mice. *Immunity.* 3:283-299.

141. Khan, W. N., P. Sideras, F. S. Rosen, and F. W. Alt. 1995. The role of Bruton's tyrosine kinase in B-cell development and function in mice and man. *Ann.N.Y.Acad.Sci.* 764:27-38.

142. Cariappa, A., T. J. Kim, and S. Pillai. 1999. Accelerated emigration of B lymphocytes in the Xid mouse. *J.Immunol.* 162:4417-4423.

143. Anderson, J. S., M. Teutsch, Z. Dong, and H. H. Wortis. 1996. An essential role for Bruton's [corrected] tyrosine kinase in the regulation of B-cell apoptosis. *Proc.Natl.Acad.Sci.U.S.A* 93:10966-10971.

144. Martin, F. and J. F. Kearney. 2000. Positive selection from newly formed to marginal zone B cells depends on the rate of clonal production, CD19, and btk. *Immunity.* 12:39-49.

145. Martin, F. and J. F. Kearney. 2000. Selection in the mature B cell repertoire. *Curr.Top.Microbiol.Immunol.* 252:97-105.

146. Ghosh, S., M. J. May, and E. B. Kopp. 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu.Rev.Immunol.* 16:225-260.

147. Siebenlist, U., K. Brown, and E. Claudio. 2005. Control of lymphocyte development by nuclear factor-kappaB. *Nat.Rev.Immunol.* 5:435-445.

148. Senftleben, U., Z. W. Li, V. Baud, and M. Karin. 2001. IKKbeta is essential for protecting T cells from TNFalpha-induced apoptosis. *Immunity.* 14:217-230.

149. Horwitz, B. H., M. L. Scott, S. R. Cherry, R. T. Bronson, and D. Baltimore. 1997. Failure of lymphopoiesis after adoptive transfer of NF-kappaB-deficient fetal liver cells. *Immunity.* 6:765-772.

150. Grossmann, M., D. Metcalf, J. Merryfull, A. Beg, D. Baltimore, and S. Gerondakis. 1999. The combined absence of the transcription factors Rel and RelA leads to multiple hemopoietic cell defects. *Proc.Natl.Acad.Sci.U.S.A* 96:11848-11853.

151. Schlissel, M. S. 2004. Regulation of activation and recombination of the murine Igkappa locus. *Immunol.Rev.* 200:215-223.

152. Gerondakis, S. and A. Strasser. 2003. The role of Rel/NF-kappaB transcription factors in B lymphocyte survival. *Semin.Immunol.* 15:159-166.

153. Claudio, E., K. Brown, S. Park, H. Wang, and U. Siebenlist. 2002. BAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells. *Nat.Immunol.* 3:958-965.

154. Cariappa, A., H. C. Liou, B. H. Horwitz, and S. Pillai. 2000. Nuclear factor kappa B is required for the development of marginal zone B lymphocytes. *J.Exp.Med.* 192:1175-1182.

155. Weih, D. S., Z. B. Yilmaz, and F. Weih. 2001. Essential role of RelB in germinal center and marginal zone formation and proper expression of homing chemokines. *J.Immunol.* 167:1909-1919.

156. Zarnegar, B., J. Q. He, G. Oganesyan, A. Hoffmann, D. Baltimore, and G. Cheng. 2004. Unique CD40-mediated biological program in B cell activation requires both type 1 and type 2 NF-kappaB activation pathways. *Proc.Natl.Acad.Sci.U.S.A* 101:8108-8113.

157. Bossen, C. and P. Schneider. 2006. BAFF, APRIL and their receptors: Structure, function and signaling. *Semin.Immunol.* 18:263-275.

158. Patke, A., I. Mecklenbrauker, and A. Tarakhovsky. 2004. Survival signaling in resting B cells. *Curr.Opin.Immunol.* 16:251-255.

159. Sasaki, Y., E. Derudder, E. Hobeika, R. Pelanda, M. Reth, K. Rajewsky, and M. Schmidt-Supprian. 2006. Canonical NF-kappaB activity, dispensable for B cell development, replaces BAFF-receptor signals and promotes B cell proliferation upon activation. *Immunity.* 24:729-739.

160. Xu, L. G., M. Wu, J. Hu, Z. Zhai, and H. B. Shu. 2002. Identification of downstream genes up-regulated by the tumor necrosis factor family member TALL-1. *J.Leukoc.Biol.* 72:410-416.

161. Cheng, P., A. Zlobin, V. Volgina, S. Gottipati, B. Osborne, E. J. Simel, L. Miele, and D. I. Gabrilovich. 2001. Notch-1 regulates NF-kappaB activity in hemopoietic progenitor cells. *J.Immunol.* 167:4458-4467.

162. Ferguson, A. R. and R. B. Corley. 2005. Accumulation of marginal zone B cells and accelerated loss of follicular dendritic cells in NF-kappaB p50-deficient mice. *BMC.Immunol.* 6:8.

163. Yu, X., L. Wang, Y. Luo, and R. G. Roeder. 2001. Identification and characterization of a novel OCA-B isoform. implications for a role in B cell signaling pathways. *Immunity.* 14:157-167.

164. Chasman, D., K. Cepek, P. A. Sharp, and C. O. Pabo. 1999. Crystal structure of an OCA-B peptide bound to an Oct-1 POU domain/octamer DNA complex: specific recognition of a protein-DNA interface. *Genes Dev.* 13:2650-2657.

165. Gstaiger, M., O. Georgiev, H. van Leeuwen, d. van, V, and W. Schaffner. 1996. The B cell coactivator Bob1 shows DNA sequence-dependent complex formation with Oct-1/Oct-2 factors, leading to differential promoter activation. *EMBO J.* 15:2781-2790.

166. Qin, X. F., A. Reichlin, Y. Luo, R. G. Roeder, and M. C. Nussenzweig. 1998. OCA-B integrates B cell antigen receptor-, C. *EMBO J.* 17:5066-5075.

167. Stevens, S., J. Ong, U. Kim, L. A. Eckhardt, and R. G. Roeder. 2000. Role of OCA-B in 3'-IgH enhancer function. *J.Immunol.* 164:5306-5312.

168. Stevens, S., L. Wang, and R. G. Roeder. 2000. Functional analysis of the OCA-B promoter. *J.Immunol.* 164:6372-6379.

169. Greiner, A., K. B. Muller, J. Hess, K. Pfeffer, H. K. Muller-Hermelink, and T. Wirth. 2000. Up-regulation of BOB.1/OBF.1 expression in normal germinal center B cells and germinal center-derived lymphomas. *Am.J.Pathol.* 156:501-507.

170. Schubart, K., S. Massa, D. Schubart, L. M. Corcoran, A. G. Rolink, and P. Matthias. 2001. B cell development and immunoglobulin gene transcription in the absence of Oct-2 and OBF-1. *Nat.Immunol.* 2:69-74.

171. Schubart, D. B., A. Rolink, K. Schubart, and P. Matthias. 2000. Cutting edge: lack of peripheral B cells and severe agammaglobulinemia in mice simultaneously lacking Bruton's tyrosine kinase and the B cell-specific transcriptional coactivator OBF-1. *J.Immunol.* 164:18-22.

172. Hess, J., P. J. Nielsen, K. D. Fischer, H. Bujard, and T. Wirth. 2001. The B lymphocyte-specific coactivator BOB.1/OBF.1 is required at multiple stages of B-cell development. *Mol.Cell Biol.* 21:1531-1539.

173. Samardzic, T., D. Marinkovic, P. J. Nielsen, L. Nitschke, and T. Wirth. 2002. BOB.1/OBF.1 deficiency affects marginal-zone B-cell compartment. *Mol.Cell Biol.* 22:8320-8331.

174. Nielsen, P. J., O. Georgiev, B. Lorenz, and W. Schaffner. 1996. B lymphocytes are impaired in mice lacking the transcriptional co-activator Bob1/OCA-B/OBF1. *Eur.J.Immunol.* 26:3214-3218.

175. Kim, U., C. S. Gunther, and R. G. Roeder. 2000. Genetic analyses of NFKB1 and OCA-B function: defects in B cells, serum IgM level, and antibody responses in Nfkb1-/-Oca-b-/- mice. *J.Immunol.* 165:6825-6832.

176. Andersson, T., A. Samuelsson, P. Matthias, and S. Pettersson. 2000. The lymphoid-specific cofactor OBF-1 is essential for the expression of a V(H) promoter/HS1,2 enhancer-linked transgene in late B cell development. *Mol.Immunol.* 37:889-899.

177. Casellas, R., M. Jankovic, G. Meyer, A. Gazumyan, Y. Luo, R. Roeder, and M. Nussenzweig. 2002. OcaB is required for normal transcription and V(D)J recombination of a subset of immunoglobulin kappa genes. *Cell* 110:575-585.

178. Samardzic, T., D. Marinkovic, C. P. Danzer, J. Gerlach, L. Nitschke, and T. Wirth. 2002. Reduction of marginal zone B cells in CD22-deficient mice. *Eur.J.Immunol.* 32:561-567.

179. Samardzic, T., J. Gerlach, K. Muller, D. Marinkovic, J. Hess, L. Nitschke, and T. Wirth. 2002. CD22 regulates early B cell development in BOB.1/OBF.1-deficient mice. *Eur.J.Immunol.* 32:2481-2489.

180. Schubart, D. B., A. Rolink, M. H. Kosco-Vilbois, F. Botteri, and P. Matthias. 1996. B-cell-specific coactivator OBF-1/OCA-B/Bob1 required for immune response and germinal centre formation. *Nature* 383:538-542.

181. Kim, U., X. F. Qin, S. Gong, S. Stevens, Y. Luo, M. Nussenzweig, and R. G. Roeder. 1996. The B-cell-specific transcription coactivator OCA-B/OBF-1/Bob-1 is essential for normal production of immunoglobulin isotypes. *Nature* 383:542-547.

182. Kim, U., R. Siegel, X. Ren, C. S. Gunther, T. Gaasterland, and R. G. Roeder. 2003. Identification of transcription coactivator OCA-B-dependent genes involved in antigen-dependent B cell differentiation by cDNA array analyses. *Proc.Natl.Acad.Sci.U.S.A* 100:8868-8873.

183. Shaffer, A. L., K. I. Lin, T. C. Kuo, X. Yu, E. M. Hurt, A. Rosenwald, J. M. Giltnane, L. Yang, H. Zhao, K. Calame, and L. M. Staudt. 2002. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity.* 17:51-62.

184. Lin, K. I., C. Angelin-Duclos, T. C. Kuo, and K. Calame. 2002. Blimp-1-dependent repression of Pax-5 is required for differentiation of B cells to immunoglobulin M-secreting plasma cells. *Mol.Cell Biol.* 22:4771-4780.

185. Calame, K. L. 2001. Plasma cells: finding new light at the end of B cell development. *Nat.Immunol.* 2:1103-1108.

186. Reimold, A. M., N. N. Iwakoshi, J. Manis, P. Vallabhajosyula, E. Szomolanyi-Tsuda, E. M. Gravallese, D. Friend, M. J. Grusby, F. Alt, and L. H. Glimcher. 2001. Plasma cell differentiation requires the transcription factor XBP-1. *Nature* 412:300-307.

187. Waldschmidt, T., K. Snapp, T. Foy, L. Tygrett, and C. Carpenter. 1992. B-cell subsets defined by the Fc epsilon R. *Ann.N.Y.Acad.Sci.* 651:84-98.

188. Sagaert, X. and C. Wolf-Peeters. 2003. Classification of B-cells according to their differentiation status, their micro-anatomical localisation and their developmental lineage. *Immunol.Lett.* 90:179-186.

189. Pillai, S., A. Cariappa, and S. T. Moran. 2004. Positive selection and lineage commitment during peripheral B-lymphocyte development. *Immunol.Rev.* 197:206-218.

190. Su, T. T., B. Guo, B. Wei, J. Braun, and D. J. Rawlings. 2004. Signaling in transitional type 2 B cells is critical for peripheral B-cell development. *Immunol.Rev.* 197:161-178.

191. Kearney, J. F. 1993. CD5+ B-cell networks. *Curr.Opin.Immunol.* 5:223-226.

192. Wang, H. and S. H. Clarke. 2004. Positive selection focuses the VH12 B-cell repertoire towards a single B1 specificity with survival function. *Immunol.Rev.* 197:51-59.

193. Vakil, M. and J. F. Kearney. 1988. Regulatory influences of neonatal multispecific antibodies on the developing B cell repertoire. *Int.Rev.Immunol.* 3:117-131.

194. Kenny, J. J., L. J. Rezanka, A. Lustig, R. T. Fischer, J. Yoder, S. Marshall, and D. L. Longo. 2000. Autoreactive B cells escape clonal deletion by expressing multiple antigen receptors. *J.Immunol.* 164:4111-4119.

195. Atencio, S., H. Amano, S. Izui, and B. L. Kotzin. 2004. Separation of the New Zealand Black genetic contribution to lupus from New Zealand Black determined expansions of marginal zone B and B1a cells. *J.Immunol.* 172:4159-4166.

196. Martin, F., A. M. Oliver, and J. F. Kearney. 2001. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity.* 14:617-629.

197. Mebius, R. E., M. A. Nolte, and G. Kraal. 2004. Development and function of the splenic marginal zone. *Crit Rev.Immunol.* 24:449-464.

198. Kraal, G. 1992. Cells in the marginal zone of the spleen. *Int.Rev.Cytol.* 132:31-74.

199. Dijkstra, C. D., E. Van Vliet, E. A. Dopp, A. A. van der Lelij, and G. Kraal. 1985. Marginal zone macrophages identified by a monoclonal antibody: characterization of immuno- and enzyme-histochemical properties and functional capacities. *Immunology* 55:23-30.

200. Kraal, G. and M. Janse. 1986. Marginal metallophilic cells of the mouse spleen identified by a monoclonal antibody. *Immunology* 58:665-669.

201. Song, H. and J. Cerny. 2003. Functional heterogeneity of marginal zone B cells revealed by their ability to generate both early antibody-forming cells and germinal centers with hypermutation and memory in response to a T-dependent antigen. *J.Exp.Med.* 198:1923-1935.

202. Gay, D., T. Saunders, S. Camper, and M. Weigert. 1993. Receptor editing: an approach by autoreactive B cells to escape tolerance. *J.Exp.Med.* 177:999-1008.

203. Li, H., Y. Jiang, E. L. Prak, M. Radic, and M. Weigert. 2001. Editors and editing of anti-DNA receptors. *Immunity.* 15:947-957.

204. Li, Y., Y. Louzoun, and M. Weigert. 2004. Editing anti-DNA B cells by Vlambdax. *J.Exp.Med.* 199:337-346.

205. Rezanka, L. J., J. J. Kenny, and D. L. Longo. 2005. 2 BCR or NOT 2. *Immunobiology* 210:769-774.

206. Rezanka, L. J., J. J. Kenny, and D. L. Longo. 2005. Dual isotype expressing B cells [kappa(+)/lambda(+)] arise during the ontogeny of B cells in the bone marrow of normal nontransgenic mice. *Cell Immunol.* 238:38-48.

207. Chen, X., F. Martin, K. A. Forbush, R. M. Perlmutter, and J. F. Kearney. 1997. Evidence for selection of a population of multi-reactive B cells into the splenic marginal zone. *Int.Immunol.* 9:27-41.

208. Chen, C., E. L. Prak, and M. Weigert. 1997. Editing disease-associated autoantibodies. *Immunity.* 6:97-105.

209. Chen, C., M. Z. Radic, J. Erikson, S. A. Camper, S. Litwin, R. R. Hardy, and M. Weigert. 1994. Deletion and editing of B cells that express antibodies to DNA. *J.Immunol.* 152:1970-1982.

210. Fermand, J. P., F. Danon, and J. C. Brouet. 1985. Characterization of a human monoclonal IgM with antibody activity to dsDNA. *Clin.Exp.Immunol.* 59:467-474.

211. Wellmann, U., A. Werner, and T. H. Winkler. 2001. Altered selection processes of B lymphocytes in autoimmune NZB/W mice, despite intact central tolerance against DNA. *Eur.J.Immunol.* 31:2800-2810.

212. Heltemes-Harris, L., X. Liu, and T. Manser. 2005. An antibody VH gene that promotes marginal zone B cell development and heavy chain allelic inclusion. *Int.Immunol.* 17:1447-1461.

213. Weaver, D., M. H. Reis, C. Albanese, F. Costantini, D. Baltimore, and T. Imanishi-Kari. 1986. Altered repertoire of endogenous immunoglobulin gene expression in transgenic mice containing a rearranged mu heavy chain gene. *Cell* 45:247-259.

214. Li, Y., H. Li, D. Ni, and M. Weigert. 2002. Anti-DNA B cells in MRL/lpr mice show altered differentiation and editing pattern. *J.Exp.Med.* 196:1543-1552.

215. Li, Y., H. Li, and M. Weigert. 2002. Autoreactive B cells in the marginal zone that express dual receptors. *J.Exp.Med.* 195:181-188.

216. Woodward, E. J. and J. W. Thomas. 2005. Multiple germline kappa light chains generate anti-insulin B cells in nonobese diabetic mice. *J.Immunol.* 175:1073-1079.

217. Radic, M. Z., J. Erikson, S. Litwin, and M. Weigert. 1993. B lymphocytes may escape tolerance by revising their antigen receptors. *J.Exp.Med.* 177:1165-1173.

218. Acevedo-Suarez, C. A., C. Hulbert, E. J. Woodward, and J. W. Thomas. 2005. Uncoupling of anergy from developmental arrest in anti-insulin B cells supports the development of autoimmune diabetes. *J.Immunol.* 174:827-833.

219. Rolf, J., V. Motta, N. Duarte, M. Lundholm, E. Berntman, M. L. Bergman, L. Sorokin, S. L. Cardell, and D. Holmberg. 2005. The enlarged population of marginal zone/CD1d(high) B lymphocytes in nonobese diabetic mice maps to diabetes susceptibility region Idd11. *J.Immunol.* 174:4821-4827.

220. Wither, J. E., A. D. Paterson, and B. Vukusic. 2000. Genetic dissection of B cell traits in New Zealand black mice. The expanded population of B cells expressing up-regulated costimulatory molecules shows linkage to Nba2. *Eur.J.Immunol.* 30:356-365.

221. Wither, J. E., C. Loh, G. Lajoie, S. Heinrichs, Y. C. Cai, G. Bonventi, and R. MacLeod. 2005. Colocalization of expansion of the splenic marginal zone population with abnormal B cell activation and autoantibody production in B6 mice with an introgressed New Zealand Black chromosome 13 interval. *J.Immunol.* 175:4309-4319.

222. Wither, J. E., V. Roy, and L. A. Brennan. 2000. Activated B cells express increased levels of costimulatory molecules in young autoimmune NZB and (NZB x NZW)F(1) mice. *Clin.Immunol.* 94:51-63.

223. Grimaldi, C. M., D. J. Michael, and B. Diamond. 2001. Cutting edge: expansion and activation of a population of autoreactive marginal zone B cells in a model of estrogen-induced lupus. *J.Immunol.* 167:1886-1890.

224. Zeng, D., M. K. Lee, J. Tung, A. Brendolan, and S. Strober. 2000. Cutting edge: a role for CD1 in the pathogenesis of lupus in NZB/NZW mice. *J.Immunol.* 164:5000-5004.

225. Martin, F. and J. F. Kearney. 2002. Marginal-zone B cells. *Nat.Rev.Immunol.* 2:323-335.

226. Oliver, A. M., F. Martin, G. L. Gartland, R. H. Carter, and J. F. Kearney. 1997. Marginal zone B cells exhibit unique activation, proliferative and immunoglobulin secretory responses. *Eur.J.Immunol.* 27:2366-2374.

227. Oliver, A. M., F. Martin, and J. F. Kearney. 1999. IgMhighCD21high lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells. *J.Immunol.* 162:7198-7207.

228. Spencer, J., M. E. Perry, and D. K. Dunn-Walters. 1998. Human marginal-zone B cells. *Immunol.Today* 19:421-426.

229. Whipple, E. C., R. S. Shanahan, A. H. Ditto, R. P. Taylor, and M. A. Lindorfer. 2004. Analyses of the in vivo trafficking of stoichiometric doses of an anti-complement receptor 1/2 monoclonal antibody infused intravenously in mice. *J.Immunol.* 173:2297-2306.

230. Attanavanich, K. and J. F. Kearney. 2004. Marginal zone, but not follicular B cells, are potent activators of naive CD4 T cells. *J.Immunol.* 172:803-811.

231. Goodnow, C. C. 1996. Balancing immunity and tolerance: deleting and tuning lymphocyte repertoires. *Proc.Natl.Acad.Sci.U.S.A* 93:2264-2271.

232. Townsend, S. E., B. C. Weintraub, and C. C. Goodnow. 1999. Growing up on the streets: why B-cell development differs from T-cell development. *Immunol.Today* 20:217-220.

233. Vinuesa, C. G., D. M. Sze, M. C. Cook, K. M. Toellner, G. G. Klaus, J. Ball, and I. C. Maclennan. 2003. Recirculating and germinal center B cells differentiate into cells responsive to polysaccharide antigens. *Eur.J.Immunol.* 33:297-305.

234. Noorchashm, H., D. J. Moore, Y. K. Lieu, N. Noorchashm, A. Schlachterman, H. K. Song, C. F. Barker, and A. Naji. 1999. Contribution of the innate immune system to autoimmune diabetes: a role for the CR1/CR2 complement receptors. *Cell Immunol.* 195:75-79.

235. Serreze, D. V., S. A. Fleming, H. D. Chapman, S. D. Richard, E. H. Leiter, and R. M. Tisch. 1998. B lymphocytes are critical antigen-presenting cells for the initiation of T

cell-mediated autoimmune diabetes in nonobese diabetic mice. *Journal of Immunology* 161:3912-3918.

236. Chan, O., M. P. Madaio, and M. J. Shlomchik. 1997. The roles of B cells in MRL/lpr murine lupus. *Ann.N.Y.Acad.Sci.* 815:75-87.

237. William, J., C. Euler, S. Christensen, and M. J. Shlomchik. 2002. Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. *Science* 297:2066-2070.

238. Bezbradica, J. S., A. K. Stanic, N. Matsuki, H. Bour-Jordan, J. A. Bluestone, J. W. Thomas, D. Unutmaz, L. Van Kaer, and S. Joyce. 2005. Distinct roles of dendritic cells and B cells in Va14Ja18 natural T cell activation in vivo. *J.Immunol.* 174:4696-4705.

239. Groom, J., S. L. Kalled, A. H. Cutler, C. Olson, S. A. Woodcock, P. Schneider, J. Tschopp, T. G. Cachero, M. Batten, J. Wheway, D. Mauri, D. Cavill, T. P. Gordon, C. R. Mackay, and F. Mackay. 2002. Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjogren's syndrome. *J.Clin.Invest* 109:59-68.

240. Carvalho, T. L., T. Mota-Santos, A. Cumano, J. Demengeot, and P. Vieira. 2001. Arrested B lymphopoiesis and persistence of activated B cells in adult interleukin 7(-/)- mice. *J.Exp.Med.* 194:1141-1150.

241. Hao, Z. and K. Rajewsky. 2001. Homeostasis of peripheral B cells in the absence of B cell influx from the bone marrow. *J.Exp.Med.* 194:1151-1164.

242. Kraus, M., L. I. Pao, A. Reichlin, Y. Hu, B. Canono, J. C. Cambier, M. C. Nussenzweig, and K. Rajewsky. 2001. Interference with immunoglobulin (Ig)alpha immunoreceptor tyrosine-based activation motif (ITAM) phosphorylation modulates or blocks B cell development, depending on the availability of an Igbeta cytoplasmic tail. *J.Exp.Med.* 194:455-469.

243. Otero, D. C., A. N. Anzelon, and R. C. Rickert. 2003. CD19 function in early and late B cell development: I. Maintenance of follicular and marginal zone B cells requires CD19-dependent survival signals. *J.Immunol.* 170:73-83.

244. Mackay, F., S. A. Woodcock, P. Lawton, C. Ambrose, M. Baetscher, P. Schneider, J. Tschopp, and J. L. Browning. 1999. Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *J.Exp.Med.* 190:1697-1710.

245. Batten, M., J. Groom, T. G. Cachero, F. Qian, P. Schneider, J. Tschopp, J. L. Browning, and F. Mackay. 2000. BAFF mediates survival of peripheral immature B lymphocytes. *J.Exp.Med.* 192:1453-1466.

246. Khare, S. D., I. Sarosi, X. Z. Xia, S. McCabe, K. Miner, I. Solovyev, N. Hawkins, M. Kelley, D. Chang, G. Van, L. Ross, J. Delaney, L. Wang, D. Lacey, W. J. Boyle, and H. Hsu. 2000. Severe B cell hyperplasia and autoimmune disease in TALL-1 transgenic mice. *Proc.Natl.Acad.Sci.U.S.A* 97:3370-3375.

247. Matsumoto, M., S. Mariathasan, M. H. Nahm, F. Baranyay, J. J. Peschon, and D. D. Chaplin. 1996. Role of lymphotoxin and the type I TNF receptor in the formation of germinal centers. *Science* 271:1289-1291.

248. Matsumoto, M., Y. X. Fu, H. Molina, G. Huang, J. Kim, D. A. Thomas, M. H. Nahm, and D. D. Chaplin. 1997. Distinct roles of lymphotoxin alpha and the type I tumor necrosis factor (TNF) receptor in the establishment of follicular dendritic cells from non-bone marrow-derived cells. *J.Exp.Med.* 186:1997-2004.

249. Ansel, K. M., V. N. Ngo, P. L. Hyman, S. A. Luther, R. Forster, J. D. Sedgwick, J. L. Browning, M. Lipp, and J. G. Cyster. 2000. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* 406:309-314.

250. Korner, H., T. H. Winkler, J. D. Sedgwick, M. Rollinghoff, A. Basten, and M. C. Cook. 2001. Recirculating and marginal zone B cell populations can be established and maintained independently of primary and secondary follicles. *Immunol.Cell Biol.* 79:54-61.

251. Guinamard, R., M. Okigaki, J. Schlessinger, and J. V. Ravetch. 2000. Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. *Nat.Immunol.* 1:31-36.

252. Girkontaite, I., K. Missy, V. Sakk, A. Harenberg, K. Tedford, T. Potzel, K. Pfeffer, and K. D. Fischer. 2001. Lsc is required for marginal zone B cells, regulation of lymphocyte motility and immune responses. *Nat.Immunol.* 2:855-862.

253. Fukui, Y., O. Hashimoto, T. Sanui, T. Oono, H. Koga, M. Abe, A. Inayoshi, M. Noda, M. Oike, T. Shirai, and T. Sasazuki. 2001. Haematopoietic cell-specific CDM family protein DOCK2 is essential for lymphocyte migration. *Nature* 412:826-831.

254. Vigorito, E., L. Gambardella, F. Colucci, S. McAdam, and M. Turner. 2005. Vav proteins regulate peripheral B-cell survival. *Blood* 106:2391-2398.

255. Pike, K. A., S. Iacampo, J. E. Friedmann, and M. J. Ratcliffe. 2004. The cytoplasmic domain of Ig alpha is necessary and sufficient to support efficient early B cell development. *J.Immunol.* 172:2210-2218.

256. Bannish, G., E. M. Fuentes-Panana, J. C. Cambier, W. S. Pear, and J. G. Monroe. 2001. Ligand-independent signaling functions for the B lymphocyte antigen receptor and their role in positive selection during B lymphopoiesis. *J.Exp.Med.* 194:1583-1596.

257. Minegishi, Y., E. Coustan-Smith, L. Rapalus, F. Ersoy, D. Campana, and M. E. Conley. 1999. Mutations in Igalpha (CD79a) result in a complete block in B-cell development. *J.Clin.Invest* 104:1115-1121.

258. Teh, Y. M. and M. S. Neuberger. 1997. The immunoglobulin (Ig)alpha and Igbeta cytoplasmic domains are independently sufficient to signal B cell maturation and activation in transgenic mice. *J.Exp.Med.* 185:1753-1758.

259. Girkontaite, I., V. Sakk, M. Wagner, T. Borggrefe, K. Tedford, J. Chun, and K. D. Fischer. 2004. The sphingosine-1-phosphate (S1P) lysophospholipid receptor S1P3 regulates MAdCAM-1+ endothelial cells in splenic marginal sinus organization. *J.Exp.Med.* 200:1491-1501.

260. Prodeus, A. P., S. Goerg, L. M. Shen, O. O. Pozdnyakova, L. Chu, E. M. Alicot, C. C. Goodnow, and M. C. Carroll. 1998. A critical role for complement in maintenance of self-tolerance. *Immunity.* 9:721-731.

261. Weintraub, B. C. and C. C. Goodnow. 1998. Immune Responses: costimulatory receptors have their say. *Curr.Biol.* 8:R575-R577.

262. Cyster, J. G. and C. C. Goodnow. 1995. Pertussis toxin inhibits migration of B and T lymphocytes into splenic white pulp cords. *J.Exp.Med.* 182:581-586.

263. Goodnow, C. C. and J. G. Cyster. 1997. Lymphocyte homing: the scent of a follicle. *Curr.Biol.* 7:R219-R222.

264. Wang, H. and S. H. Clarke. 2003. Evidence for a ligand-mediated positive selection signal in differentiation to a mature B cell. *J.Immunol.* 171:6381-6388.

265. Melchers, F. 2005. The pre-B-cell receptor: selector of fitting immunoglobulin heavy chains for the B-cell repertoire. *Nat.Rev.Immunol.* 5:578-584.

266. Melchers, F. 1995. B cell differentiation in bone marrow. *Clin.Immunol.Immunopathol.* 76:S188-S191.

267. Reth, M. 1992. Antigen receptors on B lymphocytes. *Annu.Rev.Immunol.* 10:97-121.

268. Lartigue, A., P. Courville, I. Auquit, A. Francois, C. Arnoult, F. Tron, D. Gilbert, and P. Musette. 2006. Role of TLR9 in anti-nucleosome and anti-DNA antibody production in lpr mutation-induced murine lupus. *J.Immunol.* 177:1349-1354.

269. Christensen, S. R., M. Kashgarian, L. Alexopoulou, R. A. Flavell, S. Akira, and M. J. Shlomchik. 2005. Toll-like receptor 9 controls anti-DNA autoantibody production in murine lupus. *J.Exp.Med.* 202:321-331.

270. Marshak-Rothstein, A., L. Busconi, C. M. Lau, A. S. Tabor, E. A. Leadbetter, S. Akira, A. M. Krieg, G. B. Lipford, G. A. Viglianti, and I. R. Rifkin. 2004. Comparison of CpG s-ODNs, chromatin immune complexes, and dsDNA fragment immune complexes in the TLR9-dependent activation of rheumatoid factor B cells. *J.Endotoxin.Res.* 10:247-251.

271. Viglianti, G. A., C. M. Lau, T. M. Hanley, B. A. Miko, M. J. Shlomchik, and A. Marshak-Rothstein. 2003. Activation of autoreactive B cells by CpG dsDNA. *Immunity.* 19:837-847.

272. Sultzer, B. M. 1972. Genetic control of host responses to endotoxin. *Infect.Immun.* 5:107-113.

273. Coutinho, A., G. Moller, and E. Gronowicz. 1975. Genetical control of B-cell responses. IV. Inheritance of the unresponsiveness to lipopolysaccharides. *J.Exp.Med.* 142:253-258.

274. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282:2085-2088.

275. Poltorak, A., I. Smirnova, X. He, M. Y. Liu, C. Van Huffel, O. McNally, D. Birdwell, E. Alejos, M. Silva, X. Du, P. Thompson, E. K. Chan, J. Ledesma, B. Roe, S. Clifton, S. N. Vogel, and B. Beutler. 1998. Genetic and physical mapping of the Lps locus: identification of the toll-4 receptor as a candidate gene in the critical region. *Blood Cells Mol.Dis.* 24:340-355.

276. Crawford, K. and C. A. Alper. 2000. Genetics of the complement system. *Rev.Immunogenet.* 2:323-338.

277. Nonaka, M. 2001. Evolution of the complement system. *Curr.Opin.Immunol.* 13:69-73.

278. Whaley, K. and W. Schwaeble. 1997. Complement and complement deficiencies. *Semin.Liver Dis.* 17:297-310.

279. Carroll, M. C. 1998. CD21/CD35 in B cell activation. *Semin.Immunol.* 10:279-286.

280. Cooper, N. R., B. M. Bradt, J. S. Rhim, and G. R. Nemerow. 1990. CR2 complement receptor. *J.Invest Dermatol.* 94:112S-117S.

281. Frade, R. 1990. Structure and signalling functions of C3 receptors on human B cells. *Semin.Immunol.* 2:159-164.

282. Fearon, D. T. and J. M. Ahearn. 1990. Complement receptor type 1 (C3b/C4b receptor; CD35) and complement receptor type 2 (C3d/Epstein-Barr virus receptor; CD21). *Curr.Top.Microbiol.Immunol.* 153:83-98.

283. Nemerow, G. R., M. D. Moore, and N. R. Cooper. 1990. Structure and function of the B-lymphocyte Epstein-Barr virus/C3d receptor. *Adv.Cancer Res.* 54:273-300.

284. Ahearn, J. M. and D. T. Fearon. 1989. Structure and function of the complement receptors, CR1 (CD35) and CR2 (CD21). *Adv.Immunol.* 46:183-219.

285. Holers, V. M. 1989. Complement receptors. *Year Immunol.* 4:231-240.

286. Cooper, N. R., M. D. Moore, and G. R. Nemerow. 1988. Immunobiology of CR2, the B lymphocyte receptor for Epstein-Barr virus and the C3d complement fragment. *Annu.Rev.Immunol.* 6:85-113.

287. Fearon, D. T. and M. C. Carroll. 2000. Regulation of B lymphocyte responses to foreign and self-antigens by the CD19/CD21 complex. *Annu.Rev.Immunol.* 18:393-422.

288. Matsumoto, A. K., D. R. Martin, R. H. Carter, L. B. Klickstein, J. M. Ahearn, and D. T. Fearon. 1993. Functional dissection of the CD21/CD19/TAPA-1/Leu-13 complex of B lymphocytes. *J.Exp.Med.* 178:1407-1417.

289. Hayakawa, K. and R. R. Hardy. 2000. Development and function of B-1 cells. *Curr.Opin.Immunol.* 12:346-353.

290. Pillai, S. 2005. Two lymphoid roads diverge--but does antigen bade B cells to take the road less traveled? *Immunity.* 23:242-244.

291. Wen, L., J. Brill-Dashoff, S. A. Shinton, M. Asano, R. R. Hardy, and K. Hayakawa. 2005. Evidence of marginal-zone B cell-positive selection in spleen. *Immunity.* 23:297-308.

292. Wang, H. and S. H. Clarke. 2004. Regulation of B-cell development by antibody specificity. *Curr.Opin.Immunol.* 16:246-250.

293. Tsubata, T. and T. Honjo. 2000. B cell tolerance and autoimmunity. *Rev.Immunogenet.* 2:18-25.

294. Miller, J. F. and A. Basten. 1996. Mechanisms of tolerance to self. *Curr.Opin.Immunol.* 8:815-821.

295. Ferry, H., J. C. Leung, G. Lewis, A. Nijnik, K. Silver, T. Lambe, and R. J. Cornall. 2006. B-cell tolerance. *Transplantation* 81:308-315.

296. Goodnow, C. C. 1992. B-cell tolerance. *Curr.Opin.Immunol.* 4:703-710.

297. Goodnow, C. C., J. G. Cyster, S. B. Hartley, S. E. Bell, M. P. Cooke, J. I. Healy, S. Akkaraju, J. C. Rathmell, S. L. Pogue, and K. P. Shokat. 1995. Self-tolerance checkpoints in B lymphocyte development. *Adv.Immunol.* 59:279-368.

298. Nemazee, D. and K. A. Hogquist. 2003. Antigen receptor selection by editing or downregulation of V(D)J recombination. *Curr.Opin.Immunol.* 15:182-189.

299. Rojas, M., C. Hulbert, and J. W. Thomas. 2001. Anergy and not clonal ignorance determines the fate of B cells that recognize a physiological autoantigen. *J.Immunol.* 166:3194-3200.

300. Cyster, J. G. and C. C. Goodnow. 1995. Antigen-induced exclusion from follicles and anergy are separate and complementary processes that influence peripheral B cell fate. *Immunity.* 3:691-701.

301. Fang, W., B. C. Weintraub, B. Dunlap, P. Garside, K. A. Pape, M. K. Jenkins, C. C. Goodnow, D. L. Mueller, and T. W. Behrens. 1998. Self-reactive B lymphocytes overexpressing Bcl-xL escape negative selection and are tolerized by clonal anergy and receptor editing. *Immunity.* 9:35-45.

302. Sieckmann, D. G., K. Holmes, P. Hornbeck, E. Martin, G. Guelde, S. Bondada, D. L. Longo, and J. J. Kenny. 1994. B cells from M167 mu kappa transgenic mice fail to proliferate after stimulation with soluble anti-Ig antibodies. A model for antigen-induced B cell anergy. *J.Immunol.* 152:4873-4883.

303. Goodnow, C. C., J. Crosbie, H. Jorgensen, R. A. Brink, and A. Basten. 1989. Induction of self-tolerance in mature peripheral B lymphocytes. *Nature* 342:385-391.

304. Petrenko, O., A. Beavis, M. Klaine, R. Kittappa, I. Godin, and I. R. Lemischka. 1999. The molecular characterization of the fetal stem cell marker AA4. *Immunity.* 10:691-700.

305. Norsworthy, P. J., P. R. Taylor, M. J. Walport, and M. Botto. 1999. Cloning of the mouse homolog of the 126-kDa human C1q/MBL/SP-A receptor, C1qR(p). *Mamm.Genome* 10:789-793.

306. Rolink, A. G., C. Schaniel, J. Andersson, and F. Melchers. 2001. Selection events operating at various stages in B cell development. *Curr.Opin.Immunol.* 13:202-207.

307. Rolink, A. G., J. Andersson, and F. Melchers. 1998. Characterization of immature B cells by a novel monoclonal antibody, by turnover and by mitogen reactivity. *Eur.J.Immunol.* 28:3738-3748.

308. Rolink, A. G., T. Brocker, H. Bluethmann, M. H. Kosco-Vilbois, J. Andersson, and F. Melchers. 1999. Mutations affecting either generation or survival of cells influence the pool size of mature B cells. *Immunity.* 10:619-628.

309. McKearn, J. P., C. Baum, and J. M. Davie. 1984. Cell surface antigens expressed by subsets of pre-B cells and B cells. *J.Immunol.* 132:332-339.

310. Rolink, A. G., J. Tschopp, P. Schneider, and F. Melchers. 2002. BAFF is a survival and maturation factor for mouse B cells. *Eur.J.Immunol.* 32:2004-2010.

311. Hulbert, C., B. Riseili, M. Rojas, and J. W. Thomas. 2001. B cell specificity contributes to the outcome of diabetes in nonobese diabetic mice. *J.Immunol.* 167:5535-5538.

312. Waid, D. M., G. M. Vaitaitis, and D. H. Wagner, Jr. 2004. Peripheral CD4loCD40+ auto-aggressive T cell expansion during insulin-dependent diabetes mellitus. *Eur.J.Immunol.* 34:1488-1497.

313. Judkowski, V., C. Pinilla, K. Schroder, L. Tucker, N. Sarvetnick, and D. B. Wilson. 2001. Identification of MHC class II-restricted peptide ligands, including a glutamic acid decarboxylase 65 sequence, that stimulate diabetogenic T cells from transgenic BDC2.5 nonobese diabetic mice. *J.Immunol.* 166:908-917.

314. Poulin, M. and K. Haskins. 2000. Induction of diabetes in nonobese diabetic mice by Th2 T cell clones from a TCR transgenic mouse. *J.Immunol.* 164:3072-3078.

315. Shimizu, J., O. Kanagawa, and E. R. Unanue. 1993. Presentation of beta-cell antigens to CD4+ and CD8+ T cells of non-obese diabetic mice. *J.Immunol.* 151:1723-1730.

316. Christianson, S. W., L. D. Shultz, and E. H. Leiter. 1993. Adoptive transfer of diabetes into immunodeficient NOD-scid/scid mice. Relative contributions of CD4+ and CD8+ T-cells from diabetic versus prediabetic NOD.NON-Thy-1a donors. *Diabetes* 42:44-55.

317. Pacheco-Silva, A., M. G. Bastos, R. A. Muggia, O. Pankewycz, J. Nichols, J. R. Murphy, T. B. Strom, and V. E. Rubin-Kelley. 1992. Interleukin 2 receptor targeted fusion toxin (DAB486-IL-2) treatment blocks diabetogenic autoimmunity in non-obese diabetic mice. *Eur.J.Immunol.* 22:697-702.

318. Nepom, G. T. 1990. A unified hypothesis for the complex genetics of HLA associations with IDDM. *Diabetes* 39:1153-1157.

319. Robles, D. T., P. R. Fain, P. A. Gottlieb, and G. S. Eisenbarth. 2002. The genetics of autoimmune polyendocrine syndrome type II. *Endocrinol.Metab Clin.North Am.* 31:353-vii.

320. Robles, D. T., G. S. Eisenbarth, T. Wang, H. A. Erlich, T. L. Bugawan, S. R. Babu, K. Barriga, J. M. Norris, M. Hoffman, G. Klingensmith, L. Yu, and M. Rewers. 2002. Millennium award recipient contribution. Identification of children with early onset and high incidence of anti-islet autoantibodies. *Clin.Immunol.* 102:217-224.

321. Todd, J. A. 1990. Genetic control of autoimmunity in type 1 diabetes. *Immunol.Today* 11:122-129.

322. Todd, J. A. 1990. The role of MHC class II genes in susceptibility to insulin-dependent diabetes mellitus. *Curr.Top.Microbiol.Immunol.* 164:17-40.

323. Todd, J. A. and M. Farrall. 1996. Panning for gold: genome-wide scanning for linkage in type 1 diabetes. *Hum.Mol.Genet.* 5 Spec No:1443-1448.

324. Todd, J. A. and L. S. Wicker. 2001. Genetic protection from the inflammatory disease type 1 diabetes in humans and animal models. *Immunity.* 15:387-395.

325. Serreze, D. V., H. D. Chapman, D. S. Varnum, M. S. Hanson, P. C. Reifsnyder, S. D. Richard, S. A. Fleming, E. H. Leiter, and L. D. Shultz. 1996. B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: Analysis of a new "speed congenic" stock of NOD.Ig mu(null) mice. *Journal of Experimental Medicine* 184:2049-2053.

326. Noorchashm, H., N. Noorchashm, J. Kern, S. Y. Rostami, C. F. Barker, and A. Naji. 1997. B-cells are required for the initiation of insulitis and sialitis in nonobese diabetic mice. *Diabetes* 46:941-946.

327. Greeley, S. A., D. J. Moore, H. Noorchashm, L. E. Noto, S. Y. Rostami, A. Schlachterman, H. K. Song, B. Koeberlein, C. F. Barker, and A. Naji. 2001. Impaired activation of islet-reactive CD4 T cells in pancreatic lymph nodes of B cell-deficient nonobese diabetic mice. *J.Immunol.* 167:4351-4357.

328. Noorchashm, H., Y. K. Lieu, N. Noorchashm, S. Y. Rostami, S. A. Greeley, A. Schlachterman, H. K. Song, L. E. Noto, A. M. Jevnikar, C. F. Barker, and A. Naji. 1999. I-Ag7-mediated antigen presentation by B lymphocytes is critical in overcoming a checkpoint in T cell tolerance to islet beta cells of nonobese diabetic mice. *J.Immunol.* 163:743-750.

329. Silveira, P. A., J. Dombrowsky, E. Johnson, H. D. Chapman, D. Nemazee, and D. V. Serreze. 2004. B cell selection defects underlie the development of diabetogenic APCs in nonobese diabetic mice. *Journal of Immunology* 172:5086-5094.

330. Falcone, M., J. Lee, G. Patstone, B. Yeung, and N. Sarvetnick. 1998. B lymphocytes are crucial antigen-presenting cells in the pathogenic autoimmune response to GAD65 antigen in nonobese diabetic mice. *J.Immunol.* 161:1163-1168.

331. Abiru, N., L. Yu, D. Miao, A. K. Maniatis, E. Liu, H. Moriyama, and G. S. Eisenbarth. 2001. Transient insulin autoantibody expression independent of development of diabetes: comparison of NOD and NOR strains. *J.Autoimmun.* 17:1-6.

332. Barker, J. M., K. J. Barriga, L. Yu, D. Miao, H. A. Erlich, J. M. Norris, G. S. Eisenbarth, and M. Rewers. 2004. Prediction of autoantibody positivity and progression to type 1 diabetes: Diabetes Autoimmunity Study in the Young (DAISY). *J.Clin.Endocrinol.Metab* 89:3896-3902.

333. Yu, L., D. T. Robles, N. Abiru, P. Kaur, M. Rewers, K. Kelemen, and G. S. Eisenbarth. 2000. Early expression of antiinsulin autoantibodies of humans and the NOD mouse: evidence for early determination of subsequent diabetes. *Proc.Natl.Acad.Sci.U.S.A* 97:1701-1706.

334. Eisenbarth, G. S., R. C. Nayak, and S. L. Rabinowe. 1988. Type I diabetes as a chronic autoimmune disease. *J.Diabet.Complications* 2:54-58.

335. Eisenbarth, G. S., H. Moriyama, D. T. Robles, E. Liu, L. Yu, S. Babu, M. Redondo, P. Gottlieb, D. Wegmann, and M. Rewers. 2002. Insulin autoimmunity: prediction/precipitation/prevention type 1A diabetes. *Autoimmun.Rev.* 1:139-145.

336. Schatz, D. A. and P. J. Bingley. 2001. Update on major trials for the prevention of type 1 diabetes mellitus: the American Diabetes Prevention Trial (DPT-1) and the European Nicotinamide Diabetes Intervention Trial (ENDIT). *J.Pediatr.Endocrinol.Metab* 14 Suppl 1:619-622.

337. Verge, C. F., D. Stenger, E. Bonifacio, P. G. Colman, C. Pilcher, P. J. Bingley, and G. S. Eisenbarth. 1998. Combined use of autoantibodies (IA-2 autoantibody, GAD autoantibody, insulin autoantibody, cytoplasmic islet cell antibodies) in type 1 diabetes: Combinatorial Islet Autoantibody Workshop. *Diabetes* 47:1857-1866.

338. Moore, D. J., X. Huang, M. K. Lee, M. M. Lian, M. Chiaccio, H. Chen, B. Koeberlein, R. Zhong, J. F. Markmann, and S. Deng. 2004. Resistance to anti-CD45RB-induced tolerance in NOD mice: mechanisms involved. *Transpl.Int.* 17:261-269.

339. Bach, J. F. 1993. Strategies in immunotherapy of insulin-dependent diabetes mellitus. *Ann.N.Y.Acad.Sci.* 696:364-376.

340. Hutchings, P., P. Tonks, and A. Cooke. 1997. Effect of MHC transgene expression on spontaneous insulin autoantibody class switch in nonobese diabetic mice. *Diabetes* 46:779-784.

341. Silveira, P. A., E. Johnson, H. D. Chapman, T. Bui, R. M. Tisch, and D. V. Serreze. 2002. The preferential ability of B lymphocytes to act as diabetogenic APC in NOD mice depends on expression of self-antigen-specific immunoglobulin receptors. *European Journal of Immunology* 32:3657-3666.

342. Thomas, J. W., R. P. Bucy, and J. A. Kapp. 1982. T cell-independent responses to an Ir gene-controlled antigen. I. Characteristics of the immune response to insulin complexed to Brucella abortus. *J.Immunol.* 129:6-10.

343. Whitcomb, E. A. and P. H. Brodeur. 1998. Rearrangement and selection in the developing Vkappa repertoire of the mouse: an analysis of the usage of two Vkappa gene segments. *J.Immunol.* 160:4904-4913.

344. Ewulonu, U. K., L. J. Nell, and J. W. Thomas. 1990. VH and VL gene usage by murine IgG antibodies that bind autologous insulin. *J.Immunol.* 144:3091-3098.

345. Bailey, N. C., K. N. Kasturi, T. K. Blackwell, F. W. Alt, and C. A. Bona. 1991. Complexity of the immunoglobulin light chain V kappa 1 gene family in the New Zealand black mouse. *Int.Immunol.* 3:751-760.

346. D'Hoostelaere, L. A., K. Huppi, B. Mock, C. Mallett, and M. Potter. 1988. The Ig kappa L chain allelic groups among the Ig kappa haplotypes and Ig kappa crossover populations suggest a gene order. *J.Immunol.* 141:652-661.

347. Jacobson, B. A., J. Sharon, H. Shan, M. Shlomchik, M. G. Weigert, and A. Marshak-Rothstein. 1994. An isotype switched and somatically mutated rheumatoid factor clone isolated from a MRL-lpr/lpr mouse exhibits limited intraclonal affinity maturation. *J.Immunol.* 152:4489-4499.

348. Shlomchik, M., M. Mascelli, H. Shan, M. Z. Radic, D. Pisetsky, A. Marshak-Rothstein, and M. Weigert. 1990. Anti-DNA antibodies from autoimmune mice arise by clonal expansion and somatic mutation. *J.Exp.Med.* 171:265-292.

349. Shan, H., M. J. Shlomchik, A. Marshak-Rothstein, D. S. Pisetsky, S. Litwin, and M. G. Weigert. 1994. The mechanism of autoantibody production in an autoimmune MRL/lpr mouse. *J.Immunol.* 153:5104-5120.

350. Thomas, J. W., P. L. Kendall, and H. G. Mitchell. 2002. The natural autoantibody repertoire of nonobese diabetic mice is highly active. *J.Immunol.* 169:6617-6624.

351. Brodeur, P. H. and R. Riblet. 1984. The immunoglobulin heavy chain variable region (Igh-V) locus in the mouse. I. One hundred Igh-V genes comprise seven families of homologous genes. *Eur.J.Immunol.* 14:922-930.

352. Leijon, K., A. Freitas, and D. Holmberg. 1993. Analysis of VH gene utilisation in the non-obese diabetic mouse. *Autoimmunity* 15:11-18.

353. Marrack, P., J. Kappler, and B. L. Kotzin. 2001. Autoimmune disease: why and where it occurs. *Nat.Med.* 7:899-905.

354. Nemazee, D. and M. Weigert. 2000. Revising B cell receptors. *J.Exp.Med.* 191:1813-1817.

355. Nemazee, D. 2000. Receptor editing in B cells. *Adv.Immunol.* 74:89-126.

356. Leijon, K., B. Hammarstrom, and D. Holmberg. 1994. Non-obese diabetic (NOD) mice display enhanced immune responses and prolonged survival of lymphoid cells. *Int.Immunol.* 6:339-345.

357. Andersson, A., B. Ekstrand-Hammarstrom, B. Eriksson, C. Overmo, and D. Holmberg. 1994. Neonatal treatment with monoclonal natural antibodies restores a normal pattern of VH gene utilization in the non-obese diabetic mouse. *Int.Immunol.* 6:623-630.

358. Viau, M. and M. Zouali. 2005. B-lymphocytes, innate immunity, and autoimmunity. *Clin.Immunol.* 114:17-26.

359. Dammers, P. M. and F. G. Kroese. 2005. Recruitment and selection of marginal zone B cells is independent of exogenous antigens. *Eur.J.Immunol.* 35:2089-2099.

360. Dammers, P. M., A. Visser, E. R. Popa, P. Nieuwenhuis, and F. G. Kroese. 2000. Most marginal zone B cells in rat express germline encoded Ig VH genes and are ligand selected. *J.Immunol.* 165:6156-6169.

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

362. Thien, M., T. G. Phan, S. Gardam, M. Amesbury, A. Basten, F. Mackay, and R. Brink. 2004. Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches. *Immunity.* 20:785-798.

363. Roark, J. H., A. Bui, K. A. Nguyen, L. Mandik, and J. Erikson. 1997. Persistence of functionally compromised anti-double-stranded DNA B cells in the periphery of non-autoimmune mice. *Int.Immunol.* 9:1615-1626.

364. Roark, J. H., C. L. Kuntz, K. A. Nguyen, L. Mandik, M. Cattermole, and J. Erikson. 1995. B cell selection and allelic exclusion of an anti-DNA Ig transgene in MRL-lpr/lpr mice. *J.Immunol.* 154:4444-4455.

365. Blundell, T. L., J. F. Cutfield, G. G. Dodson, E. Dodson, D. C. Hodgkin, and D. Mercola. 1971. The structure and biology of insulin. *Biochem.J.* 125:50P-51P.

366. Blundell, T. L., J. F. Cutfield, S. M. Cutfield, E. J. Dodson, G. G. Dodson, D. C. Hodgkin, and D. A. Mercola. 1972. Three-dimensional atomic structure of insulin and its relationship to activity. *Diabetes* 21:492-505.

367. Blundell, T. L., G. G. Dodson, E. Dodson, D. C. Hodgkin, and M. Vijayan. 1971. X-ray analysis and the structure of insulin. *Recent Prog.Horm.Res.* 27:1-40.

368. Jasinski, J. M. and G. S. Eisenbarth. 2005. Insulin as a primary autoantigen for type 1A diabetes. *Clin.Dev.Immunol.* 12:181-186.

369. Pinkse, G. G., O. H. Tysma, C. A. Bergen, M. G. Kester, F. Ossendorp, P. A. van Veelen, B. Keymeulen, D. Pipeleers, J. W. Drijfhout, and B. O. Roep. 2005. Autoreactive CD8 T cells associated with beta cell destruction in type 1 diabetes. *Proc.Natl.Acad.Sci.U.S.A* 102:18425-18430.

370. Wasserfall, C. H. and M. A. Atkinson. 2006. Autoantibody markers for the diagnosis and prediction of type 1 diabetes. *Autoimmun.Rev.* 5:424-428.

371. Nakayama, M., N. Abiru, H. Moriyama, N. Babaya, E. Liu, D. Miao, L. Yu, D. R. Wegmann, J. C. Hutton, J. F. Elliott, and G. S. Eisenbarth. 2005. Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. *Nature* 435:220-223.

372. Wong, F. S. 2005. Insulin--a primary autoantigen in type 1 diabetes? *Trends Mol.Med.* 11:445-448.

373. Nolte, M. A., R. Arens, M. Kraus, M. H. van Oers, G. Kraal, R. A. van Lier, and R. E. Mebius. 2004. B cells are crucial for both development and maintenance of the splenic marginal zone. *J.Immunol.* 172:3620-3627.

374. Crowley, M. T., C. R. Reilly, and D. Lo. 1999. Influence of lymphocytes on the presence and organization of dendritic cell subsets in the spleen. *J.Immunol.* 163:4894-4900.

375. Dingjan, G. M., A. Maas, M. C. Nawijn, L. Smit, J. S. Voerman, F. Grosveld, and R. W. Hendriks. 1998. Severe B cell deficiency and disrupted splenic architecture in transgenic mice expressing the E41K mutated form of Bruton's tyrosine kinase. *EMBO J.* 17:5309-5320.

376. Maas, A., G. M. Dingjan, F. Grosveld, and R. W. Hendriks. 1999. Early arrest in B cell development in transgenic mice that express the E41K Bruton's tyrosine kinase mutant under the control of the CD19 promoter region. *J.Immunol.* 162:6526-6533.

377. Porcelli, S. A. and R. L. Modlin. 1999. The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. *Annu.Rev.Immunol.* 17:297-329.

378. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki, and M. Taniguchi. 1997. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 278:1626-1629.

379. Brossay, L., M. Chioda, N. Burdin, Y. Koezuka, G. Casorati, P. Dellabona, and M. Kronenberg. 1998. CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J.Exp.Med.* 188:1521-1528.

380. Boucheix, C., P. Benoit, P. Frachet, M. Billard, R. E. Worthington, J. Gagnon, and G. Uzan. 1991. Molecular cloning of the CD9 antigen. A new family of cell surface proteins. *J.Biol.Chem.* 266:117-122.

381. Le Naour, F., S. Charrin, V. Labas, J. P. Le Caer, C. Boucheix, and E. Rubinstein. 2004. Tetraspanins connect several types of Ig proteins: IgM is a novel component of the tetraspanin web on B-lymphoid cells. *Cancer Immunol.Immunother.* 53:148-152.

382. Maecker, H. T., S. C. Todd, and S. Levy. 1997. The tetraspanin superfamily: molecular facilitators. *FASEB J.* 11:428-442.

383. Oritani, K., K. Aoyama, Y. Tomiyama, P. W. Kincade, and Y. Matsuzawa. 2000. Stromal cell CD9 and the differentiation of hematopoietic stem/progenitor cells. *Leuk.Lymphoma* 38:147-152.

384. Amano, M., N. Baumgarth, M. D. Dick, L. Brossay, M. Kronenberg, L. A. Herzenberg, and S. Strober. 1998. CD1 expression defines subsets of follicular and marginal zone B cells in the spleen: beta 2-microglobulin-dependent and independent forms. *J.Immunol.* 161:1710-1717.

385. Roark, J. H., S. H. Park, J. Jayawardena, U. Kavita, M. Shannon, and A. Bendelac. 1998. CD1.1 expression by mouse antigen-presenting cells and marginal zone B cells. *J.Immunol.* 160:3121-3127.

386. Won, W. J. and J. F. Kearney. 2002. CD9 is a unique marker for marginal zone B cells, B1 cells, and plasma cells in mice. *J.Immunol.* 168:5605-5611.

387. Widhopf, G. F., D. C. Brinson, T. J. Kipps, and H. Tighe. 2004. Transgenic expression of a human polyreactive Ig expressed in chronic lymphocytic leukemia generates memory-type B cells that respond to nonspecific immune activation. *J.Immunol.* 172:2092-2099.

388. Schiffer, L. E., N. Hussain, X. Wang, W. Huang, J. Sinha, M. Ramanujam, and A. Davidson. 2002. Lowering anti-dsDNA antibodies--what's new? *Lupus* 11:885-894.

389. Guidos, C. J. 2002. Notch signaling in lymphocyte development. *Semin.Immunol.* 14:395-404.

390. He, Y. and W. S. Pear. 2003. Notch signalling in B cells. *Semin.Cell Dev.Biol.* 14:135-142.

391. Katagiri, K., N. Ohnishi, K. Kabashima, T. Iyoda, N. Takeda, Y. Shinkai, K. Inaba, and T. Kinashi. 2004. Crucial functions of the Rap1 effector molecule RAPL in lymphocyte and dendritic cell trafficking. *Nat.Immunol.* 5:1045-1051.

392. Lo, C. G., T. T. Lu, and J. G. Cyster. 2003. Integrin-dependence of lymphocyte entry into the splenic white pulp. *J.Exp.Med.* 197:353-361.

393. Lu, T. T. and J. G. Cyster. 2002. Integrin-mediated long-term B cell retention in the splenic marginal zone. *Science* 297:409-412.

394. Cinamon, G., M. Matloubian, M. J. Lesneski, Y. Xu, C. Low, T. Lu, R. L. Proia, and J. G. Cyster. 2004. Sphingosine 1-phosphate receptor 1 promotes B cell localization in the splenic marginal zone. *Nat.Immunol.* 5:713-720.

395. Vora, K. A., E. Nichols, G. Porter, Y. Cui, C. A. Keohane, R. Hajdu, J. Hale, W. Neway, D. Zaller, and S. Mandala. 2005. Sphingosine 1-phosphate receptor agonist FTY720-phosphate causes marginal zone B cell displacement. *J.Leukoc.Biol.* 78:471-480.

396. Cariappa, A. and S. Pillai. 2002. Antigen-dependent B-cell development. *Curr.Opin.Immunol.* 14:241-249.

397. Dolmetsch, R. E., R. S. Lewis, C. C. Goodnow, and J. I. Healy. 1997. Differential activation of transcription factors induced by Ca2+ response amplitude and duration. *Nature* 386:855-858.

398. Healy, J. I., R. E. Dolmetsch, L. A. Timmerman, J. G. Cyster, M. L. Thomas, G. R. Crabtree, R. S. Lewis, and C. C. Goodnow. 1997. Different nuclear signals are activated by the B cell receptor during positive versus negative signaling. *Immunity.* 6:419-428.

399. Healy, J. I. and C. C. Goodnow. 1998. Positive versus negative signaling by lymphocyte antigen receptors. *Annu.Rev.Immunol.* 16:645-670.

400. Healy, J. I., R. E. Dolmetsch, R. S. Lewis, and C. C. Goodnow. 1998. Quantitative and qualitative control of antigen receptor signalling in tolerant B lymphocytes. *Novartis.Found.Symp.* 215:137-144.

401. Glynne, R., G. Ghandour, J. Rayner, D. H. Mack, and C. C. Goodnow. 2000. B-lymphocyte quiescence, tolerance and activation as viewed by global gene expression profiling on microarrays. *Immunol.Rev.* 176:216-246.

402. Hoyne, G. F., M. J. Dallman, B. R. Champion, and J. R. Lamb. 2001. Notch signalling in the regulation of peripheral immunity. *Immunol.Rev.* 182:215-227.

403. Kojika, S. and J. D. Griffin. 2001. Notch receptors and hematopoiesis. *Exp.Hematol.* 29:1041-1052.

404. Kuroda, K., H. Han, S. Tani, K. Tanigaki, T. Tun, T. Furukawa, Y. Taniguchi, H. Kurooka, Y. Hamada, S. Toyokuni, and T. Honjo. 2003. Regulation of marginal zone B cell development by MINT, a suppressor of Notch/RBP-J signaling pathway. *Immunity.* 18:301-312.

405. Acevedo-Suarez, C. A., D. M. Kilkenny, M. B. Reich, and J. W. Thomas. 2006. Impaired intracellular calcium mobilization and NFATc1 availability in tolerant anti-insulin B cells. *J.Immunol.* 177:2234-2241.

406. Klimecki, W. T., B. W. Futscher, and W. S. Dalton. 1994. Effects of ethanol and paraformaldehyde on RNA yield and quality. *Biotechniques* 16:1021-1023.

407. Vinuesa, C. G., M. C. Cook, M. P. Cooke, I. C. Maclennan, and C. C. Goodnow. 2002. Analysis of B cell memory formation using DNA microarrays. *Ann.N.Y.Acad.Sci.* 975:33-45.

408. Li, J., G. W. Peet, D. Balzarano, X. Li, P. Massa, R. W. Barton, and K. B. Marcu. 2001. Novel NEMO/IkappaB kinase and NF-kappa B target genes at the pre-B to immature B cell transition. *J.Biol.Chem.* 276:18579-18590.

409. Ghaleb, A. M., M. O. Nandan, S. Chanchevalap, W. B. Dalton, I. M. Hisamuddin, and V. W. Yang. 2005. Kruppel-like factors 4 and 5: the yin and yang regulators of cellular proliferation. *Cell Res.* 15:92-96.

410. Bieker, J. J. 2001. Kruppel-like factors: three fingers in many pies. *J.Biol.Chem.* 276:34355-34358.

411. Abdulkadir, S. A. 2005. Mechanisms of prostate tumorigenesis: roles for transcription factors Nkx3.1 and Egr1. *Ann.N.Y.Acad.Sci.* 1059:33-40.

412. Nagai, R., T. Suzuki, K. Aizawa, T. Shindo, and I. Manabe. 2005. Significance of the transcription factor KLF5 in cardiovascular remodeling. *J.Thromb.Haemost.* 3:1569-1576.

413. Knapska, E. and L. Kaczmarek. 2004. A gene for neuronal plasticity in the mammalian brain: Zif268/Egr-1/NGFI-A/Krox-24/TIS8/ZENK? *Prog.Neurobiol.* 74:183-211.

414. Adamson, E. D. and D. Mercola. 2002. Egr1 transcription factor: multiple roles in prostate tumor cell growth and survival. *Tumour.Biol.* 23:93-102.

415. Khachigian, L. M. and T. Collins. 1998. Early growth response factor 1: a pleiotropic mediator of inducible gene expression. *J.Mol.Med.* 76:613-616.

416. Beckmann, A. M. and P. A. Wilce. 1997. Egr transcription factors in the nervous system. *Neurochem.Int.* 31:477-510.

417. Kawamata, S., C. Du, K. Li, and C. Lavau. 2002. Overexpression of the Notch target genes Hes in vivo induces lymphoid and myeloid alterations. *Oncogene* 21:3855-3863.

418. Conne, B., A. Stutz, and J. D. Vassalli. 2000. The 3' untranslated region of messenger RNA: A molecular 'hotspot' for pathology? *Nat.Med.* 6:637-641.

419. Kruys, V., B. Beutler, and G. Huez. 1990. Translational control mediated by UA-rich sequences. *Enzyme* 44:193-202.

420. Kelley, C. M., T. Ikeda, J. Koipally, N. Avitahl, L. Wu, K. Georgopoulos, and B. A. Morgan. 1998. Helios, a novel dimerization partner of Ikaros expressed in the earliest hematopoietic progenitors. *Curr.Biol.* 8:508-515.

421. Erikson, J., M. Z. Radic, S. A. Camper, R. R. Hardy, C. Carmack, and M. Weigert. 1991. Expression of anti-DNA immunoglobulin transgenes in non-autoimmune mice. *Nature* 349:331-334.

422. Radic, M. Z., M. A. Mascelli, J. Erikson, H. Shan, and M. Weigert. 1991. Ig H and L chain contributions to autoimmune specificities. *J.Immunol.* 146:176-182.

423. D'Hoostelaere, L. A., S. R. Bauer, and F. Melchers. 1989. Restriction fragment analysis of V preB and lambda 5 within the genus Mus. *Eur.J.Immunol.* 19:37-42.

424. Bonifacio, E., M. Atkinson, G. Eisenbarth, D. Serreze, T. W. Kay, E. Lee-Chan, and B. Singh. 2001. International Workshop on Lessons From Animal Models for Human Type 1 Diabetes: identification of insulin but not glutamic acid decarboxylase or IA-2 as specific autoantigens of humoral autoimmunity in nonobese diabetic mice. *Diabetes* 50:2451-2458.

425. Dotta, F. and G. S. Eisenbarth. 1989. Type I diabetes mellitus: a predictable autoimmune disease with interindividual variation in the rate of beta cell destruction. *Clin.Immunol.Immunopathol.* 50:S85-S95.

426. Eisenbarth, G. S., R. A. Jackson, and A. Pugliese. 1992. Insulin autoimmunity: the rate limiting factor in pre-type I diabetes. *J.Autoimmun.* 5 Suppl A:241-246.

427. Noorchashm, H., S. A. Greeley, and A. Naji. 2003. The role of t/b lymphocyte collaboration in the regulation of autoimmune and alloimmune responses. *Immunol.Res.* 27:443-450.

428. Thomas, J. W. 2001. Antigen-specific responses in autoimmunity and tolerance. *Immunol.Res.* 23:235-244.

429. Parks, A. L., K. M. Klueg, J. R. Stout, and M. A. Muskavitch. 2000. Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. *Development* 127:1373-1385.

430. Gupta-Rossi, N., E. Six, O. LeBail, F. Logeat, P. Chastagner, A. Olry, A. Israel, and C. Brou. 2004. Monoubiquitination and endocytosis direct gamma-secretase cleavage of activated Notch receptor. *J.Cell Biol.* 166:73-83.

431. Koch, U., J. S. Yuan, J. A. Harper, and C. J. Guidos. 2003. Fine-tuning Notch1 activation by endocytosis and glycosylation. *Semin.Immunol.* 15:99-106.

432. Hardy, R. R., Y. S. Li, and K. Hayakawa. 1996. Distinctive developmental origins and specificities of the CD5+ B-cell subset. *Semin.Immunol.* 8:37-44.

433. Hardy, R. R. and K. Hayakawa. 1992. Developmental origins, specificities and immunoglobulin gene biases of murine Ly-1 B cells. *Int.Rev.Immunol.* 8:189-207.

434. Iuchi, S. 2001. Three classes of C2H2 zinc finger proteins. *Cell Mol.Life Sci.* 58:625-635.

435. Brown, R. S. 2005. Zinc finger proteins: getting a grip on RNA. *Curr.Opin.Struct.Biol.* 15:94-98.

436. Hall, T. M. 2005. Multiple modes of RNA recognition by zinc finger proteins. *Curr.Opin.Struct.Biol.* 15:367-373.

437. Dinkel, A., K. Warnatz, B. Ledermann, A. Rolink, P. F. Zipfel, K. Burki, and H. Eibel. 1998. The transcription factor early growth response 1 (Egr-1) advances differentiation of pre-B and immature B cells. *J.Exp.Med.* 188:2215-2224.

438. Georgopoulos, K., D. D. Moore, and B. Derfler. 1992. Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. *Science* 258:808-812.