A UNIQUE MEMORY B CELL SUBSET CORRELATES WITH ADVERSE OUTOMES IN HUMAN SLE

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ABSTRACT

MATILDA WRAY NICHOLAS: A Unique Memory B Cell Subset Correlates with Adverse Outcomes in Human SLE
(Under the direction of Stephen H. Clarke)

Systemic lupus erythematosus (SLE) is a devastating systemic autoimmune disease marked by the production of antinuclear autoantibodies whose etiology has both genetic and environmental components. We and others have shown that CD19, a positive regulator of B cell receptor (BCR) signaling, is ~20% decreased on peripheral blood (PB) naïve B cells in >95% of SLE patients (Pts). We have also identified an expanded subpopulation of IgG+ memory B cells in 25-35% of SLE Pts that display a 2-4 fold increase in CD19 expression ($CD19^{hi}$). SLE Pts with an expanded $CD19^{hi}$ population ($CD19^{hi}$ Pts) have a unique pattern of autoantibody production and increased adverse clinical outcomes, particularly end stage renal disease and neurological complications. $CD19^{hi}$ B cells have an activated phenotype, and sequencing analysis shows they are somatically hypermutated and antigen selected. Our data indicate they are most likely in G_1 phase of the cell cycle and are in the early stages of differentiation to plasma cells.

$CD19^{hi}$ cells also have a ~3 fold increase in basal levels of phosphorylated Syk (pSyk) and ERK1/2 (pERK1/2), suggesting that they have been recently activated. Although $CD19^{hi}$ cells are refractory to further increases in pSyk or pERK1/2, they phosphorylate other intermediates similarly to healthy control B cells in response to BCR stimulation.

CXCR3 expression is >14-fold elevated in $CD19^{hi}$ cells, and they chemotax effectively towards a CXCR3 ligand, suggesting they are homing to sites of inflammation. Importantly, $CD19^{hi}$ B cells are enriched for autoreactivity compared to $CD19^{lo}$ B cells from the same patient, and a 2-fold increase in this enrichment is associated with a 100-fold increase in the serum autoantibody titer, suggesting these cells are precursors to autoantibody producing plasma cells. Finally, $CD19^{hi}$ Pts are short-term or non-responders to rituximab treatment, indicating a need for a new therapy modality for these Pts.

Taken together, these results suggest that dysregulation of CD19 on B cells plays a role in the etiology and pathogenesis of SLE, and that $CD19^{hi}$ cells represent an autoreactive memory B cell subset that plays an important role in the pathology of this disease.

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LIST OF ABBREVIATIONS AND SYMBOLS

Ab	Antibody
Abs	Antibodies
Ag	Antigen
AID	Activation induced cytidine deaminase
ANA	Anti-nuclear antibody
ANCA	anti-neutrophil cytoplasmic antibody
APC	Antigen presenting cell
ASC	Antibody secreting cell
BAFF	B cell activating factor
BAFF	B cell activating factor of the TNF family
BCR	B cell receptor
Blimp-1	B lymphocyte induced maturation protein 1
BLyS	B lymphocyte stimulator
BM	Bone marrow
BSAP	B cell specific activating protein
Ca^{2+}	Calcium divalent cation
$CD19^{hi}$ cells	Memory B cells with increased CD19 expression
$CD19^{hi}$ Pts	Pts with an expanded $CD19^{hi}$ cell population in the PB
$CD19^{lo}$ cells	All non-$CD19^{hi}$ B cells (CD19+) in the PB
$CD19^{lo}$ Pts	Pts without an expanded $CD19^{hi}$ population
CDR3	Complementary determining region 3
CpG	Cytosine-phosphate-guanine motif
CR	Complement receptor
CSR	Class switch recombination
CVID	Common variable immunodeficiency
DC	Dendritic cell
dsDNA	Double-stranded DNA
ERK	Extracellular signal-regulated kinase

ESRD	End stage renal disease
Fc	Fragment crystallizable (constant) region of an antibody
FcR	Fc receptor
FDC	Follicular dendritic cell
Fo	Follicular
GC	Germinal center
H	Heavy
HC	Healthy control
IC	Immune complex
ICOS	Inducable co-stimuator
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
IV	Intravenous
JNK	c-Jun NH_2-terminal kinase
L	Light
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MAPK	Mitogen activated protein kinase
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MPO	Myeloperoxidase
MZ	Marginal zone
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor κB
NSAIDs	Non-steroidal anti-inflammatory drugs
p	Phosphorylated
PALS	Periarterial lymphoid sheath
PAMP	Pattern associated recognition motif
PB	Peripheral blood

PC	Plasma cell
PI3K	Phosphotidylinositol 3 kinase
PKC	Protein kinase C
PLC	Phospholipase C
PMN	Polymorphonuclear leukocyte
PR3	Proteinase-3
Pts	Patients
RAG	Recombination activating genes
RNP	Ribonucleoprotein
sCD21	Soluble CD21
SCR	Short consensus repeats
SHIP	Src homology-2 domain-containing inositol polyphosphate 5'-phosphatase
SHM	Somatic hypermutation
SHP-1	SH protein-tyrosine phosphatase 1
sIg	Surface immunoglobulin
SLE	Systemic lupus erythematosis
SLEDAI	SLE disease activity index
Sm	Smith antigen
snRNP	Small nuclear ribonuclear protein
ssRNA	Single stranded RNA
SVV	Small vessel vasculitis
Syk	Spleen tyrosine (Y) kinase
T regs	Regulatory T cells
T1	Transitional 1
T2	Transitional 2
T3	Transitional 3
TCR	T cell receptor
TD	T-dependent
Th	T helper
TI	T-independent
TIA	Transient ischemic attack

TLR	Toll-like receptor
TNF	Tumor necrosis factor
TTP	Thrombotic thrombocytopenic purpura
UDG	Urisil DNA glycolase
V	Variable
XBP-1	X-box protein 1
α	alpha/anti-
β	beta
ε	epsilon
γ	gamma
κ	kappa

CHAPTER 1: BACKGROUND AND INTRODUCTION

I. B CELLS AND THE IMMUNE SYSTEM

The immune system is traditionally divided into two large but overlapping arms: the innate immune system and the adaptive immune system. Whereas the innate immune system is primordial, it is none the less effective against many pathogens and is the first line of defense against invading organisms. However, it is only able to detect unchanging components common to pathogens, but rare in the host, and does not have the ability to refine its actions against a specific pathogen nor generate memory against it. The adaptive immune system, on the other hand, generates exquisite and flexible specificity, allowing it to target changing aspects of pathogens. It is also able to generate long term memory, protecting the host against future infections by the same organism. It is, however, slower to respond than the innate immune system, and, with its nearly limitless ability to adapt to any invader, also carries the risk of misdirected attack against self, or autoimmunity.

It is only recently that the level to which these two aspects of the immune system influence each other has been appreciated. We have known for some time that features of the adaptive system modify and guide the innate system, such as antibodies enhancing the phagocytosis of pathogens by opsinization, or activating complement in immune complexes (ICs). We are also beginning to understand how the innate system strongly influences the adaptive system. Particular complement components, for example, are necessary for an efficient adaptive response against certain antigens(1-4), and toll-like receptor (TLR) ligands also control and direct the adaptive response(5-15).

Herein, I will focus on the role of B cells in the normal immune response and in autoimmunity. Although antibody (Ab) production is a central role of B cells, they play

many other pivotal roles in the immune response. These roles of B cells in health and disease will be outlined in more detail below.

A. B Cells and the Innate Immune System

Traditionally, the innate immune system consists of specific cells types and soluble factors. Cell types such as epithelial cells, macrophages, polymorphonuclear leukocytes (PMNs, or neutrophils), and others are able to participate in the innate immune response. Epithelial cells can provide early warning of invading pathogens by release of chemokines, small molecules that direct macrophages, PMNs, and lymphocytes to the site of infection. These cells in turn are able to phagocytose a wide variety of pathogens and produce toxic products which indiscriminately kill pathogens. They are also able to secrete cytokines—small molecules which have specific, receptor-mediated effects on cells of the adaptive and innate immune systems.

In addition, soluble proteins, particularly complement, are key mediators of innate immunity and are able to mediate actions on their own (such as directly inducing lysis of invaders or opsinization) or to enhance the actions of other aspects of the immune system (such as modifying responses of cell types through complement receptors).

There is not a clear delineation between innate and acquired immunity, however. Innate immunity is needed for an efficient acquired response, and aspects of the acquired immune system have innate-like features. Particularly, some subsets of B cells produce what is known as "natural antibody" which acts as a first line of defense against many pathogens, and B and T cells can respond to TLR ligands.

1. Toll-like Receptors

Interestingly, many epithelial and immune cells are able to recognize pathogens through the interaction of common pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), and pattern recognition receptors (PRRs), such as toll-like receptors (TLRs)(16, 17). The TLR family contains 10 members, and this group appears to represent the first evolved type of immune defense. TLRs are highly conserved through many different species from plants to vertebrates, and recognize conserved lipid, carbohydrate, nucleic-acid and peptide structures expressed by many pathogens(18, 19). Many of the subtypes share the common MyD88 signaling pathway and ultimately exert their actions through the activation of ubiquitous transcription factor NF-κB(16, 18, 20). The recognition patterns and expression location of key TLRs is shown in Table 1.1(21-23). Like complement components, these ligand-receptor interactions can influence the acquired immune system(7, 11, 13-15, 24). TLRs, though useful in early defense and immune regulation, can also become problematic in that many also bind similar antigens derived from the host (Table 1.1), leading to a break in immunological tolerance(5, 9, 12, 22, 25).

Table 1.1. Properties of Toll-like receptors.

Receptor	Location	Ligand	Exogenous Source	Endogenous Source
TLR1	Cell Surface	Lipoproteins	Bacteria	n/a
TLR2	Cell Surface	Peptidoglycan et al.	Gram-positive Bacteria	necrotic cells
TLR3	Intracellular	dsRNA	Viruses	necrotic cells
TLR4	Cell Surface	LPS	Gram-negative Bacteria	n/a
TLR7/8	Intracellular	ssRNA	Viruses	dead/dying cells, snRNPs
TLR9	Intracellular	CpG-containing DNA	Bacteria and Viruses	dead/dying cells

a. B cells and TLRs

B cells are among the many cells in the body expressing TLRs and responding to stimulation by TLR ligands. The effects of TLR stimulation upon B cells are typically highly stimulatory, although they depend upon B cell differentiative state, presence of concurrent signaling through other receptors, and the specific ligand and receptor involved(21, 22).

In human B cells, somewhat variable TLRs expression has been reported(26-30). Most report very low expression of LPS binding TLR4 on these cells(28, 29), however, an alternate LPS receptor, RP105, may substitute in this case(31). By evaluating mRNA transcripts, TLRs1, 7, 9, and 10 are expressed in both peripheral blood (PB) and tonsillar B cells(28, 29). In tonsillar B cells, little variation in expression between naïve, germinal center (GC), and memory B cells was found in one study(28), but another showed constitutive expression only in memory B cells(26, 27). Another showed that human naïve, PB B cells respond robustly to ligands for TLR1/2, 7, and 9, suggesting presence and functional significance of these TLRs on these cells, although indirect effects via stimulation of dendritic cells (DC) cannot be ruled out in this study(6). In addition, human memory B cells can be induced to secrete Ab upon stimulation with the TLR9 ligand CpG(32). A summary of current knowledge of TLR expression in human B cells can be found in Table 1.2(30).

Much more is known of the function of TLRs in murine B cells, although differences between these B cells and human B cells are already known. Unlike human B cells, murine B cells express TLR4(10), and stimulation of these cells with LPS results in significant proliferation. Of particular import is the recent finding that concurrent stimulation of a B cell through its BCR and a TLR, particularly TLR7 and TLR9, can induce B cell activation and

differentiation to an antibody secreting cell (ASC), and can break tolerance in autoreactive B cells(5, 9, 12, 22, 25). This may be particularly important for B cells which recognize nuclear components, since these antigens are often associated with various forms of DNA or RNA that can stimulate TLRs.

Table 1.2. Expression of TLRs on human B cells.

Receptor	Naïve/Resting	Activated
TLR1	+	++
TLR2-5	+/-	+/-
TLR6-7	+	+++
TLR8	+	++
TLR9-10	+	++++

+++, strong expression; ++, moderate expression; +, low but detectable expression; +/- low or functionally controversial expression

2. The Complement Cascade and Complement Receptors

Another integral component of the innate immunity is the complement system. The complement system is composed of numerous small plasma proteins which have the ability to bind other proteins and molecules with certain properties, such as mannose or polyanionic surface structures of bacteria or apoptotic cells(1, 17, 33, 34). These proteins are proteolytically cleaved in complex cascades of activation, the details of which are beyond the scope of this dissertation. Briefly, there are three pathways for complement activation: the classical pathway, the mannose-binding lectin pathway, and the alternative pathway. The classical pathway relies on ICs formed with IgG or IgM (IgM is particularly efficient at activating complement). C1q can also directly bind certain patterns on the surface of

pathogens and apoptotic cells. C1 binds to the invariant regions of the antibodies; bound C1 cleaves C4 and C2, forming C3 convertase. C3 convertase cleaves C3, which continues the cascade, forms soluble C3a fragments, which have immunological activity, and coats the surface of the pathogen with C3b. This induces the cleavage of C5 which initiates formation of the membrane attack complex (MAC), which can directly lyse certain pathogens (Fig. 1.1)(1, 17, 33, 34).

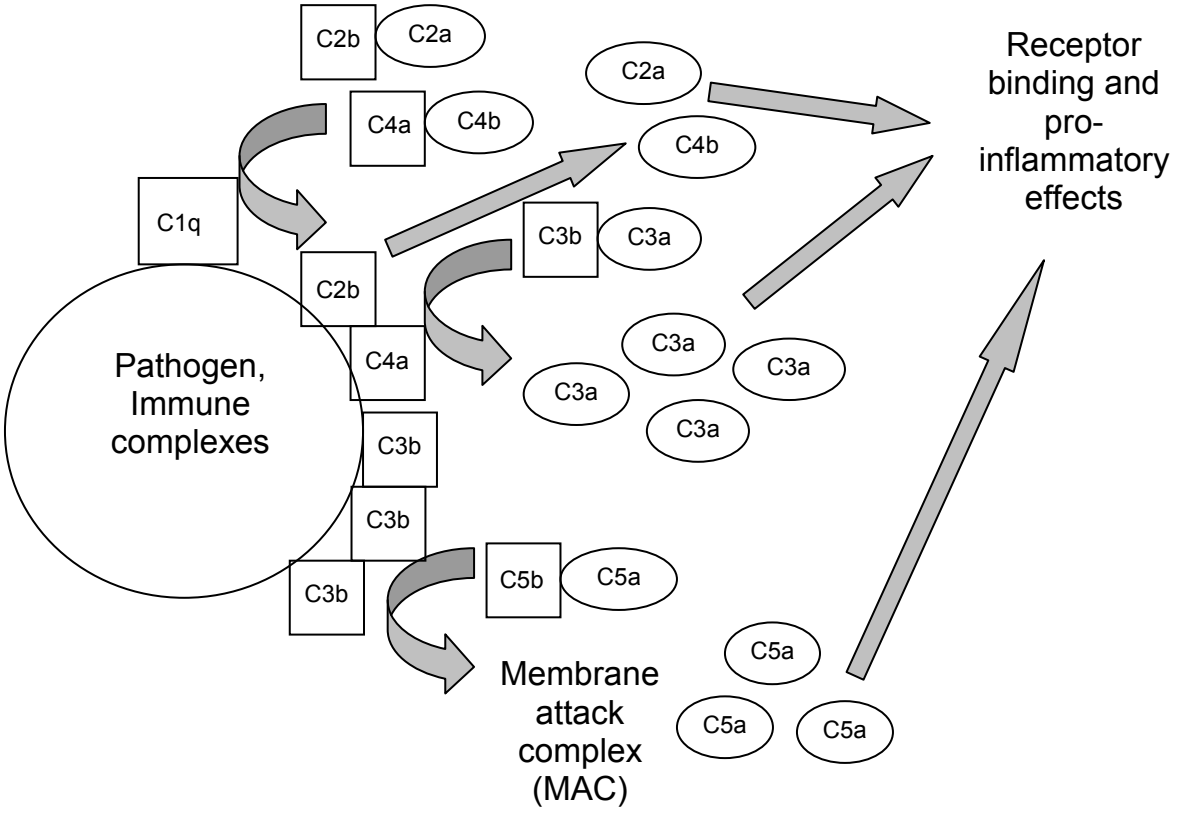


Figure 1.1. A brief overview of the classical pathway of complement.

C3a, C4b, and C5a and some of their smaller products all act as mediators of inflammation. Complement receptors (CRs) exist for many of these fragments which enhance responsiveness of the cells which express them(17, 35). Complement receptors 3

(CR3, or CD11b), and CR4 (CD11c) are expressed on macrophages and DCs, among others, and stimulate phagocytosis as well as regulating the cell response(17, 35, 36). CR2 (CD21) is a positive regulator of B cell receptor (BCR) signaling, and will be discussed in more detail in later sections. Its close relative, CR1 (CD35), has similar function and expression pattern, although it differs between mouse and man. A brief summary of complement receptors, their ligands, and expression can be found in Table 1.3(17, 35).

Table 1.3. Complement receptors, their ligands, function, and expression.

Receptor	**Ligands**	**Functions**	**Expression**
CR1 (CD35)	C3b, C4b, iC3b	Stimulates phagocytosis, transport of ICs	Erythrocytes, macrophages, monocytes, PMNs, B cells, FDCs
CR2 (CD21)	C3d, iC3b, C3dg, EBV, et al	Part of BCR co-receptor complex	B cells and FDCs
CR3 (CD11b)	iC3b	Stimulates phagocytosis	Macrophages, monocytes, PMNs, FDCs
CR4 (CD11b)	iC3b	Stimulates phagocytosis	Macrophages, monocytes, PMNs, FDCs

3. B-1 and MZ B cells and Natural Antibody

As mentioned above, some subsets of B cells straddle the divide between the innate and acquired subparts of the immune system. These subsets are responsible for T cell independent (TI) production of "natural antibody", which is typically low-affinity, cross-reactive, and IgM. Natural antibody represents the bulk of IgM in circulation, and is a key player in protection from encapsulated bacteria, septic infections, and septic shock(37-42).

B-1 cells may branch off early from the B cell development pathway(43) and are present primarily in the peritoneal and pleural cavities. They are self-renewing and express

germline Ab genes(43-45). Interestingly, autoreactive specificities appear to be positively selected into this subset(46-48). They also differ from B-2 cells (non-B-1 cells, see below), especially Fo B cells, in their activation and differentiation properties(47, 48).

MZ B cells share some of the activation properties with B-1 B cells, but belong to the B-2 B cell subset and will be discussed in more detail below.

B. B Cells and the Adaptive Immune System

The three primary cell types of the adaptive response are T lymphocytes (T cells), B lymphocytes (B cells), and antigen presenting cells (APCs). T cells, so called because they develop and mature in the thymus, have numerous subtypes which serve diverse functions. CD4+ helper T cells (Th cells) serve to regulate other cell types, and can drive the immune response towards either an inflammatory (Type 1, or Th1) or humoral (Type 2, Th2) biased response, depending on the cytokines they produce(49). CD8+ effector T cells combat intracellular pathogens by killing infected host cells(49). Finally, T regulatory cells (or T regs) appear to serve a vital role in maintaining tolerance to self in the periphery(50). The role of Th2 CD4+ T cells in B cell activation will be discussed in slightly more detail below.

Some non-lymphatic cells, such as dendritic cells (DCs) and stromal cells, have vital roles in the regulation of B and T cells. Numerous subtypes of DCs exist, and all have pivotal roles in activation, modulation, and control of the immune response(51-53), and several subtypes directly influence B cells(51, 54, 55). Follicular dendritic cells (FDCs), actually a type of stromal cell, are present in germinal centers. These cells can influence B cell activation and fate through trapping of ICs and antigen and production of cytokines, and have a debated role in lasting B cell memory, affinity maturation and the GC response(56-

59). Similarly, T cell maturation and selection in the thymus is guided by specialized thymic stromal cells.

B cells develop in the bone marrow and serve multiple functions in the immune system. As producers of Ab, they play roles in both the adaptive and innate immune systems(60). They also act as antigen presenting cells, activating T cells(61-65), and there is accumulating evidence they may act in more subtle, regulatory fashions, such as by production of pivotal cytokines(66-69).

1. Immunoglobulins

Antibodies produced by B cells are diverse in both their recognition of antigen and their effector function. An antibody, or immunoglobulin (Ig), is a protein consisting of two identical heavy chains (H) and two identical light chains (L). Both the H and L chains contain constant and variable regions. The variable regions determine antigen specificity, while the constant regions confer structural stability. The constant region of the H chain (Fc) also determines effector function and class of Ig.

a. Ig Subtypes

There are five main classes of Ig: IgM, IgD, IgA, IgE, and IgG(70). IgM exists as a pentimer and is always the first Ig class generated in any given B cell. This class makes up the bulk of the TI and natural antibody responses, and is particularly efficient at forming ICs and activating complement. IgD is still poorly understood, appears to only exist as a surface molecule, and is not secreted. IgA exists primarily as a dimer and is secreted for protection of mucosal surfaces. IgE is rarely present in its free form but instead coats the surface of mast cells and eosinophils via Fcε receptors to protect against worms and other parasites.

IgG dominates the secondary and long-term antibody responses to T-dependent antigens. Several subclasses of IgG exist, each with its own mix of effector functions. The milieu of B cell activation determines which, if any, class switch will occur when exposed to antigen and co-stimulation.

DNA splicing prevents multiple Ig classes from being expressed in a single cell at the same time. A notable exception is the co-expression of IgM and IgD which occurs commonly and for unknown purpose. Once class switch from IgM occurs, the IgM and IgD alleles are deleted from the genomic DNA and a single Ig class is expressed for the remainder of the B cell's lifespan. This change is made via the process of class switch recombination (CSR), which relies at least in part on activate-induced cytidine deaminase (AID)(71, 72).

As stated above, the effector functions of the various Ig classes are determined by the Fc region of the H chain. Three primary mechanisms are involved: formation of multimers and/or active transport via Fc-specific transporters into otherwise inaccessible areas (for example, IgA into the mucous), activation of complement by regions in the Fc (especially by IgM and subclasses of IgG), and association with receptors specific for the Fc regions of different subclasses, or Fc receptors. Some Fc receptors are inhibitory and some are stimulatory, and have profound regulatory ability on cell types such as T and B cells, dendritic cells, macrophages, eosinophils and basophils.

b. Generation of Ig Diversity and Specificity

Diversity of antigen recognition, generated by the variable regions of the H and L chains, is generated in four ways; three during development and one after activation(73-76). The variable region of the H chain consists of three gene segments, V, D, and J, while the L

chain has only V and J gene segments. There are multiple copies of the genes for H and L chains and therefore multiple copies of each variable region which can be recombined by recombination activating genes 1 and 2 (RAG1 and 2)(77-79), leading to the first method of generating variability: combinatorial diversity(73, 76, 80). During this process, nucleotides can be randomly added or deleted at the junctions between these regions. This is called junctional diversity, and can change the reading frame of the remainder of the H or L gene and therefore generates a non-productive rearrangement in 2 of every 3 attempts(76, 80).

The third process which generates diversity in the Ig repertoire is the fact that both the H and L chain have variable regions which combine to form the binding site of the antibody. Thus, a recombined H chain could have very different specificities depending on the L chain with which it associates(76).

The fourth method for diversity generation, somatic hypermutation (SHM)(81-83), occurs in the GC and requires T cell help. SHM, dependent largely upon on AID(72, 81-83), induces extensive mutation in the variable regions of Ig, allowing creation and selection of B cells with higher specificity for a given antigen. This is accomplished in part via deamination of dC residues in the variable regions of Ig genes, followed by excision with uracil-DNA glycosylase (UDG) or replacement(72, 83). The GC reaction will be discussed in more detail below.

2. Central B Cell Development

The BCR is what allows B cells to identify and respond to foreign antigen. It consists of the same two H and L chains as the antibody produced by the B cell, along with two signaling components, Igα and Igβ. The expression of this receptor guides the development and differentiation of the B cell(17, 74, 84-86), and it will be discussed in more detail below.

B cells begin as common lymphoid progenitors in the bone marrow and differentiate into pro-B cells, which upregulate the B cell lineage surface marker B220 and activate RAG1 and 2 to begin DJ rearrangement of their H chain under the control of the transcription factors E2A, EBF, and Pax-5(87-91). Pax-5 in particular is vital for pre-B cell differentiation, absolute commitment to a B cell lineage and suppression of inappropriate genes(89, 92, 93). Pre-B cells upregulate CD19, a BCR co-receptor, and proceed with V-DJ rearrangement. Once the H chain is rearranged, it is expressed with a surrogate Ig L chain to form the pre-BCR, marking the beginning of the large pre-B cell stage(74, 85). This stage determines if combinatorial and junctional diversification resulted in a functional H chain. If the rearranged H chain is not functional, the cell can attempt rearrangement a second time with the alternate H chain allele(74, 85). If the second rearrangement does not produce functional H chain, the cell undergoes apoptosis and dies (positive selection, outlined below). If successful, the large pre-B cell undergoes clonal expansion and proceeds to the small pre-B cell stage, where it rearranges its L chain gene. When the BCR containing rearranged H and L chains is finally expressed on the cell surface, the cell is considered an immature B cell, and must survive another round of positive selection(74, 85, 86).

If a functional BCR is expressed at this stage, the immature B cell leaves the bone marrow and immigrates to the spleen. There, it proceeds through several transitional stages before becoming either a MZ or mature (Fo) B cell (discussed below). Mature B cells which have not encountered their cognate antigen are called naïve B cells.

a. Positive and Negative Selection of B Cells

Developing B cells are subjected to both negative and positive selection in the bone marrow(94, 95). If the newly generated pre-BCR or BCR is incapable of signaling, it is eliminated; this constitutes positive selection, since only productive rearrangements are selected to continue. This mechanism occurs at the pre-B cell and immature B cell stages of development, and appears to require basal BCR signaling or possibly a very weak or particular interaction with self(75, 85, 95).

Whereas positive selection requires a sufficient pre-BCR or BCR signal, negative selection aims to prevent autoreactivity by eliminating immature B cells which recognize self before they leave the bone marrow. Since only self antigens should be present in the milieu of the bone marrow, if the BCR of an immature B cell transmits too strong of a signal, it is triggered to undergo receptor editing, which is a secondary rearrangement of its light chain(96). If this fails to eliminate autoreactivity, the cell undergoes apoptosis. This mechanism of regulation is known as clonal deletion(85, 95). Immature B cells that survive both negative and positive selection leave the bone marrow and migrate to the spleen to complete their differentiation(97).

3. Peripheral Development and B Cell Subsets

Newly generated immature B cells leave the bone marrow and immigrate to the spleen. The spleen is broadly divided in to red pulp, where the blood is filtered and iron recycled, and the white pulp, where lymphocytes reside(98). The white pulp is organized in sheaths around branching arterial vessels, with T cells residing largely in the periarteriolar lymphoid sheath (PALS), directly surrounding the arterioles. Adjacent to the PALS, B cells are arranged in follicles, or B cell zones, of the white pulp(98). Surrounding the PALS and follicles, dividing the red and white pulp, is the marginal zone (MZ), which contains several

distinct populations of macrophages, DCs, and MZ B cells(98). Interestingly, MZ B cells are thought to be vital in the maintenance of the MZ and the other cell types which reside there(99, 100).

a. Transitional B Cells

Immature B cells immigrating from the bone marrow are directed to the follicle by the expression of chemokine receptors, particularly CXCR5, whose ligand, CXCL13, is expressed by follicular stromal cells(98, 101-103). They then proceed through several stages wherein they are considered transitional B cells. Up until recently, three subsets of transitional cells (T1, T2 and T3) were recognized; however, recent data suggests cells previously defined as the T3 subset may in fact be anergic B cells and not transitional cells at all(104).

T1 and T2 B cells are defined by specific sets of surface markers, half life, location, and response to BCR crosslinking. As a mechanism for elimination of anti-self specificities, T1 B cells are eliminated by apoptosis upon antigen encounter. T2 cells, on the other hand, proliferate and mature when cognate antigen is encountered(75, 105, 106). This additional phase of negative selection is a vital checkpoint in the elimination of autoreactive specificities which have escaped central tolerance mechanisms(107-109).

The stage at which MZ B cells branch off and immigrate to the MZ is still unclear(74, 75). Although still under some debate in the field, numerous data suggest that the affinity displayed by the BCR determines its selection into specific subsets. Stronger signaling through the BCR, including that which results from weak reactivity to self, may positively select cells into the MZ subset, while only basal BCR signaling is required for selection of mature Fo B cells(110, 111).

b. Mature (Fo) B cells

T2 B cells primarily mature into Fo B cells. Fo B cells are responsible for mounting a high-affinity, long-lived antibody response, and also generate the memory response against a specific pathogen. Fo B cells exist in the follicle and also recirculate in the blood, lymph, and lymphoid tissues(112), and have a half-life of 2-3 months(105, 113). When Fo B cells encounter antigen, they proliferate, upregulate activation markers such as CD80, CD86, and CD40, and process and present their cognate antigen on the major histocompatibility complex (MHC) II, priming them for T cell help. When appropriate T cell help is supplied, the GC reaction is begun. This process will be discussed in more detail below.

c. MZ B cells

MZ B cells are so called because they reside in the marginal zone of the spleen, which is positioned between the red and white pulp(98). They are therefore some of the first cells to come into contact with circulating antigen and ICs(37, 114), and as such are perfectly positioned to be early immune responders. Indeed, MZ B cells are thought to produce the bulk of the early, TI response(32, 114, 115). The TI response is important in defending against blood-borne infection and such pathogens as encapsulated bacteria(37) and in the clearance of LPS, resulting in protection from endotoxic shock(40). In contrast to that of Fo B cells, the MZ B cell response is typically both short-lived and rapid; recent analysis indicates that they are molecularly poised to secrete antibody almost immediately after stimulation(116).

In addition to the TI response, MZ cells play a role in the response to T-cell dependent (TD) antigens(37, 117). They are also hyperactive compared to Fo B cells in their

processing and presentation of antigen and stimulation of T cells(37, 118, 119), and in their response to BCR crosslinking and T cell co-stimulation(118, 120, 121).

As mentioned above, multiple lines of evidence suggest that MZ B cells are positively selected by recognition of self antigen. This may be advantageous in that it may enable cross-reactivity with common pathogen-derived antigens, or aid in the clearance of apoptotic cells which could otherwise act as self-antigen (discussed below).

4. The B Cell Receptor

Unlike the T cell receptor (TCR), which respond to processed, digested pieces of antigen presented by MHC complexes, B cells are able to respond to soluble antigen via the BCR. Recently, however, it was shown that membrane-bound antigen was more effective in activating B cells(122), emphasizing a possible role for DCs in B cell activation, as will be discussed below.

The first step of activation of a Fo B cell is crosslinking of the BCR by antigen (Ag). The degree of crosslinking directly relates to the strength of the intracellular signal generated. The intensity of crosslinking is dependent upon properties of the Ag. The BCR or Ab/Ag interaction can be measured in two ways: affinity and avidity. Affinity is the strength of interaction between a single Ag epitope and a single variable region of the Ab; the higher the affinity, the stronger the binding of the variable region to the epitope. Avidity is a more complicated measure of the total strength of interaction between an Ab and the whole Ag or Ag complex at all available epitopes present, taking into account multiple antigen binding regions. Therefore, an Ag or Ag complex (such as an IC) with multiple epitopes would have higher avidity than one with a single epitope, even though the affinity of the Ab for that epitope is unchanged. For purposes of activating a Fo B cell, high avidity is most important.

Other properties which can affect efficiency of BCR crosslinking by Ag include presence of TLR ligands (described above) or complement fragments (described below) in the Ag complex. Signaling through the BCR can also differ depending on a variety of circumstances, including differentiative state, subset, previous Ag encounter, and presence of co-stimulation(85, 86, 123). Pertinent signaling will be described in the following sections.

a. Signaling Through the BCR

As mentioned above, the BCR is a membrane-bound version of the Ig currently being expressed in a given B cell. It is able to signal through its association with the transmembrane proteins Igα and Igβ, which contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tails. Crosslinking of the BCR induces the formation of the BCR signalosome, a multi-protein complex containing cytosolic and membrane-bound signaling intermediates(124, 125). The signalosome initiates a phosphorylation cascade which perpetuates and branches, culminating in the regulation of key genes.

The cascade begins with the phosphorylation of two tyrosines within the ITAM motif by Src-family kinases, particularly Lyn(85, 86, 125). This phosphorylation allows association with and phosphorylation of other tyrosine kinases which contain SH2 domains, including spleen tyrosine(Y) kinase (Syk)(86, 125, 126). Syk is a key mediator of BCR signaling, as *syk*-/- B cells are greatly impaired at both the pro- to pre-B cell and immature to mature transitions, where positive selection occurs(127-129). Through its regulation of other intermediates, Syk is essential for many downstream effects of BCR signaling, including activation of the phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ2 (PLCγ2) pathways(85, 126, 130). PLCγ2 in turn regulates activation of protein kinase C (PKC) and

the Ca^{2+} flux. The PI3K pathway is needed for efficient activation of the Ser/Thr kinase Akt, which blocks apoptosis in the stimulated cell(85).

Other key signaling molecules downstream of Syk in the BCR signaling pathway include members of the mitogen-activated protein kinase (MAPK) family, such as extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 MAPK. These molecules are key regulators of cell proliferation, survival, and differentiation. In addition, BCR signaling results in activation of transcription factors, including nuclear factor of activated T cells (NFAT) and the ubiquitously expressed NF-κB(85). A very simplified schematic, including the key member of the co-receptor complex, CD19, can be found below (Fig. 1.2).

b. The BCR Co-Receptor Complex

The BCR co-receptor complex consists of CD19, a transmembrane glycoprotein with signaling motifs in its cytoplasmic tail(131-133); CD81 (TAPA-1), which has a role in signal transduction and cell adhesion(133, 134); Leu-13, which associates with CD81 in B cells and other cell types(133); and CD21 (CR2), a complement receptor(34, 135). Together, these molecules enhance BCR signaling through several mechanisms, the most important of which is thought to involve engagement of CD21 by antigen bound to complement components(130, 132, 133). Complement-bound antigen, allowing efficient BCR and co-receptor complex interaction, amplifies the signal through the BCR and elicits a 200-10,000 fold more efficient immune response than antigen alone(1, 4, 132, 133). It also results in increased antigen processing and presentation than with BCR crosslinking alone(136). At least part of the ability of the co-receptor complex to amplify BCR signaling is explained by

the finding that crosslinking the BCR/co-receptor complex activates the stimulatory BCR signaling pathway without concurrent activation of the inhibitory pathway(137).

i. CD21

CD21 is a transmembrane protein with 15 or 16 short consensus repeats (SCRs) and a short cytoplasmic tail without known signaling motifs(39, 132, 133). CD35 shares extensive homology with CD21, and, in mice, they are simply splice variants of the same gene. In humans, each is encoded by a separate but closely related gene and display somewhat differential expression(138) as described in Table 1.2.

CD21 is unusual in that it binds several diverse ligands, including EBV, CD23, and IFN-α, in addition to the complement fragments iC3b, C3d,g and C3d for which it is named(39, 135). In mice, only binding to complement fragments has been demonstrated. All of these ligands bind in the SCR1 and 2 domains and induce a cellular response(135), indicating that binding of these ligands induces signaling. It is thought that CD19 acts as the signaling component for CD21, which has traditionally been thought to lack significant signaling motifs of its own(2, 39). However, its transmembrane domain and cytoplasmic tail have recently been found to interact with intermediates which effect antigen internalization and processing(139). CD21 is expressed on B lymphocytes and FDCs in mice, and on these cells and erythrocytes in humans.

Co-engagement of CD21 with the BCR also increases the internalization of antigen(140) and expression of stable antigen-MHC II complexes on the cell surface for presentation of antigen to T cells(3). Interestingly, complement-linked antigen is targeted to different intracellular compartments and therefore may be processed differently than antigen alone(141-143). In some B cells, complement is required to present antigen from ICs to T

cells(41, 144). CD21 also traps ICs on MZ B cells, which then transport and transfer them onto FDCs(145).

ii. CD19

CD19 is an Ig superfamily transmembrane glycoprotein expressed in B cells and FDCs(131-133). The 9 tyrosine residues in its cytoplasmic domain are highly conserved in human, mouse, and guinea-pig, suggesting a critical role in signaling(146). Indeed, these residues are phosphorylated upon crosslinking and interact with PI3K and Lyn among others(132, 147, 148). CD19 has been shown to be key in the efficient PI3K-dependent activation of Akt(149). Additionally, CD19 can augment the phosphorylation of the Src-family kinases, including Lyn, via a process known as progressive amplification, resulting in increased phosphorylation of Syk(131, 150). Signaling through CD19 also increases activation of MEK1/2, which is a kinase for ERK1/2(151). Although CD21 is thought to be required to bring CD19 into the BCR signaling complex, it is clear that CD19 has signaling roles even in the absence of CD21, given that the phenotype of CD19 deficient mice is considerably more profound than that of CD21 deficient mice(132, 133, 146). It may be that CD19 can somehow associate with the BCR in the absence of CD21; alternatively, some have proposed there may be an as of yet unidentified ligand for CD19(132, 133).

Given these findings, it is not surprising that even small increases or decreases in CD19 expression can have dramatic effects on B cell development, function, and notably tolerance in mouse and man(152-154). This will be discussed in more detail below in the context of autoimmunity.

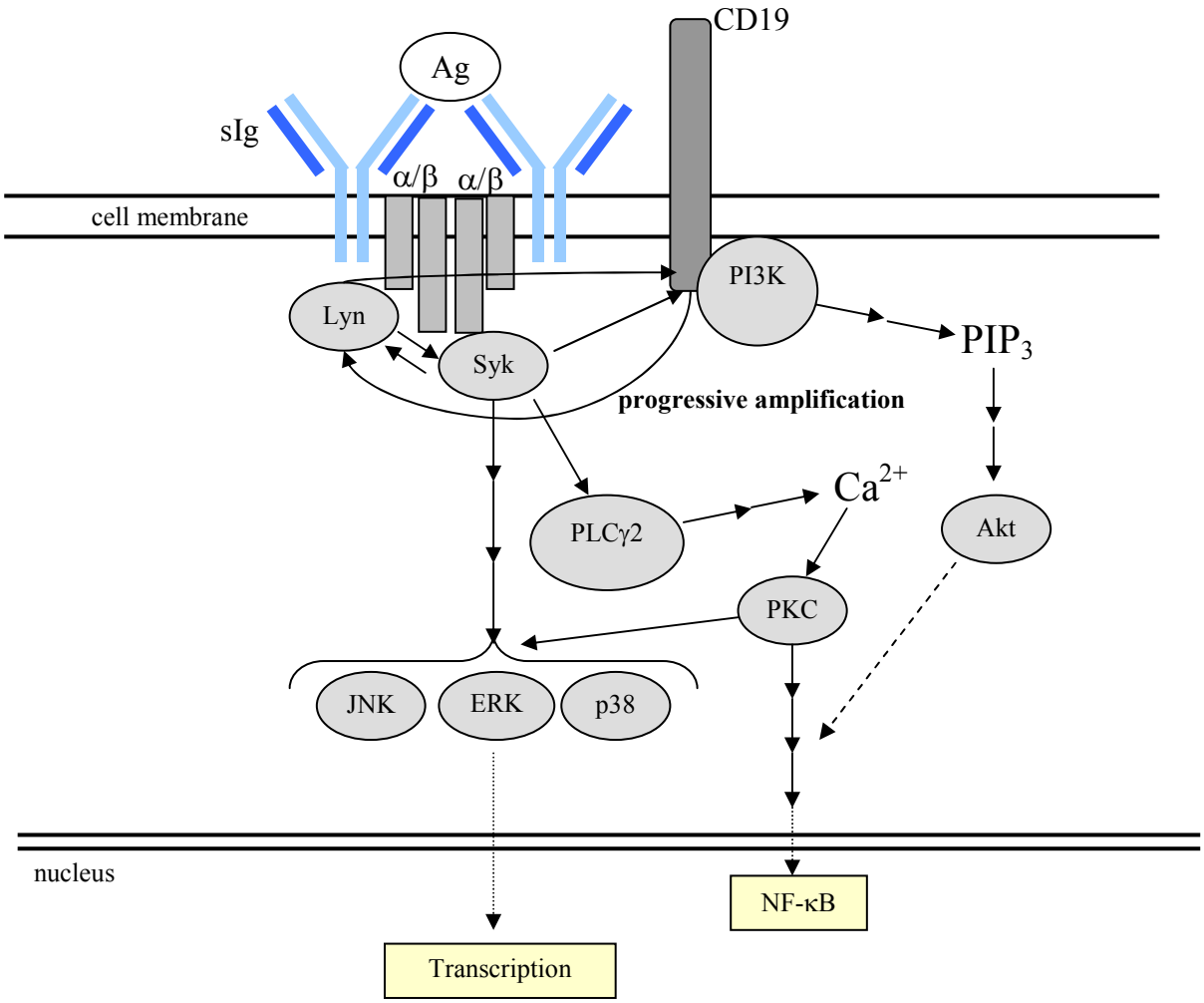


Figure 1.2. A simplified schematic depicting signaling through the BCR.

c. Other Molecules Which Regulate B Cells

Numerous additional surface receptors modulate B cell response. The most important are those provided by T cell help, as described below. In addition, B cell activation can be enhanced by the putative Ca^{2+} channel CD20(155, 156), numerous cytokines, particularly IL-21 and BLyS(157-161), and by TLR ligands, as described above.

B cell activation and differentiation can be also be inhibited by negative regulators, such as the IgG receptor FcγRIIb, CD5, and CD22(162-165). Clearly, B cells are never

simply “turned on” or “turned off”, but their response depends on the sum of many competing and overlapping signals.

5. The B Cell Response

B cells can play different roles in the immune response depending on factors such as their subset, activation state, the properties of antigen, the involvement of other cell types, and many others. As the details of the B-1 and MZ B cell response are beyond the scope of this dissertation, I will focus on the typical response mounted by mature B-2 B cells.

The response begins with engagement of the BCR by antigen. Fo B cells are highly motile, scanning the lymph and blood for soluble antigen, and interacting with FDCs in search surface-displayed antigen(103). One mechanism by which FDCs may acquire antigen is the capture of ICs containing antigen by FcRs and/or complement receptors(38, 166). Interestingly, MZ B cells can capture ICs, bring them to FDCs, and deposit them on their surface(145). In addition, compelling new data shows that FDCs can engulf, retain and present intact (non-degraded) antigen to B cells on their surface, eliciting strong BCR signaling(122, 167, 168).

Once the cognate antigen is encountered, signaling through the BCR “primes” the B cell to respond to T cell help(60, 81, 103). The antigen is internalized, processed, and presented on MHCII for recognition by antigen-specific Th cells. In addition, the B cell proliferates and upregulates the essential co-stimulatory molecules CD40, CD80, and CD86, whose roles will be discussed below. Importantly, upregulation of the chemokine receptor CCR7 allows B cells to locate to the B-T cell boundary in search of T cell help(165). If a cognate Th cell is found, a GC reaction begins.

a. B-T Cell Interactions

When an activated B cell encounters an activated Th cell whose TCR recognizes the processed antigen it is presenting, an immunological synapse is formed between the TCR and the antigen-MHCII complex(60, 81, 169, 170). This interaction is the "first signal" for activation of the T cell; the B cell's "first signal" was recognition of antigen. For efficient activation, it must be followed by the binding of CD40 on B cells with its ligand, CD40L (CD154), on T cells, without which GC formation and its downstream effects are blocked(171). This interaction, together with co-stimulation through the B7 molecules CD80 and CD86 on B cells by CD28 and CTLA-4 on Th cells, acts as the key "second signal" necessary for efficient B and T cell activation(60, 81, 172).

Multiple other cell-bound and secreted factors are also key in the stimulation of B cells by Th cells, particularly the inducible co-stimulator (ICOS) and its ligand ICOS-L, as well as IL-4, IL-21, and B cell activating factor belonging to the TNF family (BAFF), also known as B lymphocyte stimulator (BLyS)(60, 81).

These interactions provide activation and stimulatory signals not just to B cells, but also to T cells. Among other effects, the formation of these immune synapses stop T cell migration but not B cell migration, and B cells can be seen "dragging" T cells after synapse formation in *intra vital* imaging studies(102).

b. Germinal Centers

The provision of T cell help results in profound clonal expansion of the B cell. Some of the activated B cells form foci in the T cell zones (extrafollicular foci) and differentiate into short-lived antibody secreting cells (ASCs), which may class switch but do not show

signs of SHM(171, 173). The remainder of the B cells proliferate to form a region of IgD-negative cells within the primary follicle, which, once it acquires a germinal center, is termed a secondary follicle(60, 81). 7-10 days after initial priming, the secondary follicle polarizes into two regions: one near the T cell zone consisting of rapidly proliferating B cells, or centroblasts, and one consisting of quiescent B cells, or centrocytes. These regions are called the dark and light zones, respectively, and once they form, the secondary follicle is called a germinal center(60, 81).

As centroblasts divide, they also undergo SHM and CSR(60, 172, 173). They then proceed as centrocytes into the light zone for selection; competition for relatively limited supplies of antigen and a default program for cell death account for rigorous selection at this point(60, 81, 172). Cells which generate lower affinity or lose the function of their BCR die, whereas cells whose affinity is enhanced through this process are selected to re-enter the cycle or differentiate into either a long-lived plasma cell (PC) or memory cell(60, 81, 169, 171).

One of the key transcription factors expressed in GC B cells is Bcl-6. In mice lacking Bcl-6 expression, GC cannot form(174-176). Bcl-6 also acts as a transcriptional repressor of cyclin dependent kinase inhibitors, allowing the rapid proliferation of centroblasts(177, 178). It also inhibits B lymphocyte induced maturation protein 1 (Blimp-1), a transcription factor crucial for PC differentiation(174, 177). In addition to Bcl-6, Pax-5 is important in maintaining the identity of GC B cells, probably in part due to is repression of X-box binding protein 1 (XBP-1), which is upregulated upon Fo B cell activation and required for PC differentiation(178-180). Together, these proteins control the decision between exit to an

extrafollicular response vs. generation of memory, and their role in differentiation to PCs will be discussed more below.

In addition to the role of Th cells in the GC reaction, many studies have shown that FDCs play a role in survival and selection of GC B cells by means of antigen held on their surface(181, 182) and expression of surface-bound and secreted factors, particularly BLyS(57, 183-187). It was long held that the main function of CR2 on FDCs was capture of ICs, however, this idea was recently confounded by the finding that expression of CR2 on FDCs is critical, even in mice lacking any secreted Ab and therefore lacking ICs(188); this may mean that CR2 has a signaling role in FDCs. The precise role of FDCs in the GC reaction is still under debate(187).

c. Plasma Cells

Plasma cells are one of two main outcomes of the GC response and represent terminal differentiation of the B cell lineage. They are long-lived, secrete class-switched, SHM Ab, and are characterized by the downregulation of B cell specific surface markers such as the BCR, CD19, CD20, B220, MHCII and others, with concurrent upregulation of syndecan-1, or CD138(171). Cells committed to this lineage leave the GC as pre-PC, or plasmablasts, since fully differentiated PC no longer respond to chemotactic signals(171).

While the majority of plasmablasts express CXCR4, which through its interactions with its ligand CXCL12 direct PCs to the bone marrow (BM), a subset express CXCR3(180, 189-191). The CXCR3 ligands CXCL9 and 10 are expressed at sites of inflammation(192) and direct a subset of PCs there, where they terminally differentiate and become resident producers of Ab(193-196). Once formed, PCs can persist and secrete Ab for long periods of

time, likely decades, depending on their environment(197). The phenomenon of long-lived PCs residing in extra-lymphatic tissue is prominent in states of autoimmunity(193-195).

As mentioned above, complex transcriptional control is involved in the differentiation of PCs. In short, Pax-5 is downregulated, leading to a decrease in Bcl-6; the decrease in expression of these proteins relieves repression of Blimp-1, the master regulator of PC differentiation(171, 180). Blimp-1 in turn further represses Pax-5, Bcl-6, proliferation, and other B cell lineage genes, while activating XBP-1(171, 180). Recent studies indicate that commitment to this lineage also requires expression of the transcription factor IRF-4, which acts to upregulate Blimp-1 and other PC specific genes(189). A very simplified schematic depicting the interaction of these proteins can be found in Fig. 1.3(171, 180).

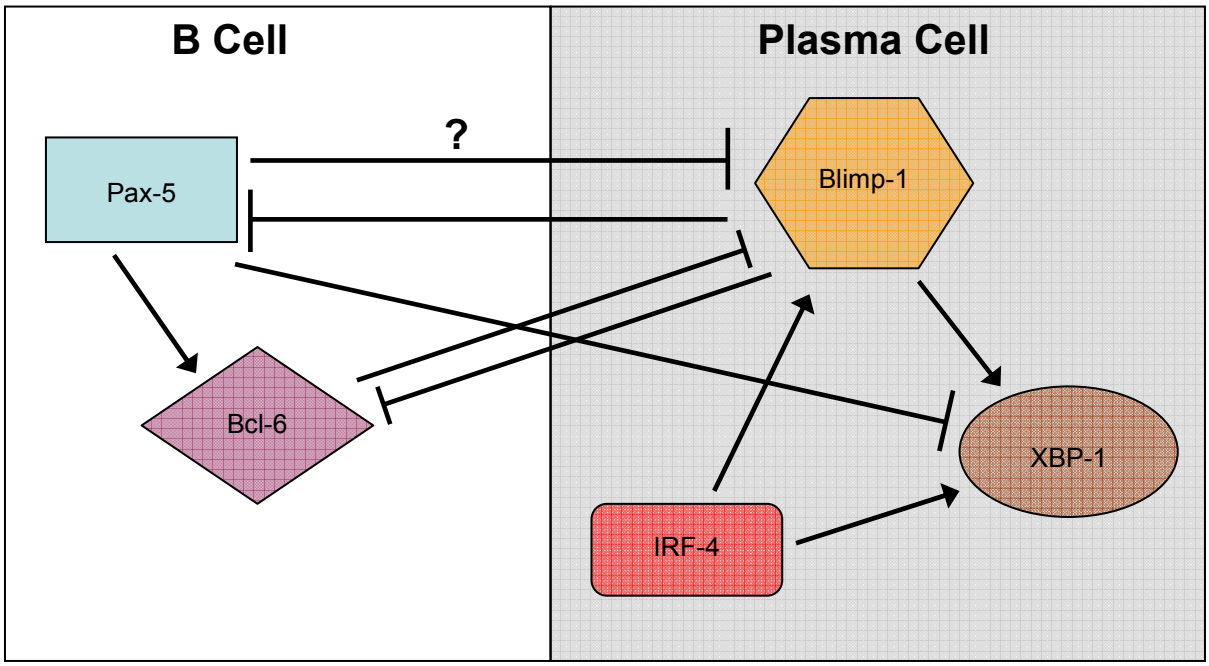


Figure 1.3. Interactions of transcriptional regulators controlling PC differentiation.

d. Memory B Cells

The other type of effector B cell generated by the GC response is the memory B cell. Memory B cells are long lived and express Ig, which has, like that of PCs, undergone SHM and typically CSR. In humans, they are defined as CD19+CD38- population which also typically, but not always, expresses CD27(197-200). Class-switched (or IgD-) and IgM memory cells have been identified, both of which show SHM of their Ig(201, 202) and, in fact, presence of mutations in the V regions of the BCR has been the "gold standard" of defining a memory population(203). Antigen-specific memory B cells can be detected for an impressive 60 years or longer after primary immunization(204).

The role of persistent antigen, in various forms, in the maintenance of this population is debated(205, 206). It was initially shown that the presence of antigen on the surface of FDCs was required for the maintenance of memory B cells(205, 207). However, in the absence of secreted Ig, which precludes IC formation, memory B cells display the same lifespan and function as those in control mice(208). In another experimental setting, memory B cells could be manipulated via a genetic switch to change BCR specificity. In this case, the memory B cells with specificity for an antigen never present in the animal persisted just as well as memory cells of the originally induced specificity(206). Therefore, the currently favored hypothesis is that that antigen is not required for maintenance of memory B cells(197).

Recently, focus has shifted from the study of memory B cells as a homogenous group to an attempt to delineate them into distinct subsets. Multiple lines of evidence suggest that memory B cell subsets with distinct functional and more subtle but detectable surface phenotypes exist(203). In mice, multiple subsets of memory B cells are beginning to be

defined based on surface receptor expression and function(209, 210). Emerging evidence suggests that this is the case in the human memory B cell pool(197-200), although specific markers largely remain to be elucidated. One possible marker is Fc receptor homologue H4 (FcRH4), which was found on a subset of largely CD27+ but class-switched and SHM B cells in human tonsil, although the significance of this finding is still unclear(200). In addition, it has recently been shown that a small subset of peripheral memory B cells in humans express CXCR3, are able to chemotax towards the corresponding ligands, and that expression of this receptor is maintained upon differentiation to a PC(190, 211); these cells may represent a distinct subset of memory B cells in humans.

Traditionally, cognate antigen and memory Th cells are thought to be required for activation of quiescent memory B cells(60, 191, 212). However, some evidence exists that polyclonal stimuli can induce the proliferation and even differentiation of human memory B cells(213), particularly "bystander" T cell help (soluble cytokines), CpG(26, 213), or stimulation with CD27 ligand (CD70) and IL-10(214). Overall, memory B cells appear to be poised to respond rapidly to activating stimuli. *In vitro*, stimulation through the BCR leads to increased secretion of Ig by memory B cells compared to naïve B cells, and memory, but not naïve, B cells are able to differentiate to ASCs upon stimulation with anti-CD40, IL-2 and IL-10 in the absence of antigen(215, 216).

Upon stimulation, memory B cells proliferate, some replenishing the memory cell pool, and others differentiating into short- or long-lived PCs(60, 191, 197, 209, 216, 217). They also strongly upregulate B7 molecules and, unlike naïve B cells, are able to effectively present antigen to T cells(215). Memory B cells retain expression of Pax-5 until their

reactivation and proliferation, after which those differentiating to a PC undergo the same transcriptional shifts as a GC B cell differentiating into a PC.

e. Other Effector Functions of B Cells

Though production of Ab is a central role of B cells, they have other pivotal roles in immune response and regulation. As mentioned above, B cells can act as APCs, and in some autoimmune diseases, particularly SLE, arthritis, and non-obese diabetes, appear to be the required APC for initial T cell activation and break in tolerance (218-220).

B cells also have the capacity for significant production of important cytokines. They have been shown to produce both Th1 and Th2 cytokines, especially IL-10, IL-6, and TNF-α(221-224). IL-10 and IL-6 have autocrine effects on B cells in addition to their effects on other cell types, and promote a Th2 response; TNF-α is one of the most potent cytokines, mediating a largely Th1 response and activating CD8+ T cells and macrophages, among others.

B cells have also been shown to produce IL-2, IL-4 and IFN-γ, though possibly to a lesser extent than those listed above(225-228). IL-2 and IFN-γ mediate Th1 responses, while IL-4 is considered a Th2 cytokine.

Like Th cells, which can differentiate to Th1 or Th2 types to mediate a Type 1 or Type 2 response, B cells can become Type 1 or Type 2 B effector cells (Be1 and Be2) which possess many of the same traits as Th1 or Th2 cells, and can control the development of Th1 and Th2 subtypes from naïve Th cells(229-231). Be1 and Be2 cells produce IL-2 at nearly equal levels. Be1 cells produce significant quantities of IFN-γ and IL-12, whereas Be2 cells produce negligible amounts of these cytokines. On the other hand, Be2 cells produce IL-4,

IL-6, and IL-10; Be1 cells produce essentially no IL-4, 4-fold less IL-6, and less than half as much IL-10 comparatively(230).

Most interestingly, Be1 and Be2 cells were able to efficiently generate Th1 or Th2 cells, respectively, from naïve CD4+ T cells, and *in vivo* responded by producing cytokines even before Th cells. These data indicate that not only do B cells produce Type 1 and 2 relevant cytokines themselves, but are able to effectively polarize the global response, and may be among the first cell types to do so(230).

Clearly, B cells play many roles in the innate and acquired immune systems, even apart from their production of Ab.

C. Generation and Maintenance of Self-Tolerance

Many mechanisms of central and peripheral tolerance via negative and positive selection have been outlined above, including receptor editing and clonal deletion. There are, however, additional mechanisms of peripheral tolerance in particular which are crucial to prevention of autoreactivity. These include anergy, block of differentiation at the pre-PC stage(232) and suppression by cytokines secreted by DCs and macrophages, which will be discussed briefly below.

1. Anergy

Anergy is a state of non-responsiveness induced by antigen encounter at an early differentiative stage or by chronic antigen exposure(233-236). Generally, whereas higher avidity for self results in receptor editing and clonal deletion, B cells which recognize autoantigens with low avidity will be regulated by anergy(233).

Anergic B cells do not proliferate, upregulate activation markers, or secrete Ab in response to BCR crosslinking(235, 237). This is due, at least in part, to dampened signaling through the BCR as measured by decreased phosphorylation of substrates and decreased Ca^{2+} flux(124, 238, 239). Interestingly, although these intracellular responses to BCR crosslinking are diminished and altered, some are basally elevated compared to naïve B cells, including intracellular Ca^{2+} levels(104, 124, 238, 239) and ERK phosphorylation(124, 240). Anergic cells also have a shorter half-life than naïve B cells(241), due in part to their increased dependence upon BAFF for survival(242).

An emerging mechanism for anergy appears to be activation of Lyn and its downstream pathways in the absence of Syk activation(233). Among other effects, this would promote the inhibitory feedback mechanisms involving the phosphatases SHP-1 and SHIP, blocking the Akt survival pathway and activation of NF-κB by Akt and Syk(233). The precise mechanisms governing anergy remain to be elucidated, and many subtle subtypes of anergy, or varying states of "non responsiveness" may exist.

2. Other Means of Peripheral Tolerance

Despite all of the above mechanisms to eliminate or silence autoreactive B cells, some still persist. Numerous additional mechanisms exist to prevent the formation of high-affinity anti-self Ab and/or memory B cells. Autoreactive B cells experience impaired selection in the GC and blocks in PC formation(243) or are excluded from the follicle ("follicular exclusion")(234). Recently a block was described for autoreactive B cells at the pre-PC stage, which represents regulation at the latest possible differentiative step(232). Ab secretion by self-specific mature B cells can also be suppressed by DCs and macrophages through the secretion of soluble mediators, particularly IL-6(244).

T cells also play a role in preventing B cell autoimmunity; the simple lack of cognate anti-self T cell help can block humeral autoimmunity. In addition, T regs can suppress B cell activation and Ab secretion(245).

II. AUTOIMMUNE DISEASE

With the phenomenal ability of B and T cells to generate receptors with nearly infinite specificity, it is not surprising that many generate receptors which recognize components of self. The immune system has therefore evolved the complex and multi-layered mechanisms described above to prevent the immune system from unleashing its considerable arsenal upon the host. Given the frequency with which autoreactive B and T cells develop, it is a testimony to these mechanisms that so little autoimmune disease occurs.

In humans, autoimmune disease takes many forms. Often, these diseases are broadly divided into those primarily mediated by either T cells or B cells. Clearly, as the immune system is a complex, interdependent system, few if any diseases are mediated by a single cell type. However, the heterogeneous family of autoimmune disorders mediated by autoantibody are considered to be chiefly B cell mediated (e.g., SLE), while diseases marked by the infiltration and destruction of tissue by T cells are considered to be chiefly T cell mediated (e.g., Type 1 Diabetes). Recently, a pivotal role for B cells, outside of their ability to produce autoantibody, has been described. It is now clear that in some systems, B cells acting as APCs are responsible for the initial break of T cell tolerance(246-248).

A summary of autoantibody-mediated diseases can be found in Table 1.4, along with their common autoantibody targets(249). Interestingly, a majority of these diseases are up to 10 times more common in women than in men, indicating that hormonal environment may play a decisive role in the development of these diseases(249, 250).

A. Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a devastating, multi-system autoimmune disease which affects millions of people worldwide. One of the most common autoimmune diseases, the prevalence of SLE in the US is approximately 1 in 2000 with an annual incidence of 1 in 10,000(251). Like most autoimmune diseases, SLE affects women much more often than men, at a ratio of about 10:1(252), and exposure to additional estrogen, such as through oral contraceptive use, can double risk of developing the disease(249), highlighting a pivotal role for this hormone in disease development. Additionally, ethnic groups are effected three times more often than Caucasians(253). The pathology of SLE is due to production of autoantibodies, primarily anti-nuclear antibodies (ANA), which are found in 95% of patients(254). The interaction of these autoantibodies with their corresponding self antigen leads to formation of ICs, which are deposited in vessels and kidneys. This deposition activates the complement cascade within tissues, causing extensive damage.

1. Autoantibodies in SLE

Although various types of ANA exist in other autoimmune diseases and even at low levels in healthy individuals, two are relatively specific for SLE: anti-dsDNA antibodies and anti-Smith (Sm) antibodies. Sm is a small nuclear ribonucleoprotein (snRNP) which is part of an RNA splicosome complex. While anti-dsDNA antibodies are sometimes found in low amounts in serum from non-SLE patients, a greatly elevated titer is considered one of the diagnostic criteria for SLE (American College of Rheumatology SLEDAI), with a sensitivity of 95%. Anti-Sm antibodies, on the other hand, are 99% specific for SLE and are considered

to be an indicator of more active disease and poorer prognosis. While they are seen in only about 30% of SLE patients, they are not seen in health or other disease states(255).

Table 1.4. Autoantibodies in human rheumatologic diseases	
Disease	**Autoantibody specificity**
Myasthenia gravis	Nicotinic acetylcholine receptor
Antiphospholipid syndrome	Phospholipid-β_2-glycoprotein 1 complex
Insulin-resistant diabetes mellitus	Insulin receptor
Pernicious anemia	Intrinsic factor
Graves' disease	TSH receptor
Wegener's granulomatosis	Proteinase-3 (ANCA)
Pemphigus vulgaris	Epidermal cadherin and desmoglein 3
Goodpasture's syndrome	Collagen IV
Systemic lupus erythematosus (SLE)	dsDNA, snRNPs
Rheumatoid arthritis (RA)	Ig
Autoimmune thrombocytopenic purpura	Platelets
Autoimmune hemolytic anemia	Rh antigens, I antigen
Hashimoto's thyroiditis	Thyroid peroxidase, thyroglobulin

The targeting of intracellular components by autoantibodies in SLE has long been considered a mystery, but recent data indicates that features of apoptosis may explain the formation of ANA. During apoptosis, numerous intracellular proteins, including nuclear components, are exposed on the surface of apoptotic blebs(256-258). Many labs have accumulated evidence that, in fact, apoptotic cells act as the stimulating antigen in SLE; apoptotic cell immunization activates autoreactive B cells and triggers autoantibody production, and defects in apoptotic cell clearance correlate with development of autoantibodies(258-261). Why these normally tolerogenic dying cells stimulate autoantibody production in lupus-prone individuals remains unclear, but is an area of active investigation.

2. Etiology of SLE

Current data strongly suggest that genetic factors predispose to SLE, but alone are not sufficient to trigger the disease; a gene-environment interaction is necessary(262). The

complexity of interaction between genes and environmental exposures has made it difficult to study. With the advent of transgenic and knockout mice, a better understanding of this interplay has become possible. Specific genes have been linked to SLE in humans and in mice, as have various environmental exposures(263, 264). Although no single gene always results in lupus, abnormalities in certain genes, such as those encoding HLA-DR2(264), Fcγ receptors(265, 266) and proteins of the complement system(253, 265) are associated with lupus in mice and man. Likewise, environmental exposures have been implicated in both the development and progression of the disease; silica(267, 268), heavy metals(262, 269, 270), chemicals(270, 271), certain viruses, especially EBV(272, 273), radiation(274, 275), and ultraviolet (UV) radiation exposure(276, 277) are among those most often linked to onset or relapse of SLE. Clearly, both genetic and environmental factors are important in the development of lupus; monozygotic twin concordance for SLE is only about 25-50%(255, 278), and no single environmental agent results in disease in all, or even most, individuals.

The complement components C1q, C2, and C4 are all intimately linked to development of SLE in humans(253, 278-280). Of all known genetic predispositions, deficiency in complement C1q or C4 have the strongest associations with the development of SLE(249). In humans, there are two genes for C4: C4A and C4B. Although deficiency in both genes, resulting in a complete lack of C4, is very rare, it is estimated that ≥75% of these individuals develop SLE(281). Heterozygous deficiency in either C4A or C4B, resulting in decreased C4 levels, is much more common and is also strongly associated with the development of SLE, occurring in 40-60% of SLE patients(282).

There is accumulating evidence that CD21 may be important in human SLE as well. A population of CD21 low B lymphocytes is present in the peripheral blood of SLE

patients(283). When B cells are activated, CD21 is shed from the cell surface in what appears to be regulated intramembrane proteolysis(284). SLE patients have decreased levels of the shed form of CD21 (soluble CD21, or sCD21) in their blood than healthy controls(285). The significance of these observations is unknown.

Why an intact complement system has a protective effect for SLE when the pathology of the disease results largely from complement activation has been the subject of much study and debate. Currently, two hypotheses are prominent: the clearance hypothesis and the tolerance hypothesis(35). The clearance hypothesis states that because the complement system is needed for effective clearance of apoptotic cells(286, 287), a deficiency in complement increases the apoptotic cell burden, and therefore available antigen, which activates autoreactive B cells. However, C3 is important in apoptotic cell clearance, and deficiencies in C3 do not predispose to SLE(279, 288-290). The tolerance hypothesis states that because complement plays an important role in B cell selection and regulation, as well as antigen processing and presentation, complement deficiency allows escape of autoreactive B cells from central and/or peripheral regulation.

It is still unclear why certain environmental exposures can act to induce or exacerbate lupus. It is likely that many mechanisms are involved; however, one property that all these exposures share is their ability to induce apoptosis, either directly or through the formation of reactive oxygen species(291-293). This has led to the hypothesis that these exposures induce ANAs by increasing exposure of autoreactive B cells to apoptotic cells, which display self antigen(294). Supporting this idea, direct intravenous (IV) injection of apoptotic cells into mice results in production of ANA(260, 295).

There have been many other abnormalities found in SLE patients. Overall decrease in CD19 expression has been shown in patients with SLE(296, 297), but subsets of B cells overexpress CD19 in these patients and in patients with common variable immunodeficiency (CVID) with autoimmune phenomena(283, 298). Increases in CD19 expression of as little as 20% can break tolerance in mice, and overexpression of CD19 has been shown in patients with the autoimmune disease systemic sclerosis(297, 299), supporting a role for CD19 dysregulation in autoimmunity.

PB B cells from SLE patients are also hyperactive and show spontaneous Ig production, reduced susceptibility to Ag-induced cell death, elevated Ca2+ responses, and hyperphosphorylation of cytosolic proteins(300-304). In addition, SLE Pts have increased numbers of activated, memory, and B-1 B cells in their PB(305-309). Interestingly, recent data implicate dysregulation of the inhibitory receptor FcγR2b on B cells, particularly memory B cells, as a possible causative factor in SLE(266, 310). The importance of these findings is still not well understood.

3. Clinical Aspects of SLE

The American Collage of Rheumatology requires the presence of at least 4 of 11 specific criteria for the diagnosis of SLE. The criteria can be found in Table 1.5(311). Among the most clinically devastating and life-threatening consequences of SLE is renal disease; almost all patients, if left untreated, will develop end-stage renal disease (ESRD) and die from this complication. Another life-threatening consequence of SLE is the

dysregulation of clotting, leading to transient ischemic attacks (TIAs), strokes, and myocardial infarctions. In addition, SLE Pts often experience a paradoxical

immunodeficiency marked by leukopenia. As can be seen in table 1.5, essentially all systems of the body can be adversely affected by this disease.

Table 1.5. Diagnostic criteria for SLE.	
Malar rash	Fixed erythema, flat or raised, over the face
Discoid rash	Erythematous circular raised patches with adherent keratotic scaling and follicular plugging
Photosensitivity	Exposure to UV light causes rash
Oral ulcers	Oral and nasopharyngeal ulcers
Arthritis	Nonerosive arthritis of two ore more peripheral joints, with tenderness, swelling or effusion
Serositis	Pleuritis or pericarditis
Renal disorder	Proteinuria or cellular casts
Neurologic disorder	Seizures or psychosis without other causes
Hematologic disorder	Hemolytic anemia, leukopenia, lymphopenia, or thrombocytopenia
Immunologic disorder	Anti-dsDNA, anti-Sm and/or anti-phospholipid
Antinuclear antibodies	An abnormally elevated ANA titer

4. Treatment of SLE

For many years, the treatment of SLE has been general immune suppression, since there is no cure for the disease. Drugs used depend largely on severity of disease, presence or absence of acute flare, and systems involved. Non-life threatening disease can be managed with mild pharmaceuticals such as non-steroidal anti-inflammatory drugs (NSAIDs). Antimalarials, such as chloroquine, also have efficacy in treatment of both symptoms and flares of SLE. They are presumed to exert their effects via blocking acidification of endosomes preventing both MHCII presentation and TLR signaling. More severe disease must be controlled with systemic glucocorticoids such as prednisone, or with

other potent immunosuppresents, which in many cases double as chemotherapeutic agents, such as azathioprine and cyclophosphamide. This is unfortunate given the frequent and severe adverse effects associated with these drugs(249).

Given the substandard efficacy and significant side effect profiles of these therapies, new treatments for SLE are constantly under investigation. The newest is rituximab (trade name, Rituxan), which was developed for use in B cell leukemias and lymphomas, particularly non-Hodgkin's lymphoma(312-314). Because its mechanism of action is depletion of B cells, rituximab has been evaluated for treatment of SLE, RA, multiple sclerosis, and other autoimmune diseases(312, 315-317). Rituximab is a monoclonal antibody which recognizes the B cell specific marker CD20. The precise mechanisms by which it accomplishes B cell depletion are unclear and are under active investigation, although it appears that this process is mediated, at least in large part, by Fc receptors(318-320).

Studies evaluating the efficacy of Rituximab in SLE have determined that response is variable: about a third respond with a extended clinical remission from the disease, a third respond to the drug short-term (less than 12 months), and a third do not respond at all(315, 321) (and Anolik and Sanz, unpublished data). Interestingly, in mouse models of Rituximab therapy, autoimmune strains were more resistant to depletion than non-autoimmune strains(322) (and Shlomchik et al, unpublished data). Therefore, Rituximab may emerge as a new therapy for a subset of SLE Pts, but new drugs with increased efficacy and fewer severe side effects are desperately needed(249, 323).

B. ANCA-Associated Small Vessel Vasculitis (ANCA-SVV)

Anti-neutrophil cytoplasmic antibodies (ANCA) recognize proteins in the granules of neutrophils and monocytes. They are present in a number of autoimmune diseases, and multiple subtypes of ANCA have been identified(249). Within the scope of this work, only ANCA-associated small vessel vasculitis (ANCA-SVV) will be discussed.

ANCA-SVV is mediated by autoantibodies directed against myeloperoxidase (MPO-ANCA)(324) or proteinase 3 (PR3-ANCA)(325, 326). Besides pathogenesis mediated by autoantibody, it shares with SLE the propensity to develop pauci-immune necrotizing glomerulonephritis, leading to rapid loss of kidney function. Other similarities to SLE include relapsing and remitting course, similar pharmaceutical treatment regimens(327, 328), and manifestations in multiple organ systems(329). Additionally, in both diseases, the severity and rate of progression can vary from an insidious course to that of fulminant, life-threatening, multiorgan disease(311, 330), and in both, at least a subset of patients can be successfully treated with Rituximab(323).

III. SUMMARY OF DISSERTATION

The data presented herein implicate CD19 dysregulation in the pathogenesis of SLE and ANCA-SVV. We show decreased CD19 expression on PB B cells in >95% of patients with either disease, and hypothesize that this decrease may allow escape of autoreactive specificities from regulation by decreasing the strength of signal generated by the interaction of the BCR and autoantigen.

Additionally, we show that CD19 expression is 2-3 fold increased on a subset of memory cells in 25-30% of SLE and ANCA-SVV patients. These $CD19^{hi}$ cells have many features of conventional memory B cells, but also appear to represent a novel and distinct subset of activated memory B cells which is enriched for autoreactive specificities. Our data

suggest that these cells travel to sites of inflammation, proliferate, and differentiate to PC; they also appear to be precursors to at least a portion of the autoantibody in circulation.

Finally, we show that patients with an expanded $CD19^{hi}$ population ($CD19^{hi}$ patients) experience an increased frequency of adverse outcomes, a unique pattern of autoreactive antibody specificities, and poor or non-responsiveness to rituximab therapy. Taken together, these data suggest that $CD19^{hi}$ patients represent a distinct subset of SLE with particular clinical features and therapeutic requirements.

CHAPTER 2: SIMILAR CD19 DYSREGULATION IN TWO AUTOANTIBODY-ASSOCIATED AUTOIMMUNE DISEASES SUGGESTING A SHARED MECHANISM OF B CELL TOLERANCE LOSS

I. ABSTRACT

We report here that dysregulation of CD19, a co-receptor that augments B cell receptor (BCR) signaling, occurs at two B cell differentiative stages in patients with systemic lupus erythematosus (SLE) and anti-neutrophil cytoplasmic antibody (ANCA) associated small vessel vasculitis (SVV). The naïve B cells of nearly all SLE and ANCA-SVV patients express ~20% less CD19 than healthy control (HC) B cells. In contrast, a subset of memory B cells of some SLE and ANCA-SVV Pts (25-35%) express 2-4 fold more CD19 than HC B cells. These $CD19^{hi}$ memory B cells are activated and exhibit evidence of antigen selection. Proteome array analysis of 67 autoantigens indicates that $CD19^{hi}$ SLE Pts exhibit a distinct autoantibody profile characterized by high levels of antibodies to small nuclear ribonucleoproteins and low levels of anti-glomerular autoantibodies. These findings have implications for autoreactive B cell activation and suggest a shared mechanism of B cell tolerance loss in these two diseases.

II. INTRODUCTION

The two most common forms of aggressive glomerulonephritis leading to the rapid loss of kidney function are glomerular immune complex deposition in systemic lupus erythematosus (SLE) and pauci-immune necrotizing glomerular diseases mediated by anti-neutrophil cytoplasmic autoantibodies (ANCA). Clinically, SLE and ANCA are rarely associated, but they do have some commonalities. Both diseases are tightly associated with well-characterized autoantibodies against intracellular proteins; SLE with autoantibodies directed against nuclear proteins, histones, and DNA(331, 332), and ANCA associated small vessel vasculitis (ANCA-SVV) with autoantibodies directed against myeloperoxidase (MPO-ANCA)(324) or proteinase 3 (PR3-ANCA)(325, 326). Moreover, both are marked by a relapsing and remitting clinical course, are treated with similar immunosuppressive regimens(327, 328), and can present with protean manifestations of inflammation involving organs such as the lungs, upper airways, skin, joints, and central and peripheral nervous systems(329). The acuity and rate of disease progression varies from an insidious course to that of fulminant, life-threatening, multiorgan disease(311, 330).

Previous studies of peripheral blood (PB) B cells in human SLE have revealed differences from normal B cells. In comparison to healthy control (HC) B cells, PB B cells in human SLE patients (Pts) are hyperactive(300). Moreover, they show spontaneous Ig production, reduced susceptibility to Ag-induced cell death, elevated Ca^{2+} responses, and hyper-phosphorylation of cytosolic proteins(300-304). SLE Pts are also reported to have a higher number of activated ($CD86^{+}$), memory ($CD27^{+}$), and B-1 ($CD5^{+}$) PB B cells(305-309). In contrast, almost nothing is known about B cells in ANCA-SVV.

Imbalances in the positive and negative influences on B cell receptor (BCR) signal strength may contribute to human autoimmunity(152). Alteration in CD19 expression has emerged as a possible mechanism for creating an imbalance in BCR signaling that leads to autoimmunity. CD19 complexes with CD81, Leu13, and CD21, the receptor for complement component C3d(g)(132, 333), and is a BCR co-receptor that enhances BCR signal transduction. CD19 functions as the signaling component for CD21(132), although it can also function independently of CD21(132, 146). Complexes of antigen and C3d(g) are at least 1000 times more potent in B cell activation than antigen alone(1). CD19 density on B cells of systemic sclerosis Pts is ~20% higher compared to B cells from HCs(297). In contrast, SLE PB B cells appear to express low levels of CD19(296, 297). A population of PB $CD19^{hi}$ B cells has been described in Pts with common variable immunodeficiency (CVID) with autoimmune phenomena, as well as in SLE Pts(283, 298). Thus, CD19 dysregulation may link several autoimmune diseases.

We report here a similar aberrant CD19 regulation in SLE and ANCA-SVV. In both diseases, naïve B cells express ~20% less CD19 than HC B cells. Some Pts also have a subset of memory B cells that expresses 2 to 4 fold more CD19 than naïve or memory HC B cells. The $CD19^{hi}$ B cells have an activated phenotype and show evidence of antigen selection. In SLE, the presence of $CD19^{hi}$ B cells is associated with anti-ribonucleoprotein (RNP) production and neurological dysfunction. These findings suggest that aberrant CD19 expression occurs at the naïve and memory stages of B cell development in human SLE and ANCA-SVV, and may contribute to disease onset and relapse.

III. MATERIALS AND METHODS

Pt and HC samples

PB samples were collected from 10 HCs and 41 SLE Pts (Table 2.1) (HC: 30 years, range: 25-30, gender: 4 female, 4 male; SLE: 37 years, range: 20-83, gender: 35 female, 4 male). SLE Pts were included in this study after informed consent in accordance with our institutional internal review board, and fulfilled at least 4 of the established American College of Rheumatology 1997 revised criteria for SLE. Clinical and serological data were gathered during routine clinic visits at the time of blood draw for B cell analysis. The SLE disease activity score (SLEDAI)(334) and British Isles Lupus Assessment Group (BILAG) index(335) scores were calculated for each Pt to reflect disease activity at the time of the sample collection.

PB samples were collected from 6 healthy donors (different from those used for the SLE Pt analysis) and 24 ANCA-SVV Pts (Table 2.2) after informed consent. Patients were classified as having active or inactive disease based on their Birmingham Vasculitis Activity Score (BVAS); any patient with a BVAS >1 was considered active. Healthy controls (HC) averaged 36.5 years and ANCA-SVV Pts averaged 47.6 (MPO-ANCA-SVV) and 53.7 years (PR3-ANCA-SVV).

HCs used for the comparison of CD19 levels on naïve and memory B cells included 7 females and 2 males, and averaged 30.5 years of age. The analysis of these samples was done at the same time and used a population of HCs distinct from those used for comparison with Pt B cells.

Renal disease was determined by renal biopsy. The Nephropathology Laboratory at the University of North Carolina performed the analysis and the results were classified by standard criteria as described(336).

Cell preparation and cell surface staining

PB mononuclear cells (PBMCs) were purified from heparinized PB samples (~20 ml) by Ficoll centrifugation. The buffy coat was washed twice in PBS and resuspended in RPMI, 2% fetal calf serum (FCS), 0.1% sodium azide for cell surface staining. Cells were stained with previously determined optimal concentrations of the following fluorochrome labeled antibodies: anti-human CD19-APC, IgD-FITC, IgG-FITC, CD38-PE, CD86-PE, CD40 PE, and MHCII-FITC (Pharmingen, San Diego, California) and then fixed with 1% paraformaldehyde.

Anti-Sm ELISA

The anti-Sm ELISA was performed as previously described(337) except that the secondary antibodies were a goat anti-human IgG (H+L) horseradish peroxidase (Jackson Immunoresearch, Westgrove, Pennsylvania) at a 1:10,000 dilution. Positive anti-Sm control serum (Immunovision, Springdale, Arizona) was used to normalize plate-to-plate variation. A positive well was defined as three times background (secondary alone) and the titer was calculated as the inversion of the most dilute sample giving a positive result.

Molecular analysis of V_H region from PB B cells

PB B cells from an SLE Pt with an expanded $CD19^{hi}$ population were stained for CD19 expression and individual $CD19^{hi}$ B cells were sorted into a 96 well plates that had been covalently coated with oligo-dT (RNAture, Irvine, California) for sequence analysis as described previously by Arnold and McCray(338). Oligonucleotide mixtures were used as reported previously, although modified by removal of the restriction sites(339) (VHL-1: 5'-TCACCATGGACTGSACCTGGA-3', VHL-2: 5'-CCATGGACACACTTTGYTCCAC-3', VHL-3: 5'-TCACCATGGAGTTTGGGCTGAGC, VHL-4: 5'-AGAACATGAAACAYCTGTGGTTCTT-3', VHL-5: 5'-ATGGGGTCAACCGCCATCCT-3', VHL-6: 5'-ACAATGTCTGTCTCCTTCCTCAT-3', CuII: 5'-CAGGAGACGAGGGGGAAAAG-3', CyII:5'-GCCAGGGGGAAGACSGATG-3'). A second nested PCR was done using products from the first PCR with the following primers (VH-1: 5'-CAGGTSCAGCTGGTRCAGTC-3', VH-2: 5'-CAGRTCACCTTGAAGGAGTCTG-3', VH-3: 5'-SAGGTGCAGCTGGTGGAGTC-3', VH-4: 5'-CAGGTGCAGCTGCAGGAGTC-3', VH-5: 5'-GARGTGCAGCTGGTGCAGTC-3', VH-6: 5'-CAGGTACAGCTGCAGCAGTCA-3', CuIII: 5'-GAAAAGGGTTGGGGCGGATGC-3', CyIII: 5'-ACSGATGGGCCCTTGGTGGA-3'). The products of the second PCR were analyzed on a 1.2% agarose gel; PCR products were cleaned and concentrated by Qiagen PCR Purification Kit (Qiagen, Chatsworth, California). DNA was sequenced using the second PCR 3' primer by the Automated DNA Sequencing Facility at UNC. V_H genes were identified using Ig BLAST (http://www.ncbi.nlm.nih.gov/igblast/).

Analysis of mutations

The action of selection on mutated Ig genes was assessed by analyzing the sequences with the program SoDA(340), which finds the best putative pre-mutation rearrangement and identifies mutations. All mutations falling into framework regions (FWR) and complementarity determining regions (CDRs) were separately classified by codon position (1, 2 or 3). In the absence of selection, mutations will be distributed uniformly over codon positions; in the presence of selection, more mutations at codon positions 1 and 2 will be purged than mutations at codon position 3, resulting in an apparent excess of mutations at position 3. Furthermore, selection differential between positions 1 and 2 may occur, depending on the relative conservation of the amino acid changes resulting on average in nucleotide changes in the two positions. Departure from the uniform distribution over codon positions was assessed by a chi-squared goodness of fit test.

Proteome array analysis

The proteome array analysis was performed as previously described(341) using HydroGel coated slides spotted with a panel of glomerular and nuclear antigens. The spotted antigens included aggencan, α-actinine, amyloid, β2-glycoprotein, β2-microglobulin, C1q, cardiolipin, CENP-A, CENP-B, chondroitin, chromatin, collagen I, collagen II, collagen III, collagen IV, ssRNA, cytochrome C, cytochrome P45, DSPG, dsDNA, elastin, fibrinogen IV, fibrinogen S, fibronectin, gliadin, glomerular basement membrane, glomerular sonicate, good pasteur antigen, histone (H) 1, H2A, H2B, H3, H4, hen egg lysozyme, hemocyanin, heparan sulfphate proleoglycan, heperin, hyaluronic acid, intrinsic factor, JO-1, Ku (p70/p80), La/SS-

B, laminin, matrigel, myosin, PL-12, PL-7, PM/Scl-100, proliferating cell nuclear antigen, proteoglycan, ribosomal phosphoprotein PO, Ro/SS-A (52KDa), Ro/SS-A(60KDa), Scl-70, ssDNA, thyroglobulin, topoisomerase, total histone, TPO, TTG, U1-snRNP-68, U1-snRNP-A, U1-snRNP-BB', U1-snRNP-C, vimentin, vitronectin, and yeast tRNA. The data were analyzed also as previously described(341). IgG and IgM serum levels were determined using human IgM and pan IgG ELISA kits (Bethyl Laboratories, Montgomery, TX).

IV. RESULTS

The majority of SLE and ANCA-SVV PB B cells were CD19^{lo}.

PBMCs were isolated from 41 SLE and 24 ANCA-SVV Pts, stained with B cell specific antibodies, and analyzed by flow cytometry. ANCA-SVV Pts were selected at random. SLE Pts were initially selected for the presence of elevated anti-Sm titers; hence there was a 50% prevalence of patients with an anti-Sm titer, rather than the expected 30%(342). A HC was stained with each experiment to control for day-to-day variation in cell surface staining.

The majority of SLE and ANCA-SVV B cells resembled HC B cells in that they comprised a single B cell population of uniform CD19 staining intensity and size (Fig. 2.1A). However, as reported previously(296, 297), SLE B cells expressed low levels of CD19. Surprisingly, the majority of B cells from ANCA-SVV Pts were also CD19^{lo}. Due to the high degree of variability of the CD19 median fluorescence index (MFI) on B cells stained on different days, comparisons were made only between a Pt and HC stained on the same day. As shown in Fig. 2.1B and C, SLE and ANCA-SVV Pt B cells stained ~18% (range: 3-45%) and ~20% (range: 15-67%) less well for CD19 than HC B cells, respectively (Fig. 2.1C) ($P<0.01$; Wilcoxon's signed-ranks test). This is unlikely to be due to treatment since B cells of untreated ANCA-SVV Pts were also CD19^{lo} ($P<0.01$; Wilcoxon's signed-ranks test) (Fig. 2.1C). The majority (>90%) of CD19^{lo} B cells were IgD^{+}, CD38^{+}, and CD27^{-} (Fig. 2.1D and data not shown) and thus appeared to be naïve B cells.

CD19^{hi} PB B cells were present in a subset of SLE and ANCA-SVV Pts.

Our analysis also revealed a $CD19^{hi}$ B cell population in a subset of SLE and ANCA-SVV Pts (Fig. 2.1A). This differs from a previous report of the presence of $CD19^{hi}$ B cells in all SLE Pts (283). Based on a criterion of more than 2 standard deviations above the mean percentage of $CD19^{hi}$ HC B cells, a $CD19^{hi}$ B cell population was detected in 14 of 41 (34.1%) SLE Pts and 6 of 24 (25%) ANCA-SVV Pts, but none of the HCs (Fig. 2.2A, B). Among the 14 SLE $CD19^{hi}$ Pts, ~7.5% PB B cells were $CD19^{hi}$ in contrast to 3.5% in the remaining SLE Pts and 2.5% in HCs. Similarly, among the 6 $CD19^{hi}$ ANCA-SVV Pts, ~11% of PB B cells were $CD19^{hi}$, in contrast to ~4% among the remaining 18 Pts. SLE and ANCA-SVV $CD19^{hi}$ B cells stained an average of 216% (range: 137-302%) and 369% (range: 274-422%) brighter, respectively, for CD19 than the $CD19^{lo}$ B cells of the same Pt (Fig. 2.2C) ($P<0.01$; Wilcoxon's signed-ranks test) (Fig. 2.2C). Four SLE pts were analyzed 2 or more times over a period of two years and, although fluctuation in frequency occurred, a $CD19^{hi}$ population was detected each time (Fig. 2.2D and data not shown). In addition, two ANCA-SVV Pts lacked a $CD19^{hi}$ B cell population on initial analysis, but developed a $CD19^{hi}$ population by the time of a second analysis (Fig. 2.2D and data not shown, Table 2). Thus, once it appears, the $CD19^{hi}$ population seems to be relatively stable over time. Herein, Pts with a $CD19^{hi}$ population will be referred to as $CD19^{hi}$ Pts, and those without a $CD19^{hi}$ population will be referred to as $CD19^{lo}$ Pts.

$CD19^{hi}$ B cells are memory B cells

To determine the differentiative stage of $CD19^{hi}$ B cells, we determined their BCR isotype and Bm classification. Approximately 43% of the $CD19^{hi}$ B cells of SLE Pts were IgG^{+}, as opposed to ~7% and ~5% of PB B cells from HCs and SLE Pts that lack a $CD19^{hi}$ B

cell population (Fig. 2.3A) (P=0.0001 and 0.0001, respectively; Student's t test). Conversely, ~37% of $CD19^{hi}$ B cells were IgD^{+}, versus 83% and 91% of PB B cells in HCs and SLE Pts that lacked a $CD19^{hi}$ B cell population (Fig. 2.3A) (P=1.14 x 10^{-5} and 2.90 x 10^{-5}, respectively; Student's t test). The $CD19^{lo}$ B cells in Pts with a $CD19^{hi}$ B cell population resembled HC B cells in both IgG and IgD expression (Fig. 2.3A). Similarly, the percent IgD^{+} B cells among $CD19^{hi}$ B cells of ANCA-SVV Pts and HCs was significantly different (56% vs.75%, respectively; P<0.5; Student's t test) (Fig. 2.3A), although the percent IgG^{+} B cells did not reach the level of significance (22% vs. 10%, respectively) owing to the small sample size (n=6) and considerable variability among Pts. Thus, the $CD19^{hi}$ B cell population is enriched in IgG^{+} B cells.

CD38 and IgD staining was used to further assess $CD19^{hi}$ B cell identity(198). Most SLE and ANCA-SVV $CD19^{hi}$ B cells were IgD^{-} $CD38^{-/low}$ memory B cells (Bm5), with a minority of IgD^{+}, $CD38^{-}$ B cells (Bm1). The Bm1 subset includes both IgD^{+} memory and naïve B cells (Fig. 2.3B and C). CD27 expression was variable among $CD19^{hi}$ B cells (data not shown). CD27 has been used as a memory B cell marker, but recent evidence indicates that some PB memory B cells are $CD27^{-}$(199), which appears to be the case here. In contrast, the $CD19^{lo}$ B cells of SLE and ANCA-SVV Pts were primarily activated naïve B cells ($CD38^{+}$ IgD^{+} Bm2), but also included memory B cells (Fig. 2.3B and C). The few B cells that fall within the $CD19^{hi}$ B cell gate in HCs and Pts lacking a $CD19^{hi}$ B cell population were IgD^{+}, $CD38^{+}$ activated naïve B cells (Bm2) (data not shown). Thus, in keeping with their BCR isotype, the majority of $CD19^{hi}$ B cells had a memory B cell phenotype, while a minority had a Bm1 phenotype and accordingly could be either naïve B cells or unswitched memory B cells.

CD19^{hi} B cells are polyclonal, somatically mutated, and antigen selected.

To assess clonality and selection within the CD19^{hi} B cell population, we determined the sequences of the expressed V_H region genes from 39 randomly selected CD19^{hi} B cells (20 IgM, 19 IgG) from an SLE Pt. Each sequence had a unique *VDJ* gene rearrangement and thus was of independent clonal origin (data not shown). V_H gene use by CD19^{hi} B cells exhibited a bias for V_H1 family genes (Fig. 2.4A), which included multiple members (data not shown), suggestive of antigen selection. This contrasts with previous reports of a bias for V_H3 (~50%) by both SLE and HC B cells(343, 344). This V_H1 bias by CD19^{hi} B cells was due to the IgG$^+$ B cells, as almost half of the γ sequences were V_H1, in contrast to just 20% of μ sequences. There was no apparent bias in complementarity determining region 3 (CDR3) length (median length: 39 bps for both μ and γ sequences) or J_H use among CD19^{hi} B cells (data not shown). Comparison to germline sequences identified by Ig BLAST indicates that the expressed V_H genes from the IgG$^+$ CD19^{hi} B cells were heavily mutated (Fig. 2.4B) (median:14 mutations/V_H), consistent with a memory B cell identity. In contrast, the V_H genes from the IgM$^+$ cells exhibited few mutations (median: 1 mutation/V_H) (Fig. 2.4B).

There was a significant bias in the distribution of H chain somatic mutations in CD19^{hi} B cells. Mutations were classified by codon position (1, 2, or 3) and analyzed for departure from a uniform distribution using a χ^2goodness of fit test. The 231 mutations in the framework encoding regions fell more frequently into the third position of the codons than would be expected by chance alone (expected distribution is 77 at each position; actual distribution is 73, 59 and 99 at codon positions 1, 2, and 3, respectively; $\chi^2 = 10.7$, $p = 2.4\times10^{-3}$). This distribution suggests selection against amino acid replacement mutations in

FWRs. In contrast, the 135 mutations in CDR 1 and 2 encoding regions occurred more frequently in codon position 2 and less frequently in position 1 than expected by chance (expected distribution is 45 at each position; actual distribution is 32, 56, and 47 mutations in codon positions 1, 2, and 3, respectively; $\chi^2 = 6.53$, $p = 0.019$; the CDR3 encoding region was excluded because of the inability to distinguish N region nucleotides and somatic mutation). This suggests a bias for amino acid replacement mutations in CDRs, consistent with antigen selection of mutant $CD19^{hi}$ B cells.

Antigen selection of $CD19^{hi}$ B cells was further suggested by the presence of shared mutations, particularly in CDR2 and FW3 (Fig. 2.4C). For example, S51I and M87T occurred in all three B cells expressing *V_H1-3*, and 6 other replacement mutations (M34I, G53A, E62K, S76T, A78V, and S82aN) occurred in two of the three cells. Altogether, there were 19 instances of the identical mutation occurring in 2 or more cells expressing the same V_H gene (Fig. 2.4C). Interestingly, 17 of these mutations occurred in *V_H1* family genes (Fig. 2.4C) reinforcing the idea of selection for *V_H1* gene use by $CD19^{hi}$ B cells.

$CD19^{hi}$ B cells have an activated phenotype.

To determine whether $CD19^{hi}$ B cells were activated, we examined the expression of activation markers. Indicative of an activated phenotype, SLE and ANCA-SVV $CD19^{hi}$ B cells expressed higher levels of CD86 and MHCII than $CD19^{lo}$ B cells from the same Pt and HC B cells analyzed on the same day (Fig. 2.5). Their larger size and increased granularity (Fig. 2.1 and data not shown) was also suggestive of activation, as was low expression levels of CD21 (Fig. 2.5)(284). In contrast, the expression of the activation marker CD40 was

lower on $CD19^{hi}$ B cells than on HC B cells or $CD19^{lo}$ B cells. Overall, these data suggest that $CD19^{hi}$ B cells are activated.

An increase in CD19 expression accompanies differentiation to a memory B cell.

As $CD19^{hi}$ B cells were predominantly memory B cells, we determined whether differentiation to a memory B cell results in increased CD19 expression. Naïve and memory B cells from 10 HCs were compared in a single experiment, and in all cases memory B cells (IgD^-, $CD38^{-/lo}$) stained ~30% (range: 11-53%) brighter for CD19 than naïve B cells (IgD^+, $CD38^+$) from the same individual ($P=3x10^{-5}$; Student's t test) (Fig. 2.6A and B). Notably, SLE and ANCA-SVV Pts with a $CD19^{hi}$ B cell population also had a population of memory B cells with levels of CD19 similar to those of HCs (Fig. 2.6A). Thus, differentiation to a memory B cell normally results in increased CD19 expression, but cannot fully explain the levels seen on $CD19^{hi}$ B cells.

Correlation of the presence of a $CD19^{hi}$ B cell population with clinical features of disease.

To determine whether the presence of a $CD19^{hi}$ B cell population was associated with more active disease or specific disease manifestations, we tested for correlations with clinical findings recorded for each Pt on the day of sample collection. No correlation with the SLEDAI or BILAG scores was observed in SLE, nor was a correlation observed with ANCA-SVV disease severity, as measured by BVAS scores (Tables 2.1 and 2.2). There was no correlation of the presence of a $CD19^{hi}$ B cell population with renal disease, as determined by biopsy, or with creatinine levels or proteinuria (data not shown). Thus, $CD19^{hi}$ B cells are present in Pts with active and inactive disease and are not an indicator of disease activity.

However, the presence of a $CD19^{hi}$ population among SLE Pts correlated significantly (P=0.01) with neurologic dysfunction (seizure or psychoses). A $CD19^{hi}$ population also correlated with a high anti-Sm titer (Table 2.1); 8 of 14 $CD19^{hi}$ Pts (mean titer of 400), but only 13 of the 29 non-$CD19^{hi}$ Pts (mean titer of zero) had an elevated anti-Sm titer (P= 0.01; Student's t test) (Fig. 2.7A). No association was found with the presence of anti-nuclear antibodies (ANAs). In addition, both anti-MPO and anti-PR3 were observed among $CD19^{hi}$ ANCA-SVV Pts (Table 2.2) indicating no association with either autoantibody.

To determine whether there were other differences in autoantibody specificities associated with $CD19^{hi}$ SLE Pts, we screened Pt serum using autoantigen proteome arrays bearing 67 nuclear and glomerular autoantigens(341). SLE Pts exhibited basal or negligible reactivity to most of these autoantigens with several important exceptions. Among these, the IgG autoantibody specificities uncovered fell into several distinct reactivity groups. 1) Three autoantibody specificities, Ro/SSA (52kD), CENP-A, and CENP-B were significantly elevated among the SLE patients, particularly those who lacked $CD19^{hi}$ B cells (Fig. 2.7B and data not shown). Whereas most of $CD19^{lo}$ patients exhibited significantly elevated titers of anti-Ro/SSA autoantibodies, only a small subset of the $CD19^{hi}$ patients exhibited elevated levels of these autoantibodies (Fig. 2.7B). Indeed, these were amongst the highest level of autoantibody specificities the SLE patients exhibited. 2) IgG autoantibodies to chromatin, dsDNA, PCNA, La/SSB, glomerular sonicates, and the core histones, H2A, H2B, H3 and H4, were equally elevated in $CD19^{lo}$ and $CD19^{hi}$ patients (Fig. 2.7B and data not shown). 3) SLE patients had reduced IgG autoantibody levels to fibrinogen, heparin sulphate, heparin,

laminin, and matrigel, compared to the healthy controls (Fig. 2.7B), and these reactivities were even lower among $CD19^{hi}$ patients. 4) Finally, it was remarkable that a small subset of autoantigens were selectively targeted by the sera from $CD19^{hi}$ SLE patients, including ribosomal phosphoprotein PO, U1snRNP-68, U1snRNP-A and U1snRNP-B/B' (Fig. 2.7B). Indeed, these appeared to be the hallmark specificities of $CD19^{hi}$ sera since the titers of these autoantibodies far exceeded the levels of all other specificities assayed in these sera and several of these autoantibodies were also significantly higher than the corresponding levels in $CD19^{lo}$ SLE patients. Notably, almost all $CD19^{hi}$ sera exhibited elevated levels of IgG autoantibodies to U1snRNP-A and U1snRNP-B/B' compared to the HCs (Fig. 2.7B).

IgM antibodies were assayed using the same autoantigen proteome arrays. Surprisingly, all autoantibody specificities exhibited a fairly similar pattern, with the levels being lower in the SLE sera compared to basal levels in the HCs (Fig. 2.7C). Interestingly, in the case of some of these specificities, including La/SSB, cardiolipin, H2B, and ribosomal phosphoprotein PO, the IgM autoantibody levels were even more depressed in $CD19^{hi}$ Pt sera. Since the IgM and IgG specificities were assayed using the same arrays, the IgG:IgM ratios for the different specificities were readily ascertained. Because all IgM specificities were depressed in the SLE sera, all autoantibodies that were uncovered in the SLE sera were heavily skewed towards the IgG isotype (data not plotted).

V. DISCUSSION

Our findings indicate a striking similarity in CD19 dysregulation by the B-cell-mediated autoimmune diseases SLE and ANCA-SVV. Low CD19 expression by naïve B cells occurs with near complete penetrance in both diseases, while high CD19 expression by a subset of memory B cells occurs with a 25-35% penetrance in both diseases. Thus, although the autoantibodies produced in SLE and ANCA-SVV mediate disease by different mechanisms—immune complex deposition and premature induction of neutrophil degranulation on vessel walls, respectively(345, 346)—SLE and ANCA-SVV Pts exhibit strikingly similar CD19 dysregulation. This suggests that diseases which are marked by production of autoantibodies, regardless of their specificity, may share a common mechanism of B cell tolerance loss.

$CD19^{lo}$ naïve B cells in SLE and ANCA-SVV

Naïve SLE and ANCA-SVV B cells stain ~20% less well for CD19 than HC B cells, as previously observed in SLE(296, 297), suggestive of low CD19 expression. Treatment is unlikely to be responsible, since naïve B cells of untreated ANCA-SVV Pts are also $CD19^{lo}$ (Fig. 2.1C). Low CD19 levels can decrease sensitivity to activation through the BCR and thus could contribute to the immunodeficiency experienced by SLE Pts(347), and could affect differentiation to the follicular, marginal zone, or B-1 cell subsets, as has been shown in mice(47). More importantly, autoreactive $CD19^{lo}$ B cells may escape tolerance induction, since it is dependent upon BCR signal strength. This could explain the higher frequency of

autoreactive B cells observed among immature and naïve B cell populations in human SLE(348). Congruent with the idea that low CD19 expression contributes to autoimmunity, *CD19* maps to a region of chromosome 16 that is linked to SLE(349), and a CD19 polymorphism associated with SLE in Japanese Pts leads to decreased CD19 mRNA stability and low CD19 expression levels(350). In addition, a single nucleotide polymorphism upstream of the human *CD19* gene is associated with high CD19 expression levels in systemic sclerosis(351).

$CD19^{hi}$ memory B cells in SLE and ANCA-SVV.

A subset of SLE and ANCA-SVV Pts (34.1% and 25%, respectively) has a $CD19^{hi}$ memory B cell population. The prevalence in SLE is likely to be somewhat lower, since SLE Pts were initially selected for having high serum anti-Sm titers, and we observe an association of $CD19^{hi}$ B cells with high circulating anti-Sm titers and other snRNP antigens (Fig. 2.7A). Among $CD19^{hi}$ SLE and ANCA-SVV Pts, $CD19^{hi}$ B cells constitute a substantial fraction of PB B cells (approximately 7.5% and 11%, respectively) (Fig. 2.1A and B). Although fluctuation in frequency occurs within Pts over time, and some ANCA-SVV Pts were observed to gain a $CD19^{hi}$ population, once it appears this population is relatively stable, as it is present during active and inactive disease and remains a significant population over time (Tables 2.1 and 2.2, and Fig. 2.2D). Most $CD19^{hi}$ B cells are IgG^{+} Bm5 memory B cells (Fig. 2.3) and, in at least one SLE Pt, are somatically mutated (Fig. 2.4B), suggesting that these cells have passed through a GC.

Consistent with a GC origin, the IgG^+ $CD19^{hi}$ B cells appear to be antigen selected. First, they exhibit a bias in V_H1 family gene expression (Fig. 2.4A), in contrast to a V_H3 bias among IgM^+ $CD19^{hi}$ B cells (Fig. 2.4A) and HC B cells(343, 344). A previous study indicated a V_H3 bias by SLE B cells(344), but it did not consider individual B cell subsets. Analysis of additional Pts is required to determine the generality of this finding and is ongoing. Second, somatic mutation among IgG^+ $CD19^{hi}$ B cells is biased in favor of CDR amino acid replacement mutations (P=0.019) and against replacement mutations in FWRs ($P=2.4x10^{-3}$), and there are multiple parallel amino acid replacement mutations in V_H1 rearrangements (Fig. 2.4C). The high frequency of V_H1 parallel mutation suggests that selection is mediated by a limited number of antigens. We think the selecting antigen(s) are likely to be self. In SLE, the selecting antigens may be Sm or other snRNP antigens, since very high levels of antibodies to these antigens appear to be the hallmark of $CD19^{hi}$ Pts (Fig. 2.7B), while in ANCA-SVV the selecting antigens may be MPO and PR3. This hypothesis is currently being tested.

The activated phenotype of $CD19^{hi}$ B cells (Figs. 2.1 and 2.5) suggests a recent or ongoing engagement with antigen, and thus these cells may be undergoing ASC differentiation. The 2-4-fold higher CD19 levels on these cells may confer increased sensitivity to activation by antigen, promoting ASC differentiation, as seen in mice with a much smaller increase (30%) in CD19 levels(152). If $CD19^{hi}$ memory B cells are indeed more sensitive to activation by antigen and are autoreactive, they likely contribute to disease progression and relapse. A precise determination of their differentiative state, in conjunction with identifying the activating antigen(s), will be important to understand their role in disease. Although not associated with increased disease severity as assessed by various

clinical scores in either SLE or ANCA-SVV, $CD19^{hi}$ B cells are associated with neurological symptoms and anti-RNP antibodies in SLE, suggesting an effect on clinical manifestations of disease. The absence of association with renal disease may be explained by the low levels of anti-glomerular antibodies (fibrinogen, laminin, heparin sulfate, Matrigel; Fig. 2.7B), which were previously observed to be associated with renal disease(341). We are currently following Pts long-term to determine whether $CD19^{hi}$ B cells are associated with a poorer outcome or more frequent relapses. Overexpression of CD19 by ~20% on systemic sclerosis B cells and by B cells in CVID Pts with autoimmune manifestations(297) suggests that elevated CD19 is significant to multiple autoimmune diseases.

Although the molecular basis for CD19 overexpression by memory B cells is unknown, our findings provide some insight. Treatment effects cannot be ruled out; but that SLE and ANCA-SVV Pts with and without a $CD19^{hi}$ population receive the same drug regimen weakens this explanation. Genetic factors may be involved, as suggested by the possible genetic linkage of CD19 and SLE(349-351). However, it is noteworthy that memory B cell differentiation results in an approximately 30% increase in CD19 expression (Fig. 2.6), and that even $CD19^{hi}$ SLE Pts have a memory B cell population with normal CD19 upregulation (Fig. 2.6). Thus, CD19hi B cells appear to represent a unique subpopulation of memory B cells in these Pts. This point, along with the finding of skewed autoreactivity in $CD19^{hi}$ patients, suggests that CD19 overexpression may be self-antigen specific, possibly requiring a combination of antigen and other activating signals, such as those through toll-like receptors (TLRs). A role for TLR signals is an enticing idea, given the recent findings strongly implicating dual activation of BCR and TLR receptors in

breaking tolerance and generating autoantibodies against nuclear antigens such as dsDNA and Sm(8, 9, 11, 352).

VI. CONCLUSION

Our data suggest that SLE and ANCA-SVV, although rarely clinically associated, share a similar mechanism for loss of B cell tolerance. We propose that CD19 dysregulation affects B cell activation at two stages. The first is at the $CD19^{lo}$ naïve B cell stage. It occurs with high penetrance in both diseases and may allow autoreactive B cells to evade tolerance induction, increasing their likelihood of activation by self. The second is at the $CD19^{hi}$ memory B cell stage. It occurs with comparatively low penetrance and potentially results in increased sensitivity for re-activation. These findings, along with those of others(283, 298), implicate CD19 dysregulation in otherwise distinct antibody-mediated autoimmune diseases and point to CD19 as a possible drug target with multiple autoimmune disease applications.

Table 2.1. SLE Patients											
ID[a]	CD19[hi b]	Meds[c]	Age[d]	Race[e]	Sex	Renal[f]	Neuro[g]	ANA[i]	α-Sm[j]	SLEDAI[k]	BILAG
1	+	PL,A	41	B	F			+	6400	2	2
2	+	PR	20	AI	F		+	+	400	2	4
10	+	PR, C	18	B	F	+		+	200	6	8
12	+	PL, PR,C	26	W	F	+		+	400	8	3
14	+	PR, CY	20	W	F	+	+	+	25600	28	48
16	+	PL, C	34	W	F	+			0	0	03
17	+	PL, PR	45	B	F				3200	6	5
24	+	PL, PR	21	W	F	+		+	0	4	7
23	+	PR, A	31	B	F			+	800	2	2
25	+	PL, PR	48	B	F			+	0	0	2
26	+	PR, M	44	B	F			+	25600	2	2
27	+	PR, CY	33	B	F	+		+	25	8	15
36	+	PR	25	B	F	+		+	400	4	14
39	+	PL, CY	27	B	F			+	800	28	24
3		PR	40	B	F			+	0	0	2
6		PL	31	B	F	+		+	800	2	6
5		PL, PR	21	B	F			+	0	1	3
4		PL, PR	33	B	F			+	25	0	3
8		PL, PR	50	W	F			+	0	0	0
7		PL	25	W	F			+	25	4	1
9		PL, PR, A	28	B	F	+		+	25600	6	6
11		PL, PR	49	B	F	+		+	0	0	8
13		C	32	W	F	+		+	25	2	0
15		C	36	B	F			+	1600	2	4

Table 2.1 Cont.											
18		none	50	B	F	+		+	25	2	7
19		C	37	B	F	+			200	2	1
20		PL, C	19	W	M	+		+	0	4	1
21		C	57	B	M	+		+	0	8	7
22		PL	26	W	F				0	0	3
28		PL	61	W	M	+		+	0	0	4
29		PL	33	B	F	+		+	200	8	6
30		PR, CY, C	41	H	F	+		+	0	4	12
31		PL	82	W	F				0	2	8
32		C	20	W	F	+			50	6	4
33		PL, M, PR	45	W	F			+	0	0	2
34		PL	23	W	F			+	0	8	5
35		PL, PR	47	W	F	+		+	25	6	6
37		PL, PR	28	W	F			+	200	7	12
38		PL, PR	33	W	M			+	50	0	3
41		CY	34	W	F	+		+	0	22	17
40		PR, A	37	B	M	+		+	400	24	18

[a]Each Pts was assigned a unique number. [b]Pts with a $CD19^{hi}$ population are indicated by a +. [c]Medications that each Pt was taking at the time of blood draw are indicated: plaquenil (PL), cellcept (C), cytoxan (CY), prednisone (PR), azathioprine (A), and methotrexate (M). [d]Age at the time of analysis is provided. [e]Race is indicated as American Indian (AI), black (B), Hispanic (H), or white (W). [f]Indicates whether the Pt has glomerulonephritis. [g]Indicates whether a Pt has ever suffered a seizure or psychoses. [h]Indicates the presence of anti-ssDNA, anti-dsDNA, or anti-Sm. [i]Indicates a positive ANA. [j]Indicates the titer of anti-Sm as measured by ELISA (illustrated in Fig. 6). [k]SLEDAI and BILAG scores were determined at the time of B cell analysis(334, 335).

Table 2.2. ANCA-SVV Patients.

ID[a]	CD19[hi b]	Meds[c]	Gender	Age[d]	Race[e]	Disease[f]	ANCA[g]	ANCA Titer[h]	Activity[I]	BVAS[J]
3	+	C, CYC	F	24	B	MPA	MPO	24.3	R	0
5	+	CY,PR	M	76	A	MPA	MPO	5.3	A	3
11a		PR, A	M	46	B	WG	PR3	3.5	R	0
11b	+	PR, A	M	49	B	WG	PR3	120.5	R	0
16	+	C, CYC	F	38	W	WG	PR3	57.9	R	0
19	+	PR	F	63	W	WG	PR3	166	R	0
23a		PR	F	24	W	WG	PR3	13	A	8
23b	+	CYC	F	25	W	WG	PR3	76.1	A	3
1		S	M	50	W	MPA	MPO	68.6	A	12
2		None	F	67	W	MPA	MPO	43.1	A	12
4a		None	F	28	W	MPA	MPO	75.9	R	0
4b		None	F	28	W	MPA	MPO	75	R	0
4c		None	F	29	W	MPA	MPO	69.9	R	3
6		PR, CYC, A	M	49	W	MPA	MPO	7.3	R	0
7		None	M	56	W	GN	MPO	29.5	A	6
8		S	M	63	W	MPA	MPO	81.4	A	20
9a		PR, A	M	63	H	MPA	MPO	103	A	20
9b		PR, A	M	63	H	MPA	MPO	32	R	0
10		None	M	66	W	MPA	MPO	71.4	R	0
12		CY, C	F	63	W	WG	PR3	12O	R	0
13a		None	F	52	B	MPA	PR3	172	A	6
13b		A	F	54	B	MPA	PR3	117	R	0
14		PR	F	45	W	WG	PR3	78.4	R	1
15		PR, CYC, C	M	57	W	WG	PR3	52	A	18
17		C	M	23	W	WG	PR3	74.3	A	11

Table 2.2 Cont.										
18		PR	M	71	W	WG	PR3	158	A	20
20		None	M	38	W	WG	PR3	116	R	0
21		S	F	55	W	WG	PR3	171	A	27
22		None	M	26	W	MPA/αGBM	PR3	4	R	0
24		S	M	77	W	WG	PR3	165	A	2

[a]Each Pt was assigned a unique number. Serial numbers of the same Pt are indicated by letters. [b]Pts with a $CD19^{hi}$ population are indicated with a +. [c]Medications that each Pt was taking at the time of blood draw are indicated: plaquenil (PL), cellcept (C), cytoxan (CY), cyclosporine (CYC), prednisone (PR), azathioprine (A), and solumedrol (S). [d]Age at time of analysis. [e]Race is indicated as asian (A), black (B), or white (W). [f]Disease classification are indicated as Wegener's granulomatosis (WG), microscopic polyangitis (MPA), or pauci-immune necrotizing and cresentic glomerulonephritis without extra-renal manifestations (GN). Pt 22 also has anti-glomerular basement membrane autoantibodies (αGBM). [g]The specificity of the ANCA (MPO or PR3) is indicated. [h]ANCA titer expressed in units/ml. A positive test corresponds to a titer >20u/ml. [i]Pts are classified as being in remission (R) or having active disease (A) based on clinical assessment and BVAS score. Pts with a BVAS score ≤1 are considered in remission; Pts with a score >1 are considered active. BVAS was calculated by a standard protocol for vasculitis activity score modified in 2003(353).

Figure 2.1. Two B cell populations are detected in SLE and ANCA-SVV Pts based on CD19 expression and size (FSC).

(A) Histograms of representative Pts and HCs. Populations of smaller $CD19^{lo}$ and larger $CD19^{hi}$ B cells are indicated. Two representative HCs, SLE Pts in which the majority of Pts that have only the $CD19^{lo}$ population (SLE), and SLE Pts that also have a $CD19^{hi}$ population (SLE $CD19^{hi}$) are shown. One representative ANCA-SVV Pt with and one Pt without a $CD19^{hi}$ population is shown. Histograms represent >10,000 $CD19^{+}$ lymphocytes. Note that the CD19 staining intensities differ between Pt samples due to unavoidable day-to-day staining variation. (B) Comparison of CD19 expression between SLE and ANCA-SVV B cells with HCs analyzed on the same day. The comparison is between the smaller $CD19^{lo}$ B cells from SLE and ANCA-SVV Pts and all $CD19^{+}$ B cells from HCs stained on the same day. Gates for this analysis are as in (A). (C) The difference in CD19 MFI between $CD19^{lo}$ B cells of SLE and ANCA-SVV Pts and HC B cells. Numbers were determined by subtracting the CD19 MFI of the HC B cells from the CD19 MFI of the $CD19^{lo}$ Pt B cells. Each symbol represents a HC/Pt comparison stained on the same day. On average $CD19^{lo}$ SLE PB B cells stain ~18% less well for CD19 than HC B cells (indicated by the horizontal dotted line). To control for medication in this analysis a group of 7 ANCA Pts not on medications at the time of analysis were compared separately. Statistical significance was determined by the Wilcoxon's signed-ranks test. (D) CD38 and IgD staining of B cells from HCs and SLE/ANCA-SVV B cells to identify the Bm designation. The subset descriptions and Bm designation are indicated. Comparison is between all $CD19^{+}$ B cells of a HC, and the $CD19^{lo}$ population from a SLE and ANCA-SVV Pt as gated in (A).

Figure 2.1

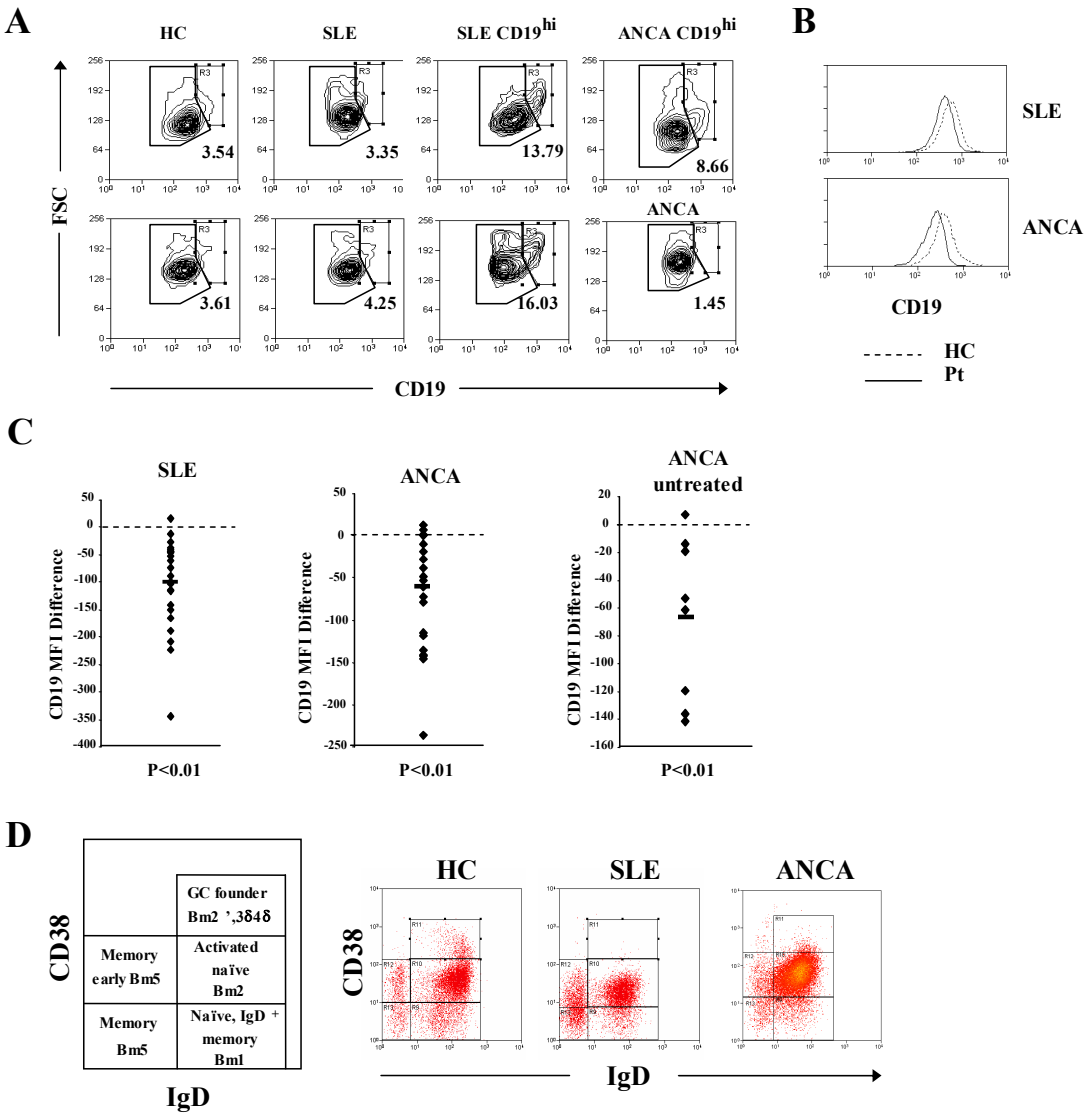

A
HC
SLE
SLE CD19hi
ANCA CD19hi
FSC
CD19
3.54
3.35
13.79
8.66
ANCA
3.61
4.25
16.03
1.45
B
SLE
ANCA
CD19
HC
Pt
C
SLE
ANCA
ANCA untreated
CD19 MFI Difference
P<0.01
P<0.01
P<0.01
D
GC founder Bm2 ',3δ4δ
Memory early Bm5
Activated naïve Bm2
Memory Bm5
Naïve, IgD + memory Bm1
CD38
IgD
HC
SLE
ANCA
CD38
IgD

Figure 2.2. CD19^{hi} B cells are present in a subset of SLE or ANCA-SVV Pts.
(A and B) The frequency of CD19hi B cells in individual SLE and ANCA-SVV Pts. Each symbol represents an individual Pt or HC. CD19hi B cells were gated as in Fig. 2.1A. The horizontal line indicates the frequency that is 2 standard deviations above the mean for HCs. Pts with a CD19hi B cell population above this point were considered positive for a CD19hi population. (C) The difference in MFI between the CD19hi population, as gated in Fig. 2.1A, and the HC B cells stained on the same day. The difference was calculated as described in Figure 2.1C. Each dot represents an individual Pt/HC comparison. Statistical significance was determined by the Wilcoxon's signed-ranks test. (D) Analysis of representative SLE and ANCA-SVV Pts at one year intervals. The SLE Pt had a CD19hi population on initial analysis that was evident at each subsequent visit, whereas the ANCA-SVV Pt did not have a CD19hi population on their initial analysis, but did on a subsequent analysis. The histograms are as described in Fig. 2.1A. The frequency of CD19hi B cells, as gated, are given.

Figure 2.2

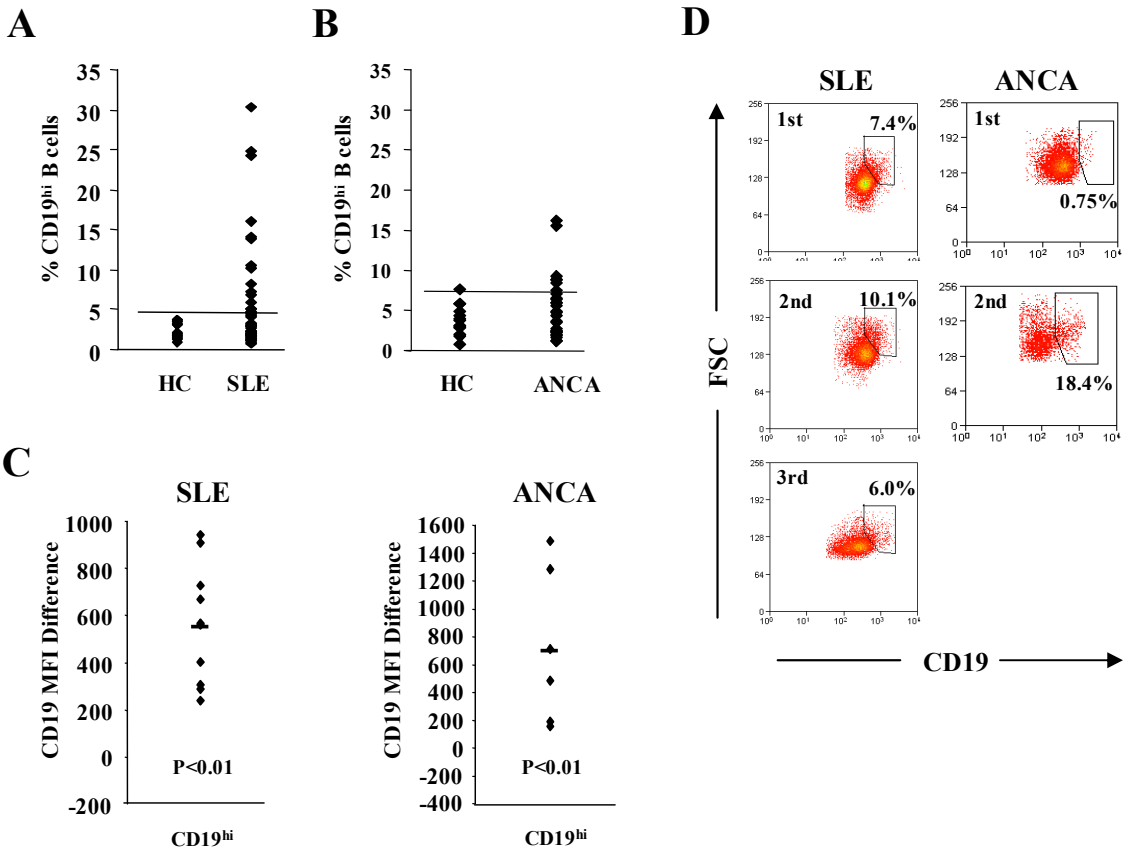

A
% CD19hi B cells
35
30
25
20
15
10
5
0
HC
SLE
B
% CD19hi B cells
35
30
25
20
15
10
5
0
HC
ANCA
C
SLE
CD19 MFI Difference
1000
800
600
400
200
0
-200
P<0.01
CD19hi
ANCA
CD19 MFI Difference
1600
1400
1200
1000
800
600
400
200
0
-200
-400
P<0.01
CD19hi
D
SLE
ANCA
1st
7.4%
1st
0.75%
2nd
10.1%
2nd
18.4%
3rd
6.0%
FSC
CD19

Figure 2.3. SLE and ANCA-SVV $CD19^{hi}$ B cells are primarily IgG+ IgD- memory B cells.

(A) The percentage of IgD^+ and IgG^+ B cells is shown for each of the following cell populations for SLE (upper) and ANCA-SVV (lower) Pts: B cells from HCs, $CD19^{lo}$ B cells from Pts and without a $CD19^{hi}$ population (labeled SLE or ANCA-SVV), and $CD19^{hi}$ B cells. P values for the differences between $CD19^{hi}$ B cells and HC B cells were calculated by the Student's t test and are shown. (B) The expression of CD38 and IgD is shown to determine the subset identity of SLE and $CD19^{hi}$ B cells. Total $CD19^+$ B cells from a HC and the $CD19^{lo}$ and $CD19^{hi}$ B cells (as defined in Fig. 2.1A) from an SLE Pt are shown. Similar results were obtained from ANCA –SVV Pts. (C) The average percentage of cells belonging to the indicated subset from all SLE and ANCA-SVV Pts and HCs analyzed based on the subset identities indicated in B, except that the memory subset is the combination of early Bm5 and Bm5. Error bars represent standard error. * P <0.05 and ** P <0.001 (Student's t test).

Figure 2.3

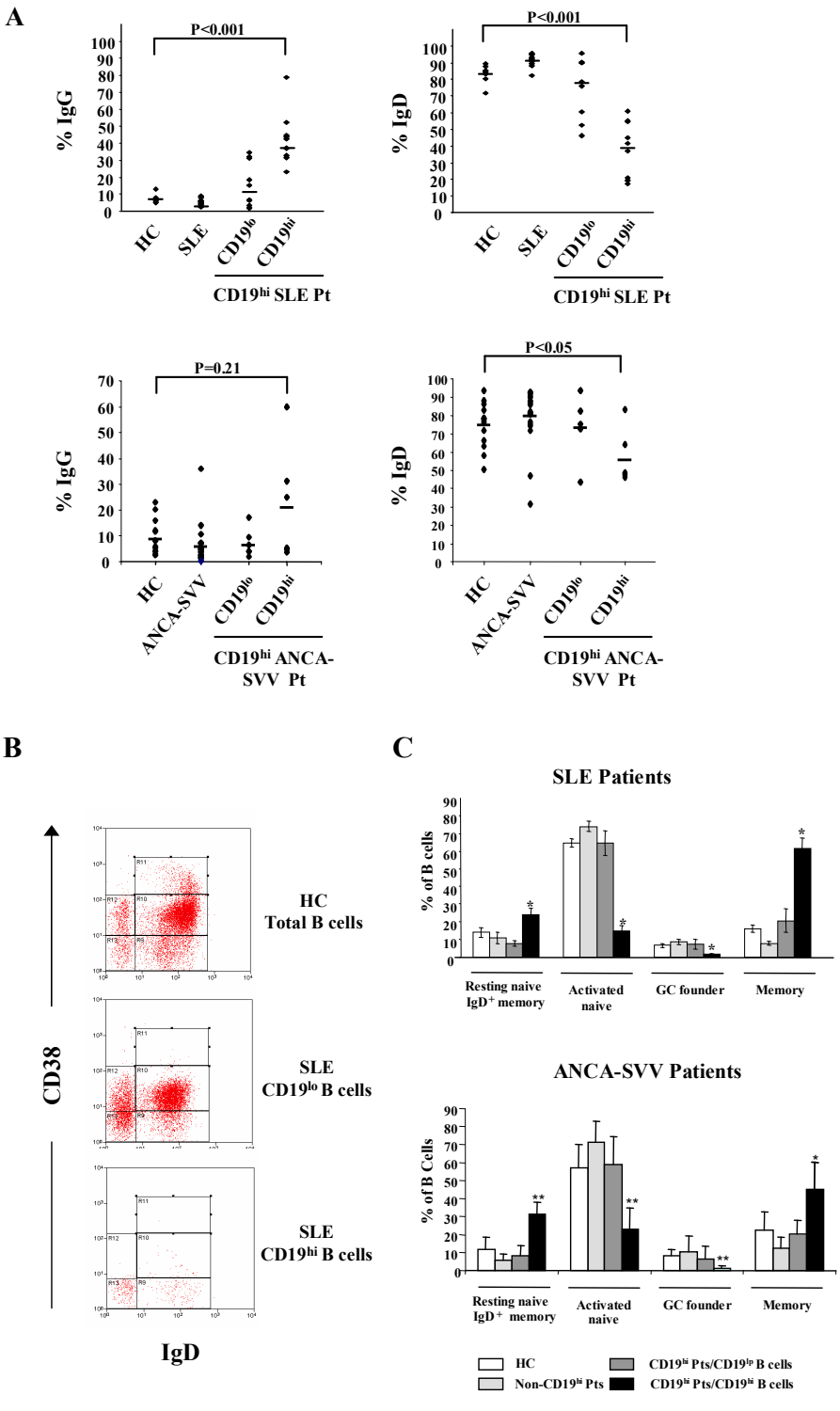

A
P<0.001
% IgG
HC
SLE
CD19lo
CD19hi
CD19hi SLE Pt
P<0.001
% IgD
CD19hi SLE Pt
P=0.21
% IgG
ANCA-SVV
CD19hi ANCA-SVV Pt
P<0.05
% IgD
CD19hi ANCA-SVV Pt
B
HC
Total B cells
SLE
CD19lo B cells
SLE
CD19hi B cells
CD38
IgD
C
SLE Patients
% of B cells
Resting naive IgD+ memory
Activated naive
GC founder
Memory
ANCA-SVV Patients
% of B Cells
HC
Non-CD19hi Pts
CD19hi Pts/CD19lo B cells
CD19hi Pts/CD19hi B cells

Figure 2.4. CD19^{hi} B cells are somatically mutated and antigen selected.

Single CD19hi B cells were sorted according to the gating shown in Fig. 2.1A. (A) Percent VH family gene use by μ (N=20) and γ (N=19) sequences is shown. (B) Summary of somatic mutations of SLE CD19^{hi} B cells are illustrated. IgM and IgG sequences are shown separately. The number of sequences with zero, 1-10, or >10 mutations is graphed. The number of sequences within each group is given. (C) Shared independent mutations are shown from sequences encoded by the same gene. The genes expressed are given and each mutation is identified with a vertical line. Unshared mutations are not shown.

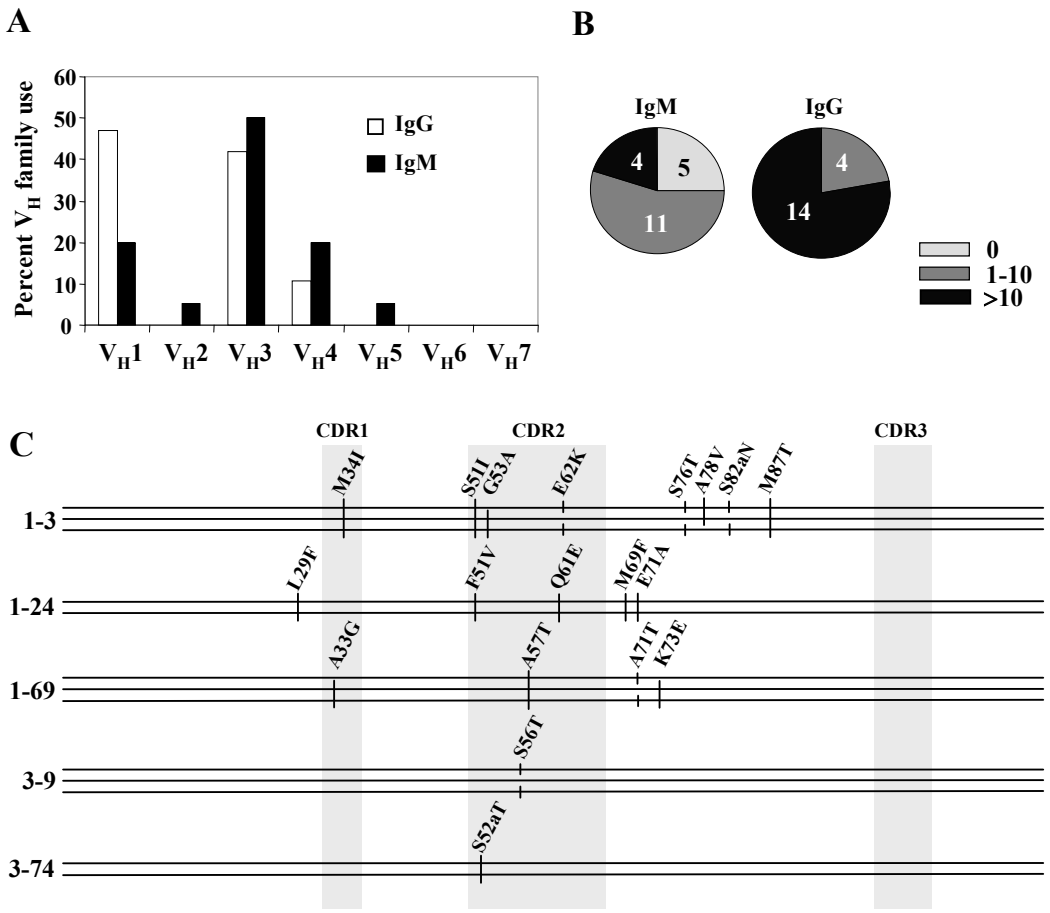


Figure 2.5. CD19^{hi} B cells of SLE and ANCA-SVV Pts have upregulated activation markers.

Overlays of the indicated cell surface molecules from a HCs and CD19^{hi} SLE Pt (top row) and ANCA-SVV Pt (bottom row). For the Pt, the CD19^{lo} and CD19^{hi} B cells are shown separately. The dashed line represents HC B cells, the thin solid line represents CD19^{lo} B cells from the Pt, and the dark solid line represents CD19^{hi} B cells from the Pt. Histograms are representative of 3 or more Pts and HCs.

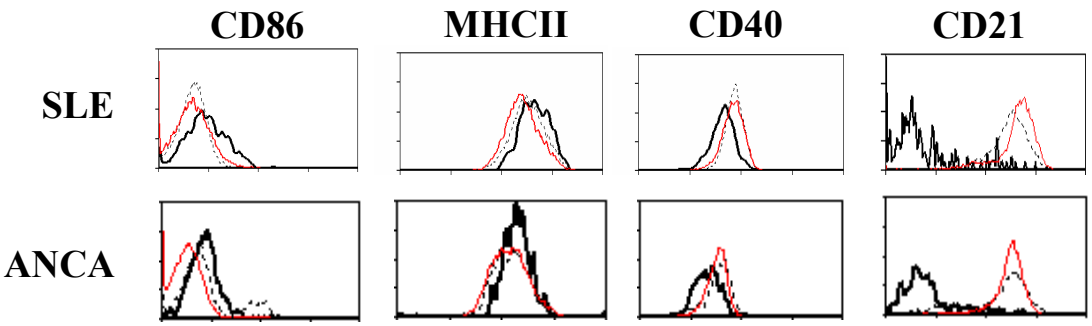


Figure 2.6. HC memory B cells have higher CD19 expression than naïve B cells.
(A) CD19 expression on naïve B cells (IgD^+ $CD38^+$, dashed line) and memory B cells (IgD^- $CD38^{lo/-}$, thin black line) from a representative HC (top panel), on naïve and memory B cells of an SLE Pt that does not have a $CD19^{hi}$ population (middle panel), and on naïve B cells (dashed line), $CD19^{lo}$ memory B cells (thin black line), and $CD19^{hi}$ memory B cells (thick black line) from an SLE and ANCA Pt with a $CD19^{hi}$ population (bottom panel) are shown. All samples were collected and stained on the same day. Vertical lines mark the CD19 MFI of the HC memory B cells and are in identical positions in all three histograms. (B) The average CD19 MFI of HC naïve and memory B cells (n=10). The difference between the two populations is significant (P=3x10^{-5}; Student's t test).

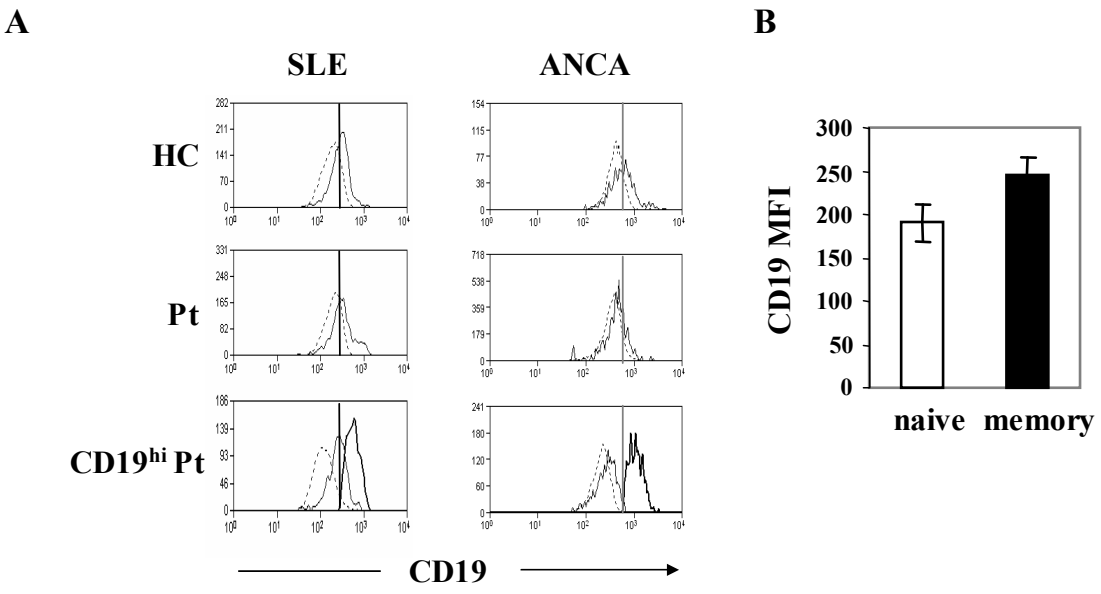
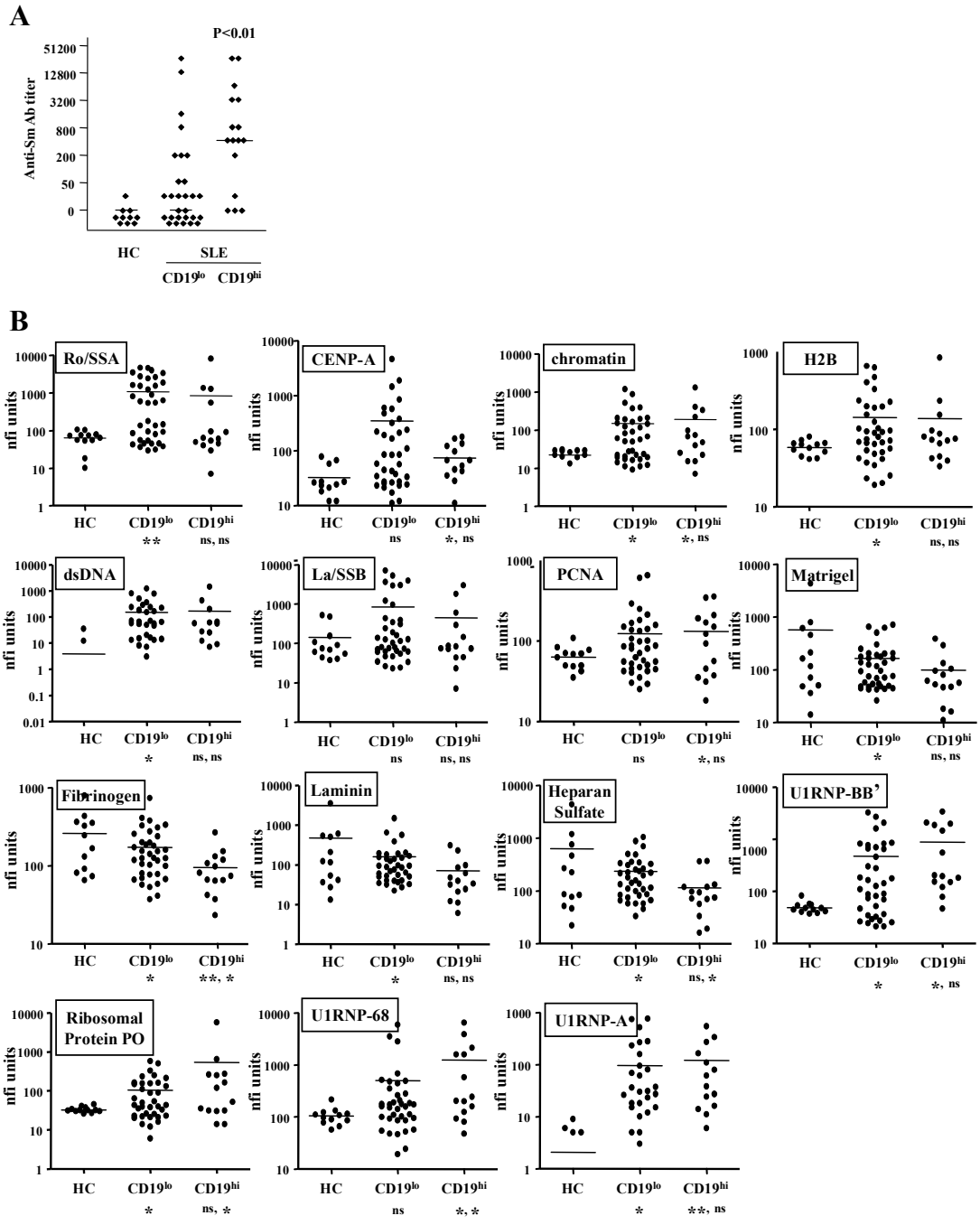


Figure 2.7. CD19^{hi} Pts have a distinct autoantibody profile.

(A) CD19hi Pts have a significantly elevated anti-Sm titer compared to CD19^{lo} Pts and HCs (P<0.01 for each comparison; Student's t test). Each symbol represents an individual Pt. The median titer for each group is marked by a horizontal line. (B) IgG autoantibody profiles were determined from sera of HCs, CD19^{lo} SLE Pts and CD19^{hi} SLE Pts using autoantigen proteome arrays bearing 67 different nuclear or glomerular autoantigens, as previously described(341). Fluorescent intensities were normalized using human IgG standard and presented as normalized fluorescent intensity (nfi) units. Each dot represents serum from an individual HC/Pt. Horizontal lines indicate group means. Listed below the x-axis are the student's test P-values when CD19^{lo} was compared to HC, or when CD19^{hi} was compared to HC or to CD19^{lo}, respectively (*, $P < 0.05$; **, $P < 0.01$; ns, not significant). The serum IgG levels for CD19^{lo} and CD19^{hi} Pts (Avg ± SD) were significantly different from HCs (P= 0.042 and 0.010, respectively, Student's t test), but not from each other. They were as follows: HCs, 5.63 ± 1.26 mg/ml; CD19^{lo} Pts, 8.64 ± 4.06 mg/ml; CD19^{hi} Pts, 8.82 ± 5.01 mg/ml. (C) IgM autoantibody levels against the same autoantigen proteome arrays as in (B). Fluorescent intensities were normalized using human IgM standard and presented as nfi units. Each dot represents serum from an individual HC/Pt. Horizontal lines indicate group means. The serum IgM levels were not significantly different by Student's t test and are given as Avg ± SD: HC, 546 ± 224 μg/ml; CD19^{lo}, 496 ± 346 μg/ml; CD19^{hi}, 455 ± 396 μg/ml.

Figure 2.7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P<0.01
Anti-Sm Ab titer
51200
12800
3200
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SLE
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CD19hi
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chromatin
H2B
dsDNA
La/SSB
PCNA
Matrigel
Fibrinogen
Laminin
Heparan Sulfate
U1RNP-BB'
Ribosomal Protein PO
U1RNP-68
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Figure 2.7, continued.

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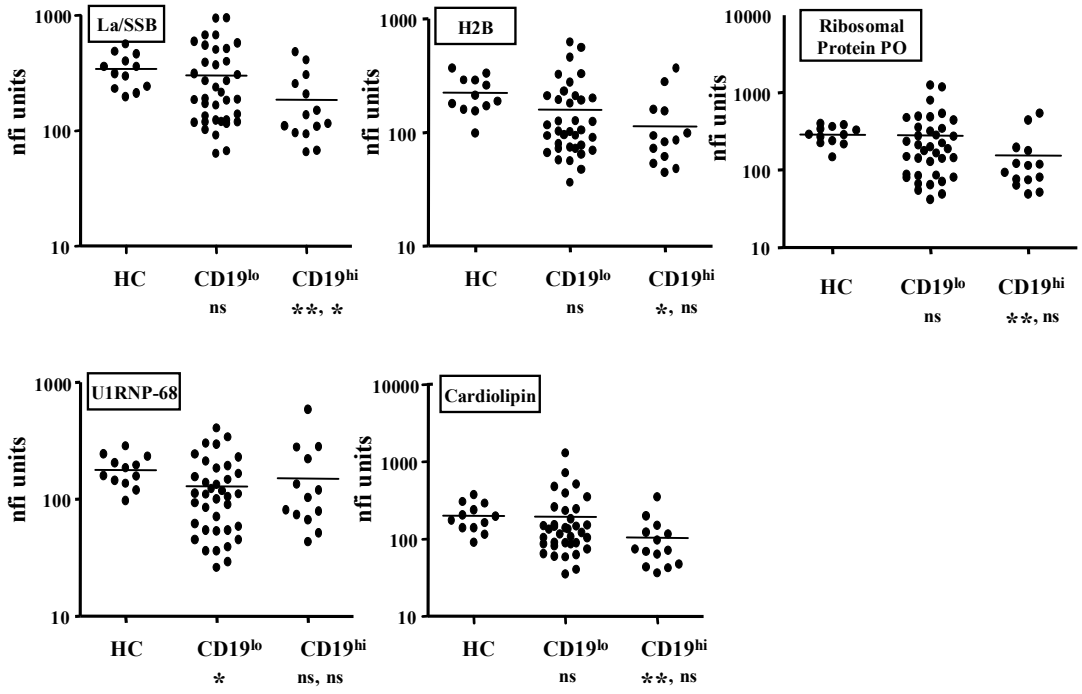


CHAPTER 3: A UNIQUE SUBSET OF MEMORY B CELLS IS ENRICHED IN AUTOREACTIVITY AND CORRELATES WITH ADVERSE OUTCOMES IN SLE

I. ABSTRACT

We previously reported that a subset of systemic lupus erythematosus (SLE) patients have an expanded population of memory B cells with >2-fold increased expression of CD19 ($CD19^{hi}$). We show here that the presence of $CD19^{hi}$ B cells correlates with long-term adverse outcomes, notably neurological complications and end stage renal disease, and with poor clinical responsiveness to treatment with rituximab. B cells specific for the Smith (Sm) autoantigen are enriched in the $CD19^{hi}$ population, and this enrichment correlates with the serum anti-Sm titer. Functional analysis and transcription factor expression suggests that $CD19^{hi}$ B cells are at an early stage in plasma cell differentiation and are not anergic. Interestingly, they have high basal levels of phosphorylated Syk and ERK1/2, indicating ongoing signaling, possibly through the B cell receptor complex. They express high levels of CXCR3, but low levels of CXCR4 and CXCR5, and chemotax towards the CXCR3 ligand CXCL9, but not the CXCR4 ligand CXCL12, suggesting that they are homing to sites of inflammation rather than to the bone marrow. We conclude that the $CD19^{hi}$ B cells are precursors to autoantibody producing plasma cells, and that this population identifies a subset of SLE patients likely to experience poor clinical outcomes.

II. INTRODUCTION

Systemic lupus erythematosus (SLE) is a severe, autoantibody-mediated disease affecting multiple organ systems. The etiology is poorly understood, but has both genetic and environmental components. Because anti-nuclear antibodies (ANA) are pathogenic in this disease(331, 332), B cells are thought to play a pivotal role in its development and progression. Apart from their role as producers of antibody, B cells may also mediate SLE development and pathogenesis through their actions as antigen presenting cells (APCs) and producers of cytokines(220, 230, 305, 332, 354-357). Thus, understanding how B cell self-tolerance is broken in SLE is important to the development of new therapeutic approaches.

No unique precursors of autoantibody producing cells have been identified in SLE patients, suggesting that autoreactive cells are indistinguishable from cells recognizing foreign antigens. Human autoantibodies are predominantly IgG and somatically mutated, suggesting that they are the product of germinal center responses. Both class-switched (IgD^-) and IgM (IgD^+) memory B cells have been described in human tonsil, spleen, and circulation, and are typically $CD38^-$ and $CD27^+$, although CD27- subsets have been described(199, 200). $CD19^{lo}CD38^{hi}$ plasmablasts have also been identified in human blood, and both of these populations are relatively expanded in SLE patients(307, 315). Upon re-encounter with their cognate antigen, memory B cells proliferate, after which some differentiate into plasma cells (PC) and others regenerate the long-lived memory population.

B cell activation is dependent on multiple factors, including specificity and affinity for antigen, and the function of co-receptor molecules that act to amplify or dampen signals through the BCR. Balance between these negative and positive modulators is important in

establishing B cell tolerance. CD19 is a BCR co-receptor that augments BCR signaling and has become of significant interest in autoimmunity. It is a component of a multimeric protein complex that includes CD21, CD81, and Leu-13(133). It also serves as a signaling partner for other receptors, including CD21, CD40, CD38, CD72, VLA-4, and FcγRIIB(132, 333, 358-360). CD19 is expressed by all B cells beginning at the pro-B stage, but is lost along with many other B cell-specific proteins upon plasma cell differentiation(358). Alterations in CD19 expression by B cells affect their function and differentiation. In the absence of CD19, B cells are hyporesponsive to BCR signaling and generate weak responses to T-dependent antigens with dramatically reduced germinal center formation; conversely, a high density of CD19 confers hypersensitivity to BCR signaling and vigorous immune responses(361, 362). In mice, as little as a 15% increase in CD19 expression induces a loss of B cell tolerance and autoantibody production(132, 152, 297). Thus, CD19 is critical to setting the threshold levels for the induction of B cell tolerance and activation.

How CD19 functions in B cell activation has been intensely investigated. Crosslinking of CD19 or the BCR results in rapid phosphorylation of the cytoplasmic tail of CD19 leading to a processive amplification of Lyn phosphorylation(363). Activated Lyn phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) on CD79a and CD79b, the signaling subunits of the BCR, which provides a binding site for the Src family tyrosine kinase Syk(85, 150, 363, 364). Syk is a key protein tyrosine kinase in B cells, since the loss of Syk results in a block in further signaling downstream of the BCR and a block in B cell development(128, 129). These initial events lead to the formation of a BCR signalsome, a complex of multiple protein tyrosine kinases and adapter proteins on the inner surface of the plasma membrane that induces in the release of intracellular Ca^{++} and the

activation of protein kinase C (PKC) and numerous downstream kinases. This cascade leads to the activation of the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase (ERK), c-JUN NH2-terminal kinase (JNK), and p38 MAPK(85), which induce the transcription of relevant genes. The profile of the genes activated by MAPKs determines cell fate by controlling proliferation, survival, and differentiation.

In addition to its role in Lyn phosphorylation, CD19 contributes to B cell activation by the recruitment phosphatidylinsoitol 3-kinase (PI3K) to the BCR. Activated PI3K is critical to the release of intracellular Ca^{++} and PKC activation, and therefore MAPK activation, and to the activation of the Akt pathway, a B cell survival and metabolic fitness pathway. CD19 signals also synergize with BCR signals by a mechanism independent of Ca^{++} and PKC to enhance the activation of the MAPK ERK1/2(151). Thus, CD19 contributes to B cell activation by inducing the activation of multiple pathways crucial to B cell function in response to antigen stimulation.

Altered CD19 expression is evident in human autoimmunity. Systemic sclerosis patients have a 20% increase in CD19 expression(297), while SLE patients have decreased CD19 expression compared to healthy control B cells(296, 297, 365). In addition, we, and others, have shown a population of B cells which have 2-3 fold increased levels of CD19 compared to other B cells from the same patient or to HC B cells($CD19^{hi}$ cells) in SLE, ANCA-SVV, and CVID patients(283, 298, 365). We previously reported that these $CD19^{hi}$ B cells have an activated memory phenotype, are class switched, and show evidence of antigen selection(365). We propose that the increased CD19 expression on $CD19^{hi}$ memory B cells decreases their activation threshold leading to enhanced proliferation, survival, and plasma cell differentiation. If the $CD19^{hi}$ memory B cell subset contains autoreactive B cells,

then a lower threshold of activation may lead to chronic activation and production of autoantibody producing plasma cells.

Herein we show that the $CD19^{hi}$ population is enriched in autoreactive B cells, and that the degree of enrichment correlates with autoantibody titers. Consistent with increased autoreactivity, their presence correlates with poor clinical outcomes and with a poor clinical response to B cell depletion by rituximab. Analysis of these cells suggests that they are at an early stage in plasma cell differentiation, are not anergic, and are likely to be homing to sites of inflammation rather than the bone marrow. Thus, $CD19^{hi}$ B cells appear to constitute a population unique to autoimmunity that contribute to autoantibody production and that may play a pivotal role in disease pathogenesis.

III. MATERIALS AND METHODS

HC and Pt Clinical Samples and Data

PB samples were collected from 10 HCs (mean age: 36.5 yrs, range: 27-54, gender: 8 female, 2 male) and 10 Pts (mean age: 37 yrs, range: 23-53, gender: 10 female); in some cases, the same HC or Pt was used in multiple occasions or experiments. Five of the 10 Pts from this study were patients continuing from the original study(365), and the other five were newly identified. Pts were included in this study after informed consent in accordance with our institutional internal review board, and fulfilled at least four of the established American College of Rheumatology 1997 revised criteria for SLE. Samples were gathered during routine clinic visits, and sera obtained from the same blood draw as that for B cell analysis.

Table 1 is a prospective analysis of clinical outcomes of patients remaining in our study since the previous publication(365) and details such as their medications can be found in that manuscript. Clinical outcomes were determined by review of patient records.

PB samples were collected into Vacutainer CPT tubes with sodium heprin (BD Biosciences) and sera and PBMCs isolated as per manufacter's protocol. PBMCs were washed once with sterile PBS before continuing with any protocol.

Flow Cytometry, Signaling Studies, and FACS

For surface staining, cells were prepared and stained as previously reported(365). Signaling and phosphoprotein studies were carried out using the BD Phosflow system as per manufacturer's Protocol 1 (BD Biosciences, San Diego, CA). Briefly, cells were resuspended at $2.5x10^6$ cells/mL in RPMI + 2% FBS and equilibrated at 37°C for at least 20 min. An equal volume of pre-warmed, 2x solution of goat F(ab')2 α-human IgG (10ug/mL final,

Southern Biotech, Birmingham, AL) or Pansorbin (0.1% final, Calbiochem, La Jolla, CA) was then added with vortexing, and cells were returned to 37°C for 10 min. Cells were then immediately fixed by addition of pre-warmed Phosflow Fix Buffer 1, and Protocol 1 followed for permeabilization and staining. Antibodies used were: pSyk-PE, pERK-PE, Syk-FITC, pan ERK, pJNK, p-p38-FITC, Ki-67-FITC, CD19-APC, CD20-PE and -APC-Cy7, and CD72-FITC (BD Biosciences, San Diego, CA); pAkt (Thr308) and pAkt (Ser473)-Alexa488 (Cell Signaling Technology, Boston, MA); CD180-PE (RP105), BR3-FITC, TLR4-PE, and TLR9-PE (eBioscience, San Diego, CA). Unlabeled antibodies were labeled using Zenon technology (Invitrogen, San Diego, CA). Appropriate pre-labeled or Zenon labeled isotype controls were used in each experiment.

For PI staining, $5x10^5$ cells were aliquoted, washed with cold PBS, resuspended in 0.5mL cold PBS and permeabilized with 4.5mL ice cold 70% ethanol for 2 hrs at -20°C. Cells were then pelleted, washed with cold PBS, and resuspended in 750uL of a solution with 0.2 mg/mL RNase A, 0.02 mg/mL propidium iodide (PI), 0.1% Triton X-100 (all from Sigma-Aldrich, St. Louis, MO) and αCD20-FITC (cytoplasmic domain, from BD Pharmingen, San Diego, CA) in PBS and incubated for 1 hr on ice. Cells were then washed with PBS and analyzed by flow cytometry.

For FACS, cells were prepared as described above and stained on ice with some or all of the following, depending on the experiment: CD19-APC, CD20-APC-Cy7, IgD-FITC, and CD38-PE-Cy7 (BD Pharmingen, San Diego, CA). Cells were sorted using settings to obtain maximum purity on one of two high-speed sorters (MoFlo, Dako Cytomation or FACSAria, BD Biosciences). Post-sort analysis determined purity of populations at >90%.

RNA Extraction and Real Time PCR

Sorted cell populations were spun down, supernatant removed, and frozen at -80°C until RNA extraction using RNeasy mini columns (Qiagen, San Deigo, CA) as per manufacturer's protocol. The reaction for generation of cDNA was carried out using a poly-A primer and SuperScriptII Reverse Transcriptase (Invitrogen, San Diego, CA) according to manufacturer's protocol.

Real time PCR was carried out using TaqMan Gene Expression Assays, with β-Actin as an endogenous control, as per manufacturer's protocol (Applied Biosystems, Foster City, CA) on an ABI Prism 7000 Sequence Detection System machine. Data were analyzed using ABI Prism 7000 software relative quantification study parameters. In each case, transcript expression, normalized to β-actin expression, in $CD19^{hi}$ cells is quantified relative to expression in $CD19^{lo}$ cells from the same Pt.

ELISpot

Anti-Sm and anti-IgG ELISpot protocols were adapted from those used in this lab for mouse B cells(232). Briefly, 96-well filter plates were coated with diluted Sm protein (Immunovision, Springdale, AZ) or anti-human IgG (Bethyl Laboratories, Montgomery, TX). Sorted cells were resuspended in DMEM supplemented with 10% FBS, 100U/mL Penicillin/Streptomycin (Gibco Invitrogen, San Diego, CA), 0.1% β-mercaptoethanol (Gibco Invitrogen, San Diego, CA), and 40ug/mL transferrin (BD Biosciences, San Diego, CA) and plated between $5x10^4$ and $6x10^5$ cells in 200uL/well either without stimulation or stimulated with 15ug/mL anti-CD40, 100U/mL rhIL-2, 100U/mL rhIL-10, (BD Pharmingen, San Diego, CA), and 0.01% Pansorbin (Calbiochem, La Jolla, CA) as previously described for maximal

stimulation of antibody secretion(366). In each plate, $CD19^{hi}$ and $CD19^{lo}$ cells, stimulated and unstimulated, were plated on both Sm and IgG coated wells and incubated for 6 days at 37°C with 5% $C0_2$. They were then washed as previously described and incubated with an anti-human IgG-HRP antibody (Bethyl Laboratories, Montgomery, TX) overnight at 4°C. The plates were then developed and read as previously described(232). Counts were normalized to spots per million cells based on the number of cells plated in that well.

Elisas

The sCD21 Elisa was carried out with frozen serum samples from the Pts and HC analyzed in our previous study(365) using a sCD21 Elisa kit (Cell Sciences, Canton, MA) according to manufacturer's protocol.

The α-Sm Elisa was carried out as previously described(365) using serum samples obtained from the same draw as the cells sorted and plated in ELISpot. All four Pt samples were run on the same plate.

Chemotaxis Assay

Chemotaxis assays were performed as previously reported for human PB B cells(211). Briefly, 5uM pore size transwell inserts (Costar, Corning, NY) were coated with human fibronectin (Sigma-Aldrich, St. Louis, MO) and washed. 100μL of $5x10^6$ cells/mL purified PBMCs in RPMI 1640 (Gibco, Invitrogen, San Deigo, CA) with 0.5% BSA (Sigma-Aldrich, St. Louis, MO) were added to the upper well and 600μL of media alone or with the appropriate chemokine were added to the lower well. Chemokines used were CXCL9/MIG at 10nM and CXCL12/SDF-1β at 100nM (R&D Systems, Minneapolis, MN). Plates were

incubated at 37°C in 5% CO_2 for 90 minutes and migration assessed by staining and flow cytometry of cells in upper and lower wells.

IV. RESULTS

The presence of CD19^{hi} B cells correlates with adverse clinical outcomes.

We previously reported that the presence of CD19^{hi} B cells did not correlate with disease activity or any specific feature of SLE except for severe neurological dysfunction (seizures or psychoses), which was seen in 2 of 14 CD19^{hi} SLE patients and none of 27 CD19^{lo} SLE patients(365). We have now followed the same patient cohort over a five-year period to determine whether there is a correlation with long-term clinical outcome. As seen in Table 1, the presence of a CD19^{hi} population was again significantly associated with severe neurological involvement, as two additional CD19^{hi} SLE patients developed this complication, whereas none of the CD19^{lo} SLE patients exhibited neurological symptoms. In addition, there was a significant correlation with end stage renal disease (ESRD), and overall presence of any one of four adverse outcomes (severe neurological complications, ESRD, thrombic thrombocytopenic purpura (TTP), or death due to disease complications). Taken together, these findings suggest that presence of the CD19^{hi} memory population identifies a subset of SLE patients that are likely to develop more severe disease, and suggests that these cells play a role in neurologic and renal pathogenesis.

CD19^{hi} patients respond poorly to rituximab.

Given the increased frequency of severe complications among CD19^{hi} patients, we determined how these patients responded clinically to B cell depletion therapy with rituximab, a humanized anti-CD20 monoclonal antibody. In SLE, the response to rituximab is variable; some patients experience long-term clinical remission, while others experience

short-term remission or no clinical response(315, 321, 367). In a survey of 17 SLE patients treated with rituximab, five possessed a $CD19^{hi}$ population either before or after treatment with rituximab based on having a frequency of $CD19^{hi}$ B cells that exceeded two-times the standard deviation above the mean percentage in healthy controls. Clinical response to rituximab was defined as an improvement in the SLAM or SLEDAI of at least 3. Clinical response duration was defined as the time point where these disease activity indexes began to increase (relapse occurred), with the SLAM and/or SLEDAI increasing by at least 3 points and/or an increase in steroids required. As shown in Fig. 1, four $CD19^{hi}$ patients were short-term responders to rituximab (9 months or less), and one was a non-responder. In contrast, almost half (5 of 12) of $CD19^{lo}$ patients experienced a 12-month or longer remission, and of these, three had an extended, ongoing clinical response to rituximab treatment. The remaining seven were short term or non-responders to treatment. Thus, rituximab may be a less efficacious treatment for SLE patients with an expanded $CD19^{hi}$ population than for SLE patients that lack this population.

The $CD19^{hi}$ population is enriched in autoreactive B cells.

To determine whether $CD19^{hi}$ B cells can contribute to autoantibody production, we tested whether the $CD19^{hi}$ population is enriched in autoreactive B cells. Since the presence of a $CD19^{hi}$ population is strongly associated with a high anti-Smith (Sm) serum titer(365), we measured the frequency of anti-Sm IgG B cells in the $CD19^{hi}$ and non-$CD19^{hi}$ ($CD19^{lo}$) populations by ELISpot. We sorted $CD19^{hi}$ and $CD19^{lo}$ B cells from four SLE patients, three of whom had elevated anti-Sm titers, and induced ASC differentiation with pansorbin, anti-CD40, IL-4, and IL-10 for 6 days on ELISpot membranes coated with purified Sm protein.

No anti-Sm antibody secreting cells were observed among the $CD19^{hi}$ or $CD19^{lo}$ B cells from the patient that lacked an anti-Sm titer. However, significantly more anti-Sm IgG secreting cells were present among $CD19^{hi}$ B cells than among $CD19^{lo}$ B cells ($p=0.01$, Fig. 2A) from the remaining three patients. Importantly, the frequency of anti-Sm ASCs within the $CD19^{hi}$ population correlated exponentially with the serum anti-Sm titer ($R^2=0.99$ and $R^2=0.4$, respectively, Fig. 2B). Since a two-fold increase in anti-Sm B cell frequency within the $CD19^{hi}$ compartment is associated with an ~100-fold increase in serum anti-Sm, not only are $CD19^{hi}$ cells likely to be direct precursors to autoantibody secreting plasma cells, but they are likely to clonally expand before differentiating, and may also play a role in skewing the immune response towards particular autoantigens, such as through activation of cognate T cells.

$CD19^{hi}$ cells have a unique basal phosphorylation state.

To determine whether increased CD19 alters BCR signaling, we measured basal and BCR stimulated levels of phosphorylated signaling molecules. We compared the levels of phosphorylated CD19, Syk, Akt, and the MAPKs ERK1/2, JNK, and p38 in $CD19^{hi}$ B cells with those of $CD19^{lo}$ B cells from the same patient and healthy control (HC) B cells. To detect phosphorylated proteins in these cells we used flow cytometry, since the limited numbers of $CD19^{hi}$ B cells that can be obtained precluded the use of standard western blotting methods. Flow cytometry had the added benefit of also determining the frequency of B cells within a population that exhibited increased phosphorylation of a given protein.

To measure basal phosphorylation levels of these proteins, we stained for their presence immediately following purification of peripheral blood monocytes (PBMCs). In

each case a HC blood sample was stained at the same time. There was a modest but significant increase in CD19 phosphorylation (pCD19) in $CD19^{hi}$ B cells compared to $CD19^{lo}$ (average=1.5 fold), but the increase in total CD19 expression was significantly greater (average=2.9 fold, Fig. 3A). Thus, a smaller proportion of CD19 was phosphorylated in $CD19^{hi}$ B cells compared to $CD19^{lo}$ cells. Because CD19 phosphorylation amplifies Syk activation(131), we were surprised to find that despite a lower frequency of CD19 phosphorylation, nearly all $CD19^{hi}$ B cells displayed significantly elevated levels of phosphorylated Syk (pSyk) compared to the $CD19^{lo}$ B cells from the same patient and from HC B cells (Fig. 3B-D). The median fluorescence intensity (MFI) of pSyk was ~3.2-fold greater than that for $CD19^{lo}$ B cells from the same patient. Likewise, nearly all $CD19^{hi}$ B cells exhibited significantly elevated levels of pERK1/2 than $CD19^{lo}$ B cells from the same patient and from HC B cells (Fig. 3B-D). $CD19^{hi}$ B cells had a MFI of pERK1/2 that was ~2.9-fold greater than $CD19^{lo}$ B cells from the same patient. Levels of total Syk (average=1.8 fold) and ERK (average=1.4 fold) protein were also elevated in $CD19^{hi}$ as compared to $CD19^{lo}$ B cells (Fig. 3C and D), but they were significantly lower than increases in pSyk and pERK1/2 levels (Fig. 3D). It should be noted that the pan-ERK antibody recognizes other ERK family members in addition to ERK1/2, and therefore the increased ERK levels detected by this antibody may have overestimated the increase in ERK1/2 levels. In contrast to Syk and ERK, we observed no significant increases in basal phosphorylation levels of Akt or the MAP kinases p38 or JNK. In the case of Akt, no increase in phosphorylation was detected at either phosphorylation site (Fig. 3D). Thus, *ex vivo* $CD19^{hi}$ B cells exhibit an unusual phenotype. Despite a lower frequency of phosphorylated CD19,

nearly all cells exhibiting increased levels of pSyk and pERK1/2, but not increased phosphorylation of other MAPKs or Akt downstream of the BCR and CD19.

$CD19^{hi}$ B cells are responsive to BCR stimulation.

Since constitutively elevated pERK levels are associated with B cell tolerance(240), we sought to determine whether crosslinking the BCR on $CD19^{hi}$ B cells results in transduction of signal and plasma cell differentiation. To determine whether BCR signaling is intact in $CD19^{hi}$ B cells, HC and SLE B cells were stimulated with anti-IgG or pansorbin for 10 minutes and the levels of phosphorylated molecules determined by flow cytometry. Although the kinetics of phosphorylation may vary for each molecule examined, we sought a time point at which phosphorylation of all measured molecules could be observed in HC and SLE B cells due to limitation of cell number, All five signaling intermediates and CD19 were phosphorylated upon BCR stimulation in HC B cells and SLE $CD19^{lo}$ B cells (Fig. 4A-C), although a somewhat higher percentage of $CD19^{lo}$ B cells than HC B cells phosphorylated these molecules (Fig. 4C) consistent with reports that SLE B cells are hyperresponsive to BCR stimulation(300-302, 304, 365). Importantly, SLE $CD19^{hi}$ B cells increased the levels of phosphorylated CD19, p38, JNK, and Akt similarly to HC and $CD19^{lo}$ cells (Fig. 4A and B). The frequency of $CD19^{hi}$ B cells that increased the levels of each phosphorylated protein was similar to that of $CD19^{lo}$ and HC B cells (Fig. 4C). In contrast, SLE $CD19^{hi}$ B cells exhibited little or no increase in the already high basal levels of pSyk and pERK1/2 (Fig. 4A and B), or in the percentage of cells positive for pSyk and pERK1/2 (Fig. 4C). Interestingly, the basal pSyk and pERK1/2 levels in $CD19^{hi}$ B cells were similar to those reached by HC and SLE $CD19^{lo}$ B cells after BCR crosslinking, suggesting that *ex*

vivo CD19^{hi} B cells have already maximally phosphorylated these molecules. Altogether, these data indicate that CD19^{hi} B cells are not refractory to BCR signaling.

To determine whether CD19^{hi} B cells can be activated to become ASCs, we determined the frequency of IgG antibody-secreting cells by an ELISpot assay before and after stimulation. As shown in Fig. 4D, an average of only 0.05% (SEM 0.03%) of CD19^{hi} cells spontaneously secreted IgG, ~7-fold less than CD19^{lo} B cells (0.34%, SEM 0.16%). However, stimulation with pansorbin, anti-CD40, IL-4, and IL-10, a cocktail previously shown to induce robust IgG secretion in memory cells(366), induced an average of 14.6% (SEM 4.51%) of CD19^{hi} B cells to secrete IgG, nearly twice that seen with CD19^{lo} B cells (7.9%, SEM 2.6%). This may be de to differences in the frequency of IgG^{+} B cells in each population or to an increased propensity of CD19^{hi} B cells to become antibody-secreting cells. Thus, the ability of CD19^{hi} B cells to signal in response to BCR ligation and their ability to differentiate to antibody-secreting cells upon stimulation suggests that CD19^{hi} B cells are not anergic.

CD21 expression is downregulated in CD19^{hi} cells.

We previously reported the CD21 expression was decreased on the cell surface of CD19^{hi} B cells, while CD21 levels were similar between HC B cells and SLE CD19^{lo} B cells (Fig. 5A)(365). Since CD19 is the signaling component for the complement receptor CD21, we wished to determine if this decreased surface expression was due to increased recycling, cleavage, or downregulation. In some settings, CD21 is cleaved from the surface of activated B cells, forming soluble CD21 (sCD21) which can be detected in serum and in culture supernatants(284, 368). We therefore compared CD21 transcript levels in sorted CD19^{hi}

cells relative to $CD19^{lo}$ B cells from the same patient using relative quantification (RQ) real time PCR (RT-PCR). CD21 transcripts were significantly decreased in all three patients examined (average RQ=0.16, Fig. 5B), suggesting downregulation, and not increased recycling or cleavage, is responsible for decreased CD21 expression by these cells. Consistent with this, although analysis of serum samples from the large cohort of $CD19^{hi}$ patients indicates that CD21 levels were lower in $CD19^{hi}$ patients compared to $CD19^{lo}$ patients and HCs, they were not statistically different (averages of 15.5U/mL, 21.6U/mL 23.2U/ml, respectively). However, sCD21 levels were significantly lower among SLE patients that had an elevated anti-Sm titer (Fig. 5C), in contrast to the previously reported general decrease in sCD21 among SLE patients compared to HCs(285).

$CD19^{hi}$ cells have a pre-plasma cell transcription profile.

Our previous analysis suggested that $CD19^{hi}$ B cells are activated memory B cells. However, the relationship between autoreactive B cell frequency and serum autoantibody titers suggests a close relationship between these cells and the plasma cell pool in these patients. Thus, we sought to determine whether these cells were at an early stage in plasma cell differentiation by measuring the levels of B cell- and plasma cells-specific transcription factors by RQ RT-PCR. RNA from sorted $CD19^{hi}$ and $CD19^{lo}$ B cells from three patients was used to determine expression levels of the B cell-specific transcription factors Pax-5 and Bcl-6, and the plasma cell-specific transcription factors Blimp-1, IRF-4 and XBP-1. During differentiation to an ASC, Pax-5 and Bcl-6 are downregulated, and IRF-4, Blimp-1, and XBP-1 are upregulated(369). As shown in Fig. 6A, $CD19^{hi}$ B cells showed significant downregulation of Pax-5 and smaller but consistent downregulation of Bcl-6 compared to

$CD19^{lo}$ B cells (average RQ=0.34 and 0.49, respectively). However, they showed no upregulation of Blimp-1, IRF-4, or XBP-1 expression (Fig 6A). Since >70% of $CD19^{hi}$ cells are IgD-CD38-(365), we sorted $IgD^{-}CD38^{-}$ memory B cells with all other $CD19^{+}$ B cells from three HCs is presented in Fig. 6B. No significant differences were observed between the cells of these populations, including in Pax-5 expression (average RQ=1.4). Thus, the low Pax-5 levels seen in $CD19^{hi}$ B cells are not a general characteristic of memory B cells, suggesting that $CD19^{hi}$ cells are at an early pre-plasma differentiative stage.

Since our findings indicate that $CD19^{hi}$ B cells may clonally expand before becoming plasma cells, we determined whether they were actively undergoing cell division. As expected, $CD19^{hi}$ B cells were not actively undergoing cell division. As shown in Fig. 6A and Table 2, $CD19^{hi}$ B cells did not express high levels of Ki-67, a protein present only in proliferating cells, or exhibit an increase in DNA content, as measured by PI staining. Thus, $CD19^{hi}$ cells are not in S phase. However, $CD19^{hi}$ B cells consistently exhibited a small (average 1.7 fold) increase in Ki-67 staining (Table 2), consistent with cells in G_1 phase or are just entering or leaving S phase(370, 371). Thus, $CD19^{hi}$ B cells are not actively undergoing cell division, indicating the clonal expansion that they undergo before becoming plasma cells occurs after they exit the circulation.

$CD19^{hi}$ B Cells have an unusual chemokine receptor expression profile.

Long-lived plasma cells reside primarily in the bone marrow, and thus to determine whether the $CD19^{hi}$ B cells are likely to be homing to the bone marrow, we compared the transcript levels of CXCR4 and CXCR5 in $CD19^{hi}$ vs. $CD19^{lo}$ cells. CXCR5 retains cells in the follicles of secondary lymphoid tissues, and is normally downregulated during plasma

cell differentiation, while CXCR4 is responsible for directing plasma cell precursors to the bone marrow and is normally upregulated during plasma cell differentiation. As shown in Fig. 7A, CXCR4 transcripts were significantly lower in $CD19^{hi}$ B cells of all three patients (average RQ=0.1), as were CXCR5 transcripts in two of the three patients (average RQ=0.36, overall average RQ=0.7). Similar to $CD19^{hi}$ B cells, HC memory B cells had lower transcript levels of CXCR4 and CXCR5 compared to non-memory B cells (average RQ=0.4 and 0.7, respectively, Fig. 7B). CXCR5 transcripts were similarly decreased in $CD19^{hi}$ and HC memory B cells relative to their respective controls (Fig. 7A and B). However, the relative decrease in CXCR4 transcripts by $CD19^{hi}$ B cells was ~4 times greater than that in HC memory B cells (Fig. 7A and B). We conclude that $CD19^{hi}$ B cells are unlikely to be homing to the bone marrow.

Since $CD19^{hi}$ B cells are unlikely to be homing to the bone marrow, we determined whether $CD19^{hi}$ B cells could be homing to sites of inflammation. CXCR3 levels are elevated on a subset of memory B cells and plasma cells(190, 194, 211), in addition to T cells(192), and directs migration sites of inflammation where CXCR3 ligands are present at high concentrations(194). As seen in Fig. 7A, CXCR3 transcript levels were dramatically higher in $CD19^{hi}$ B cells compared to $CD19^{lo}$ B cells of all three patients tested (average RQ=14.2, range= 7.6-24). CXCR3 transcripts were higher in HC memory B cells compared to non-memory B cells, but this increase was only ~2-fold (Fig. 7B). This increase in transcript levels also results in a significant increase in CXCR3 protein on the cell surface of $CD19^{hi}$ B cells (Fig. 7C). These results suggest that $CD19^{hi}$ B cells are homing to sites of inflammation.

To confirm that increased expression of CXCR3 by $CD19^{hi}$ B cells is functionally significant, we determined the ability of $CD19^{hi}$ B cells to migrate in response to CXCR3 and CXCR4 ligands. As shown in Fig 7D, $CD19^{hi}$ B cells migrated efficiently in response to the CXCR3 ligand CXCL9, but not in response to the CXCR4 ligand CXCL12 or to media alone. These data affirm that $CD19^{hi}$ B cells are unlikely to migrate to the bone marrow and indicate that the increased expression of CXCR3 on these cells is functionally relevant, supporting the idea that $CD19^{hi}$ B cells are homing to sites of inflammation.

V. DISCUSSION

$CD19^{hi}$ B cells are a novel population of memory B cells that are expanded in a subset of SLE and ANCA-vasculitis patients(365). We show here that in SLE, these B cells are a functionally and phenotypically distinct from HC memory B cells and appear to contribute to the autoantibody secreting plasma cell pool. Presence of an expanded $CD19^{hi}$ B cell population may delineate patient subgroups within these diseases, as these patients are more likely to develop neurological problems and end stage renal disease, and to show poor clinical response to rituximab treatment. These findings have implications for our understanding of autoreactive B cells in SLE and for the development of new therapies.

Relevance of $CD19^{hi}$ B cells to autoimmunity is indicated by our finding that this population is enriched 3- to10-fold in anti-Sm B cells compared to the $CD19^{lo}$ population, and that this enrichment correlates with the level of serum anti-Sm antibody. In contrast, the frequency of anti-Sm $CD19^{lo}$ B cells is not different between patients with varying degrees of anti-Sm titer elevation. $CD19^{hi}$ B cells are unlikely to be anergic, as BCR crosslinking induces phosphorylation of CD19, Akt, JNK and p38 to levels comparable to those in $CD19^{lo}$ B cells from the same patient and to HC B cells. Moreover, BCR signaling in conjunction with a CD40 signal and stimulation with IL-4 and IL-10 induces $CD19^{hi}$ IgG B cells to become antibody-secreting cells.

Since $CD19^{hi}$ B cells are present above background in only 25-30% of SLE patients, we presume that anti-self memory B cells are not limited to this population. However, our data suggest that the majority of anti-Sm B cells in the peripheral blood are $CD19^{hi}$ and therefore B cells specific for a given self-antigen may segregate to either the $CD19^{hi}$ or non-

$CD19^{hi}$ memory B cell populations, rather than being present in both. We reported previously that elevated frequencies of $CD19^{hi}$ B cells are associated with increased levels of autoantibodies against certain self-antigens, in particular small nuclear ribonucleoprotein (snRNP) antigens(365), suggesting that B cells specific for these autoantigens are also enriched in the $CD19^{hi}$ population.

Our data indicate that a doubling of the anti-Sm B cell frequency in the $CD19^{hi}$ population is associated with a ~100-fold increase in the serum anti-Sm titer, a relationship which cannot be accounted for by clonal expansion of $CD19^{hi}$ cells alone. This finding is particularly impressive given the overall small number of $CD19^{hi}$ B cells present in the blood (typically <1% of PBMC). Therefore, we hypothesize that $CD19^{hi}$ cells (1) are responsible for the majority of serum anti-Sm antibody, (2) clonally expand before differentiating into anti-Sm ASCs, and (3) also play a role in the amplification of the immune response to Sm, possibly through activation of cognate T cells. This is an intriguing idea given our previous finding that $CD19^{hi}$ cells have increased surface MHCII and CD86 expression(365) and may therefore be highly efficient T cell activators.

Our analysis of basal phosphorylation levels of signaling intermediates, transcription factor expression, and chemokine receptor expression support the hypothesis that $CD19^{hi}$ B cells are actively migrating to the tissues and undergoing plasma cell differentiation. $CD19^{hi}$ B cells have high basal levels of pSyk and pERK1/2, equivalent to those in $CD19^{lo}$ and HC B cells after strong BCR crosslinking (Fig. 4B), suggesting that they have recently received an activating signal. Since Syk phosphorylation is believed to be BCR/CD19 specific, it is likely that these cells have received a recent BCR/CD19 signal. Syk and ERK

phosphorylation in BCR stimulated human IgG B cells are normally sustained for more than two hours(372), suggesting a possible time frame for the receipt of this activating signal.

The absence of elevated basal levels of pAkt in $CD19^{hi}$ B cells seemingly contradicts the involvement of a BCR signal, since BCR signals induce Akt phosphorylation(149). The main pathway for both ERK1/2 and Akt phosphorylation is the recruitment of phosphatidylinositol 3-kinase (PI3K) to the BCR complex by CD19 and its activation by Syk(85, 373, 374). Thus, the phosphorylation of Syk and ERK1/2 suggests that the Syk/PI3K/ERK pathway is active. Our findings could be explained in several ways. Akt phosphorylation may be more transient than ERK or Syk(149) and thus, evidence of Akt phosphorylation may be lost before the cells can be analyzed. Alternatively, Akt phosphorylation may be regulated downstream or independently of PI3K activation in response to the signal received by $CD19^{hi}$ B cells. Indeed, Li and Carter(151) have demonstrated that that CD19 signals synergize with BCR signals to induce ERK1/2 activation at a point downstream of PI3K in B cells. Since Syk phosphorylation is also downstream of CD19 phosphorylation, this could explain the high levels of pSyk in these cells as well.

MAP kinases are intimately involved in cell fate decisions regarding proliferation, apoptosis, and differentiation, and the activation of different combinations of MAP kinases may result in different biological outcomes(375). Of the three MAP kinases examined, ERK1/2, JNK, and p38, only basal pERK1/2 levels were elevated in $CD19^{hi}$ B cells. In the WEHI-231 B lymphoma cell line, BCR signaling preferentially activates ERK1/2, and only weakly activates JNK and p38, whereas CD40 signaling preferentially activates the JNK and p38 MAP kinases and does not activate ERK1/2(376). Thus, the pattern of MAPK activation

in CD19^{hi} B cells is congruent with these cells having recently received a BCR signal. ERK MAP kinases are involved in regulating cell proliferation(375), and the elevated levels of pERK1/2 are consistent with our proposal that CD19^{hi} B cells proliferate before plasma cell differentiation. The absence of increased pJNK and pp38 levels in CD19^{hi} B cells may indicate that they have not received a recent CD40 signal. This could indicate that rather than being of direct germinal center origin, they are memory B cells that have been reactivated in the absence of T cell help. A better understanding of the signals that result in this pattern of MAP kinase activation would aid in illuminating the nature of the activating signals received by CD19^{hi} B cells.

The low Pax-5 expression by CD19^{hi} B cells suggests that CD19^{hi} B cells are at an early stage of plasma cell differentiation. This level of Pax-5 expression is not a characteristic of memory B cells in general, since HC memory B cells express levels of Pax-5 that are not different from non-memory B cells (Fig. 6B). Pax-5 is required for B cell differentiation and activates the expression of genes that maintain B cell identity(369), including CD19. Thus, the low levels of Pax-5 expression by CD19^{hi} B cells suggest that these cells are moving away from a B cell identity. In addition, although CD19^{hi} and CD19^{lo} B cells have similar transcript levels of XBP-1, a transcriptional activator required for plasma cell differentiation, HC memory B cells express considerably less XBP-transcript levels than control B cells, suggesting that XBP-1 has increased in CD19^{hi} B cells. Since Pax-5 represses the expression of XBP-1(377), increased XBP-1 may follow from the decreased expression of Pax-5. We did not observe evidence of increased Blimp-1 transcript levels, and since transcription of *PDRM1*, the gene for human Blimp-1, is repressed by Bcl-6(177), this is consistent with the normal expression of Bcl-6 by CD19^{hi} B cells. Although Blimp-1

is required for full plasma cell differentiation and antibody secretion, it is not required for plasmablast or pre-plasma cell formation. Blimp-1 represses the expression of a number of genes that are responsible for cell division(378), and thus the absence of increased Blimp-1 expression is compatible with the hypothesis that $CD19^{hi}$ B cells undergo multiple rounds of cell division before differentiating to plasma cells. It is important to keep in mind that these measures are relative to $CD19^{lo}$ cells from the same SLE patient (or non-memory B cells for HCs). Thus, differences in the frequency of plasmablasts between patients and HCs would tend to inflate the control levels of plasma cell-specific transcripts and diminish the control levels of B cell-specific transcripts. An increase in plasmablasts in the peripheral blood of SLE patients and other autoimmune disease has indeed been reported(283, 315, 379). Single cell RT-PCR analysis should clarify this issue, and is underway.

Chemokine receptor expression further supports the hypothesis that $CD19^{hi}$ B cells are undergoing plasma cell differentiation. $CD19^{hi}$ B cells from two of the three patients examined had less than half of the CXCR5 transcript levels seen in $CD19^{lo}$ B cells from the same patient. This is consistent with activation, and could account for their presence in peripheral blood. However, it would not appear that these cells are migrating to the bone marrow, a prominent location for long-lived plasma cells. CXCR4 transcripts, which are typically increased during plasma cell differentiation(380), are ~10% of the level present in $CD19^{lo}$ cells, and $CD19^{hi}$ B cells are not responsive to the CXCR4 ligand CXCL12.

A more likely destination for $CD19^{hi}$ B cells are sites of inflammation, guided by high levels of CXCR3. CXCR3 transcripts are ~14-fold higher in $CD19^{hi}$ B cells than in control $CD19^{lo}$ B cells, a finding that is reflected by an increase in CXCR3 surface expression. CXCR3 on these cells is functional, as they chemotax in response to the CXCR3 ligand

CXCL9. A subset of human memory B cells and plasmablasts express CXCR3, and the expression of this chemokine receptor is maintained after plasma cell differentiation(190, 194, 211). The CXCR3 ligands CXCL9, CXCL10 and CXCL11 are highly expressed in peripheral sites of inflammation, where, in autoimmunity and other highly inflammatory states, B cells can differentiate to tissue-resident plasma cells(190, 193, 194, 381, 382). For example, CXCR3 ligands are elevated in the skin of patients with cutaneous lupus(383), the synovium of RA patients(193), and the kidney and serum of SLE patients(384-386). Interestingly, CXCR3 ligands are elevated in the CNS of SLE patients with neurological involvement(387-389) and pre-plasma and plasma cells have been found in the intrathecal tissue of SLE patients with CNS involvement(389) and in the synovial tissue of patients with rheumatoid arthritis(193). Plasma cells were found to accumulate and reside in the inflamed kidneys of autoimmune NZB/W mice(382), and several studies suggest B cells can infiltrate the kidney and contribute to glomerulonephritis in SLE(390, 391). Studies of the synovium of RA patients and in the CNS during viral infection strongly support a vital role for the interaction of CXCR3 on B cells and CXCR3 ligand expression in these tissues for recruitment and retention of plasma cells(193, 195). Thus, we propose that inflamed tissue is the final destination for $CD19^{hi}$ B cells.

The elevated expression of CXCR3 and responsiveness to its ligand CXCL9 suggests a possible mechanism for the increased incidences of ESRD and severe neurological dysfunction in $CD19^{hi}$ Pts. We speculate that some $CD19^{hi}$ cells are homing to the kidney and CNS where they clonally expand and differentiate to plasma cells. Interestingly, a previous study found an association between a positive anti-Sm titer and CNS involvement in SLE(392). Our data suggests that this association may be secondary to an expansion of

$CD19^{hi}$ B cells, since such an expansion correlates with an elevated anti-Sm titer. The pathology resulting from the migration of $CD19^{hi}$ cells into peripheral sites of inflammation might arise from locally high concentrations of autoantibodies. However, B cells can contribute to pathology through mechanisms independent of antibody production, such as production of cytokines and activation of T cells. As mentioned above, the exponential association between enrichment of anti-Sm cells in the $CD19^{hi}$ compartment and the serum anti-Sm titer strongly suggests that such additional mechanisms are taking place. Thus, the presence of pre-plasma cells in these sites may be important for providing signals to other effectors of inflammation.

The presence of $CD19^{hi}$ B cells in a subset of SLE patients may have implications for prognosis and treatment. Although rituximab is a promising new therapy for SLE, not all patients have a robust clinical response. This may be because not all B cell subsets are equally susceptible to depletion. While PB B cells and follicular B cells are depleted efficiently, marginal zone and memory B cells are less responsive to depletion, and plasma cells, which do not express CD20, are not depleted at all(315, 393). We do not yet know how susceptible the $CD19^{hi}$ B cells are to rituximab depletion, but our data suggest that $CD19^{hi}$ patients are poor clinical responders to rituximab. This finding further differentiates $CD19^{hi}$ patients as a distinct subgroup of SLE patients and provides a predictive test for clinical response to rituximab. Sanz and colleagues have recently shown that SLE patients with high titers of antibodies to Sm, snRNP, Ro, and La have decreased responsiveness to rituximab(367). This is consistent with the findings reported here, since the presence of $CD19^{hi}$ B cells is associated with elevated titers of antibodies to Sm and other snRNP antigens, although anti-Ro antibodies were found to be lower in $CD19^{hi}$ patients(365).

Given the increase in adverse outcomes in these patients, it will be important to determine more efficacious therapies for treatment of their disease. Anti-CXCR3 therapy is currently under development for multiple inflammatory diseases, and we surmise that this may be a prudent choice for treatment of $CD19^{hi}$ patients.

Taken together, the data presented here and previously by this lab(365) suggest that the presence of $CD19^{hi}$ cells delineates a subgroup of SLE Pts with increased adverse clinical outcomes, a specific pattern of autoreactivity, and decreased response to Rituximab treatment. Separating heterogeneous diseases, such as SLE, into etiological and pathological subgroups is vital to developing appropriate therapeutic modalities and improving clinical outcomes.

Table 3.1. Clinical outcomes of $CD19^{hi}$ and $CD19^{lo}$ Pts.			
Outcome	**$CD19^{lo}$ Pts**	**$CD19^{hi}$ Pts**	**P vaule**
Severe Neurologic	0% (0/27)	29% (4/14)	0.01
ESRD	4% (1.27)	29% (4/14)	0.04
TTP	7% (2/27)	7% (1/14)	>0.05
Death	4% (1/27)	14% (2/14)	>0.05
Any Adverse	15% (4/27)	64% (9/14)	0.003

27 $CD19^{lo}$ and 14 $CD19^{hi}$ Pts were followed over five years for occurrence of four clinical sequelae of SLE. Fisher's exact test was used to determine significance of a 2x2 contingency table for each outcome. ESRD, end stage renal disease. TTP, thrombotic thrombocytopenic purpura.

Table 3.2. $CD19^{hi}$ cells are not in S phase.			
	Fold Ki-67 in $CD19^{hi}$ vs $CD19^{lo}$ B cells	**% PI high in $CD19^{hi}$ B cells**	**% PI high in $CD19^{lo}$ B cells**
Patient 1	1.78	2.3%	2.8%
Patient 2	1.72	3.0%	3.5%
Patient 3	1.54	2.0%	2.0%

The fold increase in the MFI for Ki-67 staining in $CD19^{hi}$ vs $CD19^{lo}$ B cells from the same patient, as well as percentage of each population falling into a PI high gate, indicating S phase.

Figure 3.1. CD19^{hi} Pts are poor responders to Rituximab treatment.

Clinical response of CD19^{hi} SLE Pts and CD19^{lo} SLE Pts to Rituximab treatment. NR=no response; OG=ongoing response.

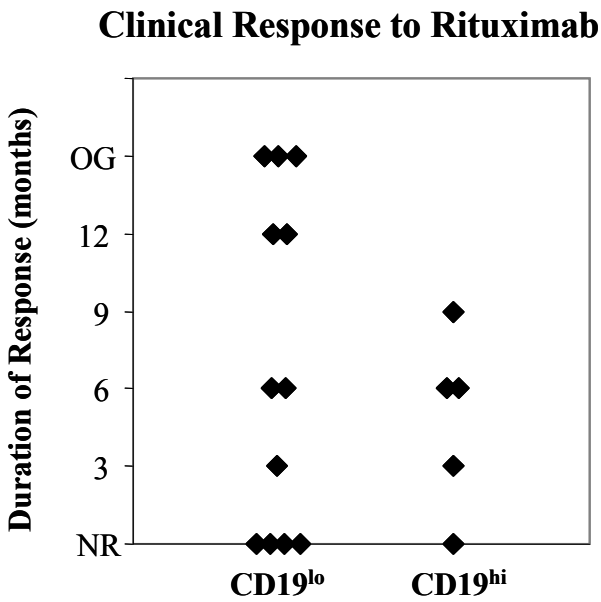


Figure 3.2. CD19^{hi} cells are enriched for autoreactivity, and the degree of autoreactivity in these cells correlates exponentially with serum autoantibody.

CD19^{hi} and CD19^{lo} cells were sorted from four Pts with differing anti-Sm titers, and ELISpot used to determine the number of anti-Sm ASCs in each group. **A**, Anti-Sm ASCs per million CD19^{hi} or CD19^{lo} cells in each of four Pts. Pt 1 did not have detectable serum anti-Sm titer, and no anti-Sm ASCs were seen in either cell group. **B**, Anti-Sm ASCs per million cells in each group vs. $\log_{10}$[anti-Sm serum titer], showing an exponential relationship between the number of anti-Sm ASCs in the CD19hi cell group and the serum titer of anti-Sm.

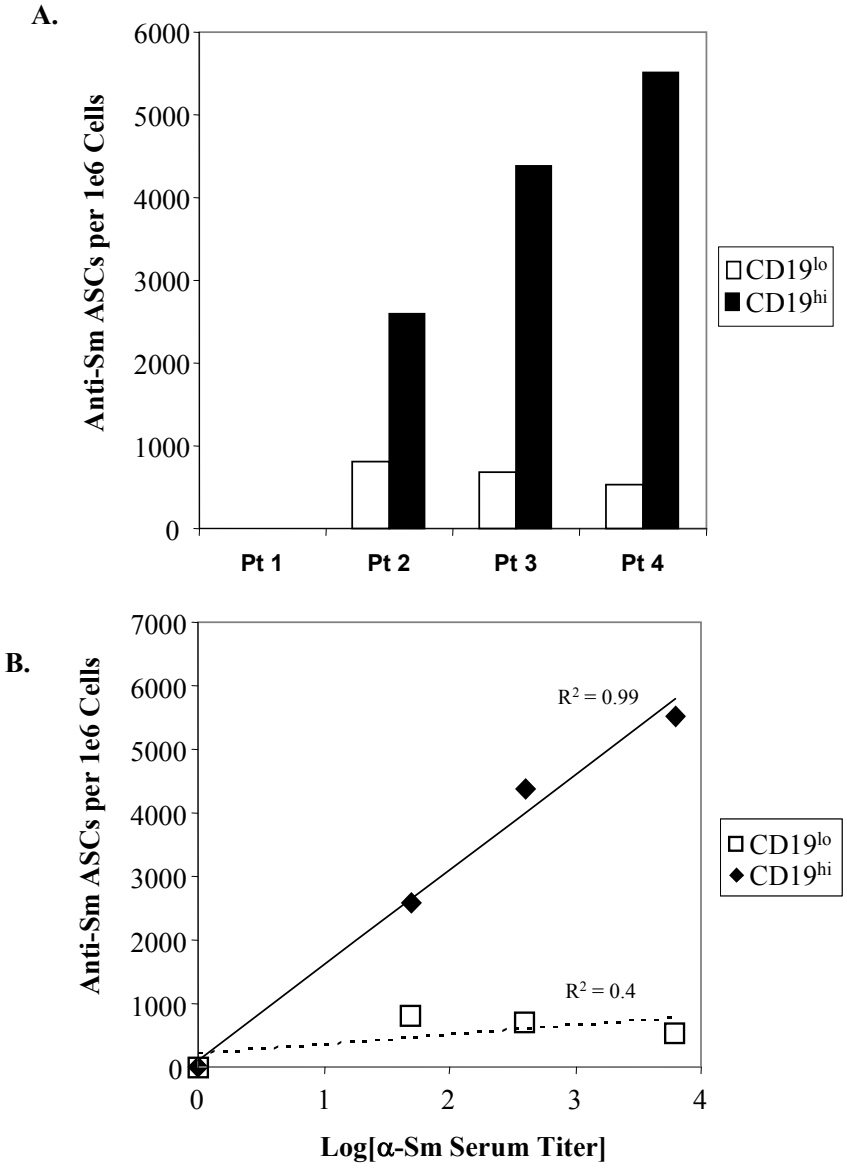
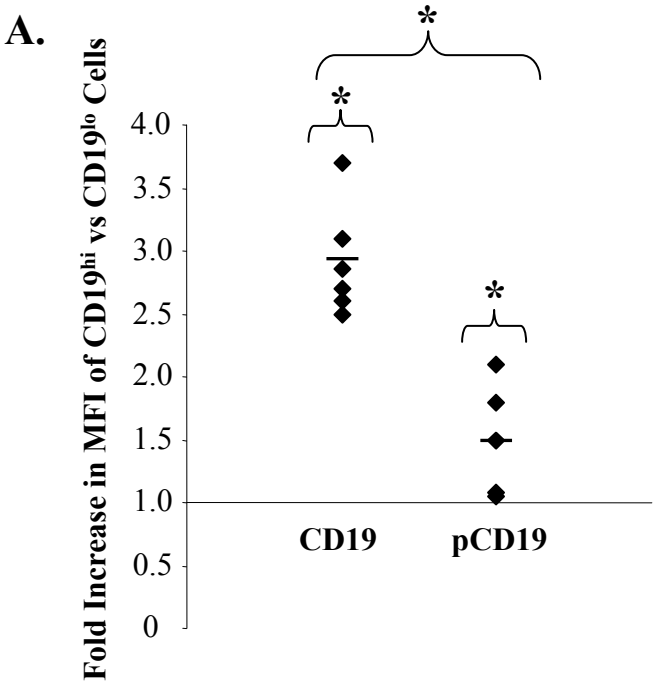


Figure 3.3. Basal phosphorylative state of CD19^{hi} cells.

PB cells were permeabilized and stained using antibodies recognizing the indicated molecules. **A**, Fold increase in MFIs of CD19 or phosphorylated CD19 (pCD19) in CD19^{hi} vs CD19^{lo} cells. **B**, Representative histograms of PB cells from a CD19^{hi} Pt and HC stained for CD19 and pSyk, pERK1/2, or an isotype control. **C**, Representative single parameter histograms showing staining for phosphorylated or total Syk and ERK1/2 in PB from a HC (shaded) and CD19^{lo} (thin line) and CD19^{hi} (thick line) B cells from the same Pt. **D**, Fold increases in MFI for the labeled signaling molecules in CD19^{lo} vs. CD19^{hi} cells from the same Pt. Each dot represents a different Pt and/or day. At least four different Pts are included for each signaling molecule. Significance for single molecules was determined using a 2-sided non parametric rank test. Significance for pCD19/CD19, pSyk/Syk and pERK1/2/ERK comparisons was determined using 2-sided Wilcoxon rank-sum tests. *p<0.05, **p<0.01.



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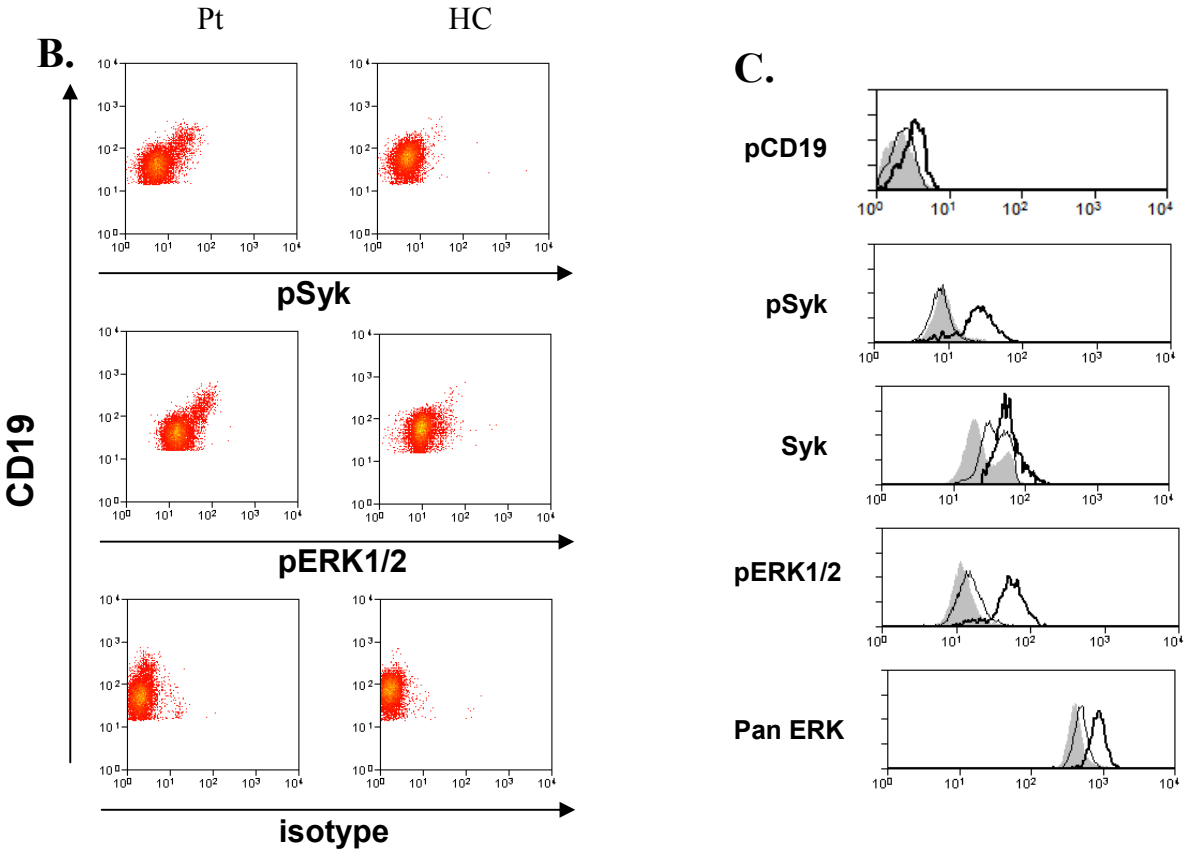


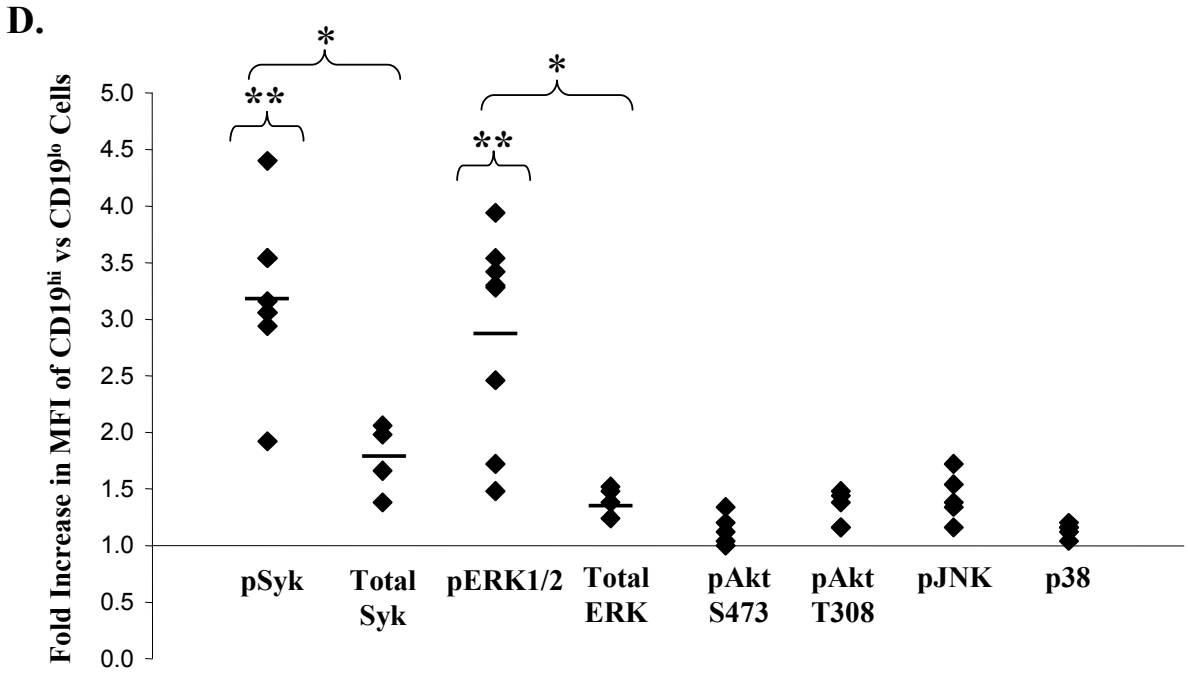


Figure 3.4. CD19^{hi} cells respond to BCR crosslinking.

A, Representative histograms of PB B cells from a CD19^{hi} Pt and a HC after incubation with media alone or with α-IgG for 10 min. Percentages indicate percent of B cells falling into each gate. **B**, Representative single parameter histograms for each of the indicated phosphorylated molecules in PB B cells from a HC and CD19^{lo} and CD19^{hi} cells from a Pt after incubation with media (shaded) or pansorbin (line) for 10 minutes. **C**, Fold change in the percentage of cells falling into the positive gate for each of the phosphorylated signaling molecules tested in cells incubated with media or pansorbin in a HC or in CD19^{lo} and CD19^{hi} cells from the same Pt. Each point represents a different individual with at least four per group. Significance was determined by Kruskal-Wallis tests. *$p<0.05$, **$p<0.01$. **D**, CD19^{hi} and CD19^{lo} cells were sorted and IgG antibody secreting cells (ASCs) determined after incubation with either media alone or with pansorbin, anti-CD40, IL-10 and IL-2 for 6 days. Each dot represents a different Pt.

Figure 3.4.

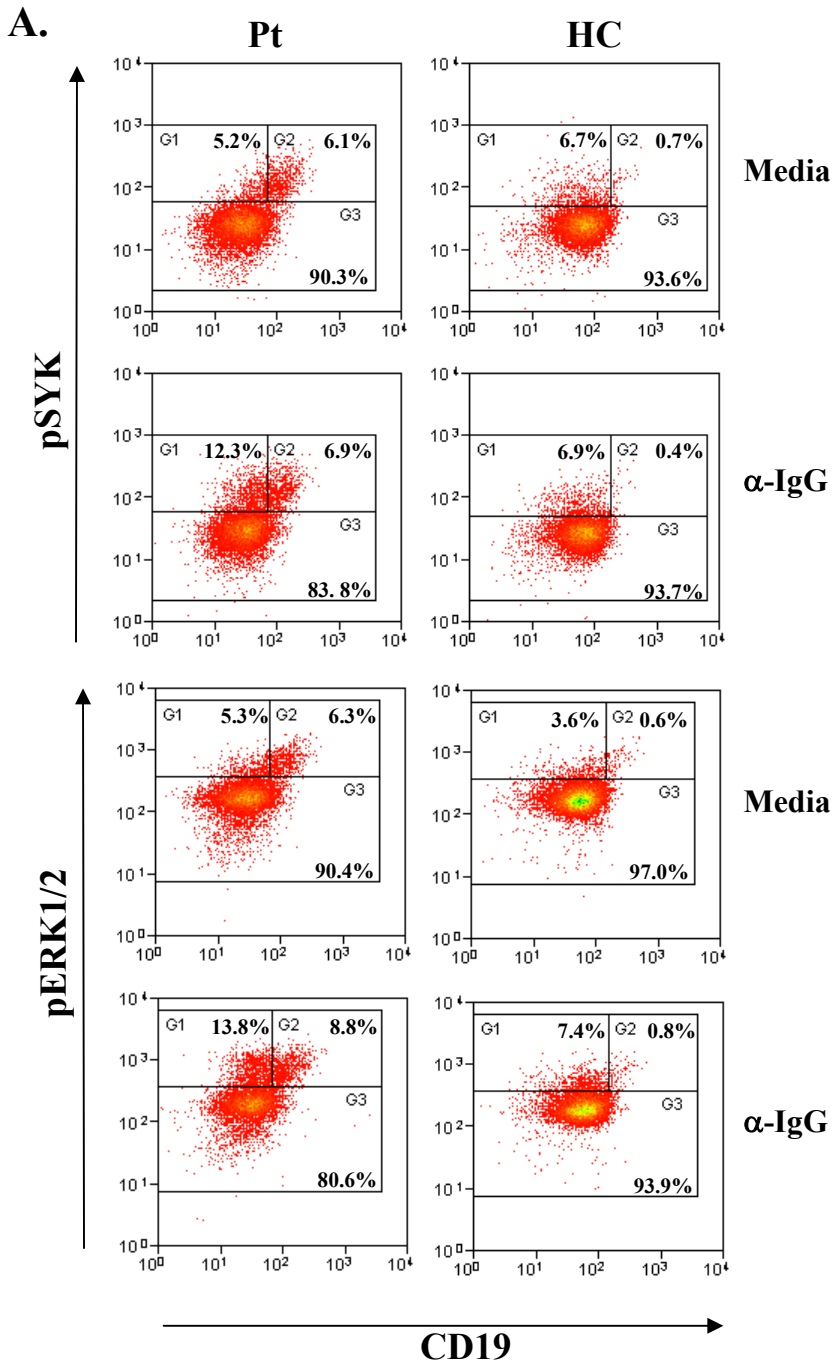
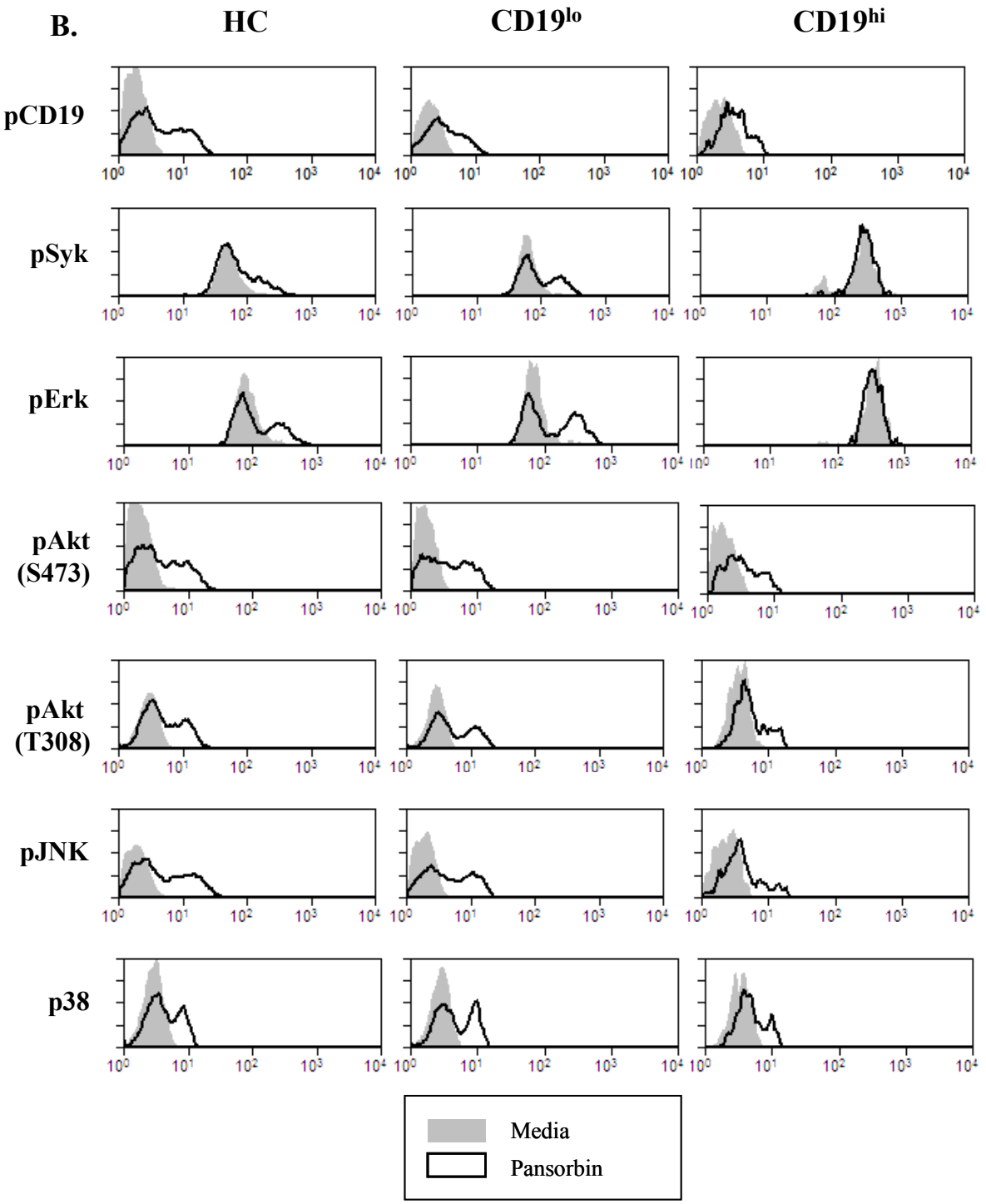

A.
Pt
HC
pSYK
pERK1/2
Media
α-IgG
Media
α-IgG
CD19
G1
G2
G3
5.2%
6.1%
90.3%
6.7%
0.7%
93.6%
12.3%
6.9%
83. 8%
6.9%
0.4%
93.7%
5.3%
6.3%
90.4%
3.6%
0.6%
97.0%
13.8%
8.8%
80.6%
7.4%
0.8%
93.9%

Figure 3.4, continued.



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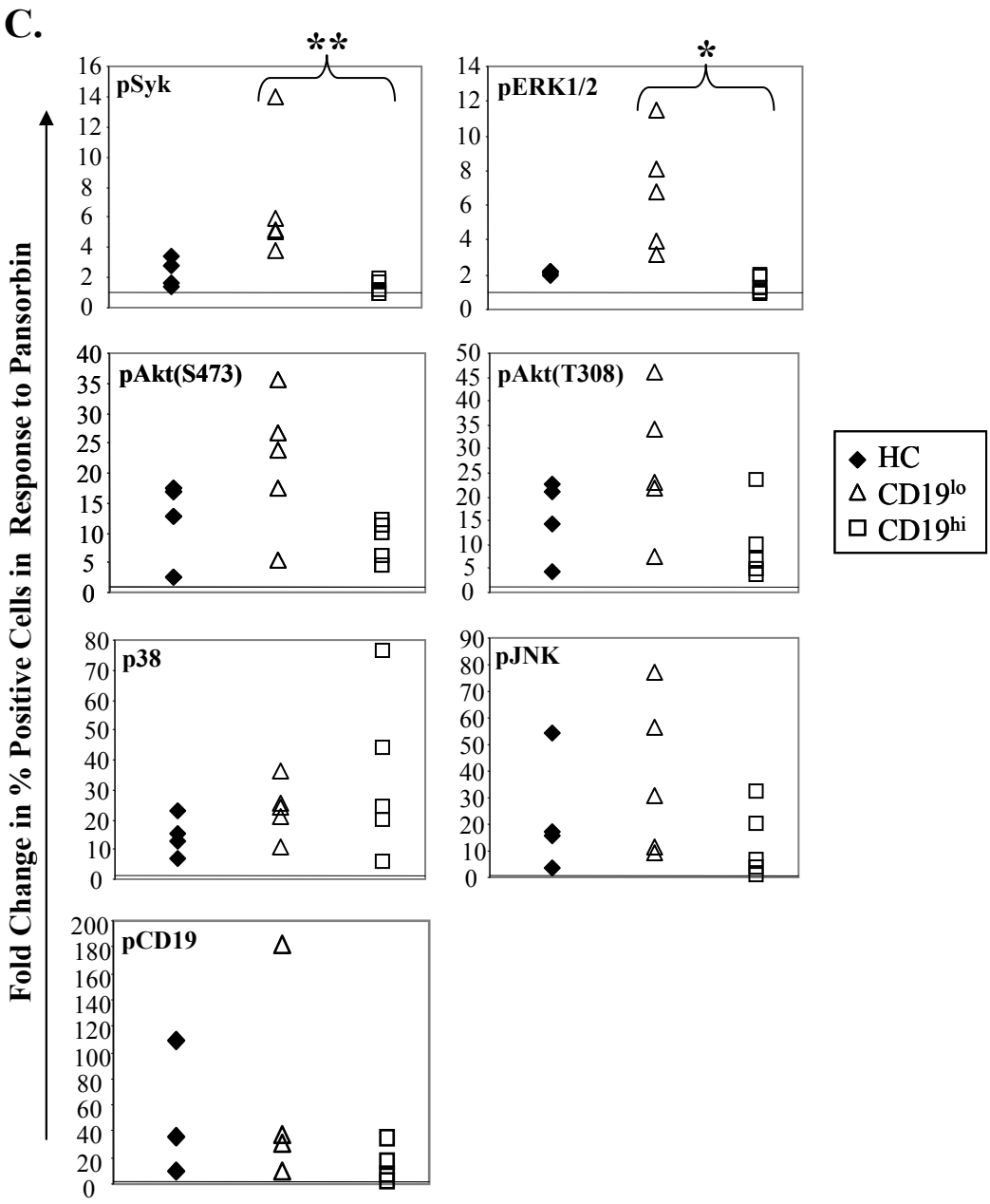

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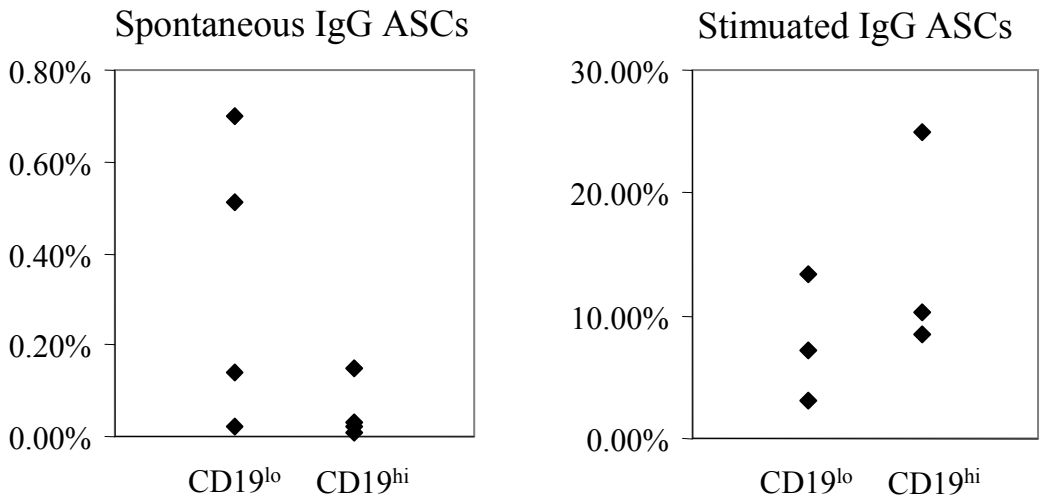


Figure 3.5. CD21 expression is downregulated in CD19^{hi} cells, and sCD21 is decreased in the serum of CD19^{hi} Pts.

A, Surface expression of CD21 as determined by flow cytometry. Histogram is representative of more than 5 Pts. **B**, CD21 transcript levels as determined by RT-PCR. CD19^{lo} and CD19^{hi} cells were sorted from three Pts and the expression of CD21 transcripts in CD19^{hi} cells determined relative to expression in CD19^{lo} cells from the same Pt. **C**, sCD21 levels in the serum of SLE Pts with and without a positive Sm titer and in HC as determined by ELISA. Significance determined by student's t test. **p<0.01.

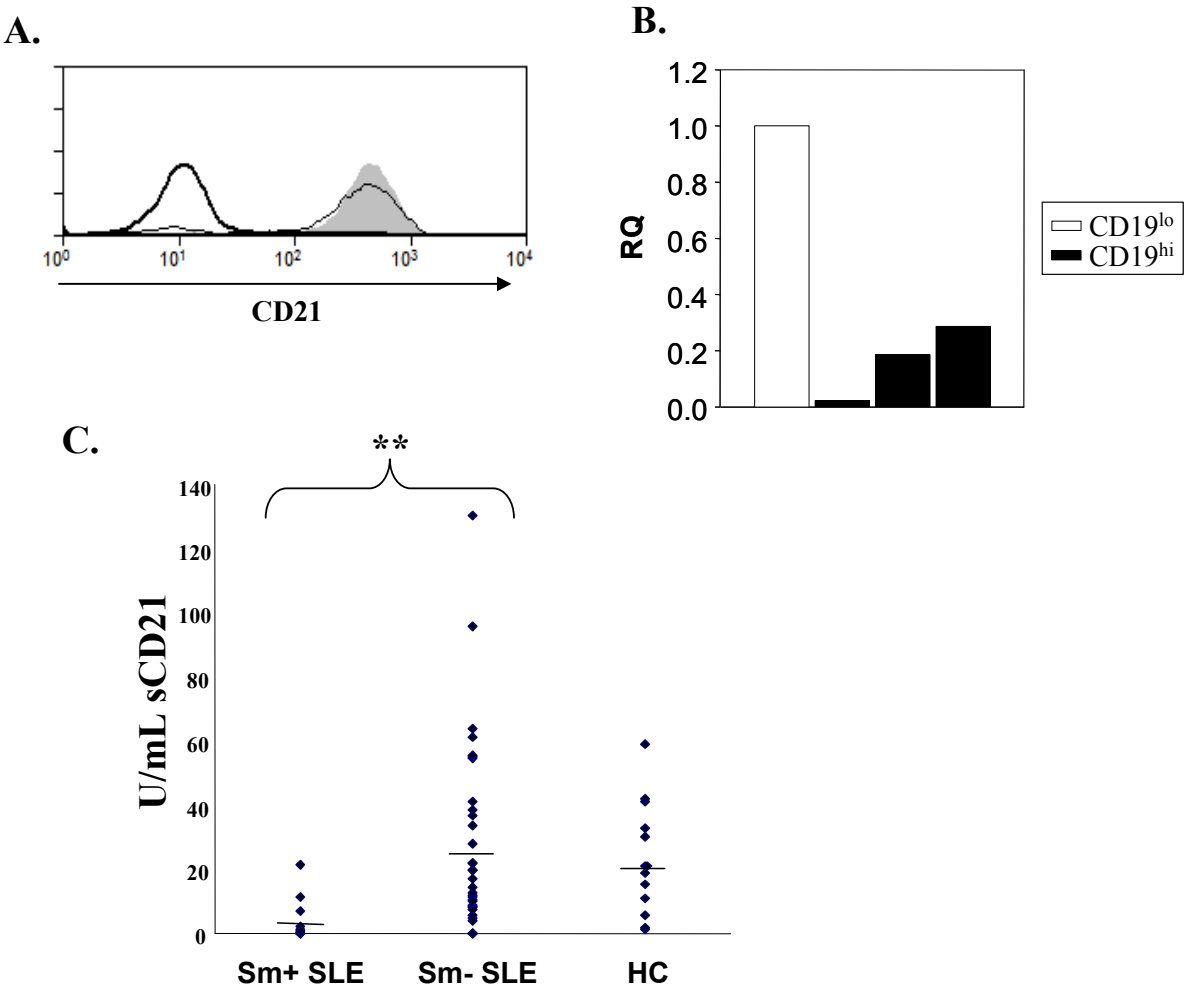


Figure 3.6. CD19^{hi} cells appear to be pre-plasma cells.

A and B, Transcription factor expression in indicated sorted B cell groups as determined by RQ RT-PCR. Each bar represents a different individual. **A**, Relative expression in CD19^{hi} cells as compared to CD19^{lo} cells from the same patient. **B**, Relative expression in CD19+CD38-IgD- memory cells as compared to all other CD19+ cells from the same HC. **A**, Representative histograms for Ki-67 staining in non-lymphoid PBMCs (shaded) and CD19^{lo} (thin line) or CD19^{hi} (thick line) Pt PB B cells and for PI staining of indicated subsets. Percentages indicate cells falling into drawn gate.

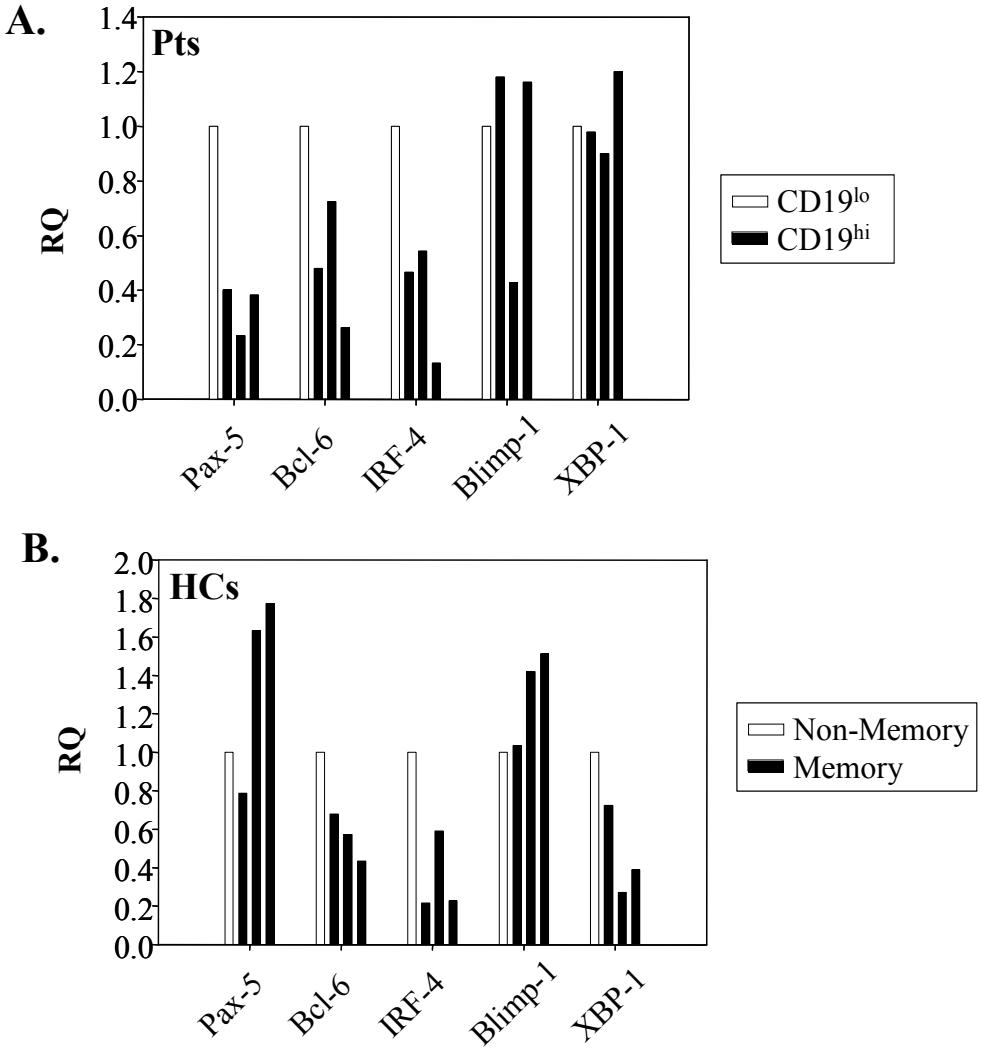


Figure 3.6, continued.

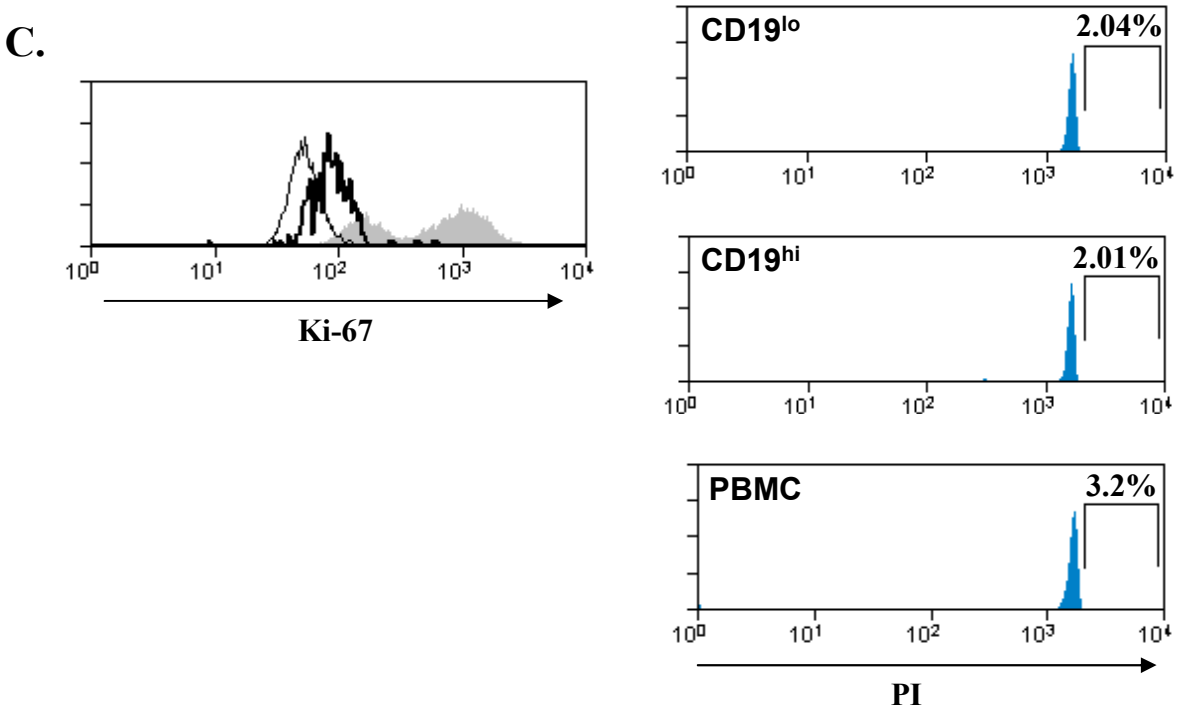


Figure 3.7. CXCR expression in CD19^{hi} and HC memory cells.

A and B, CXCR expression in indicated sorted B cell groups as determined by RQ RT-PCR. Each bar represents a different individual. **A**, Relative expression in CD19^{hi} cells as compared to CD19^{lo} cells from the same patient. **B**, Relative expression in CD19+CD38-IgD- memory cells as compared to all other CD19+ cells from the same HC. **C**, Surface CXCR3 expression determined by flow cytometry in a HC (shaded) or in CD19^{lo} (thin line) or CD19^{hi} (thick line) cells from the same Pt. Histogram is representative of three patients. **D**, Chemotaxis of CD19^{hi} cells in response to the CXCR3 ligand CXCL9, the CXCR4 ligand CXCL12, or media alone. Migration was determined by collection and staining of cells in upper and lower wells after 90 minutes.

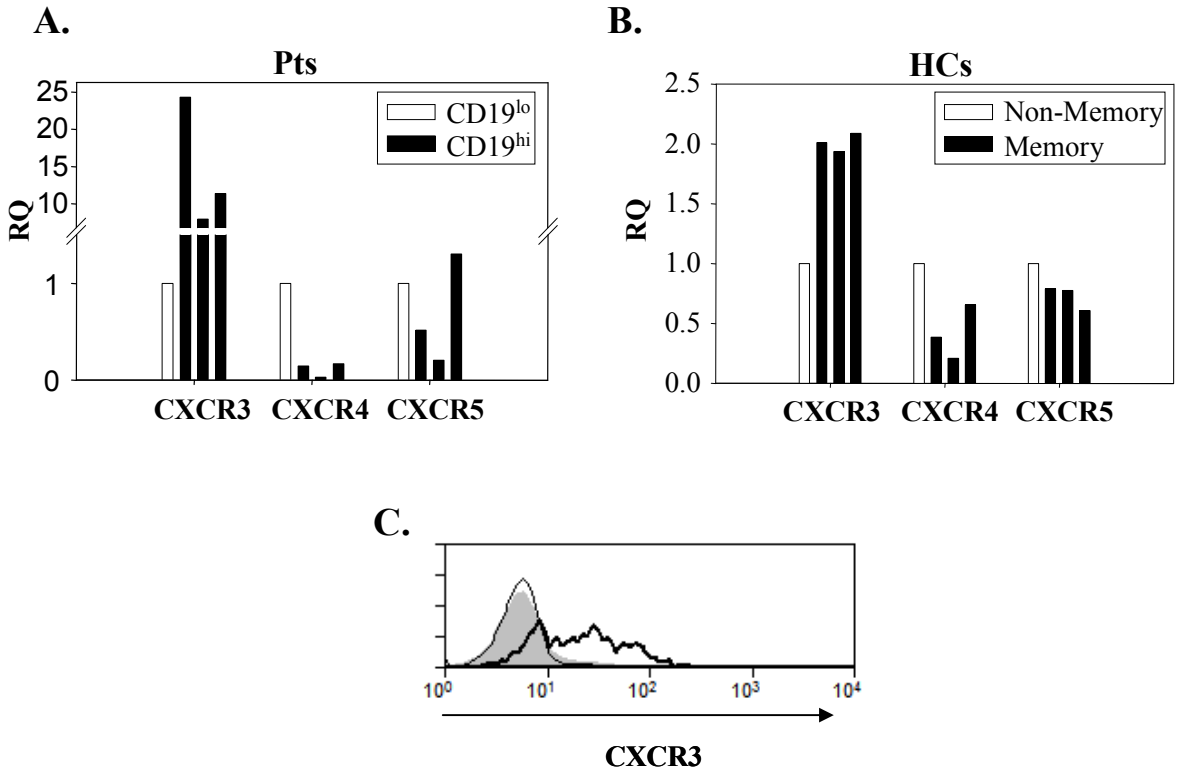
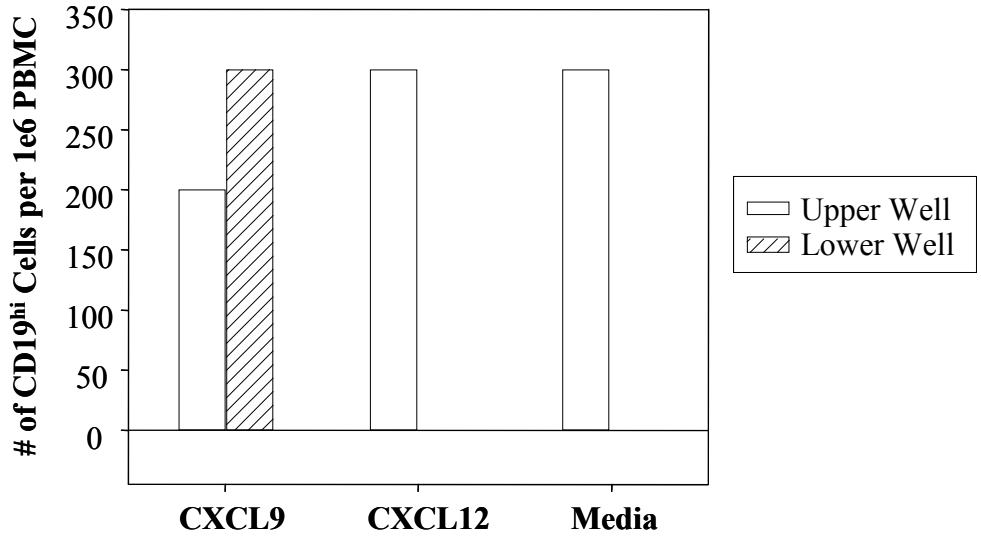


Figure 3.7, continued.

D.



CHAPTER 4: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

Autoimmune disease remains a mysterious realm in medicine. Though we continue to progress in our understanding of these devastating diseases, we still do not understand most aspects of their etiology or pathology. Because of this, we cannot cure these diseases, and must rely on palliative treatments which carry significant morbidities of their own.

SLE is often thought of as a general model for systemic autoimmune diseases. It is one of the most severe, and can affect essentially every organ system in the body. Also like other autoimmune diseases, its etiology has significant environmental and genetic components. Interestingly, even though some of these causes have been identified, how and why they contribute to the development of SLE is unknown.

The "two hit" hypothesis has been proposed to explain the complicated nature of the etiology of autoimmune disease. This hypothesis states that most of the time, a single genetic polymorphism or abnormality, or environmental exposure, may predispose to development of autoimmunity, but cannot alone lead to its development. A second "hit"—that is, a polymorphism in another gene that also predisposes to disease, or a specific environmental exposure at the appropriate time—must also be present to develop autoimmune disease.

For example, Epstein-Barr virus (EBV), although present in >80% of the general population, has been associated with SLE(272, 347). Clearly, EBV infection alone does not break self-tolerance; it must enter into a system predisposed to autoimmunity. In addition, none of the genetic polymorphisms that have been associated with SLE always result in disease. It is because of these complex and multi-parameter interactions that understanding of autoimmunity is still beyond our grasp.

Another impediment to our understanding of SLE is that, although it is heterogeneous in its etiology and pathogenesis, only one clinical diagnosis exists. An examination of even the simplified diagnostic criteria listed in Table 1.5 is enough to highlight how many varying symptoms in different combinations are diagnosed as SLE. The fact that we are most likely artificially grouping several diseases with distinct causes and pathology under a single diagnosis makes it more difficult to find the correct and relevant associations with genes and environmental exposures needed to understand SLE. For example, if all patients with a genetic alteration of FcγR2b were categorized as having "Type 1" SLE instead of lumped together with all patients diagnosed with SLE, then there would be a perfect concordance of "Type 1" SLE and FcγR2b mutations.

The lack of clinical or pathological delineation between putative subgroups of SLE impairs not only our understanding of this disease, but also our ability to effectively treat SLE patients. On the other hand, findings that are very common among all patients are useful in that they may enable developing treatments which have wide-spread efficacy. Herein I have presented data which links seemingly disparate autoimmune diseases and patients within these groups, as well as data which separates SLE patients into potential disease subgroups.

We have found that CD19 is decreased on peripheral B cells from a vast majority of patients with SLE or ANCA-SVV. This finding is very surprising, given the above discussion of heterogeneity within these diseases. In addition, the CD19 locus has been closely examined for polymorphisms which might be associated with autoimmune disease without much success. One study found an association with two CD19 polymorphisms and systemic sclerosis, although these resulted in increased CD19 expression and conferred odds

ratios of only ~2(351). Another study found a ~2-fold increased prevalence of a polymorphism in the 3' UTR of CD19, resulting in slightly decreased CD19 expression, in Japanese SLE patients(350), although this has not been successfully repeated in any other patient groups. A group of patients with hypogammaglobulinemia and immune deficiency as a result of a homozygous CD19 mutation resulting in complete lack of CD19 expression has also been described(394). As predicted, based on findings from CD19 deficient mice, these patients were immunodeficient, and did not have circulating autoantibody nor develop autoimmunity. No other associations between genetic polymorphisms of CD19 and human disease have been found, and none of these studies can explain the finding of decreased CD19 expression in >95% of SLE and ANCA-SVV patients.

It therefore seems most likely that CD19 expression is decreased on these cells as a consequence of disease pathology, rather than as a cause of it. In fact, we have generated preliminary data which suggests that soluble factors secreted by PBMCs from SLE patients are able to decrease CD19 expression on B cells from both patients and healthy controls. However, regardless of whether low CD19 expression is a cause or consequence of disease, it may have implications for perpetuation of disease. If decreased CD19 expression is present in immature or transitional B cells, it could inhibit negative selection of these cells, allowing escape of autoreactive specificities into the mature, functional B cell pool. Therefore, further investigation into the mechanism responsible for decreased CD19 expression in these diseases may provide new avenues for treatment.

The first step would be to verify the presence of a secreted factor or factors which are able to downregulate CD19 expression. Next would be to identify the cytokines and to determine the mechanism by which they decrease CD19 expression. These studies could be

carried out in several ways. Cytokine arrays could be used to compare the supernatants of PBMCs from SLE patients and healthy controls. Any cytokines with higher concentration in SLE PBMC supernatant compared to healthy control PBMC supernatant would be possible candidates. Clues as to the identity of these candidates would also be found in the literature describing cytokines found at increased concentration in the sera of SLE patients, such as IL-6, IL-10 and BAFF. These candidate cytokines could then be depleted from supernatants or blocked using monoclonal antibodies to determine which affects CD19 expression. Once the culprit is determined, the mechanism by which it accomplishes CD19 downregulation can be addressed using standard molecular biology techniques and phosflow staining to dissect signaling pathways. Identification of the players involved in this process would provide drug targets for adjunctive therapy in SLE.

We have also shown that patients with an expanded $CD19^{hi}$ memory B cell population may comprise a distinct subset of SLE patients. $CD19^{hi}$ patients differ from $CD19^{lo}$ patients in several ways. They have a unique pattern of autoreactivity, as shown in Figure 2.7. They also have an increase in adverse outcomes, particularly ESRD and severe neurological outcomes (Table 3.1). Finally, and unfortunately, show poor clinical response to rituximab treatment (Fig. 3.7). Taken together, these findings suggest that $CD19^{hi}$ cells in the PB can be used as a marker for SLE patients at high risk for ESRD, neurological dysfunction, and overall adverse outcomes, and that rituximab alone is not likely to be an effective therapy for these patients.

$CD19^{hi}$ B cells appear to be a functionally and phenotypically distinct, autoreactive subset of memory B cells. Several recent studies have implicated the memory B cell population to be important in autoimmune disease(201, 266, 307), and our data support this

idea. We do not yet know whether CD19^{hi} cells are newly generated memory B cells or memory B cells that have undergone a single or multiple rounds of reactivation. This may be a difficult question to address, although employing the IgG sequencing techniques outlined in Chapter 2 to following the CD19^{hi} clones in several patients over time might provide insight. If a limited set of clones are consistently present over time in a single patient, this would argue strongly for continued re-activation of a relatively constricted initial pool of memory cells. A constantly expanding number of clones would suggest that CD19^{hi} memory B cells are being continually generated, and are newly emerged germinal center reactions.

CD19^{hi} memory B cells are similar to non-CD19^{hi} memory B cells in their downregulation of CD38 and in the expression of mutant IgG BCRs. However, they differ in their decreased expression of CD27, CD21, and Pax-5 and their increased expression of CD19, CD20, and CXCR3. An important next step in the study of these cells would be to conduct a more thorough comparison of gene expression between these two groups, such as the employ of a gene chip analysis. In addition to determining the extent of their differences, this kind of analysis may also provide unique surface markers which could make future studies, such as the immunohistochemistry analysis of peripheral tissue, possible.

CD19^{hi} cells have a unique basal phosphorylative state, with increased basal phosphorylation of Syk and ERK1/2 but not CD19, Akt, p38 or JNK. Further exploration of functional and signaling alterations in these cells may provide the key to developing therapies for CD19^{hi} patients. Unfortunately, further studies of signaling in CD19^{hi} cells are greatly impaired by the number of B cells that can be acquired. The maximum number of B cells that can be purified from a single patient is in the range of 2-3x10^5 cells, far too few for western blot analysis. Therefore, we must rely on examination by flow cytometry and are

consequently limited by availability of antibodies and reagents which are compatible with this technique. For example, it would be useful to measure phosphorylative stages of molecules upstream of Syk, such as Lyn, and between Syk and ERK1/2 activation, such as Ras and Raf; unfortunately, antibodies that recognize these molecules are not currently available for use in flow cytometry. As this method gains favor, however, antibodies recognizing a wider variety of phosphorylated signaling molecules will be developed for this purpose and the mechanisms at work here can be further defined.

We have shown that $CD19^{hi}$ B cells are not anergic, despite their increase in autoreactive specificities. They phosphorylate CD19, Akt, p38 and JNK normally in response to stimulation with pansorbin and are able to differentiate to ASCs with pansorbin, anti-CD40, IL-10 and IL-4 stimulation. A logical next step would be to examine the proliferative potential of these cells in response to various stimuli. In addition, it will be important to further explore the response of these cells to other stimuli, particularly TLR ligands and BAFF, both of which have been implicated in autoimmunity. It would be interesting to know the minimum stimulation required to trigger the differentiation of these cells to ASCs, as this would give us hints as to how they may be activated *in vivo*. For example, is BCR crosslinking alone enough to generate ASCs in this population? If so, it would seem that they may be activated to become effector cells in the absence of T cell help. Alternatively, perhaps they do not require BCR stimulation, and the presence of certain cytokines is enough to stimulate differentiation. This would suggest that these memory B cells can be continually activated in a pro-inflammatory environment, even in the absence of cognate T cell help or significant antigen. These studies might also provide reasonable therapeutics for blocking the activation of these cells.

The presence of $CD19^{hi}$ B cells is associated with increased adverse clinical outcomes, particularly neurological dysfunction and ESRD. Importantly, only Patients with a $CD19^{hi}$ population had neurological complications in this study. We hypothesize that $CD19^{hi}$ cells home to peripheral sites of inflammation, particularly to the kidney and the CNS, where they mediate multiple effects including local production of autoantibody and possibly activation of T cells and other cells of the immune system (Fig. 3.1). B cells have already been shown to have these roles in autoimmune disease(393), and given their activated phenotype and high CXCR3 expression, $CD19^{hi}$ B cells seem particularly apt to mediate these effects. In addition, a 2-fold increase in anti-Sm reactive B cells in the $CD19^{hi}$ compartment results in a 100-fold increase in serum anti-Sm. This finding strongly supports the ideas that $CD19^{hi}$ B cells (1) are primary producers of serum antibody against certain autoantigens, (2) divide prior to differentiating, and (3) play a regulatory role in amplifying the immune response towards particular autoantigens, possibly through the activation of cognate T cells.

These cells may be particularly suited to APC function given their increased surface expression of MHCII and CD86. They have slightly decreased CD40 expression, but in many instances signaling by CD154 after interaction with CD40 is dispensable for T cell activation, and in some settings it is actually inhibitory(395, 396). It would therefore be very interesting to determine the ability of these cells to activate naïve and memory T cells; however, these studies pose some obvious technical difficulties. Ideally, one would want to determine the ability of these B cells to activate T cells for their cognate antigen; however, this would require ability to isolate and purify T cells specific for, for example, Sm. Currently, this is not feasible. However, less specific *in vitro* assays might be employed

using CD19^{hi} B cells loaded with Sm and total CD4+ T cells purified from the PB of the same patient. It will also be interesting to determine if these B cells are significant producers of cytokines, as has been reported for other B cell subsets in autoimmunity(397).

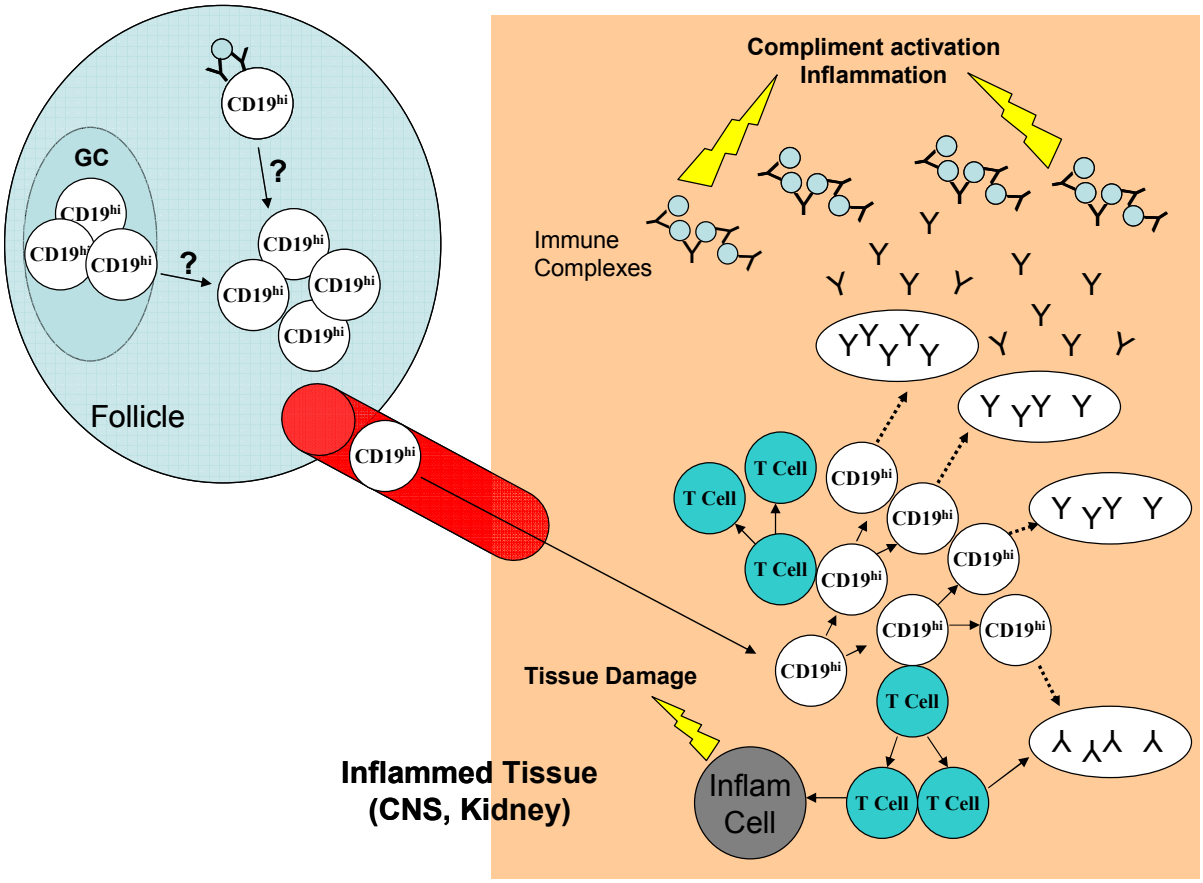


Figure 4.1. Unifying hypothesis of CD19^{hi} cell activation, homing and effects in peripheral sites of inflammation.

CD19^{hi} cells are either reactivated or newly generated memory cells which leave the follicle and home to peripheral sites of inflammation. There they proliferate and differentiate into autoantibody secreting cells. The locally produced autoantibody forms immune complexes which in turn mediate inflammatory reactions. In addition, they activate T cells, which also proliferate and mediate their own actions, such as activation of other inflammatory cell types (CD8+ T cells, for example).

Though our data support the idea that $CD19^{hi}$ B cells are important to disease pathogenesis, the only way to definitively prove this would be to transfer them to a host without autoimmunity and show that these recipients then acquire key features of disease. Clearly, such a study is not possible. One similar scenario is to show that features of disease are abrogated upon depletion of $CD19^{hi}$ B cells. If rituximab efficiently depleted these cells, one might argue that this approach would be successful. However, only depletion of $CD19^{hi}$ cells in the peripheral blood could be reasonably monitored, and our data suggest that these cells may only exist in the PB briefly before homing to sites of inflammation, where they would be highly resistant to depletion(393) and difficult to detect. To prove they have been fully depleted, sections of all relevant tissue would have to be examined, and this would be impossible in human subjects. Moreover, it appears that these cells are in fact not efficiently depleted by rituximab, although due to the reasons just described, this will be difficult to prove.

A more reasonable and practical approach to further explore our hypothesis would be to use immunohistochemistry of tissue sections of inflamed organs or tissues from SLE patients, particularly kidney. If we are correct, $CD19^{+}CD21^{-}CXCR3^{+}$ and $CD19^{-}$ $CD138^{+}$intracellular $IgG^{+}CXCR3^{+}$ cells should be present in these tissue sections. Some of the $CD19^{+}CD21^{-}$ cells should stain for Ki-67, indicating proliferation. We should also be able to stain for CXCR3 ligands in the endothelial tissue to verify their expression near accumulation of $CD19^{+}CD21^{-}$cells. If, in the future, medications are available which effectively treat $CD19^{hi}$ patients, these might be useful to determine the true role of these cells in disease.

We do not yet know what percentage of $CD19^{hi}$ cells are autoreactive. This question could be approached in two ways. First, the ELISpot technique described in Chapter 3 could be used to look for reactivity to other autoantigens. Although most patients with a $CD19^{hi}$ population are strongly snRNP reactive and have minimal reactivity to other autoantigens (Fig.2.7), some autoantibodies are also seen $CD19^{hi}$ patients, such as anti-dsDNA and anti-phospholipid antibodies. Additionally, individual Ig genes could be cloned from single-cell sorted $CD19^{hi}$ B cells, expressed in culture, and their specificity determined, using techniques recently described(201). It would be especially useful to determine if the exponential relationship between enrichment and serum autoantibody titer seen in the case of Sm is consistent for other autoantigens.

$CD19^{hi}$ patients do not have a robust clinical to rituximab, but their increased adverse outcomes dictate a particular need for treatment options. Given the high CXCR3 expression on $CD19^{hi}$ B cells, we propose that anti-CXCR3 treatment may be an efficacious treatment for these patients. Numerous lines of data suggest that blocking CXCR3 interactions with its ligands may abrogate disease in $CD19^{hi}$ patients, as discussed in Chapter 3. These studies support the idea that $CXCR3^{+}$ B cells can migrate to sites of inflammation, where, among other effector functions, they can differentiate into PCs. Therefore, blocking CXCR3 would prevent the migration of autoreactive $CD19^{hi}$ B cells into peripheral sites of inflammation, and may deny them a niche in which to differentiate to PCs and block their local effector functions(393).

In addition, a synergistic effect has been seen using rituximab together with blockade of BAFF(393). This approach may be helpful in the treatment of $CD19^{hi}$ patients, however, we have found that BAFF Receptor 3 (BR3) expression is consistently decreased on $CD19^{hi}$

cells, suggesting that these cells may have become BAFF-independent. To further explore this idea, levels of the other two BAFF receptors, TACI and BCMA, must be determined. In addition, effects of BAFF on these cells could be determined by examining survival *ex vivo* in the presence and absence of BAFF.

An overarching and necessary follow-up for the studies described in this dissertation would be to examine a much larger cohort of patients to further delineate and verify clinical and pathological differences, as well as differences in response to various treatments, in $CD19^{hi}$ SLE patients vs. SLE patients without an expanded $CD19^{hi}$ population. As SLE has always been thought of as a heterogeneous disease, studies such as these which begin to separate apparent disease subtypes are vital to developing individualized, and therefore more efficacious and less debilitating, therapeutic modalities.

REFERENCES

1. Dempsey, P. W., M. E. Allison, S. Akkaraju, C. C. Goodnow, and D. T. Fearon. 1996. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science 271:348*.

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

3. Serra, V. A., F. Cretin, E. Pepin, F. M. Gabert, and P. N. Marche. 1997. Complement C3b fragment covalently linked to tetanus toxin increases lysosomal sodium dodecyl sulfate-stable HLA-DR dimer production. *Eur J Immunol 27:2673*.

4. Villiers, M. B., C. L. Villiers, A. M. Laharie, and P. N. Marche. 1999. Amplification of the antibody response by C3b complexed to antigen through an ester link. *J Immunol 162:3647*.

5. Fields, M. L., M. H. Metzgar, B. D. Hondowicz, S. A. Kang, S. T. Alexander, K. D. Hazard, A. C. Hsu, Y. Z. Du, E. L. Prak, M. Monestier, and J. Erikson. 2006. Exogenous and endogenous TLR ligands activate anti-chromatin and polyreactive B cells. *J Immunol 176:6491*.

6. Ruprecht, C. R., and A. Lanzavecchia. 2006. Toll-like receptor stimulation as a third signal required for activation of human naive B cells. *Eur J Immunol 36:810*.

7. Boule, M. W., C. Broughton, F. Mackay, S. Akira, A. Marshak-Rothstein, and I. R. Rifkin. 2004. Toll-like receptor 9-dependent and -independent dendritic cell activation by chromatin-immunoglobulin G complexes. *J Exp Med 199:1631*.

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

9. Ehlers, M., H. Fukuyama, T. L. McGaha, A. Aderem, and J. V. Ravetch. 2006. TLR9/MyD88 signaling is required for class switching to pathogenic IgG2a and 2b autoantibodies in SLE. *J Exp Med 203:553*.

10. Hayashi, E. A., S. Akira, and A. Nobrega. 2005. Role of TLR in B cell development: signaling through TLR4 promotes B cell maturation and is inhibited by TLR2. *J Immunol 174:6639*.

11. Lau, C. M., C. Broughton, A. S. Tabor, S. Akira, R. A. Flavell, M. J. Mamula, S. R. Christensen, M. J. Shlomchik, G. A. Viglianti, I. R. Rifkin, and A. Marshak-Rothstein. 2005. RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement. *J Exp Med 202:1171*.

12. Leadbetter, E. A., I. R. Rifkin, A. M. Hohlbaum, B. C. Beaudette, M. J. Shlomchik, and A. Marshak-Rothstein. 2002. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature 416:603*.

13. Pasare, C., and R. Medzhitov. 2005. Control of B-cell responses by Toll-like receptors. *Nature 438:364*.

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

15. Vollmer, J., S. Tluk, C. Schmitz, S. Hamm, M. Jurk, A. Forsbach, S. Akira, K. M. Kelly, W. H. Reeves, S. Bauer, and A. M. Krieg. 2005. Immune stimulation mediated by autoantigen binding sites within small nuclear RNAs involves Toll-like receptors 7 and 8. *J Exp Med 202:1575*.

16. Ozinsky, A., D. M. Underhill, J. D. Fontenot, A. M. Hajjar, K. D. Smith, C. B. Wilson, L. Schroeder, and A. Aderem. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci U S A 97:13766*.

17. Janeway, C. A., Travers, P., Walport, M., and Shlomchik, M. 2005. *Immunobiology: The Immune System in Health and Disease*. Garland Science Publishing.

18. Trinchieri, G., and A. Sher. 2007. Cooperation of Toll-like receptor signals in innate immune defence. *Nat Rev Immunol 7:179*.

19. Iwasaki, A., and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. *Nat Immunol 5:987*.

20. Hayden, M. S., and S. Ghosh. 2004. Signaling to NF-kappaB. *Genes Dev 18:2195*.

21. Akira, S., and K. Takeda. 2004. Toll-like receptor signalling. *Nat Rev Immunol 4:499*.

22. Marshak-Rothstein, A. 2006. Toll-like receptors in systemic autoimmune disease. *Nat Rev Immunol 6:823*.

23. Takeuchi, O., S. Sato, T. Horiuchi, K. Hoshino, K. Takeda, Z. Dong, R. L. Modlin, and S. Akira. 2002. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol 169:10*.

24. Lang, K. S., M. Recher, T. Junt, A. A. Navarini, N. L. Harris, S. Freigang, B. Odermatt, C. Conrad, L. M. Ittner, S. Bauer, S. A. Luther, S. Uematsu, S. Akira, H. Hengartner, and R. M. Zinkernagel. 2005. Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. *Nat Med 11:138*.

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

26. Bernasconi, N. L., N. Onai, and A. Lanzavecchia. 2003. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. *Blood 101:4500*.

27. Bourke, E., D. Bosisio, J. Golay, N. Polentarutti, and A. Mantovani. 2003. The toll-like receptor repertoire of human B lymphocytes: inducible and selective expression of TLR9 and TLR10 in normal and transformed cells. *Blood 102:956*.

28. Mansson, A., M. Adner, U. Hockerfelt, and L. O. Cardell. 2006. A distinct Toll-like receptor repertoire in human tonsillar B cells, directly activated by PamCSK, R-837 and CpG-2006 stimulation. *Immunology 118:539*.

29. Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol 168:4531*.

30. Peng, S. L. 2005. Signaling in B cells via Toll-like receptors. *Curr Opin Immunol 17:230*.

31. Ogata, H., I. Su, K. Miyake, Y. Nagai, S. Akashi, I. Mecklenbrauker, K. Rajewsky, M. Kimoto, and A. Tarakhovsky. 2000. The toll-like receptor protein RP105 regulates lipopolysaccharide signaling in B cells. *J Exp Med 192:23*.

32. Lopes-Carvalho, T., J. Foote, and J. F. Kearney. 2005. Marginal zone B cells in lymphocyte activation and regulation. *Curr Opin Immunol 17:244*.

33. Barrington, R., M. Zhang, M. Fischer, and M. C. Carroll. 2001. The role of complement in inflammation and adaptive immunity. *Immunol Rev 180:5*.

34. Carroll, M. C. 1998. The role of complement and complement receptors in induction and regulation of immunity. *Annu Rev Immunol 16:545*.

35. Carroll, M. C. 2004. A protective role for innate immunity in systemic lupus erythematosus. *Nat Rev Immunol 4:825*.

36. Morelli, A. E., A. T. Larregina, W. J. Shufesky, A. F. Zahorchak, A. J. Logar, G. D. Papworth, Z. Wang, S. C. Watkins, L. D. Falo, Jr., and A. W. Thomson. 2003. Internalization of circulating apoptotic cells by splenic marginal zone dendritic cells: dependence on complement receptors and effect on cytokine production. *Blood 101:611*.

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

38. Ehrenstein, M. R., T. L. O'Keefe, S. L. Davies, and M. S. Neuberger. 1998. Targeted gene disruption reveals a role for natural secretory IgM in the maturation of the primary immune response. *Proc Natl Acad Sci U S A 95:10089*.

39. Holers, V. M. 2005. Complement receptors and the shaping of the natural antibody repertoire. *Springer Semin Immunopathol 26:405*.

40. Reid, R. R., A. P. Prodeus, W. Khan, T. Hsu, F. S. Rosen, and M. C. Carroll. 1997. Endotoxin shock in antibody-deficient mice: unraveling the role of natural antibody and complement in the clearance of lipopolysaccharide. *J Immunol 159:970*.

41. Thornton, B. P., V. Vetvicka, and G. D. Ross. 1994. Natural antibody and complement-mediated antigen processing and presentation by B lymphocytes. *J Immunol 152:1727*.

42. Reid, R. R., S. Woodcock, A. Shimabukuro-Vornhagen, W. G. Austen, Jr., L. Kobzik, M. Zhang, H. B. Hechtman, F. D. Moore, Jr., and M. C. Carroll. 2002. Functional activity of natural antibody is altered in Cr2-deficient mice. *J Immunol 169:5433*.

43. Montecino-Rodriguez, E., H. Leathers, and K. Dorshkind. 2006. Identification of a B-1 B cell-specified progenitor. *Nat Immunol 7:293*.

44. Tung, J. W., M. D. Mrazek, Y. Yang, and L. A. Herzenberg. 2006. Phenotypically distinct B cell development pathways map to the three B cell lineages in the mouse. *Proc Natl Acad Sci U S A 103:6293*.

45. Hardy, R. R. 2006. B-1 B cell development. *J Immunol 177:2749*.

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

47. Qian, Y., C. Santiago, M. Borrero, T. F. Tedder, and S. H. Clarke. 2001. Lupus-specific antiribonucleoprotein B cell tolerance in nonautoimmune mice is maintained by differentiation to B-1 and governed by B cell receptor signaling thresholds. *J Immunol 166:2412*.

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

49. Liew, F. Y. 2002. T(H)1 and T(H)2 cells: a historical perspective. *Nat Rev Immunol 2:55*.

50. Shevach, E. M. 2006. From vanilla to 28 flavors: multiple varieties of T regulatory cells. *Immunity 25:195*.

51. Ardavin, C. 2003. Origin, precursors and differentiation of mouse dendritic cells. *Nat Rev Immunol 3:582*.

52. Hackstein, H., and A. W. Thomson. 2004. Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. *Nat Rev Immunol 4:24*.

53. Colonna, M., G. Trinchieri, and Y. J. Liu. 2004. Plasmacytoid dendritic cells in immunity. *Nat Immunol 5:1219*.

54. MacLennan, I., and C. Vinuesa. 2002. Dendritic cells, BAFF, and APRIL: innate players in adaptive antibody responses. *Immunity 17:235*.

55. Balazs, M., F. Martin, T. Zhou, and J. Kearney. 2002. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. *Immunity 17:341*.

56. Tew, J. G., J. Wu, M. Fakher, A. K. Szakal, and D. Qin. 2001. Follicular dendritic cells: beyond the necessity of T-cell help. *Trends Immunol 22:361*.

57. Park, C. S., S. O. Yoon, R. J. Armitage, and Y. S. Choi. 2004. Follicular dendritic cells produce IL-15 that enhances germinal center B cell proliferation in membrane-bound form. *J Immunol 173:6676*.

58. Aydar, Y., S. Sukumar, A. K. Szakal, and J. G. Tew. 2005. The influence of immune complex-bearing follicular dendritic cells on the IgM response, Ig class switching, and production of high affinity IgG. *J Immunol 174:5358*.

59. Haberman, A. M., and M. J. Shlomchik. 2003. Reassessing the function of immune-complex retention by follicular dendritic cells. *Nat Rev Immunol 3:757*.

60. McHeyzer-Williams, M. G. 2003. B cells as effectors. *Curr Opin Immunol 15:354*.

61. Ashour, H. M., and J. Y. Niederkorn. 2006. Peripheral tolerance via the anterior chamber of the eye: role of B cells in MHC class I and II antigen presentation. *J Immunol 176:5950*.

62. Lund, F. E., M. Hollifield, K. Schuer, J. L. Lines, T. D. Randall, and B. A. Garvy. 2006. B cells are required for generation of protective effector and memory CD4 cells in response to Pneumocystis lung infection. *J Immunol 176:6147*.

63. Strutt, T. M., J. Uzonna, K. K. McKinstry, and P. A. Bretscher. 2006. Activation of thymic T cells by MHC alloantigen requires syngeneic, activated CD4+ T cells and B cells as APC. *Int Immunol 18:719*.

64. Raimondi, G., I. Zanoni, S. Citterio, P. Ricciardi-Castagnoli, and F. Granucci. 2006. Induction of peripheral T cell tolerance by antigen-presenting B cells. II. Chronic antigen presentation overrules antigen-presenting B cell activation. *J Immunol 176:4021*.

65. Raimondi, G., I. Zanoni, S. Citterio, P. Ricciardi-Castagnoli, and F. Granucci. 2006. Induction of peripheral T cell tolerance by antigen-presenting B cells. I. Relevance of antigen presentation persistence. *J Immunol 176:4012*.

66. Fillatreau, S., C. H. Sweenie, M. J. McGeachy, D. Gray, and S. M. Anderton. 2002. B cells regulate autoimmunity by provision of IL-10. *Nat Immunol 3:944*.

67. van Kooten, C., C. Gaillard, J. P. Galizzi, P. Hermann, F. Fossiez, J. Banchereau, and D. Blanchard. 1994. B cells regulate expression of CD40 ligand on activated T cells by lowering the mRNA level and through the release of soluble CD40. *Eur J Immunol 24:787*.

68. Horna, P., A. Cuenca, F. Cheng, J. Brayer, H. W. Wang, I. Borrello, H. Levitsky, and E. M. Sotomayor. 2006. In vivo disruption of tolerogenic cross-presentation mechanisms uncovers an effective T-cell activation by B-cell lymphomas leading to antitumor immunity. *Blood 107:2871*.

69. Joao, C., B. M. Ogle, and S. Geyer. 2006. Immunoglobulin promotes the diversity and the function of T cells. *Eur J Immunol 36:1718*.

70. Mix, E., R. Goertsches, and U. K. Zett. 2006. Immunoglobulins-Basic considerations. *J Neurol 253 Suppl 5:v9*.

71. Okazaki, I. M., K. Kinoshita, M. Muramatsu, K. Yoshikawa, and T. Honjo. 2002. The AID enzyme induces class switch recombination in fibroblasts. *Nature 416:340*.

72. Honjo, T., K. Kinoshita, and M. Muramatsu. 2002. Molecular mechanism of class switch recombination: linkage with somatic hypermutation. *Annu Rev Immunol 20:165*.

73. Tonegawa, S. 1983. Somatic generation of antibody diversity. *Nature 302:575*.

74. Matthias, P., and A. G. Rolink. 2005. Transcriptional networks in developing and mature B cells. *Nat Rev Immunol 5:497*.

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

76. Collins, A. M., W. A. Sewell, and M. R. Edwards. 2003. Immunoglobulin gene rearrangement, repertoire diversity, and the allergic response. *Pharmacol Ther 100:157*.

77. Brack, C., M. Hirama, R. Lenhard-Schuller, and S. Tonegawa. 1978. A complete immunoglobulin gene is created by somatic recombination. *Cell 15:1*.

78. Oettinger, M. A., D. G. Schatz, C. Gorka, and D. Baltimore. 1990. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science 248:1517*.

79. Schatz, D. G., M. A. Oettinger, and D. Baltimore. 1989. The V(D)J recombination activating gene, RAG-1. *Cell 59:1035*.

80. Kim, D. R., S. J. Park, and M. A. Oettinger. 2000. V(D)J recombination: site-specific cleavage and repair. *Mol Cells 10:367*.

81. McHeyzer-Williams, L. J., D. J. Driver, and M. G. McHeyzer-Williams. 2001. Germinal center reaction. *Curr Opin Hematol 8:52*.

82. Han, S., B. Zheng, Y. Takahashi, and G. Kelsoe. 1997. Distinctive characteristics of germinal center B cells. *Semin Immunol 9:255*.

83. Vallur, A. C., M. Yabuki, E. D. Larson, and N. Maizels. 2007. AID in antibody perfection. *Cell Mol Life Sci*.

84. Hess, J., A. Werner, T. Wirth, F. Melchers, H. M. Jack, and T. H. Winkler. 2001. Induction of pre-B cell proliferation after de novo synthesis of the pre-B cell receptor. *Proc Natl Acad Sci U S A 98:1745*.

85. Niiro, H., and E. A. Clark. 2002. Regulation of B-cell fate by antigen-receptor signals. *Nat Rev Immunol 2:945*.

86. Harnett, M. M., E. Katz, and C. A. Ford. 2005. Differential signalling during B-cell maturation. *Immunol Lett 98:33*.

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

88. Lin, H., and R. Grosschedl. 1995. Failure of B-cell differentiation in mice lacking the transcription factor EBF. *Nature 376:263*.

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

90. Urbanek, P., Z. Q. Wang, I. Fetka, E. F. Wagner, and M. Busslinger. 1994. Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell 79:901*.

91. O'Riordan, M., and R. Grosschedl. 1999. Coordinate regulation of B cell differentiation by the transcription factors EBF and E2A. *Immunity 11:21*.

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

93. Nutt, S. L., S. Vambrie, P. Steinlein, Z. Kozmik, A. Rolink, A. Weith, and M. Busslinger. 1999. Independent regulation of the two Pax5 alleles during B-cell development. *Nat Genet 21:390*.

94. Melchers, F., A. Rolink, U. Grawunder, T. H. Winkler, H. Karasuyama, P. Ghia, and J. Andersson. 1995. Positive and negative selection events during B lymphopoiesis. *Curr Opin Immunol 7:214*.

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

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

97. Levine, M. H., A. M. Haberman, D. B. Sant'Angelo, L. G. Hannum, M. P. Cancro, C. A. Janeway, Jr., and M. J. Shlomchik. 2000. A B-cell receptor-specific selection step governs immature to mature B cell differentiation. *Proc Natl Acad Sci U S A 97:2743*.

98. Mebius, R. E., and G. Kraal. 2005. Structure and function of the spleen. *Nat Rev Immunol 5:606*.

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

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

101. von Andrian, U. H., and T. R. Mempel. 2003. Homing and cellular traffic in lymph nodes. *Nat Rev Immunol 3:867*.

102. Cyster, J. G. 1999. Chemokines and cell migration in secondary lymphoid organs. *Science 286:2098*.

103. Okada, T., and J. G. Cyster. 2006. B cell migration and interactions in the early phase of antibody responses. *Curr Opin Immunol 18:278*.

104. Merrell, K. T., R. J. Benschop, S. B. Gauld, K. Aviszus, D. Decote-Ricardo, L. J. Wysocki, and J. C. Cambier. 2006. Identification of anergic B cells within a wild-type repertoire. *Immunity 25:953*.

105. Allman, D., R. C. Lindsley, W. DeMuth, K. Rudd, S. A. Shinton, and R. R. Hardy. 2001. Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation. *J Immunol 167:6834*.

106. Su, T. T., and D. J. Rawlings. 2002. Transitional B lymphocyte subsets operate as distinct checkpoints in murine splenic B cell development. *J Immunol 168:2101*.

107. Carsetti, R., G. Kohler, and M. C. Lamers. 1995. Transitional B cells are the target of negative selection in the B cell compartment. *J Exp Med 181:2129*.

108. King, L. B., A. Norvell, and J. G. Monroe. 1999. Antigen receptor-induced signal transduction imbalances associated with the negative selection of immature B cells. *J Immunol 162:2655*.

109. Sater, R. A., P. C. Sandel, and J. G. Monroe. 1998. B cell receptor-induced apoptosis in primary transitional murine B cells: signaling requirements and modulation by T cell help. *Int Immunol 10:1673*.

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

111. Kanayama, N., M. Cascalho, and H. Ohmori. 2005. Analysis of marginal zone B cell development in the mouse with limited B cell diversity: role of the antigen receptor signals in the recruitment of B cells to the marginal zone. *J Immunol 174:1438*.

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

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

114. Cyster, J. G. 2000. B cells on the front line. *Nat Immunol 1:9*.

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

116. Gunn, K. E., and J. W. Brewer. 2006. Evidence that marginal zone B cells possess an enhanced secretory apparatus and exhibit superior secretory activity. *J Immunol 177:3791*.

117. Phan, T. G., S. Gardam, A. Basten, and R. Brink. 2005. Altered migration, recruitment, and somatic hypermutation in the early response of marginal zone B cells to T cell-dependent antigen. *J Immunol 174:4567*.

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

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

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

121. Li, X., F. Martin, A. M. Oliver, J. F. Kearney, and R. H. Carter. 2001. Antigen receptor proximal signaling in splenic B-2 cell subsets. *J Immunol 166:3122*.

122. Batista, F. D., D. Iber, and M. S. Neuberger. 2001. B cells acquire antigen from target cells after synapse formation. *Nature 411:489*.

123. Jun, J. E., and C. C. Goodnow. 2003. Scaffolding of antigen receptors for immunogenic versus tolerogenic signaling. *Nat Immunol 4:1057*.

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

125. Monroe, J. G. 2006. ITAM-mediated tonic signalling through pre-BCR and BCR complexes. *Nat Rev Immunol 6:283*.

126. Sada, K., T. Takano, S. Yanagi, and H. Yamamura. 2001. Structure and function of Syk protein-tyrosine kinase. *J Biochem (Tokyo) 130:177*.

127. Turner, M., A. Gulbranson-Judge, M. E. Quinn, A. E. Walters, I. C. MacLennan, and V. L. Tybulewicz. 1997. Syk tyrosine kinase is required for the positive selection of immature B cells into the recirculating B cell pool. *J Exp Med 186:2013*.

128. Cheng, A. M., B. Rowley, W. Pao, A. Hayday, J. B. Bolen, and T. Pawson. 1995. Syk tyrosine kinase required for mouse viability and B-cell development. *Nature 378:303*.

129. Turner, M., P. J. Mee, P. S. Costello, O. Williams, A. A. Price, L. P. Duddy, M. T. Furlong, R. L. Geahlen, and V. L. Tybulewicz. 1995. Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature 378:298*.

130. Kurosaki, T. 2002. Regulation of B-cell signal transduction by adaptor proteins. *Nat Rev Immunol 2:354*.

131. Lankester, A. C., P. M. Rood, G. M. van Schijndel, B. Hooibrink, A. J. Verhoeven, and R. A. van Lier. 1996. Alteration of B-cell antigen receptor signaling by CD19 co-ligation. A study with bispecific antibodies. *J Biol Chem 271:22326*.

132. Tedder, T. F., M. Inaoki, and S. Sato. 1997. The CD19-CD21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity. *Immunity 6:107*.

133. Tedder, T. F., L. J. Zhou, and P. Engel. 1994. The CD19/CD21 signal transduction complex of B lymphocytes. *Immunol Today 15:437*.

134. Levy, S., S. C. Todd, and H. T. Maecker. 1998. CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. *Annu Rev Immunol 16:89*.

135. Asokan, R., J. Hua, K. A. Young, H. J. Gould, J. P. Hannan, D. M. Kraus, G. Szakonyi, G. J. Grundy, X. S. Chen, M. K. Crow, and V. M. Holers. 2006. Characterization of human complement receptor type 2 (CR2/CD21) as a receptor for IFN-alpha: a potential role in systemic lupus erythematosus. *J Immunol 177:383*.

136. Jacquier-Sarlin, M. R., F. M. Gabert, M. B. Villiers, and M. G. Colomb. 1995. Modulation of antigen processing and presentation by covalently linked complement C3b fragment. *Immunology 84:164*.

137. Lyubchenko, T., J. dal Porto, J. C. Cambier, and V. M. Holers. 2005. Coligation of the B cell receptor with complement receptor type 2 (CR2/CD21) using its natural ligand C3dg: activation without engagement of an inhibitory signaling pathway. *J Immunol 174:3264*.

138. Holers, V. M., and S. A. Boackle. 2004. Complement receptor 2 and autoimmunity. *Curr Dir Autoimmun 7:33*.

139. Tessier, J., A. Cuvillier, F. Glaudet, and A. A. Khamlichi. 2007. Internalization and molecular interactions of human CD21 receptor. *Mol Immunol 44:2415*.

140. Villiers, M. B., C. L. Villiers, M. R. Jacquier-Sarlin, F. M. Gabert, A. M. Journet, and M. G. Colomb. 1996. Covalent binding of C3b to tetanus toxin: influence on uptake/internalization of antigen by antigen-specific and non-specific B cells. *Immunology 89:348*.

141. Barrault, D. V., and A. M. Knight. 2004. Distinct sequences in the cytoplasmic domain of complement receptor 2 are involved in antigen internalization and presentation. *J Immunol 172:3509*.

142. Perrin-Cocon, L. A., C. L. Villiers, J. Salamero, F. Gabert, and P. N. Marche. 2004. B cell receptors and complement receptors target the antigen to distinct intracellular compartments. *J Immunol 172:3564*.

143. Cherukuri, A., P. C. Cheng, and S. K. Pierce. 2001. The role of the CD19/CD21 complex in B cell processing and presentation of complement-tagged antigens. *J Immunol 167:163*.

144. Boackle, S. A., M. A. Morris, V. M. Holers, and D. R. Karp. 1998. Complement opsonization is required for presentation of immune complexes by resting peripheral blood B cells. *J Immunol 161:6537*.

145. Ferguson, A. R., M. E. Youd, and R. B. Corley. 2004. Marginal zone B cells transport and deposit IgM-containing immune complexes onto follicular dendritic cells. *Int Immunol 16:1411*.

146. Bradbury, L. E., V. S. Goldmacher, and T. F. Tedder. 1993. The CD19 signal transduction complex of B lymphocytes. Deletion of the CD19 cytoplasmic domain alters signal transduction but not complex formation with TAPA-1 and Leu 13. *J Immunol 151:2915*.

147. Chalupny, N. J., A. Aruffo, J. M. Esselstyn, P. Y. Chan, J. Bajorath, J. Blake, L. K. Gilliland, J. A. Ledbetter, and M. A. Tepper. 1995. Specific binding of Fyn and phosphatidylinositol 3-kinase to the B cell surface glycoprotein CD19 through their src homology 2 domains. *Eur J Immunol 25:2978*.

148. Weng, W. K., L. Jarvis, and T. W. LeBien. 1994. Signaling through CD19 activates Vav/mitogen-activated protein kinase pathway and induces formation of a CD19/Vav/phosphatidylinositol 3-kinase complex in human B cell precursors. *J Biol Chem 269:32514*.

149. Otero, D. C., S. A. Omori, and R. C. Rickert. 2001. Cd19-dependent activation of Akt kinase in B-lymphocytes. *J Biol Chem 276:1474*.

150. Fujimoto, M., J. C. Poe, P. J. Jansen, S. Sato, and T. F. Tedder. 1999. CD19 amplifies B lymphocyte signal transduction by regulating Src-family protein tyrosine kinase activation. *J Immunol 162:7088*.

151. Li, X., and R. H. Carter. 1998. Convergence of CD19 and B cell antigen receptor signals at MEK1 in the ERK2 activation cascade. *J Immunol 161:5901*.

152. Inaoki, M., S. Sato, B. C. Weintraub, C. C. Goodnow, and T. F. Tedder. 1997. CD19-regulated signaling thresholds control peripheral tolerance and autoantibody production in B lymphocytes. *J Exp Med 186:1923*.

153. Sato, S., A. S. Miller, M. C. Howard, and T. F. Tedder. 1997. Regulation of B lymphocyte development and activation by the CD19/CD21/CD81/Leu 13 complex requires the cytoplasmic domain of CD19. *J Immunol 159:3278*.

154. Sato, S., D. A. Steeber, P. J. Jansen, and T. F. Tedder. 1997. CD19 expression levels regulate B lymphocyte development: human CD19 restores normal function in mice lacking endogenous CD19. *J Immunol 158:4662*.

155. Holder, M., G. Grafton, I. MacDonald, M. Finney, and J. Gordon. 1995. Engagement of CD20 suppresses apoptosis in germinal center B cells. *Eur J Immunol 25:3160*.

156. Uchida, J., Y. Lee, M. Hasegawa, Y. Liang, A. Bradney, J. A. Oliver, K. Bowen, D. A. Steeber, K. M. Haas, J. C. Poe, and T. F. Tedder. 2004. Mouse CD20 expression and function. *Int Immunol 16:119*.

157. Ettinger, R., G. P. Sims, R. Robbins, D. Withers, R. T. Fischer, A. C. Grammer, S. Kuchen, and P. E. Lipsky. 2007. IL-21 and BAFF/BLyS Synergize in Stimulating Plasma Cell Differentiation from a Unique Population of Human Splenic Memory B Cells. *J Immunol 178:2872*.

158. Ettinger, R., G. P. Sims, A. M. Fairhurst, R. Robbins, Y. S. da Silva, R. Spolski, W. J. Leonard, and P. E. Lipsky. 2005. IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. *J Immunol 175:7867*.

159. Ozaki, K., R. Spolski, R. Ettinger, H. P. Kim, G. Wang, C. F. Qi, P. Hwu, D. J. Shaffer, S. Akilesh, D. C. Roopenian, H. C. Morse, 3rd, P. E. Lipsky, and W. J. Leonard. 2004. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J Immunol 173:5361*.

160. Zhang, X., C. S. Park, S. O. Yoon, L. Li, Y. M. Hsu, C. Ambrose, and Y. S. Choi. 2005. BAFF supports human B cell differentiation in the lymphoid follicles through distinct receptors. *Int Immunol 17:779*.

161. Schneider, P. 2005. The role of APRIL and BAFF in lymphocyte activation. *Curr Opin Immunol 17:282*.

162. Rahman, Z. S., B. Alabyev, and T. Manser. 2007. FcgammaRIIB regulates autoreactive primary antibody-forming cell, but not germinal center B cell, activity. *J Immunol 178:897*.

163. Gary-Gouy, H., P. Bruhns, C. Schmitt, A. Dalloul, M. Daeron, and G. Bismuth. 2000. The pseudo-immunoreceptor tyrosine-based activation motif of CD5 mediates its inhibitory action on B-cell receptor signaling. *J Biol Chem 275:548*.

164. Fujimoto, M., and S. Sato. 2007. B cell signaling and autoimmune diseases: CD19/CD22 loop as a B cell signaling device to regulate the balance of autoimmunity. *J Dermatol Sci*.

165. Reif, K., E. H. Ekland, L. Ohl, H. Nakano, M. Lipp, R. Forster, and J. G. Cyster. 2002. Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature 416:94*.

166. Boes, M., C. Esau, M. B. Fischer, T. Schmidt, M. Carroll, and J. Chen. 1998. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J Immunol 160:4776*.

167. Delamarre, L., M. Pack, H. Chang, I. Mellman, and E. S. Trombetta. 2005. Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science 307:1630*.

168. Bergtold, A., D. D. Desai, A. Gavhane, and R. Clynes. 2005. Cell surface recycling of internalized antigen permits dendritic cell priming of B cells. *Immunity 23:503*.

169. Manser, T. 2004. Textbook germinal centers? *J Immunol 172:3369*.

170. Tarlinton, D. 1998. Germinal centers: form and function. *Curr Opin Immunol 10:245*.

171. O'Connor, B. P., M. W. Gleeson, R. J. Noelle, and L. D. Erickson. 2003. The rise and fall of long-lived humoral immunity: terminal differentiation of plasma cells in health and disease. *Immunol Rev 194:61*.

172. McHeyzer-Williams, M. G., M. J. McLean, P. A. Lalor, and G. J. Nossal. 1993. Antigen-driven B cell differentiation in vivo. *J Exp Med 178:295*.

173. McHeyzer-Williams, M. G., and R. Ahmed. 1999. B cell memory and the long-lived plasma cell. *Curr Opin Immunol 11:172*.

174. Cattoretti, G., C. C. Chang, K. Cechova, J. Zhang, B. H. Ye, B. Falini, D. C. Louie, K. Offit, R. S. Chaganti, and R. Dalla-Favera. 1995. BCL-6 protein is expressed in germinal-center B cells. *Blood 86:45*.

175. Dent, A. L., A. L. Shaffer, X. Yu, D. Allman, and L. M. Staudt. 1997. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. *Science 276:589*.

176. Ye, B. H., G. Cattoretti, Q. Shen, J. Zhang, N. Hawe, R. de Waard, C. Leung, M. Nouri-Shirazi, A. Orazi, R. S. Chaganti, P. Rothman, A. M. Stall, P. P. Pandolfi, and R. Dalla-Favera. 1997. The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation. *Nat Genet 16:161*.

177. Shaffer, A. L., X. Yu, Y. He, J. Boldrick, E. P. Chan, and L. M. Staudt. 2000. BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. *Immunity 13:199*.

178. Wakatsuki, Y., M. F. Neurath, E. E. Max, and W. Strober. 1994. The B cell-specific transcription factor BSAP regulates B cell proliferation. *J Exp Med 179:1099*.

179. Iwakoshi, N. N., A. H. Lee, and L. H. Glimcher. 2003. The X-box binding protein-1 transcription factor is required for plasma cell differentiation and the unfolded protein response. *Immunol Rev 194:29*.

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

181. Kosco, M. H., A. K. Szakal, and J. G. Tew. 1988. In vivo obtained antigen presented by germinal center B cells to T cells in vitro. *J Immunol 140:354*.

182. Kosco-Vilbois, M. H., D. Gray, D. Scheidegger, and M. Julius. 1993. Follicular dendritic cells help resting B cells to become effective antigen-presenting cells: induction of B7/BB1 and upregulation of major histocompatibility complex class II molecules. *J Exp Med 178:2055*.

183. Park, C. S., and Y. S. Choi. 2005. How do follicular dendritic cells interact intimately with B cells in the germinal centre? *Immunology 114:2*.

184. Zhang, X., L. Li, J. Jung, S. Xiang, C. Hollmann, and Y. S. Choi. 2001. The distinct roles of T cell-derived cytokines and a novel follicular dendritic cell-signaling molecule 8D6 in germinal center-B cell differentiation. *J Immunol 167:49*.

185. Pals, S. T., T. E. Taher, R. van der Voort, L. Smit, and R. M. Keehnen. 1998. Regulation of adhesion and migration in the germinal center microenvironment. *Cell Adhes Commun 6:111*.

186. Kim, H. S., X. Zhang, E. Klyushnenkova, and Y. S. Choi. 1995. Stimulation of germinal center B lymphocyte proliferation by an FDC-like cell line, HK. *J Immunol 155:1101*.

187. Kosco-Vilbois, M. H. 2003. Are follicular dendritic cells really good for nothing? *Nat Rev Immunol 3:764*.

188. Rossbacher, J., A. M. Haberman, S. Neschen, A. Khalil, and M. J. Shlomchik. 2006. Antibody-independent B cell-intrinsic and -extrinsic roles for CD21/35. *Eur J Immunol 36:2384*.

189. Sciammas, R., A. L. Shaffer, J. H. Schatz, H. Zhao, L. M. Staudt, and H. Singh. 2006. Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. *Immunity 25:225*.

190. Odendahl, M., H. Mei, B. F. Hoyer, A. M. Jacobi, A. Hansen, G. Muehlinghaus, C. Berek, F. Hiepe, R. Manz, A. Radbruch, and T. Dorner. 2005. Generation of

migratory antigen-specific plasma blasts and mobilization of resident plasma cells in a secondary immune response. *Blood 105:1614*.

191. Kalia, V., S. Sarkar, T. S. Gourley, B. T. Rouse, and R. Ahmed. 2006. Differentiation of memory B and T cells. *Curr Opin Immunol 18:255*.

192. Loetscher, M., P. Loetscher, N. Brass, E. Meese, and B. Moser. 1998. Lymphocyte-specific chemokine receptor CXCR3: regulation, chemokine binding and gene localization. *Eur J Immunol 28:3696*.

193. Tsubaki, T., S. Takegawa, H. Hanamoto, N. Arita, J. Kamogawa, H. Yamamoto, N. Takubo, S. Nakata, K. Yamada, S. Yamamoto, O. Yoshie, and M. Nose. 2005. Accumulation of plasma cells expressing CXCR3 in the synovial sublining regions of early rheumatoid arthritis in association with production of Mig/CXCL9 by synovial fibroblasts. *Clin Exp Immunol 141:363*.

194. Hauser, A. E., G. F. Debes, S. Arce, G. Cassese, A. Hamann, A. Radbruch, and R. A. Manz. 2002. Chemotactic responsiveness toward ligands for CXCR3 and CXCR4 is regulated on plasma blasts during the time course of a memory immune response. *J Immunol 169:1277*.

195. Tschen, S. I., S. A. Stohlman, C. Ramakrishna, D. R. Hinton, R. D. Atkinson, and C. C. Bergmann. 2006. CNS viral infection diverts homing of antibody-secreting cells from lymphoid organs to the CNS. *Eur J Immunol 36:603*.

196. Kunkel, E. J., and E. C. Butcher. 2003. Plasma-cell homing. *Nat Rev Immunol 3:822*.

197. Crotty, S., and R. Ahmed. 2004. Immunological memory in humans. *Semin Immunol 16:197*.

198. Bohnhorst, J. O., M. B. Bjorgan, J. E. Thoen, J. B. Natvig, and K. M. Thompson. 2001. Bm1-Bm5 classification of peripheral blood B cells reveals circulating germinal center founder cells in healthy individuals and disturbance in the B cell subpopulations in patients with primary Sjogren's syndrome. *J Immunol 167:3610*.

199. Fecteau, J. F., G. Cote, and S. Neron. 2006. A New Memory CD27-IgG+ B Cell Population in Peripheral Blood Expressing VH Genes with Low Frequency of Somatic Mutation. *J Immunol 177:3728*.

200. Ehrhardt, G. R., J. T. Hsu, L. Gartland, C. M. Leu, S. Zhang, R. S. Davis, and M. D. Cooper. 2005. Expression of the immunoregulatory molecule FcRH4 defines a distinctive tissue-based population of memory B cells. *J Exp Med 202:783*.

201. Tiller, T., M. Tsuiji, S. Yurasov, K. Velinzon, M. C. Nussenzweig, and H. Wardemann. 2007. Autoreactivity in Human IgG(+) Memory B Cells. *Immunity 26:205*.

202. Klein, U., K. Rajewsky, and R. Kuppers. 1998. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J Exp Med 188:1679*.

203. Anderson, S. M., M. M. Tomayko, and M. J. Shlomchik. 2006. Intrinsic properties of human and murine memory B cells. *Immunol Rev 211:280*.

204. Crotty, S., P. Felgner, H. Davies, J. Glidewell, L. Villarreal, and R. Ahmed. 2003. Cutting edge: long-term B cell memory in humans after smallpox vaccination. *J Immunol 171:4969*.

205. Gray, D., and H. Skarvall. 1988. B-cell memory is short-lived in the absence of antigen. *Nature 336:70*.

206. Maruyama, M., K. P. Lam, and K. Rajewsky. 2000. Memory B-cell persistence is independent of persisting immunizing antigen. *Nature 407:636*.

207. Barrington, R. A., O. Pozdnyakova, M. R. Zafari, C. D. Benjamin, and M. C. Carroll. 2002. B lymphocyte memory: role of stromal cell complement and FcgammaRIIB receptors. *J Exp Med 196:1189*.

208. Anderson, S. M., L. G. Hannum, and M. J. Shlomchik. 2006. Memory B cell survival and function in the absence of secreted antibody and immune complexes on follicular dendritic cells. *J Immunol 176:4515*.

209. Tarlinton, D. 2006. B-cell memory: are subsets necessary? *Nat Rev Immunol 6:785*.

210. McHeyzer-Williams, L. J., M. Cool, and M. G. McHeyzer-Williams. 2000. Antigen-specific B cell memory: expression and replenishment of a novel b220(-) memory b cell compartment. *J Exp Med 191:1149*.

211. Muehlinghaus, G., L. Cigliano, S. Huehn, A. Peddinghaus, H. Leyendeckers, A. E. Hauser, F. Hiepe, A. Radbruch, S. Arce, and R. A. Manz. 2005. Regulation of CXCR3 and CXCR4 expression during terminal differentiation of memory B cells into plasma cells. *Blood 105:3965*.

212. Kindler, V., and R. H. Zubler. 1997. Memory, but not naive, peripheral blood B lymphocytes differentiate into Ig-secreting cells after CD40 ligation and costimulation with IL-4 and the differentiation factors IL-2, IL-10, and IL-3. *J Immunol 159:2085*.

213. Bernasconi, N. L., E. Traggiai, and A. Lanzavecchia. 2002. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science 298:2199*.

214. Agematsu, K., H. Nagumo, Y. Oguchi, T. Nakazawa, K. Fukushima, K. Yasui, S. Ito, T. Kobata, C. Morimoto, and A. Komiyama. 1998. Generation of plasma cells from peripheral blood memory B cells: synergistic effect of interleukin-10 and CD27/CD70 interaction. *Blood 91:173*.

215. Liu, Y. J., C. Barthelemy, O. de Bouteiller, C. Arpin, I. Durand, and J. Banchereau. 1995. Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid up-regulation of B7-1 and B7-2. *Immunity 2:239*.

216. Tangye, S. G., D. T. Avery, and P. D. Hodgkin. 2003. A division-linked mechanism for the rapid generation of Ig-secreting cells from human memory B cells. *J Immunol 170:261*.

217. Tangye, S. G., D. T. Avery, E. K. Deenick, and P. D. Hodgkin. 2003. Intrinsic differences in the proliferation of naive and memory human B cells as a mechanism for enhanced secondary immune responses. *J Immunol 170:686*.

218. Taneja, V., C. J. Krco, M. D. Behrens, H. S. Luthra, M. M. Griffiths, and C. S. David. 2007. B cells are important as antigen presenting cells for induction of MHC-restricted arthritis in transgenic mice. *Mol Immunol 44:2988*.

219. Serreze, D. V., S. A. Fleming, H. D. Chapman, S. D. Richard, E. H. Leiter, and R. M. Tisch. 1998. B lymphocytes are critical antigen-presenting cells for the initiation of T cell-mediated autoimmune diabetes in nonobese diabetic mice. *J Immunol 161:3912*.

220. Chan, O. T., M. P. Madaio, and M. J. Shlomchik. 1999. The central and multiple roles of B cells in lupus pathogenesis. *Immunol Rev 169:107*.

221. O'Garra, A., G. Stapleton, V. Dhar, M. Pearce, J. Schumacher, H. Rugo, D. Barbis, A. Stall, J. Cupp, K. Moore, and et al. 1990. Production of cytokines by mouse B cells: B lymphomas and normal B cells produce interleukin 10. *Int Immunol 2:821*.

222. Van Snick, J. 1990. Interleukin-6: an overview. *Annu Rev Immunol 8:253*.

223. Ware, C. F., T. L. VanArsdale, P. D. Crowe, and J. L. Browning. 1995. The ligands and receptors of the lymphotoxin system. *Curr Top Microbiol Immunol 198:175*.

224. Pistoia, V. 1997. Production of cytokines by human B cells in health and disease. *Immunol Today 18:343*.

225. Kindler, V., T. Matthes, P. Jeannin, and R. H. Zubler. 1995. Interleukin-2 secretion by human B lymphocytes occurs as a late event and requires additional stimulation after CD40 cross-linking. *Eur J Immunol 25:1239*.

226. Okamura, H., S. Kashiwamura, H. Tsutsui, T. Yoshimoto, and K. Nakanishi. 1998. Regulation of interferon-gamma production by IL-12 and IL-18. *Curr Opin Immunol 10:259*.

227. Schultze, J. L., S. Michalak, J. Lowne, A. Wong, M. H. Gilleece, J. G. Gribben, and L. M. Nadler. 1999. Human non-germinal center B cell interleukin (IL)-12 production is primarily regulated by T cell signals CD40 ligand, interferon gamma, and IL-10: role of B cells in the maintenance of T cell responses. *J Exp Med 189:1*.

228. Sartori, A., X. Ma, G. Gri, L. Showe, D. Benjamin, and G. Trinchieri. 1997. Interleukin-12: an immunoregulatory cytokine produced by B cells and antigen-presenting cells. *Methods 11:116*.

229. Harris, D. P., S. Goodrich, K. Mohrs, M. Mohrs, and F. E. Lund. 2005. Cutting edge: the development of IL-4-producing B cells (B effector 2 cells) is controlled by IL-4, IL-4 receptor alpha, and Th2 cells. *J Immunol 175:7103*.

230. Harris, D. P., L. Haynes, P. C. Sayles, D. K. Duso, S. M. Eaton, N. M. Lepak, L. L. Johnson, S. L. Swain, and F. E. Lund. 2000. Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nat Immunol 1:475*.

231. Lee, B. O., J. Rangel-Moreno, J. E. Moyron-Quiroz, L. Hartson, M. Makris, F. Sprague, F. E. Lund, and T. D. Randall. 2005. CD4 T cell-independent antibody response promotes resolution of primary influenza infection and helps to prevent reinfection. *J Immunol 175:5827*.

232. Culton, D. A., B. P. O'Conner, K. L. Conway, R. Diz, J. Rutan, B. J. Vilen, and S. H. Clarke. 2006. Early preplasma cells define a tolerance checkpoint for autoreactive B cells. *J Immunol 176:790*.

233. Gauld, S. B., K. T. Merrell, and J. C. Cambier. 2006. Silencing of autoreactive B cells by anergy: a fresh perspective. *Curr Opin Immunol 18:292*.

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

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

236. Gauld, S. B., R. J. Benschop, K. T. Merrell, and J. C. Cambier. 2005. Maintenance of B cell anergy requires constant antigen receptor occupancy and signaling. *Nat Immunol 6:1160*.

237. Eris, J. M., A. Basten, R. Brink, K. Doherty, M. R. Kehry, and P. D. Hodgkin. 1994. Anergic self-reactive B cells present self antigen and respond normally to CD40-dependent T-cell signals but are defective in antigen-receptor-mediated functions. *Proc Natl Acad Sci U S A 91:4392*.

238. Cooke, M. P., A. W. Heath, K. M. Shokat, Y. Zeng, F. D. Finkelman, P. S. Linsley, M. Howard, and C. C. Goodnow. 1994. Immunoglobulin signal transduction guides the specificity of B cell-T cell interactions and is blocked in tolerant self-reactive B cells. *J Exp Med 179:425*.

239. Benschop, R. J., K. Aviszus, X. Zhang, T. Manser, J. C. Cambier, and L. J. Wysocki. 2001. Activation and anergy in bone marrow B cells of a novel immunoglobulin transgenic mouse that is both hapten specific and autoreactive. *Immunity 14:33*.

240. Rui, L., C. G. Vinuesa, J. Blasioli, and C. C. Goodnow. 2003. Resistance to CpG DNA-induced autoimmunity through tolerogenic B cell antigen receptor ERK signaling. *Nat Immunol 4:594*.

241. Fulcher, D. A., and A. Basten. 1994. Reduced life span of anergic self-reactive B cells in a double-transgenic model. *J Exp Med 179:125*.

242. Lesley, R., Y. Xu, S. L. Kalled, D. M. Hess, S. R. Schwab, H. B. Shu, and J. G. Cyster. 2004. Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF. *Immunity 20:441*.

243. William, J., C. Euler, N. Primarolo, and M. J. Shlomchik. 2006. B cell tolerance checkpoints that restrict pathways of antigen-driven differentiation. *J Immunol 176:2142*.

244. Kilmon, M. A., J. A. Rutan, S. H. Clarke, and B. J. Vilen. 2005. Low-affinity, Smith antigen-specific B cells are tolerized by dendritic cells and macrophages. *J Immunol 175:37*.

245. Lim, H. W., P. Hillsamer, A. H. Banham, and C. H. Kim. 2005. Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. *J Immunol 175:4180*.

246. Yan, J., B. P. Harvey, R. J. Gee, M. J. Shlomchik, and M. J. Mamula. 2006. B cells drive early T cell autoimmunity in vivo prior to dendritic cell-mediated autoantigen presentation. *J Immunol 177:4481*.

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

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

249. Fauci, A. S. 2006. *Harrison's Rheumatology*. McGraw-Hill.

250. Cohen-Solal, J. F., V. Jeganathan, C. M. Grimaldi, E. Peeva, and B. Diamond. 2006. Sex hormones and SLE: influencing the fate of autoreactive B cells. *Curr Top Microbiol Immunol 305:67*.

251. Ward, M. M., E. Pyun, and S. Studenski. 1995. Long-term survival in systemic lupus erythematosus. Patient characteristics associated with poorer outcomes. *Arthritis Rheum 38:274*.

252. Whitacre, C. C. 2001. Sex differences in autoimmune disease. *Nat Immunol 2:777*.

253. Yu, C. Y., and C. C. Whitacre. 2004. Sex, MHC and complement C4 in autoimmune diseases. *Trends Immunol 25:694*.

254. Tan, E. M. 1989. Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. *Adv Immunol 44:93*.

255. John H. Klippel, E. 2001. *Primer on the Rheumatic Diseases*. Arthritis Foundation.

256. Pellicciari, C., M. G. Bottone, and M. Biggiogera. 1999. Restructuring and extrusion of nuclear ribonucleoproteins (RNPs) during apoptosis. *Gen Physiol Biophys 18 Suppl 1:50*.

257. Miranda, M. E., C. E. Tseng, W. Rashbaum, R. L. Ochs, C. A. Casiano, F. Di Donato, E. K. Chan, and J. P. Buyon. 1998. Accessibility of SSA/Ro and SSB/La antigens to maternal autoantibodies in apoptotic human fetal cardiac myocytes. *J Immunol 161:5061*.

258. Casciola-Rosen, L. A., G. Anhalt, and A. Rosen. 1994. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J Exp Med 179:1317*.

259. Bondanza, A., V. S. Zimmermann, G. Dell'Antonio, E. D. Cin, G. Balestrieri, A. Tincani, Z. Amoura, J. C. Piette, M. G. Sabbadini, P. Rovere-Querini, and A. A. Manfredi. 2004. Requirement of dying cells and environmental adjuvants for the induction of autoimmunity. *Arthritis Rheum 50:1549*.

260. Qian, Y., H. Wang, and S. H. Clarke. 2004. Impaired clearance of apoptotic cells induces the activation of autoreactive anti-Sm marginal zone and B-1 B cells. *J Immunol 172:625*.

261. Cocca, B. A., A. M. Cline, and M. Z. Radic. 2002. Blebs and apoptotic bodies are B cell autoantigens. *J Immunol 169:159*.

262. Cooper, G. S., F. W. Miller, and J. P. Pandey. 1999. The role of genetic factors in autoimmune disease: implications for environmental research. *Environ Health Perspect 107 Suppl 5:693*.

263. Maddison, P. J. 1999. Nature and nurture in systemic lupus erythematosus. *Adv Exp Med Biol 455:7*.

264. Eroglu, G. E., and P. F. Kohler. 2002. Familial systemic lupus erythematosus: the role of genetic and environmental factors. *Ann Rheum Dis 61:29*.

265. Tsao, B. P. 2004. Update on human systemic lupus erythematosus genetics. *Curr Opin Rheumatol 16:513*.

266. Mackay, M., A. Stanevsky, T. Wang, C. Aranow, M. Li, S. Koenig, J. V. Ravetch, and B. Diamond. 2006. Selective dysregulation of the FcgammaIIB receptor on memory B cells in SLE. *J Exp Med 203:2157*.

267. Parks, C. G., G. S. Cooper, L. A. Nylander-French, W. T. Sanderson, J. M. Dement, P. L. Cohen, M. A. Dooley, E. L. Treadwell, E. W. St Clair, G. S. Gilkeson, J. A. Hoppin, and D. A. Savitz. 2002. Occupational exposure to crystalline silica and risk of systemic lupus erythematosus: a population-based, case-control study in the southeastern United States. *Arthritis Rheum 46:1840*.

268. Brown, J. M., A. J. Archer, J. C. Pfau, and A. Holian. 2003. Silica accelerated systemic autoimmune disease in lupus-prone New Zealand mixed mice. *Clin Exp Immunol 131:415*.

269. Hudson, C. A., L. Cao, J. Kasten-Jolly, J. N. Kirkwood, and D. A. Lawrence. 2003. Susceptibility of lupus-prone NZM mouse strains to lead exacerbation of systemic lupus erythematosus symptoms. *J Toxicol Environ Health A 66:895*.

270. Cooper, G. S., and C. G. Parks. 2004. Occupational and environmental exposures as risk factors for systemic lupus erythematosus. *Curr Rheumatol Rep 6:367*.

271. Cooper, G. S., C. G. Parks, E. L. Treadwell, E. W. St Clair, G. S. Gilkeson, and M. A. Dooley. 2004. Occupational risk factors for the development of systemic lupus erythematosus. *J Rheumatol 31:1928*.

272. Moon, U. Y., S. J. Park, S. T. Oh, W. U. Kim, S. H. Park, S. H. Lee, C. S. Cho, H. Y. Kim, W. K. Lee, and S. K. Lee. 2004. Patients with systemic lupus erythematosus have abnormally elevated Epstein-Barr virus load in blood. *Arthritis Res Ther 6:R295*.

273. Sundar, K., S. Jacques, P. Gottlieb, R. Villars, M. E. Benito, D. K. Taylor, and L. A. Spatz. 2004. Expression of the Epstein-Barr virus nuclear antigen-1 (EBNA-1) in the mouse can elicit the production of anti-dsDNA and anti-Sm antibodies. *J Autoimmun 23:127*.

274. Morris, M. M., and S. N. Powell. 1997. Irradiation in the setting of collagen vascular disease: acute and late complications. *J Clin Oncol 15:2728*.

275. Ross, J. G., D. H. Hussey, N. A. Mayr, and C. S. Davis. 1993. Acute and late reactions to radiation therapy in patients with collagen vascular diseases. *Cancer 71:3744*.

276. Wysenbeek, A. J., D. A. Block, and J. F. Fries. 1989. Prevalence and expression of photosensitivity in systemic lupus erythematosus. *Ann Rheum Dis 48:461*.

277. Golan, D. T., and Y. Borel. 1984. Increased photosensitivity to near-ultraviolet light in murine SLE. *J Immunol 132:705*.

278. Manderson, A. P., M. Botto, and M. J. Walport. 2004. The role of complement in the development of systemic lupus erythematosus. *Annu Rev Immunol 22:431*.

279. Pickering, M. C., and M. J. Walport. 2000. Links between complement abnormalities and systemic lupus erythematosus. *Rheumatology (Oxford) 39:133*.

280. Morgan, B. P., and M. J. Walport. 1991. Complement deficiency and disease. *Immunol Today 12:301*.

281. Walport, M. J., and P. J. Lachmann. 1990. Complement deficiencies and abnormalities of the complement system in systemic lupus erythematosus and related disorders. *Curr Opin Rheumatol 2:661*.

282. Yang, Y., E. K. Chung, B. Zhou, K. Lhotta, L. A. Hebert, D. J. Birmingham, B. H. Rovin, and C. Y. Yu. 2004. The intricate role of complement component C4 in human systemic lupus erythematosus. *Curr Dir Autoimmun 7:98*.

283. Wehr, C., H. Eibel, M. Masilamani, H. Illges, M. Schlesier, H. H. Peter, and K. Warnatz. 2004. A new CD21low B cell population in the peripheral blood of patients with SLE. *Clin Immunol 113:161*.

284. Masilamani, M., D. Kassahn, S. Mikkat, M. O. Glocker, and H. Illges. 2003. B cell activation leads to shedding of complement receptor type II (CR2/CD21). *Eur J Immunol 33:2391*.

285. Masilamani, M., R. Nowack, T. Witte, M. Schlesier, K. Warnatz, M. O. Glocker, H. H. Peter, and H. Illges. 2004. Reduction of soluble complement receptor 2/CD21 in systemic lupus erythomatosus and Sjogren's syndrome but not juvenile arthritis. *Scand J Immunol 60:625*.

286. Gaipl, U. S., S. Franz, R. E. Voll, A. Sheriff, J. R. Kalden, and M. Herrmann. 2004. Defects in the disposal of dying cells lead to autoimmunity. *Curr Rheumatol Rep 6:401*.

287. Sheriff, A., U. S. Gaipl, R. E. Voll, J. R. Kalden, and M. Herrmann. 2004. Apoptosis and systemic lupus erythematosus. *Rheum Dis Clin North Am 30:505*.

288. Sekine, H., C. M. Reilly, I. D. Molano, G. Garnier, A. Circolo, P. Ruiz, V. M. Holers, S. A. Boackle, and G. S. Gilkeson. 2001. Complement component C3 is not required for full expression of immune complex glomerulonephritis in MRL/lpr mice. *J Immunol 166:6444*.

289. Pickering, M. C., M. Botto, P. R. Taylor, P. J. Lachmann, and M. J. Walport. 2000. Systemic lupus erythematosus, complement deficiency, and apoptosis. *Adv Immunol 76:227*.

290. Einav, S., O. O. Pozdnyakova, M. Ma, and M. C. Carroll. 2002. Complement C4 is protective for lupus disease independent of C3. *J Immunol 168:1036*.

291. Wang, L., L. Bowman, Y. Lu, Y. Rojanasakul, R. Mercer, V. Castranova, and M. Ding. 2004. Essential Role of p53 in Silica-Induced Apoptosis. *Am J Physiol Lung Cell Mol Physiol*.

292. Kim, S. H., and R. P. Sharma. 2004. Mercury-induced apoptosis and necrosis in murine macrophages: role of calcium-induced reactive oxygen species and p38 mitogen-activated protein kinase signaling. *Toxicol Appl Pharmacol 196:47*.

293. Fox, D. A., M. L. Campbell, and Y. S. Blocker. 1997. Functional alterations and apoptotic cell death in the retina following developmental or adult lead exposure. *Neurotoxicology 18:645*.

294. Sturfelt, G., A. Bengtsson, C. Klint, O. Nived, A. Sjoholm, and L. Truedsson. 2000. Novel roles of complement in systemic lupus erythematosus--hypothesis for a pathogenetic vicious circle. *J Rheumatol 27:661*.

295. Mevorach, D., J. L. Zhou, X. Song, and K. B. Elkon. 1998. Systemic exposure to irradiated apoptotic cells induces autoantibody production. *J Exp Med 188:387*.

296. Sato, S., M. Fujimoto, M. Hasegawa, and K. Takehara. 2004. Altered blood B lymphocyte homeostasis in systemic sclerosis: expanded naive B cells and diminished but activated memory B cells. *Arthritis Rheum 50:1918*.

297. Sato, S., M. Hasegawa, M. Fujimoto, T. F. Tedder, and K. Takehara. 2000. Quantitative genetic variation in CD19 expression correlates with autoimmunity. *J Immunol 165:6635*.

298. Warnatz, K., C. Wehr, R. Drager, S. Schmidt, H. Eibel, M. Schlesier, and H. H. Peter. 2002. Expansion of CD19(hi)CD21(lo/neg) B cells in common variable immunodeficiency (CVID) patients with autoimmune cytopenia. *Immunobiology 206:502*.

299. Tedder, T. F., J. C. Poe, M. Fujimoto, K. M. Haas, and S. Sato. 2005. The CD19-CD21 signal transduction complex of B lymphocytes regulates the balance between health and autoimmune disease: systemic sclerosis as a model system. *Curr Dir Autoimmun 8:55*.

300. Blaese, R. M., J. Grayson, and A. D. Steinberg. 1980. Increased immunoglobulin-secreting cells in the blood of patients with active systemic lupus erythematosus. *Am J Med 69:345*.

301. Budman, D. R., E. B. Merchant, A. D. Steinberg, B. Doft, M. E. Gershwin, E. Lizzio, and J. P. Reeves. 1977. Increased spontaneous activity of antibody-forming cells in the peripheral blood of patients with active SLE. *Arthritis Rheum 20:829*.

302. Liossis, S. N., B. Kovacs, G. Dennis, G. M. Kammer, and G. C. Tsokos. 1996. B cells from patients with systemic lupus erythematosus display abnormal antigen receptor-mediated early signal transduction events. *J Clin Invest 98:2549*.

303. Suzuki, H., T. Sakurami, and H. Imura. 1982. Relationship between reduced B cell susceptibility to IgM antibodies and reduced IgD-bearing B cells in patients with systemic lupus erythematosus. *Arthritis Rheum 25:1451*.

304. Tsokos, G. C., H. K. Wong, E. J. Enyedy, and M. P. Nambiar. 2000. Immune cell signaling in lupus. *Curr Opin Rheumatol 12:355*.

305. Bijl, M., G. Horst, P. C. Limburg, and C. G. Kallenberg. 2001. Expression of costimulatory molecules on peripheral blood lymphocytes of patients with systemic lupus erythematosus. *Ann Rheum Dis 60:523*.

306. Folzenlogen, D., M. F. Hofer, D. Y. Leung, J. H. Freed, and M. K. Newell. 1997. Analysis of CD80 and CD86 expression on peripheral blood B lymphocytes reveals increased expression of CD86 in lupus patients. *Clin Immunol Immunopathol 83:199*.

307. Odendahl, M., A. Jacobi, A. Hansen, E. Feist, F. Hiepe, G. R. Burmester, P. E. Lipsky, A. Radbruch, and T. Dorner. 2000. Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus. *J Immunol 165:5970*.

308. Jacobi, A. M., M. Odendahl, K. Reiter, A. Bruns, G. R. Burmester, A. Radbruch, G. Valet, P. E. Lipsky, and T. Dorner. 2003. Correlation between circulating CD27high plasma cells and disease activity in patients with systemic lupus erythematosus. *Arthritis Rheum 48:1332*.

309. Smith, H. R., and R. R. Olson. 1990. CD5+ B lymphocytes in systemic lupus erythematosus and rheumatoid arthritis. *J Rheumatol 17:833*.

310. Su, K., H. Yang, X. Li, A. W. Gibson, J. M. Cafardi, T. Zhou, J. C. Edberg, and R. P. Kimberly. 2007. Expression Profile of Fc{gamma}RIIb on Leukocytes and Its Dysregulation in Systemic Lupus Erythematosus. *J Immunol 178:3272*.

311. Tan, E. M., A. S. Cohen, J. F. Fries, A. T. Masi, D. J. McShane, N. F. Rothfield, J. G. Schaller, N. Talal, and R. J. Winchester. 1982. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum 25:1271*.

312. Silverman, G. J., and S. Weisman. 2003. Rituximab therapy and autoimmune disorders: prospects for anti-B cell therapy. *Arthritis Rheum 48:1484*.

313. Silverman, G. J. 2007. Anti-CD20 therapy and autoimmune disease: therapeutic opportunities and evolving insights. *Front Biosci 12:2194*.

314. Sanz, I., J. H. Anolik, and R. J. Looney. 2007. B cell depletion therapy in autoimmune diseases. *Front Biosci 12:2546*.

315. Anolik, J. H., J. Barnard, A. Cappione, A. E. Pugh-Bernard, R. E. Felgar, R. J. Looney, and I. Sanz. 2004. Rituximab improves peripheral B cell abnormalities in human systemic lupus erythematosus. *Arthritis Rheum 50:3580*.

316. Dass, S., E. M. Vital, and P. Emery. 2006. Rituximab: novel B-cell depletion therapy for the treatment of rheumatoid arthritis. *Expert Opin Pharmacother 7:2559*.

317. Devauchelle-Pensec, V., Y. Pennec, J. Morvan, J. O. Pers, C. Daridon, S. Jousse-Joulin, A. Roudaut, C. Jamin, Y. Renaudineau, I. Q. Roue, B. Cochener, P. Youinou, and A. Saraux. 2007. Improvement of Sjogren's syndrome after two infusions of rituximab (anti-CD20). *Arthritis Rheum 57:310*.

318. Hatjiharissi, E., M. Hansen, D. D. Santos, L. Xu, X. Leleu, E. W. Dimmock, A. W. Ho, Z. R. Hunter, A. R. Branagan, C. J. Patterson, A. Kortsaris, S. Verselis, E. Fox, and S. P. Treon. 2007. Genetic Linkage of FcgammaRIIa and FcgammaRIIIa and Implications for Their Use in Predicting Clinical Responses to CD20-Directed Monoclonal Antibody Therapy. *Clin Lymphoma Myeloma 7:286*.

319. Clynes, R. A., T. L. Towers, L. G. Presta, and J. V. Ravetch. 2000. Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. *Nat Med 6:443*.

320. Lefebvre, M. L., S. W. Krause, M. Salcedo, and A. Nardin. 2006. Ex vivo-activated human macrophages kill chronic lymphocytic leukemia cells in the presence of rituximab: mechanism of antibody-dependent cellular cytotoxicity and impact of human serum. *J Immunother 29:388*.

321. Looney, R. J., J. H. Anolik, D. Campbell, R. E. Felgar, F. Young, L. J. Arend, J. A. Sloand, J. Rosenblatt, and I. Sanz. 2004. B cell depletion as a novel treatment for systemic lupus erythematosus: a phase I/II dose-escalation trial of rituximab. *Arthritis Rheum 50:2580*.

322. Cerny, A., M. Starobinski, A. W. Hugin, S. Sutter, R. M. Zinkernagel, and S. Izui. 1987. Treatment with high doses of anti-IgM prevents, but with lower doses accelerates autoimmune disease in (NZW x BXSB)F1 hybrid mice. *J Immunol 138:4222*.

323. Browning, J. L. 2006. B cells move to centre stage: novel opportunities for autoimmune disease treatment. *Nat Rev Drug Discov 5:564*.

324. Falk, R. J., and J. C. Jennette. 1988. Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. *N Engl J Med 318:1651*.

325. Jennette, J. C., A. S. Wilkman, and R. J. Falk. 1989. Anti-neutrophil cytoplasmic autoantibody-associated glomerulonephritis and vasculitis. *Am J Pathol 135:921*.

326. Jennette, J. C., J. R. Hoidal, and R. J. Falk. 1990. Specificity of anti-neutrophil cytoplasmic autoantibodies for proteinase 3. *Blood 75:2263*.

327. Jennette, J. C., and R. J. Falk. 1997. Small-vessel vasculitis. *N Engl J Med 337:1512*.

328. Savage, C. O., C. G. Winearls, D. J. Evans, A. J. Rees, and C. M. Lockwood. 1985. Microscopic polyarteritis: presentation, pathology and prognosis. *Q J Med 56:467*.

329. Falk, R. J., S. Hogan, T. S. Carey, and J. C. Jennette. 1990. Clinical course of anti-neutrophil cytoplasmic autoantibody-associated glomerulonephritis and systemic vasculitis. The Glomerular Disease Collaborative Network. *Ann Intern Med 113:656*.

330. Hogan, S. L., P. H. Nachman, A. S. Wilkman, J. C. Jennette, and R. J. Falk. 1996. Prognostic markers in patients with antineutrophil cytoplasmic autoantibody-associated microscopic polyangiitis and glomerulonephritis. *J Am Soc Nephrol 7:23*.

331. Hahn, B. H. 1998. Antibodies to DNA. *N Engl J Med 338:1359*.

332. Su, W., and M. P. Madaio. 2003. Recent advances in the pathogenesis of lupus nephritis: autoantibodies and B cells. *Semin Nephrol 23:564*.

333. Bradbury, L. E., G. S. Kansas, S. Levy, R. L. Evans, and T. F. Tedder. 1992. The CD19/CD21 signal transducing complex of human B lymphocytes includes the target of antiproliferative antibody-1 and Leu-13 molecules. *J Immunol 149:2841*.

334. Bombardier, C., D. D. Gladman, M. B. Urowitz, D. Caron, and C. H. Chang. 1992. Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. *Arthritis Rheum 35:630*.

335. Matsuo, T., S. Hisanaga, Y. Yamaguchi, H. Shimokawa, and H. Nakano. 1988. [Case report of a prenatal diagnosis of 46, XY, t(13q; 14q) mat, +18]. *Fukuoka Igaku Zasshi 79:927*.

336. Weening, J. J., V. D. D'Agati, M. M. Schwartz, S. V. Seshan, C. E. Alpers, G. B. Appel, J. E. Balow, J. A. Bruijn, T. Cook, F. Ferrario, A. B. Fogo, E. M. Ginzler, L. Hebert, G. Hill, P. Hill, J. C. Jennette, N. C. Kong, P. Lesavre, M. Lockshin, L. M. Looi, H. Makino, L. A. Moura, and M. Nagata. 2004. The classification of glomerulonephritis in systemic lupus erythematosus revisited. *Kidney Int 65:521*.

337. Borrero, M., and S. H. Clarke. 2002. Low-affinity anti-Smith antigen B cells are regulated by anergy as opposed to developmental arrest or differentiation to B-1. *J Immunol 168:13*.

338. Arnold, L., and S. McCray. 2004. Sequence analyses from single antigen-specific B lymphocytes using an mRNA capture plate. *American Biotechnology Laboratory:14*.

339. Wang, X., and B. D. Stollar. 2000. Human immunoglobulin variable region gene analysis by single cell RT-PCR. *J Immunol Methods 244:217*.

340. Volpe, J. M., L. G. Cowell, and T. B. Kepler. 2006. SoDA: implementation of a 3D alignment algorithm for inference of antigen receptor recombinations. *Bioinformatics 22:438*.

341. Li, Q. Z., C. Xie, T. Wu, M. Mackay, C. Aranow, C. Putterman, and C. Mohan. 2005. Identification of autoantibody clusters that best predict lupus disease activity using glomerular proteome arrays. *J Clin Invest 115:3428*.

342. Winn, D. M., J. F. Wolfe, D. A. Lindberg, F. H. Fristoe, L. Kingsland, and G. C. Sharp. 1979. Identification of a clinical subset of systemic lupus erythematosus by antibodies to the SM antigen. *Arthritis Rheum 22:1334*.

343. Brezinschek, H. P., R. I. Brezinschek, and P. E. Lipsky. 1995. Analysis of the heavy chain repertoire of human peripheral B cells using single-cell polymerase chain reaction. *J Immunol 155:190*.

344. Dorner, T., N. L. Farner, and P. E. Lipsky. 1999. Ig lambda and heavy chain gene usage in early untreated systemic lupus erythematosus suggests intensive B cell stimulation. *J Immunol 163:1027*.

345. Ewert, B. H., J. C. Jennette, and R. J. Falk. 1992. Anti-myeloperoxidase antibodies stimulate neutrophils to damage human endothelial cells. *Kidney Int 41:375*.

346. Xiao, H., P. Heeringa, P. Hu, Z. Liu, M. Zhao, Y. Aratani, N. Maeda, R. J. Falk, and J. C. Jennette. 2002. Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. *J Clin Invest 110:955*.

347. Zandman-Goddard, G., and Y. Shoenfeld. 2005. Infections and SLE. *Autoimmunity 38:473*.

348. Yurasov, S., H. Wardemann, J. Hammersen, M. Tsuiji, E. Meffre, V. Pascual, and M. C. Nussenzweig. 2005. Defective B cell tolerance checkpoints in systemic lupus erythematosus. *J Exp Med 201:703*.

349. Ord, D. C., S. Edelhoff, H. Dushkin, L. J. Zhou, D. R. Beier, C. Disteche, and T. F. Tedder. 1994. CD19 maps to a region of conservation between human chromosome 16 and mouse chromosome 7. *Immunogenetics 39:322*.

350. Kuroki, K., N. Tsuchiya, B. P. Tsao, J. M. Grossman, T. Fukazawa, K. Hagiwara, H. Kano, M. Takazoe, T. Iwata, H. Hashimoto, and K. Tokunaga. 2002. Polymorphisms of human CD19 gene: possible association with susceptibility to systemic lupus erythematosus in Japanese. *Genes Immun 3 Suppl 1:S21*.

351. Tsuchiya, N., K. Kuroki, M. Fujimoto, Y. Murakami, T. F. Tedder, K. Tokunaga, K. Takehara, and S. Sato. 2004. Association of a functional CD19 polymorphism with susceptibility to systemic sclerosis. *Arthritis Rheum 50:4002*.

352. Christensen, S. R., J. Shupe, K. Nickerson, M. Kashgarian, R. A. Flavell, and M. J. Shlomchik. 2006. Toll-like Receptor 7 and TLR9 Dictate Autoantibody Specificity and Have Opposing Inflammatory and Regulatory Roles in a Murine Model of Lupus. *Immunity 25:417*.

353. Luqmani, R. A., P. A. Bacon, R. J. Moots, B. A. Janssen, A. Pall, P. Emery, C. Savage, and D. Adu. 1994. Birmingham Vasculitis Activity Score (BVAS) in systemic necrotizing vasculitis. *Qjm 87:671*.

354. Chan, O. T., L. G. Hannum, A. M. Haberman, M. P. Madaio, and M. J. Shlomchik. 1999. A novel mouse with B cells but lacking serum antibody reveals an antibody-independent role for B cells in murine lupus. *J Exp Med 189:1639*.

355. Shlomchik, M. J., M. P. Madaio, D. Ni, M. Trounstein, and D. Huszar. 1994. The role of B cells in lpr/lpr-induced autoimmunity. *J Exp Med 180:1295*.

356. Roth, R., R. J. Gee, and M. J. Mamula. 1997. B lymphocytes as autoantigen-presenting cells in the amplification of autoimmunity. *Ann N Y Acad Sci 815:88*.

357. Mamula, M. J., S. Fatenejad, and J. Craft. 1994. B cells process and present lupus autoantigens that initiate autoimmune T cell responses. *J Immunol 152:1453*.

358. Uckun, F. M., A. L. Burkhardt, L. Jarvis, X. Jun, B. Stealey, I. Dibirdik, D. E. Myers, L. Tuel-Ahlgren, and J. B. Bolen. 1993. Signal transduction through the CD19 receptor during discrete developmental stages of human B-cell ontogeny. *J Biol Chem 268:21172*.

359. Xiao, J., Y. Messinger, J. Jin, D. E. Myers, J. B. Bolen, and F. M. Uckun. 1996. Signal transduction through the beta1 integrin family surface adhesion molecules VLA-4 and VLA-5 of human B-cell precursors activates CD19 receptor-associated protein-tyrosine kinases. *J Biol Chem 271:7659*.

360. Kitanaka, A., C. Ito, E. Coustan-Smith, and D. Campana. 1997. CD38 ligation in human B cell progenitors triggers tyrosine phosphorylation of CD19 and association of CD19 with lyn and phosphatidylinositol 3-kinase. *J Immunol 159:184*.

361. Engel, P., L. J. Zhou, D. C. Ord, S. Sato, B. Koller, and T. F. Tedder. 1995. Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. *Immunity 3:39*.

362. Sato, S., D. A. Steeber, and T. F. Tedder. 1995. The CD19 signal transduction molecule is a response regulator of B-lymphocyte differentiation. *Proc Natl Acad Sci U S A 92:11558*.

363. Fujimoto, M., Y. Fujimoto, J. C. Poe, P. J. Jansen, C. A. Lowell, A. L. DeFranco, and T. F. Tedder. 2000. CD19 regulates Src family protein tyrosine kinase activation in B lymphocytes through processive amplification. *Immunity 13:47*.

364. DeFranco, A. L., V. W. Chan, and C. A. Lowell. 1998. Positive and negative roles of the tyrosine kinase Lyn in B cell function. *Semin Immunol 10:299*.

365. Culton, D. A., M. W. Nicholas, D. O. Bunch, Q. L. Zhen, T. B. Kepler, M. A. Dooley, C. Mohan, P. H. Nachman, and S. H. Clarke. 2007. Similar CD19 Dysregulation in Two Autoantibody-Associated Autoimmune Diseases Suggests a Shared Mechanism of B-Cell Tolerance Loss. *J Clin Immunol 27:53*.

366. Tangye, S. G., Y. J. Liu, G. Aversa, J. H. Phillips, and J. E. de Vries. 1998. Identification of functional human splenic memory B cells by expression of CD148 and CD27. *J Exp Med 188:1691*.

367. Anolik, J. H., J. Barnard, T. Owen, B. Zheng, S. Kemshetti, R. J. Looney, and I. Sanz. 2007. Recovery of peripheral blood memory B cells after rituximab treatment in SLE is delayed and lags behind lymphoid tissue memory B cell reconstitution. *Arthritis Rheum In press.*

368. Fremeaux-Bacchi, V., I. Bernard, F. Maillet, J. C. Mani, M. Fontaine, J. Y. Bonnefoy, M. D. Kazatchkine, and E. Fischer. 1996. Human lymphocytes shed a soluble form of CD21 (the C3dg/Epstein-Barr virus receptor, CR2) that binds iC3b and CD23. *Eur J Immunol 26:1497*.

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

370. Lopez, F., F. Belloc, F. Lacombe, P. Dumain, J. Reiffers, P. Bernard, and M. R. Boisseau. 1991. Modalities of synthesis of Ki67 antigen during the stimulation of lymphocytes. *Cytometry 12:42*.

371. Scholzen, T., and J. Gerdes. 2000. The Ki-67 protein: from the known and the unknown. *J Cell Physiol 182:311*.

372. Irish, J. M., D. K. Czerwinski, G. P. Nolan, and R. Levy. 2006. Kinetics of B cell receptor signaling in human B cell subsets mapped by phosphospecific flow cytometry. *J Immunol 177:1581*.

373. Pogue, S. L., T. Kurosaki, J. Bolen, and R. Herbst. 2000. B cell antigen receptor-induced activation of Akt promotes B cell survival and is dependent on Syk kinase. *J Immunol 165:1300*.

374. Martin, P., A. Duran, S. Minguet, M. L. Gaspar, M. T. Diaz-Meco, P. Rennert, M. Leitges, and J. Moscat. 2002. Role of zeta PKC in B-cell signaling and function. *Embo J 21:4049*.

375. Johnson, G. L., and R. Lapadat. 2002. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science 298:1911*.

376. Sutherland, C. L., A. W. Heath, S. L. Pelech, P. R. Young, and M. R. Gold. 1996. Differential activation of the ERK, JNK, and p38 mitogen-activated protein kinases by CD40 and the B cell antigen receptor. *J Immunol 157:3381*.

377. Reimold, A. M., P. D. Ponath, Y. S. Li, R. R. Hardy, C. S. David, J. L. Strominger, and L. H. Glimcher. 1996. Transcription factor B cell lineage-specific activator protein regulates the gene for human X-box binding protein 1. *J Exp Med 183:393*.

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

379. Arce, E., D. G. Jackson, M. A. Gill, L. B. Bennett, J. Banchereau, and V. Pascual. 2001. Increased frequency of pre-germinal center B cells and plasma cell precursors in the blood of children with systemic lupus erythematosus. *J Immunol 167:2361*.

380. Hauser, A. E., G. Muehlinghaus, R. A. Manz, G. Cassese, S. Arce, G. F. Debes, A. Hamann, C. Berek, S. Lindenau, T. Doerner, F. Hiepe, M. Odendahl, G. Riemekasten, V. Krenn, and A. Radbruch. 2003. Long-lived plasma cells in immunity and inflammation. *Ann N Y Acad Sci 987:266*.

381. Cassese, G., S. Arce, A. E. Hauser, K. Lehnert, B. Moewes, M. Mostarac, G. Muehlinghaus, M. Szyska, A. Radbruch, and R. A. Manz. 2003. Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. *J Immunol 171:1684*.

382. Cassese, G., S. Lindenau, B. de Boer, S. Arce, A. Hauser, G. Riemekasten, C. Berek, F. Hiepe, V. Krenn, A. Radbruch, and R. A. Manz. 2001. Inflamed kidneys of NZB / W mice are a major site for the homeostasis of plasma cells. *Eur J Immunol 31:2726*.

383. Meller, S., F. Winterberg, M. Gilliet, A. Muller, I. Lauceviciute, J. Rieker, N. J. Neumann, R. Kubitza, M. Gombert, E. Bunemann, U. Wiesner, P. Franken-Kunkel, H. Kanzler, M. C. Dieu-Nosjean, A. Amara, T. Ruzicka, P. Lehmann, A. Zlotnik, and B. Homey. 2005. Ultraviolet radiation-induced injury, chemokines, and leukocyte recruitment: An amplification cycle triggering cutaneous lupus erythematosus. *Arthritis Rheum 52:1504*.

384. Lit, L. C., C. K. Wong, L. S. Tam, E. K. Li, and C. W. Lam. 2006. Raised plasma concentration and ex vivo production of inflammatory chemokines in patients with systemic lupus erythematosus. *Ann Rheum Dis 65:209*.

385. Narumi, S., T. Takeuchi, Y. Kobayashi, and K. Konishi. 2000. Serum levels of ifn-inducible PROTEIN-10 relating to the activity of systemic lupus erythematosus. *Cytokine 12:1561*.

386. Avihingsanon, Y., P. Phumesin, T. Benjachat, S. Akkasilpa, V. Kittikowit, K. Praditpornsilpa, J. Wongpiyabavorn, S. Eiam-Ong, T. Hemachudha, K. Tungsanga, and N. Hirankarn. 2006. Measurement of urinary chemokine and growth factor messenger RNAs: a noninvasive monitoring in lupus nephritis. *Kidney Int 69:747*.

387. Okamoto, H., Y. Katsumata, K. Nishimura, and N. Kamatani. 2004. Interferon-inducible protein 10/CXCL10 is increased in the cerebrospinal fluid of patients with central nervous system lupus. *Arthritis Rheum 50:3731*.

388. Okamoto, H., N. Iikuni, S. Kamitsuji, T. Yoshio, S. Minota, and N. Kamatani. 2006. IP-10/MCP-1 ratio in CSF is an useful diagnostic marker of neuropsychiatric lupus patients. *Rheumatology (Oxford) 45:232*.

389. Hirohata, S., and T. Miyamoto. 1986. Increased intrathecal immunoglobulin synthesis of both kappa and lambda types in patients with systemic lupus erythematosus and central nervous system involvement. *J Rheumatol 13:715*.

390. Segerer, S., B. Banas, M. Wornle, H. Schmid, C. D. Cohen, M. Kretzler, M. Mack, E. Kiss, P. J. Nelson, D. Schlondorff, and H. J. Grone. 2004. CXCR3 is involved in tubulointerstitial injury in human glomerulonephritis. *Am J Pathol 164:635*.

391. Peterson, K. S., J. F. Huang, J. Zhu, V. D'Agati, X. Liu, N. Miller, M. G. Erlander, M. R. Jackson, and R. J. Winchester. 2004. Characterization of heterogeneity in the molecular pathogenesis of lupus nephritis from transcriptional profiles of laser-captured glomeruli. *J Clin Invest 113:1722*.

392. Winfield, J. B., C. M. Brunner, and D. Koffler. 1978. Serologic studies in patients with systemic lupus erythematosus and central nervous system dysfunction. *Arthritis Rheum 21:289*.

393. Martin, F., and A. C. Chan. 2006. B cell immunobiology in disease: evolving concepts from the clinic. *Annu Rev Immunol 24:467*.

394. van Zelm, M. C., I. Reisli, M. van der Burg, D. Castano, C. J. van Noesel, M. J. van Tol, C. Woellner, B. Grimbacher, P. J. Patino, J. J. van Dongen, and J. L. Franco. 2006. An antibody-deficiency syndrome due to mutations in the CD19 gene. *N Engl J Med 354:1901*.

395. Blair, P. J., J. L. Riley, D. M. Harlan, R. Abe, D. K. Tadaki, S. C. Hoffmann, L. White, T. Francomano, S. J. Perfetto, A. D. Kirk, and C. H. June. 2000. CD40 ligand (CD154) triggers a short-term CD4(+) T cell activation response that results in secretion of immunomodulatory cytokines and apoptosis. *J Exp Med 191:651*.

396. Ozaki, M. E., B. A. Coren, T. N. Huynh, D. J. Redondo, H. Kikutani, and S. R. Webb. 1999. CD4+ T cell responses to CD40-deficient APCs: defects in proliferation and negative selection apply only with B cells as APCs. *J Immunol 163:5250*.

397. Lund, F. E., B. A. Garvy, T. D. Randall, and D. P. Harris. 2005. Regulatory roles for cytokine-producing B cells in infection and autoimmune disease. *Curr Dir Autoimmun 8:25*.