B CELL SIGNALING AND BIOINFORMATICS: REVEALING COMPONENTS OF THE MHC CLASS II ANTIGEN PROCESSING AND PRESENTATION PATHWAY

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Dedication

To Mom, Dad, Robert, and Andie

B CELL SIGNALING AND BIOINFORMATICS: REVEALING COMPONENTS OF THE MHC CLASS II ANTIGEN PROCESSING AND PRESENTATION PATHWAY

by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

March, 2005

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Acknowledgements

I first wish to thank my committee members. I have had two different committees since starting graduate school. Drs. Bill Garrard, Toru Miyazaki, and Dorothy Yuan were members of my first dissertation committee, and I thank them for their encouragement and understanding when a change in project direction forced me to choose different committee members. My current dissertation committee includes Drs. Bill Garrard, Anne Satterthwaite, and Akira Takashima. I thank them for their enthusiasm and guidance throughout my project. Bill was always quick to let me know when he came across an interesting piece of literature, Akira provided me with many resources to advance my project, and Anne always had her door open if I needed help with the direction of my project.

I especially want to thank my mentor, Dr. Richard Scheuermann. I admire his approach to science and his commitment to teaching. Graduate school is often described as a "sink or swim" situation. I can say that my experience in graduate school has benefited from Richard's commitment to teaching me how to "swim". I admire Richard's ambition and the risks he is willing to take for the things that he believes in. The lab has changed a great deal during my stay and I believe that these changes have benefited the lab enormously. It is no coincidence that the lab atmosphere and dynamics has always been wonderful, despite all of the different people that have been a part of the lab. Richard's hard work and persistence will not go unnoticed.

I thank several collaborators that were involved in the CLASSIFI and microarray analysis: Bob Sinkovits, Dennis Mock, Brian Saunders, Sangdun Choi, Shankar Subramanian, and Peng Yang.

I would like to acknowledge several people who have helped me on various projects that were not described in this dissertation. I thank Dr. Dianne DeCamp and the AfCS Protein Lab for their help with 2-D gels. I thank the AfCS Cell Lab and the AfCS Antibody Lab for their help with various techniques. Dr. John Schatzle and Jill Mooney helped me design experiments, and although my project moved in a different direction, I thank them for their time and willingness to help. I interacted with many people during lab rotations who have influenced the way I think scientifically: I thank Drs. Iwona Stroynowski, Ming Chen, Jik Ungchusri, Vinay Kumar, Michael Bennett, P.V. Sivakumar, Tad George, and Noelle Williams.

There are several people I would like to thank for their help in some of the experiments described in my dissertation. Dr. Keng Mean-Lin spent an entire afternoon helping me with the calcium flux assays. Brant Ward spent more time than he should have to help me obtain confocal microscopy images. Although I ultimately did not use these images, I thank him very much for sacrificing his own time on the confocal microscope to help me. Dr. Keith Wharton helped with fluorescence microscopy techniques. Shane Scoggin and Sterling Ortega helped me with real-time PCR.

I would like to thank all the people that I interacted with who were once part of the Scheuermann Lab. These people are not only colleagues but good friends as well. I especially thank Dr. Angus Sinclair. I learned an enormous amount from him and my

accomplishments on the project we worked on together were a direct result of his teaching. I thank Drs. Shelley Belouski, Nancy Liu, Greg Hosler, and Adrienne Hammill for their help and friendship. I thank the current members of the Scheuermann Lab basic research group: Dr. Suwan Sinha, Xin Bai, and Eva Rab for their friendship and collaboration. I also thank the members of the bioinformatics group for their friendship and help with the bioinformatics analysis and other computer issues: Jennifer Cai, Burke Squires, and Dr. Feng Luo.

I would like to thank the members of the Immunology Graduate Program and the Department of Pathology. They are too numerous to name here, but everyone I interacted with had a positive impact on my graduate school experience.

I would like to give a special thanks to my friends "outside" of the lab. Dr. Ray Lee will do anything to help you out, and always with a smile. Dr. Chia-Ying Wang is a great friend and provider of comic relief (without meaning to). Rachel Cook has been a close friend for a very long time and always manages to make herself available when I need to talk.

I would like to thank my parents for their encouragement during my education. My love of science comes from my Dad, who is always looking for anyone interested in listening to him explain how something works. My Mom taught me to be ambitions and is always excited for me when I reach a goal. I would also like to thank my future mother-in-law, Dr. Andie Hsueh, for her support and encouragement. She provided a second home for me to visit on weekends, complete with the Best Fried Chicken in the World.

Finally, I would like to thank my fiancé, Dr. Robert Hsueh. Robert has been a constant source of love and support. Without him, graduate school would have been a different story, and life would not have been so fulfilling.

B CELL SIGNALING AND BIOINFORMATICS: REVEALING COMPONENTS OF THE MHC CLASS II ANTIGEN PROCESSING AND PRESENTATION PATHWAY

Publication No._____

Jamie Ann Lee, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2005

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Stimulation of mature B lymphocytes by extracellular ligands induces phenotypic changes through complex signal transduction pathways. Gene expression is altered as a result of these changes and re-programs the cell to undergo differentiation, activation, effecter function, anergy, and/or apoptosis. Gene expression microarrays are used to determine expression levels of a large number (tens of thousands) of genes simultaneously, resulting in a gene expression profile of the experimental sample. Microarray data must be appended with biological information in order to be interesting, and this field of microarray bioinformatics is rapidly expanding. These studies prompted the development of a bioinformatics tool termed CLASSIFI (*Cl*uster *Assi*gnment *f*or Biological *I*nference), which identifies statistically significant co-clustering of genes with similar Gene Ontology annotation within microarray gene clusters. CLASSIFI was used to analyze microarray

results from two B cell projects from the Alliance for Cellular Signaling (AfCS): 1) the BAFF/CD40L project, which evaluates the effects of BAFF and CD40L on primary mouse B cells in long-term cultures, and 2) the B cell single ligand screen project, which evaluates the effects of 32 single ligands on primary mouse B cells in short-term cultures. CLASSIFI was able to identify significant overrepresentation of related genes within gene clusters for both of these data sets and facilitates hypothesis generation as to the biological process affected by a specific ligand. As CLASSIFI is strictly a statistical tool that aids in hypothesis generation, experimental validation of hypotheses was performed. The B cell single ligand screen microarray and CLASSIFI analysis followed by experimental validation revealed a biological process specific to B cell antigen receptor stimulation but not LPS or CD40L stimulation – antigen processing and presentation – and provides the groundwork for new discoveries in this field. As a result, several putative components were identified that are not currently known to play a role in antigen processing and presentation in B cells.

Table of Contents

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*contributed equally

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*contributed equally

List of Tables and Figures

Abbreviations

7AAD	-	7-amino-actinomycin D
AfCS	-	Alliance for Cellular Signaling
AIG	-	anti-IgM
anti-Ig	-	anti-immunoglobulin
APC	-	Antigen Presenting Cell
BAFF	-	B-cell activating factor of the TNF family
BCR	-	B cell receptor
BLNK	-	B cell linker protein
BrdU	-	bromodeoxyuridine
BSA	-	bovine serum albumin
CIIV	-	Class II vesicle
CLASSIFI	-	CLuster ASSIgnment For biological Inference
CLIP	-	Class II-associated invariant chain peptide
CTL	-	cytotoxic T lymphocyte
DAPI	-	4', 6-diamidino-2-phenylindole, dihydrochloride
DC	-	Dendritic Cell
DIC	-	differential interference contrast
DNA	-	deoxyribonucleic acid
DTT	-	dithiothreitol
ER	-	endoplasmic reticulum

FACS	-	fluorescence activated cell sorting
FBS	-	fetal bovine serum
FDC	-	Follicular dendritic cell
FITC	-	fluorescein isothiocyanate
G_0	-	Gap 0 phase
G_1	-	Gap 1 phase
G_2	-	Gap 2 phase
GFP	-	green fluorescence protein
GILT	-	gamma interferon-inducible lysosomal thiol reductase
GO	-	Gene Ontology
ICKA	-	immune complex kinase assay
Ig	-	Immunoglobulin
IgA	-	Immunoglobulin of the α heavy chain class
IgD	-	Immunoglobulin of the δ heavy chain class
IgE	-	Immunoglobulin of the ε heavy chain class
IgM	-	Immunoglobulin of the μ heavy chain class
Ii	-	invariant chain
ITAM	-	Immunoreceptor tyrosine-based activation motif
LPS	-	lipopolysaccharide
M	-	mitosis phase
MACS	-	Magnetic activated cell sorting
MHC	-	Major Histocompatibility Complex

MIIC	-	MHC Class II compartment
NK cell	-	Natural Killer Cell
PAMP	-	pathogen-associated molecular pattern
PBS	-	phosphate buffered saline
PE	-	phycoerythrin
PKC	-	protein kinase C
RA	-	Rheumatoid arthritis
RBC	-	red blood cell
RT-PCR	-	reverse transcriptase polymerase chain reaction
SAM	-	Significance analysis of microarrays
SLE	-	Systemic Lupus Erythematosus
Syk	-	Spleen tyrosine kinase
T1	-	First Transitional
T2	-	Second Transitional
T3	-	Third Transitional
TAP	-	transporter protein
TBS	-	tris-buffered saline
TCR	-	T Cell Receptor
TGN	-	trans-Golgi network
T_H	-	helper T cell
TLR	-	Toll-like receptor
TNF	-	tumor necrosis factor

Vps	-	vacuolar protein sorting
WBC	-	white blood cell

Chapter 1

Background

Introduction

The B cell compartment of the immune system is critical in providing specific immune responses to a variety of foreign antigens. In partnership with other antigen presenting cells (APCs) and T cells, B cells differentiate to antibody-secreting cells, providing protection against specific pathogens. In addition, B cells provide the host with long-lasting memory, ensuring a robust immune response upon re-exposure.

B cell function depends on carefully regulated developmental steps involving checkpoints that shape and mold the B cell repertoire. The fate of a B cell depends on the nature of signals through the B cell receptor (BCR), the stage of development of the B cell, and presence of other signals necessary for B cell activation. Failure of any one of these checkpoints can result in autoimmunity, where autoreactive B cells that escape tolerance lead to a disease state. B cells develop centrally in the bone marrow, then migrate to the periphery to continue development and undergo activation and differentiation in the spleen. B cell tolerance is thought to occur in these two major compartments. Differentiation of B cells to become antibody-secreting cells involves interaction with T cells and APCs in the spleen. Presentation of antigen in the context of Major Histocompatibility Complex (MHC) molecules is required for B cell interaction with T cells.

B cells are a unique APC in that they process and present antigen that bind specifically to the BCR. This is in contrast to dendritic cells and macrophages, which are also APCs but can take up antigen non-specifically through pinocytosis or phagocytosis.

Specific antigen uptake is an important component of the immune response because the BCR allows highly efficient binding and uptake of low affinity or rare antigens.

This chapter reviews B cell development and activation from bone marrow to spleen, and focuses on MHC Class II antigen processing and presentation.

B cell development and activation

B cell development in the bone marrow. The acquired immune response, which develops upon contact with an intruder and is characterized by its specificity, consists of 3 main cell types: B cells, T cells, and APCs. B cells mediate the humoral arm of immunity and their main purpose is to produce antibody, which functions to eliminate the antigen from the host. The development of B cells involves tightly regulated stages, and the goal is to populate the host with a pool of B cells capable of responding specifically to a diversity of antigens while maintaining the distinction between self antigens and foreign antigens. B cells begin development in the bone marrow as c-kit+ hematopoietic stem cells that do not express lineage markers such as B220 for B cells, Mac-1 for macrophages, and Ter-119 for red blood cells (Ikuta and Weissman 1992; Ogawa et al. 1991). These differentiate into common lymphoid progenitors, which will go on to become B, T, or natural killer (NK) cells, or common myeloid progenitors (which will go on to become dendritic cells (DCs), granulocytes, red blood cells (RBCs), and macrophages). B lineage cells develop from common lymphoid progenitors, which have their immunoglobulin (Ig) chain genes in

germline configuration. When committed to the B lineage, these cells are called pro-B cells, in which Ig heavy chain undergoes D→J rearrangement (Allman et al. 1999; Nutt et al. 1999). In the late pro-B cell stage, Ig heavy chain undergoes V→DJ rearrangement. This DNA rearrangement, called V(D)J recombination, is the process partly responsible for antigen receptor diversity. Productively rearranged Ig heavy chain then associates with the surrogate light chain to form the pre-B cell receptor (pre-BCR) in pre-B cells. Survival of developing B-lineage cells is dependent on expression of the pre-BCR (e.g. Kitamura et al. 1992; Kitamura et al. 1991) and pre-BCR signaling results in allelic exclusion, which is responsible for the clonal nature of B cells (e.g. Alt et al. 1980; Coleclough et al. 1981). Signaling through the pre-BCR drives proliferation and differentiation to small pre-B cells, during which Ig light chain rearranges and takes the place of the surrogate light chain, forming the complete BCR. These cells then become immature B cells, during which the nature of BCR signaling determines their fate.

Central tolerance. Tolerance is the state of unresponsiveness in antigen-specific B and T cells. It is crucial as a mechanism for inactivating cells that are reactive to self antigens and therefore could initiate an autoimmune response. Tolerance is divided up into two parts: central tolerance and peripheral tolerance. Central tolerance is induced during lymphocyte development in the primary lymphoid organs (bone marrow for B cells and thymus for T cells). Peripheral tolerance is induced in mature lymphocytes in peripheral lymphoid organs (spleen and lymph nodes). Central tolerance in the bone marrow includes 3 different fates of immature B cells: 1) deletion, 2) anergy, and 3) receptor editing. High

affinity binding of antigen or multimeric binding of a cell surface-bound antigen through the BCR on immature B cells typically results in deletion and is a mechanism by which self-reactive B cells that recognize and bind self-antigen in the bone marrow are eliminated (e.g. Chen et al. 1994; Hartley et al. 1991; Nemazee and Buerki 1989; Nemazee and Burki 1989; Norvell et al. 1995). On the other hand, weak binding of a soluble antigen to the BCR results in anergy (Rathmell et al. 1998), the state of antigen-specific functional unresponsiveness. An immature B cell can escape either of these fates by undergoing a process called receptor editing, during which the BCR rearranges its Ig light and/or heavy chain genes in an attempt to form a BCR of different specificity (e.g. Chen et al. 1995; Gay et al. 1993; Hertz and Nemazee 1997; Prak et al. 1994; Prak and Weigert 1995; Tiegs et al. 1993). The choice between deletion versus receptor editing may be controlled by compartment, with receptor editing favored in the bone marrow and deletion favored in the periphery (Melamed et al. 1998; Sandel and Monroe 1999). Together, these mechanisms for central tolerance exist to ensure deletion or inactivation of B cells that react to self-antigen. The result is formation of a B cell repertoire which will respond to non-self antigens and ignore self antigens.

B cell development and activation in the spleen. Immature B cells that pass education in the bone marrow emigrate to the spleen. It is unclear whether tolerance or continued differentiation occurs in transit. However, exclusion of self-reactive B cells from follicles in the spleen may be a mechanism of tolerance (Cyster and Goodnow 1995; Cyster et al. 1994). Upon entering the spleen (or in transit to the spleen), immature B cells continue to develop. Cells of the first transitional (T1) stage cells are CD23- and express IgM and low levels of

IgD. Cells in the second transitional stage (T2) become CD23+. Signaling through the BCR at the T1 and T2 stages results in death rather than proliferation (Sandel and Monroe 1999). The existence of a third late transitional stage (T3) is still controversial (Allman et al. 2001).

Transitional stage B cells are precursors that become either follicular B cells or marginal zone B cells. The decision of which pathway these precursors take is thought to be dependent on strength of signal through the BCR, with absent or weak signals driving development of marginal zone B cells and strong signals driving development of follicular B cells. Several experimental observations lead to this model. Deficiency of CD22, a negative regulator of BCR signaling, results in impaired marginal zone B cell development (Samardzic et al. 2002). Enhancement of BCR signaling strength also impairs marginal zone B cell development (Kraus et al. 2001). Conversely, deficiency of CD21/CR2, positive regulators of B cell signaling, results in over-representation of marginal zone B cells (Cariappa et al. 2001), as does a mutation in Ig light chain that abrogates BCR signaling (Sun et al. 2002). Deficiency of Aiolos, a negative regulator of BCR signaling, reduces the marginal zone compartment (Wang et al. 1998).

Development of follicular B cells is dependent on Btk, a non-receptor protein tyrosine kinase which becomes activated in response to BCR engagement and potentiates BCR signaling (Hendriks et al. 1996; Khan et al. 1995; Satterthwaite et al. 1997), while development of marginal zone B cells involves Notch signaling and NF-κB signaling (Cariappa et al. 2000; Tanigaki et al. 2002). Similar to non-classical B-1 cells in the peritoneum, marginal zone B cells have the ability to form effector cells independently of T cells to antigens such as lipopolysaccharide (LPS) and are thus considered “first responders”

in the event of infection (Martin et al. 2001; Oliver et al. 1999). They may bear autoreactive receptors and are thus implicated in autoimmunity (Grimaldi et al. 2001; Hayakawa et al. 1999; Li et al. 2002). Follicular B cells can be activated by multivalent T-independent antigens such as bacterial LPS or other polyclonal B cell activators but are mainly responsible for responding to T-dependent antigens and can recirculate and survive for months in the spleen (e.g. Allman et al. 1993; Forster and Rajewsky 1990; Hao and Rajewsky 2001). Interaction of activated follicular B cells with activated T cells results in two different differentiation pathways: 1) formation of short-lived IgM antibody-secreting cells near the T cell zone (Ho et al. 1986; Smith et al. 1996) and 2) formation of germinal center B cells. These short-lived antibody-secreting B cells serve as part of the early immune response. Production of a sustained, high-affinity immune response to antigen and generation of memory are results of the germinal center reaction.

The germinal center reaction. The germinal center is the site of B cell expansion, diversification, and generation of high-affinity antigen-specific B cells and memory B cells. Its formation is initiated by B cells that have encountered their specific antigen and their cooperating T cells and depends on migration of these cells within the splenic architecture. Activated T cells are attracted to the T cell zone by the chemokine SLC (Gunn et al. 1998; Willimann et al. 1998), facilitating interaction with B cells that have encountered their antigen. Interaction of B and T cells occur via MHC Class II/peptide on the B cell and the T cell receptor (TCR) and CD4 on the T cell. Costimulatory molecules B7 and CD40 on the B cell interact with CD28 and CD40L on the T cell and are required to avoid anergy. Activated

B cells then become centroblasts, express CXCR4 and migrate to the germinal center dark zone, aided by the chemokine SDF-1 (Allen et al. 2004). Here, they undergo somatic hypermutation, a process by which mutations occur in Ig variable region genes to increase the variety of BCRs to a particular antigen (Berek et al. 1991; Jacob et al. 1991; McHeyzer-Williams et al. 1993; Pascual et al. 1994). Centroblasts then become centrocytes and are directed by CXCR5 to the germinal center light zone, where they are tested for binding to antigen retained on the surface of follicular dendritic cells (FDCs) via antigen-antibody complexes bound to Fc receptors (Qin et al. 2000). Centrocytes with low-affinity receptors for antigen can undergo receptor editing to diversify the BCR repertoire and contribute to selection of cells with high-affinity receptors (Han et al. 1997; Papavasiliou et al. 1997). Centrocytes that receive no signal due to competition for antigen by higher-affinity cells undergo Fas-mediated apoptosis (Liu et al. 1989; Takahashi et al. 2001). This process of selecting for B cells with a high affinity to antigen is termed affinity maturation and results in an increase in the production of high-affinity antibodies. Another phenomenon that occurs during the germinal center reaction is isotype class switching, where B cells make different classes of antibodies through DNA rearrangement, such as IgE, IgA, or IgG, while maintaining the same antigenic specificity. Isotype class switching is stimulated by interaction with T cells, particularly through CD40 (Fecteau and Neron 2003).

The outcome of the germinal center reaction is production of antibody-secreting plasma cells and memory B cells. These two types of cells arise from separate differentiation pathways, perhaps through a common centrocyte progenitor (Jacob and Kelsoe 1992). These plasma cells can be long-lived (Manz et al. 1998; Slifka et al. 1998), but it is memory B cells

that are responsible for long-term immunity. Dependence on the presence of antigen for survival of memory B cells is unclear, with some studies showing that memory is regulated by persistence of FDC-bound antigen-antibody complexes (Bachmann et al. 1994a; Bachmann et al. 1994b; Qin et al. 2000) and others showing that this is not necessary for memory B cell survival (Karrer et al. 2000; Maruyama et al. 2000; Schittek and Rajewsky 1990). Re-challenge with antigen stimulates a rapid memory B cell response characterized by production of high-affinity antibody (Herzenberg et al. 1980; Okumura et al. 1976; Romano et al. 1975).

Peripheral tolerance. Induction of tolerance in mature B cells is essential in preventing the activation of autoreactive cells that may have been formed during somatic hypermutation or escaped negative selection during B cell development. Clonal anergy and clonal deletion are the classic mechanisms for tolerance in the periphery, although other mechanisms have been described, such as elimination of self-reactive B cells by exclusion from splenic follicles due to competition for space with B cells of other specificities (Cyster and Goodnow 1995; Cyster et al. 1994). Upon entering the T cell zone of the spleen or other peripheral lymphoid tissues, self-reactive B cells compete with B cells specific for foreign antigen for entry into the follicles. In the case of foreign antigen, activated T cells are available for interaction with B cells specific for that antigen, and this B-T cell interaction facilitates migration out of the T cells zone to primary follicles. Self-reactive B cells are trapped in the T cell zone due to lack of T cell help and undergo apoptosis (Cyster et al. 1994; Fulcher et al. 1996).

Induction of B cell anergy (B cell unresponsiveness) is traditionally thought of as inhibition of B cell activation in the absence of T cell help. Anergic B cells are characterized by reduced surface IgM expression and reduced signaling capacity (Eris et al. 1994; Rathmell et al. 1998). Administration of large doses of soluble antigen, which bypasses T cell engagement, contributes to B cell anergy (Adelstein et al. 1991; Hartley et al. 1991). The anergic state of B cells can be reversed by stimulation with LPS and this may contribute to development of autoimmune disease (Goodnow et al. 1991). Interestingly, anergic B cells are eliminated by Fas-dependent mechanisms in the presence of antigen and antigen-specific CD4+ T cells (Rathmell et al. 1995).

Self-reactive B cells that may be formed through somatic hypermutation of the BCR can be actively deleted during the germinal center reaction. Germinal center B cells die by apoptosis upon encounter with large doses of soluble antigen (Han et al. 1995; Linton et al. 1991; Pulendran et al. 1995). Systemic self-antigen is likely to be present in large doses, thus this may serve as a mechanism for deletion of self-reactive B cells in the germinal center.

In summary, the goal of B cell development and activation is to form a highly specific B cell compartment capable of distinguishing self from non-self, to generate antibody-secreting effector cells that produce high-affinity antibody for antigen, and to produce memory cells capable of inducing a secondary immune response upon antigen re-exposure that is faster and stronger than the primary immune response. Central tolerance is critical in selecting against maturation of autoreactive B cells, and peripheral tolerance is required to maintain self versus non-self discrimination in the face of BCR mutations occurring during

affinity maturation. Failure in any step in B cell development, differentiation, or tolerance can result in immune deficiencies and autoimmunity.

Class II MHC antigen processing and presentation

Class I vs. Class II antigen processing and presentation. Antigen processing and presentation is the process by which foreign or self antigens are degraded inside the cell and loaded onto MHC molecules, which are then exposed or "presented" on the cell surface for recognition by the appropriate T cells. Binding and recognition of MHC-peptide by T cells result in their activation. There are two types of MHC: Class I and Class II. MHC Class I is expressed in virtually all cells and is involved in the "cytosolic" or "endogenous" pathway of antigen processing and presentation. In this pathway, peptides resulting from proteolysis of cytosolic proteins are transported into the endoplasmic reticulum (ER) and loaded onto Class I molecules for presentation on the cell surface. Cytosolic proteins include self proteins that are normally degraded during protein turnover and proteins originating from intracellular pathogens localizing to the cytosol, such as viruses. Proteolysis of proteins into peptides is accomplished by the proteosome. Class I/peptide is recognized by CD8+ T cells (or CTLs-cytotoxic T lymphocytes), which function to kill infected host cells.

In contrast, MHC Class II is expressed only on antigen presenting cells and is involved in the "endocytic" or "exogenous" pathway of antigen processing and presentation. Class II molecules bind and present peptides originating from exogenous antigens that enter the cell by uptake via phagocytosis, pinocytosis, or receptor-mediated endocytosis. Degradation of antigens into peptides is accomplished in acidic endocytic compartments and involves the action of proteases. Class II/peptide is recognized by CD4+ T cells (or T_H-

helper T cells), which function to provide signals necessary for B cell differentiation into plasma cells that secrete antibody

Class I and Class II antigen processing and presentation provide a continuous "sampling" of all cellular proteins and are critical for immune recognition and responses to foreign proteins. MHC Class II presentation of peptides derived from antigen initiates development of the specific, adaptive immune response. The work presented in this dissertation focuses on an *in vitro* model of stimulation of B cells by antigen using anti-IgM antibodies to engage the BCR. B cells, along with DCs and macrophages, are known as professional APCs because of their ability to activate other cell types. Since the BCR is specific for exogenous antigens, and binding of specific antigen to the BCR can ultimately result in antibody production, this section focuses on MHC Class II antigen processing and presentation.

Overview of Class II antigen processing and presentation. The generally accepted "textbook" model of endocytic compartment trafficking during antigen processing and presentation is as follows: Antigens are internalized into endosomes, which fuse with acidic lysosomes forming endolysosomes. This acidic environment facilitates degradation of antigen into peptides by lysosomal proteases. Meanwhile, Class II/Invariant chain (Ii) molecules formed in the ER traffic to the trans-golgi network (TGN) where they leave in endosomes that fuse with antigen-containing endolysosomes to form a compartment containing MHC Class II, H2-DM (HLA-DM in humans), proteases, and other components required for peptide loading. In B cells, an additional molecule, H2-DO, regulates H2-DM-

mediated peptide loading. Following antigen proteolysis, Ii chain degradation to Class II-associated Ii peptide (CLIP), and HLA-DM-mediated peptide loading into Class II, the Class II/peptide complexes are then trafficked back to the plasma membrane for presentation to CD4+ T cells. A schematic overview of antigen processing and presentation is shown in Figure 1.1.

Antigen entry. Antigens may enter into the endosomal pathway non-specifically or specifically. Non-specific mechanisms include uptake of antigens via phagocytosis ("cell eating") or pinocytosis ("cell drinking"). Pinocytosis can be divided up into "Macropinocytosis" and "Micropinocytosis". Macropinocytosis is a form of endocytosis that is visualized as cell surface ruffling, and non-specifically takes up relatively large amounts of solute. Micropinocytosis includes uptake of small amounts of solute into small clathrin-coated or uncoated vesicles (Brunk et al. 1976; Fawcett 1965). Non-specific antigen uptake is accomplished mainly by macrophages and DCs, which may utilize receptors such as Toll-like receptors (TLRs) for recognition of bacterial cell wall components, mannose receptors for recognition of glycosylated antigens, Fc receptors, or complement receptors that recognize common pathogenic antigens, especially on bacteria, known as pathogen associated molecular patterns (PAMPs) (Blander and Medzhitov 2004; Engering et al. 1997; Griffin 1981; Tan et al. 1997). DCs are unique in that they are able to process external antigens, which are conventionally presented in a Class II-restricted manner, on Class I molecules. Mechanisms for this process, known as cross-priming or cross-presentation, have recently begun to be elucitated (Rodriguez et al. 1999). Cross-presentation has been shown

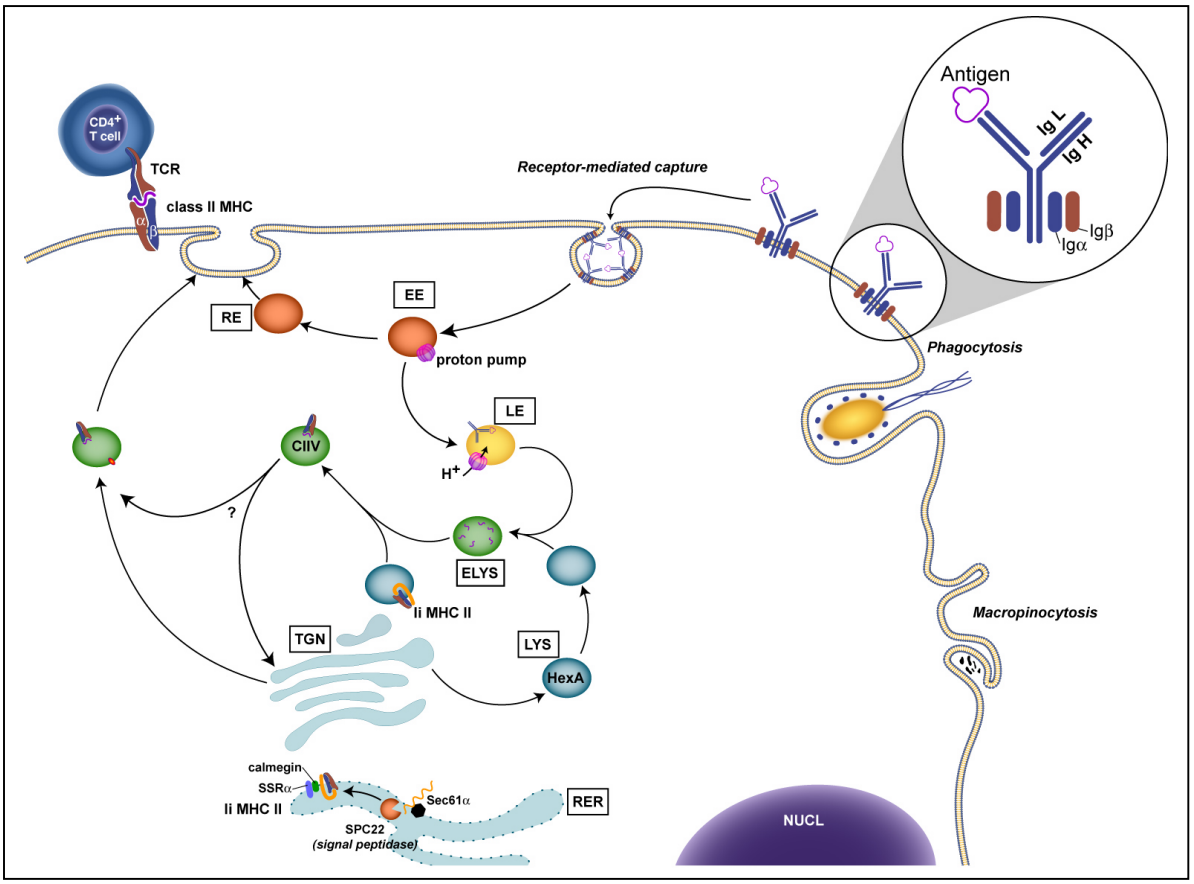

Figure 1.1. Overview of MHC Class II antigen processing and presentation. This schematic summarizes the major steps in Class II antigen processing and presentation: antigen entry via phagocytosis, pinocytosis, or receptor-mediated endocytosis, endosome trafficking (EE = early endosome, LE = late endosome), endosome fusion (LYS = lysosome, ELYS = endolysosome), peptide loading (CIIV = Class II vesicle), and transport to the cell surface for presentation. Generation and transport of invariant chain (Ii) and Class II (MHC II) from the rough endoplasmic reticulum (RER) to the trans-Golgi network (TGN) and its intersection with processed peptides in the CIIV is shown. A lesser-known recycling pathway for endosomes (RE = recycling endosome) is also shown.

to be associated with phagocytosis and pinocytosis but not with receptor-mediated endocytosis (Ackerman et al. 2003; Peppelenbosch et al. 2000). TAP and tapasin are required for cross-presentation (Chefalo et al. 2003; Garbi et al. 2000; Huang et al. 1996), suggesting that the ER membrane is involved in cross-presentation. Indeed, the ER membrane fuses with phagosomes, providing a link between Class I molecules and exogenous antigens (Gagnon et al. 2002). Alternatively, antigens taken up by phagocytosis and pinocytosis are transported to the cytosol, degraded by the proteasome, and transported back into the ER via TAP (Guermonprez et al. 2003).

Specific uptake of antigens occurs via receptor-mediated endocytosis and result in greatly increased efficiency of presentation (Lanzavecchia and Bove 1985).
A unique feature of B cells is that antigen binding to antigen-specific BCRs stimulates uptake. The specificity of the BCR enables B cells to capture low-affinity or rare antigens. This is in contrast to the capture of antigen by dendritic cells, which recognize generalized markers of infection (such as PAMPs) in locations where antigen is concentrated (in follicular microenvironments or in inflammatory environments). Depending on the cross-linking ability of the antigen, BCRs may form a cap or a patch which is then internalized into the cell (Siemasko et al. 1999; Unanue et al. 1972). In addition to binding and internalizing soluble antigen, B cells are also able to extract and internalize antigen from immobilized surfaces, such as antigen bound to complement receptors or Fc receptors on DCs (Batista et al. 2001; Batista and Neuberger 2000). Antigen has been shown to be internalized into clathrin-coated pits (Salisbury et al. 1980; Stoddart et al. 2002). Upon cross-linking, the BCR is localized to lipid rafts, cholesterol- and sphingolipid-rich detergent-insoluble

membrane microdomains thought to concentrate signaling components (Cheng et al. 1999a; Cherukuri et al. 2001; Stoddart et al. 2002). Tyrosine phosphorylation of clathrin by Src-family kinases is thought to provide a link between BCR signaling and internalization (Stoddart et al. 2002).

Targeting to endocytic compartments. In DCs, receptors that facilitate antigen uptake such as TLRs, C-type lectins (such as mannose receptors), and Fc receptors induce targeting of phagosomes/endosomes to more specialized endocytic compartments. TLR signaling stimulates actin remodeling to drive endocytosis (West et al. 2004). In the absence of TLR signaling, bacterial phagocytosis is impaired. Furthermore, phagosome maturation as measured by colocalization with acidic lysosomes is driven by TLR signaling within the phagosome (Blander and Medzhitov 2004). Mannose receptor-mediated endocytosis efficiently targets antigen to MHC Class II-containing endocytic compartments (Engering et al. 1997; Tan et al. 1997). Signaling through the Fc receptor mediates targeting to lysosomes and is dependent on Syk activation (Bonnerot et al. 1998; Crowley et al. 1997).

In B cells, signaling components are also involved in mediating targeting to endocytic compartments. Unligated BCRs are constitutively internalized through clathrin-coated pits and recycled rapidly through early endosomes for re-expression on the cell surface (Brown et al. 1999). This internalization is mediated by a conserved motif in Igα and Igβ (Cassard et al. 1998; Patel and Neuberger 1993). Propagation of the signal upon BCR cross-linking is dependent on the Igα and Igβ heterodimers that associate with Ig heavy chain, connecting the BCR to intracellular signaling components (Kim et al. 1993; Luisiri et al. 1996; Sanchez et

al. 1993). Though either chain alone is sufficient for antigen internalization, both Igα and Igβ are required for targeting of non-abundant antigens to Class II-containing endocytic compartments and antigen presentation (Siemasko et al. 1999). This trafficking pathway is accelerated by cross-linking of BCR by antigen (Brown et al. 1999; Cheng et al. 1999b) and the efficiency of trafficking depends on the nature of the BCR-antigen interaction, as some BCR-antigen interactions drive trafficking more efficiently than others (Aluvihare et al. 1997). Recruitment of Syk to phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs) of Igα is also required for sorting to Class II containing compartments (Lankar et al. 1998). Phosphorylated Igα recruits B cell linker protein (BLNK) to the BCR, linking Syk to downstream signaling pathways (Kabak et al. 2002; Siemasko et al. 2002). Phosphoinositide-3-kinase has been shown to be required for formation of antigen processing endocytic compartments (Granboulan et al. 2003). BCR engagement induces the formation of multivesicular antigen processing compartments to which Class II is concentrated and H2-DM is recruited (Lankar et al. 2002)

Class II peptide loading. Two main compartments have been described as the site where peptide is loaded onto Class II molecules. One is the Class II vesicle (CIIV) and the other is the MHC Class II compartment (MIIC). CIIVs are described to have a multivesicular structure and closely resemble endosomes (Amigorena et al. 1994; Drake et al. 1997). MIICs have a multilamellar structure (Harding and Geuze 1993; Kleijmeer et al. 1997; Kleijmeer et al. 1995; Murk et al. 2004; Peters et al. 1991). Peptide loading onto Class II can occur in either CIIVs or MIICs (Drake et al. 1999), though it has been shown to occur

less efficiently in CIIVs, presumably because of lower levels of H2-DM (Drake et al. 1999). The discovery of CIIVs and MIICs is a result of the search for a specific specialized compartment in which peptide is loaded onto Class II. A number of investigations have revealed peptide loading in a variety of compartments including MIICs, CIIVs, late endosomes, phagosomes, and early lysosomes (Amigorena et al. 1994; Arkema et al. 1991; Harding and Geuze 1993; Kleijmeer et al. 1995; Marks et al. 1995; Qiu et al. 1994; Ramachandra et al. 1999; Sanderson et al. 1994; West et al. 1994; Wubbolts et al. 1996). Since it is required for peptide loading, localization of H2-DM was thought to be the best marker for "MHC Class II peptide loading compartments". However, H2-DM was also localized to a variety of endocytic compartments including MIICs, CIIVs, and early lysosomes (Ferrari et al. 1997; Kleijmeer et al. 1997; Sanderson et al. 1994; Tan et al. 1997; Wubbolts et al. 1996). The combination of these studies indicate that the "MHC Class II peptide loading compartment" is probably not one distinct endocytic compartment, but can occur in a variety of compartments. These compartments presumably would contain all components necessary for peptide loading onto Class II molecules.

MHC Class II molecules are formed in the ER and stabilized by ER chaperones such as calnexin. Class II molecules associate in stable trimers, which are then associated with Ii trimers to form a nonameric complex that is transported to the Golgi apparatus (Cresswell 1996). Ii association blocks binding of polypeptides in the Class II peptide-binding groove in the ER until it is degraded in endosomes, thus allowing peptide loading of processed peptides (Busch et al. 1996). Class I and Class II molecules are segregated for transport in the Golgi, with Class II molecules targeted for the endocytic pathway (Peters et al. 1991). H2-DM

complexes with H2-DO in the ER and this association is required for exit from the ER and trafficking to lysosomes, suggesting that their functions are related (Liljedahl et al. 1996). Mechanisms for trafficking of these components from the Golgi to the endocytic pathway have yet to be determined in detail. Ii contains a cytoplasmic sorting signal that, when deleted, results in Ii retention in the plasma membrane (Bakke and Dobberstein 1990). The Ii sorting signal targets Class II through early endosomes to late endosomes (Lotteau et al. 1990; Romagnoli et al. 1993). Phosphorylation of Ii by protein kinase C (PKC) is involved in trafficking of Class II to endosomes and also for degradation of Ii; both of these are impaired in cells expressing Ii phosphorylation mutants (Anderson et al. 1999).

There are three main pathways of sorting from the TGN: 1) the constitutive secretory pathway, 2) the regulated secretory pathway, and 3) the endosomal pathway. The constitutive secretory pathway is the main pathway for transport from the TGN. Class I molecules, T cell receptors, integrins, and secreted cytokines all utilize this pathway. Class II molecules have also been observed to use this pathway to reach the cell surface, and then are recycled by internalization back into the endosomal pathway (Roche et al. 1993; Saudrais et al. 1998). This may be mediated by Ii sorting motifs, which are recognized by clathrin adaptor proteins for internalization from the plasma membrane and targeting to endosomes (Hofmann et al. 1999; Roche et al. 1993). Peptides may also bind to Class II for presentation during this type of recycling (Pinet et al. 1995).

The regulated secretory pathway is used for transport of secretory granules to the cell surface when triggered by receptor-ligand interaction. Examples include the exocytosis of lytic granules released by CTLs and the release of inflammatory cytokines by macrophages

in response to invasion. SNARE proteins may be involved in membrane fusion events during regulated exocytosis in mast cells (Guo et al., 1998). Class II transport is not known to be associated with this pathway.

The endosomal pathway is the main route of transport for newly-synthesized Class II molecules. Little is known about the regulators of vesicle trafficking and fusion to compartments containing antigen internalized by the APC. In yeast, vacuolar protein sorting (vps) proteins are involved in sorting into multivesicular or multilamellar endocytic compartments (Odorizzi et al. 1998; Rieder et al. 1996). In B cells, overexpression of the rab7 GTPase has been shown to increase the rate of antigen processing and presentation (Bertram et al., 2002). The endosomal pathway brings together components required for peptide loading in Class II.

Peptide loading into Class II has been extensively studied and is generally agreed to take place in MIICs, although it has been described to occur in other endocytic compartments. For simplicity, I will refer to any compartment capable of supporting Class II peptide loading as the "Class II peptide loading compartment". As internalized antigens progress through the endocytic pathway, early endosomes mature into more acidic late endosomes. This is accomplished by vacuolar proton pumps which transfers protons across the endosome membrane, resulting in a more acidic environment (Trombetta et al. 2003). Antigens begin to be degraded by proteases such as cathepsins and gamma interferon-inducible lysosomal thiol reductase (GILT) (Driessen et al. 2001; Hsieh et al. 2002; Lennon-Dumenil et al. 2002; Maric et al. 2001; Pluger et al. 2002; Riese et al. 1996; Shi et al. 1999). Ii is also degraded by cathepsins, leaving a peptide called CLIP in the peptide-binding groove

(Nakagawa et al. 1998; Nakagawa et al. 1999; Shi et al. 2000). At this point peptide loading is ready to occur. H2-DM (HLA-DM in humans) is a Class II-like accessory molecule that catalyzes the exchange of CLIP for antigenic peptides (Morris et al. 1994). H2-DM contains a cytoplasmic signaling motif for targeting to lysosomes (Copier et al. 1996; Marks et al. 1995), allowing accumulation in Class II peptide loading compartments (Sanderson et al. 1994). The peptide repertoire that binds to Class II is determined by H2-DM and can be edited; peptides that bind to Class II with lower affinity may be exchanged for peptides that bind with higher affinity (Nanda and Sant 2000) (Denzin and Cresswell 1995; Kropshofer et al. 1996; Sherman et al. 1995; Sloan et al. 1995; Weber et al. 1996). Another Class II-like accessory molecule, H2-DO (HLA-DO in humans) plays a role in determining the peptide repertoire that binds to Class II. H2-DO is expressed in thymic epithelial cells and B cells (Karlsson et al. 1991) but not in dendritic cells and macrophages. Within the B cell compartment, H2-DO expression is high in naïve B cells but lacking in germinal center B cells, which have enhanced antigen presentation capabilities (Chalouni et al. 2003; Chen et al. 2002a; Glazier et al. 2002). H2-DO blocks H2-DM function (Denzin et al. 1997; Fallas et al. 2004). In acidic compartments, H2-DO stabilizes Class II-peptide complexes better than H2-DM alone and seems to prefer loading of high-stability peptides (Kropshofer et al. 1998). H2-DM appears to limit the pH range within which H2-DM is active to more acidic late endosomal compartments. Because of its restriction to the B cell compartment, H2-DO may affect the peptide repertoire by driving preferential presentation of BCR-specific antigens, which are targeted rapidly to late endosomes, over those antigens internalized by pinocytosis (Liljedahl et al. 1998; van Ham et al. 2000).

Transport of Class II-peptide complexes to the cell surface. The least amount of information is known about this integral final step to Class II antigen processing and presentation. In dendritic cells, Class II-peptide complexes are formed from sequestered lysosomal antigens upon DC maturation, then are transported to the cell surface in non-lysosomal vesicles that contain B7 costimulatory molecules for T cell activation (Chow et al. 2002; Turley et al. 2000). Tracking of Class II by tagging with Green fluorescence protein (GFP) reveal rapid movement from lysosomes to the cell surface and fusion with the plasma membrane (Boes et al. 2002; Wubbolts et al. 1996). In B cells, fusion of multilamellar MIICs with the plasma membrane has been reported to result in the release of exosomes derived from internal MIIC membranes. These secretory exosomes contain Class II-peptide and are able to activate antigen-specific T cells. Thus, this may represent another little-known form of transport of Class II-peptide to the cell surface (Raposo et al. 1996; Stoorvogel et al. 2002). The surface of an APC interacting with a T cell (the "immunological synapse") contains concentrated Class II molecules in either lipid rafts or tetraspan microdomains. Class II molecules are associated with lipid rafts even before binding to antigenic peptide and arrival at the cell surface (Anderson et al. 2000; Poloso et al. 2004; Poloso and Roche 2004). Tetraspan microdomains are made up of tetrapan proteins such as CD63, CD81, or CD82 different from lipid rafts and are selectively enriched for Class II molecules, H2-DM, and H2-DO (Hammond et al. 1998; Vogt et al. 2002). Again, little is known about the regulators of vesicle trafficking and fusion to the plasma membrane.

Summary. B cells undergo tightly regulated pathways during development and maturation. The goal is to provide a B cell compartment that can ignore self-antigens (either by undergoing deletion or becoming anergic) and respond to foreign antigens. Activation of B cells requires recognition and uptake of antigen, processing of antigen, and presentation of antigen to the cognate T cell. They differ from other professional antigen presenting cells (dendritic cells and macrophages) in their ability to process and present specific antigens for which they express a BCR. Because of their antigen specificity, B cells contribute to immunological memory, driving a heightened response in the event of re-exposure to a specific antigen. The importance of antigen processing and presentation is illustrated by the plethora of studies that have been performed to address the mechanisms of how this process occurs. However, many aspects of antigen processing and presentation remain unclear. The majority of studies have focused on dendritic cells and macrophages, therefore less is known about antigen processing and presentation in B cells and how the presence of the BCR affects the dynamics of antigen processing and presentation.

Chapter 2

Materials and Methods

Cells

The studies described in this dissertation are related to B cell signaling. Primary mouse splenic B cells were used for microarray experiments, PCR, proliferation assays, and viability experiments. Two B cell lines were utilized: BCL_1.3B3 cells were used for apoptosis and cell cycle arrest assays, and Syk activation studies. WEHI-231 cells were used for calcium flux assays and BCR internalization and localization studies.

Primary B cell isolation and culture. Isolation of primary splenic murine B cells was performed as described in the Alliance for Cellular Signaling (AfCS)/Nature Signaling Gateway website (www.signaling-gateway.org), at http://www.signaling-gateway.org/data/cgi-bin/ProtocolFile.cgi?pid=PP00000001. C57BL/6 mice were anesthetized with CO_2, then sacrificed by cervical dislocation. Spleens were removed and placed on a 70 μm nylon cell strainer (BD Biosciences, Franklin Lakes, NJ) soaked in 5 ml of magnetic activated cell sorting (MACS) buffer (PBS/2mM EDTA) with 5 mg/ml bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) contained in a petri dish set on ice. Up to four spleens were placed in each cell strainer. Each spleen was cut into 3 sections, and splenocytes were mashed from the splenic capsules through the cell strainer using the rubber end of a plunger from a 3-cc syringe. MACS buffer from this first mashing was filtered through another cell strainer set atop a 50 ml Falcon tube kept on ice. This was repeated about 4-5 more times using 3 ml of MACS buffer each time until splenic capsules were white. Cells were pelleted at 400×g for 8 min at 4°C. MACS buffer was aspirated, leaving a

small amount in which to resuspend the cell pellet by flicking. RBC lysis buffer (155mM Ammonium chloride, 10mM potassium bicarbonate, 0.1 mM EDTA, pH to 7.2-7.4 with HCl) was added at 1 ml per spleen and incubated for 2 minutes at room temperature with gentle rolling, followed by immediate addition of chilled MACS buffer to 40 ml/tube. Cells were collected by centrifugation at 400×g for 8 min at 4°C. Supernatants were aspirated completely and cell pellets loosened by flicking. Cell pellets were resuspended in 1 ml MACS buffer per 4 spleens and filtered through a fresh cell strainer placed atop a fresh 50 ml Falcon tube. Clumps resulting from RBC lysis were also placed atop the cell strainer. Tubes were washed 3 times with 1 ml MACS buffer each time to recover all cells. Live cells were counted using a hemocytometer and resuspended at 1.1×10^5/μl with MACS buffer. Anti-CD43 microbeads (colloidal super-paramagnetic microbeads conjugated to monoclonal rat anti-mouse CD43 antibody, clone S7) were added at 1 μl per 10^6 cells and anti-Mac-1 microbeads (colloidal super-paramagnetic microbeads conjugated to rat anti-mouse human CD11b (Mac-1α) antibody, clone M1/70.15.11.5) were added at 0.5 μl per 10^6 cells (Miltenyi). This mixture was incubated at 4°C for 15 min, mixing once at 7.5 min. 40 ml of MACS buffer was added per 4 spleens to wash and cells were pelleted at 400×g for 8 min at 4°C. Supernatants were aspirated, then cells were resuspended with 1 ml MACS buffer per spleen. Cells were strained through a final fresh cell strainer placed atop a fresh 50 ml Falcon tube, and tubes were washed 3 times with 1 ml MACS buffer each to recover all cells. Cells from 4 spleens (the maximum recommended for one individual separation) result in one tube containing 7 ml of cells that are ready to separate. Cells were separated on the AutoMACS (Miltenyi Biotech, Auburn, CA) according to the manufacturer's protocol. For

each separation, when 100 μl of cells remained, an additional 5 ml of MACS buffer was added to ensure delivery of all of the cells to the column. Separations result in two fractions. The "negative" fraction contains $CD43^{-}Mac1^{-}$ cells, and the "positive" fraction contains $CD43^{+}Mac1^{+}$ cells. The negative fraction is an enriched population of resting B cells that was an average of 96% $B220^{+}$, as determined by flow cytometry (Hsueh et al. 2002).

Purified primary B cells were cultured in prewarmed, CO_2-calibrated Iscove's Modified Dulbecco's Medium supplemented with 2 mM L-glutamine, 55 μM β-mercaptoethanol, 0.025% pluronic F-68 (all from Invitrogen) and 0.1 mg/ml BSA (Sigma). Culture conditions for specific assays are described under the heading for that assay.

BCL_1.3B3 culture and maintenance. BCL_1.3B3 cells (Brooks et al. 1983), obtained from Drs. Ellen Vitetta and Jonathan Uhr, were grown in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), 25 mM HEPES (Invitrogen, Carlsbad, CA), 25 μg/ml gentamycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin/streptomycin (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), and 55 μM β-mercaptoethanol (Invitrogen). Cells were passaged by dilution to 1×10^5 cells/ml every 2-3 days. Frozen stocks of low passage number cultures were made by freezing approximately 1×10^7 cells/ml in FBS/10% DMSO for cryogenic storage.

WEHI-231 culture and maintenance. WEHI-231 cells were obtained from the AfCS and maintained in RPMI supplemented with 10% heat-inactivated fetal bovine serum, 50 μM

β-mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate, 20mM HEPES, and 100 U/ml penicillin/streptomycin (Invitrogen). Cells were passaged by dilution to 1×10^5 cells/ml every 2-3 days. Frozen stocks of low passage number cells were obtained from the AfCS.

Viable cell recovery assay

In order to evaluate the effect of ligand stimulation on cell viability, numbers of viable cells recovered from long-term cultures were determined by hemocytometry. Staining of cells using Trypan Blue (Sigma-Aldrich), a vital dye, distinguishes between live and dead cells because dead cells have lost membrane integrity and take up the blue dye. Cell count data were organized and graphed using Microsoft Excel.

BCL_1.3B3 viable cell recovery determination. BCL_1.3B3 cells were plated at 1×10^5 cells in 1 ml of growth medium per sample and incubated at 37°C with 5% CO_2. After overnight recovery, cells were stimulated with 18 μg/ml polyclonal goat-anti-IgM, μ chain specific antibody (AIG) (Jackson Immunoresearch, West Grove, PA) or left untreated. Viable cells were recognized by Trypan Blue exclusion and counted on a hemocytometer each day for 10 days.

Primary B cell recovery determination. Primary B cells were plated at 2×10^6 cells in 1 ml per sample of prewarmed, CO_2-calibrated Iscove's Modified Dulbecco's Medium

supplemented with 2 mM L-glutamine, 55 μM β-mercaptoethanol, 0.025% pluronic F-68 (all from Invitrogen) and 0.1 mg/ml BSA (Sigma) and incubated at 37°C with 5% CO_2. 62.5 ng/ml of recombinant human BAFF (Cell Sciences, Norwood, MA) or 9.73 μg/ml hamster anti-mouse CD40 monoclonal antibody (CD40L mimic), clone HM40-3 (BD Pharmingen) was added. Viable cells were recognized by Trypan Blue exclusion and counted on a hemocytometer at 4 hr, 24 hr, 48 hr, and 72 hr of culture.

Hoechst and 7AAD determination of cell cycle and apoptosis

To determine the cell cycle and apoptosis status of BCR-stimulated BCL_1.3B3, cells were stained with Hoechst 33342 (Molecular Probes, Eugene OR), a DNA-binding dye that measures DNA content, and 7-aminoactinomycin D (7-AAD)(Molecular Probes), a vital dye that is taken up by apoptotic cells which have lost membrane integrity (much like Trypan Blue). Hoechst and 7AAD have fluorescence properties that allow detection by flow cytometry.

2.5×10^5 BCL_1.3B3 cells were plated in 1 ml of growth medium in each well of a 24-well plate. The following day, AIG was added to stimulate cells through the BCR. Cells were harvested at 24 hr and washed once with PBS/1% FBS. Cells were then resuspended in 40 μl of a 400 μM solution of the vital dye 7AAD for 1 hr at 4°C. Cells were then fixed by addition of 740 μl of 0.5% paraformaldehyde in PBS (Electron Microscopy Sciences, Fort Washington, PA). 220 μl of 10 μg/ml Hoechst 33342 containing 5% Tween-20 (Sigma) was

immediately added to stain DNA. The working solution of Hoechst 33342 was made from a 1 mg/ml stock solution in methanol. Cells were incubated overnight in the dark prior to data collection by Bonnie Darnell (UTSW Pathology Flow Cytometry Core Facility) using a FACStar™ Plus (Becton Dickinson, San Diego, CA). Data were analyzed using CellQuest™ software (Becton Dickinson). Following gating of cells in Hoechst width versus area plots to exclude cell aggregates, singlet cells were analyzed for viability in dot plots of Hoechst (DNA content) versus 7AAD (membrane permeability) fluorescence. Cell cycle analysis was performed on cells falling within both the singlet gate and the viable gate by analysis of Hoechst signal area. Samples were analyzed in duplicate to ensure consistency in technique.

AnnexinV/BCR staining

In order to evaluate the effects of ligand stimulation on the induction of apoptotic cell death, levels of the membrane phospholipid, phosphatidylserine, on the cell surface was measured by staining with Annexin V, a Ca^{2+}-dependent phospholipid binding protein with high affinity for phosphatidylserine. Phosphatidylserine is translocated from the inner to the outer leaflet of the plasma membrane in early stages of apoptosis.

1×10^5 BCL_1.3B3 cells were cultured in 1 ml of growth medium with duplicate samples for each timepoint. 20 μg of fluorescein isothiocyanate (FITC)-conjugated Goat anti-mu-FITC (AIG) antibody (Jackson Immunoresearch, custom antibody) was added to cell cultures for 1, 4, 12, and 24 hr to label and cross-link the BCR. Cells were harvested and

pelleted at 200g for 5 min at room temperature, then washed once with cold PBS. As per the manufacturer's protocol, cell pellets were resuspended gently in 100 µl of cold 1X binding buffer (diluted from a sterile 10X solution containing 0.1M HEPES/NaOH pH 7.4, 1.4M NaCl, 25mM $CaCl_2$)(BD Pharmingen, San Diego, CA) and 5 µl of AnnexinV-PE (purified recombinant Annexin V conjugated to phycoerythrin for detection by flow cytometry)(BD Pharmingen, San Diego, CA) was added. Samples were incubated for 15 min in the dark, then 400 µl of cold 1X binding buffer was added. Samples were filtered through nylon mesh, then analyzed immediately by flow cytometry using the FACSCalibur™ and CellQuest™ software.

Flow cytometry

Throughout this dissertation, flow cytometry is used to detect and measure levels of cell surface proteins (i.e. Annexin V, B220, BCR, etc.), intracellular molecules (i.e. Hoechst for detecting DNA), and dyes (i.e. 7AAD). Flow cytometry is especially powerful in immunology because of the extent of knowledge about cell development, differentiation, and activation based on cell surface marker expression.

Unless otherwise noted, all flow cytometry samples were analyzed on the FACSCalibur™ or FACScan™ flow cytometers and analyzed using CellQuest™ software (BD Biosciences Immunocytometry Systems). FITC-conjugated anti-mouse CD45/B220 antibody, clone RA3-6B2 (BD Pharmingen), was used for determination of B cell purity.

MHC class II expression was measured by flow cytometric analysis using FITC-conjugated antibody against Class II I-A^d, clone 39-10-8 (BD Pharmingen). Refer to specific sections for antibodies used in specific assays.

Immunofluorescence Microscopy

In order to visualize the BCR in the context of a whole cells, immunofluorescence microscopy was used to detect BCR by labeling the BCR with fluorochrome-conjugated antibodies. All images captured by the camera for one experiment were initially stored as OpenLab LIFF files. Once images were analyzed and processed using OpenLab, they were then stored as individual TIFF files for publication.

BCL_1.3B3 BCR localization. For 3B3 imaging experiments, 3B3 cells were plated at a concentration of 1×10^5 cells/ml in a volume of 0.5 ml into each chamber of a 4-well chamber slide (Nalge Nunc, Rochester, NY). The following day, BCR was labeled by the addition of 5 μl of non-signaling monoclonal rat anti-IgM-FITC clone R6-60.2 (BD Pharmingen). This antibody was dialyzed to remove preservatives using the Slide-A-Lyzer kit for dialysis of small volumes (Pierce Biotechnology, Rockford, IL). After allowing 30 minutes for BCR labeling, AIG signaling antibody was added at a concentration of 20 μg/ml for 120, 90, 60, and 30 min prior to end-point processing. End-processing occurs as follows: growth medium was removed from each of the chambers. Cells were fixed by addition of 1

ml of 0.5% paraformaldehyde for 10 min at room temperature. Paraformaldehyde was then aspirated away and chamber walls were removed to expose a flat microscope slide surface. 12.5 μl of Vectashield (Vector Labs, Burlingame, CA) containing 1 μg/ml of the DNA dye 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes, Eugene, OR) was dropped on each chamber floor, and coverslips were positioned and sealed in place using clear fingernail polish. Slides were either analyzed immediately or stored in the dark at -20°C. Vectashield prevented photobleaching of fluorescence, allowing multiple viewings and extended viewing of each slide.

Slides were viewed and images captured using the Zeiss Axioplan 2 upright fluorescence microscope (Carl Zeiss, Inc, Thornwood, NY) and Openlab software version 4.0.1 (Improvision, Boston, MA). Two images were captured at each field of view at 20X: the first for detection of FITC fluorescence and the second for detection of DAPI fluorescence. These two images were overlayed using Openlab, allowing visualization of individual cells (via DAPI fluorescence) and BCR localization (via FITC fluorescence). Several fields of view were collected for each timepoint and approximately 100 cells, identified by the presence of nuclei, were scored for their BCR staining pattern in a blinded fashion.

WEHI-231 BCR localization. 1×10^6 WEHI cells were harvested and resuspended in 100 μl PBS containing 2% FBS. 2 μl of R6-60.2 antibody was added, and cells incubated on ice for 10 min to allow BCR staining. Following one wash in PBS/2%FBS, cells were resuspended in 100 μl phenol-red free growth medium (WEHI culture medium made with

phenol-red free IMDM, Invitrogen) and kept on ice for stimulation. Cells were stimulated by addition of ligands to the following concentrations: AIG, 20 μg/ml; anti-CD40, 9.73 μg/ml; and LPS, 40 μg/ml. Images were immediately captured at room temperature following ligand stimulation for up to 10 min (all ligands), 30 min (AIG), or 1 hr (AIG). Using the Zeiss Axioplan 2 upright fluorescence microscope, approximately 20 images were captured for each ligand and timepoint with the 63X objective under oil immersion using the Openlab software. Two images were captured for each field of view: one for detection of FITC fluorescence, and one for differential interference contrast (DIC) imaging of cells. Due to rapid photobleaching, images for FITC fluorescence were taken first, and as quickly as possible.

BrdU Proliferation Assay

In order to evaluate the effects of ligand stimulation on proliferation, an assay was used in which BrdU, a thymidine analog, was added to cell cultures ("pulse"). Proliferating cells actively synthesizing DNA will incorporate BrdU, which can then be detected in permeabilized, DNaseI-treated cells using an anti-BrdU antibody (which recognizes BrdU in single-stranded DNA) conjugated to a fluorophore to allow detection and analysis by flow cytometry.

The following ligands and concentrations were used: BAFF (62.5 ng/ml), anti-CD40 (625 ng/ml), LPS (40 μg/ml), AIG (0.3 μM), CpG, (30 μM), IL-4 (0.34 nM), S1-P (0.3 μM),

SDF (60 nM), BLC (0.2 μM), ELC (10 nM), and SLC (1.5 nM). Detailed information about these ligands can be found at http://www.signaling-gateway.org/data/cgi-bin/Protocols.cgi?cat=1. 2×10^6 primary B cells were treated with ligands in 1 ml of prewarmed, CO_2-calibrated Iscove's Modified Dulbecco's Medium supplemented with 2 mM L-glutamine, 55 μM β-mercaptoethanol, 0.025% pluronic F-68 (all from Invitrogen) and 0.1 mg/ml bovine serum albumin (Sigma). Following indicated culture times, cells were then pulsed with 25 μM BrdU (5-bromo-2'-deoxyuridine) (Roche, Basel, Switzerland) for 2 hr, harvested, and resuspended in 0.5 ml of 1% paraformaldehyde and 0.01% Tween20 (Bio-Rad, Hercules, CA) in PBS. Following overnight incubation at 4°C, cells were washed 2× in PBS/2%FBS, then incubated with 10 μg/ml anti-BrdU-FITC antibody clone B44 (BD Pharmingen) and 10 mg/ml DNaseI in PBS for 1 hr at 25°C in a final volume of 100 μl. Volumes were then adjusted to 1 ml with PBS/2% FBS, cells were filtered through nylon mesh, and data were acquired using FACSCalibur™ and analyzed using CellQuest™ software (BD Immunocytometry Systems).

Ca^{2+} flux assay

These experiments were done with the kind help of Keng-Mean Lin (AfCS, Dallas). In order to evaluate the signaling effects of anti-BCR antibodies on the WEHI-231 B cell line, Ca^{2+} flux was measured using a calcium sensitive dye following BCR crosslinking.

WEHI-231 cells were suspended at 1×10^6 cells/ml in assay medium (RPMI supplemented with 10% FBS, 50 μM β-mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate, 20 mM HEPES) containing 0.5 mM probenacid (an organic ion channel blocker)(Sigma-Aldrich) and distributed, 3 ml per well, into individual wells of a 6 well ultra-low attachment plate (Corning, Corning, NY). 18 μl of a mixture containing equal volumes of 0.3 mM Fura-2, AM (Acetoxymethyl)(Molecular Probes) and 20% Pluronic F-127 (Molecular Probes) was added to each well and mixed gently by rocking the plate. The plate was incubated at 37°C with 5% CO_2 for 30 min, with mixing every 10 min. Up to 3 wells of cells was transferred to a 15 ml conical tube. An additional 1 ml of assay medium containing 0.5 mM probenacid was used to rinse each culture well and combined in the conical tube. Cells were centrifuged at 400 × g for 5 min at room temperature. Supernatants were removed and cells were resuspended to their original volume with fresh assay medium containing 0.5 mM probenacid. Resuspended cells were re-plated in new wells of a 6-well ultra-low attachment plate. The plate was incubated at 37°C with 5% CO_2 for 30 min, with mixing every 10 min. Cells were transferred to a new tube. Wells were rinsed with 1 ml assay medium containing 0.5 mM probenacid and combined in the tube. Cells were centrifuged at 400 × g for 5 min at room temperature and washed once with 15 ml Hanks' Balanced Salt Solution (Invitrogen) with 25 mM HEPES and 1 mg/ml BSA (HBSS-BSA) containing 0.5 mM probenacid at room temperature and pelleted by centrifugation. Cells were then suspended in HBSS-BSA with 0.5 mM probenacid at 1 ml per well of cells, counted, and adjusted to 1.1×10^6 cells/ml with HBSS-BSA with 0.5 mM probenacid. Cells were kept at room temperature in the dark for no more than 2 hr before analysis. 9.9×10^4

cells (0.090 ml) were distributed into one well of a 96-well black-walled plate (Thermo Electron Corporation, Waltham, MA) and incubated in a 37°C environmental chamber (or in the fluorometer chamber) for 5 min to equilibrate temperature. The plate was transferred to the Fluoroskan Ascent Microplate Fluorometer (Thermo Electron Corporation) and baseline fluorescence was read for 5 min with filters for excitation at 340 nm and emissions at 510 nm. After the baseline measurement, 0.010 ml of 10X concentrated ligand or HBSS-BSA with 0.5 mM probenacid was added, the plate was mixed by the Fluoroskan for 1 second, then fluorescence measured continuously for 10 min. Data were analyzed using the Ascent software (Thermo-Labsystems). Cells were stimulated with 20 μg of AIG (Jackson Immunoresearch), or indicated amounts (0.125, 0.25, 0.5, or 1.0 μg) of monoclonal anti-IgM-FITC antibodies clone R6-60.2 or II-40 (BD Pharmingen).

Internalization assays

To evaluate internalization of the BCR in response to BCR cross-linking, a non-stimulating antibody was used to label the BCR, followed by cross-linking of the BCR using polyclonal antibody. Cell surface label was removed using an acid wash, and remaining fluorescence (due to protection from acid wash by internalization) was measured by flow cytometry.

$1x10^6$ WEHI-231 cells or 2×10^6 primary B cells were pre-stained for 10 min at 4°C with monoclonal antibodies to cell surface receptors: Rat anti-IgM-FITC clone R6-60.2 or

clone II/40 (BD Pharmingen), Rat anti-CD40-FITC clone 3/23 (BD Pharmingen), or Rat anti-TLR4/MD2-PE (phycoerythrin) clone MTS510 (eBiosciences). Following 2 washes in PBS/2% FBS/2mM EDTA, cells were cultured and stimulated with AIG (20 μg/ml), anti-CD40 (9.73 μg/ml) or LPS (40 μg/ml) in growth media. Following incubation at 37°C with 5% CO_2 for various time periods, cells were harvested and incubated at 4°C for 5 min in 0.2M acetic acid/0.5M NaCl to strip off staining antibodies (Tebar et al. 1999), or in PBS/2% FBS/2mM EDTA (control). Following 2 washes in 0.2M Acetic acid/0.5M NaCl or PBS/2% FBS/2mM EDTA, respectively, samples were fixed in 1% paraformaldehyde. Data were acquired using a FACSCalibur™ flow cytometer and analyzed using CellQuest™ software (Becton Dickinson Immunocytometry Systems).

Immune complex kinase assay (ICKA) and immunoblot

In order to evaluate Syk kinase activity following BCR cross-linking, we used an ICKA in which Syk was immunoprecipitated from detergent-soluble cell lysates and incubated with [γ^{32}]P-ATP. The lysates were then separated by denaturing gel electrophoresis and kinase activity was determined by the degree of autophosphorylation observed and quantitated using a phosphorimager.

5×10^6 BCL_1.3B3 or 2×10^7 primary B cells were washed once in 1 ml of PBS. Loosened cell pellets were incubated for specified amounts of time at 37°C with 20 μg of AIG to cross-link the BCR or polyclonal goat anti-ovalbumin (BT161) as a cross-linking

control. Cells were then lysed in 200 µl lysis buffer containing 5% nonidet P-40 (NP-40), 25 mM Tris-HCL pH 7.4, 150 mM NaCl, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate for 15 min on ice, then brought up to 1 ml with lysis buffer not containing NP-40 to reach a final concentration of 1% NP-40. Nuclei were pelleted by centrifugation at 14K rpm in a microfuge at 4°C and supernatants recovered. Supernatants were stored at 80°C for future use, or used immediately. Supernatants were incubated with protein-A agarose beads (Invitrogen) coated with rabbit anti-Syk antibodies (SC-929, Santa Cruz), or rabbit anti-ova antibodies (T158, gift from Drs. Ellen Vitetta and Jonathan Uhr). 4 µl beads were coated with 2 µg antibody overnight, then washed twice in wash buffer (TBS pH 7.5, 0.5% Triton X 100), then once in TBS (2 mM Tris, 13.7 mM NaCl, adjusted to pH 7.5 with HCl) for at least 1 hr or up to overnight with rotation. The beads were pelleted and washed 3 times in wash buffer. Beads were resuspended in 50 µl kinase buffer (5 mM $MgCl_2$, 5 mM $MnCl_2$, 20 mM Tris pH 7.5, 100 mM NaCl). 10 µCi of [γ-^{32}P]-ATP (Amersham Pharmacia, Piscataway, NJ) was added per sample and incubated for 15 min at room temperature. The beads were washed twice with 500 µl wash buffer and resuspended in 40 µl 2X SDS sample buffer (100 mM Tris pH 6.8, 4% SDS, 0.2% bromphenyl blue, 20% glycerol, 0.4 M DTT). Beads were heated at 95°C for 5 min, then 20 µl of supernatant per sample was resolved by SDS-PAGE on a 9% polyacrylamide gel and transferred to nitrocellulose for exposure to a phosphorimager screen and visualization using the Typhoon 8600 variable mode imager (Amersham Biosciences, Piscataway, NJ) and ImageQuaNT™ software (Amersham Biosciences).

Semiquantitative RT-PCR analysis

In order to determine relative expression levels of cell cycle-related genes found in Gene Cluster #1 of the BAFF/CD40L microarray data set to confirm microarray expression data, we used semi-quantitative RT-PCR.

RNA was isolated as described in the "Microarray Analysis and Clustering" section. cDNA was synthesized using 1 μg of total RNA treated with DNaseI (Invitrogen) to remove contaminating genomic DNA. First-strand cDNA synthesis was performed in a 20 μl reaction using 0.1 μg random hexadeoxynucleotide primers (Amersham Biosciences) and 200 U M-MLV Reverse Transcriptase, 1X first-strand buffer (diluted from a 5X stock containing 250mM Tris-HCl pH 8.3, 375 mM KCl, 15mM $MgCl_2$. 0.1M DTT), 0.1 mM DTT and 0.5 mM dNTPs (Invitrogen). 50 μl PCR reactions were performed using 1X buffer (diluted from GeneAmp 10X PCR buffer containing 15 mM $MgCl_2$, 500 mM KCl, 100mM Tris-HCl pH 8.3, 0.01% w/v gelatin) (Applied Biosystems, Foster City, CA), 0.8 mM dNTPs (0.2mM each dNTP), 2 U Platinum Taq (Invitrogen), and 20 pmol forward and reverse primers. PCR conditions include a 2 min incubation at 95°C followed by 30 amplification cycles (0.5 min at 94°C, 0.5 min at 55°C, 1 min at 72°C) followed by a final extension of 9 min at 72°C. All primers except GAPDH were designed by the Primer3 software (Whitehead Institute for Biomedical Research, http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi): Cyclin A2 forward 5'-ttggttaacattggtgctgc-3', reverse 5'-gcagtttggtttggttgctt-3'; cdc2 homolog A forward 5'-tccacttgggaaaggtgttc-3', reverse 5'-ctcggctcgttactccactc; tubulin beta forward 5'-accgaagctgagagcaacat-3', reverse 5'-

cagttggagaaagctgaggg-3'; GAPDH forward 5'-caccatggagaaggc-3, reverse 5'-tgccagtgagcttcc-3'. PCR products were resolved on 2% agarose gels and bands visualized using SYBR® Gold nucleic acid gel stain (Molecular Probes, Eugene, OR). Gels were imaged using the Typhoon 8600 variable mode imager and bands were quantified using the ImageQuaNT™ software (Amersham Biosciences). Values from BAFF- and anti-CD40-treated samples were normalized to GAPDH values, then compared to the 0 hr treated sample.

Real-time RT-PCR Analysis

Following the advent of real-time PCR technologies and instrumentation, we used real-time PCR to determine relative expression levels for genes associated with Gene Cluster #18 of the single ligand screen data set. We used the Sybr Green double-stranded DNA-binding dye to detect PCR product formation. These experiments were done to confirm microarray expression results and to test the coordinate expression hypothesis.

Total RNA was extracted as for the microarray experiments. 1 μg of total RNA was treated with DNaseI (Invitrogen), then reverse transcribed at 42°C for one hr in a 20 μl volume containing 1 μl MMLV reverse transcriptase (Invitrogen), 4 μl 5X buffer (Invitrogen), 0.5 mM dNTPs (Invitrogen), and 5 ng/μl $pd(N)_6$ (Amersham). Following inactivation at 70°C for 20 min, PCR reactions were set up in a 20 μl volume using Sybr Green Master Maker (Applied Biosystems). Thermal cycling began with a denaturation step of 10 min at 95°C,

followed by 40 cycles of 95°C for 15 seconds (denaturation) and 60°C for 1 min (annealing and extension). PCR reactions were performed in the ABI Prism 7700 Sequence Detector (PE Biosystems, Foster City, CA), and data collected and analyzed with the Sequence Detector software (PE Biosystems). Standard curves were generated using RNA isolated from RBC-depleted mouse splenocytes and serially diluted up to 1:15625. Standard curves had at least 5 points and one standard curve was generated for each gene being evaluated. PCR results from unknown samples were compared to the corresponding standard curve in order to determine a relative "quantity" value describing the amount of PCR product. Quantity values from PCR results of all samples amplified using mouse 18S rRNA primers was used for normalization. Quantity values from PCR data of treated samples were compared to untreated samples, giving values representing the fold change in gene expression relative to untreated samples. PCR primers were designed using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi): ATP6v0Bc" forward (f) 5'gaaccccagcctctttgtaa3', reverse (r) 5'cccatcttcactctggaggt3'; ATP6v1c1 (f) 5'tgcttgccaaagaggtaaca3', (r) 5'tcgctgcatgtagtttctcc3'; sorting nexin V (f) 5'gggagaaggggaaggatcta3', (r) 5'catgggtggacacagtcttc3'; vacuolar protein 29S (f) 5'ctgcagaggcagtttgatgt3', (r) 5'ggcagaacctgggttaatgt3'; ATP6v0c (f) 5'atgtcagtcatgaggccaga3', (r) 5'agcgataagtactgccacca3'; ATP6v0a1 (f) 5'tccacccagtctgtaggtga3', (r) 5'atcatgatcagggtgcagaa3'; ATP6v1h (f) 5'gatgctgctgtcccaactaa3', (r) 5'agaaatcatctgcccctgaa3'; ATP6v1a1 (f) 5'gaattatgatgcgtccgatg3', (r) 5'cgcctgggatagcagtagtt3'; 2700018N07 (f) 5'ttctgtctccccagagaagg3', (r) 5' acgtgtttccagcatagcag3'. Mouse 18S PCR primers sequences

are from (Schmittgen and Zakrajsek 2000): (f) 5'gtaacccgttgaaccccatt3', (r) 5'ccatccaatcggtagtagcg3'.

Northern Blot Analysis

We used Northern Blot analysis in order to quantify the expression of RNA processing-related genes in Gene Cluster #11 of the BAFF/CD40L microarray data set. These experiments were performed in order to confirm microarray expression results and test the coordinate expression hypothesis.

TriPure Isolation Reagent (Roche, Indianapolis, IN) was used to isolate total RNA from cultured B cells as for the microarray experiments. Typical RNA yield was at least 1 μg from 1×10^6 purified B cells. 5 μg of total RNA per sample was gel fractionated, transferred, and immobilized to Hybond™-XL membranes (Amersham Pharmacia Bioteck UK Limited) using Ambion's (Austin, TX) NorthernMax™-Gly kit. Probes for hybridization were generated by PCR using the following primers which were designed by the Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi): homolog to Exportin T forward (f) 5'-tccaggagttcatcccactc-3', reverse (r) 5'-ctgcaggaaggcgaagtaac-3'; homolog to snRNP core protein D2 (f) 5'-gatctgcgagcgaaacctac-3', (r) 5'-ccttgttgacaggcttggat-3'; U1snRNP E (f) 5'-gtgcagcccatcaaccttat-3', (r) 5'-ctttaaacacgctcaagggg-3'; Ran (f) 5'-tgtgtggcaacaaagtggat-3', (r) 5'-tcctcatctgggagagcagt-3'. PCR products were purified using Nucleospin® (Clontech, Palo Alto, CA) and labeled with [α-^{32}P]dATP (Amersham

Pharmacia) in a random priming reaction using the Strip-EZ™ DNA kit (Ambion). Between hybridizations, blots were stripped using the same kit. Membranes were exposed to phosphorimaging screens and the Typhoon 8600 variable mode imager (Amersham Biosciences) was used for detection. ImageQuaNT™ software (Amersham Biosciences) was used for data analysis.

Microarray Analysis and Clustering

Microarray experiments were performed by the AfCS. Gene expression microarray analysis was used to define common and unique biological responses of B cells to ligand stimulation. These data are made freely available to the scientific community.

Primary B cell culture and stimulation. Freshly isolated purified primary B cells were resuspended in prewarmed, CO_2-calibrated Iscove's Modified Dulbecco's Medium supplemented with 2 mM L-glutamine, 55 μM β-mercaptoethanol, 0.025% pluronic F-68 (all from Invitrogen) and 0.1 mg/ml bovine serum albumin (Sigma) at 16.7×10^6 cells/ml and 1.08 ml were plated into individual wells of a 12-well tissue culture plate. Cells were incubated for 1 hr at 37°C with 5% CO_2. 0.12 ml of 10X concentrated ligand was added to wells, resulting in final 1X concentrations. Concentrations for all ligands can be found at http://www.signaling-gateway.org/data/micro/cgi-bin/micro.cgi?expt=bref. 0.12 ml of growth medium was added to all untreated samples. Samples were incubated at 37°C with 5% CO_2 for desired times. At the end of treatment times, cells were transferred from wells to

15 ml conical tubes and centrifuged for 5 min at 400×g to pellet cells, then supernatants were removed. 1 ml of ice-cold TriPure reagent (Roche) was added to wells to recover remaining cells and 2 ml of TriPure was added to conical tubes. Cells were lysed by pipetting up and down. The combined 3 ml of lysed cells were then stored at -80°C until ready for RNA extraction.

RNA extraction. Frozen cell lysates were thawed at room temperature. Chloroform (Sigma) was added to 20% of TriPure volume and tubes were shaken for 30 seconds. Tubes were incubated at room temperature for 5 min, then centrifuged at 4°C for 15 min at 12,000×g. The colorless upper aqueous phase was transferred to a new tube. Another extraction was performed by addition of chloroform at an equal volume as the aqueous phase volume. Tubes were shaken for 30 seconds, then centrifuged immediately at 4°C for 15 min at 12,000×g. The aqueous (top) phase was transferred to a new tube and isopropanol (Sigma) was added at a volume equal to 50% of the original TriPure volume. Samples were mixed by inversion and incubated for 5 min at room temperature. Samples were centrifuged for 5 min at 4°C at 18,000×g. Supernatants were carefully removed to avoid disturbing the RNA pellet. 1 ml of 70% ethanol was added to wash pellet and samples were centrifuged briefly to anchor pellet. Supernatants were carefully removed and samples were air-dried just until RNA pellets become clear. RNA pellets were resuspended in 10 µl nuclease-free water and incubated for 20 min on ice with vortexing every 5 min. RNA concentrations were determined by diluting 1 µl of sample into 79 µl Tris-EDTA and absorbance was read at 260 nm. RNA samples were stored at -80°C.

Sample preparation and hybridization. Details for preparation of RNA samples and hybridization to Agilent inkjet-deposited cDNA arrays can be found at http://www.signaling-gateway.org/data/cgi-bin/Protocols.cgi?cat=0, protocol ID: PP00000019. Briefly, total RNA was used as starting material to generate cDNA. Following cleanup, cDNA templates were *in vitro* transcribed to amplify RNA, then RNA samples were fluorescently labeled using Cy3 (for total spleen reference RNA) and Cy5 (test sample RNA). Fluorescently-labeled RNA were then mixed and hybridized to the array slide surface by incubation in a hybridization chamber. Slides were then washed, dried, and scanned.

Microarray experiments and analysis. RNA samples from treated B cells were compared with untreated samples using a custom-printed microarray chip (Agilent Technologies, Palo Alto, CA) containing 15,494 cDNA probes printed on 15,832 spots representing 10,615 unique MGI gene matches (as of 12/31/03). 38% of the probes have not been assigned a gene name, 96% come from the RIKEN FANTOM collection, 3% from the Minoro Ko National Institute of Aging collection, and the rest from the Research Genetics and Genome Systems collections. RNA samples were used to generate Cy5- and Cy3-labeled cDNA targets (from sample RNAs and RBC-depleted total splenocyte RNA, respectively) and were hybridized together.

For BAFF/CD40L microarray experiments, arrays were scanned using the Agilent Scanner G2505A (Agilent Technologies). Image files were analyzed using the Agilent G2566AA Feature Extraction software version A.6.1.1. Spots were eliminated from analysis

if the signal was within 3 standard deviations of background fluorescence (Kadota et al. 2001). The normalized ratio of sample fluorescence to spleen fluorescence was calculated for each probe. The average normalized ratio for triplicate samples was then divided by the average normalized ratio for 0 hr untreated samples, giving values that represent the fold change in transcript levels in each sample relative to 0 hr untreated. These values were analyzed using the Cluster and Treeview programs, available at http://genome-www.Stanford.edu/~sherlock/cluster.html. The data were filtered to remove probes that showed less than a 1.8$\log_2$ fold difference in relative expression under the experimental conditions analyzed. These values were then clustered by the hierarchical clustering/average linkage method using uncentered correlation as the metric. The raw data from these experiments is available at http://www.signaling-gateway.org/data/micro/cgi-bin/micro.cgi?expt=blong.

For single ligand screen microarray experiments, all samples were run in triplicate except for 1 hr and 4 hr untreated controls, which were run in quadruplicate. Arrays were scanned using the Agilent Scanner G2505A. Image files were analyzed using the Agilent G2566AA Feature Extraction software version A.6.1.1. The raw data from these experiments is available at http://www.signaling-gateway.org/data/cgi-bin/table.cgi?cellabbr=BC. For basic filtering, spot features on each array with fluorescence intensity and $\log_2$(Cy5/Cy3) values that were saturated (flagged with Agilent "gIsSaturated"), non-uniform ("gIsFeatNonUniFOL" and "rIsFeatNonUniFOL") or below background ("gIsWellAboveBG" and "rIsWellAboveBG") were set to blank. Statistical filtering was accomplished using Significance Analysis of Microarrays (SAM) (Tusher et al. 2001).

Features found by SAM to be differentially expressed between samples and time-matched untreated controls at a false discovery rate (FDR) of 1% were included for further analysis. Input for SAM were background-subtracted, dye bias- and interarray variance-normalized Cy5 fluorescence intensity values, which represent expression level of array features. Only features with more than two replicates were used in the SAM analysis. 100 random permutations were done for each comparison of treated to time-matched control samples. Values of +1, -1, or 0 were given to genes that were found by SAM to be significantly upregulated, downregulated, or unchanged compared to time-matched untreated controls. These categorical values were used to cluster genes together based on their expression response patterns.

CLASSIFI analysis.

*Cl*uster *Assi*gnment for Biological *I*nference (CLASSIFI) was developed as a method for statistical evaluation of Gene Ontology™ (GO) annotation co-clustering. CLASSIFI is predicated on the postulate that genes involved in the same biological process are coordinately expressed. Following data transformation, filtering, normalization, standard expression clustering approaches and gene cluster membership assignment, the following steps were performed in the CLASSIFI analysis: 1) remove duplicate probe IDs, 2) extract the primary GO annotations for each gene from a probe database, 3) capture the full GO ancestry for each primary GO annotation from the Gene Ontology™ database, and 4)

calculate the solution for the cumulative hypergeometric distribution equation for every ontology in every gene cluster:

$$P = 1 - \sum_{i=0}^{n-1} \frac{\binom{f}{i}\binom{g-f}{c-i}}{\binom{g}{c}}$$

where *g*=number of probes in the data set, *c*=number of probes in the gene cluster, *f*=number of probes with a given ontology in the data set, *n*=number of probes with a given ontology in the gene cluster. The hypergeometric distribution calculates the probability (*P*) that genes with a particular GO annotation would co-cluster by chance given the proportion of genes with this annotation in the entire data set. A web interface for use of the CLASSIFI method with data derived from cDNA, oligonucleotide and Affymetrix microarrays, along with detailed information is available at **http://pathcuric1.swmed.edu/pathdb/classifi.html**.

Chapter 3

Studies in B cell antigen receptor signaling

Introduction

BCR cross-linking activates signal transduction pathways including tyrosine kinase activation and changes in intracellular calcium that result in a host of downstream cellular responses such as proliferation or apoptosis. The results in this chapter illustrate the biochemical and cellular responses of the immature B cell line BCL_1.3B3 in response to BCR cross-linking, and in particular the similarity of early signaling responses of the Syk tyrosine kinase between BCL_1.3B3 and primary splenic B cells. These experiments have been repeated extensively in the lab and the results are established, therefore this chapter serves as an experimental preface to the main study of signaling in B cells.

The murine B cell lymphoma BCL_1 spontaneously arose in an elderly Balb/c mouse (Slavin and Strober 1978). When transferred i.v. to syngeneic animals, BCL_1 cells extravasate into the spleens of recipient animals, causing palpable splenomegaly within 4 to 5 weeks (Warnke et al. 1979). BCL_1 is a monoclonal B cell-derived tumor, thus all cells share the same immunoglobulin structure (Knapp et al. 1979). Immunization of mice with BCL_1 immunoglobulins prior to challenge with a lethal dose of BCL_1 cells allows survival of syngeneic mice (George et al. 1987). The presence of a tumor-specific immune response in immunized mice controls the expansion of tumor cells; however the cells retain malignancy and expand upon transfer to naïve mice (George et al. 1987; Uhr et al. 1991). Survival of immunized animals is thought to be attributed to tumor dormancy, a phenomenon in which expansive growth of tumor cells is absent despite tumor cell presence. BCL_1 cells were established from the BCL_1 tumor for *in vitro* culture, and the resulting subclone, BCL_1.3B3, is phenotypically and functionally similar to the parent tumor, with the ability to undergo

induction of tumor dormancy (Brooks et al. 1983; Racila et al. 1995; Scheuermann et al. 1995). Our lab has used BCL_1.3B3 cells to establish mechanisms of tumor dormancy, as well as to study the biochemical events of BCR signaling.

Tyrosine kinases are components of early signal transduction events from immune receptors. Src family members (Lyn, Blk, Fyn), Tec family members (Btk) and the Syk/Zap-70 family member Syk are all involved in signal propagation from the BCR. These proteins contain SH2 (Src homology domain 2) domains that are capable of binding phophorylated tyrosines in the immunoreceptor tyrosine-based activation motifs (ITAMs) found in the cytoplasmic tails of Igα and Igβ. Syk, Spleen Tyrosine Kinase, is a non-receptor, cytoplasmic tyrosine kinase related to Zap-70. While Zap-70 expression is restricted to T cells and natural killer (NK) cells, Syk is expressed in various cells of the hematopoietic lineage (Chan et al. 1994). Upon stimulation through the BCR, Syk binds to phosphorylated ITAMs of Igα/Igβ via its Src homology 2 (SH2) domains and becomes activated (Kimura et al. 1996; Rowley et al. 1995). Syk is required for downstream phosphorylation of the adaptor molecule B cell linker protein (BLNK), which then recruits and activates PLC-γ2 and Btk, allowing for inositol-1, 4, 5-triphosphate (IP_3) production and calcium flux (Fluckiger et al. 1998; Ishiai et al. 1999; Takata et al. 1994; Xu et al. 2000).

In RAG1-deficient mice reconstituted with Syk-deficient fetal liver, pre-B cell numbers are reduced (Turner et al. 1995), suggesting that Syk is required for B cell development. Syk-deficiency in mice results in perinatal lethality (Chu and Paul 1998; Song et al. 1995; Turner et al. 1995), thus the effects of Syk-deficiency in B cells has been studied in a chicken B cell line, DT40, due to ease of gene targeting by homologous recombination

(Buerstedde and Takeda 1991). Crosslinking of the BCR by mu chain-specific antibodies induces apoptosis in DT40 cells. The lack of PLCγ2 phosphorylation and the resulting calcium flux upon BCR cross-linking in Syk-deficient DT40 demonstrates the requirement for Syk in this signaling pathway (Takata et al. 1995; Takata et al. 1994). Deletion of PLC-γ2 in DT40 cells results in absence of apoptosis, demonstrating the link between Syk signaling events and downstream cellular response (Takata et al. 1995). Deficiencies of other tyrosine kinases also negatively affect BCR signal transduction. For example, Lyn-deficiency in DT40 delays the Ca^{2+} response following BCR cross-linking (Takata et al. 1994) and splenic B cells from Btk-deficient mice fail to proliferate in response to BCR cross-linking (Khan et al. 1995). The results in this chapter focus on Syk activity following BCR cross-linking.

Results and Discussion

Multiple mechanisms are responsible for dormancy. When stimulated with polyclonal anti-IgM (AIG) to cross-link the BCR, numbers of recovered BCL_1.3B3 cells remain constant over several days of culture (Figure 3.1). This population stability in vitro may be a reflection of the stability of BCL_1 observed in dormant animals (Vitetta et al. 1997). To define the cellular responses responsible for population stability, apoptosis and cell cycle status was determined using the vital dye 7-AAD and the DNA intercalating dye, Hoechst 33342. Results are visualized using flow cytometric analysis. Apoptotic cells which have lost membrane integrity take up the vital dye, becoming 7AAD+. Hoechst 33342 gains

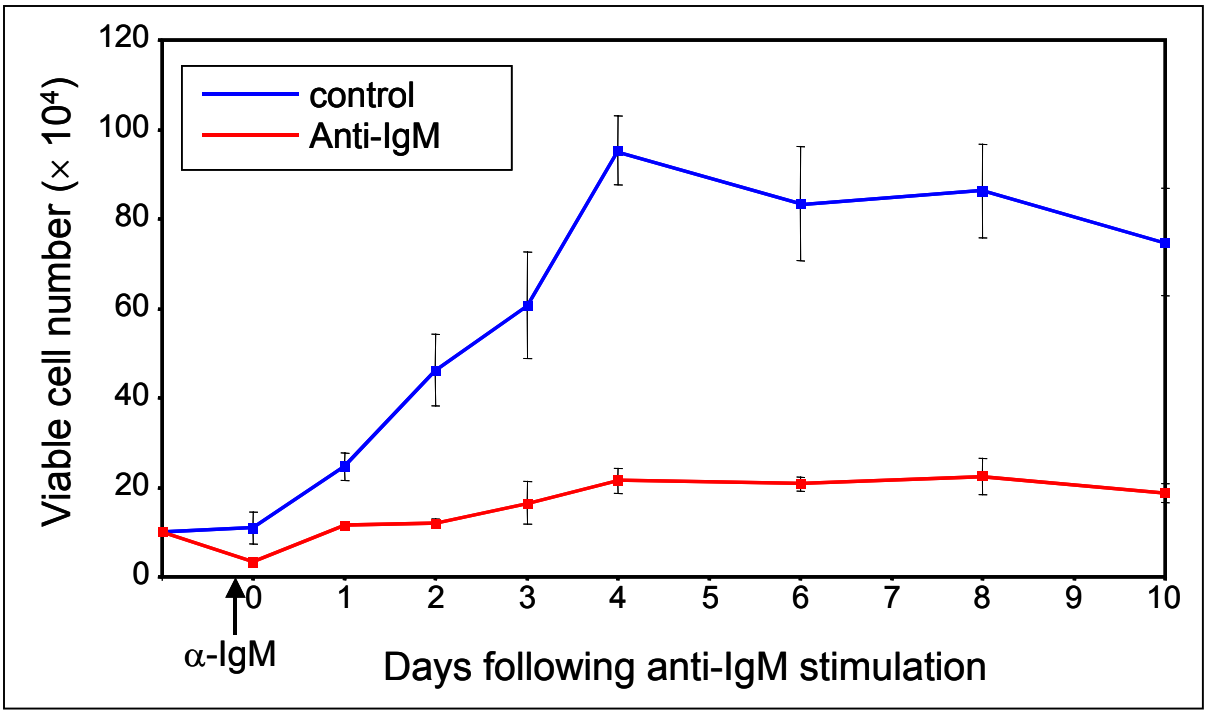

Figure 3.1. Population stability in BCL_1.3B3. Number of recovered viable BCL_1.3B3 cells as determined by trypan blue exclusion remains constant over time in culture in the presence of anti-IgM. Cells were plated at 1×10^5/ml, then live cells were counted over 10 days of culture. Unstimulated control cultures exhibit normal population cell kinetics, expanding exponentially in early days of culture, then reaching a plateau at day 4. Anti-IgM-treated cells, however, do not expand and their numbers remain constant through the course of the experiment. n=3.

access to permeabilized cells and binds to DNA. Its fluorescence intensity is proportional to cellular DNA content, allowing for determination of cell cycle position. In dot plots showing 7AAD versus Hoechst fluorescence (Figure 3.2), apoptotic cells (red) have a low Hoechst fluorescence since their DNA is being degraded and are 7AAD+ since they have lost membrane integrity, allowing uptake of 7AAD. Viable cells (green) do not take up 7AAD and are therefore 7AAD- and have a medium to high Hoechst fluorescence related to their DNA content. Viable cells in the G_0 or G_1 phase of the cell cycle have a 2n DNA content, while cells in the G_2 or M phase have a 4n DNA content. Cells with intermediate fluorescence between the 2n and 4n peaks are in S phase. Cross-linking of the BCR with AIG results in increased apoptosis (31.5%) as compared with untreated cells (6.6%), and decreased numbers of viable cells in S/G_2/M phases of the cell cycle (22.4% in untreated versus 13.8% in AIG-treated cells) (Figure 3.2). Despite the decrease in S/G_2/M phase cells, proliferation still occurs in AIG-treated cell cultures, albeit at much lower levels than in untreated cells (Hammill, unpublished data). Thus, population stability is the result of a balance between cell division, cell cycle arrest, and apoptosis.

The extent of apoptosis and cell cycle arrest is dependent on the concentration of AIG used to cross-link the BCR. The apoptotic response is maximal at 15-30 μg of AIG, while the cell cycle arrest response is maximal at 1-2 μg of AIG (Figure 3.3). Experiments to determine the kinetics of BCR-induced apoptosis and cell cycle arrest demonstrate that apoptosis occurs as early as 1 hour of AIG stimulation (Figure 3.4) whereas cell cycle arrest is not detectable until 8 hours of AIG stimulation (Hsueh, unpublished data). These data

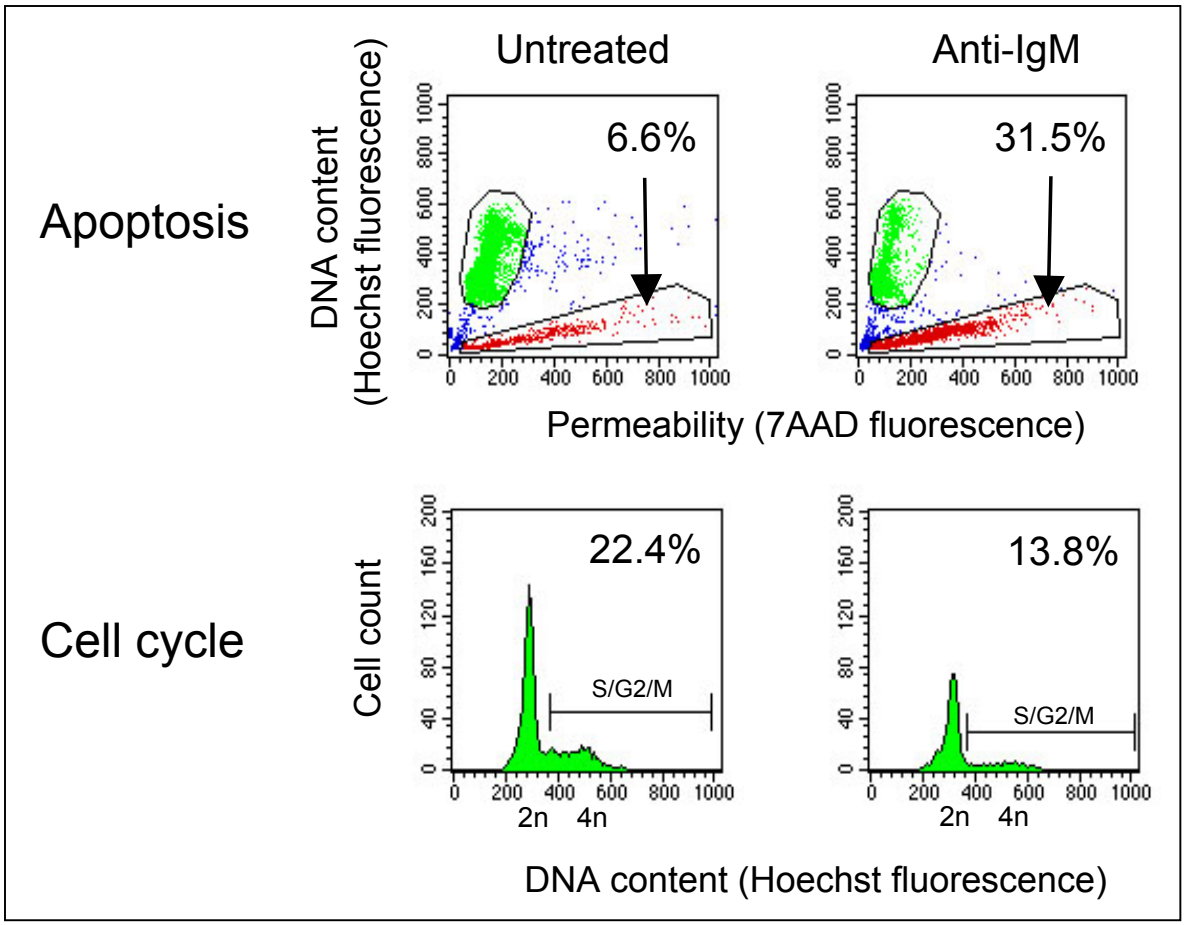

Figure 3.2. Apoptosis and cell cycle arrest induced by anti-IgM treatment in BCL_1.3B3. 2×10^5 BCL_1.3B3 cells were incubated with or without 15 μg of polyclonal goat anti-IgM antibody in 1 ml of growth medium at 37°C for 24 hr. Samples were harvested, stained with 7AAD and Hoechst 33342, and analyzed by flow cytometry. Representative dot plots (top row) distinguish viable cells (green) from apoptotis cells (red). Percentages indicate proportion of apoptotic cells. Representative histogram plots (bottom row) show DNA content of viable cells (green on dot plots). Cells with a 2n DNA content are in cell cycle G_0 or G_1 phases. Cells with a 4n DNA content are in the G_2 or M phases. Cells with DNA content between 2n and 4n are in S phase. Percentages indicate the proportion of cells in S, G_2, and M phases of the cell cycle. Cells with DNA content of <2n indicate apoptotic cells with degraded DNA.

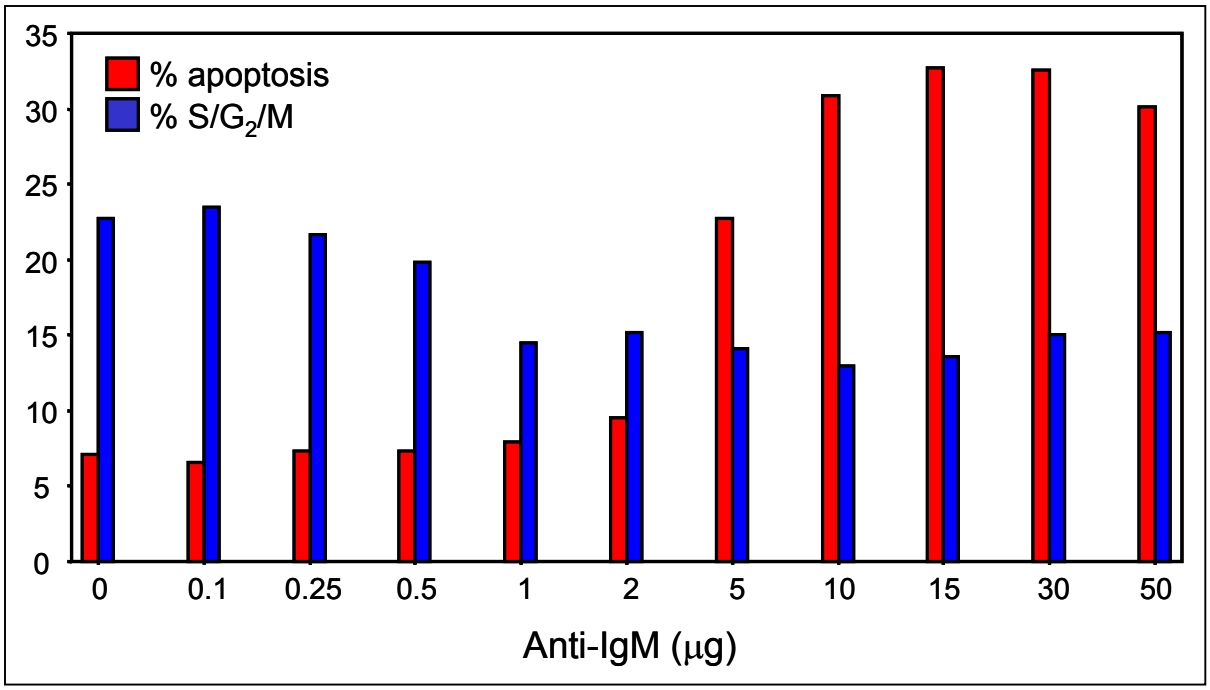

Figure 3.3. Levels of BCR cross-linking affect the decision between cell cycle arrest and apoptosis. 2×10^5 BCL_1.3B3 cells in 1 ml culture media were treated with varying amounts of polyclonal goat anti-IgM and proportions of apoptotic cells or $S/G_2/M$ phase cells were determined by Hoechst/7AAD staining and flow cytometric analysis. Cell cycle arrest (as determined by cells in G_0 or G_1 phases) is maximal at low (1 μg) amounts of anti-IgM while the apoptotic response is maximal at high (15 μg) amounts of anti-IgM.

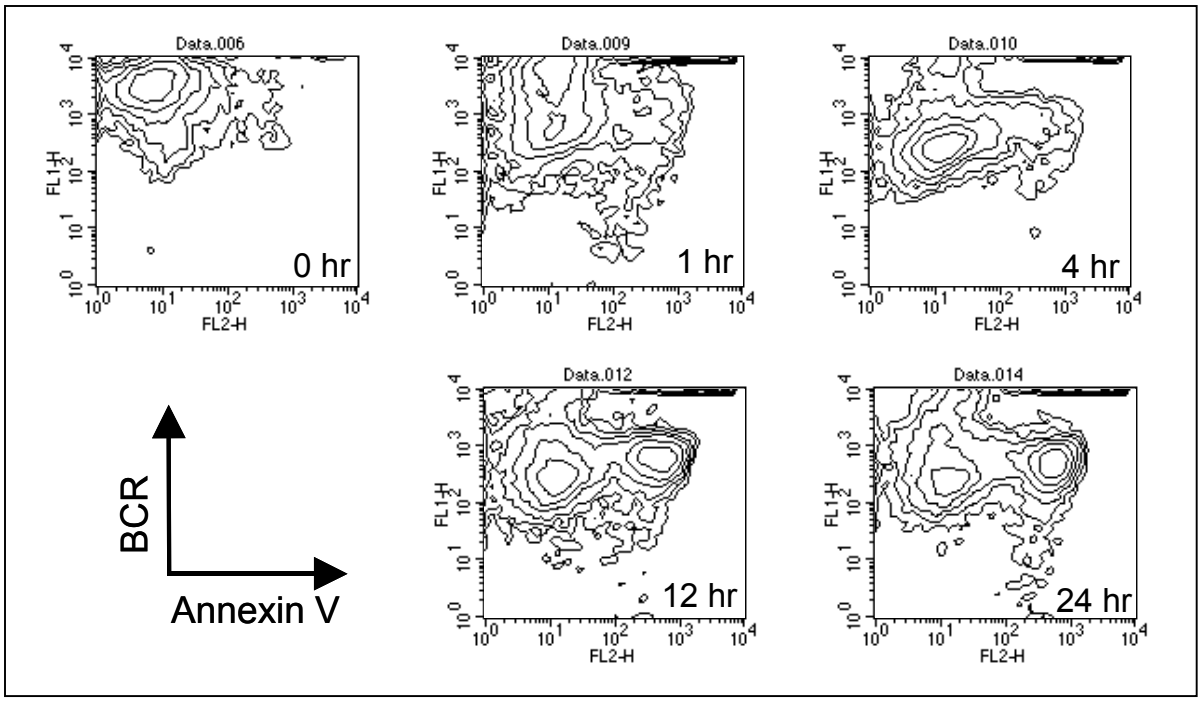

Figure 3.4. BCR levels are down-regulated upon anti-IgM treatment. BCL_1.3B3 cells were simultaneously labeled and stimulated with 20 μg polyclonal goat anti-IgM conjugated to FITC. Apoptotic cells are detected by binding of PE-conjugated Annexin V. BCR levels decrease by 1 hr of treatment and cells begin to undergo apoptosis. Apoptotic cells that are Annexin V^+ have higher BCR levels than viable cells, suggesting a link between BCR levels and apoptosis. By 24 hr of treatment, three cell populations are apparent: $BCR^+AnnexinV^-$, BCR^{high}Annexin V^+, and BCR^{low}Annexin V^+.

suggest that the kinetics and dose of AIG stimulation affects the balance between cell cycle arrest and apoptosis.

Syk activity depends on the nature of BCR cross-linking. We investigated Syk activation between BCL_1.3B3 and primary resting B cells purified from C57BL/6 mouse spleens, as Syk activity increases following BCR cross-linking and is essential for signal transduction from the BCR.

A Syk-specific immune complex kinase assay (ICKA) was used to determine Syk activity levels based on autophosphorylation following BCR cross-linking. In BCL_1.3B3, Syk kinase activity is low in unstimulated cells. An increase in Syk kinase activity is detected within 15 seconds of BCR cross-linking. Kinase activity peaks at around 30 seconds of stimulation, then drops back down to near-baseline levels (Figure 3.5A and C). This ICKA experiment was repeated using purified primary mouse splenic B cells to determine whether this early BCR signaling event occurs in a similar fashion in normal non-transformed B cells. The Syk activation response is remarkably similar in primary B cells, with kinase activity increase detected by 15 seconds and peaking at 30 seconds of BCR cross-linking (Figure 3.5B and C). To determine how levels of BCR cross-linking affects Syk kinase activity, a dose response experiment was performed in which BCL_1.3B3 or primary B cells were stimulated with anti-IgM ranging in amount from 0.1 μg to 50 μg. Again, Syk kinase activity response is remarkably similar between 3B3 and primary B cells, with Syk activity peaking at 10 μg (Figure 3.6A and B). The similarity between Syk kinase activity between BCL_1.3B3 and primary B cells would suggest that 3B3 is a good model for

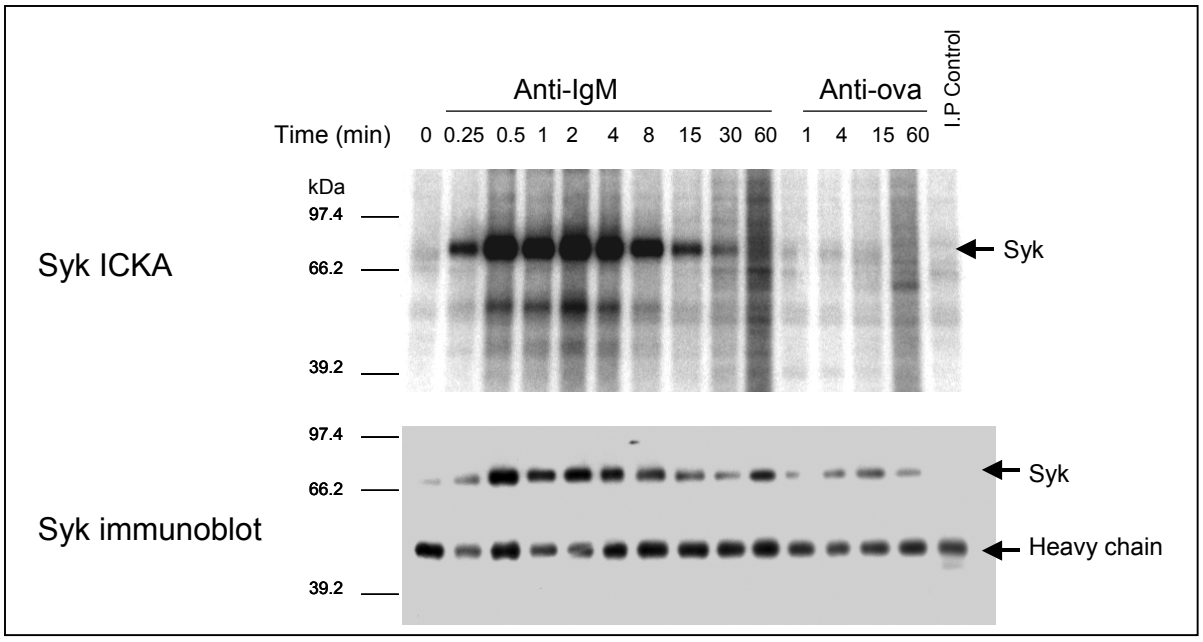

Figure 3.5A. Raw data from BCL_1.3B3 Syk ICKA. Syk, a 72 kDa protein (arrow) was immunoprecipitated from untreated or anti-IgM-treated BCL_1.3B3 cells and tested for activation by autophosphorylation in an ICKA (top). 20 μg of Goat anti-IgM was used to stimulate cells and 20 μg Goat anti-ova was used as the control treatment. Rabbit anti-ova was used for the immunoprecipitation (I.P.) control. Presence of Syk protein was confirmed by subsequent immunoblot (bottom). The varied levels of immunoprecipitated Syk protein detected may be due to the immunoprecipitating antibody or the immunoblot antibody recognizing activated Syk more readily. Past experiments normalizing Syk activity levels to Syk protein levels reveal that Syk activity still increases despite this difference in Syk protein amounts. Immunoglobulin heavy chain co-immunoprecipitation is an artifact associated with ICKA experiments in B cells.

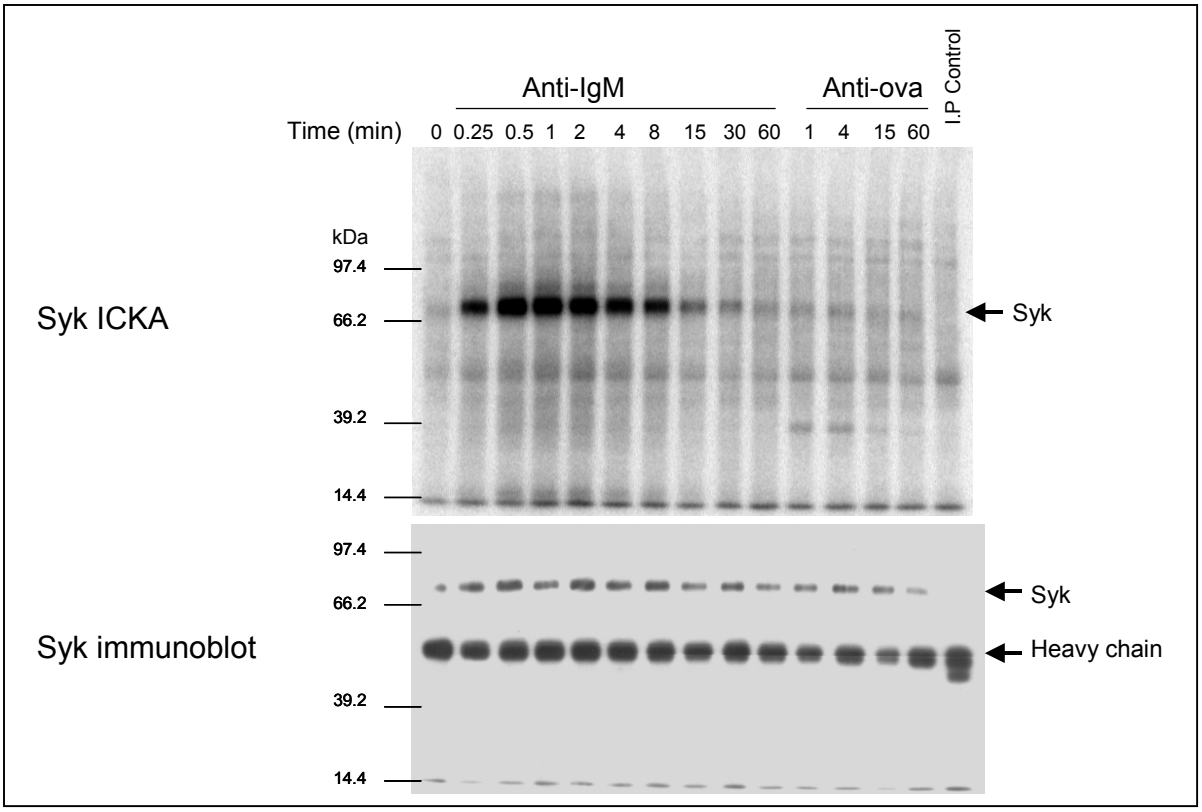

Figure 3.5B. Raw data from primary B cell Syk ICKA. Syk was immunoprecipitated from primary B cells and tested for activation in an ICKA. See Figure 3.7B for description.

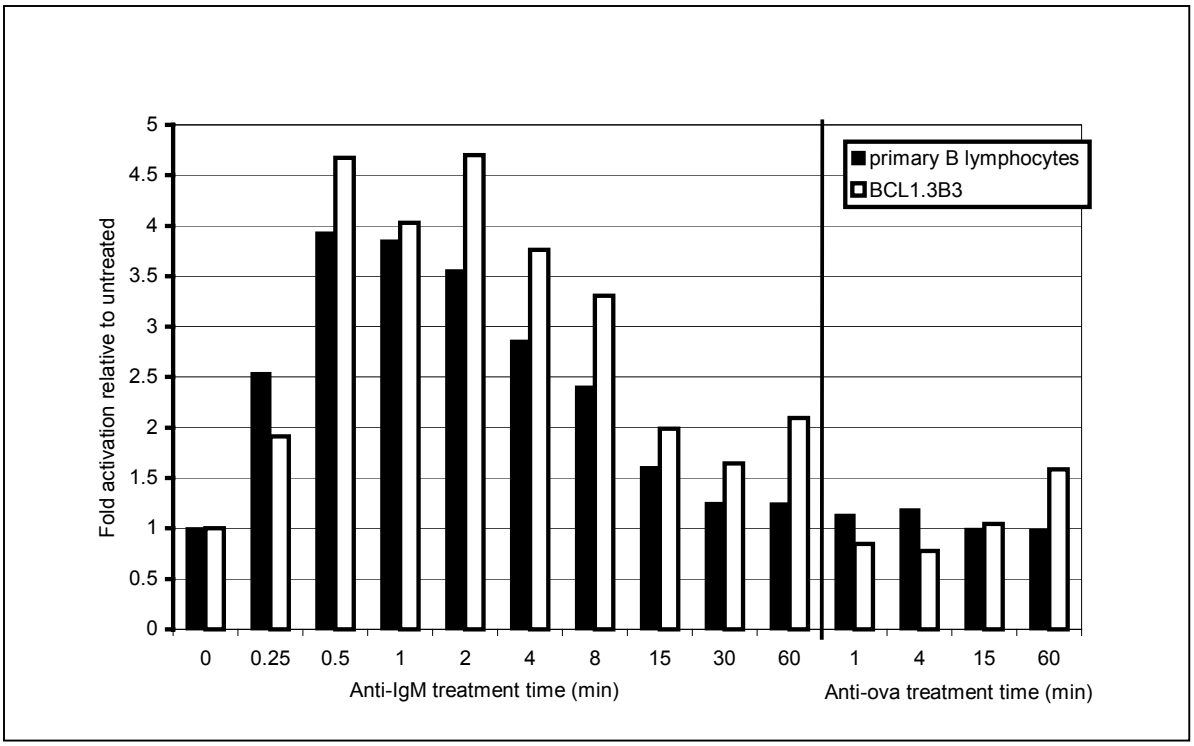

Figure 3.5C. Comparison of Syk activation kinetics in BCL_1.3B3 and purified primary splenic B lymphocytes. 20 μg of polyclonal goat anti-IgM or polyclonal goat anti-ova control antibody was added for the indicated treatment times. Syk activation kinetics were determined by immune complex kinase assay (ICKA). Syk activation is similar between BCL_1.3B3 and primary B cells, with maximal activation at around 0.5 minutes of anti-IgM treatment.

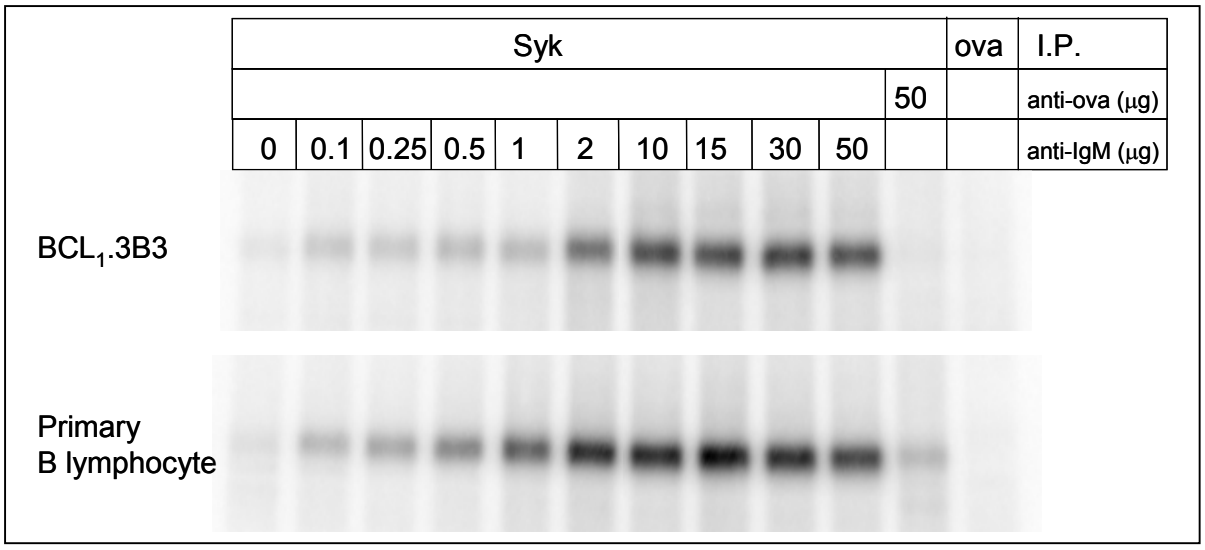

Figure 3.6A. Raw data for Syk ICKA in BCL_1.3B3 and primary B lymphocytes. Syk activation levels at 30 seconds of anti-IgM stimulation were analyzed over varying concentrations of polyclonal anti-IgM treatment. Goat polyclonal anti-ova antibodies were used for control stimulation and rabbit polyclonal anti-ova antibodies were used for control I.P.

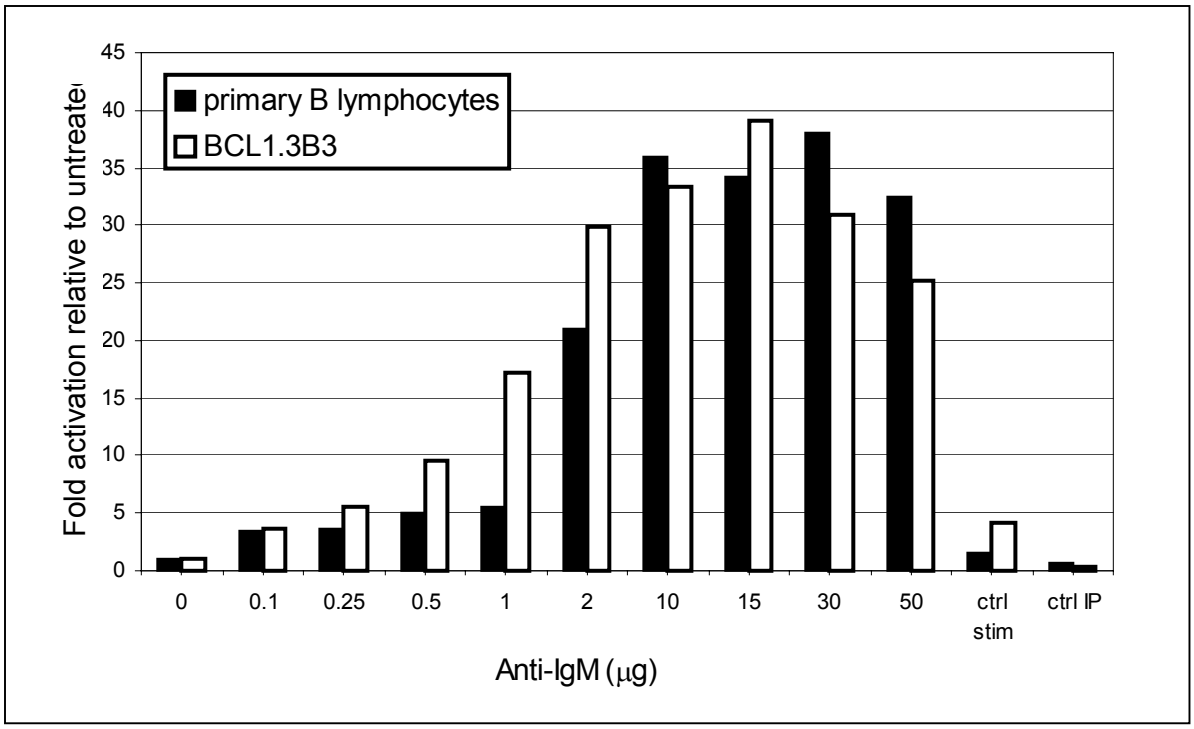

Figure 3.6B. Syk activation dose response in BCL_1.3B3 and purified primary splenic B lymphocytes. Syk activation levels at 30 seconds of anti-IgM stimulation were analyzed over varying concentrations of polyclonal anti-IgM treatment and is maximal at about 15 μg for both BCL_1.3B3 and primary B lymphocytes. Goat polyclonal anti-ova antibodies were used for control stimulation and rabbit polyclonal anti-ova antibodies were used for control I.P.

early BCR signaling events, closely resembling the response of primary B cells. Syk has been shown to respond similarly in other B cells lines such as Daudi (Hsueh, unpublished data), DT40 (Keshvara et al. 1997), Bal17 and DG75 (Peters et al. 1996). These differences in Syk activation dependent on the level and time of BCR cross-linking suggest that differences in BCR signaling may play a role in the decision between apoptosis, cell cycle arrenst, and proliferation, with higher levels of cross-linking (15 μg) favoring a high level Syk activity and apoptosis, and lower levels of crosslinking favoring a low levels of Syk activity and cell cycle arrest.

Changes in the BCR upon cross-linking. The amount and distribution of the BCR on the cell surface could be responsible for the cellular response decision because different levels of BCR would affect the amount of signaling into the cell. To determine if receptor density and apoptosis are related, cells were treated with AIG to signal through the BCR and examined for BCR expression levels and Annexin V binding as a marker for early apoptosis. Upon AIG stimulation, levels of BCR present on cell surfaces decrease. Apoptosis is induced by 1 hour of AIG treatment as evidenced by the appearance of Annexin V+ cells. As AIG stimulation time increases, the percentage of Annexin V+ cells increases. By 24 hr, three cell populations exist: 1) BCR+ AnnexinV-, 2) BCR^{hi} Annexin V+, and 3) BCR^{low} Annexin V+. The BCR^{hi} Annexin V+ population appears to partly contribute to generation of the BCR^{low} Annexin V + population (Figure 3.4). Because of the mixed cellular response

consisting of both apoptosis and ongoing cell division, one idea is that BCR expression levels are related to cell fate.

The distribution of the BCR on the cell surface of AIG-stimulated cells may be a mechanism by which dividing cells inherit different levels of BCR, thus resulting in different signaling capacities that lead to different cell fates. To examine BCR patterns in response to AIG stimulation, BCL_1.3B3 cells were labeled for BCR with a non-stimulating fluorescent antibody, then stimulated with AIG (Figure 3.7). In unstimulated BCL_1.3B3 cells, the BCR is evenly distributed about the cell surface ("diffues"). BCR rearranges upon cross-linking, and can have a "punctate" or "polarized" pattern. Cells with a punctate BCR pattern make up the majority of cells at early stimulation times (30 min) while cells with a polarized BCR pattern make up the majority of cells in later (90 min) stimulation times (Hammill, unpublished data). At even later timepoints, cells begin to cluster as "rosettes", with all BCR clustered in the center.

It is possible for BCR levels to be linked to cell fate. One hypothesis is that during mitosis, the daughter cell inheriting this BCR cap is marked for apoptosis, while the other daughter goes on to re-express BCR. This hypothesis is supported by Annexin V data in which the subset of cells expressing high levels of BCR become apoptotic (Figure 3.4). Indeed, examples of cells undergoing mitosis in which the BCR cap would clearly be inherited by one daughter have been found (Figure 3.8). Other evidence for this idea of asymmetric apoptosis has been described (Hammill, unpublished data).

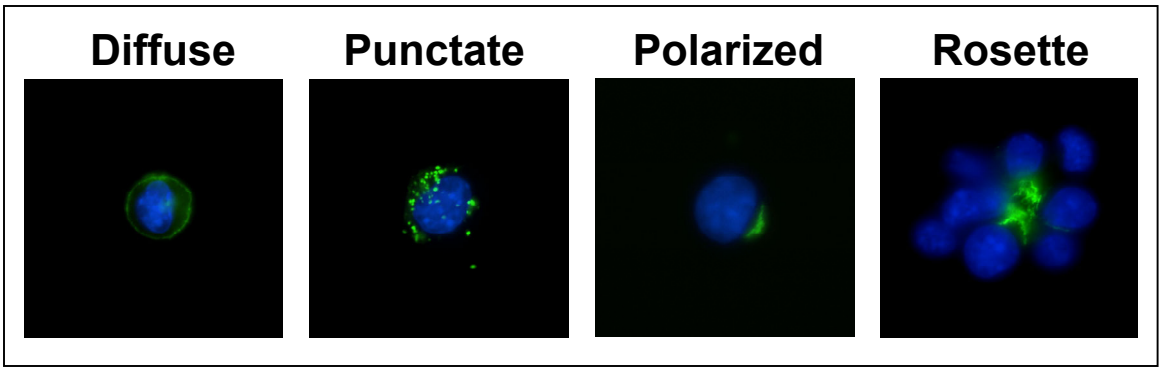

Figure 3.7. The BCR is redistributed upon treatment with anti-IgM. BCL_1.3B3 cells were labeled for the BCR with a non-stimulating FITC-conjugated monoclonal anti-IgM antibody (green). Polyclonal goat anti-IgM was added to cross-link the BCR. Cells were fixed and stained with DAPI for nuclear localization, then analyzed by fluorescence microscopy and scored for their BCR pattern (diffuse, punctate, and polarized), or for formation of cell clusters sharing a cluster of centralized BCR (rosette). Representative examples of each of these BCR patterns is shown. Untreated cells have BCR evenly distributed about the cell surface. By 30 min of treatment, the majority of cells have a punctate BCR pattern. By 60 and 120 min of treatment, the majority of cells have a polarized BCR pattern.

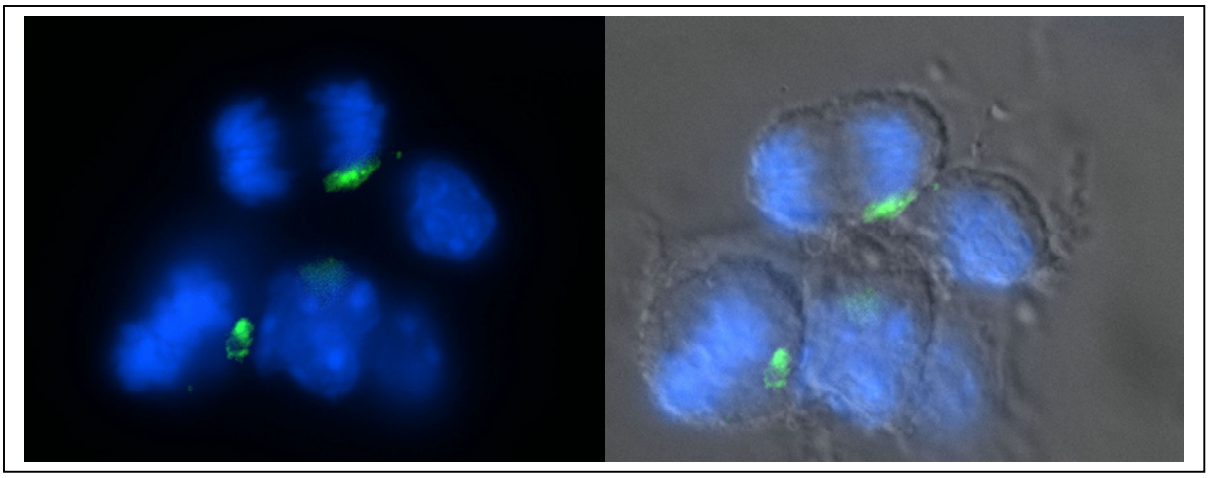

Figure 3.8. Asymmetric inheritance of the BCR. BCL_1.3B3 cells were labeled and stimulated as described in Figure 3.5. Cells undergoing mitosis as evidenced by the chromosome segregation pattern were sought and the BCR pattern was observed. Several instances could be found in which the polarized BCR would clearly be inherited by one daughter cell but not the other. The cell undergoing mitosis at the top is one example. The FITC/DAPI fluorescent image (left) was overlayed on a differential contrast (DIC) image (right) to show the cell membrane and cytoplasm boundaries.

Summary. Cellular responses to BCR engagement vary depending on the time and extent of BCR cross-linking. In the immature B cell line BCL_1.3B3, a balance of cell cycle arrest, apoptosis, and proliferation contributes to the population stability phenomenon, which may explain the mechanisms of tumor dormancy. Syk activation, which affects downstream biochemical events, is also dependent on BCR cross-linking dynamics. Syk activation dynamics are very similar between BCL_1.3B3 and primary splenic B cells, suggesting that transformed cell lines can be a good model for studying the early stages of BCR cross-linking. Taken together, the nature of BCR cross-linking affects the biochemistry of BCR signal transduction, which in turn affects the cellular response. Rearrangement of the BCR in response to cross-linking may be a physical mechanism for modulating levels of BCR signaling. A cell undergoing cell division can inherit different amounts of BCR, giving rise to two daughter cells that have different fates. This may be one mechanism that contributes to population stability in the face of a mixed cellular response. The developmental stage of a B cell also contributes to cellular responses to BCR cross-linking, with immature B cells undergoing apoptosis and mature B cells undergoing cellular activation. Tight regulatory mechanisms for BCR signaling are important since the cellular response to signaling through one receptor varies greatly depending on the circumstances. Any loss in regulation of these processes can result in immune disorders such as autoimmunity.

Chapter 4

CLASSIFI

Gene expression microarrays are a high-throughput, large-scale tool for evaluating gene expression at the level of the whole genome. It allows for simultaneous study of all genes within an organism, opening up possibilities for studying complex interplay between genes. Microarrays can be used for a variety of applications, including identification of disease states and identifying genes that differ in expression between different tissues, cell types, or treatments. Microarrays consist of immobilized nucleic acid "probes" (i.e. cDNA) on a solid support (i.e. glass slides). Tens of thousands of probes can be printed or spotted onto the solid support, allowing the evaluation of expression of a large number of genes in one experiment. Nucleic acid from fluorescently labeled samples ("targets") are then incubated with these probes, allowing complementary sequences to hybridize. The presence and amount of bound nucleic acid can then be detected based on fluorescence.

Analysis of large-scale microarray gene expression data requires computational approaches to organize the vast number of data points that are generated. Correlation metrics, e.g. Pearson correlation or uncentered correlation, can be used to measure similarity between data points (Ashburner et al. 2000; Quackenbush 2001). Different methods, e.g. k-means and hierarchical clustering, can then be used to group together genes based on these correlation metrics. These unsupervised approaches to microarray data analysis organize expression data without bias based on any preconceived understanding of the experimental variables. The goal then is to use this information to understand the cellular physiology reflected by the gene expression patterns revealed by these clustering algorithms.

In order to extract biological knowledge, integration of microarray data with information concerning protein-protein interactions (Duan et al. 2002; Troyanskaya et al.

2003), gene sequences (Bahl et al. 2003), transcriptional regulatory sequences (Fujibuchi et al. 2001; Troyanskaya et al. 2003), genetics (Ge et al. 2001; Troyanskaya et al. 2003), signal transduction (Krull et al. 2003), and clinical disease characteristics (Bumm et al. 2002) have been used. These integration approaches are facilitated by the functional annotation of the microarray probes (Ashburner et al. 2000; Bono et al. 2003; Dennis et al. 2003; Doniger et al. 2003; Draghici et al. 2003; Herrero et al. 2003; Hvidsten et al. 2001; McConnell et al. 2002; Zeeberg et al. 2003). Here we describe a method, termed CLASSIFI, that relates gene expression microarray results to biological processes using a statistical treatment of gene ontology annotation information to classify gene clusters.

CLASSIFI is based on the premise that genes whose proteins are involved in the same biological process will be coordinately regulated at the transcriptional level. The idea is that statistically significant co-clustering of genes involved in the same biological process would allow gene cluster classification. In prokaryotes, functionally-related genes are arranged in operons, ensuring linked expression via transcription of polycistronic RNA (multiple proteins encoded in a single transcript) (Jacob and Monod 1961). An example is the *E. coli* lactose (*lac*) operon, which is made up of a group of genes (*lacZ, lacY, and lacA*) that control the synthesis of β-galactosidase, β-galactoside permease, and β-galactoside transacetylase, respectively. Inducers of this operon result in transcription of all three genes, as they are expressed as a unit of coordinate regulation (Gilbert and Muller-Hill 1967). In eukaryotes, such operons do not exist, but examples of coordinate mRNA expression of interacting proteins have been described (Eisen et al. 1998; Ge et al. 2001; Grigoriev 2001; Kemmeren et al. 2002).

We used the gene description database developed by the Gene Ontology™ (GO) Consortium (www.geneontology.org) to identify genes with similar biological properties. GO descriptions fall into 3 categories – molecular function, biological process, and cellular component. Molecular function describes gene product activity at the molecular level such as "catalytic activity" or "receptor binding". Biological process describes ordered assemblies of molecular functions such as "signal transduction". Cellular component describes either anatomic location within a cell, such as "endoplasmic reticulum" or a protein complex such as "proteosome". Gene ontology terms are organized in a directed acyclic graph of parent-child relationships (Figure 4.1). (Ashburner et al. 2000; Zhong et al. 2003). For example, the molecular function of a gene may be described as "chromatin binding"(child), which in turn is a component of "DNA binding" (parent), and "helicase" (child) is a component of "enzyme" (parent) (Figure 4.1). Though similar to a hierarchy, the directed acyclic graph differs because each child (more specialized term) may have more than one parent (less specialized term) (Figure 4.1). Each of these GO terms have a given GO identifier, such as GO:0005730 for "nucleolus'. The GO description of a gene is dependent on the depth of knowledge of the function of that gene. A well characterized gene may have multiple GO identifiers, as it may be known to be involved in multiple biological processes, whereas a lesser-known gene would have fewer GO identifiers and may be described with terms relatively high up in the parental hierachy due to lack of knowledge about its function. If all of the genes in an ideal microarray data set were completely annotated based on complete knowledge of the genes, then the collection of genes in a microarray gene cluster would be sufficient for classification. However, a limitation of real microarray data sets is that they

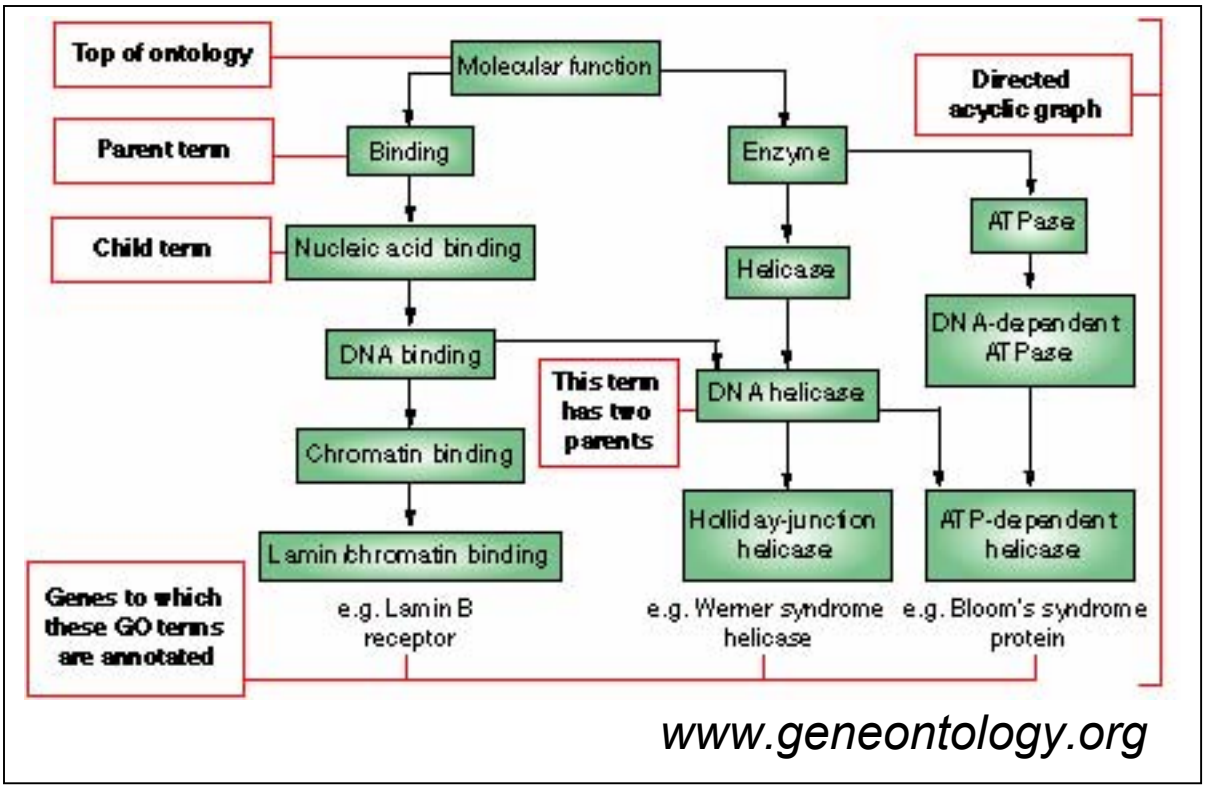

Figure 4.1. Directed acyclic graph structure. The directed acyclic graph differs from a hierarchy because a GO term may have more than one parent.

contain many genes with varying depths of knowledge and also contain experimental noise. Therefore, when using gene ontology to annotate genes and search for functional similarities, it is important to capture the entire ontology parentage in order to account for the varying depths of knowledge of the genes.

GO gene annotation has also been used by other groups as a tool for identifying biological processes relevant to gene expression profiling experiments. When the importance of developing tools to automatically append biological information to a microarray dataset became apparent, several groups developed similar software or web applications that annotate the microarray gene set with information from the Gene Ontology database. Onto-express uses gene ontology to functionally describe a group of genes in a microarray dataset and provides statistical analysis to determine significance of enriched or depleted categories based on expected number of occurrences (Draghici et al. 2003). ChipInfo retrieves all GO terms associated with each gene in an Affymetrix dataset and ranks them according to the number of associated genes (Zhong et al. 2003). MAPPFinder annotates genes using GO, then identifies GO terms associated with over- or under-represented numbers of gene expression changes (Doniger et al. 2003). GARBAN automatically classifies genes or proteins according to GO criteria and can perform clustering analysis based on GO categories (Martinez-Cruz et al. 2003). GOMiner displays user-flagged "interesting" genes within the framework of the GO hierarchy and calculates fold enrichment of GO categories associated with flagged genes (Zeeberg et al. 2003). "Graph-theoretic modeling on GO space" annotates a cluster of genes with GO terms, then transforms GO terms to GO "codes" that form the nodes of a "GO tree". Representative GO codes are then computed to determine the

extent that a gene cluster is associated with GO functional categories (Lee et al. 2004). NetAffx classifies genes represented on Affymetrix arrays with simplified GO "classes" (Cheng et al. 2004). GOTM identifies GO categories with significantly enriched gene numbers (Zhang et al. 2004). FatiGO extracts GO terms on a single level of the GO hierarchy that are over- or under-represented in a set of genes, and provides statistical analysis to determine significance (Al-Shahrour et al. 2004). GO Mapper determines the "weight" of a GO term using actual measured gene expression data, and determines the GO terms that are most representative of gene expression changes in the experiment (Smid and Dorssers 2004). GOAL offers statistical analysis of microarray expression data, assigns genes to UniGene clusters and GO terms, then calculates significance of each GO term (Volinia et al. 2004). GO::TermFinder uses the hypergeometric distribution to calculate significance of enrichment of GO terms in a gene set (Boyle et al. 2004).

Earlier tools simply provided automation for linking gene ontology information with a gene set. As the field progressed, additional analysis functions were added (determining statistical significance of GO term enrichment, for example). However, some of these tools (MAPPFinder, GARBAN, GO Mapper, GO::TermFinder) are available only as software, bringing up issues of software compatibility for users using different operating systems. Furthermore, any improvements or updates require a new software release that users must download. Web-based tools circumvent these problems, providing any user with web browsing software to utilize these tools. However, in most cases these websites (especially GARBAN and GOAL) lack explanation for the user or force the user to reference the original publications, which are often difficult for investigators with little bioinformatics

background to comprehend. Some tools work only on a particular kind of dataset (ChipInfo, NetAffx), limiting users to a microarray platform. Many of these tools are limited to analysis of one gene set, or a comparison of an "interesting" gene set with a "reference" gene set. In these cases the analysis is biased towards what the investigator determines to be "interesting". The common characteristic in all of these publications is the lack of unbiased experimental validation. Validation of these tools is limited to data sets that have already been well-characterized or those that are expected to show predictable patterns. While this does not necessarily reflect on the usefulness of these tools, it would be more convincing to demonstrate the utility of a microarray analysis tool on an uncharacterized data set and show that resulting hypotheses can be tested and experimentally validated.

CLASSIFI is applied to the results of a microarray experiment following filtering, normalization, and clustering. For example, data may be filtered to remove fluorescence values too close to background using PRIM (Preprocessing implementation for microarray) (Kadota et al. 2001), and then filtered to focus on genes that show significantly different expression levels using SAM (Significance Analysis of Microarrays) (Tusher et al. 2001). Genes passing these basic and statistical filtering tests can then be clustered using standard expression clustering algorithms (k-means, hierarchical, SOM) to yield a finite number of gene clusters containing genes with similar expression characteristics. After assigning gene cluster membership (for example the first gene cluster may be designated "1" and then next "2" and so on), CLASSIFI can be used to statistically determine the functional significance of these gene clusters by measuring the probability of co-clustering for every GO identifier in every gene cluster. The CLASSIFI analysis involves the following steps: 1) remove

duplicate probe IDs, 2) extract the primary GO annotations for each gene from a probe database, 3) capture the full GO ancestry for each primary GO annotation from the Gene Ontology™ database, and 4) calculate the solution for the cumulative hypergeometric distribution equation for every ontology in every gene cluster:

$$P = 1 - \sum_{i=0}^{n-1} \frac{\binom{f}{i}\binom{g-f}{c-i}}{\binom{g}{c}}$$

where *g*=number of probes in the data set, *c*=number of probes in the gene cluster, *f*=number of probes with a given ontology in the data set, *n*=number of probes with a given ontology in the gene cluster. The permutation combination is a component of the equation. For example, the permutation combination in the denominator is:

$$\binom{g}{c} C = \frac{g(g-1)(g-2).....(g-c+1)}{c!}$$

The hypergeometric distribution describes the process of random selection without repetition among objects of two distinct types, in this case membership or non-membership in a particular ontology category. This equation calculates the probability (*P*) that genes with a particular GO annotation would co-cluster by chance given the proportion of genes with this annotation in the entire data set. The lower the *P* value is, the less likely it is that the particular GO annotation co-clustered by chance alone, suggesting that the co-clustering resulted from coordinate regulation.

A web interface for use of the CLASSIFI method with data derived from cDNA, oligonucleotide and Affymetrix microarrays, along with detailed information is available at

http://pathcuric1.swmed.edu/pathdb/classifi.html. (Figure 4.2). CLASSIFI input files are formatted as tab-delimited text files that can be generated from Microsoft Excel spreadsheets (simply choose "Save As→text file, tab delimited"). The input file contains 3 columns: 1) probe IDs, 2) gene names and 3) Gene Cluster ID with each row corresponding to a different probe in the filtered and clustered data set (see Table 4.1 for example). The input file is uploaded to the CLASSIFI website and the query is submitted (Figure 4.3). CLASSIFI generates 3 output files: "Classifi_topfile", "Classifi_gofile", and "Classifi_outputfile" (Figure 4.4 and Tables 4.2, 4.3, 4.4). These output files can be saved as tab-delimited text files ("Save Page As→text file") to be opened and formatted in Microsoft Excel. The CLASSIFI output file is a tab-delimited text file containing all of the enumerated variables that were used in the hypergeometric distribution calculation, listing GO id, g, f, c, n, expt, prob, GO type, and GO name where GO id is the unique gene ontology identifier for the specific GO annotation, expt is the expected number of occurrences of a particular ontology based on g, f, and c given a Poisson distribution, and prob is the probability that the co-clustering of the particular ontology would have occurred by chance alone. The results are arranged by gene cluster, with p values listed from lowest to highest within each cluster. The CLASSIFI top file simply lists the GO ids giving the lowest p value in each gene cluster and is useful for a quick comparison of the lowest p values between different gene clusters. The CLASSIFI GO file lists probe IDs in the first column, gene name in the second, gene cluster assignment in the third, and all GO identifiers resulting from ontology parentage associated with primary GO annotation in the fourth column. This file is useful in identifying the probes that are responsible for gene cluster classifications.

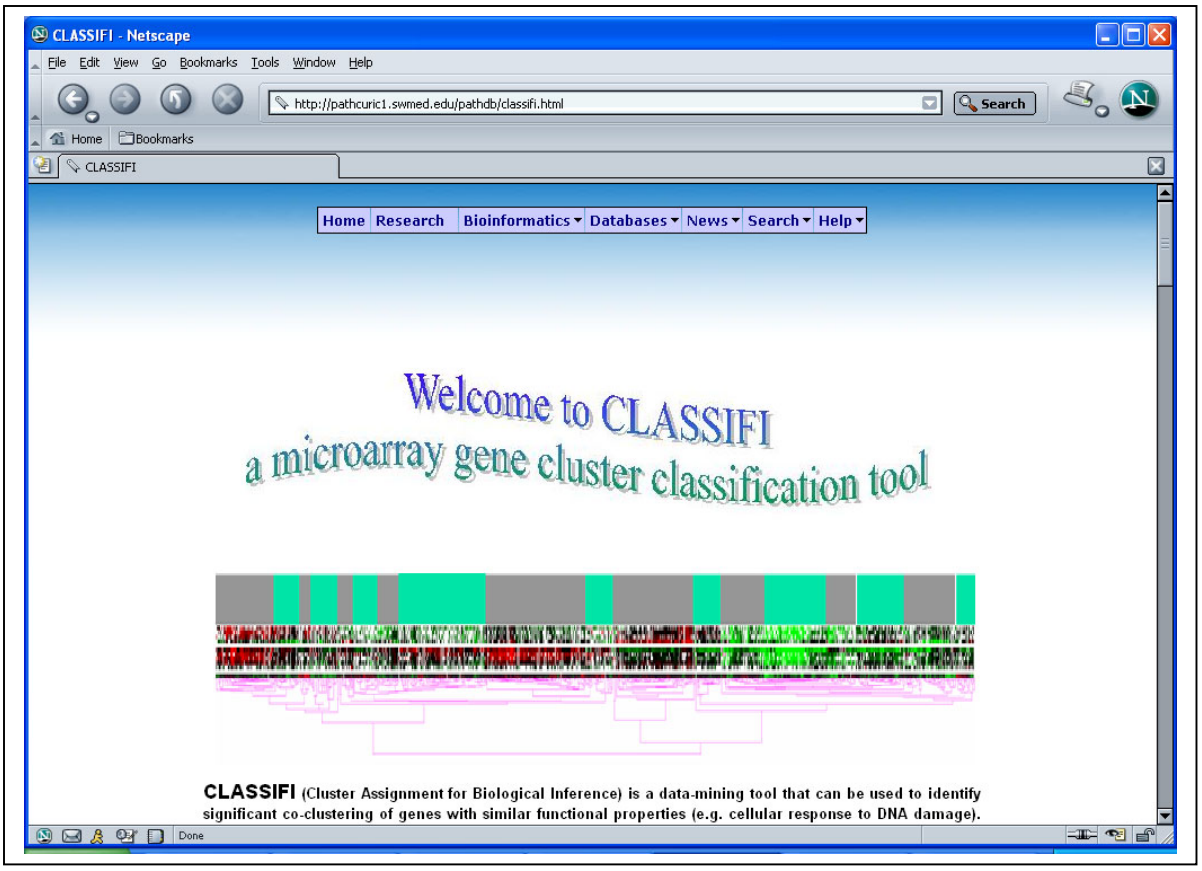

Figure 4.2. The CLASSIFI web interface. CLASSIFI homepage, located at http://pathcuric1.swmed.edu/pathdb/classifi.html.

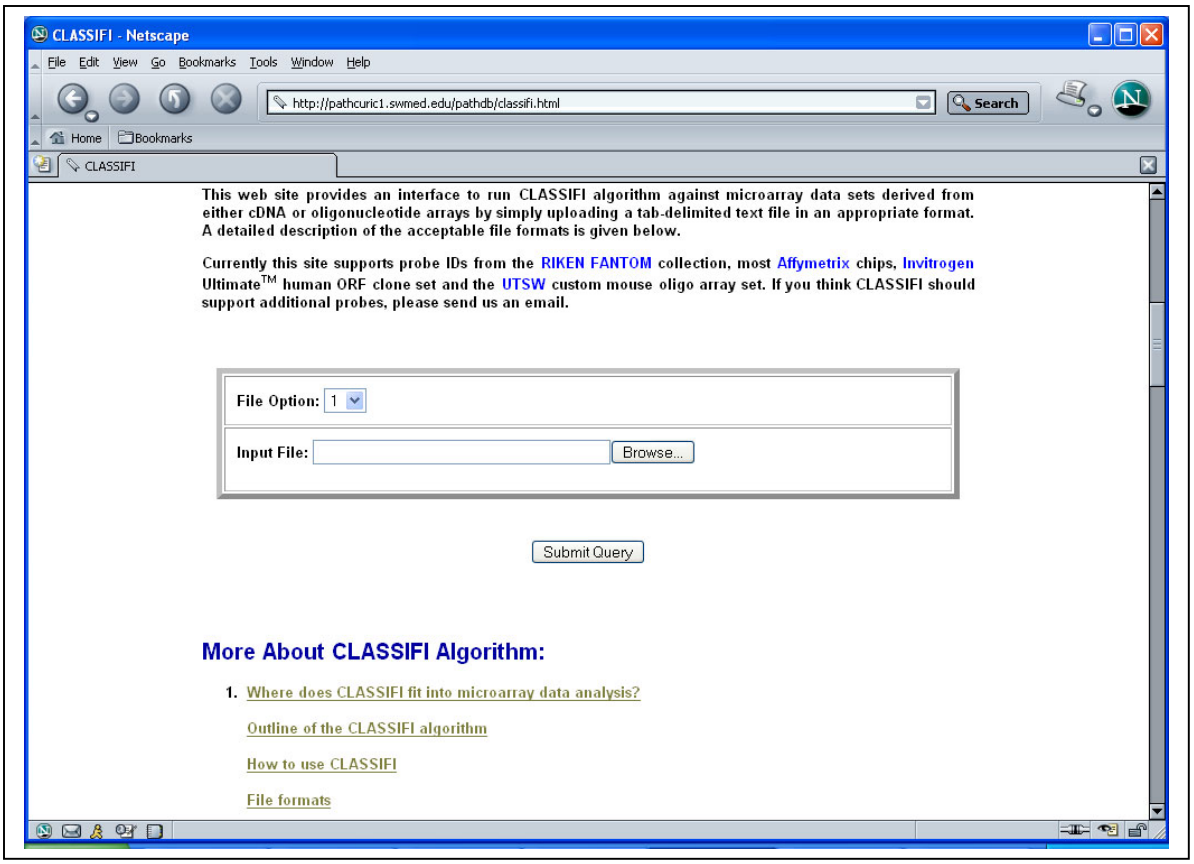

Figure 4.3. Uploading an input file for CLASSIFI. Input files can be uploaded by simple clicking on "browse", choosing the input file (which is formatted as a tab-delimited text file), then clicking on "Submit Query". Results are returned within minutes.

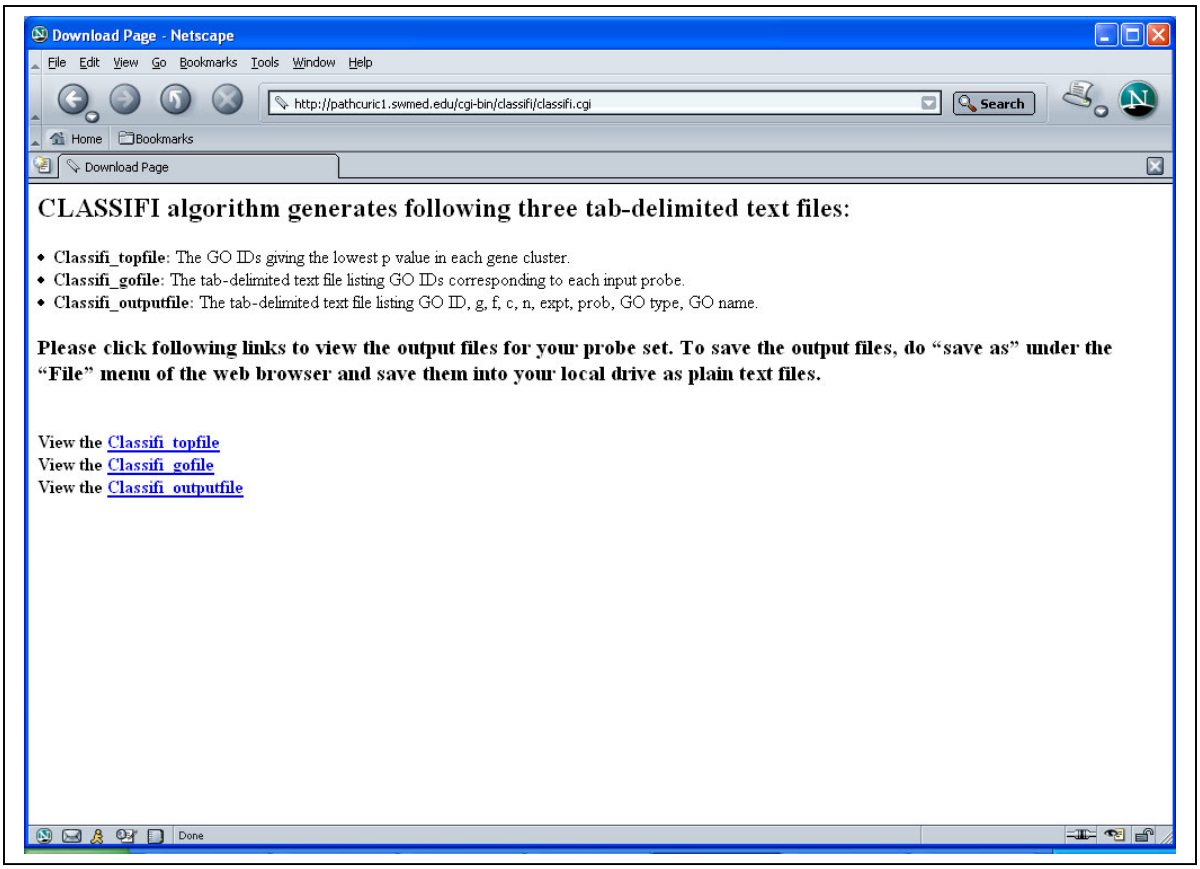

Figure 4.4. CLASSIFI output. CLASSIFI returns 3 output files which can be opened and manipulated by clicking on the links, then saving the file in Microsoft Excel format.

2410159K22	60S RIBOSOMAL PROTEIN L30 ISOLOG (MY024 PROTEIN) (RPL24) homolog	1
H3029H07	myristoylated alanine rich protein kinase C substrate	1
0610008B22	nuclear receptor binding factor 1	1
5033413D16	unknown EST	1
5730496C04	Williams-Beuren syndrome chromosome region 16 homolog (human)	1
5033417E09	arylacetamide deacetylase (esterase)	1
5730435B20	eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange pr…	1
4933404A06	embryonic large molecule derived from yolk sac	1
1500015D05	RIKEN cDNA 3110024A21 gene	1
1500002I10	CDNA FLJ13488 FIS, CLONE PLACE1003915, WEAKLY SIMILAR TO PROBA…	1

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2700088D18	RIKEN cDNA 0610025L06 gene	19
2810436J10	LENG5 PROTEIN homolog [Homo sapiens]	19
1700030C04	RIKEN cDNA 4921526K24 gene	19
4833405L11	RIKEN cDNA 4833405L11 gene	19
H3149A01	cyclin D3	19
5430428G01	weakly similar to probable glycosyl transferase [Schizosaccharomyces pombe]	19
1600021E11	RIKEN cDNA 2610042O14 gene	19
2010306L21	diazepam binding inhibitor	19
2310009M18	7ACOMP PROTEIN homolog [Rattus sp]	19
3110007F17	hypothetical protein	19

Table 4.1 Excerpt of CLASSIFI input file. Input files are generated using Microsoft Excel and list Probe ID in the first column, gene name in the second column, and cluster ID in the third column. These files are saved as tab-delimited text files for uploading at the CLASSIFI website.

GO id	g	f	c	n	expt	prob	GO type	GO name	clusterid
GO:0005634	2490	254	380	64	38.76	8.71E-06	cellular_component	nucleus	1
GO:0009058	2490	141	180	25	10.19	1.36E-05	biological_process	biosynthesis	2
GO:0008372	2490	1409	1	1	0.566	5.66E-01	cellular_component	cellular_component unknown	3
GO:0046072	2490	2	160	2	0.129	4.10E-03	biological_process	dTDP metabolism	4
GO:0009605	2490	30	3	2	0.036	4.18E-04	biological_process	response to external stimulus	5
GO:0016655	2490	14	331	11	1.861	4.94E-08	molecular_function	oxidoreductase activity, acting on NA…	6
GO:0005773	2490	12	11	4	0.053	1.00E-07	cellular_component	vacuole	7
GO:0003779	2490	15	277	9	1.669	6.30E-06	molecular_function	actin binding	8
GO:0016758	2490	6	245	4	0.59	1.17E-03	molecular_function	transferase activity, transferring hexo…	9
GO:0006417	2490	2	4	1	0.003	3.21E-03	biological_process	regulation of protein biosynthesis	10
GO:0008372	2490	1409	41	32	23.2	3.30E-03	cellular_component	cellular_component unknown	11
GO:0008047	2490	10	160	3	0.643	2.23E-02	molecular_function	enzyme activator activity	12
GO:0006397	2490	19	56	4	0.427	6.92E-04	biological_process	mRNA processing	13
GO:0005576	2490	156	183	33	11.47	7.28E-09	cellular_component	extracellular	14
GO:0046916	2490	1	4	1	0.002	1.61E-03	biological_process	transition metal ion homeostasis	15
GO:0003931	2490	3	38	2	0.046	6.74E-04	molecular_function	Rho small monomeric GTPase activity	16
GO:0004032	2490	3	188	3	0.227	4.24E-04	molecular_function	aldehyde reductase activity	17
GO:0015672	2490	10	191	7	0.767	1.38E-06	biological_process	monovalent inorganic cation transport	18
GO:0016892	2490	3	50	2	0.06	1.17E-03	molecular_function	endoribonuclease activity, producing …	19

Table 4.2. CLASSIFI top file. Example of a CLASSIFI top file returned from uploading an input file at the CLASSIFI website. Columns from left to right are: GO term ID, g (number of probes in dataset), f (number of probes with given GO term in dataset), c (number of probes in cluster), n (number of probes with given GO term in cluster), expt (expected number of occurrences of GO term in cluster), prob (the probability that the GO term ID co-clustering has occurred by chance), GO type, GO name, and Cluster ID.

2410159K22	60S RIBOSOMAL PROTEIN L3…	1	GO:0000004,GO:0003673,GO:0003674,GO:0…
H3029H07	myristoylated alanine rich protei…	1	GO:0000004,GO:0003673,GO:0003674,GO:0…
0610008B22	nuclear receptor binding factor 1	1	GO:0000004,GO:0003673,GO:0003674,GO:0…
5033413D16	unknown EST	1	GO:0000004,GO:0003673,GO:0003674,GO:0…
5730496C04	Williams-Beuren syndrome chro…	1	GO:0000004,GO:0003673,GO:0003674,GO:0…

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5430428G01	weakly similar to probable glyco…	19	GO:0003673,GO:0003674,GO:0005488,GO:0…
1600021E11	RIKEN cDNA 2610042O14 gen…	19	GO:0003673,GO:0005575,GO:0005623,GO:0…
2010306L21	diazepam binding inhibitor	19	GO:0000062,GO:0003673,GO:0003674,GO:0…
2310009M18	7ACOMP PROTEIN homolog …	19	GO:0003673,GO:0003674,GO:0003824,GO:0…
3110007F17	hypothetical protein	19	GO:0000004,GO:0003673,GO:0003674,GO:0…

Table 4.3 Excerpt of CLASSIFI GO file. Excerpt of a CLASSIFI GO file returned from uploading an input file at the CLASSIFI website. In columns from left to right, the GO file lists probe ID, gene name, cluster ID, and all associated GO term IDs.

*** Clusterid: 1 ***									
GO id	g	f	c	n	expt	prob	GO type	GO name	clusterid
GO:0005634	2490	254	380	64	38.763	8.71E-06	cellular_component	nucleus	1
GO:0005635	2490	13	380	9	1.984	1.66E-05	cellular_component	nuclear membrane	1
GO:0006412	2490	94	380	30	14.345	2.75E-05	biological_process	protein biosynthesis	1
GO:0009059	2490	94	380	30	14.345	2.75E-05	biological_process	macromolecule biosynthesis	1
GO:0006913	2490	14	380	9	2.137	4.03E-05	biological_process	nucleocytoplasmic transport	1
GO:0005622	2490	596	380	122	90.956	5.00E-05	cellular_component	intracellular	1
GO:0005643	2490	10	380	7	1.526	1.45E-04	cellular_component	nuclear pore	1
GO:0046930	2490	10	380	7	1.526	1.45E-04	cellular_component	pore complex	1
GO:0003723	2490	76	380	24	11.598	2.16E-04	molecular_function	RNA binding	1

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*** Clusterid: 19 ***									
GO id	g	f	c	n	expt	prob	GO type	GO name	clusterid
GO:0016892	2490	3	50	2	0.06	1.17E-03	molecular_function	endoribonuclease activity, …	19
GO:0000062	2490	3	50	2	0.06	1.17E-03	molecular_function	acyl-CoA binding	19
GO:0004521	2490	7	50	2	0.141	7.78E-03	molecular_function	endoribonuclease activity	19
GO:0004519	2490	9	50	2	0.181	1.30E-02	molecular_function	endonuclease activity	19
GO:0005541	2490	9	50	2	0.181	1.30E-02	molecular_function	acyl-CoA or acyl binding	19
GO:0004540	2490	10	50	2	0.201	1.60E-02	molecular_function	ribonuclease activity	19
GO:0015071	2490	1	50	1	0.02	2.01E-02	molecular_function	protein phosphatase type…	19
GO:0008420	2490	1	50	1	0.02	2.01E-02	molecular_function	CTD phosphatase activity	19
GO:0000163	2490	1	50	1	0.02	2.01E-02	molecular_function	protein phosphatase type…	19

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Table 4.4. Excerpt of CLASSIFI output file. Excerpt of a CLASSIFI output file returned from uploading an input file at the CLASSIFI website. The CLASSIFI output file lists results for every GO term ID in every gene cluster. See Table 4.2 for description of columns.

CLASSIFI circumvents two traditional limitations associated with microarray data analysis: 1) the effects of experimental and biological noise on the data and 2) the variable depth of knowledge of individual genes in the probe set. A well-characterized gene has relatively precise annotations that reach deep into the GO hierarchy, while a poorly-characterized gene is only annotated with high level terms. By capturing the entire ontology parentage of each gene, CLASSIFI overcomes limitations related to variable depth-of-knowledge associated with most gene sets. In addition, the probability calculation used in CLASSIFI is relatively robust to the addition of experimental noise, allowing the use of less stringent data filtering approaches that increase the false discovery rate. For example, we have found that increasing the false discovery rate (FDR) used during SAM filtering from 1% to 5% results in similar gene cluster classifications (Table 4.5), where additional genes that appear to be involved in the same biological processes were found to pass the initial statistical filtering and co-clustered with their relatives. For example, Gene Cluster #12 generated from data using a SAM FDR of 5% and Gene Cluster #7 generated from data using a SAM FDR of 1% are very similar. These gene clusters consist of genes with the same expression patterns (upregulated in response to AIG, downregulated in response to CD40L and LPS) and return the same GO name "vacuole" as the GO term that is found to co-cluster with the lowest probability. The difference is that more probes annotated with this GO term passed filtering when using a SAM FDR of 5% (f, c, and n are all higher). In this way, CLASSIFI allows for the reduction in the false negative rate of gene identification while controlling for the concomitant increase in false positive rates associated with lower filtering stringency.

5% SAM FDR							Gene Clusters			1% SAM FDR						
GO ID	f	c	n	prob	GO type	GO name	40L	LPS	AIG	GO ID	f	c	n	prob	GO type	GO name
GO:0009058	203	814	73	3.48E-09	BP	biosynthesis	1	1	1	GO:0005634	254	380	64	8.71E-06	CC	nucleus
GO:0042110	1	1	1	2.33E-04	BP	T-cell activation	1	1	-1							
GO:0006694	7	235	5	8.96E-06	BP	steroid biosynthesis	1	1		GO:0009058	141	180	25	1.36E-05	BP	biosynthesis
GO:0007292	4	7	3	1.06E-08	BP	oogenesis	1	-1	-1	GO:0008372	1409	1	1	5.66E-01	CC	CC unknown
GO:0005923	4	4	1	3.72E-03	CC	tight junction	1	-1								
GO:0005853	5	253	3	1.84E-03	CC	eukaryotic translation…	1		1	GO:0046072	2	160	2	4.10E-03	BP	dTDP metabolism
GO:0007292	4	1	1	9.30E-04	BP	oogenesis	1		-1	GO:0009605	30	3	2	4.18E-04	BP	response to external…
GO:0003954	24	374	16	3.03E-12	MF	NADH dehydrogenas…	1			GO:0016655	14	331	11	4.94E-08	MF	oxidoreductase activi…
GO:0008372	2499	1	1	5.81E-01	CC	CC unknown	-1	1	1							
GO:0005634	397	1	1	9.23E-02	CC	nucleus	-1	1	-1							
GO:0009416	2	6	1	2.79E-03	BP	response to light	-1	1								
GO:0005773	18	29	5	7.83E-08	CC	vacuole	-1	-1	1	GO:0005773	12	11	4	1.00E-07	CC	vacuole
GO:0003785	6	549	6	4.22E-06	MF	actin monomer binding	-1	-1	-1	GO:0003779	15	277	9	6.30E-06	MF	actin binding
GO:0004032	5	335	3	4.16E-03	MF	aldehyde reductase …	-1	-1		GO:0016758	6	245	4	1.17E-03	MF	transferase activity, tr…
GO:0015929	4	13	2	5.04E-05	MF	hexosaminidase activity	-1		1	GO:0006417	2	4	1	3.21E-03	BP	regulation of protein…
GO:0008372	2499	95	72	2.10E-04	CC	CC unknown	-1		-1	GO:0008372	1409	41	32	3.30E-03	CC	CC unknown
GO:0008372	2499	392	267	1.26E-05	CC	CC unknown	-1			GO:0008047	10	160	3	2.23E-02	MF	enzyme activator act…
GO:0007243	11	79	3	8.85E-04	BP	protein kinase cascade		1	1	GO:0006397	19	56	4	6.92E-04	BP	mRNA processing
GO:0003836	1	3	1	6.98E-04	MF	beta-galactoside alph…		1	-1							
GO:0005576	295	420	73	4.93E-15	CC	extracellular		1		GO:0005576	156	183	33	7.28E-09	CC	extracellular
GO:0046916	3	16	3	4.23E-08	BP	transition metal ion h…		-1	1	GO:0046916	1	4	1	1.61E-03	BP	transition metal ion h…
GO:0003700	53	65	4	7.99E-03	MF	transcription factor act…		-1	-1	GO:0003931	3	38	2	6.74E-04	MF	Rho small monomeri…
GO:0003998	2	328	2	5.80E-03	MF	acylphosphatase activity		-1		GO:0004032	3	188	3	4.24E-04	MF	aldehyde reductase …
GO:0006754	7	246	6	2.20E-07	BP	ATP biosynthesis			1	GO:0015672	10	191	7	1.38E-06	BP	monovalent inorganic…
GO:0006916	9	63	2	7.12E-03	BP	anti-apoptosis			-1	GO:0016892	3	50	2	1.17E-03	MF	endoribonuclease ac…

Table 4.5. Varying SAM False Discovery Rate (FDR) between 5% and 1%. Microarray expression data statistical filtering was accomplished by SAM using an FDR of 5% or 1%. Values of +1, -1, or 0 were given to genes that were found by SAM to be significantly upregulated, downregulated, or unchanged compared to time-matched controls. Clustering was accomplished using these categorical values, input files generated, and CLASSIFI analysis performed. The CLASSIFI top file for analysis done with a SAM FDR of 5% (blue) was compared to analysis done with a SAM FDR of 1% (red). Clusters giving rise to the same GO classification are highlighted in yellow.

One potential drawback to the current CLASSIFI tool is related to the microarray probe set. More than one probe may be included that are specific for the same gene. If several probes representing the same gene are found to co-cluster, the significance of identifying a biological process represented by co-clustering of related genes would be inflated because of the over-representation of one gene by several probes in calculation of the p value. However, since hypotheses must be tested and validated in order to continue a study, this is of little concern.

A schematic of a microarray analysis pipeline using CLASSIFI is shown in Figure 4.5. Future plans for the CLASSIFI web interface include providing microarray analysis tools from start to finish (from raw data to hypothesis generation). The goal is to link popular tools together so that the pipeline can be automated once the user inputs raw data.

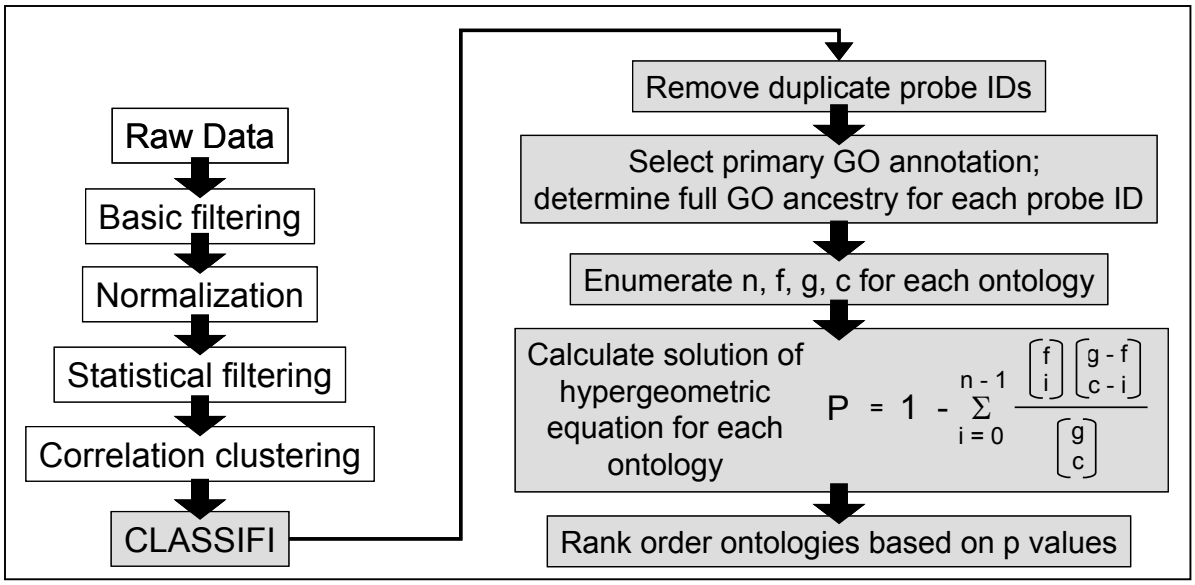

Figure 4.5. Microarray analysis pipeline. Steps taken in analysis of microarray data using CLASSIFI.

Chapter 5

Analysis of the BAFF/CD40L microarray data set

Introduction

BAFF (B-cell activating factor of the TNF family) and CD40L are both members of the TNF receptor family and are known to influence B cell physiology *in vivo* and B cell survival *in vitro*. BAFF (a.k.a. BlyS, TALL-1, THANK, TNFSF13B, zTNF4) appears to play a role in promoting the survival and differentiation of several stages of B lymphocyte development. BAFF is expressed in myeloid lineage cells, especially macrophages and dendritic cells (Moore et al. 1999; Schneider et al. 1999), and its expression is upregulated by interferon-γ and IL-10 (Nardelli et al. 2001). BAFF has 3 known receptors: TACI, BCMA, and BAFF-R. TACI and BCMA also bind other TNF family members but BAFF-R is only expressed on B cells and is specific only for BAFF (Gross et al. 2000; Thompson et al. 2001). BAFF deficiency is detrimental to B cell maturation as observed in BAFF-deficient mice (Schiemann et al. 2001), and its overexpression is associated with autoimmune phenotypes similar to Sjögren's Syndrome and Systemic Lupus Erythematosus (SLE) (Khare et al. 2000; Mackay et al. 1999). BAFF has recently been shown to prevent nuclear accumulation of PKCdelta, which contributes to spontaneous death in peripheral resting B cells (Mecklenbrauker et al. 2004). BAFF exists as a type II transmembrane protein that can be released as a soluble form by cleavage (Moore et al. 1999). Soluble BAFF has been shown to exist as a trimer (Kanakaraj et al. 2001; Schneider et al. 1999; Zhukovsky et al. 2004), and these trimers have been found in patients with systemic immune-based rheumatic diseases including SLE, rheumatoid arthritis (RA), Reiter's syndrome, psoriatic arthritis, polymyositis, and ankylosing spondylitis (Roschke et al. 2002).

CD40 is a type I membrane protein and is expressed on B cells, activated DCs, macrophages, follicular dendritic cells, thymic epithelial cells, and endothelial cells (Alderson et al. 1993; Galy and Spits 1992; Inaba et al. 1994; Stamenkovic et al. 1989). The ligand for CD40 is CD40L (CD154), a type II membrane protein expressed by activated T cells, eosinophils, basophils and NK cells. Binding of CD40L on activated T cells to CD40 on B cells drives B cells to proliferate and differentiate and promotes progression of the humoral immune response (Armitage et al. 1992; Noelle et al. 1992). CD40-CD40L interaction stimulates expression of adhesion molecules (Barrett et al. 1991; Nishioka and Lipsky 1994) and CD80/86 costimulatory molecules (Caux et al. 1994; Yang and Wilson 1996). CD40 signaling induces switch recombination and also rescues B cells from anti-Ig-induced apoptosis. CD40L signaling through the CD40 receptor is known to involve the JNK/SAP, NF-κB, and ERK/MAPK pathways (Berberich et al. 1996; Berberich et al. 1994; Li et al. 1996). CD40-CD40L interaction contributes to isotype class switching, germinal center formation, and memory B cell formation (Allen et al. 1993; Ferrari et al. 2001; Foy et al. 1994; Kawabe et al. 1994). CD40 ligation results in recruitment of TRAF2 and TRAF3 cytoplasmic adaptor proteins (Hostager et al. 2000), leading to interaction with downstream signaling molecules.

In order to evaluate the mechanisms by which BAFF and CD40L enhance cell survival, we applied CLASSIFI to gene expression microarray results from splenic B lymphocytes stimulated with BAFF versus CD40L over extended periods in serum-free culture.

Results and Discussion

In order to demonstrate the effects of BAFF and anti-CD40 (a CD40L mimic) on B cell survival *in vitro*, purified B cells were cultured with or without recombinant BAFF or anti-CD40 over several days. B cell survival was assessed by counting the number of viable cells (those that exclude Trypan Blue) recovered from culture.

BAFF and anti-CD40 enhance B cell recovery in vitro. B cells were enriched from mouse spleens using a magnetic negative selection procedure in which CD43+ and Mac-1+ cells were removed from the splenocyte cell suspension using anti-CD43 and anti-Mac-1 antibodies conjugated to magnetic microbeads (Figure 5.1). This enriched population of B cells was an average of 89.4% (±3.3%) B220+, as determined by flow cytometry (Figure 5.2). When enriched B lymphocytes were placed in culture in medium without FBS, the recovery of viable cells decreased rapidly, with a half-life of 28 hr. BAFF or anti-CD40 antibody treatment significantly increased viable cell recovery in comparison with untreated cultures (Figure 5.3). These data were confirmed by flow cytometric FSC/SSC analysis, which showed that a larger percentage of cells within a live gate were present with BAFF or anti-CD40 than with no treatment. Within the live cell gate, the proportion of blasting ("B") versus resting ("R") cells are different under BAFF versus anti-CD40 stimulation (Figure 5.4). This difference will be discussed later. Enhanced viable cell recovery could be achieved through ligand-induced effects on proliferation and/or survival. To investigate the

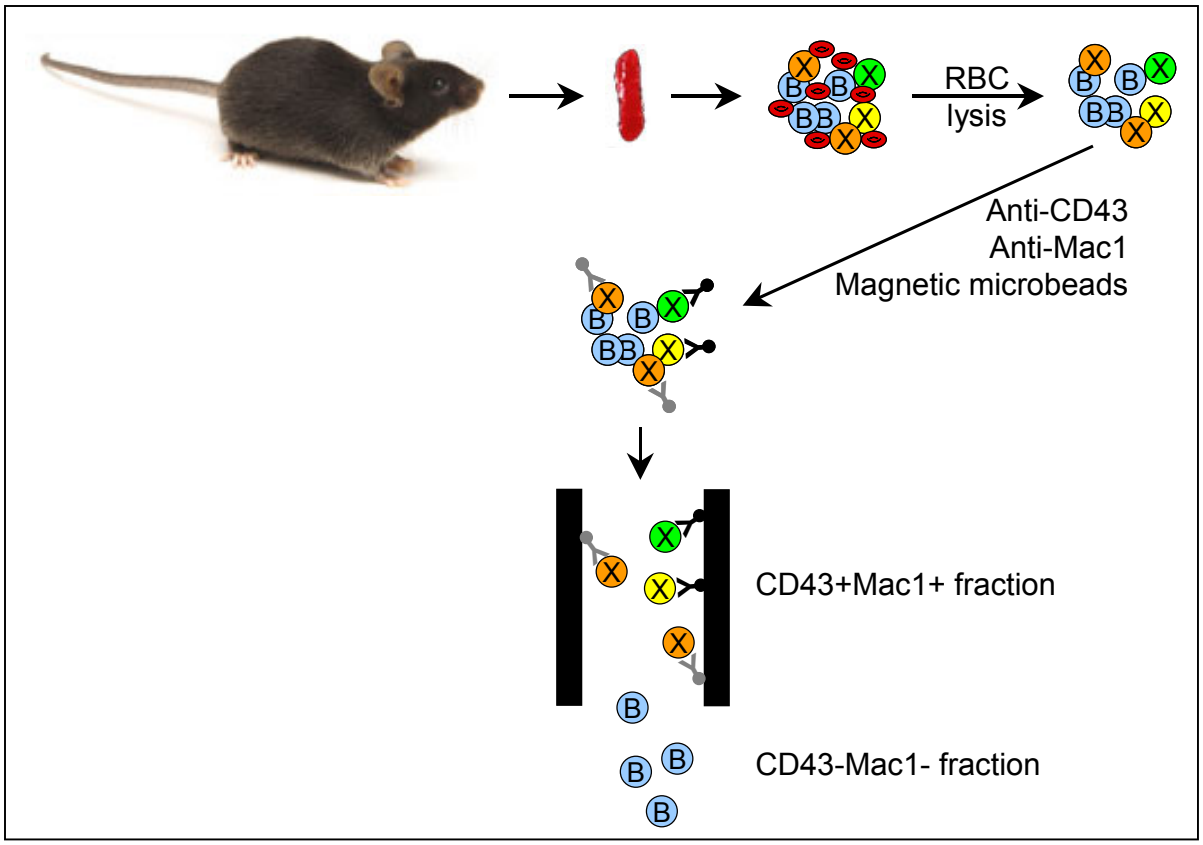

Figure 5.1. B cell purification from C57BL/6 mice. A splenocyte cell suspension is prepared from spleens harvested from mice. Splenocytes are treated for lysis of red blood cells (RBC) and remaining cells are mixed with anti-Mac-1 and anti-CD43 antibodies conjugated to magnetic microbeads. The combination of anti-Mac-1 and anti-CD43 binds to non-B cells, so that when the cell suspension is passed through a magnetic column, non-B cells which have antibody bound to them are retained in the column while unbound B cells flow through. The resulting enriched population of B cells has not been "touched" by the procedure and can then be used for experimentation.

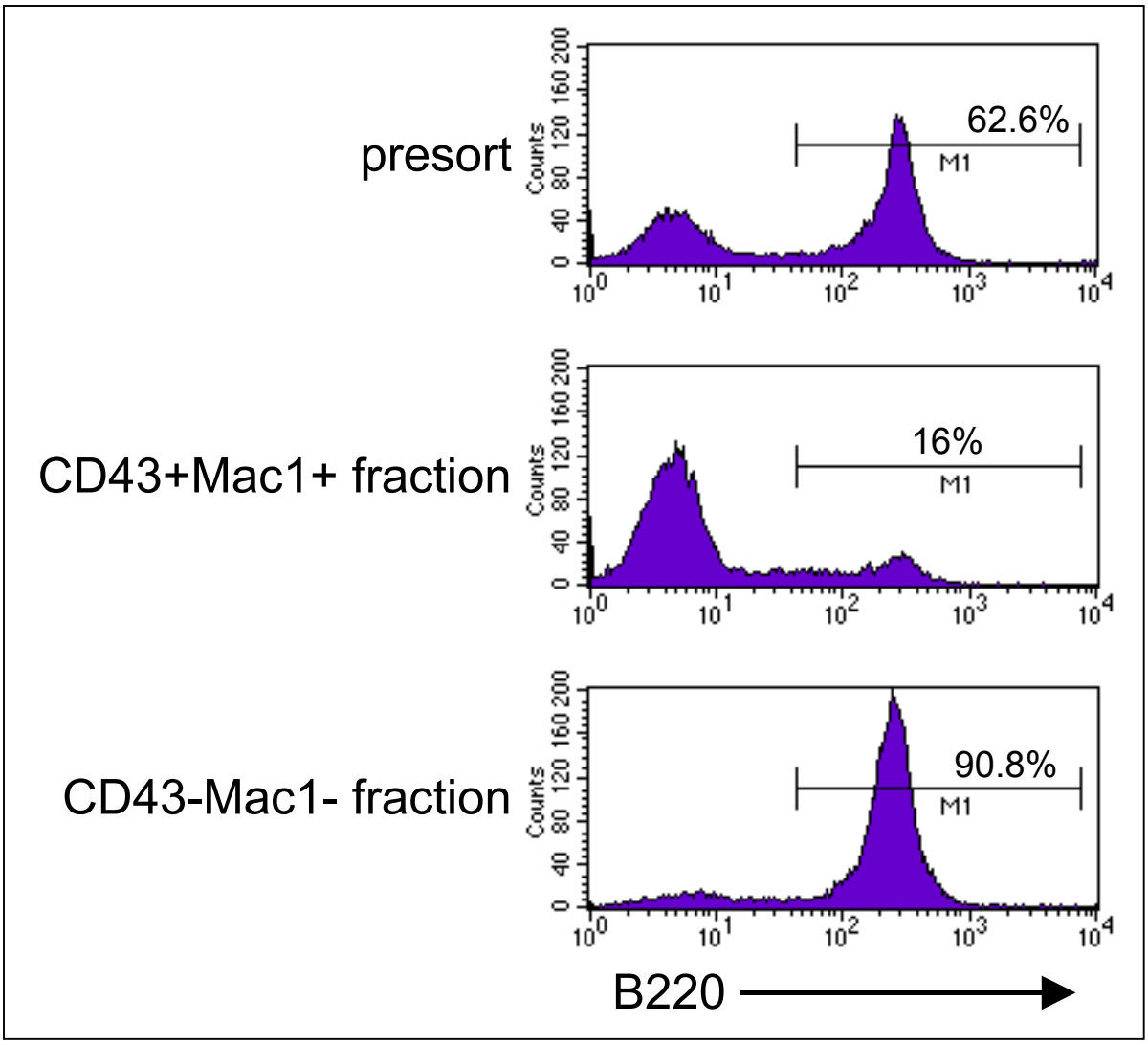

Figure 5.2. Flow cytometric assessment of B cell purity. 3 cell fractions were assessed for percentage of B220+ B cells by flow cytometry using an anti-B220 antibody conjugated to FITC. The presort fraction is RBC-removed splenocytes. Normal mouse spleens typically contain about 60% B cells. The CD43+Mac1+ fraction includes all cells that were retained in the magnetic column and are mostly non-B cells. The CD43-Mac1- fraction includes all untouched cells that flow through the magnetic column and therefore is enriched for B cells. These purified B cells, which were an average of 89.3% (±3.3%) B220+, were used for further experiments.

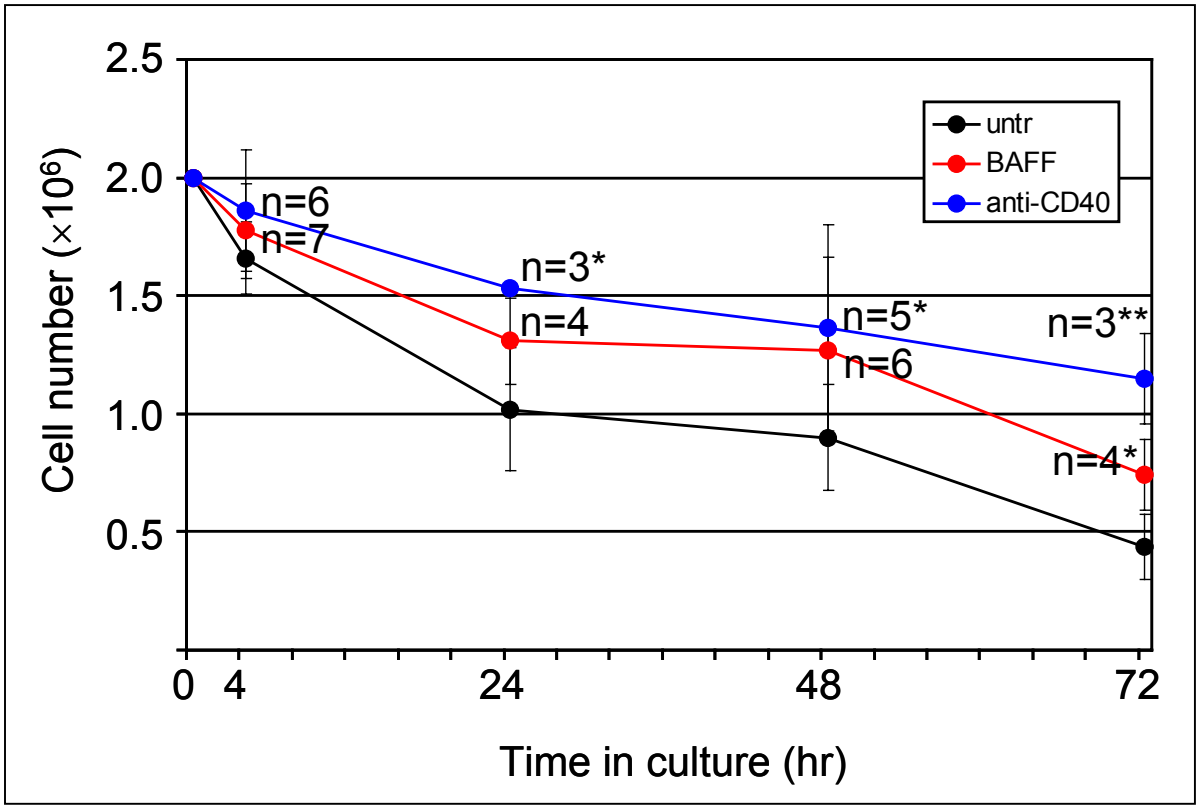

Figure 5.3. BAFF and anti-CD40 promote B cell viability *in vitro*. Viable cell recovery was determined by trypan blue exclusion and hemacytometry. The average number of cells recovered is shown. Error bars represent the standard deviation of the mean (SD). The number of determinations for each experimental condition (n) is indicated. Statistical evaluation of the results was accomplished by comparing treated sample values with time-matched control samples using the Student's *t* test.

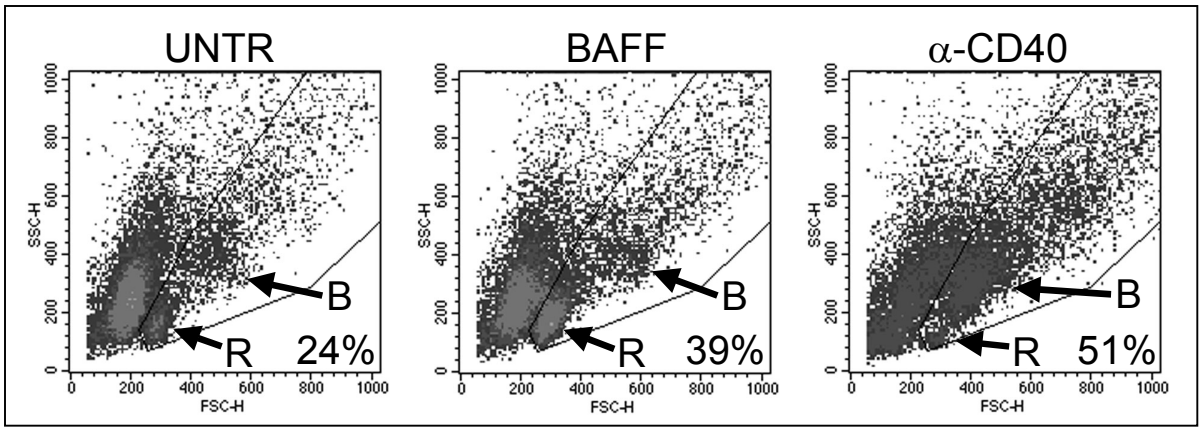

Figure 5.4. Analysis of FSC/SSC profiles of BAFF- and anti-CD40-treated B cells. Flow cytometric analysis was performed on 48 hr B cell cultures. FSC is an indication of cell size and SSC is an indication of cell complexity. Blasting cells (B) are larger than resting viable cells (R) and lie farther out on the FSC/SSC axes, while smaller apoptotic cells and cell fragments are found in the low FSC region (<200 FSC-H). Percentage of cells within the region representing viable cells (R + B) is indicated.

mechanisms responsible for enhanced B cell recovery, gene expression microarray results of BAFF- and anti-CD40-treated B cells were analyzed.

cDNA microarray analysis of BAFF- and anti-CD40-treated B lymphocytes. RNA samples were isolated from B cells treated with BAFF or anti-CD40 for varying lengths of time from 4 hr to 144 hr and compared with untreated samples using a microarray chip containing 15,833 cDNA probes. Following chip hybridization and scanning, and data filtering and normalization, values representing the fold increase or decrease in mRNA levels compared to 0 hr untreated samples were determined. 1207 probes passed filtering approaches to remove unreliable values and probes whose expression did not vary under the experimental conditions used. The $\log_2$ ratio values were then clustered using the hierarchical clustering/average linkage method with uncentered correlation as the metric, resulting in the dendrogram shown in (Figure 5.5). The frequent co-clustering of different probes for the same gene indicates that the raw data and filtering/normalization procedures have given results of sufficiently high quality to warrant further analysis (Figure 5.6).

Eighteen gene clusters were defined based on a combination of expression correlation coefficients and visual pattern inspection. The expression pattern observed in many of these clusters can be defined by kinetics and ligand-specificity (Figure 5.5). For example, Gene Clusters #8 and #9 represent genes that are upregulated relatively late in response to BAFF but not anti-CD40. Gene Cluster #11 contains genes that are upregulated early in response to anti-CD40 but not BAFF. Genes in Gene Cluster #1 are upregulated in response to both BAFF and anti-CD40, but not until 24 hr; additionally, the BAFF response appears to be

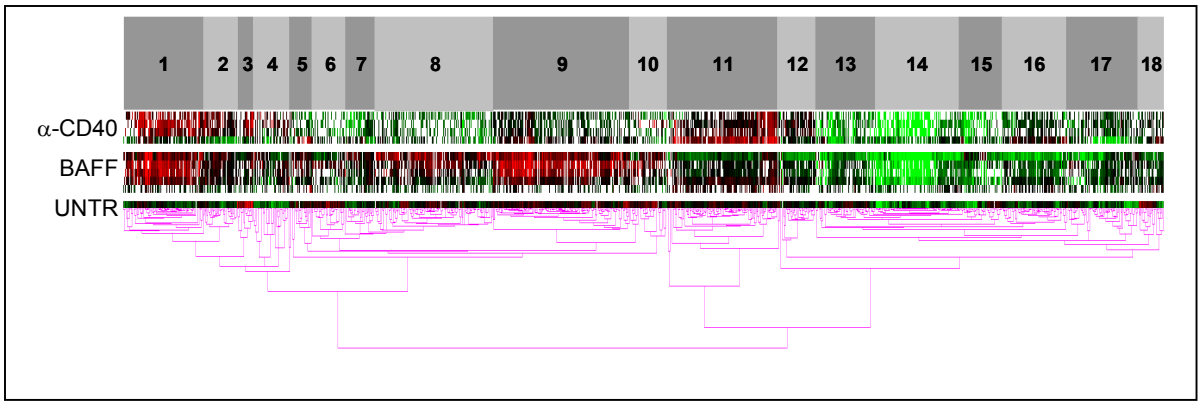

Figure 5.5. cDNA microarray data clustering from B lymphocytes treated with BAFF and anti-CD40. The order of experiments (rows, from bottom to top) is: untreated 4 hr; BAFF 4 hr, BAFF 24 hr, BAFF 48 hr, BAFF 72 hr, BAFF 144 hr; anti-CD40 4 hr, anti-CD40 24 hr, anti-CD40 48 hr, anti-CD40 72 hr. Probes that show an increase in expression in comparison to untreated samples are shown in red, a decrease in expression in green, or no change in expression in black, with color intensities reflecting the level of increase or decrease. White indicates samples that were removed from the analysis due to filtering. Probes were divided into 18 gene clusters, indicated at the top, based on correlation coefficients and visual inspection. The dendrogram (pink) is the graphical result of clustering using the hierarchical/average linkage method with uncentered correlation as the metric. The height of the bars correspond to the correlation coefficient (the shorter the bar, the higher the expression correlation).

Figure 5.6. Expression results from probes in Gene Cluster #1. Genes in this gene cluster are upregulated in response to both BAFF and anti-CD40. Different probes to the same gene would be predicted to exhibit identical expression patterns and cluster together. This is observed experimentally (probes highlighted by yellow boxes), indicating a high-quality microarray experiment.

more robust. Genes within Gene Cluster #14 exhibit a strong, sustained downregulation in response to both ligands. Thus, these gene clusters reflect gene expression patterns that correlate with the experimental variables.

CLASSIFI analysis links gene clusters to cellular physiology. CLASSIFI measures the probability of co-clustering for every gene ontology designation in every cluster given the characteristics of the data set. Expression data clustering and gene cluster membership assignment generates the CLASSIFI input file that consists of a list of probe IDs, gene names and gene cluster membership (Table 5.1). The CLASSIFI method captures all of the GO annotation information for every probe from a local database and then calculates the probability of chance co-clustering. The final CLASSIFI output is a list of every GO identifier, ranked within each gene cluster from lowest to highest probability (Table 5.2).

Our data set of 1207 probes led to the calculation of 3310 probabilities, since some probes had multiple GO identifiers. A list highlighting the lowest probabilities in select gene clusters is presented in Table 5.2. In the CD40L-specific Gene Cluster #11, the GO identifier (GO:0006396) with the lowest probability was “RNA processing”. 14 probes out of the data set of 1188 unique probes have the GO identifier “RNA processing”. Of these, 9 probes were co-clustered in Gene Cluster #11, which contains a total of 122 different probes. Intuitively, the chance occurrence of 64% of the “RNA processing” probes in a gene cluster that represents about one tenth of the entire data set would be unlikely. Using the hypergeometric distribution analysis, the calculated probability of selecting 9 probes with GO identifier “RNA processing” in 122 tries from a 1188 member set containing 14

2210009M20	vanilloid receptor-like protein 1	1
9530047P18	unclassifiable	1
2610017G21	thymidylate synthase	1
4631402F24	unclassifiable	1
5730443M15	cathepsin H	1
2810449E13	cyclin B2	1
2700047H13	unclassifiable transcript	1
2700010I01	leukemia-associated gene	1
1600009N20	growth hormone releasing hormone	1
UI-M-AN1-afg-e-07-6-UI	protein kinase C, alpha	1

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1700048O14	unclassifiable	18
2410081F06	homolog to BIFUNCTIONAL AMINOACYL-TRNA …	18
2610201K07	Ras suppressor protein 1	18
1600019A01	homolog to LEUCINE ZIPPER NUCLEAR FACTOR…	18
5730407M17	unclassifiable transcript	18
UI-M-AK0-adj-b-06-6-UI	BCL2-antagonist/killer 1	18
2610037C03	BTB/POZ domain containing protein	18
1500031P10	heat shock protein, 105 kDa	18
2410052D22	homolog to CDNA FLJ12085 FIS, CLONE HEMB…	18
2410012M21	homolog to DNA POLYMERASE DELTA SMALLE…	18

Table 5.1. Excerpt of CLASSIFI input file used in CLASSIFI analysis of BAFF/CD40L microarray data. Columns from left to right are Probe ID, gene name, and Cluster ID.

GO id*	g†	f†	c†	n†	Expt‡	Prob§	GO type*	GO name*
Gene Cluster #11 "RNA Processing"								
GO:0006396	1188	14	122	9	1.438	1.23E-06	BP	RNA processing
GO:0016070	1188	17	122	9	1.746	1.14E-05	BP	RNA metabolism
GO:0003723	1188	21	122	9	2.157	9.65E-05	MF	RNA binding
GO:0006364	1188	3	122	3	0.308	1.06E-03	BP	rRNA processing
GO:0008380	1188	6	122	4	0.616	1.35E-03	BP	RNA splicing
Gene Cluster #1 "Cytokinesis"								
GO:0016288	1188	21	93	11	1.644	6.98E-08	BP	cytokinesis
GO:0000279	1188	22	93	11	1.722	1.31E-07	BP	M phase
GO:0000280	1188	10	93	7	0.783	1.43E-06	BP	nuclear division
GO:0000087	1188	10	93	7	0.783	1.43E-06	BP	M phase of mitotic cell cycle
GO:0007067	1188	10	93	7	0.783	1.43E-06	BP	mitosis
GO:0007017	1188	10	93	7	0.783	1.43E-06	BP	microtubule-based process
GO:0007049	1188	57	93	15	4.462	1.21E-05	BP	cell cycle
Gene Cluster #8 "Protease"								
GO:0004197	1188	12	136	9	1.374	4.30E-07	MF	cysteine-type endopeptidase
GO:0008234	1188	12	136	9	1.374	4.30E-07	MF	cysteine-type peptidase
GO:0006508	1188	35	136	15	4.007	1.57E-06	BP	proteolysis and peptidolysis
GO:0008233	1188	35	136	15	4.007	1.57E-06	BP	protein degradation
GO:0008233	1188	28	136	13	3.205	2.76E-06	MF	peptidase
GO:0009057	1188	37	136	15	4.236	3.69E-06	BP	macromolecule catabolism
GO:0005773	1188	18	136	10	2.061	5.53E-06	CC	vacuole
GO:0005764	1188	18	136	10	2.061	5.53E-06	CC	lysosome
GO:0000323	1188	18	136	10	2.061	5.53E-06	CC	lytic vacuole
GO:0009056	1188	62	136	18	7.098	8.51E-05	BP	catabolism
Gene Cluster #9 "Unknown"								
GO:0005554	1188	622	160	116	83.77	1.98E-08	MF	MF unknown
GO:0000004	1188	630	160	116	84.84	5.39E-08	BP	BP unknown
GO:0008372	1188	631	160	116	84.98	6.10E-08	CC	CC unknown
GO:0003964	1188	3	160	3	0.404	2.40E-03	MF	RNA-directed DNA polymerase
GO:0007389	1188	2	160	2	0.269	1.80E-02	BP	pattern specification
GO:0001533	1188	2	160	2	0.269	1.80E-02	CC	cornified envelope

Table 5.2. Excerpts from CLASSIFI analysis of BAFF/anti-CD40 microarray results

*A unique identifier and name that corresponds to a defined molecular function (MF), biological process (BP), or cellular component (CC). †Parameters used for hypergeometric calculation - number of unique probes in entire dataset (g), number of probes with a given GO id in entire dataset (f), number of probes in a given gene cluster (c), and number of probes with a given GO id in a given gene cluster (n). ‡The expected number of occurrences of a give GO id in a given gene cluster of size (n) based on a random distribution, calculated by the equation (f×c)/g. §The probability that the GO id cluster has occurred by chance as calculated using the cumulative hypergeometric distribution analysis. Note: g is smaller than 1207 because multiple occurrences of identical probes have been removed. "MF, BP, CC unknown" annotations were assigned to probes with no GO annotation.

occurrences of "RNA processing" by chance is 1.23×10^{-6}. When we examine the other ontologies with low probabilities in Gene Cluster #11, they all have GO identifiers related to some aspect of RNA processing. Therefore, Gene Cluster #11 is classified as an "RNA processing" cluster.

In the 18 gene clusters, the lowest *P* values ranged from $\sim10^{-22}$ to 10^{-2}. The question of what is a "significant" probability was addressed using a permutation approach in which the data are randomized and the occurrences of co-clustering measured for the randomized data. This approach is more suitable than simply defining 0.05 as a significant cutoff because we are running multiple statistical tests, thus increasing the likelihood of getting a significant result by chance alone. From a simulation of 100 random data sets that maintain cluster sizes but randomizes cluster membership, *P* values less than 10^{-5} were found only twice. A similar cutoff, 1.5×10^{-5}, is derived using the relatively conservative Bonferroni correction with an *alpha* of 0.05 and an *n* of 3310 (see http://mathworld.wolfram.com/BonferroniCorrection.html and references therein). Thus, for this data set, cluster classifications with *P* values less than 10^{-5} are considered to be highly significant. Based on this cutoff, 5 gene clusters were significantly classified: Gene Cluster #11 ("RNA Processing"), Gene Cluster #8 ("Protease"), Gene Cluster #9 ("Unknown"), Gene Cluster #1 ("Cytokinesis"), and Gene Cluster #14 ("Hemoglobin").

An example of a gene cluster with relatively high *P* values is Gene Cluster #12, where the lowest *P* value, 9.08×10^{-4}, is above our significance cutoff of 10^{-5} (Table 5.2). The lowest *P* values in this cluster are associated with GO identifiers that represent several

unrelated biological processes (e.g. protein metabolism, nucleotide binding). Additionally, only minor trends in gene expression changes are observed in this gene cluster.

We have used CLASSIFI to determine the significance of gene ontology co-clustering in this data set. Significant co-clustering of related gene ontologies, such as the co-clustering of RNA processing-related gene ontologies in Gene Cluster #11, suggest that the gene cluster is made up of coordinately regulated genes involved in a common biological process.

Experimental validation of CLASSIFI. CLASSIFI should be considered a hypothesis-generating tool. Gene Cluster #11 contains genes that are rapidly up-regulated in response to anti-CD40 but not BAFF. The classification of Gene Cluster #11 as "RNA processing" leads to the hypothesis that CD40 engagement induces a global increase in the expression of genes involved in RNA processing, since CLASSIFI is based on the postulate that genes involved in the same biological process are coordinately expressed. Thus, one prediction of the CLASSIFI method is that other genes involved in the same biological process that were not subjected to the CLASSIFI analysis should show the same expression pattern. To test this hypothesis and the underlying assumptions, we chose a gene that was included in the CLASSIFI analysis, homolog to Exportin T, and compared its expression pattern to genes that were not included in the CLASSIFI analysis but are still involved in RNA processing: homolog to snRNP core protein D2, U1snRNP E, and Ran. Exportin T and Ran are involved in tRNA export; Ran, U1snRNP E, and homolog to snRNP core protein D2 are involved in UsnRNP biogenesis (Lipowsky et al. 1999; Will and Luhrmann 2001). The prediction is that

the genes not included in the CLASSIFI analysis would also be upregulated in response to anti-CD40. Northern blot analysis shows a 2 – 3 fold upregulation of homolog to Exportin T mRNA in response to anti-CD40 but not BAFF, consistent with the microarray data. Expression of the other three genes shows similar trends in upregulation in response to anti-CD40 (Figure 5.7A and B), especially the homolog to snRNP core protein D2 gene. Although U1snRNP E shows some upregulation in response to BAFF, the level of upregulation is still higher in response to anti-CD40 treatment.

These data are important for several reasons. First, they demonstrate that anti-CD40 treatment leads to a general increase in the expression of genes involved in RNA processing. Second, the fact that the hypothesis generated based on the CLASSIFI analysis was confirmed by experimentation validates this methodology for microarray data analysis. Third, these data support the underlying postulate that genes involved in the same biological process are coordinately expressed in vertebrates. Interesting and valid information concerning ligand-induced cellular responses were obtained, further demonstrating the utility of the CLASSIFI methodology.

Gene Cluster #1 classification ("Cytokinesis") leads to the hypothesis that BAFF and anti-CD40 induce mitosis and thus cell cycle progression. Cells in the S phase can be identified by measuring uptake of BrdU into their DNA. While a substantial percentage (~9%) of anti-CD40-treated B cells took up BrdU in a 2 hr pulse, few (~1%) BAFF-treated cells incorporated BrdU (Figure 5.8). The light scattering (FSC/SSC) FACS profiles support the BrdU results. Blasting cells that are preparing for cell division are larger than resting

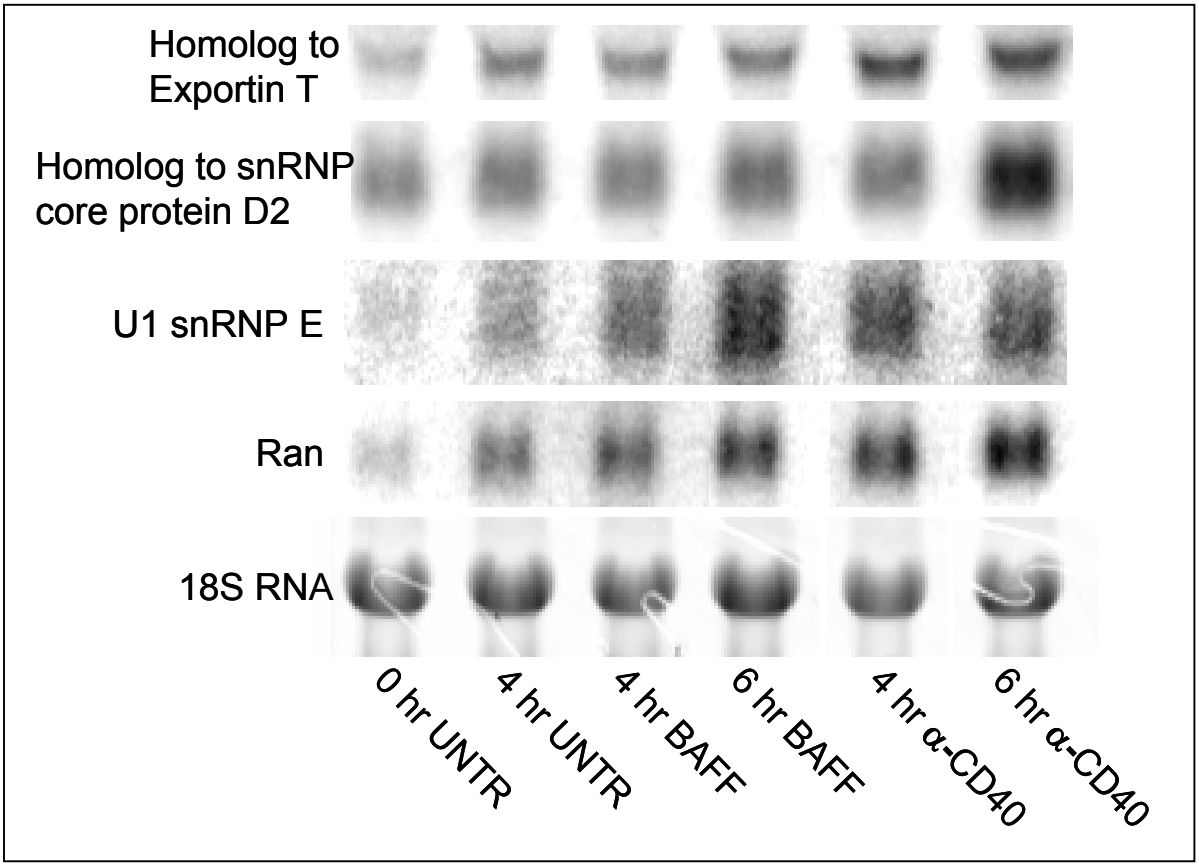


Figure 5.7A. Coordinate regulation of genes involved in RNA processing. Northern blot analysis of one gene (homolog to Exportin T) included in and three genes (homolog to snRNP core protein D2, U1 snRNP E, Ran) not included in the CLASSIFI analysis. Ethidium bromide-stained 18S RNA bands were used for normalization.

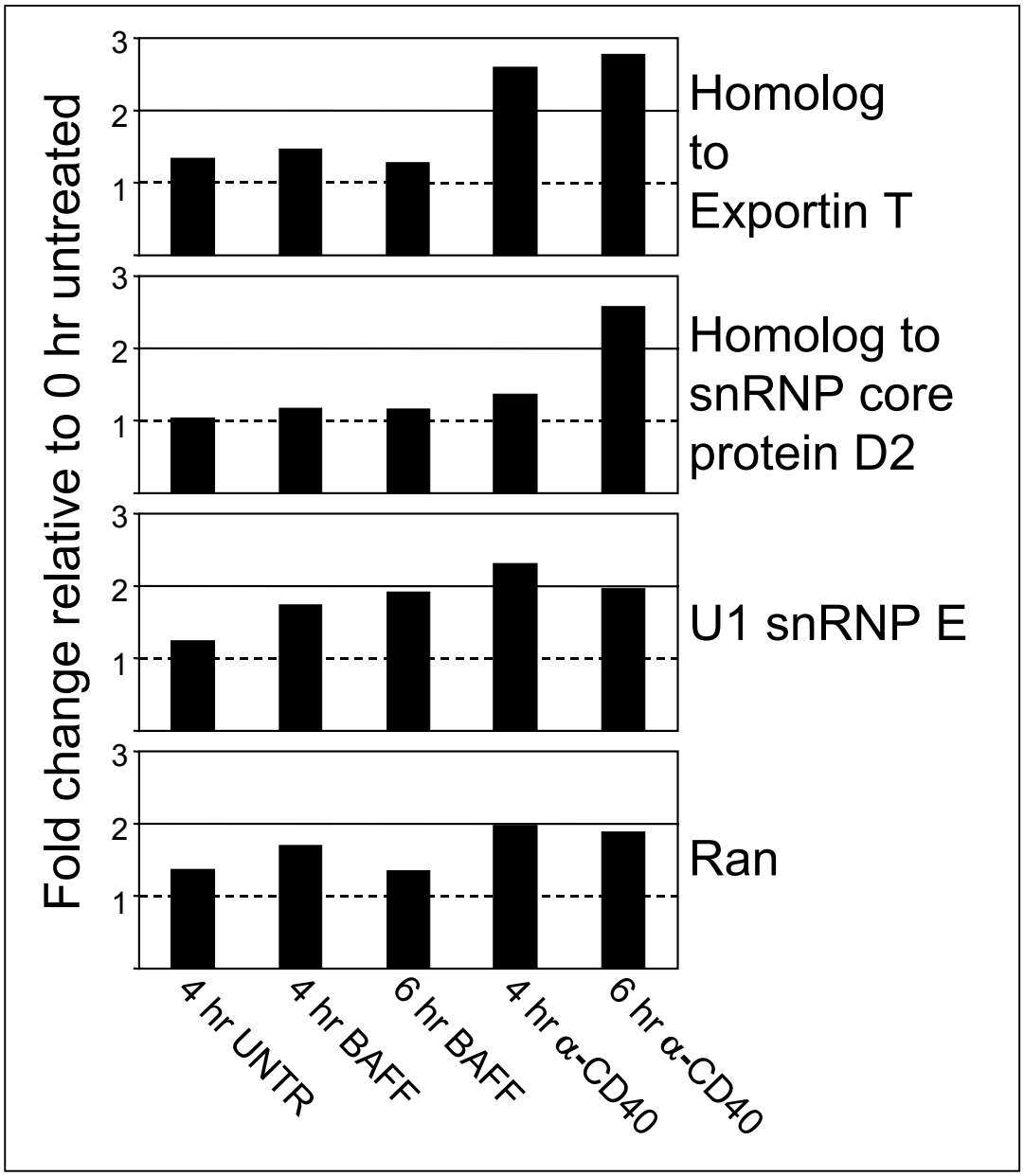


Figure 5.7B. Coordinate regulation of genes involved in RNA processing. Northern blot band intensities from Figure 5.7A were quantified by phosphorimaging software. Band intensities were first normalized to 18S RNA as a loading control, then compared to the 0 hr untreated sample. Bars represent the normalized fold change relative to the 0 hr untreated sample. Dotted lines correspond to no change in expression.

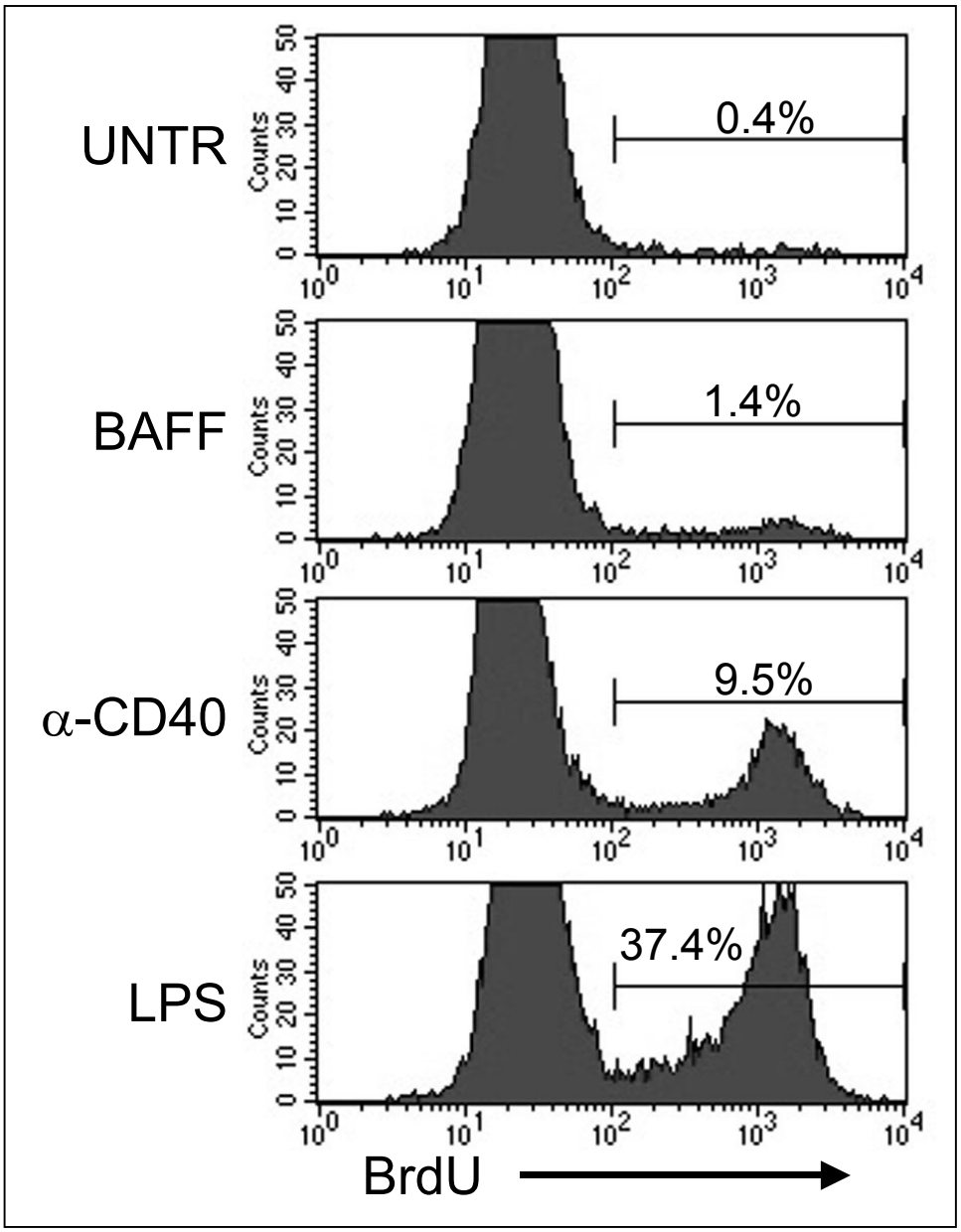


Figure 5.8. BAFF and anti-CD40 induce different degrees of proliferation. Uptake of BrdU was measured by flow cytometry in 48 hr B cell cultures that were either left untreated or were treated with BAFF or anti-CD40. Treatment with LPS, a polyclonal B cell activator, was used as a positive control for proliferation. Percentages of proliferating BrdU+ cells are indicated.

cells. The FSC/SSC profiles clearly show a greater proportion of blasting cells in anti-CD40-treated cultures than in BAFF-treated cultures (Figure 5.4). These results were unexpected given the microarray data which showed a strong upregulation of genes involved in mitosis in response to both ligands. To confirm that the mitosis genes in this cluster are indeed upregulated in response to both ligands, we performed semi-quantitative RT-PCR on a few selected genes. All of these genes were upregulated in response to both BAFF and anti-CD40, confirming the microarray expression data (Figure 5.9). Although these experiments do confirm that both BAFF and anti-CD40 enhance proliferation (albeit at different levels), the prediction that both ligands should support a robust proliferative response did not hold up to experimental testing. Thus upregulation of these mitosis genes is not sufficient to drive the cells through the complete cell cycle. This example emphasizes the importance of experimental testing of hypotheses that are generated from the leads identified using CLASSIFI. In this case, since both ligands support B cell survival, it may be that the mitosis-related genes found in Gene Cluster #1 also play important roles in promoting cell survival. The classification of Gene Cluster #11 as "RNA processing" may support the BrdU proliferation data. Since anti-CD40 induces a greater level of proliferation as measured by BrdU, the expectation would be that these cells have upregulated their metabolic machinery, including RNA processing as a component of increased protein production.

Gene Cluster #8 ("Protease") classification leads to the hypothesis that lysosomal protease activity increases in response to BAFF but not anti-CD40. This could reflect the induction of cathepsins as part of the antigen processing and presentation process (Nakagawa et al. 1999; Reinheckel et al. 2001; Shi et al. 2000), or as initiators of apoptosis

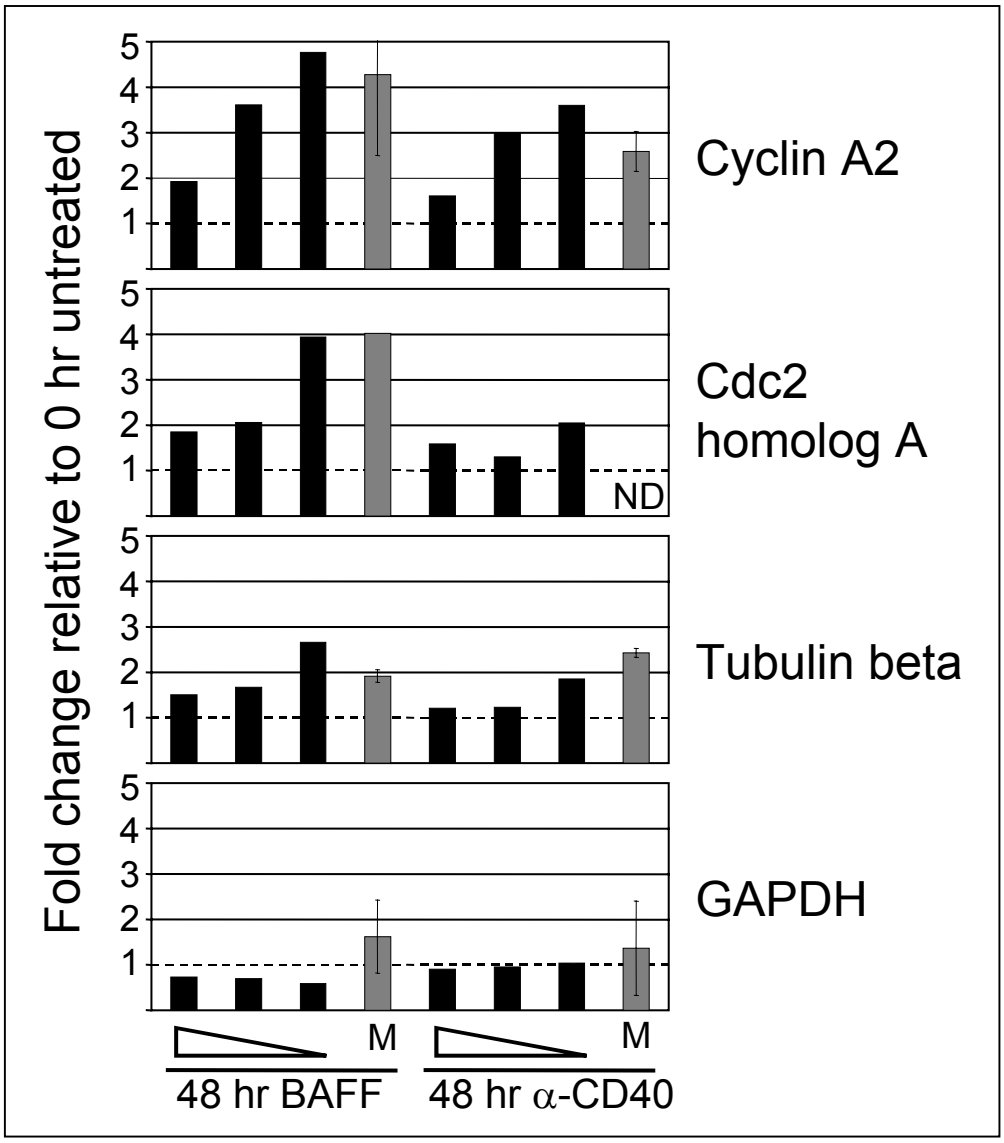


Figure 5.9. Mitosis genes are upregulated in response to both BAFF and anti-CD40. Semiquantitative RT-PCR analysis of 3 genes from Gene Cluster #1 was performed. PCR reactions were run using 10-fold serial dilutions of cDNA template (open triangles). PCR products were resolved by agarose gel electrophoresis, stained, and visualized by a fluorescence imager. Bands were quantified using ImageQuant software, giving values that were normalized to values obtained from GAPDH (housekeeping gene) PCR of corresponding samples. Normalized values were then compared to the 0 hr untreated sample to give values representing the fold change in expression relative to 0 hr untreated. Gray bars represent average microarray values for fold change relative to 0 hr untreated from every probe (including duplicates) corresponding to a specific gene. Error bars represent SD. ND=no data; data did not pass filtering.

(Johnson 2000; Stoka et al. 2001). Gene Cluster #14 ("Hemoglobin") may represent a change in cell populations that occurs during cell culture in which contaminating erythroid-lineage cells are lost over time in culture. This example brings up an important point, which is that changes in gene expression could result from real changes in RNA expression, or from changes in cell populations. These possibilities should be kept in mind when interpreting microarray results. Gene Cluster #9 is especially interesting; 116 of the 160 probes present in this gene cluster

contains "genes" that have not been previously characterized and potentially represent a biological process associated with BAFF stimulation that has not been previously characterized, and could lead to new information concerning the role of BAFF in the development of autoimmune diseases in humans (Cheema et al. 2001; Groom et al. 2002). Again, each of these hypotheses that build upon CLASSIFI data mining are amenable to experimental investigation.

Although the analysis of this data set was useful in validating the CLASSIFI analytical approach, it is not ideal. A major problem with the AfCS experimental design is the lack of unstimulated controls for all culture timepoints. These controls are crucial for helping to discriminate between ligand-specific effects and cell culture effects. B cells taken from their *in vivo* environment begin to die once placed in culture *in vitro* (See Figure 6.1 in the next chapter). By late culture timepoints, the cell death effect certainly would overwhelm any ligand-specific effects. In the next chapter, we describe the analysis of a well-controlled data set focusing on short culture timepoints to minimize undesired cell culture effects.

Chapter 6

Analysis of B cell single ligand screen microarray data set I

Introduction

Experimental validation of the BAFF/CD40L data set became difficult because the effects of cell culture over prolonged periods of time were not properly controlled for. Primary B cells begin to undergo apoptosis once placed into culture (Figure 6.1). In order to separate cell culture apoptosis effects from ligand-specific effects, it is imperative that untreated controls be included in a microarray experiment. Therefore, we decided to analyze another microarray data set from the Alliance for Cellular Signaling (AfCS) B cell single ligand screen project. The AfCS is a consortium of laboratories whose overall goal is to comprehensively understand select signaling systems by examining the relationship between cellular inputs and outputs. Data generated from the AfCS laboratories are freely provided to research communities with the idea that research could be accelerated through information sharing. Through a collaboration with Nature Publishing Group, AfCS information and data can be found at http://www.signaling-gateway.org/. The B cell single ligand screen project aims to describe the responses of primary B lymphocytes to a panel of 32 different input single ligands by measuring a series of output responses: gene expression profiles, phosphoprotein activation, and changes in concentration of cAMP and calcium (Figure 6.2). Time in culture is kept to 4 hours or less, and untreated samples are included at all timepoints. Because of the short timepoints and inclusion of untreated controls, this data set is ideal for minimizing cell culture effects and focusing on ligand-specific effects. The results in this chapter focus on three immunologically relevant ligands: AIG, CD40L, and LPS.

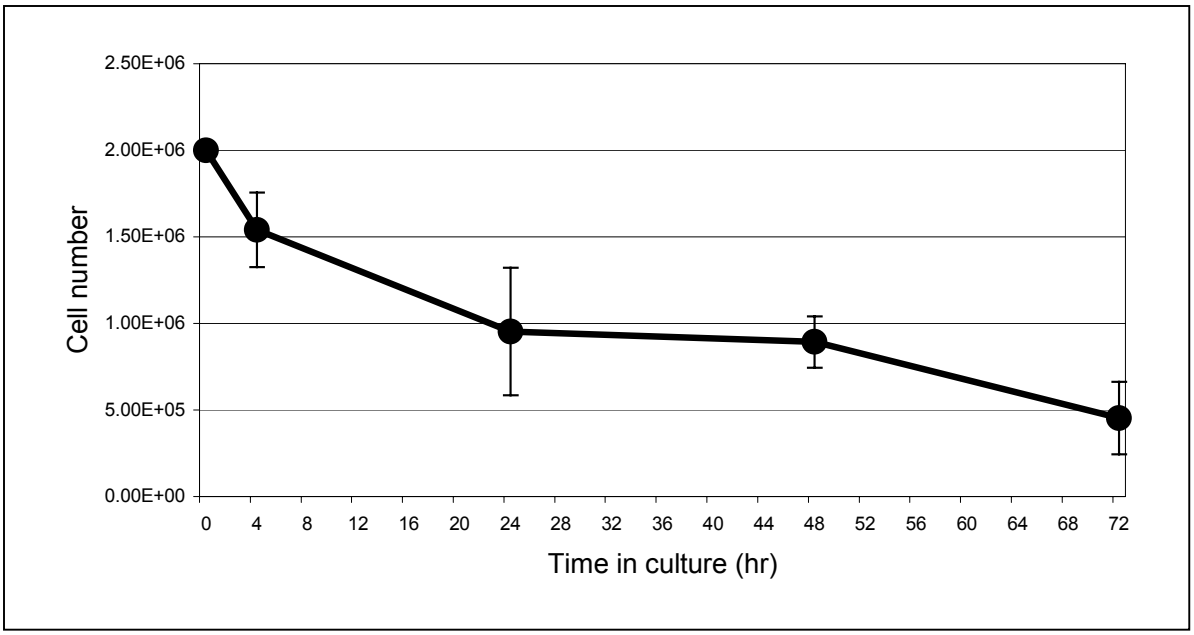


Figure 6.1. Primary B cells die over time in culture. Primary B cells were plated at 2×10^6 cells/ml and numbers of recovered viable cells were determined by Trypan blue exclusion and hemocytometry. n=4 at each timepoint. Error bars represent SD.

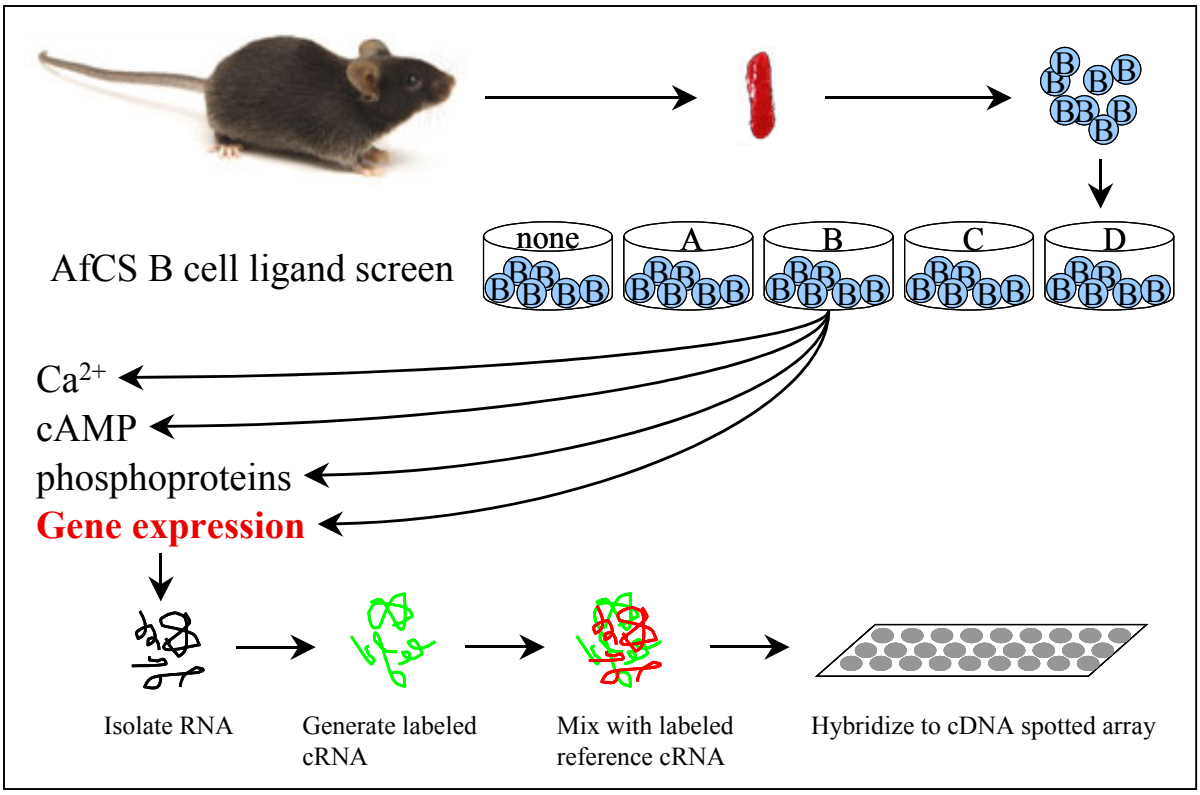


Figure 6.2. Schematic of the AfCS B cell single ligand screen microarray analysis. B cells purified from mouse spleens (See Figure 5.1) are placed into culture and left untreated or treated with 32 different ligands. The AfCS evaluates the outcome of ligand treatments in terms of changes in intracellular free calcium, cAMP, phosphoprotein analysis, and gene microarray analysis. For microarrays, RNA isolated from treated cells are labeled and mixed with labeled RNA from RBC-depleted total splenocytes as a reference. This is then hybridized to a microarray slide containing about 15,000 cDNA probes.

LPS is found in Gram negative bacteria and is a complex glycolipid component of the bacterial cell wall. It is composed of a hydrophilic polysaccharide and a hydrophobic lipid A moiety, both of which can vary among bacterial strains. Toll-like receptor 4 (TLR-4) is the LPS receptor (Poltorak et al. 1998), but successful binding of LPS and signaling depends on accessory proteins. Free LPS is bound by LPS binding protein (LBP), which is thought to aid LPS binding by forming a complex with the cell surface receptor CD14, allowing transfer of LPS to the TLR-4/MD2 complex (Hailman et al. 1994; Schumann et al. 1990; Tobias et al. 1995). Despite its proposed role, CD14-deficient mice are still able to respond to LPS, therefore there is a CD14-independent pathway for LPS binding (Haziot et al. 1996; Haziot et al. 1998). MD-2, a secreted glycoprotein, serves as an adaptor protein for LPS-induced signaling through TLR-4 (Schromm et al. 2001; Visintin et al. 2001). When complexed with the protein MD-2, TLR4 is able to recognize and bind LPS . This recognition and binding is enhanced greatly by the presence of CD14, which is present on monocytes and macrophages (Akashi et al. 2003). Signal transduction through TLR4 has been mainly studied in monocytes, macrophages, and dendritic cells. It is analogous to the IL-1 signaling pathway and involves the MyD88 signaling pathway, which results in IRAK phosphorylation and TRAF ubiquitination. Ubiquitinated TRAF binds to TAK1 and results in activation of the NF-κB, p38, and JNK pathways, resulting in transcriptional reprogramming (Cao et al. 1996; Holtmann et al. 2001; Kawai et al. 2001; Ninomiya-Tsuji et al. 1999) . Macrophages respond to LPS by secreting cytokines such as tumor necrosis factor (TNF); this is dependent on LPS recognition by the TLR-4/MD-2 receptor complex (Akashi et al. 2000). B cells respond to LPS by vigorous proliferation and antibody secretion (Kearney and Lawton

1975). Responses to LPS are reduced in TLR4-deficient mice. (Hoshino et al. 1999). Interestingly, TLR4 levels on B cells are much lower than that of macrophages (Akashi et al. 2000), suggesting that other proteins may be involved in LPS signaling in B cells. RP105 is another member of the TLR family expressed specifically on mature peripheral B cells (Miyake et al. 1994). RP105 is very similar to TLR4 and associates with MD-1, a protein homologous to MD-2 (Miura et al. 1998). RP105-deficient mice are also impaired in B cell responses to LPS, suggesting a cooperation between TLR4 and RP105 in LPS-induced B cell activation (Ogata et al. 2000).

Anti-IgM (AIG) is used as an *in vitro* mimic for antigen. It is able to cross-link the BCR due to its divalency and initiates signal transducion. BCR aggregation results in phosphorylation of ITAM motifs on Igα and Igβ by src-family kinases such as Lyn, Blk, or Fyn (Saouaf et al. 1994). Syk is recruited to phosphorylated ITAMS and becomes activated by phosphorylation (Chu et al. 1996; Weiss and Littman 1994). Syk mediates BLNK phosphorylation, then BLNK associates with PLCγ2 and Btk (Ishiai et al. 1999; Takata and Kurosaki 1996). Activation of phospholipase Cγ2 (PLCγ2) generates the second messengers inositol triphosphate (IP3) and diacylgycerol (DAG). IP_3 binds to receptors on the endoplasmic reticulum membrane and induces the release of intracellular calcium (Takata et al. 1995). Following calcium flux, transcriptional reprogramming occurs through calcium-sensitive transcription factors NF-AT (Choi et al. 1994) and NF-κB (Petro and Khan 2001). Cellular responses to AIG are varied and depend in part on the developmental state of the B cell. For example, stimulation of the BCR in mature B cells results in activation and proliferation while stimulation of the BCR in immature B cells results in apoptosis.

Results and Discussion

We are interested in the effects that ligands have on B cells. Some effects may be shared among different ligands (for example, LPS and AIG can both stimulate proliferation) while some effects are ligand-specific. We analyzed microarray data from the AfCS B cell ligand screen project to evaluate the effects of 32 different ligands on purified mouse splenic B cells and applied CLASSIFI to reveal significant co-clustering of related genes within microarray gene clusters. We were particularly interested in biological processes that are associated with a specific ligand.

Microarray analysis of ligand-treated B lymphocytes. For the AfCS microarray experiments, purified B lymphocytes were treated in culture with 32 different ligands over a timecourse of 30 min, 1 hr, 2 hr, and 4 hr. (A detailed description of the AfCS data set has been published (Zhu et al. 2004). RNA extracted from B cell cultures were used to make Cy5-labeled cDNA and were hybridized along with Cy3-labeled cDNA generated from total splenocyte RNA for normalization. Following filtering, normalization and SAM analysis, genes were identified that were differentially expressed at the 4 hr timepoint by each ligand in comparison with time-matched, untreated controls. Of the 32 ligands, CD40L, LPS, and AIG caused the most gene expression changes at the 4 hr timepoint (Figure 6.3). Further analysis focuses on these three immunologically-important ligands. Categorical values of 1, -1, and 0 (representing significantly upregulated, downregulated, or unchanged) were used to group genes together based on their expression response patterns (Table 6.1). Genes not

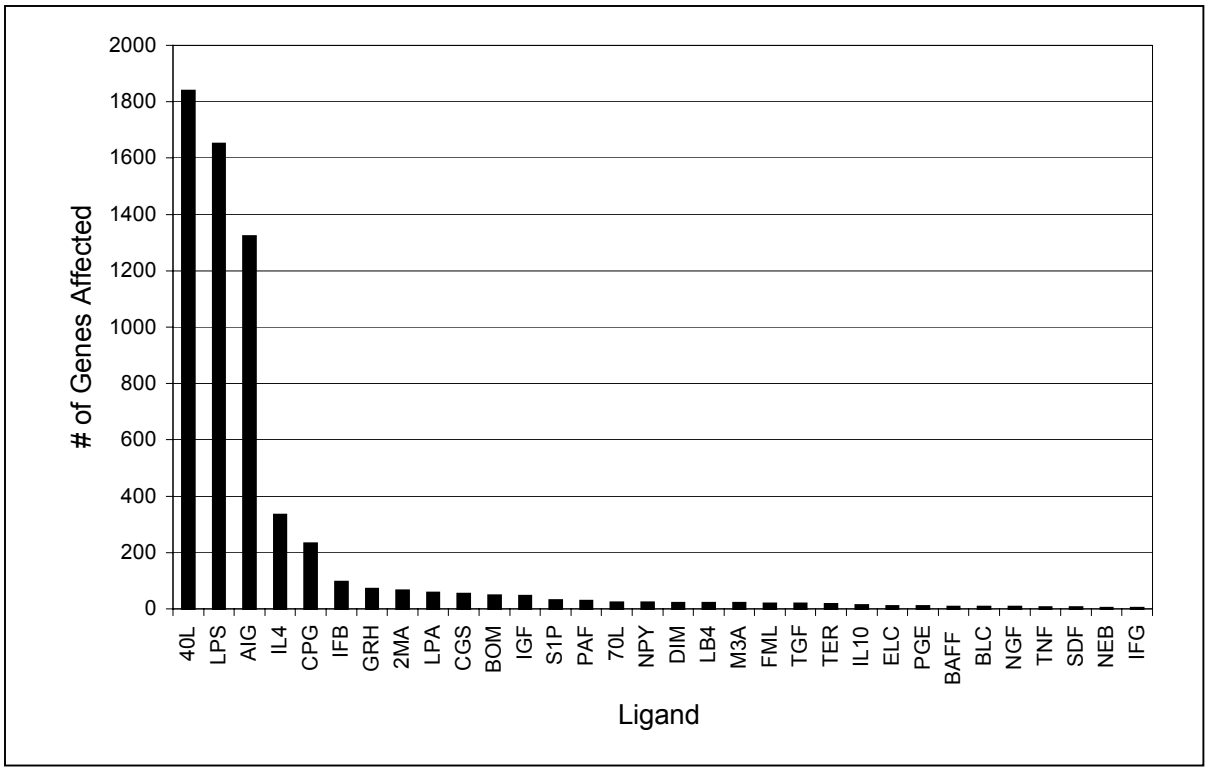


Figure 6.3. Ligand-induced gene expression changes. The number of genes on the microarray whose expression was significantly changed in 4 hr treated primary B cells relative to untreated controls as determined by SAM is shown for each of 32 ligands. Abbreviations and descriptions for each ligand can be found at http://www.signaling-gateway.org/data/cgi-bin/table.cgi?cellabbr=BC. 40L=CD40L (anti-CD40), LPS=Lipopolysaccharide, and AIG=anti-IgM.

GO ID	g	f	c	n	expt	prob	GO type	GO name	Cluster ID	anti-CD40	LPS	AIG
GO:0005634	2490	254	380	64	38.76	8.71E-06	CC	nucleus	1	1	1	1
GO:0009058	2490	141	180	25	10.19	1.36E-05	BP	biosynthesis	2	1	1	
GO:0008372	2490	1409	1	1	0.566	5.66E-01	CC	CC unknown	3	1	-1	-1
GO:0046072	2490	2	160	2	0.129	4.10E-03	BP	dTDP metabolism	4	1		1
GO:0009605	2490	30	3	2	0.036	4.18E-04	BP	response to external stimulus	5	1		-1
GO:0016655	2490	14	331	11	1.861	4.94E-08	MF	oxidoreductase activity, acting on NADH	6	1		
GO:0005773	2490	12	11	4	0.053	1.00E-07	CC	vacuole	7	-1	-1	1
GO:0003779	2490	15	277	9	1.669	6.30E-06	MF	actin binding	8	-1	-1	-1
GO:0016758	2490	6	245	4	0.59	1.17E-03	MF	transferase activity, transferring hexosyl groups	9	-1	-1	
GO:0006417	2490	2	4	1	0.003	3.21E-03	BP	regulation of protein biosynthesis	10	-1		1
GO:0008372	2490	1409	41	32	23.2	3.30E-03	CC	CC unknown	11	-1		-1
GO:0008047	2490	10	160	3	0.643	2.23E-02	MF	enzyme activator activity	12	-1		
GO:0006397	2490	19	56	4	0.427	6.92E-04	BP	mRNA processing	13		1	1
GO:0005576	2490	156	183	33	11.47	7.28E-09	CC	extracellular	14		1	
GO:0046916	2490	1	4	1	0.002	1.61E-03	BP	transition metal ion homeostasis	15		-1	1
GO:0003931	2490	3	38	2	0.046	6.74E-04	MF	Rho small monomeric GTPase activity	16		-1	-1
GO:0004032	2490	3	188	3	0.227	4.24E-04	MF	aldehyde reductase activity	17		-1	
GO:0015672	2490	10	191	7	0.767	1.38E-06	BP	monovalent inorganic cation transport	18			1
GO:0016892	2490	3	50	2	0.06	1.17E-03	MF	endoribonuclease activity, producing other than	19			-1

Table 6.1. Clustering and CLASSIFI results for data from 3 ligands.
Gene clusters (Cluster ID 1-19) resulting from categorical clustering of processed data from B cells stimulated with anti-CD40, LPS, and AIG. "1"=upregulated, "-1"=downregulated, and blank=no significant change. Following CLASSIFI analysis, the gene ontology with the lowest probability in each gene cluster is indicated. GO ID=a unique Gene Ontology identifier that corresponds to a defined molecular function (MF), biological process (BP), or cellular component (CC). g=number of probes in the data set, f=number of probes with associated GO ID in the data set, c=number of probes in the gene cluster, n=number of probes with associated GO ID in the gene cluster. Expt=the expected number of occurrences of a given GO ID in a given cluster of size (n) based on a random distribution. Prob=the probability that the GO ID co-cluster pattern has occurred by chance.

differentially expressed under at least one treatment condition were omitted from further analysis (see "Microarray analysis and clustering" section in Chapter 2 for a complete description of the analysis). A variety of different expression patterns were observed. For example, Gene Cluster #1 contains genes that are upregulated by all three ligands, whereas Gene Cluster #14 contains genes that are only upregulated in response to LPS.

CLASSIFI analysis links gene clusters to cellular physiology. To link biological function with gene expression patterns derived from microarray experiments, we performed CLASSIFI analysis of the clustered data. The filtered data set contained 2545 probes, which led to the calculation of 5036 probabilities. The GO identifier with the lowest probability for each gene cluster is shown in Table 6.1. A significance cutoff of 1×10^{-5} was established using a Bonferroni correction with an *alpha* of 0.05 and an *n* of 5036 (see http://mathworld.wolfram.com/BonferroniCorrection.html and references therein). Six gene clusters gave rise to GO identifiers with significant probabilities: Gene Clusters #1, 6, 7, 8, 14, and 18. Gene clusters identified by CLASSIFI to give significant probabilities exhibited predictable expression patterns that could be explained biologically, including all three gene clusters characterized by ligand-specific expression induction. Experimental noise may contribute to gene clusters with insignificant probabilities that show unusual expression patterns.

CLASSIFI results for some gene clusters were expected based on our current understanding of B cell physiology. For example, the GO identifier giving the lowest probability in Gene Cluster #1 is "nucleus". This gene cluster represents genes which are upregulated in

response to all three ligands: AIG, CD40L, and LPS, and includes genes involved in transcription, replication and RNA processing. All three of these ligands induce cellular activation (DeFranco 1987; Hsueh and Scheuermann 2000; Kehry 1996) and proliferation (Figure 6.4), so we would expect these kinds of nuclear genes to be highly expressed in B cells stimulated with all three ligands. The GO identifier (GO:0005576) giving the lowest probability in Gene Cluster #14 is "extracellular" which is defined by the GO database as "the space external to the outermost structure of a cell". This gene cluster represents genes upregulated specifically in response to LPS, which is a component of bacterial cell walls. Bacteria are often extracellular pathogens, therefore this classification may reflect components of the B cell response to LPS that take place outside the cell. For example, LPS binding to the LPS receptor TLR4 requires the association of TLR4 with the secreted glycoprotein MD-2. Also, LBP aids binding of free LPS to the TLR-4/MD2 complex. The GO identifier giving the lowest probability in the CD40L-specific Gene Cluster #6 is "oxidoreductase activity, acting on NADH or NADPH". It has recently been shown that stimulation through CD40 and other TNF family members results in the production of reactive oxygen species through NADPH (Ha and Lee 2004). Importantly, many of the GO identifiers giving the low probabilities in a given gene cluster are functionally related, e.g. monovalent inorganic ion transport, ion transport, transporter activity, and cation transport in Gene Cluster #18 (Table 6.2).

Validation of CLASSIFI-predicted coordinate expression. CLASSIFI analysis should be viewed as a hypothesis-generating tool. While the statistical analysis is compelling,

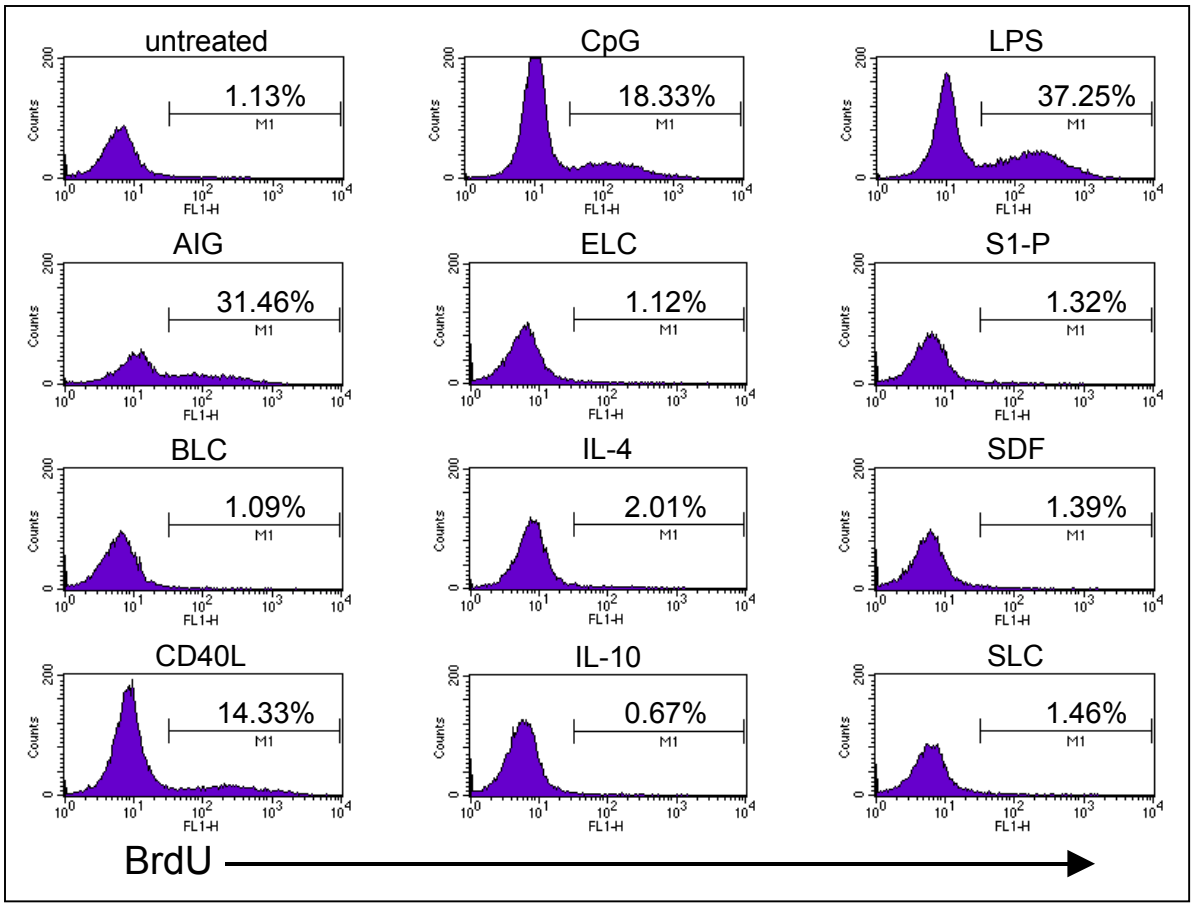


Figure 6.4. Ligand effects on proliferation. Proliferation as measured by BrdU uptake was determined for primary B cells treated for 48 hours with 12 different ligands. Percentage of BrdU+ cells is indicated. The greatest proliferative responses tended to be associated with ligands that induced changes in the greatest number of genes (See Figure 6.3).

GO ID	f	n	prob	GO type	GO name	Cluster ID
GO:0005634	254	64	8.71E-06	cellular_component	nucleus	1
GO:0005635	13	9	1.66E-05	cellular_component	nuclear membrane	1
GO:0006412	94	30	2.75E-05	biological_process	protein biosynthesis	1
GO:0009059	94	30	2.75E-05	biological_process	macromolecule biosynthesis	1
GO:0003743	20	10	2.68E-04	molecular_function	translation initiation factor activity	1
GO:0016655	14	11	4.94E-08	molecular_function	oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor	6
GO:0008137	14	11	4.94E-08	molecular_function	NADH dehydrogenase (ubiquinone) activity	6
GO:0015081	14	11	4.94E-08	molecular_function	sodium ion transporter activity	6
GO:0016651	16	11	4.61E-07	molecular_function	oxidoreductase activity, acting on NADH or NADPH	6
GO:0046873	16	11	4.61E-07	molecular_function	metal ion transporter activity	6
GO:0005773	12	4	1.00E-07	cellular_component	vacuole	7
GO:0016787	163	6	2.52E-05	molecular_function	hydrolase activity	7
GO:0005624	15	3	2.84E-05	cellular_component	membrane fraction	7
GO:0000267	18	3	5.05E-05	cellular_component	cell fraction	7
GO:0015991	3	2	5.31E-05	biological_process	ATP hydrolysis coupled proton transport	7
GO:0003779	15	9	6.30E-06	molecular_function	actin binding	8
GO:0008092	22	9	3.08E-04	molecular_function	cytoskeletal protein binding	8
GO:0003785	6	4	1.88E-03	molecular_function	actin monomer binding	8
GO:0006357	10	5	2.58E-03	biological_process	regulation of transcription from Pol II promoter	8
GO:0006355	79	17	4.85E-03	biological_process	regulation of transcription, DNA-dependent	8
GO:0005576	156	33	7.28E-09	cellular_component	extracellular	14
GO:0005578	5	4	1.33E-04	cellular_component	extracellular matrix	14
GO:0004871	50	11	7.39E-04	molecular_function	signal transducer activity	14
GO:0004888	7	4	8.29E-04	molecular_function	transmembrane receptor activity	14
GO:0007166	25	7	1.59E-03	biological_process	cell surface receptor linked signal transduction	14
GO:0015672	10	7	1.38E-06	biological_process	monovalent inorganic cation transport	18
GO:0006811	23	10	2.65E-06	biological_process	ion transport	18
GO:0005215	122	24	9.10E-06	molecular_function	transporter activity	18
GO:0006812	18	8	2.33E-05	biological_process	cation transport	18
GO:0006754	4	4	3.36E-05	biological_process	ATP biosynthesis	18

Table 6.2. Excerpt of CLASSIFI results for six gene clusters giving significant probabilities.
The five GO IDs giving the lowest probability are listed for each of six gene clusters giving significant probabilities in Table 6.1. GO ID=a unique Gene Ontology identifier that corresponds to a defined molecular function, biological process, or cellular component (GO name). f=number of probes with the associated GO ID in the data set, n=number of probes with the associated GO ID in the gene cluster. prob=the probability that the GO ID co-cluster pattern has occurred by chance.

predictions that derive from the hypotheses should be verified experimentally. We were particularly interested in the AIG-specific Gene Cluster #18 because these genes represent biological processes that are unique to antigenic stimulation rather than the general activation responses induced by all three ligands. The GO term giving the lowest probability in Gene Cluster #18 is "monovalent inorganic cation transport" (GO:0015672). An examination of the genes annotated with this GO term revealed multiple components of the vacuolar ATPase H+ pump (Table 6.3). Since stimulation through the BCR induces internalization of the receptor-antigen complex and trafficking through the endocytic system, "monovalent inorganic cation transport" could reflect the acidification of vesicles as they progress from endosomes to lysosomes during endocytosis. This AIG-specific cluster also contained an overrepresentation of genes involved in general "transporter activity" (GO:0005215), which included genes like Rab9, sorting nexin 5 (Snx5) and N-ethylmaleimide sensitive fusion protein attachment protein alpha (Napa) which are involved in endosome trafficking and vesicle fusion. These observations led us to consider the hypothesis that AIG might induce the expression of various components of the endocytic and vesicle transport pathways in a manner that is independent of its effects on the general metabolic activation and proliferation of B cells, and that this might be part of a B cell-specific antigen processing and presentation function.

To ensure that the genes identified through the CLASSIFI analysis were meaningful, we first sought to verify their differential expression patterns. The microarray expression pattern of nine probes recognizing "transporter activity" genes in unstimulated and ligand-stimulated samples were evaluated (Figure 6.5). In every case, expression of these genes was

GO ID	Probe ID	Gene Name
GO:0015672	C530010I21	ATPase, H+ transporting, V0 subunit B
GO:0015672	2310069H14	ATPase, H+ transporting, V1 subunit G isoform 1
GO:0015672	5730403E06	SIMILAR TO ATPASE, H+ TRANSPORTING, LYSOSOMAL
GO:0006811	2810002O05	sideroflexin 1
GO:0006811	1810048H22	arsA (bacterial) arsenite transporter, ATP-binding, homolog 1
GO:0006811	2310021D14	chloride channel 7
GO:0005215	1810011E08	MICROSOMAL SIGNAL PEPTIDASE 23 KDA SUBUNIT (EC 3.4.-.-)
GO:0005215	2810433C04	translocase of inner mitochondrial membrane 9 homolog (yeast)
GO:0005215	1500039N14	N-ethylmaleimide sensitive fusion protein attachment protein alpha
GO:0005215	2210039G11	fatty acid binding protein 5, epidermal
GO:0005215	5430437K12	zinc finger protein 295
GO:0005215	1810057M21	thioredoxin reductase 1
GO:0005215	2310004L13	translocase of inner mitochondrial membrane 9 homolog (yeast)
GO:0005215	2610025H03	fatty acid binding protein 5, epidermal
GO:0005215	5430413F24	RAB9, member RAS oncogene family
GO:0005215	5830417J06	24-dehydrocholesterol reductase
GO:0005215	0910001N05	sorting nexin 5
GO:0005215	2410046L22	FK506 binding protein 1a
GO:0005215	2010015D08	vacuolar protein sorting 29 (S. pombe)
GO:0005215	3010014K12	N-ethylmaleimide sensitive fusion protein attachment protein alpha

Table 6.3. Genes annotated with transport-related GO terms from Gene Cluster #18
List of characterized genes detected using specific cDNA probes (Probe ID) found in Gene Cluster #18 that are associated with GO terms "monovalent inorganic ion transport" (GO:0015672), "ion transport" (GO:0006811), and "transporter activity" (GO:0005215). N.B. GO:0015672 is a child of GO:0006811, which is a child of GO:0005215 in the GO hierarchy.

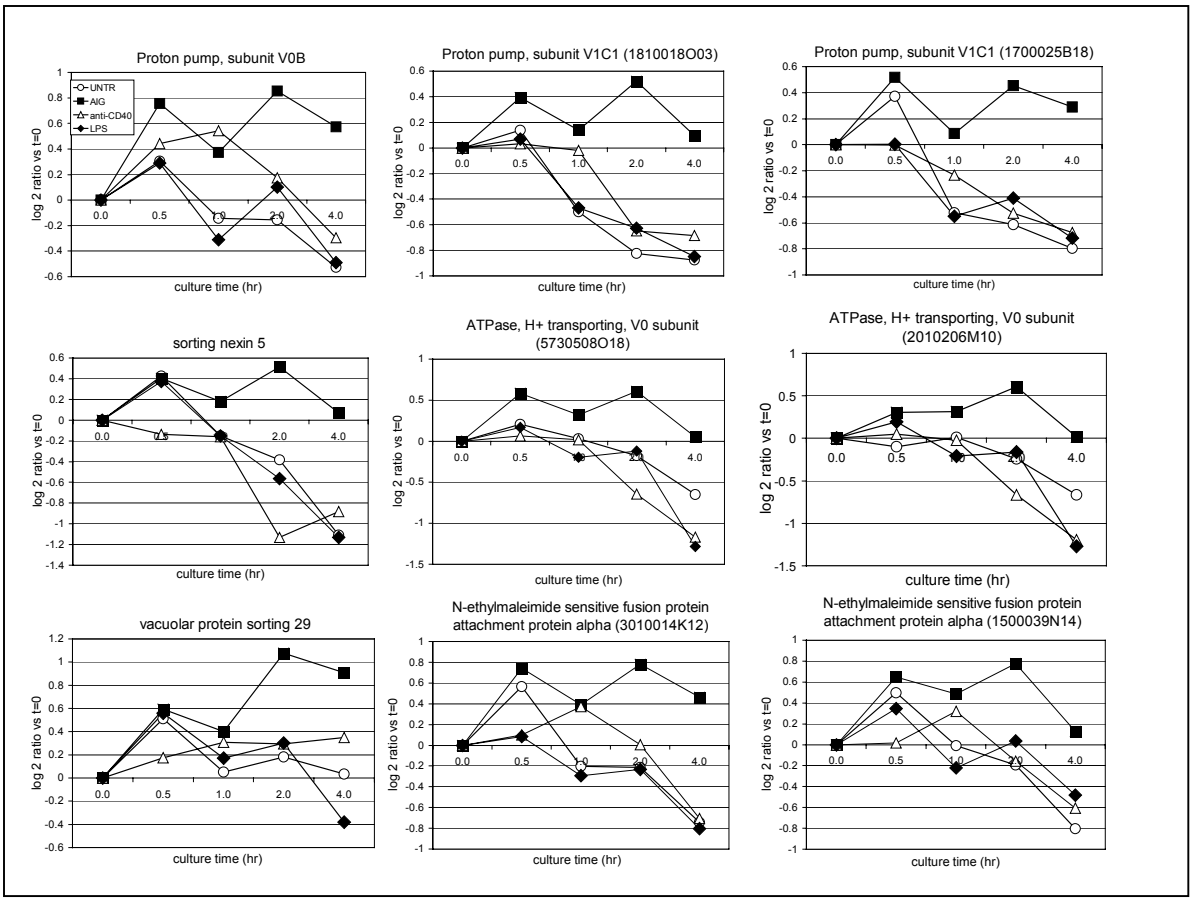


Figure 6.5. Expression data from select intracellular transport-related genes. Microarray results for expression patterns of several genes from the AIG-specific gene cluster #18 over a timecourse of 0.5 to 4 hr is shown The $\log_2$(Cy5/Cy3) values were compared to 0 hr untreated samples (t=0). Three examples of genes represented by two different probes (probe IDs in parentheses) are shown in the two right columns.

similar in all treatment conditions at 0.5 and 1 hr, but was higher in the AIG-treated samples at 2 hr and 4 hr. Examples in which the same gene is represented by two different probes show closely similar expression patterns, indicating the high quality of this microarray data set. To verify the ligand-specificity of expression, RT-PCR was used to examine the expression of a subset of these genes in independently-generated samples. Again, AIG induced enhanced expression of each of these genes compared to LPS and anti-CD40 (Figure 6.6). RT-PCR results consistently confirm microarray expression data in this data set (Zhu et al. 2004).

CLASSIFI analysis is based on the postulate that genes involved in the same biological process are coordinately expressed. Thus, one prediction of this analysis is that other genes involved in the same biological process that were not included in the microarray probe data set would show the same expression pattern. We evaluated 4 such genes that encode ATPase H+ pump complex subunits by RT-PCR. All of these genes demonstrated a preferential upregulation in response to AIG (Figure 6.7). These data not only provide experimental validation of the CLASSIFI approach to microarray data analysis, but also further support the hypothesis that AIG induces vesicle processing and transport as a biological process.

Summary. We analyzed a well-controlled microarray data set from the AfCS from mouse splenic B cells stimulated with a panel of 32 different ligands. We focused the CLASSIFI analysis on 3 immunologically important ligands: AIG, CD40L, and LPS. These ligands share the common effect of inducing proliferation at varying levels in B cells. We

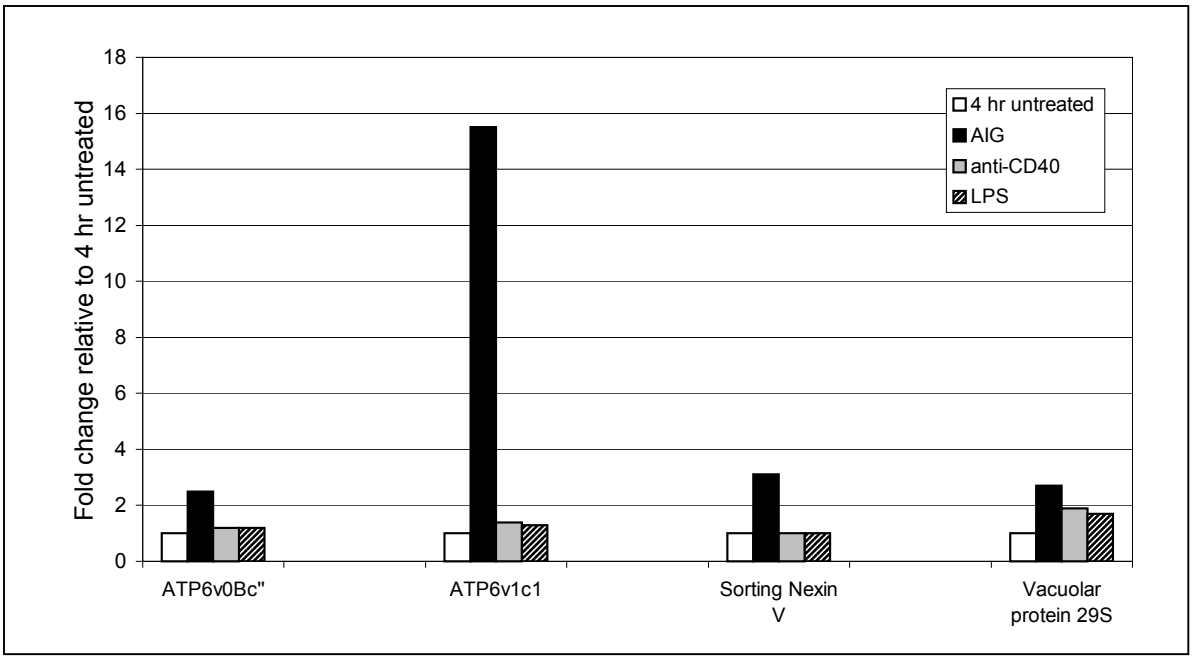


Figure 6.6. Real-time RT-PCR analysis of 4 intracellular transport-related genes found in Gene Cluster #18. Real-time RT-PCR was used to determine expression of four intracellular transport-related genes at 4 hr of stimulation that were included in the microarray data set. Data were normalized to mouse 18S rRNA as a reference and compared to 4 hr untreated samples. ATP6v0bc" and ATP6v1c1, n=2. Sorting nexin V and Vacuolar protein 29S, n=1.

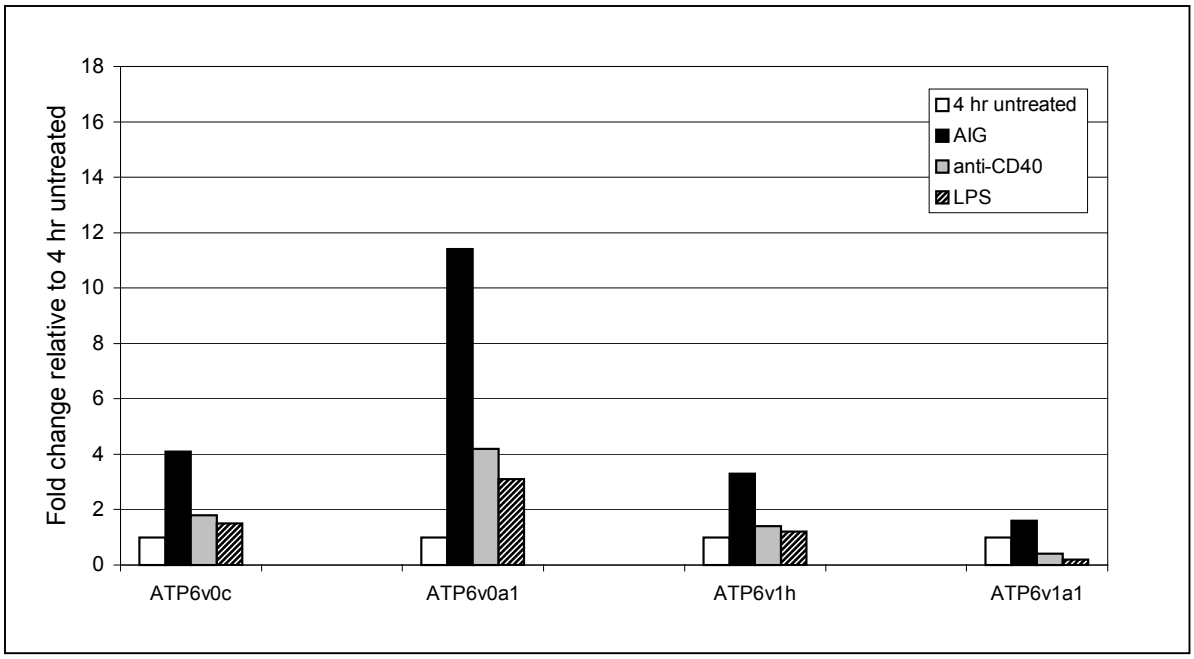


Figure 6.7. Real-time RT-PCR analysis of 4 intracellular transport-related genes not included in the microarray. Real-time RT-PCR was used to evaluate the expression of 4 intracellular transport-related genes that were not included in the microarray data set. These genes encode other subunits of the ATPase proton pump complex.

were interested in identifying ligand-specific effects. Following CLASSIFI analysis, all gene clusters containing genes specifically upregulated by one ligand show significant co-clustering of a gene ontology term: Gene Cluster #6 (CD40L-specific), Gene Cluster #14 (LPS-specific) and Gene Cluster #18 (AIG-specific). Using Gene cluster #18, we tested a CLASSIFI-based prediction that other genes involved in "monovalent inorganic cation transport" that were not included in the microarray analysis would be coordinately expressed. Indeed, ATPase H+ pump subunits not included in the analysis showed a similar expression pattern compared to those that were included in the analysis. The following chapters describe how we used this CLASSIFI analysis as a discovery tool.

Chapter 7

Analysis of the B cell single ligand screen microarray data set (II)

Introduction

The results in this chapter describe experimental validation of CLASSIFI-derived hypotheses, showing that CLASSIFI can be used to identify real biological processes defined by microarray clustering analysis. This study combining bioinformatics analysis with experimental validation is unique in that the bioinformatics analysis is done on a data set for which there are no expectatations for certain results and thus it is unbiased and provides opportunity for discovery. Other studies which have used bioinformatics tools to append microarray data with biological information were validated on a well-characterized data set with expected results.

We were particularly interested in the AIG-specific Gene Cluster #18 because these genes represent biological processes that are unique to antigenic stimulation rather than the general activation/proliferation responses induced by all three ligands.

We chose to use the mouse B lymphoma line WEHI-231 for the experiments described in this chapter. WEHI-231 is a B cell lymphoma with an immature phenotype. It lacks expression of IgD, FcR, and C3 receptors and Class II is expressed at low levels (Nossal 1983; Ralph 1979). It is similarly to BCL_1.3B3 in that it undergoes cell cycle arrest and apoptosis in response to anti-IgM stimulation (Benhamou et al. 1990; Boyd and Schrader 1981; Scott et al. 1985). Therefore, WEHI-231 has been used as a model for tolerance induction. Signaling through the BCR on WEHI-231 results in early events similar to normal B cells, including induction of tyrosine phosphorylation (Gold et al. 1990; Hutchcroft et al. 1991), activation of Blk, Lyn, Lck, and Hck (Li et al. 1992), and calcium flux (LaBaer et al.

1986). However, unlike mature B cells, calcium flux-associated PKC activation is not detected, illustrating that there are differences in late signaling events between immature and mature B cells (Sarthou et al. 1989). Signaling through CD40 rescues anti-IgM-induced apoptosis and is indicative of the requirement for costimulation to avoid tolerance induction (Tsubata et al. 1993). Unlike previous findings, one group found IgD to be expressed on WEHI-231, however no calcium mobilization was induced by IgD cross-linking (Haggerty et al. 1993). Another group found the WEHI-231 BCR to be excluded from lipid rafts upon cross-linking and detected no internalization of BCR, suggesting that this is another mechanism that contributes to differences in signaling outcomes between immature and mature B cells (Sproul et al.).

Results and Discussion

Experimental validation of CLASSIFI-derived biological predictions. The hypothesis formulated based on CLASSIFI analysis is that the over-representation of transport-related gene ontologies in the AIG-specific Gene Cluster #18 is due to AIG stimulation of endocytosis and subsequent antigen processing and presentation. This hypotheses seems reasonable based on the knowledge that B cells bind, internalize, process, and present their specific antigen very efficiently. We chose to test this hypothesis by addressing one aspect of antigen processing and presentation: antigen entry or antigen internalization. Antigen

binding and uptake by the BCR was tested by labeling the BCR, then stimulating cells with AIG and evaluating BCR fate by following the label.

We used monoclonal anti-IgM-FITC antibodies as the label. It was first imperative that the monoclonal anti-IgM-FITC antibody is non-stimulating upon binding the BCR so that we would be able to compare unstimulated cells to stimulated cells. To determine if the antibody used for labeling is indeed non-stimulating, two anti-IgM-FITC monoclonal labeling antibodies were tested for their ability to induce calcium flux in WEHI-231. One clone, R6-60.2, showed little signs of calcium flux even at the highest tested amount. Clone II/40, on the other hand, showed evidence of a delayed calcium response (Figure 7.1A and B). After allowing time for internalization, the cells are subjected to an acid wash to remove surface-bound label (Figure 7.2). Label that has been internalized is protected from the acid wash and can therefore be detected using flow cytometry. Using this method, we found that BCR internalization (arrow, Figure 7.3D) occurs with AIG stimulation, but not anti-CD40 or LPS stimulation in WEHI-231 cells (Figure 7.3A, G). Substantial internalization was found as early as 15 min post-stimulation and continued past 4 hr. Ligand-specific internalization of the BCR was also detected in primary B cells (Figure 7.4).

These data indicated that signaling through the BCR is required to stimulate BCR internalization and that other strong activators of B cells did not. To test whether receptor internalization following AIG stimulation is specific to the BCR and not other cell surface receptors, we performed internalization assays in which TLR4 (LPS receptor) or CD40 was labeled prior to AIG treatment. In both cases, no internalization of the labeled receptor was detected (Figure 7.3E, F), demonstrating that AIG induces specific internalization of the

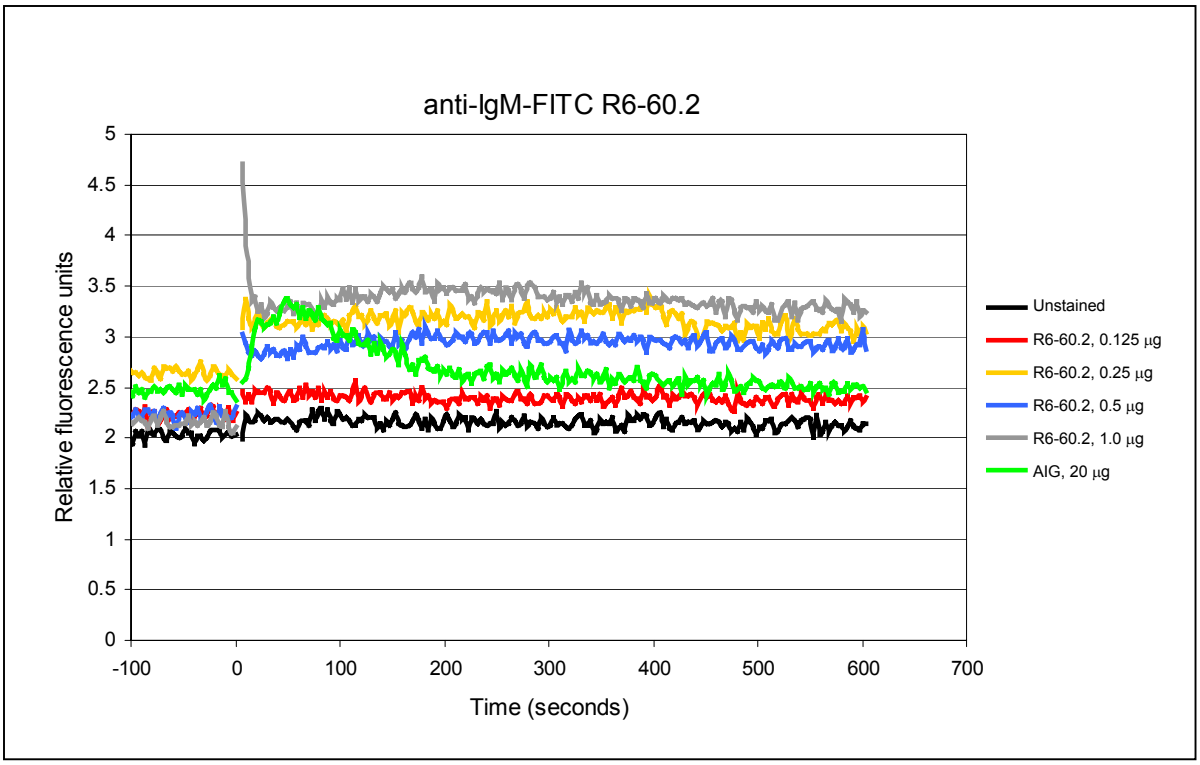


Figure 7.1A. Calcium response induced by anti-IgM-FITC clone R6-60.2. Increases in intracellular free calcium was detected by the calcium-sensitive dye Fura-2. Antibody was added at time 0 seconds. AIG stimulation using a polyclonal cross-linking antibody is included as a positive control for calcium flux. Peak in R6-60.2, 1.0 µg sample (gray) is a sampling artifact. Baseline fluorescence changes in samples with R6-60.2 antibody added is due to FITC fluorescence bleed-over into the detection filter.

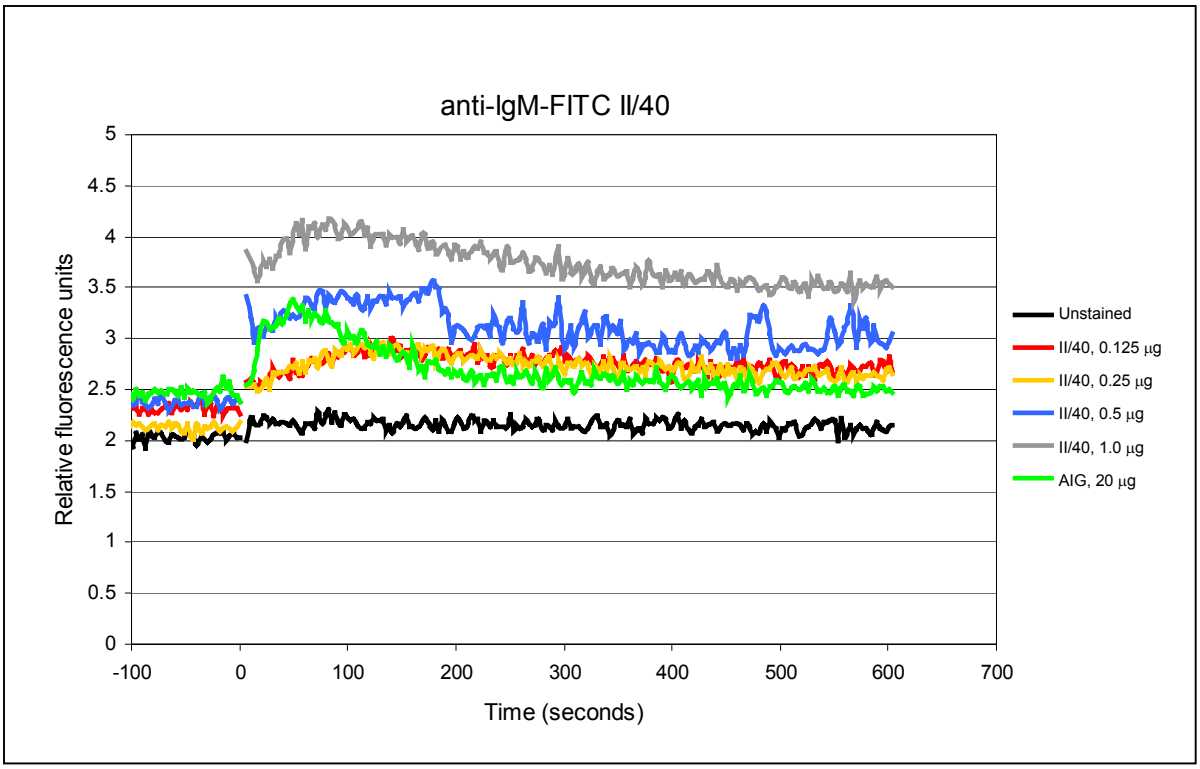


Figure 7.1B. Calcium response induced by anti-IgM-FITC clone II/40. Increases in intracellular free calcium induced by the monoclonal anti-IgM antibody clone II/40 is detected using the calcium sensitive dye Fura-2. AIG stimulation is included as a positive control for calcium flux (green).

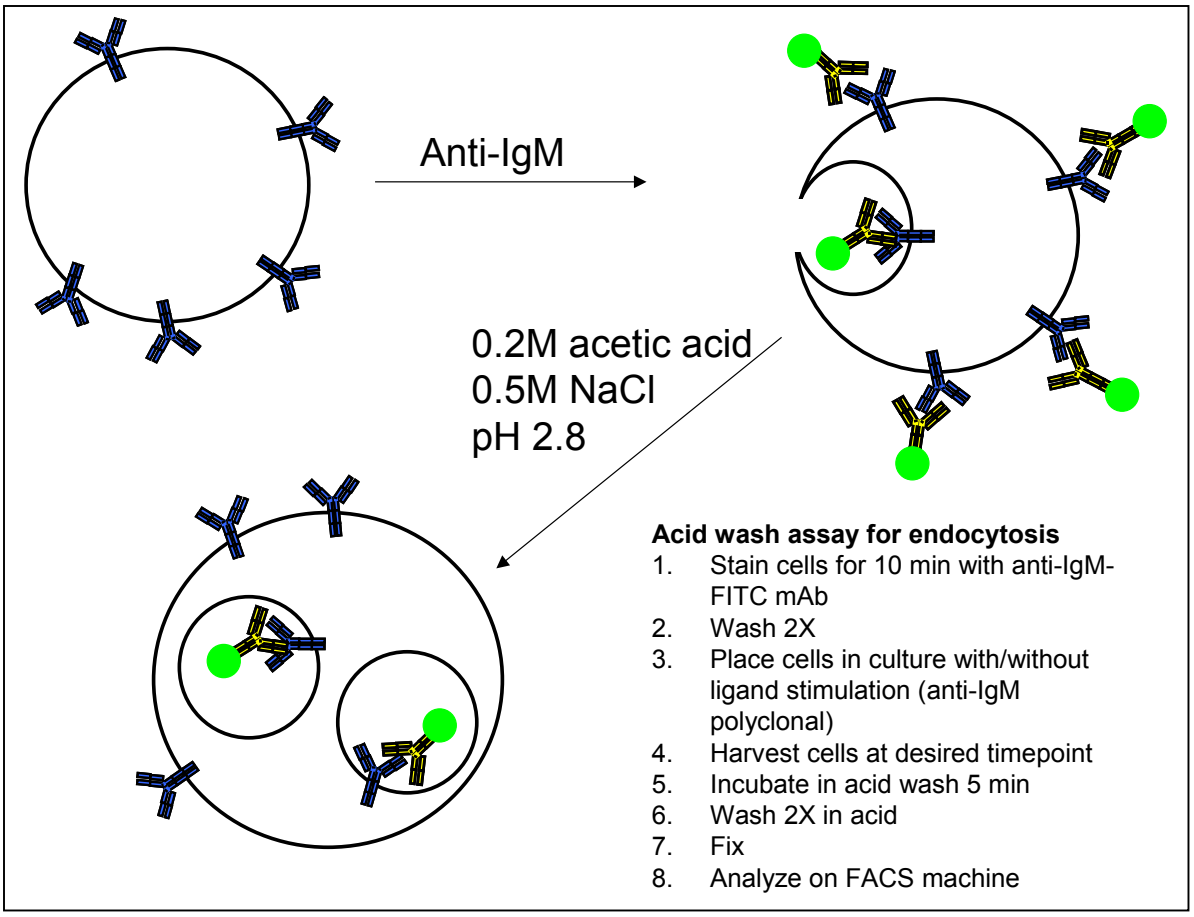


Figure 7.2. Internalization assay methodology. WEHI-231 cells labeled for the BCR with a non-stimulating anti-IgM-FITC monoclonal antibody were stimulated with AIG. Label protected from the following acid wash treatment, which strips off cell surface-bound label, is indicative of receptor internalization and can be detected by flow cytometry.

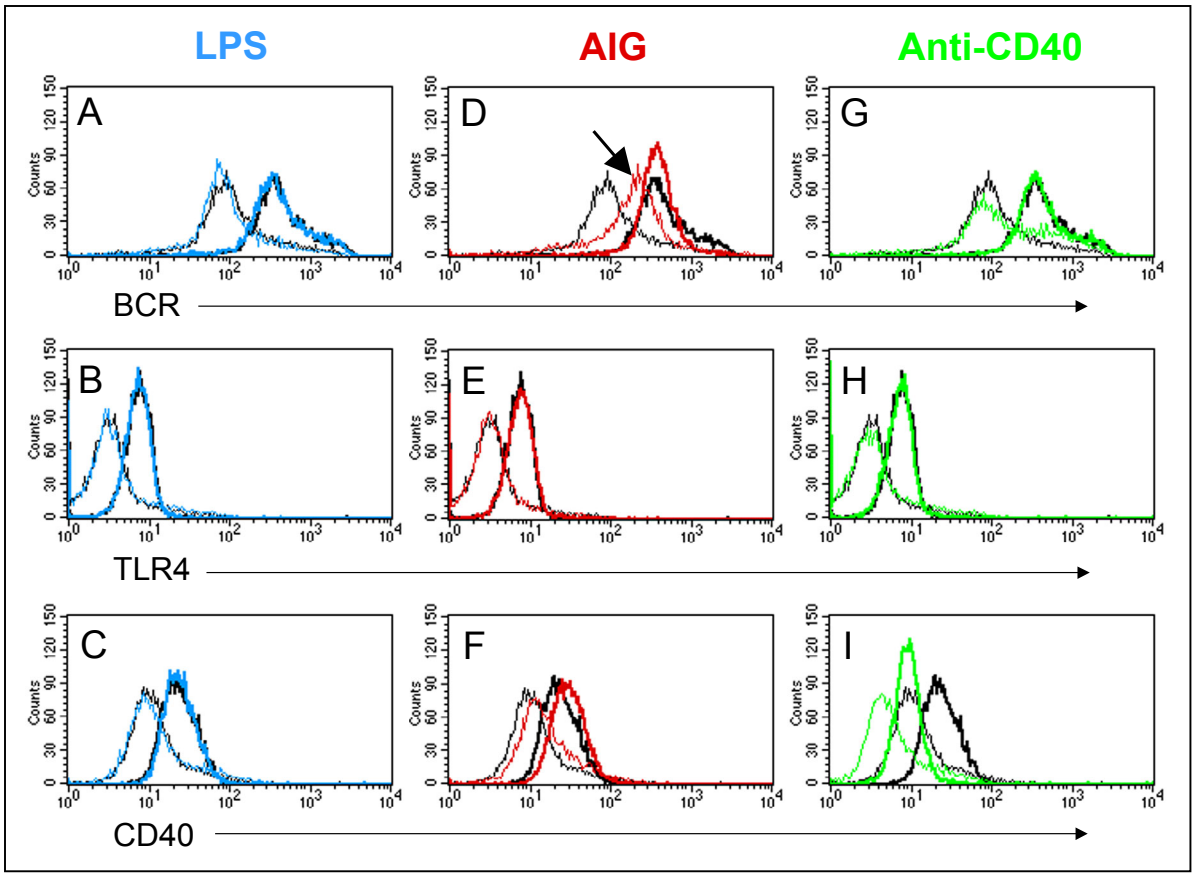


Figure 7.3. Internalization of cell surface receptors in WEHI-231. Assay for internalization of BCR, CD40, and TLR4 was performed using WEHI-231 cells. Data from 1 hr stimulations are shown. Line thickness represents treatment of cells following labeling of receptor where thin=acid treated samples and thick=no acid treatment. Black=unstimulated, blue=LPS stimulated, red=AIG stimulated, green=anti-CD40 stimulated. Arrow denotes histogram of acid-treated cells in which labeled BCR is protected from the effects of acid treatment with AIG stimulation. Ligand stimulations are labeled across the top in color, and labeled receptor is indicated on the x-axes of the histogram plots.

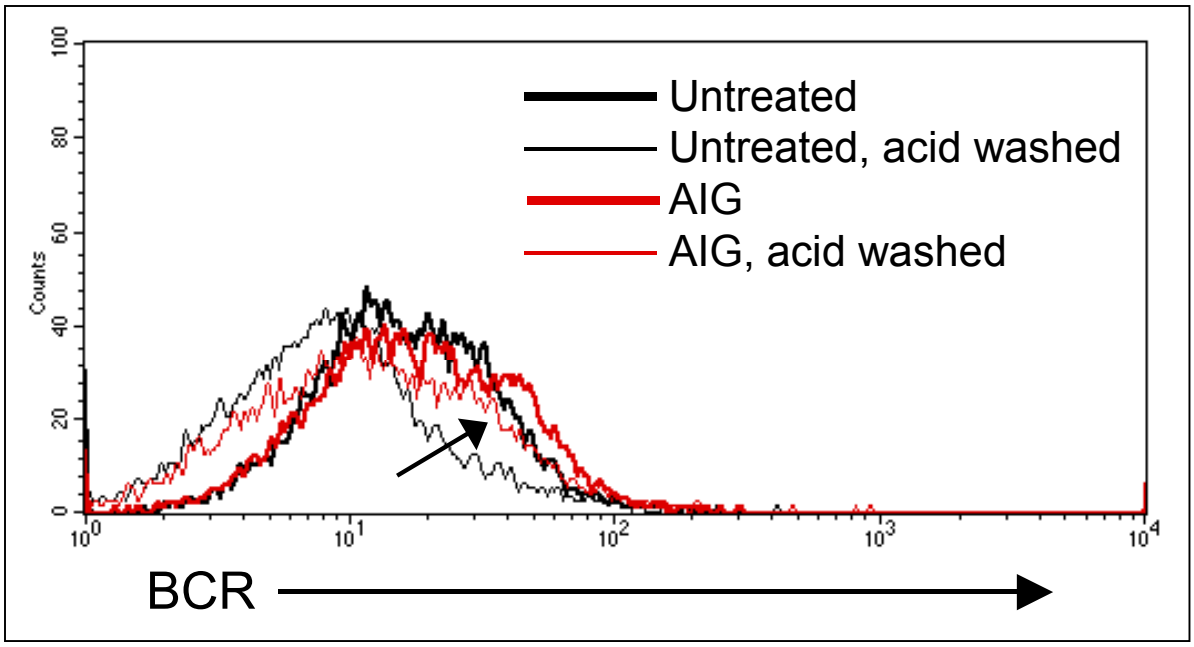


Figure 7.4. Internalization of BCR in primary B cells. The BCR of primary B cells was labeled with non-stimulating antibody, then cross-linked with polyclonal antibody (AIG). Arrow denotes shift in histogram peak in AIG, acid washed sample, indicating protection of label from acid wash.

BCR. (The slight shift in CD40 staining probably results from the upregulation of CD40 in response to AIG treatment). Finally, to test whether stimulation of TLR4 with LPS or CD40 with anti-CD40 induces TLR4 or CD40 internalization, we fluorescently labeled TLR4 or CD40, then treated cells with the corresponding ligands. In the case of TLR4 stimulation with LPS, no internalization is detected (Figure 7.3B). The anti-TLR4-MD2 antibody used to label TLR4 has been shown not to have an agonistic effect on TNFα production by peritoneal macrophages (Akashi et al. 2000). Anti-CD40 stimulation induces a rapid receptor shedding phenomenon, (Contin et al. 2003) and so it is difficult to directly compare the results of these samples with the other treatment conditions. However, no evidence for internalization was found (Figure 7.3I). Thus, AIG induces a ligand-specific, receptor-specific endocytic process that is likely designed to capture specific antigen for processing and presentation.

To provide further visual proof of BCR internalization, we used fluorescence microscopy to view FITC-labeled BCR upon stimulation with AIG. In unstimulated cells, the BCR exhibits a diffuse pattern of even distribution around the surface of the cell (Figure 7.5). Within 10 min of AIG treatment, the BCR is rapidly internalized, as evidenced by clumps of green fluorescence found distributed inside the cell. Cells treated with anti-CD40 and LPS for 10 min retain a "diffuse" even distribution of the BCR about the cell surface, thus no internalization is observed under these conditions. The internalized BCR remains visible at 30 min and 1 hr of AIG stimulation, during which WEHI-231 cells begin to clump together (Figure 7.6), possibly via upregulation of adhesions on the cell surface (Boyd et al. 1988).

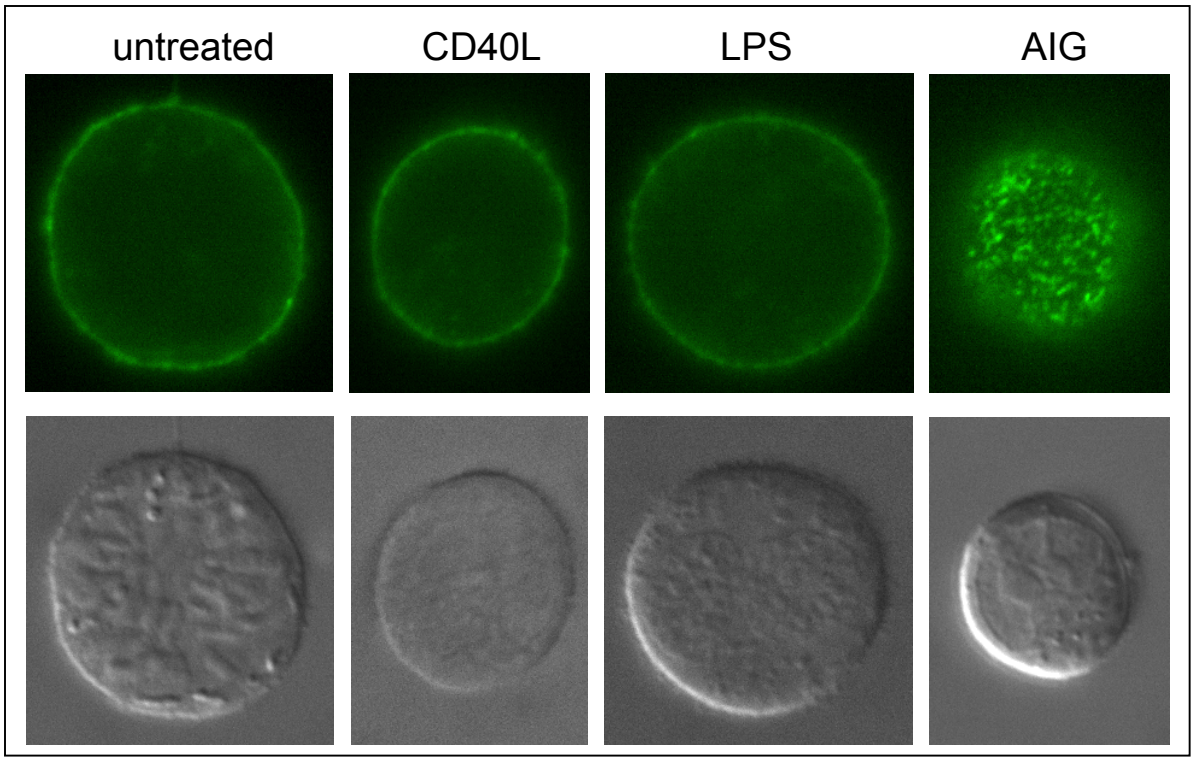


Figure 7.5. BCR localization in ligand-stimulated WEHI-231. BCR was labeled green with the non-stimulating FITC-conjugated anti-IgM antibody clone R6-60.2, then cells were stimulated with anti-CD40 (CD40L), LPS, or AIG. Images were captured during 10 min of AIG stimulation. Representative images are shown. Bottom row shows differential interference contrast (DIC) images of cells in top row.

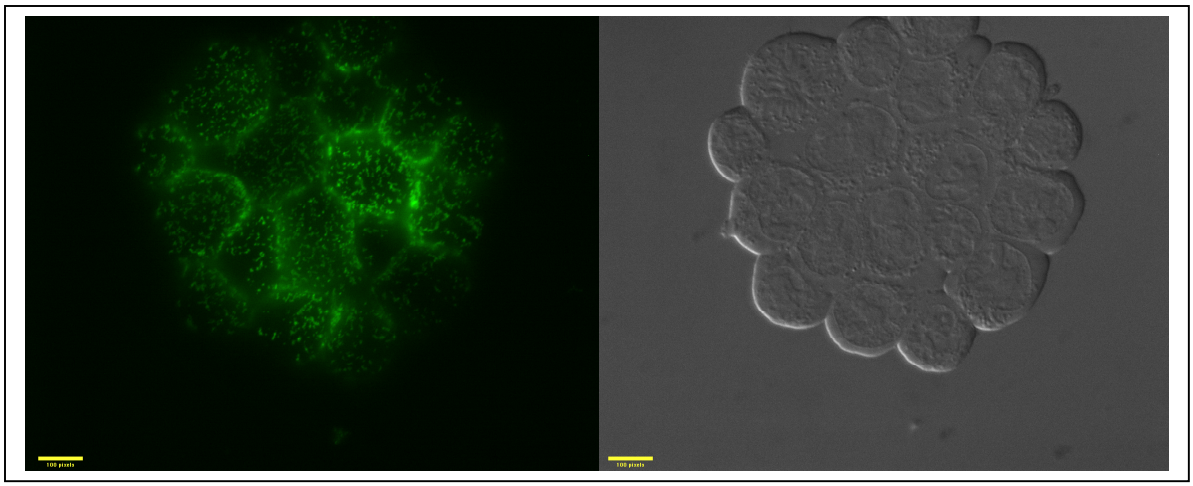


Figure 7.6. WEHI-231 clustering in response to AIG treatment. BCR was labeled green with anti-IgM-FITC antibody clone R6-60.2, then stimulated with AIG. Cell clumps form at 60 and 90 min of stimulation. An example of a cell clump is shown. The BCR is localized using FITC fluorescence (left). A DIC image of the cell clump is shown (right).

Finally, MHC class II expression would be expected to be high in B cells undergoing antigen processing and presentation as antigen activated B cells upregulate Class II expression (Benschop et al. 1999). Expression levels of Class II I-A^d in WEHI-231 were only slightly higher in AIG-treated cells compared to untreated cells. This observation consistent with data from immature B cells showing that, unlike mature B cells, Class II is not significantly upregulated in response BCR cross-linking (Tasker and Marshall-Clarke 1997). However, Class II expression was downregulated in anti-CD40- and LPS-treated cells compared to AIG-treated cells (Figure 7.7). This data is interesting because it suggests that BCR cross-linking is required to sustain Class II expression and that in the presence of other stimuli not involving the BCR, Class II is downregulated. These results provide a link between BCR cross-linking and antigen processing and presentation.

Summary. Based on the CLASSIFI results for Gene Cluster #18, we hypothesized that AIG stimulated endocytosis in B cells and that this was related to BCR-mediated antigen processing and presentation. This hypothesis was validated using WEHI-231 cells by first demonstrating that the BCR is internalized following cross-linking with AIG. Furthermore, visual proof of BCR internalization was provided by labeling the BCR and following internalization by immunofluorescence microscopy. The kinetics of BCR internalization was rapid, occurring within 10 minutes of BCR stimulation even though the cells were kept chilled on ice prior to stimulation. The hypothesized occurrence of antigen processing and presentation was supported by Class II expression levels, which were retained on AIG stimulated cells but not on CD40L or LPS stimulated cells. The significant of this study in

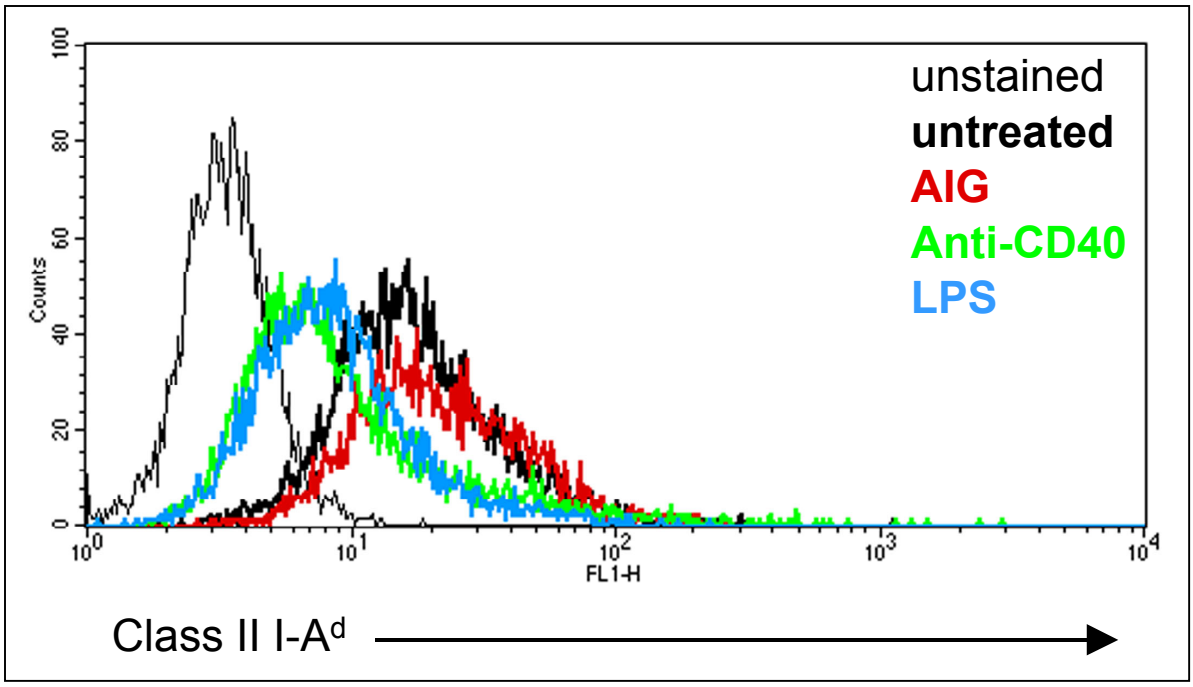


Figure 7.7. Class II MHC expression in AIG, anti-CD40, and LPS stimulated WEHI-231. Flow cytometric analysis of Class II I-A^d expression in WEHI-231 in response to overnight stimulation with AIG (red), anti-CD40 (green), and LPS (blue) or media alone (thick black line). Thin black line represents the unstained control.

the field of bioinformatics stems from a step-wise demonstration of the analysis of an uncharacterized microarray data set, the application of CLASSIFI to identify biological processes associated with specific ligands, the generation of hypotheses based on CLASSIFI results, and the validation of these hypotheses by experimentation. Experimental validation of the occurrence of a biological process identified using purely bioinformatics means is exciting and provides reason for further use of CLASSIFI in microarray data analysis. Furthermore, this study provides a means for discovery. For example, there may be genes in Gene Cluster #18 that are not currently known to be associated with BCR-mediated antigen processing and presentation. There are also expressed sequence tags (ESTs) that could encode proteins which have no known function. Further analysis of these possibilities could lead to new discoveries concerning antigen processing and presentation in B cells.

Chapter 8

2700018N07 Bioinformatics and characterization

Introduction

In order to begin investigating the functional role of an uncharacterized gene, it is helpful to have some clues as to its function. We have shown that CLASSIFI can be used to link biological processes to experimental conditions. In the case of Gene Cluster #18, which contains genes annotated with "transporter" functions, experimental validation was provided to show that AIG induces endocytosis in B cells. Furthermore, 38 genes out of this gene cluster of 193 genes have been shown to be involved in various aspects of endocytosis in different cell systems. Because of the classification of Gene Cluster 18 as "monovalent inorganic cation transport", we explored the possibility that uncharacterized genes in Gene Cluster #18 may have roles related to endocytic trafficking. We used bioinformatics tools to help generate hypotheses as to the functions of uncharacterized genes. In Gene Cluster #18, there are 10 probes derived from ESTs that could encode hypothetical proteins (Table 8.1). These are putative genes for which there are no known functions, but because they co-cluster with genes involved in endocytic trafficking, we hypothesize that these hypothetical proteins might also function in this biological process. The presence of exons and mapping to one chromosome site would suggest that these ESTs are genuine genes (Table 8.1). The intron/exon structure of 2700018N07 is well defined, supporting the idea that this is a real gene (Figure 8.1).

Probe ID	Gene name	Cluster ID	Intron/exon structure	Chromosome location
3110013H01	hypothetical protein	18	Yes	11
2700018N07	hypothetical protein	18	Yes	9
1700019D15	hypothetical protein	18	Yes	7
3830404O05	hypothetical protein	18	Yes	6
3300002A11	hypothetical protein	18	Yes	12
5730469M10	hypothetical protein	18	Yes	14
2700038E08	hypothetical protein	18	Yes	19
2610510H03	hypothetical protein	18	Yes	2, 3?
5730442P18	hypothetical protein	18	Yes	11
2600017H02	hypothetical protein	18	Yes	11

Table 8.1. List of "hypothetical protein" genes in Gene Cluster #18. A defined intron/exon structure and localization to one site on one chromosome would support the idea that these "hypothetical proteins" are genuine genes. One of these, 2610510H03, BLASTed to more than one chromosome.

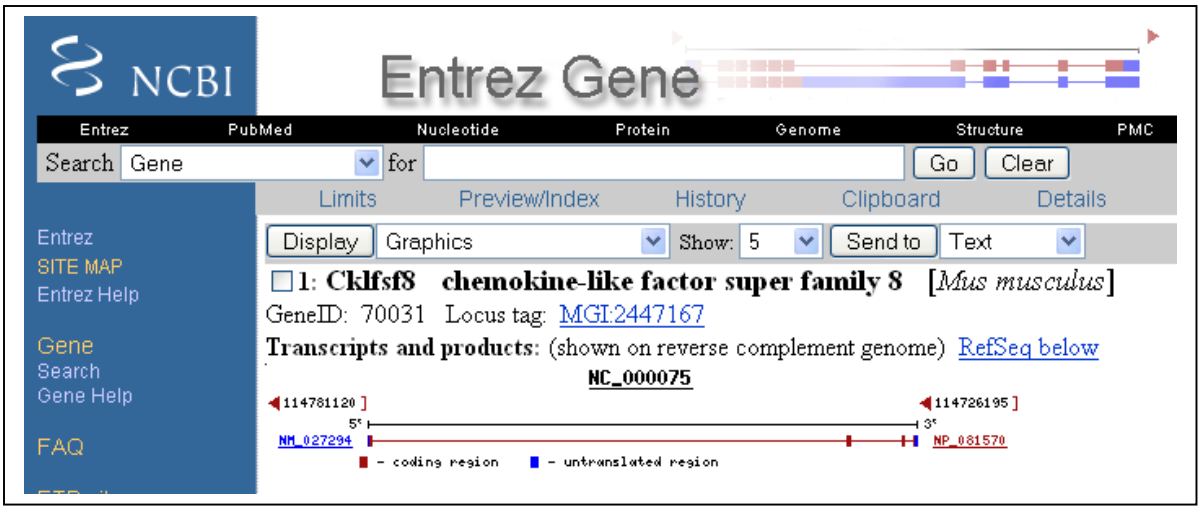


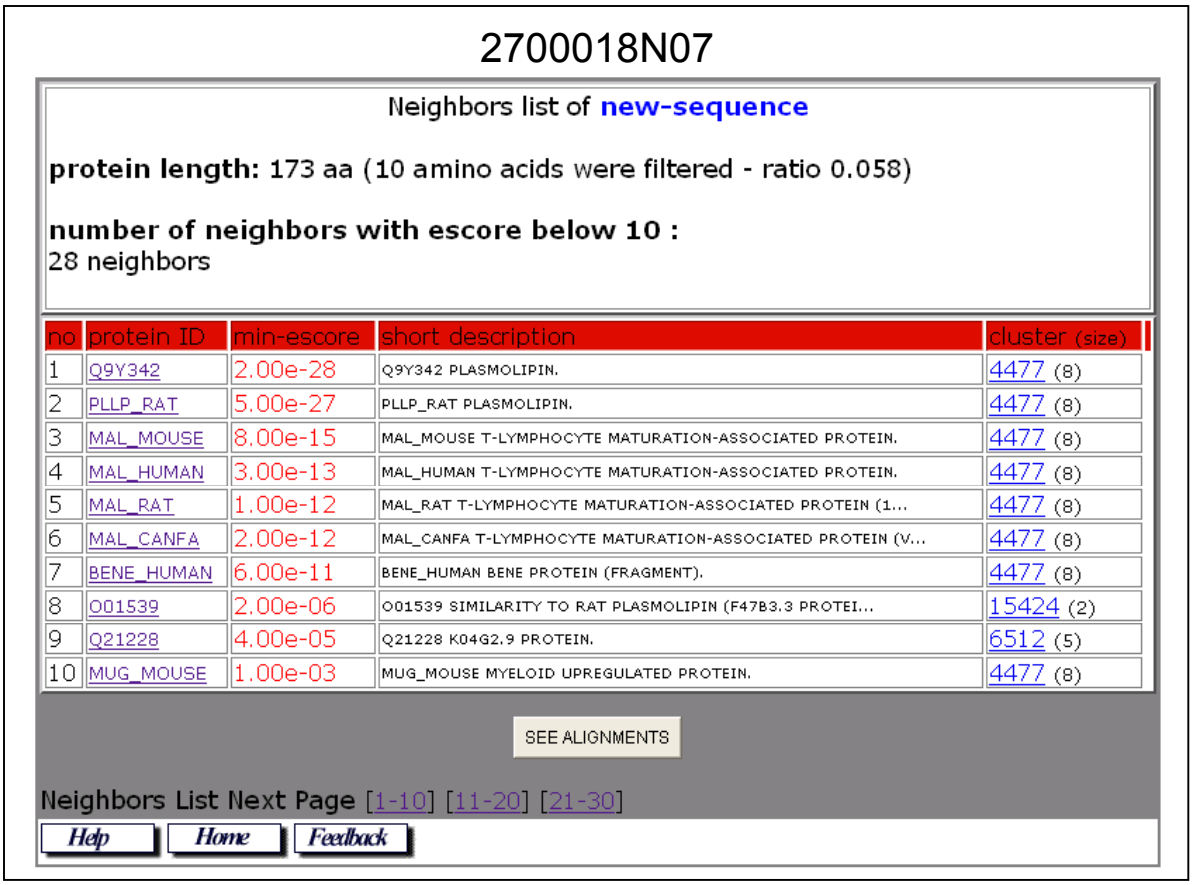
Figure 8.1. Intron/Exon structure of 2700018N07. Exon coding regions are shown in maroon boxes. Introns are shown as maroon lines. Related proteins MAL and plasmolipin also share a similar 4 exon structure.

Results and Discussion

Identification of proteins with possible endocytic functions. If these "hypothetical protein" sequences are related to known genes, that would provide clues to their putative function. We used a bioinformatics tool called ProtoMap (http://protomap.cornell.edu/index.html), which classifies all proteins in the SWISSPROT and TrEMBL databases into related groups based on whole protein sequences rather than individual domains. ProtoMap defines a protein space based on pairwise similarity between proteins and uses transitivity to identify homologous proteins because of the transitive nature of homology (if A is homologous to B and B is homologous to C, then A is homologous to C). Similarity is measured using common algorithms for protein sequence comparison (SW, FASTA, BLAST). Protein cluster classification begins at high resolution, using only connections of very high statistical significance. These clusters are then merged to form bigger and more diverse clusters in a hierarchical fashion. This results in a protein space made up of a hierarchical organization of all known proteins. Query protein sequences can be mapped to this protein space following pairwise comparisons of the query sequences and the database sequences. Thus, ProtoMap establishes putative protein classifications of a query sequence based on relatedness to known sequences in the database. Protomap analysis of the putative protein sequences (open reading frames come from the Riken database) of all ten "hypothetical protein" genes in Gene Cluster #18 was performed. The predicted protein sequence of one probe, 2700018N07, returned highly significant e scores in the e-3 to e-28 range. 2700018N07 showed high similarity with all members of the same ProtoMap protein

cluster - #4477 (Figure 8.2). Human, rat, and mouse orthologs of one protein, MAL, are present in protein cluster #4477, indicating that MAL is highly conserved between species. BENE and plasmolipin are other proteins present in this cluster.

MAL (myelin and lymphocyte protein), plasmolipin, and BENE are all members of a family of transmembrane proteins believed to be involved in endocytic trafficking. (Perez et al. 1997). MAL and related proteins contain a conserved amino acid motif-[Q/Y-G-W-V-M-F/Y-V]-that serves as a fingerprint for these proteins despite relatively low sequence identity. (Magyar et al. 1997). MAL was first cloned as a transmembrane protein expressed in intermediate-late stages of T cell differentiation (Alonso and Weissman 1987). The MAL gene is composed of 4 exons, and is predicted to be a tetraspanning membrane protein. Hydrophobicity plots reveal that MAL is very similar to bovine vacuolar H+ ATPase. Furthermore, related sequence motifs are found in MAL and *E. coli* F-ATPase as well as yeast V-ATPase (Rancano et al. 1994). MAL is lipid-raft associated and traffics between the trans-Golgi and the plasma membrane (Cheong et al. 1999; Puertollano and Alonso 1999). The plasmolipin gene also has a 4 exon structure and the protein is predicted to have 4 transmembrane regions. Protein expression is restricted to mammals and detected in several different tissues including brain, spinal cord, intestine, kidney, heart, skeletal muscle, ovary, testis, lung, thyroid, liver, and thymus. However, no plasmolipin was detected in spleen (Hamacher et al. 2001; Sapirstein et al. 1991). Plasmolipin is enriched in clathrin-coated vesicles in the brain (Sapirstein et al. 1992) and is associated with lipid rafts in rat nerve cell myelin (Hasse et al. 2002). BENE is also found to be present in lipid rafts and co-precipitates with caveolin-1. Immunofluorescence analysis revelas that BENE accumulates



2700018N07

Neighbors list of **new-sequence**

protein length: 173 aa (10 amino acids were filtered - ratio 0.058)

number of neighbors with escore below 10 :
28 neighbors

no	protein ID	min-escore	short description	cluster (size)
1	Q9Y342	2.00e-28	Q9Y342 PLASMOLIPIN.	4477 (8)
2	PLLP_RAT	5.00e-27	PLLP_RAT PLASMOLIPIN.	4477 (8)
3	MAL_MOUSE	8.00e-15	MAL_MOUSE T-LYMPHOCYTE MATURATION-ASSOCIATED PROTEIN.	4477 (8)
4	MAL_HUMAN	3.00e-13	MAL_HUMAN T-LYMPHOCYTE MATURATION-ASSOCIATED PROTEIN.	4477 (8)
5	MAL_RAT	1.00e-12	MAL_RAT T-LYMPHOCYTE MATURATION-ASSOCIATED PROTEIN (1...	4477 (8)
6	MAL_CANFA	2.00e-12	MAL_CANFA T-LYMPHOCYTE MATURATION-ASSOCIATED PROTEIN (V...	4477 (8)
7	BENE_HUMAN	6.00e-11	BENE_HUMAN BENE PROTEIN (FRAGMENT).	4477 (8)
8	O01539	2.00e-06	O01539 SIMILARITY TO RAT PLASMOLIPIN (F47B3.3 PROTEI...	15424 (2)
9	Q21228	4.00e-05	Q21228 K04G2.9 PROTEIN.	6512 (5)
10	MUG_MOUSE	1.00e-03	MUG_MOUSE MYELOID UPREGULATED PROTEIN.	4477 (8)

SEE ALIGNMENTS

Neighbors List Next Page [1-10] [11-20] [21-30]

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Figure 8.2. ProtoMap analysis of "hypothetical protein" 2700018N07. ProtoMap was used to query the 2700018N07 protein sequence and results in a list of related proteins. e scores lower than e-10 are highly significant. 8 proteins from this list were found to be members of the same protein "cluster" #4477, suggesting relatedness.

in vesicular or tubular structures (de Marco et al. 2001). Caveolin-1, MAL, and BENE have been found to co-fractionate, and they colocalize in multivesicular intracellular compartments in a prostate cancer cell line (Llorente et al. 2004). Interestingly, multivesicular compartments are also found to contain Class II antigen processing and presentation components in B cells (see Chapter 1).

In contrast to 2700018N07, ProtoMap analysis of the other "hypothetical protein" genes did not reveal any clues suggesting that the proteins may be involved in endocytosis or any other function. For example, Protomap analysis of the protein sequence of 3110013H01 results in relatively high e scores above 2e-4, and its closest member, "Q22965 cosmid F2584" exists in a singleton cluster, with no other members that might give clues to its function (Figure 8.3). ProtoMap analysis of 5730469M10 results in one protein neighbor giving a low e score of 2e-29 (Figure 8.4). Despite the significant e score, this protein also exists in a singleton cluster, with no other members to give clues as to its function. Based on these results, we have chosen to focus our current analysis on 2700018N07.

Bioinformatics analysis of 2700018N07. An amino acid sequence alignment of all proteins in cluster #4477 was generated using the MultAlin tool (http://prodes.toulouse.inra.fr/multalin/multalin.html) (Figure 8.5). The conserved fingerprint shown to be found in other MAL family members between residues 70-75 is also present in 2700018N07. Several other regions also appear to be conserved between these proteins, located at residues 28, 48, 50, 58-60, 104, 111, 124, 147, 151, and 159. Alignment of 2700018N07 with genomic DNA to determine intron/exon structure reveals that it

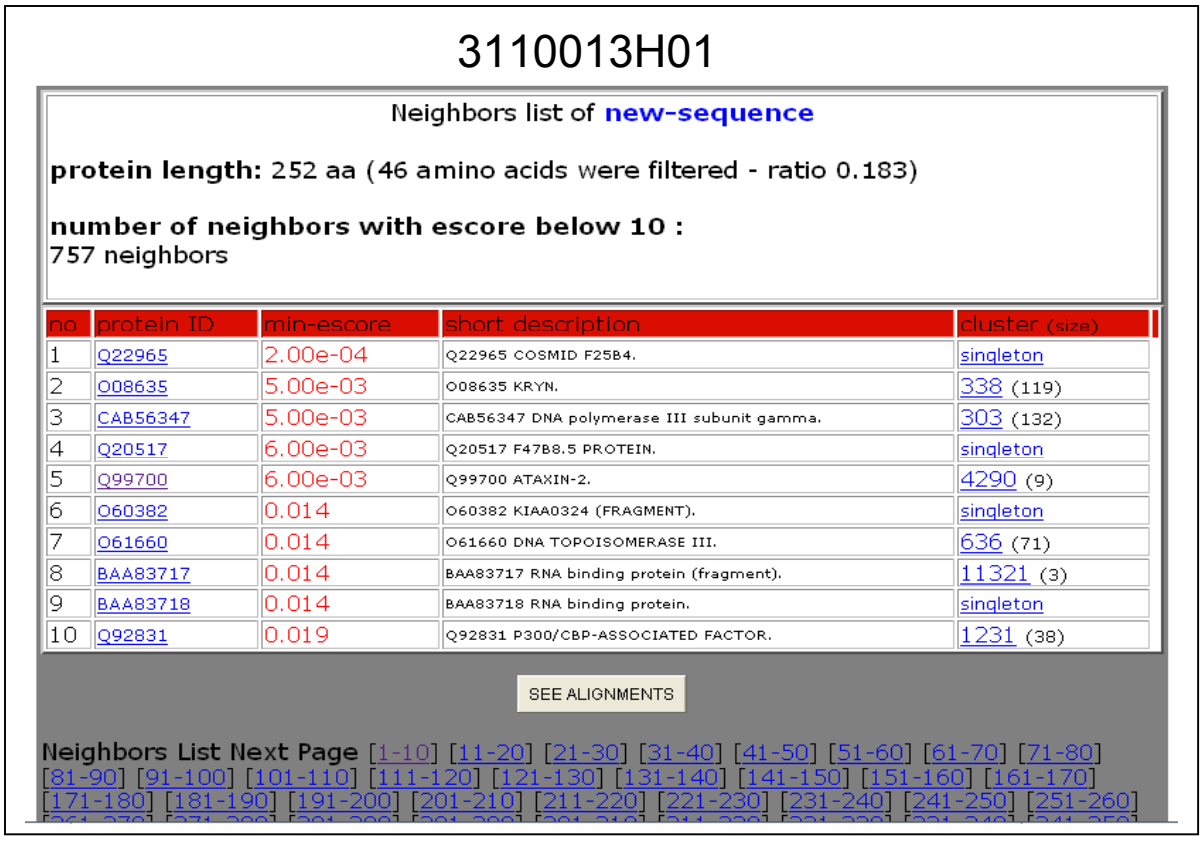

Figure 8.3. ProtoMap analysis of "hypothetical protein" 3110013H01.

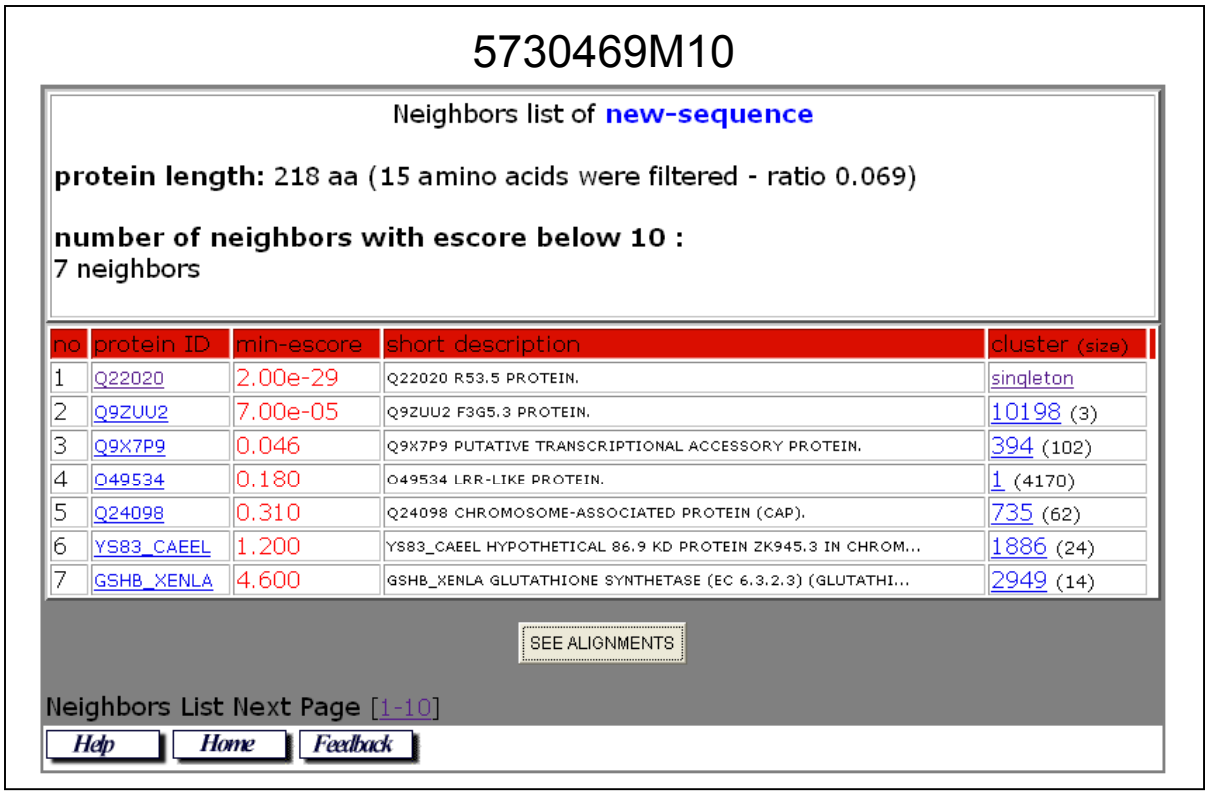

Figure 8.4. ProtoMap analysis of "hypothetical protein" 5730469M10.

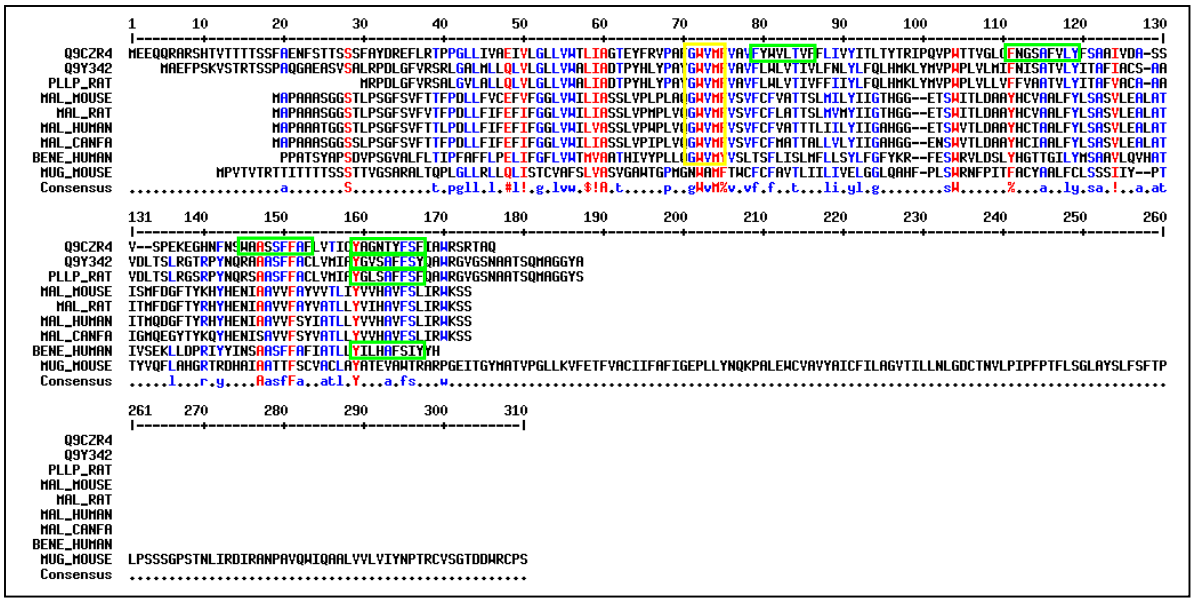

Figure 8.5. Protein sequence alignment of Protein Cluster #4477 members. QY9CZR4 is 2700018N07, and Q9Y342 is human plasmolipin. Areas of sequence homology are shown in red and blue. Caveolin-binding domains are highlighted in green. The MAL family conserved amino acid motif is highlighted in yellow.

contains 4 exons, with one long first intron (Figure 8.5). TMHMM, a tool for prediction of transmembrane helices (http://www.cbs.dtu.dk/services/TMHMM-2.0/), predicts 4 transmembrane regions in 2700018M07, human plasmolipin, and mouse MAL (Figure 8.6, 8.7, and 8.8).

2700018N07 is most similar in protein sequence to plasmolipin, a tetraspanning transmembrane proteolipid whose function is also largely unknown. Interestingly, proteolipid proteins have been found to be associated with plasma membrane H+ ATPases in yeast. The related human protein BENE has been shown to interact with caveolin-1 by co-immunoprecipitation experiments (de Marco et al. 2001) Caveolin is a structural protein involved in invagination at the plasma membrane during vesicle formation. Caveolin has been shown to interact with Btk and Bmx, non-receptor cytoplasmic tyrosine kinases involved in signaling pathways from the BCR (Vargas et al. 2002). A caveolin binding domain has been elucidated using peptide library screening: ΦXΦXXXXΦXXΦ, ΦXXXXΦXXΦ, or ΦXΦXXXXΦ where Φ is either a W (Trp), F (Phe), or Y (Tyr) residue (Couet et al. 1997). Scanning of the 2700018N07 amino acid sequence reveals four such domains that fit the caveolin binding domain rules. The last of these domains is conserved among several members of protein cluster #4477 (Figure 8.4). Furthermore, a search for conserved protein domains reveals that 2700018N07 contains a MARVEL membrane-associating domain, which is also found in other MAL family members (Figure 8.9). These bioinformatics analyses and literature searches have revealed convincing clues that 2700018N07 is a member of the MAL protein family and is involved in intracellular

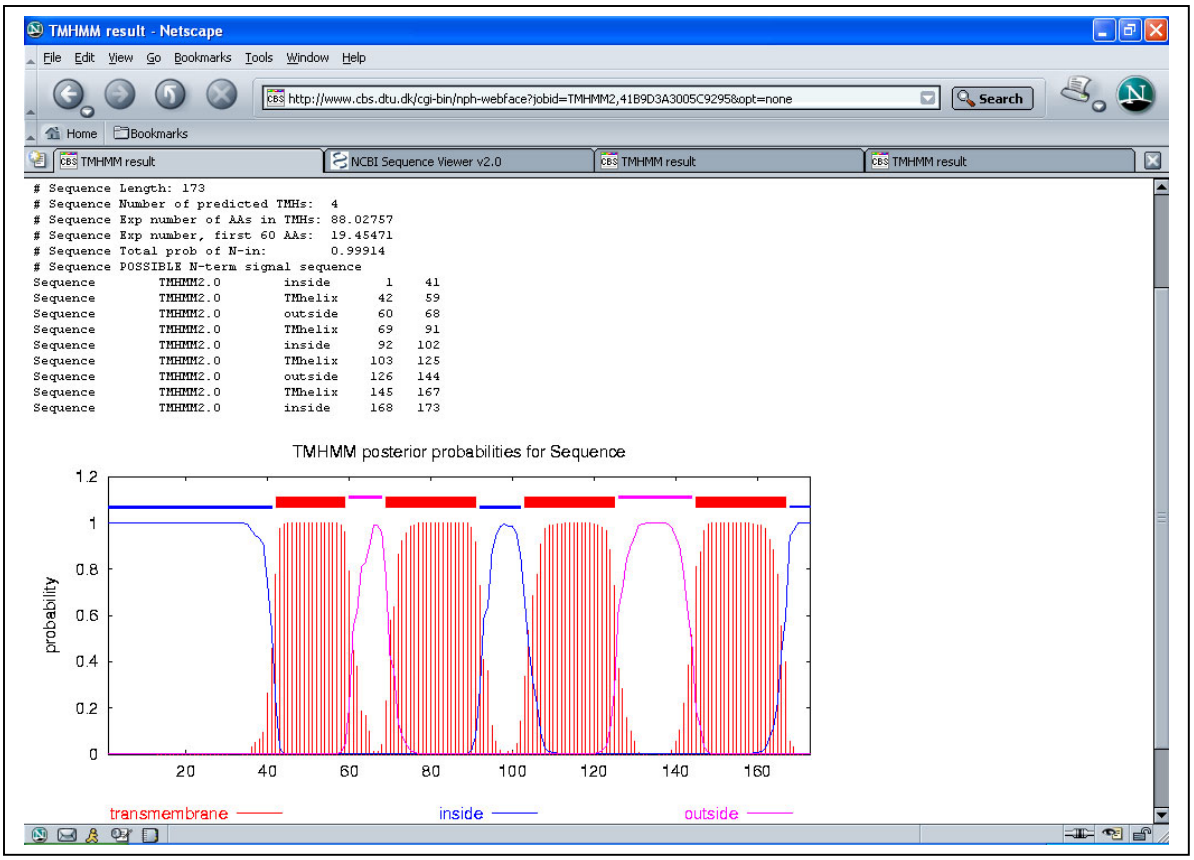

Figure 8.6. 2700018N07 predicted transmembrane regions. Protein residues with high probability of being membrane associated are shown in red.

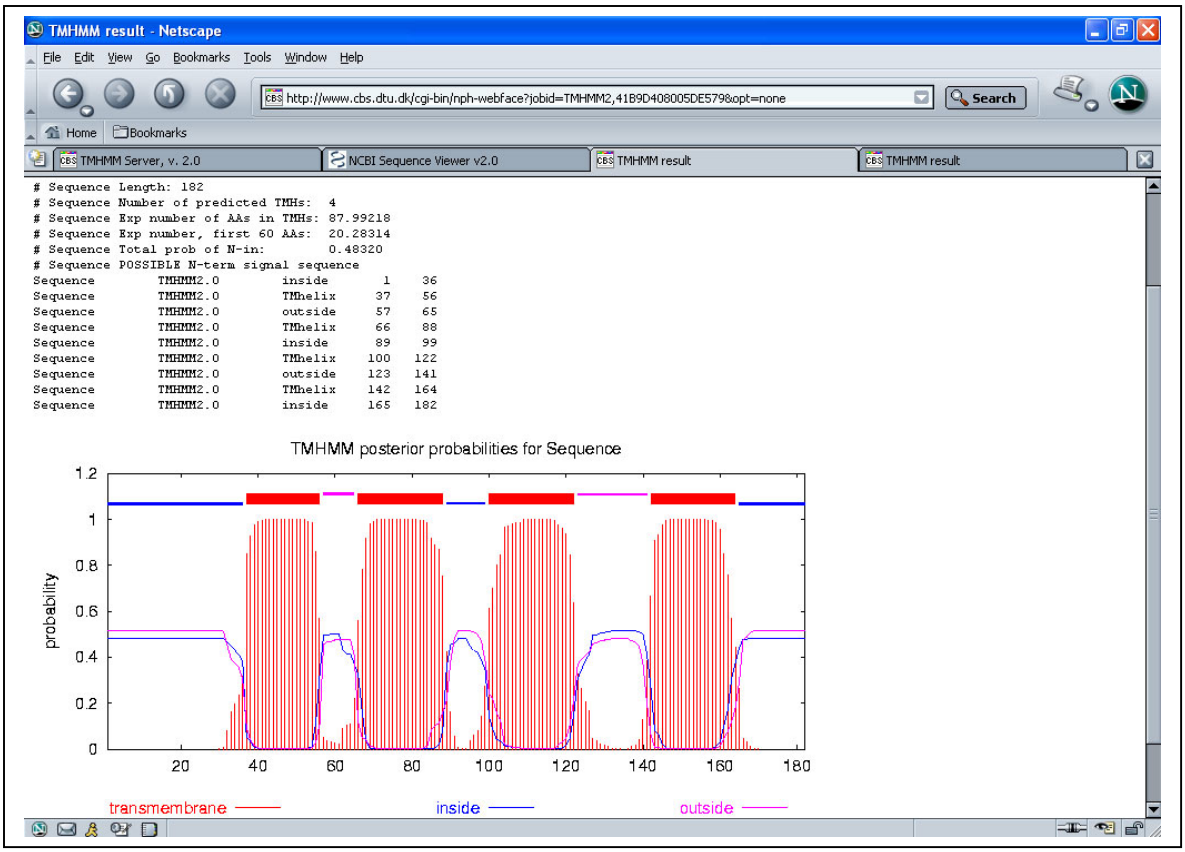

Figure 8.7. Human plasmolipin predicted transmembrane regions.

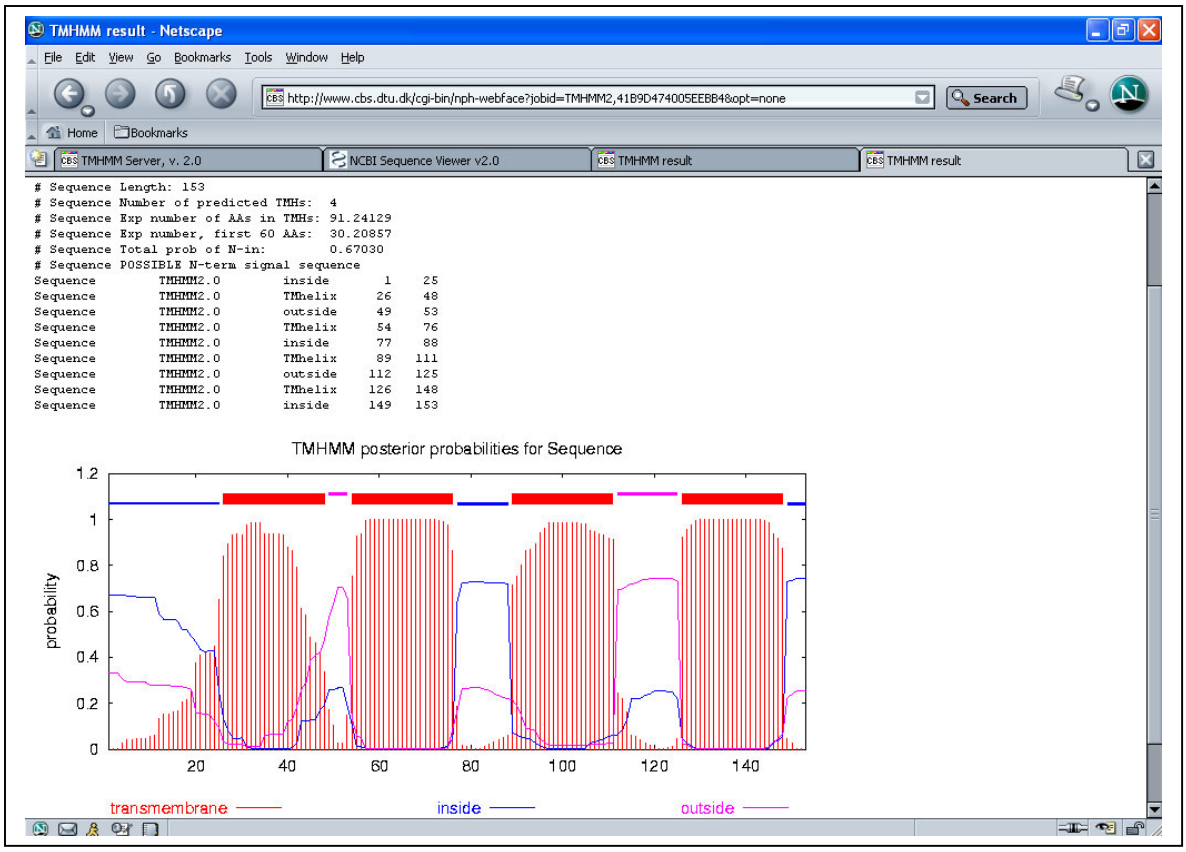

Figure 8.8. Mouse MAL predicted transmembrane regions.

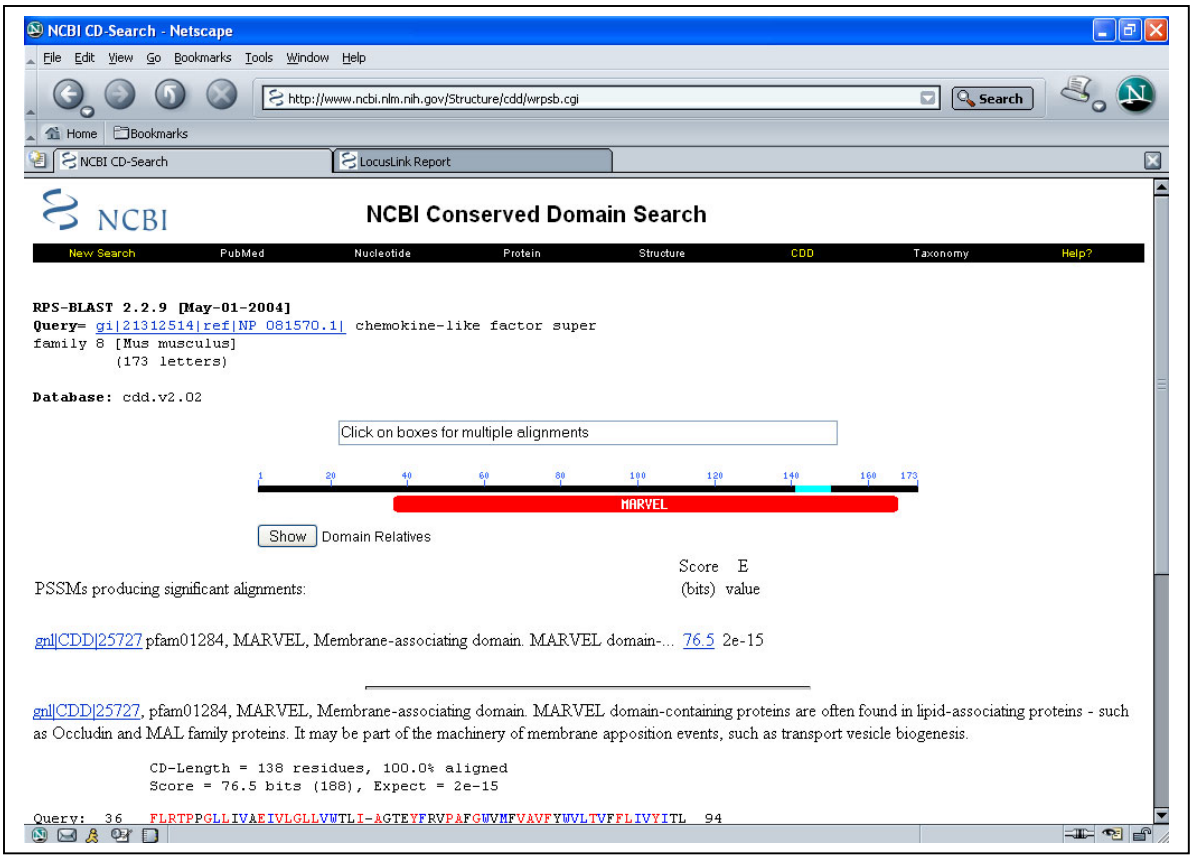

Figure 8.9. MARVEL membrane-associating domain found in 2700018N07.

trafficking in some way, possibly through an interaction with caveolin and/or proton pumps. A summary of the predicted domains in 2700018N07 is shown in (Figure 8.10).

To begin characterization of 2700018N07, ligand-specific expression patterns from the microarray experiment were first confirmed by real-time RT-PCR. 2700018N07 is upregulated in response to AIG stimulation at early (4 hr, 6 hr) timepoints, confirming the microarray expression data. This upregulation is lost by late (22 hr) timepoints (Figure 8.11). Real-time RT-PCR analysis of RNA extracted from 14 different mouse tissues/organs shows that 2700018N07 is ubiquitously expressed, with highest expression in the liver and lung. Significant expression is also seen in lymphoid organs (Figure 8.12). Further studies will be performed to determine whether 2700018N07 protein interacts with caveolin and what roles it may play in intracellular trafficking.

Summary. We have investigated the possibility that putative genes encoding "hypothetical proteins" might be related to intracellular trafficking because they co-cluster in Gene Cluster #18. Bioinformatics tools for data mining were used to extract information about one of these "hypothetical proteins" with the Riken probe ID 2700018N07. 2700018N07 was found to be remarkably similar to the MAL family of proteins which are thought to be involved in endocytic trafficking. 2700018N07 shares a 4 exon structure, 4 putative transmembrane domains, and a conserved amino acid motif with other MAL family members. In addition, putative caveolin binding domains have been found in 2700018N07. This caveolin binding domain is also found in BENE, which has been shown to interact with caveolin. Furthermore, non-receptor cytoplasmic tyrosine kinases Btk and Bmx also contain

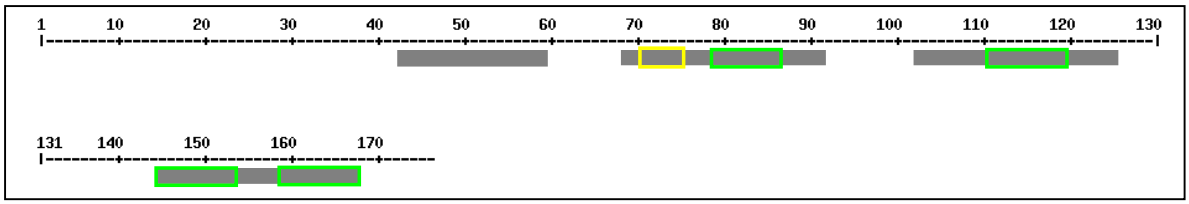

Figure 8.10. Summary of domains found in 2700018N07. Yellow box-MAL family conserved motif, Green boxes-putative caveolin-binding domains. Gray boxes-predicted transmembrane regions. All of the predicted transmembrane regions make up the MARVEL membrane associating domain.

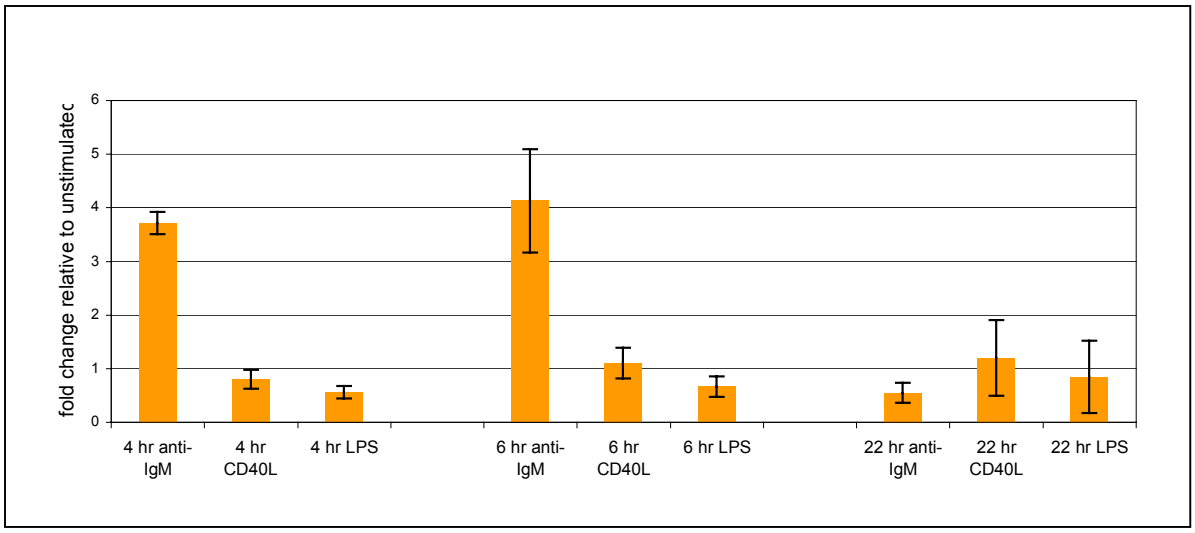

Figure 8.11. 2700018N07 expression in primary B cells. Real-time RT-PCR was used to evaluate 2700018N07 expression in primary B cells stimulated with AIG, anti-CD40 (CD40L), and LPS. Data were normalized to 18S rRNA as a reference and then compared to timepoint-matched untreated controls. Error bars indicate SD. n=3.

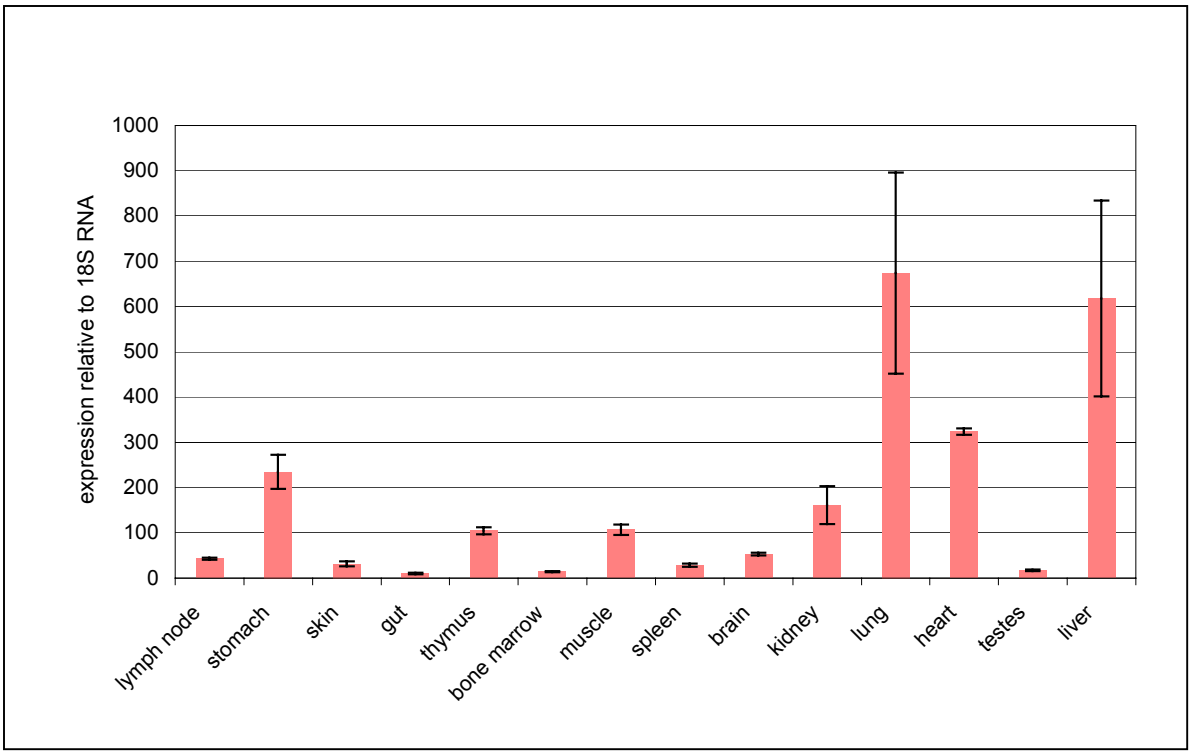

Figure 8.12. 2700018N07 expression in mouse tissues. Real-time RT-PCR was used to evaluate expression levels of 2700018N07 relative to 18S RNA expression in mouse tissues. Error bars indicate SD. n=2. Standard curves were generated using total spleen cDNA. Standards were amplified with 18S and 2700018N07 primer pairs and assigned relative values corresponding to their dilution factors. cDNA from the indicated tissues were used in a PCR reaction with 18S and 2700018N07 primer pairs. Relative expression values for these samples were then determined by extrapolation from the standard curves.

a caveolin-binding domain and have been shown to interact with caveolin. Because of the similarity of 2700018N07 to other transmembrane proteins proposed to be involved in endocytic trafficking and the presence of caveolin-binding domains that have also been found in proteins involved in BCR signaling, we hypothesize that 2700018N07 plays a role in endocytic trafficking induced by BCR signaling.

Chapter 9

Discussion

CLASSIFI Analysis

CLASSIFI, a statistical approach that uses GO annotation to identify significant co-clustering of related genes, is a useful tool to append biological information to microarray experimental results. It is robust to experimental and biological noise, a limitation inherent in microarray expression analysis. CLASSIFI distinguishes itself from other similar microarray analysis tools by its ease of use. Any investigator who wishes to apply CLASSIFI can do so by visiting the CLASSIFI website, where step-by-step instructions and analysis tips are easily found. Application of CLASSIFI to two different microarray data sets-the BAFF/CD40L dataset and the B cell single ligand screen dataset has provided results that facilitate hypothesis generation. Experimental validation of these hypotheses demonstrates that CLASSIFI is capable of identifying real biological patterns from microarray data sets.

The analysis of the B cell single ligand screen dataset is unique in that it takes a systems biology approach to advancing knowledge about a specific cell type. The goal of the AfCS is that publicly sharing the results from these systems biology experiments will generate interesting data that can be applied by investigators working in a specific field of study. Furthermore, a systems biology approach allows discovery of findings that lie outside the confines of the study (for example discovery of a new unpredicted signaling pathway). A combination of systems biology approaches and traditional scientific methods would facilitate discovery and accelerate the advance of knowledge. The B cell single ligand screen analysis began with an unbiased microarray analysis of B cells stimulated with a panel of 32

ligands. From this, focus was placed on interesting ligands and ultimately focused on a biological process, antigen processing and presentation, that occurs in AIG-treated B cells. Importantly, this analysis provided a list of genes that may play roles in antigen processing and presentation; further analysis of these genes may advance the field of B cell receptor-mediated antigen processing and presentation.

One limitation to CLASSIFI is that it depends on the accuracy of the GO database. Critical assessment of the data in the literature is the required next step to ensure the accuracy of gene annotation and to justify continued study of interesting genes in the context of the hypotheses generated from CLASSIFI results.

Identification of putative components of BCR-mediated antigen capture, processing, and presentation

Based on the genes responsible for Gene Cluster #18 classification (Table 6.3), we hypothesized that CLASSIFI results reflect BCR mediated endocytosis, vesicle acidification, vesicle trafficking, and antigen processing and presentation. We manually curated the AIG-specific cluster gene list and found a total of 38 genes, including those identified using CLASSIFI, that might be involved based on the molecular functions defined in other cell systems and biological processes from the literature (Table 9.1). Figure 9.1 shows a schematic representation of the B cell antigen processing and presentation pathway indicating the putative roles played by these genes.

	Protein description	Gene Symbol	LocusLinkID	Function
A	CD72	Cd72	12517	BCR coreceptor
	dual specificity phosphatase 14	Dusp14	56405	lymphocyte signaling
	mitogen activated protein kinase kinase kinase kinase 1	Map4k1	26411	lymphocyte signaling
	mitogen activated protein kinase kinase 3	Map2k3	26397	lymphocyte signaling
	serine/threonine kinase receptor associated protein	Strap	20901	TGFbeta signaling
	dishevelled associated activator of morphogenesis 1	Daam1	208846	Wnt signaling
	FK506 binding protein 1a	Fkbp1a	14225	BCR signaling/apoptosis
	nuclear factor of activated T-cells, cytoplasmic 2 interacting protein	Nfatc2ip	18020	BCR signaling/apoptosis
	sorting nexin 5	Snx5	79178	endocytosis
	IQ motif containing GTPase activating protein 1	Iqgap1	29875	vesicle formation
	amyotrophic lateral sclerosis 2 (juvenile) homolog (human)	Als2	74018	endosome fusion/Rab GEF
	RAB18, RAS family	Rab18	19330	endocytosis/secretory vesicle recycling
	RAB22, RAS family	Rab22a	19334	early endosome trafficking
	Proton pump, subunit V0B (21 kDa)	Atp6v0b	114143	vesicle acidification
	Proton pump, subunit V1G1 (13 kDa)	Atp6v1g1	66290	vesicle acidification
	Proton pump, subunit V1C1 (42 kDa)	Atp6v1c1	66335	vesicle acidification
	similar to Proton pump, subunit V1F		76610	vesicle acidification
	Proton pump accessory protein 2	Atp6ap2	70495	vesicle acidification
	Chloride channel 7	Clcn7	26373	vesicle pH regulation
	dystrobrevin binding protein 1 (dysbindin)	Dtnbp1	94245	lysosome formation
	cystatin B	Cstb	13014	cathepsin protease inhibitor
	hexosaminidase A	Hexa	15211	hydrolase
	ceroid lipofuscinosis, neuronal 3, juvenile (Batten, Spielmeyer-V…	Cln3	12752	lysosome function
	HSP70 binding protein	Hspbp1	66245	Ag processing/loading
	similar to HSPC135		66067	chaperone (?), GTPase (?)
	HSPC245 homolog			chaperone (?)
	N-ethylmaleimide sensitive fusion protein attachment protein alpha	Napa	108124	vesicle fusion
	RAB9, RAS family	Rab9	56382	vesicle tethering
	activating transcription factor 1	Atf1	11908	MHC expression
	Sec61 alpha	Sec61a1	53421	ER translocation
	signal sequence receptor, alpha	Ssr1	107513	MHCII chaperone
	calmegin	Clgn	12745	MHC chaperone
	similar to signal peptidase SPC22/23		76687	secretory protein processing
	vacuolar protein sorting 29	Vps29	56433	retrograde vesicle transport
	reticulon 3	Rtn3	20168	exocytosis
	tubulin, alpha 6	Tuba6	22146	cytoskeletal trafficking
	CD63	Cd63	12512	MHCII coreceptor
	source of immunodominant MHC-associated peptides	Simp	68292	MHC peptide
B	24-dehydrocholesterol reductase	Dhcr24	74754	Cholesterol biosynthesis
	fatty acid binding protein 5, epidermal	Fabp5	16592	Fatty acid intracellular transport
	phosphatidylethanolamine binding protein	Pbp	23980	Membrane lipid transport
	prostaglandin D2 synthase 2, hematopoietic	Ptgds2	54486	
	peroxisome biogenesis factor 1	Pex1	71382	Peroxisomal protein tranport
C	beclin 1 (coiled-coil, myosin-like BCL2-interacting protein)	Becn1	56208	Tumor suppression
	caspase 4, apoptosis-related cysteine protease	Casp4	12363	Apoptosis
	Cd27 binding protein (Hindu God of destruction)	Siva	30954	Apoptosis
D	polymyositis/scleroderma autoantigen 2	Pmscl2	50912	RNA processing autoantigen
	Sjogren's syndrome/scleroderma autoantigen 1 homolog (human)	Sssca1	56390	centromeric autoantigen

Table 9.1. Manual curation of Gene Cluster #18 genes

Genes found in Gene Cluster #18 with functions related to (A) intracellular trafficking, BCR signaling, vesicle processing and function, and antigen processing and presentation, (B) Genes annotated with "transporter" activity whose roles are not yet known to be associated with intracellular trafficking, (C) apoptosis, and (D) B cell-related autoimmune disease.

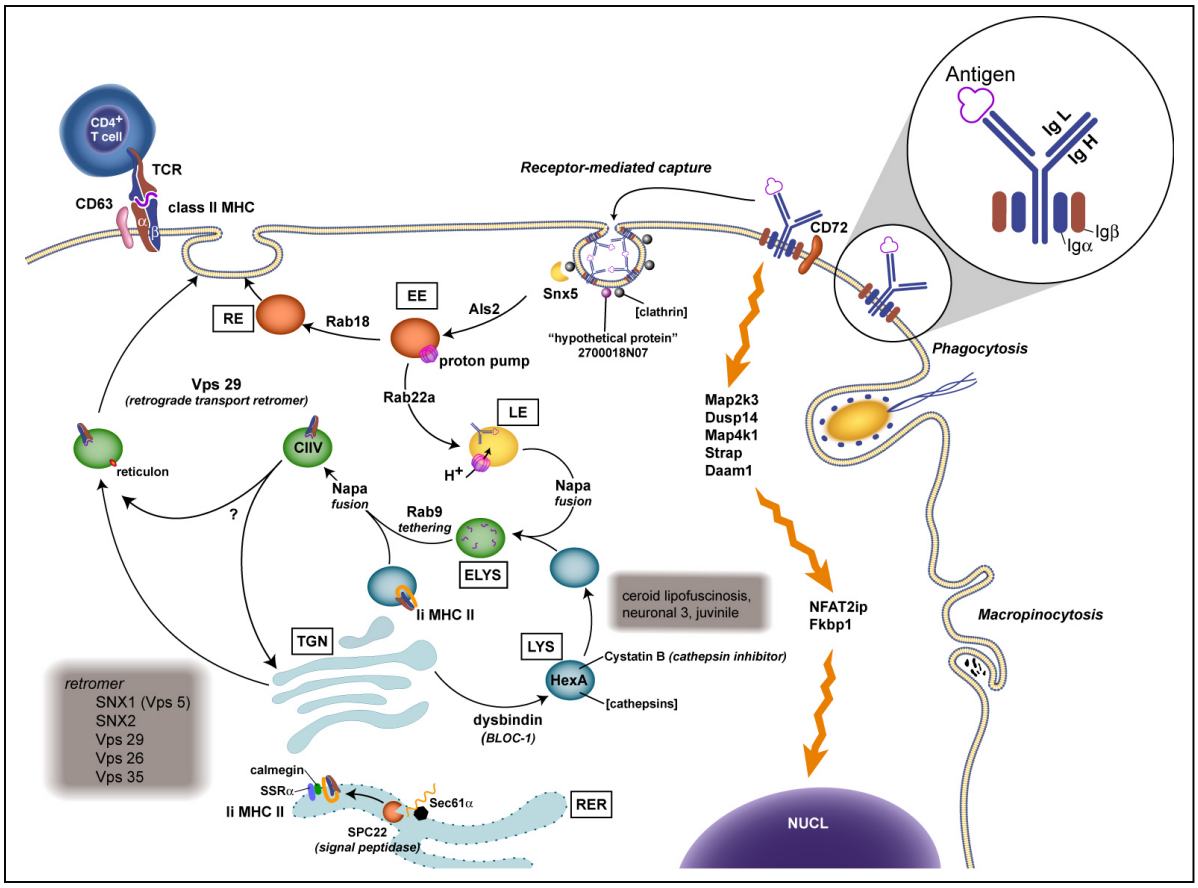

Figure 9.1. Gene Cluster #18 contains genes involved in several aspects of vesicle processing, intracellular trafficking, and antigen processing and presentation. A manual curation of the gene list comprising Gene Cluster #18 resulted in this detailed model of intracellular transport processes in B cells (refer to text for details). EE = early endosome, LE = late endosome, RE = recycling endosome, ELYS = endolysosome, LYS = lysosome, RER = endoplasmic reticulum, TGN = trans-Golgi network, NUCL = nucleus. The putatice location of "hypothetical protein" 2700018N07 at the receptor-mediated antigen entry step is shown.

Several genes known to be involved in signal transduction were found in Gene Cluster #18; some have been demonstrated to play a role in BCR signaling while others are involved in signaling pathways in other cell types and are candidates for new BCR signaling components that stimulate the endocytic process (Table 9.1A). Activation of protein kinases, including members of the MAP kinase family, in response to BCR engagement is well documented; thus Strap, Map4k1 (Hpk1) and Map2k3 may also play a role in BCR signaling. Indeed, Strap is involved in TGF-β signaling and and is upregulated during dendritic cell activation (Chen et al. 2002b; Datta et al. 1998), Map4k1 is expressed in hematopoietic cells and is required for activation-induced T cell death following TCR stimulation (Hu et al. 1996a; Hu et al. 1996b; Schulze-Luehrmann et al. 2002), and Map2k3 is required for IL-12 production by macrophages and dendritic cells, and antigen-mediated activation of T cells (Lu et al. 1999; Tanaka et al. 2002). CD72 has been found to regulate the cellular response to BCR signaling either through the recruitment of the SHP-1 phosphatase thereby dampening BCR signaling, or by cooperating with BCR signaling to prevent apoptosis in immature B cells (Adachi et al. 1998; Fujiwara et al. 2004; Hokazono et al. 2003; Wu et al. 2001). The Dusp14 dual specificity phosphatase has been found to associate with the CD28 co-receptor and dampen TCR-mediated signaling in T cells by inactivation of MAP kinases, suggesting that it may regulate BCR signaling in a similar fashion (Marti et al. 2001). Daam1 is involved in Wnt signaling in Xenopus embryogenesis (Habas et al. 2001). Whether these proteins might play a role in regulating BCR signaling remains to be determined. The NF-ATc2 isoform has been found to participate in the BCR-induced apoptosis in B-cell-derived Burkitt's lymphoma cell lines , thus the presence of Nfatc2ip and

Fkbp1 suggests that these interacting proteins may also be important in regulating Ca^{++}/calmodulin/calcineurin/NFAT signaling in B cells (Healy et al. 1997; Kondo et al. 2003; Peng et al. 2001).

A large proportion of genes in Gene Cluster #18 encode proteins with roles in endocytosis, vesicle trafficking, vesicle acidification, molecular processing and protein secretion. In most cases these roles have been defined in other cell systems. Since little is known about the molecular details of these processes in receptor-mediated antigen capture and MHC class II-dependent antigen processing and presentation in B cells, the possibility that Gene Cluster #18 has produced a putative list of proteins that coordinate these activities is intriguing.

Snx5, a member of the sorting nexin family, has been found to interact with clathrin in skeletal muscle (Teasdale et al. 2001; Towler et al. 2004). Clathrin has been implicated in facilitating membrane invagination during receptor-mediated endocytosis in B cells (Stoddart et al. 2002). The presence of Snx5 in this cluster suggests that it may help mediate the initial stage of antigen capture by the BCR. The Als2 protein acts as a guanine nucleotide exchange factor for Rab5 and Rac1, and stimulates early endosome fusion (Kunita et al. 2004; Topp et al. 2004). Rab proteins are small GTPases with homology to Ras that play key roles in vesicle transport: Rab18 has been found to localize near the apical and basolateral plasma membrane in epithelial cells of the kidney and intestine (Lutcke et al. 1994; Schafer et al. 2000; Segev 2001; Yu et al. 1993) and Rab22a has been found to localize to the plasma membrane and early endosomes (Seabra et al. 2002; Segev 2001). Iqgap1 is a scaffolding protein with multiple protein-protein interaction domains and a GTPase activation protein

(Gap) domain that stimulates the hydrolytic activity of Ras family members. Iqgap1 has also been found to localize to areas of membrane ruffling and newly formed vesicles in astrocytoma cells (Mbele et al. 2002; Roy et al. 2004).

Genes encoding homologs to several components of the vesicle-type proton pump were found in Gene Cluster #18, including Atp6v0b, Atp6v1g1, Atp6v1c1, and Atp6v1f and the proton pump accessory protein Atp6ap2. Additional proton pump components were also found to be induced in an AIG-specific manner by RT-PCR (Atp6v0c, Atp6va1, Atp6v1h and Atp6v1a1) (Figure 9.2). Together with the vesicle-specific chloride channel Clcn7, these proteins are likely to play a role in the acidification of the endocytic vesicles in preparation for their fusion with lysosomes to facilitate antigen hydrolysis (Campos-Xavier et al. 2003; Demirci et al. 2001; Kornak et al. 1999; Ludwig et al. 1998; Nishi and Forgac 2002; Smith et al. 2003; Sun-Wada et al. 2003). Proton pump components can serve as a membrane anchor point for SNARE proteins. Napa appears to mediate the tethering of these vesicles in preparation for fusion (Chae et al. 2004; Lemons et al. 1997). Rab9 also appears to play a role in vesicle fusion since mutations in Rab9 lead to the accumulation of lipid-rich vesicles (Davies et al. 1997; Walter et al. 2003).

Several proteins involved in lysosome formation and antigen processing were found in Gene Cluster #18. Dysbindin binds to dystobrevins and is a component of the biogenesis of lysosome-related organelles complex 1 in mouse liver, which regulates trafficking to lysosomal organelles (Li et al. 2003). Mutations in the Cln3 gene leads to a lysosomal storage disorder associated with the accumulation of lipopigment-laden vesicles

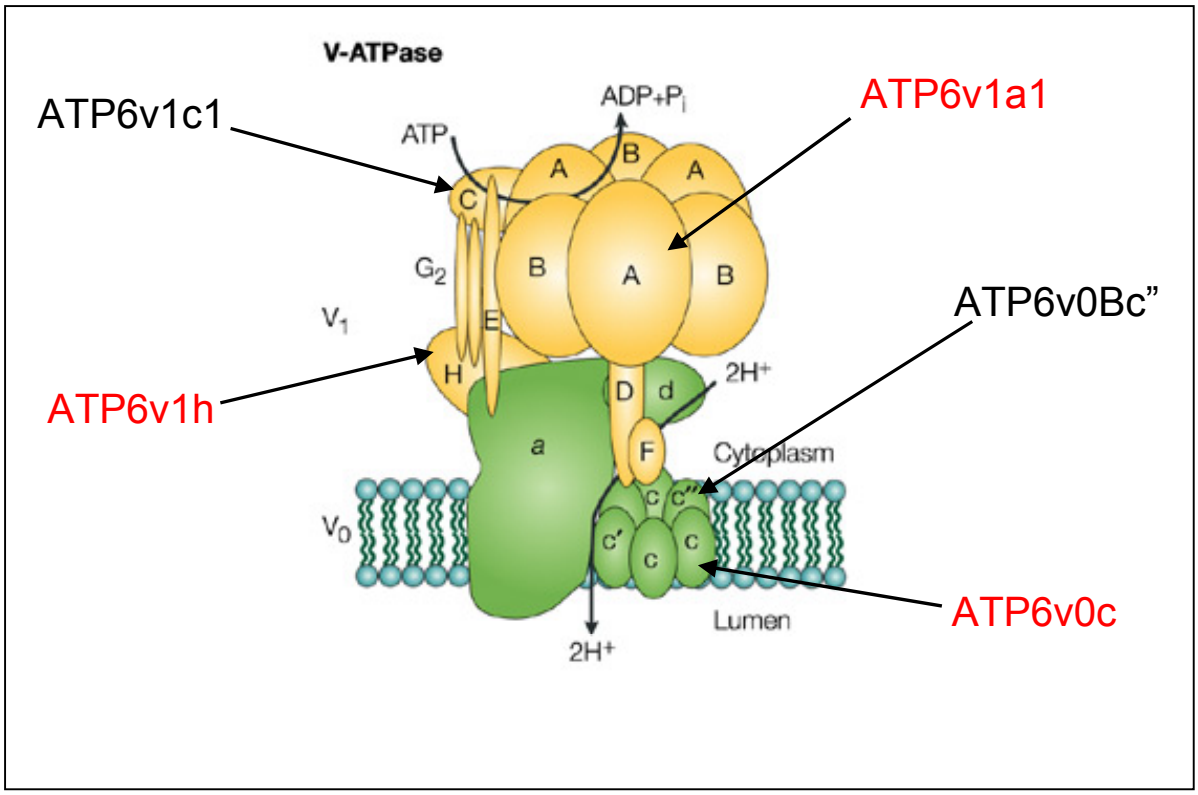

Figure 9.2. Components of the ATPase proton pump. The ATPase proton pump is made up of several components that were found in the AIG-specific Gene Cluster #18. The indicated components (arrows) were found to be coordinately expressed (See Chapter 6). Subunits labeled in red denote proton pump components that were not included in the microarray analysis. The proton pump drawing is from (Nishi et al. 2002).

(Ezaki et al. 2003). Hexosaminidase A is a pH-sensitive hydrolase (Beccari et al. 1992a; Beccari et al. 1992b). A variety of cathepsins are involved in MHC Class II antigen processing and presentation. None of the probes for these genes passed our data filtering process. However the cathepsin inhibitor Cystatin B was found in Gene Cluster #18, suggesting that it might

play a role in regulating cleavage site selection by modifying the relative activities of proteases involved in antigen processing and peptide loading (Pennacchio et al. 1996; Pennacchio and Myers 1996). Hspbp1 may facilitate antigen processing by regulating the chaperone function of Hsp70, which has been associated with the presentation of myelin basic protein though MHC class II in multiple sclerosis (Mycko et al. 2004).

Several genes involved in MHC class II expression were found in the AIG-specific gene cluster. Atf1 is a transcription factor that activates the promoter of the class II *trans*-activator (CIITA) (van der Stoep et al. 2002). Translocation of nascent MHC class II peptide chains into the lumen of the endoplasmic reticulum involves the function of the Sec61 translocation complex, the signal sequence receptor Ssr1 (a.k.a. TRAP alpha) and the signal peptidase SPC22 (Hirama et al. 1999; Romisch 1999). Calmegin (Clgn) is a Ca^{++} binding chaperone protein with significant homology to calreticulin and calnexin, an ER chaperone protein that regulates the assembly of MHC class II with the Ii invariant chain (Romagnoli and Germain 1995; Yamagata et al. 2002)..

Vesicles containing peptide-loaded MHC class II are transported to the plasma membrane for fusion and exposure on the cell surface, perhaps by traveling back through the TGN into the secretory pathway. The Vsp29 protein has been found to facilitate this kind of

retrograde TGN transport in yeast (Edgar and Polak 2000; Murk et al. 2003). In neurons, reticulon 3 is associated with synaptophysin in tubulovesicular structures and may play a role in the process of vesicle secretion (Kumamaru et al. 2004; Oertle et al. 2003). CD63, a tetraspanning membrane MHC class II co-receptor, may play a role in regulating MHC receptor compartmentalization (Duffield et al. 2003; Engering et al. 2003; Vogt et al. 2002). Tetraspan proteins have been found to be associated with Class II peptide loading compartments and are enriched at Class II-peptide complexes at the cell surface (Hammond et al. 1998; Kropshofer et al. 2002), suggesting that they may be involved in trafficking from the peptide loading compartment to the cell membrane. These proteins are especially interesting because the mechanisms of transport from Class II peptide loading compartments to the cell surface is the least understood pathway in the field of Class II antigen processing and presentation.

38 genes found in Gene Cluster #18 encode proteins that have some connection with signaling, antigen capture, vesicle transport, vesicle acidification or MHC class II expression, suggesting that activation of B cells through the BCR is inducing the expression of genes involved in MHC class II antigen processing and presentation. The activation of class II presentation by antigen receptor engagement in B cells has been described extensively (Siemasko et al. 2001). The findings reported here are novel in that microarray results combined with CLASSIFI analysis have provided a list of genes that may be involved in this important immunological process.

Several genes were annotated with “transporter” activity but their roles are not yet known to be associated with intracellular trafficking. Dhcr24 is an enzyme involved in

cholesterol biosynthesis (Waterham et al. 2001). Membrane lipid rafts are enriched in cholesterol and have been shown to cluster together B cell antigen receptor signaling components as well as antigen presentation components (Anderson et al. 2000; Cheng et al. 1999a), suggesting that Dhcr24 may be upregulated as a result of lipid raft remodeling in antigen-activated B cells. Fabp5 is a cytosolic fatty acid binding protein that may be involved in trafficking from the plasma membrane (Stremmel et al. 2001). Interestingly, epidermal-type fatty acid binding protein is found to be enriched in DCs in the splenic white pulp and in alveolar macrophages. These cell types are constantly undergoing antigen processing and presentation at a heightened level, suggesting that fabp5 may also be involved (Kitanaka et al. 2003; Owada et al. 2001). Pbp is involved in transport at the plasma membrane and has been proposed to bind lipids and participate in signal transduction from the plasma membrane (Banfield et al. 1998; Hemmaplardh et al. 1977). It has been shown to regulate G protein signaling , MAP kinase signaling and NF-κB signaling (Kroslak et al. 2001; Odabaei et al. 2004; Wang et al. 2004). Hematopoietic prostaglandin D2 synthase is expressed in dendritic cells in the spleen, thymus, and Peyer's patches and may be involved in regulating the immune response, as dendritic cells upregulate CD40, CD80, CD86, and Class II when stimulated with prostaglandin D2 (Kanaoka and Urade 2003; Steinbrink et al. 2000; Urade et al. 1989).

BCR binding to antigen is required for B cells to become activated and ultimately produce antigen-specific antibody. B cells require interaction with T cells in order to become plasma cells. This interaction is mediated by TCR binding to MHC Class II molecules containing antigenic peptide. In order for this T-B cell interaction to take place, antigen must

be taken up, processed, and presented into peptides that can be presented on Class II. For this reason, BCR signaling that results from antigen binding would induce antigen processing and presentation. This unique feature of B cells sets it apart from other antigen presenting cells, as B cells have been molded during development to express BCRs that could potentially bind specifically to any foreign antigen.

Finally, it is intriguing to note the presence of genes involved in the regulation of apoptosis and autoimmunity (Table 7.1C and D) in Gene Cluster #18, given the known function of the BCR in negative selection and the induction of autoimmunity. Beclin-1 is a tumor suppressor gene that plays a role in autophagy and apoptosis. Autophagy is a programmed response to stress such as starvation during which cellular proteins and organelles are sequestered inside autophagosomes for degradation. The autophagosome accomplishes this by acquiring lysosomal characteristics. Beclin 1-deficient embryonic stem cells are deficient in autophagy and beclin 1-deficient mice develop spontaneous tumors, demonstrating the role of beclin 1 in autophagy and tumor suppression (Yue et al. 2003). In a model of *Mycobacterium tuberculosis* infection in macrophages, beclin 1 has been shown to localize in mycobacterial phagosomes. *M. tuberculosis* inhibits phagosome maturation by disrupting delivery of H+ ATPase components from the TGN and thus prevents acidification. However, stimulation of autophagy results in increased acidification of phagosomes and has thus been proposed as a defense mechanism against *M. tuberculosis* (Gutierrez et al. 2004). Taken together, this suggests that beclin 1 may be functionally linked to both autophagy and phagosome maturation. Caspase-4, a cysteine protease involved in apoptosis, is localized to ER membranes and is activated in response to ER stress (Hitomi et al. 2004; Katayama et al.

2004). Siva binds to CD27, a member of the TNF receptor family known to provide costimulatory signals for B cell antibody production. Overexpression of Siva in a variety of cell types induces apoptosis (Prasad et al. 1997). Siva is transcriptionally activated by the tumor suppressors p53 and E2F1, demonstrating its role in apoptosis (Fortin et al. 2004).

The Pmscl2 autoantigen is a component of the polymyositis/scleroderma (PM/Scl) 3'→5' exoribonuclease complex, which is homologous to the yeast exosome. Antibodies that recognize the Pmscl2 protein product are found in individuals with polymyositis-scleroderma overlap syndrome, a mixture of connective tissue autoimmune disorders (Bliskovski et al. 2000). Sssca1 encodes an autoantigen that is recognized by a subset of anti-centromere antibodies from patients with scleroderma and/or Sjogren's sydrome, a lymphoproliferative autoimmune disorder (Muro et al. 1998).

Transcriptional positive feedback

This bioinformatics analysis combined with experimental validation has also provided evidence of transcriptional positive feedback regulation of gene expression. The changes in mRNA levels for these genes involved in vesicle trafficking might be necessary to stimulate this biological process in AIG-stimulated B cells. However, the delayed kinetics of the mRNA changes compared with the rapid induction of endocytosis suggests that this may not be the case. Elevated mRNA levels for these genes was found at 2 and 4 hr, but not at 0.5 or 1 hr post-stimulation. However, BCR endocytosis was maximally

stimulated by 15 minutes. The rapid induction of endocytosis suggests that at least the initiation of this process is activated through post-transcriptional mechanisms. This finding suggests that the AIG-specific transcriptional reprogramming observed in response to BCR stimulation may reflect positive feedback regulation in which expression of proteins that have already been activated is increased either as a means to amplify or sustain the process. Perhaps components of this important biological process are present at low levels in resting cells, and levels increase in response to appropriate environmental cues. This kind of regulatory process might allow cells to respond rapidly to a broad set of variables in their changing environment while conserving energy and materials while in the resting state. This observation emphasizes that gene expression data should not be over-interpreted, as they are not necessarily a reflection of the state of proteins in the cell due to the presence of post-transcriptional and post-translational mechanisms for regulation. However, combining gene expression data with biological information using a tool such as CLASSIFI focuses the analysis on biological processes that are affected by a change in more than one gene. Experimental testing of hypotheses generated from such a bioinformatics approach would very likely lead to new information about protein function.

Future Direction

The CLASSIFI analysis of the B cell single ligand screen data set leads to interesting observations that warrant further investigation. In particular, the identification of genes in Gene Cluster 18 that have been found to be involved in intracellular transport in various

systems, as well as the uncharacterized gene, is intriguing because of the implication that these genes also function in B cells. Further investigation of some of these candidate genes follows from this bioinformatics analysis. This section discusses the details of some experiments which could be used to investigate the functions of these candidate genes in the B cell antigen processing and presentation pathway. In addition, the AfCS B cell ligand screen has some experimental drawbacks which could be addressed with a new microarray experimental design, which is also discussed here.

Microarray Design. The AfCS B cell single ligand screen data set was one that already existed and for which we had no control over experimental design. Based on our interest in the differential effects of antigen, CD40L, and LPS, a new microarray experiment could be designed that keeps the study focused and addresses some of the caveats to experimental testing. Two caveats to the AfCS B cell single ligand screen are 1) the controversial use of AIG as an antigen mimic and 2) the effects of B cell death in the *in vitro* cultures. AIG is different from antigen in many ways and therefore may not be the best reagent for stimulating B cells in order to learn more about the effects of antigen *in vivo*. Its multivalent property results in B cell receptor crosslinking, with BCR supercrosslinking likely to occur due to the polyclonal nature of AIG. Antigen, in contrast, is not necessarily multivalent. In addition, antigen binds specifically to the variable regions of the BCR that make up the antigen combining site. These may result in different signaling properties from AIG, which is likely to also bind to the nonvariable constant regions of the BCR.

To address the two caveats mentioned above, a new microarray study can be designed using an *in vivo* system for antigen stimulation of B cells. In this study, mice whose B cells are transgenic and specifically recognize Hen Egg Lysozyme (HEL) can be used as a source of antigen-stimulated B cells. This system would be advantageous because the antigen (HEL), instead of an antigen mimic (AIG) would be used to stimulate B cells. This approach also addresses the problem of primary B cell viability *ex vivo* because B cells are *in vivo* stimulated and then can be purified from these mice and processed for RNA immediately. Another option is to use bcl-2 transgenic mice (Strasser et al. 1991), whose B cells survive for long periods of time in culture, as the source of B cells for *in vitro* stimulations. These approaches would also allow for longer stimulation times without contributing to the apoptotic effect of *in vitro* B cell culture.

The AfCS microarray experiment that we analyzed investigated the effects of individual ligands applied singly to B cell cultures. The AfCS also peformed experiments in which ligands are applied in pairs in order to investigate the crosstalk between signaling pathways. A new microarray experimental design for our interests would include ligand pairs and combinations (CD40L + HEL, LPS + HEL, CD40L + LPS, HEL + CD40L + LPS) for the study of effects of more than one ligand. B cells *in vivo* are subjected to a variety of different stimuli and the interaction of these signaling pathways determines the final cellular output response. Investigation of gene expression responses to combinations of ligands would give more information relevant to this *in vivo* situation.

Antigen processing and presentation. In order to investigate the effects of candidate genes on B cell antigen processing and presentation, an assay is needed for evaluating antigen uptake, processing, and presentation in B cells. Once such an assay is established, he effects of perturbation of genes of interest on antigen processing and presentation efficiency, such as overexpression or knockdown, could be evaluated. This assay would serve as a screen for identification of candidate genes suitable for further study, as well as provide a system for which to study the function of those genes.

In this assay, two transgenic mouse lines will be used as sources of B cells and T cells. BCR transgenic mice containing B cells specific for HEL antigen will be used as a source of antigen-specific B cells (Goodnow et at. 1988; Cooke et al. 1994). TCR transgenic mice containing cognate T cells specific for Class II-HEL presented by the transgenic B cells will be used as a source of T cells (Ho et al. 1994). B and T cells can be enriched using magnetic activated cell sorting. The antigen processing and presentation assay is performed by incubating the B cells with HEL antigen. After allowing time for antigen processing and presentation, transgenic T cells will then be added to the B cells. Alternatively, the B cells can be fixed first to "freeze" antigen processing and presentation before mixing with T cells. Following incubation of B and T cells, the concentration of IL-2 secreted by the T cells in culture media will be measured by ELISA. Alternatively, an IL-2 bioassay can be performed in which culture supernantants are added to CTLL cells, which respond to IL-2 by proliferation. Proliferation can then be measured by ^{3}[H] thymidine incorporation. The main drawback of this particular assay is the use of primary B cells, which can make studying the effects of protein perturbation on antigen processing and presentation more difficult.

To avoid the caveats associated with the use of primary cells, an antigen processing and presentation assay can be performed using B and T cell lines. The A20 murine B cell line expresses Class II I-A^d (Kim et al. 1982) and has been used in antigen processing and presentation assays with the DO-11.10 T cell hybridoma (Shimonkevitz et al. 1983), which recognizes ovalbumin peptide p323-339 in the context of I-A^d. A20 has been shown to present ovalbumin to DO-11.10 (Shimonkevitz et al. 1983, Michalek et al. 1989) and DO-BW, another ova peptide:I-A^d-recognizing T cell hybridoma (Zaliauskiene et al. 2002), albeit at low efficiency. These T cell hybridomas respond to ova peptide:I-A^d recognition by producting IL-2. The efficiency of presentation can be greatly increased by conjugating ovalbumin to something that is recognized and internalized by a B cell surface receptor. In the case of (Zaliauskiene et al. 2002), ovalbumin was conjugated to transferrin for uptake via the transferring receptor, which has been shown to intersect with MHC Class II molecules in the endocytic pathway. Since we are interested in BCR-mediated antigen processing and presentation, our experiments would involve chemical conjugation of ovalbumin to an anti-BCR antibody in order to increase the efficiency of ovalbumin processing and presentation following uptake via the BCR. 10^5 DO-11.10 cells will be incubated with 10^5 A20 cells in the presence or absence of antigen in a final volume of 200 μl of media in flat-bottom 96-well plates. Following incubation at 37°C for 24 hr, 100 μl supernatant will be removed, frozen to kill any remaining cells, and then assayed for IL-2 content by ELISA. Ultimately, the assay will be done with native ovalbumin (oval) conjugated to polyclonal anti-IgM (AIG) as the antigen using the chemical coupling protocol in (Zaliauskiene et al. 2002). Addition of this oval-AIG conjugate to A20 cells should stimulate internalization via the BCR, allowing

for antigen processing and presentation. The following will be added to the A20/DO-11.10 cultures to test for antigen processing and presentation: 1) no antigen (negative control), 2) oval alone (A20 cells have been shown to present oval without stimulation at low efficiency), 3) AIG alone (negative control), 4) oval+AIG (to test whether stimulation of A20 with AIG increases efficiency of oval presentation, 5) ova peptide p323-339 (to test ability of A20 to stimulate DO-11.10 without having to undergo antigen processing and presentation) 6) oval-AIG conjugate (this condition is expected to result in the most efficient presentation of ova peptide to DO-11.10). Once a working BCR-mediated antigen processing and presentation assay is established, the effect of perturbation of proteins of interest will be tested. The simplest perturbation approach would be to overexpress of the protein of interest by using expression constructs in A20 cells. Proteins that, when overexpressed, affect the efficiency of antigen processing and presentation (either positively or negatively) are candidates for further study. For example, overexpression of ATPase proton pump subunits might be expected to increase the efficiency of antigen processing and presentation because vesicle acidification is necessary for some proteolytic enzymes to function in the processing of native antigen. Overexpression of Vps29 might decrease the efficiency of antigen processing and presentation by driving retrograde transport of Class II-containing vesicles and preventing their intersection with antigenic peptide-containing vesicles. Thus, the overexpression studies can be used as an initial screen to identify proteins which play an important role in B cell antigen processing and presentation.

Protein colocalization studies. Several interesting proteins were identified in Gene Cluster 18 and are proposed to be involved in B cell antigen processing and presentation. To identify those genes which are likely to be involved in antigen processing and presentation, protein localization experiments can be performed. Co-localization of a candidate protein with known Class II antigen processing and presentation proteins (such as Class II or H2-DM) or with proteins found in transport organelles (such as lysosomal proteases) would suggest their involvement in B cell intracellular trafficking.

Antibodies specific to proteins of interest can be used to fluorescently label proteins for tracking inside the cell. Proteins that are relocated upon stimulation with antigen may be involved in antigen processing and presentation. Further testing of a protein's involvement in antigen processing and presentation include co-localization experiments. In these experiments, the protein of interest is labeled using one fluorochrome-conjugated antibody and another protein or organelle such as the BCR, MHC Class II, or lysosome is labeled in another fluorochrome-conjugated antibody or organelle-specific fluorescent dye. Colocalization is detected as the merge of the two different colors. Colocalization of the protein of interest with either BCR, Class II, or lysosomal proteins/markers would suggest involvement in antigen processing and presentation. All of these studies will be performed for "hypothetical protein" 2700018N07 to investigate the possibility that it is involved in B cell antigen processing and presentation. These studies will also be performed on other candidate proteins to investigate their functional properties in B cells.

The AfCS has characterized a library of organelle-specific subcellular localization markers tagged to YFP (Yellow Fluorescence Protein) in WEHI-231 cells (Chandy et al.

2003-AfCS brief communications, cited 3/30/05). Many of these markers are available from Clontech. These markers can be co-expressed with a fluorescently tagged protein of interest in order to determine localization to subcellular organelles. Expression of organelle markers has some disadvantages. The cells must be transfected in order to express the organelle marker. B cells are a particular challenge due to the extent of cell death following transfection approaches such as electroporation. In addition, overexpression of an organelle marker protein may interfere with normal signaling pathways, which could impact our studies in which signaling through the BCR drives antigen processing and presentation. Where possible, the AfCS has attempted to address this disadvantage by using markers in which the localization signal sequences were sufficient to target YFP to the target organelle. The advantage of using this organelle marker approach as opposed to an antibody staining approach is that these studies can be performed in live cells whereas antibody staining usually requires fixation and permeabilization of cell membranes in order to allow the antibody access to subcellular components. Live cell studies would allow us to visualize protein movement before and after antigen stimulation through the BCR. The AfCS has characterized several markers target organelles that are involved in the Class II antigen processing and presentation pathway. The pEYFP-ER construct available from Clontech (catalog # 6906-1) encodes the YFP tag with the calreticulon ER targeting sequence at the N-terminus and the ER retrieval sequence, KDEL, at the C-terminus. This marker would be useful to localize any proteins involved in Class II assembly in the ER. The A08XM005B1TK construct encodes the Golgin-245 GRIP domain (which binds to Golgi membranes) tagged to YFP. This marker would be useful for localizing any proteins

involved in Class II packaging and transport into the endocytic pathway. The A08XM036B1TK construct encodes YFP fused to the human K-Ras farnesylation signal and is specific for the plasma membrane. This marker would be useful for localizing any proteins involved in plasma membrane signaling or initiation of endocytosis at the plasma membrane. The pEX_EF1_YFP-VAMP3 construct encodes VAMP3 (Cellubrevin) fused to YFP and is a marker for endosomes. This marker would be useful for localization of proteins found in or on endosomes. These proteins may be involved in vesicle trafficking and antigen processing and presentation. Since these subcellular organelle markers are tagged with YFP, tagging a protein of interest with a fluorescence protein such as CFP (Cyan Fluorescence Protein) would allow for detection of co-localization by confocal microscopy. Co-localization of a protein of interest to any of these subcellular organelles involved in endocytosis would suggest the involvement of that protein in intracellular trafficking. This would warrant further study of these proteins to more closely define their role in antigen processing and presentation.

RNAi studies. RNA interference is a phenomenon in which the introduction of double-stranded RNA into a cell results in the degradation of its complementary RNA via a cellular pathway that results in cleavage of the complementary RNA. The RNAi phenomenon allows for post-transcriptional gene-specific silencing. An RNAi approach that works well in B cells would be a very useful tool for studying the effects of specific gene knockdown on antigen processing and presentation, which is evaluated using the antigen processing and presentation assay described above. These experiments can also be used to

screen candidate genes for further study. Knockdown of a gene that affects antigen processing and presentation in any way (either by increasing efficiency or decreasing efficiency) would warrant further investigation of that gene/protein.

For these experiments, dsDNA specific for the gene of interest is synthesized and introduced into B cells by various means (an example is electroporation). Knockdown of protein expression is validated by PCR or Western blot, and the B cells are used in the antigen processing and presentation assay described above. This method would definitively test whether the protein of interest is required for successful antigen processing and presentation.

Summary. Bioinformatics is a powerful approach for identifying interesting and relevant biological occurrences. For scientists, the real interest lies in further examination of the details of a biological process identified using these bioinformatics approaches in order to learn something new about biology. The experiments described in this section are approaches for further examination of the components of Gene Cluster 18, which contains genes involved in intracellular transport. Identification of a role for these genes in B cell antigen processing and presentation is exciting because it would contributes to our current knowledge of antigen processing and presentation in B cells. These studies are also important to investigators outside the field of immunology. Intracellular transport occurs in a variety of cell types and accomplish a variety of functions. Identification of regulators that determine the travel pathways of specific vesicles with specific cargo would also contribute to our overall understanding of endocytosis and intracellular transport.

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Vitae

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