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REGULATION OF dsDNA BINDING B-CELLS

by

DEVON K TAYLOR

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2003

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This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

5/28/03
Date

Linda Spatz
Chair of Examining Committee

6/6/03
Date

Kesley Davenport
Executive Officer

Linda Spatz
DCA
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Supervisory Committee

The City University of New York

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ABSTRACT

REGULATION OF dsDNA BINDING B-CELLS

by

DEVON K TAYLOR

Adviser: Professor Linda Spatz

Anti-double stranded DNA (dsDNA) autoantibodies are the hallmark of SLE. The presence of these autoantibodies correlates with disease activity such as glomerulonephritis. The absence of high affinity autoantibodies in nonautoimmune individuals suggest that B-cell regulatory mechanisms play a role in averting autoimmunity. We have used a mouse model in which mice are transgenic for the IgM heavy chain of an anti-dsDNA antibody designated R4A to study the regulation of dsDNA binding B-cells.

We demonstrated that these IgM transgenic (R4A-Cμ) mice do not spontaneously secrete transgenic autoantibody and maintain tolerance in part by anergy. These anergic B-cells preferentially utilize a Vκ1 light chain and display a functionally inactive or anergic phenotype. The dsDNA binding B-cells in these mice are unresponsive to B-cell receptor (BCR) crosslinking. However, they can be activated to secrete antibody in response to LPS or T-cell derived factors such as CD40 ligand and IL4. We therefore propose that tolerance is maintained in these dsDNA binding B-cells by blocking early events specific to BCR signaling while not affecting more downstream events common to signaling pathways induced by LPS and costimulatory T-cell derived factors.

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In this study we also compared the regulation of R4A-IgM dsDNA binding B-cells to that of R4A-IgG2b dsDNA binding B-cells. We observed that heavy chain isotype plays a role in tolerance induction and that IgG B-cells are more stringently regulated than IgM B-cells.

In an attempt to understand how varying signaling thresholds can affect tolerance induction, we bred R4A-Cμ transgenic mice to transgenic mice overexpressing hCD19 a molecule involved in lowering the threshold for B-cell activation. We demonstrated that CD19 overexpression can abrogate tolerance in transgenic IgM anti-dsDNA B-cells and lead to the spontaneous secretion of anti-dsDNA antibody. We observed that this does not require T-cell help. We also observed that transgenic anti-dsDNA B-cells are derived from the conventional B-2 cell compartment and not the B-1 subset. They reside in the marginal zone of the spleen which has recently been shown to be the residence for some autoantibody secreting cells. Finally, we have demonstrated that transgenic dsDNA binding B-cells in mice that overexpress CD19, are still unresponsive to BCR crosslinking but are hyperresponsive to BCR independent innate immune mechanisms.

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Dedicated to my wife Claudia and my parents Eunice and Dervent.

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LIST of Abbreviations

AFC	Antibody Forming Cell
AIHA	Autoimmune Hemolytic Anemia
ANA	Anti-Nuclear Antibodies
AP	Alkaline Phosphatase
ATA	Anti-Thy-1 Autoantibody
B	Biotin
BAFF	B-Cell Activating Factor
BC	Bridging Channel
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
BCR	B-cell Receptor
BLR1	Burkitt's Lymphoma Receptor 1
bp	base pair
BSAP	B-cell Specific Transcription Factor
BSA	Bovine Serum Albumin
BrdU	Bromo-2'-deoxyuridine
Btk	Bruton's tyrosine kinase
Ca	Calcium
CA	central arteriole
cDNA	complementary Deoxyribonucleic Acid
CO_2,	Carbon dioxide
Cor	lymphocyte corona
CpG	cytosine linked to a guanine by a phosphate bond
CR2	Complement Receptor type 2
CTLA	cytolytic T lymphocyte-associated antigen 4
CYC	Cychrome
D	Diversity
DME	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
dsDNA	double stranded Deoxyribonucleic Acid
DAG	diacylglycerol
DOCK2	dedicator of cytokinesis 2
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
ELISPOT	Enzyme Linked Immunosorbent Spot
ERK	extracellular receptor kinase ()
FACS	Fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
FCS	Fetal Calf Serum
FDC	follicular dendritic cells
FO	Follicular
GC	Germinal Center
HBS	Hank's Balanced solution
HC	Heavy Chain
hCD19	human CD19

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HEL	Hen Egg Lysozyme
HLA	human leukocyte antigen
HSA	Heat Stable Antigen
HSC	hematopoietic stem cells
IBD	Inflammatory bowel disease
ICAM	intercellular adhesion molecule
IDDM	insulin-dependent diabetic mellitus
Ig	Immunoglobulin
IKK	Iκ kinase α/β
IL	interleukin
i.p.	intra-peritoneal
IP3	inositol-1,4,5-triphosphate
ITAM	Immunoreceptor tyrosine based-activation motifs
ITIM	Immunoreceptor tyrosine based-inhibitory motifs
i.v.	intravenous
J	Joining
JNK	c-Jun NH_2-terminal kinase
JNK MAPK	c-Jun NH_2-terminal kinase Mitogen activated Protein
$KHCO_3$	Potassium bicarbonate
κ	Kappa
KSCN	potassium thiocyanate
LC	Light Chain
λ	Lamda
LP	Lamina propria
lpr	lymphoproliferative
LPS	lipopolysaccharide
LT	lymphotoxin
LT-TNF	lymphotoxin-tumor-necrosis factor
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
Mem	Marginal Metallophilic macrophages
mCD19	mouse CD19
me	motheaten
MFI	Mean fluorescence Intensity
μg	microgram
$MgCl_2$	Magnesium Chloride
MHC-II	Major Histocompatibility Complex Class II
mHEL	membrane Hen Egg Lysozyme
mIg	Membrane Immunoglobulin
μCi	microcurie
μM	micromolar
mM	millimolar
ml	milliliters
MZ	Marginal Zone
MZm	Marginal sinus macrophages
NaCl	Sodium Chloride

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NF	New formed
NFκB	nuclear factor kappa B
NH_4Cl	Ammonium Chloride
NTg	non transgenic
NZB	New Zealand Black
NZW	New Zealand White
NZBW/F_1	New Zealand Black x New Zealand White First generation
PALS	periarteriolar lymphoid sheath
PAMPS	pathogen associated molecular patterns
PBS	Phosphate Buffer Saline
PC	phosphorylcholine
PCR	Polymerase Chain Reaction
PE	phycoerythrin
PI 3-K	Phosphatidylinositol 3-kinase
PKC	Protein Kinase C
PLC-γ	phospholipase C γ
Pyk2	protein tyrosine kinase 2
PTK	Protein Tyrosine Kinase
r	receptor
RAG	recombinase activating genes
RBC	red blood cell
Rel	v-rel reticuloendotheliosis viral oncogene homolog (a transcription factor)
RF	rheumatoid factors
RNA	Ribonucleic Acid
RNP	ribonucleoproteins
RP	Red Pulp
RSK	cell cycle-regulated S6 protein kinase
RT	Room temperature
SH2	src-homology 2 domains
SHIP	Src homology 2-containing inositol 5'-phosphatase
SHP-1	Src homology 2-containing protein tyrosine phosphatase-1
sHEL	soluble Hen Egg Lysozyme
SL	surrogate Light chain
SLE	Systemic Lupus Erythematosus
SmRNP	Smith nuclear antigen ribonucleoprotein
Sm	Smith nuclear antigen
SnRNP	small nuclear ribonucleoprotein
Src	non-receptor protein tyrosine kinase
SSc	systemic sclerosis
ssDNA	single stranded Deoxyribonucleic Acid
T	transitional
TD	T-cell dependent
Tg	Transgenic
TdT	terminal deoxynucleotidyl transferase
TFs	transcription factors

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Th	T helper
TI	T-cell independent
TI-1	thymus independent type 1
TI-2	thymus independent type 2
TLR	Toll-Like Receptor
TNF	tumour-necrosis factor
TNFR	tumour-necrosis factor receptor
topo I	topoisomerase I
$T_{reg.}$	Regulatory T-cells
Tris	Tris(Hydroxymethyl) Aminomethane
TSK	tight-skin mouse
Tyr	Tyrosine
V	variable
VDJ	Variable-Diversity-Joining
VJ	Variable-Joining
Xid	X-chromosome-linked immune-deficient

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CHAPTER I: INTRODUCTION

Systemic Lupus Erythematosus (SLE)

Systemic Lupus Erythematosus (SLE) is an autoimmune disease characterized by immune dysregulation resulting in the production of antibodies to double stranded DNA (dsDNA). Antibodies to ribonuclear proteins and histones and other nuclear components may also be present in the serum of individuals with SLE (1). SLE is notable for unpredictable exacerbations and remissions and it is multi-systemic, affecting joints, skin, kidney, brain, lung, heart, and the gastrointestinal tract (2). The etiology of SLE remains unknown, however genetic predisposition, sex hormones, and environmental triggers may play a role. The disease disproportionately affects women in their child bearing years with a female to male ratio of 10:1 and it is 2-3 times more prevalent among African-American, Hispanic and Asian women than Caucasian women. There is an increased frequency of two histocompatibility antigens in patients with SLE, HLA-DR2 and HLA-DR3, also increased are the frequencies of the extended haplotype HLA-A1 B8 DR3 suggesting a role for genetics in SLE (3). Estrogen has been shown to play a role in eliciting the disease while androgens have been shown to have a protective effect (4-6). Environmental influences, though unclear, are highlighted by the exacerbation of skin rashes after sun exposure, exacerbation of disease after viral or bacterial infections and changes in disease activity after the administration of exogenous hormones (4).

Autoantibodies to nuclear antigens may deposit in various tissues causing inflammation and tissue damage. SLE is also associated with the impaired clearance of circulating immune complexes, decreased complement receptor type 1 (CR1) expression, defective Fc receptor function and deficiencies of early components of complement such

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as C1, C2 and C4 (7, 8). Renal failure may occur as a result of deposition of autoantibody in kidney glomeruli giving rise to a condition known as glomerulonephritis. The pathogenic antibodies in lupus are mainly of the IgG isotype, have a high affinity for dsDNA and are somatically mutated suggesting that they arise in an antigen driven process requiring T-cell help (4, 9). Disease manifestations result from recurrent vascular injury due to immune complex deposition, leukothrombosis or thrombosis.

The origin of autoantibody production in SLE is unclear but one hypothesis proposes that they arise as a result of a defect in B-cell tolerance. Immunological tolerance is maintained at multiple stages in B-cell development by antigen-induced mechanisms, which function to shape the B-cell repertoire and prevent the onset of autoimmunity.

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Antibody structure

The B-cell is the mediator of the humoral immune response. When B-cell are activated by antigen they secrete antibodies. Antibodies consist of 2 identical heavy chains (HC) held together by two inter-chain disulfide bonds and two identical light chains (LC). Disulfide bonds also hold the heavy and light chains together (Figure 1). The amino terminal ends of the heavy and light chains consist of the variable regions (V) and the carboxy terminus consists of the more conserved constant region (C). The variable regions of the HC and LC contain 3 complementary determining regions (CDRs) which comprise the binding site and determine the antibody specificity. The constant region defines the class of the HC and LC. There are 2 classes of LC; κ and λ and there are 5 classes or isotypes of HC; μ, δ, γ, α and ε.

Antibodies also serve as membrane receptors for antigen. The B-cell receptor (BCR) noncovalently associates with a heterodimer on the surface of the B-cell (Figure 1). This heterodimer is composed of an α and β chain both of which have a long cytoplasmic tail region. When an antigen binds to the BCR a signal gets transmitted through the α/β heterodimer into the cell.

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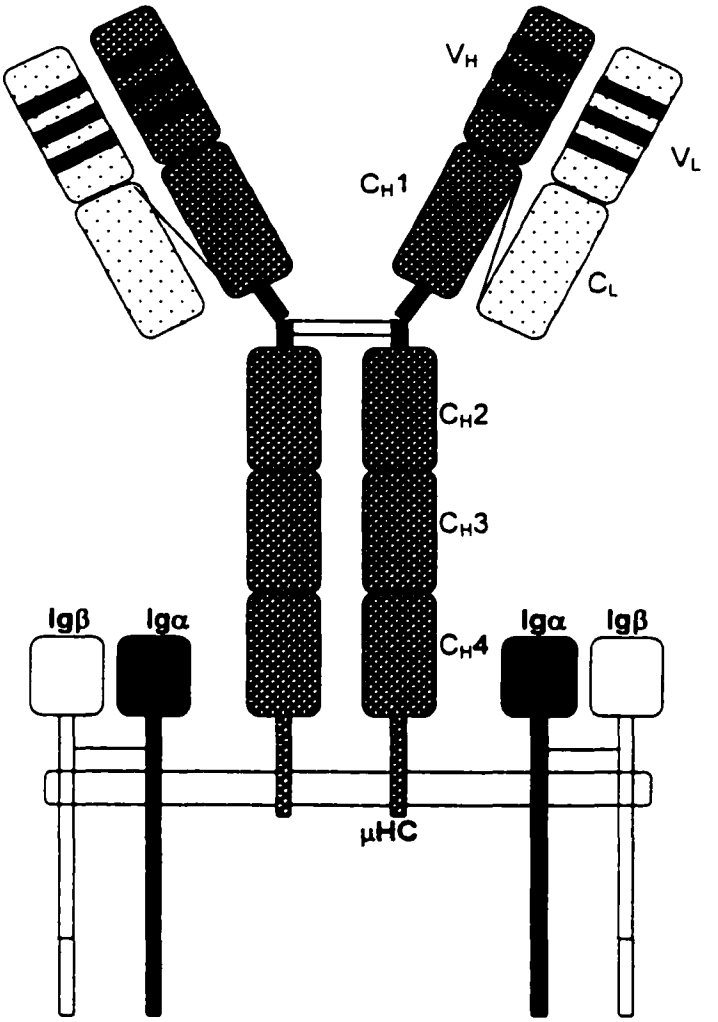

Figure 1. Antibody molecule and α/β heterodimer. The mature B-cell receptor consists of two identical heavy chains and two identical light chains. The heavy and light chains are linked by one disulfide bond while the heavy chains are linked together by two disulfide bonds in the hinge region. The α/β heterodimer also associate with the mature B-cell receptor (10).

B-cell Development

B-lymphopoiesis in the mouse is characterized by different developmental stages that are marked by changes in the rearrangement of immunoglobulin (Ig) heavy chain (HC) and light chain (LC) genes, and the expression of intracellular and cell-surface

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markers (11). B-lymphopoiesis occurs in the bone marrow and is a process, which starts with pluripotent hematopoietic stem cells, that later become B-cell lineage specific. These progenitor stem cells give rise to a differentiation pathway for B-cell development which eventually produces an immature B-cell which exits the bone marrow and enters peripheral tissue (Figure 2).

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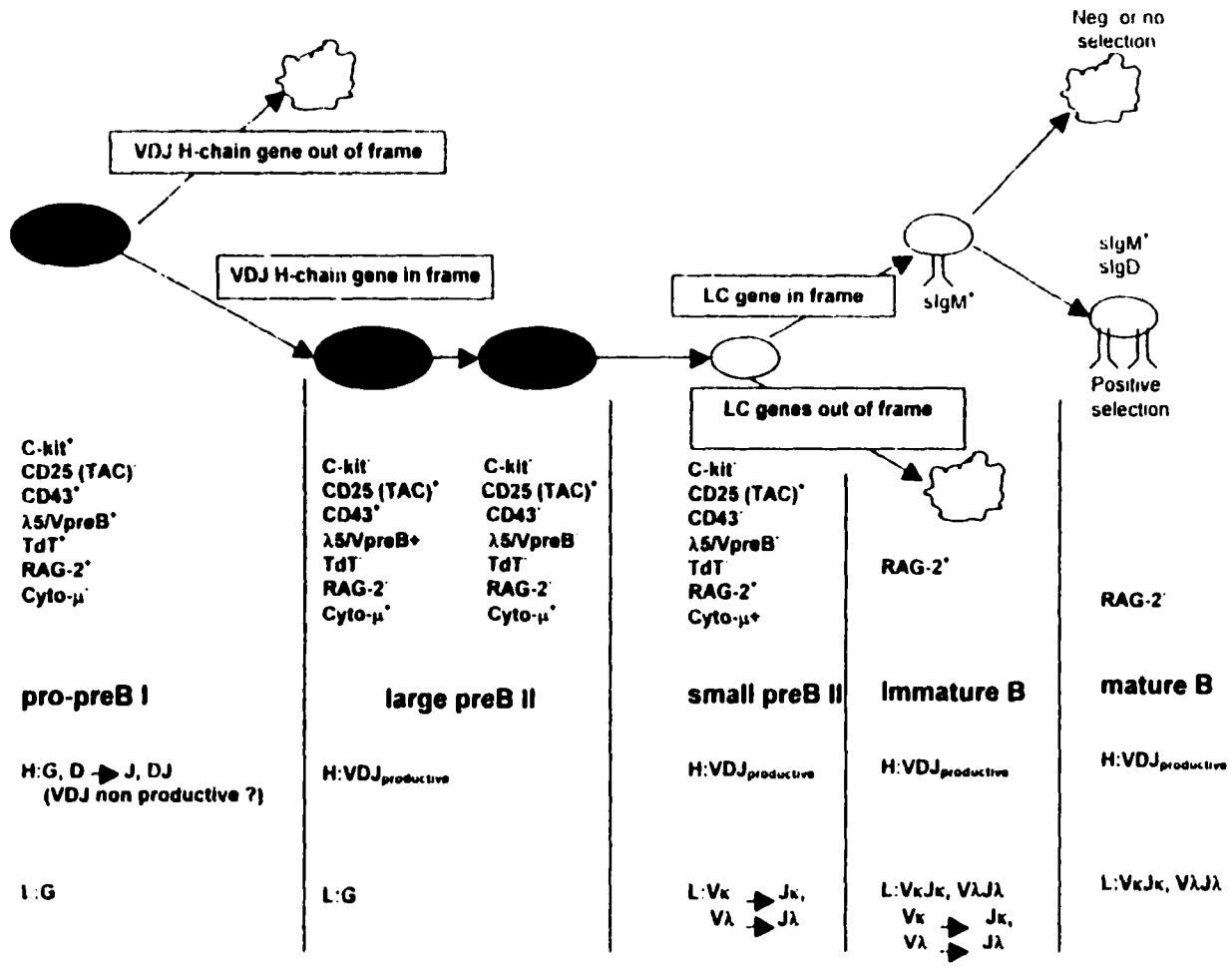

Figure 2. B-cell development in the bone marrow. Black circles represent active cycling cells; open circles represent resting cells; irregular circles represents dying cells. H-Ig heavy chain; L-Ig light chain and G-germline.

The sequential stages of B-cell development which include ***pre-proB→proB→preB-I (early preB)→large preB-II→ small preB-II (late preB) →immature B (virgin B) →mature B-cells*** are characterized by molecular markers and changes in gene expression and rearrangement of HC and LC genes (Table 1) (11-15).

Rearrangement of heavy and light chain genes

The rearrangement of immunoglobulin heavy chain and light chain genes occurs at discrete stages during B-cell ontogeny and is termed somatic recombination. The germline configuration of the heavy chain locus consists of multiple variable (V) region exons at the 5' end and the constant (C) region genes at the 3' end. Between the V and C regions separated by introns of varying lengths, are several diversity (D) and joining (J) segments. The germline configuration of the kappa light chain locus is similar to that of the heavy chain locus except that no diversity segments are present (Figure 3).

The rearrangements of the HC and LC Ig gene segments occur in precise order. The first rearrangement occurs at the HC locus during the early pro-B cell stage of development. It involves the joining of a D segment to a J segment with subsequent deletion of the intervening DNA (Figure 3A). In the late pro-B/large pre-B cell stage of development, one of the many upstream V gene segments joins to the DJ segment resulting in a rearranged VDJ gene with deletion of all D segments 5' to the rearranged D. The mechanism of Ig gene rearrangement is mediated by the recombinase activating genes, RAG1 and RAG2 and DNA repair enzymes that recognize specific recognition sequences. The recognition sequences are highly conserved heptamer and nanomer sequences separated by 12 or 22bp spacer regions. A productive VDJ rearrangement

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defines a cell committed to becoming a B lymphocyte. The μ protein is the first HC to be produced.

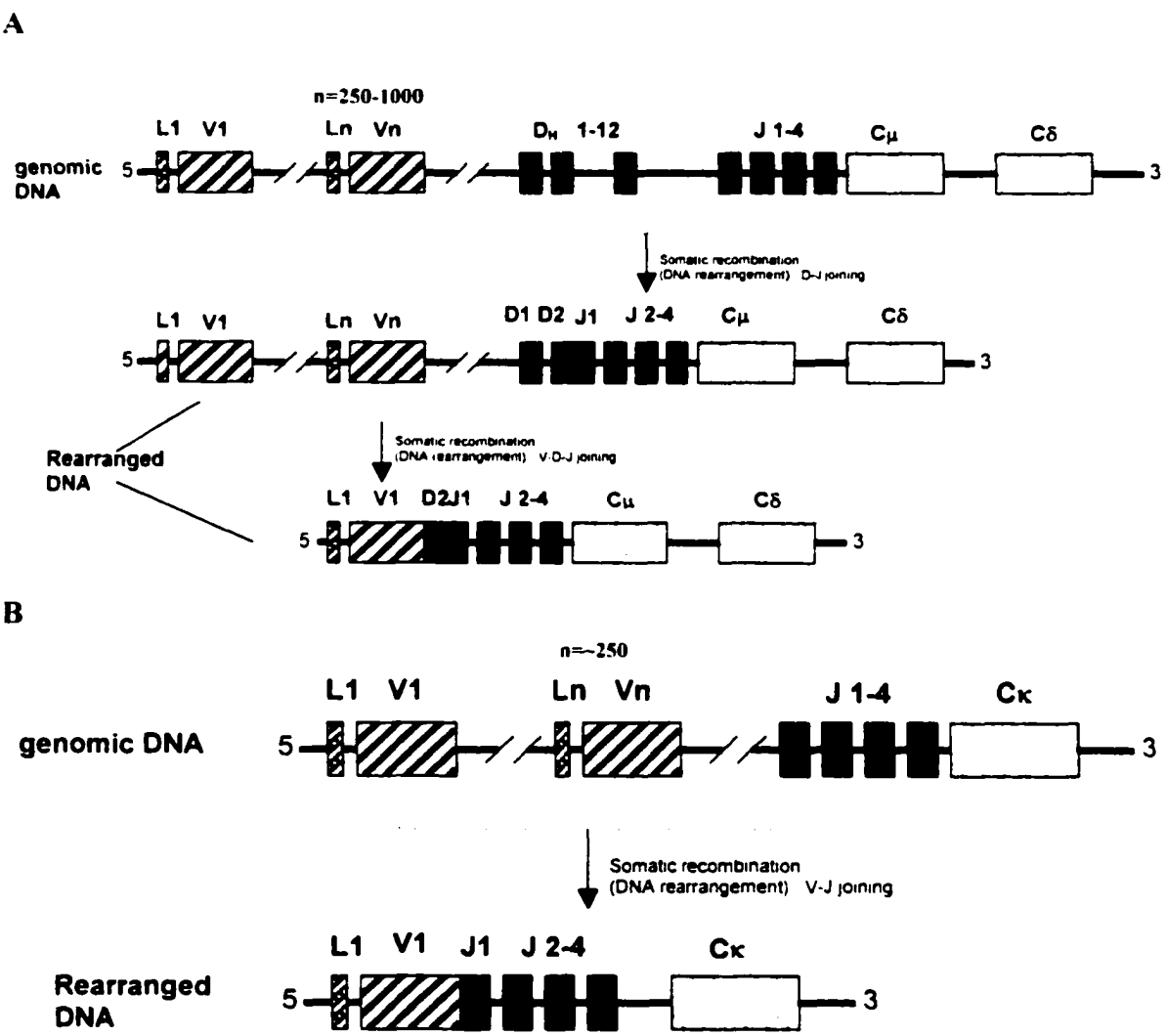

Figure 3 HC and LC gene organization and rearrangement. (A). Mouse heavy chain locus (Chromosome 12). (B). Mouse kappa light chain locus (Chromosome 6).

The production of the μ protein by the rearranged VDJ gene on one chromosome irreversibly inhibits rearrangement on the other chromosome resulting in HC allelic exclusion. If the first rearrangement is nonproductive due to deletions, insertions or a

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frameshift that leads to the generation of a stop codon, then HC rearrangement will occur on the second allele. If both alleles fail to undergo a productive rearrangement the cell dies (16-18).

Somatic DNA recombination at the light chain locus usually begins with κ chain rearrangement and proceeds to λ if the κ gene fails to rearrange productively. However recent evidence indicates that there is no precise sequence of LC rearrangement and either the κ or λ chain may rearrange first (16). Rearrangement of the LC gene follows a similar sequence as described for the HC except that it only involves the joining of a V region to a J region (Figure 3B). The productive rearrangement of either LC locus on one of the parental chromosomes prevents rearrangement of the other LC allele giving rise to light chain allelic exclusion. If LC recombination fails to result in a productive LC then rearrangement can occur on the other LC allele. If both κ or λ alleles fail to productively rearrange the cell dies. A functional κ or λ protein assembles with the previously synthesized μHC in the endoplasmic reticulum giving rise to a complete membrane IgM molecule (16-18).

Stages of B-cell development

Pre-pro B-cells represents the earliest stage in which pluripotent stem cells are committed to B-cell development (12). Commitment to this stage depends on expression of PAX5 (19). The second stage of B-cell development is the proB-cell stage. Pro-B-cells express the surface markers B220 (CD45R), CD43 and CD19. B220 and CD19 but not CD43 are expressed on all later stages of B-cells. Pro B-cells also express terminal deoxynucleotide transferase (TdT) an enzyme involved in adding nucleotides at the joining regions, c-kit$^+$, a kinase that initiates the proliferation of B-cell progenitors and

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the transcription factors E12 and E47. Pro B-cells are characterized by the rearrangement of DJ regions on the heavy chain gene and they express RAG1 and RAG2.

Pre B-cells pass through several sub stages; preB-I→large preB-II type I→large perB-II type II →small preB-II (Table 1). Pre-B-cells lose expression of CD43 but they begin to acquire other markers. Large preB-II cells undergo V to DJ rearrangement of the μ HC gene. The μ HC then associates with the surrogate light chain (SL) which consists of 2 polypeptides; VpreB and λ5, to form the pre-BCR. The pre-BCR associates with the α/β heterodimer to form the pre-BCR complex (Figure 4).

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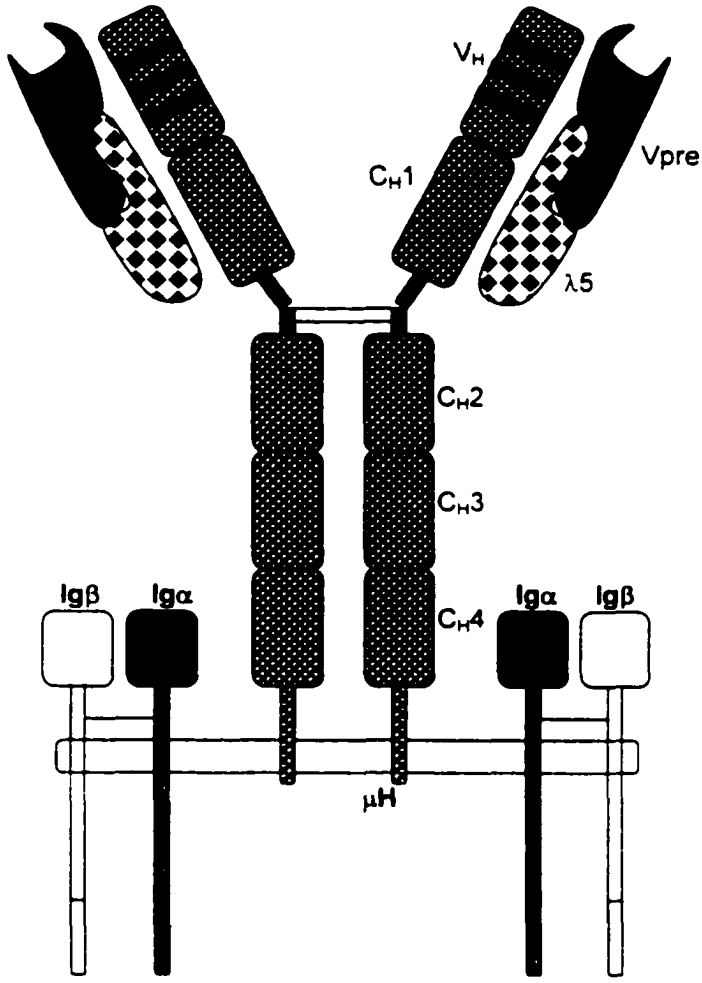

Figure 4 The pre B-cell receptor complex. The PreB-cell receptor consists of two rearranged μ heavy chains paired with two surrogate light chains (λ5 and Vpre B). The pre BCR associates non covalently with the α/β heterodimer (10).

Formation of a functional pre BCR prevents rearrangement of the second HC allele and initiates HC allelic exclusion. a mechanism which prevents the B-cells from expressing more than one HC. Large preB-II cells actively cycle. They then become small preB-cells and begin to undergo light chain rearrangement. These cells express high levels of CD24.

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Immature B-cells are marked by a successfully rearranged LC, and a μ HC. The HC and LC pair together and produce a functional IgM antibody. A functional antibody inhibits rearrangement of a second LC allele. The IgM antibody non covalently associates with the α/β heterodimer on the surface of the B-cell (Figure 1). Immature B-cells still express RAG 1 and 2 and have a phenotype that is $B220^{lo}$, HSA^{hi}, IgM^{-}. B-cell development up until this point is antigen independent and occurs strictly in the bone marrow. However once B-cells become immature they are subject to antigen induced negative selection. Those that are strongly self reactive are tolerized in the bone marrow and never make it out into the periphery. The remainder leave the bone marrow and migrate to peripheral lymphoid organs where they continue their development upon exposure to antigen. Transition from the immature to mature B-cell stage and migration from the bone marrow to the peripheral lymphoid organs such as the spleen and lymph nodes is aided by the expression of the Brutons tyrosine kinase (Btk) gene and CD40-CD40L interactions (11). The transition from an immature to a mature phenotype is gradual and involves an intermediate stage known as a transitional stage (20). Transitional B-cells are highly sensitive to tolerance induction and negative selection and can be characterized by specific phenotypic markers (Table 1) (20-22).

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Table 1. Markers that define stages of B-cell developmental

Phenotypic Markers	Pre/Pro-B	Pro/Pre-B-I	Large Pre-B-II Type I	Large Pre-B-II Type II	Small Pre-B-II	Immature	T1	T2	Mature
B220	+	+	+	+	+	+	+	+	+ (hi)
CD19	-	+	+	+	+	+	+	+	+
c-kit	+	+	-	-	-	-	-	-	-
CD43	+	+	+/-	-	-	-	+/-	-	-
SL	+	+	+	-	-	-	-	-	-
CD25	-	-	+	+	+	+/-	-	-	-
CD40	-	-	-	-	+	+	+	+	+
TdT	+	+	-	-	-	+	-	-	-
μHC	-	-	+	+	+	+	+	+	+
RAG-1/2		+	-	-	+	+			-
κ LC	-	-	-	-	+	+	+	+	+
IgM	-	-	-	-	-	+ (hi)	+ (hi)	+ (hi)	+
IgD	-	-	-	-	-	-	-	+ (hi)	+ (hi)
CD21	-	-	-	-	-	-	-	+ (hi)	+
CD23	-	-	-	-	-	-	-	+	+
CD24 (HSA)					+	+ (hi)	+ (hi)	+ (hi)	+
CD62L (L-selectin)	-	-	-	-	-	-	-	+	+

+=positive,hi=high (11, 20, 23).

Recent work on peripheral B-cell development by Loder et al. has classified two types of transitional immature B-cells; type 1 transitional B-cells (T1) and type 2 transitional B-cells (T2) (20). T1 B-cells are recent bone marrow emigrants. They are phenotypically defined as IgM^{hi} IgD^{-} $CD62L^{-}$ $CD21^{lo}$ $CD23^{-}$ $CD24^{hi}$. The T2 subset is found exclusively in the spleen and expresses a more mature phenotype. T2 cells can be distinguished from the T1 B-cells by the expression of the following markers IgM^{hi} IgD^{hi} $CD62L^{+}$ $CD21^{hi}$ $CD23^{+}$ $CD24^{hi}$ (Table 1). CD21 is also called CR2 (complement receptor type 2). It is a receptor for the C3d component of complement. CD23 is a C type lectin and CD62L (L-selectin) is an adhesion molecule which facilitates lymphocyte entry into lymph nodes. The transitional cells represent discrete stages or check points in peripheral B-cell development where decisions are made for B-cells to either enter the mature long-lived B-cell compartment or be eliminated. These decisions are BCR dependent and the outcome of signaling T1 or T2 B-cells is different (20, 24, 25). Upon BCR engagement T2 B-cells rapidly enter the cell cycle and upregulate anti-apoptotic factors such as A1/Bfl-1 and Bcl-x_L. They acquire a mature phenotype ($CD21^{lo}CD24^{lo}$) leading to positive selection and progress into the long-lived mature B-cell compartment. BCR engagement of T1 B-cells has been shown to lead to negative selection rather than proliferation (22, 24-26). These observations have helped to fuel ongoing research that is focusing on the unique roles for T1 and T2 subsets in peripheral B-cell development.

Some studies suggest that T1 precede T2 cells in development but this is controversial and it is not clear whether T2 cells can be derived directly from immature B-cells (20). The microenvironment for immature and transitional B-cell development is diverse. Immature and T1 B-cells are found in the bone marrow and in the spleen they

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are found in the T-cell rich region known as the periarteriolar lymphoid sheath (PALS) (20, 22). T2 and mature B-cells are located within B-cell rich regions of the spleen and lymph node know as the follicle. B-cells existing in a region surrounding the follicle, at the site of lymphocyte entry into the spleen, are known as marginal zone B-cells. These B-cells resemble T1 cells in that they are IgM^{hi} and $CD24^{hi}$, but they are also $CD21^{hi}$ (Table 2).

Mature B-cells express both the μHc and δHc chain on their surface due to alternate splicing of the HC RNA. IgM is down regulated relative to IgD. They are marked by the following phenotype $B220^{hi}IgM^{lo}$ $IgD^{hi}CD24^{lo}CD21^{+}CD23^{hi}$. Mature B-cells express other cell surface markers such as CD69, and MHC class II and low levels of CD80 and CD86 which allow for proper function in an immune response. They also have an increased lifespan of up to 15-20 weeks (26). They acquire the ability to recirculate in the blood and lymph and can enter the lymphoid follicles of the spleen and lymph nodes. Upon exposure to antigen, mature B-cells become activated and display an activated phenotype (Table 2). Activated follicular B-cells initiate the germinal center (GC) reaction which involves the proliferation, differentiation and affinity maturation of B-cells which then become either plasma or memory B-cells. Plasma cells are characterized by loss of expression of B220, CD19, and surface Ig. However, they upregulate CD27 which is also expressed by GC B-cells and they express CD138 (syndecan-1) (Table 2).

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Table 2 Markers of activated B-cells.

	Mature (naïve)	Activated	Plasma Cells
B220	+ (hi)	+ (hi)	-
CD19	+	+	-
IgM	+	+ (hi)	-
IgD	+ (hi)	-/+	-
CD21	+	+	-
CD23	+ (inter)	+	-
CD24 (HSA)	+	+	-
CD62L (L-selectin)	+	+	
CD80 (B7.1)	+	+ (hi)	
CD86 (B7.2)	+	+ (hi)	
CD27	-		+ (hi)
CD38	-	+	+ (hi)
CD138 (syndecan-1)	-		+ (hi)
CD44	+	+ (hi)	
CD69	+	+ (hi)	
MHCII	+	+ (hi)	+ (hi)

+=lo, inter=intermediate, hi=high (20, 23).

Mechanisms of B-cell tolerance

There are several mechanisms for maintaining B-cell tolerance. Together they serve to avert the production of autoantibodies which are antibodies to self antigens. Mechanisms of self tolerance include deletion, anergy, receptor editing, clonal ignorance and T-cell suppression. Mechanisms of tolerance operate both centrally in the bone marrow and peripherally in secondary lymphoid tissues such as the spleen and lymph nodes. Deletion involves the physical elimination of autoreactive clones because of high avidity interactions between a surface immunoglobulin (sIg) and an autoantigen (27-30). Anergy is defined as the functional inactivation whereby, an autoreactive B-cell is present but is incapable of being activated by self antigen (31, 32). Receptor editing is a process whereby an autoreactive B-cell may undergo a secondary rearrangement of its variable

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heavy and/or light chain genes to avert autoimmunity (33-35). Clonal ignorance refers to self-reactive B-cells that are present and are not anergic but fail to be activated either because the affinity of their antibody is too weak or the concentration of self antigen is too low (36-38). Suppressor T-cells can also mediate B-cell tolerance. A subset of regulatory T-cells has been identified which inhibits $CD4^+$ T helper cells and thereby prevents the activation of autoreactive B-cells (39). Failure to maintain tolerance by any one or more of these mechanisms may lead to autoimmunity.

Deletion

Clonal deletion is the primary mechanism of B-cell tolerance in the bone marrow but it also functions in the periphery. It is reported that approximately 75% of the newly generated B-cells are centrally deleted in the bone marrow, as a result of non-productive rearrangement and negative selection due to autoreactivity and only 15% make it out to the periphery (11, 20, 40). The process of deletion involves the physical elimination of self-reactive clones from a potentially protective repertoire (22, 27). This theory was initially put forth by Nossal, however, it took the advent of transgenic technology to confirm it (27, 28, 41-43). It was shown that extensive crosslinking of receptors on immature, autoreactive B-cells in the bone marrow induces their elimination (41). Evidence for central deletion has been supported by a transgenic mouse model in which mice expressing an endogenous autoantigen such as MHC class I $H\text{-}2K^k$ were made transgenic for a high affinity antibody to $H\text{-}2K^k$. In these mice, all B-cells reactive with $H\text{-}2K^k$ were deleted (27, 42). Similarly in mice transgenic for a high affinity antibody to dsDNA, anti-dsDNA B-cells were deleted (43).

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Peripheral deletion has also been demonstrated using transgenic mouse models (28, 36, 44). In one system, mice were made transgenic for peripherally expressed membrane hen egg lysozyme (mHEL) and antibody to mHEL (28). The highly multivalent mHEL was shown to cause extensive crosslinking of peripheral B-cells and thereby induce deletion of splenic B-cells (28).

Anergy

Clonal anergy is a mechanism in which autoreactive B-cells are maintained in a state of unresponsiveness. Autoantigens that bind with moderate affinity to an autoantibody can induce anergy (45). Goodnow et al. first observed evidence for anergy in mice transgenic for soluble HEL (sHEL) and antibody to HEL. These mice displayed negligible serum titers of anti-HEL antibody, but showed no evidence of B-cell deletion as their spleens and lymph nodes contained HEL binding B-cells (36, 46). These studies led them to postulate that the strength of receptor crosslinking of an immature B-cell determines whether the B-cell is anergized or deleted. Several factors can influence the strength of receptor crosslinking including the affinity of the antigen-antibody interaction, the valency of the autoantigen, the density of the Ig on the surface of the B-cell and the concentration of the antigen. According to their hypothesis, mHEL, which facilitates extensive receptor crosslinking, due to its multi-valency, induces B-cell deletion, while sHEL, which facilitates more moderate crosslinking, induces anergy. Goodnow observed that the anergic B-cells exhibit a reduced level of expression of surface immunoglobulin as a result of chronic exposure to the autoantigen (36, 46). This phenomenon is known as receptor down modulation. Erikson et al. observed similar results in mice transgenic for an antibody to ssDNA (47).

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Characterization of anergic B-cells has shown that they are unable to be activated by cross-linking their BCR with antigen or antibody to surface immunoglobulin (31, 32, 41, 46-48). Receptor crosslinking of tolerant B-cells in several models has been shown to induce proliferation but not antibody secretion even in the presence of both antigen and cognate T-cell help (31, 32, 46, 47, 49). However, some studies have demonstrated that anergic B-cells can be activated to secrete antibodies in response to the polyclonal B-cell mitogen, lipopolysaccharide (LPS) (2, 28, 48, 50, 51). This suggests that LPS can bypass a block in antigen-receptor signaling inherent to some anergic cells. Goodnow et. al. using their anti-HEL/HEL transgenic mice revealed that B-cell anergy could be reversed by *in vitro* stimulation with LPS (28, 51, 52). Similarly, Offen et al. demonstrated that anergic dsDNA binding B-cells could be activated by LPS stimulation (2). In contrast, Erikson et. al. was unable to activate their anergic population of anti-DNA B-cells to secrete anti-ssDNA antibody using LPS (47, 53).

Anergic B-cells have been shown to display a phenotype characteristic of immature B-cells. As previously described, B-cell development is defined by the expression of immunoglobulin genes and by changes in the expression of stage specific molecules. Mandik-Nayak et al. demonstrated that anergic dsDNA binding B-cells are developmentally arrested (54). By examining cell surface expression of developmental markers, they showed that these transgenic B-cells expressed the following phenotype; $B220^{lo}$, $CD24^{hi}$, $CD21/35^{lo/-}$, $CD22^{lo}$ and $CD23^{lo/-}$ characteristic of immature B-cells. Arrested development was also demonstrated for anergic HEL binding B-cells by Hartley et al. (55). Mandik-Nayak et al. observed that the phenotype of anergic B-cells is similar

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to that of chronically activated B-cells, as evidence by upregulation of CD44 and MHC Class II and downregulation of CD62L.

Anergic B-cells have been observed to have a reduced life span (54-56). The lifespan of resting B-cell is about 4-5 weeks however, developmentally arrested anergic B-cells have been observed to die within 3-4 days (40, 55, 56). It has therefore, been proposed that anergy may be an early stage of a continuum leading to deletion (40, 55).

Studies comparing the signaling in naïve and anergic B-cells following BCR crosslinking has revealed molecular and biochemical differences in them. Immunoglobulin desensitization and a block in cell signaling highlighted by diminished tyrosine kinase activation and calcium flux is observed in response to antigen crosslinking of tolerant anergic anti-HEL B-cells (48). Prior to antigen exposure, naïve B-cells display a low intracellular concentration and have a low activity of ERK, RSK, and JNK MAP kinases (57-59). The ligation of the naïve BCR by antigen leads to a large biphasic calcium response, the translocation of transcription factors to the nucleus and the activation of JNK and ERK which phosphorylate their substrates and lead to B-cell activation. However, the ligation of tolerant B-cells leads to low chronic calcium oscillations, inhibition of migration of NFκB, c-myc and JNK into the nucleus and low tyrosine kinase phosphorylation of syk kinase (57). More recent work on cell signaling demonstrates that the BCR in naïve B-cells partitions into a detergent insoluble domain containing Lyn kinase, where it is phosphorylated within 6 seconds. In anergic B-cells the BCR does not efficiently partition into the detergent insoluble domain and tyrosine phosphorylation is low (60). It has been suggested that this may be a mechanism of

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maintaining tolerance by physically separating the BCR from important downstream signaling molecules (60).

Receptor Editing

Receptor editing is defined as an immunoregulatory mechanism which immature autoreactive B-cells escape deletion by revising their HC and LC variable region genes in an attempt to produce a non-autoreactive antibody (33-35). To edit away from autoreactivity, either the variable heavy chain gene or the variable light chain gene undergoes a secondary rearrangement resulting in a V gene replacement (33-35, 43). Studies done in mice transgenic for the heavy and light chains of an anti-dsDNA antibody demonstrated that the κC of an anti-dsDNA antibody could be replaced by a different LC to yield an antibody of non-dsDNA specificity (33). Secondary rearrangement usually entails the rearrangement of an upstream V-gene with a downstream J segment. Analysis of hybridoma panels from these mice, producing non-DNA binding antibodies utilizing the transgenic heavy chain, revealed a biased use of an upstream Vκ gene along with Jκ5 a downstream Jκ segment (34). The infrequent use of more proximal Jκ genes suggested that receptor editing was occurring. Evidence for receptor editing also came from experiments by Tiegs et al (35). They identified excised circular DNAs resulting from secondary nested κ gene rearrangements and they observed increased RAG expression, which is necessary for V gene recombination in bone marrow B-cells undergoing secondary rearrangement (35).

Receptor revision is a term that refers to editing that occurs in the periphery. Secondary rearrangements that take place in the periphery may be important for the diversification and affinity maturation of an antibody response while maintaining

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immunological tolerance (61). Receptor editing in the bone marrow preserves a diverse antibody repertoire without compromising self-tolerance while receptor revision in the periphery may further increase and optimize this repertoire once B cells have been engaged in an immune response, most likely to enhance the selection of antibodies with a higher affinity for foreign antigen. However, the absence of high affinity somatically mutated pathogenic autoantibodies in normal individuals suggests that receptor revision may also play a role in averting the production of autoreactive B-cells that arise due to somatic mutation in the periphery (62-66).

Clonal Ignorance

Clonal ignorance refers to an autoreactive B-cell that is present in the immune repertoire but is not actively secreting autoantibodies either because it has not come in contact with the autoantigen and therefore has not been activated or because it has a very weak affinity for the autoantigen. In the absence of self antigen autoreactive B-cells will develop and mature in the bone marrow and migrate into the periphery. If these B-cells do not encounter self antigen because the antigen is sequestered then the B-cells will not be deleted or anergized but will remain ignorant.

Clonal ignorance attributed to low autoantigen concentration was demonstrated in two transgenic mouse model; HEL/anti-HEL and H-2K^b/anti-H-2K^b (36-38). In these models, when very low levels of sHEL (1 ng/ml) or H-2K^b were present such that the antigen caused less than 5% antigen receptor occupancy, then the autoreactive B-cells failed to be deleted or anergized (36, 38). Clonally ignorant B-cells provide a reservoir of autoreactive cells that can be activated under pathological conditions. For instance,

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the release of sequestered self antigen that may occur after tissue injury may activate circulating clonally ignorant B-cells and this may lead to autoimmunity.

T-cell suppression

Regulatory T-cells ($T_{reg.}$) are a subset of thymus derived T-cells that suppress the activation and proliferation of $CD4^+$ T helper cells (67, 68). Because they may suppress T-cells that can provide help to autoreactive B-cells they may be important in the maintenance of peripheral B-cell tolerance. B-cells can become anergized if they receive an antigenic signal in the absence of T-cell help. Disease onset has been shown to be prevented in non-obese diabetic (NOD) mice which spontaneously develop autoimmune insulin-dependent diabetic mellitus (IDDM) and in mice which develop inflammatory bowel disease (IBD), by treatment with T_{reg} cells (67). T_{reg} cells were found to be $CD4^+CD25^{hi}CD45RB/RC^{lo}CD5^{hi}RT6.1^+CD62L^+CD38^+$ (39, 67-70). The elimination of $CD4^+CD25^+$ peripheral T-cells in normal, naïve mice was observed to lead to autoimmune diseases. These could be prevented by reconstitution with $CD4^+CD25^+$ T-cells from healthy donors (71). A reduction in T_{reg} cells was observed in patients with active SLE. Upon remission of disease T_{reg} cells were found to increase (72).

The importance of T_{reg} cells in maintaining B-cell tolerance was demonstrated recently in a study by Seo et. al. (73). They demonstrated that in mice transgenic for the heavy chain of an anti-dsDNA antibody, tolerance was maintained by B-cell anergy and that anergy could be overcome if anergic B-cells were provided with T-cell help *in vivo* (73). However, when T-cell help was supplied in the presence of regulatory T-cells, then anergy was maintained and no anti-dsDNA antibody secretion was observed (73).

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B-1 B-CELLS

The majority of circulating B-cells and B-cells in peripheral lymphoid organs belongs to the conventional B-2 subset. These B-cells are responsible for most of the humoral immune responses to foreign peptide antigens. B-1 cells comprise a minor (5-10%) population of B-cells that can be distinguished from conventional B-2 cells by their surface phenotype, anatomical location, and functional characteristics. Two models have been proposed to explain the development of the B1 compartment. One model proposes that B-1 and B-2 cells originate from different precursors and as a result represent two distinct lineages of B-cells (74-78). B1 B-cells are said to be derived from hematopoietic stem cells (HSC) of the fetal liver or omentum. Conventional B-2 B-cells are initially derived from fetal HSC but later are derived from the bone marrow (74, 76-78). Adoptive transfer of embroyonic paraaortic splanchnopleura and fetal omentum progenitor cells can reconstitute recipient mice with B-1 but not B-2 cells while transfer of adult bone marrow can reconstitute the recipients fully with B2 B-cells but poorly with B-1 B-cells (75, 78).

A second model, the induced-differentiative model, proposes that B-1 cells develop from the same lineage as B2 B-cells but are induced to differentiate into a different subset. (76-81). This model states that the B-1 B-cell phenotype results from activation by T-independent type-2 (TI-2) antigens. TI-2 responses results in the production of antibodies that lack somatic mutation and are of the μ or $\gamma 3$ isotypes (76). TI-2 antigens have multiple repeating epitopes that generally consists of polysaccharide subunits. They can activate B-cells by extensive crosslinking of the BCR in the absence of T-cell help (82-84). Data suggests that BCR crosslinking by non-self antigens in the

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absence of T-cell help can lead to the upregulation of CD5 on splenic B-2 B-cells (76, 79, 80, 85). It has been shown that *in vitro* treatment with anti-IgM and IL-6 induces the acquisition of CD5, the loss of CD23 and the downregulation of IgD to levels consistent with a B-1 B-cell phenotype (79). Since these phenotypic changes were initiated by BCR crosslinking, they suggested that B-1 B-cells can arise after the appearance of IgM (79, 80, 85). Support for the induced differentiation model comes from studies demonstrating that mutations that disrupt BCR signaling such as deletion of CD19, result in the decrease of B-1 B-cell numbers while not dramatically affecting the number of B-2 B-cells (86, 87). In contrast, overexpression of CD19 enhances BCR signaling resulting in an expanded B-1 subset (88).

B-1 B-cells represent a small percent of B-cells in the spleen and peripheral blood and are practically absent from peripheral lymph nodes of mice. However they represent a large population of B-cells in the peritoneal and pleural cavities where they account for 10-40% of the total cells (74, 76, 89). They are larger and exhibit more side scatter than B-2 cells. B-1 cells are self-replenishing and are maintained by a feedback mechanism that limits their *de novo* production from progenitors (76, 90). Self-renewal of mature cells is controlled by signals generated through the CD19 complex (91). CD19 expression levels are increased on B1 cells. It is speculated that CD19 upregulation is necessary for the maintenance of B1 B-cells since their low BCR affinity may not generate a strong enough survival signal (92). The dramatic reduction of B1-cells in mice deficient in CD19 and mice treated with an anti-mouse CD19 antibody supports this (87, 88, 91, 92).

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B-1 B-cells are subdivided into two subsets; B-1a and B-1b which are distinguished by cell surface markers. Both peritoneal B-1a and B1b B-cells are $B220^{lo}Mac1^{+}IgM^{hi}IgD^{lo}CD43^{+}IL5R^{+}CD23^{-}$ (Table 3). However, only B-1a cells are $CD5^{+}$. Splenic B1 B-cells do not express Mac1. In contrast to B1 B-cells, mature B-2 cells are $Mac1^{-}CD5^{-}$ $B220^{hi}IgM^{lo}$ $IgD^{hi}IL5R^{-}$ $CD23^{+}$ (Table 3) (74-76, 78).

B1 cells are the primary source of natural IgM autoantibodies (76, 90, 93). Natural IgM autoantibodies are weak and polyreactive. They generally lack somatic mutation. They also cross-react with many common pathogen-associated carbohydrates antigens such as phosphorylcholine (PC), α-1,3 dextran and LPS (76, 94-101). These antibodies are thought to offer the first line of defense against microbial pathogens such as *Streptococcus pneumoniae* (76, 102-104).

B-1 cells also offer protection in the mucosal immune system. The transfer of total peritoneal or sorted B-1 B-cells into lethally irradiated or $RAG2^{-/-}$ mice results in IgA plasma cells in the gut lamina propria (LP) and mesenteric lymph nodes (MLN) (76, 93). This IgA antibody is dependent on exogenous antigen exposure but is independent of T-cell help (105, 106).

Studies suggest that CD5 is a negative regulatory molecule which may inhibit BCR signaling by recruiting SHP-1 (76, 107, 108). CD5 B-cells have been shown to increase the amount of antigen required for activation. It has also been observed that $CD5^{+}$ B1 B-cells have a similar phenotype to anergic B-cells. Neither $CD5^{+}$ B-1 B-cells nor anergic B-cells are able release Ca^{2+} from intracellular stores. In addition B1 B-cells and anergic B-cells fail to proliferate following BCR ligation. In mice transgenic for sHEL/anti-HEL anergic anti-HEL B-cells were found to express low levels of CD5 and

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failed to translocate NFκB to the nucleus follow antigen receptor crosslinking (58, 107-111).

Hippen et.al. demonstrated that the expression of CD5 on anergic anti-HEL binding B-cells was responsible for the anergic phenotype and the extremely low levels of circulating anti-HEL antibodies (108). They noted that breeding sHEL/anti-HEL mice with mice deficient in CD5 ($CD5^{-/-}$) anergy was lost in B-1 B-cells lacking CD5 and anti-HEL B-cells spontaneously produced high levels of circulating autoantibody (108). They therefore postulated that expression of CD5 may play a role in inhibiting autoimmune B-cells responses. Paradoxically an increase in B-1 B-cells has been observed in human autoimmune diseases such as Sjorgen's syndrome and rheumatoid arthritis and in mice that develop autoimmune conditions suggesting that the negative regulatory role of CD5 is altered in these systems, perhaps by mutations in other negative regulatory molecules that associates with CD5 (88, 112-119).

Honjo and coworkers provided data to support the role of B-1 B-cells in autoimmunity. They generated mice transgenic for the heavy and light chain of a pathogenic anti-red blood cell (RBC) antibody (120).

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Table 3 Phenotypic Characteristic of NF B-cells, B1 cells, MZ B-cell and FO B cells

Phenotypic Markers	NF/TR	B1	MZ	Follicular
IgM	hi	hi	hi	lo
IgD	lo	lo	lo	hi
CD21	lo	lo	hi	lo
CD22	lo	lo	hi	hi
CD23	lo	lo/-	lo	hi
CD11b (Mac1)	-	+	-	-
IL-5R		+		
CD43	-	+	-	-
CD9		+	+	-
CD1			hi	int

Lo=low, hi=high, int=intermediate, +=positive, -=negative, MZ=marginal zone, NF=newly formed, TD=T-cell dependent, TI=T-cell independent, TR=transitional (121-123).

The expression of transgenic anti-RBC antibody in these nonautoimmune mice resulted in tolerance induction due to anergy and deletion of the autoreactive B-cells in the spleen. However, normal numbers of transgenic B-cells remained in the peritoneal cavity of these mice (120). Interestingly, when these mice were exposed to pathogens by being removed from their germ free environment and house in a conventional facility they then developed anti-RBC antibodies and autoimmune hemolytic (124). The anti-RBC antibodies were shown to be produced by B1 cells which had escaped deletion and expanded in the peritoneum in the absence of antigen. When mice were injected intra-peritoneally, (i.p.) with antigen (mouse RBC) then the presence of the RBC antigen induced deletion of the anti-RBC B-cells and reversed anemia (125).

The role of B-1 B-cells in autoimmune disease pathogenesis was also observed in nontransgenic NZB mice. NZB mice spontaneously produce anti-RBC antibodies, an increased frequency of B-1 B-cells and develop autoimmune hemolytic anemia (AIHA) (74, 126). Selective elimination of peritoneal B-1 B-cells by repeated i.p. injections of

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water (H_2O) to induce peritoneal hypotonic cell lysis, prevented mice from spontaneously developing AIHA (127). Hypotonic lysis of peritoneal B1 B-cells also prevented murine SLE in autoimmune NZB/W F1 mice (127).

Autoimmune motheaten (*me*) and viable motheaten mice (me^v) carry a mutation which arose spontaneously in a gene encoding the protein tyrosine phosphatase, SHP-1. Autoimmunity in these mice is associated with marked expansion of the B-1 population. It is likely that the mutation in SHP-1 prevents CD5 from negatively regulating BCR signaling. Motheaten mice develop SLE like symptoms, which include elevated titers of anti-dsDNA antibodies and kidney nephritis (74, 128). An enlarged B-1 cell compartment and elevated titers of autoantibodies including anti-dsDNA antibodies are also observed in many gene targeted knockout and transgenic mice such as Lyn knockouts, and mice transgenic for CD19 (88, 112).

Splenic architecture

The development of mature B-cells occurs in the spleen from newly arriving bone marrow derived transitional B-cells. Of the $2x10^7$ immature B-cells that develop daily in the bone marrow of the mouse only 10% enter the spleen and only 1-3% of these are selected into the long-lived mature B-cell pool (11, 129). Mature B-cells enter specialized splenic compartments which serve as sites for B-cell somatic mutation, diversification and affinity maturation to circulating antigens.

The spleen is the primary site for initiation of immune responses to antigens and pathogens that have invaded the blood stream (130-132). The trafficking of lymphocytes into the spleen via the blood occurs through the splenic artery which branches into numerous arterioles that enter sinuses that supply the splenic compartments. The spleen

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is divided into the red and white pulp (Figure 5). The red pulp is a complex system of blood vessels arranged to facilitate removal of microorganisms and senescent or damaged red blood cells from the circulation via phagocytosis by macrophages and granulocytes. The white pulp refers to the lymphoid tissue of the spleen and constitutes the microenvironments for antigen trapping, presentation, cell collaboration, lymphocyte proliferation, maturation and antibody production. The major microenvironments of the white pulp are the periarteriolar lymphoid sheath (PALS), the marginal zone (MZ) and the follicular zone (FO) (Figure 5).

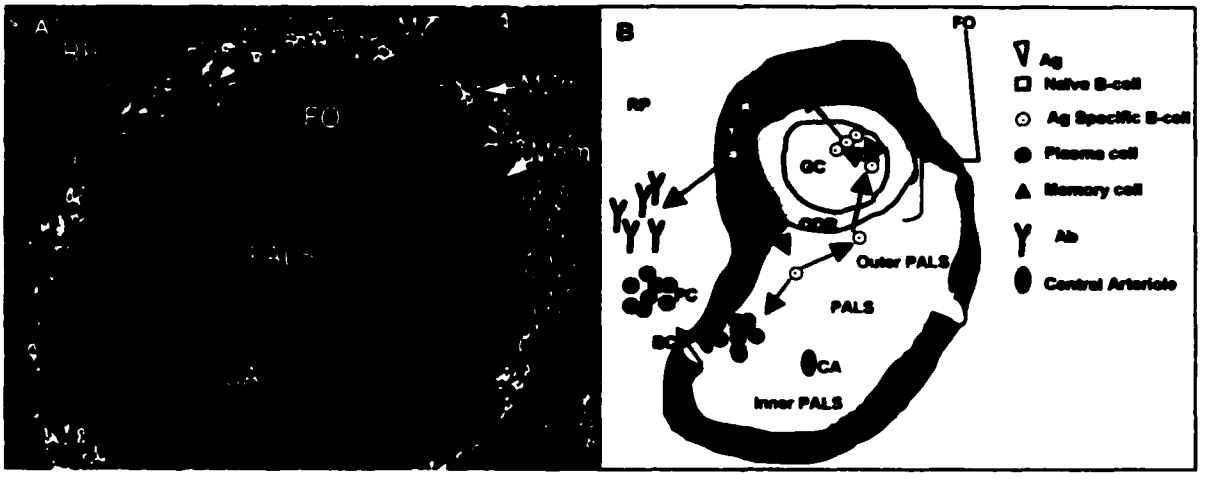

Figure 5 Splenic Compartments. **(A)** Four color histological staining showing splenic microenvironments. **(B)** Migration pattern of antigen activated marginal zone B cells. Naïve B cells enter the marginal zone (MZ) through the marginal sinus. After antigenic stimulation, activated B cells can migrate directly to the red pulp (RP) to accumulate in extra follicular zones and differentiate to antibody producing plasma cells (PC) or migrate to the PALS where they can accumulate in the outer PALS followed by clonal expansion. Activated cells can migrate to the red pulp through bridging channel (BC). Activated B cells can also migrate to the primary follicle to initiate a germinal center (GC) process with clonal expansion and affinity maturation. In the germinal center, memory B cell are generated which can directly colonize the MZ. PALS=periarteriolar lymphoid sheath, Cor=lymphocyte corona,CA=central arteriole, Mem=Marginal Metallophilic macrophages, MZm=Marginal sinus macrophages.

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The PALS is the area surrounding the central arteriole. It is mainly composed of T-cells, most of which are $CD4^+$ helper T-cells. It is also rich in a specialized antigen-presenting cell called the interdigitating dendritic cell (133, 134). The PALS is supplied with newly generated B and T cells from the marginal zone blood sinusoids. Initially, B-cells selected for the long-lived mature pool migrate through the inner PALS (T-cell rich area of the PALS) to the outer PALS (the interface between the T-cell rich inner PALS and the follicle). They then cross the B-T interface and enter the follicles (B-cell rich area). In the absence of stimulation both B and T cells re-enter the circulation. It has been shown that most of the transitional B-cells entering the PALS die within the outer PALS making it the selection site for entry of long-lived B-cells into the follicle (134-136). T-1 transitional B-cells are localized in the outer PALS while T-2 cells are localized in the follicle (20). The migratory pathways of both B and T cells are altered in the presence of foreign antigen localized on interdigitating dendritic cells in the outer PALS. T-cells that recognize such antigens are induced to stop and proliferate. B-cells that are stimulated by particular antigens here and that receive cognate T-cell help become activated and proliferate. These B-cells differentiates into antibody forming cells (AFCs) in the PALS and secrete IgM initially but later switch to other isotypes (133, 134, 137). Most of these B-cells are short lived however, a subset will migrate into the adjacent follicle to proliferate and form germinal centers where they will undergo somatic mutation and affinity maturation.

Peripheral tolerance has been shown to occur in the outer PALS (40, 54, 137-143). Using mice transgenic for both self antigen and antibody to self-antigen was observed that self reactive B-cells are stopped from migrating into B-cell follicles and die

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within the outer PALS (27, 40, 54, 56, 137-143). In sHEL/anti-HEL transgenic mice, tolerance induction in the PALS is antigen receptor-mediated and is linked to the failure of self-reactive B-cells to compete successfully with other B-cells for follicular niches in the presence of self antigen (137, 140-143). Lack of appropriate T-cell help and insufficient BCR engagement has also been shown to lead to arrest in the PALS and follicular exclusion followed by cell death (40, 139, 143). It was further demonstrated that naïve B-cells could be arrested in the PALS if BCR ligation is suboptimal (134, 139). The splenic outer PALS thus represents a unique microenvironment where B-cells undergo antigen-mediated selection, activation and deletion.

Marginal Zone (MZ)

The region encircling the white pulp which contains the PALS and B-cell follicles is called the marginal zone, (Figure 5). It is supplied by branches from the central arterioles which terminate in sheathed capillaries that are open to the marginal sinuses. It is the site of lymphocyte entry to the spleen (83, 130-132). The MZ is not fully developed in rodents until about 2-3 weeks after birth. In humans it takes 1-2 years to develop. It is present in nude and thymectomized rats, suggesting that T-cells are not necessary for its formation (121, 144-146). The MZ consists of a layer of MZ metallophilic macrophages and several concentric layers of MZ macrophages which both function during an immune response to capture blood borne particulate antigens (phagocytosis) and participate in antigen processing and presentation (121). Also present in the MZ are immature myeloid dendritic cells that participate in antigen capture, transport and presentation to both B and T cells. The naïve MZ B-cells comprise the

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major population of this microenvironment and are phenotypically defined as $IgM^{hi}IgD^{lo}CD21^{hi}CD22^{hi}CD23^{lo/-}CD1^{hi}CD9^{+}$ (Table 3) (121, 147, 148). MZ B-cells account for 5-10% of the splenic B-cell population in the adult mouse. They are non-recirculating and are larger than mature long-lived follicular B-cells (83, 144-146). MZ B-cells are important effector cells, early in the immune response. They exhibit a lower threshold for activation, proliferation and differentiation into antibody forming cells (plasma cells) than immature or recirculating B-cells (121, 147-149). They are also implicated in TI-2 responses to (lipo-) polysaccharide antigens (144-146, 150, 151). MZ B-cells share some phenotypic features with B-1 B-cells in that they are $IgM^{hi}CD23^{lo}$ and are very responsive to TI-2 antigens (Table 3). Although only a small number of $CD5^{+}$ B-cells localize to the MZ the number of B1 cells residing there may be underrepresented due to the low expression level of CD5 (83, 144, 152). Other cell types that reside in or transit through the MZ are granulocytes, plasma-cell precursors and different subsets of T-cells (83, 121, 145, 146, 150, 151, 153). Distinct extrafollicular long-lived memory B2 cells also reside in the MZ at the boundary between the red and white pulp and participate in early T cell–independent antibody responses against blood-borne particulate antigens (121, 150, 152, 154-156).

The signals and pathways involved in the development and maintenance of the MZ are very poorly understood, however with the advent of transgenic and gene knockout technology new information has arisen concerning this microenvironment. Several factors have been shown to be involved in the generation, maintenance and function of the MZ and MZ B-cells (Table 4) (121). These factors have been grouped into molecules involved in B-cell signaling molecules involved in lifespan and survival of

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B-cells, molecules involved in retention, movement and migration of B-cells, and molecules involved in B-cell transcriptional factors (121).

Signaling molecules that affect MZ formation include the BCR and its coreceptors, signal transducers and signal modulators (Table 4). It has been observed that the MZ zone is expanded in transgenic mice whose B-cells express BCR with autoreactive specificities (152, 157-159). For instance, mice transgenic for the neonatally derived BCR, V_H81x which weakly binds phosphorylcholine, have an enlarged MZ (157). Mice deficient in molecules that regulates BCR signal transduction or signaling thresholds such as CD79α, Aiolos or CD19 have a reduced MZ (147, 160, 161).

Mice deficient in molecules that affect the lifespan and survival of B-cells such as B-cell activating factor (BAFF) or lymphotoxin alpha (Ltα) lack a MZ while overexpression of these molecules results in expansion of the MZ (Table 4) (162-172).

Deficiency in molecules implicated in MZ B-cell retention, movement and migration such as protein tyrosine kinase 2 (Pyk2), dedicator of cytokinesis 2 (Dock2) and Lsc (a Rho-specific GTP exchange factor (GEF) have either markedly reduced or missing MZ B-cells (173-175). Finally in the absence of certain transcription factors such as nuclear factor-κB (NFκB) or Relb the MZ does not develop (176, 177).

MZ B-cells participate in both T-cell dependent (TD) and T-cell independent (TI) responses. MZ B-cells have been shown to capture, process and present antigens, and deliver co-stimulatory signals (MZ have increased B7.1 (CD80) and B7.2 (CD86) molecules on their cell membrane) to T-cells faster and more efficiently than FO B-cells both *in vitro* and *in vivo* (121, 148). This functional property of MZ B-cells coupled with their rapid capacity to differentiate into plasma cells and their proximal location with

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respect to blood-borne TD antigens entering the spleen, implicate them as the first responders in a TD in an immune response (121).

Table 4 Factors that influence the Generation, Formation and Maintenance of the Marginal Zone

Molecules	Observations	Refs.
Molecules involved in signaling		
BCR with autoreactive specificity i.e. anti-dsDNA, V_H81x	Expansion of MZ B-cells	(147, 157, 178, 179)
$Il7^{-/-}$, $Il7r\alpha^{-/-}$	Expansion of MZ B-cells	(180)
$CD79\alpha^{-/-}$	Reduction of MZ B-cells	(160)
$CD19^{-/-}$	Lack of MZ B-cells	(147)
$CD21^{-/-}$	Expansion of MZ	(161)
$Btk^{-/-}$ (XID), $Pi3k^{-/-}$, $Pkc\ \beta^{-/-}$	Altered selection into MZ	(147, 181-183)
$Aiolos^{-/-}$	Reduction of MZ B-cells	(184)
Molecules that Promote Life Span and cell Survival		
$Baff^{-/-}$, $Baffr^{-/-}$	Lack of MZ B-cells	(166-169)
Baff-transgenic	Expansion of MZ B cells	(170-172)
$Lt\ \alpha^{-/-}$, $Lt\ \beta^{-/-}$., $Ltr^{-/-}$	Reduction of MZ B cells	(162,163, 165, 185)
Molecules that control cell Retention, Movement and Migration		
$Pyk2^{-/-}$	Lack of MZ B cells	(173)
$Lsc^{-/-}$	Reduction of MZ B cells	(175)
$Dock2^{-/-}$	Reduction of MZ	(174)
Transcription Factors		
NF $\kappa B^{-/-}$, $RelB^{-/-}$, RelA/cRel$^{-/-}$	Reduction of MZ B cells	(176, 177, 186-188)

Adopted from (121).

Lipopolysaccharide antigens from bacteria such as *Streptococcus pneumoniae*, *Neisseria meninggitidis*, and *Haemophilus influenzae* are T-cell independent. Specialized B-cells in the splenic MZ distinct from B1 B-cells have been shown to capture and concentrate T-independent and particulate antigens circulating in the blood (189, 190). The ability to mount immune responses against TI antigens during ontogeny appears late

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and human vaccination against pneumococcal polysaccharide in children under 18 months of age does not lead to protective immunity due to the absence of splenic MZ B-cells at this time (83, 84). A direct correlation between the TI-2 response and MZ B-cells comes from studies in Pyk-2 deficient mice. These mice have no MZ and exhibit a reduced response to TI-2 antigens (173). Studies suggest that TI-2 B-cell clones are present in the MZ microenvironment in high numbers (191). They cells respond to TI blood-borne antigen in a rapid and coordinated manner. Immature myeloid dendritic cells (DC) first capture antigen and within 4-8 hours move with antigen specific MZ B-cells through the bridging channel that connects the red and white pulp (Figure 5). The MZ B-cells receive a costimulatory signal from BAFF which is present on the DC and differentiate into plasmablasts in the bridging channels and red pulp (121). In summary, MZ B-cells play a dual role in nature; they provide a rapid TD humoral immune response to blood-borne antigens and they initiate an immune response to TI-2 antigens (83, 84, 121, 173, 191, 192).

Like the peritoneal cavity, the MZ has been shown to harbor self-reactive clones. In general, studies suggest that clones displaying a lower degree of self-reactivity localize in the MZ while higher binding clones home to the peritoneal cavity (120, 147, 193). An association between MZ B-cells and autoimmunity has been observed in both animals and humans. In autoimmune, NZB/WF_1 mice, MZ B-cells increase in number just prior to preclinical or clinical signs of disease activity. In human patients with Grave's disease and in a mouse model for Sjogren's syndrome autoantibody producing B-cells have been observed to display a MZ phenotype (194, 195).

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Follicular B-cells (FO)

The splenic B-cell follicle is defined anatomically as a microenvironment in the splenic white pulp that contains a pool of long-lived recirculating B-cells that primarily participates in T-cell dependent antibody responses. The follicle is home to naïve and mature B-cells, memory cells, plasma cells, antigen specific T-cells and follicular dendritic cells (FDC) (133, 155, 196). As described earlier, immature or transitional B-cells enter the spleen via the marginal sinus and migrate across the PALS during which time they mature and differentiate into T1 and T2 cells (20). The T2 subset as defined in Table1 is located within the follicle and is the precursor for mature follicular B-cells (20). Follicular dendritic cells (FDCs) present in the follicle express the complement receptors, CR1, CR2 and CR3 and Fc receptors (130). FDCs serve as efficient antigen-capture cells that have the capacity to take up antigen and hold it on their surface for periods of more than a year. The antigen is held as an immune complex in a native unprocessed form; but the antigen may be taken from FDCs by B cells, which can process it and present it to T cells (130, 133). Phenotypically mature FO B-cells are defined as $IgM^{lo}IgD^{hi}CD21^{lo,inter}CD22^{hi}CD23^{hi}CD1^{inter}CD9^{-}$ (Table 3) and are selected for the long-lived memory B-cell pool. They participate in T-dependent antigen responses which lead to germinal center (GC) formation in the B-cell follicle (130, 155, 197). The long-lived FO B-cells have the ability to migrate between different lymphoid tissues thereby monitoring sites of antigen invasion and providing protective immunity. These cells also receive signals necessary for continued survival from FDCs in the follicle (197). For a B-cell to enter the follicle it must receive adequate T-cell help and express the putative

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chemokine receptor Burkitt's lymphoma receptor 1 (BLR1) which is important for homing from the migration of B-cell from the outer PALS to the follicle (197).

Germinal Center reaction

Upon antigen encounter and-T cell help in the outer PALS at the B-T interface, recirculating follicular B cells become activated and migrate into the follicle with antigen specific T-cells (130, 133). A germinal center reaction ensues which is defined by antigen activated FO B-cells undergoing expansion, somatic mutation, affinity maturation, isotype switching, and differentiation into memory cells or antibody-secreting plasma cells (Figure 6) (130, 136). Intensely proliferating cells differentiate into centroblasts which comprise the dark zone of the GC. High affinity clones that are selected for continued survival are called centrocytes and form the light zone of the GC. Further differentiation occurs in which cells either become plasma cells or join the memory B-cell pool thereby offering long term protective immunity (130, 155, 197, 198).

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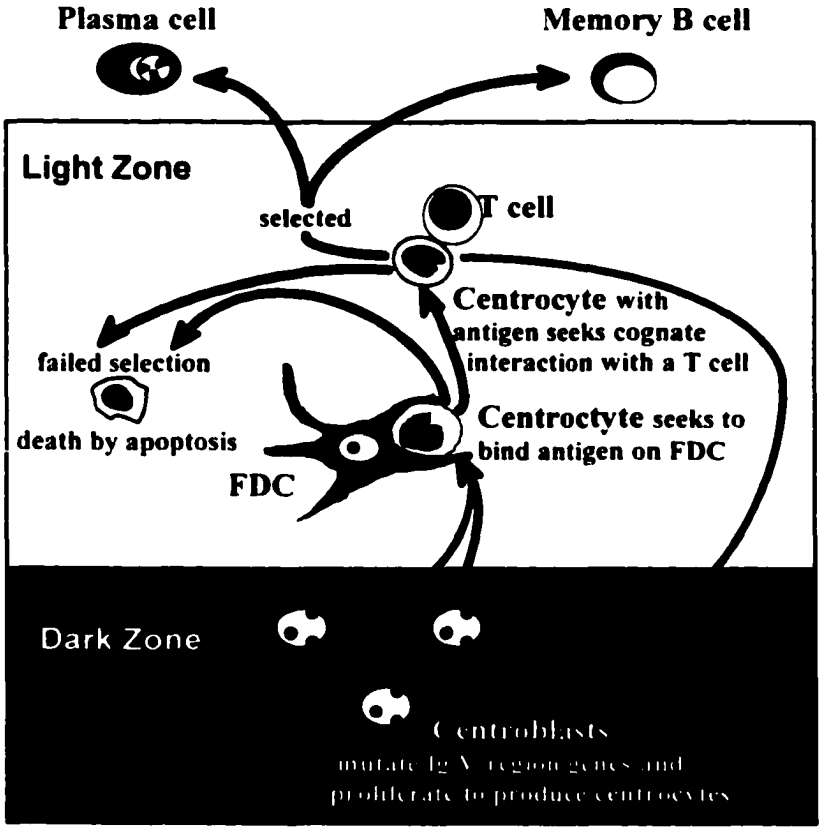

Figure 6. The events occurring in lymphoid germinal centers. Hypermutation and isotype switching occurs in the centroblasts. They differentiate into centrocytes, which die by apoptosis unless they pick up antigen from FDCs and process it and receive adequate T-cell help. Selected B-cells either differentiate into plasma or memory B cells and leave the GC. Some cells appear to remain within the GC and return to the dark zone as centroblasts (199).

B-cell localization in the MZ or peritoneum is dependent upon selection signals mediated through the BCR and its coreceptors. Emerging evidence suggests that BCR specificity and avidity are important factors in determining where B-cells go (59, 152, 159, 200-204). B-cells with some specificities such as V_H81x or M167 both of which weakly bind phosphorylcholine and are multireactive are targeted to the MZ while B-cells with specificity for HEL are targeted to the FO (46, 147, 157-159). B-cells with similar specificity but different affinity or avidity may also be targeted to different locals.

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Studies suggest that weaker binding antibodies preferentially go to the MZ while higher binding ones are more likely to move to the peritoneum. Both M167 and T15 bind phosphorylcholine but T15 which displays a much higher affinity than M167 is targeted to the peritoneum and M167 to the MZ (76, 95, 204). Furthermore B-cells with a higher density of receptor which enhances their avidity, are more likely to become B1 B-cells and home to the peritoneum than B-cell with a lower receptor density (201, 202).

Role of regulatory Signaling Molecules in Tolerance Induction

The balance between positive signaling leading to B-cell activation and the selection of a protective B-cell repertoire and negative signaling leading to tolerance induction is mediated by the B-cell antigen receptor (BCR) and its co-receptors, CD19, CD21, CD22 along with intracellular regulatory signaling molecules such as Lyn, Btk, Vav and SHP1 protein tyrosine phosphatase to name a few (Figure 7) (92, 112, 113, 128, 205-207). The mature B-cell receptor is a multiprotein complex consisting of an antigen binding membrane bound immunoglobulin (mIg) molecule that associates non-covalently with the heterodimer of CD79a (Igα) and CD79b (Igβ) (208). Ig heavy chain spans the membrane, but has a relatively short cytoplasmic tail region. This tail region was not thought to be directly involved in signaling, however, recent evidence suggests that it encodes information necessary for the immune response and BCR signal transduction (209-212). The cytoplasmic tail region of different heavy chain isotype varies in length and this may affect the signaling molecules it can interact with. The Igα/β heterodimer spans the membrane and has a long cytoplasmic tail region which is directly involved in B-cell signaling (213, 214). Both CD79α and CD79β function as the primary signal

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transducing molecules of the BCR subunit and contain amino acid sequences called immunoreceptor tyrosine based-activation motifs (ITAMs) and immunoreceptor tyrosine based-inhibitory motifs (ITIMs) (215-217). The ITAMs serve as docking sites after phosphorylation by src-family kinases, Lyn and Blk for multiple effector proteins such as Syk and Shc (218). Syk associates and activates phospholipase C γ (PLC-γ). PLC-γ is necessary for the generation of the second messengers, inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG) that regulate calcium flux and protein kinase C activation respectively. Shc can directly or via adaptor proteins interact with the G-protein Ras that leads to the activation of the MAP-kinase cascade. These responses influence the terminal BCR output and can lead to B-cell activation and proliferation (208). The docking of signaling molecules such as SHP-1 tyrosine phosphatase to ITIMs negatively regulates signaling via ITAMs. Co-receptor molecules such as CD19, CD21, CR2 and CD22 are involved in modulating the signal transduced by the BCR. A predisposition to autoimmunity can result when significant alterations of expression occur in any of the molecules involved in BCR signaling or signaling thresholds. For example deficiency in Lyn kinase or CD22 or an overexpression of CD19 can lead to autoimmunity (112, 219).

CD19

CD19 is B-cell specific. It first appears during the early proB-cell stage of development and its expression level increases with maturation. CD19 expression is turned off at the stage of plasma cell development (220-223). CD19 is a ~95-kDa transmembrane glycoprotein which is a member of the Ig gene superfamily with 2 extracellular Ig like membrane domains, a short hydrophobic domain and a intracytoplasmic tail of 242 amino acids (222). CD19 is part of a multimolecular

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complex consisting of CD81, CD21 (CR2) and leu13 (Figure 7). It physically associates via its extracellular domain with CD21, the complement receptor Type 2. CD19 also associates directly via its membrane spanning domain with CD81 a member of the tetraspans family of proteins, is implicated in the regulation of cell growth and motility and shown to be necessary for optimal surface expression of CD19 (224, 225). Leu 13 (CD225) associates indirectly with CD19 via CD81. Its function is largely unknown but it has being implicated in anti-proliferative activity and growth regulation by interferons.

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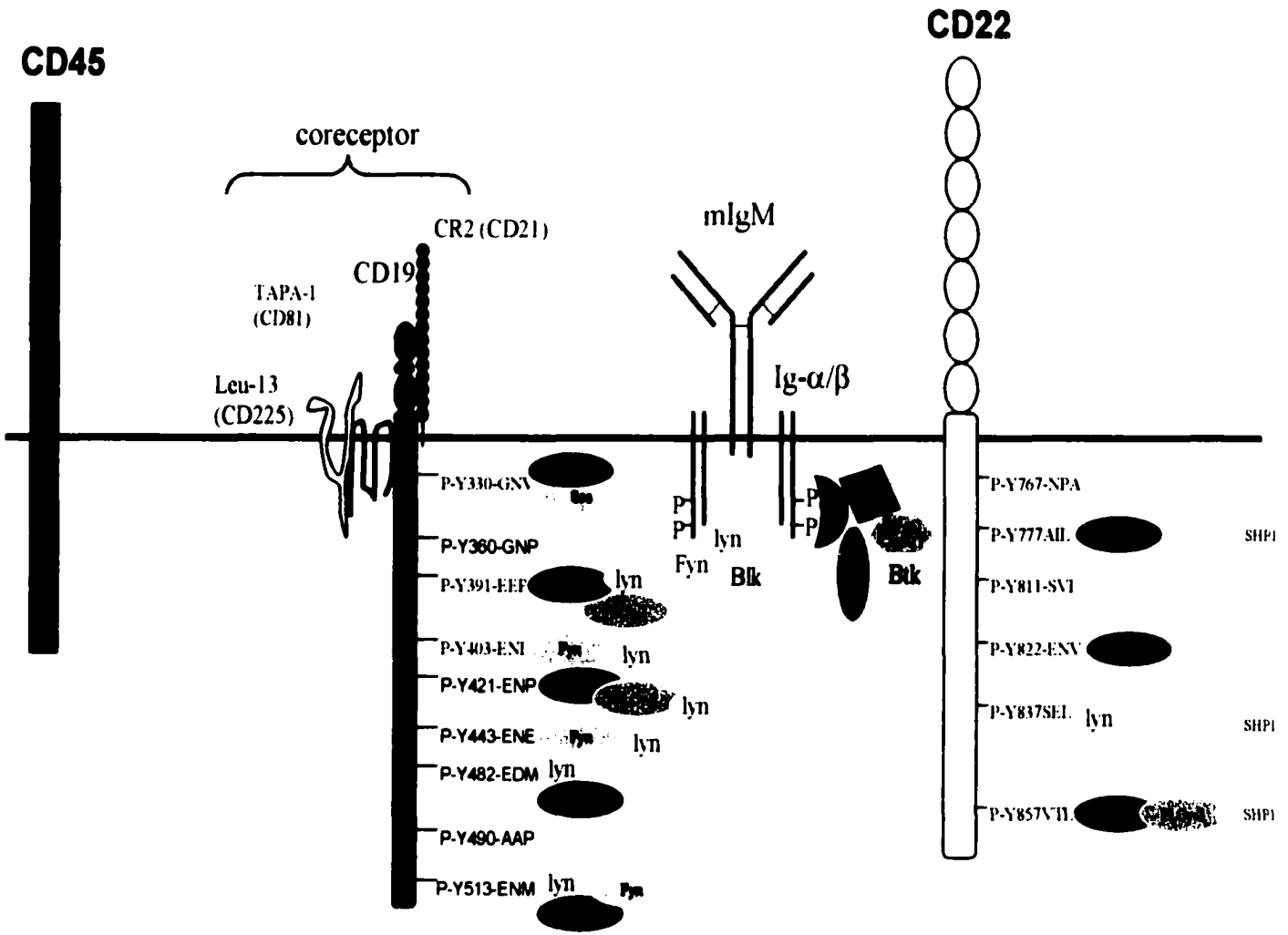

Figure 7. The B-cell receptor complex and Coreceptors.

CD19 acts as a molecular bridge that links both CD21 and CD81/leu 13. Its long cytoplasmic domain contains nine evolutionarily conserved tyrosine residues. The use of monoclonal antibodies to components of the of CD19 complex have consistently implicated CD19 in the growth regulation and activation of B-cells (226). It has been demonstrated that CD19 lowers the signaling threshold required for B-cell activation and proliferation upon BCR ligation by increasing the protein tyrosine kinase activity of Scr family kinases (227, 228). Carter et. al. showed that simultaneous engagement of membrane CD19 with anti-CD19 and the BCR with antigen in *in vitro* proliferation assays decreased the threshold for antigen receptor-dependent stimulation two fold and increased the magnitude of the B-cell response (227). The molecular mechanism by which engagement of the CD19 receptor generates biochemical signals necessary for B-cell functioning is now undergoing investigation. However evidence suggests that the recruitment of Src family protein tyrosine kinases to conserved tyrosine residues in the cytoplasmic tail domain of CD19 is important (226, 229-233). CD19 ligation has been shown to initially lead phosphorylation of one of its tyrosine residues by Lyn (228, 234, 235). This then results in subsequent phosphorylation of its other tyrosine residues by other Lyn molecules and Vav. This then lead to downstream activation of phospholipase C-γ1 (PLC- γ1), inositol phospholipid turnover, intracellular Ca^{2+} ($[Ca^{2+}]_i$) mobilization, activation of serine-specific protein kinases such as MAP kinase and activation of NFκB (92, 234).

Two models have been proposed to explain the positive signaling via the CD19 complex; the costimulatory model and the response regulator model. In the costimulatory model, which is antigen dependent, the CD21/CD19 complex is involved

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in the augmentation of B-cell proliferation, when the complex is ligated by C3d coated antigen. The antigen-C3d complexes engage both CD21 and the BCR thereby crosslinking the BCR and CD19. This coligation of CD21/CD19-BCR leads to signal amplification (230).

The response regulator model suggests that CD19 is an intrinsic regulator of signaling thresholds. It is based on the premise that CD19 ligation can provide a proliferative signal in the absence of surface Ig or CD21 engagement (231, 232, 236). It was shown in B-cell precursors lacking a functional BCR, that CD19 associates with Src PTKs upon engagement with an anti-CD19 monoclonal antibody (232). This hyper-phosphorylation of its Tyr residues results in the stimulation of several different intracellular messengers systems. It was also shown that CD19 can regulate B-cell signal transduction in mice deficient in CD21 and or C3 (237).

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TABLE 5 Functional and phenotype characteristics of CD19 deficient and overexpressing mice

	Mice deficient in CD19 ($CD19^{-/-}$)	**Mice overexpressing CD19 ($CD19^{+/+}$)**
Central B-cell development (Pre, Pro, Immature)	Near normal numbers	Severe reduction in Immature B-cells
Peripheral B-cell development (Immature/Mature)	Near normal numbers with some reduction	Significant reduction
Peritoneal B-1 B-cells	Decreased frequency	Increased frequency
Proliferation (BCR ligation and LPS)	Hyporesponsive	Hyperresponsive
Serum Ig levels in unimmunized mice	Reduced levels	Increased levels
Serum Ig levels in immunized mice (humoral response)	Significant reduction	Significant increase
Response to T-D antigens	Modest/ Significant reduction	Significant increase
Response to T-I antigens	Reduce/normal	Significant increase
Germinal Centers	Reduced number and size	Small but normal numbers

Much of what we have learned concerning the role of CD19 comes from studies using mice deficient in CD19 or mice overexpressing CD19 (transgenic for human CD19) (86, 87, 238) (Table5). Alterations in the level of CD19 expression have been shown to significantly alter B cell number and function. Mice deficient in CD19 have nearly normal B cell development in the bone marrow as determined by the number of pro, pre and immature B cells. However, in the periphery, these mice have a slight decrease in immature and mature B cell number accompanied by a reduction in serum Ig levels. It has been hypothesized that this may be due to impaired positive selection (86, 87). These mice are also hyporesponsive to transmembrane signaling and to TD antigens. Affinity maturation does not occur in the spleens of these mice and they lack germinal centers. They also have reduced responses to TI antigens which has been attributed to their dramatic reduction in peritoneal B-1 cell number (86, 173). Finally, these mice exhibit a reduction in their marginal zone suggesting the importance of CD19 in MZ formation (147, 233).

In contrast, mice overexpressing CD19 ($hCD19^{+/-}$ or $hCD19^{+/+}$), are hyperresponsive to transmembrane signaling, and have enhanced proliferation and humoral immune responses. While central B cell development is normal the number of immature and mature B cells exiting the bone marrow and the B cell number in the spleen, lymph nodes and peritoneum is severely reduced in these mice (88, 228, 239, 240). It is suggested that this is due to exaggerated negative selection. CD19 may not only augment transmembrane signals for B cell activation but for B cell deletion as well (238, 241). Despite an overall reduction in B cell frequency, CD19 overexpression increases the frequency of $CD5^{+}$ B-1 cells by approximately 50% (88, 92, 223). CD19

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overexpression also results in a significantly elevated level of serum antibody (87). It has been proposed that CD19 increases the lifespan of antibody secreting B cells but this has not been demonstrated (223). There is a significant increase in autoantibody production in mice overexpressing CD19. High titers of RH factor and IgG antibodies to DNA, histones and ribonuclear proteins are found in these mice suggesting a breakdown in tolerance (88, 242). The increase in autoantibody levels seems to correlate with the expression levels of CD19, as mice homozygous for CD19 ($hCD19^{+/+}$) seem to have higher levels of serum autoantibody than mice heterozygous for CD19 (CD19+/-) (242). The increase in $CD5^+$ B cells in these mice also seems to correlate with autoantibody production although there is no proof that these B cells are responsible for the autoantibodies produced. In fact, most of these autoantibodies are of the IgG isotype which is usually associated with B-2 cells.

The phenotype of peripheral B cells in mice overexpressing CD19 is marked by low levels of IgM receptor density, low CD21 levels and increased MHC class II (92, 223, 237). In this sense they show signs of activation and are similar in phenotype to anergic B cells that have been chronically exposed to high concentrations of soluble antigen (45, 46, 48, 92, 223). However, unlike anergic B cells that are unresponsive to BCR crosslinking these B cells are hyperresponsive.

The frequency of $CD5^+$ B cells is increased in many patients with autoimmune diseases such as SLE, rheumatoid arthritis and Sjogren's disease. Since the frequency of $CD5^+$ B cells has been shown to correlate with CD19 overexpression, this prompted investigators to examine the level of CD19 on B cells in patients with SLE and systemic sclerosis (Ssc) (242). SSc is a connective tissue disorder characterized by the deposition

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of collagen and other substances of the extracellular matrix, in the skin, internal organs and blood vessel walls. Autoantibodies are present in greater than 90% of the patients with SSc. It was observed that CD19 levels were significantly elevated on B cells from SSc patients but not on B cells from SLE patients (243). However, there was no expansion of B-1 cells in Ssc patients demonstrating the direct correlation of CD19 levels with autoantibody production, in the absence of CD5 expression. In a mouse model for SSc, known as the tight skin mouse (TSK), CD19 levels are not elevated but the basal level of CD19 tyrosine phosphorylation is augmented resulting in enhanced Ca^{2+} mobilization (243). Although the central defect in these mice is due to the duplication of the fibrillin 1 gene, the effect of hyper phosphorylation of CD19 was clearly demonstrated. When TSK mice were crossed to CD19 deficient mice the progeny displayed a significant reduction in skin fibrosis and a loss of autoantibody production.

To investigate the effect of CD19 overexpression on the regulation of autoreactive B-cells, Inaoki et. al. crossed the sHEL/anti-HEL transgenic mice which contains an anergic population of HEL binding B-cells, with mice overexpressing hCD19. They observed a breakdown to tolerance in HEL binding B-cells in mice heterozygous or homozygous for CD19 (244). They observed that 45% sHEL/anti-HEL/hCD19$^{+/-}$ and 38% sHEL/anti-HEL/hCD19$^{+/+}$ mice displayed serum levels of anti-HEL antibody that were significantly greater than sHEL/Ig^{HEL} mice wild type for CD19 at 2-3 months of age. However, by 5-10 months of age 100% of all mice displayed a breakdown in peripheral tolerance (244). It was suggested that a lowering of the signaling threshold due to CD19 overexpression was responsible for the breakdown of peripheral tolerance. It was also demonstrated that an inflammatory stimulus such injection with Freund's

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adjuvant could trigger HEL binding B-cells overexpressing CD19 to secrete autoantibody (244). An investigation of B-cell development showed that a breakdown in self tolerance in these mice was not a result of relaxed negative selection. A significant reduction in bone marrow B-cells along with a 78% reduction in circulating B-cells and a 48% reduction in splenic B-cells was still observed (244). No expansion of B1a or B1b cells was observed in two month old mice and all HEL-binding B-cells originated from the conventional B2 pool. Interestingly, the HEL binding B-cells maintained an anergic phenotype in these mice. Characteristic features of anergic HEL B-cells that were still observed in $CD19^{+/+}$ mice were a low level of Ig receptor density, coupled with an increased MHC II expression level and a failure to mobilize intracellular Ca^{2+} upon BCR crosslinking (244).

In mice transgenic for the heavy chain (2-12H) of an antibody to the ribonucleoprotein, Sm most of the Sm binding B-cells were $CD5^{+}$ B-1 cells that resided in the peritoneum. Tolerance was maintained in these mice due to clonal ignorance, presumably because of the negative regulatory affect of CD5 (193). However, when these mice were bred to mice overexpressing CD19, the serum levels of anti-Sm antibody rose indicative of breakdown in tolerance. The frequency of anti-Sm B-cells increased in these mice and all Sm B-cells were found to be $CD5^{+}$ B1 B-cells (193). In contrast, in 2-12H transgenic mice deficient in CD19, all Sm binding B-cells were found to belong to the B2 subset. It was therefore concluded that the differentiation of 2-12H cells into B-2 or B-1 cells is controlled by signal strength via the BCR which is modulated by CD19. When CD19 is eliminated, the signal strength of the BCR is reduced and B-cells preferentially differentiate into B2 B-cells, however when CD19 expression is increased,

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signal strength is increased and B-cells may differentiate into B1 B-cells. Studies in HEL transgenic mice and Sm transgenic mice demonstrate that CD19 plays a role in the regulation of both the B2 and B1 subsets of B-cells. Future studies are necessary to determine how CD19 modulates signaling in these two subsets.

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CHAPTER II: Materials and Methods

Transgene construction and generation of transgenic mice

A genomic clone containing a 3.5 kilobase (Kb) fragment consisting of the rearranged VDJ (variable-diversity-joining) region and the heavy chain enhancer region, from the anti-dsDNA producing hybridoma R4A, was ligated to a 9.9 Kb fragment obtained from a BALB/c mouse, containing all exons of the μ heavy chain gene including the secreted and membrane exons. This construct was cloned into pBluescript. The entire 13.4 kb VDJ-Cμ fragment was cut out of pBluescript using the restriction enzyme Not I and then purified by electroelution and microinjected into the male pronucleus of C57BL/6 x CBA fertilized eggs (245). The R4A-Cμ transgene was bred onto the NZW background for at least 10 generations.

Southern and Northern blot analysis

Tail DNA (15μg) was digested with 150μg of Sac I for 4 hours at 37°C, transferred to nitrocellulose after electrophoresis and hybridized with a 570bp Bam HI-Pst I bp ^{32}P labeled probe containing part of the C_H2 and C_H3 domains of the μ constant region (246). The presence of the R4A-Cm transgene was indicated by 9.5kb band.

Total RNA was isolated from the lung, kidney, heart, spleen and bone marrow of a transgenic NZW mouse and from the spleen of a nontransgenic littermate using Tri-Reagent (MRC, Cincinnati, OH). RNA was electrophoresed on a denaturing formaldehyde gel, transferred to nitrocellulose and hybridized to the Cμ probe described above or a probe specific for the S107 V_H gene family (247).

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ELISAs

Anti-dsDNA ELISA

To detect IgM^a anti-dsDNA antibodies, Immulon-2, 96 well plates (Dynatech Laboratories Inc., Chantilly, VA) were coated with salmon sperm dsDNA (dsDNA) (Calbiochem, La Jolla, CA) or calf thymus dsDNA diluted to 100 μg/ml in PBS and filtered through a 0.45 micron nitrocellulose filter (Millipore Corp., Bedford, MA) to remove single stranded DNA. Plates were dried overnight at 37°C. Prior to use, plates were rinsed for 5 minutes with 200μl of distilled water to remove excess salt and blocked in PBS, 1.0 % bovine serum albumin (BSA) pH 7.2 for 2 hours at room temperature. Wells were then incubated with serum samples collected by retro-orbital puncture diluted 1:100 in PBS, 0.1% BSA, undiluted culture supernatants from *in vitro* activation assays or hybridoma supernatants normalized to 5 μg/ml, for 2 hours at 37°C. Plates were washed 6 times in PBS, 0.05% Tween 20 and then incubated with biotinylated rat anti-mouse IgM^a antibody (B-anti-mouse IgM^a) (Pharmingen, San Diego, CA) diluted 1:200 (2.5μg/ml) in PBS, 0.1% BSA for 1 hour at 37°C. Plates were washed again 6 times and incubated with a 1:1000 dilution (1μg/ml) of alkaline phosphatase conjugated streptavidin (streptavidin-AP) (Southern, Biotechnology, Birmingham, AL) for 1 hour at 37°C and then developed with p-nitrophenyl disodium phosphate as substrate (Sigma Chemicals Co., St. Louis, MO). Plates were read at 405 nm using a Titertek Multiscan ELISA Reader (TiterTek, Huntsville, Alabama).

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Anti-IgM^a ELISA

Total IgM^a antibodies in mouse sera and in culture supernatants were detected by ELISA. Briefly, 96-well polystyrene, Falcon plates (Becton Dickinson, Franklin Lakes, NJ) were coated at 1.0 μg/well with goat anti-mouse IgM antibody (Southern Biotechnology). Plates were blocked and washed as described above and then incubated with mouse sera or culture supernatants for 1 hour at 37°C. Wells were washed again and incubated with B-anti-mouse IgM^a followed by streptavidin-AP. Plates were developed with substrate solution and read at 405 nm on a Titertek Multiscan ELISA Reader.

Antibody Quantitation by ELISA

IgM^a antibodies in mouse sera were quantitated by ELISA (248). Briefly, 96 well Falcon plates (Becton Dickinson, Lincoln, NE) were coated with goat anti mouse IgM (1:1000 or 1μg/ml) (Southern Biotechnology) in PBS and incubated at 37°C for 1 hr followed by overnight incubation at 4°C. Plates were blocked and washed as outlined above. Twofold serial dilutions of serum samples were applied to wells. Serially diluted purified mouse monoclonal IgM (Sigma) beginning at a concentration of 500ng/ml was applied to wells and used to generate a standard curve. Plates were incubated at 37°C for 1 hr, washed and biotin-conjugated goat anti-mouse IgM^a followed by streptavidin conjugated alkaline phosphatase were applied to wells as described above. Substrate solution was added and color development measured using a Titertek Multiskan ELISA reader at an OD of 405nm. A linear regression curve (optical density 405nm versus antibody concentration) was generated with purified IgM standard and the concentration of serum antibody was extrapolated from this curve.

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B-Cell Enrichment

Total splenocytes were isolated from mice and RBC lysed using 1 x RBC lysis buffer (15.5 mM NH_4Cl, 1.0 mM $KHCO_3$, 0.01 mM EDTA pH 7.2). B-cells were purified by positive selection using magnetic separation columns and magnetic labeled monoclonal rat anti-mouse CD45R (B220) or anti-mouse CD19 or by negative selection using anti-CD43 microbeads (Miltenyi Biotec, Auburn, CA). Alternatively, B-cells were enriched from splenocyte populations by depletion of T-cells using rat anti-mouse CD90 (Thy1.2). The LS+ magnetic columns were positioned on the Midi MACS magnet and washed with 3 ml of column buffer (PBS pH 7.2 supplemented with 0.5% bovine serum albumin (BSA) and 2mM EDTA). Lymphocytes were also washed and resuspended in column buffer at 10^7cells per 90µl. Cells were then incubated with 10 µl of magnetic labeled monoclonal antibody for 15 minutes at 6-12°C. Cells were then washed with 10 times the labeling volume of column buffer, centrifuged and resuspended in column buffer at a concentration of 10^8 cells per 500µl. Magnetically labeled cells were loaded onto the column and the flow through collected. The column was washed with 3 X 3 ml of column buffer. Anti-B220 and anti-CD19 (positively selected) bound cells were gently eluted in 5 ml of column buffer after removal of the column from the MidiMACS magnet. The flow through from anti-CD43 and anti-CD90 columns contained B enriched cells.

***In vitro* LPS stimulation**

Splenocytes were isolated from five, 8 to 10-wk-old mice, transgenic for the R4A-Cµ heavy chain or R4A-Cµ/hCD19 mice and RBCs lysed as described above. Cells were cultured in triplicate at $2x10^6$cells /ml in RPMI-1640 medium (Sigma) supplemented with

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10% Fetal Calf Serum (FCS) (HyClone, Logan, UT), 2mM L-glutamine (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma), 100 μM nonessential amino acids (Sigma) and 50μM B-mercaptoethanol, in 24 well tissue culture plates with and without 10 μg/ml of lipopolysaccharide (LPS) (*E. Coli.* serotype 055:B5 or 0111:B4; Sigma). After incubation for 72 hr at 37°C, 5% CO_2, cell supernatants were collected and assayed by ELISA for the presence of IgM^a antibodies to dsDNA and for total IgM^a antibodies.

***In vitro* activation and ELISPOT**

Splenocytes isolated from transgenic R4A-Cμ, R4A-Cγ2b, or R4A-Cμ/hCD19 mice were enriched for B-cells by depletion of T-cells using anti-Thy-1.2 beads or depletion of all leukocytes except immature and mature B cells using anti-CD43 magnetic beads and MACS separation columns (Miltenyi Biotec, Auburn, CA) as described above. Enriched B-cell preparations were diluted in RPMI-1640, 10% FCS, 50μM β mercaptoethanol to a concentration of 2.0×10^6 cells /ml and incubated at 37°C, 5% CO_2 in the presence of either 20 μg/ml of LPS, 10μg/ml $F(ab')_2$ fragment of goat anti-mouse IgM (ICN, Cappel, Aurora, OH), 10μg/ml of antibody to CD40 (Pharmingen, San Diego, CA), 300 units/10^6 cells of recombinant mouse IL-4 (rIL-4) (Pharmingen), 10μg/ml of anti-CD40 plus 300 units/10^6 cells of rIL-4, 200 units/10^6 cells of rIL-5 (Pharmingen), 300 units/10^6 cells of rIL-6 (Pharmingen), a cocktail of rIL-4, rIL-5 and rIL-6, 1μg/ml of CpG stimulatory sequences (TCC ATG ACG TTC CTG ACG TT) or a mixture of 6μg/ml of inhibitory CpG sequences (TCC TGG CGG GGA AGT) and 1μg/ml of stimulatory CpG (Oligos Etc, Inc, Wilsonville, OR). The inhibitory CpG was added 60 minutes prior to the addition of the stimulatory CpG. After 48 hours in culture, cells were harvested, washed and serially diluted starting at $5x10^4$/50μl, on Immulon-2

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plates coated with 100μg/ml of salmon sperm dsDNA or on Falcon plates coated with a 1:1000 dilution (1μg/ml) of anti-IgM or anti-IgG antibody (Southern Biotechnology). Prior to the incubation of cells, the plates were blocked overnight at 4°C with 2%BSA/PBS and washed three times in wash buffer (10mM Tris, 150mM NaCl, 0.1% Tween-20 pH 7.2) and twice with complete RPMI-1640 containing 20% FCS. After 8-16 hours of incubation, plates were washed 5 times with wash buffer and then B-anti-mouse IgM^a or B-anti-IgG2b (Zymed Laboratories INC, San Francisco, CA) diluted 1:300 (~1.7μg/ml) in PBS, 0.1% BSA was added to wells and plates were incubated overnight at 4°C. Plates were washed again and then incubated with a 1:1000 (1μg/ml) dilution of streptavidin-AP for three hours at room temperature. 5-bromo-4-chloro-3-indolyl phosphate (Sigma) was added as substrate and plates were allowed to develop at room temperature for 2-4 hours and the colorimetric reaction stopped by washing with distilled water. Antibody secreting cells were enumerated under a dissecting microscope using 40X magnification (249).

Flow cytometry

Single cell suspensions were prepared from the bone marrow and spleen of transgenic mice and non transgenic littermates and were depleted of RBCs by lysis with 1x RBC lysis buffer (15.5 mM NH_4Cl, 1.0 mM $KHCO_3$, 0.01 mM EDTA pH 7.2) for 4 minutes at room temperature. Cells were resuspended at a concentration of 1.0×10^6 cells per 0.1ml of PBS, 1% BSA, aliquoted in microfuge tubes or V-bottom microtiter plates and treated with Fc receptor block (CD16/CD32) (Pharmingen) for 5 minutes on ice. Cell suspensions were then surface stained by incubation with different combinations of 2 or 3 fluorescinated antibodies for 30 minutes at 4°C followed by 2

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washes with PBS, 1% BSA. The following cychrome (CYC), phycoerythrin (PE) or fluorescein isothiocyanate (FITC) conjugated antibodies were used for staining ;anti-B220, anti-IgM^a, anti-IgM^b, anti-CD24, anti-CD43, anti-CD62L (L-Selectin), anti-CD86, anti-CD80, anti-CD44, anti-MHC II, anti-CD40, anti-CD69, anti-CD27, rat anti mouse GL7, and anti-CD138 anti-CD21, anti-CD23, anti-CD1, anti-CD5 and anti-Mac1 (CD11b) (Pharmingen) and anti-kappa (Southern Biotechnology). The following antibodies were used for staining T-cells; anti-CD4, anti-CD8 and anti-CD25 (Pharmingen). In some experiments B-anti-mouse IgM^a, B-anti-mouse IgM^b, B-anti-CD24 (Pharmingen) or B -anti-mouse IgG^{2b} (Pierce Biotechnology, Rockford, IL) were used followed by streptavidin-PE (Southern Biotechnology) or streptavidin-CYC (Pharmingen). PE-anti-Rat was used to detect rat anti-mouse antibodies. Samples were analyzed on a Coulter dual laser cytometer using Elite software (Coulter corp., Hileih, FL) or a BD LSRII flow cytometer using Facsdiva software (Becton Dickinson Bioscience, Mountain View, CA). Gating was set for live lymphocytes based on forward and side scatter and 50,000-200,000 events were collected for each sample. Analysis was performed using either Elite software version 2.1 (Coulter corp., Hileih, FL) or WinMDI, version 2.8 (Scripps Institute, La Jolla CA).

Cell Sorting and ELISPOT

Single cell suspensions were combined from five to eight spleens and RBCs lysed as described earlier. B-cells were first purified using anti-mouse CD19 micro beads or enriched by T-cell depletion using anti-CD90 micro beads as described above. Cell concentrations was adjusted to $1x10^6$/100µl in 1%BSA/PBS and stained with anti-CD21-FITC and anti-CD23-PE. Follicular (CD21^{lo}CD23^{hi}) and marginal zone (CD21^{hi}CD23$^{lo/-}$)

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B-cells were separated by cell sorting based on their differential expression of CD23 and CD21 using a FACSvantage cell sorter (BD Bioscience). Cells were sorted into 1 ml of media (RPMI 1640, 20% FCS supplemented with 2% penicillin/streptomycin (200 U/ml penicillin, 200 μg/ml streptomycin). Purity after sorting was greater than 95%. Sorted cells were either used directly or stimulated with LPS (20μg/ml) for 48 hours prior to ELISPOT on dsDNA coated plates.

5-Bromo-2'-deoxyuridine (BrdU) labeling

BrdU labeling was according to Mandik-Nayak et. al (54). Briefly, NZW mice transgenic for R4A-Cμ or R4A-Cγ2b or wild type control NZW mice or C57BL/6 mice transgenic for R4A-Cμ and R4A-Cμ/hCD19 were injected intraperitoneally every 12 hours for 8 days with 200 μl of 3 mg/ml BrdU (Sigma). Splenocytes were isolated on day 8 and surface stained with CYC-anti-B220 and either B-anti-IgM^a or B-anti-IgM^b (Pharmingen) or B-anti-IgG2b (Zymed Laboratories INC, San Francisco, CA) followed by streptavidin-PE. Cells were then fixed and permeabilized in 1% paraformaldehyde plus 0.1% Tween-20 for 30 minutes on ice followed by 30 minutes at room temperature. DNA was denatured in 0.14M NaCl, 4.2 mM $MgCl_2$ buffer containing 10 μM HCl and 100 U/ml of DNase I (Roche, Indianapolis, IN) for 30 minutes at 25°C. Cells were then stained with FITC labeled anti-BrdU (Becton Dickinson) in staining buffer (0.5% Tween-20 (v/v) + 1% BSA (w/v) in PBS) for 30 minutes at room temperature in order to detect BrdU incorporation and analyzed by flow cytometry using a Coulter dual laser cytometer with Elite software (BeckmanCoulter). Gates were set on $B220^+IgM^a$, $B220^+IgM^b$ or $B220^+IgG^{2b}$ B-cells and percent FITC staining was displayed as a histogram.

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Generation of hybridomas

Spleen cells derived from (six) 8-10 wk old transgenic R4A-Cμ, NZW mice were fused with NSO cells (250). Fusion of total splenocytes was performed on unstimulated cells or cells activated with LPS for 48 hours. Splenocytes were fused with NSO cells in a 2:1 ratio by dropwise addition of 50% PEG 4000 (EM Science, Gibbstown, NJ). Cells were plated at 2 x 10^5 splenocytes/ml in HAT medium supplemented with 20% fetal calf serum (Hyclone Laboratories, Logan, UT). Hybridomas were screened for IgM^a anti dsDNA antibodies by ELISA as described above. Positive clones were expanded and subcloned on a 3.8% soft agar (FMC BioProducts, Rockland, Maine) bed made with Dulbecco's Modified Eagle's Medium (DME) (Sigma) supplemented with 10% J774 mouse macrophage tumor cell line supernatant, 20% FCS (HyClone), 2mM L-glutamine (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma) and 10% NCTC-109 medium (Sigma).

Sequencing and analysis of light chain usage

Hybridoma clones were first screened for expression of R4A-Cμ and Vκ1 genes by RNA dot blot (250). The cells were lysed in lysis buffer [2.5 M KSCN, 0.125% N-lauryl sarcosine, 12.5 mM sodium citrate and 50% DMSO]. The lysate was transferred to nitrocellulose with a BioRad (BioRad, Hercules CA) transfer apparatus and hybridized to a ^{32}P labeled 210 bp DNA probe that detects members of the S107 V_H family. A second blot was hybridized to a Vκ1 probe to detect mouse Vκ1 genes.

Sequencing the variable regions of the V_k light chain genes was according to Spatz et. al. and Kettleborough et al. (251, 252). Total RNA was isolated from $5x10^7$ hybridoma cells using Tri-reagent (Molecular Research Center, Inc, Cincinnati, OH).

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Ready Too Go™ RT-PCR beads (Amersham, Piscatway, NJ) were used to facilitate both RT-PCR and PCR in a single tube. cDNA DNA for κ light chain genes were synthesized using 1μg of total RNA and 20pM of antisense oligonucleotide primer specific for the mouse κ light chain constant region (5'-CTA CCT CGA GTT AAC ACT CAT TCC TGT TGA AGC-3') and 5' degenerate oligomers specific for framework 1 of κ light chain genes. The Vκ FR1oligomers used were; **Vkfr1a** 5'-ATA TCC ATG GCA GAC **G/A**TC **C/A**AG AT**A/G** A**T/C**C CAG **T/A**CT **C/A**CA-3' (specific for Vκ RF, 11, 9A, 9B, 10, 38C), **Vkfr1b** 5'-ATA TCC ATG GCA **G/AAC/A** ATT **G/T**TG CTG AC**T/T** CA**G/A** T**C/T**T CC-3' (specific for Vκ 21 and 23), **Vkfr1c** 5'-ATA TCC ATG GCA GAT **GT/AT/C G/T**TG ATG ACC CAA ACT CCA-3' (specific for Vκ 1 and 2), **Vkfr1d** 5'-ATA TCC ATG GCA **C/GA/GA** A**T/A**T **G/C**TT CTC **A/TC/TC/A** CAG TCT CC-3' (specific for Vκ families 4/5), **Vkfr1e** 5'-ATA TCC ATG GCA **G/AA/GC** **A/G**TT **G/CT/A**G ATG **A/T**CA CAG TC**T/G** CCA-3' (specific for Vκ 8, 22 and 12/13), **Vkfr1f** 5'-ATA TCC ATG GCA GAT ATT GTG AT**G/A** AC**G/T** CAG GC/AT **G/AC/AA**-3' (specific for Vκ 24/25), **Vkfr1g** 5'-ATA TCC ATG GCA **G/AA/GC/T** ATT GTG ATG ACC CA**G/A** **T/ACT/A** C-3' (specific for Vκ 19/28) (252). PCR was done either using a cocktail of the above degenerate primers or as individual reactions using a GeneAmp PCR system 9700 (Perkin-Elmer Applied Biosystems, Foster City, CA) programmed for 42°C at 30 minutes, 95°C at 5 minutes, followed by 35 cycles of (95°C at 30 seconds, 50°C at 30 seconds and 72°C at 1 minute) and a final extension at 72°C for 10 minutes. PCR products were cloned into Invitogen's TA pCR 2.1 plasmid (Invitrogen, Carlsbad, CA) and sequenced using the universal internal primers M13

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reverse and T7 promoter at the Gene Wiz sequencing facility (Gene Wiz North Brunswick, NJ)

Hypotonic Lysis of peritoneal B-cells

Hypotonic lysis of peritoneal B-cells was carried out as described by Murakami et al. (127). One month old hCD19$^{+/+}$R4A-Cμ C57BL/6 transgenic mice were injected intraperitoneally (i.p.) with sterile water (autoclaved and 0.2μm filtered) or 1x sterile PBS (Gibco BRL, Life Technologies, Rockville, MD) before spontaneous secretion of anti-dsDNA antibody appeared (4-6 weeks old). Injections were given weekly and sera were collected via retro-orbital puncture weekly for 12 weeks. The volume of water administered to deplete peritoneal cavity B-cells was dependent on the size of the mouse. Mice that were ≤ 4 weeks of age were given 1ml of fluid, mice 4 to 8 weeks of age received 1.5-2.0ml of fluid. Mice greater than 8 weeks of age received 2ml of water or greater until sacrificed. Depletion of CD5$^+$ B-cells was confirmed by flow cytometry (by double staining cells isolated from the spleen and lamina propria of the gut with CYC-anti-B220 and FITC-anti-CD5. Lamina propria (LP) cells were isolated according to Murakami et al. (253). First feces was removed from the gut and Peyer's patches and mesenteric lymph nodes were removed. The gut was then inverted and the epithelium stripped by treatment with Hank's Balanced solution (HBS) (Gibco BRL) followed by shaking the gut for 15 minutes at 37°C in HBS supplemented with 5mM Na_2 EDTA without Ca^{2+} and Mg^{2+}. The gut segments were then digested with 0.015% collagenase (Gibco BRL) containing 2% FSC/PBS for 75 min at 37°C. LP lymphocytes were collected via centrifugation after filtration using a 0.45μm falcon filter (Becton

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Dickinson, Franklin Lakes, NJ). Analysis by flow cytometry revealed that >98% of B-1 B-cells was depleted from the spleen and LP following hypotonic lysis.

Depletion of $CD4^+$ helper T-cells

One month old R4A-Cμ/$hCD19^{+/+}$ C57BL/6 transgenic mice were injected both ip and intravenously (iv) with 2mg of anti-CD4 GK1.5 monoclonal antibody (500μg iv followed by 500μg ip) for two consecutive days every two weeks for 12 weeks. Mice were injected with GK1.5 prior to the appearance of anti-dsDNA antibody in their serum. This treatment was sufficient to deplete mice of >98% of $CD4^+$ T-cells during the 12 week experiment. Control mice were injected with sterile 1x PBS (Gibco BRL). Depletion of $CD4^+$ T-cells was monitored by screening peripheral blood by flow cytometry as described above.

Antinuclear antibody test

A fluorescent anti-nuclear antibody (ANA) test was performed using an ANA test kit (Immuno Concepts Sacremento, CA). The test was performed as per the manufacturer's instructions. Briefly, 25ul of a 1:50 dilution of mouse sera was added to wells on slides containing Hep-2 cells and incubated at room temperature (RT) for 30 minutes in a moist chamber. Slides were then washed with PBS and incubated with a FITC labeled antibody to mouse IgM^a for 30 min. at RT. Slides were then finally washed with PBS and visualized using a Zeiss axioskop (Thornwood, NY).

***In vitro* cell proliferation assay**

Purified B-cells ($2x10^5$cells/0.1ml) were cultured for 48 hours in flat-bottom microtiter plates in the presence of either 20μg/ml of LPS, 25μg/ml $F(ab)'_2$ fragment of goat anti-mouse IgM, 1μg/ml CpG stimulatory motif (TCC ATG ACG TTC CTG ACG

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TT) or a cocktail of 6μg/ml inhibitory CpG (TCC TGG CGG GGA AGT) and stimulatory CpG (Oligos Etc, Inc, Wilsonville, OR). The inhibitory CpG was added 60 minutes prior to the addition of the stimulatory CpG in RPMI-1640 supplemented with 10% FCS, 2mM L-glutamine, 100U/ml penicillin, 100U/ml streptomycin, 100μM nonessential amino acids and 50μM β-mercaptoethanol. During the last 16 hours, the wells are pulsed-labeled with [^{3}H] thymidine (1μCi per well). Cells were harvested using a Brandel M24 harvester (Gaithersburg, MD) and thymidine quantitated using a LKB Wallac liquid scintillation counter (Perkin-Elmer, Downers Grove, IL) (254).

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CHAPTER III

ABSTRACT

One mechanism by which anti-double stranded (ds) DNA B cells are regulated is anergy. Multiple phenotypes have been attributed to anergic B cells in various transgenic models. Differences in the nature of the antigen and in the avidity of antigen-antibody interactions may account for these variations in phenotype. In the present study we describe a population of dsDNA binding B cells that display many of the features of anergic B cells but have characteristics which suggest they are partially functional as well. These B cells do not spontaneously secrete antibody nor can they be induced to secrete antibody following receptor crosslinking *in vitro*. Furthermore, they display an immature phenotype and have a shortened lifespan, characteristic of anergic B cells. However, they can be induced to secrete anti-dsDNA antibody following activation with T cell derived factors as well as with lipopolysaccharide (LPS) and they can be recovered by somatic cell hybridization even in the absence of LPS stimulation prior to fusion. These results suggest that antigen receptor signaling can be uncoupled from signaling induced by T cell derived factors or LPS and that this may be a mechanism for maintaining tolerance. This may have protective advantages because it may enable B cells to be down-regulated in response to autoantigen yet be available for recruitment in an inflammatory response. In addition, in this study we compare the phenotype and functional properties of anergic IgM dsDNA binding B-cells with that of anergic IgG dsDNA binding B-cell and observe that IgG B-cells are more stringently regulated than there IgM counterparts.

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INTRODUCTION

R4A Transgenic Mice

Understanding the regulation of autoreactive B cells has been challenging, in part, because the frequency of these B cells in the total lymphocyte population is small. Transgenic technology has diminished this problem by making it possible to generate larger numbers of autoreactive B cells. Thus, their fate can be followed and the cell fate decisions that are made in non-autoimmune and autoimmune hosts can be elucidated.

Systemic Lupus Erythematosus (SLE) is an autoimmune disease characterized by the production of IgG anti-double stranded (ds) DNA antibodies. The presence of these antibodies correlates with disease activity, especially glomerulonephritis. To understand the immunological defects in SLE and the regulation of anti-dsDNA B cells, Offen et. al., previously generated non-autoimmune, NZW mice transgenic for the γ2b heavy chain of the R4A anti-dsDNA antibody (2). The R4A anti-dsDNA antibody is encoded by an S107, V_{11} heavy chain gene and a V_k1 light chain gene, and has been shown to deposit in glomeruli of SCID mice (248, 255). Tolerance induction has previously been shown to be intact in NZW R4A-Cγ2b transgenic mice (2, 251). Negligible serum titers of IgG2b anti-dsDNA antibodies have been observed in R4A-Cγ2b mice despite the presence of R4A transgenic B-cells in the bone marrow and spleen (2, 251). Three populations of anti-DNA B cells that are differentially regulated have also been characterized in these mice (2, 251). There is an anergic population that secretes high affinity anti-dsDNA antibodies after *in vitro* stimulation with LPS. These B cells predominantly use $V_κ1$ light chain genes which are somatically mutated. There is a second subset of B cells with high affinity for dsDNA, that is targeted to deletion but can be rescued in autoimmune,

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NZB/W F1 mice transgenic for R4A-Cγ2b and in mice transgenic for both R4A-Cγ2b and the proto-oncogene, bcl-2 (251, 256). This population utilizes a broad spectrum of light chains genes that are predominantly non-$V_\kappa 1$. The inability to detect high affinity dsDNA binding B-cells producing anti-dsDNA antibodies utilizing non-Vκ1 light chains in the non-autoimmune mice suggests that these B-cells are being deleted (251, 257). The third population is not tolerized and produces antibodies that display low affinity for dsDNA and utilize a spectrum of light chain genes (251, 257, 258). This population is said to be clonally ignorant. Sequence analysis reveals that some of the low-affinity anti-DNA autoantibodies differ from their high affinity counterparts by as few as one amino acid suggesting that somatic mutation can lead to their increased affinity (258).

Other laboratories have studied the regulation of IgM anti-DNA B cells using transgenic mouse models and have observed that several mechanisms of tolerance including anergy, deletion and receptor editing, contribute to the regulation of dsDNA binding B cells. While B cells specific for single-stranded (ss) DNA have been shown to be targeted to anergy only, those specific for dsDNA have been shown to be targeted to receptor editing, anergy or deletion depending upon the affinity of the anti-dsDNA antibody for the autoantigen (33, 43, 53, 241, 259).

Although it has been demonstrated that IgG transgenic heavy chains can promote normal B cell development in mice we have been concerned that the mechanisms of tolerance induction in NZW mice transgenic for R4A-Cγ2b may not be representative of tolerance mechanisms in non transgenic animals since the expression in naïve B cells of IgG heavy chains prior to IgM heavy chains is not physiological (250, 260). This study was, therefore, undertaken to generate NZW mice transgenic for the R4A-Cμ heavy

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chain and to determine whether tolerance is maintained in these mice in a manner similar to that observed in R4A-Cγ2b mice and whether the thresholds that determine cell fate differs for IgM and IgG autoreactive B-cells. The B-cell signaling event that leads to activation and tolerance induction is transduced through the BCR, its coreceptors and the α/β heterodimer that is associated with it. However, it is not known whether the cytoplasmic tail region of the heavy chain also plays a role. The length of the IgG heavy chain cytoplasmic tail is 28 amino acids long while the IgM heavy chain is only 3 amino acids in length (261). There may be differences in the molecules that are associated with the cytoplasmic tail regions of IgM and IgG, since IgG is much longer. Functional differences may also exist between the cytoplasmic tails of IgG and IgM. The cytoplasmic tail region of the IgG heavy chain has been shown to be important in the endocytosis of antigen and antigen presentation to T-cells (210, 262). In addition, B-cells expressing mIgG have been found to be more responsive to antigen stimulation than B-cells expressing mIgM. Recently it has been shown that CD22 negatively regulates signaling through the cytoplasmic tail of IgM but not IgG. The absence of signal inhibition of IgG may play a role in the enhanced signaling of IgG (212).

The R4A-Cμ heavy chain utilizes a VDJ region that is identical to that used by the R4A-Cγ2b transgene, previously described (2). The only difference between these transgenes is that the γ2b constant region has been replaced by a μ constant region in the R4A-Cμ construct. In R4A-Cμ mice the transgenic μ heavy chain can pair with a variety of endogenous light chains to produce both dsDNA binding and non dsDNA binding antibodies. This provides a physiological environment for which to study anti-dsDNA B-

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cells and allows us to study autoreactive B-cells in the context of B cells with non-autoreactive specificities.

In this study, we observe that tolerance is maintained in the R4A-Cμ mice. Moderate to high affinity dsDNA binding B cells persist in the periphery of these mice and like their γ2b counterparts they do not spontaneously secrete antibody. These B cells display characteristics of anergic B cell including reduced surface expression of the B cell receptor (BCR), arrested development, shortened lifespan and inability to be activated by BCR cross-linking. However, unlike their γ2b counterparts they can be easily rescued by hybridoma technology in the absence of prior stimulation with LPS and can be activated to differentiate and secrete antibody in vitro by T cell derived factors. Hence there appears to be a qualitative difference in the level of anergy induced in the R4A-Cμ and the R4A-Cγ2b B cells which may be a consequence of differences in expression levels of the transgenes or may be due to differences in heavy chain isotypes. Nevertheless, the observation that R4A-Cμ dsDNA binding B cells are responsive to T cell derived factors but not BCR cross-linking suggests that these B cells are partially functional and that signaling pathways leading to their activation can be uncoupled.

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RESULTS

Generation of R4A-Cμ trangenic mice

The R4A-Cμ transgene was generated by ligating a 3.5 kb genomic fragment containing an unmutated, rearranged V_{11} gene and the heavy chain enhancer region, to a 9.9 kb genomic fragment containing both the secreted and membrane exons of Cμ. This 3.5 kb fragment containing the R4A VDJ region, is identical to the one used to generate the R4A-Cγ2b construct as previously described (2). The Cμ fragment was derived from a BALB/c mouse so it bears the a allotype. The R4A-Cμ transgene was injected into C57BL/6 x CBA F1 fertilized eggs and then backcrossed onto the NZW background for at least 10 generations. Southern blot analysis of tail DNA digested with Sac I and hybridized with a probe specific for the C_H^2-C_H^3 regions of Cμ , demonstrated integration of the transgene in a single site in the genome (Figure 8A and B). Densitometry revealed the presence of 5 copies of the transgene in the genome.

RNA expression

Expression of the R4A-Cμ transgene was demonstrated by Northern blot analysis of tissue RNA hybridized with a probe specific for the S107 V_H gene family. Elevated levels of expression of the transgene were observed in the spleen of a transgenic mouse (top panel, Figure 8C). Expression of endogenous S107 in the non transgenic spleen was too low to be detected at this exposure. Minimal levels of expression of the transgene were observed in the lung, kidney and heart of the transgenic mouse. A probe specific for the μ constant region hybridized to splenic RNA from the transgenic mouse and its non-transgenic littermate indicating that equivalent concentrations of RNA from both mice were loaded onto the gel (bottom panel, Figure 8C).

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Assays for serum immunoglobulin

Serum from 10, 7-12 week old transgenic NZW mice and 10 non-transgenic NZW littermates were assayed for IgM^a anti-dsDNA binding by ELISA (Figure 9A). Since the transgene is of the a allotype and endogenous IgM in the NZW mouse strain is of the b allotype, transgenic IgM could be distinguished from endogenous IgM using an anti-IgM antibody specific for the a allotype. A purified monoclonal IgM^a anti-dsDNA antibody (4D4) that utilizes the R4A heavy chain was used at a concentration of 1 μg/ml, as a positive control for dsDNA binding.

Serum concentrations of total IgM^a were determined by a quantitative ELISA (Figure 9B). Despite the observation that transgenic mice contain between 3-18 μg/ml of total IgM^a antibody in their sera, none of this antibody was observed to bind dsDNA. As mentioned previously, the transgenic heavy chain can pair with some endogenous light chains to produce non DNA binding antibodies which can account for the serum concentration of IgM^a antibody.

Characterization of transgenic B cells by flow cytometry

Bone marrow and splenic B cells from transgenic mice and non-transgenic littermates were examined by flow cytometry. By staining bone marrow and splenic cells with CYC-anti-B220 only or in combination with PE-anti-IgM^a and FITC-anti-IgM^b we determined the frequency of total B cells, B cells expressing the transgene (IgM^a) and B cells expressing endogenous IgM (IgM^b). Analysis of $B220^+$ gated B cells revealed that the majority of transgene expressing B cells in the bone marrow and spleen express IgM^a only and not endogenous IgM^b. This demonstrates that the transgene maintains intact allelic exclusion (Figure 10A). The splenic B cells that fail to surface stain with PE-anti-

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IgM^a or FITC-anti-IgM^b express IgG heavy chains and none of the IgG B cells co-express IgM^a (data not shown).

Table 6 summarizes the results obtained from six transgenic mice and six non transgenic littermates. While the percent of B220 B cells in the bone marrow of transgenic mice and non transgenic littermates are similar, the percent of B220 B cells is ~2 fold less in the spleens of transgenic mice. Likewise, there is ≤1.5-fold difference in the absolute number of B cells in the bone marrow of transgenic mice and non transgenic littermates while there is almost a 5 fold difference in the absolute number of B cells in the spleen of transgenic mice and non transgenic littermates. While we cannot rule out the possibility that the diminished B cell number in the spleens of transgenic mice is simply due to the presence of the rearranged transgene and unrelated to B cell specificity, a feature occasionally observed in transgenic mice, results suggest that B cell deletion may be occurring at some time in transit from the bone marrow to the spleen in transgenic mice, presumably because of autoreactivity. In addition, we observe that the relative increase in the number of B cells expressing endogenous IgM (IgM^b) from the bone marrow to the spleen in transgenic mice (32 fold) is greater than the increase of B cells expressing the transgene (4 fold) (Table 6). This may be due to a selective expansion of B cells expressing endogenous IgM or to anergy and/or deletion of autoreactive, transgenic B cells. Alternatively, receptor revision in immature B cells in the periphery may be promoting the disappearance of B cells bearing the transgenic heavy chain.

In order to determine whether B-cells expressing the IgM^a transgene undergo normal development in the bone marrow we used flow cytometry to analyze the

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expression of different developmental markers on transgenic B-cells. B-cells in different stages of maturation were identified according to the phenotypes defined by Hardy et. al., as previously discussed (263). Bone marrow cells were first triple stained with antibody to B220, IgM and CD43. FACS analysis was performed on $B220^+$ gated B-cells to demonstrate the percentage of pro B-cells ($B220^+$, $CD43^+$, IgM^-) and pre B-cells ($B220^+$, $CD43^-$, IgM^-) (Figure 11). The percentage of pro B-cells observed in both transgenic and non-transgenic littermates was very similar, 37.2% and 33.7% respectively (Figure 11). Likewise I also observed that the percentage of pre B-cells in both transgenic (42.5%) and non-transgenic (36.7%) littermates were similar (Figure 11). This reveals that the development from pro to pre B-cells is normal in transgenic mice.

Developmental Arrest

Since other studies have demonstrated that tolerized B cells are arrested in an immature stage of development, we next examined whether more B cells expressing the transgene have an immature phenotype than B cells expressing endogenous IgM (47). Immature B cells express surface IgM, have a $CD24^{hi}$ phenotype and can be detected using an antibody to CD24 (129). We stained bone marrow cells from 2 month old transgenic and non transgenic littermates with CYC-anti-B220, FITC-anti-CD24 and biotinylated-anti-IgM^a or biotinylated-anti-IgM^b followed by streptavidin PE. A higher percent of bone marrow B cells expressing the transgene are in an immature stage of development than B cells expressing endogenous IgM (Figure 12A). The ratio of $CD24^{hi}$ to $CD24^{lo}$ is 4 for B cells expressing IgM^a and 0.9 for B cells expressing IgM^b. In the spleen of transgenic mice however, we did not consistently observe an increased frequency of $CD24^{hi}$ transgene expressing B cells (Figure 12B). In fact, the majority of

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transgenic B-cells in the spleen (29.2%) displayed a mature phenotype ($B220^+$, IgM^a, $CD24^{lo}$). We speculate this reflects maturation of the non-DNA binding IgM^a B cells. The observation that at least some IgM^a B cells can acquire the mature phenotype indicates that the R4A-Cμ transgene is capable of signaling B cell maturation. The development of a reagent that can specifically track the dsDNA binding subset of transgenic B cells is necessary to accurately determine the fate of autoreactive B cells.

Density of Receptor

Studies by Goodnow et. al. demonstrated that in mice transgenic for soluble hen egg lysozyme (HEL) and antibody to HEL, tolerant HEL binding B cells exhibited an altered phenotype characterized by a reduction in membrane IgM (36). To determine whether a subset of B cells in our transgenic mice also exhibited this receptor downmodulation, we stained splenocytes from transgenic and non transgenic littermates with CYC-anti-B220, FITC-anti-kappa , and either B-anti-mouse IgM^a, or B-anti-mouse IgM^b respectively, followed by streptavidin-PE. Cells were gated on $B220^+$, IgM^a cells (transgenic mice) or $B220^+$ IgM^b cells (non transgenic mice) and mean fluorescent intensity (MFI) of kappa staining was compared for IgM^a and IgM^b B cells (Figure 13). A broad peak of kappa fluorescence was observed in transgenic mice suggesting a heterogeneous population of transgenic B-cells expressing a range of densities of $mIgM^a$. Some transgene expressing B cells displayed reduced expression of IgM^a relative to IgM^b while others displayed a level of expression of IgM^a comparable to that of IgM^b. We speculate that the transgenic B cells expressing a reduced level of IgM are dsDNA binders while those expressing levels of IgM similar to endogenous B cells are non dsDNA binders.

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In vitro activation studies

We previously demonstrated that anergic B cells in the spleens of R4A-Cγ2b transgenic mice can be activated to secrete anti-dsDNA antibody following *in vitro* stimulation with LPS. In the present study, we were interested in determining whether an anergic population of anti-dsDNA B cells also exists in the R4A-Cμ mice. We stimulated splenocytes from 5 R4A-Cμ mice *in vitro* with 20 μg/ml of LPS and assayed culture supernatants for the presence of anti-dsDNA antibody. Spleen cells cultured with LPS displayed elevated levels of IgM^a anti-dsDNA antibodies relative to unstimulated control cells (Figure 14A). Likewise, total IgM^a antibody secretion was enhanced by LPS stimulation (Figure 14B). The number of B cells secreting anti-dsDNA antibodies also increased following LPS stimulation as detected by ELISPOT (Figure 15A).

Anergic B cells have been defined as functionally inactivated B cells that fail to secrete antibody in response to cross-linking of their surface immunoglobulin (Ig) with antigen or antibody to Ig (41, 48). To determine whether the IgM DNA binding B cells are anergic or resting B lymphocytes we attempted to activate them with anti-IgM $F(ab')_2$ in the presence of an antibody to CD40 which mimics the action of CD40L thereby mediating T cell help (48, 264, 265). While the combination of these signals induced total IgM^a antibody secretion, as assayed by ELISPOT (Figure 15B), it failed to induce the production of anti-dsDNA antibody (Figure 15A) suggesting that the transgenic, dsDNA binding B cells are anergic.

It has been reported in some transgenic mouse models that anergic B cells can be induced to differentiate and secrete antibody in response to CD40L plus IL-4 while others have reported that B cells can proliferate but not secrete antibody under the same

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conditions (27-29, 500). We wished to determine whether anti-CD40 plus IL-4 could induce antibody secretion by dsDNA binding B cells from our transgenic mice. We, therefore, cultured T cell depleted splenocytes, for 48 hours in the presence of antibody to CD40 and rIL-4. We observed by ELISPOT a significant increase in the number of transgenic B cells secreting anti-dsDNA antibody, comparable to that observed with LPS (Figure 15A and C). Based on these results we also sought to determine the affect of incubating splenic B cells with a cocktail of T cell cytokines. We observed that a combination of rIL-4, rIL-5 and rIL-6 could also activate IgM^a dsDNA binding B cells to differentiate and secrete anti-dsDNA antibody (Figure 15C).

Life span measurement

Anergic B cells have been shown to have a shortened lifespan (54, 56). We assessed the lifespan of B cells expressing the R4A-Cμ transgene relative to B cells expressing endogenous IgM. B cells from transgenic mice and non transgenic littermates were continuously labeled with BrdU for 8 days and the incorporation of BrdU was measured by flow cytometry. Populations that are rapidly turning over and have a reduced life span are replaced more rapidly by labeled cells from the bone marrow and this is reflected as a higher percentage of cells that have incorporated BrdU. In three out of three experiments we observed that IgM^a B cells have a slightly higher incorporation of BrdU relative to non transgenic B cells suggesting a reduced lifespan (Table 7).

Activated Phenotype

Anergic anti-dsDNA B-cells were reported by Erikson et. al. to display a phenotype characteristic of activated B-cells as defined by the upregulation of both CD86 (B7.2) and CD69 and the down regulation of CD62L (L-selectin) due to the chronic

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exposure to self-antigen (54). We assessed transgenic B-cells from R4A-Cμ mice for their expression levels of CD86, CD69 and CD62L (L-selectin). Transgenic and non-transgenic B-cells were immunostained with antibody to B220, IgM^a or IgM^b, CD86, CD69 and CD62L. By gating on $B220^+IgM^a$ or $B220^+IgM^b$ B-cells and examining mean fluorescence intensity (MFI) for CD86, CD69 and CD62L activation markers, we observed that transgenic and non-transgenic B-cells displayed similar MFIs (Figure 16). Relative to B-cells that were activated by LPS *in vitro*, R4A-Cμ B-cells to did not display an activated phenotype (Figure 16). These results suggest that not all anergic B-cells have an activated phenotypes.

Generation of transgene-expressing IgM^a anti-DNA B cell hybridomas

We previously demonstrated that hybridomas secreting high affinity anti-DNA antibody could be obtained from R4A-Cγ2b transgenic mice only after stimulation of spleen cells with LPS prior to fusion. We were unable to generate high affinity R4A-Cγ2b anti-dsDNA hybridomas from unstimulated B cells. To analyze tolerance mechanisms in the R4A-Cμ transgenic mice, we generated hybridomas from LPS stimulated spleen cells. Approximately 750 wells from 3 fusions with LPS stimulated splenocytes showed growth of hybridoma cells; 37 displayed IgM^a dsDNA binding activity (5%) and 16 were cloned for further analysis.

Surprisingly, we were also able to generate hybridomas from unstimulated, naïve splenocytes from R4A-Cμ transgenic mice. Approximately 560 wells from 6 fusions showed growth of hybridomas. Seventeen of these clones (3%) produced high affinity IgM^a anti-dsDNA antibody. Ten of them were cloned for further analysis. These results indicate that the high affinity anti-dsDNA B cells present in the R4A-Cμ transgenic mice

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can form viable hybridomas even without prior LPS activation. Thus, they differ from R4A-Cγ2b anti-DNA B cells which can only be immortalized as hybridomas following activation with LPS. By ELISA we demonstrated that anti-dsDNA antibodies produced by hybridomas obtained from either unstimulated or LPS stimulated splenocytes show a similar binding to dsDNA (Figure 17). We also measured affinities of anti-dsDNA antibodies obtained from both LPS stimulated and unstimulated R4A-Cμ B cells by inhibition ELISA and observed that all antibodies have similar apparent affinities for dsDNA ranging from 10^7 to 10^8.

Analysis of V_κ gene usage in LPS stimulated and naïve hybridomas

In NZW R4A-Cγ2b mice, we previously observed that the anergic population of transgenic B cells predominantly utilizes a mutated V_κ1 light chain gene while the subset that is targeted to deletion utilizes non-V_κ1 genes (251). In the present study we observed by RNA dot blot, that the light chains of all of the R4A-Cμ anti-dsDNA antibodies produced by hybridomas generated following LPS activation, utilize V_κ1 genes. Similarly, the light chains from 7 of 9 anti-dsDNA antibodies produced by hybridomas generated from unstimulated B cells utilize V_κ1 genes. The light chains from 11 antibodies obtained from LPS stimulated splenocytes and 9 antibodies obtained from naïve splenocytes were sequenced to confirm these results (Table 8).

Ten of 11 antibodies produced by LPS activated B cells utilize the V_κ1-A gene and 1 utilizes the V_κ1-C gene. Only two V_κ1-A genes are mutated; one contains a somatic mutation in FR3, resulting in the substitution of a phenylalanine for a serine and one contains a substitution at the VJ joining region resulting in the replacement of a leucine with a proline. This latter change may not be the result of mutation in the

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periphery but a consequence of rearrangement of the V J regions in the bone marrow. The $V_\kappa 1C$ gene is unmutated. Seven of 9 light chains sequenced from the antibodies obtained from unstimulated anti-dsDNA B cells utilize the $V_\kappa 1A$ light chain gene. One of the $V_k 1A$ genes contains a substitution in the VJ joining region which results in the replacement of a tryptophan with a proline. Two of the light chains utilize non $V_\kappa 1$ genes ($V_k 21$-E and $V_k OX$-1), neither of which is mutated. All sequences have been reported to GenBank.

Comparison of anergic R4A IgM and anergic R4A IgG2b B cells

Once we determined that anergic dsDNA binding B-cells were present in the spleen of R4A-IgM transgenic mice and that these B-cells could be activated by anti-CD40 plus IL4, we then were interested in determining whether anergic B-cells from R4A-IgG transgenic mice could also be activated by CD40 dependent T-cell factors. We therefore set up *in vitro* cultures, where we incubated splenic B-cells from R4A-IgM or R4A-IgG mice with either LPS or anti-CD40 plus IL4 and assayed for the number of B-cells secreting anti-DNA antibody by ELISPOT. We observed that anergic IgG2b dsDNA binding B cells were less responsive to activation by anti-CD40 plus IL-4 than IgM dsDNA B-cells but their response to LPS was not significantly different (Figure 18A). Total IgM^a and total IgG2b antibody secretion from R4A-IgM and R4A-IgG mice respectively were also evaluated by ELISPOT (Figure 18B). Interestingly, total IgG2b antibody secretion was more responsive to activation with anti-CD40 and IL-4 than IgM B-cells. This was presumably due to the activation of non-dsDNA binding IgG2b B cells.

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This differential responsiveness of R4A-IgG and R4A-IgM B-cells to CD40 dependent T-cell signals led us to speculate that anergic IgG B-cells are more tightly regulated than anergic IgM B-cells.

We hypothesized that one possible explanation for differences in responsiveness between R4A-IgM and R4A-IgG B-cells could be that these transgenic B-cells express different levels of surface immunoglobulin. To check for this we determined the density of IgM^a or IgG2b surface immunoglobulin on R4A-Cμ and R4A-Cγ2b B cells respectively by immunostaining splenic B cells with antibody to B220 and either antibody to IgM^a or antibody to IgG2b and antibody to kappa light chain. By gating on either $B220IgM^a$ or B220IgG2b for B-cells from R4A-Cμ or R4A-Cγ2b mice respectively we observed that the mean fluorescent intensity (MFI) of kappa staining was similar for IgM^a and IgG2b B cells suggesting that equivalent densities of surface immunoglobulin were expressed both (Figure 19). This suggests that the differential responsiveness of anergic IgM and IgG B-cells was not due to different levels of expression of surface immunoglobulin.

Since naïve R4A-Cγ2b dsDNA binding B-cells are unable to be rescued as hybridomas unless pretreated with LPS while naïve R4A-Cμ B-cells can, we wondered whether R4A IgG B-cells have a shorter lifespan than the R4A IgM dsDNA binding B-cells. We therefore measured the lifespan of transgenic B-cells from R4A-Cγ2b and R4A-Cμ mice by BrdU labeling (as previously described). We observed that the incorporation of BrdU was higher in R4A-γ2b than R4A-Cμ B–cells suggesting that R4A-Cγ2b B-cells do have a shorter lifespan (Figure 20).

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DISCUSSION

We have observed that dsDNA binding B cells are present, but are anergic in the periphery of R4A-Cμ mice. They fail to be activated to differentiate and secrete antibody *in vitro* following crosslinking of their B cell receptor with anti-IgM $F(ab')_2$. However, they can be activated to secrete antibody by LPS, anti-CD40 as a surrogate for T cell help plus IL-4 or a combination of the cytokines IL-4, IL-5 and IL-6. We have also observed that some B cells in the transgenic mice display reduced levels of surface IgM^a, have a lag in development as evidenced by $CD24^{hi}$ expression and have a shortened lifespan. Since this phenotype is consistent with that of anergic B cells we believe that these represent the dsDNA binding B cells. We have also shown that anergic IgM dsDNA binding B-cells are more responsive to CD40 plus IL4 dependent signaling than IgG DNA binding B-cells and demonstrated that anergic IgM B-cells have a longer lifespan their than anergic IgG counterparts.

We have been able to rescue R4A IgM^a dsDNA binding B cells as hybridomas by somatic cell hybridization even in the absence of prior stimulation. Anti-dsDNA antibodies obtained from these hybridomas predominantly utilize unmutated V_k1 light chain genes. This suggests that tolerance induction is occurring in a naïve B cell population. We have previously demonstrated that the R4A heavy chain can associate with several V_κ families to produce antibodies with specificity for DNA, however, those B cells producing antibodies that utilize non $V_\kappa1$ light chains are targeted to deletion *in vivo*. Failure to obtain many anti-dsDNA producing hybridomas that utilize non $V_\kappa1$

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light chains from the R4A-Cμ mice confirms that most of these B cells are targeted to deletion.

It is intriguing that high affinity, IgM^a V_k1 dsDNA binding B cells can be rescued as hybridomas from unstimlated splenocytes since earlier studies demonstrated that high affinity, IgG2b, V_k1 dsDNA binding B cells could only be rescued if B cells were activated with LPS prior to fusion (250, 251). It is also interesting that IgM dsDNA binding B cells can be activated to secrete antibody following *in vitro* stimulation with anti-CD40 plus IL-4 while IgG2b dsDNA binding B cells from R4A-Cγ2b mice cannot be activated to secrete antibody under these conditions. The IgM dsDNA binding B cells may be partially functional relative to their IgG2b counterparts, in a weaker stage of anergy and less stringently regulated thus making them more conducive to fusion and more responsive to T cell derived factors than IgG2b anti-dsDNA B-cells. Both R4A-Cμ and R4A-Cγ2b anti-dsDNA antibodies share identical unmutated heavy chain variable regions and preferentially utilize the same Vκ1 light chains, therefore we assume their specificity is similar. They also have comparable affinities for dsDNA and the level of surface immunoglobulin expression on R4A-Cμ and R4A-Cγ2b B-cells, as demonstrated by flow cytometry is similar. Since we do not observe any differences in antigen concentration, antibody specificity or affinity and in density of surface immunoglobulin, which could account for differences in regulation of R4A-Cμ and R4A-Cγ2b dsDNA binding B-cells we speculate that differences in heavy chain isotype may play a role and that γ heavy chains may transduce qualitatively different signals leading to more exaggerated tolerance induction than μ heavy chains. A study have shown that antibodies with different heavy chain classes, but identical specificity differ in their

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ability to transduce positive activating signals (266). It has recently been shown *in vitro* that the CD22 BCR coreceptor only inhibits signaling through the IgM receptor but not through the IgG-BCR because the cytoplasmic tail of IgG prevents CD22 mediated SHP1 activation (212). It is therefore, possible that other molecules may interact with the cytoplasmic tail of IgG and alter the strength of tolerogenic signals.

Although, anergy is defined as functional unresponsiveness to antigen, the characteristics of anergic B cells seem to vary in different transgenic models. Goodnow *et al.* first demonstrated that in mice transgenic for soluble HEL and antibody to HEL, anergic HEL binding B cells display reduced levels of surface immunoglobulin known as receptor down modulation and have a shortened lifespan but are not arrested in development (46, 56). They also showed that anergic HEL B cells could be induced to proliferate and differentiate into antibody secreting cells, although at suboptimal levels, following *in vitro* activation with LPS or CD40L plus IL-4 and IL-5 as well as with an anti-sera to CD40 plus IL-4 (48, 50, 52).

Erikson and colleagues observed differences in the characteristics of anergic single stranded DNA (ssDNA) and dsDNA binding B cells (47, 54, 138, 267). They observed that anergic ssDNA binding B cells cannot be induced to secrete antibody following BCR crosslinking, however, they can be induced to proliferate following LPS stimulation or crosslinkage with CD40L plus IL-4. Furthermore, they observed that these B cells have a normal lifespan and are not arrested in development. In contrast, they observed that dsDNA binding B cells have a shortened lifespan and are arrested in development and display an antigen experienced phenotype. In addition, they do not proliferate in response to LPS or BCR crosslinking and do not differentiate into antibody

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secreting cells in response to CD40L plus IL-4 although they do proliferate in response to these T cell factors (54, 138, 267).

Our studies demonstrate that R4A-Cμ dsDNA binding B-cells display a phenotype that differs from that described above for other anergic IgM ssDNA and IgM dsDNA binding B cells. While the dsDNA binding B cells in R4A-Cμ mice show evidence of receptor downmodulation, arrested development, shortened lifespan and inability to be activated to secrete antibody following BCR crosslinking, they can be activated to secrete antibody following incubation with LPS or T cell derived factors. These results are significant because the vulnerability of some "anergic" B cells to non antigenic stimuli may provide a pathway to a breakdown in tolerance.

Differences in the avidity of interaction between autoantibodies and their autoantigen which reflects receptor affinity, surface density, and concentration of the autoantigen and differences in fine specificity, and the nature of the autoantigen may account for the range of phenotypes observed in anergic B cells. Just as the extent of receptor crosslinking determines whether autoreactive B cells are anergized, deleted or ignored, the strength and/or nature of antigen-antibody interactions may determine qualitative differences in anergy (49, 143).

The observation that R4A-Cμ dsDNA binding B cells are partially functional and can respond by antibody secretion to T cell derived factors and LPS but not BCR crosslinking suggests an uncoupling of signaling cascades. Signaling pathways transduced via the BCR, CD40 ligation, or LPS activates the transcription factor, NF-κB (268, 269). It is not clear whether these signaling pathways are distinct or have common intermediates upstream of NF-KB. PKC has been shown to be necessary for BCR but not

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CD40 or LPS induced activation of NF-KB while PI-3 kinase has been shown to be necessary for both BCR and LPS induced activation of NF-KB (268-270). Depletion or inhibition of a molecule unique to one pathway may have little affect on the other pathways. This could explain why a block in BCR signaling could be overcome by signaling through CD40 ligation or LPS. Inhibition of a factor common to all three pathways would prevent signaling following BCR crosslinking as well as CD40 ligation and LPS. Therefore, qualitative differences in anergy may be the consequence of blocking one or more signaling pathways. A paradigm for the uncoupling of signaling pathways comes from recent studies in B and T cells. Okkenhaug et. al. demonstrated that a point mutation in the co-stimulatory molecule CD28 uncouples signals required for T cell proliferation and survival (271). Bone and Williams demonstrated that an inhibitor of PKC uncouples BCR signaling from signaling through LPS by blocking a PI-3 kinase dependent pathway to NF-KB activation that is distinct for BCR signaling (270). Future studies will be aimed at determining mechanisms by which BCR uncoupling may be occurring in anergic R4A-Cμ dsDNA binding B cells.

The observations that IgM dsDNA binding B-cells from R4A-Cμ are more responsive to signaling by T-cell derived factors than IgG2b dsDNA binding B-cells from R4A-Cγ2b mice and that they have slightly longer lifespans and can be rescued as hybridomas without prior activation while IgG2b B-cells cannot, suggest that they are less stringently regulated than IgM^a B-cells. Differences in the strength of BCR induced signaling could lead to differential regulation of IgM and IgG B-cells. A recent study supporting differential signaling by IgM and IgG B-cells suggests that signaling through IgG is heightened and sustained because it cannot associate with the negative regulatory

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CD22 molecule while signaling through IgM is weaker because it does associates with CD22 (212).

Factors that could alter the strength of BCR signaling are the affinity and specificity of the Ig for its antigen and the density of membrane Ig. R4A-Cμ and R4A-γ2b anti-dsDNA antibodies share identical unmutated heavy chain variable regions and they preferentially use the same $V_{\kappa}1$ light chains, therefore, we assume their specificities are similar. They also have comparable affinities for dsDNA. Furthermore, the level of surface immunoglobulin expression on R4A-Cμ and R4A-γ2b B-cells, as demonstrated by surface staining and flow cytometry is very similar. Since we do not observe any differences in antigen concentration, antibody specificity or affinity and in density of surface immunoglobulin, which could alter receptor cross-linking and account for differences in the regulation of R4A-Cμ and R4A-γ2b dsDNA binding B-cells. We conclude that heavy chain class must play a role.

In summary, this study describes a novel phenotype for IgM dsDNA binding B cells; one in which B cells can be activated to differentiate and secrete antibody in response to T cell derived factors or LPS but are unresponsive to signaling through the BCR. The persistence of these "partially functional" B cells due to the uncoupling of the BCR signaling pathway from other signaling pathways may offer the immune system a protective advantage by enabling B cells cross reactive to both self and foreign antigen to be downregulated in response to autoantigen while still being available for recruitment in an inflammatory response. In addition, in this study we observe that in the presence of autoantigen, IgM anti-dsDNA B-cells are less stringently regulated than their IgG counterparts. This may be because mIgM delivers a weaker tolerogenic signal than mIgG

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and because the threshold of tolerance induction is lower in IgG than IgM B-cells. Since IgG antibodies can penetrate tissue and potentially cause more damage in this way than IgM antibodies it may be necessary to keep IgG antibodies under tighter control.

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Figure 8. Map and expression of the R4ACμ heavy chain transgene. **(A)** A 3.5 Kb EcoRI fragment containing the rearranged VDJ (variable-diversity-joining) and heavy chain enhancer (E_H) regions was ligated to a 9.9 Kb fragment containing the μ constant region including the membrane exons (m_μ). White boxes depict exons. Dark boxes depict introns. $C_{H(1-4)}$- constant region exons, E-EcoR 1, B- BamH 1, N-Not 1, S-Sac 1, P-Pst 1, sμ-secretion coding exon, m_μ-membrane coding exons. **(B) Southern blot of tail DNA digested with *Sac* 1 and hybridized to a Cμ probe.** Lanes: 1, DNA from a nontransgenic mouse; 2, DNA from a transgenic littermate (the transgene is indicated by a band at 9.7 Kb). **(C) Tissue-specific expression of the R4A-Cμ transgene.** RNA isolated from different organs from a R4A-C_μ transgenic (Tg) mouse (lanes 1-4) was hybridized with a probe specific for the S107 V_H gene family (top panel) and a probe specific for the Cμ constant region (bottom panel). RNA from the spleen of a nontransgenic littermate (NTg) was used as control (lane 5).

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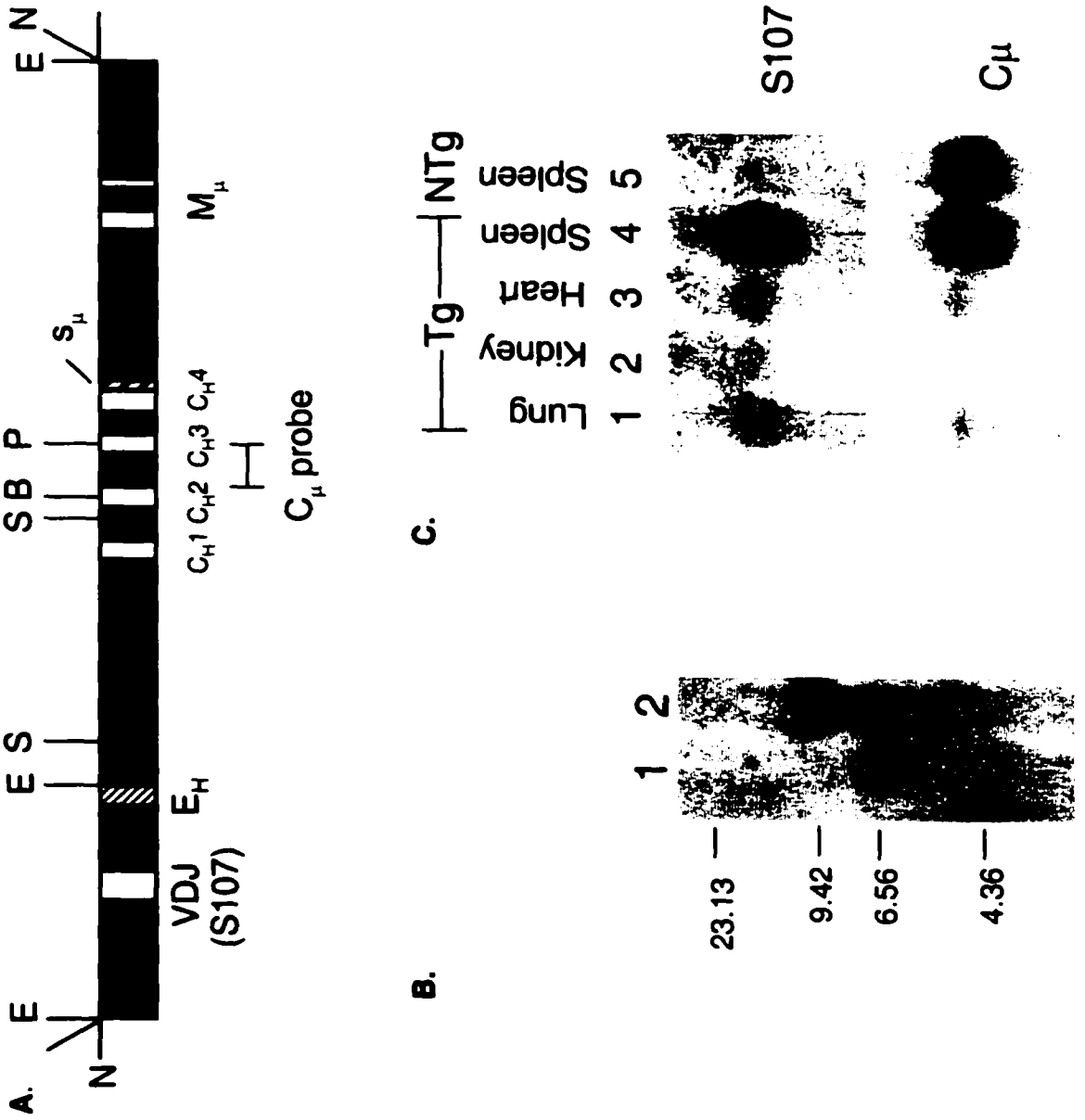

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Figure 9. Measurement of R4A-Cμ anti-dsDNA antibody in the sera of transgenic mice and their nontransgenic littermates. (A)Sera from 10 transgenic mice (Tg) and 10 nontransgenic littermates (NTg) were diluted 1/100 and then screened by ELISA for binding of IgM^a antibody to salmon sperm dsDNA coated plates. (B) The concentration of total IgM^a antibody in the sera of Tg and NTg mice was quantitated by ELISA. Mice ranged in age from 7-12 weeks. Mean values are indicated by a bar. Purified IgM^a anti-dsDNA antibody designated 4D4, obtained from a hybridoma line generated from R4A-Cμ mice, was diluted to 1μg/ml and used as a positive control.

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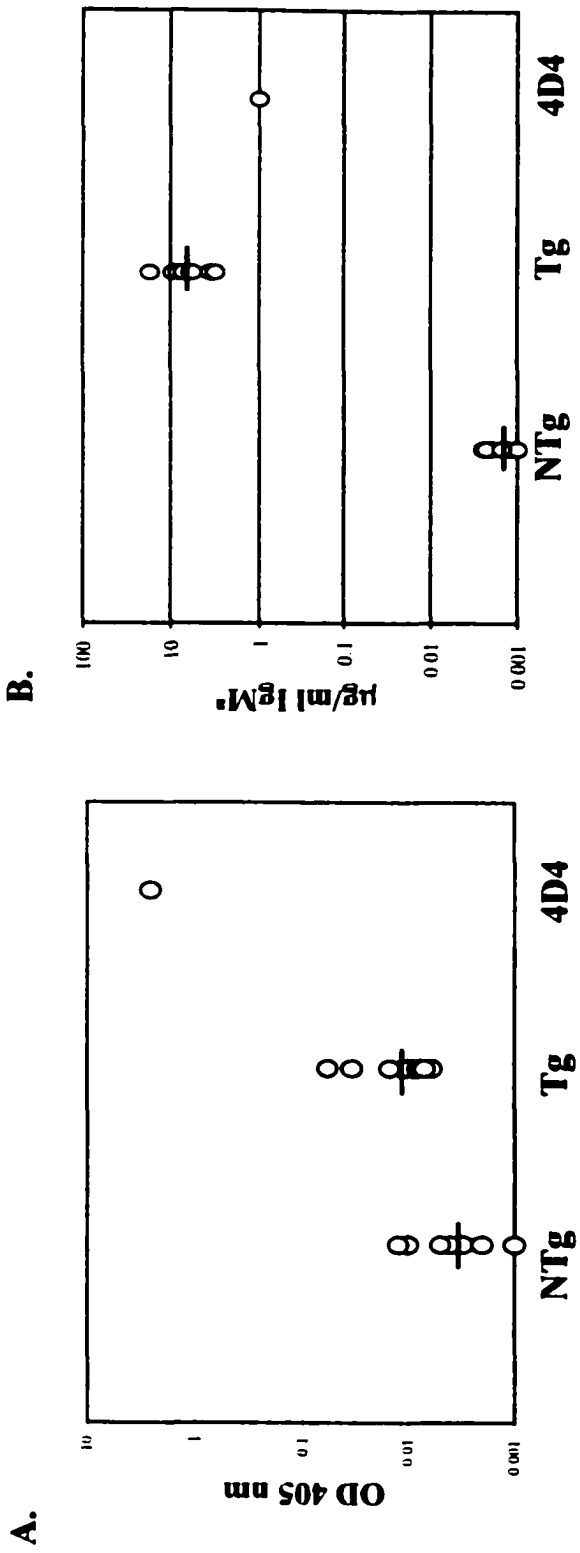

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Figure 10. FACS analysis of transgenic and nontransgenic mice. Heavy chain allelic exclusion; bone marrow and splenic B cells from Tg and NTg mice were tripled stained with CYC-anti-B220, PE-anti-IgM^a, and FITC-anti-IgM^b. Gates were set on $B220^+$ cells and percent IgM^a, IgM^b and IgM^{ab} B cells are displayed by dot plot.

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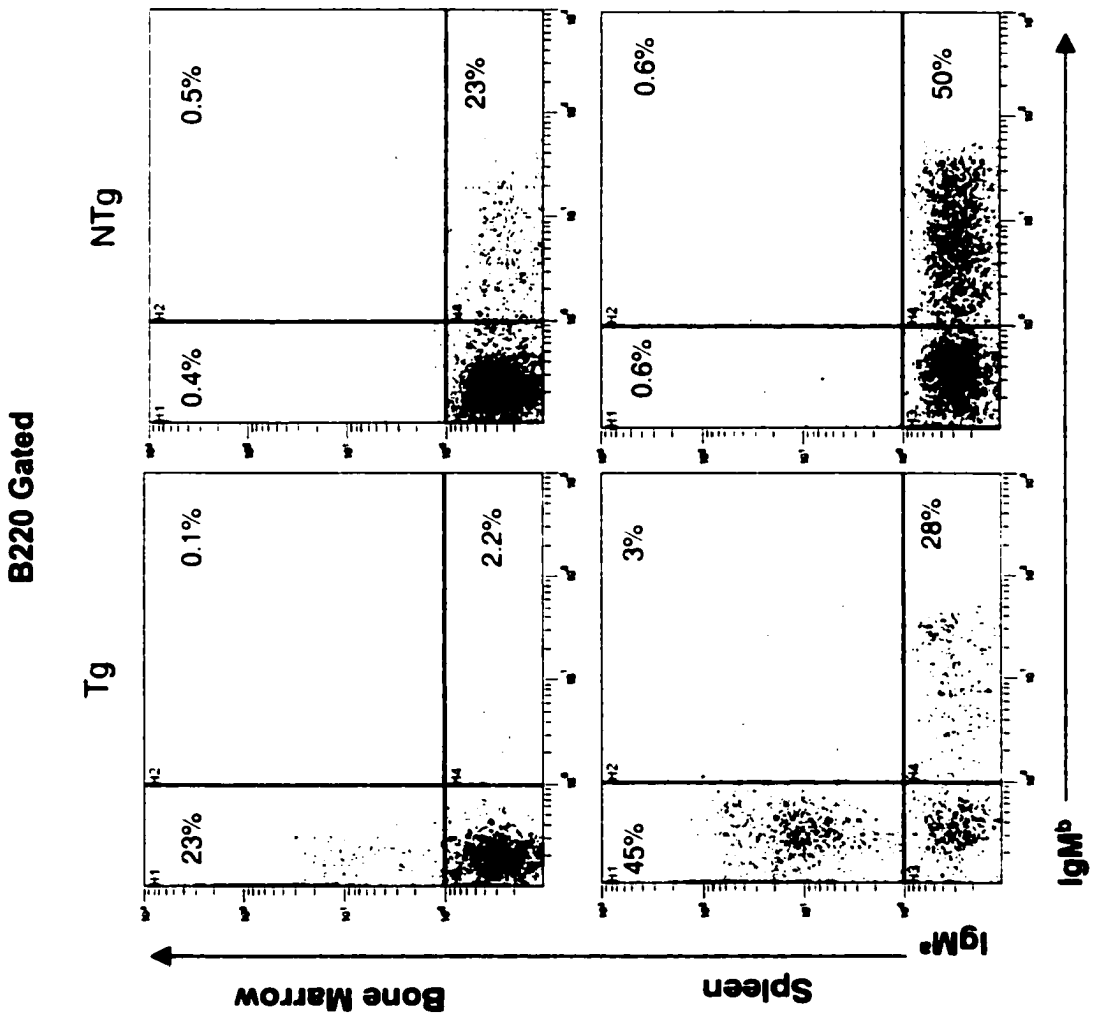

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TABLE 6: Frequency of bone marrow and splenic B cells in transgenic mice and nontransgenic littermates

	B220 *Number of cells x 10^6* (%)		*B220, IgM^a* Number of cells x 10^5 (%)*		*B220, IgM^b* Number of cells x 10^5 (%)†	
	Bone Marrow	**Spleen**	**Bone Marrow**	**Spleen**	***Bone Marrow***	**Spleen**
Tg	3.4± 1.8	3.9 ± 1.3	4.4 ± 1.8	18.1 ± 10.8	0.3 ± 0.1	9.6 ± 5.0
	(71.2 ±10.2)	(18.1 ± 5.2)	(10 ± 2.4)	(7.0 ± 2.5)	(0.7 ± 0.3)	(4.7 ± 2.1)
NTg	5.0 ± 2.0	18.5 ± 5.7	NA	NA	13.1 ± 7.5	122.8 ± 36.6
	(70.3 ±12.0)	(39.5 ± 5.5)			(18.6 ± 4.5)	(25.4 ± 6.2)

Analysis was performed by flow cytometry. Mice were 2-3 months old. Results represent the mean ± SD from 6 transgenic (Tg) mice and 6 non transgenic (NTg) littermates. Splenocytes were labeled with antibody to B220 and either antibody to IgM^a to detect B cells expressing the transgene or antibody to IgM^b to detect B cells expressing endogenous IgM.

*Percent of lymphocytes that are $B220^+$, IgM^a

†Percent of lymphocytes that are $B220^+$, IgM^b

NA- not applicable

Figure 11. Maturational stage of development of transgenic B cells. Bone marrow cells from Tg and NTg mice were triple stained with CYC-anti-B220, FITC-anti-CD43 and B-anti-IgM followed by streptavidin PE. Gates were set on $B220^+$ B cells. Percentage of pro B-cells ($B220^+$, $CD43^+$, IgM^-) and pre B-cells ($B220^+$, $CD43^-$, IgM^-) are indicated.

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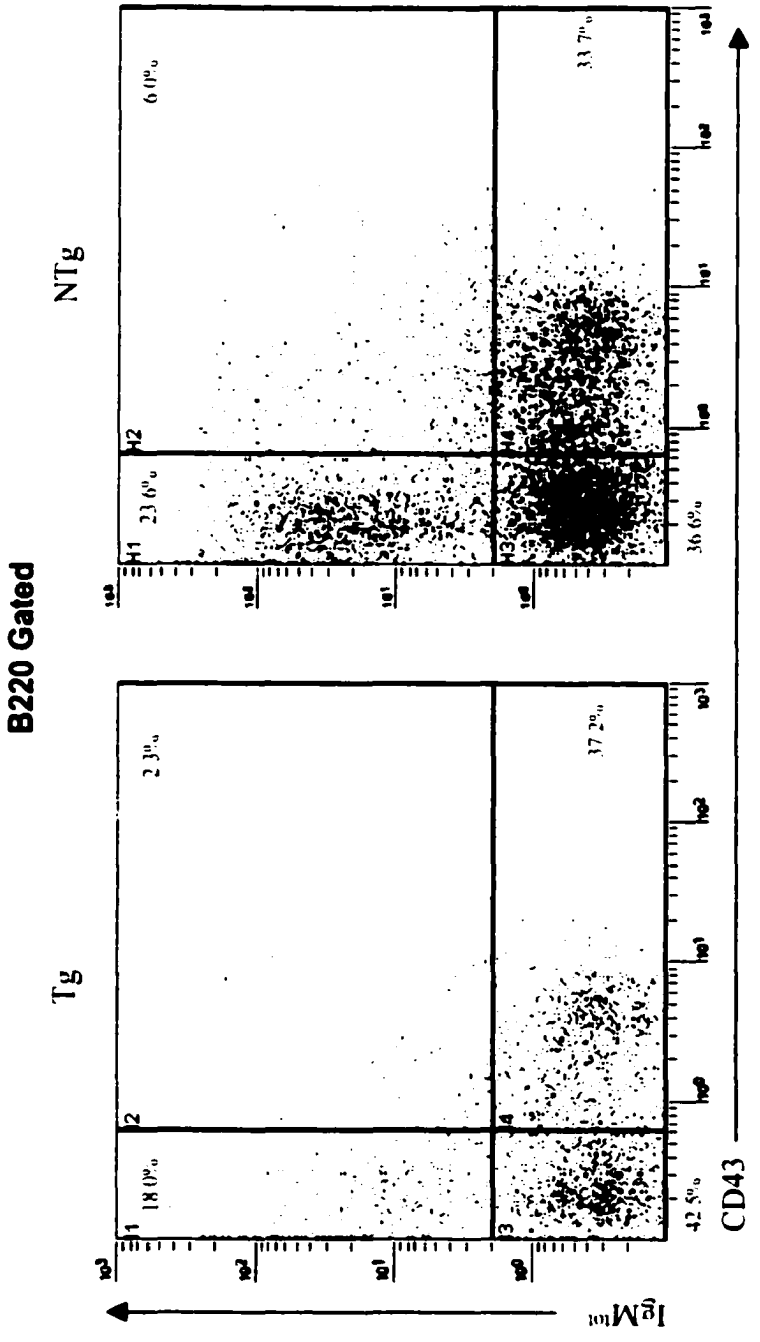

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Figure 12. Developmental status of transgenic B cells in Bone Marrow and Spleen. **(A)** Bone marrow cells from Tg and NTg mice were triple stained with CYC-anti-B220, FITC-anti-CD24 and either B-anti-IgM^a or B-anti-IgM^b followed by streptavidin PE. Gates were set on $B220^+$ B cells. Percentages of $CD24^{hi}$ and $CD24^{lo}$ B cells are indicated. This experiment is representative of 4 others. The ratio of $CD24^{hi}/CD24^{lo}$ (immature B-cells/mature B-cells) is 4.0 and 0.9 for R4A-Cμ transgenic and nontransgenic littermate respectively. This experiment is representative of 4 others. (B) Maturational analysis of R4A-Cμ transgenic B-cells in the spleen. Splenic B cells from Tg and NTg mice were triple stained and analyzed as described in A.

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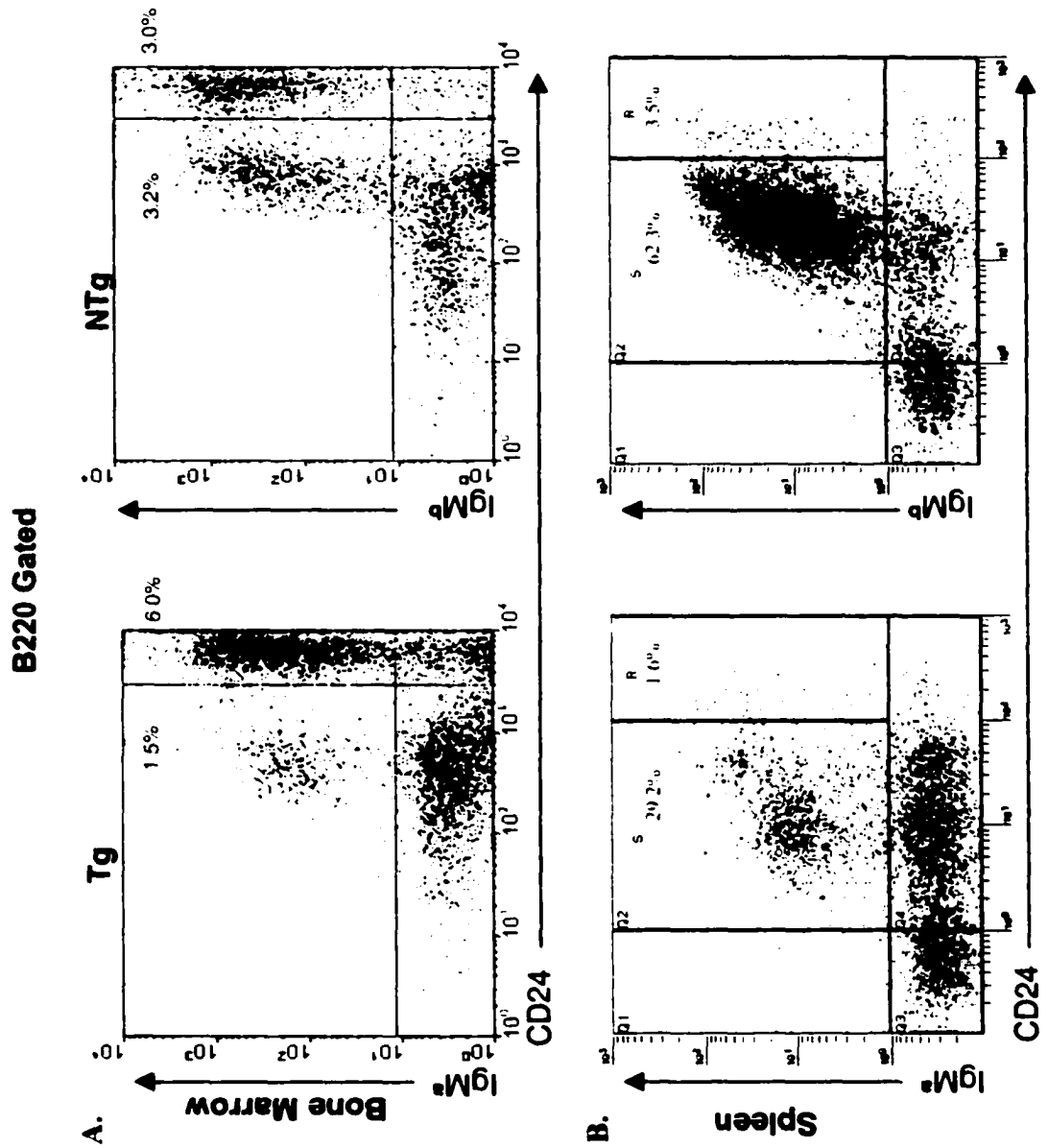

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Figure 13. Reduced expression of surface IgM^a. Splenocytes from R4A-Cμ transgenic mice and non transgenic littermates were triple stained with PE-anti-IgM^a or PE-anti-IgM^b respectively and CYC-anti-B220 and FITC-anti-kappa. A gate was set on either B220$^+$IgM^a or B220$^+$IgM^b cells for splenocytes from R4A-Cμ or non transgenic mice respectively, and kappa staining was analyzed by histograms. Results are representative of 5 experiments.

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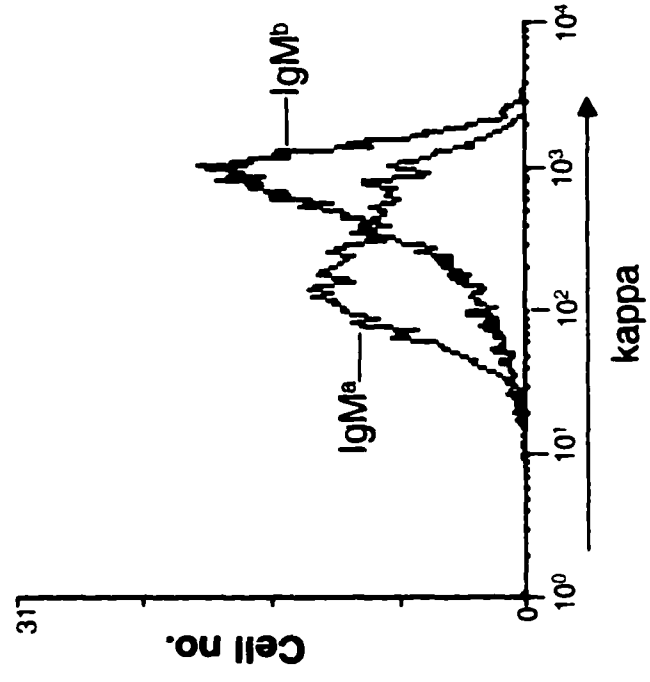

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Figure 14. *In vitro* LPS stimulation of IgM^a dsDNA binding B cells. Splenocytes from five (#1-#5) 8-10 week old R4A-Cμ transgenic mice were cultured for 3 days with (open bars) and without (cross-hatched bars) 10 μg/ml LPS. Supernatants were tested by ELISA for (A) IgM^a anti-dsDNA antibody secretion and (B) total IgM^a antibody secretion. Triplicate samples from each mouse were analyzed.

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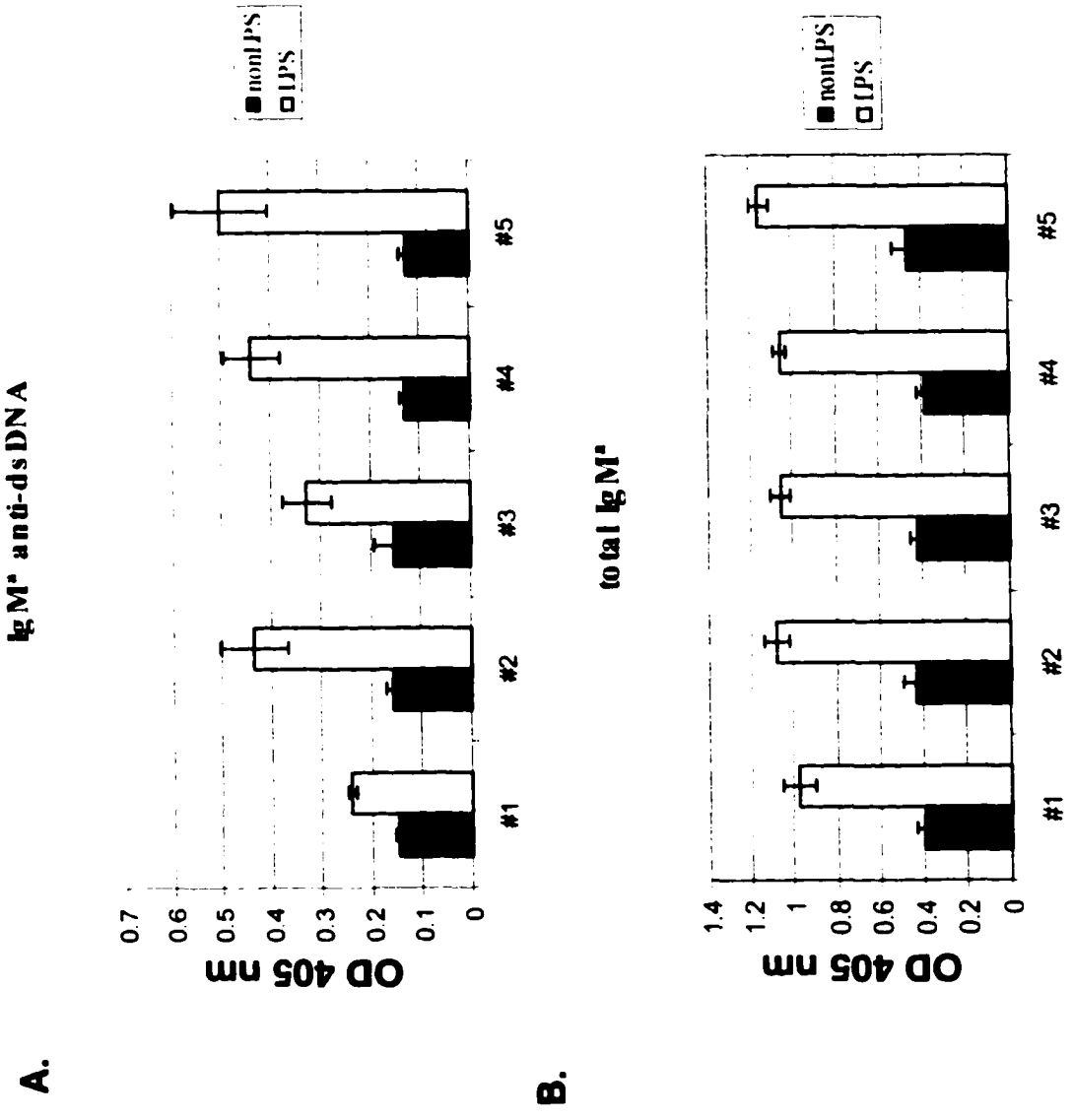

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Figure 15. ***In vitro*** **activation of IgM^a dsDNA binding B cells.** Splenocytes from R4A-C_μ transgenic mice that were enriched for B cells by negative depletion of T cells were incubated *in vitro* at a concentration of 2.0 x 10^6 cells/ml for 48 hours at 37°C in medium alone or with either 20 μg/ml of LPS, 10 μg/ml of antibody to CD40 plus 10 μg/ml of F(ab')$_2$ anti-IgM, or 10 μg/ml of anti-CD40 plus 300 units of rIL-4. DNA binding B cells and total IgM^a B cells were detected by ELISPOT on microtiter plates coated with dsDNA **(A)** or antibody to IgM **(B)** respectively followed by incubation with B-anti-IgM^a and streptavidin-AP. A hybridoma cell line, 4D4 producing IgM^a anti-dsDNA antibody was used as a positive control. Results are representative of 4 experiments. **(C)** B cell enriched splenocytes were cultured with rIL-4, rIL-5, rIL-6 or a cocktail of all three cytokines and dsDNA binding B cells were detected by ELIspot. Results are representative of 3 experiments.

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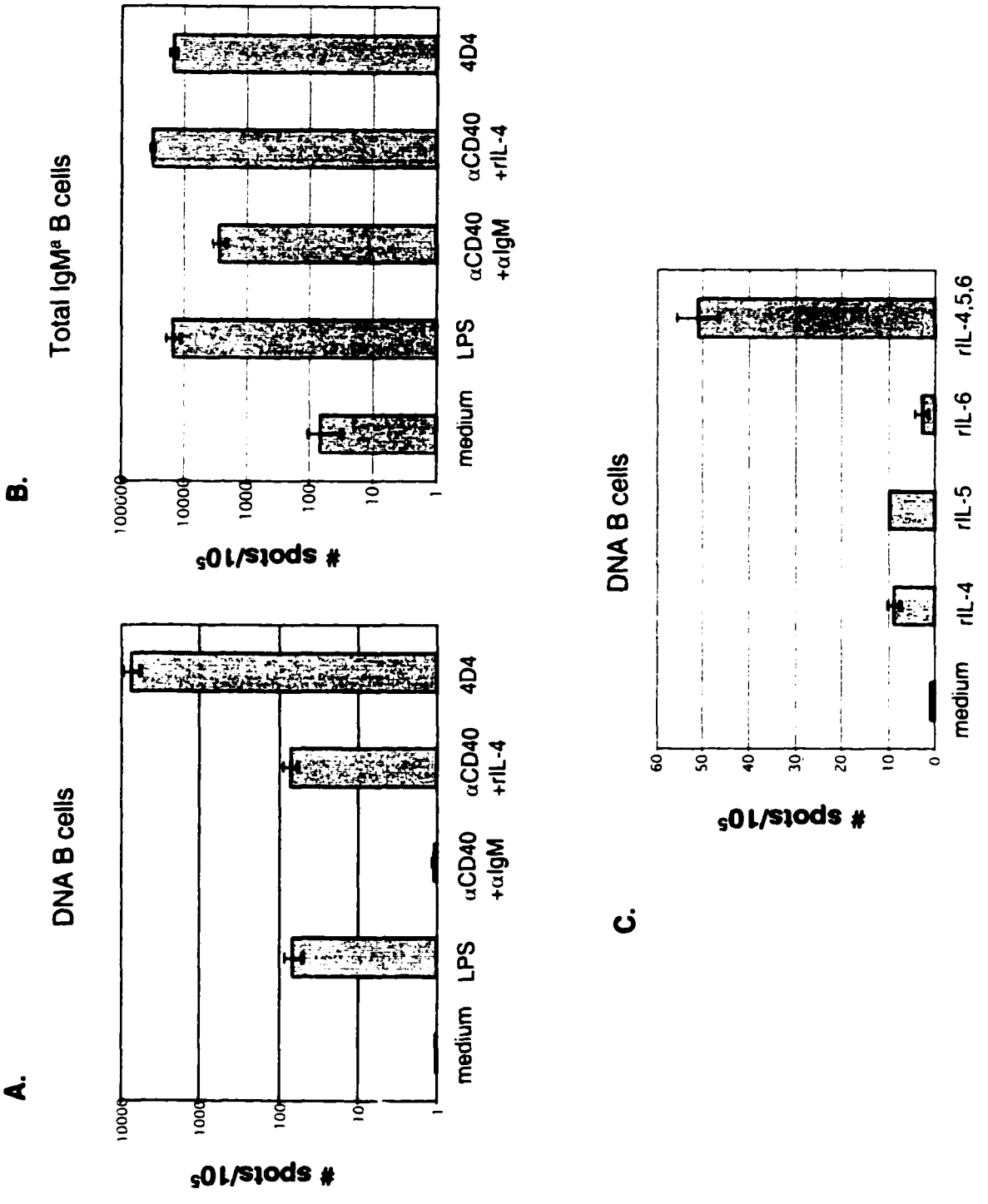

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TABLE 7: BrdU incorporation in splenic B cells

	BrdU splenic IgM^a B cells in R4A-Cμ mice (%)	BrdU splenic IgM^b B cells in non-transgenic mice (%)
# 1	24	14
# 2	14	9
# 3	13	7

BrdU incorporation is measured by percent FITC anti-BrdU staining in $B220^+IgM^a$, and $B220^+IgM^b$ gated B cell populations from R4A-Cμ and non-transgenic mice respectively following 8 days of injection with BrdU. Results from 3 separate experiments are presented. Analysis was by flow cytometry.

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Figure 16. Transgenic B-cells do not bear an activated phenotype. B-cells were stained with anti-B220-CYC, anti-IgM^a-PE or anti IgM^b-PE, and either anti-CD86, CD69 or CD62L-FITC. Activation markers were analyzed on $B220^+$ gated IgM^a or IgM^b B-cells and histograms were established for IgM^a transgenic B-cells (red line), IgM^b B-cells in transgenic mice, (green line) and IgM^b non-transgenic B-cells in a non-transgenic littermate, (blue line).

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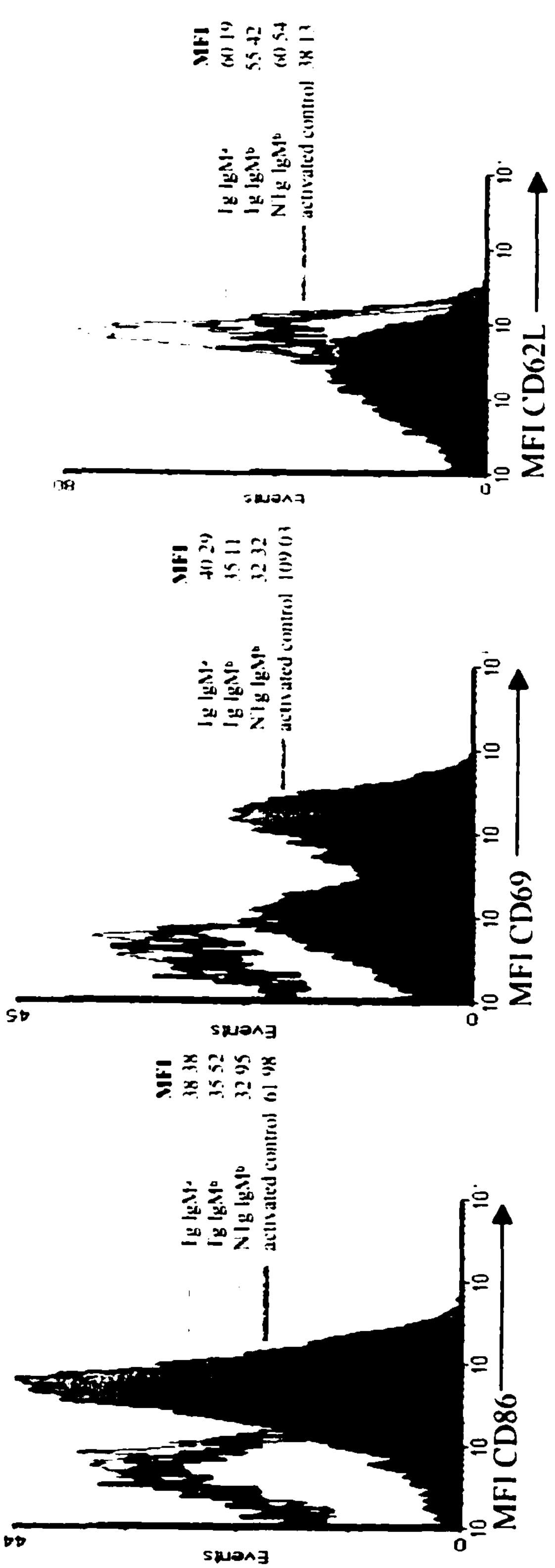

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Figure 17. DNA binding activity in hybridoma supernatants Normalized supernatants (5μg/ml) from hybridomas from LPS stimulated spleen cells (dotted bars) and naïve spleen cells (open bars), were measured for dsDNA binding activity by ELISA. μk is an IgM antibody without specificity for dsDNA. 2D2 is an IgM anti-DNA antibody of known pathogenic potential.

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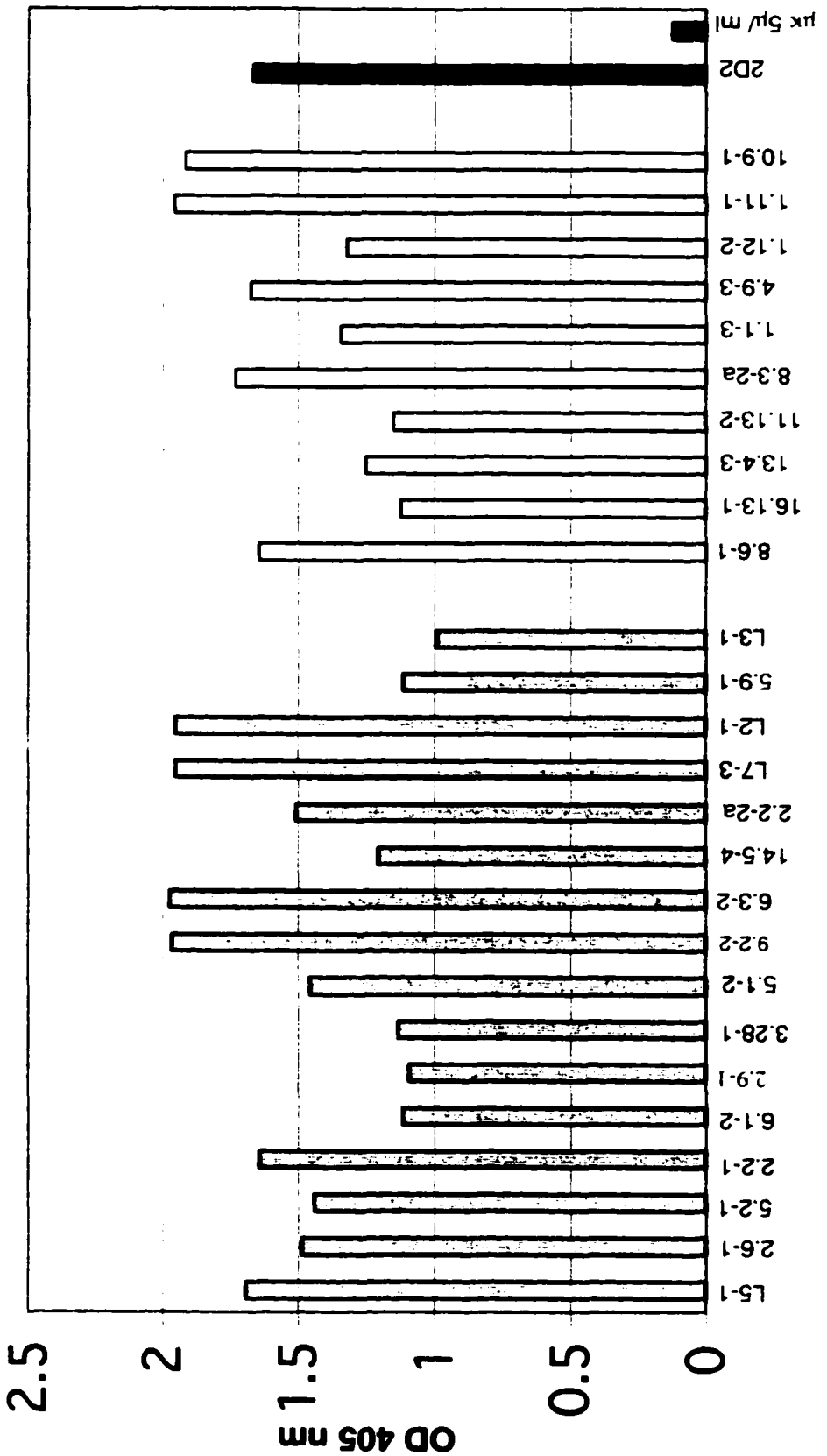

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Table 8. Light Chain analysis of R4A transgenic dsDNA binding B-cells obtained from LPS stimulated and naïve hybridoma.

Hybridomas	Vκ1	Non Vκ1	Jκ1	Jκ2	Jκ4	Jκ5	Mutated
[Ψ]LPS stimulated	11/11	0	---	7	2	2	2
[Ω]Naïve	7/9	2	2	6	---	1	1

[Ψ]Hybridomas were generated from splenocytes obtained from R4A-Cμ transgenic mice and stimulated *in vitro* for 48 hours with 10μg/ml of LPS prior to fusion.

[Ω]Hybridomas were generated from unstimulated (naïve) splenocytes from R4A-Cμ transgenic mice.

Figure 18. *In vitro* activation of dsDNA binding IgM^a and IgG2b B cells as detected by ELISPOT. Purified B cells from R4A-Cμ or R4A-Cγ2b transgenic mice were incubated for 48 hours at 37°C at a concentration of 2.0 x 10^6 cells/ml, in medium alone or with 10 μg/ml of LPS, or 10 μg/ml of anti-CD40 plus 300 units of rIL-4. IgM^a or IgG2b dsDNA binding B cells **(A)** and total IgM^a or IgG2b B cells **(B)** were detected by ELISPOT on microtiter plates coated with salmon sperm dsDNA, or anti-IgM or anti-IgG2b respectively followed by incubation with B-anti-IgM^a or B-anti-IgG2b plus streptavidin-AP. Crosshatched bars designate IgM^a B cells from R4A-Cμ mice and open bars designate IgG2b B cells from R4A-Cγ2b mice. Results are representative of three experiments.

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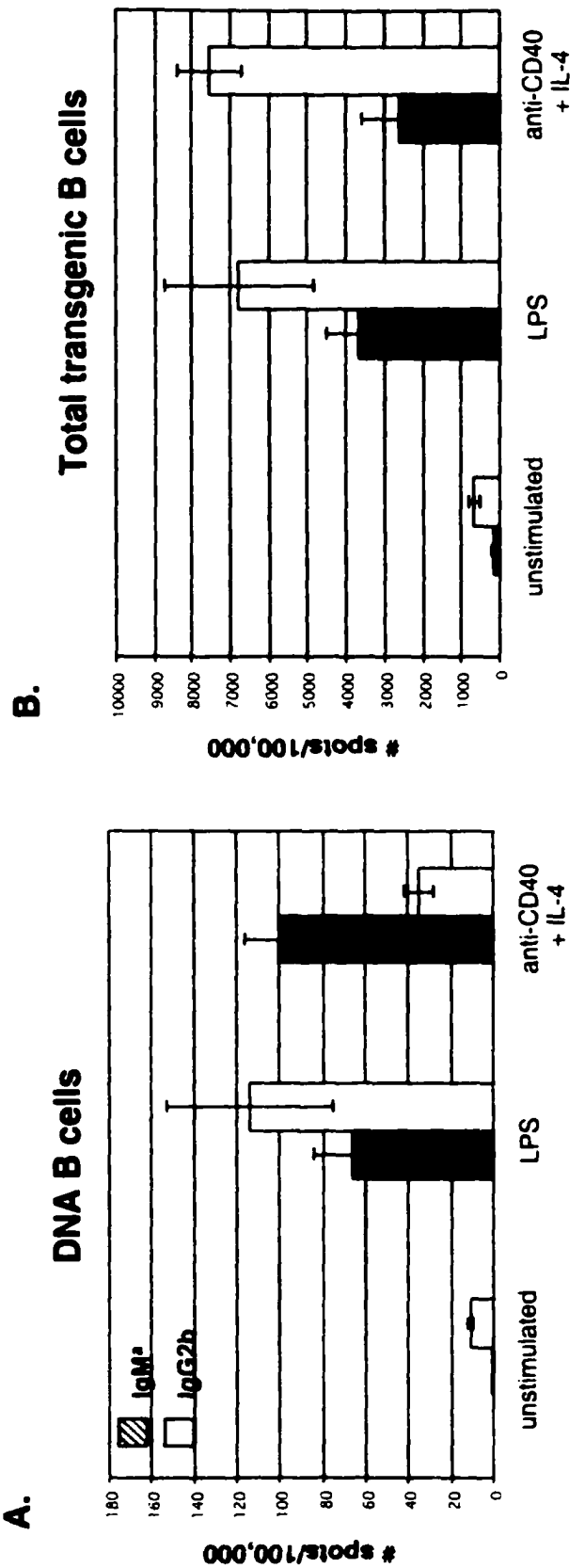

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Figure 19. Density of surface immunoglobulin receptors. Splenocytes from R4A-Cμ and R4A-Cγ2b transgenic mice were triple stained with PE-anti-IgM^a or PE-anti-IgG2b respectively and CYC-anti-B220 and FITC-anti-kappa. A gate was set on either $B220^+IgM^a$ or $B220^+$IgG2b cells for splenocytes from R4A-Cμ or R4A-Cγ2b mice respectively, and kappa staining was analyzed by histograms. Shaded histogram shows kappa staining of IgM^a B cells from R4A-Cμ mouse. Bold histogram shows kappa staining of IgG2b B cells from R4A-Cγ2b mouse. Dotted histogram represents negative control. MFI = mean fluorescent intensity. Results are representative of 3 experiments.

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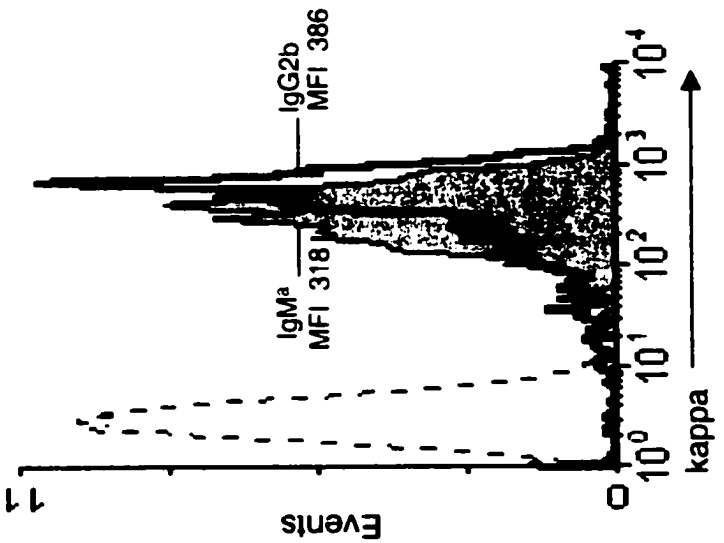

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Figure 20. Reduced lifespan of R4A-Cγ2b B-cells compared to R4A-Cμ B-cells. Following 8 days of BrdU incorporation, spleen cells from R4A-Cμ, R4A-Cγ2b and NTg mice were immunostained with anti-B220-CYC and biotin conjugated anti-IgM^a, IgG2b or IgM^b followed by streptavidin PE. Cells were gated for either $B220IgM^a$ for B-cells from R4A-Cμ mice, $B220IgM^b$ for B-cells from NTg mice, or B220IgG2b for B-cells from R4A-Cγ2b mice. Percentage BrdU incorporation in IgM^a (red), IgM^b (blue) or IgG2b (green) B-cells is indicated on histograms.

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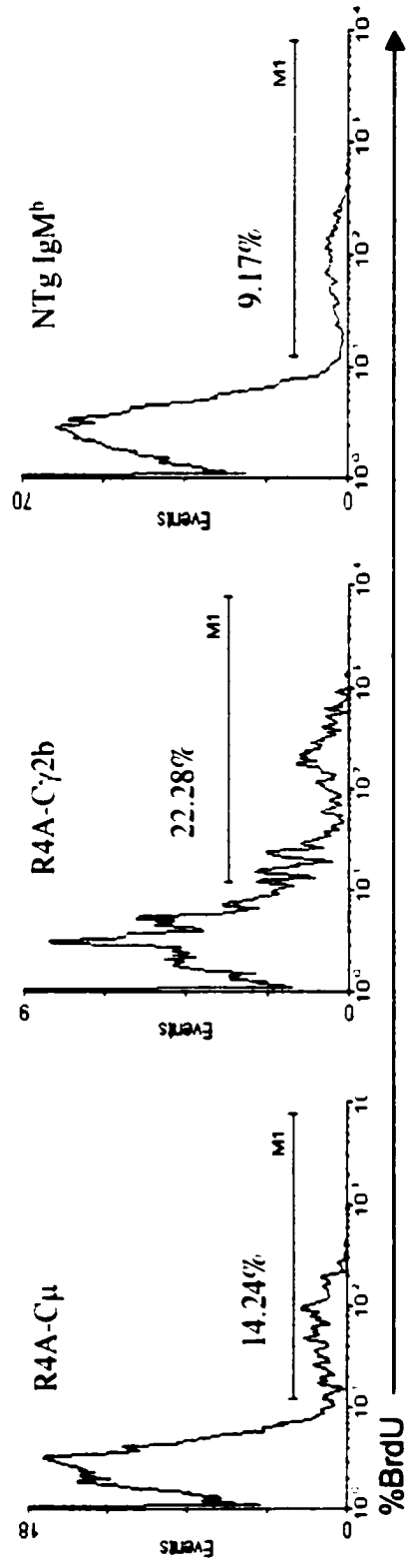

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CHAPTER IV

ABSTRACT

The B-cell receptor complex (BCR) is important for the signaling involved in B-cell activation and the induction of self-tolerance. Thresholds for B-cell activation and tolerance are mediated by B-cell specific molecules, such as CD19. B-cells from mice overexpressing CD19 have a lowered threshold of activation and spontaneously secrete autoantibodies. B-cells overexpressing CD19 also display heightened negative selection in the bone marrow. CD19 overexpressing mice have an increased population of B1 B-cells which are thought to be the source of autoantibody production in these mice. We recently demonstrated that mice transgenic for the R4A-Cμ heavy chain of an anti-dsDNA antibody, maintain tolerance by anergy and deletion. To determine whether CD19 overexpression affects the induction of tolerance in these mice, we bred R4A-Cμ transgenic mice with mice transgenic for human CD19 (hCD19). We generated R4A-Cμ mice heterozygous and homozygous for hCD19 and observed that B-cells from these mice seem to spontaneously secrete high titers of transgenic anti-dsDNA antibody indicative of a breakdown in tolerance. Despite this, these B-cells in these mice still maintain phenotypic characteristics of anergic B-cells which include reduced surface immunoglobulin density, shortened lifespan and arrest in an immature stage of development. Futhermore, these transgenic dsDNA binding B-cells are unresponsive to BCR crosslinking. However, they are hyperresponsive to LPS activation *in vitro* compared to B cells from R4A-Cμ nonautoimmune mice. We observed that these dsDNA binding B-cells are derived from the B-2 and not the B-1 subset in these mice. We also observed that $CD4^+$ T-cells are not required for the spontaneous anti-dsDNA

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secretion by these B-cells suggesting that the breakdown in tolerance is T-cell independent. The marginal zone (MZ) is expanded in mice overexpressing CD19 and we have demonstrated that R4A-dsDNA binding B-cells reside in the MZ of these mice. Finally, we have observed that CpG sequences can activate dsDNA binding B-cells in R4A-Cμ mice transgenic for CD19 but not in R4A-Cμ mice. This indicates that B-cells overexpressing CD19 are hyperresponsive to signaling through CpG and suggests that innate immune mechanisms may play a role in triggering B-cells with lowered signaling thresholds.

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INTRODUCTION

The autoimmune disease Systemic Lupus Erythematosus (SLE) is characterized by the production of IgG antibodies against self-antigens, including dsDNA, histones and ribonucleoproteins (RNP) (1). Although the etiology of SLE remains unknown there is evidence to support a defect in B-cell tolerance as a possible cause. In addition to B-cell dysregulation, genetic factors, sex hormones, and environmental factors may also predispose to SLE. Anergy, deletion, receptor editing, clonal ignorance and T-cell suppression are mechanisms which regulate autoreactive B-cells (37, 39). Factors which influence these mechanisms of regulation are the affinity of the BCR for self-antigen, the nature and the concentration of the self-antigen and the density of the Ig receptor (27, 28, 44, 46, 143, 203, 272). Thresholds of B-cell signaling that lead to B-cell activation or tolerance are mediated by the B-cell coreceptors and intracellular molecules that are important in B-cell signal transduction. Together these molecules are known as the signalosome (24, 57-59, 141, 205, 273-275).

Alterations in the expression level of some molecules involved in the signalosome have been shown to disrupt the regulation of autoreactive B-cells and cause autoimmunity. For example, mice deficient in CD22, SHP-1 or Lyn are hyperresponsive to BCR signaling and develop autoantibodies including anti-dsDNA antibodies (112, 114, 128, 276-279). The increase in autoantibody production in CD22 and SHP-1 deficient mice also corresponds to an increase in B-1 B-cells. Mice overexpressing human CD19 (hCD19), a member of the B-cell co-receptor complex, expressed on all B-cells fail to display tolerance and produce high titers of IgG anti-DNA autoantibodies and rheumatoid factor. B -cells in these mice are hyperresponsiveness to BCR induced transmembrane

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signaling (87, 88, 92). These mice also have an increased frequency of $CD5^+$ B1a B-cells which seems to correlate with autoantibody levels. CD19 functions as a specialized adapter protein which lowers the threshold for BCR induced activation and functions by amplifying the activity of Src family protein tyrosine kinases (228). It also facilitates the interaction of multiple signaling pathways (280, 281). CD19 has also been shown to be important in the formation of the splenic marginal zone, the generation of B-1 B-cells, and in the induction of allelic exclusion (86-88, 147, 237, 282). It has been proposed that CD19 ligation can provide a proliferative signal in the absence of surface immunoglobulin ligation or CD21 engagement and may be able to signal in an antigen independent manner (231, 232, 236).

Patients with systemic sclerosis (SSc) an autoimmune connective tissue disorder characterized by increased levels of autoantibodies, have been observed to have an increased level of CD19 expressed on their B-cells (242, 243, 283). A mouse model for human SSc known as the tight skin mouse (TSK/+) exists. B-cells from TSK mice have an increased level of CD19 phosphorylation and exhibit augmented CD19 signaling (243).

Mice transgenic for hCD19 have B cells that mature normally in the bone marrow with normal numbers of pro and pre B cells but a significant reduction in the number of immature B cells. This is thought to be due to heightened negative selection at the immature stage of development (87, 205). Peripheral development has been shown to be normal in these mice despite a significant reduction in the number of mature splenic and peritoneal B-cells compared to wild type mice. Splenic B-cells in mice overexpressing

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CD19 display a chronically activated phenotype consistent with anergic cells but are not anergic as they can mobilize intracellular Ca^{2+} upon BCR crosslinking (88, 92, 223).

Inaoki et. al. observed that overexpression of CD19 in mice transgenic for soluble hen egg lysozyme (sHEL) and antibody to HEL led to a breakdown of tolerance and the spontaneous secretion of anti-HEL antibody by conventional B-2 cells (244). Qian et. al. also demonstrated a loss of tolerance in CD19 overexpressing mice transgenic for the heavy chain of an antibody to Sm. However, they observed that spontaneous secretion of autoantibody was by the B-1 subset of B-cells (193).

We previously generated mice transgenic for the IgM heavy chain of an anti-dsDNA antibody designated R4A and showed that these mice fail to spontaneously secrete anti-dsDNA antibodies and maintain tolerance in part by anergy (284). Transgenic B-cells in R4A-Cμ mice fail to be activated to secrete antibody following BCR crosslinking with anti-IgM and display an anergic phenotype characterized by immunoglobulin receptor downmodulation, an immature phenotype and reduced lifespan (284).

To study how overexpression of CD19, affects the induction of tolerance in DNA binding B-cells we mated C57BL/6 mice transgenic for the R4A-IgM heavy chain (R4A-Cμ) with mice transgenic for hCD19. In this study, we observed that tolerance is broken in R4A-Cμ mice overexpressing hCD19. We have characterized the dsDNA binding B-cells in these mice, both phenotypically and functionally. We demonstrated that the breakdown of tolerance in these mice is T-cell independent and that B-cells secreting transgenic anti-dsDNA antibody belong to the B-2 subset and are localized in the splenic marginal zone. We also demonstrated that these B-cells still maintain an anergic

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phenotype and are unable to be activated by BCR signaling. However due to lowered signaling thresholds mediated by CD19, they are hyperresponsive to innate immune mechanisms of signaling.

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RESULTS

Spontaneous Secretion of anti-dsDNA in R4A-Cμ/hCD19 Transgenic Mice

To determine if peripheral tolerance of transgenic IgM anti-dsDNA B-cells is lost as a result of hCD19 overexpression we generated R4A-Cμ mice heterozygous (R4AhCD19$^{+/-}$) or homozygous (R4AhCD19$^{+/+}$) for hCD19 overexpression. These mice were generated by breeding C57BL/6 R4A-Cμ transgenic mice with C57BL/6 mice homozygous for the human CD19 transgene (hCD19) kindly provided by Thomas Tedder at Duke University (238). hCD19 transgene expression was shown to be B-cell specific and was able to restore normal function in mice lacking endogenous CD19 (223, 238). ELISAs were performed to detect the presence of transgenic anti-dsDNA antibody in the sera of transgenic mice using an anti-IgM^a antibody specific for the allotype of the transgene. The R4A-IgM^a transgene was initially isolated from BALB/c mice which bears the a allotype. This can be distinguished from endogenous IgM in the C57BL/6 mice which bear the b allotype. By assaying R4A-Cμ/hCD19 mice at one month intervals over a period of four months for IgM^a anti-dsDNA antibody, we observed that transgenic antibody began to be spontaneously secreted at between two and three months of age (Figure 21A). We also observed that in some but not all mice the titer of IgM^a anti-dsDNA antibody increased with age. Approximately 50-70% of all mice overexpressing CD19 developed a significant titer of transgenic anti-dsDNA antibody compared to R4A-Cμ mice deficient in hCD19 (Figure 21B). Quantitation of total serum anti-IgM^a transgenic antibodies revealed a 4 fold increase in antibody concentration in R4A mice homozygous and heterozygous for hCD19 (R4A-Cμ/hCD19$^{+/+}$ and R4A-Cμ/hCD19$^{+/-}$) compared to R4A-Cμ mice (Figure 21C).

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To confirm the specificity of transgenic anti-dsDNA antibody in the sera of R4A-Cμ/hCD19$^{+/+}$ mice, we performed an Anti-Nuclear Antibody assay (ANA) by immunostaining slides containing commercially prepared human epithelial cells (Hep-2) with mouse sera. We observed a homogenous pattern of staining of the nuclei of Hep-2 cells with R4A-Cμ/hCD19$^{+/+}$ sera, which is usually indicative of anti-dsDNA antibodies (Figure 22). No staining was observed with control sera from wild type mice. Sera from autoimmune NZB/W F_1 was used as a positive control.

These observations demonstrated that CD19 overexpression can lead to a breakdown of tolerance of transgenic anti-dsDNA B-cells resulting in the spontaneous secretion of anti-dsDNA antibodies.

Frequency of Transgenic B-cells in Mice Overexpressing hCD19

We examined the frequency of B220$^+$ B-cells in the bone marrow, spleen and peritoneum of R4A-Cμ/hCD19 mice. By staining cells with antibody to B220, a B-cell specific marker and performing Facs analysis we determined the percentage and absolute number of B220$^+$ B-cells in nontransgenic (NTg) control, R4A-Cμ and R4A-Cμ mice overexpressing hCD19 (Table 9). We observed that there was a dramatic overall reduction in the total number of B-cells in the bone marrow, spleen and peritoneum of hCD19 mice compared to NTg mice. We previously observed that due to deletion of autoreactive B-cells there was a reduction in B-cell numbers in R4A-Cμ mice. A further reduction in B-cells was observed in R4A-Cμ mice overexpressing hCD19. Therefore, negative selection was heightened by the combination of the CD19 transgene and the presence of the R4A-Cμ transgene. The two together had a synergistic effect.

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In order to determine which subset or subsets of B-cells (proB-cells, preB-cells or immature/mature B-cells) was reduced in these mice and to investigate if normal B-cell development was intact, we stained bone marrow cells with antibodies to B220, CD43 and IgM. By flow cytometry we observed that the percentage of proB-cells ($B220^+IgM^-CD43^+$) in R4A-Cμ/$hCD19^{+/+}$ mice (2.3%) was similar to that in NTg (1.1%), R4A-Cμ (2.1%), and $hCD19^{+/+}$ mice (1.0%) (Figure 23). As expected, the percentage of preB-cells ($B220^+IgM^-CD43^-$) was elevated in R4A-Cμ mice (64.4%) compared to NTg mice (35.4%) because of the presence of the rearranged transgene. Interestingly, however, the percentage of preB-cells was also elevated in $hCD19^{+/+}$ mice (54.0%) relative to NTg mice (35.4%) and further elevated in R4A-Cμ/$hCD19^{+/+}$ mice (74.3%). A dramatic reduction in the percentage of immature/mature B-cells ($B220^+IgM^+CD43^-$) was observed in the bone marrow (Figure 23) of hCD19 mice(44.0%) compared to that in NTg mice (63.0%) consistent with earlier reports (87, 238). We observed that the percentage of immature/mature B-cells was further reduced in R4A-Cμ (33.3%) and R4A-Cμ/$hCD19^{+/+}$ mice (23.2%) (Figure 23). These results indicate that the reduction in B-cells observed in R4A-Cμ mice, hCD19 mice and R4A-Cμ/$hCD19^{+/+}$ mice occurs at the immature/mature B-cell stage of development.

Since R4A-Cμ transgenic mice overexpressing hCD19 spontaneously secrete transgenic IgM^a anti-dsDNA antibody, we wanted to assess the frequency of IgM^a B-cells by flow cytometry in order to determine if there was a correlation between elevated titers of IgM^a anti-dsDNA secretion and the number of transgenic B-cells. Cells were stained with antibodies to B220 and IgM^a to detect transgenic B-cells. We observed that there was no increase in the absolute number and percentage of $B220^+IgM^{a+}$ B-cells in the

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bone marrow, spleen or peritoneum of R4A-Cμ/hCD19$^{+/+}$ mice compared to R4A-Cμ mice (Table 9). In fact, despite the increased level of IgM^a anti-dsDNA antibody in the serum of R4A-Cμ/hCD19$^{+/+}$ mice there was a significant reduction in the number and percentage of transgenic B-cells in the bone marrow (9.2%) and spleen (2.9%) of these mice compared to the bone marrow and spleen of R4A-Cμ mice (14.0% and 11.3% respectively) (Figure 24).

Evidence for Allelic Exclusion

Allelic exclusion is defined as the expression of a single BCR with a single antigen specificity on any one B-cell. It occurs at both the HC and LC loci and arises following a successful rearrangement of one allele of the antigen receptor gene which in turn suppresses the other parental allele (285). It has been observed in several transgenic mouse models that some autoreactive transgenic B-cells lack allelic exclusion (159, 178, 250). Both Iliev et. al. and Li et. al. observed that dsDNA binding B-cells lacking heavy chain allelic exclusion are present in the spleen of mice transgenic for an anti-dsDNA antibody (178, 250). Expression of dual receptors may be a mechanism by which autoreactive B-cells escape clonal deletion. A study by Shivtiel et.al. suggested that CD19 is necessary to maintain allelic exclusion in B-cells (282). We were curious to see whether CD19 overexpression could promote better heavy chain allelic exclusion in R4A-Cμ mice. We therefore triple stained bone marrow and splenic B-cells with antibodies to B220, the IgM^a transgene and endogenous IgM^b and analyzed B220$^+$ gated B-cells by flow cytometry. The majority of bone marrow B-cells from R4A-Cμ, R4A-Cμ/hCD19$^{+/-}$ and R4A-Cμ/hCD19$^{+/+}$ transgenic mice displayed allelic exclusion and expressed either the transgene (IgM^a) or endogenous IgM (IgM^b) (Figure 25). Less than

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0.5% of transgenic B-cells failed to display allelic inclusion and expressed both IgM^a and IgM^b. Previous studies suggested that the only transgenic B-cells that failed to display heavy chain allelic exclusion in R4A mice were dsDNA binders while all non dsDNA binding B-cells maintained intact allelic exclusion.

In the spleen we observed that 6% of transgenic B-cells in R4A-Cμ mice failed to display allelic exclusion and expressed both transgenic IgM^a and nontransgenic IgM^{b+} Ig receptors (Figure 25). We also observed that in mice overexpressing CD19, a smaller percent of splenic B-cells failed to display allelic exclusion than in R4A-Cμ mice (Figure 25). In fact, the percent of B-cells lacking allelic exclusion and expressing both IgM^a and IgM^b decreased as CD19 levels increased. In R4A-Cμ/hCD19$^{+/-}$ mice, (1.1%) of splenic B-cells lacked allelic exclusion and in R4A-Cμ/hCD19$^{+/+}$mice, only (0.4%) of splenic B-cells lacked allelic exclusion (Figure 25). These results support the study that suggests that CD19 plays a role in maintaining allelic exclusion (282).

Frequency of B-1 B-cells in Spleen and Peritoneum of Mice Overexpressing hCD19

CD19 has been shown to be important in the generation and maintenance of B-1 B-cells which have been implicated in autoimmunity (88, 91, 112). There are two subsets of B1 cells; B1a and B1b cells. B1a cells can be identified by expression of the cell surface molecule CD5. It was previously demonstrated that CD19 overexpression specifically leads to an expansion of the $CD5^+$ B1a B-cell subset in the peritoneum and that expansion of this subset correlates with autoimmunity (87, 88, 242). By immunostaining peritoneal B-cells from hCD19 mice with anti-B220 and anti-CD5 and performing flow cytometry we observed that 9.2% of peritoneal B-cells in hCD19 mice were $CD5^+$ while 2.3% were $CD5^+$ in NTg mice (Figure 26). In order to determine if the

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B-1 compartment was expanded in R4A-Cμ mice overexpressing hCD19, we evaluated the frequency of $CD5^+$ expressing B-1 cells in these mice. We observed that the relative ratio of the percent of $B220^+CD5^+$ B-cells to $B220^+CD5^-$ B-cells was increased in the peritoneum of R4A-Cμ/$hCD19^{+/+}$ mice (6.8%/6.4%) compared to R4A-Cμ mice (7.0%/23.4%) and NTg mice (2.3%/41.3%). Similar results were observed in spleen as well. We also observed a slight increase in the relative ratio of $CD5^+$ transgenic B-cells (IgM^aCD5^+) in the peritoneum of R4A-Cμ/$hCD19^{+/+}$ mice ($CD5^+/CD5^-$=0.5/2.4=0.21) compared to that in R4A-Cμ mice (0.5/11.2=0.04). However, no increase in the relative ratio was observed in the spleen of CD19 overexpressing mice (Figure 27).

Maintenance of an Anergic Phenotype

Previously we demonstrated that anti-dsDNA B-cells present in R4A-Cμ transgenic mice are targeted to anergy. These B-cells display an anergic phenotype as characterized by receptor downmodulation, developmental arrest and reduced lifespan (284). We were interested in determining whether CD19 overexpression prevents anti-dsDNA B-cells from becoming anergic. We first characterized transgenic B-cells from R4A-Cμ/hCD19 mice by flow cytometry to see if they displayed an anergic phenotype. By immunostaining B-cells with antibodies to B220, IgM^a and kappa light chain (κ LC), we demonstrated a reduced expression of the IgM^a receptor on transgenic B-cells in the bone marrow and spleen of R4A-Cμ mice and R4A-Cμ mice overexpressing hCD19 relative to B-cells from NTg mice (Figure 28). The mean fluorescent intensity (MFI) of κ LC staining of B220, IgM B-cells from the bone marrow of NTg mice was 267 compared to MFIs of 101, 83.3 and 63.6 for IgM^a B-cells from R4A-Cμ, R4A-Cμ/$hCD19^{+/-}$ and R4A-Cμ/$hCD19^{+/+}$ mice respectively (Figure 28). Similarly the MFI of

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splenic B-cells from NTg mice was 281 while the MFIs of R4A-Cμ, R4A-Cμ/hCD19$^{+/-}$ and R4A-Cμ/hCD19$^{+/+}$ mice were 172, 94 and 96 respectively indicating a reduction in Ig surface density with CD19 overexpression. It has previously been shown that the density of mIgM is reduced on B-cells from mice overexpressing CD19 (92). We have reproduced these results in our study demonstrating that the MFI of IgM B-cells in the bone marrow of hCD19 mice is 127 and that in the spleen is 149 (about 2 fold less than NTg control). Despite this, the level of IgM^a is further reduced in R4A-Cμ/hCD19$^{+/-}$ and R4A-Cμ/hCD19$^{+/+}$ mice. These results suggest that the additional receptor down modulation in R4A-Cμ mice overexpressing hCD19 is due to anergy.

We previously demonstrated that anergic R4A-Cμ B-cells were arrested at the immature stage of development as characterized by their CD24^{hi} phenotype (Figure 29 and Table 10) (284). Using flow cytometry to assess the percentage of transgenic and nontransgenic B-cells that expressed high levels of CD24 we were able to show that CD19 overexpression did not alter the lag in development of transgenic B-cells. In fact, the ratio of immature to mature (CD24^{hi}/CD24^{lo}) bone marrow B-cells was even greater for transgenic IgM^a B-cells from R4A-Cμ/hCD19$^{+/+}$ mice (88.5%/9.9%) than B-cells from R4A-Cμ mice (70.2/20.4%), the ratio of CD24^{hi} to CD24^{lo} bone marrow B-cells in hCD19 mice (42.4%/62.6%) is similar to NTg mice (35.9%/62.7%) indicating that although CD19 expression induces a reduction in these 2 subsets of B-cells it does not alter their relative ratios (Figure 29). Similar observations were made in the spleen. Results which are an average of 4 experiments are summarized in Table 10. These results suggest that some IgM^a B-cells are still arrested in the immature stage of development in mice overexpressing CD19 and that CD19 does not seem to rescue them.

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Anergic B-cells have been shown to display a shortened lifespan with respect to naïve B-cells (40, 54-56). Since hCD19 mice have a greater frequency of autoreactive B-cells in the periphery we were interested in determining whether hCD19 overexpression increases the lifespan of anergic B-cells thereby making them more eligible for activation. To measure the lifespan of splenic B-cells, we injected mice intraperitoneally (i.p.) with 5-bromo-2'-deoxyuridine (BrdU) for 12 days. BrdU is an analog of thymidine (derivative of uridine) and can be incorporated specifically into DNA in place of thymidine. Cells can be pulse-labeled with BrdU, and those cells that are synthesizing DNA will incorporate BrdU into the DNA. The loading of B-cells with BrdU can be used to determine their lifespan. A B-cell population that is slowly renewed from bone marrow precursors shows a lower percentage of BrdU incorporation which reflects a longer lifespan. In contrast, B-cells that are rapidly turning over are replaced more quickly by labeled precursor cells from the bone marrow. This is reflected by a higher percent of BrdU incorporation corresponding to a shorter lifespan. Using anti-BrdU antibody and flow cytometry cells that undergo DNA synthesis during exposure to BrdU can be determined. Splenic B-cells were immunostained with anti-BrdU-FITC, anti-B220-CYC and either anti-IgM^a-PE or anti-IgM^b-PE. Flow cytometric analysis was performed and B220, IgM cells were gated and percent BrdU staining measured. We observed that BrdU incorporation was higher for R4A-Cμ mice (37.1%) than for hCD19 mice (26.2%) or NTg mice (17.0%) suggesting a shorter lifespan (Figure 30). BrdU incorporation for R4A-Cμ/$hCD19^{+/+}$ mice (41.1%) was similar to that of R4A-Cμ mice indicating that CD19 overexpression did not increase the lifespan of R4A transgenic B-cells (Figure 30).

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In summary, evidence of receptor downmodulation, developmental arrest and shortened lifespan indicate that CD19 overexpression does not rescue transgenic IgM^a B-cells from an anergic phenotype. In fact, hCD19 seems to augment the anergic characteristics of transgenic B-cells which may be due to more exaggerated signaling by self-antigen.

Activation Status of R4A-Cμ B-cells overexpressing hCD19

It has been reported that anergic anti-dsDNA B-cells display an activated phenotype due to chronic exposure to self-antigens (54). Peripheral B-cells from mice overexpressing hCD19 have been shown to display elevated levels of MHC class II but to no other activation molecules (Figure 31) (92). Transgenic R4A-Cμ B-cells in mice that overexpress hCD19 were examined by flow cytometry for expression of several cell surface molecules such as CD44, CD80 (B7.1), and MHC class II used as markers of B-cell activation (286, 287). A slight upregulation in MHC class II was observed on B-cells from R4A-Cμ/hCD19$^{+/-}$ mice (MFI=54.9) and R4A-Cμ/hCD19$^{+/+}$ mice (MFI=62.1) compared to NTg (MFI=44.6), R4A-Cμ (MFI=38.2) and activated control mice (MFI=370.8) (Figure 31). CD80 (B7.1) was also slightly upregulated on some transgenic B-cells in R4A-Cμ mice overexpressing CD19 (Figure 31). However, CD44 was not upregulated on transgenic B-cells in these mice (Figure 31). These results indicate that R4A-Cμ B-cells overexpressing hCD19display a slightly activated phenotype suggesting that they are receiving some tonic signals.

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Hyperresponsiveness of R4A-Cμ/hCD19$^{+/+}$ B-cells to LPS

B-cells from mice overexpressing hCD19 have been shown to have a heightened *in vitro* proliferative response to IgM cross-linking and to LPS activation (92, 223). This hyperresponsiveness to transmembrane signaling is thought to be due to CD19 mediated lowered signaling thresholds making these B-cells more susceptible to activation (92). To determine if transgenic B-cells in R4A-Cμ/hCD19 mice are hyperresponsive to BCR crosslinking and BCR independent signaling, we isolated splenic B-cells from R4A-Cμ/hCD19$^{+/+}$ mice and activated them *in vitro* for 72 hours with either LPS, anti-IgM F(ab')$_2$, or anti-IgM F(ab')$_2$ plus anti-CD40. Serum IgM^a anti-dsDNA antibody was measured by ELISA (Figure 32A). Our results demonstrated that R4A-Cμ/hCD19$^{+/+}$ transgenic B-cells were more responsive to LPS signaling than R4A-Cμ transgenic B-cells. However, transgenic anti-dsDNA B-cells still remained unresponsive to crosslinking with anti-IgM F(ab')$_2$ despite hCD19 overexpression. Addition of anti-CD40 as a surrogate for CD40L in combination with anti-IgM F(ab')$_2$ still failed to activate transgenic B-cells.

The number of transgenic dsDNA binding B-cells present in R4A-Cμ and R4A-Cμ/hCD19$^{+/+}$ mice following activation by BCR crosslinking or LPS were quantitated by ELISPOT. We observed that there were 10-fold more B-cells spontaneously secreting IgM^a anti-dsDNA antibody in R4A-Cμ/hCD19$^{+/+}$ mice than R4A-Cμ mice (Figure 32B). Stimulation with LPS induced further activation of IgM^a anti-dsDNA B-cells in R4A-Cμ/hCD19$^{+/+}$ as evidenced by a 3-fold increase in the number of spots. Activation by BCR crosslinking using IgM F(ab')$_2$ plus anti-CD40 did not elicit an increase in the number of anti-dsDNA binding B-cells despite the fact that total transgenic IgM^a B-cells

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were increased (Figure 32B and C). These results suggests that functional anergy is still maintained in IgM^a dsDNA binding B-cells in R4A-Cμ/hCD19$^{+/+}$ mice, as defined by lack of responsiveness to BCR crosslinking.

Distribution of R4A-Cμ dsDNA Binding B-cells

CD19 has been shown to be important in the formation of the marginal zone (MZ). Self-reactive B-cells including anti-dsDNA B-cells in both normal and autoimmune mouse models have been found to reside in the MZ (147, 158, 178, 179, 288, 289). The MZ is the site of B-cells that are the first line of defense against blood-borne pathogens. Most of these B-cells respond to type-2 T-cell independent antigens. We were interested in determining the splenic distribution of anti-dsDNA B-cells in R4A-Cμ mice overexpressing hCD19. We first used flow cytometry to determine whether the percent of B-cells belonging to different splenic compartments are altered as a consequence of CD19 overexpression. Mature B-cells in the spleen reside in the MZ or the follicular zone (FO) and are characterized by distinct phenotypes; MZ B-cells are $CD21^{hi}CD23^{lo/-}$ and FO B-cells are $CD21^{lo\text{-}inter}CD23^{hi}$. We immunostained splenic B-cells from NTg, R4A-Cμ, hCD19 and R4A-Cμ/hCD19$^{+/+}$ mice with antibodies to IgM^a or IgM^b, CD21, and CD23 and analyzed them by flow cytometry. We gated on $CD19^+IgM^{a+}$ or $CD19^+IgM^{b+}$ for transgenic or nontransgenic splenic B-cells respectively. We observed that the frequency of MZ B-cells was increased in R4A-Cμ (26.0%), hCD19 (38.4%) and R4A-Cμ/hCD19$^{+/+}$ (22.0%) mice compared to C57BL/6 NTg mice (6.8%) (Figure 33). The majority of B-cells in C57BL/6 mice (78.0%) are FO B-cells. The skewing of B-cells to the MZ in R4A-Cμ mice and mice overexpressing CD19 may be a consequence of an increased population of B-cells with autoreactive specificities in

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these mice. On average, mice that are transgenic for both R4A and CD19 have a higher ratio of MZ B-cells to FO B-cells (25.6%/24.8%) than R4A-Cμ mice (29.9%/45.2%) (Table 11)

In order to determine whether B-cells spontaneously secreting transgenic anti-dsDNA antibody in R4A-Cμ/hCD19$^{+/+}$ mice are MZ or FO B-cells, we sorted on these 2 populations to ≥95% purity (Figure 34A) and determined the number of B-cells secreting IgM^a anti-dsDNA antibody from each subset by ELISPOT. We observed that B-cells derived from the MZ subset were the source of spontaneously secreted IgM^a anti-dsDNA antibody in R4A-Cμ/hCD19$^{+/+}$ mice (Figure 34B).

Role of T-cells

Seo et. al. recently demonstrated that loss of B-cell tolerance in transgenic anti-dsDNA binding B-cells in autoimmune mice may be due to increased T-cell help and the lack of suppressor T-cells (T_{reg}) (73). We were interested in determining whether increased production of autoantibody in mice overexpressing CD19 may be a consequence of an alteration in T-cell subsets. We therefore examined the percent of CD4$^+$ T helper cells and CD4$^+$CD25$^+$ T suppressor cells in R4A-Cμ mice overexpressing CD19. We observed that the number of CD4$^+$ T-cells in R4A-Cμ/hCD19$^{+/+}$ mice was similar to that in R4A-Cμ mice and that there was no increase in the CD4:CD8 ratio (Figure 35A). In addition, we did not observe a reduction in the percent of T_{reg} cells (CD4$^+$CD25$^+$) in R4A-Cμ/hCD19$^{+/+}$ mice compared to R4A-Cμ mice (Figure 35B). These results suggest that the increase in anti-dsDNA antibody secretion in R4A-Cμ/hCD19$^{+/+}$ and R4A-Cμ/hCD19$^{+/+}$ is not due to an increase in CD4$^+$ helper T-cells or a decrease in CD4$^+$CD25$^+$ suppressor T-cells.

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Although there was no increase in $CD4^+$ T-cell number in mice overexpressing CD19, we were interested in determining whether existing CD4 T-cells were involved in activating dsDNA binding B-cells in these mice. T dependent B-cells require 2 signals for activation: one signal is derived from BCR crosslinking and the other signal is derived from cognate interaction between CD40 on the B-cell and CD40 ligand (CD40L) on the activated helper T-cell (290, 291). $CD4^+$ T helper cells have been shown to be important for the production of anti-dsDNA antibodies in the autoimmune mouse strains, NBZW/F_1 and MRL-lpr/lpr. Treatment of these mice with anti-CD4 monoclonal antibodies which induce depletion of $CD4^+$ T-cells was found to lead to a profound reduction in autoantibody production (292, 293). R4A-Cμ/hCD19 mice displayed signs of an activated phenotype such as MHC class II upregulation. In an attempt to determine whether CD4 T-cells are involved in the breakdown in B-cell tolerance in these mice, we used anti-CD4 antibody (GK1.5) to deplete these mice of $CD4^+$ T-helper cells. Mice 4-5 weeks old were injected every two weeks for 3 months with a total of 2mg of anti-CD4 antibody or PBS as control and the serum titer of anti-dsDNA antibody was evaluated monthly by dsDNA ELISA. The depletion of $CD4^+$ T-helper cells was monitored by flow cytometry of peripheral blood. The treatment regiment was sufficient to deplete mice of >99% of $CD4^+$ T-cells (Figure 36A). Serum titer of anti-dsDNA antibody (Figure 36B) revealed that R4A-Cμ/hCD19$^{+/+}$ mice were still able to spontaneously secrete anti-dsDNA IgM^a antibody regardless of whether they were treated with anti-CD4 antibody or not. No significant difference in anti-dsDNA secretion existed among anti-CD4 treated, PBS treated and untreated R4A-Cμ/hCD19$^{+/+}$ mice. The failure of $CD4^+$ T helper cell depletion to abrogate spontaneous anti-dsDNA secretion in R4A-Cμ/hCD19$^{+/+}$

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mice suggests that CD19 overexpression leads to activation of anergic dsDNA binding B-cells in a T-cell independent manner.

Hypotonic Lysis of Peritoneal B-cells

Sato et. al. demonstrated that CD19 overexpression induces an increase in $CD5^+$ B-cell number which correlates with autoantibody production (88). Studies by Murakami et. al. demonstrated that peritoneal, $CD5^+$, B-1 B-cells are responsible for the secretion of anti-RBC antibody in anti-RBC transgenic mice (125). They also demonstrated that depletion of peritoneal cells by hypotonic lysis from autoimmune NZB mice, which spontaneously produce anti-RBC autoantibodies, abolishes autoantibody production (127). Based on these studies we were interested in determining whether peritoneal B-1 B-cells are responsible for anti-dsDNA autoantibody production in R4A-Cμ mice overexpressing hCD19. We induced hypotonic lysis of peritoneal B-cells by injecting 8 week old R4A-Cμ/hCD19$^{+/+}$ mice, i.p. with sterile water for 7 weeks (Figure 37). Control mice were injected with PBS. Serum samples were collected weekly and subjected to an anti-dsDNA ELISA. Results demonstrated that hypotonic lysis of peritoneal B1 cells failed to abrogate the secretion of R4A IgM^a anti-dsDNA antibody suggesting that B1 cells are not responsible for the spontaneous secretion of transgenic anti-dsDNA antibody in these mice.

CpG activation

Conserved molecular sequences derived from microbial antigens and known as PAMPS (pathogen associated molecular patterns) are recognized by receptors of the innate immune system. The Toll like family of receptors (TLRs) are expressed on the

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surface of cells of the immune system. Upon recognition of PAMPs they activate signaling pathways. Two PAMPs; LPS and CpG have been shown to activate B-cells through their Toll receptors TLRs (TLR 4 for LPS and TLR 9 for CpG) and induce B-cell proliferation and differentiation into antibody producing cells (294-296). CpG sequences are stretches of hypomethylated DNA present in bacteria and mammalian promoter elements and may be released by apoptotic cells (297, 298). Since 30-50% of R4A-Cμ mice overexpressing hCD19 do not spontaneously produce IgM^a anti-dsDNA antibodies and since BCR crosslinking cannot activate dsDNA binding B-cells in these mice, we wondered whether signaling by CpG sequences might trigger these B-cells to start secreting anti-dsDNA antibody. Since CpG sequences are released by apoptotic cells we reasoned that they may be good candidates for activating dsDNA binding B-cells in R4A-Cμ/hCD19$^{+/+}$ mice. We therefore cultured B-cells from R4A-Cμ/hCD19$^{+/+}$ and R4A-Cμ mice *in vitro* for 48 hours with stimulatory CpG (1μg/ml). As demonstrated in Figure 38A, stimulatory CpG sequences were able to induce proliferation of total IgM^a B-cells in both R4A-Cμ and R4A-Cμ hCD19 overexpressing mice. In addition, CpG induced an increase in the total number of IgM^a producing B-cells from R4A-Cμ and R4A-Cμ/hCD19$^{+/+}$ mice as detected by ELISPOT (Figure 38C). However, the number of dsDNA binding B-cells secreting antibody from R4A-Cμ/hCD19$^{+/+}$ mice was dramatically increased upon CpG activation compared to the number in R4A-Cμ mice (Figure 38B). Furthermore, an inhibitory CpG motif that was recently identified was able to suppress the effect of the stimulatory CpG sequences and prevent B-cell proliferation and differentiation (299). These results suggest that the stimulatory CpG sequences may play a role in the activation of anergic dsDNA binding B-cells in R4A-Cμ/hCD19$^{+/+}$

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mice. Lowering of the signaling the threshold in these mice due by CD19 overexpression may not be enough to activate these B-cells. Likewise, CpG sequences may not provide enough of a signal to activate anergic B-cells in R4A-Cμ mice. However the combination of CD19 overexpression plus CpG stimulation may be enough to trigger a break down in tolerance in this anergic population.

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DISCUSSION

The CD19 molecule is an integral member of the BCR co-receptor complex and functions to lower the threshold for antigen receptor stimulation thereby amplifying signaling through the BCR (92, 227, 228, 235, 280, 300, 301). This study demonstrates that CD19 overexpression in R4A-Cμ dsDNA binding B-cells abrogates self tolerance leading to the spontaneous secretion of IgM^a anti-dsDNA antibody. Surprisingly, however, dsDNA binding B cells in these mice still maintain the phenotypic and functional characteristics of anergic B cells. They are arrested in the immature stage of development, have reduced levels of membrane Ig, and have a shortened lifespan. In addition, they fail to be activated by BCR crosslinking. Similar observations were made by Inaoki et. al. in hCD19 overexpressing mice transgenic for HEL/anti-HEL (244). A loss of tolerance was observed in these mice but HEL binding B cells still maintained an anergic phenotype. Previous studies in mice overexpressing CD19 demonstrate that these mice have an overall reduction in immature and mature B cells. This was attributed to exaggerated negative selection, although it was never determined if the cells that were negatively selected had autoreactive specificities. Evidence that tolerance can be induced in mice overexpressing hCD19 seems to contradict the observation that elevated levels of autoantibodies are produced in these mice. Studies in hCD19 mice suggest that appropriate negative selection occurs in the B-2 subset of B cells but that there is an expansion of peritoneal $CD5^+$ B-1 cells, correlating with increased autoantibody production in these mice. Increased negative selection and elevated titers of autoantibodies due to an expansion of B-1 cells has been observed in mice deficient in Lyn kinase or CD22 (112-114, 278, 279, 302-305). Many spontaneous mouse models of

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SLE, and transgenic mouse models have demonstrated that pathogenic autoantibodies are derived from the B-1 pool (88, 114, 117, 124, 193, 253, 306). However, conventional, B-2 cells have also been implicated as autoantibody producing cells in mouse models of SLE (244, 307-309).

In this present study, our data suggests that B cells secreting transgenic anti-dsDNA antibody in R4A-Cμ/hCD19$^{+/+}$ mice are derived from the B-2 rather than the B-1 subset. We only observed a slight expansion of CD5^{+} B cells in these mice compared to that in R4A-Cμ mice and hypotonic lysis of peritoneal B-1 cells failed to deplete transgenic anti-dsDNA secreting B cells from R4A-Cμ/hCD19$^{+/+}$ mice. However, proof that B-1 B cells are not the source of IgM^{a} anti-dsDNA antibody in mice overexpressing CD19 will require cell sorting to purify B-1 B cells and activating them *in vitro* to see if they can produce anti-dsDNA antibodies. Inaoki et. al. similarly observed that the source of anti-HEL antibody in HEL/anti-HEL mice is B-2 cells. These results challenge the idea that CD19 overexpression only leads to dysregulation of B-1 cells. Studies by Clarke et. al. suggest that signal strength determines whether B cells differentiate to B-1 or B-2 cells and that B cells with strong signals favor B-1 differentiation (81, 193). Therefore, the determining factor in whether CD19 overexpression rescues B-1 or B-2 cells may reflect signal strength.

It was recently reported that light chain allelic exclusion was impaired in mice lacking CD19 because this resulted in an incompetent BCR with a weakened signal that failed to prevent light chain rearrangement on the other allele (282). In R4A-Cμ mice, we observed that transgenic dsDNA binding B cells fail to display heavy chain allelic exclusion while transgenic non dsDNA binding B cells display intact allelic exclusion. In

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the present study, we observed by FACS analysis, that a higher percent of B cells in R4A-Cμ/hCD19$^{+/+}$ mice display intact heavy chain allelic exclusion than in R4A-Cμ mice. However, it is not yet known whether intact allelic exclusion is maintained in the dsDNA binding B cell population in these mice. This awaits the generation of dsDNA binding B cell hybridomas from these mice. It is also not clear what role CD19 might have in facilitating better heavy chain allelic exclusion since a productive heavy chain on one allele is enough to stop rearrangement on the other allele. Light chain allelic exclusion on the other hand, is mediated by a complete antibody molecule and this may need to associate with CD19 to optimally inhibit rearrangement of the other allele.

We observed an expansion of MZ B cells in both R4A-Cμ and R4A-Cμ mice overexpressing CD19. However, spontaneously secreting anti-dsDNA B cells could only be isolated from MZ B cells sorted from R4A-Cμ/hCD19$^{+/+}$ mice. It is likely that transgenic dsDNA binding B cells from R4A-Cμ mice also reside in the MZ but we speculate that since they are anergic and not spontaneously secreting antibody they could not be detected. The MZ does not appear in the mouse until 2-3 weeks after birth. It is interesting to note that serum levels of IgM^a anti-dsDNA antibody were negligible prior to this time in mice overexpressing CD19. The MZ has been shown to expand and be the source of pathogenic autoantibodies in NZB/W F1 autoimmune mice (310-312). It has also been shown that in estrogen treated R4A-IgG2b mice, dsDNA binding B cells localize to the MZ (179). This hormonal treatment was shown to lead to the upregulation of CD22 and SHP-1 which results in lowered signaling strength. Autoreactive B cells expressing dual receptors were also found to reside in the MZ (189). Expression of two receptors on the surface of a B cell may also serve to lower the signal strength.

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Although the exact signal, that guides B-cells to become MZ B cells is largely unknown, recent studies have indicated that the strength of the signal transmitted through the BCR along with molecules that modulate signal thresholds such as CD19, molecules that influence life span and survival such as BAFF and lymphotoxin, molecules that affect B-cell retention movement and migration such as pyk2 and Vav 1, and transcription factors such as NFκB, and Rel B, are involved in MZ generation and maintenance (76, 121, 147, 151, 193, 313). Studies from several laboratories have provided experimental evidence which suggests that B-cells require an intermediate signal for follicular localization and a weaker signal for MZ localization (20, 147, 161, 218). Strong signals seem to guide B cells to the peritoneum (218). Positive selection of antibodies with certain specificities may also play a role in MZ localization (147, 158). It has been proposed that weak, self-reactive B-cells in the MZ may constitute a dominant low-affinity autoreactive subset which functions to clear the body of self antigen (158, 314-317). In addition, since many MZ B cells produce polyspecific IgMs and are hyperresponsive to mitogen activation they may function as the first line of defense to quickly rid the host of bacterial antigen (148, 158).

The transgenic anti-dsDNA B cells targeted to anergy and deletion in R4A-Cμ and R4A-Cμ/hCD19$^{+/+}$ mice have been shown to produce antibodies with moderate to high affinities for dsDNA. Why then would these B cells localize to the MZ since this is the preferred site for B cells with weak affinities? The answer may be that these B cells have a reduced density of surface immunoglobulin which can lead to a lowered signaling strength. Reduced surface Ig is characteristic of anergic B cells and B cells which

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overexpress CD19. In addition, if these dsDNA binders express dual receptors than that can account for lowered signaling strength as well.

Spontaneous secretion of IgM^a anti-dsDNA antibody in R4A-Cμ/hCD19$^{+/+}$ mice did not seem to require T cell help. Depletion of $CD4^+$ T cells from these mice failed to prevent the loss of tolerance. T-cell help has been shown to be important for autoantibody production in NZB/W F1 mice and CD4 depletion or co-stimulatory blockade has been shown to slow autoimmunity and promote survival in these mice (292, 293, 318). However, autoantibody production has also been observed when pre-B cells from NZB/W F1 mice were adoptively transferred into mice lacking T cells (SCID or RAG-2 deficient) indicating that some B cells from NZB/W F1 mice are capable of secreting autoantibodies even in the absence of T cells (117, 319). As previously discussed, the MZ is a known reservoir for T-independent B-cells with autoreactive specificities. Our observation that B-cells responsible for the spontaneous secretion of IgM^a anti-dsDNA antibody in R4A-Cμ/hCD19$^{+/+}$ mice reside in the MZ and are T-cell independent is consistent with this. However, ultimate proof that T cells do not play a role in the production of IgM^a anti-dsDNA antibodies in R4A-Cμ/hCD19$^{+/+}$ mice may require breeding these mice onto a T cell deficient background and observing the affect that this has on anti-dsDNA antibody secretion.

B cells in mice overexpressing CD19 have been shown to be hyperresponsive to both BCR dependent and BCR independent mechanisms of transmembrane signaling (87, 92, 240). Overexpression of CD19 has been proposed to lead to an increase in Src kinase activation following transmembrane signaling which serves to lower the threshold of B cell activation (228, 235). While anti-dsDNA B-cells from R4A-Cμ/hCD19$^{+/+}$ mice

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could not be activated to secrete antibody upon BCR crosslinking they could be activated by LPS and were hyperresponsive to signaling by stimulatory CpG sequences. Hypomethylated CpG sequences are present in bacterial DNA and eukaryotic promoter regions. CpG activation of B cells is mediated through TLR9 and the MyD88-dependent signaling pathway (229, 296, 320, 321).

It is not clear why CD19 overexpression does not render IgM^a dsDNA binding B cells hyperresponsive to BCR crosslinking. Our data suggests that these B cells are still in a state of anergy. It may be that because of the receptor downregulation characteristic of anergic B cells that there are not enough IgM molecules present on the cell surface to associate with CD19 and thereby deliver an activating signal. It is also unclear whether, the breakdown of tolerance and spontaneous secretion of anti-dsDNA antibody in R4A-Cμ/hCD19$^{+/+}$ mice is due to the activation of clonally ignorant, deleted or anergic B cells. We have previously defined subsets of B cells targeted to anergy, deletion or clonal ignorance on the basis of the light chain used by the anti-dsDNA antibody they produce (251, 257, 258). Although CD19 overexpression may not be able to rescue anergic B cells it may lower the threshold of activation enough to activate clonally ignorant B cells. Future studies to determine which subset/subsets of B cells are rescued by CD19 will involve generating IgM^a anti-dsDNA producing hybridomas from R4A-Cμ/hCD19$^{+/+}$ mice, and sequencing the light chains of the antibodies they produce to determine light chain usage.

The observation that anergic B cells from R4A-Cμ/hCD19$^{+/+}$ were more responsive to stimulatory CpG sequences (*in vitro*) than B cells from R4A-Cμ mice suggests that the CpG signaling pathway may converge with the intrinsic CD19 signaling

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pathway to amplify the signal for activation in a BCR independent manner. Signaling mediated through CD19 leads to PLC phosphorylation, inositol phospholipid turnover, Ca^{2+} mobilization, stimulation of serine-specific protein kinases such as PKC, activation of NFκB, and activation of the MAPK pathways (231, 281, 322-324). Convergence with non BCR, innate pathways such as CpG may occur at the level of Iκ kinase α/B (IKK) degradation, activation of NFκB or at the level of activation of the MAP kinases such as ERK, p38, and JNK (92, 228, 235, 294, 296, 301, 320, 321).

Not all R4A-Cμ mice overexpressing CD19 have elevated serum titers of transgenic anti-dsDNA antibody indicative of a loss of tolerance. This combined with the knowledge that CpG sequences can activate anergic dsDNA binding B cells, led us to speculate that a second signal is needed *in vivo* to trigger the activation of dsDNA binding B cells in R4A-Cμ/hCD19$^{+/+}$ mice. We therefore, propose that either exposure to bacteria or increased apoptosis, due to heightened negative selection in CD19 overexpressing mice, could lead to an ample source of hypomethylated CpG DNA available to deliver activating signals to anergic B cells. This together with intrinsic signaling through CD19 could be sufficient to activate anergic B cells in these mice. Intrinsic signaling of CD19 can also be amplified by engagement of C3d coated antigen with CD21, the receptor for C3d that associates with CD19 in the B cell co-receptor complex (325). This can occur in a BCR independent manner.

In summary, CD19 overexpression has been shown to lead to a breakdown of tolerance of transgenic anti-dsDNA B cells. These B cells are derived from the conventional B cell pool and reside in the MZ. Activation of these B cells does not require T cell help. Anti-dsDNA B cells in CD19 overexpressing mice still maintain the

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phenotypic and functional characteristics of anergic B cells but can be activated by combined signaling mediated through CD19 and innate signaling pathways.

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Figure 21. Measurement of R4A-IgM^a anti-dsDNA antibody and quantitation of total R4A-IgM^a antibody in the sera of transgenic mice overexpressing hCD19. **(A)** The level of anti-dsDNA R4A IgMa antibody was determined at 1 month intervals over a 4 months time period. Results of these 3 mice are representative of others. Open symbols ($\triangle \circ \square$) represent 3 individual R4A-Cμ/hCD19$^{+/+}$ mice. Closed circle represents an R4A-Cμ mouse. **(B)** Sera from 10, 12 week old R4A-Cμ/hCD19$^{+/+}$, R4A-Cμ/hCD19$^{+/-}$, R4A-Cμ, and 10 NTg mice were screened by ELISA for binding of IgM^a antibody to dsDNA coated plates. Mean values indicated by bars. Dashed line indicates 4 standard deviations (SD) above the mean $p<0.01$ **(C)** Quantitation of total IgM^a antibody in R4A-Cμ/hCD19$^{+/+}$, R4A-Cμ/hCD19$^{+/-}$, R4A-Cμ, and 10 NTg mice.

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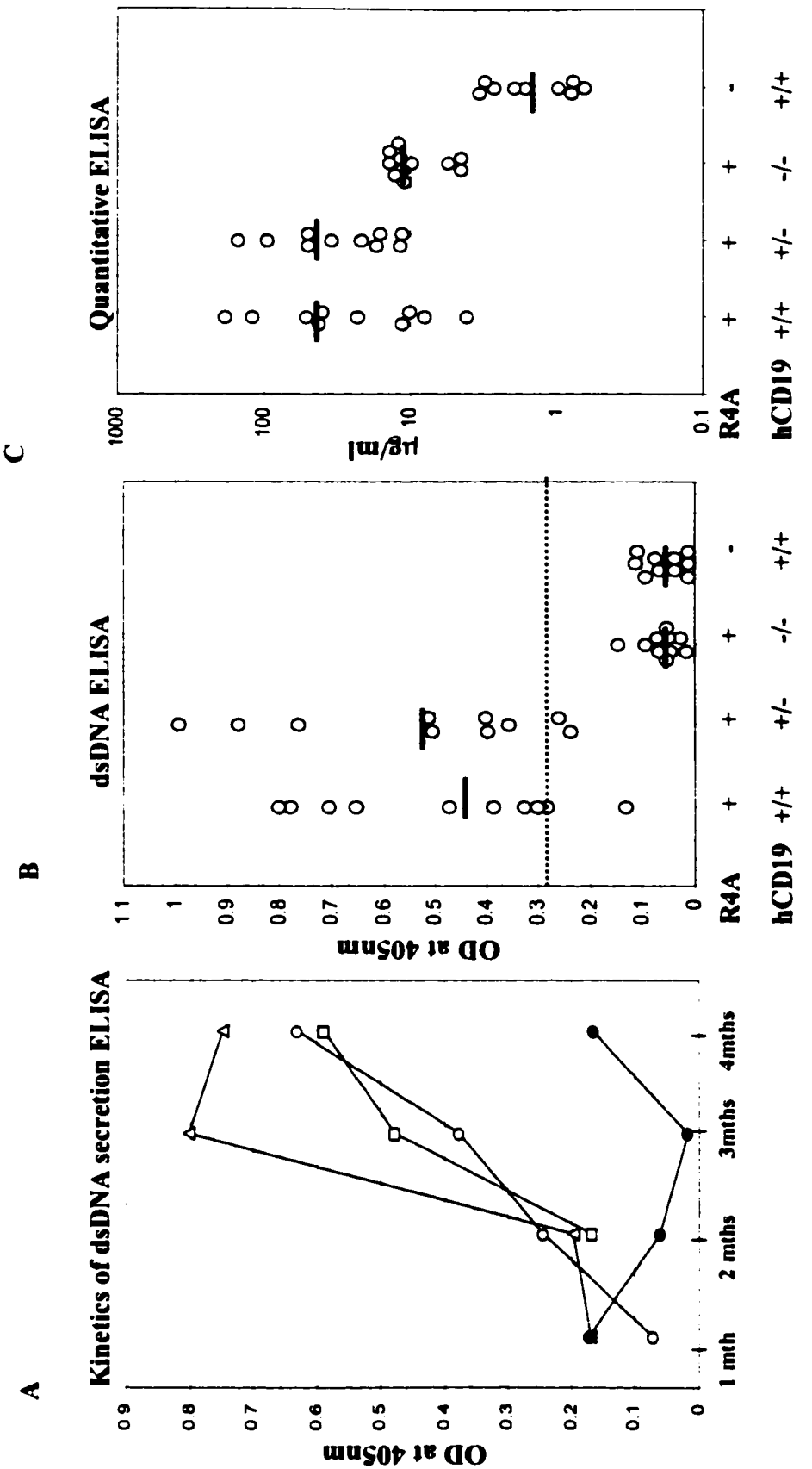

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Figure 22. Anti-Nuclear antibody (ANA) staining with R4A-Cμ/hCD19$^{+/+}$serum. The specificity of serum from R4AhCD19$^{+/+}$ transgenic mice was assessed by immunofluorescent staining. Serum samples diluted 1:50 were incubated with human epithelial cells (HEp-2) followed by FITC labeled (green) antibody to mouse IgM^{a} used to detect bound ANAs. The samples were visualized under a fluorescent microscope. Control staining was done with serum from a wild-type mouse and a NZB/W F1 mouse.

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TABLE 9. Frequency of bone marrow, splenic and peritoneum Conventional B-2 B cells in R4A transgenic mice overexpressing hCD19 and littermate controls.

	Number of B220 cells x10^6 (%)			*Number of B220, IgM^a cells x10^6 (%)**		
	Bone Marrow	Spleen	Peritoneum	Bone Marrow	Spleen	Peritoneum
NTg	6.3± 2.9 (78.6±5.1)	33± 5.0 (62.3±8.1)	0.2± 0.1 (53.7±9.4)	NA	NA	NA
R4A	2.9±0.9 (62.9±3.7)	6.1±2.1 (28.6±3.3)	0.08±0.03 (33.7±11.3)	0.7±0.3 (15.3±1.1)	2.3±0.6 (12.0±1.4)	0.004±0.002 (2.5±1.2)
hCD19	2.6± 1.7 (45.2±4.8)	8.0±3.7 (26.1±1.2)	0.06±0.01 (21.8±9.9)	NA	NA	NA
R4A-Cμ/hCD19$^{+/+}$	1.4±0.4 (46.2±2.1)	1.3±0.5 (10.5±3.1)	0.01±0.008 (9.4±5.7)	0.2±0.07 (8.1±0.7)	0.3±0.09 (2.0±0.6)	0.0003±0.00 02 (0.2±0.1)

Analysis was performed by flow cytometry. Mice were 3-4 months old. Results represent the mean ± SD from 4 mice in each group. Lymphocytes were labeled with antibody to B220 and antibody to IgM^a to detect B cells expressing the R4A transgene.

*Transgenic lymphocytes are B220$^+$, IgM^a

NA- not applicable

Figure 23. B-cell maturation in mice overexpressing CD19. Bone marrow cells from NTg, hCD19, R4A-Cμ, and R4A-Cμ/hCD19$^{+/+}$ mice were triple stained with CYC-anti-B220, FITC-anti-CD43 and PE-anti-IgM. Gates were set on B220^{+} B cells. Percentage of pro B-cells (B220^{+}, CD43^{+}, IgM^{-}), pre B-cells (B220^{+}, CD43^{-}, IgM^{-}) and immature/mature (B220^{+}, CD43^{-}, IgM^{+}) are indicated. These are representative dot plots from n=3 mice of each genotype.

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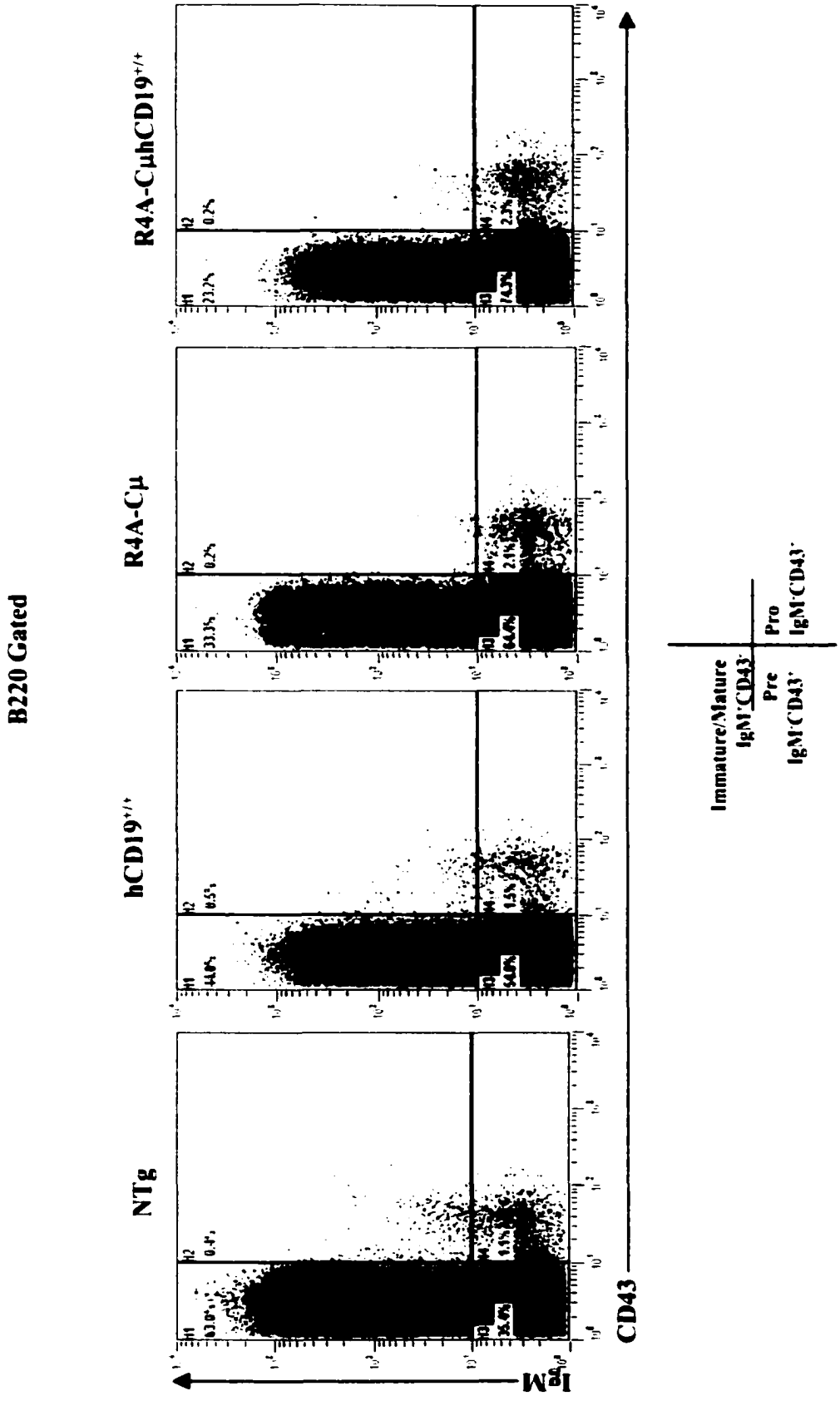

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Figure 24. Frequency of transgenic B-cells in mice overexpressing hCD19. Percent of bone marrow and splenic lymphocytes expressing the R4A-IgM^a transgene in R4A-Cμ and R4A-Cμ/hCD19$^{+/+}$ mice overexpressing hCD19 were determined by two-color flow cytometry. Transgenic B-cells were detected by staining with anti-B220 and anti-IgM^a. Dot plots are representative of n=5 mice of each genotype.

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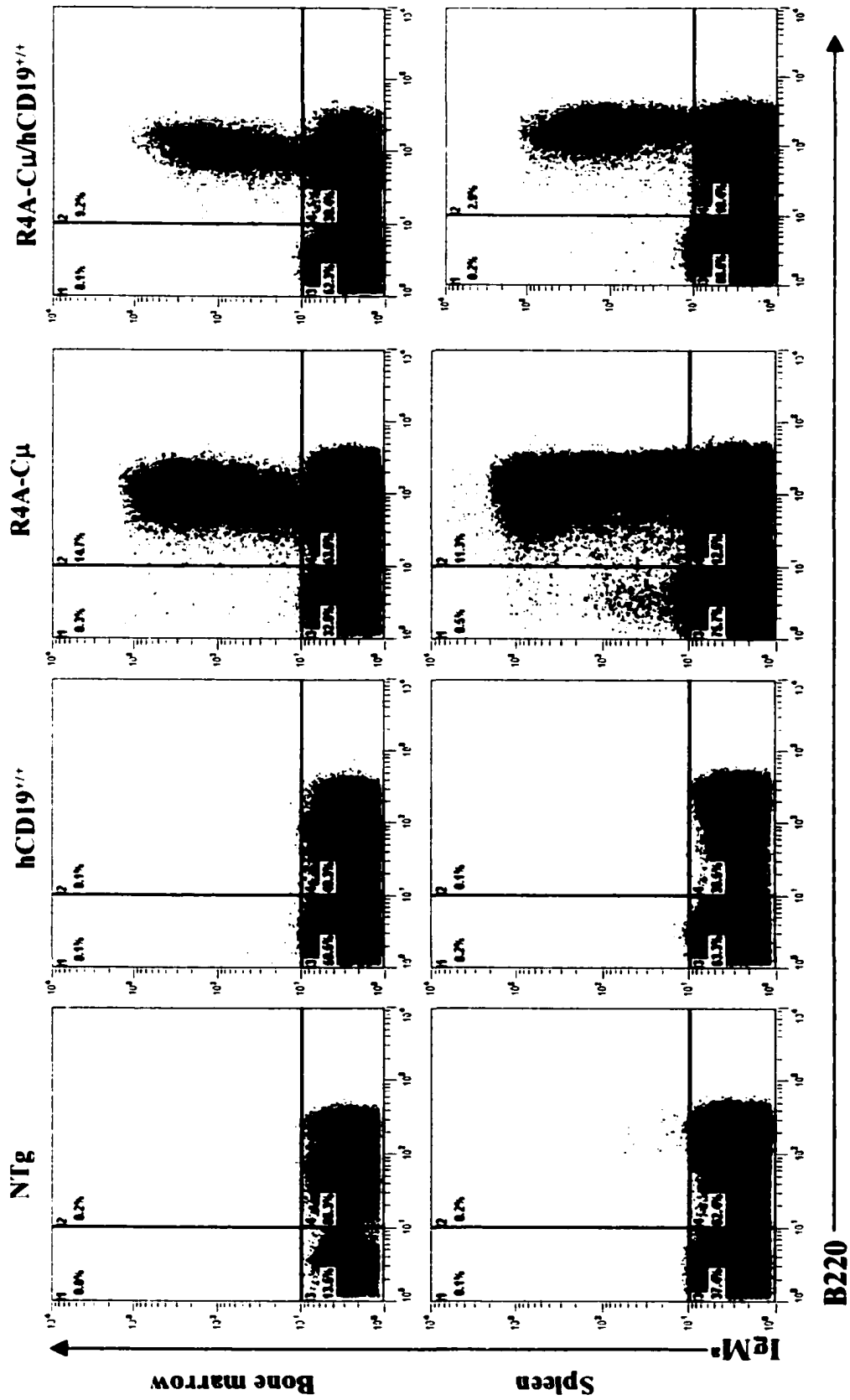

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Figure 25. hCD19 overexpression improves heavy chain allelic exclusion. Flow cytometric analysis of bone marrow and splenic B-cells from R4A-Cμ mice, and R4A-Cμ mice overexpressing hCD19 and nontransgenic mice were stained for B220 and surface expression of IgM^a and IgM^b allotypes. Gates were set on $B220^+$ cells and percent of splenic IgM^a, IgM^b and IgM^{ab} B-cells displayed by dot plots. This figure is representative of n=3 mice of each genotype.

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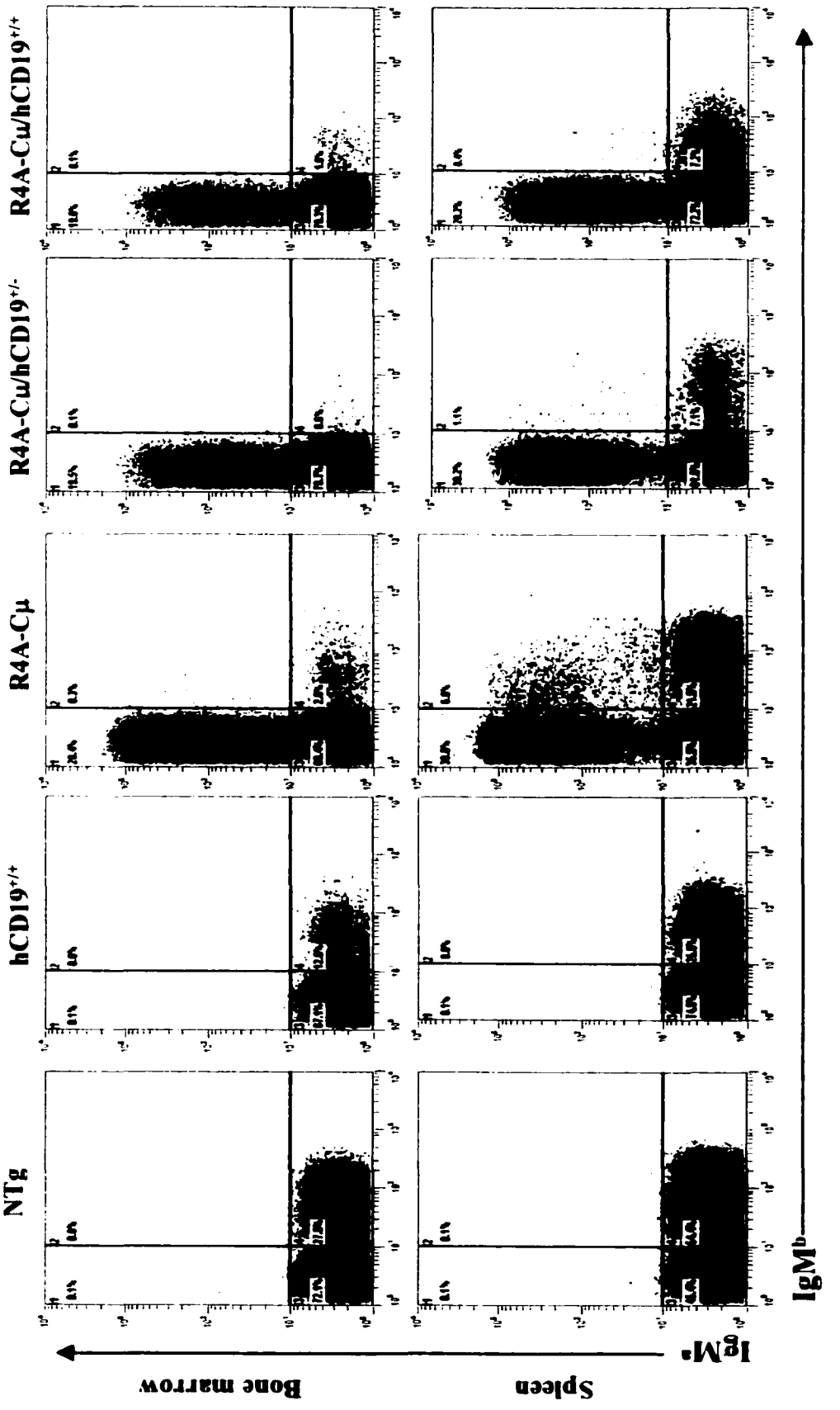

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Figure 26. B1 B-cell frequency in the spleen and peritoneum of R4A mice overexpressing hCD19. Peritoneal cells isolated by lavage and splenocytes from R4A-Cμ and R4A-Cμ/hCD19$^{+/+}$ were immunostained with anti-B220 and anti-CD5 antibodies and analyzed by dual color flow cytometry. This figure is representative of n=3 mice of each genotype.

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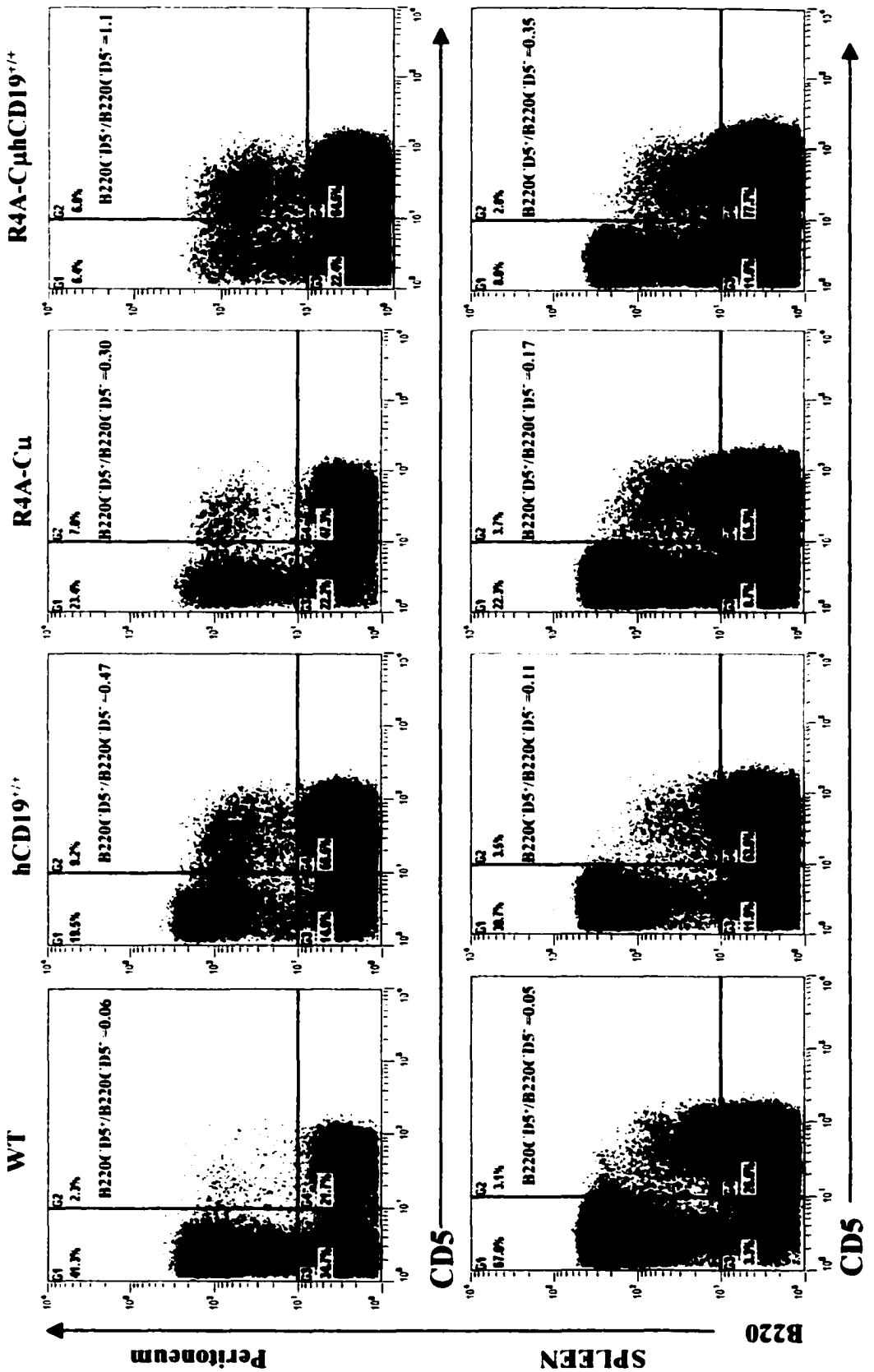

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Figure 27. Frequency of transgenic IgM^a B-cells in the peritoneum and spleen of R4A mice overexpressing hCD19. Isolated peritoneal and splenic cells were immunostained with anti-B220, anti-CD5 and anti-IgM^a antibodies and analyzed by flow cytometry. Gates were set on B220$^+$ cells and the percent of splenic CD5$^+$IgM^a B1a B-cells displayed by dot plot. Figures are representative of n=3 mice of each genotype.

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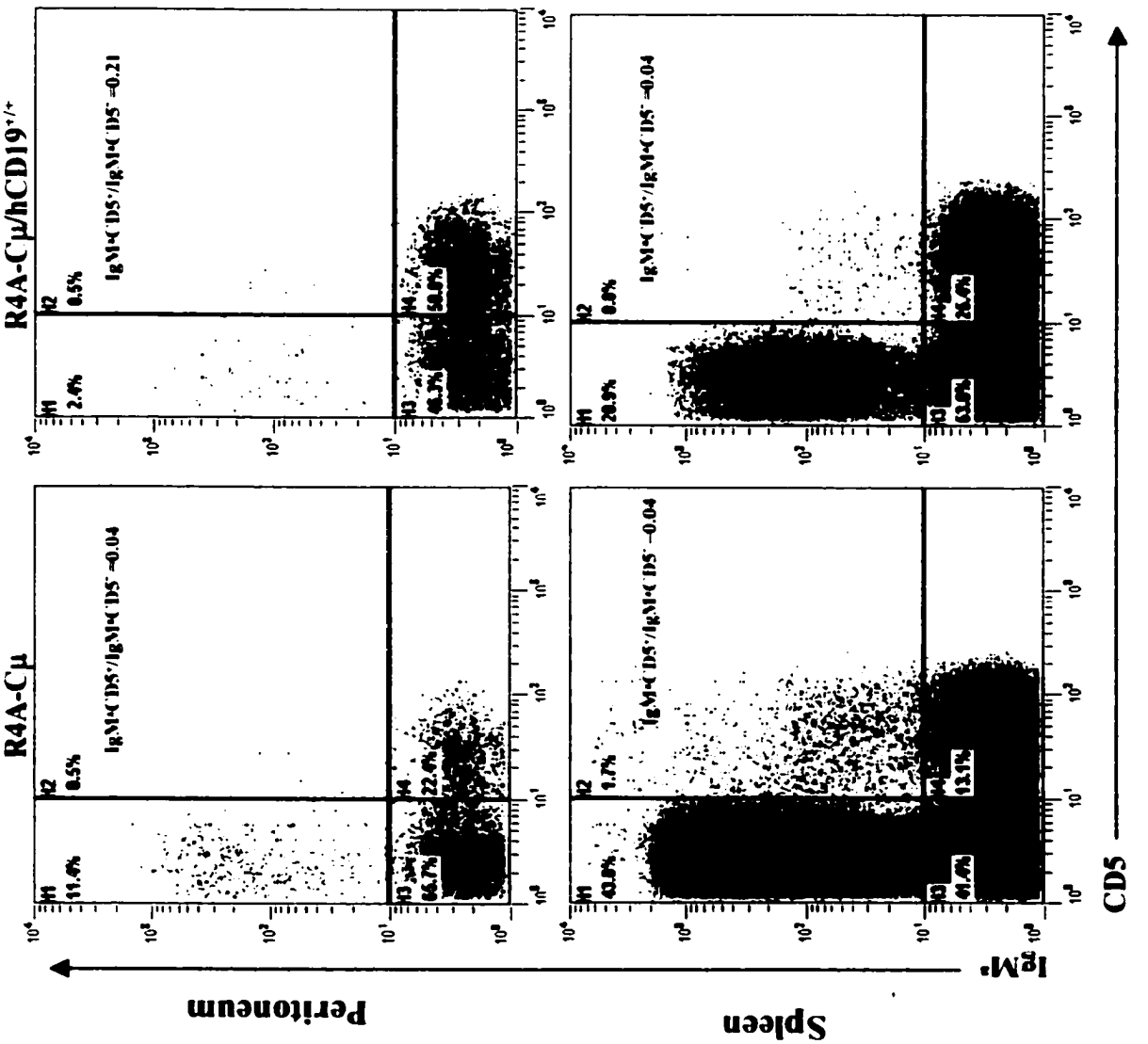

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Figure 28. Reduced expression of surface IgM receptor on R4A transgenic B-cells in hCD19 overexpressing mice. Splenic B-cells from NTg, hCD19, R4A-Cμ, R4A-Cμ/hCD19$^{+/-}$, and R4A-Cμ/hCD19$^{+/+}$mice were triple stained with anti-B220, anti-Igκ and either anti-IgM^{a} or anti-IgM^{b} antibodies and analyzed by flow cytometry. Gates were set for Tg B-cells on B220^{+}IgM^{a+} cells and for NTg B-cells on B220^{+}IgM^{b+} cells and κ staining displayed as histograms. Dotted line represents MFI of wild type mouse and solid line represents MFI of hCD19 transgenic mouse. These are representative histograms from n=3 mice of each genotype.

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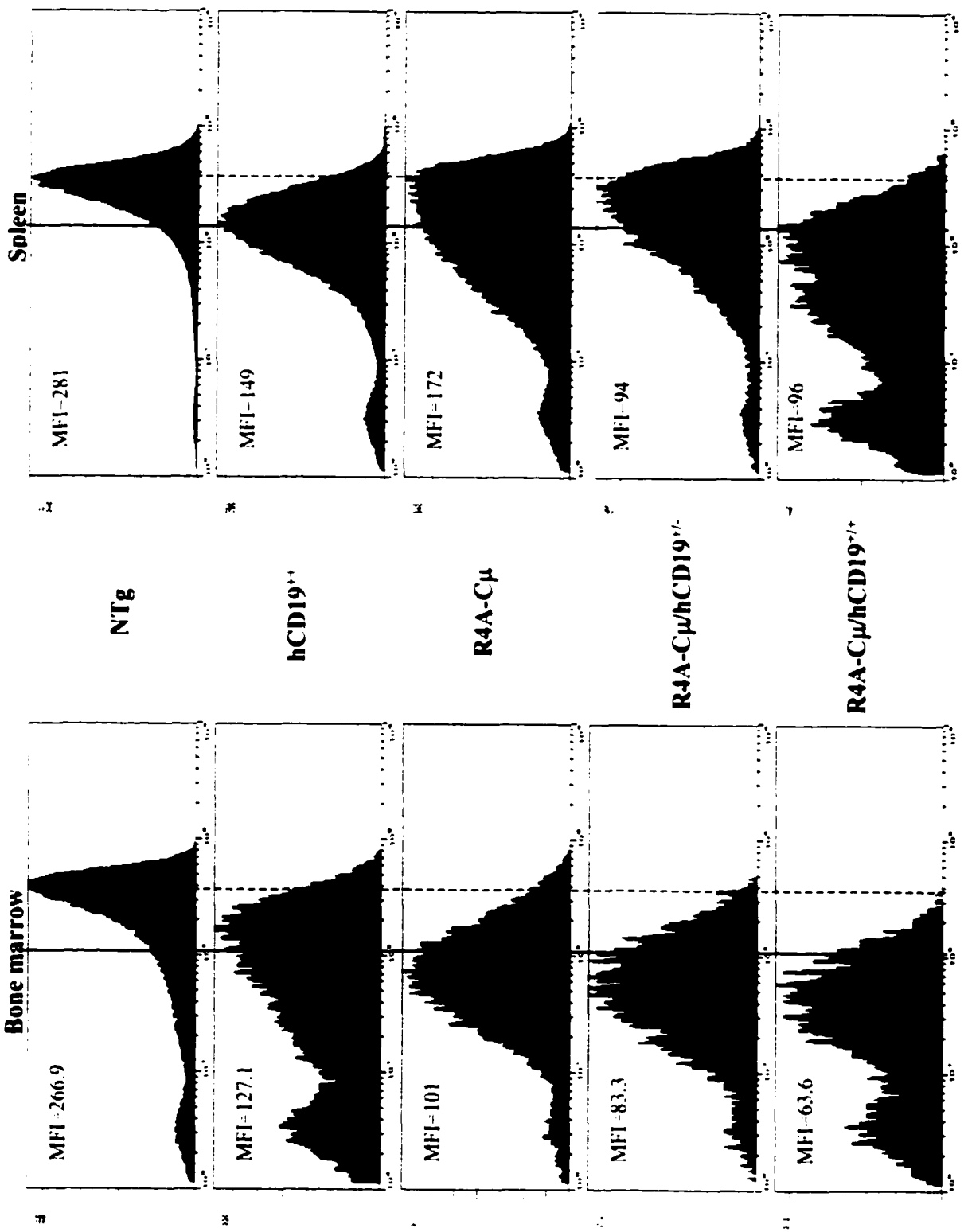

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Figure 29. Developmental arrest of transgenic R4A IgM^a B-cells in mice overexpressing hCD19. Bone marrow and Splenic B-cells from NTg, hCD19, R4A-Cμ, R4A-Cμ/hCD19$^{+/-}$ and R4A-Cμ/hCD19$^{+/+}$ mice were triple stained with anti-B220-CYC, anti-CD24-FITC and either anti-IgM^a-PE or anti-IgM^b-PE antibodies and analyzed by flow cytometry. Gates were set on B220$^+$IgM^{a+} for Tg B-cells and on B220$^+$IgM^{b+} for NTg B-cells. CD24 staining was displayed as a histogram. CD24^{hi} represents immature and CD24^{lo} represents mature B-cell subsets. Histograms are representative of n=3 mice of each genotype.

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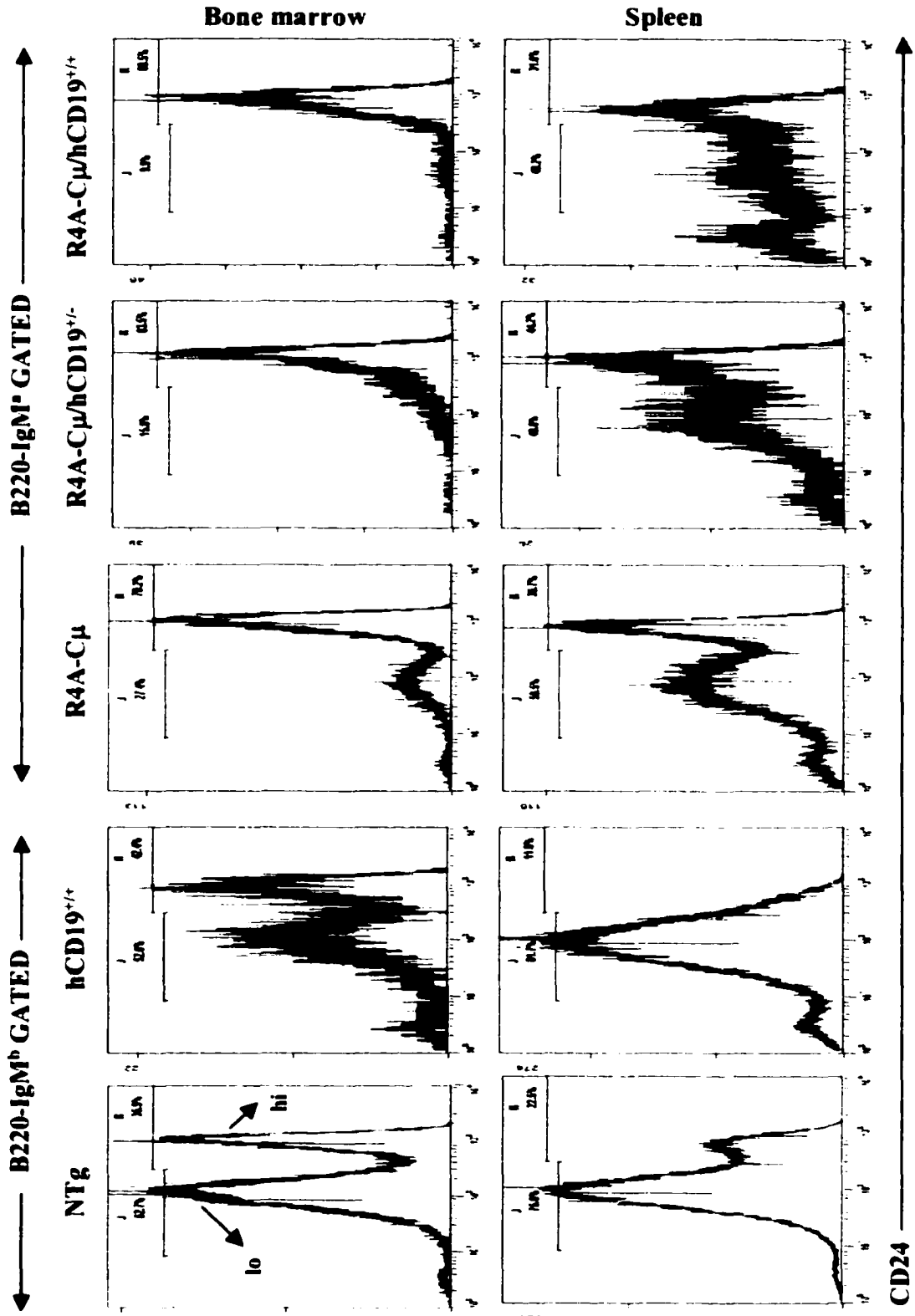

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Table 10. Ratio of immature to mature B-cells

		Average %		
	MICE	$CD24^{hi}$ (immature)	$CD24^{lo}$ (mature)	Ratio of immature:mature B-cells
Bone Marrow	NTg	29.4± 5.6	64.7 ± 3.0	0.5
	$hCD19^{+/+}$	53.3 ± 15.4	46.1 ± 9.3	1.2
	R4A-Cμ	67.6 ± 4.8	29.8 ± 6.2	2.3
	R4A-Cμ/$hCD19^{+/+}$	82.1 ± 9.1	16.6 ± 9.4	4.9
Spleen	NTg	21.6 ± 6.0	76.5 ± 5.0	0.3
	$hCD19^{+/+}$	11.6 ±0.8	68.3 ± 19.0	0.2
	R4A-Cμ	38.1 ± 4.6	55.6 ± 1.6	0.7
	R4A-Cμ/$hCD19^{+/+}$	35.6 ± 5.6	42.1 ± 8.6	0.8

Analysis was performed by flow cytometry. Mice were 3-4 months old. Results represent the mean ± SD from 4 mice in each group. Lymphocytes were labeled with antibody to B220 and either antibody to IgM^a to detect B cells expressing the R4A transgene or antibody to IgM^b to detect nontransgenic B-cells and CD24 to differentiate immature from mature B-cell subsets.

Figure 30. Life Span of R4A transgenic B-cells overexpressing hCD19. Mice were given 3mg/ml a day of BrdU by i.p. injection for 12 days and splenocytes were isolated and double stained with anti-B220-CYC and PE conjugated anti-IgM^a or IgM^b. The incorporated BrdU was detected with anti-BrdU-FITC. Gates were set on either $B220^+IgM^{a+}$ for Tg B-cells and on $B220^+IgM^{b+}$ for NTg B-cells and the percentage of BrdU staining displayed as a histogram. Histograms are representative of n=3 mice of each genotype.

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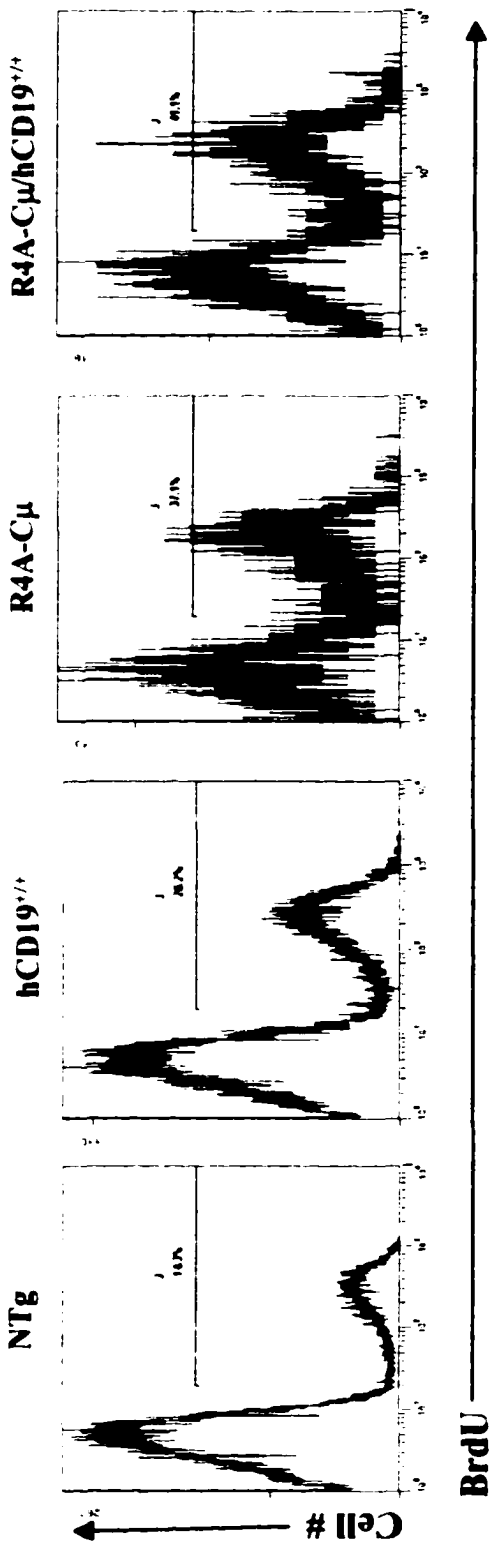

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Figure 31. Analysis of activated phenotype of splenic B-cells from R4A-Cμ mice overexpressing hCD19. Isolated splenocytes were immunostained with CYC conjugated anti-B220, PE-conjugated anti-IgM^a or PE-conjugated anti-IgM^b-PE and either FITC conjugated anti-CD44, CD80 or MHC class II. Cells were gated on either $B220^+IgM^{a+}$ to detect transgenic B-cells or $B220^+IgM^{b+}$ to detect nontransgenic B-cells and displayed by histogram for respective activation markers. Splenic B-cells stimulated *in vitro* for 48 hours with LPS plus anti-IgM $F(ab')_2$ were used as activated controls. Histograms are representative of n=3 mice for each group.

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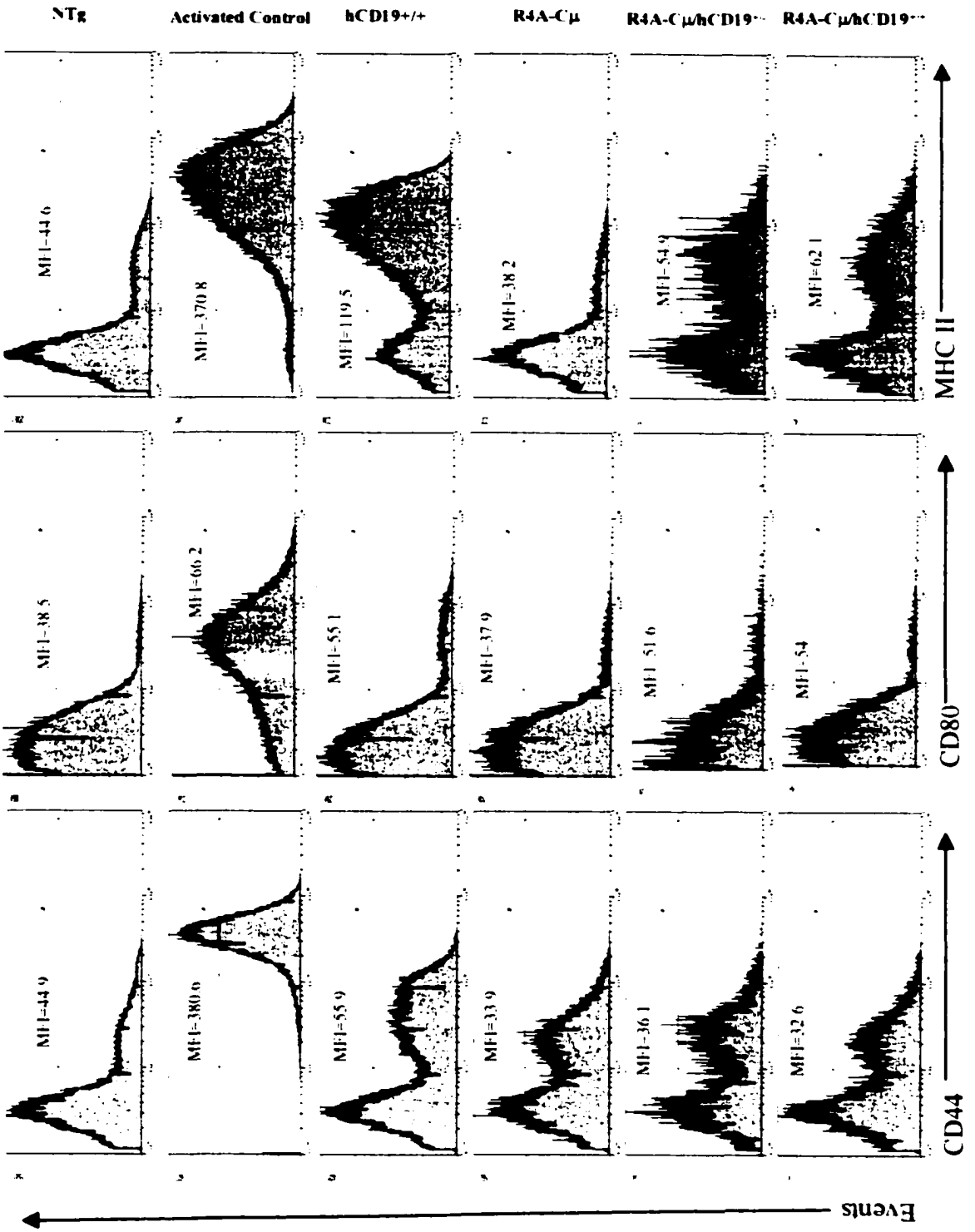

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Figure 32. *In vitro* activation of IgM^a dsDNA binding B-cells in R4A-Cμ mice overexpression CD19. Splenic B-cells were obtained from R4AhCD19$^{+/+}$ (▨) and R4A-Cμ (■) transgenic mice by positive selection using anti-CD19 magnetic beads. **(A)** $2x10^6$ cells/ml from 2 month old mice were activated *in vitro* for 6 days in media alone or with 20μg/ml of LPS, 10 μg/ml anti-CD40, 10 μg/ml anti-IgM F(ab')2 or anti-CD40 plus anti-IgM F(ab')2. Supernatants were tested by ELISA for IgM^a anti-dsDNA antibody secretion. **(B-C)** $2x10^6$ cells/ml from 3 mth old mice were activated *in vitro* for 48 hrs as described above. IgM^a anti-dsDNA B-cells (B) and total IgM^a B-cells (C) were detected by ELISPOT on dsDNA and IgM coated microtiter plates respectively. Results are representative of n=3 experiments.

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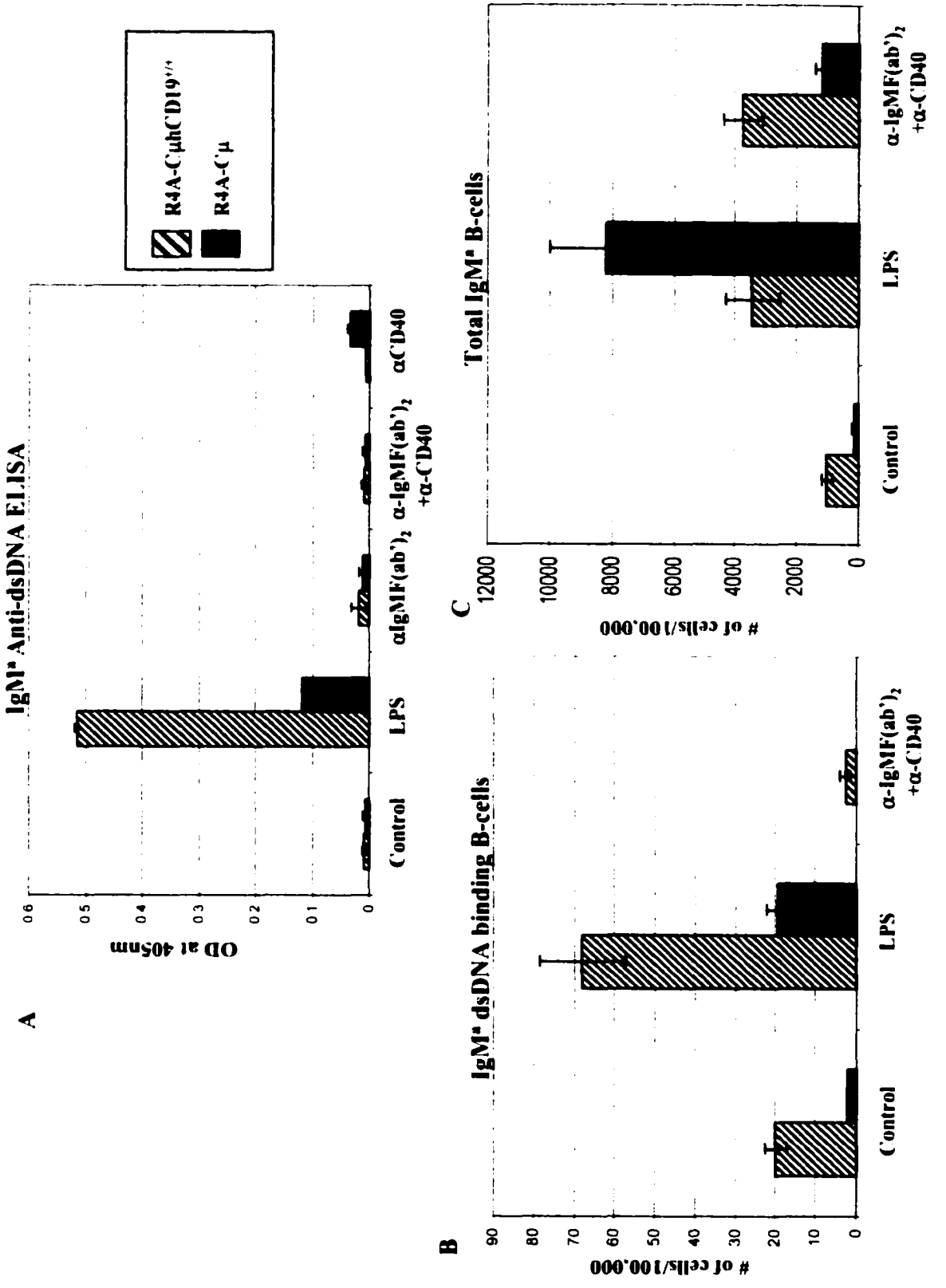

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Figure 33. Splenic distribution of R4A-Cμ B-cells. Purified splenic B-cells were triple stained with anti-CD21, anti-CD23 and either anti-IgM^a or anti-IgM^b and analyzed by flow cytometry. Gates were set on $B220^+IgM^{a+}$ for Tg B-cells or $B220^+IgM^{b+}$ for NTg B-cells and displayed as dot plots. MZ B-cells ($CD21^{hi}CD23^{lo/-}$), FO B-cells ($CD21^{lo/inter}$ $CD23^+$). Result is representative of n=3 mice of each genotype.

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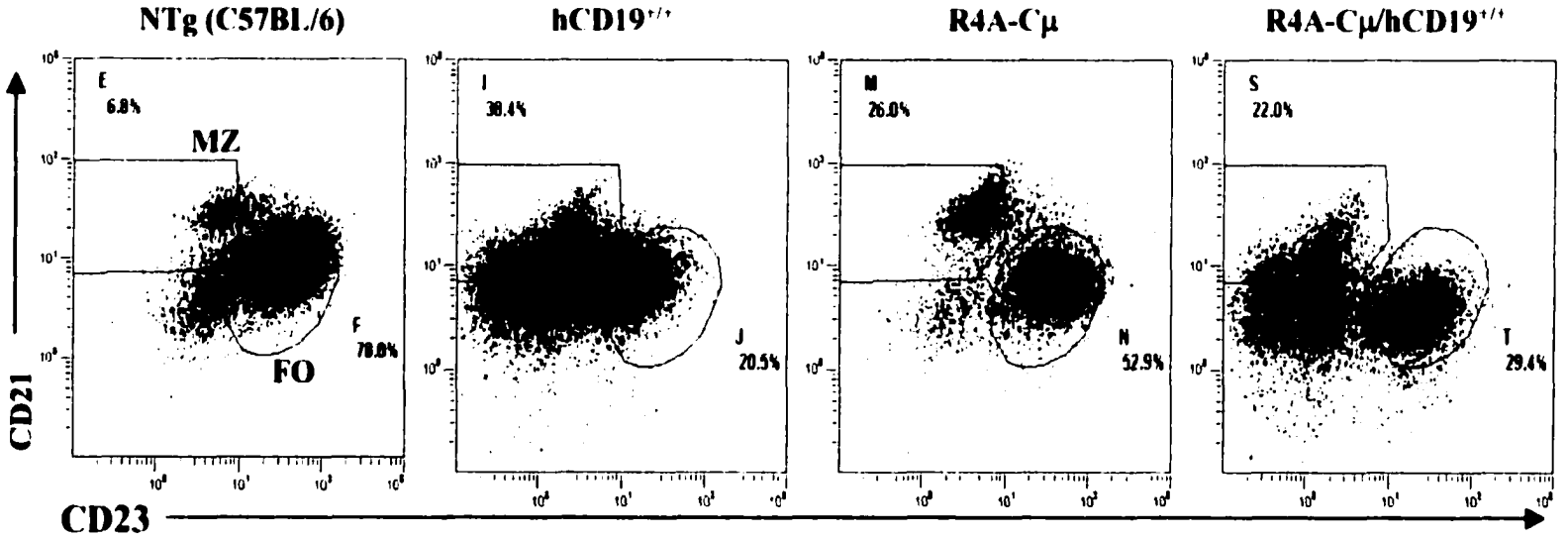

Table 11. Percentage of Marginal zone and Follicular B-cell subsets

	NTg (C57BL/6)	hCD19$^{+/+}$	R4A-Cμ	R4A-Cμ/hCD19$^{+/+}$
MZ	6.6±0.3	34.3 ±5.9	29.9 ±5.5	25.6 ±5.0
FO	78.4 ±0.6	23.2 ±3.7	45.2 ±10.9	24.8 ±6.5
Ratio of MZ:FO	0.08 ±0.004	1.5 ±0.500	0.70 ±0.300	1.1 ±0.500

Results represent the mean ± SD from n=4 mice in each group

Figure 34. IgM^a anti-dsDNA B-cells are derived from the marginal zone. **(A)** Purified B-cells from R4A-Cμ/hCD19$^{+/+}$ (▨) and R4A-Cμ (■) mice were immunostained with anti-CD21 and anti-CD23 and sorted for MZ (CD21^{hi}CD23$^{lo/-}$) and FO (CD21$^{lo/inter}$ CD23^{+}) B-cells respectively using a FACSVantage cell sorter. **(B)** ELISPOT was performed using sorted subsets from R4A-Cμ or R4A-Cμ/hCD19$^{+/+}$ on dsDNA coated plates. Result is representative of n=3 mice of each genotype.

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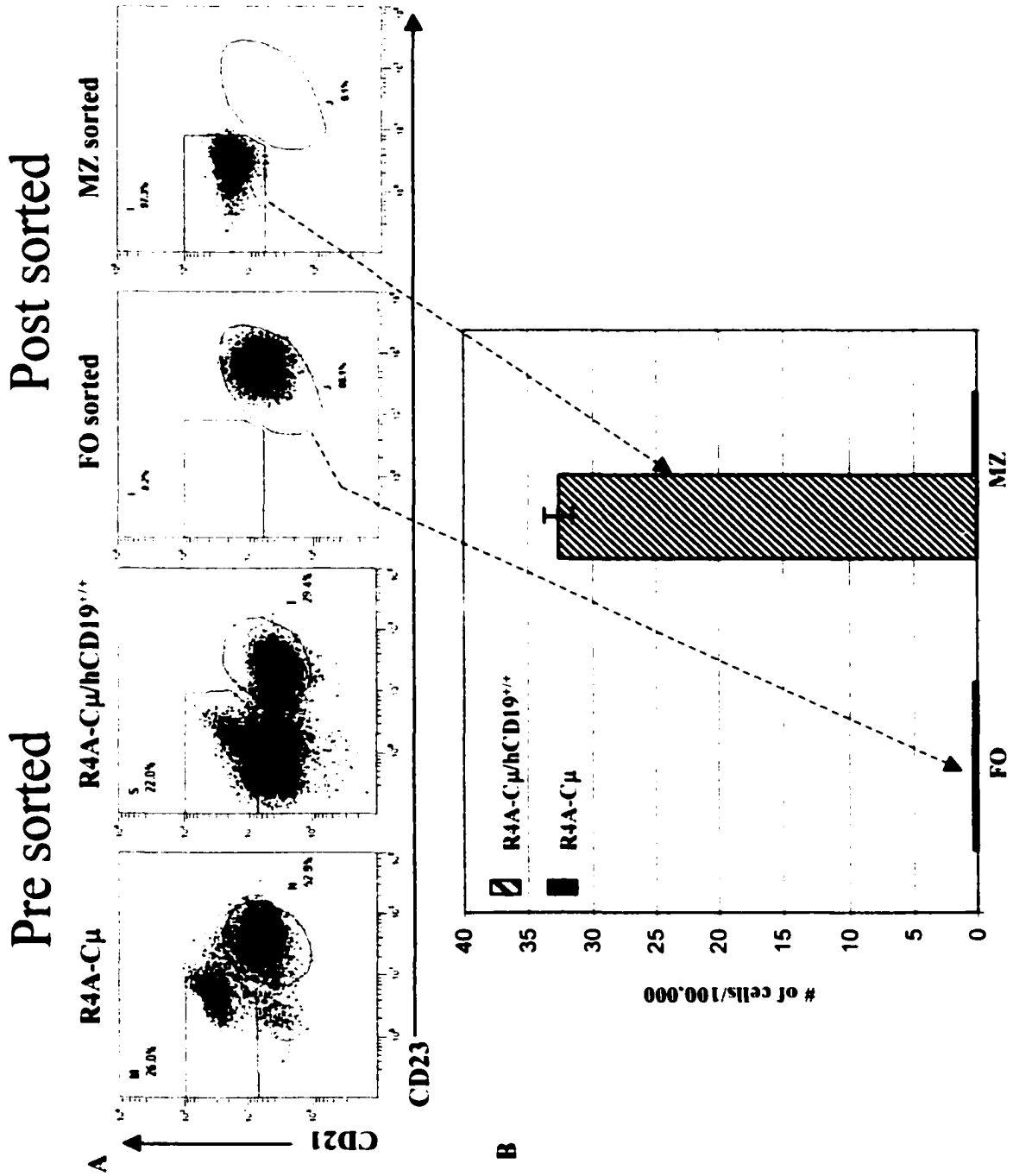

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Figure 35. Frequency of $CD4^+$ and $CD4^+CD25^+$ (T_{reg}) T-cells in R4A-Cμ mice overexpressing CD19. (A). Splenocytes from NTg, $hCD19^{+/+}$, R4A-Cμ/$hCD19^{+/-}$ and R4A-Cμ/$hCD19^{+/+}$ were doubled stained with PE-anti-CD8 and FITC- anti-CD4 and T-cell subsets analyzed by flow cytometry. Ratios of CD4/CD8 T-cells are indicated. **(B).** Above splenocytes were double stained with PE-anti-CD4 and FITC-anti-CD25 to measure the frequency of $CD4^+CD25^+$, T_{reg} cells. This experiment is representative on n=3 mice in each group.

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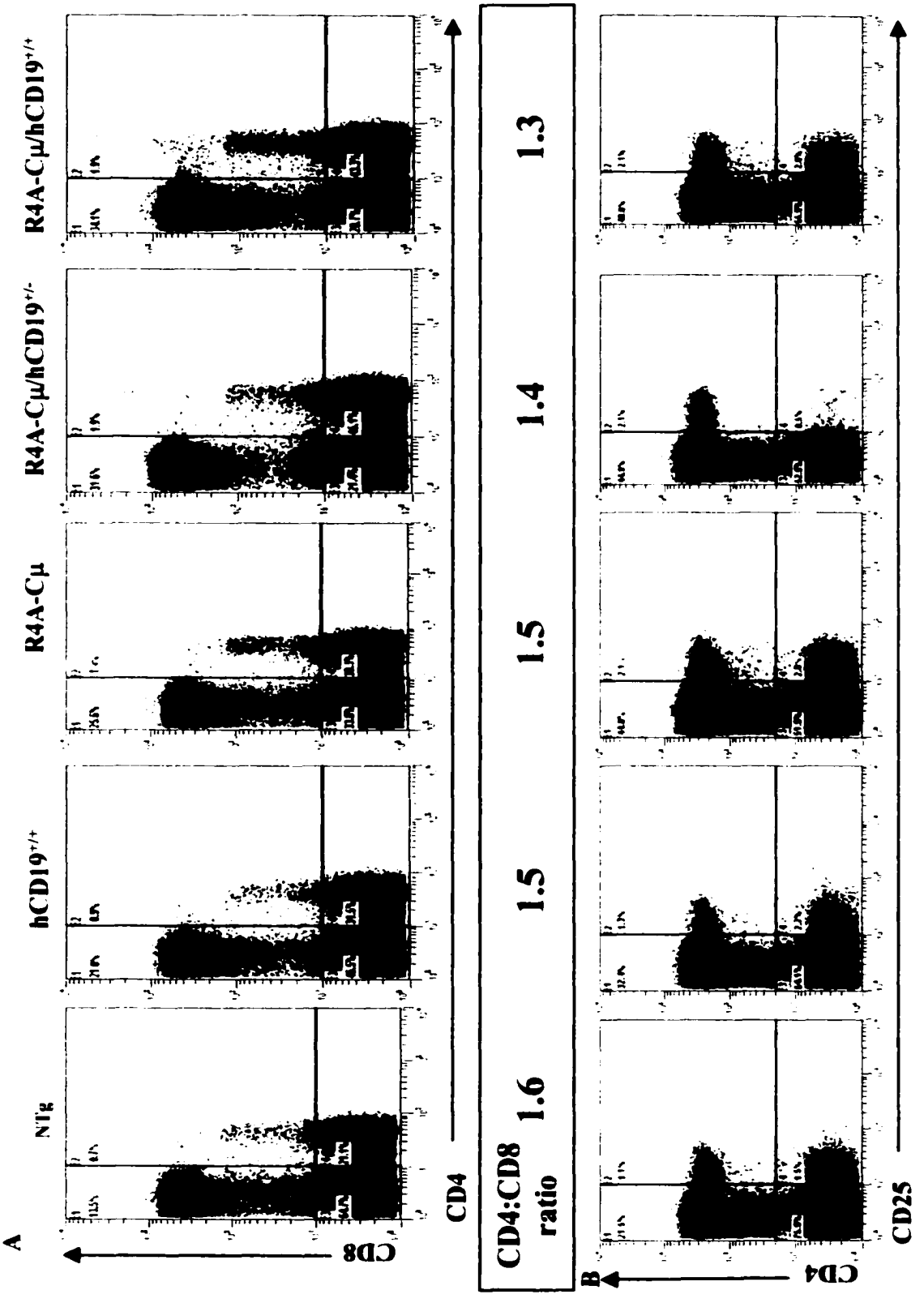

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Figure 36. Depletion of CD4$^+$ T-cells fails to abrogate anti-dsDNA antibody secretion. R4A-Cμ/hCD19$^{+/+}$ transgenic mice received injections of 2 mg of anti-CD4 antibody (GK1.5) (●) or sterile PBS (■) for two consecutive days bi-weekly for 12 weeks. Uninjected (Δ) mice were used as controls. **(A)** Flow cytometry was performed on peripheral blood to assess CD4 T-cell depletion. Cells were dual stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8. This experiment is representative of n=6 mice in each group. **(B)** Serum were collected every two weeks via retro-orbital puncture and serum titer from R4A-Cμ/hCD19$^{+/+}$ mice evaluated by ELISA for IgM^a anti-dsDNA antibody. Vertical arrows indicate injection points.

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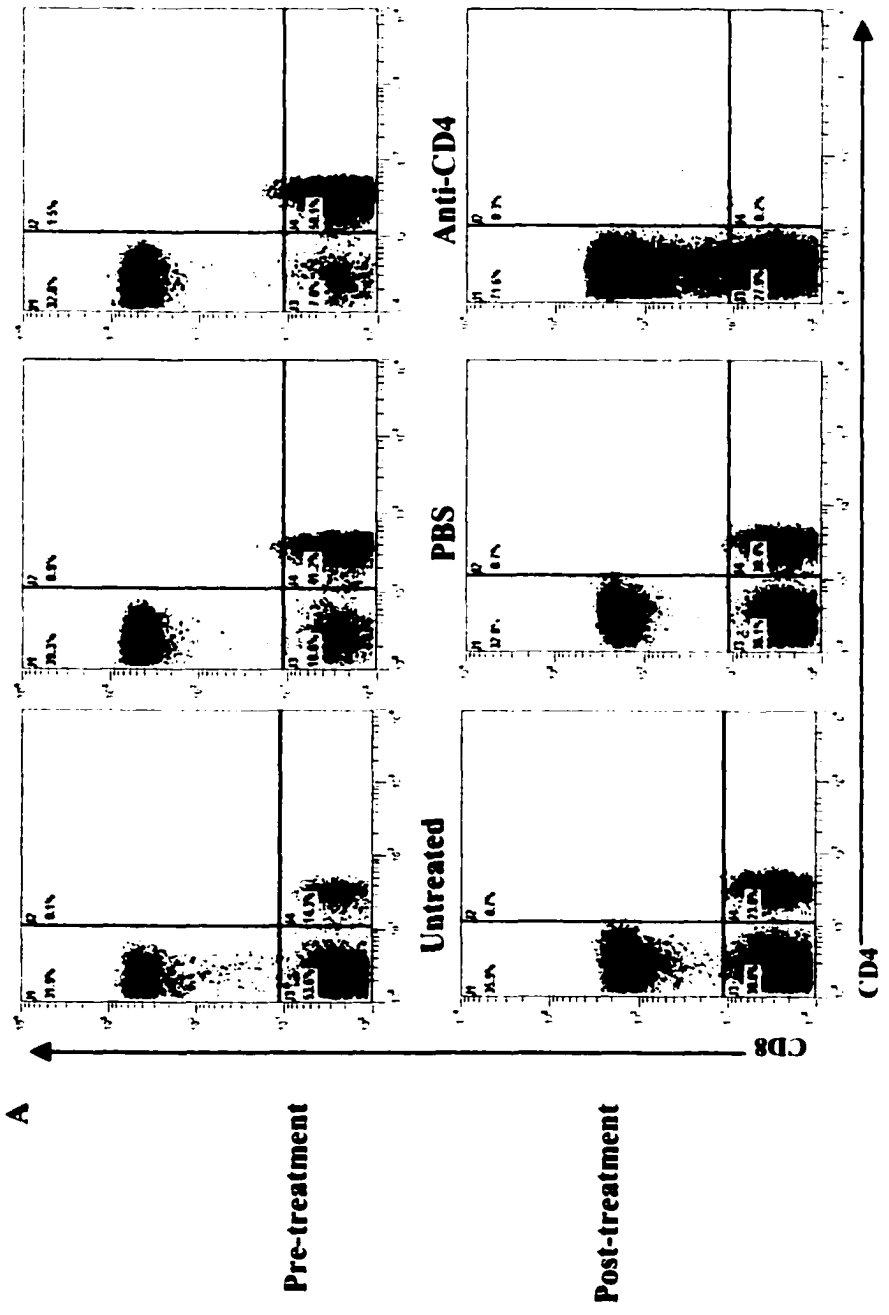

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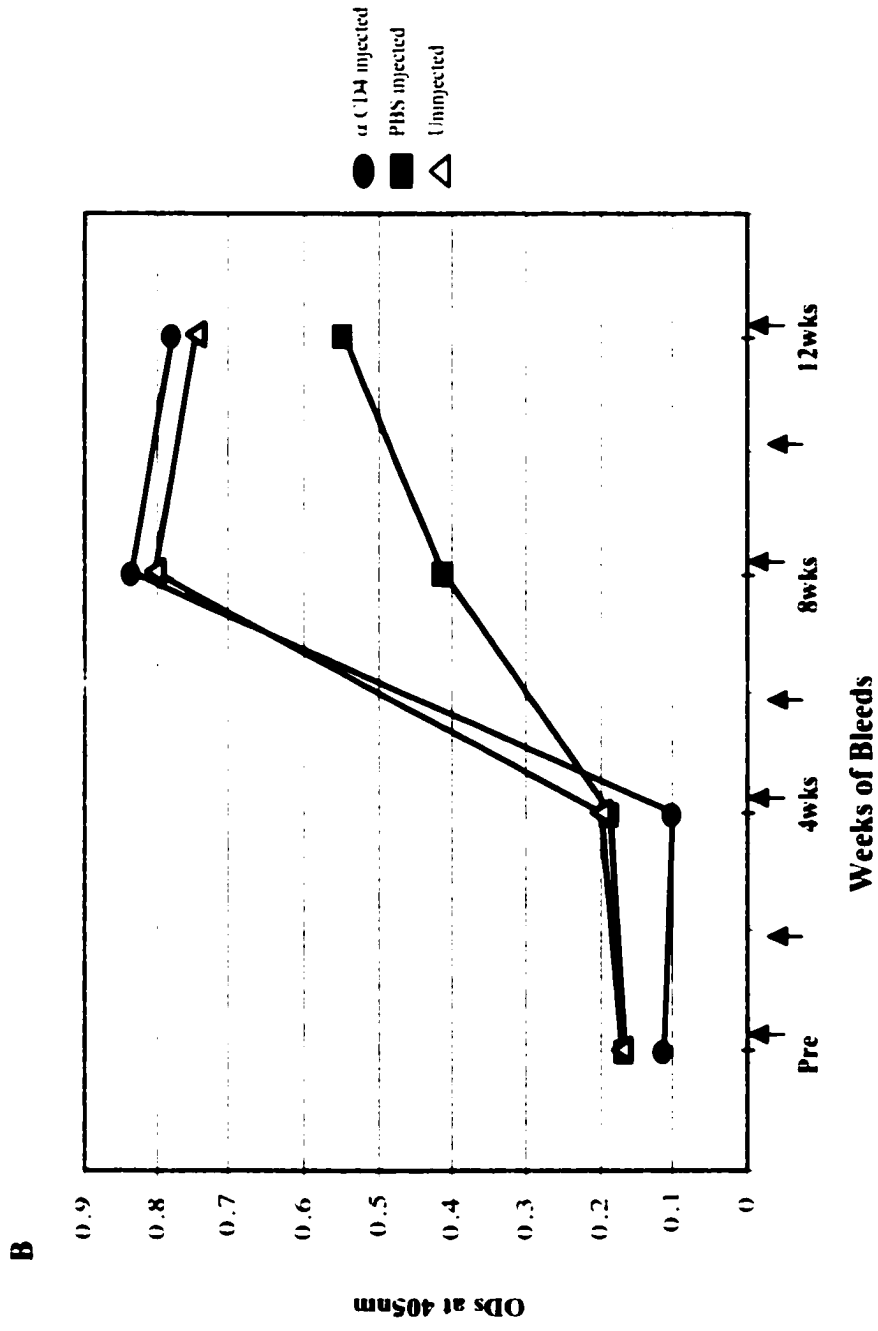

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Figure 37. Depletion of peritoneal B-cells by hypotonic lysis does not abrogate IgM^a anti-dsDNA antibody secretion. R4A-Cμ/R4AhCD19$^{+/+}$ mice received weekly i.p. injections of sterile water (●) or PBS (□) for 7 weeks. Serum was collected before each injection, via retro-orbital puncture and serum titer of IgM^a anti-dsDNA antibody was determined by ELISA. Results are representative of n=4 mice in each group. Vertical arrows indicate weekly injection points.

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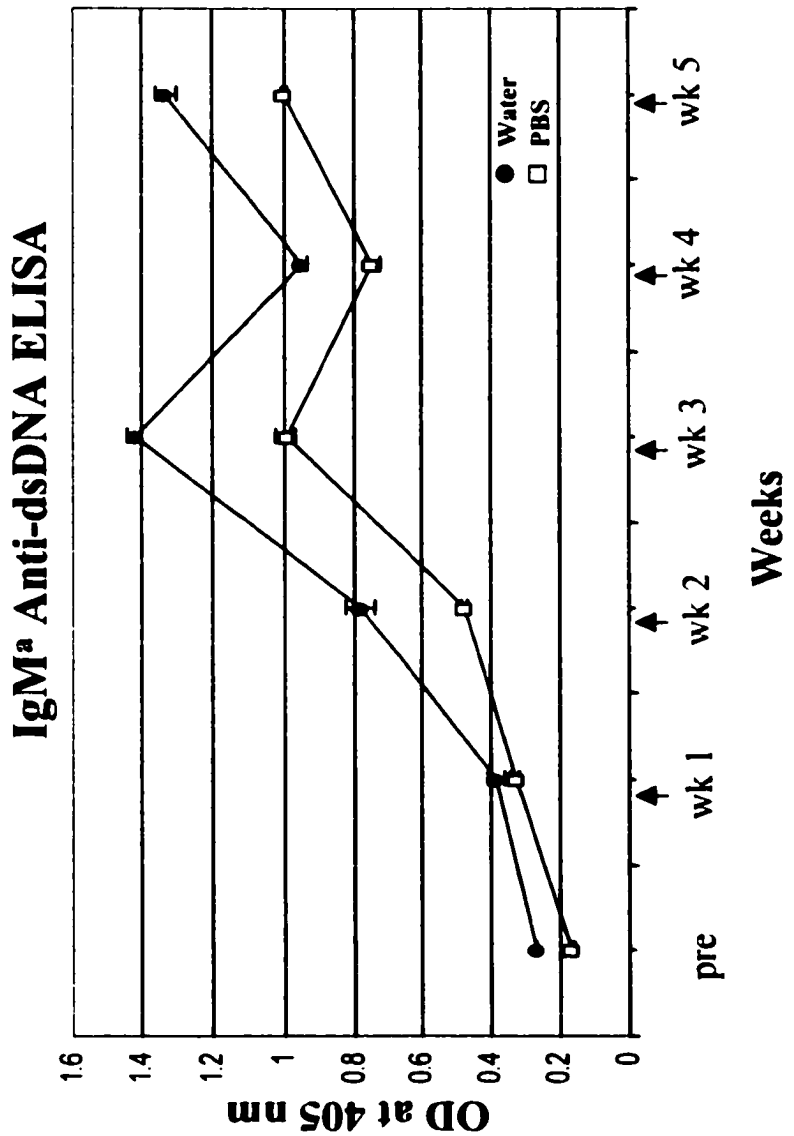

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Figure 38. CpG triggers activation of dsDNA binding B-cells overexpressing hCD19. Purified B-cells were obtained from R4A-Cμ/hCD19$^{+/+}$ (▨) and R4A-Cμ (■) mice. **(A)** 2x10^5 cells/100μl cells from 2-3 month old mice were activated *in vitro* for 48 hours in media alone or with 10μg/ml of CpG stimulatory motif, 20 μg/ml LPS, 12 μg/ml of an inhibitory CpG motif or a cocktail of stimulatory and inhibitory CpG sequences. Cells were pulsed with 1μci/well of ^{3}H for 16 hrs and proliferation quantitated. **(B-C).** 2x10^6 cells/ml from 3 mth old mice were activated *in vitro* for 48 hrs as described above. IgM^a anti-dsDNA and total IgM^a B-cells were detected by ELISPOT.

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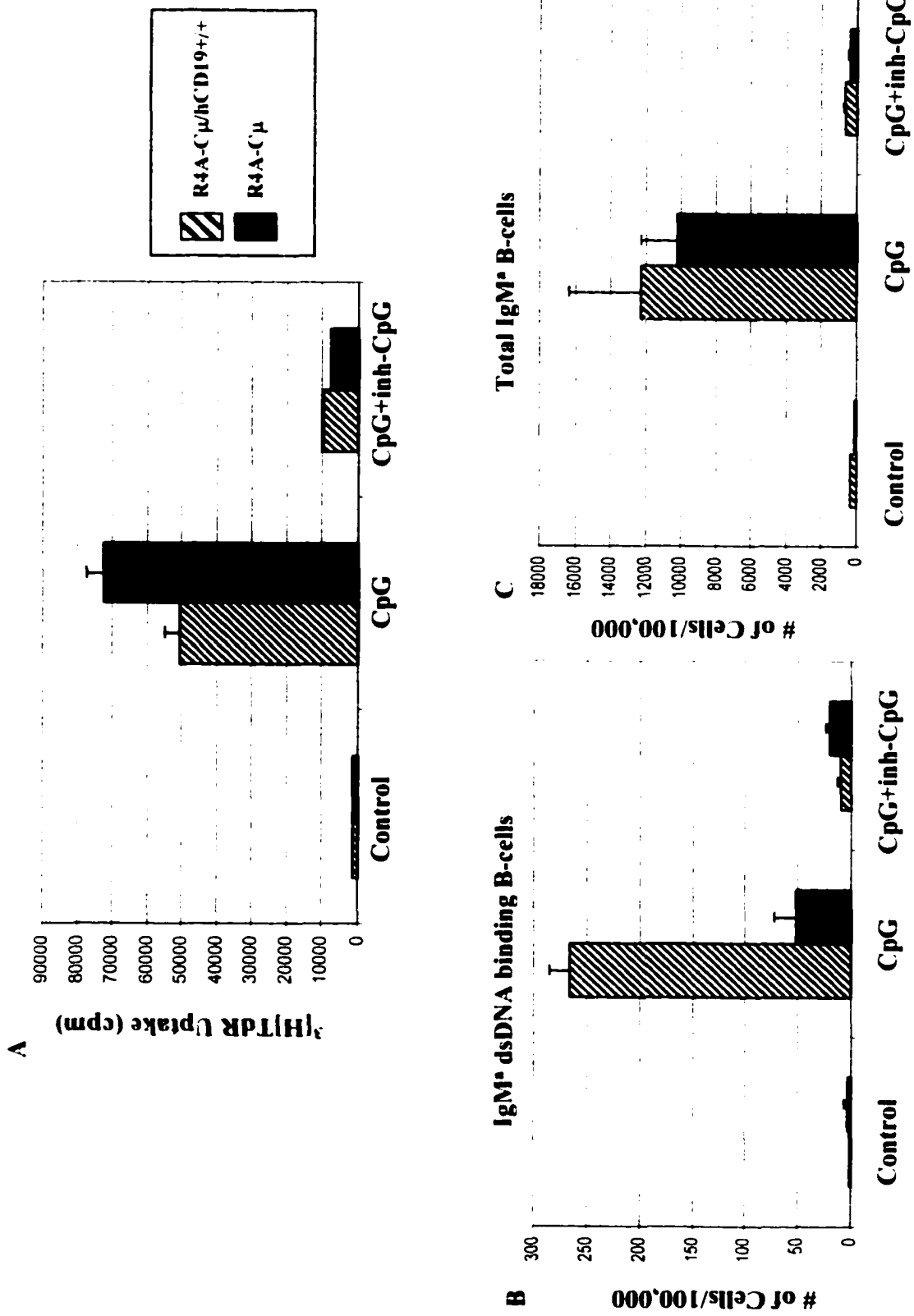

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CHAPTER V: Chapter III and IV Summary

Understanding the development and progression of autoimmune diseases still remain a complex task as ongoing research reveals that the onset is often due to multiple factors. Transgenic mouse models carrying rearranged immunoglobulin receptor genes with autoreactive specificities have provided a valuable system in which to study tolerance induction. These transgenic models have an increased frequency of autoreactive B-cells which makes it easier to track the fate of these cells. Using transgenic mouse models three dominant mechanisms of tolerance have emerged; anergy, deletion and receptor editing. A breakdown in any one of these mechanisms can lead to an autoreactive disease such as SLE.

Using nonautoimmune mice transgenic for the R4A-Cμ heavy chain we have demonstrated that tolerance is maintained in part by anergy and deletion. We have observed that dsDNA binding B-cells persist in the periphery of these mice and are targeted to anergy. They display the phenotype characteristic of anergic cells which includes Ig receptor down modulation, reduced lifespan and developmental arrest. BCR crosslinking *in vitro* fails to activate or elicit the production of transgenic anti-dsDNA antibody yet treatment with LPS or surrogate T-cell factors such as anti-CD40 plus IL4 or a cocktail of IL4, 5 and 6 results in differentiation into antibody secreting cells. These results suggest that anergy is maintained in these B-cells by blocking early events specific for BCR engagement while not affecting more downstream signaling events that are common to and converge with other signaling pathways (i.e. CD40L and/or LPS signaling). Hence the block in signaling in these mice uncouples BCR signaling from other signaling pathways. Blocking BCR signaling while preserving other signaling

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pathways may represent a mechanism by which anergic anti-dsDNA B-cells maintain non-responsiveness to autoantigen while being available for recruitment in inflammatory episodes.

We also compared tolerance induction in R4A-Cμ and R4A-Cγ2b mice and observed that γ2b dsDNA binding B-cells are more stringently regulated than IgM B-cells. R4A-Cμ but not R4A-Cγ2b anergic B-cells could be activated with anti-CD40 plus IL4 to differentiate into antibody secreting cells. In addition anergic IgM but not IgG dsDNA binding B-cells could be rescued as hybridomas from unstimulated splenocytes. These observations suggest that there are qualitative differences in anergy between IgM and IgG dsDNA B-cells. We propose that heavy chain isotypes may influence the level of anergy induction and γ heavy chains may transduce qualitatively different tolerizing signals than μ heavy chains.

To investigate how signaling thresholds affect the induction of self tolerance, we bred nonautoimmune C57BL/6 mice transgenic for the R4A-IgM^a heavy chain (R4A-Cμ) with mice overexpressing hCD19. We observed that tolerance was broken as a result of CD19 overexpression. However, CD19 overexpression did not reverse the anergic phenotype as R4A-Cμ/hCD19$^{+/+}$ B-cells were still developmentally arrested at the immature stage of development, expressed reduced receptor density and had a shortened lifespan. Furthermore these B-cells failed to be activated by BCR crosslinking suggesting that they are still anergic. We also observed that conventional B-2 B-cells are most likely to be responsible for the secretion of transgenic autoantibody in R4A-Cμ/hCD19$^{+/+}$ mice, not CD5$^+$ B1 B-cells. However, proof of this will require sorting the B-2 and B-1 subsets from R4A-Cμ/hCD19$^{+/+}$ mice, putting these B-cells in culture and

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determining which subset contains B-cells that can be activated by LPS or CpG to secrete anti-dsDNA antibody.

We demonstrated that B-cells secreting anti-dsDNA antibodies from R4A-Cμ/hCD19$^{+/+}$ mice do not require T-cell help and reside in the marginal zone. The marginal zone is emerging as an important splenic compartment where many T-independent B-2 cells with autoreactive specificities can be found. Finally we demonstrated that R4A-Cμ dsDNA binding B-cells in mice overexpressing CD19 could be activated *in vitro* to secrete antibody following LPS stimulation or stimulation by CpG sequences. Anergic B-cells from these mice are more responsive to non-BCR mechanisms of signaling than anergic B-cells from R4A-Cμ mice.

CD19 lowers the threshold of activation for both BCR and BCR independent mechanisms of signaling. Since anergic B-cells have low levels of mIg receptors, CD19 overexpression may not be sufficient to activate them through BCR associated mechanisms. However, in anergic B-cells overexpressing CD19, signals delivered through Toll receptors by LPS or CpG may synergize with intrinsic CD19 signaling and this may be enough to induce activation. Hence altered expression of molecules involved in B-cell signaling may predispose autoimmune B-cells to escape tolerance but signaling via innate immune mechanisms may be required to trigger autoimmunity.

Understanding how overexpression of CD19 can alter signaling threshold and lead to a breakdown in tolerance will aid in the design of therapeutic strategies that can prevent B-cell autoimmunity.

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