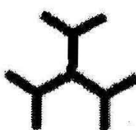
Autoantibodies Directed Against Apoptotic Cells: Antigenic and Biological Analysis

Thesis Submitted to
JAWAHARLAL NEHRU UNIVERSITY
In the partial fulfillment of requirement for the degree of

Doctor of Philosophy

Joy T Das



Immunoendocrinology Laboratory
National Institute of Immunology
New Delhi
India
2006

राष्ट्रीय प्रतिरक्षाविज्ञान संस्थान
NATIONAL INSTITUTE OF IMMUNOLOGY

CERTIFICATE

This is to certify that the thesis titled "**Autoantibodies Directed Against Apoptotic Cells: Antigenic and Biological Analysis**" submitted by **Joy T Das** in partial fulfillment of the degree of **Doctor of Philosophy** from Jawaharlal Nehru University, embodies the work done by the candidate under my guidance at the National Institute of Immunology, New Delhi. This work is original and has not been submitted in part or full for any other degree or diploma of any university.

Dr. Rahul Pal PhD (Supervisor)
Staff Scientist
National Institute of Immunology
New Delhi

अरूणा आसफ अली मार्ग, नई दिल्ली - 110067 फैक्स : 91-11-26162125, 26177626 तार : IMMUNOLOGY
Aruna Asaf Ali Marg, New Delhi - 110067 Fax : 91-11-26162125, 26177626 Grams : IMMUNOLOGY
दूरभाष (Telephones) EPABX : 26717121 - 45 & 26717010 - 19

Table of Contents

Acknowledgements	
Abbreviations	
Introduction	**1-3**
Review of Literature	**4-35**
Materials and Methods	**36-50**
Results	**51-70**
Discussion	**71-85**
Summary and Conclusions	**86-87**
Bibliography	
Appendix	

ACKNOWLEDGEMENTS

The successful completion of this research work towards my Ph.D thesis is due to combined efforts and contributions of numerous people. I gratefully acknowledge their presence on this Ph.D. journey and know these words do not suffice to express my gratitude to them. The fiery enthusiasm of my Ph.D. mentor Dr. Rahul Pal fuelled my interest in this work on Lupus. His constructive criticisms, long discussions and his unfailing support, in terms of time and ideas together with his amazing zest for life, provided me with an excellent environment to do research. His interests ranging from arts, music and science always gave me an opportunity to appreciate life in many spheres and learn something new everyday.

I wish to thank the present and former Director of NII Dr. A. Surolia and Dr. S. K. Basu, for providing me the exceptional facilities at this research institute. I gratefully acknowledge Dr. Om Singh, Head of the IEL lab and Dr. Apurba Sau, Senior Scientist at the IEL lab for their support, scientific discussions and contribution of ideas. Special thanks to Dr. Ayub Qadri, Scientist at the Hybridoma lab, for the lengthy discussions and humor which helped to generate an extensive perspective about my research work and experimental design. I thank Dr Devinder Sehgal for immense help with the antibody sequence analysis and the FAF organization, specially the picnics. I also thank Mr. Akhilesh Aggarwal (Academic Cell) and Mr. P. L. Dahra, Administrative Officer at NII for continued support during the entire duration of my Ph.D. and Prof. G. P. Talwar for providing me a generous travel bursary.

I gratefully acknowledge the constructive criticisms and suggestions from all my Doctoral Committee members; Dr. J. K. Batra, Dr. Vineeta Bal, Dr. Chandrima Shaha and Dr. Subrata Sinha.

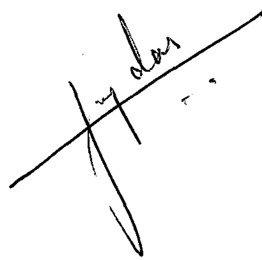
I am grateful to Dr. Satyajit Rath for the B16Flt3 cell line, Dr Amitabha Mukhopadhyay for providing me usage of the confocal microscope and to Shruti, Nitin,

Ajay, Ruchir for help in microscopy work. Dr. Lalit Garg and his lab members, Divya and Neeraj for permitting me to use the GRL lab facilities at unearthly hours for the time-course experiments. Mr. Sundar Bisht for sequencing. Nagarjuna for his expertise in flow cytometry and his friendship. Mr Jaspal for his help in the Animal House and Dr. Ramesh Juyal for all matters animal.

Mr Ashok Kumar, Mr Dayanand, Mr Ramesh Chand, IEL lab technicians for animal handling and for their invaluable help in the lab. To Donald, Beena, Praveen, Rafi and Elishba for their continued assistance. Harshita for being a great junior and a concerned neighbour, Priyanka and Pooja for being great lab mates. Roopali, for teaching me various experimental techniques and being a great friend. My friends at NII, Nagesh for his 'high pressure' shayari, Senthil for intellectual (PM) and scientific discussions, Zeeshan, for all the fun and all my batchmates at NII. To Srikanth for unlimited supply of Jurkat, Ashutosh for THP-1 and various other support. Naeha for LPC, Naveen and Rohini for Jurkat, THP-1 and lots more. Neha for the 'Falcon', Manu and Ankush for the steady supply of really competent competent cells. Tulika for the support and consideration at difficult times.

To the numerous people who have patiently borne my eccentricities and love me for the crazy person that I am; Atul, Ajay, Akhil, KK, BN, Jan, Prashant, Dhananjay and the entire Goa Gang and the oldest friend I got Avik.

Finally, for the unconditional love, support and faith throughout this journey; my Mother, Father and Grandfather and last but not the least, to Life; for letting me live it in fifth gear.



ABBREVIATIONS

β_2-GP1	β_2-Glycoprotein 1
ACAMPs	Apoptotic Cell Associated Molecular Patterns
ADCC	Antibody Dependent Cell-Mediated Cytotoxicity
AICD	Activation Induced Cell Death
AIHA	Autoimmune Haemolytic Anemia
ALPS	Autoimmune Lympho-Proliferative Syndrome
APC	Antigen Presenting Cell
APP	Amyloid Precursor Protein
BSA	Bovine Serum Albumin
CDR	Complementary Determining Region
CFA	Complete Freund's Adjuvant
cFLIP	Cellular FADD like Interleukin-1 β Converting Enzyme like Protease Inhibitor Proteins
CNBr	Cynogen Bromide
CNS	Central Nervous System
CREB	cAMP Response Element-Binding Protein
CSF	Cerebrospinal Fluid
CTL	Cytotoxic T Lymphocyte
Cyt c	Cytochrome c
DAB	Diaminobenzidine
DD	Death Domain
DED	Death Effector Domain
DIABLO	Direct Inhibitor of Apoptotic Proteins Binding Protein with Low PI
DISC	Death Induced Signaling Complex
DMEM	Dulbecco's Modified Eagle's Medium
DTT	Dithiothreitol

EBV	Epstein Barr Virus
ELISA	Enzyme Linked Immunosorbant Assay
ERK	Extracellular Signal Regulated Protein Kinase
FACS	Fluorescence Associated Cell Scanning
FADD	Fas Associated Death Domain
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
FRW	Frame Work Region
HLA	Human Leukocyte Antigen
HRPO	Horseradish Peroxidase
IAP	Inhibitor of Apoptotic Protein
IFA	Incomplete Freund's Adjuvant
IL-10	Interleukin-10
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
MLR	Mixed Lymphocyte Reaction
NMDA	N-Methyl-D-Aspartate
NPSLE	Neuropsychiatric Systemic Lupus Erythematosus
NZB	New Zealand Black
NZM	New Zealand Mixed
NZW	New Zealand White
p53RDL1	p53-Regulated Receptor for Death and Life.
PAMPs	Pathogen Associated Molecular Patterns
PARP	Poly (ADP-ribose) Polymerase
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with 0.05% Tween
PCR	Polymerase Chain Reaction
PHA	Phytohaemagglutanin
PI	Propidium Iodide

PI(3)K	Phospho Inositide 3 kinase
PMA	Phorbol 12-Myristate13-Acetate
PSR	Phosphotidylserine Receptor
RNP	Ribonucleoprotein
RPMI	Rosewell Park Memorial Institute
SAP	Serum Amyloid Protein
SDS	Sodium Dodecyl Sulphate
SLE	Systemic Lupus Erythematosus
Smac	Second Mitochondria Derived Activator of Caspases
TEMED	N'N'N'N-Tetramethylethylenediamine
TGF-β	Transforming Growth Factor-Beta
TNF-α	Tumor Necrosis Factor-Alpha
TRAIL	Tumor Necrosis Factor Related Apoptosis Inducing Ligand
TT	Tetanus Toxoid
UV	Ultraviolet
vFLIP	Viral FADD like Interleukin-1 β Converting Enzyme like Protease Inhibitor Proteins
v/v	Volume by volume
w/v	Weight by volume

Introduction

INTRODUCTION

Systemic lupus erythematosus (SLE), the prototypic non-organ specific autoimmune disorder, is characterized by presence of self-reactive B and T cells. More than a hundred different antibodies are described, and multiple organs are affected; the kidneys, lungs, skin and blood are frequently targeted, and the involvement of the central nervous system is not infrequent. Given the extent and variety of pathology, the reasons for morbidity are many; mortality, on the other hand, occurs mainly due to kidney failure. The disease has a strong sex bias; women in the reproductive age group are more prone to disease.

Pathology in SLE is thought to be primarily antibody-mediated. Dominant "self" specificities include double-stranded DNA (dsDNA), a variety of ribonucleoproteins components, and several phospholipids. Many of these specificities have been associated with marked pathogenic outcomes. For instance, anti-dsDNA reactivity has been implicated in kidney damage, whereas antibodies to the ribonucleoproteins Ro and La, when present in pregnant SLE patients, are linked to neonatal lupus. Antibodies to the Ribosomal P Protein are associated with neuro-psychaitric manifestations of the disease (such as psychoses and suicidal behaviour) and antibodies to various phospholipids (which are also found in anti-phospholipid syndrome [APLS]) may contribute to recurrent fetal loss.

The etiology of SLE remains a matter of speculation and investigation. Human and murine studies implicate both genetic and environmental factors. For a better understanding of disease, a multitude of murine animal models have been generated - transgenics, knockouts, as well as animals bred to be naturally susceptible to lupus-like disease. One of the best-characterized animal models for SLE is the NZB/W F1 mouse; disease develops spontaneously as animals age, and females show a heightened incidence. Organ pathologies and antibody specificities mimic human disease; antibodies to dsDNA, ribonucleoproteins and phospholipids are all found. Genetic analysis has

implicated several individual loci that appear to act in concert to precipitate fulminant disease.

The characteristics and progression of humoral autoimmune responses have been studied in great detail. Frequently, at the initiation of autoimmunity, antibodies typically target a few epitopes on a few on antigens. With the progress of disease, a phenomenon referred to as "epitope spreading" occurs; antibodies arise against multiple epitopes on individual antigens, and against multiple antigens. Most severe pathological outcomes of disease occur when the spectrum of reactivity is at its broadest. Frequently, due to unexplained reasons, individual antibodies exhibit extensive cross-reactivity. It has been repeatedly demonstrated that some monoclonal antibodies have the capacity to bind to different classes of molecules; for example, antibodies to dsDNA have been shown to bind the NR2 glutamate receptor, as well as to a protein on the kidney basement membrane. The idiotypic network has been implicated in lupus initiation and progression. Most work in this regard has been done with anti-dsDNA antibodies; immunization with antibodies of this specificity containing the pathogenic idiotype 16/6 can precipitate disease.

Recent theories on lupus etio-pathogenesis have implicated aberrance in the apoptotic machinery. As cells die, nuclear and cytoplasmic moieties are transported onto the surface. Enhanced apoptosis, as well as the improper clearance of apoptotic cells, would make available excess "self" antigen. Such antigens may express caspase-generated epitopes against which no tolerance exists, and excessive exposure could lead to a state of autoimmunity. In support of this line of argument, it has been demonstrated that immunization of non-autoimmune prone animals with apoptotic cells leads to the generation of autoimmunity. In addition, when molecules involved in the clearance of apoptotic cells are deleted, autoimmune disease frequently results.

Since apoptotic cells are thought to be the original antigenic insult, and since antibodies have been implicated in the pathogenesis of systemic autoimmune responses, this study sought to characterize humoral immune response directed against apoptotic cells. Six

murine monoclonal antibodies were generated, using B cells sourced from two autoimmune strains of mice. Antibodies were characterized for antigenic recognition and assessed for their effects on the phagocytosis of apoptotic cells. Based upon the recognition of biologically important lipids by these antibodies, effects of neutralization of these moieties were assessed. Since idiotypy is postulated to play a role is systemic autoimmune disease, antibodies were immunized into mice and the generation of anti-self responses assessed. Attempts were made to identify possibly peptidic mimotopes recognized by two antibodies, by use of random peptide phage display libraries. Finally, the heavy and light chains of the antibodies were sequenced in an effort to determine whether somatic mutations were required in the generation of apoptotic cell specificity. Knowledge of the antigenic reactivity patterns, genetic profiles and biological effects mediated by antibodies that specifically recognize the products of cell death would serve to increase understanding of the intriguing phenomenon that is systemic autoimmunity.

Review of Literature

REVIEW OF LITERATURE

APOPTOSIS

Introduction and Overview

The phenomenon of apoptosis was first described by C Vogt in 1842. Apoptosis is a central mechanism in the development organisms. At the fetal stage, it aids the organogenesis and sculpting of multi-cellular tissues, resulting in the metamorphosis of the embryo into a fully developed individual. In adults, it is principally involved in the clearance of damaged, mutated or infected cells. Cellular apoptotic clearance is thought to be a quiescent process and is associated with the secretion of anti-inflammatory cytokines (Huynh et al 2002). The term 'apoptosis' itself was coined by Kerr et al (1972). Morphologically, apoptosis is characterized by presence of a condensed nucleus, DNA degradation and plasma membrane blebbing (Kerr et al 1972). Any aberrance in apoptotic mechanisms can lead to multifarious pathological manifestations; cancer, immunodeficiency or autoimmunity can arise as a consequence. The apoptotic cascade is well-orchestrated, and is mainly mediated by Death Receptors (DRs), chemical agents (e.g. anti-cancer compounds) and physical agents (e.g. ultraviolet radiation). A multitude of DRs are characterized, as discussed below. DR ligation leads to the activation of a group of proteases called 'caspases' (Alnemri et al 1996), so called because they are cysteinyl aspartate proteases; the enzymes mediate cleavage after an aspartic acid residue. Post-ligation of the ligand, a cascade of events are initiated involving multiple proteins of both pro- and anti-apoptotic nature, as described below. Decisions on cell death and survival depend upon the relative dominance of these signals. In certain cells, the mitochondria assume central importance, releasing pro-apoptotic molecules and leading to an amplification of death-inducing signals.

Caspases

Fourteen caspases are currently known, which are categorized under three groups, namely Inflammatory, Activator (or Initiator), and Executioner (or Effecter) caspases (Table I) (Fan et al 2005; Larvik et al 2005 a).

Caspases are present as inactive enzymes or zymogens, and are activated by proteolytic cleavage. Inactive caspases contain a 'pro' domain which contains a 80-100 amino acid Death Domain (DD). Death Effecter Domain (DED) and Caspase Recruitment Domain (CARD) in caspases are implicated in the recruitment of the initiator caspases to the signaling complexes (see below) leading to their activation (Fesik 2000). Caspase activation is thought to be autocatalytic, though the exact mechanism is not very clear. It is thought that caspases have very low basal level activity and, when aided by co-factors, these molecules come in proximity and autocatalytic activation occurs (Thornberry et al 1998; Boatright et al 2003). Activation of the initiator caspases leads to the downstream activation of executioner caspases like Caspase 3, 6 and 7. Caspase 3 is thought to be the main executioner caspase; amongst its substrates is I-CAD which is normally associated with CAD (Caspase Activated Dnase), causing its inactivation (Enari et al 1998). I-CAD cleavage leads to the activation of CAD, resulting in nucleosomal cleavage. Caspases also act upon antigens commonly targeted in autoimmune disorders, like the U1RNP, 70 KDa protein, La and PARP (poly ADP ribose polymerase).

Death Receptors

A total of eight members of the death receptor family have been characterized so far; tumor necrosis factor receptor-1 (TNFR-1; also known as DR-1, CD-120a, p55 and p60), CD95 (DR-2, APO-1 and Fas), DR-3 (APO-3, LARD, TRAMP, and WSL-1), TNF-related apoptosis inducing ligand receptor 1 (TRAIL-1; also known as DR-4, APO-2), TRAIL-R2 (also known as DR-5, KILLER, TRICK-2), DR-6, ectodysplasin A receptor (EDAR) and nerve growth factor receptor (NGFR) (Lavrik et al 2005). These receptors can be distinguished by a cytoplasmic tail of ~ 80 amino acid residues which is termed the Death Domain (DD), as well as by extracellular regions containing a number of cysteine-rich domains. Apart from signalling receptors, there also exist decoy receptors which lack the cytosolic signalling domain and therefore do not recruit the signalling molecules discussed below. Four decoy receptors are thus far known (TRAIL-R3 or DcR-1, TRAIL-R4 or DcR-2, DcR-3 and osteoprotegrin or OPG).

Sub-family	Role	Members
Group I	Inflammatory Mediators	Caspase 1 Caspase 4 Caspase 5 Caspase 11 Caspase 12 Caspase 13 Caspase 14
Group II	Apoptosis Activators	Caspase 2 Caspase 8 Caspase 9 Caspase 10
Group III	Apoptosis Executioners	Caspase 3 Caspase 6 Caspase 7

Table I: Classification of the fourteen known caspases

Post-ligation of the receptor by its cognate ligand, two distinct signalling events lead to initiation of the death cascade. Stimulation of CD95, TRAIL-R1 or TRAIL-R2 leads to the formation of a Death Inducing Signalling Complex (DISC; Lavrik et al 2005 b), discussed in detail below. Signalling cascades occurring via TNF-R1, DR-3, and EDAR, on the other hand, can recruit separate molecules which can transduce both survival and death signals.

CD95 is the best-characterized receptor of the TNF superfamily (Peter et al 2003). It is present on the cell surface as pre-associated homo-trimers (Papoff et al 1999; Siegel et al 2000). TRAIL R1 also demonstrates similar features of receptor homo-trimerization (Chan et al 2000). Siegel et al (2000) used FRET (fluorescent resonance energy transfer) to shed further light on CD95; they implicated a region to the N- terminal of the first cysteine rich motif in the process of homo-trimerization. Disruption of this domain, named PLAD (pre-ligand binding assembly domain), led to diminished anti-CD95 antibody-mediated apoptosis. Mice carrying mutants of this domain exhibit autoimmune lymphoproliferative disorder (ALPS; Seigel 2000) indicating the importance of the pre-associated receptor in physiological CD95-mediated signaling.

Ligand binding to the death receptor leads to a multitude of events, the first being the formation of DISC (Kischkel 1995). Immuno-precipitation of CD95 receptor complex followed by two-dimensional gel electrophoresis revealed four associated proteins, initially named CAP 1-4 (Cytotoxicity-dependent Apo-1 Associated Protein; Kischkel 1995); subsequently, CAP 5 and CAP 6 were also elucidated (Medema et al 1997) which were later identified to be the pro-domains of Procaspase 8a and 8b. CAP 1 and CAP 2 were revealed to be the native and phosphorylated forms of Fas Associated Death Domain (FADD; see below) (Chinnaiyan et al 1995; Boldin et al 1995), while CAP 4 turned out to be Procaspase 8a (Muzio et al 1996). CAP 3 was recently found to be an intermediate cleavage product of Procaspase 8a which was generated at the DISC (Golks et al 2006).

FADD was identified by yeast two-hybrid screens and was found to be a Fas-interacting protein. FADD contains an 80 amino acid DD similar to that found in TNF-R and Fas (Chinnaiyan et al 1995). FADD interacts with Fas with its DD and with Caspase 8 (which is also called FLICE (FADD like ICE)) (Muzio et al 1996) or MACH 1α (Boldin et al 1996) with its Death Effecter Domain (DED; Medema et al 1997). This interaction is homotypic in nature, indicating presence of a DED in Caspase 8 (Boldin et al 1996). Caspase 8 has two isoforms (Caspase 8a and 8b) and both are recruited to the DISC (Scaffidi et al 1997). Other components of the DISC include Caspase 10, cellular-FLICE like inhibitory protein (c-FLIP; see below), Fas associated phosphatase-1 (FAP) and Daxx. FADD has also been implicated in TNF-R and TRAIL-R mediated death signal (Chinnaiyan et al 1996, Sprick et al 2000). It is thought that FADD interacts with TNF-R1 via TRADD (Wallach et al 1999).

Caspase 10 is thought to interact via its DED with the DISC. It is thought to be an initiator caspase (Kischkel et al 2001; Wang et al 2001) which is then activated upon recruitment. Recent data also demonstrate recruitment and activation of Caspase 2 in the DISC; the molecule, however, was incapable of initiating apoptosis in Caspase 8 $^{(-/-)}$ cell lines on stimulation of CD95 (Lavrik et al 2006).

v-FLIP (viral-FLICE like inhibitory protein), identified in γ-herpesvirus and molluscipoxvirus, inhibited CD95-mediated apoptosis by binding to the DISC (Bertin et al 1997; Thome et al 1997). v-FLIP interacts with the DED of FADD and interferes with Caspase 8 binding; alternatively, it can also interact with the DED of Caspase 8 and block caspase recruitment to the DISC. Two cellular homologs of the v-FLIP, identified later, were named c-FLIP$_S$ (short) and c-FLIP$_L$ (long) (Irmler et al 1997). c-FLIP$_L$ contains tandem DEDs and a protease-like domain homologous to Caspase 8; several amino acids necessary for the caspase activity are, however, perturbed. It is thought that recruitment of c-FLIP$_L$ can lead to generation of an inactive protease, hence arresting apoptosis (Irmler et al 1997).

The Role of Mitochondria in Apoptosis

Depending on the efficiency of DISC formation, cells can be broadly classified as Type I or Type II (Scaffidi et al 1998). In Type I cells, sufficient activation of Caspase 8 at the DISC leads to adequate activation of Caspase 3. On the contrary, in Type II cells, there is little activation of Caspase 8 because of poor formation of DISC. Apoptosis in these cells is more dependent on the Caspase 8-mediated cleavage of the BH3 family protein Bid to t-Bid, which leads to the release of cytochrome c from the mitochondria, the activation of Caspase 9 and further down-stream events in the cascade (Scaffidi et al 1998).

In the case of an intracellular apoptotic stimulus like DNA damage or oxidative stress, mitochondrial activation precedes DISC formation. Mitochondria harbor a multitude of pro-apoptotic proteins which are released during apoptosis and can lead to the activation of effector caspases like Caspase 3, 6 and 7, which in turn can act on a multitude of substrates. The permeability of the mitochondria is influenced by the Bcl (B cell lymphoma) family of proteins, individual members of which can be either anti-apoptotic and pro-apoptotic in nature. A critical balance of pro- and anti-apoptotic Bcl family members acts as a rheostat and can either lead to cell death or survival (Danial et al 2004). Bcl-2, Bcl-X_L, Bcl-W, MCL-1 are anti-apoptotic, while pro-apoptotic members include Bid, Bak, Bad, Bax (Danial et al 2004). Cytochrome c, essential for the electron transport chain in mitochondria, is bound to the inner mitochondrial membrane and is one of the key proteins released from mitochondria during apoptosis (Ott et al 2002). Initially called Apoptotic Protease Activating Factor (Apaf-2), upon release it interacts with Apaf-1 (Zou et al 1997), Caspase 9 (initially called Apaf-3 (Li et al 1997)), dATP (Liu et al 1996) and ATP. The complex, referred to as the Apoptosome, in turn leads to the activation of effecter caspases (Acehan et al 2002). Smac, also known as DIABLO (Direct IAP binding protein with low pI) (Du et al 2000; Verhagen et al 2000) and Apoptosis Inducing Factor (AIF) are other molecules of significance released by the mitochondria. Smac/DIABLO acts by repressing the activity of cellular-Inhibitor of Apoptotic Proteins (c-IAPs), molecules sharing homology with viral XIAP; it is believed

that c-IAPs inhibited the activity of Caspases 3, 7 and 9 (Roy et al 1997; Deveraux et al 1997; Chai et al 2001; Riedl et al 2001).

Clearance of Apoptotic Cells

As mentioned above, apoptosis has a central role to play in a whole gamut of physiological processes. Equally important are the mechanisms by which apoptotic debris is cleared. Clearance mechanisms primarily involves three main steps which are (1) exposure (and secretion) of ligands on the surface of dying cells, (2) migration of phagocytes to the site of apoptosis and (3) specific recognition of apoptotic cells by phagocytes, followed by their ingestion. Clearance is essentially manifested by professional phagocytes like macrophages but non-professional phagocytes are also implicated (Henson et al 2006).

The clearance of apoptotic cells is a specific process where multiple ligands, receptors and bridging molecules are involved (Figure I).

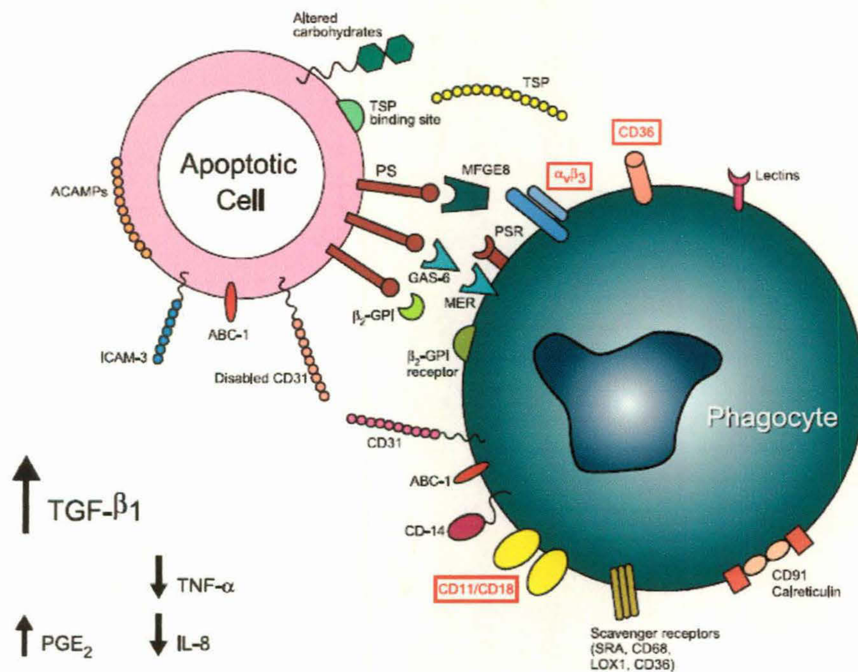


Figure I: Surface-associated and bridging molecules implicated in the phagocytic uptake of dying cells. See text for details.

One of the best characterized ligands on the surface of apoptotic cells is phosphatidylserine (PS), an anionic phospholipid. On healthy cells, this lipid is present in the inner leaflet of the plasma membrane; during apoptosis, it flips to the outer membrane, leading to a loss of plasma membrane asymmetry (Fadok et al 1992). The precise mechanism of this flip is not very well established. A role of aminophospholipid translocase is implicated (Verhoven et al 1995) in the inward flip of PS from the outer membrane; phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are also thought to be internalized, but to a lesser extent (Zachowski et al 1986). Significantly, Verhoven et al (1995) demonstrated loss of aminophospholipid translocase function during apoptosis. However, there has been controversy regarding the caspase dependence of the flip of PS (Martin et al 1996), and some evidence exists linking it to other phenotypes of apoptosis like nuclear degradation (Bratton et al 1997). Bratton et al (1997) also demonstrated a clear role of extracellular (and not intracellular) calcium in the loss of membrane asymmetry during apoptosis. Along with the absence of PS on the external membranes of dying cells, the homotypic interaction of CD31 may also contribute to this discrimination. On healthy cells and phagocytes, CD31 molecules interact in a homotypic fashion, inducing a detachment signal. In contrast, apoptotic cells appear to be incapable of transducing such a signal, leading to engulfment by phagocytes (Brown et al 2002). Other molecules are also exposed on the surface of the apoptotic cells. These include nuclear material, ribonucleoproteins, various cytosolic proteins, as well as other moieties not yet documented or characterized; together, these molecules are designated Apoptotic Cell Associated Molecular Patterns (ACAMPs; Franc et al 1999; Gregory et al 2000).

The phagocytic receptors and bridging molecules are better characterized than are the ACAMPs. The phosphatidylserine receptor (PSR) was one of the first receptors specifically implicated in clearance of apoptotic cells (Fadok et al 2000) though other receptors like CD14 and scavenger receptors (which also bound other ligands) were known to play a role in clearance (Gregory et al 2000). Ingestion of apoptotic cells was shown to lead to expression of anti-inflammatory cytokines like TGF-β (Fadok et al 1998

a). Liposomes containing PS were also phagocytosed and could induce the secretion of the cytokine. Further, the uptake of apoptotic cells could revert the inflammatory effects of LPS (Lipopolysaccharide) (Cvetanovic et al 2004). Some other phagocyte receptors implicated in the uptake of dying cells include CD36, vitronectin receptor (Fadok et al 1998 b), complement receptors CR3 and CR4 (Mevorach et al 1998 a) and c-mer, a receptor tyrosine kinase (Scott et al 2001). Many bridging molecules are also implicated; MFG-E8 (Milk Fat Globule Protein Epidermal Growth Factor-8; Hanayama et al 2002), Gas-6 (Growth Arrest Specific Gene-6; Nakano et al 1997) are the best characterized. Gas-6 bridges PS to the receptor tyrosine kinase c-mer; ablation of c-mer inhibits the uptake of apoptotic cells (Scott et al 2001). MFG-E8 bridges PS and the $\alpha_5\beta_3$ integrin and macrophages from MFG-E8$^{-/-}$ animals are also deficient in the phagocytosis of dying cells (Hanamaya et al 2004). Components of complement (Verbovetski et al 2002) as well as "normal" IgMs (Peng et al 2005; Kim et al 2002) have also been found to influence the uptake of apoptotic cells. SAP (Serum Amyloid Protein; Bijl et al 2003) is implicated in the clearance of the nuclear and chromatin debris generated during apoptosis.

It is apparent that *in vivo*, phagocytes would need to "seek out" dying cells in order to ingest them. It has recently become clear that dying cells secrete substances that act as chemo-attractants. In cells dying via apoptosis, calcium independent phospholipase A_2 (I-PLA_2) is activated, leading to the production and secretion of lysophosphatidylcholine (LPC), a molecule that induces the migration of phagocytic cells (Lauber et al 2003). Membrane blebs, which form and pinch off from the surface of apoptotic cells, also constitute a chemotactic factor (Segundo et al 1999).

The efficient disposal of dying cells is critical for immune homeostasis. As is discussed in the following sections, aberrance in the mechanisms of clearance of apoptotic debris can lead to the onset of autoimmunity.

AUTOIMMUNITY

Introduction

Autoimmunity is defined as a state where T cells or antibodies recognize immunological "self". Though autoimmunity itself is probably not uncommon, pathological manifestations of autoreactivity can lead to autoimmune disorders. Autoimmune diseases can be broadly classified in two main categories, namely organ-specific and non-organ specific. Type I diabetes is an example of an organ-specific autoimmune disease, where T cell-mediated destruction of pancreatic islet cells occurs. Similarly, in multiple sclerosis, T cells directed against myelin basic protein and other antigens cause neuronal damage. In Hashimoto's thyroiditis, antibodies target the thyroid gland, causing its destruction.

In contrast, in non-organ specific (or systemic) autoimmune disorders, multiple organs are targeted. One of the best known examples of systemic autoimmunity is SLE (systemic lupus erythematosus). In SLE, multiple organs are targeted; the brain, kidneys, heart, and joints are frequently affected. Lymphocytopenia, anemia, photosensitivity and neuro-psychiatric manifestations are relatively common occurrences. Possibly the most serious manifestation of disease, and one most often associated with both morbidity and mortality, is glomerulonephritis.

Given its myriad manifestations, the diagnosis of SLE is often difficult. Diagnostic criteria for SLE, put forth by the American College of Rheumatology (ACR), are described in Table II. Individuals manifesting any four criteria simultaneously or during any period of observation are diagnosed as having the disease.

Pathogenesis in SLE is thought to be primarily mediated by autoantibodies; over a hundred autoantibody specificities have been described. Some prominent autoantigens include double stranded DNA (dsDNA), Sm, Ro, La, and various phospholipids. Pathologies are associated with each of these specifcities, and these are discussed in detail below. Anti-dsDNA and anti-Sm antibodies are specific to SLE, and are therefore

used for diagnosis. Antibodies to other ribonucleoproteins are found in autoimmune diseases like Rheumatoid Arthritis and Sjogren's Syndrome as well.

Item	Definitions
1. Malar rash	Fixed erythema, flat or raised, over the malar eminences sparing the naso labial fold
2. Discoid rash	Erythmatosus raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occure in older lesions.
3. Photosensitivity	Skin rashes as a result of unusual reaction to sunlight, by patient history or physician observation.
4. Oral ulceration	Oral or nasopharyngeal ulceration usually painless observed by physician.
5. Non erosive arthritis	Involving 2 or more peripheral joints, characterized by tenderness, swelling or effusion.
6. Pleuritis or pericarditis	Pleuritis: convincing history of pleuritis or rub heard by a physician or evidence of pleural effusion. OR Pericarditis: documented by ECG or rub or evidence of pericardial effusion.
7. Renal disorder	Persistent proteinuria greater than 0.5 g/day or greater than 3+ if quantitative not performed. OR Cellular casts- may be red cells, hemoglobin, granular, tubular or mixed.
8.) Seizures or psychosis	Seizures- in the absence of offending drugs or known metabolic derangement: e.g. uremia, ketoacidosis, or electrolyte imbalance. OR Psychosis- in the absence of offending drugs or known metabolic derangement: e.g. uremia, ketoacidosis, or electrolyte imbalance.
9.) Hematologic disorder	Hemolytic anemia with reticulocytosis OR Leukopenia- less than $4000/mm^3$ on 2 occations OR Lymphopenia- less that $1500/mm^3$ on 2 occations OR Thrombocytopenia- less than $100,000/mm^3$ in the absence of offending drug.
10.) Immunologic disorder	Anti-DNA: antibodies to native DNA in abnormal titres.OR Anti-Sm: presence of antibodies to Sm nuclear antigen OR Positive finding of anti- phospholipid antibodies based on: An abnormal level of IgG or IgM anti-cardiolipin antibodies. A positive test for lupus anticoagulant using standard methods. Or A false positive test for at least 6 months and confirmed by *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test.
11.) Positive antinuclear antibody	An abnormal titre of antinuclear antibody by immunofluorescence or an equivalent assay at any point of in time in the absence of drug.

Table II: Clinical criteria set by American College of Rheumatology for the diagnosis of SLE

Antibody Specificities in SLE

Anti-dsDNA Antibodies

Anti-dsDNA antibodies are a hallmark of SLE; the reactivity is very specific to SLE and is not found in any other autoimmune disorder (Isenberg et al 1985). Anti-dsDNA antibodies are found in about 70% of the lupus patients (Cervera et al 1993; Savarese et al 2006; Kumar et al 2005). Presence of this specificity also reflects disease activity (Swaak et al 1979; Okamura et al 1993). Even though these autoantibodies were first described in 1957 (Holborow et al 1957), their etiology is still not clear. Despite the fact that affinity-matured, isotype-switched anti-dsDNA antibodies are found in SLE patients (Diamond et al 1992), immunization with native DNA does not lead to generation of an anti-DNA response (Plescia et al 1964).

Molecular mimicry is hypothesized to play a role in the genesis of anti-dsDNA responses. Individuals harboring pneumococcal infection can demonstrate serum anti-dsDNA reactivity (Grayzel et al 1991). A single point mutation in the anti-phosphorylcholine antibody S107 (which is protective against lethal pneumococcal infection) can lead to loss of phosphorylcholine reactivity and gain of anti-dsDNA reactivity (Diamond et al 1984). Anti-dsDNA antibodies also demonstrate cross-reactivity to ribonucleoproteins (Koren et al 1995) and lipids like cardiolipin (Lafer et al 1981). Cross-reactivity between DNA and other molecules may indicate that the initiating antigen need not necessarily be DNA. Certain idiotypes of anti-dsDNA antibody, however, demonstrate lesser cross reactivity to other antigens. One such idiotype is F4 (Davidson et al 1989) which, unlike idiotypes like 3I and 8.12 (Grayzel et al 1991), is not present in the non-autoimmune anti-pneumococcal antibodies but is highly represented in lupus patients (Manheimer-Lory et al 1997). These authors demonstrated that such anti-dsDNA antibodies show different patterns of somatic mutation in autoimmune individuals than those found in non-autoimmune individuals. Differences between individuals who are prone or protected from autoimmunity may also depend upon the differential pairing of heavy and light chains, and in the mechanisms that control the induction of tolerance. Spatz et al (1997) described mice (BALB/C, NZW and autoimmune NZB/W) transgenic for heavy chain of

the anti-dsDNA antibody R4A. This antibody uses a Vκ1 light chain. This study demonstrated the existence of two variants of anti-dsDNA reactive antibodies with respect to light chain usage. While the non-autoimmune strains preferentially used the Vκ1 light chain, no anti-dsDNA reactivity was detected in serum, a finding indicative of anergy. NZB/W animals, on the other hand, preferentially used the non-Vκ1 light chains and antibodies utilizing both Vκ1 and non-Vκ1 light chains were observed in serum. Ill-defined mechanisms under genetic control may therefore determine the quantum of pathogenic autoantibody production (Spatz et al 1997).

Anti-dsDNA antibodies are found to be cationic in nature. Often, positively charged amino acids like arginine are found in the complementarity determining regions (CDR) of anti-dsDNA antibodies (Radic et al 1993; Wellmann et al 2005). Arginine residues are thought to be involved in the formation of hydrogen bonds with DNA (Seeman et al 1976). Reverse mutation studies have delineated the specific mutations necessary for anti-dsDNA (Radic et al 1993; Wellmann et al 2005).

The idiotypic network has been implicated in the generation of anti-dsDNA antibodies. Immunization with the DNA binding protein Fus-1 led to the generation of anti-DNA reactivity (Desai et al 1993). The following scenario is envisaged in this and other similar instances: Anti-Fus-1 antibodies, arising upon immunization, would endogenously generate an anti-idiotypic antibody response; a sub-population of such antibodies would behave like "internal images" of Fus-1 and would therefore have to capacity to bind DNA. The involvement of the idiotypic network in the generation of anti-DNA responses is also supported by other studies. Immunization with an anti-dsDNA (referred to as 'Ab1') antibody bearing the 16/6 idiotype (a "public" idiotype, so called because its relatively abundant presence in both human lupus patients and lupus-prone mice) leads to generation of anti-dsDNA antibodies (Mendlovic et al 1988); in this case, the generation of 'Ab2' upon immunization endogenously generates 'Ab3' which, like 'Ab1', can bind DNA.

Anti-dsDNA antibodies have been implicated in kidney damage and glomerulonephritis, both in human patients and murine lupus models (Madaio et al 1987; Vlahakos et al 1992; Suzuki et al 1993). It is thought that antibodies to dsDNA can lead to immune complex formation and deposition in the kidneys, resulting in inflammation and glomerulonephritis. In support of this hypothesis, anti-DNA antibodies and anti-histone antibodies can be eluted from diseased kidneys (Amoura et al 1994; Minota et al 1996). Another view suggests cross-reactivity between particular kidney antigens and dsDNA. Two such antigens against which anti-dsDNA antibodies cross-react are heparin sulfate (located in the glomerular basement membrane; Ohnishi et al 1994) and alpha actinin (Deocharan et al 2002; Zhao et al 2005). Antibodies present in the sera of lupus mice (Deocharan et al 2002) and in patients of lupus nephritis also demonstrate reactivity to alpha actinin, in contrast antibodies in the sera of lupus patients not exhibiting nephritis (Mason et al 2004).

Immunization with a peptide mimotope of an anti-DNA antibody (identified by screening a random peptide phage display library) led to generation of an anti-dsDNA response in non-autoimmune BALB/C mice. Though immune complex deposition in the kidneys was also observed, no disease pathology was demonstrated (Putterman et al 1998). The peptide mimotope inhibited antibody-induced glomerular deposition in severe combined immunodeficient (SCID) mice, providing further evidence of functional mimicry (Gaynor et al 1997). Recent data implicates anti-dsDNA reactivity in neuronal damage; mimotope-generated antibodies cross-reacted with the NR_2 glutamate receptor present on neuronal cells (DeGiorgio et al 2001). On labilization of the blood brain barrier, these antibodies caused neuronal damage, leading to cognitive deficits (Kowal et al 2004; Huerta et al 2006).

Anti-dsDNA antibodies can potentially affect disease pathogenesis by activation of Toll-like receptors (TLRs). Enhanced apoptosis and/or aberrant clearance of apoptotic cells in the lupus (see below) potentially makes available shredded DNA. Immune complexes of such DNA with anti-DNA antibodies have been shown to activate TLR-9, leading to

secretion of various inflammatory cytokines like interferon-alpha (IFN-α) by plasmacytoid dendritic cells (pDCs) (Means et al 2005). DNA eluted from such immune complexes was sized at approximately 160-180 bp, as expected if it was sourced as a cleavage product during apoptosis (Cabrespines et al 1998). Immune complex containing ribonucleoproteins are similarly implicated in the generation of IFN-α and IL-6 from pDCs (Vollmer et al 2005). Both of these cytokines are implicated in the pathogenesis of SLE (Tackey et al 2004); lupus patients often show a heightened IL-6 and IFN-α levels (Linker-Israeli et al 1991; Bennett et al 2003) and it is thought that IFN-α promotes differentiation of pDCs (Blanco et al 2001).

Anti-Ribonucleoprotein Antibodies

Ribonucleoproteins (complexes of RNA and protein) are implicated in RNA splicing. In autoimmune diseases like Sjogren's Syndrome, Rheumatoid Arthritis and SLE, antibodies to ribonucleoprotein are a common occurrence. The etiology of these responses is not very well established; it is believed that aberrant apoptotic mechanisms may be involved. As discussed below, evidence for a viral origin also exists. Some of the antigens to which antibodies are found are SS-A (Ro60 and Ro52), SS-B (La), the U1-snRNP proteins and Smith (Sm). Of these, only anti-Sm antibodies are specific to SLE, and are present in about 30% of patients (Savarese et al 2006; Mahler et al 2005). Anti-Ro60 and anti-Ro52 antibodies have a marginally higher incidence rate in Sjogren's Syndrome (60%) than in SLE (50%). Anti-La reactivity is found in 20% of SLE patients, while incidences in Sjogren's Syndrome can be as high as 60-80% (Wahren-Herlenius et al 1999).

Recombinant fragments and synthetic peptides have been employed to determine major B cell auto-antigenic determinants. PPPGMRPP and PPPGIRGP from SmB appear to be immuno-dominant (James et al 1995). Regions representing amino acids 145-164, 289-308, 301-320 and 349-368 of the La protein seems to form major epitopes. Homology of the La epitope represented by amino acids 147 to 154 have been described with myelin basic protein and DNA topoisomerase II (Tzioufas et al 1997).

Often, the antibody response to Ro52 is directed against the linear epitope in the putative leucine zipper (Frank et al 1994), and the antibody response to Ro60 is generally directed to tertiary epitopes. Kurien et al (2001) hypothesized that antibodies to the tertiary structure of Ro60 could bind to the native epitope of Ro52 derived from its leucine zipper region. Often, however, reactivities to different ribonucleoproteins appear to be linked even in the absence of demonstrable cross-reactivity; for example, antibodies to Ro60 are often seen in patients expressing anti-La (Meilof et al 1997). Many of these molecules are present as components of supra-molecular complexes. The initiation of an immune response to one component (by deliberate immunization, aberrant apoptotic mechanisms or pathogen-mediated molecular mimicry as discussed below) would involve the activation of T cells. Such cells would be capable of providing help to B cells directed against the associated antigen, were such B cells to internalize the whole complex and present the relevant T cell epitope. In several experimental animal models, both intra-molecular as well as inter-molecular "epitope spreading" has been shown to occur. For example, PPPGMRPP and PPPGIRGP from SmB appear to be immuno-dominant, and immunization with the peptides can lead to diversification of the autoimmune response to other molecules (James et al 1995). Similar results have been reported in the Ro/La system (Topfer et al 1995). In idiopathic inflammatory myopathy, anti-Ro52 and anti-Jo1 reactivity seems to be associated even though there was no evidence of cross-reactivity (Rutjes et al 1997). It is apparent that such diversification could contribute to pathology during disease progression. Interestingly, in immunization studies, anti-Ro52 and anti-Ro60 antibody responses appear to be associated as well (Keech et al 1996), though no clear evidence exists for their physical association (Boire et al 1995); it is speculated that apoptosis or viral infections may induce interaction (Casciola-Rosen et al 1994 a).

Other evidence for a viral etiology for anti-ribonucleoprotein responses has accumulated. McClain et al (2005) demonstrated that the region comprising amino acids 169-180 of Ro60 appeared antigenically dominant, and demonstrated that antibodies to this peptide cross-reacted with a amino acids 58-72 of the Epstein Barr virus nuclear antigen-1 (EBNA-1). Immunization with this peptide led to appearance antibodies to Ro60.

Antibodies to SmB were also observed, possibly as a result of epitope spreading. Interestingly, the SmB peptide PPPGMRPP discussed above also demonstrates cross reactivity to EBNA-1 (Sabbatini et al 1993). In further evidence of a possible viral etiology of anti-ribonucleoprotein autoimmune responses, the Ro60 peptide AIALREYRKKMDIPA, which lies near the C-terminal of the protein, cross-reacts with the Vesicular Somatitis Virus (VSV) nucleocapsid protein containing a similar sequence (ERYKKLMD; Scofield et al 1991), and antibodies to VSV can be found in the sera of lupus patients (Hardgrave et al 1993). Ro60 also demonstrates cross reactivity to the Coxsackie virus 2B protein (Stathopoulou et al 2005). Molecular mimicry may thus play an important role in the appearance of these antibody reactivities.

Studies have been carried out using sequential sera obtained from lupus patients to determine the time lag between the appearance of anti-self reactivity and the clinical onset of the disease. Anti-ribonucleoprotein reactivity was the first to appear, approximately 3.4 yr prior disease; in one instance, the antibody appeared approximately 8 yr prior to the disease onset. The first autoimmune responses were directed at Ro60. When disease is clinically apparent, antibodies in serum express fulminant autoreactivity with no subsequent diversification (Arbuckle et al 2003).

Anti-Sm antibodies have not been demonstrated to have a pathogenic role in SLE. Maternal anti-Ro antibodies, on the other hand, have been associated with neonatal lupus and congenital heart block; almost all neonates who exhibit congenital heart block are born to mothers expressing anti-Ro reactivity, but not all children born to mothers with anti-Ro are afflicted (Julkunen et al 1998). It is believed that anti-Ro/La antibodies can react with Ro or La expressed on the surface of apoptotic cardiac myocytes, leading to activation of macrophages and release of inflammatory cytokines like TNF-α (Miranda carús et al 2000). Immunization of mice with Ro and La, which are subsequently rendered pregnant, led to cardiac block in the fetus (Miranda carús et al 1998). Trans-placental migration of infused anti-Ro and anti-La antibodies has been demonstrated to occur, leading to binding with fetal cardiac tissue (Tran et al 2002). Anti-Ro reactivity has also been implicated in neutropenia; cross-reactivity with D1, a 64 KDa membrane

protein, is thought to mediate the decrease in granulocytopenia numbers (Kurien et al 2000). Similarly, anti-Ro reactivity has also been associated with lymphopenia (Harley et al 1989). New evidence that autoantibody responses to ribonucleoproteins may perpetuate disease; immune complexes of U1 snRNP and Sm activated pDCs and led to the secretion of IFN-α. Nucleic acid-mediated activation of TLR ligands is thought to be responsible (Savarese et al 2006).

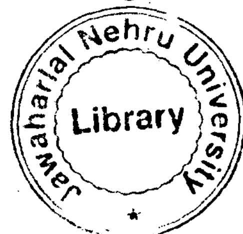
The biological roles of many of these ribonucleoproteins are not very clear. Ro60 has been implicated in the editing of misfolded RNA (O'Brien et al 1994). Genetic deletion of Ro60 leads to development of a lupus-like syndrome in mice, a finding that still requires adequate explanation (Xue et al 2003). Over-expression of Ro52 in Jurkat cells leads to enhanced production of IL2 upon CD3 and CD28 stimulation. Studies on the IL2 promoter demonstrated that Ro52 is involved in the CD28-NFκB signaling pathway (Ishii et al 2003). Ro52 has been found to belong to a family of Ring-B-box-coiled-coil or tripartite motif family of proteins and is also known as TRIM 21 (Reymond et al 2001); many of these proteins have E3 ligase activity. Espinosa et al (2006) formally demonstrated that Ro52 is an E3 ligase, involved in transfer of ubiquitin to proteins, and showed that its over-expression led to reduced cell growth and enhanced apoptosis.

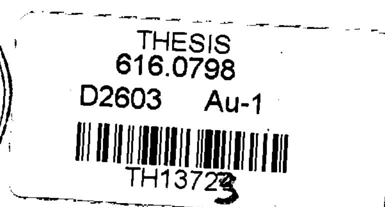
TH-13723

Anti-Phospholipid antibodies

Anti-phospholipid (APL) antibodies are found in about 40% of SLE patients (Shoenfeld 2003) and are more prevalent in autoimmune anti-phospholipid syndrome (APLS) or Hughes' syndrome. There is an over-lap of these antibodies with the so-called "lupus anticoagulant", which is a misnomer; though some anti-phospholipid antibody specificities can delay the clotting cascade *in vitro*, they are thought responsible for an increase in thrombotic events *in vivo* (Shoenfeld 2003). Initially, such antibodies were found in syphilis patients and were used as a diagnostic tool; it was later found that about 50% of these subjects did not show any additional clinical symptoms of syphilis (Lahita et al 1999). Consequently, a false positive test for *Treponema palladium* was included in the diagnostic criteria for SLE by the American College of Rheumatology.

TH-13723





Historically, a description of their reactivity pattern was prompted by the observation that there was an enhancement of binding of antibodies towards cardiolipin in presence of bovine serum; the serum factor responsible for this effect was identified to be β2-Glycoprotein 1 (β2-GPI; McNeil et al 1990), a 50 KDa protein with approximately 17% carbohydrate content, present in normal human plasma at concentrations of about 200 μg/ml (Schultze et al 1961). Present in the lipoprotein fraction, it is also known as apolipoprotein H and is found to bind various negatively charged phospholipids (Wurm 1984). It is thought that such antibodies interact with cryptic epitopes on β2-GPI which are exposed only upon phospholipids binding (Matsuura et al 1994). Kasahara et al (2006) delineated a cryptic epitope present in the domain IV of the β2-GPI which seems to be naturally hidden by the domain V of β2-GPI. Alternatively, the interaction of β2-GPI to anionic phospholipids may increase the local concentration of β2-GPI, leading to an increased ability of antibodies to bind β2-GPI; in the latter case, exposure of cryptic epitopes need not be a necessary requirement (Sheng et al 1998). The thrombotic activity of anti-phospholipid antibodies may be related to the fact that β2-GPI inhibits intrinsic blood coagulation prothrombinase activity (Nimpf 1986).

APL antibodies have been associated with recurrent fetal loss. Passive infusion of anti-cardiolipin antibodies in the mice has been demonstrated to lead to reduced fecundity and decreased number of offspring per animal (Blank et al 1991). Antibodies of this specificity have been associated with various infectious agents like cytomegalovirus, HIV, hepatitis C virus (HCV) (Shoenfeld et al 2003). Panning of a random peptide phage display library using a pathogenic APL antibody H-3 (which could induce experimental anti-phospholipid syndrome and endothelial cell activation) yielded a hexapeptide which could inhibit these biological effects (Blank et al 1999). This peptide mimotope was homologous to proteins from *H. influenzae* and *N. gonorrhoeae* and immunization with these pathogens led to development of an APL antibody response (Blank et al 2002). These studies implicate pathogenic microbes and molecular mimicry in the development of anti-lipid reactivity; such data assumes higher relevance, given the fact that DNA and

phospholipids can be dually recognized by some cross-reactive antibodies (Lafer et al 1981).

APL antibodies have been shown to bind to apoptotic cells; ingestion of opsonization apoptotic cells by macrophages can result in the secretion of inflammatory cytokines like TNF-α (Manfredi et al 1998). Chang et al (2004) demonstrated the exposure of oxidized lipids on the surface of apoptotic cells. They also demonstrated that immunization with apoptotic cells (but not with viable or necrotic cells) led to generation of anti-phospholipid specificities. Both molecular mimicry and apoptosis may therefore play important roles in the genesis of APL antibody responses.

Animal Models

Animal models of disease are useful in precise dissection of disease pathology. Essentially, models can broadly be classified as spontaneous, induced or transgenic. Some significant animal models of SLE are briefly described below.

Spontaneous Models

NZB/W: The NZB mouse was one of the first in which spontaneous autoimmunity similar to SLE were documented; mice had hemolytic anemia but only rarely showed glomerulonephritis (Bielschowsky et al 1964). This strain, however, did not show extended SLE-linked immunological features like abnormal B cell tolerance or B cell hyper-proliferation. Mice demonstrate anti-ssDNA antibodies but not high titres of anti-dsDNA, or anti-histones antibodies in the first year of life. NZB/W (NZB x NZW) mice and SNF1 (NZB x Swiss-Webster [SWR]) mice proved to be better models; these animals show accelerated glomerulonephritis and circulating anti-DNA antibodies (Manny et al 1979). As in human disease, females show a higher disease incidence than males At least 99% NZB/W F1 females suffer from disease; these animals start showing anti-dsDNA antibodies by 6-8 months along with symptoms of renal disease, and die within a year.

These animal models have also helped in the genetic elucidation of the disease. It was found that H-$2^{d,z}$ animals showed greater symptoms of renal disease and anti-DNA antibodies than the homozygous H-$2^{d,d}$ MHC, indicating a role for particular MHC haplotypes in disease manifestation (Carlsten et al 1993). Animals transgenic for the same MHC haplotypes (but on different backgrounds) did not demonstrate disease (Rozzo et al 1999), indicating once again the vital contribution of background genes.

MRL$^{lpr/lpr}$: These mice have a complex genome, comprising contributions from the following strains in the indicated proportions: LG/J (75%), AKR (12.1%), C3H (12.1%) and C57BL/6 (0.3%). Animals are deficient in Fas (CD95), causing an acute lymphoproliferative syndrome. B and T cells are not deleted during development and, as a result, autoreactive cells exist in the periphery. The symptoms of SLE are manifested, with circulating anti-DNA antibodies and renal disease. A higher degree of double negative T cells (CD4- CD8- CD3+ and B220+) are observed (Morse et al 1982). It has been demonstrated that even though the absence of Fas is crucial to the observed phenotype, other background genes contribute significantly towards disease. In this context, it should be mentioned that in humans, the absence of Fas is not significantly associated with SLE, but instead with autoimmune lymphoproliferative syndrome (ALPS). Like MRL$^{lpr/lpr}$ animals, human lupus patients also show a higher double negative T cell population.

BXSB: This strain was derived by crossing C57/BL6 females and SB/Le males. The autoimmune potential is primarily contributed by SB/Le parent. Unlike in other models, male animals of this strain fall sick earlier than females; the mean survival time for males is 20 weeks and for females 68 weeks. This is attributed to the presence of *yaa* (Y chromosome linked autoimmune accelerator). The crucial influence of background genes has once again been highlighted in experiments carried out upon this strain. The presence of *yaa* in C57/BL6 mice does not result in a pathological outcome. Backcrosses with NZW result in mice that are autoimmune-prone (Izui et al 1988).

Induced Models

Idiotypy: As discussed previously, the idiotypic network has also been implicated in SLE onset and pathogenesis. Breakage of tolerance against proteins that interact with DNA (for example histones, transcription factors, or foreign proteins) would lead to the generation of antibodies (Ab1) against these components. Spontaneously generated anti-idiotypic antibodies (Ab2) may contain so-called "internal image" antibodies that bind DNA and cause pathology.

C3H and BALB/c animals, when injected with an anti-dsDNA antibody bearing the 16/6 idiotype, generate anti-ssDNA and anti-dsDNA antibodies. Along with anti-idiotypic antibodies to 16/6 idiotype, and these animals also develop anti-anti-idiotypes, some of which bear the 16/6 idiotype; lethal glomerulonephritis results (Mendlovic et al 1988). The 16/6 idiotype is found in about 54% of lupus patients expressing anti-DNA antibodies (Waisman et al 1997).

Pristane: Injection of the branched alkane Pristane (2,6,10,14-tertamethylpentadecane), routinely used for priming animals for the production of ascites, induces symptoms associated with SLE; for example, glomerular deposition of immunoglobulins and complement, and antibodies reactive to ssDNA are observed. Significantly, however, no anti-dsDNA antibodies are seen. Again, genetic background seems to play a role in determining antibody specificity; BALB/C mice ($H\text{-}2^d$) generate anti-DNA antibodies, whereas in SJL mice ($H\text{-}2^s$), other specificities of antinuclear antibodies are produced (Satoh et al 1995). It is hypothesized that Pristane may cause expansion of CD5+ B1 cells. This subset of cells makes low affinity antibodies (usually of the IgM isotype, against ssDNA, for example) without the need for T cell help. However, Pristane-primed animals also generate antibodies of the IgG isotype against autoantigens like Sm and Ku. These observations indicate the existence of multiple mechanisms and pathways, involving multiple cell types, for autoantibody generation (Satoh et al 1995).

Knockouts

Knockout models help in directly testing the significance of individual genes in disease pathology. A few of them are described here.

C1q: Complement proteins are involved in the clearance of apoptotic cells; deficiency of complement C1q has been implicated in human SLE (Bowness et al 1994). C1q knockout animals were constructed on different genetic backgrounds. 129/O1a x C57/BL6 animals were found to be more prone to the generation of anti-nuclear antibodies (ANA) and crescent glomerulonephritis (GN) than the 129/O1a animals. The disease did not exhibit a sex bias. ANA, but no anti-ssDNA or anti-histone antibodies, were observed. These animals also showed high levels of apoptotic-body deposition in the kidneys, implying either enhanced apoptosis or impaired clearance of apoptotic bodies. This data is in support of other information that implicates faults in the apoptotic machinery in the initiation and/or pathology of SLE (Botto et al 1998).

DNase 1: SLE patients and NZB/W animals (Macanovic et al 1997) exhibit a lower DNase 1 activity with respect to normal individuals. DNase$^{-/-}$ female mice demonstrated higher ANA titers than did males; these animals also showed anti-ssDNA and anti-dsDNA antibodies, with lower reactivity towards isolated histones and ribosomal P protein. Animals also showed deposition of complement C3 and leukocyte infiltration in the kidneys. Lupus-like disease was observed at 6-8 months, to which animals finally succumbed. The incidence of GN was higher in females in homozygous vs heterozygous DNase 1 knockouts (31% vs 19%). These data suggest that DNase1 may be necessary for the removal of nuclear debris from sites of rapid cellular turn over, and this process may be crucial for preventing immune responses against these molecules (Napirei et al 2000).

Secreted IgM: Boes et al (1998) demonstrated that secretory IgM (sIgM) knockout animals exhibited hyper-proliferation of the B-1 (CD5+, B220low, IgM high, Mac1^{+}) subset of cells in the peritoneal lavage. Sub-optimal dosage of antigen resulted in humoral hypo-responsiveness, implying a role for natural IgM in the initiation of

antibody production. sIgM knockouts exhibited anti-dsDNA antibodies in sera by 12 to 18 months of age, with IgG deposition in the kidney (Ehrenstein et al 2000). MRL$^{lpr/lpr}$ carrying the same genotype showed significantly greater kidney deposition of immune complexes at 3 months, compared with un-manipulated MRL$^{lpr/lpr}$ animals or C57/BL6 sIgM knockout animals. Some data suggest that IgM antibodies recognize apoptotic cells, and may be involved in their clearance (Kim et al 2002). However, selective IgM deficiency is poorly correlated with autoimmunity in humans, unlike deficiency of IgA (Rankin et al 1997).

SAP: Serum amyloid protein (SAP) was so named because of its presence on amyloid deposits. This protein can bind to chromatin by displacing histone H1. SAP has been shown to bind to the surface of apoptotic cells and mediate phagocytosis (Bijl et al 2003), probably via recognition of chromatin-containing blebs; it also binds nuclear debris (Breathnach et al 1989). SAP was thus postulated to have a role in the handling and disposal of apoptotic cells. SAP knockout animals developed antibodies against chromatin by 3 months. Animal developed severe proliferative GN with typical immune complex deposition, with the skew towards female predominance being more pronounced than for DNase1 knockouts. Immunization of chromatin led to the development of anti-DNA antibodies in both control and SAP knockouts, but titers in the latter case were much higher. Further, SAP knockouts showed a higher rate of chromatin degradation. These data suggest that SAP may act to stabilize and solubilize the chromatin present in plasma. It is thought that degradation of SAP-linked chromatin and C-reactive protein (CRP) associated ribonucleoproteins like Sm takes place in the liver (Burlingame et al 1996); absence of SAP therefore renders chromatin immunogenic (Bickerstaff et al 1999).

c-Mer: c-Mer is a receptor tyrosine kinase, implicated in the clearance of apoptotic cells. It was shown that, were the kinase domain of c-mer (c-mer^{kd}) deleted, while the binding of apoptotic cells to phagocytes was preserved, subsequent internalization was abolished (Scott et al 2001). Anti-chromatin antibodies and rheumatoid factor were documented;

these animals also showed lupus-like diffuse GN, mostly in females (Cohen et al 2002). This study, therefore, essentially indicates that improper clearance of apoptotic cells can lead to a pathological state like lupus.

Ro60: Ro60 is a known auto-antigen in many autoimmune diseases including SLE. Ro60 is thought to function in the quality control of the 5S r-RNA biogenesis (O'Brien et al 1994). Ro knockout in *D. radiodurans* leads to decreased survival after UV treatment (Chen et al 2000). A Ro60 knockout model was created in the 129/Sv X C57BL/6 strain of mice; animals demonstrated membranoproliferative GN and showed a mortality rate of up to 28% by 12 months of age. Back crossing into C57BL/6 background led to reduced mortality rates but the kidneys still demonstrated hyper-cellularity and immune complex deposition. The sera demonstrated reactivity to nucleosomes, ssDNA and dsDNA. Anti-Sm, anti-La and anti-Ro antibodies were absent, but anti-ribosome antibodies were present. The precise reasons for the development of autoimmunity in this model are at present unclear. In the absence of Ro60, misfolded RNA may be exposed to the immune system, leading to exposure of cryptic epitopes (Xue et al 2003).

G2A Receptor: G2A is an orphan family of G-protein coupled receptors (GPCR) and is thought to bind lysophosphatidylcholine (LPC) and cause chemotaxis (Radu et al 2004). Knockout of this receptor led to lymphoid organ enlargement and polyclonal expansion of lymphocytes. Animals demonstrate anti-nuclear antibodies and T cells showed hyper-proliferative responses (Le et al 2001).

MFG-E 8: As indicated earlier, MFG-E8 has been implicated in the clearance of apoptotic cells. MGF-E8 is secreted by the macrophages and acts as a bridging molecule between apoptotic cells and phagocytes; on the former, it binds phosphatidylserine and on the latter, the $\alpha_v\beta_3$ integrin (Hanayama et al 2002). MFG-E8$^{-/-}$ mice demonstrated splenomegaly and demonstrated enhanced apoptosis of macrophages in the spleen. Animals demonstrated anti-dsDNA reactivity and anti-nuclear antibodies at around 40 weeks (Hanayama et al 2004), once again re-iterating the importance of efficient

apoptotic cell clearance. Further, a mutation (D89E) in the RGD motif of MFG-E8 not only inhibited clearance of the apoptotic cells *in vitro* but also led to generation of auto-antibodies in mice infused with the mutant protein, possibly because of accumulation of apoptotic cells (Asano et al 2004).

Apoptosis and SLE

As discussed above, a huge body of data now implicates aberrance in apoptotic mechanisms with SLE. It is thought that inefficient clearance of apoptotic debris might lead to enhanced exposure of self antigens to the immune system which in turn may lead to breach of tolerance and subsequently autoimmunity. Often, there are increased number of apoptotic cells in the blood of lupus patients; this enhancement correlates with disease activity (Jin et al 2004). The increased number of dying cells can be caused by enhanced apoptosis and/or by the impaired clearance of apoptotic cells. Evidence in support of both phenomena has accumulated (Cohen et al 2002; Boes et al 1998; Bickerstaff et al 1999).

The clearance of apoptotic cells is a very efficient process and in thymus where at least 90% of cells are dying, it is almost impossible to score for apoptotic cells unless very sensitive techniques like TUNEL are used (Surh et al 1994). When clearance is improper, secondary necrosis would result and excess availability of self-antigen may trigger activation of the immune system. As outlined above, in various animal models, abrogation (by genetic deletion) of mechanisms involved in the clearance apoptotic debris leads to experimental autoimmunity.

Immunization of apoptotic thymocytes was shown to lead to generation of anti-ribonucleoprotein and anti-nuclear antibodies (Mevorach et al 1998 b) as well as to anti-phospholipid antibodies (Chang et al 2004). As mentioned earlier, antibodies of such specificity are often found the sera of SLE patients.

During apoptosis, the action of caspases might result in the formation and exposure of "neo-epitopes", determinants that are otherwise cryptic. Under conditions of secondary necrosis, which would occur were the uptake of apoptotic debris impaired, these determinants, against which no tolerance exists, might be rendered immunogenic (Casciola-Rosen et al 1999). Pan et al (2006) made animals transgenic for human La and immunized non-transgenic animals with either healthy and apoptotic thymocytes derived from these animals. Reactivity to human La appeared much earlier in animals immunized with apoptotic cells. A multitude of auto-antigens are known to be cleaved during apoptosis, including La (Huang et al 2005), PARP (poly ADP ribose polymerase; Casiano et al 1996), the U1RNP 70 KDa protein (Casciola-Rosen et al 1994 b) and human RNA helicase (Takeda et al 1999). Often, auto-antibodies are directed towards cleaved products.

Exposure of these auto-antigens to the immune systems is also achieved, particularly in situations of aberrant phagocytic uptake. Many auto-antigens have been shown to be exposed on the surface of apoptotic cells, accumulating into discrete vesicles called apoptotic blebs. Ro60, Sm and nucleosomal material are present on the "large" blebs while Ro52, the ribosomal P protein and fodrin are components of "small" blebs (Casciola-Rosen et al 1994 a). Autoantibodies reactive to DNA and ribonucleoproteins can access these surface structures on apoptotic cells (Cocca et al 2002; Radic et al 2004). Such autoreactive responses may serve to perpetuate disease by causing further decreases in the phagocytic uptake of dying cells, as has been demonstrated using sera from diseased lupus mice (Licht et al 2004), as well as with a human monoclonal antibody (Gandhi et al 2006). Dendritic cells (DCs) may also contribute in the initiation of anti-self responses in situations where early apoptotic cells are not efficiently cleared; DCs have been shown to ingest cells undergoing secondary necrosis, leading to generation of anti-dsDNA antibody responses (Ma et al 2005).

The fact that most antibodies in lupus are reactive to intracellular antigens is interesting, and may be related to the more efficient deletion of B cells reactive to surface antigens.

Intracellular antigens fail to induce B cell tolerance and can lead to formation of B1 cells which can produce IgM antibodies in a T-independent manner (Ferry et al 2003).

Genetic Factors in SLE

Though the etiology of SLE is not clear, genetic predisposition and environmental factors are both implicated in the initiation of disease. The worldwide incidence rate of SLE is approximately 64 per 100,000 individuals. In the female reproductive age group, the female to male ratio is approximately 9:1 (Wakeland et al 2001). As discussed previously, in lupus-prone mice, the heavy chain transgene of an anti-DNA antibody pairs with a distinct set of light chains than does the transgene in non-autoimmune prone animals. In the latter case, B cells also appear to be anergic, since no antibodies are found in serum (Spatz et al 1997). This data implicates genetics in the generation of autoantibody specificity as well as in the activation status of autoreactive cells. The genetic basis of SLE is also supported by the fact that the disease has a strong familial aggregation and siblings of SLE patients are at more risk for disease than the population as a whole (Lawrence et al 1987). Further, there is 10-fold higher concordance rate of SLE in monozygotic (34%) over dizygotic (3%) twins (Deapen et al 1992).

Many candidate genes have been identified in humans and in murine lupus models. In humans, HLA, complement components, and low affinity receptor for IgG has been implicated in predisposition to SLE (Wakeland et al 2001). The DR-B1 alleles DR2 and DR3 have shown consistent associations with SLE and DR/DQ alleles show stronger association with the autoantibody profile observed (Schur et al 1995). The loci for TAP genes, HSP 70, TNF-α are also implicated (Wakeland et al 2001). African-American SLE patients heterozygous or homozygous for the low binding Fcγ RIIa-R131 allele have a higher prevalence of nephritis (Salmon et al 1996). Fcγ RIIIa, which is implicated in clearance of the immune complexes, has also been implicated in the SLE nephritis (Wu et al 1997). As previously discussed, complement also plays a very significant role in the development of SLE. Deficiency of C1q, C2 and C4 has been correlated with development of SLE (Schur et al 1995), and homozygous deletion of C1q in animal

models leads to development of SLE (Botto et al 1998). Complement proteins are encoded by the genes in the HLA Class III region (Wakeland et al 2001). In human SLE subjects, approximately 48 gene loci have been identified which show association with the development of disease (Moser et al 1998). Polymorphisms in the region 16q13 also showed strong relation to SLE (Gaffney et al 1998), a region also associated with Crohn's disease (Hugot et al 2001).

The genetics of lupus has also been studies in various murine models and various loci have been identified. Some of the best-characterized strains in this regard are NZM2410, NZB/W, BXSB and $MRL^{lpr/lpr}$. Approximately 30 chromosomal regions has been implicated in murine lupus on various regions on chromosomes 1, 4, 7 (Wakeland et al 2001). *Sle1* is strongly associated with production of autoantibodies to nuclear antigens (Vyse et al 1996 a), while *Nba2* has been implicated in severe glomereulonephritis (Vyse et al 1996 b). To delineate the exact contribution of different gene loci, these stretches have been transferred to non-autoimmune prone C57/BL6 mice. Transfer of *Sle1* leads to a breach of tolerance to nuclear antigens, with antibodies arising to histones 2A and 2B, as well as to DNA (Mohan et al 1998), *Sle2* has been implicated in the polyclonal activation of B cells (Mohan et al 1997) and *Sle3* been implicated in the dysregulation of polyclonal IgG antibodies and decreased activation-induced cell death in $CD4^+$ T cells (Mohan et al 1999). Transfer of the *Sle3* loci to normal animals also leads to augmented anti-microbial response to pneumonia and intra-abdominal sepsis. There is a marked increase in neutrophil numbers, a fact that has been attributed to reduced apoptosis (Mehrad et al 2006). While the congenic stains for individual loci in C57/BL6 do not express severe disease, the co-expression of all three loci leads to development of severe pathology (Morel et al 2000). These findings strongly implicate genetic pre-disposition in susceptibility to lupus.

Cytokines in SLE

Cytokines are important mediators of the immune system against invading pathogens, and a fine balance of various inflammatory, regulatory or suppressive cytokines aid in the

coordinated execution of immune responses. Disruption of the balance may lead to (or be associated with) various pathological states, including autoimmunity.

Cytokines are significantly associated with lupus pathogenesis. TNF-α and the type I interferons (IFN-α and IFN-β, referred to as IFN-α/β) have been implicated; TNF-α also plays a major role in rheumatoid arthritis (Banchereau et al 2004). A bias towards Th2 cytokines is found in SLE, a finding that is consonant with the plethora of antibodies found in the disease. Reduced TNF-α has been associated with reduced lupus nephritis (Magnusson et al 2001). Along with IFN-α, lupus patients have also been demonstrated to exhibit increased levels of IL-6 (Linker-Israeli et al 1991; Bennett et al 2003). Interestingly, autoimmunity can be induced by IFN-α/β therapy (Ronnblom et al 1991). Bennett et al (2003) further demonstrated a IFN-α "signature" in lupus patients; genes known to be activated by the cytokine were transcribed to a greater extent. Significantly, glucocorticoid treatment of patients abrogated the interferon signature genes. Blanco et al (2001) demonstrated that IFN-α present in the sera of lupus patients could lead to maturation of monocytes into pDCs which could then capture apoptotic cells and present self antigens to T cells. Further, deletion of the IFN regulatory factor 4-binding (IRF-4-binding) protein lead to a disease similar to SLE (Fanzo et al 2006).

In an autoimmune scenario, aberrant processing of apoptotic debris may result in heightened production of IFN-α. In situations where the uptake of dying cells is compromised, nuclear material may be systemically released during apoptosis; immune complex formation can then occur, due to the presence of anti-DNA (Means et al 2005) or anti-ribonucleoprotein (Vollmer et al 2005; Savarese et al 2006) antibodies. Such immune complexes can interact with (and be internalized by) pDCs, leading to production of IFN-α and IL6; lupus sera, due to the presence of such complexes, can elicit cytokine release (Blanco et al 2001). Immune complexes can thus lead to enhanced production of cytokines, and can, as described above, promote the generation of pDC, thereby potentially enhancing the presentation of self-antigens. The pathogenic role of IFN-α has been further elucidated in NZB mice in which the α-chain of the IFN-α/β

receptor has been deleted; reduced anti-erythrocyte autoantibodies, kidney disease and mortality resulted (Santiago-Raber et al 2003).

Therapy for SLE

Since the etiology of SLE remains unclear, non-specific therapies are the treatments of choice. Life expectancy of SLE patients can currently be increased by 10 years in about 90% of cases (Goldblatt et al 2005). As a primary pharmacological therapy for SLE, especially when an early diagnosis is achieved, the anti-malarial hydroxychloroquine is employed (Morand et al 1992); the drug is thought to act by inhibiting antigen presentation and phagocytosis and is well tolerated (Fox 1993). Corticosteroids and immune suppressants like cyclosporine are also frequently employed (Dostal et al 1998). Other therapies include intravenous immunoglobulin (IVIg) which contains immunoglobulins purified from several thousand individuals (Francioni et al 1994); the mechanism of action remains speculative, and may include perturbations of the idiotypic network. Mycophenolate mofetil selectively suppresses T and B cells proliferation by inhibition of inosine monophosphate dehydrogenase and consequently leads to suppression of antibody synthesis (Allison et al 1996).

Alternate therapies include strategies for B cell depletion, autologous haematopoietic stem cell transplant, and biological therapies for blockade of co-stimulation and cytokines through antibodies or soluble receptors. Rituximab, a chimeric antibody to CD20 (a molecule expressed on B cells) has been employed for the treatment of SLE and non-Hodgkin's lymphoma (NHL; Hainsworth et al 2000). Treatment with the antibody results in B cell depletion by complement-mediated lysis and antibody-dependent cell mediated cytotoxicity (ADCC); evidence suggesting that the antibody can also induce apoptosis and inhibit cell growth also exists (Reff et al 1994). Combination treatment of Rituximab and traditional pharmacological agents like cyclophosphamide and corticosteroids results in higher efficacy (Leandro et al 2002).

The first heaematopoietic stem cell transfer (HSCT) was carried out in a SLE patient in 1997 (Marmont et al 1997). Currently, HSCT is attempted in lupus patients non-

responsive to conventional therapy or in those exhibiting acute side-effects from such treatment. The procedure has been quite successful; most patients show reduced disease activity and can discontinue immunosuppressant treatment after 12 months (Traynor et al 2002).

In order to dampen the immune response, the specific blockade of co-stimulatory molecules has also been attempted. CD40 ligand/CD40 or CD28/CD86 blockade can lead to down modulation of the immune response; CTLA4-Ig has been used to treat Rheumatoid Arthritis (Emery et al 2003).

Given the accumulating data implicating IFN-α in lupus pathogenesis (some of which is discussed above, antagonists of IFN-α might hold great promise as therapeutics (Blanco et al 2001).

Materials and Methods

MATERIALS AND METHODS

Cell lines, Animals and Cell Culture

Cultures were maintained at 37°C in a 5% CO_2 atmosphere. All media were supplemented with 10% Fetal Calf Serum (FCS; Biological Industries) and a penicillin-streptomycin mixture (Gibco-BRL) unless otherwise indicated. Jurkat (a human T cell line) and THP-1 (a human monocytic cell line) were maintained in RPMI-1640 (Gibco). SP2/O, used as the fusion partner for generation of the hybridomas as described below, was maintained in DMEM (Gibco-BRL). Peritoneal macrophages were cultured in DMEM or X-Vivo 10 serum-free media (Bio-Whittaker).

Mice used in the study were maintained at the animal facility of National Institute of Immunology. The following strains were employed:

NZB x NZW (F1; referred to as NZB/W)
C57/BL6$^{lpr/lpr}$
C57/BL6
BALB/C
C57/BL6 x BALB/C (F1)
NZB/W F1 x BALB/C (F1)
FVB

All other reagents were of analytical grade and were obtained from Sigma, unless otherwise indicated.

Hybridoma

Fusion

Spleens from aging autoimmune prone NZB/W or C57/BL6$^{lpr/lpr}$ were "teased" between frost-ended slides and the residual tissue discarded, resulting in a single-cell suspension. Erythrocytes were lysed by brief hypotonic shock with water followed reconstitution of

isotonicity with phosphate buffer saline (PBS). SP2/O and the spleenocytes were extensively washed with serum-free DMEM by brief centrifugations at 400 g, and mixed in a ratio of 1:4 (SP2/O: spleenocytes). A solution (50% w/v) of polyethylene glycol (PEG; Mw ≈ 1500 Da) added to the cells over a period of 30 sec, and the tube gently tapped for another 30 sec. After additional, static incubation for 30 sec, 10 ml serum-free DMEM was added over a period of 90 sec. After further washes to remove PEG, cells were resuspended in DMEM supplemented with 20% FCS and hypoxanthine (H) (100 μM), aminopterin (A) (400 nM), and thymidine (T) (16 μM) (HAT; Sigma). Cells were plated at a density of 5 x 10^5 cells/well in 24-well polystyrene plates (Nunc). Individually plated SP2/O and spleenocytes served as negative controls for the fusion as well as selection events. From the seventh day onwards, DMEM supplemented with 20% FCS, H (100 μM) and T (16 μM) was employed. Hybrids were visualized as dividing groups of cells by the fourth or fifth day post-fusion, by which time SP2/O cells (being $HGPRT^{-/-}$) had succumbed to the action of aminopterin. Cultures of growing cells were transferred to 25 cm^2 flasks. At appropriate times, cells were cryopreserved (in liquid nitrogen vapours) in FCS containing 10% dimethylsulfoxide at 2-20 x 10^6 per aliquot. Supernatants were analyzed for reactivity towards healthy, permeabilized and apoptotic cells by FACS, as described below. Hybrids secreting antibodies of interest were rapid-thawed, allowed to recover by 24 hrs in culture and then subcloned by limiting dilution.

Subcloning

The number of viable hybrid cells in a particular culture was determined by the ability of cells to exclude the vital dye Trypan Blue. Cells were dispensed into flat-bottomed 96-well plates (Falcon) at 10, 5, 2.5, 1.25 and 0.625 cells/well. As cells multiplied, wells containing single clones (as morphologically apparent) were identified by microscopic examination. Culture supernatant from each such well was screened for desired reactivity as described below. To ensure monoclonality, three sequential subcloning procedures were carried for each hybridoma. Six monoclonal antibodies were generated, designated as 2C11 (IgMκ), 2H8 (IgMκ), 1B4 (IgMκ), 2C3 (IgG2bκ), 1B3 (IgG2aκ) and 1B1 (IgG2aκ).

Ascites

C57/BL6 x BALB/C (F1) animals were employed for generating ascites for the hybrids generated from C57/BL6$^{lpr/lpr}$ animals, while NZB/W x BALB/C (F1) animals were used for hybrids generated from NZB/W animals. 8-12 week old mice were administered an intra-peritoneal (i.p.) injection of 500 μl Incomplete Freund's Adjuvant (IFA, Difco). Ten days later, hybridoma cells (10^7/animal), re-suspended in serum-free DMEM, were infused, also i.p. The animals were regularly assessed for abdominal distension. Ascites fluid was "tapped" from the peritoneal cavity in as sterile environment, using an 18G needle. The fluid was clarified by passage through glass wool, aliquoted and stored at -70°C.

Induction of Apoptosis

For analysis of the specificity and reactivity of the monoclonal antibodies, apoptotic target cells were generated. Jurkat cells as well as murine thymocytes were employed for this purpose. Jurkat cells, at a density of 3-5 x 10^6 cells/ml, were exposed to ultraviolet (UV) radiation (312 nm) for 15 min followed by culture at a density of 10^6 cells/ml. Thymocytes, obtained from 4-6 week old C57/BL6 mice, were prepared by "teasing" the thymus between frosted-ended slides and were plated at a density of 5 x 10^6 cells/ml. In this case, apoptosis was induced by addition of 10 μM dexamethasone (Sigma). For both Jurkat cells and thymocytes, post-apoptotic induction incubation times ranged from 6 to 24 hr, during which the extent of apoptosis was assessed the binding of cells to Annexin-V (Pharmingen) and/ or Propidium Iodide (PI; Pharmingen).

Preparation of Apoptotic Blebs

Apoptotic blebs were prepared by differential centrifugation by a modification of the protocol described by Casciola-Rosen et al 1994. Apoptosis was induced by UV in Jurkat cells as described above. Cells were centrifuged at 400 g for 5 min at 4°C and the resulting supernatant re-centrifuged for 10 min at 1000 g at 4°C. Once again, the resulting supernatant was further centrifuged at 17000 g for 30 min at 4°C. The pellet, which contained apoptotic blebs, was washed thrice with PBS by repeated centrifugation.

Flowcytometry and Immunofluorescence Microscopy

2 x 10^5 healthy, permeabilized or apoptotic cells were incubated with quantified amounts of purified antibody (or diluted culture supernatant or ascites fluid) for 1 hr at 4°C in 96-well round-bottomed plates (Nunc). Unbound antibody was removed by three 1 min washes (carried out by centrifugations at 400 g at 4°C) with FACS buffer. The secondary reagent (goat anti-mouse Ig coupled to FITC or PE, Jackson ImmunoResearch) was added after appropriate dilution in FACS buffer and an incubation was carried out as before. Following further washes with FACS buffer, cells were re-suspended in PBS containing 0.1% paraformaldehyde. Samples were analyzed on a LSR flowcytometer (Becton-Dickenson).

Reactivity to Propidium Iodide (PI, Sigma) was assessed after a 10 min incubation of cells with the DNA-binding agent. For assessment of Annexin-V (BD Pharmingen) reactivity, cells were resuspended in Annexin-V binding buffer (Appendix) and incubated with the recommended concentration of Annexin-V at room temperature (RT) for 20 min; samples were analyzed within 1 hr.

Immuno-localization studies were carried out on HeLa cells. 2 x 10^5 cells were dispensed on chamber slides (Falcon) and allowed to expand and adhere by overnight incubation. Both healthy and permeable cells (obtained by brief incubation in chilled methanol containing 0.001% Triton X-100) were employed in order to ascertain both cell surface and internal antibody recognition. The protocol followed was essentially as described above for FACS analysis. Digital fluorescence images were acquired on a Canon microscope.

Confocal microscopy was carried out using on Jurkat cells at different times after the induction of apoptosis, essentially following a protocol described by Cocca et al (2002). Cells were fixed by incubation for 10 min in freshly prepared ice-cold paraformaldehyde (2%) in PBS. This was followed by "blocking" by a 10 min incubation in PBS containing

1% BSA. The cells were then processed as for FACS staining. The images were acquired in a Carl Zeiss confocal microscope.

Western Blotting

Preparation of lysates and electrophoresis

Whole-cell lysates of Jurkat or SP2/O cells were prepared. The cells were harvested and extensively washed with PBS by repeated centrifugation at 400 g at 4°C. Cells were then incubated in TKM buffer (Appendix) containing 0.1% NP40 (Appendix) for 30 min on ice, followed by three rounds of snap freeze (in liquid nitrogen) and thaw. The homogenate was centrifuged at 12000 g for 5 min at 4°C to precipitate non-soluble material. The supernatant (now referred to as the lysate) was treated with denaturing Sampling Loading Buffer (Appendix) and loaded on a 12% SDS-PAGE (Appendix). Electrophoresis was carried out at a constant current of 40 mA. Apoptotic blebs, prepared as described above, were similarly processed.

Western Transfer

Nitrocellulose membrane (MDI, India), previously soaked in transfer buffer (Appendix), was placed on a gel containing the electrophoresed proteins. This assembly was encased by a layer of Whatman filter sheets and supportive sponges on both the ends and enclosed in the transfer cassette. The cassette was inserted in the transfer apparatus which was then filled with the Transfer Buffer (Appendix). Transfer of proteins from the gel to the nitrocellulose membrane was achieved by the application of an electric current (300 mA for 3 hr at 4°C). The efficiency of transfer was gauged by the extent of binding of the resolved proteins with the dye Ponceau S.

Immunoblotting

Excess reactive binding sites upon the nitrocellulose membrane containing the resolved proteins were "blocked" by overnight incubation at 4°C in Blocking Buffer (Appendix). The membrane was then cut into strips, each of which was incubated with a dilution (made in Dilution Buffer, Appendix) of antibody for 2 hr at room temperature. Strips

were extensively washed with Wash Buffer (Appendix) with 10 min incubations in the buffer between each wash. An anti-mouse Ig-HRP (Jackson ImmunoResearch) secondary antibody (appropriately diluted in Dilution Buffer as suggested by the manufacturer) was then added to the nitrocellulose strips and a further incubation for an hour carried out at room temperature. After further washes, reactive moieties were revealed on X-ray film by enhanced chemi-luminescence (ECL; Amersham Biosciences).

Enzyme Linked Immunosorbant Assay (ELISA)

Ribonucleoproteins and Peptides

Recombinant autoantigens were diluted in 0.1 M carbonate buffer, pH 9.2 (Appendix). 50 μl of buffer containing 500 ng antigen (or 3 μg of MAP or MAP-synthetic peptide) was dispensed in each well of a 96-well ELISA plate (Nunc). Plates were incubated overnight at 4°C. After washes with Wash Buffer (Appendix), un-occupied binding sites were "blocked" by incubation with 200 μl Blocking Buffer (Appendix). All subsequent incubations were at 37°C for 2 hr. 50 μl of primary antibodies, appropriately diluted in Dilution Buffer (Appendix) were dispensed into the wells and a further incubation carried out. After further washes, 50 μl of secondary antibody (Goat anti-mouse Ig-HRP; Jackson ImmunoResearch), appropriately diluted in Dilution Buffer, was added to each well. Following incubation, wells were extensively washed. Enzymatic reactivity was visualized with a TMB substrate solution (Appendix). Absorbance was recorded at 450 nm in a Bio-Tek Elx 800 ELISA plate reader.

Phospholipids

The following phospholipids were employed: Lysophosphatidylcholine (LPC), Lysophosphatidic Acid (LPA), Phosphatidylserine (PS), Phosphatidylethanolamine (PE), Phosphatidylcholine (PC), Phosphatidic Acid (PA) and Cardiolipin (CL). Phospholipids were resuspended in 1:4 choloroform : methanol at a concentration of 50 μg/ml. 30 μl of this solution was dispensed into wells of a 96-well ELISA plate. Evaporation of the solvent occurred during incubation at 16 hr at 4°C. Un-occupied binding sites were

"blocked" by incubation with PBS containing 5% low fat milk powder or 0.1% polyvinyl alcohol (PVA). The incubation and washing procedures were as mentioned above.

Phagocytosis

CFSE Labeling of Apoptotic Thymocytes

Thymocytes were isolated as described earlier. Cells were counted in a Neubauer's chamber, washed twice with sterile PBS by centrifugation at 400 g at 4°C and re-suspended to a density of 10^7 cells/ml. Carboxy fluorescein succinimdyl ester diacetate salt (CFSE-DA) mixed isomer (Molecular Probes) was added to a final concentration of 10 nM and an incubation was carried out for 20 min at 37°C. Cells were washed twice with RPMI-1640 and plated at a density of 5 x 10^6 cells/ml. Apoptosis was induced by the addition of dexamethasone as described earlier.

Phagocytic Uptake

For generation of "elicited" macrophages, C57/BL6 mice were injected intraperitoneally with 1 ml of a 4% thioglycolate solution. Peritoneal macrophages were "tapped" 72 hr post-thioglycolate injection. Cells were resuspended in DMEM and allowed to adhere to the plastic substrate of tissue culture plates for 2 hr. Non-adherent cells were removed by repeated flushing by medium. CFSE-labeled apoptotic thymocytes were added to adhered phagocytes in a ratio of 1:4 (phagocyte: apoptotic cells) in presence or absence of varying concentrations of antibodies. Cultures were incubated for 2 hr at 37°C. Plates were then transferred to 4°C to stop further uptake of apoptotic cells. In certain instances, the supernatant from phagocytosis wells were harvested for subsequent cytokine analysis. The cells were "harvested" by extensive flushing or scrapping. Cells were then stained with an anti-Mac1-phycoerythrin (PE; BD Pharmingen) conjugate (as per protocols outlined above) to label the macrophages population. FACScan analysis was then carried out. The Phagocytic Index was assessed as the number of dually-stained cells (macrophages [red] that had ingested apoptotic cells [green]) over total number of macrophages (red).

Inflammation

One of the apoptotic-cell specific antibodies (2C11 [IgMκ], see below) was reactive towards Lysophosphatidylcholine (LPC; Avanti Polar). LPC is a bioactive lipid with anti-inflammatory effects (Yan et al 2004). To delineate the anti-inflammatory effects of LPC, thioglycolate-elicited peritoneal macrophages were cultured in X-Vivo 10 serum-free media and allowed to adhere for 2-3 hr at 37°C. Non-adherent cells were removed, as before. 100 ng/ml of *S typhi* LPS was then added to the cells, in presence of varying concentrations of LPC. Phosphatidylcholine (PC, Avanti Polar) served as the negative control. An incubation was then carried out for 4 hr at 37°C. TNF-α was quantified in all cultures by ELISA (BD Pharmingen). To assess the potential of the LPC-reactive antibody 2C11 (as opposed to an apoptotic cell-specific but LPC non-reactive antibody 1B4 [IgMκ]) to revert the anti-inflammatory effects of LPC, normalized concentrations of ascites (of antibodies 2C11 or 1B4) were adsorbed on wells of an ELISA plate by incubation for 16 hr at 4°C. Un-occupied sites were "blocked" by incubation of the wells with a 2 hr incubation with PBS supplemented with 5% low fat milk. Different concentrations of LPC, prepared in X-Vivo 10 serum free media, were then dispensed into antibody 2C11 or 1B4 adsorbed wells (or antibody-free wells just "blocked" with low fat milk as control) and an incubation carried out for 2 hr at room temperature. Supernatants (containing unbound LPC) were added onto cultures of peritoneal macrophages derived from C57/BL6 mice (isolated as described above) in presence of 100 ng/ml of *S typhi* LPS (Sigma). Mouse TNF-α was estimated by ELISA after an incubation for 4 hr at 37°C.

Transmigration

Apoptotic cells are thought to secrete LPC, a lipid which acts as a chemo-attractant for phagocytic cells (Lauber et al 2003). It is also believed that small membranous vesicles release by apoptotic cells have similar properties (Segundo et al 1999). The antibody 2C11 bound both LPC and apoptotic blebs (see below). The ability of the antibody to inhibit the transmigration of THP-1 cells towards supernatant obtained from apoptotic cell cultures was assessed. An anti-TNP antibody (IgMκ; BD Pharmingen) was employed

as a negative control. Apoptosis was induced in Jurkat cells as described earlier. Supernatants were harvested by centrifugation at 1200 g for 10 min. 300 μl of apoptotic cell culture supernatant (supplemented with 1 μg 2C11 or the control antibody) was added to the lower chamber of the transmigration wells. THP-1 cells (50 μl of a suspension of 2 x 10^6 cells/ml) were added to the top chamber of the cell culture inserts (BD Pharmingen). Inserts were then placed over the wells and an incubation carried out for 90 min under standard culture conditions. Transmigration was assessed by harvesting (by centrifugation) and counting the cells in the lower chamber.

Anti-Idiotypic Responses

Antibody Purification

The antibody 2C11 (IgMκ) was purified from ascites by affinity purification on Ultra Link Mannan Binding Protein (MBP) affinity columns (Pierce). Ascites fluid was dialyzed against 20 mM Tris pH 7.5. 500 μl of dialyzed ascites was diluted to 3 ml with Binding Buffer (10 mM Tris, 20 mM $CaCl_2$, pH 7.4) and added to the MBP column. A 16 hr incubation was carried out at 4°C after stopping flow. After resuming flow, the column was washed with 20 column volumes of Binding Buffer at the same temperature. The column was then transferred to room temperature and elution buffer (0.1 M Tris, 10 mM EDTA, pH 7.4) added. A 30 min incubation was then carried out at room temperature after stopping flow. Elution was then achieved by resuming flow. The protein was concentrated using Amicon Ultra (Millipore; with a cut-off of 30 KDa) and quantified by UV absorbance at 280 nm (1.18 $OD_{280} \approx$ 1mg/ml IgM). Purity of antibodies was assessed by SDS-PAGE (under denaturing conditions), followed by silver staining. Bands were compared with those obtained upon Western blots when anti-mouse Ig reagents were used to reveal resolved heavy and light chains.

The antibodies 1B1 (IgG2aκ) and 2C3 (IgG2bκ) were purified using Protein-G Sepharose affinity columns (GE Healthcare). Protein-G beads (2 ml) were packed into a column. Equilibration was carried out with 5-7 column volumes of PBS. Ascites was pre-dialyzed against PBS and 500 μl of ascites was aliquoted into the column, which was

then sealed at both ends and incubated overnight at 4°C on an end-to-end rocker. The column was washed with 30 column volumes of PBS to wash off unbound protein. The wash was checked for the presence of protein by the Coomassie Plus™ protein assay reagent (Pierce). Glycine-HCl Buffer (Appendix) was added to the column and again incubated at room temperature on an end- to-end rocker for 30 min. 5 ml fractions were then collected which were neutralized by addition of Neutralization Buffer (1M Tris; pH 9.1). Protein content in the eluate fractions was assessed; fractions containing protein were concentrated using Amicon Ultra (Millipore; with a cut-off of 30 KDa). The concentrated antibodies were dialyzed against PBS and sterilized by passage through 0.2 micron syringe filters (MDI, India). Protein content was estimated by the Bichinchonic acid (BCA; Pierce) method.

Immunization

C57/BL6 mice were employed for antibodies 2C11 and 2C3, and NZB/W mice for antibody 1B1. Animals were sub-cutaneous (s.c) injections of 50 μg antibody emulsified in Incomplete Fruend's Adjuvant (IFA); each animal received 100 μl at multiple sites. Two booster injections were administered at fortnightly intervals and blood samples were collected one week after each booster. Anti-self reactivity in the sera was assessed by FACS analysis on permeabilized and non-permeabilized Jurkat cells, by indirect immunofluorescence assays on HeLa cell, by Western blots using cellular lysates as substrate, and by ELISA using a panel of recombinant autoantigen.

Effect of Antibodies on Pregnancy

The FVB strain of mice was chosen for this experiment because of its high (>80%) successful pregnancy rate at the National Institute of Immunology Animal Facility. Female mice were co-habited with males; individuals in which intercourse had occurred were identified by presence of vaginal plugs. Such animals were infused the respective antibodies - 1B1, 2C3, or an isotype control antibody. Intraperitoneal injections (100 μg in 100 μl PBS) were administered every alternate day, and a total of four injections were given to each animal. A fourth group received just PBS.

Antibody-Reactive Mimotopes

Antibodies 2C11 (IgMκ) and 2C3 (IgG2bκ) were used as probes in these experiments.

Panning of a 12-mer Random Phage Library

To identify antibody-reactive mimotopes, purified antibody (2.5 μg in Coating Buffer) was adsorbed onto a well of a 96-well tissue culture plate (Falcon) by a 16-hr incubation at 4°C. Unoccupied binding sites in antibody containing wells were "blocked" by incubation with 1% BSA (prepared in Coating Buffer); some "blank" wells not containing antibody were also similarly "blocked". 10^{10} phages contained in an unconstrained 12-mer phage display random peptide library (PhD-12; New England Biolabs) were re-suspended in 100 μl Tris buffered saline containing 0.1% Tween 20 (TBST) and added to "blank" wells (to eliminate BSA-reactive as well as potential plastic-reactive phages) and an incubation carried out for 1 hr room temperature. Unbound phages were transferred to fresh "blank" wells, and the procedure repeated two additional times. Unbound phages were then added to antibody-adsorbed wells for a further incubation. Unbound phages were removed and the well was washed extensively with TBST. The bound phages were then eluted at low pH using 100 μl of Elution Buffer (Appendix). The pH of the solution of eluted phages was neutralized by the addition of 1M Tris, pH 9.2. Eluted phages were titrated, amplified and precipitated as described subsequently. This protocol (i.e. three rounds panning on "blank" wells followed by panning on antibody-coated wells) was sequentially carried out four times. In rounds 2 to 4, the Tween-20 concentration in TBS (in the washing steps) was increased from 0.1% to 0.5% for increased stringency. After the last round of panning, individual plaques were picked (see below) and amplified, and glycerol stocks were prepared. Binding of the phages to respective antibodies were confirmed by ELISA.

Phage Titration

For titration, the phages were serially diluted 10^{10} fold (subsequent to amplification, see below) or 10^{8} fold (subsequent to panning) in LB broth. 10 μl of diluted phage and 20 μl of a culture of *E coli* ER 2837 (overnight grown culture) were added to a 0.7% molten

soft agarose solution maintained approximately at 50°C. The soft agar was then poured on a petri-plate containing X-Gal/IPTG/Tetracycline supplemented LB agar. An overnight incubation was then carried out. Phage titre was estimated by counting blue plaques at several dilutions.

Phage Amplification and Precipitation

A "starter culture" was inoculated in 2 ml LB broth containing 20 μg/ml tetracycline and an overnight incubation was carried out at 37°C in an orbital shaker (120 rpm). 100 μl of culture was used to inoculate 20 ml tetracycline-containing LB broth in a 150 ml conical flask. Cultures were incubated as before till the mid-log phase growth was achieved. The phage-containing solution was then added to the bacterial culture and a further incubation carried out for another 4.5 to 5 hr. The bacterial culture was centrifuged at 10000 g for 10 minutes at 4°C and the supernatant was centrifuged again, and the top 80% of the supernatant collected. Phages were precipitated by overnight incubation at 4°C, subsequent to addition of 1/6th the volume of 20% polyethylene glycol (PEG) in 2.5 M NaCl. After centrifugation at 10000 g for 10 min at 4°C, the phage pellet was re-suspended in 1 ml Tris buffered saline (TBS). Phages were re-precipitated by addition of 200 μl of PEG, an incubation at 4°C for 2 hrs and centrifugation at 10000 g for 10 min. After the supernatant was dispensed, brief centrifugation at 10000 g was carried out to discard residual supernatant. The phage pellet was re-suspended in 100 μl TBS. The precipitated phages were titrated as described above. Individual plaques lifted after four rounds of panning were similarly treated, except that the culture volume was scaled down to 2 ml, out of which 1 ml was utilized for the phage ELISA (described below). Phages in the other milliliter were cryopreserved at -20°C after addition of glycerol to a final concentration of 20%.

ELISA

Reactivity of individual phages to respective antibodies was delineated. Wells of an ELISA plate were adsorbed with 2.5 μg/well of antibody, following the protocol described above. "Blocking" of unbound sites was carried out by incubation with Phage

Blocking Buffer (Appendix). Wells not containing antibody and just "blocked" with BSA served as negative controls. 50 μl of amplified phage supernatant was added in each well and an incubation was carried out for 2 hr at room temperature. Wells were "washed" extensively with TBST containing 0.5% Tween 20. Horse radish peroxidase (HRP)-conjugated anti-M13 antibody (Amersham Pharmacia), diluted 1:4000 in TBST, was then dispensed and further incubation carried out for 1 hr at room temperature. After further washes, enzymatic reactivity was assessed upon addition of a TMB substrate solution as described earlier. Absorbance was recorded at 450 nm.

DNA Isolation

Phages were precipitated by the addition of PEG as described before. The pellet was re-suspended in 100 μl of Iodide Buffer (Appendix) and 250 μl of ethanol. An incubation was carried out for 10 min at room temperature. Phage DNA was precipitated by centrifugation at 12000 g for 10 min. DNA was "washed" twice with 70% ethanol. The DNA pellet was dried and re-suspended in 20 μl sterile water. DNA sequencing was carried out at the Sequencing Facility of the National Institute of Immunology using the -96 primer provided with the Phage PhD-12 kit.

Immunization

Antibody-reactive peptidic mimotopes, identified by screening the random phage display library, were commercially synthesized on a 8-branch multiple antigenic peptide (MAP) backbone (GL Biochem, China). Non-autoimmune prone BALB/C mice were immunized with 100 μg MAP-peptide conjugate (or just the MAP backbone) after emulsification Complete Freund's Adjuvant (CFA). Each animal received a 100 μl s.c. injection. Two subsequent boosters were administered at weekly intervals, for which IFA was employed. Blood samples were collected every seven days and sera were analyzed for peptide and protein reactivity by ELISA.

Antibody Variable Region Genes

RNA Isolation

RNA was isolated from the extensively subcloned hybridoma cultures secreting monoclonal antibodies. Cells, grown in T-75 flasks, and were harvested by "flushing" with medium. 1 ml of TRIzol (Invitrogen, USA) was added to 5-10 x 10^6 cells and an incubation was carried out for 5 min at room temperature. 200 μl of chloroform was added per ml of TRIzol and the mix further incubated at room temperature for another 3 min. Tubes were centrifuged at 12000 g for 15 min at 4°C. The colorless aqueous phase was collected. RNA was precipitated by addition of 0.5 ml iso-propanol and incubation for 10 min at room temperature. Tubes were centrifuged at 12000 g for 10 min at 4°C. The RNA precipitate was washed twice with 75% ethanol. RNA was finally dissolved in di-ethyl pyrocarbonate (DEPC) treated sterile water.

cDNA Preparation

cDNA was prepared using oligo dT primers and avian myeloblastosis virus (AMV) reverse transcriptase (Promega). RNA and the oligo dT primer (Promega) were incubated at 70°C for 10 min followed by instantaneously cooling of the tube on ice. Additional pre-mixed components (as indicated in the Appendix) were added and the mixture incubated on a thermal cycler (Perkin Elmer) for 60 min at 48°C. Reverse transcriptase activity was subsequently quenched by heating 70°C for 5 min. The cDNA preparation was also treated with RNase H (Promega) for 37°C for 30 min to degrade residual RNA.

Polymerase Chain Reaction (PCR)

cDNA was used to amplify the antibody genes by PCR. A mouse Ig primer kit (Novagene) containing primers (the sequences for which are indicated in the Appendix) for the murine light and heavy chain genes was employed. Forward and reverse primers (5 pM) were added in a total reaction volume of 25 μl; the concentrations and volumes of other components are indicated in Appendix. Amplification (for 30 cycles) was carried out on a thermal cycler. The cycling parameters were as follows:

Hot start	94°C/ 5 min
Denaturation	94°C/ 45 sec
Annealing	60°C/ 45 sec
Extension	72°C/ 45 sec
Cycles	30 cycles
Final extension	72°C/ 7 min
Storage	4°C

Cloning, Sequencing and Analysis

The PCR product was eluted after electrophoresis 1% agarose (Pronadiska) using the Qiagen gel extraction kit. The PCR product was ligated to the pGEMT vector using the pGEMT ligation kit (Promega). The DH5 α strain of *E coli* was transformed with the ligated plasmid using standard protocols. The transformed cells were selected on LB ampicillin (100μg/ml) containing X-gal and Iso-propyl thio-galactopyranose (IPTG). Following day only the white colonies were picked up and individual colonies were picked and grown overnight in 3 ml LB broth at 37°C with shaking at 200 rpm for plasmid isolation. Plasmids were isolated using Qiagen plasmid isolation kit. The size of the insert was assessed by digestion with EcoRI (New England Biolabs) followed by agarose gel electrophoresis; a fragment of ≈ 500 bps was expected. The cloned antibody gene inserts were sequenced by using M13 (forward and reverse) primers, corresponding to sequences present bordering the multiple cloning site of the pGEMT vector. Automated sequencing was carried out at the Centralized Sequencing Facility of the National Institute of Immunology. Analysis of antibody variable region gene sequences was carried out using the NCBI and IMGT germline databases for mouse antibodies.

Statistical Analysis

Statistical analysis was carried out by using the Student's unpaired t-test.

Results

RESULTS

Generation of Murine Monoclonal Autoantibodies

The presence of antibodies directed towards various cellular and nuclear antigens is a well-documented phenomenon in systemic autoimmunity. As discussed earlier, aberrant apoptotic mechanisms have been implicated in the initiation of SLE; excessive apoptosis has been documented, as has the deficient uptake of apoptotic cells.

Two autoimmune strains of mice were employed in this study - NZB/W and C57/BL6$^{lpr/lpr}$. Aging animals belonging to the former strain are known to express frank autoimmune responses and lupus-related pathology (Lambert et al 1968; Howie et al 1968; Burnet et al 1965; Helyer et al 1963), while mice of the latter strain exhibit the presence of some autoreactive specificities (Cohen et al 1991). As expected, older animals (at 56 weeks) exhibited higher serum IgM levels compared with younger animals (at 5 weeks, Figure 1a). Sera from young and old NZB/W were also assessed for self-reactivity on Jurkat cells by flow cytometry (Figure 1b). An interesting dichotomy was observed as far as self-reactivity was concerned. Sera from older animals demonstrated heightened IgG anti-self reactivity on permeabilized Jurkat cells (Figure 1b top left panel); IgM antibodies exhibited no such reactivity (Figure 1b, top right panel). IgG anti-self reactivity towards non-permeabilized cells was not appreciably enhanced in the sera of older animals (Fig 1b, lower left panel); IgM reactivity towards such cells was significant (Figure 1b, lower right panel).

Sera from young and old NZB/W animals were also assessed for the presence of anti-nuclear antibodies (ANA), a common autoimmune specificity in both human and murine lupus. Sera from older animals exhibited this specificity (Figure 2, right panel), while sera from younger animals did not (Figure 2, left panel).

Hybridomas were generated using spleen cells from both strains of mice (NZB/W and C57/BL6$^{lpr/lpr}$). In both instances, SP2/O was employed as the fusion partner. A total of

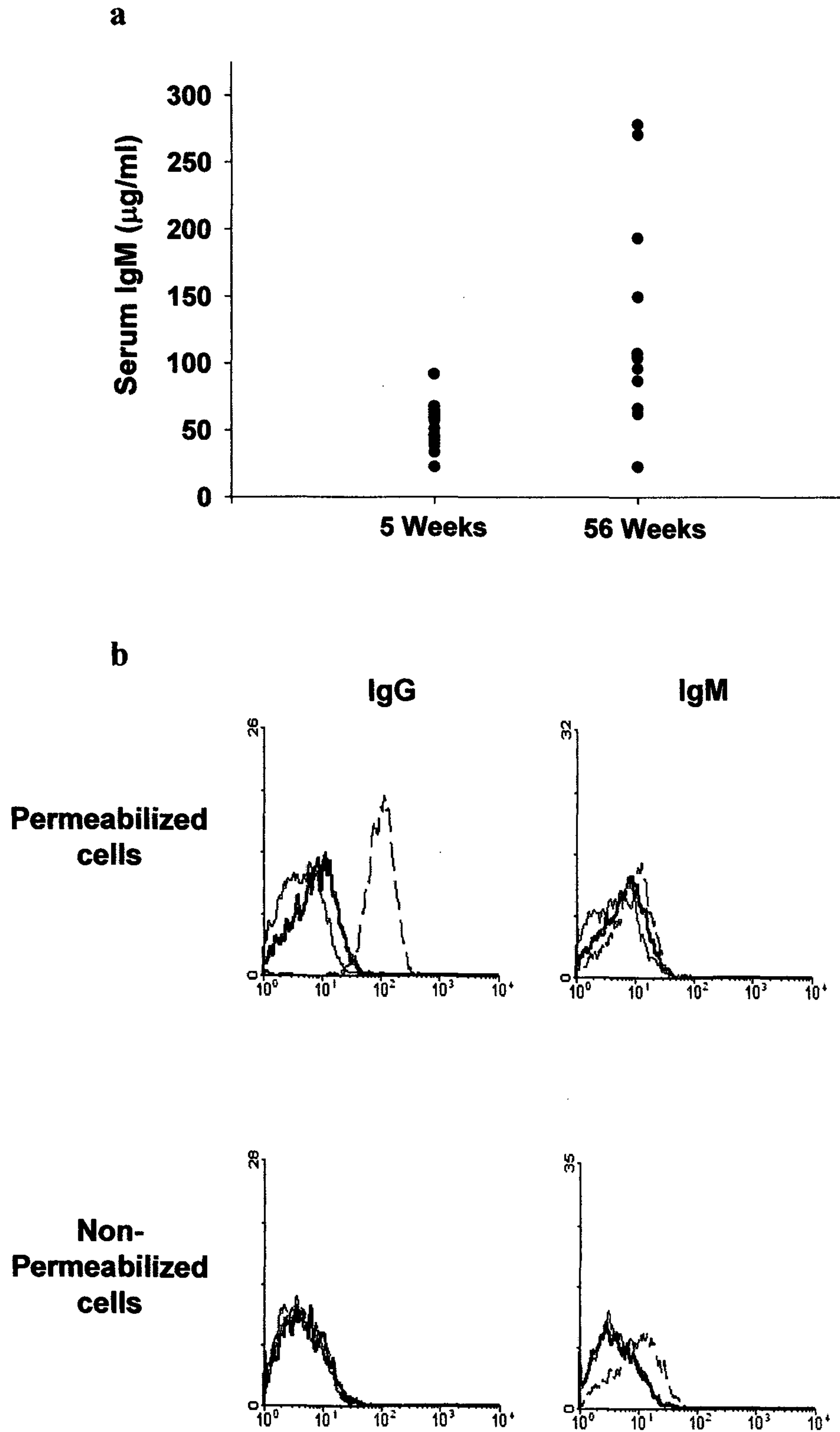
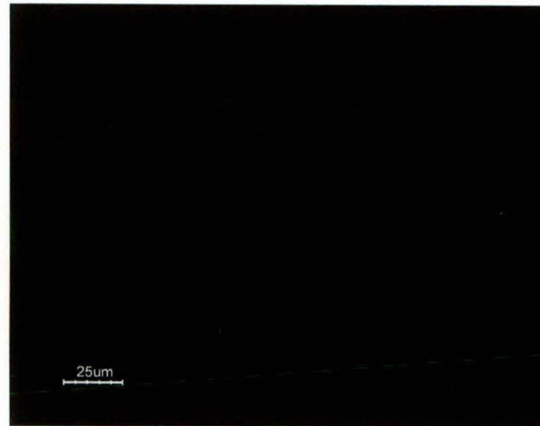


Figure 1: (a) Serum IgM titers of young (5 weeks) and old (56 weeks) NZB/W F1 mice. **(b)** Flowcytometry profile of serum IgG and IgM reactivity on permeabilized and non-permeabilized Jurkat cells. Broken lines represent sera from 56 weeks old animals while thick solid lines from 5 week old animals. Thin solid lines represent negative controls, where only second antibody was employed.

a



b

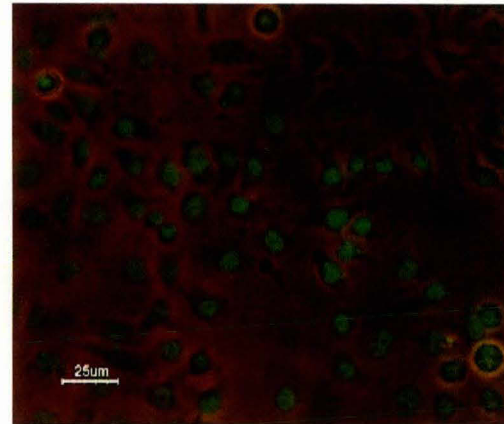


Figure 2: Immunofluorescence on HeLa cells. **(a)** Antibodies in sera from young (5 weeks) NZB/W F1 animals demonstrate no recognition **(b)** Anti-nuclear antibodies were evident in the sera of old (56 weeks) NZB/W F1 animals.

465 independent hybrids were generated. As an initial measure of anti-self reactivity, hybridoma supernatants were screened for the presence of antibodies that were reactive towards permeabilized cells and non-reactive towards non-permeabilized, healthy cells, both by FACS analysis and on indirect immunofluorescence. Supernatants from hybridomas containing antibodies satisfying these criteria were assessed for specificity towards apoptotic cells (see below). Hybridomas secreting antibodies of interest were subcloned at least three times. Six monoclonal antibodies specifically recognizing apoptotic cells were generated, three of the IgM (2C11, 2H8 and 1B4) and three of the IgG (2C3, 1B1 and 1B3) isotype. Table 1 describes the strain of the animal from which each individual antibody was generated as well as the respective Ig isotypes.

Reactivity Towards Non-apoptotic Cells

Figure 3 indicates the reactivity of the antibodies towards non-permeabilized and permeabilized Jurkat cells by FACS analysis. It is apparent that the antibodies were non-reactive to antigens present on the surface of healthy cells while exhibiting reactivity towards internal (cytoplasmic and/or nuclear) antigens. Interestingly, the antibodies recognized such antigens on many cell types and across two species (humans and mice), indicating evolutionary and lineage antigenic conservation.

Cellular reactivity patterns as observed on FACS analysis were extended and confirmed in indirect immunofluorescence assays, using HeLa cells as substrate. As expected, none of the six apoptotic cell-specific antibodies recognized healthy, non-permeabilized cells to a significant extent (Figure 4a). All cells demonstrated frank recognition of permeabilzed HeLa cells (Figure 4b); variations in reactivity patterns were apparent amongst the different antibodies. While antibodies 2C11 and 2H8 appeared to predominantly bind cytoplamic antigen(s), antibodies 1B3 and 1B1 recognized nuclear moieties. Antibodies 2C3 and 1B4 bound antigen(s) in both the cytoplasm and the nucleus.

Antibody	Cellular Specificity	Isotype	Strain
2C11	Intracellular/ Apoptotic	IgMκ	C57/BL6$^{lpr/lpr}$
2H8	Intracellular/ Apoptotic	IgMκ	C57/BL6$^{lpr/lpr}$
1B4	Intracellular/ Apoptotic	IgMκ	NZB/W F1
2C3	Intracellular/ Apoptotic	IgG2bκ	NZB/W F1
1B3	Intracellular/ Apoptotic	IgG2aκ	NZB/W F1
1B1	Intracellular/ Apoptotic	IgG2aκ	NZB/W F1

Table 1: Description of nomenclature, cellular specificity, isotype and animal source of the monoclonal antibodies generated in this study.

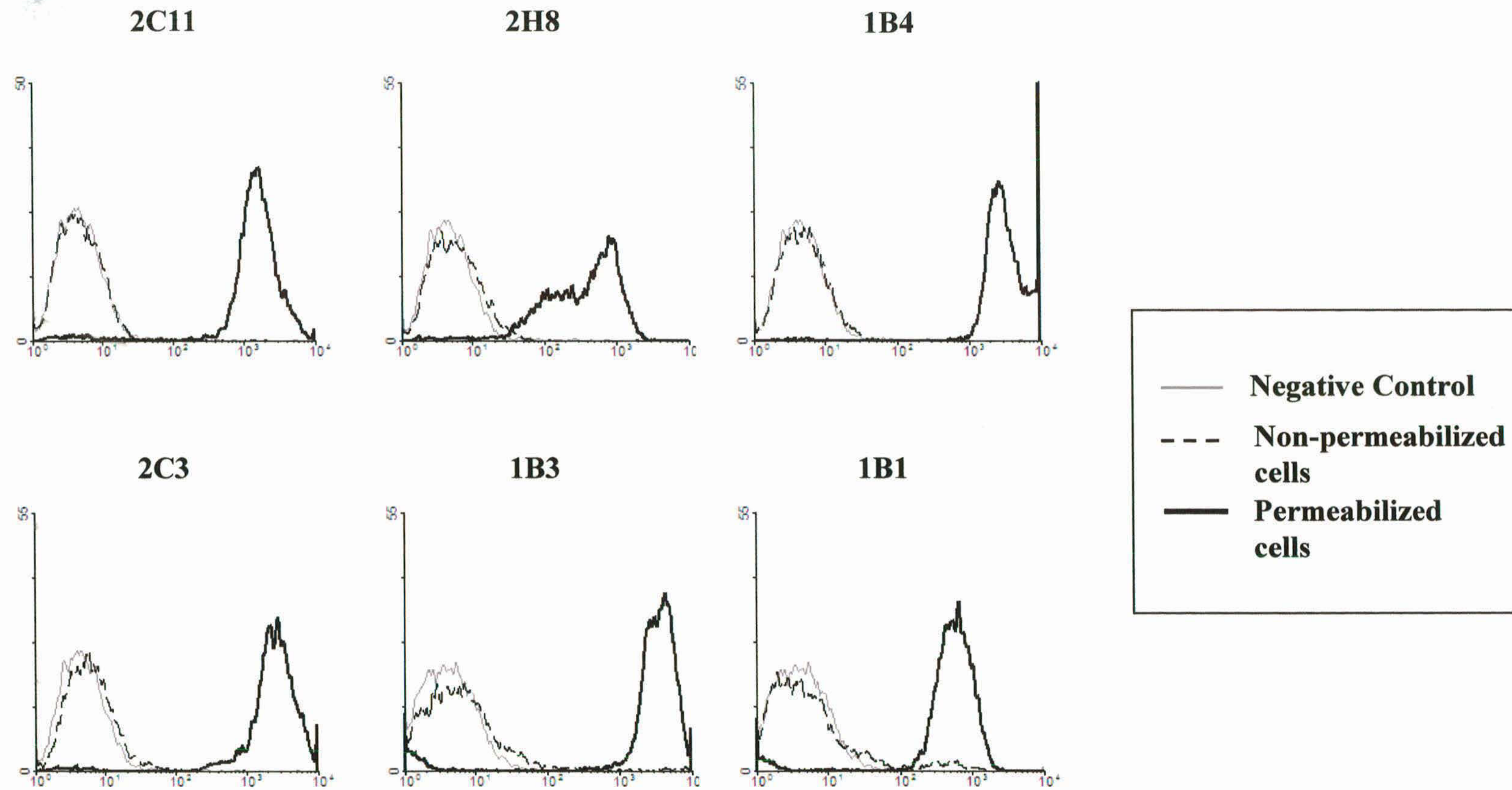


Figure 3: Recognition of permeabilized Jurkat cells by the IgM (2C11, 2H8, 1B4) and IgG (2C3, 1B3, 1B1) monoclonal antibodies. Non-permeabilized cells were not recognized.

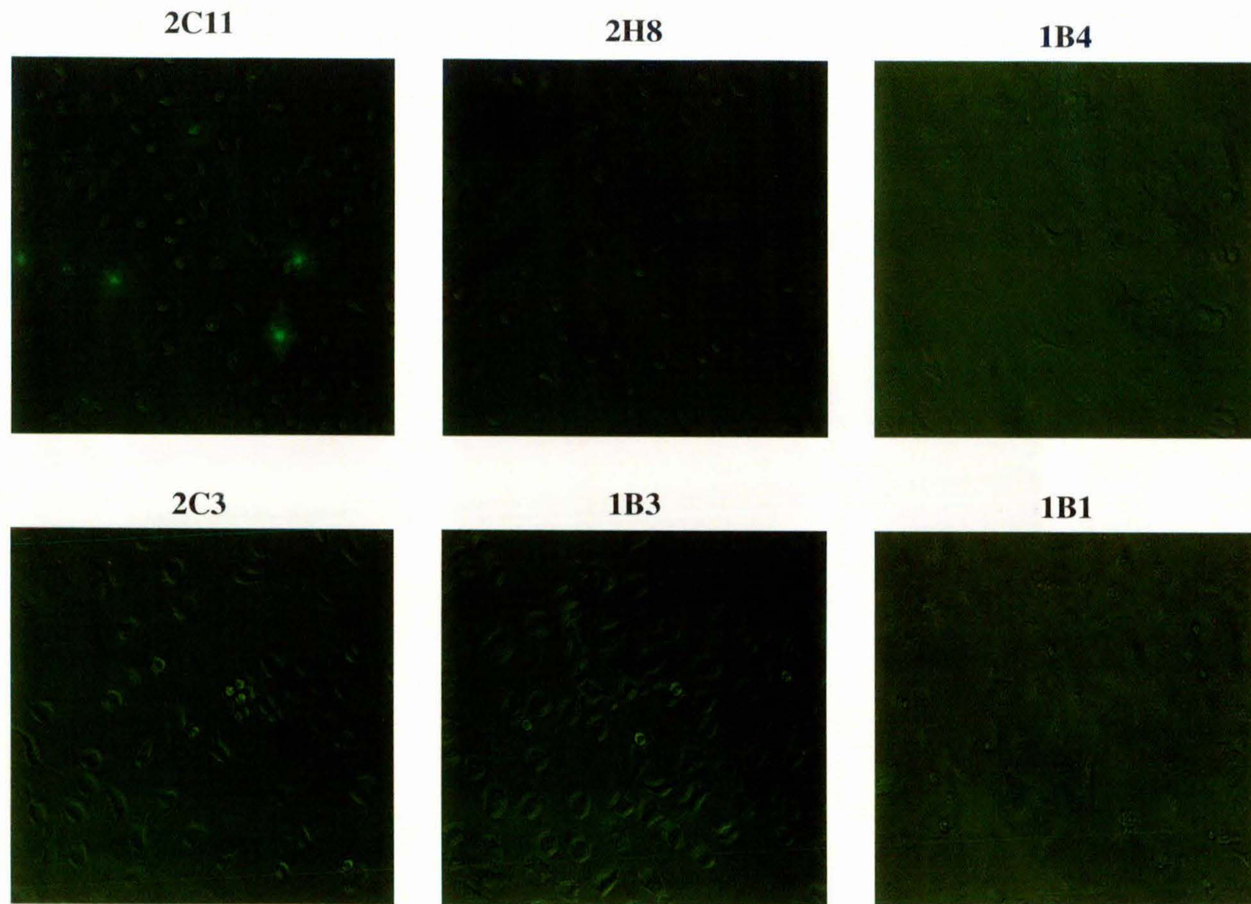
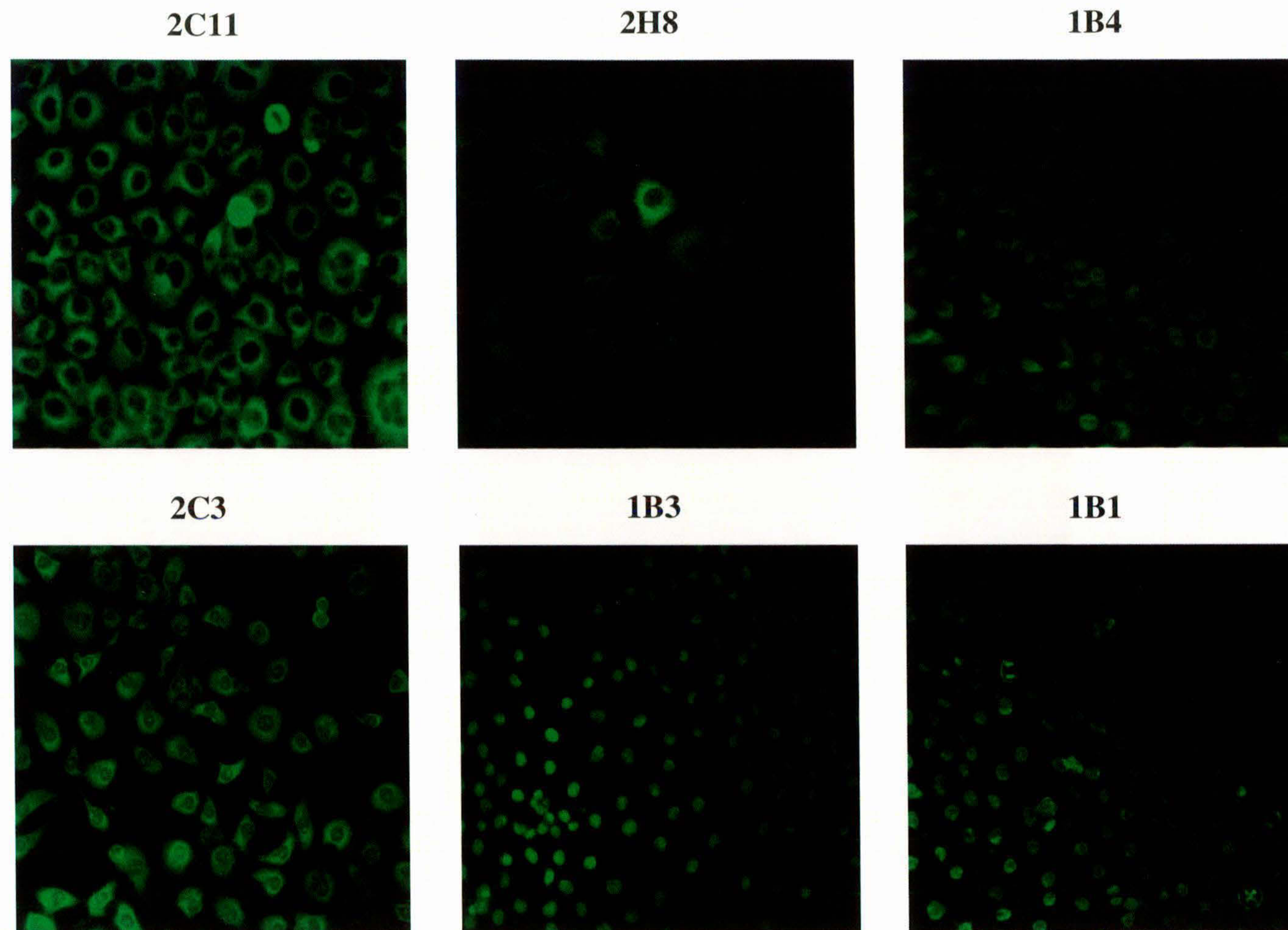


Figure 4a: Reactivity of the monoclonal antibodies on non-permeabilized HeLa cells. Except for minor, punctate binding by the monoclonal antibody 2C11, no reactivity was seen.



<u>Figure 4b</u>: Reactivity of the monoclonal antibodies on permeabilized HeLa cells. 2C11 and 2H8 bound cytoplasmic antigens, 1B3 and 1B1 recognized nuclear moieties. 2C3 and 1B4 bound both cytoplasmic and nuclear antigens.

Reactivity Towards Apoptotic Cells

FACS analysis

Apoptosis was induced in Jurkat cells by exposure to ultra-violet (UV) light, as described in the Methods section. After an 18 hr incubation, cells were dually stained with the monoclonal antibodies and Annexin-V. Data was collected on a FACS machine. Similar results were obtained for both IgG and IgM isotypes, as depicted in Figure 5a. Cells that bound antibody also bound Annexin-V (top right quadrants) and cells not binding Annexin-V were also not bound by antibody (as reflected by the relative lack of cells in the bottom right quadrants). There were cells that bound Annexin-V but not antibody (top left quadrants), as well as healthy calls that bound neither reagent (bottom left quadrants). To further establish the apoptotic specificity of the antibodies, the pan-caspase inhibitor z-VAD-fmk was employed; in all cases, there was dramatic reduction in both Annexin-V as well as antibody reactivity; in this case too, the cells recognized by the antibodies also bound Annexin-V (Figure 5b). Figure 6 depicts quantification of the same data, represented as percentage reactivity achieved (over control cultures) in antibody, Annexin-V and Propidium Iodide (PI) binding in the presence of the inhibitor. The fact that antibodies bound only Annexin-V positive cells, as well as demonstrated significant decreases in binding upon use of a caspase inhibitor testified to their being specifically targeted at moieties exposed on apoptotic cells.

The kinetics of antibody recognition was then investigated, and assessed in relation to Annexin-V and PI binding, the interaction of which with cells delineates relatively early and late events respectively in the apoptotic cascade. Results are shown in Figure 7a. Significant Annexin-V reactivity was observed at six hours post-apoptosis induction, with the number of cells recognizing the phosphotidylserine-recognizing protein reaching a plateau at about twelve hours. The binding of PI to cells demonstrated slower kinetics, as did the reactivity of the antibodies. No significant differences were seen between the IgM and IgG isotypes. Antibody recognition, therefore, appeared to be a relatively late event. Similar results were obtained using a variety of cell lines.

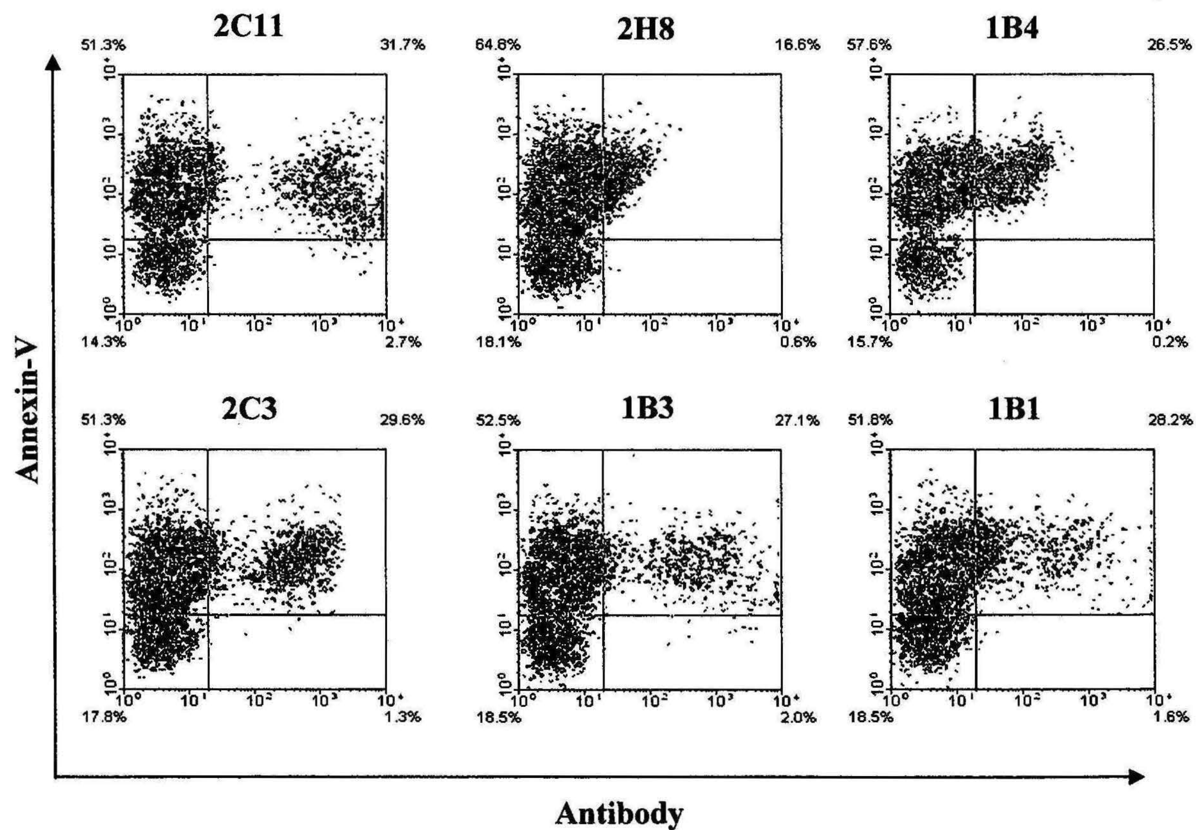


Figure 5a: Preferential recognition of Annexin-V positive Jurkat cells by the monoclonal antibodies after induction of apoptosis by UV. Some Annexin-V positive cells remained unbound by the antibodies.

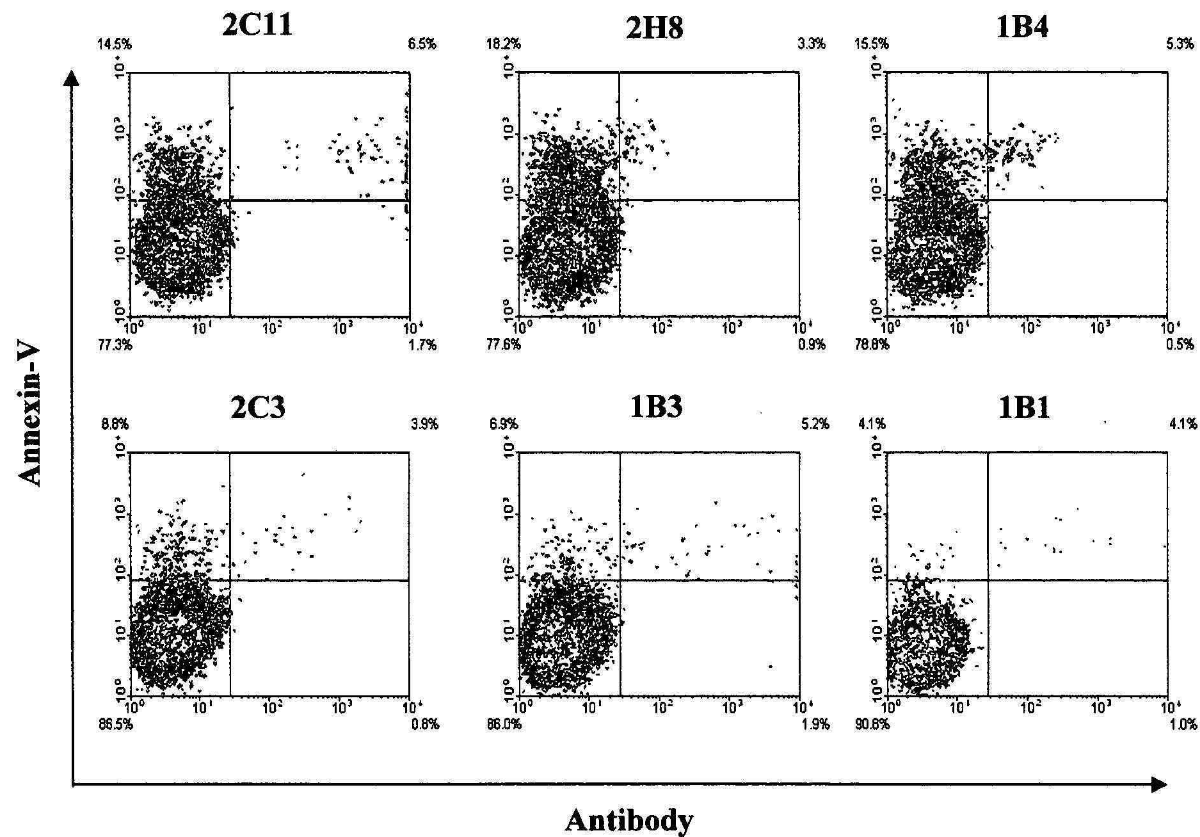


Figure 5b: Reduction of monoclonal antibody reactivity (in relation to Figure 5a) upon Jurkat cells induced to undergo apoptosis in the presence of pan-caspase inhibitor z-VAD-fmk. The total number of Annexin-V positive cells were also reduced, as expected.

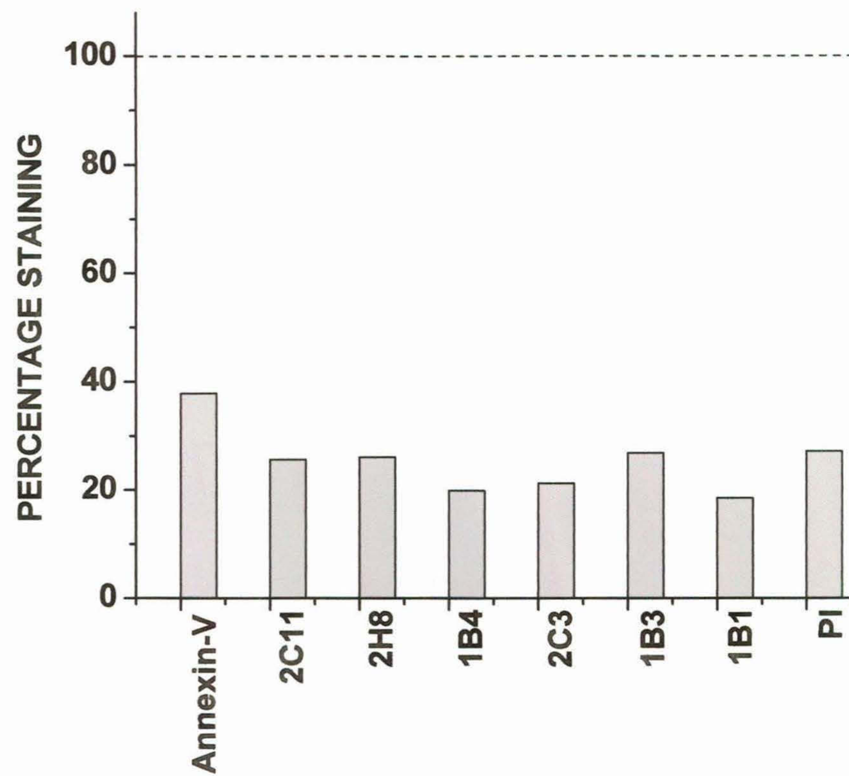
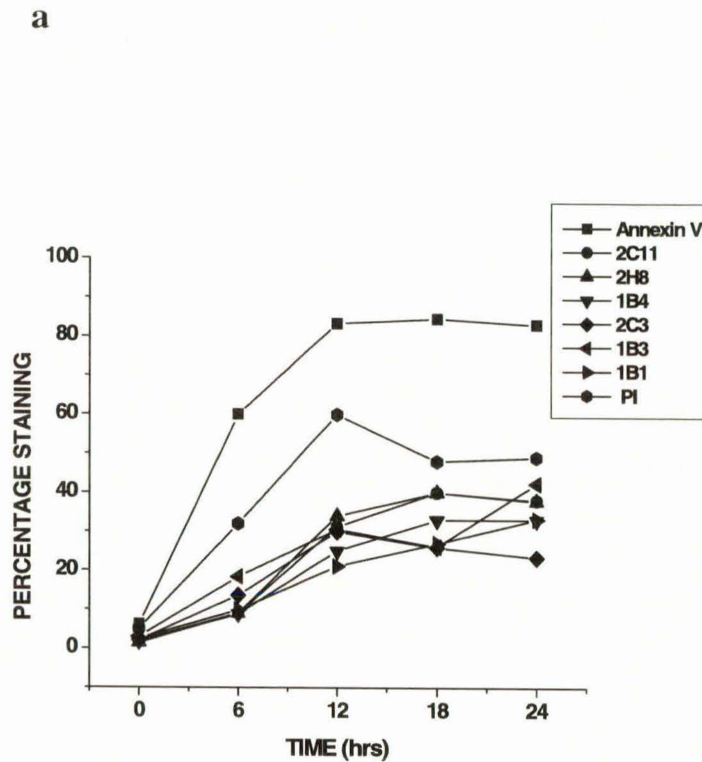


Figure 6: Quantitation of binding (over respective controls) of the antibodies (2C11, 2H8, 1B4, 2C3, 1B3, 1B1) to Jurkat cells induced to undergo apoptosis in the presence of the pan-caspase inhibitor z-VAD-fmk. Annexin-V and Propidium Iodide (PI) demonstrated the expected decrease in reactivity.

a



b

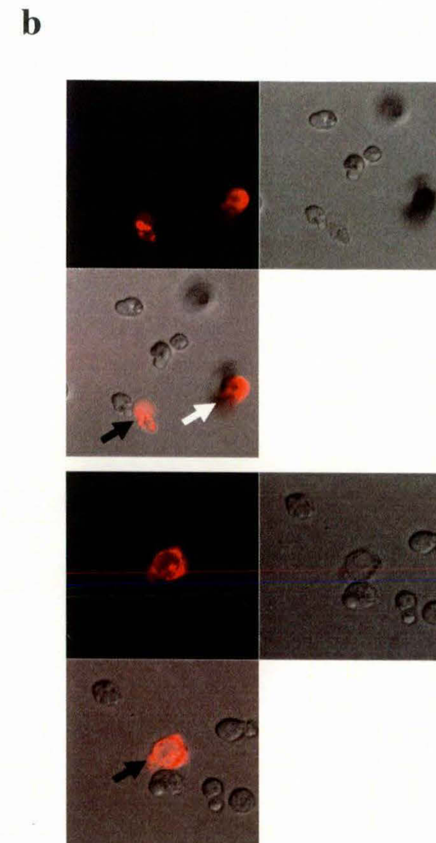


Figure 7: (**a**) Kinetics of binding of the monoclonal antibodies to apoptotic cells. Reactivity of Annexin-V and Propidium Iodide (PI) are also shown. (**b**) Co-staining of apoptotic cells with PI and Trypan Blue. The top panel indicates a cell binding PI and not Trypan Blue (black arrow) and a cell binding both Trypan Blue and PI (white arrow). The bottom panel indicates a cell with surface localization of PI, with no Trypan Blue staining (black arrow). In all cases, healthy cells remained unbound by both dyes.

Significantly, reactivity to Trypan Blue remained low through the course of the experiment, indicating that the plasma membrane retained significant integrity. Cells at a late stage of apoptosis were assessed for PI as well Trypan Blue reactivity (Figure 7b). While some cells internalized Trypan Blue as well as bound PI, others demonstrated the capacity to bind PI while still excluding Trypan Blue. Microscopic and immunofluorescence data together indicate that cell-surface binding of PI could occur before true membrane permeability was apparent.

The kinetic parallels between PI binding and antibody recognition upon apoptotic cells prompted two-colour FACS analysis. Figure 8 indicates the results. Data obtained was similar to that obtained when Annexin-V was employed (Figure 5a). Antibody-bound cells were all PI-positive, whereas there existed cells that bound PI but not antibody, as well as healthy cells that were not recognized by either reagent.

Immunofluorescence

Figures 9-15 depicts confocal analysis. Annexin-V (FITC, green) (except in Figure 9b, see below) and the various antibodies (Texas Red, red) were co-localized on either healthy cells (Figure 9), or cells at different stages of apoptosis (Figures 10-15). None of the antibodies recognized healthy cells; as a representative example, Figure 9a depicts results obtained for the IgM antibody 1B4. The murine monoclonal antibody 1D1 (IgG3κ, previously generated in the lab) was employed as a positive control for cell surface binding. Figure 9b shows the interaction of 1D1 on healthy Jurkat cells; just in this figure, green fluorescence represents the binding of cholera toxin (and not Annexin-V as in the other figures) to the cell surface. Distinctive membrane staining was observed.

Figures 10-12 show results for the IgM monoclonal antibodies 2C11, 2H8 and 1B4 respectively. In every instance, though Annexin-V reactivity appeared to be essentially distributed all across the cell surface, antibody reactivity appeared confined to either restricted regions of the membrane (2C11: Figure 10a; 2H8: Figures 11a and 11c; 1B4: Figure 12a) or to surface protrusions or blebs on the cell surface (2C11: Figure 10c; 2H8:

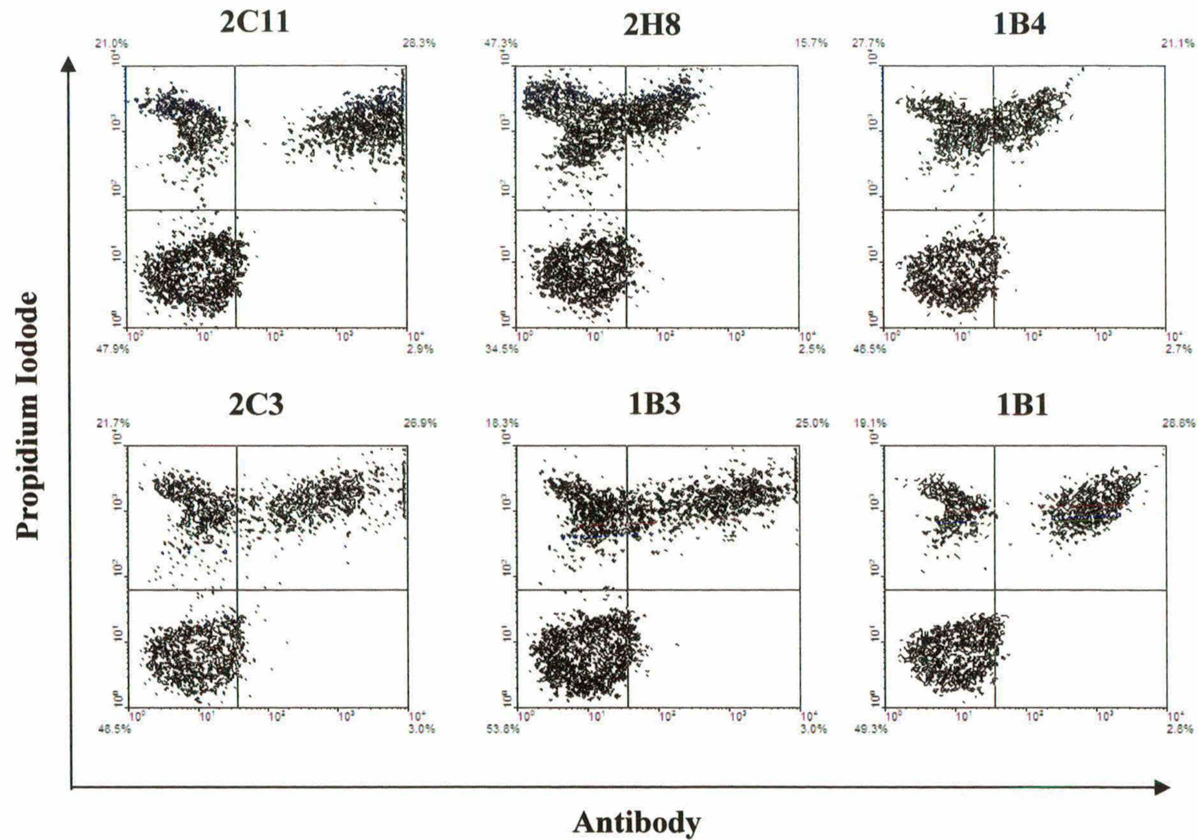


Figure 8: Preferential recognition of PI-positive Jurkat cells after induction of apoptosis by UV. Some PI-positive cells remained unbound by the antibodies.

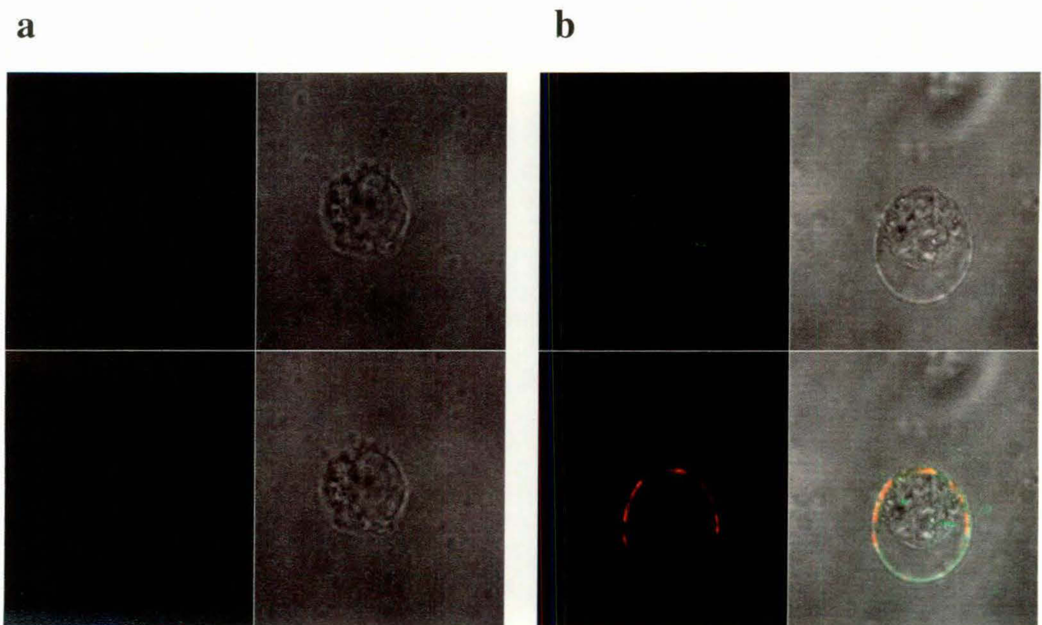


Figure 9: Confocal microscopy on healthy Jurkat cells. (**a**) Binding of the apoptotic cell-specific antibody 1B4 (Texas Red) and Annexin-V (FITC). No recognition was observed. (**b**) Binding of antibody 1D1 (Texas Red) and cholera toxin-B (FITC). The antibody bound the cell surface.

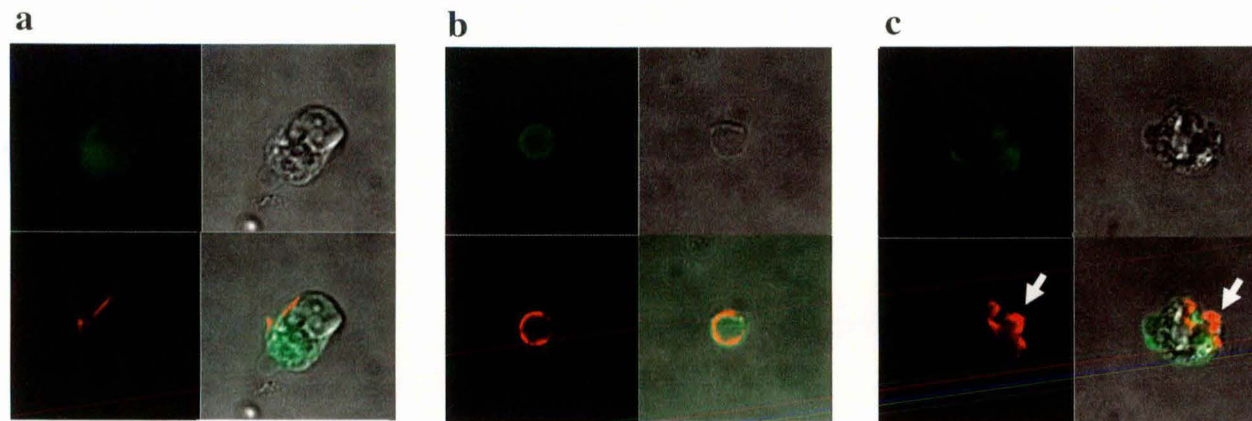


Figure 10: Confocal microscopy on apoptotic Jurkat cells with Annexin-V (FITC) and monoclonal antibody 2C11 (Texas Red). **(a, b)** In most instances, antibody bound discrete areas of the membrane unlike Annexin-V, whose binding was more uniform. **(c)** Preferential localization upon bleb-like projections (arrows) at a late stage of apoptosis. (Top Right: Phase Contrast; Top Left: Annexin-V; Bottom Left: Antibody; Bottom Right: Merged Image).

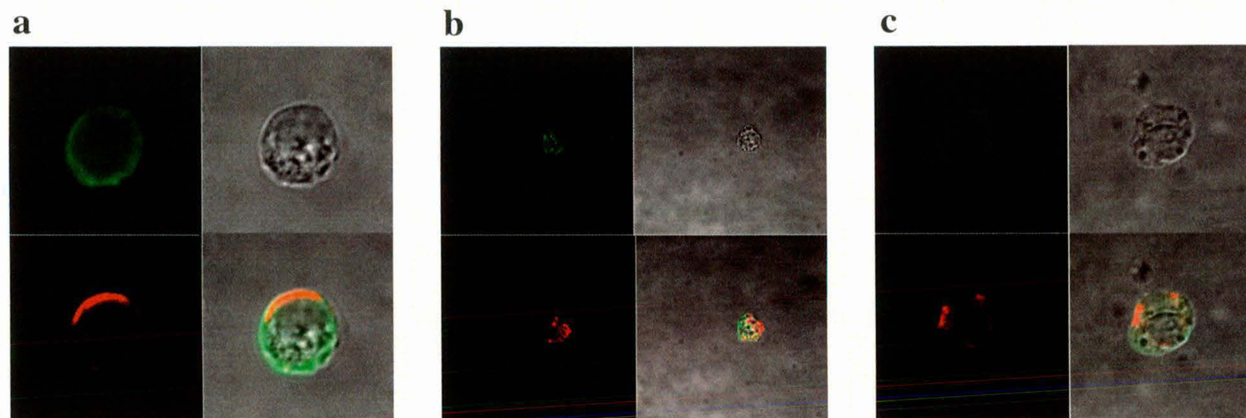


Figure 11: Confocal microscopy on apoptotic Jurkat cells with Annexin-V (FITC) and monoclonal antibody 2H8 (Texas Red). **(a, c)** The antibody either bound discrete areas on the surface of the cell or **(b)** appeared to bind bleb-like projections on the surface. Panel descriptions as in Figure 10.

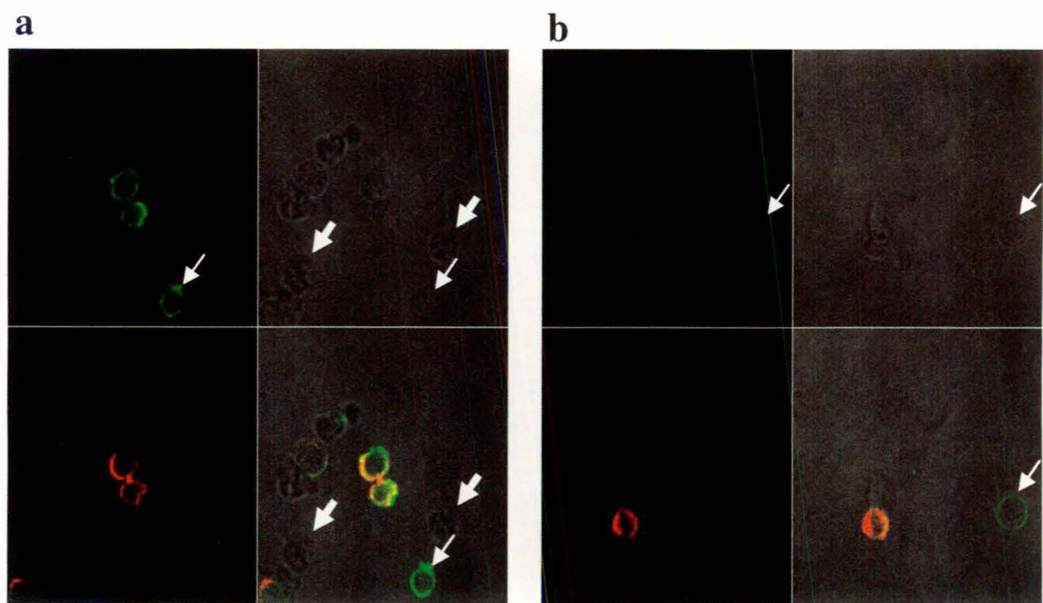


Figure 12: Confocal microscopy on apoptotic Jurkat cells with Annexin-V (FITC) and 1B4 (Texas Red). Extensive co-localization (observed as yellow or orange) of the antibody and Annexin-V was observed. **(a)** Several healthy cells remained unbound to both reagents (thick arrows). Some cells bound only Annexin-V (thin arrows).

Figure 11b). Region of overlap between Annexin-V and antibody reactivity appeared as yellow or orange in merged images.

Figures 13-15 depict data for the IgG antibodies 2C3, 1B3 and 1B1 respectively. Essentially similar results were obtained as for the IgM antibodies described above, with discrete membrane localization on non-permeable cells being a prominent observation.

Reactivity towards apoptotic blebs

Experiments carried out thus far revealed that monoclonal antibodies recognized internal (cytoplasmic and/or nuclear) antigens on permeablized cells, did not bind to the surface of healthy cells and bound the surface of apoptotic cells in a spatially restricted manner. Nuclear and cytoplasmic antigens are known to be transported onto the cell membrane as cells die, and are packaged into "blebs" that are externalized (Casciola-Rosen *et al* 1994 a). Many prominent autoantigens appear to be packaged into such blebs, and these structures are thought to constitute a dominant antigenic insult in the onset of lupus in genetically susceptible individuals.

The fact that immunofluorescence assays revealed the preferential recognition of bleb-like structures by some antibodies prompted enquiry into whether the antibodies could also recognize the antigen(s) contained within these blebs. Blebs pinched off from apoptotic Jurkat cells were isolated by differential centrifugation, as described in the Materials section, and visualized by scanning electron microscopy (Figure 16a). The antibody 2C11 was incubated with such blebs. After washes by repeated centrifugation, proteins (and bound antibody) contained in the bleb preparation were resolved by SDS-PAGE. After Western transfer, antibody heavy and light chains were revealed subsequent to incubation with a goat anti-mouse IgM-HPR conjugate (Figure 16b, Lane 1). To provide additional evidence of the capability of antibodies to recognize antigens present upon the blebs, these structures were isolated either from the supernatants of apoptotic SP2/O or apoptotic 2C11 hybridoma cells; after SDS-PAGE and Western blot procedures,

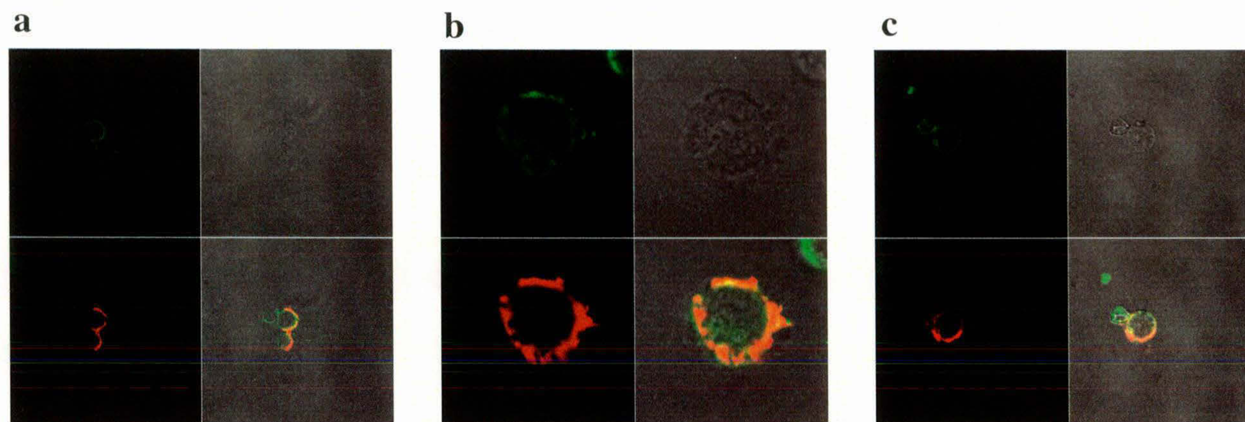


Figure 13: Confocal microscopy on apoptotic Jurkat cells with Annexin-V (FITC) and monoclonal antibody 2C3 (Texas Red). Extensive co-localization of the antibody and Annexin-V was observed (observed as yellow or orange in **a, b** and **c**). Panel descriptions as in Figure 10.

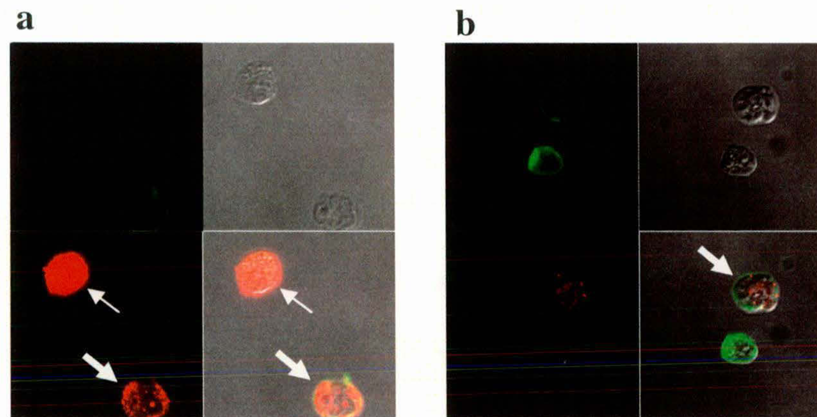


Figure 14: Confocal microscopy on apoptotic Jurkat cells with Annexin-V (FITC) and monoclonal antibody 1B3 (Texas Red). **(a)** A permeabilized cell is indicated by the thin arrows, upon which the antibody bound internal antigens. **(a, b)** 1B3 also bound the cell surface (thick arrows). Panel descriptions as in Figure 10.

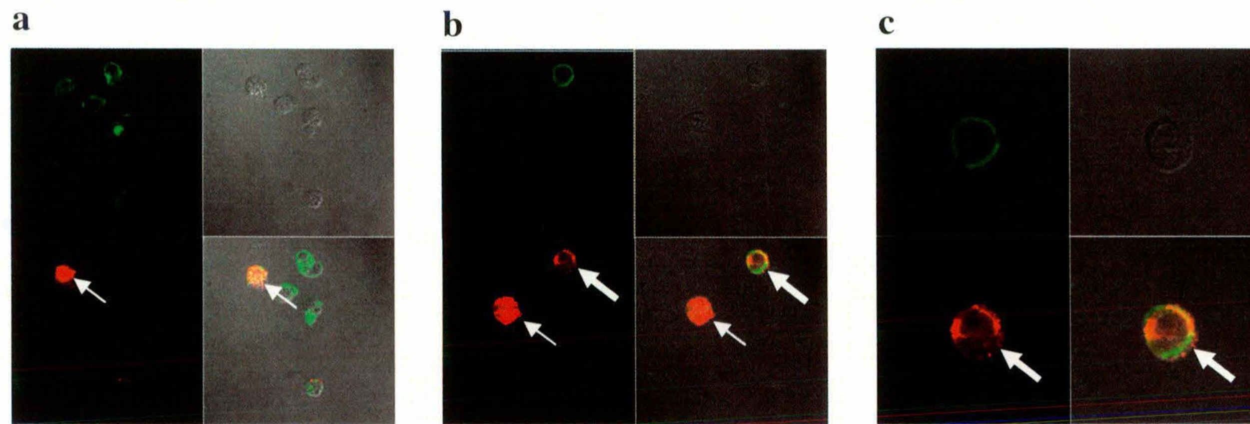
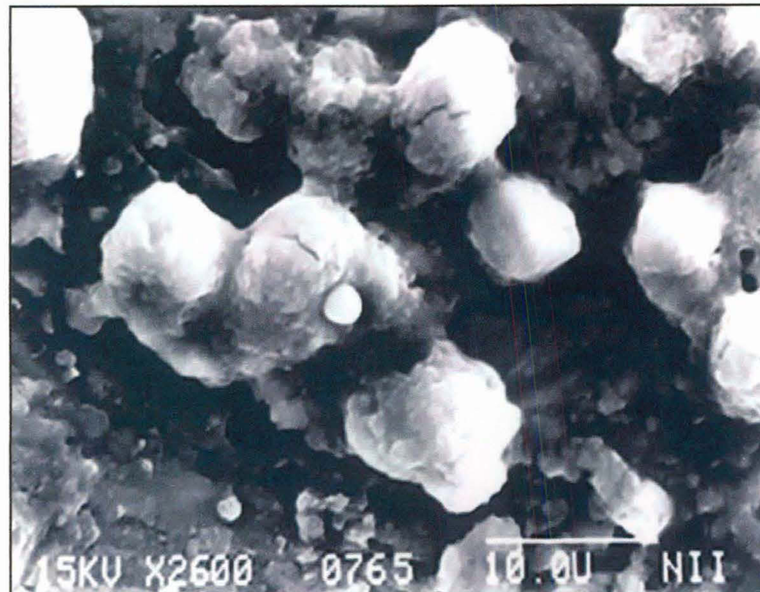


Figure 15: Confocal microscopy on apoptotic Jurkat cells with Annexin-V (FITC) and monoclonal antibody 1B1 (Texas Red). **(a, b)** Permeabilized cells are indicated by the thin arrows, upon which the antibody bound internal antigens. **(b, c)** 1B3 also bound the cell surface (thick arrows). Panel descriptions as in Figure 10.

a



b

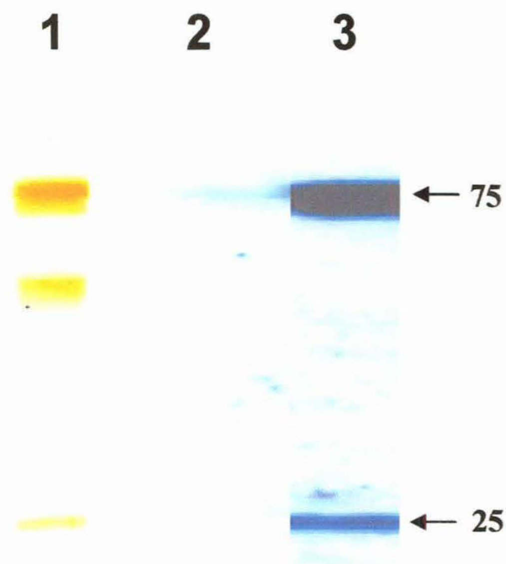


Figure 16: **(a)** Electron micrograph of purified blebs **(b)** Lane 1: Binding of antibody 2C11 to purified blebs as revealed by Western blot using goat anti-mouse IgM-HRP. Bands corresponding to the heavy chain (75 KDa) and light chain (25 KDa) were seen along with a degradation product. Lanes 2 & 3: Blebs isolated from either apoptotic SP2/O cells (Lane 2) or apoptotic 2C11 hybridoma cells (Lane 3) cells were processed for Western blot. The presence of the heavy chain (75 KDa) and light chain (25 KDa) was observed in Lane 3.

antibody heavy and light chains were detected only in the latter preparation (Figure 16b, Lane 3 vs Lane 2).

Antigenic Specificity

Western Blot

To delineate the antigens recognized by the monoclonal apoptotic cell-specific antibodies, Western blots were carried out on lysates prepared from either healthy Jurkat cells or from purified apoptotic blebs. Figure 17 depicts results obtained when the IgM antibodies were employed. It was apparent that, despite the monoclonal nature of the antibodies, multiple moieties were recognized, indicating that the antibodies were polyreactive. While multiple bands were observed in the 40 – 100 KDa region upon cellular lysates (Figure 17a), reactivity on apoptotic bleb lysates appeared more restricted and included reactivity to lower molecular weight moieties (Figure 17b, thin and thick arrows). Polyreactivity was a characteristic hallmark even when the IgG antibodies were employed, though distinct recognition patterns were observed (Figure 18). Most significantly, antibodies 2C3 and 1B1 demonstrated recognition of moieties in the 132 – 214 KDa region on cellular lysates (Figure 18a), unlike the IgM antibodies; 1B3 appeared poorly reactive. Reactivity on the apoptotic bleb lysates was once again distinct (Figure 18b). Antibodies 2C3 and 1B1 recognized moieties of 30 KDa or lower, as indicated by the arrow heads. Significantly, antibody 1B3, essentially non-reactive upon the lysate derived from healthy cells, bound three distinct moieties on apoptotic cell lysates (Figure 18b).

These findings assume significance in light of the fact that proteolytic cleavage, resulting from the activation of the caspase cascade during apoptosis, is known to alter antigenecity and/or immungenecity of several autoantigens in lupus (for example, La, PARP, 70K), possibly because of the exposure of neo-epitopes during this process (Huang et al 2005; Casiano et al 1996; Casciola-Rosen et al 1994b). Such events, followed by subsequent antigenic diversification, could play significant roles in disease pathology.

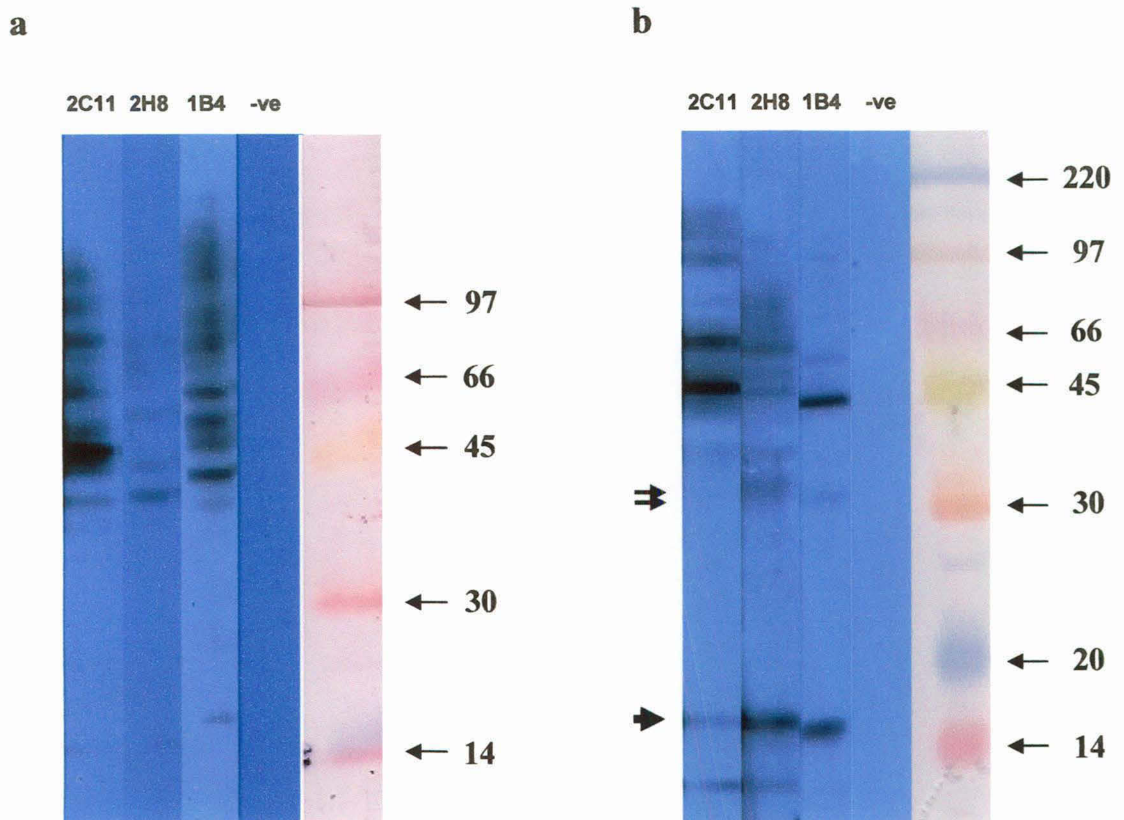


Figure 17: Western blots on lysates of **(a)** healthy Jurkat cells or **(b)** apoptotic blebs, using the IgM monoclonal antibodies. All antibodies were poly-reactive, recognizing moieties in the 40-100 KDa range upon healthy cell lysates. Upon blebs, several lower molecular weight moieties were recognized (for example, thin arrows for 2H8 and 1B4 and the thick arrow for 1B4, 2H8 and 2C11) possibly indicating specific interaction toward apoptotic products. –ve: Negative Controls, where just the goat-anti mouse IgM-HRP was employed.

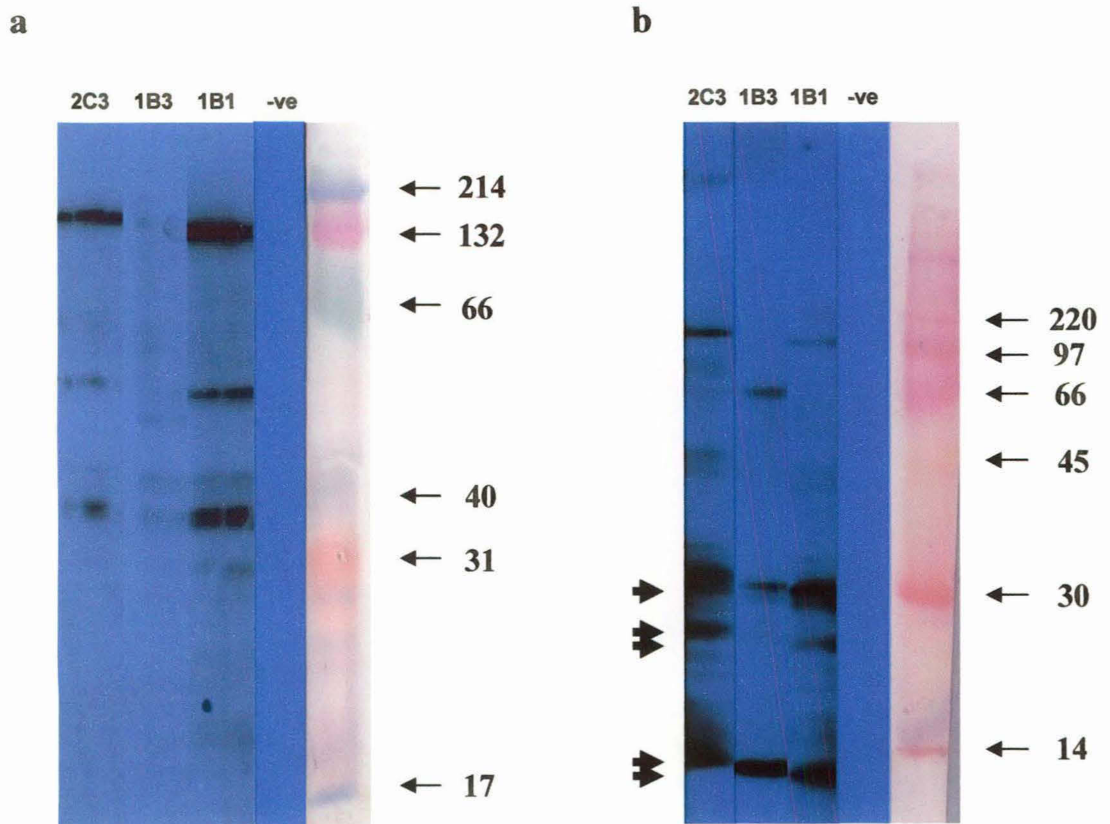


Figure 18: Western blot on lysates of **(a)** healthy Jurkat cells or **(b)** apoptotic blebs, using the IgG monoclonal antibodies. 2C3 and 1B1 antibodies were poly-reactive, recognizing moieties in the 35-140 KDa range upon healthy lysates; 1B3 was poorly reactive. Upon blebs, several lower molecular weight moieties were specifically recognized (arrows). –ve: Negative Controls, where just the goat-anti mouse IgG-HRP was employed.

ELISA

Ribonucleoproteins

Ribonucleoproteins (RNPs) are dominant auto-antigens in systemic auto-immune disorders, and are known to be packaged into apoptotic blebs as cells die. ELISA assays were carried out on a panel of RNPs, obtained as purified recombinant proteins from Dr. Shu Man Fu at the University of Virginia. Reiterating observations of polyreactivity upon Western blots, the IgM antibodies appeared to be reactive towards multiple RNPs (Figure 19a). Antibody 2C11 predominantly bound Ro52 and the U1RNP A protein, while antibody 2H8 bound Ro60 to the most significant extent, but also demonstrated recognition of Ro52, SmB, SmD and the U1RNP A protein. Antibody 1B4 recognized Ro52 and Ro60, while demonstrating minor reactivity towards all the other proteins with the exception of La. Similar (but distinctive) polyreactivity was observed with the IgG antibodies (Figure 20a). Antibodies 2C3 and 1B1 bound Ro60 and SmD in a dominant fashion, while 1B3 exclusively recognized SmD. 1D1, the antibody that recognized antigen(s) present upon healthy cells, was used as control, and was essentially non-reactive.

Lipids

Antibodies against phospholipids are frequently found in Antiphopholipid Syndrome (APS), in SLE, and in patients suffering from Recurrent Spontaneous Abortion (RSA) (Blank et al 1991). In addition, antibodies to oxidized phospholipids have been shown to bind to the apoptotic cell surface and induce the release of pro-inflammatory cytokines (Manfredi et al 1998). Reactivity of the apoptotic cell-specific antibodies towards a panel of lipids was therefore analyzed. The IgM antibody 2C11 specifically bound lysophosphatidylcholine (LPC, Figure 19b). LPC, at certain concentrations, is anti-inflammatory in nature (Yan et al 2004). It has been shown to be secreted by apoptotic cells, and to act as a chemo-attractant for phagocytes (Lauber et al 2003). Possibly as a consequence, mice lacking G2A (the LPC receptor) exhibit systemic autoimmunity (Le et al 2001). The IgG antibody 2C3 appeared to be exhibit broad cross-reactivity towards the

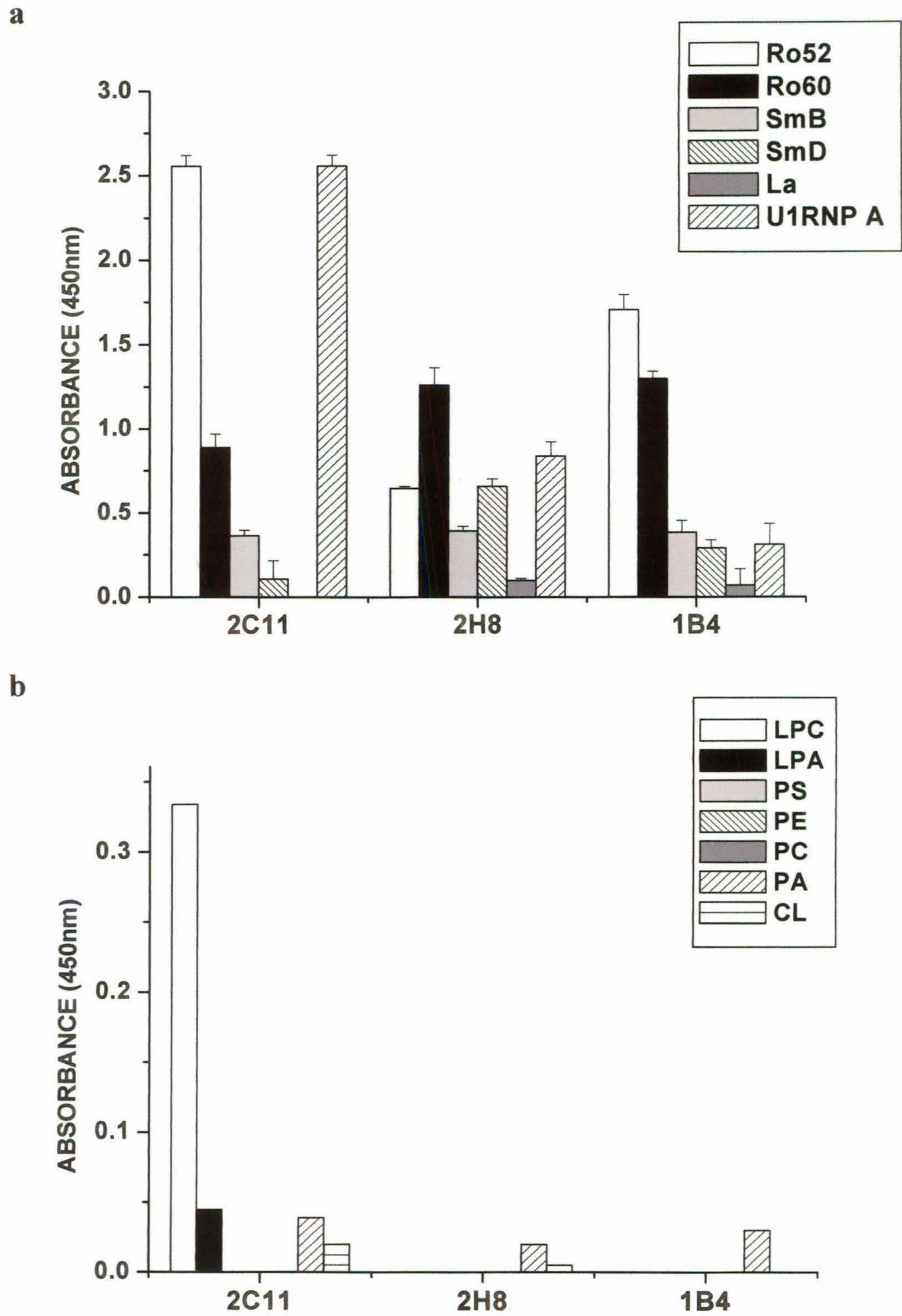


Figure 19: Reactivity of the IgM monoclonal antibodies towards **(a)** recombinant ribonucleoprotein auto-antigens and **(b)** phospholipids by ELISA. All antibodies demonstrated poly-reactivity towards the former. 2C11 additionally bound the bio-active lipid LPC.

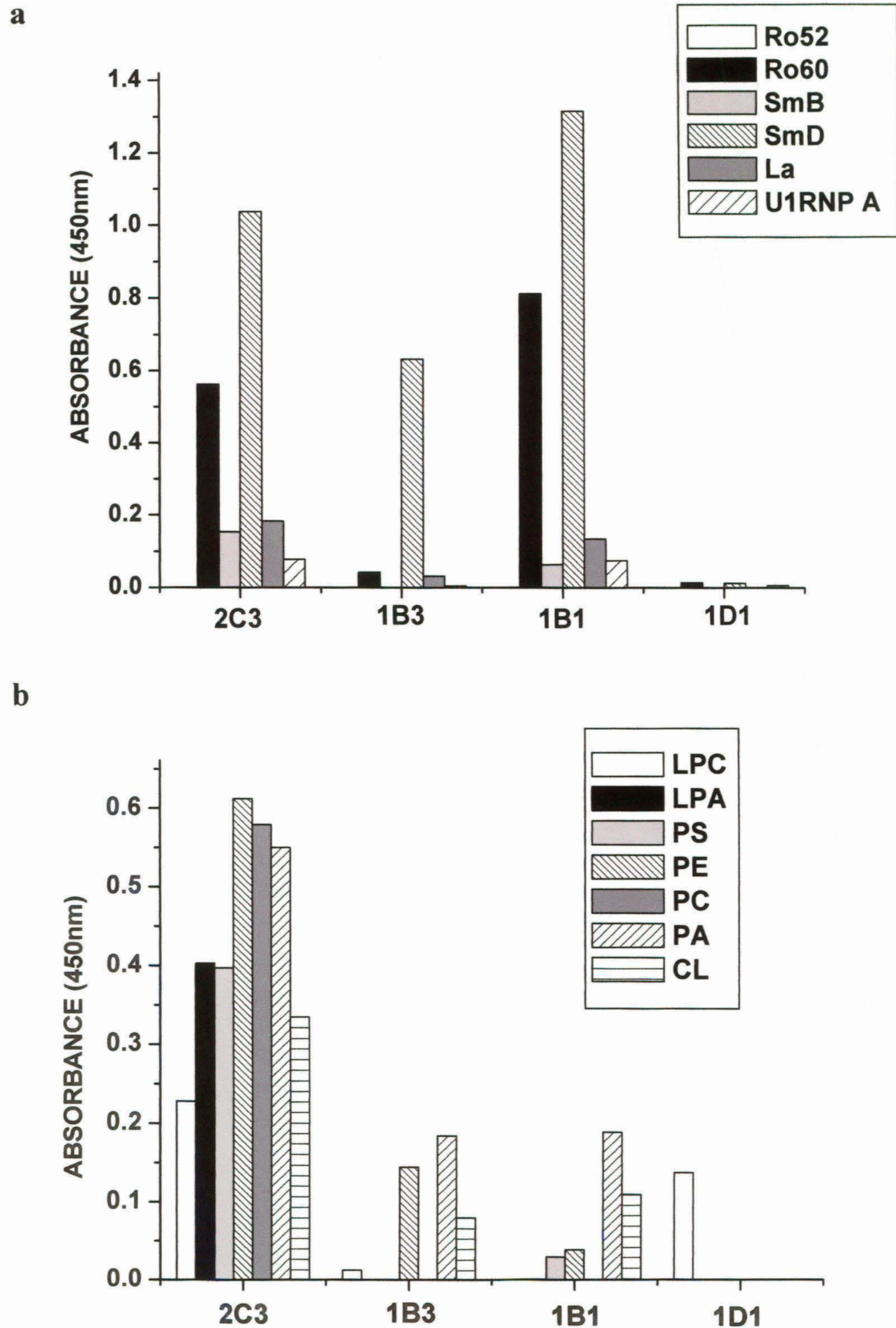


Figure 20: Reactivity of the IgG monoclonal antibodies towards **(a)** recombinant ribonucleoprotein auto-antigens and **(b)** phospholipids by ELISA. All antibodies demonstrated poly-reactivity towards the former. 2C3 was additionally cross-reactive to many lipids. Antibody 1D1 was employed as a negative control.

lipids, binding them all but to variable extents. The other antibodies were poorly reactive (Figure 20b).

Autoreactivity to lipids and ribonucleoproteins has been previously demonstrated. However, the fact that monoclonal auto-antibodies specific to apoptotic cells exhibit frank cross-reactivity between the two chemically dissimilar biological molecules is a finding of some significance.

Biological Effects

Effects on Phagocytic Uptake of Apoptotic Cells

The phagocytic clearance of apoptotic cells is believed to be a very efficient process (Surh et al 1994). In both mice (Mevorach et al 1998; Chang et al 2003; Licht et al 2004) and humans (Jin et al 2004), defective uptake of cellular debris has been associated with the onset of systemic autoimmunity. Since apoptotic cells are thought to be the antigenic source in lupus, the influence of apoptotic cell-specific antibodies on the phagocytic uptake of dying cells was assessed. Figure 21 depicts the results. Figure 21a indicates data for the IgG antibodies 2C3, 1B3 and 1B1; the antibody 1D1 was employed as an isotype negative control. The Phagocytic Index was calculated as described in the Materials section. It was apparent that all antibodies caused significant increases in the uptake of apoptotic cells. Figure 21b depicts results obtained with the IgM antibodies 2C11, 2H8 and 1B4; in this case, an IgM monoclonal antibody recognizing the Vi molecule of *Salmonella typhi* was employed as control. In contrast to the effects of the IgG antibodies, all IgM antibodies caused dose-dependent decreases in phagocytic uptake. Data from Figure 21 is depicted once again in Figure 22, here represented as fold-change over respective isotype control antibodies.

Neutralization of the Anti-Inflammatory Environment During Apoptosis

The fact that the IgM antibody 2C11 recognized LPC was of interest since, as indicated earlier, LPC has been demonstrated to mediate a multitude of biological functions. Of particular significance is that fact that LPC is generated during the process of apoptosis

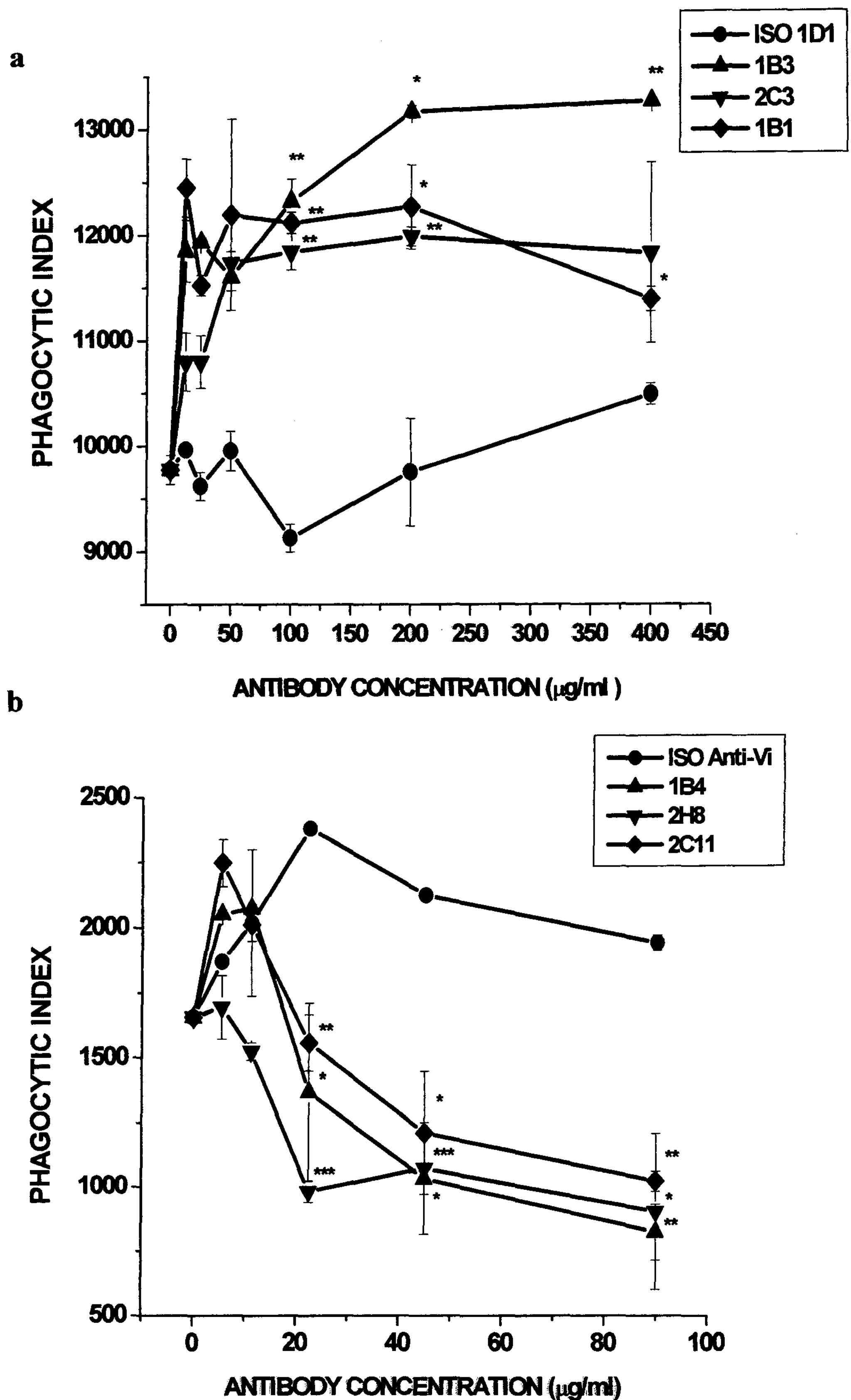
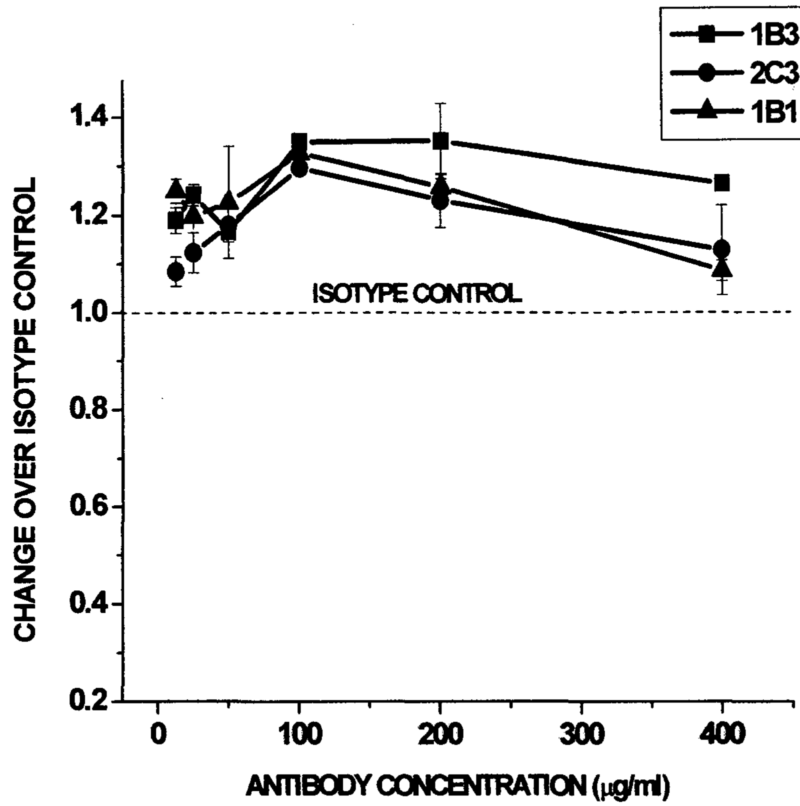


Figure 21: Phagocytic uptake of apoptotic thymocytes in presence of **(a)** IgG and **(b)** IgM monoclonal antibodies by peritoneal macrophages. The IgGs demonstrated enhanced uptake (**, $p \leq 0.009$; *, $p \leq 0.5$) while the IgMs demonstrated diminished uptake (***, $p \leq 0.0009$; **, $p \leq 0.008$; *, $p \leq 0.5$) of apoptotic cells. The Phagocytic Index was calculated as described in the text. ISO 1D1 and ISO anti-Vi are isotype control antibodies.

a



b

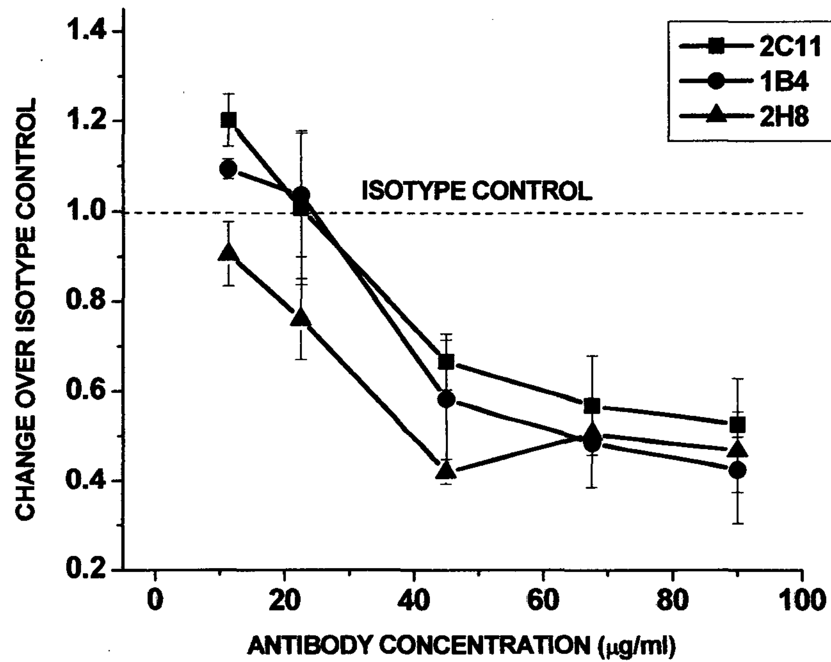


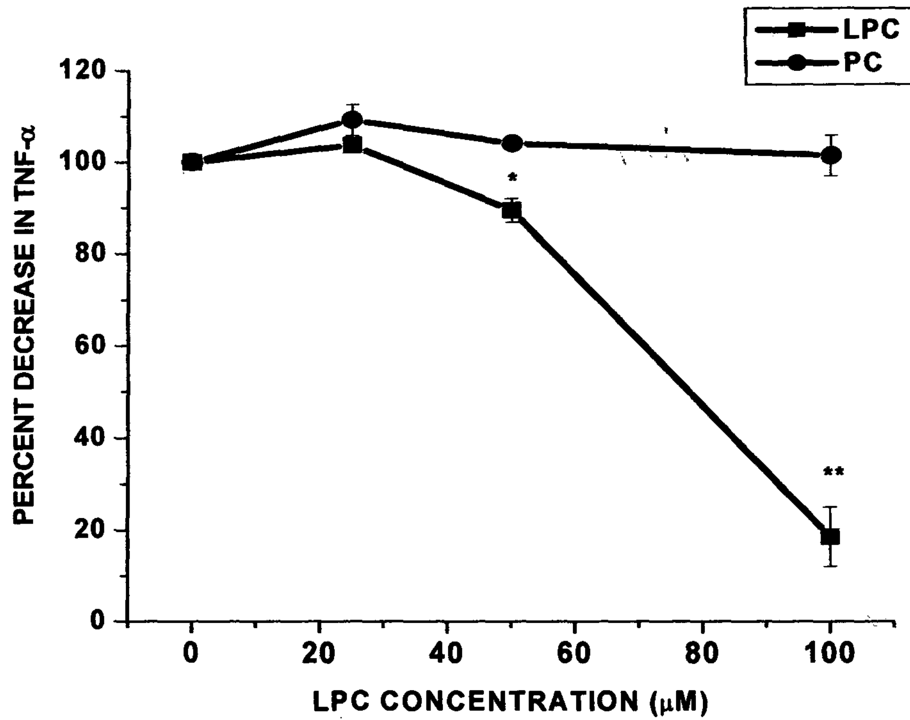
Figure 22: Normalization (fold change over respective isotype controls) of data presented in Figure 21. **(a)** IgG and **(b)** IgM monoclonal antibodies.

(subsequent to the activation of I-PLA_2), and may contribute the anti-inflammatory nature of the surrounding milieu, thereby reducing the chances of aggressive, autoreactive responses (Lauber et al 2003; Yan et al 2004). Figure 23a reveals the dose-dependent inhibitory effect of LPC on lipopolysaccharide (LPS) induced TNF-α production by peritoneal macrophages. Expectedly, Phosphatidylcholine (PC), used as a control, had no such effect. The capacity of the LPC-reactive antibody 2C11 to revert LPC mediated anti-inflammatory effects was then investigated. Results are shown in Figure 23b. 1B4 (a non-LPC reactive antibody of the IgM isotype) was employed as negative control. The antibodies were incapable of stimulating TNF-α production on their own. LPC, or LPC incubated with the control antibody 1B4, caused significant (and equivalent) dose-dependent decreases in TNF-α production. Incubation of LPC with 2C11 led to significant decrease in the capacity of LPC to suppress the generation of TNF-α (in relation with the "LPS alone" control), particularly when the experiment was carried out at low LPC concentrations (50 μM and 75 μM). A relative lack of effect at 100 μM LPC was attributed to an excess of lipid in relation to the binding capacity of the amount of antibody employed. Neutralization of naturally-produced LPC by aberrant auto-reactive antibodies which can bind the lipid could be instrumental in initiation or perpetuation of autoreactivity by reducing the normally anti-inflammatory character of the phagocytic milieu.

Inhibition of the Transmigratory Ability of Macrophages

Lauber et al (2003) and Segundo et al (1999) demonstrated that the supernatant derived from apoptotic cells has chemo-attractant properties for phagocytes. Lauber et al (2003) attributed the chemo-attractant nature of apoptotic cell supernatant to LPC, while Segundo et al (1999) discovered that micro-vesicles released form the apoptotic cells also have similar properties. Thus, dying cells secrete components which, in addition to being anti-inflammatory, also help phagocytes "seek" them out (Lauber et al 2003; Yan et al 2004). Since 2C11 fulfilled both these criteria (it bound apoptotic blebs as well as demonstrated reactivity towards LPC), the effect of its addition in transmigration assays was ascertained. Results are depicted in Figure 24. Apoptotic-cell supernatant (designated

a



b

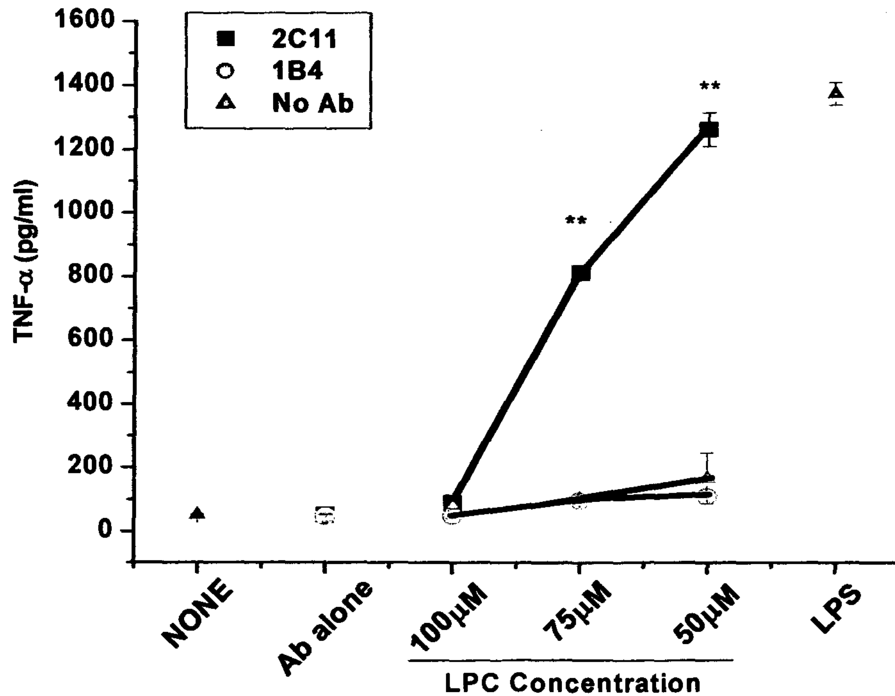


Figure 23: **(a)** Anti-inflammatory effect of lysophosphatidylcholine (LPC) on LPS-stimulated peritoneal macrophages. A dose-dependent decrease in the generation of the TNF-α was observed. Phosphatidylcholine (PC) was employed as a negative control (*, $p<0.05$; **, $p<0.005$). **(b)** Neutralization of the anti-inflammatory effects of LPC by LPC-reactive antibody 2C11. 1B4 was employed as a negative control (** $p<0.00003$).

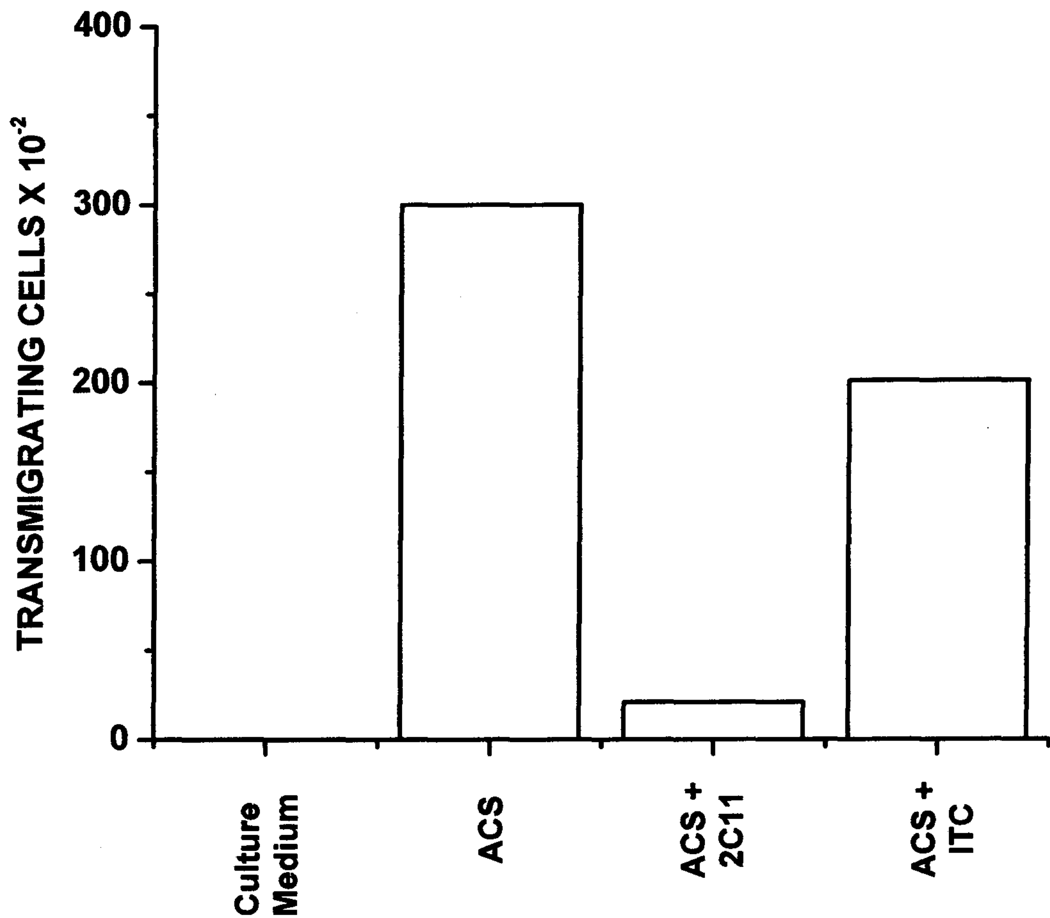


Figure 24: Transmigration of phagocytes (THP-1) towards culture medium or apoptotic cell supernatant (ACS). The LPC-reactive monoclonal antibody 2C11 (1 μg) caused substantial decreases in transmigration in comparison with an isotype control (ITC; 1 μg) antibody. Representation of one of three independent experiments.

"ACS"), added to the lower wells of a transmigration chamber, caused significant enhancement (in comparison with fresh culture medium) in the movement of THP-1 cells. The monoclonal antibody 2C11 caused significant diminution in this migration, reducing it almost to background levels. The monoclonal antibody employed as the isotype control (designated "ITC") did not cause equivalent effects. Neutralization by antibodies (such as 2C11) of LPC secreted by apoptotic cells could thus blind phagocytic cells *in vivo*, adversely affecting the clearance of debris.

Characterization of Anti-idiotypic Responses

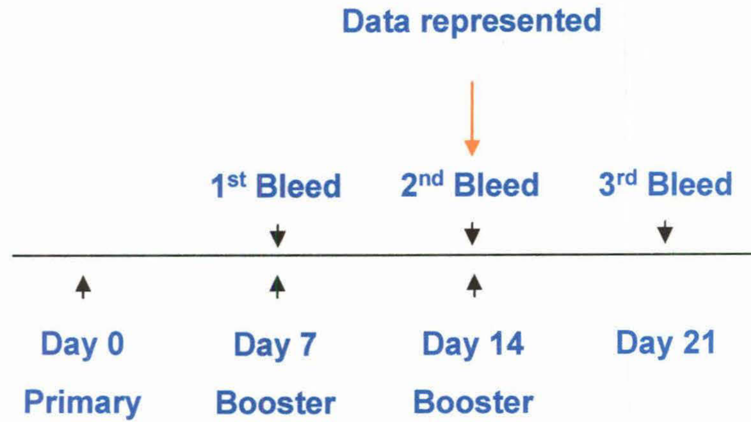
In naturally-occurring systemic autoimmune disease in humans as well in mice, autoreactive antibodies are initially directed at a few restricted epitopes upon autoantigens. With the progress of time, an increasing number of epitopes and antigens are targeted. This phenomenon is referred to as epitope or determinant spreading, and is also observed in non-autoimmune prone animals that are externally immunized with self-antigens (Deshmukh et al 2005). How this might occur could be easily postulated; in a scenario where self-antigens are continually processed and presented, the breaking of T cell tolerance against even a single determinant on a self-molecule could conceivably "help" in the generation of a large diversity of autoreactive antibodies, given the fact that B cells can function as antigen presenting cells.

The network theory postulates that a subset of anti-idiotypic antibodies (Ab2) arising upon immunization with antibody (Ab1) behave as "internal images" of the antigen, which can then be instrumental in the generation of Ab3. Ab1 and Ab3 could share antigenic specificity, which could lead to perpetuation of immune responses. Such amplification could be additionally significant as far as auto-reactive antibodies are concerned. Most work in this regard has been carried out with anti-dsDNA antibodies; immunization with an anti-dsDNA antibody bearing the 16/6 idiotype leads to the generation of an anti-dsDNA response in non-autoimmune prone strains of mice (Mendlovic *et. al* 1988).

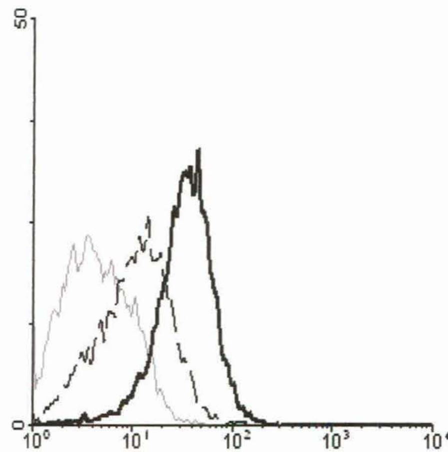
We theorized that the idiotypic network may also be involved in the generation of diversity via epitope or determinant spreading. It can be postulated that, were the autoantigen (to which Ab1 binds) to exist complexed to an interacting moiety (as part of a macro-molecular complex *in situ*), Ab2 might acquire the additional capacity to bind to the interacting moiety, a capacity that Ab1 may not possess. This would constitute antigenic spreading.
Non-autoimmune prone C57/BL6 mice were immunized with 2C11 (the IgMκ, LPC-reactive antibody) or with adjuvant alone. Figure 25a describes the immunization protocol and the blood sampling schedule. As an initial assessment of self-reactivity, antibodies in sera were assessed for binding to antigens on permeabilized Jurkat cells by FACS. As shown in Figure 25b, sera from animals immunized with 2C11 (designated "anti-2C11 sera") demonstrated enhanced presence of IgG antibodies reactive towards self antigen(s), in comparison with sera generated by adjuvant-alone immunization (designated "control sera"); antibodies of the IgM isotype did not exihibit such reactivity (Figure 25c). The anti-idiotypic response arising in 2C11-immunized mice was further characterized by immuno-fluorescence microscopy on permeabilized HeLa cells (Figure 26). As described earlier, antibody 2C11 demonstrated predominantly cytosolic recognition, with the nucleus remaining unbound (Figure 26c). Antibodies in animals immunized with 2C11, on the other hand, essentially bound nuclear antigens, with some peri-nuclear recognition as well (Figure 26b). Results were interpreted with respect to two controls. Figure 26a depicts results obtained when control sera (obtained from animals immunized with adjuvant alone) was employed; no significant reactivity was seen. Reference human SLE serum, previously characterized and catalogued as containing anti-nuclear antibodies (obtained from the Center for Disease Control and Prevention, Atlanta) served as positive control (Figure 26e), with the relevant negative control is depicted in Figure 26d.

Antibodies generated upon immunization with 2C11 were also assessed for reactivity by Western blots using SP2/O cellular lysate as substrate. Recognition of a specific moiety (≈ 40 KDa) was observed, with control sera not demonstrating such reactivity (Figure

a



b IgG



c IgM

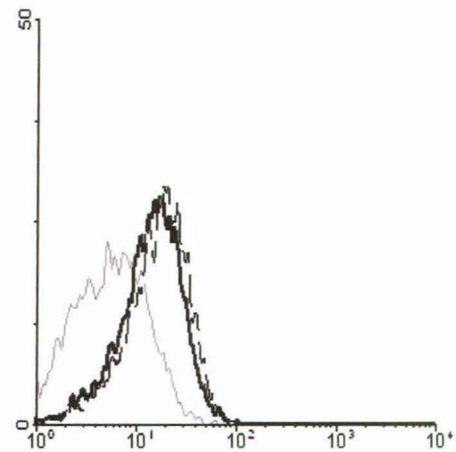


Figure 25: Generation of anti-self reactivity upon active immunization of the poly-reactive antibody 2C11. **(a)** Immunization and blood sampling schedule. **(b)** Significantly increased IgG anti-self reactivity was observed in the sera of animals immunized with 2C11 (designated "anti-2C11 sera") in comparison with adjuvant-immunized mice (designated "control sera"). **(c)** Lack of such anti-self reactivity in antibodies of the IgM isotype. Gray profiles represent negative controls where only the respective second antibodies were employed.

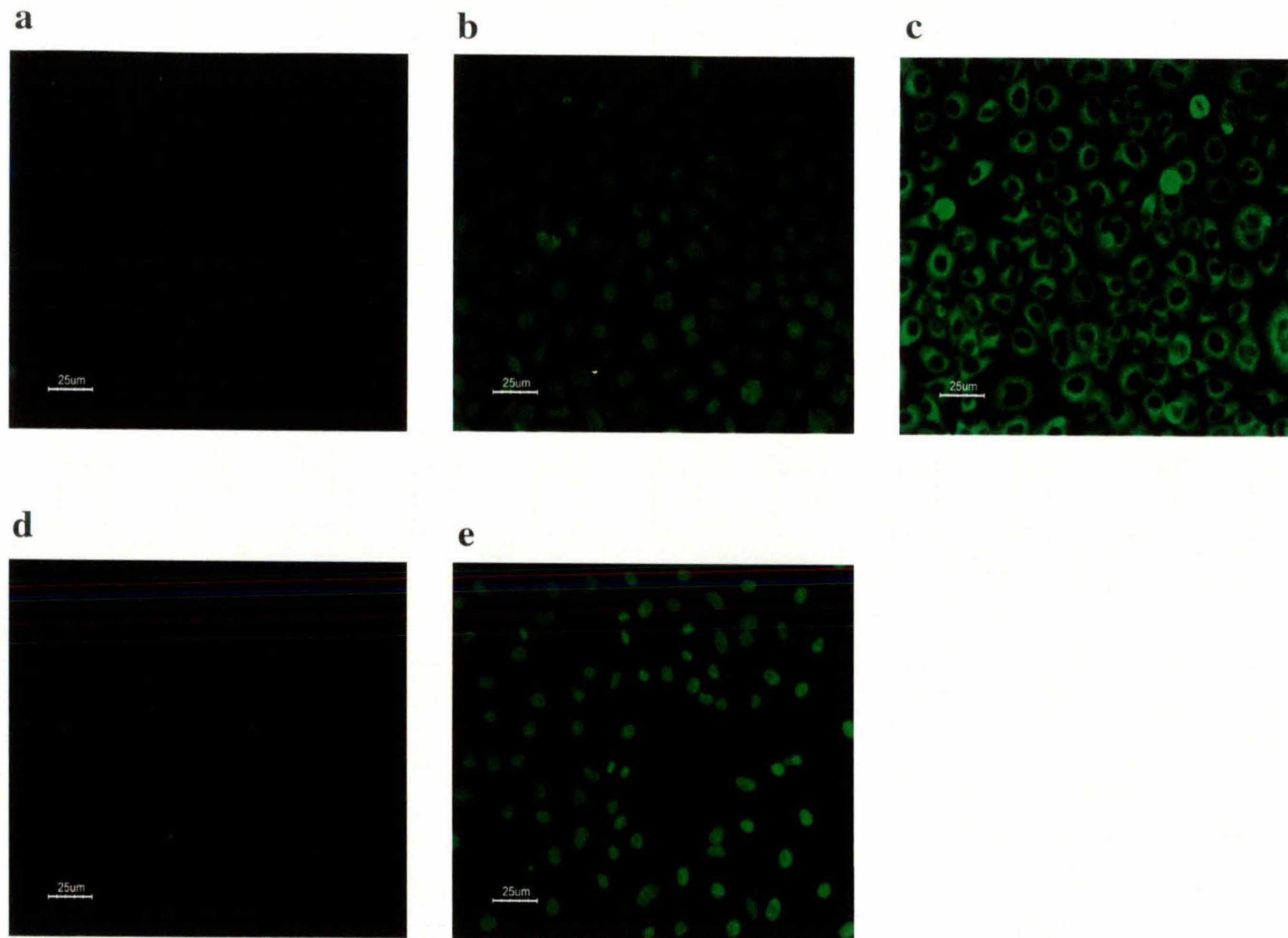


Figure 26: Reactivity of antibodies in **(a)** control sera and in **(b)** anti-2C11 sera upon HeLa cells by immunoflourescence. Anti-2C11 sera demonstrated reactivity towards both nuclear and cytoplasmic moieties **(c)** As previously described, immunogen (2C11) displayed essentially cytoplasmic reactivity. **(d)** Reactivity of goat-ant-human IgG FITC. **(e)** Reactivity of ANA-containing human sera, obtained from the CDC (Altlanta), which was employed as a positive control.

27a). A band common to both sera at ≈ 50Kda, (indicated by '*' in the figure) possibly represents a component of the anti-self response known to be generated by immunization with aggressive adjuvants like IFA.

Immune and control sera were also assessed for reactivity against a panel of recombinant auto-antigens by ELISA (Figure 27c). Immune sera demonstrated enhanced recognition of La and the U1RNP A protein over control sera. Interestingly, control sera demonstrated significant reactivity towards Ro52 and the U1RNP A Protein, a fact possibly related to the immuno-stimulatory properties of IFA. While this was expected, the reason why control sera showed enhanced reactivity towards Ro52, compared with anti-2C11 sera, was not immediately apparent, and possible reasons are discussed later. The appearance of antibodies to La in immune sera was perhaps of most significance, since neither 2C11 (the reactivity of which has been reproduced in Figure 27b for the sake of comparison) nor control sera exhibited reactivity towards this autoantigen.

Immunofluorescence, Western blot and ELISA assays all revealed that immunization with 2C11 led to the generation of autoantigenic specificities in serum not exhibited by 2C11. Stimulation of the idiotypic network could thus result in epitope spreading.

An anti-idiotypic anti-self response was obtained upon immunization of the monoclonal antibody 1B1 as well. This antibody (IgG2aκ) was immunized in lupus-prone NZB/W F1 mice; Figure 28a describes the immunization schedule. A significant IgG anti-self response was generated in mice immunized with 1B1 (Figure 28b; designated "anti-1B1 sera") in comparison with mice immunized with adjuvant (designated "control sera). As with antibody 2C11 immunization, IgM anti-self responses were not observed (Figure 28c). The anti-self immune response generated by 1B1 immunization was further characterized by upon Western blots using SP2/O (Figure 29a) and Jurkat (Figure 29b) cell lysates. Recognition of distinct moieties by the anti-idiotypic sera was documented; on the SP2/O lysate, two moieties of ≈ 80 KDa and 90 KDa were recognized (arrows in Figure 29a); some reactivities arising due to the presence of adjuvant are indicated by '*'.

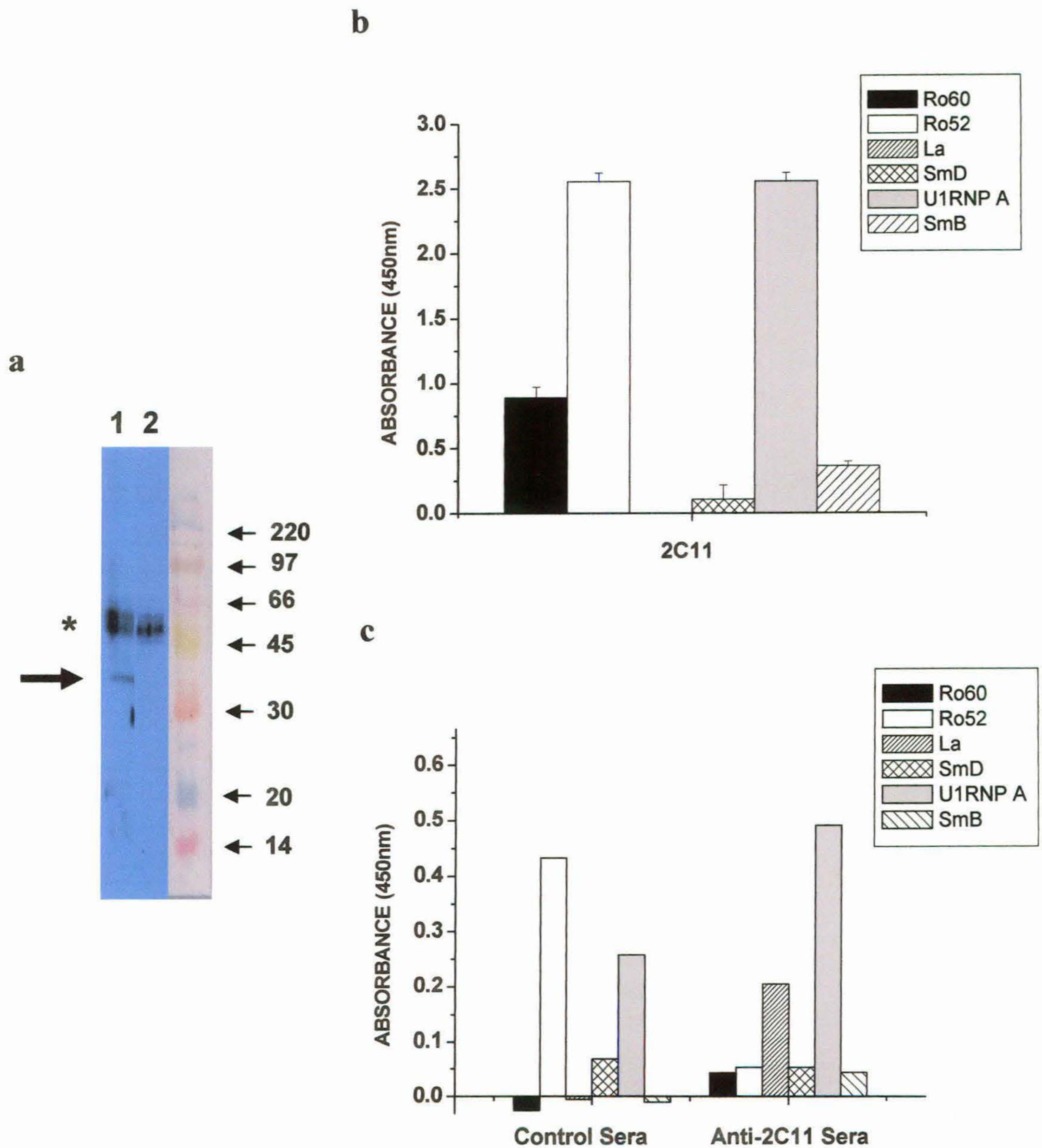


Figure 27: Analysis of anti-2C11 sera and adjuvant-immunized (control) sera. **(a)** Western blot upon Sp2/O lysate. Anti-2C11 sera (Lane 1) demonstrated reactivity (arrow) not observed in control sera (Lane 2). Adjuvant-elicited reactivities are indicated by *. **(c)** ELISA upon recombinant antigens. Antibodies in control sera demonstrated reactivity towards Ro52 and the U1RNP A protein. Anti-2C11 sera principally demonstrated reactivity towards La and the U1RNP A protein. **(b)** Reactivity pattern of 2C11 is reproduced for comparison.

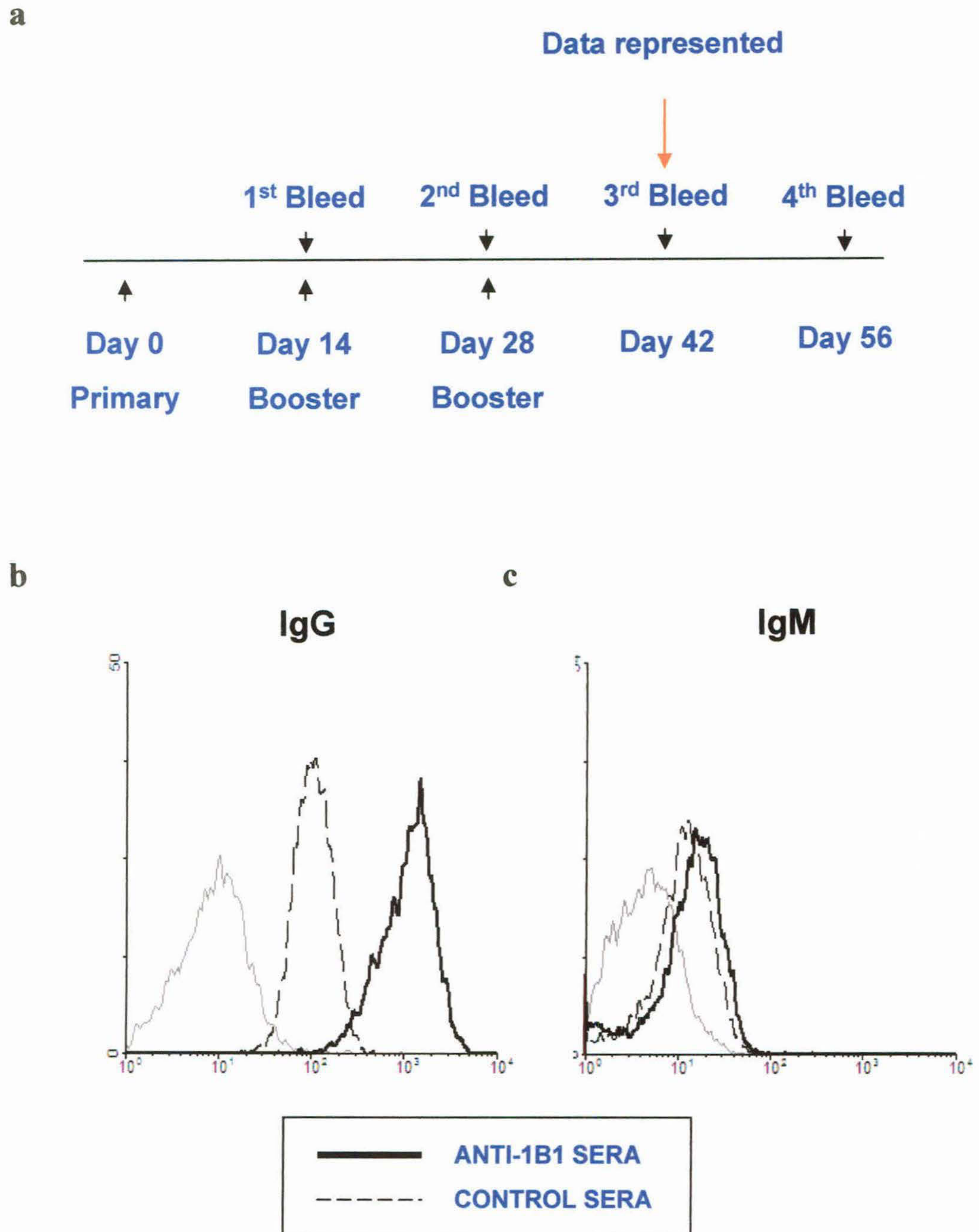


Figure 28: Generation of anti-self reactivity upon active immunization of the poly-reactive antibody 1B1. **(a)** Immunization and blood sampling schedule. **(b)** Significantly increased IgG anti-self reactivity was observed in the sera of animals immunized with 1B1 (designated "anti-1B1 sera") in comparison with adjuvant-immunized mice (designated "control sera"). **(c)** Lack of such anti-self reactivity in antibodies of the IgM isotype. Gray profiles represent negative controls where only the respective second antibodies were employed.

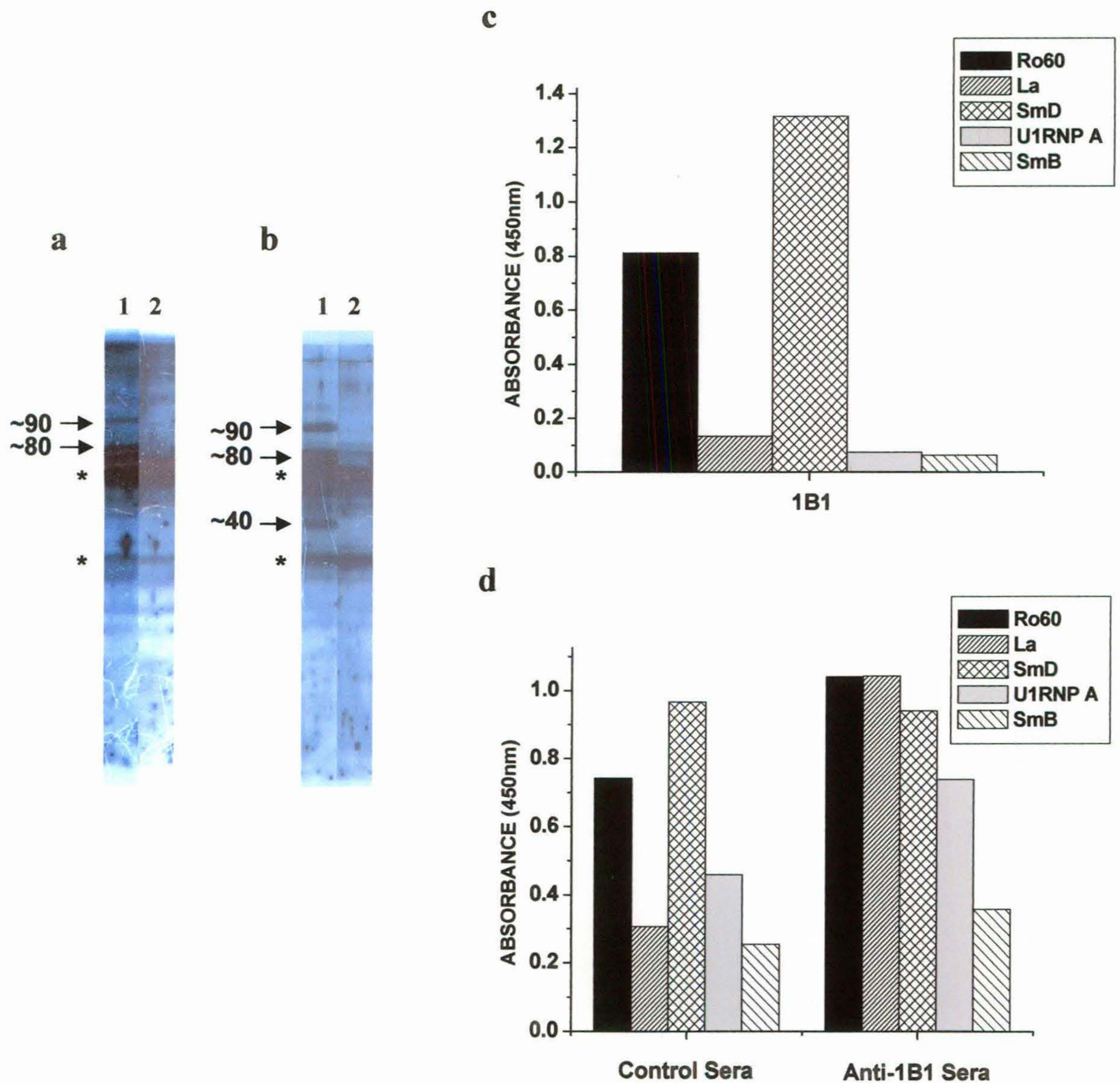


Figure 29: Analysis of anti-1B1 sera and adjuvant-immunized (control) sera. Western blot upon **(a)** Sp2/O and **(b)** Jurkat cell lysates. Anti-2C11 sera (Lanes 1) demonstrated reactivity (arrows) not observed in control sera (Lanes 2). Some adjuvant-elicited reactivities are indicated by *. **(d)** ELISA upon recombinant antigens. Antibodies in control sera demonstrated predominant reactivity towards Ro60 and the SmD. Anti-1B1 sera principally demonstrated enhanced reactivity towards La and the U1RNP A protein. **(b)** Reactivity pattern of 1B1 is reproduced for comparison.

On the Jurkat lysate, moieties of similar molecular weight were recognized, along with additional specific reactivity towards a ≈ 40 KDa moiety (arrows in Figure 29b); adjuvant-induced reactivities were apparent in this case as well, as indicated. ELISA was then carried out on the panel of recombinant auto-antigens (Figure 29d). Antibodies in 1B1 anti-idiotypic sera demonstrated significantly enhanced recognition of La and the U1RNP A Protein over antibodies in control sera. Significantly, neither of these antigens were bound to an appreciable extent by 1B1 (Figure 29c). These data appear to indicate that immunization with 1B1, like with 2C11, leads to the appearance of newer specificities.

We have previously reported the diversification of the autoimmune response when rats were immunized with a human IgM monoclonal antibody that recognized late-stage apoptotic cells (Gandhi et al 2006), indicating that such epitope spreading was not unique to 2C11 or 1B1. However, no significant IgG or IgM anti-self responses were observed when 2C3 (the IgG2bκ antibody) was similarly immunized (Figure 30), indicating that initiation of self-reactivity via idiotypic stimulation might not be a generalized phenomenon. The rules that govern the generation of anti-idiotype responses directed against "self" moieties needs further investigation.

Effects on Fertility

Antibodies directed to ribonucleoproteins and phospholipids have been implicated in aberrant pregnancy outcomes and pregnancy loss. Anti-phospholipid antibodies are known to cause recurrent spontaneous abortion (Blank et al 1991), while antibodies to ribonucleoproteins are thought to cause neonatal lupus and congenital heart block by reacting to antigens on the fetal heart (Julkunen et al 1998). To evaluate the potential of the apoptotic cell-specific monoclonal antibodies to affect pregnancy outcomes, antibodies were passively infused into pregnant mice, as described in the Materials section. Antibody 2C3 and antibody 1B1 were employed; both antibodies predominantly bound the ribonucleoproteins Ro60 and SmD, while the former also bound many phospholipids. As depicted in Table 2, administration of antibody 2C3 as well as the

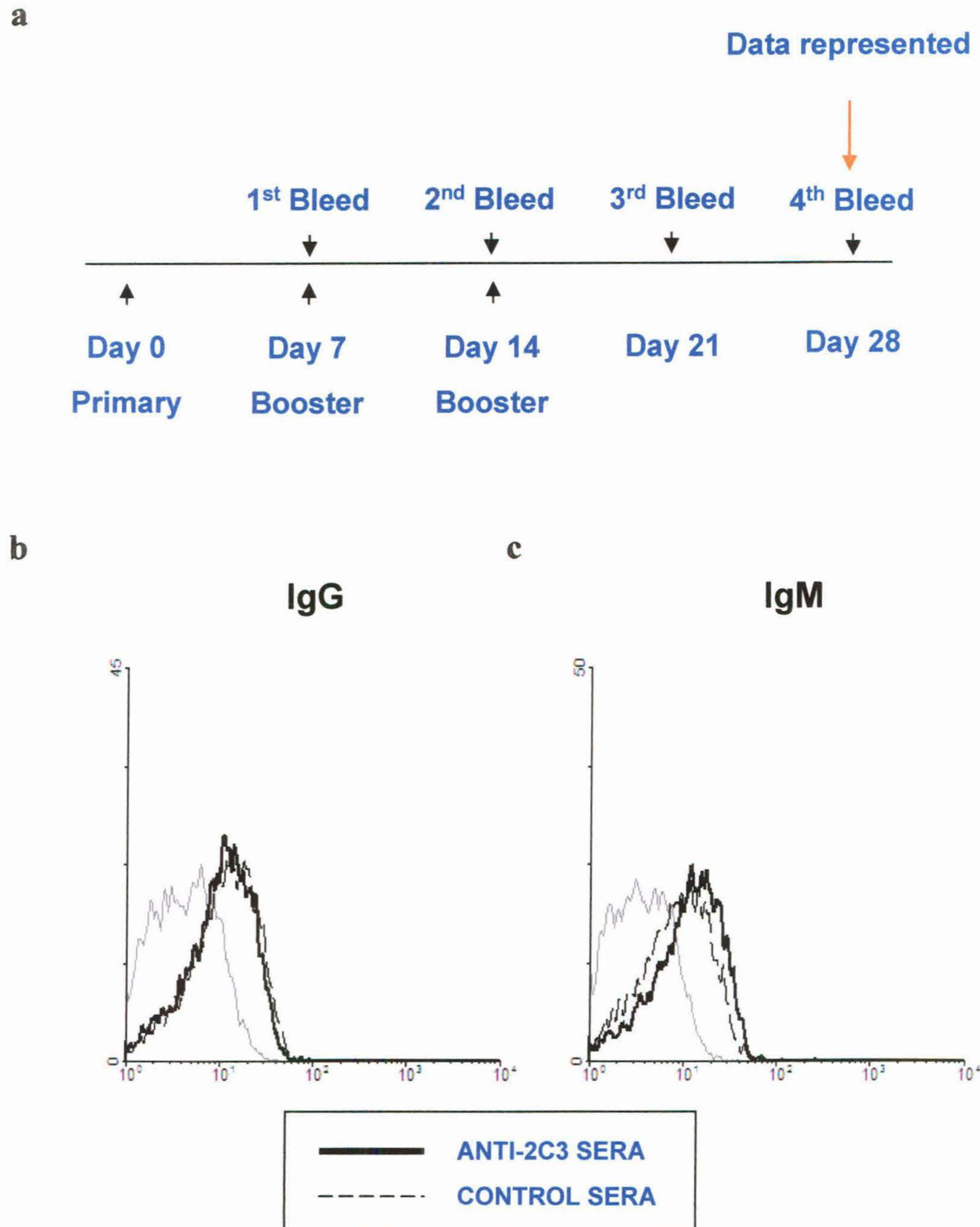


Figure 30: Lack of anti-self reactivity upon active immunization of the poly-reactive antibody 2C3. **(a)** Immunization and blood sampling schedule. No significant **(b)** IgG or **(c)** IgM anti-self reactivity was observed in the sera of animals immunized with 2C3 (designated "anti-2C3 sera") in comparison with adjuvant-immunized mice (designated "control sera"). Gray profiles represent negative controls where only the respective second antibodies were employed.

Treatment	No. of Animals	No. Pregnant	No. Pups	Pups / Animal
1B1	6	1	0	0
2C3	6	6	49	~8.17
IS0TYPE CONTROL	6	5	38	~7.6
PBS	6	5	42	~8.4

Table 2: Treatment of pregnant FVB mice with various antibodies. Control animals received the vehicle (PBS). Antibody 1B1 caused inhibition of fertility.

isotype control antibody resulted in pregnancy rates that approximated those seen in animals administered the vehicle (PBS); the number of live births were also comparable. In animals administered antibody 1B1, however, parameters of fertility were dramatically reduced; though one pregnancy was observed (as diagnosed by gross observation), no live births were recorded. After a rest period of three weeks, five 1B1-treated female mice were co-habited with males once again. Three animals experienced a return to fertility, with a total of twenty-six live births recorded. These results ruled out intrinsic defects in the animals and lent further credence to the claim that anti-fertility effects were due to extrinsically administered antibody.

The reasons for these observations are at present unclear; *aprioi*, anti-fertility effects would have been expected in animals treated with antibody 2C3 (and not 1B1), given its extensive phospholipid reactivity. Other, unidentified reactivities may play a role, and the data therefore merits further investigation. It remains significant, however, that an auto-antibody specifically targeting apoptotic cells can mediate fertility loss, a significant pathology associated with systemic autoimmume responses.

Identification of Mimotopes

"Panning" of a Phage Display Library Using Antibody 2C11

In order to further antibody reactivity, antibody-reactive peptidic mimotopes were identified. The monoclonal antibody 2C11 was purified to homogeneity by Mannan Binding Protein (MBP) affinity chromatography. The antibody was used as a probe to "pan" a 12-mer unconstrained phage-display random peptide library. Four rounds of sequential panning and amplification were carried out as described in the Materials section. Phages, amplified from individual plaque lifts, were assessed for reactivity to 2C11 by ELISA, with wells adsorbed with BSA (the "blocking" agent) serving as the negative control. All phages (with the exception of Phage 16) specifically bound to 2C11, while displaying with minimal reactivity towards BSA (Figure 31). Phage DNA was isolated and sequenced (as described in the Materials section), using primers provided by the manufacturer. Phage insert amino acid sequences were translated from the obtained

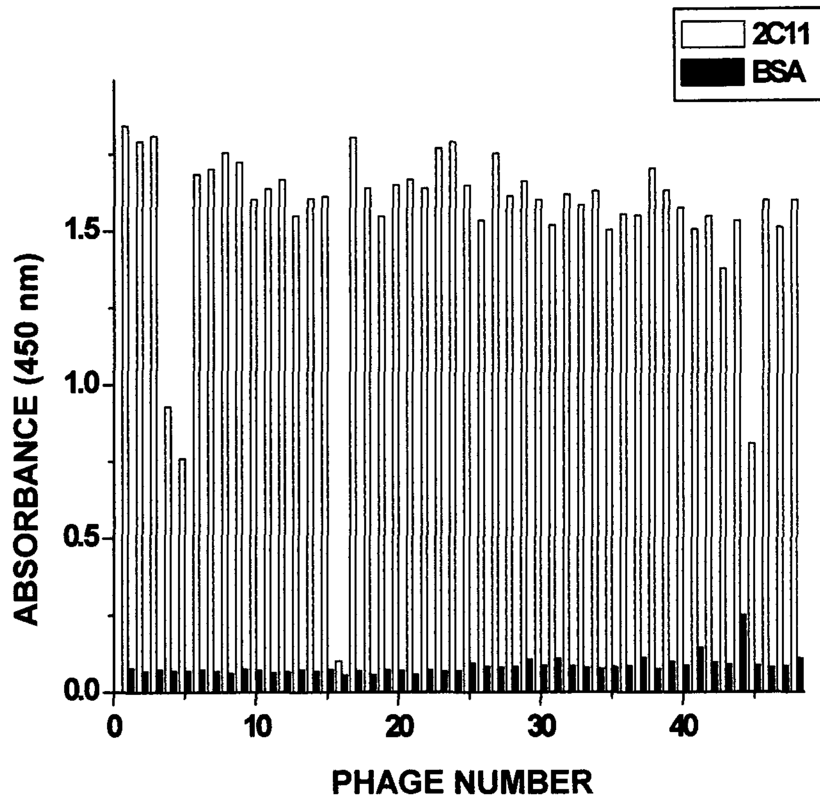


Figure 31: ELISA reactivity of phages (after four sequential rounds of panning and amplification) towards 2C11. All phages (except Phage 16) were reactive. Blank wells blocked with bovine serum albumin (BSA) served as negative controls.

nucleotide sequence; Figure 32 depicts sequences obtained for twenty-two individual phages. Significantly, the motif **QXPXXL** (where X was a varying amino acid) was found in all sequences. Twelve of twenty-two phage inserts contained **Q(L/I)PXHL**, while an additional four had the sequence **Q(L/I)PXXL** (where the second position of the motif was either Leucine or Isoleucine, amino acids of similar nature). The remaining six phage inserts contained the sequence **QXPXWL**. The sequence **QTPLHL** is found in Ro52 (amino acids 387-406) and **RIPTHL** in Ro60 (amino acids 112-130), two RNPs towards which 2C11 demonstrates reactivity. When mapped against the entire Ro52 and Ro60 sequences, most peptides aligned with amino acids 385-406 for Ro52 (Figure 33a) and 110-103 for Ro60 (Figure 33b), once again re-iterating the importance of the core motif for recognition and possibly elucidating a sequential epitope on these two proteins. In the case of La, a protein not recognized by the antibody and which does not contain the core recognition sequence, no single region of the molecule exhibited such dominant recognition (Figure 33c). In the U1RNP A Protein (towards which 2C11 also demonstrates reactivity) sequence, a similar motif **(QILDIL)** was found.

Antigenecity

The peptides **KYEAGTYPQTPLHLQVPPCQ** and **FKAVSEVCRIPTHLFTFIQ** (corresponding to the core peptides in Ro52 and Ro60 respectively, each flanked by amino acids against which weaker homologies were observed) were synthesized on 8-branched MAP backbone. A core consensus peptide **QIPTHL** (chosen on the basis of amino acid predominance in sequenced inserts) was also similarly synthesized. Figure 34a reveals that though antibody 2C11 was strongly reactive towards both the MAP-Ro60 peptide and the MAP-Ro52 peptide on Day 1 (the day the peptides were resuspended in dimethylformamide (DMF) and employed in the assay), the MAP-Ro60 peptide had lost antigenecity by Day 5. The reasons for this remain unclear, but possibly reflect a change in conformation that affected subsequent immunogenecity experiments with MAP-Ro60. Figure 34b reveals that antibody 2C11 strongly bound both the MAP-Ro52 peptide and MAP-QIPTHL; none of the other antibodies exhibited such interaction. These results indicate the specificity of the panning process in the elucidation of the

```
2C11P1          -TN-QHQIPSHLFR----  12
2C11P23         -TYPQQQIPLHLH-----  12
2C11P8          ---NIVQIPRHLALF---  12
2C11P29         ---NYTQIPQHLTAS---  12
2C11P24         -GMTNSQIPTHLW-----  12
2C11P5          ---GTSQLPIHLLES---  12
2C11P27         --GGSSQLPFHLRT----  12
2C11P6          TLTLNPQLPSHL------  12
2C11P2          ---SSVQLPKHLYPH---  12
2C11P19         -THQSNQLPPHLM-----  12
2C11P3          -VPTETQLPAHLH-----  12
2C11P4          ---TMIQLPTHLATP---  12
                      *:* **

2C11P15         ----VYQFPPWLHLEP-   12
2C11P30         IQRSISQSPPWL------  12
2C11P17         --DHWAQWPLWLEK----  12
2C11P26         SPTHPPQVPRWL------  12
2C11P25         NAGTLHQMPTWL------  12
2C11P28         KPLPVTQAPTWL------  12
                      * * **

2C11P21         -KPLDSQLPHWLR-----  12
2C11P10         --HYNTQIPPRLIS----  12
2C11P7          ------QIPMFLLRTPSH  12
2C11P18         ------QIPWFLNGASSR  12
                      *:*  *
```

Figure 32: Alignment of 2C11-reactive peptide sequences, determined after the sequencing of phage inserts. A minimum consensus sequence of QXPXXL was observed. Peptides were further divided in three groups based on additional homologies (:) and identities (*), as indicated by the color code.

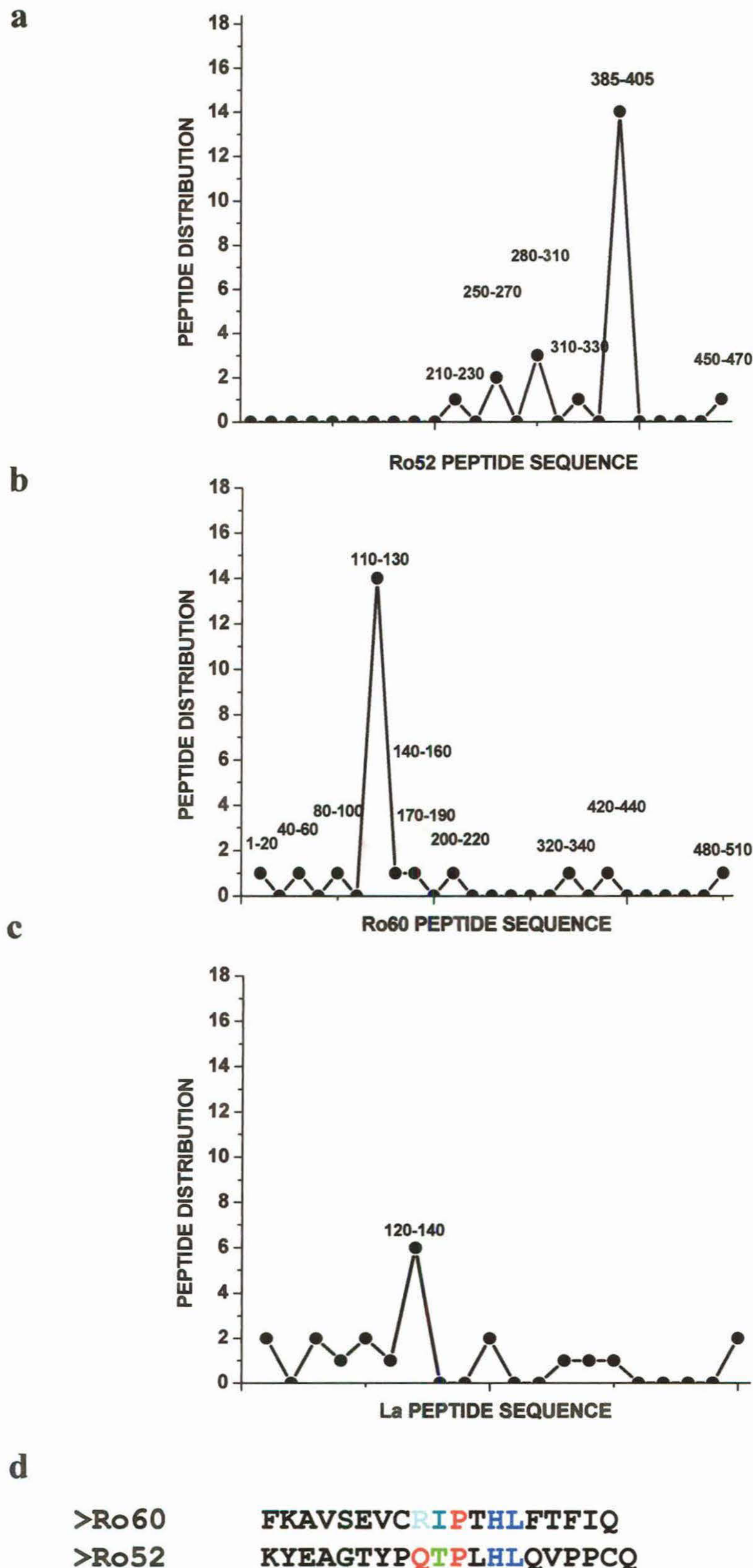


Figure 33: Alignment of all 2C11-reactive peptides (described in previous figure) with the sequence of 2C11-reactive (**a**, Ro52 and **b**, Ro60) and non-reactive (**c**, La) antigens. A significant number of sequences aligned with amino acid 385-405 from Ro52 and 110-130 from Ro60. **(d)** The identified peptide regions on Ro60 and Ro52 contain mimics (indicated in colour code) of 2C11-reactive consensus sequence. Refer to the text for details.

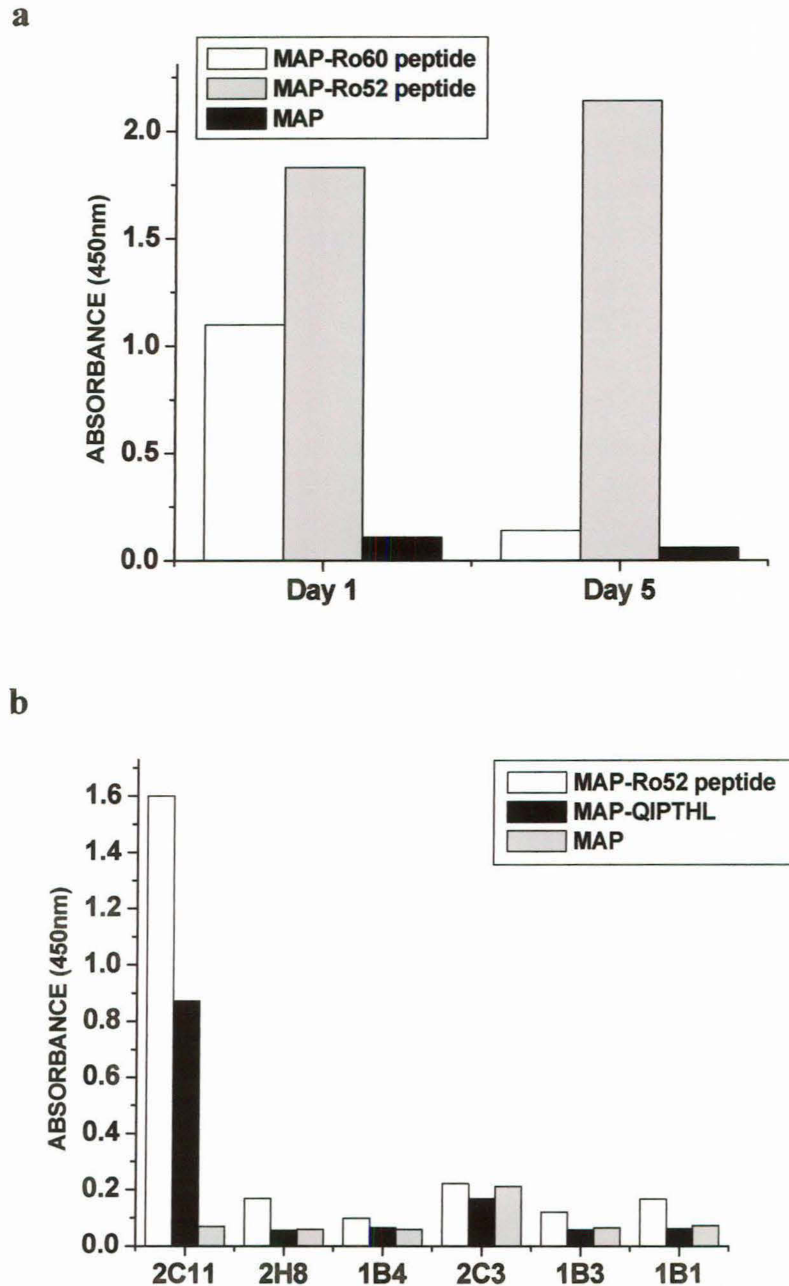


Figure 34: (a) Early loss of reactivity of 2C11 towards MAP-Ro60 peptide, in comparison with that towards MAP-Ro52. MAP served as negative control. **(b)** Reactivity of the apoptotic cell-specific monoclonal antibodies towards the MAP-Ro52 peptide, MAP-QIPTHL and the MAP backbone. Only 2C11 specifically bound both the peptides, while demonstrating no reactivity towards the MAP backbone.

2C11-reactive peptide, and also showed that the peptides were adequately antigenic when represented on a MAP backbone.

Immununogenecity

The individual MAP-Ro52 and MAP-Ro60 peptides, as well as the core consensus sequence on a MAP backbone (MAP-QIPTHL) were immunized in non-autoimmune prone BALB/C mice, as described in the Materials section. Animals immunized with just the MAP backbone (without attached peptides) served as controls. Figure 35a depicts the immunization schedule. Sera were analyzed for reactivity to peptides and whole protein. Sera from Ro52 peptide-immunized animals demonstrated reactivity to the Ro52 peptide, but sera from Ro60 peptide or MAP-immunized animals did not (Figure 35b). Sera from both Ro52 peptide-immunized animals and Ro60 peptide-immunized animals bound the Ro60 peptide, but to a greatly reduced extent; sera from MAP-immunized animals did not exhibit even this minimal reactivity (Figure 35b). The loss of antigenicity (to 2C11) of the Ro60 peptide discussed above, if indicative of an altered state, could possibly have affected its immunogenic and/or antigenic potential in these experiments. A complete lack of reactivity was observed towards the MAP backbone irrespective of the immunogen employed (Figure 35c). Sera from Ro52 peptide-immunized animals bound both Ro52 and Ro60 (Figure 35d), indicating that the region representative of the core consensus peptide was adequately antigenic on both molecules. Since association between the two molecules has not been demonstrated (negating the possibility of antigenic spreading), these observations of cross-reactivity may lay the basis for the association of anti-Ro60 and anti-Ro52 responses in systemic autoimmunity previously described by Keech et al (1996).

"Panning" of a Phage Display Library Using Antibody 2C3

As described previously, monoclonal antibody 2C3 (IgG2bκ) demonstrated extensive cross-reactivity to lipids and ribonucleoproteins. A 12-mer phage display library was panned using 2C3 as the probe, using analogous techniques as described above for 2C11. Figure 36 represents the ELISA with individual phages on purified 2C3; BSA-coated

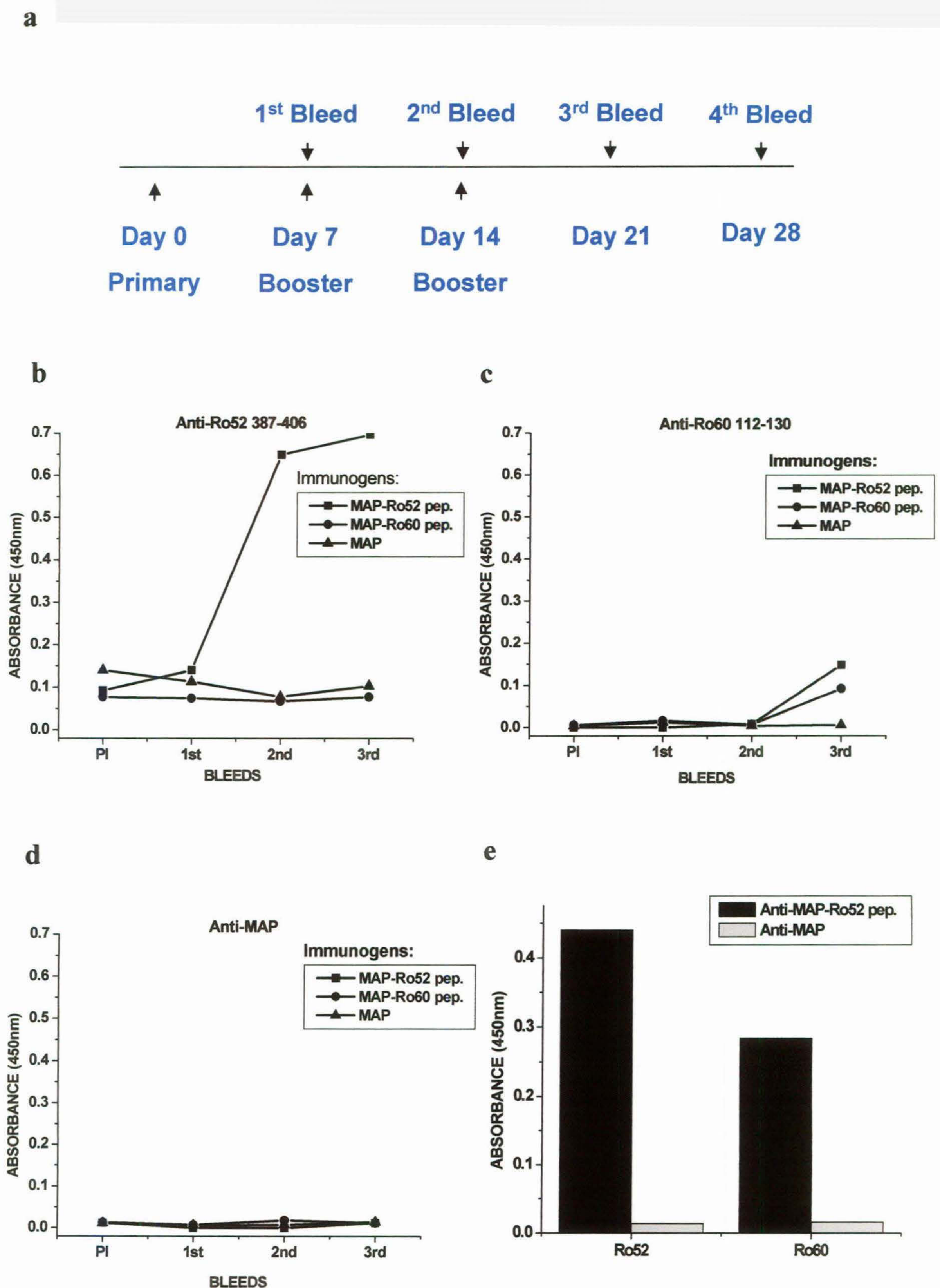


Figure 35: Reactivity of sera generated upon MAP-Ro52 peptide (pep.), MAP-Ro60 pep. and MAP. **(a)** Immunization and blood sampling schedule. **(b)** Reactivity against MAP-Ro52 pep. MAP-Ro52 pep.immunized animals generated reactive antibodies. **(c)** Reactivity against MAP-Ro60 pep. No significant reactivity was seen. **(d)** Reactivity against MAP. No reactivity was observed against the backbone. **(e)** Antibodies generated against Ro52 peptide bound both Ro60 and Ro52, whereas the antibodies generated against the MAP backbone did not (Data from 3rd bleed).

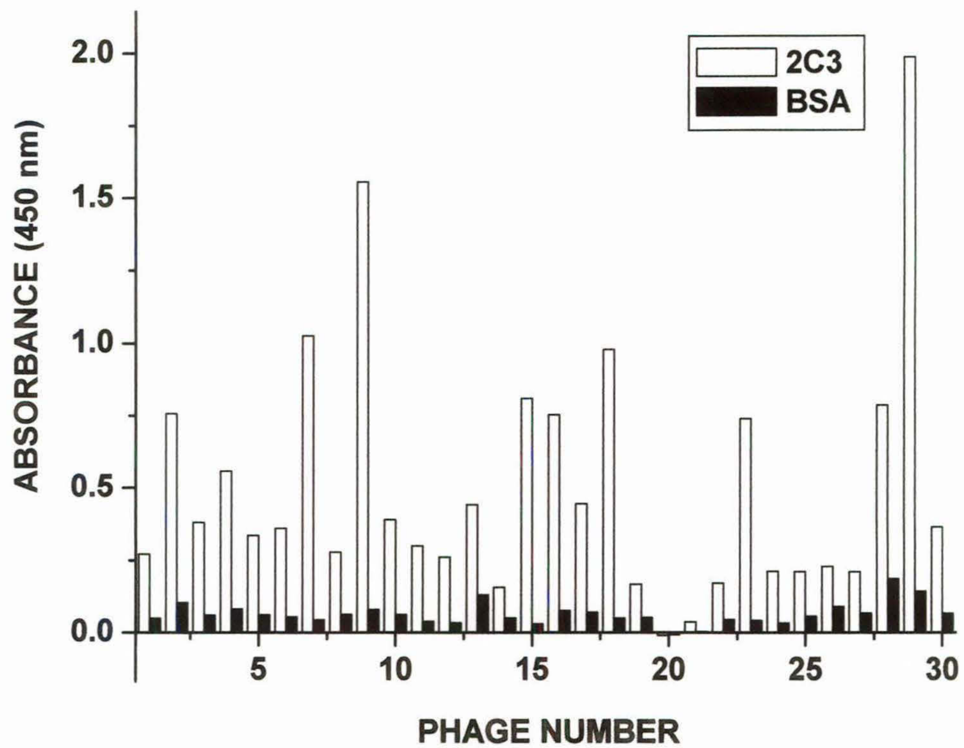


Figure 36: ELISA reactivity of phages (after four sequential rounds of panning and amplification) towards 2C3. All phages (except phages 20 and 21) were reactive. Blank wells blocked with bovine serum albumin (BSA) as negative controls.

wells served as negative controls. Thirty phages were screened, and nineteen ultimately sequenced to identify reactive peptides; Figure 37 depicts the results. Unlike 2C11, 2C3 did not demonstrate a minimal core recognition motif. Instead, 2C3 demonstrated reactivity towards a set of three repeating sequences. The sequences **HSVSNIRPMFPS** was repeated the maximum number of times, and represented the insert in 42% of the total phages sequenced; no homologies to the other identified sequences were apparent (Figure 37). The immunogenecity of these peptides is under assessment; the sera of animals immunized with the peptides in isolation or in combination would be assessed for anti-self reactivity and anti-phospholipid responses.

Genetic Characterization of Antibodies

The heavy and light chain variable region genes were sequenced, as described in the Materials section. Briefly, cDNA was prepared by reverse transcribing mRNA isolated from the hybridomas. PCR amplification was carried out using immunoglobulin-specific primers and PCR products were cloned and sequenced. Comparisons were made to closest germline sequences, Somatic mutations and variations arising due to junctional changes were enumerated.

The antibody 2C11 heavy chain (Figure 38a) belonged to the VH9 family and demonstrated the closest homology with the VH9.15 germline gene. Except for one replacement mutation in FWR4 (a G to T shift), no other somatic mutations were seen in either the complementarity determining regions (CDRs) or framework regions (FWRs). The heavy chain demonstrated two junctional variations, one at V-D (Y to S) and the other at D-J (Y to F). The D segment used in the 2C11 heavy chain was DFL16.1, with a five base deletion (TTTAT) at its 5' terminal; the JH1 segment was employed. The light chain of antibody 2C11 (Figure 38b) also demonstrated a germline configuration, with cp9 being the nearest germline gene. A variation (W to P) at the V-J junction was observed. Jκ1 was employed, with a three base deletion (GTG) at it 5' terminal.

P14	-MDAKNPLGPTTY--	12
P24	-MDAKNPLGPTTY--	12
P3	--HSVSNIRPMFPS-	12
P5	--HSVSNIRPMFPS-	12
P11	--HSVSNIRPMFPS-	12
P15	--HSVSNIRPMFPS-	12
P16	--HSVSNIRPMFPS-	12
P23	--HSVSNIRPMFPS-	12
P30	--HSVSNIRPMFPS-	12
P8	--HSVSNIRPMFPP-	12
P26	--SQNWMPMRVATP-	12
P27	--SQNWMPMRVATP-	12
P12	---DRAPLIPFASQH	12
P13	--SPRPMMRRIRKQ-	12
P6	LLADTTHHRPWT---	12
P22	--TSPASASPRTLH-	12
P18	-ALAEPQLTAILP--	12
P29	--HQAPKHVALLVK-	12
P28	--WGYEHAMTYKAA-	12

HSVSNIRPMFPS

MDAKNPLGPTTY

SQNWMPMRVATP

Figure 37: Alignment of 2C3-reactive peptide sequences, derived after the sequencing of the phage inserts. Of the 19 sequences, 8 sequences were identical. Two additional sets of sequences, each indicated by a different color-code were also identified.

a

```
                 <-----------------------------------------FWR1-----------------------------------
                  Q  I  Q  L  V  Q  S  G  P  E  L  K  K  P  G  E  T  V  K  I  S  C  K  A  S  GY
2C11 HEAVY       CAGATCCAGTTGGTACAGTCTGGACCTGAGCTGAAGAAGCCTGGAGAGACAGTCAAGATCTCCTGCAAGGCTTCTGGGTA
                  Q  I  Q  L  V  Q  S  G  P  E  L  K  K  P  G  E  T  V  K  I  S  C  K  A  S  GY
VH9.15           ..............................................................................

                 ---------> <-----CDR1----> <-------------------FWR2-----------------> <-----------
                  T  F  T    T  Y  G  M  S   W  V  K  Q  A  P  G  K  G  L  K  W  M  G   W  I  N  T
                 TACCTTCACA ACCTATGGAATGAGC TGGGTGAAACAGGCTCCAGGAAAGGGTTTAAAGTGGATGGGC TGGATAAACACCT
                  T  F  T    T  Y  G  M  S   W  V  K  Q  A  P  G  K  G  L  K  W  M  G   W  I  N  T
                 .......... ............... .......................................... .............

                 -----------CDR2---------------------> <----------------------------------------
                  Y  S  G  V  P  T  Y  A  D  D  F  KG   R  F  A  F  S  L  E  T  S  A  S  T  A  Y
                 ACTCTGGAGTGCCAACATATGCTGATGACTTCAAGGGA CGGTTTGCCTTCTCTTTGGAAACCTCTGCCAGCACTGCCTAT
                  Y  S  G  V  P  T  Y  A  D  D  F  KG   R  F  A  F  S  L  E  T  S  A  S  T  A  Y
                 ...................................... ..........................................

                 -----FWR3-------------------------------------------> <---------------CDR3-----
                  L  Q  I  N  N  L  K  N  E  D  T  A  T  Y  F  C  A  R   S  Y  Y  G  S  S  F  Y  W
                 TTGCAGATCAACAACCTCAAAAATGAGGACACGGCTACATATTTCTGTGCAAGA AGCTACTACGGTAGTAGCTTCTACTG
                  L  Q  I  N  N  L  K  N  E  D  T  A  T  Y  F  C  A  R   Y  Y  Y  G  S  S  Y  Y  W
                 ...................................................... ---................-......

                 -----------> <-------------FWR4-------------->
                  Y  F  D  V   W  G  T  G  T  T  V  T  V  S  S
                 GTACTTCGATGTC TGGGGCACAGGGACCACGGTCACCGTCTCCTCA
                  Y  F  D  V   W  G  A  G  T  T  V  T  V  S  S
                 ............. ......G...................T......
```

b

```
                    Y  Y  Y  G  S  S  Y
DFL16.1          TTTATTACTACGGTAGTAGCTAC

                   Y  W  Y  F  D  V  W  G  A  G  T  T  V  T  V  S  S
JH1              CTACTGGTACTTCGATGTCTGGGGCGCAGGGACCACGGTCACCGTCTCCTCAG
```

__Figure 38a__: **(a)** Heavy chain sequence of antibody 2C11. Homologies in the closest germline gene are indicated as dots. Nucleotide or amino acid mutations (or junctional changes) are indicated in **red**. Dashes indicate junctions. **(b)** Germline sequences of the D and J segments employed.

a

```
             <-----------------------------FWR1-----------------------------> <----------
              D  I  Q  M  T  Q  T  T  S  S  L  S  A  S  L  G  D  R  V  T  I  S  C   S  A  S  Q
2C11 LIGHT   GATATCCAGATGACACAGACTACATCCTCCCTGTCTGCCTCTCTGGGAGACAGAGTCACCATCAGTTGC AGTGCAAGTCA
              D  I  Q  M  T  Q  T  T  S  S  L  S  A  S  L  G  D  R  V  T  I  S  C   S  A  S  Q
cp9          ...................................................................... ...........

             ----CDR1------------> <--------------------FWR2-----------------> <--------CDR2
              G  I  S  N  Y  L  N   W  Y  Q  Q  K  P  D  G  T  V  K  L  L  I  Y   Y  T  S  S
             GGGCATTAGCAATTATTTAAAC TGGTATCAGCAGAAACCAGATGGAACTGTTAAACTCCTGATCTAT TACACATCAAGTT
              G  I  S  N  Y  L  N   W  Y  Q  Q  K  P  D  G  T  V  K  L  L  I  Y   Y  T  S  S
             ...................... ............................................. .............

             -------> <--------------------------------------------FWR3--------------------
             L  H  S   G  V  P  S  R  F  S  G  S  G  S  G  T  D  Y  S  L  T  I  S  N  L  E  P
             TACACTCA GGAGTCCCATCAAGGTTCAGTGGCAGTGGGTCTGGGACAGATTATTCTCTCACCATCAGCAACCTGGAACCT
             L  H  S   G  V  P  S  R  F  S  G  S  G  S  G  T  D  Y  S  L  T  I  S  N  L  E  P
             ........ ........................................................................

             ----------------------> <----------CDR3-----------> <------------------FWR4------
              E  D  I  A  T  Y  Y  C   Q  Q  Y  S  K  L  P  P  T   F  G  G  G  T  K  L  E  I  K
             GAAGATATTGCCACTTACTATTGT CAGCAGTATAGTAAGCTTCCTCCGACG TTCGGTGGAGGCACCAAGCTGGAAATCAA
              E  D  I  A  T  Y  Y  C   Q  Q  Y  S  K  L  P  W  T   F  G  G  G  T  K  L  E  I  K
             ........................ ......................--.... .............................

             --->
              R
             ACG

             ...
```

b

```
               W  T  F  G  G  G  T  K  L  E  I  K
Jk1          GTGGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAAC
```

Figure 38b: **(a)** Light chain sequence of antibody 2C11. Homologies in the closest germline gene are indicated as dots. Nucleotide or amino acid mutations (or junctional changes) are indicated in **red**. Dashes indicate junctions. **(b)** Germline sequence of the J segment employed.

The antibody 2H8 heavy chain (Figure 39a) belonged to the VH1 family and showed the highest homology with the J558.67.166 germline gene. No replacement mutations were observed till the end of FWR3; one silent mutation was observed in CDR2 (C to T), and another at the end of FWR3 (A to G). The D segment this antibody was unidentifiable, as only one amino acid (E) was ascribable to this region; the JH3 segment was employed, with possibly an eight base deletion (CCTGGTTT) at its 5' terminal. A mutation in the CDR3 was observed within the J chain (Y to F). For the antibody 2H8 light chain (Figure 39b), the closest germline gene was 19-23 was utilized with possibly an eight base deletion (GCTATCCT) at its 3' terminal. Essentially, the germline configuration was maintained; one replacement mutation (T to P) was observed in the FWR3. Jκ5 was utilized. Three non-encoded amino acids (N, F and P) were added at the V-J joining region.

The antibody 1B4 heavy chain (Figure 40a) was a member of the VH1 family and was most homologous to the J558.84.190 germline gene. The sequence demonstrated replacement mutations in the CDR1 (S to T), FWR2 (K to R), CDR2 (G to A). Two non-encoded amino acids (I and S) were added at the V-D junction, The D segment used was DSP2.2 and the entire segment was utilized, and was fully conserved. Three non-coded G residues were observed at the D-J junction. The JH4 segment was employed with a five nucleotide deletion (ATTAC) at its 5' terminal. The antibody 1B4 light chain (Figure 40b) demonstrated the closest homology with the 23-48 germline gene. The FWR2 region contained a silent mutation. The Jκ4 segment was employed.

The heavy chain of antibody 2C3 (Figure 41a) belonged to the VH5 family, with VH7183.3b being the closest germline gene, with a single base missing at its 3' terminal (Figure 41a). This antibody demonstrated extensive replacement somatic mutations. While one mutation was observed in CDR1 (T to A), four were found in CDR2 (T to Y, G to S, G to R and N to S). FRW2, FRW3 and FRW4 all contained single mutations. The DSP2.5 segment was employed, with three bases (TCT) deleted from its 3' end. The V-J junction contained four non-encoded amino acids (R, G, R, R). The JH4 segment was

a

```
                 <----------------------------------------FWR1----------------------------------
                  Q  V  Q  L  Q  Q  P  G  A  E  L  V  K  P  G  A  S  V  K  L  S  C  K  A  S  G  Y
2H8 HEAVY        CAGGTCCAACTGCAGCAGCCTGGGGCTGAGCTGGTAAAGCCTGGGGCTTCAGTGAAGTTGTCCTGCAAGGCTTCTGGCTA
                  Q  V  Q  L  Q  Q  P  G  A  E  L  V  K  P  G  A  S  V  K  L  S  C  K  A  S  G  Y
J558.67.166      ................................................................................

                 ---------> <-----CDR1----> <-------------------FWR2-----------------> <-----------
                  T  F  T    S  Y  W  M  H   W  V  K  Q  R  P  G  Q  G  L  E  W  I  G   M  I  H  P
                 CACTTTCACC AGCTACTGGATGCAC TGGGTGAAGCAGAGGCCTGGACAAGGCCTTGAGTGGATTGGA ATGATTCATCCTA
                  T  F  T    S  Y  W  M  H   W  V  K  Q  R  P  G  Q  G  L  E  W  I  G   M  I  H  P
                 .......... ............... ......................................... .............

                 -----------CDR2----------------------> <-----------------------------------------
                 N  S  G  S  T  N  Y  N  E  K  F  K  S   K  A  T  L  T  V  D  K  S  S  S  T  A  Y
                 ATAGTGGTAGTACTAACTATAATGAGAAGTTCAAGAGC AAGGCCACACTGACTGTAGACAAATCCTCCAGCACAGCCTAC
                 N  S  G  S  T  N  Y  N  E  K  F  K  S   K  A  T  L  T  V  D  K  S  S  S  T  A  Y
                 ...................C.................. ..........................................

                 -----FWR3-----------------------------------------> <--CDR3-> <----------FWR4--
                  M  Q  L  S  S  L  T  S  E  D  S  A  V  Y  Y  C  A  R   E  A  F   W  G  Q  G  T  L
                 ATGCAACTCAGCAGCCTGACATCTGAGGACTCTGCGGTCTATTACTGTGCGAGA GAGGCCTTC TGGGGCCAAGGGACTCT
                  M  Q  L  S  S  L  T  S  E  D  S  A  V  Y  Y  C  A  R      A  Y   W  G  Q  G  T  L
                 ...................................................A... ---..T.A. .................

                 --------------->
                  V  T  V  S  A
                 GGTCACTGTCTCTGC
                  V  T  V  S  A
                 ...............
```

b

```
D                SEGMENT UNIDENTIFIABLE (E)

                   W  F  A  Y  W  G  Q  G  T  L  V  T  V  S  A
JH3              CCTGGTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCAG
```

Figure 39a: **(a)** Heavy chain sequence of antibody 2H8. Homologies in the closest germline gene are indicated as dots. Nucleotide or amino acid mutations (or junctional changes) are indicated in **red**. Dashes indicate junctions. **(b)** Germline sequences of the D and J segments employed.

a

```
            <-----------------------------FWR1----------------------------> <---------
             D  I  V  M  T  Q  S  H  K  F  M  S  T  S  V  G  D  R  V  S  I  T  C   K  A  S  Q
2H8 LIGHT   GACATTGTGATGACCCAGTCTCACAAATTCATGTCCACATCAGTAGGAGACAGGGTCAGCATCACCTGC AAGGCCAGTCA
             D  I  V  M  T  Q  S  H  K  F  M  S  T  S  V  G  D  R  V  S  I  T  C   K  A  S  Q
19-23       ..................................................................... ...........

            ----CDR1-------------> <-------------------FWR2-----------------> <--------CDR2
             D  V  G  T  A  V  A   W  Y  Q  Q  K  P  G  Q  S  P  K  L  L  I  Y   W  A  S  T
            GGATGTGGGTACTGCTGTAGCC TGGTATCAACAGAAACCAGGGCAATCTCCTAAATTATTGATTTAC TGGGCATCCACCC
             D  V  G  T  A  V  A   W  Y  Q  Q  K  P  G  Q  S  P  K  L  L  I  Y   W  A  S  T
            ...................... ..................................C..C....... .............

            -------> <---------------------------------------------FWR3--------------------
            R  H  T   G  V  P  D  R  F  T  G  S  G  S  G  T  D  F  P  L  T  I  S  N  V  Q  S
            GGCACACT GGAGTCCCTGATCGCTTCACAGGCAGTGGATCTGGGACAGATTTCCCTCTCACCATTAGCAATGTGCAGTCT
            R  H  T   G  V  P  D  R  F  T  G  S  G  S  G  T  D  F  T  L  T  I  S  N  V  Q  S
            ........ .............................................A..........................

            -----------------------> <-----------CDR3---------> <-------------FWR4------------>
             E  D  L  A  D  Y  F  C   Q  Q  Y  S  N  F  P  L  T   F  G  A  G  T  K  L  E  L  K
            GAAGACTTGGCAGATTATTTCTGT CAGCAATATAGCAATTTTCCGCTCACG TTCGGTGCTGGGACCAAGCTGGAGCTGAAAC
             E  D  L  A  D  Y  F  C   Q  Q  Y  S           L  T   F  G  A  G  T  K  L  E  L  K
            ........................ .............-------....... ...............................
```

b

```
             L  T  F  G  A  G  T  K  L  E  L  K
JK5         GCTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAAC
```

Figure 39b: **(a)** Light chain sequence of antibody 2H8. Homologies in the closest germline gene are indicated as dots. Nucleotide or amino acid mutations (or junctional changes) are indicated in **red**. Dashes indicate junctions. **(b)** Germline sequence of the J segment employed.

a

```
             <----------------------------------------FWR1---------------------------------
              Q  V  Q  L  Q  Q  S  G  A  E  L  A  R  P  G  A  S  V  K  L  S  C  K  A  S  G  Y
1B4 HEAVY    CAGGTTCAGCTGCAGCAGTCTGGAGCTGAGCTGGCGAGGCCTGGGGCTTCAGTGAAGCTGTCCTGCAAGGCTTCTGGCTA
              Q  V  Q  L  Q  Q  S  G  A  E  L  A  R  P  G  A  S  V  K  L  S  C  K  A  S  G  Y
J558.84.190  .................................................................................

             ----------> <-----CDR1----> <------------------FWR2-----------------> <-----------
              T  F  T    T  Y  G  I  S    W  V  R  Q  R  T  G  Q  G  L  E  W  I  G    E  I  Y  P
             CACCTTCACA ACTTATGGTATAAGC TGGGTGAGGCAGAGAACTGGACAGGGCCTTGAGTGGATTGGA GAGATTTATCCT
              T  F  T    S  Y  G  I  S    W  V  K  Q  R  T  G  Q  G  L  E  W  I  G    E  I  Y  P
             .......... .GC............ ......A................................... ............

             ------------CDR2----------------------> <-----------------------------------------
              R  S  A  N  T  Y  Y  N  E  K  F  K  G   K  A  T  L  T  A  D  K  S  S  S  T  A  Y
             AGAAGTGCTAATACTTACTACAATGAGAAGTTCAAGGGC AAGGCCACACTGACTGCAGACAAATCCTCCAGCACAGCGTA
              R  S  G  N  T  Y  Y  N  E  K  F  K  G   K  A  T  L  T  A  D  K  S  S  S  T  A  Y
             .......G............................... .........................................

             ------FWR3-------------------------------------------> <----------------CDR3----
              M  E  L  R  S  L  T  S  E  D  S  A  V  Y  F  C  A  R   I  S  Y  Y  D  Y  D  G
             CATGGAGCTCCGCAGCCTGACATCTGAGGACTCTGCGGTCTATTTCTGTGCAAGA ATCTCCTACTATGATTACGACGGAG
              M  E  L  R  S  L  T  S  E  D  S  A  V  Y  F  C  A  R
             ....................................................... -----................----

             -------------------> <---------------FWR4------------->
              G  G  Y  A  M  D  Y   W  G  Q  G  T  S  V  T  V  S  S
             GTGGCTATGCTATGGACTAC TGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA
                    Y  A  M  D  Y   W  G  Q  G  T  S  V  T  V  S  S
             ----................ .................................
```

b

```
               Y  Y  D  Y D
DSP2.2       TCTACTATGATTACGAC

                Y  Y  A  M  D  Y  W  G  Q  G  T  S  V  T  V  S  S
JH4          ATTACTATGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAG
```

<u>Figure 40a</u>: **(a)** Heavy chain sequence of antibody 1B4. Homologies in the closest germline gene are indicated as dots. Nucleotide or amino acid mutations (or junctional changes) are indicated in **red**. Dashes indicate junctions. **(b)** Germline sequences of the D and J segments employed.

a

```
              <------------------------------FWR1------------------------------> <---------
               D  I  L  L  T  Q  S  P  A  I  L  S  V  S  P  G  E  R  V  S  F  S  C   R  A  S  Q
1B4 LIGHT     GACATCTTGCTGACTCAGTCTCCAGCCATCCTGTCTGTGAGTCCAGGAGAAAGAGTCAGTTTCTCCTGC AGGGCCAGTCA
               D  I  L  L  T  Q  S  P  A  I  L  S  V  S  P  G  E  R  V  S  F  S  C   R  A  S  Q
23-48         ..................................................................... ...........

              ----CDR1--------------> <--------------------FWR2------------------> <--------CDR
                 S  I  G  T  S  I  H   W  Y  Q  Q  R  T  N  G  S  P  R  L  L  I  K   Y  A  S  E
              GAGCATTGGCACAAGCATACAC TGGTACCAGCAAAGAACAAATGGTTCTCCAAGGCTTCTCATAAAG TATGCTTCTGAG
                 S  I  G  T  S  I  H   W  Y  Q  Q  R  T  N  G  S  P  R  L  L  I  K   Y  A  S  E
              ...................... .....T....................................... ............

              2-------> <-------------------------------------------------FWR3------------------
               S  I  S   G  I  P  S  N  G  S  P  R  L  L  I  K  Y  A  S  E  S  I  S  G  I  P  S
              TCTATCTCT GGGATCCCTTCAAATGGTTCTCCAAGGCTTCTCATAAAGTATGCTTCTGAGTCTATCTCTGGGATCCCTTC
               S  I  S   G  I  P  S  N  G  S  P  R  L  L  I  K  Y  A  S  E  S  I  S  G  I  P  S
              ......... .........................................................................

              ------------------------> <---------CDR3----------> <-------------FWR4-----------
                E  D  I  A  D  Y  Y  C   Q  Q  S  N  S  W  P  F  T   F  G  S  G  T  K  L  E  I
              TGAAGATATTGCAGATTATTACTGT CAACAAAGTAATAGCTGGCCATTCAC GTTCGGCTCGGGGACAAAGTTGGAAATAA
                E  D  I  A  D  Y  Y  C   Q  Q  S  N  S  W  P  F  T   F  G  S  G  T  K  L  E  I
              ......................... ........................... .............................

              -->
              K
              AAC
              K
              ...
```

b

```
                 F  T  F  G  S  G  T  K  L  E  I  K
JK4           ATTCACGTTCGGCTCGGGGACAAAGTTGGAAATAAAAC
```

Figure 40b: **(a)** Light chain sequence of antibody 1B4. Homologies in the closest germline gene are indicated as dots. The nucleotide mutation is indicated in **red**. **(b)** Germline sequence of the J segment employed.

a

```
             <----------------------------------------FWR1--------------------------------
              E  V  K  L  V  E  S  G  G  G  L  V  K  P  G  G  S  L  K  L  S  C  A  A  S  G  F
2C3 HEAVY    GAAGTGAAGCTGGTGGAGTCTGGGGGAGGCTTAGTGAAACCTGGAGGGTCCCTGAAACTCTCCTGTGCAGCCTCTGGATT
              E  V  K  L  V  E  S  G  G  G  L  V  K  P  G  G  S  L  K  L  S  C  A  A  S  G  F
VH7183.3b    ......................................G.........................................

             ---------> <-----CDR1----> <-------------------FWR2-----------------> <------------
              T  F  S    S  Y  A  M  S    W  V  R  Q  T  P  A  K  R  L  E  W  V  A   Y  I  S  S
             CACTTTCAGT AGCTATGCCATGTCT TGGGTTCGCCAGACTCCGGCGAAGAGGCTGGAGTGGGTCGCA TACATTAGTAGTC
              T  F  S    S  Y  T  M  S    W  V  R  Q  T  P  E  K  R  L  E  W  V  A   T  I  S  G
             .......... ......A........ ...................A...................... AC.......G..G

             -----------CDR2---------------------> <----------------------------------------
             R  G  G  S  T  Y  Y  P  D  S  V  K  G    R  F  T  I  S  R  D  N  A  K  N  T  L  Y
             GTGGTGGTAGCACCTACTATCCAGACAGTGTAAAGGGC CGATTCACCATCTCCAGAGACAATGCCAAGAACACCCTGTAC
             G  G  G  N  T  Y  Y  P  D  S  V  K  G    R  F  T  I  S  R  D  N  A  K  N  T  L  Y
             .........A.....................G.....T ..........................................

             -----FWR3---------------------------------------------> <----------CDR3--------> <-
              L  L  M  S  S  L  R  S  E  D  T  A  M  Y  Y  C  A  R   R  G  R  R  T  M  D  Y   W
             CTGCTAATGAGCAGTCTGAGGTCTGAGGACACGGCCATGTATTACTGTGCAAGA AGGGGACGGAGGACTATGGACTAC TG
              L  Q  M  S  S  L  R  S  E  D  T  A  M  Y  Y  C  A  R               T  M  D  Y   W
             ....A................................................. ------------............ ..

             -------------FWR4------------>
              G  Q  G  T  S  G  T  V  S  S
             GGGTCAGGGAACCTCGGGCACCGTCTCCTCA
              G  Q  G  T  S  V  T  V  S  S
             ......A........A.T.............
```

b

```
              S  T  M  V  T
DSP2.5       TCTACTATGGTAACTAC

                Y  Y  A  M  D  Y  W  G  Q  G  T  S  V  T  V  S  S
JH4-01       ATTACTATGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAG
```

Figure 41a: **(a)** Heavy chain sequence of antibody 2C3. Homologies in the closest germline gene are indicated as dots. Nucleotide or amino acid mutations (or junctional changes) are indicated in **red**. Dashes indicate junctions. **(b)** Germline sequences of the D and J segments employed.

a

```
                 <-----------------------------FWR1-----------------------------> <---------
                     V  M  T  Q  T  P  L  S  L  S  V  S  L  G  D  Q  A  S  I  S  C   R  S  S  Q
2C3 LIGHT        ----CTGTGATGACCCAAACTCCACTCTCCCTGTCTGTCAGTCTTGGAGATCAAGCCTCCATCTCTTGC AGGTCTAGTCA
                  D  A  V  M  T  Q  T  P  L  S  L  P  V  S  L  G  D  Q  A  S  I  S  C   R  S  S  Q
bl1              GATG.........................C................................ ...........

                 -----------CDR1---------------------> <---------------------FWR2---------------
                   S  L  E  N  S  N  G  N  T  Y  L  N   W  Y  L  Q  K  P  G  Q  S  P  Q  L  L  I
                 GAGCCTTGAAAACAGTAATGGAAACACCTATTTGAAT TGGTACCTCCAGAAACCAGGCCAGTCTCCACAGCTCCTGATC
                   S  L  E  N  S  N  G  N  T  Y  L  N   W  Y  L  Q  K  P  G  Q  S  P  Q  L  L  I
                 ....................................C .........................................

                 --> <--------CDR2-------> <---------------------------------------------FWR3-----
                  Y   R  A  S  N  R  F  S   G  V  L  D  R  I  S  G  S  G  S  G  T  D  F  T  L  K  I
                 TAC AGGGCTTCCAACCGATTTTCT GGGGTCCTAGACAGGATCAGTGGTAGTGGATCAGGGACAGATTTTACACTGAAAAT
                  Y   R  V  S  N  R  F  S   G  V  L  D  R  F  S  G  S  G  S  G  T  D  F  T  L  K  I
                 ... ....T.............. ...............T.........................C..........

                 ---------------------------------------> <----------CDR3------------> <----------
                   S  R  V  E  A  E  D  L  G  V  Y  F  C   L  Q  V  S  H  V  P  Y  T   F  G  G  G
                 TAGCAGAGTGGAGGCTGAGGATTTGGGAGTTTATTTCTGC CTCCAAGTTTCACATGTCCCGTACACGT TCGGAGGGGGGA
                   S  R  V  E  A  E  D  L  G  V  Y  F  C   L  Q  V  T  H  V  P  Y  T   F  G  G  G
                 C....................................... .........A.........-....... ............

                 -FWR4------------->
                 T  K  L  E  I  K  R
                 CCAAGCTGGAAATAAAACG
                 T  K  L  E  I  K
                 ...................
```

b

```
                  Y  T  F  G  G  G  T  K  L  E  I  K
JK2              TACACGTTCGGAGGGGGGACCAAGCTGGAAATAAAACGT
```

Figure 41b: **(a)** Light chain sequence of antibody 2C3. Homologies in the closest germline gene are indicated as dots. Nucleotide or amino acid mutations (or junctional changes) are indicated in **red**. Dashes indicate junctions. **(b)** Germline sequence of the J segment employed.

used, with fourteen bases (ATTACTATGCTATG) deleted from its 5' end. The light chain (Figure 41b) was most homologous to the bl1 germline gene. Only two replacement mutations were observed in the CDR regions; V to A in CDR2 and T to S in CDR3. The Jκ2 segment was used in its entirety

The antibody 1B3 heavy chain (Figure 42a) demonstrated extensive mutations both in the CDRs and FWRs, with a preponderance of mutations in CDR2 and CDR3. The gene belonged to the VH1 family, with the nearest germline gene being J558.26.116. The DSP2.2 segment was employed, with a thirteen base deletion (TCTACTATG) at its 3' end. Two non-encoded amino acids (R, E) occurred at the V-J junction. JH4 was employed, and exhibited several mutations. The light chain (Figure 42b) demonstrated the closest homology towards the ba9 germline gene. This sequence too demonstrated the presence of mutations in the CDRs as well as the FWRs. While two replacements to K residues (from N and S) occurred in CDR1, a mutation from A to T was found in CDR2. The Jκ2 segment was employed, possibly with a two base (TG) deletion at its 5' end.

The heavy chain of antibody 1B1 (Figure 43a) belonged to the VH5 family and demonstrated closest homology with the VH7183.10 germline gene. Mutations were distributed over the CDR and FRW regions. CDR1 contained a single change (Y to A), while CDR2 exhibited two changes (Y to N and G to D). Except for a stretch of non-encoded amino acids, the CDR3 contained no mutations. The D region was unidentifiable (possible due to extensive mutations), and therefore a stretch of six amino acids is listed as arising due to non-encoded additions in the Figure. The JH2 segment was employed, possibly with a two base deletion (AC) at its 5' end. The light chain of antibody 1B1 (Figure 43b) exhibited closest homology to the 8-19 germline gene and did not manifest many mutations; one replacement mutation was observed in CDR2 (E to K) and one in the FWR3 (T to A). Jκ2 was employed, possibly with a single base deletion (T) at its 5' end.

a

```
            <----------------------------------------FWR1-------------------------------
             E  V  Q  L  Q  Q  S  G  P  E  L  V  K  P  G  T  S  V  E  I  S  C  K  A  S  G  F
1B3 HEAVY    GAGGTCCAGTTGCAACAATCTGGACCTGAACTGGTGAAGCCTGGGACTTCAGTTGAAATATCCTGTAAGGCTTCTGGATT
             E  V  Q  L  Q  Q  S  G  P  E  L  V  K  P  G  A  S  V  K  I  S  C  K  A  S  G  Y
J558.26.116 .........C..................G...............G.......GA.G.....................A

             ---------> <-----CDR1----> <-------------------FWR2-----------------> <-----------
              T  F  I    D  Y  Y  I  N   W  V  K  Q  S  H  E  K  N  L  E  W  I  G   D  I  Y  P
             CACGTTCATT GACTACTACATAAAC TGGGTGAAGCAGAGCCATGAAAAGAACCTTGAATGGATAGGA GATATTTATCCTA
              T  F  T    D  Y  Y  M  N   W  V  K  Q  S  H  G  K  S  L  E  W  I  G   D  I  N  P
             ........C. ...........G... ...................G.....G......G.....T... ......A......

             -----------CDR2---------------------> <-----------------------------------------
             N  N  G  D  A  D  Y  N  Q  K  F  R  G   K  A  T  L  T  V  D  K  S  S  G  T  A  Y
             ACAATGGTGATGCTGATTACAACCAGAAGTTCAGGGGC  AAGGCCACATTGACTGTTGACAAGTCTTCCGGCACAGCCTAC
             N  N  G  G  T  S  Y  N  Q  K  F  K  G   K  A  T  L  T  V  D  K  S  S  S  T  A  Y
             .........G.A..AGC................A....  .................A........C...A..........

             -----FWR3--------------------------------------------> <-------------CDR3-------
              M  M  L  H  S  L  T  S  E  D  S  A  V  Y  Y  C  A  R   R  E  L  R  R  F  Y  S  L
             ATGATGCTCCACAGCCTGACATCTGAGGACTCTGCGGTCTATTACTGTGCAAGG AGGGAATTACGACGTTTTTATTCTTT
              M  E  L  R  S  L  T  S  E  D  S  A  V  Y  Y  C  A            L  R  H  Y  Y  A  M
             ...GA.....G.....................A.................A -----........A..AC...G..A.

             ------> <------------FWR4------------->
              D  Y    W  G  Q  G  T  S  V  T  V  S
             GGACTAC TGGGGTCAAGGAACCTCTGTCACCGTCTCCT
              D  Y    W  G  Q  G  T  S  V  T  V  S
             ....... .................A.............
```

b

```
              L  L  *  L  R
DSP2.2       TCTACTATGATTACGAC

                Y  Y  A  M  D  Y  W  G  Q  G  T  S  V  T  V  S  S
JH4          ATTACTATGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAG
```

Figure 42a: **(a)** Heavy chain sequence of antibody 1B3. Homologies in the closest germline gene are indicated as dots. Nucleotide or amino acid mutations (or junctional changes) are indicated in **red**. Dashes indicate junctions. **(b)** Germline sequences of the D and J segments employed.

a

```
               <--------------------------------FWR1------------------------------> <--------
                D  I  Q  M  T  Q  S  P  S  S  I  Y  T  S  L  G  E  R  V  T  I  T  C   K  A  S
1B3 LIGHT      GACATCCAGATGACCCAGTCTCCATCCTCCATATATACATCTCTAGGAGAGAGAGTCACTATCACTTGC AAGGCGAGTC
                D  I  K  M  T  Q  S  P  S  S  M  Y  A  S  L  G  E  R  V  T  I  T  C   K  A  S
ba9            ......A..................T.....G...G.............................  ..........

               -----CDR1-------------> <--------------------FWR2------------------> <--------CDR
               Q  D  I  K  K  Y  L  S   W  F  Q  Q  K  P  G  N  S  P  K  T  L  I  Y   R  T  N  R
               AGGACATTAAAAAATATTTAAGT TGGTTCCAGCAGAAACCAGGAAATTCTCCTAAGACCCTGATCTAT CGTACAAATAGA
               Q  D  I  N  S  Y  L  S   W  F  Q  Q  K  P  G  K  S  P  K  T  L  I  Y   R  A  N  R
               ..........T.GC.......C ..................G..A.................... ...G....C...

               2-------> <---------------------------------------------FWR3-------------------
                L  V  D   G  V  P  S  R  F  S  G  S  G  S  G  P  D  Y  S  L  T  I  I  S  L  D  Y
               TTGGTAGAT GGGGTCCCATCAAGGTTCAGTGGCAGTGGATCTGGGCCAGATTATTCTCTCACCATCATCAGCCTGGACTA
                L  V  D   G  V  P  S  R  F  S  G  S  G  S  G  Q  D  Y  S  L  T  I  S  S  L  E  Y
               ......... ................................A..................G.........G..

               ------------------------> <-------------CDR3--------> <--------------FWR4---------
                E  D  M  G  I  Y  Y  C   L  Q  Y  D  E  L  P  Y  T   F  G  G  G  T  K  L  E  I
               TGAAGATATGGGAATTTATTACTGT TTACAGTATGATGAACTTCCGTACACG TTCGGAGGGGGGACCAAGCTAGAAATAA
                E  D  M  G  I  Y  Y  C   L  Q  Y  D  E  L  P  Y  T   F  G  G  G  T  K  L  E  I
               ....................T... C............GT....T......  ...................G.......

               ---->
               K R
               AACG
               K
               ....
```

b

```
                 Y  T  F  G  G  G  T  K  L  E  I  K
JK2            TGTACACGTTCGGAGGGGGGACCAAGCTGGAAATAAAAC
```

Figure 42b: **(a)** Light chain sequence of antibody 1B3. Homologies in the closest germline gene are indicated as dots. Nucleotide or amino acid mutations are indicated in **red**. **(b)** Germline sequence of the J segment employed.

a

```
                 <------------------------------------------FWR1-------------------------------
                           L  V  E  S  G  G  G  L  V  K  P  G  G  S  L  K  L  S  C  A  A  S  G  F
1B1 HEAVY        -------AGCTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTCCCTGAAACTCTCCTGTGCAGCCTCTGGATT
                  D  V  K  L  V  E  S  G  G  G  L  V  K  L  G  G  S  L  K  L  S  C  A  A  S  G  F
VH7183.10        GACGTGA....C.........................T....................................

                 ---------> <-----CDR1----> <-------------------FWR2----------------> <-----------
                  T  F  S   S  Y  A  M  S   W  V  R  Q  T  P  E  K  R  L  E  W  V  A   A  I  N  S
                 TACTTTCAGT AGCTATGCCATGTCT TGGGTTCGCCAGACTCCAGAGAAGAGGCTGGAGTGGGTCGCA GCCATTAATAGTA
                  T  F  S   S  Y  Y  M  S   W  V  R  Q  T  P  E  K  R  L  E  L  V  A   A  I  N  S
                 C......... ......TA...... .................................T...... ............

                 --------------CDR2----------------------> <------------------------------------
                 N  G  G  S  T  Y  N  P  D  T  V  K  D   R  F  T  I  S  R  D  S  A  K  N  T  L
                 ATGGTGGTAGCACCTACAATCCAGACACTGTGAAGGAC CGATTCACCATCTCCAGAGACAGTGCCAAGAACACCCTG
                 N  G  G  S  T  Y  Y  P  D  T  V  K  G   R  F  T  I  S  R  D  N  A  K  N  T  L
                 ................T.................G. ......................A...............

                 --------FWR3--------------------------------------------> <---------------CDR3---
                  Y  L  Q  M  S  S  L  R  S  E  D  T  A  L  Y  Y  C  A  R   R  A  K  N  V  R  Y  F
                 TACCTGCAAATGAGTAGTCTGAGGTCTGAGGACACAGCCTTGTATTACTGTGCAAGA CGAGCGAAAAACGTCCGGTACTT
                  Y  L  Q  M  S  S  L  K  S  E  D  T  A  L  Y  Y  C  A  R
                 ..............C.......A................................. .-----------------.....

                 ------> <-------------FWR4-------------->
                  D  Y   W  G  Q  G  T  T  L  T  V  S  S
                 TGACTAC TGGGGCCAAGGCACCACTCTCACAGTCTCCTCA

                 ....... .................................
```

b

```
                                    R  A  K  N  V  R
D SEGMENT        UNIDENTIFIABLE    CGAGCGAAAAACGTCCGG

                    Y  F  D  Y  W  G  Q  G  T  T  L  T  V  S  S
JH2              ACTACTTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCAG
```

Figure 43a: **(a)** Heavy chain sequence of antibody 1B1. Homologies in the closest germline gene are indicated as dots. Nucleotide or amino acid mutations (or junctional changes) are indicated in **red**. Dashes indicate junctions. **(b)** Germline sequences of the D and J segments employed.

a

```
                <------------------------------FWR1------------------------------> <---------
                 D  I  V  M  T  Q  S  P  S  S  L  T  V  T  A  G  E  K  V  T  M  S  C   K  S  S  Q
1B1 LIGHT       GACATTGTGATGACACAGTCTCCATCCTCCCTGACTGTGACAGCAGGAGAGAAGGTCACTATGAGCTGC AAGTCCAGTCA
                 D  I  V  M  T  Q  S  P  S  S  L  T  V  T  A  G  E  K  V  T  M  S  C   K  S  S  Q
8-19            ..................................................................... ...........

                ------------CDR1---------------------> <--------------------FWR2-------------
                  S  L  L  N  S  G  N  Q  K  N  Y  L  T   W  Y  Q  Q  K  P  G  Q  P  P  K  L  L
                GAGTCTGTTAAACAGTGGAAATCAAAAGAACTACTTGACC TGGTACCAGCAGAAACCAGGGCAGCCTCCTAAACTGTTGA
                  S  L  L  N  S  G  N  Q  K  N  Y  L  T   W  Y  Q  Q  K  P  G  Q  P  P  K  L  L
                ........................................ .......................................

                ----> <--------CDR2-------> <----------------------------------------------FW
                I  Y   W  A  S  T  R  K  S   G  V  P  D  R  F  T  G  S  G  S  G  A  D  F  T
                TCTAC TGGGCATCCACTAGGAAATCT GGGGTCCCTGATCGCTTCACAGGCAGTGGATCTGGAGCAGATTTCACTC
                I  Y   W  A  S  T  R  E  S   G  V  P  D  R  F  T  G  S  G  S  G  T  D  F  T
                ..... ...............G..... ....................................A............

                R3--------------------------------------------> <-----------CDR3----------> <----
                L  T  I  S  S  V  Q  A  E  D  L  A  V  Y  Y  C   Q  N  D  Y  S  Y  P  Y  T   F  G
                TCACCATCAGCAGTGTGCAGGCTGAAGACCTGGCAGTTTATTACTGT CAGAATGATTATAGTTATCCGTACACGT TCGGA
                L  T  I  S  S  V  Q  A  E  D  L  A  V  Y  Y  C   Q  N  D  Y  S  Y  P  Y  T   F  G
                ............................................... ............................ .....

                ----------FWR4------------>
                 G  G  T  K  L  E  I  K  R
                GGGGGGACCAAGCTGGAAATAAAACG
                 G  G  T  K  L  E  I  K  R
                ..........................
```

b

```
                   Y  T  F  G  G  G  T  K  L  E  I  K
JK2             TGTACACGTTCGGAGGGGGGACCAAGCTGGAAATAAAAC
```

<u>Figure 43b</u>: **(a)** Light chain sequence of antibody 1B1. Homologies in the closest germline gene are indicated as dots. Nucleotide or amino acid mutations are indicated in **red**. **(b)** Germline sequence of the J segment employed.

	Heavy Chain	Junctional Additions	Light Chain	Junctional Additions
2C11	VH9.15 DFL 16.1 JH1	2	cp9 Jκ1	1
1B4	J558.84.190 DSP2.2 JH4	5	23-48 Jκ4	0
2H8	J558.67.166 U.I. JH3	1	19-23 Jκ5	3
2C3	VH7183.3b DSP2.5 JH4	4	bl1 Jκ2	0
1B3	J558.26.116 DSP2.2 JH4	2	ba9 Jκ2	0
1B1	VH7183.10 U.I. JH2	6	8-19 Jκ2	0

Table 3: Description of antibody germline gene usage (listed as V, D and J regions for Heavy Chains and V, J regions for Light Chains) and the number of junctional variations. U.I. : Unidentifiable.

Table 3 summarizes data on the germline segments employed by the antibodies, as well indicates the number of junctional additions (at V-D and at D-J for heavy chains and at V-J for light chains) observed. A more detailed analysis of the antibody genes and the relative frequencies of somatic mutations is included in the Discussion.

Discussion

DISCUSSION

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disorder characterized by the presence of antibodies to various self antigens and by multi-organ pathology. Targeted organs include the kidneys, the brain, the lungs, the skin and the blood.

SLE is the only autoimmune disorder in which antibodies to dsDNA are found. Anti-dsDNA reactivity has been implicated in renal damage and glomereulonephritis (Madaio et al 1987; Vlahakos et al 1992; Suzuki et al 1993). Often, kidney damage is the cause of death in SLE. Antibodies to various phospholipids are also found and are associated with thrombotic events and with recurrent spontaneous abortion (Shoenfeld 2003; Blank et al 1991). Various ribonucleoproteins are targeted, with anti-Ro, anti-La and anti-Sm being the prominent specificities. Anti-Ro and anti-La antibodies have been implicated in neonatal lupus and congenital heart block (Julkunen et al 1998; Tran et al 2002). Antibodies to the Ribosomal P Protein have been linked to neuropsychaitric lupus erythematosus (NPSLE; Reichlin 2006). It is thought that the disease initiates with antibodies against a very few specificities, and subsequent reactivities become apparent in the course of time (Arbuckle et al 2003). Only when all the specificities are evident is fulminant disease expressed. The etiology of most autoimmune reactivities in SLE is unknown. Many hypotheses have been put forth, encompassing various phenomena like molecular mimicry, polyclonal activation of cells, epitope spreading and elicitation of the idiotypic network.

Systemic and excessive exposure of self-antigens might also contribute to the initiation and/or progression of disease. A recent hypothesis concerning the breakage of tolerance implicates faulty apoptotic mechanisms. Apoptosis is a centrally involved in organismal development. The sculpting of organs, the development of immune system and the clearance of virally infected or mutated cells are all critically dependent on the efficient elimination and clearance of unwanted or harmful cells. Apoptotic cells express certain surface molecules that mediate recognition and clearance by professional phagocytes.

One of the best characterized markers on apoptotic cells is phosphatidylserine (PS) which, when bound by the phosphotidylserine receptor on phagocytes, mediates ingestion. PS can also bind the $\alpha_5\beta_3$ integrin and the membrane-associated kinase mer via the bridging molecules MFG-E8 (Hanayama et al 2002) and Gas 6 (Nakano et al 1997) respectively. Other receptors like CD14 and CD36 aid in tethering the apoptotic cells to the phagocyte. Normally, phagocytic uptake of dying cells is efficient and specific, and is immunologically quiescent (Surh et al 1994); the anti-inflammatory cytokine TGF-β is secreted by phagocytes on uptake or contact with apoptotic cells (Fadok et al 1998a). Uptake of apoptotic cells can also cause suppression of LPS-induced inflammatory responses by macrophages (Cvetanovic et al 2004).

Defects in clearance can result in non-ingested cells progressing to secondary necrosis and lysis, leading to exposure of self antigens. Data from humans (Jin et al 2004) and from a variety of animal models (Cohen et al 2002; Boes et al 1998; Bickerstaff et al 1999; Licht et al 2004) suggest that enhanced apoptosis as well as defective clearance of apoptotic are linked with disease. Apoptosis is associated with activation of caspases and cleavage of a multitude of cellular proteins. It is often found that autoantibodies are directed against these cleaved or modified auto-antigens (Pan et al 2006); caspase activity is known to lead to the exposure of cryptic epitopes which, by definition, are foreign to the immune system. Surface exposure of nucleosomal and ribonucleoprotein moieties occur in an antibody-accessible manner on apoptotic cells in a state of secondary necrosis (Cocca et al 2002; Radic et al 2004). Sera from lupus-prone mice and antibodies directed to apoptotic cells derived from human SLE patients can lead to diminished uptake of apoptotic cells (Licht et al 2004; Gandhi et al 2006). Lupus patients often show presence of higher number of apoptotic cells, a phenomenon that correlates with disease activity (Jin et al 2004). Immunization with apoptotic cells in absence of any adjuvant can also lead to generation of anti-self responses, directed at nucleosomes and oxidized phospholipids (Mevorach et al 1998; Chang et al 2004).

In this study, the autoimmune antibody response against apoptotic cells was evaluated, using monoclonal antibodies as probes. The autoimmune strains C57/BL6$^{lpr/lpr}$ and NZB/W were employed, and a total of six (three IgG and three IgM) monoclonal antibodies were generated. Antibodies did not bind healthy cells, bound cytoplasmic and/or nuclear antigens in cells that had been previously permeabilized, and bound a sub-population of Annexin-V-reactive apoptotic cells. As mentioned above, as cells undergo secondary necrosis, (as would occur in cell culture in the absence of phagocytic cells) cytoplasmic and nuclear moieties are transported to the cell surface, prior to cellular lysis (Casciola-Rosen et al 1994a). Distinct binding to cell surface protrusion (or "blebs") was observed upon confocal microscopy. Dual recognition with PI was also observed. Cell-surface binding of PI was observed in apoptotic cells (particularly in bleb like projection known to contain nucleic acids) in cells that retained the capacity to exclude Trypan Blue. Recognition of all antibodies was shown to be caspase-dependent.

To carry out preliminary characterization of the antigens recognized by the antibodies, Western blots were carried out. As indicated above, cellular moieties undergo modifications as cells undergo apoptosis, due to the action of caspases and other enzymes. Accordingly, lysates derived from healthy cells as well as from apoptotic blebs were used as antigenic substrates; lysates from apoptotic cell cultures were not employed, as antigen contributions from any remaining healthy cells in the cultures would complicate analysis. The fact that the antibodies were poly-reactive was apparent; antibodies of both the IgG and IgM isotype (and irrespective of the whether they carried somatic mutations in their Complementarity Determining Regions (CDR), as discussed later) bound several moieties on the extracts. Poly-reactivity of both human and murine monoclonal antibodies is a well-recognized phenomenon (Koren et al 1995; Lafer et al 1981), but no such data currently exists with regard to antibodies specifically targeting apoptotic cells. It was also apparent that the reactivity patterns upon lysates derived from healthy cells were distinct from those upon lysates derived from apoptotic blebs; both IgG and IgM antibodies recognized moieties with molecular weights lower than 30 KDa range upon bleb lysates. For the IgG antibody 1B3, the contrast between the two

antigenic preparations was particularly dramatic; while no specific binding was observed when healthy whole cell lysates were employed, distinct binding to a single moiety at ≈ 10 KDa was observed on lysates prepared from apoptotic blebs. Since such blebs are known to contain cytoplasmic and nuclear components that have may have undergone proteolytic processing (Casciola-Rosen et al 1994a; Casciola-Rosen et al 1994b; Casiano et al 1996; Casciola-Rosen et al 1999), this data provides strong circumstantial evidence of the recognition of products arising as a result of the apoptotic cascade. Formal demonstration that the antibody-binding moieties in the bleb preparation are derived upon processing of antigens present in healthy cells (except possible for antibody 1B3, as discussed above) would provide proof.

Antibodies specificity was further studied on a panel of prominent autoantigens. Significantly, both IgG and IgM antibodies appeared cross-reactive across several of the antigens tested. Antibody 2H8 predominantly bound Ro60, but also Ro52, SmB, SmD and the U1RNP A protein. 2C11 reacted with Ro52 and the U1RNP A protein in a dominant fashion, and 1B4 recognized Ro52 and Ro60, while binding all the other proteins with the exception of La to a minor extent. Distinct patterns of recognition were demonstrated by IgG antibodies; 1B1 and 2C3 both predominantly recognized Ro60 and SmD, while 1B3 was specific in its reactivity towards SmD. The poly-reactivity observed upon Western blot analysis was therefore mirrored in these assays. These data appear similar to that reported by other investigators (Pal et al 2005). In those studies, extensive poly-reactivity to other ribonucleoproteins was observed in human monoclonal antibodies initially selected to bind to Ro60. Interestingly, this study also demonstrated intra-molecular cross-reactivity, using peptides and recombinant sub-fragment of Ro60. A human monoclonal antibody specifically targeting apoptotic cells also demonstrated cross-reactivity between Ro60 and Ro52 (Gandhi et al 2006). The extensive cross-reactivity of anti-ribonucleoprotein responses has also been demonstrated in murine model systems (Kurien et al 2001).

Examples of cross-reactivity across different classes of molecules also occur. For example, antibodies to dsDNA have been shown to bind peptides and phospholipids (Putterman et al 1998; Lafer et al 1981). The reactivity of the antibodies to lipids was assessed. 2C11 (IgMκ) bound LPC, while and 2C3 (IgG2bκ) appeared to be more broadly reactive. The common perception that IgM antibodies tend to be more poly-reactive owing to their "sticky" nature therefore appeared not to hold true in this case. Antibodies to phosphospholipids (including LPC) are a common occurrence in SLE and anti-phospholipid syndrome (APS) (Shoenfeld 2003) and are associated with thrombotic events and Recurrent Spontaneous Abortion. The recognition of LPC by an apoptotic cell-specific antibody was additionally significant. LPC is made (and secreted) by apoptotic cells by the action of calcium independent phosholipase-A_2 (I-PLA_2) and has two important biological effects. Firstly, in certain contexts, it has anti-inflammatory properties (Yan et al 2004), and may contribute to the anti-inflammatory milieu of the apoptotic cell-phagocyte cell environment. Secondly, it acts as a chemo-attractant for phagocytes (Lauber et al 2003), probably helping them locate dying cells *in vivo*. Perturbation of these effects would be expected to have severe physiological consequences. Antibody 2C11 could considerably dampen the anti-inflammatory ability of LPC to suppress LPS-stimulated TNF-α production by macrophages. LPC-reactive antibodies could thus hinder an important biological role the molecule mediates. In an environment already rendered pro-inflammatory due to the presence of a bacterial infection, these antibodies would serve to amplify inflammatory mediators. 2C11 could also inhibit the transmigration of the human monocytic cell line THP-1 towards supernatant derived from an apoptotic cell culture. Such supernatant, in addition to containing LPC, would also contain apoptotic blebs which too are described as possessing chemo-attractant properties for phagocytes (Segundo et al 1999); significantly, antibody 2C11 demonstrated the capability to bind to purified blebs as well.

Sera from lupus-prone mice, as opposed to sera from normal mice, have been shown to inhibit the uptake of apoptotic cells (Licht et al 2004). The auto-antigens responsible for this inhibition remain ill-defined, however. The LPC-reactive antibody 2C11, as well as

the other IgM antibodies (2H8 and 1B4), were very efficient in inhibiting the uptake of apoptotic thymocytes by peripheral macrophages, mimicking results previously obtained with a human monoclonal antibody which specifically bound apoptotic cells (Gandhi et al 2006). Antibodies like 2C11 could therefore be envisaged to potentially be powerful disease perpetuators; on the one hand, while in circulation, they would "blind" the phagocyte by preventing the sensing of an important chemotactic agent while on the other, at the very site of apoptotic cell-phagocyte interaction, they could prevent the efficient uptake. Chang et al (1999) have shown that antibodies against low-density lipoproteins on apoptotic cells can also inhibit phagocytosis. "Normal" (non-immune) IgM has been shown to bind the surface of apoptotic cells, possibly aiding in their clearance (Kim et al 2002). This finding, coupled with the fact that serum IgM knock-out mice demonstrate many features of autoimmune disease (Boes et al 1998), indicates a potential physiological role for normal IgM in the maintenance of immune homoeostasis. These results differ from ours, however. Many of our experiments included a normal IgM control, and reactivity equivalent to those reported for the three IgM reported in this study has never been observed.

The IgG antibodies generated in this study (2C3, 1B1, 1B3), on the other hand, enhanced the internalization of apoptotic cells, possibly by FcγR mediated mechanisms. Anti-phospholipid antibodies have also been shown to increase the uptake of apoptotic cells (Manfredi et al 1998). Therefore, distinct phagocytic outcomes can result, depending on the nature of the targeted ACAMP and the isotype of the elicited antibody.

In addition to enhancing the uptake of apoptotic cells, anti-phophoplipid can also induce the release of TNF-α by phagocytes during the process (Manfredi et al 1998). SLE sera, or immune complexes purified from such sera, have been shown to stimulate the production of produce inflammatory cytokines like IFN-α and IL6 by pDCs (Vollmer et al 2005, Means et al 2005, Savarese et al 2006). Purified snRNP has also been demonstrated to cause the enhanced secretion of IFN-α, with lesser efficiency (Vollmer et al 2005). In the present study, supernatants of cultured phagocytes which had ingested

apoptotic cells (in the presence or absence of individual antibodies) were assessed for a variety of cytokines (IL6, MCP-1, IFN-γ, TNF-α, IL10 and IL12p70). In no instances were antibodies found to influence cytokine production (data not shown). These results suggest that not all antibodies directed towards nucleic acid-containing compounds (such as ribonucleoproteins or DNA) provide an inflammatory stimulus. The reasons for these differences are not clear; fine specificity of the antibodies may be a determining factor, as might antibody affinity.

Evidence exists implicating the idiotypic network in the initiation and progression of autoimmune responses. The idiotypic network theory postulates that a subset of anti-idiotypic antibodies (Ab2) arising upon immunization with antibody (Ab1) behave as "internal images" of the antigen, which can then be instrumental in the generation of Ab3. Ab1 and Ab3 could share antigenic specificity, which could lead to perpetuation of immune responses. Such amplification could be additionally significant as far as auto-reactive antibodies are concerned. Most work in this regard has been carried out with anti-dsDNA antibodies; immunization with an anti-dsDNA antibody bearing the 16/6 idiotype leads to the generation of an anti-dsDNA response in non-autoimmune prone strains of mice (Mendlovic et al 1988). Not much information exists concerning anti-idiotyic responses generated by ribo-nucleoprotein reactive auto-antibodies. Once such report, arising from our lab, described data obtained upon immunization with a human monoclonal antibody that recognized Ro60 and Ro52; antigenic spreading occurred, with novel auto-antigens being targeted. In addition, anti-idiotypic bound the surface of healthy cells, unlike the immunizing antibody which specifically bound apoptotic cells (Gandhi et al 2006). We have now addressed this issue in a system that offers an increased level of stringency; immunization of mice with murine antibodies would be expected to generate a purely anti-idiotypic response, un-like immunization with human antibodies, which necessitate extensive absorptions to rid the engendered antibodies of isotypic and allotypic reactivity. Three antibodies were employed in these experiments: 2C11 (IgMκ), 1B1 (IgG2aκ) and 2C3 (IgG2bκ). Antibodies 2C11 and 1B1 were capable of engendering anti-self responses, as adjudged by FACS analysis. In addition, Western

blot analysis revealed the appearance of antibodies of additional specificity in the sera of animals immunized with the antibodies, in comparison with those in the sera of mice immunized with adjuvant. 2C11 predominantly bound cytoplasmic antigens, whereas the anti-idiotypic antibodies arising upon its immunization appeared to recognize nuclear moieties. Finally, in both instances, anti-idiotypic antibodies demonstrated enhanced recognition of some recombinant auto-antigens than did antibodies generated against adjuvant. Anti-2C11 anti-idiotypes bound La and anti-1B1 anti-idiotypes bound La and the U1RNP A protein. These proteins were not recognized by the respective immunizing antibodies in a dominant fashion; 2C11 principally bound Ro52 and U1RNP A protein, and 1B1 principally bound Ro60 and SmD respectively.

It is important to mention that these ELISA assays, conducted upon a restricted list of recombinant auto-antigens, are only indicative in nature, and more comprehensive antigenic analysis would obviously be beneficial. Nevertheless, the data provide evidence that the idiotypic network can mediate epitope spreading in anti-ribonucleoprotein responses. One way in which these responses might arise can be postulated. Ribonucleoproteins often exist as components of macromolecular complexes. Antibodies reactive towards one component could conceivably generate "internal image" anti-idiotypic antibodies. Such antibodies would have the capacity to bind to other component molecule(s) of the complex to which the inciting antigen belongs. While such a scenario is possible (and indeed probable), all specifities that arise in experiments such as these cannot be predicted *apriori*, on the basis of knowledge of known interactions. For example, while the human monoclonal antibody described by Gandhi et al (2006) bound Ro52 and Ro60, engendered anti-idiotypic antibodies bound not just La (as *apriori* reasoning would predict, based on its known association with Ro60), but also a wide spectrum of other antigens. Similarly, while the anti-1B1 anti-idiotypic specificity could conceivably be predicted (based on the known associations of Ro60 with La, and of SmD with the U1RNP complex), such forecasts for 2C11 would not hold true. Unexpected reactivity also arises upon immunization with anti-DNA antibodies (Shoenfeld 1994). It is conceivable that an unappreciated level of idiotypic connectivity exists amongst auto-

immune antibody responses, which results in such effects. Anti-idiotype mediated epitope spreading may not be a general phenomenon, however; immunization with antibody 2C3 did not result in the generation of anti-self reactivity. Further work, employing antibodies of differing specificity and idiotype, would be required for a clearer appreciation of the rules that govern such responses.

Anti-phospholipid antibodies are implicated in thrombotic events and in Recurrent Spontaneous Abortion (Blank et al 1999), while anti-Ro and anti-La antibodies are associated with neonatal lupus and congenital heart block (Tran et al 2002; Julkunen et al 1998). Infusion into pregnant mice of anti-Ro and anti-La antibodies derived from human lupus patients leads to congenital heart block in the fetus. It is believed that these antibodies bind to the surface of apoptotic cardiac myocytes, causing the activation of macrophages which then secrete the inflammatory cytokine TNFα (Miranda carús et al 2000). Experiments were conducted to ascertain whether two of the antibodies that were generated in the present study affected fertility when passively administered to mice. Animals of the FVB strain were employed, since they demonstrated fertility rates in excess of 80% (after confirmed coitus) at the institutional animal facility. Antibodies 1B1 and 2C3 were chosen the experiment; while the former demonstrated minor reactivity towards phosphatidic acid and cardiolipin, the latter was broadly cross-reactive across all the lipids tested (lysophosphatidylcholine, lysophosphatidic acid, phosphotidylserine, phosphatidylethanolamine, phosphatidylcholine, phosphatidic acid and cardiolipin). Control animals were administered either a non-reactive antibody of the IgG isotype or PBS. Successful pregnancies and live births were observed (at expected rates) in animals receiving PBS and the control antibody. Interestingly, while administration of 2C3 resulted in no diminution in these parameters, animals injected with 1B1 demonstrated a dramatic reduction in the number of pups born. To assess whether animals would return to fertility status, animals treated with 1B1 were rested for three weeks, after which they were co-habited with males once again; pregnancy resulted in four out of five animals. These results validated the anti-fertility effects of 1B1 treatment. However, the reasons for the observed effects are far from clear at this time. Given the extensive anti-

phospholipid recognition 2C3 demonstrated, this was the antibody expected to mediate any anti-fertility effects. Differences in the anti-fertility effects were also not due obvious difference in ribonucleoprotein reactivity; both 1B1 and 2C3 predominantly bound Ro60 and SmD, although there could be differences in reactivity towards yet undiscovered moieties. Further experimentation would be required before these results can be adequately explained. Nevertheless, the fact that an antibody specifically directed at apoptotic cells can elicit anti-fertility effects when passively administered to non-autoimmune prone animals is significant.

Molecular mimicry has also been implicated in the initiation of autoimmune responses. Often, anti-self antibodies cross-react with antigens from microbial pathogens (Grayzel et al 1991). Such cross-reactivity can encompass different classes of molecules, as has been elegantly demonstrated with anti-dsDNA antibodies. A peptide mimic, identified upon panning of a random peptide phage display library using an anti-dsDNA antibody as probe, led to generation of anti-DNA (and subsequently, anti-ribonucleoprotein reactivity) upon immunization (Putterman et al 1998). In this study, we attempted to identify mimotpes of two of the apoptotic cell-specific antibodies; 2C11 (IgMκ) and 2C3 (IgG2bκ). Both antibodies demonstrated cross-reactive (but distinct) recognition of ribonucleoproteins and lipids. For 2C11, a minimal consensus sequence of **QXPXXL** was identified; interestingly, analogous sequences were present in Ro52 (**QTPLHL**; amino acids 387-406), Ro60 (**RIPTHL**; amino acids 112-130), two ribonucleoproteins towards which the antibody demonstrated reactivity. Based upon the relative abundance of the reactive peptides, the minimum consensus core recognition sequence was ascertained to be **QIPTHL**. Specific reactivity of 2C11 towards this hexapeptide confirmed it as the antibody mimotope; 2H8 and 1B4, the two other IgM antibodies previously shown to bind both Ro52 and Ro60, did not bind the peptide, and neither did the three IgG antibodies. Regions of Ro52 (**KYEAGTYPQTPLHLQVPPCQ**) and Ro60 (**FKAVSEVCRIPTHLFTFIQ**) encompassing the putative recognition regions were synthesized on a MAP backbone. The Ro60 peptide lost antigenecity within five days, due to unknown reasons; since the peptide contained cysteine residue, we postulate the

formation of inter-chain di-sulphide bonds could be responsible. The Ro52 peptide, on the other hand, elicited antibodies which bound the peptide, as well as Ro52 and Ro60. The reasons for reactivity towards Ro60 are not known at the present time, but can be speculated upon. The most obvious reason would be cross-reactive recognition of the protein, arising due to identified consensus motif. Epitope spreading, with or without stimulation of the idiotypic network could be alternate explanations. Epitope (or determinant) spreading refers to the increasing diversification of the auto-immune response with the passage of time. In the humoral response, antibodies initially target a few epitopes on a few antigens; other epitopes on the same antigen become targets ("intra-molecular spreading") as do epitopes on associated molecules ("inter-molecular spreading") (James et al 1995). Mechanisms by which this may occur have been discussed in the Review of Literature. Epitope spreading has also been shown to occur between antigens not associated *in situ*, like Ro52 and Ro60 (Keech et al 1996); the processes involved in such cases remain unclear. In this study, and in previous work (Gandhi et al 2006), we have described diversification of the immune responses arising due to stimulation of the idiotypic network. Such processes could be involved in the generation of anti-Ro60 reactivity.

These studies also re-iterate that antigenecity does not imply **equivalent** immunogenecity. The minimal consensus core peptide (**QIPTHL**) was found to be immunogenic, in that immunization resulted in anti-peptide antibodies (data not shown). However, anti-sera did not contain antibodies reactive to Ro52, Ro60 or LPC.

Unlike with antibody 2C11, when the antibody 2C3 was used to probe the phage display library, a consensus motif could not be identified. Rather, three peptide sequences were elucidated, which were present at different frequency. The antigenecity and immunogenecity of these sequences is currently under investigation.

V region analysis of the antibodies was carried out, essentially to determine whether somatic mutations were present (or even essential) in antibodies specifically targeting

apoptotic cells. Our analysis revealed that somatic mutations were not a mandatory requirement. A detailed mutational analysis of the antibody genes is described in Table 4. Replacement and silent mutations have been separately tabulated for the CDR and FWR regions of the heavy and light chains; this analysis ignores variations arising due to V-D-J and V-J junctional additions. At the outset, heavy chains appeared to carry more mutations. In some instances (for example, the 2C11 and 1B4 heavy chains), the replacement to silent (R/S) ratio has been listed as ∞ (infinity) to indicate an absence of silent mutation. In most instances the R/S ratio was higher for the CDR than the FWR; the 2H8 and 1B1 light chains were exceptions. The highest R/S ratio (ignoring ∞) was observed in the heavy chain of antibody 1B3. Interestingly, this antibody appeared to be the least polyreactive, based upon Western blot, and ELISA data.
Since the CDR and FWR regions are variable in length, replacement mutations per unit length were assessed for these regions. As Table 4a and Table 4b reveal, ratios of these values for CDR regions to those for FWR regions yielded values greater than one in all cases, except for the light chains of antibodies 2C11 and 1B4. These results indicated the preferential presence (in most instances) of somatic mutations in the CDR regions, as would be expected in an antigen driven response.

Many studies have been carried out to understand germline gene usage as well as to evaluate the somatic mutations auto-antibodies express. Most available information concerns anti-dsDNA, and some of these studies have been referred to earlier. Arginine residues in the CDR have previously been demonstrated to be important for anti-DNA reactivity; "reverse" mutation of these residues can lead to loss of recognition (Radic et al 1993; Wellmann et al 2005). Many of these antibodies have been reported to cross-react with lipids and ribonucleoproteins (Lafer et al 1981; Koren et al 1995). Anti-Sm antibodies generated from lupus-prone strain MRL$^{lp/lpr}$ mice indicated that while most heavy chains employed J558 (or VH1) family, no preferred pattern of mutations was observed; a higher number of arginine residues were documented CDR3 (Bloom et al 1993). Retter et al (1996) too reported the presence of an increased number of arginine residues in the CDRs in anti-Sm antibodies. Some of these antibodies were additionally

Antibody	Replacement CDR , FWR	Silent CDR , FWR	Replacement/ Silent CDR , FWR	Mutations per U. L. CDR , FWR	Mutations per U. L. CDR/ U. L. FWR
2C11 HEAVY	2 , 1	0 , 1	∞ , 1	0.057 , 0.013	5.04
2C11 LIGHT	0 , 0	0 , 0	0 , 0	0 , 0	0
2H8 HEAVY	1 , 0	1 , 0	1 , 0	0.043 , 0	∞
2H8 LIGHT	0 , 1	0 , 2	0 , 0.5	0 , 0.012	0
1B4 HEAVY	2 , 1	0 , 0	∞ , ∞	0.054 , 0.011	4.9
1B4 LIGHT	0 , 0	0 , 1	0 , 0	0 , 0	0

Table 4a: Mutations in the framework and complimentarity determining regions of the IgM monoclonal antibodies. ∞ indicates division by zero. U.L. : Unit Length

Antibody	Replacement CDR , FWR	Silent CDR , FWR	Replacement/ Silent CDR , FWR	Mutations per U. L. CDR , FWR	Mutations per U. L. CDR/ U. L. FWR
2C3 HEAVY	**5 , 3**	**2 , 3**	**2.5 , 1**	**0.16 , 0.034**	**4.7**
2C3 LIGHT	**2 , 2**	**1 , 2**	**2 , 1**	**0.06 , 0.025**	**2.4**
1B3 HEAVY	**9 , 7**	**1 , 11**	**9 , 0.63**	**0.27 , 0.081**	**3.37**
1B3 LIGHT	**3 , 6**	**6 , 5**	**0.5 , 1.2**	**0.22 , 0.074**	**3.003**
1B1 HEAVY	**3 , 4**	**0 , 2**	**∞ , 2**	**0.093 , 0.047**	**1.9**
1B1 LIGHT	**1 , 1**	**1 , 0**	**1 , ∞**	**0.03 , 0.012**	**2.5**

Table 4b: Mutations in the framework and complimentarity determining regions of the IgG monoclonal antibodies. ∞ indicates division by zero. U.L. : Unit Length

reactive to DNA while others not. These authors went on to demonstrate that "reverse" mutation of these residues led to abrogation of anti-Sm reactivity. If the unmutated antibody variable region was grafted upon an IgM backbone, anti-Sm reactivity was at least partially retained. Therefore, certain antibodies can, in germline configuration, bind specific autoantigens, an observation that the present study also makes. Quite possibly, such antibodies can contribute to autoimmune pathology upon subsequent somatic mutational events.

Arginine residues have also been implicated in anti-phospholipid reactivity. Kita et al (1993) demonstrated that in MRL$^{lpr/lpr}$ mice, most anti-cardiolipin antibodies demonstrating lupus anti-coagulant activity were derived from the J558 (or VH1) family and these antibodies possessed a significant number arginine residues in CDR3. No specific D or J segments appeared to contribute to antibody specificity, and no specific pattern of mutation could be discerned. Addition of arginine in the CDR of an antibody recognizing DNA and phosphatidylserine led to enhanced binding, providing additional proof of the amino acid's impact on antibody specificity (Cocca et al 2001). Besides arginine, anti-phospholipid antibodies are also thought to accumulate other basic residues like lysine and asparagines (Giles et al 2003). Modeling studies on these antibodies have demonstrated the surface accessibility of arginine residues, further implicating their role in antigen binding (Giles et al 2003). Positively-charged amino acids are also acquired on antibodies binding U1RNA; Hoet et al (1999) demonstrated presence of arginine, lysine and histidine residues in the CDR1 and 2 of U1RNA-binding antibodies. These authors also demonstrated that the light chain usage also had an important role to play in this specificity; shuffling of the light chain could lead to a shifting of epitopic specificity upon U1RNA (Hoet et al 1999).

Interestingly, in the present study, several arginine residues were observed in the heavy chain CDR3 regions of antibodies 2C3 and 1B3, arising either due to somatic mutations or to non-encoded junctional additions. Antibody 2C3 demonstrates reactivity to cardiolipin and many other phospholipids like phosphatidic acid, while also binding to

SmD. Antibody 1B3, though not demonstrating substantial reactivity to phospholipids, binds SmD. The IgM antibodies demonstrate reactivity to ribonucleoproteins (and, for 2C11, reactivity to the lipid LPC) but do not exhibit the presence of arginine residues in the CDR regions.

Sequence alignments with other genes submitted to the NCBI database were also carried out, and a few significant observations are highlighted below. The light chain of the 2C11 antibody was found highly homologous to that of an anti-influenza hemaglutinin antibody and to an anti-nuclear antibody (ANA) derived from a lupus-prone strain of mice (Kavaler et al 1990; Portanova et al 1995). However, 2C11 did not demonstrate ANA reactivity, but rather bound peri-nuclear and cytoplasmic antigen(s); the heavy of the antibody conceivably affected specificity.

The antibody 2H8 heavy chain appeared homologous to an anti-(4-hydroxy-3-nitrophenylacetyl) antibody (Furukawa et al 2006); however, no autoantibody in the database appeared significantly similar. The 2H8 light chain demonstrated homology to an antibody predicted to bind ssDNA (Tillman et al 1992).

Unlike antibodies 2C11 and 2H8, which demonstrated few mutations or junctional changes in the CDR regions, antibody 1B4 demonstrated the addition of two and three non-encoded amino acids at the V-D and D-J junctions respectively. The heavy chain demonstrated homology to an antibody exhibiting anti-nuclear reactivity derived from the tight skin mouse. It is interesting that antibody 1B4 did demonstrate faint recognition of nuclear antigens.

The heavy chain of antibody 2C3 demonstrated 96.6% identity to an anti-nucleosomal and an anti-Sm antibody derived from MRL$^{lpr/lpr}$ mice (Bloom et al 1993). As mentioned above, the antibody bound Sm as well as a variety of phospholipids. No homologies to sequences of anti-phospholipid antibodies in the database were observed, however. The

2C3 light chain demonstrated high homology with a light chain derived from an anti-dsDNA antibody generated from NZB/W animals (Tillman et al 1992).

The heavy chain of antibody 1B3 demonstrated considerable homology to an anti-β2 Glycoprotein-I antibody (Monestier et al 1996), a finding of significance, since the molecule binds to negatively-charged phosphoplipids. The light chain of 1B3 demonstrated similarity with antibodies reactive anti-HIV Tat protein and anti-apolipoprotein B.

The heavy chain of antibody 1B1, like that of antibody 2H8, also contained a D region which was unidentifiable. Non-encoded amino acids at the V-D junction contained two arginine residues. The database search revealed homologies to antibodies reactive to dsDNA derived from NZB/W F1 animals (Tillman et al 1992). The light chain also demonstrated similarities to anti-DNA and anti-hemaglutinin antibodies (Krishnan et al 1996; Clarke et al 1990). As discussed above, antibodies of the latter specificity were also found homologous to the light chain of antibody 2C11.

It is interesting that, in many instances, homologies in the heavy and/or light chain sequences were found with antibodies previously characterized as having anti-self reactivity. The fine specificities of the antibodies would obviously additionally be influenced by light chain – heavy chain pairing. Nevertheless, such similarity was probably to be expected, since the antibodies reported on here were selected against apoptotic cells, considered the primary antigenic insult in systemic autoimmune diseases.

Summary and Conclusions

SUMMARY AND CONCLUSIONS

Six monoclonal antibodies (2C11 (IgMκ), 2H8 (IgMκ), 1B4 (IgMκ), 2C3 (IgG2bκ), 1B3 (IgG2aκ), 1B1 (IgG2aκ)) were generated, using B cells sourced from two auto-immune prone strains of mice ($C57/BL6^{lpr/lpr}$ and NZB/W).

Antibodies did not bind plasma membrane antigens on the surface of healthy cells, recognized cytoplasmic and/or nuclear antigens on permeabilized cells, and interacted with moieties exposed on the membrane of dying cells in a caspase-dependent manner. All antibodies almost exclusively bound cells also bound by Annexin-V and Propidium Iodide, indicating recognition of antigens characteristic of a relatively late stage of apoptosis.

Polyreactivity was evident; all antibodies, irrespective of isotype, bound several moieties upon Western blots using whole cell- or apoptotic bleb- lysates, and (except for antibody 1B3) bound several recombinant autoantigens. Antibody 2C11 also recognized lysophosphatidylcholine (LPC), and antibody 2C3 many lipids.

While the IgM antibodies specifically suppressed the phagocytosis of apoptotic cells, the IgG antibodies appeared to cause enhancement, possibly by Fcγ receptor-mediated mechanisms. These effects could have disease-modifying consequences *in vivo*.

LPC has anti-inflammatory and chemotactic properties. The LPC reactive antibody 2C11 suppressed LPC-mediated down-modulation of LPS-induced TNF-α synthesis by peritoneal macrophages. In addition, the antibody decreased the migration of phagocytes towards supernatant obtained from cultures of apoptotic cells. In the context of disease, such antibodies could contribute to pathogenesis by neutralizing the anti-inflammatory environment in the vicinity of phagocytotic uptake, as well as by preventing the "seeking" out of dying cells by phagocytes.

Immunization of mice with antibodies 211 and 1B1 (but not with antibody 2C3) led to the generation of anti-self responses, as adjudged by Western blot and FACS analysis. In addition, antisera bound antigens not recognized by the immunizing antibodies. These results indicate possible epitope spreading which is mediated via the idioypic network, a phenomenon that may serve to expand the repertoire of targeted self antigens.

Passive administration of antibody 1B1 into FVB animals caused significant decreases in fertility. This finding is under further investigation in order to more fully understand the causative factors. Nevertheless, the results assume significance in light of the fact that recurrent spontaneous abortion is a serious pathological outcome of systemic autoimmune diseases like SLE and anti-phospholipid syndrome.

A random peptide phage display library was screened using the antibody 2C11 as probe. A consensus hexapeptide motif was identified which, when synthesized, specifically bound 2C11, to the exclusion of the other antibodies. Closely homologous peptide sequences were found in Ro50 and Ro52 (two RNPs bound by 2C11) and not in La (an RNP not recognized by 2C11), possibly laying a basis of the observed cross-reactivity. Immunization with an extended peptide (on a MAP backbone) representing the hexapeptide sequence led to the generation of anti-Ro52 and Ro60 antibodies.

The heavy and light chain variable region genes of all antibodies were sequenced. Sequences demonstrated homologies to several different gene families. While some antibody genes appeared non-mutated with respect to the closest respective germline sequences, others (particularly the heavy chains) exhibited extensive mutations in their CDR regions. These observations suggest that somatic mutations are not a necessary requirement for anti-apoptotic cell specificity.

Bibliography

BIBLIOGRAPHY

Acehan D, Jiang X, Morgan DG, Heuser JE, Wang X, Akey CW. Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. Mol Cell. 2002 Feb;9(2):423-32.

Allison AC, Eugui EM. Purine metabolism and immunosuppressive effects of mycophenolate mofetil (MMF). Clin Transplant. 1996 Feb;10(1 Pt 2):77-84.

Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J. Human ICE/CED-3 protease nomenclature. Cell. 1996 Oct 18;87(2):171.

Amoura Z, Chabre H, Koutouzov S, Lotton C, Cabrespines A, Bach JF, Jacob L. Nucleosome-restricted antibodies are detected before anti-dsDNA and/or antihistone antibodies in serum of MRL-Mp lpr/lpr and +/+ mice, and are present in kidney eluates of lupus mice with proteinuria. Arthritis Rheum. 1994 Nov;37(11):1684-8.

Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA, Harley JB. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. N Engl J Med. 2003 Oct 16;349(16):1526-33.

Asano K, Miwa M, Miwa K, Hanayama R, Nagase H, Nagata S, Tanaka M. Masking of phosphatidylserine inhibits apoptotic cell engulfment and induces autoantibody production in mice. J Exp Med. 2004 Aug 16;200(4):459-67.

Banchereau J, Pascual V, Palucka AK. Autoimmunity through cytokine-induced dendritic cell activation. Immunity. 2004 May;20(5):539-50.

Bennett L, Palucka AK, Arce E, Cantrell V, Borvak J, Banchereau J, Pascual V. Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. J Exp Med. 2003 Mar 17;197(6):711-23.

Bertin J, Armstrong RC, Ottilie S, Martin DA, Wang Y, Banks S, Wang GH, Senkevich TG, Alnemri ES, Moss B, Lenardo MJ, Tomaselli KJ, Cohen JI. Death effector domain-containing herpesvirus and poxvirus proteins inhibit both Fas- and TNFR1-induced apoptosis. Proc Natl Acad Sci U S A. 1997 Feb 18;94(4):1172-6.

Bickerstaff MC, Botto M, Hutchinson WL, Herbert J, Tennent GA, Bybee A Mitchell DA, Cook HT, Butler PJ, Walport MJ, Pepys MB. Serum amyloid P component controls chromatin degradation and prevents antinuclear autoimmunity. Nat Med. 1999 Jun;5(6):694-7.

Bielschowsky M, Bielschowsky F. Observations on NZB/B1 mice; differential fertility in reciprocal crosses and the transmission of the auto-immune haemolytic anaemia to nzb/b1 x nzc/b1 hybrids. Aust J Exp Biol Med Sci. 1964 Aug;42:561-8.

Bijl M, Horst G, Bijzet J, Bootsma H, Limburg PC, Kallenberg CG. Serum amyloid P component binds to late apoptotic cells and mediates their uptake by monocyte-derived macrophages. Arthritis Rheum. 2003 Jan;48(1):248-54.

Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. Science. 2001 Nov 16;294(5546):1540-3.

Blank M, Cohen J, Toder V, Shoenfeld Y. Induction of anti-phospholipid syndrome in naive mice with mouse lupus monoclonal and human polyclonal anti-cardiolipin antibodies. Proc Natl Acad Sci U S A. 1991 Apr 15;88(8):3069-73.

Blank M, Krause I, Fridkin M, Keller N, Kopolovic J, Goldberg I, Tobar A, Shoenfeld Y. Bacterial induction of autoantibodies to beta2-glycoprotein-I accounts for the infectious etiology of antiphospholipid syndrome. J Clin Invest. 2002 Mar;109(6):797-804.

Blank M, Shoenfeld Y, Cabilly S, Heldman Y, Fridkin M, Katchalski-Katzir E. Prevention of experimental antiphospholipid syndrome and endothelial cell activation by synthetic peptides. Proc Natl Acad Sci U S A. 1999 Apr 27;96(9):5164-8.

Bloom DD, Davignon JL, Retter MW, Shlomchik MJ, Pisetsky DS, Cohen PL, Eisenberg RA, Clarke SH. V region gene analysis of anti-Sm hybridomas from MRL/Mp-lpr/lpr mice. J Immunol. 1993 Feb 15;150(4):1591-610.

Boatright KM, Renatus M, Scott FL, Sperandio S, Shin H, Pedersen IM, Ricci JE, Edris WA, Sutherlin DP, Green DR, Salvesen GS. A unified model for apical caspase activation. Mol Cell. 2003 Feb;11(2):529-41.

Boes M, Esau C, Fischer MB, Schmidt T, Carroll M, Chen J. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. J Immunol. 1998 May 15;160(10):4776-87.

Boire G, Gendron M, Monast N, Bastin B, Menard HA. Purification of antigenically intact Ro ribonucleoproteins; biochemical and immunological evidence that the 52-kD protein is not a Ro protein. Clin Exp Immunol. 1995 Jun;100(3):489-98.

Boldin MP, Goncharov TM, Goltsev YV, Wallach D. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell. 1996 Jun 14;85(6):803-15.

Boldin MP, Varfolomeev EE, Pancer Z, Mett IL, Camonis JH, Wallach D. A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. J Biol Chem. 1995 Apr 7;270(14):7795-8.

Botto M, Dell'Agnola C, Bygrave AE, Thompson EM, Cook HT, Petry F, Loos M, Pandolfi PP, Walport MJ. Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies. Nat Genet. 1998 May;19(1):56-9.

Bowness P, Davies KA, Norsworthy PJ, Athanassiou P, Taylor-Wiedeman J, Borysiewicz LK, Meyer PA, Walport MJ. Hereditary C1q deficiency and systemic lupus erythematosus. QJM. 1994 Aug;87(8):455-64.

Bratton DL, Fadok VA, Richter DA, Kailey JM, Guthrie LA, Henson PM. Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. J Biol Chem. 1997 Oct 17;272(42):26159-65.

Breathnach SM, Kofler H, Sepp N, Ashworth J, Woodrow D, Pepys MB, Hintner H. Serum amyloid P component binds to cell nuclei in vitro and to in vivo deposits of extracellular chromatin in systemic lupus erythematosus. J Exp Med. 1989 Oct 1;170(4):1433-8.

Brown S, Heinisch I, Ross E, Shaw K, Buckley CD, Savill J. Apoptosis disables CD31-mediated cell detachment from phagocytes promoting binding and engulfment. Nature. 2002 Jul 11;418(6894):200-3.

Burlingame RW, Volzer MA, Harris J, Du Clos TW. The effect of acute phase proteins on clearance of chromatin from the circulation of normal mice. J Immunol. 1996 Jun 15;156(12):4783-8.

Burnet, F. M., and M. C. Holmes. 1965. The natural history of the NZB/NZW F1 hybrid mouse: a laboratory model of systemic lupus erythematosus. *Australas. Ann. Med.* 14:185.

Carlsten H, Tarkowski A. Histocompatibility complex gene products and exposure to oestrogen: two independent disease accelerating factors in murine lupus. Scand J Immunol. 1993 Oct;38(4):341-7.

Casciola-Rosen L, Andrade F, Ulanet D, Wong WB, Rosen A. Cleavage by granzyme B is strongly predictive of autoantigen status: implications for initiation of autoimmunity. J Exp Med. 1999 Sep 20;190(6):815-26.

Casciola-Rosen LA, Anhalt G, Rosen A. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. J Exp Med. 1994a Apr 1;179(4):1317-30.

Casciola-Rosen LA, Miller DK, Anhalt GJ, Rosen A. Specific cleavage of the 70-kDa protein component of the U1 small nuclear ribonucleoprotein is a characteristic biochemical feature of apoptotic cell death. J Biol Chem. 1994b Dec 9;269(49):30757-60.

Casiano CA, Martin SJ, Green DR, Tan EM. Selective cleavage of nuclear autoantigens during CD95 (Fas/APO-1)-mediated T cell apoptosis. J Exp Med. 1996 Aug 1;184(2):765-70.

Cervera R, Khamashta MA, Font J, Sebastiani GD, Gil A, Lavilla P, Domenech I, Aydintug AO, Jedryka-Goral A, de Ramon E, et al. Systemic lupus erythematosus: clinical and immunologic patterns of disease expression in a cohort of 1,000 patients. The European Working Party on Systemic Lupus Erythematosus. Medicine (Baltimore). 1993 Mar;72(2):113-24.

Chai J, Shiozaki E, Srinivasula SM, Wu Q, Datta P, Alnemri ES, Shi Y. Structural basis of caspase-7 inhibition by XIAP. Cell. 2001 Mar 9;104(5):769-80.

Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, Lenardo MJ. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. Science. 2000 Jun 30; 288 (5475):2351-4.

Chang MK, Bergmark C, Laurila A, Horkko S, Han KH, Friedman P, Dennis EA, Witztum JL. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. Proc Natl Acad Sci U S A. 1999 May 25;96(11):6353-8.

Chang MK, Binder CJ, Miller YI, Subbanagounder G, Silverman GJ, Berliner JA, Witztum JL. Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory. J Exp Med. 2004 Dec 6; 200 (11):1359-70.

Chen X, Quinn AM, Wolin SL. Ro ribonucleoproteins contribute to the resistance of Deinococcus radiodurans to ultraviolet irradiation. Genes Dev. 2000 Apr 1; 14 (7):777-82.

Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell. 1995 May 19;81(4):505-12.

Chinnaiyan AM, Tepper CG, Seldin MF, O'Rourke K, Kischkel FC, Hellbardt S, Krammer PH, Peter ME, Dixit VM. FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factorreceptor-induced apoptosis. J Biol Chem. 1996 Mar 1;271(9):4961-5.

Clarke SH, Staudt LM, Kavaler J, Schwartz D, Gerhard WU, Weigert MG. V region gene usage and somatic mutation in the primary and secondary responses to influenza virus hemagglutinin. J Immunol. 1990 Apr 1;144(7):2795-801.

Cocca BA, Cline AM, Radic MZ. Blebs and apoptotic bodies are B cell autoantigens. J Immunol. 2002 Jul 1;169(1):159-66.

Cocca BA, Seal SN, D'Agnillo P, Mueller YM, Katsikis PD, Rauch J, Weigert M, Radic MZ. Structural basis for autoantibody recognition of phosphatidylserine-beta 2 glycoprotein I and apoptotic cells. Proc Natl Acad Sci U S A. 2001 Nov 20;98(24):13826-31.

Cohen PL, Caricchio R, Abraham V, Camenisch TD, Jennette JC, Roubey RA, Earp HS, Matsushima G, Reap EA. Delayed apoptotic cell clearance and lupus-like autoimmunity in mice lacking the c-mer membrane tyrosine kinase. J Exp Med. 2002 Jul 1;196(1):135-40.

Cohen PL, Eisenberg RA. Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. Annu Rev Immunol. 1991;9:243-69.

Cvetanovic M, Ucker DS. Innate immune discrimination of apoptotic cells: repression of proinflammatory macrophage transcription is coupled directly to specific recognition. J Immunol. 2004 Jan 15;172(2):880-9.

Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell. 2004 Jan 23;116(2):205-19.

Davidson A, Smith A, Katz J, Preud'Homme JL, Solomon A, Diamond B. A cross-reactive idiotype on anti-DNA antibodies defines a H chain determinant present almost exclusively on IgG antibodies. J Immunol. 1989 Jul 1;143(1):174-80.

Deapen D, Escalante A, Weinrib L, Horwitz D, Bachman B, Roy-Burman P, Walker A, Mack TM. A revised estimate of twin concordance in systemic lupus erythematosus. Arthritis Rheum. 1992 Mar;35(3):311-8.

DeGiorgio LA, Konstantinov KN, Lee SC, Hardin JA, Volpe BT, Diamond B. A subset of lupus anti-DNA antibodies cross-reacts with the NR2 glutamate receptor in systemic lupus erythematosus. Nat Med. 2001 Nov;7(11):1189-93.

Deocharan, B., Xiaoping, Q., Lichauco, J., Putterman, C. Alpha-actinin is a cross-reactive renal target for pathogenic anti-DNA antibodies. J Immunol. 2002 Mar 15;168(6):3072-8.

Desai DD, Krishnan MR, Swindle JT, Marion TN. Antigen-specific induction of antibodies against native mammalian DNA in nonautoimmune mice. J Immunol. 1993 Aug 1;151(3):1614-26.

Deshmukh US, Bagavant H, Lewis J, Gaskin F, Fu SM. Epitope spreading within lupus-associated ribonucleoprotein antigens. Clin Immunol. 2005 Nov;117(2):112-20.

Deveraux QL, Takahashi R, Salvesen GS, Reed JC. X-linked IAP is a direct inhibitor of cell-death proteases. Nature. 1997 Jul 17;388(6639):300-4.

Diamond B, Katz JB, Paul E, Aranow C, Lustgarten D, Scharff MD. The role of somatic mutation in the pathogenic anti-DNA response. Annu Rev Immunol. 1992;10:731-57.

Diamond B, Scharff MD. Somatic mutation of the T15 heavy chain gives rise to an antibody with autoantibody specificity. Proc Natl Acad Sci U S A. 1984 Sep;81(18):5841-4.

Dostal C, Tesar V, Rychlik I, Zabka J, Vencovsky J, Bartunkova J, Stejskalova A, Tegzova D. Effect of 1 year cyclosporine A treatment on the activity and renal involvement of systemic lupus erythematosus: a pilot study. Lupus. 1998;7(1):29-36.

Du C, Fang M, Li Y, Li L, Wang X. Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell. 2000 Jul 7;102(1):33-42.

Ehrenstein MR, Cook HT, Neuberger MS. Deficiency in serum immunoglobulin (Ig)M predisposes to development of IgG autoantibodies. J Exp Med. 2000 Apr 3;191(7):1253-8.

Emery P. The therapeutic potential of costimulatory blockade with CTLA4Ig in rheumatoid arthritis. Expert Opin Investig Drugs. 2003 Apr;12(4):673-81.

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391:43–50.

Espinosa A, Zhou W, Ek M, Hedlund M, Brauner S, Popovic K, Horvath L, Wallerskog T, Oukka M, Nyberg F, Kuchroo VK, Wahren-Herlenius M. The

Sjogren's syndrome-associated autoantigen Ro52 is an E3 ligase that regulates proliferation and cell death. J Immunol. 2006 May 15;176(10):6277-85.

Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J Clin Invest. 1998a Feb 15;101(4):890-8.

Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, Henson PM. A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature. 2000 May 4;405(6782):85-90.

Fadok VA, Warner ML, Bratton DL, Henson PM. CD36 is required for phagocytosis of apoptotic cells by human macrophages that use either a phosphatidylserine receptor or the vitronectin receptor (alpha v beta 3). J Immunol. 1998b Dec 1;161(11):6250-7.

Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol. 1992 Apr 1;148(7):2207-16.

Fan TJ, Han LH, Cong RS, Liang J. Caspase family proteases and apoptosis. Acta Biochim Biophys Sin (Shanghai). 2005 Nov;37(11):719-27.

Fanzo JC, Yang W, Jang SY, Gupta S, Chen Q, Siddiq A, Greenberg S, Pernis AB. Loss of IRF-4-binding protein leads to the spontaneous development of systemic autoimmunity. J Clin Invest. 2006 Mar;116(3):703-14.

Ferry H, Jones M, Vaux DJ, Roberts IS, Cornall RJ. The cellular location of self-antigen determines the positive and negative selection of autoreactive B cells. J Exp Med. 2003 Nov 3;198(9):1415-25.

Fesik SW. Insights into programmed cell death through structural biology. Cell. 2000 Oct 13;103(2):273-82.

Fox RI. Mechanism of action of hydroxychloroquine as an antirheumatic drug. Semin Arthritis Rheum. 1993 Oct;23(2 Suppl 1):82-91.

Franc NC, White K, Ezekowitz RA. Phagocytosis and development: back to the future. Curr Opin Immunol. 1999 Feb;11(1):47-52.

Francioni C, Galeazzi M, Fioravanti A, Gelli R, Megale F, Marcolongo R. Long-term i.v. Ig treatment in systemic lupus erythematosus. Clin Exp Rheumatol. 1994 Mar-Apr;12(2):163-8.

Frank MB, Itoh K, McCubbin V. Epitope mapping of the 52-kD Ro/SSA autoantigen. Clin Exp Immunol. 1994 Mar;95(3):390-6.

Furukawa K, Manabe A, Furukawa A, Kuba H, Okajima T, Azuma T. Initial repertoire of anti-(4-hydroxy-3-nitrophenylacetyl) antibodies as potential donors for effective affinity maturation. Mol Immunol. 2006 Apr;43(11):1751-60.

Gaffney PM, Kearns GM, Shark KB, Ortmann WA, Selby SA, Malmgren ML, Rohlf KE, Ockenden TC, Messner RP, King RA, Rich SS, Behrens TW. A genome-wide search for susceptibility genes in human systemic lupus erythematosus sib-pair families. Proc Natl Acad Sci U S A. 1998 Dec 8;95(25):14875-9.

Gandhi R, Hussain E, Das J, Handa R, Pal R. Anti-idiotype-mediated epitope spreading and diminished phagocytosis by a human monoclonal antibody recognizing late-stage apoptotic cells. Cell Death Differ. 2006 Feb 10; [Epub ahead of print]

Gaynor B, Putterman C, Valadon P, Spatz L, Scharff MD, Diamond B. Peptide inhibition of glomerular deposition of an anti-DNA antibody. Proc Natl Acad Sci U S A. 1997 Mar 4;94(5):1955-60.

Giles IP, Haley J, Nagl S, Latchman DS, Chen PP, Chukwuocha RU, Isenberg DA, Rahman A. Relative importance of different human aPL derived heavy and light chains in the binding of aPL to cardiolipin. Mol Immunol. 2003 Sep;40(1):49-60.

Goldblatt F, Isenberg DA. New therapies for systemic lupus erythematosus. Clin Exp Immunol. 2005 May;140(2):205-12.

Golks A, Brenner D, Schmitz I, Watzl C, Krueger A, Krammer PH, Lavrik IN. The role of CAP3 in CD95 signaling: new insights into the mechanism of procaspase-8 activation. Cell Death Differ. 2006 Mar;13(3):489-98.

Grayzel A, Solomon A, Aranow C, Diamond B. Antibodies elicited by pneumococcal antigens bear an anti-DNA—associated idiotype. J Clin Invest. 1991 Mar;87(3):842-6.

Gregory CD. CD14-dependent clearance of apoptotic cells: relevance to the immune system. Curr Opin Immunol. 2000 Feb;12(1):27-34.

Hainsworth JD, Burris HA 3rd, Morrissey LH, Litchy S, Scullin DC Jr, Bearden JD 3rd, Richards P, Greco FA. Rituximab monoclonal antibody as initial systemic therapy for patients with low-grade non-Hodgkin lymphoma. Blood. 2000 May 15;95(10):3052-6.

Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. Identification of a factor that links apoptotic cells to phagocytes. Nature. 2002 May 9;417(6885):182-7.

Hanayama R, Tanaka M, Miyasaka K, Aozasa K, Koike M, Uchiyama Y, Nagata S. Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. Science. 2004 May 21;304(5674):1147-50.

Hardgrave KL, Neas BR, Scofield RH, Harley JB. Antibodies to vesicular stomatitis virus proteins in patients with systemic lupus erythematosus and in normal subjects. Arthritis Rheum. 1993 Jul;36(7):962-70.

Harley JB, Sestak AL, Willis LG, Fu SM, Hansen JA, Reichlin M. A model for disease heterogeneity in systemic lupus erythematosus. Relationships between histocompatibility antigens, autoantibodies, and lymphopenia or renal disease. Arthritis Rheum. 1989 Jul;32(7):826-36.

Helyer, B. J., and J. B. Howie. 1963. Renal disease associated with positive lupus erythematosus tests in a cross-bred strain of mice. *Nature (Lond.).* 197:197.

Henson PM, Hume DA. Apoptotic cell removal in development and tissue homeostasis. Trends Immunol. 2006 May;27(5):244-50.

Hoet RM, Pieffers M, Stassen MH, Raats J, de Wildt R, Pruijn GJ, van den Hoogen F, van Venrooij WJ. The importance of the light chain for the epitope specificity of human anti-U1 small nuclear RNA autoantibodies present in systemic lupus erythematosus patients. J Immunol. 1999 Sep 15;163(6):3304-12.

Holborow EJ, Weir DM, Johnson GD. A serum factor in lupus erythematosus with affinity for tissue nuclei. Br Med J. 1957 Sep 28;(5047):732-4.

Howie, J. B., and B. J. Helyer. 1968. The immunology and pathology of NZB mice. *Adv. Imrnunol.* 9:215.

Huang M, Ida H, Kamachi M, Iwanaga N, Izumi Y, Tanaka F, Aratake K, Arima K, Tamai M, Hida A, Nakamura H, Origuchi T, Kawakami A, Ogawa N, Sugai S, Utz PJ, Eguchi K. Detection of apoptosis-specific autoantibodies directed against granzyme B-induced cleavage fragments of the SS-B (La) autoantigen in sera from patients with primary Sjogren's syndrome. Clin Exp Immunol. 2005 Oct;142(1):148-54.

Huerta PT, Kowal C, DeGiorgio LA, Volpe BT, Diamond B. Immunity and behavior: antibodies alter emotion. Proc Natl Acad Sci U S A. 2006 Jan 17;103(3):678-83.

Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tysk C, O'Morain CA, Gassull M, Binder V, Finkel Y, Cortot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahbatou M, Thomas G. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature. 2001 May 31;411(6837):599-603.

Huynh ML, Fadok VA, Henson PM. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. J Clin Invest. 2002 Jan;109(1):41-50.

Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschopp J. Inhibition of death receptor signals by cellular FLIP. Nature. 1997 Jul 10;388(6638):190-5.

Isenberg DA, Shoenfeld Y, Walport M, Mackworth-Young C, Dudeney C, Todd-Pokropek A, Brill S, Weinberger A, Pinkas J. Detection of cross-reactive anti-DNA antibody idiotypes in the serum of systemic lupus erythematosus patients and of their relatives. Arthritis Rheum. 1985 Sep;28(9):999-1007.

Ishii T, Ohnuma K, Murakami A, Takasawa N, Yamochi T, Iwata S, Uchiyama M, Dang NH, Tanaka H, Morimoto C. SS-A/Ro52, an autoantigen involved in CD28-mediated IL-2 production. J Immunol. 2003 Apr 1;170(7):3653-61.

Izui S, Higaki M, Morrow D, Merino R. The Y chromosome from autoimmune BXSB/MpJ mice induces a lupus-like syndrome in (NZW x C57BL/6)F1 male mice, but not in C57BL/6 male mice. Eur J Immunol. 1988 Jun;18(6):911-5.

James JA, Gross T, Scofield RH, Harley JB. Immunoglobulin epitope spreading and autoimmune disease after peptide immunization: Sm B/B'-derived PPPGMRPP and PPPGIRGP induce spliceosome autoimmunity. J Exp Med. 1995 Feb 1;181(2):453-61.

Jin O, Sun LY, Zhou KX, Zhang XS, Feng XB, Mok MY, Lau CS. Lymphocyte apoptosis and macrophage function: correlation with disease activity in systemic lupus erythematosus. Clin Rheumatol. 2005 Apr;24(2):107-10.

Julkunen H, Kaaja R, Siren MK, Mack C, McCready S, Holthofer H, Kurki P, Maddison P. Immune-mediated congenital heart block (CHB): identifying and counseling patients at risk for having children with CHB. Semin Arthritis Rheum. 1998 Oct;28(2):97-106.

Kasahara H, Matsuura E, Kaihara K, Yamamoto D, Kobayashi K, Inagaki J, Ichikawa K, Tsutsumi A, Yasuda S, Atsumi T, Yasuda T, Koike T. Antigenic structures recognized by anti-beta2-glycoprotein I auto-antibodies. Int Immunol. 2005 Dec;17(12):1533-42.

Kavaler J, Caton AJ, Staudt LM, Schwartz D, Gerhard W. A set of closely related antibodies dominates the primary antibody response to the antigenic site CB of the A/PR/8/34 influenza virus hemagglutinin. J Immunol. 1990 Oct 1;145(7):2312-21.

Keech CL, Gordon TP, McCluskey J. The immune response to 52-kDa Ro and 60-kDa Ro is linked in experimental autoimmunity. J Immunol. 1996 Oct 15;157(8):3694-9.

Kerr, J.F., Wyllie, A.H. and Currie, A.R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer 1972 26, 239–257.

Kim SJ, Gershov D, Ma X, Brot N, Elkon KB. I-PLA(2) activation during apoptosis promotes the exposure of membrane lysophosphatidylcholine leading to binding by natural immunoglobulin M antibodies and complement activation. J Exp Med. 2002 Sep 2;196(5):655-65.

Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J. 1995 Nov 15;14(22):5579-88.

Kischkel FC, Lawrence DA, Tinel A, LeBlanc H, Virmani A, Schow P, Gazdar A, Blenis J, Arnott D, Ashkenazi A. Death receptor recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. J Biol Chem. 2001 Dec 7;276(49):46639-46.

Kita Y, Sumida T, Ichikawa K, Maeda T, Yonaha F, Iwamoto I, Yoshida S, Koike T. V gene analysis of anticardiolipin antibodies from MRL-lpr/lpr mice. J Immunol. 1993 Jul 15;151(2):849-56.

Koren E, Koscec M, Wolfson-Reichlin M, Ebling FM, Tsao B, Hahn BH, Reichlin M. Murine and human antibodies to native DNA that cross-react with the A and D SnRNP polypeptides cause direct injury of cultured kidney cells. J Immunol. 1995 May 1;154(9):4857-64.

Kowal C, DeGiorgio LA, Nakaoka T, Hetherington H, Huerta PT, Diamond B,Volpe BT. Cognition and immunity; antibody impairs memory.Immunity. 2004 Aug;21(2):179-88.

Krishnan MR, Jou NT, Marion TN. Correlation between the amino acid position of arginine in VH-CDR3 and specificity for native DNA among autoimmune antibodies. J Immunol. 1996 Sep 15;157(6):2430-9.

Kumar S, Bunting KA, Kalsi J, Hinks JA, Latchman DS, Pearl LH, Isenberg DA. Lupus autoantibodies to native DNA preferentially bind DNA presented on PolIV. Immunology. 2005 Mar;114(3):418-27.

Kurien BT, Chambers TL, Thomas PY, Frank MB, Scofield RH. Autoantibody to the leucine zipper region of 52 kDa Ro/SSA binds native 60 kDa Ro/SSA: identification of a tertiary epitope with components from 60 kDa Ro/SSA and 52 kDa Ro/SSA. Scand J Immunol. 2001 Mar;53(3):268-76.

Kurien BT, Newland J, Paczkowski C, Moore KL, Scofield RH. Association of neutropenia in systemic lupus erythematosus (SLE) with anti-Ro and binding of an immunologically cross-reactive neutrophil membrane antigen. Clin Exp Immunol. 2000 Apr;120(1):209-17.

Lafer EM, Rauch J, Andrzejewski C Jr, Mudd D, Furie B, Furie B, Schwartz RS, Stollar BD. Polyspecific monoclonal lupus autoantibodies reactive with both polynucleotides and phospholipids.J Exp Med. 1981 Apr 1;153(4):897-909.

Lahita, R. G. (1999) *Systemic Lupus Erythematosus. III Edition. Academic Press.* pp 813-827

Lambert, P. H., and F. J. Dixon. 1968. Pathogenesis of glomerulonephritis of NZB/W mice.J. *Exp. Med.* 127:507.

Lauber K, Bohn E, Krober SM, Xiao YJ, Blumenthal SG, Lindemann RK, Marini P, Wiedig C, Zobywalski A, Baksh S, Xu Y, Autenrieth IB, Schulze-Osthoff K, Belka C, Stuhler G, Wesselborg S. Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. Cell. 2003 Jun 13; 113(6):717-30.

Lavrik I, Golks A, Krammer PH. Death receptor signaling. J Cell Sci. 2005a Jan 15;118 :265-7.

Lavrik IN, Golks A, Baumann S, Krammer PH. Caspase-2 is activated at the CD95 Death-Inducing Signaling complex in the course of CD95-induced apoptosis. Blood. 2006 Mar 23; [Epub ahead of print]

Lavrik IN, Golks A, Krammer PH. Caspases: pharmacological manipulation of cell death. J Clin Invest. 2005b Oct;115(10):2665-72.

Lawrence JS, Martins CL, Drake GL. A family survey of lupus erythematosus. 1. Heritability. J Rheumatol. 1987 Oct;14(5):913-21.

Le LQ, Kabarowski JH, Weng Z, Satterthwaite AB, Harvill ET, Jensen ER, Miller JF, Witte ON. Mice lacking the orphan G protein-coupled receptor G2A develop a late-onset autoimmune syndrome. Immunity. 2001 May;14(5):561-71.

Leandro MJ, Edwards JC, Cambridge G, Ehrenstein MR, Isenberg DA. An open study of B lymphocyte depletion in systemic lupus erythematosus. Arthritis Rheum. 2002 Oct;46(10):2673-7.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell. 1997 Nov 14;91(4):479-89.

Licht R, Dieker JW, Jacobs CW, Tax WJ, Berden JH. Decreased phagocytosis of apoptotic cells in diseased SLE mice. J Autoimmun. 2004 Mar;22(2):139-45.

Linker-Israeli M, Deans RJ, Wallace DJ, Prehn J, Ozeri-Chen T, Klinenberg JR. Elevated levels of endogenous IL-6 in systemic lupus erythematosus. A putative role in pathogenesis. J Immunol. 1991 Jul 1;147(1):117-23.

Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell. 1996 Jul 12;86(1):147-57.

Ma L, Chan KW, Trendell-Smith NJ, Wu A, Tian L, Lam AC, Chan AK, Lo CK, Chik S, Ko KH, To CK, Kam SK, Li XS, Yang CH, Leung SY, Ng MH, Stott I, MacPherson GG, Huang FP. Systemic autoimmune disease induced by dendritic cells that have captured necrotic but not apoptotic cells in susceptible mouse strains. Eur J Immunol. 2005 Nov;35(11):3364-75.

Macanovic M, Lachmann PJ. Measurement of deoxyribonuclease I (DNase) in the serum and urine of systemic lupus erythematosus (SLE)-prone NZB/NZW mice by a new radial enzyme diffusion assay. Clin Exp Immunol. 1997 May;108(2):220-6.

Madaio MP, Carlson J, Cataldo J, Ucci A, Migliorini P, Pankewycz O. Murine monoclonal anti-DNA antibodies bind directly to glomerular antigens and form immune deposits. J Immunol. 1987 May 1;138(9):2883-9.

Magnusson V, Nakken B, Bolstad AI, Alarcon-Riquelme ME. Cytokine polymorphisms in systemic lupus erythematosus and Sjogren's syndrome. Scand J Immunol. 2001 Jul-Aug;54(1-2):55-61.

Mahler M, Fritzler MJ, Bluthner M. Identification of a SmD3 epitope with a single symmetrical dimethylation of an arginine residue as a specific target of a subpopulation of anti-Sm antibodies. Arthritis Res Ther. 2005;7(1):R19-29.

Manfredi AA, Rovere P, Galati G, Heltai S, Bozzolo E, Soldini L, Davoust J, Balestrieri G, Tincani A, Sabbadini MG. Apoptotic cell clearance in systemic lupus erythematosus. I. Opsonization by antiphospholipid antibodies. Arthritis Rheum. 1998 Feb;41(2):205-14.

Manheimer-Lory AJ, Zandman-Goddard G, Davidson A, Aranow C, Diamond B. Lupus-specific antibodies reveal an altered pattern of somatic mutation. J Clin Invest. 1997 Nov 15;100(10):2538-46.

Manny N, Datta SK, Schwartz RS. Synthesis of IgM by cells of NZB and SWR mice and their crosses. J Immunol. 1979 Apr;122(4):1220-7.

Marmont AM, van Lint MT, Gualandi F, Bacigalupo A. Autologous marrow stem cell transplantation for severe systemic lupus erythematosus of long duration. Lupus. 1997;6(6):545-8.

Martin SJ, Finucane DM, Amarante-Mendes GP, O'Brien GA, Green DR. Phosphatidylserine externalization during CD95-induced apoptosis of cells and cytoplasts requires ICE/CED-3 protease activity. J Biol Chem. 1996 Nov 15;271(46):28753-6.

Mason LJ, Ravirajan CT, Rahman A, Putterman C, Isenberg DA. Is alpha-actinin a target for pathogenic anti-DNA antibodies in lupus nephritis? Arthritis Rheum. 2004 Mar;50(3):866-70.

Matsuura E, Igarashi Y, Yasuda T, Triplett DA, Koike T. Anticardiolipin antibodies recognize beta 2-glycoprotein I structure altered by interacting with an oxygen modified solid phase surface. J Exp Med. 1994 Feb 1;179(2):457-62.

McClain MT, Heinlen LD, Dennis GJ, Roebuck J, Harley JB, James JA. Early events in lupus humoral autoimmunity suggest initiation through molecular mimicry. Nat Med. 2005 Jan;11(1):85-9.

McNeil, H.P., Simpson, R.J, Chesterman C.N. & Krilis.S.A. Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta 2-glycoprotein I (apolipoprotein H). Proc Natl Acad Sci U S A. 1990 Jun;87(11):4120-4.

Means TK, Latz E, Hayashi F, Murali MR, Golenbock DT, Luster AD. Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. J Clin Invest. 2005 Feb;115(2):407-17.

Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH, Peter ME. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). EMBO J. 1997 May 15;16(10):2794-804.

Mehrad B, Park SJ, Akangire G, Standiford TJ, Wu T, Zhu J, Mohan C. The lupus-susceptibility locus, Sle3, mediates enhanced resistance to bacterial infections. J Immunol. 2006 Mar 1;176(5):3233-9.

Meilof JF, Veldhoven CH, Swaak AJ, Smeenk RJ. Production of anti-Ro/SS-A and anti-La/SS-B autoantibodies is closely coordinated in systemic lupus erythematosus and independent of anti-dsDNA production. J Autoimmun. 1997 Feb;10(1):67-75.

Mendlovic S, Brocke S, Shoenfeld Y, Ben-Bassat M, Meshorer A, Bakimer R, Mozes E. Induction of a systemic lupus erythematosus-like disease in mice by a common human anti-DNA idiotype. Proc Natl Acad Sci U S A. 1988 Apr;85(7):2260-4.

Mevorach D, Mascarenhas JO, Gershov D, Elkon KB. Complement-dependent clearance of apoptotic cells by human macrophages. J Exp Med. 1998a Dec 21;188(12):2313-20.

Mevorach D, Zhou JL, Song X, Elkon KB. Systemic exposure to irradiated apoptotic cells induces autoantibody production. J Exp Med. 1998b Jul 20;188(2):387-92.

Minota S, Yoshio T, Iwamoto M, Takeda A, Masuyama J, Mimori A, Yamada A, Kano S. Selective accumulation of anti-histone antibodies in glomeruli of lupus-prone lpr mice. Clin Immunol Immunopathol. 1996 Jul;80(1):82-7.

Miranda-Carus ME, Askanase AD, Clancy RM, Di Donato F, Chou TM, Libera MR, Chan EK, Buyon JP. Anti-SSA/Ro and anti-SSB/La autoantibodies bind the surface of apoptotic fetal cardiocytes and promote secretion of TNF-alpha by macrophages. J Immunol. 2000 Nov 1;165(9):5345-51.

Miranda-Carus ME, Boutjdir M, Tseng CE, DiDonato F, Chan EK, Buyon JP. Induction of antibodies reactive with SSA/Ro-SSB/La and development of congenital heart block in a murine model. J Immunol. 1998 Dec 1;161(11):5886-92.

Mohan C, Alas E, Morel L, Yang P, Wakeland EK. Genetic dissection of SLE pathogenesis. Sle1 on murine chromosome 1 leads to a selective loss of tolerance to H2A/H2B/DNA subnucleosomes. J Clin Invest. 1998 Mar 15;101(6):1362-72.

Mohan C, Morel L, Yang P, Wakeland EK. Genetic dissection of systemic lupus erythematosus pathogenesis: Sle2 on murine chromosome 4 leads to B cell hyperactivity. J Immunol. 1997 Jul 1;159(1):454-65.

Mohan C, Yu Y, Morel L, Yang P, Wakeland EK. Genetic dissection of Sle pathogenesis: Sle3 on murine chromosome 7 impacts T cell activation, differentiation, and cell death. J Immunol. 1999 Jun 1;162(11):6492-502.

Monestier M, Kandiah DA, Kouts S, Novick KE, Ong GL, Radic MZ, Krilis SA. Monoclonal antibodies from NZW x BXSB F1 mice to beta2 glycoprotein I and cardiolipin. Species specificity and charge-dependent binding. J Immunol. 1996 Apr 1;156(7):2631-41.

Morand EF, McCloud PI, Littlejohn GO. Continuation of long term treatment with hydroxychloroquine in systemic lupus erythematosus and rheumatoid arthritis. Ann Rheum Dis. 1992 Dec;51(12):1318-21.

Morel L, Croker BP, Blenman KR, Mohan C, Huang G, Gilkeson G, Wakeland EK. Genetic reconstitution of systemic lupus erythematosus immunopathology with polycongenic murine strains. Proc Natl Acad Sci U S A. 2000 Jun 6;97(12):6670-5.

Morse HC 3rd, Davidson WF, Yetter RA, Murphy ED, Roths JB, Coffman RL. Abnormalities induced by the mutant gene Ipr: expansion of a unique lymphocyte subset. J Immunol. 1982 Dec;129(6):2612-5.

Moser KL, Neas BR, Salmon JE, Yu H, Gray-McGuire C, Asundi N, Bruner GR, Fox J, Kelly J, Henshall S, Bacino D, Dietz M, Hogue R, Koelsch G, Nightingale L, Shaver T, Abdou NI, Albert DA, Carson C, Petri M, Treadwell EL, James JA, Harley JB. Genome scan of human systemic lupus erythematosus: evidence for linkage on chromosome 1q in African-American pedigrees. Proc Natl Acad Sci U S A. 1998 Dec 8;95(25):14869-74.

Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. Cell. 1996 Jun 14;85(6):817-27.

Nakano T, Ishimoto Y, Kishino J, Umeda M, Inoue K, Nagata K, Ohashi K, Mizuno K, Arita H. Cell adhesion to phosphatidylserine mediated by a product of growth arrest-specific gene 6. J Biol Chem. 1997 Nov 21;272(47):29411-4.

Napirei M, Karsunky H, Zevnik B, Stephan H, Mannherz HG, Moroy T. Features of systemic lupus erythematosus in Dnase1-deficient mice. Nat Genet. 2000 Jun;25(2):177-81.

Nimpf J, Bevers EM, Bomans PH, Till U, Wurm H, Kostner GM, Zwaal RF. Prothrombinase activity of human platelets is inhibited by beta 2-glycoprotein-I. Biochim Biophys Acta. 1986 Oct 29;884(1):142-9.

O'Brien CA, Wolin SL. A possible role for the 60-kD Ro autoantigen in a discard pathway for defective 5S rRNA precursors. Genes Dev. 1994 Dec 1;8(23):2891-903.

Ohnishi K, Ebling FM, Mitchell B, Singh RR, Hahn BH, Tsao BP. Comparison of pathogenic and non-pathogenic murine antibodies to DNA: antigen binding and structural characteristics. Int Immunol. 1994 Jun;6(6):817-30.

Okamura M, Kanayama Y, Amastu K, Negoro N, Kohda S, Takeda T, Inoue T. Significance of enzyme linked immunosorbent assay (ELISA) for antibodies to double stranded and single stranded DNA in patients with lupus nephritis: correlation with severity of renal histology. Ann Rheum Dis. 1993 Jan;52(1):14-20.

Ott M, Robertson JD, Gogvadze V, Zhivotovsky B, Orrenius S. Cytochrome c release from mitochondria proceeds by a two-step process. Proc Natl Acad Sci U S A. 2002 Feb 5;99(3):1259-63.

Pal R, Deshmukh US, Ohyama Y, Fang Q, Kannapell CC, Gaskin F, Fu SM. Evidence for multiple shared antigenic determinants within Ro60 and other lupus-related ribonucleoprotein autoantigens in human autoimmune responses. J Immunol. 2005 Dec 1;175(11):7669-77.

Pan ZJ, Davis K, Maier S, Bachmann MP, Kim-Howard XR, Keech C, Gordon TP, McCluskey J, Farris AD. Neo-epitopes are required for immunogenicity of the La/SS-B nuclear antigen in the context of late apoptotic cells. Clin Exp Immunol. 2006 Feb;143(2):237-48.

Papoff G, Hausler P, Eramo A, Pagano MG, Di Leve G, Signore A, Ruberti G. Identification and characterization of a ligand-independent oligomerization domain in the extracellular region of the CD95 death receptor. J Biol Chem. 1999 Dec 31;274(53):38241-50.

Peng Y, Kowalewski R, Kim S, Elkon KB. The role of IgM antibodies in the recognition and clearance of apoptotic cells. Mol Immunol. 2005 May;42(7):781-7.

Peter ME, Krammer PH. The CD95 (APO-1/Fas) DISC and beyond. Cell Death Differ. 2003 Jan;10(1):26-35.

Plescia OJ, Braun W, Palczuk NC. Production of antibodies to denatured deoxyribonucleic acid (DNA). Proc Natl Acad Sci U S A. 1964 Aug;52:279-85.

Portanova JP, Creadon G, Zhang X, Smith DS, Kotzin BL, Wysocki LJ. An early post-mutational selection event directs expansion of autoreactive B cells in murine lupus. Mol Immunol. 1995 Feb;32(2):117-35.

Putterman C, Diamond B. Immunization with a peptide surrogate for double-stranded DNA (dsDNA) induces autoantibody production and renal immunoglobulin deposition. J Exp Med. 1998 Jul 6;188(1):29-38.

Radic M, Marion T, Monestier M. Nucleosomes are exposed at the cell surface in apoptosis. J Immunol. 2004 Jun 1;172(11):6692-700.

Radic MZ, Mackle J, Erikson J, Mol C, Anderson WF, Weigert M. Residues that mediate DNA binding of autoimmune antibodies. J Immunol. 1993 Jun 1;150(11):4966-77.

Radu CG, Yang LV, Riedinger M, Au M, Witte ON. T cell chemotaxis to lysophosphatidylcholine through the G2A receptor. Proc Natl Acad Sci U S A. 2004 Jan 6;101(1):245-50.

Rankin EC, Isenberg DA. IgA deficiency and SLE: prevalence in a clinic population and a review of the literature. Lupus. 1997;6(4):390-4.

Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, Newman RA, Hanna N, Anderson DR. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. Blood. 1994 Jan 15;83(2):435-45.

Retter MW, Cohen PL, Eisenberg RA, Clarke SH. Both Sm and DNA are selecting antigens in the anti-Sm B cell response in autoimmune MRL/lpr mice. J Immunol. 1996 Feb 1;156(3):1296-306.

Reymond A, Meroni G, Fantozzi A, Merla G, Cairo S, Luzi L, Riganelli D, Zanaria E, Messali S, Cainarca S, Guffanti A, Minucci S, Pelicci PG, Ballabio A. The tripartite motif family identifies cell compartments. EMBO J. 2001 May 1;20(9):2140-51.

Riedl SJ, Renatus M, Schwarzenbacher R, Zhou Q, Sun C, Fesik SW, Liddington RC, Salvesen GS. Structural basis for the inhibition of caspase-3 by XIAP. Cell. 2001 Mar 9;104(5):791-800.

Ronnblom LE, Alm GV, Oberg KE. Autoimmunity after alpha-interferon therapy for malignant carcinoid tumors. Ann Intern Med. 1991 Aug 1;115(3):178-83.

Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J. 1997 Dec 1;16(23):6914-25.

Rozzo SJ, Vyse TJ, David CS, Palmer E, Izui S, Kotzin BL. Analysis of MHC class II genes in the susceptibility to lupus in New Zealand mice. J Immunol. 1999 Mar 1;162(5):2623-30.

Rutjes SA, Vree Egberts WT, Jongen P, Van Den Hoogen F, Pruijn GJ, Van Venrooij WJ. Anti-Ro52 antibodies frequently co-occur with anti-Jo-1 antibodies in sera from patients with idiopathic inflammatory myopathy. Clin Exp Immunol. 1997 Jul;109(1):32-40.

Sabbatini A, Bombardieri S, Migliorini P. Autoantibodies from patients with systemic lupus erythematosus bind a shared sequence of SmD and Epstein-Barr virus-encoded nuclear antigen EBNA I. Eur J Immunol. 1993 May;23(5):1146-52.

Salmon JE, Millard S, Schachter LA, Arnett FC, Ginzler EM, Gourley MF, Ramsey-Goldman R, Peterson MG, Kimberly RP. Fc gamma RIIA alleles are heritable risk factors for lupus nephritis in African Americans. J Clin Invest. 1996 Mar 1;97(5):1348-54.

Santiago-Raber ML, Baccala R, Haraldsson KM, Choubey D, Stewart TA, Kono DH, Theofilopoulos AN. Type-I interferon receptor deficiency reduces lupus-like disease in NZB mice. J Exp Med. 2003 Mar 17;197(6):777-88.

Satoh M, Kumar A, Kanwar YS, Reeves WH. Anti-nuclear antibody production and immune-complex glomerulonephritis in BALB/C mice treated with pristane. Proc Natl Acad Sci U S A. 1995 Nov 21;92(24):10934-8.

Savarese E, Chae OW, Trowitzsch S, Weber G, Kastner B, Akira S, Wagner H, Schmid RM, Bauer S, Krug A. U1 small nuclear ribonucleoprotein immune complexes induce type I interferon in plasmacytoid dendritic cells through TLR7. Blood. 2006 Apr 15;107(8):3229-34.

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME. Two CD95 (APO-1/Fas) signaling pathways. EMBO J. 1998 Mar 16;17(6):1675-87.

Scaffidi C, Medema JP, Krammer PH, Peter ME. FLICE is predominantly expressed as two functionally active isoforms, caspase-8/a and caspase-8/b. J Biol Chem. 1997 Oct 24;272(43):26953-8.

Schultze HE. Glycoproteins of human plasma. Bull Schweiz Akad Med Wiss. 1961 Oct;17:77-91.

Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S, Peter ME. Apoptosis signaling by death receptors. Eur J Biochem. 1998 Jun 15;254(3):439-59.

Schur PH. Genetics of systemic lupus erythematosus. Lupus. 1995 Dec;4(6):425-37.

Scofield RH, Dickey WD, Jackson KW, James JA, Harley JB. A common autoepitope near the carboxyl terminus of the 60-kD Ro ribonucleoprotein: sequence similarity with a viral protein. J Clin Immunol. 1991 Nov;11(6):378-88.

Scott RS, McMahon EJ, Pop SM, Reap EA, Caricchio R, Cohen PL, Earp HS, Matsushima GK. Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature. 2001 May 10;411(6834):207-11.

Seeman NC, Rosenberg JM, Rich A. Sequence-specific recognition of double helical nucleic acids by proteins. Proc Natl Acad Sci U S A. 1976 Mar;73(3):804-8.

Segundo C, Medina F, Rodriguez C, Martinez-Palencia R, Leyva-Cobian F, Brieva JA. Surface molecule loss and bleb formation by human germinal center B cells undergoing apoptosis: role of apoptotic blebs in monocyte chemotaxis. Blood. 1999 Aug 1;94(3):1012-20.

Sheng Y, Kandiah DA, Krilis SA. Anti-beta 2-glycoprotein I autoantibodies from patients with the "antiphospholipid" syndrome bind to beta 2-glycoprotein I with low affinity: dimerization of beta 2-glycoprotein I induces a significant increase in anti-beta 2-glycoprotein I antibody affinity. J Immunol. 1998 Aug 15;161(4):2038-43.

Shoenfeld Y. Idiotypic induction of autoimmunity: a new aspect of the idiotypic network. FASEB J. 1994 Dec;8(15):1296-301.

Shoenfeld Y. Etiology and pathogenetic mechanisms of the anti-phospholipid syndrome unraveled. Trends Immunol. 2003 Jan;24(1):2-4.

Siegel RM, Frederiksen JK, Zacharias DA, Chan FK, Johnson M, Lynch D, Tsien RY, Lenardo MJ. Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. Science. 2000 Jun 30;288(5475):2354-7.

Spatz L, Saenko V, Iliev A, Jones L, Geskin L, Diamond B. Light chain usage in anti-double-stranded DNA B cell subsets: role in cell fate determination. J Exp Med. 1997 Apr 7;185(7):1317-26.

Sprick MR, Weigand MA, Rieser E, Rauch CT, Juo P, Blenis J, Krammer PH, Walczak H. FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. Immunity. 2000 Jun;12(6):599-609.

Stathopoulou EA, Routsias JG, Stea EA, Moutsopoulos HM, Tzioufas AG. Cross-reaction between antibodies to the major epitope of Ro60 kD autoantigen and a homologous peptide of Coxsackie virus 2B protein. Clin Exp Immunol. 2005 Jul;141(1):148-54.

Surh CD, Sprent J. T-cell apoptosis detected in situ during positive and negative selection in the thymus. Nature. 1994 Nov 3;372(6501):100-3.

Suzuki N, Harada T, Mizushima Y, Sakane T. Possible pathogenic role of cationic anti-DNA autoantibodies in the development of nephritis in patients with systemic lupus erythematosus. J Immunol. 1993 Jul 15;151(2):1128-36.

Swaak AJ, Aarden LA, Statius van Eps LW, Feltkamp TE. Anti-dsDNA and complement profiles as prognostic guides in systemic lupus erythematosus. Arthritis Rheum. 1979 Mar;22(3):226-35.

Tackey E, Lipsky PE, Illei GG. Rationale for interleukin-6 blockade in systemic lupus erythematosus. Lupus. 2004;13(5):339-43.

Takeda Y, Caudell P, Grady G, Wang G, Suwa A, Sharp GC, Dynan WS, Hardin JA. Human RNA helicase A is a lupus autoantigen that is cleaved during apoptosis. J Immunol. 1999 Dec 1;163(11):6269-74.

Thome M, Schneider P, Hofmann K, Fickenscher H, Meinl E, Neipel F, Mattmann C, Burns K, Bodmer JL, Schroter M, Scaffidi C, Krammer PH, Peter ME, Tschopp J. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature. 1997 Apr 3;386(6624):517-21.

Thornberry NA, Lazebnik Y. Caspases: enemies within. Science. 1998 Aug 28;281(5381):1312-6.

Tillman DM, Jou NT, Hill RJ, Marion TN. Both IgM and IgG anti-DNA antibodies are the products of clonally selective B cell stimulation in (NZB x NZW)F1 mice. J Exp Med. 1992 Sep 1;176(3):761-79.

Topfer F, Gordon T, McCluskey J. Intra- and intermolecular spreading of autoimmunity involving the nuclear self-antigens La (SS-B) and Ro (SS-A). Proc Natl Acad Sci U S A. 1995 Jan 31;92(3):875-9.

Tran HB, Macardle PJ, Hiscock J, Cavill D, Bradley J, Buyon JP, Gordon TP. Anti-La/SSB antibodies transported across the placenta bind apoptotic cells in fetal organs targeted in neonatal lupus. Arthritis Rheum. 2002 Jun;46(6):1572-9.

Traynor AE, Barr WG, Rosa RM, Rodriguez J, Oyama Y, Baker S, Brush M, Burt RK. Hematopoietic stem cell transplantation for severe and refractory lupus. Analysis after five years and fifteen patients. Arthritis Rheum. 2002 Nov;46(11):2917-23.

Tzioufas AG, Yiannaki E, Sakarellos-Daitsiotis M, Routsias JG, Sakarellos C, Moutsopoulos HM. Fine specificity of autoantibodies to La/SSB: epitope mapping, and characterization. Clin Exp Immunol. 1997 May;108(2):191-8.

Vlahakos DV, Foster MH, Adams S, Katz M, Ucci AA, Barrett KJ, Datta SK, Madaio MP. Anti-DNA antibodies form immune deposits at distinct glomerular and vascular sites. Kidney Int. 1992 Jun;41(6):1690-700.

Verbovetski I, Bychkov H, Trahtemberg U, Shapira I, Hareuveni M, Ben-Tal O, Kutikov I, Gill O, Mevorach D. Opsonization of apoptotic cells by autologous iC3b facilitates clearance by immature dendritic cells, down-regulates DR and CD86, and up-regulates CC chemokine receptor 7. J Exp Med. 2002 Dec 16;196(12):1553-61.

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell. 2000 Jul 7;102(1):43-53.

Verhoven B, Schlegel RA, Williamson P. Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. J Exp Med. 1995 Nov 1;182(5):1597-601.

Vollmer J, Tluk S, Schmitz C, Hamm S, Jurk M, Forsbach A, Akira S, Kelly KM, Reeves WH, Bauer S, Krieg AM. Immune stimulation mediated by autoantigen binding sites within small nuclear RNAs involves Toll-like receptors 7 and 8. J Exp Med. 2005 Dec 5;202(11):1575-85.

Vyse TJ, Drake CG, Rozzo SJ, Roper E, Izui S, Kotzin BL. Genetic linkage of IgG autoantibody production in relation to lupus nephritis in New Zealand hybrid mice. J Clin Invest. 1996a Oct 15;98(8):1762-72.

Vyse TJ, Morel L, Tanner FJ, Wakeland EK, Kotzin BL. Backcross analysis of genes linked to autoantibody production in New Zealand White mice. J Immunol. 1996b Sep 15;157(6):2719-27.

Wahren-Herlenius M, Muller S, Isenberg D. Analysis of B-cell epitopes of the Ro/SS-A autoantigen. Immunol Today. 1999 May;20(5):234-40.

Waisman A, Ruiz PJ, Israeli E, Eilat E, Konen-Waisman S, Zinger H, Dayan M, Mozes E. Modulation of murine systemic lupus erythematosus with peptides based on complementarity determining regions of a pathogenic anti-DNA monoclonal antibody. Proc Natl Acad Sci U S A. 1997 Apr 29;94(9):4620-5.

Wakeland EK, Liu K, Graham RR, Behrens TW. Delineating the genetic basis of systemic lupus erythematosus. Immunity. 2001 Sep;15(3):397-408.

Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, Boldin MP. Tumor necrosis factor receptor and Fas signaling mechanisms. Annu Rev Immunol. 1999;17:331-67.

Wang J, Chun HJ, Wong W, Spencer DM, Lenardo MJ. Caspase-10 is an initiator caspase in death receptor signaling. Proc Natl Acad Sci U S A. 2001 Nov 20;98(24):13884-8.

Wellmann U, Letz M, Herrmann M, Angermuller S, Kalden JR, Winkler TH. The evolution of human anti-double-stranded DNA autoantibodies. Proc Natl Acad Sci U S A. 2005 Jun 28;102(26):9258-63.

Wu J, Edberg JC, Redecha PB, Bansal V, Guyre PM, Coleman K, Salmon JE, Kimberly RP. A novel polymorphism of FcgammaRIIIa (CD16) alters receptor function and predisposes to autoimmune disease. J Clin Invest. 1997 Sep 1;100(5):1059-70.

Wurm H. beta 2-Glycoprotein-I (apolipoprotein H) interactions with phospholipid vesicles. Int J Biochem. 1984;16(5):511-5.

Xue D, Shi H, Smith JD, Chen X, Noe DA, Cedervall T, Yang DD, Eynon E, Brash DE, Kashgarian M, Flavell RA, Wolin SL. A lupus-like syndrome develops in mice lacking the Ro 60-kDa protein, a major lupus autoantigen. Proc Natl Acad Sci U S A. 2003 Jun 24;100(13):7503-8.

Yan JJ, Jung JS, Lee JE, Lee J, Huh SO, Kim HS, Jung KC, Cho JY, Nam JS, Suh HW, Kim YH, Song DK. Therapeutic effects of lysophosphatidylcholine in experimental sepsis. Nat Med. 2004 Feb;10(2):161-7.

Zachowski A, Favre E, Cribier S, Herve P, Devaux PF. Outside-inside translocation of aminophospholipids in the human erythrocyte membrane is mediated by a specific enzyme. Biochemistry. 1986 May 6;25(9):2585-90.

Zhao Z, Weinstein E, Tuzova M, Davidson A, Mundel P, Marambio P, Putterman C. Cross-reactivity of human lupus anti-DNA antibodies with alpha-actinin and nephritogenic potential. Arthritis Rheum. 2005 Feb;52(2):522-30.

Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell. 1997 Aug 8;90(3):405-13.

Appendix

APPENDIX

Annexin-V Binding Buffer (10X)

Hepes	100 mM
Sodium Chloride (NaCl)	1.4 M
Calcium Chloride ($CaCl_2$)	25 mM

Polymerase Chain Reaction

cDNA Synthesis Mix

Annealed RNA	20 ul
Rnasin	1.0 µl
5X RT buffer	5.0 µl
10 mM dNTP mix	1.0 µl
100 mM DTT	2.0 µl
Reverse Transcriptase	1.0 µl

PCR Amplification Mix (Heavy and Light Chain)

cDNA	2.5 µl
dNTPs (10 mM)	0.5 µl
10X buffer	2.5 µl
Forward Primer	0.5 µl (5 pM)
Reverse Primer	0.5 µl (5 pM)
H_2O	18.0 µl
Taq Polymerase	0.5 µl (5 U)

Ligation Mixture

2X Ligation Buffer	7.5 µl
pGEMT Easy Vector	1.0 µl
DNA	5.5 µl
T4 DNA Ligase	1.0 µl

***FACS Buffer* (pH 7.4)**

PBS	10.0 mM
BSA	1.0 % w/v
NaN_3	0.2 % w/v

Lysis Buffer

Tris HCl (pH 7.4)	50 mM
KCl	25 mM
$MgCl_2$	5 mM
EDTA	1 mM

SDS-PAGE

Monomer Stock Solution

Acrylamide	60 g
Bis-acrylamide	1.60 g

***4X Resolving Gel Buffer* (pH 8.8)**

Tris (Basse)	1.50 M

***4X Stacking Gel Buffer* (pH 6.8)**

Tris (Base)	50 mM

6X Sample Loading Buffer

4X Tris Base (pH 6.8)	35 mM
Glycerol	30.0 % v/v
SDS	10.0 % w/v
DTT	9.3 % w/v
Bromophenol Blue	1.2 mg/10 ml

Electrophoresis Buffer

Tris	25 mM
Glycine	192 mM
SDS	0.1 % w/v

Resolving Gel (12.5%)

30% Acrylamide stock	8.0 ml
H_2O	6.0 ml
1 M Tris-HCl, pH 8.8	5.0 ml
10% SDS	0.2 ml
10% APS	0.2 ml
TEMED	20.0 µl

Stacking Gel (3.0%)

30% Acrylamide stock	1.3 ml
H_2O	6.0 ml
1 M Tris-HCl, pH 6.8	2.5 ml
10% SDS	0.1 ml
10% APS	0.1 ml
TEMED	10.0 µl

Western Blotting

Transfer Buffer

Tris	25 mM
Glycine	192 mM
Methanol	20 % v/v

Immunoblotting

Blocking Buffer

PBS (pH 7.4)	10 mM
Lactogen 1	5 % w/v
BSA	1 % w/v

Dilution Buffer

PBST 0.05% (pH 7.4)	10 mM
BSA	1 % w/v

Wash Buffer (PBST)

PBS (pH 7.4)	10 mM
Tween 20	0.05 %

IgG Purification

Binding Buffer

PBS (pH 7.4)	10 mM

Elution Buffer

Glycine HCl (pH 2.5)	100 mM

Neutralization Buffer

Tris Base (pH 9.2)	1 M

IgM Purification

Equilibriation Buffer

Tris Base (pH 7.4)	10 mM

Binding Buffer

Tris Base (pH 7.4)	10 mM
$CaCl_2$	20 mM

Elution Buffer

Tris Base (pH 7.4)	10 mM
EDTA	10 mM

Bacterial Culture

***1 Litre LB Agar* (pH 7.0)**

NaCl	10 g
Tryptone	10 g
Yeast Extract	5 g
Agar	20 g

LB plates were supplemented with tetracycline 20 μg/ml or ampicillin 100 μg/ml for appropriate use.

X-Gal IPTG Stock

IPTG	1.25 g
X-Gal	1.0 g
DMF	25 ml (Total Volume)

1 Litre LB Agar with X-Gal/ IPTG

LB Agar	
X-Gal IPTG stock	1 ml

***1 Litre LB Broth* (pH 7.0)**

NaCl	10 g
Tryptone	10 g
Yeast Extract	5 g

1 Litre Soft Top Agar

LB Broth	1000 ml
$MgSO_4$	10 mM
Agarose	7.2 g

ELISA

Blocking Buffer

PBS (pH 7.4)	10 mM
BSA	2 % w/v
OR	
Poly Vinyl Alcohol (PVA)	0.1 % w/v (For Phospholipid ELISA)

***Carbonate-Bicarbonate Buffer* (pH 9.6)**

Na_2CO_3	15 mM
$NaHCO_3$	35 mM

***Citrate-Phosphate Buffer* (pH 5.0)**

Citric acid	22.1 mM
NaH_2PO_4	51.4 mM

Dilution Buffer

PBST 0.05% (pH 7.4)	10 mM
BSA	1 % w/v

TMB Substrate stock

3, 3', 5, 5',-Tetra Methyl benzidine	41 mM
Tetra Methyl Ammonium Borohydride	8.2 mM
N, N-Dimethyl Acetamide	10 ml (Total Volume)

TMB Substrate

200 μl of TMB stock + 8 μl H_2O_2 in 8ml Citrate-Phosphate Buffer

Wash Buffer

PBS (pH 7.4)	10 mM
Tween 20	0.05 % v/v

Agarose Gel Electrophoresis

50X* Tris *Acetate EDTA Buffer (TAE)

Tris Acetate	40 mM
EDTA	1 mM

Phage Display

***Coating Buffer* (pH 9.2)**

Na_2CO_3	15 mM
$NaHCO_3$	35 mM

Blocking Buffer

Coating Buffer containing 1% BSA

Wash Buffer

Tris Base (pH 7.4)	50 mM
Sodium Chloride	140 mM
Tween 20	0.5 % v/v

Iodide Buffer

Tris HCl (pH 8.0)	10 mM
Sodium Iodide (NaI)	4 M
EDTA	1 mM

Name	Bases	Degeneracy	aa position	Sequence (5'-3')
MuIgVH5'-A	33	512	-20 to -13	GGGAATTCATGRASTTSKGGYTMARCTKGRTTT
MuIgVH5'-B	34	64	-20 to -13	GGGAATTCATGRAATGSASCTGGGTYWTYCTCTT
MuIgVH5'-C	39	-	-20 to -11	ACTAGTCGACATGGACTCCAGGCTCAATTTAGTTTTCCT
	36	48	-20 to -12	ACTAGTCGACATGGCTGTCYTRGBGCTGYTCYTCTG
	39	24	-20 to -11	ACTAGTCGACATGGVTTGGSTGTGGAMCTTGCYATTCCT
MuIgVH5'-D	36	8	-20 to -12	ACTAGTCGACATGAAATGCAGCTGGRTYATSTTCTT
	36	32	-20 to -12	ACTAGTCGACATGGRCAGRCTTACWTYYTCATTCCT
	36	-	-20 to -12	ACTAGTCGACATGATGGTGTTAAGTCTTCTGTACCT
MuIgVH5'-E	36	8	-20 to -12	ACTAGTCGACATGGGATGGAGCTRTATCATSYTCTT
	33	24	-20 to -13	ACTAGTCGACATGAAGWTGTGGBTRAACTGGRT
	35	64	-20 to -13	ACTAGTCGACATGGRATGGASCKKIRTCTTTMTCT
MuIgVH5'-F	35	32	-20 to -13	ACTAGTCGACATGAACTTYGGGYTSAGMTTGRTTT
	35	-	-20 to -13	ACTAGTCGACATGTACTTGGGACTGAGCTGTGTAT
	33	-	-20 to -13	ACTAGTCGACATGAGAGTGCTGATTCTTTTGTG
	38	-	-20 to -12	ACTAGTCGACATGGATTTTGGGCTGATTTTTTTTATTG
MuIgMVH3'-1	32	-	125 to 118	CCCAAGCTTACGAGGGGGAAGACATTTGGGAA
MuIgGVH3'-2	35	32	126 to 119	CCCAAGCTTCCAGGGRCCARKGGATARACIGRTGG
MuIgκVL5'-A	32	32	-20 to -13	GGGAATTCATGRAGWCACAKWCYCAGGTCTTT
MuIgκVL5'-B	33	-	-20 to -13	GGGAATTCATGGAGACAGACACACTCCTGCTAT
MuIgκVL5'-C	39	8	-20 to -11	ACTAGTCGACATGGAGWCAGACACACTSCTGYTATGGGT
MuIgκVL5'-D	42	16	-20 to -10	ACTAGTCGACATGAGGRCCCCTGCTCAGWTTYTTGGIWTCTT
	41	128	-24 to -14	ACTAGTCGACATGGGCWTCAAGATGRAGTCACAKWYYCWGG
MuIgκVL5'-E	39	4	-20 to -11	ACTAGTCGACATGAGTGTGCYCACTCAGGTCCTGGSGTT
	41	32	-15 to -5	ACTAGTCGACATGTGGGGAYCGKTTTYAMMCTTTTCAATTG
	38	-	-20 to -11	ACTAGTCGACATGGAAGCCCCAGCTCAGCTTCTCTTCC
MuIgκVL5'-F	36	32	-20 to -12	ACTAGTCGACATGAGIMMKTCIMTTCAITTCYTGGG
	36	96	-20 to -12	ACTAGTCGACATGAKGTHCYCIGCTCAGYTYCTIRG
	35	8	-20 to -12	ACTAGTCGACATGGTRTCCWCASCTCAGTTCCTTG
	37	-	-16 to -8	ACTAGTCGACATGTATATATGTTTGTTGTCTATTTCT
MuIgκVL5'-G	39	-	-19 to -10	ACTAGTCGACATGAAGTTGCCTGTTAGGCTGTTGGTGCT
	39	8	-22 to -13	ACTAGTCGACATGGATTTWCARGTGCAGATTWTCAGCTT
	37	12	-15 to -7	ACTAGTCGACATGGTYCTYATVTCCTTGCTGTTCTGG
	37	24	-15 to -7	ACTAGTCGACATGGTYCTYATVTTRCTGCTGCTATGG
MuIgκVL3'-1	30	-	122 to 116	CCCAAGCTTACTGGATGGTGGGAAGATGGA
MuIgλVL5'-A	33	128	-20 to -13	GGGAATTCATGGCCTGGAYTYCWCTYWTMYTCT
MuIgλVL3'-1	32	32	125 to 118	CCCAAGCTTAGCTCYTCWGWGGAIGGYGGRAA

List of primers used for amplification of the antibody genes.